Validation of Forensic DNA Technologies:

Impacts of Thresholds on the Interpretation of Low-Template Mixtures

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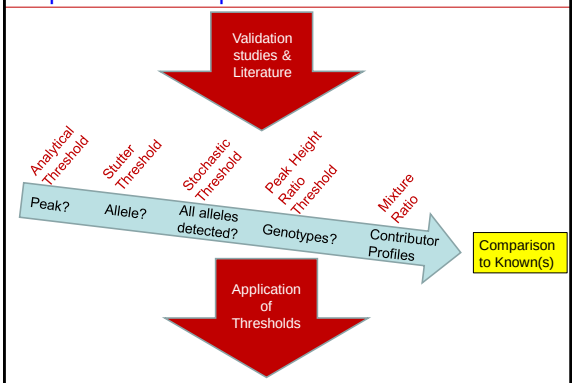
August 6, 2014
Boston, MA

Steps in DNA Interpretation

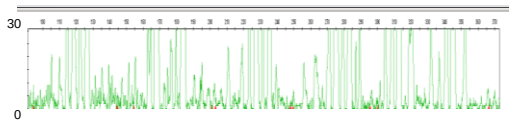


Principles Behind Thresholds

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 noise
Limit of Linearity (e.g. 5000 RFU)	Above this value, the CCD 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 is single-source samples are assumed 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 Threshold (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

Type I Error – False labeling of noise

Analytical Threshold: Peak or Noise?

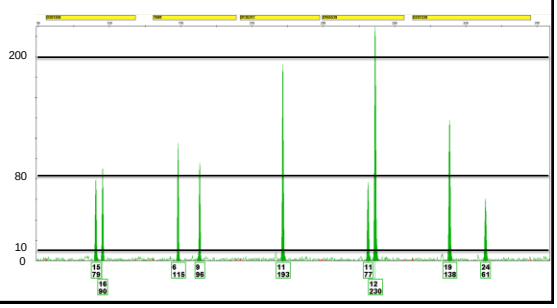


Analytical Thresholds are ***applied*** to ensure peaks are originating from sources other than noise,

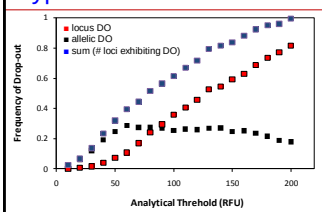
they are ***determined*** via close examination of noise during (and possibly after) validation

Type II Error – False non-labeling of alleles (Drop-out)

Single source 0.125ng, 1ul 3130 prep volume



Type II Error – False non-labeling of alleles



$$freqDO(locus) = \frac{\#hetloci(2alleleDO)}{total\#hetloci}$$

$$freqDO(allele) = \frac{\#hetloci(1alleleDO)}{total\#hetloci}$$

-As AT increases, locus DO increases, while allele DO stabilizes after 50 RFU then starts to decrease after AT of ~150 RFU.

-Although a higher AT (i.e. >150 RFU) begins to decrease the number of loci where allele DO occurs (less stochastic variation),

-Locus DO increases, resulting in an overall increase in DO with AT for Low-template samples

What is our goal of validating an AT?

Analytical Thresholds are ***applied*** to
1) ensure peaks are originating from sources other than noise and,
2) minimize unnecessary allele/locus drop-out

(Stochastic thresholds, PHR thresholds and Pr(D)'s can handle the rest)!

They are ***determined*** via close examination of noise during (and possibly after) validation

Analytical Threshold: How is it determined?

