Development of Biochemical Assays for the Screening, Diagnosis, and Prognosis of Treatable Inborn Errors of Metabolism

Xinying Hong

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2020

Reading Committee:

Michael H. Gelb, Chair

Jesse G. Zalatan

Joshua C. Vaughan

Program Authorized to Offer Degree:

Department of Chemistry

University of Washington

Abstract

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Xinying Hong

Chair of the Supervisory Committee:
Professor Michael H. Gelb
Department of Chemistry

Inborn errors of metabolism (IEM) are a phenotypically and genetically heterogenous group of disorders caused by defective enzymes, cofactors or transporters in metabolic pathways, which lead to dysfunctional metabolism and/or the accumulation of toxic intermediate metabolites. Although individually rare, they are common as a group with a combined incidence rate of 1 in 800. A subset of these disorders have viable treatments and novel therapies are being developed at rapid pace for an increasing number of IEM. However, to achieve the optimal therapeutic outcome, it is essential to initiate treatments prior to the onset of irreversible symptoms. Therefore, treatable inborn errors of metabolism are excellent candidates for newborn screening

program, which is a broad public health prevention program that aims at identifying a number of conditions for which early intervention can reduce premature mortality and morbidity.

Tandem mass spectrometry (MS/MS) is the most comprehensive platform for quantifying enzymatic function and biomarkers in dried blood spots (DBS), which are the sample specimens used in newborn screening programs. An enormous effort has been made to develop high-throughput and robust MS/MS-based biochemical assays for newborn screening. Herein, we report (1) a liquid chromatography MS/MS (LC-MS/MS) assay to improve the current screening test for classic galactosemia; (2) a multiplexed ultra-performance liquid chromatography MS/MS (UPLC-MS/MS) assay for the screening of 18 IEM; (3) a large-scale study to investigate the feasibility of newborn screening of metachromatic leukodystrophy (MLD). Apart from these biochemical assays for screening purposes, a high-performance diagnostic test for MLD was developed. To better understand the clinical outcomes of the MLD-related variants, a transient gene expression system was designed to study these variants systematically.

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ACKNOWLEDGEMENTS

I would like to thank my advisor Michael H. Gelb for his constant support and guidance; my committee members for their advice and encouragement; the Chemistry Department of the University of Washington and the National Institutes of Health for financial support throughout graduate school. My research would not have been possible without the help of numerous lab members, peers, and personnel from the Department of Chemistry.

Chapter 1. Introduction

1.1 Inborn errors of metabolism

Sir Archibald Edward Garrod (1857-1936) first coined the term inborn errors of metabolism in early 1900s, followed by his extensive studies on alkaptonuria, cystinuria, pentosuria, and porphyria.[1, 2] Inborn errors of metabolism (IEM) are a phenotypically and genetically heterogenous group of disorders caused by defective enzymes, cofactors or transporters in metabolic pathways, leading to dysfunctional metabolism and/or the accumulation of toxic intermediate metabolites.[1] This group of disorders includes amino acid disorders, urea cycle defects, organic acidemias, fatty acid oxidation defects, mitochondrial disorders, carbohydrate metabolism disorders, peroxisomal disorders, purine and pyrimidine metabolism disorders, neuro-transmitter disorders, transport and mineral disorders, cholesterol and neural lipid metabolism disorders, lipid storage disorders, lysosomal storage disorders, and other miscellaneous defects.[1] To date, more than 1,000 inborn errors of metabolism have been identified and described.[3] Although individually rare, inborn errors of metabolism are common as a group and have a cumulative incidence rate of up to 1 in 800.[3]

Inborn errors of metabolism can present from the fetal stage to advanced age, and it can affect any cell type or organ, or can occur in combinations, with protean and often non-specific clinical presentations.[1] Untreated diseases can be highly detrimental, resulting in severe disabilities and fatalities.[4] Many of the inborn errors of metabolism are amenable to dietary intervention and and/or supplementation of deficient metabolites, which alleviate of metabolic stress and remove toxic metabolites.[1] Innovative treatments, including hemopoietic stem cell transplant, chaperone treatment, substrate reduction therapy, enzyme replacement therapy and gene therapy, are also advancing at rapid pace. However, the prognosis of these treatable inborn errors of metabolism is

only favorable when treatment is initiated prior to the onset of irreversible symptoms.[5, 6] Therefore, early diagnosis is crucial for optimal therapeutic outcomes.

1.2 Newborn screening of treatable inborn errors of metabolism

The newborn screening program in the U.S. dated back to early 1960s, when Dr. Robert Guthrie introduced a population screening program for phenylketonuria using a bacterial inhibition assay for the detection of phenylalanine in blood collected from newborns by heel stick and dried onto special filter paper.[7] Dr. Guthrie was prompted to pursue such testing because treatment for this otherwise devastating neurometabolic disorder had become available, and it had been shown that early initiation of treatment led to optimal outcome. The use of filter paper for the collection and analysis of human blood specimens also made cost-effective large-scale screening feasible. Newborn screening for phenylketonuria has been expanded into a broad public health prevention program, which aims at identifying an increasing number of conditions for which early intervention can prevent premature mortality, morbidity, and disability.[8] Today, more and more diseases are being included into the U.S. Recommended Uniform Screening Panel, nominated by the Secretary of the Department of health and Human Services, for states to screen as part of their state universal newborn screening program. As of July 2018, there are 35 core conditions and 26 secondary conditions on the Recommended Uniform Screening Panel (Table 1.1 and Table 1.2), the majority of which are inborn errors of metabolism.

Table 1.1. Core conditions on the Recommended Uniform Screening Panel (as of July 2018)[a]

Metabolic disorder			Endocrine disorder	Hemoglobin disorder	Other disorder
Organic acid condition	Fatty acid oxidation disorders	Amino acid disorders			
Propionic Acidemia	Carnitine Uptake Defect/Carnitine Transport Defect	Argininosuccinic Aciduria	Primary Congenital Hypothyroidism	S,S Disease (Sickle Cell Anemia)	Biotinidase Deficiency
Methylmalonic Acidemia (methylmalonyl-CoA mutase)	Medium-chain Acyl-CoA Dehydrogenase Deficiency	Citrullinemia, Type I	Congenital adrenal hyperplasia	S, βeta-Thalassemia	Critical Congenital Heart Disease
Methylmalonic Acidemia (Cobalamin disorders)	Very Long-chain Acyl-CoA Dehydrogenase Deficiency	Maple Syrup Urine Disease		Hemoglobin S-C Disease	Cystic Fibrosis
Isovaleric Acidemia uria	Long-chain L-3 Hydroxyacyl-CoA Dehydrogenase Deficiency	Homocystinuria			Classic Galactosemia
3-Methylcrotonyl-CoA Carboxylase Deficiency	Trifunctional Protein Deficiency	Classic Phenylketonuria			Glycogen Storage Disease Type II (Pompe)
3-Hydroxy-3-Methyglutaric Aciduria		Tyrosinemia, Type I			Hearing Loss
Holocarboxylase Synthase Deficiency					Severe Combined Immunodeficiency
ß-Ketothiolase Deficiency					Mucopolysaccharidosis Type 1
Glutaric Acidemia Type I					X-linked Adrenoleukodystrophy
					Spinal Muscular Atrophy due to homozygous deletion of exon 7 in SMN1

[a] Modified from https://www.hrsa.gov/advisory-committees/heritable-disorders/rusp/index.html

Table 1.2. Secondary conditions on the Recommended Uniform Screening Panel (as of July 2018)[a]

Metabolic disorder			Hemoglobin disorder	Other disorder
Organic acid condition	Fatty acid oxidation disorders	Amino acid disorders		
Methylmalonic acidemia with homocystinuria	Short-chain acyl-CoA dehydrogenase deficiency	Argininemia	Various other hemoglobinopathies	Galactose epimerase deficiency
Malonic acidemia	Medium/short-chain L-3-hydroxyacylCoA dehydrogenase deficiency	Citrullinemia, type II		Galactokinase deficiency
Isobutyrylglycinuria	Glutaric acidemia type II	Hypermethioninemia		T-cell related lymphocyte deficiencies
2-Methylbutyrylglycinuria	Medium-chain ketoacyl-CoA thiolase deficiency	Benign hyperphenylalaninemia		
3-Methylglutaconic aciduria	2,4 Dienoyl-CoA reductase deficiency	Biopterin defect in cofactor biosynthesis		
2-Methyl-3-hydroxybutyric aciduria	Carnitine palmitoyltransferase type I deficiency	Biopterin defect in cofactor regeneration		
	Carnitine palmitoyltransferase type II deficiency	Tyrosinemia, type II		
	Carnitine acylcarnitine translocase deficiency	Tyrosinemia, type III		

[a] Modified from https://www.hrsa.gov/advisory-committees/heritable-disorders/rusp/index.html

Many inborn errors of metabolism have been added into the mandatory screening panel over the years, most of which satisfy Wilson-Jungner criteria, including (1) the condition should be important; (2) there must be a recognizable latent or early symptomatic stage; (3) natural course of condition, including development from latent to declared disease, should be adequately understood; (4) suitable test or examination available; (5) test acceptable to population; (6) case finding should be continuous (not just a "once and for all" project); (7) accepted treatment for patients with recognized disease; (8) facilities for diagnosis and treatment available; (9) Agreed policy concerning whom to treat as patients; and (10) costs of case finding (including diagnosis and treatment of patients diagnosed) economically balanced in relation to possible expenditures on medical care as a whole.[9, 10] Detecting these disorders, which mainly present in childhood and are treatable, have great direct benefits to infants and their families.[1] As a result of the populational screening, the percentage of mental retardation caused by inborn errors of metabolism drastically reduced from 16.5% in 1950 to 0.005% in 2000.[11]

1.3 General strategies for newborn screening of treatable genetic conditions

General strategies for screening genetic disorders usually fall into one of these three categories: (1) quantifying the relevant biochemical markers in dried blood spots (DBS); (2) quantifying the enzymatic activity of the relevant enzyme in DBS, if the disease is caused by a deficient enzyme; and (3) quantifying the abundance of relevant proteins in DBS, typically via immunoassays.[4] The metabolic disorders targeted by the Recommended Universal Screening Panel (Table 1.1 and Table 1.2) are screened by measuring the relevant biomarkers in DBS by tandem mass spectrometry (MS/MS). Endocrine disorders are screened by quantifying the relevant biomarkers using immunoassays. Hemoglobin disorders are screening by detecting the presence or absence of hemoglobin F, A, S, C, E, and D in DBS using high throughput high-pressure liquid

chromatography (HPLC). Critical congenital heart disease and hearing loss are screened by bedside tests that do not involve DBS. Cystic fibrosis is screened by measuring the abundance of a relevant protein by immunoassay. Severe combined immunodeficiencies, T-cell related lymphocyte deficiencies, and spinal muscular atrophy caused by homozygous deletion of exon 7 in *SMN1* gene are screened by real-time PCR. Biotinidase deficiency, Pompe disease, and mucopolysaccharidosis type I (MPS-I) are screened by measuring the activities of the relevant enzymes in DBS. Classic galactosemia can be screened either by quantifying the relevant biomarker or the activity of the relevant enzyme in DBS. Neonates with galactose epimerase deficiency and galactokinase deficiency can be identified if the relevant biomarker for classic galactosemia (total galactose in DBS) is measured as the screening test.

Adding a second-tier test to follow up subjects with abnormal screening results is crucial to reduce the false-positive rate. The cost to perform the two-tier screening is moderately increased under these circumstances, but it is counterbalanced by fewer recalls, less clinical follow-ups, and a reduction in unnecessary anxiety for families.[12] Two-tier testing strategy has been implemented in the screening for cystic fibrosis,[13] medium-chain acyl-CoA dehydrogenase deficiency,[14] galactosemia,[14] and congenital adrenal hyperplasia,[12] inborn errors of propionate, methionine, and cobalamin metabolism,[15] and Krabbe disease[16]. Subjects with abnormal results for both primary and secondary tests will be contacted for clinical visit and confirmatory testing. It is believed that more second-tier tests will be implemented as part of routine screening when they become available, as they can improve both the sensitivity and the specificity of the screening.[17] Second-tier tests are not typically used in primary screening because they usually have lower sample throughput and are more expensive to carry out.[17]

Currently, genetic testing does not suffice as the primary screening test, due to the incomplete understanding of genotype-phenotype correlations, especially for rare diseases of which variant annotations are limited, albeit it may be the most intuitive way to detect genetic disorders. For example, genetic testing can often uncover variations of unknown significance, and therefore lead to inconclusive results. This can cause uncertainty and anxiety throughout the life of the subjects.

1.4 The use of tandem mass spectrometry for newborn screening of treatable genetic disorders

Currently, tandem mass spectrometry (MS/MS) and fluorometry are two most common detection platform for screening treatable genetic conditions. MS/MS is by far the most comprehensive detection platform for biochemical analysis for analytes in DBS, including enzymatic functions,[18-22] biomarkers,[23-26] and proteins of low abundance.[27] Fluorometric assays, while useful for measuring some enzymatic activities in DBS,[28] have limited applications in biomarker analysis. Immunoassays are useful for measuring abundant proteins and biomarkers in DBS, yet fall short of detecting proteins of extremely low concentration. They can also give rise to false negative errors in the case of properly folded, but enzymatically-inactive proteins.[29]

In newborn screening laboratories, the most common platform for MS/MS analysis is flow-injection MS/MS, where samples are introduced to the electrospray ionization (ESI) source directly by a continuous solvent flow without chromatography. Analytes are converted into ions in the heated ESI source and analyzed by MS/MS. Currently, all the metabolic disorders on the Recommended Universal Screening Panel (Table 1.1 and Table 1.2) are screened using flow-injection MS/MS, together with X-linked adrenoleukodystrophy, Mucopolysaccharidosis Type I

and Pompe disease, which are most recently included. However, flow-injection MS/MS analysis is inadequate to detect analytes of low abundance and to accurately quantify analytes when isobaric interferences are present. The alternative to flow-injection MS/MS is liquid chromatography (LC) MS/MS, where the in-line LC column provides additional analytical separation. LC-MS/MS also offers improved sensitivity over flow-injection MS/MS as the column reduces ionization suppression effect and improves the signal-to-noise ratio, especially for low concentration analytes. LC-MS/MS based assays have been implemented in newborn screening laboratories as well,[30, 31] although not as common due to the compromised throughput (2 minute vs 1 minute per sample for a typical LC-MS/MS analysis vs a typical flow-injection MS/MS analysis).

The Gelb Laboratory has been focused on developing MS/MS-based enzymatic and biomarker assays over the past decades. The key advantages of MS/MS-based assays include but not limited to: (1) they allow the use of natural substrates or close analogues, therefore have lower false-negative or false-positive rate compared to fluorometric assays, which use artificial substrates;[32] (2) they allow direct quantification of biomarkers without immuno-precipitation, which are required in fluorometry-based biomarker assays; (3) they are highly quantitative due to the use of internal standards, which are heavy-isotope labeled analytes; (4) they can simultaneously detect various analytes (multiplexing).

Shown in Fig. 1.1 are typical workflows for MS/MS-based enzymatic assays and biomarker assays. For a typical MS/MS-based enzymatic assay, assay cocktail containing enzymatic substrates and internal standards (isotope-labeled version of enzymatic products) in assay buffer is added to a 3-mm DBS punch. The assay cocktail typically consists of multiple pairs of substrate and internal standard, as MS/MS-based enzymatic assays are usually carried out in multiplexed manner so that multiple disorders can be screened simultaneously. Additives, i.e.

inhibitors, activators, and precipitators, are also included in the assay cocktail to improve the assay performance. The assay is then incubated at 37 °C for hours (usually from 3 to 20 hours) before a liquid-liquid extraction is performed to quench the reaction and clean up the sample by removing the majority of salts and ionic detergent. The organic layer that contains the analytes of interest is transferred out, dried down and reconstituted with proper solvent before analyzing by flow-injection MS/MS or LC-MS/MS analysis (Fig. 1.1a). The enzymatic activity can be calculated by quantifying the amount of enzymatic product generated after the incubation using the chemically-identical but isotopically-distinguished version of the enzymatic product. MS/MS-based assays are highly quantitative due to the use of internal standards, as any downstream loss of the analytes as well as suppression at the ionization source can be accounted for.

In a typical MS/MS-based biomarker assay, biomarkers in one 3-mm DBS punch were extracted with proper organic solvent (mainly methanol) containing suitable internal standards (isotope-labeled version of the biomarkers) for 0.5-4 hours. Derivatization is sometimes required to improve the MS/MS response of particular analytes, e.g. amino acids, acyl-carnitines and succinylacetone.[33] After minimal sample workup, the biomarkers or the biomarker derivatives are ready for flow-injection MS/MS or LC-MS/MS analysis (Fig. 1.1b).

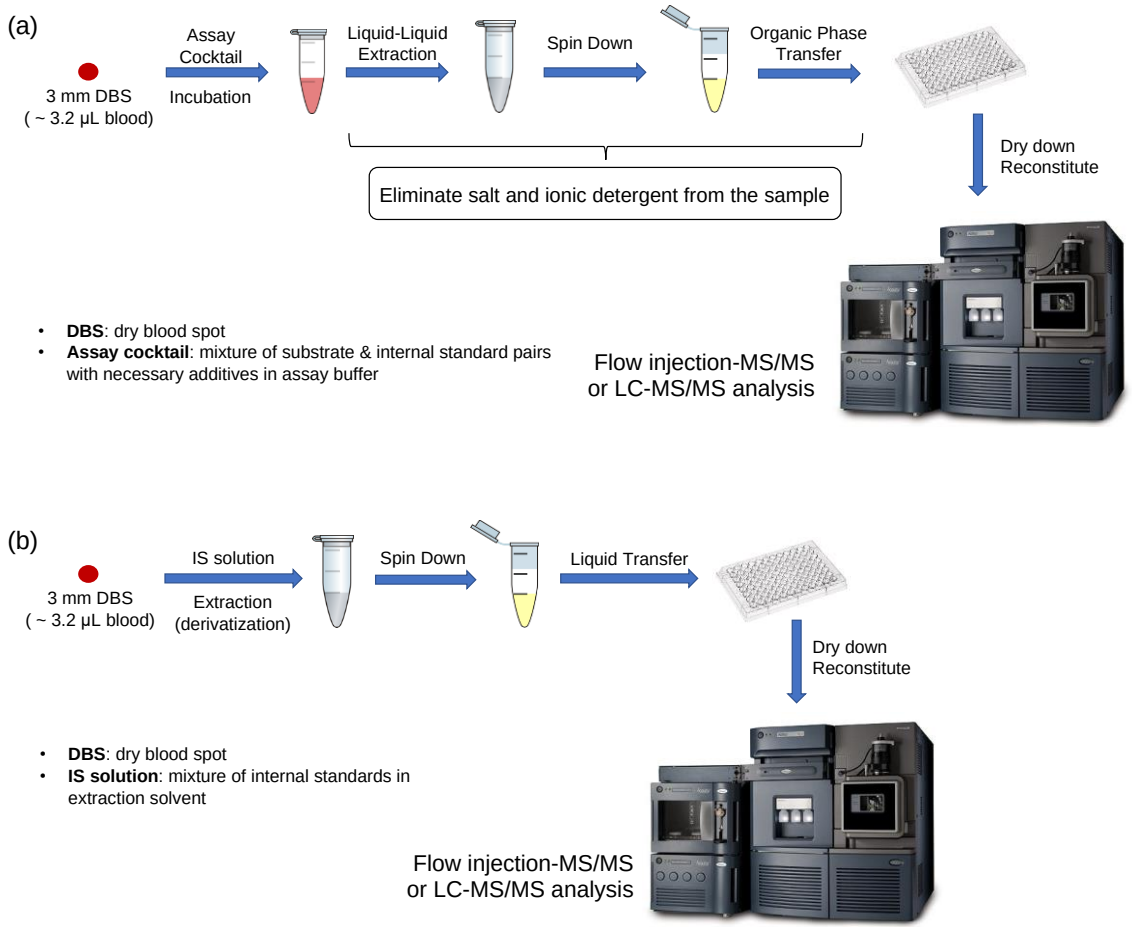


Figure 1.1 General workflow for (a) MS/MS-based enzymatic assay; and (b) MS/MS-based biomarker assay.

Chapter 2. Developing multiplexed assay for the screening of treatable genetic disorders

2.1 Developing a 5-plex HPLC-MS/MS assay for newborn screening of classic galactosemia, biotinidase deficiency, X-linked adrenoleukodystrophy, mucopolysaccharidosis type I, and Pompe disease[34]

The work presented in this section is published in Molecular Genetics and Metabolism: Hong et al., Multiplex tandem mass spectrometry assay for newborn screening of X-linked adrenoleukodystrophy, biotinidase deficiency, and galactosemia with flexibility to assay other enzyme assays and biomarkers. Mol Genet Metab ***2018,*** *124 (2), 101-108.*

All states in the United States are testing for classic galactosemia and biotinidase deficiency in their newborn screening programs. Classic galactosemia is caused by deficiency of galactose-1-phosphate uridyltransferase (GALT), one of the three galactose metabolic enzymes, and is the major inborn error of galactose metabolism. Although there is no cure for classic galactosemia or approved medication to replace the deficient enzyme, it can be treated with low-galactose diet. Most newborn screening laboratories are screening for this disorder by assaying GALT activity in DBS using quantitative Beutler fluorometric assay, in which a DBS punch is incubated with assay cocktail containing galactose-1-phosphate, UDP-glucose and $NADP^+$.[35] In the presence of GALT, glucose-1-phosphate and UDP-galactose are generated and phosphoglucomutase converts glucose-1-phosphate to glucose-6-phosphate, which is further converted to 6-phospho-D-glucono-1,5-lactone by glucose-6-phosphate dehydrogenase (G6PD) and then to ribulose-5-phosphate by 6-phosphogluconate dehydrogenase. These latter three enzymes are endogenous in human and the last 2 steps generate 2 equivalents of NADPH, which is highly florescent and can be detected by

fluorometry. However, this assay may give rise to false-positive results if the tested subject has G6PD deficiency, which is one of the most common genetic disorders in human.[36] Other GALT assays, which directly quantify one of the enzymatic product, UDP-galactose, through LC-MS/MS require lengthy LC analysis to separate UDP-galactose from its isobaric form, UDP-glucose,[37, 38] and therefore are not suitable for high throughput screening.

Biotinidase deficiency is an autosomal recessive metabolic disorder in which the body is unable to recycle and reuse the vitamin biotin and can be treated through daily oral supplementation of biotin. Screening laboratories are screening for this disorder by measuring the activity of biotinidase in newborn DBS using colorogenic or fluorogenic substrates.[39, 40] This requires a separate DBS punch from the one required for classic galactosemia.

X-linked adrenoleukodystrophy (X-ALD), mucopolysaccharidosis type I (MPS-I), and Pompe disease are the three disorders added to the Recommended Uniform Screening Panel most recently. X-ALD is caused by mutations in *ABCD1*, a gene located on the X chromosome that encodes a peroxisomal membrane transporter protein. Currently, X-ALD can be treated with allogeneic hematopoietic stem cell transplant, which can halt disease progression if initiated prior to the onset of irreversible symptoms.[41] X-ALD can be screened by measuring C26:0-lyso-phosphocholine (C26:0-LPC) in DBS using either flow-injection MS/MS or LC-MS/MS.[24, 42] Flow-injection MS/MS enables faster sample turnaround time compared to the LC-MS/MS method (1 min vs 2 min), but it also results in a 1.8% false-positive rate due to the endogenous isobaric interferences.[24, 43, 44] This necessitates a second-tier LC-MS/MS analysis to resolve the false-positive cases.[43, 45] On the other hand, if the LC-MS/MS analysis is used as the first-tier screening test for X-ALD, the second-tier test will not be required, but this may slow down the throughput, thus not suitable for laboratories with extremely high volume. MPS-I and Pompe

disease are caused by the deficiency of α-L-iduronidase (IDUA) and acid α-glucosidase (GAA), respectively. MPS-I can be treated with hematopoietic stem cell transplant and enzyme replacement therapy. Pompe disease can be treated with enzyme replacement therapy. These two disease can be screening either using flow-injection MS/MS in a multiplexed manner or digital microfluidics.[18, 28, 46]

In this study, we aimed to develop a rapid GALT assay that is not affected by G6PD, and to multiplex it with a new assay for biotinidase so that only one 3 mm DBS punch would be required to screen for these two disorders. The GALT-biotinidase duplex assay was further combined with the existing assays for X-ALD, MPS-I and Pompe disease for newborn screening to minimize the instrumentation burden introduced by the expanding screening panel.

2.1.1 Development of a new GALT assay

To develop a new GALT assay for screening purposes that is also not affected by G6PD, signal detected should not rely on any other endogenous enzymes other than GALT. Since the separation of UDP-Gal and UDP-Glu requires an over 10-minute gradient,[37, 38] the only way forward to separate these two compound in a high throughput manner was to distinguish them by mass. The idea was to introduce a mass modification that was highly specific to UDP-Gal, therefore the derivative could have a different mass from UDP-Glu. Ideally, the derivative retained better on reverse-phase columns, making it multiplexable with other assays that were typically analyzed using reverse-phase LC.. To this end, a bacterial enzyme, α-1,4-galactosyltransferase (LgtC) from *Neisseria meningitidis*, was used to transfer the galactosyl group from UDP-galactose (donor) to a lactosyl group of a receptor (Fig. 2.1).[47] The LgtC enzyme transfers the galactosyl group from UDP-galactose to the acceptor with high specificity,[47] leaving the otherwise isobaric UDP-glucose

untouched. The final enzymatic product could be detected through LC-MS/MS with an appropriate internal standard.

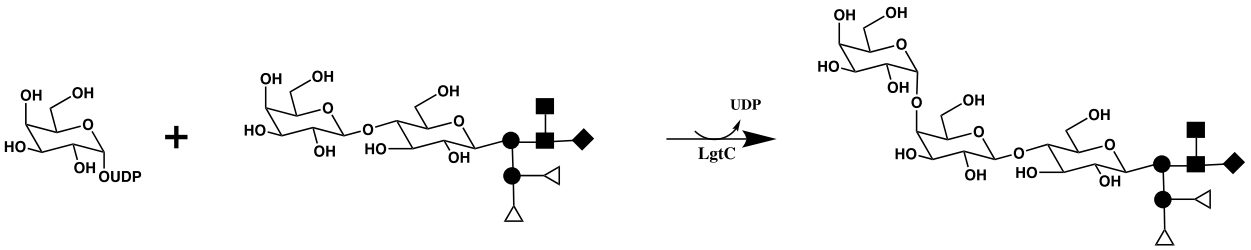


Figure 2.1 LgtC catalyzes the transfer of galactose from UDP-Gal to the 4- position of a terminal lactose.

We envisioned a coupled GALT-LgtC assay in which UDP-Gal was converted into Gal-LgtC-acceptor by LgtC enzyme in the presence of a LgtC-acceptor. The final product, Gal-LgtC-acceptor, had a different mass from UDP-Glu, therefore chromatographic separation of UDP-Gal and UDP-Glu was no longer required for accurate quantification. GALT activity could thus be quantified by MS/MS using an isotope-labeled UDP-Gal that went through the same LgtC derivatization as internal standard. It should be noted that saturated amount of LgtC enzyme should be used in the assay so the final formation of the Gal-LgtC-acceptor is limited by the GALT step. This new GALT-LgtC assay preserves the Beutler assay part, as this original fluorometry assay quantifies the other enzymatic product, Glu-1-P. With this dual test strategy, physicians can have preliminary results for classic galactosemia from the Beutler assay on the same day the samples are tested, with confirmative results from the GALT-LgtC assay that is not affected by G6PD available on day 2 (Fig. 2.2). Alternatively, for subjects with positive Beutler assay results, laboratories personnel can prioritize them for a same day GALT-LgtC assay analysis. This way, physicians and families only receive the confirmative results unaffected by G6PD.

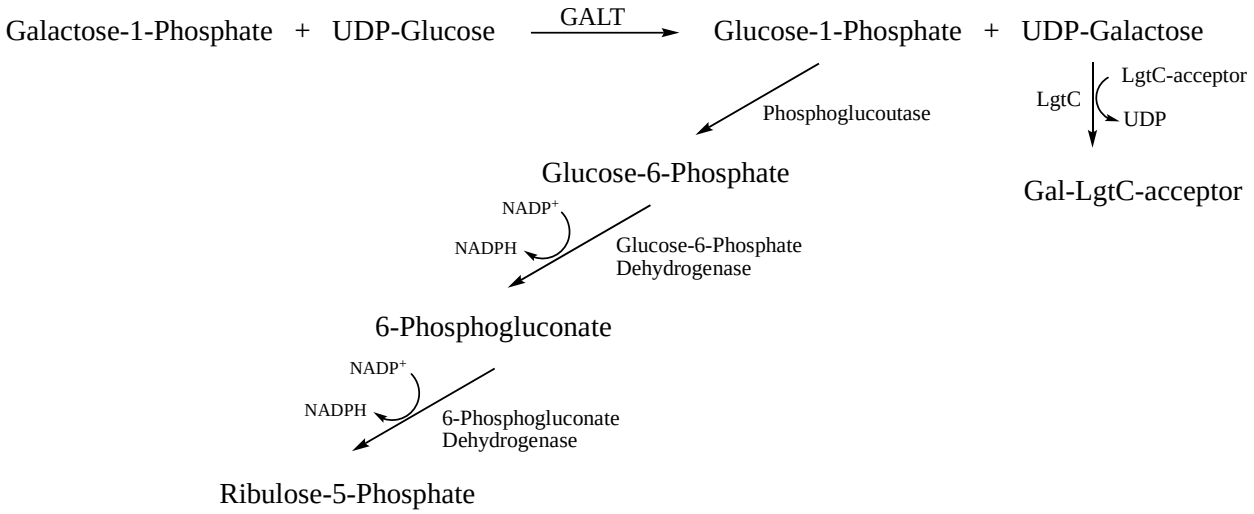
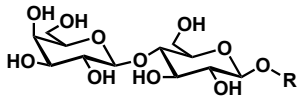
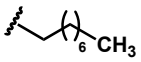
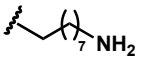
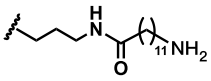
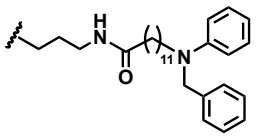


Figure 2.2 Scheme of the dual test for GALT.

Dr. Arun Kumar in the Gelb laboratory synthesized a series of LgtC acceptors, in search of the optimal structure of which the final enzymatic product retained on the reverse phase column, and was also compatible with liquid-liquid extraction and other assays (Table 2.1). Liquid-liquid extraction is a crucial sample clean-up step as it minimizes the amount of salt and ionic detergent from going into the injection sample, thus keeping the mass spectrometry cleaner. We intended to multiplex the GALT-LgtC assay with assays for X-ALD, biotinidase (Section 2.1.2), MPS-I and Pompe disease, which can all be analyzed on reverse-phase columns. The final enzymatic product for the GALT-LgtC was likely to elute closely with analytes for biotinidase, MPS-I, and Pompe on column based on its hydrophobicity. Since these analytes were detected in ESI positive mode, therefore the final enzymatic product for the GALT-LgtC needed to be protonated easily in the ESI source. The final enzymatic products all retained well on a C8-based reverse-phase column. By installing an amine group in the acceptor, we were able to detect the final enzymatic product in ESI positive mode, making it compatible with other assays of interest. By making the aglycon of the acceptor more hydrophobic, the final enzymatic product was finally compatible with the liquid-liquid extraction with ethyl acetate and water.

Table 2.1 Structural optimization of LgtC acceptor.

General structure of the LgtC-acceptor	R group			
				
Property of the final enzymatic product				
Retention on reverse phase column	✓	✓	✓	✓
Compatibility with liquid-liquid extraction	X	X	X	✓
Compatibility with other assays	X	✓	✓	✓

The structure of the optimized LgtC-acceptor was shown in Fig. 2.3a. The very hydrophobic aglycon of the acceptor made the final product of this coupled assay compatible with liquid-liquid extraction. It was shown that Gal-LgtC-acceptor was not generated when either one of glucose-1-phosphate, UDP-galactose, LgtC enzyme, or LgtC-acceptor was depleted from the assay cocktail, demonstrating that the final product could only be formed when UDP-galactose was formed via the action of GALT on galactose-1-phosphate and UDP-glucose (Fig. 2.3b). It also indicated that patients with G6PD deficiency would result in a normal reading with this GALT-LgtC coupled assay, as they can convert Glu-1-P and UDP-Gal into Gal-1-P and UDP-Glu like healthy controls.

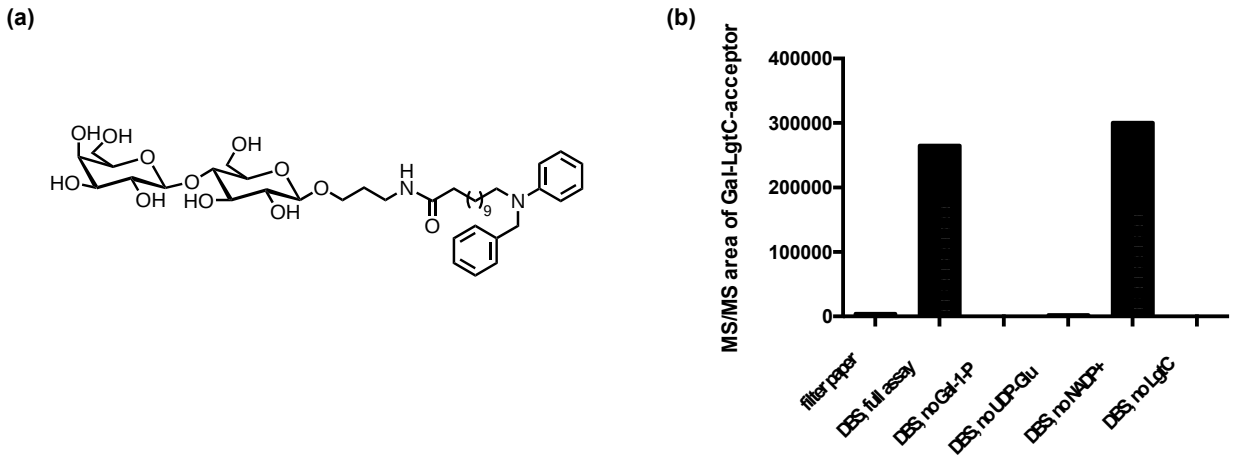


Figure 2.3 (a) Structure of the optimized LgtC-acceptor; and (b) results of a controlled experiment for the GALT-LgtC assay.

With the optimized LgtC-acceptor in hand, we further optimized the assay conditions, including the amount of UDP-Glu, $NADP^+$, Gal-1-P, LgtC enzyme and LgtC-acceptor used in the assay (Fig. 2.4). Saturating amount of UDP-Glu, $NADP^+$, and Gal-1-P were used to ensure that the GALT and other endogenous enzymes required for the Beutler assay were operating at maximum rates. Saturating amount of LgtC enzyme and LgtC-acceptor were also used such that the formation of the final enzymatic product, Gal-LgtC-acceptor, was limited by GALT. The optimized assay cocktail consisted of 1.8 mM Gal-1-P, 1.6 mM UDP-Glu, 3.3 mM $NADP^+$,1 mM LgtC-acceptor, and 0.6 μg LgtC enzyme in assay buffer (0.25 M Tris-HCl, pH 7.5 buffer containing 2.5 g/L sodium taurocholate). Sodium taurocholate was included in the assay buffer to help solubilize the LgtC-acceptor.

