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References

I. Materials.

(Z)-Pugnac ((Z)-O-(2-acetamido-2-deoxy-D-glucopyranosylidene)amino N-phenylcarbamate) was from Santa Cruz Biotechnology (Cat. sc-204415). Ammonium formate (Cat. 156264-2.5KG), ammonium acetate (A1542-250G), barium acetate (243671-100G), and cerium acetate (529559-10G) were from Sigma. Quality control DBS were obtained from the CDC (1) or from Perkin Elmer Life Sciences. Solvents for flow injection- and LC-MS/MS and formic acid are Optima grade from Fisher Scientific. DBS from random newborns were obtained from Prof. Christiane Auray-Blais, University of Sherbrooke, Quebec. The DBS were kept at ambient temperature for 1 week after generation, then stored at -20°C for ~2 years. DBS from MPS-affected patients were obtained with the help of the MPS Society. DBS were generated and received at the Univ. of Washington within 3 days, then stored at -20°C for up to 1 yr. All human samples were obtained and processed in compliance with our IRB Protocol at the Univ. of Washington.

II. Preparation of recombinant human IDUA.

Cell growth. CHO cells overexpressing human IDUA into the culture medium were obtained from Prof. E. Neufeld (UCLA) (2). Growth medium is 1:1 DMEM:F12-Ham (Invitrogen Cat. 11885084 and 11765054) containing 10% heat inactivated fetal bovine serum, 20 mM HEPES, non-essential amino acids (1 mM final, Sigma M7145), 300 µg/mL Geneticin (Invitrogen Cat. 10131-035) Pen-Strep, nucleosides (10 mg/mL each of guanosine, adenosine, uridine, cytidine, hypoxanthine, thymidine (Sigma G6264, A4036, U3003, C4654, H9636, and T1895), pH adjusted to 6.8 and filter sterilize. Production medium is growth medium but with fetal bovine serum reduced to 2.5% and additives (10 mg/L human insulin (Santa Cruz Sc-360248), 6.7 µg/L selenium, added as sodium selenite, Sigma S9133, 2 mg/L ethanolamine). The stock solution of sodium selenite was prepared according to the manufacturer's instructions.

Gelatin coated microcarrier beads were prepared as follows. Beads (1.2 gram at 1 g/L, Sigma M9418) were suspended in PBS without calcium and magnesium and left for 1 hr then autoclaved at 120 °C for 20 min. The beads were allowed to settle by gravity and washed with 100 mL of PBS and then repeated with growth medium. Store the suspension at 4 °C for up to 30 days (keep sterile).

CHO cells were grown to 7-90% confluency in growth medium in an incubator (37 °C, 5% CO₂) in 10 cm Nunc dishes (split with trypsin-EDTA). In a 3L spinner flask 600 mL of pre-warmed growth medium plus 1.2 g of beads was added followed by 1.2-2.4 x 10⁸ cells. This was stirred at 30-40 rpm. About 28 hr later another 600 mL of pre-warmed growth medium was added to bring the final volume to 1.2 L. We continued to culture the cells, counting them every other day. To count, 1 mL of medium with beads was removed and the supernatant removed after the beads settled in the tube. The beads were washed once with pre-warmed PBS, then add 0.45 mL of pre-warmed trypsin-EDTA was added, and the mixture incubated 37 °C in a water bath for 30 min (or until all cells detach from beads). A aliquot of the supernatant was submitted to cell counting with Trypan blue. The cells should reach a density of ~10⁶ in 1 week. After that, the beads were allowed to settle for ~20 min, and 800 ml of medium was removed and replaced with 800 ml of pre-warmed production medium. After removing 800 mL of medium, it was added to 1 M sodium phosphate, pH 5.8 to give 10 mM final. The solution was stored at 4 °C. About 9-12 hours after adding production medium, the beads were allowed to settle, and 800 mL of medium was removed and replaced with 800 mL production medium as above. Phosphate was added to the harvested medium as above. Each batch of production medium was checked for IDUA enzymatic activity as described below, and the above process

was continued until the level of activity started to fall (typically we collected 800 mL of the original growth medium and 4x800 mL of production medium).

Partial purification of IDUA. Each 800 mL portion of medium (see above) was filtered with a Nalgene filter sterilizer unit to remove particulate. All batches of filtered medium were combined and dialyzed (30 kDa MW cutoff tubing) at 4 °C against 10 mM sodium phosphate, 20 mM NaCl, pH 5.8 (7-9 volumes of buffer, 3 times). The dialyzed medium is lyophilized. To the powder was added 75 mL of purified water (Milli-Q, Millipore Corp.) per 400 mL of dialyzed medium. The solution was centrifuged at ~6,000xg at 4 °C for 20 min and the supernatant filtered through a Nalgene sterilizing filter unit. Store remaining lyophilized powder at -80 °C until processed.

The ice-cold solution is loaded at 1.2 mL/min with a peristaltic pump onto a Hi-Trap Heparin Sepharose column (5 mL, Pharmacia 17-0407-01) (the column was on the bench at room temperature but the sample solution and buffers were chilled in ice). The column was pre-equilibrated with buffer A (10 mM sodium phosphate, 100 mM NaCl, pH 5.8). After sample loading, the column was washed with 50 mL of buffer A, then with 25 mL of buffer A with 200 mM NaCl (both at 1.2 mL/min). Elute the IDUA with buffer A with 0.6 M NaCl at 1.0 mL/min. Most of the IDUA elutes, but we typically run an additional 15 mL of this buffer to make sure all IDUA is obtained. Fractions are assayed for IDUA (see below), pooled and concentrated and buffer exchanged by ultrafiltration (Amicon Ultra15, 30 kDa MW cutoff) into 10 mM sodium phosphate, 100 mM NaCl, pH 5.8 (3 rounds of buffer exchange, the column eluant was concentrated 5-fold). This partially purified IDUA was stored in aliquots at -20 °C. The column matrix can be re-used 4-5 times if it is recycled according to the manufacturer's procedure. We typically use up to 10 mL of Heparin Sepharose to process CHO cell medium from 4 L of culture. The yield of partially purified IDUA is 4.5-5.0 million Units (see below) from 800 mL of growth medium plus the 4x800 mL portions of production medium (see above).

IDUA assay. The 4MU substrate has been published (3). An aliquot of stock solution in methanol was transferred to a glass tube, and solvent was completely removed with a stream of oil-free air or using a centrifugal vacuum concentrator. The residue was taken up in assay buffer (50 mM sodium formate, pH 2.8) to give 0.2 mM substrate. To 40 µL of this assay cocktail was added 1-2 µL of CHO cell medium. The mixture was incubated at 37 °C for 1 hr in a capped Eppendorf tube. The reaction was quenched by adding 0.25 mL of glycine-carbonate buffer, pH 10.5 (to 85 mM glycine, adjust to pH 10.5 with sodium carbonate). The fluorescence was read with excitation at 365 nm and emission at 450 nm in a plate reader. The assay was calibrated by adding known amounts of free 4MU (Sigma M1381) to buffer. 1 Unit of IDUA is the amount that generates 1 nmole of product per hr under the above conditions.

III. Preparation of recombinant β-NGA.

Bacterial growth and enzyme purification. A synthetic *NdeI/XhoI* gene fragment for β-NGA (sequence shown below) was prepared by Genscript corporation. The restriction fragment was ligated into the pET28C expression vector. After transfection of into *E. coli* BL21-DE3, cells were grown on LB medium containing 50 µg/mL kanamycin. The plasmid encodes β-NGA but with the peptide segment MVNRKQKTISFQLLVWSMMMILILQPLC deleted from the N-terminus (to improve protein expression).

E. coli was grown at 25 °C with swirling in 1 L of medium plus antibiotic until the OD600 reached ~2.0. Medium (250 mL) was removed and replaced with 250 mL of fresh medium containing 1 mM IPTG, and the culture was continued for 4 hr. Cells were pelleted by centrifugation at 5000-6000xg for 15 min at 4 °C, and the pellet was immediately lysed. To the

pellet was added 50 mL of ice-cold lysis buffer A (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.5) containing one EDTA-free protease inhibitor table (Pierce 88266), 1 mM PMSF and 5 mM β -mercaptoethanol. The pellet was stirred in lysis buffer for 10 min on ice, and then sonicated with a probe sonicator (10 times using a cycle of 20 pulses on then 10 sec off). The sample was centrifuged at 11,000xg for 20 min at 4 °C. The supernatant was loaded onto 4-5 ml of packed Ni-NTA gel (Qiagen) that was pre-equilibrated in lysis buffer in a 2.6 cm diameter glass column. The column was capped and mixed end-over-end on a wheel at 4 °C overnight. The column was mounted vertically to allow the resin to settle. The outlet of the column was opened to allow the excess buffer to elute. The resin was washed with 50-60 mL of buffer A plus 1 mM PMSF and 5 mM β -mercaptoethanol (both freshly added). The column was then washed with 30 mL of buffer A plus 50 mM imidazole plus 5 mM β -mercaptoethanol (freshly added). The enzyme was eluted with buffer A plus 250 mM imidazole plus 5 mM β -mercaptoethanol (freshly added). Fractions were analyzed by SDS-PAGE, and those containing β -NGA were pooled (the Ni-NTA resin can be regenerated according to the manufacturer's instructions). The enzyme pool was dialyzed against 25 mM HEPES, 150 mM NaCl, 1 mM DTT, pH 7.5 at 4 °C. The dialyzed enzyme was stored in aliquots at -20 °C. The yield was ~13 mg per L of culture. The purity is >90% as judged by SDS-PAGE.

Enzyme assay. The activity of β -NGA was measured using 0.2 mM of the MPS-IVA internal standard compound (4) in 50 mM sodium acetate, pH 6.0 for 1 hr at 37 °C. The mixture was quenched with 250 mL of glycine-carbonate, pH 10.5 buffer (see above), and fluorescence was measured as above. The assay was calibrated with free 4MU. One Unit of enzyme is the amount that generates 1 nmole of product per hr.

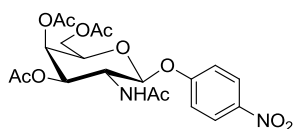
NdeI/XhoI gene fragment coding for β -NGA:

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IV. Synthesis of MS/MS substrates, products and internal standards for GALNS and ARBS assays.

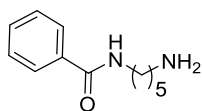
Synthesis of MS/MS substrates, products and internal standards for GALNS and ARBS assays.



