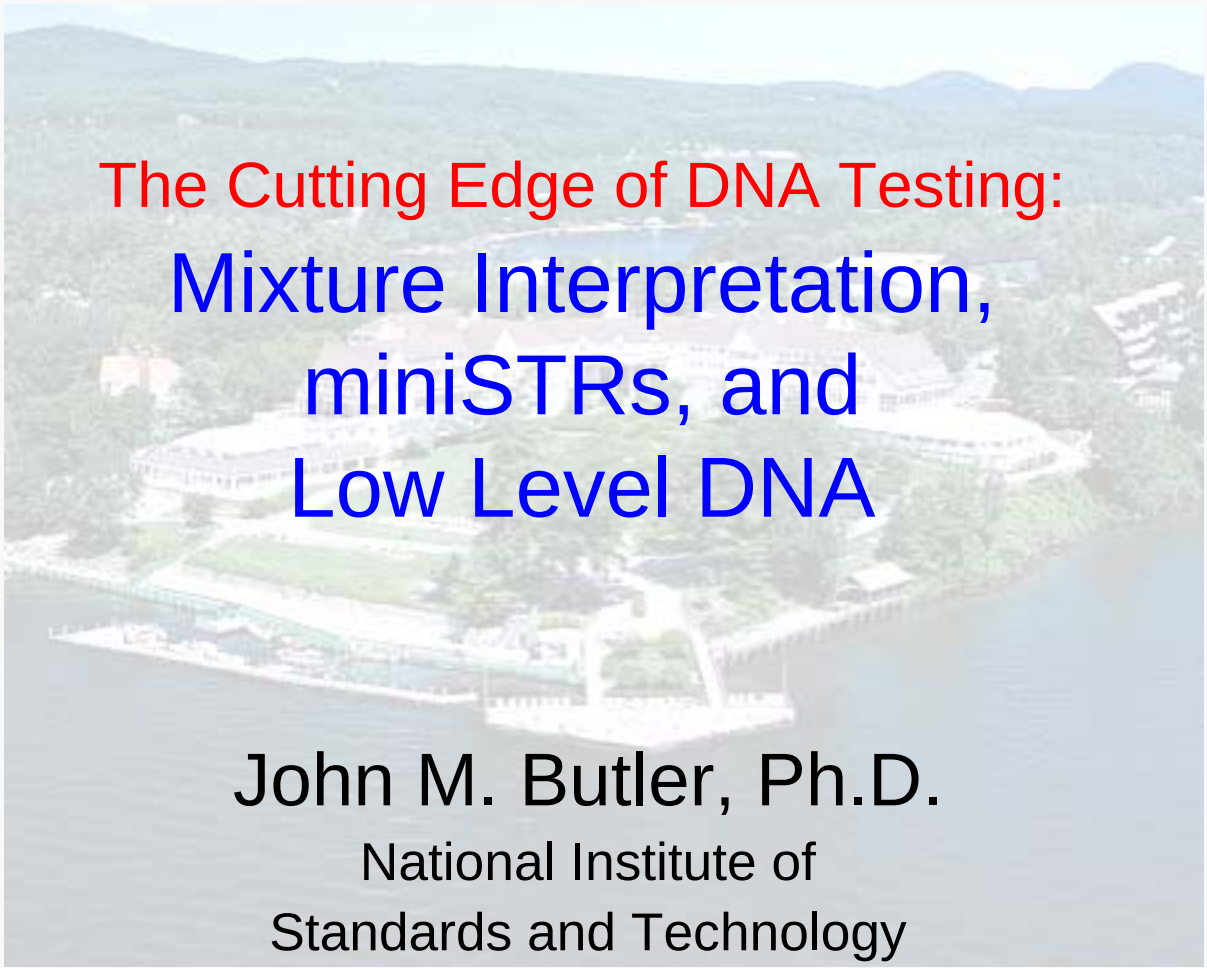
2007 Workshop

Northeastern Association of Forensic Scientists



The Cutting Edge of DNA Testing:

Mixture Interpretation, miniSTRs, and Low Level DNA

John M. Butler, Ph.D.
National Institute of Standards and Technology

November 2-3, 2007
Bolton Landing, NY

NIST

The Cutting Edge of DNA Testing:
Mixtures, miniSTRs, and Low Level DNA

Mixture Interpretation

NEAFS 2007 Workshop
Bolton Landing, NY
November 2-3, 2007

Dr. John M. Butler

Northeastern Association of Forensic Scientists

john.butler@nist.gov

qPCR Workshop

- AAFS (February 18, 2008)
  - **Human DNA Quantification Using Real-Time PCR Assays**
  - Peter Vallone (NIST)
  - Margaret Kline (NIST)
  - Eric Buel (Vermont)
  - Jan Nicklas (Vermont)
  - Marie Allen (Uppsala)
  - Mark Timken (CA DOJ)
  - David Foran (Michigan State)
  - Melanie Richard (CFS – Toronto)
  - Toni Diegoli (AFDIL)

Mixture Interpretation Workshop

- AAFS (February 19, 2008)
  - **DNA Mixture Interpretation:** Principles and Practice in Component Deconvolution and Statistical Analysis
  - John Butler (NIST)
  - Ann Gross (MN)
  - George Carmody (Carleton U.)
  - Gary Shutler (WA)
  - Joanne Sguelia (MA)
  - Angela Dolph (Marshall U./NIST)
  - Tom Overson (retired USACIL)

Outline

- Case numbers – how often are mixtures seen?
- German mixture classification categories
- **NIST MIX05 Study**
- **ISFG Recommendations on Mixtures**
- Available Computer Tools

CFS Toronto Case Summary Data

Case type	N = 276		# contributors: 1	2	3	4	>4
Sexual Assault	N = 152		42%	52%	7%	1%	--
High Volume	N = 56		69%	16%	16%	--	--
Major Crime	N = 68		59%	34%	7%	--	--

Single source | Mixtures

MN BCA Case Summary Data

Case type	N = 273		# contributors: 1	2	3	4	>4
Sexual Assault	N = 117		60%	37%	3%	--	--
High Volume	N = 82		70%	20%	9%	1%	--
Major Crime	N = 74		50%	39%	10%	1%	--

Single source | Mixtures

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…

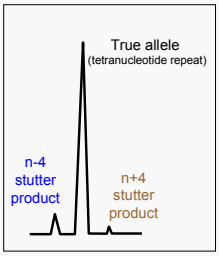
- **Type of sample (biological material if ID'd)**
- **Type of substrate**
- **Quantity amp'd**
- **Minimum # of contributors (1, 2, 3, 4, or >4)**
- **Predominant type (major profile) determined?**
- **Stats reported**
- **Comments**

We would love to have your lab mixture numbers…

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

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		N+4		N+4 Stutter % of
	allele	rfu	'allele'	rfu	
6.43%	19	4684	20	57	1.22%

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

Two Parts to Mixture Interpretation

- **Deduction of alleles present in the evidence** (compared to victim and suspect profiles)
- **Providing some kind of statistical answer** regarding the weight of the evidence
  - An ISFG DNA Commission (Peter Gill, Bruce Weir, Charles Brenner, etc.) has evaluated the statistical approaches to mixture interpretation and made recommendations

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

Mixture Classification Scheme

(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

SWGDAM Mixture Committee considering plan to reorder classifications and change designations to α (alpha), β (beta), and γ (gamma)

Rechtsmedizin 2006, 16 : 401 - 404


Rechtsmedizin 2006 · 16:401–404
DOI 10.1007/s00194-006-0411-1
Online publiziert: 16. November 2006
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See Handout

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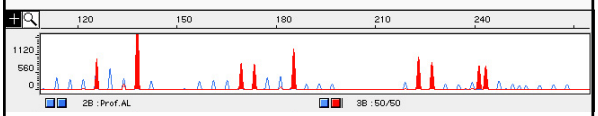
General recommendations of the stain commission on the interpretation of DNA results from mixed stains

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

Mixtures



- Mixed stain: more than two alleles per locus in at least two DNA systems
- Inference on the number of contributors:
  - up to 4 alleles: at least 2 contributors
  - up to 6 alleles: at least 3 contributors
  - more than 6 alleles: no meaningful interpretation possible

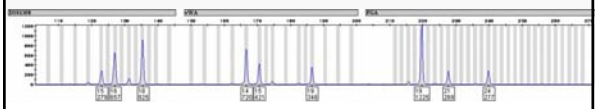
Slide from Peter Schneider (presented at EDNAP meeting in Krakow in April 2007)

Classification of mixtures

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

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

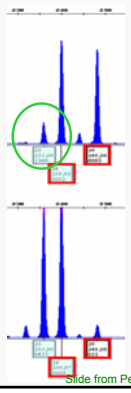
Stochastic phenomena



- May lead to allele and locus drop-out and drop-in effects
- Occur when using „low copy number" conditions
  - e,.g. with increased no. of PCR cycles,
  - BUT ALSO using standard conditions and DNA amounts < 200pg (e.g. as minor component in a mixture!)

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

Stutter effects



- The following criteria have to be considered in case of *stutter peaks*:
  - the relative *stutter* intensities within the alleles of a locus, as well as between loci of a multiplex amplification,
  - the possibility that a stain allele is in the position of a *stutter peak*.

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

Stutter effects

- In case of doubt a suspicious peak in the position of a *stutter* band has to be considered as a true allele and part of the DNA profile, and should be included into the biostatistical interpretation.

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

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)

NIST MIX05 Summary

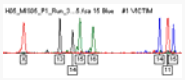
MIX05 Study Design and Purpose

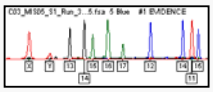
Interlab studies provide a "big picture" view of the community

- 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

MIX05 Case Scenarios

Based on Identifiler 15 STR loci

Genomic DNA samples with specific allele combinations ("evidence") were mixed in the following ratios:	#alleles N all	#alleles N unq	#loci with #alleles 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

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

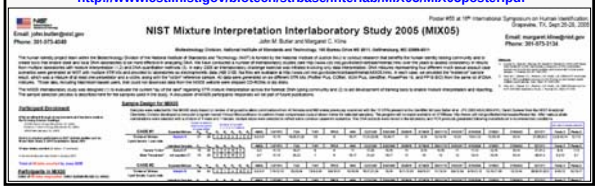
- **34 ProfilerPlus/COfiler**
- **10 PowerPlex 16**
- **7 PP16 BIO**
- **5 Identifiler**
- **2 SGM Plus**
- **1 All ABI kit data**
- **9 Various combinations**

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

LabID	Kits Used	Case 1 Caucasians	ASCLD-LAB accredited?	Solved loci listed?
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

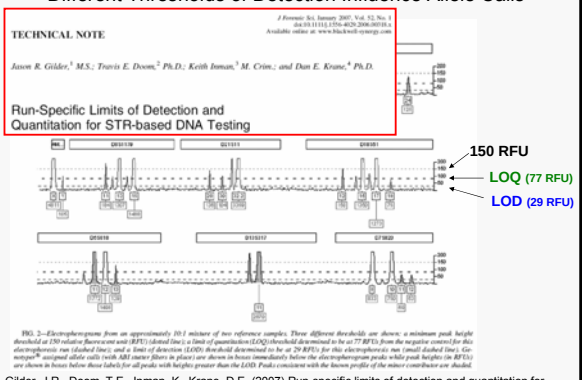
Different Stats Used

Combined Probability of Exclusion

Random Match Probability on Deduced Profiles

- **Lab 9** (4.14 x 10^7) used 1/CPI
- **Lab 6** (4.0 x 10^7) used selected loci and summed all possible genotypes for loci not completely deduced
- **Lab 90** (1.18 x 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

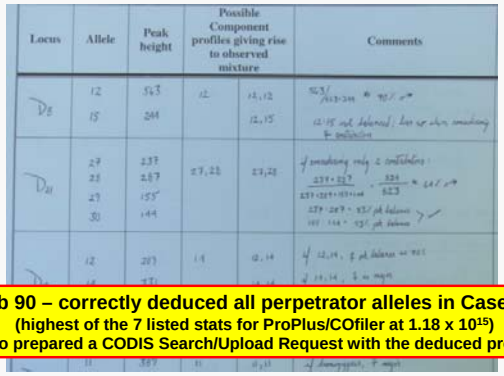
LabID	Kits Used	Case 1 Caucasians	
90	ProPlus/Cofiler	1.18E+15	**75 RFUs**; all 13 STRs; **all results correct**
34	ProPlus/Cofiler	2.40E+11	**Not stated**; 8 STRs, 2 partial, 3 INC
33	ProPlus/Cofiler	2.94E+08	**75 RFUs**; no deduced alleles reported
6	ProPlus/Cofiler	40,000,000	**Not provided**; 3 STRs, 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…"

Questions for Consideration

- Do you look at the evidence data first without considering the suspect's profile?
- Without a suspect, does your lab proceed with mixture interpretation?
- Do you have a decision point whereby you consider a mixture too complicated and do not try to solve it? If so, is the case declared inconclusive?
- What kind of training materials would benefit your lab in improving consistency in mixture interpretation?

