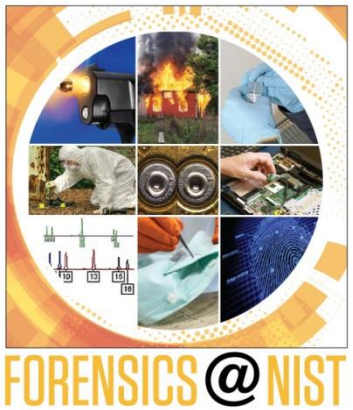


DNA Mixture Interpretation Webcast

April 12, 2013

http://www.nist.gov/oles/forensics/dna-analyst-training-on-mixture-interpretation.cfm

http://www.cstl.nist.gov/strbase/mixture.htm

Introduction to Interpretation Issues

John M. Butler

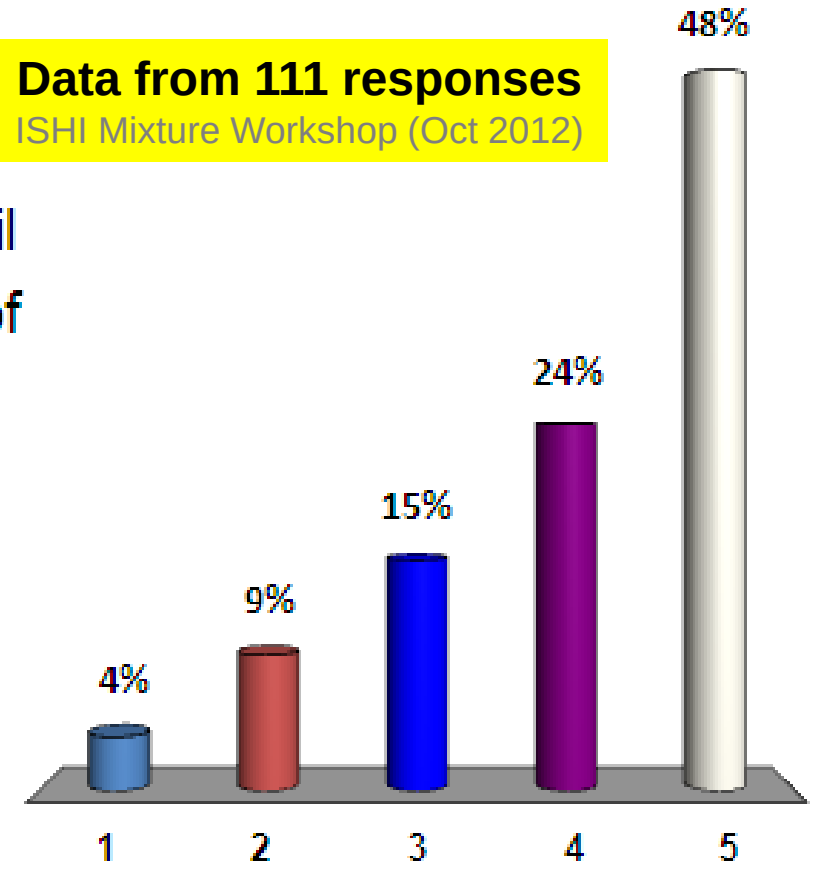
National Institute of Standards and Technology

2012 Response at ISHI Workshop

Which of the topics below would be your first choice for additional training?

1. Relevant literature
2. How to validate thresholds in more detail
3. Reporting and the use of assumptions
4. **Interpretation of low level mixtures**
5. **Likelihood ratios and other statistical approaches**

Data from 111 responses
ISHI Mixture Workshop (Oct 2012)



~75% want more information on these topics

Planned Presentation Outline

- Overview/thoughts on interpretation & statistics
- SWGDAM 2010 interpretation guidelines
- Thoughts on setting thresholds
- Problems with CPI/CPE statistics
- Take home messages

Steps Involved in Process of Forensic DNA Typing

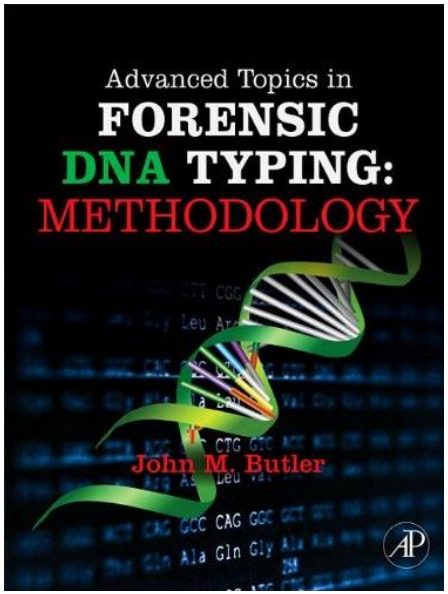
1) Data Interpretation
2) Statistical Interpretation

Gathering the Data **Understanding the Data**

Collection/Storage/ Characterization	Extraction/ Quantitation	Amplification/ Marker Sets	Separation/ Detection	Interpretation	Report

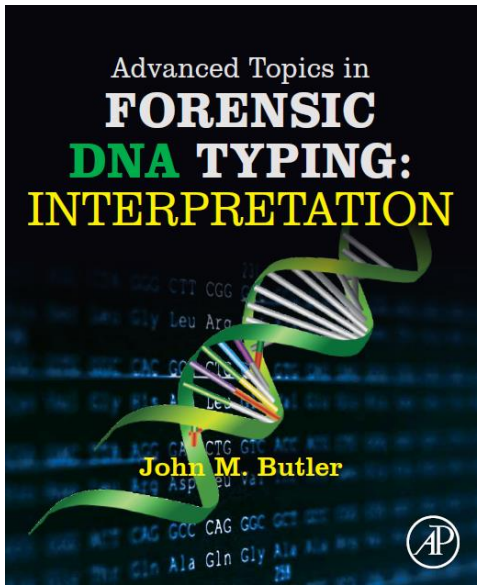
Advanced Topics: Methodology

Published August 2011 (2012)



Advanced Topics: Interpretation

Hopefully Fall 2014 *(delayed)*



Importance of Improved Understanding Regarding DNA Mixture Interpretation

- Each DNA analyst may think his or her approach is correct – but misinterpretations have given rise to a variety of approaches being undertaken today, some of which are not correct…

- I believe that **a better understanding of general principles will aid consistency and quality of work being performed**

What We Hope to Accomplish with this NIST Webcast

Desired Learning Outcomes:

- Explore how the analytical threshold and stochastic threshold affect data analysis, interpretation, conclusions and statistical calculations in mixed DNA profiles
- Examine approaches for establishing one or more analytical thresholds and stochastic thresholds for casework
- Enhance knowledge of mixture interpretation and presentation of results, conclusions and opinions

Many Labs are in the Process of Changing their Protocols

Perhaps lowering the expected peak height ratio (PHR) from 70% down to 55% when interpreting DNA mixtures?

Using **Ideal Data** to Discuss Principles

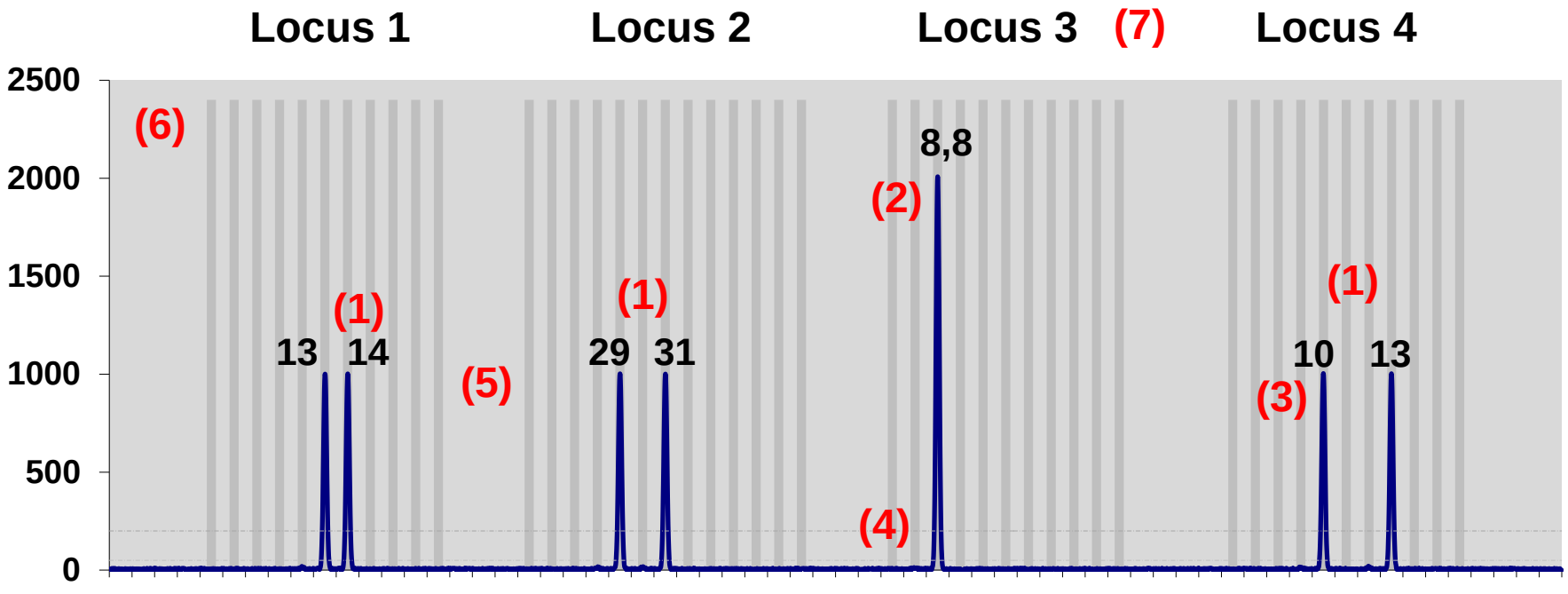


image created with EPG Maker(SPM v3)
kindly provided by Steven Myers (CA DOJ)

(1) 100% PHR between heterozygous alleles
(2) Homozygotes are exactly twice heterozygotes due to allele sharing
(3) No peak height differences exist due to size spread in alleles (any combination of resolvable alleles produces 100% PHR)
(4) No stutter artifacts enabling mixture detection at low contributor amounts
(5) Perfect inter-locus balance
(6) Completely repeatable peak heights from injection to injection on the same or other CE instruments in the lab or other labs
(7) Genetic markers that are so polymorphic all profiles are fully heterozygous with distinguishable alleles enabling better mixture detection and interpretation

Challenges in Real-World Data

- **Stochastic (random) variation** in sampling each allele during the PCR amplification process
  - This is highly affected by DNA quantity and quality
  - Imbalance in allele sampling gets worse with low amounts of DNA template and higher numbers of contributors
- **Degraded DNA** template may make some allele targets unavailable
- **PCR inhibitors** present in the sample may reduce PCR amplification efficiency for some alleles and/or loci
- **Overlap of alleles** from contributors **in DNA mixtures**
  - Stutter products can mask true alleles from a minor contributor
  - Allele stacking may not be fully proportional to contributor contribution