(2R,3R,4R,5R,6S)-5-acetamido-2-(acetoxymethyl)-6-(4-nitrophenoxy)tetrahydro-2H-pyran-3,4-diyl diacetate (1) Pyridine (60 mL) was added to nitrogen back flushed flask containing D-galactosamine hydrochloride (5 g, 23.2 mmol) and the resultant slurry was cooled on an ice bath. To the cooled mixture acetic anhydride (25 g,

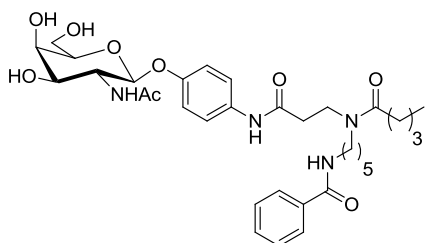
245 mmol) was added dropwise and allowed to warm to room temperature followed by stirring at this temperature for 16 hours. The reaction mixture was quenched with the addition of methanol (15 mL) and let to stir for 20 minutes. The resultant mixture was concentrated under reduced pressure and the residue was dissolved in 20% methanol in chloroform with the aid of warming the mixture. This solution was washed with 1N HCl solution followed by brine solution. The resultant organic layer was dried using anhydrous sodium sulfate and concentrated under reduced pressure. The residue was taken in nitrogen back flushed flask equipped with a dropping funnel. Anhydrous dichloromethane (100 mL) was added to this residue and the resultant slurry was cooled on an ice bath. In the dropping funnel titanium(IV) chloride (6.5 g, 42.1 mmol) was dissolved in anhydrous dichloromethane (40 mL) and the resulting solution was added dropwise to the cooled solution. The reaction mixture was warmed to 50°C in an oil bath and left to stir at this temperature for 48 hours. The reaction mixture was cooled back on an ice bath

and saturated sodium bicarbonate solution was added dropwise with vigorous shaking. The resultant mixture was extracted between chloroform and saturated sodium bicarbonate solution. The organic layer was dried using anhydrous sodium sulfate and concentrated under reduced pressure. The resultant residue was dissolved in acetone (60 mL) and added slowly to a cooled mixture of 4-nitrophenol (16.1 g, 116 mmol) in acetone (130 mL) and 4N KOH aqueous solution (23.2 mL) at 0°C. The reaction was left to stir at room temperature for 48 hours and concentrated under reduced pressure to dryness. The residue was redissolved in 10% methanol in chloroform by the aid of warming the mixture. This solution was extracted between 1N NaOH and chloroform and the chloroform layer was further washed with brine solution. The organic layer was dried using anhydrous sodium sulfate and concentrated under reduced pressure. The crude product thus obtained was purified by silica flash chromatography using 3% methanol in dichloromethane as the elution mixture. The fractions with the desired compound, as determined by TLC, were combined and concentrated under reduced pressure to get **1** (3.29 g, 30%). ¹H NMR (300 MHz, CDCl₃) δ 8.20 (d, *J* = 9.1 Hz, 2H), 7.09 (d, *J* = 9.1 Hz, 2H), 5.61 (d, *J* = 8.0 Hz, 1H), 5.56 – 5.39 (m, 3H), 4.32 – 4.07 (m, 4H), 2.18 (s, 3H), 2.07 (s, 3H), 2.04 (s, 3H), 1.97 (s, 3H). MS (ESI⁺) for [M + Na]⁺; calculated: 491.1, found: 491.2.



***N*-(5-aminopentyl)benzamide (2).** To methyl benzoate (1.0 g, 7.34 mmol), pentane-1, 5-diamine (0.75 g, 7.34 mmol) and water (0.37 mL) were added, and the mixture was heated to 100°C for 24 hours under constant stirring. The reaction mixture was cooled to room temperature and directly loaded on to a short silica column. Upon elution with 10 to 20% of methanol (with 5% aq. NH₄OH solution)

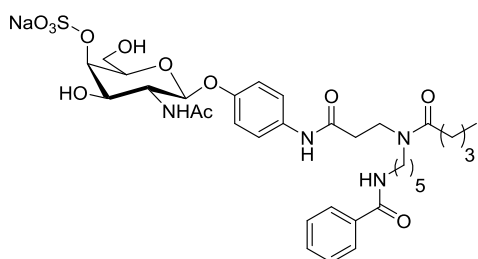
in chloroform the desired mono-benzoylated product **2** was obtained (0.80 g, 53%) as a pale yellow oil. ¹H NMR (300 MHz, MeOD) δ 7.83 (d, *J* = 7.4 Hz, 2H), 7.58 – 7.31 (m, 4H), 3.41 (t, *J* = 7.0 Hz, 2H), 2.76 (t, *J* = 7.2 Hz, 2H), 1.74 – 1.21 (m, 6H). MS *m/z* 207.2 (M+H)⁺.



***N*-(5-(*N*-(3-((4-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)phenyl)amino)-3-oxopropyl)pentanamido)pentyl)benzamide (GalNAc-C5/C5-Bz, ARSB Product)** To a solution of **1** (3.5 g, 7.47 mmol) in anhydrous methanol (90 mL), cooled on an ice bath, 0.5 M sodium methoxide solution in methanol (3 mL, 1.50 mmol) was added dropwise and allowed to warm to

room temperature. After 2 hours formic acid (0.1 mL) was added to the reaction mixture and concentrated to dryness under reduced pressure. To the resulting residue methanol (135 mL), water (15 mL) and 10% palladium on activated carbon (125 mg) was added and let to stir under a hydrogen atmosphere at room temperature for 16 hours. Water was added dropwise to the reaction mixture till the entire white residue was completely dissolved. The reaction mixture was filtered and the filtrate was cooled on an ice bath. To it pyridine (2 mL) was added and followed by the dropwise addition of a solution of acryloyl chloride (2.1 g, 23.2 mmol) in dichloromethane (50 mL). The reaction was let to stir on the ice bath for 30 minutes and then warmed to room temperature and continued for 2 hours. Sodium carbonate powder (3.0 g) was added to the reaction mix and let to stir for 15 minutes and filtered. The filtrate was concentrated under reduced pressure and further dried under high vacuum. The residue was dissolved in 2-propanol (50 mL) and water (6.6 mL) mixture and to it *N*-(5-aminopentyl)benzamide **2** (2.0 g, 9.69 mmol) was added and let to stir for 40 hours at 65°C. The reaction mixture was concentrated to dryness under reduced pressure and

redissolved in methanol (70 mL). Upon cooling this mixture on an ice bath, triethylamine (2.5 mL) was added followed by the dropwise addition of a solution of pentanoyl chloride (2.7 g, 22.4 mmol) in dichloromethane (50 mL). The reaction was left to stir on the ice bath for 30 minutes and then warmed to room temperature and continued for 16 hours. The reaction mixture was concentrated under reduced pressure and subjected to purification by silica flash chromatography using 15% methanol in dichloromethane as the elution mixture to yield **GalNAc-C5/C5** (2.96 g, 60%). ¹H NMR (300 MHz, MeOD) δ 7.84 (d, $J = 7.1$ Hz, 2H), 7.59 - 7.35 (m, 5H), 7.09 - 6.91 (m, 2H), 5.00 (d, $J = 8.4$ Hz, 1H), 4.28 - 4.09 (m, 1H), 3.97 - 3.55 (m, 7H), 3.46 - 3.24 (m, 4H), 2.72 - 2.51 (m, 2H), 2.49 - 2.28 (d, $J = 7.3$ Hz, 2H), 2.02 (s, 3H), 1.78 - 1.49 (m, 6H), 1.49 - 1.22 (m, 4H), 0.94 (t, $J = 7.1$ Hz, 3H). MS (ESI⁺) for [M + H]⁺; calculated: 657.3, found: 657.5.

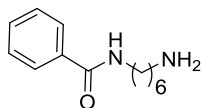


sodium (2R,3R,4R,5R,6S)-5-acetamido-6-(4-(3-(N-(5-benzamidopentyl)pentanamido)propanamido)phenoxy)-4-hydroxy-2-(hydroxymethyl)tetrahydro-2H-pyran-3-yl sulfate (GalNAc-4-S-C5/C5-Bz, ARSB Substrate).

To a cooled (0°C) solution of **ARSB Product** (25 mg, 38.1 μ mol) in anhydrous pyridine (0.5 mL), benzoyl chloride (4.9 μ L, 41.9 μ mol) was added. After 1 hour at room temperature the solution was

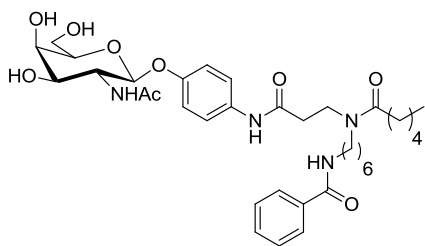
cooled back to 0°C and another portion of benzoyl chloride (4.9 μ L, 41.9 μ mol) was added and left to stir for 2 hours at room temperature. Upon checking the TLC and mass spec analysis of the reaction mixture, more small portions of benzoyl chloride can be added until most of the starting material was converted to the dibenzoylated product. The reaction was extracted between 1 M HCl solution and chloroform. The chloroform layer was further washed with a mixture of water and brine solution (1:1). The organic layer was concentrated and purified by flash silica column chromatography using 5% methanol in DCM as the eluent. The desired fractions were concentrated under reduced pressure and further under high vacuum. The resultant residue was dissolved in anhydrous pyridine and sulfur trioxide pyridine complex (8.3 mg, 52.1 μ mol) was added to it at room temperature. The resulting mixture was heated to 45°C for 16 hours followed by the addition of methanol (0.5 mL) and stirred for further 10 mins. The reaction mixture was concentrated under reduced pressure and further under high vacuum. The resulting residue was re-dissolved in anhydrous methanol (5.0 mL) and cooled to 0°C. To this cooled solution 0.5 M solution of sodium methoxide in methanol (0.5 mL) was added dropwise and let stir for 16 hours. The reaction was quenched by the addition of 1 M aqueous solution of sodium phosphate monobasic (1.0 mL) and subjected to semi-preparative reverse phase HPLC purification (gradient water/methanol system) to yield **GalNAc-4-S-C5/C5-Bz** (5.8 mg, 20%). The **GalNAc-4-S-C5/C5-Bz** was further purified by ion-exchange chromatography. A fritted thin column was loaded with 2 mL of Q-Sepharose fast flow (GE Healthcare, product code: 17-0510-01) and washed with methanol (50 mL). A solution of the **GalNAc-4-S-C5/C5-Bz** in methanol (2 mL) was loaded on to this column and the column was further washed with methanol (100 mL). The **GalNAc-4-S-C5/C5-Bz** was then eluted with 1M ammonium formate in methanol solution (100 mL) and concentrated under reduced pressure at temperature less than 25°C. The resultant residue was dissolved in DI-water and loaded on a C18 cartridge (Resprep cat # 26034, which was pre-activated with methanol and washed with DI-water) by applying negative pressure in the bottom. The cartridge was further washed with DI water (4x25 mL) and the substrate was eluted from the

cartridge using methanol (4x25 mL). The methanol fraction was concentrated under reduced pressure at temperature less than 25°C to afford the pure **GalNAc-4-S-C5/C5-Bz**. ¹H NMR (300 MHz, MeOD) δ 7.80 (dd, *J* = 7.0, 1.2 Hz, 2H), 7.58 – 7.38 (m, 5H), 7.03 – 6.94 (m, 2H), 5.01 (dd, *J* = 8.4, 1.1 Hz, 1H), 4.75 (d, *J* = 3.1 Hz, 1H), 4.13 (dd, *J* = 10.9, 8.4 Hz, 1H), 3.95 – 3.61 (m, 6H), 3.45 – 3.34 (m, 4H), 2.61 (dd, *J* = 16.1, 7.0 Hz, 2H), 2.47 – 2.31 (m, 2H), 1.97 (s, 3H), 1.73 – 1.49 (m, 6H), 1.43 – 1.23 (m, 5H), 0.91 (td, *J* = 7.3, 2.2 Hz, 3H). MS *m/z* 735.4 [M – Na⁺].



