Low Template (LT) DNA Analysis

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Technical Leaders Meeting

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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
  - Approaches to genotyping low template DNA
  - 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

- Work with **<100 pg genomic DNA** (~15-17 diploid copies of nuclear DNA markers such as STRs)
- Data below stochastic threshold level where PCR amplification is not as reliable (determined by each laboratory; typically 150-250 RFUs)
- Enhancing sensitivity of detection (34 cycles instead of 28 cycles)
- Having too few copies of DNA template to ensure reliable PCR amplification
- Often the minor component of mixture samples consists of low level DNA template amounts

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

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

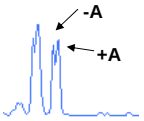
Impact of DNA Amount into Multiplex PCR Reaction

We generally aim for 0.5-2 ng

DNA amount
(log scale)

High levels of DNA create interpretation challenges (more artifacts to review)

100 ng



10 ng

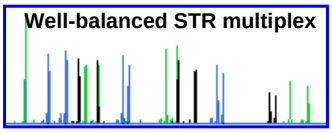
Too much DNA
- Off-scale peaks
- Split peaks (+/-A)
- Locus-to-locus imbalance

2.0 ng

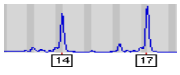
1 ng

STR Kits Work Best in This Range

0.5 ng



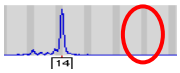
0.1 ng



100 pg template

Too little DNA
- Heterozygote peak imbalance
- Allele drop-out
- Locus-to-locus imbalance

0.01 ng



5 pg template

Stochastic effects when amplifying low levels of DNA can produce allele dropout

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

http://www.cstl.nist.gov/biotech/strbase/pub_pres/AAFS2006_qPCR_LCN.pdf

Technical Aspects:
LT-DNA Challenges, Approaches for Testing, and Example Data

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)

Reagent contamination

- Data interpretation is more complicated (due to stochastic variation during PCR amplification):
  - Allele drop-out
  - Allele drop-in
  - Increased stutter products

LT-DNA profiles may be interpreted with careful guidelines

- May not be able to associate DNA profile with bloodstain or other visual evidence

Comparison of STR Kit Amplification SOP with LT-DNA

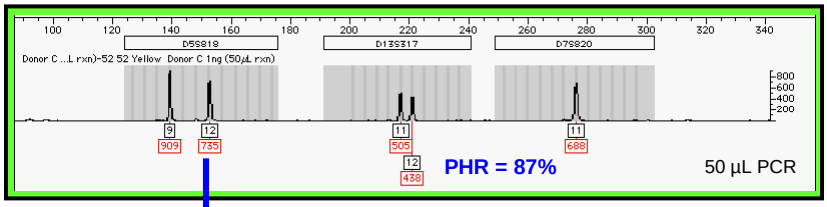
Using the Same DNA Donor

Input DNA

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

SOP

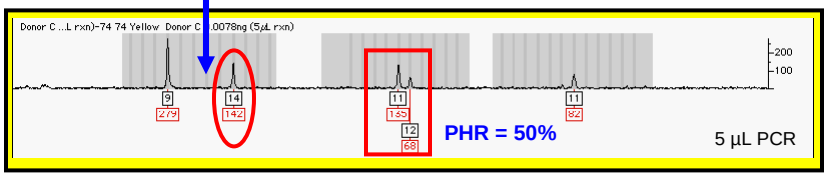
1ng



Allele Drop Out

LCN

8pg



Allele Drop In

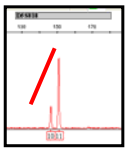
Heterozygote Allele Imbalance

Stochastic (Random) Effects with Low DNA

When Combined with Higher Sensitivity Techniques

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

Heterozygote Peak Imbalance



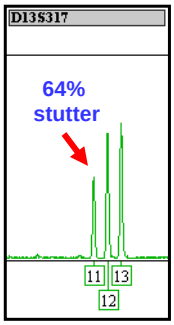
Identifiler, 30 pg DNA, 31 cycles

Allelic **Drop-out**

Identifiler, 30 pg DNA, 31 cycles

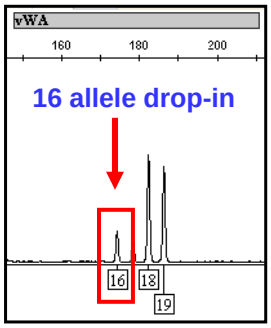
*Gain of False Signal (**False Positive**)*

