NIST National Institute of Standards and Technology
http://www.cstl.nist.gov/biotech/strbase/newSTRs.htm
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A New STR 26plex

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Development of a New Autosomal STR 26plex to Address Challenges in Human Identity Testing

PP23

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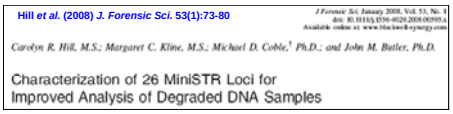
A new short tandem repeat (STR) multiplex assay has been successfully designed and developed with 25 non-CODIS (COmbined DNA Index System) markers plus the sex-typing marker amelogenin for a total of 26 loci in a single polymerase chain reaction (PCR) reaction. These 25 non-CODIS (NC) loci span unused chromosomal locations across the 22 autosomes and have been previously characterized in our laboratory. These loci were originally developed as reduced size miniSTRs with product sizes below 140 base pairs (bp) intended for recovery of information from degraded DNA typically present in missing persons or mass disaster samples. The PCR primer pairs for the majority of these NC markers have been redesigned to fit into a single amplification reaction with product sizes ranging from 65 to 400 bp and five-dye fluorescent detection. The present 26plex has the potential to significantly benefit the forensic community for reference purposes and complex relationship testing in situations including kinship analysis, criminal paternity testing, parentage testing, immigration testing, and for use in missing persons/mass disaster cases.

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

25 of the 26 loci described in the above publication were used in this multiplex. The D8S1115 locus was not included because multiple primer sets that were attempted (15 total) were not compatible in this multiplex. The sex-typing marker amelogenin was also added for a total of 26 loci.

NC Locus Information

- **New autosomal NC loci**: Ref (1 – 3)
  http://www.cstl.nist.gov/biotech/strbase/newSTRs.htm
  http://www.cstl.nist.gov/biotech/strbase/miniSTR.htm
- **Allele frequencies**: (1)
  http://www.cstl.nist.gov/biotech/strbase/NISTpopdata/Allele_Frequencies_for_26miniSTRs.pdf
- **Heterozygosities**: (1, 2)
  http://www.cstl.nist.gov/biotech/strbase/populationdata.htm
  http://www.cstl.nist.gov/biotech/strbase/NISTpop.htm
- **Concordance Study**: (3)
  http://www.promega.com/geneticidproc/ussymp18proc/oralpresentations/Butler.pdf
- **Mutation Rate Study**: (3)
  http://www.promega.com/geneticidproc/ussymp18proc/oralpresentations/Butler.pdf
- **Amelogenin**: (4)
  http://www.cstl.nist.gov/biotech/strbase/Amelogenin.htm
- **Reference Genotypes (9947A, 9948, 007, K562)**: (1)
  http://www.cstl.nist.gov/biotech/strbase/miniSTR/miniSTR_NC_loci_types.htm

Primer Design for the Multiplex

1. Allele ranges and size ranges were determined from previous miniSTR information and U.S. population data (1,2).
2. Layout the Multiplex design by size and dye color (6FAM, VIC, NED, and PET) (5,6)
   - Each locus size range is separated by at least 10 bp
   - The smallest 7 loci are the original miniSTR sizes and have not been redesigned (1)
   - Amelogenin was added to the Multiplex at ~ 80 and 83 bp (4)
   - The remaining 18 loci were all redesigned (< 400 bp)
3. Primers were designed using the Primer3 Software (7).
4. Primer to primer inter-comparisons were performed with AutoDimer (8) and BLAST searches (9).
5. Forward primers were labeled at the 5' end with either 6FAM, VIC, NED, and PET and reverse primers were unlabeled with a guanine (G) base added to the 5' end to promote full adenylation.

