# Development of a New Autosomal STR Multiplex with Additional Loci to Benefit Human Identity Testing

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## Disclaimers

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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 did I use and why?**
  - Why additional loci are important
  - Characterization of additional STR loci
- **How did I combine these new loci into one multiplex?**
  - The "Autoplex" 5-dye single amplification reaction
- **How can this be useful?**
  - SRM 2391b has been updated with certified and reference values for new loci

## Which Loci Were Used and Why?

Going Beyond the CODIS Loci

## 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**

## Characterizing New Loci

- New loci were chosen based on the following characteristics:
  - Genomic Position
  - Polymorphic Content
  - Span/Range of observed alleles
- Details about the characterization process have all been previously reported at length:

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  - *Eighteenth International Symposium on Human Identification*. See http://www.promega.com/geneticidproc/
  - John Butler's talk at the 18th International Symposium on Human Identification (Promega 2007), "New Autosomal and Y-Chromosome STR Loci: Characterization and Potential Uses"

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

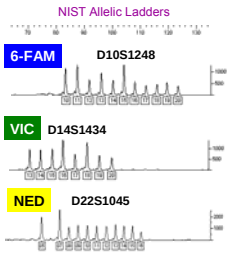
- Characterization of **26** new autosomal loci
- Primer sequences, GeneMapper bins and panels, genotypes on common samples, and allele frequency information **already available on STRBase**

**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.

## NC Miniplexes

| NC01 | NC02 | NC03 |
|---|---|---|
| D10S1248 | D1S1677 | D3S3053 |
| D14S1434 | D2S441 | D6S474 |
| D22S1045 | | |

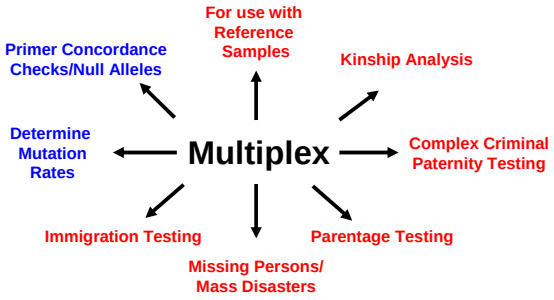
**26 total new loci**

| NC04 | NC05 | NC06 | NC10 |
|---|---|---|---|
| D1GATA113 | D1S1627 | D3S4529 | D3S3053 |
| D2S1776 | D8S1115 | D9S2157 | D6S474 |
| D4S2408 | D9S324 | D10S1430 | D20S482 |

| NC07 | NC08 | NC09 |
|---|---|---|
| D9S1112 | D17S1301 | D10S2327 |
| D12ATA63 | D18S8534 | D11S4463 |
| D14S1280 | D20S1082 | D17S974 |

