NIST Validation Studies on the 3500 Genetic Analyzer

https://products.appliedbiosystems.com/ab/en/US/htdocs/productMgr/images/3500_family_big.jpg

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U.S. National Institute of Standards and Technology

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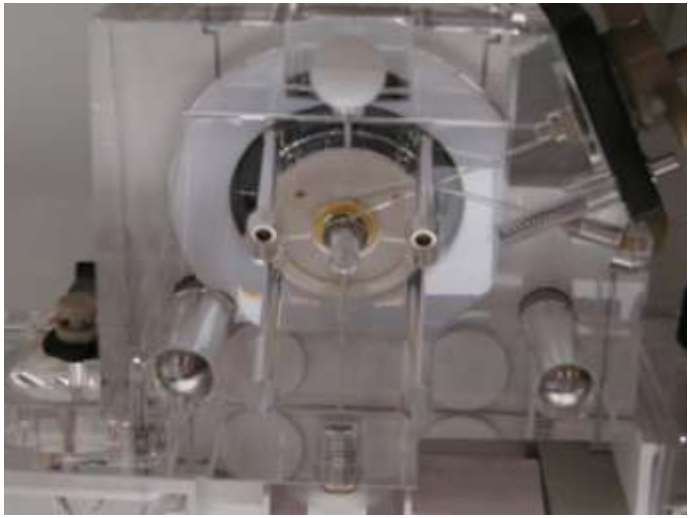
Outline

- Details of the ABI 3500 Genetic Analyzer
- Validation design and results with Identifiler Plus and PowerPlex 16 HS and
  - Injection parameters and reaction setup
  - Precision and size standard comparison
  - Concordance and mixture evaluation
- Methodology of setting analytical and stochastic thresholds
- Brief overview of signal normalization
- Consumable usage comments from a research laboratory standpoint
- SRM2391c Update

Details of the ABI 3500

No lower pump block
(Fewer air bubbles)



Improved sealing for better temperature control

Primary Differences

	31xx Platforms	**3500 Platforms**
Laser	Argon ion (AR+) with 488/514 nm wavelength	Single-line 505 nm, solid-state, long-life laser
Power Requirement	220V	110V
File Generated	.fsa files	.hid files
Normalization	None	Instrument-to-instrument; only with AB kits
Optimal Signal Intensity	1500-3000 RFU	~4x greater than 31xx platforms

What is Validation?

Section 1.1 (SWGDAM Revised Validation Guidelines) Validation is the process by which the scientific community acquires the necessary information to:

(a) Assess the ability of a procedure to obtain reliable results.

(b) Determine the conditions under which such results can be obtained.

(c) Define the limitations of the procedure.

The validation process identifies aspects of a procedure that are critical and must be carefully controlled and monitored.

Reliability, Reproducibility, Robustness

Experimental Summary

	Test	Types of Samples Used	Number Examined
Reliability	**Size Standard Comparison**	15 Allelic Ladders per size standard (LIZ 500 vs. LIZ 600 v2.0)	30
Reliability	**Size Standard Dilution**	Samples heterozygous at 15 loci plus Amelogenin at 1.0 ng DNA input	16
Reliability		Amplified Extraction Blanks	
Reliability	**Injection Parameters***	3 male samples heterozygous at 15 loci 1 ng DNA input	15 3 samples per injection
Reproducibility	**Precision***	Allelic Ladders*	24
Reproducibility		3 male samples heterozygous at 15 loci	6
Reproducibility	**Concordance***	50 genomic DNA samples	60
Reproducibility		SRM 2391b: 10 genomic DNA samples	
Robustness	**Sensitivity***	Dilution series of 3 male samples heterozygous at 15 loci	84 4 replicates of each dilution series
Robustness	**Mixtures***	Mixture dilution series of 2 male samples heterozygous at 15 loci at 1.0 ng DNA input (1:1, 1:2, 1:3, 1:5, 1:7, 1:9, 1:10, 10:1, 9:1, 7:1, 5:1, 3:1, 2:1)	28
	Total Number of Samples: Identifiler Plus		**247**
	Total Number of Samples: PowerPlex 16 HS		**227**

*Identical experiments for Identifiler Plus and PowerPlex 16 HS

n=30 allelic ladders (ILS Precision)
n=15 samples (Injection Parameters)

Identifiler Plus: Reliability

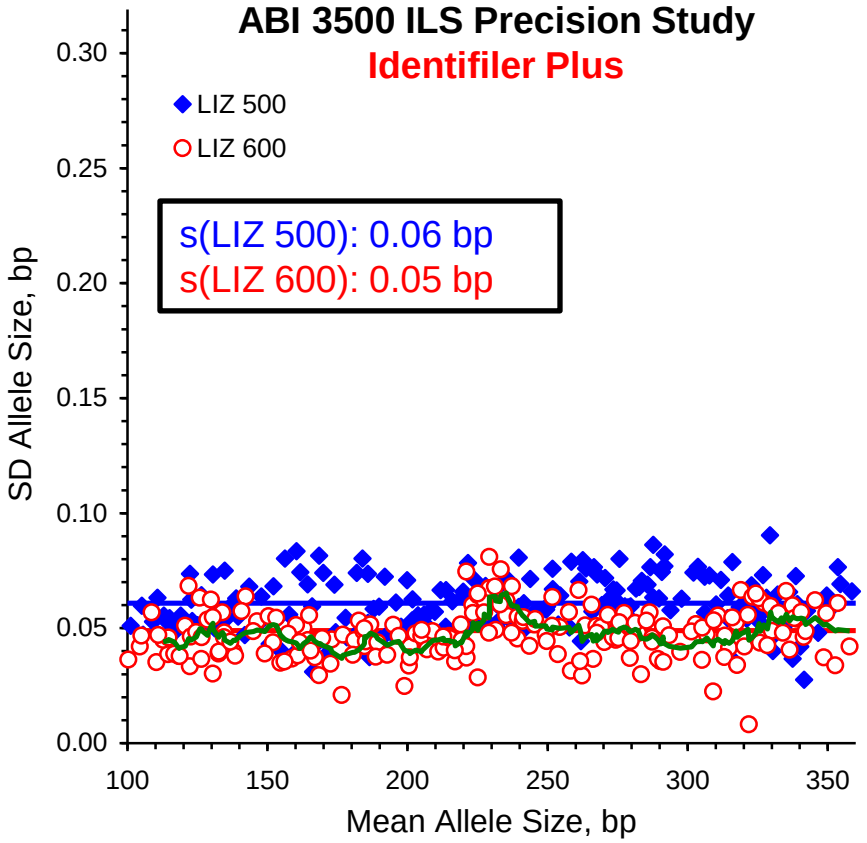
- No significant difference between the LIZ500 and LIZ600 v2.0 size standards

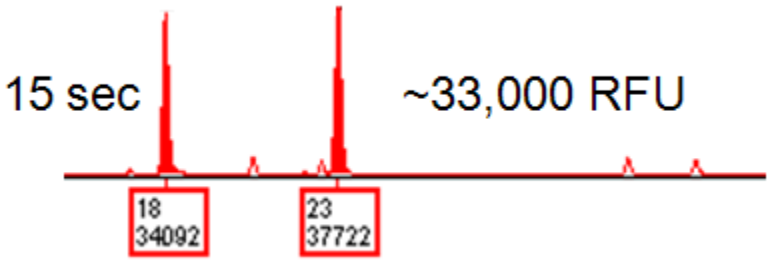
- Injection parameters set for ½ PCR reactions:

