Michigan Department of Community Health

MDCH

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Semi-Quantitative Method for Determining Biotinidase Deficiency: Improving Performance Metrics, Evaluation of Cutoffs and Consideration for Age-Based Cutoffs

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Study Objectives

To improve performance metrics in screening individuals with profound and partial biotinidase deficiency by utilizing genotyping to evaluate borderline positive cutoff activities.

- Determine the correlation between biotinidase activity and age of child at the time of sample collection.
- Establish an age-related cutoff activity that differentiates between individuals with partial biotinidase deficiency and those with carrier-like activity.

Background

Newborn screening for biotinidase deficiency is conducted in essentially all of the United States and in many countries with a worldwide incidence of about 1:61,000. The Michigan newborn screening laboratory used the modified qualitative colorimetric assay of Wolf from 1987 – 2011. Actively screening for partial BD led to the highest overall detection rate in Region 4 in 2009; 1:38,540 with profound biotinidase deficiency and 1:9,635 with partial biotinidase deficiency; with an overall rate of 1:7,708. The subjectivity in visual reading and variability in filter paper resulted in a false positive rate of 0.21%. In anticipation that another method would reduce the false positive rate, we began using the semi-quantitative fluorometric Neonatal Biotinidase kit from PerkinElmer in August, 2011.

Table 1: 2009 - Biotinidase Deficiency Detection Rate for Region 4

State	Screens (N)	Confirmed Partial (BW)	Detection Rate (BW)	Confirmed Profound (BB)
Michigan	115,619	12	1:9,635	3
Illinois	167,659	1	1:167,659	1
Indiana	87,520	2	1:43,760	1
Kentucky	55,594	6	1:9,266	1
Minnesota	70,426	3	1:23,475	1
Ohio	145,517	2	1:72,759	1
Wisconsin	70,090	0	-	1
Nation	4,084,186	168	1:24,311	48

The Neonatal Biotinidase kit measures enzyme activity. The mutations associated with decreases in enzyme activity have various degrees of pathogenicity. For simplicity in correlating genotypes and phenotypes, Michigan's laboratory developed the following terminology for classifying mutations.

- "B" mutations cause profound biotinidase deficiency (<10% activity) when homozygous or compound heterozygous (BB). A carrier is a patient possessing one "B" mutation and one normal copy of the gene (BN).
- "W" mutation describes the D444H mutation. Individuals who are homozygous (WW) for this mutation have carrier-like activity (>30% activity). This mutation is not as deleterious to enzyme activity as is a "B" mutation.
- "BW" patients carry one "B" mutation and the "W" mutation. These are classified as having partial biotinidase deficiency (10 – 30% activity).

Method

Validation of the PerkinElmer Neonatal Biotinidase kit was performed by analyzing approximately 5,000 normal and 28 infants confirmed to have biotinidase deficiency.

The PerkinElmer Neonatal Biotinidase kit's numeric result directly correlates with biotinidase activity in each sample. Per the package insert (PerkinElmer 07/2011): "The Neonatal Biotinidase kit should not be used to distinguish partial from profound biotinidase deficiency". However, these semi-quantitative numeric results have allowed our laboratory to set cutoff activity to detect both profound and partial biotinidase deficiency. The strong positive (S+) cutoff, historically, was used to identify individuals with profound biotinidase deficiency. This cutoff activity, set above the biotinidase activity measured in samples from individuals with confirmed profound biotinidase deficiency, is 34 U (1 U = 1 nmol/min/dL). A borderline positive (B+) cutoff activity was used to identify partial biotinidase deficiency. This cutoff activity, set by the same means, was 50 U. It has recently been adjusted to 65 U. A borderline presumptive positive result generates a request for a second (repeat) sample from the child. If the repeat sample is also B+, then the child is referred for confirmatory diagnostic testing.

We found that many initial samples reported as B+ had slightly higher biotinidase activity on the second (repeat) sample, which was just above the B+ cutoff activity. It was also evident that a higher percentage of subsequent samples had activities greater than that of the highest standard. Initial samples from individuals with profound biotinidase deficiency, partial biotinidase deficiency, those with carrier-like activity, and unaffected infants had biotinidase activities ranging from 23-36 U, 29-55 U, 30-54 U and 40->364 U, respectively. In repeat samples, the biotinidase activities ranged from 43-50 U, 34-68 U, 60-132 U and 120->364 U, respectively. This led to the conclusion that biotinidase activity increases with age. As illustrated, overlap between multiple categories exists, making it impossible to determine phenotype based on the assay results. In response, the laboratory investigated an age-related cutoff activity to better identify those with partial deficiency.