Higher Stutter



Identifiler, 10 pg DNA, 31 cycles

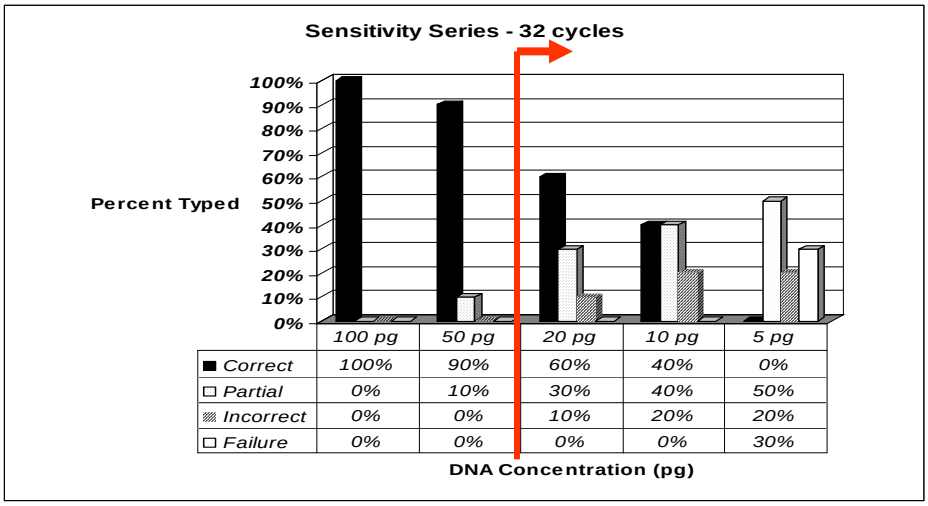
Allelic Drop-in



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



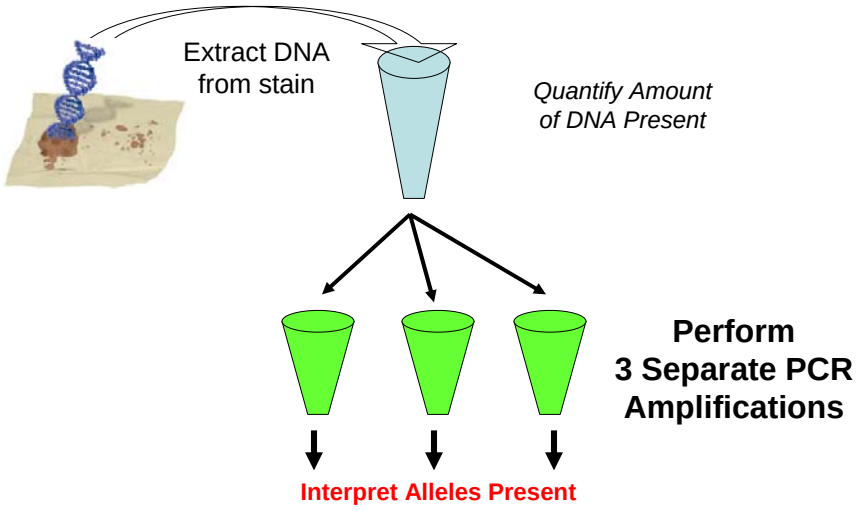
	100 pg	50 pg	20 pg	10 pg	5 pg
Correct	100%	90%	60%	40%	0%
Partial	0%	10%	30%	40%	50%
Incorrect	0%	0%	10%	20%	20%
Failure	0%	0%	0%	0%	30%

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

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]

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[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")

NIST Example LT-DNA Data

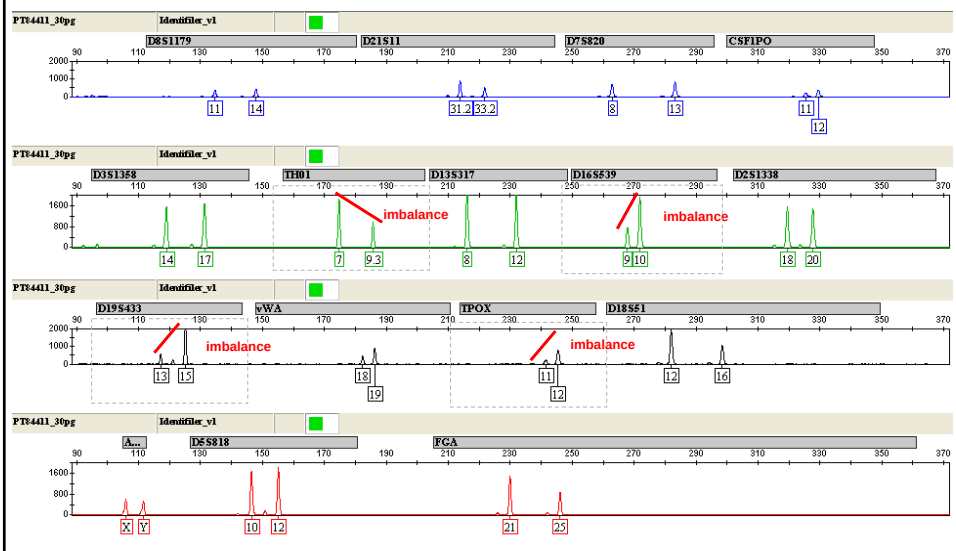
Experimental Design to Study LT-DNA Issues

