The Copenhagen Forensic Genetic Summer School
Advanced Topics in STR DNA Analysis
June 27-28, 2012

Low Template (LT) DNA Analysis

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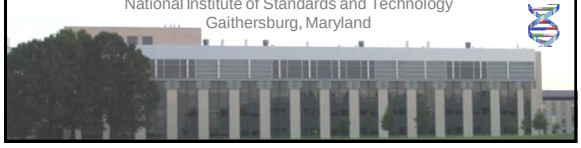
National Institute of Standards and Technology
Gaithersburg, Maryland



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)
- Conclusions and summary

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

Amounts of DNA Required

1985-1995 **RFLP/VNTRs** **50 ng – 1000 ng**

↓

1991-present (kits since 1996) **PCR/STRs** **0.5 – 2 ng**

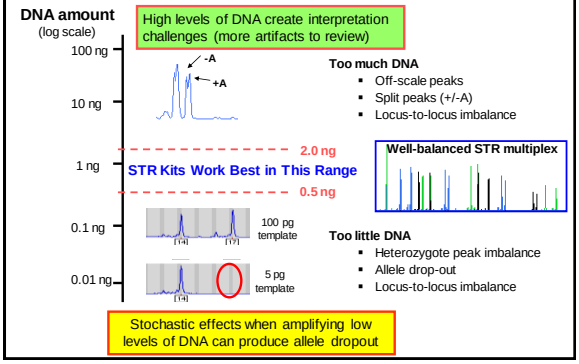
↓

1999-present **LT-DNA/STRs** **<0.1 ng**

LT-DNA testing extends the range of samples that may be attempted with DNA testing

Impact of DNA Amount into Multiplex PCR Reaction

We generally aim for 0.5-2 ng



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

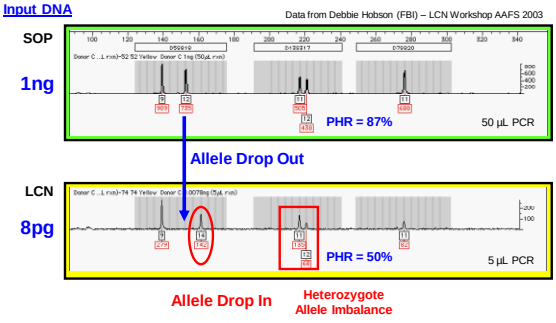
Gill, P. (2001) *Croatian Med. J.* 42(3): 229-232

- 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



Stochastic (Random) Effects with LT-DNA

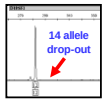
When Combined with Higher Sensitivity Techniques

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

Heterozygote Peak Imbalance

Identifiler, 30 pg DNA, 31 cycles

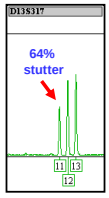
Allelic **Drop-out**



Identifiler, 30 pg DNA, 31 cycles

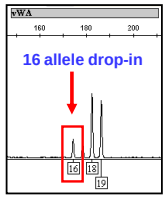
Gain of False Signal (***False Positive***)

Higher Stutter



Identifiler, 10 pg DNA, 31 cycles

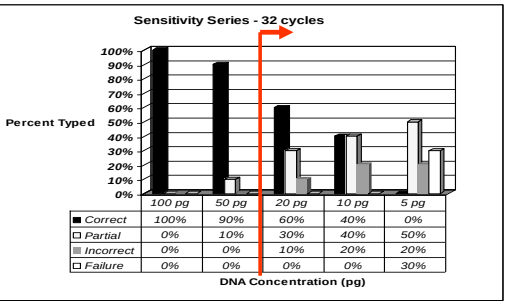
Allelic Drop-in



Identifiler, 10 pg DNA, 31 cycles

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

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

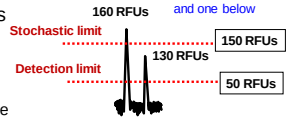
Setting Stochastic Thresholds with LT-DNA

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

Straddle Data

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

One allele peak above the stochastic threshold and one below



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**

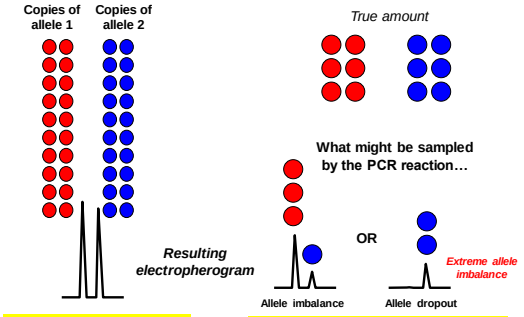
- **When stochastic fluctuation is present, interpreting data becomes problematic due to the potential for:**
  - **Allele dropout**
  - **Poorly defined mixture ratios**
  - **Low template DNA**
- Bottom line: Input levels of DNA should be sufficiently high to avoid straddle data. Mixture interpretation 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

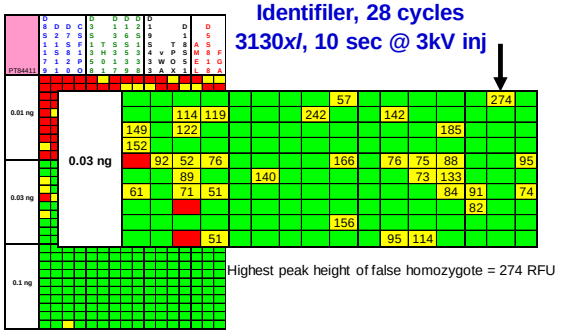
Issues with Data Below the Stochastic Threshold

- 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

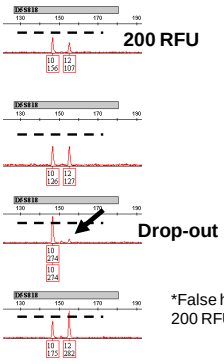
- Set based on data collected from your system
- Multiple samples, replicates, and concentrations are ideal to get a feel for how the system is working
  - We used 3 fully heterozygous samples with 10 replicates at 2 ng, 1 ng, 800 pg, 500 pg, 400 pg, 300 pg, 200 pg, 100 pg, 30 pg, & 10 pg
- Stochastic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

Setting Stochastic Thresholds



Stochastic Threshold for this data set = 275 RFU

Setting Stochastic Thresholds

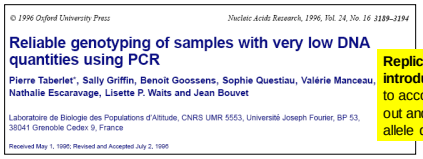


- **Stochastic threshold** – point at which data is considered reliable
- **"Level of risk"**: the higher you go, the less risk you have but you start to lose more data for statistics

