Understanding Enzyme Assays for Lysosomal Storage Disorders

Part II: How to Measure Product Formation

The availability of new technologies and new tests for newborn screening of treatable childhood conditions provides the exciting promise of helping more children, but also confers significant challenges to the screening programs that must implement these new tests. Today, most states screen for over 30 different conditions using a combination of hospital based physiological tests (hearing screening, pulse oximetry) and laboratory based biochemical tests (immunoassays, separation assays, molecular assays, metabolite analysis and enzyme measurement). Enzyme assays for lysosomal storage disorders (LSDs) are currently being implemented in several screening programs. Here, we elucidate the differences between LSD enzyme analysis workflows on digital microfluidics and tandem mass spectrometry (MS/MS) platforms. Additionally, we also highlight the significant difference between products of an enzymatic reaction measured with MS/MS and direct measurement of metabolites with MS/MS.

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MS/MS Detection of Metabolites

Tandem mass spectrometry (MS/MS), pioneered by Prof. Millington, revolutionized newborn screening for inborn errors of metabolism in the 1990’s by enabling multiplexed (multi-analyte, multi-disease) metabolite measurement from dried blood spot (DBS) samples, and it remains the ‘gold standard’ technology for metabolite newborn screening today. The original derivatized MS/MS protocol for metabolite profiling requires around 12 manual steps and provides a similar screening capacity to the non-derivatized protocol (500 samples per instrument per day)\(^1\). Figure 1a illustrates a typical workflow for metabolite measurement using non-derivatized MS/MS, which consists of 4 steps\(^2\). The results of both MS/MS metabolite protocols are reported as concentration of metabolite in micromoles/unit volume of blood.

MS/MS Detection of Enzyme Activity

In addition to metabolite analysis, MS/MS has been used to measure enzyme activity to screen for LSDs. The workflow for MS/MS enzyme activity analysis is different from the MS/MS metabolite workflow and requires an additional enzymatic reaction and a multi-step protocol to process the cleaved product(s) prior to mass analysis. A published workflow for a 6-plex enzyme activity MS/MS (flow injection) protocol\(^3\) is shown in Figure 1b. In this example, a single punch from a DBS sample is incubated overnight with the enzyme substrates, inhibitors, cofactors and internal standards, and the products of the enzymatic reaction are then processed via multiple organic extraction, drying and reconstitution steps and injected directly into a triple quadrupole mass spectrometer. All six enzyme reactions in this 6-plex protocol are performed in the same sample well using a common buffer and (after processing) are injected into the mass analyzer together; the product formed by each enzyme is measured specifically based on the different masses and fragmentation patterns of their respective substrates. The results of MS/MS enzyme analysis are reported as concentration of product/unit time and are a measure of the enzyme activity as opposed to metabolite concentration. The total time to result for this MS/MS enzyme assay (6 enzymes) is around two full working days (including the overnight enzyme incubation and overnight MS/MS analysis) for approximately 500 samples per instrument per day. The time component of the unit of measurement (micromoles/liter/hour) and the addition of a substrate to the reaction mixture differentiate MS/MS enzymatic activity assays from the MS/MS metabolite assays that are commonplace in newborn screening laboratories today. Additionally, the disease states in metabolite assays result in elevated concentrations of the analyte, whereas in the enzyme activity assays such as for LSDs, the disease state translates to lower activity due to enzyme deficiencies.

Digital Microfluidic Detection of Enzyme Activity

The digital microfluidic (DMF) platform is a small volume, automated liquid handling system that performs high throughput enzyme activity analysis using fluorimetric substrates. For enzyme activity analysis of LSDs, a 4-enzyme protocol has been described (Figure 1c)\(^4\). Similar to the non-derivatized MS/MS
metabolite protocol, DMF enzyme analysis requires a short DBS extraction procedure prior to loading the samples onto the DMF cartridge. DBS extracts from one 96-well microtiter plate are used to fill two DMF cartridges; once the cartridge is loaded into the instrument, all subsequent reaction steps are automated without further user intervention. The fluorescent enzyme activity assays take place simultaneously within discrete reaction droplets on the same disposable cartridge, which allows each enzymatic reaction to occur under optimal reaction conditions. Product formation is measured based on the fluorescent output of each substrate after incubation and is reported as concentration of product/unit time (micromoles/liter/hour). The total time to result for each DMF enzyme assay (4 enzymes) is approximately 3.5 hours and approximately 500 samples can be analyzed per workstation per day.

Additional Considerations

Preliminary prospective population screening studies indicate that both MS/MS and DMF can effectively be used for enzyme activity measurements in LSD newborn screening\(^5\),\(^6\). There are however, additional technical limitations that are likely to affect the successful implementation of each platform in newborn screening laboratories. Most notably, additional equipment must be acquired and installed to support both DMF and MS/MS platforms. DMF is a relatively new technology that is not yet used for other tests in public health laboratories. Fortunately, DMF equipment is low cost, has a small footprint and can be installed in minutes, with no facility modifications (HVAC) required. By contrast, MS/MS equipment is already installed in all newborn screening laboratories for metabolite screening; however, fundamental differences in assay protocols and workflows between MS/MS metabolite and enzyme assays prevent these two methods from being combined efficiently on a shared piece of equipment. In many cases, implementation of MS/MS enzyme activity measurement therefore also requires the purchase and installation of additional equipment, including back-up equipment and maintenance contracts for all these equipment, and usually necessitates facility modifications to maintain a healthy working environment. The digital microfluidic platform performs high throughput enzyme activity analysis using a simplified workflow and provides a cost-effective alternative to MS/MS.

References

## DETAILED WORKFLOWS

### Mass Spectrometry Metabolic
1. **Punch**: 3.2 mm DBS into 96-well plate
2. **Extract**: Add Extraction Solution with internal standards, cover and extract
3. **Transfer & Measure**

### Mass Spectrometry Enzymatic
1. **Punch**: 3.2 mm DBS into 96-well plate
2. **Extract & Add Cocktail**: Add 30 μL Cocktail (substrates & internal standards), seal
3. **Shake & Incubate**: 37°C, 400 rpm (18 ± 2 hours)
4. **Quench & Mix**: Add 100 μL 50:50 MeOH:EA, mix with pipette
6. **Separate Layers**: Deep 96-well plate centrifuge, 2 minutes
7. **Transfer Top Layer**: Transfer 100 μL of top layer to sampling plate
8. **Dry**
9. **Add Flow Solvent**: Add 100 μL Flow Solvent
10. **Shake**: Room temperature, 750 rpm, 10 minutes
11. **Transfer & Measure**

### Digital Microfluidics Enzymatic
1. **Punch**: 3.2 mm DBS into 96-well plate
2. **Extract**: Add Extraction Buffer, cover and extract
3. **Load & Measure**