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

Identifiler, 100 pg @ 31 cycles, ½ Reaction

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

Identifiler, 30 pg @ 31 cycles, ½ Reaction

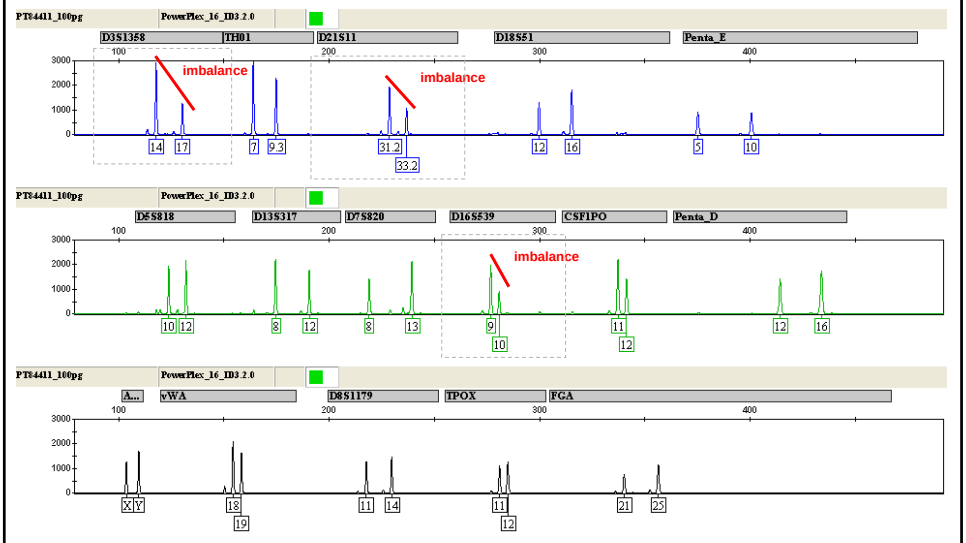


*Slight allelic drop-out in replicates, significant peak height imbalance

Identifiler, 10 pg @ 31 cycles, ½ Reaction

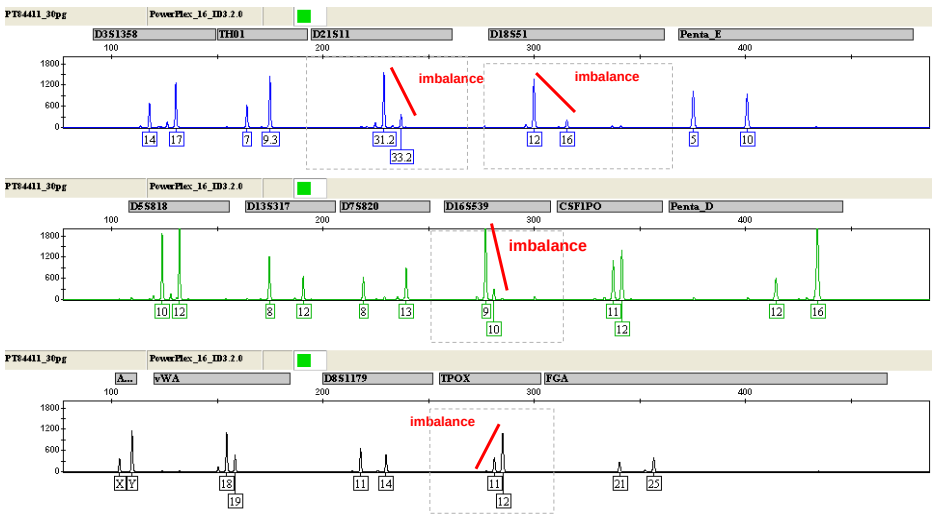
*Significant allelic drop-out in replicates, high stutter and allelic drop-in

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



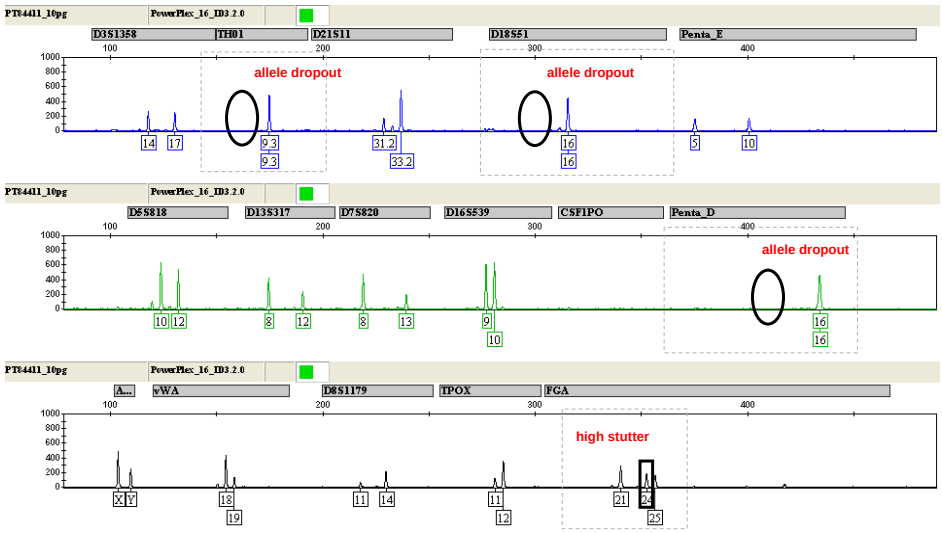
*No drop-out, slight peak height imbalance, full profiles in all replicates

