Multiplexed Measurement of Enzyme Activities Associated with Seven Lysosomal Storage Disorders from a Dried Blood Spot via LC-MS/MS

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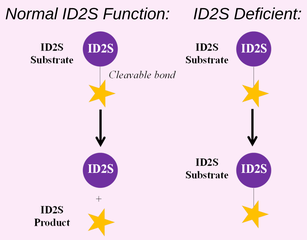
1 Introduction

- Lysosomal storage disorders (LSD) are a class of diseases initiated by inherited gene mutations that result in a disruption of lysosomal function. These mutations often cause a deficiency in activity of a particular lysosomal enzyme, resulting in the accumulation of cellular components.
- The development of newborn screening (NBS) assays for LSDs provide the ability to identify pre-symptomatic individuals, allowing for more effective therapeutic interventions.
- The goal of this study was to determine if a seven-plex enzyme activity assay could be developed for two types of LSDs: mucopolysaccharidoses (MPS) and neuronal ceroid lipofuscinoses.
- DBS collected from various age groups were also analyzed in order to begin to understand the relationship between age and enzyme activity.

2 Assay Background

- The assay centers around the ability of each enzyme to convert a **substrate** to a **product**.



- Enzymatic reactions are carried out by incubating dried blood spots (DBS) within a cocktail containing substrates and internal standards in a buffered environment allowing for proper enzyme activity.
- The high specificity of each substrate allows for multiplexed measurements.

Table 1. Concentrations of enzyme specific substrates and internal standards included in assay cocktail and buffer composition.

Substrate	Concentration (µM)	Internal Standard	Concentration (µM)
ID2S	500	ID2S	5
NAGLU	500	NAGLU	5
GALNS	1000	GALNS	5
bGAL	500	bGAL	5
ARSB	1000	ARSB	5
GUSB	500	GUSB	10
TPP1	200	TPP1	15

Buffer Composition	
Reagent	**Concentration (mM)**
Ammonium Acetate (pH 5)	60
Cerium Acetate	7
NAG-thiazoline	0.1

- The activity of the following enzymes will be measured directly by (MS/MS):
  - Iduronate 2-sulfatase (ID2S)
  - α-N-acetylglucosaminidase (NAGLU)
  - N-acetylgalactosamine 6-sulfatase (GALNS)
  - β-galactosidase (bGAL)
  - Arylsulfatase B (ARSB)
  - β-glucuronidase (GUSB)
  - Tripeptidyl peptidase 1 (TPP1)

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3 Assay Procedure

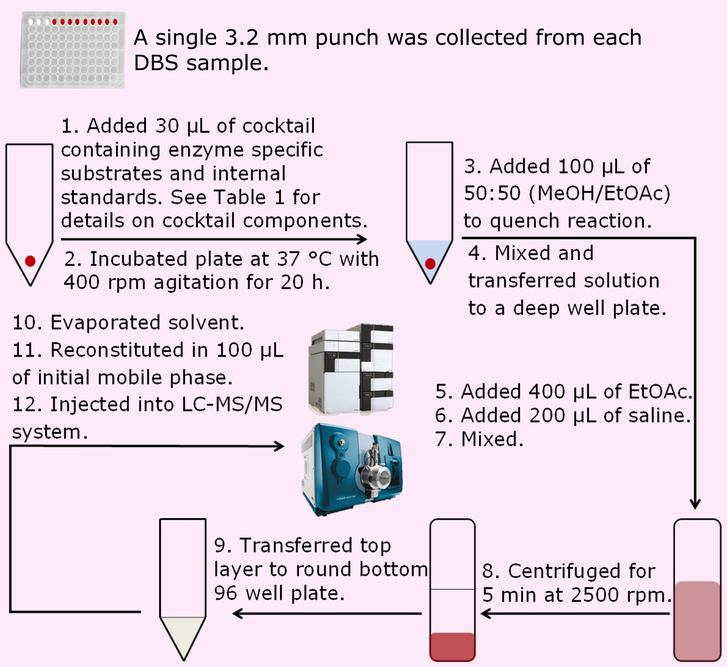

Figure 1. Overview of sample preparation for seven-plex lysosomal enzyme activity assay.

