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High-throughput Multiplex Newborn Screening Assay for Six Lysosomal Storage Disorders (LSDs) using Dried Blood Spots and UPLC-Tandem Mass Spectrometry

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Lysosomal Storage Disorder (LSD)

- Lysosome – an intracellular organelle containing many enzymes that degrade complex molecules.
- The absence or loss of function of an enzyme/protein along the pathway results in the accumulation of complex molecules that are normally degraded within lysosomes.
- The progressive accumulation of these products leads to cellular dysfunction and eventually causes tissue and organ dysfunction.

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LSD (cont'd)

- LSD represents about 50 genetically heterogeneous disorders.
- Almost all LSDs are inherited as autosomal recessive traits except for the X-linked Fabry and Hunter diseases.
- Individually, the incidences of these diseases are rare. However, collectively, LSDs are far more common (1 in 8000)
- Pre-symptomatic diagnosis will be beneficial for babies.

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LSD Testing Timeline

2007– Legislative mandate for five LSDs (Krabbe, Pompe, Fabry, Gaucher, Niemann-Pick A/B)

2010 – Pilot screening for Pompe, Fabry & Gaucher using microfluidic platform

- 8,012 DBS screened
- Two had abnormal GAA activities, confirmed negative by second-tier tests

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LSD Testing Timeline (cont'd)

2011 – Legislative mandate expanded to seven LSDs (addition of Hurler and Hunter), with the following provisions before screening:

- A method either cleared by the US Food and Drug Administration (FDA) or validated under the Clinical Laboratory Improvement Amendments (CLIA)
- Availability of quality control and proficiency testing materials
- Appropriate equipment for high-volume screening
- Adequate funding

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LSD Testing Timeline (cont'd)

2011 – Decision made to switch from microfluidic platform to tandem mass spectrometry

- Microfluidic platform did not have substrates for all LSDs.
- Microfluidic platform lacked throughput for Illinois' volume (~170,000 newborns per year).
- Recent developments with multiplex MS/MS promised adequate testing throughput for more disorders and with less staff.

2014 – Method validation and limited pilot testing

2015 – Statewide testing expected to begin 1ST quarter

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Multiplex LC-MS/MS Assay

Modification of method developed at the University of Washington for six LSDs: Krabbe, Pompe, Fabry, Gaucher, Niemann-Pick (A/B), Hurler (MPS I).

- Single DBS punch
- Single buffer
- In-line chromatographic purification (no solid-phase or liquid extraction)

- Three-hour incubation (maintains work flow).
- UPLC column separates product/ISTD pairs and removes salt, detergent, & phospholipids by valving.
- 2.5 minute injection cycle, 500 injections/instrument/day, >10,000 injections/PM.

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6-Plex Assay

Final Composition of Assay Cocktail & Assay Conditions*

Ammonium formate **Sodium cholate** **Acarbose** **N-Acetyl-α-galactoseamine**	**0.1 M, pH 4.4** **10 g/L** **0.08 M** **50 mM**
IDUA Substrate (S), Internal Standard (IS) GLA S, IS GAA S, IS ASM S, IS (d7-C6 Ceramide) GALC S, IS (d7-C8 Ceramide) ABG S, IS (d7-C12 Ceramide)	 500 µM, 3.5 µM 600 µM, 1.2 µM 200 µM, 2.0 µM 150 µM, 2.5 µM 450 µM, 2.5 µM 300 µM, 2.5 µM
3 h/17 h incubation at 37 ^{0}C	
• Reaction was quenched with 200 µL acetonitrile (ACN) and centrifuged for 5 min at 1000 x g.	• 100 µL top layer was transferred to a glass-lined plate, and 100 µL MS-grade water was added to each well.

*Spacil Z, Tatipaka H, Barcenas M, Scott CR, Turecek F, Gelb MH. Clin Chem. 2013 Mar;59(3):502-11

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Acquity TQD Instrument

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Retention Times (RT, min) for Substrates and Products of GAA, GALC, and ABG

Enzyme	Substrate RT	Product RT
GAA	0.53	0.59
GALC	0.86	0.96
ABG	1.08	1.23

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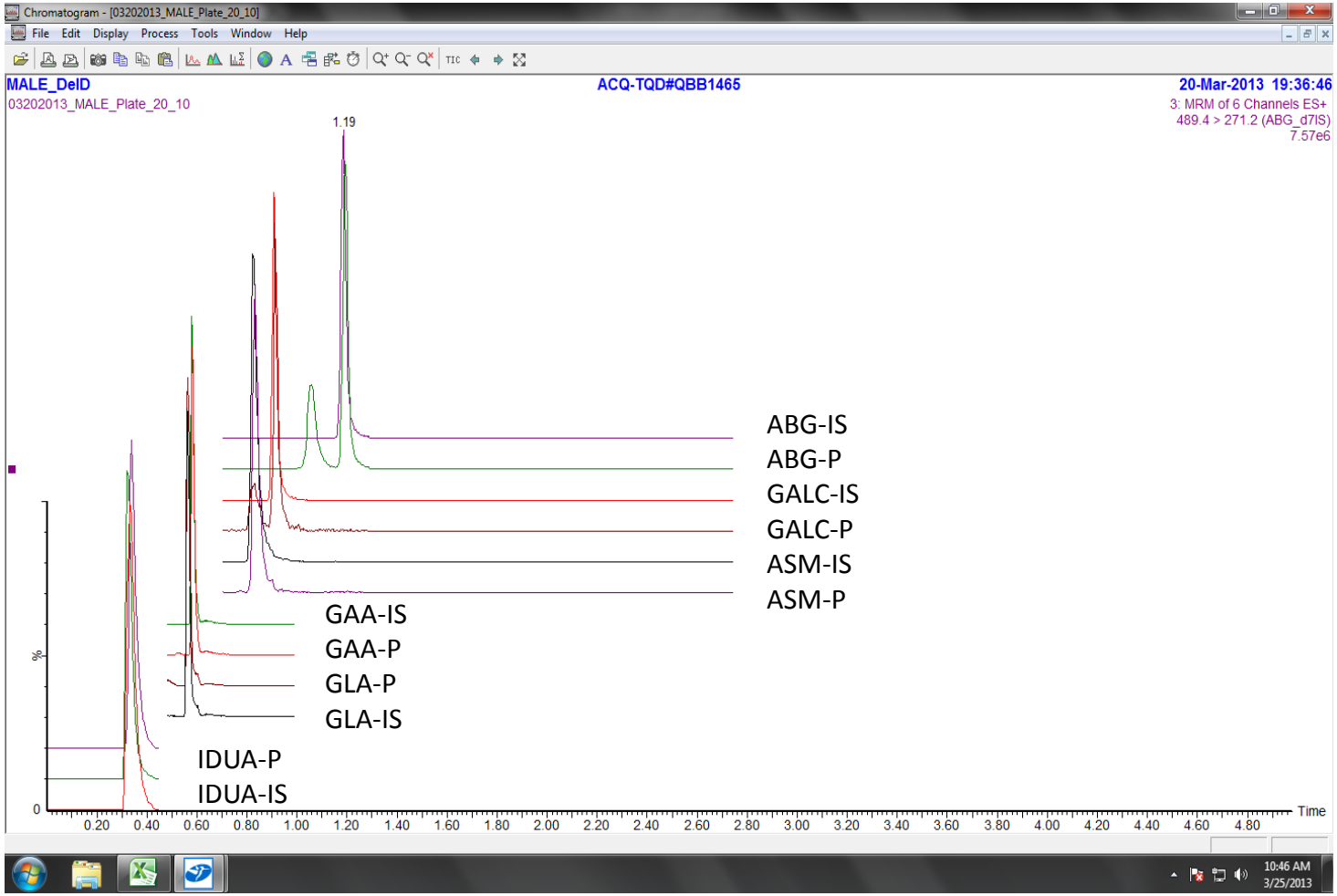
UPLC Chromatogram



