A Comparative Analysis of Low Template (LT) DNA Testing

Becky Hill and John Butler
National Institute of Standards and Technology

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Outline of Topics to Discuss

- Introduction to Low Template (LT) DNA
  - Brief description and historical perspective
- Challenges and Limitations of LT-DNA testing
  - Approaches to genotyping low template DNA
- Signal enhancement techniques: NIST data and results
  - Increasing PCR cycling with 3 extra cycles
  - MinElute PCR Purification Kit on PCR products
- Summary and conclusions

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

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

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

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

Stochastic (Random) Effects with LT-DNA

When Combined with Higher Sensitivity Techniques

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

Heterozygote Peak Imbalance

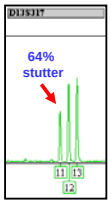
Identifiler, 30 pg DNA, 31 cycles

Allelic **Drop-out**

Identifiler, 30 pg DNA, 31 cycles

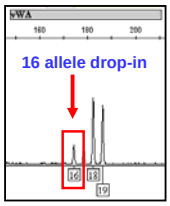
*Gain of False Signal (**False Positive**)*

Higher Stutter



Identifiler, 10 pg DNA, 31 cycles

Allelic Drop-in

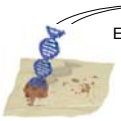


Identifiler, 10 pg DNA, 31 cycles

Suggestions for Optimal Results with LT-DNA

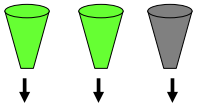
- Typically at least 2 – 3 PCR amplifications from the same DNA extract are performed to obtain consensus profiles
- An allele cannot be scored (considered real) unless it is present at least twice in replicate samples
- Extremely sterile environment is required for PCR setup to avoid contamination from laboratory personnel or other sources

Replicate Testing and Consensus Profiles



Extract DNA from stain

Quantify Amount of DNA Present



Perform 2 or 3 Separate PCR Amplifications

Interpret Alleles Present

Develop a Consensus Profile

(based on replicate consistent results)

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

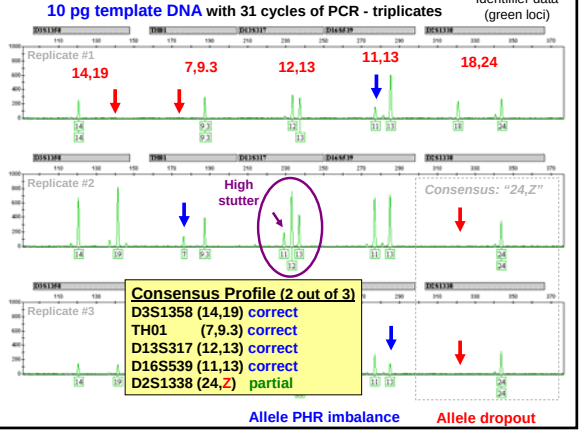
	Amelo	D19	D3	D8	THO	VWA	D21	FGA	D16	D18	D2
CONTROL	X X	14,14	18,18	15,15	7 9.3	19,19	28 32.2	20,23	9,12	12,16	17,23
Sample											
1	--	14 F'	--	15 F'	--	--	28 32.2	20 F'	--	16 F'	--
2	X F'	--	18 F'	15 F'	--	19 F'	--	--	12 F'	--	--
3	X F'	--	--	15 F'	--	--	--	--	--	--	17 F'
4	X F'	14 F'	18 F'	--	--	--	--	--	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")

10 pg template DNA with 31 cycles of PCR - triplicates

Identifiler data (green loci)



Consensus Profile (2 out of 3)

D3S1358 (14,19) correct
TH01 (7,9.3) correct
D13S317 (12,13) correct
D16S539 (11,13) correct
D2S1338 (24,Z) partial

Allele PHR imbalance

Allele dropout

Comparison of Approaches

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

What "LCN Labs" Are Doing

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)

