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# LC-MS/MS: Multiplexed Assay to Detect Five (actually six) Lysosomal Storage Disorders

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**April 16, 2015**

# LSD Testing Timeline for the State of Illinois

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, shown to be negative for Pompe by second-tier tests
- Five confirmed-positive Fabry (GLA)
- Two confirmed-positive Gaucher (ABG)

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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.

2013 – Acquisition of equipment and staff; method development

2014 – Method validation and limited pilot testing

2015 – Statewide testing expected to begin mid-year

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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** | **0.1 M, pH 4.4** |
|---|---|
| **Sodium cholate** | **10 g/L** |
| **Acarbose** | **0.08 M** |
| **N-Acetyl-α-galactosamine** | **50 mM** |
| IDUA Substrate (S), Internal Standard (IS) | 500 µM, 3.5 µM |
| GLA S, IS | 600 µM, 1.2 µM |
| GAA S, IS | 200 µM, 2.0 µM |
| ASM S, IS (d7-C6 Ceramide) | 150 µM, 2.5 µM |
| GALC S, IS (d7-C8 Ceramide) | 450 µM, 2.5 µM |
| ABG S, IS (d7-C12 Ceramide) | 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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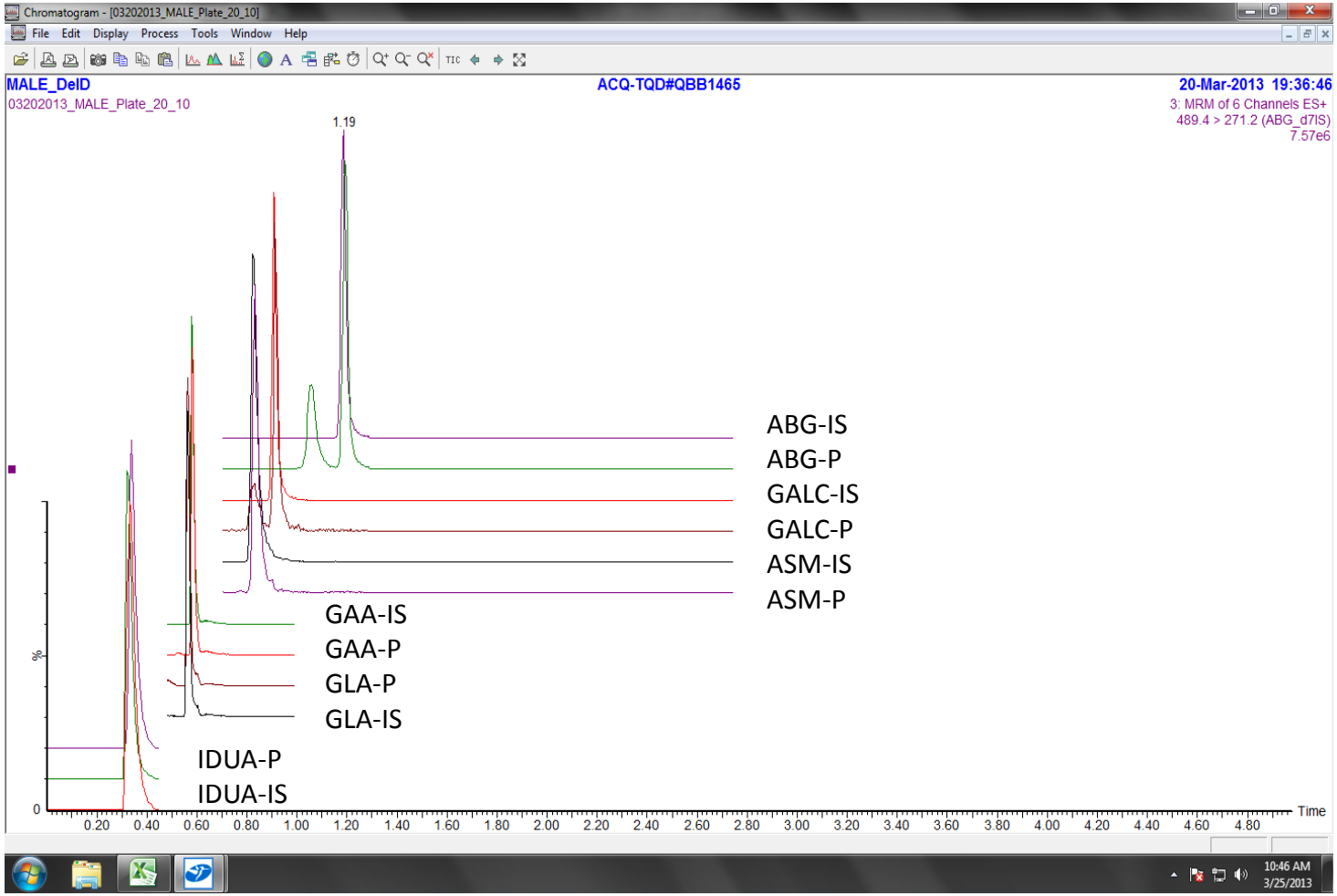
# UPLC Chromatogram

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# Validation of Final Method

- 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.
- Refine cutoff values
- Exchange specimens with a qualified testing laboratory to establish comparability of results.

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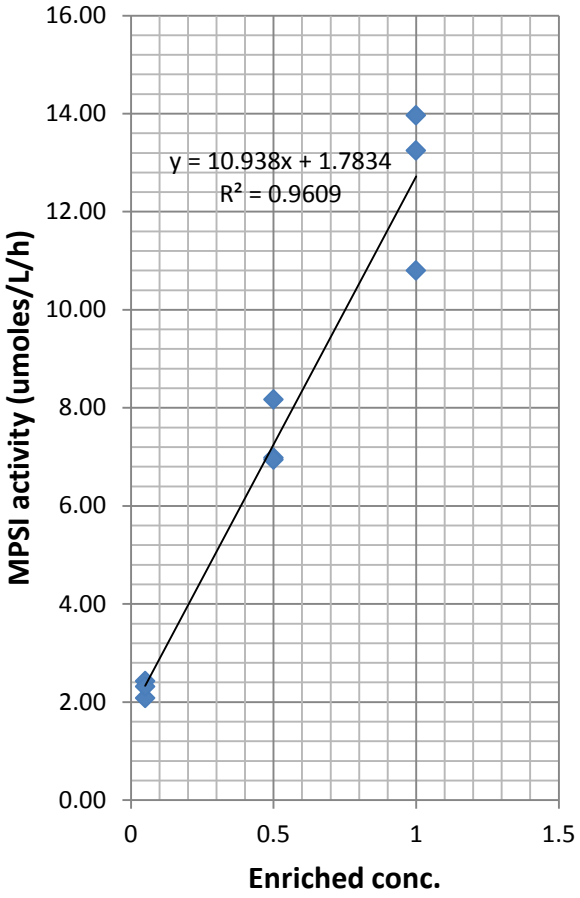
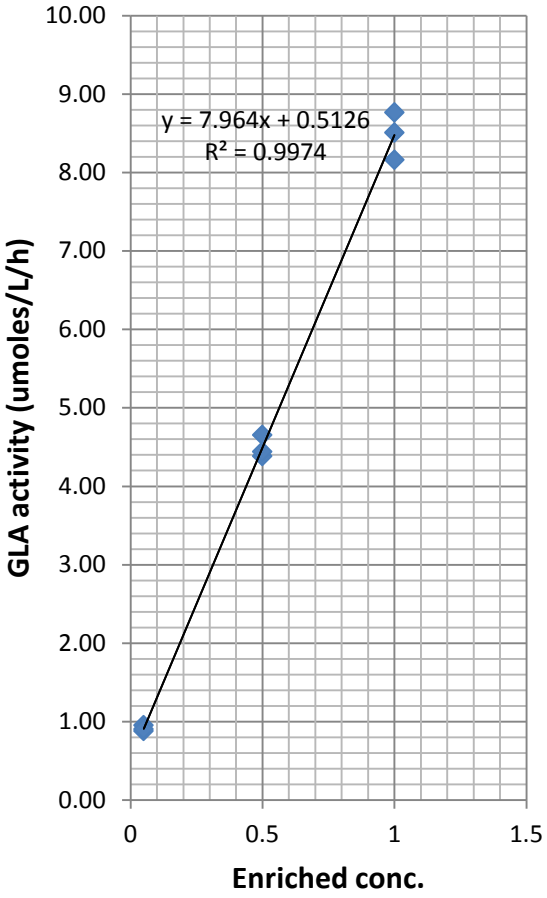
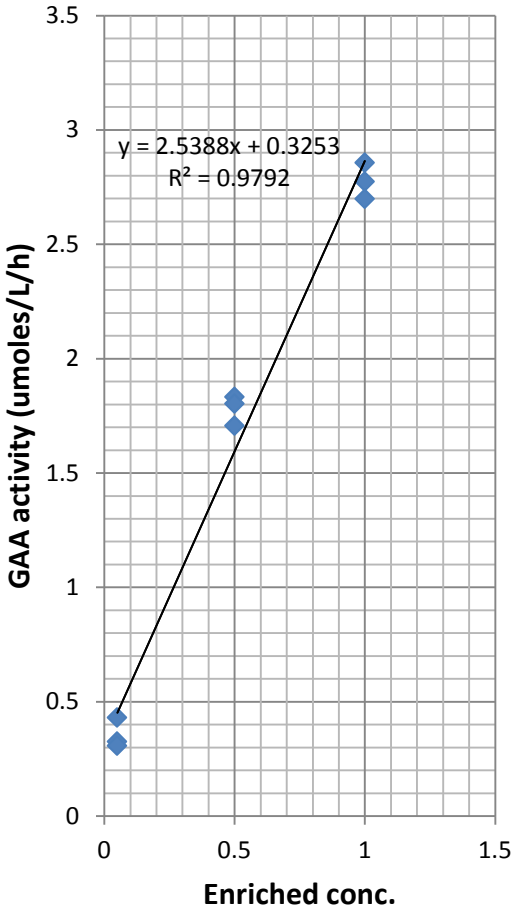
# CDC QC levels for IDUA, GLA & GAA