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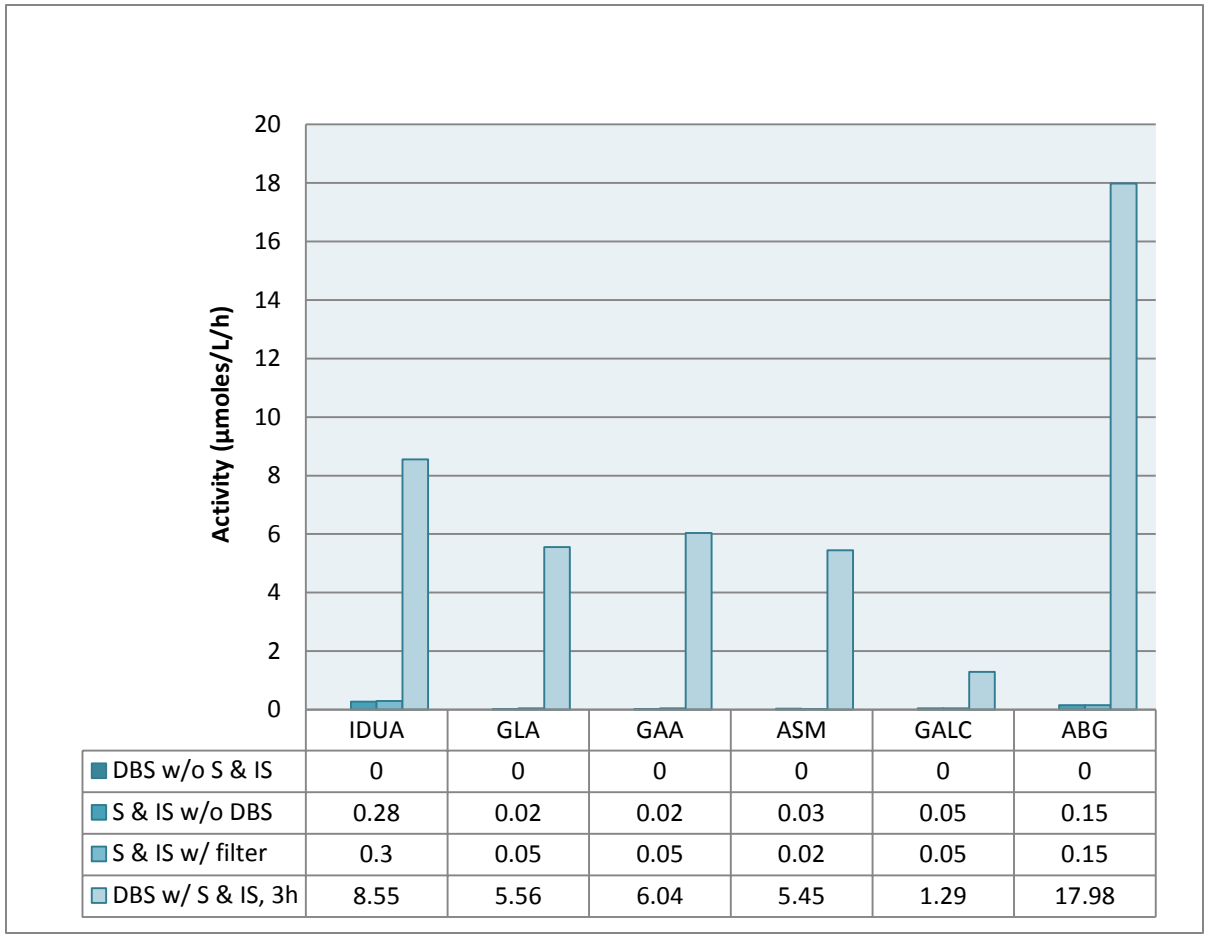
DBS Median Activities for Six Enzymes



	IDUA	GLA	GAA	ASM	GALC	ABG
DBS w/o S & IS	0	0	0	0	0	0
S & IS w/o DBS	0.28	0.02	0.02	0.03	0.05	0.15
S & IS w/ filter	0.3	0.05	0.05	0.02	0.05	0.15
DBS w/ S & IS, 3h	8.55	5.56	6.04	5.45	1.29	17.98

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Method Validation

- Evaluate different levels of Quality Control samples (Low, Medium, and High).
- Perform precision studies.
- Perform accuracy studies.
- Participate in the CDC pilot Proficiency Testing (PT) program for Pompe (and Krabbe).
- Obtain DBSs from confirmed cases.

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Method Validation (cont'd)

- Test de-identified specimens from male, female, low birth weight, and 7+ day-old babies.
- Study the effects of detergents and DBS storage conditions on LSD enzyme activities.
- Determine cut-off values.
- Exchange specimens with a qualified testing laboratory to establish comparability of results.

