CAC Fall Meeting (Sacramento, CA) – October 25, 2011

CALIFORNIA ASSOCIATION OF CRIMINALISTS

Challenges with Low-Level DNA and Mixture Interpretation

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Gaithersburg, Maryland

NIST

Low-Level DNA

Data from Becky Hill (NIST)

Name Change…

LT DNA

low template 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)

Low Template DNA Testing

- **Every lab faces samples with low template DNA**
  - Do you choose to attempt an "enhanced interrogation technique" such as increasing the cycle number, desalting samples, etc.?
  - **Next generation kits coming from manufacturers are capable of greater sensitivity – will they be misused without appropriate caution and validation?**

- **At what point do you draw a line and not attempt to analyze data below this line?**
  - A certain amount of input DNA (based on what data?)
  - A pre-determined stochastic threshold (based on what data?)

I Do Read *The CAC News* !

The CACNews • 3rd Quarter 2010, pp. 40-42

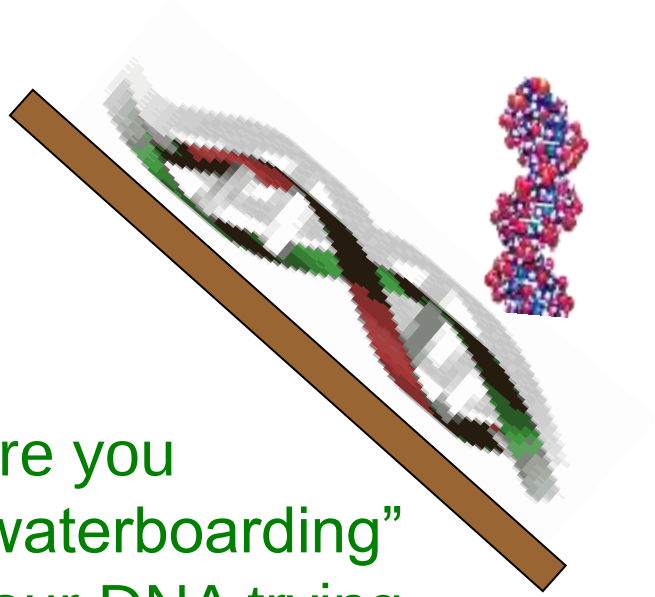
norah rudin & keith inman • the proceedings of lunch

www.forensicdna.com • norah@forensicdna.com • kinman@ix.netcom.com

How low can you go?
Should you just say no?

Since we began meeting "halfway in between" in San Mateo a few years ago, the food scene seems to have picked up. Taking a break from our usual "office" at Astaria, we had a couple of wonderful meals at Capellini's over the winter. For this meeting, we decide to try a new location, Aquapazza. We

"Enhanced Interrogation" Techniques to Improve Sensitivity



Are you "waterboarding" your DNA trying to get more information from the sample?

- **Increased PCR cycle number**

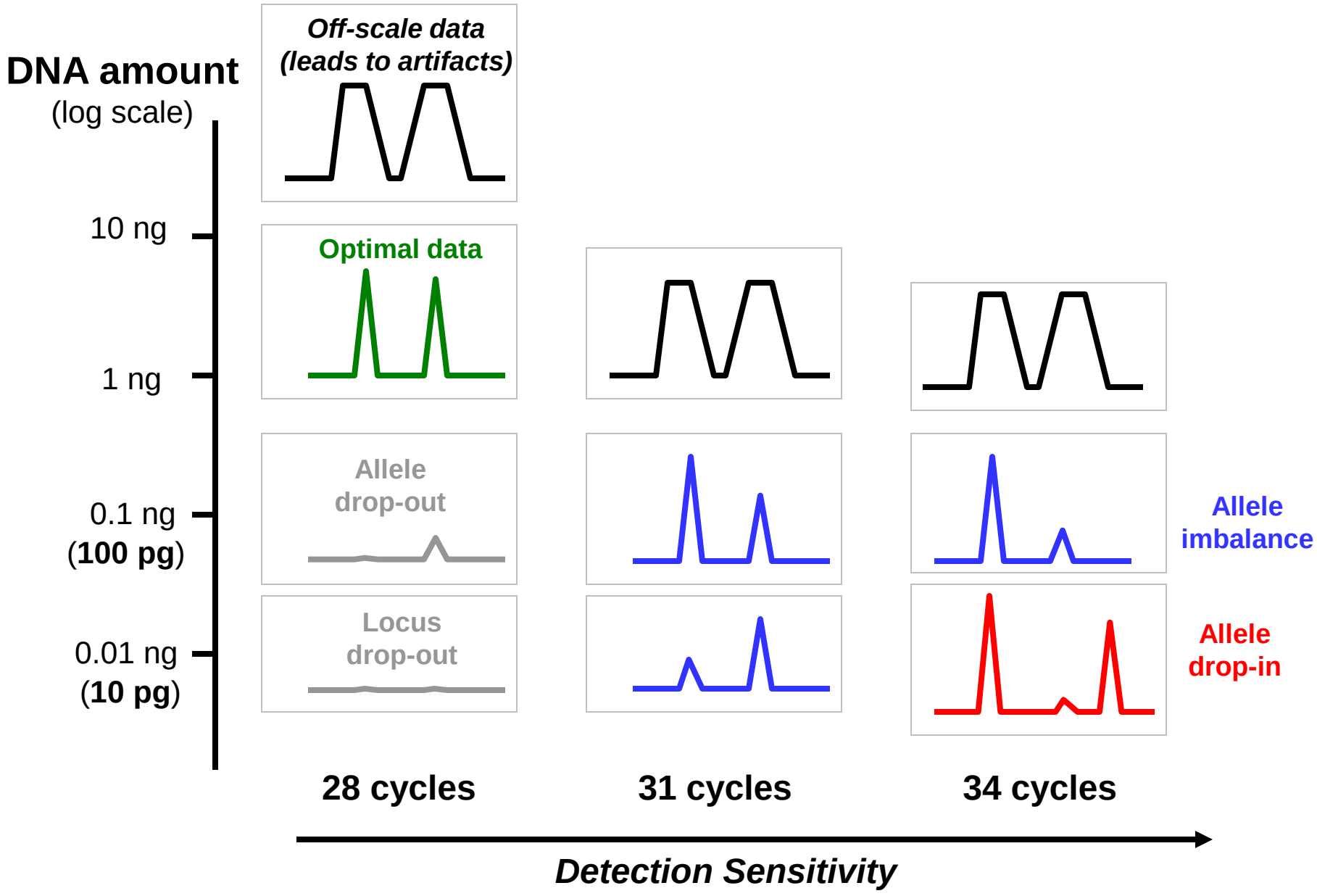
  With 100% efficiency:

  - 28 cycles = 67 million copies
  - 31 cycles = 1 billion copies (x16)
  - 34 cycles = 4 billion copies (x64)

- Reduced volume PCR
- Sample desalting (e.g., MinElute) prior to CE
- Extended CE injections

Requires validation to determine appropriate thresholds for reliability

Illustration of Potential Results at a Heterozygous Locus



http://www.promega.com/profiles/

http://www.promega.com/profiles/1301/1301_02.html

Profiles in DNA

Profiles in DNA Article on Low Level DNA

Article Type: Meetings

Scientific Issues with Analysis of Low Amounts of DNA

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April 2010 issue
Follow-up to ISHI 2009 panel debate

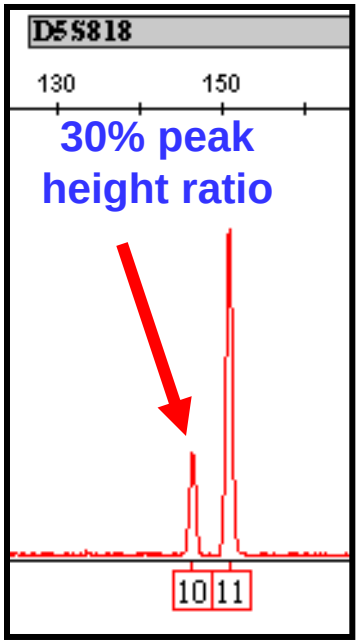
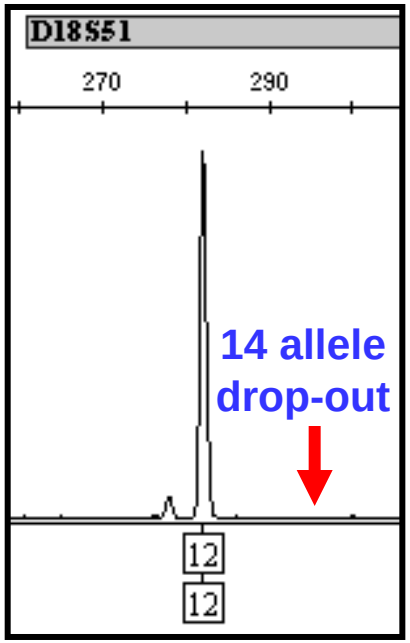
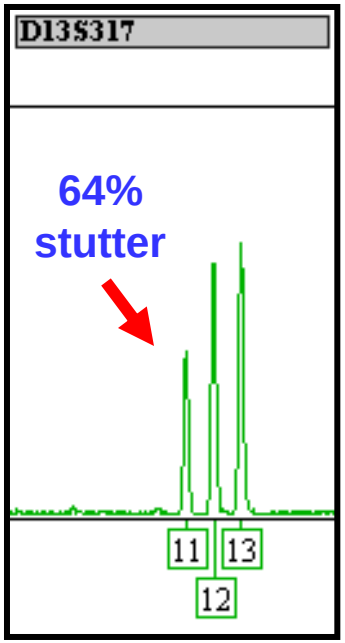
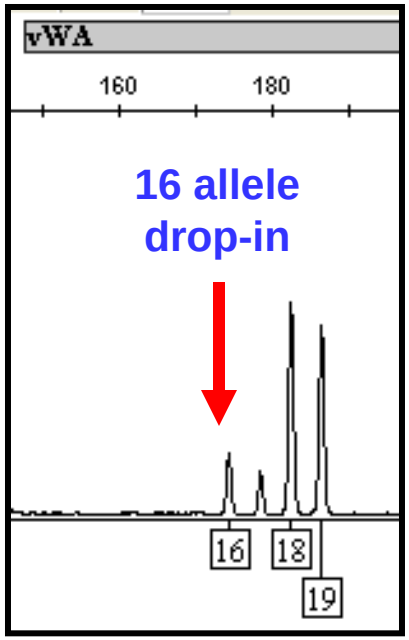
Faced with limited evidence that yield low amounts of DNA, forensic analysts will continually have to confront the question of how far to push DNA-testing techniques. Low copy number (LCN) analysis, also known as low template DNA (LT-DNA) testing, involves enhancing detection sensitivity usually through increasing the number of PCR cycles. Stochastic effects inherent with analysis of low amounts of DNA yield allele or locus drop-out. Additionally, increasing detection sensitivity can result in a greater potential for contamination or allele drop-in. Validation studies with replicate testing of low amounts of DNA were performed to assess the level of allele and locus drop-out and allele drop-in using 10, 30 and 100 picograms with several commercially available STR-typing kits under both standard and increased number of PCR cycles. The results with pristine, fully heterozygous samples demonstrate that a replicate testing approach can produce reliable information with single-source samples when consensus profiles are created.