Primer Designs for Each Locus

Locus	# of Primers Tested Forward	# of Primers Tested Reverse	Total Primers Tested per Locus	Forward Dye Label	GenBank Accession	Observed Allele Range
D1GATA113	1	1	2	PET	Z97987	7 - 13
D1S1627	2	2	4	NED	AC093119	10 - 16
D1S1677	2	4	6	6FAM	AL513307	9 - 19
D2S441	1	1	2	NED	AC079112	9 - 17
D2S1776	1	1	2	VIC	AC009475	7 - 15
D3S3053	2	4	6	VIC	AC099259	7 - 17
D3S4529	1	1	2	NED	AC117452	11 - 18
D4S2364	1	1	2	VIC	AC022317	7 - 11
D4S2408	1	1	2	NED	AC110763	7 - 13
D5S2500	3	3	6	VIC	AC008791	14 - 24
D6S474	1	1	2	6FAM	AL357514	11 - 20
D6S1017	1	1	2	NED	AL035588	7 - 14
D8S1115	15	14	29	PET	AC090739	9 - 20
D9S1122	1	1	2	VIC	AL161789	9 - 17
D9S2157	7	7	14	NED	AL162417	7 - 19
D10S1248	1	1	2	6FAM	AL391869	8 - 19
D10S1435	1	1	2	VIC	AL354747	5 - 19
D11S4463	2	2	4	6FAM	AP002806	10 - 17
D12ATA63	1	2	3	6FAM	AC009771	10 - 20
D14S1434	2	2	4	PET	AL121612	9 - 17
D17S974	7	7	14	NED	AC034303	5 - 12
D17S1301	1	1	2	PET	AC016888	9 - 15
D18S853	3	3	6	PET	AP005130	9 - 16
D20S482	1	1	2	PET	AL121781	9 - 19
D20S1082	9	10	19	6FAM	AL158015	8 - 17
D22S1045	1	1	2	6FAM	AL022314	8 - 19
Amelogenin	1	1	2	PET	M55418, M55419	X,Y
Total designed	70	75	145			
Total Used	26	26	52			
Total Unused	44	49	93			

58% First Pass Success Rate

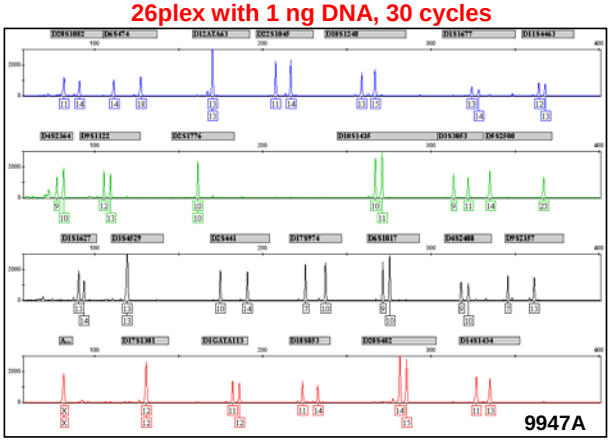
The primer sets for 11 of the loci had to be redesigned multiple times and the number of redesigns are listed in this table. The marker highlighted in red was ultimately not used in the 26plex (D8S1115). The dye channel, GenBank accession number, and allele ranges are also listed in the above table. The final primer sequences will be listed in a forthcoming publication.

References

1. Hill, C.R., Kline, M.C., 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.
2. 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.
3. Butler, J.M., Hill, C.R., Decker, A.E., Kline, M.C., Reid, T.M., Vallone, P.M. (2007) New autosomal and Y-chromosome STR loci: characterization and potential uses. *Proceedings of the Eighteenth International Symposium on Human Identification*. See http://www.promega.com/geneticidproc/
4. Haas-Rochholz H, Weiler G. (1997) Additional primer sets for an amelogenin gene PCR-based DNA-sex test. *Int. J. Legal Med.*, 110: 312-15.
5. Schoske, R., Vallone, P.M., Ruitberg, C.M., Butler, J.M. (2003) Multiplex PCR design strategy used for the simultaneous amplification of 10 Y chromosome short tandem repeat (STR) loci. *Anal. Bioanal. Chem.*, 375: 333-343.
6. Butler, J.M. (2005) Constructing STR multiplex assays. *Methods in Molecular Biology: Forensic DNA Typing Protocols* (Carracedo, A., ed.), Humana Press: Totowa, New Jersey, 297: 53-66.
7. Rozen S, Skaletsky HJ. Primer3 (primer3_www.cgi v 0.2). 1998 Code available at http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi.
8. Vallone PM, Butler JM. AutoDimer: a screening tool for primer-dimer and hairpin structures. Biotechniques 2004;37(2):226-31.
9. BLAST-nucleotide (BLASTn) search: http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi.