N-(6-aminohexyl)benzamide (3). To methyl benzoate (25.0 g, 183.6 mmol), hexane-1,6-diamine (21.3 g, 183.6 mmol) and water (9.25 mL) were added and the mixture was heated to 100°C for 24 hours under constant stirring. The reaction mixture was cooled to room temperature and directly loaded on to a short silica

column. Upon elution with 10 to 20% of methanol (with 5% aq. NH₄OH solution) in chloroform the desired mono-benzoylated product **3** was obtained (19.8 g, 49%) as a pale yellow oil. ¹H NMR (300 MHz, MeOD) δ 7.81 – 7.78 (m, 2H), 7.52 – 7.40 (m, 3H), 3.38 (t, *J* = 7.1 Hz, 2H), 2.78 – 2.69 (m, 2H), 1.64 – 1.38 (m, 8H). MS *m/z* 221.1 (M+H⁺).

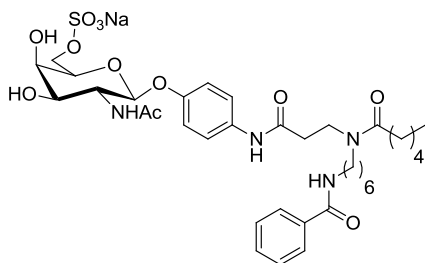


N-(6-(N-(3-((4-(((2S,3R,4R,5R,6R)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)oxy)phenyl)amino)-3-

oxopropyl)hexanamido)hexyl)benzamide (GalNAc-C6/C6-Bz, GALNS Product) To a solution of **1** (0.27 g, 0.576 mmol) in anhydrous methanol (9 mL), cooled on an ice bath, 0.5 M sodium methoxide solution in methanol (0.3 mL, 0.15 mmol) was added dropwise and allowed to warm to room temperature. After 2 hours formic acid (10

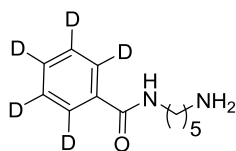
μL) was added to the reaction mixture and concentrated to dryness under reduced pressure. To the resulting residue methanol (13.5 mL), water (1.5 mL) and 10% palladium on activated carbon (12.5 mg) were added and let to stir under a hydrogen atmosphere at room temperature for 16 hours. Water was added dropwise to the reaction mixture till the entire white residue was completely dissolved. The reaction mixture was filtered and the filtrate was cooled on an ice bath. To it pyridine (0.16 mL) was added and followed by the dropwise addition of a solution of acryloyl chloride (0.16 g, 1.76 mmol) in dichloromethane (4 mL). The reaction was let to stir on the ice bath for 30 minutes and then warmed to room temperature and continued for 2 hours. Sodium carbonate powder (0.3 g) was added to the reaction mix and let to stir for 15 minutes and filtered. The filtrate was concentrated under reduced pressure and further dried under high vacuum. The residue was dissolved in 2-propanol (6.3 mL) and water (0.7 mL) mixture and to it *N*-(6-aminohexyl)benzamide **3** (0.17 g, 0.77 mmol) was added and let to stir for 40 hours at 65°C. The reaction mixture was concentrated to dryness under reduced pressure and redissolved in methanol (12 mL). Upon cooling this mixture on an ice bath triethylamine (0.25 mL) was added followed by the dropwise addition of a solution of hexanoyl chloride (0.24 g, 1.78 mmol) in dichloromethane (4 mL). The reaction was left to stir on the ice bath for 30 minutes and then warmed to room temperature and continued for 16 hours. The reaction mixture was concentrated under reduced pressure and subjected to purification by silica flash chromatography using 15% methanol in dichloromethane as the elution mixture to yield **GalNAc-C6/C6-Bz** (0.23 g, 58%). ¹H NMR (300 MHz, MeOD) δ 7.80 (d, *J* = 6.9 Hz, 2H), 7.56 – 7.39 (m, 5H), 6.99 (d, *J* = 9.1 Hz, 2H), 4.96 (d, *J* = 8.4 Hz, 1H), 4.17 (dd, *J* = 10.7, 8.4 Hz, 1H), 3.96 – 3.51 (m, 7H), 3.44 – 3.33 (m, 4H), 2.69 – 2.50 (m, 2H), 2.48 – 2.28 (m, 2H), 1.98 (s, 3H),

1.72 – 1.49 (m, 6H), 1.49 – 1.21 (m, 9H), 0.89 (dt, $J = 8.7, 4.8$ Hz, 3H). MS (ESI⁺) for [M + H]⁺; calculated: 685.4, found: 685.5.



Sodium ((2R,3R,4R,5R,6S)-5-acetamido-6-(4-(3-(N-(6-benzamidohexyl)hexanamido)propanamido)phenoxy)-3,4-dihydroxytetrahydro-2H-pyran-2-yl)methyl sulfate (GalNAc-6-S-C6/C6-Bz, GALNS Substrate) To GALNS **Product** (99 mg, 0.144 mmol) under nitrogen, anhydrous pyridine (5 mL) was added. To this solution sulfur trioxide pyridine complex (34 mg, 0.214 mmol) was added and let to stir for 5 hours at room temperature. The reaction was quenched with the addition of methanol (0.5 mL) and

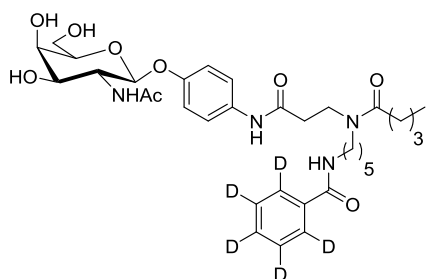
stirring for 30 minutes. The reaction mixture was concentrated under reduced pressure and redissolved in water and subjected to reversed phase (C18) HPLC purification using water-methanol gradient system to get **GalNAc-6-S-C6/C6-Bz, GALNS Substrate** (36 mg, 32%). The **GalNAc-6-S-C6/C6-Bz** was further purified by ion-exchange chromatography. A fritted thin column was loaded with 2 mL of Q-Sepharose fast flow (GE Healthcare, product code: 17-0510-01) and washed with methanol (50 mL). A solution of **GalNAc-6-S-C6/C6-Bz** in methanol (2 mL) was loaded on to this column and the column was further washed with methanol (100 mL). The **GalNAc-6-S-C6/C6-Bz** was then eluted with 1M ammonium formate in methanol solution (100 mL) and concentrated under reduced pressure at temperature less than 25°C. The resultant residue was dissolved in DI-water and loaded on a C18 cartridge (Resprep cat # 26034, which was pre-activated with methanol and washed with DI-water) by applying negative pressure in the bottom. The cartridge was further washed with DI water (4x25 mL) and the substrate was eluted from the cartridge using methanol (4x25 mL). The methanol fraction was concentrated under reduced pressure at temperature less than 25°C to afford the pure **GalNAc-6-S-C6/C6-Bz**. ¹H NMR (300 MHz, MeOD) δ 7.81 (d, $J = 6.9$ Hz, 2H), 7.59 – 7.35 (m, 5H), 7.00 (d, $J = 9.0$ Hz, 2H), 4.93 (d, $J = 8.4$ Hz, 1H), 4.32 – 4.10 (m, 3H), 4.03 – 3.90 (m, 2H), 3.83 – 3.60 (m, 3H), 3.44 – 3.33 (m, 4H), 2.61 (q, $J = 7.0$ Hz, 2H), 2.49 – 2.29 (m, 2H), 1.98 (s, 3H), 1.72 – 1.49 (m, 6H), 1.48 – 1.22 (m, 9H), 0.98 – 0.81 (m, 3H). MS (ESI) for [M – Na⁺]⁺; calculated: 763.3, found: 763.7.



N-(5-aminopentyl)benzamide-2,3,4,5,6-d₅ (6) To a solution of *t*-butyl (5-aminopentyl)carbamate (0.15 g, 0.741 mmol) and triethylamine (0.3 mL) in dichloromethane (10 mL), cooled on an ice bath, benzoyl chloride-d₅ (119 mg, 0.817 mmol) was added dropwise and warmed to room temperature. This mixture was let to stir for 4 hours and quenched with addition of methanol (1 mL) and concentrated to dryness under reduced pressure. The residue was

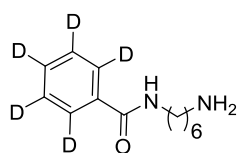
resuspended in dichloromethane (2 mL) and 4M HCl in dioxane (0.75 mL, 3 mmol) was added to it dropwise and let to stir at room temperature for 16 hours. The reaction mixture was concentrated to dryness under reduced pressure and redissolved in methanol (10 mL) and to it sodium bicarbonate powder (0.3 g) was added and let to stir for 15 minutes. The resultant slurry was filtered and the filtrate was

concentrated to dryness under reduced pressure to yield **6** and used for next step without further purification.



