Microtiter plate fluorometry

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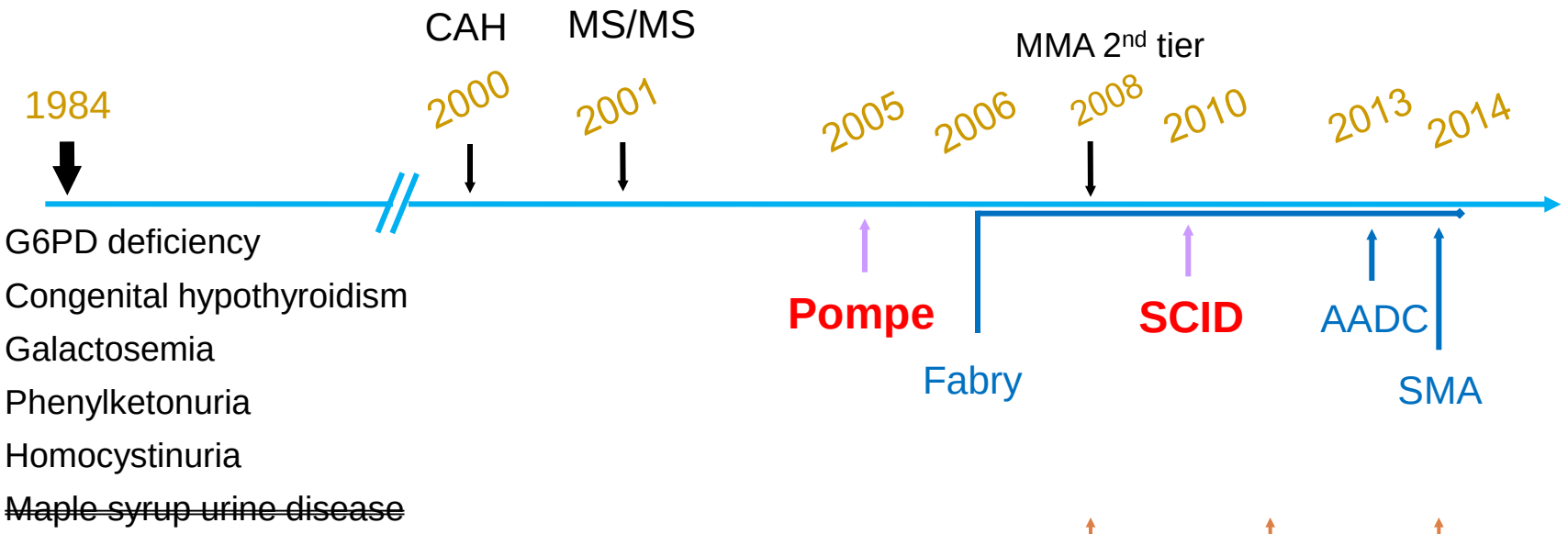
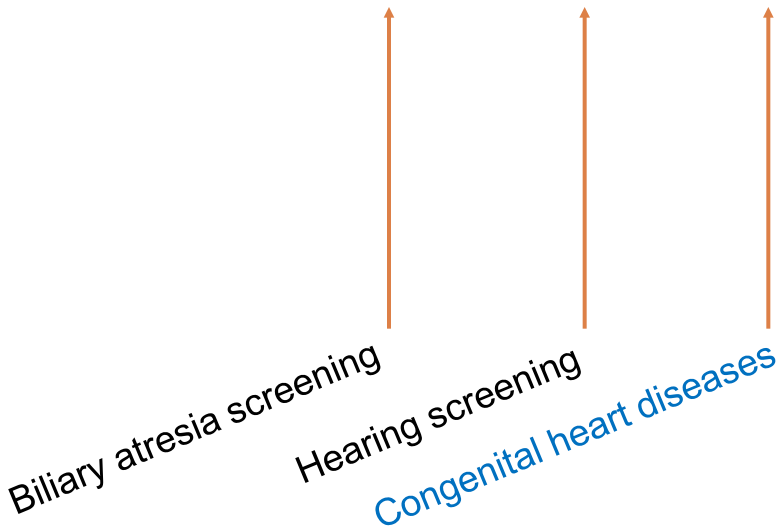
Development of Newborn Screening in National Taiwan University Hospital (NTUH)

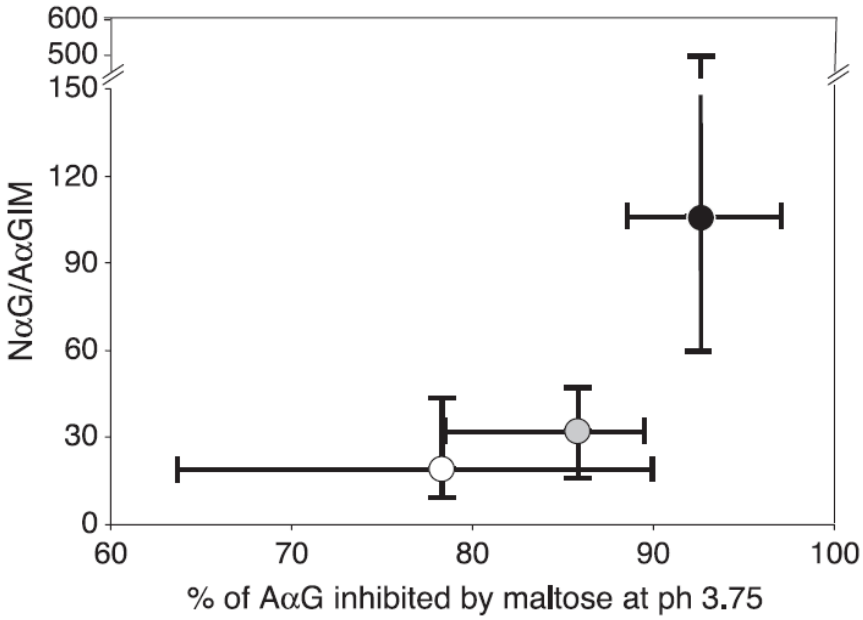


Abbreviations:
G6PD: Glucose-6-phosphate dehydrogenase
CAH: congenital adrenal hyperplasia
MS/MS: Tandem mass spectrometry screening
MMA: methylmalonic acidemia
SCID: severe combined immunodeficiency
AADC: Aromatic L-amino acid decarboxylase deficiency
SMA: spinal muscular atrophy

Diseases Color notes:
Black: recommend and partially sponsored by government
Red: self-paid add-in items
Blue: pilot



4-methylumbelliferone (4-MU) assay in GAA DBS



○ open circles, healthy
◉ shadow circles, obligate carriers
● closed circles, GSD II patients

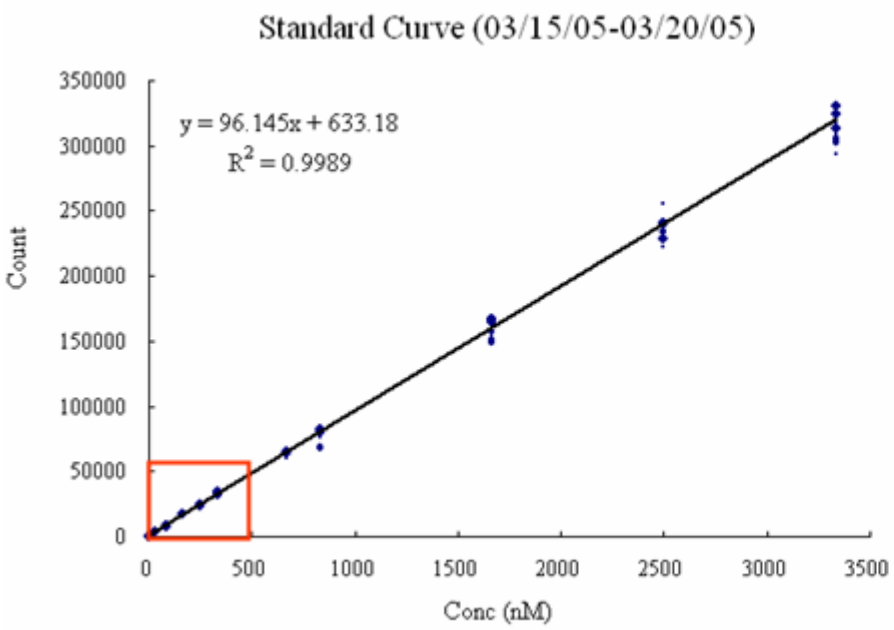
- 3 enzymes activities are measured
  - tGAA
    - Acid α-glucosidase, pH 3.8, in the abscence of acarbose
  - GAA
    - Acid α-glucosidase, pH 3.8, in the presence of acarbose
  - NAG
    - Neutral α-glucosidase, pH 7.0
- 2 dimension cutoffs are applied
  - NAG/GAA ratio
  - % Acarbose inhibition
    - (tGAA – GAA)/tGAA*100%

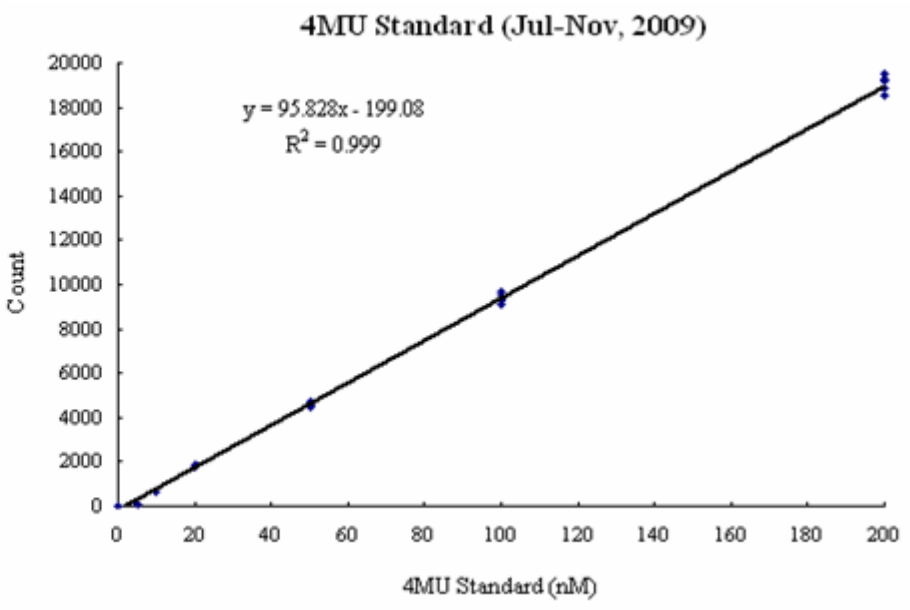
Chamoles, et al. Clin Chim Acta 2004;347, 97-102