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

26plex with 1 ng DNA, 30 cycles



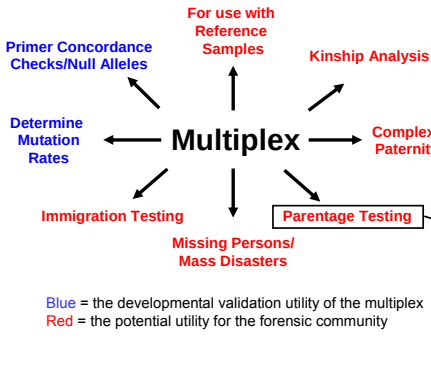
PCR Master Mix

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

Thermal Cycling Parameters

ABI 9700 in 9600 emulation mode: 20 µL reaction volumes
95ºC Hot Start for 11 min, 30 cycles of 94ºC for 45 sec, 59ºC for 2 min, 72ºC for 1 min
60ºC soak for 60 min, then 25ºC hold (∞)

Forensic Utility of the 26plex



Blue = the developmental validation utility of the multiplex
Red = the potential utility for the forensic community

NC Loci Compared to CODIS Loci

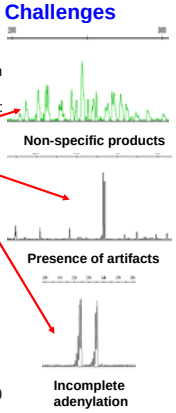
CODIS Loci	Repeat Motif	Chromosomal Location	Chromosome Position	Heterozygosity (Overall)*	
FGA	CTTT	4q31.3	155.866 Mb	0.886	1
D18S51	AGAA	18q21.33	59.100 Mb	0.876	2
D21S11	[TCTA][TCTG]	21q21.1	19.476 Mb	0.844	3
VWA	[TCTG][TCTA]	12p13.31	5.963 Mb	0.826	
D7S820	GATA	7q21.11	83.433 Mb	0.806	
D8S1179	[TCTA][TCTG]	8q24.13	125.976 Mb	0.774	
D16S539	GATA	16q24.1	84.944 Mb	0.766	
D3S1358	[TCTG][TCTA]	3p21.31	45.557 Mb	0.763	
D13S317	TATC	13q31.1	81.620 Mb	0.747	
CSF1PO	TAGA	5q33.1	149.436 Mb	0.745	
TH01	TCAT	11p15.5	2.149 Mb	0.745	
D5S818	AGAT	5q23.2	123.139 Mb	0.721	
TPOX	GAAT	2p25.3	1.472 Mb	0.707	
NC Loci	**Repeat Motif**	**Chromosomal Location**	**Chromosome Position**	**Heterozygosity (Overall)***	
D9S2157	ATA	9q34.2	133.065 Mb	0.844	4
D12ATA63	[TAA][CAA]	12q23.3	106.825 Mb	0.829	5
D10S1248	GGAA	10q26.3	130.567 Mb	0.792	
D22S1045	ATT	22q12.3	35.779 Mb	0.784	
D2S441	TCTA	2p14	68.214 Mb	0.774	
D10S1435	TATC	10p15.3	2.233 Mb	0.766	
D2S1776	AGAT	2q24.3	169.471 Mb	0.763	
D3S4529	ATCT	3p12.1	85.935 Mb	0.761	
D6S474	[AGAT][GATA]	6q21	112.986 Mb	0.761	
D5S2500	[GGTA][GACA] [GATA][GATT]	5q11.2	58.735 Mb	0.747	
D1S1627	ATT	1p21.1	106.676 Mb	0.746	
D1S1677	TTCC	1q23.3	160.747 Mb	0.746	
D6S1017	ATCC	6p21.1	41.785 Mb	0.740	
D3S3053	TATC	3q26.31	173.234 Mb	0.739	
D9S1122	TAGA	9q21.2	76.918 Mb	0.734	
D17S974	CTAT	17p13.1	10.459 Mb	0.732	
D11S4463	TATC	11q25	130.338 Mb	0.730	
D4S2408	ATCT	4p15.1	30.981 Mb	0.722	
D18S853	ATA	18p11.31	3.981 Mb	0.711	
D20S1082	ATA	20q13.2	53.299 Mb	0.696	
D14S1434	[CTGT][CTAT]	14q32.13	93.298 Mb	0.696	
D20S482	AGAT	20p13	4.454 Mb	0.691	
D1GATA113	GATA	1p36.23	7.377 Mb	0.668	
D8S1115	ATT	8p11.21	42.656 Mb	0.663	
D17S1301	AGAT	17q25.1	70.193 Mb	0.649	
D4S2364	ATTC	4q22.3	93.976 Mb	0.511	

