



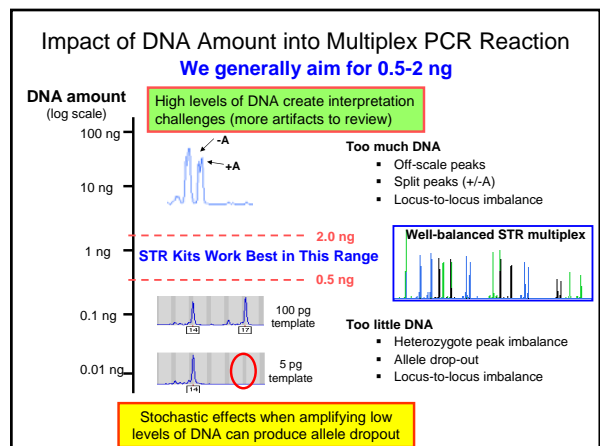
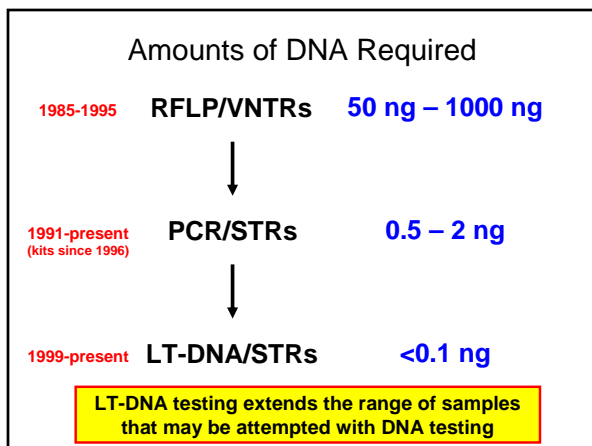
Low Template (LT) DNA Analysis

Becky Hill and Becky Mikulasovich
 National Institute of Standards and Technology
 Office of the Chief Medical Examiner, NYC
 Green Mountain DNA Conference
 Burlington, VT
 July 26, 2010

- ### Outline of Topics to Discuss
- Introduction to Low Template (LT) DNA
 - Historical perspective of LT-DNA testing
 - Technical Aspects of LT-DNA testing
 - Challenges and limitations with LT-DNA testing
 - Validation and Setting Stochastic Thresholds
 - Approaches to genotyping low template DNA
 - NIST LT-DNA data and Peak Height Ratios (PHR)
 - History of LT-DNA testing at OCME
 - Conclusions and recommendations for setting up an LT-DNA testing lab

Introduction to Low Template (LT) DNA

- ### Some Definitions of Low Template (LT) DNA
- Working with **<100-200 pg genomic DNA**
 - Considered to be data below stochastic threshold level where PCR amplification is not as reliable (determined by each laboratory; typically 150-250 RFUs)
 - Enhancing the sensitivity of detection (increasing PCR cycles, PCR product clean-up, increasing CE injection/voltage)
 - Having too few copies of DNA template to ensure reliable PCR amplification (allelic or full locus drop-out)
 - Can often be the minor component of mixture samples consisting of low level DNA template amounts



Low Template DNA situations exist in many samples

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

Historical Perspective of LT-DNA Testing

LT-DNA is not a "new" technique...

- 1996 – Taberlet *et al.* describe "reliable genotyping of samples with very low DNA quantities using PCR"
- 1997 – Findlay *et al.* report single cell STR analysis
- 1999 – Forensic Science Service begins LT-DNA casework in UK (as an alternative to mtDNA)
- 2001 – Budowle and FBI co-authors urge caution with using LT-DNA
- 2005 – NY State Commission of Forensic Science with the recommendation of NY State DNA subcommittee approve NYC OCME to use protocols for LT-DNA testing

Low Template DNA 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

Previous Presentations on LT-DNA Issues

- AAFS Feb 2003 LCN workshop
- AAFS Feb 2006 Advanced Topics in STRs workshop
- MAAFS May 2006 LCN workshop
- NEAFS Nov 2007 Cutting Edge workshop
- MAAFS May 2009 Advanced Forensics DNA Concepts workshop
- Promega Oct 2009 Technical Leaders workshop
- AAFS Feb 2010 presentation
- Bode East and West 2010 presentations

<http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm#Presentations>

LT-DNA Challenges

Challenges of LT-DNA Testing

Gill, P. (2001) *Croatian Med. J.* 42(3): 229-232

- Increased chance for contamination (want a sterile lab environment to reduce staff contamination)
- Data interpretation is more complicated (due to stochastic variation during PCR amplification):
 - Heterozygote peak imbalance
 - Allele drop-out
 - Allele drop-in
 - Increased stutter products

LT-DNA profiles should be interpreted with careful guidelines

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

Data from Debbie Hobson (FBI) – LCN Workshop AAFS 2003

Input DNA

SOP

1ng

50 µL PCR

PHR = 87%

LCN

8pg

5 µL PCR

PHR = 50%

Allele Drop Out

Allele Drop In Heterozygote Allele Imbalance

Stochastic (Random) Effects with LT-DNA When Combined with Higher Sensitivity Techniques

**Loss of True Signal
(False Negative)**

Heterozygote Peak Imbalance

30% PHR

Identifiler, 30 pg DNA, 31 cycles

Allelic Drop-out

14 allele drop-out

Identifiler, 30 pg DNA, 31 cycles

**Gain of False Signal
(False Positive)**

Higher Stutter

64% stutter

Identifiler, 10 pg DNA, 31 cycles

Allelic Drop-in

16 allele drop-in

Identifiler, 10 pg DNA, 31 cycles

Problems with Obtaining Correct Allele Calls at Low DNA Levels

From John Butler May 3, 2006 MAAFS LCN Workshop presentation (Richmond, VA)
 Available at http://www.cstl.nist.gov/biotech/strbase/pub_pres/LCNintro_MAAFSworkshop_May2006.pdf

Sensitivity Series - 32 cycles

	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%

DNA Concentration (pg)

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

Setting Stochastic Thresholds

Types of Results at Low Signal Intensity (Stochastic amplification potential)

Straddle Data

- Only one allele in a pair is above the laboratory stochastic threshold

Stochastic limit

Detection limit

One allele peak above the stochastic threshold and one below

160 RFUs

150 RFUs

130 RFUs

50 RFUs

At low levels of input DNA, the potential for straddle data is high.

The issue is best avoided by re-amplifying the sample at higher input DNA

Otherwise **straddle data makes locus inconclusive**

Straddle data may be caused by degradation, inhibition and low copy issues.