To clarify the cutoff activity between individuals with partial biotinidase deficiency and those with carrier-like activities, samples with initial B+ results and repeat sample results ≤ 100 U were sent for genotyping. The results of this analysis were integral in establishing the age-related borderline positive cutoff activity.

Evaluation of the Primary Cutoff

As described above, approximately 5,000 normal and 28 true positive partial and profound biotinidase deficient samples were analyzed during the method validation.

The 0.01 percentile for these 5,000 patients was 34 U, and was thus used for the S+ cutoff activity. All true positive profound biotinidase deficient samples in the validation had values ≤ 34 U. We found that, due to overlap of populations, the S+ cutoff activity cannot be used to solely detect individuals with profound biotinidase deficiency. There have been samples from individuals with partial biotinidase deficiency that are in the S+ category. However, a lower activity means that the infant has a higher probability of having biotinidase deficiency. Therefore, a S+ category is still utilized.

The laboratory was reporting approximately 36 B+ initial samples per year using a B+ cutoff of 50 U. However, due to the uncertainty of the selectivity of the B+ cutoff activity, we reported B+ on initial samples between 50 and 60 U. In total, 17 initial samples and 8 repeat samples were therefore reported as B+. Of these, six were confirmed as having partial biotinidase deficiency by genotyping, and one was homozygous for the D444H mutation. At the beginning of 2013, the B+ cutoff was adjusted to 65 U.

Initially, the laboratory incorporated a single B+ cutoff activity. An age-related B+ cutoff activity was investigated after observing that biotinidase activity increases with age.

Biotinidase Activity and Age at Collection

Biotinidase Activity begins increasing at approximately 3 days of age. It steadily increases until about 11 days of age. Our laboratory observed an increase of 80 U over this age range.

Samples with activities greater than the highest standard (currently 361 U) are repeated using a single punch to acquire a valid activity. Samples with activities ≤ 90 U (equivocal zone) are repeated in duplicate for confirmation because they exhibit lower than normal activity.

Figure 1: BTD vs Age

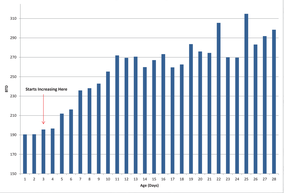


Table 2: Result Statistics - Number of Specimens Equivocal Zone and > Highest Calibrator

	Number ≤ 72 hours	Percent of Total		Number > 72 hours	Percent of Total		Total
Result ≤ 90 U (x2 Repeat)	859	93.37%		61	6.63%		920
Result > STD F (x1 Repeat)	384	14.67%		2234	85.33%		2618

- Only 6.63% of samples with activities ≤ 90 U were repeat samples. Repeat samples generally have values >90 U.
- 85.33% of samples with activities greater than the highest standard are repeat samples. This indicates that activities increase with age. If activities did not change with age, the percentage of repeats that were greater than the highest standard would not have increased at such a rate.
- This increase in activity illustrates the need for an age-related cutoff activity.

Due to the observed increase, the age-related cutoff activity must start at the age where the activity increase begins, cover all ages up to the plateau activity and be based on activities at the plateau.

Figure 2 - Histogram consists of 2013 patient data for initial specimens. The S+, B+ and equivocal zone cutoffs are illustrated using colored lines.

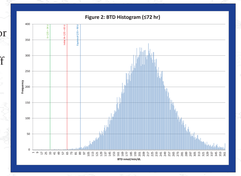
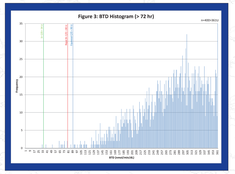


Figure 3 - Histogram consists of 2013 patient data for repeat specimens. As previously described, the mean activity is greater for older infants. The histogram shows a noticeable shift in patient activity when compared to figure 2. The S+, B+ and equivocal zone cutoffs are illustrated using colored lines. As illustrated, few repeat specimens have activity less than the equivocal cutoff.

Age-related cutoff activity calculations were performed using patient mean data. Assuming an increase to 270 U, calculations are as follows:

- From 0 to 72 hours the patient mean is 190 U. The current cutoff activity is 65 U.
- At the plateau, the mean activity increases to 270 U.
- The calculated cutoff activity is 92 U (190/65 = 270/x).
- This activity is higher than the equivocal zone cutoff activity.
- Patient data supports a lower cutoff.



Mutation Analysis

Eighteen repeat specimens, with activities between 59 and 100 U, that were 'closed on repeat' meaning the repeat sample was as reported negative for biotinidase deficiency, were genotyped.