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

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

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)

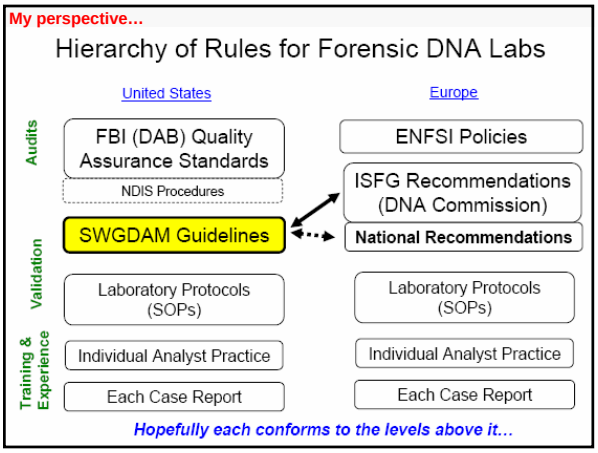
Conclusions from the MIX05 Study
(Opportunities for Improvement)

- 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

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

My perspective...



Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Forensic Science International
Forensic Science International 160 (2006) 90–101

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]

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Received 4 April 2006; accepted 10 April 2006
Available online 5 June 2006

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

ISFG (2006) Recommendations

- Recommendation 1: **The likelihood ratio is the preferred approach to mixture interpretation.** The RMNE (probability of exclusion) 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.

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

ISFG (2006) Recommendations

- 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.

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

ISFG (2006) Recommendations

- Recommendation 3: The methods to calculate likelihood ratios of mixtures (not considering peak area) described by Evett *et al.* (*J. Forensic Sci. Soc.* 1991;31:41-47) and Weir *et al.* (*J. Forensic Sci.* 1997;42:213-222) are recommended.

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

ISFG (2006) Recommendations

- 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.* (*Forensic Sci. Int.* 1998;91:55-70).

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

ISFG (2006) Recommendations

- 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 defense. The prosecution and defense both seek to maximize 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).

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

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

ISFG (2006) Recommendations

- Recommendation 7: If drop-out of an allele is required to explain the evidence under Hp: (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 Hp 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

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

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

Available Computer Tools to Aid Mixture Interpretation

Software Programs (~~Expert Systems~~) for Mixture Deconvolution

These programs do not supply stats (only attempt to deduce mixture components)

- Linear Mixture Analysis (LMA)
  - **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)
  - Described by T. Wang (University of Tennessee) at Oct 2002 Promega meeting
  - Available for use at **https://lsd.lit.net/**
- 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

The DNA Mixture Conundrum: Exploring the Applicability of Mixture Deconvolution Tools

Angela M. Dolph

National Institute of Standards and Technology

August 15, 2007

PENDULUM (i-STReam)

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]

[a]The Forensic Science Service, Trident Court, Solihull Parkway, Birmingham Business Park, Solihull B37 7YN, UK
[b]Department of Statistics, University of Waikato, Private Bag 3105, Hamilton, New Zealand
[c]The Forensic Science Service, Sanbeck Way, Audby Lane, Wetherby, West Yorkshire, LS227DN, UK
[d]ESR, Private Bag 92021, Auckland, New Zealand

- FSS-i³
- Primary purpose is to use the heterozygote balance and mixture proportion guidelines to eliminate unreasonable genotype combinations
- Uses least squares method to estimate the mixture proportion of two contributors

Bill M, et al. PENDULUM: a guideline-based approach to the interpretation of STR mixtures. *Forensic Sci Int.* 2005:181-189.

i-STReam

- Heterozygote balance:

$$Hb_3 = \frac{\phi_1}{\phi_2}$$

Choice of peaks 1 and 2 is arbitrary

0.6 < Hb < 1.66

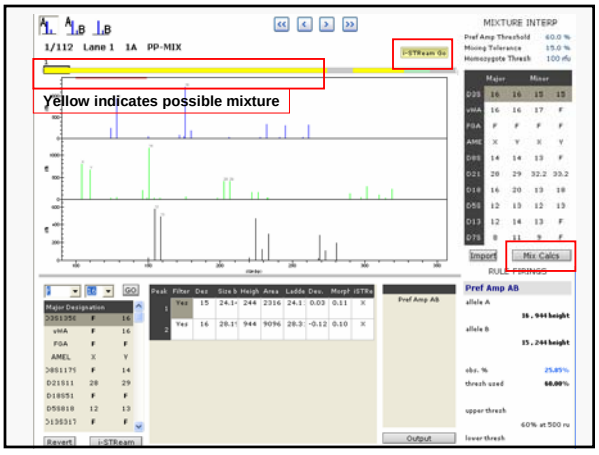
- Mixture proportion (Mx):
  - The ratio of the major and minor contributors
  - Vary Mx until minimum residual between observed peak data and calculated peak data is found
  - Mass proportion, x, in LSD
  - Variance between loci ± 0.35

Bill M, et al. PENDULUM: a guideline-based approach to the interpretation of STR mixtures. *Forensic Sci Int.* 2005:181-189.

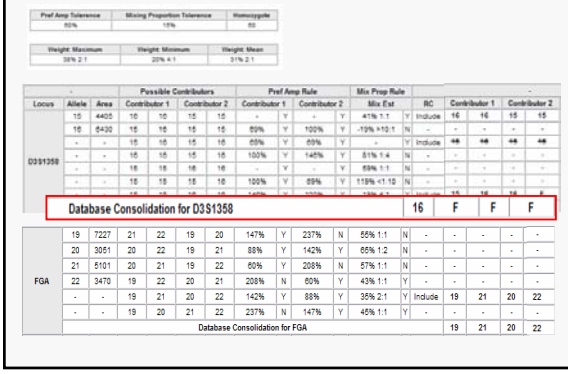
i-STReam

- Step 1: List all of the possible genotype combinations without considering peak data
- Step 2: Average Mx calculated for whole profile
- Step 3: Genotypes evaluated on per locus basis with respect to Hb
  - 0.6 < Hb < 1.66 are retained
- Step 4: Mx calculated independently for all loci
  - Must be within ± 0.35 of profile average
- Step 5: Only those genotypes that pass the Hb and Mx are listed as possible genotypes
  - If multiple alleles possible, F designations
- Step 6: Analyst reviews genotypes

Bill M, et al. PENDULUM: a guideline-based approach to the interpretation of STR mixtures. *Forensic Sci Int.* 2005:181-189.



i-STReam Summary Sheet



i-STRess vs. i-STReam

Major Designation		
D3S1358	F	16
vWA	F	16
FGA	F	F
AMEL	X	Y
D8S1179	F	14
D21S11	28	29
D18S51	F	F
D5S818	12	13
D13S317	F	F
D7S820	F	F

Revert i-STReam

Major contributor profile **before** i-STReam calculations

Major Designation		
D3S1358	16	16
vWA	16	16
FGA	F	F
AMEL	X	Y
D8S1179	14	14
D21S11	28	29
D18S51	16	20
D5S818	12	13
D13S317	12	14
D7S820	8	11

Revert i-STReam

Major contributor profile **after** i-STReam calculations and imported into i-STRess

Least-Square Deconvolution

J Forensic Sci, November 2006, Vol. 51, No. 6
doi:10.1111/j.1556-4029.2006.00268.x
Available online at: www.blackwell-synergy.com

Tsewei Wang,[1] Ph.D.; Ning Xue,[1] M.Sc.; and J. Douglas Birdwell,[2] Ph.D.

Least-Square Deconvolution: A Framework for Interpreting Short Tandem Repeat Mixtures*

- Before LSD calculation, proper allele calls required
  - GeneMapper ID (v3.2)
  - remove artifacts
- No peak saturation or high degradation
  - Mass proportions are effected
- Ax = b

A = profile matrix
x = mass proportion vector
b = allele peak height vector

Wang T, Xue N, Birdwell J. Least-square deconvolution: a framework for interpreting short tandem repeat mixtures. *J Forensic Sci.* 2006:51(6):1284-1297.

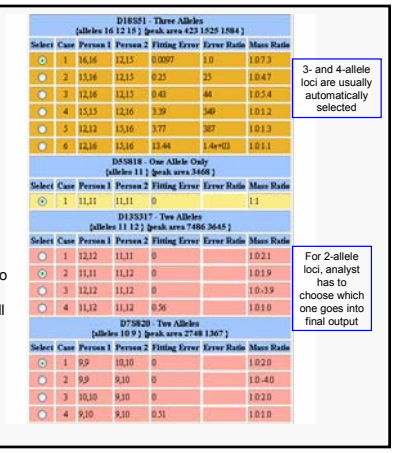
Least-Square Deconvolution

- Considered a filter
  - Gives most likely genotype combinations
- Only inputs are allele designation and peak height/area
- Looks at each locus separately
- Calculates best-fit mass proportions and error residuals for all possible genotype combinations
- LDS results reviewed by analyst who applies heuristic guidelines to create final profiles
- This study utilized Web-LSD available at https://lsd.lit.net/
  - Also available in DNA_DataAnalysis mixture interpretation tool

Wang T, Xue N, Birdwell J. Least-square deconvolution: a framework for interpreting short tandem repeat mixtures. *J Forensic Sci.* 2006:51(6):1284-1297.

LSD Output

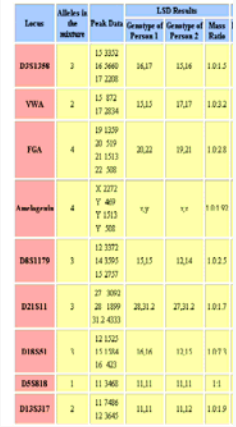
- 3- and 4-allele loci:
  - Small fitting error
  - Mass ratio constant across all loci
    - Error ± 0.35
    - Subjective
- 2-allele loci:
  - Math begins to fail
  - Mass proportion ratio comparable
  - May need to keep all possibilities
  - Check reference



3- and 4-allele loci are usually automatically selected

For 2-allele loci, analyst has to choose which one goes into final output

LSD Final Output

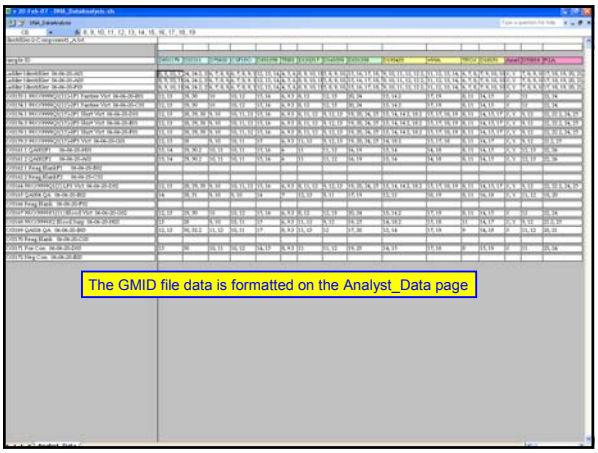


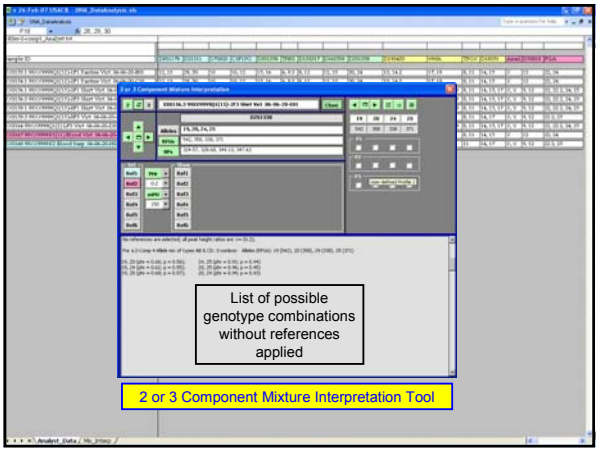
Person 1 = minor contributor

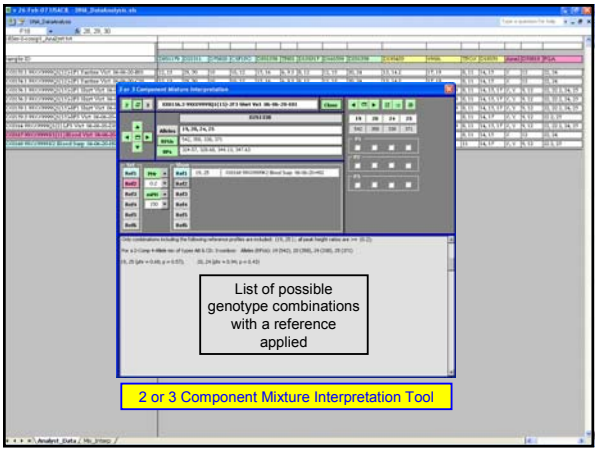
Person 2 = major contributor

DNA_DataAnalysis

- U.S. Army Criminal Investigation Laboratory (USACIL)
- Developed by Tom Overson
- Mouse-driven program that was written in Visual Basic and runs in Microsoft Excel 2003
- NOT an expert system – DNA data interpretation tool to aid analysts
  - Check controls, ladders
  - Matching
  - Statistics
    - RMNE, LR, PI
  - Mixture Interpretation
- Requires proper allele calls and output table from GMID

