# New Autosomal STR Loci to Address Challenges in Human Identity Testing

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June 11, 2008

## Disclaimers

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

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

## Questions to Address

- **Which loci were selected?**
  - Utility of additional loci
  - Characterization of additional STR loci
- **How was the STR multiplex developed?**
  - Initial work with smaller "miniplexes"
  - The "Autoplex" single amplification reaction
- **How can this information be useful?**

## Aren't the Current STR Loci Good Enough?

- For general forensic matching of evidence to suspect, the 13 CODIS STR loci are sufficient
- For other human identity/relationship testing questions, more autosomal loci can be beneficial or even necessary

## More Loci are Useful in Situations Involving Relatives

- **Missing Persons** and Disaster Victim Identification (kinship analysis)
- Immigration Testing (often limited references)
  - Recommendations for 25 STR loci
- Deficient Parentage Testing
  - often needed if only one parent and child are tested

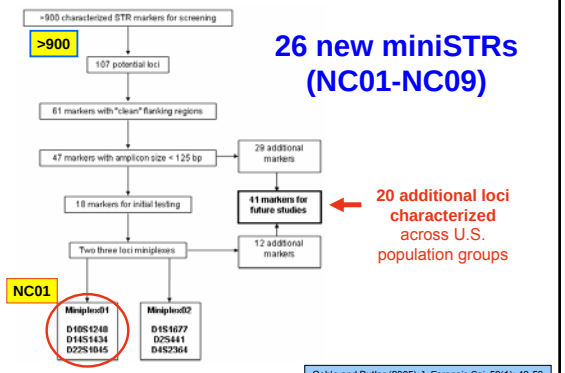
Relationship testing labs are being pushed to answer more difficult genetic questions…and **we want to make sure the right tools are in place**

## Selection of New Autosomal Loci

- Aim to have candidate sets for optimal miniSTRs
- Using ~900 STR loci with some literature data as a starting point…
  - Loci with high heterozygosities (>0.7)
  - Loci with small allele ranges (<24 bp) – low mutation?
  - Tetra (some tri-)nucleotide repeats without variants
  - Clean flanking regions (PCR products <140 bp)
- **26 loci** met criteria and fully characterized…

Coble and Butler (2005) Characterization of new miniSTR loci to aid analysis of degraded DNA *J. Forensic Sci.* 50(1): 43-53

## Initial Testing Results with Potential miniSTR Loci




**26 new miniSTRs (NC01-NC09)**

**20 additional loci characterized** across U.S. population groups

Coble and Butler (2005) *J. Forensic Sci.* 50(1): 43-53

## Characterizing New Loci

- ✓ Select genetic loci
- ✓ Design primers – optimize multiplex assay
- ✓ Type population samples to examine variation
- ✓ Sequence alleles to establish nomenclature
- ✓ Develop bins and panels for genotyping
- ✓ Construct allelic ladders
- ✓ Evaluate RMP or ability to separate common types
- ✓ Perform mutation rate studies
- ✓ Perform concordance studies (when applicable)
- ✓ Calibrate genotypes with NIST SRM components
- ✓ Work with companies/collaborators
- ✓ Publish details on loci and assays

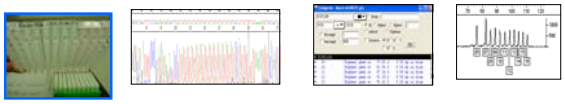
## Characterization of New Loci

**"Computer Work"**

Candidate STR marker selection (e.g. literature searches) → Pull down sequence data from the web (e.g. NCBI) → Identify Chromosome Location (e.g. Human BLAT Search ) → Screen for PCR Primers (e.g. Primer3) → Test primers for Multiplex-ability (e.g. AutoDimer - NIST )

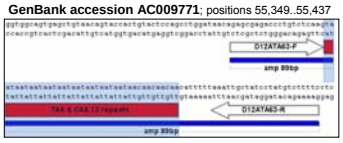
**"Laboratory Work"**

Test Markers on Population samples → Sequence homozygotes to determine allele sizes → Build Macros for Genotyping → Construct Allelic Ladders