Of these 18 specimens, three had partial biotinidase deficiency (BW) and two had novel mutations in addition to one copy of the D444H mutation.

The remaining 13 specimens were characterized as WW. WW individuals have biotinidase activities similar to that of heterozygous individuals (BN), who are asymptomatic and are not treated with biotin. Such cases are considered false positives and should be minimized.

Table 3: BTD DNA Mutation Analysis Specimens

Case	Mutation 1	Mutation 2	Additional Variant	Genotype
1	Q456H	D444H		Partial - BW
2	c.933delT	D444H		Partial - BW
3	R211C	D444H	L215L	Partial - BW
4	p.R209H; c.626G>A	D444H		Unknown - VW
5	p.T196A; c.586A>G	D444H		Unknown - VW
6	D444H	D444H		WW
7	D444H	D444H		WW
8	D444H	D444H		WW
9	D444H	D444H		WW
10	D444H	D444H		WW
11	D444H	D444H		WW
12	D444H	D444H		WW
13	D444H	D444H		WW
14	D444H	D444H		WW
15	D444H	D444H		WW
16	D444H	D444H		WW
17	D444H	D444H		WW
18	D444H	D444H		WW

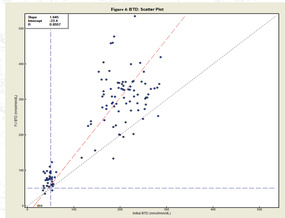
Presumptive B+ with Age-Related (>72 hours) Cutoff ≤ 80 U.

Patient Data: Repeat Results vs. Initial Results

The following graph compares biotinidase activities of the initial specimens to that of the repeat specimens. As illustrated by the comparison of the specimens (blue dots) to the gray dotted line (1:1 ratio), activities increase by a factor of 1.6 from the initial to the repeat screen.

Initial BTD refers to the activities of the initial specimens, FU BTD to the activities of the repeat specimens. The dotted blue lines refer to the initial B+ cutoff activity of 50 U. The dotted red line is the best fit line for the data points with a slope of 1.645.

The following graph focuses on the individuals with low enzyme activities, specifically those confirmed to be positive. Normal, WW, BW and BB specimens are differentiated by color



- The vertical blue line represents the cutoff activity of 65 U for initial specimens.
- The horizontal blue line represents the cutoff activity of 80 U for repeat specimens (age >72 hours).

As shown by the graph, biotinidase activities of affected individuals increase with age. However, the activity increase for individuals with a genotype of WW is generally greater than that of those with BW. This data supports the use of an age-related cutoff activity for selectively identifying individuals with partial biotinidase deficiency. Using this strategy, many individuals homozygous for D444H are not referred for diagnostic testing. However, some overlap is evident between BW and WW individuals.

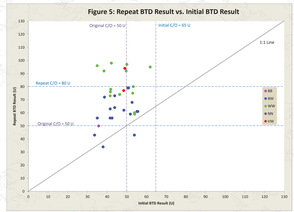


Table 4: Result Comparison and Change

Case	Genotype	Initial Result (U)	Repeat Result (U)	Difference in Result (U)
1	Partial - BW	39	72	33
2	Partial - BW	50	79	29
3	Partial - BW	44	73	29
4	Partial - BW	42	63	21
5	Partial - BW	44	64	20
6	Partial - BW	43	56	13
7	Partial - BW	55	61	6
8	Partial - BW	53	68	15
9	Partial - BW	35	56	21
10	Partial - BW	54	43	-11
11	Partial - BW	34	-	-
12	Partial - BW	38	34	-4
13	Partial - BW	31	-	-
14	Partial - BW	30	-	-
15	Partial - BW	29	-	-
16	Partial - BW	34	43	9
17	Partial - BW	49	62	13
18	Partial - BW	52	59	7
19	Partial - BW	56	61	5
20	Unknown - VW	48	77	29
21	Unknown - VW	49	94	45
22	WW	42	78	36
23	WW	62	95	33
24	WW	42	98	56
25	WW	50	97	47
26	WW	53	80	27
27	WW	42	73	31
28	WW	35	96	61

Case	Genotype	Initial Result (U)	Repeat Result (U)	Difference in Result (U)
29	WW	54	59	5
30	WW	42	76	34
31	WW	53	75	22
32	WW	50	92	42
33	WW	38	92	54
34	WW	46	74	28
35	WW	54	60	6
36	WW	32	-	-
37	Normal	160	253	93
38	Normal	234	330	96
39	Normal	194	330	136
40	Normal	194	374	180
41	Normal	196	374	178
42	Normal	250	374	124
43	Normal	201	374	173
44	Normal	265	374	109
45	Normal	187	225	38
46	Normal	149	212	63
47	Normal	125	206	81
48	Normal	171	263	92
49	Normal	138	253	115
50	Normal	130	298	168
51	Normal	211	261	50
52	Normal	138	213	75
53	Normal	120	316	196
54	Normal	161	284	123
55	Normal	155	203	48
56	Normal	208	327	119
Presumptive B+ with Age-Related (>72 hours) Cutoff ≤ 80 U.				
Change > 10 U.				