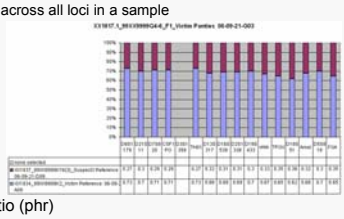






DNA_DataAnalysis: Mixture Calculations

- Mixture proportion (p)
  - Fairly stable across all loci in a sample



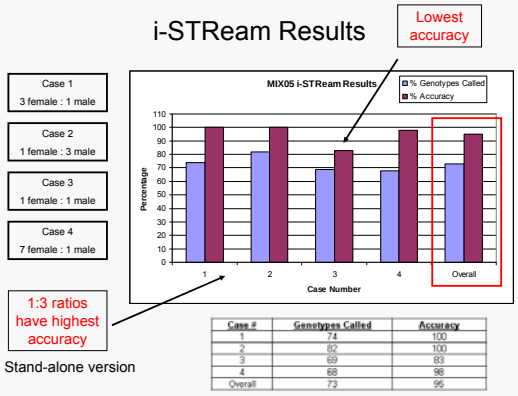
- Peak height ratio (phr)
  - Peak height ratios for a locus want to be one

Experiment 1 – MIX05 Data Mixture Deconvolution

Materials and Methods

- Several STR Kits:
  - SGM+, Profiler Plus, Identifiler, COfiler, Powerplex 16
- 3130xl
- Data already collected and profiles in GMID v3.2
- Mixture deconvolution tools:
  - i-STReam
  - LSD

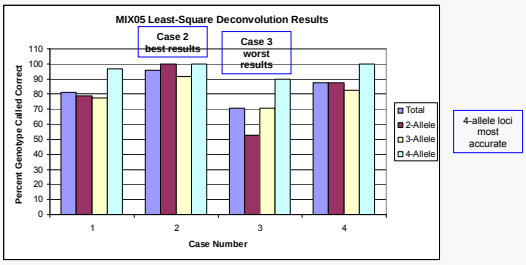
i-STReam Results



Stand-alone version

Case #	Genotypes Called	Accuracy
1	74	100
2	82	100
3	69	83
4	68	98
Overall	73	95

LSD Results



Case #	% Genotype Correct	% 2 Allele Correct	% 3 Allele Correct	% 4 Allele Correct
1	81	79	77	97
2	96	100	92	100
3	70	53	71	90
4	88	88	82	100

Experiment 2 – Replicates and Ratios
Mixture Deconvolution

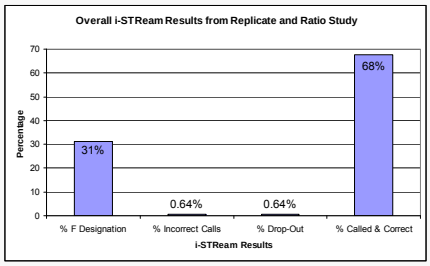
Materials and Methods

- Identifiler, COfiler, Profiler Plus
- 1:2, 1:3, 1:5, and 1:8 mixture ratios
- 6-7 amplification replicates
  - PCR variation
- How does i-STReam handle this variation?
  - Different results for the same mixture?
  - Incorrect calls?

Some i-STReam Observations…

- GeneMapper ID minus A and stutter filters set at zero to allow all alleles into FSS-i^3
  - Some minor alleles filtered out as stutter and not called
- Some incorrect calls
  - Incorrect calls can be explained by variation in peak height ratios
  - 26 / 4080 alleles
- Very conservative
  - F designations allow the program to not make a call

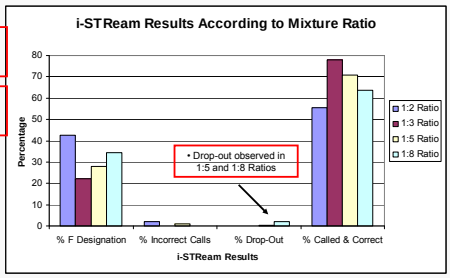
Total i-STReam Results



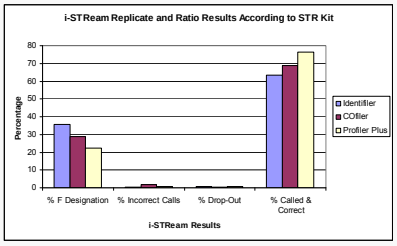
Total # Alleles	% 4-Allele	% 3-Aallele	% 2-Allele	% 1-Allele
4080	25	33	38	4

i-STReam Results According to Mixture Ratio

- 1:2 ratio worst results
  56% Called and Correct Alleles
- 1:3 ratio best results
  78% Called and Correct Alleles



i-STReam Results According to STR Kit



Identifiler	Total # Alleles	% 4-Allele	% 3-Allele	% 2-Allele	% 1-Allele
	2352	29	33	33	5

Profiler Plus	Total # Alleles	% 4-Allele	% 3-Allele	% 2-Allele	% 1-Allele
	1000	35	25	35	5

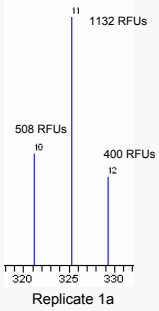
COfiler	Total # Alleles	% 4-Allele	% 3-Allele	% 2-Allele	% 1-Allele
	728	0	43	57	0

PCR Variation and Incorrect Calls

- 26 incorrect calls out of 4080 alleles
- Plotted peak height ratios for replicates
  - According to kit and ratio

Identifiler 1 : 2 Ratio

Genotypes:
Major: 11,11
Minor: 10,12



Locus CSF

Most Common i-STReam Call:
Major: F,F
Minor: F,F

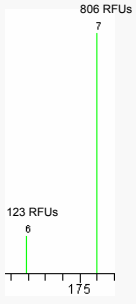
1a	1.3 : 2.8 : 1.0	
2a	1.5 : 3.1 : 1.0	
3a	1.4 : 3.4 : 1.0	
4a	1.7 : 3.6 : 1.0	10,11 11,12
5a	1.1 : 2.4 : 1.0	
6a	1.1 : 2.5 : 1.0	
7a	1.8 : 4.1 : 1.0	10,11 11,12

Incorrect call once 11 passes certain height threshold

COfiler 1 : 5 Ratio

Locus TH01

Genotypes:
Major: 7,7
Minor: 6,7

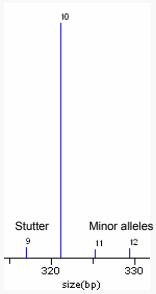


1b	1.0 : 5.2	
2b	1.0 : 3.6	7,7 6,6
3b	1.0 : 6.6	
4b	1.0 : 4.9	
5b	1.0 : 11.3	
6b	1.0 : 6.8	
7b	1.0 : 6.1	

Average Ratio:
1.0 : 6.4 ± 2.4

Most Common i-STReam Call:
Major: 7,7
Minor: 6,F

Stutter Problems



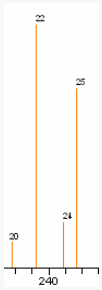
Identifiler Locus CSF

- Stutter introduces problems, lower minor contributor ratios (1:8)
  - Stutter peaks may be higher than allele peaks
  - Peak Heights:
    - 9 = 252 RFUs
    - 11 = 201 RFUs
    - 12 = 225 RFUs
- International Society of Forensic Genetics (ISFG) Recommendation:
  - When minor alleles same size as stutter, they are indistinguishable
  - Must include both in LR

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

Stutter Problems

- Stutter introduces problems, lower ratios (1:8)
  - If minor allele in stutter position, may cause heterozygote peak imbalance and genotype will not pass the pref amp rule (Hb)
  - F Designations



Peak Heights of Minor Alleles:

20 = 317 RFUs

24 = 589 RFUs

60% of allele 24 is 353 RFUs

Identifiler 1:8 Locus FGA

Conclusions

- LSD about 84% accuracy
- i-STReam above 95% accuracy
- Amplification variability can lead to different and/or incorrect calls
- **Only certain mixture ratios are solvable**
  - Window of opportunity around 1:3 - 1:8
  - Influence mathematics
- Optimization of program parameters very important
  - Filter settings, threshold settings, etc.

Thank you for your attention…

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

Questions?

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

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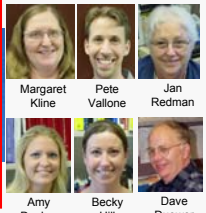

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Funding from the **National Institute of Justice (NIJ)** through NIST Office of Law Enforcement Standards

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The Cutting Edge of DNA Testing:
Mixtures, miniSTRs, and Low Level DNA

miniSTRs

NEAFS 2007 Workshop
Bolton Landing, NY
November 2-3, 2007

Dr. John M. Butler

Northeastern Association of Forensic Scientists

john.butler@nist.gov

Outline for This Section

- NIST projects funded by NIJ
- Background on miniSTRs
- MiniFiler kit and concordance studies performed
- New non-CODIS (NC) miniSTR loci

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

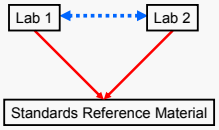
Standard Reference Materials

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

Traceable standards to ensure accurate measurements in our nation's crime laboratories

SRM 2391b – CODIS STRs
SRM 2392-I – mtDNA
SRM 2395 – Y-STRs
SRM 2372 – DNA quantitation

Helps meet DAB Std. 9.5 and ISO 17025



Calibration with SRMs enables confidence in comparisons of results between laboratories

Information Resource

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

Includes information on:

- Core STR loci
- Validation
- STR reference list
- NIST publications
- miniSTRs
- Forensic SNPs
- Variant STR alleles
- Population data resources
- Addresses of scientists

Provides up-to-date information and has been used in court cases to support application of DNA technology

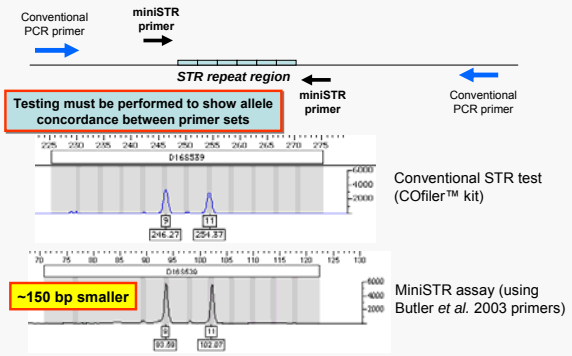
Recent STRBase Updates…

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

Technology: Research Programs

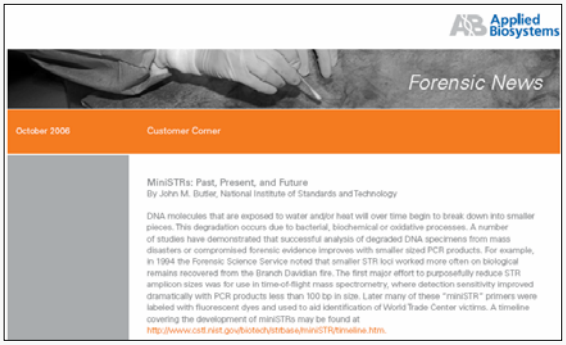
- miniSTRs
- Y-chromosome STRs
- mtDNA
- SNPs
- qPCR for DNA quantitation
- DNA stability studies
- Variant allele characterization and sequencing
- Software tools
- Expert System review
- Assay development with collaborators

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

Recent Publications on miniSTRs

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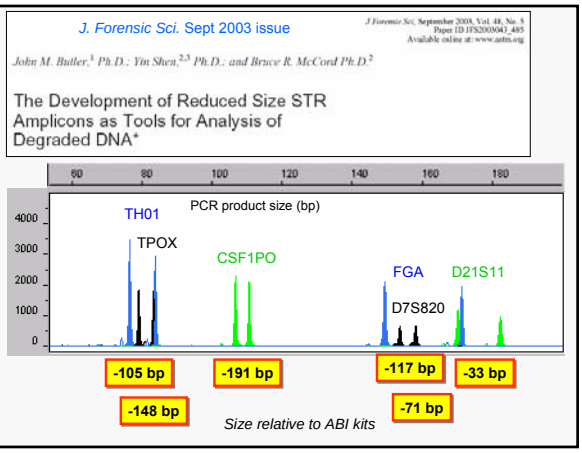
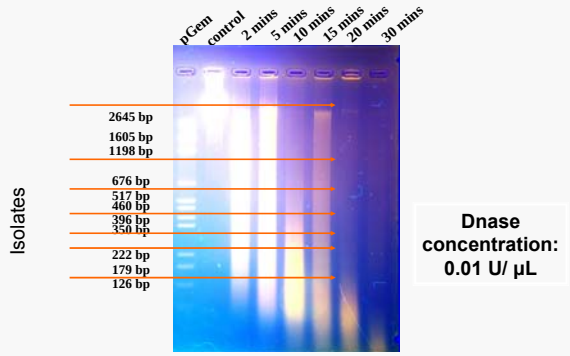
http://www.cstl.nist.gov/biotech/strbase/miniSTR.htm
http://www.cstl.nist.gov/biotech/strbase/miniSTR/timeline.htm

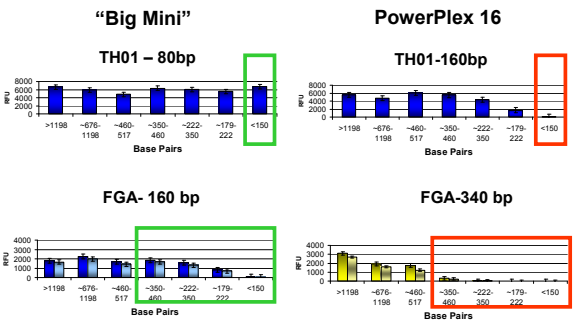


DNA Degraded With DNase I



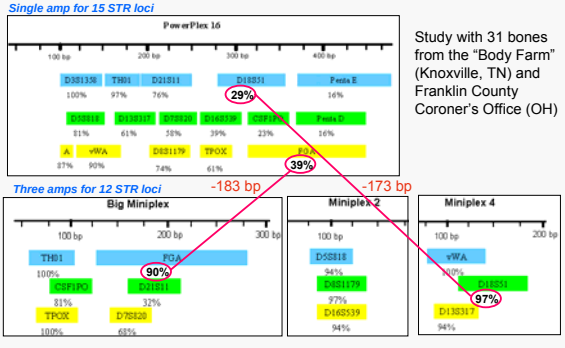
Chung, D.T., Drabek, J., Opel, K.L., Butler, J.M., McCord, B.R. (2004) A study on the effects of degradation and template concentration on the efficiency of the STR miniplex primer sets. *J. Forensic Sci.* 49(4): 733-740.

