NIST Validation Studies of the 3500 Genetic Analyzer

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National Institute of Standards and Technology

Mid-Atlantic Association of Forensic Scientists
May 27, 2011
### Information on ABI Genetic Analyzers

<table>
<thead>
<tr>
<th>ABI Genetic Analyzer</th>
<th>Years Released for Human ID</th>
<th>Number of Capillaries</th>
<th>Laser</th>
<th>Polymer delivery</th>
<th>Other features</th>
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<tbody>
<tr>
<td>310</td>
<td>1995-</td>
<td>1</td>
<td>10 mW Ar+ (488/514 nm)</td>
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<td>Mac operating system &amp; Windows NT (later)</td>
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<tr>
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<td>25 mW Ar+ (488/514 nm)</td>
<td>syringe</td>
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<td>3130</td>
<td>2003-2011</td>
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<td>pump</td>
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<tr>
<td>3130xI</td>
<td>2003-2011</td>
<td>16</td>
<td>25 mW Ar+ (488/514 nm)</td>
<td>pump</td>
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<tr>
<td>3500</td>
<td>2010-</td>
<td>8</td>
<td>10-25 mW diode (505 nm)</td>
<td>new pump</td>
<td>110V power; RFID-tagged reagents; normalization &amp; 6-dye detection possible</td>
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<tr>
<td>3500xI</td>
<td>2010-</td>
<td>24</td>
<td>new pump</td>
<td></td>
<td></td>
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<tr>
<td>3700</td>
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<td>cuvette-based</td>
<td>Split beam technology</td>
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<tr>
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<td>96</td>
<td>25 mW Ar+ (488/514 nm)</td>
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**Table 6.1, Advanced Topics in Forensic DNA Typing: Methodology (J.M. Butler, 2011).** Information courtesy of Michelle S. Shepherd, Applied Biosystems, LIFE Technologies.

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No ABI 3100/3100-Avant instruments after Dec 31, 2011
No more sales of ABI 3130/3130xI after June 30, 2011
Details of the new ABI 3500

No lower pump block
(less polymer waste)

Improved sealing for better temperature control

Reagents prepackaged with RFID tags

Better seal around the detector
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 3130xl?
  - 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

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

**Total Number of Samples** 249

Identical experiments for **Identifiler** and **Identifiler Plus**
# Reaction Setup

<table>
<thead>
<tr>
<th><strong>Identifiler</strong></th>
<th><strong>Identifiler Plus</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>• PCR Volume: 12.5 µL</td>
<td>• PCR Volume: 12.5 µL</td>
</tr>
<tr>
<td>– Primer Mix</td>
<td>– Primer Mix</td>
</tr>
<tr>
<td>– Master Mix</td>
<td>– Master Mix</td>
</tr>
<tr>
<td>– Taq Gold Polymerase</td>
<td>– No separate Taq/enzyme added</td>
</tr>
<tr>
<td>• 28 cycles</td>
<td>• 28 cycles</td>
</tr>
<tr>
<td>• 1 ng DNA target input unless otherwise stated</td>
<td>• 1 ng DNA target input unless otherwise stated</td>
</tr>
</tbody>
</table>

**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 3130xl and 3500 simultaneously
Size Standard Comparison

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

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>LIZ 500</td>
<td>LIZ 600 v2.0</td>
</tr>
<tr>
<td>B</td>
<td>LIZ 600 v2.0</td>
<td>LIZ 500</td>
</tr>
<tr>
<td>C</td>
<td>LIZ 500</td>
<td>LIZ 600 v2.0</td>
</tr>
<tr>
<td>D</td>
<td>LIZ 600 v2.0</td>
<td>LIZ 500</td>
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<tr>
<td>E</td>
<td>LIZ 500</td>
<td>LIZ 600 v2.0</td>
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<tr>
<td>F</td>
<td>LIZ 600 v2.0</td>
<td>LIZ 500</td>
</tr>
<tr>
<td>G</td>
<td>LIZ 500</td>
<td>LIZ 600 v2.0</td>
</tr>
<tr>
<td>H</td>
<td>LIZ 600 v2.0</td>
<td>LIZ 500</td>
</tr>
</tbody>
</table>

Injected twice on 3130xl:
- 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.

n=16 Allelic Ladders
Size Standard Comparison

LIZ 500

The 250 bp and sometimes 340 bp peaks do not size reproducibly and are often not designated as standard peaks

LIZ 600 v2.0

LIZ 600 v2.0 is designed for use on the 3500 and with the normalization feature in data collection and GeneMapper IDX v1.2 software
LIZ 600 v2.0 generated the most linear results on both the 3130x/ and 3500 and was used as the size standard on both instruments for remaining testing.