Stochastic (Random) Effects with LT-DNA

When Combined with Higher Sensitivity Techniques

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

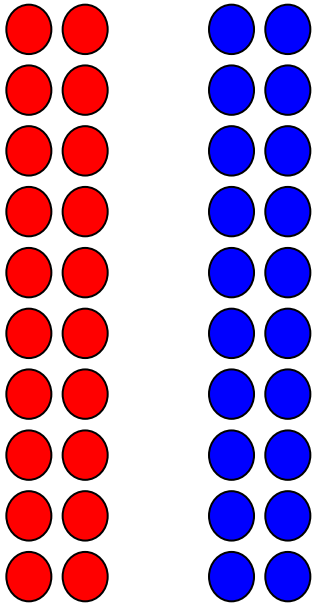
*Gain of False Signal (**False Positive**)*

Severe **Peak Imbalance**	Allelic **Drop-out**	**High Stutter**	Allelic **Drop-in**
			
Identifiler, 30 pg DNA, 31 cycles	Identifiler, 30 pg DNA, 31 cycles	Identifiler, 10 pg DNA, 31 cycles	Identifiler, 10 pg DNA, 31 cycles
Correct genotype: **10,11**	**12,14**	**12,13**	**18,19**

High copy number

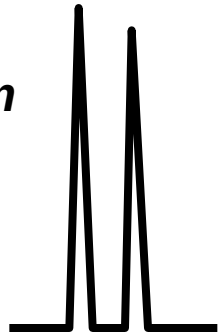
>20 copies per allele

Copies of allele 1 **Copies of allele 2**



What is sampled is consistent with the true amount present in the sample

Resulting electropherogram

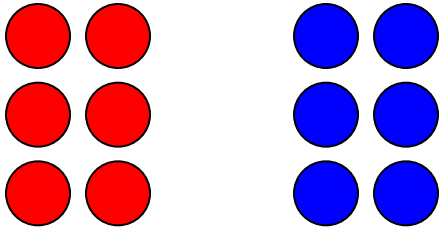


Complete (and correct) genotype

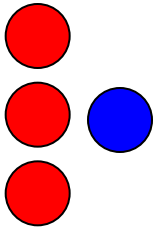
Low copy number

6 copies per allele

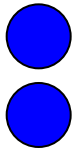
True amount



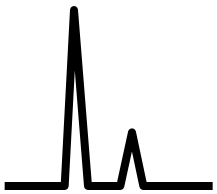
What might be sampled by the PCR reaction…



OR



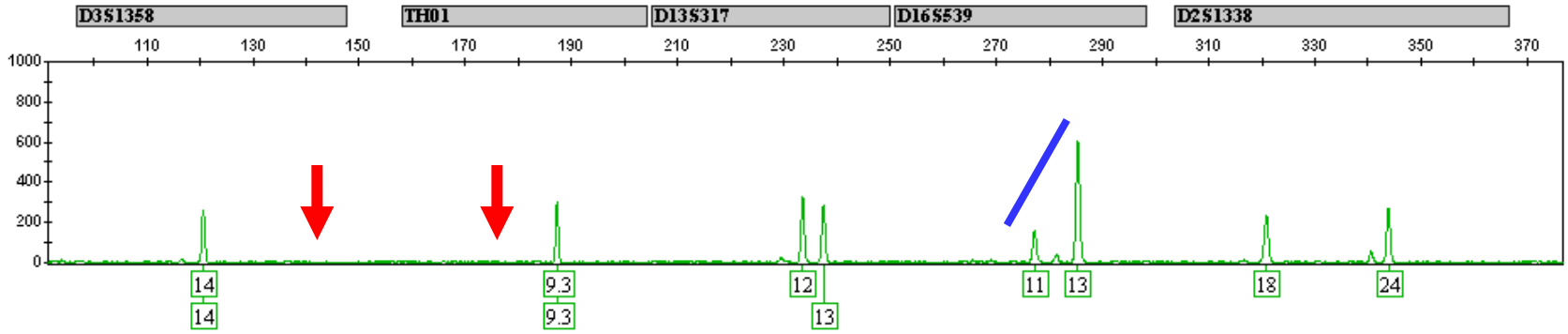
Extreme allele imbalance



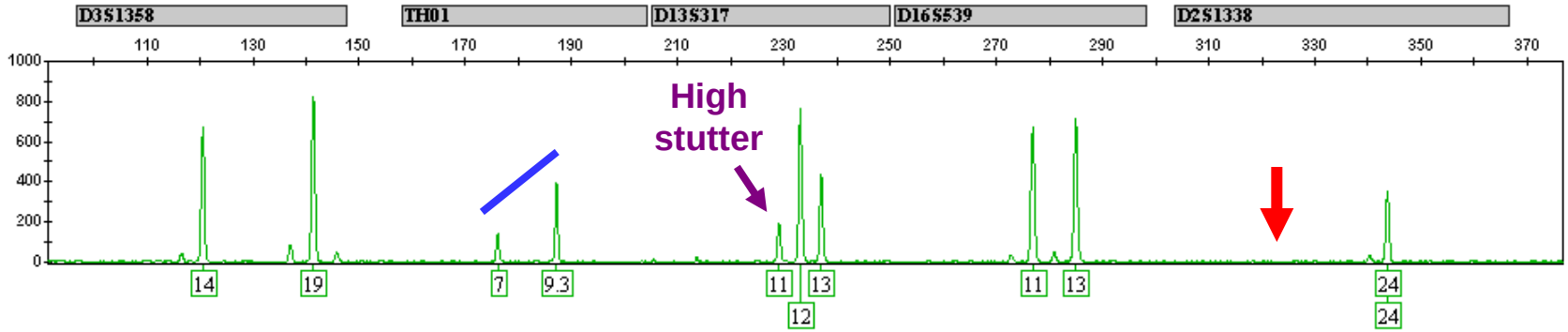
Allele imbalance

Allele dropout

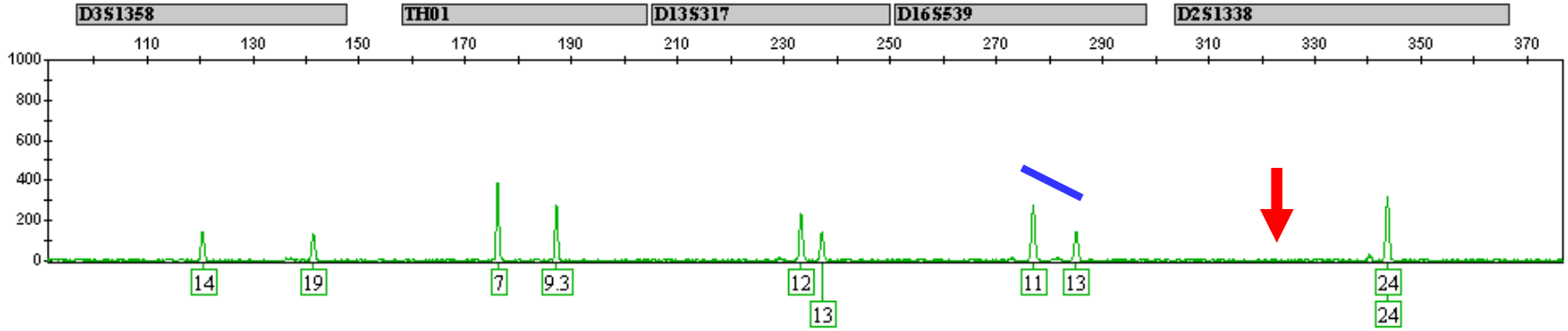
Replicate #1



Replicate #2



Replicate #3



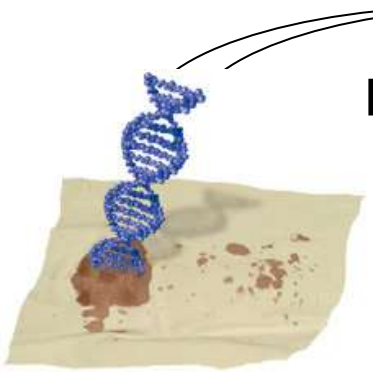
Consensus Profile: **14,19** **7,9.3** **12,13** **11,13** **24,Z**

