NIST Validation Studies of the 3500 Genetic Analyzer

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

Erica L.R. Butts, Carolyn R. Hill, Margaret C. Kline, David L. Duewer, John M. Butler, Kristen L. O'Connor, Michael D. Coble, Peter M. Vallone

National Institute of Standards and Technology

Mid-Atlantic Association of Forensic Scientists
May 27, 2011

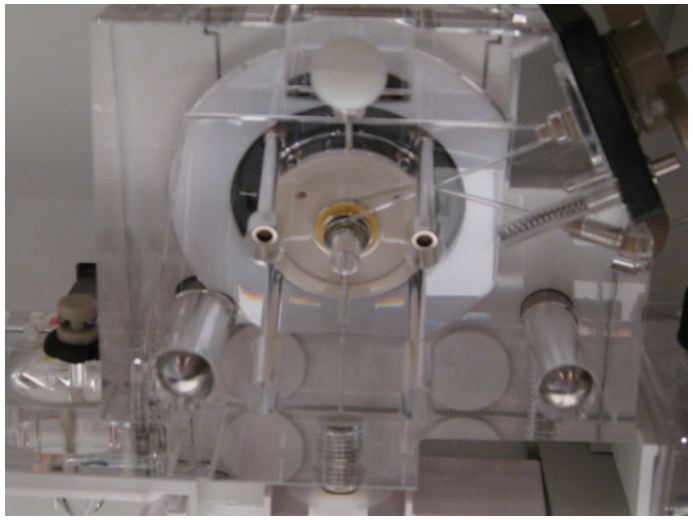
Information on ABI Genetic Analyzers

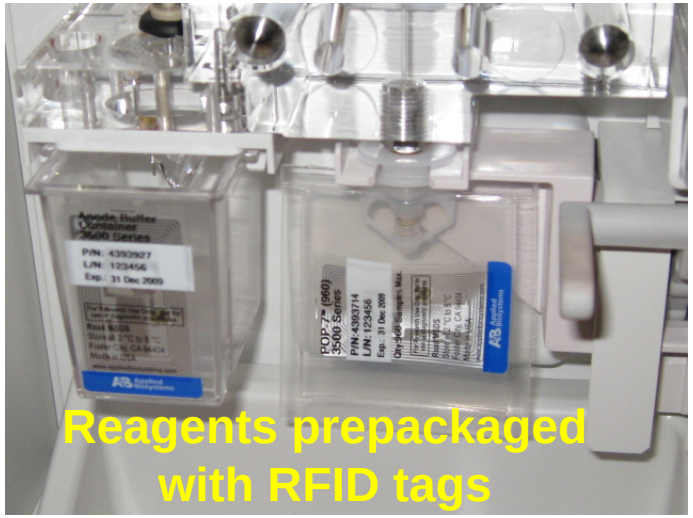
ABI Genetic Analyzer	Years Released for Human ID	Number of Capillaries	Laser	Polymer delivery	Other features
No ABI 3100/3100-Avant instruments after Dec 31, 2011 **No more sales of ABI 3130/3130xl after June 30, 2011**					
310	1995-	1	10 mW Ar+ (488/514 nm)	syringe	Mac operating system & Windows NT (later)
3100	2000-2005	16	25 mW Ar+ (488/514 nm)	syringe	
3100-Avant	2002-2007	4	25 mW Ar+ (488/514 nm)	syringe	
3130	2003-2011	4	25 mW Ar+ (488/514 nm)	pump	
3130xl	2003-2011	16	25 mW Ar+ (488/514 nm)	pump	
3500	2010 -	8	10-25 mW diode (505 nm)	new pump	110V power; RFID-tagged reagents; normalization & 6-dye detection possible
3500xl	2010 -	24			
3700	2002-2003	96	25 mW Ar+ (488/514 nm)	cuvette-based	Split beam technology
3730	2005-	48	25 mW Ar+ (488/514 nm)	pump	
3730xl	2005-	96	25 mW Ar+ (488/514 nm)	pump	

Table 6.1, *Advanced Topics in Forensic DNA Typing: Methodology* (J.M. Butler, 2011). Information courtesy of Michelle S. Shepherd, Applied Biosystems, LIFE Technologies.

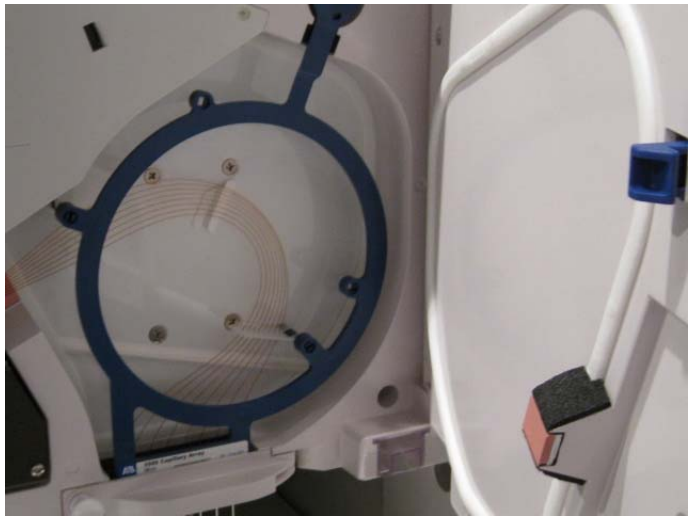
Details of the new ABI 3500

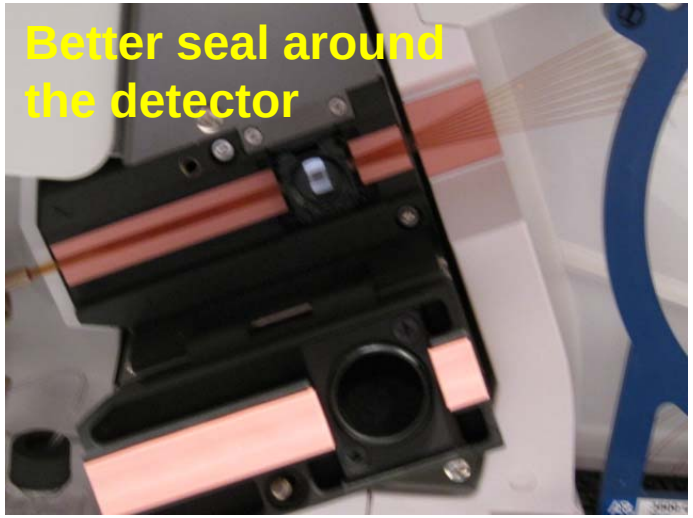
No lower pump block
(less polymer waste)