4-MU substrate for GAA test

- Advantages
  - 4-MU substrate is a sensitive assay
    - The low detection limit is down to 1 nM
  - It is convenient to do tGAA and NAG at the same run
- Drawbacks
  - High background of the 4-MU substrate
    - Poor quality of substrate
  - Interfered (quenching) by hemoglobin
  - Not suitable for multiplex assay

4-MU sensitivity

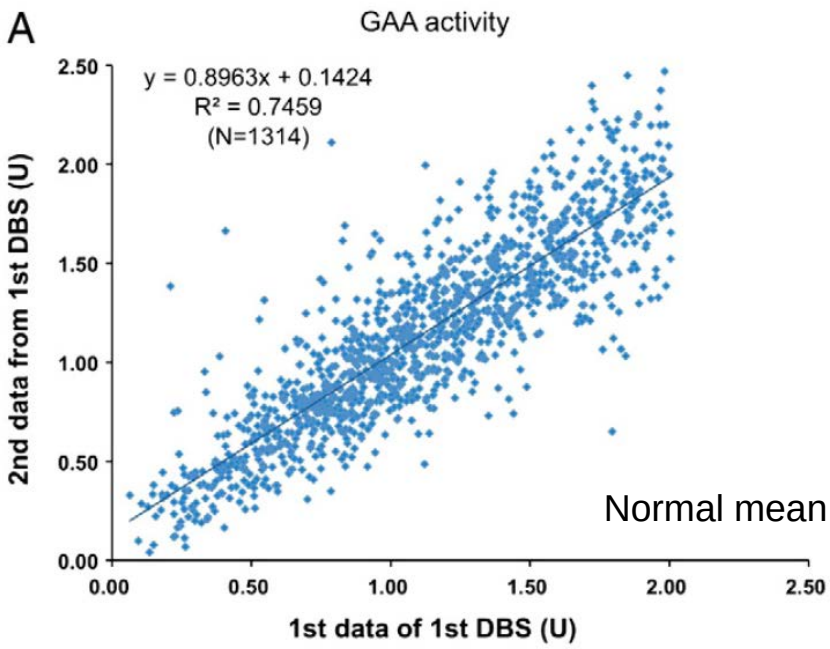




Drawbacks of 4MU assays
Substrate background and hemoglobin quenching

- Substrate background
  - Substrate blank: 7,000 counts
  - Normal: 20,000-30,000 counts
  - Patient: 6,000-6,800 counts
  - Sample blank: 4400-5300 counts
  - Cutoffs for suspected patients: 8,000 counts
- Source of variations
  - Variation of hemoglobin concentration in DBS
  - Variation in pipetting
- Solutions for decreasing variations
  - Individual blank
  - Accurate automatic pipetting station

Results after introducing our approaches
Accuracy of the fluorescence assay



Normal mean: 10.5 U

Correlation between results from duplicated GAA assays (N=1, 314). Only the low activity region is shown. X-axis: X-axis: 1st data; Y-axis: 2nd data.

Chiang SC, et al. Mol Genet Metab. 2012;106 (3): 281-6

Problems in DBS assay

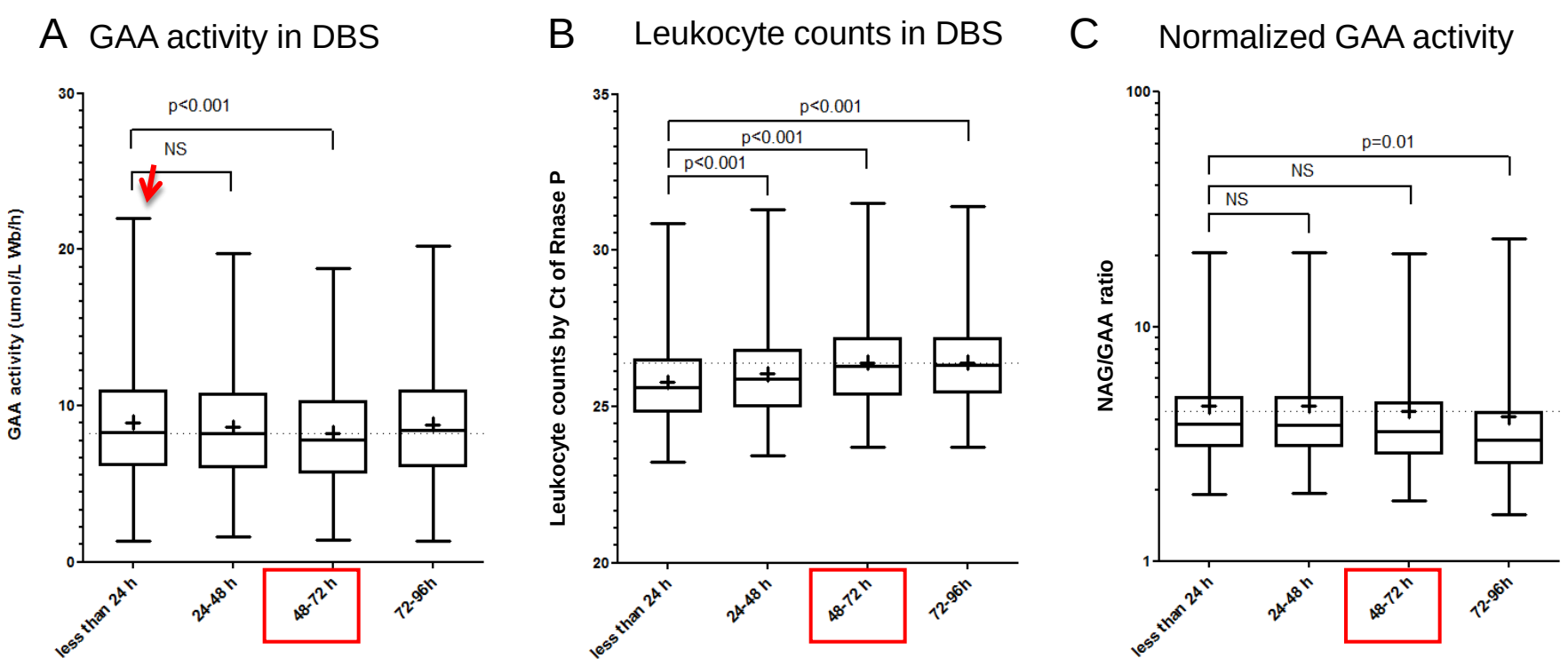
- Technical problems
  - Absorption may be varied in the preparation of DBS
  - Stability of the enzyme after storage and transportation
  - Not dry, too dry, hemolysis, clot (also affect the efficiency of elution)
- Physiological problems
  - Variation in leukocyte counts in babies
  - Stimulation of GAA activity by stress (?)
  - Jaundice (poor elution)

Comparison between DBS and lymphocytes assays

- We always measure protein concentration and a control enzyme in lymphocytes assay
- Why don't we do those for DBS assay?
  - When the range of affected activity overlaps with non-affected activity, it may be due to elevation of lymphocytes counts or elevation of all LSD enzymes.

  ➔ false negative!

Age/leukocyte count effects on GAA activity in DBS



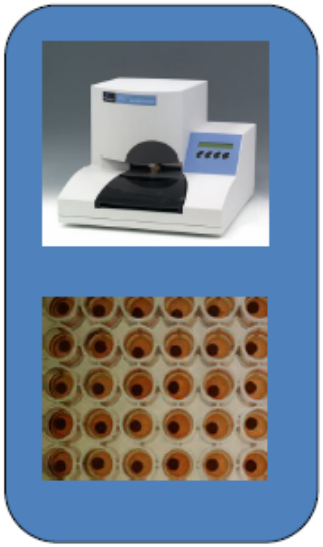
Box: 25th and 75th percentile, with median bar; +: mean; whisker: 1st and 99th percentile.
Suggested sample collection age: 48-72h after birth

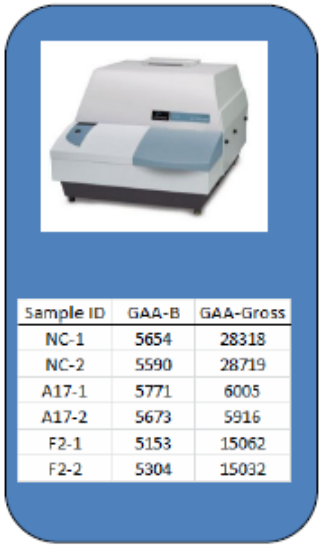
Chiang SC, et al. Mol Genet Metab. 2012;106 (3): 281-6

The current work flow

- First tier test: Two assays (GAA and NAG, each with individual blank)
  - Elutes→ Dispensing into 4 replicate plates
- Second tier test: Three assays (tGAA, GAA and NAG, each with individual blank)
  - Elutes → Dispensing into 6 replicate plates
- Daily work load
  - 6 plates/day (498 samples), 24 working plates
  - Procedures: adding substrates, overnight incubation, stop reaction, read florescence /calculation
  - Equipments:
    - One Beckmann, one plate centrifuge, two shakers, one reader
  - Two technicians

