Published in final edited form as:
Clin Biochem. 2013 December ; 46(18): 1889–1891. doi:10.1016/j.clinbiochem.2013.09.003.

Novel application of digital microfluidics for the detection of biotinidase deficiency in newborns

Carrie Graham[a], **Ramakrishna S Sista**[a], **Jairus Kleinert**[a], **Ning Wu**[a], **Allen Eckhardt**[a], **Deeksha Bali**[b], **David S Millington**[b], and **Vamsee K. Pamula**[a]

[a]Advanced Liquid Logic, Inc. (an Illumina Company), PO Box 14025, Research Triangle Park, NC, 27709 USA

[b]Division of Medical Genetics, Department of Pediatrics, Duke University Medical Center, 801 Capitola Dr, Suite 6, Durham, NC, 27713 USA

Abstract

Objective—Newborn screening for biotinidase deficiency can be performed using a fluorometric enzyme assay on dried blood spot specimens. As a pre-requisite to the consolidation of different enzymatic assays onto a single platform, we describe here a novel analytical method for detecting biotinidase deficiency using the same digital microfluidic cartridge that has already been demonstrated to screen for five lysosomal storage diseases (Pompe, Fabry, Gaucher, Hurler and Hunter) in a multiplex format.

Methods—A novel assay to quantify biotinidase concentration in dried blood spots (DBS) was developed and optimized on the digital microfluidic platform using proficiency testing samples from the Centers for Disease Control and Prevention. The enzymatic assay uses 4-methylumbelliferyl biotin as the fluorogenic substrate. Biotinidase deficiency assays were performed on normal (n=200) and deficient (n=7) newborn DBS specimens.

Results—Enzymatic activity analysis of biotinidase deficiency revealed distinct separation between normal and affected DBS specimens using digital microfluidics and these results matched the expected activity.

Conclusions—This study has demonstrated performance of biotinidase deficiency assays by measurement of 4-methylumbelliferyl product on a digital microfluidic platform. Due to the inherent ease in multiplexing on such a platform, consolidation of other fluorimetric assays onto a single cartridge may be realized.

Keywords

Newborn screening; biotinidase deficiency; digital microfluidics; dried blood spot; multiplex assay

Correspondence: Vamsee K. Pamula, PhD, Advanced Liquid Logic, Inc. (an Illumina Company), PO Box 14025, Research Triangle Park, NC 27709 USA, vpamula@liquid-logic.com; Phone: +1-(919)-287-9010; Fax: +1-(919)-287-9011.

1. Introduction

Newborn screening (NBS) via dried blood spots (DBS) identifies a number of diseases that, if left untreated, can have devastating consequences. Biotinidase deficiency is one such disease that results in an inability to recycle biotin, which can lead to multiple carboxylase deficiency [1]. Symptoms of biotinidase deficiency can include rash, seizures, respiratory ailments and developmental delays. Early diagnosis and a straightforward treatment with pharmacological biotin can thereby provide newborns with a positive health outcome.

Assays for biotinidase deficiency were originally described in the 1980's, and newborn bloodspot screening for biotinidase deficiency is routinely performed using either colorimetric [2, 3] or fluorometric [4] enzymatic assays. Screening for biotinidase deficiency is now universally and fully implemented in all U.S. states, although many states still perform manual assays [5]. Processing times for screening range from 90 minutes to up to 4 hours for automated processing and overnight incubation for manual processing.

As new high-throughput technologies are developed and more diseases are added to the recommended universal screening panel, NBS programs can greatly benefit from consolidation of several assays into integrated, multiplexed platforms. The benefits of multiplexing, such as better utilization of testing personnel, efficient use of dried blood spot punches and the ability to perform additional assays without further complicating workflows, are evident from the introduction of mass spectrometry for NBS [6].

We recently demonstrated digital microfluidic (DMF) multiplex screening for Pompe, Fabry, Gaucher, Hurler and Hunter lysosomal storage diseases (LSDs) using fluorescence enzymatic assays [7] that require 100 nL of extract per reaction out of a total of 100 μL extract obtained from a single DBS punch. A disposable DMF cartridge used with the analyzer has 48 reservoirs for samples, quality controls and calibrants. The cartridge also includes 10 reagent reservoirs. The DMF cartridges are inherently flexible so that assays requiring different reaction conditions can be programmed together. In this article, we present a novel analytical application of a rapid and automated fluorometric assay for biotinidase deficiency on the same digital microfluidic cartridge used for multiplex LSD screening.

2. Materials and Methods

2.1 Dried Blood Spot Samples

Under a material transfer agreement, we obtained de-identified dried blood spots (NBS cards; n=200) from the North Carolina State Laboratory of Public Health (NCSLPH), Newborn Screening Section. These newborn DBS specimens had normal activity for biotinidase deficiency, as analyzed by NCSLPH. These leftover specimens were approximately 2 weeks old and were stored at -20 °C upon receipt. Under an IRB-approved protocol, Michigan Department of Community Health Bureau of Laboratories, Neonatal Biobank provided de-identified DBS spots (n=7) from newborns with known biotinidase-deficiency. We obtained biotinidase proficiency testing (PT) DBS specimens (deficient and normal) from the Centers for Disease Control and Prevention (CDC).

