

## Supplemental File

### Preparation of substrates and internal standards for assay of GAA in DBS.

All substrates and internal standards were obtained from H. Zhang and R. Vogt (CDC, Atlanta, GA). The GAA CDC vial contained 7.91 mg of GAA substrate and 0.06 mg of GAA internal standard. The vial was reconstituted by vortex mixing in 18mL of GAA buffer. The GAA buffer of each vial contained 1.8 mL of 100g/L CHAPS (Sigma-Aldrich, C9426) in water, 15.9 mL of citrate phosphate buffer (0.34 M sodium phosphate (monobasic) (Sigma-Aldrich, S0751) and 0.17 M sodium citrate (tribasic) dehydrate (Sigma-Aldrich, C8532), pH 4.0), and 0.3 mL of 0.8 mM acarbose (Sigma-Aldrich, A8980) in water. The vial was vortex mixed in 1.8 mL of 100g/L 3-[(3-cholamidopropyl)dimethylammonio-1-propanesulfonate (CHAPS) (Sigma-Aldrich, C9426) in water. Then, 15.9 mL of GAA buffer (0.34 M sodium phosphate (monobasic) (Sigma-Aldrich, S0751) and 0.17 M sodium citrate (tribasic) dehydrate (Sigma-Aldrich, C8532), pH 4.0) was added, and the mixture was vortexed. Finally, 0.3 mL of 0.8 mM acarbose (Sigma-Aldrich, A8980) in water was added. The reconstituted vials were aliquoted and stored <1 month at -20°C.

The GLA CDC vial contained 38.72 mg of GLA substrate and 0.06 mg of GLA internal standard. The vial was reconstituted by vortex mixing in 18mL of GLA buffer. The GLA buffer of each vial contains 0.45 mL of aqueous sodium taurocholate (120 g/L sodium taurocholate, ≥95% purity, Sigma-Aldrich, T4009), 14.67 mL of 0.174M sodium acetate buffer (pH4.6, Kanto chemical, Cat: 37093), and 2.88 mL of 1 M N-acetylgalactosamine (Sigma-Aldrich, A2795) in water. The reconstituted vials were aliquoted and stored <1 month at -20 °C.

The ABG CDC vial contained 7.72 mg of ABG substrate and 0.12 mg of ABG internal standard. The vial was reconstituted by vortex mixing in 18mL of ABG buffer. The ABG buffer of each vial contained 2.4 mL of aqueous sodium taurocholate (120 g/L sodium taurocholate, ≥95% purity, Sigma-Aldrich, T4009) and vortexed. Then, 15.6 mL of ABG buffer (0.72 M sodium

phosphate (monobasic) (Sigma-Aldrich, S0751) and 0.36 M sodium citrate (tribasic) dehydrate (Sigma-Aldrich, C8532), pH 5.1) was added, and the mixture was vortexed. The reconstituted vials were aliquoted and stored <1 month at -20 °C.

The IDUA CDC vial contained 17.0 mg of IDUA substrate and 0.08 mg of IDUA internal standard. The vial was reconstituted by vortex mixing in 18mL of IDUA buffer. The IDUA buffer of each vial contained 0.5 mL of inhibitor solution (3.0 mM D-saccharic acid 1,4 lactone monohydrate, Sigma-Aldrich, S0375 in water) and 17.5 mL of sodium formate buffer (0.11 M sodium formate, Sigma-Aldrich, 71541, and 0.16 M formic acid, Merck, CAS 64-18-6, pH 3.6). The reconstituted vials were aliquoted and stored <1 month at -20 °C.

#### **Preparation of substrates and internal standards for assay of GAA in DBS using 6-plex substrate/internal standard mix**

The PerkinElmer 6plex vial contained 0.80 mg of GAA substrate and 0.041mg GAA internal standard, 0.94 mg of ABG substrate and 0.026mg ABG internal standard, 1.45 mg of ASM substrate and 0.024 mg ASM internal standard, 1.93 mg of GALC substrate and 0.015 mg GALC internal standard, 2.77 mg of GLA substrate and 0.038 mg GLA internal standard, 0.44 mg of IDUA substrate and 0.021 mg IDUA internal standard, and 4.29 mg of sodium oleate.