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

n=16 Allelic Ladders

Size Standard Comparison

LIZ 500: 0.128 bp
LIZ 600: 0.049 bp

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

LIZ 500: 0.061 bp
LIZ 600: 0.049 bp
Injection Parameters

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

Standard injection parameters set based on samples with:
1. No pull-up present
2. No drop out present
Sizing Precision

<table>
<thead>
<tr>
<th></th>
<th>Identifiler</th>
<th>Identifiler Plus</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Identifiler</td>
<td>EB</td>
</tr>
<tr>
<td>B</td>
<td>Neg</td>
<td>Identifiler</td>
</tr>
<tr>
<td>C</td>
<td>Identifiler</td>
<td>EB</td>
</tr>
<tr>
<td>D</td>
<td>Neg</td>
<td>Identifiler</td>
</tr>
<tr>
<td>E</td>
<td>Identifiler</td>
<td>EB</td>
</tr>
<tr>
<td>F</td>
<td>Neg</td>
<td>Identifiler</td>
</tr>
<tr>
<td>G</td>
<td>Identifiler</td>
<td>Sample</td>
</tr>
<tr>
<td>H</td>
<td>Sample</td>
<td>Identifiler</td>
</tr>
</tbody>
</table>

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 3130xl 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


- 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

“Mixture Interpretation: Principles, Protocols, and Practice” workshop at the 21st International Symposium on Human Identification (San Antonio, TX), October 11, 2010
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**: $2 \times (Y_{\text{max}} - Y_{\text{min}})$
- **Method 4 & 5**: Dilution series of positive controls – Data Not Shown
- **Method 6**: Average RFU + 10 Standard Deviations

“Mixture Interpretation: Principles, Protocols, and Practice” workshop at the 21st International Symposium on Human Identification (San Antonio, TX), October 11, 2010
Calculations Using Negative Controls

<table>
<thead>
<tr>
<th></th>
<th>Average RFU</th>
<th>Stdev</th>
<th>Min RFU</th>
<th>Max RFU</th>
<th>Method 1</th>
<th>Method 2</th>
<th>Method 3</th>
<th>Method 4</th>
<th>Method 5</th>
<th>Method 6</th>
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<tbody>
<tr>
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<td>9</td>
<td>3</td>
<td>2</td>
<td>22</td>
<td>19</td>
<td>19</td>
<td>44</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Green</td>
<td>13</td>
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<td>5</td>
<td>27</td>
<td>24</td>
<td>23</td>
<td>54</td>
<td></td>
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<td>34</td>
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<td>49</td>
<td>48</td>
<td>100</td>
<td></td>
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<table>
<thead>
<tr>
<th></th>
<th>Average RFU</th>
<th>Stdev</th>
<th>Min RFU</th>
<th>Max RFU</th>
<th>Method 1</th>
<th>Method 2</th>
<th>Method 3</th>
<th>Method 4</th>
<th>Method 5</th>
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<tr>
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<td>3</td>
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<td>108</td>
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<td>99</td>
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</tbody>
</table>

Tentative analytical threshold set for **Identifiler**: 100 RFU

Tentative analytical threshold set for **Identifiler Plus**: 100 RFU
<table>
<thead>
<tr>
<th>Identifier</th>
<th>Identifier Plus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>3130x/</strong></td>
<td><strong>3500</strong></td>
</tr>
<tr>
<td>50 RFU</td>
<td>100 or 150 RFU</td>
</tr>
<tr>
<td>Data Not Shown</td>
<td>Data Not Shown</td>
</tr>
</tbody>
</table>
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

Setting Stochastic Thresholds

• Dilution series of three heterozygous samples at 15 loci plus Amelogenin to evaluate where dropout 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

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

Identifier: 28 cycles

Standard Injection on 3500:
7 sec @ 1.2 kV inj

Highest peak height of false homozygote = 404 RFU

Stochastic Threshold for this data set = 450 RFU
**Stochastic Threshold**

**Identifier 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

n=84 Samples
Concordance

- 50 unique male blood samples
- SRM 2391b: 10 genomic DNA Samples
- All 60 samples concordant between 3130x/ and 3500
- Total of 1689 alleles examined
Mixture Experimental Design

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<td>Ladder</td>
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<td>Ladder</td>
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<td>Ladder</td>
<td>Ladder</td>
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<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
<td>1:1</td>
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</tr>
<tr>
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<td>2:1</td>
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<td>1:2</td>
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<td>1:2</td>
</tr>
<tr>
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<td>3:1</td>
<td>1:3</td>
<td>1:3</td>
<td>3:1</td>
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<td>1:10</td>
<td>10:1</td>
<td>10:1</td>
<td>1:10</td>
<td>1:10</td>
</tr>
</tbody>
</table>

- 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

n=28 Mixtures
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

NF = 1.959
Signal increases almost by a factor of 2

With Normalization

NF = 0.596
Signal decreases by almost half

LIZ 600 v2.0
Peak Height Average:
1021 RFU

LIZ 600 v2.0
Peak Height Average:
3192 RFU
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

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

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301-975-5107

**NIST Disclaimer:** Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

**Points of view are mine** and do not necessarily represent the official position of the National Institute of Standards and Technology.