## Characterization of miniSTR **D12ATA63**

**GenBank accession AC009771**; positions 55,349..55,437




**[FAM]** – GAGCGAGACCCTGTCTCAAG
GAAAAGACATAGGATAGCAATTT

**Chr 12** 106.825 Mb (12q23.3)

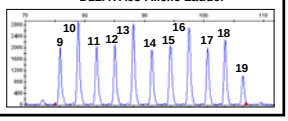
**Trinucleotide**
[TAA][CAA] repeat
**76 -106 bp**
Alleles 9 -19

| Allele | Caucasian (N = 260) | African Am (N = 259) | Hispanic (N = 140) |
|---|---|---|---|
| 9 | -- | -- | 0.0036 |
| 10 | 0.0019 | 0.0154 | 0.0036 |
| 11 | 0.1385 | 0.1525 | 0.1500 |
| 12 | 0.2154 | 0.1004 | 0.1786 |
| 13 | 0.0173 | 0.1564 | 0.0286 |
| 14 | 0.1615 | **0.3340** | 0.2214 |
| 15 | 0.0577 | 0.0772 | 0.0714 |
| 16 | **0.2981** | 0.1004 | **0.2643** |
| 17 | 0.0981 | 0.0521 | 0.0679 |
| 18 | 0.0096 | 0.0058 | 0.0071 |

**Heterozygosity Values**

| | |
|---|---|
| U.S. Caucasian | **0.842** |
| African American | **0.788** |
| U.S. Hispanic | **0.879** |

**D12ATA63 Allelic Ladder**




## European Labs Have Adopted the NIST-Developed NC (non-CODIS) miniSTRs

*FSI* (2006) **156(2):** 242-244

Short communication

The evolution of DNA databases—Recommendations for new European STR loci

Peter Gill[a,*], Lyn Fereday[b], Niels Morling[c], Peter M. Schneider[d]

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Received 25 May 2005; accepted 26 May 2005

**…recommended that existing multiplexes are re-engineered to enable small amplicon detection, and that three new mini-STR loci with alleles <130 bp (D10S1248, D14S1434 and D22S1045) are adopted as universal. This will increase the number of European standard Interpol loci from 7 to 10.**

## In Jan 2008 Issue of *J. Forensic Sci.*

**J. Forensic Sci., Jan 2008, 53(1):73-80**

*J Forensic Sci*, January 2008, Vol. 53, No. 1
doi: 10.1111/j.1556-4029.2008.00595.x
Available online at: www.blackwell-synergy.com

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Characterization of 26 MiniSTR Loci for Improved Analysis of Degraded DNA Samples

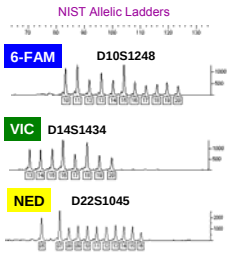
- Characterization of **26** new autosomal loci
- Primer sequences, GeneMapper bins and panels, genotypes on common samples, and allele frequency information **already available on STRBase**

**http://www.cstl.nist.gov/div831/strbase/miniSTR.htm**
**http://www.cstl.nist.gov/div831/strbase/newSTRs.htm**

## Multiple Miniplexes

- **26 characterized loci** divided into 10 miniplexes
- One locus per dye color
- Allelic ladders created
- **Amplicons <140 bp**
- miniSTRs
- Work with 100 pg DNA
- **For degraded samples**
  **(bones in missing persons cases)**

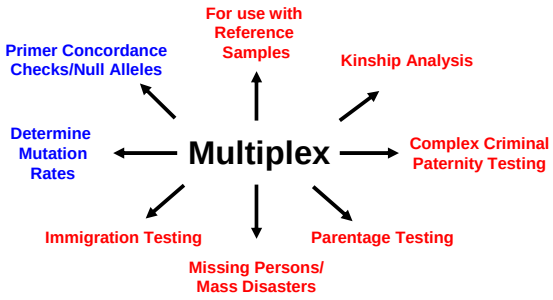
**NC = Non-CODIS or non-core**




**NC01 Loci**

See Dixon et al. (2006) *Forensic Sci. Int.* 164: 33-44.