Correct Profile: *14,19* *7,9.3* *12,13* *11,13* *18,24*

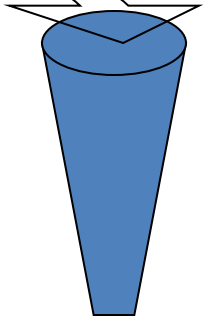
Suggestions for Optimal Results with LT-DNA

- 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

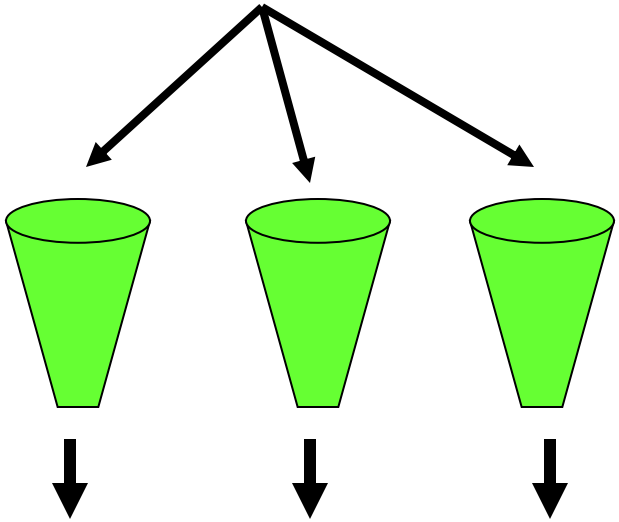
Typical LT-DNA Analysis Procedure



Extract DNA
from stain



Quantify Amount of DNA Present



Perform 3 Separate PCR Amplifications

Interpret Alleles Present

Develop a Consensus Profile

(based on replicate consistent results)

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)

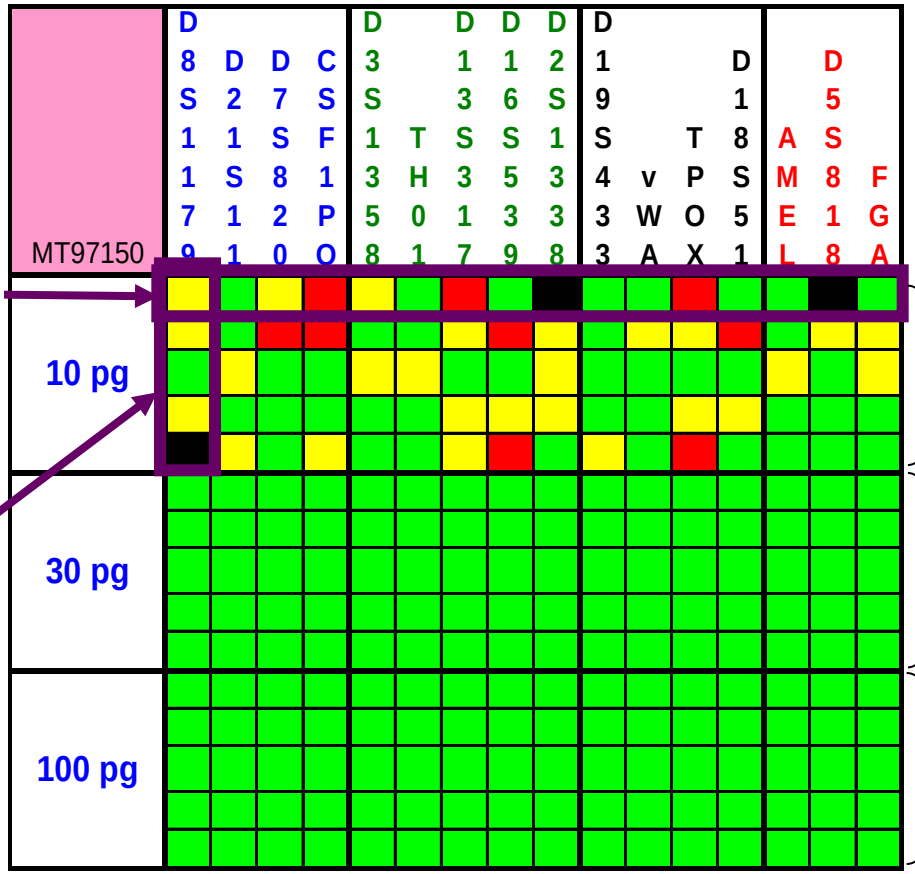
Experimental Design to Study LT-DNA Issues

- 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)

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*



A single profile slice

A replicate slice

Results broken down by locus

5 replicates of each [DNA]

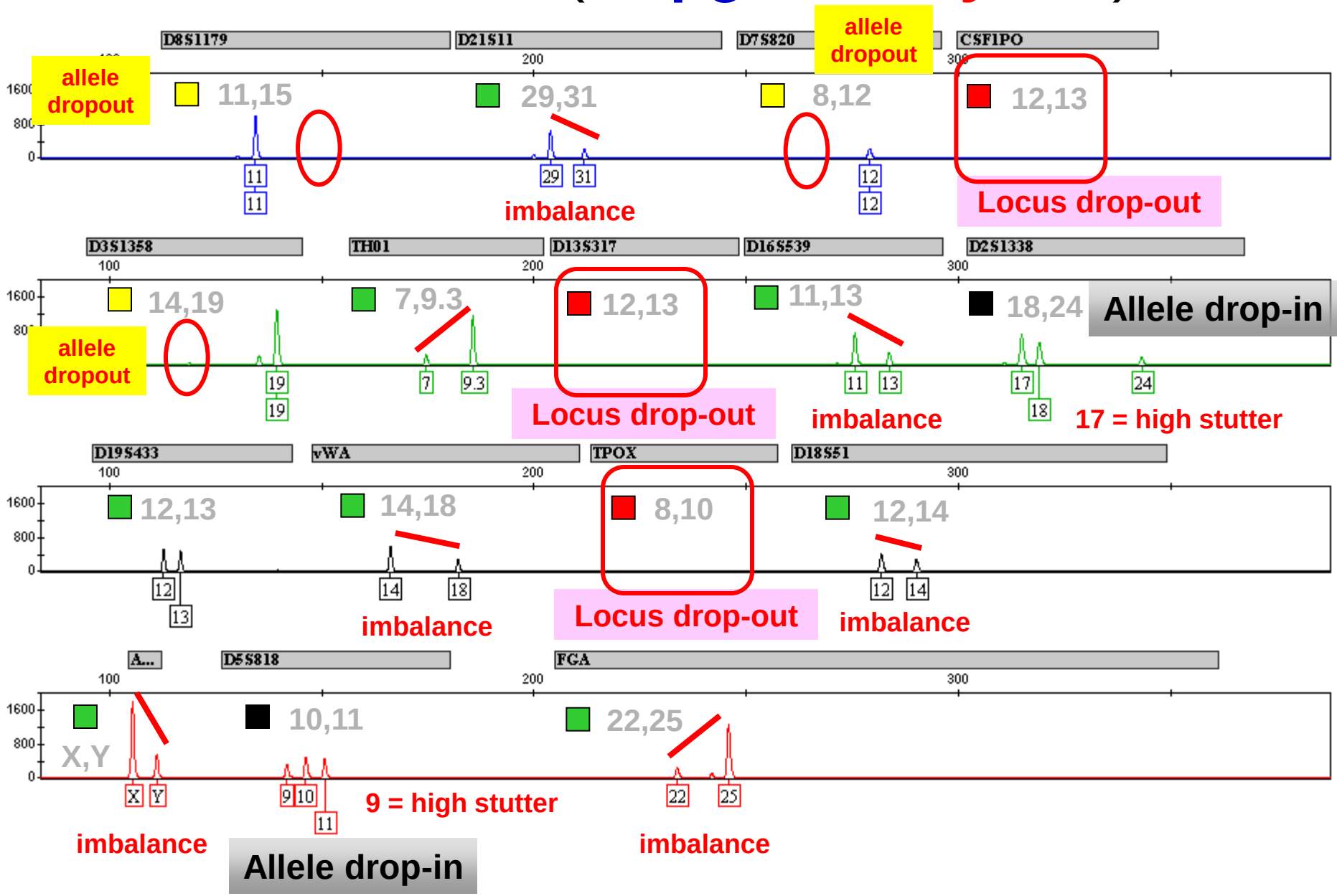
5 replicates of each [DNA]

5 replicates of each [DNA]

10 pg (~2 cells)
30 pg (~6 cells)
100 pg (~18 cells)

32 Cycles

Identifiler Plus (**10 pg** @ **32 cycles**)