Signal Enhancement Techniques

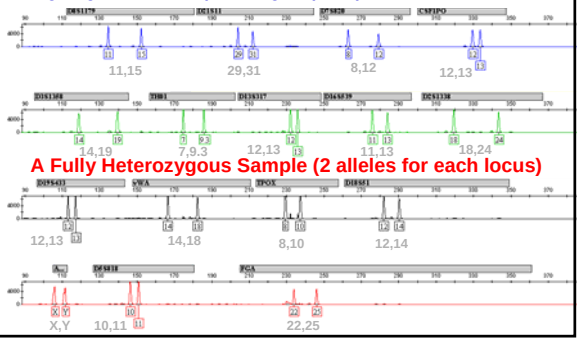
- Additional PCR cycles
- More sensitive kits (Identifiler Plus and PowerPlex 16 HS)
- Microcon cleanup to remove salts that interfere with electrokinetic injection (MinElute PCR Purification Kit from Qiagen)
- Lower PCR volume (concentrates amplicon)
- Increase TaqGold/enzyme concentration
- Longer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

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)

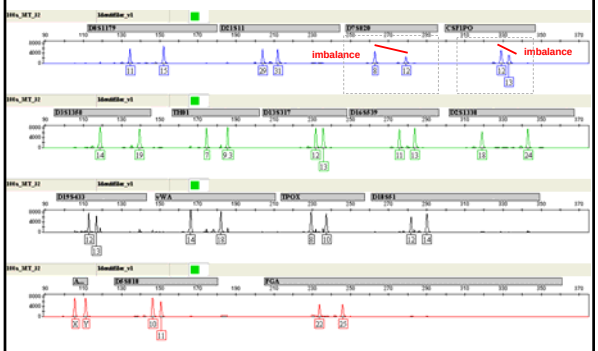
Identifiler Plus (½ Reaction)
1 ng @ 28 cycles

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



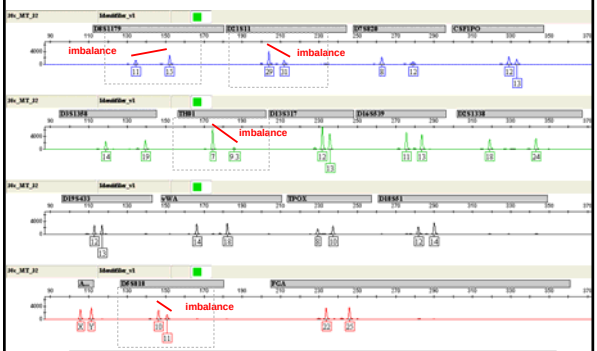
A Fully Heterozygous Sample (2 alleles for each locus)

Identifiler Plus, 100 pg @ 32 cycles, ½ Reaction



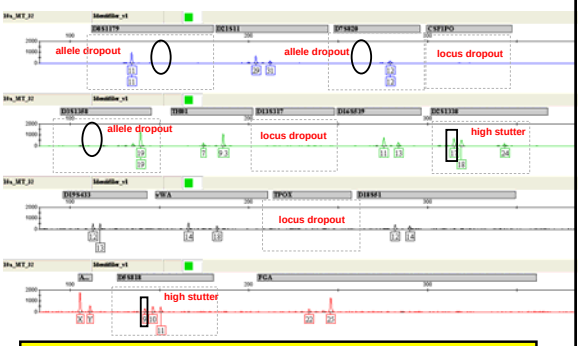
***No drop-out, slight peak height imbalance, full profiles in all replicates**

Identifiler Plus, 30 pg @ 32 cycles, ½ Reaction



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

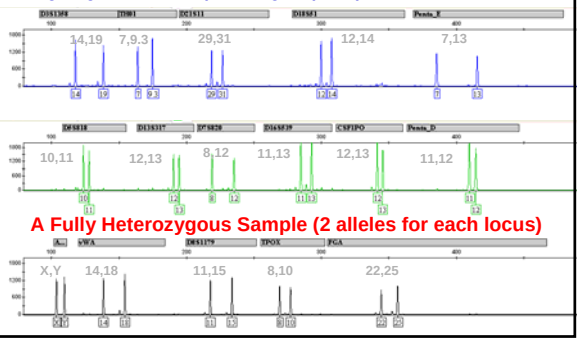
Identifiler Plus, 10 pg @ 32 cycles, ½ Reaction



