STRs, CE, and Mixtures

Florida Statewide DNA Training

Indian Rocks Beach (Largo), FL

May 12-13, 2008

Dr. John M. Butler
National Institute of Standards and Technology

john.butler@nist.gov

Topics and Techniques for Forensic DNA Analysis

Introductions

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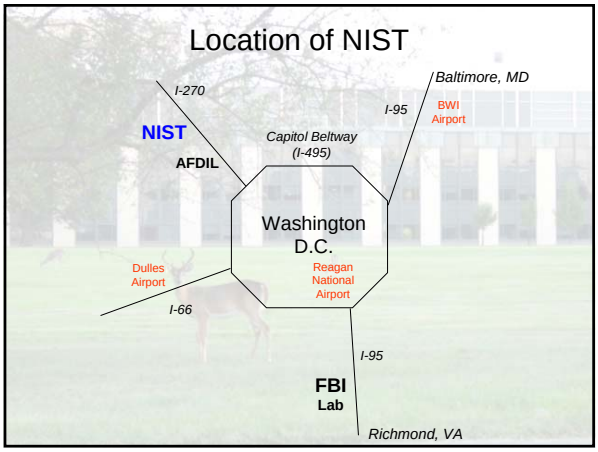
NIST History and Mission

- National Institute of Standards and Technology (NIST) was created in 1901 as the National Bureau of Standards (NBS). The name was changed to NIST in 1988.
- NIST is **part of the U.S. Department of Commerce** with a mission to develop and promote measurement, standards, and technology to enhance productivity, facilitate trade, and improve the quality of life.
- NIST supplies over 1,300 Standard Reference Materials (SRMs) for industry, academia, and government **use in calibration of measurements**.
- **NIST defines time for the U.S.**

$603 for 3 jars


DNA typing standard

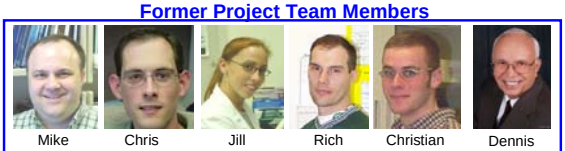
Location of NIST



NIST Human Identity Project Team

John Butler | Margaret Kline | Jan Redman | Pete Vallone | Becky Hill | Amy Decker | Dave Duewer

Former Project Team Members



Mike Coble — AFDIL
Chris DeAngelis — Medical School
Jill Appleby — NC SBI
Rich Schoske — Air Force
Christian Ruitberg — Pharma
Dennis Reeder — Retired/ABI

National Institute of Justice

The Research, Development, and Evaluation Agency of the U.S. Department of Justice

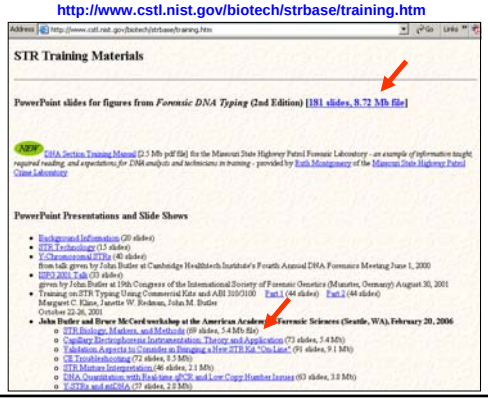
Current Areas of NIST Effort with Forensic DNA

- **Standards**
  - Standard Reference Materials
  - Standard Information Resources (STRBase website)
  - Interlaboratory Studies
- **Technology**
  - Research programs in SNPs, miniSTRs, Y-STRs, mtDNA, qPCR
  - 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

Training Materials Available on STRBase

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



Contributors to These Workshop Slides

Bruce McCord

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miniSTRs

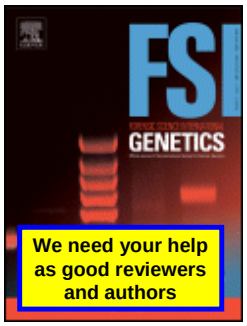
Angie Dolph

Marshall U./ NIST

mixtures

Forensic Science International: Genetics

http://www.fsigenetics.com/



Editor-in-Chief:
Angel Carracedo (Spain)
Associate Editors:
Peter M. Schneider (Germany)
John M. Butler (USA)

FSI: Genetics is a new journal dedicated exclusively to the field of forensic genetics. It has been launched in 2007 by Elsevier Publishers in affiliation with the International Society of Forensic Genetics. **All members of the ISFG receive a free subscription of this journal** (print and online version) as part of their membership benefits.

Primary Sources for Material Covered in this Workshop

- Butler, J.M., Buel, E., Crivellente, F., and McCord, B.R. (2004) Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis. *Electrophoresis* 25: 1397-1412
- Butler, J.M. (2006) Genetics and genomics of core STR loci used in human identity testing. *J. Forensic Sci.* 51(2): 253-265
- McCord, B. (2003) Troubleshooting capillary electrophoresis systems. *Profiles in DNA* 6(2): 10-12 (Promega Corporation); available at http://www.promega.com/profiles/602/ProfilesInDNA_602_10.pdf
- Butler, J.M. (2005) *Forensic DNA Typing, 2nd Edition: Biology, Technology, and Genetics of STR Markers*. Elsevier Science/Academic Press
- NIST STRBase website: http://www.cstl.nist.gov/biotech/strbase/

These workshop materials will be made available at http://www.cstl.nist.gov/biotech/strbase/training.htm

Outline for Workshop

Day 1

- STRs and Artifacts
- miniSTRs
- CE Troubleshooting

LUNCH

- Dawn Herkenham (Legal Issues)

Day 2

- Mixture Interpretation
- Mixture Examples

LUNCH

- Mixture Stats
- Interlab Studies
- Company presentations

My Goal is to Answer YOUR Questions – So Please Ask Them…

Understanding the Audience Here

- Where is everyone from?
  - State lab?
  - Local lab?
  - Private lab?
- Experience level?
  - Less than 1 year?
  - 1-3 years?
  - >3 years?
- STR kits in use?
  - Profiler Plus/COfiler
  - Identifiler
  - PowerPlex 16
  - Y-STRs?
- Instrumentation is use?
  - ABI 310
  - ABI 3100/3130xl
  - Other?
- Software in use?
  - GeneScan/Genotyper
  - GeneMapperID
  - Other?

NIST and NIJ 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 mine and do not necessarily represent the official position or policies of the US Department of Justice or the National Institute of Standards and Technology.

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

Topics and Techniques for Forensic DNA Analysis

STRs and Molecular Biology Artifacts

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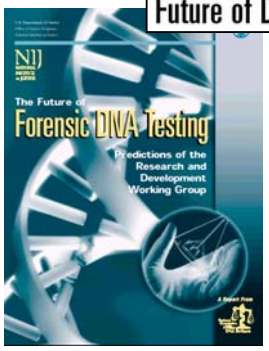
National Commission on the Future of DNA Evidence



http://www.ojp.usdoj.gov/nij/pubs-sum/183697.htm

- Report published in Nov 2000
- Asked to estimate where DNA testing would be 2, 5, and 10 years into the future

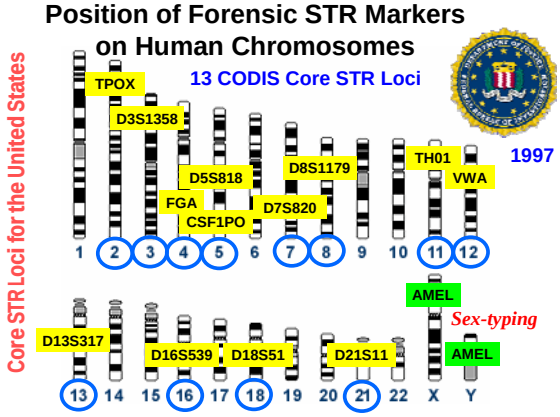
Conclusions

STR typing is here to stay for a few years because of DNA databases that have grown to contain millions of profiles

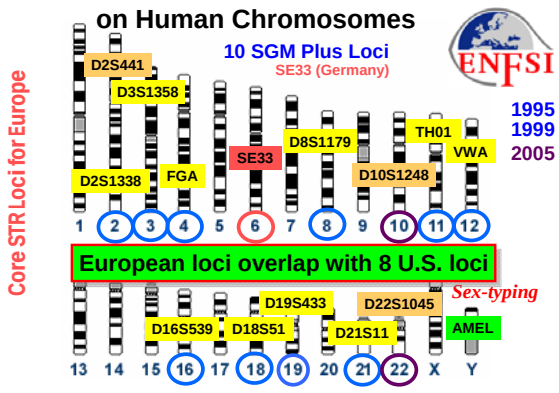
Advantages for STR Markers

- Small product sizes are generally compatible with degraded DNA and PCR enables recovery of information from small amounts of material
- Multiplex amplification with fluorescence detection enables high power of discrimination in a single test
- Commercially available in an easy to use kit format
- Uniform set of core STR loci provide capability for national and international sharing of criminal DNA profiles

Position of Forensic STR Markers on Human Chromosomes



Position of Forensic STR Markers on Human Chromosomes



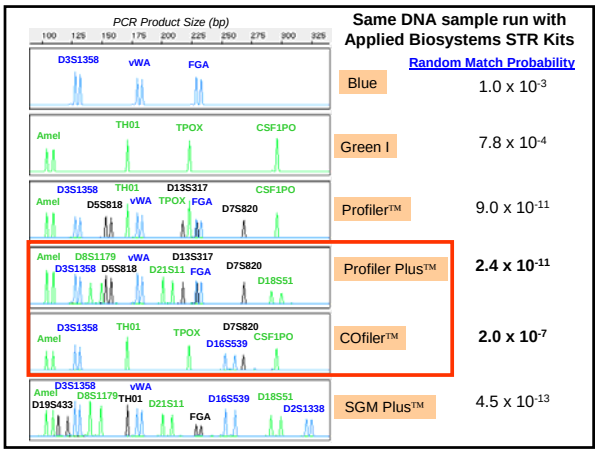
Value of STR Kits

Advantages

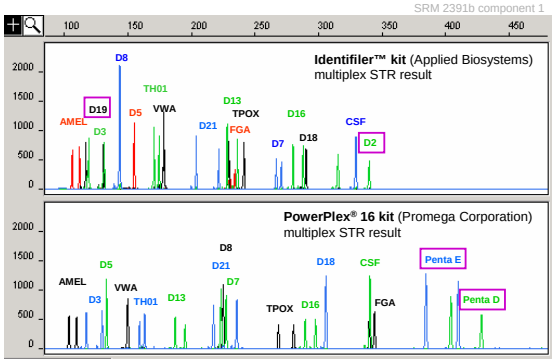
- Quality control of materials is in the hands of the manufacturer (saves time for the end-user)
- Improves consistency in results across laboratories – same allelic ladders used
- Common loci and PCR conditions used – aids DNA databasing efforts
- Simpler for the user to obtain results

Disadvantages

- Contents may not be completely known to the user (e.g., primer sequences)
- Higher cost to obtain results



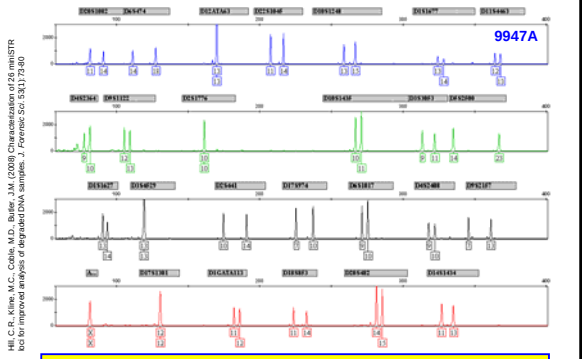
Commercial STR 16plex Kits



From 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.*

NIST "Autoplex" (26plex)

See Hill et al. AAFS 2008 talk (Washington, DC) and poster PP50 at DNA in Forensics 2008 meeting (Ancona)



Gender identification + 25 autosomal STR loci in a single amplification

How many STRs in the human genome?

- The efforts of the Human Genome Project have increased knowledge regarding the human genome, and hence there are many more STR loci available now than there were 10 years ago when the 13 CODIS core loci were selected.
- **More than 20,000 tetranucleotide STR loci have been characterized in the human genome** (Collins et al. An exhaustive DNA micro-satellite map of the human genome using high performance computing. Genomics 2003;82:10-19)
- There may be more than a million STR loci present depending on how they are counted (Ellegren H. Microsatellites: simple sequences with complex evolution. Nature Rev Genet 2004;5:435-445).
- STR sequences account for approximately 3% of the total human genome (Lander et al. Initial sequencing and analysis of the human genome. Nature 2001;409:860-921).

Butler, J.M. (2006) Genetics and genomics of core STR loci used in human identity testing. *J. Forensic Sci.* 51(2): 253-265.

Types of STR Repeat Units

Requires size based DNA separation to resolve different alleles from one another

- **Di**nucleotide (CA)(CA)(CA)(CA)
- **Tri**nucleotide (GCC)(GCC)(GCC)
- **Tetra**nucleotide (AATG)(AATG)(AATG)
- **Penta**nucleotide (AGAAA)(AGAAA)
- **Hexa**nucleotide (AGTACA)(AGTACA)

Short tandem repeat (STR) = microsatellite = simple sequence repeat (SSR)

Categories for STR Markers

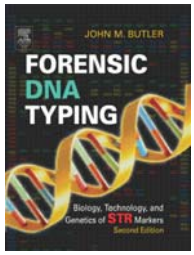
Category	Example Repeat Structure	13 CODIS Loci
Simple repeats – contain units of identical length and sequence	(GATA)(GATA)(GATA)	TPOX, CSF1PO, D5S818, D13S317, D16S539
Simple repeats with non-consensus alleles (e.g., TH01 9.3)	(GATA)(GAT-)(GATA)	TH01, D18S51, D7S820
Compound repeats – comprise two or more adjacent simple repeats	(GATA)(GATA)(GACA)	VWA, FGA, D3S1358, D8S1179
Complex repeats – contain several repeat blocks of variable unit length	(GATA)(GACA)(CA)(CATA)	D21S11

These categories were first described by **Urquhart *et al.* (1994) *Int. J. Legal Med.* 107:13-20**

Biological "Artifacts" of STR Markers

- Stutter Products
- Non-template nucleotide addition
- Microvariants
- Tri-allelic patterns
- Null alleles
- Mutations

Chapter 6 covers these topics in detail



Stutter Products

- Peaks that show up primarily one repeat less than the true allele as a result of strand slippage during DNA synthesis
- Stutter is less pronounced with larger repeat unit sizes (dinucleotides > tri- > tetra- > penta-)
- Longer repeat regions generate more stutter
- Each successive stutter product is less intense (allele > repeat-1 > repeat-2)
- Stutter peaks make mixture analysis more difficult

STR Alleles with Stutter Products

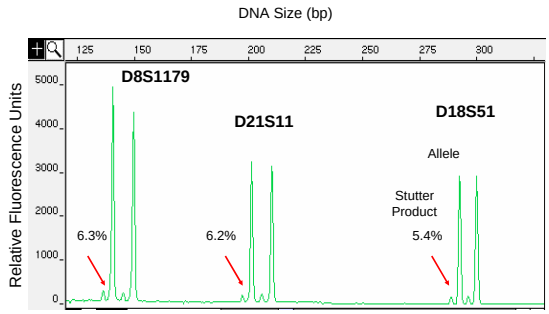
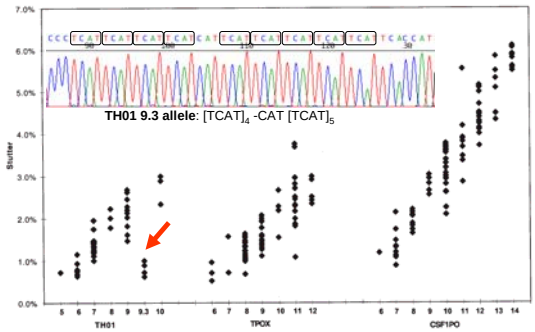


Figure 6.1, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

Measured Stutter Percentages

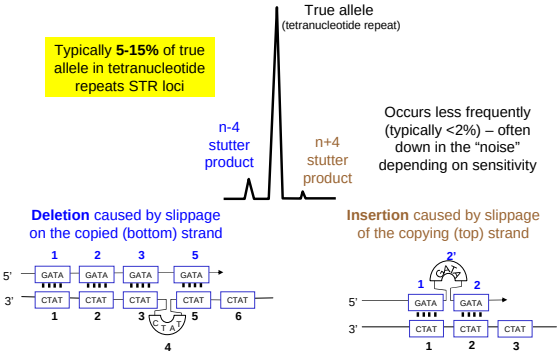
Variable by Allele Length and Composition



Holt CL, Buoncristiani M, Wallin JM, Nguyen T, Lazaruk KD, Walsh PS. TWGDAM validation of AmpFISTR PCR amplification kits for forensic DNA casework. *J Forensic Sci* 2002; 47(1): 66-96.

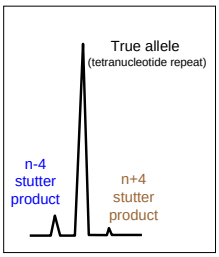
Stutter Product Formation

Repeat unit bulges out when strand breathing occurs during replication



N+4 Stutter Evaluation Summaries

- **Mass State Police DNA Lab**
- **Trying to collect data from as many laboratories as possible** to characterize N + 4 stutter percentages in various platforms.
- Please email information to **rebecca.post@pol.state.ma.us**



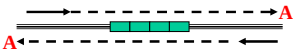
N-4 Stutter % of	main allele: allele	main allele: rfu	N+4: 'allele'	N+4: rfu	N+4 Stutter % of
6.43%	19	4664	20	57	1.22%

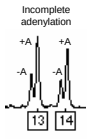
http://www.cstl.nist.gov/biotech/strbase/validation/N+4_stutter_spreadsheet.xls

Non-Template Addition

- Taq polymerase will often add an extra nucleotide to the end of a PCR product; most often an "A" (termed "adenylation")
- **Dependent on 5'-end of the reverse primer; a "G" can be put at the end of a primer to promote non-template addition**
- Can be enhanced with extension soak at the end of the PCR cycle (e.g., 15-45 min @ 60 or 72 ºC) – to give polymerase more time
- Excess amounts of DNA template in the PCR reaction can result in incomplete adenylation (not enough polymerase to go around)

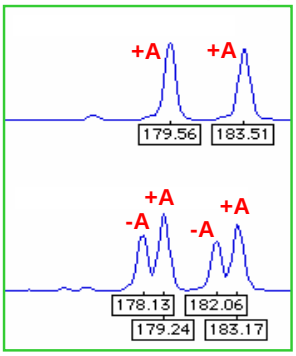
Best if there is NOT a mixture of "+/- A" peaks
(desirable to have full adenylation to avoid split peaks)





D8S1179

Impact of the 5' Nucleotide on Non-Template Addition



5'-ACAAG…

Last Base for Primer Opposite Dye Label

(PCR conditions are the same for these two samples)

5'-CCAAG…

Promega includes an ATT sequence on the 5'-end of many of their unlabeled PP16 primers to promote adenylation
see Krenke *et al.* (2002) *J. Forensic Sci.* 47(4): 773-785
http://www.cstl.nist.gov/biotech/strbase/PP16primers.htm

Higher Levels of DNA Lead to Incomplete Adenylation

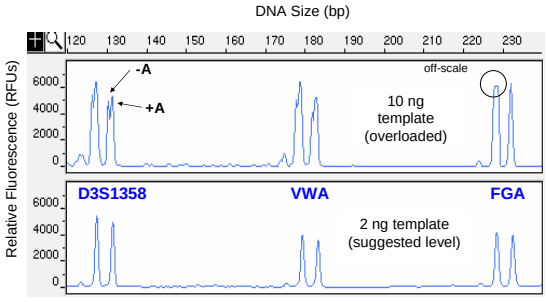
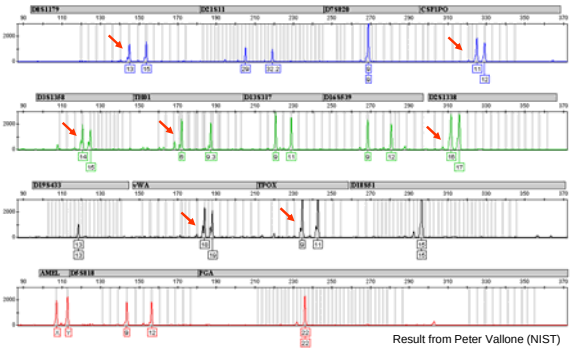


Figure 6.5, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

Identifiler – Rapid PCR (36 min total time)

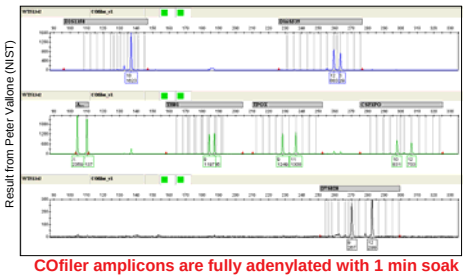
with 1 min 60 ºC adenylation soak (using different polymerases)



Result from Peter Vallone (NIST)

Rapid PCR Work and Adenylation

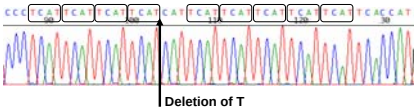
- **Poor adenylation (presence of –A peaks) is locus-specific and impacted by number of loci amplified**



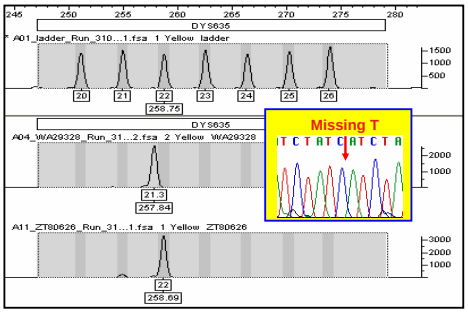
COfiler amplicons are fully adenylated with 1 min soak

Microvariant "Off-Ladder" Alleles

- Defined as alleles that are not exact multiples of the basic repeat motif or sequence variants of the repeat motif or both
- Alleles with partial repeat units are designated by the number of full repeats and then a decimal point followed by the number of bases in the partial repeat (Bar *et al. Int. J. Legal Med.* 1994, 107:159-160)
- Example: **TH01 9.3 allele**: $[TCAT]_4$ -CAT $[TCAT]_5$



An Example of an "Off-Ladder" Microvariant at the Yfiler Locus DYS635

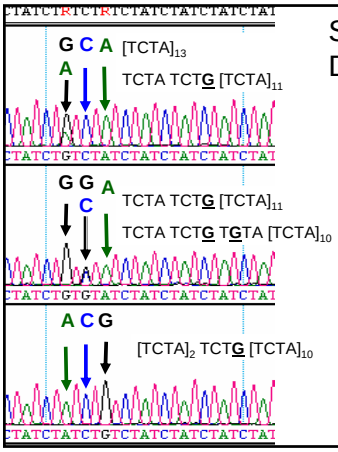


Allele 22 bin
258.75 +/- 0.5 = 258.25 to 259.25

Allele 21.3
257.84
(-0.41 from bin)

$[TCTA]_4(TGTA)_2[TCTA]_2(TGTA)_2[TCTA]_2(TGTA)_2[TCTA]_5$ TC-A $[TCTA]_2$

SNPs within the D8S1179 repeat



Repeat is TCTA
Three NIST samples have genotypes 13,13.

Analysis by Mass Spec indicates the presence of SNPs (Tom Hall, IBIS)
Confirmation of the Mass Spec by sequencing at NIST indicates:

There are **4** different 13 alleles in these 3 samples.

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

Lab Resources and Tools

- Addresses for scientists working with STRs
- Training Materials
- STR Allele Sequencing

STRbase has a summary of alleles that have been submitted and sequenced, **if** the submitting agency agrees to share the information.

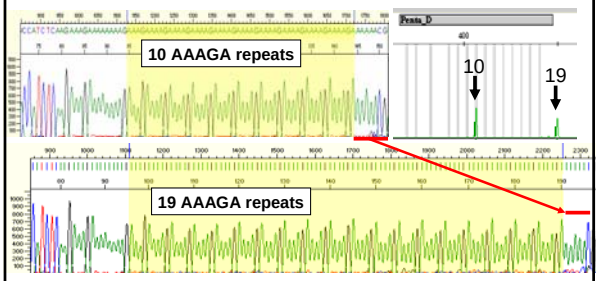
We require a minimum of 10 ng for the sequencing.
We request copies of the electropherograms demonstrating the variant allele.
The more information we have up front the better.
Please have patience we will get to your samples!

Sample Submissions

- For those that desire more assurances of confidentiality we can have MOUs signed.
- We generally re-type the samples at NIST prior to starting sequencing.
- We may run a monoplex assay (single locus).
- We return results as PowerPoint slides.
- We thank all of those agencies that have used this free service (thanks to NIJ)!

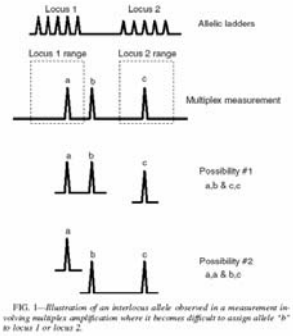
- Contact Margaret Kline: **margaret.kline@nist.gov**

Penta D 10, Variant Allele 19



All sequenced bases align before and after the repeat region. The 19 allele has been previously reported in STRBase. The Penta D ladder has Alleles 2.2, 3.2, 5, 7 – 17 represented.

Characterizing a Variant Allele That Occurs Between Two Loci

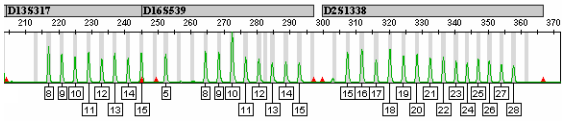


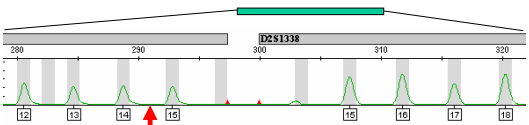
- Use a different multiplex STR kit with different locus combinations
- Test singleplex for each putative locus
- Example: Identifiler D16S539 and D2S1338

Butler, J.M. (2006) Genetics and genomics of core STR loci used in human identity testing. *J. Forensic Sci.* 51(2): 253-265

Steps to Detection
of Which Locus an Out-of-Range Allele Belongs With…

- Consider locus heterozygosities – heterozygote is likely from locus with higher heterozygosity (e.g., D16 = 0.766 while D2 = 0.882)
- Remember that tri-allelic patterns and homozygotes are less common than heterozygotes – thus two heterozygotes are more likely than a homozygote next to a tri-allelic pattern
- Check STRBase for variant alleles reported previously by other labs (e.g., D16 has no >16 alleles while D2 has several <15 alleles)
- Consider genotype frequencies observed for the various possible combinations (e.g., D16 11,11 = 10.7% while D2 20,20 = 0.92%)





D16S539
"14.2" = 291 bp

A state lab submitted to STRBase a new tri-allele:
D16S539 10, 12, 14.2 (Identifiler)

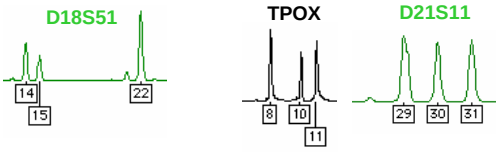
Likely a D2S1338 allele 11

D2S1338 alleles
11 = 291 bp
12 = 295 bp
13 = 299 bp
14 = 303 bp
15 = 307 bp

SWGDAM July 2007 (Doug Hares): search of NDIS for D16 tri-alleles with single D2 alleles found **25 profiles**

Three-Peak Patterns

Clayton *et al.* (2004) A genetic basis for anomalous band patterns encountered during DNA STR profiling. *J Forensic Sci.* 49(6):1207-1214



"Type 1"

Sum of heights of two of the peaks is equal to the third

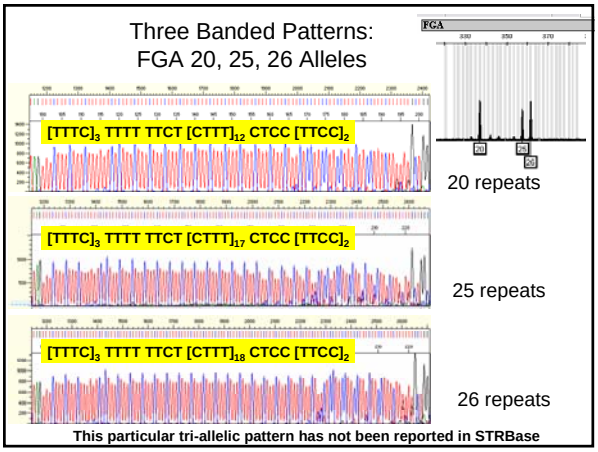
Most common in D18S51 and …..

"Type 2"

Balanced peak heights

Most common in TPOX and D21S11

Three Banded Patterns:
FGA 20, 25, 26 Alleles



This particular tri-allelic pattern has not been reported in STRBase

TPOX Tri-Allelic Patterns

FSI Genetics 2008; 2(2): 134–137

Available online at www.sciencedirect.com
ScienceDirect
Forensic Science International: Genetics 2 (2008) 134–137

The nature of tri-allelic TPOX genotypes in African populations

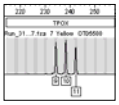
A.B. Lane

Division of Human Genetics, Room 212 James Gear Building, National Health Laboratory Service and University of the Witwatersrand, Corner of Hospital and De Korte Streets, Braamfontein, Johannesburg 2001, South Africa

Received 18 June 2007; received in revised form 8 October 2007; accepted 9 October 2007

Approximately 2.4% of indigenous South Africans have three rather than two TPOX alleles. Data collected during routine paternity testing revealed that **the extra allele is almost always allele 10** and that it segregates independently of those at the main TPOX locus. Approximately twice as many females as males have tri-allelic genotypes which suggested that **the extra allele is on an X chromosome.**

TPOX Tri-Allelic Patterns Reported on STRBase



http://www.cstl.nist.gov/biotech/strbase/var_TPOX.htm#Tri

- 6,8,**10** (4x)
- 6,9,**10** (5x)
- 6,**10**,11 (4x)
- 6,**10**,12 (1x)
- 7,8,**10** (2x)
- 7,9,**10** (1x)
- 7,**10**,11 (2x)
- 8,9,**10** (14x)
- **8,9,11 (1x)**
- 8,**10**,11 (19x)
- 8,**10**,12 (4x)
- **8,11,12 (3x)**
- 9,**10**,11 (11x)
- 9,**10**,12 (2x)
- **10**,**10**,11 (1x)
- **10**,11,12 (4x)

TPOX 10 freq
In NIST U.S. pop
Af Am 8.9%
Cau 5.6%
Hisp 3.2%

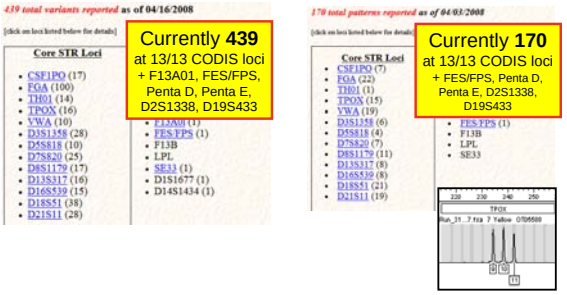
In 78 observations of 16 different TPOX tri-allelic patterns, only 4 times (5%) is allele "10" not present

Variant Alleles Cataloged in STRBase

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

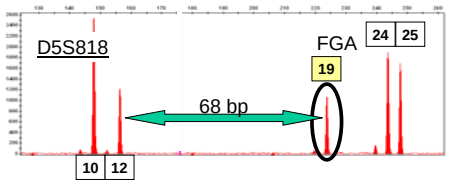
Off-Ladder Alleles

Tri-Allelic Patterns



Is this an FGA - Tri-allelic pattern identified using Identifiler?

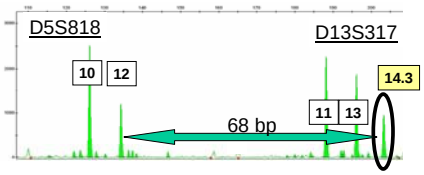
PK HT Ratio
12/10 - 0.48



PK HT Ratio
19/24 - 0.55
25/24 – 0.89

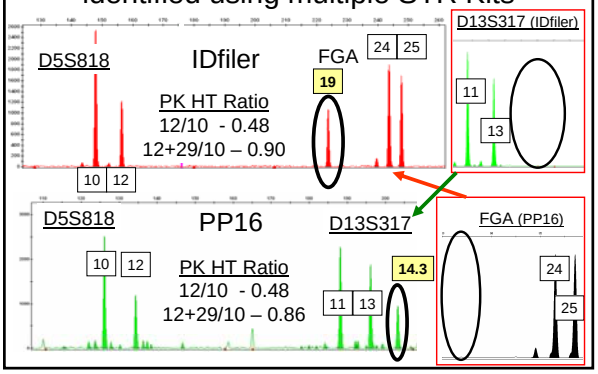
Or is this a D13S317 - Tri-allelic pattern identified using Powerplex 16?

PK HT Ratio
12/10 - 0.48

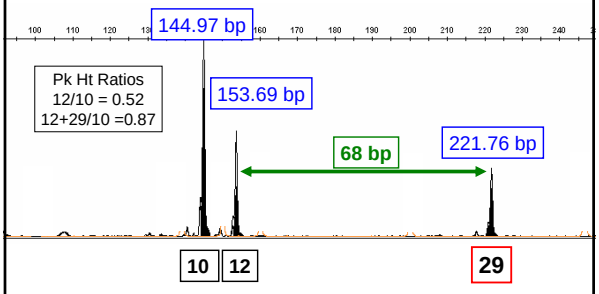


PK HT Ratio
13/11 - 0.83
14.3/11 – 0.42

It's really a D5S818 Tri-allelic pattern identified using multiple STR Kits

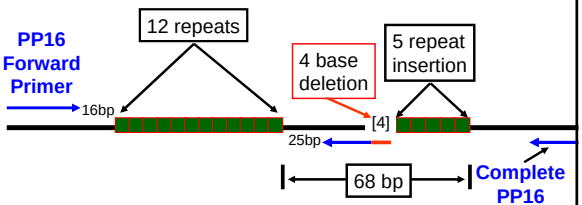


D5S818 monoplex results



The 68 bp size difference between the 12 allele and the variant allele sizing as an **"apparent 29"** allele.

D5S818 Apparent 29 Allele Sequencing Results



There is a 4 bp deletion, the last 4 bases of the PP16 reverse primer binding site, followed by an insertion of 5 repeats.
The 10 and 12 alleles of this sample have been sequenced and have the expected sequences.

Are there other large D5S818 alleles?

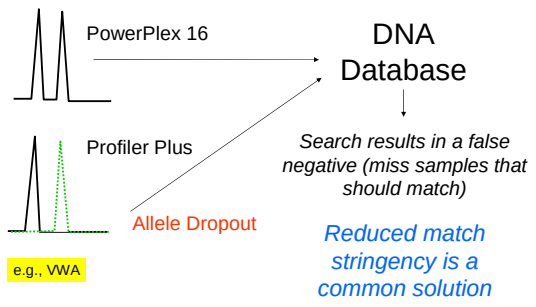
- STRBase Tri-allelic reports for FGA for 19,*,* patterns with AB amplification kits.
  - 5 reports :
  - 19,20,21; 19,20,23; 19,20,24; 19,22,23; 19,24,25
  - But there we have sequenced true tri-allelic FGA samples
- STRBase Tri-allelic reports for D13S317 for *,*, OL patterns with PP16 amplification kits.
  - NO tri-allelic patterns with Off-Ladder alleles reported

Null Alleles

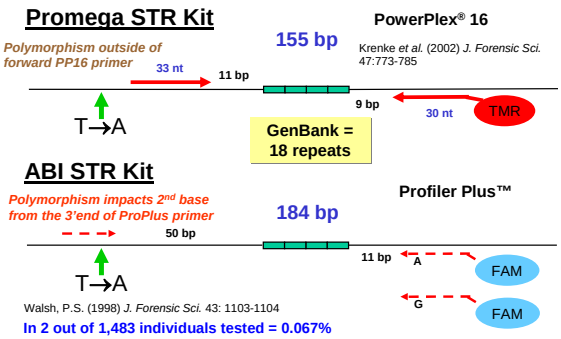
- Allele is present in the DNA sample but fails to be amplified due to a **nucleotide change in a primer binding site**
- Allele dropout is a problem because a heterozygous sample appears falsely as a homozygote
- Two PCR primer sets can yield different results on samples originating from the same source
- This phenomenon impacts DNA databases
- Large concordance studies are typically performed prior to use of new STR kits

For more information, see J.M. Butler (2005) *Forensic DNA Typing, 2nd Edition*, pp. 133-138

Concordance between STR primer sets is important for DNA databases



vWA Primer Position Comparisons



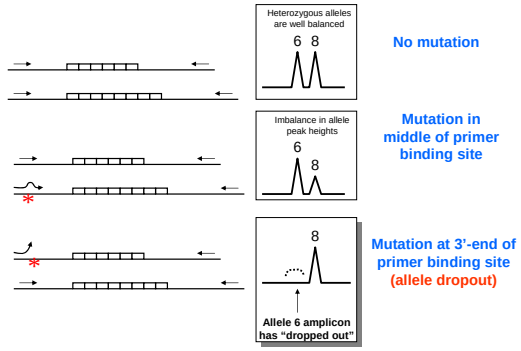
Krenke *et al.* (2002) *J. Forensic Sci.* 47:773-785

Walsh, P.S. (1998) *J. Forensic Sci.* 43: 1103-1104

In 2 out of 1,483 individuals tested = 0.067%

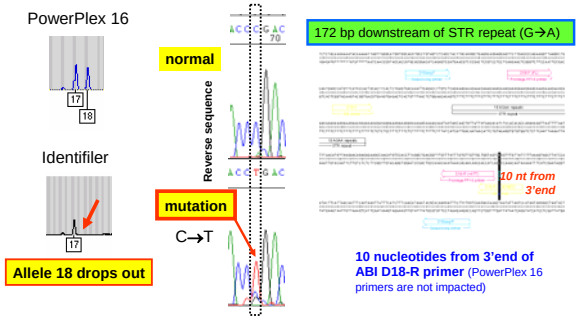
Lazaruk *et al.* (2001) *Forensic Sci Int.* 119:1-10

Impact of DNA Sequence Variation in the PCR Primer Binding Site



Butler, J.M. (2005) *Forensic DNA Typing, 2nd Edition,* Figure 6.9, ©Elsevier Academic Press

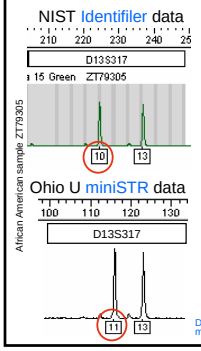
D18S51 Null Allele from Kuwait Samples with ABI Primers



Clayton et al. (2004) **Primer binding site mutations affecting the typing of STR loci contained within the AMPFlSTR SGM Plus kit.** *Forensic Sci Int.* 139(2-3): 255-259

D13S317 Flanking Region Deletion

A 4 bp deletion *outside* the miniSTR primers causes the commercial kit produced allele to appear one repeat smaller…



Sequence analysis identified two regions where 4 bp deletions occur to cause this 1 repeat variation

Drabek, J., Chung, D.T., Butler, J.M., McCord, B.R. (2004) Concordance study between miniplex STR assays and a commercial STR typing kit, *J. Forensic Sci.* 49(4): 859-860.

Apparent Null Alleles Observed During Concordance Studies

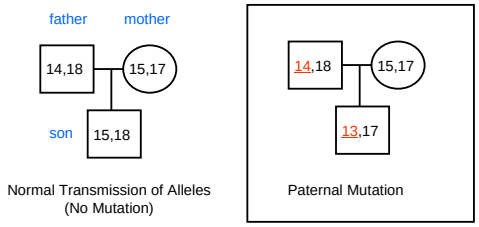
10/13 CODIS loci affected so far

New Section of STRBase (launched to track MiniFiler discordance and allele dropout frequency):
http://www.cstl.nist.gov/biotech/strbase/NullAlleles.htm

Locus	STR Kits/Assays	Results	Reference
D13S317	Identifiler vs miniplexes	Shift of alleles 10 and 11 due to deletion outside of **miniplex assay**	Butler et al. (2003), Drabek et al. (2004)
D16S539	PP1.1 vs PP16 vs COfiler	Loss of alleles with **PP1.1**; fine with PP16 and COfiler	Nelson et al. (2002)
D8S1179	PP16 vs ProPlus	Loss of alleles 15, 16, 17, and 18 with **ProPlus**; fine with PP16	Budowle et al. (2001)
FGA	PP16 vs ProPlus	Loss of allele 22 with **ProPlus**; fine with PP16	Budowle and Sprecher (2001)
D18S51	SGM vs SGM Plus	Loss of alleles 17, 18, 19, and 20 with **SGM Plus**; fine with SGM	Clayton et al. (2004)
CSF1PO	PP16 vs COfiler	Loss of allele 14 with **COfiler**; fine with PP16	Budowle et al. (2001)
TH01	PP16 vs COfiler	Loss of allele 9 with **COfiler**; fine with PP16	Budowle et al. (2001)
D21S11	PP16 vs ProPlus	Loss of allele 32.2 with **PP16**; fine with ProPlus	Budowle et al. (2001)

From Table 6.2 in J.M. Butler (2005) *Forensic DNA Typing, 2nd Edition*, p. 136

Mutation Observed in Family Trio



Butler, J.M. (2001) *Forensic DNA Typing*, Figure 6.9, ©Academic Press

STR Measured Mutation Rates

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

STR Locus	Maternal Meioses (%)	Paternal Meioses (%)	Either Parent	Total Mutations	Rate
CSF1PO	70/179,353 (0.04)	727/504,342 (0.14)	303	1,100/683,695	**0.16%**
FGA	134/238,378 (0.06)	1,481/473,924 (0.31)	495	2,110/712,302	**0.30%**
TH01	23/189,478 (0.01)	29/346,518 (0.008)	23	75/535,996	**0.01%**
TPOX	16/299,186 (0.005)	43/328,067 (0.01)	24	83/627,253	**0.01%**
VWA	133/400,560 (0.03)	907/646,851 (0.14)	628	1,668/1,047,411	**0.16%**
D3S1358	37/244,484 (0.02)	429/336,208 (0.13)	266	732/580,692	**0.13%**
D5S818	84/316,102 (0.03)	537/468,366 (0.11)	303	924/784,468	**0.12%**
D7S820	43/334,886 (0.01)	550/461,457 (0.12)	218	811/796,343	**0.10%**
D8S1179	54/237,235 (0.02)	396/264,350 (0.15)	225	675/501,585	**0.13%**
D13S317	142/348,395 (0.04)	608/435,530 (0.14)	402	1,152/783,925	**0.15%**
D16S539	77/300,742 (0.03)	350/317,146 (0.11)	256	683/617,888	**0.11%**
D18S51	83/130,206 (0.06)	623/278,098 (0.22)	330	1,036/408,304	**0.25%**
D21S11	284/258,795 (0.11)	454/306,198 (0.15)	423	1,161/564,993	**0.21%**
Penta D	12/18,701 (0.06)	10/15,088 (0.07)	21	43/33,789	**0.13%**
Penta E	22/39,121 (0.06)	58/44,152 (0.13)	55	135/83,273	**0.16%**
D2S1338	2/25,271 (0.008)	61/81,960 (0.07)	31	94/107,231	**0.09%**
D19S433	22/28,027 (0.08)	16/38,983 (0.04)	37	75/67,010	**0.11%**
F13A01	1/10,474 (0.01)	37/65,347 (0.06)	3	41/75,821	**0.05%**
FES/FPS	3/18,918 (0.02)	79/149,028 (0.05)	None reported	82/167,946	**0.05%**
F13B	2/13,157 (0.02)	8/27,183 (0.03)	1	11/40,340	**0.03%**
LPL	0/8,821 (<0.01)	9/16,943 (0.05)	4	13/25,764	**0.05%**
SE33 (ACTBP2)	0/330 (<0.30)	330/51,610 (0.64)	None reported	330/51,940	**0.64%**

13 CODIS core loci

*Data used with permission from American Association of Blood Banks (AABB) 2002 Annual Report.

Summary of STR Mutations

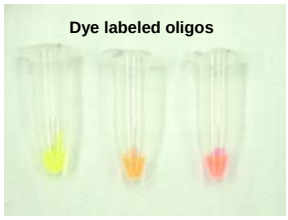
Mutations impact paternity testing and missing persons investigations but not forensic direct evidence-suspect matches…

- Mutations happen and need to be considered
- Usually 1 in ~1000 meioses
- Paternal normally higher than maternal
- VWA, FGA, and D18S51 have highest levels
- TH01, TPOX, and D16S539 have lowest levels

Primer Synthesis and Dye Blobs

- Oligonucleotide primers are synthesized from a 3'-to-5' direction on solid-phase supports using phosphoramidite chemistry
- The fluorescent dye is attached at 5'end of the primer (it is the last component added)
- The coupling reaction at each step of primer synthesis is not 100%, which can lead to some minor level impurities
- Left-over dye molecules that are not removed by post-synthesis purification can be carried through the PCR amplification step and injected onto the capillary to produce "dye blobs" or "dye artifacts" in CE electropherograms (wider than true allele peaks)

PCR Primer Quality Control

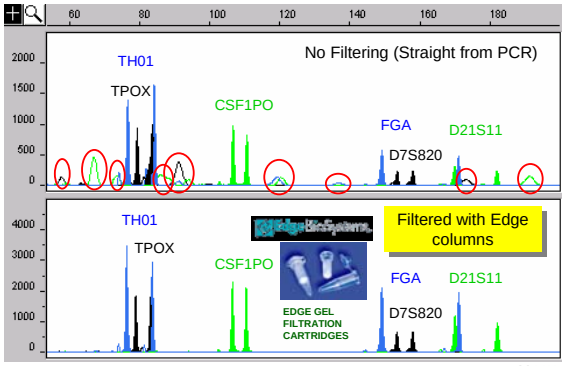


6FAM (yellow), **VIC** (orange), **NED** (red)

- UV Spec to determine concentration
- HPLC to evaluate purity
- TOF-MS to confirm correct sequence
- CE (ABI 310) to determine presence of residual dye molecules ("dye blobs")

Butler *et al.* (2001) *Forensic Sci. Int.* 119: 87-96

Problems with Dye Artifacts from Fluorescent Primers



STRBase

Short Tandem Repeat DNA Internet Database

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

General Information
- Intro to STRs (downloadable PowerPoint)
- STR Fact Sheets
- Sequence Information
- Multiplex STR Kits
- Variant Allele Reports
- Training Slides

Forensic Interest Data
- FBI CODIS Core Loci
- DAB Standards
- NIST SRMs 2391
- Published PCR Primers
- Y-Chromosome STRs
- Population Data
- Validation Studies
- miniSTRs

Supplemental Info
- Reference List **>3000**
- Technology Review
- Addresses for Scientists
- Links to Other Web Sites
- DNA Quantitation
- mtDNA
- New STRs

New information is added regularly…

Thank you for your attention…

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


Margaret Kline
STR allele sequencing


Jan Redman
Variant allele cataloging


Becky Hill
miniSTRs and 26plex work


Amy Decker
Y-STRs


Pete Vallone
Rapid PCR

http://www.cstl.nist.gov/biotech/strbase
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Mike Coble (AFDIL)

J Forensic Sci, March 2006, Vol. 51, No. 2
doi:10.1111/j.1556-4029.2006.00046.x
Available online at: www.blackwell-synergy.com

John M. Butler,[1] *Ph.D.*

Genetics and Genomics of Core Short Tandem Repeat Loci Used in Human Identity Testing

ABSTRACT: Over the past decade, the human identity testing community has settled on a set of core short tandem repeat (STR) loci that are widely used for DNA typing applications. A variety of commercial kits enable robust amplification of these core STR loci. A brief history is presented regarding the selection of core autosomal and Y-chromosomal STR markers. The physical location of each STR locus in the human genome is delineated and allele ranges and variants observed in human populations are summarized as are mutation rates observed from parentage testing. Internet resources for additional information on core STR loci are reviewed. Additional topics are also discussed, including potential linkage of STR loci to genetic disease-causing genes, probabilistic predictions of sample ethnicity, and desirable characteristics for additional STR loci that may be added in the future to the current core loci. These core STR loci, which form the basis for DNA databases worldwide, will continue to play an important role in forensic science for many years to come.

KEYWORDS: forensic science, DNA typing, short tandem repeat, mutation rate, CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, D2S1338, D19S433, Penta D, Penta E, SE33, CODIS, national DNA databases, Y-STR, Y-chromosome, DYS19, DYS385, DYS389I/II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS635, Y-GATA-H4

It has been almost a decade since the 13 genetic markers that form the core of the FBI Laboratory's Combined DNA Index System (CODIS) were selected in November 1997. Because of their use in the U.S. national DNA database (NDNAD) as well as other criminal justice databases around the world, these short tandem repeat (STR) loci dominate the genetic information that has been collected to date on human beings (1–3). In the U.S. and U.K. alone, more than 5 million profiles now exist in criminal justice DNA databases that contain information from these core loci or a subset (4,5). In addition, almost 1 million samples are run annually with core STR loci as part of parentage testing (6).

The 13 CODIS loci used in the U.S. are CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11 (7). The U.K. and much of Europe utilize 10 core loci that include the additional markers D2S1338 and D19S433 along with eight overlapping loci FGA, TH01, VWA, D3S1358, D8S1179, D16S539, D18S51, and D21S11. These loci have become the common currency of data exchange for human identity testing both in forensic casework and paternity testing largely because of their ease of use in the form of commercial STR kits. Missing persons investigations and mass disaster victim identification typically also involve the same STR markers and kits (8,9).

This review article describes what has been learned over the past few years about these commonly used STR markers in terms of their population genetic variation and genomic locations. Their precise chromosomal information only recently became available with the completion of the Human Genome Project. The past few years have seen over a 1000 population studies performed—usually with data included in the FOR THE RECORD section of the *Journal of Forensic Sciences* or an Announcement of Population Data in *Forensic Science International*. Available Internet resources for further information on these commonly used STR markers are reviewed. In addition, controversial issues such as potential disease gene linkage and probabilistic predictions of sample ethnicity are discussed. Finally, commonly used Y-chromosome STR loci are briefly reviewed.

Historical Perspective on STR Marker Selection

STR markers were first described as effective tools for human identity testing in the early 1990s (10,11). The Forensic Science Service (FSS) began to aggressively search for new loci and study population variation with a number of STR candidates (12). The Royal Canadian Mounted Police (RCMP) also contributed to early efforts with STR typing (13) along with a number of European labs. The first FSS multiplex applied to forensic casework included the four loci TH01, VWA, FES/FPS, and F13A1 (14). A second generation multiplex (SGM) followed with the loci TH01, VWA, FGA, D8S1179, D18S51, and D21S11 (15). The U.K. NDNAD was launched in April 1995 utilizing the SGM loci and the amelogenin sex-typing test (16).

Seeing the promise of STR typing technology and the success being obtained in the U.K., the FBI Laboratory led U.S. efforts to establish core STR loci that would form the backbone of CODIS, the U.S. national database system. Fueled through funding provided by the Congressional DNA Identification Act of 1994, a community-wide STR Project was launched in April 1996 (7).

[1]National Institute of Standards and Technology, Gaithersburg, MD 20899-8311.

 Sources of Support: National Institute of Justice funded this work in part through interagency agreement 2003-IJ-R-029 with the NIST Office of Law Enforcement Standards.

Received 25 April 2005; and in revised form 31 Aug. 2005; accepted 1 Oct. 2005; published 9 Feb. 2006.

This project, which lasted for approximately 18 months, involved 22 DNA typing laboratories that collectively evaluated 17 candidate loci, which were available as commercial or preliminary kits from either Promega Corporation (Madison, WI) or Applied Biosystems (Foster City, CA). Performance studies and protocol evaluations were performed, population databases were established, and forensic validation was conducted on the various STR systems investigated. While early work with STRs involved detection on silver-stained polyacrylamide gels (17), the community has embraced fluorescence detection methods involving first gel electrophoresis (10,12,13) and then capillary electrophoresis with such instruments as the ABI 310 and ABI 3100 Genetic Analyzers (18). Over the years, the ABI 373 and 377 gel-based DNA sequencers have also played a significant role in forensic DNA typing (19).

For the STR Project, Promega Corporation provided F13A1, F13B, FES/FPS, and LPL as part of an "FFFL" multiplex and CSF1PO, TPOX, TH01, VWA, D16S539, D7S820, D13S317, and D5S818 as part of the PowerPlex kit (20). Applied Biosystems had the AmpFlSTR Blue kit consisting of D3S1358, VWA, and FGA and the AmpFlSTR Green I kit with TH01, TPOX, CSF1PO, and the sex-typing system amelogenin. AmpFlSTR Yellow multiplex with D5S818, D13S317, and D7S820 along with the AmpFlSTR Green II multiplex consisting of D8S1179, D21S11, D18S51, and amelogenin were also made available to participants in the STR evaluation project. Eventually AmpFlSTR Blue, Green I, and Yellow were combined to form the AmpFlSTR Profiler kit, and the Blue, Green II, and Yellow loci were eventually combined to create the AmpFlSTR Profiler Plus kit (21,22).

At the STR Project meeting held on November 13–14, 1997, the 13 STR loci were announced as the core CODIS markers required for the U.S. national database (7). In the late 1990s, Applied Biosystems began providing the Profiler Plus and COfiler kits to enable coverage of the 13 core loci for use on their instrument platforms (22). Promega Corporation developed the PowerPlex 2.1 kit to cover the additional loci not present in their PowerPlex 1.1 kit for use on the FMBIO detection platform (1,20,23).

Table 1 summarizes the various STR kits that have become available in the past decade. Since the turn of the century, new multiplex assays have been developed that amplify all 13 CODIS core loci in a single reaction. The PowerPlex 16 kit, which was released by the Promega Corporation in May 2000, amplifies the 13 core loci, amelogenin, and two pentanucleotide loci referred to as Penta D and Penta E (24). Applied Biosystems released their 16plex Identifiler kit in July 2001, which amplifies the 13 core loci, amelogenin, and two tetranucleotide loci D2S1338 and D19S433 (25).

The Penta loci were discovered and characterized by Promega scientists in an effort to find loci with high variability yet exhibiting low amounts of stutter product formation (26,27). Although Penta D and Penta E are not officially required loci for any NDNADs, they are considered as "core loci" for the purposes of this paper because of their presence in widely used commercial STR kits. The D2S1338 and D19S433 STR markers were identified in searches for new tetranucleotide loci in the late 1990s (28,29). The extra two STR loci in the PowerPlex 16 and Identifiler kits provide an increased power of discrimination and enable improved mixture interpretation (in the case of the low stutter penta loci) or increased overlap with European STR systems (in the case of D2S1338 and D19S433).

After it became available in 1999, the U.K. and much of Europe adopted a commercial STR kit from Applied Biosystems known as SGM Plus, which contains the original SGM loci and amelogenin plus D3S1358, D16S539, D2S1338, and D19S433 (30). When Germany established its NDNAD in 1998, the highly polymorphic STR locus SE33 (also known as ACTBP2) was included as a core locus because of its previous use in casework applications (31,32). Both Promega Corporation and Applied Biosystems (along with several German companies) now supply kits that include SE33 (Table 1).

TABLE 1—*Summary of available commercial STR kits that are commonly used.*

Kit Name	STR Loci Included	Random Match Probability with Author's Profile*
	Promega Corporation	
PowerPlex 1.1 and 1.2	CSF1PO, TPOX, TH01, VWA, D16S539, D13S317, D7S820, D5S818	7.4×10^{-10}
PowerPlex 2.1 (for Hitachi FMBIO users)	D3S1358, TH01, D21S11, D18S51, VWA, D8S1179, TPOX, FGA, Penta E	3.4×10^{-11}
PowerPlex ES	FGA, TH01, VWA, D3S1358, D8S1179, D18S51, D21S11, SE33, amelogenin	1.3×10^{-10}
PowerPlex 16	CSF1PO, FGA, TPOX, TH01, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, Penta D, Penta E, amelogenin	1.2×10^{-18}
PowerPlex 16 BIO (for Hitachi FMBIO users)	CSF1PO, FGA, TPOX, TH01, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, Penta D, Penta E, amelogenin	1.2×10^{-18}
	Applied Biosystems	
AmpFlSTR Blue	D3S1358, VWA, FGA	1.0×10^{-3}
AmpFlSTR Green I	Amelogenin, TH01, TPOX, CSF1PO	7.8×10^{-4}
AmpFlSTR Cofiler (CO)	D3S1358, D16S539, Amelogenin, TH01, TPOX, CSF1PO, D7S820	2.0×10^{-7}
AmpFlSTR Profiler Plus (Pro)	D3S1358, VWA, FGA, Amelogenin, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820	2.4×10^{-11}
AmpFlSTR Profiler Plus ID	D3S1358, VWA, FGA, Amelogenin, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820 (extra unlabeled D8-R primer)	2.4×10^{-11}
AmpFlSTR Profiler	D3S1358, VWA, FGA, Amelogenin, TH01, TPOX, CSF1PO, D5S818, D13S317, D7S820	9.0×10^{-11}
AmpFlSTR SGM Plus (SGM)	D3S1358, VWA, D16S539, D2S1338, Amelogenin, D8S1179, D21S11, D18S51, D19S433, TH01, FGA	4.5×10^{-13}
AmpFlSTR Sefiler (SE)	FGA, TH01, VWA, D3S1358, D8S1179, D16S539, D18S51, D21S11, D2S1338, D19S433, SE33, amelogenin	5.1×10^{-15}
AmpFlSTR Identifiler (ID)	CSF1PO, FGA, TPOX, TH01, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, D2S1338, D19S433, amelogenin	7.2×10^{-19}

*Allele frequencies used for random match probability calculations (to unrelated individuals) from U.S. Caucasian population data associated with Butler et al. (37), Reid et al. (38), and Levadokou et al. (39). Subpopulation structure adjustments (θ corrections) were not made with these calculations (i.e., only p^2 and $2pq$ were used).

STR, short tandem repeat.

Thus, the creation of commercial STR kits has been historically driven by selection of loci that have become part of NDNADs. However, in some cases loci were selected for inclusion in databases based on which ones were available in commercial kits or already previously in use for forensic casework (e.g., SE33). It is also important to realize that patents play a role in the cost of STR kits and their commercial availability (33–36).

Locus Information

Information regarding the repeat structure and number of observed alleles for each core STR locus is available in Table 2. The first article describing each STR locus is also listed in Table 2 under the original reference column (40–55). Note that many of these loci were selected from genetic markers under evaluation by the Cooperative Human Linkage Center (CHLC) (see http://www.chlc.org).