The current work flow





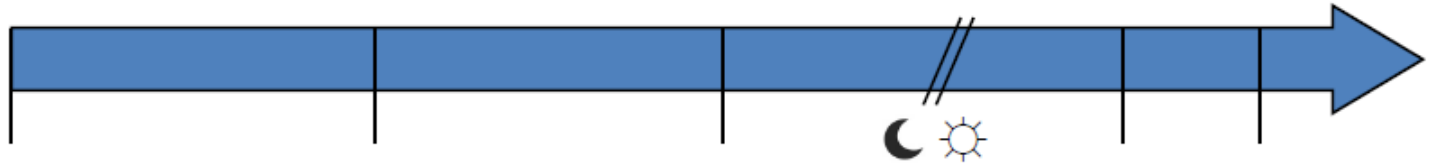
Sample ID	GAA-B	GAA-Gross
NC-1	5654	28318
NC-2	5590	28719
A17-1	5771	6005
A17-2	5673	5916
F2-1	5153	15062
F2-2	5304	15032

DBS Punch
Extraction

Aliquot
4-MU added

Incubation

Stop & Reading
Reporting

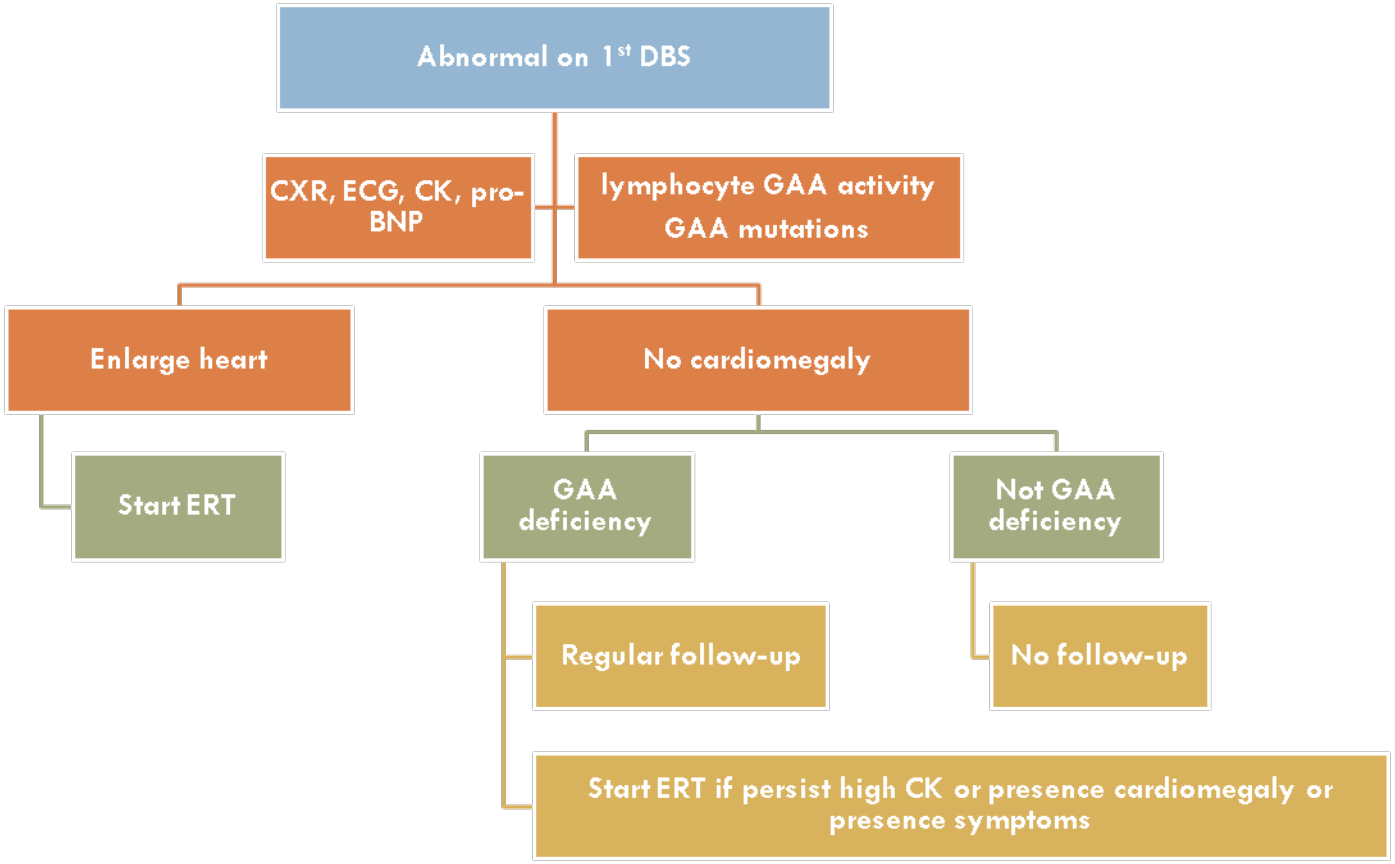


08:00 11:00 12:00 08:00 10:00

~ 26 hrs for 498 DBS per day

Confirmation algorithm

- Treat IOPD ASAP



Our 4-MU Std QC performance
CV no greater than 5% in all concentrations

Intraplate, 6 repeats/plate, 6 concentrations

Date	Count 1	Count 2	Count 3	Count 4	Count 5	Count 6	Mean	SD	CV (%)
0 nM	0	0	0	0	0	0	0	0.00	
50 nM	4413	4798	4358	4729	4341	4259	4483	223.88	**4.99**
100 nM	8302	8463	8085	8108	7869	8023	8142	210.59	**2.59**
200 nM	16983	17433	15954	15855	15891	16326	16407	658.36	**4.01**
500 nM	39370	39079	37481	37806	37433	38111	38213	825.70	**2.16**
1000 nM	75260	74927	71102	72494	71939	74733	73409	1777.86	**2.42**

Interplate, 6 repeats/plate, 15 plates/9 days

nM	Mean	SD	CV (%)
0	0		
50	4617	245.98	**5.33**
100	8221	297.30	**3.62**
200	16392	520.58	**3.18**
500	38140	1023.60	**2.68**
1000	74010	2002.29	**2.71**

CV: Coefficient of variation

Data collected in Mar. 2015

Our assays QC performance
CV less than 10% in normal activity ranges

Normal Control	GAA conc.	GLA conc.	NAG conc.	NAG/GAA	NAG/GLA
Mean	10.07	3.88	22.36	2.14	5.93
SD	0.83	0.53	2.39	0.16	0.47
CV (%)	**8.23**	13.68	**10.69**	7.38	7.90

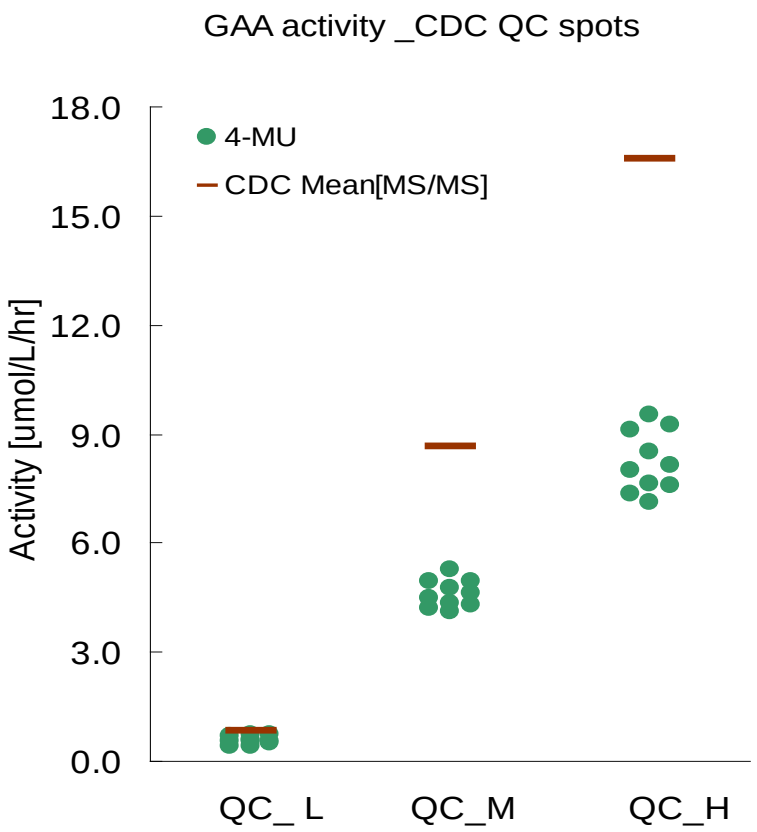
GAA Control	GAA conc.	GLA conc.	NAG conc.	NAG/GAA	NAG/GLA
Mean	0.28	4.80	25.07	92.66	5.34
SD	0.07	0.31	1.46	25.80	0.21
CV (%)	***23.59***	6.40	**5.83**	27.84	3.93

GLA Control	GAA conc.	GLA conc.	NAG conc.	NAG/GAA	NAG/GLA
Mean	4.44	0.23	21.21	4.62	102.68
SD	0.32	0.06	1.26	0.23	32.30
CV (%)	**7.10**	28.00	**5.92**	4.94	31.46