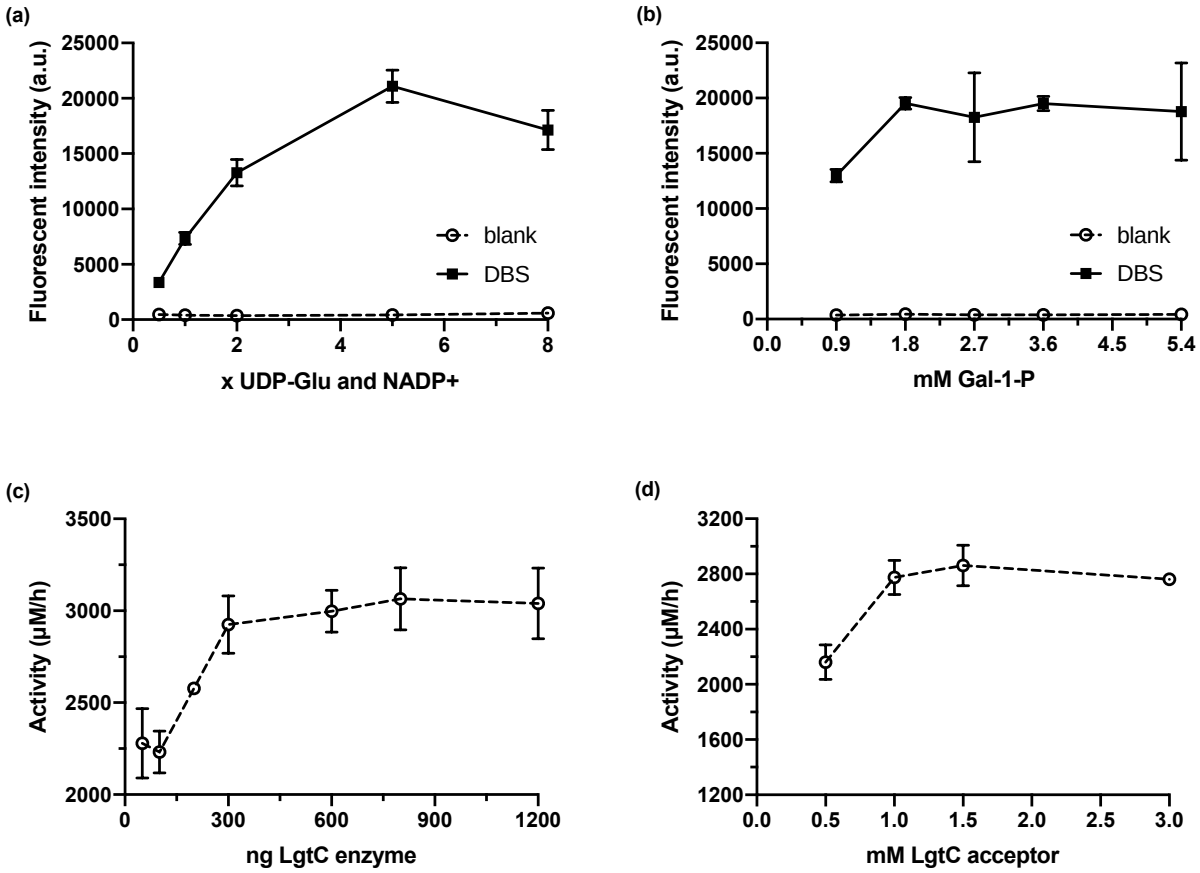


Figure 2.4 Assay optimization for the GALT-LgtC coupled assay: optimization for (a) UDP-Glu and NADP+, 1x indicates 0.32 mM UDP-Glu and 0.66 mM $NADP^+$; (b) the Gal-1-P; (c) amount of LgtC enzyme; and (d) amount of LgtC acceptor. Error bars are calculated from triplicate measurements.

Using the optimized condition, the GALT-LgtC assay was validated on quality control DBS provided by Centers for Disease Control and Prevention (CDC) in a dual-test manner. Shown in Fig. 2.5a was the result using the fluorometry readout (Beutler assay), and in Fig. 2.5b was the result from using MS/MS readout (GALT-LgtC assay). Both the fluorescent and MS/MS signal increased proportionally with the GALT activity (in U/g Hb) provided by CDC, indicating the assay had good linearity. It was also found that the fluorescent readout had a larger variation than the MS/MS readout, which was due to the nature of fluorometry.

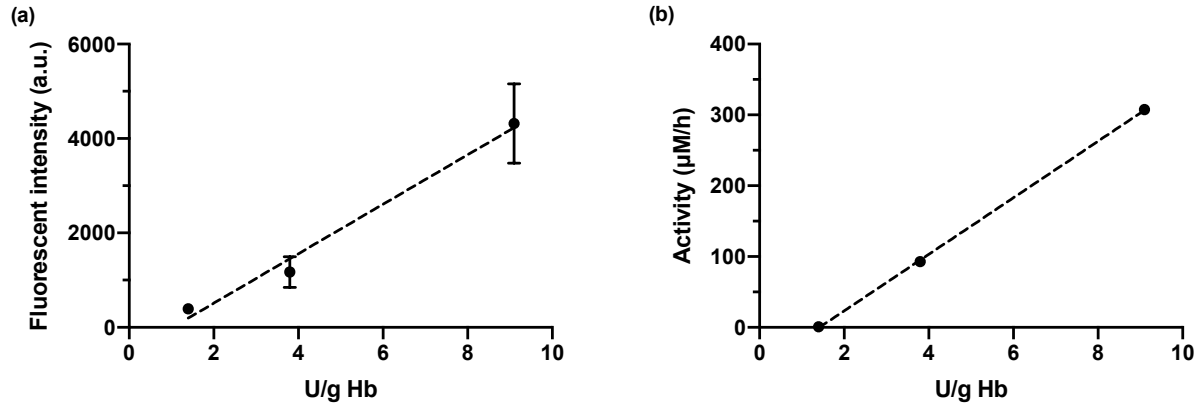


Figure 2.5. (a) Fluorometry and (b) MS/MS readout of the GALT-LgtC coupled assay using CDC QC DBS samples. The U/g Hb value was provided by the CDC. Error bars are calculated from triplicate measurements.

2.1.2 Consolidating the GALT-LgtC coupled assay with a new biotinidase assay

Currently, all states in the United States are screening for biotinidase deficiency using colorogenic or fluorogenic substrates[39, 40]. These substrates, however, eliminate the possibility of multiplexing the biotinidase assay with the fluorogenic Beutler GALT assay as both enzymatic products share similar fluorescent spectrums. Dr. Arun Kumar in the Gelb laboratory developed a new generation of biotinidase substrate that was MS/MS compatible with no fluorescent background (Fig. 2.6). Biotinidase acts on the new substrate to produce the substituted aniline biotinidase product, which can be quantified by LC-MS/MS with the use of a structurally identical but isotopically substituted internal standard. All previously reported substrates used to detect biotinidase in DBS contain an aromatic-amine as the leaving group, presumably because substrates with aliphatic-amines are more sluggish substrates (poorer leaving group)[39, 48].

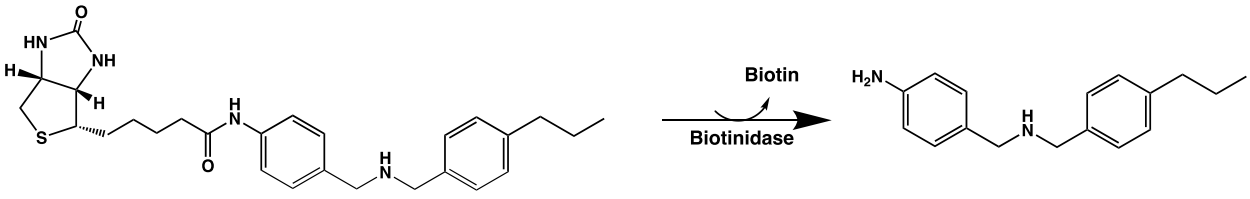


Figure 2.6. Enzymatic reaction with the new biotinidase substrate.

A substrate dosage optimization was carried out for the new biotinidase assay. As shown in Fig. 2.7, the biotinidase activity reached maximum when 200 μM substrate was used. Beyond 200 μM, the activity decreased gradually with increasing amount of substrate, probably because the substrate was added into the cocktail by diluting a DMSO stock solution and the DMSO slightly inhibited the enzyme.

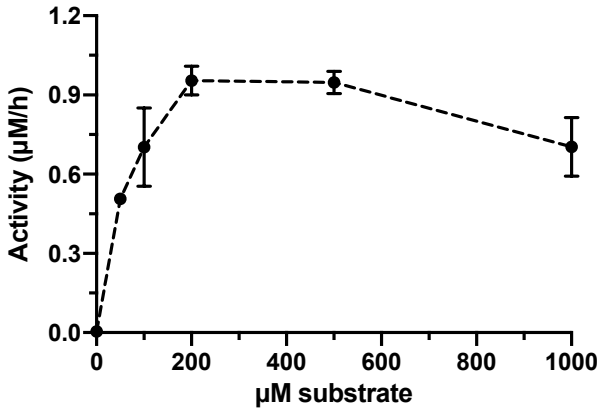


Figure 2.7. Substrate dosage optimization for the biotinidase assay.

Since the DBS on newborn screening card are a limited resource, we sought to consolidate the GALT-LgtC assay with the biotinidase assay such that only one instead of two DBS punch was required to screen for these two disorders. This was a reasonable approach, since GALT, LgtC, and biotinidase all operate under basic conditions. Furthermore, since the new biotinidase substrate and product afforded no fluorescent background, they could be tested in the same reaction mixture as the dual-test for GALT without interfering the fluorescent readout of the Beutler assay. Efforts were made in search of a buffer system that work for the GALT-biotinidase duplex assay (Fig. 2.8). The screening criteria was that the buffer system should result in blood to no-blood ratios above 20 for all three tests (GALT-LgtC, Beutler and biotinidase assays). The blood to no-blood ratio was calculated by dividing the mean product to internal standard ratio of a 3-mm DBS punch from a healthy adult to that of a filter paper punch. This criteria was chosen arbitrarily, but it was found previously in our laboratory that assays with blood to no-blood ratios above 20 usually

resulted in good reproducibility. After screening more than 20 different buffers, it was found that only the Tris buffer at pH 7.5 met the criteria and was used for all the further studies.

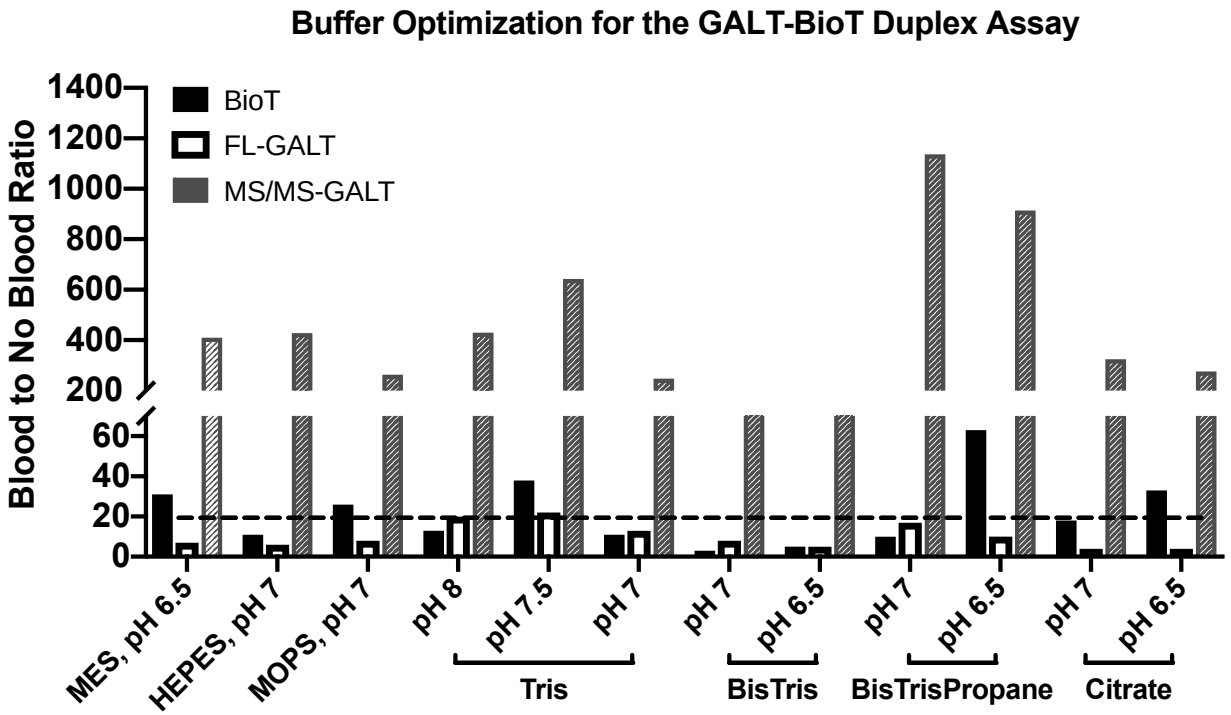


Figure 2.8. Buffer optimization for the GALT-biotinidase duplex assay. The dash line was the screening criteria with a blood to no-blood ratio of 20. Blood to no-blood ratio was calculated by dividing the mean product to internal standard ratio of a 3-mm DBS punch from a healthy adult to that of a filter paper punch. Blood to no blood ratio was calculated based on triplicate measurements.

To validate the new GALT-biotinidase duplex assay with patient samples, DBS from three newborns with classical galactosemia and one newborn with biotinidase deficiency as well as 30 random newborns from Washington State were tested. Enzymatic activities of the MS/MS-based assays (GALT-LgtC and biotinidase) were calculated using appropriate internal standards, and the activities of the fluorescent Beutler assay were calculated with a 3-point standard curve using the quality control samples from CDC (Fig. 2.5a). The patients displayed clear deficits in the relevant enzymatic activity (Fig. 2.9). The classic galactosemia patients had essentially no GALT activity detected, either with the fluorescent or MS/MS readout, whereas they displayed normal biotinidase activity (Fig. 2.9). Noted that the patients with classic galactosemia had higher biotinidase activity

compared to the random newborns (Fig. 2.9c), which was probably because the newborn DBS had been stored at room temperature for over 2 months prior to the analysis while the patient samples were relatively fresh. The biotinidase patient displayed undetectable biotinidase activity while the GALT activity was normal (Fig. 2.9). This suggested that the duplex assay was highly specific.

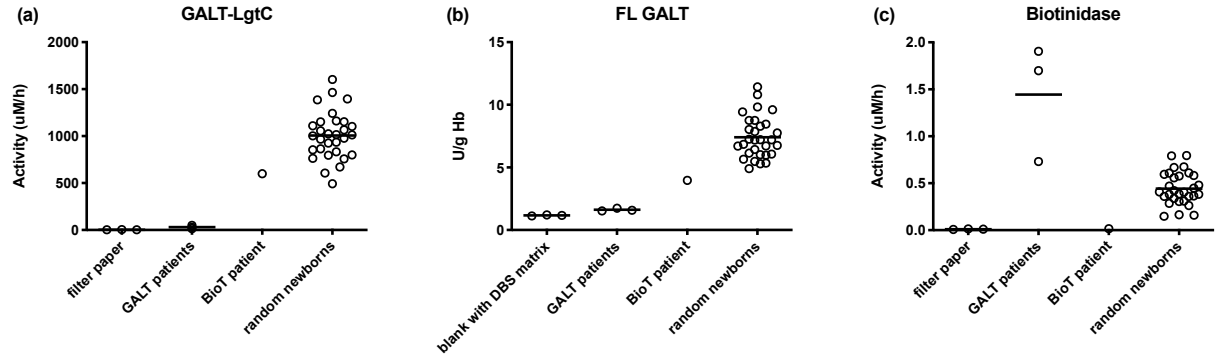


Figure 2.9. Results of (a) GALT-LgtC coupled assay; (b) quantitative Beutler assay; and (c) biotinidase assay of 3 classical galactosemia patients, 1 biotinidase deficiency patient and 30 random newborns.

2.1.3 Expansion to a 5-plex assay for the screening of classic galactosemia, biotinidase deficiency, X-ALD, MPS-I and Pompe disease

The new GALT-biotinidase duplex assay was then combined with the well validated assay for X-ALD,[24] MPS-I and Pompe diseases,[18] the three conditions added into the Recommended Uniform Screening Panel most recently. Although it would be ideal to assay the four enzymes using one DBS punch, the accompanying enzyme compatibility problems hindered this strategy. The biotinidase and GALT assay needed a separate punch as these two enzymes operate under near-neutral pH and are inactive at acidic pH, which is the optimum for the lysosomal enzymes, i.e. IDUA for MPS-I and GAA for Pompe.[34] MPS-I and Pompe disease could be detected with 1 punch using a reported protocol.[49] X-ALD required an additional punch as it relied on a biomarker assay. Nonetheless, we envisioned that the new GALT-biotinidase duplex assay could be consolidated with assays for X-ALD, MPS-I and Pompe diseases by combing the samples together after their individual incubation/extraction (Fig. 2.10). Therefore, only one instead of three LC-MS/MS

analysis was required. This greatly reduced the instrumentation burden.

Shown in Fig. 2.10 is the typical workflow for the 5-plex HPLC-MS/MS assay for the screening of GALT, biotinidase, X-ALD, MPS-I and Pompe. A total of 3 DBS punches were required to perform the assay. The first DBS punch was used for the GALT-Biotinidase duplex assay. After a 3-hour incubation, the assay was quenched and a small portion was transferred out for fluorometry analysis (quantitative Beutler assay). This provided the day-one result for GALT. The second DBS punch was used for the MPS-I-Pompe duplex assay with a 3-hour incubation. The MPS-I-Pompe sample was combined with the remaining sample from the GALT-biotinidase assay, and a liquid-liquid extraction was performed to clean up the sample. The third DBS punch was used for biomarker analysis where the analytes were extracted with methanol solution for 3 hours. After centrifuged the sample, the supernatant was transferred out and combined with the purified sample containing GALT, biotinidase, MPS-I, and Pompe analytes. A single HPLC-MS/MS analysis was performed.

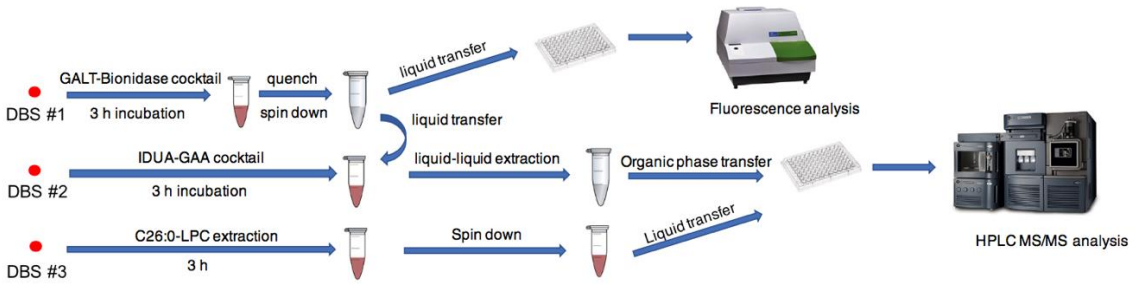


Figure 2.10. Typical workflow for the 5-plex HPLC-MS/MS assay for the screening of GALT, biotinidase, X-ALD, MPS-I and Pompe.

The injection-to-injection time of this 5-plex assay is 2.3 minutes with a baseline separation achieved between each substrate and product pair and also C26:0-LPC, the biomarker for X-ALD, and its endogenous isobaric interference (Fig. 2.11). This sample turnaround time allowed more than 500 samples to be analyzed per instrument per day, which was suitable for high volume

laboratories.

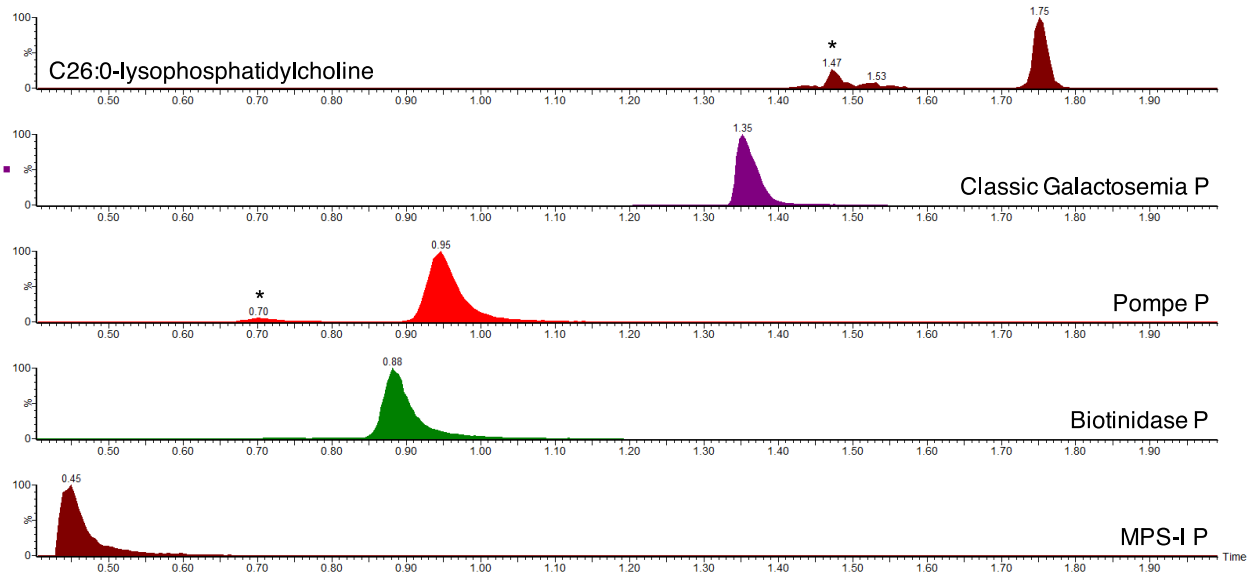


Figure 2.11. HPLC-MS/MS chromatogram of the GALT, biotinidase, X-ALD, MPS-I, and Pompe 5-plex assay. The x axis is time (min) and the y axis is the MS/MS signal after normalization to the largest peak in the MRM channel. Asterisks (*) indicate products from substrates in-source breakdown or endogenous isobaric interference.

Next, a reproducibility study for the 5-plex assay was carried out. Since DBS from healthy adults contained little C26:0-LPC, the biomarker was spiked into whole blood from a healthy adult to the level that was typically seen in X-ALD patients (500 nM).[24] Table 2.2 lists the results of the study, where the reproducibility was assessed by pentaplicate measurements on filter paper punches (no blood controls), and punches from spiked adult DBS (positive control). Large variations were observed in the no blood controls, as they contained essentially no enzymatic products or C26:0-LPC, and the poor reproducibility was the result of integrating noise from the HPLC-MS/MS chromatogram. On the other hand, the 5-plex assay displayed good reproducibility (CV < 20%) with the positive control (Table 2.2).

Table 2.2 Reproducibility study of the 5-plex assay.

		blank (n=5)	adult DBS spiked with 0.5 μM C26:0-LPC (n=5)
Beutler GALT assay	Fluorescent intensity (a.u.)	200.47	9644.60
	s.d. (a.u.)	14.68	1485.35
	% CV	7	15
GALT-LgtC	Averaged activity (μM/h)	0.73	350.46
	s.d. (μM/h)	0.61	17.51
	% CV	84	5
Biotinidase	Averaged activity (μM/h)	0.01	0.54
	s.d. (μM/h)	0.00	0.08
	% CV	13	14
MPS-I	Averaged activity (μM/h)	0.02	0.51
	s.d. (μM/h)	0.00	0.03
	% CV	31	6
Pompe	Averaged activity (μM/h)	0.01	0.66
	s.d. (μM/h)	0.00	0.04
	% CV	44	6
X-ALD	nM C26:0-LPC in blood	0.46	528.35
	s.d. (nM)	0.55	32.42
	% CV	118	6

2.1.4 Discussions and conclusions

The new multiplex reported here may be useful for newborn screening for classic galactosemia, biotinidase deficiency, X-ALD, MPS-I and Pompe diseases. Further studies are needed to fully validate the assay in newborn screening laboratories. One advantage of this new multiplex is that it allows assays to be consolidated with or without addition of lysosomal storage diseases, thus reducing overall costs. The lack of interference from G6PD is an important factor in regions where there are a relatively high incidence of this deficiency.

In our previous MS/MS assays for lysosomal enzymes, an overnight incubation was typically used,[19, 49] whereas in the current study only a 3-hour incubation was performed. The overnight incubation is needed only if the enzyme (GALC) relevant to Krabbe disease is included,

since it has a relatively low specific activity. If Krabbe disease is omitted, a short incubation period of 3 hours is more than sufficient.

Furthermore, this assay is can be easily expanded to include 3 additional lysosomal enzymes that are relevant to Fabry, Gaucher, and Krabbe diseases (data not shown). This 9-plex assay requires ultra-performance liquid chromatography (UPLC), and can be done on most modern LC systems (e.g. the Waters AQUITY system). This may be of future interest as treatments for these disorders are currently being developed and evaluated in ongoing clinical trials and they may be included in the universal screening panel.

2.1.5 Experimental Details

2.1.5.1 Protocol for the GALT, biotinidase, X-ALD, MPS-I, and Pompe 5-plex assay[34]

The 5-plex assay (GALT, biotinidase, X-ALD, MPS-I and Pompe) was carried out using three 3-mm DBS punches. The first DBS punch was used for the GALT-biotinidase duplex assay, the second one was for MPS-I and Pompe, and the third one was used to extract the biomarker relevant to X-ALD (Fig. 2.10).

To the first punch, 30 μL of GALT-biotinidase cocktail (1 mM LgtC-acceptor, 1.8 mM Gal-1-P, 1.6 mM UDP-Glu, 3.3 mM $NADP^+$, 20 μM $^{13}C_6$-UDP-Gal, 200 μM biotinidase substrate, 1 μM biotinidase internal standard in 0.25 M Tris-HCl, pH 7.5 buffer containing 2.5 g/L sodium taurocholate) was added, together with 10 μL LgtC working solution (0.06 μg/μL LgtC enzyme in 20 mM HEPES-NaOH, pH 7.5, 0.1% (w/v) bovine serum albumin, 50 mM KCl, 5 mM $MnCl_2$, 5 mM dithiothreitol). The assay was then incubated in an orbital shaker at 37 °C for 3 hours.

To the second punch, 30 μL of MPS-I-Pompe cocktail (0.22 mM MPS-I substrate, 1.5 μM MPS-I internal standard, 0.37 mM GAA substrate, 2.5 μM GAA internal standard in 89 mM

succinate buffer, pH 4.7, 2.5 g/L sodium taurocholate, 8 μM acarbose, 42 μM saccharic acid lactone). The assay was then incubated in an orbital shaker at 37 °C for 3 hours.

To the third punch, 100 μL of 18.75 nM d_4-C26:0-LPC in methanol was added. The assay was then extracted in an orbital shaker at 37 °C for 3 hours.

After the incubation and extraction were finished, the X-ALD assay was centrifuged at 3000 g for 5 minutes, and 50 μL upper layer was transferred out. A total of 100 μL 50:50 (v:v) ethylacetate:methanol was added to quench the GALT-biotinidase assay. After being centrifuged at 3000 g for 5 minutes, 30 μL upper layer was transferred out and diluted into 200 μL water in a black fluorometry plate. The fluorescent NADPH was detected with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. From the rest of the quenched GALT/biotinidase assay, 75 μL was transferred out to quench the MPS-I/Pompe assay, and 400 μL ethylacetate and 200 μL 0.5 M NaCl in water was added subsequently. After mixing vigorously by pipetting, the sample was then centrifuged at 3000 g for 5 minutes. 200 μL upper layer was transferred out into the plate containing the X-ALD analyte, and was dried under a stream of nitrogen. To the residue, 100 μL 65:35 (v:v) methanol:water with 5 mM ammonium acetate was added for reconstitution.

LC-MS/MS was carried out on a Waters Xevo-TQ tandem mass spectrometry instrument coupled to a Waters ACQUITY binary solvent system. An XBridge BEH C8 XP column (2.5 μm, 2.1 x 50 mm, Waters Corp., Cat. 186006041) connected to an XBridge BEH C8 XP VanGuard Cartridge (2.5 μm, 2.1 x 50 mm, Waters Corp., Cat. 186007781) was used for the analysis. The column was held at 40 °C in the column oven. The flow rate was 0.45 mL/min. Solvent A was 1/1 (v/v) water/methanol with 5mM ammonium acetate, and solvent B was 1/1 (v/v) methanol/ acetonitrile with 5 mM ammonium acetate. The ammonium acetate was added to the mobile phases

by diluting a 5 M ammonium acetate stock solution by 1000-fold. The solvent program started with 25% solvent B from 0 to 0.8 min, then increased linearly to 100% solvent B from 0.8–0.85 min, then remained at 100% solvent B from 0.85–1.75 min, then jumped back to 25% solvent B at 1.75 min and remained at 25% solvent B until 1.85 min. The inject-to-inject was 2.3 min with the "load ahead" function enabled and the "loop offline time" set at 0.5 min. The weak and strong needle wash solvents for the autosampler were 9/1 (v/v) water/acetonitrile and 47.5/47/5 (v/v/v) methanol/isopropanol/water, respectively. 10 μL was injected per sample using the full-loop method with a loop overfill factor of 1.5-fold. ESI source parameters and MS/MS parameters are listed in Tables 2.3 and 2.4. A standard diversion valve was programed to divert the void volume and the post-C26-LPC eluent to waste to help maintain ESI source cleanliness.

Table 2.3. ESI source parameters on Xevo TQ for the 5-plex assay.

Parameter		
Polarity	ES+	ES-
Capillary Voltage (kV)	3.3	3.0
Source temperature (°C)	150	150
Desolvation temperature (°C)	500	500
Cone gas flow (L/hr)	30	30
Desolvation gas flow (L/hr)	1000	1000
Collision gas	Argon	Argon

Table 2.4. MS/MS parameters on Xevo TQ for the 5-plex assay.

Analyte	ESI polarity	Parent mass (m/z)	Daughter mass (m/z)	Cone voltage (V)	Collision energy (V)
Biotinidase-P	+	283.3	91	26	32
Biotinidase-IS	+	287.2	91	26	32
IDUA-P	+	426.2	317.2	30	14
IDUA-IS	+	431.2	322.2	30	14
GAA-P	+	498.5	398.4	16	15
GAA-IS	+	503.5	403.4	16	15
C26:0-Lyso-PC	+	636.9	104.1	46	26
d_4-C26:0-Lyso-PC	+	640.7	104.1	46	26
GALT-P	+	925.6	439.3	42	38
GALT-IS	+	928.7	439.3	42	38

2.1.5.2 LgtC-25H Purification

Plasmid containing the LgtC gene was provided by Dr. Withers (University of British Columbia) as a generous gift. An overnight culture of E. coli (BL21, DE3), harboring the pET29a (+) plasmid containing the LgtC-25 gene in Luria broth was diluted 100-fold into Luria broth containing kanamycin (50 μg/mL). The culture was shaken at 37°C until the OD600 reached 0.6, then isopropylthiogalactoside was added to a final concentration of 0.5 mM, and the culture was continued for 6 hours. Cells were harvested by centrifugation at 3000 g for 20 minutes at 4 °C.

All subsequent steps were done on ice or in a room at 4 °C. The cell pellet from a 1 L culture was resuspended in 30 mL of cold binding buffer (50 mM sodium phosphate, 300 mM NaCl, 10mM imidazole, 10% (v/v) glycerol, 10mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and EDTA-free protease inhibitor cocktail (Roche Cat. 11873580001), pH 8.0). The suspension was magnetically stirred on ice for 15–20 minutes. The suspension was probe sonicated on ice (6–8 cycles of 20 on-off cycles). The lysate was centrifuged at 15,000 g for 20 minutes at 4 °C.

Ni-NTA Agarose (3 mL resin) from Qiagen (Cat. 30230) was washed with purified water (Milli-Q, EMD-Millipore) and then equilibrated in binding buffer. The supernatant from the cell lysate (about 30 mL) was added to the washed gel in a 50 mL plastic tube. The tube was rotated end-over-end for 1–2 hours at 4 °C. The tube content was poured into a 1.6 × 15 cm column, and eluent was collected by gravity. The column was washed with 30 mL of binding buffer, then with 20 mL of binding buffer containing 50 mM imidazole, then with binding buffer containing 110 mM imidazole, and finally with binding buffer containing 300 mM imidazole. 1 mL fractions were collected. LgtC eluted in the 110 mM imidazole fraction. Aliquots of fractions were analyzed by SDS- PAGE (apparent MW ~ 29 kDa), and those containing essentially pure LgtC were combined.

The solution was dialyzed at 4 °C against 20 mM Tris-HCl, pH 7.5 (2 L twice over 4–5 hours, then 1 L once overnight). The amount of protein was estimated using the BCA protein assay (ThermoFisher, Cat. 23250) using bovine serum albumin as a standard. The typical yield was 20 mg protein per liter culture (purity was checked by SDS-PAGE and the amount was determined using bicinchoninic (BCA) acid assay (ThermoFisher, Cat. 23250).

2.2 One-step chemoenzymatic synthesis of isotope-labeled globotriaosylsphigosine (lyso-Gb3)[50]

The work presented in this section is published in Molecular Genetics and Metabolism: Hong et al., One-step synthesis of carbon-13-labeled globotriaosylsphingosine (lyso-Gb3), an internal standard for biomarker analysis of Fabry disease. Mol Genet Metab ***2018,*** *125 (3), 292-294.*

Fabry disease is a treatable lysosomal storage disease caused by deficiency of the lysosomal enzyme alpha-galactosidase A (GLA), which is involved in the breakdown of sphingolipids. Newborn screening for Fabry disease is typically done by measuring residual GLA enzymatic activity in DBS by tandem mass spectrometry or fluorometry.[18, 28] Deficiency of GLA enzyme leads to accumulation of globotriaosylceramide and its hydrolyzed form, globotriaosylsphigosine (lyso-Gb3). Lyso-Gb3 is typically quantified by LC-MS/MS in the presence of an internal standard, which in most cases is an analogue of lyso-Gb3.[51, 52] However, in any LC-MS/MS assay it is always better to use a structurally-identical, but isotopically substituted, internal standard so that the internal standard and analyte of interest co-migrate on column. This way the ionization suppression in the ESI source of the mass spectrometry by multiple components in the sample will be identical for analyte and internal standard, which is usually not the case if the analyte and its internal standard elute from the LC column at different retention times. Also, any losses of lyso-

Gb3 due to sample handling are best mimicked by the use of a structurally identical internal standard. Although a multi-step synthesis of heavy isotopic lyso-Gb3 has been reported[53], this labor-intense method makes it impractical for worldwide distribution. Herein we took the advantage of the biochemical function of the LgtC enzyme and the structure of lyso-Gb3 and envisioned a simple one-step chemoenzymatic synthesis of carbon-13-labeled lyso-Gb3.

2.2.1 Results and discussions

The bacterial enzyme LgtC transfers a galactosyl unit from UDP-galactose to the terminal 4-hydroxyl of lactosyl-glycosides.[47] The enzyme is highly specific for galactosyl as the donor and lactosyl-glycosides as the acceptor.[47] Since lactosyl-sphingosine and UDP-Galactose labeled uniformly in all 6 carbons of the galactosyl-unit with carbon-13 are both commercially available, we envisioned a one-step synthesis of isotope-labeled lyso-Gb3 (Fig. 2.12). The enzymatic reaction proceeded in high yield (> 90%). A simple solid-phase extraction method using a C18 Sep-Pak cartridge could be used to remove the salt and ionic detergent in the buffer. However, this method cannot remove the excess lactosyl-sphingosine (Fig. 2.13). If chemically pure labeled lyso-Gb3 is required, the enzymatic reaction mixture is submitted to preparative HPLC to fully purify lyso-Gb3 from the excess lactosyl-sphingosine (Fig. 2.14). The final yield after the HPLC purification is about 50%. Subsequently UPLC-MS/MS analysis of the HPLC-purified material showed ~0.04% lactosyl-sphingosine as a contaminant. No unlabeled lyso-Gb3 was detected in the final product. The heavy isotope labeled lysoGb3 was then used in the multiplex study detailed below.

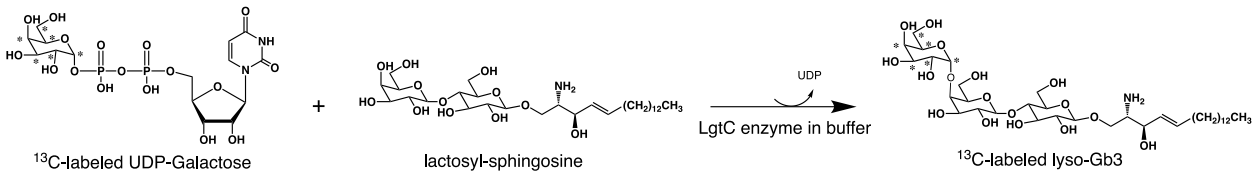


Figure 2.12. One-step chemoenzymatic synthesis of ^{13}C-labeled lyso-Gb3. The asterisks (*) indicate ^{13}C.

