Clinical Chemistry 56:12
1854–1861 (2010)

A Tandem Mass Spectrometry Triplex Assay for the Detection of Fabry, Pompe, and Mucopolysaccharidosis-I (Hurler)

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BACKGROUND: We sought to develop a tandem mass spectrometry assay in which the enzymatic activities of 3 lysosomal enzymes (α-glucosidase, α-galactosidase A, and α-L-iduronidase) could be quantified in dried blood spots by using a single assay buffer.

METHODS: A 3-mm dried blood spot punch was incubated in a single assay buffer with 3 different substrates and internal standards. The sample was processed by a simple liquid-liquid extraction by using ethyl acetate. The extract was dried down and resuspended in solvent for injection into the tandem mass spectrometer. Products and internal standards were monitored by multiple reaction monitoring.

RESULTS: Assay for the 3 lysosomal enzymes was successfully achieved with acceptable statistics. The assay can be performed by using a minimal quantity of disposable supplies and equipment. The entire procedure fits into a 48-h cycle including data analysis. Data from 5990 anonymous newborn dried blood spots showed an approximate bell-shaped distribution of enzymatic activities (mean values of 19.0, 11.5, and 3.5 $\mu mol \cdot h^{-1} \cdot (L\ blood)^{-1}$ for α-glucosidase, α-galactosidase A, and α-L-iduronidase, respectively. Blank values obtained in the absence of blood were 0.13, 0.24, and 0.45 $\mu mol \cdot h^{-1} \cdot (L\ blood)^{-1}$, respectively). By assaying 3 enzymes at once, problematic samples are spotted for reanalysis if enzyme activity values are low for all enzymes (for example, if insufficient blood is present in the assay).

CONCLUSIONS: This method demonstrates that a triplex assay in a single buffer and with minimal supplies and labor can be adapted to a high-throughput newborn screening laboratory for the analysis of Pompe, Fabry, and mucopolysaccharidosis-I (Hurler) diseases.

There is interest in expanding newborn screening programs to include a subset of lysosomal storage diseases for which early recognition and treatment has been shown to be effective. On the basis of the literature and our experience, we selected Pompe, Fabry, and mucopolysaccharidosis-I (Hurler) (MPS-IH)[5] diseases as the most likely conditions that would meet the standard criteria for newborn screening programs. We previously developed tandem mass spectrometry with electrospray ionization (ESI-MS/MS) for the assay of the enzymatic activities in dried blood spots (DBSs) for Fabry, Pompe, Niemann-Pick-A/B, Gaucher, Krabbe, and Hurler syndromes *(1–5)*. The assay for Pompe disease was made possible by our recognition that acarbose selectively inhibits the interfering enzyme, maltase glucoamylase, present on the surface of granulocytes *(2, 6, 7)*. This procedure has been validated independently by an international panel of experts *(6)*. Newborn screening for Krabbe disease using ESI-MS/MS has been initiated in New York state *(8)*, and plans are underway to screen for other lysosomal storage diseases. Our assays for Pompe and Fabry diseases have been piloted in newborn screening laboratories *(9–10)*.

Previously, each enzyme assay made use of a separate buffer that gave optimal enzyme activity. The sample workup involved extracting each reaction mixture with organic solvent and passing the organic ex-

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Received June 21, 2010; accepted September 20, 2010.
Previously published online at DOI: 10.1373/clinchem.2010.152009

[5] Nonstandard abbreviations: MPS-IH, mucopolysaccharidosis-I (Hurler); ESI-MS/MS, tandem mass spectrometry with electrospray ionization; DBS, dried blood spot; GLA, α-galactosidase A; GLA-IS and GLA-S, internal standard and substrate for the Fabry assay, respectively; GAA, α-glucosidase; GAA-IS and GAA-S, internal standard and substrate for the Pompe assay, respectively; IDUA, α-L-iduronidase; IDUA-IS and IDUA-S, internal standard and substrate for the MPS-IH assay, respectively; QC, quality control; GalNAc, *N*-acetylgalactosamine.

tract through a small plug of silica gel to remove buffer salts and detergents before infusion into the mass spectrometer. It became apparent that this solid-phase extraction step was not ideal for the screening laboratory because of the multiple pipetting steps, adding a full day to the workflow. We set out to optimize our ESI-MS/MS assays by exploring buffer conditions that would allow multiple lysosomal enzymes to be assayed with a single DBS punch in a single assay well, plus a sample workup that no longer required solid-phase extraction. In the current study, we focus on a triplex assay to detect Fabry, Pompe, and MPS-IH. These disorders were selected because each may be difficult to recognize clinically, and recent studies have shown enzyme replacement therapy or bone marrow transplantation to improve the natural history of the disease.