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.

Recent Work with Telogen Hair Shafts

Electrophoresis 2007, 28, 2835–2842

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Received October 13, 2006
Revised January 12, 2007
Accepted January 24, 2007

Research Article

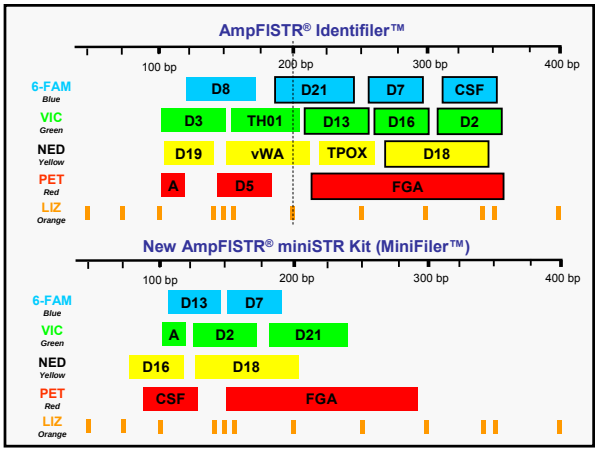
Improved STR typing of telogen hair root and hair shaft DNA

Today the STR typing of telogen hair and hair shafts is regarded as a challenge. The small DNA quantity in the hair is highly degraded. Another problem are PCR inhibitors in the hair. In particular hair pigments, the melanins, are known to inhibit PCR. Hairs are exposed to sunlight and partly to chemical oxidation processes, which make them even more difficult to analyze. To increase the chances of a correct typing of hair, the small amount of DNA must be successfully isolated and the inhibitors have to be removed or neutralized. Furthermore, miniSTR typing improves the analysis of stains with degraded DNA like it is the case with hair. We introduce a nonorganic extraction method and in addition a miniSTR concept which is promising in typing stains with little and degraded DNA, especially hairs. The miniSTR concept including five database STRs (SE33, VWA, TH01, FGA, D351358) and the gender typing system Amelogenin was optimized for the amplification of hair DNA. Compared to commercial STR kits, this approach resulted in considerably higher success rates.

Table 3. Success rates of the miniSTR typing results subdivided into categories

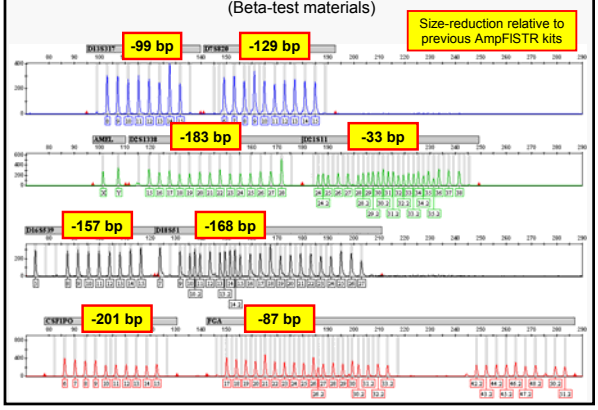
Results/probes	Telogen hair root		Hair shaft	
	Undyed (%)	Dyed (%)	Undyed (%)	Dyed (%)
Complete profile	63.0	22.1	18.6	2.7
At least two complete systems	31.9	48.4	57.1	20.6
Less than two complete systems	4.3	29.5	24.2	76.7

miniSTRs provide a higher success rate…



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

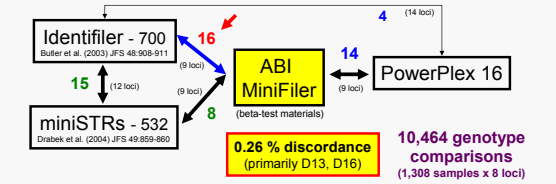
1,308 samples **Allele concordance = 10,437/10,464 = 99.7%**

- **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**

Concordance Conducted at NIST

27 Discordant Calls

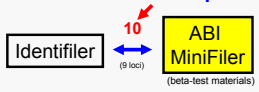
656 NIST U.S. population samples



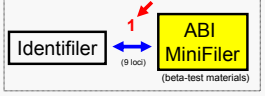
0.26 % discordance (primarily D13, D16)

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

481 father-son samples



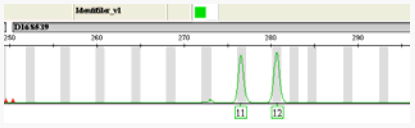
171 ABI samples



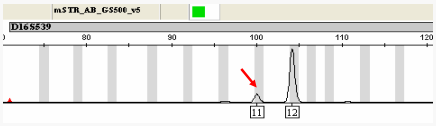
Concordance Studies Reveal Potential Primer Binding Site Mutations with Different Primer Sets

D16S539

Identifiler



miniSTR Kit (beta-test)

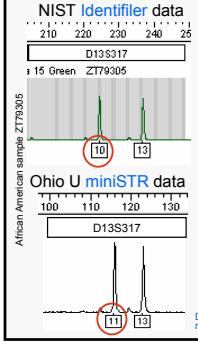


Appears to be an allele 11 dropout/reduction due to primer binding site mutation

D13S317 Flanking Region Deletion

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

NIST Identifiler data



Ohio U miniSTR data

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

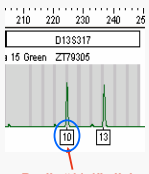
Drabek, J., Chung, D.T., Butler, J.M., McCord, B.R. (2004) Concordance study between miniplex STR assays and a commercial STR typing kit, *J. Forensic Sci.* 49(4): 859-860.

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

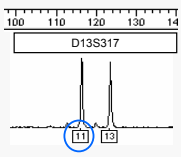


Really "11-1" allele

10,13

Reverse primer is outside deletion

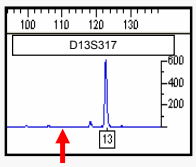
Ohio U miniSTR data



11,13

Reverse primer is inside deletion

AB miniSTR beta-test

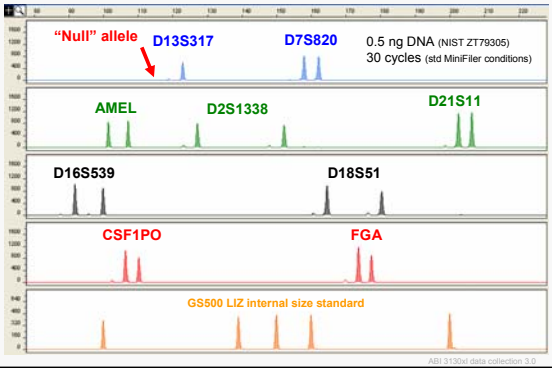


"Null" allele

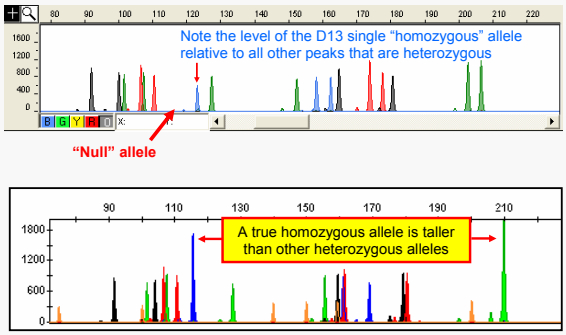
13,13

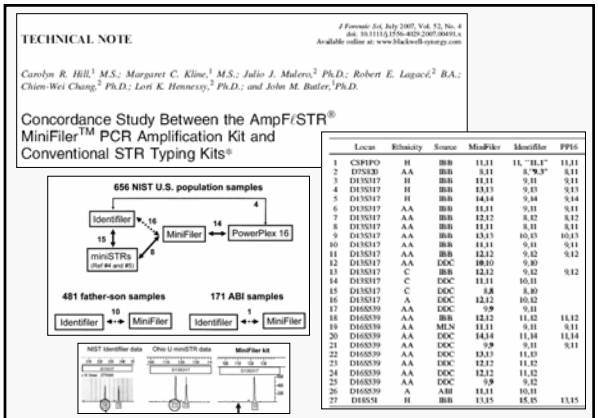
Reverse primer is on top of deletion

Full MiniFiler Profile for NIST Sample with D13S317 Allele Dropout



Note the Relative D13 Peak Heights
(Suggests Allele Dropout)



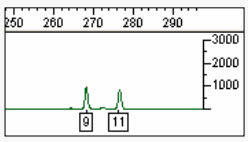


Hill, C.R., Kline, M.C., Mulero, J.J., Lagace, R.E., Chang, C.-W., Hennessy, L.K., Butler, J.M. (2007) Concordance study between the AmpFISTR MiniFiler PCR Amplification Kit and conventional STR typing kits. *J. Forensic Sci*. 52(4): 870-873.

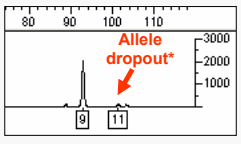
SRM 2391b Genomic 8 with D16S539

All allele calls with MiniFiler for CSF1PO, D7S820, D13S317, D18S51, D21S11, FGA, and D16S539 (with the exception noted below) ***match previously certified values.***

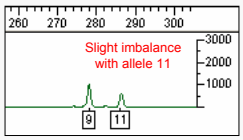
Identifiler



MiniFiler



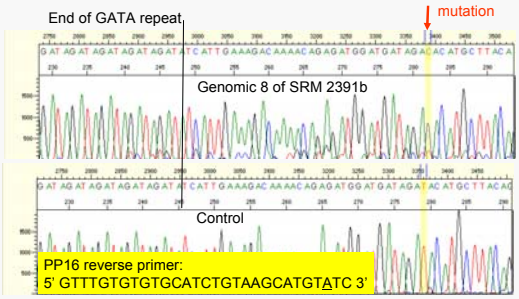
PowerPlex 16



Due to primer binding site mutation

D16S539 SRM 2391b Genomic 8

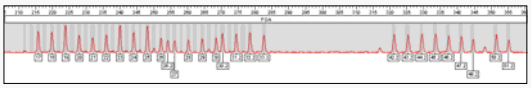
T→C mutation 34 bp downstream of the repeat



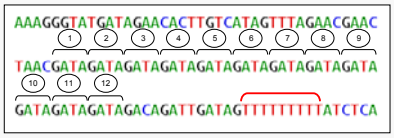
Position of the T→C probably affects the reverse primer of Minifiler and is the 3rd base found the 3' end of the Reverse PP16 primer. This could explain the imbalance of the allele seen when using PP16.

Why Go Beyond the CODIS Loci?

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



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



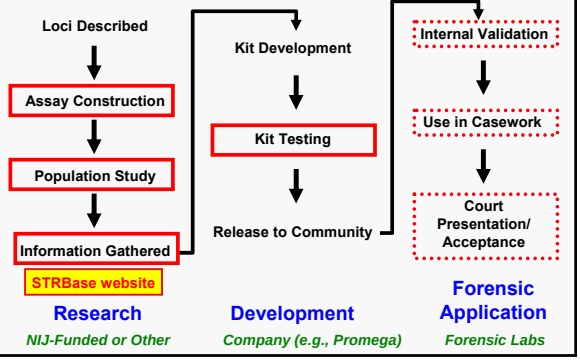
Butler, JM, Shen, Y., McCord, BR (2003) *JFS* 48(5): 1054-1064

How Would Additional STR Loci Be Useful?

- **Databases**: More loci to help resolve relatives in growing national DNA databases (UK went from 6 to 10 STRs in 1999; future Pan-European database will include >10 loci)
- **Casework**: Obtaining additional information with degraded DNA samples (miniSTRs); rapid screening of multiple crime scene samples
- **Identity/Relationship Testing**: Kinship analysis, parentage testing, complex criminal paternity, **missing persons/mass disasters**, immigration testing

Steps in Adopting Genetic Markers

Role of the NIST Human Identity Project Team



Justice for All Act of 2004

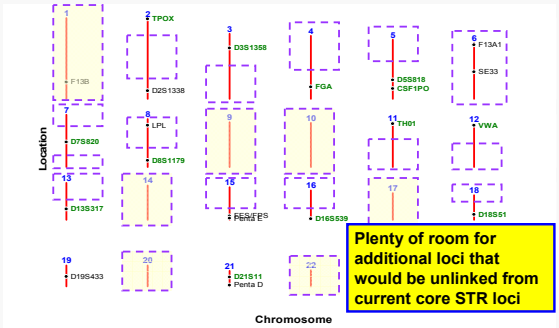
- **If additional loci are desired as core or supplementary loci on the national DNA database, the FBI must inform Congress six months prior to doing so…**
- "REPORT TO CONGRESS- If the Department of Justice plans to modify **or supplement the core genetic markers** needed for compatibility with the CODIS system, it shall notify the Judiciary Committee of the Senate and the Judiciary Committee of the House of Representatives in writing not later than 180 days before any change is made and explain the reasons for such change." (Section 203f)

Primary Characteristics in New STRs

- Genomic position
  - Adequate spacing from other (and current) loci to enable product rule use with autosomal markers
- Avoid known disease genes or linkage
  - To protect privacy concerns
- Polymorphic content (high heterozygosity)
  - More variable markers mean less can be used to reach desired rarity in full profile

Unused Chromosomal Locations

(relative to CODIS 13 STRs)



Plenty of room for additional loci that would be unlinked from current core STR loci

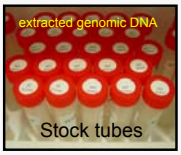
Butler, J.M. (2006) Genetics and genomics of core STR loci used in human identity testing. *J. Forensic Sci.* 51(2): 253-265.