This simple procedure to produce labeled lyso-Gb3 will allow reference laboratories to use this as a proper internal standard for the quantification of the biomarker, lysoGb3, for Fabry disease. This is more reliable than use of structural-related lysoGb3 analogs that do not exactly co-migrate with lysoGb3 during UPLC. The isotope-labeled lyso-Gb3, prepared with the chemoenzymatic synthesis described in this study, has already been made commercially available by Avanti Polar Lipids, Inc.

2.2.2 Experimental Details

Methanol was completely removed from stock solutions of lactosyl-sphingosine and sodium taurocholate to leave 1 mg lipid and 1.25 mg detergent in a glass vial. The residue was then reconstituted in 0.5 mL of reaction buffer (20 mM HEPES-NaOH, pH 7.5, 50 mM KCl, 5 mM $MnCl_2$, 0.1% bovine serum albumin, 5 mM dithiothreitol). The buffer also contained 1.4 mg of $^{13}C_6$- UDP-galactose. LgtC enzyme was recombinantly expressed and purified as described in Section 2.1.5.2. A total of 28 μg LgtC enzyme was added into the mixture, and the capped vial was incubated overnight at 37 °C on an orbital shaking platform (250 rpm).

After the overnight incubation, the reaction mixture was purified on a 3 mL C18 Sep-Pak cartridge (J.T. Baker Cat. 7020–02), which was conditioned with 10 mL of methanol followed by 10 mL of purified water (Milli-Q, EMD Millipore). The crude enzymatic reaction mixture was then loaded onto the cartridge, which was then washed with 5 mL of water, 5 mL of

water/acetonitrile (3/1, v/v), 5 mL of water/acetonitrile (3/2, v/ v), 5 mL of water/acetonitrile (1/4, v/v), and finally 5 mL of pure acetonitrile. Slight air pressure was used to force the solvent to pass through the cartridge, and 1 mL fractions were collected. A typical Sep-Pak elution profile is shown in Fig. 2.13. About 80% of the lyso-Gb3 eluted in the water/acetonitrile (3/2, v/v) wash, and fractions #11–#20 were combined. Solvent was removed with a vacuum, centrifugal concentrator at room temperature. This procedure provided labeled lyso-Gb3 that also contained some lactosyl-sphingosine and is suitable for most purposes. A typical yield of 0.9 mg (70%) lysoGb3 was obtained using 1 mg lactosyl-sphingosine.

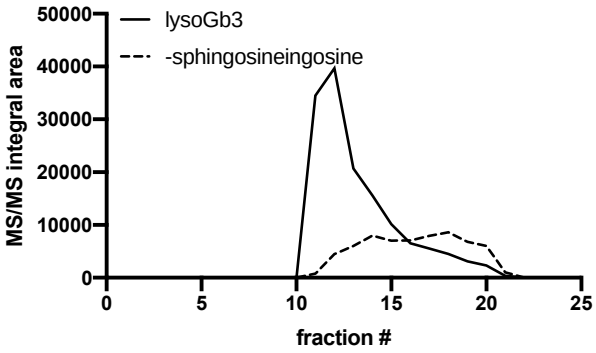


Figure 2.13. Sep-Pak separation of lyso-Gb3 and lactosyl-sphingosine.

An alternate workup scheme was to use preparative HPLC using a Vydac 218TP C18 column (1.0 × 25 cm, VWR Scientific, Cat. 218TP1010) at room temperature. Solvent A was 100% water with 0.1% formic acid, and solvent B was 100% acetonitrile with 0.1% formic acid (all were Optima Grade from Fisher Scientific). The crude enzymatic reaction mixture was mixed with methanol to reach a methanol/water ratio of 2:3 (v/v). The sample was then filtered through a 0.2 μm Nylon-66 cartridge filter prior to injection onto the HPLC. The gradient program started at 30% B for 2 min, then to 45% B from 2 to 45 min, then to 55% B from 45 to 55 min, then to 100% B from 55 to 60 min, then hold at 100% B from 60 to 69 min, then to 30% B from 69 to 70 min, then hold at 30% B from 70 to 80 min, all linear gradients. The flow rate was 6 mL/min, and 45 s fractions were collected. A typical HPLC chromatogram is shown in Fig. 2.14. Each fraction

was diluted 100-fold and analyzed by UPLC-MS/MS detailed below to examine the purity. Pure lysoGb3 fractions (fraction #49–#52 eluting at 36–39 minute) were combined and concentrated to dryness in a vacuum, centrifugal concentrator. A typical recovery rate after HPLC purification was 65%, and the lactosyl-sphingosine contamination is < 0.1%.

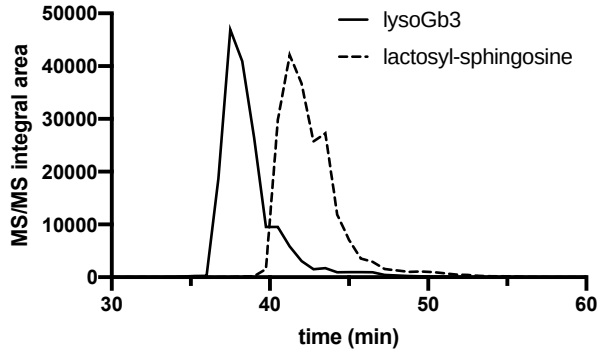


Figure 2.14. HPLC separation between lysoGb3 and lactosyl-sphingosine.

UPLC-MS/MS was carried was carried out on a Waters Xevo-TQ MS/MS instrument coupled to a Waters Aquity binary solvent system. Separation of lactosyl-sphingosine and lyso-Gb3 was achieved on an ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm, Waters Corp., Cat. 186002350) connected to an ACQUITY UPLC BEH C18 VanGuard Pre-column (1.7 μm, 2.1 × 5 mm, Waters Corp., Cat. 186003975). The column was held at 40 °C and the flow rate was 0.6 mL/min. Solvent A was 100% water with 0.1% formic acid and solvent B was 100% acetonitrile, 0.1% formic acid. The weak needle wash and strong needle wash were 90/10 (v/v) water/acetonitrile, with 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively. The gradient started with 50% solvent B, and was ramped to 65% B from 0 to 1.5 min, then jumped to 100% B and held until 2.0 min, then jumped back to 50% until 2.5 min for re-equilibration, all linear gradients. MS/MS was carried out in ESI positive mode and ESI source parameters and MS/MS parameters are given in Tables 2.3 and Table 2.5, respectively. UPLC solvents were all Optima grade from Fisher Scientific.

Table 2.5. MS/MS parameters for lysoGb3 and lactosyl-sphingosine.

Analyte	parent mass (m/z)	daughter mass (m/z)	cone voltage (V)	collision energy (V)
lactosyl-sphingosine	624.6	282.1	38	28
lysoGb3	786.6	282.3	36	34
$^{13}C_6$-lysoGB3	792.7	282.3	36	34

Unlabeled and labeled lyso-Gb3 were quantified by quantitative ^{1}H-NMR using dimethylformamide as an internal standard (a recycle delay between NMR pulses of ~ 10 s was used). A typical ^{1}H-NMR spectrum of lyso-GB3 is shown in Fig. 2.15. In a typical experiment, 3.22 μmole DMF and nominally 1.8 mg lyso-Gb3 in CD_3OD solvent was used.

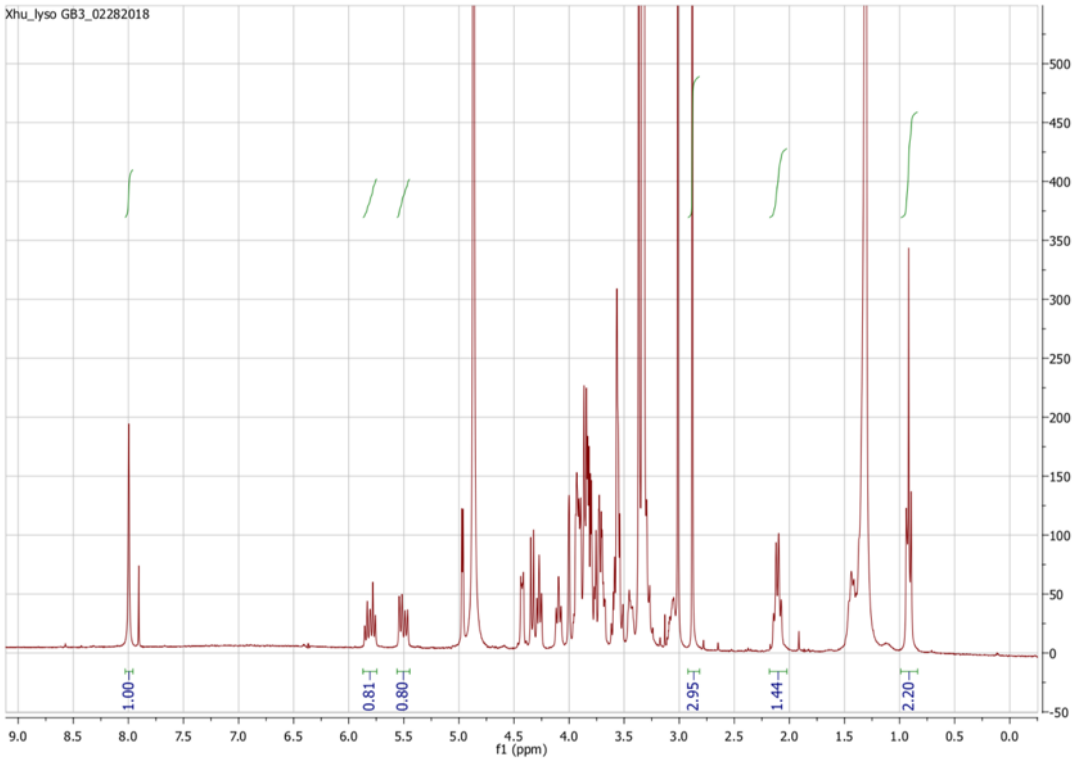


Figure 2.15. ^{1}H-NMR spectrum of lyso-Gb3.

2.3 A Highly multiplexed biochemical assay for analytes in dried blood spots: application to newborn screening and diagnosis of lysosomal storage disorders and other inborn errors of metabolism

The work presented in this section has been accepted at Genetics in Medicine: Hong et al., A Highly Multiplexed Biochemical Assay for Analytes in Dried Blood Spots: Application to Newborn Screening and Diagnosis of Lysosomal Storage Disorders and Other Inborn Errors of Metabolism.

Every year, millions of newborns are screened for a panel of genetic disorders that are best treated if therapy starts prior to the onset of irreversible symptoms. Expansion of the newborn screening panel is inevitable due to the rapid development of treatments for genetic diseases, and there has been widespread discussion of how to expand the panel in a flexible, comprehensive and affordable manner. It is clear that the biochemical methodology will be the main way forward in the coming years, as genotyping-based screening cannot be currently adopted as a first-tier screen due to incomplete understanding of genotype/phenotype correlations, especially for rare diseases of which variant annotations are limited.

Over the past decade, our laboratory has developed multiple MS/MS-compatible enzymatic substrates that are suitable for high-throughput screening, including reagents for α-L-iduronidase (IDUA) for MPS-I, acid α-glucosidase (GAA) for Pompe disease, α-galactosidase A (GLA) for Fabry disease, galactocerebrosidase (GALC) for Krabbe disease, β-glucosidase (GBA) for Gaucher disease, acid sphingomyelinase (ASM) for Nieman-Pick A/B disease, tripeptidyl peptidase 1 (TPP1) for neuronal ceroid lipofuscinosis 2 (CLN-2), iduronate-2-sulfatase (I2S) for MPS-II, α-N-acetyl-glucosaminidase (NAGLU) for MPS-IIIB, N-acetylgalactosamine-6-sulfatase (GALNS) for MPS-IVB, N-acetylgalactosamine-4-sulfatase (ARSB) for MPS-VI, β-glucuronidase (GUSB) for MPS-VII, palmitoyl protein thioesterase I (PPT1) for neuronal ceroid

lipofuscinosis 1 (CLN-1), lysosomal acid lipase (LAL) for Wolman disease, galactose-1-phosphate uridylyltransferase (GALT) for classic galactosemia, and biotinidase for biotinidase deficiency.[18-21, 54, 55] These substrates were strategically designed so that they are (1) highly enzyme specific;[20, 32, 54] (2) highly sensitive as they ionize well in the ESI source and fragment through one major pathway in the gas phase;[46, 56] and (3) highly multiplexable through MS/MS. In this study, we are also interested in biomarkers which have been reported to be useful disease indicators, including C26:0-lyso-phosphotidylcholine (C26:0-LPC) for X-linked adrenoleukodystrophy (X-ALD),[57] and C16:0-sulfatide for metachromatic leukodystrophy (MLD).[25] Furthermore, lyso-sphingomyelin (lyso-SM) for Niemann-Pick A/B disease and lyso-globotriaosylceramide (lyso-Gb3) for Fabry disease may be useful secondary biomarkers to resolve the false-positive cases identified by the relevant enzymatic assays.[51, 58] These biomarkers accumulate in DBS when the relevant proteins are deficient.

Herein, we reported a multiplex DBS assay for the detection of 18 genetic diseases using UPLC-MS/MS. Treatments for these conditions are either available or are being developed and evaluated in ongoing clinical trials. We believe that LC-MS/MS is continuously expandable to include additional disorders and is the only platform that is capable of keeping up with the expected expansion of newborn screening panels brought by the rapid advancements in treatments.

2.3.1 Results

The 18 disorders targeted in this multiplex assay are listed in Table 2.6 together with their relevant proteins and/or biomarkers. All conditions except X-ALD and MLD were tested by measuring the activity of the relevant enzyme. Classic galactosemia could be screened enzymatically in a dual test manner, where the first-tier test was the traditional quantitative Beutler assay (result ready on

day 1), and the second-tier test was the novel GALT-LgtC coupled assay (result ready on day 2).[34] X-ALD and MLD were screened based on the abundance of the relevant biomarkers. For X-ALD, a functional assay of the relevant lipid transporter was not feasible. For MLD, the thermal instability of the relevant enzyme and the high frequency of pseudodeficiency variants posed additional challenges for enzymatic activity-based screening.[59] Lyso-SM and lyso-Gb3 were also included in the assay and could serve as secondary disease indicators along with the enzymatic activity tests.

Table 2.6 The disorders and their relevant enzymes and biomarkers included in the 18-plex assay.

Disease	Protein defected	Biomarker
MPS I	α-L-iduronidase (IDUA)	
Pompe disease	Acid α-glucosidase (GAA)	
Fabry disease	α-Galactosidase A (GLA)	Lyso-Gb3
Krabbe disease	Galactocerebrosidase (GALC)	
Gaucher disease	β-Glucosidase (GBA)	
Niemann–Pick type A and B	Acid sphingomyelinase (ASM)	Lyso-SM
Neuronal ceroid lipofuscinosis 2 (CLN-2)	Tripeptidyl peptidase 1 (TPP1)	
MPS II	Iduronate-2-sulfatase (I2S)	
MPS IIIB	α-N-Acetyl-glucosaminidase (NAGLU)	
MPS IVA	N-Acetylgalactosamine 6-sulfatase (GALNS)	
MPS VI	N-Acetylgalactosamine 4-sulfatase (ARSB)	
MPS VII	β-Glucuronidase (GUSB)	
Neuronal ceroid lipofuscinosis 1 (CLN-1)	Palmitoyl protein thioesterase I (PPT1)	
Wolman disease	Lysosomal acid lipase (LAL)	
Metachromatic leukodystrophy	Arylsulfatase A (ARSA)	C16:0-sulfatide
X-linked adrenoleukodystrophy	ABCD1	C26:0-LPC
Classic galactosemia	Galactose-1-phosphate uridylyltransferase (GALT)	
Biotinidase deficiency	Biotinidase	

Combining multiple assays into the same reaction mixture minimizes the number of DBS punches needed as well as the pre-MS/MS steps. Although it is ideal to assay all the enzymes using one DBS punch, the accompanying substrate solubility and enzyme compatibility problems hindered this strategy. LAL and PPT1 are strongly inhibited by some blood components, therefore the assays for these enzymes required a DBS extraction step to alleviate the inhibition.[54, 55] The

biotinidase and GALT assay needed a separate punch as these two enzymes operate under near-neutral pH and are inactive at acidic pH, which is the optimum for the lysosomal enzymes.[34] Nonetheless, we envisioned that different assays could be combined together after their individual incubation/extraction and subjected to one single UPLC-MS/MS analysis to reduce instrumentation burden.

Since DBS on newborn screening cards are a limited resource, the assay was further consolidated to keep the number of DBS punch required to minimum. As only a small portion of the DBS extract is required for LAL and PPT1 assays, we hypothesized that it was possible to assay these two enzymes together with those for MPS-II, -IIIB, -IVA, -VI, -VII and CLN2 by splitting the blood extract. The enzymes from the DBS punch were extracted into water, and a small portion of the extract was transferred out for LAL and PPT1, while the rest of the extract and the punch was used for MPS-II, -IIIB, -IVA, -VI, -VII and CLN2. Although three separate incubations were performed, only one instead of three DBS punches was needed. Since the proposed blood splitting approach diluted the substrate concentration for the multiplex assay for MPS-II, -IIIB, -IVA, -VI, -VII and CLN2, comparison studies between the whole punch method and the splitting method were carried to test its feasibility. As shown in Fig. 2.16, TPP1, NAGLU and ARSB activities were not affected by the blood extract splitting, indicating that the dilution of enzymatic substrates had little impact on their enzyme kinetics (saturating amounts of substrates are being used). Intriguingly, I2S, GALNS and GUSB activities increased by 20-100% with the splitting method, which was speculated to be due to the dilution of some inhibitory blood components during the process. Together, the data indicated that LAL and PPT1 could be assayed together with MPS-II, -IIIB, -IVA, -VI, -VII and CLN2 using one DBS punch without compromising their performance.

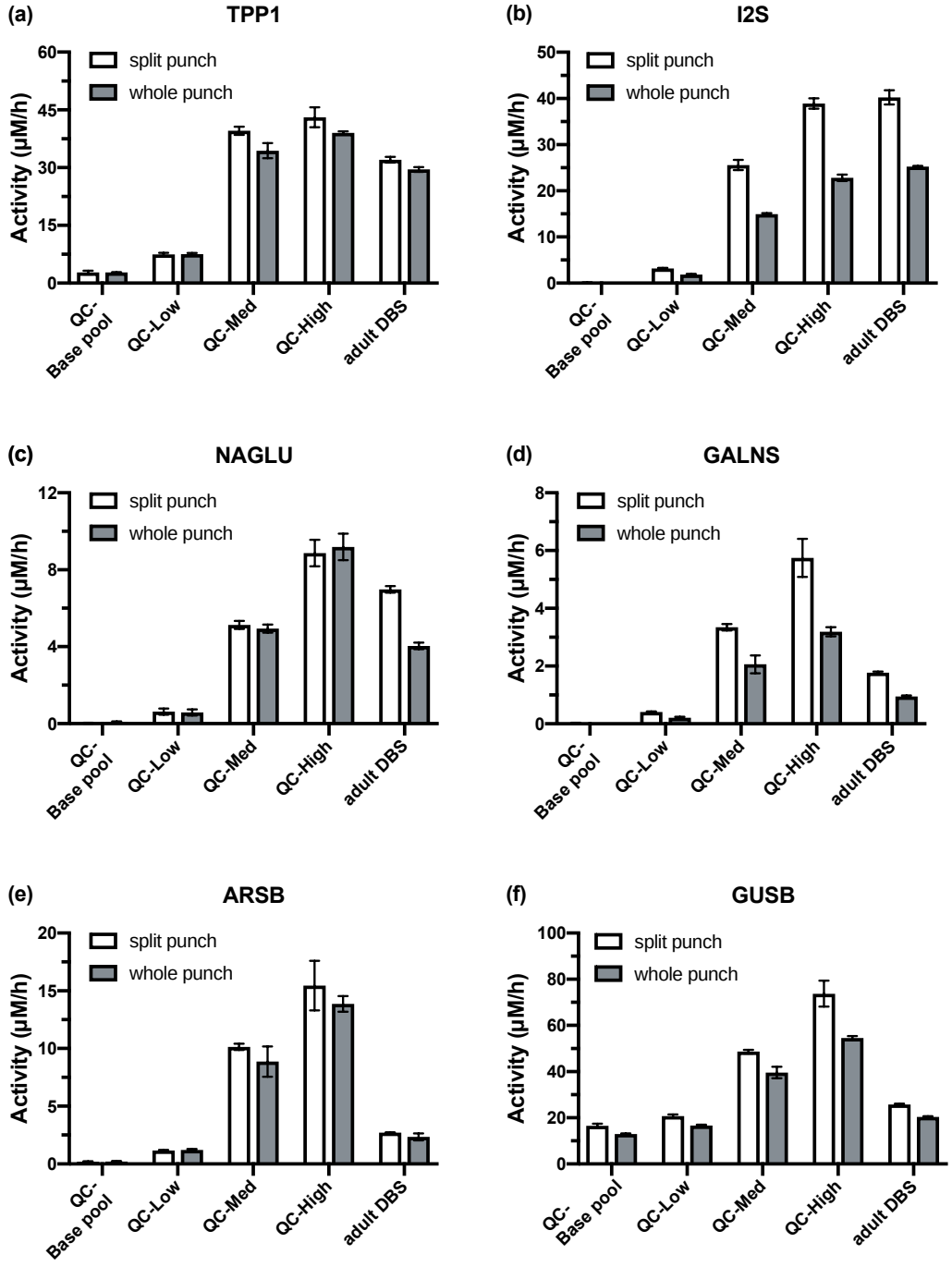


Figure 2.16 Enzymatic activity for (a) TPP1, (b) I2S, (c) NAGLU, (d) GALNS, (e) ARSB, and (f) GUSB assayed with whole DBS punch or split DBS punch. The whole DBS punch method was as reported previously.[19] The split method was described in Section 2.3.3. Error bars were standard deviations based on triplicate measurements.

Shown in Fig. 2.17 is the UPLC-MS/MS chromatogram of the 18-plex assay. Each substrate and product pair as well as C26:0-LPC and its endogenous isobars were baseline

resolved. The charged stationary phase of the column allowed separation between the sulfated substrates and the de-sulfated products of I2S, GALNS, and ARSB without the use of ion-pairing reagents. Even though the heat-labile sulfated substrates underwent partial thermal breakdown in the heated ESI source and contributed significantly to their relevant product signal, it was of no concern as they were completely separated from their respective enzymatic products by chromatography. Column carryover and autosampler carryover were assessed and were negligible. The MRM channels were scheduled based on the retention time of each analyte to improve the duty cycle, though it was found that scanning 32 channels at the same time with a 5 millisecond dwell time and inter-channel delay did not affect the signal significantly on our platform (data not shown). Columns from different batches were tested, with no significant difference found in the peak height, peak shape, and retention time for each analyte. Columns with high injection numbers displayed slightly higher backpressure (about 500 psi) and about 20% loss in peak height when compared to a brand new column, with minimum shift in retention time. Nonetheless, chromatographic variations introduced by columns had negligible impact on the results, due to the use of chemically-identical but isotopically-distinguished internal standards. The sample injection-to-injection time of this 18-plex assay was 2.7 min, allowing more than 500 samples to be analyzed per day per instrument.

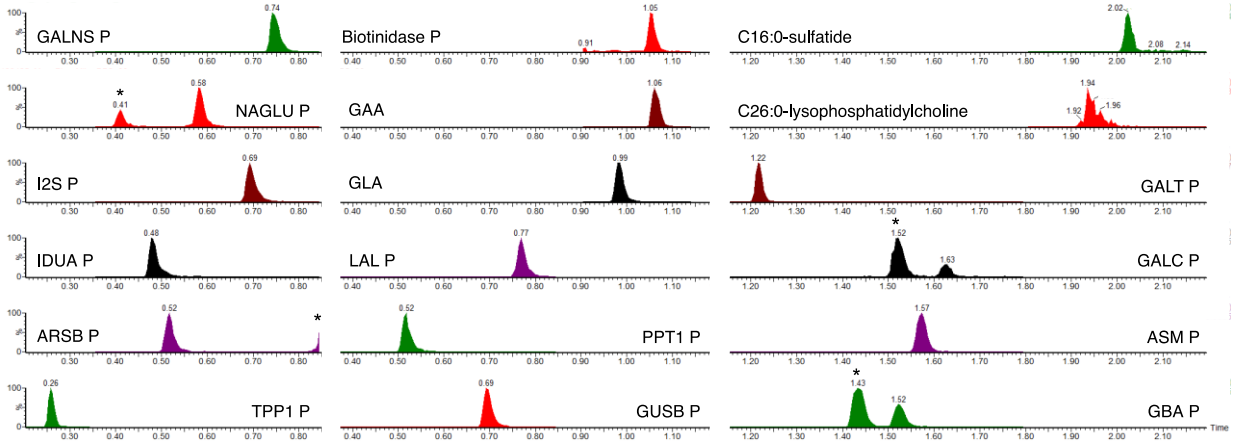


Figure 2.17. UPLC-MS/MS chromatogram of the 18-plex assay. The x-axis is time (min) and the y-axis is the MS/MS intensity after normalization to the largest peak in the channel. Asterisks (*) indicate products from substrates in-source breakdown.

Kinetics and linearity studies as well as validations with patient samples were previously conducted for each assay, and were not repeated in the current study. There have been reports detailing the variations of DBS-based analysis introduced by hematocrit and sampling location.[60, 61] The bias introduced by sampling location was investigated by comparing the results obtained from 10 center and 10 peripheral punches from DBS prepared from whole blood spiked with biomarkers. As shown in Fig. 2.18, no statistically significant difference was found for all the analytes, suggesting that the chromatographic effect of the filter paper was negligible. The reproducibility and precision of the assay was assessed by analyzing QC-Low, QC-High, and spiked adult DBS in pentaplicates over 5 days (Table 2.7). All 14 lysosomal enzymes displayed good inter-day and intra-day reproducibility at both the low and high end. The QC DBS did not constitute a positive control for the non-lysosomal enzymes (GALT and biotinidase) and the biomarkers except for C16:0-sulfatide, thus spiked adult DBS was included as positive control for these disorders. Good reproducibility was found for the GALT and biotinidase activity as well as for the biomarkers with the spiked adult DBS (Table 2.7).

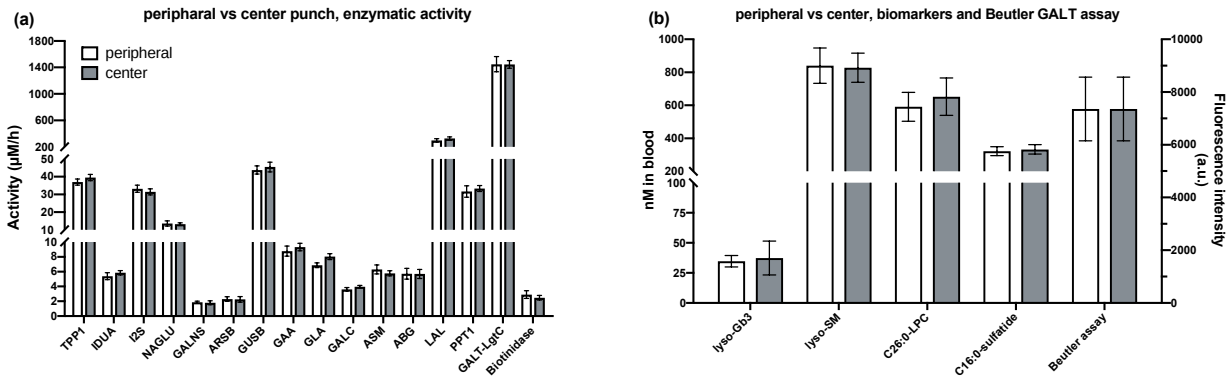


Figure 2.18 Results for (a) the enzymatic assays, and (b) the biomarker assays and the Beutler GALT assay. Ten replicates of peripheral punches and center punches were used in the study.

Table 2.7 Reproducibility study for the 18-plex assay. Each sample was repeated in pentaplicate on 5 days.

	IDUA activity (µM/h)			GAA activity (µM/h)			GLA activity (µM/h)			GALC activity (µM/h)			GBA activity (µM/h)			ASM activity (µM/h)		
		% CV			% CV			% CV			% CV			% CV			% CV	
	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay
QC-Low	0.35	22	23	0.73	18	23	0.98	11	9	0.64	11	11	0.64	17	16	0.47	10	10
QC-High	7.01	6	6	9.44	5	4	13.6	4	5	9.41	4	4	8.53	8	9	4.89	9	8
Spiked adult DBS	5.91	11	9	9.61	8	6	7.6	13	11	4.22	7	8	6.44	13	12	7.18	8	8

	TPP1 activity (µM/h)			I2S activity (µM/h)			NAGLU activity (µM/h)			GALNS activity (µM/h)			ARSB activity (µM/h)			GUSB activity (µM/h)		
		% CV			% CV			% CV			% CV			% CV			% CV	
	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay
QC-Low	5.53	11	16	4.36	15	8	0.65	23	11	0.13	26	26	0.40	26	26	22.8	7	5
QC-High	55.1	6	4	28.3	12	5	5.20	13	10	3.06	18	8	5.69	18	11	53.5	6	5
Spiked adult DBS	37.7	7	4	31.0	14	5	12.2	16	10	2.46	20	7	2.92	15	9	46.0	5	4

	PPT1 activity (µM/h)			LAL activity (µM/h)			GALT-LgtC activity (µM/h)			Biotinidase activity (µM/h)			nM C26:0-LPC in blood			nM C16:0-sulfatide in blood		
		% CV			% CV			% CV			% CV			% CV			% CV	
	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay
QC-Low	9.44	8	10	14.6	22	22	1523	7	4	-0.04	-150	-43	7.55	98	143	265.6	8	9
QC-High	99.9	12	7	204.9	18	9	1678	10	6	0.17	39	29	46.7	37	40	97.9	14	15
Spiked adult DBS	32.2	11	7	270.4	14	11	1451	11	5	3.71	21	7	549.6	16	15	336.5	15	7

	GALT FL activity (Fluorescence intensity)			nM lyso-Gb3 in blood			nM lyso-SM in blood		
		% CV			% CV			% CV	
	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay	mean	intra-assay	inter-assay
QC-Low	6250	10	6	0	168	37	36.8	73	64
QC-High	10685	12	5	-0.04	50	185	69.3	53	58
Spiked adult DBS	9350	13	3	33.9	16	17	497.4	15	16

2.3.2 Discussions and conclusions

Flow-injection MS/MS was used as the detection platform for our early assays for lysosomal storage disorders.[18] Flow-injection MS/MS works well for enzymatic assays for which the substrates undergo minimal thermal breakdown to enzymatic products in the ESI source. In-source conversion of substrates to products is not an issue with LC-MS/MS since the substrates can be chromatographically separated from enzymatic products, and thus this technique is more generally useful and most appropriate for highly multiplexed assays such as the 18-plex reported in this study. Flow-injection MS/MS is often problematic for detection of low-abundant analytes in complex mixtures such as DBS due to the presence of one or more isobaric species. With flow-injection MS/MS, all of the material extracted from DBS passes into the ESI source, resulting in the need for routine ESI source cleaning. This is a particularly important issue with newborn screening given the large volume of samples being analyzed. A key advantage of LC-MS/MS is that the vast majority of the mass of material in DBS elutes from the LC column in the void volume and can thus be diverted away from the ESI source with a valve. LC-MS/MS usually allows the analyte to be resolved on the column from isobaric interferences. LC-MS/MS is more sensitive than flow-injection MS/MS since analytes suffer less ionization suppression due to matrix effects.

Recently, our laboratory and other groups have started to develop LC-MS/MS-based screening assays for newborn screening.[19, 62, 63] The addition of LC to the MS/MS workflow does not significantly increase instrumentation complexity. Flow-injection MS/MS requires a single solvent delivery pump, whereas LC-MS/MS requires a second pump to allow for solvent gradients. An LC column is inserted into the same solvent line between the autosampler and the mass spectrometer as used in flow-injection MS/MS. All other components of the system are virtually identical, and the cost of the LC column is insignificant since one column can be used for several

thousand newborns. LC-MS/MS based assays are currently used in some newborn screening laboratories including Illinois, Washington, and Taiwan.[30, 31, 64] It has been our experience in the Washington newborn screening laboratory that LC-MS/MS requires less routine cleaning of ESI source and internal mass spectrometer components (i.e. ion focusing lens, quadrupoles, and collision cells).

The assay reported here is highly multiplexed, with the ability to screen for 18 disorders simultaneously in a high throughput manner. It is also highly robust as demonstrated by the reproducibility study and the consistency with columns from different batches and with different numbers of injections. Moreover, this 18-plex assay is continuously expandable for future addition of tests for new conditions, as the gradient covers a wide range of analytes with various hydrophobicity.

Most of our assays included a liquid-liquid extraction step to remove water soluble salts and detergents from the sample. However, it has been reported that LC-MS/MS based enzymatic assays were able to work robustly in high volume laboratories without the liquid-liquid extraction as the salts and detergents were diverted to waste.[30] Nevertheless, liquid-liquid extraction is still recommended in our multiplex assay as it can be hard to insert waste diversion windows when 18 analytes are eluting during most of the span of the run. In this study, 12-channel manual pipettes were used for all liquid transfers. Automation is possible for high volume laboratories, and therefore the assay can be carried out by one single laboratory worker.

Lyso-SM and lyso-Gb3 were included in our multiplexed panel as potential secondary disease indicators for Niemann-Pick A/B and Fabry disease, respectively. These markers were extracted together with the sulfatide and C26:0-LPC and were analyzed with the other analytes

without adding additional complexity to the assay. Use of second-tier tests is often important for reducing the false-positive rates of the primary tests, including psychosine for Krabbe disease,[16] the ratio of creatine/creatinine for Pompe disease,[65] and glycosaminoglycans for MPS-I. Implementing lyso-Gb3 as a secondary screening filter may also reduce the false-negative rate of Fabry disease, a rare X-linked disorder caused by the deficiency of the GLA enzyme. Due to random X-chromosomal inactivation, female carriers can have manifestation ranging from asymptomatic to as severely affected as males.[66] It was reported that the GLA activity was normal or slightly decreased in at least 40% of the carriers.[66] On the contrary, lyso-Gb3 was reported to be substantially elevated in symptomatic female carriers.[66, 67] Nevertheless, it remains to be proven if lyso-SM and lyso-Gb3 are indeed good biomarkers for Niemann-Pick A/B and Fabry disease, respectively. If so, by incorporating the additional biomarkers into the panel as secondary screening criteria, the screen-positive and screen-negative rates can be reduced. Psychosine is an excellent biomarker for following up newborns with abnormally low GALC activities.[68] However, it is not included in the current assay as our method cannot separate psychosine from its endogenous isobar, glucosyl-sphingosine (lyso-Gb1), which may also be a good biomarker for following up newborns with abnormal GBA activities. Moreover, psychosine analysis in DBS requires a top-end mass spectrometer, therefore a separate analysis may be more appropriate for this assay.

Amino acid, acylcarnitine and succinylacetone analyses in DBS are crucial parts of newborn screening. Some laboratories derivatize these analytes prior to flow-injection MS/MS analysis.[33] These derivatives, however, cannot be analyzed on our multiplex platform as some of them are highly hydrophilic and elute in the void volume. We are currently working on new derivatization methods to be compatible with the LC-MS/MS method described in this study. By

incorporating the amino acid, acylcarnitine and succinylacetone analyses into our multiplex assay, instrumentation burden can be further reduced.

Probably the most significant hurdle to adopt LC-MS/MS in newborn screening laboratories is the sample turnaround time. An injection-to-injection time below 2 minute is usually preferred, especially in laboratories with extremely high volume. Nonetheless, the increased turnaround time of the LC-MS/MS based assay is offset by the consolidation of many individual assays into a single analysis, which are currently analyzed separately. We feel that consolidation is critical as newborn screening continues to expand due to the increasing rate of development of new treatments including gene therapies.

