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Multiplex newborn screening for Pompe, Fabry, Hunter, Gaucher, and Hurler diseases using a digital microfluidic platform

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ABSTRACT

Purpose: New therapies for lysosomal storage diseases (LSDs) have generated interest in screening newborns for these conditions. We present performance validation data on a digital microfluidic platform that performs multiplex enzymatic assays for Pompe, Fabry, Hunter, Gaucher, and Hurler diseases.
Methods: We developed an investigational disposable digital microfluidic cartridge that uses a single dried blood spot (DBS) punch for performing a 5-plex fluorometric enzymatic assay on up to 44 DBS samples. Precision and linearity of the assays were determined by analyzing quality control DBS samples; clinical performance was determined by analyzing 600 presumed normal and known affected samples (12 for Pompe, 7 for Fabry and 10 each for Hunter, Gaucher and Hurler).
Results: Overall coefficient of variation (CV) values between cartridges, days, instruments, and operators ranged from 2 to 21%; linearity correlation coefficients were ≥0.98 for all assays. The multiplex enzymatic assay performed from a single DBS punch was able to discriminate presumed normal from known affected samples for 5 LSDs.
Conclusions: Digital microfluidic technology shows potential for rapid, high-throughput screening for 5 LSDs in a newborn screening laboratory environment. Sample preparation to enzymatic activity on each cartridge is less than 3 h.

1. Introduction

Newborn screening (NBS) is generally performed for those diseases for which both an inexpensive screening test and treatment are available and the disease, if untreated, leads to profound morbidity or mortality. Nationally, newborn screening began with a screen for phenylketonuria [1], and has since expanded to include >50 conditions [2] due to available treatments and technological advances in screening, such us multiplex tandem mass spectrometry (MS/MS) [3]. Lysosomal storage diseases (LSDs) have been identified as diseases where patients can greatly benefit from newborn screening. Although individual LSDs are rare, as a group they can lead to devastating consequences, including infant mortality [4], and many can be screened using dried blood spots (DBSs) [5,6]. The combined incidence rate of LSDs is reported to be as high as 1:2315 live births [7]. Recent advances in treatment, including enzyme replacement therapy [8], for certain lysosomal storage diseases have generated renewed interest in newborn screening for individual LSDs [9,10]. Here, we focus on a multiplex method using a single DBS punch to screen for five LSDs with proven treatments: Pompe disease (glycogen storage disease type II, caused by acid α-glucosidase (GAA) deficiency), Fabry disease (α-galactosidase (GLA) deficiency), Hunter disease (mucopolysaccharidosis type II, iduronate-2-sulfatase (IDS) deficiency), Gaucher disease (glucocerebrosidase (GBA) deficiency), and Hurler disease (mucopolysaccharidosis type I, α-iduronidase (IDU) deficiency).

At present, only 5 states in the USA (including New York, Illinois, Missouri, New Mexico, and New Jersey) have mandated newborn

Abbreviations: LSDs, lysosomal storage diseases; DBS, dried blood spot; NBS, newborn screening; GAA, acid α-glucosidase; GLA, acid α-galactosidase; IDS, acid α-L-iduronate-2-sulfatase; GBA, acid β-D-glucosidase; IDU, acid α-L-iduronidase; ALL, advanced liquid logic, Inc.; CDC, Centers for Disease Control and Prevention; 4-MU-α-Gal, 4-methylumbelliferyl α-D-galactopyranoside; GalNac, N-acetyl-D-galactosamine; 4-MU-α-gluc, 4-methylumbelliferyl α-D-glucopyranoside; 4-MU-β-Gluc, 4-methylumbelliferyl β-D-glucopyranoside; 4-MU, 4-methyl umbelliferone; D-Sac, D-saccharic acid 1,4 lactone; DMSO, dimethyl sulfoxide; 4-MU-α-IDU, 4-methylumbelliferyl α-L-iduronide; 4-MU-α-IDS, 4-methylumbelliferyl α-L-iduronate-2-sulfate; β-MBCD, methyl-β-cyclodextrin; RFU, relative fluorescence units; CLSI, Clinical and Laboratory Standards Institute; QCH, quality control high, cord blood adjusted to 50.5% hematocrit on filter paper; QCM, quality control medium, 50% cord blood + 50% leukoreduced adult blood adjusted to 50.5% hematocrit on filter paper; QCL, quality control low, 5% cord blood + 95% leukoreduced adult blood adjusted to 50.5% hematocrit on filter paper; QCBP, quality control base pool, 0% cord blood + 100% leukoreduced adult blood adjusted to 50.5% hematocrit on filter paper.