Improved sealing for better temperature control





Primary Differences Between 31xx and 3500

31xx Instruments

- Argon ion (Ar+) lasers with 488/514 nm wavelengths for fluorescence excitation
- 220V power requirement
- Optimal signal intensity 1500-3000 RFU
- Currently validated and operational in most forensic laboratories

3500 Instruments

- Single-line 505 nm, solid-state long-life laser
- Smaller footprint
- 110V power requirement
- Optimal signal intensity can approach 20,000-30,000 RFU
- Normalization of instrument-to-instrument signal variability
  - Ability to increase or decrease overall signal
- Requires the use of GeneMapper IDX 1.2

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

Aspects of Validation

- Reliability
  - Size Standard Comparison
    - What is the difference between LIZ 500 and LIZ 600 v2.0?
  - Injection Parameters
    - What are the best injection parameters for typable data?
    - Adjustment alongside PCR reaction volume?
- Reproducibility
  - Precision
    - Is the precision comparable to the 3130*xl*?
  - Concordance
    - Are the correct allele calls being made?
- Robustness
  - Sensitivity
    - How do the thresholds affect the analysis of data?
  - Mixtures
    - How often is the minor component identified?

Experimental Summary

Test	Types of Samples Used	Number Examined
Size Standard Comparison	16 Allelic Ladders per size standard (LIZ 500 vs. LIZ 600 v2.0)	32
Injection Parameters	3 samples heterozygous at all loci including Amelogenin 1 ng DNA input	3 samples/injection time Total 15 samples
Precision	Allelic Ladders	24
	3 samples heterozygous at all loci including Amelogenin	6
Sensitivity	Dilution series of 3 samples heterozygous at all loci including Amelogenin	4 Replicates of each dilution series Total 84 samples
Concordance	50 genomic DNA samples	60
	SRM 2391b: 10 genomic DNA samples	
Mixtures	Mixture dilution series of 2 samples heterozygous at all loci including Amelogenin	28
	Total Number of Samples	**249**

Identical experiments for Identifiler and Identifiler Plus

Reaction Setup

Identifiler

- PCR Volume: 12.5 µL
  - Primer Mix
  - Master Mix
  - Taq Gold Polymerase
- 28 cycles
- 1 ng DNA target input unless otherwise stated

Identifiler Plus

- PCR Volume: 12.5 µL
  - Primer Mix
  - Master Mix
  - No separate Taq/enzyme added
- 28 cycles
- 1 ng DNA target input unless otherwise stated

Identifiler Plus is optimized to overcome inhibition with an improved buffer mix
Cleaner baseline and improved heterozygote peak balance

Sample Preparation for Capillary Electrophoresis

- 17.4 µL HiDi + 0.6 µL LIZ 600 v2.0 per sample
- 2 µL Sample or Allelic Ladder
- Centrifuge for 1 min
- Aliquot 10 µL into a separate plate
  - Centrifuge both plates 1 min
- Plates run on 3130*xl* and 3500 simultaneously

n=16 Allelic Ladders

Size Standard Comparison

	1	2
A	LIZ 500	LIZ 600 v2.0
B	LIZ 600 v2.0	LIZ 500
C	LIZ 500	LIZ 600 v2.0
D	LIZ 600 v2.0	LIZ 500
E	LIZ 500	LIZ 600 v2.0
F	LIZ 600 v2.0	LIZ 500
G	LIZ 500	LIZ 600 v2.0
H	LIZ 600 v2.0	LIZ 500

Individual master mixes created for LIZ 500 and LIZ 600 v2.0 with Identifiler/Identifiler Plus allelic ladders

Injected twice on 3130*xl*

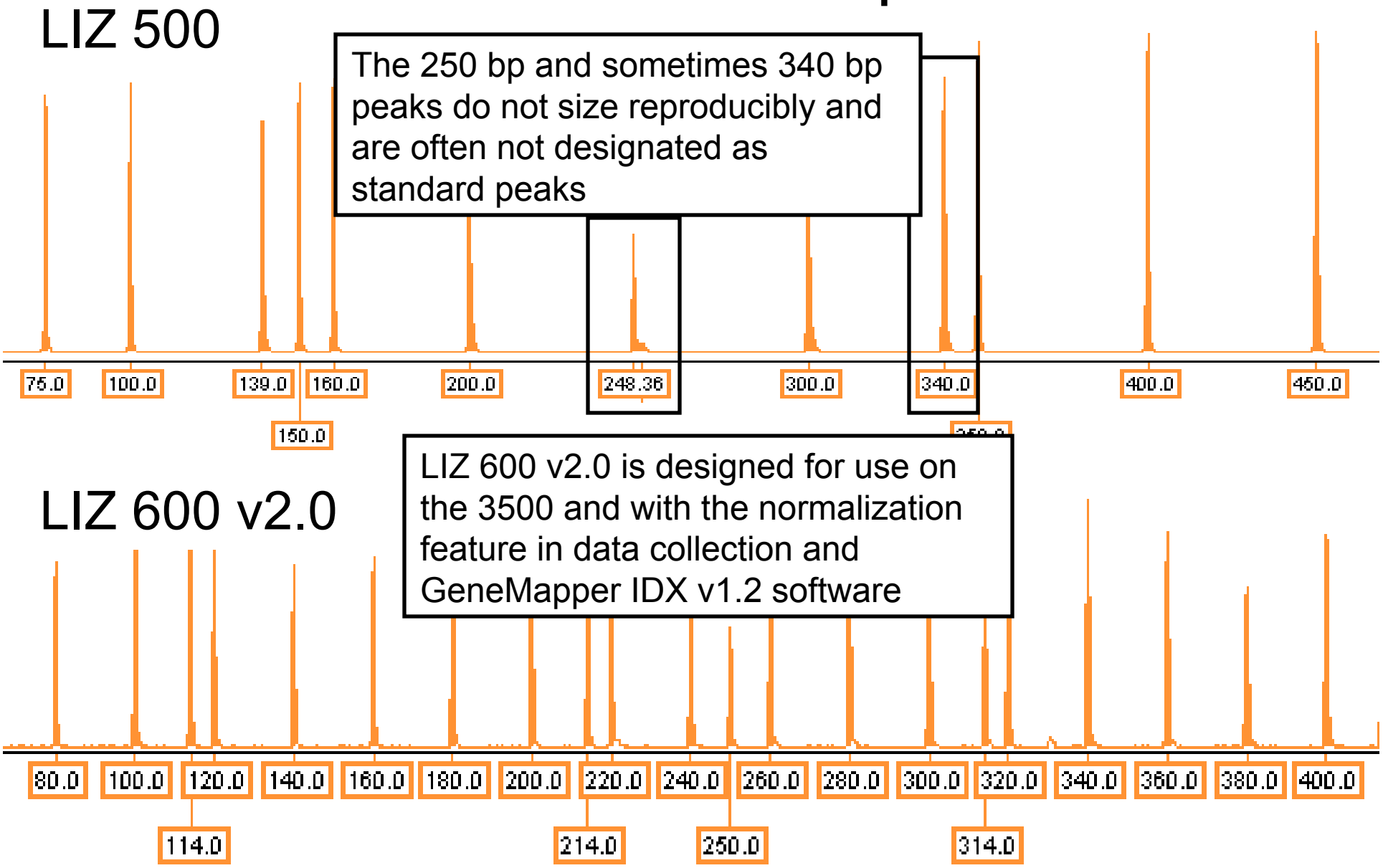
- Standard injection of 3 kV for 10 seconds