2.3.3 Standard operating procedure for the 18-plex UPLC-MS/MS assay

4 DBS punches are required to assay the 18 diseases:

- 1 DBS for MPS-I, Pompe, Fabry, Krabbe, Gaucher and Niemann-Pick A/B
- 1 DBS for MPS-II, -IIIB, -IVA, -VI, -VII, CLN1, CLN2 and Wolman
- 1 DBS for classic galactosemia and biotinidase deficiency
- 1 DBS for MLD (C16:0-sulfatide), X-ALD (C26:0-LPC), lyso-SM (Niemann-Pick A/B), and lyso-Gb3 (Fabry)

5 incubations and 2 extractions are required to assay the 18 diseases:

- 3-hour incubation for Wolman (ready on day 1)
- 3-hour incubation for classic galactosemia and biotinidase deficiency (ready on day 1)
- 3-hour biomarker extraction for MLD, X-ALD, Niemann-Pick A/B, and Fabry (ready on day 1)
- 1-hour DBS extraction for MPS-II, -IIIB, -IVA, -VI, -VII, CLN2, LAL, and CLN1
- Overnight incubation for MPS-I, Pompe, Fabry, Krabbe, Gaucher and Niemann-Pick A/B (NeoLSD, ready on day 2)
- Overnight incubation for MPS-II, -IIIB, -IVA, -VI, -VII, CLN2 (ready on day 2)

- Overnight incubation for CLN1 (ready on day 2)

Assay Setup:

The first DBS punch is used to assay the activity of the enzymes responsible for MPS-I, Pompe, Fabry, Krabbe, Gaucher and Niemann-Pick A/B (NeoLSD MSMS Kit, PerkinElmer).

- To each 3 mm DBS punch, add 30 µL PE 6-plex cocktail, ensure the DBS is fully immersed in the cocktail by centrifuging the plate at 3000 xg for 1 minute. Seal the plate.
- Incubate overnight for 16 hours at 37 °C with shaking (e.g. 400 rpm on a shaking radius of 3 mm).

The second DBS punch is used to assay the activity of the enzymes responsible for MPS-II, -IIIB, -IVA, -VI, -VII, CLN1, CLN2 and Wolman disease, with 3 separate incubations.

- To each 3 mm DBS punch, add 30 µL water, ensure the DBS is fully immersed in the water by centrifuging the plate at 3000 xg for 1 minute. Seal the plate.
- Shake at room temperature for 1 hour.
- Pipette up and down the blood extract ~ 5 x to ensure homogeneity before transferring 10 µL blood extract out and diluting it into 40 µL water in another plate (50 µL total diluted blood extract). Mix by pipetting up and down ~ 5 x.
- From the diluted blood extract, transfer 10 µL out to another plate, add 30 µL LAL cocktail. Ensure the solutions are well mixed by centrifuging the plate at 3000 xg for 1 minute. Seal the plate.
- Incubate the LAL plate at 37 °C for 3 hours with shaking.
- To the rest of the diluted blood extract (~ 40 µL), add 15 µL CLN1 cocktail and 2 µL S-methyl methanethiosulfonate (MMTS) working solution. Ensure the solutions are well mixed by centrifuging the plate at 3000xg for 1 minute. Seal the plate.
- Incubate the CLN1 plate at 37 °C overnight for 16 hours with shaking.
- To the rest of the undiluted blood extract (with DBS punch inside), add 30 µL MPS-5plex with CLN2 cocktail, ensure the DBS is fully immersed in the cocktail by centrifuging the plate at 3000xg for 1 minute. Seal the plate.
- Incubate MPS-5plex with CLN2 plate at 37 °C overnight for 16 hours with shaking.

The third DBS punch is used to assay the activity of the enzymes responsible for classic galactosemia and biotinidase deficiency.

- To each 3 mm DBS punch, add 30 µL GALT-BioT cocktail and 10 µL LgtC working solution, ensure the DBS is fully immersed in the cocktail by centrifuging the plate at 3000 xg for 1 minute. Seal the plate.
- Incubate at 37 °C for 3 hours with shaking.

The fourth DBS punch is used to extract biomarkers for MLD (C16:0-sulfatide), X-ALD (C26:0-LPC). Lyso-Gb3 and lyso-SM are optional.

- To each 3 mm DBS punch, add 100 µL IS working solution (18.75 nM d_4-C26:0-LPC, 30 nM d_5-C16:0-sulfatide, 10 nM $^{13}C_6$-lysoGb3, 10 nM d_7-lyso-SM in methanol). Seal the plate.
- Extract at 37 °C for 3 hours with shaking.

<u>Assay workup (day 1):</u>

- Spin down the biomarker plate at 3000 x g for 5 minutes after the 3-hour extraction.
- Transfer 50 µL upper layer into a shallow 96 well plate, and store in a -20 °C freezer.
- To the GALT-BioT plate, add 100 µL 1/1 (v/v) ethyl acetate/methanol to quench the reaction, and mix well by pipetting up and down 2 to 3 times.
- Spin down the quenched GALT-BioT plate at 3000 x g for 5 minutes.
- Transfer 30 µL from the quenched assay, and dilute it into 200 µL water in a black, 96-well, fluorometer plate.
- Read the fluorescence signal with excitation at 355 nm and emission at 460 nm.
- Transfer the quenched GALT-BioT plate to a -20 °C freezer.
- Transfer the LAL plate to a -20 °C freezer after the 3-hour incubation.

<u>Assay workup (day 2):</u>

- Remove the plates from day 1 (biomarker plate, quenched GALT-BioT plate, and LAL plate) from the freezer and let them warm up to room temperature.
- Quench the CLN1 plate with 400 µL 1/1 (v/v) ethyl acetate/methanol. Mix well by pipetting up and down.

- From the quenched CLN1 plate, transfer 100 µL and add to NeoLSD plate to quench it. Mix well by pipetting up and down 2 to 3 times.
- From the quenched NeoLSD plate, transfer 100 µL out to quench the MPS 5-plex with CLN2 plate. Mix well by pipetting up and down 2 to 3 times.
- From the quenched MPS 5-plex with CLN2 plate, transfer 100 µL out to the quenched GALT-BioT plate. Mix well by pipetting up and down 2 to 3 times.
- From the quenched GALT-BioT plate, transfer 100 µL to quench the LAL plate. Mix well by pipetting up and down 2 to 3 times.
- To the quenched LAL plate, add 400 µL ethyl acetate and 200 µL 0.5 M NaCl in water. Mix well by pipetting up and down 10 times.
- Spin down the LAL plate at 3000 x g for 5 minutes.
- Transfer 200 µL upper ethyl acetate layer into the biomarker plate (final sample plate).
- Remove the organic solvent in the final sample plate with a stream of nitrogen at room temperature.
- The dried down final sample plate can be store under -20 °C until the UPLC-MS/MS analysis.

UPLC-MS/MS analysis:

An ACQUITY UPLC system coupled to a Xevo TQ tandem mass spectrometer (Waters Corp.), operating in multiple reaction monitoring (MRM) mode, is used for the analysis. The ESI source parameters are listed in Table 2.3. Note that these source temperatures are higher than those typically used for flow-injection MS/MS due to the higher solvent flow rate.

An ACQUITY UPLC CSH C18 column (1.7 µm, 2.1 mm x 50 mm, Waters Corp. 186005296) coupled to an ACQUITY UPLC CSH C18 VanGuard pre-column (1.7 µm, 2.1 mm x 5 mm, Waters Corp. 186005303) is used for the separation. The column is held at 55 °C, and the flow rate is 0.8 mL/min. Mobile phase A is 70/30 (v/v) water/acetonitrile with 0.1% formic acid, and mobile phase B is 65/35 (v/v) isopropanol/acetonitrile with 0.1% formic acid. The weak needle wash is 90/10 (v/v) water/acetonitrile with 0.1% formic acid, and the strong needle wash is

47.5/47.5/5 (v/v/v) isopropanol/methanol/water. The 18-plex assay is analyzed using a 2.2 min gradient starting with 0.5% solvent B, which is ramped up to 25% B over 0.75 min, to 60% B at 1.0 min, to 75% B at 1.5 min, to 100% B at 1.8 min (all linear gradients). From 1.8 min to 2.15 min, the composition is held at 100% B and is ramped down back to 0.5% B at 2.15 min. The sample is reconstituted with 100 µL 1/1 (v/v) water/methanol, and 10 µL is injected onto the column with an overfill factor of 1.5-fold. Load ahead function is enabled with a loop offline time of 0.5 minute to improve the throughput of the assay. The injection-to-injection time is 2.7 minute. The MRM transitions are scheduled based on the retention time of the analyte to maximize the dwell time of each channel (Table 2.8). Eluent coming out between 0-0.2 min, 0.85-0.9 min, and 2.15-2.20 min is diverted to waste.

Table 2.8 MS/MS parameters for the 18-plex assay.

scan time (min)	Analyte	ESI	parent mass (m/z)	daughter mass (m/z)	dwell time (s)	cone voltage (V)	collision energy (V)
0-0.35	TPP1 P	+	350.3	250.3	0.138	12	12
	TPP1 IS	+	359.3	251.2	0.138	12	12
0.35-0.8	LAL-P	+	219.4	190.1	0.012	40	26
	LAL-IS	+	223.3	194.1	0.012	40	26
	PPT1-MMTS-P	+	401.3	177.2	0.012	20	25
	PPT1-MMTS-IS	+	405.2	181.2	0.012	20	25
	MPS-IIIB P	+	420.2	311.2	0.012	25	25
	MPS-IIIB IS	+	423.2	314.2	0.012	25	25
	IDUA-P	+	426.2	317.2	0.012	30	14
	IDUA-IS	+	431.3	322.2	0.012	30	14
	MPS-VII P	+	434.4	325.3	0.012	25	15
	MPS-VII IS	+	441.4	332.3	0.012	25	15
	MPS-II P	+	644.3	359.2	0.012	34	23
	MPS-II IS	+	649.3	364.3	0.012	34	23
	MPS-VI P	+	657.3	345.2	0.012	26	24
	MPS-VI IS	+	662.4	350.2	0.012	26	24
	MPS-IVA P	+	685.4	373.2	0.012	26	25
	MPS-IVA IS	+	690.4	378.3	0.012	26	25
0.9-1.35	Biotinidase-P	+	283.3	91.0	0.018	26	32
	Biotinidase-IS	+	287.2	91.0	0.018	26	32

	lyso-SM	+	465.6	184.1	0.018	30	22
	d7-lyso-SM	+	472.6	184.1	0.018	30	22
	GLA-P	+	484.5	384.5	0.018	18	15
	GLA-IS	+	489.5	389.5	0.018	18	15
	GAA-P	+	498.5	398.4	0.018	16	15
	GAA-IS	+	503.5	403.4	0.018	16	15
	lysoGb3	+	786.6	282.3	0.018	36	34
	13C6-lysoGB3	+	792.7	282.3	0.018	36	34
	GALT-P	+	925.6	439.3	0.018	42	38
	GALT-IS	+	931.8	439.3	0.018	42	38
1.35-1.8	ABG-P	+	384.4	264.5	0.042	14	19
	ABG-IS	+	391.4	271.5	0.042	14	19
	ASM-P	+	398.5	264.4	0.042	13	20
	ASM-IS	+	405.5	264.4	0.042	13	20
	GALC-P	+	412.6	264.4	0.042	12	20
	GALC-IS	+	417.6	264.4	0.042	12	20
1.8-2.2	C26:0-LPC	-	620.6	395.4	0.035	57	30
	d4-C26:0-LPC	-	624.8	399.5	0.035	57	30
	C16:0 sulfatide	-	778.5	96.9	0.035	100	65
	d5 C16:0 sulfatide	-	783.5	96.9	0.035	100	65

Data analysis:

Analytes and internal standards are detected by MRM. The peaks are integrated automatically by the TargetLynx software (Waters Corp.) and inspected manually.

The enzymatic activity in DBS (μM/h) can be calculated by multiplying the ion ratio of the product to the internal standard (blank subtracted) by the amount of internal standard (μmoles) added to the assay and dividing by the incubation time (h) and the volume of the blood (L), assuming each 3 mm DBS punch contained 3.2 μL blood.

The biomarker concentration in DBS (nM) can be calculated by multiplying the ion ratio of the analyte to the internal standard (blank subtracted) by the amount of internal standard (nmoles) added to the assay and dividing by the volume of blood (L), assuming each 3 mm DBS punch contained 3.2 μL blood.

NeoLSD MSMS Kit (Perkin Elmer):

Assay cocktail: 0.22 mM IDUA-S, 0.37 mM GAA-S, 1.3 mM GLA-S, 1.02 mM GALC-S, 0.52 mM ABG-S, 0.78 mM ASM-S, 14.6 µM IDUA-IS, 24.5 µM GAA-IS, 23.9 µM GLA-IS, 10.9 µM GALC-IS, 20.4 µM ABG-IS and 17.9 µM ASM-IS in assay buffer (89 mM sodium succinate, pH 4.7, with 8 µM acarbose, 51 mM N-acetylgalactosamine, 42 µM saccharic acid lactone, 14.8 mg/mL sodium taurocholate, 0.601 mM $ZnCl_2$). The assay cocktail is typically prepared prior to usage by adding the assay buffer the powder of substrates, internal standards and sodium oleate (can be stored in aliquots at -20 °C). The kit can be purchased from PerkinElmer. The sources for additional reagents are listed in Table 2.9.

Table 2.9. Sources for additional reagents used in the MPS-II, IIIB, IVA, VI, VII, CLN2 assay.

Compound	Source	Cat No.
Acarbose	CarboSynth	OA00002
N-acetylgalactosamine	CarboSynth	MA04390
Saccharic acid lactone	Sigma	S0375
Sodium taurocholate	CarboSynth	FS45308
Succinic acid	Sigma	S7501
$ZnCl_2$	Sigma	22997
Sodium hydroxide	Sigma	221465

MPS-II, -IIIB, -IVA, -VI, -VII, CLN2 assay:

Assay cocktail: 1 mM MPS-II substrate, 0.5 mM MPS-IIIB substrate, 1 mM MPS-IVA substrate, 1 mM MPS-VI substrate, and 0.5 mM MPS-VII substrate, 0.2 mM CLN2 substrate, 20 µM MPS-II internal standard, 10 µM MPS-IIIB internal standard, 7.5 µM MPS-IVA internal standard, 15 µM MPS-VI internal standard, and 10 µM MPS-VII internal standard, 15 µM CLN2 internal standard, 100 µM NAG-thiazoline in assay buffer (50 mM sodium acetate, 5 mM cerium (III) acetate, pH 5.0). The assay cocktail is typically prepared prior to use by adding the assay buffer to the solid residue of substrates, internal standards and NAG-thiazoline (solid residues can be made in aliquots and stored in aliquots at -20 °C). The substrates and internal standards are synthesized in the Gelb lab.[19, 46] All MPS reagents are also available from PerkinElmer, and CLN2 reagents

are available from PerkinElmer and GelbChem, LLC. The sources for additional reagents are listed in Table 2.10.

Table 2.10. Sources for additional reagents used in the MPS-II, IIIB, IVA, VI, VII, CLN2 assay.

Compound	Source	Cat No.
NAG-Thiazoline	TRC Chemicals, Toronto	T293625
Sodium acetate	J.T. Baker	3460-01
Cerium (III) acetate	Sigma	529559

GALT-Biotinidase assay:

Assay cocktail: 200 µM BioT-S, 1 µM BioT-IS, 1 mM LgtC acceptor, 1.8 mM Gal-1-P, 1.6 mM UDP-Glu, 3.3 mM $NADP^+$, 20 µM $^{13}C_6$-UDP-Gal in buffer (0.25 M Tris-Acetate, 2.5 g/L sodium taurocholate, pH 7.5).

The assay cocktail is typically prepared fresh by adding the assay buffer to the dried down solid residue of biotinidase internal standard and the LgtC acceptor (can be stored in aliquots at -20 °C). Stock solution of biotinidase substrate is prepared in DMSO at 20 mM and is stored at 20 °C. Stock solution of 180 mM Gal-1-P, 160 mM UDP-Glu, 330 mM $NADP^+$, and 2 mM $^{13}C_6$-UDP-Gal is prepared in 20 mM sodium acetate, pH 5.5 and stored at -20 °C. These two stock solutions are added to the cocktail by diluting 100-fold.

LgtC working solution: 0.06 µg/µL LgtC enzyme in 20 mM Tris-Acetate, pH 7.5, with 0.1% (w/v) BSA, 50 mM KCl, 5 mM $MnCl_2$, 5 mM DTT. The LgtC working solution is typically prepared by diluting the LgtC stock solution (0.6 µg/µL) to the buffer, followed by the addition of DTT (DTT needs to be added fresh by diluting 100-fold from 0.5 M DTT). The LgtC stock solution is be stored at -20 °C and can be freeze-thaw for up to 5 cycles.

The LgtC acceptor, biotinidase substrate and internal standard are synthesized in the Gelb lab.[34] The LgtC enzyme is cultured in the Gelb lab.[34] The sources for additional reagents are listed in Table 2.11.

Table 2.11. Sources for additional reagents used in the GALT-biotinidase assay.

Compound	Source	Cat No.
Gal-1-P	Carbosynth	MG06733
UDP-Glu	Carbosynth	MU089060
$^{13}C_6$-UDP-Gal	Omicron Biochemicals	NTS-005
$NADP^+$	Sigma	N5755
$MnCl_2$	Sigma	450995
dithiothreitol	Goldbio	DTT100
KCl	Fisher	P217
Tris-base	Amresco	0497
BSA	Sigma	A6003

LAL assay:

Assay cocktail: To 9 volumes of assay buffer (0.1 M sodium acetate buffer, pH 4.5, with 2.5 mM sodium taurodeoxycholate hydrate), add 1 volume of 0.5% (w/v) bovine heart cardiolipin in ethanol containing 3 mM LAL-S and 50 µM LAL-IS. The assay cocktail is typically prepared prior to use by diluting the ethanol solution containing the substrate, internal standard, and cardiolipin (can be stored at -20 °C) into the assay buffer. The LAL substrate and internal standard are synthesized in the Gelb lab.[54] They are also available from GelbChem, LLC. The sources for additional reagents are listed in Table 2.12.

Table 2.12. Sources for additional reagents used in the LAL assay.

Compound	Source	Cat No.
sodium taurodeoxycholate hydrate	Sigma	287245
bovine heart cardiolipin	Sigma	C1649

CLN1 assay:

Assay cocktail: 0.3 mM CLN1 S, 20 µM CLN1 IS, 30 mM nonyl-glucoside in assay buffer (0.4 M phosphate-citrate buffer, pH 4.5). MMTS working solution: 23 mM MMTS, 7 mM nonyl-glucoside in assay buffer (0.4 M phosphate-citrate buffer, pH 4.5). The assay cocktail is typically prepared by adding the assay buffer to the powder of substrate, internal standard, and the nonyl-glucoside (can be stored in aliquots at -20 °C). The MMTS solution can be stored at 4 °C. The

CLN1 substrate and internal standard are synthesized in the Gelb lab.[69] They are also available from GelbChem, LLC. The sources of additional reagents are listed in Table 2.13.

Table 2.13. Sources for additional reagents used in the CLN1 assay.

Compound	Source	Cat No.
nonyl-glucoside	Avanti	850510P
MMTS	Sigma	208795

Biomarker (C16:0-sulfatide, C26:0-LPC, lyso-SM, lyso-GB3) assay:

IS working solution: 18.75 nM d_4-C26:0-LPC, 30 nM d_5-C16:0-sulfatide, 10 nM $^{13}C_6$-lyso-Gb3, 10 nM d_7-lyso-SM in methanol. The IS working solution can be stored at -20 °C. D_5-C16:0-Sulfatide and $^{13}C_6$-lyso-Gb3 were synthesized in the Gelb lab and are also available from GelbChem, LLC. The isotope-labeled lysoGb3 can also be purchased from Avanti Polar Lipids. The sources for d_7-lyso-SM and d_4-C26:0-LPC are listed in the Table 2.14.

Table 2.14. Source for the labeled lyso-SM and C26:0-LPC used in the biomarker assay.

Compound	Source	Cat No.
d_7-lyso-SM	Avanti	860639
d_4-C26:0-LPC	Avanti	860389

Chapter 3. Metachromatic leukodystrophy: from screening to diagnosis and prognosis

3.1 Introduction

Metachromatic leukodystrophy (MLD, OMIM #250100) is an autosomal recessive lysosomal storage disorder where the de-sulfation of 3-sulfo-galactosylceramides (sulfatide) is impaired. The de-sulfation of sulfatides requires the combined action of arylsulfatase A (ARSA) and its activator protein, saposin B. Therefore, MLD is caused by the deficiency of ARSA, or more rarely, saposin B.[70] Sulfated glycolipids occur in the myelin sheaths of the central and peripheral nervous system and to a lesser extent in visceral organs like kidney, gallbladder, and liver.[71] Therefore, in MLD patients, the sulfated glycolipids accumulate in lysosomes of these tissues and are responsible for their metachromatic staining.[71] The clinical and histopathologic manifestations of MLD are dominated by the demyelination observed in the central and peripheral nervous system.[71]

The overall incidence rate of MLD ranges from 1:40,000 to 1:170,000 and varies greatly between ethnic groups.[71] The clinical onset and severity of MLD displays great variations. MLD is characterized clinically by symptoms related to progressive demyelination and is classified according to age of onset as late-infantile (< 30 months), juvenile (2.5 to 16 years) and adult (> 16 years).[71] Late-infantile form is the most common form of MLD (50-60%), and symptoms involve deterioration of gross motor function.[71] Death usually occurs within 5 years after the onset of clinical symptoms.[71] In the milder form (juvenile and adult onsets), initial symptoms usually consist of cognitive problems.[71] The majority of patients with juvenile MLD die before age of 20 years, whereas patients with adult MLD can live from a few years to several decades after the disease onset.[71]

Currently, no standardized treatment protocol is available for MLD patients, but several approaches have been studied clinically.[71] Hematopoietic stem cell transplantation can benefit children with presymptomatic late-infantile MLD or minimally symptomatic juvenile MLD[72]. Innovative treatments, including *ex vivo* and *in vivo* gene therapies and enzyme replacement therapy, for MLD are being evaluated in ongoing clinical trials[73]. Given the facts that new treatments for MLD are advancing at rapid pace and the presymptomatic diagnosis may secure an optimum treatment outcome, newborn screening for MLD may be considered in the near future.

3.2 Development of enzymatic activity assay for arylsulfatase A in leukocytes and DBS[74]

The work presented in this section is published in Analytical Chemistry: Hong et al., Leukocyte and Dried Blood Spot Arylsulfatase A Assay by Tandem Mass Spectrometry. Anal. Chem., ***2020,*** *just accepcted.*

Current major diagnostic tests for MLD include genetic and biochemical testing. Up to now, more than 160 pathogenic variants have been characterized in the *ARSA* gene, of which only a few occur with high frequency.[71] Therefore, while *ARSA* gene sequencing can confirm the diagnosis if two known pathogenic variants are found in *trans*, it can often lead to inconclusive results if novel variants are found. These novel variants, or so-called variations of unknown significance (VOUS), can largely complicate the interpretation of sequencing results.

The biochemical testing typically includes measurement of ARSA activity in leukocyte or fibroblast lysate and measurement of sulfatide in urine. However, the traditional colorimetric and fluorometric ARSA assays use generic sulfatase substrates[75, 76]. Since these substrates are not specific to ARSA, these assays either require two assays performed in parallel, one with an ARSA

specific inhibitor and one without, or require the removal of isoenzymes (mostly arylsulfatase B, ARSB) by ion-exchange chromatography[75, 76]. There have been multiple reports detailing the pitfalls of the current ARSA assays using generic fluorometric substrates, and the delayed diagnosis due to the confusions caused by these incompetent ARSA assays[77, 78]. Recently, a new spectrophotometric ARSA assay was developed using sulfatide, the natural substrate to ARAS, as the assay substrate[79]. However, this assay quantified the amount of enzymatic substrate instead of the enzymatic product, therefore it may not be accurate at the lower end. While the urine sulfatide assay is the most definitive test for diagnosing MLD patients, testing ARSA activity is also informative as it can help to differentiate MLD patients that are due to dysfunctional ARSA from those due to dysfunctional saposin B.

Hence, we aimed to develop an *in vitro* leukocyte assay for ARSA with high accuracy. With such an assay, we want to assess if ARSA activity in leukocyte lysate is predicative of phenotype, i.e. if lower ARSA activity corelates with earlier symptom onset and thus more severe form of the disease. If so, this will be very helpful in stratifying MLD patients, as little correlation has been found between ARSA activity measured with the artificial substrates, urine sulfatide, and genotype to phenotype. In previous studies with two other lysosomal enzymes, acid α-glucosidase (enzyme deficient in Pompe disease) and galactocerebrosidase (enzyme deficient in Krabbe disease), it was demonstrated that the residual activity correlated with the severity of the disease[80, 81]. Therefore, with the high performance ARSA leukocyte assay, we want to test the hypothesis that the severity of MLD, including the age of onset, can be predicted based on the level of residual ARSA activity in leukocyte lysates.

During 2016 to 2019, we carried out a large-scale pilot newborn screening study for MLD by measuring sulfatides abundance in DBS from random newborns using a reported method[25].

After screening approximately 50,000 random newborns, it became apparent that a second-tier test was required to reduce the number of false positives. Therefore, we also sought to develop an assay to measure ARSA enzymatic activity in DBS, so that the test can be performed onsite as part of the screening process without the need to contact families to obtain a different sample specimen like whole blood or urine.

Herein, we report an *in vitro* assay for ARSA in leukocytes lysate that utilized its natural substrate for specificity. The new ARSA leukocyte assay had an exceptionally high sensitivity and precision such that trace amounts of residual enzymatic activity could be detected with statistical significance. An ARSA enzymatic activity assay in DBS was also developed in this study, which was conducted for the first time without an anti-ARSA antiserum. These new ARSA assays will also be useful for diagnosing patients with multiple sulfatase deficiency (MSD), where defect in the formylglycine generating enzyme prohibits the crucial modification on the active site of all sulfatases[82].

3.2.1 Development of a leukocyte ARSA enzymatic assay

Recently, Han et al. reported the detection of ARSA enzymatic activity when a natural substrate, C18:0-sulfatide, was incubated with leukocyte lysate in buffer containing sodium taurodeoxycholate as detergent[83]. In this study, we used deuterium labeled sulfatide, d_3-C18:0-sulfatide, as the substrate, so that the enzymatic product (d_3-C18:0-galactosyl-ceramide) also carried the three deuterium labels, and could be selectively quantified without interference form endogenous galactosyl-ceramide. Using this approach, we were able to detect ARSA enzymatic activity in lysates of mixed leukocytes (mainly lymphocytes) by LC-MS/MS. As shown in Fig. 3.1, the de-sulfated product peak eluted at 1.07 min, and was fully separated from the peak of the

sulfated substrate eluting at 1.5 min. The separation was achieved using a column with mixed mechanism of anion exchange chromatography and reserve-phase chromatography where the negatively charged enzymatic substrate, sulfatide, retained more than the uncharged enzymatic product. If leukocyte lysate was omitted, no discernable product peak was observed above the baseline noise (not shown).

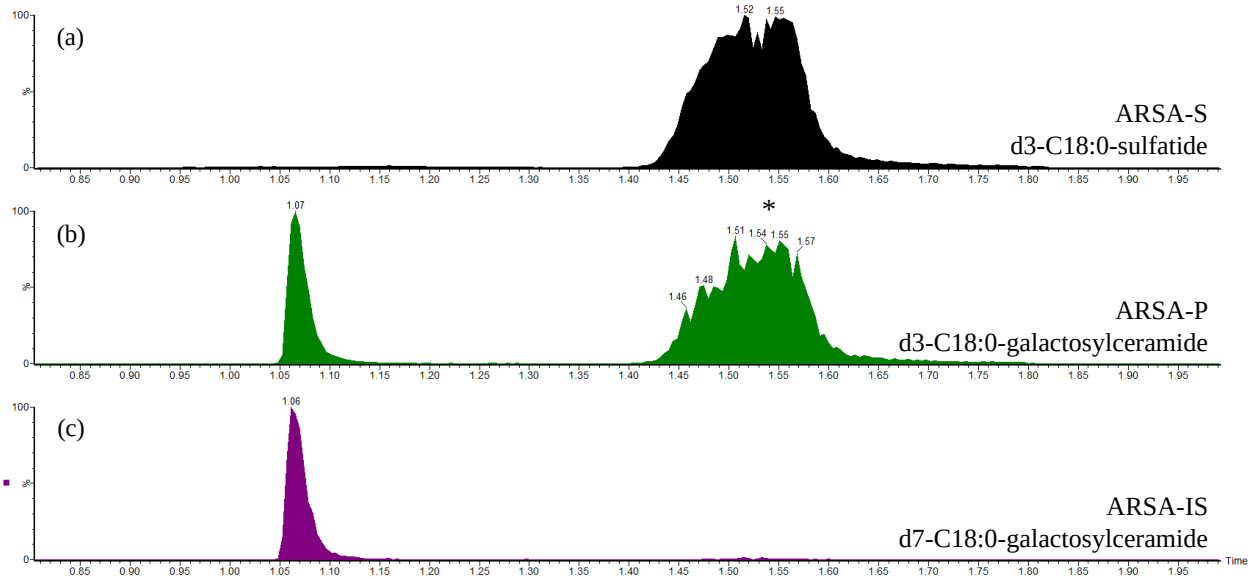


Figure 3.1. LC-MS/MS chromatography of the (a) ARSA substrate; (b) ARSA enzymatic product; and (c) ARSA internal standard channel. The asterisk in the ARSA enzymatic product channel was from substrate breakdown at the ESI source. The Y-axis is the ion counts in the MRM channel after being normalized to maximum (100%).

The enzymatic rate displayed hyperbolic kinetics as the concentration of substrate varied, giving a K_M of 83 μM (Fig. 3.2), which agreed to the K_M reported by Morena et al., where they used spectrophotometry to detect the amount of sulfatide depleted in the presence of ARSA[79]. The substrate concentration used in the following studies was 150 μM.

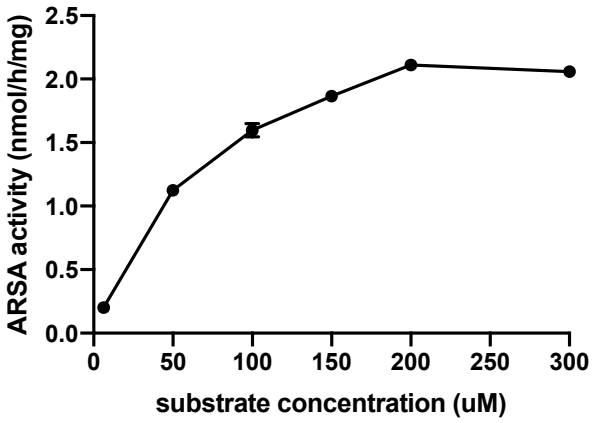


Figure 3.2. Substrate dosage optimization for the ARSA leukocyte assay. A total of 2 μg protein was used per assay with each point measured in triplicates. Error bars were standard deviations based on the triplicate measurements.

The ARSA activity was highly pH dependent with an optimal pH of 4.5 (Fig. 3.3a). The pH optimum of ARSA in our study agreed with the previous reports[79, 83]. The ARSA activity showed a sigmoidal dependence on the detergent concentration (Fig. 3.3b), and a concentration of 2.0 g/L sodium taurodeoxycholate gave maximal activity and was used in all following studies. Since detergent was present in the assay buffer, MLD patients that have dysfunctional saposin B protein will not be detected with this assay, but these variants are very rare among MLD patients (< 5%)[70]. In the previous report, $MnCl_2$ was added to the buffer at a concentration of 33 mM[83]. This was based on an earlier paper suggesting that this metal ion lowers the critical micellar concentration of the inhibitory ionic form of the bile salt and allows the formation of mixed micelles of taurodeoxycholate and sulfatide[84]. However, as shown in Fig. 3.3c, we found a decrease of ARSA activity with increasing amount of $MnCl_2$ in assay buffer, thus $MnCl_2$ was omitted from the final assay conditions. Barium or cerium sulfates are often used to assay sulfatases in complex matrices since these metals cause precipitation of the inorganic sulfate and phosphate ions, which are potent inhibitors of sulfatases[85]. However, ARSA activity was found to decrease as the concentration of $Ce(Acetate)_3$ was increased from 0-20 mM (Fig. 3.3d), therefore this salt was omitted from the standard ARSA assay. The reaction progress curve shows a falloff from linearity over 20 hours (Fig. 3.3e). Even though the progress curve is not linear, the amount

of enzymatic product formed after 16 hr incubation increased linearly with the amount of leukocyte protein used (Fig. 3.3f).

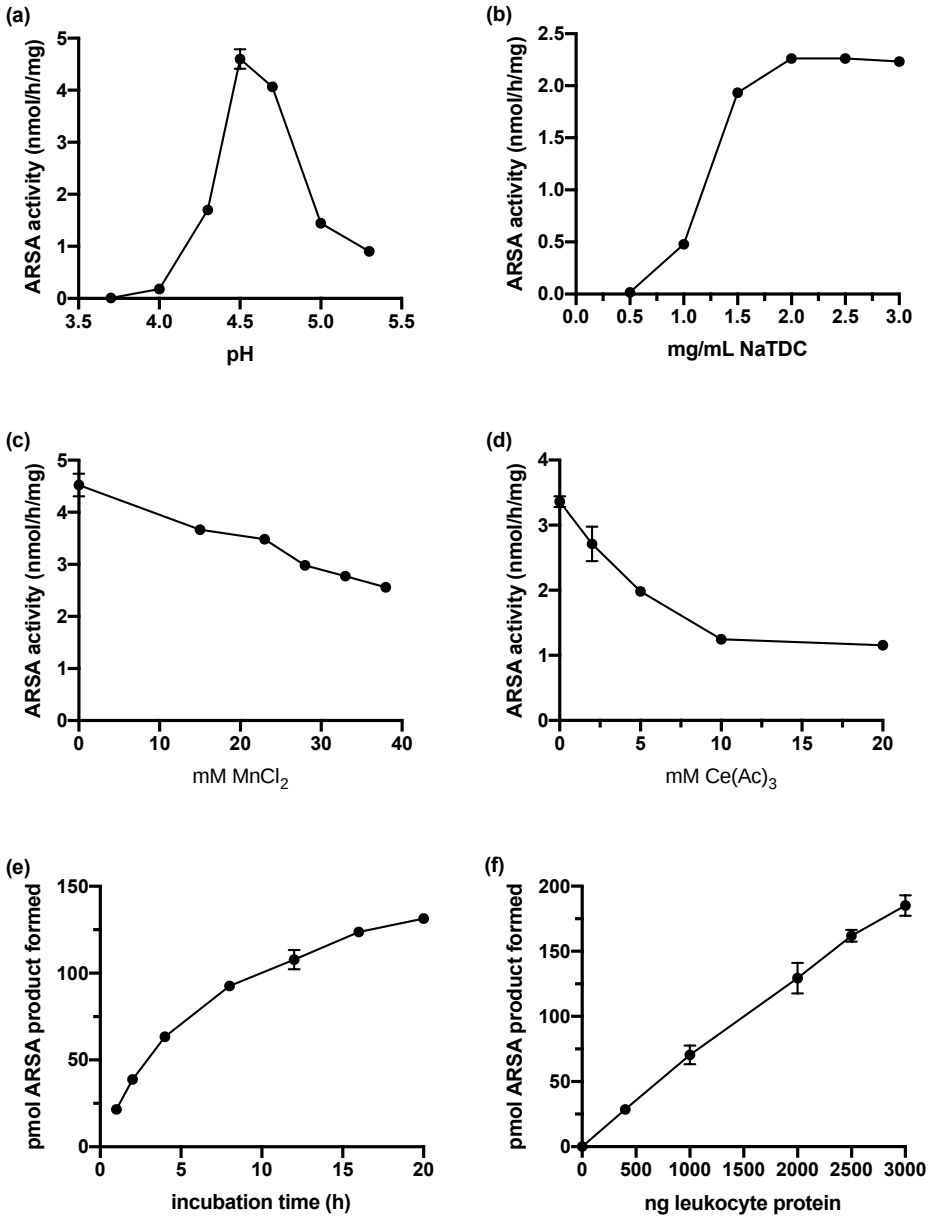


Figure 3.3. Optimization for the leukocyte ARSA assay: (a) pH of the assay buffer; (b) amount of sodium taurodeoxycholate in the assay buffer; (c) amount of $Ce(acetate)_3$ in the assay buffer; (d) amount of $MnCl_2$ in the assay buffer; (e) incubation time course; (f) amount of leukocyte protein used per assay. A total of 2 μg protein was used per assay with each point measured in triplicates. Error bars are standard deviations based on the triplicate measurements.