*False homozygote because it is above the 200 RFU stochastic threshold

Approaches for LT-DNA Testing

Early Work on Replicate Testing with Low Levels of DNA



© 1996 Oxford University Press Nucleic Acids 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

ELSEVIER Forensic Science International 112 (2000) 17–40

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]

[a]Forensic Science Service, Priory House, Gooch Street North, Birmingham B36QQ, UK
[b]ESR, Private Bag 92021, Auckland, New Zealand

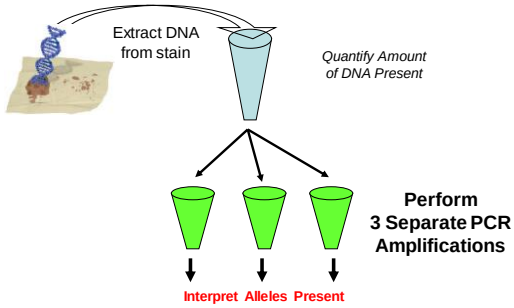
Received 9 December 1999; received in revised form 12 February 2000; accepted 15 February 2000

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

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

Typical LT-DNA Analysis Procedure



Develop a Consensus Profile
(based on replicate consistent results)

New Interpretation Rules Required for LT-DNA

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]

[a]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

Replicate LT-DNA Test Results from FSS

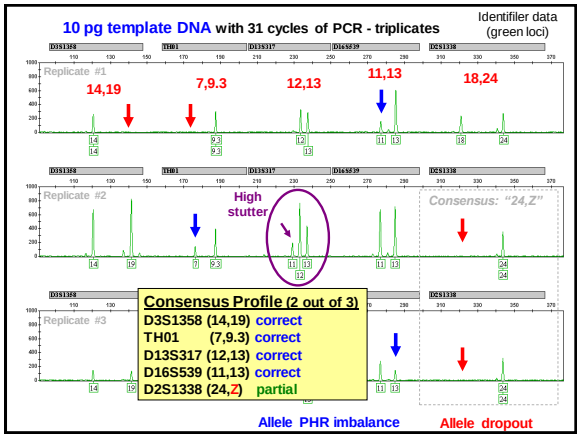
Gill, P. (2002) Role of short tandem repeat DNA in forensic casework in the UK--past, present, and future perspectives. *BioTechniques* 32(2): 366-385.

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

	Amelo	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'	--	15 F'	--	--	28 32.2	20 F'	--	16 F'	--
2	X F'	--	18 F'	15 F'	--	19 F'	--	--	12 F'	--	--
3	X F'	--	--	15 F'	--	--	--	--	--	--	17 F'
4	X F'	14 F'	18 F'	--	--	--	--	--	9 12	--	--
5	X F'	--	18 F'	--	--	18 F'	--	--	--	--	--
6	X F'	14 F'	--	--	--	19 F'	28 32.2	20 F'	--	12 F'	--
Consensus	X F'	14 F'	18 F'	15 F'	--	19 F'	28 32.2	20 F'	12 F'	--	--

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

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



Comparison of Approaches

What "LCN Labs" Are Doing

Replicate Amplification with Consensus Profile

Low amount of DNA examined

Stochastic effects

Amplification #1
Amplification #2
Amplification #3

Consensus Profile Developed
(from repeated alleles observed)

Interpretation Rules Applied
(based on validation experience)
e.g., specific loci may dropout more

Result can be and usually is Reliable & Reproducible

Single Amplification

Low amount of DNA examined

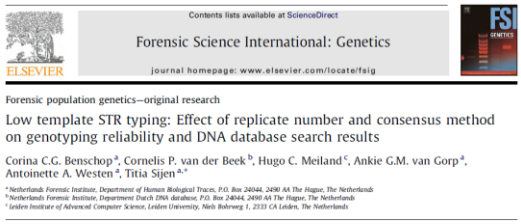
Stochastic effects

Amplification #1
(only a single test)

Result can be Unreliable

Individual results may vary but a consensus profile is reproducible
(based on our experience with sensitivity studies and replicate amplifications)

Recent Publication (2011) on LT-DNA Consensus Testing


Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

Forensic population genetics—original research

Low template STR typing: Effect of replicate number and consensus method on genotyping reliability and DNA database search results

Corina C.G. Benschop [a], Cornelis P. van der Beek [b], Hugo C. Meiland [c], Ankie G.M. van Gorp [a], Antoinette A. Westen [a], Titia Sijen [a,*]

[a] Netherlands Forensic Institute, Department of Human Biological Traces, P.O. Box 24044, 2490 AA The Hague, The Netherlands
[b] Netherlands Forensic Institute, Department Dutch DNA database, P.O. Box 24044, 2490 AA The Hague, The Netherlands
[c] Leiden Institute of Advanced Computer Science, Leiden University, Niels Bohrweg 1, 2333 CA Leiden, The Netherlands

A very thorough, comprehensive article describing the consensus method when using low template amounts of DNA: The best general approach is n/2 (2 of 3 reps, 2 of 4 reps, and 3 of 6 reps)

Consensus Approach = n/2

Table 5
Percentage detected alleles in the consensus profile for three consensus methods (n = number of PCR amplifications and x = requested level of reproducibility) per category of percentage detected alleles in standard STR typing.

Number of PCR amplifications (n)	Requested reproducibility (x)	LT method	Percentage detected alleles in standard STR typing			
			0–10%	10–25%	25–50%	>50%
			Average % detected alleles in consensus			
n = 3	x = 2	9 kV	38% ± 13%	82% ± 4%	97% ± 1%	99% ± 1%
n = 3	x = 2	28 + 6	57% ± 16%	88% ± 3%	98% ± 1%	99% ± 1%
n = 4	x = 2	9 kV	46% ± 14%	91% ± 3%	99% ± 1%	100% ± 0%
n = 4	x = 2	28 + 6	64% ± 16%	95% ± 2%	100% ± 0%	100% ± 0%
n = 6	x = 3	9 kV	42% ± 16%	90% ± 4%	99% ± 1%	100% ± 0%
n = 6	x = 3	28 + 6	63% ± 16%	96% ± 2%	100% ± 0%	100% ± 0%

4 replicates (n=4) with alleles present (reproducibility) in 2 of the 4 replicates shows improvement over 3 replicates and raising the replicates to 6 only shows little additional value

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

TECHNICAL NOTE

David Sweet,[1] D.M.D, Ph.D.; Miguel Lorente,[2] M.D., Ph.D.; José A. Lorente,[2] M.D., Ph.D.; Aurora Valenzuela,[2] M.D., Ph.D., B.D.S.; and Enrique Villanueva,[2] M.D., Ph.D.