D.N.A. Approach to Understanding

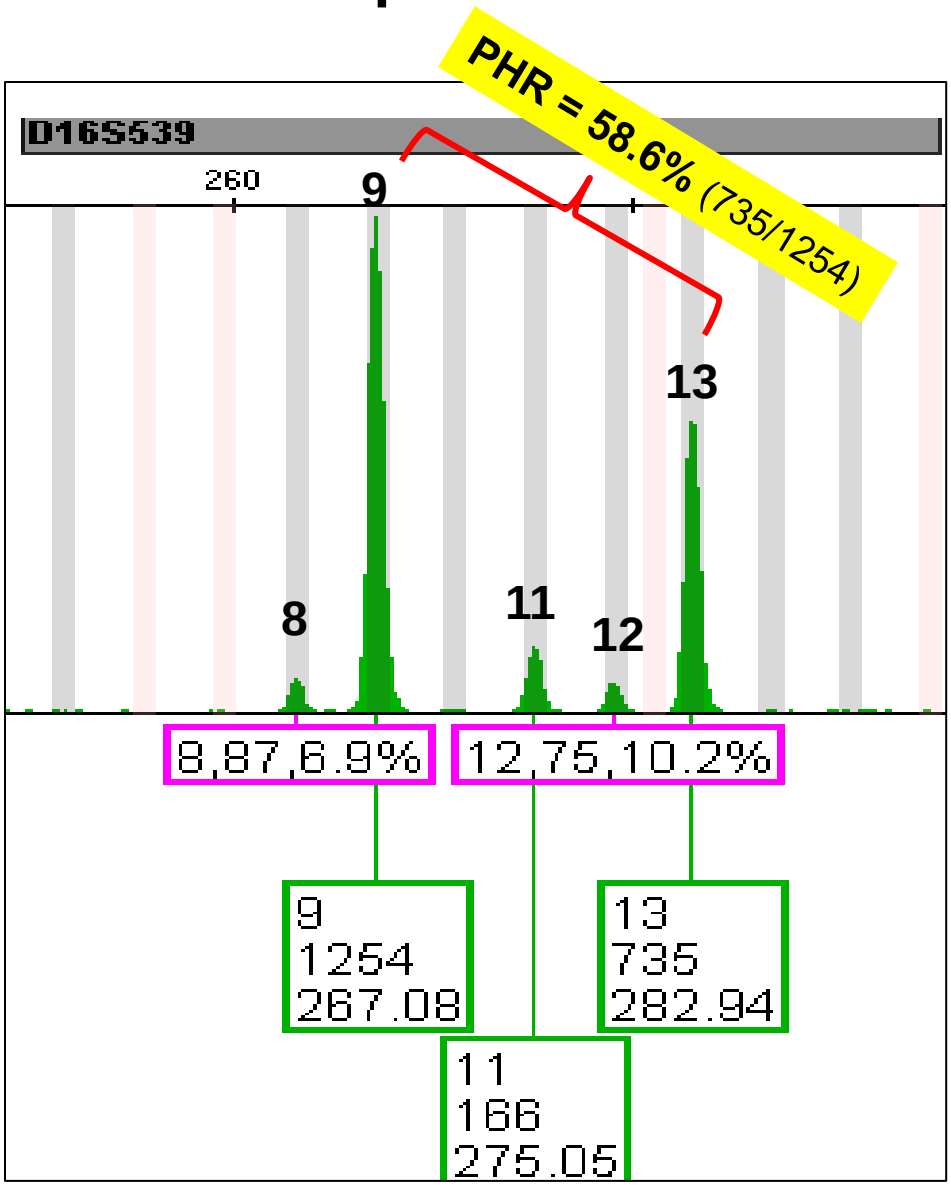
- **D**octrine or Dogma (why?)
  - A fundamental law of genetics, physics, or chemistry
    - Offspring receive one allele from each parent
    - Stochastic variation leads to uneven selection of alleles during PCR amplification from low amounts of DNA templates
    - Signal from fluorescent dyes is based on …
- **N**otable Principles (what?)
  - The amount of signal from heterozygous alleles in single-source samples should be similar
- **A**pplications (how?)
  - Peak height ratio measurements can associate alleles into possible genotypes

Results Depend on Assumptions

- "Although courts expect one simple answer, statisticians know that **the result depends on how questions are framed and on assumptions tucked into the analysis**."
  - Mark Buchanan, Conviction by numbers. *Nature* (18 Jan 2007) 445: 254-255

- SWGDAM 2010 Interpretation Guideline 3.6.5
  - "Because **assumptions** regarding the origin of evidence or the number of contributors to a mixture **can impact comparisons**, the laboratory should **establish guidelines for documenting any assumptions that are made when formulating conclusions**"

Example: D16S539 from Profile 1

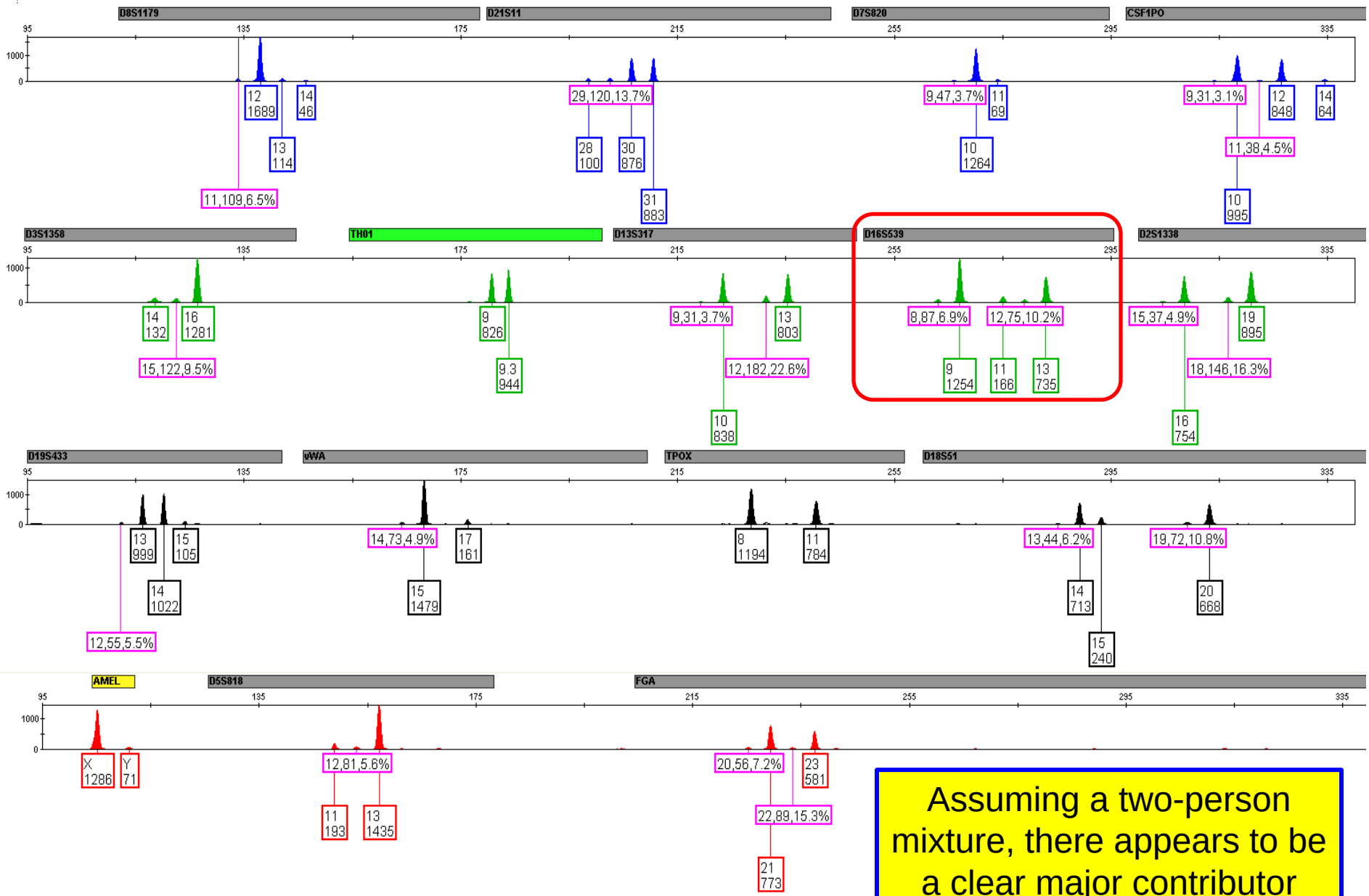


PHR = peak height ratio; also known as heterozygote balance (Hb)

Some Observations:

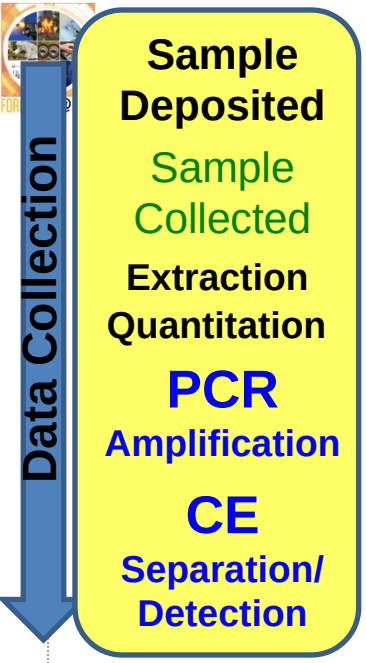
- Depending on expected PHR, alleles 9 and 13 may or may not be associated into a genotype (<60%)
- Allele 11 could be paired with 8, 9, 12, or 13 or itself (11,11 homozygote) depending on stochastic threshold
- Alleles 8 and 12 could be stutter products or possibly be paired with allele 11

Profile 1 (stutter filter off)



Assuming a two-person mixture, there appears to be a clear major contributor

Steps in DNA Interpretation



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

A threshold is a value used **to reflect reliability of information** (generally you are more confident of data above a threshold than below)

Signal observed

Data Interpretation

- Analytical Threshold → Peak
- Stutter Threshold → Allele
- Stochastic Threshold → ***All Alleles Detected?***
- Peak Height Ratio → Genotype(s)
- Mixture Ratio → Contributor profile(s)

Comparison to Known(s)
Weight of Evidence (Stats)

Overview of the SWGDAM 2010 Interp Guidelines

http://www.swgdam.org/Interpretation_Guidelines_January_2010.pdf

1. Preliminary evaluation of data – **is something a peak and is the analysis method working properly?**
2. Allele designation – **calling peaks as alleles**
3. Interpretation of DNA typing results – **using the allele information to make a determination about the sample**
    1. Non-allelic peaks
    2. Application of peak height thresholds to allelic peaks
    3. Peak height ratio
    4. Number of contributors to a DNA profile
    5. Interpretation of DNA typing results for mixed samples
    6. Comparison of DNA typing results
4. Statistical analysis of DNA typing results – **assessing the meaning (rarity) of a match**

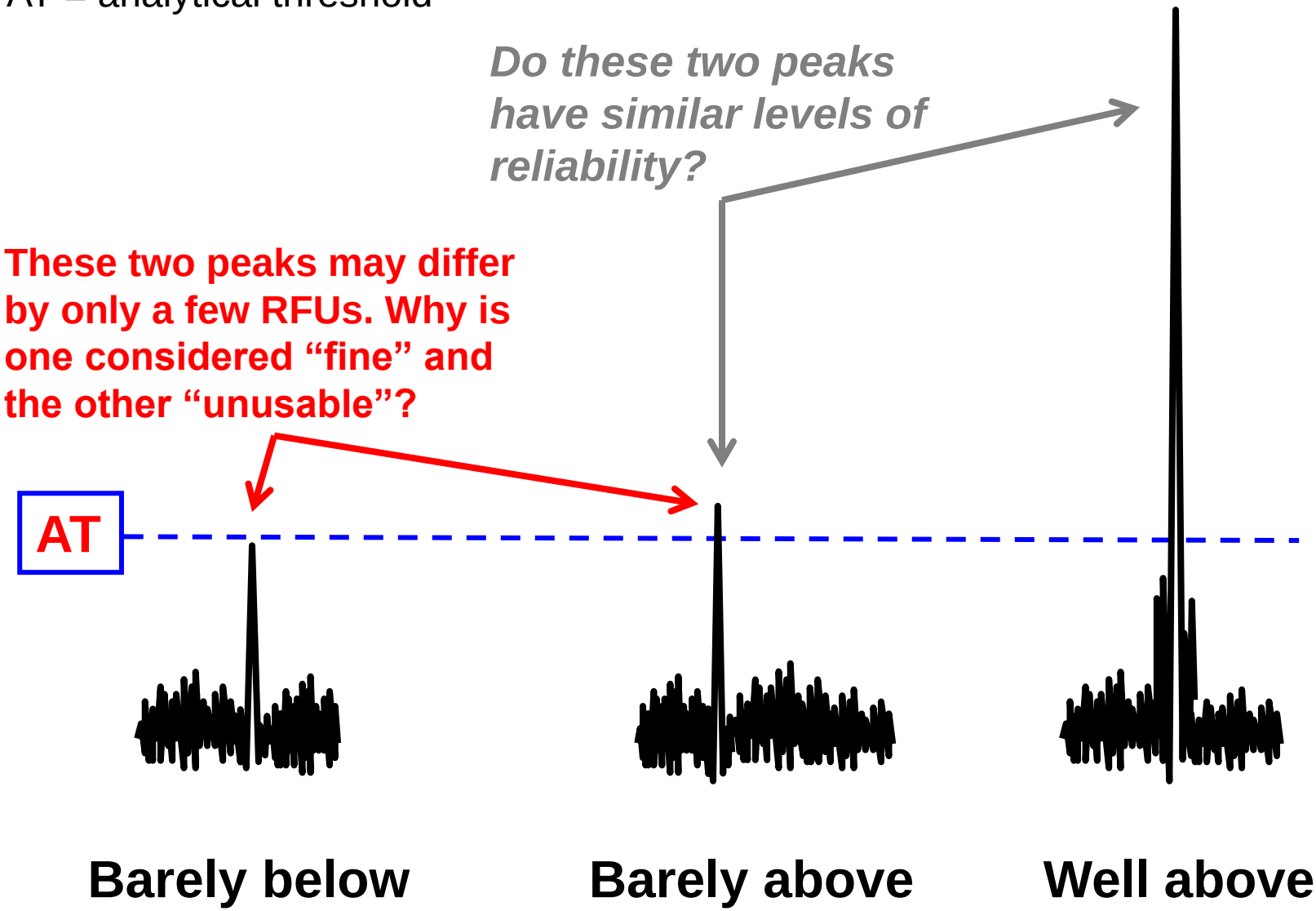
Other supportive material: statistical formulae, references, and glossary

Principles Behind Thresholds

Thresholds (example values)	**Principles Behind** *(if properly set based on lab- & kit-specific empirical data)*
Analytical Threshold (e.g., 50 RFU)	Below this value, observed peaks cannot be reliably distinguished from instrument noise (baseline signal)
Limit of Linearity (e.g., 5000 RFU)	Above this value, the CCD camera can become saturated and peaks may not accurately reflect relative signal quantities (e.g., flat-topped peaks) and lead to pull-up/ bleed-through between dye color channels
Stochastic Threshold (e.g., 250 RFU)	Above this peak height value, it is reasonable to assume that allelic dropout of a sister allele of a heterozygote has not occurred at that locus; single alleles above this value in single-source samples are assumed to be homozygous
Stutter Threshold (e.g., 15%)	Below this value, a peak in the reverse (or forward) stutter position can be designated as a stutter artifact with single-source samples or some mixtures (often higher with lower DNA amounts)
Peak Height Ratio (e.g., 60%)	Above this value, two heterozygous alleles can be grouped as a possible genotype (often lower with lower DNA amounts)
Major/Minor Ratio (e.g., 4:1)	When the ratio of contributors is closer than this value in a two-person mixture, it becomes challenging and often impossible to correctly associate genotype combinations to either the major or minor contributor

What is the meaning of a threshold?