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

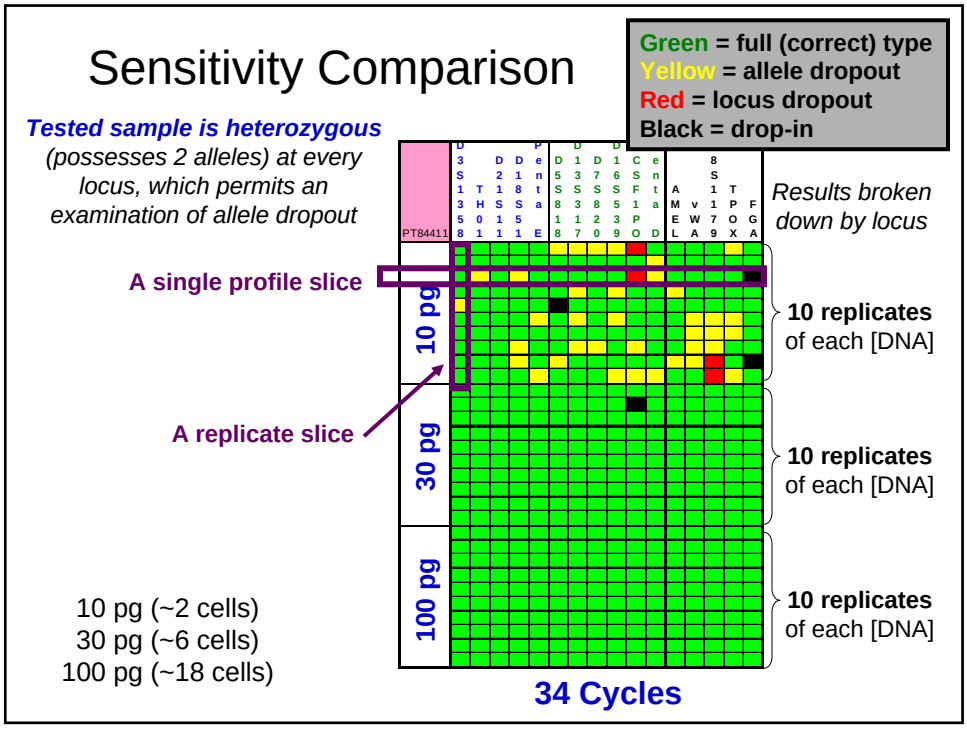


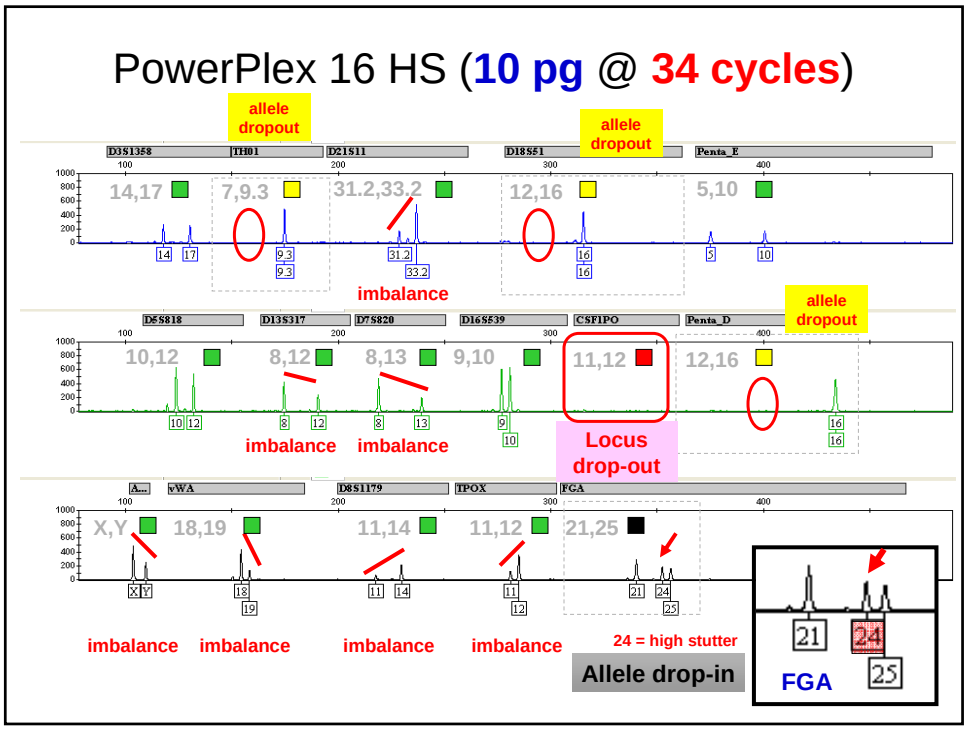
*Slight allelic drop-out in replicates, significant peak height imbalance

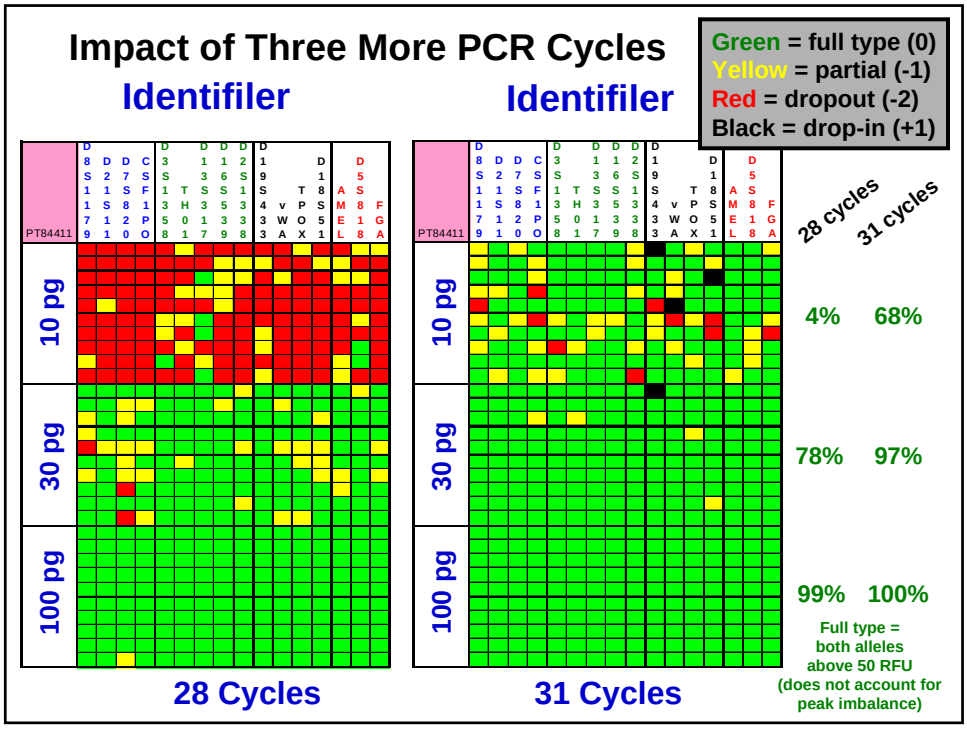
PowerPlex 16 HS, 10 pg @ 34 cycles, ½ Reaction