An Improved Method to Recover Saliva from Human Skin: The Double Swab Technique

REFERENCE: Sweet D, Lorente M, Lorente JA, Valenzuela A, Villanueva E. An improved method to recover saliva from human skin: The double swab technique. J Forensic Sci 1997;42(2): 320–322.

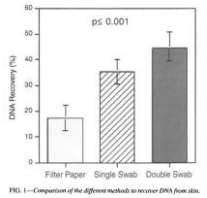


FIG. 1—*Comparison of the different methods to recover DNA from skin.*

Extraction Efficiency Results in the Literature

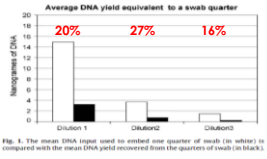


Fig. 1. The mean DNA input used to embed one quarter of swab (in white) is compared with the mean DNA yield recovered from the quarters of swab (in black).

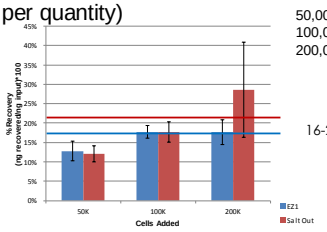
A. Colussi et al. "**Efficiency of DNA IQ System in recovering semen from cotton swab.**" Forensic Science International: Genetics Supplement Series 2 (2009) 87-88.

Liquid Blood Dilutions	Volume of Liquid Blood Extracted (µL)	BioRobot® EZ1, DNA (ng)	BioRobot® EZ1 with cRNA, DNA (ng)	Organic Extraction, DNA (ng)
1:10	0.1	8.025	10.000	7.900
1:50	0.02	0.215	2.250	1.840
1:250	0.004	0.050	0.260	0.263
1:1250	0.0008	0.000	0.040	0.038
1:2500	0.0004	0.000	0.013	0.000
		33%		33%

R. Kishore et al. "**Optimization of DNA Extraction from Low-Yield and Degraded Samples Using the BioRobot EZ1 ad BioRobot M48.**" J Forensic Sci, September 2006, Vol. 51, No 5.

Extracted Cell Line Efficiency

Swabbed 100 µL of a solution containing human epithelial cells in a Teflon tube (n=12 per quantity)



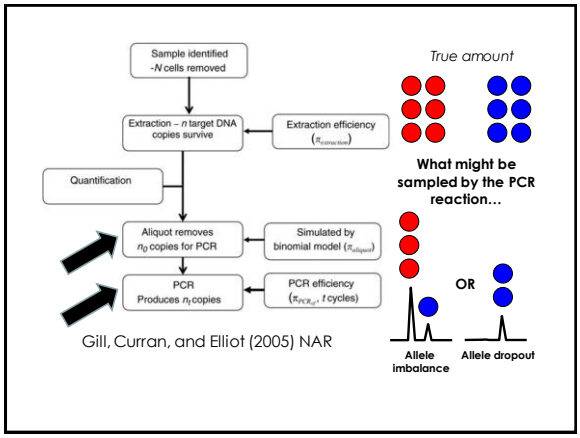
50,000 cells (300 ng)
100,000 cells (600 ng)
200,000 cells (1200 ng)

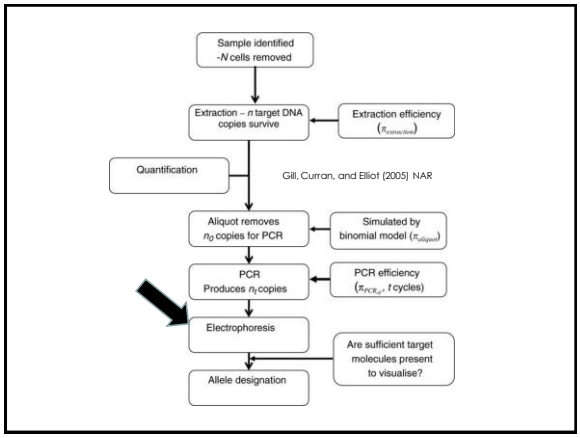
16-21% avg. recovery

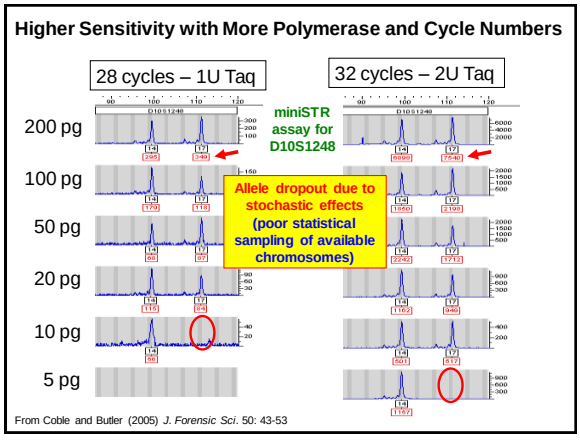
Why Does This Matter?

- Low extraction efficiency could lower sample quantity into the Low Template DNA (LT-DNA) range

1 ng → Extraction process, ~ 70-80% sample loss → 200 - 300 pg


Sample identified
-N cells removed
Extraction – n target DNA copies survive
Extraction efficiency
Quantification
Aliquot removes n0 copies for PCR
Simulated by binomial model
PCR
Produces n1 copies
PCR efficiency
t cycles)
Gill, Curran, and Elliot (2005) NAR
True amount
What might be sampled by the PCR reaction...
OR
Allele imbalance
Allele dropout


Sample identified
-N cells removed
Extraction – n target DNA copies survive
Extraction efficiency
Quantification
Gill, Curran, and Elliot (2005) NAR
Aliquot removes n0 copies for PCR
Simulated by binomial model
PCR
Produces n1 copies
PCR efficiency
t cycles)
Electrophoresis
Are sufficient target molecules present to visualise?
Allele designation