2.2 Reagents

4-methylumbelliferyl biotin (4-MU biotin; n-D-biotinyl-7-amino-4-methylcoumarin) was purchased from Toronto Research Chemicals (Toronto, Canada; http://www.trc-canada.com). Sodium bicarbonate, potassium phosphate (monobasic and dibasic), DL-dithiothreitol (DTT) and Tween 20 were all obtained from Sigma-Aldrich Corp. (St. Louis, MO; www.sigmaaldrich.com). Molecular grade water was purchased from Fisher Scientific

(Pittsburgh, PA; www.fishersci.com). 5cSt silicone oil was obtained from Gelest Inc., (Morrisville, PA; www.gelest.com).

2.3 Sample and Reagent Preparation

Dried blood spots were punched and stored at -20 °C as follows. Briefly, for each of CDC proficiency DBS, normal DBS (n=200) and biotinidase-deficient DBS (n=7), one 3 mm punch was collected in a 96-well plate. Reagent was comprised of 0.1 mmol/L 4 MU-biotin, 1.1 mmol/L DTT and 0.1% Tween 20 in 0.15 mol/L KH_2P0_4, pH 6.5. Ready-to-use reagent aliquots were prepared and stored at -80 °C. Extraction buffer (water with 0.1% Tween 20) and stop buffer (0.6 mol/L $NaHC0_3$ pH 11.0 with 0.01% Tween 20) solutions were prepared and stored at room temperature.

2.4 Digital Microfluidic Enzyme Assay for Detection of Biotinidase Deficiency

2.4.1 Assay Protocol—The experiments described herein were performed on disposable, single use digital microfluidic cartridges that can accept up to 48 samples and calibrants. This cartridge was previously used to demonstrate fluorometric assays for Pompe, Fabry, Gaucher, Hunter and Hurler diseases [7]. The protocol for these 5-plex assays was previously described [7] and has essentially been kept the same for the biotinidase assay.

Briefly, prior to the start of the assay, a four point calibration curve was generated in duplicate on the cartridge with the following concentrations of 4-MU in stop buffer droplets: 0.0375 μmol/L, 0.075 μmol/L, 0.15 μmol/L and 0.3 μmol/L. A 3 mm punch from each DBS was subject to extraction in 100 μL extraction buffer (0.1% (w/v) Tween 20 in water) for 30 minutes at room temperature in a 96 well plate. During this time, a DMF cartridge was inserted into the analyzer. After extraction was complete, 3.5 μL DBS extract was loaded into each of the 44 reservoirs on the cartridge via a multi-channel pipette along with loading of 12 μL each of fluorescence reagent and stop buffer in reagent reservoirs. All subsequent droplet handling operations were automated on the cartridge by the digital microfluidic platform.

To begin the reaction, one droplet (~100 nL) of DBS extract from each of the 44 reservoirs was dispensed and mixed with one droplet (~100 nL) of reagent (4-MU biotin at pH 6.5) to form 44 reaction droplets (~200 nL each). The reaction droplets were incubated for one hour at 37 °C on the DMF cartridge. The reactions were stopped when a droplet of stop buffer (~100 nL) was dispensed and merged with each reaction droplet. Endpoint fluorescence was measured at 360 nm excitation and 460 nm emission. Enzymatic activity was reported as μmoles of 4-MU produced per liter of blood per hour of incubation using the 4-MU calibration curve.

The protocol for biotinidase deficiency screening using digital microfluidics was first optimized using PT samples (deficient (n=9) and normal (n=26)) obtained from the CDC. The performance of the assay was assessed using biotinidase-deficient DBS specimens (n=7) from Michigan Neonatal Biobank and normal DBS specimens (n=200) from the NCSLPH over a three day period. The samples from the Michigan Neonatal Biobank were known to be deficient for biotinidase prior to the study; the samples from NCSLPH were presumed to be normal and were confirmed normal by NCSLPH after the study was completed.

2.4.2 Enzymatic Activity Variability—Variability in measurement of biotinidase activity was assessed by determining intra-cartridge variability essentially as described previously [8]. Briefly, blood from normal adults was pooled together and spotted on DBS cards. Adult blood was used because biotinidase activity in older NBS samples may be

significantly reduced. Several punches were taken from this set of DBS prepared from a single pool of blood. The punches were extracted and then pooled. The same pool of DBS extract was loaded into three separate cartridges; these cartridges were run consecutively on one instrument by the same operator in a single day. All reagents made in a single batch were used for experiments on all three cartridges. Inter-cartridge variability was also assessed from these results.

3. Results and Discussion

The feasibility of applying digital microfluidic technology to a fluorometric enzymatic assay for the detection of biotinidase deficiency was initially demonstrated by analysis of CDC's PT samples. These specimens were analyzed in a blinded fashion and the results, summarized in Figure 1, clearly demonstrate that the method correctly identified deficient and normal PT samples. Reproducibility of the assay was determined by measuring intra-cartridge (40 samples per cartridge) and inter-cartridge variability using DBS punches from normal adult bloodspots. The inter-cartridge CV between all cartridges was 11.0%, and the median intra-cartridge CV was 6.6%.