Use data from negatives (i.e. samples with no DNA)

- Method 1.
  - Kaiser (IUPAC 1976)
    - Long & Winefordner 1983 and Krane 2007
- Method 2.
  - Currie (IUPAC 1995)
    - Long & Winefordner 1983
- Method 3.
  - Example in SWGDAM Guidelines
- Method 4.
  - Percentile Rank

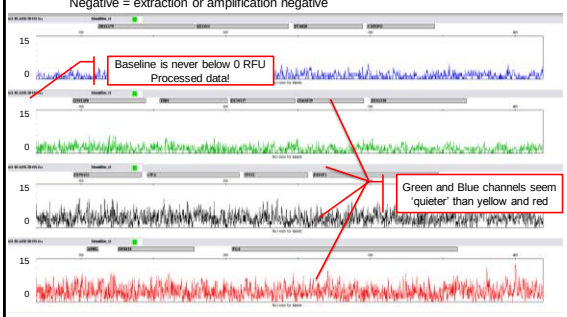
Use data from DNA dilution series

- Method 5.
  - Miller & Miller. *Statistics for Analytical Chemistry (Ellis Horwood & Prentice Hall)*
    - IUPAC 1997 ElectroAnalytical Committee
- Method 6.
  - 1997 IUPAC ElectroAnalytical Committee Recommendations
- Method 7.
  - Performance Measurements (i.e., ROC Analysis)
  - 2012 Rakay et. al. FSI: Genetics

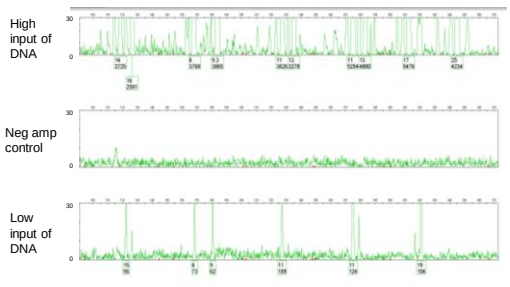
Analytical Threshold: Color Dependent?

-Negative sample run with an internal size standard (not shown) using manufacturer's recommended protocol

Negative = extraction or amplification negative

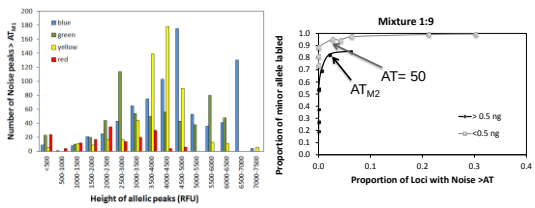


Analytical Threshold: Template Dependent?



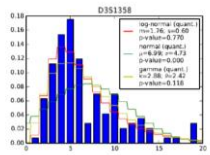
Template Dependence

- This is not instrument baseline/noise
- Single source DNA data amplified from 0.0625 – 2 ng
  - Differentiated 'noise' from artifact
    - -A, pull-up, stutter (+ or -), spikes, dye artifacts
- Plotted RFU of the known/expected peak versus the highest 'noise' peak
- High noise with >0.5 ng of DNA, higher AT needed for higher-template samples



Noise Distribution

- Is the noise normally distributed?
  - Performed Kolmogorov-Smirnov and Chi-squared tests on ID & IDPlus noise obtained after GeneMapper analysis at various targets
  - In most instances, it is best described as log-normal



Summary of Results

Method	Validation Sample Type	Analytical Threshold for Green 5s injection example	Assumptions
1	Negatives	7	Noise is Gaussian
2	Negatives	4	Noise is Gaussian
3	Negatives	18	Noise is Gaussian
4	Negatives	6	None
5	DNA Series	31	Signal is linear wrt template and Gaussian
6	DNA Series	39	Signal is linear wrt template and Gaussian
7	DNA Series (Low-Template)	13	None
7	DNA Series (All-Templates)	50	

Analytical Threshold: Conclusions

Baseline Noise is;
- -Color dependent (low-templates)
- -Target dependent (high-templates)
- -Described by a log-normal

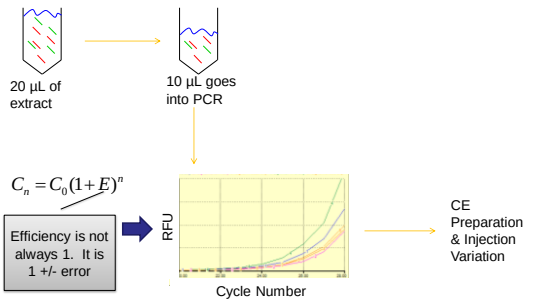
To determine an AT;
- -Amplify numerous samples at all targets typically seen in casework (do not only use negatives or blanks)
- -Analyze at 1 RFU and determine the AT
- -Method 7 has become the method of choice for us
  - -No distribution assumption
  - -Directly measures performance of different ATs and gives the proportion of false positives AND negatives

What is the purpose of an ST?

