Multiplex Enzyme Assay Screening of Dried Blood Spots for Lysosomal Storage Disorders by Using Tandem Mass Spectrometry

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BACKGROUND: Reports of the use of multiplex enzyme assay screening for Pompe disease, Fabry disease, Gaucher disease, Niemann-Pick disease types A and B, and Krabbe disease have engendered interest in the use of this assay in newborn screening. We modified the assay for high-throughput use in screening laboratories.

METHODS: We optimized enzyme reaction conditions and procedures for the assay, including the concentrations of substrate (S) and internal standard (IS), assay cocktail compositions, sample clean-up procedures, and mass spectrometer operation. The S and IS for each enzyme were premixed and bottled at an optimized molar ratio to simplify assay cocktail preparation. Using the new S:IS ratio, we validated the modified assay according to CLSI guidelines. Stability of the S, IS, and assay cocktails were investigated. Dried blood spots from 149 healthy adults, 100 newborns, and 60 patients with a lysosomal storage disorder (LSD) were tested using the modified assay.

RESULTS: In our study, the median enzyme activity measured in adults was generally increased 2–3–fold compared to the original method, results indicating higher precision. In the multiplex format, each of the 5 modified enzyme assays enabled unambiguous differentiation between samples from healthy individuals (adult and newborn) and samples from patients with LSDs.

CONCLUSIONS: The modified multiplex enzyme assay with premixed S and IS is appropriate for use in high-throughput screening laboratories.

1 Nonstandard abbreviations: NP A/B, Niemann-Pick disease types A and B; LSD, lysosomal storage disorder; GAA, acid α-glucosidase; GLA, acid α-galactosidase; GBA, acid glucocerebrosidase; ASM, acid sphingomyelinase; GALC, galactocerebrosidase; DBS, dried blood spots; MS/MS, tandem mass spectrometry; S, substrate; IS, internal standard; CHAPS, 3-(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate.
15.9 mL of 0.3 mol/L of phosphate citrate buffer and 0.3 mL of 0.8 mmol/L acarbose (Toronto Research Chemicals) in water were added, and the vial was vortex-mixed. The final GAA assay cocktail contained 0.67 mmol/L of GAA-S, 6.67 mmol/L of GAA-IS, 10 g/L CHAPS, 13.3 mmol/L acarbose, 0.3 mol/L phosphate, and 0.15 mol/L citrate, pH 4.0. Preparation of the other 4 assay cocktails is described in the online Data Supplement.

The multiplex enzyme assay was adapted to a 96-well plate. Chloroform was replaced with ethyl acetate. The composition and volume of solvents used in liquid/liquid- and solid-phase extractions were modified. We used two 3.2-mm DBS punches instead of one 5-mm punch. One punch was incubated directly in the GALC assay cocktail, thereby eliminating the enzyme elution step. Complete details of the modified assay protocol are in the online Data Supplement. We monitored all analytes by using selected-reaction monitoring, rather than constant neutral loss or parent-ion scanning (3), with an API 4000 triple-quadrupole MS/MS (MDS Sciex). The enzyme activity of each sample was calculated from the ion abundance ratio of product to IS measured by MS. All enzyme activities were blank subtracted (details in the online Data Supplement). Ion suppression was effectively controlled, as demonstrated by the regression analysis of calibration curves collected during the validation study (Table 1 in the online Data Supplement).

With use of the previously reported method (3), ASM activities measured in samples from adults and patients with NP A/B overlapped. Zn$^{2+}$ is required for optimal activity of both secreted and lysosomal ASMs (4–6), which are deficient in NP A/B patients. We chose the 2 highest-activity NP A/B patient samples and 3 low-activity adult samples and monitored their activities with increasing concentrations of ZnCl$_2$. ASM activities in the adult samples increased with ZnCl$_2$, with maximal distinction between the normal and disease samples at 0.5–1.5 mmol/L ZnCl$_2$.

The previously reported GALC assay gave high CVs attributable to low activity values, i.e., the minimum activity in an adult sample was 0.6 mmol/h/L in whole blood. When we varied pH, S, taurocholate, and oleate concentrations, GALC activity increased in adult samples. The S concentration was increased 5-fold to 1 mmol/L (Supplemental Fig. 1 in the online Data Supplement). We determined that GALC activity was preserved by eluting samples directly into the GALC assay cocktail.

With the use of maltose or acarbose as an inhibitor of maltase glucoamylase, DBS can be used in the diagnosis of Pompe disease (3, 7). With the fluorogenic substrate 4-methylumbelliferyl-$\alpha$-glucoside, acarbose concentrations of 3–9 mmol/L are sufficient to inhibit maltase glucoamylase activity in the assay (8, 9). We found that with the synthetic substrate in this assay, 8 mmol/L acarbose provided sufficient inhibition of maltase glucoamylase and maintenance of GAA activity (Supplemental Fig. 2 in the online Data Supplement). To minimize the effect of detergent on the MS/MS, Triton X-100 was replaced by CHAPS, which is more effectively removed by solid-phase extraction and results in an increase in GAA activity.

