Supplemental Material

I. Supplemental Table 1. Some comparative features of the MS/MS versus digital microfluidics LSD enzyme assay methods. See Supplemental Materials for a more detailed analysis.

	Flow injection-MS/MS	Digital microfluidics fluorimetry
LSDs covered	MPS-I, MPS-II, MPS-IIIA-D, MPS-IVA, MPS-VI, Fabry, Gaucher, Krabbe, Niemann-Pick-A/B, Pompe	MPS-I, MPS-II, Fabry, Gaucher, Pompe
Pre-instrument steps	5 liquid transfers, 1 centrifugation, 1 dry down	7 liquid transfers, 1 centrifugation
Space required[1]	Table top centrifuge: 9.2" x 10.2" x 7.75" MS/MS: 23x32" footprint, 14 cu ft	Table top centrifuge: 9.2" x 10.2" x 7.75" digital microfluidics workstation: 20x30" footprint, 8 cu ft
Technician time	1 full time laboratory technician	1 full time laboratory technician

[1]The volume space requirment of the MS/MS includes the vacuum pump that would typically sit underneat the MS/MS unit. This is why the volume space requirment for the MS/MS is larger than the digital microfluidics workstation eventhough the footprints of both are similar.

Detailed explanation of Supplemental Table 1.

1. The diseases listed are those for which a published enzyme assay exists.

2. Liquid transfers refer to both liquid addition and liquid transfer from one sample container to another (both done by manual pipetting, typically with a multi-channel pipettor). For a 96-well plate, the MS/MS method requires 5 liquid transfers: 1) Addition of assay cocktail (using a 96-channel Rainin liquidator); 2) Addition of water after the incubation; 3) Addition of ethyl acetate; 4) transfer of a portion of the ethyl acetate layer to a new plate; 5) Addition of flow injection solvent. The digital microfluidics method

requires 7 liquid transfers: 1) Addition of DBS extraction solvent (using a 96-channel Rainin liquidator; 2) Transfer of the extract to the microfluidics chip (each chip accepts 48 samples so this requires 2 tranfers); Addition of reagents to two chips; 4) Addition of stop buffer to two chips.

3. Centrifugation refers to a ~10 min centrifugation of the 96-well plate at ~3,000 rpm.

4. Dry down refers to organic solvent removal with a multi-jet evaporator (~15 min).

5. Space requirements:

a. The table top centrifuge is a contemporary mini-centrifuge capable of holding 2x96-well plates at a time (i.e. Alkali Scientific, Inc.).

b. The 96-well plate incubator is a standard in many NBS labs.

c. A single MS/MS instrument is required to handle ~ 400-600 samples per day. The inject-to-inject time is 115 sec for the 6-plex LSD assay, and thus 400 samples can be analyzed in 12.8 hrs (overnight run). The digital microfluidics workstation includes 4 stand alone chip handling instruments. Each digital microfluidics analyzer handles 48 samples in about 2 hrs, thus a workstation of 4 analyzers is needed to handle 400 samples per day. Based on these estimates, it is reasonable to suggest that a NBS laboratory charged with the task of running 400 samples per day can function with a single MS/MS or a single digital microfluidics work station. Space for the DBS puncher and manual pipetting of liquids is not included since they are the same for both methods.

6. Laboratory technician:

Manpower is based on ~400 samples per day. Punching of the blood spots is not included since it is the same for both methods. For the MS/MS method, after obtaining 4 x 96-well plate with DBS punches, it takes about 1 hr to prepare the reagents and add them to 4 plates. The plates are placed in the incubator (~5 min). After unassisted incubation, the plates are charged with water and ethyl acetate (~30 min), and after a 10-15 min centrifugation step the upper layers are transfered to a new plate and placed

on the solvent dry down manifold (~30 min). After ~15 min of solvent removal, the plates are charged with flow injection solvent and placed on the MS/MS autosampler (~30 min). Thus it seems clear that a single full-time laboratory technician can handle 4 x 96-well plates per day. We do not have accurate manpower time estimates for the digital microfluidics sample processing, but the data in Table 1 suggests that it is similar to the time for the MS/MS analysis.