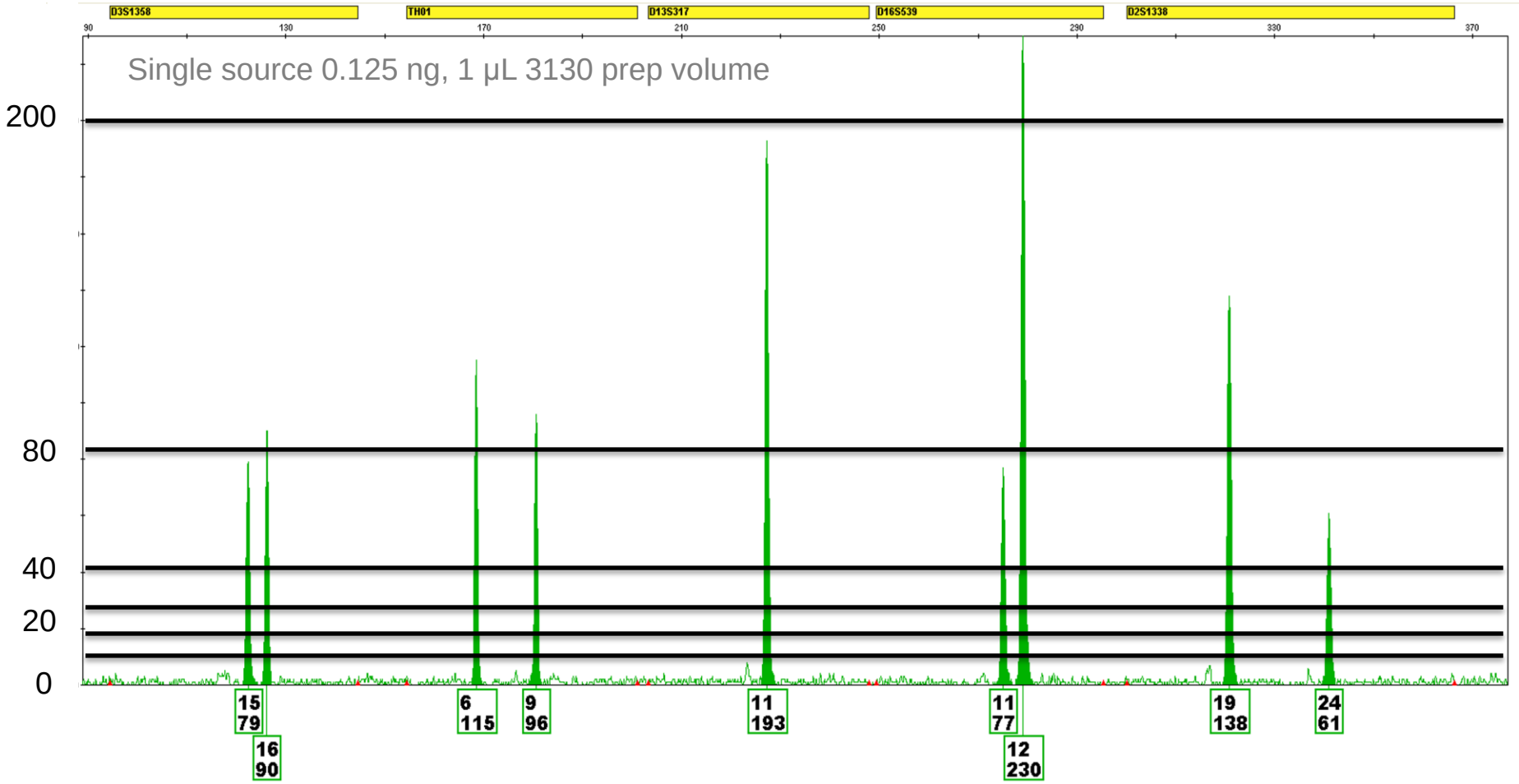
AT = analytical threshold



Impact of Various Analytical Thresholds

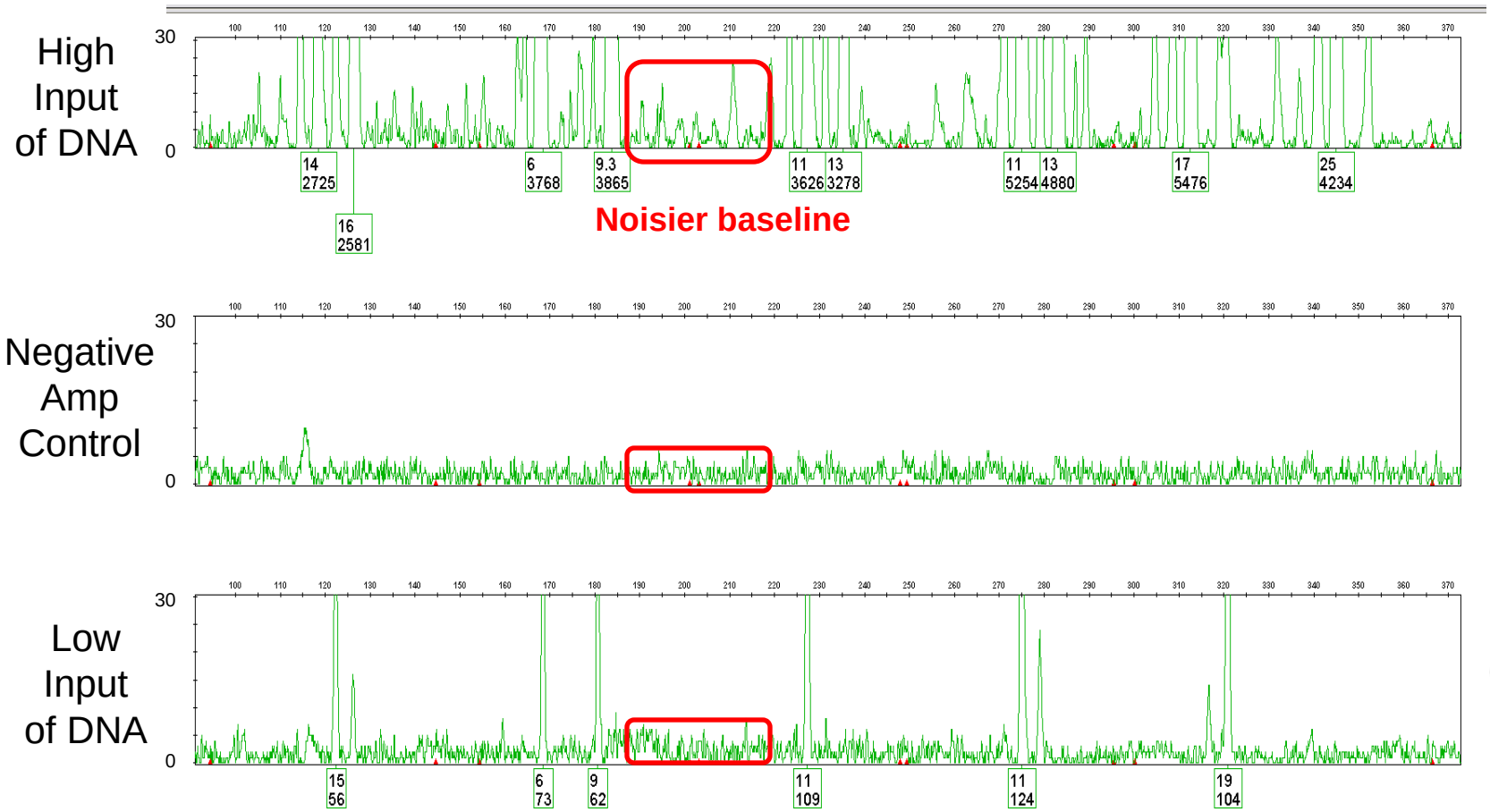
Rakay et al. (2012) Maximizing allele detection: effects of analytical threshold and DNA levels on rates of allele and locus drop-out. *Forensic Sci. Int. Genet.* 6: 723-728.

Bregu et al. (2013) Analytical thresholds and sensitivity: establishing RFU thresholds for forensic DNA analysis. *J. Forensic Sci.* 58(1): 120-129.



Data and slide courtesy of Catherine Grgicak (Boston U.)

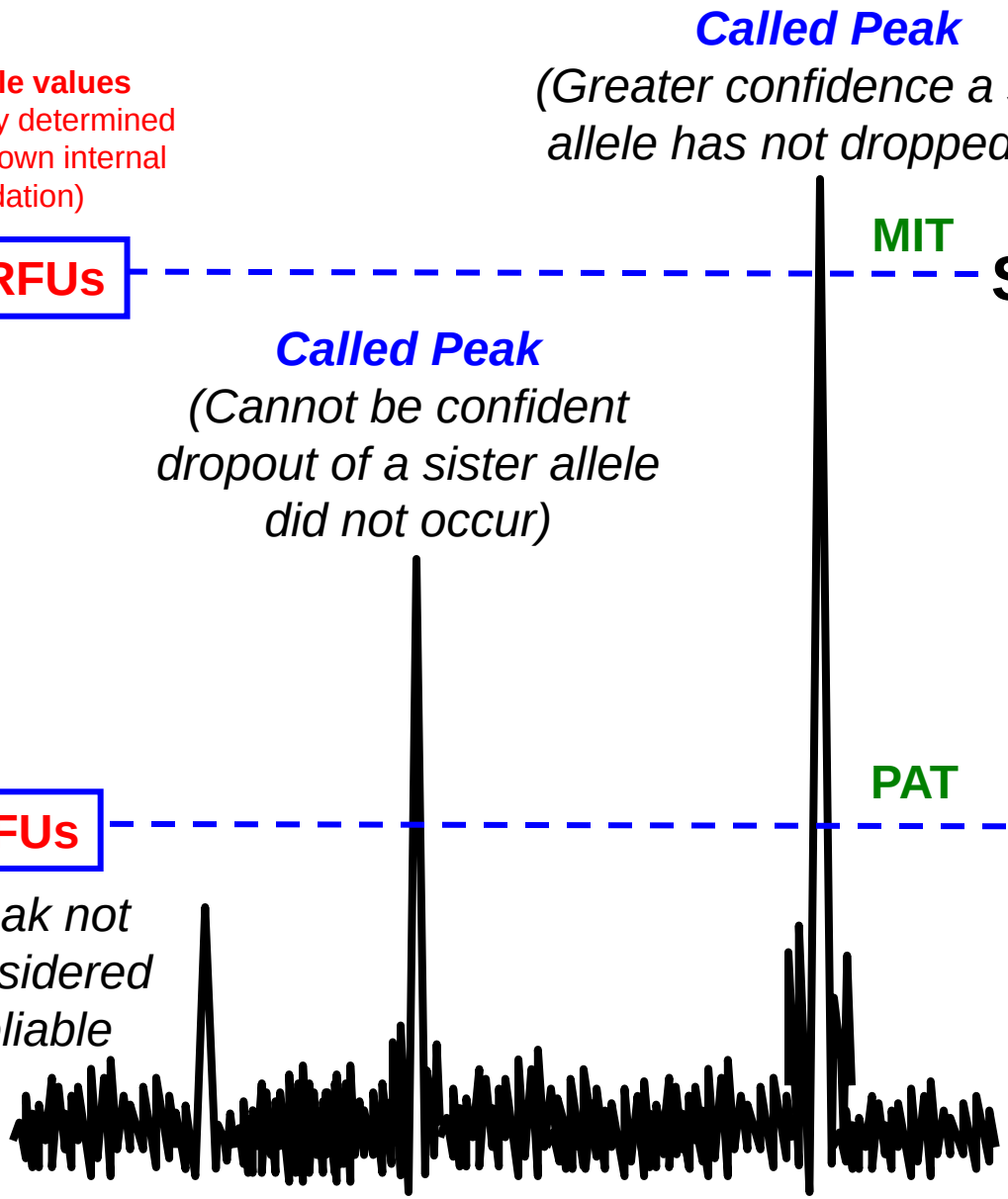
Baselines Positives ≠ Baselines Negatives



Bregu et al. (2013) Analytical thresholds and sensitivity: establishing RFU thresholds for forensic DNA analysis. *J. Forensic Sci.* 58(1): 120-129.