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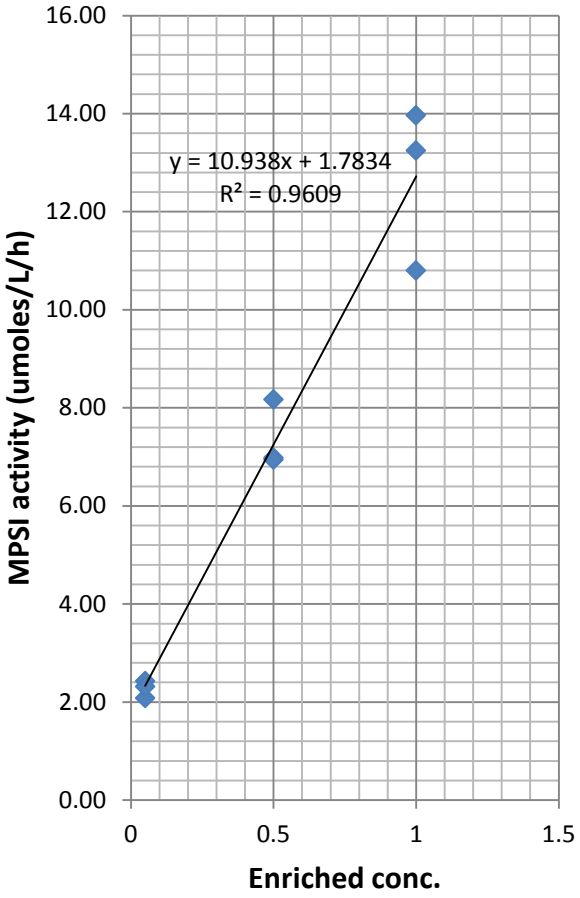
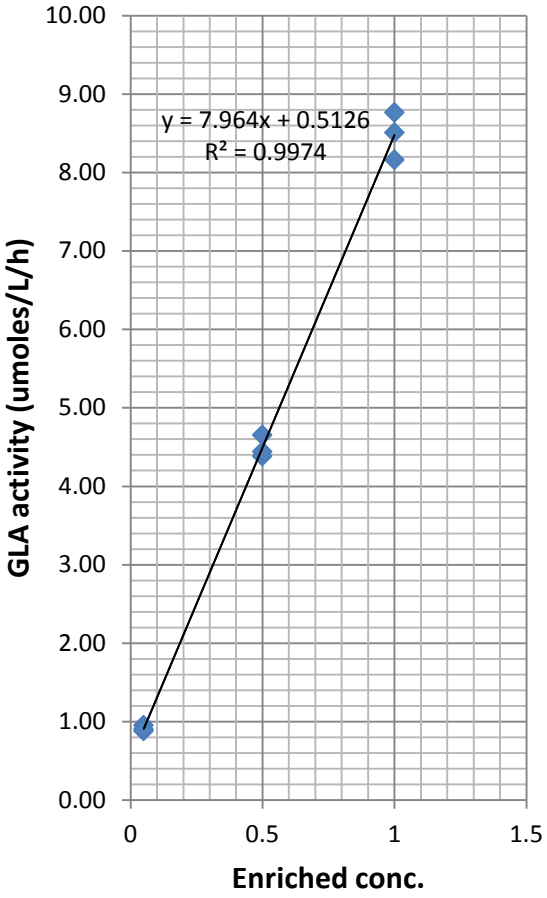
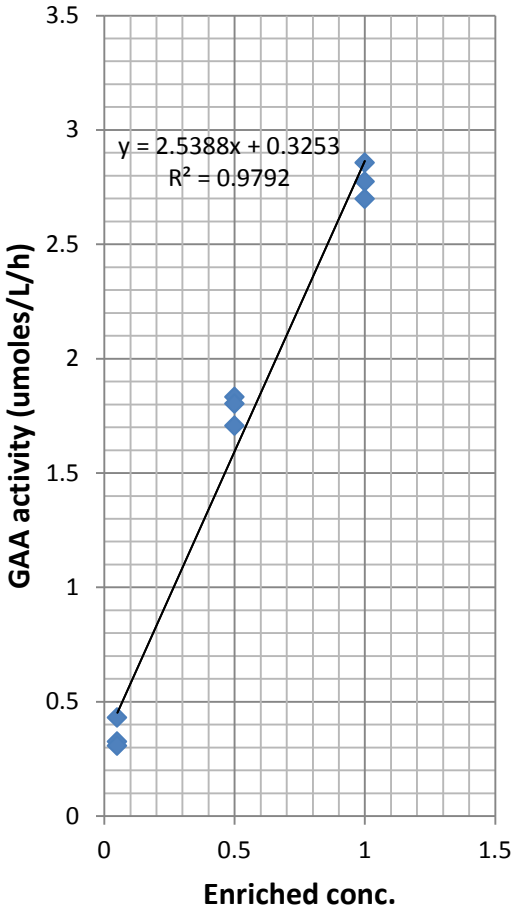
CDC QC levels for IDUA, GLA & GAA

(Hurler) (Fabry) (Pompe)







