DNA Interpretation Workshop 1

Validation and Thresholds

Michael D. Coble, PhD
U.S. National Institute of Standards and Technology (NIST)

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

ISFG Pre-Conference Workshop
Melbourne, Australia
September 2-3, 2013

NIST and NIJ Disclaimer

Funding: Interagency Agreement between the National Institute of Justice and NIST Office of Law Enforcement Standards

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

DNA Mixture Training (2010-13)

- NIJ Forensic Science Training Development and Delivery Program
- NIJ Grant # 2008-DN-BX-K158, awarded to Biomedical Forensic Science Program at Boston University School of Medicine
- Focus on the ISHI (Promega) Meeting

DNA Mixture Training (2010-13)


Robin Cotton
Boston University


Catherine Grgicak
Boston University


John Butler
NIST

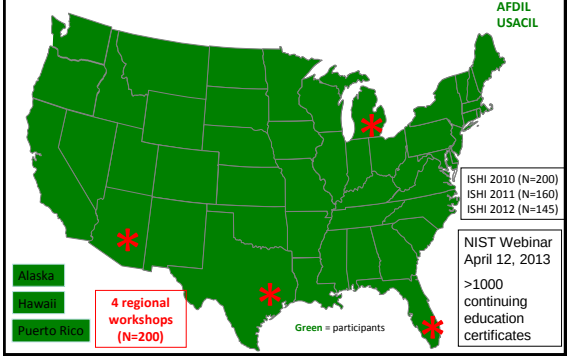

Mike Coble
NIST


Charlotte Word
Consultant

Mixture Workshop Attendees

50 states and 25 other countries

Federal Labs
FBI
ATF
AFDIL
USACIL



ISHI 2010 (N=200)
ISHI 2011 (N=160)
ISHI 2012 (N=145)

NIST Webinar
April 12, 2013

>1000 continuing education certificates

Changes in DNA Testing in Recent Years

- Case and Sample Acceptance Policies
  - Then: High profile cases, homicides, sexual assaults
    - Lots of DNA, single source, two-person mixtures
  - Now: Burglaries, Car jackings, Possession
    - Handled items with "touch" DNA, small amount of DNA (Low Template DNA), complex mixtures, clothing ("wearer" DNA)

Now accepting samples that would never have been accepted in the early STR testing days

Changes in DNA Testing in Recent Years (cont.)

- Increased Sensitivity of PCR test kits
  - Use of enhancement techniques
- Many more STR test kits available
- Options for types of tests
  - Autosomal STR
  - Y (male) STR
  - mini-STR (degraded DNA)
  - May use all 3 tests on a sample if sufficient DNA

Changes in DNA Testing in Recent Years

- Existing SOPs may not be adequate
  - Low Template (LT) DNA
  - Complex Mixtures
  - Relatives in mixtures
  - Enhancement techniques

Likely need to modify SOPs and do additional validation studies

- SWGDAM Interpretation Guidelines issued in 2010 (for single source and 2 person mixtures)
  - Need defined analytical and stochastic thresholds
  - Need interpretation methods that fit with available statistical methods

Validation Options

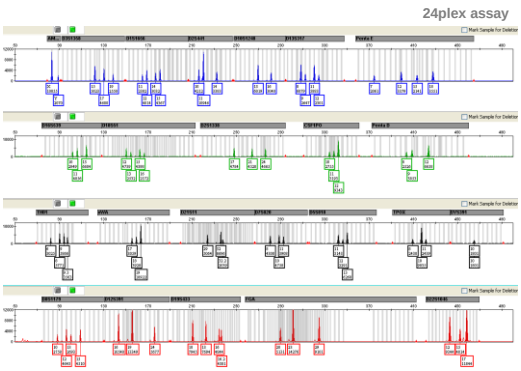
- New Extraction Kits and Columns
  - Manual
  - Automated
- Automated/Robotic instrumentation, software, documentation

Validation Options (cont.)

- New Quantification Kits
  - Human and Y
  - Human, Y and degradation
- New Amplification Kits
  - Higher sensitivity
    - Identifiler® Plus, NGM® SElect, PowerPlex® 16 HS
  - More loci
    - PowerPlex® Fusion (Promega)
    - GlobalFiler™ (Life Technologies)

More time needed for analysis, interpretation and technical review

DNA Mixture with **PowerPlex Fusion** (Promega)



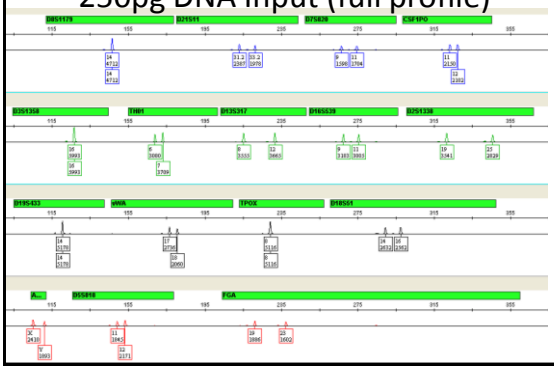
Validation Options (cont.)

- New Amplification Kits
  - Y STRs
    - Yfiler®, Yfiler® Plus
    - PowerPlex® Y23
    - Rapidly mutating Y loci?
  - MiniSTRs
    - MiniFiler™
  - Phenotypes
    - IrisPlex (hair and eye color)
  - In/Del? (DIPlex, Qiagen)
  - Rapid DNA?