Materials and Methods

MATERIALS

Fabry internal standard (α-galactosidase A [GLA]-IS), Fabry substrate (GLA-S), Pompe internal standard (α-glucosidase [GAA]-IS), and Pompe substrate (GAA-S) were from Drs. H. Zhou and V. De Jesus (CDC, Atlanta, Georgia, USA). Hurler internal standard (α-L-iduronidase [IDUA]-IS) and Hurler substrate (IDUA-S) were from Drs. K. Zhang and J. Keutzer (Genzyme, Cambridge, Massachusetts, USA) *(2, 4)* or from H. Zhou (CDC, Atlanta, Georgia, USA). These were originally designed and synthesized by our group, but the present studies used reagents synthesized by Genzyme in Liestal, Switzerland, approved for use as analyte specific reagents by the U.S. Food and Drug Administration and provided to the CDC for distribution.

All experiments were conducted in compliance with institutional review board guidelines. DBSs from patients diagnosed with Fabry, Pompe, and MPS-IH were obtained from Genzyme (J. Keutzer) or from our clinical program as anonymous samples, in compliance with institutional review board requirements. The Fabry samples were from affected males only, the Pompe samples were from both infantile and late-onset clinical forms, and the MPS-IH samples were from patients with early childhood presentations (Hurler). For assay development, DBSs were obtained as anonymous samples from the Washington state newborn screening laboratory. DBSs were obtained from birthing centers and kept at ambient temperature during shipment (<10 days). For assays carried out in the Washington state newborn screening laboratory, DBSs were used after all routine testing was performed (i.e., leftover DBSs) and were up to approximately 6 months old and kept at ambient laboratory temperature.

TRIPLEX ASSAY

Experimental details for assays carried out during the optimization phase of the project at the University of Washington are provided in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue12. Below are the experimental details for the triplex assay carried out in the Washington state newborn screening laboratory.

PREPARATION OF ASSAY BUFFER

Ammonium formate (1.24 g, >99.0%, Sigma-Aldrich) was added to water (200 mL, DI Nanopure, Barnstead CAP type 1) and stirred to dissolve the powder. Formic acid (0.2 mL, 97%, Alfa Aesar) was added, and the pH was adjusted to 4.4 with ammonium hydroxide or formic acid (not hydrochloric acid or sodium hydroxide). The solution was brought up to 250 mL with water, sterile filtered, and stored at 2–8 °C for up to 6 months in a glass bottle. The final formate concentration was 0.1 mol/L (pH 4.4).

PREPARATION OF QUENCH BUFFER

Ammonium acetate (3.45 g, >99.0%, Sigma-Aldrich) was added to water (400 mL) and stirred to dissolve the powder. Acetic acid (0.3 mL, glacial, JT Baker) was added, and the pH was adjusted to 5.5 with ammonium hydroxide or acetic acid (not hydrochloric acid or sodium hydroxide). The solution was brought up to 500 mL with water, sterile filtered, and stored at 2–8 °C for up to 6 months in a glass bottle. The final acetate concentration was 0.1 mol/L (pH 5.5).

PREPARATION OF ASSAY COCKTAIL

The IDUA and GAA substrate/internal standard vials from Genzyme and the CDC were dissolved in methanol (6 mL). The GLA substrate/internal standard vials from the CDC were dissolved in methanol (10 mL). Portions of the contents of the vials were then transferred to a new vial (1 mL each of IDUA, GAA, and GLA solutions). This was repeated 4 times to generate 5 vials total of the mixture of IDUA, GAA, and GLA substrates and internal standards. The methanol was evaporated with a stream of N_2 with slight heating (<40 °C). The solution of the remaining GLA substrate/internal standard vial was further distributed into 4 additional vials (1 mL each). The methanol was removed as above, and the vials were stored at −20 °C for later use by addition of IDUA and GAA vial reagents as above.