4 Analysis Method

- Analysis was performed using a Shimadzu® Nexera® X2 UPLC system coupled with a Sciex® 5500 QTRAP™ MS/MS.
- The mass spectrometer was operated in MRM mode with positive polarity.

Parameter	
Column	Waters® X-select™ CSH C18 column (50 x 2.1 mm; 3.5 µm)
Guard column	Waters® Vanguard™ CSH pre-column (10 x 2.1 mm; 3.5 µm)
Column Temperature (° C)	35
Flow Rate (0.5 mL/min)	0.5
Injection Volume (µL)	1.0
Mobile Phase A	0.1% formic acid
Mobile Phase B	0.1% formic acid in acetonitrile

Time (min)	% A	% B
0.01	70	30
0.50	70	30
1.00	60	40
2.00	60	40
2.50	15	85
3.00	15	85
3.01	70	30
3.50	70	30

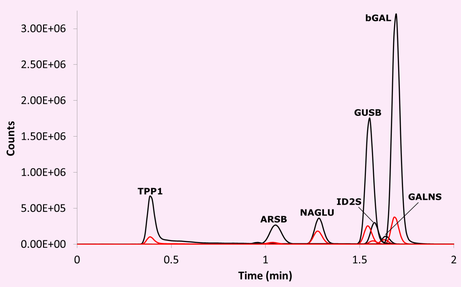

Figure 2. Sample chromatogram of a high activity quality control sample. Black traces represent the product formed by each enzyme. Red traces represent internal standards.

- As seen in figure 2, the response of each product and internal standard was monitored via tandem mass spectrometry. Enzyme activities were then calculated using equation 1.

$$Enzyme\ activity\ (\mu M/h) = \frac{\frac{P_s}{IS_s} - \frac{P_b}{IS_b} \quad (C_{IS}) \times (V_{IS})}{(V_b) \times (t)}$$

Equation 1. Calculation of enzyme activity. Where, (P_s/IS_s) represents the area ratio in a sample; (P_b/IS_b) represents the area ratio in a blank; C_{IS} represents the IS concentration in units of µM; V_{IS} represents volume of IS solution in units of µL; V_b represents the volume of blood in units of µL; and t represents the incubation time in units of h. A blood volume of 3.1 µL was assigned to a 1/8 inch DBS punch.

5 Results

Table 4. Presumed normal patient groups.

Group	Age Range	Number of Patients
1	≤ 7 days	508
2	6 mo – 2 y	17
3	3-5 y	20
4	6-10 y	20
5	11-15 y	20
6	16-20 y	20

- Apparently normal samples were analyzed from six different age groups in order to establish normal activity ranges and observe any relationship between activity and age.
- Confirmed low activity patient samples were randomly distributed among presumed healthy samples for all seven disorders.