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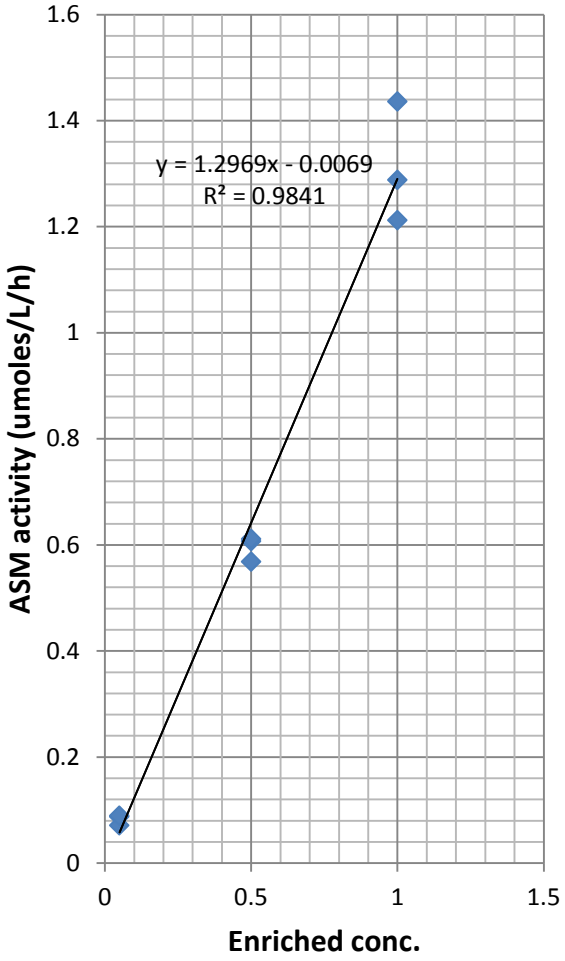
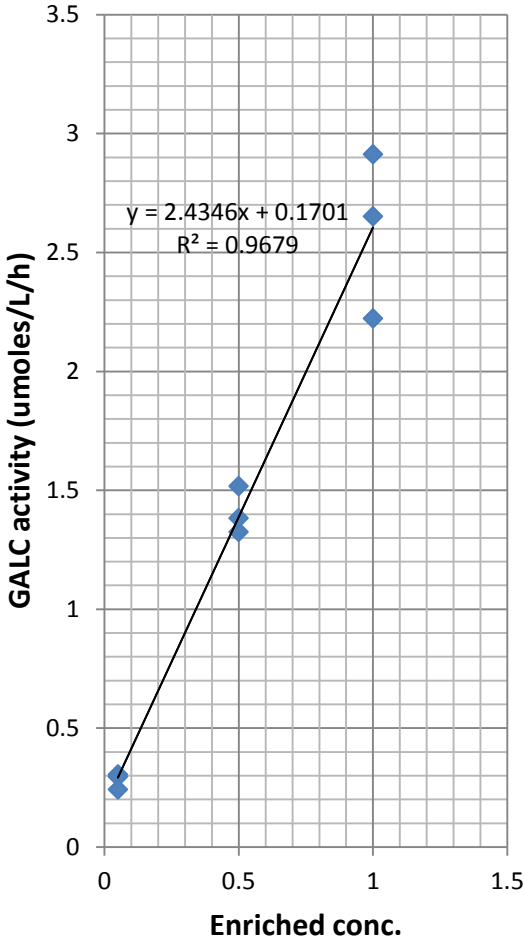
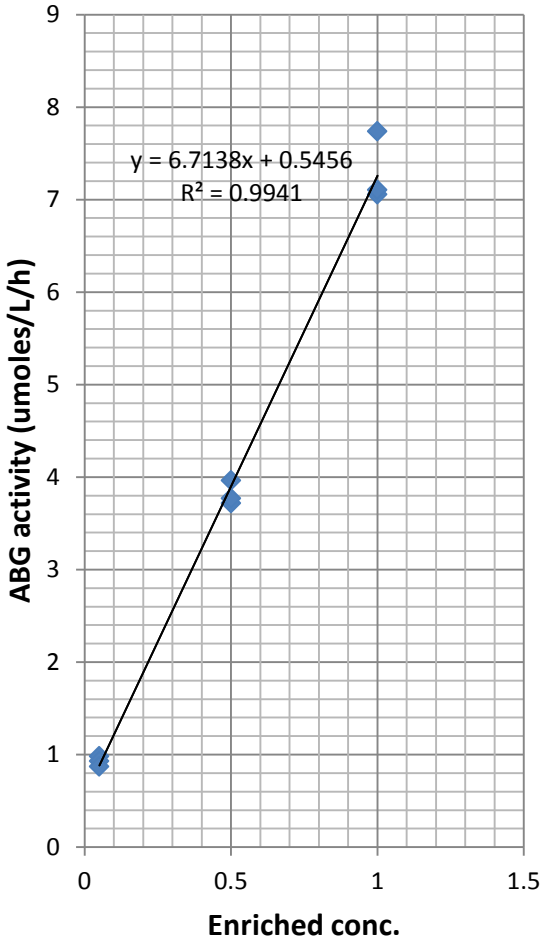
CDC QC Levels for ASM, GALC & ABG

(Niemann-Pick) (Krabbe) (Gaucher)







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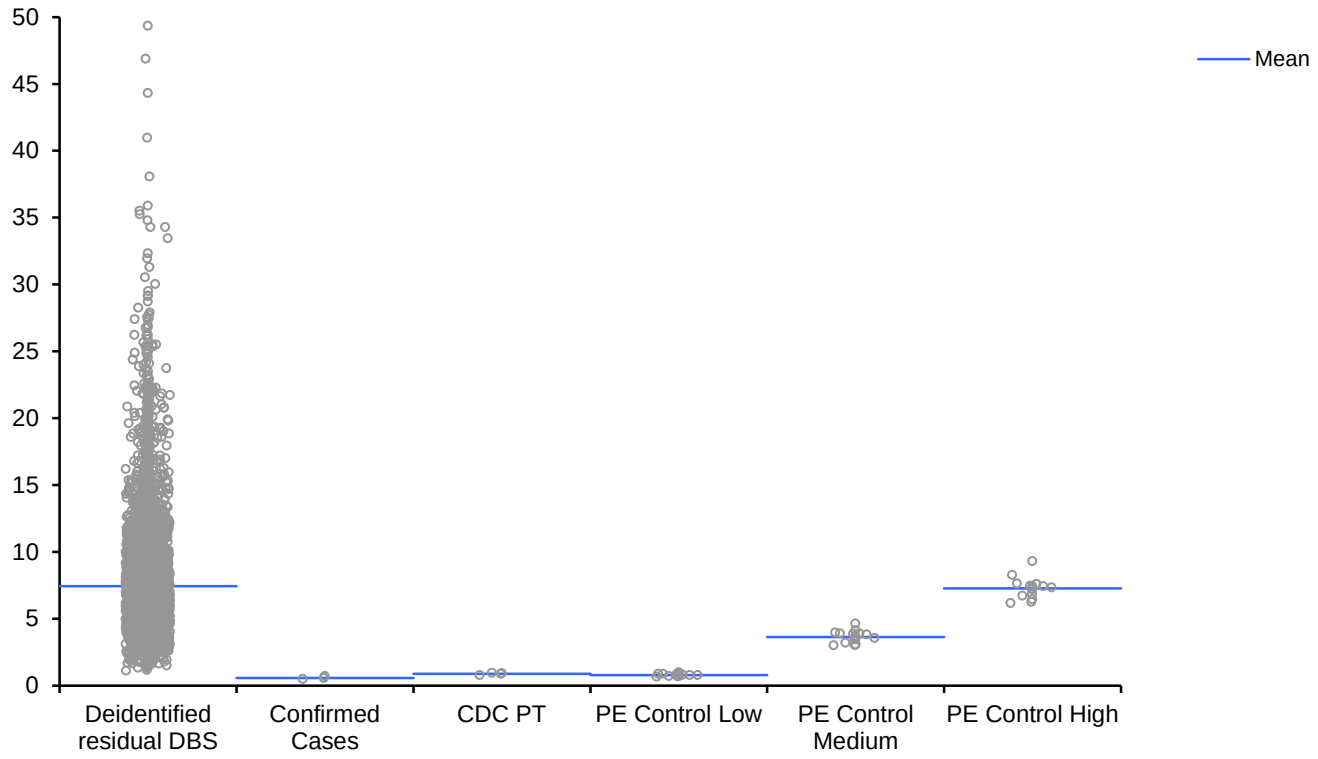
Pompe Results for de-ID DBSs, Confirmed Cases, PTs, and Quality Controls