On the day of the assay, the residue in a vial was reconstituted in assay buffer (9.9 mL, 0.1 mol/L ammonium formate, pH 4.4), and acarbose (Sigma Aldrich, A8980-1G) was added (0.1 mL, 0.8 mmol/L in water). The solution was stirred with a magnetic stir bar until the residue was fully dissolved (slight heating, <50 °C,

should be used to ensure that all material is dissolved). The final assay cocktail contained 0.48 mmol/L IDUA-S, 3.1 μmol/L IDUA-IS, 0.2 mmol/L GAA-S, 2.0 μmol/L GAA-IS, 0.6 mmol/L GLA-S, 1.2 μmol/L GLA-IS, and 8 μmol/L acarbose. Excess assay buffer could be stored at 4 °C and could also be used the following day without loss of activity. The other 4 vials of dried and mixed reagents were stored at −20 °C and reconstituted on the day of the assay.

ASSAY SETUP AND INCUBATION

Three DBS punches (1/8 inch, 3.2 mm) collected with a blood spot puncher (BSD Technologies International, BSD 600 Duet) were taken from each newborn sample. We avoided punching near the edge of the blood region because such punches may not be completely saturated with blood. One punch was placed in a single well of a 96–deep well plate (1 mL, Costar, Fisher Scientific) and used for the triplex assay. The 2 extra punches were placed in a separate 96-well plate, sealed with aluminum plate-sealing film (VWR), and stored in refrigeration in a gasket-sealed plastic storage container. These extra punches may be used for confirmatory testing if necessary. Each 96–deep well plate also contained 6 wells with a blank filter paper punch and 2 wells of each of the CDC quality control (QC) DBS samples (base, low, medium, and high, obtained from Drs. H. Zhou and V. De Jesus at the CDC in Atlanta, stored at −20 °C in a Ziploc plastic bag). The blanks and QC samples were aliquoted on the first and last columns of the plate. From top to bottom, there were 2 blanks, then QC base, QC low, QC medium, QC high, an adult DBS, and then another blank. QC samples were manually punched with a 1/8-inch whole punch. Assay cocktail (30 μL) was added to each well in the 96–deep well plate by using a Rainin Liquidator 96-tip pipette. The plate was sealed with aluminum plate-sealing film (VWR) and incubated at 37 °C for 16 h (overnight) with orbital shaking at 225 rpm.

ASSAY WORKUP

After 16 h, the assay was quenched with ammonium acetate buffer (100 μL, 0.1 mol/L, pH 5.5) transferred directly into the 96–deep well plate by using a Rainin Liquidator 96-tip pipette. Ethyl acetate (400 μL) was added, and the wells were mixed by aspirating the liquid 5 times by using the Rainin Liquidator 96-tip pipette. The plate was covered with foil and centrifuged for 5 min at 3000*g* to separate the layers. The plate was returned to the Rainin Liquidator 96-tip pipette, and 200 μL of the top layer (ethyl acetate) was transferred to a new 96–shallow well plate (0.5 mL, Axygen Scientific and VWR). The ethyl acetate was evaporated by using a stream of air (SPE Dry 96 Dual Argonaut sample concentrator system, Biotage) with a flow rate of 40-80 psi of air and heating <35 °C (typically <30 min). The residue in the wells was resuspended in the mass spectrometry mobile phase (100 μL, 80% acetonitrile:20% water containing 0.2% formic acid) by using the Rainin Liquidator 96-tip pipette. After addition of the mobile phase, the plate was mixed for 5 min in an orbital shaker. The plate was covered with aluminum foil (not sealing foil, since acetonitrile can dissolve the glue) and placed in the autoinjector tray for mass spectrometric analysis.