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screening for select LSDs. Currently, the technologies available to perform DBS assays for lysosomal storage diseases are tandem mass spectrometry [11], microplate fluorometry [12], immunoassays [13] and, more recently, digital microfluidic fluorometry [14]. As more states move to adopt LSD screening, there is a need to develop rapid, efficient and economical high-throughput methods to screen for multiple LSDs simultaneously [15–17]. Orsini et al. reported using a 4 + 1 multiplex tandem MS/MS platform to screen for Gaucher, Pompe, Krabbe, Fabry, and Niemann-Pick A/B that reduced processing time and test complexity [18]. Mechtler et al. reduced tandem MS/MS incubation times to less than 4 h using a multiplex assay for Pompe, Fabry, Hunter, Gaucher, and Niemann-Pick A/B [15].

We have previously demonstrated that digital microfluidics can be used to screen for Hunter, Pompe and Fabry diseases using low throughput disposable cartridges [14,19,20]. A prototype cartridge designed by Advanced Liquid Logic, Inc. (ALL) that performed 3 assays (Pompe, Fabry and Gaucher) on up to 12 samples was recently pilot tested in Illinois Department of Public Health laboratories [21]. From a total of 8012 DBS samples screened, seven cases of Fabry and two cases of Gaucher disease were confirmed.

In this report, we demonstrate the capabilities of a new digital microfluidic system to meet the high throughput and rapid turnaround requirements of a newborn screening laboratory by means of a 5-plex fluorometric enzymatic assay for Pompe, Fabry, Hunter, Gaucher and Hurler diseases on a cartridge that accepts 44 specimens. The platform utilizes a single disposable cartridge that automates all liquid handling steps and reduces the overall time to result to <3 h, using a single 3 mm DBS punch from each of the 44 specimens.

2. Materials and methods

2.1. Dried blood spot samples

We obtained presumed normal, de-identified dried blot spots (NBS cards) from the North Carolina Division of Public Health NBS laboratories under a material transfer agreement. These spots were 2–3 months old and were stored at −20 °C upon receipt. Duke University Biochemical Genetics Laboratory (Durham, NC), Shire Human Genetic Therapies, Inc. (Lexington, MA) and the Centers for Disease Control and Prevention (Atlanta, GA; CDC) laboratories provided de-identified affected DBS for Pompe (n = 12), Fabry (n = 7), Hunter (n = 10), Gaucher (n = 10) and Hurler (n = 10) diseases under an IRB-approved protocol. These affected spotted specimens were from disease patients (not from newborns) that had not received enzyme replacement therapy.

2.2. Reagents

4-Methylumbelliferyl α-D-galactopyranoside-(4MU-α-Gal), N-acetyl-D-galactosamine (GalNac), 4-methylumbelliferyl-α-D-glucopyranoside (4-MU-α-Gluc), acarbose, 4-methylumbelliferyl-β-D-glucopyranoside (4-MU-β-Gluc), 4-methylumbelliferone sodium salt (4-MU), D-saccharic acid 1,4-lactone monohydrate (D-Sac), sodium acetate (99% pure), sodium bicarbonate, sodium taurocholate, ethylenediaminetetraacetic acid, dimethyl sulfoxide (DMSO), citrate phosphate, and Tween 20 were all from Sigma-Aldrich Corp. (St. Louis, MO; www.sigmaaldrich.com). 4-Methylumbelliferyl-α-L-iduronide sodium salt (4-MU-α-IDU) was from Affymetrix (Santa Clara, CA; http://www.affymetrix.com). 4-Methylumbelliferyl-α-L-iduronide-2-Sulfate (4-MU-IDS) was from Moscerdam Substrates (The Netherlands; www.moscerdam.com). Triton X-100 was obtained from Mallinckrodt (Hazelwood, MO; www.mallinckrodt.com). Molecular grade water and methyl β-cyclodextrin (β-MBCD) were from Fisher Scientific (Pittsburgh, PA; www.fishersci.com), and acetic acid (glacial) was from Fluka (Sigma-Aldrich). 5cSt silicone oil was from Gelest (Morrisville, PA; www.gelest.com).