Standard U.S. Population Dataset

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

260 Caucasians, 260 African Americans, 140 Hispanics, 3 Asians = **663 males**

DNA extracted from whole blood (anonymous; self-identified ethnicities) received from Interstate Blood Bank (Memphis, TN) and Millennium Biotech Inc. (Ft. Lauderdale, FL)



To date: (>100,000 allele calls)

Identifiler (15 autosomal markers + Amelogenin) (10,608)
Roche Linear Arrays (HV1/HV2 10 regions) (6,630)
Y STRs 22 loci—27 amplicons (17,388)
Y STRs 27 new loci (14,535)
Yfiler kit 17 loci (11,237)
Y SNPs 50 markers on sub-set of samples (11,498)
Orchid 70 autosomal SNPs on sub-set (13,230)
miniSTR testing-new loci and CODIS concordance (9,228)
New miniSTR loci – for 26 loci, 17,238 genotypes
mtDNA full control region sequences by AFDIL

Genotypes with various human identity testing markers

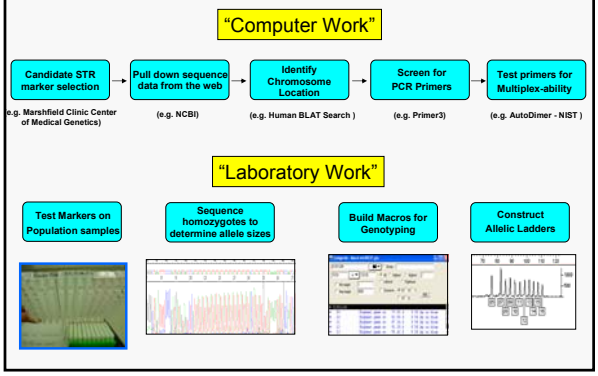
Valuable Characteristics in New STRs

- Span/Range of observed alleles
  - Impacts electrophoretic real-estate
  - Tighter range makes differential amplification less likely
- Clean flanking region
  - To enable primer design near repeat (miniSTRs)
- Mutation rate known when trying to address multi-generational questions
- Provides benefit to haplotype resolution (Y-STRs)

Steps We Use in Characterizing New Loci

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

Characterization of New miniSTR Loci

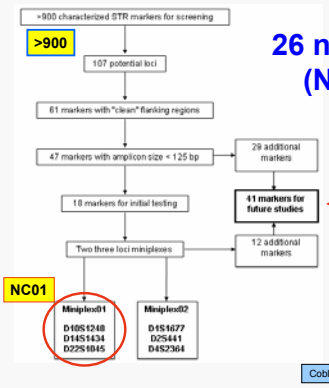


Selection of New Autosomal Loci

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

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

Initial Testing Results with Potential miniSTR Loci

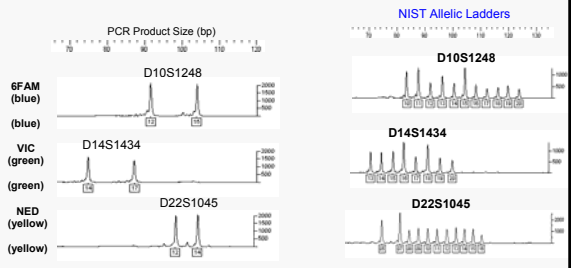


26 new miniSTRs (NC01-NC09)

20 additional loci characterized across U.S. population groups

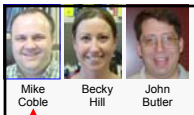
Coble and Butler (2005) *J. Forensic Sci.* 50(1): 43-53

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



Mike Coble, Becky Hill, John Butler

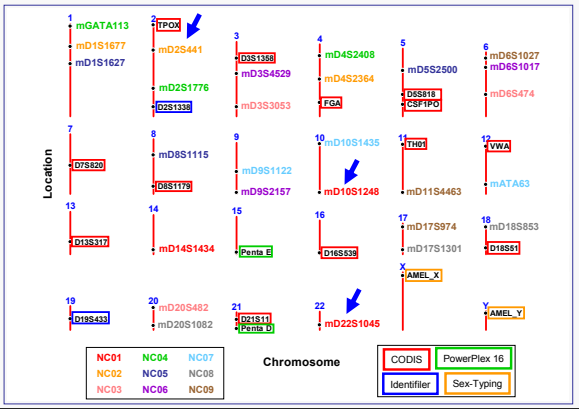
No longer at NIST (AFDIL Research Section Chief since April 2006)

New miniSTR Non-CODIS (NC) Loci

- 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

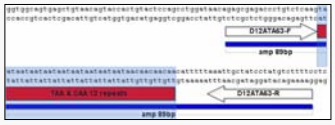
Chromosomal Locations of New miniSTR Loci



Characterization of New Autosomal Loci
(miniSTR **D12ATA63**)

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

[FAM] – GAGCGAGACCCTGTCTCAAG
GGAAAAGACATAGGATAGCAATTT



Chr 12 106.825 Mb
(12q23.3)

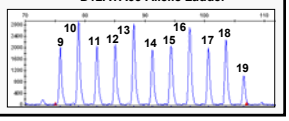
Trinucleotide
[TAA][CAA] repeat
76 -106 bp
Alleles 9 -19

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	
12	0.2154	0.109	
13	0.0173		0.0286
14		0340	0.2214
15		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

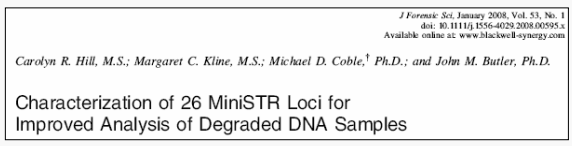
~660 U.S. population samples

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

D12ATA63 Allelic Ladder



To Appear in Jan 2008 Issue of *J. Forensic Sci.*



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, GeneMapper bins and panels, genotypes on common samples, and allele frequency information **already available on STRBase**

Comparison of heterozygosity values on 26 non-CODIS loci across the U.S. samples examined in this study.

Locus	N	Heterozygosity (Overall)	Rank	African American	Caucasian	Hispanic	
D9S2157	661	0.844	1	0.884	0.840	0.779	
ATA63 (D12)	659	0.829	2	0.788	0.842	0.879	
D10S1248 (NC01)	663	0.792	3	0.825	0.785	0.743	European recommended loci
D22S1045 (NC01)	663	0.784	4	0.817	0.785	0.721	
D2S441 (NC02)	660	0.774	5	0.798	0.780	0.721	
D10S1435	663	0.766	6	0.798	0.770	0.700	
D2S1776	654	0.763	7	0.740	0.801	0.734	
D3S4529	660	0.761	8	0.752	0.723	0.829	
D6S474	648	0.761	9	0.765	0.802	0.679	
D5S2500	664	0.747	10	0.757	0.747	0.729	
D1S1627	660	0.746	11	0.783	0.737	0.693	
D1S1677 (NC02)	660	0.746	12	0.743	0.749	0.743	
D6S1017	664	0.740	13	0.807	0.698	0.693	
D3S3053	648	0.739	14	0.713	0.724	0.814	
D9S1122	659	0.734	15	0.753	0.742	0.686	
D17S974	664	0.732					
D11S4463	664	0.730					
D4S2408	654	0.722					
D18S853	664	0.711					
D20S1082	664	0.696					
D14S1434 (NC01)	663	0.696					
D20S482	648	0.691					
GATA113 (D1)	654	0.668	23	0.673	0.632	0.727	
D8S1115	664	0.663	24	0.629	0.660	0.729	
D17S1301	664	0.649	25	0.626	0.717	0.564	
D4S2364 (NC02)	660	0.511	26	0.385	0.551	0.664	

>17,000 genotypes collected to measure these relative heterozygosities

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]

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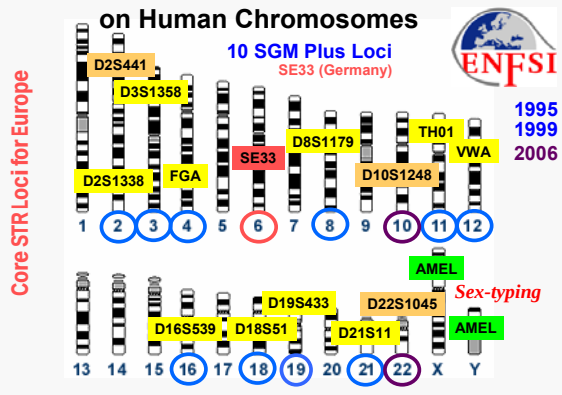
Received 25 May 2005; accepted 26 May 2005

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

(D14 has been replaced with D2S441 from NC02)

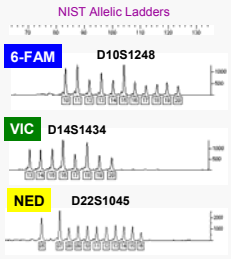
Position of Forensic STR Markers on Human Chromosomes



Multiple Miniplexes

- **26 characterized loci** divided into nine 3plexes
- One locus per dye color
- Allelic ladders created
- **Amplicons <140 bp**
- miniSTRs
- Work with 100 pg DNA
- **For degraded samples**
  (bones in missing persons cases)

NC = Non-CODIS or non-core



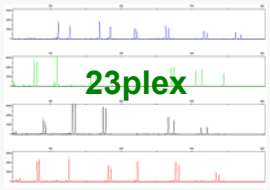
NC01 Loci

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

Single Megaplex

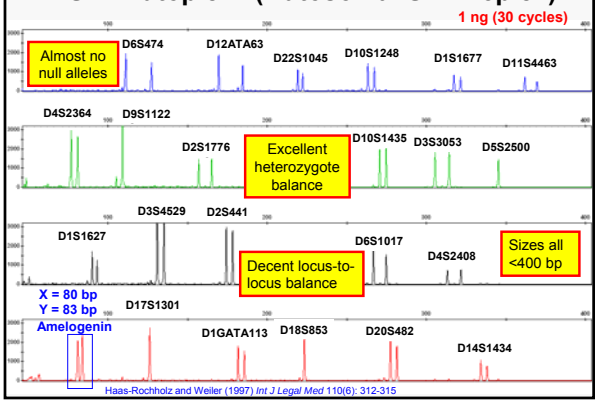
- So far **22 STRs and amelogenin** in single multiplex (Eventual goal to have all 26 loci)
- Multiple loci in four dye channels
- **Amplicons 70 to 400 bp**
  (No longer 'miniSTRs')
- Typically use 1 ng DNA
- **For reference samples**
  (a missing person's relatives)

"Autoplex" or "miniMegaplex"



All loci unlinked from core (CODIS) STRs

NIST "Autoplex" (Autosomal STR 23plex)



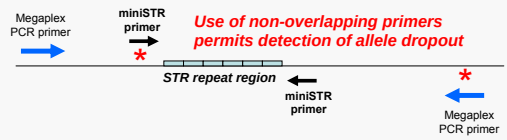
Evaluation of Autoplex (23plex)

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

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

Concordance Study to Check for Null Alleles

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



"Autoplex" vs miniSTRs
639 samples compared
Total types (639 x 22 loci): 14,058
28 types discordant (0.20%)*
99.80% concordance
**discordance not confirmed yet with sequencing*

Identifiler vs MiniFiler
1308 samples compared
Total types (1308 x 8 loci): 10,464
27 types discordant (0.26%)
99.74% concordance
Hill et al. (2007) JFS 52(4): 870-873

Conclusions: (1) Our PCR primers have been well-designed and have almost no primer binding site mutations. (2) Roughly half of dropout is from megaplex primers – flanking regions near STR repeat do not appear to have a higher level of mutation

Mutation Rates Measured for New STRs

- **395 father/son pairs** (790 samples total)
- 22 STR loci examined
- 8690 allelic transfers
- Only **6 mutations** were observed in total

- **0.069%**
- (2-3 times less than typical 0.2% for common STRs)

Mutation rates generally track with heterozygosity (locus variability)

Locus	Mutation Rate
SE33	0.64%
FGA	0.28%
D18S51	0.22%
…	…
TPOX	0.01%

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

Conclusions: Mutation rates are lower than commonly used STRs likely due to selection of loci for miniSTR application with tighter allele ranges, more moderate heterozygosities, and more stable flanking regions.