## Why Build a Large Multiplex?

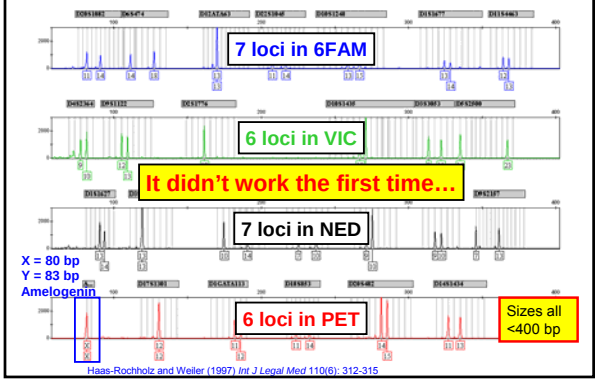



## The Multiplex Design Process

## The Design of the Multiplex

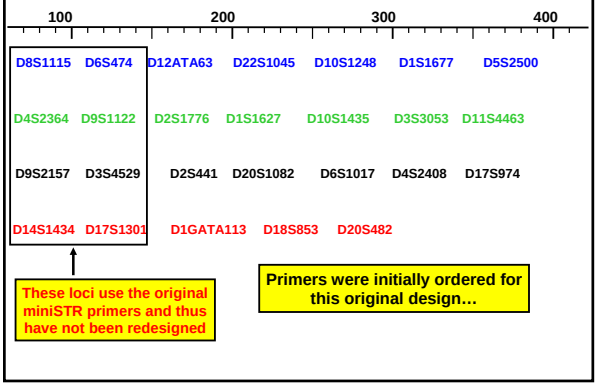
- **Goal**: A single amplification multiplex combining the 26 autosomal loci + Amelogenin in one reaction (27plex)
- How was this achieved?
  - PCR amplicons labeled with 6FAM, VIC, NED, and PET dyes
  - Primer redesign for non "miniSTR loci"
  - Empirical trial and error of primer compatibility, as well as balancing the primer concentrations

## Current Autoplex – 26 Loci

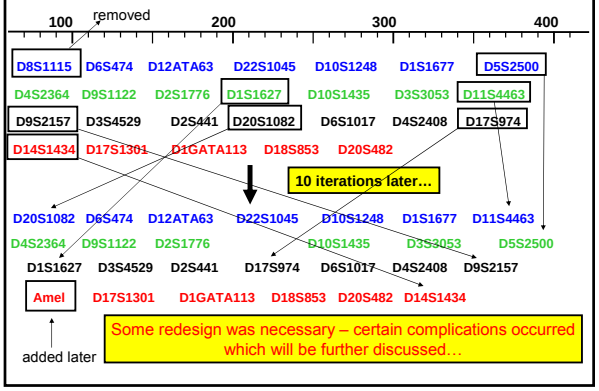



Haas-Rochholz and Weiler (1997) *Int J Legal Med* 110(6): 312-315

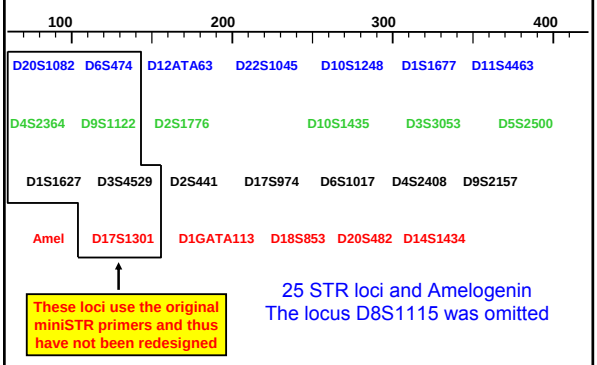
## 1st Autoplex Design



## Some Loci Had to be Rearranged




## Current Autoplex Design

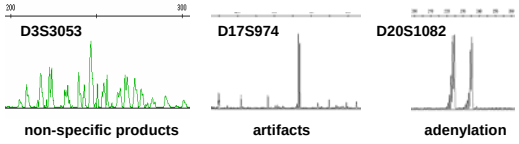



## Where do we begin?

- PCR primers were designed using Primer3
- Primers were screened with the AutoDimer software
- Designed primers were mapped to confirm amplicon size and ensure primers flank the repeat
- Primers ordered
  - Forward fluorescent dye-labeled from AB
  - Reverse non-dye labeled from Operon