Data and slide courtesy of Catherine Grgicak (Boston U.)

Overview of Two Thresholds



Example values (empirically determined based on own internal validation)

200 RFUs

MIT

Stochastic Threshold

The value above which it is reasonable to assume that allelic dropout of a sister allele has not occurred

30 RFUs

PAT

Analytical Threshold

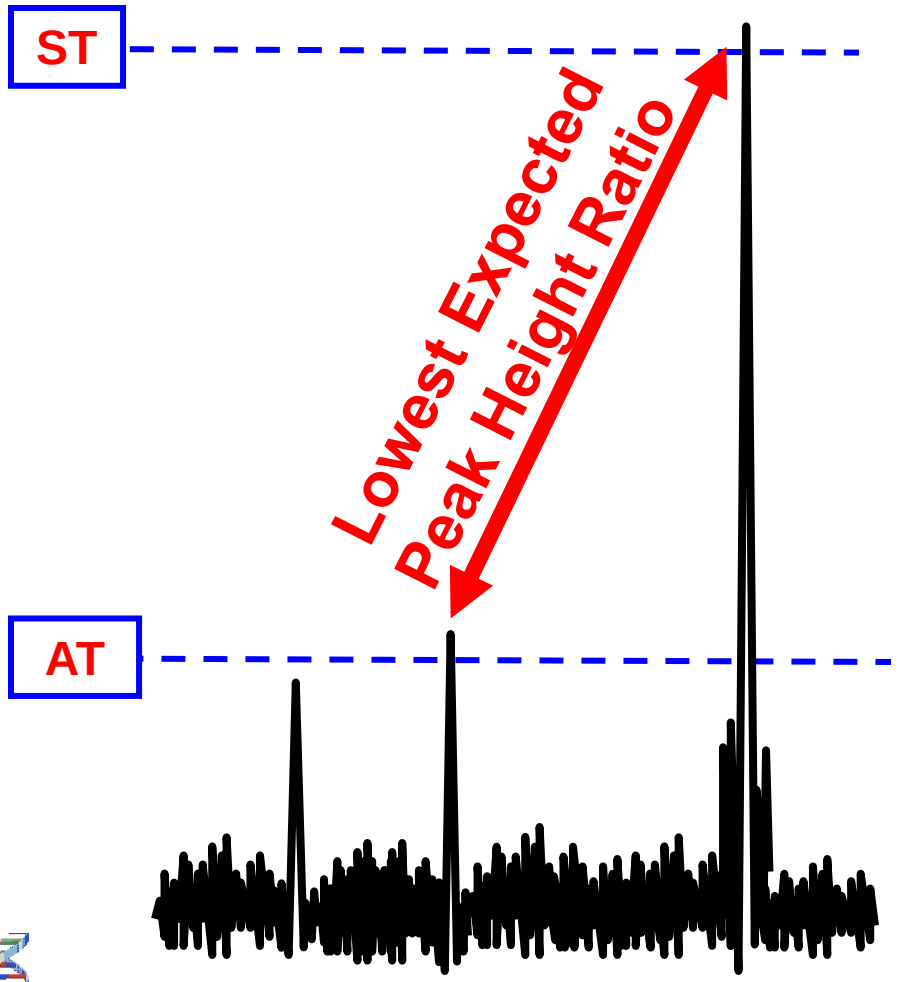
Minimum threshold for data comparison and peak detection in the DNA typing process

Noise

Butler, J.M. (2010) *Fundamentals of Forensic DNA Typing*. Elsevier Academic Press: San Diego.

Stochastic and Analytical Thresholds

Impact Lowest Expected Peak Height Ratio



The lower you go trying to analyze low-level data… (i.e., more sensitive STR kits)

the worse your expected peak height ratios for single-source samples

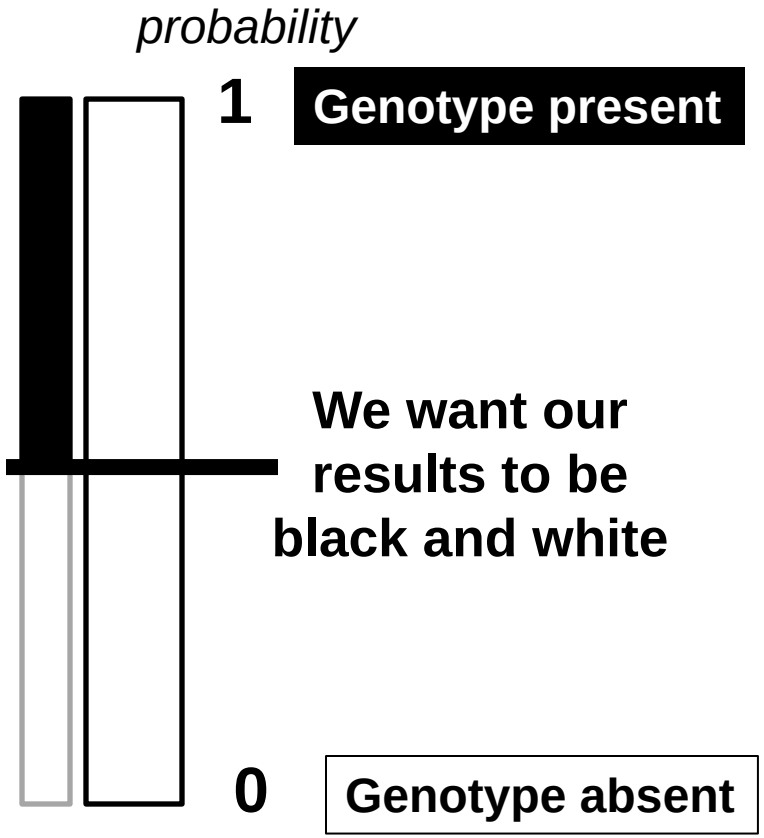
Therefore, there is greater uncertainty with associating genotypes of contributors in mixtures (or even determining that you have a mixture)

Keep in Mind…

"The use of bounds **applied to data that show continuous variation** is common in forensic science and is often a pragmatic decision. However it should be borne in mind that applying such bounds has arbitrary elements to it and that **there will be cases where the data lie outside these bounds**."

Bright, J.A., et al. (2010). Examination of the variability in mixed DNA profile parameters for the Identifiler multiplex. *Forensic Science International: Genetics, 4,* 111-114.

Approaches to Data Interpretation:
Binary vs Probabilistic



Binary Approach

Probabilistic Approach

Is There Uncertainty in the Data?

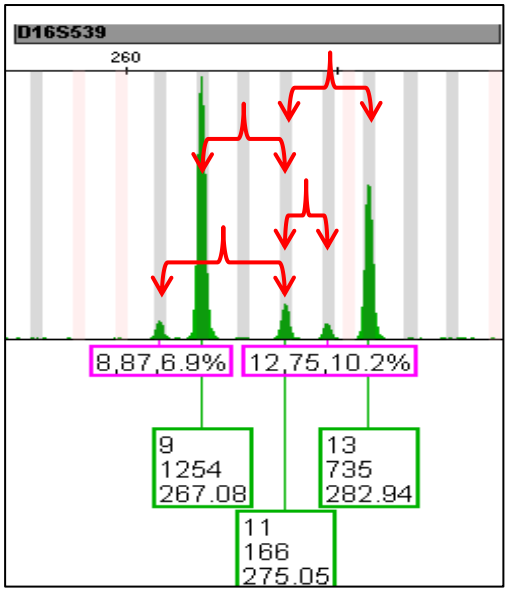
- **If allele dropout is a possibility** (e.g., in a partial profile), then there is uncertainty in whether or not an allele is present in the sample…and therefore what genotype combinations are possible

- **If different allele combinations are possible** in a mixture, then there is uncertainty in the genotype combinations that may make up the mixture result…

Minor contributor at D16S539 could be:

8,11 or
9,11 or
11,12 or
11,13 or
11,11

Possible allele pairing with the 11 allele



Uncertainty and Probability

- "Contrary to what many people think, **uncertainty is present throughout any scientific procedure**."
  - Dennis V. Lindley, in his foreword to Aitken & Taroni (2004) *Statistics and the Evaluation of Evidence for Forensic Scientists, Second Edition*

- "It is now recognized that **the only tool for handling uncertainty is probability**."
  - Dennis V. Lindley, in his foreword to Aitken & Taroni (2004) *Statistics and the Evaluation of Evidence for Forensic Scientists, Second Edition*

Conference Held in Rome in April 2012

http://www.oic.it/ForensicGenetics/immagini/img-home.jpg

Peter Gill

University of Oslo, Norway

- “If you are going to have a threshold, at least try to associate it with a level of risk. You can have a threshold any where you like, but the lower the [stochastic] threshold, the greater the risk is of wrongful designation [of genotypes]. The higher the threshold, the more likely you will have an inconclusive result.”

David Balding

- “In ideal analysis, we would never use thresholds, but in practice they are useful. I don’t think we have sophisticated enough models in many situations to understand all of the details of the data. **Thresholds provide a simplification.** That is reasonable as long as they are backed up by calibration evidence.”

Rome meeting, April 27-28, 2012: The hidden side of DNA profiles: artifacts, errors and uncertain evidence

Bruce Budowle

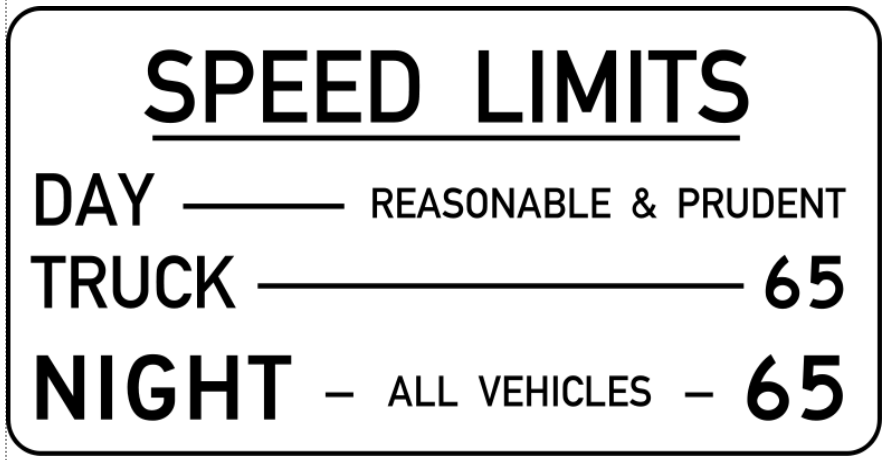
University of North Texas Health Science Center

- "**We put thresholds in place to help protect us from risk of making wrong decisions**. They have value."