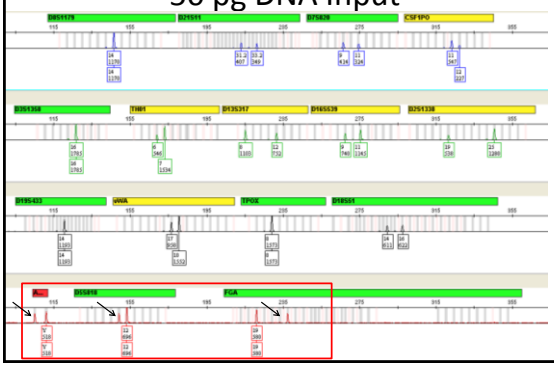
Validations Needed

- New Capillary Electrophoresis Genetic Analyzer
  - ABI 3500
  - Different data collection software
  - Optimal peak heights MUCH higher than with previous CEs (e.g., 6000-14,000)
  - Need to define analytical thresholds and stochastic thresholds
    - May be different for different colors

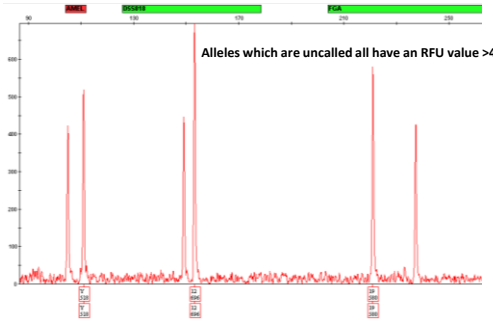
250pg DNA Input (full profile)



50 pg DNA Input



50 pg DNA Input
AT = 500 RFU



Validations Needed

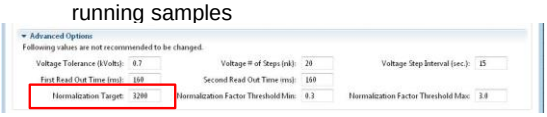
- New Capillary Electrophoresis Genetic Analyzer
  - ABI 3500
  - Different data collection software
  - Optimal peak heights MUCH higher than with previous CEs (e.g., 6000-14,000)
  - Need to define analytical thresholds and stochastic thresholds
    - May be different for different colors
  - Requires different GeneMapper ID-X software
  - Normalization?

Normalization of Data

- Recommended to compare signal between instruments
- Motivation mainly for large laboratories with many instruments
  - Correct for signal variation between instruments
- Can be used with a single instrument
  - Correct for signal variation between single and multiple injections

Normalization Definitions

- Normalization Target (NT)
  - Requires the use of LIZ 600 v2.0 size standard
  - Average peak heights of 11 peaks within LIZ 600 v2.0 selected for peak height consistency across lots
  - Applied within data collection software prior to running samples

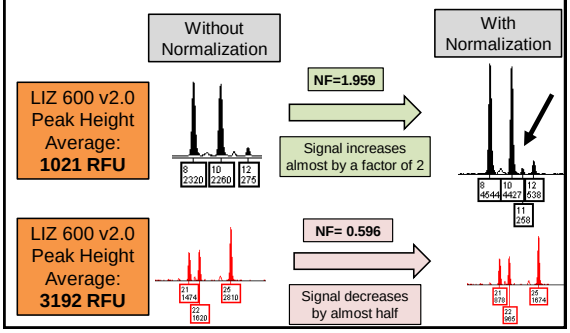


Normalization Definitions

- Normalization Factor (NF)
  - Adjustment needed for individual samples to reach the Normalization Target value
  - Full signal adjustment (baseline, peaks, artifacts, etc)
    - Either **increase** or **decrease** signal

Normalization Example

Theoretical Normalization Target: 2000 RFU



Validations – More Data Needed

- Sensitivity Studies
  - Better understanding of Low Template (LT) DNA and Stochastic Effects
    - Single dilution series NOT adequate
  - Aid in establishing one or more analytical thresholds and stochastic thresholds
    - Low amount of DNA vs. high amounts of DNA
- Mixture Studies
  - Complex mixtures, if accepting and interpreting samples with >2 contributors

Validations – More Data Needed

- Enhancement Techniques for LT DNA
  - Decreased amplification volume
  - Increased amplification cycles
  - Increased injection time or voltage
  - Increased product in sample prep for CE
  - Post-amplification clean-up
- Must do validation studies for ALL conditions with all kits

Validations Options

- Casework vs. Databasing
  - Direct amplification kits (no extraction or quantification)
  - Small amounts of DNA vs. higher amounts
  - Mixtures vs. single source
- Interpretation for database entry vs. case work interpretation
  - How different are they?

Validations need to include:

Evaluation of all aspects of testing procedures

1) Technology performance (kits, instruments)
2) Assessment of data with known contributor(s)
   - ➢ Limitations of each aspect of the test system
3) Development of SOPs that reflect validation done, ***including interpretation guidelines***

Testing of samples from known individuals that reflect casework acceptance policies

1) Low Template DNA
2) Complex Mixtures

New Validation Studies

- Technical leader will need lots of ***help and time*** to conduct and evaluate appropriate studies
- Multiple samples will need to be tested
- May need additional training or assistance to evaluate data (statistics)
- Interpretation SOPs will be much longer and more complicated and detailed

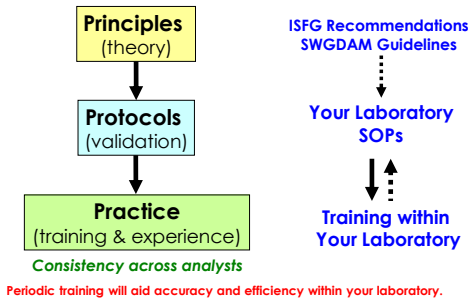
Considerations

- Review samples received and test results
  - – Successes vs. inconclusives
- Review case acceptance policies
  - – Limit sample number
  - – Limit samples with low likelihood of results
- What tests are really needed?
  - – What does your lab need to validate vs. outsource? (e.g., Y STRs, MiniFiler)