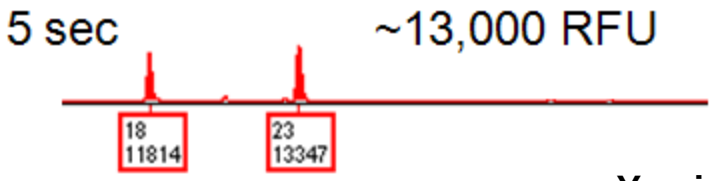
  28 cycles

  – 3500 Default: 1.2 kV for 15 s

  – **Identifiler Plus**: 1.2 kV for 5 s







Y-axis: 35,000 RFU

n=16 allelic ladders (Size Standard Dilution)
n=15 samples (Injection Parameters)

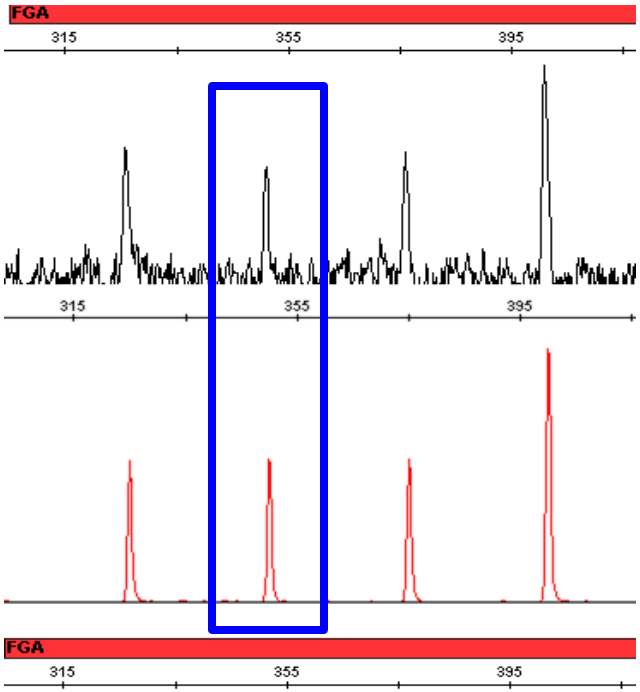
PowerPlex 16 HS: Reliability

- Dilution of ILS 600 to eliminate bleed-through of the size standard into the yellow dye channel
  - 0.25 µl ILS 600 + 9.75 µl HiDi per sample corrected the bleed-through
- Injection parameters set for ½ PCR reactions:

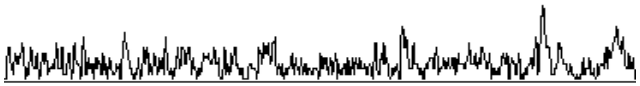
30 cycles

  - 3500 Default: 1.2 kV for 15 s
  - **PowerPlex 16 HS**: 1.2 kV for 10 s

0.5 µl ILS 600 / sample



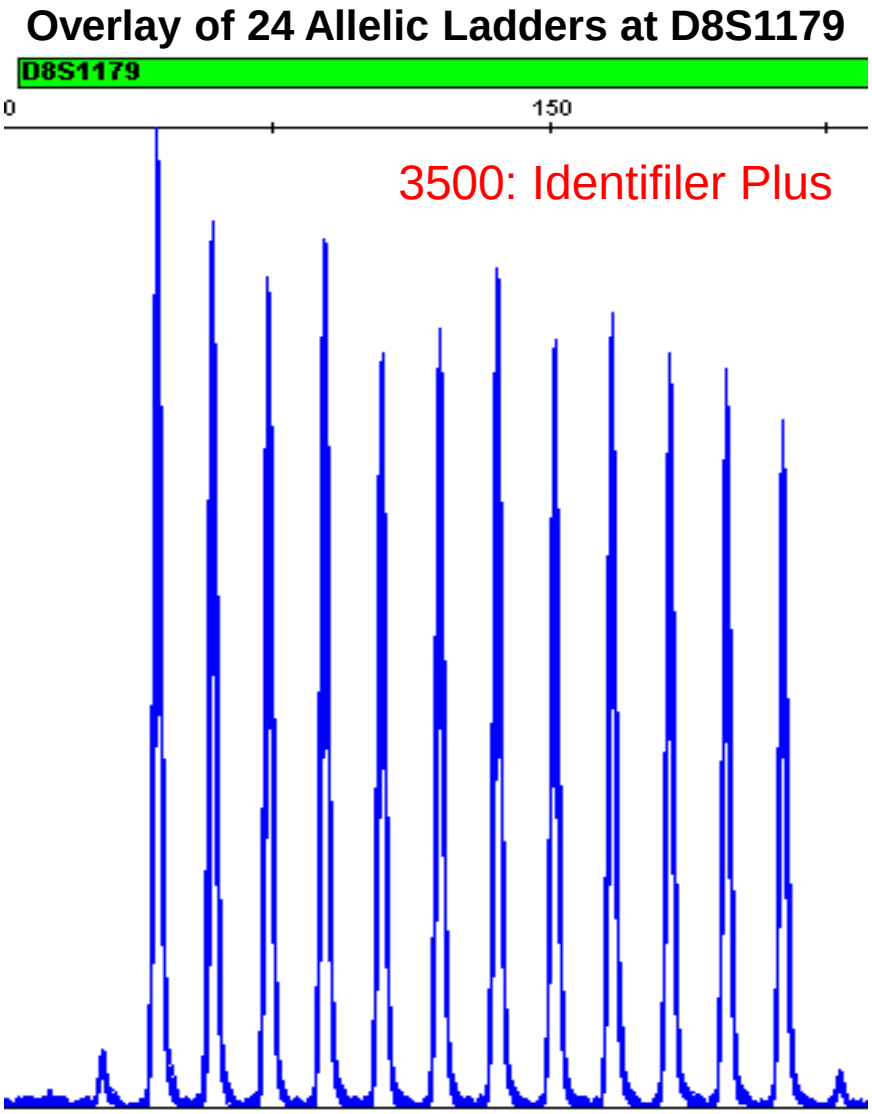
0.25 µl ILS 600 / sample



Y-axis: 50 RFU

Identifiler Plus: Reproducibility

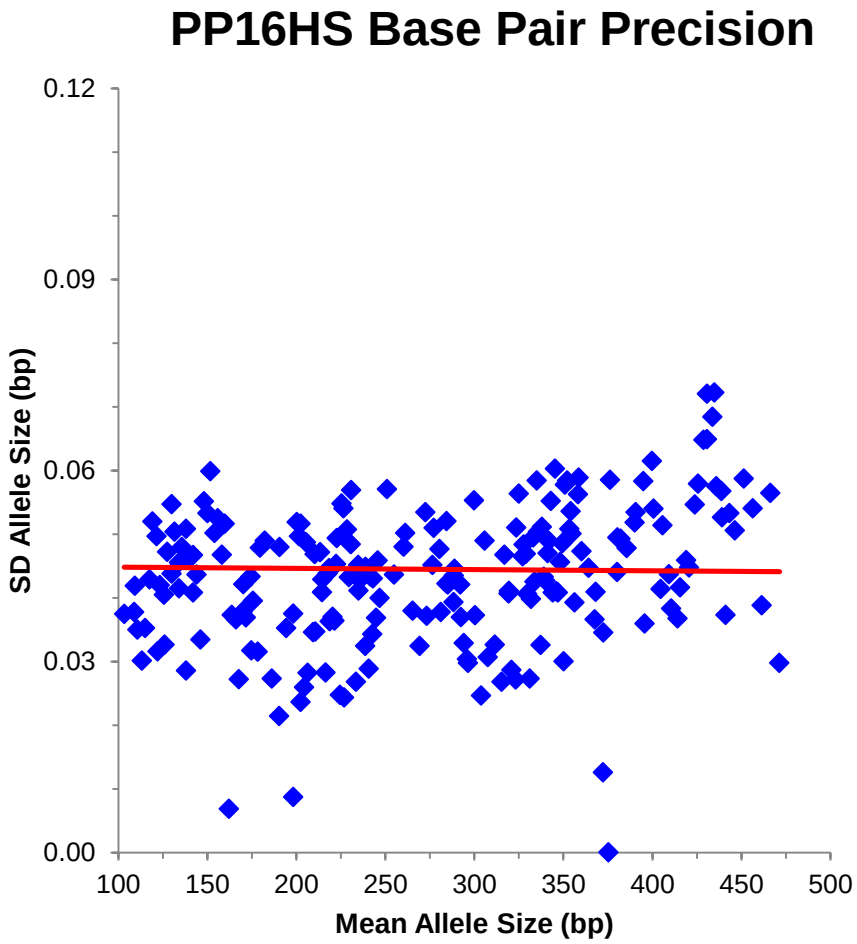
- 60 samples concordant between 3130*xl* and 3500
  - Total of 1689 alleles examined
- Precision of base pair sizing ±0.04 bp between allelic ladders and samples tested
  - No significant difference between the 3130*xl* and 3500



n=24 allelic ladders (Precision)

PowerPlex 16 HS: Reproducibility

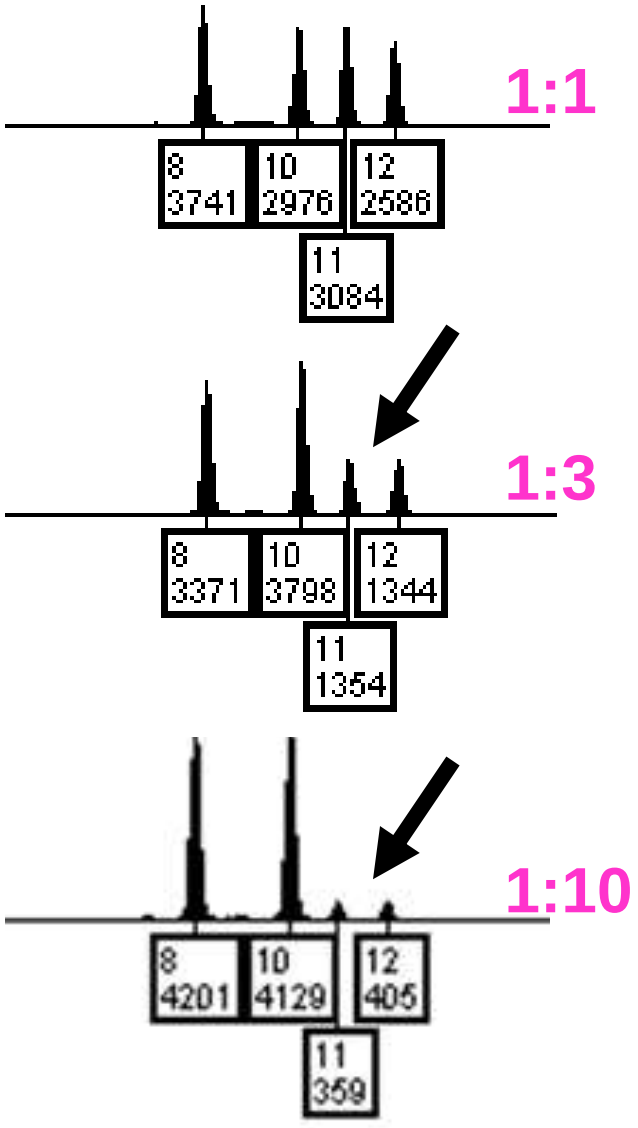
- 60 samples concordant between 3130*xl* and 3500
  - Total of 1688 alleles examined
- Precision of base pair sizing ±0.05 bp between allelic ladders and samples tested
  - No significant difference between PowerPlex 16 HS and Identifiler Plus



n=28 samples (Mixture Study)
n=84 samples (Sensitivity)

Identifiler Plus: Robustness

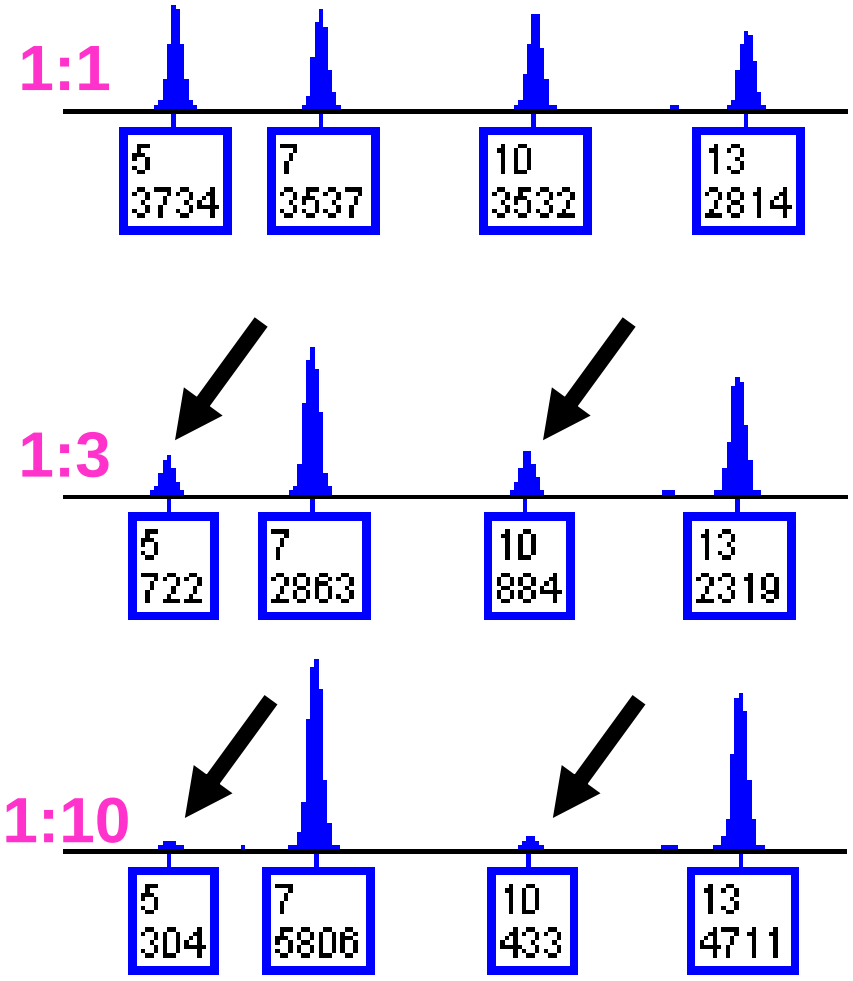
- Minor component identified correctly in a 1:10 mixture ratio
- Sensitivity data examined to set analytical and stochastic thresholds
  - Full (correct) profiles observed from 1.0 ng to 100 pg