(MPS-I, Hurler) (Fabry) (Pompe)

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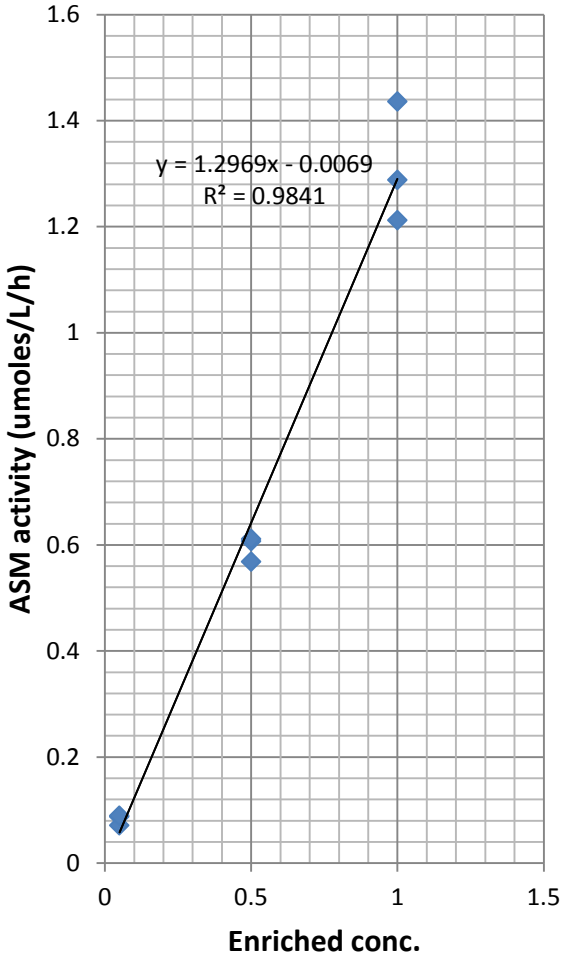
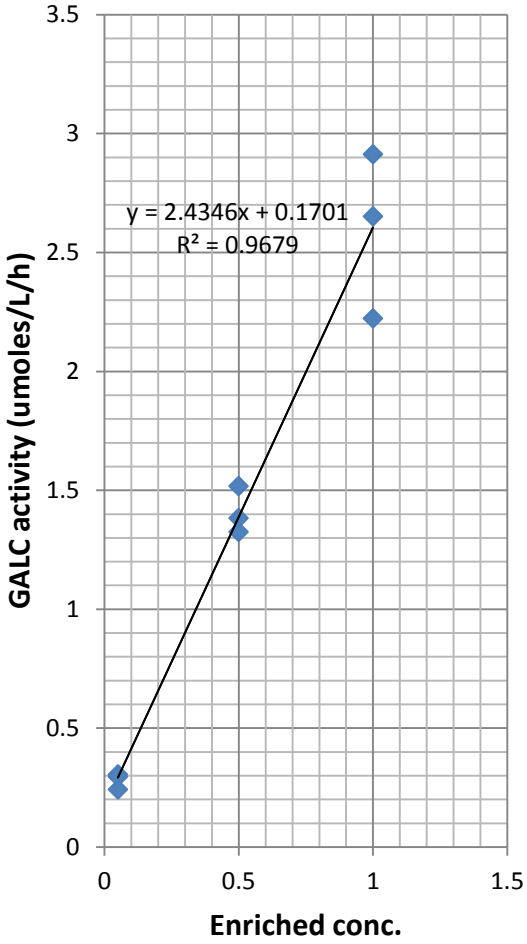
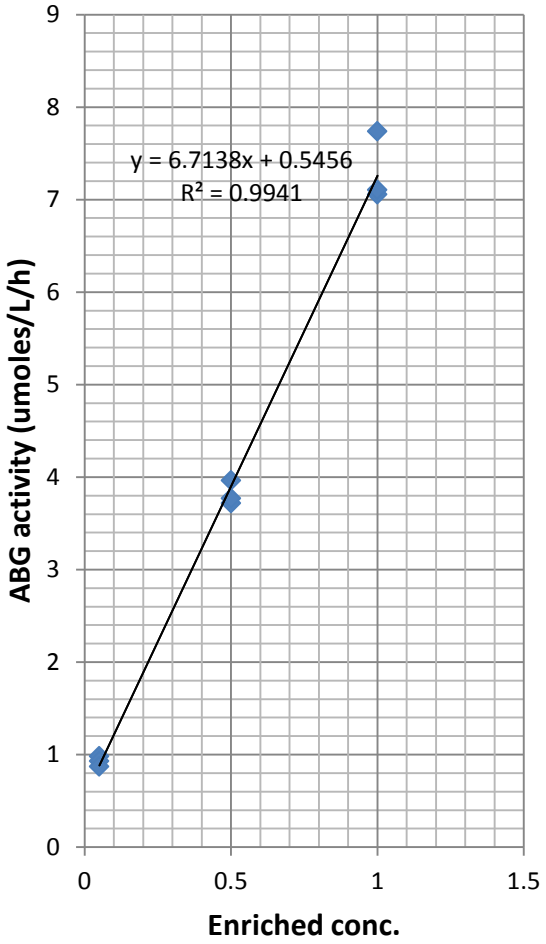
# CDC QC Levels for ASM, GALC & ABG

(Niemann-Pick) (Krabbe) (Gaucher)

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# Normal and Abnormal Ranges as Percent of Daily Median Activity

| | Normal Range | 1st Cut-off (Percentile) | Borderline (Percentile) | 2nd Cut-off: presumptive positive (Percentile) |
|---|---|---|---|---|
| **IDUA** | > 31% | ≤ 35% (0.25) | > 28 and ≤ 31% (0.12) | ≤ 28% (0.09) |
| **GLA** | > 18% | ≤ 20% (0.10) | > 13 and ≤ 18% (0.09) | ≤ 13% (0.03) |
| **GAA** | > 28% | ≤ 30% (0.40) | > 23 and ≤ 28% (0.27) | ≤ 23% (0.16) |
| **ASM** | > 15% | ≤ 20% (0.03) | > 11 and ≤ 15% (0.02) | ≤ 11% (N/A) |
| **GALC** | > 13% | ≤ 20% (0.32) | No Borderline | ≤ 15% (0.11) |
| **ABG** | > 20% | ≤ 25% (0.15) | > 17 and ≤ 20% (0.09) | ≤ 17% (0.05) |

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# Specimen Exchange

- Once confidence in the method has been established, test identified specimens prospectively and send those with activities below a generous first cutoff to a qualified (i.e., CLIA-certified) laboratory.
- Compare second cutoff results with results from the certified laboratory.
- Send reports from the certified laboratory to submitters and short-term follow-up program.
- Reconsider the role of other stakeholders and the value of communication in this process.

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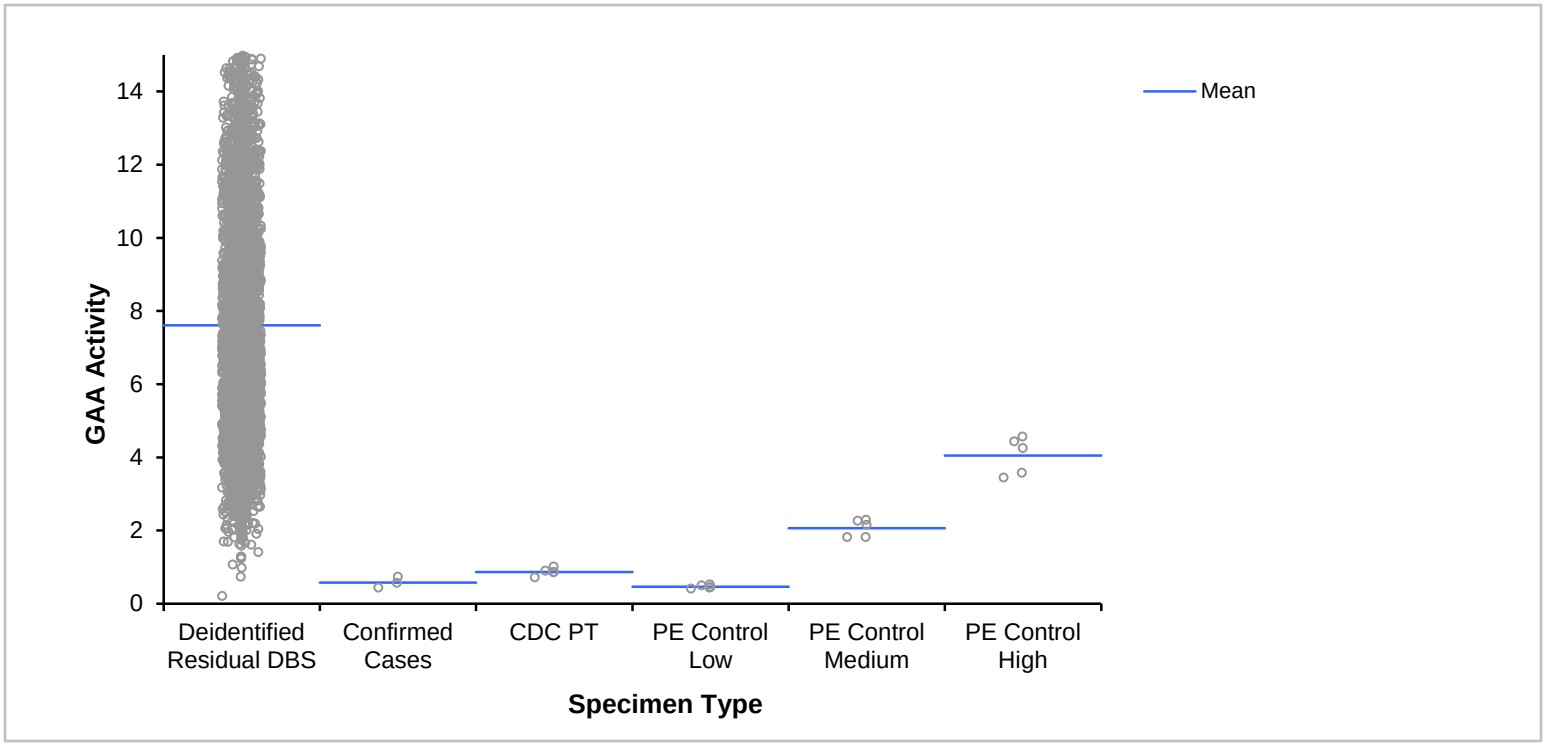
# Assay Results for GAA (Pompe)




| | n | Min | Mean | Max |
|---|---|---|---|---|
| Deidentified Residual DBS | 12392 | 0.21 | 7.605 | 83.96 |
| Confirmed Cases | 3 | 0.43 | 0.575 | 0.73 |
| CDC PT | 5 | 0.71 | 0.866 | 1.01 |
| PE Control Low | 5 | 0.41 | 0.461 | 0.52 |
| PE Control Medium | 5 | 1.81 | 2.065 | 2.29 |
| PE Control High | 5 | 3.44 | 4.051 | 4.56 |