Scientific Reasoning behind the Stochastic Threshold

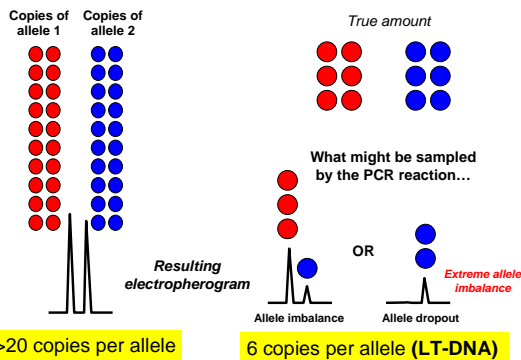
- When stochastic fluctuation is present, interpreting data becomes problematic due to the potential for:
 - Allele dropout
 - Poorly defined mixture ratios
 - Low template DNA
- Bottom line: Input levels of DNA should be sufficiently high to avoid straddle data. Mixture interpretation must be done cautiously on low level data as peak intensities are highly variable.

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



Stochastic Effect

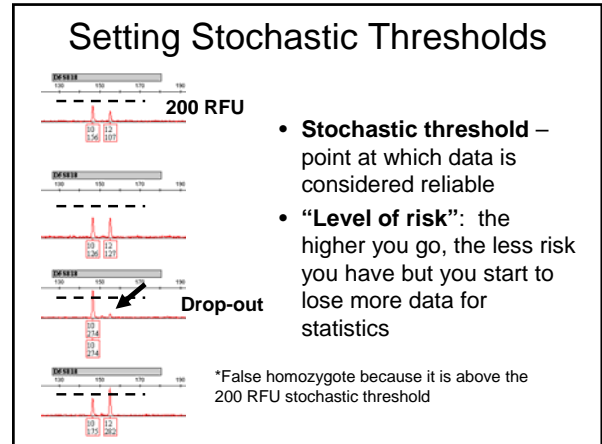
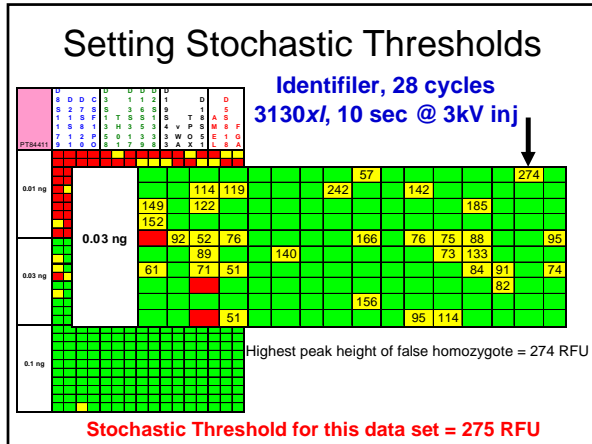
- Sometimes called “preferential amplification” – not really a correct term since either allele may be amplified if the other drops-out...not related to allele size
- Stutter product amounts may go up...
 - If in an early cycle of PCR, the stutter product is amplified more (due to sampling effect)
-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

Issues with Data Below the Stochastic Threshold

- PCR artifacts and stutter become prevalent
- Low levels of bleed through are possible
- Instrument spikes are more numerous
- -A peaks may appear
- Dye blobs become more significant in overall e-gram
- Low level 2nd contributors may show peaks

Setting Stochastic Thresholds

- Set based on data collected from your system
- Multiple samples, replicates, and concentrations are ideal to get a feel for how the system is working
 - We used 3 fully heterozygous samples with 10 replicates at 2 ng, 1 ng, 800 pg, 500 pg, 400 pg, 300 pg, 200 pg, 100 pg, 30 pg, & 10 pg
- Stochastic thresholds are not perfect or “cut and dry”
 - Can vary between loci and dye channels



Approaches for LT-DNA Testing

Early Work on Replicate Testing with Low Levels of DNA

© 1996 Oxford University Press. *Nucleic Acid Research*, 1996, Vol. 24, No. 16, 3189-3194

Reliable genotyping of samples with very low DNA quantities using PCR
 Pierre Taberlet*, Sally Griffin, Benoit Goossens, Sophie Questiau, Valérie Manceau, Nathalie Escaravage, Lisette P. Waits and Jean Bouvet

Laboratoire de Biologie des Populations d'Altitude, CNRS UMR 5553, Université Joseph Fourier, BP 53, 38041 Grenoble Cedex 9, France

Received May 1, 1996; Revised and Accepted July 2, 1996

Replicate testing introduced (up to 7 times) to account for allele drop-out and avoid miscalling allele drop-in

In conjunction with interpretation rules, duplication of observed alleles in replicates was shown to correctly define the original sample

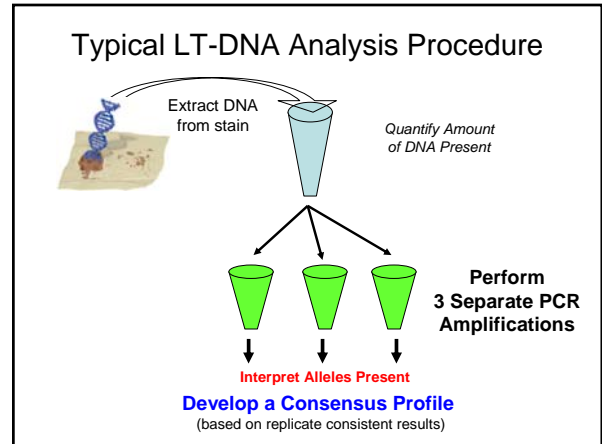
An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA

Peter Gill*, Jonathan Whitlock*, Christine Flocas*, Nick Brown*, John Buckleton*


*Forensic Science Service, Priory House, South Street North, Birmingham B10QJ, UK
 *ES, Priory Bldg 1020, Jordanhill, New Zealand

Received 9 December 1995; received in revised form 11 February 1996; accepted 13 February 1996

- ### Suggestions for Optimal Results with LT-DNA
- Typically at least 2 – 3 PCR amplifications from the same DNA extract are performed to obtain **consensus profiles**
 - 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



New Interpretation Rules Required for LT-DNA



Forensic Science International
 112 (2000) 17–40
 www.elsevier.com/locate/forensicint

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

^aForensic Science Service, Priory House, Gooch Street North, Birmingham B560Q, UK
^bESR, Private Bag 92021, Auckland, New Zealand

