Development and Evaluation of Quality Control Dried Blood Spot Materials in Newborn Screening for Lysosomal Storage Disorders

Victor R. De Jesus,1* X. Kate Zhang,2 Joan Keutzer,2 Olaf A. Bodamer,3 Adolf Mühl,3 Joseph J. Orsini,4 Michele Caggana,4 Robert F. Vogt,1 and W. Harry Hannon1

BACKGROUND: Lysosomal storage disorders (LSDs) comprise more than 40 genetic diseases that result in the accumulation of products that would normally be degraded by lysosomal enzymes. A tandem mass spectrometry (MS/MS)-based method is available for newborn screening for 5 LSDs, and many laboratories are initiating pilot studies to evaluate the incorporation of this method into their screening panels. We developed and evaluated dried blood spot (DBS) QC materials for LSDs and used the MS/MS method to investigate their suitability for LSD QC monitoring.

METHODS: We incubated 3.2-mm punches from DBS controls for 20–24 h with assay cocktails containing substrate and internal standard. Using MS/MS, we quantified the resulting product and internal standard. Samples were run in triplicate for 3 consecutive days, and results were reported as product-to-internal standard ratios and enzyme activity units (μmol/L/h).

RESULTS: Enzyme activity interday imprecision (CV) for the high, medium, and low series were 3.4%–14.3% for galactocerebroside α-galactosidase, 6.8%–24.6% for acid α-galactosidase A, 7.36%–22.1% for acid sphingomyelinase, 6.2%–26.2% for acid α-glucocerebrosidase, and 7.0%–24.8% for lysosomal acid α-glucosidase (n = 9). In addition, DBS stored at −20 °C and 4 °C showed minimal enzyme activity loss over a 187-d period. DBS stored at 37 °C and 45 °C had lower activity values over the 187-day evaluation time.

CONCLUSIONS: Suitable QC materials for newborn screening of LSDs were developed for laboratories performing DBS LSD screening. Good material linearity was observed, with goodness-of-fit values of 0.953 and higher. The QC materials may be used by screening laboratories that perform LSD analysis by MS and/or more conventional fluorescence-based screening methods.

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Lysosomal storage disorders (LSDs)5 include more than 40 unique genetic diseases that result in the accumulation of macromolecular substrates that would normally be degraded by lysosomal (and in some cases nonlysosomal) enzymes involved in lysosomal metabolism (1, 2). Although individual LSDs are rare, their combined incidence has been estimated at 1 per 7700 live births (1). Most LSDs are inherited in an autosomal recessive manner, with the exception of X-linked inherited Fabry, Hunter, and Danon diseases (3). In addition, LSDs exhibit heterogeneous clinical phenotypes that involve multiple organs and can manifest from birth to adulthood.

Diagnosis of LSDs usually entails assaying the enzyme of interest by means of an artificial substrate with a fluorescent tag, such as 4-methylumbelliferone, or a natural substrate mimic, in which a fragment of the substrate is labeled depending on the detection scheme in serum, leukocytes, or cultured skin fibroblasts (4). The wide availability of dried blood spot (DBS) specimens from newborn screening laboratories impelled the development of assays that use DBS collection devices. As a result, many screening diagnostic laboratories have developed and adapted fluorescence assays

1 Newborn Screening and Molecular Biology Branch, Centers for Disease Control and Prevention, Atlanta, GA; 2 Genzyme, Framingham, MA; 3 Division of Biochemical and Paediatric Genetics, University Children’s Hospital, Vienna, Austria; 4 New York Department of Health, Wadsworth Center, Albany, NY, USA
* Address correspondence to this author at: Newborn Screening and Molecular Biology Branch, Centers for Disease Control and Prevention, 4770 Buford Highway, Mail Stop F-19, Atlanta, GA 30341. Fax (770) 488-7459; e-mail vdejesus@cdc.gov

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5 Nonstandard abbreviations: LSD, lysosomal storage disorder; DBS, dried blood spot; MS/MS, tandem mass spectrometry; ABG, acid α-glucocerebrosidase; GLA, acid α-galactosidase A; GAA, lysosomal acid α-glucosidase; GALC, galactocerebroside α-galactosidase; ASM, acid sphingomyelinase; LC, liquid chromatography.
that rely on DBS for detection of Pompe (5), mucopolysaccharidosis type I (6), Fabry (7), and Gaucher and Niemann-Pick type A/B (8) diseases. Immuno-capture activity assays have also been developed for Pompe (9), Fabry (10), mucopolysaccharidosis type I (11), and Gaucher (12) diseases, although the availability of specific antibody reagents is a limiting factor in their use.