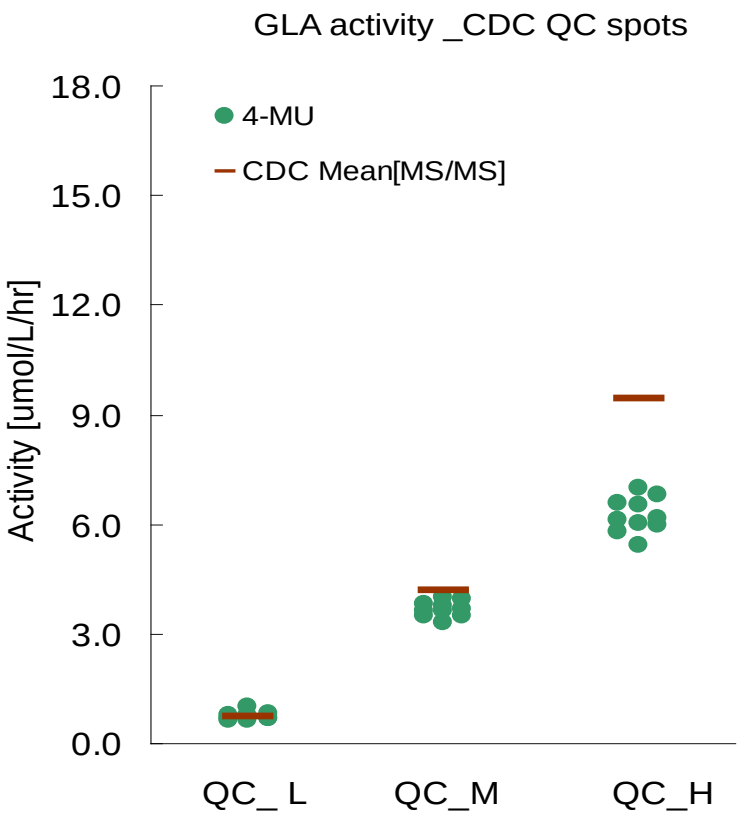
conc.=concentration, μmol/L/h
CV: Coefficient of variation

data collected in Feb 2011

Our performance in CDC-QC spots



CV%=8.1~18%



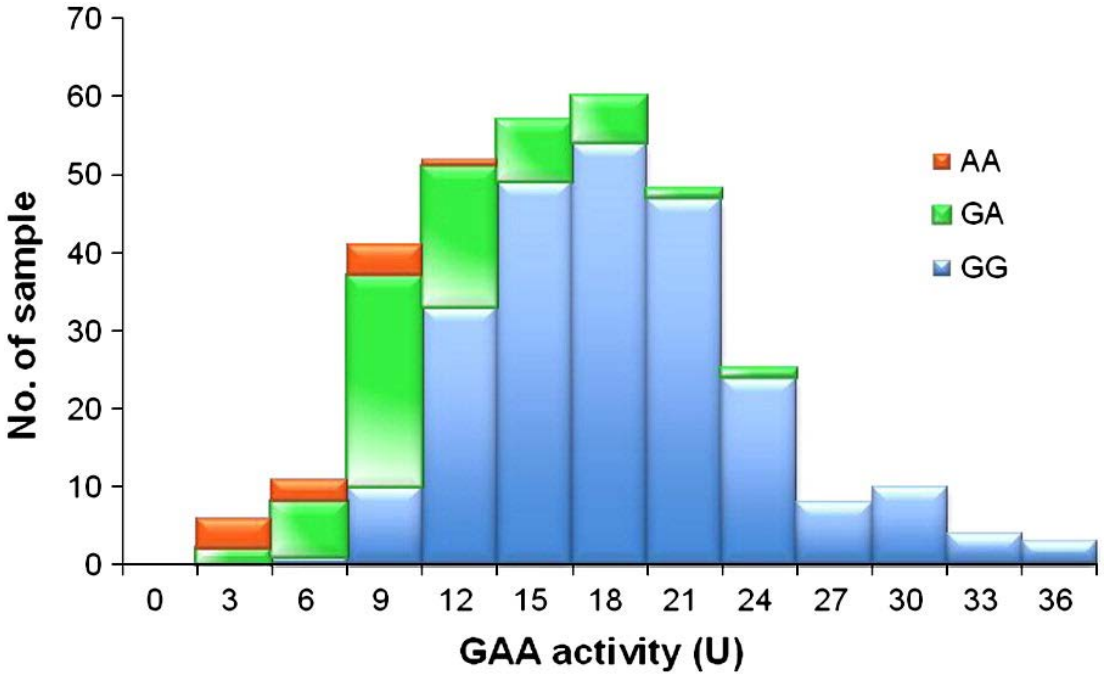
CV%=5.9~15.2%

data collected in Feb 2015

GAA pseudodeficiency

Pseudodeficiency homozygotes may contain **6-80%** of normal mean GAA activity in DBS

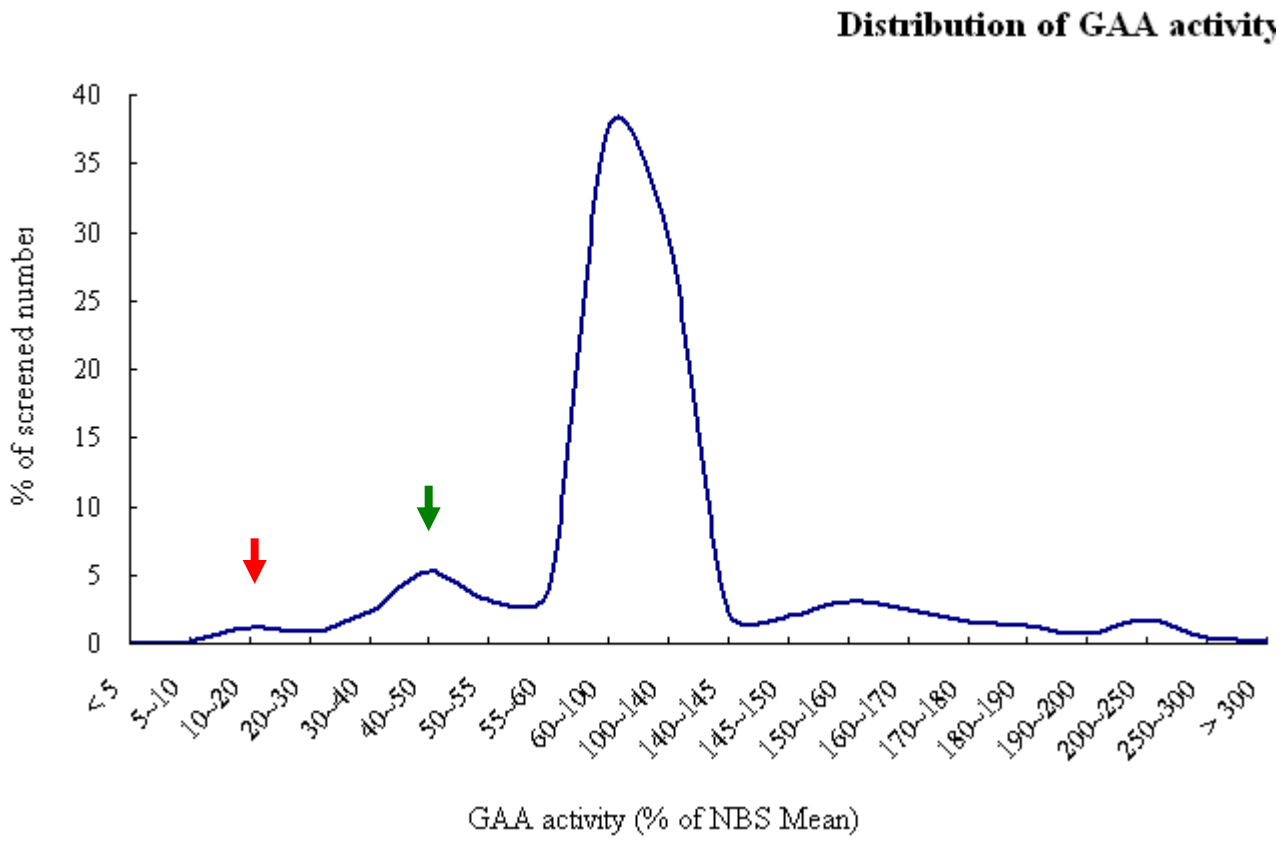
The Pseudodeficiency allele: c.1726 G>A (p.G576S)



- Population frequencies
  - Allele frequency: 14.5%
  - Homozygosity frequency: **3.69%**

Chiang SC, et al. Mol Genet Metab. 2012;106 (3): 281-6

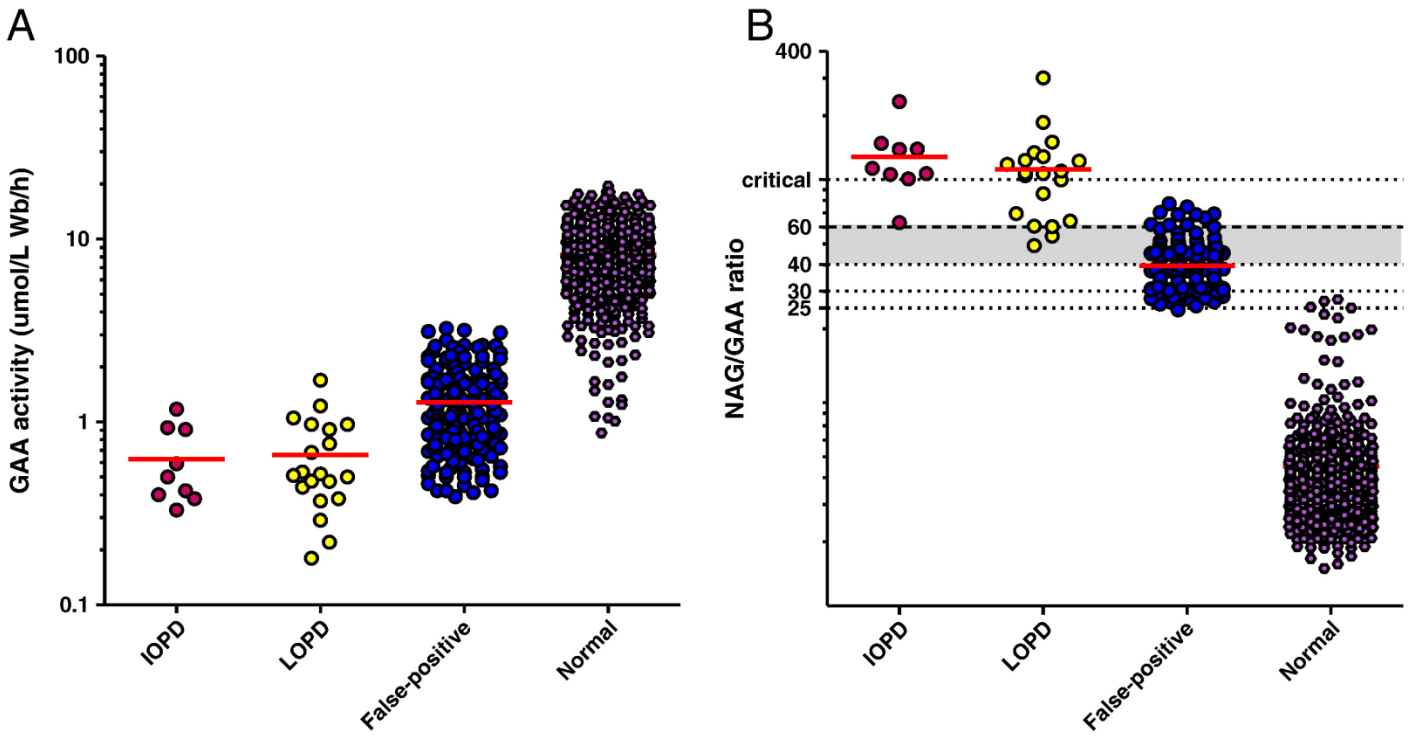
Distribution of DBS GAA activity in Taiwanese babies



The distibution of newborns (n=91,591) was collected in 2006.