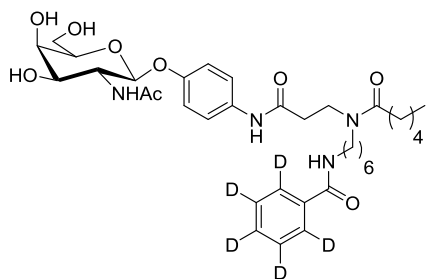
***N*-(5-(*N*-(3-((4-(((2*S*,3*R*,4*R*,5*R*,6*R*)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)phenyl)amino)-3-oxopropyl)pentanamido)pentyl)benzamide-2,3,4,5,6-*d*₅ (ARSB Internal Standard)** By extending the synthetic procedure outlined for the synthesis of **ARSB Product** and substituting the use of *N*-(5-aminopentyl)benzamide **2** with the *d*₅-deuterated amine **6** the desired **ARSB Internal Standard** (142 mg, 51%) was obtained as a white amorphous solid. ¹H NMR (300 MHz, MeOD) δ 7.43 (dd, *J*

= 9.0, 2.1 Hz, 2H), 6.99 (dd, *J* = 9.1, 2.4 Hz, 2H), 4.96 (d, *J* = 8.4, 1H), 4.17 (dd, *J* = 10.7, 8.4 Hz, 1H), 3.89 (d, *J* = 3.1 Hz, 1H), 3.85 – 3.59 (m, 6H), 3.44 – 3.33 (m, 4H), 2.61 (dd, *J* = 15.4, 7.0 Hz, 2H), 2.47 – 2.29 (m, 2H), 1.98 (s, 3H), 1.73 – 1.49 (m, 6H), 1.46 – 1.23 (m, 5H), 0.90 (td, *J* = 7.3, 1.8 Hz, 3H). MS (ESI⁺) for [M + H]⁺; calculated: 662.4, found: 662.7.



***N*-(6-aminoethyl)benzamide-2,3,4,5,6-*d*₅ (**7**)**. To a solution of Boc-1,6-diaminohexane·HCl salt (0.2 g, 0.791 mmol) and triethylamine (0.4 mL) in methanol (5 mL), cooled on an ice bath, benzoyl chloride-*d*₅ (345 mg, 2.36 mmol) solution in anhydrous dichloromethane (1 mL) was added dropwise and warmed to room temperature. This mixture was let to stir for 4 hours and concentrated to dryness under reduced pressure. The residue was redissolved

in 10% methanol in DCM and the resulting solution was washed with 1M aqueous NaOH solution and followed by brine-water (1:1) mixture. The organic layer thus obtained was concentrated to dryness under reduced pressure. The resultant residue was resuspended in dichloromethane (2 mL) and 4M HCl in dioxane (0.75 mL, 3 mmol) was added to it dropwise and let to stir at room temperature for 16 hours. The reaction mixture was concentrated to dryness under reduced pressure and redissolved in methanol (10 mL) and to it sodium bicarbonate powder (0.3 g) was added and let to stir for 15 minutes. The resultant slurry was filtered and the filtrate was concentrated to dryness under reduced pressure to yield **7** and used for next step without further purification.



***N*-(6-(*N*-(3-((4-(((2*S*,3*R*,4*R*,5*R*,6*R*)-3-acetamido-4,5-dihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl)oxy)phenyl)amino)-3-oxopropyl)hexanamido)hexyl)benzamide-2,3,4,5,6-*d*₅ (GALNS Internal Standard)** By extending the synthetic procedure outlined for the synthesis of **GALNS Product** and substituting the use of *N*-(6-aminoethyl)benzamide **3** with the *d*₅-deuterated amine **7** the desired **GALNS Internal Standard** (39.5 mg, 52%) was obtained as a

white amorphous solid. ^1H NMR (300 MHz, MeOD) δ 7.43 (d, $J = 9.0$, 2H), 6.99 (dd, $J = 9.1$, 2.4 Hz, 2H), 4.96 (d, $J = 8.4$ Hz, 1H), 4.24 – 4.12 (m, 1H), 3.89 (d, $J = 3.1$ Hz, 1H), 3.85 – 3.60 (m, 6H), 3.42 – 3.33 (m, 4H), 2.61 (q, $J = 6.8$ Hz, 2H), 2.47 – 2.29 (m, 2H), 1.98 (s, 3H), 1.70 – 1.49 (m, 6H), 1.49 – 1.21 (m, 9H), 0.89 (td, $J = 6.7$, 4.0 Hz, 3H). MS (ESI⁺) for [M + H]⁺; calculated: 690.4, found: 690.5.

V. Quantification of compounds by ^1H -NMR.

We used NMR to accurately determine the amount of reagent in stock solutions so that the comparison between MS/MS and fluorimetric assays could be done starting from assay cocktails with known concentrations of substrates. This also allowed us to determine relative ionization efficiencies (MS/MS ion counts in the appropriate single reaction monitoring channel) when the same number of moles of 2 different analytes were analyzed by LC-MS/MS. The reagent was dissolved in 0.75 mL of CD₃OD and 6-9 μL of 1 or 10% DMF in CD₃OD was added as an internal standard. ^1H -NMR was acquired with an extended time between pulses of 15 sec to obtain accurate peak integrals. The integrals of the NMR peaks for the analyte and internal standard were used to determine the absolute moles of analyte. After quantitative NMR, the samples were concentrated to dryness using a vacuum centrifuge and re-dissolved in methanol to give 5 mM stock solutions. The samples were serially diluted in 10-fold increments to give 500 nM solutions, which were submitted to flow injection MS/MS to measure the single reaction monitoring ion signals for the analytes in triplicate. Single reaction monitoring parameters (cone voltage and collision energy) were optimized for each analyte.

V. MS/MS assays.

Flow-injection analysis (FIA)-MS/MS assays of GALNS and ARSB with β -NGA and ethyl acetate extraction.

Substrates and internal standards were prepared as stock solutions in methanol and stored in glass vials with Teflon-lined screw caps at -20 °C. After transferring stock solution to a glass tube, methanol was completely removed in a vacuum centrifuge (important to remove all solvent) prior to adding assay buffer. The assay buffer is 50 mM ammonium acetate, 7.5 mM Ba(OAc)₂ and 5.0 mM Ce(OAc)₃, pH 5.0 (adjusted with acetic acid after metal salts added, stored 4 °C). The final cocktail, made fresh, contained 2 mM (Z)-Pugnac (added from a stock in water), 8 Units/ μL β -NGA, 1 mM substrate, and 5 μM internal standard. Assays were carried out with 3 mm DBS punches in 30 μL of assay cocktail in polypropylene 96-well plates (Sigma Cat. CLS3363) sealed with a storage mat (Sigma Cat. CLS3080) and shaken at 250 rpm at 37 °C for 16 hr. Assays were quenched by adding 0.3 mL of 1 g/L sodium taurocholate (to prevent emulsions), followed by 0.4 mL of reagent grade ethyl acetate. After mixing up and down ~5 times with the pipettor the plate was centrifuged (Allegra X-12R, Beckmann) for 20 min at ~2100g to cause complete separation of the solvent layers. A 0.2 mL aliquot of the upper phase was transferred to a new 96-well plate, and solvent was completely removed at room temperature with a stream of nitrogen. Residues were taken up in 0.1 mL of 1:1 water:acetonitrile for FIA-MS/MS. The FIA solvent is 80% acetonitrile/20% water/5 mM ammonium formate.

LC-MS/MS assays of GALNS and ARSB.

Samples for LC-MS/MS were setup as for FIA assays except β -NGA was omitted. Incubated assays were quenched by adding 120 μL of acetonitrile. The plate was sealed as

above and centrifuged at ~3,000 rpm for 5 min. Purified water (120 μ L) was added to wells of a new 96-well plate, and 120 μ L aliquots of the supernatant from the centrifuged plate was transferred to the wells containing water. The LC column is a Chromolith FastGradient RP-18 encapped 50-2 HPLC column (EMD Millipore Cat. 152007) fitted with a Uniguard Cartridge Holder (Thermo Scientific Cat. 850-00) and a Hypersil Drop-In Guard Cartridge C18 (3 μ m, 10L x 2.1 mm I.D., Fisher Scientific 30103-012101). The LC flow rate was 0.4 mL/min. Ten μ L of sample were injected per run. The isocratic LC solvent is 45% water/25% methanol/30% acetonitrile/0.1% formic acid, prepared from Optima grade solvents (Fisher Scientific).

MPS-II Assays

The assay cocktail (made fresh) contained 1 mM substrate, 0.8 Units/ μ L IDUA, 5 μ M internal standard in the same buffer as above. The assay was setup, processed and analyzed as described above for FIA-MS/MS. LC-MS/MS assays were carried out as above in the presence and absence of IDUA.

Triplex Assays

The assay cocktail (made fresh) contained 0.5 mM I2S substrate, 1 mM ARSB and GALNS substrates, 5 μ M of each of the three internal standards, and other buffer components as above for the LC-MS/MS assay of ARSB and GALNS. After incubation with the DBS punch as above, the mixture was quenched with 0.1 mL of methanol/ethyl acetate (1/1). After mixing up and down 4-5 times with the Pipettor, the liquid was transferred to a new deep-well, 96-well plate followed by addition of 0.4 mL of ethyl acetate then 0.2 mL of 44 mM citric acid in water. After mixing up and down 5-10 times with the Pipettor, the plate was sealed as above and centrifuged for 5 min at 3,000 rpm at room temperature. A 0.2 mL portion of the top layer was transferred to a new plate, and solvent was removed with a jet of oil-free nitrogen. Residues were taken up in 0.1 mL of acetonitrile/water (1/1) with 0.02% formic acid. After mixing 5-10 times with the Pipettor, samples were subjected to LC-MS/MS as above.

VI. 4MU fluorimetric assays.

α -L-Iduronidase, acid α -glucosidase, I2S, GALNS, ARSB

α -L-Iduronidase assay buffer is 100 mM sodium formate, 75 mM D-saccharic acid-1,4-lactone (Santa Cruz Biotechnology Cat. ss-221521), pH 3.4. Acid α -glucosidase buffer is 89 mM succinic acid, 8 μ M acarbose (Carbosyn Cat. OA00002), pH 4.7. I2S buffer is as for MS/MS buffer (maint text) except without (Z)-Pugnac. Assay cocktails were prepared fresh from methanol stock solutions of substrates. Methanol was completely removed by vacuum centrifuge or a stream of N₂ before buffer addition. The substrates are: 1) 1 mM 4MU- α -L-iduronide (Carbosynth Cat. EM58440); 2) 1 mM 4MU- α -D-glucopyranoside (Toronto Research Chemicals Cat. M334495); 3) 1 mM of the 4MU-containing I2S substrate published previously (5).

A 3 mm DBS punch was incubated with 30 μ L of assay cocktail in sealed 96-well plates (see main text). Reactions were quenched with 0.25 mL of 0.1 M sodium bicarbonate, pH 10.7, the plates were centrifuged for 5 min at 3,000 rpm, 0.2 mL of supernatant was transferred to a black, 96-well plate, and the fluorescence was measured (355 nm excitation and 460 nm emission). If bubbles were seen in the plate wells, the black plate was centrifuged as above to remove bubbles.