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

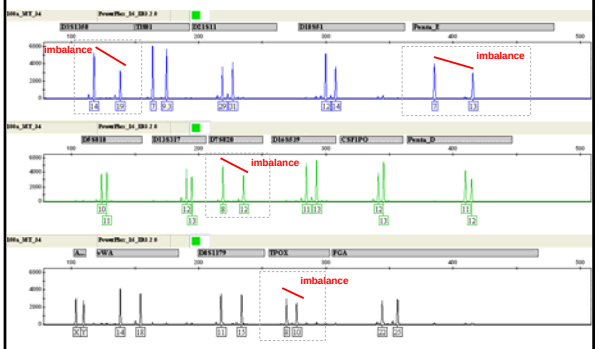
PowerPlex 16 HS (½ Reaction)
1 ng @ 30 cycles

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



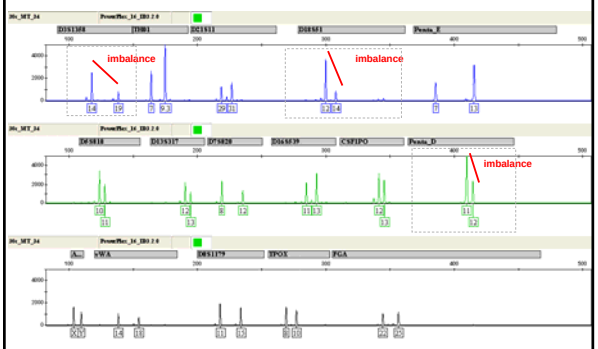
A Fully Heterozygous Sample (2 alleles for each locus)

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



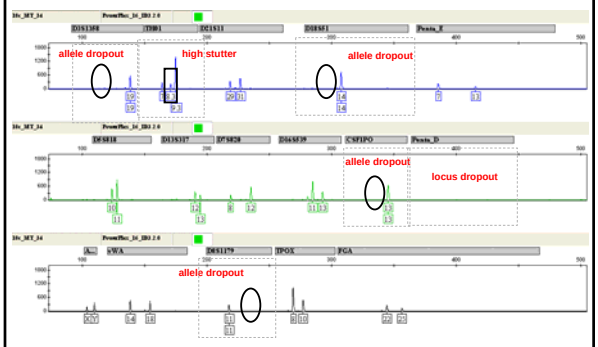
*No drop-out, slight peak height imbalance, full profiles in all replicates

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

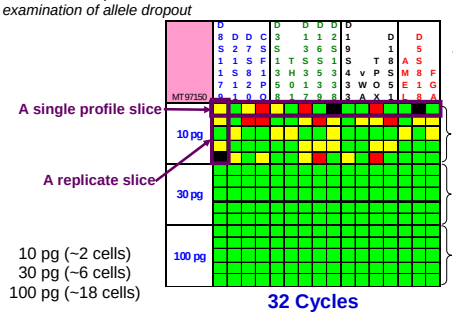


*Significant allelic drop-out in replicates, high stutter and allelic drop-in

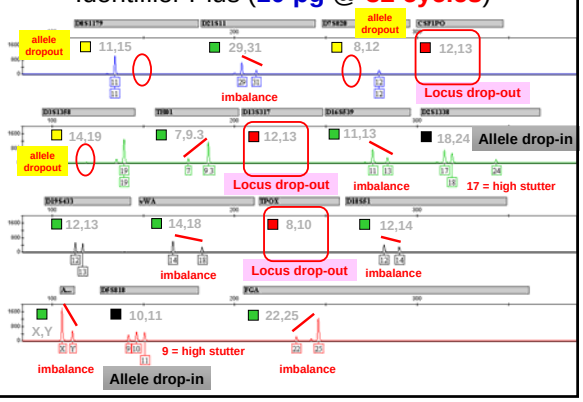
Sensitivity Comparison

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)