X-axis means percentage of GAA mean activity (Mean of NBS=11.38, SD=4.99, unit: μmol/L Whole blood/h) and Y-axis means the percentage of the screened cases.

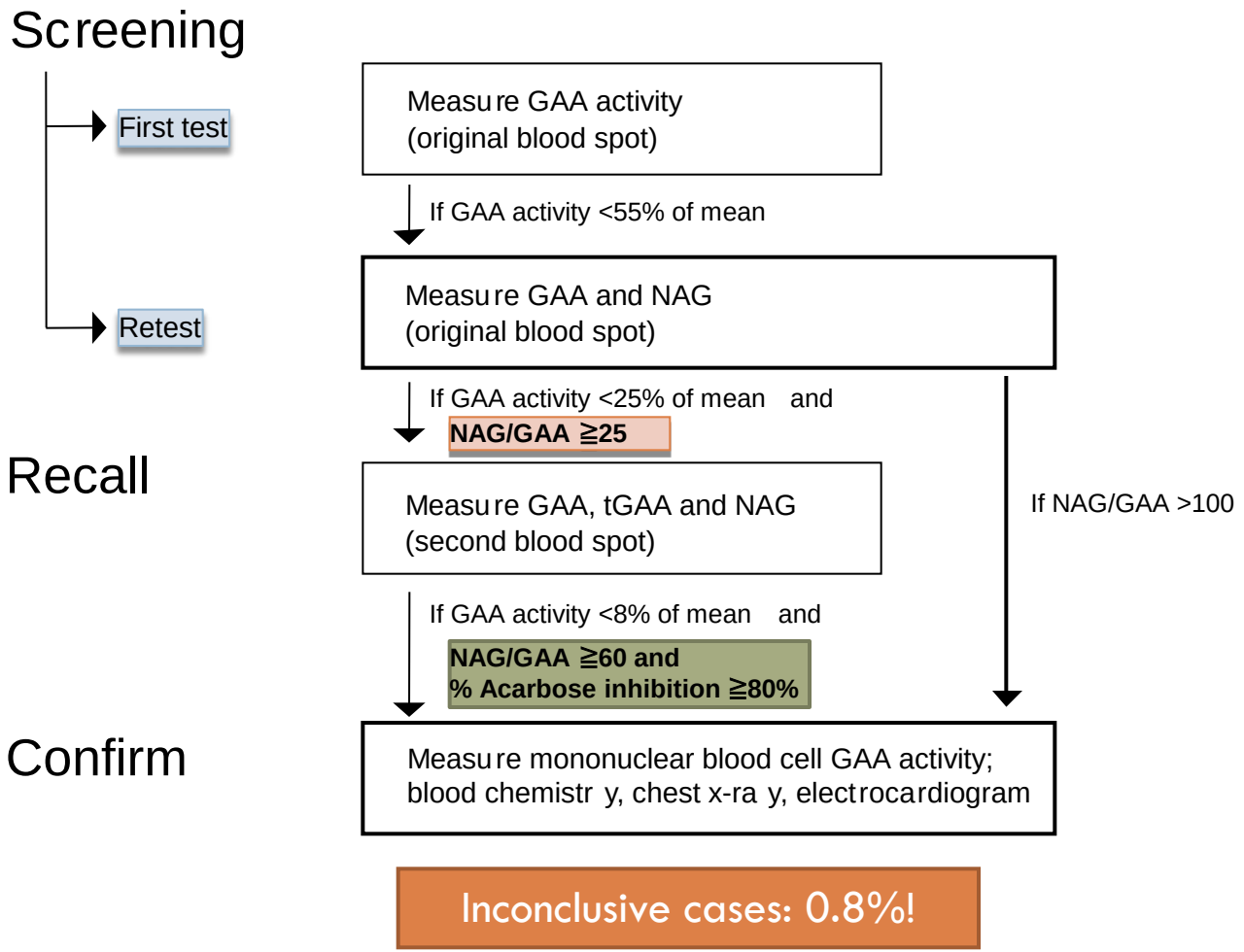
NAG/GAA ratio, but not GAA activity, can be an effective cutoff



Comparison between the GAA activity and the NAG/GAA ratio as a cutoff in newborn screening.
The GAA activity (panel A) and NAG/GAA ratio (panel B) of the 1st DBS are depicted for the patients (IOPD and LOPD), false-positive cases, and normal newborns (n = 920, 180, and 498 respectively). Wb: Whole blood.

Chiang SC, et al. Mol Genet Metab. 2012;106 (3): 281-6

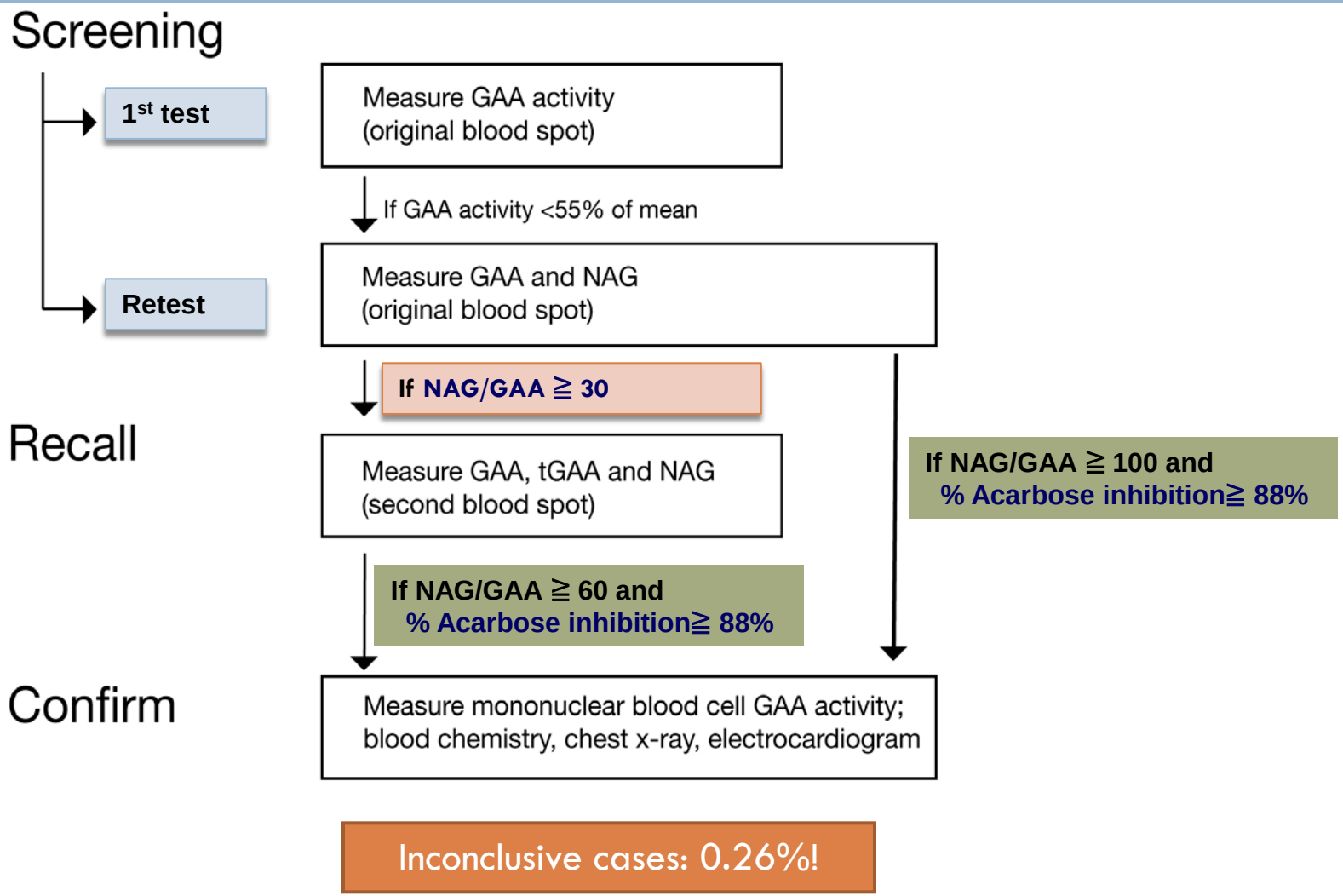
The Pilot Screening Algorithm (2005-2007) - Not knowing the pseudodeficiency impact