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

Replicate LT-DNA Test Results from FSS

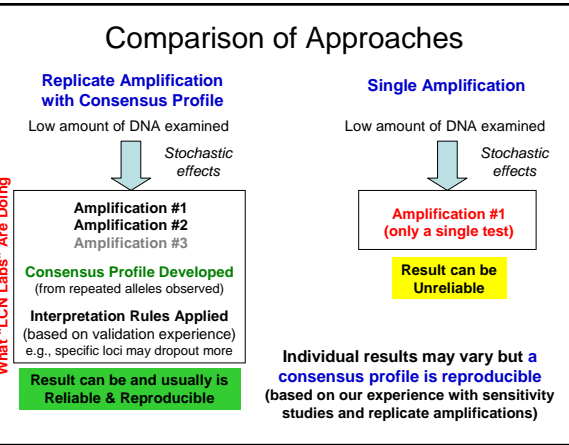
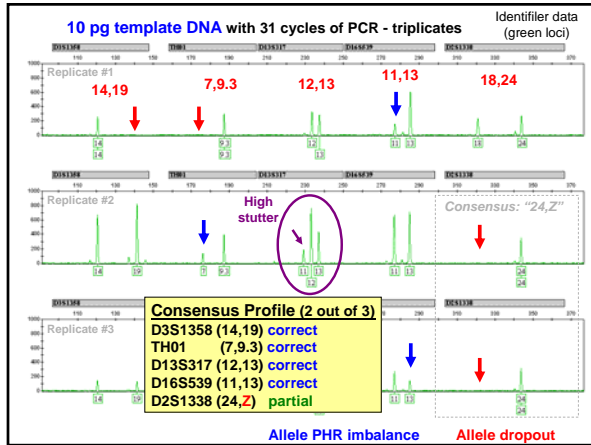
Gill, P. (2002) Role of short tandem repeat DNA in forensic casework in the UK—past, present, and future perspectives. *BioTechniques* 32(2): 366-365.

Table 2. Result of Six Replicate PCR Tests of a Sample Under Low Copy Number Analysis Conditions Compared to the Control Sample

	Ameio	D19	D3	D8	THO	VWA	D21	FGA	D16	D18	D2
CONTROL	X X	14,14	18,18	15,15	7,9,3	19,19	28,32,2	20,23	9,12	12,16	17,23
Sample											
1	—	14 F ^o	—	15 F ^o	—	—	28,32,2	20 F ^o	—	16 F ^o	—
2	X F ^o	—	18 F ^o	15 F ^o	—	19 F ^o	—	—	12 F ^o	—	—
3	X F ^o	—	—	15 F ^o	—	—	—	—	—	—	17 F ^o
4	X F ^o	14 F ^o	18 F ^o	—	—	—	—	—	9,12	—	—
5	X F ^o	—	18 F ^o	—	—	18 F ^o	—	—	—	—	—
6	X F ^o	14 F ^o	—	—	—	19 F ^o	28,32,2	20 F ^o	—	12 F ^o	—
Consensus	X F ^o	14 F ^o	18 F ^o	15 F ^o	—	19 F ^o	28,32,2	20 F ^o	12 F ^o	—	—

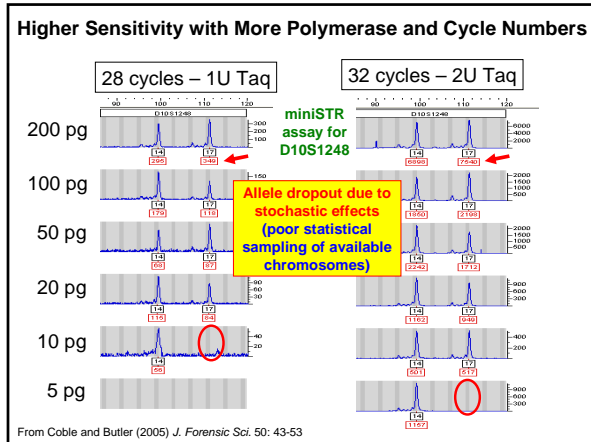
The consensus result is reported, provided that an allele is observed at least twice. If only one allele is observed, then an F^o designation is given to denote the possibility of allele drop-out.

F^o used to designate that allele drop-out of a second allele cannot be discounted when only a single allele is observed (OCME uses "Z")



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?

Signal Enhancement Techniques

- **Additional PCR cycles**
- **More sensitive kits** (Identifiler Plus and PowerPlex 16 HS)
- **Microcon cleanup** to remove salts that interfere with electrokinetic injection (MinElute PCR Purification Kit from Qiagen)
- Lower PCR volume (concentrates amplicon)
- Increase TaqGold/enzyme concentration
- Longer CE injection times and voltage
 - 10 s @ 3 kV = 30
 - 5 s @ 2 kV = 10

Reduced Volume PCR

- Possibility of lower volume PCR to effectively concentrate the amount of DNA in contact with the PCR reagents
 - Gaines *et al.* (2002) *J. Forensic Sci.* 47(6):1224-1237
 - Leclair *et al.* (2003) *J. Forensic Sci.* 48: 1001-1013
- Can samples be concentrated or can extraction volume be reduced?

Leclair et al. (2003) JFS 48:1001-1013

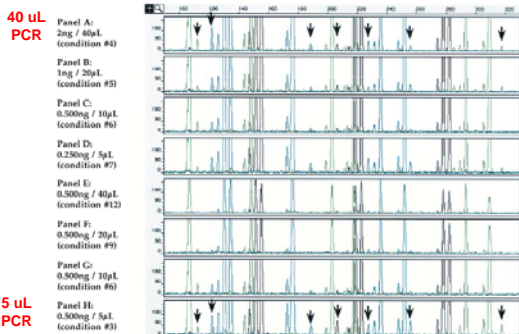


FIG. 5—Effects of a reduction of PCR reaction volume and DNA template concentration on amplification of a casework sample with a minor profile representing 2% of the total mixture.