The vial was reconstituted by vortex mixing in 1.82 mL of GAA buffer. The GAA buffer of each vial contained 0.18 mL of 100g/L CHAPS (Sigma-Aldrich, C9426) in water, 1.61 mL of citrate phosphate buffer (0.34 M sodium phosphate (monobasic) (Sigma-Aldrich, S0751) and 0.17 M sodium citrate (tribasic) dehydrate (Sigma-Aldrich, C8532), pH 4.0), and 0.03 mL of 0.8 mM acarbose (Sigma-Aldrich, A8980) in water. The concentration of GAA substrate was identical to CDC GAA cocktail buffer, but the concentration of internal standard was different since this was controlled by the substrate/internal standard ratio in the PerkinElmer 6plex reagent mixture.

**Supplemental Table 1.** Data for patients shown in Figure 1 of the main text. 4MU activity values are not blank corrected.

	Patient Number	gender	4MU enzyme activity	MS/MS enzyme activity	genotype
Infantile Pompe	1	female	0.45	0.01	c.424_440del17/ c.1726 G>A, c.1935 C>A
	2	male	0.43	0.13	c.1396C>T/c.1935C>A
	3	female	0.42	0.10	c.1726 G>A, c.1935 C>A / c.1726 G>A, c.1935 C>A
	4	male	0.45	0.09	c.1726 G>A, c.1935 C>A / c.2274insC
	Patient Number	gender	4MU enzyme activity	MS/MS enzyme activity	genotype
Suspected late-onset Pompe	1	female	0.44	0.08	c.424_440del17 / c.1958C>A
	2	female	0.85	0.42	c.671G>A / c.752C>T;c.761 C>T
	3	female	0.49	0.10	c.752C>T, c.761 C>T/ c.1004 G>A
	4	female	0.61	0.01	c.752C>T;c.761 C>T/ c.1075+1G>T
	5	male	1.08	0.46	c.752C>T;c.761 C>T/ c.1726 G>A, c.1935 C>A
	Patient Number	gender	4MU enzyme activity	MS/MS enzyme activity	genotype
Pseudodef. and Carriers	1	female	1.15	1.20	c.1726 G>A/WT
	2	male	0.84	1.34	c.693-5 C>T / c.1726 G>A
	3	female	1.26	1.21	c.752C>T;c.761 C>T/c.1726 G>A
	4	male	0.67	1.09	c.752C>T;c.761 C>T/c.1726 G>A
	5	male	0.78	1.40	c.752C>T;c.761 C>T/c.1726 G>A
	6	male	1.10	1.18	c.1726 G>A/c.1726 G>A
	7	female	1.00	1.00	c.1726 G>A/c.1726 G>A
	8	male	1.06	0.83	c.1726 G>A / c.1726 G>A, c.1935C>A
	9	female	0.98	0.83	c.1726 G>A/c.2815 2816del

**Supplemental Table 2.** Data for patients shown in Figure 2 of the main text.

	Patient Number	Gender	GAA (MS/MS) umole/hr/L	genotype	CK (U/L)
Infantile Pompe Disease	1	Female	0.01	c.424_440del17/ c.1726 G>A, c.1935 C>A	237
	2	Male	0.09	c.872 T>C/c.1935 C>A; *c.1726 G>A, heter	458
	3	Male	0.04	c.1082C>T/ c.1726 G>A, c.1935C>A	809
	4	Female	0.08	c.1194+2T>C/ c.1935C>A; *c.1726 G>A, heter	385
	5	Male	0.13	c.1396 C>T/c.1935C>A	443
	6	Female	0.02	c.1726 G>A, c.1935 C>A / c.1726 G>A, c.1935 C>A	709
	7	Female	0.08	c.1726 G>A, c.1935 C>A / c.1726 G>A, c.1935 C>A	766
	8	Female	0.10	c.1726 G>A, c.1935 C>A / c.1726 G>A, c.1935 C>A	380
	9	Male	0.09	c.1726 G>A, c.1935 C>A / c.2274insC	483
	10	Male	0.34	c.1935 C>A / c.1935C>A	542
	11	Male	0.15	c.1935C>A/c.2303C>T; *c.1726 G>A, heter	662