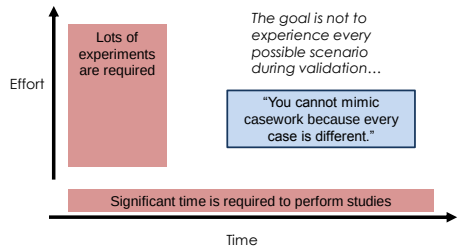
Considerations

- Implement a plan for evaluation of reported cases when interpretation SOPs change
  - – Minor or significant change in SOP leading to minor or significant change in interpretation?
  - – Change in conclusions (e.g., inclusion to inconclusive or exclusion – most likely)
  - – Possible options:
    - Sampling of 10-20% of cases → form plan
    - Re-review when discovery requested and/or when requested to testify
    - When additional testing being done in a case

Elements of DNA Mixture Interpretation



Common Perceptions of Validation



Validation Studies

- Information from validation studies should be used to set laboratory-specific
  - Minimum Peak Heights (detection thresholds)
  - Stutter %
  - Heterozygote balance (Peak Height Ratios)
  - Relative balance across loci
- These values are all dependent on amount of input DNA
  - If low-level DNA is amplified, stutter % may be higher and peak height ratios may be lower

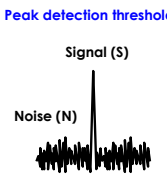
Setting Thresholds

- **Analytical (detection) threshold** — what is a peak?
  - **Dependent on *instrument sensitivity***
  - **~50 RFU**
  - **Impacted by instrument baseline noise**
- **Stochastic (drop-out) threshold** — what is reliable PCR data?
  - **Dependent on *biological sensitivity***
  - **~150-200 RFU**
  - **Impacted by assay and injection parameters**

Validation studies should be performed in each laboratory

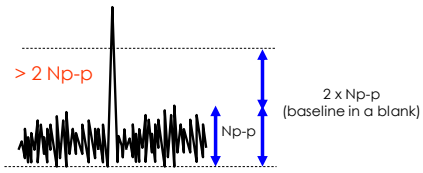
Analytical Threshold

- **The laboratory should establish an analytical threshold based on signal-to-noise analyses of internally derived empirical data.**

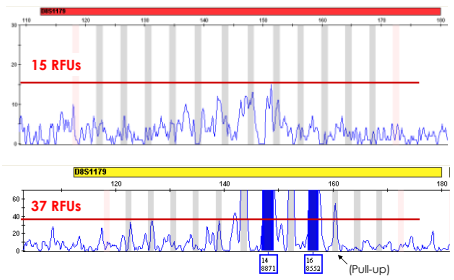


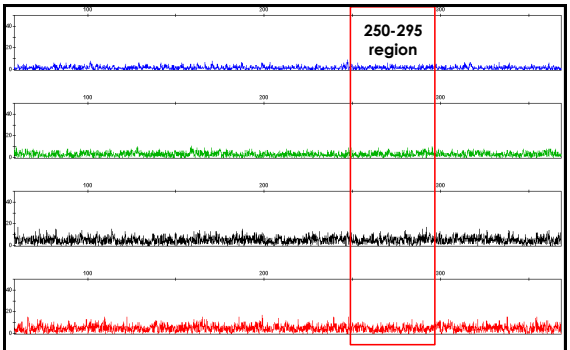
Analytical Threshold

- **As an example, an analytical threshold may be based on two times the intensity difference between the highest peak and lowest trough within the instrumental noise data. *Other scientific methods may be used.***



Sample Source – Negatives? Positives?





Analytical Thresholds can be determined for each dye channel

How to set an analytical threshold (AT)? Some Examples…

SWGDAM: Two times the intensity difference between the highest peak and lowest trough (as an example).

Three times the highest peak.

Gilder et al. (2007): Determined LOD by examining Pos, Neg, RB from 150 cases.

$$LOD = \mu_b + 3\sigma_b$$

J Forensic Sci, January 2007, Vol. 52, No. 1
doi:10.1111/j.1556-4029.2006.00318.x
Available online at: www.blackwell-synergy.com

TECHNICAL NOTE

Jason R. Gilder,[1] M.S.; Travis E. Doom,[2] Ph.D.; Keith Inman,[3] M. Crim.; and Dan E. Krane,[4] Ph.D.

Run-Specific Limits of Detection and Quantitation for STR-based DNA Testing

Gilder et al. (2007)

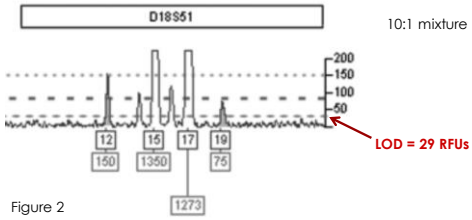


Figure 2

Gilder et al. (2007)

TABLE 1—*Maximum, minimum, and average baseline levels observed in the set of reagent blanks, negative controls, and positive controls (determined from controls in 50 different runs).*

	μ_b	σ_b	$\mu_b+3\sigma_b$	$\mu_b+10\sigma_b$
Positive Control				
Maximum	6.7	6.9	27.4	75.7
Average	5.0	3.7	16.1	42.0
Minimum	3.7	2.4	10.9	27.7
Negative Control				
Maximum	13.4	13.2	53.0	145.4
Average	5.4	3.9	17.1	44.4
Minimum	4.0	2.6	11.8	30.0
Reagent Blank				
Maximum	6.5	11.0	39.5	116.5
Average	5.3	4.0	17.3	45.3
Minimum	4.0	2.6	11.8	30.0
All three controls averaged				
Maximum	7.1	7.3	29.0	80.1
Average	5.2	3.9	16.9	44.2
Minimum	3.9	2.5	11.4	28.9

All values are in RFUs.

How to set an analytical threshold (AT)? Some Examples…

SWGDAM: Two times the intensity difference between the highest peak and lowest trough (as an example).

Three times the highest peak.

Gilder et al. (2007): Determined LOD by examining Pos, Neg, RB from 150 cases.