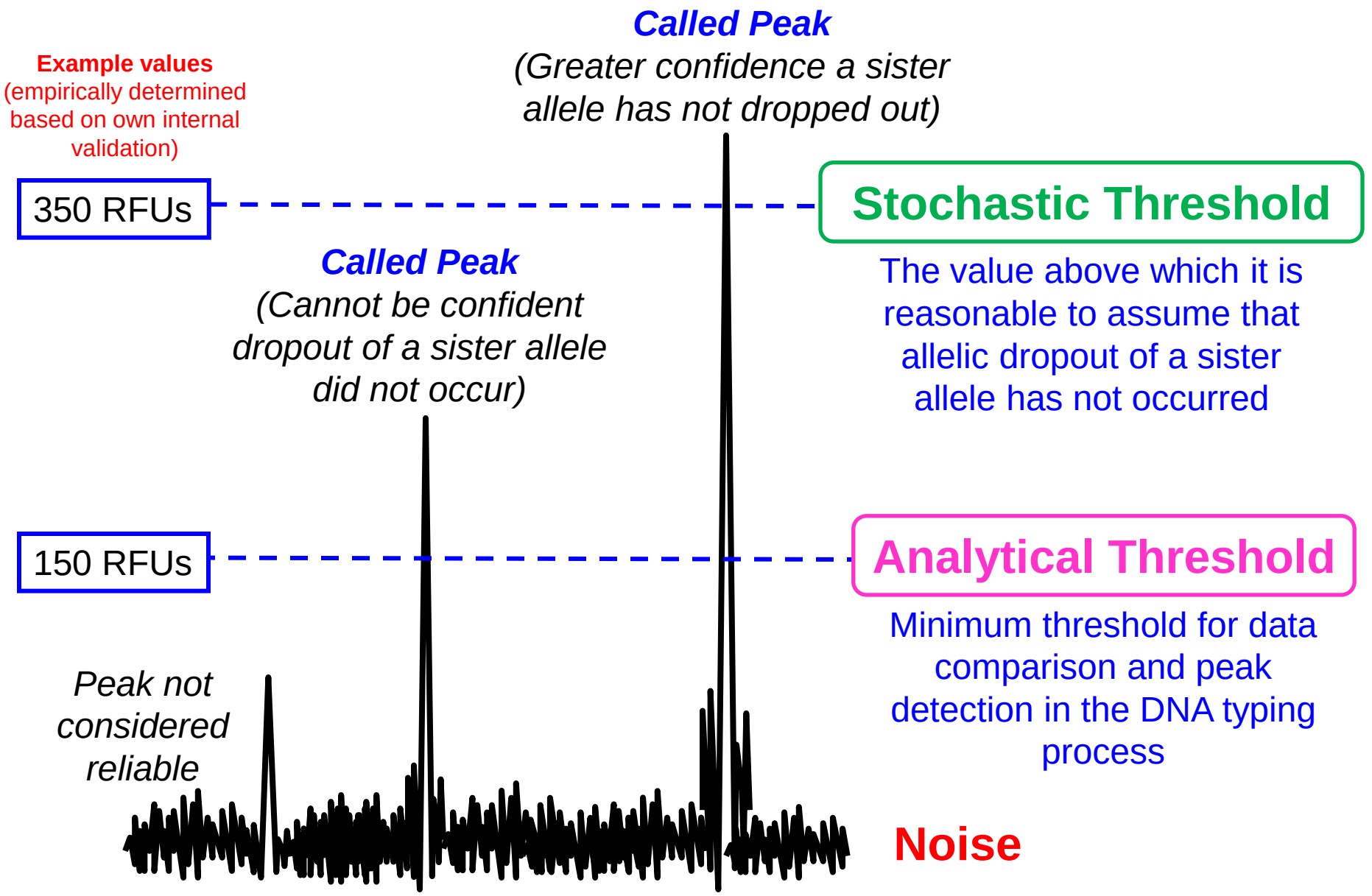
n=28 samples (Mixture Study)
n=84 samples (Sensitivity)

PowerPlex 16 HS: Robustness

- Minor component identified correctly in a 1:10 mixture ratio
- Sensitivity data examined to set analytical and stochastic thresholds
  - Full (correct) profiles observed from 1.0 ng to 100 pg



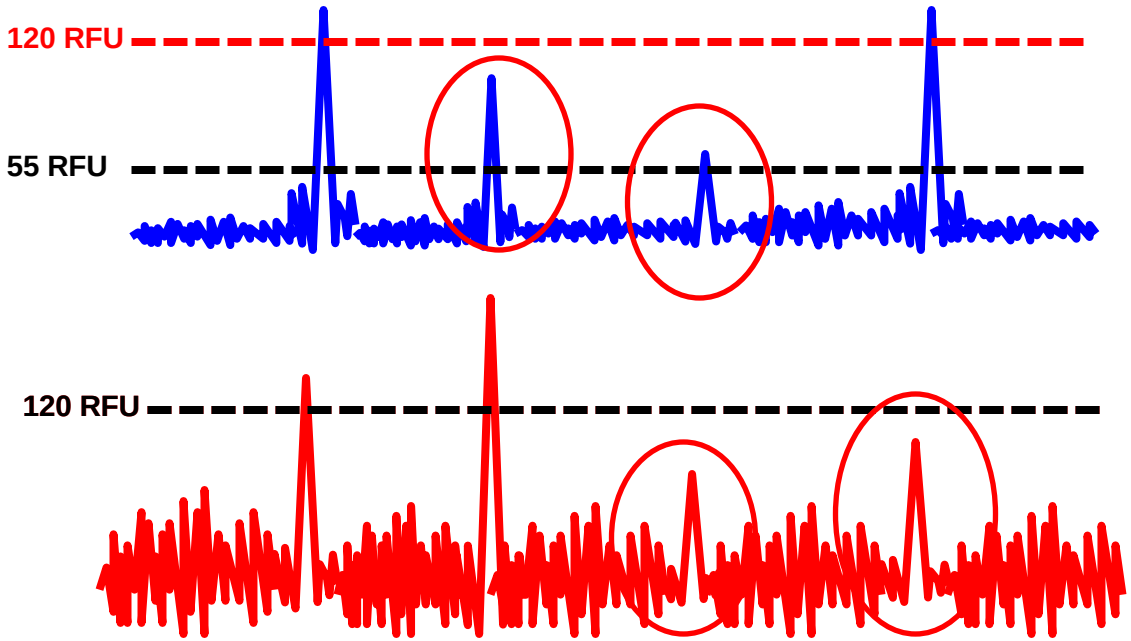
Different Threshold Overview



Butler, J.M. (2009) *Fundamentals of Forensic DNA Typing*. Elsevier Academic Press: San Diego.