Controls were carried out by adding 15 μ L of buffer without substrate to a well containing a 3 mm DBS punch. A second well contained 30 μ L of complete assay cocktail (buffer +

substrate) but substrate was 2 mM and the well lacked a DBS punch. Samples were incubated as above, then 15 μ L of the 30 μ L well was transferred to the well with the DBS punch. Samples were immediately quenched and processed as above. All assays were calibrated using standard 4MU replacing 4MU-substrate and prepared and processed as for the control reactions. In this way, the calibration, contains blood, which significantly quenches the 4MU fluorescences, and this is representative of the quenching of 4MU in the enzymatic assays.

GALNS and ARSB fluorimetric assays with 4MU-GalNAc-6-S and 4MU-GalNAc-4-S were carried out as described (Kumar, A. H., Spacil, Z., Ghomashchi, F., Masi, S., Sumida, T., Ito, M., Turecek, F., Scott, C. R., and Gelb, M. H. Fluorimetric Assays for N-Acetylgalactosamine-6-Sulfatase and Arylsulfatase B Based on the Natural Substrates for Confirmation of Mucopolysaccharidoses Types IVA and VI, submitted to Clin. Chim. Acta). ARSB assays with 4MU-sulfate were prepared as follows. Assay buffer (15 μ L, same as I2S buffer above) containing 5 mM 4MU-sulfate was added to a 3 mm DBS punch in a well of a 96-well, polypropylene plate. After 16 hr incubation at 37 ° with shaking (250 rpm), a 15 μ L portion of assay buffer (no substrate) was added, and the mixture was quenched with 200 μ L of 0.1 M sodium bicarbonate, pH 10.7. 4MU fluorescence was read as above.

VIII. Conversion of assay response to enzyme activities.

Enzyme activities in units of μ mol product per hour per L of blood were calculated using the amount of product formed in the complete assay with the DBS punch minus that formed in the control assay divided by the incubation time in hours and divided by the volume of blood in the DBS punch (taken as 3.1×10^{-6} L).

IX. Calculation of analytical range values in main text Table 1.

For MS/MS assays, possible contributors to the enzyme-independent assay response are: 1) buffer alone; 2) product present as an impurity in the substrate; 3) breakdown of substrate in buffer without blood to give the product; 4) cleavage of the substrate in the electrospray ionization source to give product; 5) components of the blood other than product that give rise to the same MS/MS response in the product channel. Contributor 5 is taken as zero since we do not see any detectable signal in the product MS/MS channel if we leave substrate out of the assay with DBS. Contributors 1-4 will occur in a control in which substrate is incubated in buffer without DBS. Thus the analytical range is calculated as [(assay response in the complete assay) - (assay response in the no DBS control)] divided by (assay response in the no blood control). Note that the no blood control lacks compounds in the blood that suppress the product MS/MS signal, whereas such suppressors are present in the complete assay with DBS. Since the internal standard is chemically identical to the product but contains deuterium, suppression of product and internal standard has to be identical. We take the assay response as the MS/MS signal for the product divided by that for the internal standard, so differential suppression in complete and control assays does not contribute to the calculated analytical range.

X. Calculation of the values in main text Table 2.

Percent free 4MU in the 4MU-substrates.

The fluorescence of 4-MU glycosides has to be pH independent in the range of 3 to 10.7 since there is no functional groups in the molecules that can ionize in this range. Any increase

in fluorescence observed when the pH is shifted from 3 to 10.7 has to be due to free 4MU contamination (the presence of other fluorescent impurities was ruled out by showing that the emission and excitation wavelength maxima fit those published for 4MU, not shown). The increase in fluorescence for the pH 3 to 10.7 shift was used along with the fluorescence per mole of the anion 4-MU-O⁻ at pH 10.7 (measured with standard 4MU) to obtain the moles of free 4MU present in the 4MU-substrate. The mole % of free 4MU is then calculated using the moles of 4MU-substrate present.

Using the mole % of free 4MU and the 4MU-OH/4MU-O⁻ emission ratio (see main text), the contribution to the fluorescence of 4MU-glycoside at pH 3.0 from the 4MU-OH contaminant is obtained. This value is used to correct the observed fluorescence of 4MU-glycoside at pH 3.0 to obtain the fluorescence of the pure 4MU-glycoside. From these values we obtain the ratio (4MU-O⁻ emission per mole)/(4MU-glycoside emission per mole).

XI. Calculation of the analytical range of the enzyme assays.

IDUA, acid a-glucosidase, I2S

The complete assay is set up by adding 30 μ L of buffer plus substrate to a 3 mm quality control high DBS punch and incubating for the desired time. A second DBS punch is mixed with 15 μ L of buffer without substrate (sample A), and a second sample is set up containing 30 μ L of buffer without a DBS punch and with substrate at a 2-fold higher concentration than in the complete assay (sample B). Samples A and B are incubated along with the complete assay sample. After incubation, 15 μ L of sample B is transferred to sample A, and the mixture is quenched immediately with pH 10.7 buffer. The complete assay sample is also quenched with pH 10.7 buffer. In this way, both the complete and control assays contain the 4MU-glycoside, any 4MU present in 4MU-glycoside as a contaminant and any 4MU formed from non-enzymatic hydrolysis of 4MU-glycoside in assay buffer during the incubation. The fluorescence for the complete assay is due to fluorescence from the enzyme-dependent and -independent events, whereas the fluorescence for the control is due only to the enzyme-independent events, thus the analytical response is obtained as:

$$\frac{[(\text{fluorescence of complete assay}) - (\text{fluorescence of control assay})]}{(\text{fluorescence of control assay})}$$
Note that the analytical range is not influenced by quenching of fluorescence by blood components such as hemoglobin since blood is present in both the complete and control assays at the same concentration. The fluorescence measured for 20 μ M 4MU in buffer with blood is only 21.7% of that measured in buffer alone. Since this quenching is substantial, we did not measure the enzyme-independent assay response by incubating 4MU-substrate in buffer without blood since the signal would be anomalously high when compared to that for the complete assay, which contains blood.

GALNS and ARSB assays.

To measure the analytical range, we measured the fluorescence in complete assay with blood, substrate, (Z)-Pugnac and β -NGA to that measured in an identical solution but lacking β -NGA. The enzyme β -NGA releases 4MU only after the sulfate is removed from the GALNS and ARSB substrates (Kumar, A. H., Spacil, Z., Ghomashchi, F., Masi, S., Sumida, T., Ito, M., Turecek, F., Scott, C. R., and Gelb, M. H. Fluorimetric Assays for N-Acetylgalactosamine-6-Sulfatase and Arylsulfatase B Based on the Natural Substrates for Confirmation of Mucopolysaccharidoses Types IVA and VI, submitted to Clin. Chim. Acta). Only in the presence of β -NGA do the GALNS and ARSB sulfatases contribute to the increase in fluorescence. Any

4MU present as an impurity in the substrates or formed by non-enzymatic breakdown of the substrates occurs in the absence of β -NGA. Both the plus and minus β -NGA samples contain blood and thus are quenched to the same extent. If there is non-sulfated 4MU-GalNAc present as an impurity in the substrates or if the sulfated substrates can lose sulfate in the absence of sulfatases during incubation, these factors will lead to an increase in fluorescence in the plus β -NGA sample (but not in the minus β -NGA sample) that is sulfatase-independent, and thus the analytical range will be overestimated. To measure the amount of non-sulfated 4MU-GalNAc in the substrates and the amount of non-enzymatic substrate desulfation, we measured the increase in fluorescence when the substrates in buffer without blood were treated with β -NGA since this enzyme liberates 4MU only from the non-sulfated material (see above). No detectable increase in fluorescence was observed with the GALNS and ARSB substrates (not shown) indicating essentially no non-sulfated material in the substrates or formed by non-enzymatic desulfation during incubation. This justifies the use of the minus β -NGA assay response to calculate the analytical range. Thus, the analytical range is calculated as: [(assay response with β -NGA)-(assay response without β -NGA)]/(assay response without β -NGA).

Supplemental Table 1. MS/MS instrumentation parameters.

MS/MS was carried out on a Waters Xevo TQD (Waters, Milford, MA) using separate instrumentation settings for flow injection and LC MS/MS (Supplemental material). Ion count peak areas were determined using the Waters TargetLynx software. FIA and LC were done on a Waters Acquity UPC with 2D technology (Sample Manager, Binary Solvent Manager, Column Manager).

FIA and LC-MS/MS assays.

Parameter (units)	FIA	LC-MS/MS
Polarity	ES+	ES+
Capillary voltage (V)	3000	3500
Extractor (V)	3.00	3.00
Source temperature (°C)	90	150
Desolvation temperature (°C)	150	500
Cone Gas Flow (L/h)	50	30
Desolvation Gas Flow (L/h)	450	1000
LM 1 Resolution	2.6	2.9
HM 1 Resolution	15.0	15.0
Ion Energy 1	0.5	0.0
Collision Cell Entrance Potential (V)	0.50	0.5
Collision Cell Exit Potential (V)	0.50	0.5
LM 2 Resolution	2.8	2.8
HM 2 Resolution	14.7	14.7
Ion Energy 2	0.6	0.6
Multiplier (V)	493.04	493.04
Collision Gas	Argon	Argon

Supplemental Table 2. MS/MS Single reaction monitoring values for I2S, GALNS and ARSB.

Compound	Alternate name ¹	Precursor [m/z]	Product [m/z]	Cone [V]	Collision energy [eV]
C5/C5-Bz aglycone	ARSB P aglycone	454.27	345.21	25	17
C5/C5-Bz-d5 aglycone	ARSB IS aglycone	459.30	350.25	25	17
C5/C6-Bz aglycone	I2S P aglycone	468.29	359.23	26	17
C5/C6-Bz-d5 aglycone	I2S IS aglycone	473.32	364.26	26	17
C6/C6-Bz aglycone	GALNS P aglycone	482.30	373.19	27	17
C6/C6-Bz-d5 aglycone	GALNS IS aglycone	487.33	378.28	27	17
Ida-C5/C6-Bz	I2S P	644.32	359.23	34	23
Ida-C5/C6-Bz-d5	I2S IS	649.35	364.26	34	23
GalNAc-C5/C5-Bz	ARSB P	657.35	345.21	26	24
GalNAc-C5/C5-Bz-d5	ARSB IS	662.38	350.25	26	24
GalNAc-C6/C6-Bz	GALNS P	685.38	373.25	26	25
GalNAc-C6/C6-Bz-d5	GALNS IS	690.41	378.28	26	25
Ida-2-S-C5/C6-Bz	I2S S	724.27	359.23	20	25
GalNAc-4-S-C5/C5-Bz	ARSB S	737.31	345.21	15	24
GalNAc-6-S-C6/C6-Bz	GALNS S	765.34	373.25	15	24

¹S, P, and IS stand for substrate, product, and internal standard, respectively.