Higher Sensitivity with More Polymerase and Cycle Numbers
28 cycles – 1U Taq
32 cycles – 2U Taq
200 pg
100 pg
50 pg
20 pg
10 pg
5 pg
miniSTR assay for D10S1248
Allele dropout due to stochastic effects (poor statistical sampling of available chromosomes)
From Coble and Butler (2005) J. Forensic Sci. 50: 43-53

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

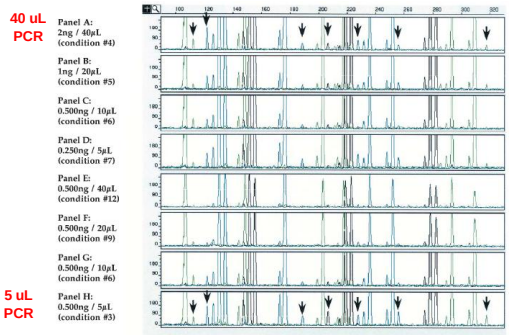


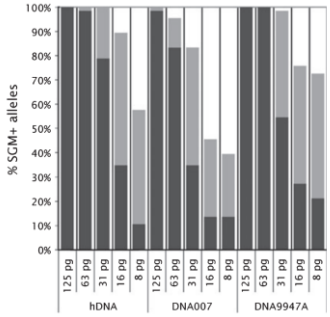
FIG. 5—*Effects of a reduction of PCR reaction volume and DNA template concentration on amplification of a casework sample with a minor profile representing 2% of the total mixture.*

Modified Procedures to Increase Sensitivity

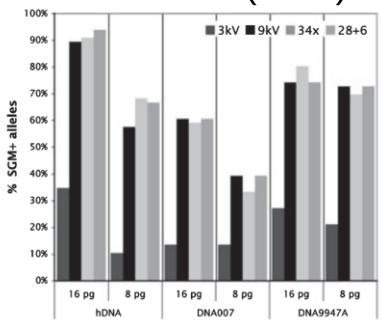
- Increased CE injection/voltage
  - Advantages: More amplicons are electrophoretically injected into the capillary
  - Disadvantages: Can increase the analytical and stochastic thresholds

- Westen *et al.* (2009) *J. Forensic Sci.* 54: 591-59 Higher Capillary Electrophoresis Injection Settings as an Efficient Approach to Increase the Sensitivity of STR Typing

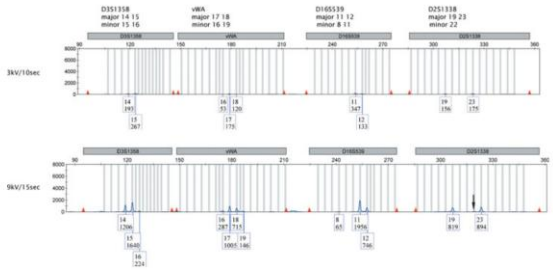
Westen et al. (2009)



Westen et al. (2009)



Westen et al. (2009)

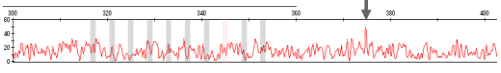


10:1 mixture SGM+ 28 cycles

Changes in Thresholds…

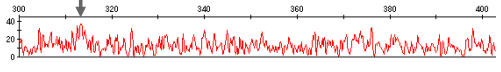
ABI 3500
1.2 kV, 15 sec (Default)

50 RFUs



37 RFUs

ABI 3500
1.2 kV, 10 sec



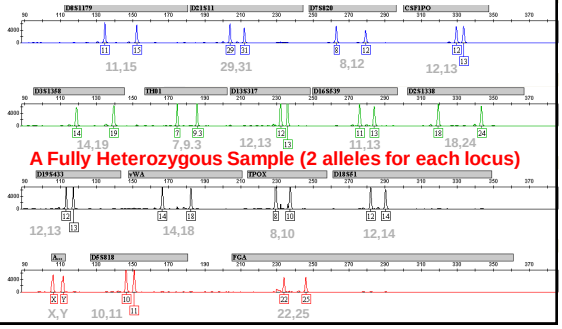
NIST Example LT-DNA Data

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)
  - PowerPlex16 HS (**31 cycles and 34 cycles**; 30 for 1 ng)

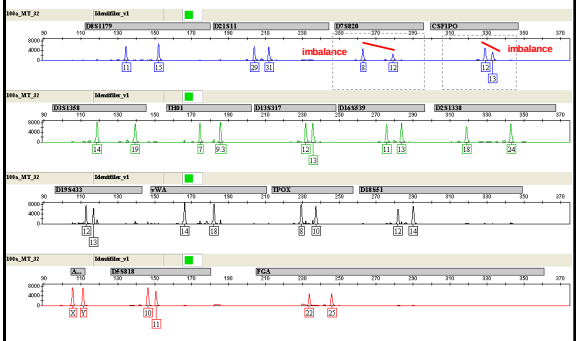
Identifiler Plus (½ Reaction)
1 ng @ 28 cycles

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



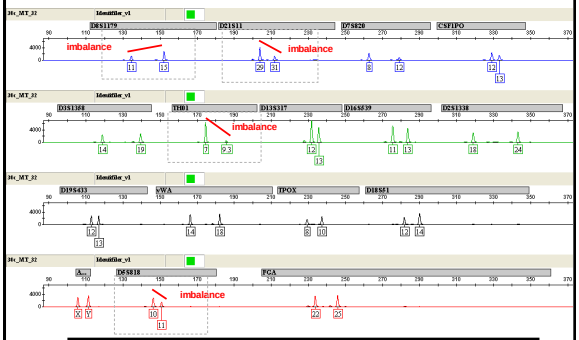
A Fully Heterozygous Sample (2 alleles for each locus)

Identifiler Plus, 100 pg @ 32 cycles, ½ Reaction



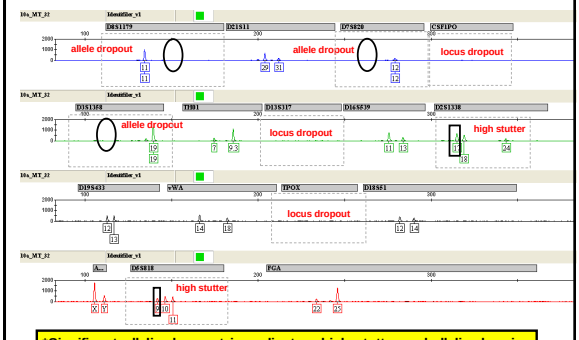
*No drop-out, slight peak height imbalance, full profiles in all replicates