Injected 3 times on 3500

- Default Injection of 1.2 kV for 15 seconds

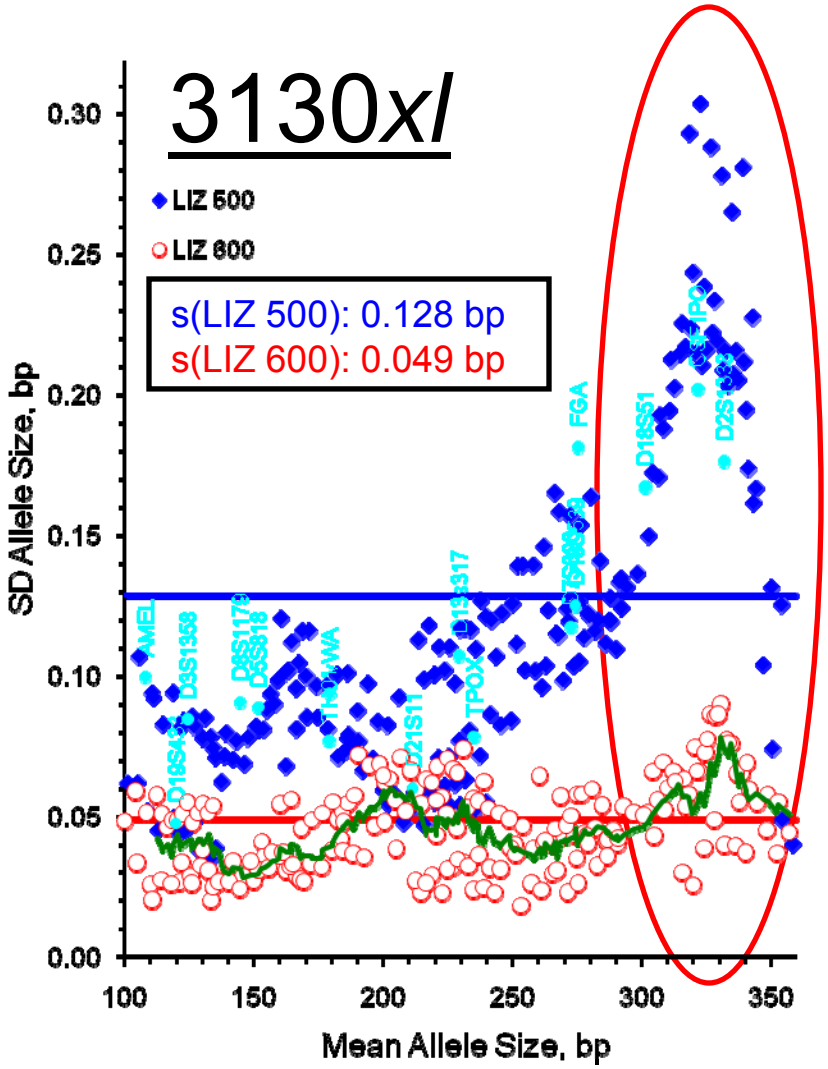
It is important to determine if one size standard can be used consistently on both the 3130xl and 3500 for proper comparison