- **Compares thresholds to speed limits**, which are set for safety reasons

Do you leave thresholds and protocols up to “analysts’ discretion”?

http://korsgaardscommentary.blogspot.com/2011/10/its-time-to-put-brakes-on-speed-limit.html



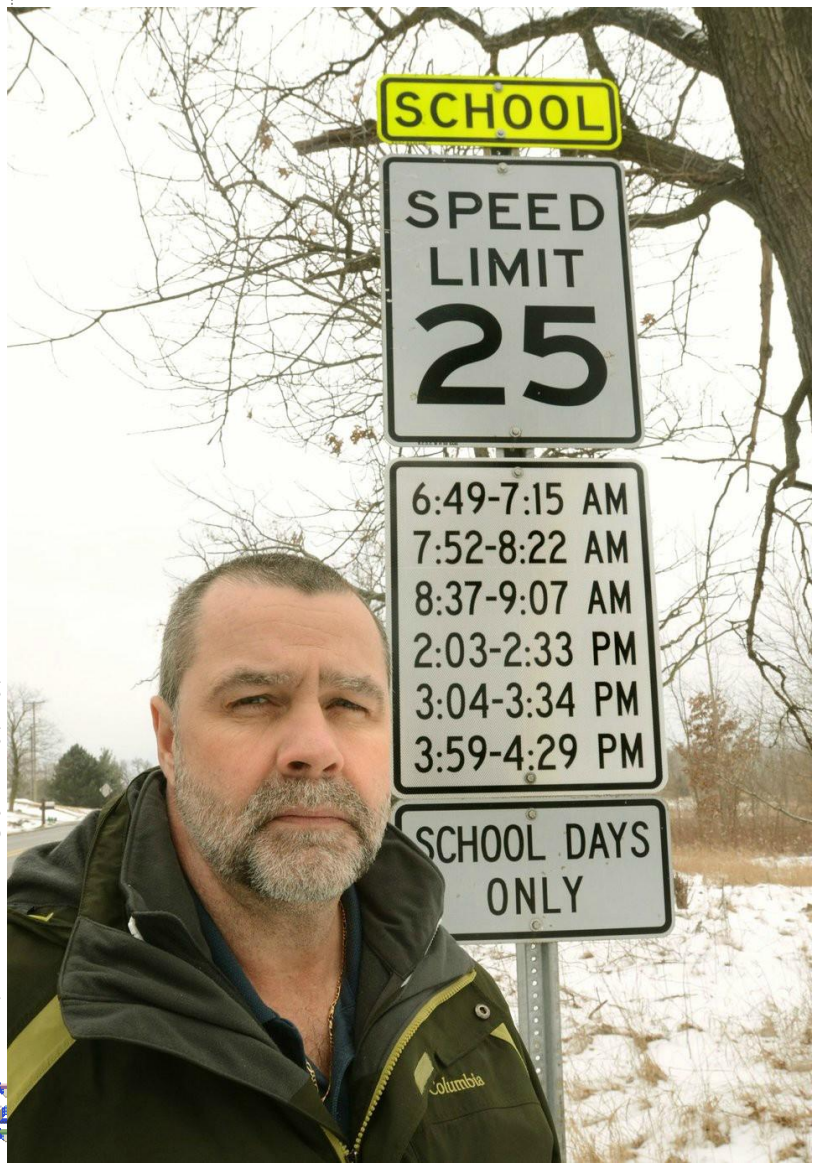
http://en.wikipedia.org/wiki/File:MONTANA-PR.svg

Typical speed limit sign that one would see at the Montana state line from December 1995 to June 1999

http://1.bp.blogspot.com/-5gagl4xZbT0/TdvMBGODBZI/AAAAAAAAJYo/Pj9MRqANvvs/s400/speed-limit-change-sign-537.jpg

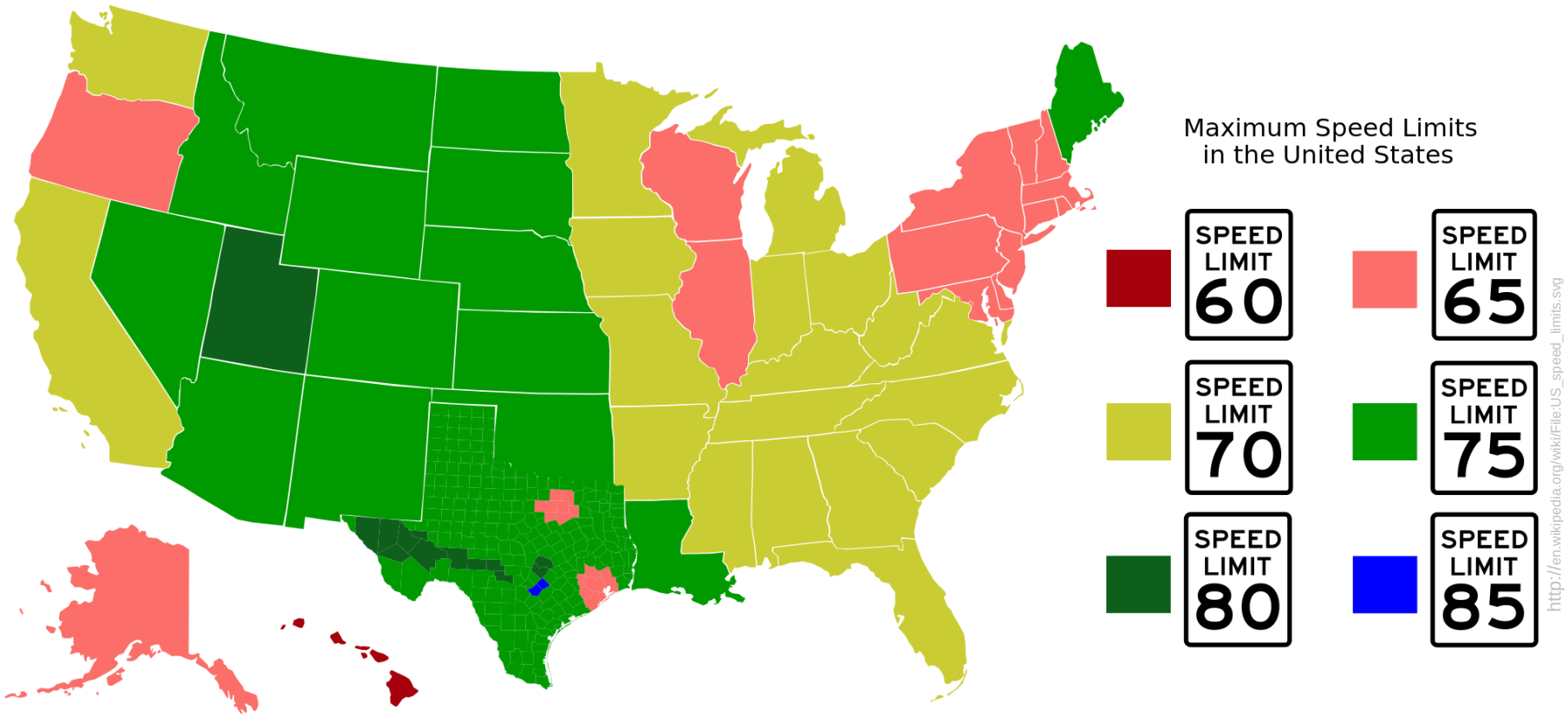
A Potential Outcome!

Do you carefully try to regulate everything with specific protocols?



Truly **a protocol with specificity**…. we even have **an auditor**, the local chief of police!

A variety of approaches exist for how protocols and thresholds are set…

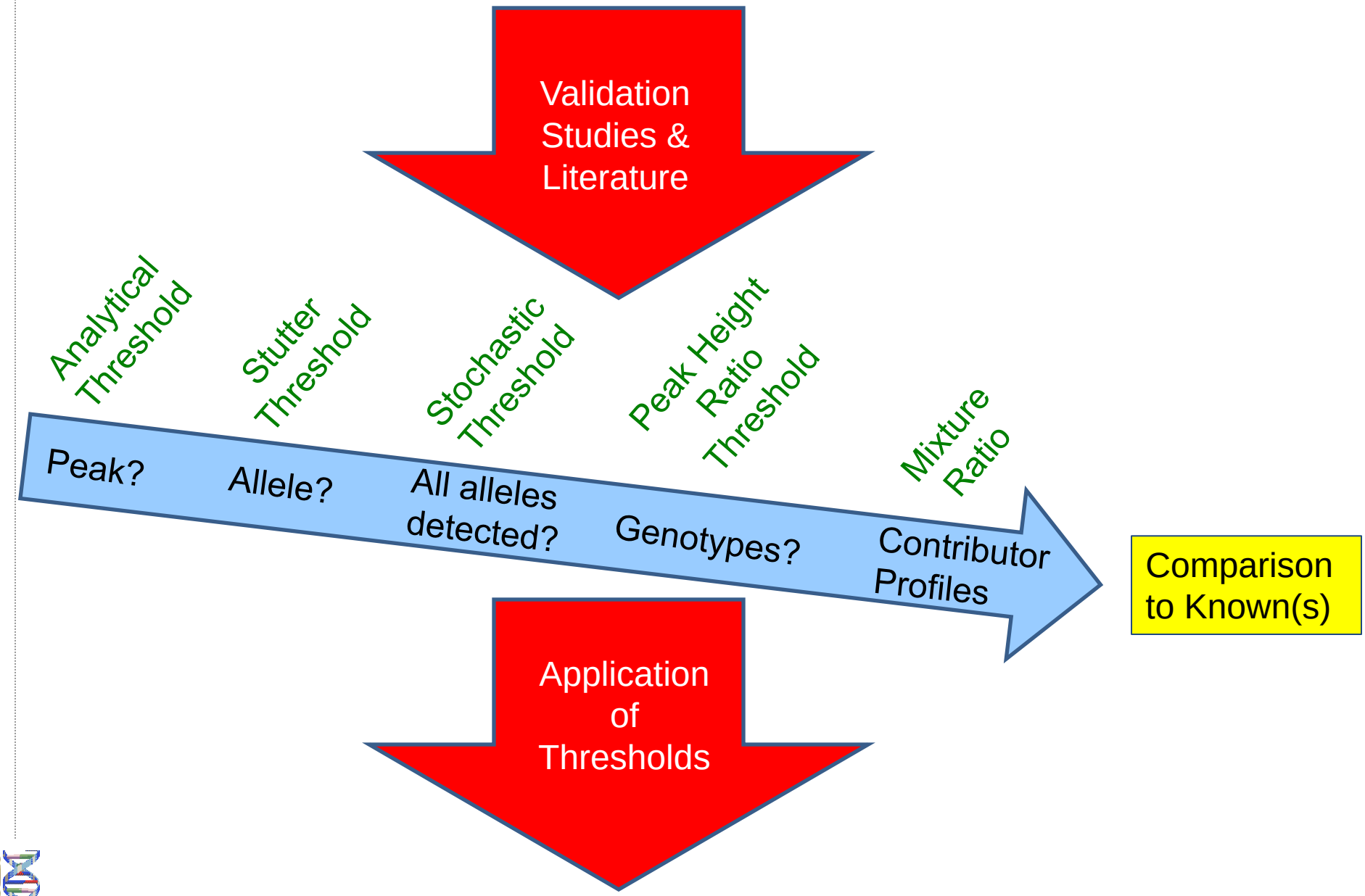


http://en.wikipedia.org/wiki/Speed_limits_in_the_United_States

Threshold Decisions

Thresholds to Determine	Decisions to Make (lab & kit specific)	Useful Validation Data
Analytical = _____ RFU	Single overall value or color specific	Noise levels in negative controls or non-peak areas of positive controls
Stochastic = _____ RFU	Minimum peak height RFU value or alternative criteria such as quantitation values or use of a probabilitistic genotype approach	Level where dropout occurs in low level single-source heterozygous samples under conditions used (e.g., different injection times, post-PCR cleanup)
Stutter filter = ___%	Profile, locus, or allele-specific	Stutter in single-source samples (helpful if examined at multiple DNA quantities)
Peak Height Ratio = ___%	Profile, locus, or signal height (quantity) specific	Heterozygote peak height ratios in single-source samples (helpful if examined at multiple DNA quantities)
Major/Minor Ratio = _____	When will you attempt to separate components of a mixture into major and minor contributors for profile deductions?	Defined mixture ratios (e.g., 1:1, 1:3, 1:9) with known samples to observe consistency across loci and to assess ability to deduce correct contributor profiles

Steps in DNA Interpretation



How Speed Limits Are Set?

http://www.crab.wa.gov/LibraryData/REPORTS/EngineerAnswers/Article03-04SpeedLimits.pdf

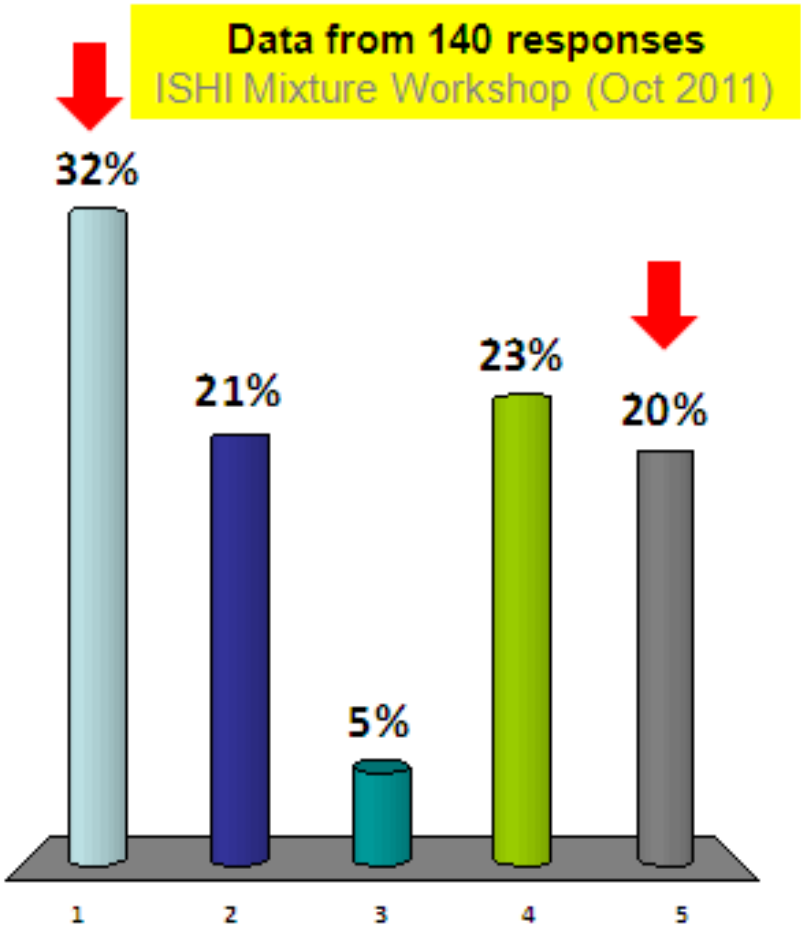
The posted speed limit for a road is set in slightly different ways in different counties. The most common way though, is to use **the "85th percentile" speed**. 85 out of 100 drivers will choose this speed no matter what the signs say. Many studies have shown this method to be safe, practical and enforceable. It also doesn't depend on the opinion of one person.