Identifiler Plus, 30 pg @ 32 cycles, ½ Reaction



*No allelic drop-out in replicates, significant peak height imbalance

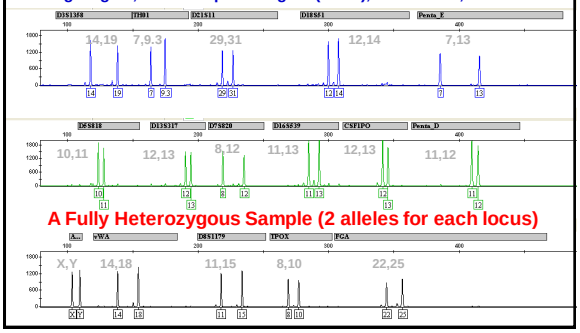
Identifiler Plus, 10 pg @ 32 cycles, ½ Reaction



*Significant allelic drop-out in replicates, high stutter and allelic drop-in

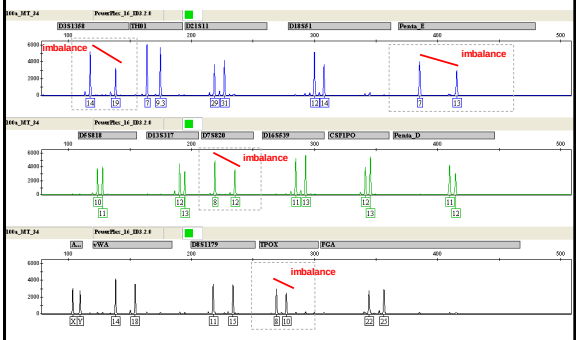
PowerPlex 16 HS (½ Reaction)
1 ng @ 30 cycles

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



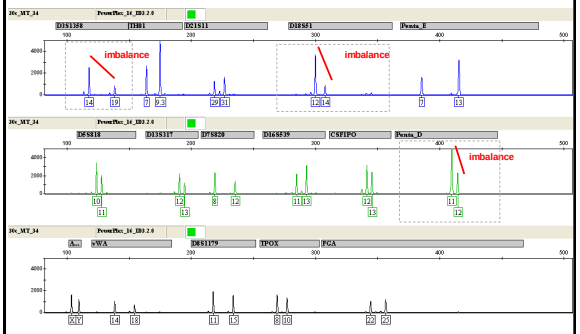
A Fully Heterozygous Sample (2 alleles for each locus)

PowerPlex 16 HS, 100 pg @ 34 cycles, ½ Reaction



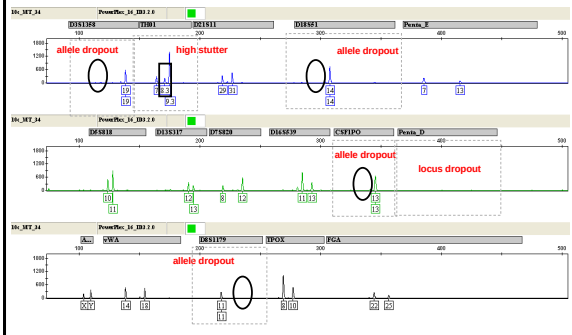
*No drop-out, slight peak height imbalance, full profiles in all replicates

PowerPlex 16 HS, 30 pg @ 34 cycles, ½ Reaction



*No allelic drop-out in replicates, significant peak height imbalance

PowerPlex 16 HS, 10 pg @ 34 cycles, ½ Reaction

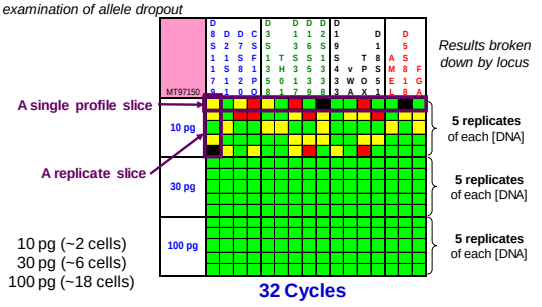


*Significant allelic drop-out in replicates, high stutter and allelic drop-in

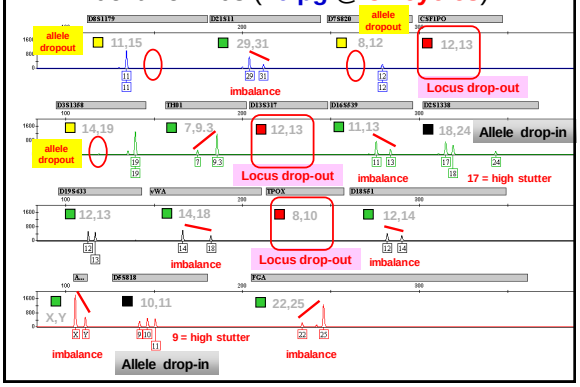
Sensitivity Comparison

Green = full (correct) type
Yellow = allele dropout
Red = locus dropout
Black = drop-in

Tested sample is heterozygous *(possesses 2 alleles) at every locus, which permits an examination of allele dropout*



Identifiler Plus (10 pg @ 32 cycles)

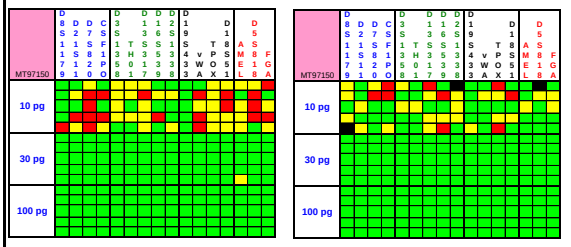


Impact of Three More PCR Cycles

Identifiler Plus

Green = full (correct) type
Yellow = allele dropout
Red = locus dropout
Black = drop-in

*Full type = both alleles above 50 RFU
(does not account for peak imbalance)



29 Cycles 32 Cycles

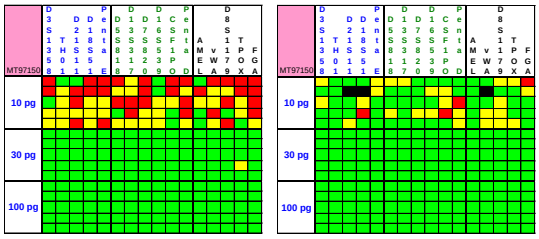
33% vs. 53% full profiles

38% improvement with 3 extra cycles

Sensitivity & Performance

PowerPlex 16 HS

Green = full (correct) type
Yellow = allele dropout
Red = locus dropout
Black = drop-in