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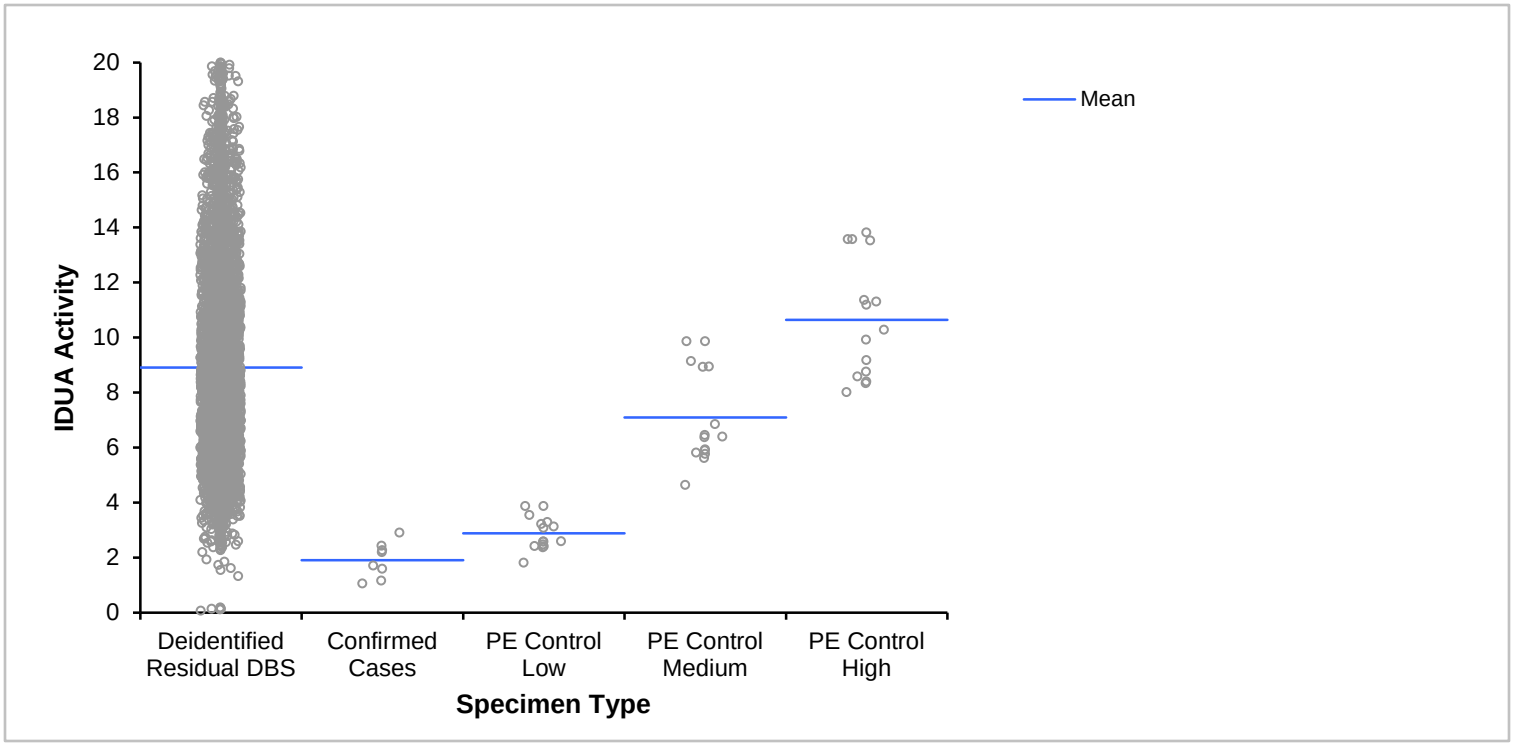
# Assay Results for IDUA (MPS-I)




| | n | Min | Mean | Max |
|---|---|---|---|---|
| Deidentified Residual DBS | 12396 | 0.06 | 8.912 | 130.31 |
| Confirmed Cases | 8 | 1.05 | 1.906 | 2.90 |
| PE Control Low | 15 | 1.809 | 2.8751 | 3.859 |
| PE Control Medium | 15 | 4.630 | 7.0926 | 9.857 |
| PE Control High | 15 | 8.011 | 10.6463 | 13.806 |

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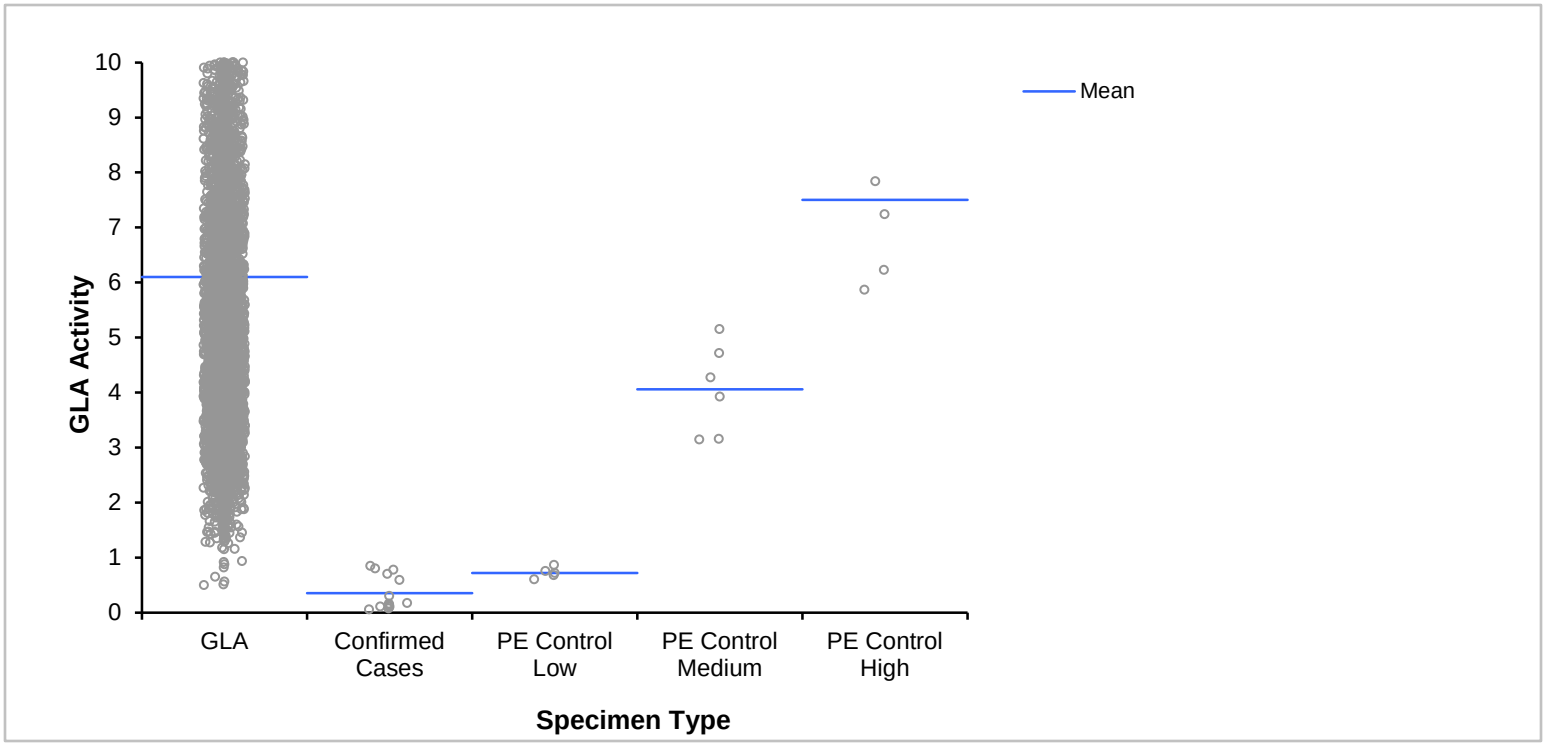
# Assay Results for GLA (Fabry)




| | n | Min | Mean | Max |
|---|---|---|---|---|
| Deidentified Residual DBS | 12391 | 0.49 | 6.098 | 646.51 |
| Confirmed Cases | 14 | 0.06 | 0.350 | 0.84 |
| PE Control Low | 5 | 0.60 | 0.721 | 0.86 |
| PE Control Medium | 6 | 3.14 | 4.059 | 5.15 |
| PE Control High | 5 | 5.86 | 7.505 | 10.37 |