To improve assay precision and facilitate assay automation, the GLA assay was modified to use a 15-$\mu$L instead of a 2.5-$\mu$L assay cocktail. Optimization of N-acetyl galactosamine, buffer, and detergent concentrations increased GLA activity and decreased assay variation. Changes to the GBA assay were limited to increasing the S concentration. The modifications of

### Table 1. Assay cocktail compositions used in the multiplex assay.

<table>
<thead>
<tr>
<th>Assay</th>
<th>DBS punch</th>
<th>Assay volume, $\mu$L</th>
<th>[S] mmol/L</th>
<th>[IS] mmol/L</th>
<th>Buffer</th>
<th>Detergent</th>
<th>Additional components</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAA</td>
<td>1/7*</td>
<td>25</td>
<td>0.4</td>
<td>4</td>
<td>0.18 mol/L citrate-phosphate, pH 4.0</td>
<td>6 g/L CHAPS</td>
<td>8 $\mu$mol/L acarbose</td>
</tr>
<tr>
<td>GLA</td>
<td>1/7*</td>
<td>25</td>
<td>2</td>
<td>4</td>
<td>0.09 mol/L sodium acetate, pH 4.6</td>
<td>1.8 g/L sodium taurocholate</td>
<td>96 mmol/L N-acetyl galactosamine</td>
</tr>
<tr>
<td>GBA</td>
<td>1/7*</td>
<td>25</td>
<td>0.4</td>
<td>8</td>
<td>0.37 mol/L citrate-phosphate, pH 5.1</td>
<td>9.6 g/L sodium taurocholate</td>
<td>None</td>
</tr>
<tr>
<td>ASM</td>
<td>1/7*</td>
<td>25</td>
<td>0.2</td>
<td>4</td>
<td>0.55 mol/L sodium acetate, pH 5.7</td>
<td>0.6 g/L sodium taurocholate</td>
<td>0.36 mmol/L zinc chloride</td>
</tr>
<tr>
<td>GALC</td>
<td>1$^b$</td>
<td>30</td>
<td>1</td>
<td>6.7</td>
<td>0.18 mol/L citrate-phosphate, pH 4.4</td>
<td>9.6 g/L sodium taurocholate</td>
<td>1.2 g/L oleic acid</td>
</tr>
</tbody>
</table>

* 10 of 70 $\mu$L of DBS extractant was used in the assay, equivalent to 1/7 of 1 whole DBS.

$^b$ One whole DBS was used in the assay.
The 5 assays are summarized in Table 1, and their comparison with the previous assay are summarized in Supplemental Table 2 in the online Data Supplement.

The assay was validated according to the CLSI standard protocol (10). For each enzyme assay, within-plate imprecision was calculated using 20 punches from 1 healthy adult sample and 1 corresponding patient sample. The within-plate CVs for the adult sample in the GAA, GLA, GBA, ASM, and GALC assays were 8.3%, 9.8%, 11.9%, 12.5%, and 5.8%, respectively, and for the disease sample were 10.1%, 24.8%, 24.8%, 23.1%, and 12.3%, respectively. Overall CVs in the GAA, GLA, GBA, ASM, and GALC assays were 7.5%, 14.0%, 11.7%, 13.7%, and 8.2%, respectively. The limits of the blank for the GAA, GLA, GBA, ASM, and GALC assay were 0.02, 0.09, 0.32, 0.25, and 0.02 μmol/h/L in whole blood, respectively. The limits of detection (defined as the 1.65 times the SD above the limits of the blank) of the GAA, GLA, GBA, ASM, and GALC assays were 0.15, 0.29, 0.50, 0.66, and 0.07 μmol/h/L in whole blood, respectively. We verified that the GAA, GLA, GBA, ASM, and GALC assay results were linear in the range between the limits of detection and 7.2, 4.9, 5.3, 7.6 and 4.7 μmol/h/L in whole blood, respectively. Analysis performed with assay cocktails stored for 14 days at 37 °C, 30 days at 25 °C, and 45 days at 2–8 °C resulted in measured activities that were within 20% of those for once-frozen assay solutions (Supplemental Figs. 2–5 in the online Data Supplement). There was no detectable MS/MS carryover in the assay.

Each of the 5 modified enzyme assays in the multiplex format showed an unambiguous distinction between samples from healthy individuals (adult and newborn) and corresponding samples from patients with LSDs (Fig. 1 and Supplemental Table 3 in the online Data Supplement). The median activities in samples from healthy adults (n = 149) were increased 2–3-fold relative to the original method. In the GAA and GALC assays, the separation between normal and disease samples became even more pronounced, and the disease sample with the highest percentage of mean normal activity decreased from 15% to 5% and 19% to 15%, respectively. The limits of detection of each assay were at least 2-fold below the maximum observed disease activity.

The availability of S and IS premixed for each enzyme assay has simplified cocktail preparation. The modified assay proved to be reproducible and reliable for the differentiation of normal and disease-specific samples. Transfer of the high-throughput multiplex enzyme assay to newborn screening laboratories should be a straightforward process.

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or revising the article for intellectual content; and (c) final approval of the published article.

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**References**


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