ESI-MS/MS

ESI-MS/MS analysis was performed on a Waters Acquity TQD Ultra Performance tandem quadrupole mass spectrometer by using the positive mode multiple reaction monitoring mode and flow injection. A total of 10 μl of the 100-μL sample was injected for each analysis at a flow rate of 0.1 mL/min by using a Waters 2777C Sample Manager via flow injection with 80/20 acetonitrile/water with 0.2% formic acid for 1.10 min and then 0.5 mL/min for 0.4 min. Most of the sample eluted during the 1.10-min phase, and the faster flow phase was used to reduce the time of sample clearance in preparation for the next injection. Data were collected during 1.5 min of infusion, and the signal returned to the background level before the next injection.

The mass transitions used for multiple reaction monitoring were as follows: IDUA-IS: *m/z* 377.2 → *m/z* 277.1; IDUA-P: *m/z* 391.2 → *m/z* 291.2; GLA-P: *m/z* 484.2 → *m/z* 384.3; GLA-IS: *m/z* 489.4 → *m/z* 389.2; GAA-P: *m/z* 498.4 → *m/z* 398.4; and GAA-IS: *m/z* 503.4 → *m/z* 403.4. Other ESI-MS/MS parameters are given in the online Data Supplement. The amount of product was calculated from the ion abundance ratio of the product to the internal standard for the sample minus that of the blank (filter paper only punch, mean of 6 blanks was used), multiplied by the amount of added internal standard and divided by the response factor ratio of the product to internal standard. The response factor was determined from a calibration curve obtained from standards containing ratios of product and internal standard from 0.0 to 5.0 for GAA, GLA (obtained from Drs. H. Zhou and V. De Jesus at the CDC), and IDUA (obtained from Drs. K. Zhang and J. Keutzer at Genzyme Corporation). The enzyme activity in units of $\mu mol \cdot h^{-1} \cdot (L\ blood)^{-1}$ was calculated from the measured amount of product assuming the 3-mm DBS punch contains 3.2 μL blood.

Results

OPTIMIZATION OF ASSAY BUFFER COMPONENTS

The goal of the first set of studies was to carefully examine the original buffer conditions reported previ-

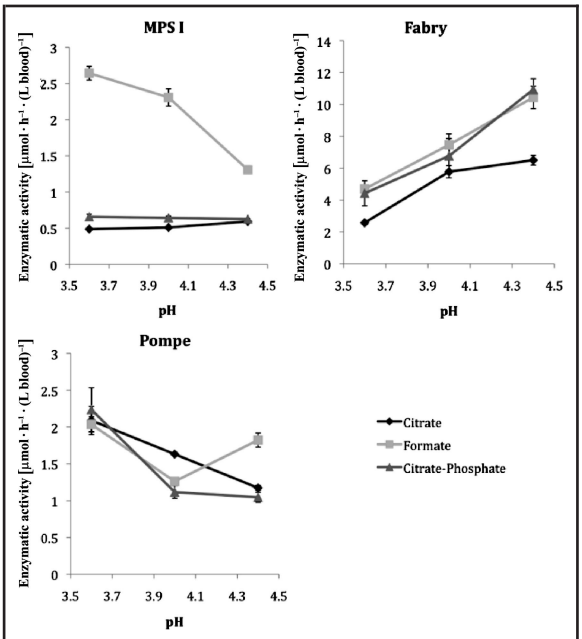

Fig. 1. Enzyme activity versus pH and buffer components.
Assays were performed with DBSs from a healthy adult. Data are plotted as the mean and SD of triplicate assays carried out as described in the online Data Supplement.

Table 1. Effect of GalNAc on GLA activity in DBSs.