Universal vs. Dye-specific

- Universal analytical threshold assumes all dye channels have the **same** amount of noise
- Dye-specific analytical threshold take into consideration that all dye channels do not have the same level of noise



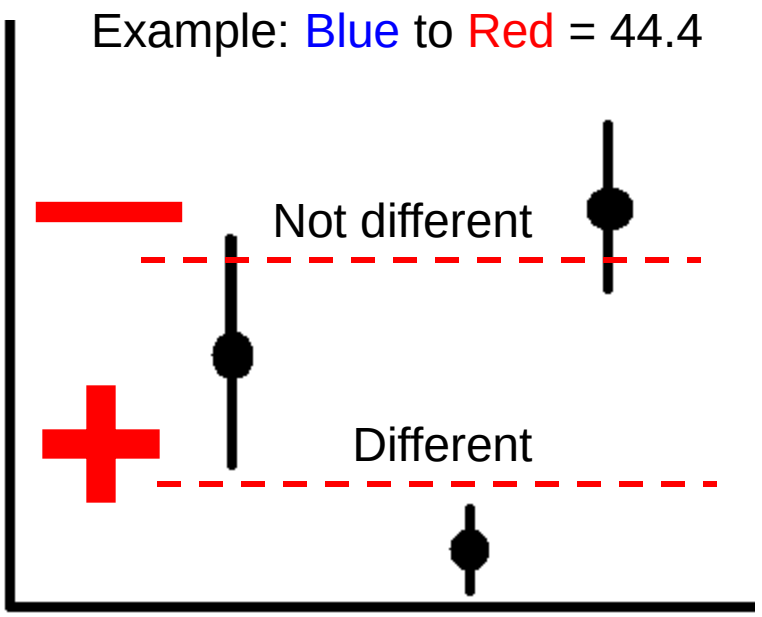
Statistical Difference Calculation

- Evaluation of data to determine the statistical difference between dye channel analytical thresholds

- Calculated statistical difference using a z-test

- If negative: **Not** statistically different
  - Error bars overlap
  - One standard analytical threshold can be applied to all dyes

- If positive: Statistically different
  - Error bars do not overlap
  - Dye specific analytical thresholds need to be applied



n=84 samples

Statistical Difference Calculation

- Dye channels were **not statistically different** for PowerPlex 16 HS
  - Ability to use one universal threshold for all dye channels

- Dye channels were **statistically different** for Identifiler Plus
  - Each dye channel mush be treated independently

Analytical Threshold Methodology

- Baseline noise values calculated with data from the sensitivity study (DNA dilution series)
  - Threshold set at 1 RFU for all dye channels
  - Remove calls for all alleles and artifacts (stutter, n+4, pull-up, etc.)

- 4 methods to evaluate analytical thresholds calculated

- **Analytical Threshold**: Average RFU + (10 x Standard Deviation)

n=84 samples

Analytical Threshold Calculation

Identifiler Plus						
Dye Channel	**Average RFU**	**Stdev**	**Min RFU**	**Max RFU**	**Calculated Noise (RFU)**	**Dye-Specific**
Blue	10	4.6	3	68	55	55
Green	16	5.6	3	78	72	75
Yellow	24	7.9	7	63	103	105
Red	31	8.9	7	81	120	120

Universal Threshold: 120 RFU

Dye-Specific: Rounded to nearest 5 RFU

PowerPlex 16 HS					
Dye Channel	**Average RFU**	**Stdev**	**Min RFU**	**Max RFU**	**Calculated Noise (RFU)**
Blue	16	17.4	2	99	190
Green	20	19.3	2	99	212
Yellow	18	119.3	2	99	211

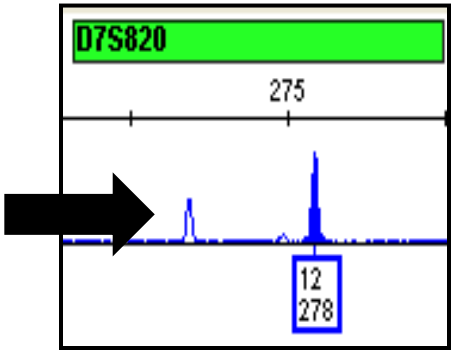
Universal Threshold: 215 RFU

n=560 alleles

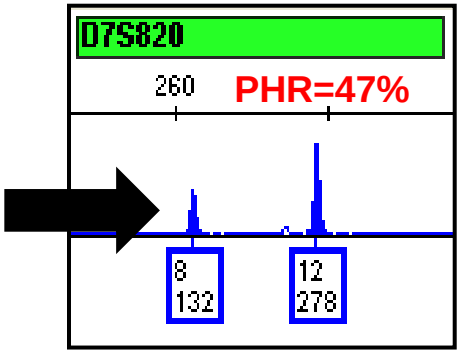
Threshold Comparison:
Identifiler Plus

Total of 560 alleles examined (50 pg, 30 pg, and 10 pg) where dropout was observed

Single Threshold (140 RFU)



Dye-Specific Thresholds



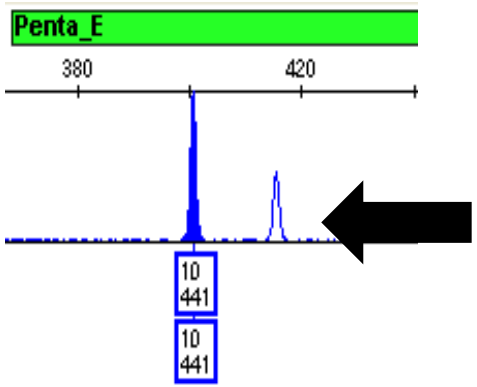
22.0% of the total possible allele calls were lost using a single threshold rather than using dye-specific thresholds with **Identifiler Plus**

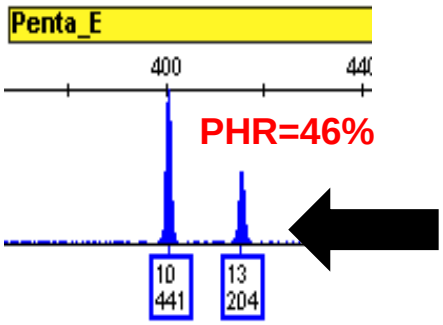
n=560 alleles

Threshold Comparison:
PowerPlex 16 HS

Total of 560 alleles examined (50 pg, 30 pg, and 10 pg) where dropout was observed

Single Threshold (215 RFU)	Dye-Specific Thresholds





Employing a **dye-specific** threshold recovered **less than 1.0%** of the possible allele calls which were lost with a universal threshold.