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Statistical Analysis of Pompe Assay Results for DBSs

	n	Mean	95% CI		SE	SD
De-identified residual DBS	10003	7.45	7.37	7.52	0.04	3.64
Confirmed Cases	3	0.58	0.31	0.85	0.06	0.11
CDC PT	4	0.88	0.76	1.00	0.04	0.08
Control Low	16	0.78	0.73	0.83	0.02	0.09
Control Medium	16	3.63	3.40	3.87	0.11	0.44
Control High	15	7.28	6.84	7.73	0.21	0.80

	n	Min	Median	95% CI		Max
De-identified residual DBS	10003	1.12	6.70	6.64	6.76	49.3
Confirmed Cases	3	0.48	0.57	N/A		0.69
CDC PT	4	0.77	0.90	N/A		0.94
Control Low	16	0.65	0.77	0.71	0.87	0.99
Control Medium	16	3.01	3.62	3.19	3.90	4.63
Control High	15	6.16	7.34	6.72	7.60	9.30

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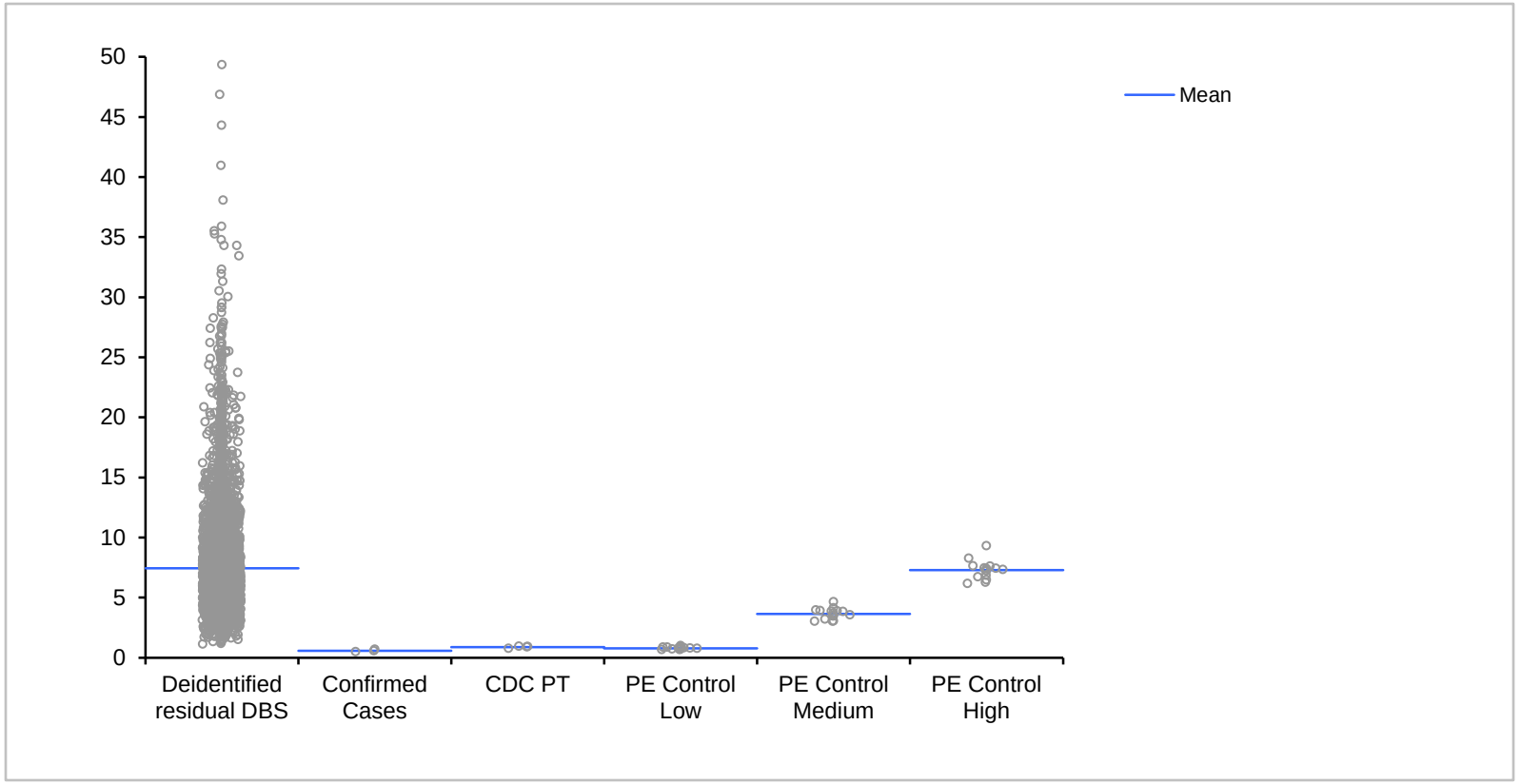
Krabbe Results for de-ID DBSs, Confirmed Cases, PTs, and Quality Controls



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Statistical Analysis of Krabbe Assay Results for DBSs

	n	Mean	95% CI		SE	SD
Deidentified Residual DBS	12222	1.49	1.47	1.52	0.01	1.43
Confirmed Cases	7	0.14	0.10	0.18	0.02	0.04
CDC PT	5	0.12	0.09	0.14	0.01	0.02
PE Control Low	21	0.11	0.09	0.12	0.01	0.03
PE Control Medium	20	0.35	0.31	0.38	0.02	0.07
PE Control High	13	0.58	0.48	0.69	0.05	0.17

Groups	n	Min	Median	95% CI		Max
Deidentified Residual DBS	12222	0.07	1.16	1.14	to 1.17	34.49
Confirmed Cases	7	0.07	0.16	0.07	to 0.18	0.18
CDC PT	5	0.10	0.11	-	-	0.15
PE Control Low	21	0.07	0.10	0.09	to 0.12	0.18
PE Control Medium	20	0.23	0.34	0.28	to 0.42	0.48
PE Control High	13	0.40	0.52	0.41	to 0.71	0.92