Supplemental Table 3. Comparison of Gal-6-S versus GalNAc-6-S substrates.

Substrate	Substrate concentration	GALNS activity ($\mu\text{mole hr}^{-1} \text{L}^{-1}$)
Gal-6-S-C7/C6-Bz	1.0 mM	0.039
GalNAc-6-S-C6/C6-Bz	1.0 mM	1.90
Gal-6-S-C7/C6-Bz + GalNAc-C6/C6-Bz	0.5 mM each	0.0053 (Gal-6-S) 1.13 (GalNAc-6-S)

Injection of 0.5 pmole each of Gal-C7/C6-Bz and GalNAc-C6/C6-Bz showed that the former gave a 1.26-fold higher response by LC-MS/MS than the latter.

Supplemental Table 4. Action of recombinant human-HexA on GalNAc-4-S-C5/C5-Bz

recombinant human-HexA	(Z)-Pugnac	GalNAc-C5/C5-Bz ion counts	Aglycone-C5/C5-Bz ion counts
0 ng	0 mM	174	2789
3.1 ng	0 mM	138	26128
3.1 ng	0.5 mM	708	6744
3.1 ng	1 mM	154	3562

All assays were done with standard assay conditions and analyzed by LC-MS/MS.

Supplemental Table 5. Action of HexA in DBS on GalNAc-4-S-C5/C5-Bz and GalNAc-6-S-C6/C6-Bz.

Substrate	Sample	(Z)-Pugnac	GalNAc-C5/C5-Bz ion counts	Aglycone-C5/C5-Bz ion counts
GalNAc-4-S-C5/C5-Bz	Filter paper	0 mM	3426	33818
GalNAc-4-S-C5/C5-Bz	DBS	0 mM	423801	380478
GalNAc-4-S-C5/C5-Bz	Filter paper	1 mM	3368	28376
GalNAc-4-S-5/C5-Bz	DBS	1 mM	327075	102466
GalNAc-6-S-C5/C5-Bz	Filter paper	0 mM	820	14466
GalNAc-6-S-C5/C5-Bz	DBS	0 mM	110155	1106009
GalNAc-6-S-C5/C5-Bz	Filter paper	1 mM	757	15363
GalNAc-6-S-5/C5-Bz	DBS	1 mM	110828	55262

All assays were done with standard assay conditions and analyzed by LC-MS/MS. DBS was obtained from a single adult and filter paper is a blood-free newborn screening card, a 3 mm punch was used in both cases.

Supplemental Table 6. Action of β -NGA on GalNAc-C5/C5-Bz

Substrate	β -NGA	(Z)-Pugnac	GalNAc-C5/C5-Bz ion counts	Aglycone-C5/C5-Bz ion counts
GalNAc-C5/C5-Bz	0 Units	0 mM	206941	25465
GalNAc-C5/C5-Bz	118 Units	0 mM	101781	372629
GalNAc-C5/C5-Bz	118 Units	0.5 mM	110088	352827
GalNAc-C5/C5-Bz	118 Units	1 mM	123159	379031

All assays were done with standard assay conditions using a 3 mm punch of filter paper (no blood) and analyzed by LC-MS/MS.

Supplemental Table 7. Action of β -NGA on GalNAc-4-S-C5/C5-Bz and GalNAc-6-S-C5/C5-Bz

Substrate	β -NGA	GalNAc-C5/C5-Bz ion counts	Aglycone-C5/C5-Bz ion counts
GalNAc-4-SC5/C5-Bz	0 Units	1842	13395
GalNAc-4-S-C5/C5-Bz	118 Units	132	22281
GalNAc--6-S-C5/C5-Bz	0 Units	1097	16405
GalNAc-6-S-C5/C5-Bz	118 Units	253	19787

All assays were done with standard assay conditions using a 3 mm punch of filter paper (no blood) and analyzed by LC-MS/MS.

Supplemental Table 8. LC-MS/MS assay results for GALNS.

Sample	P ion cts	IS ion cts	P/IS	Activity $\mu\text{mole h}^{-1} \text{L}^{-1}$
NB ctrl #01	11053	59182	0.187	0.555
NB ctrl #02	7923	69934	0.113	0.333
NB ctrl #03	10904	69926	0.156	0.462
NB ctrl #04	7489	75152	0.100	0.292
NB ctrl #05	7105	86012	0.083	0.240
NB ctrl #06	12341	95225	0.130	0.382
NB ctrl #07	24468	89978	0.272	0.813
NB ctrl #08	18621	93486	0.199	0.593
NB ctrl #09	31676	86738	0.365	1.095
NB ctrl #10	17443	102301	0.171	0.506
NB ctrl #11	12341	107864	0.114	0.336
NB ctrl #12	17457	92381	0.189	0.562
NB ctrl #13	17332	144382	0.120	0.354
NB ctrl #14	20983	147035	0.143	0.422
NB ctrl #15	31981	155581	0.206	0.612
NB ctrl #16	15532	150387	0.103	0.303
NB ctrl #17	27872	148073	0.188	0.560
NB ctrl #18	29502	157291	0.188	0.558
NB ctrl #19	19284	155663	0.124	0.365
NB ctrl #20	20031	144771	0.138	0.409
NB ctrl #21	21438	149363	0.144	0.425
NB ctrl #22	23597	151711	0.156	0.461
NB ctrl #23	19127	153239	0.125	0.368
NB ctrl #24	20282	164934	0.123	0.362
NB ctrl #25	14227	143961	0.099	0.289
NB ctrl #26	32529	151650	0.215	0.639
NB ctrl #27	22224	163931	0.136	0.400
NB ctrl #28	25289	152062	0.166	0.493
NB ctrl #29	18617	167033	0.111	0.328
NB ctrl #30	29607	160807	0.184	0.547
NB ctrl #31	28039	161098	0.174	0.517
NB ctrl #32	28062	153535	0.183	0.543
NB ctrl #33	23924	155191	0.154	0.457
NB ctrl #34	19715	156159	0.126	0.372
NB ctrl #35	42090	167638	0.251	0.750
NB ctrl #36	25194	171485	0.147	0.435
NB ctrl #37	20196	148481	0.136	0.402
NB ctrl #38	16065	146980	0.109	0.321
NB ctrl #39	11852	166717	0.071	0.205

NB ctrl #40	11564	166942	0.069	0.200
NB ctrl #41	16736	160962	0.104	0.305
NB ctrl #42	25585	163185	0.157	0.465
NB ctrl #43	39940	163277	0.245	0.730
NB ctrl #44	30127	159231	0.189	0.563
NB ctrl #45	36823	163590	0.225	0.671
NB ctrl #46	23234	172584	0.135	0.398
NB ctrl #47	33232	160093	0.208	0.618
NB ctrl #48	25397	168030	0.151	0.448
NB ctrl #49	23307	159626	0.146	0.432
NB ctrl #50	13939	171796	0.081	0.236
MPS_4A pat#01	963	136974	0.007	0.012
MPS_4A pat#02	4230	135619	0.031	0.085

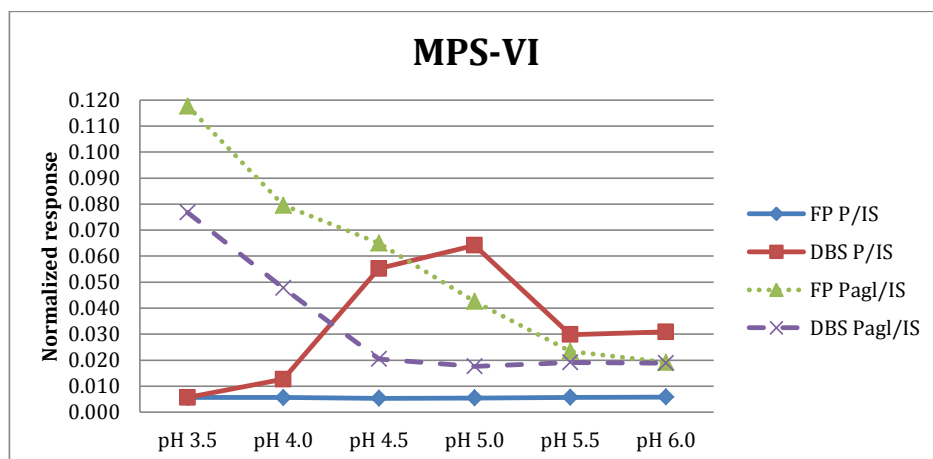
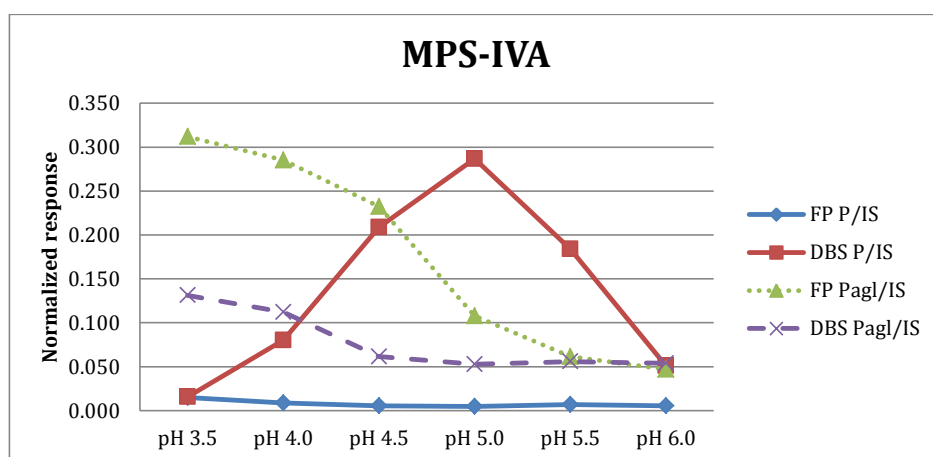
Supplemental Table 9. LC-MS/MS assay results for ARSB.