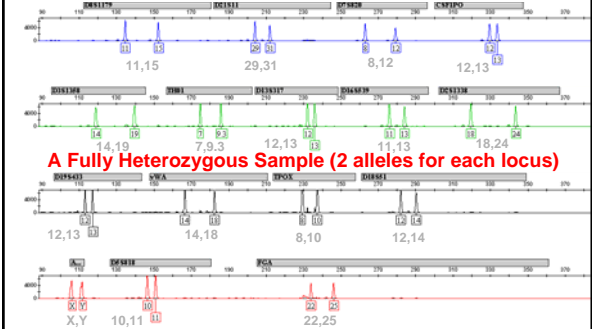
NIST Example LT-DNA Data

Experimental Design to Study LT-DNA Issues

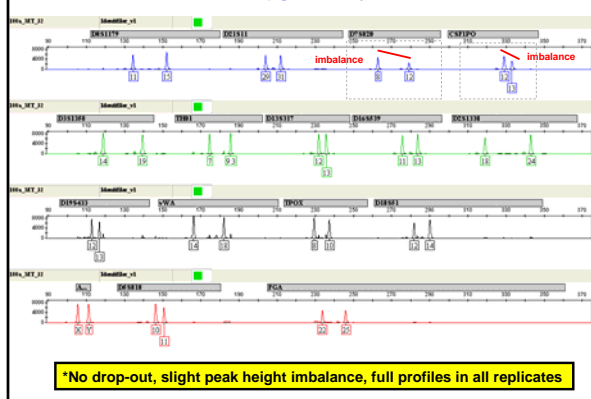
- Pristine DNA Samples
 - 2 single-source samples
 - **heterozygous for all loci tested** (permits peak height ratio studies)
- Low DNA Template Amounts
 - Dilutions made after DNA quantitation against NIST SRM 2372
 - **100 pg, 30 pg, and 10 pg** (1 ng tested for comparison purposes)
- Replicates
 - **5 separate PCR reactions** for each sample
- STR Multiplex Kits
 - **Identifiler Plus and PowerPlex 16 HS** (half-reactions)
- Increased Cycle Number
 - Identifiler Plus (**29 cycles and 32 cycles**; 28 for 1 ng)
 - PowerPlex 16 HS (**31 cycles and 34 cycles**; 30 for 1 ng)

Identifiler Plus (½ Reaction)
 1 ng @ 28 cycles

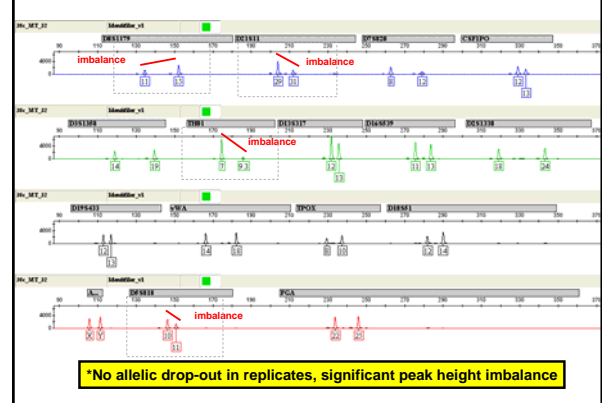
High signal, balanced peak heights (>0.80), no artifacts, low stutter



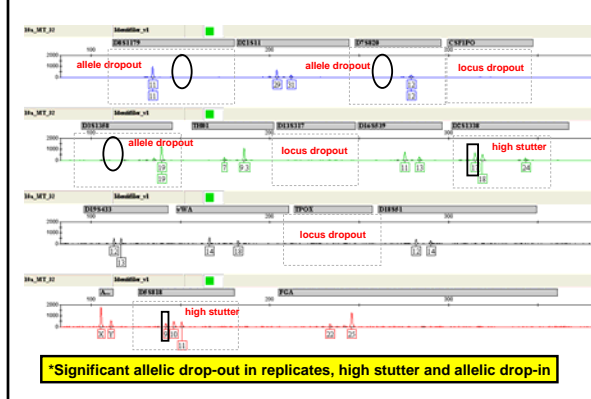
Identifiler Plus, 100 pg @ 32 cycles, ½ Reaction



Identifiler Plus, 30 pg @ 32 cycles, ½ Reaction

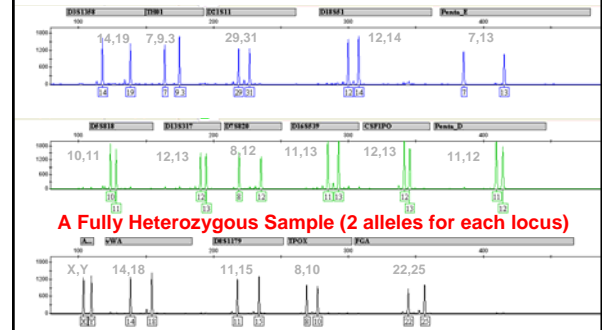


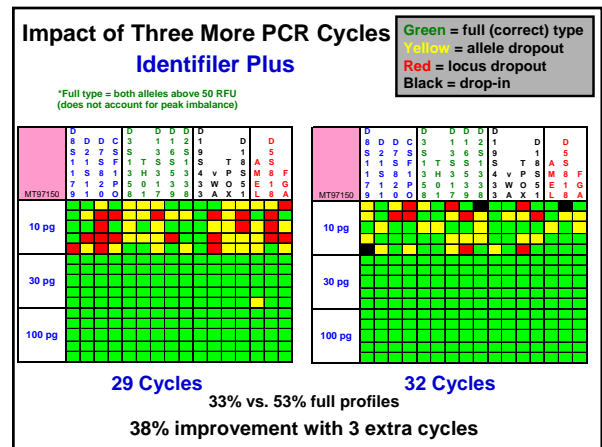
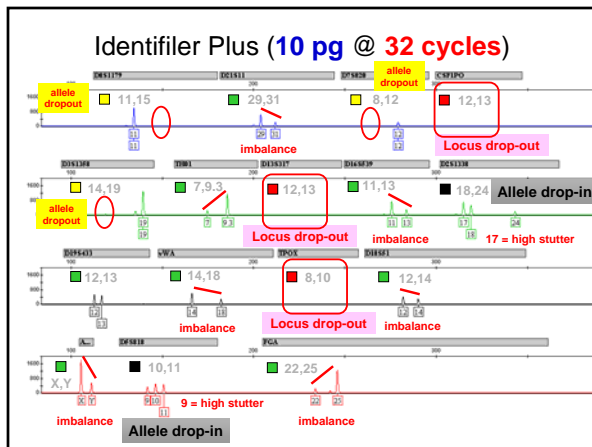
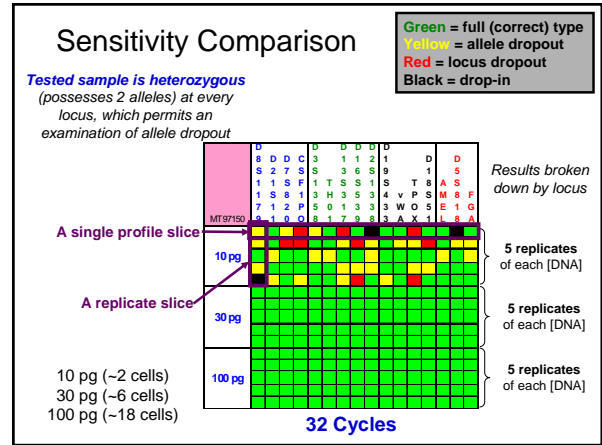
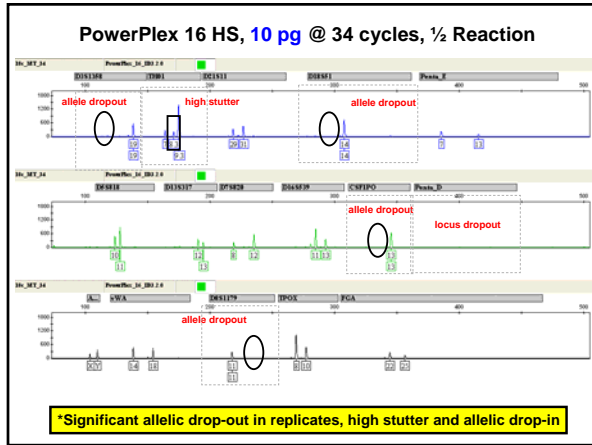
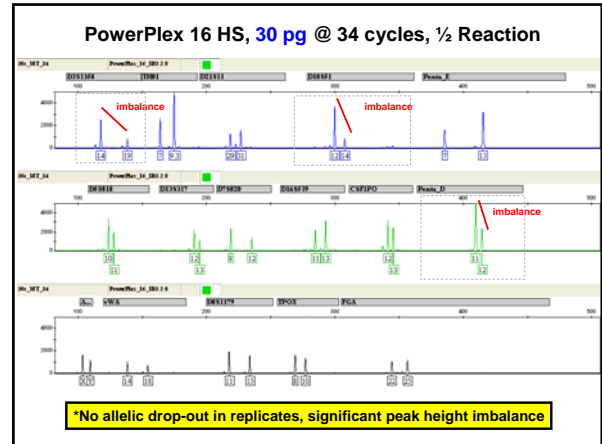
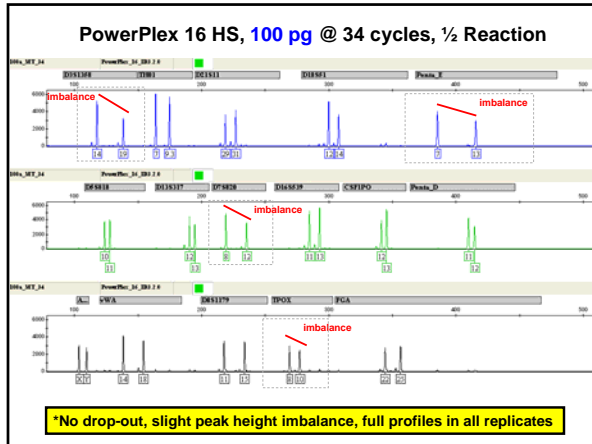
Identifiler Plus, 10 pg @ 32 cycles, ½ Reaction