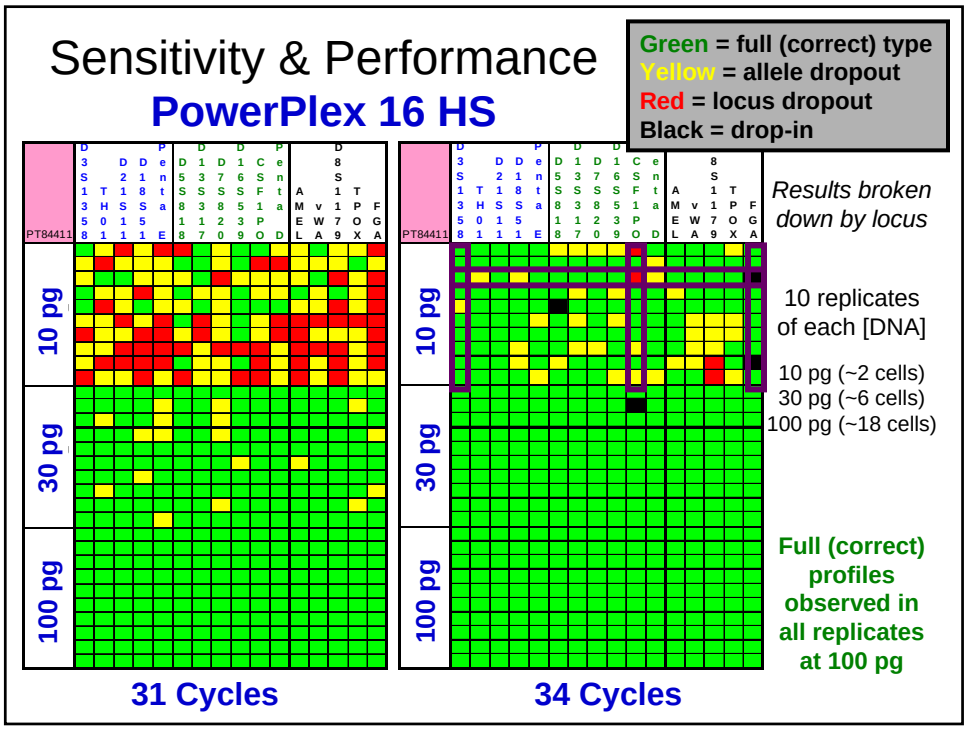


*Significant allelic drop-out in replicates, high stutter and allelic drop-in


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
Results broken down by locus
A single profile slice
A replicate slice
10 pg
30 pg
100 pg
10 replicates of each [DNA]
10 replicates of each [DNA]
10 replicates of each [DNA]
10 pg (~2 cells)
30 pg (~6 cells)
100 pg (~18 cells)
34 Cycles


PowerPlex 16 HS (10 pg @ 34 cycles)
allele dropout
allele dropout
14,17
7,9.3
31.2,33.2
12,16
5,10
imbalance
allele dropout
10,12
8,12
8,13
9,10
11,12
12,16
imbalance
imbalance
Locus drop-out
X,Y
18,19
11,14
11,12
21,25
imbalance
imbalance
imbalance
imbalance
24 = high stutter
Allele drop-in
FGA

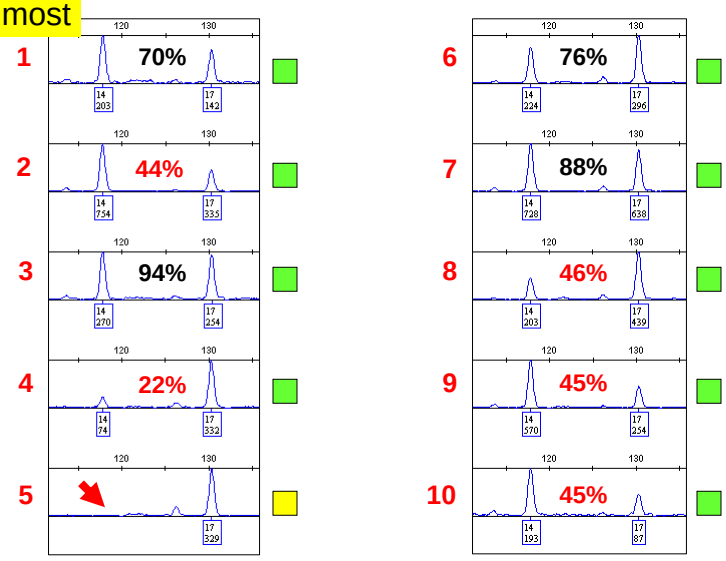

Impact of Three More PCR Cycles
Identifiler
Identifiler
Green = full type (0)
Yellow = partial (-1)
Red = dropout (-2)
Black = drop-in (+1)
PT84411
10 pg
30 pg
100 pg
28 cycles
31 cycles
4%
68%
78%
97%
99%
100%
Full type = both alleles above 50 RFU (does not account for peak imbalance)
28 Cycles
31 Cycles


Sensitivity & Performance
PowerPlex 16 HS
Green = full (correct) type
Yellow = allele dropout
Red = locus dropout
Black = drop-in
PT84411
10 pg
30 pg
100 pg
Results broken down by locus
10 replicates of each [DNA]
10 pg (~2 cells)
30 pg (~6 cells)
100 pg (~18 cells)
Full (correct) profiles observed in all replicates at 100 pg
31 Cycles
34 Cycles

D3S1358 works better than most

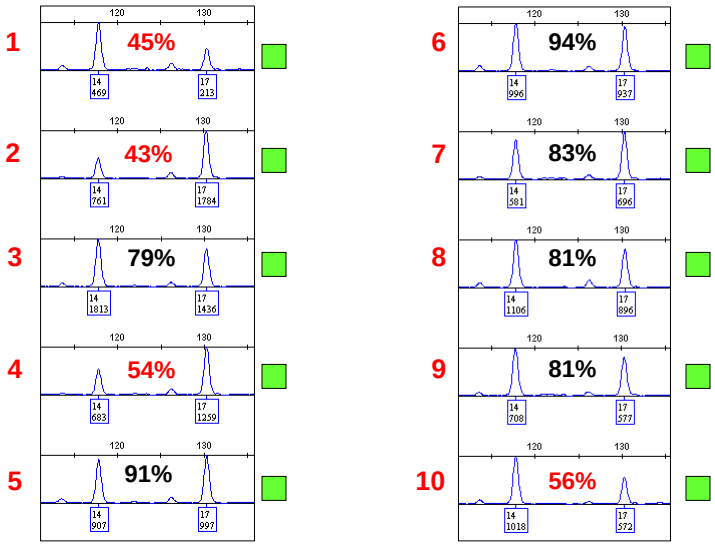
D3S1358 Replicates

PowerPlex 16 HS (**10 pg** @ 34 cycles)