## What Next?

- 1st step: primers were amplified in singleplex
  - If the primers fail in singleplex, they will most likely fail in multiplex – Candidates for redesign
  - Initially, 7 loci out of 26 failed – 19 worked in singleplex
- How do we define failure?
  - Incomplete adenylation, presence of artifacts, low signal, non-specific products, or no PCR product

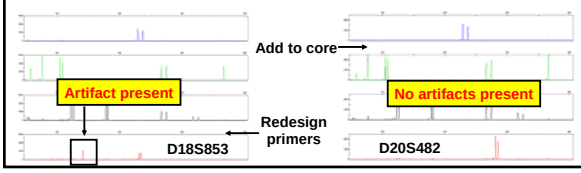



## Building the Multiplex

- Successful primers were tested in multiplex – 19 loci were combined together
- Then, the minimum amount of loci that work well together were determined
  - Can isolate by dye color – amplify loci in one dye channel at a time to hone in on failures
  - 9 loci: add remaining loci one at a time


## Trial and Error

- Serially add a locus to the "core-9" multiplex and determine compatibility
  - Check for artifacts and adenylation
- Increase the size of the multiplex
  - Continue adding to a 'core' multiplex and redesign incompatible primers



## Finalizing the Multiplex

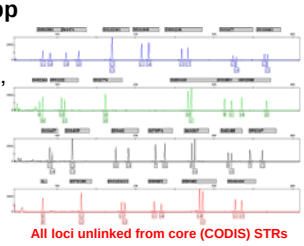
- The size of the multiplex increased
  - 18, 19, and 23plex
- Locus to locus primer balancing was performed with each multiplex
- Concordance and Mutation Rate studies were performed with a 23plex
- Currently, the multiplex has all but one locus (D8S1115) 26plex

## Lessons Learned from Primer Redesign

- Some loci had to be redesigned with PCR products in a different size range
  - If artifacts are present or if it is a noisy baseline
- The fluorescent dye label can be switched to the reverse primer to mask an artifact
  - Can check forward and reverse primers separately
- Adenylation issues: a 'PIGTAIL' (GTTTCTT) can be added to the 5' end of reverse primers
  - D1S1677, D3S3053, D11S4463, and D12ATA63
- Dye artifacts can be filtered out with post-PCR cleanup (Edge Columns)
  - Especially in the PET dye channel

## Autoplex

- **25 STRs and amelogenin** in single multiplex
  Multiple loci in four dye channels
- **Amplicons 70 to 400 bp**
  (No longer 'miniSTRs')
- Typically use 1 ng DNA, 30 cycles



**26plex**

All loci unlinked from core (CODIS) STRs

## PCR Parameters

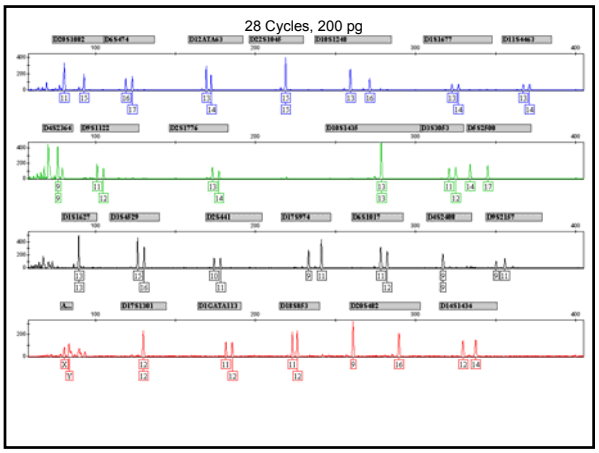
- Master Mix
  - 2 mM $MgCl_2$
  - 1x PCR Buffer
  - 1 Unit TaqGold
  - 0.2 µM Primer mix
  - 250 mM dNTPs
  - 0.16 mg/mL BSA
- 20µL reaction volume = 19µL MM + 1µL DNA sample (~1ng)