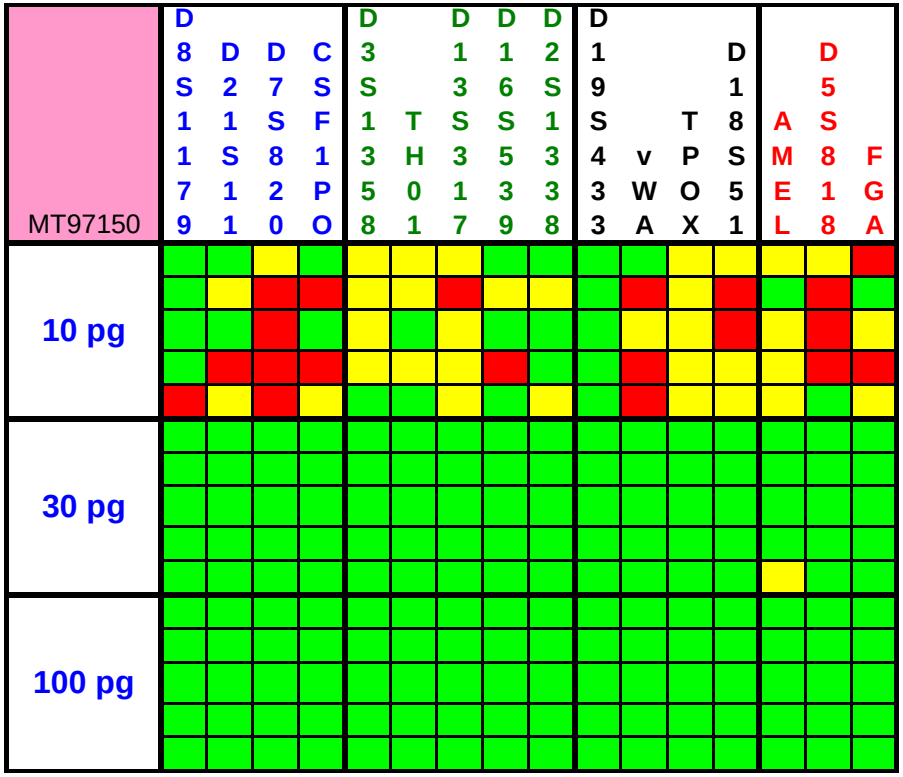


Impact of Three More PCR Cycles

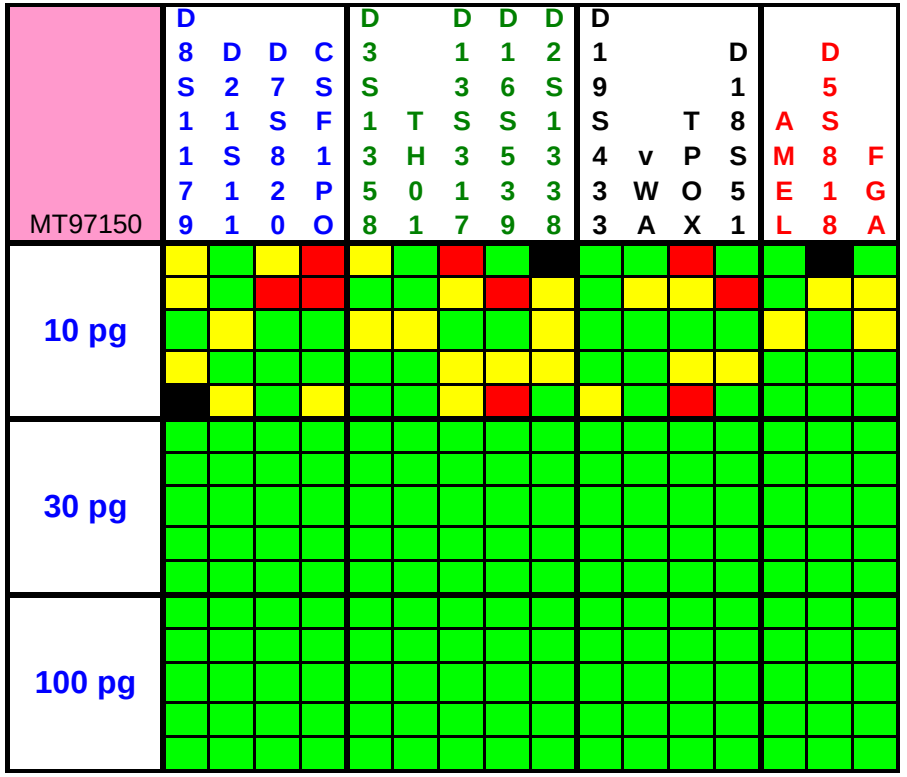
Identifiler Plus

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

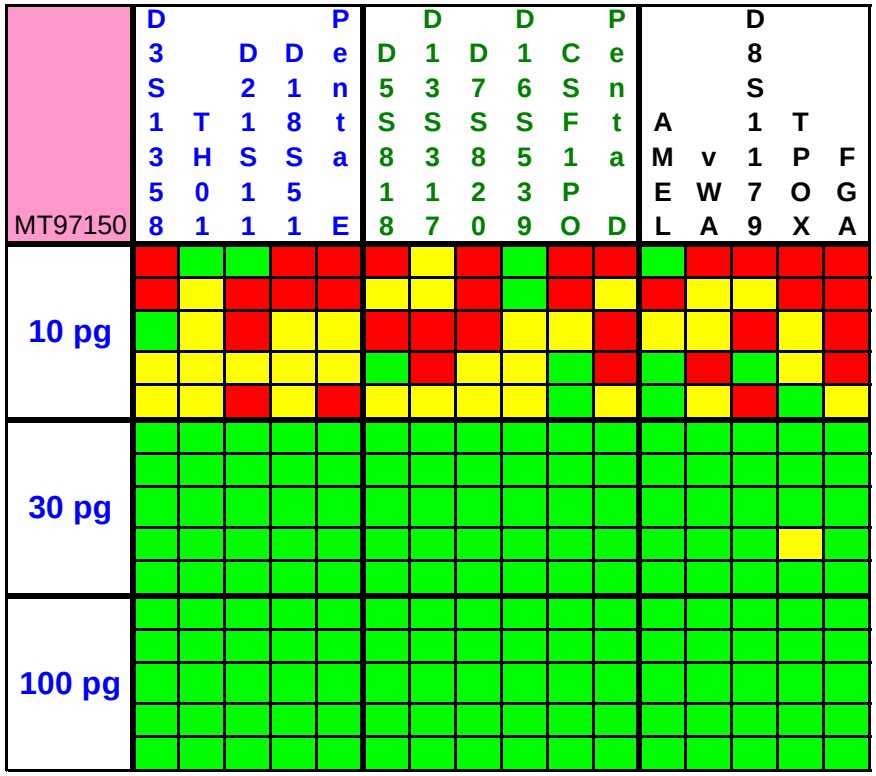
33% vs. 53% full profiles

38% improvement with 3 extra cycles

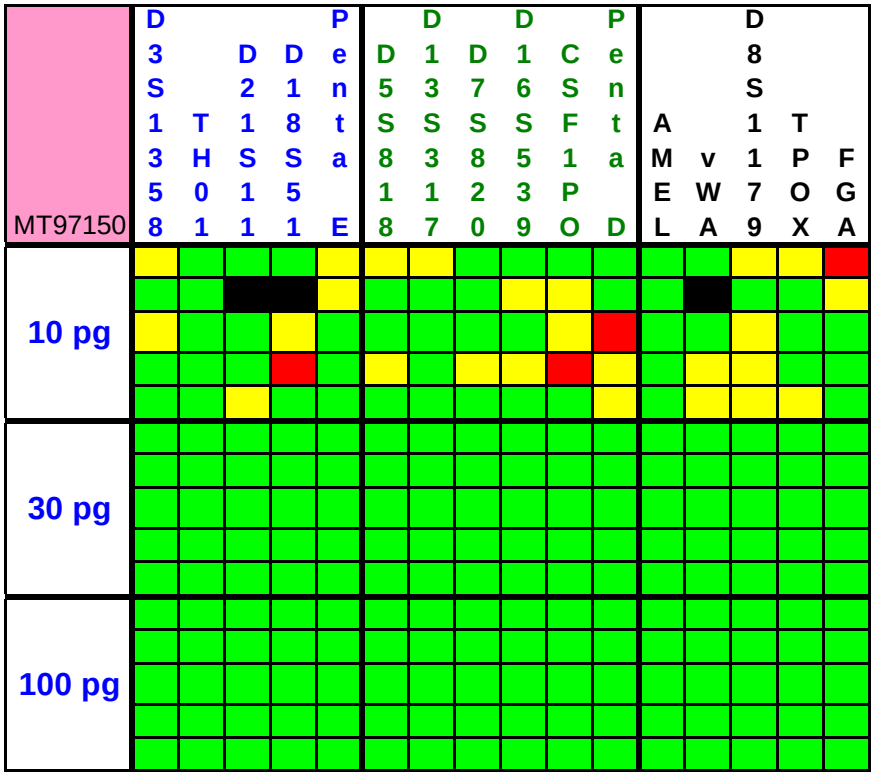
Sensitivity & Performance

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

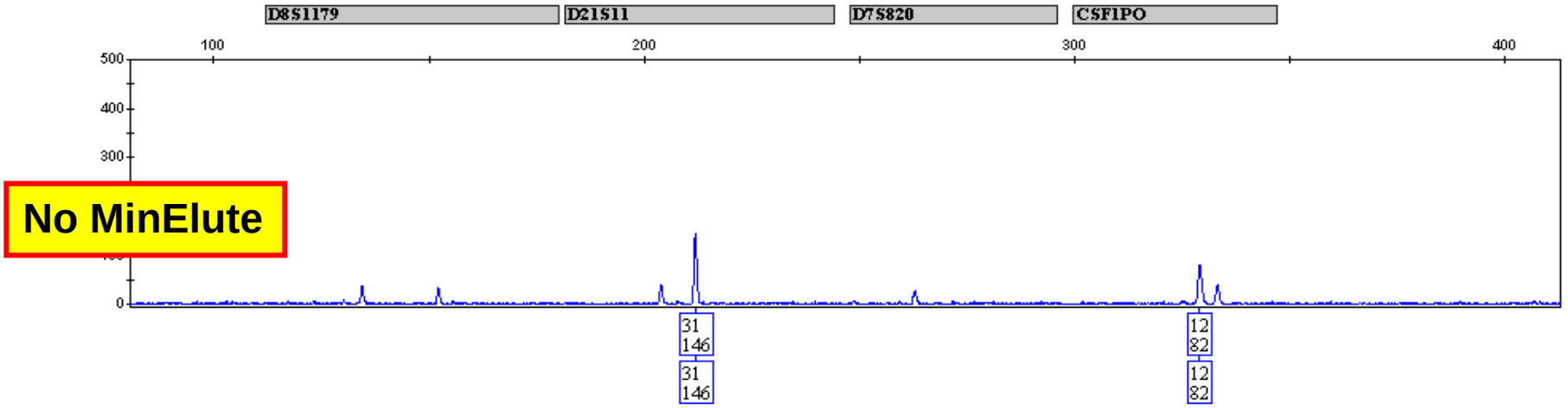
Summary of LT-DNA Testing

- More and more labs are “pushing the envelope” and attempting LT-DNA testing.
- LT-DNA testing has been “generally accepted as reliable” in many recent court cases.
- Our results demonstrate that replicate testing can produce reliable information with single source samples at low levels of DNA when consensus profiles are created.

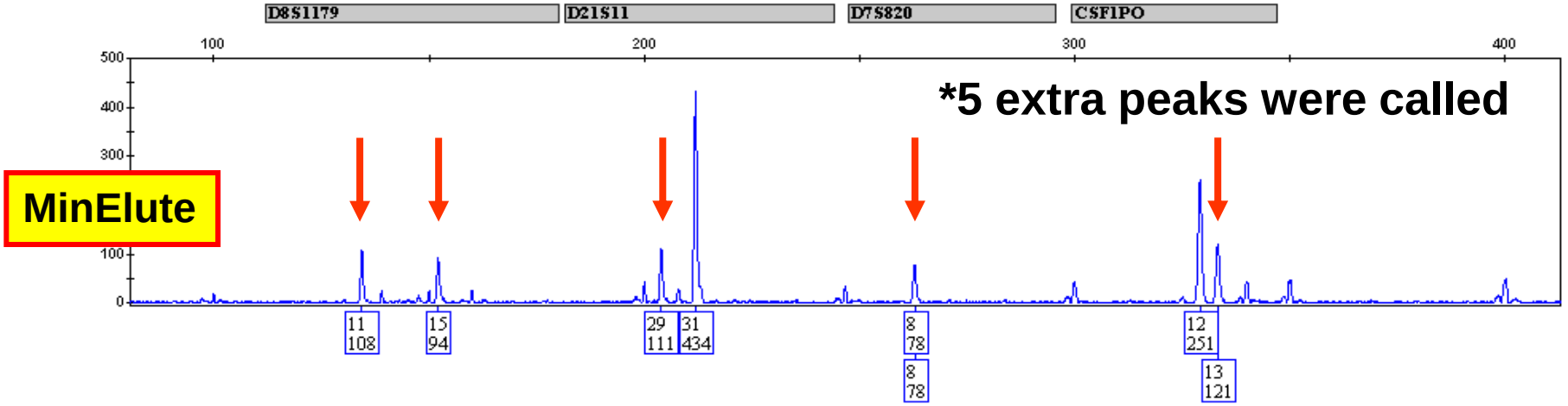
MinElute PCR Purification Kit

*96 well plates with vacuum protocol used

Identifiler Plus, 29 cycles, 10 pg



No MinElute



MinElute

Signal Improvement:	**~66%**	**~67%**

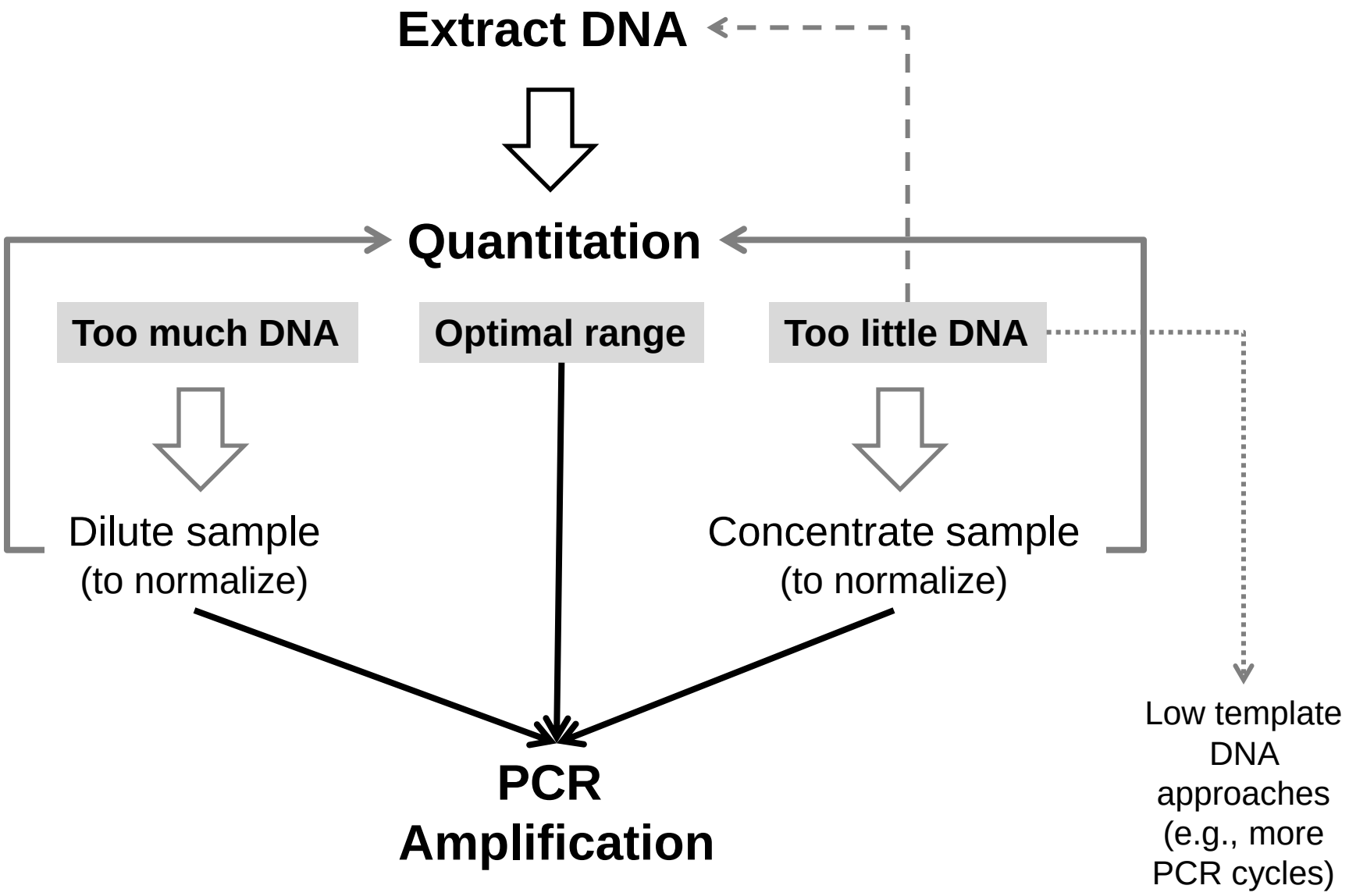
Comments on DNA Quantitation

- qPCR has enabled lower amounts of DNA to be quantified in recent years – providing in some cases a false sense of confidence in accuracy at these low levels

- Remember that **qPCR is also subject to stochastic effects** and thus DNA quantitation will be less accurate and exhibit more variation at the low end…

- **Next generation STR kits** with their greater sensitivity and ability to overcome inhibition **have the potential to make the current qPCR DNA quantitation kits obsolete as an appropriate gatekeeper** to whether or not to continue with a low level, compromised DNA sample