2.3. Sample and reagent preparation

For each dried blood spot from normal newborns and affected newborns, a single 3 mm punch was obtained and stored in a separate 96-well plate at −20 °C. Formulations for Pompe, Fabry, and Hunter reagents and inhibitor solutions have been published previously [14,19]. Briefly, Pompe and Fabry reagents were prepared as described earlier [14] with the addition of 20.0 mmol/l methyl β–cyclodextrin (β-MBCD). Hunter reagent was prepared in assay buffer as described earlier [19] with the addition of 20.0 mmol/l β-MBCD. Gaucher reagent was prepared in assay buffer (0.1/0.2 mol/l citrate phosphate buffer pH 5.2 with 0.01% Tween 20 and 1.5% sodium taurocholate) in the absence of inhibitor to a final reagent concentration of 16 mmol/l of 4-MU-β-Gluc with 1.5% sodium taurocholate. Hurler reagent was prepared in assay buffer (0.04 mol/l pH 3.5 acetate buffer with 20 mmol/l β-MBCD and 300 mmol/l NaCl and 0.01% Tween 20) in the presence of 3.0 mmol/l of D-Saccharolactone inhibitor to a final concentration of 2 mmol/l 4-MU-α-IDU with 3.0 mmol/l D-Sac and 20 mmol/l β-MBCD. Ready-to-use reagent aliquots containing the substrate, assay buffer, inhibitors and other additives were prepared ahead of time and stored at −80 °C; working solutions were prepared just before use for each experiment. Other buffers, such as the assay extraction buffer (0.1% (w/v) Tween 20 in water, labeled as EXT) and stop buffer (0.2 mol/l $NaHCO_3$, pH 10.0, 0.01% (w/v) Tween 20) solutions were prepared and stored at room temperature.

2.4. Description and loading protocol for a 48 sample-input cartridge

In order to achieve the sample throughput required by newborn screening laboratories, we developed a disposable, single use digital microfluidic cartridge that has 48 input reservoirs for DBS extracts, 4-Methyl Umbelliferone (4-MU) calibrants, extraction buffer and quality control (QC) spot extracts, 5 input reservoirs for enzymatic substrates in assay buffers, 5 input reservoirs for stop buffers, and one large output reservoir (waste reservoir) to collect all of the droplets after incubation and detection (Fig. 1, digital microfluidic cartridge). The sample reservoirs are labeled as per the 96-well format with rows (horizontal) labeled with letters and columns (vertical) labeled with numbers to assist in transferring the extracts from the 96-well plate. The entire space between the top plastic layer and the printed circuit board of the cartridge is filled with silicone oil prior to loading the reagents and DBS extracts to prevent evaporation of the droplets during the incubation process.

DBS punches (3 mm diameter) were eluted with extraction buffer (100 μl) in standard deep-well 96-well plates on an orbital shaker for 30 min at ambient temperature. Prior to sample loading, the cartridge was inserted into a deck on a desktop analyzer as described earlier in [22]. The analyzer houses all the electrical components required to perform microfluidic operations, a heated deck that can be programmed to the required temperature, and a fluorometer capable of detecting 4-MU at wavelengths of 370 nm for excitation and 460 nm for emission. The extracts from the dried blood spot samples (1.6 μl) were then transferred to the sample input wells on the digital microfluidic cartridge (Fig. 1) using a multi-channel pipette. The prepared reagents, calibrators and stop buffers were equilibrated to ambient temperature and transferred to the appropriate wells on the digital microfluidic cartridge. The protocol used to perform the enzymatic analysis on-cartridge is briefly described in the following section.

2.4.1. Five-plex, 48 sample cartridge on-chip protocol

Once the cartridge had been loaded with the samples, buffers and reagents, all of the subsequent steps described in this protocol were performed entirely under software control and required no operator