The repeat motif for each STR marker is listed according to the International Society of Forensic Genetics (ISFG) recommendation that the repeat sequence motif be defined so that the first 5′-nucleotides on the GenBank forward strand define the repeat motif used (56). Observed allele ranges for each locus are also included in Table 2 along with PCR product sizes and dye labels for the various STR kits described in Table 1. It is important to remember that STR allele sizes are measured relative to an internal size standard during electrophoresis and, depending on the DNA strand that is dye labeled, may have a different apparent measured size than the actual DNA sequence (see (18)).

A detailed synopsis of each marker including the PCR product sizes generated with the various STR kits is available in Chapter 5 of *Forensic DNA Typing* (1). A full description of the allele range and number of alleles reported to date for each locus is contained in Appendix I of *Forensic DNA Typing* (1). Note that the most complex loci, D21S11 and SE33, contain a number of alleles with internal sequence variation that can only be fully appreciated through DNA sequence analysis of the STR repeat region. For example, Rolf et al. (57) found 102 different SE33 alleles upon sequencing a total of 33 different length variants.

Genomic Information

The Human Genome Project officially came to a successful completion in April 2003 with the announcement of a "finished" reference sequence of the human genome (58). However, the finished sequence continues to be refined and several compilations exist, which differ from one another. Using the BLAST-like alignment tool (BLAT) that is available at http://genome.ucsc.edu, each of the core STR loci has been located within the reference human genome sequence. Table 3 lists the 18 core loci in terms of their chromosomal locations. In addition, an evaluation of the physical position of these STR loci has been performed in the National Center for Biotechnology Information (NCBI) build 34 (July 2003) vs. NCBI build 35 (May 2004) versions of the human genome sequence. Reference sequences for the STR loci used for this BLAT search are available at http://www.cstl.nist.gov/biotech/strbase/seq_ref.htm. In some cases, the reverse complement of the GenBank accession sequence was used in order to have the forward strand possess the traditional repeat motif listed in Table 2.

With the exceptions noted below, the core loci are located on separate chromosomes and therefore expected to segregate independently of one another during meiosis. This independent segregation enables use of the product rule in estimating random match probabilities with DNA profiles generated from multiple STR loci (59). As can be seen in Table 3, CSF1PO and D5S818

TABLE 2—*Characteristics of alleles observed in 18 core STR loci. See Ref. 1, Appendix I for a more complete description of the number of alleles seen.*

Locus Name	First Reference	Repeat Motif ISFG format	Allele Range	Applied Biosystems STR Kits PCR Product Sizes (Dye Label)	Promega STR Kits PCR Product Sizes (Dye Label)	Number of Alleles Seen
CSF1PO	(40)	TAGA	5–16	CO: 276–320 bp (JOE) ID: 276–320 bp* (6FAM)	1.1: 287–331 bp (TMR) 16: 317–361 bp (JOE)	20
FGA	(41)	CTTT	12.2–51.2	Pro: 196–348 bp (5FAM) SGM/SE: 196–348 bp (NED) ID: 196–348 (PET)	2.1/16/ES: 308–464 bp (TMR)	80
TH01	(42)	TCAT	3–14	CO: 160–204 bp (JOE) SGM/SE: 160–204 bp (NED) ID: 160–204 bp (VIC)	1.1: 171–215 bp (TMR) 2.1/16/ES: 152–196 bp (FL)	20
TPOX	(43)	GAAT	4–16	CO: 209–257 bp (JOE) ID: 209–257 bp* (NED)	1.1: 216–264 bp (TMR) 2.1/16: 254–302 bp (TMR)	15
VWA	(44)	[TCTG][TCTA]	10–25	Pro/SGM: 152–212 bp (5FAM) ID: 152–212 bp (NED) SE: 152–212 bp (6FAM)	1.1/16/ES: 123–183 bp (TMR)	29
D3S1358	(45)	[TCTG][TCTA]	8–21	Pro/CO: 97–149 bp (5FAM) ID: 97–149 bp (VIC) SE: 97–149 bp (6FAM)	2.1/16/ES: 99–151 bp (FL)	25
D5S818	(46)	AGAT	7–18	Pro: 134–178 bp (NED) ID: 134–178 bp (PET)	1.1: 119–163 bp (FL) 16: 119–163 bp (JOE)	15
D7S820	(47)	GATA	5–16	Pro/CO: 253–297 bp (NED) ID: 253–297 bp (6FAM)	1.1: 211–255 bp (FL) 16: 211–255 bp (JOE)	30
D8S1179	(48)	[TCTA][TCTG]	7–20	Pro: 123–175 bp (JOE) ID: 123–175 bp (6FAM) SE: 123–175 bp (VIC)	2.1/16/ES: 203–255 bp (TMR)	15
D13S317	(49)	TATC	5–16	Pro: 193–237 bp (NED) ID: 193–237 bp* (VIC)	1.1: 157–201 bp (FL) 16: 157–201 bp (JOE)	17
D16S539	(50)	GATA	5–16	CO/SGM: 233–277 bp (5FAM) ID: 233–277 bp* (VIC) SE: 233–277 bp (6FAM)	1.1: 264–308 bp (FL) 16: 264–308 (JOE)	19
D18S51	(51)	AGAA	7–39.2	Pro/SGM: 264–394 (JOE) ID: 264–394 (NED) SE: 264–394 (PET)	2.1/16/ES: 286–416 bp (FL)	51
D21S11	(52)	Complex [TCTA][TCTG]	12–41.2	Pro/SGM: 138–256 bp (JOE) ID: 138–256 bp (6FAM) SE: 138–256 bp (PET)	2.1/16/ES: 155–273 bp (FL)	89
D2S1338	(53)	[TGCC][TTCC]	15–28	SGM: 289–341 bp (5FAM) ID: 289–341 bp* (VIC) SE: 289–341 bp (6FAM)	N/A	17
D19S433	(54)	AAGG	9–17.2	SGM/ID/SE: 106–140 bp (NED)	N/A	26
Penta D	(26)	AAAGA	2.2–17	N/A	16: 376–449 bp (JOE)	29
Penta E	(26)	AAAGA	5–24	N/A	2.1/16: 379–474 bp (FL)	33
SE33 (ACTBP2)	(55)	Complex AAAG	4.2–37	SE: 203–333 bp (VIC)	ES: 203–333 bp (JOE)	>100

Note that the more complex loci have internal variation, which can only be fully characterized with DNA sequence analysis. PCR product sizes are listed for the corresponding allele ranges and STR kits (see Table 1 for kit abbreviations) based on actual sequence length without any nontemplate addition, which may differ from observed size relative to an internal size standard. Five loci in the Identifiler STR kit are marked with an asterisk (*) since mobility modifiers have been added in order to adjust their apparent size during electrophoresis.

STR, short tandem repeat.

TABLE 3—*Genomic locations of core STR loci.*

Locus (UniSTS)	GenBank Accession (Allele Repeat #)	Chromosomal Location	Physical Position (July 2003; NCBI Build 34)	Physical Position (May 2004; NCBI Build 35)
TPOX (240638)	M68651 (11)	2p25.3 thyroid peroxidase, 10th intron	Chr 2 1.436 Mb	Chr 2 1.472 Mb
D2S1338 (30509)	AC010136 (20)	2q35	Chr 2 219.082 Mb	Chr 2 218.705 Mb
D3S1358 (148226)	AC099539 (16)	3p21.31	Chr 3 45.543 Mb	Chr 3 45.557 Mb
FGA (240635)	M64982 (21)	4q31.3 αfibrinogen, 3rd intron	Chr 4 156.086 Mb	Chr 4 155.866 Mb
D5S818 (54700)	AC008512 (11)	5q23.2	Chr 5 123.187 Mb	Chr 5 123.139 Mb
CSF1PO (156169)	X14720 (12)	5q33.1 c-fms proto-oncogene, 6th intron	Chr 5 149.484 Mb	Chr 5 149.436 Mb
SE33 (ACTBP2) (none reported)	V00481 (26.2)	6q14 β-actin related pseudogene	Chr 6 88.982 Mb	Chr 6 89.043 Mb
D7S820 (74895)	AC004848 (13)	7q21.11	Chr 7 83.401 Mb	Chr 7 83.433 Mb
D8S1179 (83408)	AF216671 (13)	8q24.13	Chr 8 125.863 Mb	Chr 8 125.976 Mb
TH01 (240639)	D00269 (9)	11p15.5 tyrosine hydroxylase, 1st intron	Chr 11 2.156 Mb	Chr11 2.149 Mb
VWA (240640)	M25858 (18)	12p13.31 von Willebrand Factor, 40th intron	Chr 12 19.826 Mb	Chr 12 5.963 Mb
D13S317 (7734)	AL353628 (11)	13q31.1	Chr 13 80.520 Mb	Chr 13 81.620 Mb
Penta E (none reported)	AC027004 (5)	15q26.2	Chr 15 95.104 Mb	Chr 15 95.175 Mb
D16S539 (45590)	AC024591 (11)	16q24.1	Chr 16 86.168 Mb	Chr 16 84.944 Mb
D18S51 (44409)	AP001534 (18)	18q21.33	Chr 18 59.098 Mb	Chr 18 59.100 Mb
D19S433 (33588)	AC008507 (16)	19q12	Chr 19 35.109 Mb	Chr 19 35.109 Mb
D21S11 (240642)	AP000433 (29)	21q21.1	Chr 21 19.476 Mb	Chr 21 19.476 Mb
Penta D (none reported)	AP001752 (13)	21q22.3	Chr 21 43.912 Mb	Chr 21 43.880 Mb

Results with two different builds of the human genome are shown in order to illustrate that the physical position within the reference genome may shift slightly as new information becomes available. UniSTS is a comprehensive database of sequence tagged sites (STSs) available on the NCBI Web site: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi? db, units.

STR, short tandem repeat.

are both found on chromosome 5 and are separated by approximately 26.3 megabases (Mb). Likewise, Penta D and D21 are both located on chromosome 21 separated by approximately 24.4 Mb. However, the occurrence of loci on the same chromosome that are many millions of base pairs apart should not impact reliable use of the product rule as even loci less than a million bases apart can be shuffled separately because of recombination hot spots and patterns of linkage disequilibrium (60). To date, hundreds of population studies involving D5S818 and CSF1PO (see, e.g., the listing at http://www.cstl.nist.gov/biotech/strbase/population/PopSurvey.htm) conducted on unrelated individuals have failed to show any signs of significant linkage between these two loci.

The amelogenin locus that occurs on both the X and Y chromosomes and enables sex typing (61) was also located within the reference human genome sequence. AMELX is located on the X chromosome at 10.676 Mb (July 2003) and 11.075 Mb (May 2004). AMELY is located on the Y chromosome at 6.441 Mb (July 2003) and 6.781 Mb (May 2004).

In addition to the determination of physical locations of various DNA sequences, the cytogenetic map has been integrated with the human genome sequence to permit a more precise approximation of band locations (62). Table 3 contains the most up-to-date chromosomal band locations available for the core loci. For example, TPOX is found at 2p25.3 rather than the previously used span of "2p23-2pter."

Population Variation

Allele Range and Variants

STR typing is typically performed using size comparisons with standardized allelic ladders that possess the most common alleles, which have been sequenced to reveal the true number of repeats (63). Different STR kit manufacturers may supply allelic ladders with slightly different allele ranges. Note that in Table 2 the observed allele ranges for the core loci are listed based on a review of the published literature rather than available allelic ladders (see also Appendix I in Ref. (1)).

As more samples are run with STR loci, new alleles are constantly being discovered that do not size exactly with the ladder alleles. These "off-ladder" alleles can be variants with more or less of the core repeat unit than present in the common alleles found in the commercially available allelic ladder. Alternatively, these variant alleles may contain partial repeats or insertions/deletions in the flanking region close to the repeat.

A good example of an insertion/deletion event that creates off-ladder alleles is found in D7S820, which can contain 8, 9, or 10 adjacent T nucleotides starting 12 nucleotides downstream of the GATA repeat (64). This flanking region insertion/deletion gives rise to the 9.1, 9.3, 10.1, 10.3, etc., alleles observed in D7S820 (Table 4). In addition, new alleles can be discovered that occur outside the range defined by the commercially available allelic ladder. In many instances, these alleles are simply classified as greater than the largest allele (or smaller than the smallest allele) in the ladder rather than attempting to extrapolate to a predicted number of repeats. Table 4 contains a list of variant or "off-ladder" alleles that have been reported to the NIST STRBase Web site as of April 2005.

Triallelic patterns have been observed for many of the core STR loci and recorded on the NIST STRBase Web site (Table 5). Clayton et al. (65) have described possible reasons for triallelic patterns, which can occur as an imbalance in amounts between the three alleles (type 1) or equal amounts of all three alleles (type 2). A type 1 tri-allelic pattern imbalance is typically a situation where the sum of the peak heights for two of the alleles is approximately equivalent to the third allele (65). It is interesting to note that TPOX, which occurs closest to the tip of a chromosome (see Table 3), has the highest number of observed tri-allelic patterns—most

TABLE 4–*Variant or "off-ladder" alleles reported in STRBase for commonly used STR loci.*

STR Locus	Number Reported	Variant Alleles Reported as of April 2005
CSF1PO	11	5, 7.3, 8.3, 9.1, 9.3, 10.1, 10.2, 10.3, 11.1, 12.1, 16
FGA	69	12.2, 13.2, 14, 14.3, 15, 15.3, 16, 16.1, 16.2, "< 17", 17, 17.2, 18.2, 19.1, 19.2, 19.3, 20.1, 20.2, 20.3, 21.1, 21.2, 21.3, 22.1, 22.2, 22.3, 23.1, 23.2, 23.3, 24.1, 24.2, 24.3, 25.1, 25.2, 25.3, 26.1, 26.2, 26.3, 27.3, 29.2, 30.2, 31, 31.2, 32.1, 32.2, 33.1, 34.1, 34.2, 35.2, 41.1, 41.2, 42.1, 42.2, 43.1, 43.2, 44, 44.1, 44.2, 44.3, 45.1, 45.2, 46.1, 46.2, 47.2, 48.2, 49, 49.1, 49.2, 50.2, 50.3
TH01	7	4, 7.3, 8.3, 9.1, 10.3, 11, 13.3
TPOX	7	4, 5, 7.3, 13.1, 14, 15, 16
VWA	6	16.1, 18.3, 22, 23, 24, 25
D3S1358	18	8, 8.3, 9, 10, 11, 15.1, 15.2, 15.3, 16.2, 17.1, 17.2, 18.1, 18.2, 18.3, ">19", 20, 20.1, 21.1
D5S818	5	10.1, 11.1, 12.3, 17, 18
D7S820	22	5, 5.2, 6.3, 7.1, 7.3, 8.1, 8.2, 8.3, 9.1, 9.2, 9.3, 10.1, 10.3, 11.1, 11.3, 12.1, 12.2, 12.3, 13.1, 14.1, 15, 16
D8S1179	4	7, 15.3, 18, 20
D13S317	10	5, 6, 7, 7.1, 8.1, 11.1, 11.3, 13.3, 14.3, 16
D16S539	10	6, 7, 9.3, 11.3, 12.1, 12.2, 13.1, 13.3, 14.3, 16
D18S51	30	7, 8, 9, 11.2, 12.2, 12.3, 13.1, 13.3, 14.2, 15.1, 15.2, 16.1, 16.2, 16.3, 17.2, 17.3, 18.1, 18.2, 19.2, 20.1, 20.2, 21.2, 22.1, 22.2, 23.2, 24.2, 27, 28.1, 28.3, 40
D21S11	24	24.3, 25.1, 25.2, 25.3, 26.2, 27.1, 27.2, 28.1, 28.3, 29.1, 29.3, 30.3, 31.1, 31.3, 32.1, 33.1, 34.1, 34.3, 35.1, 36.1, 36.2, 37, 37.2, 39
Penta D	14	6, 6.4, 7.1, 7.4, 9.4, 10.3, 11.1, 11.2, 12.2, 12.4, 13.2, 13.4, 14.1, 14.4
Penta E	13	9.4, 11.4, 12.1, 12.2, 13.2, 14.4, 15.2, 15.4, 16.4, 17.4, 18.4, 19.4, 23.4
D2S1338	3	13, 23.2, 23.3
D19S433	11	6.2, 7, 8, "< 9", 11.1, 12.1, 13.2, 18, 18.2, 19.2, 20
SE33	0	None reported yet in STRBase

These 264 alleles were as of April 2005. For up-to-date information, see http://www.cstl.nist.gov/biotech/strbase/var_tab.htm. Many of these variant alleles have been seen more than once. Note that some of these alleles may be present in allelic ladders from commercial kits not used by laboratories reporting these variants.
STR, short tandem repeat.

of which are type 2 with equal intensity alleles (Table 5). Thus, it is possible that this section of chromosome 2 is more likely to be duplicated in some individuals for telomere maintenance to keep the end of the chromosome intact (66,67).

Characterizing a Variant Allele That Occurs Between Two Loci

Occasionally a variant allele can occur with a size between two loci in a multiplex STR electropherogram making it difficult to assign the allele to the appropriate locus without further characterization, such as individual locus amplification (Fig. 1). Unfortunately, some manufacturers only provide STR kits in multiplex format preventing easy single locus amplification with the same PCR primers. However, a different STR kit, which has the loci assembled in a different configuration in terms of size and dye label (see Table 2), can be used in some cases to effectively assign an unusual allele to the appropriate locus. Alternatively, single STR locus PCR amplification primers are available from Promega Corporation or can be synthesized based on locus-specific information recorded in the STR Fact Sheets on the NIST STRBase Web site.

There are several points of consideration that can be made in order to help ascertain to which locus an extremely off-ladder and interlocus allele belongs. First, if one of the loci contains two alleles and the other one only one allele within the common allele range, then it is likely that the interlocus allele belongs to the apparent homozygote. It is also worth checking if any new variant alleles have been reported previously by other labs (see Table 4).

In a situation such as is illustrated in Fig. 1, where the sample has a locus1 with only an allele "a" and locus2 only has an allele "c" with an allele "b" occurring between the two loci, the possible

TABLE 5—*A total of 62 tri-allelic patterns observed and reported on STRBase (http://www.cstl.nist.gov/biotech/strbase/tri_tab.htm).*

STR Locus	Number Reported	Tri-Allelic Patterns Reported as of April 2005
CSF1PO	2	9/11/12; 10/11/12
FGA	10	19/20/21; 19/22/23; 19/24/25; 20/21/22; 20/21/24; 20/23/24; 21/22/23; 21/25/26; 22/24/25; 22.2/23/23.2
TH01	1	7/8/9
TPOX	13	6/8/10; 6/9/10; 6/10/11; 6/10/12; 7/9/10; 7/10/11; 8/9/10; 8/10/11; 8/10/12; 8/11/12; 9/10/11; 9/10/12; 10/11/12
VWA	8	11/16/17; 12/18/19; 14/15/17; 14/15/18; 14/16/18; 14/17/18; 15/16/17; 18/19/20
D3S1358	4	15/16/17; 15/17/18; 16/17/19; 17/18/19
D5S818	2	10/11/12; 11/12/13
D7S820	2	8/9/12; 8/10/11
D8S1179	5	10/12/13; 10/12/15; 12/13/14; 12/13/15; 13/15/16
D13S317	3	8/11/12; 10/11/12; 10/12/13
D16S539	1	12/13/14
D18S51	7	12/13/15; 12/14/15; 12/16/17; 14/15/22; 15/16/20; 16/17/20; 19/22.2/23.2
D21S11	4	28/29/30; 28/30.2/31.2; 29/31/32; 30/30.2/31
Penta D	0	None reported yet in STRBase
Penta E	0	None reported yet in STRBase
D2S1338	0	None reported yet in STRBase
D19S433	0	None reported yet in STRBase
SE33	0	None reported yet in STRBase

Many of these tri-allelic patterns have been observed more than once.
STR, short tandem repeat.

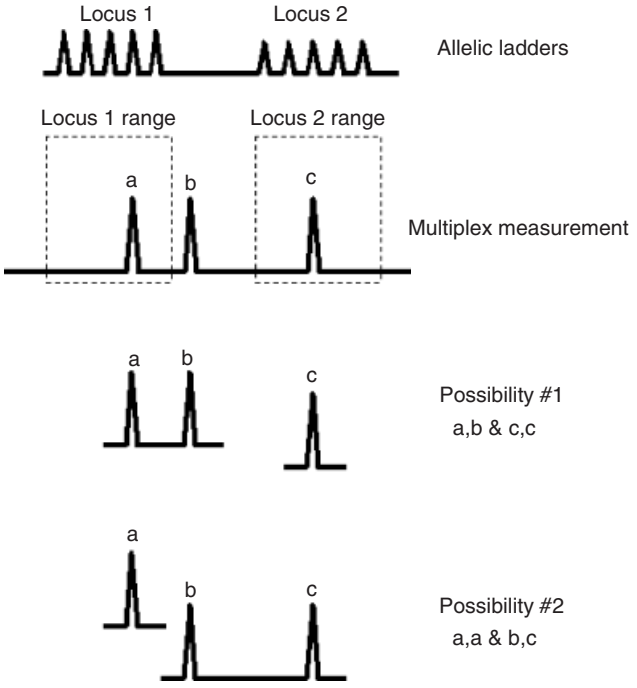

FIG. 1—*Illustration of an interlocus allele observed in a measurement involving multiplex amplification where it becomes difficult to assign allele "b" to locus 1 or locus 2.*

genotypes are as follows: locus1 (a,b) and locus2 (c,c) or locus1 (a,a) and locus2 (b,c). Heterozygosities of the two loci in question can be considered to predict which locus is more likely to be a heterozygote and possess two different alleles. For example, if a green-colored peak occurs between D16S539 and D2S1338 in the Identifiler kit and only a single allele is observed in each of the D16 and D2 normal allele ranges, then the interlocus allele more likely belongs to D2S1338 because D2 has a higher heterozygosity. The STRBase, variant allele section (or Table 4) can be examined to see if any other laboratories have observed extremely large D16 or extremely small D2 alleles. In this example, no large D16S539 alleles have been reported in STRBase, whereas several D2S1338 allele 13 observations have been noted. Finally, genotype frequencies can be examined to see if a locus1 or a locus2 homozygote is more common. For example, in an Identifiler genotype database (e.g., http://www.cstl.nist.gov/biotech/strbase/NISTpopdata/JFS2003IDresults.xls) a D16S539 11,11 homozygote occurs 10.7% of the time while a D2S1338 20,20 homozygote occurs only 0.92% of the time. Thus, it is more likely that the interlocus allele is a D2S1338 allele 13 rather than a D16S539 allele 17. While these considerations can help advise a laboratory on the best way to proceed with associating interlocus off-ladder alleles, it is recommended that final confirmation be performed with single locus amplification for each of the two adjacent STR loci.

Null Alleles with Commercial STR Kits

Sequence variation does occur in the flanking regions surrounding STR loci, and some PCR primers have been noted to be impacted by a primer binding site mutation, which can lead to allele dropout. For example, allele dropout at the VWA locus with the Applied Biosystems kits was reported (68) and ascribed to a point mutation near the 3'end of the forward PCR primer used (69). Potential null alleles resulting from allele dropout can often be predicted through statistical evaluation of STR typing data via comparison of the observed number of homozygotes to those expected based on Hardy-Weinberg equilibrium (70,71).

Because of the fact that different assays or commercial STR kits have primers that anneal to different flanking region sequences around a particular STR locus, concordance studies are conducted to detect possible null alleles. An examination of over 2000 samples comparing the PowerPlex 16 kit to the Profiler Plus and COfiler kit results found 22 examples of allele dropout because of a primer mismatch at seven of the 13 core STR loci in common (72,73). In addition, mutations under primer-binding sites have impacted the detection of D5S818 (74), D16S539 (75), and D18S51 (76) alleles with various PCR primer sets. The use of an extra or "degenerate" primer to account for possible sequence variation under a primer-binding site has been done with VWA (77), D16S539 (21), and D8S1179 (78) in some STR kits.

Mutation Rates

In situations where a direct comparison between evidence and a suspect is being made, mutation rates are not important. However, with comparisons between relatives in parentage testing and kinship analysis, such as may be applied in mass disaster victim identification, mutational events can play a significant role (79). Table 6 summarizes mutation rate data collected by the American Association of Blood Banks (AABB) as part of their 2003 annual report. These data come from several paternity testing laboratories. Not surprisingly, the loci with the highest mutation rate, e.g., SE33, FGA, D18S51, are the most polymorphic and possess the highest number of alleles (see Table 2). An exception to this observation is the complex repeat STR locus D21S11 where internal sequence variation may go undetected in size-based separations.

Population Studies

The literature contains over 1000 papers with information on STR allele frequencies observed in various population groups from around the world. An attempt to encapsulate many of these studies into a helpful list based on the commercial STR kits from which the data were generated has been made by Brian Burritt of the San Diego Police Department. As of early 2005, this list contains 365 population studies based on 183 literature references. This information has been made available on the internet at http://www.cstl.nist.gov/biotech/strbase/population/PopSurvey.htm.

In addition, Brian Burritt has developed a Microsoft Excel-based program called OmniPop that permits calculation of a user-inputted profile's frequency using allele frequencies from 166 published population surveys. OmniPop can be downloaded at http://www.cstl.nist.gov/biotech/strbase/population/OmniPop150.4.2.xls.

While most population studies include only 100–150 samples (see (80)), a few reported data sets have included thousands of individuals (81,82). A widely used population set is that published by Budowle et al. (83). Allele frequencies between small- and large-sized population databases (for the same or similar population group) rarely differ significantly for common alleles. Large data sets typically identify a greater number of rare alleles as more individuals in a population are included in the analysis. These rare alleles can be reliably accounted for through use of a minimum allele frequency as recommended by the National Research Council report (59).

TABLE 6—*Summary of apparent mutations observed at core STR loci in the course of parentage testing.*

STR System	Maternal Meioses (%)	Paternal Meioses (%)	Number from Either	Total Number of Mutations	Mutation Rate (%)
CSF1PO	95/304,307 (0.03)	982/643,118 (0.15)	410	1487/947,425	0.16
FGA	205/408,230 (0.05)	2210/692,776 (0.32)	710	3125/1,101,006	0.28
TH01	31/327,172 (0.009)	41/452,382 (0.009)	28	100/779,554	0.01
TPOX	18/400,061 (0.004)	54/457,420 (0.012)	28	100/857,481	0.01
VWA	184/564,398 (0.03)	1482/873,547 (0.17)	814	2480/1,437,945	0.17
D3S1358	60/405,452 (0.015)	713/558,836 (0.13)	379	1152/964,288	0.12
D5S818	111/451,736 (0.025)	763/655,603 (0.12)	385	1259/1,107,339	0.11
D7S820	59/440,562 (0.013)	745/644,743 (0.12)	285	1089/1,085,305	0.10
D8S1179	96/409,869 (0.02)	779/489,968 (0.16)	364	1239/899,837	0.14
D13S317	192/482,136 (0.04)	881/621,146 (0.14)	485	1558/1,103,282	0.14
D16S539	129/467,774 (0.03)	540/494,465 (0.11)	372	1041/962,239	0.11
D18S51	186/296,244 (0.06)	1094/494,098 (0.22)	466	1746/790,342	0.22
D21S11	464/435,388 (0.11)	772/526,708 (0.15)	580	1816/962,096	0.19
Penta D	12/18,701 (0.06)	21/22,501 (0.09)	24	57/41,202	0.14
Penta E	29/44,311 (0.065)	75/55,719 (0.135)	59	163/100,030	0.16
D2S1338	15/72,830 (0.021)	157/152,310 (0.10)	90	262/225,140	0.12
D19S433	38/70,001 (0.05)	78/103,489 (0.075)	71	187/173,490	0.11
SE33 (ACTBP2)	0/330 (<0.30)	330/51,610 (0.64)	None reported	330/51,940	0.64

Includes compilation of multiple years from American Association of Blood Banks (AABB) 2003 annual report (see http://www.aabb.org/About_the_AABB/Stds_and_Accred/ptannrpt03.pdf, Appendix 2); see also http://www.cstl.nist.gov/biotech/strbase/mutation.htm.

Web Resources

A number of Internet resources regarding STR markers and their use in human identity testing applications are listed in Table 7. More information on some of these resources is described below.

STRBase

One of the most comprehensive and widely used Internet resource on core STR loci involved in human identity testing is the National Institute of Standards and Technology Short Tandem Repeat Internet Database, which is commonly referred to as STRBase (http://www.cstl.nist.gov/biotech/strbase/). This site was created in 1997 by John Butler and Dennis Reeder (84) and has been described by Ruitberg et al. (85). New information is regularly added including variant alleles, triallelic patterns, and addresses for scientists working with STRs. In the past year, new sections of STRBase have been created to describe ongoing efforts with miniSTRs, validation procedures, single nucleotide polymorphisms of forensic interest, Y-chromosome markers and databases, and population data summaries.

Profile Frequency Estimates

Calculations for the rarity of a particular STR profile using core STR loci may be performed over the Internet using several different Web sites. The European Network of Forensic Science Institutes (ENFSI) has sponsored a site that enables different calculations and frequency estimates of an inputted STR profile against 24 different European populations using the SGM Plus kit loci (see http://www.str-base.org/calc.php).

STR profile frequency estimates can also be calculated using Canadian population databases generated by the Centre for Forensic Sciences and the RCMP (along with FBI Caucasian, African American, and Apache databases). This Web site can be accessed at http://www.csfs.ca/pplus/profiler.htm and an index of the available databases can be found at http://www.csfs.ca/databases/index.htm. The FBI raw STR data are publicly available for download at http://www.fbi.gov/hq/lab/fsc/backissu/july1999/dnaloci.txt.

Potential Linkage to Disease Genes

It is important to keep in mind that even though medical genetic researchers claim to have shown linkage between a particular disease gene and a core STR marker, these types of findings are often tentative and should not prevent the continued use of the STR locus in question. In fact, many times these linkage "findings" can later be proven false with further studies, such as with TH01 (86,87). To date there has only been a single call to remove an infrequently used STR marker from future consideration in human

TABLE 7—*Web resources regarding STR markers and forensic DNA typing.*

Short Tandem Repeat Internet Database (STRBase) with details on STR loci http://www.cstl.nist.gov/biotech/strbase
STR profile frequency calculations with SGM Plus loci http://www.str-base.org/index.php
STR profile frequency calculations with Profiler Plus and COfiler loci http://www.csfs.ca/pplus/profiler.htm
The Distribution of the Human DNA-PCR Polymorphisms http://www.uni-duesseldorf.de/WWW/MedFak/Serology/database.html
Y-Chromosome Haplotype Reference Database (YHRD) http://www.yhrd.org/index.html
Progress in Forensic Genetics 9 and 10 (Conference Proceedings of the ISFG) http://www.ics-publishing.com/periodicals/ics
Conference Proceedings of the International Symposium on Human Identification http://www.promega.com/geneticidproc/
Denver District Attorney's Office with DNA court case summaries http://www.denverda.org
FBI's Combined DNA Index System (CODIS) http://www.fbi.gov/hq/lab/codis/index1.htm
Forensic Science Service http://www.forensic.gov.uk
International Society of Forensic Genetics (ISFG) http://www.isfg.org
European DNA Profiling Group (EDNAP) http://www.isfg.org/ednap/ednap.htm
European Network of Forensic Science Institutes (ENFSI) DNA Working Group http://www.enfsi.org/ewg/dnawg

identity testing (88). The X-chromosome STR locus HumARA (11) is a CAG repeat located in a coding region (androgen receptor gene, exon 1) that has been directly linked to several genetic diseases (see (88)). It is probably worthwhile to reiterate that none of the 18 core loci shown in Table 2 and widely used in human identity testing are located in a gene coding region (i.e., exon) or are trinucleotide repeats, which can be prone to expansions that cause genetic defects (89).

An STR profile is simply a string of numbers that provides a unique genetic identifier to a tested sample. Yet because this information ultimately may be linked back to an individual, privacy concerns have been raised as to whether or not predisposition to a genetic disease can be ascertained from the presence of a particular STR allele. In some jurisdictions, there is a perceived problem with using genetic loci that are linked in some form to a genetic disease. Regions of the human genome are being explored with microsatellite (i.e., STR) markers to ascertain disease gene locations through linkage as demonstrated with family studies of effected individuals (90). Colin Kimpton et al. (91) and coworkers from the European DNA Profiling Group recognized early on in the application of STRs for human identity testing that "it is likely that many or possibly most STRs will eventually be shown to be useful in following a genetic disease or other genetic trait within a family and therefore this possibility must be recognized at the outset of the use of such systems."

Indeed, a number of the core STR loci described in this review have been reported to be useful in tracking various genetic diseases through loss of heterozygosity or allelic imbalance. For example, D8S1179 was used to localize a gene connected to Meckel–Gruber syndrome, which is the most common monogenic cause of neural tube defects (92). Another study employing 401 STR markers showed that D8S1179 was the most closely examined locus associated with the gene responsible for urinary microalbuminuria, which impairs kidney function and can lead to an elevated risk for cardiovascular disease (93).

The reason that suspected linkages are even reported in the first place for some of the core STR loci is that many of them are utilized in genome-wide scans in searches for disease-causing genes. For example, the Marshfield panel of more than 400 STRs (Weber set 10) that are spaced across the human genome includes TPOX, D7S820, D8S1179, D13S317, D16S539, and D19S433 (90). It is important to keep in mind that many of the early selections for candidate STR loci by the FSS (12) and by Promega Corporation (94) came from CHLC loci (http://www.chlc.org) that form the basis for genome scans used today for genetic linkage studies. Thus, many of the core STR loci in current use have a common origin to loci widely used for human disease gene linkage analysis studies.

One core STR locus that has gotten a bad reputation over the years for supposed linkage to genetic diseases is TH01, which occurs in the first intron of the tyrosine hydroxylase gene (see Table 3). Allele associations with particular TH01 alleles have been noted for individuals with schizophrenic (95,96) and bipolar disorders (97). However, other researchers failed to confirm these associations (98,99). Likewise, a reported association between TH01 alleles 9.3 and 10 with hypertension (100) was not found with further testing (86). A recent study claims that individuals possessing TH01 allele 7 have less nicotine dependence and are less likely to smoke in a dependent manner, although the data are far from definitive (101).

Trisomy-21, otherwise known as Down's syndrome, can often be detected by the presence of three alleles in any polymorphic marker found on chromosome 21 (102). Certainly, the core STR locus D21S11 qualifies as a useful test for trisomy-21 (103). Likewise, trisomy-18 (Edwards' syndrome) assessment from prenatal samples has been performed with D18S51 (104). In addition, loss of heterozygosity or extreme allelic imbalance is also considered to demonstrate linkage to cancer in some instances (105–107).

Probabilistic Predictions of Sample Ethnicity

Information regarding the probable ethnicity of an unknown offender has the potential to assist investigators in narrowing their search for the true perpetrator, provided that the information is reliable. Since early in the use of DNA typing, efforts have been made to infer ethnic origin from DNA profiles (108). The approach that is generally taken is to examine alleles present in the evidentiary profile and compare them with allele frequencies found in various population data sets. Likelihood ratios can then be created based on competing hypotheses (i.e., that the profile could have come from one population vs another).

Of course this approach requires a number of assumptions, including that the population data sets are representative of individuals coming from a particular ethnic background (109). While any population database with individuals of self-declared ethnicity cannot be regarded as "ethnically pure" and therefore poor calibrators of ethnic origin, efforts have been made to provide a probabilistic prediction with commonly used STR loci (110–112).

Studies involving hundreds of STR loci have found that there are STRs that are more likely to have drastic allele frequency differences between various population groups (see (113,114)). However, it is important to keep in mind that ambiguity is introduced by the relatively high rate of mutation with STR loci (see Table 6), which makes it challenging to separate alleles that are identical by state from those identical by descent (115). Typically single nucleotide polymorphisms (SNPs) or *Alu* insertion elements are more likely to be used for estimating ethnic origin because of their lower mutation rate and the likelihood that a particular allele becomes fixed in a certain population (116–118).

Additional STRs Beyond the Current Core Loci

The efforts of the Human Genome Project have increased knowledge regarding the human genome, and hence there are many more STR loci available now than there were 10 years ago. In fact, more than 20,000 tetranucleotide STR loci have been characterized in the human genome (119) and there may be more than a million STR loci present depending on how they are counted (120). STR sequences account for approximately 3% of the total human genome (121). Yet as noted in the historical perspective at the beginning of this article, even if the initial set of STR loci screened was not substantial, an effective DNA database could only be constructed by generating genotypes with a common set of genetic markers. The current core loci have played and will continue to play a vital role in human identity testing. Commercial STR kits exist, which have further increased the use of these STR loci.

With the fact that millions of DNA samples have now been examined across the core STR loci discussed in this article, it is perhaps worth taking a brief retrospective evaluation and asking the question, are these loci the best available? And if not, what characteristics would be beneficial to future applications in human identity testing? With 20/20 hindsight, are there characteristics for or lessons learned that could be applied in developing additional loci to complement current STR systems (and possibly become part of core loci of the future)?

TABLE 8—*Characteristics of core Y-chromosome STR loci.*

STR Marker	Position (Mb)	Repeat Motif	Allele Range	Mutation Rate (%)	STR Diversity
DYS393	3.17	AGAT	8–17	0.05	0.363
DYS19	10.12	TAGA	10–19	0.20	0.498
DYS391	12.54	TCTA	6–14	0.40	0.552
DYS439	12.95	AGAT	8–15	0.38	0.639
DYS389I/II	13.05	[TCTG] [TCTA]	9–17/24–34	0.20, 0.31	0.538/0.675
DYS438	13.38	TTTTC	6–14	0.09	0.594
DYS390	15.71	[TCTA] [TCTG]	17–28	0.32	0.701
DYS385 a/b	19.19, 19.23	GAAA	7–28	0.23	0.838
DYS392	20.97	TAT	6–20	0.05	0.596

Positions in megabases (Mb) along the Y-chromosome were determined with NCBI build 35 (May 2004) using BLAT. Allele ranges represent the full range of alleles reported in the literature. Mutation rates summarized from YHRD (http://www.yhrd.org; accessed 6 April 2005). The listed STR diversity values are calculated from 244 U.S. Caucasian males (see ref. (134)) and can be helpful in ranking the relative informativeness of the loci.

STR, short tandem repeat.

Certainly for most applications in human identity testing, where a high degree of polymorphism in a marker is advantageous, it is desirable to have loci with better allele frequency distributions than TPOX and TH01. The most common alleles for these two loci can occur at frequencies of greater than 60% in some populations. However, as noted in Table 6, these less polymorphic loci have lower mutation rates, which can make them more useful in some parentage testing situations. Thus, because of different needs, not all human identity testing applications may desire the same characteristics or select the same core STR marker set.

Simple repeat loci are desirable over highly complex loci, such as D21S11 and SE33, with internal sequence variation that can potentially add ambiguity to results and that can only be fully characterized through sequence analysis (rather than PCR product size measurements). However, it should be noted that because of matches at additional loci being tested, it is highly unlikely that a case (i.e., suspect to evidence match) would ever be impacted by potential internal sequence variation at a complex locus such as D21S11. Thus, in practice, forensic DNA testing does not require the sequencing of specific STR alleles to confirm a length-based match discovered at a single complex locus during multiplex STR analysis.

STR loci with a large allele span, such as FGA that possesses alleles spreading across almost 40 repeat units or 160 bp (see Table 2), consume a great deal of potential electrophoretic real estate in STR multiplexes. Two or three moderately polymorphic STR loci on separate chromosomes would be more powerful when the product rule was applied and would easily fit into the same PCR product space. In addition, if a higher molecular weight FGA allele is present in a sample, it undoubtedly will not be amplified as well as a companion lower molecular weight allele. This allele imbalance could even result in allele dropout, particularly in DNA examined from environmentally traumatized samples.

A number of studies have shown what is theoretically predicted—that DNA types can be recovered more effectively from degraded DNA samples when the PCR products are smaller (122–124). Therefore, future loci for consideration in forensic casework applications should contain a more compact allele range and be able to be amplified as small PCR products (125). Unfortunately, core loci such as FGA cannot be made much smaller because of their enormous allele range (125). STR loci that are sufficiently polymorphic and possess a smaller size range do exist and are beginning to be characterized (126).

As new assays that incorporate desirable STR markers (e.g., (126)) are developed, they may still meet some resistance by those who wish to maintain consistency to legacy data in national databases that already contain millions of DNA profiles generated with the previously established core loci. However, it is possible to attach information from additional markers to current STR tests (127); much like the FSS did in the late 1990s, as they added four new STR loci (D2S1338, D3S1358, D16S539, and D19S433) when the U.K. NDNAD went from the six STRs of SGM to the 10 STRs of SGM Plus. As has been noted, mass disaster investigations, which do not rely on large databases constructed over time in many different laboratories, may be more amenable to adopting new loci and assays (128). Recently, there has been a recommendation to adopt three new miniSTR loci (D10S1248, D14S1434, and D22S1045) as part of the standard European loci (127).

Y-Chromosome STR Loci

Although the primary focus of this review is on autosomal STR loci that are widely used for human identity testing, Y chromosome STR loci are growing in popularity and are briefly considered here. The Y chromosome is found only in males, and therefore genetic markers along the Y chromosome can be specific to the male portion of a male–female DNA mixture such as is common in sexual assault cases. Y chromosome markers can also be useful in missing persons investigations, some paternity testing scenarios, historical investigations, and genetic genealogy, because of the fact that most of the Y chromosome (barring mutation) is passed from father to son without changes.

A core set of Y-chromosome STR (Y-STR) loci is widely used in laboratories worldwide for human identity testing and genetic genealogy (129). The minimal haplotype loci (MHL) were selected in the late 1990s from a meager set of available Y-STRs (130,131). The MHL include DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, and the polymorphic, multi-copy marker DYS385. In 2003, the Y-chromosome subcommittee of the Scientific Working Group on DNA Analysis Methods (SWGDAM) recommended two additional Y-STRs named DYS438 and DYS439 for inclusion in the U.S. minimal haplotype (132).

Table 8 contains information on these Y-STR loci, including their chromosomal location, allele ranges, and mutation rates. Alleles observed with Y-STR markers are concatenated to form a haplotype for each examined DNA sample. Y-STR results from individual loci cannot be combined with the product rule, because the core Y-STR loci are all on the nonrecombining portion of the Y chromosome. To date, almost 200 studies have been conducted to examine Y-STR haplotype variation, including one with 2443 male individuals from five North American population groups

TABLE 9—*A summary of locus configuration in Y-chromosome STR typing kits from Promega Corporation and Applied Biosystems.*

PowerPlex Y (kit released in September 2003 by Promega Corporation)			
Dye Label	Locus	Alleles in Ladder	PCR product sizes (Based on DNA Sequence; ref. (136)) (bp)
FL	DYS391	6,8–13	90–118
	DYS389I	10–15	148–168
	DYS439	8–15	203–231
	DYS389II	24–34	256–296
JOE	DYS438	8–12	101–121
	DYS437	13–17	183–199
	DYS19	10–19	232–268
	DYS392	7–18	294–327
TMR	DYS393	8–16	104–136
	DYS390	18–27	191–227
	DYS385 a/b	7–25	243–315
Yfiler (kit released in December 2004 by Applied Biosystems)			
Dye Label	Locus	Alleles in Ladder	PCR product sizes (relative to GS500 LIZ size standard; ref (137)) (bp)
6-FAM	DYS456	13–18	105–124
	DYS389I	10–15	143–165
	DYS390	18–27	192–228
	DYS389II	24–34	253–294
VIC	DYS458	14–20	131–156
	DYS19	10–19	176–211
	DYS385 a/b	7–25	243–318
NED	DYS393	8–16	100–132
	DYS391	7–13	151–176
	DYS439	8–15	198–225
	DYS635	20–26	246–271
	DYS392	7–18	291–327
PET	GATA H4	8–13	122–142
	DYS437	13–17	183–198
	DYS438	8–13	224–249
	DYS448	17–24	280–325

STR, short tandem repeat.

(133). Additional Y-STR markers are also being examined beyond the core loci in order to determine the value of expanding haplotypes generated in the future (134,135).

A number of online databases exist, which permit a comparison of a Y-STR haplotype to those haplotypes already observed in various populations (for a summary of databases, see http://www.cstl.nist.gov/biotech/strbase/y_strs.htm). The largest of these databases is the Y-chromosome haplotype reference database (YHRD; http://www.yhrd.org), which contains over 28,000 haplotypes run with the minimal haplotype loci. Commercial Y-STR kits are now available that amplify the entire set of core Y-STR loci in a single, robust multiplex assay (Table 9). These kits can produce male-specific amplification even in the presence of more than a 1000-fold excess of female DNA (138).

Conclusions

STR markers have become important tools for human identity testing and will continue to be widely used for many years because of their high degree of variability, ease of use in multiplex amplification formats, and implementation in NDNADs (139). Utilization of a uniform set of core STR loci provides the capability for national and international sharing of criminal DNA profiles.

The core loci currently employed in human identity testing have demonstrated their usefulness in aiding the resolution of numerous criminal and parentage testing cases over the past dozen years. Robust commercial STR kits permit reliable amplification of these core loci from small amounts of starting DNA template. Resulting STR profiles enable high powers of discrimination to be achieved among both related and unrelated individuals.

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Topics and Techniques for Forensic DNA Analysis

miniSTRs

Florida Statewide Training Meeting

Indian Rocks Beach, FL
May 12-13, 2008

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National Institute of Justice
The Research, Development, and Evaluation Agency of the U.S. Department of Justice

Current Areas of NIST Effort with Forensic DNA

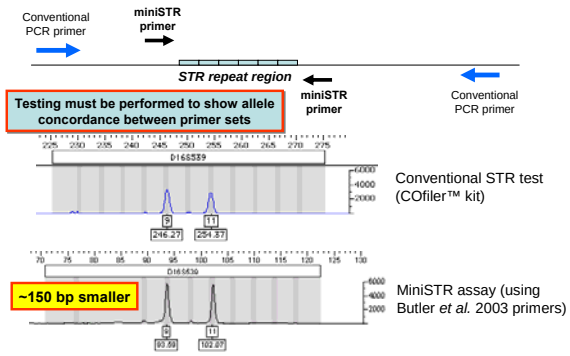
- **Standards**
  - Standard Reference Materials
  - Standard Information Resources (STRBase website)
  - Interlaboratory Studies
- **Technology**
  - Research programs in SNPs, miniSTRs, Y-STRs, mtDNA, qPCR
  - Assay and software development, expert system review
- **Training Materials**
  - Review articles and workshops on STRs, CE, validation
  - PowerPoint and pdf files available for download

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

Technology: Research Programs

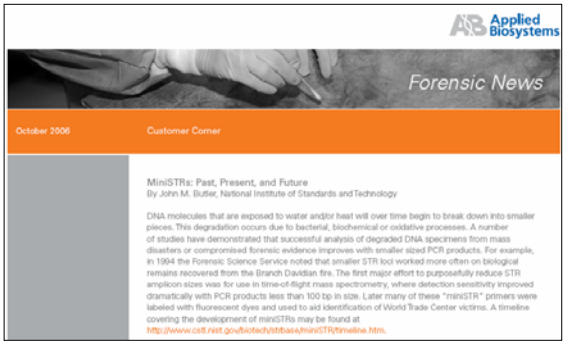
- miniSTRs
- Y-chromosome STRs
- mtDNA
- SNPs
- qPCR for DNA quantitation
- DNA stability studies
- Variant allele characterization and sequencing
- Software tools
- Expert System review
- Assay development with collaborators

A miniSTR is a reduced size STR amplicon that enables higher recovery of information from degraded DNA samples



Butler, J.M. (2005) *Forensic DNA Typing, 2nd Edition*, Figure 7.2, ©Elsevier Science/Academic Press

miniSTR Overview Article



http://marketing.appliedbiosystems.com/images/enews/ForensicNews_Vol7/PDF/02A_CustomerCorner_Butler.pdf

Timeline for miniSTRs

and Demonstrating the Value of Using Reduced Size Amplicons for Degraded DNA

- 1994 – FSS finds that smaller STR loci work best with burned bone and tissue from Branch Davidian fire
- 1997 – New primers developed for time-of-flight mass spectrometry to make small STR amplicons
- 2001 – Work at NIST and OhioU with CODIS STRs; BodePlexes used in WTC investigation starting 2002
- 2004 – Work at NIST with **non-CODIS (NC) miniSTRs**
- 2007 – Applied Biosystems releases 9plex MiniFiler

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

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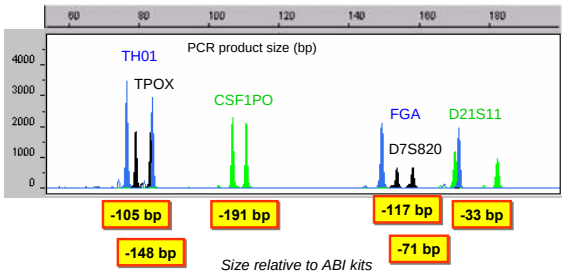
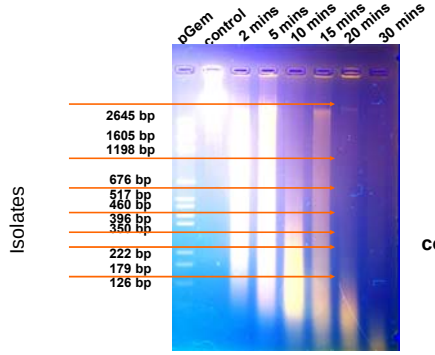
John M. Butler,[1] Ph.D.; Yin Shen,[2,3] Ph.D.; and Bruce R. McCord Ph.D.[2]

The Development of Reduced Size STR Amplicons as Tools for Analysis of Degraded DNA*

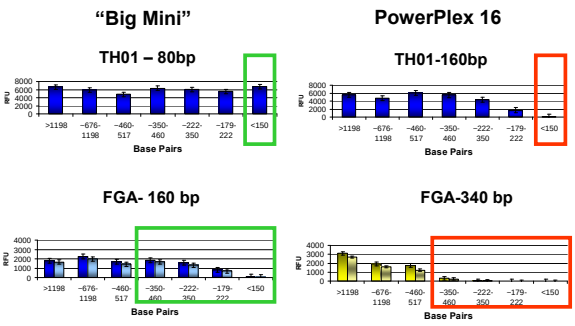


DNA Degraded With DNase I



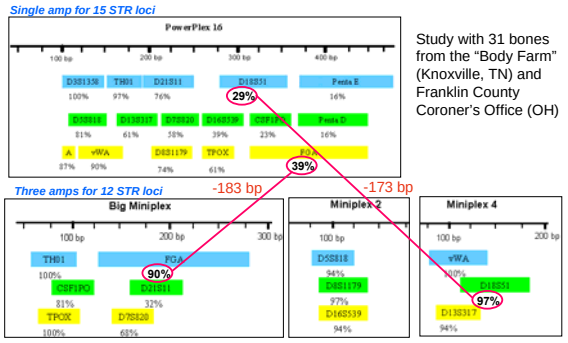
Chung, D.T., Drabek, J., Opel, K.L., Butler, J.M., McCord, B.R. (2004) A study on the effects of degradation and template concentration on the efficiency of the STR miniplex primer sets. *J. Forensic Sci.* 49(4): 733-740.

Miniplexes improve detection of degraded DNA



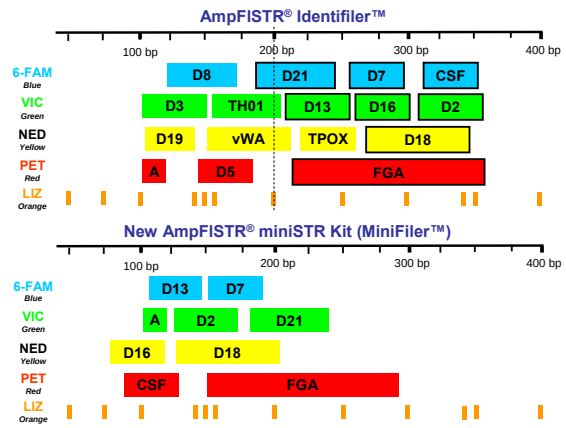
Chung, D.T., Drabek, J., Opel, K.L., Butler, J.M., McCord, B.R. (2004) A study on the effects of degradation and template concentration on the efficiency of the STR miniplex primer sets. *J. Forensic Sci.* 49(4): 733-740.

Comparison of PCR Amplification Success Rates with Commercial Kit vs. miniSTR Assays



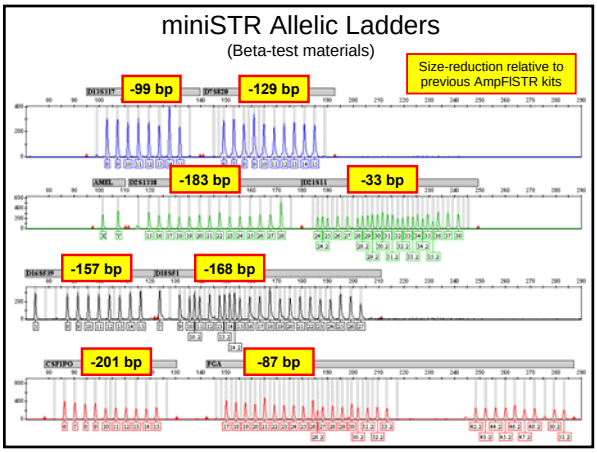
Study with 31 bones from the "Body Farm" (Knoxville, TN) and Franklin County Coroner's Office (OH)

Opel K. L.; Chung, D. T.; Drábek, J.; Tatarek, N. E.; Jantz, L. M.;. McCord, B.R. (2006) The Application of Miniplex Primer Sets in the Analysis of Degraded DNA from Human Skeletal Remains. *J. Forensic Sci.* 51(2): 351-356.



miniSTR Allelic Ladders

(Beta-test materials)



Summary of Samples Typed with ABI MiniFiler kit at NIST and ABI

- Primarily only population samples examined – no extensive sensitivity or degraded DNA tests were performed

1,308 samples Allele concordance = 10,437/10,464 = 99.7%

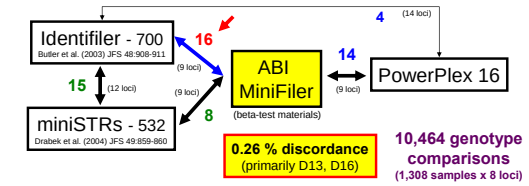
- **656 NIST U.S. population samples**
  - 260 Caucasian, 253 African American, 140 Hispanic, 3 Asian
  - Previously examined with **Identifiler; also with PowerPlex 16**
  - Also tested with Butler *et al.* (2003) **published miniSTR primers**
  - http://www.cstl.nist.gov/biotech/strbase/NISTpop.htm
- **481 father-son pairs**
  - 184 Caucasian, 196 African American, 101 Asian samples (provided by paternity testing company DDC)
  - Previously examined with **Identifiler**
- **171 samples from Applied Biosystems**

Hill *et al.* (2007) Concordance study between the AmpFlSTR MiniFiler PCR Amplification Kit and conventional STR typing kits. *J. Forensic Sci.* 52(4): 870-873.

Concordance Conducted at NIST

27 Discordant Calls

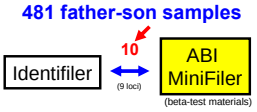
656 NIST U.S. population samples



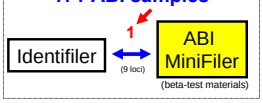
0.26 % discordance (primarily D13, D16)

10,464 genotype comparisons (1,308 samples x 8 loci)

481 father-son samples



171 ABI samples

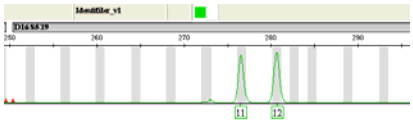


Hill *et al.* (2007) Concordance study between the AmpFlSTR MiniFiler PCR Amplification Kit and conventional STR typing kits. *J. Forensic Sci.* 52(4): 870-873.

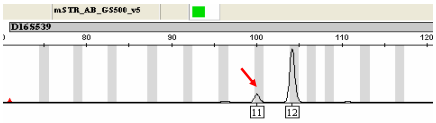
Concordance Studies Reveal Potential Primer Binding Site Mutations with Different Primer Sets

D16S539

Identifiler



miniSTR Kit (beta-test)



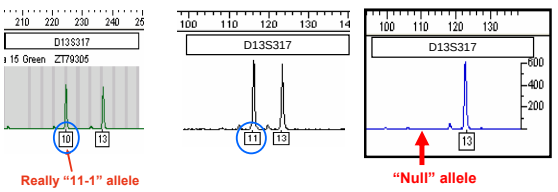
Appears to be an allele 11 dropout/reduction due to primer binding site mutation

Examination of **D13S317** Concordance:

African American sample ZT79305

Drabek, J., Chung, D.T., Butler, J.M., McCord, B.R. (2004) Concordance study between miniplex STR assays and a commercial STR typing kit. *J. Forensic Sci.* 49(4): 859-860.

NIST Identifiler data | Ohio U miniSTR data | **AB miniSTR beta-test**

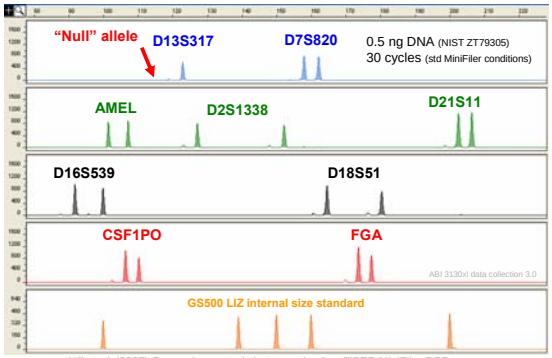


10,13 **11,13** **13,13**

Reverse primer is outside deletion | **Reverse primer is inside deletion** | **Reverse primer is on top of deletion**

Hill *et al.* (2007) Concordance study between the AmpFlSTR MiniFiler PCR Amplification Kit and conventional STR typing kits. *J. Forensic Sci.* 52(4): 870-873.

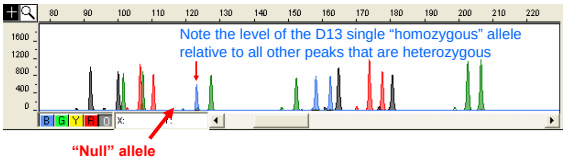
Full MiniFiler Profile for NIST Sample with D13S317 Allele Dropout

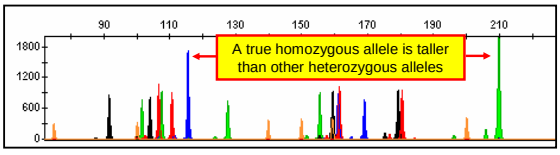


Hill *et al.* (2007) Concordance study between the AmpFlSTR MiniFiler PCR Amplification Kit and conventional STR typing kits. *J. Forensic Sci.* 52(4): 870-873.