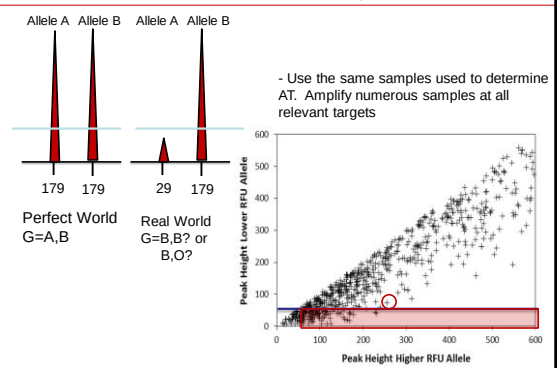
Stochastic Thresholds are ***applied*** to
1) help us determine whether we can infer a homozygous genotype when we observe 1 peak
2) minimize unnecessary false heterozygotes (i.e. too many 2p's).

They are ***determined*** via close examination of allele drop-out during (and possibly after) validation

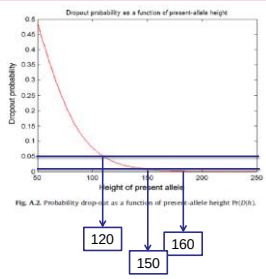
Stochastic Threshold – Why do we need this?



Stochastic Threshold - Max Height



Stochastic Threshold - Method 2 (Pr(D))



Gill et al. FSI Genetics, 2009, 3, 104-111.

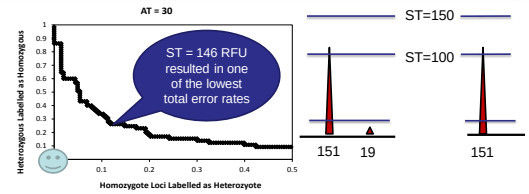
Methods 1 and 2 minimizes the chance of wrongly deciding a heterozygous locus is homozygous

Stochastic Threshold - Method 3

Minimizing the error of incorrectly concluding a heterozygous locus is homozygous

Minimizing the error of incorrectly concluding a homozygous locus is potentially heterozygous

At various STs, for all heterozygous loci, determine proportion of heterozygous loci falsely labeled as homozygous
For all homozygous loci, determine the proportion of homozygous loci falsely considered possible heterozygotes. Plot the proportions against each other.



Stochastic Threshold: Conclusions

To determine an ST;

- -Amplify numerous samples at all targets typically seen in casework (can be same set you used to determine the AT)
- -Analyze at your AT (not AT=1) and determine the ST
- -Method 3 has become the method of choice at BU
  - -Unlike ATs, STs do not seem to substantially change when determined via 3 different methods
  - -No distribution assumption
  - -Directly measures performance of STs and gives the proportion of false positives AND negatives

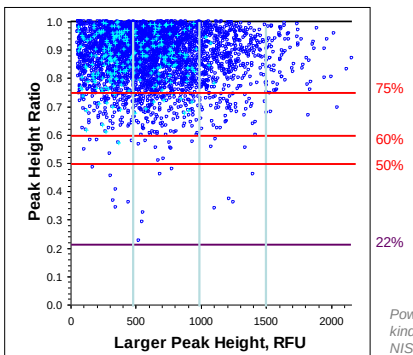
Peak Height Ratio Thresholds

Evaluate PHRs at various DNA template levels (e.g., dilution series of DNA). Can use the same data set used to determine AT and ST.

STs and PHRs are related.

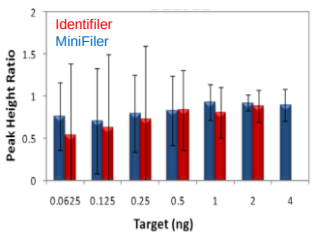
Different PHR expectations at different peak height ranges may be established.