Setting Stochastic Methodology

- Analyzed data from the sensitivity study (DNA dilution series)
  - Dye-specific analytical thresholds for Identifiler Plus
  - Universal analytical threshold for PowerPlex 16 HS

- Examined sample amounts where dropout was observed (50 pg, 30 pg, 10 pg)
  - Used to examine stochastic effects including severe imbalance of heterozygous alleles and allele dropout

- **Stochastic Threshold**: Determined by the best fit of the data produced within the sensitivity study (DNA dilution series)

Stochastic Threshold Calculations

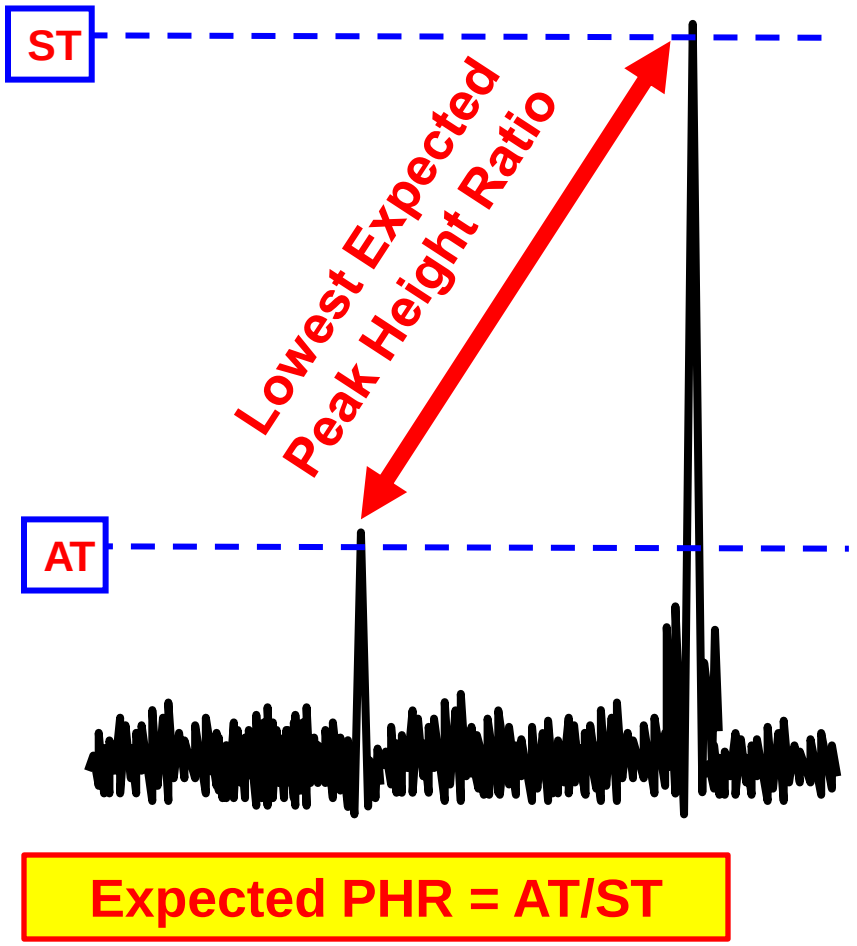
	No false homozygote calls above **415 RFU**		No false homozygote calls above **935 RFU**	
	Identifiler Plus ST (RFU)	**% False Homozygote Calls**	**PowerPlex 16 HS ST (RFU)**	**% False Homozygote Calls**
AVG RFU	160	36.9	365	37.4
AVG + 1SD	235	16.4	515	12.9
AVG + 2SD	301	4.9	665	6.8
AVG + 3SD	385	0.8	810	2.1
MAX RFU	415	0.0	935	0.0
	Injection: 1.2 kV for 5 sec **PCR Cycles**: 28		**Injection**: 1.2 kV for 10 sec **PCR Cycles**: 30	

n=84 samples

Summary of Thresholds

Identifiler Plus 5 sec @ 1.2 kV (28 cycles)			
	AT (RFU)	ST (RFU)	Lowest Expected PHR
Blue	55	290	19%
Green	75	385	19%
Yellow	105	415	25%
Red	120	265	45%

PowerPlex 16 HS 10 sec @ 1.2 kV (30 cycles)			
	AT (RFU)	ST (RFU)	Lowest Expected PHR
All Dyes	215	665	32%



Validation Conclusions

- The 3500 has proven to be reliable, reproducible and robust
  - Out of 247 samples with Identifiler Plus only 3 required reinjection
- Universal analytical thresholds may result in more allelic and full locus dropout (kit and condition dependent)
- Stochastic thresholds are linked to analytical thresholds
  - If the analytical threshold is adjusted, the stochastic threshold **should be reevaluated** along with expected peak height ratios
    - Requires **consideration for overall interpretation workflow** which we are still evaluating
- There **may** be occasions where the data falls outside the bounds of the stochastic threshold leading to potential false homozygote calls
- Increase of cycle number can impact the observed stochastic effects

What is Normalization and how does it work?

Normalization of Data

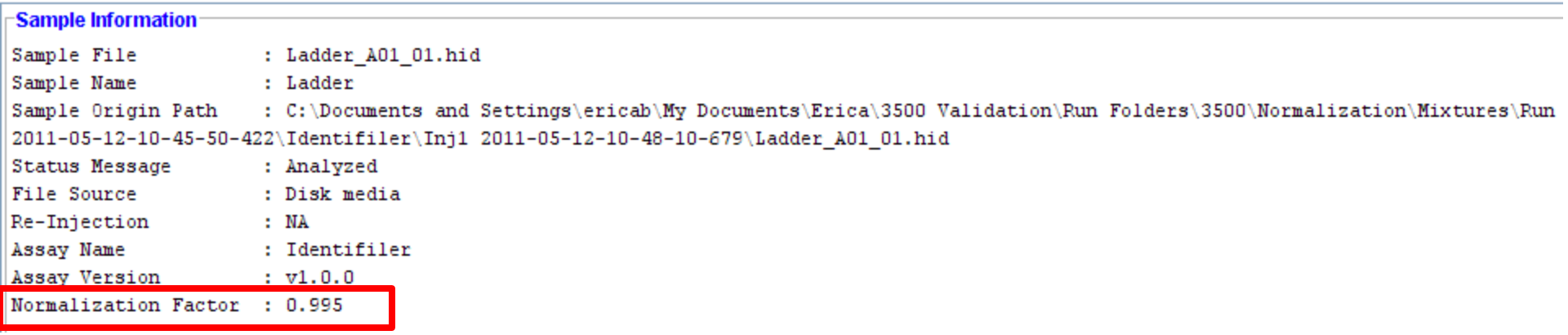
- Recommended to compare signal between instruments
- Motivation mainly for large laboratories with many instruments
  - Correct for signal variation between instruments
- Can be used with a single instrument
  - Correct for signal variation between single and multiple injections

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



Sample Information

Sample File : Ladder_A01_01.hid
Sample Name : Ladder
Sample Origin Path : C:\Documents and Settings\ericab\My Documents\Erica\3500 Validation\Run Folders\3500\Normalization\Mixtures\Run 2011-05-12-10-45-50-422\Identifiler\Inj1 2011-05-12-10-48-10-679\Ladder_A01_01.hid
Status Message : Analyzed
File Source : Disk media
Re-Injection : NA
Assay Name : Identifiler
Assay Version : v1.0.0
Normalization Factor : 0.995