Note the Relative D13 Peak Heights

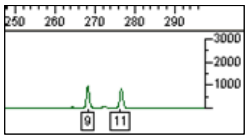
(Suggests Allele Dropout)



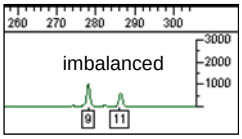


D16S539 SRM 2391b Genomic 8

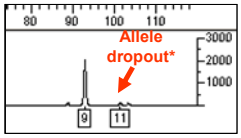
Identifiler



PowerPlex 16



MiniFiler



Due to primer binding site mutation

Example of a SNP in a primer region causing peak imbalances and Allele dropout

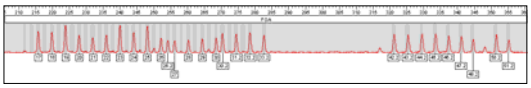
More Loci are Useful in Situations Involving Relatives

- **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

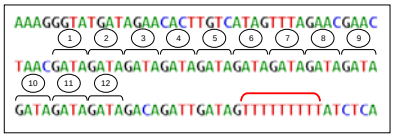
Relationship testing labs are being pushed to answer more difficult genetic questions…and **we want to make sure the right tools are in place**

Why Go Beyond the CODIS Loci?

(1) Large Allele Ranges (e.g. FGA)



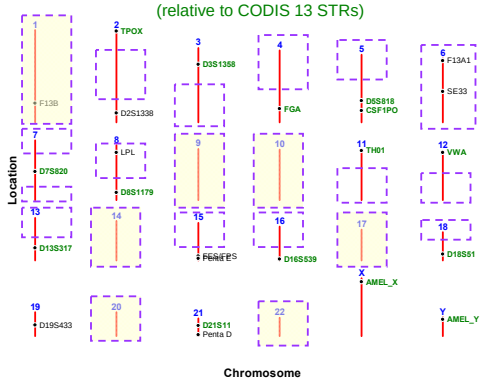
(2) "Unclean" Flanking Sequences (e.g. D7S820)

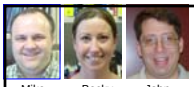


Butler, JM, Shen, Y., McCord, BR (2003) *JFS* 48(5): 1054-1064

Locations of Focus for New miniSTR Loci

(relative to CODIS 13 STRs)





New miniSTR Non-CODIS (NC) Loci

No longer at NIST (AFDIL Research Section Chief since April 2006)

- 32 STR loci tested on NIST **665 U.S. population samples**
- **26 STR loci with allele sizes below 140 bp and good heterozygosities (above TPOX level)**
- All new STR loci are **physically unlinked** to the 13 CODIS core loci
- **Submitted articles** regarding primer sequences and locus characterization including population statistics
- **SRM 2391b components are being certified** through sequencing for D10S1248, D2S441, D22S1045; for reference purposes, genotypes for standard samples (9947A, 9948, 007, K562) will be made available on STRBase

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

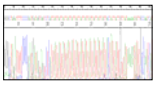
Characterization of New miniSTR Loci

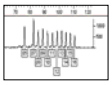
"Computer Work"

Candidate STR marker selection (e.g. Marshfield Clinic Center of Medical Genetics) → 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)

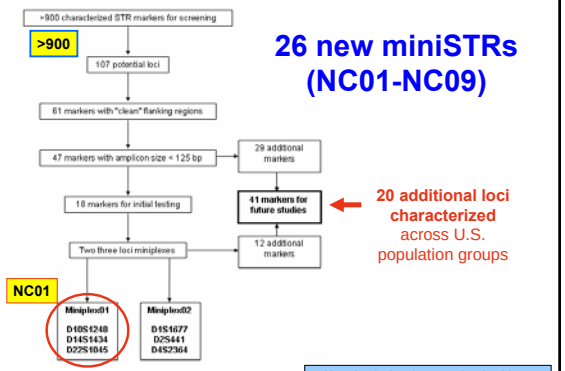
"Laboratory Work"

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



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

New STR Loci Characterized

Hill *et al.* (2008) *J. Forensic Sci.* 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

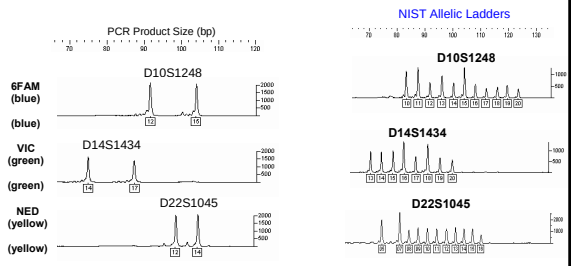
Carolyn R. Hill, M.S.; Margaret C. Kline, M.S.; Michael D. Coble,† Ph.D.; and John M. Butler, Ph.D.

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

- Primer sequences (for miniplexes), GeneMapper bins and panels, genotypes on common samples, and allele frequency information **available on STRBase**

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

Miniplex "NC01"



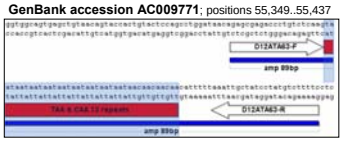
http://www.cstl.nist.gov/biotech/strbase/miniSTR.htm

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

Characterization of miniSTR **D12ATA63**

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

[FAM] – GAGCGAGACCCTGTCTCAAG
GAAAAGACATAGGATAGCAATTT



Chr 12 106.825 Mb (12q23.3)

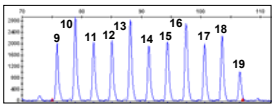
Trinucleotide [TAA][CAA] repeat
76 -106 bp
Alleles 9 -19

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
19	0.0019	0.0058	0.0036

Heterozygosity Values

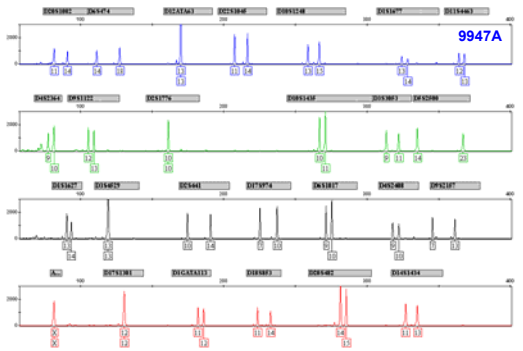
U.S. Caucasian	**0.842**
African American	**0.788**
U.S. Hispanic	**0.879**

D12ATA63 Allelic Ladder



"Autoplex" (26plex)

See Hill et al. AAFS 2008 talk (Washington, DC) and poster PP50 at DNA in Forensics 2008 meeting (Ancona)



Gender identification + 25 autosomal STR loci in a single amplification

European Labs Have Adopted the NIST-Developed NC 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]

[a] Forensic Science Service, Birmingham, UK
[b] Forensic Science Service, London, UK
[c] Department of Forensic Genetics, Institute of Forensic Medicine, University of Copenhagen, Denmark
[d] Institute of Legal Medicine, University of Cologne, Germany

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.

(D14 has been replaced with D2S441 from NC02)

Summary of miniSTRs

- **Reduced size amplicons improve success rates with degraded DNA** or samples possessing PCR-inhibitors – European leaders view **miniSTRs as "the way forward"**
- **MiniFiler concordance** testing performed
- **New miniSTR loci are being characterized** at NIST – 26 loci developed

Thank you for your attention…

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

Becky Hill

miniSTRs and 26plex work

Bruce McCord

Early miniSTR work

Mike Coble

Original NC miniSTR work

Margaret Kline

STR allele sequencing

http://www.cstl.nist.gov/biotech/strbase
john.butler@nist.gov
301-975-4049

Collaborators from ABI
Lori Hennessy
Julio Mulero
Rob Lagace
Chien-Wei Chang

TECHNICAL NOTE

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Carolyn R. Hill,[1] *M.S.; Margaret C. Kline,*[1] *M.S.; Julio J. Mulero,*[2] *Ph.D.; Robert E. Lagacé,*[2] *B.A.; Chien-Wei Chang,*[2] *Ph.D.; Lori K. Hennessy,*[2] *Ph.D.; and John M. Butler,*[1] *Ph.D.*

Concordance Study Between the AmpFℓSTR® MiniFiler™ PCR Amplification Kit and Conventional STR Typing Kits*

ABSTRACT: The AmpFℓSTR® MiniFiler™ polymerase chain reaction amplification kit developed by Applied Biosystems enables size reduction on eight of the larger STR loci amplified in the Identifiler® kit, which will aid recovery of information from highly degraded DNA samples. The MiniFiler™ Kit amplifies CSF1PO, FGA, D2S1338, D7S820, D13S317, D16S539, D18S51, and D21S11 as well as the sex-typing locus amelogenin. A total of 1308 samples were evaluated with both the MiniFiler™ and Identifiler® STR kits: 449 African American, 445 Caucasian, 207 Hispanic, and 207 Asian individuals. Full concordance between Identifiler and MiniFiler Kits was observed in 99.7% (10,437 out of 10,464) STR allele calls compared. The 27 differences seen are listed in Table 1 and encompass the loci D13S317 (n = 14) and D16S539 (n = 10) as well as D18S51 (n = 1), D7S820 (n = 1), and CSF1PO (n = 1). Genotyping discrepancies between the Identifiler and MiniFiler kits were confirmed by reamplification of the samples and further testing using the PowerPlex® 16 kit in many cases. DNA sequence analysis was also performed in order to understand the nature of the genetic variations causing the allele dropout or apparent repeat unit shift.

KEYWORDS: forensic science, DNA profiling, short tandem repeats, DNA typing, miniSTR, concordance, CSF1PO, FGA, D7S820, D13S317, D16S539, D18S51, D21S11, D2S1338, amelogenin, U.S. Caucasian, African American, Hispanic, Asian

Short tandem repeat (STR) markers are the primary means used today for human identity and forensic DNA testing (1). STRs are highly polymorphic and capable of generating typing results from very little material through multiplex amplification using the polymerase chain reaction (PCR). However, with highly degraded DNA specimens a loss of signal is typically observed with larger-sized STR products, either due to PCR inhibitors present in the forensic evidence or fragmented DNA molecules.

Size reduction of STR markers, and thus improved success rates with degraded or inhibited DNA samples, may be accomplished by moving PCR primers in as close as possible to the STR repeat region (2–4). A major advantage of these smaller STRs, or "miniSTRs," is that database compatibility may be maintained with convicted offender samples processed using commercial STR megaplexes. Concordance studies examining current assays compared to new ones on the same DNA samples are necessary in order to identify potential allele dropout situations due to primer binding site mutations (1,4–6). This report contains a summary of concordance results obtained on over 1300 samples run with a new miniSTR assay.

[1]National Institute of Standards and Technology, Biochemical Science Division, 100 Bureau Drive, Mail Stop 8311, Gaithersburg, MD 20899.
[2]Applied Biosystems, 850 Lincoln Centre Drive, Foster City, CA 94404.

*Sources of Support: Portions of this work was funded by the National Institute of Justice (NIJ) through interagency agreement (2003-IJ-R-029) with the National Institute of Standards and Technology (NIST) Office of Law Enforcement Standards.

Received 2 Dec. 2006; and in revised form 18 Mar. 2007; accepted 18 Mar. 2007; published 4 June 2007.

Materials and Methods

Anonymous liquid blood samples with self-identified ethnicities were purchased from Interstate Blood Bank (Memphis, TN) and Millennium Biotech, Inc. (Ft. Lauderdale, FL) and extracted, quantified, and typed with the Applied Biosystems AmpFℓSTR® Identifiler® PCR Amplification Kit (Applied Biosystems, Foster City, CA) as previously described (7). Additional anonymous samples were obtained as father/son pairs from DNA Diagnostics Center (Fairfield, OH) in the form of buccal swabs. The swabs were manually extracted using the DNA IQ™ system (Promega Corporation, Madison, WI) and quantified with an Alu-based quantitative PCR assay (8).

Beta-test materials (primer sequences are the same in the manufactured product) of the AmpFℓSTR® MiniFiler™ PCR Amplification Kit (Applied Biosystems) were used in this study. This miniSTR kit permits size reduction on eight of the larger STR loci amplified in the Identifiler® kit, including CSF1PO, FGA, D2S1338, D7S820, D13S317, D16S539, D18S51, D21S11 as well as amelogenin (9). All miniSTR assays were run in accordance with the manufacturer's recommendation. The PCR reaction contains 10 μL AmpFℓSTR® MiniFiler™ Master Mix, 5 μL AmpFℓSTR® MiniFiler™ Primer Set, 1 μL of DNA template (0.5 ng/μL), and 9 μL of 10 mmol/L Tris 0.1 mmol/L EDTA (TE^{-4}). Thermal cycling was performed in a GeneAmp® PCR System 9700 (Applied Biosystems) operating in the 9600 emulation mode with the following cycling parameters: an 11-min incubation at 95°C; followed by 30 cycles of 20 sec at 94°C, 2 min at 59°C, and 1 min at 72°C; and concluded with a 45-min incubation at 60°C. A final hold at 4°C was added until samples were removed.

Additional tests with the Identifiler® and PowerPlex® (Promega) 16 kits followed manufacturer recommended conditions with the exception of half reaction volumes being used.

Following PCR amplification, 1 μL of each sample was diluted in 8.7 μL Hi-Di™ formamide (Applied Biosystems) and 0.3 μL GeneScan™-500 LIZ® internal size standard (Applied Biosystems) and analyzed with an ABI PRISM® 3130*xl* Genetic Analyzer (Applied Biosystems) using Data Collection v3.0, POP-4™ or POP-6™ polymer (Applied Biosystems), and a 36-cm array. All genotyping was performed with GeneMapper® *ID* v3.2 software (Applied Biosystems) using manufacturer provided allelic ladders and bins and panels. Allele comparisons for concordance purposes were made with in-house Perl scripts written at Applied Biosystems and Excel macros created at NIST.

DNA sequencing of the discordant alleles was performed by first amplifying the target sequences for 28 cycles of PCR with the locus-specific primers. The PCR products were cloned using the TOPO TA cloning® kit for Sequencing (Invitrogen, Carlsbad, CA) and sequenced with the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) using the M13 forward and reverse primers following the recommendations of the manufacturers. The sequencing reactions were carried out using ~200 ng of plasmid DNA purified with a QIAprep Spin Miniprep kit (Qiagen, Valencia, CA). Unincorporated dye terminators were removed using the DyeEx 2.0 Spin kit (Qiagen). Samples were electrophoresed on the ABI PRISM® 3130*xl* Genetic Analyzer using Performance Optimized Polymer (POP-4™ polymer) on a 36-cm capillary array. The sequences were analyzed using the DNA Sequencing Analysis software v5.2 (Applied Biosystems).

Results and Discussion

A total of 1308 samples were evaluated with both the MiniFiler™ and Identifiler® STR kits: 449 African American, 445 Caucasian, 207 Hispanic, and 207 Asian individuals. Full concordance between Identifiler® and MiniFiler™ kits was observed in 99.7% (10,437 out of 10,464) STR allele calls compared. The 27 differences seen are listed in Table 1 and encompass the loci D13S317 (n = 14) and D16S539 (n = 10) as well as D18S51 (n = 1), D7S820 (n = 1), and CSF1PO (n = 1). The other three STR loci, D2S1338, FGA, and D21S11, and the sex determining locus amelogenin were fully concordant at all samples examined in this study.

Three of the null alleles detected in this study (Table 1) were from children of fathers also possessing the mutation impacting the primer-binding site. Sample no. 15 is the child of sample no. 14 (receiving the D13S317 null allele 10), sample no. 20 is the child of sample no. 21 (receiving the D16S539 null allele 11), and sample no. 22 is the child of sample no. 23 (receiving the D16S539 null allele 11). Thus, our data demonstrate Mendelian inheritance of the primer binding site mutation for these D13S317 and D16S539 null alleles.

Genotyping discrepancies between the Identifiler® and MiniFiler™ kits were confirmed by reamplification of the samples and further testing using the PowerPlex® 16 kit in 17 cases. DNA sequence analysis was also performed in order to understand the nature of the genetic variations causing the allele dropout or apparent repeat unit shift (Table 1).

Insertions or deletions in the flanking region outside of the MiniFiler™ kit primer binding sites give rise to differences in allele calls

TABLE 1—*Summary of 27 discordant STR profiling results observed in this study between the Identifiler® and MiniFiler™ kits for 449 different AA, 445 C, 207 H, and 207 A samples.*

	Locus	Ethnicity	Source	MiniFiler	Identifiler	PP16	Genetic Variation
1	CSF1PO	H	IBB	11,11	11, "**11.1**"	11,11	One base insertion in Identifiler amplicon outside of MiniFiler and PP16 primers
2	D7S820	AA	IBB	8,11	8,"**9.3**"	8,11	5 base deletion in Identifiler amplicon outside of MiniFiler and PP16 primers
3	D13S317	H	IBB	**11**,11	9,11	9,11	4 base deletion in the reverse MiniFiler primer binding region
4	D13S317	H	IBB	**13**,13	9,13	9,13	*(same as sample no. 3)*
5	D13S317	H	IBB	**14**,14	9,14	9,14	*(same as sample no. 3)*
6	D13S317	AA	IBB	**11**,11	9,11	9,11	*(same as sample no. 3)*
7	D13S317	AA	IBB	**12**,12	8,12	8,12	*(same as sample no. 3)*
8	D13S317	AA	IBB	**11**,11	8,11	8,11	*(same as sample no. 3)*
9	D13S317	AA	IBB	**13**,13	10,13	10,13	*(same as sample no. 3)*
10	D13S317	AA	IBB	**11**,11	9,11	9,11	*(same as sample no. 3)*
11	D13S317	AA	IBB	**12**,12	9,12	9,12	*(same as sample no. 3)*
12	D13S317	AA	DDC	**10**,10	9,10		
13	D13S317	C	IBB	**12**,12	9,12	9,12	*(same as sample no. 3)*
14	D13S317	C	DDC	**11**,11	10,11		
15	D13S317	C	DDC	8,**8**	8,10		
16	D13S317	A	DDC	**12**,12	10,12		
17	D16S539	AA	DDC	9,**9**	9,11		
18	D16S539	AA	IBB	**12**,12	11,12	11,12	A/G SNP in MiniFiler primer binding site
19	D16S539	AA	MLN	**11**,11	9,11	9,11	*(same as sample no. 18)*
20	D16S539	AA	DDC	**14**,14	11,14	11,14	*(same as sample no. 18)*
21	D16S539	AA	DDC	9,**9**	9,11	9,11	*(same as sample no. 18)*
22	D16S539	AA	DDC	**13**,13	11,13		
23	D16S539	AA	DDC	**12**,12	11,12		
24	D16S539	AA	DDC	**12**,12	11,12		
25	D16S539	AA	DDC	9,**9**	9,12		
26	D16S539	A	ABI	**11**,11	10,11		G/A SNP in MiniFiler primer binding site
27	D18S51	H	IBB	13,15	**15**,15	13,15	Allele 13 C/T SNP in Identifiler primer binding site

Sample sources include IBB, MLN, DDC, and ABI. With only three exceptions (see samples no. 1, 2, 27), PowerPlex® 16 (PP16) results agree with the Identifiler® results for these samples. DNA sequencing was performed to ascertain the genetic variation responsible for the discordance of the impacted allele (shown in bold font). Note that sample *no.* 15 is the child of sample no. 14, sample no. 20 is the child of sample no. 21, and sample no. 22 is the child of sample no. 23.

AA, African American; C, Caucasian; H, Hispanic; A, Asian; MLN, Millennium; IBB, Interstate Blood Bank; DDC, DNA Diagnostic Center; ABI, Applied Biosystems.

between the Identifiler® kit and the MiniFiler™ kit for CSF1PO (Table 1, sample no. 1) and D7S820 (Table 1, sample no. 2). Likewise, an Identifiler® kit primer-binding site mutation in D18S51 can cause allele dropout (Table 1, sample no. 27). The PowerPlex® 16 kit was run on a subset of our samples and found to exhibit 14 discordant calls (10 for D13S317 and 4 for D16S539) relative to the MiniFiler™ kit (see Table 1 and Fig. 1). By way of comparison, for the samples examined, there were a total of four discordant results between the PowerPlex® 16 and Identifiler® kits (Fig. 1).

Comparisons were also made to previous miniSTR primer sets described by Butler et al. (4) and the concordance reported by Drabek et al. (5). As illustrated in Fig. 2, the reverse primer for D13S317 in the MiniFiler™ kit is in a different place relative to the primer reported in Butler et al. (4) causing a different amplification outcome. Data from this study are available at http://www.cstl.nist.gov/biotech/strbase/NISTpop.htm.

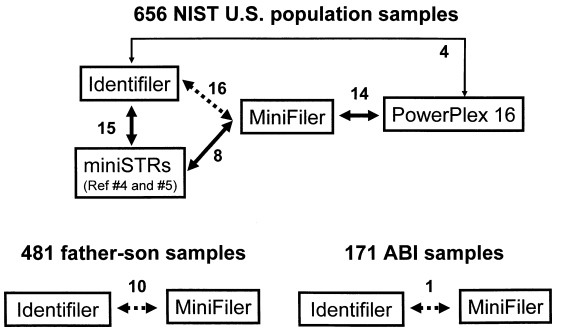

FIG. 1—*A schematic representation of the various comparisons conducted in this study. The number of discordant genotypes is shown for each comparison, which is illustrated by the double-headed arrow between the Identifiler®, MiniFiler™, and PowerPlex® 16 kits. The 27 discordant calls noted in this work are a composite of the dashed arrow comparisons.*

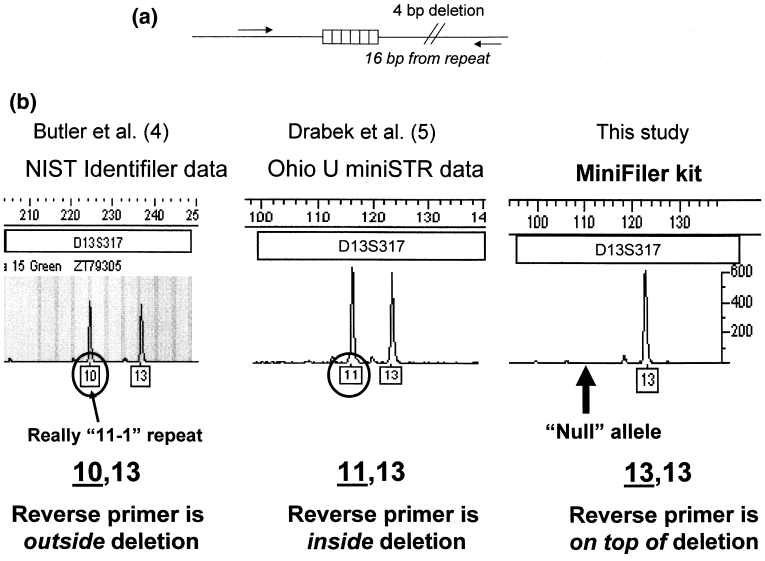

FIG. 2—*(a) Illustration of 4 bp deletion found near the D13S317 repeat region. (b) Different primer sets produce different genotyping results on the same DNA sample due to the relative positions of the reverse primer compared to the 4 bp deletion. A repeat shift in the Identifiler® kit "10" allele is observed with the Drabek et al. (5) result on the same sample, whereas the allele dropped out in this study due to the MiniFiler™ kit primer being on top of the deletion.*

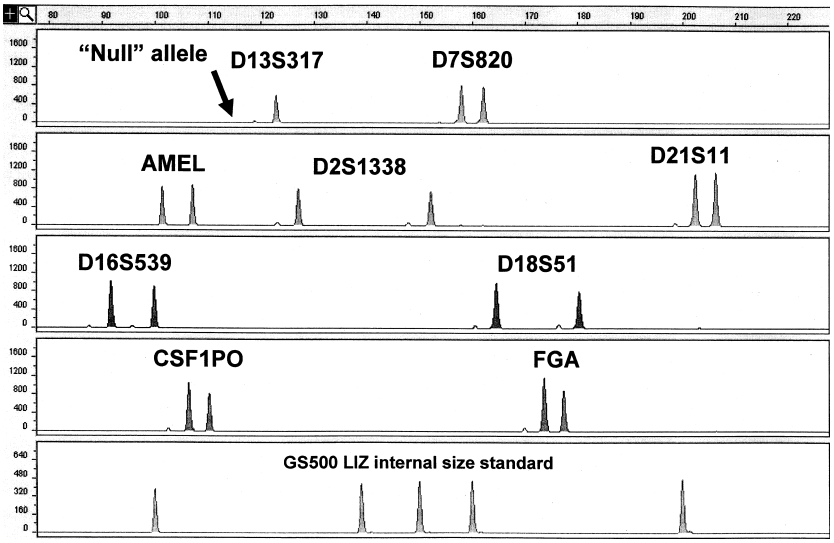

FIG. 3—*Genotyping results using MiniFiler™ kit from the same sample shown in Fig. 2 with the arrow indicating the allele dropout.*

With a multiplex amplification that produces well-balanced PCR product yields across loci, it is possible to detect allele dropout at a locus by noting when an apparent "homozygous" allele is similar in peak height to the two alleles present in a neighboring heterozygous locus (Fig. 3). A section of the NIST STRBase website has been established to collect information on allele dropout "observed" between different STR testing systems such as Mini Filer™ and Identifiler® or Identifiler® and PowerPlex® 16 kits (see http://www.cstl.nist.gov/biotech/strbase/NullAlleles.htm). Laboratories observing these null alleles are invited to submit data so that up-to-date null allele frequencies can be made available to the community.

Acknowledgments

We acknowledge the efforts of Jan Redman, Richard Schoske, Peter Vallone, and Amy Decker in preparing many of the DNA samples used at NIST as well as Tom Reid from DNA Diagnostics Center for providing the father/son samples. Madison Jordan assisted with sequencing some of the DNA samples at Applied Biosystems.

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Topics and Techniques for Forensic DNA Analysis

Capillary Electrophoresis Fundamentals and Troubleshooting

Florida Statewide Training Meeting

Indian Rocks Beach, FL
May 12-13, 2008

Dr. John M. Butler
National Institute of Standards and Technology

john.butler@nist.gov

Questions?

- What are your biggest challenges with keeping your ABI 310/3100/3130xl running?
- What kind of signal intensity variation are you seeing between your different instruments?
- Have anyone seen uneven injection across a sample plate? (We believe this to be an autosampler calibration issue…e.g., position G10 or H12 does not inject properly)

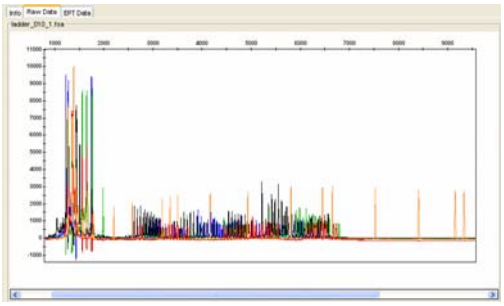
Planned Promega 2008 Meeting Troubleshooting Workshop

- Title: **"Principles of Interpretation and Troubleshooting of Forensic DNA Typing Systems"**
- Instructors: **John Butler (NIST) and Bruce McCord (FIU)**
- Date: **October 16, 2008** with Promega Int. Symp. Human ID

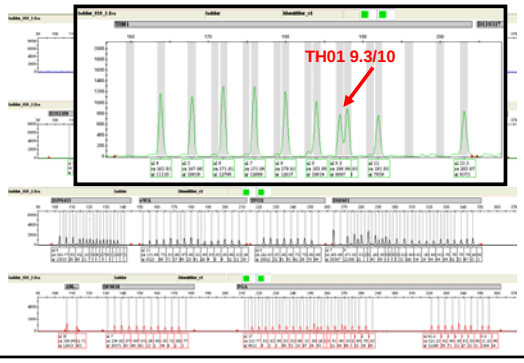
The workshop will consist of three parts:
(1) a through examination of theoretical issues with capillary electrophoresis PCR amplification of short tandem repeat markers
(2) a discussion of how to properly set instrument parameters to interpret data (including mixtures), and
(3) **a review of specific problems seen by labs** submitting problematic data and commentary on possible troubleshooting solutions.

Seeking input of problems observed with CE systems

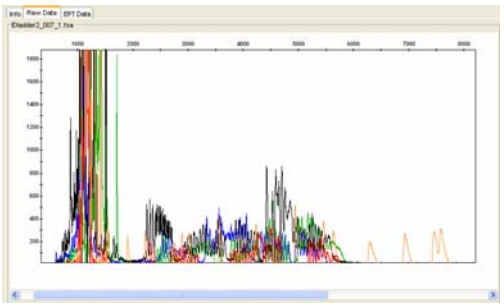
Identifiler Allelic Ladder March 14, 2007



Identifiler Allelic Ladder March 14, 2007

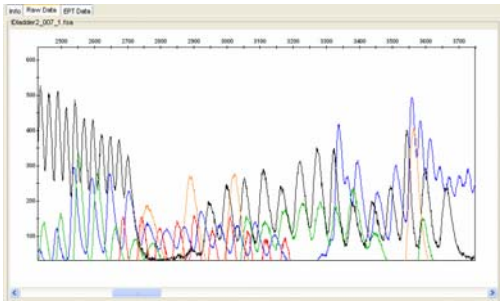


Identifiler Allelic Ladder March 23, 2007

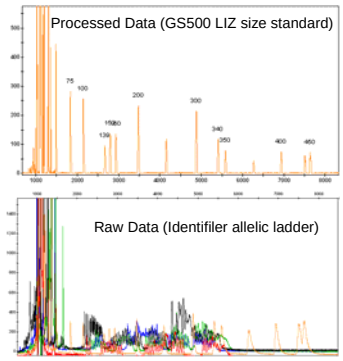


Same capillary array, same POP6 polymer, …

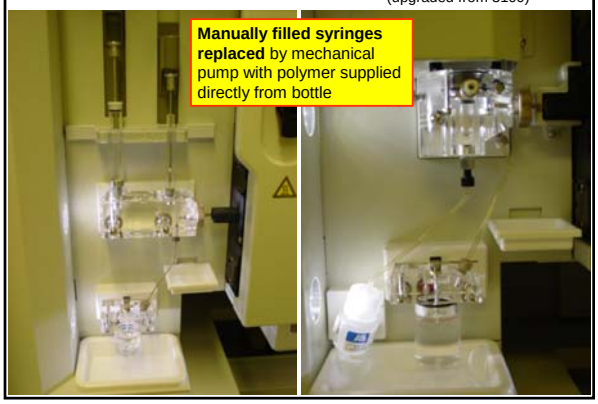
Examination of Resolution in TH01 Region



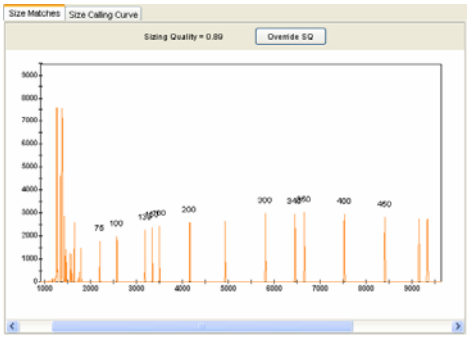
Examine the Size Standard…



ABI 3100 / ABI 3130xl (upgraded from 3100)



The Size Standard Provides an Excellent Indicator of Performance on Every Sample



Review Article on STRs and CE

pdf available from http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm

Electrophoresis 2004, *25*, 1397–1412

Review

John M. Butler[1]
Eric Buel[2]
Federica Crivellente[3]*
Bruce R. McCord[3]

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Forensic DN
using the AB
for STR anal

DNA typing with shor
applications includin
such as the ABI Prism
for many laboratories
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results using CE syst
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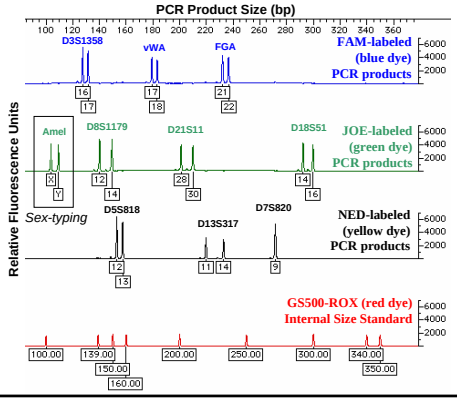
throughput and ease

Contents

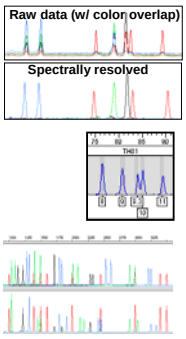
Genotype Results with Profiler Plus™ kit



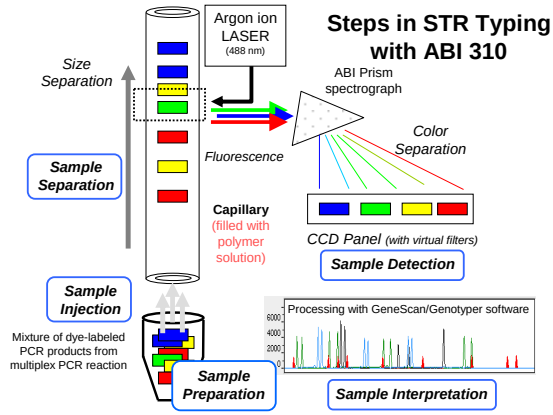
Analytical Requirements for STR Typing

Butler *et al.* (2004) Electrophoresis 25: 1397-1412

- Fluorescent dyes must be **spectrally resolved** in order to distinguish different dye labels on PCR products
- PCR products must be **spatially resolved** – desirable to have single base resolution out to >350 bp in order to distinguish variant alleles
- High **run-to-run precision** – an internal sizing standard is used to calibrate each run in order to compare data over time



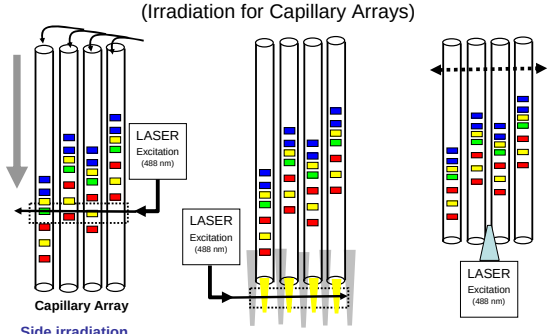
Steps in STR Typing with ABI 310



Butler, J.M. (2005) *Forensic DNA Typing, 2nd Edition*, Figure 13.8, © Elsevier Science/Academic Press

Detection with Multiple Capillaries

(Irradiation for Capillary Arrays)



Process Involved in 310/3100 Analysis

- Separation
  - Capillary – 50um fused silica, 43 cm length (36 cm to detector)
  - POP-4 polymer – Polydimethyl acrylamide
  - Buffer - TAPS pH 8.0
  - Denaturants – urea, pyrolidinone
- Injection
  - electrokinetic injection process (formamide, water)
  - importance of sample stacking
- Detection
  - fluorescent dyes with excitation and emission traits
  - CCD with defined virtual filters produced by assigning certain pixels

Ohm's Law

- V = IR (where V is voltage, I is current, and R is resistance)
- Current, or the flow of ions, is what matters most in electrophoresis
- CE currents are much lower than gels because of a higher resistance in the narrow capillary
- CE can run a higher voltage because the capillary offers a higher surface area-to-volume ratio and can thus dissipate heat better from the ion flow (current)

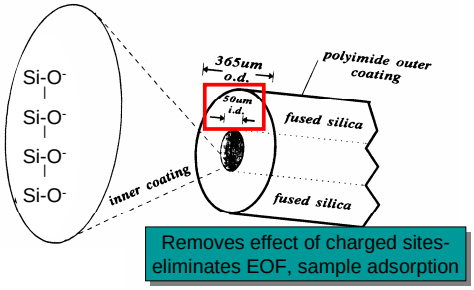
Separation Issues

- Electrophoresis buffer –
  - Urea for denaturing and viscosity
  - Buffer for consistent pH
  - Pyrolidinone for denaturing DNA
  - EDTA for stability and chelating metals
- Polymer solution -- POP-4 (but others work also)
- Capillary wall coating -- dynamic coating with polymer
  - Wall charges are masked by methyl acrylamide
- Run temperature -- 60 ºC helps reduce secondary structure on DNA and improves precision. (Temperature control affects DNA sizing)

Why TAPS instead of Tris-borate (TBE) buffer?

- TBE is temperature/pH sensitive
  - as temperature increases, the pH decreases (0.02 pH units with every 1 °C); this is the principle by which TaqGold activation works
- At lower pH, fluorescence emission of dyes decreases
  - see Singer and Johnson (1997) *Proceedings of the Eighth International Symposium on Human Identification*, pp. 70-77
- Thus when running at 60 °C on the ABI 310, if Tris-borate was used, fluorescent intensity of PCR products would be lower

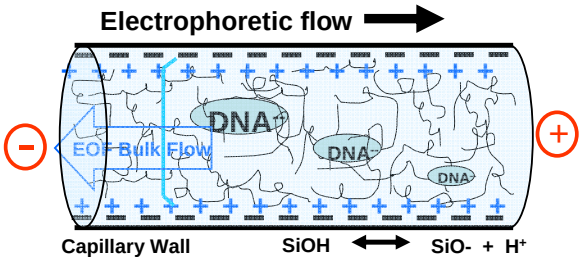
Capillary Coating



Removes effect of charged sites-eliminates EOF, sample adsorption

Dynamic coating of charged sites on fused silica capillary is accomplished with POP-4 polymer

Capillary Wall Coatings Impact DNA Separations



Electroosmotic flow (EOF)

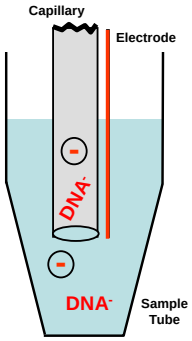
Solvated ions drag solution towards cathode in a flat flow profile

How to Improve Resolution?

1. **Lower Field Strength**
2. **Increase Capillary Length**
3. **Increase Polymer Concentration**
4. **Increase Polymer Length**

All of these come at a cost of longer separation run times

Electrokinetic Injection Process



Amount of DNA injected is inversely proportional to the ionic strength of the solution

Salty samples result in poor injections

Sample Conductivity Impacts Amount Injected

$$[DNA_{inj}] = \frac{Et(\pi r^2)\,(\mu_{ep} + \mu_{eof})[DNA_{sample}]\,(\lambda_{buffer})}{\lambda_{sample}}$$

$[DNA_{inj}]$ is the amount of sample injected

E is the electric field applied

t is the injection time

r is the radius of the capillary

μ_{ep} is the mobility of the sample molecules

μ_{eof} is the electroosmotic mobility

$[DNA_{sample}]$ is the concentration of DNA in the sample

λ_{buffer} is the buffer conductivity

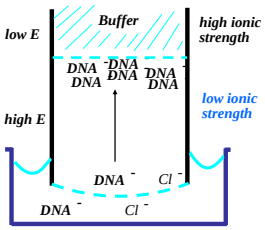
λ_{sample} is the sample conductivity

Cl- ions and other buffer ions present in PCR reaction contribute to the sample conductivity and thus will compete with DNA for injection onto the capillary

Butler *et al.* (2004) Electrophoresis 25: 1397-1412

Two Major Effects of Sample Stacking

1. Sample is preconcentrated. Effect is inversely proportional to ionic strength
2. Sample is focused. Ions stop moving in low electric field
3. Mobility of sample = μ_{ep} = velocity/ electric field



Steps Performed in Standard Module

See J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition; Chapter 14

- **Capillary fill** – polymer solution is forced into the capillary by applying a force to the syringe
- **Pre-electrophoresis –** the separation voltage is raised to 10,000 volts and run for 5 minutes;
- **Water wash of capillary – capillary is dipped several times in deionized water to remove buffer salts that would interfere with the injection process**
- **Sample injection** – the autosampler moves to position A1 (or the next sample in the sample set) and is moved up onto the capillary to perform the injection; a voltage is applied to the sample and a few nanoliters of sample are pulled onto the end of the capillary; the default injection is 15 kV (kilovolts) for 5 seconds
- **Water wash of capillary** – capillary is dipped several times in waste water to remove any contaminating solution adhering to the outside of the capillary
- **Water dip** – capillary is dipped in clean water (position 2) several times
- **Electrophoresis** – autosampler moves to inlet buffer vial (position 1) and separation voltage is applied across the capillary; the injected DNA molecules begin separating through the POP-4 polymer solution
- **Detection** – data collection begins; raw data is collected with no spectral deconvolution of the different dye colors; the matrix is applied during Genescan analysis

Comments on Sample Preparation

- Use high quality formamide (<100 μS/cm)!
  - ABI sells Hi-Di formamide
  - regular formamide can be made more pure with ion exchange resin
- Deionized water vs. formamide
  - Biega and Duceman (1999) *J. Forensic Sci.* 44: 1029-1031
  - *Crivellente, Journal of Capillary Electrophoresis* **2002,** *7 (3-4),* 73-80.
  - water works fine but samples are not stable as long as with formamide; water also evaporates over time…
- Denaturation with heating and snap cooling
  - use a thermal cycler for heating and cold aluminum block for snap cooling
  - heat/cool denaturation step is necessary only if water is substituted for formamide...

January 6, 2005 Letter from Applied Biosystems to ABI 310 Customers

- "Testing has shown that Hi-Di Formamide denatures DNA **without the need to heat samples**…"
- In other words, no heat denaturation and snap cooling needed!

Applied Biosystems Okays Use of Deionized Water for DNA Sequencing

Technical Bulletin #1 **Issued August 2006**

Applied Biosystems 3730/3730*xl* DNA Analyzer

Subject: Influence of Sequencing Injection Solution on 3730/3730xl DNA Analyzer Performance

In this Bulletin:

- Three Loading Solutions Tested on Page 1
- Loading Solution Test Data on Page 2
- Recommendations on Page 6
- Guidelines for Use on Page 6

Three Loading Solutions Tested

Loading Solution Background

Applied Biosystems presently recommends the use of Hi-Di™ Formamide as the sample-loading solution for all Applied Biosystems DNA sequencers to ensure sample preservation and resistance to evaporation. However, many users of the 3730 choose either deionized water or dilute EDTA solutions. These choices are driven largely by cost and safety/hazardous material considerations.

Detection Issues

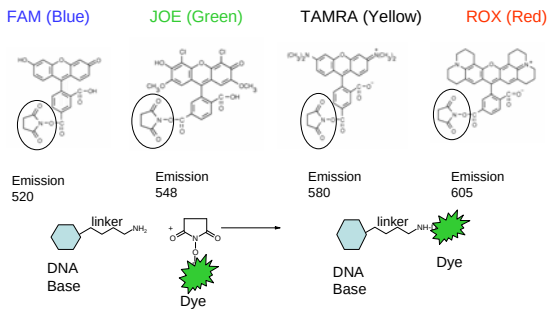
- Fluorescent dyes
  - spectral emission overlap
  - relative levels on primers used to label PCR products
  - dye "blobs" (free dye)
- Virtual filters
  - hardware (CCD camera)
  - software (color matrix)

Filters determine which wavelengths of light are collected onto the CCD camera

Fluorescent Labeling of PCR Products

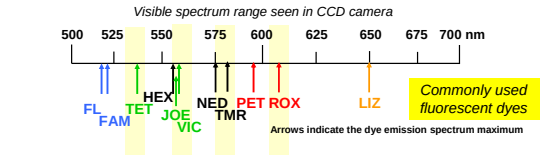
- Dyes are attached to one primer in a pair used to amplify a STR marker
- Dyes are coupled to oligonucleotides (primers) through NHS-esters and amine linkages on the 5'end of the primer: Dye-$(CH_2)_6$-primer
- Dye-labeled oligonucleotides are incorporated during multiplex PCR amplification giving a specific color "tag" to each PCR product
- PCR products are distinguished using CCD imaging on the 310

Amine Reactive Dyes used in Labeling DNA



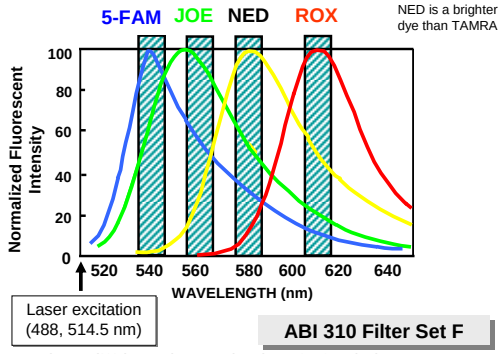
The succinimidyl ester reacts rapidly with amine linkers on DNA bases

Virtual Filters Used in ABI 310



	Blue	Green	Yellow	Red	Orange	Used with These Kits
Filter A	FL	JOE	TMR	CXR		PowerPlex 16
Filter C	6FAM	TET	HEX	ROX		in-house assays
Filter F	5FAM	JOE	NED	ROX		Profiler Plus
Filter G5	6FAM	VIC	NED	PET	LIZ	Identifiler

Fluorescent Emission Spectra for ABI Dyes

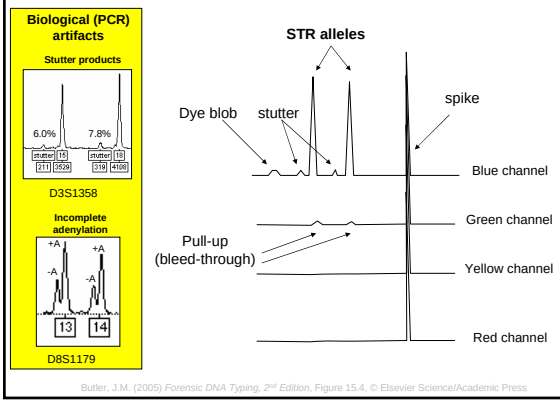


Butler, J.M. (2001) *Forensic DNA Typing*, Figure 10.4, ©Academic Press

Please Note!

- There are no filters in a 310
- Its just the choice of pixels in the CCD detector
- All the light from the grating is collected
- You just turn some pixels on and some off

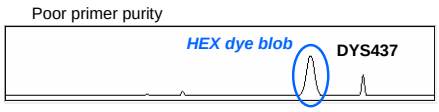
Deciphering Artifacts from the True Alleles



Butler, J.M. (2005) *Forensic DNA Typing, 2nd Edition*, Figure 15.4, © Elsevier Science/Academic Press

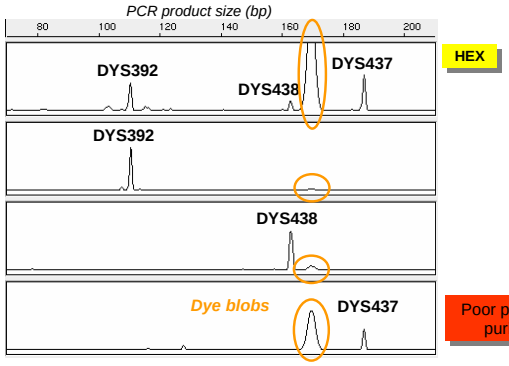
Dye Blobs ("Artifacts")

- Free dye (not coupled to primer) can be injected into the CE capillary and interfere with detection of true STR alleles
- **Dye blobs are wider and usually of less intensity** than true STR alleles (amount depends on the purity of the primers used)
- Dye blobs usually appear at an apparent size that is unique for each dye (e.g., FAM ~120 bp, PET ~100 bp)

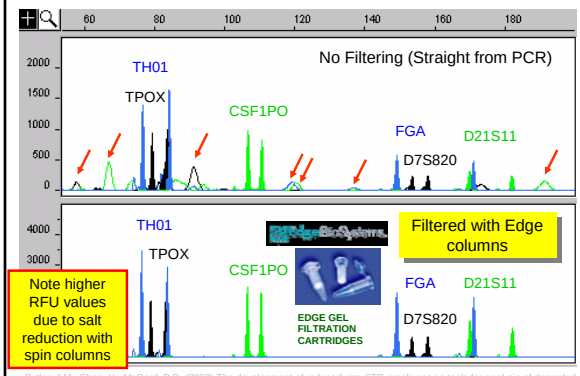


Dye Blob Problems with Some PCR Primers

Individual Y-STR Locus Amplifications



Removal of Dye Artifacts Following PCR Amplification



Butler, J.M., Shen, Y., McCord, B.R. (2003) The development of reduced size STR amplicons as tools for analysis of degraded DNA. *J. Forensic Sci* 48(5) 1054-1064.

Conclusions

DNA typing by capillary electrophoresis involves:

1) The use of entangled polymer buffers
2) Injection by sample stacking
3) Multichannel laser induced fluorescence
4) Internal and external calibration

Practical Aspects of ABI 310/3100 Use

ABI Genetic Analyzer Usage at NIST

- ABI 310 x 2 (originally with Mac, then NT)
  - 1st was purchased in 1996
  - 2nd was purchased in June 2002
- ABI 3100 (Data collection v1.0.1)
  - Purchased in June 2002
  - Original data collection software retained
- ABI 3130xl upgrade (Data collection v3.0)
  - Purchased in April 2001 as ABI 3100
  - Upgraded to ABI 3130xl in September 2005
  - Located in a different room

Jan 2007 – upgraded to 3130xl with data collection v3.0

Our Use of the ABI 3100

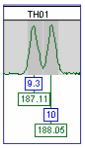
- Data collection software, version 1.0.1
- **POP-6** with 36 cm capillary array

- STR kits and in-house assays for autosomal STRs, Y-STRs, and miniSTRs
- SNaPshot assays for mtDNA SNPs, Y-SNPs, and autosomal SNPs
- DNA sequencing for mtDNA and STR repeat sequencing

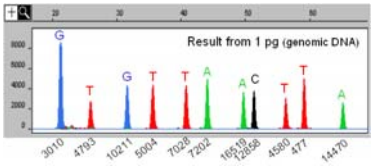
We can routinely get more than 400 runs per capillary array by not changing the polymer between applications

NIST ABI 3100 Analysis Using POP-6 Polymer

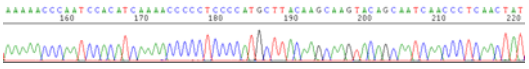
High Resolution STR Typing



SNaPshot SNP Typing
(Coding Region mtSNP 11plex minisequencing assay)

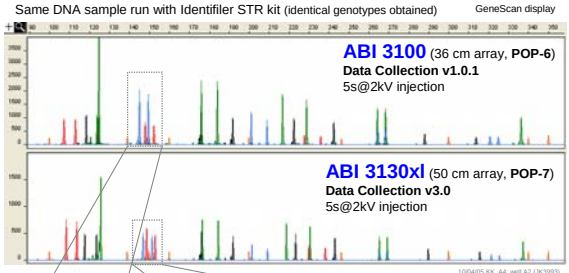


mtDNA Sequencing (HV1)



Comparison of ABI 3100 Data Collection Versions

Same DNA sample run with Identifiler STR kit (identical genotypes obtained) GeneScan display



v1.0.1 v3.0

Relative peak height differences are due to "variable binning" with newer ABI data collection versions.

Difference in the STR allele relative mobilities (peak positions) are from using POP-6 vs. POP-7.

Consumables for ABI 310/3100

What we use at NIST

- A.C.E.™ Sequencing Buffer 10X (Amresco)
  - $155/L = $0.0155/mL 1X buffer **(costs 20 times less!)**
  - http://www.amresco-inc.com
- 3700 **POP-6 Polymer** (Applied Biosystems)
  - $530 / 200 mL = $2.65/mL **(costs 20 times less!)**

What ABI protocols suggest

- 10X Genetic Analyzer Buffer with EDTA
  - $78/25 mL = $0.312/mL 1X buffer (ABI)
- 3100 POP-4 Polymer
  - $365 / 7 mL = $52/mL

2004 prices

Maintenance of ABI 310/3100/3130

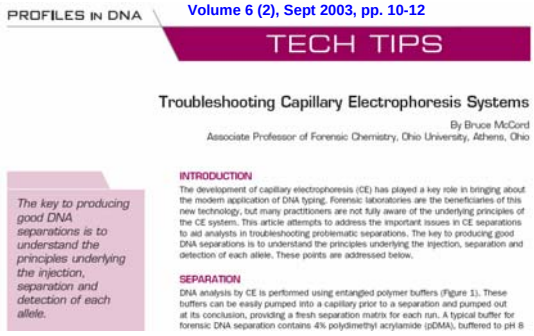
- Syringe – leaks cause capillary to not fill properly
- Capillary storage & wash – **it dries, it dies!**
- Pump block – cleaning helps insure good fill
- Change the running buffer regularly

YOU MUST BE CLEAN AROUND A CE!

Overall Thoughts on the ABI 310/3100/3130

- Settling on a common instrument platform has been good for the forensic DNA community in terms of data consistency (this is also true with the use of common STR kits)
- I am concerned that the community is very dependent primarily on one company…
- I really like using the instrument and can usually get nice data from it
- Like any instrument, it has its quirks…

Bruce McCord's *Profiles in DNA* Article



PROFILES IN DNA

Volume 6 (2), Sept 2003, pp. 10-12

TECH TIPS

Troubleshooting Capillary Electrophoresis Systems

By Bruce McCord
Associate Professor of Forensic Chemistry, Ohio University, Athens, Ohio

The key to producing good DNA separations is to understand the principles underlying the injection, separation and detection of each allele.

INTRODUCTION

The development of capillary electrophoresis (CE) has played a key role in bringing about the modern application of DNA typing. Forensic laboratories are the beneficiaries of this new technology, but many practitioners are not fully aware of the underlying principles of the CE system. This article attempts to address the important issues in CE separations to aid analysts in troubleshooting problematic separations. The key to producing good DNA separations is to understand the principles underlying the injection, separation and detection of each allele. These points are addressed below.

SEPARATION

DNA analysis by CE is performed using entangled polymer buffers (Figure 1). These buffers can be easily pumped into a capillary prior to a separation and pumped out at its conclusion, providing a fresh separation matrix for each run. A typical buffer for forensic DNA separation contains 4% polydimethyl acrylamide (pDMA), buffered to pH 8

CE Troubleshooting

Bruce McCord, AAFS 2006 Workshop (Seattle, WA)
February 20, 2006

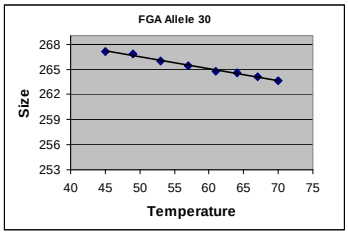
Outline for This Section

1. Chemistry/molecular biology problems – stutter, -A, degradation, inhibition, low copy #
2. Sample and buffer problems – formamide, urea, water, salt concentration, free dye ("dye blobs")
3. External factors – power supply, room temperature, cleanliness, voltage leaks
4. Instrument problems – optical system, capillary clogging, air bubbles, syringe leaks
5. Troubleshooting benchmarks/QC monitoring

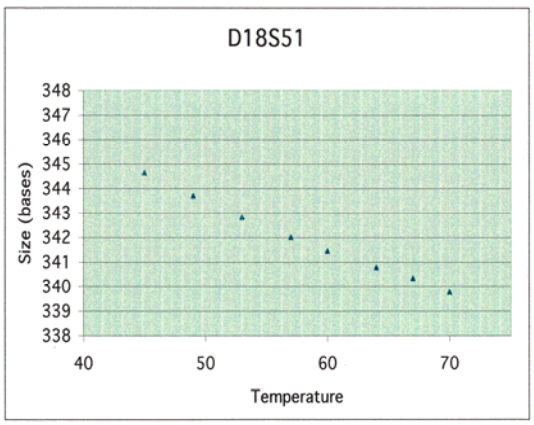
3. External Factors

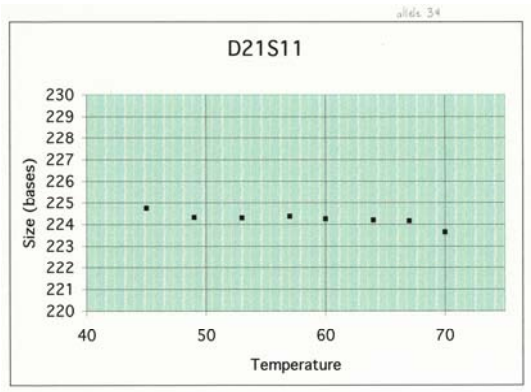
- Room temperature
  - Variations in room temperature can cause mobility shifts with band shifts and loss of calibration
  - Temperature is also important due to effects of high humidity on electrical conductance
- Cleanliness
  - Urea left in sample block can crystallize and catalyze further crystal formation causing spikes, clogs and other problems.
  - Best bet is to keep polymer in system and not remove or change block until polymer is used up.

Effect of Temperature on allele size

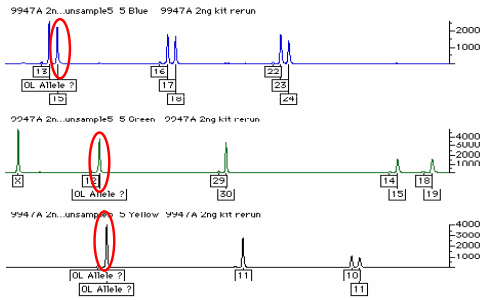


Slope is 0.14 bases/degree centigrade
Therefore a small change in temperature has a big effect
(A 1-2 degree shift in temperature of the heat plate can produce an OL allele)

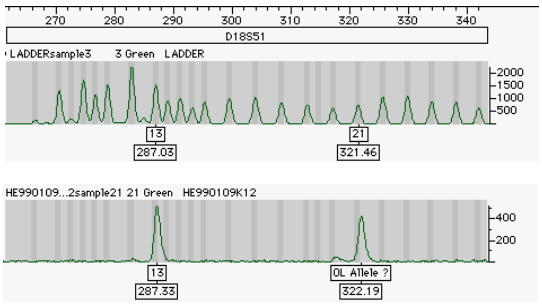




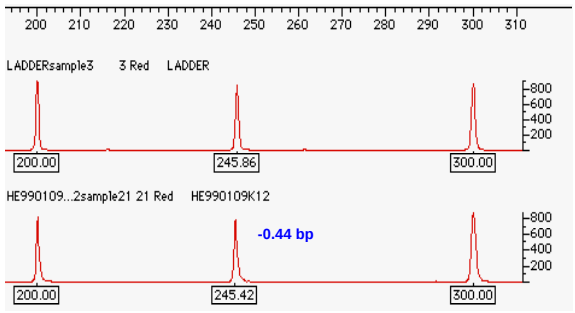
Temperature Effects: "OL" Alleles



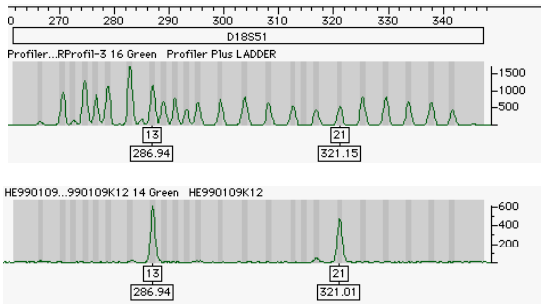
"OL Alleles"



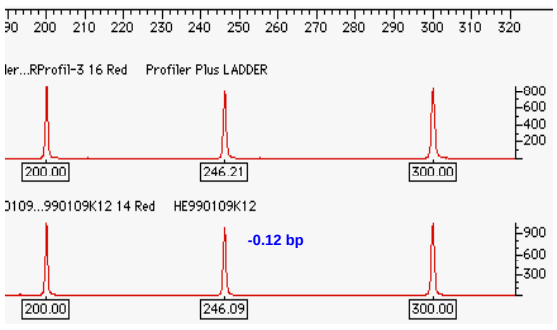
"OL alleles " - look at the 250 peak



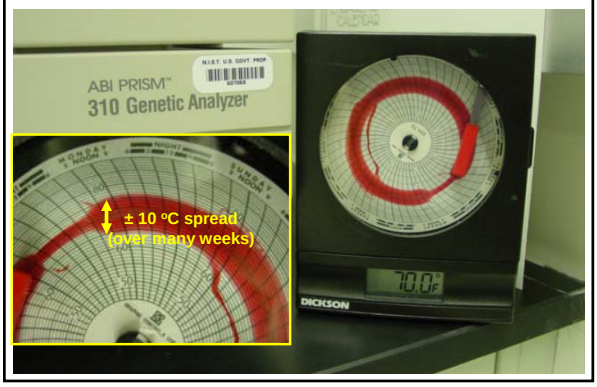
"OL allele re-injected"



And the 250 peak...