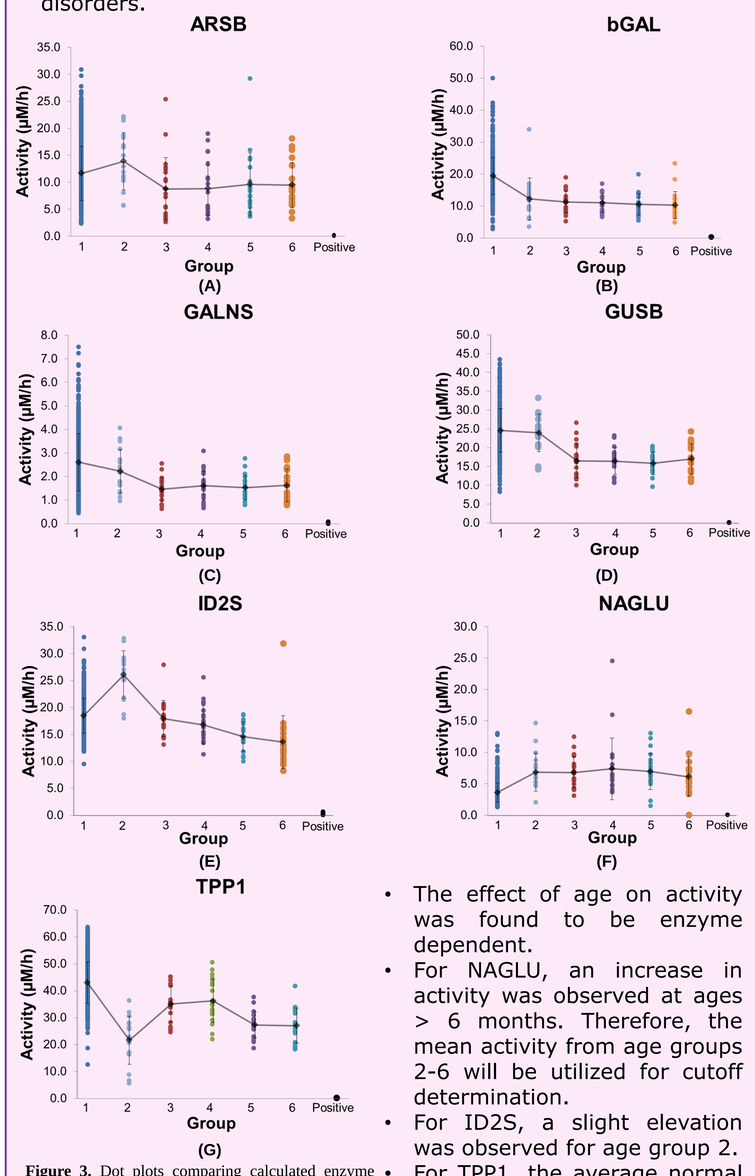

Figure 3. Dot plots comparing calculated enzyme activities of (A) ARSB, (B) bGAL, C) GALNS, (D) GUSB, (E) ID2S, (F) NAGLU, and (G) TPP1. Black traces display the mean enzyme activity within each age group.

Note: For TPP1, the linear range only extends to 23 µM/h. Any values > 23 µM are inaccurate and included for reference only.

- The effect of age on activity was found to be enzyme dependent.
- For NAGLU, an increase in activity was observed at ages > 6 months. Therefore, the mean activity from age groups 2-6 will be utilized for cutoff determination.
- For ID2S, a slight elevation was observed for age group 2.
- For TPP1, the average normal activity is outside the linear range of this enzyme.
- For all remaining enzymes, the use of newborn samples (group 1) is appropriate for establishing initial cutoffs.

Table 5. Proposed initial cutoffs for screening samples up to age 20 y.

Enzyme	Activity (µM/h)	Description
ARSB	2.35	20 % of newborn mean
bGAL	2.94	15 % of newborn mean
GALNS	0.39	15 % of newborn mean
GUSB	4.95	20 % of newborn mean
ID2S	5.21	20 % of group 2 mean
NAGLU	1.39	20 % of groups 3-6 mean
TPP1	9.88	Average of low activity contrived control

6 Conclusion

- An assay has been successfully developed that allows for the direct measurement of the activity of seven lysosomal enzymes from DBS for patients age 0-20 y. This assay is used as a primary screening tool. For identified positives, sequencing is performed for confirmation.
- For TPP1, activity was found to be linear within the clinically relevant region, however presumed normal activities are well outside of the linear range. Therefore, the mean normal activity cannot be used for cutoff determination. In this case, the cutoff has initially been set at the average activity for the low quality control. This cutoff may be adjusted in the future.

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