Peak Height Ratios



Power Plex 16 data kindly provided by NIST,>8000 alleles

Peak Height Ratios



<0.5 ng (500RFU)	>0.5 ng (500RFU)
0.2	0.5

Stutter

True allele
(tetranucleotide repeat)

Typically **5-15%** of true allele in tetranucleotide repeats STR loci

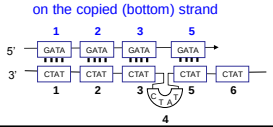
Occurs less frequently (typically <2%)

n-4 stutter product

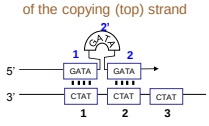
n+4 stutter product

Walsh, P.S., et al. (1996). Sequence analysis and characterization of stutter products at the tetranucleotide repeat locus vWA. *Nucleic Acids Research, 24*, 2807-2812.

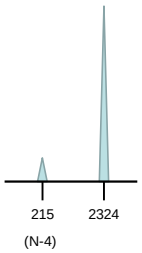
Deletion caused by slippage on the copied (bottom) strand



Insertion caused by slippage of the copying (top) strand



Stutter



$$\text{Stutter \%} = \frac{\text{N-4 peak}}{\text{allele peak}} = \frac{215}{2324} = 9.25\%$$

Stutter Filters

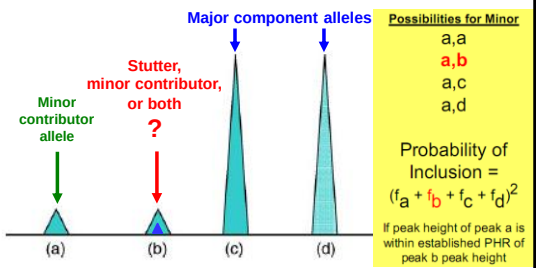


Fig. 4. *c* and *d* are unambiguous alleles, *b* is a minor allele in a stutter position and *a* is an unambiguous minor allele.

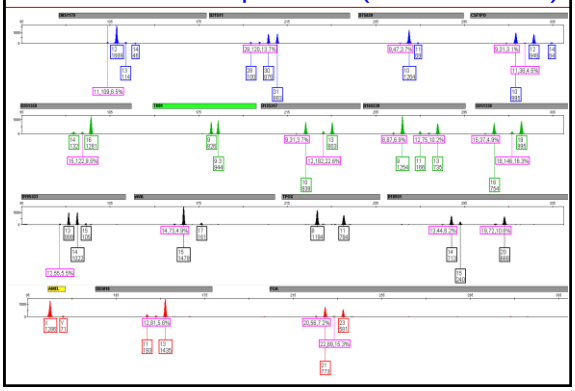
Gill *et al.* (2006) DNA Commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. *Forensic Sci. Int.* 160: 90-101

Stutter Threshold from manufacturer's validation data – locus dependent

- Dependent on the length of the allele
- Variance of stutter ratios may increase with target
- Use the same data set used to determine PHR, ST and AT to establish stutter thresholds

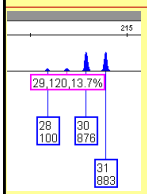
Locus	Stutter Threshold
CSF1PO	9.2%
D2S1338	11.1%
D3S1358	10.7%
D5S818	6.8%
D7S820	8.2%
D8S1179	8.2%
D13S317	8.0%
D16S539	10.4%
D18S51	17.0%
D19S433	13.3%
D21S11	9.4%
FGA	14.7%
TH01	5.1%
TPOX	4.8%
vWA	12.6%

Thresholds & Interpretation (stutter filter off)



D21S11

AT	30RFU	
ST	150RFU	
Stutter Filter	9.4%	
PHR	0.2 (<500RFU)	0.5 (>500RFU)
Major:Minor	4:1	



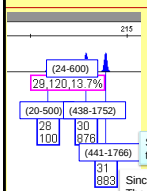
Possible genotype combinations (28,29,30,31)	
Person 1	Person 2
28,29	30,31
28,30	29,31
28,31	29,30
29,30	28,31
29,31	28,30
30,31	28,29