**Removed because they were problematic**

## Why Build a Large Multiplex?

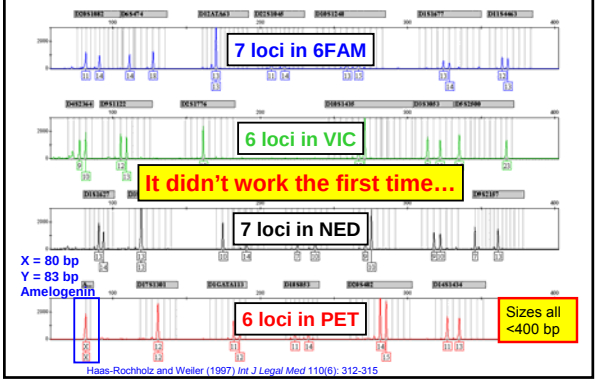



## The Multiplex Design Process

## The Design of the Multiplex

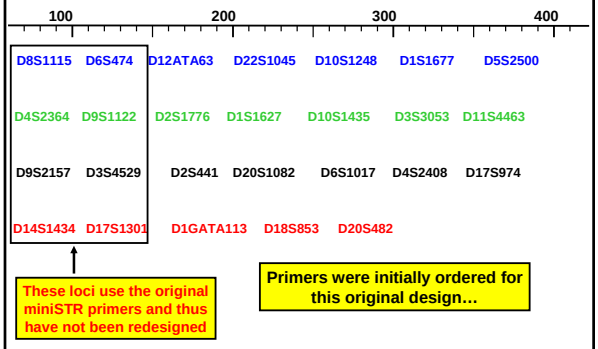
- **Goal**: A single amplification 5-dye multiplex to combine the 26 new autosomal loci + Amelogenin in one reaction (27plex)
- How can this be achieved?
  - Initial placement of all loci within 6FAM, VIC, NED, and PET dye channels (the size standard is in the LIZ channel)
  - Primer redesign for all but 7 of the original miniSTR loci
  - Trial and error of primer compatibility, as well as balancing for all working primers

## Current Autoplex – 26 Loci

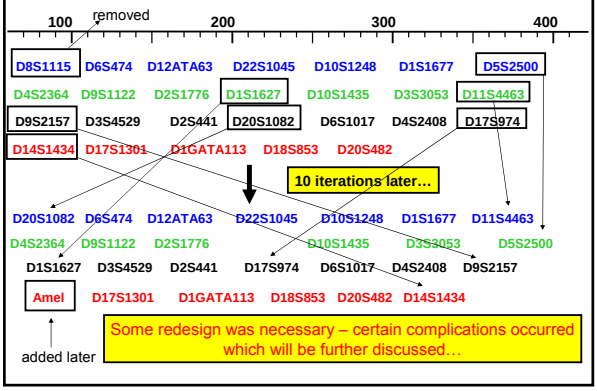



Haas-Rochholz and Weiler (1997) *Int J Legal Med* 110(6): 312-315

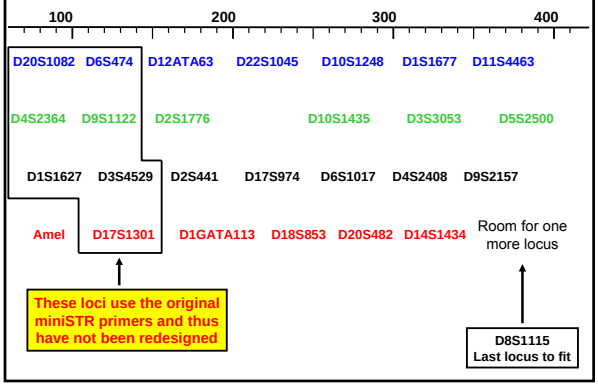
## 1st Autoplex Design




## Some Loci Had to be Rearranged




## Current Autoplex Design

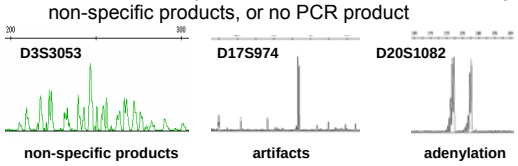



## Where do we begin?

- Once the initial design has been laid out, primers were designed using Primer3
- Next, they were tested for multiplex-ability with AutoDimer developed by Pete Vallone
- Finally, the newly designed primers were mapped in LaserGene to confirm amplicon size and ensure primers flank the repeat
- Primers were then ordered
  - Forward flourescent dye-labeled from ABI
  - Reverse non-dye labeled from Operon

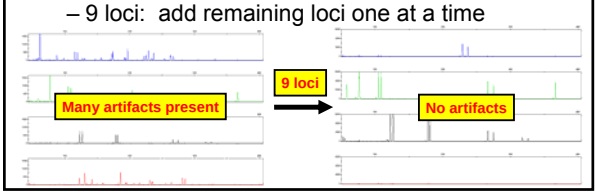
## What Next?