	Patient Number	Gender	GAA (MS/MS) umole/hr/L	Genotype	CK (U/L)
Suspected Late Onset Pompe Disease	1	Female	0.54	c.424_440del17/ c.1840 A>G, c.2647-23delT	72
	2	Female	0.08	c.424_440del17 / c.1958C>A	109
	3	Female	0.42	c.671G>A / c.752C>T;c.761 C>T	140
	4	Female	0.10	c.752C>T;c.761 C>T / c.752C>T;c.761 C>T	66
	5	Female	0.47	c.752C>T;c.761 C>T / c.752C>T;c.761 C>T	68
	6	Female	0.10	c.752C>T, c.761 C>T/ c.1004 G>A	103
	7	Male	0.22	c.752C>T;c.761 C>T/ c.1054 C>T	89
	8	Female	0.01	c.752C>T;c.761 C>T/ c.1075+1G>T	80
	9	Male	0.44	c.752C>T;c.761 C>T / c.1411_1414 del, c.1726 G>A	140
	10	Male	0.06	c.752C>T;c.761 C>T/ c.2662G>T	670
	11	Male	0.46	c.752C>T;c.761 C>T/ c.1726 G>A, c.1935 C>A	86
	12	Male	0.43	c.1726 G>A, c.1935 C>A / c.1726 G>A, c.2482-5 T>C	29

	Gender	GAA (MS/MS)	Genotype
One Pompe mutation	Male	0.97	c.1726 G>A, c.1935 C>A / WT
	Male	1.34	c.693-5 C>T, c.1726 G>A / WT
One pseudodeficiency mutation	Female	1.07	c.1726 G>A /WT
	Female	1.35	c.1726 G>A /WT
	Female	1.19	c.1726 G>A /WT
	Female	1.20	c.1726 G>A /WT
	Male	1.26	c.1726 G>A /WT
	Male	1.29	c.1726 G>A /WT
	Male	1.56	c.1726 G>A /WT
	Male	1.40	c.1726 G>A /WT
	Male	1.04	c.1726 G>A /WT
	Male	0.69	c.1726 G>A /WT
	One Pompe allele plus one pseudodeficiency allele	Male	0.41
Male		1.53	c.424 440del / c.1726G>A
Male		0.94	c.424 440del / c.1726G>A
Female		1.16	c.460 465del / c.1726 G>A
Female		0.97	c.503 G>C /c.1726 G>A
Female		0.75	c.569 G>A / c.1726 G>A
Male		1.44	c.569 G>A / c.1726 G>A
Male		1.50	c.569 G>A / c.1726 G>A
Female		0.68	c.671 G>A / c.1726 G>A
Male		1.34	c.693-5 C>T / c.1726 G>A
Male		0.98	c.701 C>T / c.1726 G>A
Female		0.68	c.752C>T; c.761 C>T / c.1726 G>A
Female		0.89	c.752C>T; c.761 C>T / c.1726 G>A
Female		0.93	c.752C>T; c.761 C>T / c.1726 G>A
Female		0.97	c.752C>T; c.761 C>T / c.1726 G>A
Female		1.01	c.752C>T; c.761 C>T / c.1726 G>A
Female		1.08	c.752C>T; c.761 C>T / c.1726 G>A
Female		1.13	c.752C>T; c.761 C>T / c.1726 G>A
Female		1.20	c.752C>T; c.761 C>T / c.1726 G>A
Female		1.21	c.752C>T; c.761 C>T / c.1726 G>A
Female		1.28	c.752C>T; c.761 C>T / c.1726 G>A
Female		1.32	c.752C>T; c.761 C>T / c.1726 G>A
Female		1.38	c.752C>T; c.761 C>T / c.1726 G>A
Male		0.85	c.752C>T; c.761 C>T / c.1726 G>A
Male		0.99	c.752C>T; c.761 C>T / c.1726 G>A
Male		1.00	c.752C>T; c.761 C>T / c.1726 G>A