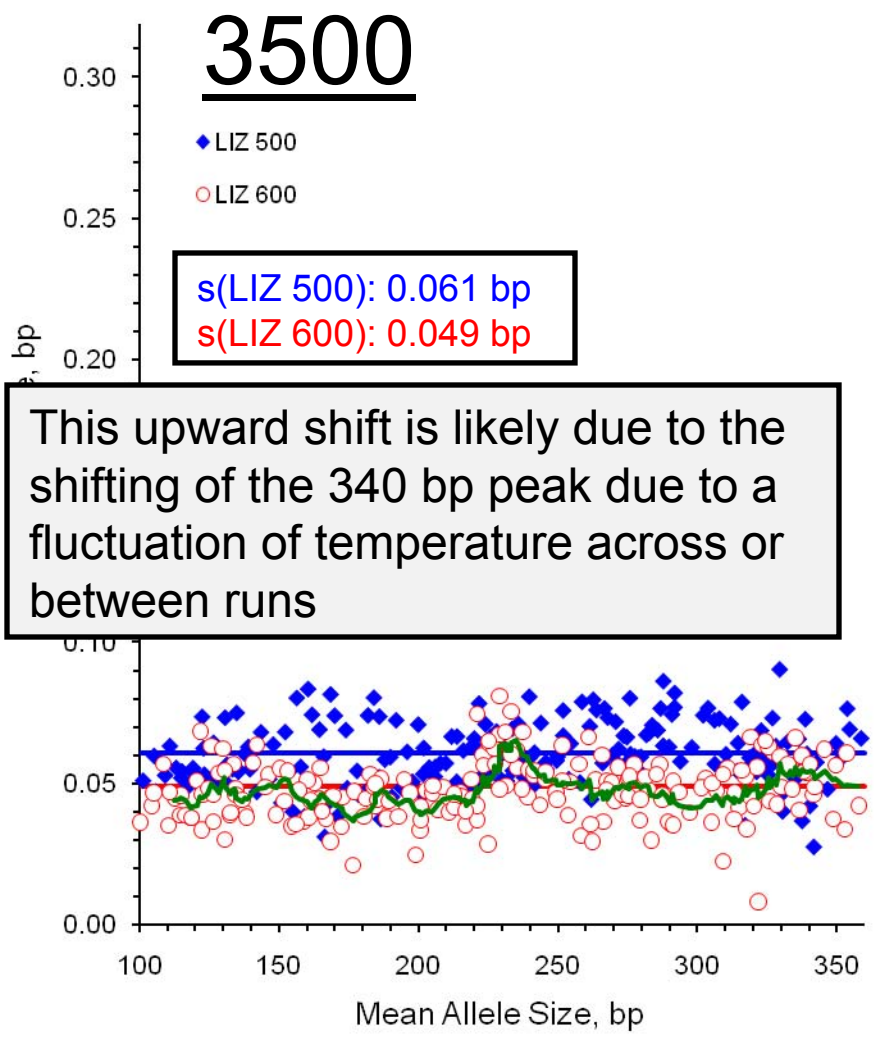
Size Standard Comparison



n=16 Allelic Ladders

Size Standard Comparison





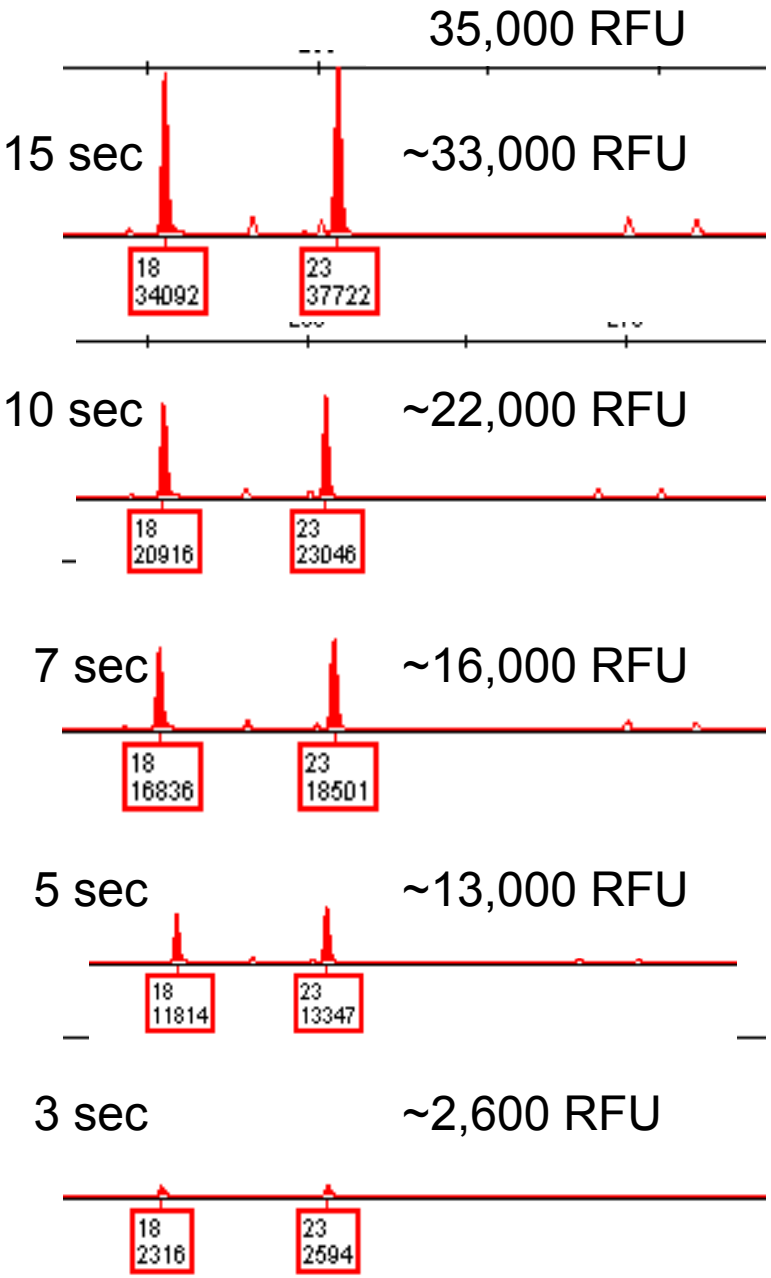
This upward shift is likely due to the shifting of the 340 bp peak due to a fluctuation of temperature across or between runs

LIZ 600 v2.0 generated the most linear results on both the 3130*xl* and 3500 and was used as the size standard on both instruments for remaining testing

n=20: Identifiler n=15: Identifiler Plus

Injection Parameters

- Injection voltage/time:
  - 1.2 kV for 15 sec
  - 1.2 kV for 10 sec
  - 1.2 kV for 7 sec ➡ Identifiler
  - 1.2 kV for 5 sec ➡ Identifiler Plus
  - 1.2 kV for 3 sec



Standard injection parameters set based on samples with:

1. No pull-up present
2. No drop out present

n=24 Allelic Ladders, n=6 Samples

Sizing Precision

	Identifiler		Identifiler Plus	
	1	2	3	4
A	Identifiler	EB	Identifiler Plus	EB
B	Neg	Identifiler	Neg	Identifiler Plus
C	Identifiler	EB	Identifiler Plus	EB
D	Neg	Identifiler	Neg	Identifiler Plus
E	Identifiler	EB	Identifiler Plus	EB
F	Neg	Identifiler	Neg	Identifiler Plus
G	Identifiler	Sample	Identifiler Plus	Sample
H	Sample	Identifiler	Sample	Identifiler Plus