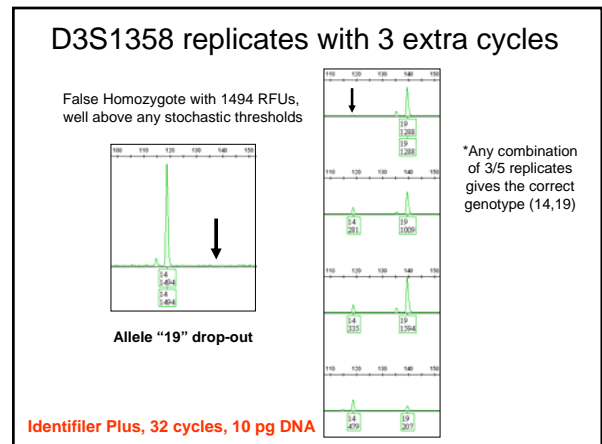
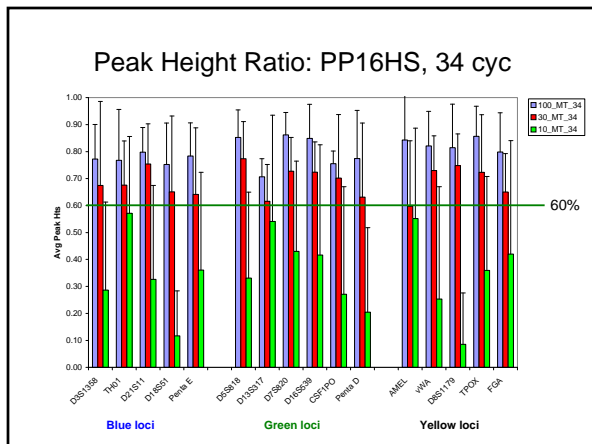
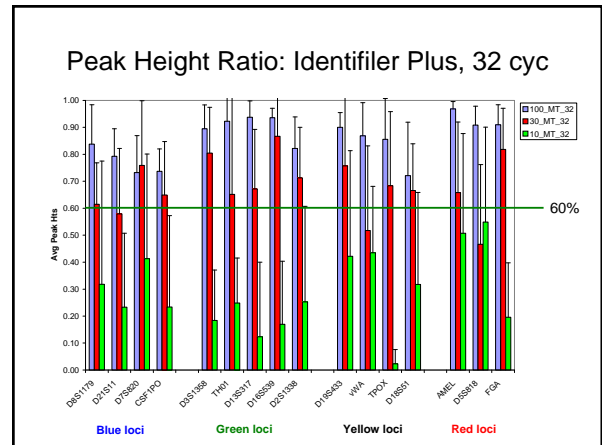
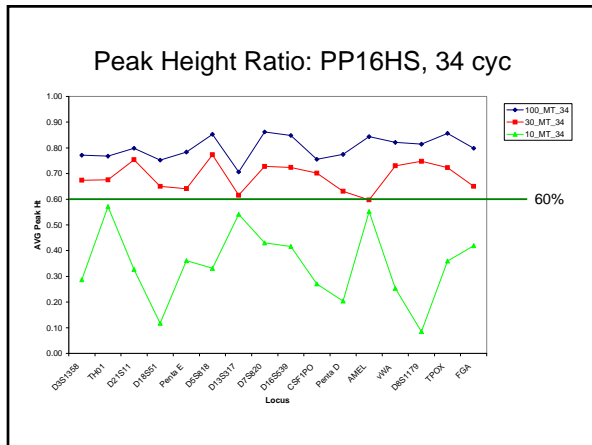
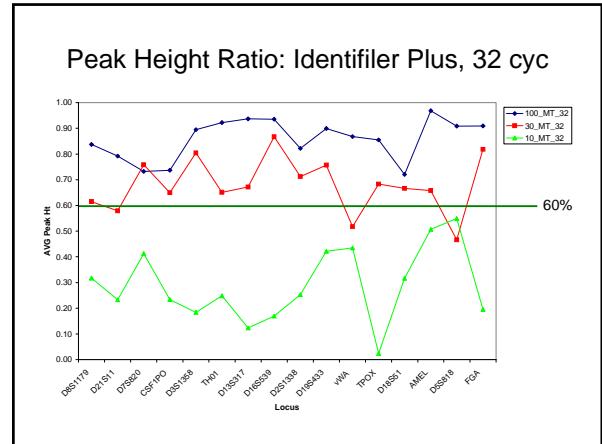
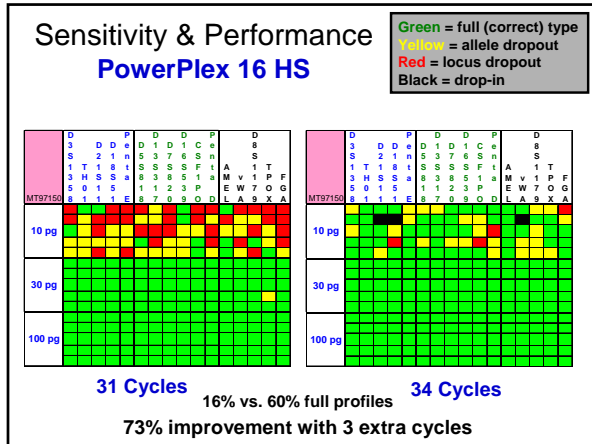