1. No. of contributors? 2.
2. Is the 29 an allele? Yes.
2. Possible non-observed allele if 2 contributors? No.

List out all alleles:
28, 29, 30, 31 and consider all possible pairs – assuming 2 contributors

D21S11

AT	30RFU	
ST	150RFU	
Stutter Filter	9.4%	
PHR	0.2 (<500RFU)	0.5 (>500RFU)
Major:Minor	4:1	



$$PH_{Stutter29} = 100(0.2) \text{ to } \frac{100}{0.2}$$

$$PH_{Stutter29} = 20 \text{ to } 500 \text{ RFU}$$

Since PH_{29}=120 is btw 20-500, then 28,29 is possible combination

Since PH_{30}=876 is NOT btw 20-500 Then 28,30 is NOT a possible combination

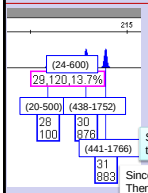
$$\frac{100+120}{100+120+876+883} = 0.11$$

D21S11 Inferred Genotypes	
Major	30,31
Minor	28,29

Possible genotype combinations (28,29,30,31)	
Person 1	Person 2
28,29	30,31
~~28,30~~	~~29,31~~
~~28,31~~	~~29,30~~
~~29,30~~	~~28,31~~
~~29,31~~	~~28,30~~
30,31	28,29

D21S11

AT	100RFU	
ST	500RFU	
Stutter Filter	9.4%	
PHR	0.2 (<500RFU)	0.5 (>500RFU)
Major:Minor	4:1	



$PH_{Stutter28} = \frac{100(0.2)}{0.2}$ to $\frac{100}{0.2}$

$PH_{Stutter28} = 20$ to 500 RFU

Since PH_{28}=120 is btw 20-500, then 28,29 is possible combination

Since PH_{30}=876 is NOT btw 20-500 Then 28,30 is NOT a possible combination

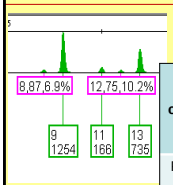
$$\frac{100+120}{100+120+876+883} = 0.11$$

D21S11 Inferred Genotypes	
Major	30,31
Minor	28,29

Possible genotype combinations (28,29,30,31)	
Person 1	Person 2
28,29	30,31
~~28,30~~	~~29,31~~
~~28,31~~	~~29,30~~
~~29,30~~	~~28,31~~
~~29,31~~	~~28,30~~
30,31	28,29

D16S539

AT	30RFU	
ST	150RFU	
Stutter Filter	10.4%	
PHR	0.2 (<500RFU)	0.5 (>500RFU)
Major:Minor	4:1	



1. No. of Contributors? 2. Are 8 and 12 alleles? Maybe.
2. Possible non-observed alleles if 2 contributors? No.

List out all possible alleles: 8, 9, 11, 12, 13 and consider all possible pairs – assuming 2 contributors

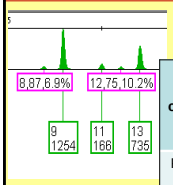
Possible genotype combinations if 8 contained allele (8,9,11,13)	
Person 1	Person 2
8,9	11,13
8,11	9,13
8,13	9,11
9,11	8,13
9,13	8,11
11,13	8,9

Possible genotype combinations if 12 contained allele (9,11,12,13)	
Person 1	Person 2
9,11	12,13
9,12	11,13
9,13	11,12
11,12	9,13
11,13	9,12
12,13	9,11

Possible genotype combinations if 8 and 12 were stutter (9,11,13)	
Person 1	Person 2
9,9	11,13
9,11	9,13 or 11,13 or 13,13
9,13	9,11 or 11,11 or 11,13
11,11	9,13
11,13	9,9 or 9,11 or 9,13
13,13	9,11

D16S539

AT	30RFU	
ST	150RFU	
Stutter Filter	10.4%	
PHR	0.2 (<500RFU)	0.5 (>500RFU)
Major:Minor	4:1	



D16S11 Inferred Genotypes	
Major	9,13
Minor	8,11 or 11,12 or 9,11 or 11,11 or 11,13