The 85th percentile speed is easily determined with special traffic counters that check the traffic on the roadway. The speed limit can then be set at the next lower 5 miles per hour. For example, if the traffic counters show 38 mph, the limit would be set at 35 mph. The speed limit may be set another 5 mph lower if there are features not obvious to the driver. These may include unusual roadside or traffic conditions including a high number of accidents.

2011 Response from ISHI Workshop

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

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!

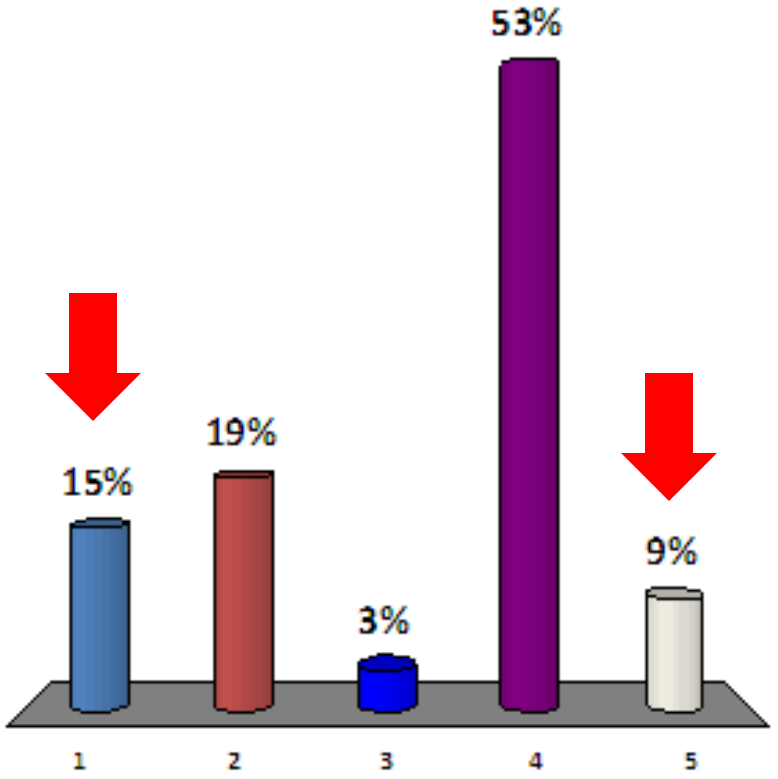


2012 Response from ISHI Workshop

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

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!

Data from 120 responses
ISHI Mixture Workshop (Oct 2012)



A Few Slides Were Kindly Provided by the Life Technologies/Applied Biosystems Validation Group Showing Data Variation between ABI 3130xl and ABI 3500

Dynamic Range of 3130*xl* vs. 3500 Genetic Analyzer

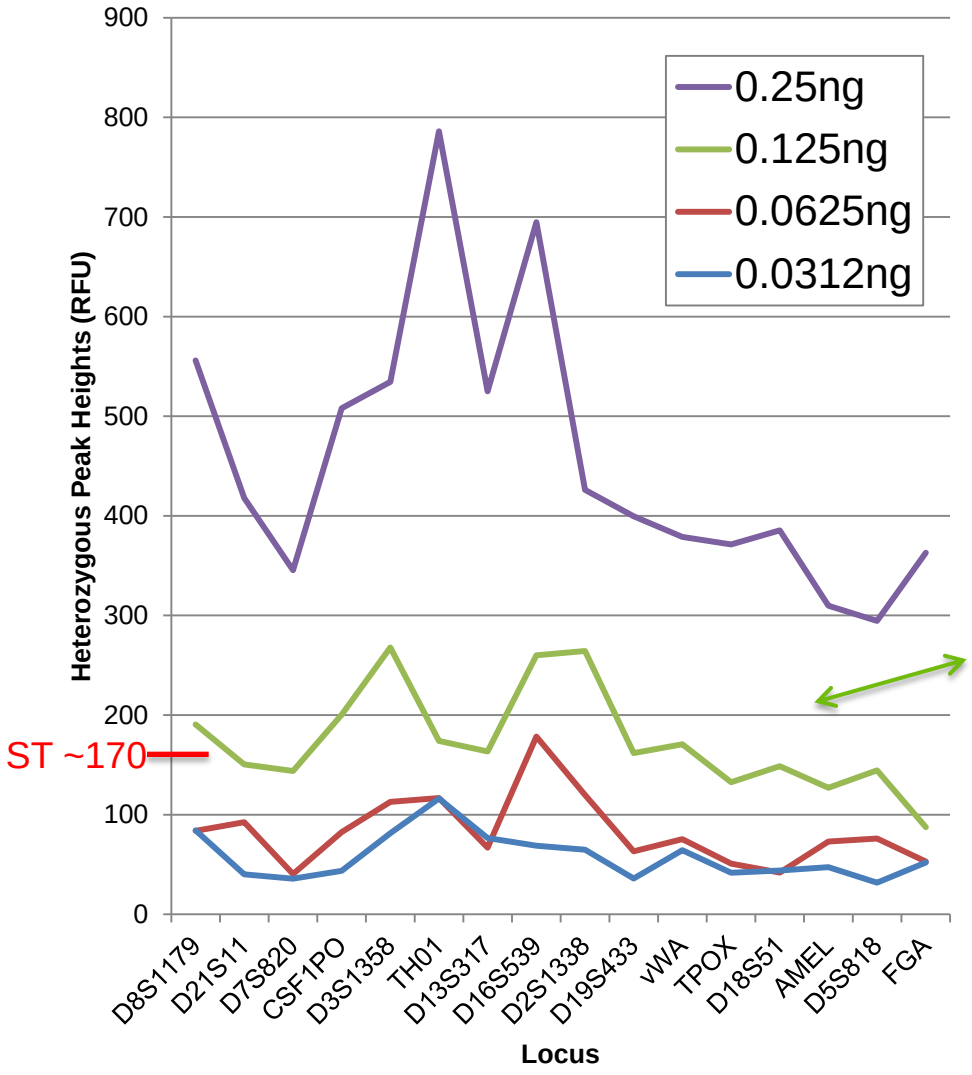
	3500	3130*xl*
Saturation	~8,000	~30,000 – 32,000
	3000 RFU	12,000 RFU
Optimal Target Range (RFU)	Heterozygote ~1,500	Heterozygote ~6,000
	1000 RFU	3000 RFU
Stochastic Threshold	Peak Height Ratio Imbalance Low Template DNA ?	Peak Height Ratio Imbalance Low Template DNA ?

Slide kindly provided by Joanne B. Sgueglia and Jennifer L. Elliott (Life Technologies, HID Professional Services)

Stochastic Threshold Considerations

Identifiler® Plus on a 3130*xl* Genetic Analyzer

Sensitivity Dilution Series (Low Level Samples)



Peak Height Ratios for Heterozygous Loci (%)

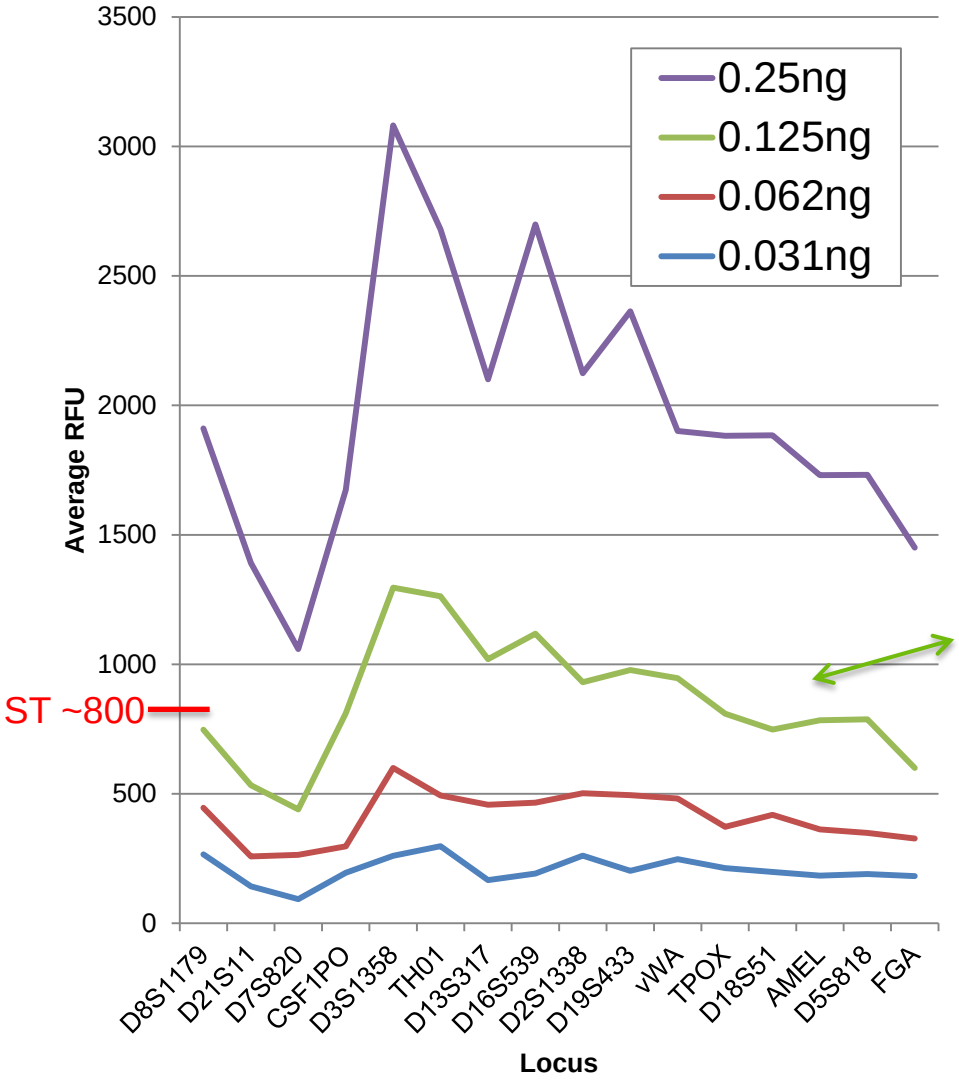
Input (ng)	Rep	D8S1179	D21S11	CSF1PO	D3S1358	TH01	D19S433	TPOX	D18S51	AMEL	D5S818	FGA
1	1	98.85	93.71	99.60	93.73	82.76	95.04	86.05	84.05	85.24	85.19	98.76
	2	61.14	71.21	85.29	83.58	96.74	72.95	99.73	99.57	91.78	88.79	84.95
	3	97.03	81.28	92.36	81.99	76.06	95.01	74.28	81.76	89.32	93.80	90.71
0.5	1	81.09	91.38	91.06	76.22	77.14	80.95	89.56	97.39	99.27	91.67	81.66
	2	86.35	59.08	79.69	68.36	86.11	77.15	88.84	74.51	73.03	90.72	79.23
	3	96.72	94.82	81.30	78.05	92.68	70.51	77.42	83.36	89.69	61.67	86.78
0.25	1	89.43	83.33	95.71	82.47	86.13	86.05	68.44	66.98	70.23	88.75	63.13
	2	85.35	98.04	97.48	83.43	54.59	84.73	97.91	77.19	78.53	98.08	80.54
	3	98.15	88.83	94.42	99.15	78.76	63.98	84.73	97.67	66.99	93.87	75.86
0.125	1	77.51	81.44	80.40	64.04	91.20	46.50	38.22	86.49	56.34	81.60	93.24
	2	92.42	80.29	73.57	88.29	75.52	76.16	85.50	81.31	45.58	95.26	96.15
	3	52.86	93.63	91.88	90.88	20.63	76.27	73.20	77.24	42.36	94.87	83.51
0.0625	1	34.21	29.88	78.48	58.14	18.46	45.45	86.96	73.33	64.96	79.22	52.81
	2	87.67	65.54		63.64	93.40	67.31	41.25	41.82	43.90		
	3	85.22	64.41	27.00		21.43	52.50	88.61	53.23	60.76	34.51	86.84
0.0312	1	79.27	97.14	100.00	43.10	78.38		30.49		60.61		
	2	83.33	63.08	77.59	54.10		70.00	55.56	84.62			
	3	72.13				75.66		97.73	33.33	36.27		