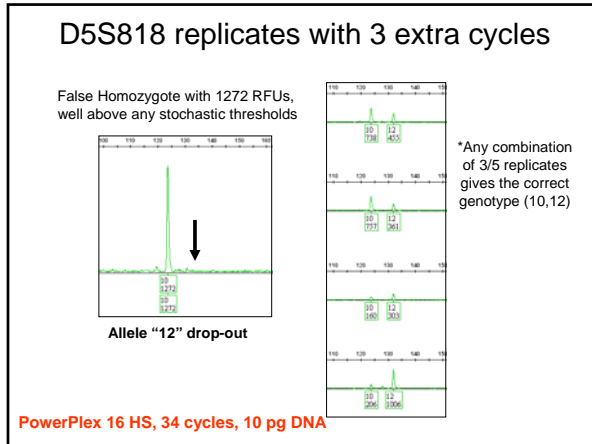
PowerPlex 16 HS (½ Reaction)
 1 ng @ 30 cycles

High signal, balanced peak heights (>0.80), no artifacts, low stutter

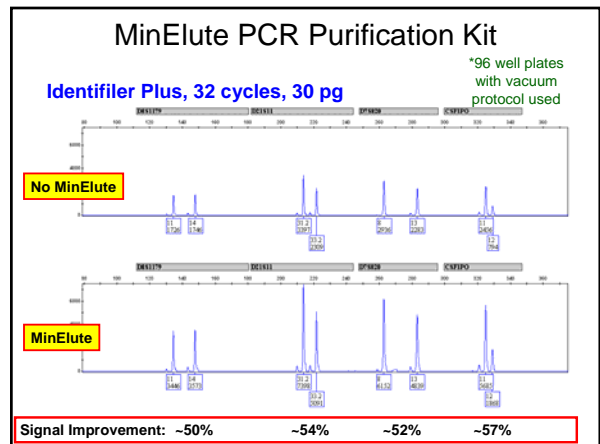
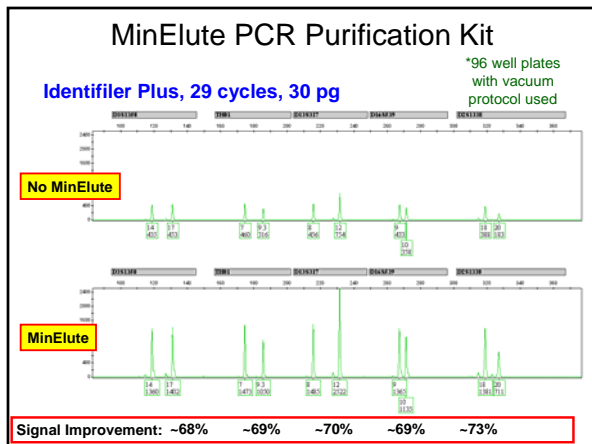
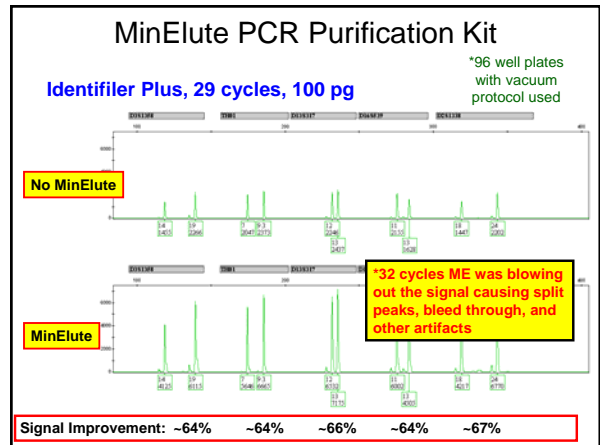
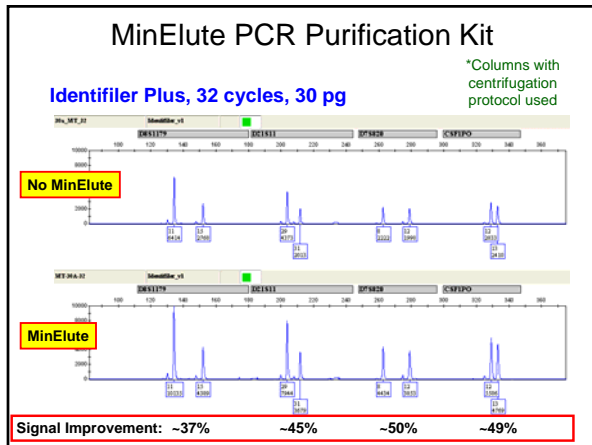