Catherine Grgicak (Boston U.) presentation at the 2010 ISHI (Promega) mixture workshop. (http://www.cstl.nist.gov/biotech/strbase/mixture.htm)

Multiple methods for determining AT

- **Method 1.**
  - **Kaiser (IUPAC 1976)**
    - **Winefordner 1983 and Krane 2007**
- **Method 2.**
  - **Currie (IUPAC 1995)**
    - **Winefordner 1983**
- **Method 3.**
  - **Example in SWGDAM Guidelines**
- **Method 4.**
  - **Miller & Miller. *Statistics for Analytical Chemistry (Ellis Horwood & Prentice Hall)***
    - **IUPAC 1997 ElectroAnalytical Committee**
- **Method 5.**
  - **1997 IUPAC ElectroAnalytical Committee Recommendations**

Negative Controls (at least 20)

DNA Dilution Series

Courtesy of Catherine Grgicak

Multiple methods for determining AT

$$AT_{M1} = \bar{Y}_{bl} + ks_{bl}$$

$$AT_{M2} = \bar{Y}_{bl} + t_{1-\alpha,v}\frac{s_{bl}}{\sqrt{n}}$$

$$AT_{M3} = 2(Y_{\max} - Y_{\min})$$

Negative Controls (at least 20)

$$AT_{M4} = b + 3S_y$$

$$AT_{M5} = b + t_{n-1,\alpha}S_y$$

(http://www.cstl.nist.gov/biotech/strbase/mixture.htm)

Courtesy of Catherine Grgicak

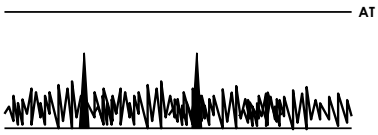
Multiple methods for determining AT

Method	Origin	Analytical Threshold for green 5s injection example
1	Negatives	7
2	Negatives	4
3	Negatives	20
4	DNA Series	31
5	DNA Series	39

Courtesy of Catherine Grgicak

What about peaks below the AT?

- The Analytical Threshold is the "floor" of the EPG. Peaks below the AT by definition should not be trusted!



Stochastic Thresholds
Some thoughts…

Conference Held in Rome in April 2012

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."

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

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

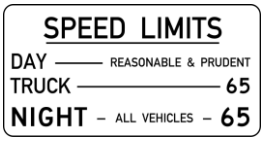
Bruce Budowle

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

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

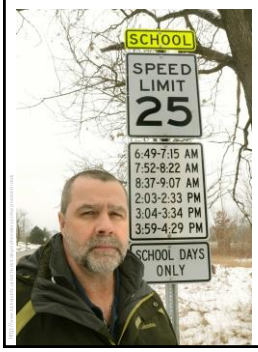
Do you leave thresholds and protocols up to "analysts' discretion"?



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

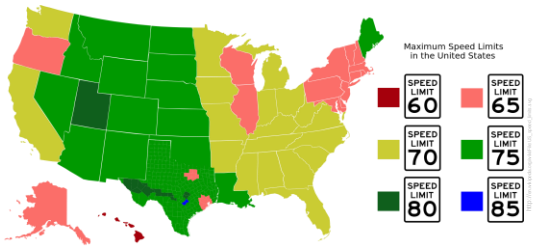
A Potential Outcome!

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…

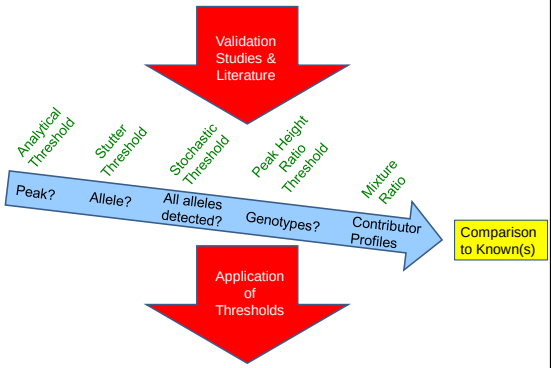


http://en.wikipedia.org/wiki/Speed_limits_in_the_United_States

Threshold Decisions

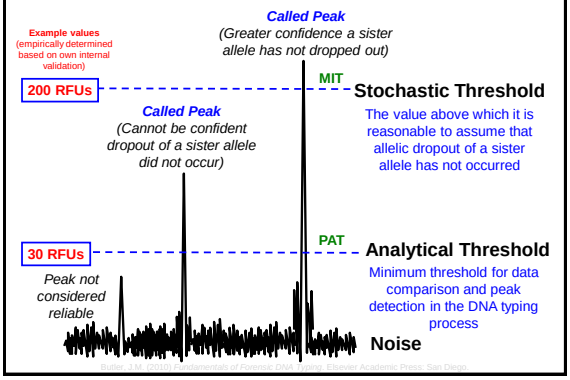
Thresholds to Determine	Decisions to Make (lab & kit specific)	Useful Validation Data
Analytical = ____ RFU	Single overall value or color specific	Noise levels in negative controls or non-peak areas of positive controls
Stochastic = ____ RFU	Minimum peak height RFU value or alternative criteria such 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



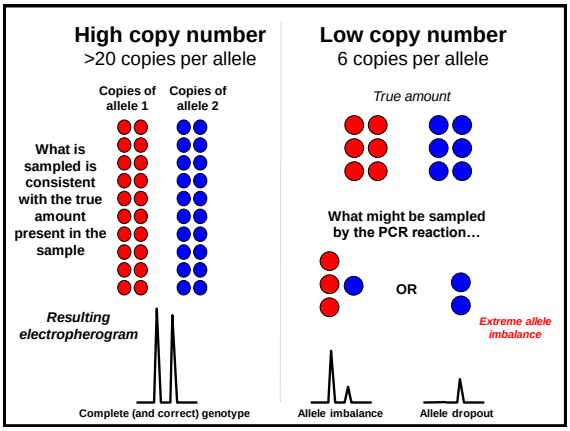
Approaches to Setting a Stochastic Threshold

Overview of Two Thresholds



General Definition of Stochastic

- Stochastic is synonymous with "random." The word is of Greek origin and means "pertaining to chance". … Stochastic is often used as counterpart of the word "deterministic," which means that random phenomena are not involved. Therefore, stochastic models are based on random trials, while deterministic models always produce the same output for a given starting condition.
- http://mathworld.wolfram.com/Stochastic.html

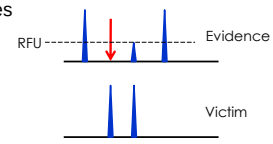


How can we characterize variation?