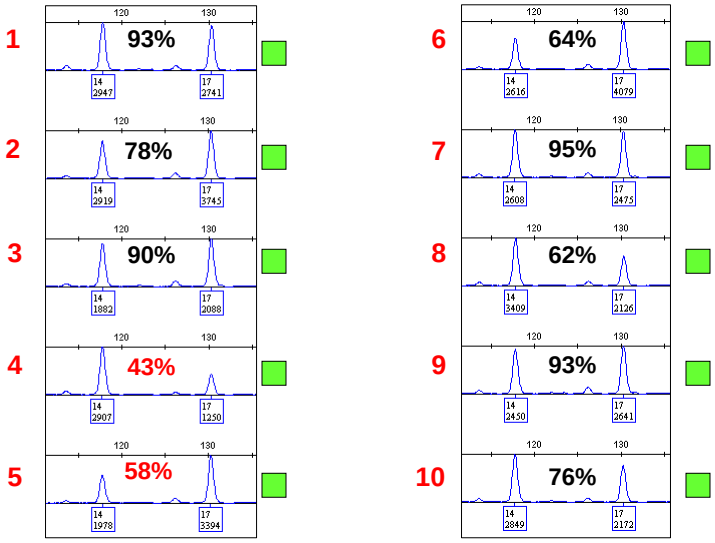
D3S1358 Replicates

PowerPlex 16 HS (**30 pg** @ 34 cycles)



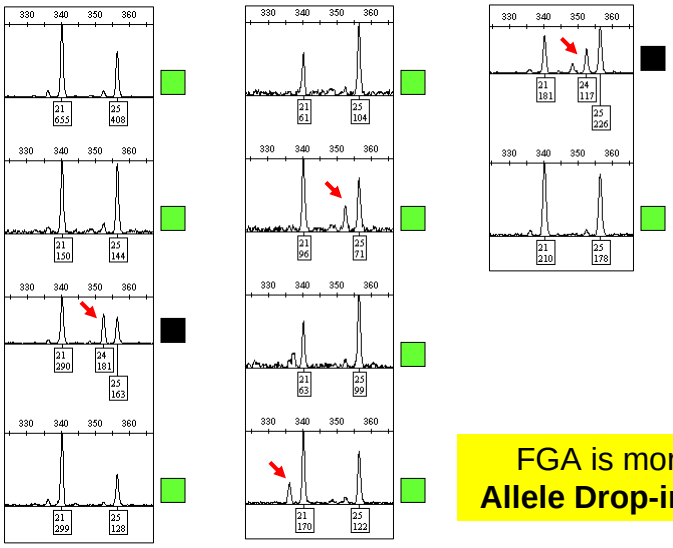
D3S1358 Replicates

PowerPlex 16 HS (**100 pg** @ 34 cycles)



FGA Replicates

PowerPlex 16 HS (**10 pg** @ 34 cycles)

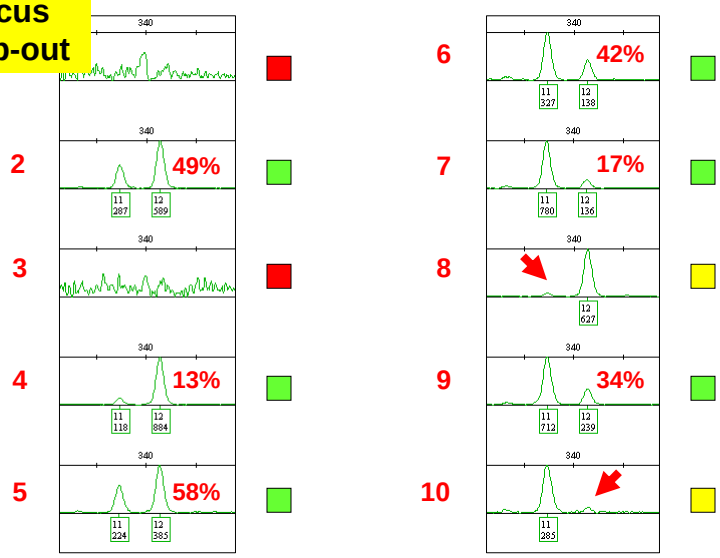


FGA is more prone to **Allele Drop-in** from stutter

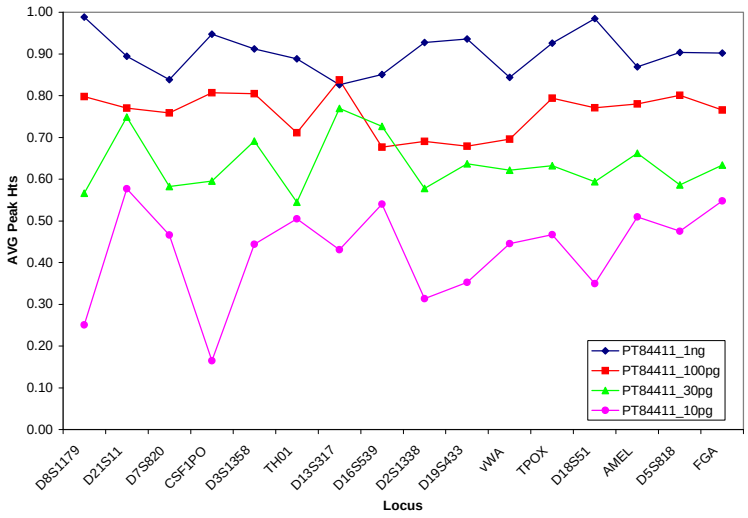
CSF1PO is more prone to **Locus Drop-out**