The introduction of tandem mass spectrometry (MS/MS) in newborn-screening laboratories has resulted in a rapid expansion of newborn-screening activities in both private and state-sponsored laboratories (13–17). Specifically, MS/MS has enabled laboratories to screen for several diseases in a multiplex fashion (e.g., for aminoacidopathies and inborn errors of fatty acid oxidation), allowing for increased throughput. Consequently, MS/MS-based assays offer an advantage over other methods owing to their ability to multiplex several analytes at once, thereby reducing substantially the costs incurred per patient sample. In addition, MS/MS technology is currently available in newborn screening laboratories around the world, and as a result many resources are available that are designed to ensure that high-quality data is produced by MS/MS system operators. Screening for LSDs by use of MS/MS technology was first described by Li et al. (18) for the direct multiplex assay that uses DBS to screen for Fabry, Gaucher, Krabbe, Niemann–Pick A/B, and Pompe diseases. The assay calls for the incubation of DBS collected from newborns by use of buffers containing substrates for the 5 lysosomal enzymes, followed by MS/MS of all 5 enzymatic products relevant to the diagnosis of the 5 diseases.

To support the quality of newborn-screening tests performed worldwide, the Newborn Screening Quality Assurance Program at the US CDC provides services to more than 73 domestic and 58 international newborn-screening laboratories. The CDC prepares and distributes more than 500,000 DBSs per year. The QC program enables screening laboratories to achieve high levels of technical proficiency and continuity that transcend changes in commercial assay reagents while maintaining high-volume specimen throughput. The DBS materials manufactured at CDC simulate as closely as possible the actual specimens tested by the participating laboratories.

The Newborn Screening Translation Research Initiative at the CDC provides reagents to be used for newborn screening to detect selected LSDs by MS/MS. QC materials for LSDs were developed and evaluated by use of the MS/MS method at 3 independent domestic laboratories and an international laboratory to investigate their suitability for LSD QC monitoring.

Materials and Methods

LSD MS/MS Multiplex Assay Cocktail Preparation
Vials containing substrate and internal standard for assaying deficient enzymes for Gaucher disease [acid α-glucocerebrosidase (ABG) deficiency], Fabry disease [acid α-galactosidase A (GLA) deficiency], Pompe disease [lysosomal acid α-glucosidase (GAA) deficiency], Krabbe disease [galactocerebrosidase α-galactosidase (GALC) deficiency], and Niemann–Pick disease, type A/B [acid sphingomyelinase (ASM) deficiency] were obtained from Kate Zhang (Genzyme). Appropriate buffer and detergent solutions were added to known lyophilized amounts of substrate and internal standard to create disorder-specific cocktails. On mixing, aliquots of the cocktails were then stored at −20 °C until use. Two one-eighth-inch (3.2 mm) punches from a DBS were used for the MS/MS multiplex assay (one for GALC and one for the other 4 assays).

DBS QC Materials Preparation
A base pool was created to serve as a very-low lysosomal enzyme activity pool for all 5 disorders. The base pool was prepared with leukocyte-reduced blood obtained from a regional blood bank (Tennessee Blood Services) and heat-inactivated, charcoal-stripped serum (SeraCare Life Sciences) mixed to achieve a mean (SD) 55% (5%) hematocrit level. In addition, unprocessed cord blood was purchased from the New Jersey Stem Cell Resource at the Coriell Institute for Medical Research (Camden, NJ) and used for high enzyme activity QC materials. The base pool and the unprocessed cord blood were mixed in different ratios to achieve a large range of enzyme activity levels, thus serving as low and medium activity controls.

All blood pools were spotted onto Whatman 903® filter paper in 100-μL aliquots, then dried overnight at room temperature and stored in airtight bags with desiccant to minimize moisture levels (19).