*n = 648 - 664 samples

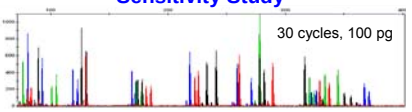
Top 5 Het. Rankings

Multiplex Design Challenges

- There were multiple iterations (as many as 11 different versions) of the multiplex design
- Problematic primer sets were defined if the following occurred:
  - Non-specific products
  - Presence of artifacts
  - Incomplete adenylation
- Primers must be completely redesigned, often times in different size ranges, or modified to have the locus amplify in multiplex:
  - A "pigtail" (GTTTCTT) was added to the 5' end to help promote adenylation (D1S1677, D3S3053, D11S4463, and D12ATA63)



Sensitivity Study



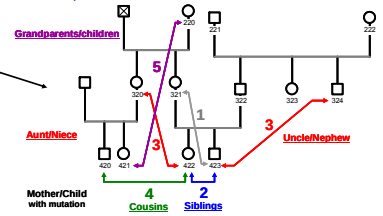
- A highly characterized sample was used for this study at a wide range of concentrations (conc.):
  - **2 ng, 1 ng, 750 pg, 500 pg, 400 pg, 300 pg, 250 pg, 200 pg, 100 pg, 50 pg, and 25 pg**
- 3 different PCR cycles were tested:
  - **28, 30, and 32 cycles**
- Ideal DNA concentration and cycling:
 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

Extended Family Study*

Testing Multiple Familial Relationships

*These studies were performed with a 23plex (22 autosomal loci + amelogenin). The 3 remaining loci of the 26plex were added after these evaluations.

Example



Comparison of Likelihood Ratios (LR)

Relationship Examined		15 STRs (Identifiler, ID15)	ID15 + Autoplex 22 STRs = 37 loci (A37)
Mother/Child* (*with single mutation)	1	0.214	**5,200,000**
Siblings	2	477	**113,000**
Uncle/Nephew	3	824	**247,000**
Cousins	4	0.45	**2.25**
Grandparents/ Grandchildren	5	0.53	**1.42**

LR calculations performed by Tom Reid at DNA Diagnostics Center

- Additional autosomal STR loci can be beneficial in close family relationships. See examples 1 - 3
- Longer distance multi-generational questions cannot always be solved with extra autosomal loci. See examples 4 & 5

Updates to U.S. NIST SRM 2391b

- Genotyping and sequencing have been performed with SRM 2391b components #1-12 for all 26 additional loci (using miniSTR primers)
- Certified and reference values have been assigned to all resulting alleles
- The Certificate of Analysis has been updated for all 26 loci
- 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

Poster available for download from STRBase: http://www.cstl.nist.gov/biotech/strbase/pub_pres/Hill_Ancona2008poster.pdf

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