Male	1.05	c.752C>T; c.761 C>T / c.1726 G>A
Male	1.17	c.752C>T; c.761 C>T / c.1726 G>A
Male	1.26	c.752C>T; c.761 C>T / c.1726 G>A
Male	1.28	c.752C>T; c.761 C>T / c.1726 G>A
Male	1.40	c.752C>T; c.761 C>T / c.1726 G>A
Male	1.46	c.752C>T; c.761 C>T / c.1726 G>A
Male	1.56	c.752C>T; c.761 C>T / c.1726 G>A
Male	1.57	c.752C>T; c.761 C>T / c.1726 G>A
Female	1.20	c.752C>T; c.761 C>T / c.1726 G>A
Female	0.56	c.784 G>A /c.1726 G>A
Male	1.56	c.841C>T / c.1726G>A
Female	0.99	c.872 T>C / c.1726 G>A
Male	0.67	c.872 T>C / c.1726 G>A
Male	0.47	c.872 T>C / c.1726 G>A
Female	1.20	c.1082 C>T / c.1726 G>A
Female	1.51	c.1222 A>G / c.1726 G>A
Female	1.46	c.1411 1414 del / c.1726 G>A
Male	1.12	c.1411 1414del / c.1726G>A
Male	0.83	c.1461C>G / c.1726G>A
Female	1.02	c.1508 1510 del / c.1726 G>A
Male	0.82	c.1508 1510 del / c.1726 G>A
Male	0.68	c.1634 C>T / c.1726 G>A
Male	1.03	c.1726 G>A / c.1822 C>T
Male	1.05	c.1726 G>A / c.1843 G>A
Female	1.23	c.1726 G>A / c.1843 G>A
Female	1.13	c.1726 G>A / c.1850 T>G
Female	0.80	c.1726 G>A / c.1935 C>A
Female	0.86	c.1726 G>A / c.1935 C>A
Male	1.14	c.1726 G>A / c.1935 C>A
Male	1.48	c.1726 G>A / c.1935 C>A
Female	1.60	c.1726 G>A / c.1958 C>A
Male	0.88	c.1726 G>A / c.1958 C>A
Male	0.91	c.1726 G>A / c.1958 C>A
Male	0.98	c.1726 G>A / c.1958 C>A

	Female	0.70	c.1726 G>A / c.1935 C>A
	Female	0.63	c.1726 G>A / c.2024 2026del
	Female	0.85	c.1726 G>A / c.2024 2026del
	Male	1.05	c.1726 G>A / c.2024 2026del
	Male	0.72	c.1726 G>A / c.2024 2026del
	Female	0.77	c.1726 G>A / c.2185delC
	Female	0.92	c.1726 G>A / c.2185delC
	Female	0.93	c.1726 G>A / c.2236 T>G
	Female	1.31	c.1726 G>A / c.2236T>G
	Male	0.96	c.1726 G>A / c.2236T>G
	Male	0.47	c.1726 G>A / c.2238 G>T
	Female	0.99	c.1726 G>A / c.2431dupC
	Male	1.31	c.1726 G>A / c.2662 G>T
	Male	1.42	c.1726 G>A / c.2662 G>T
	Female	0.57	c.1726 G>A / c.2662 G>T
	Female	0.83	c.1726 G>A / c.2815 2816del
	Male	0.72	c.1726 G>A / c.2815 2816del
	Female	1.38	c.1726 G>A / c.2815 2816del
One pompe allele plus two pseudodeficiency alleles	Female	1.47	c.546+5 G>T, c.1726 G>A / c.1726 G>A
	Male	0.90	c.546+5 G>T, c.1726 G>A / c.1726 G>A
	Male	1.21	c.546+5 G>T, c.1726 G>A / c.1726 G>A
	Male	0.72	c.752C>T; c.761 C>T, c.1726 G>A / c.1726 G>A
	Female	1.27	c.1222 A>G, c.1726 G>A / c.1726 G>A
	Female	0.50	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Female	0.56	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Female	0.58	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Female	0.69	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Female	0.79	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Female	0.90	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Female	0.91	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Female	0.93	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Female	0.95	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Female	0.97	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Female	0.99	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Female	1.08	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Female	1.09	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Female	1.10	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Female	1.30	c.1726 G>A / c.1726 G>A, c.1935 C>A
Female	1.31	c.1726 G>A / c.1726 G>A, c.1935 C>A	
Female	1.33	c.1726 G>A / c.1726 G>A, c.1935 C>A	
Female	1.34	c.1726 G>A / c.1726 G>A, c.1935 C>A	
Female	1.38	c.1726 G>A / c.1726 G>A, c.1935 C>A	
Female	1.39	c.1726 G>A / c.1726 G>A, c.1935 C>A	
	Female	1.48	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.48	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.53	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.59	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.61	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.63	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.64	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.66	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.69	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.70	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.73	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.74	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.83	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.84	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.85	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.88	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.90	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.92	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.93	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.94	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.99	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.01	c.1726 G>A / c.1726 G>A, c.1935 C>A