CSF1PO Replicates

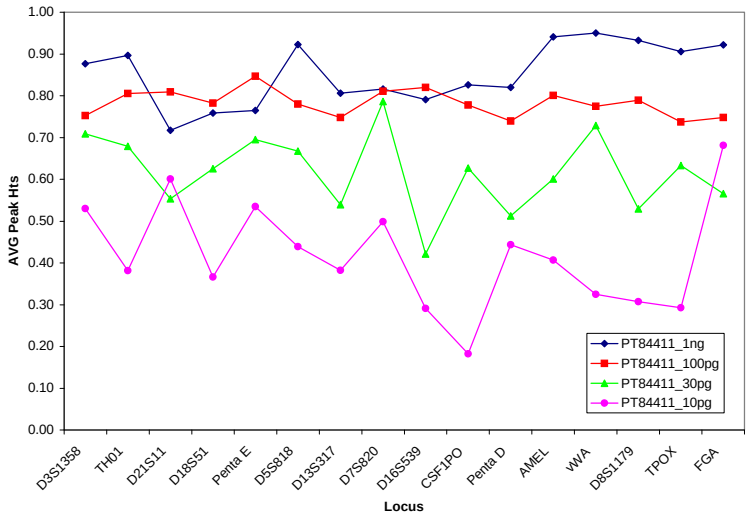
verPlex 16 HS (**10 pg** @ 34 cycles)