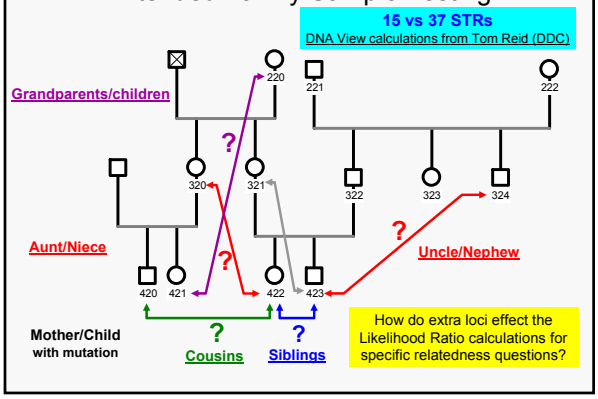
Population Data on New STRs

- **~660 samples** with three major U.S. populations on **all 26 autosomal STR loci**
  - Available on STRBase
    - **http://www.cstl.nist.gov/biotech/strbase/NISTpopdata/Allele_Frequencies_for_26miniSTRs.pdf**
- **>3,000 samples tested world-wide** (Spain, Italy, Japan, Malaysia, Korea) on **first 6 loci** (NC01 & NC02)
  - **D2, D10, D22 now recommended European loci**

Gill *et al.* (2006) *Forensic Sci Int* **156(2):** 242-244

Can these new STRs help in missing persons cases or other forms of relationship testing?

Extended Family Sample Testing



Comparison of Likelihood Ratios

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

Conclusions: Longer distance multi-generational questions cannot usually be solved with additional autosomal STRs… **need lineage markers like mtDNA or Y-STRs**

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"** (Gill et al. 2006)
- Applied Biosystems **MiniFiler kit** now available
  - concordance testing done at NIST (Hill et al. 2007)
- **26 miniSTR loci** characterized at NIST (Hill et al. 2008)

Enhanced Detection Capabilities with miniSTRs **will Extend Labs into Low Level DNA Work**

1: Fa Yi Xue Za Zhi. 2007 Aug;23(4):304-6.

[Application of MiniFiler kit in forensic DNA testing of low copy number template]

[Article in Chinese]

Tang JP, Wu D, Zhang C, Zhou HG.

Institute of Forensic Science, Shanghai Municipal Public Security Bureau, Shanghai 200083, China.

OBJECTIVE: To detect low copy number of DNA samples by using a newly launched commercial miniSTR detection kit (MiniFiler) in forensic practice. METHODS: Low concentration and/or challenged forensic DNA samples were analyzed according to protocols provided by the manufacturer (Applied Biosystems, Foster City, USA). RESULTS: DNA samples as low as 10 pg could be amplified by MiniFiler kit, and the optimal DNA quantity was 40 pg or above. CONCLUSION: MiniFiler kit can be used for analysis of low copy number STR.

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Gill, P., Fereday, L., Morling, N., Schneider, P.M. (2006) Letter to editor -- New multiplexes for Europe-amendments and clarification of strategic development. *Forensic Sci Int.* 163:155-157.

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Yong, R.Y., Gan, L.S., Coble, M.D., Yap, E.P. (2007) Polymorphism studies of four miniSTR loci for three ethnic populations in Singapore. *Legal Med (Tokyo)* 9(5):278-281.

Applied Biosystems MiniFiler Kit

October 2006 Forensic News:
http://marketing.appliedbiosystems.com/images/enews/ForensicNews_Vol7/PDF/00_ForensicNews.pdf

MiniFiler product information: http://minifiler.appliedbiosystems.com

Hill, C.R., Kline, M.C., Mulero, J.J., Lagace, R.E., Chang, C.-W., Hennessy, L.K., Butler, J.M. (2007) Concordance study between the AmpFlSTR MiniFiler PCR Amplification Kit and conventional STR typing kits. *J. Forensic Sci.* 52(4): 870-873.

Further Information from NIST Human Identity Project Team

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

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

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

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

Becky Hill poster at 58th Annual Meeting of the American Academy of Forensic Sciences (Seattle, WA), February 24, 2006, "Development of 27 New miniSTR Loci for Improved Analysis of Degraded DNA Samples" [.pdf]

Becky Hill poster at 17th International Symposium on Human Identification (Nashville, TN), October 10-12, 2006, "Characterization of 26 New miniSTR Loci" [.pdf]

Margaret Kline poster at 17th International Symposium on Human Identification (Nashville, TN), October 10-12, 2006, "NIST SRM Updates: Value-added to the Current Materials in SRM 2391b and SRM 2395" [.pdf]

Mike Coble talk at the International Society of Forensic Genetics meeting (Ponta Delgada, Azores, Portugal), September 14, 2005, "Characterization and performance of new miniSTR loci for typing degraded samples" [.pdf]

Mike Coble on-line presentation for the Forensic E-symposium (http://www.forensic.e-symposium.com/humid/), February 28, 2006, "miniSTR's for low copy number and degraded DNA" [.pdf]

Mike Coble presentation at the NIJ DNA Grantees meeting (Crystal City, VA), June 26, 2006, "Development, Characterization and Performance of New miniSTR Loci for Typing Degraded Samples (on behalf of NIST)" [.pdf]

The Cutting Edge of DNA Testing:
Mixtures, miniSTRs, and Low Level DNA

Low Level DNA Testing

NEAFS 2007 Workshop
Bolton Landing, NY
November 2-3, 2007

Dr. John M. Butler

Northeastern Association of Forensic Scientists

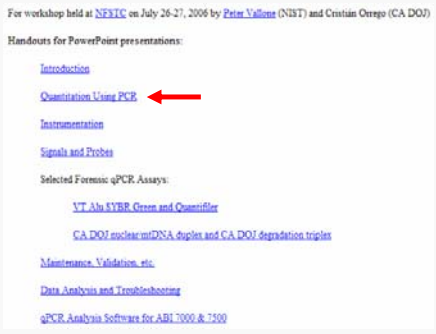
john.butler@nist.gov

Earlier Workshop on This Topics

- **qPCR workshop by Vallone and Orrego** (July 2006) – slides available on STRBase
  - **http://www.cstl.nist.gov/biotech/strbase/qPCRworkshop.htm**
- **LCN workshop by Butler, Caragine, and Gill** (May 2006) – Butler slides available on STRBase
  - **http://www.cstl.nist.gov/biotech/strbase/training.htm**

qPCR Workshop Materials

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


For workshop held at NFSTC on July 26-27, 2006 by Peter Vallone (NIST) and Cristian Orrego (CA DOJ)

Handouts for PowerPoint presentations:

- Introduction
- Quantitation Using PCR
- Instrumentation
- Signals and Probes

Selected Forensic qPCR Assays:

- VT Alu SYBR Green and Quantifiler
- CA DOJ nuclear/mtDNA duplex and CA DOJ degradation triplex

- Maintenance, Validation, etc.
- Data Analysis and Troubleshooting
- qPCR Analysis Software for ABI 7000 & 7500

Purpose of Human-Specific DNA Quantitation

- All sources of DNA are extracted when biological evidence from a crime scene is processed to isolate the DNA present.
- Thus, non-human DNA such as bacterial, fungal, plant, or animal material may also be present in the total DNA recovered from the sample along with the relevant human DNA of interest.
- For this reason, the DNA Advisory Board **(DAB) Standard 9.3 requires human-specific DNA quantitation** so that appropriate levels of human DNA can be included in the subsequent PCR amplification.
- **Multiplex STR typing works best with a fairly narrow range of human DNA** – typically 0.5 to 2.0 ng of input DNA works best with commercial STR kits.

Higher quality data saves time and money

Why Do We Care About Quantitating DNA?

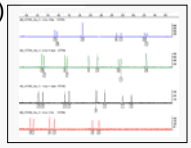
- If we can confidently determine the amount of DNA in an extract we can then ask questions:
  - Will mitochondrial sequencing be required (skip STR analysis)
  - Should we use a miniSTR assay?
  - Should we use low copy number LCN methods for STRs?
  - Re-extract the sample?
  - If problems occur in the STR typing process we can have confidence that the DNA template is not the source (CE, cycler, kit)

qPCR

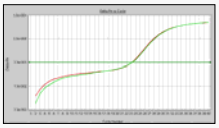
- qPCR is a recently developed technique
  - Developed by Higuchi in 1993
  - Used a modified thermal cycler with a UV detector and a CCD camera
  - Ethidium bromide was used as intercalating reporter As [dsDNA] increased fluorescence increased
- First paper on qPCR:
  - Higuchi, R.; Fockler, C.; Dollinger, G.; Watson, R. “Kinetic PCR analysis: real-time monitoring of DNA amplification reactions” Biotechnology (N Y). 1993 Sep;11(9):1026-30

PCR/qPCR What is the Difference?

- In the PCR the products are analyzed after the cycling is completed (static)
  - gel, CE, UV, fluorimeter
  - End point assay



- qPCR the products are monitored as the PCR is occurring (dynamic)
  - Once per thermal cycle
  - Fluorescence is measured
  - Kinetics of the system



Why Real Time qPCR?

Advantages

- The availability of commercial qPCR kits (labs are beginning to switch over to this method)
- Higher throughput and reduced user intervention
  - Automated set up
  - Simple data analysis
  - Experimental data rapidly analyzed in software; interpolating into the calibration curve
- qPCR will be sensitive to the same inhibitors as faced in a traditional STR test (both PCR based)

Why Real Time qPCR?

Advantages

- No post PCR manipulation (reduced contamination issues)
- High sensitivity (down to a single copy number ?)
- Large dynamic range: ~30 pg to 100 ng
- Assays are target specific (autosomal, mito, Y) and can be multiplexed – to a degree…

Why Real Time qPCR?

Challenges

- qPCR is subject to inhibition
  - internal PCR controls (IPC) can help
- qPCR quantitation precision suffers at low copy numbers (below 30 pg by a factor of 2)
- When working below 100 pg qPCR is still subject to variability and uncertainty

Why Real Time qPCR?

Challenges

- qPCR quantitates specific target sequences, it does not quantify "DNA"
  - In highly degraded samples, assays that amplify short target sequences will detect and measure more DNA than assays that amplify long target sequences (relevant to STR typing)
- Accurate qPCR quantitation assumes that each unknown sample is amplified at the same efficiency as the Calibrant sample in the dilution series
- Results are relative to the Calibrant (these can vary)

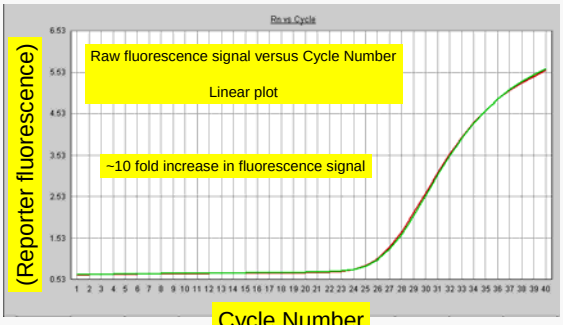
PCR Amplification

- 4 phases of PCR amplification
  - Lag (doubling, but not detected)
  - Exponential (doubling)
  - Linear (less than doubling)
  - Plateau (little change)

Efficiency is dropping < 100%

- The exponential phase is where we make our qPCR measurements

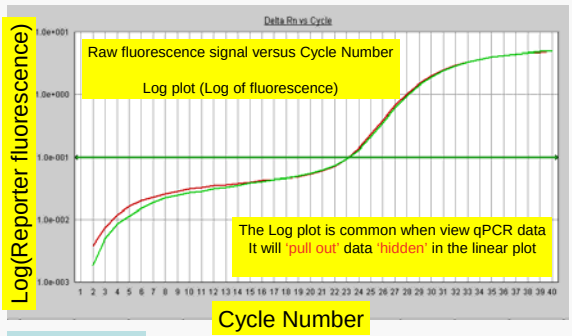
qPCR Real Time Curves



Y scale 0.5 to 5.5

Quantifiler Data

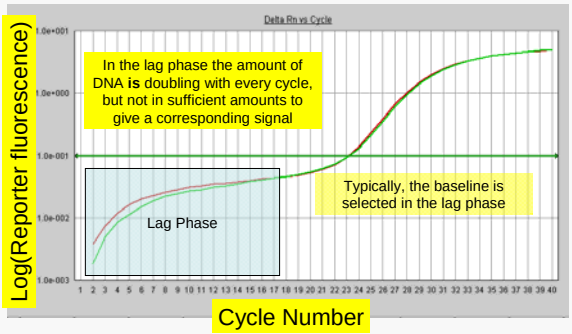
qPCR Real Time Curves



Y scale 0.001 to 10

Quantifiler Data

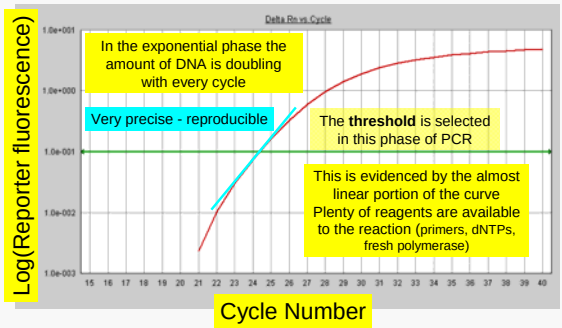
Lag Phase



Cycles ~ 1 - 20

Quantifiler Data

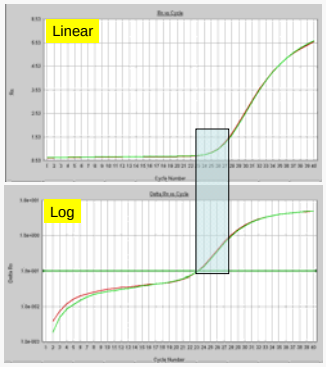
Exponential Phase



Cycles ~ 20 - 27

Quantifiler Data

qPCR Real Time Curves



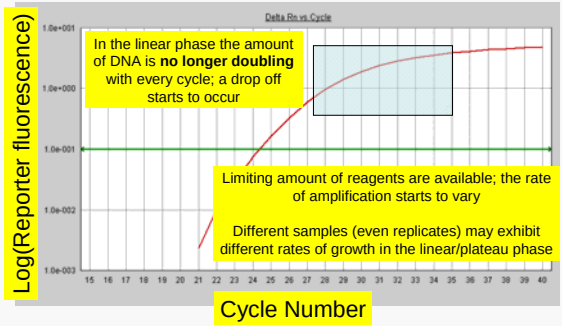
These plots are on the same X scale (Cycle Number)