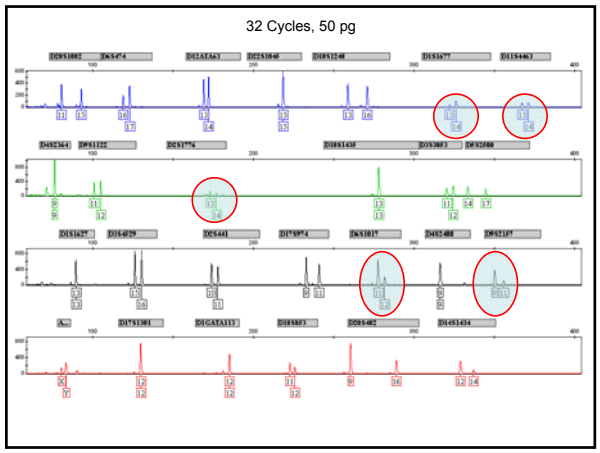
## Thermal Cycling Conditions

- Conditions for ABI 9700 in 9600 emulation mode
- 95˚C Hot Start for 11 min
- 30 cycles
  - 94˚C for 45 sec Denaturation
  - 59˚C for 2 min Annealing
  - 72˚C for 1 min Elongation
- 60˚C soak for 60 min
- 25˚C hold (∞)

## Sensitivity Study

- A highly characterized sample was used for this study at a wide range of concentrations:
  - **2 ng, 1 ng, 750 pg, 500 pg, 400 pg, 300 pg, 250 pg, 200 pg, 100 pg, 50 pg, and 25 pg**
- 3 different PCR cycles were tested:
  - **28, 30, and 32 cycles**
- Ideal DNA concentration and cycling:
  - **1 ng DNA for 30 cycles**
- Multiplex sensitivity (lowest concentration where all peaks were detected above 50 RFUs):
  - **28 cycles, 200 pg**
  - **30 cycles, 100 pg**
  - **32 cycles, 50 pg**




## Further Work with the Autoplex

Studies were Performed with the 23plex

## Evaluation of Autoplex (23plex)

- **660 U.S. population samples**
  - U.S. Caucasian, African American, Hispanic
  - **Concordance testing** compared to miniSTR results
- **790 father/son samples**
  - U.S. Caucasian, African American, Hispanic, Asian
  - **Mutation rate determination**
- 12 samples for **extended family testing**

**>1450 samples examined so far**
(multiple primer batches prepared)

## Concordance Study to Check for Null Alleles

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

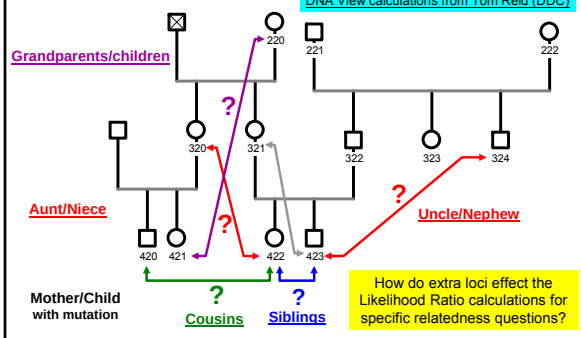
- **639 samples** compared
- 14,058 total types (639 x 22 loci)
- 28 types discordant (0.20%)
- **99.80%** concordance
- Discordance has not yet been confirmed by sequencing

## Mutation Rates Measured for New STRs

- **395 father/son pairs** (790 samples total)
- 22 STR loci examined
- 8690 allelic transfers
- Only **6 mutations** were observed in total
- **0.069%** mutation rate
- 2-3 times less than typical 0.2% for common STRs

## Extended Family Sample Testing

**15 vs 37 STRs**
DNA View calculations from Tom Reid (DDC)




How do extra loci effect the Likelihood Ratio calculations for specific relatedness questions?