Important Role of DNA Quantitation



3,068 casework samples

↓ EZ1 DNA extraction (no inhibitors seen)

DNA quantitation

↓ **Quantifiler** (performed twice and results averaged)

STR amplification

↓ **Nanoplex^{QS}** and **SEfiler** (with up to 500 pg DNA added)

	Group 1	**Group 2**	**Group 3**	**Group 4**
	0-5 pg/µL	5-10 pg/µL	10-30 pg/µL	>30 pg/µL
	1564 samples	279 samples	371 samples	854 samples
No results	96%	67%	26%	3%
Full profile	**3%**	**23%**	**67%**	**96%**
Partial profile	1%	10%	7%	1%

Kremser et al. 2009

1564 Samples
with ‘Zero’ Quantifiler Results (pg/µL)

	0,0	**0,>0**	**>0,>0**
Number of Samples	750	478	336
Positive results	0%	7%	27%
Negative results	100%	93%	63%

When both Quantifiler results were zero, then all subsequent STR testing failed to obtain a result

Kremser et al. 2009

The 2009 LCN Debates

ISFG Session - September 2009

Promega LCN Panel - October 2009

UK Court Decision – December 2009

ISFG LCN Session – September 18, 2009

Adrian Linacre (UK)

Bruce Budowle (US)

Peter Gill (UK)

Articles have been published in *Forensic Sci. Int. Genet. Suppl. Series* (*Progress in Forensic Genetics 13: Proceedings of the 23rd International ISFG Congress*); freely available at **http://www.fsigeneticssup.com/**

1. Linacre, A. (2009) **Review of low template DNA typing**. *Forensic Sci. Int. Genetics Suppl. Ser.* 2: 549-550.
2. Budowle, B., Eisenberg, A., van Daal, A. (2009) **Low copy number has yet to achieve "general acceptance".** *Forensic Sci. Int. Genetics Suppl. Ser.* 2: 551-552.
3. Gill, P. and Buckleton, J. (2009) **Low copy number typing -- where next?** *Forensic Sci. Int. Genetics Suppl. Ser.* 2: 553-555.