The log plot is useful to see where the amount of DNA is doubling with cycle number

This can not be readily observed in the linear plot

Quantifiler Data

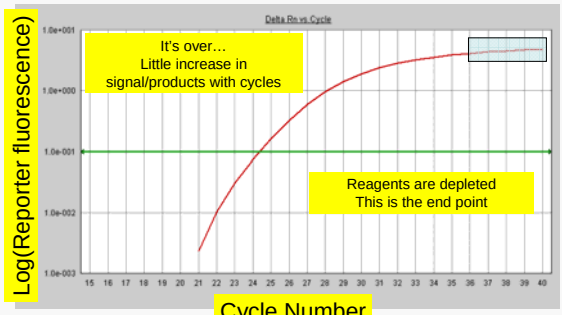
Linear Phase



Cycles ~ 27 - 35

Quantifiler Data

Plateau Phase

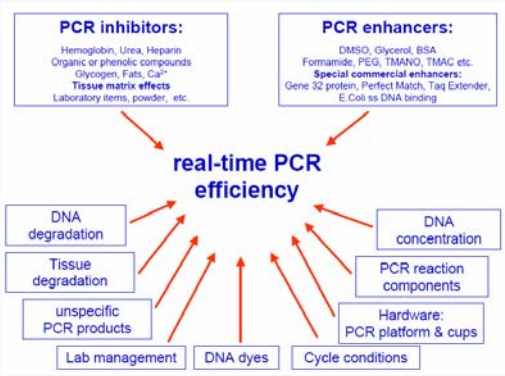


Cycles ~ 36 - end

Quantifiler Data

PCR Efficiency

- How is the PCR progressing?
- Is the PCR running at maximum efficiency?
- Is there some factor (environmental) inhibiting the reaction?
- Are we at the optimal annealing-extension temperatures (during assay development)?
- Are the unknowns amplifying with the same E as the Calibrants?



http://www.gene-quantification.info/ Michael W. Pfaffl: pfaffl@wzw.tun.de

PCR Efficiency

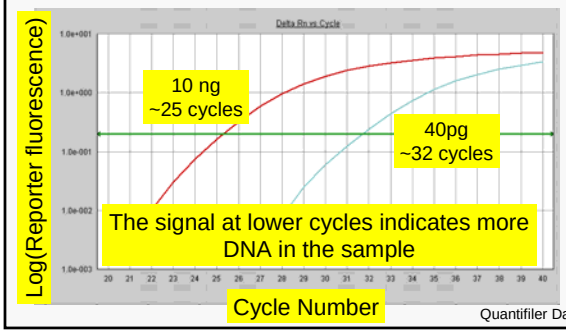
- Taking our previous relationship 2^N
- The efficiency of the PCR can be represented as:
- $X_N = X_0 (1 + E)^N$
  - X_N predicted copies
  - X_0 starting copy number
  - E efficiency (0 to 1)
  - N number of cycles

PCR Efficiency

- Starting with 100 copies and 100% and 28 cycles
  $X_N = 100(1 + 1)^{28}$
  2.68×10^{10} copies
- 90%
  $X_N = 100(1 + 0.9)^{28}$
  6.38×10^9 copies
- 80%
  $X_N = 100(1 + 0.8)^{28}$
  1.40×10^9 copies

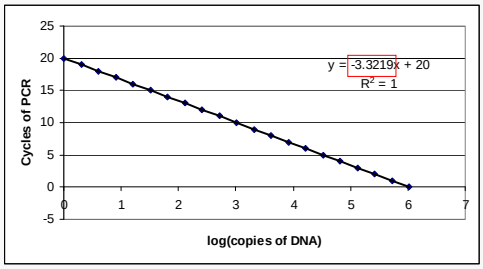
PCR Efficiency

- When applied to qPCR the relationship is the inverse (the signal at lower cycles indicates more DNA in the sample)



PCR Efficiency

- When applied to qPCR the relationship is the inverse (the signal at lower cycles indicates more DNA in the sample).
- The line has a negative slope



PCR Efficiency

- A optimal reaction should be between 90% to 110% slope = -3.58 to -3.10
- The slope may vary even more when looking at more complex (multiplex) qPCR assays; multiplex probes, targets, copies etc

Importance of the Calibrant!

- Things to keep in mind about Calibrants
- The Calibrant is usually a pristine well-characterized DNA sample
  - Not extracted
  - Not subjected to the same environment as your unknown(s)
  - Will not contain inhibitors, Ca^{++} etc
  - May be from a cell line or mixed source sample
  - **May exhibit lot-to-lot variation (monitor this)**

Varying the C_T Value

- Relative small changes in C_T result in large variations in concentration

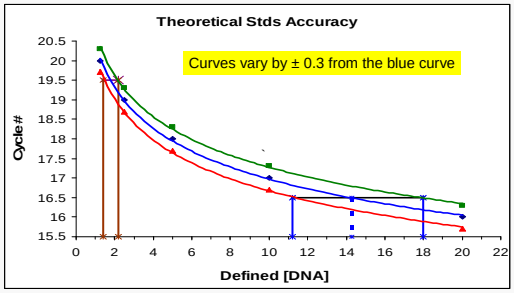
m	b	CT	[DNA]	%	delta
-3.3219	26	25.1	1.87	6.70	0.13
-3.3219	26	25	2.00		
-3.3219	26	24.9	2.14	6.70	-0.14
-3.3219	26	20.1	59.72	6.70	4.29
-3.3219	26	20	64.00		
-3.3219	26	19.9	68.60	6.70	-4.59

± 0.1 C_T

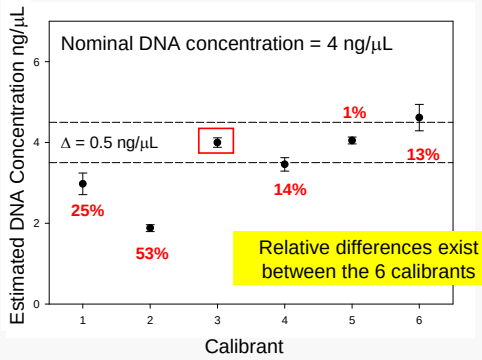
m	b	CT	[DNA]	%	delta
-3.3219	26	25.3	1.62	18.77	0.38
-3.3219	26	25	2.00		
-3.3219	26	24.7	2.46	18.77	-0.46
-3.3219	26	20.3	51.99	18.77	12.02
-3.3219	26	20	64.00		
-3.3219	26	19.7	78.80	18.77	-14.79

± 0.3 C_T

Importance of the Calibrant!



Differences Between Calibrants



NIST Lessons Learned from Real Time-qPCR Assays

Using ABI 7500 (early work with ABI 7000 and some Roche LightCycler)

- Results are RELATIVE to standards used
- Single source and mixed source samples with same UV concentrations differ with RT-qPCR assays
- Need to keep instrument clean to avoid background fluorescence problems
- Assay reagent costs:
  - Quantifiler: $2.46/sample (only permits 2 µL/sample)
  - SYBR Green: $0.80/sample (up to 10 µL/sample)
  - QuantiBlot: $0.54/sample (5 µL/sample)

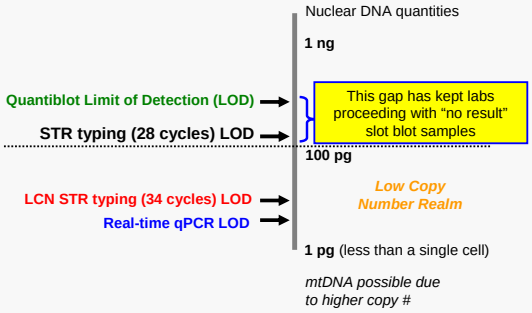
http://www.cstl.nist.gov/biotech/strbase/DNAquant.htm

Proceeding with Testing when "No DNA" Detected

If the qPCR results indicate that there is no detectable DNA, will you stop testing or will you proceed with attempting STR typing?

- The practice of proceeding even with a "no result" Quantiblot was because the STR typing assay was more sensitive than the quantification method.
- What types of experiments might be done to satisfy you that "no result" from a qPCR assay is truly "no DNA"?

Difference in DNA Quantitation Capability vs. STR Typing Sensitivity



DNA Quantitation Summary

- RT-qPCR is a homogeneous PCR based method that enables human specific quantification
  - Is easily automated, provides electronic storage of data
  - SYBR green or targeted probes can be used
- Results give quantity of amplifiable DNA – not necessarily overall quantity
  - PCR inhibition can be detected
  - Multiplexing can be used
- Big advantages are speed and dynamic range
- Commercial kits are now available

MAAFS DNA Workshop

Introduction to Low Copy Number (LCN) DNA Testing Issues

John M. Butler, PhD

john.butler@nist.gov
NIST

Richmond, VA
May 3, 2006

Some Definitions of Low-Copy Number (LCN)

- Work with **<100 pg genomic DNA** (~15-17 diploid copies of nuclear DNA markers such as STRs)
- Below stochastic threshold level where PCR amplification is not as reliable (determined by each laboratory; typically 150-250 pg)
- Enhancing sensitivity of detection (34 cycles instead of 28 cycles)
- Too few copies of DNA template to ensure reliable PCR amplification
- Other terms for LCN:
  - Low-level DNA
  - Trace DNA
  - Touch DNA

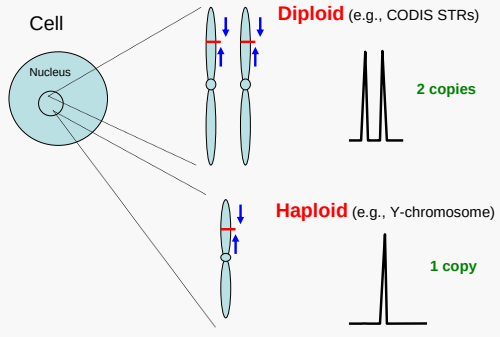
LCN is dependent on the amount of DNA present NOT the number of PCR cycles performed; LCN conditions may exist with 28 or 34 cycles

Low-Copy Number (LCN) Work

- **Early work on touched objects and single cells**:
  - van Oorschot, R. A. and Jones, M. K. (1997) DNA fingerprints from fingerprints. *Nature*. 387(6635): 767
  - Findlay, I., Taylor, A., Quirke, P., Frazier, R., and Urquhart, A. (1997) DNA fingerprinting from single cells. *Nature*. 389(6651): 555-556
- **Application to routine forensic casework was pioneered by the Forensic Science Service**:
  - Gill, P., Whitaker, J., Flaxman, C., Brown, N., and Buckleton, J. (2000) An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. *Forensic Sci. Int.* 112(1): 17-40
  - Whitaker, J. P., Cotton, E. A., and Gill, P. (2001) A comparison of the characteristics of profiles produced with the AMPFISTR SGM Plus multiplex system for both standard and low copy number (LCN) STR DNA analysis. *Forensic Sci. Int.* 123(2-3): 215-223
  - Gill, P. (2001) Application of low copy number DNA profiling. *Croatian Medical Journal* 42(3): 229-32

DNA quantity in samples

Diploid vs. Haploid



Calculation of the Quantity of DNA in a Cell

1. **Molecular Weight of a DNA Base Pair = 618 g/mol**

   A = 313 g/mol; T = 304 g/mol; A-T base pairs = 617 g/mol
   G = 329 g/mol; C = 289 g/mol; G-C base pairs = 618 g/mol

2. **Molecular Weight of DNA = 1.98 x10^{12} g/mol**

   There are **3.2 billion base pairs** in a haploid cell ~3.2 x 10^9 bp
   (~3.2 x 10^9 bp) x (618 g/mol/bp) = 1.98 x 10^{12} g/mol

3. **Quantity of DNA in a Haploid Cell = 3 picograms**

   1 mole = 6.02 x 10^{23} molecules
   (1.98 x 10^{12} g/mol) x (1 mole/6.02 x 10^{23} molecules)
   = 3.3 x 10^{-12} g = 3.3 picograms (pg)
   A diploid human cell contains ~6.6 pg genomic DNA

4. **One ng of human DNA comes from ~152 diploid cells**

   1 ng genomic DNA (1000 pg)/6.6pg/cell = **~303 copies of each locus**
   (2 per 152 diploid genomes)

Adapted from D.N.A. Box 3.3, J.M. Butler (2005) Forensic DNA Typing, 2nd Edition (Elsevier Academic Press), p. 56

At the 2003 AAFS LCN Workshop (Chicago,IL), **Robin Cotton** from Orchid Cellmark presented a talk entitled "Are we already doing low copy number (LCN) DNA analysis?"