	Male	1.03	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.05	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.07	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.08	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.10	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.11	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.13	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.17	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.18	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.19	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.20	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.24	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.25	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.33	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.34	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.38	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.41	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.42	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.43	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.49	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.53	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.58	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.60	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	1.24	c.1726 G>A / c.1726 G>A, c.1935 C>A
	Male	0.61	c.1726 G>A / c.1726 G>A, c.2014 C>T
	Male	0.91	c.1726 G>A / c.1726 G>A, c.2214 G>A
	Male	0.75	c.1726 G>A / c.1726 G>A, c.2238 G>C
	Male	0.86	c.1726 G>A / c.1726 G>A, c.2238 G>C
	Female	0.89	c.1726 G>A / c.1726 G>A, c.2238 G>C
	Male	1.59	c.1726 G>A / c.1726 G>A, c.2238 G>C
	Male	0.60	c.1726 G>A / c.1726 G>A, c.2238 G>C
	Female	1.23	c.1726 G>A / c.1726 G>A, c.2238 G>C
Two pseudodeficiency alleles	Female	0.39	c.1726 G>A / c.1726 G>A
	Female	0.71	c.1726 G>A / c.1726 G>A
	Female	0.75	c.1726 G>A / c.1726 G>A
	Female	0.83	c.1726 G>A / c.1726 G>A
	Female	0.83	c.1726 G>A / c.1726 G>A
	Female	0.84	c.1726 G>A / c.1726 G>A
	Female	0.97	c.1726 G>A / c.1726 G>A
	Female	1.00	c.1726 G>A / c.1726 G>A
	Female	1.02	c.1726 G>A / c.1726 G>A
	Female	1.10	c.1726 G>A / c.1726 G>A



**Supplemental Table 3. Multiple reaction monitoring and electrospray source parameters for MS/MS analysis.**

Name	Parent ion	Daughter ion	Collision energy
GAA-P	498.3	398.3	16
GAA-IS	503.3	403.3	16