Assay conditions, including substrate dosage and buffer composition, was optimized before performance tests were carried out. When using artificial substrates, it was well known that

arylsulfatase B (ARSB), the enzyme deficient in MPS-VII disease can also recognize and digest the substrate unless inhibitors are used[75]. Yet with this new assay using natural substrate, no product was generated when using recombinant ARSB without the addition of any inhibitors, showing this assay is highly specific to ARSA (Fig. 3.4).

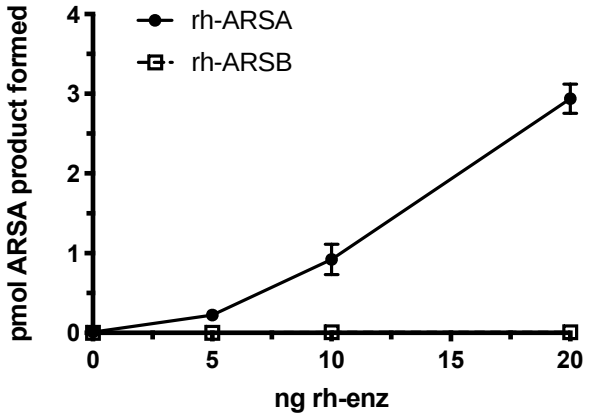


Figure 3.4. The amount of ARSA enzymatic product formed when incubated with recombinant human ARSA or ARSB. Error bars are standard deviations based on the triplicate measurements.

To study the minimal amount of residual ARSA activity that can be detected by this LC-MS/MS assay, various amount of lysate from ARSA-containing lymphoblast (Coriell Institute Repository, GM14603) was mixed with lysate from ARSA-lacking lymphoblast (GM23097), 2 μg protein total. With 100% ARSA-deficient cells lysate, no ARSA activity was observed (Fig. 3.5). ARSA activity increased proportionally with the percentage of ARSA-containing cells lysate in the mixture. From the inset of Fig. 3.5, it was clear that down to 0.1% residual ARSA activity could be detected above baseline with statistical significance.

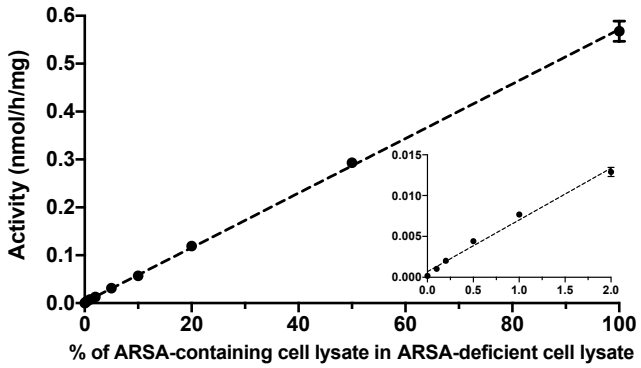


Figure 3.5. ARSA activity as a function of the fraction of ARSA-containing lymphoblast lysate (GM14603) added to ARSA-deficient lymphoblast lysate (GM23097). A total of 2 μg protein was used per assay with each point measured in triplicates. Error bars were standard deviations based on the triplicate measurements. The insert is an expansion of the plot at the lower end.

Next we studied the stability of ARSA in whole blood under optimized assay conditions. K_2EDTA-treated venous blood was kept at room temperature or 4 °C for up to 4 days prior to the preparation of leukocytes. As shown in Fig. 3.6a, about 14% and 32% of the ARSA activity was lost over the first 24 hr when the whole blood was stored at 4 °C or room temperature, respectively. The ARSA activity continued to drop with blood storage time at both 4 °C and room temperature, with approximately 45% remaining after 4 days. We thus recommend that whole blood be shipped overnight at 4 °C followed by immediate leukocyte preparation and freezing of the cell pellet. ARSA activity decreased only slightly with the number of freeze/thaw cycles of the mixed leukocyte, with about 20% activity lost after 5 cycles (Fig. 3.6b).

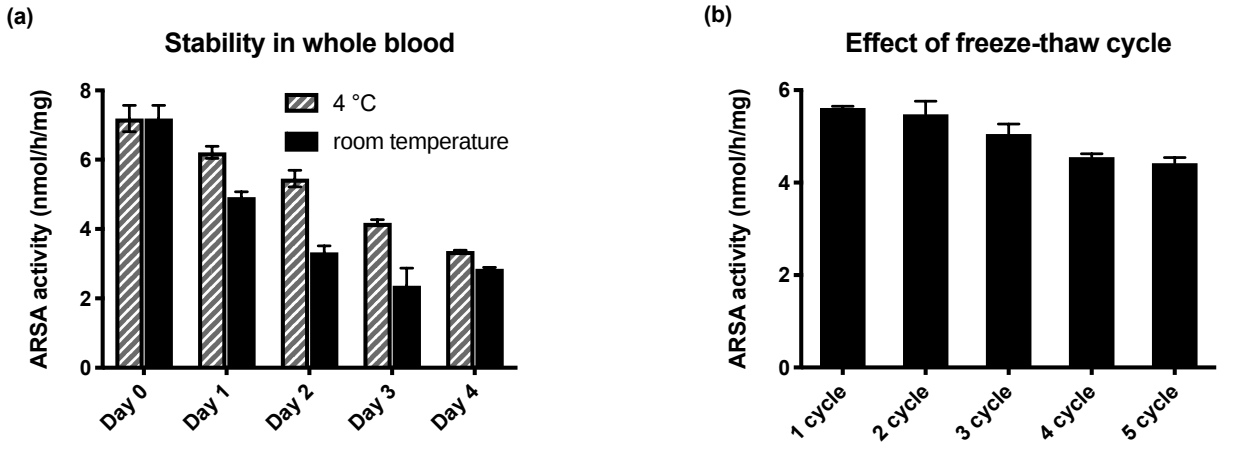


Figure 3.6. (a) Stability of ARSA in whole blood when stored at 4 °C or room temperature, and (b) the effect of freeze-thaw cycle on ARSA in cell lysate. A total of 2 μg protein was used per assay with each point measured in triplicates. Error bars are standard deviations based on the triplicate measurements.

3.2.2 Results of ARSA activity in leukocytes from MLD and MSD patients

Table 3.1 shows the ARSA activity in leukocyte lysates from 22 MLD patients and 1 MSD patient. Genotype information and age of onset (categorized by gross motor or cognitive symptoms), if available, are also provided. ARSA activity in leukocyte lysate was expressed as nmol/h/mg protein or as a percentage of the activity measured in the leukocyte lysate from a single healthy adult donor. MLD patient 21 and 22 were identified through their affected siblings, and were asymptomatic at the time of sampling, therefore their ages of disease onset were unavailable. MLD patient 5 also had Type I Gaucher disease, therefore the MLD variants were not the only contributing factor to the age of onset. The most severe MLD patients (late-infantile onset) in our cohort displayed symptoms between 12-35 months, and had residual ARSA activity in the range of 0.03-5.8% compared to the healthy adult. MLD patient 13 and 14, two late infantile patients, displayed higher residual ARSA activity (4.0% and 5.8%) than the rest of the late-infantile patient cohort. Four MLD patients with juvenile onset (MLD patient 15-19) had residual ARSA activity below 0.4% when compared to the healthy adult. One MLD patient (MLD patient 20) showed initial symptoms at 100 months and displayed 0.18% residual ARSA activity. Therefore, with this limited dataset, there appeared to be no correlation between residual ARSA activity in leukocyte lysate and the age of disease onset. The patient genotypes were not predictive of the age of disease onset as well. For example, MLD patient 7 and 15 had the same genotype (c.459+1G>A//c.1277C>T) and similar residual leukocyte ARSA activity (0.20% and 0.17% of the healthy adult), yet their age of onset was 45 months apart.

An additional multiplexed assay was used to measure the activity of iduronate-2-sulfatase (I2S), *N*-acetylgalactosamine-6-sulfatase (GALNS), *N*-acetylgalactosamine-4-sulfatase (ARSB), *α-N*-acetylglucosaminidase (NAGLU), and lysosomal *β*-glucuronidase (GUSB) in these leukocyte

lysates containing 2 μg protein using a previously published protocol[86]. All MLD patients had activities above 50% when compared to the healthy adult donor, whereas the MSD patient had sulfatase (I2S, GALNS and ARSB) activities below 2%, consistent with the diagnosis of MSD (data not shown).

Table 3.1. Summary of ARSA activity in leukocyte lysates from 22 MLD patients and 1 MSD patient[a].

	Genotype	age of onset	ARSA activity (nmol/h/mg protein)	% to adult control	Note
MLD patient 1		late infantile	0.0011 ± 0.0005	0.03	
MLD patient 2	c.370G>A+c*96A>G//c.685-1G>A+c.1055A>G	late infantile	0.0071 ± 0.0002	0.18	
MLD patient 3	c.465+1G>A//c.1108-3C>G	late infantile	0.0055 ± 0.0007	0.14	
MLD patient 4	c.449C>T//c.474C>A	12 month	0.0067 ± 0.0002	0.17	
MLD patient 5	c.178C>T//c.178C>T	13 month	0.0081 ± 0.0007	0.21	patient also has Gaucher
MLD patient 6		14 month	0.0055 ± 0.0031	0.14	
MLD patient 7	c.459+1G>A//c.1277C>T	15 month	0.0064 ± 0.0002	0.17	
MLD patient 8		18 month	0.0198 ± 0.0018	0.52	
MLD patient 9		18 month	0.0056 ± 0.0011	0.15	
MLD patient 10		18 month	0.0052 ± 0.0022	0.14	
MLD patient 11	p.E198A199del//c.848+1G>A	25 month	0.0078 ± 0.0018	0.20	
MLD patient 12	c.1175G>A//c.442G>A	29 month	0.0128 ± 0.0003	0.33	
MLD patient 13	c.136T>C//c.115G>A	30 month	0.1533 ± 0.0108	3.99	
MLD patient 14	c.1144G>A//c.459 + 1G>A	35 month	0.2212 ± 0.0142	5.75	
MLD patient 15	c.459+1G>A//c.1277C>T	60 month	0.0078 ± 0.0011	0.20	
MLD patient 16	c.465+1G>A//c.869G>A	60 month	0.0139 ± 0.0005	0.36	
MLD patient 17	c.931G>A//c.931G>A	early juvenile	0.0040 ± 0.0005	0.10	
MLD patient 18	c.465+1 G>A//c.869 G>A	early juvenile	0.0057 ± 0.0006	0.15	
MLD patient 19	c.1136C>T//c.1136C>T	early juvenile	0.0063 ± 0.0013	0.16	
MLD patient 20		100 month	0.0068 ± 0.0018	0.18	
MLD patient 21	c.459+1G>A//c.1277C>T		0.008 ± 0.0004	0.21	
MLD patient 22	c.459+1G>A//c.1277C>T		0.0067 ± 0.0002	0.17	
MSD patient 1		18 month	0.0046 ± 0.0015	0.12	
Adult control			3.8438 ± 0.0971		

[a] ARSA activity was measured in triplicate on three aliquots from the same batch of leukocyte lysate.

3.2.3 Assaying arylsulfatase A activity in DBS after purification by immuno-precipitation

Initial attempts to detect ARSA enzymatic activity in DBS were unsuccessful. When a 3 mm DBS punch was incubated in 30 μL of assay cocktail (same as used in the leukocyte assay), no ARSA activity could be detected by LC-MS/MS (Fig. 3.7). When leukocyte lysate was added to the assay containing a 3 mm DBS punch, no ARSA activity was observed, whereas activity was seen when the lysate was incubated with a filter paper punch lacking blood matrix (Fig. 3.7). This suggested the presence of some ARSA inhibitor(s) present in whole blood. It should be noted that addition of 5 mM $Ce(Acetate)_3$ or $Pd(Acetate)_2$ to the assay buffer as precipitators for inorganic sulfate and phosphate ions did not rescue ARSA activity in DBS, suggesting that the inhibitor(s) in whole blood were not merely inorganic sulfate or phosphate ions.

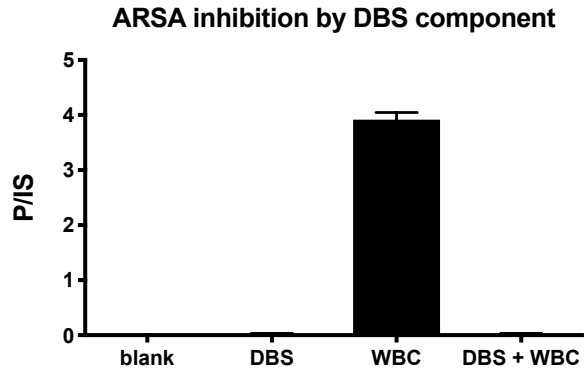


Figure 3.7. The ion ratio of ARSA product to internal standard in a blank ARSA assay, as well as when DBS and leukocyte (WBC) lysate was incubated separately and together (DBS+WBC).

In an earlier study, Tan et al. were able to detect ARSA enzymatic activity in DBS after immuno-precipitating the ARSA protein in the DBS extract and measuring the activity using the generic fluorometric substrate for sulfatases, 4-methylumbelliferyl sulfate (4MUS)[59]. Inspired by their work, we purified the blood extract from DBS by immuno-precipitation and measured the ARSA activity using 4MUS as the substrate (Fig. 3.8). It was found that adult control had a 10% increase in the fluorescent signal when compared to the blank and the MLD patient, despite the high background (Fig. 3.8). This suggested that ARSA activity was rescued from the blood matrix

and was consistent with the previous report and indicated that the ARSA inhibitor(s) from whole blood were removed during the immuno-precipitation process.

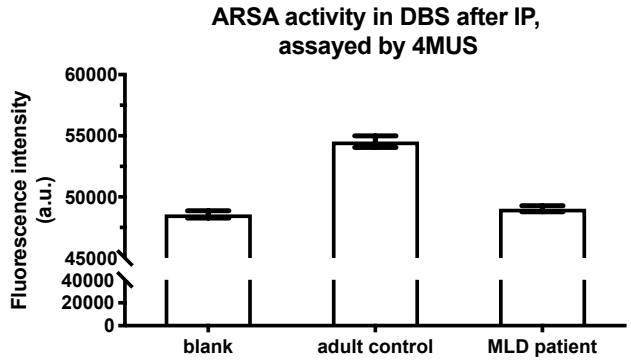


Figure 3.8. ARSA activity in DBS measured by 4MUS after immuno-precipitation purification. Error bars are standard deviations based on the triplicate measurements.

Given that the fluorogenic substrate resulted in a high background (Fig. 3.8), we then assayed the ARSA activity in DBS using deuterated sulfatide as was for the leukocyte ARSA assay after the immune-precipitation purification. Fig 3.9 shows the ARSA enzymatic activity in DBS from 4 MLD patients (median: 0.007 μM/h, range: 0.005-0.011 μM/h), 1 MSD patient (0.087 μM/h), and 7 healthy adults (0.63 μM/h, range: 0.39-1.30 μM/h) after the DBS extract was purified by immune-precipitation. All MLD patients had barely detectable ARSA activity, and the MSD patient displayed 14% ARSA activity when compared to the mean activity of the healthy adults, indicating these patients had essentially no residual ARSA activity.

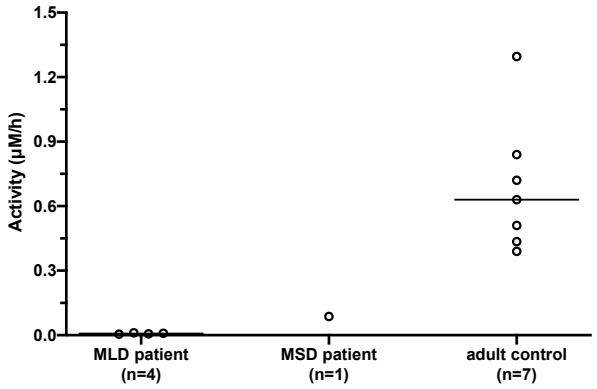


Figure 3.9. ARSA activity in DBS from 4 MLD patients (median: 0.007 μM/h, range: 0.005-0.011 μM/h), 1 MSD patient (0.087 μM/h), and 7 healthy adults (0.63 μM/h, range: 0.39-1.30 μM/h), after immuno-precipitation purification. The horizontal bar indicates the median of each group.

The stability of ARSA in DBS was investigated using the ARSA enzymatic assay after immune-precipitation (Fig. 3.10). When stored at room temperature, ARSA activity dropped to

95% after 7 days and to 60% after 14 days. Intriguingly, additional ARSA enzymatic activity loss was minimal over the next 100 days. ARSA activity remained stable in DBS when kept under 4 °C or -20 °C for over 3 months (Fig. 3.10). These results suggested that DBS should not be kept at room temperature over extended period. It should be noted that the DBS used in the study was stored with desiccant, and it was found out later that DBS stored under unideal conditions (i.e. without desiccant or long exposure to sunlight) had difficulties extracting the protein from the filter paper, therefore resulting in lower ARSA enzymatic activity.

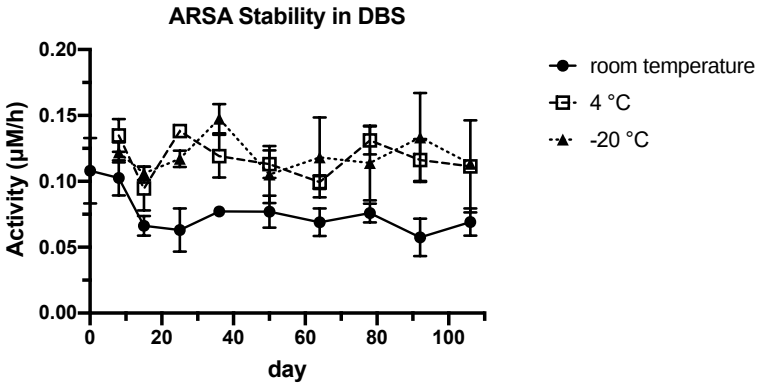


Figure 3.10. ARSA stability in DBS from a healthy adult, stored at room temperature, 4 °C and – 20 °C, after immuno-precipitation purification. Error bars are standard deviations based on the triplicate measurements.

However, other commercially available anti-ARSA serums, including one polyclonal and five monoclonal antiserums, were tested, but no ARSA activity could be recovered after immuno-precipitation (Fig. 3.11).

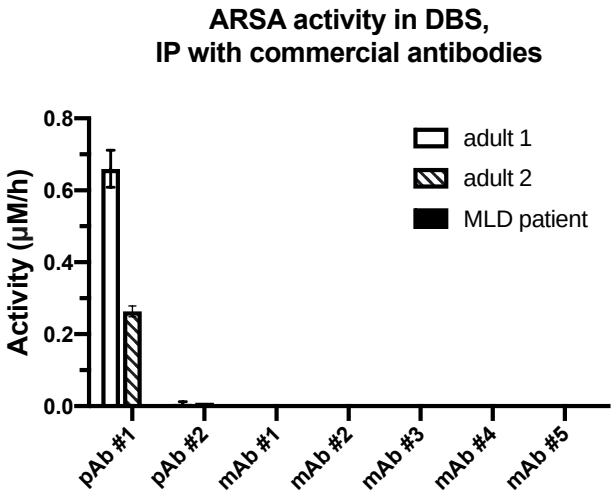


Figure 3.11. ARSA activity in DBS from 2 healthy adults and 1 MLD patient, after immuno-precipitation by 2 different polyclonal anti-ARSA serum and 5 monoclonal anti-ARSA serum. pAb #1 was from R&D System (Cat. AF2485) and was used in the previous studies.

3.2.4 Assaying arylsulfatase A activity in DBS after purification by size-exclusion chromatography

Given that the ARSA activity in DBS could only be measure after immuno-precipitation by a commercially available antibody, we sought to alternative purification methods that does not rely on a reagent in limiting quantity. Initial attempts were based on ion exchange chromatography as it was previously reported to purify recombinantly expressed ARSA or to purify endogenous ARAS from body fluids before assaying its activity[76, 87]. The DBS was extracted in acidic or basic buffer, and the extract was applied to a Pierce Strong Cation Exchange Spin Column (ThermoFisher, Cat. 90008) or Pierce Strong Anion Exchange Spin Column (ThermoFisher, Cat. 90010), followed by elusion with acidic or basic buffer containing 0.5 M NaCl, respectively, following vendor's instruction. No ARSA activity in DBS extract or leukocyte lysate was observed after the purification by ion exchange chromatography (data not shown), probably because NaCl is a strong inhibitor to ARSA at high concentration[88].

We then pursued size-exclusion chromatography to purify ARSA from DBS matrix after eluting the DBS punch with ARSA assay buffer. The extract was applied to pre-conditioned Zeba

Spin Desalting Column with a MW cutoff of 7 k Da or 40 k Da (ThermoFisher, Cat. 89882 or Cat. 87766), and purified by centrifugation at 1500 g for 2 min, as per vendor's instruction. ARSA activity was observed after the purification (Fig. 3.12). Given the success of this method, we tested a lower-cost size-exclusion method where Sephadex G-25 and G50 resin (GE Lifesciences, Cat. 17003201 and 17004201) with a MW cutoff of 5 k and 30 k resin were loaded into wells of a 96-well fritted plate. Fig. 3.12 showed the ARSA activity in DBS from a healthy adult was recovered using size exclusion resins with different MW cutoff. The Sephadex G-25 resin with a MW cutoff of 5 k gave optimal result and was used for all additional studies. Since the Sephadex G-25 resin can only remove molecules below 1000 Da, it confirms our hypothesis that small molecule inhibitor(s) from blood is inhibiting ARSA.

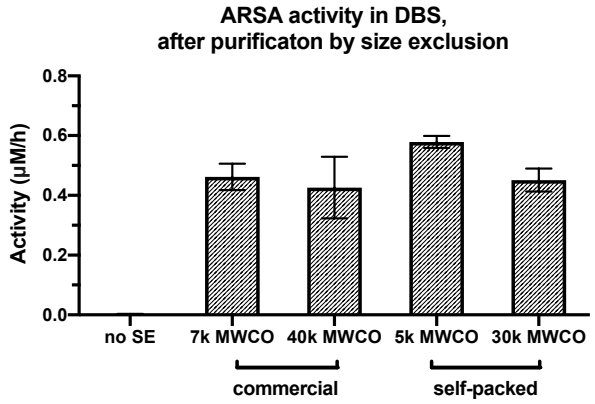


Figure 3.12. ARSA activity in DBS from 1 healthy adults, after purification by size-exclusion chromatography with resin with a MW cutoff of 7 kDa (Zeba Spin Desalting Column, 7 k MWCO, ThermoFisher, Cat. 89882); 40 kDa (Zeba Spin Desalting Column, 40 k MWCO, ThermoFisher, Cat. 87766); 5 kDa (Sephadex G-25, GE Lifesciences, Cat. 17003201); 30 kDa (Sephadex G-50, GE Lifesciences, Cat. 17004201). The Sephadex G25 and G50 resin were self-packed into a fritted well-plate.

Next the extraction protocol of ARSA from the DBS punch was optimized. It was found that the color of the extract from poorly stored DBS punch was pale compared to extract from DBS stored under good conditions, and the ARSA activity measured in such pale extract was substantially lower, suggesting not all the protein was extracted from the punch (Fig. 3.13). To improve the protein recovery from DBS punch, different elution buffers were tested, including ARSA buffer, ammonium hydroxide in water and ammonium hydroxide in ARSA buffer, with a 50% increase in ARSA recovery when 0.5% ammonium hydroxide was present in the ARSA

buffer (Fig. 3.13d). The use of ammonium hydroxide was based on an earlier study showing improved extraction of proteins from DBS using this additive[89].

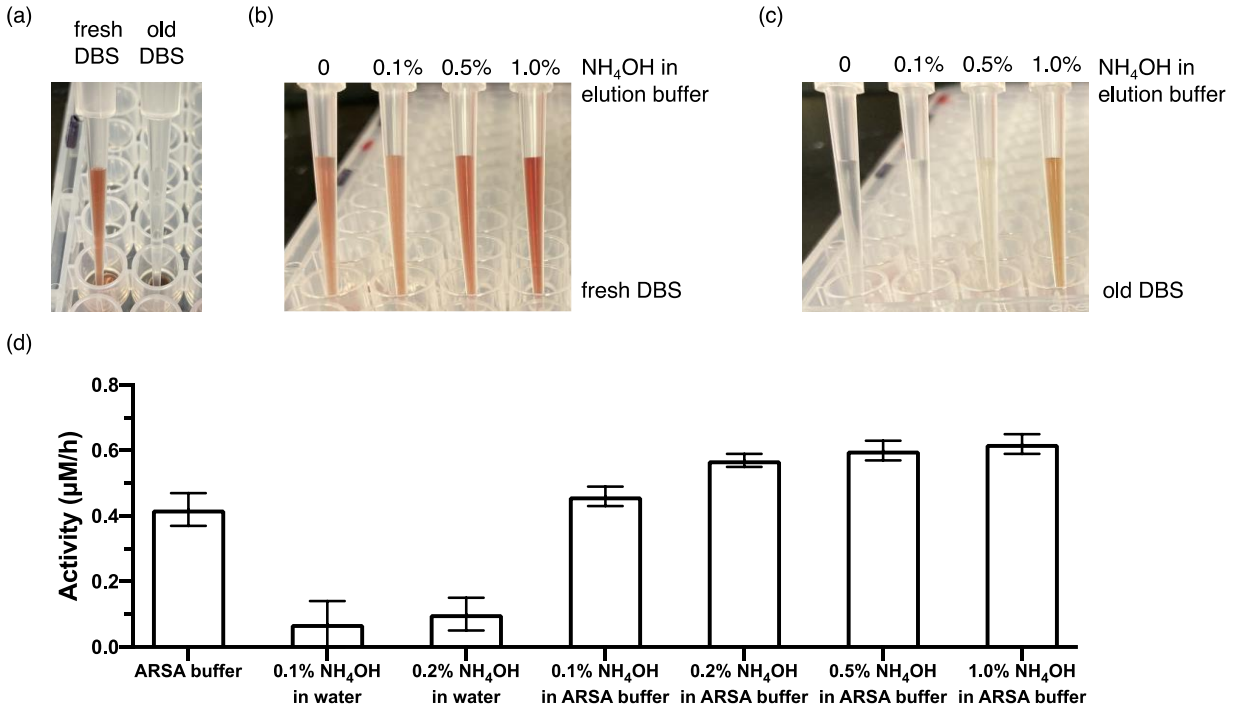


Figure 3.13. (a) Extract from a fresh and old DBS; (b) extract from fresh DBS, with 0 to 1.0% NH_4OH in the elution buffer; (c) extract from old DBS, with 0 to 1.0% NH_4OH in the elution buffer; (d) ARSA activity in DBS after purification by size-exclusion chromatography, with the DBS eluted with 50 μL ARSA buffer; 0.1% and 0.2% NH4OH in water; 0.1%, 0.2%, 0.5% and 1.0% NH4OH in ARSA buffer, respectively.

Further optimization of the amount of ammonium hydroxide in the ARSA buffer gave the final DBS extraction buffer condition (0.8 % ammonium hydroxide in ARSA buffer) (Fig. 3.14a). The extraction time was optimized, and 4 hr at room temperature was chosen for optimal extraction (Fig. 3.14b).

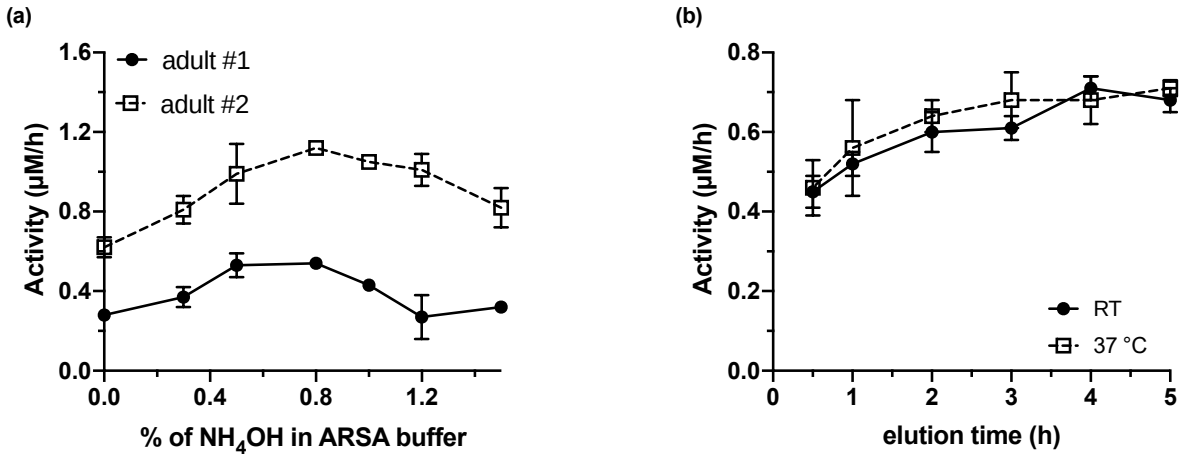


Figure 3.14. ARSA activity in DBS after purification by size-exclusion chromatography. (a) DBS punches from two healthy adults were eluted with 50 μL 0%, 0.3%, 0.5%, 0.8%, 1.0%, 1.2% and 1.5% NH4OH in ARSA buffer at 37 °C for 1 hr; (b) DBS punch from one healthy adult was eluted with 50 μL 0.8% NH4OH in ARSA buffer for 1-5 hr at room temperature or at 37 °C, respectively.

We also varied the amount of resin used per assay for the size-exclusion chromatography purification and found that 60 mg dry resin per assay gave better consistency compared to 40 mg per assay, though at the cost of a 20% loss in activity (Fig. 3.15).

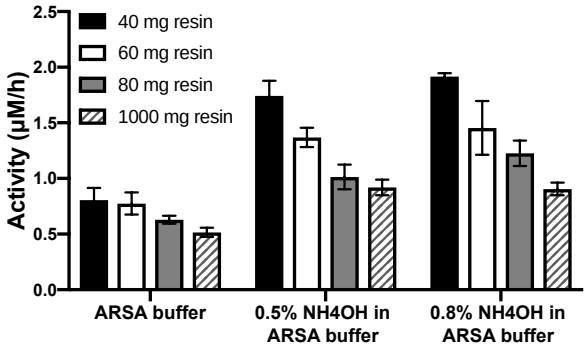


Figure 3.15. ARSA activity in DBS from one healthy adult after purification by size-exclusion chromatography with various amount of resin packed in the fritted well. The DBS punches were extracted with 50 μL ARSA buffer, 0.5% and 0.8% NH4OH in ARSA buffer at room temperature for 4 hr.

With the final ARSA DBS assay in hand, we studied the linearity and reproducibility of the assay using a series of Quality Control DBS for lysosomal storage disorders from the CDC (the base pool (0.023 ± 0.003 μM/h), low (0.048 ± 0.007 μM/h), medium (0.21 ± 0.02 μM/h), and high (0.37 ± 0.02 μM/h) standards) (Fig. 3.16). These QC samples were prepared by mixing various amounts of unprocessed cord blood with base pool blood (prepared from leukocyte-reduced blood and heat-inactivated, charcoal-stripped serum)[90]. Fig. 3.16 showed good linear

response of the ARSA activity to the fraction of high control in the Quality Control DBS and good reproducibility with 20 replicates at each point (< 15% CV). It should be noted that there was finite ARSA activity in the base pool sample (non-zero intercept of Fig. 3.16), showing that not all the ARSA activity was depleted in the base pool DBS.

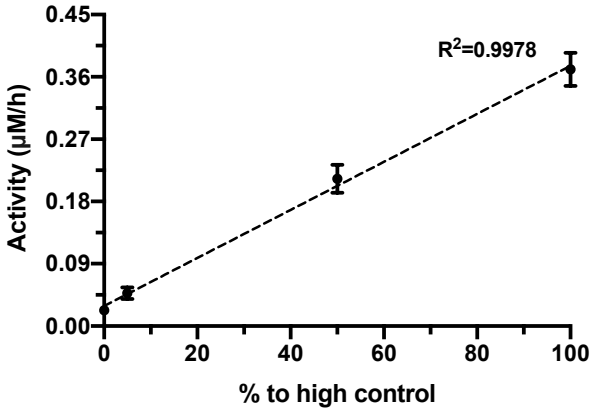


Figure 3.16. ARSA activity after size-exclusion chromatography purification in CDC Quality Control DBS samples, including base pool (0.023 ± 0.003 μM/h), low (0.048 ± 0.007 μM/h), medium (0.21 ± 0.02 μM/h), and high (0.37 ± 0.02 μM/h), each representing 0, 5%, 50% and 100% to the high control, respectively. Each point was measured in 20 replicates. Error bars were standard deviations based on the 20 measurements.

Having fully optimized and validated the ARSA DBS assay, we measured ARSA activity in DBS from 34 MLD patients (median: 0.0015 μM/h, range: 0-0.18 μM/h), 3 MSD patients (median: 0.032 μM/h, range: 0.028-0.076 μM/h), 10 healthy adults (median: 0.80 μM/h, range: 0.45-1.3 μM/h), and 294 presumed random newborns (median: 0.27 μM/h, range: 0.082-0.65 μM/h) (Fig. 3.17a). The two MLD patients that had the highest ARSA DBS activity (0.11 and 0.18 μM/h) were MLD patient 13 and 14 (Table 3.1), respectively. It should be noted that the random newborn DBS were stored for 1-2 months at room temperature. Presumably about 40% of the ARSA activity had been lost in these samples (based on DBS stability data, Fig. 3.10), thus the range of activities in random newborns showed in Fig. 3.17a was lower than what would be expected in fresh newborn DBS. The patients and normal adult DBS were stored at -20 °C shortly after DBS collection with presumably minimal loss of ARSA activity. Since it was extremely hard to obtain large amount of fresh newborn DBS, an aging experiment was carried out as a compromise. When fresh DBS from 18 MLD (including DBS from MLD patient 13 and 14, Table

3.1) and 2 MSD patients were aged at room temperature for 1 month, over 50% of the residual ARSA activity in DBS from MLD patient 13 and 14 was lost (Fig. 3.17b). The result demonstrated that patients can be completely distinguished from the normal (random newborns) based on the ARSA DBS activity if they had similar storage condition (Fig. 3.17b).

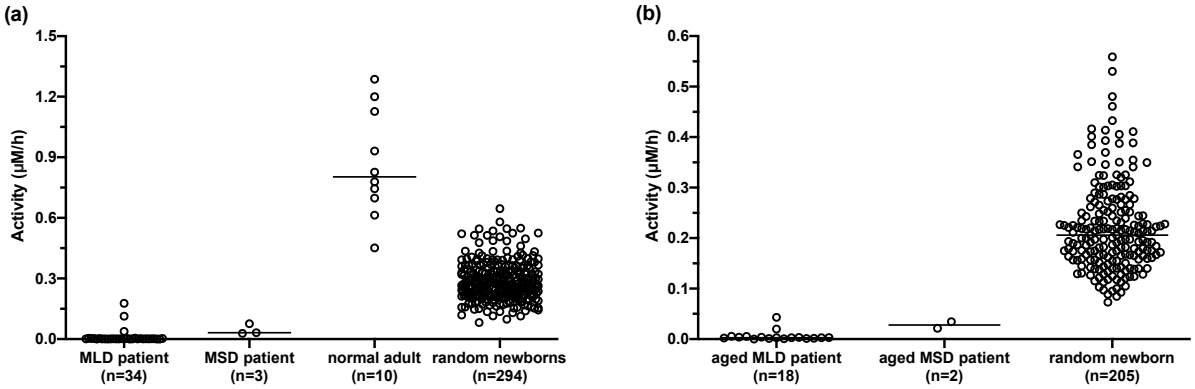


Figure 3.17. (a) ARSA activity after size-exclusion chromatography purification in DBS from 34 MLD patients (median: 0.0015 μM/h, range: 0-0.18 μM/h), 3 MSD patients (median: 0.032 μM/h, range: 0.028-0.076 μM/h), 10 healthy adults (median: 0.80 μM/h, range: 0.45-1.3 μM/h) and 294 random newborns (median: 0.27 μM/h, range: 0.082-0.65 μM/h). Fresh DBS from patients and healthy adults were used. (b)ARSA activity in aged DBS (stored at room temperature for 1 month before processing) from 18 MLD patients (median: 0.003 μM/h, 0-0.043 μM/h), 2 MSD patients (0.021 and 0.035 μM/h), and 205 random newborns (median: 0.21 μM/h, 0.073-0.56 μM/h. The horizontal bars indicate the median of each group.