Identifiler and Identifiler Plus allelic ladders in checkerboard pattern

Neg: PCR blank
- PCR primers + water

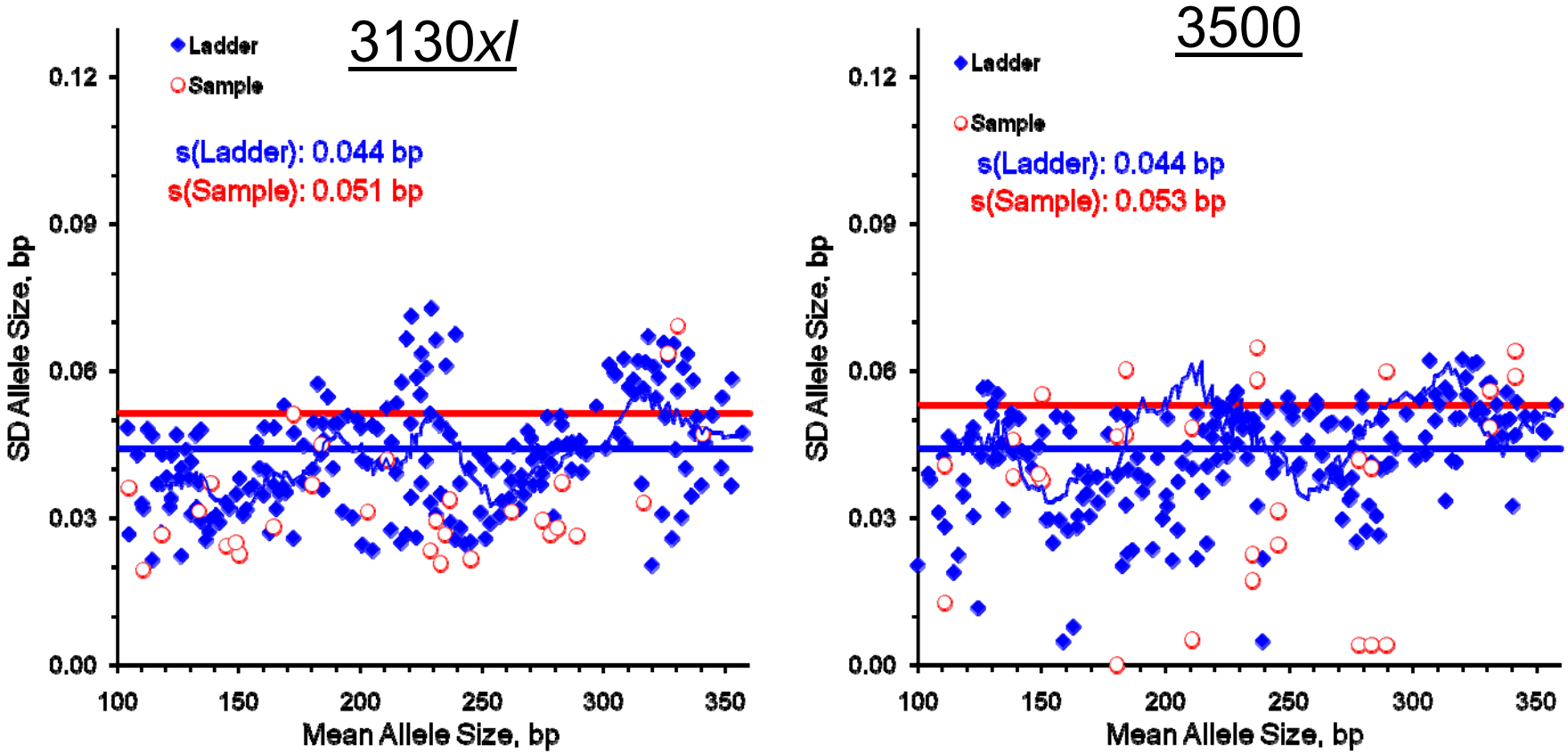
EB: Extraction blank
- PCR primers + extraction eluent

Sample: 1 ng heterozygous sample at 15 loci plus Amelogenin

Injected 3 times with the newly determined injection parameters

n=24: Allelic Ladders, n=6 Samples

Precision of Base Pair Sizing



No significant difference between 3130*xl* and 3500

No significant difference between Identifiler and Identifiler Plus

Setting Analytical Thresholds

- Analytical Threshold (AT)
  - Minimum threshold for data comparison and peak detection in the DNA typing process

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

- Several methods to calculate AT
  - 5 methods mentioned in Catherine Grgicak's module of ISHI 2010 Mixture Workshop

- AT values calculated using negative controls
  - Analyze with threshold set at 1 RFU
  - Calculate average RFU noise per dye channel

Methods For Calculation

- Method 1: Average RFU + 3 Standard Deviations
- Method 2: Average RFU + $t_{1-\alpha,v}$ from student t-table x Standard Deviation
- Method 3: 2 x ($Y_{max} - Y_{min}$)
- Method 4 & 5: Dilution series of positive controls
  - Data Not Shown
- Method 6: Average RFU + 10 Standard Deviations

Calculations Using Negative Controls

Identifiler										
	Average RFU	Stdev	Min RFU	Max RFU	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6
Blue	9	3	2	22	19	19	44			42
Green	13	4	5	27	24	23	54			49
Yellow	20	5	8	31	35	34	62			69
Red	27	7	10	50	49	48	100			99

Identifiler Plus										
	Average RFU	Stdev	Min RFU	Max RFU	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6
Blue	9	3	3	20	18	18	40			39
Green	13	3	4	26	23	23	52			47
Yellow	20	5	7	37	36	35	74			72
Red	28	7	11	54	49	48	108			99

Tentative analytical threshold set for Identifiler: 100 RFU

Tentative analytical threshold set for Identifiler Plus: 100 RFU

Tentative Analytical Threshold Summary

	3130*xl*	**3500**
Identifier	50 RFU Data Not Shown	100 *or* 150 RFU Data Not Shown
Identifiler Plus	50 RFU Data Not Shown	100 *or* 150 RFU Data Not Shown

Setting Stochastic Thresholds

Stochastic Threshold (ST)

- Detection level on an instrument (31xx or 3500) where a potential sister allele of detected peak may fall below the analytical threshold
- The value above which it is reasonable to assume that allelic dropout of a sister allele has not occurred

