High-throughput Multiplex Newborn Screening Assay for Six Lysosomal Storage Disorders (LSDs) using Dried Blood Spots and UPLC-Tandem Mass Spectrometry

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Lysosomal Storage Disorder (LSD)

- Lysosome – an intracellular organelle containing many enzymes that degrade complex molecules.
- The absence or loss of function of an enzyme/protein along the pathway results in the accumulation of complex molecules that are normally degraded within lysosomes.
- The progressive accumulation of these products leads to cellular dysfunction and eventually causes tissue and organ dysfunction.
LSD (cont’d)

- LSD represents about 50 genetically heterogeneous disorders.
- Almost all LSDs are inherited as autosomal recessive traits except for the X-linked Fabry and Hunter diseases.
- Individually, the incidences of these diseases are rare. However, collectively, LSDs are far more common (1 in 8000)
- Pre-symptomatic diagnosis will be beneficial for babies.
LSD Testing Timeline

2007 – Legislative mandate for five LSDs (Krabbe, Pompe, Fabry, Gaucher, Niemann-Pick A/B)

2010 – Pilot screening for Pompe, Fabry & Gaucher using microfluidic platform
  o 8,012 DBS screened
  o Two had abnormal GAA activities, confirmed negative by second-tier tests
LSD Testing Timeline (cont’d)

2011 – Legislative mandate expanded to seven LSDs (addition of Hurler and Hunter), with the following provisions before screening:

- A method either cleared by the US Food and Drug Administration (FDA) or validated under the Clinical Laboratory Improvement Amendments (CLIA)
- Availability of quality control and proficiency testing materials
- Appropriate equipment for high-volume screening
- Adequate funding
2011 – Decision made to switch from microfluidic platform to tandem mass spectrometry
  o Microfluidic platform did not have substrates for all LSDs.
  o Microfluidic platform lacked throughput for Illinois’ volume (~170,000 newborns per year).
  o Recent developments with multiplex MS/MS promised adequate testing throughput for more disorders and with less staff.

2014 – Method validation and limited pilot testing

2015 – Statewide testing expected to begin 1ST quarter
Multiplex LC-MS/MS Assay

Modification of method developed at the University of Washington for six LSDs: Krabbe, Pompe, Fabry, Gaucher, Niemann-Pick (A/B), Hurler (MPS I).

- Single DBS punch
- Single buffer
- In-line chromatographic purification (no solid-phase or liquid extraction)
  - Three-hour incubation (maintains work flow).
  - UPLC column separates product/ISTD pairs and removes salt, detergent, & phospholipids by valving.
  - 2.5 minute injection cycle, 500 injections/instrument/day, >10,000 injections/PM.
# 6-Plex Assay

## Final Composition of Assay Cocktail & Assay Conditions*

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration/Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium formate</td>
<td>0.1 M, pH 4.4</td>
</tr>
<tr>
<td>Sodium cholate</td>
<td>10 g/L</td>
</tr>
<tr>
<td>Acarbose</td>
<td>0.08 M</td>
</tr>
<tr>
<td>N-Acetyl-α-galactoseamine</td>
<td>50 mM</td>
</tr>
<tr>
<td>IDUA Substrate (S), Internal Standard (IS)</td>
<td>500 µM, 3.5 µM</td>
</tr>
<tr>
<td>GLA S, IS</td>
<td>600 µM, 1.2 µM</td>
</tr>
<tr>
<td>GAA S, IS</td>
<td>200 µM, 2.0 µM</td>
</tr>
<tr>
<td>ASM S, IS (d7-C6 Ceramide)</td>
<td>150 µM, 2.5 µM</td>
</tr>
<tr>
<td>GALC S, IS (d7-C8 Ceramide)</td>
<td>450 µM, 2.5 µM</td>
</tr>
<tr>
<td>ABG S, IS (d7-C12 Ceramide)</td>
<td>300 µM, 2.5 µM</td>
</tr>
</tbody>
</table>


- Reaction was quenched with 200 µL acetonitrile (ACN) and centrifuged for 5 min at 1000 x g.
- 100 µL top layer was transferred to a glass-lined plate, and 100 µL MS-grade water was added to each well.