Monitoring Room Temperature Over Time



Temperature Probes

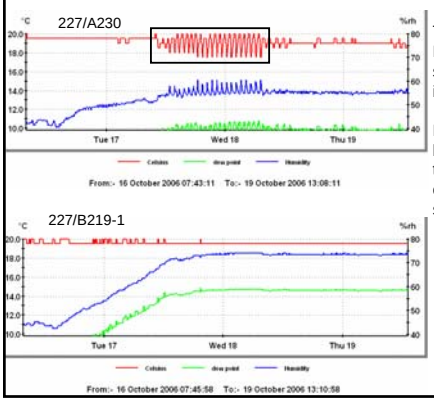
Refrigerator and freezer monitoring

Frig/Freeze Monitors $240
#DT-23-33-80 – USB Temperature Datalogger PLUS Software $79.00 (#DT-23-33-60)

Room Monitors, # DT-23039-52 – USB Temperature-Humidity Datalogger $91.00
(Cole Parmer, Vernon Hills IL)

Room temperature monitoring

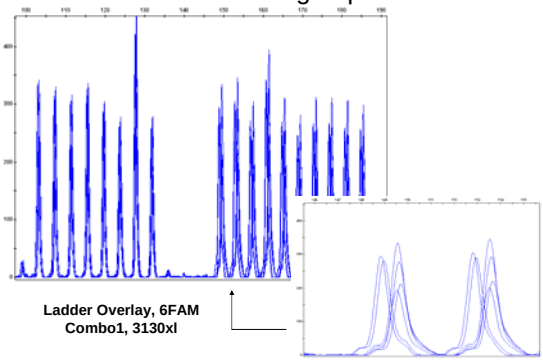
Monitoring Instrument Room Temperature Fluctuations



Temperature Monitoring of two separate instrument rooms.

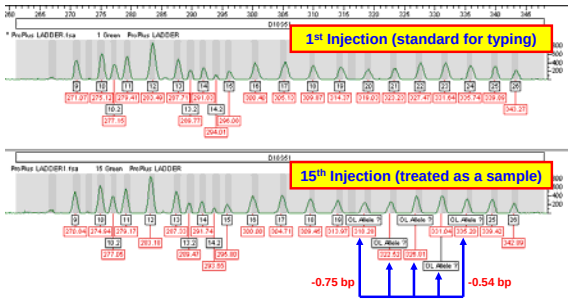
Box area is a 24 hour period where temperature control is not stable.

Poor Temperature Control Causes DNA Sizing Imprecision



Ladder Overlay, 6FAM Combo1, 3130xl

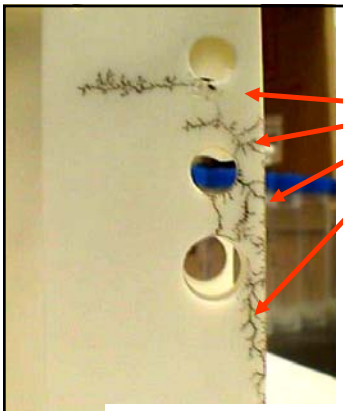
Use of Second Allelic Ladder to Monitor Potential Match Criteria Problems



These alleles have drifted outside of their genotyping bins due to temperature shifting over the course of the sample batch

Cleanliness

- Urea sublimates and breaks down to ionic components - these find a path to ground
- Similarly wet buffer under a vial creates paths to ground
- Capillary windows must be clear or matrix effects will occur
- Laser will often assist in this process
- Vial caps will transfer low levels of DNA to capillary



Carbon Trails

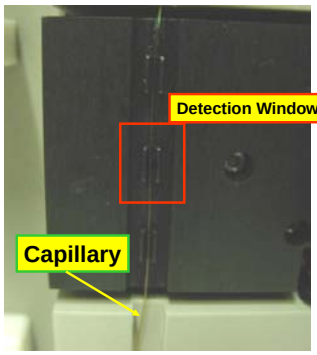
High Humidity or wet buffer vials can create other paths to ground

Keep Your System Clean!

4. Instrumental Factors

- Optical System
  - Sensitivity changes with age, capillary diameter, capillary cleanliness, instrument calibration
- Fluidic System
  - Effects of bubbles, dust, urea crystals, leaks in syringe and capillary ferrule
- Matrix Calculations
  - Changes in buffer, optics, sample dye can alter the software calibrations
- Capillary Problems
  - Chemisorbed materials on capillary surface can produce osmotic flow, DNA band broadening and inconsistent resolution (meltdowns)

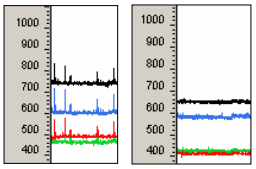
The Detection Window



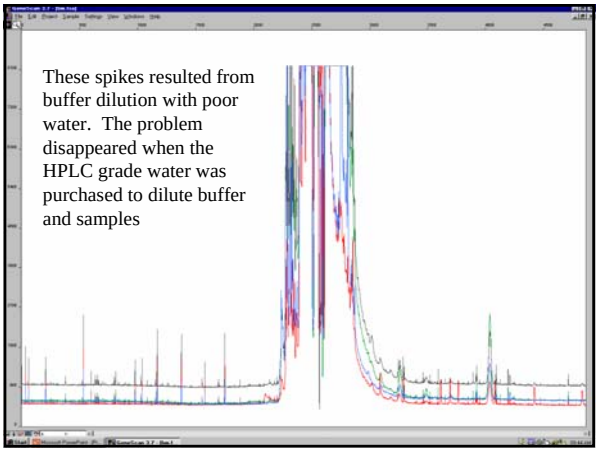
Make sure that the capillary window is lined up (if it is not, then no peaks will be seen)

Window may need to be cleaned with ethanol or methanol

Review Start of Raw Data Collection

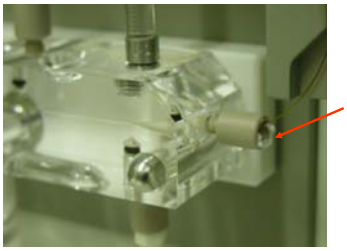


Little spikes indicate need to change buffer… check current



These spikes resulted from buffer dilution with poor water. The problem disappeared when the HPLC grade water was purchased to dilute buffer and samples

Beware of Urea Crystals



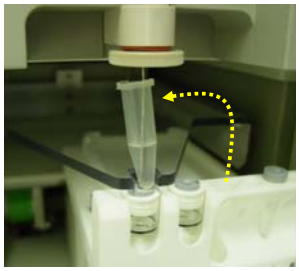
Urea crystals have formed due to a small leak where the capillary comes into the pump block

Urea sublimates and can evaporate to appear elsewhere

Use a small balloon to better grip the ferrule and keep it tight

Pump block should be well cleaned to avoid problems with urea crystal formation

Storage when ABI 310 is not in use



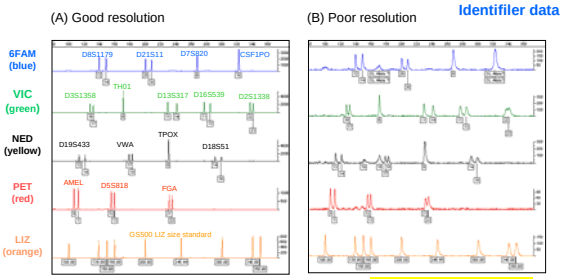
Remember that the water in the open tube will evaporate over time…

- Keep inlet of capillary in water…if it dries out then urea crystals from the polymer will clog the opening
- The waste vial (normally in position 3) can be moved into position
- A special device can be purchased from Suppelco to rinse the capillary off-line
- Store in distilled water
- Note that the laser is on when the instrument is on

Buffer Issues

- The buffer and polymer affect the background fluorescence- affecting the matrix
- Urea crystals and dust may produce spikes
- High salt concentrations may produce reannealing of DNA
- High salt concentrations affect current
- Low polymer concentrations affect peak resolution

Capillary Meltdowns



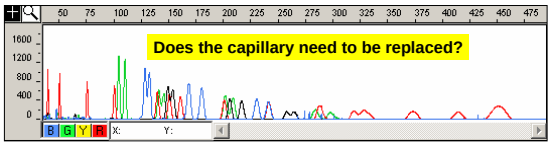
Good Capillary in 3100 Array

Bad Capillary in 3100 Array

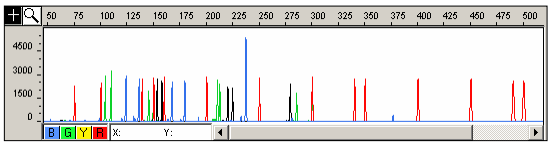
Butler, J.M., Buel, E., Crivellente, F., McCord, B.R. (2004) Forensic DNA typing by capillary electrophoresis: using the ABI Prism 310 and 3100 Genetic Analyzers for STR analysis. *Electrophoresis*, 25: 1397-1412.

Meltdowns can be permanent or transitory

as we have seen these may result from sample contamination effects



No! The next injection looks fine…



Meltdowns may be the result of

- Bad formamide
- Excess salt in sample/renaturation
- Water in the polymer buffer
- **Syringe leak** or bottom out
- Poisoned capillary
- Conductive polymer buffer due to urea degradation
- Crack/shift in capillary window
- Detergents and metal ions

5. Troubleshooting benchmarks

- **Monitor run current**
- Observe syringe position and movement during a batch
- Examine ILS (ROX) peak height with no sample
- Observe “250 bp” peak in GS500 size standard
- Monitor resolution of TH01 9.3/10 in allelic ladder and size standard peak shapes
- **Keep an eye on the baseline signal/noise**
- Measure formamide conductivity
- Reagent blank – are any dye blobs present?
- See if positive control DNA is producing typical peak heights (along with the correct genotype)

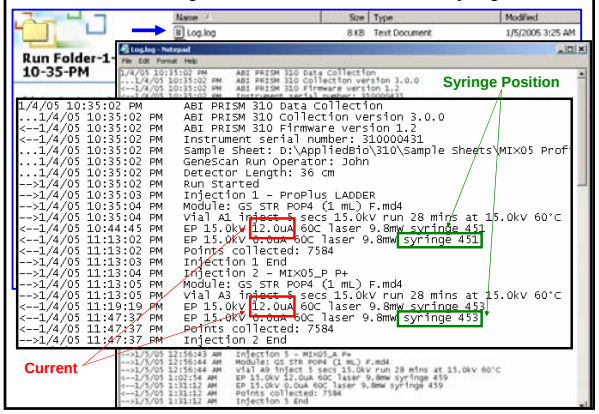
Measurement of Current

- V/I = R where R is a function of capillary diameter, [buffer], and buffer viscosity
- In a CE system the voltage is fixed, thus changes in resistance in the capillary will be reflected in the current observed
- Air bubbles, syringe leaks, alternate paths to ground, changes in temperature, changes in zeta potential, and contamination, will be reflected in the current
- A typical current for a CE system with POP4 buffer is **8-12 µA** (microamps)

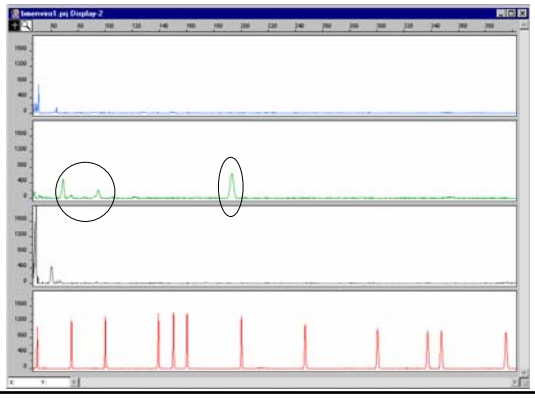
Syringe Travel

- The ABI 310 instrument also keeps track of the position of the syringe (in the log file)
- Depending on the resistance to flow, the syringe will travel different lengths
- Syringe leaks may be reflected in a longer distance traveled prior to each injection
- These leaks occur around the barrel of the syringe and at the connection to the capillary block

Use of ABI 310 Log File to Monitor Current and Syringe Travel



Dye Blobs in the Negative Control Sample



Measuring Formamide Conductivity

(not this way)

The key is to measure the bottle when it comes in or buy the good stuff and immediately pipette it out into small tubes with or without ROX already added. Then freeze the tubes.

Do not ever open a cold bottle of formamide. Water will condense inside and aid in the formation of conductive formic acid.

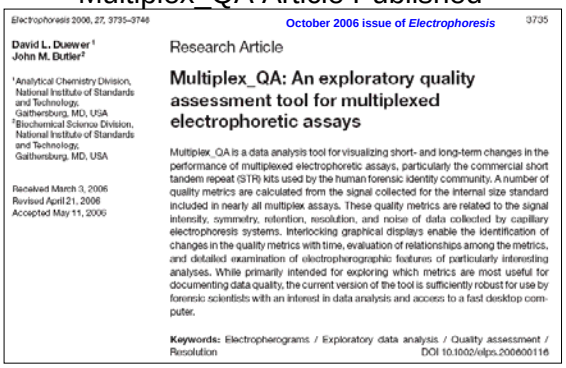
Conclusion:

Troubleshooting is more than following the protocols

It means keeping watch on all aspects of the operation

1. Monitoring conductivity of sample and formamide
2. Keeping track of current and syringe position in log.
3. Watching the laser current
4. Watching and listening for voltage spikes
5. Monitoring room temperature and humidity

Multiplex_QA Article Published



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Research Article

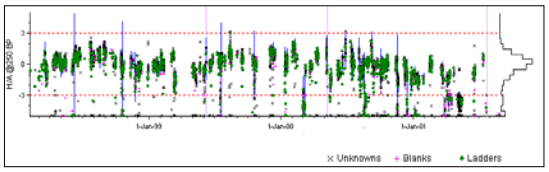
Multiplex_QA: An exploratory quality assessment tool for multiplexed electrophoretic assays

Multiplex_QA is a data analysis tool for visualizing short- and long-term changes in the performance of multiplexed electrophoretic assays, particularly the commercial short tandem repeat (STR) kits used by the human forensic identity community. A number of quality metrics are calculated from the signal collected for the internal size standard included in nearly all multiplex assays. These quality metrics are related to the signal intensity, symmetry, retention, resolution, and noise of data collected by capillary electrophoresis systems. Interlocking graphical displays enable the identification of changes in the quality metrics with time, evaluation of relationships among the metrics, and detailed examination of electropherographic features of particularly interesting analyses. While primarily intended for exploring which metrics are most useful for documenting data quality, the current version of the tool is sufficiently robust for use by forensic scientists with an interest in data analysis and access to a fast desktop computer.

Keywords: Electropherograms / Exploratory data analysis / Quality assessment / Resolution — DOI 10.1002/elps.200600116

User manual (127 pages) available for download from STRBase

Multiplex_QA Overview



- **Research tool** that **provides quality metrics to review instrument performance over time** (e.g., examines resolution and sensitivity using internal size standard peaks)
- Runs with Microsoft Excel macros. Requires STR data to be converted with NCBI's BatchExtract program into numerical form.

Available for download from STRBase:
http://www.cstl.nist.gov/biotech/strbase/software.htm

Acknowledgments

NIST Human Identity Project Team

Leading the Way in Forensic DNA…

John Butler (Leader) | Margaret Kline | Pete Vallone | Jan Redman | Amy Decker | Becky Hill | Dave Duewer

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Many wonderful collaborators from industry, university, and government laboratories.

Bruce McCord (Florida International University) for many of the slides

Thank you for your attention…

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

Questions?

See also http://www.dna.gov/research/nist

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Review

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Forensic DNA typing by capillary electrophoresis using the ABI Prism 310 and 3100 genetic analyzers for STR analysis

DNA typing with short tandem repeat (STR) markers is now widely used for a variety of applications including human identification. Capillary electrophoresis (CE) instruments, such as the ABI Prism 310 and ABI 3100 Genetic Analyzers, are the method of choice for many laboratories performing STR analysis. This review discusses issues surrounding sample preparation, injection, separation, detection, and interpretation of STR results using CE systems. Requirements for accurate typing of STR alleles are considered in the context of what future analysis platforms will need to increase sample throughput and ease of use.

Keywords: Capillary electrophoresis / DNA typing / Forensic science / Review / Short tandem repeat
DOI 10.1002/elps.200305822

CE and CEC

Contents

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1 Introduction

1.1 General aspects

The law enforcement community has greatly benefited from recent developments in the area of DNA testing. Forensic laboratories may now match minuscule amounts of biological evidence from a crime scene to the perpetrator and can reliably exclude falsely accused individuals. In the past two decades, numerous advances in DNA testing technologies have occurred, most notably among them the development of polymerase chain reaction (PCR)-based typing methods [1–2].

Today, the forensic DNA typing community has standardized on the use of short tandem repeat (STR) markers [1–4]. In November 1997, the Federal Bureau of Investigation (FBI) selected 13 STR markers to serve as the core of its Combined DNA Index System (CODIS) [5]. These markers are CSF1PO, FGA, TH01, TPOX, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, and D21S11 (Table 1). Multiplex PCR amplification of all or a subset of these STR markers is possible with a variety of commercial STR kits using spectrally resolvable fluorescent dyes (Table 2). The availability of commercial STR kits has greatly simplified the use of STRs in recent years and aided the

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Table 1. Information on 13 STR markers used in the FBI's CODIS DNA database and other STR markers contained in commercial kits

Locus name	Chromosomal location	Repeat motif	GenBank accession	Allele range[a)]	Number of alleles seen[b)]
CSF1PO	5q33.1 c-fms proto-oncogene, 6th intron	TAGA	X14720	6–16	15
FGA	4q31.3 α-Fibrinogen, 3rd intron	CTTT	M64982	15–51.2	69
TH01	11p15.5 Tyrosine hydroxylase, 1st intron	TCAT	D00269	3–14	20
TPOX	2p25.3 Thyroid peroxidase, 10th intron	GAAT	M68651	6–13	10
VWA	12p13.31 von Willebrand factor, 40th intron	[TCTG][TCTA]	M25858	10–24	28
D3S1358	3q21.31	[TCTG][TCTA]	NT_005997	9–20	20
D5S818	5q23.2	AGAT	G08446	7–16	10
D7S820	7q21.11	GATA	G08616	6–15	22
D8S1179	8q24.13	[TCTA][TCTG]	G08710	8–19	13
D13S317	13q31.1	TATC	G09017	5–15	14
D16S539	16q24.1	GATA	G07925	5–15	10
D18S51	18q21.33	AGAA	L18333	7–27	43
D21S11	21q21.1	Complex [TCTA][TCTG]	AP000433	24–38	70
Other STRs included in kits from Applied Biosystems or Promega					
Penta D	21q22.3	AAAGA	AP001752	2.2–17	14 alleles
Penta E	15q26.2	AAAGA	AC027004	5–24	21 alleles
D2S1338	2q35	[TGCC][TTCC]	G08202	15–28	14 alleles
D19S433	19q12	AAGG	G08036	9–17.2	15 alleles
SE33	6q15	AAAG	V00481	4.2–37	>50 alleles

a) Numbers in this column refer to the number of repeat units present in the alleles.
b) See Appendix 1 in [1]

development of large and effective DNA databases [6]. A report by the National Commission on the Future of DNA Evidence [7] concludes that STR typing will likely be the primary means of forensic DNA analysis for the next 5–10 years because of the need for consistency in national and international DNA databases. STR markers offer a number of advantages over previously used methods for DNA typing including the ability to obtain results from degraded DNA samples and extremely small amounts of DNA [1]. The process is fairly rapid and results may routinely be obtained in less than one working day.

Figure 1 illustrates how an STR marker within a DNA template is targeted with a forward and reverse PCR primer that anneal on either side of the repeat region. One of the primers is labeled on the 5'-end with a fluorescent dye that enables detection of the resulting PCR product following amplification. The position of the primers defines the overall PCR product size as does the number of repeats present in the STR region. PCR products are separated by size and dye color using electrophoresis followed by laser-induced fluorescence with multiwavelength detection. An internal standard, containing DNA fragments of known size and labeled with a different dye color, is typically coelectrophoresed with each sample to calibrate sizes from run to run. The collected data in the form of multicolored electropherograms are analyzed by software that automatically determines STR allele sizes based on a standard curve produced from the internal size standard. STR genotyping is performed by comparing the allele sizes in each sample to the sizes of alleles present in an allelic ladder, which contains common alleles that have been previously sequenced [8]. On a capillary electrophoresis (CE) system, the allelic ladder is

Table 2. Commonly used STR kits for analysis on ABI Prism 310 Genetic Analyzer

STR kit name	Source	Dye color	STR markers amplified in kit (shown in order of increasing PCR product size)
AmpFlSTR® Profiler Plus™	Applied Biosystems	B	D3S1358, VWA, FGA
		G	Amelogenin, D8S1179, D21S11, D18S51
		Y	D5S818, D13S317, D7S820
AmpFlSTR COfiler™	Applied Biosystems	B	D3S1358, D16S539
		G	Amelogenin, TH01, TPOX, CSF1PO
		Y	D7S820
AmpFlSTR SGM Plus™	Applied Biosystems	B	D3S1358, VWA, D16S539, D2S1338
		G	Amelogenin, D8S1179, D21S11, D18S51
		Y	D19S433, TH01, FGA
AmpFlSTR Identifiler™ (5-dyes)	Applied Biosystems	B	D8S1179, D21S11, D7S820, CSF1PO
		G	D3S1358, TH01, D13S317, D16S539, D2S1338
		Y	D19S433, VWA, TPOX, D18S51,
		R	Amelogenin, D5S818, FGA
AmpFlSTR SEfiler™ (5-dyes)	Applied Biosystems	B	D3S1358, VWA, D16S539, D2S1338
		G	Amelogenin, D8S1179, SE33
		Y	D19S433, TH01, FGA
		R	D21S11, D18S51
PowerPlex® 1.2	Promega	B	D5S818, D13S317, D7S820, D16S539
		Y	VWA, TH01, Amelogenin, TPOX, CSF1PO
PowerPlex 16	Promega	B	D3S1358, TH01, D21S11, D18S51, Penta E
		G	D5S818, D13S317, D7S820, D16S539, CSF1PO, Penta D
		Y	Amelogenin, VWA, D8S1179, TPOX, FGA
PowerPlex ES	Promega	B	D3S1358, TH01, D21S11, D18S51
		G	SE33 (ACTBP2)
		Y	Amelogenin, VWA, D8S1179, FGA
PowerPlex Y	Promega	B	DYS391, DYS389I, DYS439, DYS389II
		G	DYS438, DYS437, DYS19, DYS392
		Y	DYS393, DYS390, DYS385 a/b
Y-PLEX™ 6	ReliaGene Technologies	B	DYS393, DYS19, DYS389II
		Y	DYS390, DYS391, DYS385 a/b
Y-PLEX 5	ReliaGene Technologies	B	DYS389I, DYS389II
		G	DYS439
		Y	DYS438, DYS392
Y-PLEX 12	ReliaGene Technologies	B	DYS392, DYS390, DYS385 a/b
		G	DYS393, DYS389I, DYS391, DYS389II
		Y	Amelogenin, DYS19, DYS439, DYS438

An internal size standard is typically run in the fourth or fifth dye position. Dye colors, blue (B), green (G), yellow (Y), or red (R). See [78] for more information on the Y-STR loci and kits.

run along with the internal size standard in one injection, and sample alleles with the same internal size standard are run in subsequent injections on the capillary in a sequential fashion [9].

In order to accurately genotype STR markers using multicolor fluorescence detection, a separation and detection technique must exhibit the following characteristics: (i) Methods for reliable sizing over a 75–500 bp size range; (ii) high run-to-run precision between processed samples to permit comparison of allelic ladders to sequentially processed STR samples; (iii) effective color separations of different dye sets used to avoid bleed through between four or five different colors; (iv) resolution of at least 1 bp to approximately 350 bp to permit reliable detection of microvariant alleles.

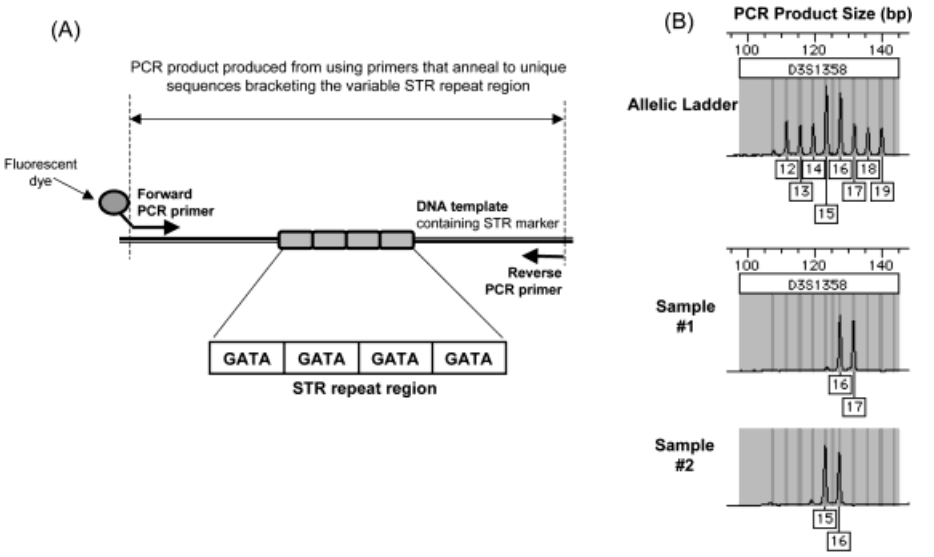


Figure 1. (A) Schematic of PCR primer positions for amplification of a STR DNA marker. The single-headed arrows represent the primer positions. The double-headed arrows illustrate the overall PCR product size using a particular set of primers. The PCR product size is measured and converted back to the number of repeat units present in the sample for genotyping purposes. A fluorescent dye is present on one of the primers in order to label the PCR product with a specific color. (B) Allelic ladder for the STR marker D3S1358 shown above two heterozygous DNA samples exhibiting different genotypes. Values below the peaks indicate the number of tandem repeats present in the measured allele.

Early work with STR markers used polyacrylamide gels [3, 4, 10]. However, CE, where the DNA molecules are separated in a narrow glass tube, has become increasingly popular for STR typing because it eliminates the need to pour gels and to load the DNA samples onto the gel. CE offers greater automation at the injection and detection phases of DNA analysis. In addition, CE consumes only a small portion of the actual sample so that it can be retested if needed. This article will review the use of CE for DNA analysis and its application to STR typing. The primary focus will be on the chemistry, hardware, and software used with the ABI Prism 310 Genetic Analyzer from Applied Biosystems as it is the most widely used instrument today for STR analysis. Higher throughput approaches for STR typing will also be discussed including the 16-capillary ABI 3100 Genetic Analyzer.

1.2 Early work with CE

Since the first description of electrophoresis in small diameter tubes [11, 12], CE has been identified as a powerful analytical technique capable to replace slab gel-based electrophoresis of nucleic acids. In CE the separation takes place in a capillary with an internal diameter of 50–100 μm). The narrow capillary enables the application of high electric fields, and thus faster run times, without overheating problems associated with the high voltages used. In addition, the capillary can be easily manipulated for automated injections. CE has been shown to be a versatile technique and has been used for a variety of forensic applications including analysis of gunshot residues, explosive residues, and drugs as well as DNA typing [13]. Since 1996, CE results have been admissible in courts of law [14].

Early work with CE and STR typing used instruments having UV detection [15] or laser-induced fluorescence detection of a single color [16]. In these cases, dual internal size standards had to bracket the allelic ladder or amplified alleles in order to accurately type the STR alleles [17]. The advent of the ABI Prism 310 Genetic Analyzer in July 1995 with its multicolor fluorescence detection capabilities opened a whole new world to STR typing. The ability to examine more than one wavelength simultaneously during electrophoresis permits a higher density of genetic information to be obtained. CE systems have played a vital role in other applications such as sequencing the human genome [18]. Thousands of CE instruments are in use around the world now for DNA sequencing and genotyping. A search of the PubMed database in October 2003 located more than 1300 references with keywords of DNA and CE.

The ABI 310 Genetic Analyzer instrument is probably the most widely used platform for STR testing today. DNA samples are processed in a serial fashion at a rate of approximately one sample per 30 min on this single-capillary instrument. The multi-capillary ABI 3100 became available in the spring of 2001 and has become

the instrument of choice for many laboratories needing an increased level of throughput. The steps for processing DNA samples through size and color separations are illustrated in Fig. 2. Issues impacting sample injection, separation, detection, and interpretation for reliable STR typing are addressed below (Fig. 3).

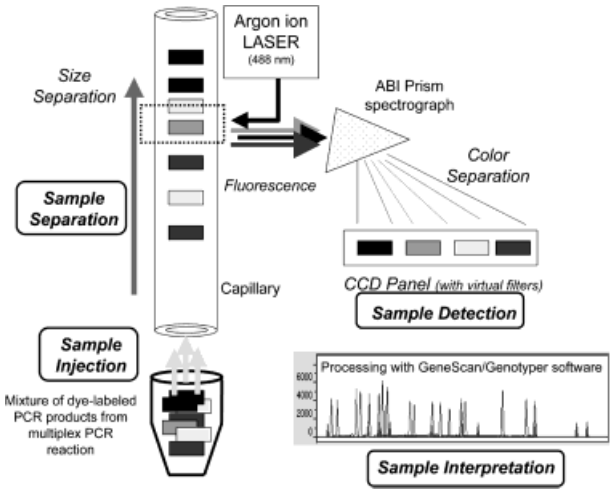


Figure 2. Schematic illustration of the separation and detection of STR alleles with an ABI Prism 310 Genetic Analyzer.

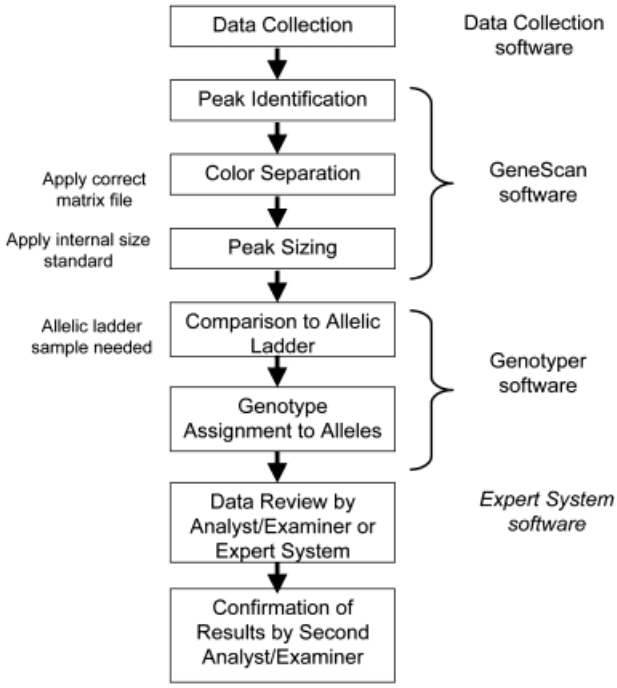


Figure 3. Sample interpretation and genotyping process for STR allele determination (see [1]). Software packages for DNA fragment analysis and STR genotyping perform much of the actual analysis, but extensive review of the data by trained analysts/examiners is often required.

2 Sample preparation and injection

A major advantage of CE is that samples can be loaded onto the separation medium in an automated fashion from a sample plate. Traditional gel electrophoresis techniques require careful manual loading of samples prior to initiating electrophoresis although some methods for comb loading with robotic spotting have been described [19]. Samples for CE separation are usually prepared by diluting a small portion of the PCR product into water or deionized formamide. Another significant advantage for CE in the context of forensic analysis is that only a small portion of the actual sample is examined each time. It may be reinjected additional times if needed for retesting purposes.

Most CE systems utilize electrokinetic injection, where a voltage is applied for a defined time, to move charged molecules from the sample into the capillary. As DNA is negatively charged, a positive voltage is applied to draw the DNA molecules into the capillary. Electrokinetic injections produce narrow injection zones, but are highly sensitive to the sample matrix. In general, the quantity of DNA injected onto a CE column ($[Q_{inj}]$) is a function of the electric field (E), the injection time (t), the true concentration of DNA in the sample ($[DNA_{sample}]$), the area of the capillary opening (πr^2), and the ionic strength of the sample (λ_{sample}) *versus* the buffer (λ_{buffer}). This can be described by the following equation [20]:

$$[DNA_{inj}] = Et(\pi r^2)(\mu_{ep} + \mu_{eof})[DNA_{sample}](\lambda_{buffer}/\lambda_{sample}) \quad (1)$$

where r is the radius of the capillary, μ_{ep} is the mobility of the sample molecules, and μ_{eof} is the electroosmostic mobility, which is hopefully negligible in a coated capillary.

However, this equation assumes no interfering ions are present. The addition of ions such as Cl^- from the PCR reaction mixture will compete with DNA and reduce the total amount of DNA injected onto the capillary because the sample conductivity (λ_{sample}) will be higher. As Cl^- ions are smaller than DNA molecules, they will have a higher charge/mass ratio and subsequently a higher sample mobility (μ_{ep}). Likewise, smaller DNA molecules, such as remaining PCR primers, will travel more quickly into the capillary opening from the sample solution than the larger PCR products.

To reduce this sample bias problem with electrokinetic injection, PCR samples can be purified by means of dialysis [16, 21], spin columns [15, 22, 23] or ethanol precipitation [24]. The dialysis step appears to be the most effective for removing excess salt, while the spin columns are more effective at removing primer peaks, enzyme and deoxy nucleotide triphosphates (dNTPs). However, early in the development of DNA testing with CE, it was demon-

strated that a simple dilution of the sample in water or deionized formamide can be an effective method for sample preparation because the sample ionic strength is reduced relative to the buffer ionic strength [17].

Since formamide is a strong denaturant, it is commonly used in the preparation of single-stranded DNA samples for CE. Merely placing a sample in formamide is sufficient to denature it. However, rapid heating to 95°C and snap-cooling on ice is commonly performed to ensure that the denaturation process has occurred. Use of high-quality formamide with a low conductivity is important. Formamide produces ionic decomposition products including formic acid, which is negatively charged at a neutral pH and will be preferentially injected into the capillary. The formamide by-products can cause problems in both sensitivity and resolution [25]. The quality of formamide can be easily measured using a portable conductivity meter and should be 80 μS or less to obtain the best results. Many laboratories buy ultrapure formamide and freeze aliquots immediately to ensure sample quality. Water has also been successfully used in the preparation of STR samples for CE analysis instead of formamide [17, 26]. Use of deionized water can eliminate the health hazard and the cost of formamide as well as problems with disposal. While studies have shown that water gives fully concordant results with formamide, long-term sample stability suffers because DNA molecules will renature in water after a few days.

A useful method for keeping the sample zone narrow and improving the amount of analyte placed onto the column during an injection involves a process commonly called sample stacking [27, 28]. Stacking, also called field-amplified injection, occurs when the ionic strength of the sample zone is lower than that of the buffer. This is in effect what is happening when a sample is diluted in deionized water or formamide. As the current through the system is constant, the lack of charge carriers in the sample zone produces a strong electric field that ends abruptly at the interface between the sample zone and the buffer inside the capillary. DNA molecules mobilized by this field move rapidly towards the capillary as the injection voltage is applied and "stack" in a narrow zone at the interface. Stacking allows a large sample zone to be loaded onto the capillary with a minimum of band broadening. Stacking also aids in producing efficient separations. With sharp injection zones, shorter capillaries and less gel media is required to effect a separation. The key to producing a good stacking interaction is to produce a zone of low conductivity immediately in front of the sample. This is facilitated in many CE systems by dipping the capillary in water just prior to sample injection. Other methods can also be utilized such as on-line sample dialysis or buffer neutralization with NaOH [29], but these are more difficult to implement. In forensic analyses these methods are typically not employed since sufficient sample stacking occurs through the dilution of the amplified sample.

3 Sample separation

Besides the width of the sample injection zone, there are several other components that impact DNA separations within CE systems: the polymer used for enabling the separation, the capillary, the electrophoresis buffer, and the field strength [30]. STR allelic ladders are useful tools for monitoring system resolution (see Fig. 4).

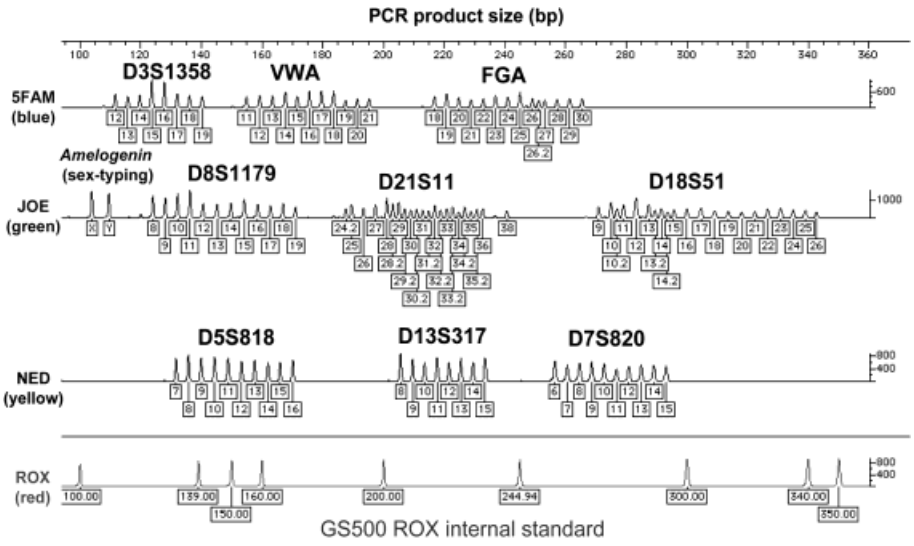


Figure 4. Allelic ladders present in the Profiler Plus STR kit from Applied Biosystems. Note the clean color separation (*i.e.*, no pull-up between dye colors).

3.1 The polymer separation matrix

There are several different types of sieving media utilized in electrophoretic separations, depending on the physical characteristics of the media. Chemical gels such as the common polyacrylamide gels used in denaturing slab-gel electrophoresis are rigid cross-linked materials whose porous structure is linked together by strong covalent bonds. Agarose produces physical gels. This material's shape is the result of weaker intermolecular forces produced *via* entanglement of the various strands of different agarose molecules. Entangled polymers are the third type of sieving media. Similar to physical gels, these materials are also characterized by intermolecular interactions. However, such substances are not true gels, as they cannot hold their shape unless placed in some container such as a capillary. Entangled polymers are characterized by a rapid increase in viscosity as the polymer concentration reaches a certain threshold value. The viscosity of these materials is also dependent on the polymer's molecular weight. All of the above types of materials have been used in CE separations, and thus there is nothing especially novel about the CE method of electrophoresis other than the convenience of containing the gel in a capillary and the enhanced heat dissipation which results from the small cross sectional area of the capillary.

Early attempts to apply CE to the size separation of biomolecules were based on gel-filled capillaries (*e.g.*, cross-linked polyacrylamide or agarose) [31]. However, gel-filled capillaries presented several disadvantages: air bubble formation during the filling of the capillary as well as in the process of shrinkage of the gel during polymerization, limited their applications. Moreover gels, in particular acrylamide, suffer from degradation by hydrolysis, particularly at the alkaline pH commonly used to separate biopolymers. This degradation leads to short lifetimes for gel-filled capillaries. Currently, gel-filled capillaries play a minor role in DNA separation applications [32].

Capillary cross-linked gel systems have been replaced with entangled polymer solutions such as linear (uncross-linked) polyacrylamide [33]. The idea of using polymer solutions to separate biopolymers is not new, as it was proposed years ago by Bode [34, 35]. However, it only became popular in combination with CE, because the very efficient anticonvective and heat dissipation properties of thin capillaries permit separation in fluids without loss of resolution. Grossman and Soane [36, 37] demonstrated that by using a dilute, low-viscosity polymer solution as the separation medium, high-resolution separations of DNA mixtures could be achieved. Barron *et al.* [38] found that dilute solutions of hydroxyethylcellulose well below the entanglement threshold have the ability to separate large DNA fragments from 2000 to 23 000 bp. However, in a systematic study with small double-stranded DNA, the entangled polymer solutions gave superior separations over dilute solutions [39]. Therefore, for many high-resolution applications, such as DNA sequencing and genotyping, the properties of an entangled polymer network are needed.

Even though a great number of polymers exist which could potentially be used as a separation matrix for biological molecules, not all of them are suitable for standard CE systems. Especially in the new multicapillary devices, a low viscosity is needed to keep the technical sophistication low. Therefore, the ideal polymer should have at least the same separation properties as classical gels, combined with a low viscosity that would allow easy replacement. These conditions have been achieved with the performance optimized polymers, POP™-4 and POP™-6, from Applied Biosystems [40]. POP-4 is commonly used for DNA fragment analysis including STR typing while the POP-6 polymer, which is the same polydimethylacrylamide polymer present at a higher concentration, is capable of higher resolution to meet the single-base resolution needs of DNA sequencing.

3.2 The buffer

The buffer that is used to dissolve the polymer in CE systems is important as it stabilizes and solubilizes the DNA, provides charge carriers for the electrophoretic current, and can enhance injection. If the buffer concentration and concomitant conductivity are too high, then the column will overheat resulting in a loss of resolution. In the process of electrophoresis, the composition of the anode and the cathode buffers may change due to electrolysis and migration of buffer ions. Thus, to avoid problems with poor size calibration of the system over time, it is a good policy to periodically replace the CE buffers with fresh solution.

The Genetic Analyzer buffer commonly used with the ABI 310 is 100 mM TAPS and 1 mM EDTA, adjusted to pH 8.0 with NaOH [43]. TAPS is short for *N*-tris-(hydroxymethyl) methyl-3-aminopropane-sulfonic acid. TAPS is used instead of Tris-borate-EDTA (TBE) since TBE is temperature and pH-sensitive. As analysis temperature is increased with TBE, the pH decreases at a rate of 0.02 pH units with every 1°C. As pH decreases so does the fluorescence emission of many dyes [46].

The forensic community primarily uses the ABI 310 for the analysis of STRs. Under the analysis parameters typically employed for STR analysis, the amplified DNA fragments must remain denatured. To accomplish this DNA denaturation, the capillary column run temperature is set

to a higher than room temperature, and buffer additives such as formamide, urea, and 2-pyrrolidinone are added to keep the DNA from reannealing [43]. Even under strong denaturing conditions, DNA molecules can sometimes assume various conformations due to intramolecular attractions and capillary run temperatures of 60°C are commonly employed to help reduce secondary structure in DNA [43]. Thus, high concentrations of urea and elevated temperatures are used to keep the various STR alleles uniformly denatured, since the mobility of DNA fragments can be affected by its conformation. Even with these measures, the operator must take care to maintain their system at a stable ambient temperature, as temperature variations can have profound effects on allele migration [47]. Many laboratories assess an internal standard peak (such as the 250 peak in the ABI GS500 internal standard, see Figs. 4 and 5), which is particularly sensitive to temperature variation to demonstrate that their CE systems are stable and well calibrated [47]. CE analysis of DNA fragments at elevated pH conditions, where the DNA molecule is predominately denatured, suggests that DNA secondary structure is responsible for the variations observed in DNA size determinations with fluctuating temperatures [48–50]. By carefully controlling the run conditions, *i.e.*, pH, buffer, denaturants, and temperature, variations within and between runs can be minimized and overall run precision improved. Run-to-run precision can also be enhanced using a global Southern sizing algorithm rather than the traditional local Southern sizing [47, 51].

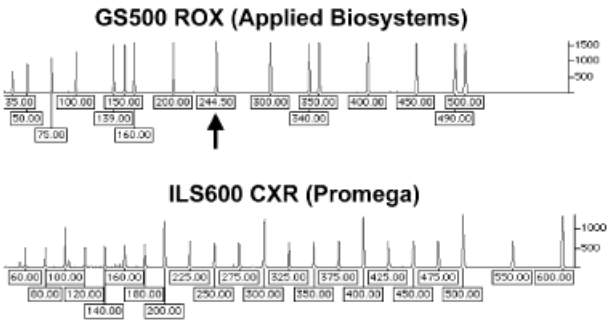


Figure 5. Two different internal size standards commonly used with STR typing. The ~245 bp peak (arrow) in the GS500 ROX standard is not included in the software calculations.

3.3 The capillary

The capillary column is central to the separation capabilities of CE. In uncoated capillary columns, residual charges on the silica surface induce a flow of the bulk solution toward the negative electrode. This process known as electroosmotic flow (EOF) creates problems for reproducible DNA separations because the velocity of the DNA molecules can change from run to run. Capillary and microchip channel walls, which contain charged silanol groups, are chemically modified [41] or dynamically coated [42, 43] to prevent EOF in DNA separations.

One method to accomplish EOF suppression in a fused-silica channel or capillary is to mask the charged sites on the wall by adsorption of neutral linear polymers that provide a viscous layer on the capillary surface [40, 42]. The commercially available poly-dimethylacrylamide POP-4 and POP-6 are successfully used in DNA genotyping by CE because they provide a sieving matrix for the separation of single-stranded DNA and, at the same time, suppress the EOF [43]. POP-4 consists of 4% linear dimethylacrylamide, 8 M urea, 5% 2-pyrrolidinone [43, 44]. For STR analysis, the run temperature is typically set at 60°C to further help keep the DNA strand denatured.

When using the ABI 310 Genetic Analyzer, an operator simply loads a batch of samples and leaves the instrument unattended. If a capillary failure occurs, all the subsequent analysis will be ruined. Thus, it is important to understand the potential issues involved in the breakdown of a capillary or series of analyses. Often, the causes of a capillary failure are unknown but they can result in loss of valuable time and effort. As capillary failures occur, migration times can shift or peaks can broaden (Fig. 6). Determining at which point the failure occurred is critical, as separations may be affected several runs prior to the perceived failure. To avoid this problem, it is common practice to dispose of capillaries before their useful lifetime has expired.

Failure to obtain successful results with CE may also occur due to capillary wall effects, which are the results of adsorption of sample and buffer components on the capillary surface. The theory of gel-based separations in CE generally ignores the capillary wall as a contributor to the separation, but under certain conditions the wall can play a major role in the quality of the separation [45]. One effect, which could lead to this type of behavior, is EOF. Under normal conditions this phenomena does not occur because the viscous polymer solution masks charged sites on the wall and resists the bulk flow. However, with continued operation, the buildup of contaminants gradually over the course of many separations can produce active sites along the wall. These sites produce a charge double layer along the capillary wall, which can induce bulk flow, destroying the reproducibility of the migration times and making the resultant data unreadable. Another potential problem with the buildup of active sites on a capillary wall is the adsorption of the DNA molecules resulting in loss of resolution as sample bands become diffuse.

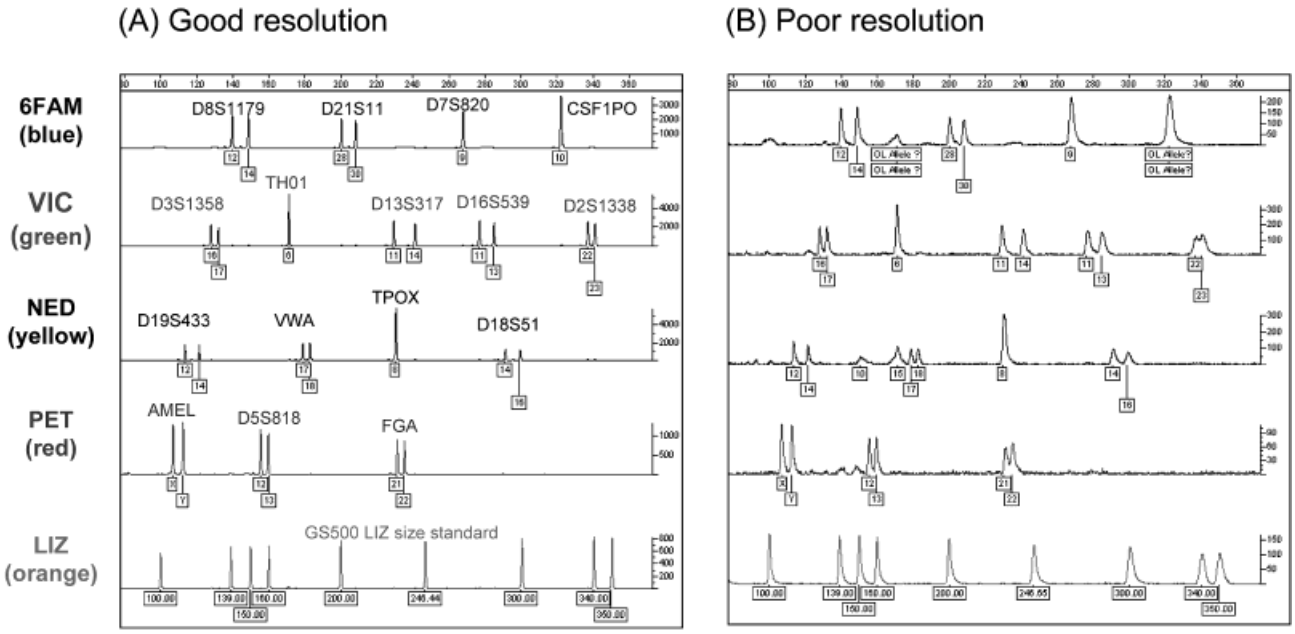
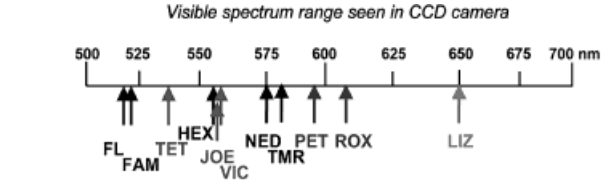


Figure 6. Comparison of same sample with (A) good resolution and (B) poor resolution due to a bad capillary. The STR kit used was the AmpFlSTR® Identifiler™ (5-dyes).

Manufacturers of capillaries often suggest replacing a capillary at around 100 injections to avoid problems with resolution failure. Capillary lifetimes can be improved by rinsing the capillary with consecutive washes of water, tetrahydrofuran, hydrochloric acid, and polymer solution [40]. Unfortunately, the ABI 310 instrument does not permit an on-the-instrument wash so the capillary must first be removed to conduct the rinsing procedure. With good sample preparation, many forensic laboratories see capillary lifetimes extend far past the 100 injections recommended by the manufacturer. Through effective monitoring of sample resolution [30] columns can be replaced when resolution declines. As the capillary column washing step is a manual procedure with the ABI 310, most forensic laboratories view capillary life spans of two to three hundred in number as acceptable, and hence columns with a large number of injections are viewed as expendable items.

4 Sample detection

Multiwavelength detection has expanded the capabilities of DNA analysis beyond a single-dye color and permitted greater multiplexing for STR markers. The key to the utilization of this technology is to covalently bind a different dye onto the 5'-(nonreactive) end of each primer or set of primers [52]. These dyes have a number of interesting properties. They are all excited by a single argon-ion laser tuned to 488 nm, yet fluoresce in different regions of the spectra. A multiwavelength analyzer, such as a charged-coupled device (CCD) camera, can then be used to determine which dye is present, based on the emission of each fragment as it passes the detector window. This technique permits the analysis of fragments of DNA that overlap in size as long as they are labeled with different dyes, which fluoresce at different wavelengths. The ABI 310 Genetic Analyzer uses virtual filters to collect the light striking the CCD camera at particular wavelength intervals. Figure 7 illustrates the fluorescence emission spec-



	Blue	Green	Yellow	Red	Orange	Used with These Kits
Filter A	FL	JOE	TMR	CXR		PowerPlex 16
Filter C	6FAM	TET	HEX	ROX		in-house assays
Filter F	5FAM	JOE	NED	ROX		Profiler Plus
Filter G5	6FAM	VIC	NED	PET	LIZ	Identifiler

Figure 7. Schematic of fluorescent dye emission maxima and virtual filters used in ABI 310 with various STR kits.

tra of the different dyes used to label the DNA and the position of several common virtual filters used in the ABI 310. The correct filter needs to be selected to match the fluorescent dye combinations in use in order to maximize sensitivity.

Note that in spite of the difference in emission wavelengths of the various dyes, there is still some overlap between them. To eliminate this spectral overlap between the dyes, a computer algorithm known as a matrix calculation is utilized to deconvolute the overlapping dyes and produce peaks that can be attributed to one fluorescent dye. Users of this technology must be careful to properly prepare and evaluate the matrix to calibrate their instruments to prevent what is known as "cross-talk", "bleed through", or "pull-up" between the different spectral channels. This "pull-up" problem is easily recognized as it results in the production of small peaks of a different color that occur at exactly the same size as a major peak in a different color. In addition, several artifacts peaks may also occur in some electropherograms such as residual dye "blobs" and spikes [53].

5 Sample interpretation

5.1 Software used

There are three software programs used to process data from the ABI 310 and produce STR genotypes: ABI 310 data collection software, GeneScan®, and Genotyper® (see Fig. 3). These programs were originally written for Macintosh computers but more recently have been adapted to run on Microsoft Windows NT. Applied Biosystems also has developed another program called GeneMapper® that combines the functions of GeneScan and Genotyper. The 310 data collection software [54] performs three primary functions: control of electrophoresis run conditions, control of which wavelengths of light will be examined on the CCD camera through the use of "virtual filters", and enables sample sheets and injection lists to be created whereby the sample name and processing order are specified. The user inputs the name of each sample and which dye colors are present in a sample sheet. The injection list controls the order in which each sample is injected onto the capillary as well as the time and voltage for the electrokinetic injection and electrophoresis voltage and run temperature. The virtual filter is also designated in the injection list depending on the dyes present in the sample being analyzed (Fig. 7). The output from the data collection program is "raw data" that comes in the form of relative fluorescence units on the *y*-axis and number of data points collected on the *x*-axis. The GeneScan and Genotyper programs are necessary to convert the raw data into the appropriately colored peak and to generate STR genotyping information.

GeneScan software [55] also performs three primary functions. It calls peaks based on threshold values specified by the user; it separates the peaks into the appropriate dye color based on a matrix file; and it sizes the STR allele peaks based on an internal size standard labeled with a different colored dye that is run in every sample. Typically, the internal standard is labeled with the red dye ROX while the STR alleles are labeled with blue, green, and yellow dyes (see Table 2). Different internal size standards may be used (Fig. 5). It is important to be consistent in the use of an internal size standard because all STR allele peaks are measured relative to this internal size standard. The default sizing algorithm, and one most commonly used, with the GeneScan program is the local Southern method [47, 56, 57]. The local Southern method measures the size of an unknown peak relative to its position from two peaks in the internal standard that are larger than the unknown peak and two that are smaller than the unknown peak. GeneScan software contains six different screens that may be used as part of data analysis and evaluation: processed data (color-separated), size standard curve, electrophoresis history, sample information, raw data (no color separation), and an analysis log file.

The Genotyper software program [58] takes GeneScan data and converts the sized peaks into genotype calls. Genotyping is performed by comparison of allele sizes in an allelic ladder to the sample alleles. The manufacturer of a particular STR kit normally provides Genotyper macros in order to make the allele calls from the allelic ladders. These macros can be designed to filter out stutter peaks (see [59]) that may interfere with sample interpretation.

5.2 Assessing resolution of DNA separations

Determining the resolution of an electropherogram allows the analyst to evaluate the performance of the CE system [30]. These resolution measurements can be useful in evaluating casework data, or assessing system modifications that may alter electrophoretic conditions. In the review of casework, or in the appraisal of variations made upon the system, resolution measurements can be applied as part of the evaluation process in conjunction with other assessments to judge system performance.

Before forensic laboratories report casework data, electopherograms and supporting data must undergo considerable review. Most laboratories conduct at least a

qualitative resolution assessment of an electropherogram through a visual inspection of peak shape, breadth and separation. Peaks that are poorly shaped, overly broad, merged or lack appropriate baseline separation indicate deteriorated system performance. For example, Fig. 6 compares a good and poor resolution DNA separation with the same STR sample. Such visual inspections offer an excellent qualitative gauge of the system.

Resolution measurements can be conducted if a nonsubjective approach is desired to evaluate casework electropherograms. For casework analysis this may take the form of evaluating the resolution of the allelic ladders typically bracketing casework samples or by evaluating the samples themselves. The allelic ladder typically contains multiple peaks that span the breadth of the electrophoretic run and are consistently applied from run to run. These factors make the allelic ladder an excellent sample to assess the performance of the system. Assessing individual sample resolution may be approached by evaluation the sample peaks or through the assessment of an internal marker.

Due to the vagaries of crime scene samples, much variation would be expected in the resultant sample peaks found in these electropherograms. However, most laboratories include in the preparation of each sample for CE an internal lane standard (ILS) for determining sample peak base sizes. When the amplified sample and internal lane standard are co-injected, the variations of sample-to-sample injections may be evaluated and appropriate sizing conducted along with an assessment of the samples resolution based upon the ILS.

6 Applications of forensic DNA testing

With the analytical aspects of forensic DNA typing considered using CE systems, we can examine the two primary applications of this technology – forensic casework and DNA databasing. Each application has issues and challenges.

6.1 Forensic casework

As with any technology that is applied to forensic casework, the use of CE to determine DNA profiles must be rigorously evaluated through a comprehensive validation program [23, 60]. The DNA Advisory Board through the publication of DNA standards has established the basis for this validation that forensic laboratories are obliged to follow [1, 2]. These validation experiments reveal the operational parameters that are employed in the assessment of peaks detected during CE analysis. The forensic community primarily uses CE for STR analysis although it is used to a lesser extent in mitochondrial DNA sequencing [61].