II. Cost analysis.

The table below gives cost estimates for the MS/MS method to screen 6 LSDs (6-plex analysis of Fabry, Gaucher, Krabbe, MPS-I, Niemann-Pick-A/B, and Pompe diseases). Costs are per newborn per disease (per enzyme). The text below the table gives the details of the cost calculations.

Supplemental Table 2. Estimated cost for MS/MS NBS of 6 LSDs.

Item	Cost per newborn per enzyme (US cents)
MS/MS Instrumentation	7.7
Liquidator manual multi-channel pipettor	0.26
96-Well plates and pipet tips.	5
6-Plex kit components (substrates, internal standards, buffer, solvents, quality control samples)	10

The triple quadrupole MS/MS with autosampler costs $220,000 (Waters TQD or the equivalent), the nitrogen generator costs $19,000, and we assume $10,000 to run a vent conduit from the MS/MS to the existing fume hood (or vent from other MS/MS instruments in existence in the same lab). Electricity is estimated at $100 per month. We assume that the MS/MS and the nitrogen generator last 10 yrs and require an

annual service contract fee of $30,000 (MS/MS) and $2,000 (nitrogen). The inject-to-inject time for the MS/MS analysis is 115 sec, and thus 470 samples can be run in 15 hrs, and this is 420 newborn samples if we analyze 50 quality control samples as well. The number of newborns analyzed per year is approximately 50 weeks x 6 nights per week x 420 samples per night = 126,000. The MS/MS cost per year per newborn sample (not including the technician and space) is thus: [$23,900 (instrumentation) + $32,000 (servicing) + $1,000 (vent) + $1,200 (electricity)] divided by 126,000 = $0.46. Since all 6 LSDs are analyzed in the same 115 sec run, the cost per newborn per disease is 7.7 cents.

The Rainin Liquidator 96-channel pipettor costs $10,000, and servicing is $1,000 per year so the cost per year per newborn per enzyme is (assuming the instrument lasts 10 years): [$1,000 (instrument) + $1,000 (service)] divided by 126,000 divided by 6 = 0.26 cents.

All required plastic 96-well plates and tips cost $37,800 for 126,000 newborns which gives 5 cents per newborn per enzyme.

The MS/MS reagent costs (substrates, internal standards and buffer) is ~10 cents per newborn per LSD. This is based on actual USA manufacturing costs including quality control analysis. Pricing will drop overtime since this estimate includes the costs for process chemistry development, and that has to be done only once per compound.

The total costs in Supplemental Table 1 comes to $0.23 per newborn per LSD analyzed. Manpower is not included in this cost analysis since it is expected to be the same for the MS/MS and digital microfluidics assay methods. It is clear from this analysis that the cost for LSD NBS by MS/MS is expected to be below $1.00 per newborn per LSD. This is inline with current costs for NBS of other diseases currently carried out by NBS laboratories. We do not have accurate cost estimates for the digital microfluidics reagents and microfllluidics chips at this time, but it is reasonable to expect

that costs will also be close to $1.00 per newborn per LSD. This estimate is mainly based on the fact that the costs cannot be much more than the costs for NBS of other diseases, and it cannot be much less either because of the costs of developing these methods at the research stage and to seek FDA approval. The upfront costs for the MS/MS method are higher because of instrumentation purchasing, but the actual annual costs per newborn per LSD is as stated in Supplemental Table 1. Often NBS labs lease the MS/MS instruments from the company that provides servicing.