The highest activity reported for an individual with partial biotinidase deficiency was 79 U in this study. This led us to institute a cutoff activity of 80 U for individuals >72 hours of age. Had this cutoff activity been in place, the program would still have identified 7 of the 13 WWs.

The age-related cutoff activity is used to differentiate between BW and WW individuals. Distinguishing between individuals with partial and profound biotinidase deficiency using this type of cutoff activity has not been investigated because of lack of data.

Patient Data: Confirmed Partial Biotinidase Deficiency

The following table consists of all of the cases confirmed to have partial biotinidase deficiency since the program began using the PerkinElmer Neonatal Biotinidase kit in August 2011.

The Michigan Newborn Screening program, incorporating selective genotyping, has identified 20 individuals with partial biotinidase deficiency and two individuals with novel mutations and one copy of D444H.

Table 5: MI Confirmed Partial BD

Case	Mutation 1	Mutation 2	Initial Result (U)	Repeat Result (U)	Age (hrs)	Repeat Result Difference from C/O (U)
1	Q456H	D444H	39	72	226	9
2	Q456H	D444H	42	63	155	0
3	c.933delT	D444H	50	79	150	-16
4	R211C	D444H	44	73	330	23
5	Q456H	D444H	44	64	230	1
6	Results not available.		43	56	496	6
7	Results not available.		55	61	226	-2
8	R157H	D444H	53	68	207	5
9	Y414-V417del	D444H	35	56	155	6
10	Results not available.		54	43	175	-7
11	P253S	D444H	34	-	26	-
12	A171T;D444H	D444H	38	34	138	-16
13	I106V	D444H	31	-	26	-
14	Q456H	D444H	30	-	26	-
15	A171T;D444H	D444H	29	-	25	-
16	A171T;D444H	D444H	34	43	205	-7
17	R211C	D444H	49	62, 75	278,849	12,25
18	L215F	D444H	30	-	24	-
19	R53fC	D444H	52	59	230	9
20	Results not available.		56	61	144	11
				Difference Range		-16 to +25

Patient Data: Confirmed D444H Homozygous

The following table contains nine patients confirmed to be homozygous for the D444H mutation with results ≤ 80 U. Two were referred for confirmatory testing by the newborn screening laboratory. Seven were included in the specimens sent for genotyping. Under the age-related cutoff activity, all of the samples would have been referred for confirmatory testing.

Table 6: MI Confirmed D444H Homozygous

Case	Initial Result (U)	Repeat Result (U)	Age (hrs)	Repeat Result Difference from C/O (U)
1	42	78	303	15
2	53	80	129	30
3	42	73	229	23
4	54	59,113	130,396	9,63
5	42	76	272	26
6	53	75	182	25
7	54	60	183	-3
8	32	-	-	-
9	46	74	544	24
		Difference Range		-3 to +63

Patient Data: Result Overlap

The following table consists of all of the cases that were referred for confirmatory testing based on a S+ result in 2012.

As illustrated, there is overlap between the populations. The S+ cutoff does not identify profound BD exclusively.

- BB range = 23 – 36 U on initial specimen and up to 50 U on repeat specimen.
- BW range = 29 – 55 U on initial specimen and up to 79 U on repeat specimen.
- WW range = 30 – 62 U on initial specimen and up to 113 U on repeat specimen.

This overlap is the reason that it is difficult to differentiate between individuals with profound and partial biotinidase deficiency as well as those with partial biotinidase deficiency and D444H homozygous on the initial sample. The age-related cutoff activity is a useful screening tool because it takes advantage of the increasing activity rates for WW compared to BW. However, it cannot be used as a definitive tool for differentiation because all WW specimens do not increase at a rate greater than BW specimens.