Specimen Shipment and Interlaboratory Evaluation Setup
Specimen cards containing 15 DBSs each were blinded and shipped unrefrigerated (samples shipped in November 2007) via overnight delivery to all the participants the week before the scheduled material evaluation. On specimen receipt, the participating laboratories were instructed to analyze the specimens in triplicate for 3 consecutive days (n = 9 per specimen; 3 specimens), following their own protocols for the assays performed at each location. In addition, the laboratories were asked to analyze calibration samples to ascertain their MS response to the analytes (data not shown). Specimens analyzed at the CDC were not
shipped, and served as indicators of lysosomal enzyme stability during shipment.

**SAMPLE WORKUP**

The method described by Li et al. (18) was followed, with modifications. Briefly, 70 µL of 20 mmol/L sodium phosphate monobasic (NaH₂PO₄), pH 7.1, were added to one 3.2-mm DBS punch, and the specimen was then extracted at 37 °C in a thermostated incubator (Boekel Jitterbug) for 60 min. After extraction, 10 µL of extract were added to 15 µL of GAA, GLA, ABG, and ASM cocktails in separate wells. In separate wells 30 µL GALC cocktail was added to a second 3.2-mm DBS punch. The 96-well plates were then sealed with aluminum film and incubated in a thermostated orbital shaker at 225 rpm and 37 °C for 20–24 h.

The enzymatic reactions were quenched with 100 µL of a solution containing 50% ethyl acetate/methanol. After quenching, the 5 assay reactions were then combined into a single deep well, where 400 µL each of ethyl acetate and water were added and mixed. The deep-well plate was then centrifuged, and a 200–300 µL aliquot of the organic top layer was transferred into a clean deep-well plate. The organic phase was dried under a gentle nitrogen stream, and reconstituted with 100 µL of a 95% ethyl acetate/methanol solution. After reconstitution, the samples were subjected to a solid-phase extraction step performed with 100-mg silica gel and washing with 1.6 mL 95% ethyl acetate/methanol. The extract and the washes were dried under nitrogen and resuspended with 200 µL of an 80/20/0.2% acetonitrile/water/formic acid solution before liquid chromatography (LC)-MS/MS analysis or 100 µL of 80/20/5 mmol/L acetonitrile/water/ammonium formate.

**LC-MS/MS ANALYSIS**

Samples were analyzed at the CDC on an API 3200 LC-MS/MS system (Applied Biosystems). Analysis at the laboratory at Genzyme was done on an API 4000 LC-MS/MS system (Applied Biosystems), whereas analyses at the New York Department of Health and the University Children’s Hospital (Vienna, Austria) were performed on a Waters Quattro micro LC-MS/MS system and an API 2000 LC-MS/MS system (Applied Biosystems), respectively. The electrospray source operated in positive mode, and the analytes were interrogated in multiple reaction–monitoring mode. Samples were introduced to the mass spectrometer by flow injection. Blank filter paper spots were analyzed for background correction. Enzyme activities (in units of µmol/L/h) were determined by calculating the ion abundance ratio of product:internal standard ratios, multiplied by the amount of added internal standard and divided by the response factor ratio of product to internal standard, sample incubation time, and sample blood volume.

### Table 1. Mean enzyme activity (n = 9) for interlaboratory evaluation of low-QC materials.

<table>
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<th>Laboratory</th>
<th>Enzyme activity, µmol/L/h</th>
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<td></td>
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*NE, not evaluated

### Table 2. Mean enzyme activity (n = 9) for interlaboratory evaluation of medium-QC materials.

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*NE, not evaluated
Results

DBS QC MATERIAL EVALUATION

The blinded samples consisted of unprocessed cord blood (high QC), a 50/50 mixture of the cord blood and a leukocyte-reduced base pool (medium QC), and a 5% cord blood in base pool (low QC). The laboratories were instructed to provide product:internal standard ratios (see Supplemental Tables 1–3 available with the online version of this article at http://www.clinchem.org/content/vol55/issue1) and blank-corrected enzyme activities for each sample (Tables 1–3). Product:internal standard ratios were requested to compare instrumental response to all analytes, given that enzymatic activity calculations take into account each laboratory's specific value for blood volume in a blood-spot punch. All samples were received as scheduled, except for those sent to the University Children’s Hospital, which were delayed by 2 days in transit. All assays were linear up to Product:internal standard ratio = 5; assay performance characteristics have been described elsewhere (18). Enzymatic activity interday precision (CV) for the high, medium, and low series were 3.4%–14.3% for GALC, 6.8%–24.6% for GLA, 7.36%–22.1% for ASM, 6.2%–26.2% for ABG, and 7.0%–24.8% for GAA (see Supplemental Figs. 1–3).