- Look at total amount of variation at end of process
  - Follow the positive control over time
- Experimentally break process into components and characterize using appropriate statistics
  - e.g., separate amplification variation from injection variation
- Analyze existing or new validation data, training sample data, SRM data, kit QC data
- Use casework data
  - e.g., variation between knowns (victim's DNA profile within an intimate sample) and matching single-source evidence profiles

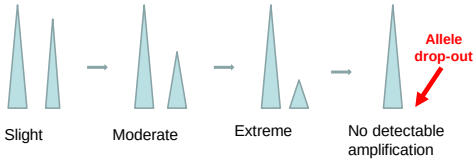
Using "Real World" Data (Courtesy of Dr. Robin Cotton)

- Examine sexual assault casework data from known heterozygous loci using:
  - two person mixtures
  - one component is consistent with a known victim
  - loci with 4 alleles



Problem with Stochastic Effects

- **Allele drop-out** is an extension of the amplification disparity that is observed when heterozygous peaks heights are unequal
  - Occurs in single-source samples and mixtures
  - Analyst is unable to distinguish complete allele drop-out in a true heterozygote from a homozygous state



What is Allele Drop Out?

- Scientifically
  - **Failure to detect** an allele within a sample or failure to amplify an allele during PCR. *From SWGDAM Guidelines, 2010*
  - Note that: Failure to detect ≠ failure to amplify
- Operationally
  - Setting a threshold(s) or creating a process, based on validation data and information in the literature, which allows assessment of the likelihood of drop-out of an allele or a locus.

Stochastic Effects and Stochastic Threshold

SWGDAM 2010 Interpretation Guidelines glossary:

- **Stochastic effects**: the observation of intra-locus peak imbalance and/or allele drop-out resulting from random, disproportionate amplification of alleles in low-quantity template samples
- **Stochastic threshold**: the peak height value above which it is reasonable to assume that, at a given locus, allelic dropout of a sister allele has not occurred

http://www.fbi.gov/about-us/lab/codis/swgdam-interpretation-guidelines

Important Principle: With many casework sample, we cannot avoid stochastic effects and allele or locus drop-out.

Why ?

We do not know the number of contributors to a sample or the true contributor ratio in a mixture!

Sample Mixture Ratio Impacts Amount of DNA Available for PCR Amplification

Assume sample is a 3:1 mixture of two sources:

Amount of DNA	~ # of cells from major component	~ # of cells from minor component
1 ng	107	36
0.5 ng	53	18
0.25 ng	27	9
0.125 ng	12	4
0.063 ng	7	2

Stochastic effects expected with PCR amplification from <20 cells

Setting Stochastic Thresholds (some examples)

Setting Stochastic Thresholds (NIST)

- Multiple samples, replicates, and concentrations are ideal to get a feel for how the system is working
  - We used 3 fully heterozygous samples with 10 replicates at 2 ng, 1 ng, 800 pg, 500 pg, 400 pg, 300 pg, 200 pg, 100 pg, 30 pg, & 10 pg

Sample Selection

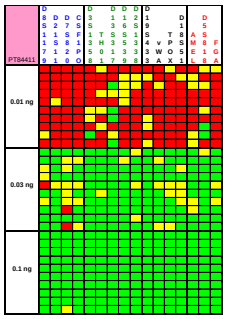
Description	CSF1PO	D3S1358	D5S818	D7S820	D8S1179	D13S317	D16S539	D18S51	D21S11	FGA	THO1	TPOX	vWA	Penta D	Penta E	D2	D19
Genomic 8	10, 12	15, 18	12, 13	9, 10	12,14	9, 13	9, 11	15,18	30,31	24, 28	7, 8	8,12	15,17	8, 9	5,10	22,22	12.2,15
9947A	10, 12	14, 15	11, 11	10, 11	13,13	11, 11	11, 12	15,19	30,30	23, 24	8, 9.3	8,8	17,18	12,12	12,13	19,23	14,15

9947A – 5/13 loci are homozygous

Setting Stochastic Thresholds

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

Setting Stochastic Thresholds



Identifiler, 28 cycles

3130*xl*, 10 sec @ 3kV inj

Both alleles are present

One allele has dropped out

Locus drop out

Slide courtesy of Becky Hill (NIST)

Setting Stochastic Thresholds

Identifiler, 28 cycles

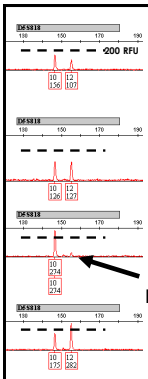
3130*xl*, 10 sec @ 3kV inj

0.03 ng									57						274		
			114	119				242			142						
	149		122										185				
	152																
		92	52	76					166		76	75	88				95
			89				140					73	133				
	61		71	51									84	91		74	
														82			
									156								
				51							95	114					

Highest peak height of "false homozygote" = 274 RFU

Allelic drop-out is prevalent at 30 – 50 pg DNA

Slide courtesy of Becky Hill (NIST)



Setting Stochastic Thresholds

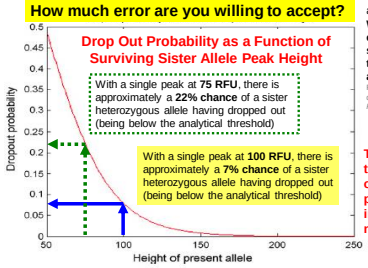
- Stochastic threshold – point at which data is considered reliable
- "Level of risk": the higher you go, the less risk you have of calling a false homozygote - but you start to lose more data for statistics

*False homozygote if the stochastic threshold is above set at 200 RFU.