## Comparison of Likelihood Ratios

| Relationship Examined | **15 STRs** (Identifiler, ID15) | ID15 + **Autoplex 22** STRs = **37 loci** (A37) |
|---|---|---|
| Mother/Child* (*with single mutation) | 0.214 | **5,200,000** Extra loci help… |
| Siblings | 477 | **113,000** Extra loci help… |
| Uncle/Nephew | 824 | **247,000** Extra loci help… |
| Cousins | 0.45 | **2.25** |
| Grandparents/ Grandchildren | 0.53 | **1.42** |

**Conclusions:** Longer distance multi-generational questions cannot usually be solved with additional autosomal STRs…

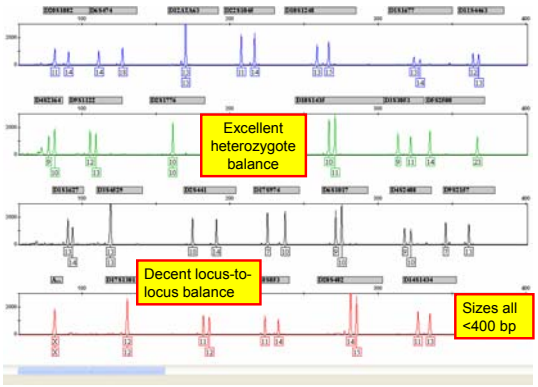
## SRM 2391b: DNA Profiling Standard Certificate of Analysis Update

- Genotyping and sequencing have been performed with SRM 2391b components (#1-12) for all 26 additional loci
- Certified and reference values have been assigned to all resulting alleles
- The Certificate of Analysis is in the process of being updated for all 26 loci (coming up in near future…)
- Using these values, bins and panels have been written in GeneMapper*ID*
- **Purpose**: No commercial allelic ladders are available, but all genotypes are certified for the components of SRM 2391b

http://www.cstl.nist.gov/biotech/strbase/miniSTR/miniSTR_NC_loci_types.htm

## Current Autoplex (26 loci)

Comp 9
9947A




## Summary/Conclusions

- **26 unlinked loci** have been characterized and we have developed multiple miniplexes and an Autoplex (26plex)
- The Autoplex is a robust single amplification 5-dye multiplex reaction that can benefit the forensic community for reference purposes and relationship testing
- **NIST SRM 2391b** will include certified and reference values on these 26 additional autosomal STR loci

## In the Future…

- All information will be available on STRBase
- A manuscript is currently being prepared for submission to a forensic journal
- **Use loci in new applications such as Rapid PCR**

## Further Evaluation of the Loci for Rapid PCR

- 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

## 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 ºC/sec ramp rate (9600 emulation)
    - Utilize the 9700 4 ºC/sec ramp rate
  - Using commercial polymerases that are 'faster'

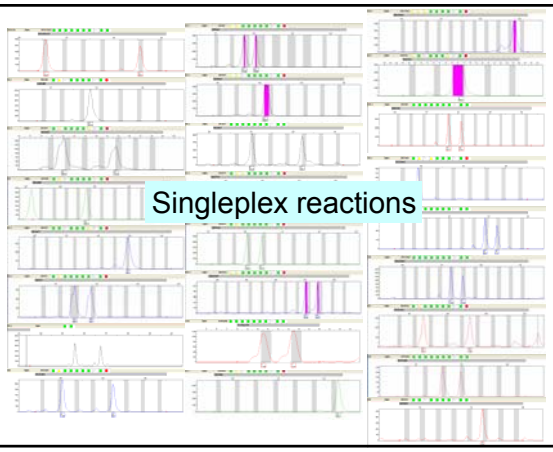
Obtain results in less than 45 minutes
Trying simple things first…