Sample	P ion cts	IS ion cts	P/IS	Activity $\mu\text{mole h}^{-1} \text{L}^{-1}$
NB ctrl #01	334134	121063	2.760	8.337
NB ctrl #02	122755	121034	1.014	3.058
NB ctrl #03	107617	112816	0.954	2.875
NB ctrl #04	99382	117635	0.845	2.545
NB ctrl #05	293926	118904	2.472	7.466
NB ctrl #06	167472	114661	1.461	4.408
NB ctrl #07	180650	121997	1.481	4.469
NB ctrl #08	181599	128933	1.408	4.250
NB ctrl #09	313116	116856	2.679	8.094
NB ctrl #10	193886	122340	1.585	4.783
NB ctrl #11	104020	116572	0.892	2.689
NB ctrl #12	228105	126872	1.798	5.428
NB ctrl #13	183396	121898	1.504	4.540
NB ctrl #14	164821	120633	1.366	4.122
NB ctrl #15	142788	120927	1.181	3.561
NB ctrl #16	177605	116091	1.530	4.617
NB ctrl #17	157385	120535	1.306	3.939
NB ctrl #18	193366	125628	1.539	4.645
NB ctrl #19	205698	131130	1.569	4.734
NB ctrl #20	180305	127868	1.410	4.255
NB ctrl #21	134713	122656	1.098	3.312
NB ctrl #22	225243	126645	1.779	5.369
NB ctrl #23	161685	114446	1.413	4.263
NB ctrl #24	253135	124759	2.029	6.127
NB ctrl #25	116840	118962	0.982	2.961
NB ctrl #26	364299	127322	2.861	8.643
NB ctrl #27	149503	150249	0.995	3.000
NB ctrl #28	202392	128559	1.574	4.752
NB ctrl #29	97083	136801	0.710	2.137
NB ctrl #30	159646	120631	1.323	3.993
NB ctrl #31	283990	138661	2.048	6.184
NB ctrl #32	267620	126674	2.113	6.380
NB ctrl #33	219169	129646	1.691	5.103
NB ctrl #34	122823	137218	0.895	2.697
NB ctrl #35	401611	134195	2.993	9.041
NB ctrl #36	234728	134686	1.743	5.261
NB ctrl #37	145171	95604	1.518	4.583
NB ctrl #38	91795	90175	1.018	3.069
NB ctrl #39	70944	91099	0.779	2.346

NB ctrl #40	86576	94464	0.916	2.762
NB ctrl #41	105875	108698	0.974	2.936
NB ctrl #42	120056	88728	1.353	4.082
NB ctrl #43	212687	82549	2.576	7.782
NB ctrl #44	124531	101207	1.230	3.712
NB ctrl #45	198482	121683	1.631	4.923
NB ctrl #46	124756	106206	1.175	3.543
NB ctrl #47	284389	102605	2.772	8.373
NB ctrl #48	90606	100861	0.898	2.707
NB ctrl #49	220360	134542	1.638	4.944
NB ctrl #50	142504	129641	1.099	3.315
MPS_6 pat#01	24035	123199	0.195	0.580
MPS_6 pat#03	32688	173161	0.189	0.561

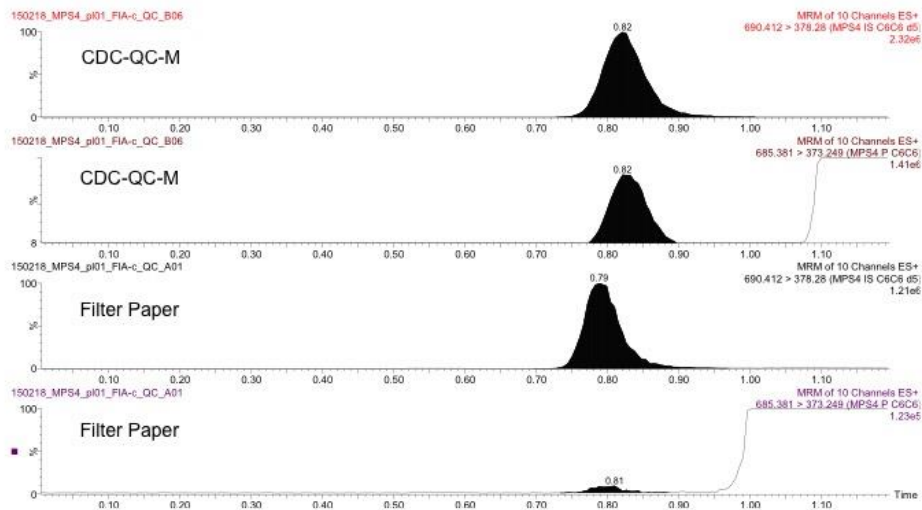
Supplemental Figure 1. pH Dependence of (Top Panel) GalNAC-6-S-C6/C6/-Bz and (Bottom Panel) GalNAC-4-S-C5/C5-Bz enzyme-independent and -dependent reactions.

Shown is the ion count peak area for GalNac-containing product divided by that of the internal standard (P/IS) or the ion count peak area for aglycone divided by that of the internal standard (Pagl/IS) as a function of the indicated buffer pH during incubation for samples that contained a filter paper punch (FP) or a DBS punch (from a healthy adult). Buffers were 50 mM ammonium acetate adjusted to the desired pH with 5% acetic acid. Samples were analyzed by LC-MS/MS.

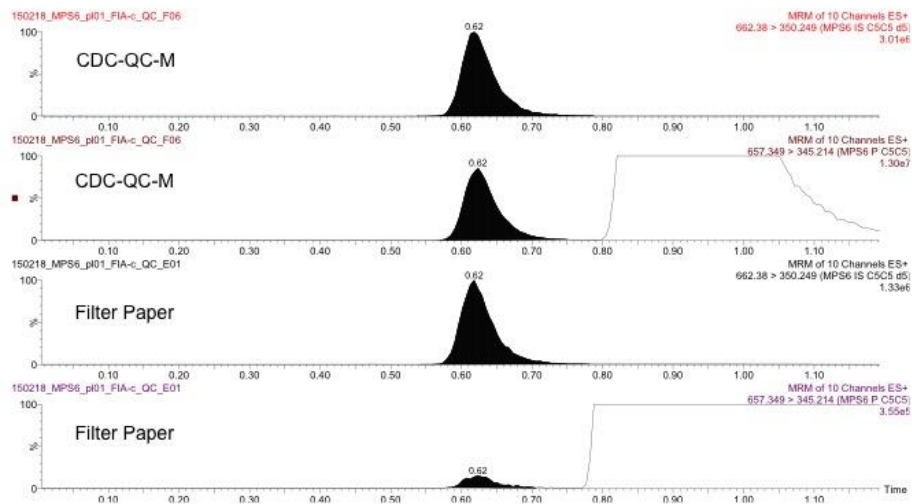


Supplemental Figure 2. LC-MS/MS ion traces. MPS-IVA (LC) and MPS-VI (LC) are the selective MRM traces for GALNS and ARSB LC-MS/MS assays, respectively. For both, the bottom panel is the product MRM trace for filter paper, second from bottom is internal standard MRM for filter paper, third from bottom is product for the CDC QC M DBS and top is the internal standard MRM for the CDC QC M DBS. MPS-II (FIA-IDUA) is the flow injection-MS/MS assay for I2S using IDUA, MPS-II (LC-IDUA) is the I2S LC-MS/MS assay with IDUA, and MPS-II (LC) is the I2S LC-MS/MS assay without IDUA. The substrate peaks are visible in the ion traces. The retention times are: MPS-IVA (1.14 min); MPS-VI (0.93 min); MPS-II IDUA method (1.04 min); MPS-II (1.17 min).

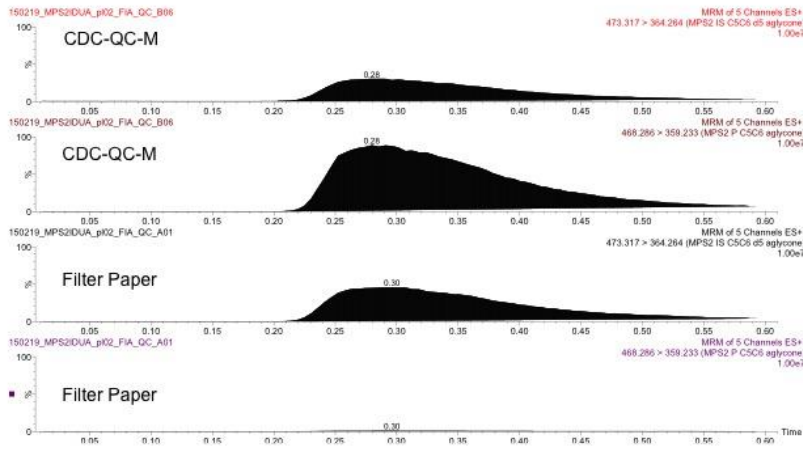
MPS-IVA (LC)



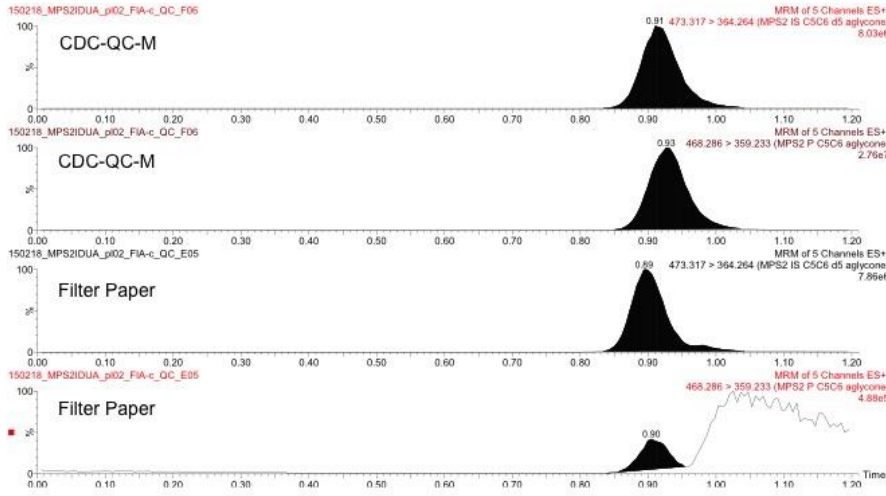
MPS-VI (LC)