For those involved with STR analysis, many parameters must be determined that are typically based upon the STR system employed. Commercial kits are available which allow the user to amplify many STR loci simultaneously (Table 2). The analysis of this amplified product may be done in one or two electrophoretic runs depending upon the kit. The evaluation of the peaks derived from this amplification is to some extent kit-dependent, where the amplification product yields fragments interpreted as a "colored" peak by the CE. The assessment of these peaks must take into consideration a number of factors inherent in the amplification such as peak imbalance, stochastic effects, stutter and *n*-1 peaks [1]. The analysis of these parameters must be done with an understanding of the limitations of the CE unit. For example, there is a linear fluorescent range for the instrument that should be well understood to be able to calculate meaningful heterozygote peak ratios. These ratios are important in the determination of alleles in a possible mixture and must be calculated within the operational range of the instrument. Likewise it is important to understand the sensitivity of the system to allow the analyst to develop a threshold fluorescence value above which peaks would be assigned as an allele.

In addition, a properly assigned matrix is critical to the evaluation of observed peaks. As discussed in a previous section, the fluorescent dyes employed in STR analysis have some spectral overlap and with a poorly assigned matrix, peaks of one color will be observed and misinterpreted as a peak of another color. This "pull-up" may yield peaks that could be mistaken as true alleles and hence it is important to review peaks to determine if they are detected in more than one wavelength. Such electropherograms that show a considerable "pull-up" may be revalued with a new matrix.

As discussed previously, artifacts such as spikes and dye "blobs" may be observed in an electrophoretic run. These artifacts may yield peaks in the allelic range and could be initially interpreted as an allele. The experienced operator should be able to review the peak shape and possible multifluorescent attributes of these artifacts to identify these as such and not as true allelic peaks. Through a good understanding of the CE system, appropriate DNA profile determinations can be obtained. The analysis of validation samples is an important mechanism, which provides operators with the opportunity to examine the system and to learn the criteria necessary to make appropriate interpretations especially for challenging samples containing mixtures or degraded DNA profiles [1, 62].

6.2 DNA databasing

DNA databasing has become a useful forensic tool and as more samples are added to the database the probability of a case-to-case match or case to convicted offender match increases. One problem facing most forensic laboratories in the United States concerning the database is the backlog of convicted offender samples waiting to be processed and entered into the database. Most US laboratories do not have the staff or instrumentation necessary to process the volume of samples collected, and hence these laboratories typically out source their samples to commercial laboratories. Many of these laboratories have developed highly automated systems to handle this demand. Some of the CE systems employed for this high-throughput typing is detailed below.

Another problem encountered by forensic laboratories engaged in databasing is the need to perform a second reading of the electropherograms prior to loading the profiles into the database. Typically, the commercial laboratory will perform their analysis and requisite quality control analysis and forward the profiles to the sending laboratory for their review. The process involved in this second review is very time-consuming and delays the uploading of convicted offender profiles into the database. Much work has been conducted to assist in this second review through the use of what have been termed "expert systems". These systems evaluate the electropherogram using specific criteria detailed by the examining laboratory to make allelic determinations from the electropherogram. Once fully validated, the system could be used to read the electropherogram and make the allelic calls and "flag" those samples that require human intervention. Some states have begun validation efforts with these systems and may soon be in a position to implement them for database use.

7 Increasing sample throughput

7.1 Capillary array electrophoresis systems

The ABI 310 uses a single capillary and as such cannot match the parallel processing potential throughput of a multilane slab-gel system. At its maximum capacity, the ABI 310 can run about 48 samples in a 24 h time period since each run takes close to 30 min. However, a number of capillary array electrophoresis (CAE) instruments are now commercially available [18]. These CAE systems offer from 8 to 384 capillaries run in parallel (Table 3). Thus, sample throughputs can be greatly increased by running many samples in parallel. However, it should be kept in mind that each capillary is an independent environment and thus not directly analogous to a multilane slab gel.

Table 3. Size of arrays in commercial CAE systems

	No. of capillaries
Applied Biosystems (Foster City, CA, USA)	
ABI 3100 Avant	4
ABI 3100	16
ABI 3700	96
ABI 3730	96
Amersham Biosciences (Piscataway, NJ, USA)	
MegaBACE 500	48
MegaBACE 1000	96
MegaBACE 4000	384
SpectruMedix Corporation (State College, PA, USA)	
SCE 2410	24
SCE 9610	96
SCE 19210	192
Beckman Coulter (Fullerton, CA, USA)	
CEQ 8800	8

STR typing by CAE has been reported in a number of publications. Early demonstrations of CAE for STR typing were performed in the laboratory of Rich Mathies at UC-Berkeley [63, 64] and at Molecular Dynamics [65, 66]. CAE systems have used different detection formats including a sheath flow cuvette, moving capillaries over a fixed laser beam, moving laser beam and detector over the capillaries, and a split beam approach to illuminate all of the capillaries simultaneously. Since the ABI 310 has been so widely used by the forensic DNA community, many labs will likely look to the ABI 3100 (16-capillary) and ABI 3700 or ABI 3730 (96-capillary) instruments in order to increase their sample throughput capabilities [67].

Precision studies conducted on the ABI 3100 [68] and the ABI 3700 [67] demonstrates that reliable results can be obtained with a multicapillary CE system. Table 4 illustrates the high degree of precision observed with more than 4600 allele measurements across all 16 capillaries over a six-month period on the same ABI 3100 instrument [69]. Note that the maximum spread in observed allele sizes was 0.83 bases for DYS389II allele 30 with 215 measurements. Most of the standard deviations for these Y-STR allele measurements are below 0.10 bases.

A high degree of resolution is needed with STR typing in spite of the fact that most of the markers are tetranucleotide repeats with expected nearest-neighbor alleles being

Table 4. Summary of 4651 Y-chromosome STR allele measurements observed on an ABI 3100 Genetic Analyzer across all 16 capillaries over a six-month period

Loci	Allele	Observed range (bp size relative to GS500 LIZ)	Spread in bp size	Sample No. (*N*)	Mean (bp)	SD (bp)
DYS19	13	243.36–243.81	0.45	47	243.62	0.096
	14	247.30–247.89	0.59	316	247.64	0.097
	15	251.38–251.88	0.50	198	251.68	0.079
	16	255.50–255.90	0.40	69	255.73	0.073
	17	259.65–260.02	0.37	50	259.81	0.068
DYS385	10	248.20–248.55	0.35	10	248.33	0.092
	11	251.78–252.42	0.64	275	252.21	0.083
	12	255.90–256.33	0.43	46	256.13	0.082
	13	259.89–260.28	0.39	104	260.05	0.078
	14	263.71–264.19	0.48	302	263.95	0.083
	15	267.42–268.13	0.71	156	267.89	0.098
	16	271.60–272.03	0.43	138	271.82	0.079
	17	275.49–276.03	0.54	118	275.76	0.088
	18	279.51–279.96	0.45	69	279.72	0.084
	19	283.44–283.85	0.41	30	283.65	0.102
	20	287.35–287.59	0.24	9	287.50	0.117
DYS388	10	148.96–149.13	0.17	10	149.04	0.055
	12	155.10–155.58	0.48	537	155.43	0.089
	13	158.29–158.71	0.42	55	158.58	0.088
	14	161.42–161.81	0.39	46	161.67	0.096
	15	164.63–164.86	0.23	19	164.76	0.059
	16	167.55–167.88	0.33	11	167.74	0.123
DYS389I	12	152.35–152.95	0.60	126	152.74	0.115
	13	156.53–157.22	0.69	421	157.00	0.103
	14	160.79–161.38	0.59	128	161.16	0.103
	15	165.22–165.36	0.14	8	165.28	0.049
DYS389II	26	262.23–262.54	0.31	3	262.44	0.179
	28	270.24–270.91	0.67	91	270.55	0.152
	29	274.21–275.03	0.82	230	274.63	0.147
	30	278.35–279.18	0.83	215	278.78	0.154
	31	282.52–283.20	0.68	108	282.90	0.155
	32	286.77–287.32	0.55	22	286.99	0.156
	33	291.11–291.22	0.11	4	291.17	0.046
DYS390	20	200.76–200.93	0.17	5	200.83	0.071
	21	204.56–205.09	0.53	157	204.86	0.085
	22	208.63–209.12	0.49	70	208.84	0.104
	23	212.57–213.09	0.52	138	212.82	0.112
	24	216.54–217.13	0.59	243	216.83	0.127
	25	220.52–221.10	0.58	67	220.84	0.109

Adapted from [69], Table 4.8

4 bp apart. In a recent population study involving approximately 12 000 allele measurements at 15 autosomal STRs [70], we observed 160 instances where heterozygous alleles were present that required a 1, 2, or 3 bp resolution up to about 300 bp due to microvariant alleles. Figure 8 shows several examples of these closely spaced alleles.

7.2 Microchip CE systems

Running single or multiple samples faster may also increase sample throughputs. By micromachining channels in glass, researchers have miniaturized CE systems with demonstrated DNA separations of less than a minute [71]. A major reason that microchip CE systems can achieve

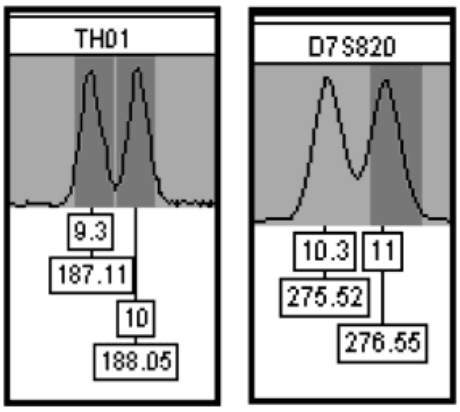


Figure 8. Resolution of STR variant alleles containing single-nucleotide spacing on ABI 3100 with POP-6 polymer. These TH01 alleles 9.3/10 and D7S820 alleles 10.3/11 were observed when typing samples in a previous study [70].

faster separation times is that the injection plug can be kept extremely small. Unfortunately, as of December 2003, no group has succeeded in producing routine and reliable STR typing data with 4 or 5-dye detection on a microchip CE device that is ready for "prime time" in a forensic laboratory setting. Caliper Technologies and Agilent Technologies both sell microchip CE devices such as the Agilent Bioanalyzer 2100, but these systems do not have the resolution or the multiwavelength detection capability necessary to perform modern STR typing. Rich Mathies' group at UC-Berkeley [72–74] and Dan Ehrich's group at the Whitehead Institute have made progress in this area [71, 75, 76].

7.3 Future methods for DNA typing with STR markers

Future analysis systems that wish to enable more rapid or easier STR typing will need to match or exceed the capabilities of currently available analytical systems such as the ABI 310 single-capillary CE system or the multicapillary ABI 3100. These capabilities include analysis of PCR reactions that contain at least four or five spectrally resolvable fluorescent dyes without significant pull-up between the various colors. Many current microchip CE platforms fall short in this regard. Future STR typing systems must maintain single-base resolution over a size range that extends from 50 bp to 250 bp or even 500 bp. Time-of-flight mass spectrometry approaches, while making substantial strides in recent years [77], currently fail in this regard.

Future electrophoretic systems need to maintain good temperature control to enable a high degree of precision from run to run. Throughput must be increased without compromising data quality. Due to the time invested in validating current STR kits and typing methodologies, many forensic laboratories will likely be reluctant or slow to change to a new technology even if substantial improvements can be demonstrated [7]. Rather an evolution to a multicapillary environment on a familiar platform is more likely than a radical change in technologies.

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Mixture Interpretation Questions

Homework for Monday Night

Name (*leave blank if you want to be anonymous*): _____ Email address:_____

Interpretation Guidelines

What would you like to see in national guidelines on how to perform DNA mixture interpretation and statistical analysis?

How does your lab handle reference samples during interpretation of evidence? Do you try to solve the mixture entirely without looking at either victim or suspect profiles?

What kind of pre-case assessment do you perform when approaching a case where a possible mixture is involved?

Does your lab attempt statistics on a minor component? If so, what types of statistics are used?

Do you have a decision point whereby you consider a mixture too complicated and do not try to solve it? How do you know when to stop in terms of mixture interpretation?

Are composite profiles acceptable – e.g., high injection for minor component and low injection for major component allele identification?

How do you report mixture statistics in court?

Would a flowchart for mixture interpretation be helpful?

Validation and Training

For your lab validation studies of a new STR kit or instrument, how many mixtures should be evaluated? How do you decide on what combination of alleles to include in such a study?

What kind of training materials would be beneficial to help your laboratory more effectively solve mixtures?

Suggestions for training staff to have more analyst consistency within your lab:

Other Topics

What percentage of time is spent in a case trying to deduce the mixture components?

Have you seen performance differences between various STR typing kits that would impact mixture interpretation?

Is your lab using Y-STRs to help with mixtures?

What kinds of software features would be valuable to aid mixture interpretation?

What are the biggest obstacles you face in your lab in terms of mixture interpretation?

Mixture Interpretation Discussion

Florida Statewide Training Meeting

Indian Rocks Beach, FL
May 12-13, 2008

Dr. John M. Butler
National Institute of Standards and Technology

john.butler@nist.gov

NIST and NIJ Disclaimer

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

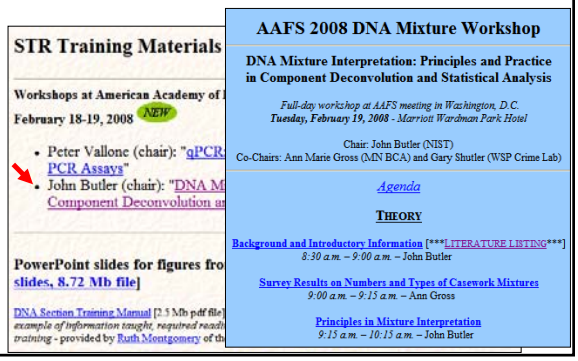
Points of view are mine and do not necessarily represent the official position or policies of the US Department of Justice or the National Institute of Standards and Technology.

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.

SWGDAM Disclaimer…

Training Information Available on STRBase

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



AAFS 2008 Workshop Presenters

Ann Marie Gross
MN BCA

John M. Butler
NIST

George Carmody
Carleton University/ Statistical Consultant

Gary Shutler
Wash State Police Crime Lab

Angie Dolph
Marshall University (NIST Summer Intern)

Joanne B. Sgueglia
Mass State Police Crime Lab

Tim Kalafut
US Army Crime Lab

Purpose for Teaching AAFS Workshop

We hope that participants:

- Gain a better understanding of the current approaches being used throughout the community for mixture interpretation
- See worked examples of mixture component deconvolution and statistical analysis
- Come away with ideas to improve your laboratory's interpretation guidelines and training regarding mixtures in forensic casework

AAFS Workshop Morning Agenda - Theory

Background and Introductory Information
8:30 a.m. – 9:00 a.m. – John Butler

Survey Results on Numbers and Types of Casework Mixtures
9:00 a.m. – 9:15 a.m. – Ann Gross

Principles in Mixture Interpretation
9:15 a.m. – 10:15 a.m. – John Butler

***10:15 a.m. – 10:30 a.m.* BREAK**

Strategies for Mixture Deconvolution with Worked Examples
10:30 a.m. – 11:30 a.m. – John Butler

Different Approaches to Statistical Analysis of Mixtures
11:30 a.m. – 12:00 p.m. – George Carmody

***12:00 p.m. – 1:15 p.m.* LUNCH**

Afternoon Agenda – **Practical Application**

Real Case Example – Importance of Properly Stating Your Conclusions
1:15 p.m. – 1:30 p.m. – Gary Shutler

Variability between Labs in Approaches & Mixture Interlaboratory Studies
1:30 p.m. – 2:15 p.m. – John Butler

Validation Studies and Preparing Mixture Interpretation Guidelines
2:15 p.m. – 2:45 p.m. – Joanne Sgueglia

***2:45 p.m. – 3:00 p.m.* BREAK**

Testing of Mixture Software Programs
3:00 p.m. – 3:15 p.m. – Angela Dolph

DNA_DataAnalysis Software Demonstration
3:15 p.m. – 4:00 p.m. – Tim Kalafut

Training Your Staff to Consistently Interpret Mixtures
4:00 p.m. – 4:45 p.m. – Panel Discussion with Ann Gross, Gary Shutler, Joanne Sgueglia

***4:45 p.m. – 5:00 p.m.* – Questions and Answers as needed**

Recent Mixture Workshops
Conducted by John Butler

Helpful feedback obtained from workshop participants

Southern Association of Forensic Scientists (SAFS)
September 11, 2007 (Atlanta, GA)
- **Mixture Interpretation (theory)**
- Along with Software discussion (Rhonda Roby) and demonstration (Tom Overson/Tim Kalafut)
- **33 attendees from 13 different labs**

Northeastern Association of Forensic Scientists (NEAFS)
November 2-3, 2007 (Bolton Landing, NY)
- **The Cutting Edge of DNA Testing: Mixture Interpretation, miniSTRs, and Low Level DNA**
- **42 attendees from 13 different labs**

NEAFS Workshop materials (70 pages) available on STRBase:
http://www.cstl.nist.gov/biotech/strbase/pub_pres/NEAFS2007_CuttingEdgeDNA.pdf

Mixture Basics

From J.M. Butler (2005) *Forensic DNA Typing, 2nd Edition*, p. 154

- Mixtures arise when two or more individuals contribute to the sample being tested.
- Mixtures can be challenging to detect and interpret without extensive experience and careful training. **Even more challenging with poor quality data when degraded DNA is present…**
- Differential extraction can help distinguish male and female components of many sexual assault mixtures. **Y-chromosome markers can help here in some cases…**

More on Mixtures...

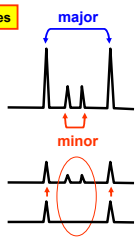
Most mixtures encountered in casework are 2-component mixtures arising from a combination of victim and perpetrator DNA profiles

Ann Gross will discuss some recent collected casework summaries

Torres *et al.* (2003) *Forensic Sci. Int.* 134:180-186 examined 1,547 cases from 1997-2000 containing **2,424 typed samples** of which **163 (6.7%) contained a mixed profile** with **only 8 (0.3%) coming from more than two contributors**

95.1% (155/163) were 2-component mixtures

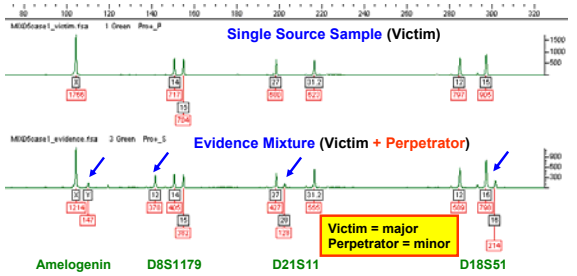
Ratios of the various mixture components stay fairly constant between multiple loci enabling deduction of the profiles for the major and minor components



Some mixture interpretation strategies involve using victim (or other reference) alleles to help isolate obligate alleles coming from the unknown portion of the mixture

http://www.cstl.nist.gov/biotech/strbase/interlab/MIX05.htm
MIX05 Case #1; Profiler Plus green loci

Example Mixture Data (MIX05 Study-Profiler Plus)



Obligate Alleles (not present in the victim reference)

Y	12	28	16

True "Perpetrator" Profile

X,Y	12,12	28,31.2	15,16

Sources of DNA Mixtures

- **Two (or more) individuals** contribute to the biological evidence examined in a forensic case (e.g., sexual assault with victim and perpetrator or victim, consensual sexual partner, and perp)

  Victim Reference and Spouse or Boyfriend Reference
- **Contamination** of a single source sample from
  - evidence collection staff
  - laboratory staff handling the sample
  - Low-level DNA in reagents or PCR tubes or pipet tips

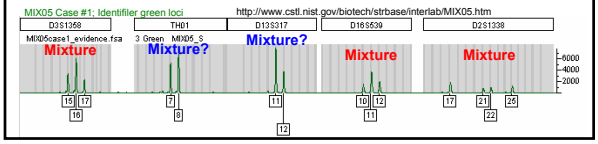
  Examine Staff Profiles (Elimination Database), etc.

Reference elimination samples are useful in deciphering both situations due to possibility of intimate sample profile subtraction

Mixtures: Issues and Challenges

From J.M. Butler (2005) *Forensic DNA Typing, 2nd Edition*, p. 155

- The probability that a mixture will be detected improves with the use of more loci and genetic markers that have a high incidence of heterozygotes.
- The detectability of multiple DNA sources in a single sample relates to the ratio of DNA present from each source, the specific combinations of genotypes, and the total amount of DNA amplified.
- Some mixtures will not be as easily detectable as other mixtures.

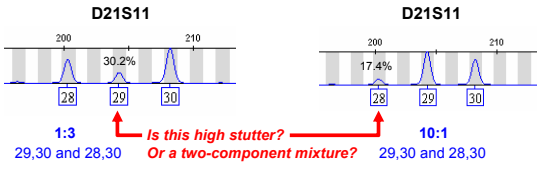


Detecting Mixtures

- Review and compile information from the entire profile – **don't just focus on a single locus!**
- **Tri-allelic patterns exist** in single source samples
  - **145 different tri-alleles recorded for the 13 core CODIS loci** on STRBase as of Jan 22, 2008
  - CSF1PO (5), FGA (22), TH01 (1), TPOX (15), VWA (18), D3S1358 (6), D5S818 (4), D7S820 (7), D8S1179 (11), D13S317 (8), D16S539 (8), D18S51 (21), D21S11 (19)
- A mixture often declared when **>2 peaks in ≥2 loci**

Mixtures: Issues and Challenges

- Artifacts of PCR amplification such as stutter products and heterozygote peak imbalance complicate mixture interpretation
- Thus, only a limited range of mixture component ratios can be solved routinely



1

Responses to Questions
from a Previous Mixture Workshop (Fall 2007)

What are the biggest obstacles you face in your lab in terms of mixture interpretation?

- Trying to be consistent in my interpretation and with coworkers
- Consistency between analysts
- No consistency – based on analysts discretion/experience; due to lack of consistent training
- Vague SOP leading to inconsistency between analysts due to differences in how "conservative" or not each analyst is
- There is a lot of "individual interpretation" in our lab
- Varying opinions between interpreting analysts due to lack of uniform guidelines
- Resistance to change from other analysts/supervisors
- Getting management to commit to guidelines that will be followed by everyone

2

Responses to Questions
from a Previous Mixture Workshop (Fall 2007)

What are the biggest obstacles you face in your lab in terms of mixture interpretation?

- Where to draw the line without throwing away valuable data
- Partial minor contributors
- Stochastic effects in minor components
- STATS and presenting them in court so that the jury will understand them
- When to do stats and what stats to do in different cases
- Lack of concrete/uniform guidelines from statisticians

DNA Mixture Interpretation:
Principles and Practice in Component Deconvolution and Statistical Analysis

Numbers and Types of Casework Mixtures

Handouts available on STRBase at
http://www.cstl.nist.gov/biotech/strbase/training/AAFS2008_MixtureWorkshop.htm

AAFS 2008 Workshop #16
Washington, DC
February 19, 2008

Ann Marie Gross
ann.gross@state.mn.us

Mixtures……

- How often are mixtures obtained
- What types of mixtures are we seeing
  - Where should we focus our attention for training
  - What info can we give to the forensic community regarding mixtures
- What types of samples most often yield mixtures

Torres et al. 4 year Spanish study

- Four year study (1/1997 to 12/2000)
- 2412 samples typed
  - 955 samples from sexual assaults
  - 1408 samples from other offenses
  - 49 samples from human remains identifications
- 163/2412 samples (6.7% showed mixed profile)

Spreadsheet Information Requested

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

Labs requested to also provide info on kit, PCR volume used, etc.

- **Case#**
- **Item#**

This information retained by lab and not returned…

- **Type of sample (biological material if ID'd)**
- **Type of substrate**
- **Quantity amp'd**
- **Minimum # of contributors (1, 2, 3, 4, or >4)**
- **Predominant type (major profile) determined?**
- **Stats reported**
- **Comments**

We would love to have your lab mixture numbers…

Email information to Ann.Gross@state.mn.us

12 Labs Submitted Data
(prior to AAFS meeting)

- **Palm Beach Sheriff's Office Crime Lab, Florida**
- Centre for Forensic Science, Toronto
- Connecticut State Police
- Washington State Police
- New Jersey State Police
- Georgia Bureau of Investigation
- Royal Canadian Mounted Police, Ottawa
- USACIL, Georgia
- Michigan State Police
- Kern County Crime Lab, California
- CAL DOJ
- Minnesota Bureau of Criminal Apprehension

We would still like to collect more case summary data…

All Laboratory Data Combined

Case type	N = 3106	1	2	3	4	>4
			# contributors			
Sexual Assault	N = 1408	51%	40%	8%	--	--
Major Crime	N = 1388	66%	24%	8%	2%	--
High Volume	N = 310	43%	37%	19%	1%	--
		Single source	Mixtures			

Overall Summary – 3106 samples

- 57% of samples from all types of cases are single source
- 43% of samples from all types of cases are mixtures
  - 33% of mixtures of at least two contributors
  - 9% of mixtures of at least three contributors
  - 1% of mixtures of at least four contributors

Focus in training materials will be on two-person mixtures as they presently predominate

Principles of Mixture Interpretation

Topics for Discussion

- SWGDAM Mixture Interpretation Committee progress
- Different statistical approaches: CPE or LR
- ISFG Mixture Interpretation Recommendations
  - UK response
  - German categories for mixtures
- Validation as it relates to mixture interpretation
  - Stochastic threshold vs analytical threshold
- Low-level DNA and mixtures
- Important elements of interpretation guidelines

SWGDAM Mixture Interpretation Subcommittee

- **John Butler** (NIST) - chair
- **Gary Sims** (CA DOJ) - co-chair
- Mike Adamowicz (CT)
- Jack Ballantyne (UCF/NCFS)
- George Carmody (Carleton U)
- Cecelia Crouse (PBSO)
- Allison Eastman (NYSP)
- Roger Frappier (CFS-Toronto)
- Ann Gross (MN BCA)
- Phil Kinsey (MT)
- Jeff Modler (RCMP)
- Gary Shutler (WSP)

Everyone not at every meeting…

Have met 3 times:
Jan 2007
July 2007
Jan 2008

Through the Jan 2008 meeting we have also had to deal with Y-STR issues – which has limited our focus on mixtures

Additional Participants (Jan 2008)
Bruce Heidebrecht (MD)
Steve Lambert (SC)

Started in January 2007

Progress and Plans for Mixture Committee

- **Guidelines in process** of being discussed and written
- Collecting data on number and type of mixture cases observed in various labs
- Plan to create a training workbook with worked examples
- Considering flow charts to aid mixture interpretation
- Have discussed responses to ISFG Recommendations

I invite your input as to what should be included in the guidelines…

Your HOMEWORK…

Elements of DNA Mixture Interpretation

Principles (theory)

↓

Protocols (validation)

↓

Practice (training & experience)

Consistency across analysts

ISFG Recommendations
SWGDAM Guidelines

⋮

Your Laboratory SOPs

↕

Training within Your Laboratory

We discussed and would advocate periodic training to aid accuracy and efficiency within your laboratory.

ISFG Recommendations on Mixture Interpretation

July 13, 2006 issue of *Forensic Science International*

Our discussions have highlighted a significant need for continuing education and research into this area.

ELSEVIER

Forensic Science International 160 (2006) 90–101

www.elsevier.com/locate/forsciint

DNA commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures

P. Gill [a,*], C.H. Brenner [b], J.S. Buckleton [c], A. Carracedo [d], M. Krawczak [e], W.R. Mayr [f], N. Morling [g], M. Prinz [h], P.M. Schneider [i], B.S. Weir [j]

[a] Forensic Science Service, Trident Court, 2960 Solihull Parkway, Birmingham, UK
[b] Forensic Science Group, School of Public Health, University of California, Berkeley, CA 510-330-1911, USA

Abstract

The DNA commission of the International Society of Forensic Genetics (ISFG) was convened at the 21st congress of the International Society for Forensic Genetics held between 13 and 17 September in the Azores, Portugal. The purpose of the group was to agree on guidelines to encourage best practice that can be universally applied to assist with mixture interpretation. In addition the commission was tasked to provide guidance on low copy number (LCN) reporting. Our discussions have highlighted a significant need for continuing education and research into this area. We have attempted to present a consensus from experts but to be practical we do not claim to have conveyed a clear vision in every respect in this difficult subject. For this reason, we propose to allow a period of time for feedback and reflection by the scientific community. Then the DNA commission will meet again to consider further recommendations.

Keywords: STR typing; Biostatistical analysis; Likelihood ratio; Probability of exclusion; Mixtures; ISFG DNA commission

Who is the ISFG
and why do their recommendations matter?

International Society of Forensic Genetics
http://www.isfg.org/

- An international organization responsible for the promotion of scientific knowledge in the field of genetic markers analyzed with forensic purposes.
- Founded in 1968 and represents more than 1100 members from over 60 countries.
- **A DNA Commission regularly offers recommendations on forensic genetic analysis.**

DNA Commission of the ISFG

- DNA polymorphisms (1989)
- PCR based polymorphisms (1992)
- Naming variant alleles (1994)
- Repeat nomenclature (1997)
- Mitochondrial DNA (2000)
- Y-STR use in forensic analysis (2001)
- Additional Y-STRs - nomenclature (2006)
- **Mixture Interpretation (2006)**
- Disaster Victim Identification (2007)

http://www.isfg.org/Publications/DNA+Commission

ISFG Executive Committee

President
Niels Morling
(Copenhagen, Denmark)

Vice-President
Peter Schneider
(Köln, Germany)

Working Party Representative
Mecki Prinz
(New York City, USA)

Treasurer
Leonor Gusmão
(Porto, Portugal)

Secretary
Wolfgang Mayr
(Vienna, Austria)

Angel Carracedo
***FSI Genetics* Editor-in-Chief**
(former ISFG President, VP)
(Santiago de Compostela, Spain)

Authors of ISFG Mixture Article

Peter Gill
Pioneer of forensic DNA techniques and applications
UK's Forensic Science Service (1978-2008)
University of Strathclyde (Apr 2008 – present)

The Statisticians

Charles Brenner
DNA-View,
Berkeley, CA, USA

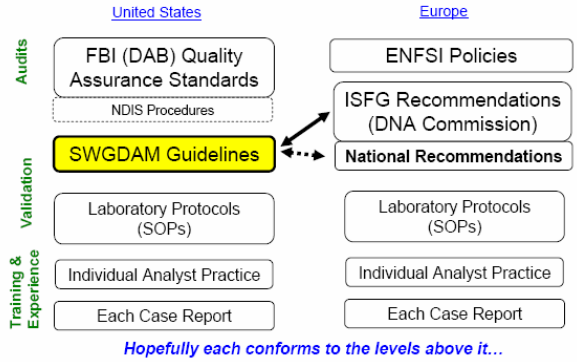
John Buckleton
ESR,
Auckland, New Zealand

Michael Krawczak
Christian-Albrechts-University,
Kiel, Germany

Bruce Weir
U. Washington,
Seattle, USA

My perspective…

Hierarchy of Rules for Forensic DNA Labs



Hopefully each conforms to the levels above it…

UK Response to ISFG Mixture Recommendations

Gill, P., et al. (2008) National recommendations of the technical UK DNA working group on mixture interpretation for the NDNAD and for court going purposes. *FSI Genetics* 2(1): 76–82

Using the published UK response as a model, let us review the nine ISFG Recommendations on mixture interpretation…

From Report to the **Virginia Scientific Advisory Committee** by the DNA Subcommittee – Addendum January 8, 2008

(authored by Dr. Norah Rudin and Dr. Artie Eisenberg)

- "Among the many reasons that Forensic DNA analysis has become the gold standard for forensic science is the relatively discrete nature of the data. For strong, single source samples, a profile can readily be determined, and is subject to little or no analyst judgment. **However, ambiguity may arise when interpreting more complex samples, such as those containing multiple contributors, of poor quality (e.g. degraded or inhibited DNA), of low quantity (e.g. contact samples), or various combinations of these challenging situations**…"

http://www.dfs.virginia.gov/about/minutes/saCommittee/20080108.pdf

From Report to the **Virginia Scientific Advisory Committee** by the DNA Subcommittee – Addendum January 8, 2008

(authored by Dr. Norah Rudin and Dr. Artie Eisenberg)

- "…**These kinds of samples are encountered with increasing frequency, as the sensitivity of the technology has increased, and as law enforcement has become more sophisticated about the kinds of samples they submit for analysis**. Difficult samples are also frequently encountered when reanalyzing historical cases, in which samples were not collected and preserved using the precautions necessary for DNA analysis…"

"Cold cases" or Innocence Project samples…

http://www.dfs.virginia.gov/about/minutes/saCommittee/20080108.pdf

From Report to the **Virginia Scientific Advisory Committee** by the DNA Subcommittee – Addendum January 8, 2008

(authored by Dr. Norah Rudin and Dr. Artie Eisenberg)

- "It is for these types of challenging samples, where the evidence profile may not exactly "match" a reference profile, that confirmation bias becomes a concern. **The interpretation of an evidentiary DNA profile should not be influenced by information about a subject's DNA profile.** Each item of evidence must be interpreted independently of other items of evidence or reference samples. Yet forensic analysts are commonly aware of submitted reference profiles when interpreting DNA test results, creating the opportunity for confirmatory bias, despite the best intentions of the analyst…"

http://www.dfs.virginia.gov/about/minutes/saCommittee/20080108.pdf

DNA Mixture Interpretation:

Principles and Practice in Component Deconvolution and Statistical Analysis

Principles in Mixture Interpretation

Handouts available on STRBase at
http://www.cstl.nist.gov/biotech/strbase/training/AAFS2008_MixtureWorkshop.htm

AAFS 2008 Workshop #16
Washington, DC
February 19, 2008

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Two Parts to Mixture Interpretation

- Determination of alleles present in the evidence and **deconvolution of mixture components** where possible
  - Many times through comparison to victim and suspect profiles
- **Providing some kind of statistical answer** regarding the weight of the evidence
  - There are multiple approaches and philosophies

Software tools can help with one or both of these…

Status of Software for Mixture Interpretation

- NIJ Expert System Testbed (**NEST**) Project
  - Evaluating software programs for DNA analysis of single-source (Phase I) and mixtures (Phase II)
  - **http://forensics.marshall.edu/NEST/NEST-Intro.html**
- US Army Crime Laboratory (**USACIL**)
  - Commonly deal with complex sexual assaults
  - Developed software for aiding mixture interpretation and statistical analysis

Steps in the Interpretation of Mixtures

(Clayton *et al.* 1998)

Step #1 Identify the Presence of a Mixture

Step #2 Designate Allele Peaks

Step #3 Identify the Number of Potential Contributors

Step #4 Estimate the Relative Ratio of the Individuals Contributing to the Mixture

Step #5 Consider All Possible Genotype Combinations

Step #6 Compare Reference Samples


Analysis and interpretation of mixed forensic stains using DNA STR profiling

Clayton *et al.* (1998) *Forensic Sci. Int.* 91:55-70

Adapted from Peter Schneider slide (presented at EDNAP meeting in Krakow in April 2007)

Mixture Classification Scheme

Schneider *et al.* (2006) *Rechtsmedizin* 16:401-404

(German Stain Commission, 2006):

- **Type A**: no obvious major contributor, no evidence of stochastic effects
- **Type B**: clearly distinguishable major and minor contributors; consistent peak height ratios of ***approximately 4:1*** (major to minor component) for all heterozygous systems, no stochastic effects
- **Type C**: mixtures without major contributor(s), evidence for stochastic effects

Type A **Type B** **Type C**

Type of mixture and interpretation

- **Type A:** Mixed profile without stochastic effects, a biostatistical analysis has to be performed
- **Type B:** Profile of a major contributor can be unambiguously described and interpreted as a profile from an unmixed stain
- **Type C:** due to the complexity of the mixture, the occurrence of stochastic effects such as allele and locus drop-outs have to be expected:
  - a clear decision to include or exclude a suspect may be difficult to reach, thus a biostatistical interpretation is not appropriate.

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

Biostatistical approaches

- Calculation of the **probability of exclusion** for a randomly selected stain donor* [P(E)]
  (*RMNE - "random man not excluded")
- Calculation of the **likelihood ratio** [LR] based on defined hypotheses for the origin of the mixed stain

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

Which approach should be used?

- If the basis for clearly defined and mutually exclusive hypotheses is given, i.e.:
  - the number of contributors to the stain can be determined,
  - unambiguous DNA profiles across all loci are observed (type A mixtures, or type B, if the person considered as "unknown" contributor is part of the minor component of the mixture),

  then the calculation of a likelihood ratio is appropriate.

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

Which approach should be used?

- If major/minor contributors cannot be identified based on unambiguous DNA profiles, or if the the number of contributors cannot be determined, then the calculation of the probability of exclusion is appropriate.
- The calculation of P(E) is always possible for type A and type B mixtures.

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

Not acceptable …

- … is the inclusion of a genotype frequency of a non-excluded suspect into the report, if the given mixed stain does not allow a meaningful biostatistical interpretation.
  - this would lead to the wrongful impression that this genotype frequency has any evidentiary value regarding the role of the suspect as a contributor to the mixed stain in question.

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

Conclusions

- The likelihood ratio has a significant weight of evidence, as it relates directly to the role of the suspect in the context of the origin of the stain.
- The exclusion probability makes a general statement without relevance to the role of the suspect.
- However, this does not imply that P(E) is always more "conservative" in the sense that the weight of evidence is not as strong compared to the LR.

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

GEDNAP 32

Mixture interpretation exercise:

- 3 person mixture without major contributor
- Person A from group of reference samples was not excluded
- Allele frequencies for eight German database systems provided for exercise
- German-speaking GEDNAP participants invited to participate based on published recommendations

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

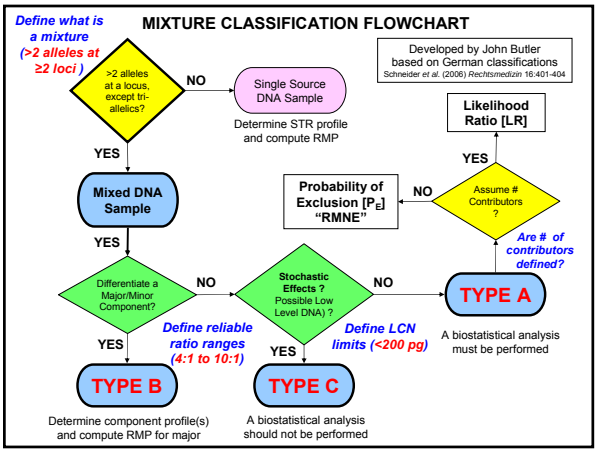
GEDNAP 32

Results:

- 22 labs submitted results (from approx. 80 German-speaking GEDNAP participants)
- Calculations submitted were all correct and consistent:
  - 15x LR approach:
    - Person A + 2 unknown vs. 3 unknown contributors
  - 11x RMNE calculation
- Will be offered again next time

Training and Specific Guidelines/Classification Schemes yielded consistent results among laboratories

Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

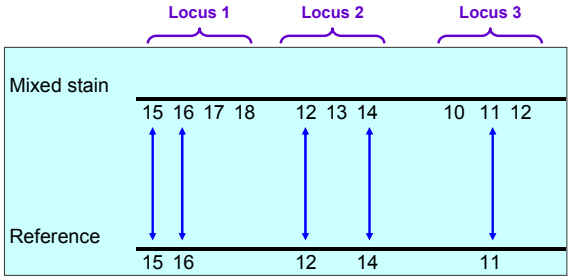


German Type A,B, and C mixture classifications

- **Type A**, where major/minor contributors cannot be deduced, require stats
  - LR
  - RMNE
- **Type B** enables major contributor to be deduced
  - RMP (which is 1/LR)
- **Type C** no stats should be attempted because of the possibility of failure to account for allele dropout due to stochastic effects with low level DNA samples

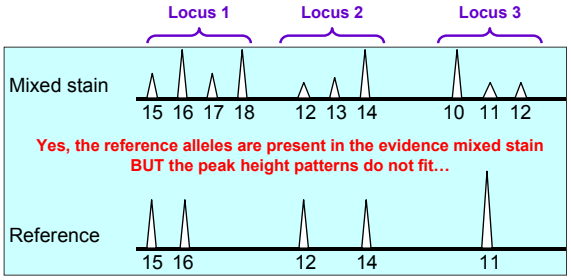
Mixture Example

Comparing Alleles Only



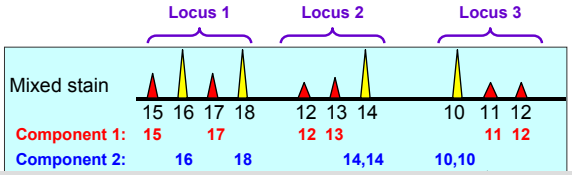
Mixture Example

Showing Importance of Using Peak Height Information



Mixture Example

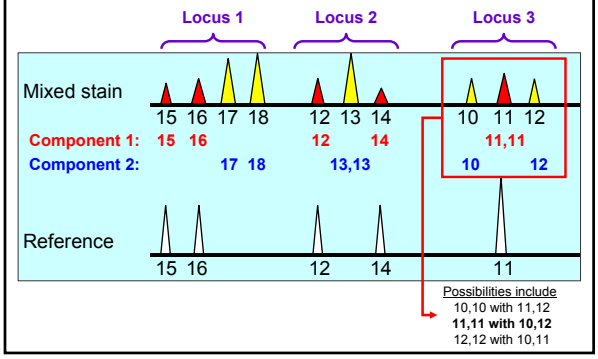
Solving Components Prior to Comparison to Suspect Reference



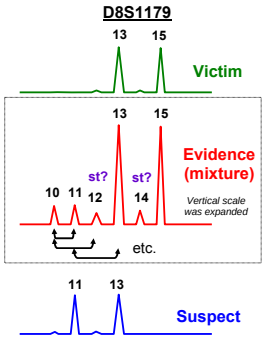
Reference (suspect) does not match either component of the mixed stain and therefore could not have contributed to the evidence sample

Mixture Example

Different Evidence Sample…



Another Mixture Example



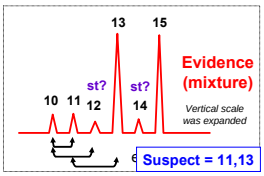
Conclusions from the evidence:

1. **Major contributor = 13,15 (victim) –** *to be expected with an intimate sample like a fingernail or vaginal swab*
2. Alleles 12 and 14 are **likely stutter products** of the major contributor's 13 and 15 alleles but could also be masking minor contributor alleles
3. A number of minor contributor combinations are possible (e.g., 10,11 or 10,12 or 10,13 or 11,13, etc.)
4. Could have more than two contributors present in this mixture

"Suspect cannot be excluded" BUT statement needs to be qualified by statistics because a large percentage of the population might also not be able to be excluded…

Probability of Exclusion Calculation
for a Single STR Locus

The case may grow stronger against a suspect with information from additional STR loci…



From VA DFS STR Allele Frequencies
http://www.dfs.virginia.gov/manuals/manuals.cfm?id=5

D8S1179 alleles	AA (n=384)	C (n=346)	H (n=366)
10	0.0287	0.1069	0.0820
11	0.0495	0.0925	0.0465
12	0.1094	0.1416	0.1093
13	0.2422	0.3093	0.3224
14	0.2969	0.1965	0.2623
15	0.1849	0.0896	0.1202
SUM	0.9115	0.9364	0.9426
Sq SUM = PI	0.8308	0.8769	0.8886
PE = 1-PI	0.1692	0.1231	0.1114
PE (%)	**16.9%**	**12.3%**	**11.1%**
	African Am.	Caucasians	Hispanics

"Suspect cannot be excluded" BUT we would expect to see, for example, only 11.1% of Hispanics excluded (or 88.9% cannot be excluded) based on results at this one locus

The fact that in this case a suspect is included is not very informative because ~9 out of 10 people examined from any population could potentially be included in the evidence mixture…

The Statistic (Determining the Weight of the Evidence) Should Be **Calculated from the Evidence**

Evidence (partial profile):

	Type	Statistic
Locus 1	16,17	1 in 9
Locus 2	17,18	1 in 9
Locus 3	21,22	1 in 12
Locus 4	12,14	1 in 16
Locus 5	28,30	1 in 11

		Product = 1 in 171,000

Match Observed at All Loci that May Be Compared

Reference (full profile):

	Type	Statistic
Locus 1	16,17	1 in 9
Locus 2	17,18	1 in 9
Locus 3	21,22	1 in 12
Locus 4	12,14	1 in 16
Locus 5	28,30	1 in 11
Locus 6	14,16	1 in 26
Locus 7	12,13	1 in 9
Locus 8	11,14	1 in 31
Locus 9	9,9	1 in 32
Locus 10	9,11	1 in 14
Locus 11	6,6	1 in 19
Locus 12	8,8	1 in 3
Locus 13	10,10	1 in 21

		Product = 1 in 665 trillion

The reference sample is still a "match" – just not as much information is available from the evidence for comparison

Statistical Approaches with Mixtures

See Ladd *et al.* (2001) *Croat Med J.* 42:244-246

- **Inferring Genotypes of Contributors** - Separate major and minor components into individual profiles and compute the random match probability estimate as if a component was from a single source
- **Calculation of Exclusion Probabilities** - CPE/CPI (RMNE) – The probability that a random person (unrelated individual) would be excluded as a contributor to the observed DNA mixture
- **Calculation of Likelihood Ratio Estimates** – Comparing the probability of observing the mixture data under two (or more) alternative hypotheses; in its simplest form LR = 1/RMP

RMNE = Random Man Not Excluded (same as CPE)
CPE = Combined Probability of Exclusion (CPE = 1 – CPI)
CPI = Combined Probability of Inclusion (CPI = 1 – CPE)

Advantages and Disadvantages

RMNE (CPE/CPI)

- **Advantages**
  - Does not require an assumption of the number of contributors to a mixture
  - Easier to explain in court
- **Disadvantages**
  - Weaker use of the available information (robs the evidence of its true probative power because this approach does not consider the suspect's genotype)
  - Likelihood ratio approaches are developed within a consistent logical framework

Likelihood Ratios (LR)

- **Advantages**
  - Enables full use of the data including different suspects
- **Disadvantages**
  - More difficult to calculate

John Buckleton, *Forensic DNA Evidence Interpretation*, p. 223

Assumptions for CPE/CPI Approach

- There is no allele dropout (i.e., all alleles are above stochastic threshold) – low-level mixtures can not reliably be treated with CPE
- All contributors are from the same racial group (i.e., you use the same allele frequencies for the calculations)
- All contributors are unrelated
- Peak height differences between various components are irrelevant (i.e., component deconvolution not needed) – this may not convey all information from the available sample data…

Likelihood Ratio (LR)

- Provides ability to express and evaluate both the prosecution hypothesis, H_p (the suspect is the perpetrator) and the defense hypothesis, H_d (an unknown individual with a matching profile is the perpetrator)

$$LR = \frac{H_p}{H_d}$$

- **The numerator, H_p, is usually 1** – since in theory the prosecution would only prosecute the suspect if they are 100% certain he/she is the perpetrator
- The denominator, $\mathbf{H_d}$, is typically the profile frequency in a particular population (based on individual allele frequencies and assuming HWE) – i.e., **the random match probability**

LR is not a probability but a ratio of probabilities

DAB Recommendations on Statistics

February 23, 2000
Forensic Sci. Comm. 2(3); available on-line at
http://www.fbi.gov/hq/lab/fsc/backissu/july2000/dnastat.htm

"The DAB finds either one or both PE or LR calculations acceptable and strongly recommends that one or both calculations be carried out whenever feasible and a mixture is indicated"

- Probability of exclusion (PE)
  - Devlin, B. (1993) Forensic inference from genetic markers. *Statistical Methods in Medical Research*, 2, 241–262.
- Likelihood ratios (LR)
  - Evett, I. W. and Weir, B. S. (1998) *Interpreting DNA Evidence*. Sinauer, Sunderland, Massachusetts.

ISFG DNA Commission on Mixture Interpretation

Gill *et al.* (2006) DNA Commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. *Forensic Sci. Int.* 160: 90-101

Available for download from the ISFG Website:
http://www.isfg.org/Publication;Gill2006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DNA commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures

P. Gill, C.H. Brenner, J.S. Buckleton, A. Carracedo, M. Krawczak, W.R. Mayr, N. Morling, M. Prinz, P.M. Schneider, B.S. Weir

Gill *et al.* (2006) DNA Commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. *Forensic Sci. Int.* 160: 90-101

Summary of ISFG Recommendations on Mixture Interpretation

1. The likelihood ratio (LR) is the preferred statistical method for mixtures over RMNE
2. Scientists should be trained in and use LRs
3. Methods to calculate LRs of mixtures are cited
4. Follow Clayton et al. (1998) guidelines when deducing component genotypes
5. Prosecution determines H_p and defense determines H_d and multiple propositions may be evaluated
6. When minor alleles are the same size as stutters of major alleles, then they are indistinguishable
7. Allele dropout to explain evidence can only be used with low signal data
8. No statistical interpretation should be performed on alleles below threshold
9. Stochastic effects limit usefulness of heterozygote balance and mixture proportion estimates with low level DNA

Gill *et al.* (2006) DNA Commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. *Forensic Sci. Int.* 160: 90-101

Thoughts by Peter Gill on Recommendation #5

(ENFSI meeting, Krakow, Poland, April 19, 2007)

- Prosecution and defense each want to maximize their respective probabilities
- Recommendation 5 places ownership for each hypothesis.
- In order to perform the LR calculation(s), the forensic scientist decides on both the prosecution and defense hypotheses.
- Since the forensic scientists usually cannot discover the defense hypothesis before the trial (as they are typically working with the prosecution if the DNA matches…), assumptions must be clearly stated with the important caveat that you cannot perform calculations on the stand! (For example, you need three weeks warning to make and check calculations.)
- By anchoring the respective hypotheses to each side, the defense can change their hypothesis but the prosecution does not need to change theirs…
- It is worth noting that the likelihood ratio always goes up if the defense lowers their hypothesis (H_d gets lower with more possible combinations)

ISFG (2006) Recommendations

- Recommendation 6: If the crime profile is a major/minor mixture, **where minor alleles are the same size (height or area) as stutters of major alleles, then stutters and minor alleles are indistinguishable.** Under these circumstances alleles in stutter positions that do not support H_p should be included in the assessment.
- In general, stutter percentage is <15%

Gill *et al.* (2006) DNA Commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. *Forensic Sci. Int.* 160: 90-101

Consideration of Peak in Stutter Position

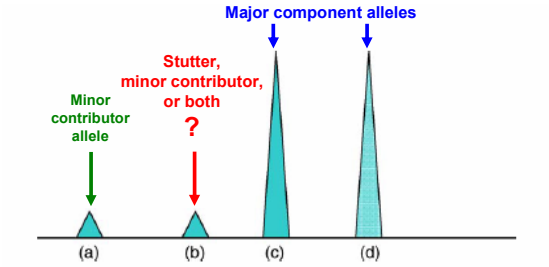


Fig. 4. *c* and *d* are unambiguous alleles, *b* is a minor allele in a stutter position and *a* is an unambiguous minor allele.

Gill *et al.* (2006) DNA Commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. *Forensic Sci. Int.* 160: 90-101

UK Response

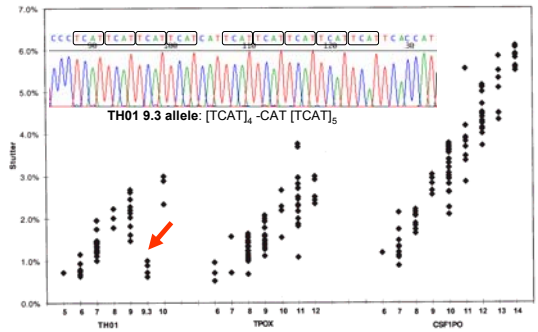
Gill *et al.* (2008) *FSI Genetics* 2(1): 76–82

Recommendation 6:

- Stutters are locus-dependent…
- It is recommended that laboratories make their own maximum experimentally observed stutter sizes per locus determinations since the effects may be technique dependent.
- It is recommended that [maximum stutter percentages be] evaluated per locus.

Measured Stutter Percentages

Variable by Allele Length and Composition



Holt CL, Buoncristiani M, Wallin JM, Nguyen T, Lazaruk KD, Walsh PS. TWGDAM validation of AmpFISTR PCR amplification kits for forensic DNA casework. *J Forensic Sci* 2002; 47(1): 66-96.

UK Response

Gill *et al.* (2008) *FSI Genetics* 2(1): 76–82

- ***Characterization of +4 base stutters***

We agreed to review +4 bp stutters, however, we note that their presence often relates to over-amplified samples. Preliminary experimental work suggests that they are low level and **generally less then 4% the size of the progenitor allele** (Rosalind Brown, personal communication). Note that 4 bp and +4 bp stutter cannot be distinguished from genetic somatic mutation without experimental work—furthermore, somatic mutations may give rise to peaks that are larger than those caused by stutter artifacts.

ISFG (2006) Recommendations

- Recommendation 7: If drop-out of an allele is required to explain the evidence under H_p: (S = ab; E = a), then the allele should be small enough (height/area) to justify this. Conversely, if a full crime stain profile is obtained where alleles are well above the background level, and the probability of drop-out approaches Pr(D) ≈ 0, then H_p is not supported.

Gill *et al.* (2006) DNA Commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. *Forensic Sci. Int.* 160: 90-101

UK Response

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Recommendation 7:

- We recommend slight rewording…[with mention of companion allele]
- If a full crime-stain profile is obtained where alleles are well above the background level, and the probability of dropout Pr(D) approaches zero, then H_p is not supported (Figure 6).

Hypothetical Examples

Gill *et al.* (2008) *FSI Genetics* 2(1): 76–82

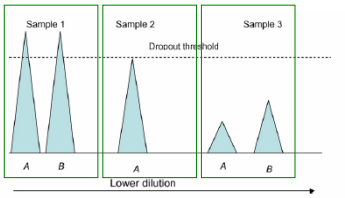

Fig. 4. Results from serial dilutions of the same sample genotype *AB*. The first result (sample 1) shows a locus where both alleles are represented in the profile. One or both of these alleles are above the dropout threshold and consequently are always present in the epg. The second result shows a result where dropout has occurred – the survivor allele is just below the dropout threshold hence this is a rare event, but not impossible. If *A* was just above the dropout threshold we would determine it to be a homozygote *AA* genotype. In the third sample, both alleles are well below the dropout threshold – it is an unambiguous, albeit unbalanced heterozygote. If only one allele was present, then we would have to consider the possibility of dropout of the partner. The same rationale can be applied to any analytical regime, e.g. 28 and 34 PCR cycles.

If Below Dropout Threshold…

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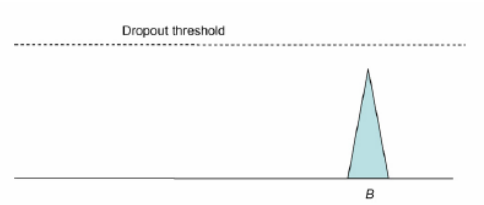

Fig. 5. In this example allele *B* is below the dropout threshold, hence we cannot be confident that it is from a homozygote *BB* individual. It could also be from an individual who is heterozygote, where the missing allele is any other allele. The probability *B*|unknown, H_d is 2Pr(*BF*), where the '*F*' designation is assigned a probability of 1 to take account of the possibility that any allele could have dropped out.

If Above Dropout Threshold…

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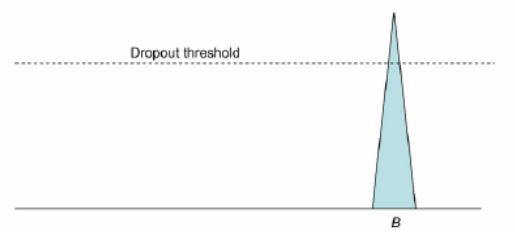

Fig. 6. In this example allele *B* is above the dropout threshold, hence we can be confident that it is from a homozygote *BB* individual. The probability of B|unknown, H_d is $\Pr(B)^2$.

Setting Thresholds

- **Detection (analytical) threshold**
  - Dependent on instrument sensitivity
  ~50 RFU
  - Impacted by instrument baseline noise
- **Dropout (stochastic) threshold**
  - Dependent on biological sensitivity
  ~150-200 RFU
  - Impacted by assay and injection parameters

Determining the Dropout (Stochastic) Threshold

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- The dropout threshold can be determined experimentally for a given analytical technique from a series of pre-PCR dilutions of extracts of known genotype technique (it will probably vary between analytical methods). These samples can be used to determine the point where allelic dropout of a heterozygote is observed relative to the size of the survivor companion allele. The threshold is the maximum size of the companion allele observed. This is also the point where Pr(D) approaches zero (Fig. 4).

Dropout threshold will change depending on instrument and assay conditions (e.g., longer CE injection will raise dropout threshold)

ISFG (2006) Recommendations

- Recommendation 8: If the alleles of certain loci in the DNA profile are at a level that is dominated by background noise, then a biostatistical interpretation for these alleles should not be attempted.

Gill *et al.* (2006) DNA Commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. *Forensic Sci. Int.* 160: 90-101

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Recommendation 8:

- If there is a band below the experimental threshold where background noise might be prevalent, and it is distinct and clear from the background, then it should be recorded and available on the case file.

ISFG (2006) Recommendations

- Recommendation 9: In relation to low copy number, stochastic effects limit the usefulness of heterozygous balance and mixture proportion estimates. In addition, allelic drop-out and allelic drop-in (contamination) should be taken into consideration of any assessment.

Gill *et al.* (2006) DNA Commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. *Forensic Sci. Int.* 160: 90-101

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Recommendation 9:

- Case pre-assessment is necessary in order to determine the best scientific method to process a sample. To facilitate this, it is recommended that wherever possible, this should include quantification. Quantification is used to determine the optimum method to process—if low-level DNA, a sample would benefit from procedures to enhance sensitivity of detection. There may be reasons where quantification is not practicable, especially if low levels of DNA are expected, since the result itself may be compromised if a portion of the sample is sacrificed. At low DNA levels, the accuracy of the quantification test itself may be inefficient.

UK Response

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Recommendation 9 (cont):

- It is possible that a given DNA profile may simultaneously comprise both 'conventional' and 'low-level' loci: for example, if degradation has occurred then low molecular weight loci may be above the dropout threshold, whereas high molecular weight loci may be below the dropout threshold.
- Similarly, if the sample is a mixture, then at a given locus there may be some alleles that are above the dropout threshold (from a major contributor) and others that are below the dropout threshold (from a minor contributor), i.e. different interpretation rationale may be simultaneously applied to different contributors within a locus.

Thank you for your attention…

Questions
or Comments?