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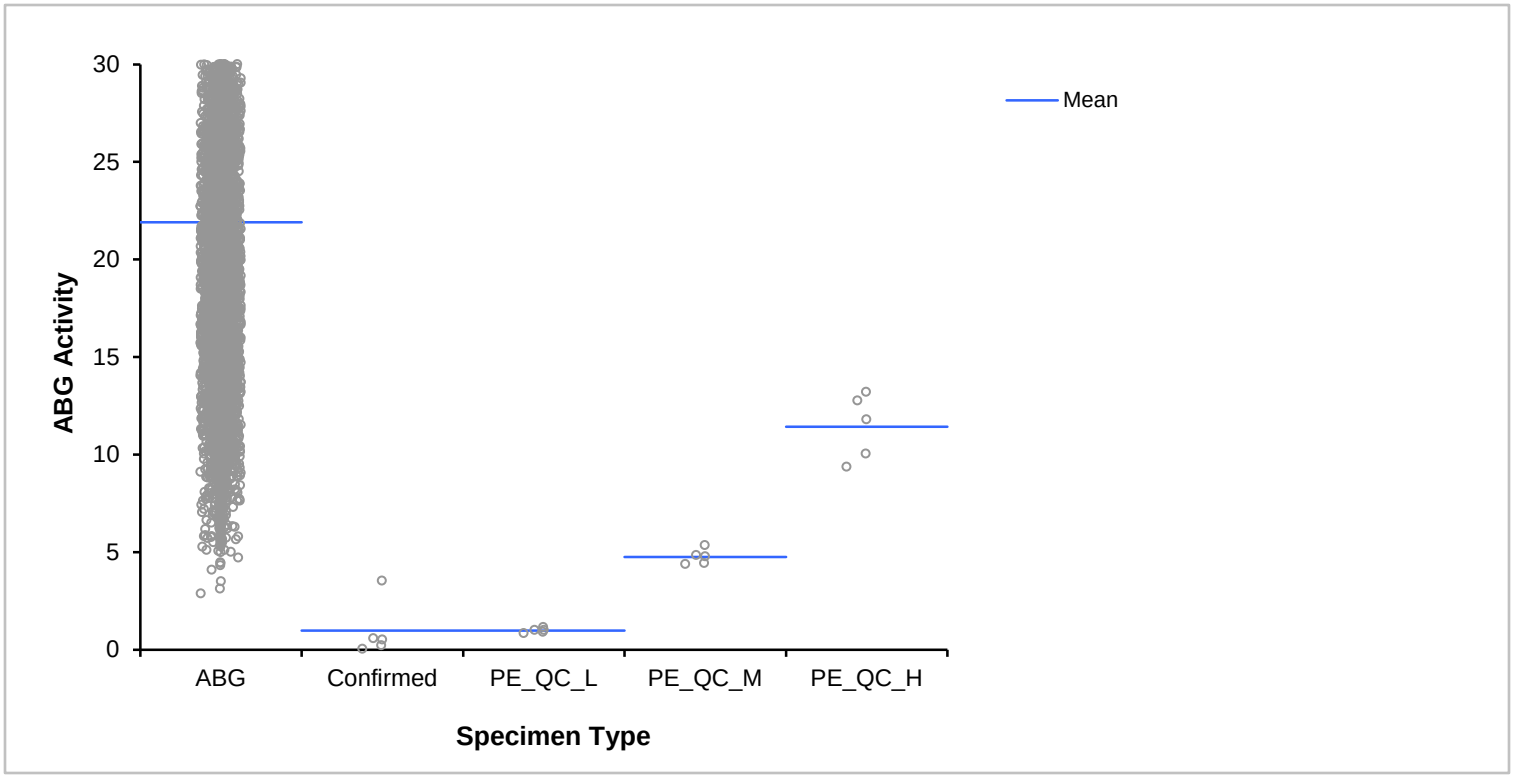
# Assay Results for ABG (Gaucher)




| | n | Min | Mean | Max |
|---|---|---|---|---|
| Deidentified Residual DBS | 12387 | 2.87 | 21.905 | 553.02 |
| Confirmed Cases | 5 | 0.04 | 0.976 | 3.53 |
| PE Control Low | 5 | 0.84 | 0.978 | 1.14 |
| PE Control Medium | 5 | 4.38 | 4.756 | 5.35 |
| PE Control High | 5 | 9.37 | 11.435 | 13.20 |

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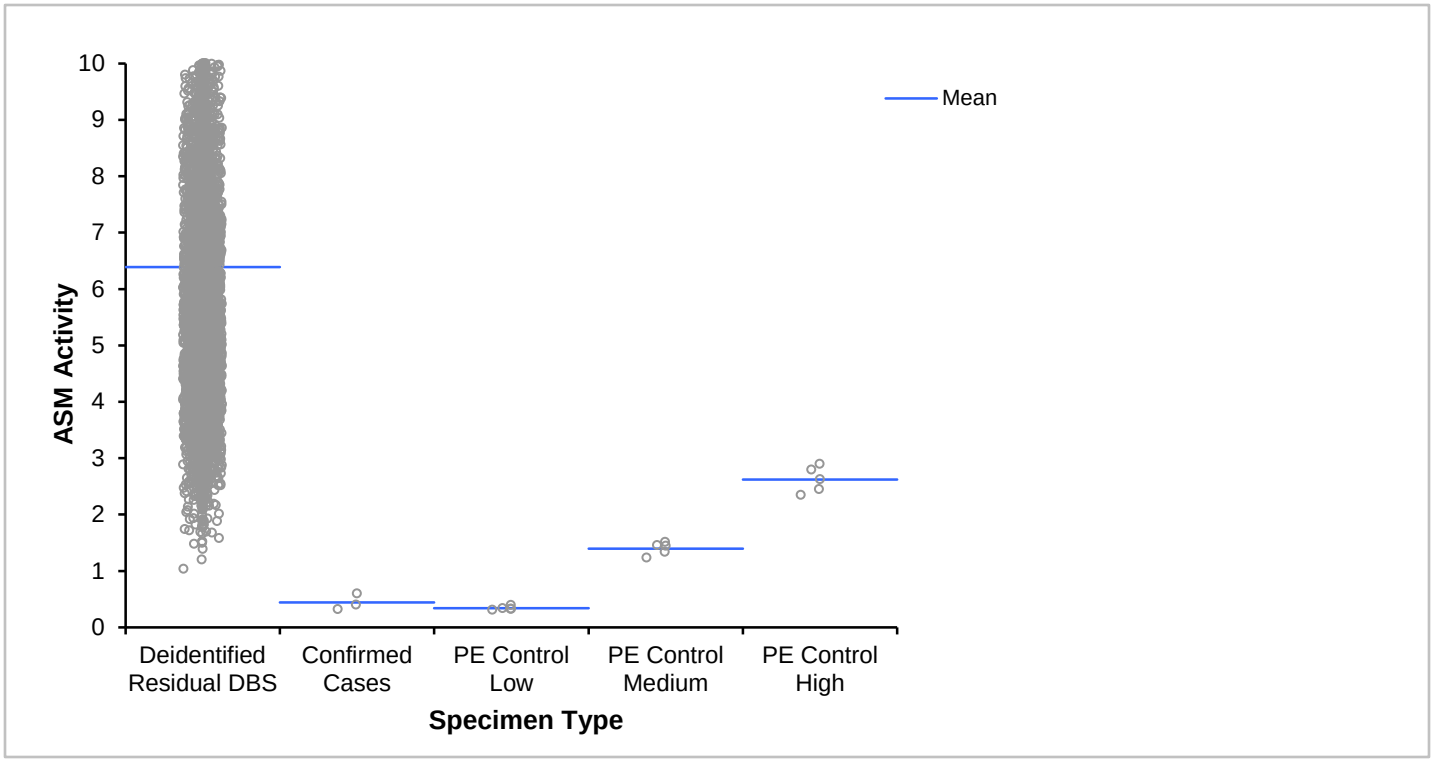
# Assay Results for ASM (Niemann-Pick A/B)




| | n | Min | Mean | Max |
|---|---|---|---|---|
| Deidentified Residual DBS | 12385 | 1.04 | 6.391 | 6317.28 |
| Confirmed Cases | 3 | 0.32 | 0.440 | 0.60 |
| PE Control Low | 5 | 0.311 | 0.3370 | 0.392 |
| PE Control Medium | 5 | 1.234 | 1.3954 | 1.510 |
| PE Control High | 5 | 2.346 | 2.6216 | 2.894 |

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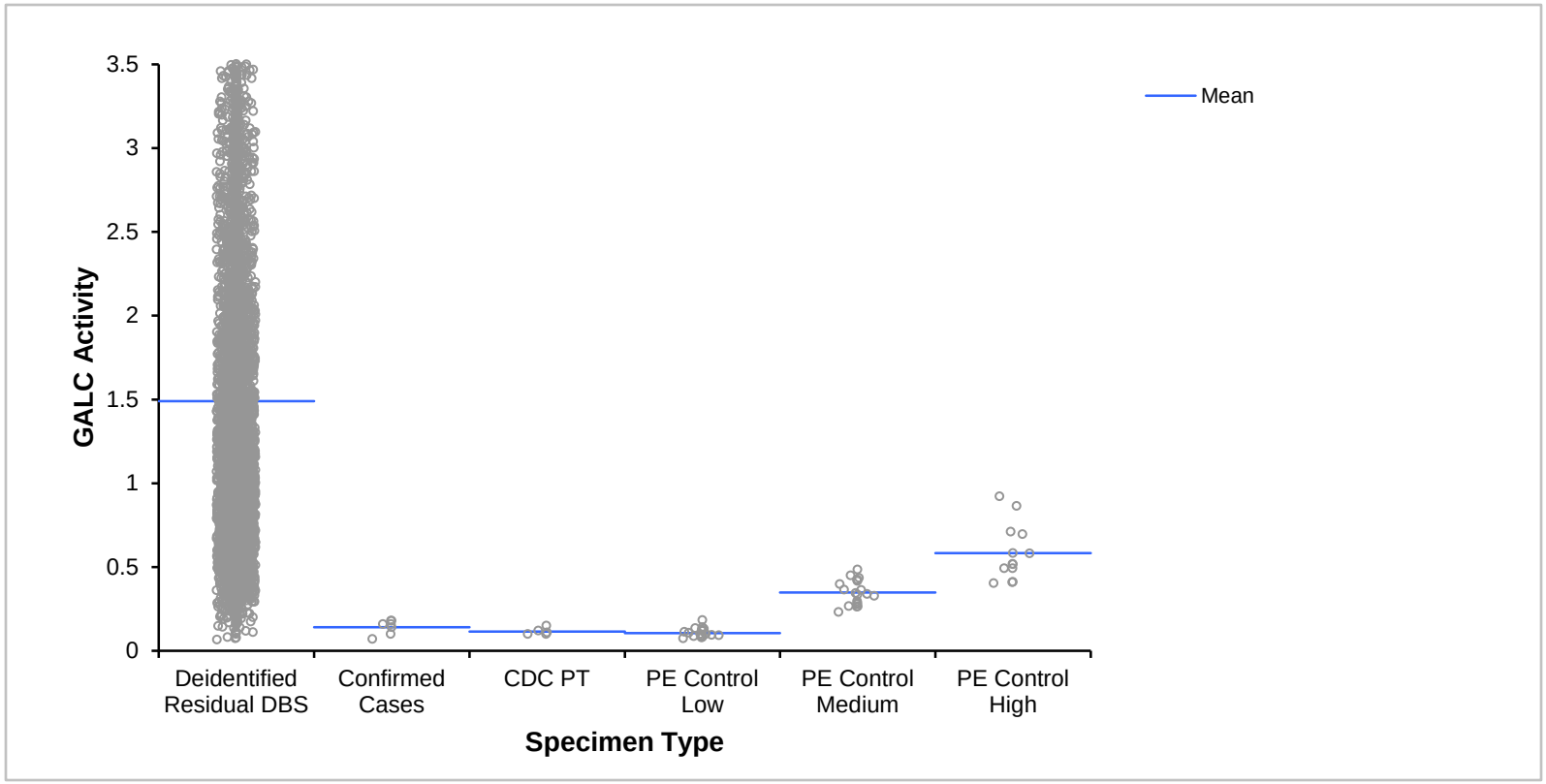
# Assay Results for GALC (Krabbe)