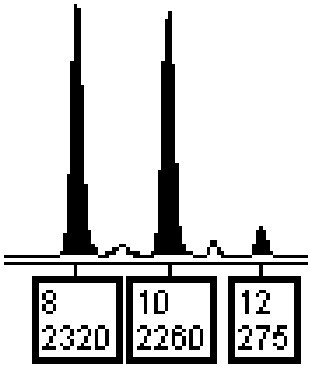
Normalization Example

Theoretical Normalization Target: 2000 RFU

Without Normalization

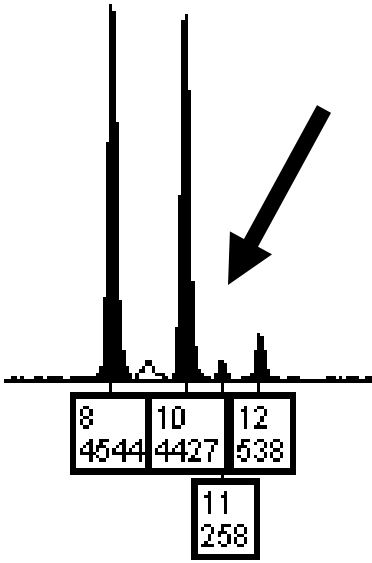
With Normalization

LIZ 600 v2.0 Peak Height Average: **1021 RFU**

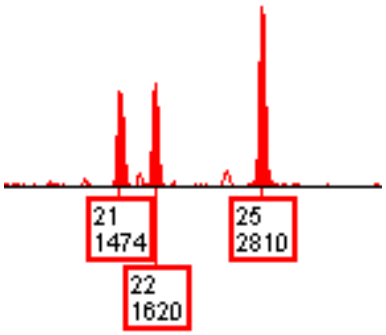


NF=1.959

Signal increases almost by a factor of 2

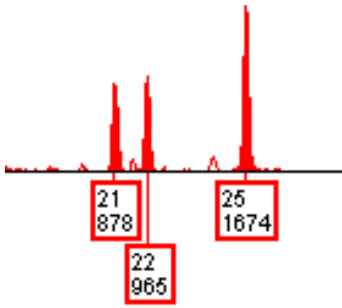


LIZ 600 v2.0 Peak Height Average: **3192 RFU**



NF= 0.596

Signal decreases by almost half

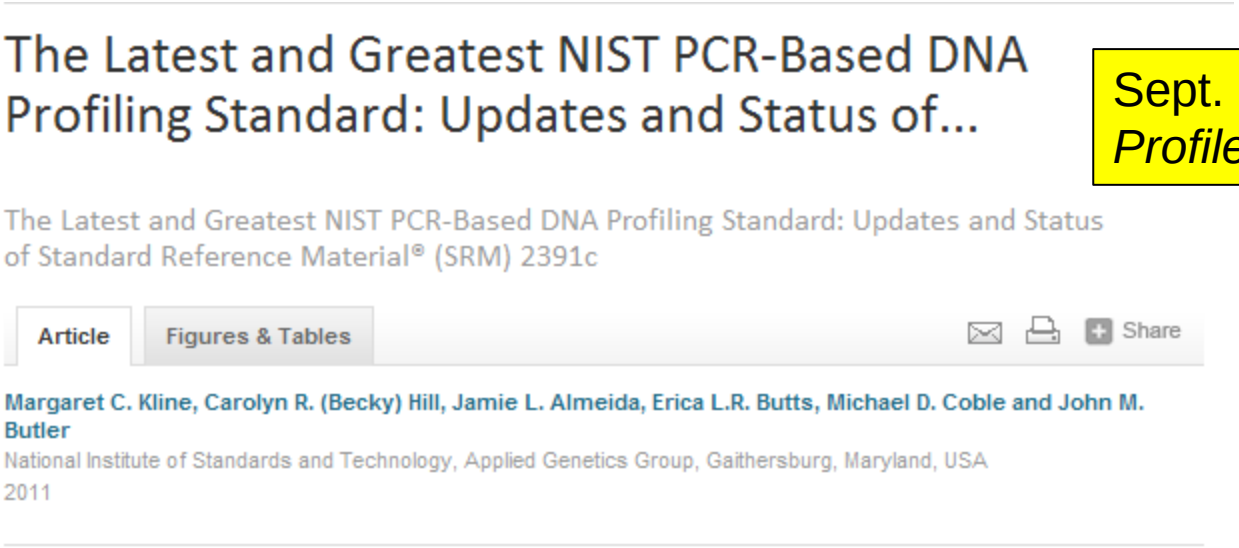


Consumable RFID Tracking Experience Within a Research Laboratory

Consumable RFID Tracking Limits

	RFID Hard Stops	Usage Comments From a Research Laboratory Standpoint
Array	None	1. Very easy to change between HID and sequencing 2. Array from validation was stored at least twice and reinstalled on 3500 during validation
Buffer	Expiration Date 7 Days on Instrument # Injections	1. Can no longer use in-house buffer 2. Very easy to change on the instrument (snap-and-go)
Polymer	Expiration Date # Samples # Injections	1. Hard stop with the expiration date has caused us to discard unused polymer we would have otherwise kept on the instrument 2. ~50% of total polymer remains in the pouch after "consumption" 3. Expiration dates have changed purchasing strategy (smaller batches, based on ongoing project needs)

SRM 2391c Update



The Latest and Greatest NIST PCR-Based DNA Profiling Standard: Updates and Status of...

The Latest and Greatest NIST PCR-Based DNA Profiling Standard: Updates and Status of Standard Reference Material® (SRM) 2391c

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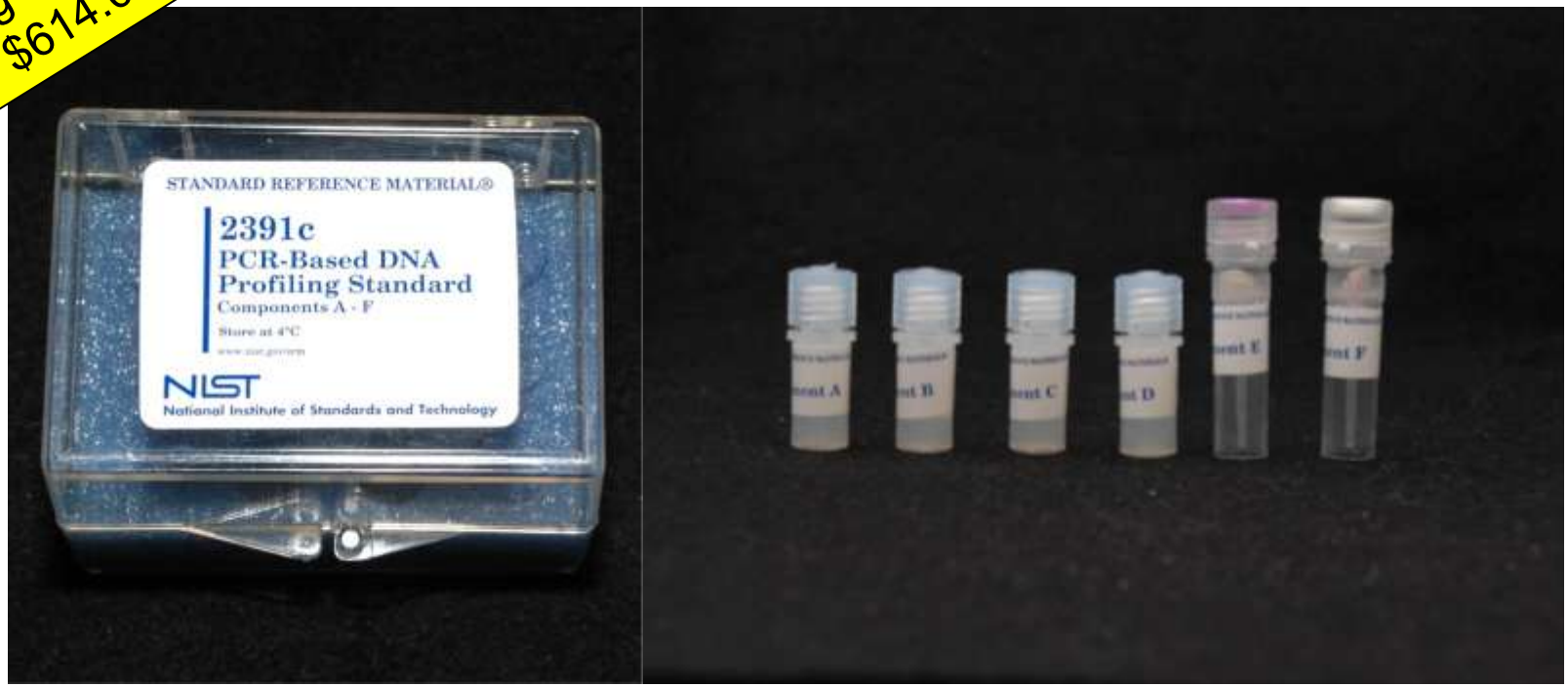
2011