Slide courtesy of Becky Hill (NIST)

Setting a Stochastic Threshold is Essentially Establishing a Risk Assessment

How much error are you willing to accept?



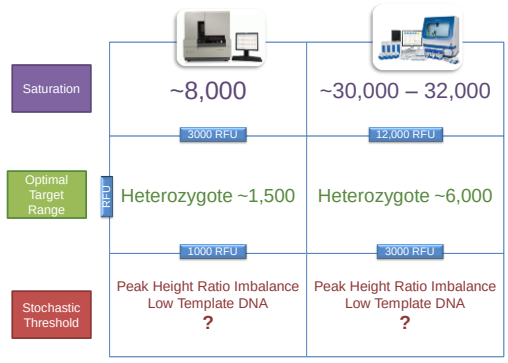
"Currently, most laboratories use an arbitrary stochastic threshold. **When a protocol is changed, especially if it is made more sensitive to low-level DNA, then the stochastic threshold must also change.**"
Puch-Solis R, et al. (2011). Practical determination of the low template DNA threshold. *Forensic 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.

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

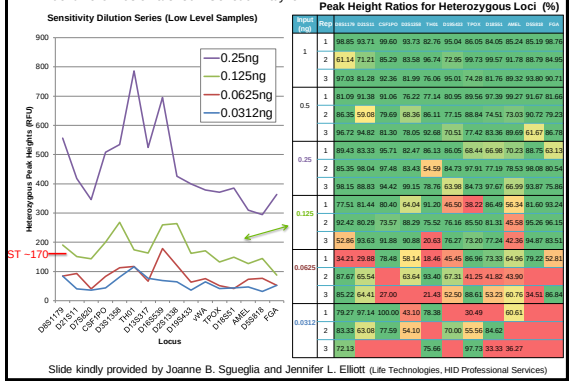
Dynamic Range of 3130*xl* vs. 3500 Genetic Analyzer



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

Stochastic Threshold Considerations

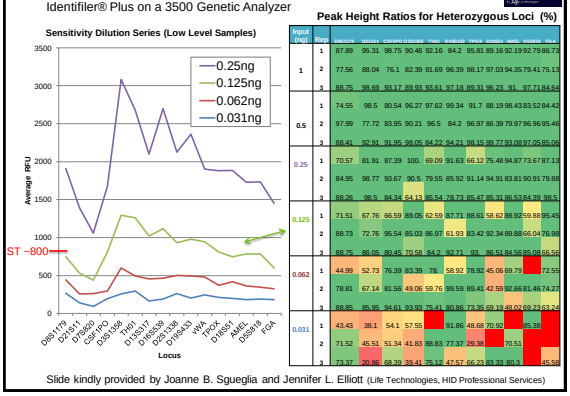
Identifiler® Plus on a 3130*xl* Genetic Analyzer



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

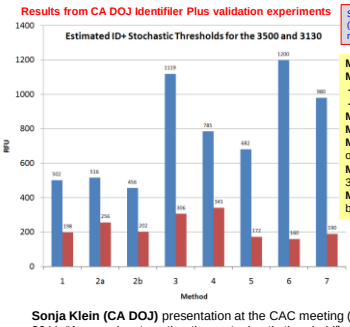


Slide kindly provided by Joanne B. Sgueglia and Jennifer L. Elliott (Life Technologies, HID Professional Services)

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"

Forensic Science International: Genetics 4 (2010) 111–114

Contents lists available at ScienceDirect

Forensic Science International: Genetics

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

Examination of the variability in mixed DNA profile parameters for the Identifiler™ multiplex

Jo-Anne Bright, Jnana Turkington, John Buckleton *

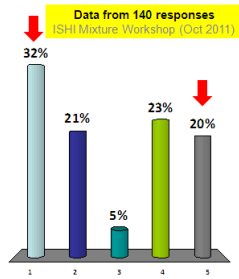
ESR, 120 Mt Albert Road, PB 92021, Auckland, New Zealand

"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."

2011 Response from ISHI Workshop

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

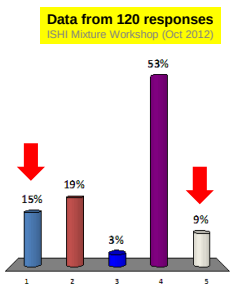
1. Same value as our analytical threshold (**we don't use a ST**)
2. About twice as high as our AT (e.g., AT = 50 and ST = 100 RFU)
3. Less than twice as high as our AT
4. Greater than twice as high as our AT
5. I don't know!



2012 Response from ISHI Workshop

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

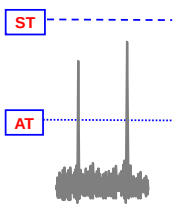
1. Same value as our analytical threshold (**we don't use a ST**)
2. About twice as high as our AT (e.g., AT = 50 and ST = 100 RFU)
3. Less than twice as high as our AT
4. Greater than twice as high as our AT
5. I don't know!



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

Stutter Thresholds

Review of the Literature

Study	Kit	Measured	TH01	vWA	D18S51
Greenspoon *et al.* (2004)	PP16 BIO	mean + 3SD	5	14	13
Krenke *et al.* (2002)	PP16	mean + 1SD	3	10	9
Moretti *et al.* (2001)	Pro+/CoFiler	mean + 3SD	15.9	11.7	13.9
Mulero *et al.* (2008)	MiniFiler	max %	-	-	**17.3**
Hill et al. (2010)	PP ESX	mean + 3SD	4.2	14.6	14.6
User Manual	Identifiler	max%	5.1	12.6	17
User Manual	IDfiler Direct	mean + 3SD	4.7	11.9	12.8
User Manual	IDfiler Plus	mean + 3SD	4	12.4	13.6