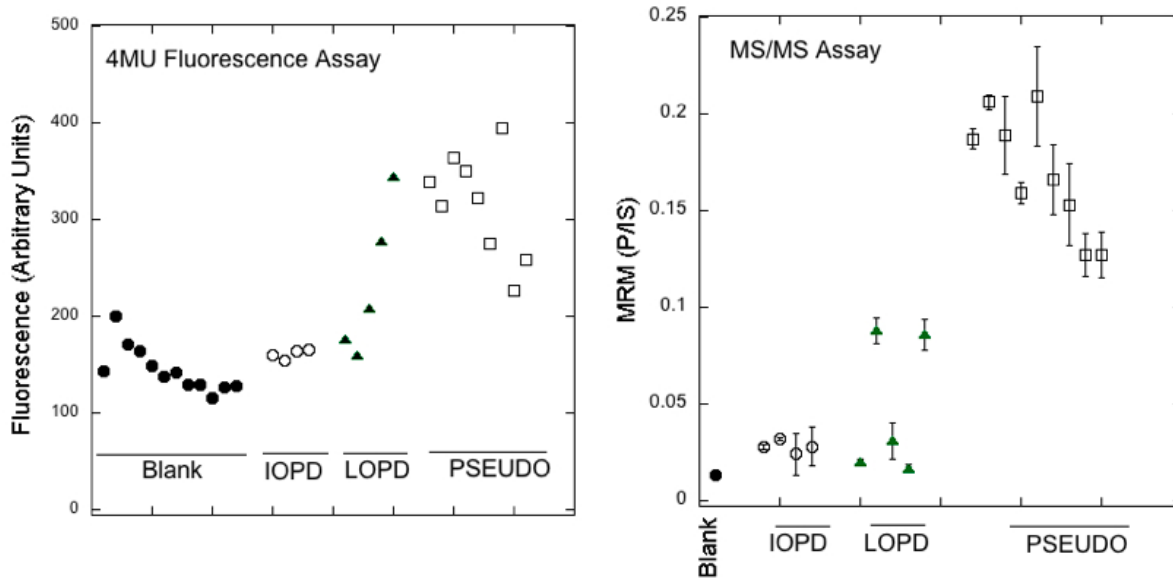
Device	Value
Polarity	ES+
Spray Voltage	3800
Vaporizer Temperature	200 °C
Sheath Gas Pressure	10
Ion Sweep Gas Pressure	0
Aux Gas Pressure	15
Collision Pressure	1.1
Q1 Peak Width	0.7
Q3 Peak Width	0.7
Capillary Temperature	270 °C



**Supplemental Material Table 4.** An identical set of DBS was analyzed by MS/MS using either liquid-liquid extraction with ethyl acetate after assay incubation (LL extraction) or with liquid-liquid extraction followed by solid-phase extraction (SPE extraction) after assay incubation.

	GAA Activity (MS/MS) (umole/L/Hr)	
	LL extraction	SPE extraction
CDC-BP	0.15	0.14
CDC-Low	0.59	0.59
CDC-Med	2.46	5.85
CDC-High	4.36	8.51
IOPD 1	0.35	0.01
IOPD 2	0.37	0.08
IOPD 3	0.25	0.15
Pseudo 1	1.61	1.42
Pseudo 2	1.16	1.08
Pseudo 3	1.18	1.06
Pseudo 4	1.17	0.92
Pseudo 5	0.99	0.64
Pseudo 6	1.78	1.37
Pseudo 7	1.29	1.51
Pseudo 8	0.77	0.65
Pseudo 9	1.87	1.40
Pseudo 10	1.35	1.56
Pseudo 11	1.11	1.29
Pseudo 12	0.79	0.99
Normal 1	9.07	18.48
Normal 2	9.22	18.04
Normal 3	11.04	21.35
Normal 4	3.14	9.85
Normal 5	9.78	21.97
Normal 6	5.10	9.61
Normal 7	6.53	12.81
Normal 8	6.27	6.56
Normal 9	6.02	11.61
Normal 10	12.40	25.99
Normal 11	8.84	15.70
Normal 12	10.80	20.72

**Supplemental Material Figure 1.** Same data as in Figure 1 of the main text, but plotted are the fluorometer plate reader values including 12 repetitions of the blank. Also, the MS/MS data shows the standard deviations of measurements for three separate punches from the same DBS. The fluorescence assay blanks were carried out by incubating DBS extract in 1 well and buffer, substrate, and acarbose in a second well. After overnight incubation, the well contents were combined and quenched immediately prior to reading on the plate reader.



**Supplemental Material Figure 2.** IOPD, LOPD, pseudodeficiency and normal DBS were analyzed by the MS/MS assay with solid-phase extraction workup using either the CDC GAA substrate (CDC SPE) or the PerkinElmer 6plex substrate/internal standard mixture (PE SPE). The upper right plot shows the correlation of the two GAA activity values measured with the two substrates. The bottom right plot shows the separation of GAA activities for the various types of DBS using the PerkinElmer 6plex substrate/internal standard mixture.