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

MIXTURE CLASSIFICATION FLOWCHART

Define what is a mixture (>2 alleles at ≥2 loci)

Developed by John Butler
based on German classifications
Schneider *et al.* (2006) *Rechtsmedizin* 16:401-404

>2 alleles at a locus, except tri-allelics?

NO

Single Source DNA Sample

Determine STR profile and compute RMP

YES

Mixed DNA Sample

YES

Differentiate a Major/Minor Component?

NO

Stochastic Effects ? Possible Low Level DNA) ?

Define reliable ratio ranges (4:1 to 10:1)

Define LCN limits (<200 pg)

YES

TYPE B

Determine component profile(s) and compute RMP for major

YES

TYPE C

A biostatistical analysis should not be performed

NO

TYPE A

A biostatistical analysis must be performed

Are # of contributors defined?

Assume # Contributors ?

YES

Likelihood Ratio [LR]

NO

Probability of Exclusion [P_E] "RMNE"

Summary of ISFG Recommendations on Mixture Interpretation

1. The likelihood ratio (LR) is the preferred statistical method for mixtures over RMNE
2. Scientists should be trained in and use LRs
3. Methods to calculate LRs of mixtures are cited
4. Follow Clayton et al. (1998) guidelines when deducing component genotypes
5. Prosecution determines H_p and defense determines H_d and multiple propositions may be evaluated
6. When minor alleles are the same size as stutters of major alleles, then they are indistinguishable
7. Allele dropout to explain evidence can only be used with low signal data
8. No statistical interpretation should be performed on alleles below threshold
9. Stochastic effects limit usefulness of heterozygote balance and mixture proportion estimates with low level DNA

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DNA commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures

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Abstract

The DNA commission of the International Society of Forensic Genetics (ISFG) was convened at the 21st congress of the International Society for Forensic Genetics held between 13 and 17 September in the Azores, Portugal. The purpose of the group was to agree on guidelines to encourage best practice that can be universally applied to assist with mixture interpretation. In addition the commission was tasked to provide guidance on low copy number (LCN) reporting. Our discussions have highlighted a significant need for continuing education and research into this area. We have attempted to present a consensus from experts but to be practical we do not claim to have conveyed a clear vision in every respect in this difficult subject. For this reason, we propose to allow a period of time for feedback and reflection by the scientific community. Then the DNA commission will meet again to consider further recommendations.

Keywords: STR typing; Biostatistical analysis; Likelihood ratio; Probability of exclusion; Mixtures; ISFG DNA commission

1. The general approaches used to interpret DNA profiles

There are two different methods in common use to report DNA profiles: these are the classical profile probability approach and the likelihood ratio approach. See Buckleton [1] and Balding [2] for a full discussion of the various methods to interpret evidence.

1.1. The profile probability approach

In the forensic context the profile probability approach presents the probability of the evidentiary DNA profile (E) under a stated hypothesis (H_o). This hypothesis may be as simple as saying that the DNA profile is from a person unrelated to the suspect. The probability is written formally as $\Pr(E|H_o)$, where Pr is an abbreviation for 'probability' and the vertical line, or conditioning bar, is an abbreviation for 'given'. For a single-contributor stain, under the approximation that profiles from unrelated people are independent, this probability is the frequency of occurrence of the profile in the population.

1.2. The likelihood ratio

An extension of the profile probability approach works with the probabilities of the evidence under two or more alternative hypotheses about the source(s) of the profile. A typical analysis of a crime sample has the prosecution hypothesis (H_p) and the defence hypothesis (H_d). For a profile with more than one

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contributor, the prosecution may hypothesise that the suspect (S) and one unknown (U) person were the contributors, whereas the defence may hypothesise that there were two unknown contributors U_1 and U_2. The likelihood ratio (LR) compares the probabilities of the evidence under these alternative hypotheses:

$$\mathrm{LR} = \frac{\Pr(E|H_\mathrm{p})}{\Pr(E|H_\mathrm{d})}$$

If the LR is greater than one, then the evidence favours H_p but if it is less than one then the evidence favours H_d.

In the single-contributor case, the probability of the evidence profile under H_p (the suspect is the contributor) is one and the LR reduces to the reciprocal of the probability of the stain profile if it did not come from the suspect. Ignoring the possibility of relatives and population structure this is just the population frequency of the profile as would have been given by the profile probability approach.

But, it is worth noting that under certain easily defined circumstances, involving low level crime stain profiles, the probability of the numerator $\Pr(E|H_\mathrm{p})$ is less than one. When this happens the LR gives a number that is less than that obtained using the profile probability approach. Examples are given in Appendix A (stutter) and Appendix B (drop-out).

To evaluate mixtures population genetics principles are applied—to the extent that the suspect (if innocent) and the perpetrator are suspected to be from the same sub-population then an F_St correction is desirable.

1.3. Types of alleles

There are three kinds of alleles in a crime stain profile:

A. alleles that are unmistakeable;
B. alleles that may be masked by an artefact such as a stutter;
C. alleles that have dropped out completely and are therefore not detected.

We emphasise the need to carry out appropriate biochemical and genetic tests—e.g. the analysis of multiple stains in order to obtain the best results possible before carrying out the statistical analysis.

2. A comparison of the probability of exclusion method versus the LR method

The probability of exclusion $\Pr(Ex)$, or random man not excluded (RMNE) [3,4] or the complementary probability of inclusion $\Pr(I)$ entails a binary view of alleles, meaning that alleles are only present or absent, and further if present are observed. Using the method therefore entails the implicit assumption that all alleles are either in category A or at least – and this necessitates counting all artefacts that might mask an allele in the RMNE calculation – in category A or B. In particular it is problematical to apply the method when there are loci which, under the hypothesis being considered of the suspect at hand, appear to have alleles in category C. We have seen many instances in which laboratories do just this, usually by omitting from the RMNE calculation the inconvenient loci. Such a calculation implies, certainly incorrectly, that among the ''random men'' considered for comparison by the calculation only the same loci would be used for inculpation/exculpation as those being considered for the present suspect. It fails to acknowledge that choosing the omitted loci is suspect-centric and therefore prejudicial against the suspect. (If, on the other hand, a locus is eliminated from analysis simply because it is a poor result showing no alleles at all, then of course there is no prejudice in ignoring it.)

Consequently the exclusion method may be justified under the following circumstances:

1. It is known that all relevant alleles are in category A.
   Or:
2. It is known that all relevant alleles are in category A or B.
3. All of the suspect's alleles are present and the report is conditional, e.g. ''The suspect is not excluded as being a major (or salient) contributor, whereas x% of random men would be''.

The method is usually quite conservative provided it is properly applied as described above.

The advantage of the LR framework is that stutter and drop-out can be assessed probabilistically [5–7] (Appendices A and B), and it is the only way to provide a meaningful calculation based on the probability of the evidence under H_p and H_d. The RMNE method has considerable intuitive appeal but usually entails an unrealistically simple model of DNA evidence and is therefore restricted in its use to unambiguous profiles. Even in those cases RMNE has the further shortcomings as it does not make full use of the evidence.

A likelihood ratio approach is therefore preferred. There is a broad consensus view on this point that originates from the original recommendation of the NRC II report [8].

Various advantages and disadvantages have been suggested in relation to the LR and RMNE approaches; summarised by Clayton and Buckleton [9]. In particular, Weir [10] states that exclusion probabilities ''often rob the items of any probative value'' and Brenner [11] states ''the exclusion probability usually discards a lot of information compared to the correct likelihood ratio approach''. Michael Krawczak states: ''In my view, this is not a question of 'correct' and 'incorrect', but of 'efficient' and 'inefficient'. The RMNE simply does not use as much of the information included in the data as the LR approach but, conceptually, they are equivalent. The RMNE is based on a different statistical model than the LR approach. So the two methods are bound to give different answers in one and the same case. The RMNE result is still correct, given the model, but is not an optimal result since the model does not make efficient use of the available information''.

However, if the model is used outside the constraints of its working limitations, then there is no reason to suppose that the concept of 'conservativeness' still applies. An example follows:

Consider a genetic marker, such as a SNP that has only two alleles *a* and *b* in the population. For a two-contributor stain with both alleles (*ab*), no-one in the population is excluded so the RMNE probability is one. However, if the suspect is of type *aa*, and it is a common type, then the LR assuming two contributors is less than one. Although unlikely to concern STR multiplexes in current use, this would extend to the multi-allele case when nearly all of the allele types at the locus are present in the stain.[1]

Clayton and Buckleton [9] report two advantages for the RMNE approach: (a) it does not require an assumption of the number of contributors to a mixture and (b) it is easier to explain in court. Otherwise the RMNE usually results in an underestimate of the strength of evidence in numerical terms (except for unusual situations where all or most alleles are present at a locus). Nevertheless, this may be an important consideration. The US DNA Advisory Board [3] states: ''The calculation is particularly useful in complex mixtures, because it requires no assumptions about the identity or number of contributors to a mixture''.

- *Recommendation* 1: The likelihood ratio is the preferred approach to mixture interpretation. The RMNE approach is restricted to DNA profiles where the profiles are unambiguous. If the DNA crime stain profile is low level and some minor alleles are the same size as stutters of major alleles, and/or if drop-out is possible, then the RMNE method may not be conservative.

3. Court acceptance of the LR approach

In addition, an argument that may be put forward is that courts are unwilling to accept the LR method. Whereas we recognise that there are restrictions that are placed upon scientists by legal systems, we recommend that the scientist should always prepare his/her evidence using the LR method wherever possible. We accept that the court may not wish to hear the evidence presented in this way, but this does not preclude it from being present on the case-file. Neither is the scientist precluded from drawing the courts attention to the preferred method before presenting evidence in line with the court requirements. The court may be unaware of the method if the scientist does not attempt to introduce it. In the O.J. Simpson case [12], the prosecution wished to use LRs, the defence advocated use of RMNE but the final result was that the court heard both methods—the judge finally ruled that the LR method was preferable.

- *Recommendation* 2: Even if the legal system does not implicitly appear to support the use of the likelihood ratio, it is recommended that the scientist is trained in the methodology and routinely uses it in case notes, advising the court in the preferred method before reporting the evidence in line with the court requirements. The scientific community has a responsibility to support improvement of standards of scientific reasoning in the court-room.

4. The likelihood ratio method using the unrestricted combinatorial approach (not taking account of peak height/areas)

This method examines all possible sets of genotypes consistent with the alternative hypotheses of H_p and H_d [13,14]. We assume uniform assumptions (such as number of contributors) across loci. For example, suppose we have four alleles *a*, *b*, *c* and *d* at a locus. If we assume that there are two contributors, then an exhaustive list of all of the possible genotype combinations is given in Table 1. The probabilities are calculated for each combination, e.g. in the first row the probability of genotype *ab* (assuming Hardy–Weinberg equilibrium) is assigned as $2p_ap_b$ and the probability of *cd* is $2p_cp_d$. Multiplying the two together to calculate the probability of *ab* and *cd* gives $4p_ap_bp_cp_d$. This is repeated for each row, then all of the probabilities are summed together to give $\Pr(E|H_d) = 24p_ap_bp_cp_d$.

$\Pr(E|H_p)$ is calculated separately. If the suspect (*S*) is *ab*, the unknown individual (*U*) must be *cd*, then $\Pr(E|H_p) = 2p_cp_d$, hence:

$$\mathrm{LR} = \frac{2\,p_c\,p_d}{24\,p_a\,p_b\,p_c\,p_d} = \frac{1}{12\,p_a\,p_b}$$

The evaluation of two- or three-banded loci is more complex but follows the same rationale [13,14].

- *Recommendation* 3: The methods to calculate likelihood ratios of mixtures (not considering peak area) described by Evett et al. [13] and Weir et al. [14] are recommended.

5. The likelihood ratio method using the restricted combinatorial approach (taking account of peak height/areas)

A typical mixture may consist of major/minor components (Fig. 1). Provided that there is sufficient difference in peak height between the two pairs of alleles and the major components are sufficiently represented so that stochastic effects leading to substantial heterozygous imbalance can be discounted, then they may be separated according to size. Hence in the example above, it may be appropriate to designate *ab* major and *cd* minor components if the profile is derived from a two person mixture.

Interpretation is easiest if the genotype of interest (attributed to the suspect under H_p) corresponds to the major alleles *ab* of the mixture. If the genotype of interest is the minor component

[1] For a two-allele locus with allele frequencies p_a and $p_b = 1 - p_a$ the probability of *ab* under H_p that the contributors were an *aa* suspect and one unknown person is $1 - p_a^2$. The probability of *ab* under H_d that the contributors were two unknown people is $1 - p_a^4 - (1 - p_a)^4$. The LR is less than one when p_a is greater than about 0.4. The RMNE probability is 1 since no-one is excluded from the mixture. For a four-allele locus with allele frequencies p_a, p_b, p_c, p_d, suspect *ab* and crime profile evidence *abcd*, then the LR for H_p: suspect and one unknown versus H_d: two unknowns is $1/(12p_ap_b)$. This is less than one when *ab* is a common genotype, whereas the RMNE probability is one since no-one is excluded from the mixture. The probability of the DNA profile evidence increases with the number of contributors in this case.

Table 1
Evaluation of $\Pr(E|H_d)$; two person mixture with four discrete alleles present

Individual 1	Individual 2	Genotype probability
ab	*cd*	$4p_ap_bp_cp_d$
ac	*bd*	$4p_ap_bp_cp_d$
ad	*bc*	$4p_ap_bp_cp_d$
cd	*ab*	$4p_ap_bp_cp_d$
bd	*ac*	$4p_ap_bp_cp_d$
bc	*ad*	$4p_ap_bp_cp_d$
Sum		$24p_ap_bp_cp_d$

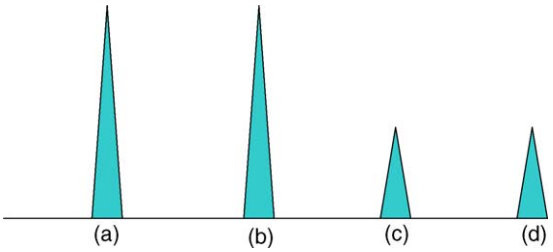

Fig. 1. A four-allele mixture, showing major *ab* and minor *cd* contributions.

cd, then interpretation is more complex since other considerations include drop-out, stutter and masking by major alleles. If the mixture is composed from two contributors, and the suspect is *ac* (i.e. one major and one minor allele), and the unknown contributor is *bd*, this combination would be accepted using the unrestricted combinatorial approach, but would be rejected, or excluded using the restricted approach under H_p. This means that defaulting to the unrestricted combinatorial approach is not necessarily conservative. If the profile is a mixture and the contributors are roughly 50:50 then the restricted approach converges to the unrestricted approach at all four peak loci and approaches it at the other loci. This convergence is most marked if the crime profile is low level as more combinations must be allowed under H_d.

A good understanding of the characteristics of H_b (heterozygote balance) and M_x (the mixture proportion) are needed to properly implement either approach [9,15–18].

5.1. An example of masking—three alleles at a locus

If the genotypes of two persons are *ab* and *bc*, then they share the *b* allele. The contributions are assumed to be additive. Given a mixture ratio of 2:1 as an example, we expect the proportions of $a{:}b{:}c = 2{:}3{:}1$ (Fig. 2). The mixture ratio is approximately the same across loci.

The profile is no longer balanced and consequently the interpretation is more difficult but more informative. The major component (*ab*) can be identified. The minor component is *bc*. Other combinations might be considered reasonable, such as *bb*, *ac*. The principle followed is to assess the combinations that would be expected to give a reasonable fit to the peak areas, eliminating those that are unreasonable. To do this it is necessary to make an assessment in relation to the heterozygote balance (H_b) and mixture proportion (M_x) [9,15–17].

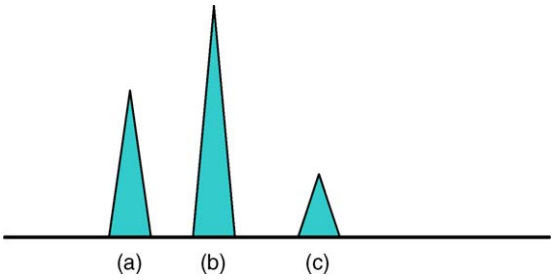

Fig. 2. A three-allele profile showing masking.

5.2. The restricted combinatorial (binary) model

The restricted combinatorial (binary) model [9,16] starts from the position that all alternatives (Table 1) are considered possible unless the combination gives a poor fit to the peak height/areas. For example, in Fig. 1, the combination of a minor allele <60% the peak height/area of the major allele when there is reasonable quantity of DNA analysed (at least 500 pg) is unrealistic given experimental data on heterozygote balance (H_b) [19]. Consequently, the peak height/areas are unlikely given a combination such as (*ac*, *bd*), hence $\Pr(E|ac, bd) \approx 0$. All of the alternatives that give low probabilities for the areas are discounted based on an assessment of whether the genotype combinations are explicable in relation to mixture proportion (M_x) and heterozygote balance (H_b). This assessment is easiest when the loci are four-banded, but can also be carried out when there is masking of alleles, i.e. three- and two-allele mixtures where there are two contributors [20]. The implementation of such an approach in routine casework, in particular when using a computer-based expert system for mixture interpretation, requires an extensive validation of the variable parameters such as H_b and M_x, as well as appropriate guidelines for all laboratory procedures.

Clayton and Buckleton [9] assess the limitations of the restricted combinatorial (binary) model. The method is robust provided that the H_p propositions give a reasonable fit to the peak heights/areas. From the example above, if the suspect was *ac* then this would not give a good fit to the data. Both numerator and denominator need to be separately assessed and this is linked to the formulation of propositions and the number of contributors (Appendix C).

5.3. The steps to interpret a mixture

These guidelines are modified from Clayton et al. [17]. They are widely used and are summarised here as a way to interpret mixture profiles.

5.3.1. Step 1: Identify the presence of a mixture

If more than two allelic bands per locus are present, a mixture may be inferred. Note extra bands may also be present because of somatic/genetic polymorphism and stutters. In addition, allele asymmetry occurs because shared alleles result in ‘masking’. The profile appears unbalanced as a result.

5.3.2. Step 2: Designation of allelic peaks

(1) Alleles should be within ±0.5 bp of the designated control allele ladder marker.
(2) The band shift for each allele, relative to the control allelic ladder marker, should be approximately constant.

5.3.3. Step 3: Identify the number of contributors in the mixture

The number of alleles observed per locus, circumstances of the case, and the possibility of related contributors go into deciding how many contributors to condition on.

When all loci of the crime stain profile (from a cosmopolitan population) are taken into consideration to calculate the LR, often, but not always, the probability of the evidence under H_p and H_d is maximised when the number of contributors is minimised. This applies to STR multiplexes in current use but cannot be applied to SNPs.[2]

5.3.4. Step 4: Estimation of the mixture proportion or ratio of the individuals contributing to the mixture

At this stage, it may be possible to separate major/minor contributors to the mixture. If DNA templates are mixed, then the ratio/proportion of contributors are approximately preserved throughout the mixture at each locus. The mixture proportion (M_x) or ratio (M_r) can be approximately assessed [16,20]. For example, the approximate value of M_x for a four-banded profile conditioned on two contributors, where two minor component alleles *a* and *b* are present with two major component alleles *c* and *d* is:

$$M_x = \frac{\phi_a + \phi_b}{\phi_a + \phi_b + \phi_c + \phi_d}$$

where ϕ_i is the peak height or peak area of the *i*th allele.

More robust methods have been developed that calculate a single $\hat{M}_x$ across all loci by calculating least squares residuals [20]. Experimentation has shown that the error in the estimation of $\hat{M}_x$ is within ±0.35 [9]. Note that the variance of this parameter may differ between processes, e.g. when different STR multiplexes, DNA amounts, and PCR conditions are used—it is given here as an example only.

[2] Other things being equal, the aim of the defense is to maximize the probability of the evidence under H_d. Similarly, the prosecution aims is to maximize the probability of the evidence under H_p, consistent with their theory of the case. The number of contributors is a secondary consideration; usually, but not always, this coincides with the fewest number of contributors required to explain the crime stain profile. It does not assist the defense case to postulate more contributors than necessary, if it reduces $\Pr(H_d)$—but exceptions are possible: consider a crime stain profile $E = a, b, c, d$; for simplicity we assume that the allele frequencies are the same (p_x). The probability given two individuals ($n_c = 2$) under H_d: two unknown individuals is $24x^4$ whereas for three individuals this probability equals $1560x^6$. The latter ($n_c = 3$) is larger than the former ($n_c = 2$) when $p_x > 0.124$. Whereas it is easy to show an exception to the generalisation at a single locus, when it does occur: (a) the impact on the LR of very common alleles on a single locus, is minimal (b) it is unlikely to have any impact when all other loci in the crime stain profile are taken into consideration since much rarer alleles will be prevalent in STR multiplexes in standard use. The overall effect will be to maximize $\Pr(H_d)$ concurrent with minimizing the number of contributors.

The second parameter under consideration is heterozygote balance (H_b)

$$H_b = \frac{\phi_a}{\phi_b} \quad (\text{where } \phi_a \text{ is the smallest peak in height or area}).$$

Experimental observation showed that under conditions where the DNA was undegraded and present in quantities >500 pg, $H_b > 0.6$ [19], hence a genotype where $H_b < 0.6$ would not be supported (we denote the threshold as $H_{b\ min} = 0.6$). Note that for low levels of DNA, stochastic effects reduce the $H_{b\ min}$ threshold. Degradation disproportionately affects high molecular weight alleles more than low molecular weight alleles, this can have a substantial effect in reducing H_b when alleles differ greatly in molecular weight (such as the HUMFIBRA/FGA locus).

5.3.5. Step 5: Consideration of all possible genotype combinations

The next step is to consider all combinations of the unrestricted combinatorial list of genotypes (Table 1) in relation to the mixture proportion (M_x) and the heterozygote balance (H_b) across all loci and their verified experimental tolerances [9]. Taking the example in Fig. 1 where there are two major alleles *ab* and two minor alleles *cd*: if the estimated $\hat{M}_x = 0.7 \pm 0.35$ across loci and $H_{b\ min} = 0.6$, a mixture can be assessed by considering each of the possible genotype combinations, per locus, with respect to these two parameters (Table 2).

Those combinations that are not supported by guidelines formulated around these two parameters are considered to have a low posterior probability and are removed. The final list of genotypes comprises those allelic combinations that are well supported by experimental observations. For example, to explain the combination *ac*, *bd*, this would require a low heterozygous balance that has not been observed in experimental data. In Table 2, only *ab*, as the major contributor, and *cd*, as the minor contributor, are feasible combinations.

These guidelines are not 'all or nothing'. If a genotype combination is borderline or uncertain, then it should be included under H_d since this will increase $\Pr(E|H_d)$, but inclusion of a borderline result is problematic under H_p because the restricted combinatorial (binary) model assumes that conditional genotypes are reasonable fits to the peak height/

Table 2
Assessment of major (*ab*)/minor (*cd*) genotypes of a mixture of two contributors relative to $\hat{M}_x$ and H_b calculated using $\phi_a = 1200$ rfu, $\phi_b = 100$ rfu, $\phi_c = 400$ rfu, $\phi_d = 380$ rfu, where rfu is relative fluorescence units (allele peak height)

Genotypes		M_x|major, minor genotypes	Heterozygous balance		Comment
Major	Minor		$H_{b\ major}$	$H_{b\ minor}$	
ab	*cd*	0.70	0.9	0.9	Passes H_b, $\hat{M}_x$
ac	*bd*	0.53	0.3	0.3	Fails H_b
ad	*bc*	0.51	0.3	0.3	Fails H_b
cd	*ab*	0.30	0.9	0.9	Fails $\hat{M}_x$
bd	*ac*	0.48	0.3	0.3	Fails H_b
bc	*ad*	0.49	0.3	0.3	Fails H_b

areas under this hypothesis. In this extreme example, if the suspect is *ac* and the unknown genotype is *bd* then the H_p propositions are unreasonable.

5.3.6. Step 6: Compare reference samples

It is important that steps 1–5 take place without considering the reference samples. This is because we demonstrably avoid the possibility of bias. If the genotype of a suspect matches a well-supported combination in the list, then there is evidence to suggest that the individual has contributed to the mixture. When the comparisons of the crime profiles and the reference samples are made, it may be necessary to refine the propositions [21]. For example, if the initial propositions suggest H_p: the stain contains the DNA of the suspect (S) and two victims (V_1, V_2), and comparison of the profile with reference samples suggests H_p: the suspect (S), one of the victims (V_1) and one unknown (U), then additional propositions may be considered.

The calculation of the likelihood ratio is exactly the same as described above (Table 1) except that in the summation of probabilities, only those that are well supported are included under H_p and H_d.

Irrespective of the principles outlined in step 3, where conditioning on the minimum number of contributors, maximises $\Pr(E|H_p)$ and $\Pr(E|H_d)$ it may still be necessary to consider multiple propositions at the final stage of analysis. It will be for the court to decide those that are relevant for consideration, bearing in mind that perhaps several different LR calculations are relevant.

- *Recommendation* 4: If peak height or area information is used to eliminate various genotypes from the unrestricted combinatorial method, this can be carried out by following a sequence of guidelines based on Clayton et al. [17].
- *Recommendation* 5: The probability of the evidence under H_p is the province of the prosecution and the probability of the evidence under H_d is the province of the defence. The prosecution and defence both seek to maximise their respective probabilities of the evidence profile. To do this both H_p and H_d require propositions. There is no reason why multiple pairs of propositions may not be evaluated (Appendix C).

6. Treatment of stutter

The characteristics of stutter bands (one tandem repeat less than the parent allele) have been evaluated in relation to the size of the associated parent allele [22,23]. The stutter peak area or height is measured as a proportion (St_p) of the parent allele peak area or height.

$$St_p = \frac{\phi_{stutter}}{\phi_{allele}}$$

In general $St_p < 0.15$.

Suppose there are minor alleles *ab* and two major alleles *cd* where *b* is in a stutter position and is within the range of experimental observations of St_p (Fig. 3). It is not known if the band in the stutter position is an allele, a stutter, or a mixture of both. The genotypes of the minor contributor to consider are *ab* (if *b* is not a stutter, or an allele with a stutter) and *ac*, *ad* and *aa* (if *b* is a stutter). If the suspect is *ab* and the victim is *cd*, then calculation of the LR is conservative if genotype combinations include bands in stutter positions under H_d. However, if the suspect is *aa* and the victim is *cd* such that the explanation under H_p is conditional upon *b* being a stutter, then the probability of stutter must be considered in the numerator. Further advice and examples are given in Appendix A.

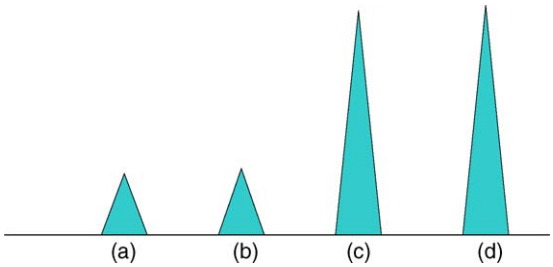

Fig. 3. Two minor alleles, *a* and *b*, with two major alleles, *c* and *d*, where allele *b* is in a stutter position.

- *Recommendation* 6: If the crime profile is a major/minor mixture, where minor alleles are the same size (height or area) as stutters of major alleles, then stutters and minor alleles are indistinguishable. Under these circumstances alleles in stutter positions that do not support H_p should be included in the assessment.

7. Drop-out

The consideration of drop-out is analogous to stutter. Suppose an allele *a* is present in a mixture at close to background level, indicating a contributor who made a small contribution. There is a substantial probability that *a*'s partner allele has dropped out completely. This has implications for an *ab* suspect when *b* is not seen. It may be net evidence against the suspect of strength approximately $1/2p_a$. But as the intensity of the *a* allele increases, the probability of drop-out $p(D)$ continually decreases until the point at which the $p(D)$ is zero and the suspect is excluded and the LR at the locus is zero [7]. Consequently, for slightly lesser *a* intensities, the net evidential value of the locus must be in favour of the suspect, i.e. LR is less than one. Therefore, it would be prejudicial to calculate a likelihood ratio of one or greater or to omit the locus because that amounts to taking LR = 1. If the hypothesised genotype is *ab* and the crime stain profile includes *a* but not *b*, then drop-out is very plausible if allele *a* is close to the background level. If allele *a* is significant in size (i.e. at a level where drop-out would not be expected), then the probability of drop-out is less likely, i.e. the possibility that the source is *aa* is more likely. See Appendix B for further considerations.

A point is reached where the background noise of the electropherogram is equivalent to the signal strength of the DNA profile. The negative controls will be particularly useful to ascertain this level. A biostatistical interpretation of an evidential

profile that is dominated by background noise is inadvisable—in the case of a major/minor mixture, only the contribution by the low level minor contributor(s) is compromised, while the major contributor is unaffected and the interpretation of this component of the mixture is not compromised.

- *Recommendation* 7: If drop-out of an allele is required to explain the evidence under H_p: ($S = ab$; $E = a$), then the allele should be small enough (height/area) to justify this. Conversely, if a full crime stain profile is obtained where alleles are well above the background level, and the probability of drop-out approaches $\Pr(D) \approx 0$, then H_p is not supported.
- *Recommendation* 8: If the alleles of certain loci in the DNA profile are at a level that is dominated by background noise, then a biostatistical interpretation for these alleles should not be attempted.

8. Low copy number

The operational definition of low copy number PCR is the manifestation of stochastic effects leading to allelic imbalance, drop-out and increased prevalence of laboratory-based contamination. Consequently, the conventional rules of heterozygous balance and other characteristics of DNA profiling do not apply [6] in the same way.

Low copy number is usually associated with a low amount of DNA (less than 200 pg). The method is typically associated with an elevated PCR cycle number, but it is important to realise that the effects may occur at 28 PCR cycles, typically with a major/minor mixture where the minor component alleles are subject to drop-out and may be the same size as stutter alleles. There are a number of caveats associated with LCN reporting [24]. LCN alleles are not necessarily category A (unambiguous). Therefore, LCN mixture analysis will have to allow for stochastic events (drop-out, heterozygous imbalance and contamination) [6].

- *Recommendation* 9: In relation to low copy number, stochastic effects limit the usefulness of heterozygous balance and mixture proportion estimates. In addition, allelic drop-out and allelic drop-in (contamination) should be taken into consideration of any assessment.

9. Definition of contamination

DNA introduced after the crime has happened and from a source that is unrelated to the crime scene: for example, the investigating officer, laboratory technicians, laboratory plasticware [25,26].

10. Training

We recognise that scientists should be trained to a level appropriate to carry out the necessary calculations. Training schedules are required for accreditation under standards such as ISO17025. There is clearly a need for comprehensive training schedules to become widely available.

11. Future

A future approach would elaborate the combinatorial approaches by taking into account all aspects including stutter, contamination and other artefacts, allelic drop-out, such as by using a probabilistic weighting for each possible genotype rather than just using a weighting of zero or one, as is inherent in the restricted combinatorial (binary) approach.

12. Accreditation and expert systems

We note that accrediting standards such as ISO17025 require traceability, which may be interpreted as excluding ''black boxes''. This is a consideration in using expert system computer programs.

Acknowledgement

The authors are grateful to James Curran for clarifying a number of issues in this paper.

Appendix A

A.1. Stutters

The interpretation of allelic components of the minor component of a mixture can be compromised:

Stutters (from a major contributor) may be the same height/peak area as the minor contributor to the mixture. This means (Fig. 4) that those bands in stutter positions may be allele only, allele plus stutter, or stutter only. In Fig. 4, bands *a*, *b* are minor alleles that are very similar in height/area. Band *b* is in a stutter position and we must assume that it could be from an unknown contributor under H_d. Consequently, if we condition on the number of contributors = 2, then the possible minor contributor genotypes are *aa*, *ac*, *ad* (where *b* is a stutter), or *ab* (where *b* is an allele either with or without a stutter).

Taking a simple scenario H_p: the stain contains the DNA of the suspect and the victim versus H_d: the stain contains the DNA of the victim and an unknown individual. If the genotype of $V = cd$, then under H_d, the possible genotypes for U include

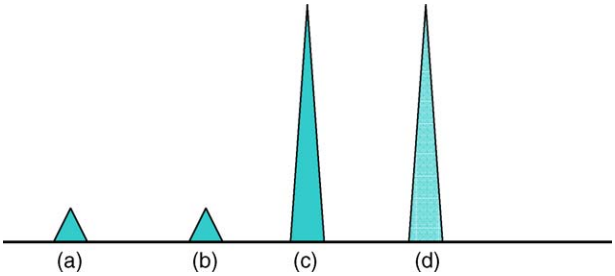

Fig. 4. *c* and *d* are unambiguous alleles, *b* is a minor allele in a stutter position and *a* is an unambiguous minor allele.

ab, since the stutter *b* is a possible allele, and $\Pr(E|H_d) = 2p_a p_b + 2p_a p_c + 2p_a p_d + p_a^2$, where p_i is the allele probability for the *i*th allele. If $S = ab$, then the LR is computed conservatively by including *ab* among the choices for *U* in the denominator, whereas if $S = aa$ or *ac* (i.e. does not have an allele in a stutter position) then it may not be conservative to include *ab* among the choices for *U* [5]. This is because $\Pr(E|H_p)$ has the probability of stutter Pr(St) as a factor, i.e. the numerator is less than one. Under H_d, we multiply by Pr(St) the combinations that can be explained if a stutter has occurred. If stutter has not occurred, then the only possibility is *ab* but we must multiply by the probability $\Pr(\overline{\text{St}})$ that stutter has not occurred where $\Pr(\overline{\text{St}}) = 1 - \Pr(\text{St})$. The formula is now:

$$\text{LR} = \frac{\Pr(\text{St})}{[p_a^2 + 2p_a p_b + 2p_a p_c + 2p_a p_d]\Pr(\text{St}) + [2p_a p_b]\Pr(\overline{\text{St}})}$$

$$\text{LR} = \frac{\Pr(\text{St})}{[p_a^2 + 2p_a p_c + 2p_a p_d]\Pr(\text{St}) + [2p_a p_b]}$$

$$\text{LR} = \frac{1}{p_a^2 + 2p_a p_c + 2p_a p_d + (2p_a p_b / \Pr(\text{St}))}$$

Which obviously approaches zero monotonically as Pr(St) approaches zero (Fig. 5).

The probability Pr(St) can be determined experimentally from a known population of samples where the proportion ϕ_{St}/ϕ_a is calculated; ϕ_{St} is the peak area/height of a stutter and ϕ_a is the peak area/height of an allele.

If ϕ is either peak area or height (it does not matter which so long as we are consistent throughout), then we can calculate the probability from data of experimental observations of probability of observing a stutter of a given proportion conditioned on the size of the 'parent' allele.

It is possible to generalise that stutters are rarely observed when $\phi_{St}/\phi_a > 0.15$ [22,23]. This means that when the allele in the stutter position is larger than this, $\Pr(\text{St}) \approx 0$.

To summarise, if the suspect is *aa*, and there is an allele *b* present, which is in a stutter position, and allele *b* is too large to be only a stutter, then $\Pr(\text{St}) \approx 0$ (from experimental observations). This means that the LR is close to zero and the H_p proposition is unsupported.

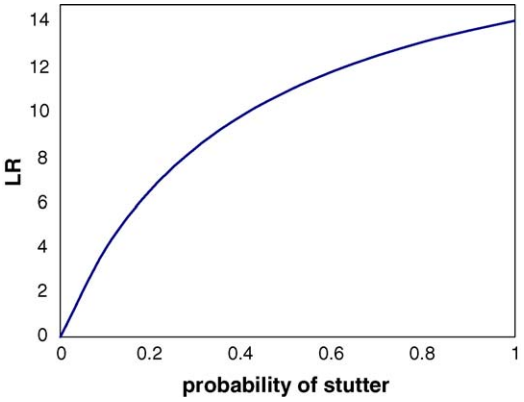

Fig. 5. Plot of Pr(St) vs. $1/(p_a^2 + 2p_a p_c + 2p_a p_d + (2p_a p_b/\Pr(\text{St})))$, where $p_a = p_b = p_c = p_d = 0.1$. The suspect is a minor contributor *aa*, the victim is (major) *cd* and allele *b* is present at the stutter position.

If ϕ is either peak area or height (it does not matter which so long as we are consistent throughout), then we can calculate the probability from data of experimental observations of probability of observing a stutter of a given proportion conditioned on the size of the 'parent' allele.

Appendix B

B.1. Further considerations of drop-out

Allele drop-out is an important consideration whenever a homozygote is observed in a DNA profile. Is the genotype of the contributor homozygous, or is it heterozygous and an allele has dropped out, giving a 'false' homozygote? Many laboratories have carried out experimentation to determine a threshold, T_{rfu} (either peak height or peak area) to signify the upper limit where allele drop-out has been observed in a heterozygote (Fig. 6). Provided that $\phi_a > T_{rfu}$ (ϕ_a is the peak height/area of allele *a*) then the probability of drop-out $\Pr(D) \approx 0$. If a homozygote is observed where $\phi_a < T_{rfu}$ then $\Pr(D) < 1$. Furthermore, the smaller ϕ_a then the greater Pr(*D*) becomes (Fig. 6).

If the suspect (*S*) is *ab* and the crime stain profile is *a*, then under H_p we must consider the probability of drop-out Pr(*D*). If the $\Pr(D) \approx 0$, as $\phi_a > T_{rfu}$, then the proposition that the suspect is a donor is not supported and an exclusion is likely to be the best conclusion. If $\Pr(D) < 1$, then the term Pr(*D*) must appear in the numerator of the likelihood ratio:

$$\text{LR} \approx \frac{\Pr(D)}{p_a(p_a + 2\Pr(D)(1 - p_a))} \quad \text{from [7]} \tag{1}$$

The correct formulae have been described for non-mixtures [6,7], but their complexity has led to the use of approximations; an example is the '*F*' designation which represents the situation where an allele may have failed to amplify. In such a circumstance the genotype may be signified by *aF* which describes a genotype containing the *a* allele and any other allele. It is customary to assign the probability of the profile as $2\Pr(a)$. This is often termed the $2p$ rule.

However, this formula may overestimate the strength of the evidence. An example where the '*F*' designation is not conservative, for non-mixtures, occurs whenever Pr(*D*) appears in the numerator (as above), i.e. the suspect is *ab*, the stain is *a* and $\Pr(D) < 0.5$ (excluding sub-population corrections).

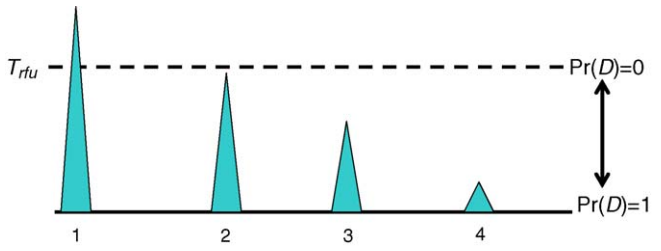

Fig. 6. Alleles 1–4 are phenotypic homozygotes of decreasing size. The probability of drop-out Pr(*D*) increases as the size of the surviving peak decreases.

Table 3
Evaluation of the crime stain profile $E = acd$, $S = ab$ and $U = cd$

Hypothesis	M_j	$\Pr(M_j)$	$\Pr(E\|M_j)$	Comments
H_p	cd	$2p_c p_d$	$p(D)\,p(\bar{D})^3$	One drop-out event
H_{d1}	Any combination that carries $acdQ$	$24p_a p_c p_d p_Q$	$p(D)\,p(\bar{D})^3$	One drop-out event (with Q allele)
H_{d2}	Any combination that carries acd	$12\ p_a p_c p_d(p_a + p_c + p_d)$	$p(\bar{D})^4$	No drop-out event

M_j is a "genotype" or a collection of ordered alleles representing a genetic combination we might wish to consider as having gone into the crime scene stain.

If it is not necessary to invoke drop-out to explain the evidence—if the suspect is a donor under H_p, then the F designation is always conservative (unless F_{St} and $\Pr(D)$ are high).

$$\mathrm{LR} \approx \frac{\Pr(D)}{p_a(p_a + 2\Pr(D)(1 - p_a))} \geq \frac{1}{2p_a} \quad \text{from [7]} \tag{2}$$

Expansion of these concepts to mixtures is complex and this is the reason why they are not generally used. Programmed solutions have recently appeared however that use a modified (improved) concept instead of 'F' [27]. This is called the 'Q' virtual allele concept: if there are n alleles visible in a mixture and drop-out has occurred, we can calculate a ceiling for the frequency of any missing allele:

$$\Pr(Q) = 1 - \sum_{i}^{k_p} p_i \tag{3}$$

where k_p is the number of alleles present at the locus in the crime stain and p_i is the population frequency of the ith allele.

We include below a summary of a further evaluation using the 'F' designation compared to the model incorporating $\Pr(D)$ for a number of scenarios for a simple mixture:

$$\mathrm{LR} = \frac{\Pr(E|S + U)}{\Pr(E|U_1 + U_2)} \tag{4}$$

where S is the suspect, U the unknown and E is the crime stain profile DNA evidence. No sub-population correction is made in this example. We make the simplified assumption that $\Pr(D)$ is the same for S and U.

B.2. Example 1

We assume that the probability of drop-out is the same for all alleles. The crime stain profile $E = acd$, $S = ab$ and $U = cd$. This means that under H_p, allele b has dropped out. To calculate H_d we consider separately the conditions of drop-out and no drop-out. Under H_{d1}, drop-out is invoked. We simultaneously incorporate the virtual allele Q to describe all pairwise combinations (M_j) from alleles a, c, d, Q. Alternatively, under H_{d2} drop-out is not invoked, in which case combinations (M_j) from the visible alleles a, c, d are evaluated. Summing H_{d1} and H_{d2} gives the denominator of the LR (Table 3).

$$\mathrm{LR} = \frac{p(D)}{6p_a\{2p(D)p_Q + p(\overline{D})(p_a + p_c + p_d)\}} \tag{5}$$

Using Eq. (5), if $p_a = p_c = p_d = 0.1$ then the resultant LRs are shown in Table 4.

The evidence favours H_d, unless $p(D) > 0.6$, when it is neutral. If the 'F' designation is used, the numerator = 1, then:

$\mathrm{LR} = (1/12p_a) = 0.83$ which corresponds to $p(D) \approx 0.3$. Note that if $p(D)$ is smaller, this has a relatively minor effect, e.g. LR = 0.41 when $p(D) = 0.1$.

If $p_a = p_c = p_d = 0.02$, then the resultant LRs are shown in Table 5.

The biological model ('F' designation) returns LR = 4.17, consistent with $\Pr(D) \approx 0.3$. The LR is relatively insensitive to $\Pr(D)$ in this example.

Conclusion: The 'F' designation is conservative provided $\Pr(D) > 0.3$ (approximately).

B.3. Example 2

As usual, we assume that the probability of drop-out is the same for all alleles. Consider a low level profile $E = abd$, $S = ab$ and $U = d$. Because the profile is low level, it is possible that allele drop-out has occurred, although both alleles pertaining to S are observed. Under H_d, we should assume that an allele may have dropped out. In such a case we should

Table 4
LRs generated from Eq. (6) where $p_a = p_c = p_d = 0.1$

$\Pr(D)$	$\Pr(E\|H_p)$	$\Pr(E\|H_d)$	LR
0.1	0.1000	0.246	0.41
0.2	0.2000	0.312	0.64
0.3	0.3000	0.378	0.79
0.4	0.4000	0.444	0.90
0.5	0.5000	0.510	0.98
0.6	0.6000	0.576	1.04
0.7	0.7000	0.642	1.09
0.8	0.8000	0.708	1.13
0.9	0.9000	0.774	1.16

Table 5
LRs generated from Eq. (6) where $p_a = p_c = p_d = 0.02$

$\Pr(D)$	$\Pr(E\|H_p)$	$\Pr(E\|H_d)$	LR
0.1	0.1000	0.029	3.44
0.2	0.2000	0.051	3.93
0.3	0.3000	0.073	4.13
0.4	0.4000	0.095	4.23
0.5	0.5000	0.116	4.30
0.6	0.6000	0.138	4.34
0.7	0.7000	0.160	4.37
0.8	0.8000	0.182	4.40
0.9	0.9000	0.204	4.42

Table 6
Evaluation of a low level profile where $E = abd$, $S = ab$ and $U = d$

Hypothesis	M_j	$\Pr(M_j)$	$\Pr(E\|M_j)$	Comments
H_p	ad, bd or dd	$2p_a p_d + 2p_b p_d + p_d^2$	$\Pr(\bar{D})^4$	No drop-out event
H_{d1}	Any combination that carries $abdQ$	$24p_a p_b p_d p_Q$	$\Pr(D)\Pr(\bar{D})^3$	One drop-out event (with Q allele)
H_{d2}	Any combination that carries abd	$12p_a p_b p_d(p_a + p_b + p_d)$	$\Pr(\bar{D})^4$	No drop-out events

Table 7
LRs generated from Eq. (7) where $p_a = p_c = p_d = 0.1$

$\Pr(D)$	$\Pr(E\|H_p)$	$\Pr(E\|H_d)$	LR
0.1	0.4500	0.04920	9.1
0.2	0.4000	0.06240	6.4
0.3	0.3500	0.07560	4.6
0.4	0.3000	0.08880	3.4
0.5	0.2500	0.10200	2.5
0.6	0.2000	0.11520	1.7
0.7	0.1500	0.12840	1.2
0.8	0.1000	0.14160	0.7
0.9	0.0500	0.15480	0.3

Table 8
LRs generated from Eq. (7) where $p_a = p_c = p_d = 0.02$

$\Pr(D)$	$\Pr(E\|H_p)$	$\Pr(E\|H_d)$	LR
0.1	0.0900	0.00116	77.5
0.2	0.0800	0.00204	39.3
0.3	0.0700	0.00291	24.1
0.4	0.0600	0.00378	15.9
0.5	0.0500	0.00466	10.7
0.6	0.0400	0.00553	7.2
0.7	0.0300	0.00640	4.7
0.8	0.0200	0.00728	2.7
0.9	0.0100	0.00815	1.2

invoke Q, where Q is any allele other than a, b, d. Under H_p, it is not necessary to invoke Q to explain S, hence the simplest explanation of U that maximises $\Pr(E|H_p)$ is either ad, bd or dd. Under H_d, $\Pr(E|H_d)$ is the same as in the previous example, hence the LR is calculated (Table 6):

$$\mathrm{LR} = \frac{\Pr(\overline{D})(2p_a + 2p_b + p_d)}{12p_a p_b\{2\Pr(D)p_Q + \Pr(\overline{D})(p_a + p_b + p_d)\}}. \quad (6)$$

When $p_a = p_b = p_d = 0.1$, then the resultant LRs are shown in Table 7.

Note that under H_p, drop-out is not invoked. Under H_d, there are two scenarios—one assumes drop-out, whereas the other does not. The LR is greatest when $p(D)$ is low. If $p(D)$ is high, then the LR is low since it is more likely that two bands will survive.

Invoking the 'F' designation produces:

$$\mathrm{LR} = \frac{2p_a + 2p_b + p_d}{12p_a p_b(2\Pr(F) + p_a + p_b + p_d)} = 1.8 \quad (7)$$

The LR corresponds approximately to $p(D) \approx 0.6$.

We now calculate (Table 8) using a rare allele probability ($p_a = p_b = p_d = 0.02$):

The 'F' designation gives LR = 10.11, corresponding to $\Pr(D) \approx 0.5$.

Conclusion: Although both S alleles are present, it is reasonable to postulate drop-out under H_d if $\phi_a < T_{rfu}$. The 'F' designation is conservative if $\Pr(D) < 0.5$. If $\phi_d > T_{rfu}$, then there is no need to use 'F' under H_d since the best supported explanation for U is homozygote dd.

B.4. Example 3

The profile is cd and $S = ab$; both S alleles have dropped out. Under H_p, $U = cd$, but under H_{d1}, U_1 and U_2 incorporate any combination of alleles Q, c and d where Q is any allele except for c and d. In addition, H_{d2} can invoke any combination of two alleles c, d without Q. However, the probability of a two-allele model is several orders of magnitude lower than the Q model and is consequently not included in this example (Table 9).

If $p_c = p_d = 0.1$, then:

$$\mathrm{LR} \approx \frac{1}{6p_Q} = 0.21 \quad (\text{independent of } p(D)) \quad (8)$$

The LR always favours H_d, independent of $p(D)$. Substituting with the 'F' designation results in:

$$\mathrm{LR} = \frac{1}{6\Pr(F)(p_c + p_d + \Pr(F))} = 0.14 \quad (9)$$

If the scenario changes so that U has dropped out, then the numerator ≈ 1, as U could be any allelic combination. The LR is:

$$\mathrm{LR} \approx \frac{1}{12p_a p_b p_Q(p_a + p_b + p_Q)} \quad (10)$$

LR $\approx$ 10.4 (when $p_c = p_d = 0.1$).

Substituting 'F' instead of Q gives LR = 6.9.

Table 9
Evaluation of a DNA profile where $E = cd$ and $S = ab$; both S alleles have dropped out

Hypothesis	M_j	$\Pr(M_j)$	$\Pr(E\|M_j)$	Comments
H_p	cd	$2p_c p_d$	$\Pr(D)^2\Pr(\bar{D})^2$	Two drop-out events
H_{d1}	Any combination that carries cdQ	$12p_c p_d p_q(p_c + p_d + p_Q)$	$\Pr(D)^2\Pr(\bar{D})^2$	Two drop-out events (with Q alleles)

Conclusion: The evidence strongly favours H_d. The 'F' designation gives a slightly lower LR.

B.5. Generalised conclusions

A further generalisation can be made. Whenever a correction factor such as 'F' is used, the effect is to increase the probability. Therefore caution is required whenever this is used in the numerator. For example, if $S = ab$, $U = c$, $E = abc$ and $\phi_{abc} < T_{rfu}$, then drop-out may or may not have happened. Whereas it is reasonable to include 'F' in the denominator to achieve Pr_{max}, it is not necessary in the numerator. U can be conservatively assigned as genotype cc, which is always less then $Pr(cF)$.

Appendix C

C.1. The formulation of propositions

It is not always easy to specify hypotheses in complex cases where multiple perpetrators or victims may be present. The DNA result itself may indicate that different explanations are possible. Furthermore, it is possible that H_p and H_d could be very different from each other. For example, under H_p we might consider a victim and suspect to be the contributors ($V + S$), whereas under H_d we might examine more complex scenarios such as three unknowns being the contributors to the stain ($U_0 + U_1 + U_2$). There is a common misconception that the numbers of contributors under H_p and H_d should be the same. There is no requirement for this.

C.2. Formulation of H_p and H_d

In principle, H_p is the province of the prosecutor and H_d is the province of the defence. Both are constrained by what is known about the circumstances of the case. The forensic scientist usually formulates both H_p and H_d. In a typical example, H_p may propose that the DNA is a mixture of the suspect (S) and an unknown (U_1) individual. Under H_d, S is substituted by U_0. However, the defence may alter H_d (but not H_p), for example, if the number of contributors is contested. Consequently, some dialogue between the forensic scientist and defence is desirable in order to establish H_d. If this cannot be carried out pre-trial, the analyst may acknowledge in the report that the defence may offer alternative propositions which will require additional calculations.

C.3. Number of contributors

The number of contributors under H_p and H_d may be different. The most parsimonious explanations (the smallest number of unknown contributors needed to explain the evidence) are usually the ones that maximise the respective likelihoods [12]. But further research is needed to clarify, hence it may be wise explore options for different numbers of contributors.

C.4. Relevance of propositions

It follows that some propositions may be redundant if they only serve to reduce $Pr(E|H_d)$. This will be especially true in many circumstances where H_d incorporates more unknown individuals than required to maximise this probability.

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Glossary

Allele drop-in: Contamination from a source unassociated with the crime stain manifested as one or two alleles.

Allele drop-out: Low level of DNA insufficiently amplified to give a detectable signal.

Conservative: 1. An assignment for the weight of evidence that is believed to favour the defence. 2. When the evidence is very powerful in one direction, assigning the weight as less than our belief in that direction. 3. Lack of conservativeness will often result when the assumptions that underpin a statistical model are seriously violated.

Contamination: Extraneous DNA from a source unassociated with the crime stain—e.g. plastic-ware can be contaminated at manufacturing source.

Continuous approach: The allelic intensity information is used to give a variable, probability, weight to the validity of each genotype set as an explanation, rather than merely binary weights as in the combinatorial approaches.

Exclusion: Exclusion from a stain: 1. a decision (by the expert) that a particular reference DNA profile does not represent a contributor to the stain; 2. (jargon) situation in which the reference profile is ''excluded (3)'' from the stain at one or more loci. *Exclusion at a locus*: 3. (jargon) pattern of the assumed genotypes at a locus that some allele seen in a particular reference DNA profile is not observed in a stain.

Exclusion probability: The probability that a randomly selected DNA profile would be excluded (2).

Frequency: Rate at which an event occurs. For example, *sample frequency of an allele* is the number of occurrences of the allele in a population sample, divided by the sample size; *population frequency of a DNA profile* is the (unknown) number of times that the profile occurs in the population, divided by the population size.

Likelihood: Conditional probability of an event, where the event is considered as an outcome corresponding to one of several conditions or hypotheses. An example of an event is the DNA profile evidence from a crime stain. The probability of the event is conditional upon the hypothesis that may vary. If the DNA profile is a mixture, a typical prosecution hypothesis may be suspect and victim. This is written as $\Pr(E|H)$, where E is the event, the vertical bar in between the two terms means ''given'', and H is the hypothesis.

Likelihood ratio: Ratio of two likelihoods, i.e. the ratio of two probabilities of the same event (E) under different hypotheses (H_1, H_2). Written as $\mathrm{LR} = (E|H_1)/(E/H_2)$. Typically H_1 corresponds to the prosecution hypothesis and H_2 corresponds to the defence hypothesis. If H_1 consists of suspect and victim, then the alternative H_2 is unknown and victim.

Probability: Long-term rate of occurrence of an event in a conceptually repeatable experiment. Same as *expected* frequency, the expectation evaluated over cases described by the probability condition. Or: a coherent assignment of a number between zero and one that reflects in a fair and reasonable way our belief that the event is true.

Propositions: The hypothesis of the defence or prosecution arguments that are used to formulate the likelihood ratio.

Restricted combinatorial method: Elaboration of the unrestricted method in which allelic intensity (peak height/area) information is used to restrict the sets of genotypes that are considered plausible explanations.

Stutter: An allelic artefact cause by 'slippage' of the *Taq* polymerase enzyme. It is always four bases less than the allele that causes the stutter. Stutters are always found in allelic positions and can compromise interpretation of minor contributors to mixtures.

Unrestricted combinatorial method: The simple likelihood ratio method of evaluating mixture evidence described in Weir et al. [14] and Clayton and Buckleton [9]. The method assumes a list of all alleles in the mixture, and considers competing hypotheses that various known or unknown profiles are the constituents of the mixture. It uses no information about allelic intensities, hence one set of genotypes whose allele sets are coincident with the mixture is considered to be as valid an explanation of the mixture as any other set.

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Letter to the Editor

National recommendations of the Technical UK DNA working group on mixture interpretation for the NDNAD and for court going purposes

Abstract

The Technical UK DNA working group comprises representatives from all of the major suppliers of the UK and Ireland who contribute to the UK national DNA database. The group has the following terms of reference:To act as a peer review body.To agree experimental designs, to provide advice to the custodian to facilitate the development of the NDNAD.To support the CJS by the development of a coordinated UK strategy.To be inclusive, rather than exclusive, with regard to the introduction and use of methods.To define best scientific practice.To define guidelines for analysis and interpretation of evidence.To produce guidance that can be used by the UK Accreditation Services (UKAS).The group falls under the European Network of Forensic Science Institutes (ENFSI) umbrella. We will feed back recommendations to the ENFSI group for further discussion in order to facilitate European Policy.

The group recently met in order to consider in detail the ISFG DNA Commission recommendations on the interpretation of mixtures, to place them in the context of the UK jurisdictions.

Keywords: ISFG DNA commission; Mixtures; Technical UK DNA working group

1. Introduction

This group recognises that a diversity of (statistical) results will be achieved that are dependent upon the precise method used in the analysis of a sample for DNA profiling purposes. These statistical differences inevitably result from the efficiency or the sensitivity of the methods used: e.g. extraction protocols, injection times, PCR cycle number, can all contribute to differences in the resultant DNA profile. For a given crime stain, this means that complete or partial profiles may be obtained between laboratories and consequently the statistical results will also differ between laboratories.

However, we do not intend to standardise on particular methodology, neither do we intend to be prescriptive, recognising that all processes are subject to continuous improvement. It is the province of individual laboratories to drive change and to decide their protocols. Rather, our aim is to derive a set of simple guidelines that can be applied to all DNA profiles independent of the method used. Over time it will be necessary to update the recommendations.

Whereas differences in statistical results will still remain between methods and laboratories, the intent is to produce consistency such that different scientists who analyse results for a given DNA profile will produce similar statistical results. Standardisation of interpretation methodology demonstrates peer acceptance, and consequently gives the courts confidence that methods are widely accepted. Our aim is to facilitate peer review via the ENFSI group and the other major scientific bodies.

Key to achieving this is development of guidelines and defining their use. Guidelines are currently applied in association with thresholds. These thresholds are determined experimentally and are specific to each process or method used and may be specific to a particular laboratory. The most important is the 'dropout' threshold. This is applied whenever dropout has to be invoked to support a prosecution hypothesis (H_p) such as suspect alleles = *ab*; crime-stain allele = *a*. The evidence can only be explained under H_p if allele *b* has dropped out. However, in turn, this proposition can only be justified if the survivor allele is small enough such that the probability of dropout is less than one. Conversely, if Pr(*D*) approaches zero then the suspect is excluded since the conclusion must be that the donor is *aa*. The determination of this threshold is derived experimentally. The threshold is a guideline.

The second guideline is in relation to the interpretation of stutters. Here the problem is similar—if the suspect is *aa* and the crime-stain is *ab*, where *b* is in a stutter position, then clearly a consideration is required whether the peak can be a stutter, an allele or both. Again, experimentation is required to determine a 'stutter threshold' that can be used relative to associated guidelines. Stutter thresholds may also be technique dependent.

We have considered the International Society of Forensic Genetics (ISFG) DNA commission recommendations below in order to agree the UK recommendations for DNA reporting and submission of samples to the National DNA database—we have taken into account our 'local' considerations; court-going

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experiences; and appeal court recommendations in arriving at our stated position.

2. Response to the ISFG DNA commission 'recommendations on the interpretation of mixtures [1]'

2.1. Recommendation 1

''The likelihood ratio is the preferred approach to mixture interpretation. The RMNE approach is restricted to profiles where the profile is unambiguous. If the DNA crime-stain profile is low level and some minor alleles are the same size as stutters of major alleles, and/or if dropout is possible, then the RMNE method may not be conservative''.