| | n | Min | Mean | Max |
|---|---|---|---|---|
| Deidentified Residual DBS | 12222 | 0.07 | 1.49 | 34.49 |
| Confirmed Cases | 7 | 0.07 | 0.14 | 0.18 |
| CDC PT | 5 | 0.10 | 0.12 | 0.15 |
| PE Control Low | 21 | 0.07 | 0.11 | 0.18 |
| PE Control Medium | 20 | 0.23 | 0.35 | 0.48 |
| PE Control High | 13 | 0.40 | 0.58 | 0.92 |

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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<br>2 Carrier<br>1 Normal | 5 PD<br>1 Normal<br>1 Lost to follow-up | | 1 PD<br>1 Carrier |

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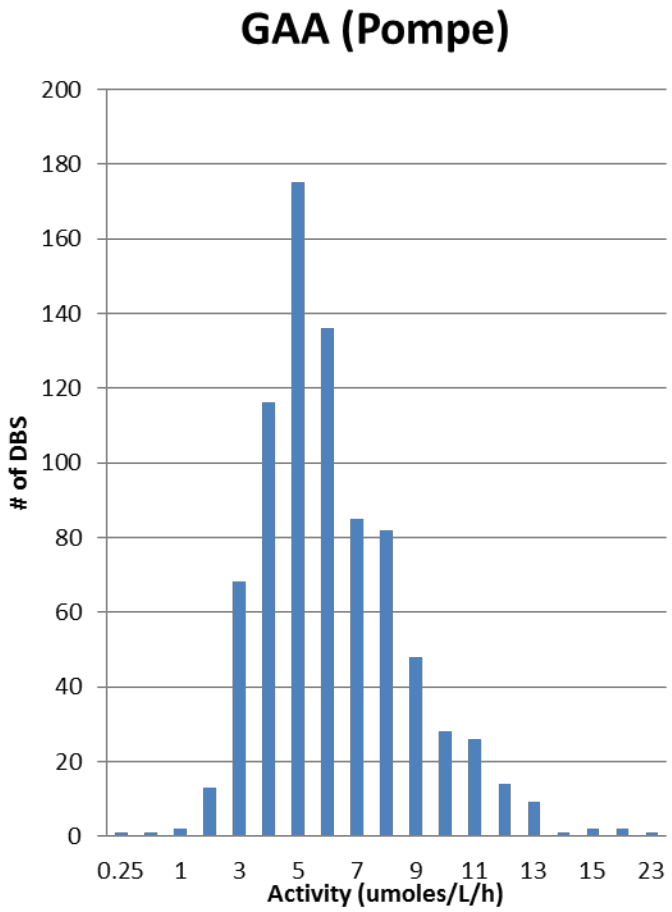
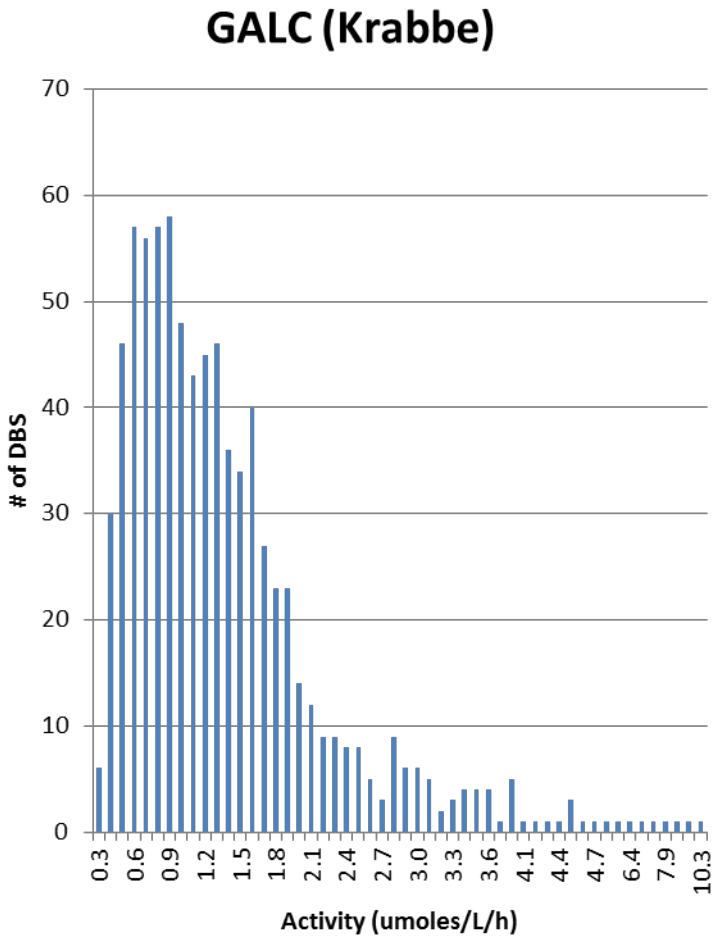
# Specific Activity Distribution for GALC and GAA

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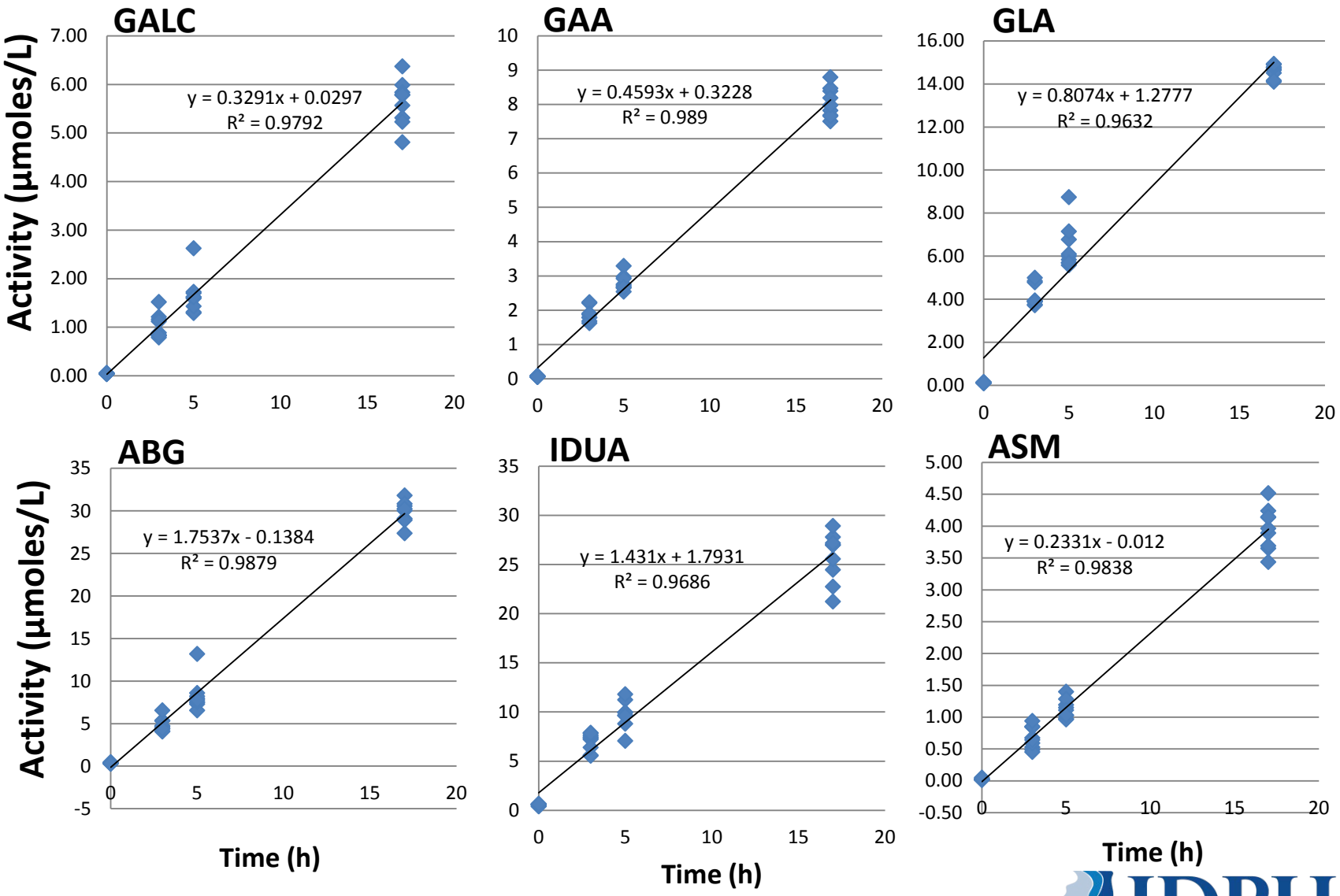
# Linearity of Enzyme Reactions