III. Factors that affect the ratio of mean lysosomal enzymatic activity of random newborns versus that of disease-affected newborns. The time courses for product formation versus assay incubation time for the MS/MS and fluorimetric assays are essentially linear. Thus, the ratio of activity of any two DBS samples should be independent of the incubation time as long as the same time is used for both samples. Also the specific activities for the action of the lysosomal enzyme on the MS/MS and fluorimetric substrates may be different, but again the ratio of activies for any 2 DBS samples should be the same. These are true even if the assay has a significant signal coming from the sample in the absence of enzymatic product (blank signal) since time courses for the LSD-affected and non-affected samples should start at the same assay signal. The most likely explanation for the much higher ratio of mean activity for random newborns to activity for LSD-affected newborns between the MS/MS and fluorimetric assay (Table 1, main text) is that the background for the fluorimetric assay is higher. When substrate and internal standard are omitted from the MS/MS assay using DBS, the ion counts in the product and internal standard MRM windows are essentially 0 (supplemental material Table 3) showing that there are no components of the DBS unrelated to substrate-derived product and internal standard that contribute to the MRM signals. If substrate is included but the sample not incubated to allow product formation,

there is a measureable amount of product by MS/MS due to a small amount of conversion of substrate to product in the heated electrospray ionization source, but this amount of product only represents 1% or less of the amount of product for the complete and incubated assay (Table 1, main text). On the other hand all biological samples including blood will have a significant amount of fluorescence (autofluorescence) unrelated to the fluorescent product derived from the fluorimetric substrate. If the fluorimemtric substrate has some intrinsic fluorescence, this will also contribute to the blank (this is very small for glycosides of 4MU). Supplemental Material Figure 1 shows that the observed assay response for the non-affected sample (true enzymatic activity response + blank) divided by the observed assay response the affected sample (true enzymatic activity + blank) increases as the ratio of true enzymatic activity of the affected sample divided by the blank increases. In this analysis, blank refers to the fluorescence coming from the blood and from the fluorimetric substrate in the absence of fluorescent product for the fluorimetric assay or product MRM ion counts coming from in-source substrate-to-product conversion in the MS/MS assay (since there is essentially no MRM ion counts coming from the sample alone, Supplemental Material Table 3).

Significant blank contribution to the assay signal is more problematic if it is derived from the biological sample since it is unlikely to be constant for all samples. This is the case for fluorescence assays where significant autofluorescence coming from the sample alone contributes to the observed assay response. On the other hand, for the MS/MS assay, the blank comes mainly from in-source conversion of substrate to product, and this does not vary with biological sample. If the sample-dependent blank is significant, the most reliable assay will require that the blank be determined for each sample so that it can be subtracted from the observed assay response for the complete assay.

Fluorescence background data analogous to that in Supplemental Table 3 for conventional fluorimetric and digital microfluidics fluorimetric assays have not been published (to the best of our knowledge). Thus a more detailed analysis of this issue is not possible presently.

Supplemental Material Table 3.

	ABG-P	**ABG-IS**	**ASM-P**	**ASM-IS**	**GALC-P**	**GALC-IS**	**IDUA-P**	**IDUA-IS**	**GLA-P**	**GLA-IS**	**GAA-P**	**GAA-IS**
FP with S/IS	1200	114e3	44	56e3	301	77e3	1400	54e3	2300	32e3	1200	75e3
DBS with S/IS	73e3	99e3	14e4	29e3	22e3	34e3	179e3	39e3	111e3	12e3	86e3	24e3
FP no S/IS	0	13	13	30	2	0	0	17	26	9	0	0
DBS no S/IS	0	28	6	0	18	0	0	4	0	7	4	0

Supplemental Material Figure 1. The Y-axis is the observed assay response with a DBS from a non-affected individual divided by that for a DBS from an LSD-affected individual. The X-axis is the observed assay response for a DBS from an LSD-affected individual divided by the assay response in the blank (complete assay with DBS and substrate but no product). The plot was generated by using a fixed values of 10 and 1 arbitrary units for the component of the assay response due to enzymatic product formation with the non-affected and LSD-affected sample, respectively and a range of blank values of 0.1 - 30 arbitrary units.