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

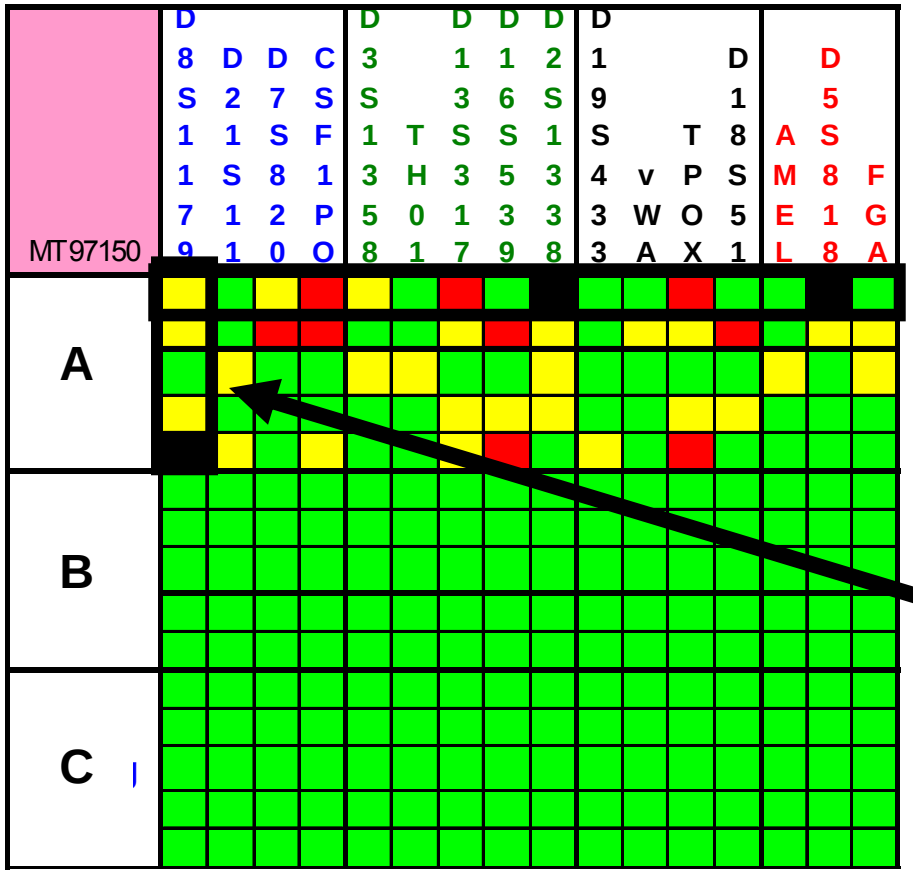
Setting Stochastic Thresholds

- Dilution series of three heterozygous samples at 15 loci plus Amelogenin to evaluate where drop out is first observed
- Total DNA input: 1.0 ng, 0.5 ng, 0.25 ng, 0.10 ng, 50 pg, 30 pg,10 pg in 4 replicates
- Determine RFU value of highest surviving false homozygous peak
  - RFU value is then rounded up to the nearest 50 to set the stochastic threshold

Heat Map Explanation

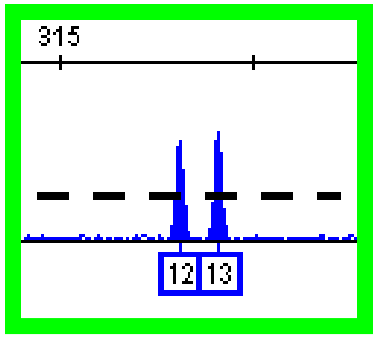
Results broken down by locus

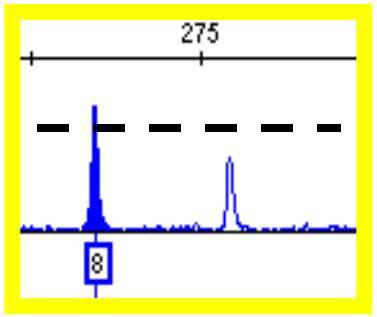
Green = full (correct) type
Yellow = allele dropout
Red = locus dropout
Black = drop-in