The assay was also tested on newborn DBS specimens collected in state public health laboratories. One set of DBS from newborns with a confirmed diagnosis of biotinidase deficiency (n=7) was compared with another set of newborn DBS known to be normal (n=200). The results, summarized in Figure 1, showed complete discrimination between the normal and affected patient samples. In Figure 1, the horizontal black line represents the median and the error bars represent the inter-quartile range. The range of enzymatic activity for the normal samples was 4.6 - 41.9 (mean 11.7) μmol/L/h and range for affected samples was 2.3 - 3.2 μmol/L/h (mean 2.7). No overlap in enzymatic activity was observed with clear separation between normal and affected samples.

4. Conclusions

This study has demonstrated the successful translation of a fluorescent enzymatic assay for biotinidase deficiency onto a digital microfluidic platform that correctly identified normal and affected samples from newborn dried blood spots. The assay protocol for biotinidase deficiency screening using digital microfluidics is essentially identical to that previously described for multiplex screening of lysosomal storage diseases [7], suggesting that this platform is capable of performing multiple types of enzymatic assays from a single newborn dried blood spot specimen. This represents a critical step toward a single, easy to use, inexpensive and automated platform applicable to a variety of enzymatic newborn screening assays from dried blood spots.

Acknowledgments

We acknowledge Drs. Shu Chaing and Scott Zimmerman (North Carolina State Laboratory of Public Health) for providing de-identified normal DBS, the Centers for Disease Control and Prevention for providing proficiency testing samples and the Michigan Neonatal Biobank for providing affected DBS. Research reported in this publication was partly supported by the *Eunice Kennedy Shriver* National Institute of Child Health & Human Development of the National Institutes of Health under award number R44HD057713. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Non Standard Abbreviations

NBS	Newborn bloodspot screening
DBS	Dried blood spot
DMF	Digital microfluidic platform
LSD	Lysosomal storage diseases
NCSLPH	North Carolina State Laboratory of Public Health
PT	Proficiency testing
CDC	Centers for Disease Control and Prevention
4-MU- Biotin	4-methylumbelliferyl biotin
DTT	DL-dithiothreitol

Highlights

- Novel fluorometric enzymatic assay for biotinidase deficiency newborn screening.
- Biotinidase deficiency assay performed with an incubation time of 1 h.
- Digital microfluidic technology can potentially multiplex NBS enzymatic assays.

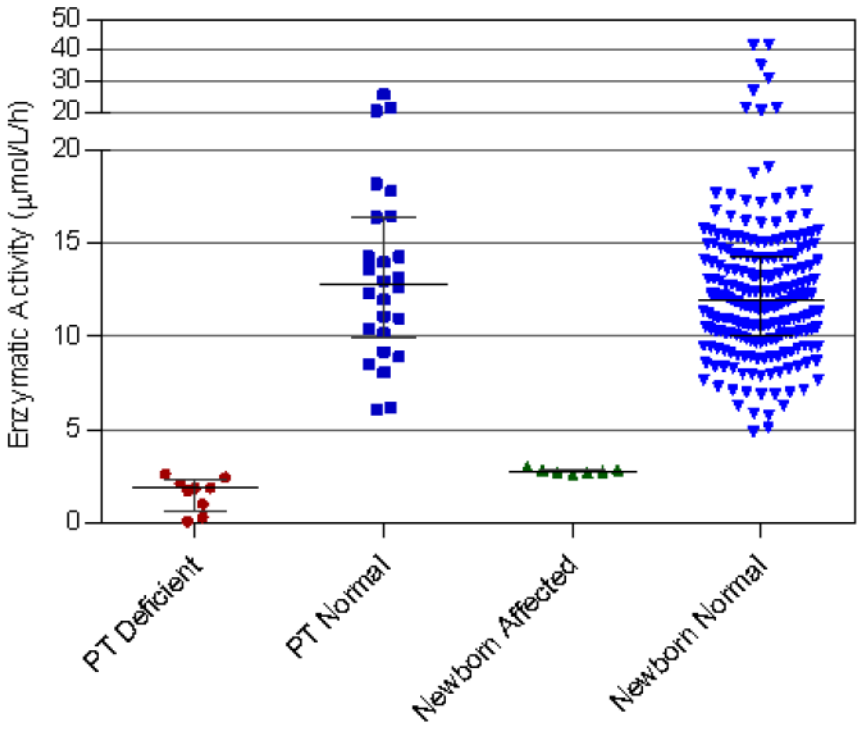

Figure 1.

Enzymatic activity results for biotinidase deficiency using a digital microfluidic cartridge. Deficient (n=9) and normal (n=26) proficiency testing (PT) samples and confirmed affected (n=7) and normal (n=200) newborn patient samples are represented. The horizontal black line in the middle represents the median while the error bars represent the inter-quartile range.