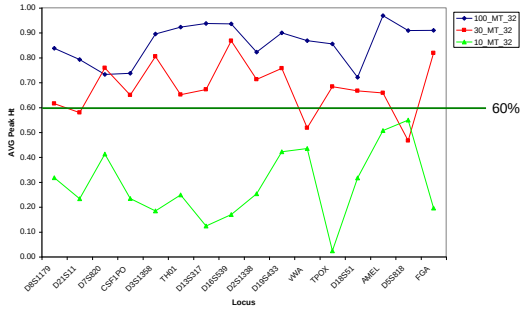


31 Cycles 34 Cycles

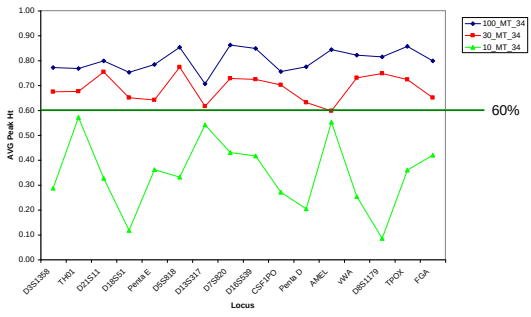
16% vs. 60% full profiles

73% improvement with 3 extra cycles

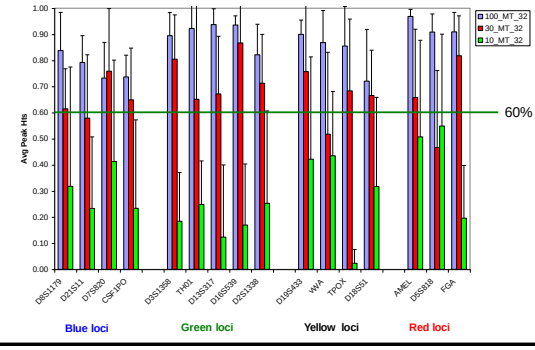
Peak Height Ratio: Identifiler Plus, 32 cyc



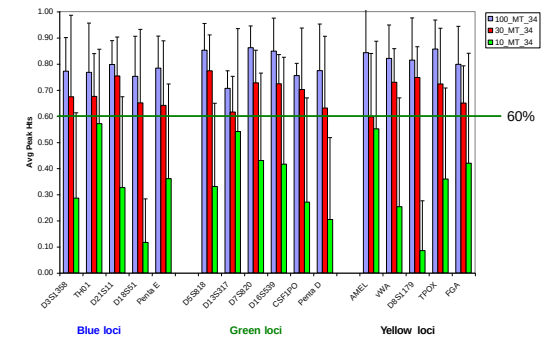
Peak Height Ratio: PP16HS, 34 cyc



Peak Height Ratio: Identifiler Plus, 32 cyc

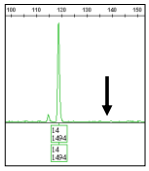


Peak Height Ratio: PP16HS, 34 cyc

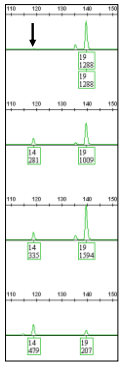


D3S1358 replicates with 3 extra cycles

False Homozygote with 1494 RFUs, well above any stochastic thresholds



Allele "19" drop-out

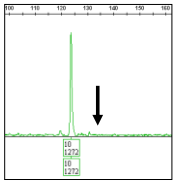


*Any combination of 3/5 replicates gives the correct genotype (14,19)

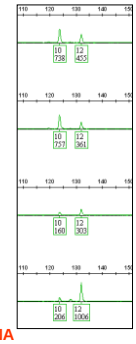
Identifiler Plus, 32 cycles, 10 pg DNA

D5S818 replicates with 3 extra cycles

False Homozygote with 1272 RFUs, well above any stochastic thresholds



Allele "12" drop-out



*Any combination of 3/5 replicates gives the correct genotype (10,12)

PowerPlex 16 HS, 34 cycles, 10 pg DNA

Additional Methods of LT-DNA Testing and Future Studies at NIST

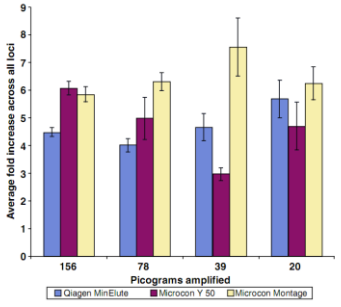
- Signal enhancing techniques
  - MinElute PCR purification kit (Qiagen) for salt removal in final product – results shown
  - Increasing CE injection voltage and time
  - Reduced volume PCR (concentrates amplicon)
- Degraded DNA studies
- LT-DNA mixture studies (results shown)

Modified Procedures to Increase Sensitivity

- Post-PCR Removal of Salts
  - Advantages: Less competition of charged ions and amplicons electrophoretically injected into the capillary
  - Disadvantages: Can increase the stochastic threshold, added expense and time for processing

○ Smit Simplified Low-Copy-Number DNA Analysis by Post-PCR Purification *Sci.* 52: 820-829

Smith and Ballantyne (2007)



Fold-increase in signal strength

Smith and Ballantyne (2007)

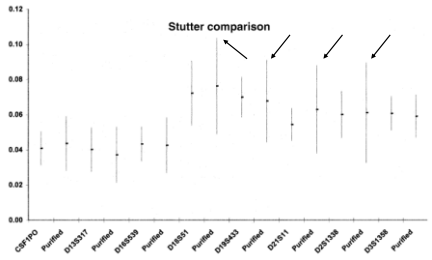
TABLE 1—*Increased sensitivity with post-PCR purification.*

PCR product	156 pg	78 pg	39 pg	20 pg	10 pg	5 pg
1.5 µL unpurified	30	15–25	5–9	0–1	0	0
1.5 µL purified	30	30	27–28	9–19	5–13	0–5
Entire purified product	N/D	30	30	30	22–28	12–27

PCR, polymerase chain reaction.

The number of alleles detected out of 30 possible alleles. Data indicate the range of alleles detected from four amplifications (two extractions amplified in duplicate). Complete profiles with or without purification were obtained for all samples amplified with 625–312 pg of DNA.