* * *
Acquity TQD Instrument
### Retention Times (RT, min) for Substrates and Products of GAA, GALC, and ABG

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate RT</th>
<th>Product RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAA</td>
<td>0.53</td>
<td>0.59</td>
</tr>
<tr>
<td>GALC</td>
<td>0.86</td>
<td>0.96</td>
</tr>
<tr>
<td>ABG</td>
<td>1.08</td>
<td>1.23</td>
</tr>
</tbody>
</table>
UPLC Chromatogram

Abnormalities in the Chromatogram:
- ABG-IS
- ABG-P
- GALC-IS
- GALC-P
- ASM-IS
- ASM-P
- GAA-IS
- GAA-P
- GLA-P
- GLA-IS
- IDUA-P
- IDUA-IS

Date: 28-Mar-2013
Time: 19:36:46
DBS Median Activities for Six Enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>DBS w/o S &amp; IS</th>
<th>S &amp; IS w/o DBS</th>
<th>S &amp; IS w/ filter</th>
<th>DBS w/ S &amp; IS, 3h</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDUA</td>
<td>0</td>
<td>0.28</td>
<td>0.3</td>
<td>8.55</td>
</tr>
<tr>
<td>GLA</td>
<td>0</td>
<td>0.02</td>
<td>0.05</td>
<td>5.56</td>
</tr>
<tr>
<td>GAA</td>
<td>0</td>
<td>0.02</td>
<td>0.05</td>
<td>6.04</td>
</tr>
<tr>
<td>ASM</td>
<td>0</td>
<td>0.03</td>
<td>0.02</td>
<td>5.45</td>
</tr>
<tr>
<td>GALC</td>
<td>0</td>
<td>0.05</td>
<td>0.05</td>
<td>1.29</td>
</tr>
<tr>
<td>ABG</td>
<td>0</td>
<td>0.15</td>
<td>0.15</td>
<td>17.98</td>
</tr>
</tbody>
</table>
Method Validation

• Evaluate different levels of Quality Control samples (Low, Medium, and High).
• Perform precision studies.
• Perform accuracy studies.
• Participate in the CDC pilot Proficiency Testing (PT) program for Pompe (and Krabbe).
• Obtain DBSs from confirmed cases.
Method Validation (cont’d)

• Test de-identified specimens from male, female, low birth weight, and 7+ day-old babies.
• Study the effects of detergents and DBS storage conditions on LSD enzyme activities.
• Determine cut-off values.
• Exchange specimens with a qualified testing laboratory to establish comparability of results.
CDC QC levels for IDUA, GLA & GAA

(Hurler) (Fabry) (Pompe)

MPSI activity (umoles/L/h)

GLA activity (umoles/L/h)

GAA activity (umoles/L/h)

- MPSI: $y = 10.938x + 1.7834, R^2 = 0.9609$
- GLA: $y = 7.964x + 0.5126, R^2 = 0.9974$
- GAA: $y = 2.5388x + 0.3253, R^2 = 0.9792$
CDC QC Levels for ASM, GALC & ABG

(Niemann-Pick) (Krabbe) (Gaucher)

ASM activity (umoles/L/h)

\[ y = 1.2969x - 0.0069 \]
\[ R^2 = 0.9841 \]

GALC activity (umoles/L/h)

\[ y = 2.4346x + 0.1701 \]
\[ R^2 = 0.9679 \]

ABG activity (umoles/L/h)

\[ y = 6.7138x + 0.5456 \]
\[ R^2 = 0.9941 \]
Pompe Results for de-ID DBSs, Confirmed Cases, PTs, and Quality Controls
## Statistical Analysis of Pompe Assay Results for DBSs

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>De-identified residual DBS</td>
<td>10003</td>
<td>7.45</td>
<td>7.37</td>
<td>7.52</td>
<td>0.04</td>
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<tr>
<td>Confirmed Cases</td>
<td>3</td>
<td>0.58</td>
<td>0.31</td>
<td>0.85</td>
<td>0.06</td>
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<tr>
<td>CDC PT</td>
<td>4</td>
<td>0.88</td>
<td>0.76</td>
<td>1.00</td>
<td>0.04</td>
</tr>
<tr>
<td>Control Low</td>
<td>16</td>
<td>0.78</td>
<td>0.73</td>
<td>0.83</td>
<td>0.02</td>
</tr>
<tr>
<td>Control Medium</td>
<td>16</td>
<td>3.63</td>
<td>3.40</td>
<td>3.87</td>
<td>0.11</td>
</tr>
<tr>
<td>Control High</td>
<td>15</td>
<td>7.28</td>
<td>6.84</td>
<td>7.73</td>
<td>0.21</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Min</th>
<th>Median</th>
<th>95% CI</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>De-identified residual DBS</td>
<td>10003</td>
<td>1.12</td>
<td>6.70</td>
<td>6.64</td>
<td>6.76</td>
</tr>
<tr>
<td>Confirmed Cases</td>
<td>3</td>
<td>0.48</td>
<td>0.57</td>
<td>N/A</td>
<td>0.69</td>
</tr>
<tr>
<td>CDC PT</td>
<td>4</td>
<td>0.77</td>
<td>0.90</td>
<td>N/A</td>
<td>0.94</td>
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<tr>
<td>Control Low</td>
<td>16</td>
<td>0.65</td>
<td>0.77</td>
<td>0.71</td>
<td>0.87</td>
</tr>
<tr>
<td>Control Medium</td>
<td>16</td>
<td>3.01</td>
<td>3.62</td>
<td>3.19</td>
<td>3.90</td>
</tr>
<tr>
<td>Control High</td>
<td>15</td>
<td>6.16</td>
<td>7.34</td>
<td>6.72</td>
<td>7.60</td>
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Krabbe Results for de-ID DBSs, Confirmed Cases, PTs, and Quality Controls
# Statistical Analysis of Krabbe Assay