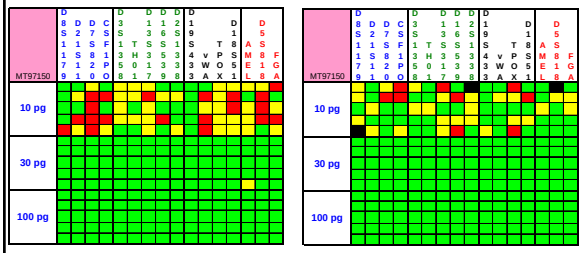


Impact of Three More PCR Cycles

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

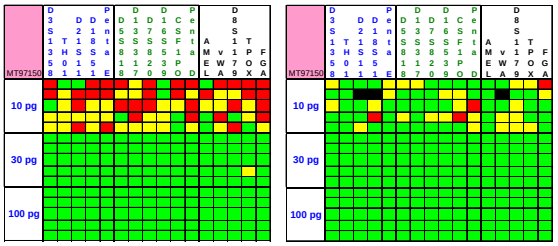
33% vs. 53% full profiles

38% improvement with 3 extra cycles

Sensitivity & Performance

PowerPlex 16 HS

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

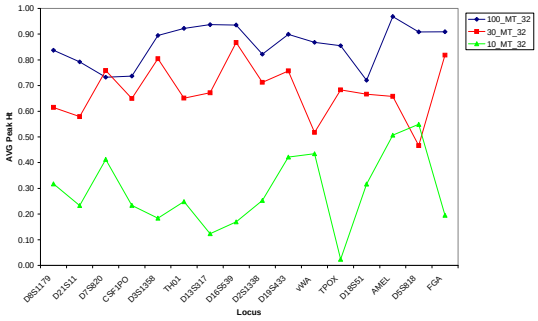


31 Cycles | 34 Cycles

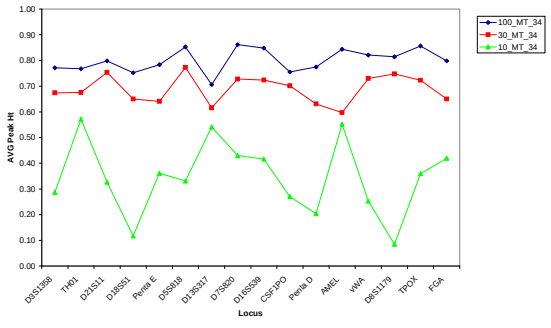
16% vs. 60% full profiles

73% improvement with 3 extra cycles

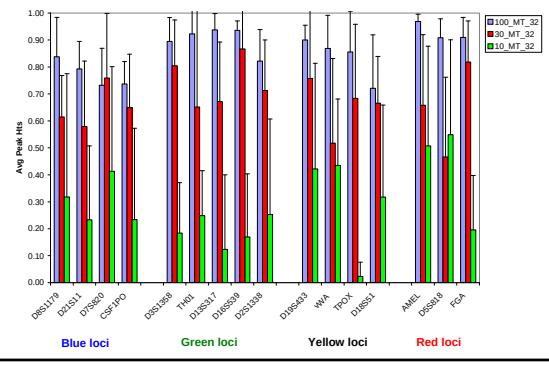
Peak Height Ratio: Identifiler Plus, 32 cyc



