NIST Validation Studies on the 3500 Genetic Analyzer

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Outline

• Details of the ABI 3500 Genetic Analyzer

• Validation design and results with Identifiler and Identifiler Plus
  – Injection parameters and reaction setup
  – Precision and size standard comparison
  – Concordance and mixture evaluation

• Methodology of setting analytical and stochastic thresholds
Details of the ABI 3500

- No lower pump block (Fewer air bubbles)
- Improved sealing for better temperature control
- Reagents prepackaged with RFID tags
- Improved seal around the detector

8-capillary instrument at NIST
## Primary Differences

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

SWGDAM: FBI Laboratory’s Scientific Working Group on DNA Analysis Methods
## 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 15 loci plus Amelogenin 1 ng DNA input</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 samples per injection</td>
</tr>
<tr>
<td><strong>Precision</strong></td>
<td>Allelic Ladders</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>3 samples heterozygous at all 15 loci plus Amelogenin</td>
<td>6</td>
</tr>
<tr>
<td><strong>Concordance</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><strong>Sensitivity</strong></td>
<td>Dilution series of 3 samples heterozygous at 15 loci plus Amelogenin</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4 replicates of each dilution series</td>
</tr>
<tr>
<td><strong>Mixtures</strong></td>
<td>Mixture dilution series of 2 samples heterozygous at 15 loci plus Amelogenin</td>
<td>28</td>
</tr>
<tr>
<td><strong>Total Number of Samples</strong></td>
<td></td>
<td>249</td>
</tr>
</tbody>
</table>

Identical experiments for **Identifiler** and **Identifiler Plus**
Injection parameters set for \( \frac{1}{2} \) PCR reactions at 28 cycles:
- Default: 1.2 kV for 15 s
- Identifiler: 1.2 kV for 7 s
- Identifiler Plus: 1.2 kV for 5 s

No significant difference between the LIZ500 and LIZ600 v2.0 size standards.
Validation Results: Reproducibility

• 60 samples concordant between 3130x/ and 3500
  – Total of 1689 alleles examined

• Precision of base pair sizing ±0.05 bp between allelic ladders and samples tested
  – No significant difference between the 3130x/ and 3500
  – No significant difference between Identifiler and Identifiler Plus
Validation Results: 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

Example values
(empirically determined based on own internal validation)

350 RFUs

 Called Peak
(Greater confidence a sister allele has not dropped out)

150 RFUs

 Called Peak
(Cannot be confident dropout of a sister allele did not occur)

Peak not considered reliable

Stochastic Threshold
The value above which it is reasonable to assume that allelic dropout of a sister allele has not occurred

Analytical Threshold
Minimum threshold for data comparison and peak detection in the DNA typing process

Noise

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)
Different Thresholds

Single thresholds for all dye channels assumes all dye channels have the same amount of noise.

Dye-specific thresholds take into consideration that all dye channels do not have the same level of noise.

Can increase the amount of data that is callable.
n=84 samples

## Analytical Threshold Calculation

<table>
<thead>
<tr>
<th>Dye Channel</th>
<th>Average RFU</th>
<th>Stdev</th>
<th>Min RFU</th>
<th>Max RFU</th>
<th>Calculated Noise (RFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td>9</td>
<td>8.4</td>
<td>1</td>
<td>66</td>
<td>93</td>
</tr>
<tr>
<td>Green</td>
<td>13</td>
<td>11.5</td>
<td>3</td>
<td>84</td>
<td>128</td>
</tr>
<tr>
<td>Yellow</td>
<td>22</td>
<td>11.6</td>
<td>4</td>
<td>88</td>
<td>138</td>
</tr>
<tr>
<td>Red</td>
<td>28</td>
<td>8.8</td>
<td>10</td>
<td>80</td>
<td>116</td>
</tr>
</tbody>
</table>

### Identity Plus

<table>
<thead>
<tr>
<th>Dye Channel</th>
<th>Average RFU</th>
<th>Stdev</th>
<th>Min RFU</th>
<th>Max RFU</th>
<th>Calculated Noise (RFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td>10</td>
<td>4.6</td>
<td>3</td>
<td>68</td>
<td>55</td>
</tr>
<tr>
<td>Green</td>
<td>16</td>
<td>5.6</td>
<td>3</td>
<td>78</td>
<td>72</td>
</tr>
<tr>
<td>Yellow</td>
<td>24</td>
<td>7.9</td>
<td>7</td>
<td>63</td>
<td>103</td>
</tr>
<tr>
<td>Red</td>
<td>31</td>
<td>8.9</td>
<td>7</td>
<td>81</td>
<td>120</td>
</tr>
</tbody>
</table>

- Statistical difference was calculated between dye channels using a z-test
- Statistically each dye channel is different for both `Identifier` and `Identifier Plus`
  - Must be treated independently
n=560 alleles

Threshold Comparison

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

14.8% of the total possible allele calls were lost using a single threshold rather than using dye-specific thresholds with **Identifiler**

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

- Analyzed data from the sensitivity study (DNA dilution series) analyzed with dye specific analytical thresholds.

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

- **Stochastic Threshold**: The RFU value of highest surviving false homozygous peak per dye channel.
n=84 samples

## Summary of Thresholds

### Identifier: 7 sec @ 1.2 kV (28 cycles)

<table>
<thead>
<tr>
<th></th>
<th>AT (RFU)</th>
<th>Highest Surviving Peak (RFU)</th>
<th>ST (RFU)</th>
<th>Lowest Expected PHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td>95</td>
<td>344</td>
<td>345</td>
<td>28%</td>
</tr>
<tr>
<td>Green</td>
<td>130</td>
<td>435</td>
<td>435</td>
<td>30%</td>
</tr>
<tr>
<td>Yellow</td>
<td>140</td>
<td>409</td>
<td>410</td>
<td>34%</td>
</tr>
<tr>
<td>Red</td>
<td>120</td>
<td>309</td>
<td>310</td>
<td>39%</td>
</tr>
</tbody>
</table>

### Identifier Plus: 5 sec @ 1.2 kV (28 cycles)

<table>
<thead>
<tr>
<th></th>
<th>AT (RFU)</th>
<th>Highest Surviving Peak (RFU)</th>
<th>ST (RFU)</th>
<th>Lowest Expected PHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td>55</td>
<td>288</td>
<td>290</td>
<td>19%</td>
</tr>
<tr>
<td>Green</td>
<td>75</td>
<td>383</td>
<td>385</td>
<td>19%</td>
</tr>
<tr>
<td>Yellow</td>
<td>105</td>
<td>414</td>
<td>415</td>
<td>25%</td>
</tr>
<tr>
<td>Red</td>
<td>120</td>
<td>265</td>
<td>265</td>
<td>45%</td>
</tr>
</tbody>
</table>

Both AT and ST values rounded to the nearest 5 RFU value.

Expected peak height ratio (PHR) is assuming the possibility of having one peak at the AT and one peak at the ST.

Expected PHR = AT/ST.
# Consumable RFID Tracking Limits

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

- The 3500 has proven to be reliable, reproducible and robust
  - Out of 498 samples between Identifiler and Identifiler Plus only 5 required reinjection

- Dye specific analytical thresholds resulted in less allelic and full locus dropout than applying one analytical threshold to all dyes

- 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

- RFID tracking decreases flexibility in our research experience
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