Table 7: MI Initial S+ Result (≤ 34 U) and Classifications

Case	Result (U)		Classification
1	30		Profound - BB
2	23		Profound - BB
3	25		Profound - BB
4	Initial Result	Repeat Result	Profound - BB
	36	50	
5	34		Partial - BW
6	34		Partial - BW
7	31		Partial - BW
8	30		Partial - BW
9	29		Partial - BW
10	34		Partial - BW
11	30		Partial - BW
12	32		WW

The Next Step: Considerations for Future Testing

The Michigan Newborn Screening Laboratory employs 2nd tier tests for MMA, MCA and HCY screening. These analytes are used to screen for several diseases/deficiencies, such as Homocystinuria, PA, MMA, Cobalamin metabolism and maternal nutritional deficiency, MTHFR deficiency and Vitamin B12 deficiency. This improves laboratory performance metrics because these samples are reported positive only if the 2nd tier test is positive.

Eventually we would like to consider employing this referral process for Biotinidase Deficiency testing. A 2nd tier DNA targeted-mutation test would increase the selectivity and performance metrics. This would decrease the number of WW individuals referred for diagnostic testing, using only the dried blood spots submitted for Newborn Screening.

Logistics: The DNA from the filter paper card would be extracted in the newborn screening laboratory. This would be completed on all second B+ specimens. S+ samples are and will continue to be directly referred for diagnostic testing. The DNA would then be shipped to a reference laboratory for targeted mutation analysis. The reference laboratory would be specifically looking for number of D444H copies, as well as the D444H combined mutations (Q95X, R157H, A171T, N214S and F403V).

The findings of this project suggest that all of the second B+ specimens will be either BW or WW (none of the samples sent for genotyping were BB). If a specimen has two copies of the D444H mutation and no combined mutation, the sample is designated WW and will not be referred for diagnostic testing. All other samples could be BB or BW and will be referred for confirmatory testing.

Table 8: Targeted Mutation Analysis on Repeat B+ Specimens - Possible Phenotypes and Follow-up Action

Copies of D444H	With Combined Mutation (Q95X, R157H, A171T, N214S and F403V)	Without Combined Mutation
0	Profound, Carrier, Not Affected **Follow-up Recommended**	Profound, Carrier, Not Affected **Follow-up Recommended**
1	Profound, Partial, Carrier, W Carrier **Follow-up Recommended**	Partial, W Carrier **Follow-up Recommended**
2	Profound, Partial **Follow-up Recommended**	Homozygous W **Follow-up Not Recommended**

Conclusion

Implementation of the semi-quantitative fluorometric assay reduced our false-positive rate from 0.21% to 0.03%. Positive predictive value improved from 10.8% to 35.3%.

The Michigan Newborn Screening Program is screening for profound and partial biotinidase deficiency. Identified infants are treated. The age-related cutoff activity of 80 U will assist in the identification of infants with partial biotinidase deficiency. However, we expect our performance metrics to decline slightly because of the overlap between the BW and WW populations.

As demonstrated, the increase in cutoff activity (from 65 U to 80 U for infants >72 hours of age), will allow us to efficiently detect BW individuals. No NN or BN individuals were identified in this study. Therefore, we do not expect that any of our referred infants will be genotypically normal.

Targeted-mutation DNA analysis is an effective 2nd tier test to decrease the false positive rate by identifying individuals with WW genotypes. However, for financial reasons, this has not been considered a viable option.

Table 9: 2009 - 2012 Michigan Biotinidase Deficiency Yearly Performance Metrics

Birth Dates	Screens (N)	Positive	Confirmed	PPV (%)	FPR (%)	Detection Rate
01/05/08-12/31/08: Qualitative Colorimetric Method						
Borderline	119,661	134	7	5.2	0.11	1:14,958
Strong		25	3	12.0		
Overall		159	10	6.3		
01/01/09-12/31/09: Qualitative Colorimetric Method						
Borderline	115,619	120	11	9.1	0.11	1:7,708
Strong		10	4	40		
Overall		130	15	10.8		
01/01/10-12/31/10: Qualitative Colorimetric Method						
Borderline	112,386	222	7	3.2	0.19	1:8,691
Strong		2	2	100		
Overall		224	13	5.8		
01/01/11-08/24/11: Qualitative Colorimetric Method						
Borderline	70,010	147	5	3.4	0.21	1:11,668
Strong		9	1	11.1		
Overall		156	6	3.8		
08/15/11-12/31/11: Semi-Quantitative Fluorometric Method						
Borderline	42,512	11	5	45.5	0.01	1:8,502
Strong		0	0			
Overall		11	5	45.5		
01/01/12-12/31/12: Semi-Quantitative Fluorometric Method						
Borderline	115,544	45	14	31	0.03	1:6,197
Strong		11	4	36.4		
Overall		56	18	32.1		

** We are consider false positives in the screening process.