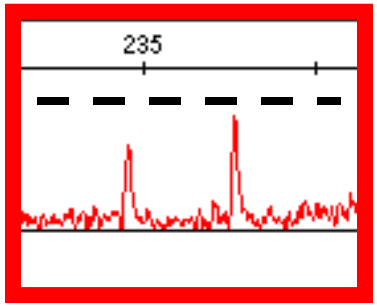


A single profile slice

A replicate slice







This is an easy way to look at a lot of data at once

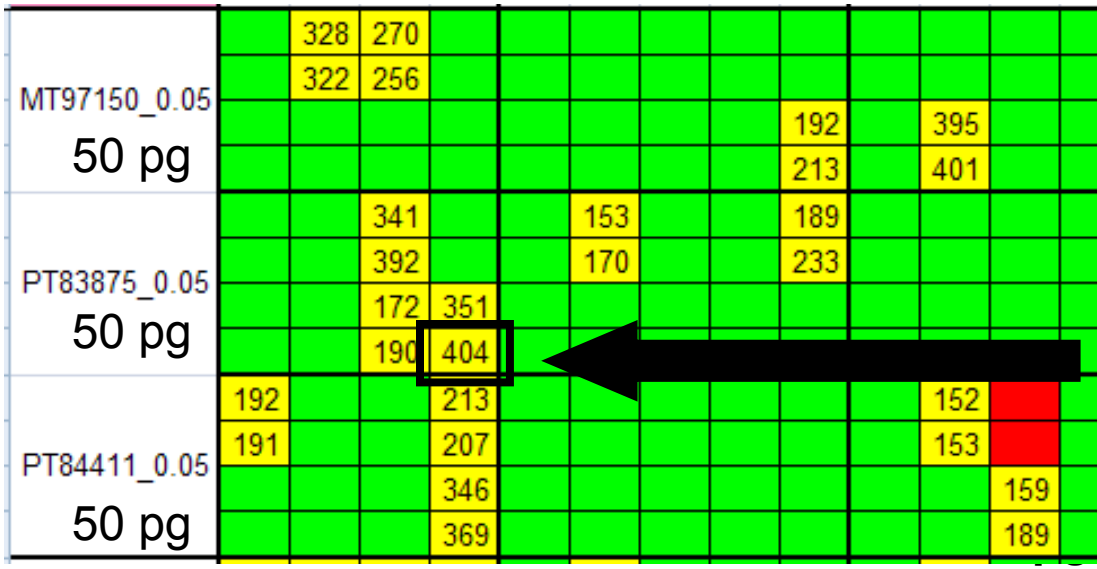
n=84 Samples

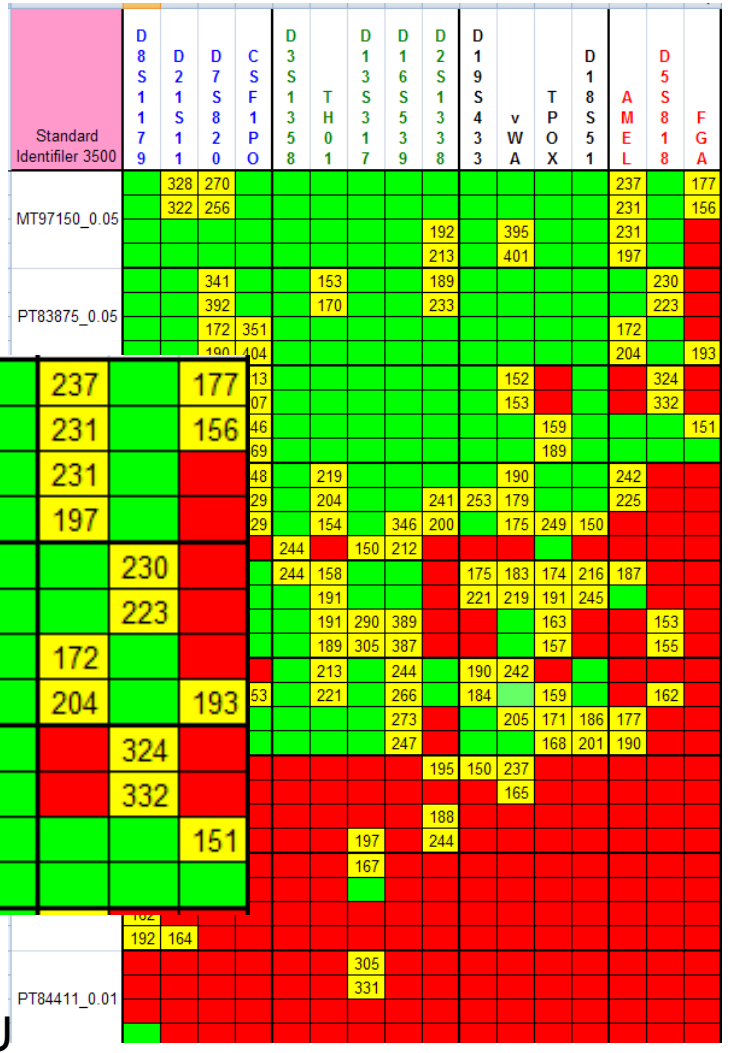
Stochastic Threshold

Identifiler: 28 cycles

Standard Injection on 3500:
7 sec @ 1.2 kV inj

50 pg





Highest peak height of false homozygote = 404 RFU

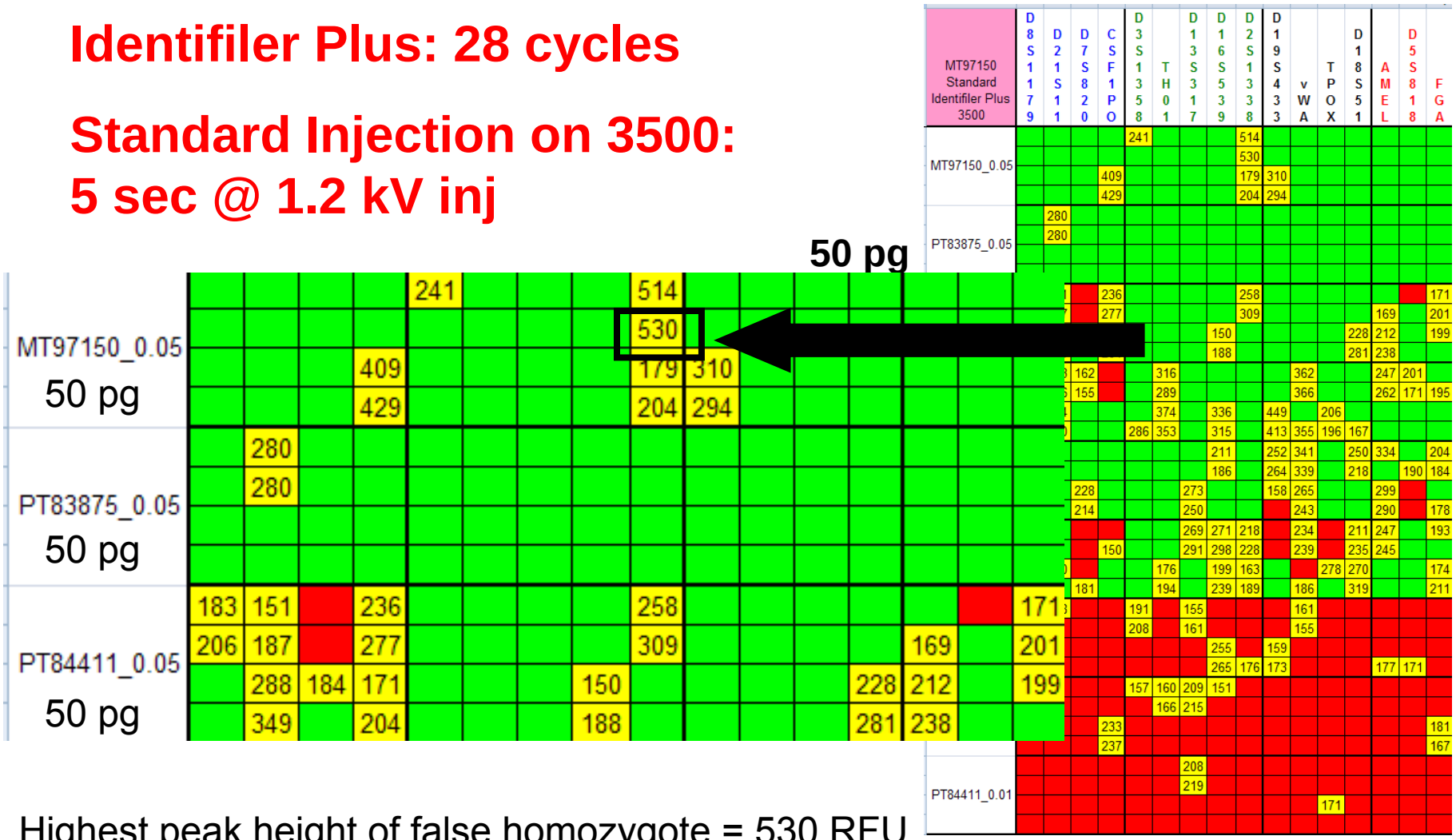
Stochastic Threshold for this data set = 450 RFU