20TH INTERNATIONAL SYMPOSIUM ON
HUMAN IDENTIFICATION

THE REAL DEAL

LAS VEGAS, NEVADA • OCTOBER 12–15, 2009

JW Marriott
Las Vegas Resort & Spa
at Summerlin

Sponsored by
Promega Corporation

LCN Panel

Articles planned for publication in March 2010 issue of Promega's *Profiles in DNA*; freely available at **http://www.promega.com/profiles/**

Promega LCN Panel – October 15, 2009

<u>Questions Addressed</u>:

(1) How do you define or use the term "LCN"? – **Theresa and Bruce**
(2) Has PCR testing of small amounts of DNA been appropriately validated and accepted in non-forensic DNA testing? – **Gillian and Angela**
(3) What do you see as the biggest scientific challenge with "LCN" testing? – **Bruce and Theresa**
(4) Can single-source DNA samples with low amounts of DNA be interpreted reliably? – **Bruce and Gillian**
(5) What advice do you have to offer to forensic scientists working with attorneys on cases that may be considered "LCN" cases? – **Brad, Theresa, Bruce**
(6) Is it better to consume a sample with a single amplification vs. replicate amplifications? – **Angela and Gillian**
(7) Where do we go next with "LCN" testing? – **Bruce, Theresa, Brad, John, Angela, Gillian**

Some LT-DNA Court Rulings

- "…a challenge to the validity of the method of analysing Low Template DNA by the LCN process should no longer be permitted at trials where the quantity of DNA analysed is above the stochastic threshold of 100-200 picograms…"
  - United Kingdom: Crown vs. Reed & Reed, Dec. 21, 2009

- LT-DNA testing is "…generally accepted as reliable in the forensic scientific community under the standard enunciated in Frye…"
  - NYC OCME: People vs. Megnath, Feb. 8, 2010

- "LCN DNA evidence is not inherently unreliable."
  - New Zealand: Crown vs. Wallace, Mar. 3, 2010

The judge in the Wallace case quotes from John Butler's *Fundamentals of Forensic DNA Typing* in drawing the court's conclusion

Literature Debates

- A number of letters to the editor went on-line in *FSI Genetics* with back and forth arguments between Peter Gill & John Buckleton and Bruce Budowle

- These contentious opinion articles were terminated with a January 2011 editorial in FSI Genetics when the letters were all published in a single issue

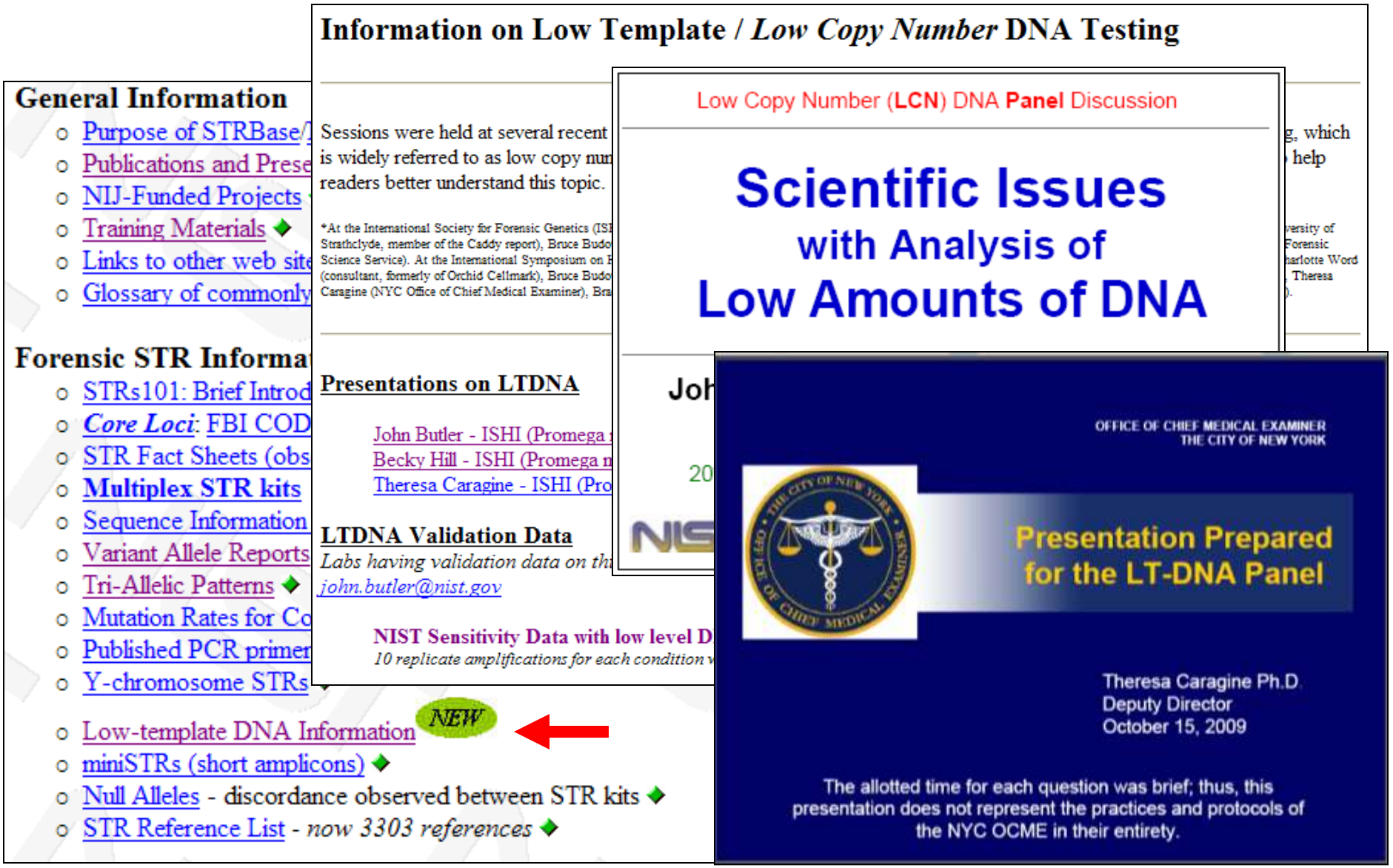
LT-DNA Section of STRBase

- Launched October 30, 2009
  - **http://www.cstl.nist.gov/biotech/strbase/LTDNA.htm**
  - Low-template DNA = LTDNA (not LCN!)

- Includes:
  - **Presentations from Promega 2009 LCN Panel and Technical Leader's meeting**
  - **Validation data from NIST 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

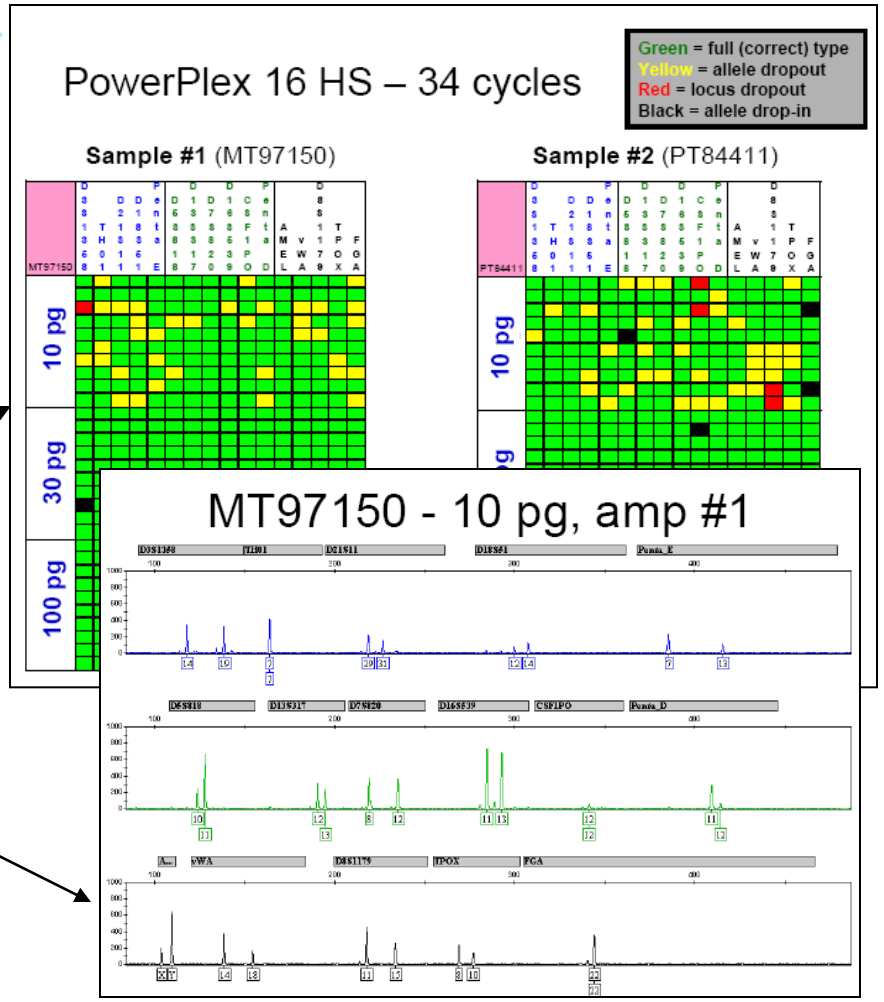
http://www.cstl.nist.gov/biotech/strbase/LTDNA.htm