2.1.1. Response

Conservativeness applies in the 'criminal context' only—civil disputes (such as paternity) should not be biased towards either the complainant or the defendant.

RMNE is a recognised and advocated interpretation method. The likelihood ratio and match probability methods are interchangeable—however, the wording of the match probability is equally acceptable for understanding in court. In addition, a frequency calculation can be used, e.g. ''I have calculated that the chance of observing this combination of DNA markers is about in 1 in X of the UK population'' or ''the chance that a person picked at random from the general UK population would have this combination of DNA markers is about 1 in X''.

If a profile can be identified with confidence from a mixture then the match probability statement may be preferable. A non-exhaustive list of examples is as follows:

(a) There is a major/minor mixture where the major contributor can be easily separated from the minor contributor(s) by virtue of the differences in peak height/area of the alleles.
(b) It may be possible to condition on one contributor, e.g. a victim, and to subtract this profile from the mixture, to leave a single contributor that can be reported separately. The contributors may be even, or major/minor. If the evidential profile is not major then it is inevitable that the conditioned major profile will mask some of the minor contributor alleles. Consequently, if a match probability is reported, some of the minor contributor alleles will be masked by the major contributor. The LR method may be preferred if this is the case.
(c) When conditioning is used to subtract a profile, then this should be made clear in the statement. If conditioning is challenged, then it may be appropriate to recalculate the strength of the evidence using the LR approach. A caveat can be included in the statement to make this point clear.

2.2. Recommendation 2

''Even if the legal system does not implicitly appear to support the use of the likelihood ratio, it is recommended that the scientist is trained in the methodology and routinely uses it in case notes, advising the court in the preferred method before reporting the evidence in line with the court requirements. The scientific community has a responsibility to support improvement of standards of scientific reasoning in the court-room''.

2.2.1. Response

Accepted—albeit we prefer to think in terms of advising the justice system rather than the court or court-room.

2.3. Recommendation 3

''The methods to calculate likelihood ratios of mixtures (not considering peak area) described by Evett et al. and Weir et al. are recommended'' (see [1] for the references cited).

2.3.1. Response

All laboratories in the UK consider peak height/area in their assessments. The formulae are fundamental to all mixture interpretation with or without peak height/area consideration.

2.4. Recommendation 4

''If peak height or area information is used to eliminate various genotypes from the unrestricted combinatorial method, this can be carried out by following a sequence of guidelines based on Clayton et al.'' (see [1] for the reference cited).

2.4.1. Response

Accepted.

2.5. Recommendation 5

''The probability of the evidence under H_p is the province of the prosecution and the probability of the evidence under H_d is the province of the defence. The prosecution and defence both seek to maximise their respective probabilities of the evidence profile. To do this both H_p and H_d require propositions. There is no reason why multiple pairs of propositions may not be evaluated''.

2.5.1. Response

Accepted.

2.6. Recommendation 6

If the crime profile is a major/minor mixture, where minor alleles are the same size (height or area) as stutters of major alleles, then stutters and minor alleles are indistinguishable. Under these circumstances alleles in stutter positions that do not support H_p should be included in the assessment.

2.6.1. Response

Stutters are locus-dependant. The Applied Biosystems SGM plus manual lists maximum experimentally observed stutter sizes per locus (St_{max}) where St_{max} is also utilised as the stutter threshold (described below). It is recommended that laboratories make their own St_{max} determinations since the effects

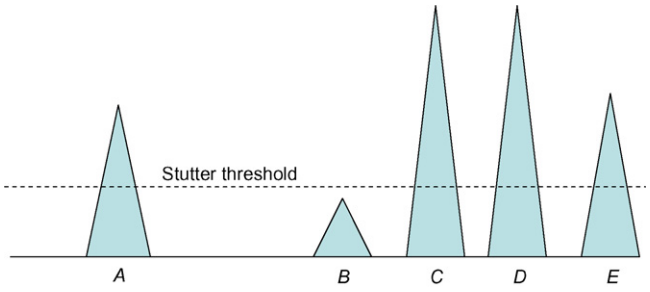

Fig. 1. A two-person mixture with major peaks *C*, *D* and minor peaks *A*, *E*. There is an additional peak present in a stutter position (*B*).

may be technique dependent. It is recommended that St_{max} is evaluated per locus.

We agreed to review stutter guidelines at a subsequent meeting.

2.6.2. How to use stutter guidelines

An evaluation of a mixture proceeds by a preliminary assessment to determine the number of contributors. This may include a consideration of the casework circumstances as well as an examination of the electropherogram (epg). If a simple two-person mixture is apparent, then interpretation can proceed as follows.

In the first example (Fig. 1), we condition on a two-person mixture, assuming that an assessment of the remaining loci justifies this position. Peaks *A* and *E* are minor contributors and are not in stutter positions. Peak *B* is below the stutter guideline (St_{max}), and can therefore be unambiguously designated as a stutter and discounted from the interpretation.

If allele *A* is above the dropout threshold (Fig. 2), and allele *B* is below the dropout threshold and below the stutter threshold, and differences in peak height/area are sufficient to discount the possibility of a heterozygote ($Hb_{obs} < Hb_{min}$) (see appendix for definition of *Hb*) then it may be designated *AA*. If the *C*, *D* allelic combination is unbalanced ($Hb_{obs} < Hb_{min}$) then it may be necessary to include *AC* and *AD* as potential minor contributors in the denominator of a likelihood ratio calculation, as masking may have occurred.

If *A* is low level (Fig. 3), equivalent in size to the stutter peak, then *B* may be an allele, or it may be an allele/stutter composite (contributor is *AB*) or it may be a stutter (the contributor is *AA*). Low-level alleles would usually be below the dropout threshold, hence the *AF* designation would be appropriate

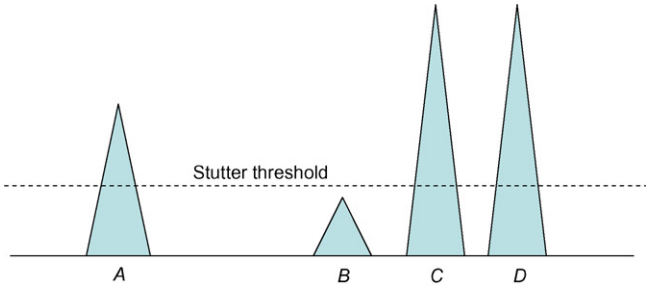

Fig. 2. A two person mixture with major peaks *C*, *D* and minor peaks *A*. There is an additional peak present in a stutter position (*B*).

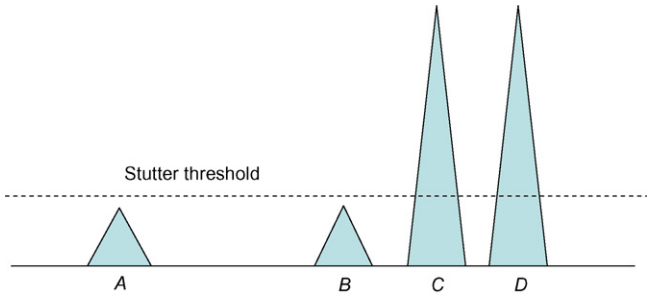

Fig. 3. A two person mixture with major peaks *C*, *D* and minor peaks *A*, *B*, where *B* is in a stutter position.

(see Section 2.7), since this encompasses the possibility of allele *B* in the stutter position. See Appendix A of the ISFG DNA commission report (pp. 96–97) on a method to calculate the likelihood ratio. Provided that the suspect is *AB*, then it is always conservative to compute the likelihood ratio including all possible combinations in the denominator, whereas if the suspect is a homozygote, so that the evidence is only explained if we condition on *B* as a stutter under H_p, then this must *a priori* be demonstrated to be a reasonable proposition—i.e. the size of allele *B* must be less than the stutter guideline (St_{max}) for the given locus. It is always good practice to repeat analyses showing potentially ambiguous results, if this is possible to do.

2.6.3. Characterisation of +4 base stutters

We agreed to review +4 bp stutters, however, we note that their presence often relates to over-amplified samples. Preliminary experimental work suggests that they are low level and generally less then 4% the size of the progenitor allele (Rosalind Brown, personal communication).

Note that −4 bp and +4 bp stutter cannot be distinguished from genetic somatic mutation without experimental work—furthermore, somatic mutations may give rise to peaks that are larger than those caused by stutter artefacts.

2.7. Recommendation 7

"If dropout of an allele is required to explain the evidence under H_p: ($S = ab$; $E = a$) then the allele should be small enough (height/area) to justify this. Conversely, if a full crime-stain profile is obtained where alleles are well above the background level, and the probability of dropout $Pr(D) \approx 0$, then H_p is not supported".

2.7.1. Response

We recommend slight rewording (including underlined below): If dropout of an allele is required to explain the evidence under H_p: ($S = ab$; $E = a$), then the *companion* allele should be small enough (height/area) to justify this (Figs. 4–6).

"Small enough" equates to a peak that is below the predetermined dropout threshold, i.e. Pr(*D*) is more than zero (Fig. 5).

Conversely, if a full crime-stain profile is obtained where alleles are well above the background level, and the probability of dropout Pr(*D*) approaches zero, then H_p is not supported (Fig. 6).

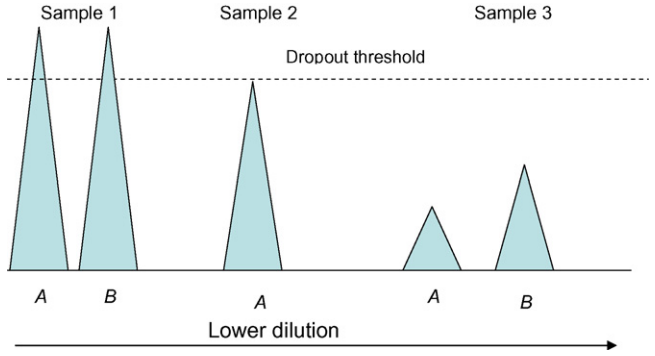

Fig. 4. Results from serial dilutions of the same sample genotype *AB*. The first result (sample 1) shows a locus where both alleles are represented in the profile. One or both of these alleles are above the dropout threshold and consequently are always present in the epg. The second result shows a result where dropout has occurred – the survivor allele is just below the dropout threshold hence this is a rare event, but not impossible. If *A* was just above the dropout threshold we would determine it to be a homozygote *AA* genotype. In the third sample, both alleles are well below the dropout threshold – it is an unambiguous, albeit unbalanced heterozygote. If only one allele was present, then we would have to consider the possibility of dropout of the partner. The same rationale can be applied to any analytical regime, e.g. 28 and 34 PCR cycles.

From the above example: allele *b* may either dropout completely, or it could be present at such low level that a statistical calculation is not supported by Section 2.8 because it is at a level where background noise could be prevalent.

The dropout threshold can be determined experimentally for a given analytical technique from a series of pre-PCR dilutions of extracts of known genotype technique (it will probably vary between analytical methods). These samples can be used to determine the point where allelic dropout of a heterozygote is observed relative to the size of the survivor companion allele. The threshold is the maximum size of the companion allele observed. This is also the point where Pr(D) approaches zero (Fig. 4).

Note that for custodian purposes it is sufficient to unambiguously designate a homozygote locus for databasing purposes provided that it is above the dropout threshold. To apply a statistical analysis, the guidelines provided in this paper will assist to ensure that application of the '*F*' designation is conservative (or nearly so), remembering that care is required only when dropout must be invoked under H_p.

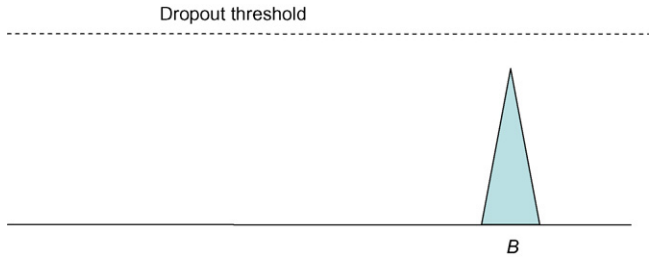

Fig. 5. In this example allele *B* is below the dropout threshold, hence we cannot be confident that it is from a homozygote *BB* individual. It could also be from an individual who is heterozygote, where the missing allele is any other allele. The probability $B|\text{unknown}, H_d$ is $2\Pr(BF)$, where the '*F*' designation is assigned a probability of 1 to take account of the possibility that any allele could have dropped out.

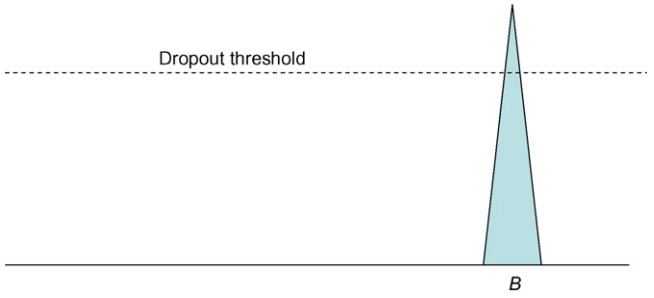

Fig. 6. In this example allele *B* is above the dropout threshold, hence we can be confident that it is from a homozygote *BB* individual. The probability of $B|\text{unknown}, H_d$ is $\Pr(B)^2$.

It is always good practice to repeat analyses with potentially ambiguous results, if this is possible to do. For example, duplication of the test may assist in to determine if dropout is a consideration in the interpretation of the evidence.

2.7.2. Implications of Bates

The appeal court, Bates [2], was asked to consider whether a partial DNA profile was admissible as evidence on the grounds that the DNA profile was incomplete and therefore did not match the defendant at every locus. At two loci (D2 and D8) alleles were missing. The missing alleles were called ''voids'' by the judge. The defence asserted that there was no accepted method to report partial profiles: ''the inability to take account of the potential exculpatory effect of voids invalidates any match probability''.

The Bates ruling specifically examined the implications of reporting a partial DNA profile where some alleles were missing or dropped out and the '*F*' designation was used. The judgement considered:

> ''Such voids are potentially significant because, if the missing allele did not match either of the alleles at that locus of the person under investigation, it would establish conclusively that he (or she) had not provided that sample of DNA. Every partial profile carries within it, therefore, the possibility that the missing information excludes the person under investigation, but there is currently no means of calculating the statistical chances of that being the case''.

The judgement goes on to conclude:

> ''What are the consequences of the impossibility of assigning a statistical weight to the voids? The alternatives are to exclude the evidence entirely or to admit it subject to an appropriate warning to the jury of the limitations of the evidence, and particularly highlighting the fact that although what was found was consistent with Bates' DNA profile, the voids at D2 and D18 in particular may have contained an allele or alleles, the presence of which would have been wholly exculpatory.

In arriving at the correct conclusion it is important to remember that scientific evidence frequently only provides a partial answer to a case. However, the test of admissibility is

not whether the answer is complete, but whether science can properly and fairly contribute to the matter in question. . .''

In the context of our discussions above (especially in relation to a consideration of Section 2.7 when $S = ab$ and $E = a$), we conclude that it is reasonable to assign dropped out alleles or ''voids'' as neutral events provided that the survivor allele is small enough, and below the designated dropout threshold so that the loss of the *b* allele is a reasonably plausible explanation. Appendix B of reference [1] gives a number of worked examples to illustrate this point.

Furthermore, it is advisable to carry out additional work in order to resolve this apparent ambiguity. A 'zoom' of the baseline may reveal the 'missing' allele to be present but sub-threshold? Alternatively, a re-amplification of the DNA extract (if there is sufficient) may reveal the presence of the missing *b* allele.

If both alleles have dropped out at a locus, then there is no information that can be adduced, and this must be regarded as neutral.

2.8. Recommendation 8

''If the alleles of certain loci in the DNA profile are at a level that is dominated by background noise then a biostatistical interpretation should not be attempted''.

2.8.1. Response

If there is a band below the experimental threshold where background noise might be prevalent, and it is distinct and clear from the background, then it should be recorded and available on the case file.

2.9. Recommendation 9

In relation to profiles derived from the amplification of low amounts of template DNA, stochastic effects may limit the usefulness of heterozygous balance and mixture proportion estimates. In addition, allelic dropout and allelic drop-in (contamination) should be taken into consideration of any assessment.

2.9.1. Response

Case pre-assessment is necessary in order to determine the best scientific method to process a sample. To facilitate this, it is recommended that wherever possible, this should include quantification. Quantification is used to determine the optimum method to process—if low-level DNA, a sample would benefit from procedures to enhance sensitivity of detection. There may be reasons where quantification is not practicable, especially if low levels of DNA are expected, since the result itself may be compromised if a portion of the sample is sacrificed. At low DNA levels, the accuracy of the quantification test itself may be inefficient.

Based on manufacturers guidelines we can define a low-level sample as one that contains ca. <200 pg DNA. At this level we might expect stochastic effects to occur, including:

(a) locus dropout,
(b) allele dropout,
(c) extreme heterozygote imbalance.

These are consequences that are universally observed at 28–34 + PCR cycles. Duplication of the test can aid to interpret profiles with *Hb* imbalance and dropout.

Since the introduction of CE, sub-200 pg amounts of amplifiable DNA can be visualised by multiple methods—where increased cycle number, increased injection time etc (or a combination of the two) can be used to achieve the same effect. We have demonstrated experimentally that some laboratories achieve results from ca. 50 pg of DNA using standard 28 PCR cycles.

Since these consequences are common to all methods of DNA analysis, and are not restricted to 34 cycles, we do not consider the LCN label for 34 cycles work to be useful, or particularly helpful, and propose to abandon it as a scientific concept, because a clear definition cannot be formulated. Rather, our aim is to recommend generic guidelines that can be universally applied to all DNA profiles that are independent of the method utilised. It is important to consider that where the profile is well amplified and fully represented, without allele dropout, then special considerations are not required since interpretation is standard and straightforward.

Therefore, we can easily define a 'conventional' result as one where the alleles are above the dropout threshold (determined by experimentation). Reporting of the locus is normally straightforward because the alleles are unambiguous. The cycle no. used is irrelevant since the dropout threshold may be separately determined for any given protocol.

Conversely, we define a 'low-level' result as one where the alleles are below the dropout threshold. Special considerations are then applied.

It is possible that a given DNA profile may simultaneously comprise both 'conventional' and 'low-level' loci: for example, if degradation has occurred then low molecular weight loci may be above the dropout threshold, whereas high molecular weight loci may be below the dropout threshold.

Similarly, if the sample is a mixture, then at a given locus there may be some alleles that are above the dropout threshold (from a major contributor) and others that are below the dropout threshold (from a minor contributor), i.e. different interpretation rationale may be simultaneously applied to different contributors within a locus.

Appendix. Guidance note on the use of the heterozygote balance guideline

For a well-amplified heterozygote from good quality DNA >0.5 ng, the heterozgote balance is defined as the proportion of the lower peak height/area divided by the higher peak height/area:

$$Hb = \frac{\text{lower peak height or area}}{\text{higher peak height or area}}$$

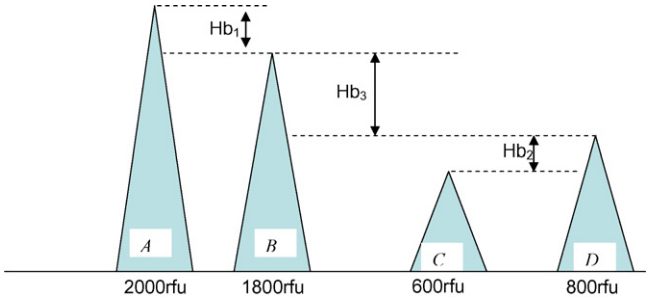

Fig. 7. A typical major(*AB*)/minor(*CD*) mixture.

The distribution of *Hb* generally ranges between ca. 0.5 and 1 for a well-amplified DNA profile. This parameter is used to evaluate DNA profiles. It is particularly useful to determine if mixtures are present and to determine whether respective alleles can be associated with a given contributor.

If a single profile is present, then Hb_{obs} (the observed *Hb*) should be greater than Hb_{min} (the minimum *Hb* from the observed experimental distribution for 'conventional' DNA is usually not less than 0.5—*this parameter may vary between laboratories*).

Consider the mixture in Fig. 7. All of the alleles are above the dropout threshold. Can allele *A* be paired with allele *B* and can allele *C* be paired with allele *D*? $Hb_1 = 1800/2000 = 0.9$; $Hb_2 = 600/800 = 0.8$, i.e. both parameters >0.5 (Hb_{min}). Could alleles *B* and *D* be considered to be from a single contributor? $Hb_3 = 800/1800 = 0.44$, i.e. $Hb_3 < 0.5$. These three calculations provide strong evidence to support the contention that alleles *A* and *B* are a pair of heterozygous alleles from a major contributor and alleles *C* and *D* are a pair of alleles from a minor contributor.

Some care is needed with using the heterozygote balance guideline. As the quantity of DNA declines, then the Hb_{min} also falls, hence it is desirable to understand the relationship between Hb_{min} and the size (height/area) of the respective alleles if this guideline is to be used below 0.5, otherwise, under the defence hypothesis H_d, it is always conservative to include more allelic combinations than necessary in the assessment. To formulate the prosecution hypothesis H_p, it is anti-conservative to include too many combinations here and the opposite applies—if in doubt then do not include the combination. Allele dropout is an extreme form of heterozygote balance and is equivalent to $Hb_{min} = 0$.

Thus, in the above example in Fig. 7, an ultra-conservative assessment would ignore the peak height/area information to formulate the defence hypothesis $\Pr(H_d)$. Suppose that we are evaluating suspect (*S*) and an unknown (*U*) under the prosecution hypothesis (H_p) and two unknown people (U_1 and U_2) under the defence hypothesis (H_d). If the suspect = *AB*, our most conservative evaluation will comprise $2pCpD$ (pC is the frequency of allele *C* in the relevant population) in the numerator (noting that if *A*, *B*, *C*, *D* were all equivalent in peak area then this would still be appropriate). Conversely, under H_d we would include combinations *AB*:*CD*; *AC*:*BD*; *AD*:*BC* (along with reverse options) as viable options using the classic likelihood ratio formulation. The LR = $1/12pApB$.

Given the peak height/area considerations, we can conclude that the major/minor contributors can be separated and consequently the minor contributor can be subtracted from the evidential profile, to allow the major profile to be reported as a match probability. $P_m = 1/2pApB$ which gives a figure that is greater than the LR formulation.

References

[1] P. Gill, C.H. Brenner, J.S. Buckleton, A. Carracedo, M. Krawczak, W.R. Mayr, N. Morling, M. Prinz, P.M. Schneider, B.S. Weir, DNA commission of the International Society of Forensic Genetics: recommendations on the interpretation of mixtures, Forens. Sci. Int. 160 (2006) 90–101.

[2] R.v. Bates, Neutral Citation Number: (2006) EWCA Crim 1395, Case No. 395200503241C3.

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12 July 2007

Mixture Deconvolution

Florida Statewide Training Meeting

Indian Rocks Beach, FL
May 12-13, 2008

Dr. John M. Butler
National Institute of Standards and Technology

john.butler@nist.gov

Outline

- Points for Consideration
  - DNA quantity and quality
- Deconvolution steps by Clayton *et al.* (1998)
- Worked Example – using NEST data
- Software programs introduced

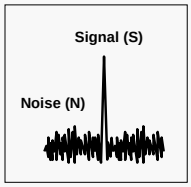
Final version available at
http://www.cstl.nist.gov/biotech/strbase/training/AAFS2008_MixtureWorkshop.htm

Points for Consideration

- Peak height vs peak area
- Thresholds – analytical vs stochastic levels
- Other lab-specific values:
  - Heterozygote peak height balance
  - Locus-specific stutter percentage
- DNA quantity and quality
  - problems with low-level or degraded DNA

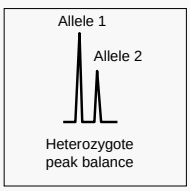
What is a true peak (allele)?

Peak detection threshold



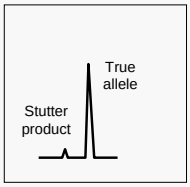
Signal > 3x sd of noise

Peak height ratio (PHR)



PHR consistent with single source Typically above 60%

Stutter percentage



Stutter location below 15%

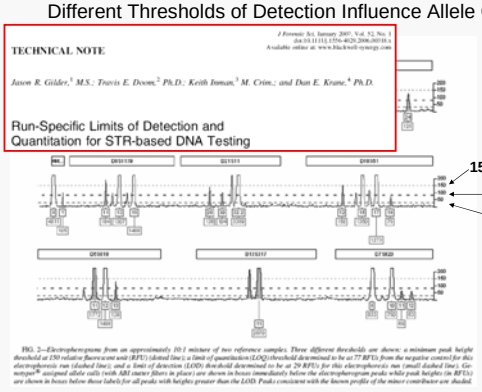
Validation Studies

- Information from validation studies should be used to set laboratory-specific
  - Stutter %
  - Peak Height Ratios
  - Minimum Peak Heights (detection thresholds)
  - Relative balance across loci
- These values are all dependent on amount of input DNA
  - If low-level DNA is amplified, stutter % may be higher and peak height ratios may be lower

Thresholds

- Validation studies should be performed in each laboratory
- Some labs have set two thresholds:
  - Analytical thresholds – what is a peak? (50 RFU)
  - Stochastic thresholds – what is reliable PCR data? (150 RFU)

Different Thresholds of Detection Influence Allele Calls

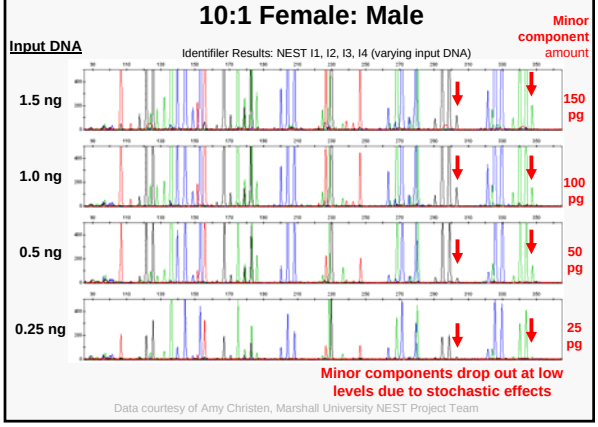


Gilder, J.R., Doom, T.E., Inman, K., Krane, D.E. (2007) Run-specific limits of detection and quantitation for STR-based DNA testing. *J. Forensic Sci.* 52(1): 97-101.

The Scientific Reasoning behind the Concept of an Analytical Threshold (limit of detection)

- This is fundamentally an issue of reliability
- **For a peak intensity three times the standard deviation of the noise there is a limited chance that such a signal is the result of a random fluctuation**
- This is because 99.7 percent of all noise signals fall below this value (from the definition of a Gaussian curve)
- **Below this point the very real possibility exists that what you think is a peak is simply a statistical fluctuation in the baseline noise**.

10:1 Female: Male



Steps in the Interpretation of Mixtures

(Clayton *et al.* 1998)

Step #1 Identify the Presence of a Mixture

Step #2 Designate Allele Peaks

Step #3 Identify the Number of Potential Contributors

Step #4 Estimate the Relative Ratio of the Individuals Contributing to the Mixture

Step #5 Consider All Possible Genotype Combinations

Step #6 Compare Reference Samples

Clayton *et al.* (1998) *Forensic Sci. Int.* 91:55-70

Will review each step with a worked example

Step #1: Is a Mixture Present in an Evidentiary Sample?

- Examine the number of peaks present in a locus
  - More than 2 peaks at a locus (except for tri-allelic patterns at perhaps one of the loci examined)
- Examine relative peak heights
  - Heterozygote peak imbalance <60%
  - Peak at stutter position >15%
- Consider all loci tested

Is a DNA Profile Consistent with Being a Mixture?

From J.M. Butler (2005) *Forensic DNA Typing, 2nd Edition*, pp. 156-157

If the answer to any one of the following three questions is yes, then the DNA profile may very well have resulted from a mixed sample:

- Do any of the loci show more than two peaks in the expected allele size range?
- Is there a severe peak height imbalance between heterozygous alleles at a locus?
- Does the stutter product appear abnormally high (e.g., >15-20%)?

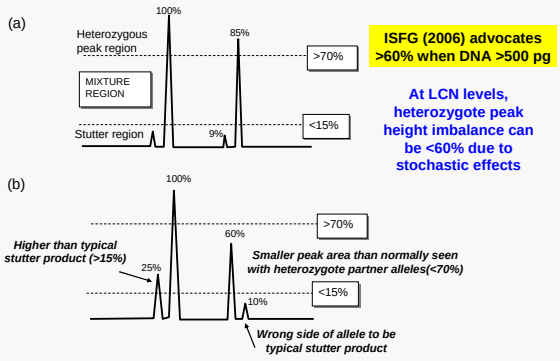


Figure 7.3, J.M. Butler (2005) *Forensic DNA Typing*, 2nd Edition © 2005 Elsevier Academic Press

Step #2: Designate Allele Peaks

- Use regular data interpretation rules to decipher between true alleles and artifacts
- Use stutter filters to eliminate stutter products from consideration (although stutter may hide some of minor component alleles at some loci)
- Consider heterozygote peak heights that are highly imbalanced (<60%) as possibly coming from two different contributors

Step #3: Identifying the Potential Number of Contributors

- **Important for some statistical calculations**
- Typically if 2, 3, or 4 alleles then 2 contributors
- If 5 or 6 alleles per locus then 3 contributors
- If >6 alleles in a single locus, then >4 contributors

- JFS Nov 2005 paper by Forensic Bioinformatics on number of possible contributors
  - Relies on maximum allele count alone
  - Does not take into account peak height information

Forensic Bioinformatics Article

http://www.bioforensics.com/articles/empirical_mixtures.pdf

J Forensic Sci, Nov. 2005, Vol. 50, No. 6
Paper ID JFS2004475
Available online at: www.astm.org

David R. Paoletti,[1] M.S.; Travis E. Doom,[1,2] Ph.D.; Carissa M. Krane,[3] Ph.D.; Michael L. Raymer,[1,2] Ph.D.; and Dan E. Krane,[4] Ph.D.

Empirical Analysis of the STR Profiles Resulting from Conceptual Mixtures

TABLE 2—*Count and percent of three-person mixtures in which a particular number of unique alleles was the maximum observed across all loci, both for the original and randomized individuals*[a].

Unique Alleles	Count	Percent (%)
2	0	0.00%
3	78	0.00%
4	4,967,034	3.39%
5	93,037,010	63.49%
6	48,532,037	33.12%

Using 959 complete 13-locus STR profiles from FBI dataset

146,536,159 possible combinations **with 3-person mixtures**

3.39 % (4,967,034 combinations) would only show a maximum of four alleles (i.e., appear based on maximum allele count alone to be a 2-person mixture)

Recent Article by Buckleton *et al.*

Available online at www.sciencedirect.com
ScienceDirect
Forensic Science International: Genetics 1 (2007) 20–28
FSI GENETICS
www.elsevier.com/locate/fsig

Towards understanding the effect of uncertainty in the number of contributors to DNA stains

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Abstract

DNA evidence recovered from a scene or collected in relation to a case is generally declared as a mixture when more than two alleles are observed at several loci. However, in principle, all DNA profiles may be considered to be potentially mixtures, even those that show not more than two alleles at any locus. When using a likelihood ratio approach to the interpretation of mixed DNA profiles it is necessary to postulate the number of potential contributors. However, this number is never known with certainty. The possibility of a, say three-person mixture, presenting four or fewer peaks at each locus of the CODIS set was explored by Paoletti et al. [D.R. Paoletti, T.E. Doom, C.M. Krane, M.L. Raymer, D.E. Krane, Empirical analysis of the STR profiles resulting from conceptual mixtures, J. Forensic Sci. 50 (2005) 1361–1366]. In this work we extend this analysis to consider the profiler plus and SGM plus multiplices. We begin the assessment of the risk associated with current practice in the calculation of LR's. We open the discussion of possible ways to surmount this ambiguity.

Two-Person Mixtures for Simulated Profiles:

Probability by Locus of A Particular Number of Alleles Being Observed

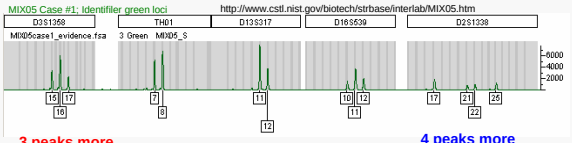
Table 1
The probability of observing a given number of alleles in a two-person mixtures for simulated profiles at the SGM+™ loci

Loci	No. of alleles			
	1	2	3	4
D3	0.011	0.240	0.559	0.190
vWA	0.008	0.194	0.548	0.250
D16	0.016	0.287	0.533	0.164
D2	0.003	0.094	0.462	0.441
D8	0.011	0.194	0.521	0.274
D21	0.007	0.147	0.505	0.341
D18	0.003	0.095	0.472	0.430
D19	0.020	0.261	0.516	0.203
THO	0.016	0.271	0.547	0.166
FGA	0.003	0.116	0.500	0.381

Buckleton et al. (2007) Towards understanding the effect of uncertainty in the number of contributors to DNA stains. *FSI Genetics* 1:20-28

Levels of Locus Heterozygosity Impact Number of Alleles Observed in Mixtures

Loci	No. of alleles			
	1	2	3	4
D3	0.011	0.240	0.559	0.190
vWA	0.008	0.194	0.548	0.250
D16	0.016	0.287	0.533	0.164
D2	0.003	0.094	0.462	0.441



3 peaks more common for D3

4 peaks more common for D2

Three-Person Mixtures for Simulated Profiles:

Probability by Locus of A Particular Number of Alleles Being Observed

Table 2
The probability of observing a given number of alleles in a three-person mixtures for simulated profiles at the SGM+™ loci

Loci	No. of alleles showing					
	1	2	3	4	5	6
D3	0.000	0.053	0.366	0.463	0.115	0.002
vWA	0.000	0.037	0.285	0.468	0.194	0.016
D16	0.001	0.086	0.397	0.411	0.100	0.005
D2	0.000	0.008	0.104	0.385	0.393	0.110
D8	0.001	0.041	0.258	0.436	0.236	0.029
D21	0.000	0.023	0.192	0.428	0.302	0.055
D18	0.000	0.007	0.109	0.392	0.396	0.096
D19	0.003	0.078	0.352	0.401	0.152	0.014
THO	0.001	0.074	0.395	0.439	0.088	0.002
FGA	0.000	0.012	0.144	0.424	0.346	0.074

Buckleton et al. (2007) Towards understanding the effect of uncertainty in the number of contributors to DNA stains. *FSI Genetics* 1:20-28

Number of Alleles Observed with Simulated Four-Person Mixtures

- The simulation of four person mixtures suggests that 0.014% of four person mixtures would show four or fewer alleles and that 66% would show six or fewer alleles for the SGM Plus loci.
- The results for the Profiler Plus loci were 0.6% and 75%.
- The equivalent values for the CODIS set from Paoletti et al. were 0.02% showing four or fewer and 76.35% showing six or fewer.

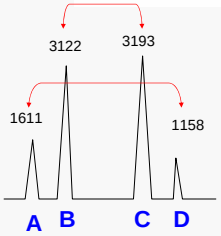
Buckleton et al. (2007) Towards understanding the effect of uncertainty in the number of contributors to DNA stains. *FSI Genetics* 1:20-28

Step #4: Estimation of Relative Ratios for Major and Minor Components to a Mixture

- Mixture studies with known samples have shown that the mixture ratio between loci is fairly well preserved during PCR amplification
- Thus it is generally thought that the peak heights (areas) of alleles present in an electropherogram can be related back to the initial component concentrations
- Start with loci possessing 4 alleles…

Estimating Mixture Proportion (M_x) or Mixture Ratio (M,)

$$M_x = \frac{\phi_a + \phi_b}{\phi_a + \phi_b + \phi_c + \phi_d}$$



$$\frac{A + D}{A + D + B + C}$$

$$\frac{1611 + 1158}{1611 + 1158 + 3122 + 3193}$$

= 2769/9084 = 0.305

Step #5: Consider All Possible Genotype Combinations

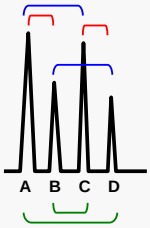
Table 3
Pairwise combinations of two, three and four alleles

Four alleles (a,b,c,d)		Three alleles (a,b,c)		Two alleles (a,b)	
a,b	c,d	a,a	b,c	a,a	a,b
a,c	b,d	b,b	a,c	a,b	a,b
a,d	b,c	c,c	a,b	a,a	b,b
c,d	a,b	a,b	a,c	a,b	b,b
b,d	a,c	b,c	a,c	a,b	a,a
b,c	a,d	a,b	b,c	b,b	a,a
		b,c	a,a	b,b	a,b
		a,c	b,b		
		a,b	c,c		
		a,c	a,b		
		a,c	b,c		
		b,c	a,b		

Key: bold entries represent reciprocal combinations.

Clayton *et al. Forensic Sci. Int.* 1998; 91:55-70

Considering Genotype Combinations



AC
BD
AB
CD
BC
AD

Depends on PHR

Peak Height Ratios (PHR)
Minimum Peak Height (mPH)
Proportion (p) or mixture proportion (M_x)

Step #6: Compare Reference Samples

- If there is a suspect, a laboratory must ultimately decide to include or exclude him…
- **If no suspect is available for comparison, does your laboratory still work the case?** (Isn't this a primary purpose of the national DNA database?)
- Victim samples can be helpful to eliminate their allele contributions to intimate evidentiary samples and thus help deduce the perpetrator

Worked Example

NIJ Expert Systems Testbed (NEST) Project

http://www.promega.com/profiles/1002/ProfilesInDNA_1002_13.pdf

Profiles in DNA (September 2007) 10(2): 13-15 PROFILES IN DNA

EXPERT SYSTEMS

Validating Expert Systems: Examples with the FSS-i³™ Expert Systems Software

By Rhonda K. Roby[1] and Amy D. Christen[2]
[1]Technical Consultant, National Institute of Justice
[2]Research Analyst, Marshall University

NIJ National Institute of Justice

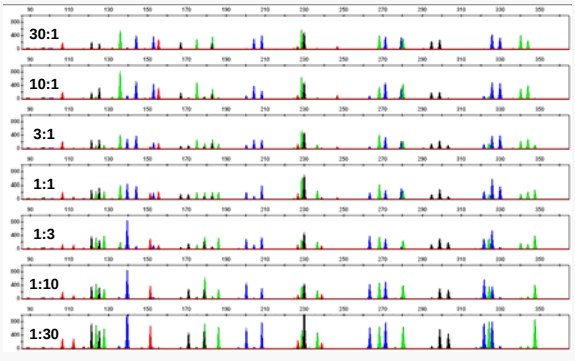
MARSHALL UNIVERSITY FORENSIC SCIENCE CENTER

NEST Project Mixture Sample Set

- NIJ Expert Systems Testbed (NEST) Project
  - Marshall University with Rhonda Roby (NIJ consultant)
- Phase II Mixture Sample Analysis
  - **Amy Christen** (Marshall University) produced a dataset while interning at Forensic Science Service in Summer 2006
  - Data to be used for evaluating "expert systems"
- Mixtures tested (280 total samples)
  - **2 different female/male sample combinations**: A:X and B:Y
  - **4 input DNA amounts**: 1.5 ng, 1.0 ng, 0.5 ng, 0.25 ng
  - **5 kits**: Identifiler, ProfilerPlus, COfiler, PowerPlex 16, SGM Plus
  - **7 mixture ratios**: 30:1, 10:1, 3:1, 1:1, 1:3, 1:10, 1:30

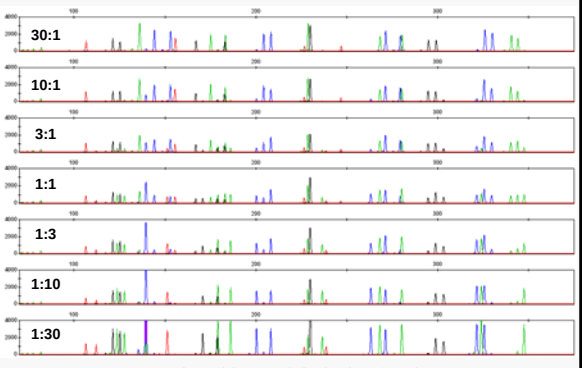
I will focus on a subset of this data… e.g., B:Y, 1.0 ng, Identifiler, 3:1

Identifiler Results: NEST H4 – N4 (**0.25 ng input DNA**)



Data courtesy of Amy Christen, Marshall University NEST Project Team

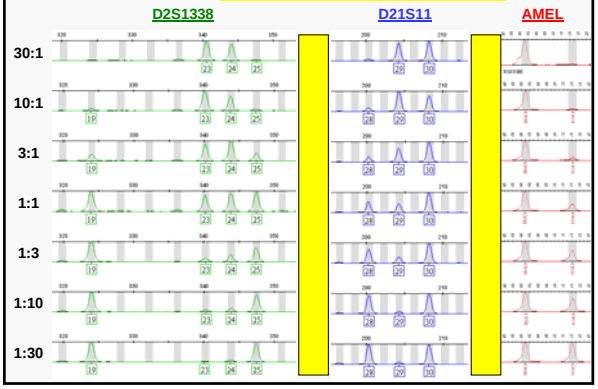
Identifiler Results: NEST H2 – N2 (**1.0 ng input DNA**)



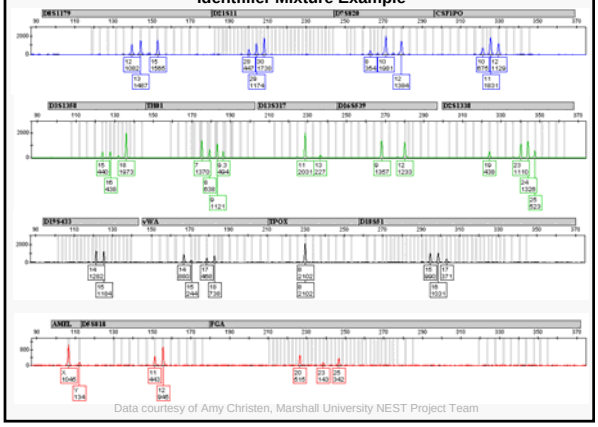
Data courtesy of Amy Christen, Marshall University NEST Project Team

Identifiler Results: NEST H2 – N2 (1.0 ng input DNA)

Calculate ratios based on peak heights

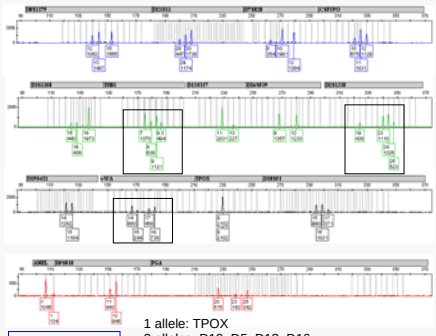


Identifiler Mixture Example



Data courtesy of Amy Christen, Marshall University NEST Project Team

Profile Overview



Evaluation Notes:

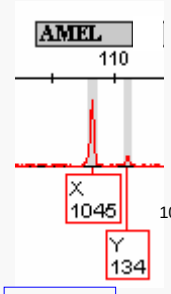
1. Loci seen with 1,2,3,&4 alleles (**a mixture** with at least 2 contributors)
2. Imbalance at amelogenin (female & male mixture with female as major)
3. Decent overall signal with D8 in ~1500 RFU (out of stochastic range)
4. Large MW loci have decent signal with D18 in ~1000 RFU range (degradation unlikely)
5. Ratio of **major to minor around 3:1** (from amelogenin X/Y ratios)

1045/134 = 7.8
~3 female (X,X):
1 male (X,Y)

1 allele: TPOX
2 alleles: D19, D5, D13, D16
3 alleles: D8, D21, D7, CSF, D3, D18, FGA
4 alleles: TH01, D2, VWA

Amelogenin Ratio

In many cases, amelogenin provides a helpful guide to assessing the mixture ratio



Female/Male ratio = X:X / X:Y

X/3 = 1045/3 = 348

348/134 = 2.6 (closest to 3 parts female to 1 part male)

1045/134 = 7.80

Chart of Expected Ratios

F:M	Chr ratio
1:1	3X:1Y
2:1	5X:1Y
3:1	7X:1Y
4:1	9X:1Y

1045/134 = 7.8
~3 female (X,X):
1 male (X,Y)

Potential problems with X or Y amplicon deletions

Anomalous Amelogenin Alleles

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

- **Males possessing only a single X amelogenin amplicon (Y null)** - *a male DNA sample will falsely look like a female DNA sample*:
  - Santos et al. (1998) reported a rare deletion of the amelogenin gene on the Y-chromosome
  - Y-STR typing can be performed to verify that other portions of the Y-chromosome are present
- **Males possessing only a single Y amelogenin amplicon (X null)**:
  - Shewale et al. (2000) observed loss of the X chromosome amplicon in three our of almost 7,000 males examined
  - while this phenomenon should not result in a gender misclassification (as the Y null situation might), its occurrence can impact the expected X and Y amplicon ratios in a mixture (see NIST MIX05 interlab study, case #3)

Running reference samples from suspect and/or victim may help discover potential amelogenin anomalies

Locus-by-Locus Breakdown…

- Start with 4 allele loci…
  - Assume two person mixture
  - With non-overlapping heterozygotes
  - Pair peaks with similar peak heights

Possible but not as likely depending on ratios

Possible Genotype Combinations

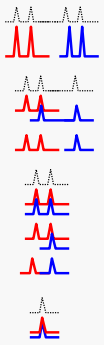
See Butler, J.M. (2005) *Forensic DNA Typing, 2nd Edition*, pp. 156-157

Four Peaks **(4 allele loci)**
- heterozygote + heterozygote, no overlapping alleles (genotypes are unique)

Three Peaks **(3 allele loci)**
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

Two Peaks **(2 allele loci)**
- heterozygote + heterozygote, two overlapping alleles (genotypes are identical)
- heterozygote + homozygote, one overlapping allele
- homozygote + homozygote, no overlapping alleles (genotypes are unique)

Single Peak **(1 allele loci)**
- homozygote + homozygote, overlapping allele (genotypes are identical)



MUST ALSO CONSIDER STUTTER POSITION

Population Database Used for STR Allele Frequencies

- U.S. population data contained in J.M. Butler (2005) *Forensic DNA Typing, 2nd Edition*, Appendix II (pp. 577-583)
- Published in Butler *et al.* (2003) *J. Forensic Sci.* 48(4): 908-911
- Available at http://www.cstl.nist.gov/biotech/strbase/NISTpop.htm
- Will focus on Caucasians for simplicity

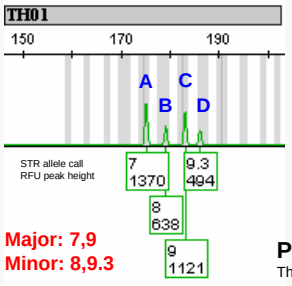
TH01

Allele	Caucasian N = 302	African-American N = 258	Hispanic N = 140
5	0.00166*	0.00388*	
6	0.23179	0.12403	0.21429
7	0.19040	**0.42054**	0.27857
8	0.08444	0.19380	0.09643
9	0.11424	0.15116	0.15000
9.3	**0.36755**	0.10465	0.24643
10	0.00828	0.00194*	0.01429*
11	0.00166*		

Remember that different population databases will have different allele frequencies because they are based on different samples

Stats

4 Allele Locus: TH01



Allele	Frequency
7	0.190
8	0.084
9	0.114
9.3	0.368

$PI = (P_A + P_B + P_C + P_D)^2$
$= (0.190 + 0.084 + 0.114 + 0.368)^2$
$= (0.756)^2$
$= 0.572$

Major: 7,9
Minor: 8,9.3

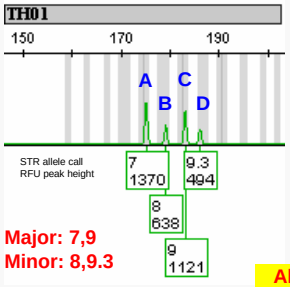
PE = 1 – PI = 1 – 0.572 = 0.428

Thus ~43% of Caucasian population can be excluded from contributing to this mixture (primarily because allele 6 is missing)

Four Peaks **(4 allele loci)**
- heterozygote + heterozygote, no overlapping alleles (genotypes are unique)

PHRs

4 Allele Locus: TH01



Major: 7,9
Minor: 8,9.3

Consider all possible combinations:

B/A = 638/1370 = 0.466

B/C = 638/1121 = 0.569

C/A = 1121/1370 = 0.818 **major**

D/B = 494/648 = 0.774 **minor**

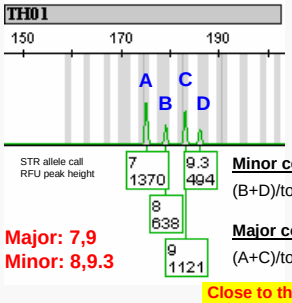
D/C = 494/1121 = 0.441

All other combinations <0.60 (60% heterozygote Peak Height Ratio)

Four Peaks **(4 allele loci)**
- heterozygote + heterozygote, no overlapping alleles (genotypes are unique)

Mix Ratio

4 Allele Locus: TH01



Major: 7,9
Minor: 8,9.3

Total of all peak heights
= 1370 + 638 + 1121 + 494
= 3623 RFUs

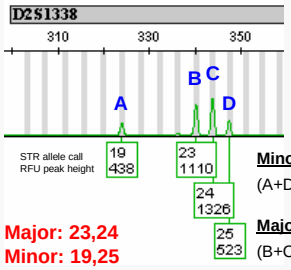
Minor component:
(B+D)/total = (638+494)/3623 = **0.312**

Major component:
(A+C)/total = (1370+1121)/3623 = **0.688**

Close to the ~3:1 predicted by amelogenin X/Y allele ratio – thus major component = female

Four Peaks **(4 allele loci)**
- heterozygote + heterozygote, no overlapping alleles (genotypes are unique)

Mix Ratio
4 Allele Locus: D2S1338



Total of all peak heights
= 438 + 1110 + 1326 + 523
= 3397 RFUs

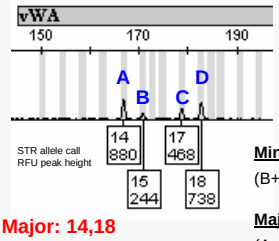
Minor component:
(A+D)/total = (438+523)/3397 = **0.283**

Major component:
(B+C)/total = (1110+1326)/3397 = **0.717**

Major: 23,24
Minor: 19,25

Four Peaks **(4 allele loci)**
- heterozygote + heterozygote, no overlapping alleles (genotypes are unique)

Mix Ratio
4 Allele Locus: vWA



Total of all peak heights
= 880 + 244 + 468 + 738
= 2330 RFUs

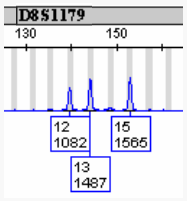
Minor component:
(B+C)/total = (244+468)/2330 = **0.306**

Major component:
(A+D)/total = (880+738)/2330 = **0.694**

Major: 14,18
Minor: 15,17

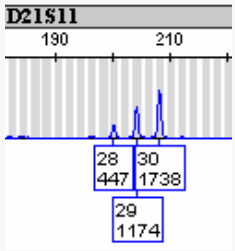
Four Peaks **(4 allele loci)**
- heterozygote + heterozygote, no overlapping alleles (genotypes are unique)

3 Allele Locus: D8S1179



Three Peaks **(3 allele loci)**
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

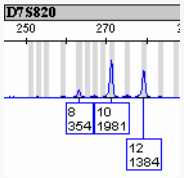
3 Allele Locus: D21S11



Allele	Frequency
28	0.159
29	0.195
30	0.278

Three Peaks **(3 allele loci)**
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

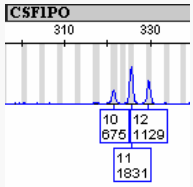
3 Allele Locus: D7S820



Allele	Frequency
8	0.151
10	0.243
12	0.166

Three Peaks **(3 allele loci)**
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

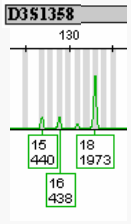
3 Allele Locus: CSF1PO



Allele	Frequency
10	0.217
11	0.301
12	0.361

Three Peaks **(3 allele loci)**
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

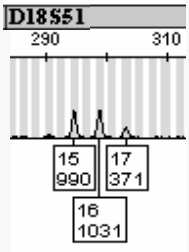
3 Allele Locus: D3S1358



Allele	Frequency
15	0.262
16	0.253
18	0.152

Three Peaks **(3 allele loci)**
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

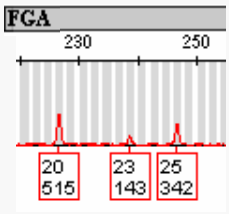
3 Allele Locus: D18S51



Allele	Frequency
15	0.159
16	0.139
17	0.126

Three Peaks **(3 allele loci)**
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

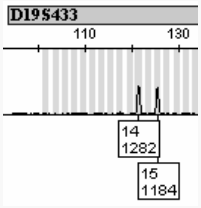
3 Allele Locus: FGA



Allele	Frequency
20	0.127
23	0.134
25	0.071

Three Peaks **(3 allele loci)**
- heterozygote + heterozygote, one overlapping allele
- heterozygote + homozygote, no overlapping alleles (genotypes are unique)

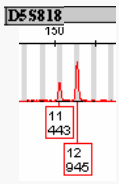
2 Allele Locus: D19S433



Allele	Frequency
14	0.369
15	0.152

Two Peaks **(2 allele loci)**
- heterozygote + heterozygote, two overlapping alleles (genotypes are identical)
- heterozygote + homozygote, one overlapping allele
- homozygote + homozygote, no overlapping alleles (genotypes are unique)

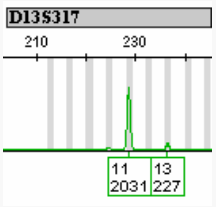
2 Allele Locus: D5S818



Allele	Frequency
11	0.361
12	0.384

Two Peaks **(2 allele loci)**
- heterozygote + heterozygote, two overlapping alleles (genotypes are identical)
- heterozygote + homozygote, one overlapping allele
- homozygote + homozygote, no overlapping alleles (genotypes are unique)

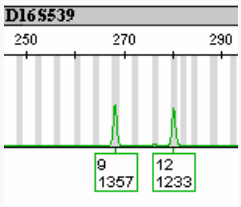
2 Allele Locus: D13S317



Allele	Frequency
11	0.339
13	0.124

Two Peaks **(2 allele loci)**
- heterozygote + heterozygote, two overlapping alleles (genotypes are identical)
- heterozygote + homozygote, one overlapping allele
- homozygote + homozygote, no overlapping alleles (genotypes are unique)

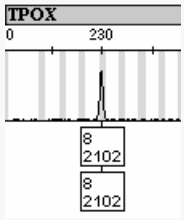
2 Allele Locus: D16S539



Allele	Frequency
9	0.113
12	0.326

Two Peaks **(2 allele loci)**
- heterozygote + heterozygote, two overlapping alleles (genotypes are identical)
- heterozygote + homozygote, one overlapping allele
- homozygote + homozygote, no overlapping alleles (genotypes are unique)

1 Allele Locus: TPOX



Allele	Frequency
8	0.535

Single Peak **(1 allele loci)**
- homozygote + homozygote, overlapping allele (genotypes are identical)

Profiles Used In Mixture Samples

	Victim	Suspect
D8S1179	13,15	12,12
D21S11	29,30	28,30
D7S820	10,12	8,10
CSF1PO	11,12	10,11
D3S1358	18,18	15,16
TH01	7,9	8,9.3
D13S317	11,11	11,13
D16S539	9,12	9,12
D2S1338	23,24	19,25
D19S433	14,15	14,15
vWA	14,18	15,17
TPOX	8,8	8,8
D18S51	15,16	16,17
AMEL	X,X	X,Y
D5S818	12,12	11,11
FGA	20,25	20,23

Software Programs (~~Expert Systems~~) for Mixture Deconvolution

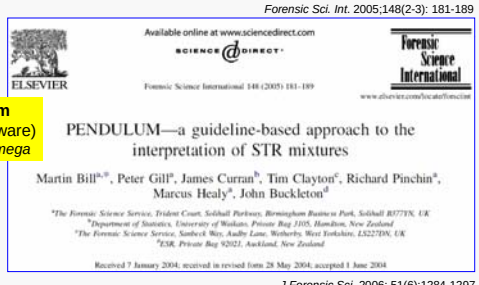
These programs do not supply stats (only attempt to deduce mixture components)

- Linear Mixture Analysis (LMA) **U.S. Patent 6,807,490**
  - **Part of TrueAllele system** developed by Mark Perlin (Cybergenetics)
  - Perlin, M. W. and Szabady, B. (2001) Linear mixture analysis: a mathematical approach to resolving mixed DNA samples. *J.Forensic Sci.* 46(6): 1372-1378
- Least Squares Deconvolution (LSD)
  - Available for use at **https://lsd.lit.net/**
  - Wang, T., Xue, N., Birdwell, J.D. (2006) Least-square deconvolution: a framework for interpreting short tandem repeat mixtures. *J Forensic Sci.* 51(6):1284-1297.
- PENDULUM
  - **Part of FSS i-3 software suite (i-STReam)**
  - Bill, M., Gill, P., Curran, J., Clayton, T., Pinchin, R., Healy, M., and Buckleton, J. (2005) PENDULUM-a guideline-based approach to the interpretation of STR mixtures. *Forensic Sci.Int.* 148(2-3): 181-189

USACIL program developed by Tom Overson called **DNA_DataAnalysis**

Forensic Sci. Int. 2005;148(2-3): 181-189

i-STReam (FSS-i³ software) *Sold by Promega*


Available online at www.sciencedirect.com
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ELSEVIER
Forensic Science International

PENDULUM—a guideline-based approach to the interpretation of STR mixtures

Martin Bill[a,*], Peter Gill[a], James Curran[b], Tim Clayton[c], Richard Pinchin[a], Marcus Healy[a], John Buckleton[d]

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J Forensic Sci. 2006; 51(6):1284-1297

Available for use over internet at **https://lsd.lit.net/**

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Available online at: www.blackwell-synergy.com

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Least-Square Deconvolution: A Framework for Interpreting Short Tandem Repeat Mixtures*

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- Tim Kalafut (USACIL)

Mixture Statistics

Florida Statewide Training Meeting

Indian Rocks Beach, FL
May 12-13, 2008

Dr. John M. Butler
National Institute of Standards and Technology

john.butler@nist.gov

German Type A,B, and C mixture classifications

- **Type A**, where major/minor contributors cannot be deduced, require stats
  - LR
  - RMNE
- **Type B** enables major contributor to be deduced
  - RMP (which is 1/LR)
- **Type C** no stats should be attempted because of the possibility of failure to account for allele dropout due to stochastic effects with low level DNA samples

Statistical Interpretation of DNA Mixtures


CROATIAN MEDICAL JOURNAL 42(3):244-246, 2001

CMJ FORENSIC SCIENCES

Interpretation of Complex Forensic DNA Mixtures

Carll Ladd, Henry C. Lee, Nicholas Yang, Frederick R. Bieber[1]

[1]State of Connecticut Department of Public Safety, Division of Scientific Services, Forensic Science Laboratory, Conn; and [2]Departments of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, Mass, USA

Ladd et al. 2001. *Croatian Medical Journal* 43(3): 244-246

1. **Qualitative statement ('..cannot exclude..')**
2. **Interpret as single source from peak height differences, differential extraction, etc. and calculate random match probability (RMP)**
3. **Calculate probability of exclusion (CPE)**
4. **Calculate likelihood ratio (LR)**

Random Man Not Excluded (RMNE)

- = Probability of Exclusion (PE)
- John Buckleton (*Forensic DNA Evidence Interpretation*, p. 222) quotes Laszlo Szabo of Tasmania Forensic Science Laboratory: "Intuitively, RMNE is easier to explain to a jury and express in reports than the likelihood ratio, and is probably closer to what the court wants—e.g., the suspect matches the mixture, but what if this is the wrong person– then what is the probability that someone else in the population would also match the mixture (i.e., not be excluded as a contributor)."
- Buckleton (*Forensic DNA Evidence Interpretation*, p. 222) also quotes Bruce Weir: that exclusion probabilities "often rob the items of probative value"

Probability of Exclusion (RMNE)

- **Advantages**
  - Does not require an assumption of the number of contributors to a mixture
  - Easier to explain in court
- **Disadvantages**
  - Weaker use of the available information (robs the evidence of its true probative power because this approach does not consider the suspect's genotype)
  - Likelihood ratio approaches are developed within a consistent logical framework

John Buckleton, *Forensic DNA Evidence Interpretation*, p. 223

RMNE (CPE)

- Statements from DAB Recommendations on Statistics (FDT2e, p. 617)
- CPE provides a calculation of the estimated proportion of individuals from a defined population group **that can be excluded as a contributor** to an observed DNA mixture

Probability of Exclusion

The probability that a random person (unrelated individual) would be excluded as a contributor to the observed DNA mixture

For each locus, 1 minus the square of the sum of frequencies for the observed alleles

$$PE_i = 1 - \left(\sum_{i=1}^{n} p(A_i) \right)^2$$

Buckleton (2005) *Forensic DNA Evidence Interpretation*, p. 219

Across multiple loci (i.e., **combined probability of exclusion**, CPE):

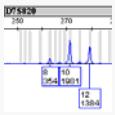
$$PE = 1 - \prod_{l} (1 - PE_l)$$

Buckleton (2005) *Forensic DNA Evidence Interpretation*, p. 221

Combined Probability of Exclusion (CPE)

Each locus is calculated separately and then combined for CPE

$CPE = 1 - (1 - PE_1)(1 - PE_2)(1 - PE_3)\ldots(1\text{-}PE_N)$



Probability of exclusion at a single locus:

- ***The combined frequency of alleles detected (P)***
  P = frequency of allele 1 + frequency of allele 2 + frequency of allele 3, … N

US Caucasian Data

Allele	Frequency
8	0.151
10	0.243
12	0.166

P = 0.151 + 0.243 + 0.166 = **0.56**

- ***The combined frequency of alleles not detected (Q)***
  Q = 1 – P

Q = 1 – 0.56 = **0.44**

- **PE = $Q^2 + 2Q(1-Q)$**

CPI = 1 – CPE

PE = $(0.44)^2 + 2(0.44)(1\text{-}0.44)$ = 0.1936 + 0.4928
PE = 0.686

Calculation from CPI Perspective

Each locus is calculated separately and then combined for CPE

CPI or $P_{profile}$ = (P_{locus1}) (P_{locus2}) (P_{locus3}) … ($P_{locus(N)}$)

Probability of inclusion at a single locus:

- ***Individual frequencies are summed and then squared***
  PI or $P_{locus} = (p_1 + p_2 + p_3 + \ldots + p_N)^2$
  Alleles present in the mixture; Remaining possible alleles in the population

- PE = 1 – P_{locus} = 1 – PI
- **PE = $Q^2 + 2Q(1-Q)$**

Essentially $P^2 + 2PQ + Q^2 = 1$
PI PE

P + Q = 1 so
P = 1 – Q and
Q = 1 – P

Provides probability of an unrelated individual in the population is a contributor to the mixture at the loci examined

Likelihood Ratios

Basic Math Terms

- When '+' is used, this means 'OR'
- When 'x' is used, this means 'AND'
- Pr. is shorthand for probability

- Therefore…
  - the probability of *a* 'AND' *b* happening together is **Pr(*a* and *b*) = *a* x *b***
  - the probability of *a* 'OR' *b* happening together is **Pr(*a* or *b*) = *a* + *b***

Slide information from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

Conditioning

- **Probabilities are conditional**, which means that the probability of something is based on a hypothesis

- In math terms, conditioning is denoted by a vertical bar
  - Hence, **Pr(a|b)** means 'the probability of *a* ***given*** that *b* is true"

- The probability of an event *a* is dependent upon various assumptions—and these assumptions or hypotheses can change…

Slide information from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

Probability Example – Will It Rain? (1)

Defining the Event and Assumptions/Hypotheses

- Let's suppose that *a* is the probability of an event (e.g., **will it rain?**)
- What is the probability that it will rain in the afternoon – **Pr(a)**?
- This probability is dependent upon assumptions
  - We can look at the window in the morning and observe if it is sunny (s) or cloudy (c)
  - Pr(a) **if** it is sunny (s) is less than Pr(a) **if** it is cloudy (c)
- We can write this as **Pr(a|s)** and **Pr(a|c)**
  - Since sunny or cloudy are the only possibilities, Pr(s) + Pr(c) = 1
  - or **Pr(s) = 1 – Pr(c)**

Slide information from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

Probability Example – Will It Rain? (2)

Examining Available Data

- Pr(a|s) and Pr(a|c) can be calculated from data
- How often does it rain in the afternoon when its sunny in the morning?
  - 20 out of 100 observations so **Pr(a|s) = 0.2**
- How often does it rain in the afternoon when it is cloudy in the morning?
  - 80 out of 100 observations so **Pr(a|c) = 0.8**

Slide information from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

Probability Example – Will It Rain? (3)

Formation of the Likelihood Ratio (LR)

- The LR compares two probabilities to find out which of the two probabilities is the most likely

The probability that it will rain in the afternoon when it is cloudy in the morning or **Pr(a|c)** is divided by the probability that it will rain in the afternoon when it is sunny in the morning or **Pr(a|s)**

$$LR = \frac{\Pr(a \mid c)}{\Pr(a \mid s)} = \frac{0.8}{0.2} = 4$$

Slide information from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

Probability Example – Will It Rain? (4)

Explanation of the Likelihood Ratio

$$LR = \frac{\Pr(a \mid c)}{\Pr(a \mid s)} = \frac{0.8}{0.2} = 4$$

- The probability that it will rain is 4 times more likely **if** it is cloudy in the morning than **if** it is sunny in the morning.
- The word **if** is very important here. It must always be used when explaining a likelihood ratio otherwise the explanation could be misleading.