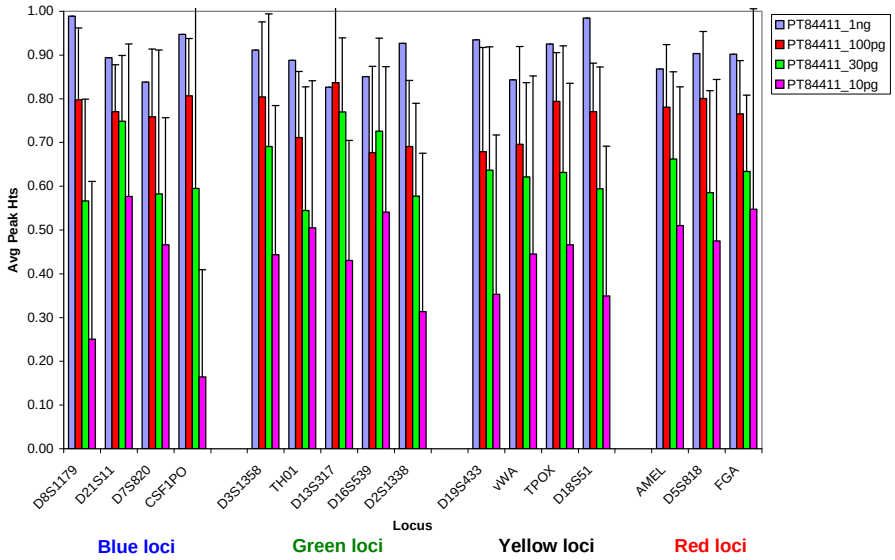
Peak Height Ratio: Identifiler, 31 cyc



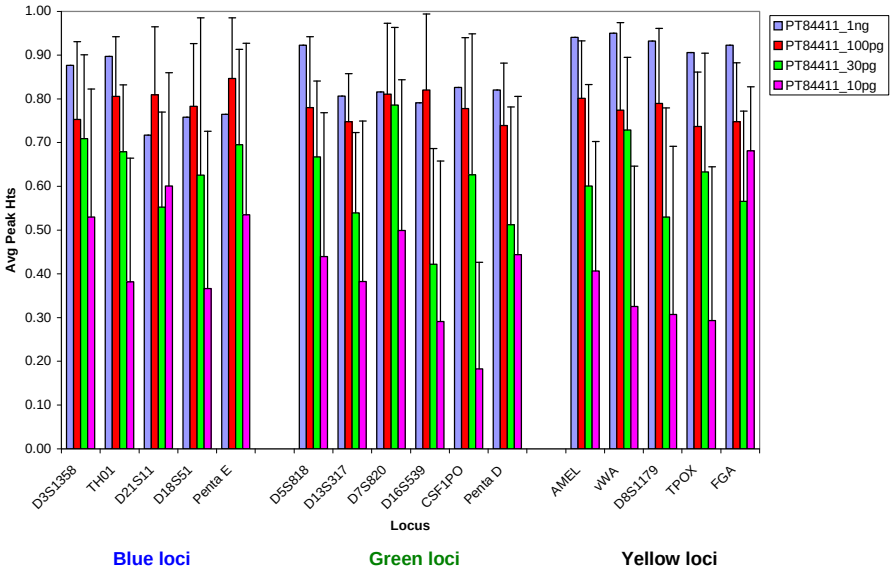
Peak Height Ratio: PP16HS, 34 cyc



Peak Height Ratio: Identifiler, 31 cyc



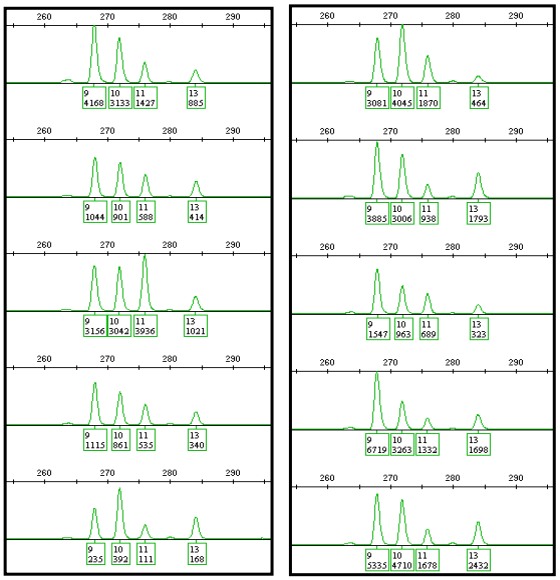
Peak Height Ratio: PP16HS, 34 cyc



Mixture Samples

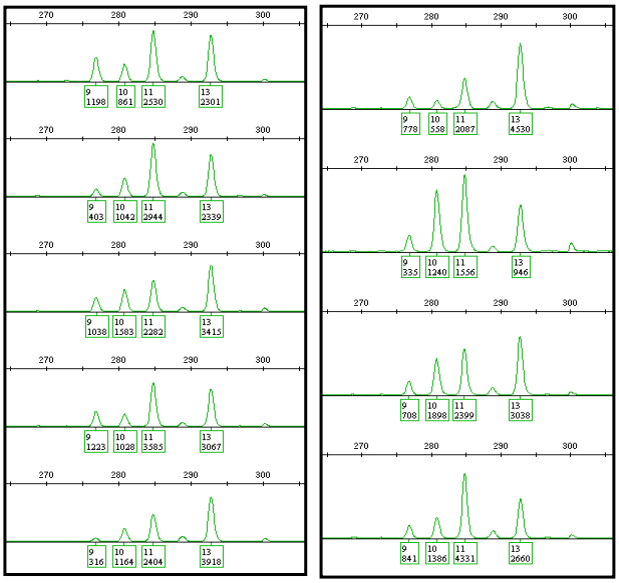
- 2 samples heterozygous at all loci were mixed together at 1:3 and 3:1 ratios – 100 pg total DNA
- Identifiler (31 cycles) and PowerPlex 16 HS (31 and 34 cycles) kits were tested (half reactions)
- 10 replicates
- Variability of peak heights in replicates was observed in both kits

D16S539 (Identifiler, 31 cycles)
10 replicates of a 1:3 mixture (100 pg total)



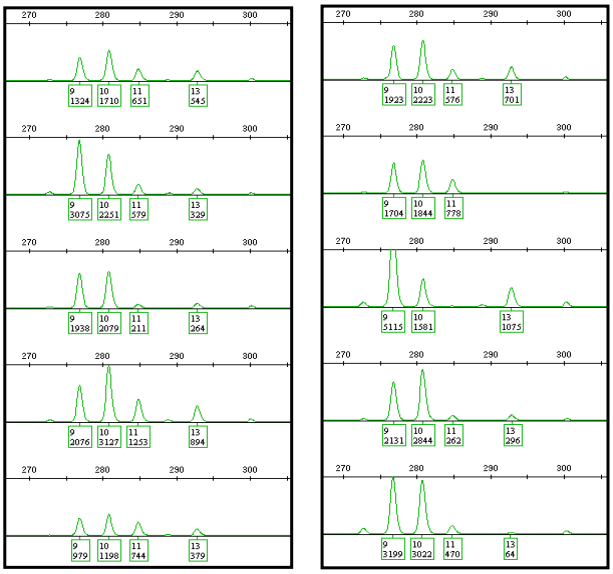
Major: 9,10
Minor: 11,13

D16S539 (Identifiler, 31 cycles)
10 replicates of a 3:1 mixture (100 pg total)



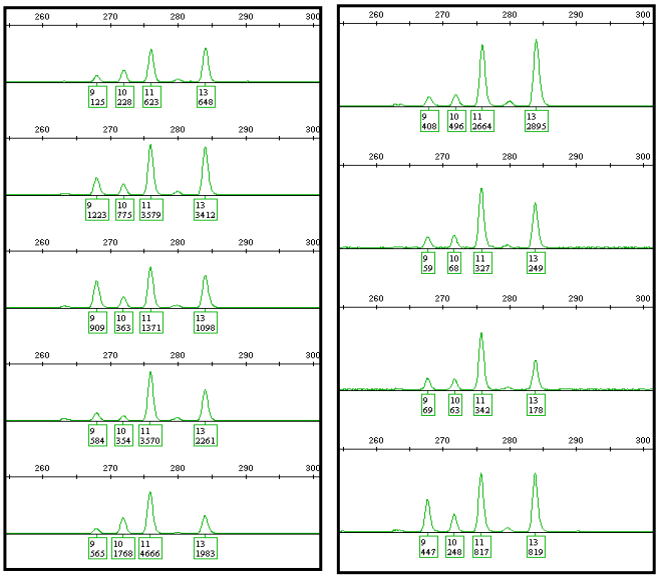
Major: 11,13
Minor: 9,10

D16S539 (PowerPlex 16 HS, 34 cycles)
10 replicates of a 1:3 mixture (100 pg total)



Major: 9,10
Minor: 11,13

D16S539 (PowerPlex 16 HS, 34 cycles)
10 replicates of a 3:1 mixture (100 pg total)



Major: 11,13
Minor: 9,10

Summary of Data Observed

- Increasing the cycle number creates a higher number of full profiles (note: at both 31 and 34 cycles, 100 pg results were all correct)
- Certain loci are more prone to allele and locus drop-out (depends on kit and PCR product sizes; i.e. CSF1PO)
- Identifiler with 31 cycles and PowerPlex 16 HS with 34 cycles were comparable in performance with low-level DNA analysis.

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

Summary

- Low-template DNA (LT-DNA), often referred to as low-copy number (LCN), is often defined as <100-200 pg input DNA.
- In order to improve sensitivity, the number of PCR cycles is often increased (e.g., 31 or 34 cycles instead of 28 cycles) when amplifying DNA with conventional STR kits.
- While increasing the assay sensitivity enables lower amounts of DNA to be detected, these “enhanced interrogation techniques” are prone to stochastic amplification effects that are exhibited in the form of allele drop-out and drop-in.
- To improve result reliability, replicate amplifications are typically compared from low-level DNA samples and consensus profiles developed. Cautious data interpretation rules are also applied based on validation studies.
- Identifiler with 31 cycles and PowerPlex 16 HS with 34 cycles were comparable in performance with low-level DNA analysis.

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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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