Sept. 29, 2011
Profiles in DNA

http://www.promega.com/resources/articles/profiles-in-dna/2011/the-latest-and-greatest-nist-pcr-based-dna-profiling-standard/

Selling since
Aug 16, 2011
$614.00

SRM 2391c



Produced with an entirely new set of genomic DNA samples.

9947a & 9948 are NOT included.

https://www-s.nist.gov/srmors/view_detail.cfm?srm=2391C

Description of Components in SRM 2391c

Component	Description	Quantity [a]
A	50 µL of anonymous female genomic DNA	1.4 – 1.9 ng DNA/µL
B	50 µL of anonymous male genomic DNA	1.3 – 1.5 ng DNA/µL
C	50 µL of anonymous male genomic DNA	1.3 – 2.0 ng DNA/µL
D	50 µL of mixed-source (Components A and C)	1.4 – 2.0 ng DNA/µL
E	Two 6 mm punches of CRL-1486 cells spotted on 903 paper	7.5 x10^4 cells per punch
F	Two 6 mm punches of HTB-157 cells spotted on FTA paper	7.5 x10^4 cells per punch

[a] DNA concentrations and cell counts are nominal values and are **not** intended for use as quantitative standards.

STR Genotyping kits and primer mixes used at NIST to certify SRM 2391c

Kit Provider			Primer Mixes
Life Technologies	*Promega*	*Qiagen*	*NIST*
Identifiler	Powerplex 16	ESSplex	26plex
Identifiler Plus	Powerplex 16 HS	IDplex	miniSTRs
NGM	Powerplex ESX 17		
NGM SElect	Powerplex ESI 17		
COfiler	Powerplex ES		
Profiler	Powerplex S5		
Profiler Plus	Powerplex Y		
Profiler Plus ID	FFFL		
SGM Plus			
SEfiler			
MiniFiler			
Yfiler			

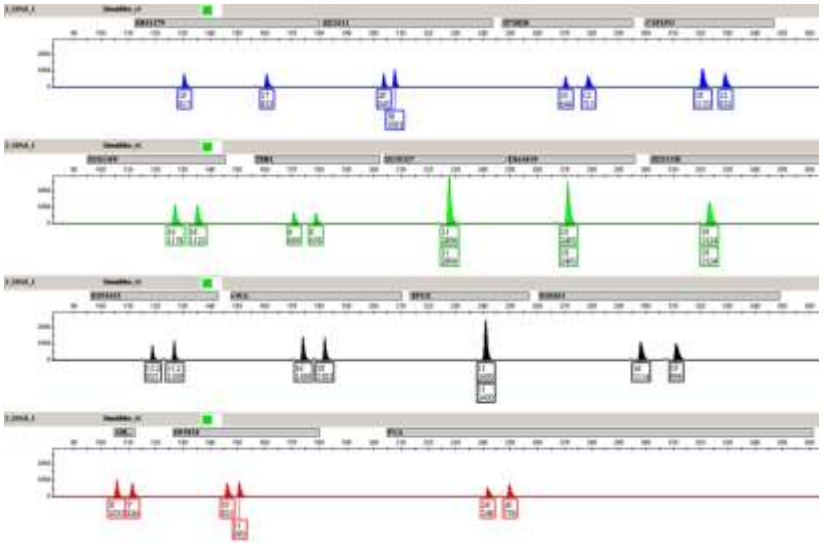
All results are concordant across all kits.

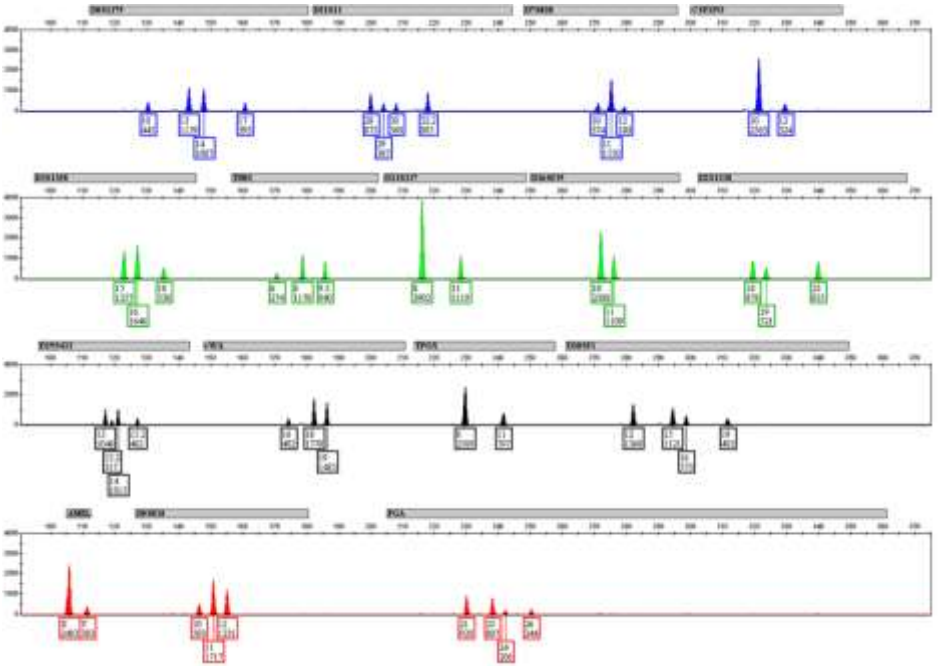
In total there is data for 51 autosomal STRs and 17 Y-STRs

Component D

3 part A

1 part C





The certified ratio for Component D, the mass of Component A relative to that of Component C, is **3.1 ± 0.1 Component A / Component C.**

Acknowledgments

Forensic DNA Team


John Butler


Mike Coble


Becky Hill


Margaret Kline

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DNA Biometrics Team


Pete Vallone


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Jeff Sailus with Applied Biosystems

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Presentation made available on STRbase