	GLA activity [μmol · h^{-1} · (L blood)$^{-1}$]	
	No GalNAc	96 mmol/L GalNAc
Blank[a,b]	0.26 (0.08)	0.26 (0.01)
QC base	0.53 (0.05)	0.61 (0.05)
QC low	1.2 (0.3)	0.99 (0.06)
QC medium	4.6 (0.5)	4.6 (0.2)
QC high	8.9 (0.6)	8.6
Fabry patient 1	0.54	0.63
Fabry patient 2	0.58	0.54
Fabry patient 3	0.50	0.42
Fabry patient 4	0.44	0.31
Fabry patient 5	0.51	0.33

[a] Values reported are the mean (SD) for triplicate runs of different punches from the same DBS.
[b] Blank assays were carried out with the assay cocktail incubated with a filter paper punch that does not contain blood.

ously for each assay (Fabry, Pompe, and MPS-IH) *(2, 4, 7)* to determine the buffer components that are most important for maintaining high enzymatic activity. The original buffer systems for the individual assays ranged from pH 3.4 to 4.6 and used formate, citrate-phosphate, and acetate buffering salts. The assays were conducted in sodium formate, sodium citrate, and sodium citrate-phosphate buffers at pH 3.6, 4.0, and 4.4 on a single DBS by using GLA-S, GAA-S, IDUA-S, GLA-IS, GAA-IS, and IDUA-IS (Fig. 1). The enzyme activity of IDUA was low in sodium citrate and sodium citrate-phosphate buffers at all pH values, so sodium formate was chosen as the preferred buffer salt. The enzyme activity of GLA was substantially higher at the higher pH, and the blank values obtained with all assay components except the DBS punch for all the assays were lower at higher pH, so pH 4.4 was used for further optimizations.

The Pompe and Fabry enzyme activity assays described previously used CHAPS and sodium taurocholate, respectively *(2)*. Sodium taurocholate reduced the activity of all 3 enzymes at all concentrations tested (see Table 3 in the online Data Supplement). Likewise, CHAPS reduced the activity of all 3 enzymes at 4–6 g/L CHAPS, whereas at 2 g/L CHAPS, the enzyme activities were similar to the activities without detergent. In an effort to keep the assay mixtures as simple as possible, subsequent assays were conducted without detergent.

In the original GLA enzyme assay, *N*-acetylgalactosamine (GalNAc) was added as an inhibitor for *N*-acetyl-galactoseaminidase, since the GLA substrate could potentially be a substrate for this enzyme resulting in product formation in Fabry patients with low α-galactosidase activity. The activity of GLA in normal DBS punches and in DBS punches from Fabry patients was determined by using the triplex assay conditions with and without GalNAc added. GLA enzyme activity was similar in normal DBS punches with and without the GalNAc added (Table 1). More importantly, the GLA enzyme activity in 5 punches obtained from male patients with Fabry disease were 0.33–0.63 μmol · h^{-1} · (L blood)$^{-1}$ with GalNAc and 0.44–0.58 μmol · h^{-1} · (L blood)$^{-1}$ without the GalNAc added. Thus, there is minimal conversion of the GLA substrate to product by *N*-acetyl-galactoseaminidase under these conditions, and the measured activity is from α-galactosidase.

The substrate concentration must be high enough to provide enzyme activities with a large enough difference between the normal newborn blood with low activity and affected patient blood. Also, the use of substrate concentrations well above the K_m minimizes effects of competitive inhibitors that may be present in blood. On the other hand, higher substrate concentrations increase the load on the mass spectrometer and lead to a higher background signal and to higher reagent costs. The assays were conducted at a substrate

concentration lower than the apparent K_m of the enzymes, so the activity of the enzymes should double when the substrate concentration is doubled. This would be true if DBS samples did not contain competitive inhibitors or if the amount of competitive inhibitors was similar in all samples. Thus, we measured the variation in GLA, GAA, and IDUA activity when the concentration of the 3 substrates was varied 2-fold, and this analysis was carried out with 30 DBSs from different individuals. The enzyme activity approximately doubles for all 3 enzymes when the substrate concentration doubles (see Fig. 1 in the online Data Supplement). In addition, the same 30 DBSs were incubated in the assay buffer without the 3 enzyme substrates. Analysis of these samples did not detect an ion for the enzymatic product. The data show that differential competitive inhibition is not a problem and that DBSs do not contain substances that give rise to false product signals in the ESI-MS/MS analysis (i.e., all product ESI-MS/MS signal comes from enzymatic conversion of added substrates).