NIST Sensitivity Data with low level DNA templates

10 replicate amplifications for each condition with two fully heterozygous, single-source samples

Click on links to see summaries and DNA profiles observed

STR kit - PCR conditions	Sample 1	Sample 2
Identifiler - 28 cycles	100 pg	100 pg
	30 pg	30 pg
	10 pg	10 pg
Identifiler - 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**

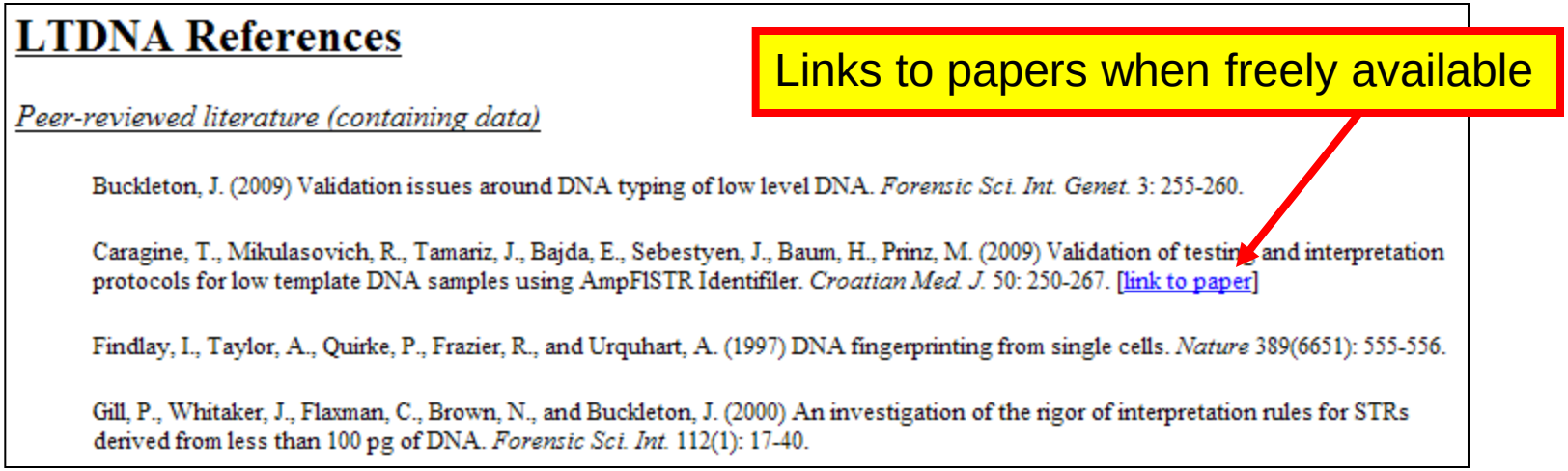


Literature Listing on LT-DNA (LCN)

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)*



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

Is LCN Effort Worthwhile?
Thoughts to Consider…

- Success rates are often low
- Requires dedicated “clean” facilities and extreme care to avoid limit contamination
- Complex interpretation procedure – requires more experienced analysts to do
- Significance of a DNA match?? – intelligence information but likely not to be probative due to unknown time when sample may have been deposited…

From John Butler May 3, 2006 MAAFS LCN Workshop presentation (Richmond, VA)
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The Wisdom of Obi Wan Kenobi

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

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

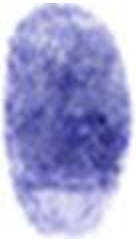
WE MUST BE CAUTIOUS!"

Mixtures

All TrueAllele data from Mike Coble (NIST)

Stochastic threshold 3500 data from Erica Butts (NIST)

The Forensic Institute



Forensic e-Symposium™

The Forensic Institute

April 14, 2005

"If you show 10 colleagues a mixture, you will probably end up with 10 different answers."

- Dr. Peter Gill

"Don't do mixture interpretation unless you have to"

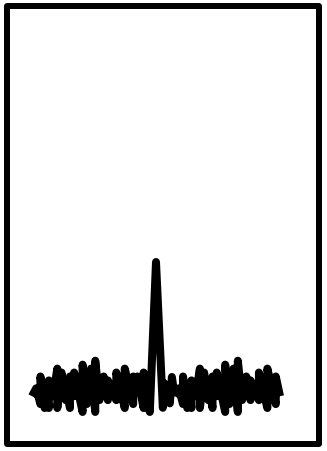
- Dr. Peter Gill (1998)

Mixture Interpretation Protocols Build on Single-Source Sample Information

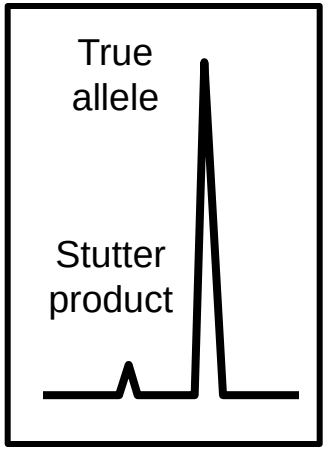
The Steps of Data Interpretation

Peak (vs. noise) → **Allele** (vs. artifact) → **Genotype** (allele pairing) → **Profile** (genotype combining)

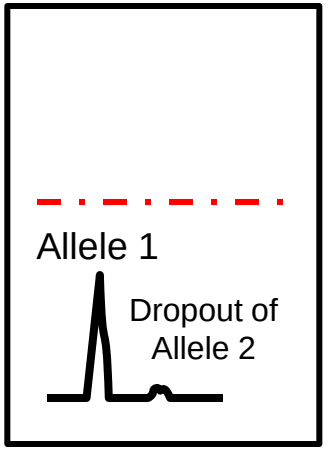
Analytical Threshold



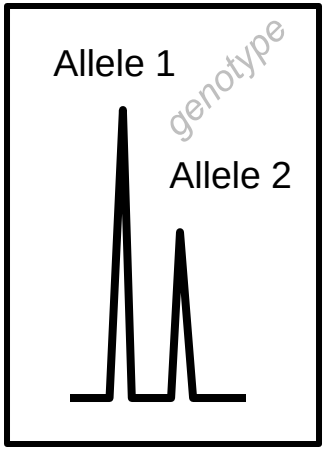
Expected Stutter %



Stochastic Threshold



Peak Height Ratio (PHR)



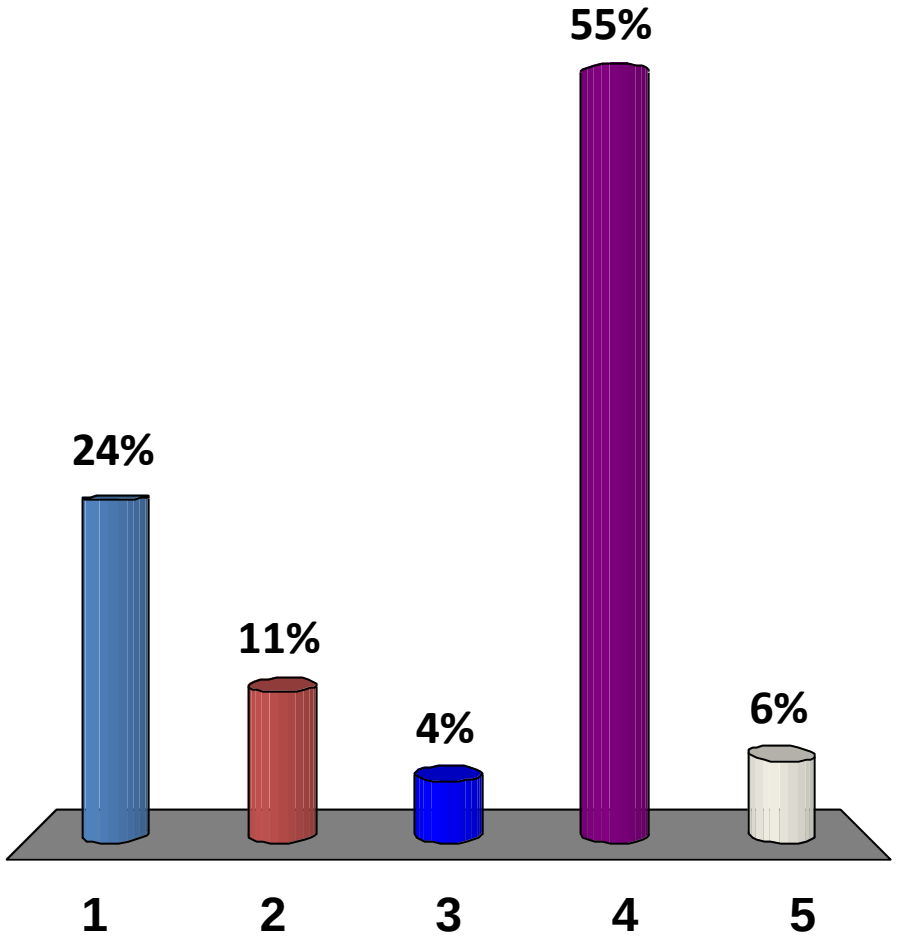
Next step: Examine feasible genotypes to deduce possible contributor profiles