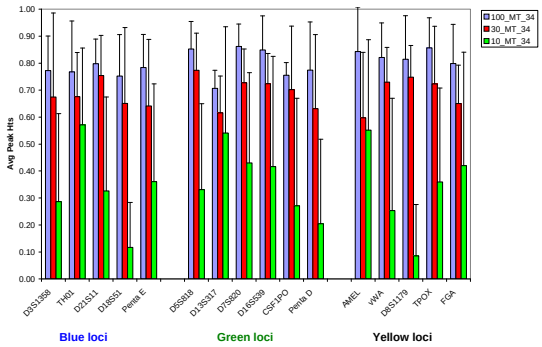
Peak Height Ratio: PP16HS, 34 cyc



Peak Height Ratio: Identifiler Plus, 32 cyc

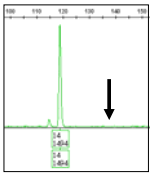


Peak Height Ratio: PP16HS, 34 cyc

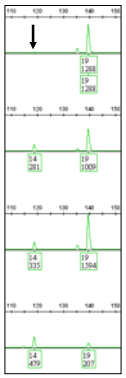


D3S1358 replicates with 3 extra cycles

False Homozygote with 1494 RFUs, well above any stochastic thresholds



Allele "19" drop-out

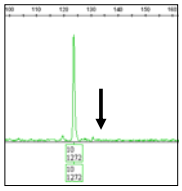


*Any combination of 3/5 replicates gives the correct genotype (14,19)

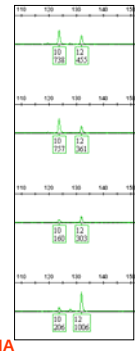
Identifiler Plus, 32 cycles, 10 pg DNA

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

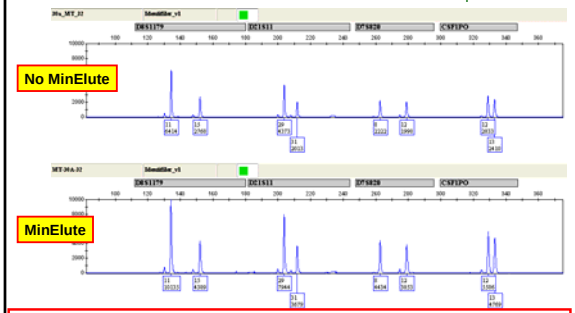
Additional Methods of LT-DNA Testing and Future Studies at NIST