The solid-phase extraction step was eliminated (as will be shown below), so there is the potential for nonvolatile buffer salts that are extracted into ethyl acetate to build up in the mass spectrometer source and also to suppress the electrospray ionization process (although most of the salts will remain in the aqueous phase). Thus, volatile buffer components were evaluated. The enzyme activities for GAA, GLA, and IDUA were compared in sodium formate and ammonium formate buffers at pH 4.4 (see Fig. 2 in the online Data Supplement). IDUA enzyme activity was slightly higher in ammonium formate than in sodium formate, and GAA and GLA enzyme activities were comparable for the 2 buffers. Therefore, the volatile buffer ammonium formate was used as the buffer for the triplex assay.

OPTIMIZATION OF ASSAY MANIPULATIONS

We explored the possibility of eliminating the solid-phase extraction step *(1, 2, 7)*. Our concern was that using only the liquid-liquid extraction with ethyl acetate would lead to higher amounts of enzyme substrates in the mass spectrometer infusion solvent. Since substrates may fragment to give products in the source of the mass spectrometer, we would expect higher product amounts in control reactions. A mixture of the internal standards and substrates for GAA, GLA, and IDUA were prepared in sodium formate buffer and purified without incubation by using both liquid-liquid extraction alone and liquid-liquid extraction plus solid-phase extraction on silica gel. The samples purified by solid-phase extraction had a lower background signal for the products of all 3 enzymes than those with just the liquid-liquid extraction workup, but the background signal of the samples purified without solid-phase extraction is very small compared with the signal observed from enzyme activity in DBS punches (see Table 4 in the online Data Supplement). The amount of in-source fragmentation of the substrate to product was kept to a minimum by carefully tuning the mass spectrometric parameters (cone voltage and collision energy, Table 1 in the online Data Supplement) while still maintaining a high signal for the production.

We sought to minimize the number of liquid transfer steps required, thereby reducing the number of plates and pipette tips used, as well as reducing the overall assay time and the potential for error. We placed the DBS punch directly into the well of a 96–deep well plate followed by addition of the assay cocktail. This eliminated the step in which the DBS punch was extracted with buffer before assay initiation. The use of the deep well plate allowed the liquid-liquid extraction to be carried out in the same well as the incubation. After incubation, quench buffer and ethyl acetate were added directly to the assay well. After extraction, the top ethyl acetate layer was transferred to a 96–shallow well plate, and solvent was removed with a stream of air. The residue was taken up in the mobile phase for infusion into the mass spectrometer. This plate was used directly in the autosampler. In total, the new method used two 96-well plates and 5 boxes of pipette tips to assay the activity of 3 enzymes, as opposed to the seven 96-well plates, a 96-well filter plate, and 18 boxes of pipette tips for the same 3 enzymes in the previous method *(2)*.

ANALYSIS OF PATIENT BLOOD

By using the optimized assay conditions, patient blood from 11 Pompe patients, 8 MPS-IH patients, and 5 male Fabry patients was tested, along with QC DBSs from the CDC (Table 2). Blood collected from affected patients had low activity of the enzyme corresponding to the disease but normal activity for the other 2 enzymes assayed. For example, Fabry patients had low GLA enzyme activity but normal activity for GAA and IDUA. The activity of the affected enzyme of each of the patient blood samples screened was below the QC low sample, which represented approximately 5% of the mean activity measured in a population screen.

STUDIES IN THE WASHINGTON STATE NEWBORN SCREENING LABORATORY

The entire protocol fits within a 48-h period, including data analysis (Fig. 2). The analysis of 4 plates containing 320 newborn DBS samples, 40 QC DBS samples, and 24 blanks in this 48-h period requires the items listed in Table 3. The following equipment was required: a DBS punch machine, a manually operated 96-tip pipette, an incubator for 96-well plates, a centri-

Table 2. Enzyme activities in DBSs.