3.2.5 Discussions and conclusions

Recently, Han et al. reported an ARSA leukocytes assay similar to the one reported in the current study[83]. They used non-deuterated C18:0-sulfatide as substrate and commercially available d_{35}-C18:0-galactosyl-ceramide as internal standard[83]. In this study, we used deuterium labeled sulfatide as the enzymatic substrate so that the deuterated product can be differentiated from the endogenous galactosyl-ceramide. Moreover, d_{35}-C18:0-galactosyl-ceramide does not co-elute with the enzymatic product due to the 35 deuterium labels, therefore in this study we used the in-house synthesized d_7-C18:0-galactosyl-ceramide that co-eluted with the enzymatic product as the internal standard. Co-elution of the analyte and its internal standard is important for LC-MS/MS analysis as there could be different matrix effects on analytes eluting at different retention time.

Surprisingly, the ARSA activity in leukocyte lysate from healthy adults reported by Han et al. were approximately 100-fold higher than those measured in the current study. We speculated that the enzymatic product measured by Han et al. was a combination of enzymatic breakdown of sulfatide and the endogenous galactosyl-ceramide present in the sample. We also note that the activities reported by Han et al. were in general 30 to 100-fold higher than the activities of other lysosomal enzymes measured in leukocytes[91-93].

The LC-MS/MS ARSA assay developed here is expected to be more accurate and precise than the traditional colorimetric and fluorometric ARSA assays, where generic artificial sulfatase substrates were used[75, 76]. Since these generic substrates are not specific to ARSA, these assays either required two assays performed in parallel, one with an ARSA specific inhibitor and one without, or required the removal of isoenzymes by ion-exchange chromatography[75, 76]. Inhibition of ARSA is only partial in these earlier methods as the inhibitors used are neither highly potent, nor ARSA-specific, which compromises the reliability of the assay, especially at the lower end. Usage of assay buffer that favors ARSA activity has been reported, but it is not clear if the residual activity is due to ARSA, or other off-target sulfatases, or both. This is a serious concern as proper evaluation of trace amounts of residual ARSA activity is crucial when diagnosing potential patients. Recently, a new spectrophotometric ARSA assay was developed using sulfatide as substrate[79]. However, this assay quantified the enzymatic activity by measuring the decrease of substrate instead of the formation of product, therefore it will not be accurate to measure trace amounts of residual ARSA activity as well. It is also possible to use generic sulfatase substrates to selectively assay ARSA after immuno-precipitation purification[59], but this requires a polyclonal antiserum that is available in limited quantities, and also relies on the assumption that no off-target sulfatases are being pulled down during the process. The current LC-MS/MS-based ARSA assay

was highly ARSA-specific due to the usage of the natural substrate and was highly precise and sensitive as trace amounts of residual activity could be detected with statistical significance (Fig. 2.5). It should be noted that the methods described in this study are for research only and do not meet The Clinical Laboratory Improvement Amendments (CLIA) validation guidelines for the development of laboratory developed tests (LDTs). Furthermore, studies of a larger cohort of patients and normal controls are needed to define a reference range. The fluorometric and LC-MS/MS assays may both be adequate for diagnostic purposes, where measurement of nominally low enzymatic activity is potentially sufficient for diagnosis when the patient exhibits symptoms that are characteristic of the disease. However, due to its high performance, we believe this new ARSA leukocyte assay can be beneficial for evaluating potential patients who are identified by newborn screening program and may be at risk to develop MLD but are so far asymptomatic. This is especially important in the case of MLD where there is a high frequency of pseudodeficiency variants[59].

We were able to detect ARSA activity in DBS only if the enzyme is purified or partially purified from the matrix. Immunoprecipitation of ARSA was useful in this context, but to date we have not been able to find a monoclonal antibody that works in the enzymatic assay. Reliance on a commercially-available polyclonal anti-ARSA antiserum is problematic for long-term sustainability of the assay. Fortunately, we found that a simple size-exclusion procedure was sufficient to remove the inhibitor(s) and make ARSA detectable in the DBS matrix. Furthermore, the protocol was simple and inexpensive to carry out. With automation, it is also appropriate for high throughput screening.

Currently, the ARSA enzymatic activity assay in DBS is implemented as the second-tier test for our ongoing large-scale de-identified pilot study for the newborn screening of MLD, where

the ARSA enzymatic assay is performed on samples with abnormal sulfatide results. With this two-tier algorithm, the false positive rate is largely reduced and so is the accompanying anxiety to families in a real-world scenario. It should be noted that the ARSA activity in newborns reported here cannot be used as a normal range as these DBS were stored at room temperature for 1-2 months prior to analysis, and ARSA is known to be unstable under this storage condition. Since the ARSA enzymatic assay in DBS is conducted in a high throughput manner with a turnaround time of 2 min per sample, we are also exploring the option of using this assay as the first-tier screening test for MLD. More details are provided in Section 3.3.

In summary, a novel ARSA leukocyte and DBS assay by LC-MS/MS was developed. Both assays used deuterated natural sulfatide as substrate, therefore are highly specific to ARSA. This is essential for accurately diagnosing MLD and MSD patients. Implementation of this new ARSA DBS assay as a second-tier test is crucial for our on-going de-identified pilot study for the newborn screening of MLD. This high throughput assay may also serve as the first-tier screening test for MLD.

3.2.6 Experimental details

3.2.6.1 ARSA leukocyte assay

Leukocyte was prepared typically from 1 mL of whole blood (collected in EDTA tube). To each volume of well mixed whole blood, three volume of chilled RBC lysis (Qiagen, Cat. 158904) was added. The mixture was gently inverted 10 times and incubated at room temperature for 10 minutes. The mixture was inverted for another 10 times after the incubation and was centrifuged at 600 g for 5 minutes. The supernatant was removed, and 5 mL of water was added, then vortexed 10 s before adding 5 mL 1.8% NaCl in water. After vortexing for 10 seconds, the mixture was then spun down at 600 g for 5 minutes. The supernatant was removed again, and 5 mL of water was

added, vortexed, followed by 5 mL of 1.8% NaCl in water, and vortexed again. After centrifuging at 600 g for 5 minutes, the supernatant was removed, leaving ~ 100 μL above the leukocyte pellet, and transfer to – 80 °C freezer. The leukocyte was lysed during the freeze-thaw.

The amount of protein in the leukocyte lysis was determined using a bicinchoninic (BCA) acid assay that is compatible with reducing reagent (ThermoFisher, Cat. 23250) using bovine serum albumin (BSA) as standards as per vendor's instruction. Briefly, the frozen leukocyte was thawed on ice, and spun down at 10,000 g for 1 minute. 9 μL supernatant was used in the BCA assay. To 9 μL leukocyte lysate or 9 μL BSA standard, 4 μL compatibility reagent was added, and then incubated in an orbital shaker (400 rpm on a 3 mm radius) at 37 °C for 15 minutes. Then 260 μL BCA working reagent (prepared by mixing 50 part of BCA Reagent A and 1 part of BCA Reagent B) was added to each sample, and then incubated in an orbital shaker (400 rpm on a 3 mm radius) at 37 °C for 30 minutes. The sample was then cooled down at room temperature for 5 min and absorbance at 562 nm was measured on a spectrophotometer.

Assay cocktail was 150 μM d_3-C18:0-sulfatide as substrate and 2 μM d_7-C18:0-galactosyl-ceramide as internal standard in assay buffer (80 mM sodium acetate (J.T. Baker, Cat. 3460-01), 2.0 g/L sodium taurodeoxycholate (Carbosyth, Cat. FS45995), pH 4.5 ± 0.02). The internal standard, d_7-C18:0-galactosyl-ceramide, was synthesized by Dr. Arun Kumar in the Gelb laboratory. It was prepared by mixing proper amount of substrate and internal standard stock solution, then removing the organic solvent with a centrifugal-concentration or a jet of oil-free air at room temperature, and reconstituting with assay buffer with a vortex mixer.

Leukocyte protein concentration was adjusted to 0.2 μg/μL using 0.9% NaCl in water as diluent. The assay was set up by using 10 μL of leukocyte lysate (2 μg protein) and 30 μL of assay

cocktail in a 96-well, polypropylene deep-well plate sealed with a silicone matt. The plate was centrifuged for 1 minute at 3000 g to ensure that all liquid was at the bottom of each well. The assay was then placed on an orbital shaker (400 rpm on a 3 mm shaking radius) at 37 °C for 16 hours. Reactions were quenched by addition of 300 μL of methanol, and the plate was centrifuged for 5 minutes at 3000 g. 150 μL supernatant was transferred out and diluted into 50 μL water in a autosampler plate, and placed in the cooled (8 °C) autosampler chamber of the LC-MS/MS instrument.

UPLC-MS/MS was carried out on a Waters Xevo TQ-S micro mass spectrometer coupled to a Waters AQUITY UPLC I-Class system using multiple reaction monitoring (MRM). MRM settings and ESI source conditions are listed in Table 3.2 and 3.3.

Table 3.2. ESI source parameters on TQ-S micro for the ARSA assay

Parameter	
Polarity	ES+
Capillary Voltage (kV)	3.5
Source temperature (°C)	150
Desolvation temperature (°C)	550
Cone gas flow (L/hr)	50
Desolvation gas flow (L/hr)	750
Collision gas	Argon

Table 3.3. MS/MS parameters for the ARSA assay on Xevo TQ-S micro

Analyte	ESI	parent mass (m/z)	daughter mass (m/z)	dwell time (s)	cone voltage (V)	collision energy (V)
ARSA-P	+	731.7	264.3	0.054	25	40
ARSA-IS	+	735.8	264.3	0.054	25	40
ASRA-S	+	811.5	264.3	0.054	25	40

Separation of the enzymatic substrate and product was achieved at 30 °C using an ACQUITY UPLC CSH Fluoro Phenyl column (1.7 μm, 2.1 mm x 50 mm, Waters Corp., Cat. 186005351) connected to an ACQUITY UPLC CSH Fluoro Phenyl VanGuard Pre-column (1.7

μm, 2.1 mm x 5 mm, Waters Corp., Cat. 186005358) at a flow rate of 0.7 mL/min. Mobile phase A was 50/50 water/acetonitrile (v/v) with 0.1 % formic acid; mobile phase B was 50/50 acetonitrile/isopropanol (v/v) with 0.1% formic acid. The weak needle wash and the strong needle wash for the autosampler were 25/25/50 (v/v/v) methanol/isopropanol/water, and 47.5/47.5/5 (v//v/v) methanol/isopropanol/water, respectively. The linear gradient was as follow: 0-0.5 min, 0% solvent B; 0.5-1.0 min, 0 to 90% solvent B; 1.0-1.5 min, 90 to 100% solvent B, 1.5-1.95 min, 100% solvent B, 1.95-2.0 min, 100 to 0% solvent B.

An alternative separation was achieved at 30 °C using an CORTECS UPLC C18+ Column (1.6 μm, 2.1 mm x 100 mm, Waters Corp., Cat. 186007116) connected to an CORTECS UPLC C18+ VanGuard Pre-column (1.6 μm, 2.1 mm x 5 mm, Waters Corp., Cat. 186007125) at a flow rate of 0.5 mL/min using the same mobile phases and washes as above. The gradient was as follow: 0-4.0 min, 50 to 70% solvent B (linear); 4.0-11 min, 70-80% solvent B (linear); 11-12 min, 80-100% solvent B (step); 12-14 min, 100-50% solvent B (step)).

3.2.6.2 ARSA DBS assay by immuno-precipitation.

Polyclonal anti-ARSA (R&D System, Cat. AF2485) was immobilized on a high binding plate (PerkinElmer, Cat. 1244-550) at 1 μg anti-ARSA per well. The antibody was coated by adding 100 μL anti-ARSA (10 μg/mL) in 0.2 M sodium phosphate, pH 6.8 in each well, then incubating at room temperature overnight. The solution was aspirated off, washed with 300 μL 0.9% NaCl in water and aspirated off again. 250 μL blocking buffer (0.05 M Tris-HCl, pH 7.8, 0.9% NaCl, 0.05% NaN_3, 6.0% D-sorbitol, 1.0% bovine serum albumin, 1.0 mM $CaCl_2$) was then added, and incubated on an orbital shaker at room temperature overnight. The solution was aspirated off, and

washed with 300 μL 0.9% NaCl, followed by aspiration. The coated plate can be stored at 4 °C for at least 6 months.

To each 3 mm DBS punch in a 96-well plate, 120 μL elution buffer (0.1 M sodium acetate, 0.1% (w/v) bovine serum albumin, pH 5.0) was added. The plate was then shaken on an orbital shaker at 37 °C for 1 hour. 100 μL DBS eluent was transferred onto an anti-ARSA coated well and shaken on an orbital shaker at room temperature for 1 hour. The solution was aspirated off, and 300 μL 20 mM sodium acetate, pH 5.0 was added to wash the well, followed by aspiration. To each well, 50 μL assay cocktail (150 μM d_3-C18:0-sulfatide as substrate and 0.2 μM d_7-C18:0-galactosyl-ceramide as internal standard in assay buffer) was added. The plate was centrifuged for 1 minute at 3000 g to ensure that all liquid was at the bottom of each well. The assay was then placed on an orbital shaker (400 rpm on a 3 mm shaking radius) at 37 °C for 16 hours. Reactions were quenched by addition of 300 μL of methanol, and the plate was centrifuged for 5 minutes at room temperature at 3000 g. 150 μL supernatant was transferred out and diluted into 50 μL water in a autosampler plate, and placed in the cooled (8 °C) autosampler chamber of the LC-MS/MS instrument.

UPLC-MS/MS was carried out on a Waters Xevo TQ-S micro mass spectrometer coupled to a Waters AQUITY UPLC I-Class system using multiple reaction monitoring (MRM). MRM settings and ESI source conditions are given in Table 3.2 and 3.3. The UPLC method was the same as described in section 3.2.6.1.

3.2.6.3 ARSA DBS assay by size-exclusion chromatography.

To each 3 mm DBS punch in a 96-well plate, 50 μL elution buffer (0.8% NH_4OH (Millipore, Cat. AX1303) in assay buffer) was added. The plate was then shaken on an orbital shaker at room

temperature for 4 hours. Sephadex G-25 resin (GE Healthcare Life Sciences, Cat. 17003201) was swollen in Milli-Q water at 10 mL water per g resin for 3 hours prior to use. In a 96-well fritted plate (ThermoFisher, Cat. 278011), 600 μL resin slurry (equivalent to 60 mg resin) was added per well. The water was removed by centrifuging the plate at 800 g for 1 minute, and the resin was washed with 600 μL assay buffer for 3 times, each time the assay buffer was removed by centrifugation at 800 g for 1 minute. After the third wash, 30 μL DBS eluent was loaded on to the resin, followed by 15 μL assay buffer as a stacker. The loaded resin was then centrifuged at 800 g for 1 minute, and the eluent was collected. To the purified DBS eluent, 10 μL assay cocktail (450 μM d_3-C18:0-sulfatide as substrate and 1 μM d_7-C18:0-galactosyl-ceramide as internal standard in assay buffer) was added. The plate was centrifuged for 1 minute at 3000 g to ensure that all liquid was at the bottom of each well. The assay was then placed on an orbital shaker (400 rpm on a 3 mm shaking radius) at 37 °C for 16 hours. Reactions were quenched by addition of 300 μL of methanol, and the plate was centrifuged for 5 minutes at 3000 g. 150 μL supernatant was transferred out and diluted into 50 μL water in a autosampler plate, and placed in the cooled (8 °C) autosampler chamber of the LC-MS/MS instrument.

UPLC-MS/MS was carried out on a Waters Xevo TQ-S micro mass spectrometer coupled to a Waters AQUITY UPLC I-Class system using multiple reaction monitoring (MRM). The parameters were the same as in Table 3.2 and 3.3. he UPLC method was the same as described in section 3.2.6.1.

3.3 A large-scale pilot study for the newborn screening of metachromatic leukodystrophy

Manuscript in preparation: Hong. et al., Newborn Screening of Metachromatic Leukodystrophy: Results from a Large-Scale Study.

Given the facts that innovative treatments for MLD are advancing at rapid pace and that treatment is much more beneficial if carried out prior to full onset of symptoms,[94, 95] newborn screening for MLD may be considered in the near future.

Multiplex biochemical assays using dried blood spots (DBS) have been developed to support newborn screening of MLD using various strategies, including the quantification of (1) sulfatides,[25] (2) ARSA protein abundance (immunoassays),[59] and most recently, (3) ARSA activity.[74] Screening for MLD based on the ARSA activity was not previously considered because such DBS assay was not available until recently and the thermal instability of ARSA and the high frequency of pseudodeficiency variants may pose additional challenges for enzymatic activity based screening.[59] Ridsdale et al. conducted a screening study of nearly 100,000 de-identified newborns using an immunoassay to quantify the abundance of ARSA in DBS.[96] A total of 73 newborns had abnormally low ARSA protein abundance in DBS, none of whom was considered at risk after *ARSA* gene sequencing and sulfatide measurement.[96] However, the sensitivity of the immunoassay is in question as it gives rise to false-negative errors regarding properly folded, but enzymatically deficient proteins, which was the case when quantifying the abundance of acid α-glucosidase in DBS and plasma for the diagnosis of Pompe disease.[29] Moreover, the specificity of the immunoassay assay is also compromised by the fact that one of the most common ARSA pseudodeficiency variants, c.*96A>G, causes the reduction of ARSA mRNA and hence ARSA protein by about 90% due to the polyadenylation defect.[97, 98] On the contrary, MLD newborns

(newborns who developed MLD later in life) had elevated sulfatide in blood when compared to normal newborns.[25] Individuals with ARSA pseudodeficiency had normal sulfatide levels,[25] suggesting that sulfatide analysis might be a better screening tests than an immunoassay.

Hence, our laboratory initiated a large-scale newborn screening study of MLD by measuring sulfatide abundance in DBS from de-identified newborns. Even though the sulfatide assay resulted in a 0.7% screen-positive rate due to the overlap between the affected and normal ranges, most of the false positives could be identified by implementing the ARSA activity assay as the second-tier test. This two-tier screening strategy can also identify MSD patients. MSD patients have defects in the formylglycine generating enzyme, therefore also display high sulfatide and low ARSA activity in blood.[99] In conclusion, this study demonstrated that newborn screening for MLD, and even MSD, is feasible, with a good balance between sensitivity and specificity.

3.3.1 Sulfatide analysis in DBS from MLD newborns

Samples from MLD newborns (newborns that develop MLD later in life) are a valuable resources in this study to help establish the screening cut-off, as it was found that the blood sulfatide level increased with increasing age in MLD patients as well as normal controls. A total of 15 archived DBS from MLD newborns were acquired throughout the study from the California Department of Health, the Children's Hospital of Pittsburgh, and the Meyer Children's Hospital (Florence, Italy). Sulfatide analysis on these DBS samples were performed as detailed in Section 3.3.8. Results from the random newborns and the 15 MLD newborns are summarized in Table 3.4.

Table 3.4. Summary of the results from the 15 MLD newborns and the random newborns acquired throughout the study.

ID	age of onset (year)	C16:0-sulfatide (μM)	sum sulfatide (μM)	Normalized C16:0-sulfatide	Note
MLD newborn 1	0.0	0.309	0.926	n/a	
MLD newborn 2	1.3	0.338	0.859	n/a	
MLD newborn 3	1.5	0.424	1.206	n/a	
MLD newborn 4	2.5	0.403	1.173	1.401	Acquired in 2019
MLD newborn 5	2.5	0.400	0.706	1.400	Acquired in 2019
MLD newborn 6	late infantile	0.278	0.722	n/a	Saposin B deficiency
MLD newborn 7	late infantile	0.356	0.877	n/a	
MLD newborn 8	late infantile	0.468	1.233	n/a	
MLD newborn 9	late infantile	0.410	1.107	1.478	Acquired in 2019
MLD newborn 10	late infantile	0.297	0.789	1.072	Acquired in 2019
MLD newborn 11	5.4	0.321	0.771	n/a	
MLD newborn 12	6 y	0.186	0.498	0.685	Acquired in 2019
MLD newborn 13	6.5 y	0.195	0.517	0.677	Acquired in 2019
MLD newborn 14	juvenile	0.293	0.696	n/a	
MLD newborn 15	juvenile	0.287	0.692	n/a	
Random newborn[a]	n/a	0.100 (0.055-0.187, n=27,335)	0.245 (0.115-0.510, n=21,551)	0.345 (0.186-0.617, n=27,335)	

[a] Data for random newborns are expressed as median (1-99 percentile, number of newborns

MLD newborn 12 and 13, diagnosed with the juvenile form of MLD, had the lowest blood sulfatide levels and overlapped with normal range (Table 3.4). The sulfatide assay was repeated on punches from a second set of DBS with the same result. An ARSA protein abundance assay and an ARSA enzymatic activity assay were also performed on the DBS, with essentially no ARSA protein and ARSA activity detected (data not shown). Together, these results suggested that the correct archived DBS were pulled out of storage for the study. These archived DBS were stored at -20 °C, and sulfatides should be relatively stable under this storage condition for at least 1 year based on our experience with adult DBS.

3.3.2 Analysis of total sulfatide in DBS

In our previous study, it was observed that at least four sulfatide species were elevated in DBS from MLD patients compared to healthy controls. C16:0- and C16:0-OH-sulfatide accounted for most of the sulfatides in blood along with a relatively small amount of C16:1-OH- and C18:0-sulfatide.[25] Therefore, we initiated a large-scale newborn screening study of MLD by monitoring the total amount of these four sulfatide species in DBS using high throughput UPLC-MS/MS. Shown in Fig. 3.18 is a typical chromatogram of C16:1-OH, C16:0-OH, C16:0, and C18:0-sulfatide in the DBS from a random newborn. The sample injection-to-injection time was 2.5 min, allowing more than 500 samples to be analyzed per day per instrument.

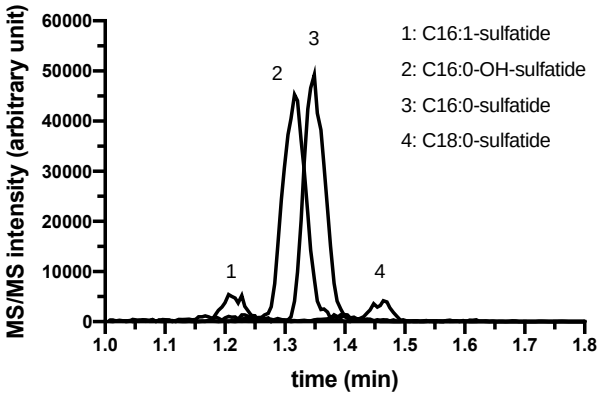


Figure 3.18. UPLC-MS/MS chromatogram of the four sulfatide species in DBS from a random newborn. The x-axis is time (min) and the y-axis is the MS/MS intensity. Peak 1 at 1.22 min is C16:1-OH-sulfatide, peak 2 at 1.31 min is C16:0-OH-sulfatide, peak 3 at 1.35 min is C16:0-sulfatide and peak 4 at 1.46 min is C18:0-sulfatide.

The screening cut-off at 0.62 μM total sulfatides was based on the results from the 9 MLD newborns available at that time (Table 3.4, acquired prior to 2019) to achieve 100% sensitivity. Among these 9 MLD newborns, there were 6 late-infantile patients and 3 juvenile patients. Fig. 3.19 shows the total sulfatide abundance in the 15 MLD newborns as well as in 2,000 random newborns. The dash line indicates the screening cut-off at 0.62 μM. A total of 21,551 random newborns were screened using this strategy, of which 27 (0.13%) had total sulfatide level above the screening cut-off (Table 3.5).

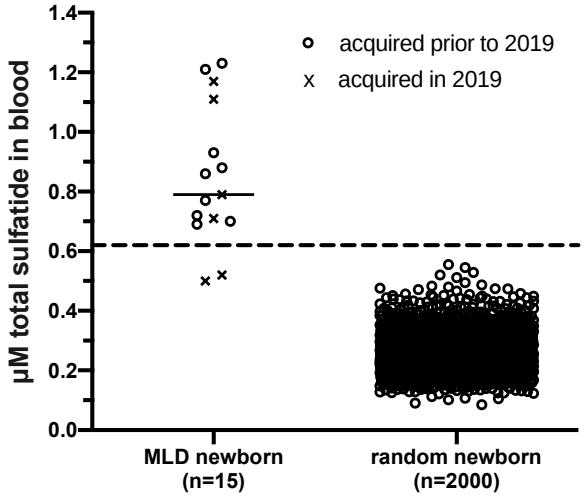


Figure 3.19. The total sulfatide abundance (μM) in blood from 15 MLD newborns and 2000 random newborns. The dash line is the screening cut-off at 0.62 μM. The open circles indicate samples from MLD newborns and random newborns acquired prior to 2019, and the crosses indicate samples from MLD newborns acquired in 2019.

Table 3.5. Summary of the results using different screening strategies.

Screening method	No. of newborns screened	Screening cut-off	No. of newborns with abnormal 1st tier results	No. of newborns with abnormal 2nd tier results
Total sulfatide	21,551	0.62 μM[a]	27	N/A
C16:0-sulfatide	27,335	0.64 after normalization	195	2 out of 122[b]
ARSA activity	2,287	20% daily mean activity	3	0 out of 3[c]

[a] The 0.62 μM cut-off was selected based on results from the MLD newborns acquired prior to 2019.
[b] The 2nd tier test was to measure ARSA activity in DBS. Only 122 out of the 195 newborns with abnormal C16:0-sulfatide results were submitted for the ARSA activity assay. The rest were not tested for ARSA activity as the DBS were too old for the test.
[c] The 2nd tier test was to measure C16:0-sulfatide in DBS.

However, we decided to stop monitoring the total sulfatide level in DBS because a systematic shift in the total sulfatide level was observed immediately after a new UPLC column was installed, while C16:0-sulfatide level remained stable (Fig. 3.20). It was also observed that the retention time for the four sulfatide species all shifted forward dramatically by 0.4 min on a new column, and shifted gradually backward when injections were being made before stabilizing after about 1000 injections. Same pattern of retention time shift was observed on additional columns with different solid phases. Since C16:0-OH-, C16:1-OH- and C18:0-sulfatide did not coelute with the universal internal standard (d_5-C16:0-sulfatide), a shift in retention time would result in a change in the relative matrix effect, hindering the accurate quantification of these three species. On the contrary, the shift in retention time had minimal effect on the quantification of C16:0-sulfatide as the change in matrix effect was accounted for by the coeluting internal standard. Therefore, an isotope-labeled version of C16:0-OH-sulfatide, the second most abundant sulfatide species in blood, was in need for its quantification. Yet it was then found out that the DBS matrix contained endogenous species that were isobaric with the potential internal standards (+3, +5, +6, +7, and +9) and these isobars eluted closely to the C16:0-OH-sulfatide (data not shown), making a robust quantification of C16:0-OH-sulfatide hardly possible. Hence, we decided to continue the

study by monitoring only the C16:0-sulfatide in DBS, since it was elevated in all the 15 MLD newborns acquired throughout the study (Table 3.4).

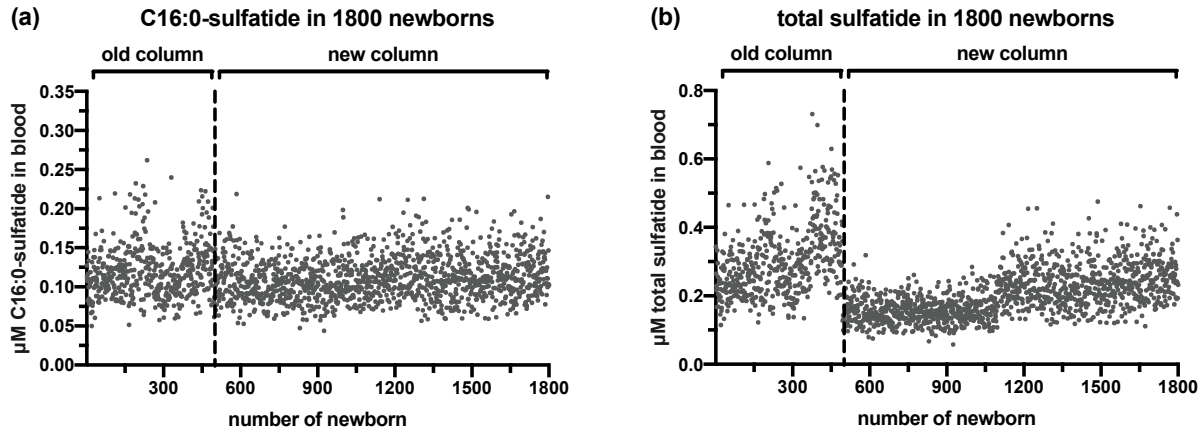


Figure 3.20. The (a) C16:0-sulfatide abundance and (b) total sulfatide abundance in the same set of newborns. The first 500 newborns were analyzed on an old UPLC column, and the rest of the 1300 newborns were analyzed on a new UPLC column.

3.3.3 Analysis of C16:0-Sulfatide in DBS.

During this phase of the study, we received six additional newborn DBS from diagnosed MLD patients. Total sulfatide and C16:0-sulfatide level were both substantially lower in two of these patients compared to the rest of the cohort (MLD newborn 12 and 13, Table 3.4). They would have be considered as "screen negatives" if the original cut-off based on data prior to 2019 was used (Fig. 3.19). Thus, to ensure a 100% assay sensitivity, the screening cut-off was lowered to 0.17 μM C16:0-sulfatide. This lead to a significant increase in the screen-positive rate due to the slight overlap between the C16:0-sulfatide concentration in MLD newborns and random newborns (Fig. 3.21).

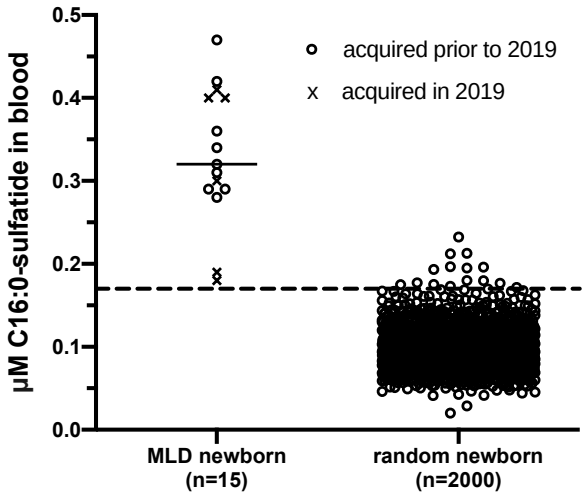


Figure 3.21. C16:0-sulfatide abundance (µM) in blood from 15 MLD newborns and 2000 random newborns. The dash line is the screening cut-off at 0.17 µM. The open circles indicate samples from MLD newborns and random newborns acquired prior to 2019, and the crosses indicate samples from MLD newborns acquired in 2019.

To further harmonize the results, a single-point external calibration strategy was adopted where the analyte to internal standard ion ratio for C16:0-sulfatide of a newborn was normalized to that of a neat C16:0-sulfatide standard extracted at the same time as the DBS. Shown in Fig. 3.22 is the C16:0-sulfatide level in groups of 500 newborns collected over 5 months with or without this normalization. Results with normalization were more consistent between groups, suggesting that the variations were mostly introduced from internal standard batch-to-batch differences as well as fluctuations of the mass spectrometry, which could be accounted for by the normalization to the external calibrator.

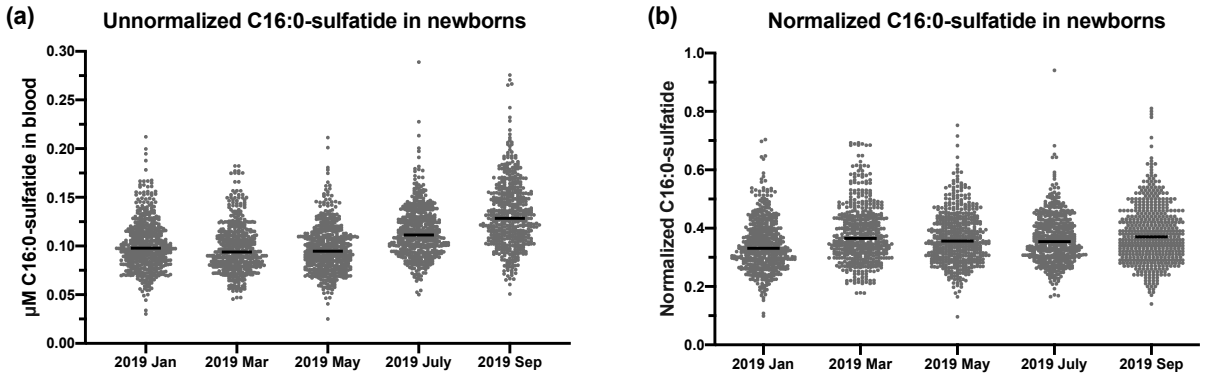


Figure 3.22. The (a) Unnormalized and (b) normalized C16:0-sulfatide abundance in the same sets of newborns. Each group consists of data from 500 newborns collected over five separate months. The horizontal bars indicate the median of the group.

Our final approach for the screening of MLD was to quantify C16:0-sulfatide in newborns using d_5-C16:0-sulfatide as internal standard and then to normalize the result to the external calibrator (C16:0-sulfatide standard extracted at the same time as the DBS). The screening cut-off was set at 0.64 after the normalization and corresponded to 0.17 μM C16:0-sulfatide in blood (Fig. 3.23). This was based on results from the 6 MLD newborns acquired in 2019 (Fig. 3.23). Since these 6 MLD newborns included the two with lowest sulfatide concentration (MLD newborn 12 and 13, Table 3.4), the screening cut-off at 0.64 after the normalization would be valid for all 15 MLD newborns.

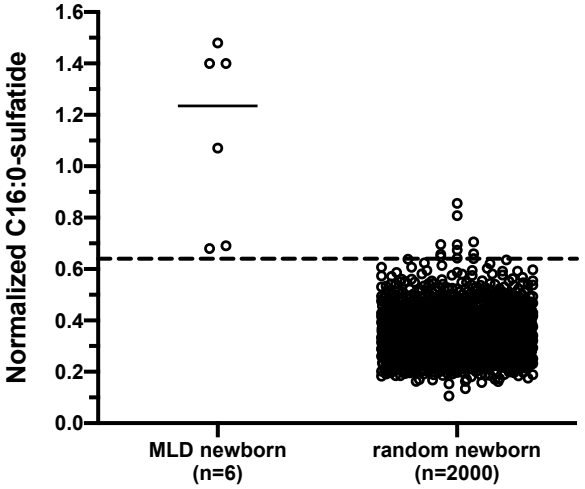


Figure 3.23 Normalized C16:0-sulfatide level in blood from 6 MLD newborns and 2000 random newborns. These 6 MLD newborns were all acquired in 2019. The dash line is the screening cut-off at 0.64 after normalization.

A total of 27,335 random newborns were screened using this strategy, of which 195 had normalized C16:0-sulfatide level above the cut-off (Table 3.5). The distributions of the C16:0-sulfatide abundance in the 27,335 newborns with or without the normalization are shown in Fig. 3.24. Results of the 195 newborns with abnormal C16:0-sulfatide level are summarized in Appendix A.

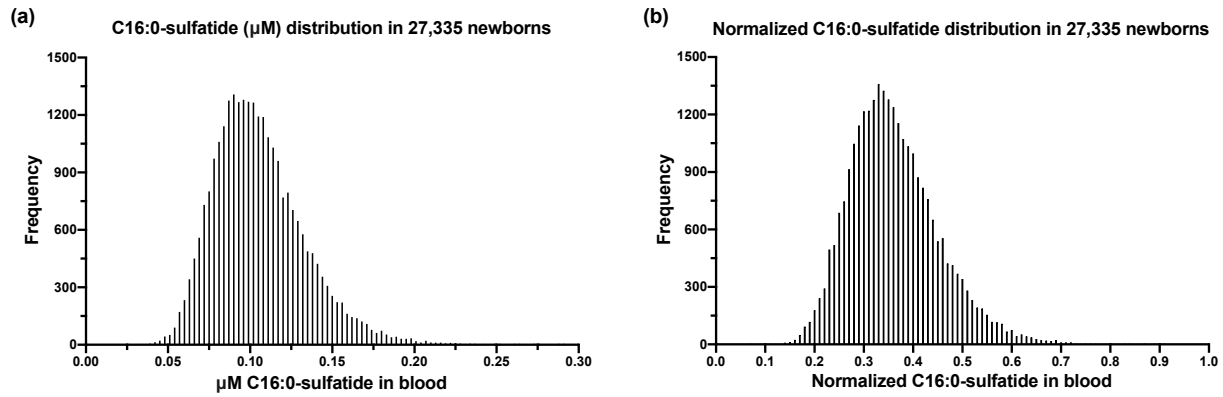


Figure 3.24. The distribution of (a) unnormalized C16:0-sulfatide and (b) normalized C16:0-sulfatide in 27,335 random newborns.