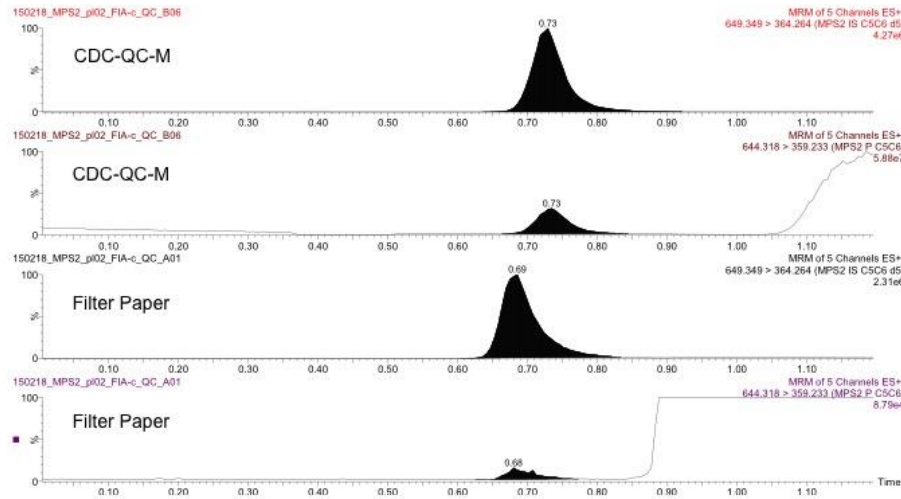
MPS-II (FIA-IDUA)



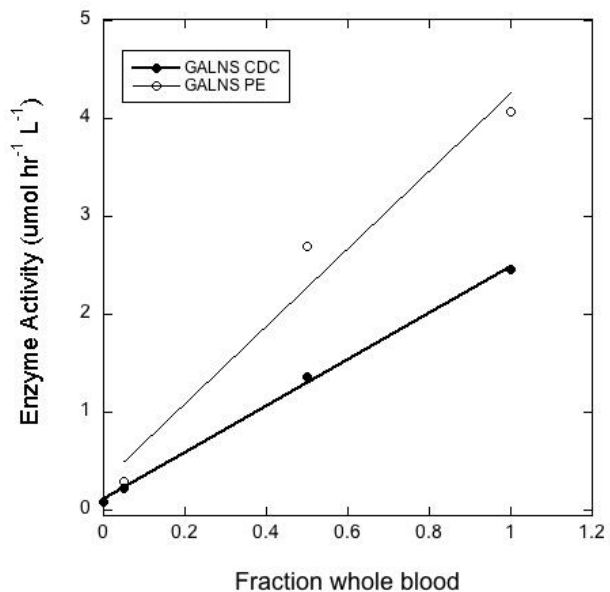
MPS-II (LC-IDUA)

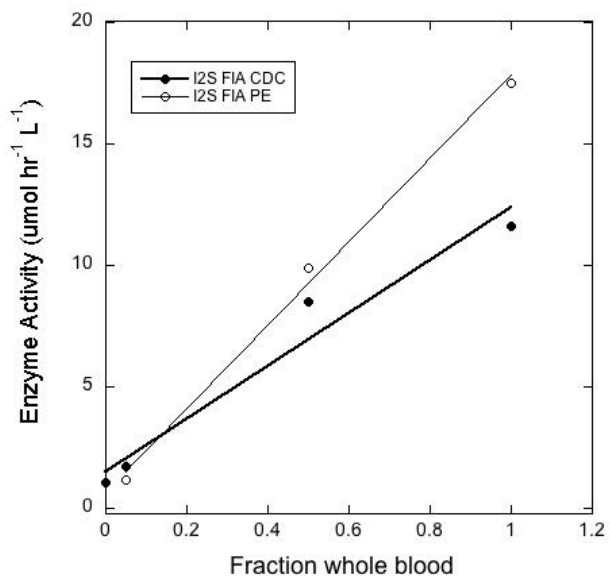
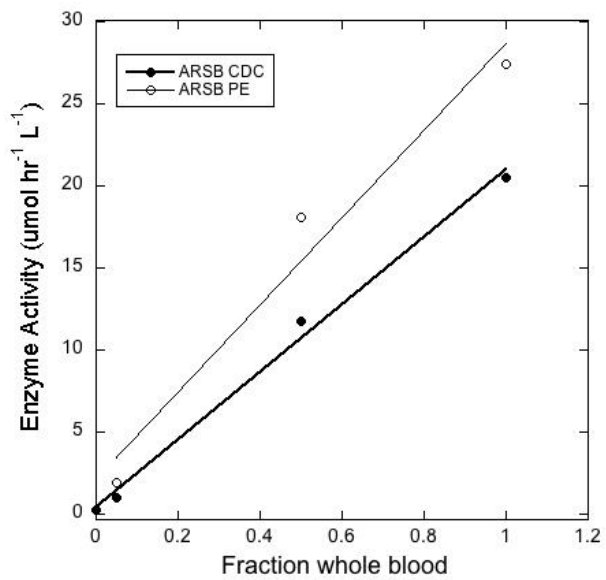


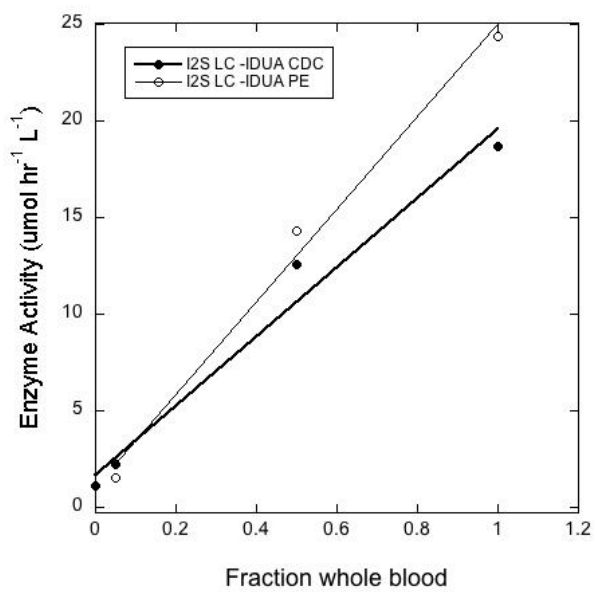
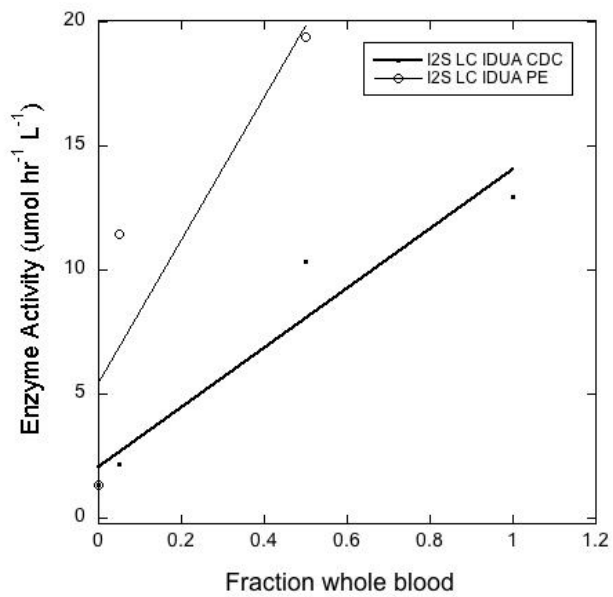
MPS-II (LC)

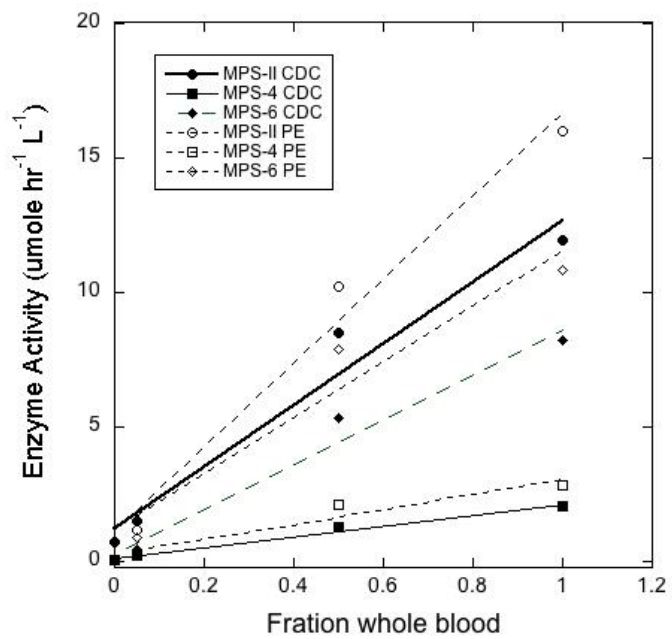


Supplemental Figure 3. Enzyme activity versus the fraction of whole blood for the quality control DBS. The DBS are made by mixing leukocyte-depleted blood with whole blood (fraction given on the X-axis). In the case of the CDC quality control DBS, there is finite activity at 0 fraction whole blood showing that there is some residual lysosomal enzyme in the leukocyte-depleted blood. In the case of the Perkin Elmer (PE) quality control DBS, no 0 fraction whole blood samples were available. First panel is GALNS LC-MS/MS assay, second panel is ARSB LC-MS/MS assay, third panel is I2S flow injection-MS/MS assay with IDUA, fourth panel is I2S LC-MS/MS assay with IDUA, fifth panel is I2S LC-MS/MS assay without IDUA, and sixth panel is the triplex for I2S, GALNS, and ARSB using ethyl acetate liquid-liquid extraction followed by LC-MS/MS.









References

1. De Jesus VR, Zhang XK, Keutzer J, Bodamer O, Muhl A, Orsini JJ, et al. Development and evaluation of quality control dried blood spot materials in newborn screening for lysosomal storage disorders. *Clin Chem* 2009;55:158-64.
2. Kakkis ED, Joans MA, Neufeld EF. Overexpression of the human lysosomal enzyme alpha-l-iduronidase in chinese hamster ovary cells. *Protein Expr Purif* 5:255-32.
3. Blanchard S, Sadilek M, Scott CR, Turecek F, Gelb MH. Tandem mass spectrometry for the direct assay of lysosomal enzymes in dried blood spots: Application to screening newborns for mucopolysaccharidosis i. *Clin Chem* 2008;54:2067-70.
4. Duffey TA, Sadilek M, Scott CR, Turecek F, Gelb MH. Tandem mass spectrometry for the direct assay of lysosomal enzymes in dried blood spots: Application to screening newborns for mucopolysaccharidosis vi (maroteaux_lamy syndrome). *Anal Chem* 2010;82:9587-91.
5. Wolfe BJ, Blanchard S, Sadilek M, Scott CR, Turecek F, Gelb MH. Tandem mass spectrometry for the direct assay of lysosomal enzymes in dried blood spots: Application to screening newborns for mucopolysaccharidosis ii (hunter syndrome). *Anal Chem* 2011;83:1152-6.

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