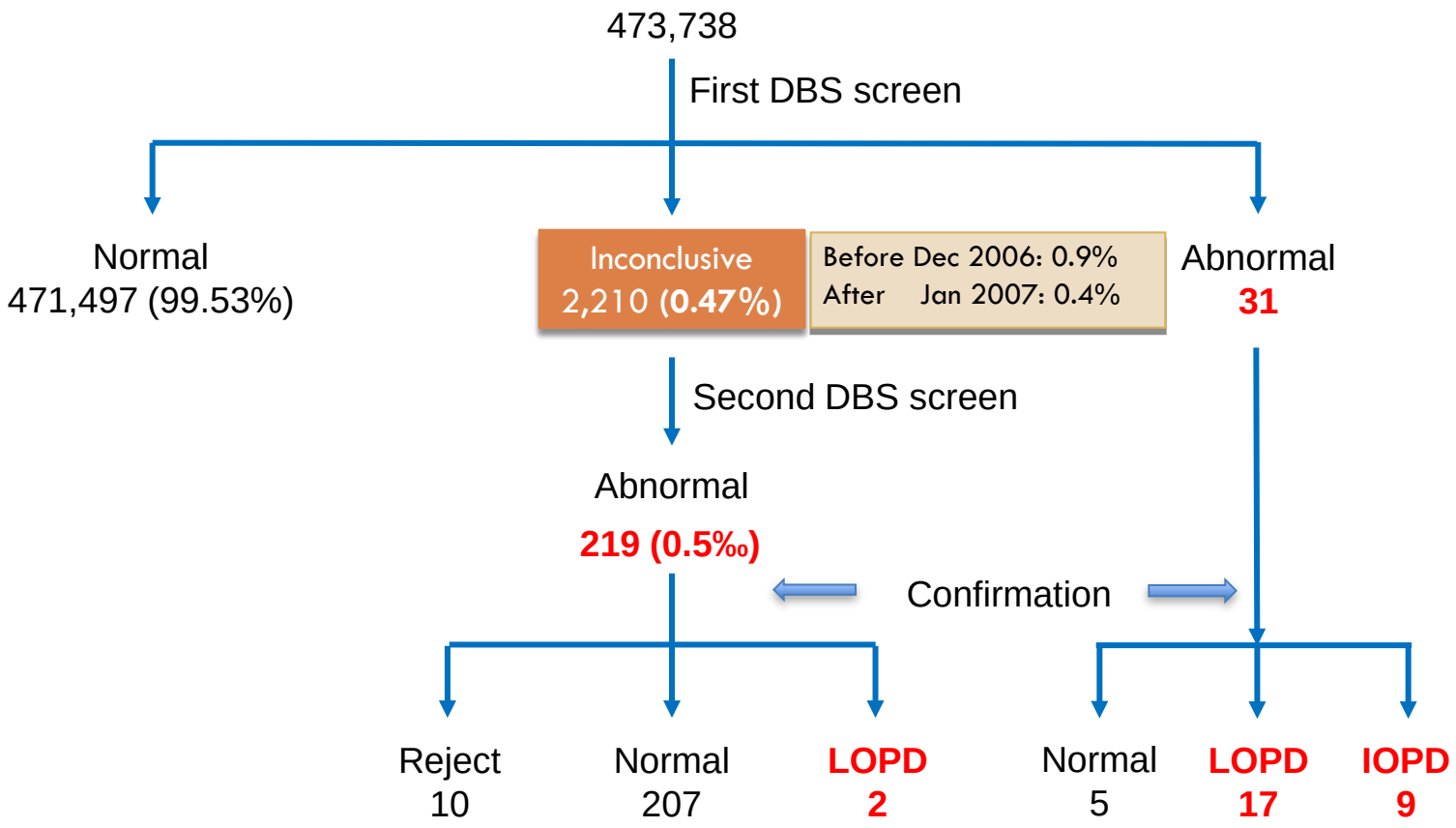
Inconclusive cases: 0.8%!

Chien YH, et al. Pediatrics. 2008 Jul;122(1):e39-45.

The First Revised Screening Algorithm (2008-2012)



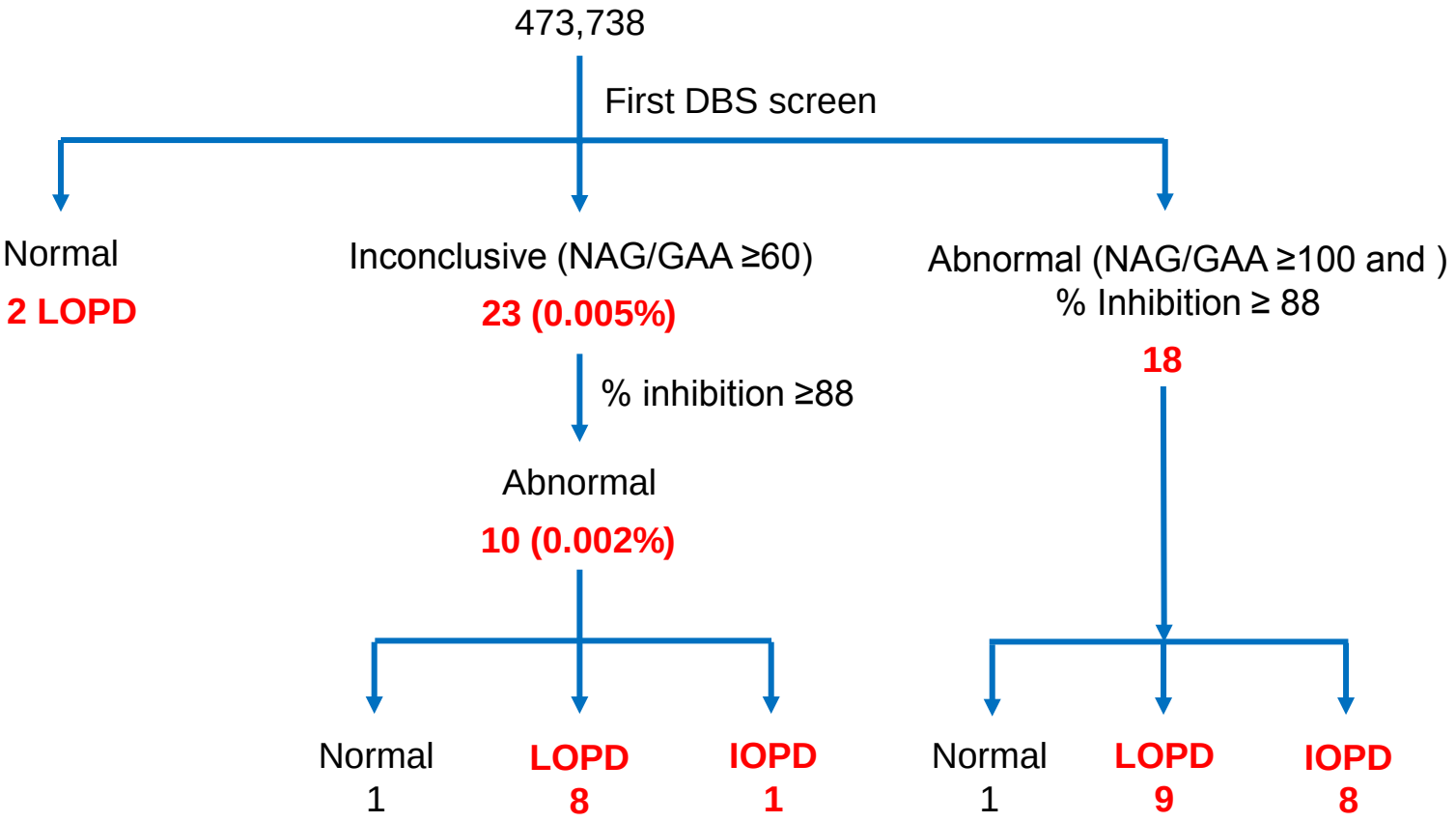
Overall results (2005-2011)