Slide kindly provided by Joanne B. Sgueglia and Jennifer L. Elliott (Life Technologies, HID Professional Services)

Stochastic Threshold Considerations

Identifiler® Plus on a 3500 Genetic Analyzer

Sensitivity Dilution Series (Low Level Samples)



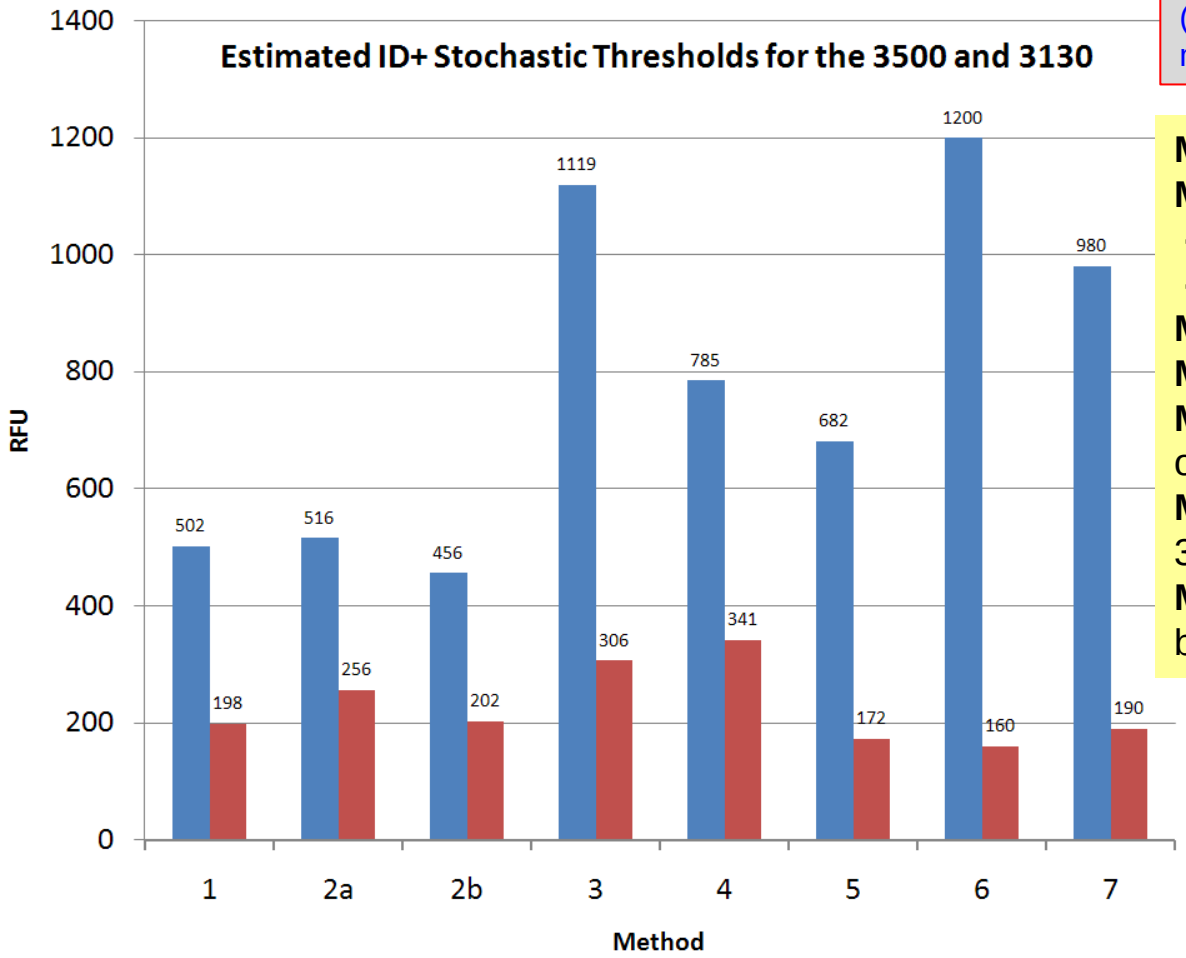
Peak Height Ratios for Heterozygous Loci (%)

Input (ng)	Rep	D8S1179	D21S11	CSF1PO	D3S1358	TH01	D19S433	TPOX	D18S51	AMEL	D5S818	FGA
1	1	87.89	95.31	98.75	90.46	92.16	84.2	95.81	89.16	92.19	92.79	86.73
	2	77.56	88.04	76.1	82.39	91.69	96.39	98.17	97.03	94.35	79.41	75.13
	3	88.75	98.69	93.17	89.93	93.61	97.18	89.31	96.23	91.	97.71	84.64
0.5	1	74.55	98.5	80.54	96.27	97.62	99.34	91.7	88.19	98.43	83.52	84.42
	2	97.99	77.72	83.95	90.21	96.5	84.2	96.97	86.39	79.97	96.96	95.46
	3	88.41	92.91	91.95	98.05	84.22	94.21	98.15	99.77	93.08	97.05	85.06
0.25	1	70.57	81.91	87.39	100.	69.09	91.63	66.12	75.48	94.87	73.67	87.13
	2	84.95	98.77	93.67	90.5	79.55	85.92	91.14	94.91	83.81	90.91	79.88
	3	88.26	98.5	84.34	64.13	85.54	78.73	85.47	85.31	86.53	84.39	98.5
0.125	1	71.51	67.76	66.59	89.05	62.59	87.71	88.61	58.62	88.92	59.88	95.45
	2	88.73	72.76	95.54	85.03	86.97	61.93	83.42	92.34	89.88	66.04	76.98
	3	88.75	86.05	80.45	70.58	84.2	92.71	93.	86.51	84.56	85.08	66.56
0.062	1	44.99	52.73	76.39	83.39	78.	58.92	78.92	45.06	69.79		72.55
	2	78.81	67.14	81.56	49.06	59.76	99.59	89.41	42.59	92.66	81.46	74.27
	3	88.85	85.95	94.61	93.93	75.41	80.86	73.35	69.19	48.02	69.23	63.24
0.031	1	43.43	38.1	54.1	57.55		91.86	48.68	70.92		85.38	
	2	71.52	45.51	51.34	41.83	88.83	77.37	29.38		70.51		
	3	73.37	20.86	68.39	39.41	75.12	47.57	66.23	83.33	80.3		45.58

Slide kindly provided by Joanne B. Sgueglia and Jennifer L. Elliott (Life Technologies, HID Professional Services)

Comparison of Different Approaches to Determining a Stochastic Threshold

Results from CA DOJ Identifiler Plus validation experiments



Studied 3 DNA samples with serial dilutions (1, 0.5, 0.25, 0.125, 0.062, 0.031, 0.016 ng), multiple amps of each template quantity

Method 1: tallest false homozygote
Method 2: false homo. ave. +3SD
- **2a:** using most relevant input amount
- **2b:** using all observed false homo.

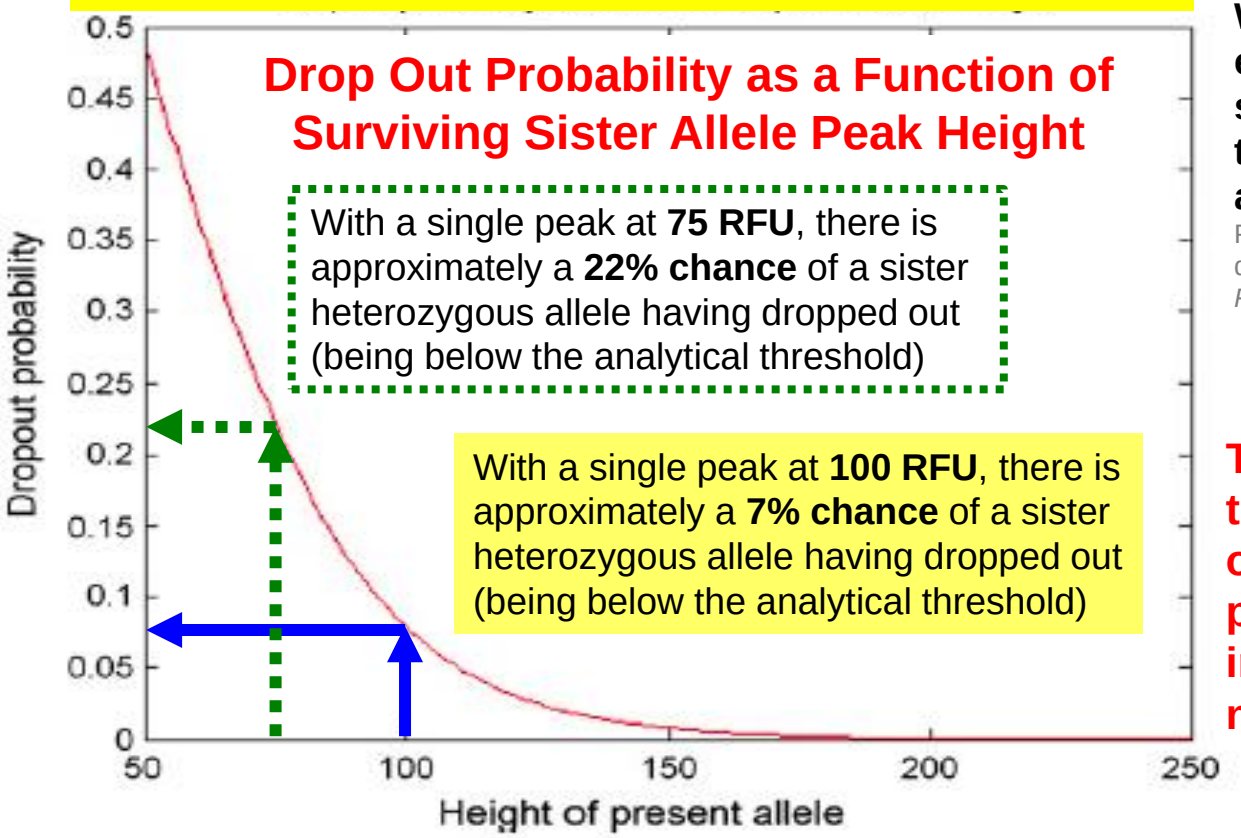
Method 3: average PH het. +3 SD
Method 4: ave. PHR -3 SD vs. signal
Method 5: AT divided by minimum observed PHR
Method 6: partial profile at ~150 pg and 3x AT
Method 7: where majority of PHRs fall below 60%

Blue bars: 3500 ST
Red bars: 3130 ST

Sonja Klein (CA DOJ) presentation at the CAC meeting (Sacramento, CA), October 25, 2011: "Approaches to estimating a stochastic threshold"

Setting a Stochastic Threshold is Essentially Establishing a Risk Assessment

How much error are you willing to accept?



"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.

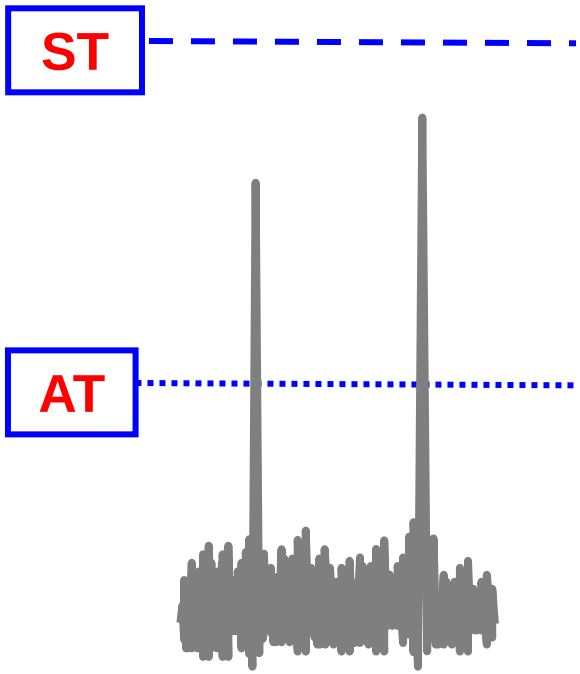
The position and shape of this curve may change based on anything that can impact peak detection (e.g., CE injection time, PCR cycle number, post-PCR cleanup).

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.

Limitations of Stochastic Thresholds

- The possibility of allele sharing with a complex mixture containing many contributors may make a stochastic threshold meaningless
- “Enhanced interrogation techniques” to increase sensitivity (e.g., increased PCR cycles) may yield false homozygotes with >1000 RFU
- **New turbo-charged kits with higher sensitivity will need to be carefully evaluated to avoid allele drop-out and false homozygotes**

Can This Locus Be Used for Statistical Calculations?



It depends on your assumption as to the number of contributors!

If you assume a single-source sample, then you can assume that the detection of two alleles fully represents the heterozygous genotype present at this locus.