Moving from individual locus genotypes to profiles of potential contributors to the mixture is dependent on mixture ratios and numbers of contributors

If your laboratory uses a stochastic threshold (ST), it is:

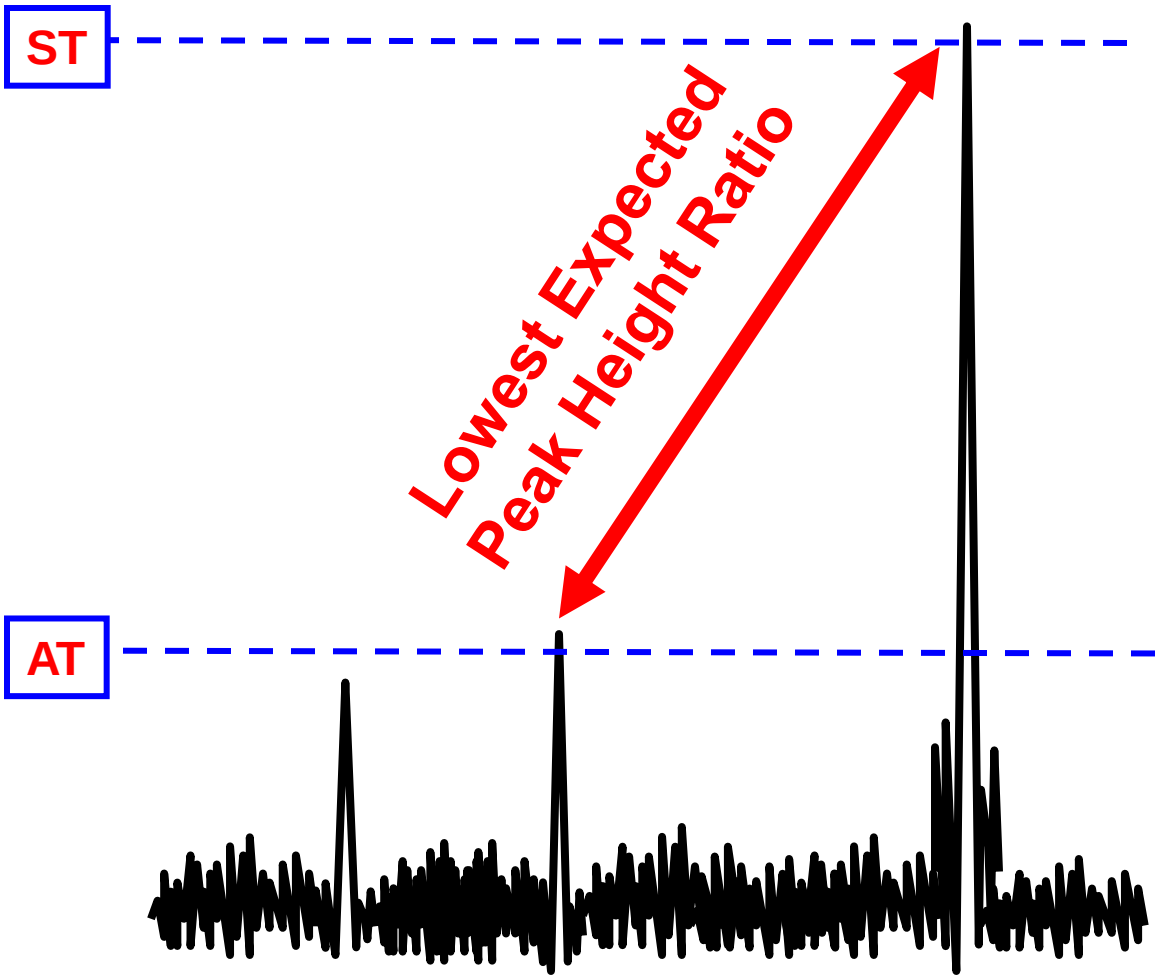
Responses from 140 participants in ISHI 2011 Workshop

1. Same value as our analytical threshold (we don't use a ST)
2. About twice as high as our AT (e.g., AT = 50 and ST = 100 RFU)
3. Less than twice as high as our AT
4. Greater than twice as high as our AT
5. I don't know!



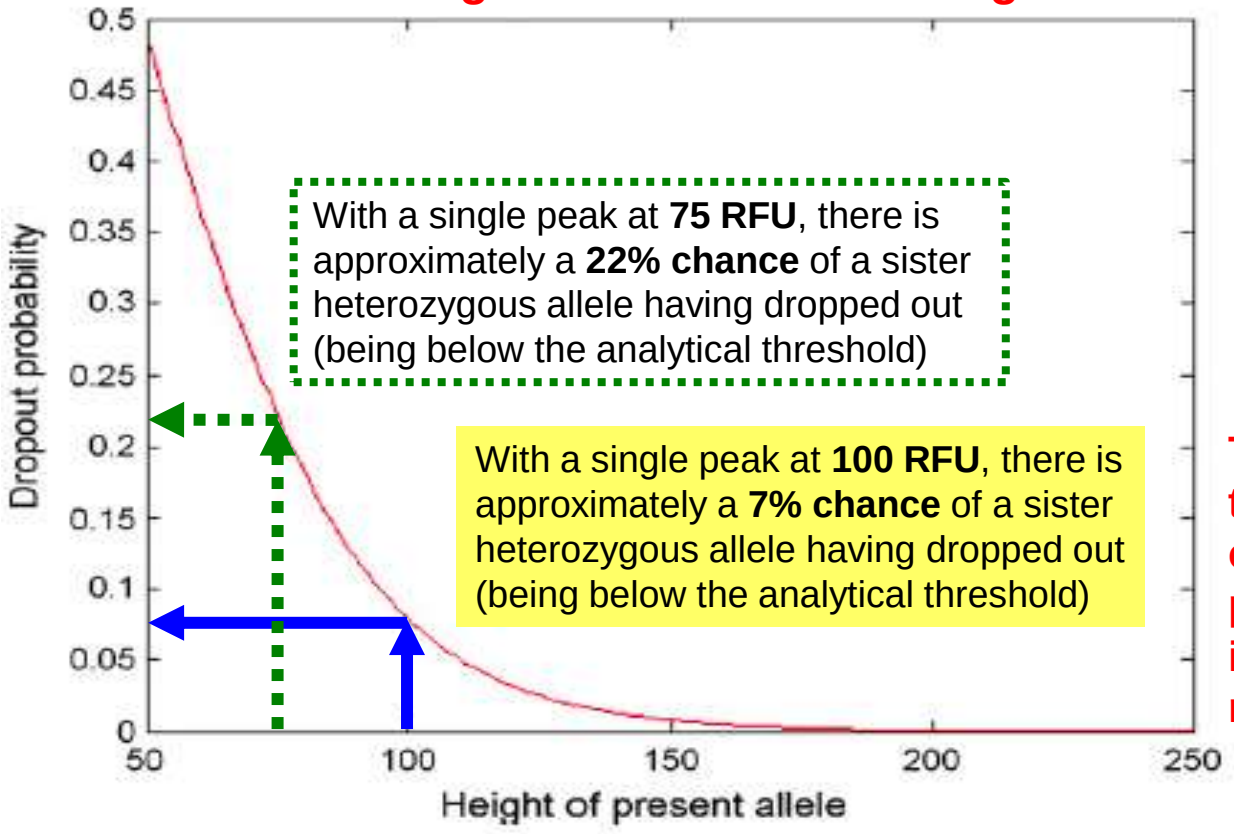
Stochastic and Analytical Thresholds

Impact Lowest Expected Peak Height Ratio



Setting a Stochastic Threshold is Essentially Establishing a Risk Assessment

Drop Out Probability as a Function of Surviving Sister Allele Peak Height



"Currently, most laboratories use an arbitrary stochastic threshold. **When a protocol is changed, especially if it is made more sensitive to low-level DNA, then the stochastic threshold must also change**."

Puch-Solis R, et al. (2011). Practical determination of the low template DNA threshold. *Forensic Sci. Int. Genet.* 5(5): 422-427.

The position and shape of this curve may change based on anything that can impact peak detection (e.g., CE injection time, PCR cycle number, post-PCR cleanup).

Gill, P., et al. (2009). The *low-template* (stochastic) threshold-Its determination relative to risk analysis for national DNA databases. *FSI Genetics*, 3, 104-111.

Acknowledgments

- For additional information, see
  http://www.cstl.nist.gov/biotech/strbase/mixture.htm

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

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