IOPD: Infantile-onset Pompe disease; **LOPD**: Later-onset Pompe disease

Chiang SC, et al. Mol Genet Metab. 2012;106 (3): 281-6

Proposed revised algorithm - should have higher positive-prediction rate

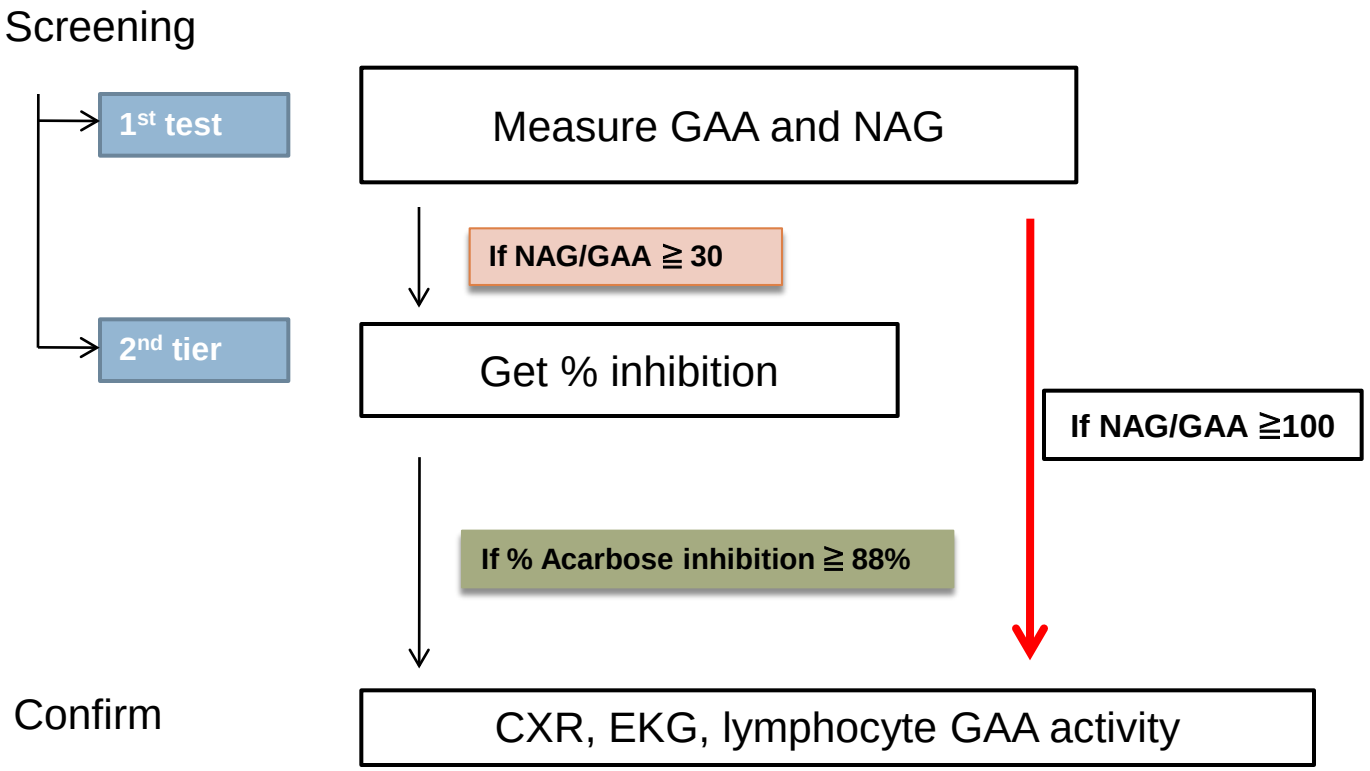


IOPD: Infantile-onset Pompe disease; **LOPD**: Later-onset Pompe disease

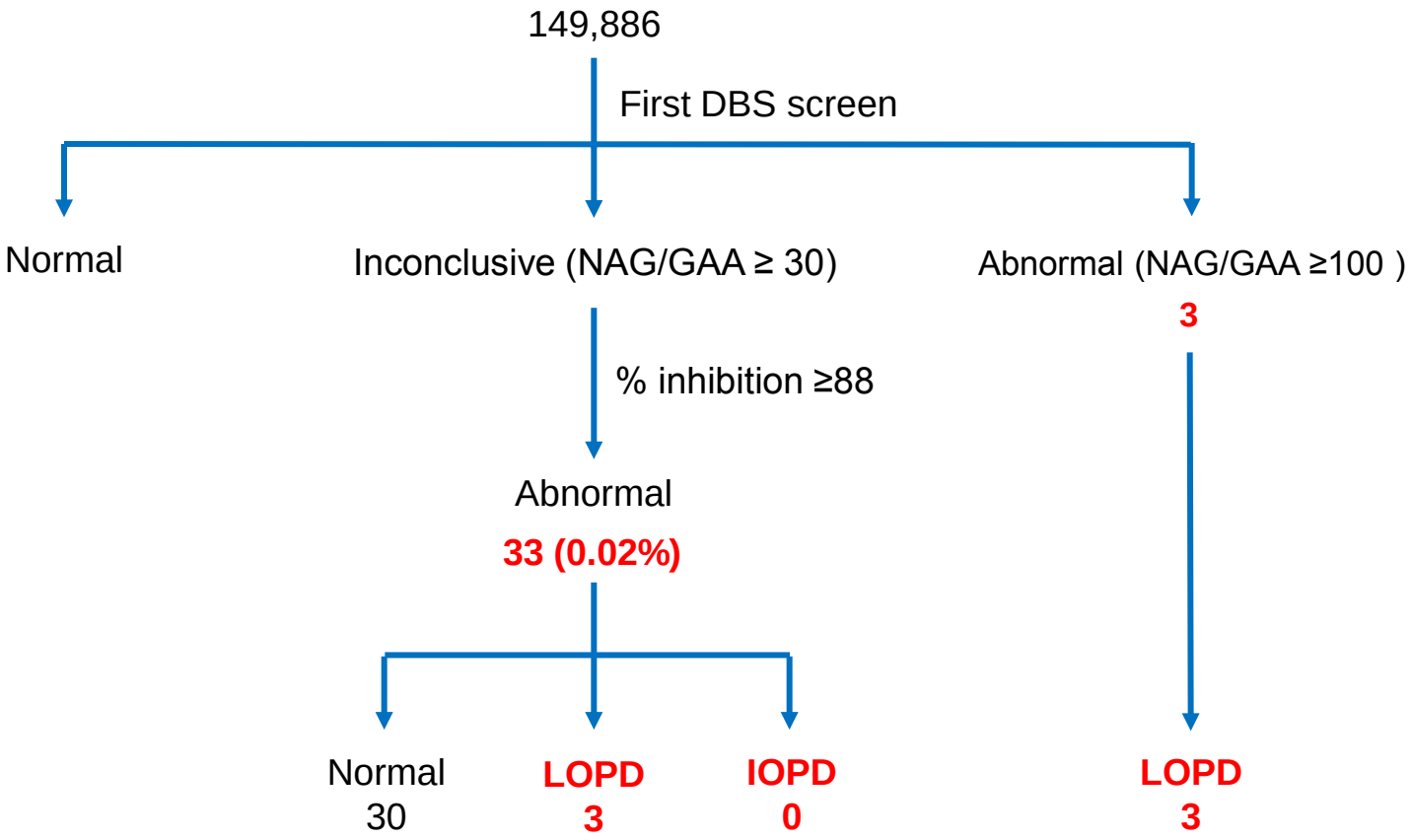
Chiang SC, et al. Mol Genet Metab. 2012;106 (3): 281-6

Current Screening Algorithm (2013- now)

- for better positive prediction rate and faster screening results



Current result (2013-2014)



IOPD: Infantile-onset Pompe disease; **LOPD**: Later-onset Pompe disease

False positive or false negative

- University of Washington
  - False positive: some are related to pseudodeficiency

Pompe disease (n = 4):		
2.53	IVS1-13t>g	
	IVS1-13t>g	
1.70	c.365T>A	p.Met122Lys
	c.1925T>A	p.Val642Glu
1.57	IVS1-13t>g	
	IVS1-13t>g	
1.43	IVS1-13t>g	
	c.1-17C>T	p.Ser(-6)Phe
Unaffected with low GAA activity (n = 13):		
2.45-2.20	carrier/wt	n = 4
2.38-2.20	carrier/pseudo def	n = 3
2.44-1.49	pseudo def/wt	n = 6

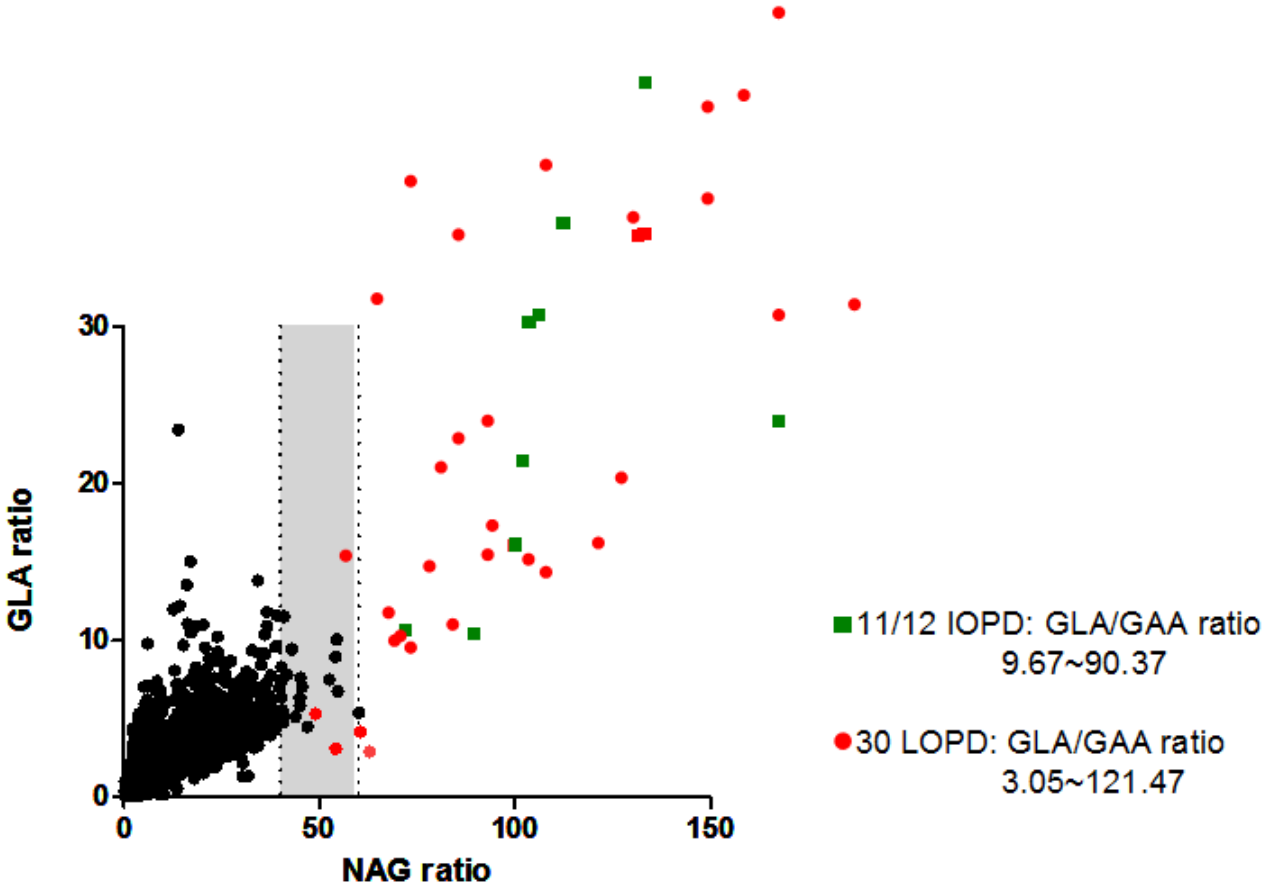
Scott CR, et al, J Pediatr. 2013 Aug;163(2):498-503

False positive or false negative - NTUH

- False positive
- False negative
  - A newborn, with c.-32-13 t>g / c.2214 G>A (p.W748X) compound heterozygotes , was positive for GAA screening
    - NAG/GAA ratio: 60.11; % of inhibition: 88.30
    - Tracing back his family history, his elder sister has the same mutations but was negative when screened
      - NAG/GAA ratio: 81.48; % of inhibition: 79.53
  - By our current cut-off, we would likely not detect these two siblings

Thanks for your attention !!!