If you assume (from examining other loci in the profile as a whole) that the sample is a mixture of two or more contributors, then there may be allele drop-out and all alleles may not be fully represented.

Stochastic Threshold Summary

- A stochastic threshold (ST) may be established for a specific set of conditions to reflect possibility of allele drop-out, which is essential for a CPE/CPI stats approach
- ST should be re-examined with different conditions (e.g., higher injection, sample desalting, increase in PCR cycles)
- ST will be dependent on the analytical threshold set with a method and impacts the lowest expected peak height ratio
- Assumptions of the number of contributors is key to correct application of ST

Stats Required for Inclusions

SWGDAM Interpretation Guideline 4.1:

> "**The laboratory must perform statistical analysis in support of any inclusion** that is determined to be relevant in the context of a case, irrespective of the number of alleles detected and the quantitative value of the statistical analysis."

Buckleton & Curran (2008): "There is a considerable aura to DNA evidence. Because of this aura **it is vital that weak evidence is correctly represented as weak or not presented at all**."

Buckleton, J. and Curran, J. (2008) A discussion of the merits of random man not excluded and likelihood ratios. *Forensic Sci. Int. Genet.* 2: 343-348.

Coupling of Statistics and Interpretation

- **The CPE/CPI approach** for reporting an inclusionary statistic **requires that all alleles be observed** in the evidence sample

- If allele drop-out is suspected at a locus, then any allele is possible and the probability of inclusion goes to 100% -- in other words, the locus is effectively dropped from consideration for statistical purposes

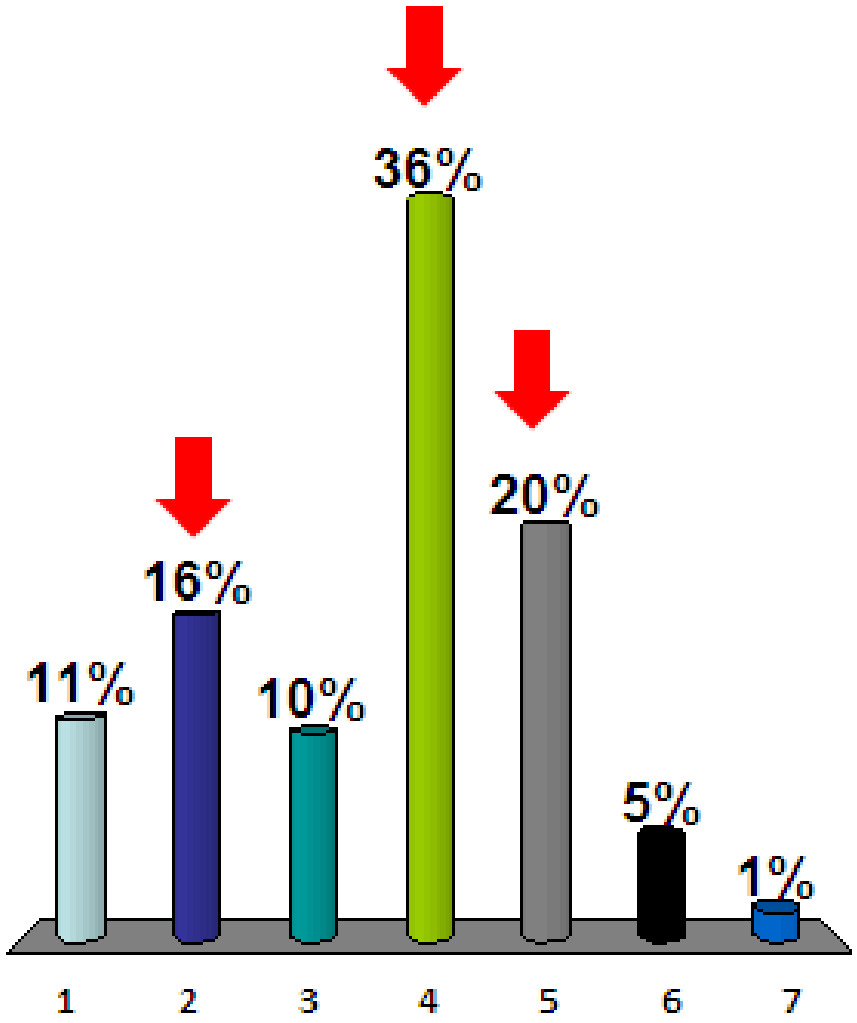
- If alleles are seen below the established stochastic threshold, then the locus is typically eliminated ("INC" – declared inconclusive) in many current lab SOPs

2011 Response from ISHI Workshop

What kind of mixture statistic does your lab use?

1. LR
2. CPE (RMNE, CPI)
3. RMP
4. CPE or RMP
5. Other combinations
6. Probabilistic modeling (e.g., TrueAllele)
7. We don't use stats (contradicting the new guidelines – section 4.1)

72% using CPI



Data from 138 responses
ISHI Mixture Workshop (Oct 2011)

CPE/CPI (RMNE) Limitations

- A CPE/CPI approach assumes that ***all alleles*** **are present** (i.e., cannot handle allele drop-out)
- Thus, statistical analysis of low-level DNA CANNOT be correctly performed with a CPE/CPI approach because some alleles may be missing
- Charles Brenner in his AAFS 2011 talk addressed this issue
- Research is on-going to develop allele drop-out models and software to enable appropriate calculations

Notes from Charles Brenner's AAFS 2011 talk

The Mythical "Exclusion" Method for Analyzing DNA Mixtures – Does it Make Any Sense at All?

1. The claim that it requires **no assumption about number of contributors** is mostly wrong.
2. The supposed **ease of understanding** by judge or jury is really an illusion.
3. **Ease of use** is claimed to be an advantage particularly for complicated mixture profiles, those with many peaks of varying heights. The truth is the exact opposite. **The exclusion method is completely invalid for complicated mixtures.**
4. The exclusion method is only **conservative** for guilty suspects.

Conclusion: "Certainly no one has laid out an explicit and rigorous chain of reasoning from first principles to support the exclusion method. It is at best guesswork."

Brenner, C.H. (2011). The mythical "exclusion" method for analyzing DNA mixtures – does it make any sense at all? *Proceedings of the American Academy of Forensic Sciences*, Feb 2011, Volume 17, p. 79

ISFG Recommendations on Mixture Interpretation

http://www.isfg.org/Publication;Gill2006

1. The likelihood ratio (LR) is the preferred statistical method for mixtures over RMNE
2. Scientists should be trained in and use LRs
3. Methods to calculate LRs of mixtures are cited
4. Follow Clayton et al. (1998) guidelines when deducing component genotypes
5. Prosecution determines H_p and defense determines H_d and multiple propositions may be evaluated
6. When minor alleles are the same size as stutters of major alleles, then they are indistinguishable
7. Allele dropout to explain evidence can only be used with low signal data
8. No statistical interpretation should be performed on alleles below threshold
9. Stochastic effects limit usefulness of heterozygote balance and mixture proportion estimates with low level DNA

Gill *et al.* (2006) DNA Commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. *Forensic Sci. Int.* 160: 90-101

Clayton et al. (1998)
ISFG (2006) Rec. #4

Step #1

Identify the Presence of a Mixture

Step #2

Designate Allele Peaks

Step #3

Identify the Number of Potential Contributors

Step #4

Estimate the Relative Ratio of Contributors

Step #5

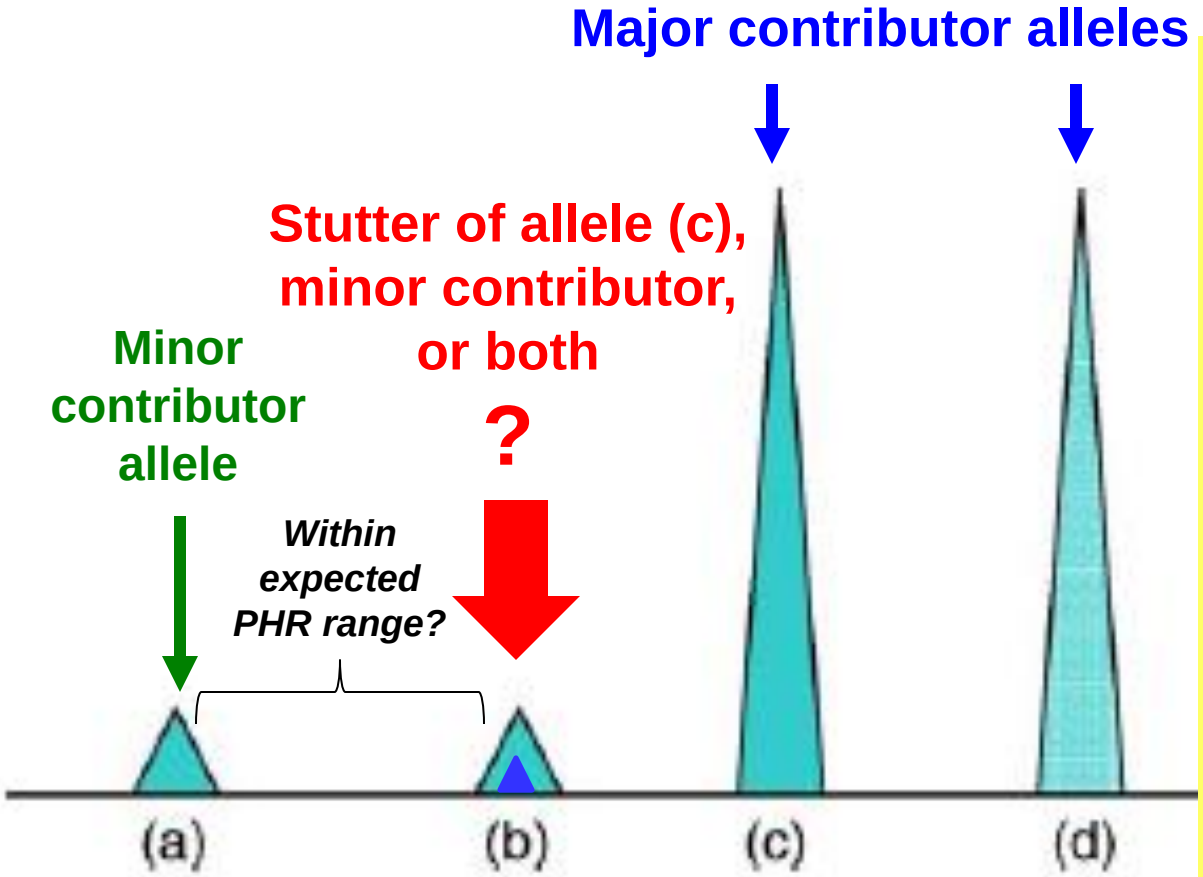
Consider All Possible Genotype Combinations

Step #6

Compare Reference Samples

ISFG Recommendation #6

Indistinguishable from Stutter



Possibilities for Minor

a,a

a,b

a,c

a,d

Probability of Inclusion =

$(f_a + f_b + f_c + f_d)^2$

If peak height of peak a is within established PHR of peak b peak height

Fig. 4. *c* and *d* are unambiguous alleles, *b* is a minor allele in a stutter position and *a* is an unambiguous minor allele.

Gill *et al.* (2006) DNA Commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. *Forensic Sci. Int.* 160: 90-101

Likelihood Ratio (LR)

- Provides ability to express and evaluate both the prosecution hypothesis, H_p (the suspect is the perpetrator) and the defense hypothesis, H_d (an unknown individual with a matching profile is the perpetrator)

$$LR = \frac{\Pr(E \mid H_p)}{\Pr(E \mid H_d)}$$

- **In the simplest case, the numerator, H_p, is 1** – since in theory the prosecution would only prosecute the suspect if they are 100% certain the suspect is the perpetrator

- The denominator, **H_d**, is typically the profile frequency in a particular population (based on individual allele frequencies and assuming unrelated individuals in Hardy-Weinberg equilibrium) – i.e., **the random match probability**

Take Home Messages

- Inclusionary statements (including “cannot exclude”) need statistical support to reflect the relevant weight-of-evidence
- Stochastic thresholds are necessary if using CPI statistics to help identify possible allele dropout
- CPI is only conservative for guilty suspects as this approach does a poor job of excluding the innocent
- Uncertainty exists in scientific measurements and increases with complex mixtures (low level DNA and/or >2 contributors)
- An increasing number of poor samples are being submitted to labs – labs may benefit from developing a complexity threshold

President John F. Kennedy

Yale University commencement address (June 11, 1962)

"For the greatest enemy of truth is very often not the lie – deliberate, contrived and dishonest – but the myth – persistent, persuasive, and unrealistic. Too often we hold fast to the clichés of our forebears. We subject all facts to a prefabricated set of interpretations. **We enjoy the comfort of opinion without the discomfort of thought**."

http://www.jfklibrary.org/Research/Ready-Reference/Kennedy-Library-Miscellaneous-Information/Yale-University-Commencement-Address.aspx