Slide information from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

Likelihood Ratios in Forensic DNA Work

- We evaluate the evidence (*E*) relative to alternative pairs of hypotheses
- Usually these hypotheses are formulated as follows:
  - The probability of the evidence if the crime stain originated with the suspect or Pr(*E*|*S*)
  - The probability of the evidence if the crime stain originated from an unknown, unrelated individual or Pr(*E*|*U*)

$$LR = \frac{\Pr(E \mid S)}{\Pr(E \mid U)}$$

The numerator

The denominator

Slide information from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

The Likelihood Ratio Must Be Stated Carefully

- The probability of the evidence is *x* times more likely **if** the stain came from the suspect Mr. Smith than **if** it came from an unknown, unrelated individual.
- It is not appropriate to say: "The probability that the stain came from Mr. Smith." because we must always include the conditioning statement – i.e., **always make the hypothesis clear in the statement.**
- Always use the word '**if**' when using a likelihood ratio to avoid this trap

Slide information from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

Likelihood Ratio (LR)

- Provides ability to express and evaluate both the prosecution hypothesis, H_p (the suspect is the perpetrator) and the defense hypothesis, H_d (an unknown individual with a matching profile is the perpetrator)

$$LR = \frac{H_p}{H_d}$$

- **The numerator, H_p, is usually 1** – since in theory the prosecution would only prosecute the suspect if they are 100% certain he/she is the perpetrator
- The denominator, **H_d**, is typically the profile frequency in a particular population (based on individual allele frequencies and assuming HWE) – i.e., **the random match probability**

Relationship between Likelihood Ratio (LR) and Random Match Probability (RMP)

- For single source samples or deduced major component profiles in a mixture…

$$LR = \frac{1}{RMP} \quad \text{or} \quad RMP = \frac{1}{LR}$$

Example #1

A Single Locus from a 2-Person Mixture

- Consider a simple **two person mixture** with one locus consisting of two heterozygotes with non-overlapping alleles
- If the suspect is *ab*, then there must be another (unknown person) who is *cd*

Suspect = a,b

Forget peak heights for the time being

Adapted from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

Example #1

The Two Hypotheses Are Formed…

- **Prosecution (H_p)**: The DNA result has come from the suspect and one unknown person, or **Pr(*E*|*S*,*U*)**
- **Defense (H_d)**: The DNA result has come from two unknown people, or **Pr(*E*|U_1,U_2)**

$$LR = \frac{\Pr(E \mid S,U)}{\Pr(E \mid U_1,U_2)}$$

Suspect = a,b

Forget peak heights for the time being

Adapted from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

Example #1

Formulating the Numerator
(Prosecution Hypothesis)

- If the prosecution hypothesis is true, then we would expect genotype *ab* to be present with 100% probability or Pr=1.
- The chance of seeing an unknown person of type *cd* is the frequency of that type in the population or $2p_cp_d$, where p_c is the allele frequency for allele c.
- **Pr(*E*|*S*,*U*)** = 1 x $2p_cp_d$ **= $2p_cp_d$**

Suspect = a,b

Forget peak heights for the time being

Adapted from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

Example #1

Formulating the Denominator
(Defense Hypothesis)

- The defense claims that the evidence could come from any two random individuals
- We must work out **all possible pairwise combinations** from alleles *abcd* and their probabilities (genotype frequencies)

Multiplied because you are considering individual #1 **AND** individual #2

Added because you are considering any of the possibilities (combination 1,2,3,4,5, **OR** 6)

Individual #1	Individual #2	Products
ab	cd	$2p_ap_b \times 2p_cp_d$ → $4p_ap_bp_cp_d$
ac	bd	$2p_ap_c \times 2p_bp_d$ → $4p_ap_bp_cp_d$
ad	bc	$2p_ap_d \times 2p_bp_c$ → $4p_ap_bp_cp_d$
cd	ab	$2p_cp_d \times 2p_ap_b$ → $4p_ap_bp_cp_d$
bd	ac	$2p_bp_d \times 2p_ap_c$ → $4p_ap_bp_cp_d$
bc	ad	$2p_bp_c \times 2p_ap_d$ → $4p_ap_bp_cp_d$
Sum of products		**$24p_ap_bp_cp_d$**

Reverse combinations

Pr(*E*|U_1,U_2) = $24p_ap_bp_cp_d$

Adapted from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

Example #1

Formulating the Likelihood Ratio

- The numerator and denominator are combined to form the LR
- And common elements in both numerator and denominator are eliminated to simplify the algebraic equation…

$$LR = \frac{\Pr(E \mid S,U)}{\Pr(E \mid U_1,U_2)} = \frac{\cancel{2}\,p_c p_d}{\cancel{24}^{12}\,p_a p_b p_c p_d} = \frac{1}{12\,p_a p_b}$$

All LR Calculations Follow the Same Basic Rules Just Shown

- Form hypotheses
  - Keep in mind what you are conditioning on
- The LR numerator belongs to the prosecution
- The LR denominator belongs to the defense
- Numerator and denominator are combined and equation is simplified
- Allele frequency values are placed into the equation for each locus
- **The LR from each locus is combined through multiplication if the loci are independently inherited (i.e., the product rule) to form a LR for the entire profile**

Example #2

Another Example…

- The evidentiary mixture profile is from a semen stained vaginal swab and possesses alleles a, b, c, and d.
- The suspect is a,b and the victim is c,d.
- Because it is reasonable to assume that the victim's alleles would be present on the swab (i.e., an intimate sample), we can condition on this…

Suspect = a,b
Victim = c,d

Example #2

With an Intimate Sample, the Hypothesis Changes…

- **Prosecution (H_p)**: The DNA result has come from the suspect and the victim, or **Pr(*E*|*S,V*)**
- **Defense (H_d)**: The DNA result has come from the victim and one unknown person, or **Pr(*E*|*U,V*)**

$$LR = \frac{\Pr(E \mid S,V)}{\Pr(E \mid U,V)}$$

Suspect = a,b
Victim = c,d

Example #2

Formulating the Numerator (Prosecution Hypothesis)

- The prosecution hypothesis (S+V) is completely explains the evidence. Hence, the probability is Pr=1
- **Pr(*E*|*S,V*) = 1 x 1 = 1**

Suspect = a,b
Victim = c,d

Example #2

Formulating the Denominator (Defense Hypothesis)

- The defense hypothesis is that the presence of alleles *a* and *b* are the result of an unknown person – and they concede that alleles *c* and *d* come from the victim
- Since the frequency of an unknown, unrelated individual possessing alleles *a* and *b* in the population is $2p_ap_b$, where p_a is the allele frequency for allele *a* and p_b is the allele frequency for allele *b*, then
- **Pr(*E*|*U,V*)** = $2p_ap_b$ x 1 = **$2p_ap_b$**

Suspect = a,b
Victim = c,d

Example #2

Formulating the Likelihood Ratio

- The numerator and denominator are combined to form the LR

$$LR = \frac{\Pr(E \mid S,V)}{\Pr(E \mid U,V)} = \frac{1}{2p_a p_b}$$

- **Note that this LR is the same as for a non-mixed sample comprising the suspect alone.**
- This example then is an illustration of simplification by "subtraction" (victim's alleles are being removed from mathematical consideration…).

Adapted from Peter Gill (ISFG 2007 workshop, Copenhagen, August 20-21, 2007)

Forming the Denominator (H_d) for the LR…

Evidence (Mixture)	Victim	Suspect	LR
A_1, A_2, A_3	A_2, A_3	A_1, A_2	$\frac{1}{p_1(2p_2+2p_3+p_1)}$
8,10,12	10,12	8,10	

Potential Combinations:

If victim is A_2,A_3, then perpetrator could be

	Type	Frequency (probability)
H_d	A_1,A_2	$2p_1p_2$
	A_1,A_3	$2p_1p_3$
	A_1,A_1	p_1^2

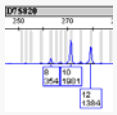
Other possible genotypes contributing to the evidence

Determine joint probability through summing individual probabilities

$2p_1p_2 + 2p_1p_3 + p_1^2 \longrightarrow p_1(2p_2 + 2p_3 + p_1)$

Likelihood Ratio (LR) Calculations

Evidence (Mixture)	Victim	Suspect	LR
A_1, A_2, A_3	A_2, A_3	A_1, A_2	$\frac{1}{p_1(2p_2+2p_3+p_1)}$
8,10,12	10,12	8,10	



US Caucasian Data

Allele	Frequency
A_1 8	p_1 0.151
A_2 10	p_2 0.243
A_3 12	p_3 0.166

$$LR = \frac{1}{(0.151)[(2)(0.243)+2(0.166)+(0.151)]}$$

LR = 6.83

Does not consider peak height information

The prosecution hypothesis (that the suspect is the perpetrator) is 6.83 times more likely than the defense hypothesis (that an unknown, unrelated individual is the perpetrator).

Likelihood Ratios for the Following Hypotheses

H_p: The mixture contains the DNA of the victim and the suspect

H_d: The mixture contains the DNA of the victim and an unknown, unrelated individual

Evidence (Mixture)	Victim	Suspect	LR
A_1, A_2, A_3, A_4	A_1, A_2	A_3, A_4	$\frac{1}{2p_3p_4}$
A_1, A_2, A_3	A_1, A_2	A_1, A_3 or A_2, A_3 or A_3, A_3	$\frac{1}{p_3(2p_1+2p_2+p_3)}$
A_1, A_2, A_3	A_1, A_1	A_2, A_3	$\frac{1}{2p_2p_3}$
A_1, A_2	A_1, A_2	A_1, A_1 or A_1, A_2 or A_2, A_2	$\frac{1}{(p_1+p_2)^2}$
A_1, A_2	A_1, A_1	A_1, A_2 or A_2, A_2	$\frac{1}{p_2(2p_1+p_2)}$
A_1, A_1	A_1, A_1	A_1, A_1	$\frac{1}{p_1^2}$

Adapted from Buckleton (2005) *Forensic DNA Evidence Interpretation*, Table 7.1, p. 229

DAB Recommendations on Statistics

February 23, 2000

Forensic Sci. Comm. 2(3); available on-line at http://www.fbi.gov/hq/lab/fsc/backissu/july2000/dnastat.htm

"The DAB finds either one or both PE or LR calculations acceptable and strongly recommends that one or both calculations be carried out whenever feasible and a mixture is indicated"

- Probability of exclusion (PE)
  - Devlin, B. (1993) Forensic inference from genetic markers. *Statistical Methods in Medical Research*, 2, 241–262.
- Likelihood ratios (LR)
  - Evett, I. W. and Weir, B. S. (1998) *Interpreting DNA Evidence*. Sinauer, Sunderland, Massachusetts.

Topics and Techniques for Forensic DNA Analysis

Interlaboratory Mixture Studies

Florida Statewide Training Meeting

Indian Rocks Beach, FL
May 12-13, 2008

Dr. John M. Butler
National Institute of Standards and Technology

john.butler@nist.gov

Outline

- Purpose of Interlaboratory Studies
- Overview of Mixture Studies and Lessons Learned
- NIST MIX05 Study Results

Interlaboratory Studies

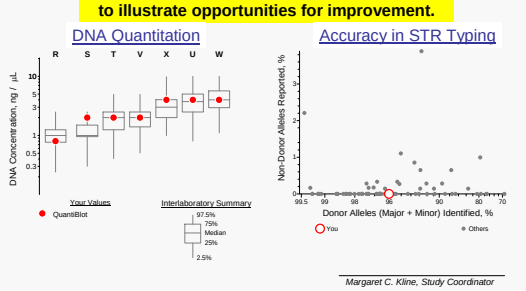
- Purpose…
  - Not a proficiency test
  - Most labs see them as opportunity to anonymously directly compare themselves to others
- STRBase section on interlab studies
  - http://www.cstl.nist.gov/biotech/strbase/interlab.htm

A High Degree of Variability Currently Exists with Mixture Interpretation

- **"If you show 10 colleagues a mixture, you will probably end up with 10 different answers"**
  - Peter Gill, Human Identification E-Symposium, April 14, 2005
- **Interlaboratory studies help to better understand why variability may exist between laboratories**
- Most analysts are only concerned about their own lab protocols and do not get an opportunity to see the big picture from the entire community that can be provided by a well-run interlaboratory study

The National Institute of Standards and Technology
Gratefully Acknowledges the Participation of the

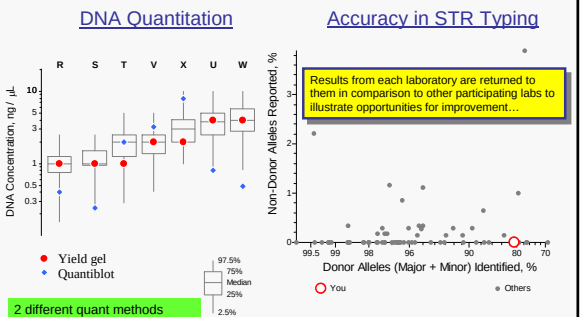
This feedback can be helpful to a laboratory to know where they stand relative to other labs to illustrate opportunities for improvement.



Margaret C. Kline, Study Coordinator

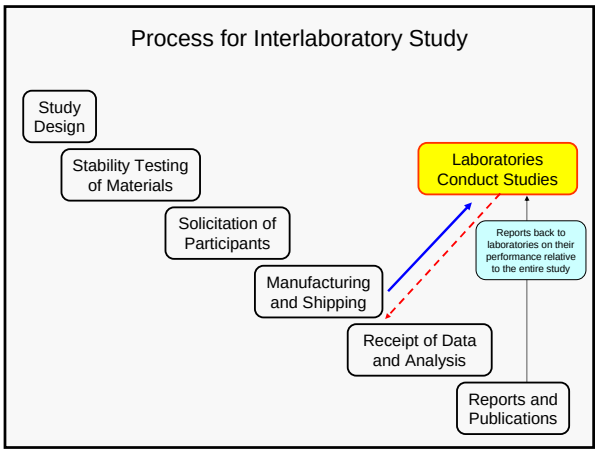
See Kline, M.C., *et al.* (2003) *Anal. Chem.* 75: 2463-2469

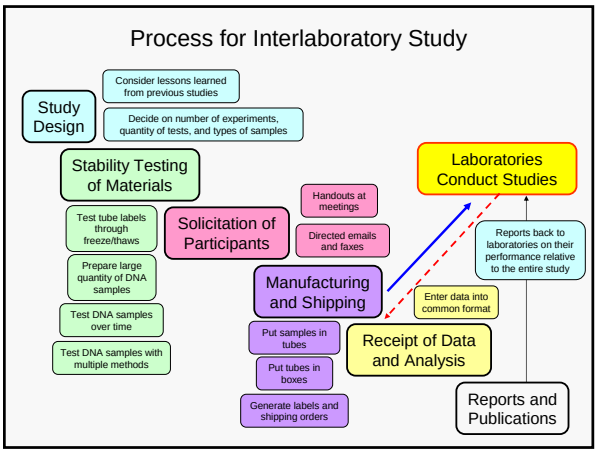
Individual Performance in an Interlaboratory Study



2 different quant methods gave different results; this lab followed the Quantiblot results

See Kline, M.C., *et al.* (2003) *Anal. Chem.* 75: 2463-2469





NIST Initiated Interlaboratory Studies

Studies involving STRs	# Labs	Publications
Evaluation of CSF1PO, TPOX, and TH01	34	Kline MC, Duewer DL, Newall P, Redman JW, Reeder DJ, Richard M. (1997) Interlaboratory evaluation of STR triplex CTT. *J. Forensic Sci.* 42: 897-906
Mixed Stain Studies #1 and #2 (Apr–Nov 1997 and Jan–May 1999)	45	Duewer DL, Kline MC, Redman JW, Newall PJ, Reeder DJ. (2001) NIST Mixed Stain Studies #1 and #2: interlaboratory comparison of DNA quantification practice and short tandem repeat multiplex performance with multiple-source samples. *J. Forensic Sci.* 46: 1199-1210
MSS3 **Mixed Stain Study** #3 (Oct 2000-May 2001)	74	Kline, M.C., Duewer, D.L., Redman, J.W., Butler, J.M. (2003) NIST mixed stain study 3: DNA quantitation accuracy and its influence on short tandem repeat multiplex signal intensity. *Anal. Chem.* 75: 2463-2469. Duewer, D.L., Kline, M.C., Redman, J.W., Butler, J.M. (2004) NIST Mixed Stain Study #3: signal intensity balance in commercial short tandem repeat multiplexes, *Anal. Chem.* 76: 6928-6934.
DNA Quantitation Study (Jan-Mar 2004) QS04	80	Kline, M.C., Duewer, D.L., Redman, J.W., Butler, J.M. (2005) Results from the NIST 2004 DNA Quantitation Study, *J. Forensic Sci.* 50(3):571-578
Mixture Interpretation Study (Jan - Aug 2005) **MIX05**	**69**	***Several presentations made ...*** **Poster at 2005 Promega meeting (Sept 2005); available on STRBase**

Overall Lessons Learned from NIST MSS 1,2,&3

- Laboratories have instruments with different sensitivities
- **Different levels of experience and training plays a part in effective mixture interpretation**
- Amount of input DNA makes a difference in the ability to detect the minor component (labs that put in "too much" DNA actually detected minor components more frequently)

NIST MIX05 Summary

Purpose of MIX05 Study

- **Goal is to understand the "lay of the land" regarding mixture analysis across the DNA typing community**
- One of the primary benefits we hope to gain from this study is **recommendations for a more uniform approach to mixture interpretation** and training tools to help educate the community

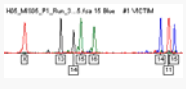
MIX05 Study Design and Purpose

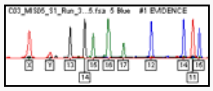
Interlab studies provide a "big picture" view of the community

- Permit a large number of forensic practioners to **evaluate the same mixture data**
- Provide multiple cases representing a range of mixture scenarios
- Generate data from multiple STR kits on the same mixture samples to compare performance for detecting minor components
- The primary variable should be the laboratory's interpretation guidelines rather than the DNA extraction, PCR amplification, and STR typing instrument sensitivity
- **Are there best practices in the field that can be advocated to others?**

Mixture Interpretation Interlab Study (MIX05)

- **Only involves interpretation of data – to remove instrument detection variability and quantitation accuracy issues**
- **94 labs enrolled** for participation
- **69 labs have returned results** (17 from outside U.S.)
- Four mock cases supplied with "victim" and "evidence" electropherograms (GeneScan .fsa files – that can be converted for Mac or GeneMapper; gel files made available to FMBIO labs)
- Data available with Profiler Plus, COfiler, SGM Plus, PowerPlex 16, Identifiler, PowerPlex 16 BIO (FMBIO) kits
- Summary of results will involve training materials to illustrate various approaches to solving mixtures





Perpetrator Profile(s) ??

Along with reasons for making calls and any stats that would be reported

Requests for Participants in MIX05

Mixtures representing four different case scenarios have been generated at NIST with multiple STR kits and provided to laboratories as electropherograms.

We would like to receive the following information:

1) **Report the results as though they were from a real case** including whether a statistical value would be attached to the results. *Please summarize the perpetrator(s) alleles in each "case" as they might be presented in court—along with an appropriate statistic (if warranted by your laboratory standard operating procedure) and the source of the allele frequencies used to make the calculation.* Please indicate which kit(s) were used to solve each case.

2) **Estimate the ratio for samples present in the evidence mixture** and how this estimate was determined.

3) **Provide a copy of your laboratory mixture interpretation guidelines** and a brief explanation as to why conclusions were reached in each scenario

A MIX05 Participant Noted…

"Things we do not do:

- **Calculate mixture ratios for casework**
  - **Calculation used for this study:** Find loci with 4 alleles (2 sets of sister alleles). Make sure sister alleles fall within 70%, then take the ratio of one allele from one sister set to one allele of the second sister set, figure ratios for all combinations and average. Use peak heights to calculate ratios.
- **Provide allele calls in reports**
- **Provide perpetrator(s) alleles or statistics in court without a reference sample to compare to the DNA profile obtained from the evidence. We will try to determine the perpetrator(s) profile for entry into CODIS."**

We recognize that some of the information requested in this interlab study may not be part of a lab's standard operating procedure

MIX05 Case Scenarios

Genomic DNA samples with specific allele combinations ("evidence") were mixed in the following ratios:

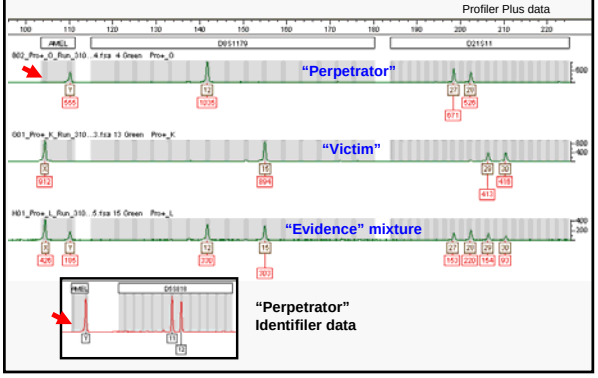
Based on Identifiler 15 STR loci

	#alleles		#loci with #alleles				
	N all	N unq	N 1	N 2	N 3	N 4	N 5
Case #1 – victim is major contributor (3F:1M)	39	26	2	6	5	2	0
Case #2 – perpetrator is major contributor (1F:3M)	55	52	0	1	4	10	0
Case #3 – balanced mixture (1F:1M) • Male lacked amelogenin X	48	37	0	3	8	4	0
Case #4 – more extreme mixture (7F:1M) • Male contained tri-allelic pattern at TPOX	50	42	0	3	7	4	1

Female victim DNA profile was supplied for each case

Labs asked to deduce the perpetrator DNA profile – suspect(s) not provided

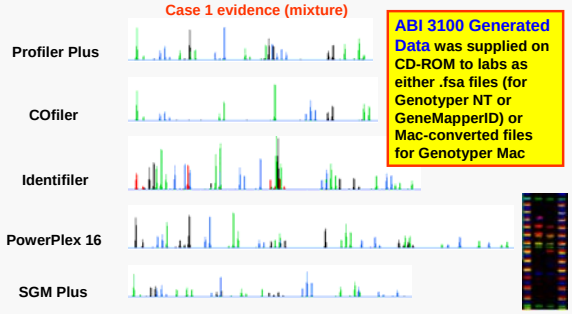
Amelogenin X allele is missing in male perpetrator DNA sample for MIX05 Case #3



MIX05 Results on Multiple Kits

http://www.cstl.nist.gov/biotech/strbase/interlab/MIX05.htm

Case 1 evidence (mixture)



ABI 3100 Generated Data was supplied on CD-ROM to labs as either .fsa files (for Genotyper NT or GeneMapperID) or Mac-converted files for Genotyper Mac

FMBIO data was also made available upon request

Summary of MIX05 Responses

94 labs enrolled for participation

69 labs returned results (17 from outside U.S.)

50 labs made allele calls
39 labs estimated ratios
29 labs provided stats

All participants were supplied with all data and could choose what kits to examine based on their experience and lab protocols

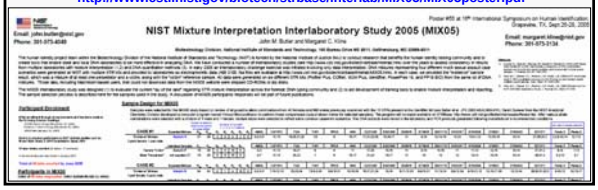
STR kit results used
- 34 ProfilerPlus/COfiler
- 10 PowerPlex 16
- 7 PP16 BIO
- 5 Identifiler
- 2 SGM Plus
- 1 All ABI kit data
- 9 Various combinations

Generally Identifiler data was of poorer quality in the electropherograms we provided...which caused some labs to not return results (they indicated a desire for higher quality data through sample re-injection to reduce pull-up prior to data interpretation)

What MIX05 Participants Have Received Back from NIST…

- Certificate of participation in the interlab study
- Copy of the poster presented at the Promega Sept 2005 meeting displaying "correct" results for the perpetrator in each case scenario as well as an explanation of study design and preliminary results

http://www.cstl.nist.gov/biotech/strbase/interlab/MIX05/MIX05poster.pdf


NIST Mixture Interpretation Interlaboratory Study 2005 (MIX05)

When is a Sample a Potential Mixture?

According to several MIX05 participant interpretation guidelines

- Number of Observed Peaks
  - Greater than two peaks at a locus
  - More than two alleles are present at two or more loci, although three banded patterns can occur
  - Presence of 3 alleles at a single locus within a profile
  - 4 peaked patterns (if observed at any locus), 3 peaked patterns (if observed at two or more loci), significant imbalances (peak height ratios <60%) of alleles for a heterozygous genotype at two or more loci with the exception of low template amplifications, which should be interpreted with caution
- Imbalance of heterozygote alleles
  - thresholds range from 50-70%
- Stutter above expected levels
  - generally 15-20%

Detection thresholds also varied in the range of 50-200 RFUs

These protocol differences can lead to variation in reported alleles and therefore the deduced profile and resulting statistics

Summary of Some MIX05 Reported Results

Case #2 has perpetrator as major component and thus is the easiest to solve...

CASE #2		D3S1358	VWA	FGA	AMEL	D8S1179	D21S11	D18S51	D5S818	D13S317	D7S820	D16S539	TH01	TPOX	CSF1PO
True Perp	Z779619	15,15	15,15	20,24	X,Y	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
LabID	Kit Used														
16	ProPlus/Cofiler	--	--	--	--	--	--	--	--	--	--	--	--	--	--
6	ProPlus/Cofiler	15	15	20,24	X,Y	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
91	SGM Plus	15	15	20,24	X,Y	11,13	28,32.2	17,18				10,11	7,9.3		
46	PP16	--	--	--	--	--	--	--	--	--	--	--	--	--	--
37	ProPlus/Cofiler	--	15	20	X,Y	13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
2	PP16	15	15,15	20,24	X,Y	11,13	28,32.2	17,18	8,13	INC	8,10	10,11	7,9.3	9,10	7,10
13	PP16 & Identifiler	15	15	20,24	--	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
34	ProPlus/Cofiler	15	15	20,24	--	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
70	Identifiler	15	15	20,24	X,Y	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
55	ProPlus/Cofiler	15	15	20,24	--	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
21	ProPlus/Cofiler	15,15	15,15	20,24	X,Y	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
73	ProPlus/Cofiler	15,15	15,15	20,24	X,Y	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
29	Identifiler	15	15	20,24	X,Y	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
54	All Kits	15,15	15,15	20,24	X,Y	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
90	ProPlus/Cofiler	15	15	20,24	X,Y	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
9	ProPlus/Cofiler	15	15	20,24	X,Y	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
4	ProPlus/Cofiler	15	15	20,24	X,Y	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
33	ProPlus/Cofiler	--	--	--	--	--	--	--	--	--	--	--	--	--	--
12	ProPlus/Cofiler	15	15	20,24	X,Y	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
67	PP16	15	15,16	20,24	X,Y	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
86	ProPlus/Cofiler	15,15	15,15	20,24	--	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
79	ProPlus/Cofiler	15,15	15,15	20,24	--	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
77	Identifiler	--	--	--	--	--	--	--	--	--	--	--	--	--	--
60	PP16	15	15	20,24	X,Y	11,13	28,32.2	17,18	8,13	12,14	8,10	10,11	7,9.3	9,10	7,10
61	Identifiler	--	--	--	--	--	--	--	--	--	--	--	--	--	--

Most calls were correct (when they were made)

Some Mixture Ratios Reported in MIX05

Many labs do not routinely report the estimated ratio of mixture components

LabID	Case1 (F:M)	Case2 (M:F)	Case3 (M:F)	Case4 (F:M)
13	2	5	<2	10
34	1.8--3.6	3.9--6.7	1.6--1.8	6.2--7.6
70				
55	68%:32%	85%:15%	64%:36%	
21				
73	2:1	6:1	2:1	not determined
29				
54	2:1	6:1	2:1	6:1
90	male23-39%	not determined	male64-71%	
9	3 or 4:1	4 or 5:1	1.4:1	~10:1
4	10:1	6:1	1:1	not determined
33	male60-78%	male80-90%	male58-71%	victim86%
12	male25%	male85%	male40-45%	unknown10%
67	1:2.3	6.4:1	2:1	1:6.8
86	2:1	6-6.5:1	1.6-2:1	4-4.5:1
79	~3:1 to ~2:1	~6:1 to ~4:1	~2:1*	a lot of victim
77				
60	2:1	5:1	2:1	10:1
61				

Some Reported Stats for MIX05 Case #1

Many of the 29 labs providing statistics used PopStats 5.7

		Case1		
LabID	Kits Used	Caucasians	African Americans	Hispanics
77	Identifiler	PE calculated	PE calculated	PE calculated
73	ProPlus/Cofiler	none provided	none provided	none provided
4	ProPlus/Cofiler	none provided	none provided	none provided
12	ProPlus/Cofiler	none provided	none provided	none provided
29	Identifiler	none provided	none provided	none provided
90	ProPlus/Cofiler	1.18E+15	2.13E+14	3.09E+15
34	ProPlus/Cofiler	2.40E+11	7.00E+09	9.80E+10
46	PP16	5.60E+09	3.80E+11	none provided
33	ProPlus/Cofiler	2.94E+08	1.12E+08	1.74E+09
6	ProPlus/Cofiler	40,000,000	3,500,000	280,000,000
9	ProPlus/Cofiler	1.14E+07	1.97E+07	1.54E+08
61	Identifiler	1.50E+06	260,000	2.40E+07
79	ProPlus/Cofiler	930,000	47,900	1,350,000
16	ProPlus/Cofiler	434,600	31,710	399,100

Which loci are included in each calculation?

Some Differences in Reporting Statistics

		Case1		
LabID	Kits Used	Caucasians	African Americans	Hispanics
90	ProPlus/Cofiler	1.18E+15	2.13E+14	3.09E+15
34	ProPlus/Cofiler	2.40E+11	7.00E+09	9.80E+10
33	ProPlus/Cofiler	2.94E+08	1.12E+08	1.74E+09
6	ProPlus/Cofiler	40,000,000	3,500,000	280,000,000
9	ProPlus/Cofiler	4.14E+07	1.97E+07	1.54E+08
79	ProPlus/Cofiler	930,000	47,900	1,350,000
16	ProPlus/Cofiler	434,600	31,710	399,100

~10 orders of magnitude difference (10^5 to 10^{15}) based on which alleles were deduced and reported

Remember that these labs are interpreting the same MIX05 electropherograms

Further Examination of These 7 Labs

		Case 1	ASCLD-LAB accredited?	Solved loci listed?
LabID	Kits Used	Caucasians		
90	ProPlus/Cofiler	1.18E+15	Yes	Yes
34	ProPlus/Cofiler	2.40E+11	Yes	Yes
33	ProPlus/Cofiler	2.94E+08	Yes	No
6	ProPlus/Cofiler	40,000,000	Yes	Yes
9	ProPlus/Cofiler	4.14E+07	No	No (CPE)
79	ProPlus/Cofiler	930,000	Yes	Yes
16	ProPlus/Cofiler	434,600	Yes	No

Possible Reasons for Variability in Reported Statistics:

- Different types of calculations (CPE vs RMP)
- Different loci included in calculations (due to different thresholds used)
- Different allele frequency population databases (most use PopStats)
- Use of victim (e.g., major component in Case 1) profile stats

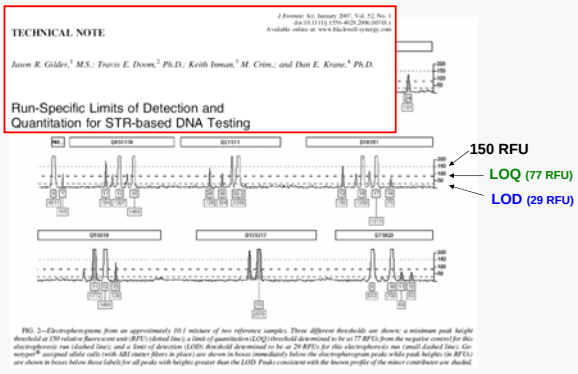
Different Stats Used

Combined Probability of Exclusion

Random Match Probability on Deduced Profiles

- **Lab 9** (4.14×10^7) used 1/CPI
- **Lab 6** (4.0×10^7) used selected loci and summed all possible genotypes for loci not completely deduced
- **Lab 90** (1.18×10^{15}) used theta value of 0.03 and deduced alleles at all 13 loci (***correctly deduced all perpetrator alleles***)

Different Thresholds of Detection Influence Allele Calls



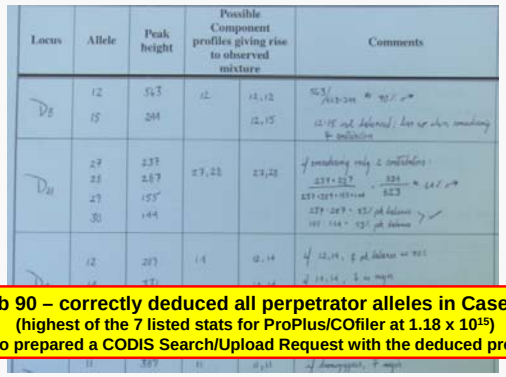
Gilder, J.R., Doom, T.E., Inman, K., Krane, D.E. (2007) Run-specific limits of detection and quantitation for STR-based DNA testing. *J. Forensic Sci.* 52(1): 97-101.

Different Detection Thresholds Used

		Case 1	
LabID	Kits Used	Caucasians	
90	ProPlus/Cofiler	1.18E+15	**75 RFUs**; all 13 STRs; **all results correct**
34	ProPlus/Cofiler	2.40E+11	**Not stated**; 8 STRs, 2 partial, 3 INC
33	ProPlus/Cofiler	2.94E+08	**75 RFUs**; no deduced alleles reported
6	ProPlus/Cofiler	40,000,000	**Not provided**; 3 STRs, 6 partial, 4 INC
9	ProPlus/Cofiler	4.14E+07	**100 RFUs**; no deduced alleles reported
79	ProPlus/Cofiler	930,000	**150 RFUs**; 2 STR, 5 partial, 6 INC
16	ProPlus/Cofiler	434,600	**Not stated**; no deduced alleles reported

- **Lab 90** has **specific, detailed mixture interpretation guidelines** with worked examples and a fabulous flowchart
- **Lab 16** has **vague guidelines** that begin with "mixture interpretation is not always straightforward. Analysts must depend on their knowledge and experience…"

Manually Solving Mixture Component Profiles



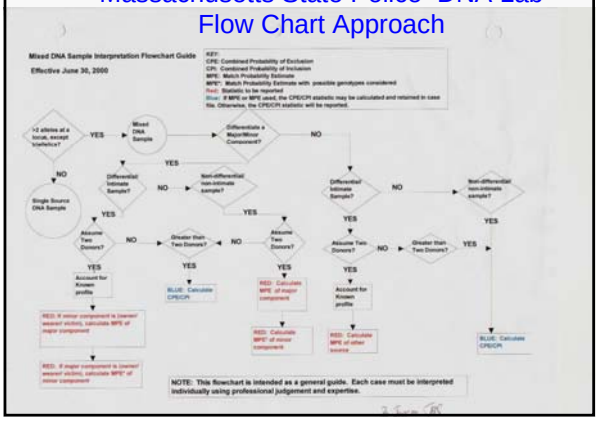
Lab 90 – correctly deduced all perpetrator alleles in Case #1
(highest of the 7 listed stats for ProPlus/COfiler at 1.18 x 10^{15})
Also prepared a CODIS Search/Upload Request with the deduced profile

A Model Report of Analysis…

- "The Profiler Plus and COfiler sample files were evaluated by **four different analysts**, using both NT and MAC analysis platforms. **The analysts checked for concordance, and a single conclusion for each mock case has been issued**."
- They detailed all assumptions made outside the course of routine casework:
  - Assumed intimate samples
  - That a comparison of deduced "foreign" alleles had been made with the perpetrator's known standard in order to calculate the significance of the inclusion with the evidentiary profile
- For Case #4: "A **Combined Probability of Inclusion was calculated** and reported for only those loci where all the alleles were above threshold [75 RFUs]. However, a minor profile(s) could not be deduced from this sample. **Please note that our laboratory may employ strategies to gain more information from the sample, such as a 10 second injection of the CE and Y-STR analysis**.

Lab 90

Massachusetts State Police DNA Lab Flow Chart Approach



Quotes from One Lab's MIX05 Report

- Case 1: STR typing results from the Evidence sample indicate a DNA mixture profile. The victim cannot be excluded as a possible donor of the genetic material in the Evidence sample. No statistics will be generated at this time.
- The Evidence samples would have to be rerun in order to verify any alleles called in the final profiles. This is true for any mixed sample profiles as per our laboratory guidelines.
- **Our laboratory does not "pull out" any profile from a mixture for interpretation or statistical purposes.** The exception to this is for CODIS profiles where the alleles that can be unambiguously attributed to the victim are removed.
- **We currently do not calculate and report statistics on mixture samples.**

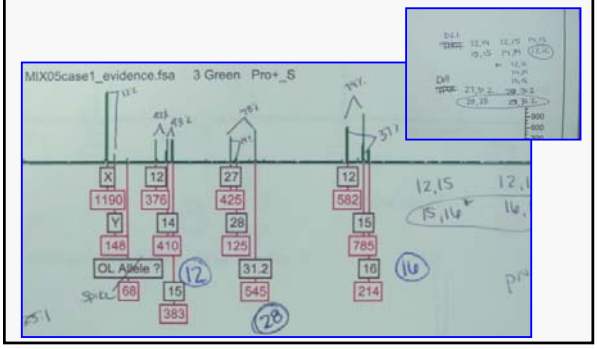
Lab 88

Examples of MIX05 Report Formats

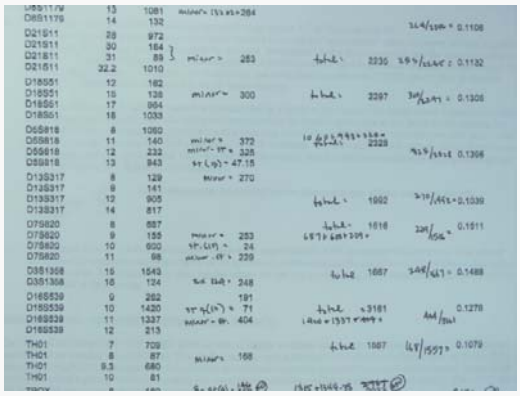
All examples with Case #1

(~3:1 mixture with female victim as the major component – and victim profile is provided)

Manual Solving of MIX05 Peak Ratios and Possible Mixture Combinations



Another MIX05 Participant Manually Solving a Mixture



Semi-Automated Locus-by-Locus Interpretation Performed by One MIX05 Participant

D21S11

Peak	A	B	C	D
Allele	28	30	31	32.2
RFU	988	167	92	1025

Known type: K1 30, K2 31

Possible combinations					Thresholds 70%	Thresholds 60%	Htrzyg. Pair 1	Htrzyg. Pair 2	Mixture ratio(1/2)	
28	30	AND	31	32.2	N	N	16.90%	8.98%		
28	31	AND	30	32.2	N	N	9.31%	16.29%		
28	32.2	AND	30	31	N	N	96.39%	55.09%		Best fit

D18S51

Peak	A	B	C	D
Allele	12	15	17	18
RFU	163	139	972	1047

Known type: K1 12, K2 15

Possible combinations					Thresholds 70%	Thresholds 60%	Htrzyg. Pair 1	Htrzyg. Pair 2	Mixture ratio(1/2)	
12	15	AND	17	18	Y	Y	85.28%	92.84%	0.15	Known present
12	17	AND	15	18	N	N	16.77%	13.28%		
12	18	AND	15	17	N	N	15.57%	14.30%		

Excel spreadsheet used to examine possible component combinations

Different Reporting Formats for MIX05 Data

Different Reporting Formats for MIX05 Data

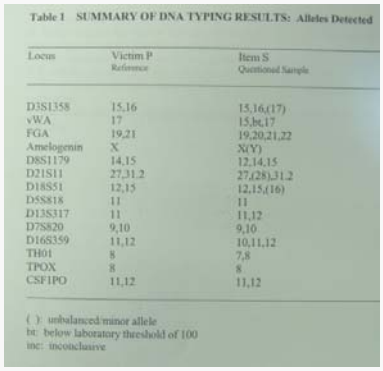


Table 1 SUMMARY OF DNA TYPING RESULTS: Alleles Detected

Locus	Victim P Reference	Item S Questioned Sample
D3S1358	15,16	15,16,(17)
vWA	17	15,bt,17
FGA	19,21	19,20,21,22
Amelogenin	X	X(Y)
D8S1179	14,15	12,14,15
D21S11	27,31.2	27,(28),31.2
D18S51	12,15	12,15,(16)
D5S818	11	11
D13S317	11	11,12
D7S820	9,10	9,10
D16S359	11,12	10,11,12
TH01	8	7,8
TPOX	8	8
CSF1PO	11,12	11,12

( ): unbalanced minor allele
bt: below laboratory threshold of 100
inc: inconclusive

No attempt to deduce perpetrator alleles (foreign profile)

Different Reporting Formats for MIX05 Data

Profile that would be put into CODIS

LOCI	CODIS ENTRY * obligate allele	OTHER ALLELE'S IN SUSPECT'S POSSIBLE PROFILE
D3S1358	17	16,17
VWA	15*	15,17
FGA	20,22	20,22
D8S1179	12	12,12
D21S11	28*	28,31.2
D18S51	15*	15,16
D5S818	--	
D13S317	12	12,12
D7S820	--	10
D16S539	10,11*	10,11
THO1	7*	7,8 maybe
TPOX	8	8 maybe
CSF1PO	--	11,12 maybe

Kits – Profiler Plus and Cofiler
Ratio - 1:2 (perpetrator:victim)

Different Reporting Formats for MIX05 Data

	Items	
Locus	"S" Case 1 Evid.	"P" Case 1 Victim
D3S1358	15, 16, *	15, 16
D16S539	(10), 11, (12)	11, 12
AMEL	X, *	X
THO1	(7), 8	8
TPOX	8	8
CSF1PO	11, 12	11, 12
D7S820	9, 10	9, 10
vWA	(15), 17	17
FGA	19, 20, 21, 22	19, 21
D8S1179	12, 14, 15	14, 15
D21S11	27, 31.2, *	27, 31.2
D18S51	12, 15, (16)	12, 15,
D5S818	11	11
D13S317	11, 12	11

() indicates apparent minor peaks in a mixture.
"*" indicates peaks below the VFL threshold (150 rfus) for reporting.

Different Reporting Formats for MIX05 Data

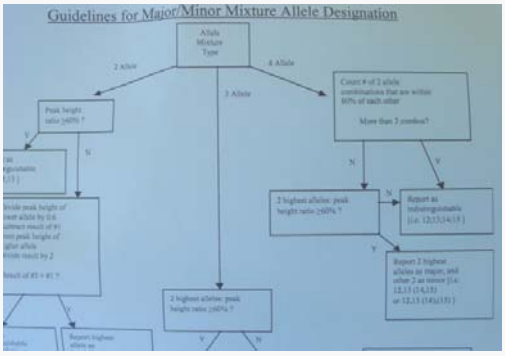
Case 1:

Item: description	D3S1358	VWA	FGA	AMEL	D8S1179	D21S11	D18S51	D5S818	D13S317	D7S820	D16S539	TH01	TPOX	CSF1PO
Pro+/CO_S: evid 1	15,16 (17)	15|17	19|20| 21|22	X,X (Y)	12|14|15	27,31.2 (28)	12,15 (16)	11,11	11|12	9|10	10|11 |12	7|8	8,8	11,12
Pro+/CO_P: victim 1 reference	15,16	17,17	19,21	X,X	14,15	27,31.2	12,15	11,11	11,11	9,10	11,12	8,8	8,8	11,12
Male interpreted from evidence 1	17	15,15 15,17	20,22	X,Y	12,12	28	16	11,11	12,12	Nd	10,11	7,7 7,8	Nd	Nd

Two allele values separated by a comma represent a genotype. Genotype calls assume biallelic donors with no null alleles.
() Indicates minor allele detected.
Single numbers and numbers separated by "|" represent an allele only designation rather than a genotype.
Interpreted profile assumes that the victim is present in the evidence mixture of two people. More than one genotype may be listed where a single genotype could not be conclusively determined. Nd=not determined due to level of results.

The community would benefit from more uniform reporting formats and mixture solving strategies…

Some Protocols Have Flow Charts to Help Make Decisions in Mixture Resolution



Value of the MIX05 Study

http://www.cstl.nist.gov/biotech/strbase/interlab/MIX05.htm

- Data sets exist with multiple mixture scenarios and a variety of STR kits that **can be used for training purposes**
- A wide variety of approaches to mixture interpretation have been applied on the **same data sets evaluated as part of a single study**
- **Interpretation guidelines from many laboratories are being compared to one another for the first time in an effort to determine challenges facing future efforts to develop "expert systems" for automated mixture interpretation**
- **We are exploring the challenges of supplying a common data set to a number of forensic laboratories** (e.g., if a standard reference data set was ever desired for evaluating expert systems)

Conclusions from the MIX05 Study (Opportunities for Improvement)

- It is worth taking a closer look at protocol differences between labs to see the impact on recovering information from mixture data
- Training should help bring greater consistency
- Expert systems (when they become available and are used) should help aid consistency in evaluating mixtures and help produce more uniform reporting formats

NIST Software Programs to Aid Mixture Work

Excel-based programs developed by David Duewer (NIST)

- **mixSTR** *(developed at request of Palm Beach Sheriff's Office)*
  - Does not interpret data (relies on user inputted alleles following STR data review)
  - Aids in the organization of STR mixture information
  - Considers only the presence/absence of alleles (no peak heights used)
- **Virtual MixtureMaker** *(developed to aid MIX05 sample selection)*
  - Creates mixture combinations through pairwise comparisons of input STR profiles
  - Returns information on the number of loci possessing 0,1,2,3,4,5, or 6 alleles in each 2-person mixture (also reports number of loci in each sample with 0,1,2, or 3 alleles)
  - Useful for selection of samples in mixture or validation studies with various degrees of overlapping alleles in combined STR profiles
  - Useful in checking for potentially related individuals in a population database

Programs can be downloaded from NIST STRBase web site:
http://www.cstl.nist.gov/div831/strbase/software.htm

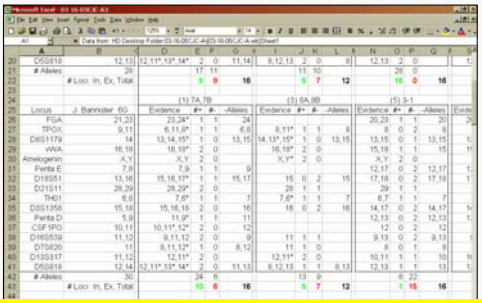
mixSTR Program

Comparisons are made between

- suspect and evidence (S/E) alleles,
- suspect and suspect (S/S) alleles **(to look for potential close relatives),**
- evidence and other evidence (E/E) sample(s) alleles **(to see how various evidentiary samples compare to one another),** and
- controls to evidence (C/E) and controls to suspect (C/S) alleles **(as a quality control contamination check).**

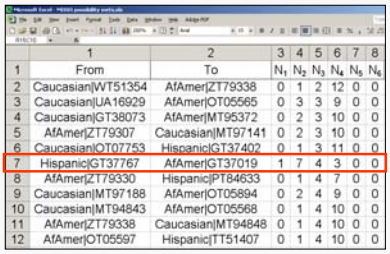
mixSTR S/E output

Data from Palm Beach County Sheriff's Office Case
Supplied by Catherine Cothran



Example of suspect to evidence (S/E) comparisons made in this case. Note that the suspect is 21,23 at FGA while the evidence contains 23,24* (* indicates that allele 24 is a minor component). Thus this suspect has allele 23 in common and is missing allele 24 in the evidence.

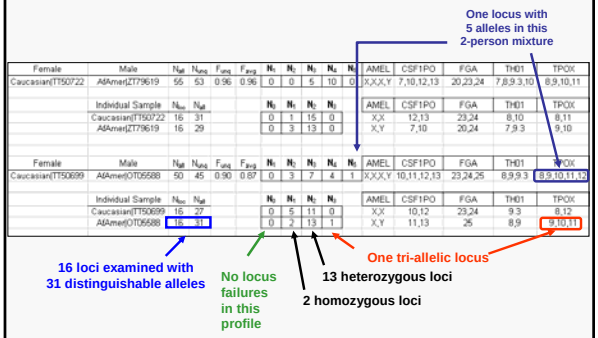
Virtual MixtureMaker Output



	1	2	3	4	5	6	7	8
1	From	To	N_1	N_2	N_3	N_4	N_5	N_6
2	Caucasian|WT51354	AfAmer|ZT79338	0	1	2	12	0	0
3	Caucasian|UA16929	AfAmer|OT05565	0	3	3	9	0	0
4	Caucasian|GT38073	AfAmer|MT95372	0	2	3	10	0	0
5	AfAmer|ZT79307	Caucasian|MT97141	0	2	3	10	0	0
6	Caucasian|OT07753	Hispanic|GT37402	0	1	3	11	0	0
7	Hispanic|GT37767	AfAmer|GT37019	1	7	4	3	0	0
8	AfAmer|ZT79330	Hispanic|PT84633	0	1	4	7	0	0
9	Caucasian|MT97188	AfAmer|OT05894	0	2	4	9	0	0
10	Caucasian|MT94843	AfAmer|OT05568	0	1	4	10	0	0
11	AfAmer|ZT79338	Caucasian|MT94848	0	1	4	10	0	0
12	AfAmer|OT05597	Hispanic|TT51407	0	1	4	10	0	0

When the STR profiles for these two individuals are combined to create a 2-person mixture, the mixture profile will contain 1 locus with a single allele, 7 loci with two alleles, 4 loci with three alleles, and 3 loci with four alleles (and no loci with 5 or 6 alleles, which is only possible if one or both samples possess tri-allelic patterns at the same STR locus).

Virtual MixtureMaker Output



Some Final Thoughts…

- It is of the highest importance in the art of detection to be able to recognize out of a number of facts, which are incidental and which vital. Otherwise your energy and attention must be dissipated instead of being concentrated (Sherlock Holmes, *The Reigate Puzzle*).
- "**Don't do mixture interpretation unless you have to**" (Peter Gill, Forensic Science Service, 1998).
- Mixture interpretation consumes a large part of DNA analysts' time – software tools that improve consistency in analysis will speed casework reporting and hopefully cases solved

Conclusion

ELSEVIER — Forensic Science International

DNA mixtures in forensic casework: a 4-year retrospective study

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Received 27 March 2003; accepted 1 April 2003

"Mixture interpretation theory is well established and used in forensic laboratories. Most mixtures detected in casework are satisfactorily solved. But from this revision we can conclude that the behaviour of each mixed sample can be different and multifactorial and occasionally its interpretation turns out to be complicated—sometimes paralleling the importance of the evidence in the resolution of the case. In some casework mixtures our experience has proved that theoretical assumptions from studies with laboratory samples, albeit very useful, can turn out to be impracticable. **We consider that more sharing of day to day forensic laboratory problems is needed to refine our technical procedures in the resolution of specially difficult evidence."**

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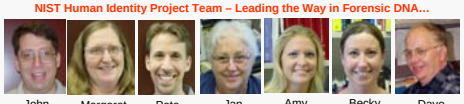
NIST Human Identity Project Team – Leading the Way in Forensic DNA…



John Butler, Margaret Kline, Pete Vallone, Jan Redman, Amy Decker, Becky Hill, Dave Duewer

Role in MIX05

- Margaret Kline (running study, sample prep, data review)
- John Butler (study design and data review)
- Becky Hill (GeneMapper*ID* data review)
- Jan Redman (Access database entry, shipping)
- Dave Duewer (*Virtual MixtureMaker* to aid sample selection; **mixSTR program**)
- Chris Tomsey & Frank Krist (FMBIO Mac data)
- Kermit Channel & Mary Robnett (FMBIO NT data)

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