- 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

MinElute PCR Purification Kit

*Columns with centrifugation protocol used

Identifiler Plus, 32 cycles, 30 pg

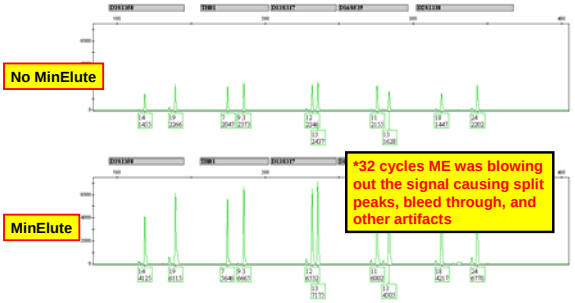


Signal Improvement:	~37%	~45%	~50%	~49%

MinElute PCR Purification Kit

*96 well plates with vacuum protocol used

Identifiler Plus, 29 cycles, 100 pg

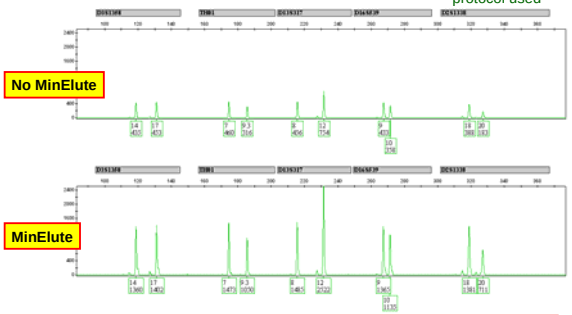


Signal Improvement:	~64%	~64%	~66%	~64%	~67%

MinElute PCR Purification Kit

Identifiler Plus, 29 cycles, 30 pg

*96 well plates with vacuum protocol used

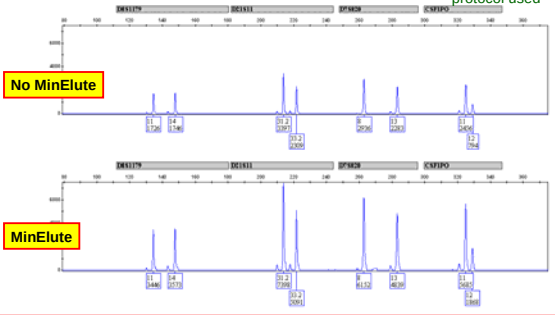


Signal Improvement: ~68% ~69% ~70% ~69% ~73%

MinElute PCR Purification Kit

Identifiler Plus, 32 cycles, 30 pg

*96 well plates with vacuum protocol used

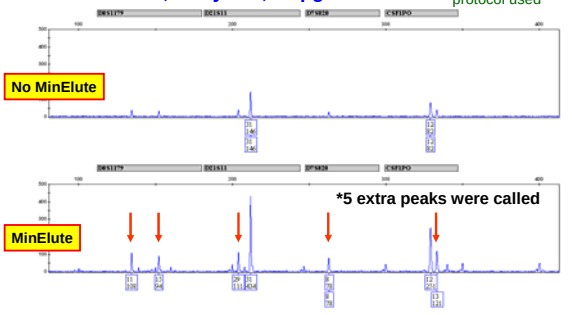


Signal Improvement: ~50% ~54% ~52% ~57%

MinElute PCR Purification Kit

Identifiler Plus, 29 cycles, 10 pg

*96 well plates with vacuum protocol used

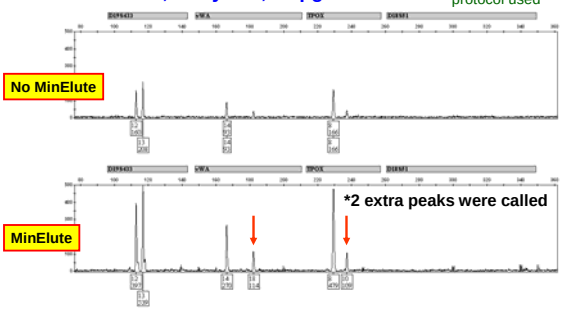


Signal Improvement: ~66% ~67%

MinElute PCR Purification Kit

Identifiler Plus, 29 cycles, 10 pg

*96 well plates with vacuum protocol used

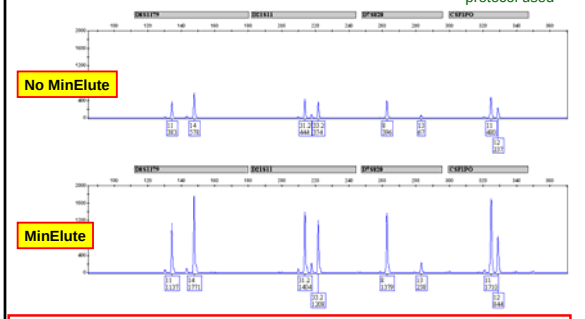


Signal Improvement: ~60% ~66% ~65%

MinElute PCR Purification Kit

Identifiler Plus, 32 cycles, 10 pg

*96 well plates with vacuum protocol used



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

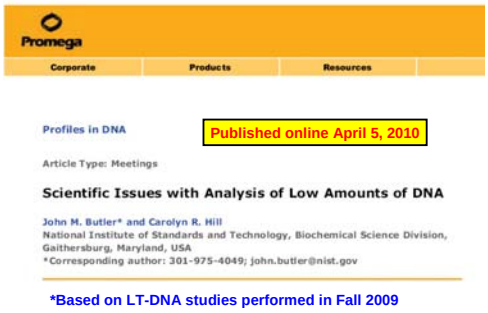
Conclusions

- 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.

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

Publication on Scientific Issues of LT-DNA


Promega

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Profiles in DNA

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Scientific Issues with Analysis of Low Amounts of DNA

John M. Butler* and Carolyn R. Hill
National Institute of Standards and Technology, Biochemical Science Division, Gaithersburg, Maryland, USA
*Corresponding author: 301-975-4049; john.butler@nist.gov

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

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NIST Team for This Work


John Butler


Dave Duewer

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