Many labs just use a flat 15%

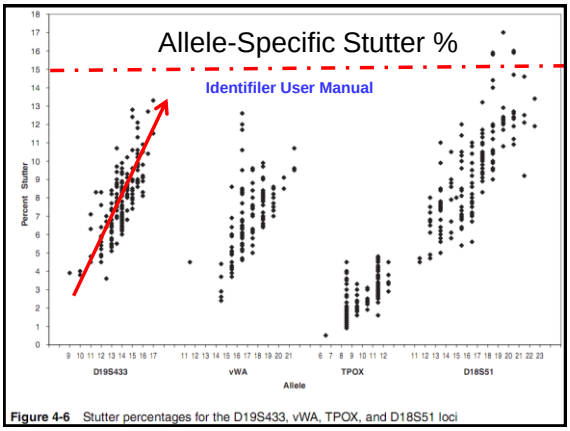
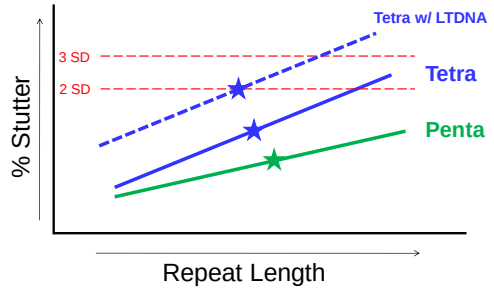


Figure 4-6 Stutter percentages for the D19S433, vWA, TPOX, and D18S51 loci

Developing Stutter Filter Values

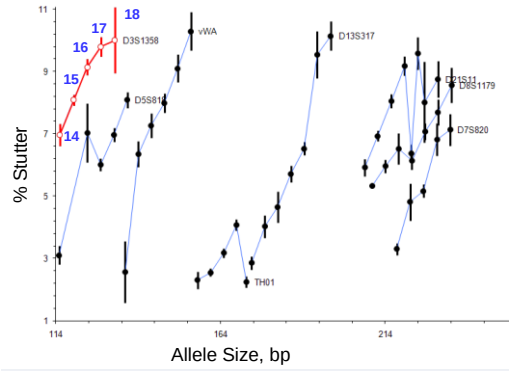
- Samples – Ideally at least 5 observations of each stutter product per locus from relevant populations (e.g. longer repeats in FGA alleles are observed mostly among African Americans).
- Use typical DNA input quantities (0.5 – 2.0ng), but may want to assess stutter at lower levels (e.g. <150pg). Excessive DNA (5-10ng) can skew your average percentages.

Stutter Trends

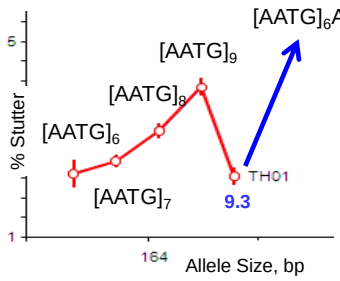


D3S1358 – TCTA[TCTG]$_N$[TCTA]$_N$

			Stutter		
Locus	Allele	Size	#	Median	MADe
D3S1358	14	115.2	26	7.0	0.9
	15	119.4	66	8.1	0.7
	16	123.5	47	9.1	0.9
	17	127.7	47	9.8	1.1
	18	131.9	41	10.0	3.4
		Avg	227	8.8	1.7
		SD		1.3	



TH01 - $[AATG]_N$



"LUS"
Longest Uninterrupted Stretch

A good predictor for stutter ratio

Brookes C, Bright J-A, Harbison S, Buckleton J. Characterising stutter in forensic STR multiplexes. Forensic Science International: Genetics. 2012;6(1):58-63.

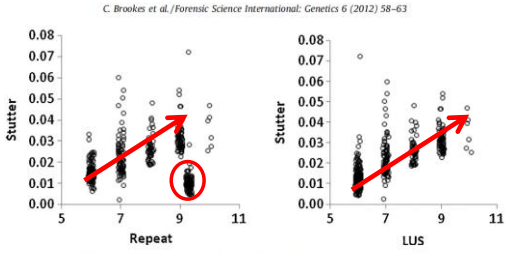


Fig. 7. A comparison of stutter ratio vs. allele designation and LUS for the locus TH01.

Interpretation of Potential Stutter Peaks in a Mixed Sample

- For mixtures in which minor contributors are determined to be present, a peak in stutter position (generally n-4) may be determined to be 1) a stutter peak, 2) an allelic peak, or 3) indistinguishable as being either an allelic or stutter peak.

ISFG Recommendation #6 Example

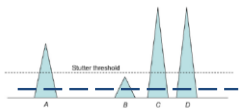


Fig. 2. A two person mixture with major peaks *C, D* and minor peaks *A*. There is an additional peak present in a stutter position (*B*).

Likely a AA
(homozygote)

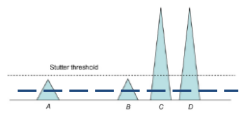


Fig. 3. A two person mixture with major peaks *C, D* and minor peaks *A, B*, where *B* is in a stutter position.

Possibly AB
(heterozygote)

Could also be AC, AD, AA, or A,? (dropout)

Stutter effects

- In case of doubt a suspicious peak ***in the position of a stutter band*** has to be considered as a true allele and part of the DNA profile, and should be included into the biostatistical interpretation.

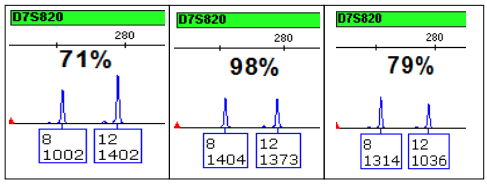
Slide from Peter Schneider
(presented at EDNAP meeting in Krakow in April 2007)

Summary

- Stutter can vary across profiles, loci, or alleles.
- Stutter becomes especially problematic for mixtures when samples are at low [DNA] levels.
- Labs should decide when is it appropriate to turn off stutter filters, especially when the minor component alleles are nearly the same height as stutter peaks.