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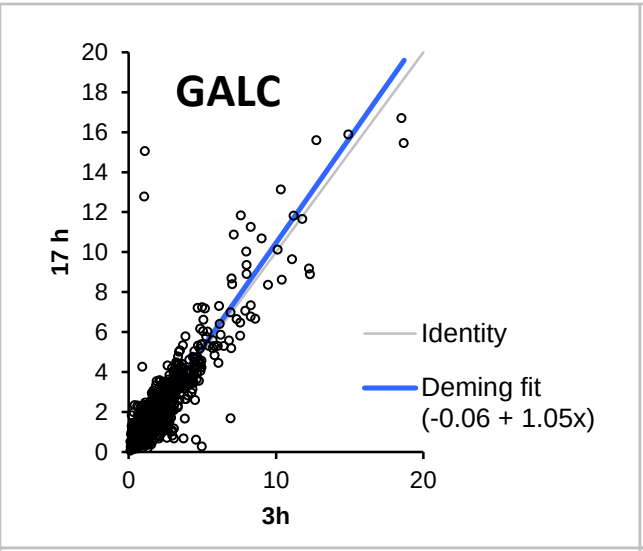
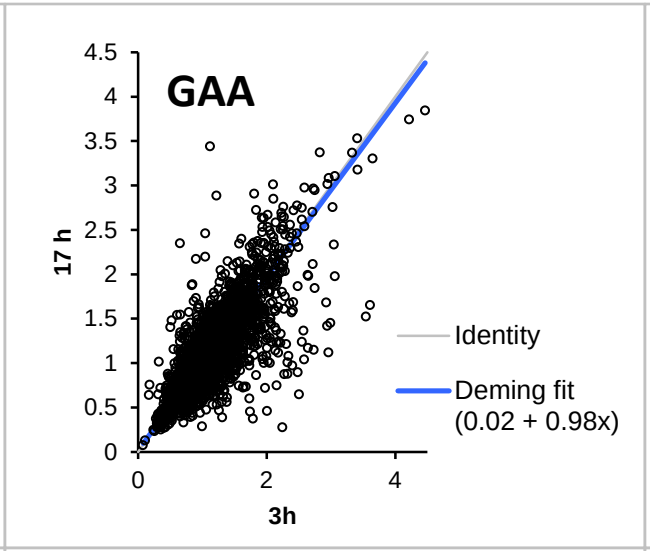
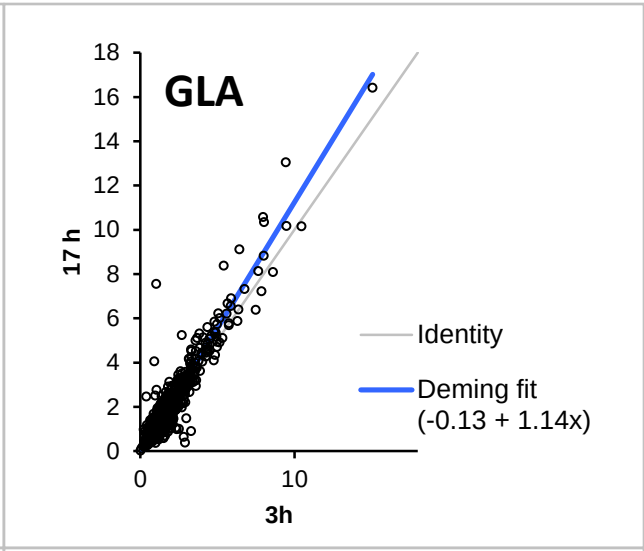
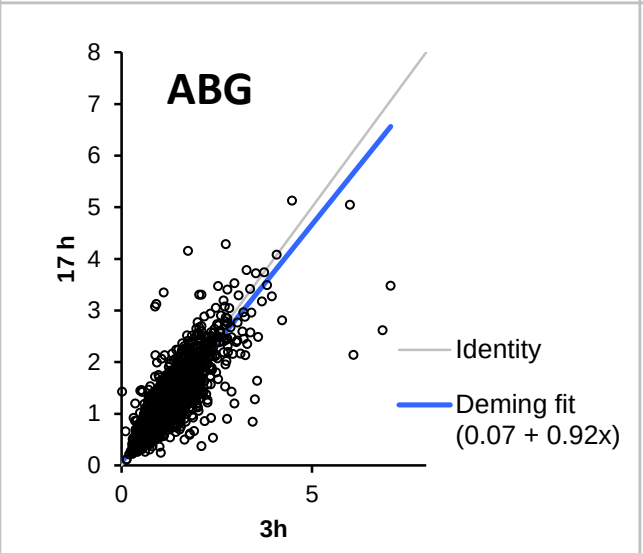
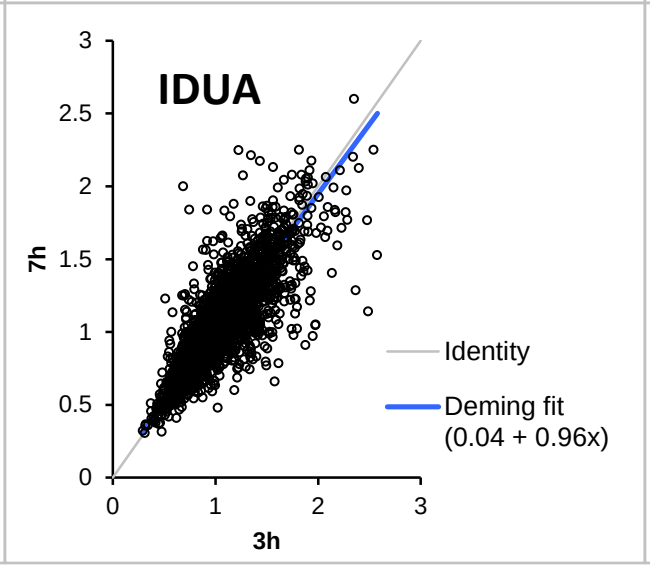
# 3 h vs 17 h Assays – Percent of Median Activities

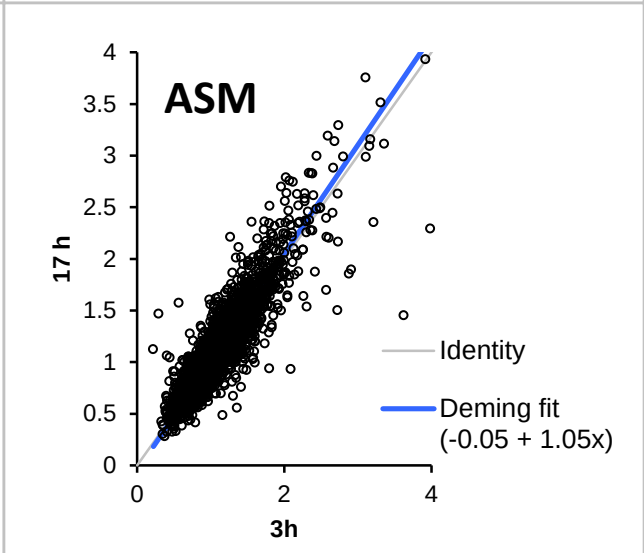
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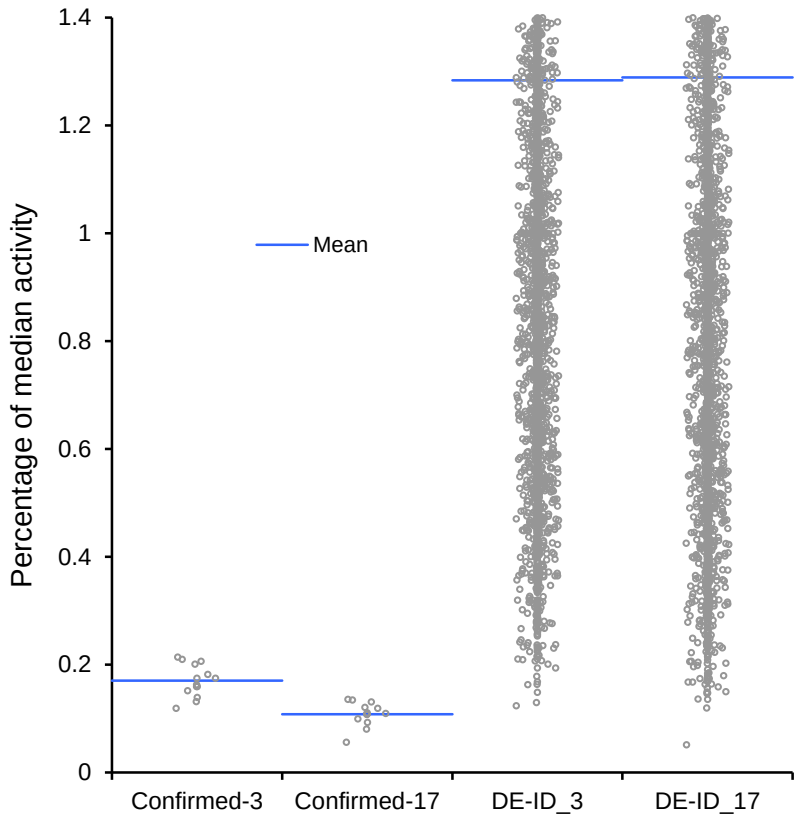
# Comparison of 3 h to 17 h incubation for GALC




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

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# 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 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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# Acknowledgments


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# THANK YOU

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# Update on Testing for Lysosomal Storage Disorders from the Illinois Newborn Screening Program – Pilot Testing and Beyond

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**Atlanta, GA**
**April 17, 2015**