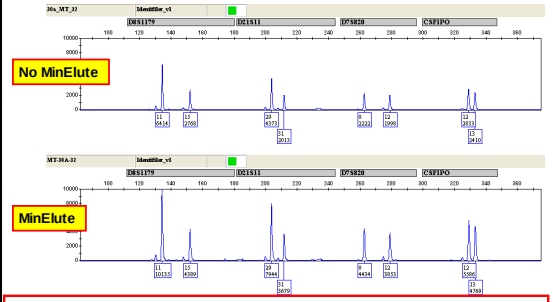
Smith and Ballantyne (2007)



MinElute PCR Purification Kit

*Columns with centrifugation protocol used

Identifiler Plus, 32 cycles, 30 pg

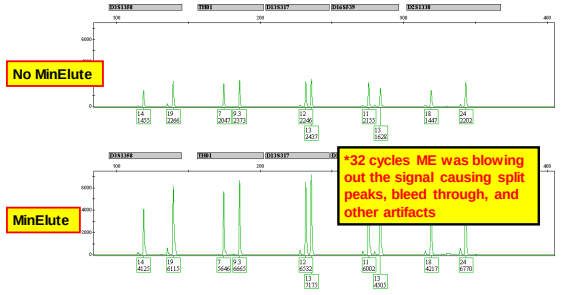


Signal Improvement:	~37%	~45%	~50%	~49%

MinElute PCR Purification Kit

*96 well plates with vacuum protocol used

Identifiler Plus, 29 cycles, 100 pg



Signal Improvement:	~64%	~64%	~66%	~64%	~67%

MinElute PCR Purification Kit

*96 well plates with vacuum protocol used

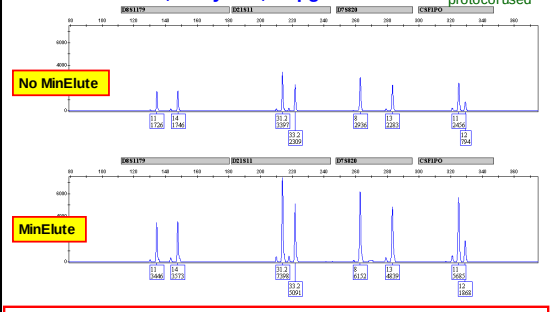
Identifiler Plus, 29 cycles, 30 pg

Signal Improvement:	~68%	~69%	~70%	~69%	~73%

MinElute PCR Purification Kit

*96 well plates with vacuum protocol used

Identifiler Plus, 32 cycles, 30 pg

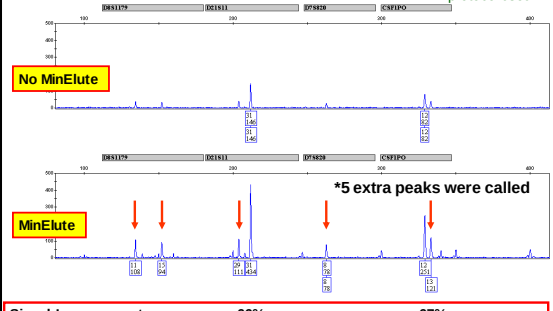


Signal Improvement:	~50%	~54%	~52%	~57%

MinElute PCR Purification Kit

*96 well plates with vacuum protocol used

Identifiler Plus, 29 cycles, 10 pg

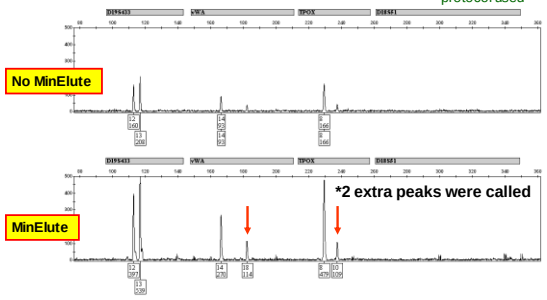


Signal Improvement:	~66%	~67%

MinElute PCR Purification Kit

*96 well plates with vacuum protocol used

Identifiler Plus, 29 cycles, 10 pg

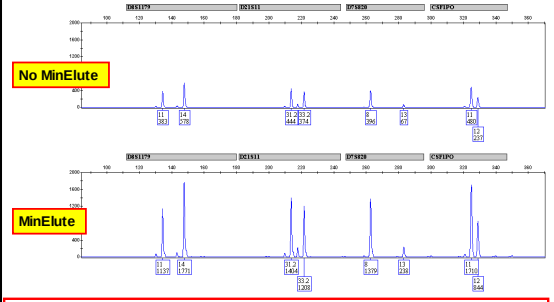


Signal Improvement: ~60% ~66% ~65%

MinElute PCR Purification Kit

*96 well plates with vacuum protocol used

Identifiler Plus, 32 cycles, 10 pg



Signal Improvement: ~67% ~69% ~71% ~72%

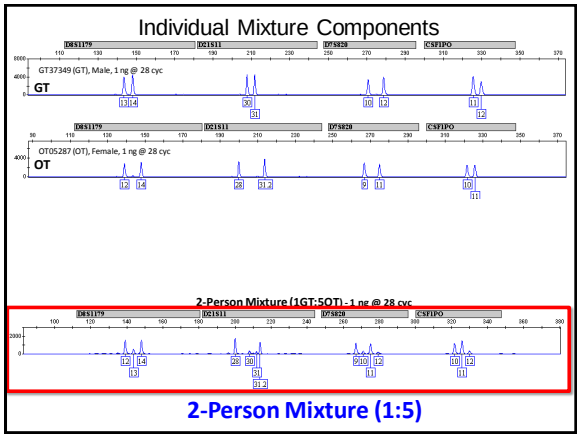
Summary of Data Observed

- 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.