NAG as a better control than GLA



- Newborns with pseudodeficiency Ho +Fabry variant (3% * 1/800= ~1/30 000)

Chiang SC, et al. Mol Genet Metab. 2012;106 (3): 281-6

Newborn Screening for LSDs

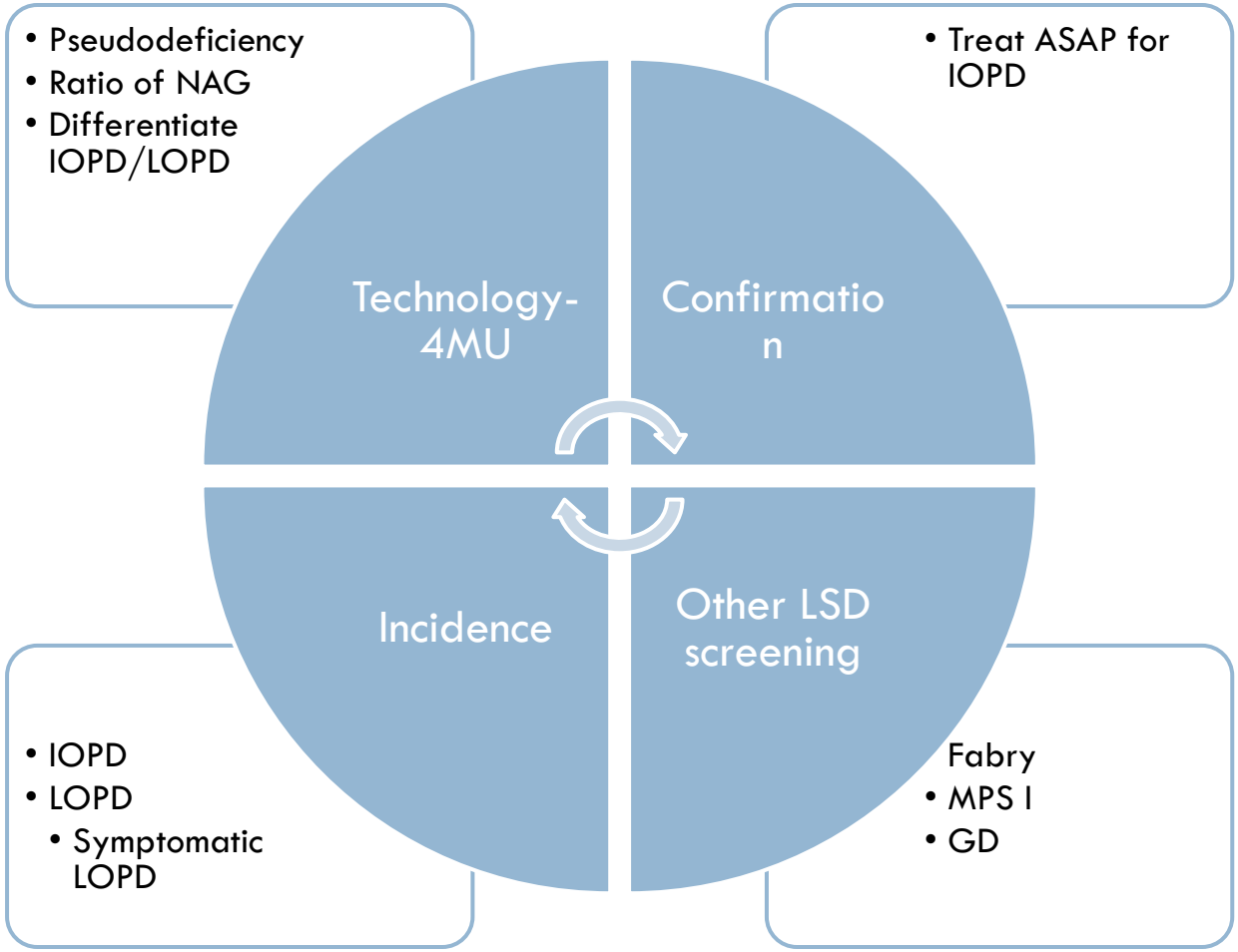
- NTUH Fabry screening- 2006~2014
  - Enzyme assay may detect both classic and variants
    - Molecular test revealed an incidence of 1 in 800 of males with variants (IVS4+919 g>a)
    - Current enzyme assay can't detect all males with variants
    - Current enzyme assay can't detect females with mutations
  - Nature history of variants is unknown
    - Value of newborn screening for late-onset variants is questionable
    - Long-term caring system for the asymptomatic variants is incomplete
- The other 2 regional NBS labs have additional pilot LSD NBS programs
  - Fabry, Gaucher, MPS I

Treatment

- Infantile-onset Pompe disease
  - 12 patients
  - All treated immediately
- Suspicious later-onset Pompe disease
  - 31 patients
    - Confirm with fibroblast GAA activity and found 2 mutations
  - 6 treated currently
    - Due to elevation of CK and/or muscle weakness

Conclusion

- Newborn screening can detect Pompe disease
  - The fluorescence substrate together with a reference enzyme is a suitable method to screen for Pompe disease
- Newborn screening improves the outcome of infantile-onset Pompe disease
  - The presence of residual disease needs to be watched
  - Value for later-onset Pompe disease remains to be determined
- Specific genotype may cause difficulties in the screening
  - Pseudodeficiency in the Asian population
  - IVS-1 splicing mutation in North America?


• Pseudodeficiency
• Ratio of NAG
• Differentiate IOPD/LOPD
• Treat ASAP for IOPD
Technology-4MU
Confirmation
Incidence
Other LSD screening
• IOPD
• LOPD
• Symptomatic LOPD
Fabry
• MPS I
• GD

Fibroblast GAA activity is better to differentiate IOPD from LOPD

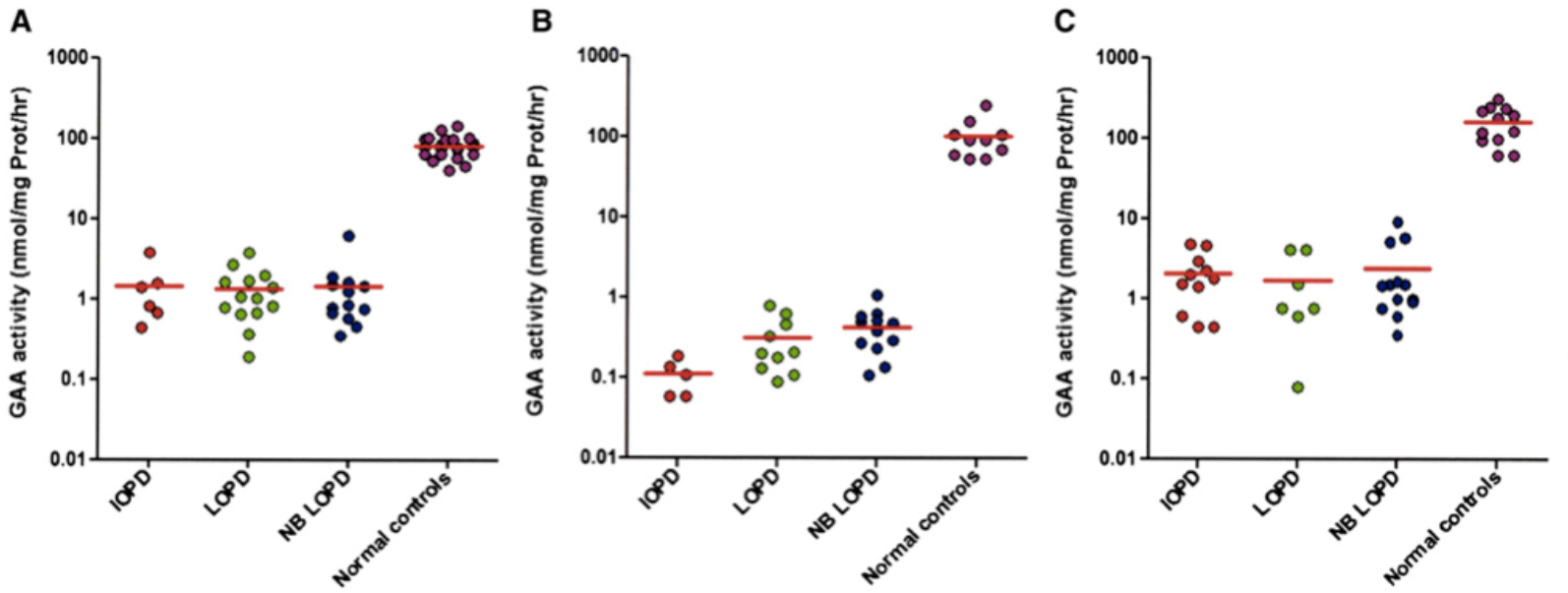
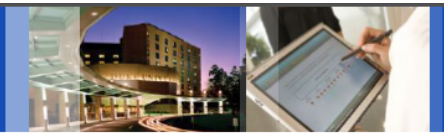


Figure 2. GAA activity of newborns with infantile-onset Pompe disease *(IOPD)*, patients with later-onset Pompe disease *(LOPD)*, newborns with later-onset Pompe disease *(NB LOPD)*, and control subjects. **A,** Lymphocyte GAA activity assayed with 4-methylumbelliferyl-α-D-glucopyranoside *(4MU)*; **B,** fibroblast GAA activity assayed with 4MU; **C,** fibroblast GAA activity assayed with glycogen.

Chien YH *et al*. J Pediatri. 2011 Jun;158(6):1023-1027.e1

Test Performance

Duke Clinical Research Institute



Newborn Screening for Pompe Disease—Summary

	Univ of Washington	Missouri NBS	Taiwan NBS
Incidence	**1 in 27,800**	**1 in 8,657**	**1 in 16,919**
Positive Rate	**0.015%**	**0.03%**	**0.053%**
Positive Predictive Value	**24%**	**33%**	**>90%**
Screening method	MS/MS	Digital Microfluidics	Fluorescence Assay
Total samples screened	111,544	25,971	473,738
Total True Pompe Cases	4	3	28
Infantile-onset with CMP	0	*1*	*9*
Infantile-onset without CMP	0	*1*	
Late-onset	*4*	*1*	*19*

Alex Kemper. Newborn Screening for Pompe Disease: Summary of the Condition Review Workgroup Report. Discretionary Advisory Committee on Heritable Disorders in Newborns and Children. First Meeting May 16-17, 2013.
http://www.hrsa.gov/advisorycommittees/mchbadvisory/heritabledisorders/meetings/2013/first/pompediseasepresentation.pdf