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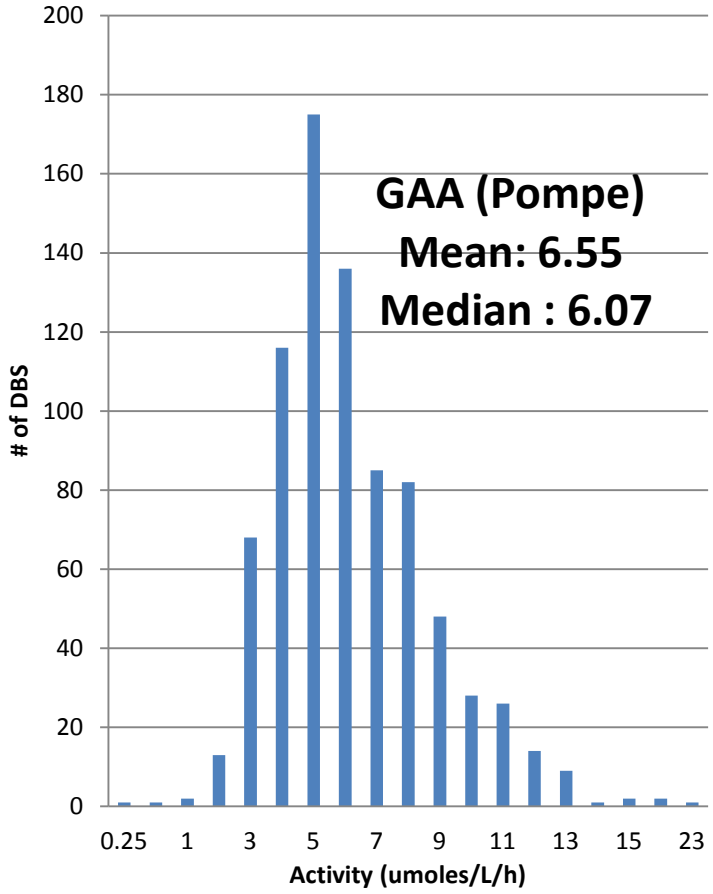
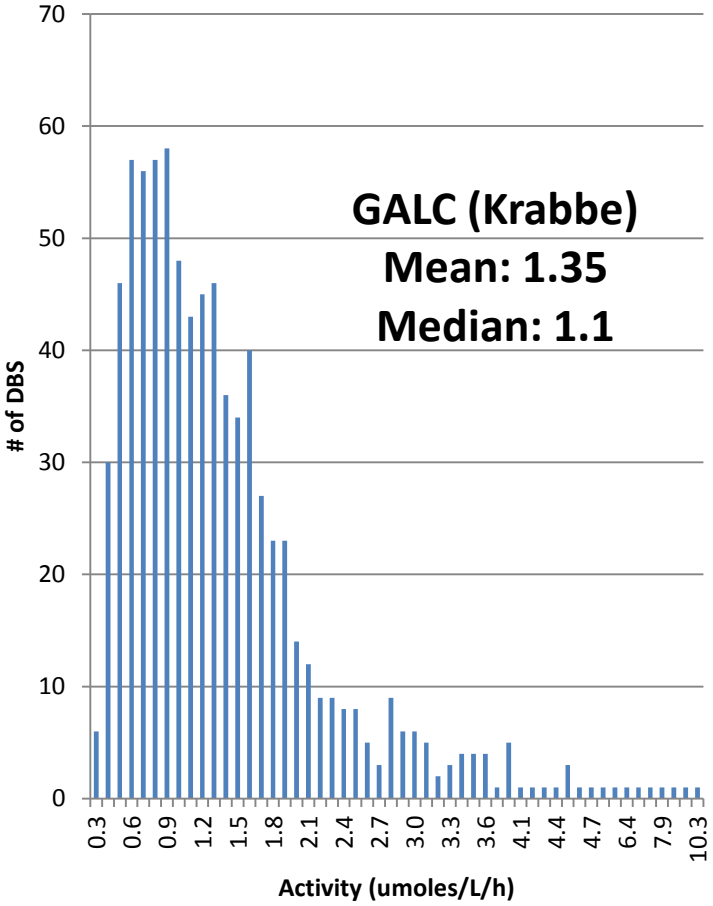
Enzyme Activity Distribution for GAA and GALC





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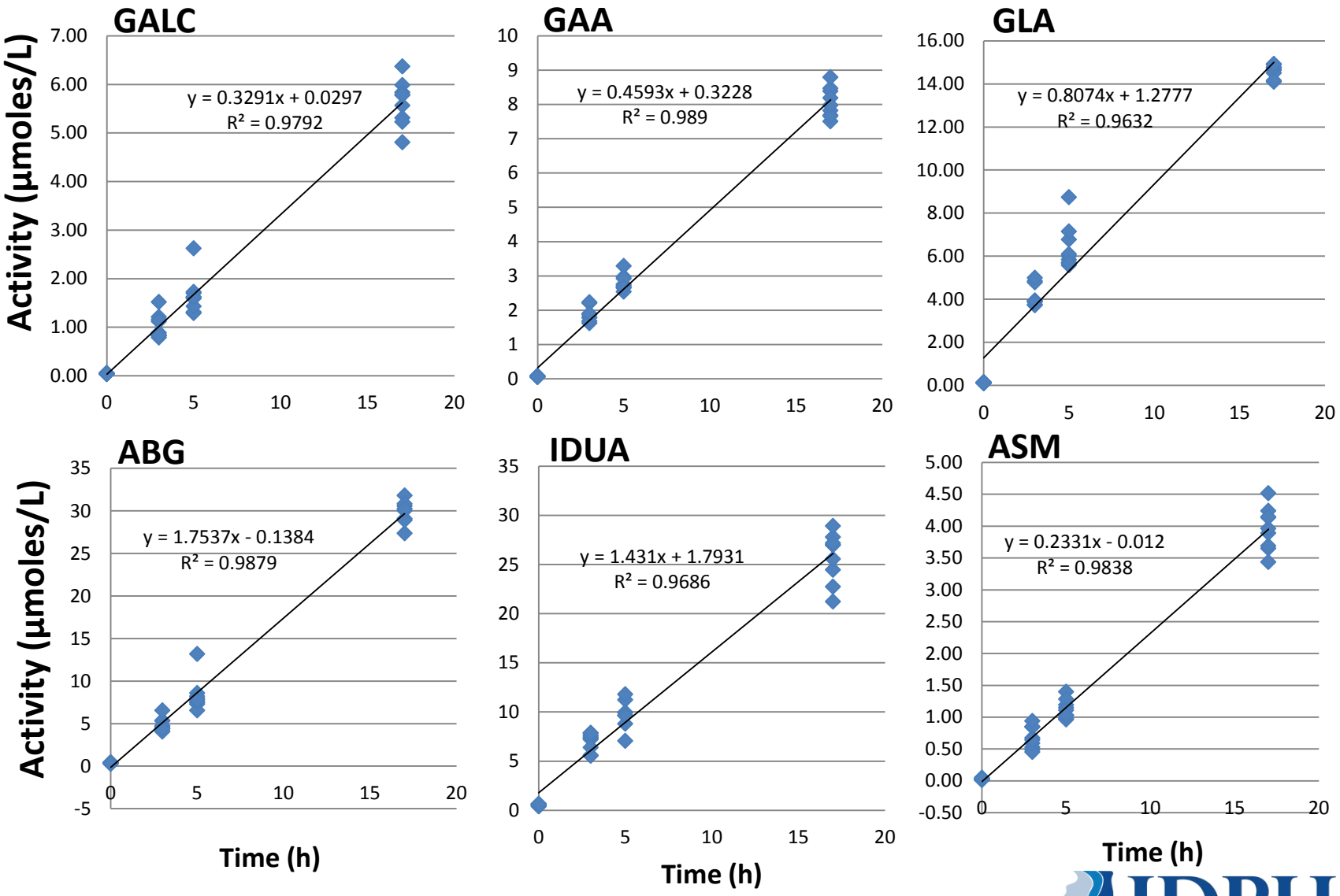
Linearity of Enzyme Reactions