3.3.4 Implementation of ARSA enzymatic activity as a second-tier test.

A two-tier screening algorithm was proposed given that the C16:0-sulfatide analysis resulted in a 0.7% screen-positive rate (Fig. 3.25). An ARSA activity assay in DBS was implemented as the second-tier test using an additional 3 mm DBS punch.[74] Newborns with abnormal C16:0-sulfatide but normal ARSA activity were considered to be screen negative, whereas newborns with abnormal C16:0-sulfatide (above 0.64 after normalization) and ARSA activity (below 20% to the mean activity of the matching newborns) were considered to be screen positive. In the latter case, three additional sulfatases (I2S, GALNS, and ARSB) were measured to distinguish potential MLD patients from MSD patients.[19]

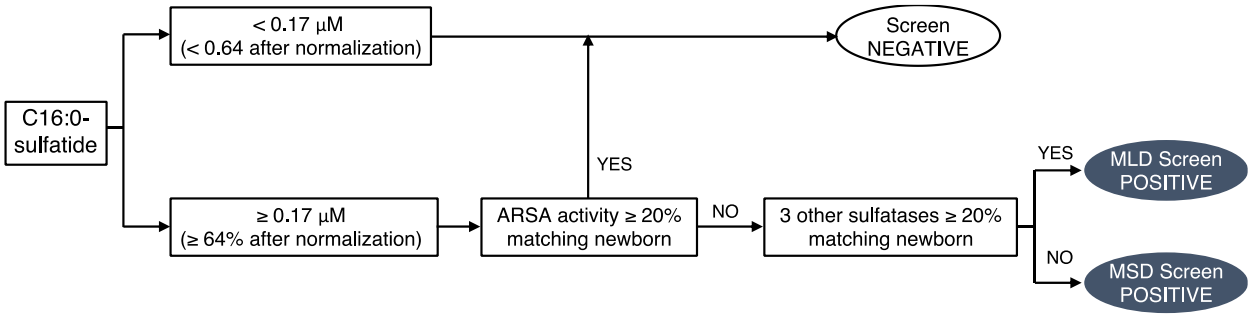


Figure 3.25. The proposed screening algorithm for MLD.

Among those 195 newborns with C16:0-sulfatide abundance above 0.64 after normalization, 122 were submitted for ARSA activity assay (Table 3.5 and Appendix A). The rest

were not tested for ARSA activity as the DBS were too old (more than 3 months at room temperature) for the test. All but two newborns with C16:0-sulfatide level above the cut-off had normal ARSA activity (> 20% to their matching newborns), suggesting they were all false positives identified by the sulfatide assay (Table 3.5). Between the two newborns with abnormal sulfatide and ARSA activity level, one displayed C16:0-sulfatide at 0.86 after normalization and ARSA activity at 0% of the matching newborns (MLD hit 24, Appendix A), while the other displayed C16:0-sulfatide at 0.72 after normalization and ARSA activity at 8% of the matching newborns (MLD hit 128, Appendix A). Activity of I2S, GALNS and ARSB were only measured in MLD hit 128, with the activities all above 20% of the mean activities of its matching newborns, ruling out the possibility of MSD. Even though there were no I2S, GALNS and ARSB data on MLD hit 24, a third punch from these two newborns were both submitted for ARSA gene sequencing, since MSD is much rarer than MLD.

3.3.5 Results of *ARSA* gene sequencing

Next generation sequencing of the *ARSA* gene was conducted using reserved 3 mm DBS punches from the 2 newborns (MLD hit 24 and 128, Appendix A) that were considered at risk of MLD at Utah Public Health Laboratory.

Two known pathogenic variants were found in MLD hit 24 (Appendix A): p.Tyr431Ser// p.Pro428Leu, together with one pseudodeficiency variant, p.Thr393Ser.[70] Therefore, MLD hit 24 (Appendix A) was interpreted as a MLD-affected patient. On the other hand, one pathogenic variant, p.Arg392Trp, was identified in MLD hit 128 (Appendix A), together with one pseudodeficiency variant, p.Thr393Ser, in heterozygous and one pseudodeficiency variant, p.Asn352Ser, in homozygous.[70] Unfortunately, we did not have a good coverage for the other

common pseudodeficiency variant, c.*96A>G, for both subjects. Even though MLD is inherited recessively, it has been reported that one pathogenic variant and a pseudodeficiency variant in *trans* could lead to some neurological and psychiatric symptoms.[100] Therefore, the genetic sequencing result for MLD hit 128 (Appendix A) was deemed inconclusive (see Section 3.3.7 for discussions).

3.3.6 ARSA enzymatic assay as the first-tier screening test for MLD.

Since the sulfatide assay resulted in a high false-positive rate , the possibility of using the ARSA activity assay as the primary screening test and the sulfatide assay as the second-tier test for the newborn screening of MLD was also explored. ARSA activity in DBS from 2,287 newborns were screened using a reported protocol.[74] Three out of the 2,287 newborns had ARSA activity below 20% of the daily mean activity and secondary punches from these three subjects were submitted for sulfatide analysis (Table 3.5). The C16:0-sulfatide level in these low ARSA activity subjects were below the 0.64 cut-off after normalization. Therefore, they were not considered to be at risk of MLD. Results from the 2,287 random newborns and the three subjects with abnormally low ARSA activity are summarized in Table 3.6.

Table 3.6 Results from the three newborns with ARSA activity below the cut-off (20% of daily mean activity) and the 2,287 newborns screened based on the ARSA DBS activity.

	ARSA activity (μM/h)	% to daily mean	C16:0-sulfatide (μM)	Normalized C16:0-sulfatide
subject 1	0.035	19	0.092	0.330
subject 2	0.012	13	0.162	0.586
subject 3	0.004	2	0.128	0.463
random newborns[a]	0.157 (0.041-0.430, n=2,287)	n/a	n/a	n/a

[a] Data for random newborns are expressed as median (1-99 percentile, number of newborns).

In the summer of warm states like Florida and Texas, the transport conditions of DBS can be relatively harsh. Thus, the ARSA stability under extreme conditions was assessed by studying

the ARSA activity in DBS stored at room temperature and at 37 °C with different relative humidity levels for a period of up to 7 days. As shown in Fig. 3.26, less than 50% of the ARSA activity was lost in the first 3 days under all conditions. The activity continued to decrease, with 25% remaining when the DBS were stored at 37 °C with a relative humidity of 33% for 7 days. Intriguingly, it appeared that a higher relative humidity helped to preserve ARSA in DBS, as over 50% ARSA activity remained when DBS were stored at 37 °C with a relative humidity of 84% for 7 days.

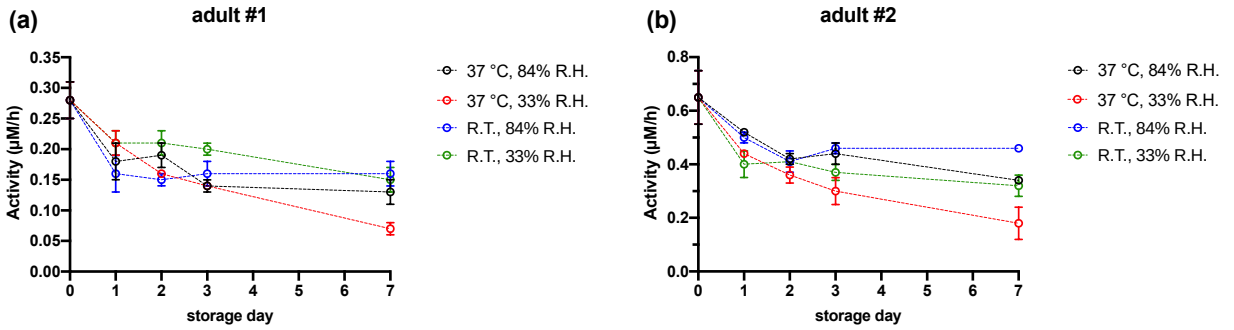


Figure 3.26. The ARSA stability in DBS from two adults under different storage conditions. R.T. stands for room temperature and R.H. stands for relative humidity. Error bars were standard deviations based on triplicate measurements.

3.3.7 Discussions and conclusions

MLD newborns can be distinguished from normal newborns based on C16:0-sulfatide abundance in DBS with statistical significance (Fig. 3.21 and Fig. 3.23). To improve the assay performance, it is required to use an isotope-labeled internal standard that is otherwise chemically identical to the analyte of interest and external calibration on a routine basis. Having an internal standard that coelutes with the analyte is critical as changes in retention times can have a large impact on ionization suppression, as was found in the study of total sulfatide analysis in DBS. It should be noted that using an external calibration curve with matrix would be more ideal in this case, but during the time of the study, we did not find any sulfatide-free blood matrix for spiking purposes.

Later, it was found that blood from mouse, trout, and chicken contained essentially no sulfatide, therefore could serve as analyte-free matrices (data not shown).

The screening cut-off used in the study was established based on the results from the 15 MLD newborns to achieve a 100% sensitivity. Obtaining more DBS from MLD newborns can help to better assess the sensitivity of our method. However, these samples are extremely rare. Hopefully, with increasing awareness of MLD and future perspective screening studies, more DBS from MLD newborns will become available. It is possible but highly unlikely that out current cut-off is higher than optimum, which will lead to false-negative cases. Under such circumstances, the screening cut-off needs to be lowered accordingly to minimize the false-negative rate, and the false-positive rate will increase consequently. Nevertheless, we believe that most of the false-positive cases can be identified through the second-tier test, which was the case in our pilot study.

Implementation of a second-tier test is often in need to reduce the false-positive rates of primary screening tests.[17] Over the years, many second-tier tests were developed, including the measurement of psychosine for Krabbe disease,[16] the ratio of creatine/creatinine to adjust acid α-glucosidase activity for Pompe disease,[65] and total homocysteine, methylmalonic acid, and methylcitric acid for inborn errors of propionate, methionine, and cobalamin metabolism.[15] By implementing a second-tier test that can be performed on DBS as part of the screening process, false-positive cases can be resolved without the need to contact families. This greatly reduces family anxiety as well as the burden to medical system

This strategy is extremely relevant in the case of MLD where the amount of blood sulfatide in normal newborns slightly overlaps with that in MLD newborns (Fig. 3.19, 3.21, 3.23). Since the screening cut-off is based on the results from the 15 MLD newborns for a 100% sensitivity, the

specificity of the assay was compromised with a screen-positive rate of 0.71%. The prevalence of MLD is between 1:40,000 to 1:160,000, therefore almost all the newborns with abnormally high C16:0-sulfatide abundance were false positives.[71] This necessitated a second-tier test to identify the false-positive cases. To improve the specificity of the test, ARSA enzymatic activity assay in DBS was implemented as the second-tier test to identify the false-positive cases. Out of the 122 newborns with abnormal C16:0-sulfatide in DBS, 120 were deemed false-positives as they displayed ARSA activity higher than the cut-off (Table 3.5). This underlines the power of second-tier tests as they can greatly reduce the false-positive rate if implemented properly.

However, it should be noted that MLD patients that are due to dysfunctional saposin B protein would be missed with this two-tier algorithm as they displayed abnormal sulfatide level but normal ARSA activity.[70] Although it was reported that bone marrow transplantation could benefit patients with saposin B deficiency, they are extremely rare.[41, 70] If MLD patients that are due to saposin B deficiency is of interest of newborn screening, immunoassay for saposin B can be used as a secondary test to follow up newborns with abnormal sulfatide results. Such assay has been reported using plasma as sample specimen, and should be straightforward to implement on DBS samples.[101]

Between the two high-risk newborns identified in our study due to abnormally high C16:0-sulfatide abundance and low ARSA activity in DBS, one was identified as a MLD-affected patient (MLD hit 24, Appendix A) while the other appeared to be a carrier (MLD hit 128, Appendix A) based on *ARSA* gene sequencing. A Canadian patient with adult MLD was reported to have the same combination of pathogenic variants (p.Tyr431Ser// p.Pro428Leu) as MLD hit 24 (Appendix A).[102] This Canadian patient with adult MLD initially presented with behavioral problems and progressed to seizures and cognitive deterioration.[102] However, we cannot predict if MLD hit 24

will have similar disease progression as the Canadian patient, as considerable clinical heterogeneity has been found among patients with the same genotype and genotype-phenotype correlation only becomes obvious when groups of patients are examined.[103-105] Furthermore, although ARSA pseudodeficiency variants are not associated with MLD *per se*, they can occur in *cis* with pathogenic variants and exacerbate the MLD phenotype.[106, 107] Therefore, MLD hit 24 (Appendix A) is an MLD patient based on the genotype, but his/her clinical phenotype is hard to predict. On the other hand, only one pathogenic variant was found in MLD hit 128 (Appendix A), thus this subject should be interpreted as a carrier. However, three additional copies of pseudodeficiency variants were found (p.Thr393Ser in heterozygous and p.Asn352Ser in homozygous) in the *ARSA* gene. This well explains the abnormally low ARSA activity (8% to its matching newborns, Appendix A), but also complicates the interpretation of the genotyping result. Hohenschutz et al. reported a patient with compound heterozygote of ARSA pathogenic variant and pseudodeficiency variant, who displayed intermediate ARSA activity between adult MLD and subjects with pseudodeficiency, slightly elevated urine sulfatide, and more importantly, neuropsychiatric symptoms.[100] It should be noted that this patient developed the first symptom at the age of 36, and did not deteriorate until 16 years later.[100] Also, the compound heterozygote status was confirmed through western blotting.[100] We did not have a good coverage for the other common pseudodeficiency variant, c.*96A>G, which was reported to reduce ARSA activity to 10% of control *in vivo*.[97, 98] Even though c.*96A>G and p.Asn352Ser usually occur in cis, it was also found that p.Asn352Ser could appear independently, especially in African and East Asians.[70, 108] Therefore, the appearance of p.Asn352Ser in MLD hit 128 (Appendix A) strongly suggests the presence of c.*96A>G but is not confirmative. If c.*96A>G is indeed present in this subject, it may result in some clinical consequences, as this polyadenylation defect together with the

pathogenic variant may reduce the residual ARSA activity below a certain critical threshold.[109] Hence, it is possible that MLD hit 128 (Appendix A), an apparent MLD carrier, will display some mild symptoms later in life, although this is not in the interest of newborn screening programs.

Taken these together, our two-tier screening algorithm identified two high-risk cases after screening 27,335 neonates, of which one was a MLD-affected patient, while the other was a carrier with inconclusive genotyping result. This demonstrates that our two-tier screening algorithm is highly specific (50% or 100%), with an exceptionally low false-positive rate (0.0037% or 0%). The particular number depends on how to interpret the result of MLD hit 128 (Appendix A). In either case, our strategy outperforms almost all the screening methods reported so far.[30, 64, 110, 111]

To date, lysosomal storage diseases that are due to malfunctioning enzymes are typically screened using assays that measure enzymatic activity. However, ARSA activity assays in DBS were not available until recently.[74] This screening strategy was further discredited due to two major concerns. The first concern was the potential false-positive problem caused by high prevalence of pseudodeficiency variants.[59] Individuals who were homozygous for the pseudodeficiency variants (1-2% in European population) or compound heterozygous of a pseudodeficiency variant and a pathogenic variant (ca. 1:2300) were reported to have essentially zero ARSA activity in DBS when assayed with an artificial sulfatase substrate, making them indistinguishable from MLD patients.[59] This conclusion, however, remains to be assessed using the new ARSA activity assay which utilizes the natural substrate, sulfatide, and has better specificity and sensitivity.[74] The second concern was the thermal instability of ARSA in DBS.[59, 74] As shown in Fig. 3.26, 50% of the ARSA activity was lost when the DBS was stored under extreme conditions for three days. Since DBS should be delivered to the screening laboratories within 3 days upon sample collection, the ARSA thermal instability may not be a major issue; however, it remains to be seen how

problematic this will be in warmer parts of the world. Nonetheless, given the high false-positive rate (0.71%) of the sulfatide assay, ARSA activity assay may be adequate as the primary test. This is supported by the lower false-positive rate (0.13%) resulted from the small-scale study on 2,287 newborns (Table 3.5). Currently, the ARSA assay is more complex than the sulfatide assay; nevertheless, with automation, the ARSA activity and sulfatide assays can be carried out by a single laboratory person and multiplexed with other assays.

In conclusion, the large-scale study presented here demonstrates that newborn screening for MLD is feasible if a two-tier screening strategy is adopted. We propose that newborns be screened based on the abundance of blood sulfatide and ARSA activity be measured on those with abnormal sulfatide. This two-tier screening approach is crucial to the balance between the sensitivity and the specificity of the test. Alternatively, it may also be possible to screen for MLD using the ARSA activity assay as the primary test, and the sulfatide assay as the secondary test.

3.3.8 Standard Operating Procedure for the DBS Sulfatide Assay

<u>Preparation of controls: adult DBS and external calibrator:</u>

- Preparation of adult DBS:

  Whole blood from a healthy adult donor is collected into a K_2EDTA blood collection tube and is mixed by inversion to distribute the anti-coagulant. Pipet the K_2EDTA blood onto a Whatmann 903 protein saver card (Sigma, Cat. WHA10534612), about 50 μL per spot. Let the DBS air dry on bench for 3 hours before storing in ZipLock bags inside a capped jar with desiccant in -20 °C freezer.

<u>Preparation of the external calibrator (14.4 nM C16:0 sulfatide in methanol):</u>

- C16:0-sulfatide is purchased from Matreya, LLC (Cat. 1875) and quantified by quantitative ^{1}H-NMR in the Gelb laboratory. The sulfatide powder is dissolved in 2:1 (v:v) chloroform:methanol. The solution is then diluted to 9 μM in 2:1 (v:v) chloroform:methanol.

Aliquot the 9 μM stock solution in glass vials with Teflon-septum screw caps. Parafilm the vials and store in a -80°C freezer.

- To prepare the external calibrator (14.4 nM C16:0 sulfatide in methanol), remove 9 μM C16:0-sulfatide stock solution from the -80°C freezer and allow it to warm to room temperature. In a 50 ml volumetric flask, add about 40 ml of methanol. Add 80 μL of 9 μM C16:0 sulfatide using a P100 pipette. Fill the volumetric flask with methanol to the calibration mark. Cap the flask with the stopper and mix by inverting 5 to 10 times.
- The external calibrator is aliquoted into 5 mL glass vials with Teflon-septum screw caps. Parafilm the vial and store the aliquoted calibrator into a -20°C freezer.

Preparation of the internal standard working solution (10 nM d5-C16:0-sulfatide in methanol):

- d_5-C16:0-sulfatide is synthesized in the Gelb laboratory as previously describe and quantified by quantitative ^{1}H-NMR.[25] D_5-C16:0-sulfatide powder is dissolved in 2:1 (v:v) chloroform:methanol. The solution is then diluted to 5 μM in 2:1 (v:v) chloroform:methanol. Aliquot the 5 μM stock solution in glass vials with Teflon-septum screw caps. Parafilm the vials and store in a -80°C freezer.
- To prepare the internal standard working solution (10 nM d_5-C16:0-sulfatide in methanol), remove the 5 μM d_5-C16:0 sulfatide stock solution from the -80°C freezer. Allow the vial to warm to room temperature. In a 200 mL volumetric flask, add about 150 mL methanol. Add 400 μl 5 μM d_5-C16:0 sulfatide with a P200 pipette. Fill the volumetric flask with methanol to the calibration mark. Cap the flask with the stopper and mix by inverting 5 to 10 times. Parafilm the flask and store it in the -20°C freezer.

Assay setup:

- Sample inspection, and the addition of controls and external calibrators:
  - Newborn DBS are typically in column 2 to 11 (Table 3.7) of a deep well plate (Costar, Cat. 3959). Inspect all wells that contains 3 mm DBS punch, and make note of any wells that contain a white spot (filter paper, blank) or blood spot that is insufficient for testing. Insufficient for testing means any DBS punch that is not a completely circular or is not fully saturated with blood.
  - Filter paper punch (3 mm) is added to the plate in duplicate. Adult DBS punch (3 mm) is added to the plate in triplicate. External calibrator (100 μL 14.4 nM C16:0 sulfatide in

methanol) is added to the plate in triplicate. Methanol (200 μL) is added to the plate in duplicate as inter-plate blank. See Table 3.7 for a typical plate layout.

Table 3.7. Typical plate layout for the newborns, controls and calibrators.

	1	2	3	4	5	6	7	8	9	10	11	12
A	FP[a]	nb[b]	nb	nb	nb	nb	nb	nb	nb	nb	nb	MeOH
B	FP	nb	nb	nb	nb	nb	nb	nb	nb	nb	nb	MeOH
C	Adult	nb	nb	nb	nb	nb	nb	nb	nb	nb	nb	
D	Adult	nb	nb	nb	nb	nb	nb	nb	nb	nb	nb	
E	Adult	nb	nb	nb	nb	nb	nb	nb	nb	nb	nb	
F	calibrator	nb	nb	nb	nb	nb	nb	nb	nb	nb	nb	
G	calibrator	nb	nb	nb	nb	nb	nb	nb	nb	nb	nb	
H	calibrator	nb	nb	nb	nb	nb	nb	nb	nb	nb	nb	

[a] FP stands for filter paper
[b] NB stands for newborn

- Sulfatide extraction from DBS
  - Remove the internal standard working solution from freezer, and warm up to room temperature. Pour proper amount of IS working solution (about 30 mL per plate) into a plastic tray (VWR, Cat. 490015-114).
  - Add 300 μL internal standard solution to each well using a 12-channel pipette (30-300 μL). Discard the remaining solution in the tray. Seal the plate with Easy Pierce Heat Sealing Foil (Thermo Scientific, Cat. AB3720). Visually inspect if all wells are sealed and every DBS is immersed in solution. If not, centrifuge the plate for 1 minute at 3000 g.
  - Place the sealed plate in the 37 °C incubator with orbital shaking (e.g. 400 rpm on a 3 mm shaking radius). Extract for 4 hours.
  - After the 4-hour incubation, centrifuge the plate for 5 minutes at 3000 g at room temperature. Remove the cover foil. Use a liquidator (Mettler Toledo) to transfer 200 μL supernatant from the DBS-containing deep well plate to a clean shallow plate (Greiner, Cat. M7310). Visually inspect if all wells contain enough sample. If not, transfer additional supernatant manually. Seal plate with Easy Pierce Heat Sealing Foil.
  - Sample is ready for UPLC-MS/MS analysis. Alternatively, it can be stored in a -20°C freezer for up to 1 week.

UPLC-MS/MS analysis:

- UPLC-MS/MS analysis was carried out on a Waters Xevo TQ-S micro mass spectrometry coupled to a Waters AQUITY UPLC I-Class system using multiple reaction monitoring (MRM) in ESI negative mode. The ESI source parameters and MRM parameters are listed in Table 3.8 and 3.9. Only the LC eluate coming out from 0.25-1.7 minute goes into MS/MS. The rest is diverted into waste. The ESI source cone is cleaned daily.

Table 3.8. ESI parameters for the DBS sulfatide assay.

Parameter (units)	ES+	ES-
Capillary (kV)	3.4	2.5
Source Temperature (°C)	150	150
Desolvation Temperature (°C)	550	550
Cone Gas Flow (L/Hr)	30	30
Desolvation Gas Flow (L/Hr)	1000	1000

Table 3.9. ESI parameters for the DBS sulfatide assay.

Analyte	ESI	dwell time (s)	SRM transition (m/z)	Cone Voltage (V)	Collision energy (V)
C16:0-sulfatide	-	0.054	778.2 > 96.9	150	68
d_5-C16:0-sulfatide	-	0.054	783.6 > 96.9	150	68
C16:1-OH-sulfatide	-	0.054	792.5 > 96.9	150	68
C16:0-OH-sulfatide	-	0.054	794.2 > 96.9	150	68
C18:0-sulfatide	-	0.054	806.3 > 96.9	150	68

- The analysis is carried out on an ACQUITY UPLC HSS T3 column (1.8 μm, 2.1 x 50 mm, Waters Crop., Cat. 186003538) connected to an ACQUITY UPLC HSS T3 VanGuard Pre-column (1.8 μm, 2.1 x 5 mm, Waters Crop., Cat. 186003976) at 40 °C. The guard column is changed every 3,300 injection, and the column is changed every 10,000 injection. Mobile phases and wash solvents are listed in Table 3.10. The gradient uses a flow rate of 0.5 mL/min. Elution starts with 50% of mobile phase B and linearly increases to 95% mobile phase B over 1 min, then linearly increases to 100% B from 1 min to 1.5 min, then jumps immediately back to 50% B until 2 min.

Table 3.10. Composition of mobile phases and wash solvents[a].

Solvent	Composition
Mobile phase A	50:50 (v:v) water:acetonitrile, with 0.1% formic acid
Mobile phase B	80:20 (v:v) isopropanol:acetonitrile, with 0.1% formic acid
Weak needle wash	50:25:25 (v:v:v) water:methanol:isopropanol
Strong needle wash	47.5:47.5:5 (v:v:v) methanol:isopropanol:water

[a]All solvents should be Fisher Optima grade. Formic acid is from Fisher Scientific (Cat. A117-5).

- 10 μL sample is injected on the column, with an overfill factor of 1.5 fold. The autosampler chamber is set at 4 to 8 °C. The volume of weak needle wash and strong needle wash is 800 μL and 400 μL, respectively. The load ahead function is enabled, with a loop offline time of 0.5 minute.

Post UHPLC-MS/MS analysis:

- The chromatography peaks are integrated automatically by TargetLynx (Waters Corp.) after being smoothed by 1 iteration and are inspected manually to ensure the integration boundaries are chosen properly (from peak valley to peak valley).
- The concentration of C16:0-sulfatide in blood (μM) can be calculated by multiplying the ion ratio of the analyte to the internal standard by the μmole of the internal standard added to the assay, then dividing by the volume of blood (L), assuming each 3 mm DBS punch contained 3.2 μL blood.
- The amount of C16:0-sulfatide in blood can be normalized to the external calibrator by dividing the analyte/internal standard ion ratio of C16:0-sulfatide of the DBS sample to the averaged analyte/internal standard ion ratio of the three calibrators in the plate.
- Newborns with normalized C16:0-sulfatide above 0.64 are considered to be at risk of MLD. A second punch of these newborns, along with punches from 6-8 newborns with sulfatide level below 0.64 after normalization and similar storage conditions (matching newborns) are submitted for ARSA enzymatic assay using the reported protocol.[74]
- A third punch from newborns with normalized C16:0-sulfatide above 0.64 and ARSA activity below 20% of the mean activity of its matching newborns is submitted for I2S, GALNS, ARSB enzymatic assay using the reported protocol.[19]
- Genetic sequencing of ARSA or SUMF1 gene is conducted accordingly.

3.4 A genotype-phenotype study of ARSA

In collaboration with BioMarin Pharmaceutical. Manuscript in preparation.

Up to now, 200 pathogenic variants have been characterized in the *ARSA* gene, of which only a few occur with high frequency.[70] The majority of ARSA pathogenic variants are rare or private.[70]

It is expected to uncover novel ARSA variants when evaluating potential MLD patients, especially if newborn screening for MLD is initiated. This may lead to confusion and anxiety to families. Therefore, in collaboration with BioMarin Pharmaceutical, we are carrying out a genotype-phenotype study to better understand the novel variants with unknown clinical significance.

Transient gene expression system is commonly used to study the clinical significance of novel variants.[112-114] However, such system usually suffers from large variation introduced by difference in cell number, transfection efficiency and expression efficiency across samples.[114] Therefore, we intend to design a new system that are less suspectable to these variations and use this novel system to evaluate the ARSA variants systematically. We also note that it was observed that enzyme activity is reduced in constructs in which one or more polymorphisms are in *cis* with a pathogenic variant ("haplotype effect").[114] Although we are not intending to study the haplotype effect in the current study, we believe that the transient gene expression system developed here can serve as a general tool to study them. Previous studies on novel variants using transient gene expression usually focused on missense mutations in exons.[112-114] However, in this study we intend to evaluate mutations both in exons and introns. Hopefully, this study can help to interpret genotyping results collected in asymptomatic at-risk subjects.

3.4.1 Development of a transient gene expression system for ARSA and beta-lactamase

Shown in Fig. 3.27 is the designed transient gene expression system used for the ARSA genotype-phenotype study. The system utilized a human cell line, HEK293, thus providing the authentic machinery for posttranslational modifications. In order to evaluate trace amount of residual ARSA activity with high accuracy, a ARSA knockout HEK293 cell line was in need so that the background ARSA activity in the un-transfected cells was essentially zero. ARSA variants were

driven by CMV promoter, while beta-lactamase, the reporter, was driven by EF1a promoter. Plasmid encoding an ARSA variant and a reporter gene was transfected into ARSA knockout HEK293 cells. Noted that the plasmid also encoded a reporter protein, the signal of which could be used as a calibrator to correct the variations introduced by different transfection and expression level across the well. The transfected cells were harvested after culturing for 3 to 5 days. Signal coming the mutant ARSA protein and the reporter protein are measured using the cell lysate. Signal from the mutant ARSA protein was then normalized to that from the reporter protein.

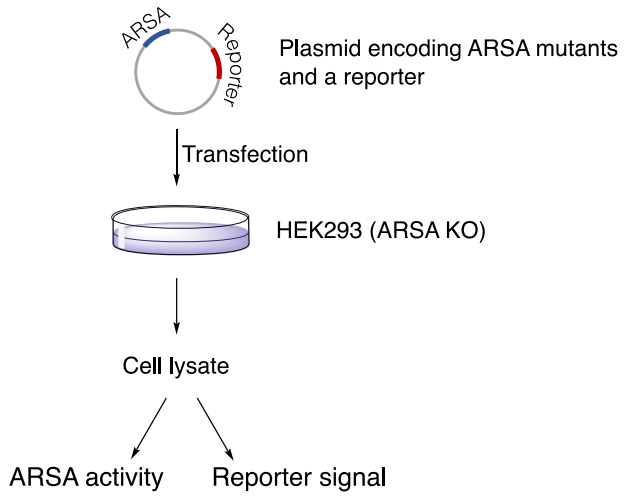


Figure 3.27. Experimental design for the transient gene expression system used for the ARSA genotype-phenotype study.

Beta-lactamase (BLA), a small 29 kDa enzyme from bacteria, was chosen as the reporter protein as mammalian cells have no homologous proteins to it, therefore no endogenous background from HEK293 cells. Furthermore, BLA requires no cofactor or metal for activity, making it an ideal reporter gene. A commercial BLA assay kit based on fluorescence resonance energy transfer (FRET) (ThermoFisher, Cat. K1150) was first tested in HEK293 cells with or without the transfection with recombinant BLA gene.[115] However, transfection with BLA-encoding plasmid resulted in less than 2-fold increase in terms of BLA response ratio, which was defined as the signal from transfected cells normalized by that from the un-transfected cells (mock) (Fig. 3.28). This might be caused by various reasons, including (1) the assay is not sensitive enough

for our system; (2) assay conditions were not optimized; (3) low level of transfection and/or expression; and (4) interfering signal coming from cell matrix. This indicated that the FRET-based BLA assay was not suitable for our study. Normalization to the BLA signal generated by this assay might introduce large variations when evaluating the residual ARSA activity of ARSA variants.

We then developed a LC-MS/MS-based assay for BLA using deuterated penicillin-G as the enzymatic substrate, since it has been our experience that LC-MS/MS assays are usually more sensitive than their fluorometry counterparts. BLA activity was measured by quantifying the formation of the enzymatic product, deuterated benzyl penicilloic acid, by LC-MS/MS using non-deuterated benzyl penicilloic acid as internal standard. The two BLA assays were compared side by side, and the LC-MS/MS assay outperformed the FRET assay by at least 10-fold in terms of response ratio (Fig. 3.28). This might be because the MS/MS assay directly measures the amount of enzymatic product whereas the FRET assay measures the fluorescent signal to which cellular matrix can introduce background enhancement or quenching. This new LC-MS/MS-based BLA assay was used in the following studies.

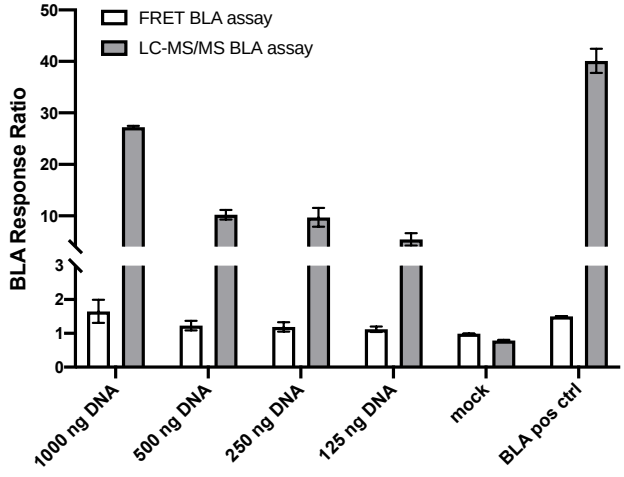


Figure 3.28. BLA response ratio in cells transfected with various amount of plasmid, un-transfected cells (mock), and Jurkat cells that are constitutively expressing BLA (BLA pos ctrl) using FRET-based BLA assay and LC-MS/MS-based BLA assay. Response ratio was calculated by dividing the signal from transfected cells by that from the un-transfected cell. Error bars were standard deviations based on triplicate measurements.

Two ARSA KO HEK293 cell lines were generated (I16 and G13, Fig. 3.29). Clone I16 and G13 had a 4 bp and 25 bp deletion, respectively, both resulting in stop codon. No endogenous

ARSA activity was detected in the ARSA KO clones, while wild type (WT) HEK293 had considerable endogenous ARSA activity (Fig. 3.29). Transfecting with plasmids encoding ARSA pre-mRNA (with introns) and ARSA cDNA (without introns) resulted in significant increase in ARSA activity in I16, G13, and WT HEK293 cells, although transfection with pre-mRNA constructs resulted in approximately 5-fold less activity compared to the cDNA constructs (Fig. 3.29). Based on these results, I16 cell clone was used in the furthering studies, as it afforded no endogenous ARSA activity and also higher ARSA activity after the transfection. cDNA constructs were used to evaluate missense ARSA variants, and the pre-mRNA constructs were used to evaluate variants that might affect splicing.

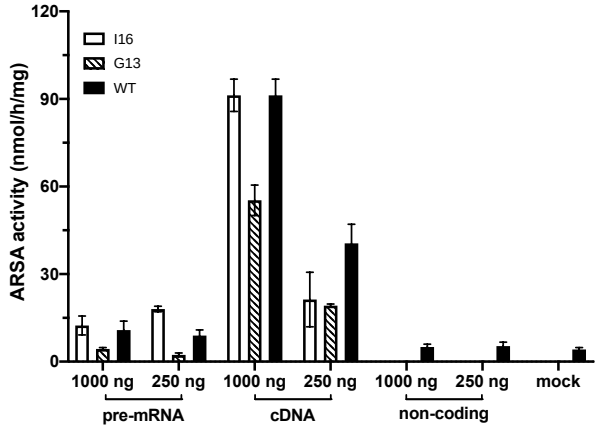


Figure 3.29. ARSA activity in transfected and un-transfected (mock) ARSA KO HEK293 cells (I16 and G13) and WT HEK293 cells. Plasmids encoding ARSA pre-mRNA, ARSA cDNA, non-coding transcript were included. Error bars were standard deviations based on triplicate measurements.

The effectiveness of the normalization strategy was evaluated by measuring ARSA activity in transfected cells with or without normalizing to BLA activity. When the ARSA activity was normalized by the amount of protein used, cells transfected with 1000 ng plasmid displayed higher ARSA activity compared to the ones transfected with lower amounts of plasmid, probably because higher transfection efficiency was achieved when more plasmid was used (Fig. 3.30a). However, upon normalization to BLA activity, similar normalized ARSA activity independent of the amount of plasmid used was observed (Fig. 3.30b). This demonstrated that normalization to a reporter

could indeed account for differences in cell number, transfection efficiency, and expression efficiency across samples.