Heterozygote Balance (Peak Height Thresholds)

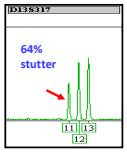
Variation Among Replicates



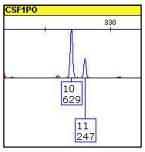
Peak heights will vary from sample-to-sample, even for the same DNA sample amplified in parallel

Causes of Peak Height Imbalance
Single Source Samples

- LT DNA and stochastic effects
  - Elevated Stutter – artifact, not true allele
  - Unequal sampling of true alleles – the two alleles are not sampled and amplified equally



12,13



0.25 ng

How to calculate Peak Height Ratios?

From Validation Studies

- Sensitivity Series at different amounts of DNA
- Non-probative single-source samples with good quality profiles amplified with different amounts of DNA (or at least with different peak height ranges)
- Perform for each kit validated as PHRs may vary for the same locus amplified with different kits

Courtesy of Charlotte Word
(http://www.cstl.nist.gov/biotech/strbase/mixture.htm)

How to calculate Peak Height Ratios?

From Casework and Training samples

- Known standards and single-source samples with good quality profiles amplified with different amounts of DNA (or at least with different peak height ranges)
- Database samples (as long as same procedures being used for casework)

Courtesy of Charlotte Word
(http://www.cstl.nist.gov/biotech/strbase/mixture.htm)

How to calculate Peak Height Ratios?

- Use a sufficient number and variety of samples to get representative data from each locus, especially for loci with a wide range of alleles and HMW markers (e.g., FGA, D18).

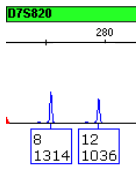
Courtesy of Charlotte Word
(http://www.cstl.nist.gov/biotech/strbase/mixture.htm)

Two Approaches to Determine Hb

$$h = \frac{\phi_{smaller}}{\phi_{larger}}$$

ϕ = peak height

(most common)



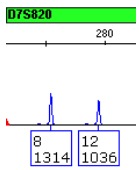
$$\frac{1036}{1314} = 79\%$$

Kelly H, Bright J-A, Curran J, Buckleton J. Modelling heterozygote balance in forensic DNA profiles. Forensic Science International: Genetics. 2012; 6: 729-734.

Two Approaches to Determine Hb

$$h = \frac{\phi_{HMW}}{\phi_{LMW}}$$

HMW = High MW
LMW = Low MW



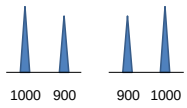
$$\frac{1314}{1036} = 1.268$$

Kelly H, Bright J-A, Curran J, Buckleton J. Modelling heterozygote balance in forensic DNA profiles. Forensic Science International: Genetics. 2012; 6: 729-734.

Advantages of the HMW/LMW method

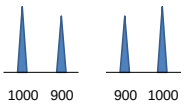
- Preserves information

$h = \frac{\phi_{smaller}}{\phi_{larger}}$



= 0.90 = 0.90

$h = \frac{\phi_{HMW}}{\phi_{LMW}}$



= 0.90 = 1.11

Provides "positional" information

Hb Data

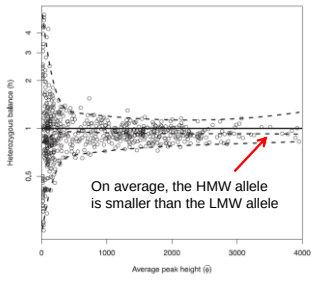


Fig. 1. Heterozygous balance versus average peak height.

Kelly H, Bright J-A, Curran J, Buckleton J. Modelling heterozygote balance in forensic DNA profiles. Forensic Science International: Genetics. 2012; 6: 729-734.

Validation Studies - Revisited

- Some have critiqued that validation studies based on single-source pristine DNA samples are being used to develop SOPs for casework mixtures.
- This issue was addressed by Bright *et al.* (2012).

Forensic Science International: Genetics 6 (2012) 180–184

A comparison of stochastic variation in mixed and unmixed casework and synthetic samples

Jo-Anne Bright [a,*], Kurt McManus [a], SallyAnn Harbison [a], Peter Gill [b,c], John Buckleton [a]

[a] ESR, Private Bag 92021, Auckland, New Zealand
[b] Institute of Forensic Medicine, Oslo University, Norway
[c] Centre for Forensic Science, University of Strathclyde, Glasgow, UK

Focus on Hb and mixture ratio

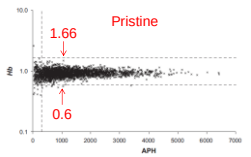


Fig. 1. Heterozygote balance versus APH data from single source pristine ($N = 2889$). The horizontal dotted lines are at Hb = 0.6 and 1.66 and the vertical dotted line is at APH = 300 RFU.

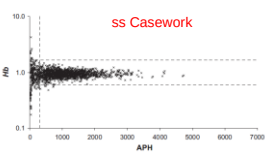


Fig. 2. Heterozygote balance versus APH data from single source casework ($N = 1810$).

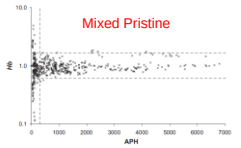


Fig. 3. Heterozygote balance versus APH data from mixed source pristine DNA ($N = 559$).

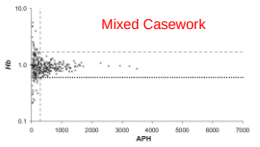


Fig. 4. Heterozygote balance versus APH data from mixed source casework DNA ($N = 299$).

Summary

- Validation studies are necessary to establish thresholds for mixture interpretation.
- In addition to testing only single source samples, mixtures should also be a part of the validation study.
- Bright et al. (2012) did not observe a difference between the use of pristine and casework samples for Hb and mixture ratio parameters.

Acknowledgements

- Becky Hill, Erica Butts, Peter Vallone, Dave Duewer and John Butler (NIST)
- Catherine Grgicak and Robin Cotton (Boston U.)
- Charlotte Word (Charlotte Word Consulting)
- Joanne Sgueglia and Jennifer Elliott (Life Tech)