- 1st step: Once received, all new 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



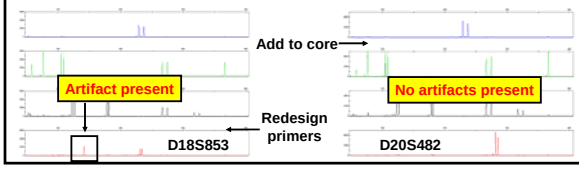

## Begin Building the Multiplex

- Next, 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

- When a locus was added to the "core-9" multiplex, decide whether it works or not
  - Check for artifacts and adenylation
- Then, build the multiplex from there
  - Keep adding to the core multiplex and redesign all primers that fail




## Finalizing the Multiplex

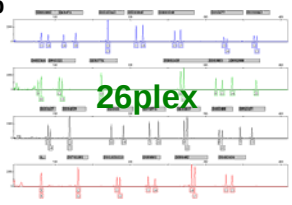
- Eventually the multiplex gained more loci with clean profiles: 18, 19, and 23plex
- Locus to locus primer balancing was performed with each multiplex
- Concordance and Mutation Rate studies were performed with the 23plex
- Currently, the multiplex has all but one locus (D8S1115) and we are working on redesigning further out (possibly > 400 bp)

## Lessons Learned from Primer Redesign

- Some loci had to be redesigned in a completely different location
  - 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
- With adenylation issues, a PIGTAIL (GTTTCTT) can be added to the 5' end of reverse primers
  - D1S1677, D3S3053, D11S4463, and D12ATA63
- Dye blobs can be filtered out with Edge Columns
  - Especially in the PET dye channel

## Autoplex

- So far **25 STRs and amelogenin** in single multiplex (Eventual goal to have all 26 loci)
- Multiple loci in four dye channels
- **Amplicons 70 to 400 bp**
  (No longer 'miniSTRs')
- Typically use 1 ng DNA, 30 cycles
- **For reference samples**
  **(a missing person's relatives)**
  **"Autoplex" or "miniMegaplex"**



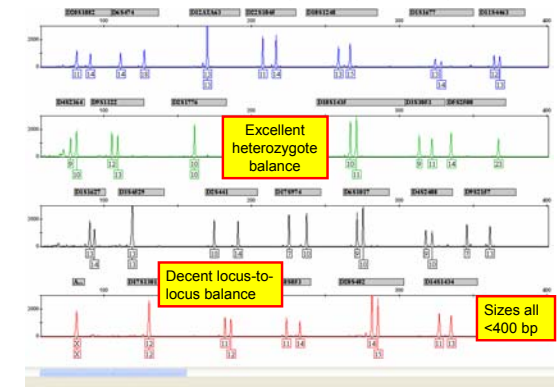
## PCR Parameters

- Master Mix (MM)
  - 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 (∞)

## Current Autoplex (26 loci)




## 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

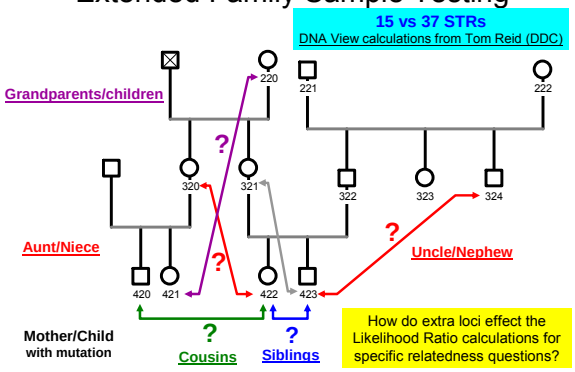
http://www.cstl.nist.gov/biotech/strbase/NullAlleles.htm

- **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




## 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
- Cert… ed to all re… **More information on this to come in Margaret Kline's presentation on Saturday morning…**
- 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

## In the Future…

- The D8S1115 will be redesigned to fit into the Autoplex – 27plex goal
- Allelic ladders for the final Autoplex will be prepared (made available to interested companies)
- All information will be available on STRBase
- A comprehensive publication is currently being written for submission to a forensic journal
- Use in new applications such as Rapid PCR (initial findings to be presented by Pete Vallone on Saturday morning)

## 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

## Thank you for your attention…

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

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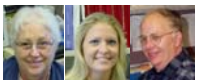
Jan Redman | Amy Decker | Dave Duewer

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**Collaborators**

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

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