# Prospective Pilot

- Limited pilot started in late 2014 with four hospitals; later expanded to eight in order to provide statistically valid daily median
- Database/LIMS considerations for lab, follow-up program, and submitters. Largest submitter on HL7 messaging
- Decision was made to begin pilot without Krabbe screening because of inability to arrange timely DNA testing
- Follow-up protocols in place for all disorders

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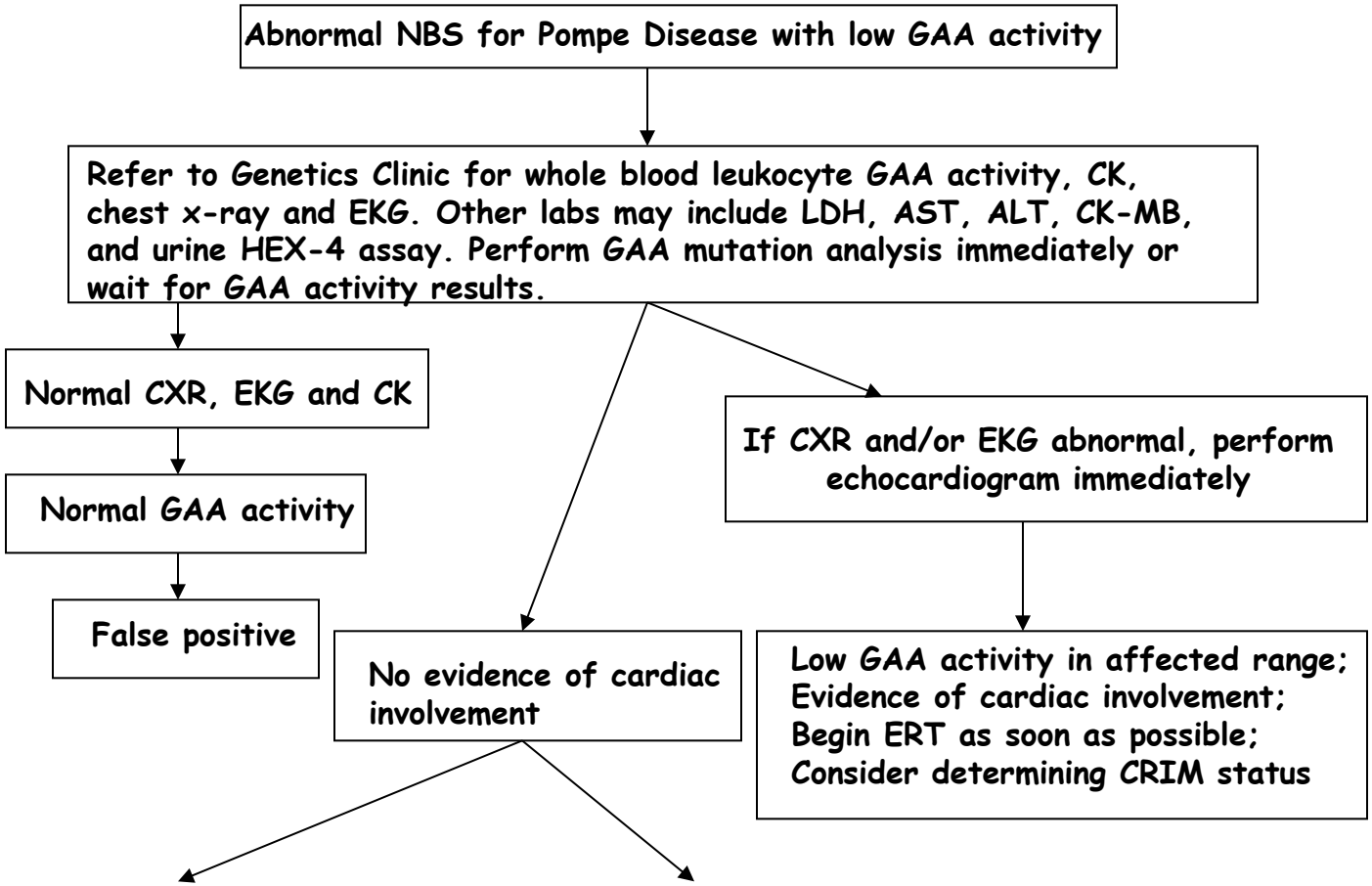
# Pompe Disease Follow-up Algorithm




**(See Follow-up Algorithm, continued)**

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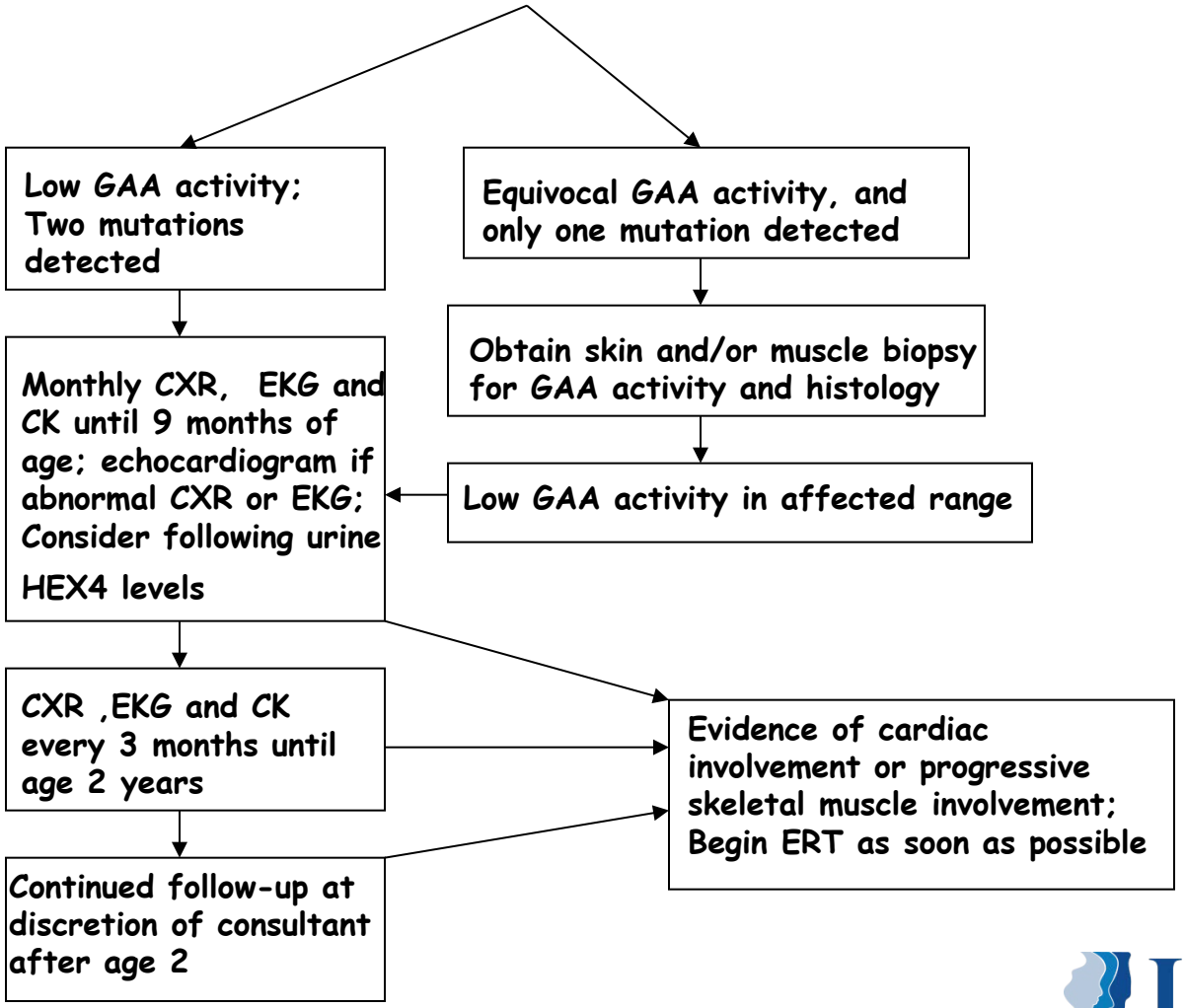
# Pompe Disease Follow-up Algorithm (Continued)

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# New York State Proposed Timeline: Analytical Testing

| SUN | MON | TUE | WED | THU | FRI | SAT |
|---|---|---|---|---|---|---|
| 1 **Infant born** | 2 **NBS collected & mailed** | 3 **NBS received, testing begins** | 4 | 5 **Prelim Krabbe results, DNA started** | 6 **Krabbe, DNA results; referral made** | 7 |
| 8 | 9 **Infant seen, blood sent** | 10 **Blood received** | 11 **Low activity confirmed; HLA, identity done** | 12 **Infant admitted** | 13 **Work-up complete, UCB unit found (?)** | 14 **Family Decision** |
| 15 | 16 **Family arrives at transplant center** | 17 | 18 **Chemo prep begins** | 19 | 20 | 21 |
| 22 | 23 | 24 | 25 | 26 | 27 | 28 **Stem cell infusion** |

# Decision to Postpone Krabbe Screening

- Limited options for DNA analysis
- Contract required to ensure uninterrupted second-tier analysis
- Insistence by follow-up specialists that DNA results accompany enzyme reporting, although concern is more with the number of unnecessary call outs rather than maintaining timeline for transplantation