Where does low copy number start?

<100 pg template DNA

(Butler, 2001, Fregeau & Fourney 1993, Kimpton *et al* 1994)

Amount of DNA	~ # of cells
1 ng	152
0.5 ng	76
0.25 ng	38
0.125 ng	19
0.0625 ng	10

Values for # of cells adjusted to reflect updated DNA quantitation numbers

Robin Cotton, AAFS 2003 LCN Workshop
"Are we already doing low copy number (LCN) DNA analysis?"

Assume sample is from a **single source**:

Amount of DNA	Total Cells in sample	~ # of copies of each allele if het.
1 ng	152	152
0.5 ng	76	76
0.25 ng	38	38
0.125 ng	19	19
0.0625 ng	10	10

Robin Cotton, AAFS 2003 LCN Workshop
"Are we already doing low copy number (LCN) DNA analysis?"

Assume sample is a **1:1 mixture** of two sources:

Amount of DNA	Total Cells in sample	~ # of cells from each component
1 ng	152	76
0.5 ng	76	38
0.25 ng	38	19
0.125 ng	19	10
0.0625 ng	10	5

Robin Cotton, AAFS 2003 LCN Workshop
"Are we already doing low copy number (LCN) DNA analysis?"

Assume sample is a **1:3 mixture** of two sources:

Amount of DNA	~ # of cells from **major component**	~ # of cells from **minor component**
1 ng	114	38
0.5 ng	57	19
0.25 ng	28	10
0.125 ng	14	5
0.0625 ng	7	2

Robin Cotton, AAFS 2003 LCN Workshop
"Are we already doing low copy number (LCN) DNA analysis?"

Assume sample is a **1:9 mixture** of two sources:

Amount of DNA	~ # of cells from **major component**	~ # of cells from **minor component**
1ng	137	15
0.5ng	68	8
0.25ng	34	4
0.125ng	17	2
0.0625ng	9	1

Robin Cotton, AAFS 2003 LCN Workshop
"Are we already doing low copy number (LCN) DNA analysis?"

Low copy number situations exist in many samples

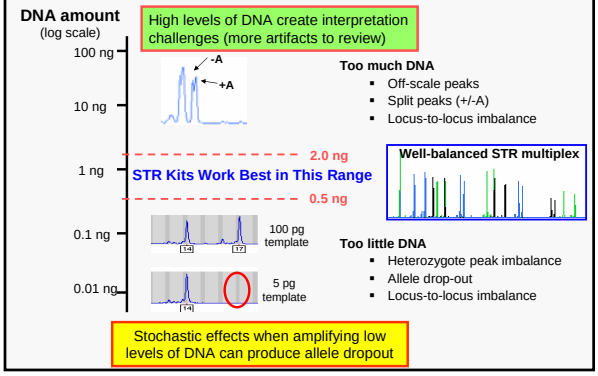
- In a 1:1 mixture, each DNA source is at LCN when the total amount of DNA in the amplification reaction is ~ 0.125 ng.
- In a 1:9 mixture, the minor component could be at LCN **even when the total amount of DNA in the amplification is 1 ng**.

Two different amplifications would be useful with a 1:9 mixture situation:
Normal level of total DNA (e.g., 1 ng) so that major component is on-scale
High level of total DNA (e.g., 5 ng) so that minor (e.g., ~500 pg) is out of LCN realm – yes, the major component will be off-scale…

Robin Cotton, AAFS 2003 LCN Workshop
"Are we already doing low copy number (LCN) DNA analysis?"

Impact of DNA Amount into Multiplex PCR Reaction

We generally aim for 0.5-2 ng



Stochastic PCR amplification

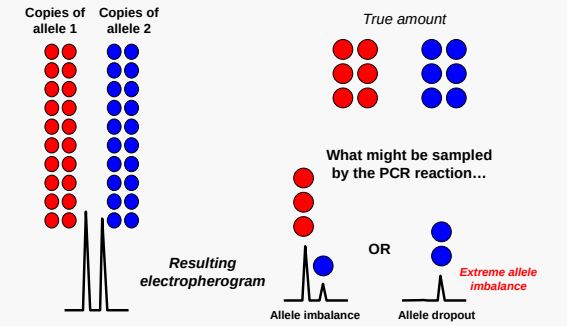
Stochastic = random selection

Stochastic Fluctuation Effects

- Unequal sampling of the two alleles present in a heterozygous individual can occur when low levels of input DNA are used (**results in allele drop-out**)
- PCR reactions **with <100 pg** (~17 diploid copies)
- Walsh *et al.* (1992) – propose avoiding stochastic effect by adjusting the number of PCR cycles in an assay so that the sensitivity limit is around 20 or more copies of target DNA (i.e., a full profile is obtained with ~125 pg)

Walsh PS, Erlich HA, Higuchi R. Preferential PCR amplification of alleles: Mechanisms and solutions. *PCR Meth Appl* 1992; 1:241-250.

Stochastic Statistical Sampling

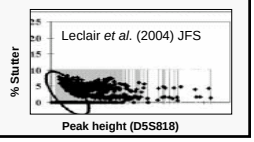


>20 copies per allele

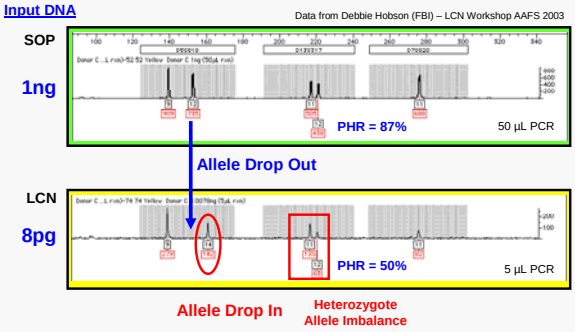
6 copies copies per allele **(LCN)**

Stochastic Effect

- Sometimes called "preferential amplification" – not really a correct term since either allele may be amplified if the other drops-out…not related to allele size
- Stutter product amounts may go up…
  - If in an early cycle of PCR, the stutter product is amplified more (due to sampling effect)
- Contaminating DNA can also be amplified giving rise to allele "drop-in" or a mixture



Comparison of STR Kit Amplification SOP with LCN
Using the Same DNA Donor

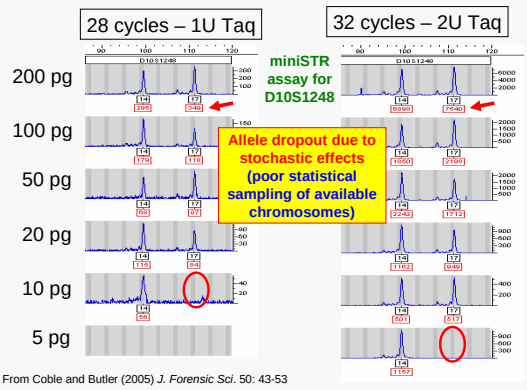


Balance of Assay Sensitivity and Potential for Stochastic Effects

- **One of the ways that assays can be made more sensitive is by increasing the number of PCR amplification cycles**
- Optimal cycle number will depend on desired assay sensitivity
- The number of PCR cycles was set to 28 for ABI STR kits to limit their sensitivity for generating full profiles to ~125 pg or 20 cells
- Sensitivity is a combination of fluorescent dye characteristics (relative to the instrument and laser excitation used) and PCR amplification conditions such as primer concentration and amount of polymerase used

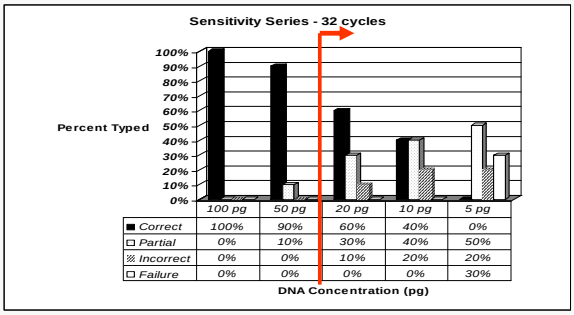
Note that Promega STR kits use higher numbers of cycles to generate roughly equivalent sensitivity to ABI kits because they have less efficient dye labels and lower primer and polymerase concentrations

Higher Sensitivity with More Polymerase and Cycle Numbers



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

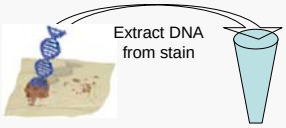
Problems with Obtaining Correct Allele Calls at Low DNA Levels



	100 pg	50 pg	20 pg	10 pg	5 pg
Correct	100%	90%	60%	40%	0%
Partial	0%	10%	30%	40%	50%
Incorrect	0%	0%	10%	20%	20%
Failure	0%	0%	0%	0%	30%

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

Typical LCN Procedure



Extract DNA from stain

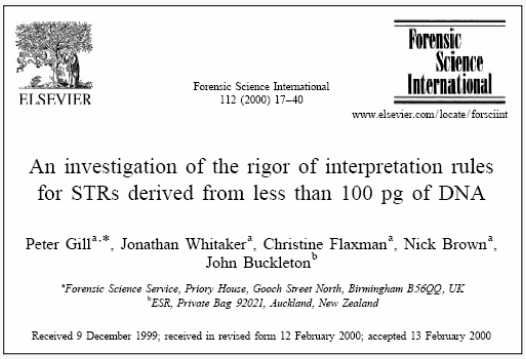
Quantify Amount of DNA Present

Perform 3 Separate PCR Amplifications

Interpret Alleles Present

Develop a Consensus Profile
(based on replicate consistent results)

New Interpretation Rules Required for LCN



ELSEVIER

Forensic Science International
112 (2000) 17–40

Forensic Science International

www.elsevier.com/locate/forsciint

An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA

Peter Gill[a,*], Jonathan Whitaker[a], Christine Flaxman[a], Nick Brown[a], John Buckleton[b]

*Forensic Science Service, Priory House, Gooch Street North, Birmingham B56QQ, UK
[b]ESR, Private Bag 92021, Auckland, New Zealand

Received 9 December 1999; received in revised form 12 February 2000; accepted 13 February 2000

Suggestions to Optimal Results with LCN

- At least two* PCR amplifications from the same DNA extract (if enough DNA is present to do more than 4-5 amplifications, then most likely a single aliquot would be run under standard STR typing conditions)
- An allele cannot be scored (considered real) unless it is present at least twice in replicate samples
- Extremely sterile environment is required for PCR setup to avoid contamination from laboratory personnel or other sources

*five is better; results are typically viewed as investigative

Other methods for higher sensitivity and signal enhancements

Improving Sensitivity

- Improved recovery of biological material and DNA extraction
- Longer injection on CE
- Salt removal from CE sample – enhances electrokinetic injection
- Reduced volume PCR – concentrates amplicon
- Increase number of cycles in PCR and/or TaqGold concentration
- Use miniSTRs – shorter amplicons amplify better; **MiniFiler**
- Use mtDNA – higher copy number per cell

Modifications in DNA Analysis Process to Improve LCN Success Rates

- **Collection** – better swabs for DNA recovery
- **DNA Extraction** – into smaller volumes
- **DNA Quantitation** – qPCR helps with low DNA amounts
- **PCR Amplification** – increased number of cycles
- **CE Detection** – longer electrokinetic injection; more sensitive fluorescent dyes
- **Interpretation** – composite profile from replicate analyses with at least duplicate results for each reported locus
- **Match** – is it even relevant to the case?

miniSTRs and LCN

- miniSTR assays are typically more sensitive than conventional STR kits currently in use
- Labs will start “pushing the envelope” in order to try and get a result with more sensitive assays including future miniSTR assays and kits
- Labs may move into the LCN realm without realizing it or adopting the careful LCN interpretation rules such as replicate analyses with duplicate results prior to reporting alleles

DNA Profiles are An Investigative Tool

- **Finding DNA indicates contact.**
- **Lack of a DNA profile is inconclusive.**

LCN is analogous to a bigger, more powerful magnifying glass

Theresa Caragine (AAFS 2003 LCN Workshop)

The Wisdom of Obi Wan Kenobi

http://www.starwars.com/kids/explore/lore/img/news20000902_1.jpg

Just before entering the Mos Eisley spaceport in Episode IV, Ben (Obi Wan) Kenobi warned Luke Skywalker, "You will never find a more wretched hive of scum and villainy…
WE MUST BE CAUTIOUS!"

Thank you for your attention…

Questions?

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

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

Low-Copy Number DNA Reference List

General Information

Budowle, B., Hobson, D.L., Smerick, J.B., Smith, J.A.L. (2001) Low copy number – consideration and caution. *Proceedings of the Twelfth International Symposium on Human Identification*. Available at http://www.promega.com/geneticidproc/ussymp12proc/contents/budowle.pdf.

Buckleton, J. and Gill, P. (2005) Low copy number. Chapter 8 in *Forensic DNA Evidence Interpretation* (Eds. J. Buckleton, C.M. Triggs, S.J. Walsh) CRC Press: Boca Raton, FL, pp. 275-297.

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