DBS QC MATERIAL STABILITY

High-QC control DBSs were placed in airtight bags with desiccant packs at 5 different temperatures (–20, 4, 25, 37, and 45 °C) to examine enzyme stability in DBS during storage at the CDC laboratories for 187 days. Results suggest that lysosomal enzymes are stable as shown in Figure 1.

Table 1. Mean enzyme activity \((n = 9)\) for interlaboratory evaluation of high-QC materials.

<table>
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<tr>
<th>Laboratory</th>
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<th>ASM</th>
<th>ABG</th>
<th>GAA</th>
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*a* NE, not evaluated

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**Fig. 1.** Lysosomal enzyme stability in DBS at $-20 ^\circ C$. 
in DBS at $-20$ and $4 \, ^{\circ}C$, with minimal enzymatic activity loss observed (Figs. 1, 2). Enzymatic activity loss was minimal also at $25 \, ^{\circ}C$, except for GLA, which exhibited a 42% loss of activity when compared to day 0 levels (see Supplemental Fig. 4). Storage at 37 (Fig. 3) and 45 $^{\circ}C$ resulted in lower activity values ranging from 4.5% to 61% activity loss over the 187-d evaluation time (see Supplemental Fig. 5).
Discussion

The Newborn Screening Quality Assurance Program developed QC materials for LSD screening because many newborn-screening laboratories are considering or currently performing these assays. The development of QC materials for newborn screening of LSDs resulted in suitable linear dose–response blood pools for use in screening laboratories. Good linearity for the 5 queried enzymes was observed, with goodness-of-fit values of 0.953 and higher. The QC materials may be used by screening laboratories that perform LSD analysis by MS.

All 4 laboratories achieved comparable data using different instrumentation, with 1 exception. ABG results from 1 laboratory were increased, and after investigation it was determined that the instrument used for the analysis was not sufficiently sensitive to query the ABG enzymatic product. Although proper instrument optimization resulted in analytical values that were closer to the other 3 laboratories’ mean values, this exception may be a concern to laboratories that operate older instrumentation. Consequently, it is recommended that laboratory personnel ascertain their mass spectrometer performance using the QC materials provided by the CDC during their assay validation experiments to avoid potential false-positive screening samples. In addition, shipment of the QC materials with desiccant to a European destination in the winter months did not affect the integrity of the samples, given that analytical results obtained at the CDC laboratory were comparable to those from the 3 outside participating laboratories.

The highest CV values were observed in the low-activity pool. The results may be attributed to differences in peak integration during data analysis, as well as instrumental variation among laboratories. Furthermore, low enzyme activity values may be confounded by the creation of product molecules during sample introduction into the mass spectrometer by a process called insource fragmentation, attributable mainly to incomplete removal of the enzymatic substrate during sample workup. This effect is an important consideration for laboratories considering implementing LSD screening, because an LSD screen-positive sample would likely exhibit some residual enzymatic activity. Proper assay validation calls for the complete characterization of insource fragmentation, which may be achieved with the use of the QC materials described in this report. The low-QC pools may also be used to examine analytical method limit of detection and carryover characteristics for the instruments, as well as the sample workup process by monitoring for the presence of substrate in the samples. The low-QC blood pool was designed to mimic a potential screen-positive sample. CV values for the medium- and high-QC controls were below 21% for all reactions. However, population-based studies are needed to establish useful clinical reference intervals for affected and nonaffected individuals.

DBS samples were shown to be stable for 6 months under dry storage conditions at −20 and 4 °C, with minimal loss of enzyme activity. QC DBS materials are prepared and stored at the CDC at −20 °C, thus ensuring DBS sample integrity before shipment. However, the data suggests that QC samples should be distributed refrigerated, to eliminate any enzyme activity losses during transport. Current work continues to evaluate QC DBS material stability to ascertain sample integrity during extended storage and shipment conditions.

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References