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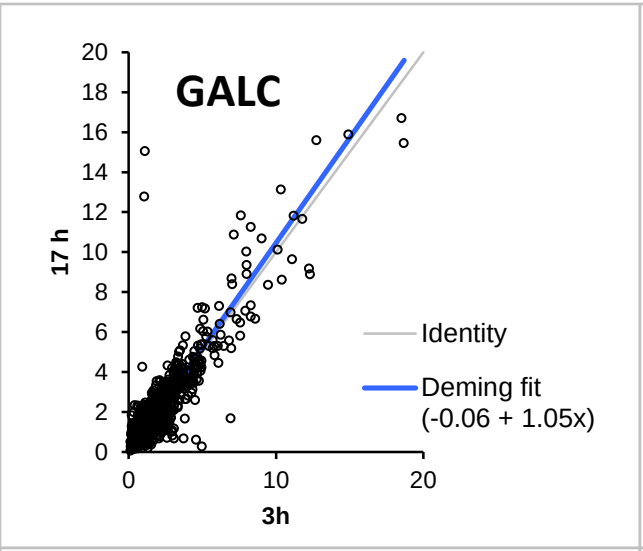
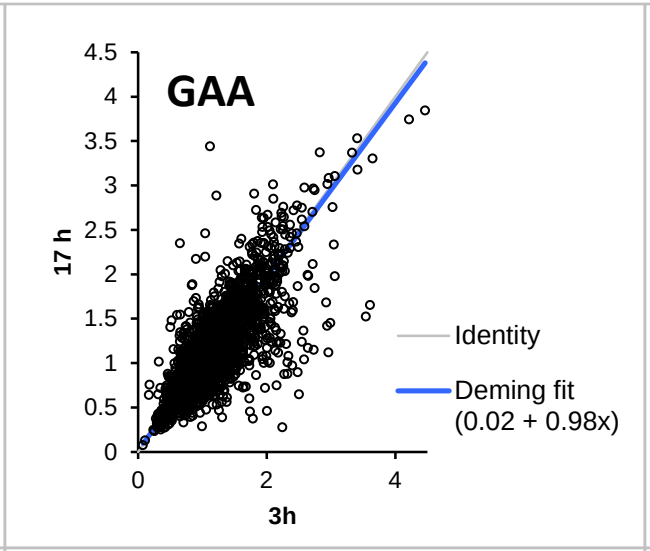
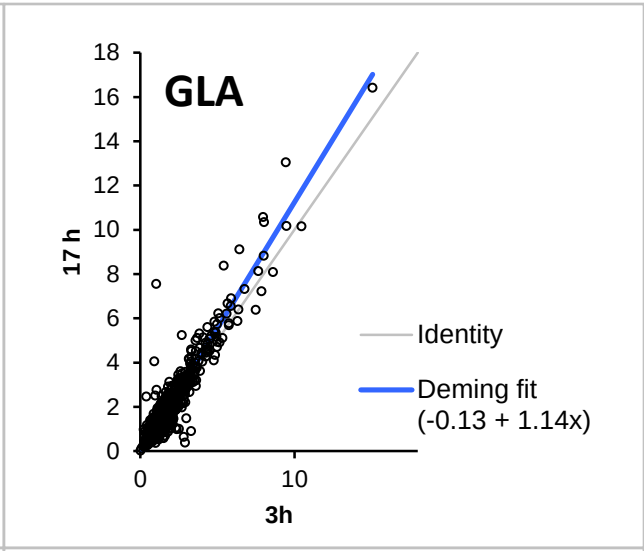
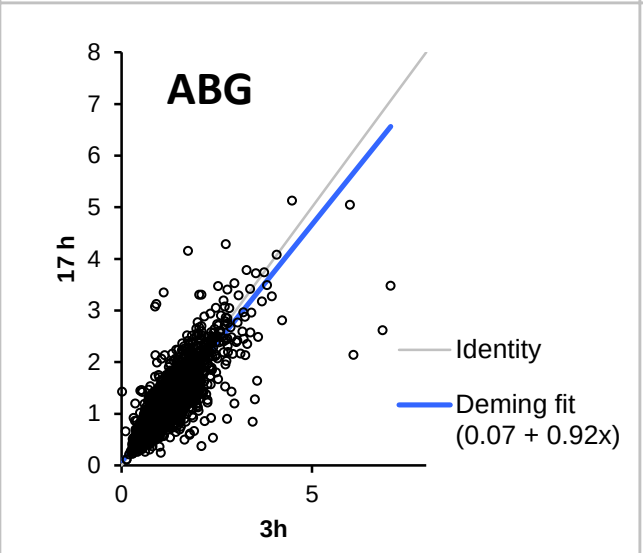
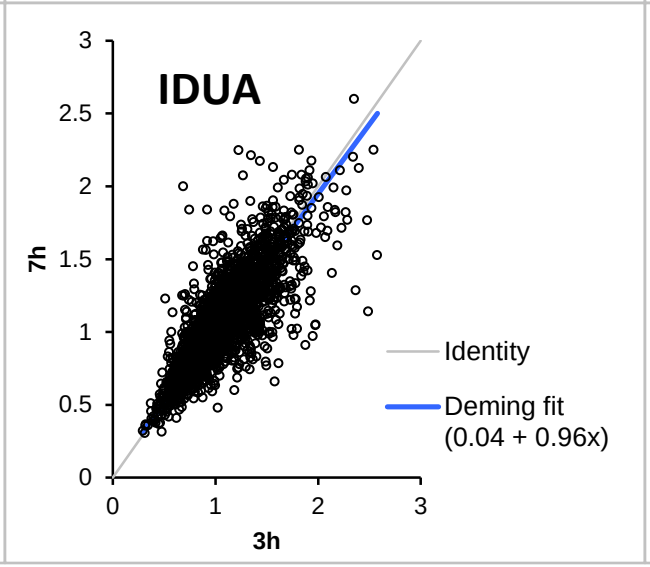
3 h vs 17 h Assays – Percent of Median Activities