## Thermal Cycling

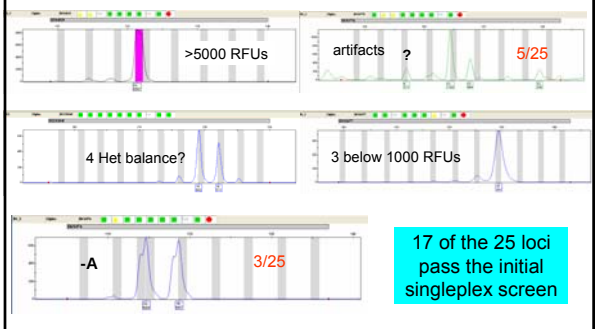
| Parameter | Unit | Trad | Rapid | Difference (min) | % |
|---|---|---|---|---|---|
| **Hot Start** | Min | 10 | 1 | 9.0 | 6.3 |
| **Hold** | Sec | 60 | 5/10 | 72.3 | 50.6 |
| **Soak** | Min | 60 | 1 | 59.0 | 41.2 |
| **Ramp rate** | (deg/sec) | 1 | 4 | 22.4 | 15.7 |
| **Cycles** | | 28 | 28 | | |
| **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




## Singleplex Evaluation




17 of the 25 loci pass the initial singleplex screen

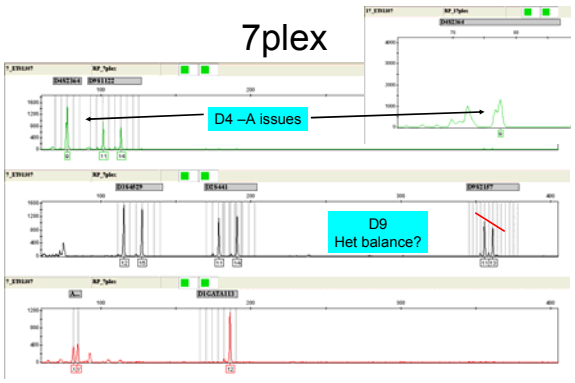
## Testing 4 Multiplexes

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

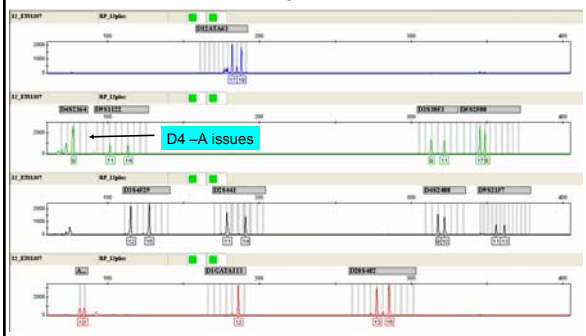
  } Subset of the 17plex

- Run under rapid cycling conditions
- 1 ng DNA, 28 cycles

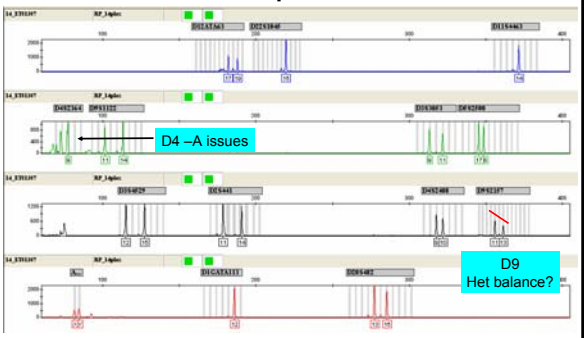
## 7plex




## 12plex




## 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)
- Evidence that heterozygote imbalance does not directly track with amplicon size

Future: continuing developing rapid PCR protocols

## Thank you for your attention…

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

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

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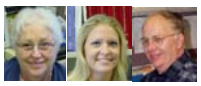

Jan Redman | Amy Decker | Dave Duewer

Funding from the **National Institute of Justice (NIJ)** through NIST Office of Law Enforcement Standards

**Collaborators**

**Mike Coble** (now AFDIL) – early miniSTR work

**Tom Reid** (DDC) - father/son samples