## Results for DBSs

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Mean</th>
<th>95% CI</th>
<th>SE</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deidentified Residual DBS</td>
<td>12222</td>
<td>1.49</td>
<td>1.47 to 1.52</td>
<td>0.01</td>
<td>1.43</td>
</tr>
<tr>
<td>Confirmed Cases</td>
<td>7</td>
<td>0.14</td>
<td>0.10 to 0.18</td>
<td>0.02</td>
<td>0.04</td>
</tr>
<tr>
<td>CDC PT</td>
<td>5</td>
<td>0.12</td>
<td>0.09 to 0.14</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>PE Control Low</td>
<td>21</td>
<td>0.11</td>
<td>0.09 to 0.12</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>PE Control Medium</td>
<td>20</td>
<td>0.35</td>
<td>0.31 to 0.38</td>
<td>0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>PE Control High</td>
<td>13</td>
<td>0.58</td>
<td>0.48 to 0.69</td>
<td>0.05</td>
<td>0.17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Min</th>
<th>Median</th>
<th>95% CI</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deidentified Residual DBS</td>
<td>12222</td>
<td>0.07</td>
<td>1.16</td>
<td>1.14 to 1.17</td>
<td>34.49</td>
</tr>
<tr>
<td>Confirmed Cases</td>
<td>7</td>
<td>0.07</td>
<td>0.16</td>
<td>0.07 to 0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>CDC PT</td>
<td>5</td>
<td>0.10</td>
<td>0.11</td>
<td>-</td>
<td>0.15</td>
</tr>
<tr>
<td>PE Control Low</td>
<td>21</td>
<td>0.07</td>
<td>0.10</td>
<td>0.09 to 0.12</td>
<td>0.18</td>
</tr>
<tr>
<td>PE Control Medium</td>
<td>20</td>
<td>0.23</td>
<td>0.34</td>
<td>0.28 to 0.42</td>
<td>0.48</td>
</tr>
<tr>
<td>PE Control High</td>
<td>13</td>
<td>0.40</td>
<td>0.52</td>
<td>0.41 to 0.71</td>
<td>0.92</td>
</tr>
</tbody>
</table>
Enzyme Activity Distribution for GAA and GALC

GAA (Pompe)
Mean: 6.55
Median: 6.07

GALC (Krabbe)
Mean: 1.35
Median: 1.1
Linearity of Enzyme Reactions

- **GALC**
  - $y = 0.3291x + 0.0297$
  - $R^2 = 0.9792$

- **GAA**
  - $y = 0.4593x + 0.3228$
  - $R^2 = 0.989$

- **GLA**
  - $y = 0.8074x + 1.2777$
  - $R^2 = 0.9632$

- **ABG**
  - $y = 1.7537x - 0.1384$
  - $R^2 = 0.9879$

- **IDUA**
  - $y = 1.431x + 1.7931$
  - $R^2 = 0.9686$

- **ASM**
  - $y = 1.7537x - 0.1384$
  - $R^2 = 0.9879$

- **GLPH**
  - $y = 0.2331x - 0.012$
  - $R^2 = 0.9838$
3 h vs 17 h Assays – Percent of Median Activities

- **GALC**: Deming fit: $(-0.06 + 1.05x)$
- **GAA**: Deming fit: $(0.02 + 0.98x)$
- **GLA**: Deming fit: $(-0.13 + 1.14x)$
- **ABG**: Deming fit: $(0.07 + 0.92x)$
- **IDUA**: Deming fit: $(0.04 + 0.96x)$
- **ASM**: Deming fit: $(-0.05 + 1.05x)$
Comparison of 3 h to 17 h incubation for GALC