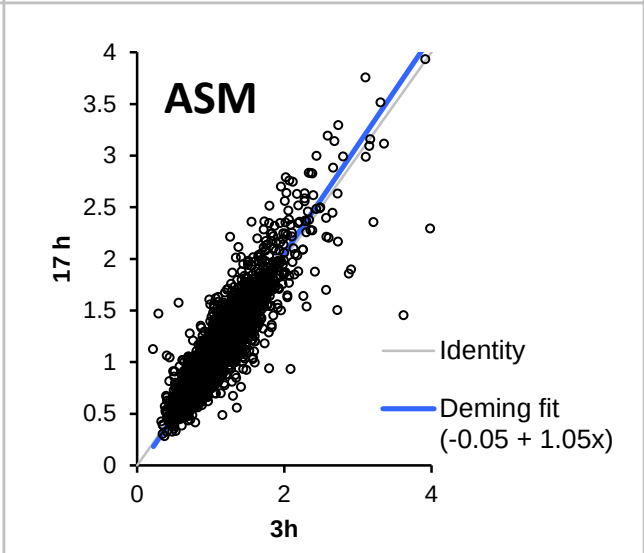












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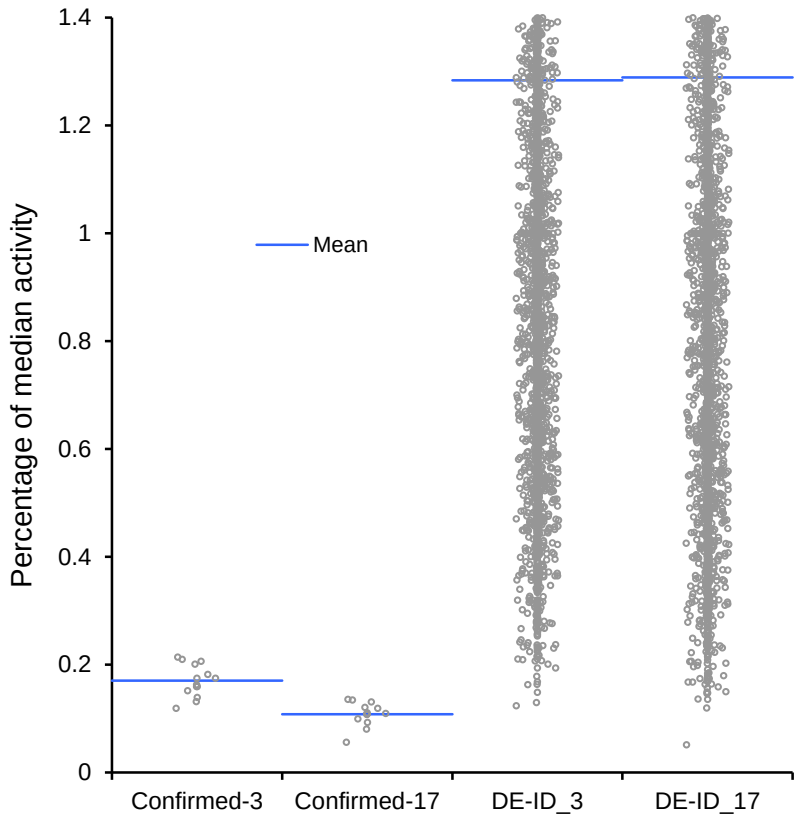
Comparison of 3 h to 17 h incubation for GALC



Longer incubation improves discrimination between confirmed-positive and presumed-negative specimens, increasing specificity.

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Normal and Abnormal Ranges as Percent of Daily Median Activity

	Normal Range	1st Cut-off	Borderline	2nd Cut-off (presumptive positive)
IDUA	> 31%	≤ 35%	> 28 and ≤ 31	≤ 28%
GLA	> 18%	≤ 20%	> 13 and ≤ 18	≤ 13%
GAA	> 28%	≤ 30%	> 23 and ≤ 28	≤ 23%
ASM	> 15%	≤ 20%	> 11 and ≤ 15	≤ 11%
GALC	> 13%	≤ 18%	No Borderline	≤ 13%
ABG	> 20%	≤ 25%	> 17 and ≤ 20	≤ 17%

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Summary of IDPH-CLIA Laboratory Comparison (n=12,000)

	FABRY	GAUCHER	KRABBE	MPS I	NIEMANN PICK A/B	POMPE
Number of Normal Specimens sent to CLIA Lab	69	66	72	54	74	62
Number of Specimens Below 1st Cut-off sent to CLIA Lab	6	9	37	21	1	13
Positives and Borderlines Determined by IDPH	4	4	8	16	1	9
Positives Confirmed by CLIA Laboratory	0	1	4	7	1	2
Diagnosed Cases	0	1	0	0	1	0
Other Resolutions (PD: Pseudodeficiency)			1 PD 2 Carrier 1 Normal	5 PD 1 Normal 1 Pending		1 PD 1 Carrier

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Lessons Learned

- Many different individuals with a wide range of skills need to work together to successfully develop a complex, high-throughput analytical assay.
- The process will take longer than initially anticipated; regular interactions and good communications are vital.
- MS/MS platform permits expanded test menu and multiplexing with a single injection.

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Lessons Learned (cont'd)

- There are many challenges in adapting a research procedure to a high-throughput newborn screening assay (e.g., analytical, personnel, physical plant, and IT). FDA-cleared tests are vastly preferable.
- If at all possible for mandated testing, have legislation or administrative rules written to permit adequate preparation and milestones (e.g., method validation, QC and PT availability, acquisition of high volume equipment, & funding).

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Conclusions

- Very useful for high-throughput newborn screening for six lysosomal enzymes
- Can be adopted to screen 1-6 enzymes depending upon laboratory requirements
- Using 3 hour incubation, first screening results can be obtained within 24 hours of specimen receipt, and positive results can be released after an additional 24 hours.
- For Krabbe, 17 hour incubation should be used for evaluating second cut-off.

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