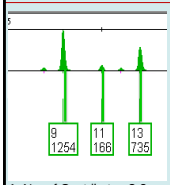
Possible genotype combinations if 8 contained allele (8,9,11,13)	
Person 1	Person 2
~~8,9~~	~~11,13~~
8,11	9,13
~~8,13~~	~~9,11~~
~~9,11~~	~~8,13~~
9,13	8,11
~~11,13~~	~~8,9~~

Possible genotype combinations if 12 contained allele (9,11,12,13)	
Person 1	Person 2
~~9,11~~	~~12,13~~
~~9,12~~	~~11,13~~
9,13	11,12
11,12	9,13
~~11,13~~	~~9,12~~
~~12,13~~	~~9,11~~

Possible genotype combinations if 8 and 12 were stutter (9,11,13)	
Person 1	Person 2
~~9,9~~	~~11,13~~
9,11	~~9,13~~ or 11,13 or 13,13
9,13	9,11 or 11,11 or 11,13
11,11	9,13
11,13	~~9,9~~ or 9,11 or 9,13
~~13,13~~	~~9,11~~

D16S539

AT	100RFU	
ST	500RFU	
Stutter Filter	10.4%	
PHR	0.2 (<500RFU)	0.5 (>500RFU)
Major:Minor	4:1	



1. No. of Contributors? 2.
2. Possible non-observed alleles if 2 contributors?
Yes.
List out all possible alleles:
9, 11, 13, O and consider all possible pairs – assuming 2 contributors

D16S11 Inferred Genotypes	
Major	9,13
Minor	9,11 or 11,11 or 11,13 or 11,O

Possible genotype combinations if 8 and 12 were stutter (9,11,13)

Person 1	Person 2
~~9,9~~	~~11,13~~
9,11	~~9,13~~ or ~~11,13~~ or ~~13,13~~
9,13	9,11 or 11,11 or 11,13
11,11	9,13
11,13	~~9,9~~ or ~~9,11~~ or 9,13
~~13,13~~	~~9,11~~

Possible genotype combinations if DO (9,11,13,O)

Person 1	Person 2
~~9,11~~	~~13,O~~
9,13	11,O
~~9,O~~	~~11,13~~
~~11,13~~	~~9,O~~
11,O	9,13
~~13,O~~	~~9,11~~

Profile 1. – Minor Genotype Possibilities

Given 30 AT Threshold and interpretation standard operating procedures, the genotypes of the minor are…….

Description	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338
Inferred Genotypes of Minor	13,14	28,29	9,11 or 10,11 or 11,11 or 11,O	9,14 or 10,14 or 11,14 or 12,14 or 14,14 or 14,O	14,14 or 14,15 or 14,16	9,9 or 9,9.3 or 9.3,9.3 or 9,O or 9.3,O or O,O	9,12 or 10,12 or 12,12 or 12,13	8,11 or 9,11 or 11,11 or 11,12 or 11,13	15,18 or 16,18 or 18,18 or 18,19 or 18,O
Known	13,14	28,29	10,11	10,14	14,16	9,9.3	12,13	11,13	18,19

Description	vWA	TPOX	D18S51	AMEL	D5S818	FGA
Inferred Genotypes of Minor	14,17 or 15,17 or 17,17	8,8 or 8,11 or 11,11 or 8,O or 11,O O,O	14,15 or 15,15 or 15,19 or 15,20	X,Y	11,11 or 11,12 or 11,13	20,22 or 21,22 or 22,22 or 22,23 or 22,O
Known	14,15	8,11	15,20	X,Y	11,13	22,23

O= any other allele (not observed)

Conclusions

- Thresholds should be set to minimize false positives AND false negatives.
- These thresholds can be applied in a systematic way in order to deduce genotypes of 1- and 2- person mixtures
  - In particular, peak height ratio thresholds can be used to examine possible genotype combinations
- The same validation data-set of single-source samples of known genotype can be used to establish all thresholds. If possible, use actual samples (not dilution series from concentrated stock).
- Thresholds obtained for a given method must be applied only to evidence obtained using the same method (i.e. kit, injection time, etc).