	Enzyme activity [μmol · h^{-1} · (L blood)$^{-1}$]		
	MPS-IH: IDUA	Fabry: GLA	Pompe: GAA
CDC QC blank[a,b]	0.45 (0.03)[a]	0.24 (0.02)	0.13 (0.01)
CDC QC base[a]	0.28 (0.02)	0.78 (0.10)	0.28 (0.10)
CDC QC low[a]	0.74 (0.02)	1.5 (0.1)	1.4 (0.1)
CDC QC medium[a]	5.4 (0.6)	8.4 (0.6)	9.8 (2.6)
CDC QC high[a]	9.4 (1.0)	16.1 (1.0)	14.7 (0.5)
Pompe patient 1	7.63	9.52	0.83
Pompe patient 2	3.35	6.92	0.46
Pompe patient 3	6.04	8.77	0.71
Pompe patient 4	4.05	6.47	0.46
Pompe patient 5	6.74	7.07	0.66
Pompe patient 6	4.66	8.15	0.56
Pompe patient 7	2.47	6.15	0.41
Pompe patient 8	3.91	6.60	0.68
Pompe patient 9	2.64	2.80	0.17
Pompe patient 10	1.53	4.09	0.47
Pompe patient 11	4.75	3.71	0.36
MPS-IH patient 1	0.48	5.10	14.13
MPS-IH patient 2	0.58	5.33	20.14
MPS-IH patient 3	0.30	6.61	9.69
MPS-IH patient 4	0.28	5.17	7.04
MPS-IH patient 5	0.29	3.52	5.83
MPS-IH patient 6	0.74	7.35	11.31
MPS-IH patient 7	0.35	2.10	2.77
MPS-IH patient 8	0.34	5.01	8.99
Fabry patient 1	4.35	0.61	8.29
Fabry patient 2	3.28	0.52	8.31
Fabry patient 3	5.38	0.80	12.83
Fabry patient 4	2.83	0.52	6.69
Fabry patient 5	2.99	0.60	5.58

[a] Values reported are the mean (SD) for triplicate runs of different punches from the same DBS.
[b] Blank assays were carried out with the assay cocktail incubated with a filter paper punch that does not contain blood.

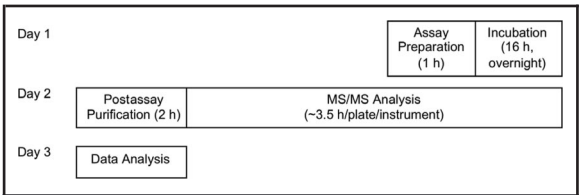

Fig. 2. Typical work flow of the assay run in a newborn screening laboratory.
Assay results are obtained within 48 h of the beginning of the procedure.

fuge to spin 96-well plates, a simple solvent evaporation system for 96-well plates, an autosampler, and an ESI-MS/MS instrument. No robotics were necessary.

Fig. 3 shows the distribution of GLA, GAA, and IDUA activities, respectively, for 5990 DBSs submitted to the triplex assay. The activity of GLA and GAA was similar to the activity previously documented by using a citrate-phosphate or acetate buffer. The mean activity of IDUA was approximately 50% less than prior determinations (pH 3.4) but still clearly separated affected patients from unaffected newborns with the use of formate buffer at pH 4.4.

QC samples for inclusion in the DBS enzyme assay were obtained from the CDC. These were artificially prepared blood samples that represented the range of enzyme activity expected from newborn blood spots submitted for screening. They were expected to approximate 5%, 50%, and 90% of the normal range. The inclusion of 2 QC samples for each activity level in each 96-well plate served as a reliable indicator of assay integrity. Table 5 in the online Data Supplement summarizes the enzyme activity data obtained for 80 punches of the low, medium, and high QC samples from the CDC (8 punches per day, distributed 2 per 96-well plate in 4 plates) over a 10-day period. The coefficients of variation were generally higher for the low QC samples, as expected, since the medium enzyme activity in these samples was approximately 10% of those for the high QC samples. The coefficients of variation were in the range 7.9–13.5% for the high QC samples. These values

Table 3. Items required for triplex enzyme assay of four 96-well plates in a 48-h period.[a]

Item	Required amount
GAA-S/GAA-IS	1.5 mg/0.012 mg
GLA-S/GLA-IS	4.5 mg/0.007 mg
IDUA-S/IDUA-IS	3.3 mg/0.014 mg
Acarbose	0.060 mg
96-well, deep-well plates	4
96-well, shallow-well plates	4
Large volume pipette tips (Liquidator pipette tips)	8 boxes of 96 tips
200-μL pipette tips	16 tips
Technician	1
Data analyst	0.5–1

[a] The table does not include buffer salts, ethyl acetate, and 96-well plate sealers, but the cost of these is small.