Longer incubation improves discrimination between confirmed-positive and presumed-negative specimens, increasing specificity.
## Normal and Abnormal Ranges as Percent of Daily Median Activity

<table>
<thead>
<tr>
<th></th>
<th>Normal Range</th>
<th>1st Cut-off</th>
<th>Borderline</th>
<th>2nd Cut-off (presumptive positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IDUA</td>
<td>&gt; 31%</td>
<td>≤ 35%</td>
<td>&gt; 28 and ≤ 31</td>
<td>≤ 28%</td>
</tr>
<tr>
<td>GLA</td>
<td>&gt; 18%</td>
<td>≤ 20%</td>
<td>&gt; 13 and ≤ 18</td>
<td>≤ 13%</td>
</tr>
<tr>
<td>GAA</td>
<td>&gt; 28%</td>
<td>≤ 30%</td>
<td>&gt; 23 and ≤ 28</td>
<td>≤ 23%</td>
</tr>
<tr>
<td>ASM</td>
<td>&gt; 15%</td>
<td>≤ 20%</td>
<td>&gt; 11 and ≤ 15</td>
<td>≤ 11%</td>
</tr>
<tr>
<td>GALC</td>
<td>&gt; 13%</td>
<td>≤ 18%</td>
<td>No Borderline</td>
<td>≤ 13%</td>
</tr>
<tr>
<td>ABG</td>
<td>&gt; 20%</td>
<td>≤ 25%</td>
<td>&gt; 17 and ≤ 20</td>
<td>≤ 17%</td>
</tr>
</tbody>
</table>
### Summary of IDPH-CLIA Laboratory Comparison (n=12,000)

<table>
<thead>
<tr>
<th></th>
<th>FABRY</th>
<th>GAUCHER</th>
<th>KRABBE</th>
<th>MPS I</th>
<th>NIEMANN PICK A/B</th>
<th>POMPE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of Normal Specimens</strong></td>
<td>69</td>
<td>66</td>
<td>72</td>
<td>54</td>
<td>74</td>
<td>62</td>
</tr>
<tr>
<td>sent to CLIA Lab</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Number of Specimens Below</strong></td>
<td>6</td>
<td>9</td>
<td>37</td>
<td>21</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>1st Cut-off sent to CLIA Lab</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Positives and Borderlines</strong></td>
<td>4</td>
<td>4</td>
<td>8</td>
<td>16</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Determined by IDPH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Positives Confirmed by CLIA</strong></td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>7</td>
<td>1</td>
<td>2</td>
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<td>Laboratory</td>
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<tr>
<td><strong>Diagnosed Cases</strong></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Other Resolutions</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(PD: Pseudodeficiency)</td>
<td>1 PD</td>
<td>2 Carrier</td>
<td>1 Normal</td>
<td>5 PD</td>
<td>1 Normal</td>
<td>1 Pending</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Note:**
- PD: Pseudodeficiency
- 1 PD
- 2 Carrier
- 1 Normal
- 1 Pending
- 1 PD
- 1 Carrier
Lessons Learned

- Many different individuals with a wide range of skills need to work together to successfully develop a complex, high-throughput analytical assay.
- The process will take longer than initially anticipated; regular interactions and good communications are vital.
- MS/MS platform permits expanded test menu and multiplexing with a single injection.
Lessons Learned (cont’d)

- There are many challenges in adapting a research procedure to a high-throughput newborn screening assay (e.g., analytical, personnel, physical plant, and IT). FDA-cleared tests are vastly preferable.

- If at all possible for mandated testing, have legislation or administrative rules written to permit adequate preparation and milestones (e.g., method validation, QC and PT availability, acquisition of high volume equipment, & funding).
Conclusions

- Very useful for high-throughput newborn screening for six lysosomal enzymes
- Can be adopted to screen 1-6 enzymes depending upon laboratory requirements
- Using 3 hour incubation, first screening results can be obtained within 24 hours of specimen receipt, and positive results can be released after an additional 24 hours.
- For Krabbe, 17 hour incubation should be used for evaluating second cut-off.
Acknowledgments

IDPH

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• Pearlie Gardley – Clinical Laboratory Technologist
• Tamara Simulick – Clinical Laboratory Technologist

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• PerkinElmer Corporation
THANK YOU

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