n=84 Samples

Stochastic Threshold

Identifiler Plus: 28 cycles

Standard Injection on 3500:
5 sec @ 1.2 kV inj



Highest peak height of false homozygote = 530 RFU

Stochastic Threshold for this data set = 550 RFU

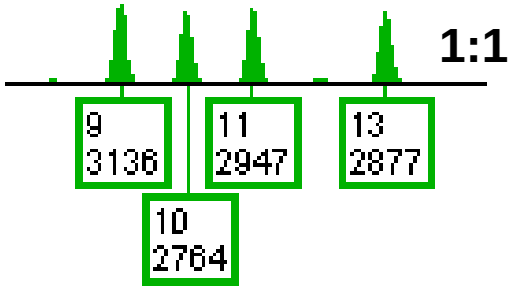
Concordance

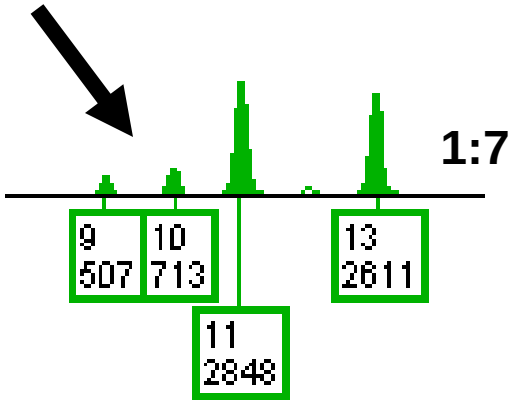
- 50 unique male blood samples
- SRM 2391b: 10 genomic DNA Samples
- All 60 samples concordant between 3130*xl* and 3500
- Total of 1689 alleles examined

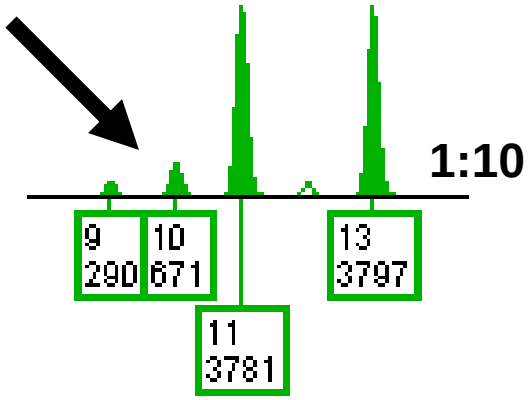
n=28 Mixtures

Mixture Experimental Design

	1	2	3	4	5	6	7	8
A	Ladder	Ladder	Ladder	Ladder	Ladder	Ladder	Ladder	Ladder
B	1:1	1:1	1:1	1:1	1:1	1:1	1:1	1:1
C	2:1	2:1	1:2	1:2	2:1	2:1	1:2	1:2
D	3:1	3:1	1:3	1:3	3:1	3:1	1:3	1:3
E	5:1	5:1	1:5	1:5	5:1	5:1	1:5	1:5
F	7:1	7:1	1:7	1:7	7:1	7:1	1:7	1:7
G	9:1	9:1	1:9	1:9	9:1	9:1	1:9	1:9
H	10:1	10:1	1:10	1:10	10:1	10:1	1:10	1:10
	Identifiler				Identifiler Plus			







- 2 samples heterozygous at 15 loci plus Amelogenin
- Mixture ratios from 1:1 to 1:10 (and inverse)
- Samples were injected twice

Minor component identified correctly in a 1:10 mixture ratio

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 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

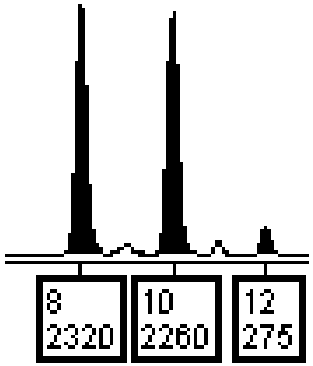
Normalization Example

Theoretical Normalization Target: 2000 RFU

Without Normalization

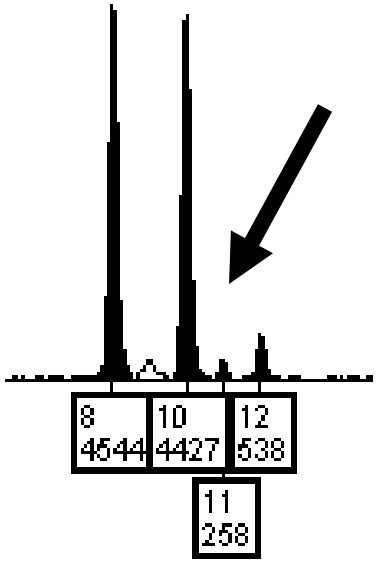
With Normalization

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

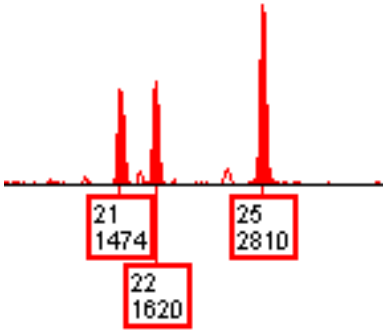


NF=1.959

Signal increases almost by a factor of 2

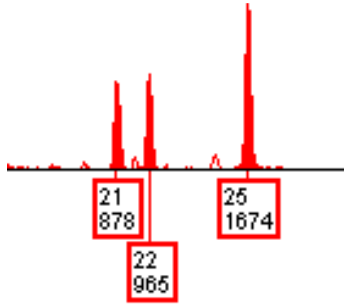


LIZ 600 v2.0 Peak Height Average: **3192 RFU**



NF= 0.596

Signal decreases by almost half



Conclusions

- The 3500 has proven to be reliable, reproducible and robust
  - Out of 498 samples between Identifiler and Identifiler Plus only 5 required reinjection
  - Precision within about 0.05 base pairs
- Tentative Analytical Threshold: 100 or 150 RFU
- Tentative Stochastic Threshold: 450 RFU (Identifiler) or 550 RFU (Identifiler Plus)
- Minor contributor successfully identified in as low as a 1:10 mixture

Future Work

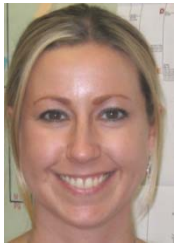
- Validation of additional kits (PowerPlex 16 HS)
- More extensive review of thresholds and how they are applied
  - What is the impact of dye specific thresholds?
  - Impact on interpretation
- More extensive review of normalization
  - Do thresholds change when employing normalization?

Acknowledgments

Forensic DNA Team


John Butler


Mike Coble


Becky Hill


Margaret Kline

Data Analysis Support


Dave Duewer

DNA Biometrics Team


Pete Vallone


Kristen Lewis O'Connor

Funding from the **National Institute of Justice (NIJ)** through NIST Office of Law Enforcement Standards

Funding from the **FBI S&T Branch** through NIST Information Access Division

Contact Info:

erica.butts@nist.gov

301-975-5107