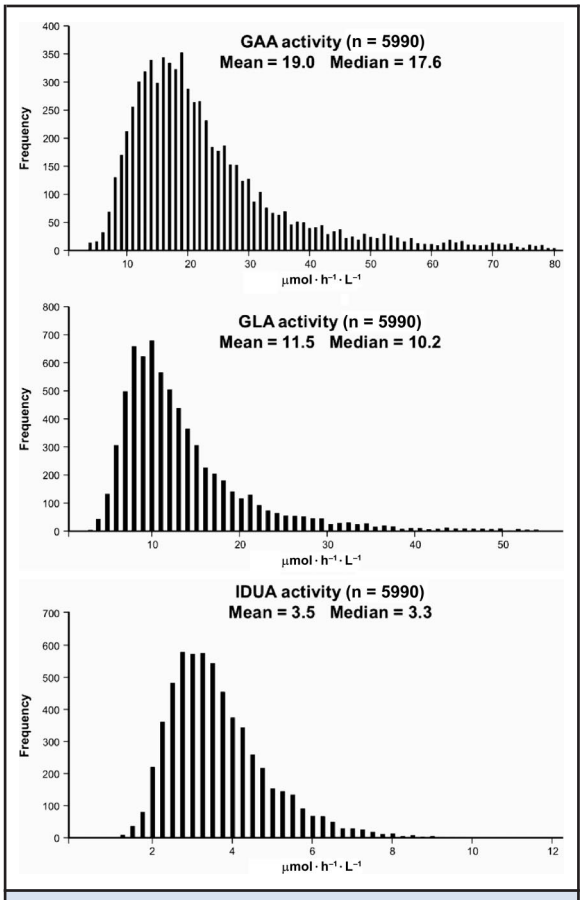

Fig. 3. Activity distribution of GLA, GAA, and IDUA from 5990 anonymous newborn blood spots.

Assays were performed in the Washington State newborn screening laboratory by using the triplex assay.

included all variation from the assay procedure and from punching the DBS at different spots, since multiple punches were taken from each DBS.

Discussion

The triplex assay has the added benefit of having an internal quality control. In the newborn screening laboratory setting, it is possible to obtain DBS punches that are not fully saturated with blood. When tested for a single enzyme, these samples appear to come from affected patients. In the triplex, such a DBS would have low activity in all 3 enzymes, flagging an error in the testing procedure.

Since Fabry disease is an X-linked disease, females will have a wide spectrum of α-galactosidase activity due to random X-chromosome inactivation. Thus, the assay reported here, or any enzyme activity assay, will primarily detect hemizygous males with absent or low α-galactosidase activity.

In conclusion, the enzyme activities of 3 separate enzymes can be determined in a single incubation of a DBS with the appropriate enzyme substrates and internal standards. The new assay reliably distinguishes blood from affected patients with Fabry, Pompe, and MPS-IH from unaffected newborns. In addition, because 3 enzyme activities are measured simultaneously, the assay has an internal control for integrity that minimizes the occurrence of false-positives. The new procedure is simplified from the original published procedures because it requires fewer manipulations and resources.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: M.H. Gelb, Genzyme; C.R. Scott, Genzyme.
Stock Ownership: None declared.
Honoraria: C.R. Scott, Genzyme.
Research Funding: M.H. Gelb, NIH grants (DK67859 and HHSN26700603429) and Genzyme grants; C.R. Scott, NIH grants (DK67859 and HHSN26700603429) and Genzyme grants; F. Turecek, NIH grants (DK67859 and HHSN26700603429) and Genzyme grants.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We are grateful to Santosh Shaunak, Jessica Daiker, and Stand Kosciow of the Washington State Newborn Screening Program for continued cooperation and technical assistance.

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