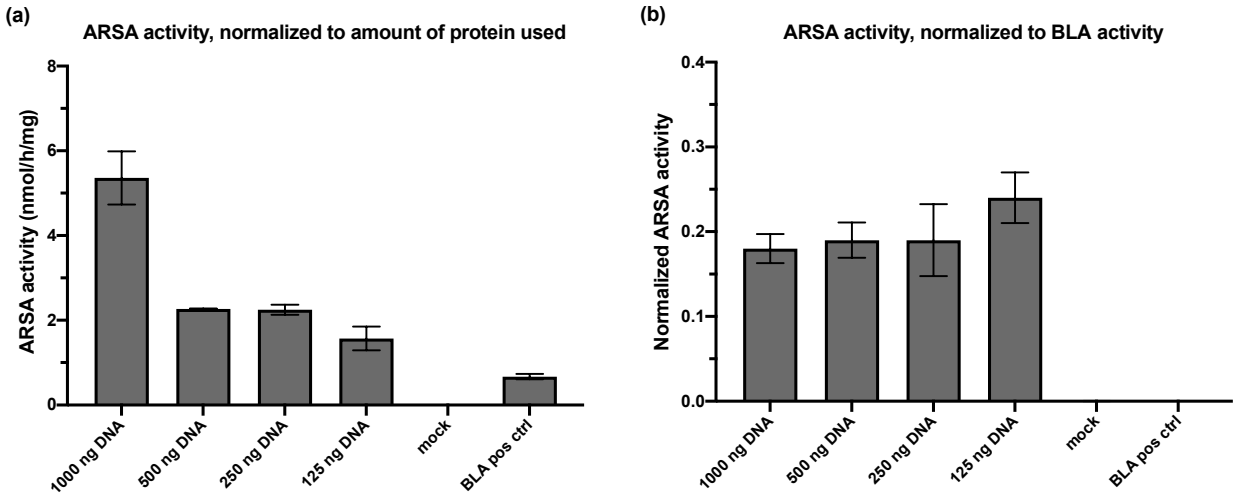


Figure 3.30. ARSA activity normalized by (a) amount of protein used; and (b) beta-lactamase activity in ARSA KO HEK293 cells transfected with various amount of plasmids and un-transfected cell (mock). Error bars were standard deviations based on triplicate measurements.

3.4.2 Screening missense ARSA variants with unknown significance

The transient gene expression system was used to screen ARSA variants of unknown clinical significance. The quality of the transfected cells was assessed by the protein concentration as well as the BLA activity. Repeat experiments were performed on cells with protein concentration below 0.2 μg/μL and/or BLA activity below 50 nmol/h/mg protein. These two filters were applied to ensure the quality of the data, as low protein concentration was indicative to cell toxicity and low BLA activity suggested low transfection and expression efficiency, thus we could not ascertain if the low ARSA activity measured under there two circumstances was due to the mutation or to the poor sample quality.

Known pathogenic missense ARSA variants causing severe, moderate and mild clinical severity were first evaluated using our system as a test run using the cDNA construct (Fig. 3.31). Seven out of the ten severe variants had undetectable ARSA activity, which was in line with the

annotation. Among the four moderate variants, E384K had residual activity comparable to WT (Fig. 3.31). In silico analysis suggests that this mutation may affect splicing, therefore its biochemical consequence needs to be evaluated using the pre-mRNA construct. Preliminary data showed that E384K had residual ARSA activity of 18% compared to the WT when evaluated using the pre-mRNA construct (data not shown). P428L , a moderate variants, displayed essentially no ARSA activity, despite the fact that homozygous P428L usually leads to juvenile or adult MLD. The P428L was believed to destabilize the protein by impairing oligomerization , making it more susceptible to degradation in lysosomes by cathepsin L.[116] However, adding leupeptin, an inhibitor to cathepsin L, to the lysis buffer did not result in detectable ARSA activity in the cell lysates (data not shown). We are still investigating the cause of the discrepancy between the annotation and the experimental result. The three mild variants included in the trial displayed 5-10% residual ARSA activity compared to the WT (Fig. 3.31), which was in line with their annotation.

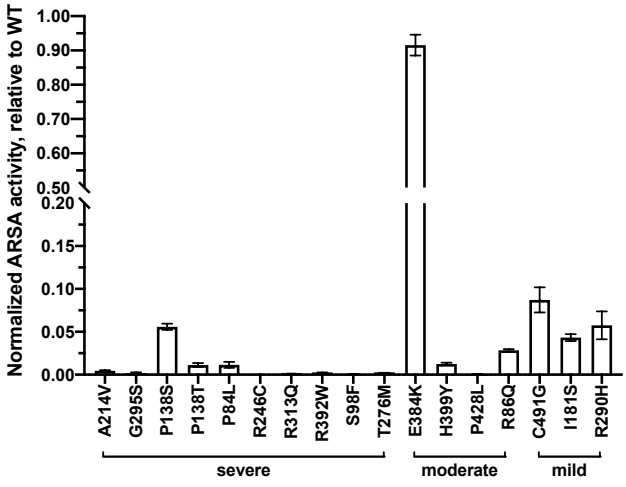


Figure 3.31. Normalized ARSA activity measured in known pathogenic ARSA variants, including severe, moderate, and mild variants. Error bars were standard deviations based on triplicate measurements.

Next, we carry out a screening study on all the missense ARSA variants with unknown clinical significance reported in database and literature using the cDNA construct. Only variants that do not affect splicing according to in silico analysis were included in this phase of the study. The screening is demonstrated in Fig. 3.32. We established our screening cut-off based on the

results from the pathogenic variants and high frequency variants that were not indicative to disease (likely to be "pseudodeficiency"). Variants with residual ARSA activity below 10% to WT were indicative to be pathogenic. A total of 194 variants have been characterized in our study, of which 56 had residual ARSA activity below 10% to WT (Appendix B). Splice-site variants together with missense variants that might affect splicing according to in silico analysis will be evaluated in the next phase of the study using the pre-mRNA construct.

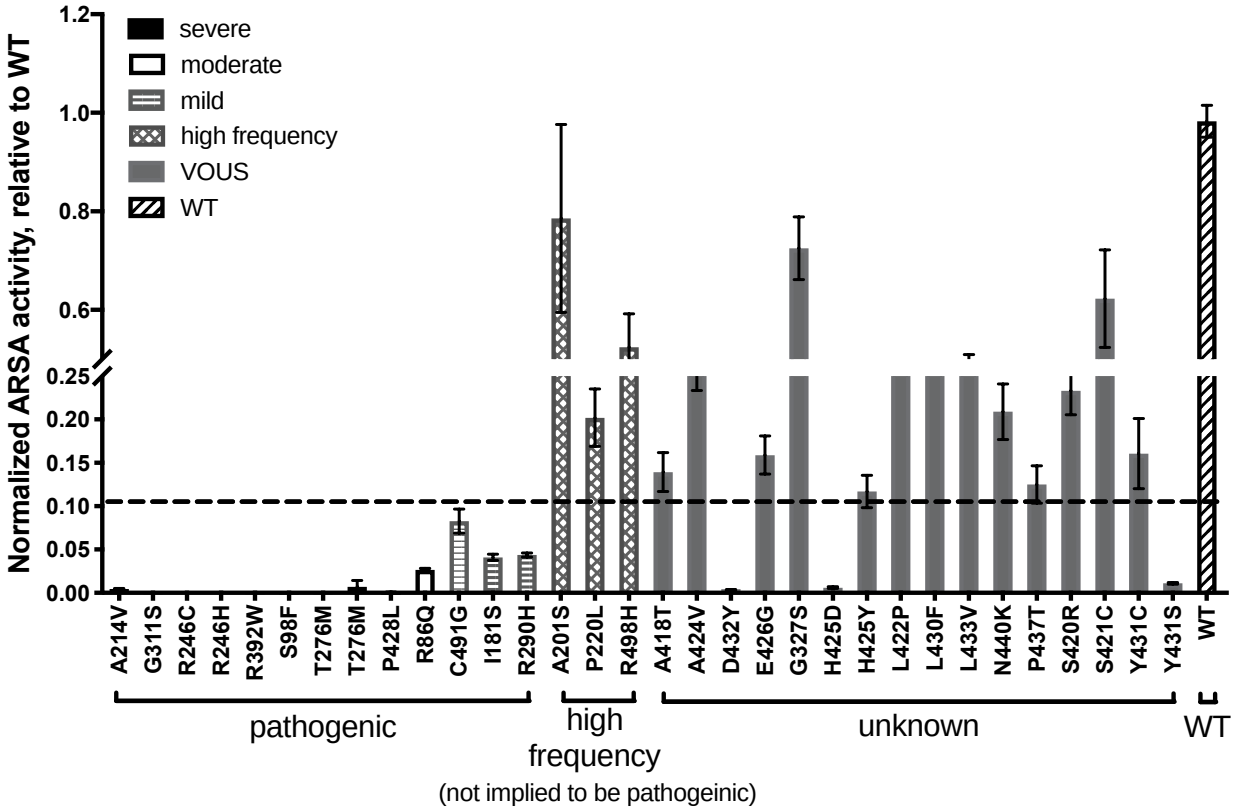


Figure 3.32. Demonstration of the ARSA variants screening. ARSA activity normalized to BLA activity was measured in pathogenic variants, variants occurring at high frequency therefore not implied to be pathogenic, variants of unknown significance and WT ARSA. A cut-off was established based on the results from the pathogenic variants. Unknown variants with normalized ARSA activity below the cut-off were suggestive to be pathogenic. Error bars were standard deviations based on triplicate measurements.

3.4.3 Experimental details

Cell lysis: To each well containing transfected HEK293 cells, added 60 μL lysis buffer (20 mM Tris-HCl, 2.5 g/L CHAPS, pH 7.5±0.02). The plate was sealed and centrifuged at 3000 g for 1

minute before being placed on an orbital shaker at room temperature and lysed for 15 minutes. The plate was centrifuged at 3000 g for 15 minutes after the lysis. The supernatant was used for BCA protein assay, beta-lactamase assay, and arylsulfatase A assay.

BCA protein assay: The concentration of protein was determined by the Pierce™ BCA Protein Assay Kit (ThermoFisher, Cat. 23227), using bovine serum albumin as the standard.

BLA assay: D_7-penicillin G and 5R,6R-benzylpenicilloic acid were both purchased from Toronto Research Chemicals (Cat. B288600 and B288593). BLA assay cocktail consisted of 200 μM d_7-penicillin G as substrate and 5 μM 5R,6R-benzylpenicilloic acid as internal standard in BLA assay buffer (50 mM Tris-HCl, pH 7.5±0.02). Noted that d_7-penicillin G and 5R,6R-benzylpenicilloic acid should be stored in powder in aliquot under argon, as these compounds are easily oxidized. During the enzymatic reaction, BLA hydrolyzed d_7-penicillin G into d_7-5R,6R-benzylpenicilloic acid, the enzymatic product. The amount of enzymatic product generated during the reaction was quantified by the internal standard, 5R,6R-benzylpenicilloic acid.

To 10 μL cell lysate supernatant, added 30 μL BLA assay cocktail. The plate was sealed, centrifuged at 3000 g for 1 minute, and placed on an orbital shaker at 37 °C for 1 hour. The reaction was quenched by addition of 150 μL acetonitrile, and the plate was centrifuged at 3000 g for 5 minutes. Supernatant (75 μL) was transferred to a new plate, followed by addition of 75 μL of water. The sample was ready for subsequent UPLC-MS/MS analysis.

UPLC-MS/MS analysis was carried on a Xevo TQ mass spectrometer coupled to an Acquity UPLC system (Waters Corp.). Separation of the enzymatic product (d_7-5R,6R-benzylpenicilloic acid) and the enzymatic substrate (d_7-penicillin G) was achieved using an ACQUITY UPLC HSS T3 column (1.8 μm, 2.1 x 50 mm, Waters Crop., Cat. 186003538)

connected to an ACQUITY UPLC HSS T3 VanGuard Pre-column (1.8 μm, 2.1 x 5 mm, Waters Crop., Cat. 186003976) at 40 °C. Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% formic acid. The weak needle wash was 90:10 (v:v) water:acetonitrile with 0.1% formic acid, and the strong needle wash was acetonitrile with 0.1% formic acid. The flow rate was 0.8 mL/min and the linear gradients were as followed: 0-0.2 min, 10% solvent B; 0.2-1.2 min, 10%-45% solvent B; 1.2-1.4 min, 45%-100% solvent B; 1.4-1.7 min, 100% solvent B; 1.7-1.71 min, 100%-10% solvent B, 1.71-2.0 min, 10% solvent B. 5R,6R-benzylpenicilloic acid and d_7-penicillin G eluted at 0.98 minute and 1.32 minute, respectively. Enzymatic product (d_7-5R,6R-benzylpenicilloic acid) and internal standard (5R,6R-benzylpenicilloic acid) were detected through multiple reaction monitoring (MRM) using the following transitions in ESI positive mode: 360.4 > 160.0 and 353.3 > 160.0. The cone voltage and collision energy were 20 V and 15 V, respectively. The ESI source condition was the same as listed in Table 2.3.

BLA activity in cell lysate (nmol/h/mg protein) was calculated by multiplying the ion ratio of BLA product to BLA internal standard (blank subtracted) by the nanomoles of internal standard added to the assay, then dividing by the incubation time (h) and the amount of protein used (mg).

ARSA assay: The ARSA assay was modified from the ARSA leukocyte assay detailed in Section 3.2. A total of 5 μL cell lysate supernatant was used in for the assay. The rest was the same as detailed in Section 3.2.

ARSA activity in cell lysate (nmol/h/mg protein) was calculated by multiplying the ion ratio of ARSA product to ARSA internal standard (blank subtracted) by the nanomoles of internal

standard added to the assay, then dividing by the incubation time (h) and the amount of protein used (mg).

Normalized ARSA activity in cell lysate was calculated by dividing the ion ratio of ARSA product to ARSA internal standard (blank subtracted) by the ion ratio of BLA product to BLA internal standard (blank subtracted).

Normalized ARSA activity in cell lysate relative to WT was calculated by diving the normalized ARSA activity measured in the unknown variant by the mean of normalized ARSA activity measured in the WT included in the transfection.

Appendix A Summary of the results from all the newborns with C16:0-sulfatide above the screening cut-off (0.64 after normalization).

Sample ID	C16:0-sulfatide (μM)	Normalized C16:0-sulfatide	2nd tier ARSA activity (μM/h)	% of ARSA to mean matching newborn
MLD hit 1	0.22	0.70	N/A	N/A
MLD hit 2	0.23	0.65	N/A	N/A
MLD hit 3	0.21	0.64	N/A	N/A
MLD hit 4	0.21	0.66	N/A	N/A
MLD hit 5	0.21	0.64	N/A	N/A
MLD hit 6	0.21	0.64	N/A	N/A
MLD hit 7	0.23	0.68	N/A	N/A
MLD hit 8	0.23	0.67	N/A	N/A
MLD hit 9	0.28	0.89	0.06	35
MLD hit 10	0.20	0.67	N/A	N/A
MLD hit 11	0.23	0.76	0.09	55
MLD hit 12	0.20	0.68	N/A	N/A
MLD hit 13	0.18	0.67	N/A	N/A
MLD hit 14	0.20	0.71	N/A	N/A
MLD hit 15	0.21	0.70	N/A	N/A
MLD hit 16	0.18	0.64	N/A	N/A
MLD hit 17	0.19	0.70	N/A	N/A
MLD hit 18	0.20	0.64	N/A	N/A
MLD hit 19	0.19	0.65	N/A	N/A
MLD hit 20	0.22	0.67	N/A	N/A
MLD hit 21	0.21	0.64	N/A	N/A
MLD hit 22	0.21	0.69	N/A	N/A
MLD hit 23	0.23	0.74	0.16	129
MLD hit 24[1]	0.26	0.86	0	0
MLD hit 25	0.23	0.81	0.09	68
MLD hit 26	0.20	0.71	N/A	N/A
MLD hit 27	0.19	0.64	N/A	N/A
MLD hit 28	0.20	0.70	N/A	N/A
MLD hit 29	0.19	0.66	N/A	N/A
MLD hit 30	0.18	0.66	N/A	N/A
MLD hit 31	0.17	0.64	N/A	N/A
MLD hit 32	0.16	0.65	N/A	N/A
MLD hit 33	0.16	0.66	N/A	N/A
MLD hit 34	0.17	0.68	N/A	N/A
MLD hit 35	0.18	0.69	N/A	N/A
MLD hit 36	0.17	0.65	N/A	N/A

MLD hit 37	0.18	0.68	N/A	N/A
MLD hit 38	0.18	0.69	N/A	N/A
MLD hit 39	0.16	0.67	N/A	N/A
MLD hit 40	0.17	0.69	N/A	N/A
MLD hit 41	0.17	0.71	N/A	N/A
MLD hit 42	0.20	0.81	0.16	91
MLD hit 43	0.16	0.65	N/A	N/A
MLD hit 44	0.18	0.68	N/A	N/A
MLD hit 45	0.17	0.69	N/A	N/A
MLD hit 46	0.16	0.65	N/A	N/A
MLD hit 47	0.18	0.69	N/A	N/A
MLD hit 48	0.16	0.67	N/A	N/A
MLD hit 49	0.17	0.68	N/A	N/A
MLD hit 50	0.16	0.64	N/A	N/A
MLD hit 51	0.16	0.65	N/A	N/A
MLD hit 52	0.16	0.64	N/A	N/A
MLD hit 53	0.18	0.70	N/A	N/A
MLD hit 54	0.17	0.68	N/A	N/A
MLD hit 55	0.17	0.68	N/A	N/A
MLD hit 56	0.18	0.71	N/A	N/A
MLD hit 57	0.16	0.64	N/A	N/A
MLD hit 58	0.17	0.64	N/A	N/A
MLD hit 59	0.18	0.68	N/A	N/A
MLD hit 60	0.19	0.71	N/A	N/A
MLD hit 61	0.25	0.95	N/A	N/A
MLD hit 62	0.18	0.68	N/A	N/A
MLD hit 63	0.18	0.68	N/A	N/A
MLD hit 64	0.19	0.71	N/A	N/A
MLD hit 65	0.18	0.65	N/A	N/A
MLD hit 66	0.19	0.67	N/A	N/A
MLD hit 67	0.23	0.90	0.17	91
MLD hit 68	0.22	0.83	0.3	200
MLD hit 69	0.20	0.80	0.15	91
MLD hit 70	0.22	0.78	0.11	69
MLD hit 71	0.20	0.77	0.23	149
MLD hit 72	0.19	0.75	0.18	97
MLD hit 73	0.19	0.76	N/A	N/A
MLD hit 74	0.22	0.74	N/A	N/A
MLD hit 75	0.20	0.73	N/A	N/A
MLD hit 76	0.21	0.71	N/A	N/A
MLD hit 77	0.18	0.70	N/A	N/A
MLD hit 78	0.21	0.68	N/A	N/A
MLD hit 79	0.19	0.68	N/A	N/A
MLD hit 80	0.17	0.67	N/A	N/A

MLD hit 81	0.16	0.65	N/A	N/A
MLD hit 82	0.17	0.65	N/A	N/A
MLD hit 83	0.16	0.64	N/A	N/A
MLD hit 84	0.17	0.64	N/A	N/A
MLD hit 85	0.19	0.64	N/A	N/A
MLD hit 86	0.21	0.75	0.74	197.4
MLD hit 87	0.19	0.69	0.47	126.6
MLD hit 88	0.19	0.69	0.48	129.3
MLD hit 89	0.18	0.68	0.4	110.2
MLD hit 90	0.18	0.65	0.43	113.8
MLD hit 91	0.20	0.75	0.14	62
MLD hit 92	0.18	0.67	0.2	89
MLD hit 93	0.17	0.65	0.28	99
MLD hit 94	0.18	0.65	0.24	111
MLD hit 95	0.17	0.65	0.22	78
MLD hit 96	0.18	0.64	0.17	80
MLD hit 97	0.23	0.89	0.25	81
MLD hit 98	0.18	0.68	0.27	91
MLD hit 99	0.21	0.75	0.2	86
MLD hit 100	0.20	0.72	0.18	77
MLD hit 101	0.17	0.64	0.21	75
MLD hit 102	0.18	0.64	0.19	83
MLD hit 103	0.24	0.87	0.45	129.85
MLD hit 104	0.18	0.71	0.25	123
MLD hit 105	0.20	0.69	0.19	77
MLD hit 106	0.18	0.68	0.24	115
MLD hit 107	0.17	0.67	0.47	137.65
MLD hit 108	0.17	0.66	0.41	158
MLD hit 109	0.18	0.66	0.35	104
MLD hit 110	0.18	0.65	0.08	23
MLD hit 111	0.17	0.64	0.26	128
MLD hit 112	0.17	0.64	0.23	59.65
MLD hit 113	0.19	0.70	0.22	120
MLD hit 114	0.17	0.65	0.31	197
MLD hit 115	0.22	0.79	0.11	48
MLD hit 116	0.22	0.72	0.19	120
MLD hit 117	0.19	0.71	0.16	102
MLD hit 118	0.21	0.69	0.28	135
MLD hit 119	0.21	0.68	0.18	123
MLD hit 120	0.18	0.67	0.11	61
MLD hit 121	0.17	0.66	0.12	88
MLD hit 122	0.19	0.64	0.14	67
MLD hit 123	0.24	0.94	0.14	126
MLD hit 124	0.23	0.86	0.1	91

MLD hit 125	0.26	0.84	0.12	131
MLD hit 126	0.23	0.78	0.1	103
MLD hit 127	0.21	0.77	0.13	137
MLD hit 128[2]	0.19	0.72	0.01	8
MLD hit 129	0.21	0.68	0.11	81
MLD hit 130	0.20	0.67	0.15	87
MLD hit 131	0.18	0.65	0.17	144
MLD hit 132	0.20	0.65	0.1	64
MLD hit 133	0.20	0.64	0.14	113
MLD hit 134	0.23	0.68	0.29	185
MLD hit 135	0.29	0.94	0.14	79
MLD hit 136	5.16	0.77	0.13	70
MLD hit 137	0.22	0.75	0.22	101
MLD hit 138	0.21	0.73	0.16	72
MLD hit 139	0.21	0.73	0.14	67
MLD hit 140	0.21	0.71	0.12	55
MLD hit 141	0.22	0.68	0.26	144
MLD hit 142	0.21	0.67	0.22	124
MLD hit 143	0.20	0.67	0.23	107
MLD hit 144	0.21	0.66	0.09	48
MLD hit 145	0.20	0.65	0.11	79
MLD hit 146	0.23	0.64	0.2	115
MLD hit 147	0.20	0.64	0.17	114
MLD hit 148	0.22	0.66	0.13	66
MLD hit 149	0.23	0.69	0.08	65
MLD hit 150	0.24	0.73	0.12	96
MLD hit 151	0.22	0.67	0.2	161
MLD hit 152	0.23	0.66	0.21	141
MLD hit 153	0.20	0.65	0.12	74
MLD hit 154	0.23	0.71	0.3	179
MLD hit 155	0.21	0.67	0.16	80
MLD hit 156	0.21	0.66	0.29	143
MLD hit 157	0.20	0.68	0.17	104
MLD hit 158	0.21	0.66	0.12	60
MLD hit 159	0.20	0.64	0.16	64
MLD hit 160	0.31	0.93	0.13	110
MLD hit 161	0.27	0.76	0.05	66
MLD hit 162	0.23	0.75	0.1	121
MLD hit 163	0.23	0.74	0.04	44
MLD hit 164	0.23	0.71	0.05	62
MLD hit 165	0.23	0.68	0.11	49
MLD hit 166	0.23	0.67	0.09	123
MLD hit 167	0.20	0.67	0.08	98
MLD hit 168	0.20	0.66	0.13	151

MLD hit 169	0.23	0.66	0.09	91
MLD hit 170	0.21	0.66	0.06	81
MLD hit 171	0.23	0.66	0.07	31
MLD hit 172	0.20	0.66	0.14	181
MLD hit 173	0.23	0.65	0.09	40
MLD hit 174	0.23	0.65	0.66	304
MLD hit 175	0.23	0.65	0.09	118
MLD hit 176	0.22	0.65	0.08	98
MLD hit 177	0.22	0.64	0.1	118
MLD hit 178	0.19	0.64	0.07	84
MLD hit 179	0.22	0.69	0.02	30
MLD hit 180	0.19	0.68	0.03	26
MLD hit 181	0.20	0.71	0.06	61
MLD hit 182	0.18	0.65	0.08	102
MLD hit 183	0.20	0.66	0.05	59
MLD hit 184	0.22	0.72	0.1	145
MLD hit 185	0.28	0.81	0.06	66
MLD hit 186	0.27	0.80	0.16	176
MLD hit 187	0.27	0.79	0.12	161
MLD hit 188	0.27	0.79	0.11	194
MLD hit 189	0.24	0.72	0.07	56
MLD hit 190	0.24	0.71	0.07	62
MLD hit 191	0.27	0.71	0.09	120
MLD hit 192	0.23	0.68	0.14	117
MLD hit 193	0.25	0.67	0.05	87
MLD hit 194	0.24	0.67	0.11	100
MLD hit 195	0.23	0.64	0.05	77

[1] MLD hit 24 had 0% ARSA activity compared to the mean of its matching newborns. Activities of I2S, GALNS and ARSB were not measured.

[2] MLD hit 128 had 8% ARSA activity compared to the mean of its matching newborns. Activities of I2S, GALNS and ARSB were measured, with the activity of 222%, 59%, and 23%, respectively, to the mean of its matching newborns.

Appendix B Summary of the results from the ARSA variants (pathogenic and unknown) included in the ARSA genotype-phenotype study.

Index	Mutation	Severity	Normalized ARSA activity, relative to WT	Standard deviation
1	C491G	Mild	0.0872	0.0147
2	I181S	Mild	0.0432	0.0040
3	R290H	Mild	0.0575	0.0163
4	E384K	Moderate	0.9155	0.0305
5	H399Y	Moderate	0.0125	0.0014
6	P428L	Moderate	0.0005	0.0001
7	R86Q	Moderate	0.0283	0.0015
8	A214V	Severe	0.0045	0.0006
9	G295S	Severe	0.0017	0.0010
10	P138S	Severe	0.0558	0.0036
11	P138T	Severe	0.0112	0.0023
12	P84L	Severe	0.0114	0.0035
13	R246C	Severe	0.0001	0.0000
14	R313Q	Severe	0.0010	0.0002
15	R392W	Severe	0.0020	0.0005
16	S98F	Severe	0.0006	0.0001
17	T276M	Severe	0.0021	0.0001
18	A110T	Unknown	1.0622	0.1190
19	A135D	Unknown	1.3431	0.1956
20	A14T	Unknown	1.0675	0.1600
21	A167S	Unknown	1.2587	0.3342
22	A167V	Unknown	0.0148	0.0031
23	A185S	Unknown	1.4679	0.0813
24	A191V	Unknown	1.6240	0.2541
25	A201S	Unknown	0.8392	0.2034
26	A201V	Unknown	0.8014	0.0571
27	A207D	Unknown	0.0120	0.0008
28	A207T	Unknown	0.8544	0.0517
29	A214D	Unknown	0.0016	0.0007
30	A214T	Unknown	0.0125	0.0018
31	A226V	Unknown	0.0798	0.0028
32	A325T	Unknown	1.3920	0.1573
33	A406S	Unknown	1.2052	0.3423
34	A418T	Unknown	0.1486	0.0244
35	A424V	Unknown	0.2999	0.0505
36	A457T	Unknown	1.2850	0.0962

37	A466V	Unknown	0.8648	0.5002
38	A480V	Unknown	0.8103	0.2492
39	A486T	Unknown	2.2997	0.3105
40	A5T	Unknown	1.0314	0.1500
41	A5V	Unknown	1.6117	0.1004
42	C158*	Unknown	0.0049	0.0000
43	C158F	Unknown	0.0134	0.0014
44	C174R	Unknown	0.4512	0.0777
45	C71*	Unknown	0.0058	0.0005
46	D154N	Unknown	0.0225	0.0014
47	D171N	Unknown	0.4243	0.0475
48	D171Y	Unknown	0.9421	0.0379
49	D213N	Unknown	0.5720	0.0427
50	D257H	Unknown	0.0001	0.0000
51	D257N	Unknown	0.0339	0.0041
52	D337V	Unknown	0.0002	0.0001
53	D359N	Unknown	0.7951	0.1166
54	D432Y	Unknown	0.0039	0.0005
55	D484H	Unknown	0.0937	0.0109
56	E133K	Unknown	0.9318	0.1788
57	E242A	Unknown	0.8730	0.0398
58	E255K	Unknown	0.0013	0.0004
59	E287Q	Unknown	0.0227	0.0038
60	E309K	Unknown	0.0136	0.0011
61	E314D	Unknown	0.1468	0.0332
62	E426G	Unknown	0.1349	0.0085
63	F146L	Unknown	0.0596	0.0019
64	F164L	Unknown	0.1670	0.0366
65	F206V	Unknown	0.2524	0.0438
66	F319C	Unknown	0.2107	0.0301
67	F389S	Unknown	0.0654	0.0121
68	F401L	Unknown	0.1876	0.0014
69	F61L	Unknown	0.4345	0.0383
70	G117D	Unknown	2.0452	0.3005
71	G124S	Unknown	0.0016	0.0000
72	G148R	Unknown	0.0004	0.0002
73	G172C	Unknown	0.1770	0.0310
74	G172S	Unknown	0.7898	0.1080
75	G198R	Unknown	0.8961	0.1107
76	G247R	Unknown	0.0023	0.0007
77	G261R	Unknown	0.4379	0.0271
78	G268R	Unknown	1.0407	0.0856
79	G298S	Unknown	0.1123	0.0024
80	G327S	Unknown	0.7744	0.0677

81	G347R	Unknown	0.7506	0.1326
82	G387A	Unknown	0.0719	0.0205
83	G394R	Unknown	0.4665	0.0551
84	G39D	Unknown	0.0148	0.0019
85	G39R	Unknown	0.3300	0.0494
86	G438S	Unknown	0.9701	0.0444
87	G438V	Unknown	0.0196	0.0019
88	G445V	Unknown	1.2329	0.0395
89	G449R	Unknown	0.9235	0.0466
90	G475S	Unknown	0.4564	0.0195
91	G482D	Unknown	0.2316	0.0348
92	G482S	Unknown	0.4315	0.0865
93	G4R	Unknown	0.9964	0.1743
94	G619A	Unknown	0.7076	0.0409
95	G81R	Unknown	0.0017	0.0011
96	G81S	Unknown	0.2255	0.0197
97	G92D	Unknown	0.1483	0.0305
98	H140D	Unknown	0.3907	0.0104
99	H140Y	Unknown	1.1792	0.1604
100	H208Y	Unknown	1.0614	0.0441
101	H228D	Unknown	0.1808	0.0293
102	H330R	Unknown	0.1628	0.0212
103	H399Q	Unknown	0.2068	0.0075
104	H425D	Unknown	0.0065	0.0006
105	H425Y	Unknown	0.2271	0.0330
106	I149T	Unknown	0.6097	0.0630
107	I25V	Unknown	1.2346	0.1486
108	I267T	Unknown	0.3487	0.0928
109	I267V	Unknown	0.7915	0.0352
110	I279M	Unknown	0.9415	0.1342
111	I324T	Unknown	0.4008	0.0434
112	K459E	Unknown	0.1482	0.0380
113	K459R	Unknown	0.0049	0.0000
114	L10F	Unknown	1.3146	0.1417
115	L113P	Unknown	0.0013	0.0003
116	L210H	Unknown	0.2818	0.0375
117	L272Q	Unknown	1.4170	0.3208
118	L299P	Unknown	0.5400	0.0394
119	L300F	Unknown	0.3614	0.0264
120	L342V	Unknown	0.7750	0.1150
121	L422P	Unknown	0.3614	0.0341
122	L430F	Unknown	0.6200	0.0126
123	L433V	Unknown	0.4830	0.0613
124	L54V	Unknown	1.2153	0.0699

125	L94V	Unknown	0.3524	0.0495
126	L9F	Unknown	1.2015	0.4234
127	L9V	Unknown	1.2164	0.4489
128	M211I	Unknown	1.2103	0.1207
129	M254T	Unknown	1.3013	0.2263
130	M289T	Unknown	0.0254	0.0034
131	N352H	Unknown	0.5453	0.0309
132	N352S	Unknown	0.6537	0.0920
133	N440K	Unknown	0.2233	0.0341
134	N442S	Unknown	0.1987	0.0056
135	P138L	Unknown	0.0662	0.0045
136	P139L	Unknown	0.9773	0.0115
137	P150L	Unknown	0.0008	0.0002
138	P166L	Unknown	0.8929	0.0985
139	P193T	Unknown	0.0064	0.0004
140	P194A	Unknown	0.8824	0.1613
141	P194L	Unknown	0.0093	0.0019
142	P220L	Unknown	0.2014	0.0331
143	P220S	Unknown	0.8094	0.0067
144	P220T	Unknown	0.0261	0.0028
145	P233A	Unknown	0.0000	0.0000
146	P286T	Unknown	0.0014	0.0002
147	P379L	Unknown	0.1221	0.0049
148	P379Q	Unknown	0.8941	0.0932
149	P437T	Unknown	0.1334	0.0231
150	P452L	Unknown	0.7362	0.0733
151	P485S	Unknown	0.4704	0.0451
152	Q155H	Unknown	0.0027	0.0020
153	Q159H	Unknown	0.8990	0.0305
154	Q215H	Unknown	0.5242	0.0513
155	Q373P	Unknown	0.9007	0.1722
156	Q460E	Unknown	0.3990	0.0203
157	R116G	Unknown	2.3387	0.1741
158	R145Q	Unknown	0.9511	0.2013
159	R202C	Unknown	0.2214	0.0518
160	R202H	Unknown	0.7264	0.0750
161	R219C	Unknown	0.5688	0.0197
162	R219H	Unknown	0.1718	0.0081
163	R243C	Unknown	0.8697	0.0720
164	R243H	Unknown	0.1319	0.0176
165	R293Q	Unknown	0.6496	0.0119
166	R301Q	Unknown	0.0984	0.0023
167	R372Q	Unknown	0.2329	0.0269
168	R372W	Unknown	0.0029	0.0003

169	R386C	Unknown	0.1352	0.0056
170	R386G	Unknown	0.3160	0.0333
171	R386H	Unknown	0.0033	0.0028
172	R392G	Unknown	0.0945	0.0056
173	R392L	Unknown	0.0928	0.0129
174	R392Q	Unknown	0.4169	0.0733
175	R481Q	Unknown	0.3306	0.0188
176	R481W	Unknown	0.0831	0.0049
177	R82P	Unknown	0.0008	0.0004
178	R99P	Unknown	0.8621	0.0797
179	R99Q	Unknown	1.0896	0.0720
180	S152T	Unknown	0.2573	0.0080
181	S227C	Unknown	0.0000	0.0000
182	S292F	Unknown	1.1402	0.0922
183	S405F	Unknown	0.0014	0.0001
184	S405P	Unknown	0.0012	0.0005
185	S408N	Unknown	0.0130	0.0028
186	S408T	Unknown	0.0132	0.0018
187	S420R	Unknown	0.2489	0.0301
188	S421C	Unknown	0.6653	0.1061
189	T162S	Unknown	0.5805	0.0423
190	T288P	Unknown	0.8078	0.0395
191	T354S	Unknown	0.8563	0.1160
192	T393A	Unknown	0.7150	0.0501
193	T393S	Unknown	0.1466	0.0085
194	T410I	Unknown	0.0291	0.0109
195	T48I	Unknown	0.0045	0.0021
196	V109M	Unknown	0.4385	0.0921
197	V189M	Unknown	0.7882	0.0712
198	V19A	Unknown	1.5840	0.2121
199	V278F	Unknown	1.0854	0.2901
200	V479M	Unknown	1.3715	0.1323
201	V66A	Unknown	1.6827	0.2259
202	V95M	Unknown	0.1383	0.0161
203	W195C	Unknown	0.2286	0.0124
204	W320G	Unknown	0.0198	0.0035
205	Y224C	Unknown	0.0977	0.0049
206	Y225C	Unknown	0.9316	0.0790
207	Y381H	Unknown	0.9072	0.0638
208	Y41*	Unknown	0.0080	0.0012
209	Y41C	Unknown	0.8108	0.1584
210	Y431C	Unknown	0.1986	0.0116
211	Y431S	Unknown	0.0120	0.0011

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