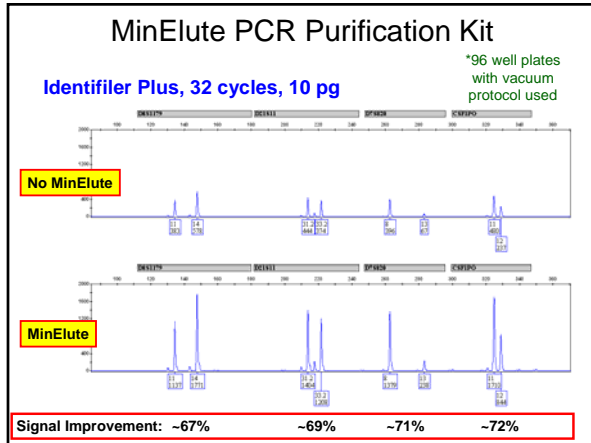
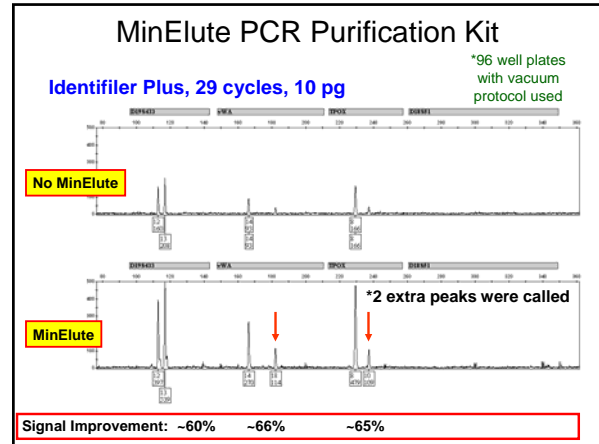
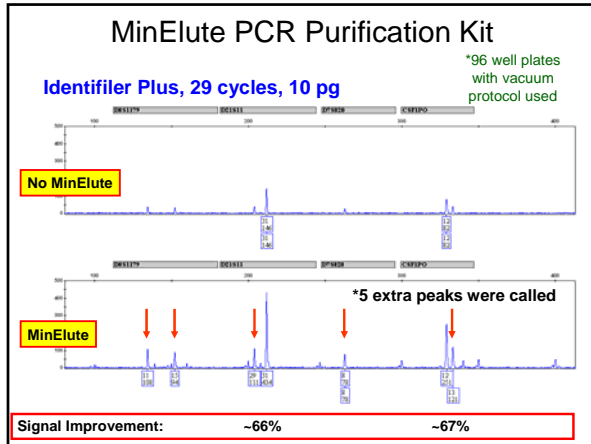






- ### Additional Methods of LT-DNA Testing and Future Studies at NIST
- Signal enhancing techniques
 - MinElute PCR purification kit (Qiagen) for salt removal in final product – **results shown**
 - Increasing CE injection voltage and time
 - Reduced volume PCR (concentrates amplicon)
 - Degraded DNA studies
 - LT-DNA mixture studies (**results shown**)

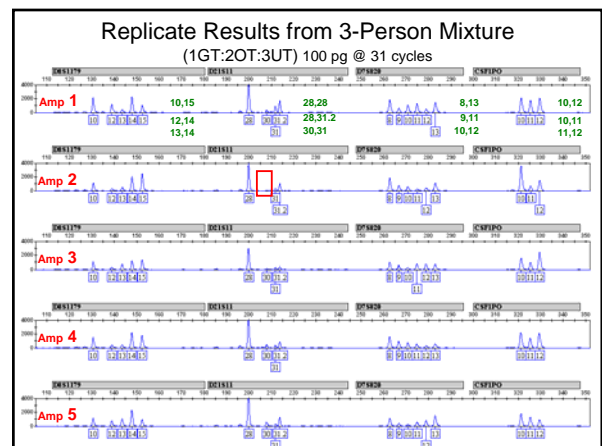
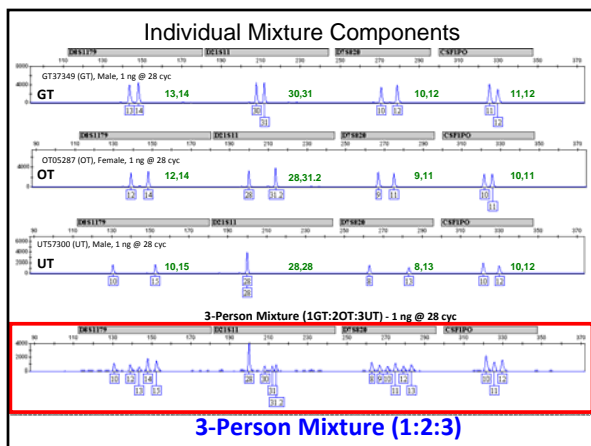
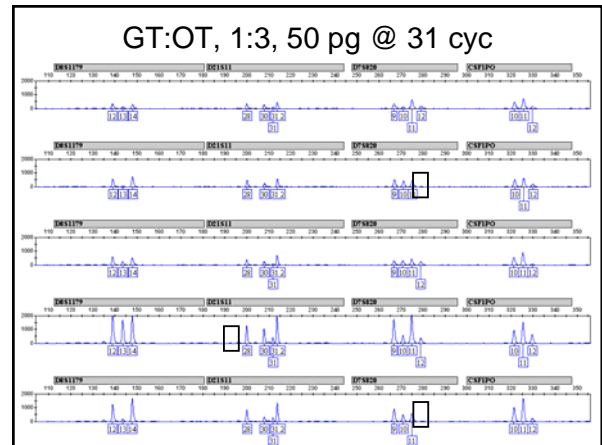
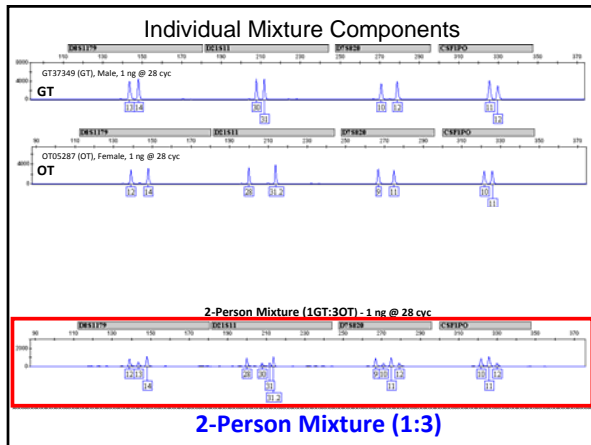
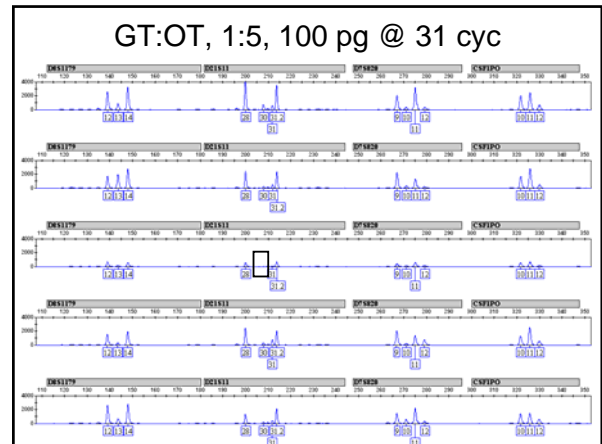
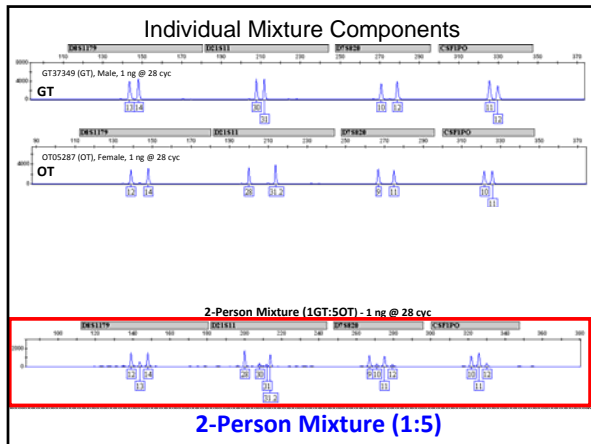