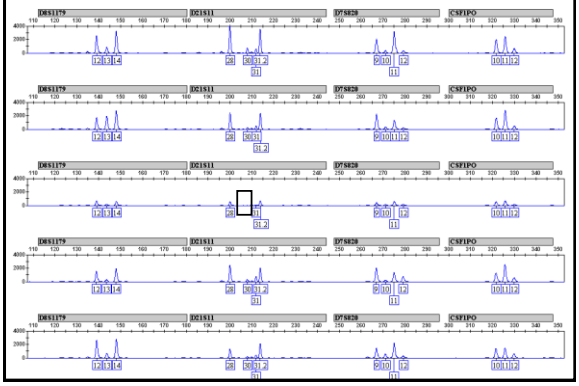
Examination of LT-DNA Mixtures

LT-DNA Mixture Samples

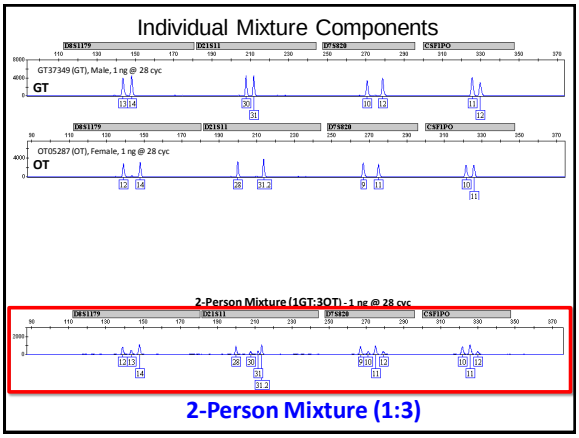
- 2 samples (male and female) were mixed together at 1:3 and 1:5 – 1 ng (1:3 and 1:5) or 100 pg (1:5) or 50 pg (1:3) total DNA
- 3 person mixture (2 males and female) were mixed together at 1:2:3 – 1 ng or 100 pg total DNA
- Identifiler Plus (28 and 31 cycles) was tested (half reactions)
- 5 replicates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



GT:OT, 1:5, 100 pg @ 31 cyc

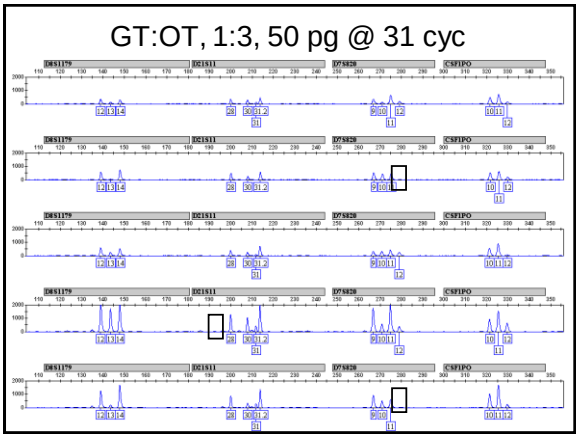


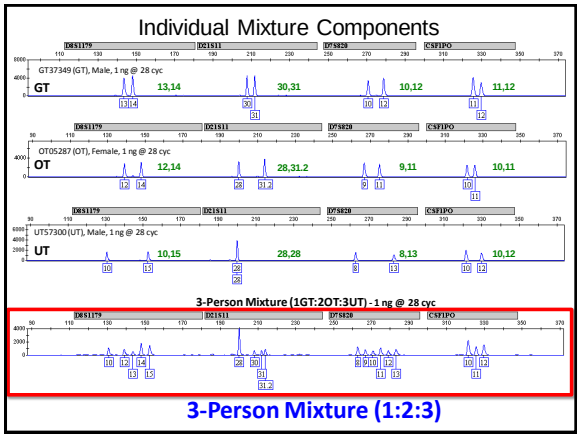
Individual Mixture Components

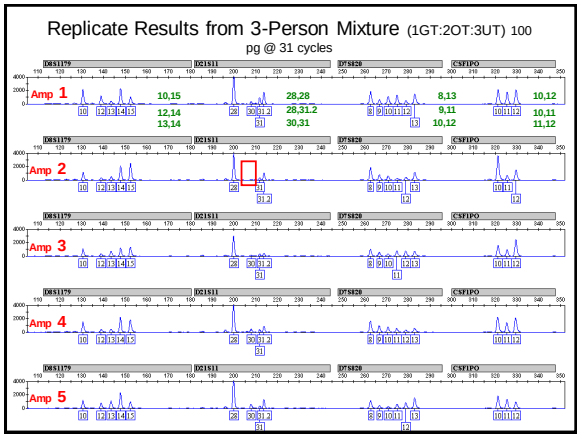


2-Person Mixture (1:3)

GT:OT, 1:3, 50 pg @ 31 cyc







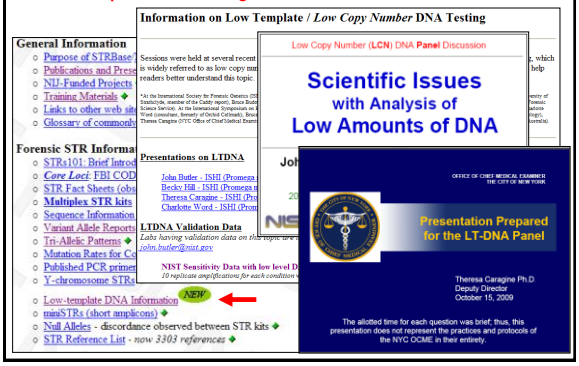
Recent Updates to STRBase

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

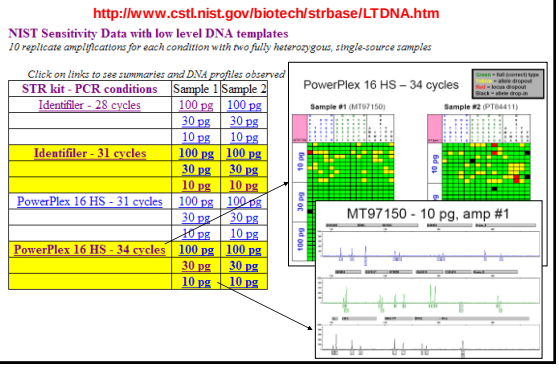
New STRBase Website on LT-DNA (LCN)

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



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

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

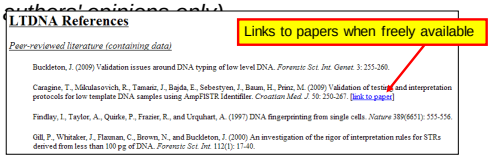


Literature Listing on LT-DNA (LCN)

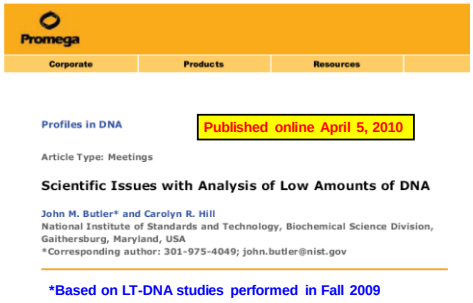
http://www.cstl.nist.gov/biotech/strbase/LTDNA.htm

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)*



Publication on Scientific Issues of LT-DNA



***Based on LT-DNA studies performed in Fall 2009**

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 SElect
- Technology keeps improving…

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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.

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Thank you for your attention

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