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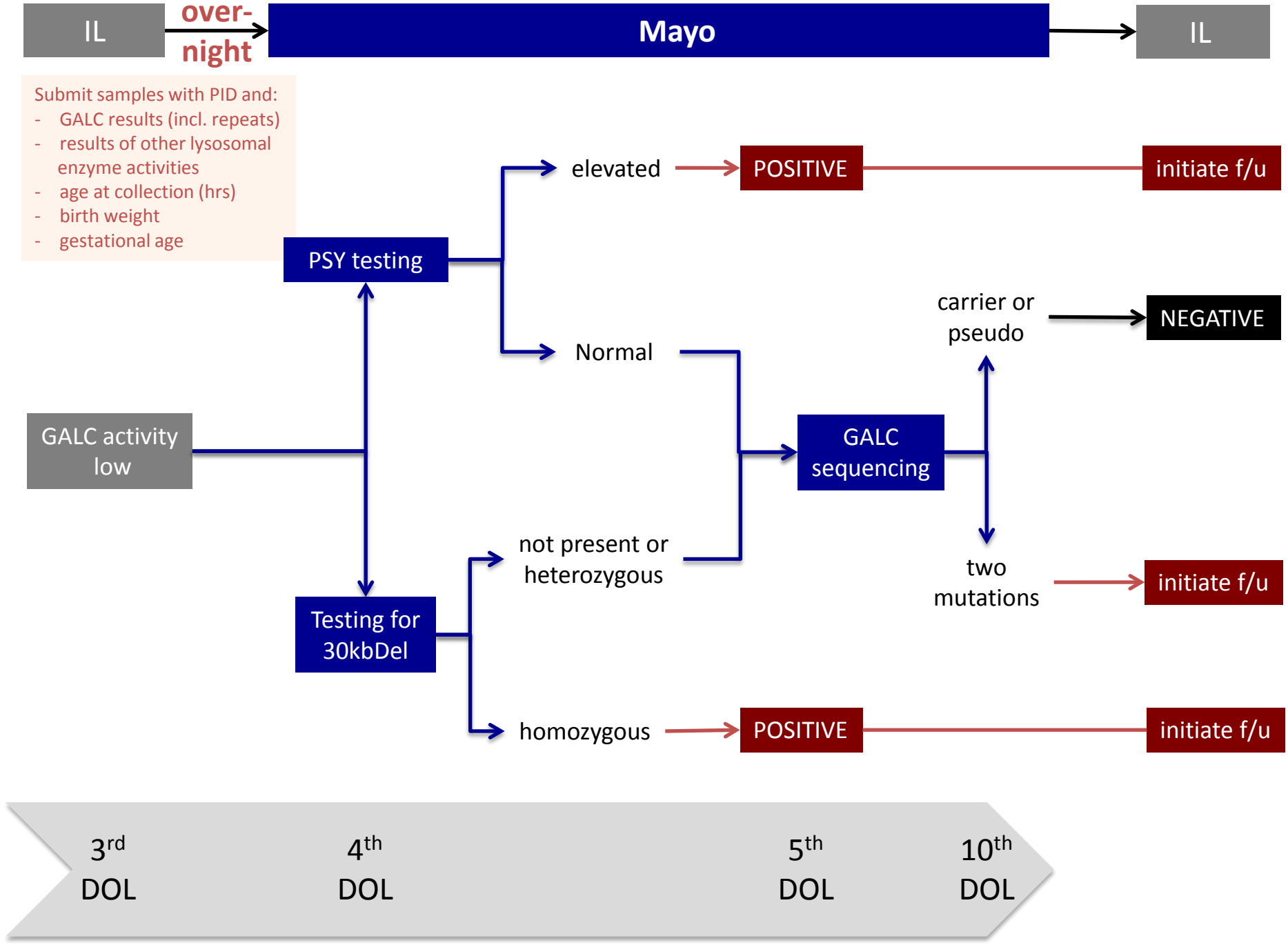
IL
over-night
Mayo
IL
Submit samples with PID and:
- GALC results (incl. repeats)
- results of other lysosomal enzyme activities
- age at collection (hrs)
- birth weight
- gestational age
PSY testing
elevated
POSITIVE
initiate f/u
Normal
carrier or pseudo
NEGATIVE
GALC activity low
GALC sequencing
not present or heterozygous
two mutations
initiate f/u
Testing for 30kbDel
homozygous
POSITIVE
initiate f/u
3rd DOL
4th DOL
5th DOL
10th DOL

# Six-day Workflow for LSD Testing

| Monday (4) | Tuesday (4) | Wednesday (5) | Thursday (6) | Friday (5) | Saturday |
|---|---|---|---|---|---|
| Specimen receipt, punch, incubate O/N | Process incubation, load MS/MS to run O/N | Print repeat list, punch repeats, incubate O/N, send out for Krabbe DNA | Process incubation, load repeats on MS/MS to run O/N | Call out positives, call/email about Krabbe DNA | |
| | Specimen receipt, punch, incubate O/N | Process incubation, load MS/MS to run O/N | Print repeat list, punch repeats, incubate O/N, send out for Krabbe DNA | Process incubation, load repeats on MS/MS to run O/N | Call out positives, call/email about Krabbe DNA |
| Call out positives, call/email about Krabbe DNA | | Specimen receipt, punch, incubate O/N | Process incubation, load MS/MS to run O/N | Print repeat list, punch repeats, incubate O/N, send out for Krabbe DNA | Process incubation, load repeats on MS/MS to run until Monday |
| incubate O/N, send out for Krabbe DNA | Process incubation, load repeats on MS/MS to run O/N | Call out positives, call/email about Krabbe DNA | Specimen receipt, punch, incubate O/N | Process incubation, load MS/MS to run O/N | Print repeat list, punch repeats, refrigerate until Monday |

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# Six-day Workflow for LSD Testing (Continued)

| | Monday (4) | Tuesday (4) | Wednesday (5) | Thursday (6) | Friday (5) | Saturday |
|---|---|---|---|---|---|---|
| | Print repeat list, punch repeats, incubate O/N, send out for Krabbe DNA | Process incubation, load repeats on MS/MS to run O/N | Call out positives, call/email about Krabbe DNA | | Specimen receipt, punch, incubate O/N | Process incubation, load MS/MS to run until Monday |
| MS/MS Activity Started that Day | O/N Run | O/N Run | O/N Run | O/N Run | O/N Run | Run until Monday |
| Content of Finished MS/MS Runs | Nothing | Monday specimens & Thurs, Fri repeats | Tuesday specimens, no repeats | Wed specimens & Mon repeats | Thursday specimens & Tues repeats | Friday specimens & Wed repeats |

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# Five LSD Pilot Study Summary n=10,108

| Enzyme | Disorder | DBS Repeated TBC | % Repeat Rate | Presumptive Positive called | % Positive Rate | BL | Confirmation-Positive Callout | % Confirmed cases | Range Positive Callout (% daily median) | BL Repeat |
|---|---|---|---|---|---|---|---|---|---|---|
| IDUA | MPS-I | 25 | 0.25 | 7 | 0.07 | 1 | 4 Normal; 3 Pending | 0 | 18-28 | 1 Repeat Normal |
| GLA | Fabry | 10 | 0.10 | 3 | 0.03 | 2 | 2 Normal; 1 pending | 0 | 8.4-11 | 2 Repeat Normal |
| GAA | Pompe | 38 | 0.38 | 11 | 0.11 | 6 | 4 Normal; 7 Pending | 0 | 18.3-22.7 | 2 Repeat Normal; 1 Repeat Abnormal; 1 Expired; 2 Pending |
| ASM | Niemann Pick A/B | 2 | 0.02 | 0 | 0.00 | 1 | -- | 0 | -- | 1 Repeat Normal |
| ABG | Gaucher | 15 | 0.15 | 3 | 0.03 | 2 | 2 Normal Repeat; 1 Pending | 0 | 12.5-16.3 | 2 Repeat Normal |

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# IDPH Consent Form

Please indicate if you are willing to share information regarding the newborn screening test result or blood specimen from you or your child. This information will NOT include names.

If you are willing to share this information, please indicate your consent by initialing beside the information you agree to share below:

___ I consent to my child's/my newborn screening diagnostic testing results and treatment information, which is provided to the Illinois Department of Public Health, being shared with medical specialists and public health experts.

___ I consent to have my child's/my residual newborn screening blood spot shared by the Illinois Department of Public Health with one or more laboratories for test review, development and improvement purposes.

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# Current/Future Developments

- Change Cutoffs
  - Krabbe
  - Gaucher
  - Others ongoing
- Expand testing for five LSDs state-wide June 1, 2015
- Change Buffer
- Evaluate current Perkin Elmer S/ISTD & LC to FIA
- Add back Krabbe

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# Changes to Cutoffs Based on Validation and Pilot (Percentage of Daily Median Activities)

- Krabbe
  - Validation: 1st cutoff ≤20%
    2nd cutoff ≤15%
  - Future testing: 1st cutoff ≤15%
    2nd cutoff ≤12%
- Pompe
  - Valid. & Pilot: 1st cutoff ≤30%
    Borderline ≤28%
    2nd cutoff ≤23%
  - Future testing: 1st cutoff ≤26%
    Borderline ≤23%
    2nd cutoff ≤18%

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# Comparison of IDPH to PerkinElmer Buffer for Multiplex Assay

**IDPH Substrates and Internal Standards**

| **Ammonium formate**<br>**Sodium cholate**<br>**Acarbose**<br>**N-Acetyl-α-galactosamine** | **0.1 M, pH 4.4**<br>**10 g/L**<br>**0.08 M**<br>**50 mM** |
|---|---|
| IDUA Substrate (S), Internal Standard (IS)<br>GLA S, IS<br>GAA S, IS<br>ASM S, IS (d7-C6 Ceramide)<br>GALC S, IS (d7-C8 Ceramide)<br>ABG S, IS (d7-C12 Ceramide) | 500 µM, 3.5 µM<br>600 µM, 1.2 µM<br>200 µM, 2.0 µM<br>150 µM, 2.5 µM<br>450 µM, 2.5 µM<br>300 µM, 2.5 µM |

**PerkinElmer Buffer**

| | |
|---|---|
| Acarbose | 8 µmol/L |
| N-Acetylgalactosamine | 50 mmol/L |
| D-Saccharic acid-1,4-lactone monohydrate | 40 µmol/L |
| Sodium taurocholate | 28 mmol/L |
| Zinc chloride | 0.6 mmol/L |
| Succininc Acid, pH 4.7 | 85 mmol/L |

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# Future Developments

- Evaluate current PerkinElmer S/ISTD and FIA
- Switch from LC to FIA – parallel test
- Add back Krabbe
  - Establish contract with reference laboratory
  - Develop in-house test for psychosine, 30 kb deletion, sequencing

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# THANK YOU

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