- Summary of Data Observed**
- The results with pristine full heterozygous samples demonstrate that replicate testing can produce reliable information with single source samples at low levels of DNA when consensus profiles are created.
 - Identifiler Plus with 32 cycles and PowerPlex 16 HS with 34 cycles were comparable in performance with low-level DNA analysis.
 - With 3 extra cycles, there was better recovery at 10 pg of DNA using both kits including less allelic and full locus drop-out. However, there is a greater potential for allele drop-in or high stutter.
 - MinElute PCR Purification Kits were successful in significantly increasing the signal for LT-DNA PCR products and resulted in extra peaks being called at 10 pg DNA samples.

Examination of LT-DNA Mixtures

- LT-DNA Mixture Samples**
- 2 samples (male and female) were mixed together at 1:3 and 1:5 – 1 ng (1:3 and 1:5) or 100 pg (1:5) or 50 pg (1:3) total DNA
 - 3 person mixture (2 males and female) were mixed together at 1:2:3 – 1 ng or 100 pg total DNA
 - Identifiler Plus (28 and 31 cycles) was tested (half reactions)
 - 5 replicates with 3 extra cycles
 - Variability of peak heights in replicates was observed
 - More minor contributor peaks were called with 3 extra cycles



Recent Updates to STRBase

- ### New Section of STRBase on LT-DNA
- Recently launched webpage
 - <http://www.cstl.nist.gov/biotech/strbase/LTDNA.htm>
 - Low-template DNA = LT-DNA
 - The LT-DNA section includes:
 - **Presentations from past LT-DNA talks and workshops**
 - **Validation data from our sensitivity studies** to illustrate problems and consensus profile solution to low levels of DNA testing
 - **Literature listing of pertinent articles** to help explain the issues involved in this topic

New STRBase Website on LT-DNA (LCN)

<http://www.cstl.nist.gov/biotech/strbase/LTDNA.htm>

The screenshot shows the website's navigation structure with categories like 'General Information', 'Forensic STR Informa...', and 'Information on Low Template / Low Copy Number DNA Testing'. A central banner features a presentation by Theresa Caragine, Ph.D., Deputy Director, dated October 19, 2009.

Complete Set of NIST Sensitivity Data Available on New LT-DNA Website

<http://www.cstl.nist.gov/biotech/strbase/LTDNA.htm>

NIST Sensitivity Data with low level DNA templates
 10 replicate amplifications for each condition with two fully heterozygous, single-source samples

STR kit - PCR conditions	Sample 1	Sample 2
Identifier - 28 cycles	100 pg	100 pg
	30 pg	30 pg
	10 pg	10 pg
Identifier - 31 cycles	100 pg	100 pg
	30 pg	30 pg
	10 pg	10 pg
PowerPlex 16 HS - 31 cycles	100 pg	100 pg
	30 pg	30 pg
	10 pg	10 pg
PowerPlex 16 HS - 34 cycles	100 pg	100 pg
	30 pg	30 pg
	10 pg	10 pg

Literature Listing on LT-DNA (LCN)

<http://www.cstl.nist.gov/biotech/strbase/LTDNA.htm>

Subdivided into categories

- Peer-reviewed literature (*containing data*)
- Reports (*evaluating the methodology*)
- Review articles (*commenting on other's data*)
- Non-peer reviewed literature (*representing the authors' opinions only*)

LT-DNA References

Peer-reviewed literature (containing data)

Links to papers when freely available

Buckleton, J. (2009) Validation issues around DNA typing of low level DNA. *Forensic Sci. Int. Genet.* 3: 235-240.

Casper, T., Mikolajczyk, S., Tamazi, J., Bayle, E., Behrman, J., Basso, R., Pinar, M. (2009) Validation of revised and interpretation protocols for low template DNA samples using AmpFISTR Identifiler. *Criminal Just. J.* 30: 205-217. [Link to paper](#)

Fendley, J., Taylor, A., Quake, P., Frazier, R., and Orphan, A. (1997) DNA fingerprinting from single cells. *Nature* 389(6612): 555-558.

Old, P., Whitlow, J., Flanagan, C., Brown, N., and Buckleton, J. (2005) An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. *Forensic Sci. Int.* 152(1-2): 17-40.

Publication on Scientific Issues of LT-DNA

The screenshot shows the Promega website navigation (Corporate, Products, Resources) and a featured article titled 'Scientific Issues with Analysis of Low Amounts of DNA' by John M. Butler* and Carolyn R. Hill, published online April 5, 2010. The authors are from the National Institute of Standards and Technology, Biochemical Science Division, Gaithersburg, Maryland, USA.

Additional Thoughts

- “Pay attention to your data”
 - Validate your individual PCR conditions
 - Set appropriate thresholds and implement interpretation guidelines
- DNA quantitation plays an important role
 - Anchor to NIST SRM 2372 or a traceable material
- Protocols for interpretation should reflect validation data

Future of LT-DNA

- New kits with increased sensitivity and resistance to inhibitors
 - PowerPlex 16 HS
 - Identifiler Plus
 - MiniFiler
 - PowerPlex ESX/ESI 16/17 Systems
 - NGM
- Technology keeps improving...

Acknowledgments

NIST Funding: Interagency Agreement 2008-DN-R-121 between the [National Institute of Justice](#) and NIST Office of Law Enforcement Standards

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

NIST Team for This Work



John Butler



Dave Duewer

**For LT-DNA
Collaborations
NYC OCME
Kyra McKay
Becky Mikulasovich
Theresa Caragine**

**A special thanks
to Applied
Biosystems and
Promega for
providing the kits
used in this study**

Contact Info: becky.hill@nist.gov, 301-975-4275