Adaptation of Neonatal Dried Blood Spot Assays for Use on Automated Microplate Instruments

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INTRODUCTION:

Every year millions of babies undergo newborn bloodspot screening (NBS) to identify the risk of several rare but serious genetic, endocrine and metabolic disorders. These conditions can affect a baby's normal development and life expectancy. NBS uses a few drops of blood obtained by a pinprick from a 24-48 hour old baby's heel deposited on a filter paper card. Once the blood spot has dried it can be easily sent to the laboratory for testing. Due to the large number of samples received automating this testing can be very advantages to a clinical laboratory. In this study we show how several newborn tests can be successfully processed using the Awareness Technology ChemWell-2 openly programmable automated ELISA microplate processing system.

ABSTRACT:

Background: Newborn screening (NBS) is routinely conducted to identify various inherited metabolic disorders which can affect a child's long-term health or survival. The assay methods used include enzyme-linked immunosorbent assays (ELISA) as well as enzymatic biochemical assays. Dried blood spots (DBS) prepared by applying whole blood to filter paper cards are typically used as the sample for these screening assays. In this study we have adapted an openly programmable automated ELISA processing instrument to perform these types of assays with a high level of accuracy and precision.

Method: The Awareness Technology (Palm City, FL) ChemWell-2 instrument was chosen for this study. This instrument is a two plate ELISA processing system which has the capability of performing the DBS extraction in an uncoated round bottom 96-well microplate and transferring a small volume of extracted sample to be assayed to a second flat bottom 96-well microplate. The following enzymatic assay kits manufactured by ZenTech (Liège, Belgium) were used in this study: total galactose, phenylalanine, glucose-6-phosphate dehydrogenase (G-6-PD) and biotinidase.

Results: In order to evaluate the assay performance controls and additional standard DBS were run as samples. Three to four different sample concentration levels were run four to five times in each assay. The runs were repeated 4 times each to determine inter-assay (between run) precision. The results are summarized in the table below:

	Results of 4 Assay Runs with 4 Sample Replicates	
Assay	Inter-assay Precision Range (% C.V.)	Sample Recovery Range (%)
Total Galactose	5.6 – 8.9	92.2 – 94.8
Phenylalanine	4.5 – 10.3	100.5 – 118.2
G-6-PD	3.0 – 3.1	99.9 – 107.5
Biotinidase	5.7 – 9.8	88.2 – 95.4

Conclusion: Enzymatic NBS methods for total galactose, phenylalanine, G-6-PD and biotinidase including the DBS sample extraction step can be processed with clinically useful accuracy and precision using the Awareness Technology ChemWell-2 automated analyzer.

METHOD:

1. Instrumentation:

The Awareness Technology (Palm City, FL) ChemWell-2 (Model 5100) is an openly programmable 2-plate ELISA processing system. The 2-plate format allows one to perform the blood spot sample extraction in a round bottom 96-well microplate (Greiner Bio-One, 650101) and transfer an aliquot of this extract to a second flat bottom microplate (Greiner Bio-One, 655101) to perform the enzymatic reaction. Since this instrument was primarily designed for use with ELISA methods the base unit contains the following filters: 405, 492, 450 and 630 nm (630 nm for differential reading) which are the commonly used wavelengths for ELISA methods. To run the ZenTech enzymatic neonatal tests 2 additional filters were added to the instrument: 550 nm and 700 nm. The violet formazan chromophore used in these methods has an absorbance peak at 550 nm. The 700 nm filter used as a differential filter was shown to improve the assay precision in this instrument. For the biotinidase assay 630nm was used as the differential filter for the azo-dye does not have any significant absorbance at this wavelength.

Some additional software was developed by Awareness Technology to process these assays with accurate incubation timing and to extract the sample using a minimal amount of probe dip to prevent interference from the blood spots in the extraction well. In addition to this a special calculation mode was also created to express the G-6-PD results directly in U/g Hemoglobin based on the sample hemoglobin absorbance at 405 nm. These software features are now available to anyone using the ChemWell-2 instrument.

2. Automated Assay Protocols:

A. Total Galactose:
Plate 1: Two 3mm DBS + 100 µl Elution Buffer (3% trichloroacetic acid) -> Mix 30 minutes -> Transfer 40 µl from plate 1 to plate 2
Plate 2: + 100 µl Galactose Enzyme-Coenzyme (alkaline phosphatase + galactose dehydrogenase + NAD) -> Incubate 30 minutes -> + 80 µl Color Reagent (tetrazolium salt) -> Incubate 15 minutes -> Read (550/700 nm)

B. Phenylalanine:
Plate 1: Two 3mm DBS + 100 µl Elution Buffer (3% trichloroacetic acid) -> Mix 30 minutes -> Transfer 40 µl from plate 1 to plate 2
Plate 2: + 100 µl Phenylalanine Enzyme-Coenzyme (phenylalanine dehydrogenase + NAD) -> Incubate 30 minutes -> + 80 µl Color Reagent (tetrazolium salt) -> Incubate 15 min -> Read (550/700 nm)

C. Galactose 6-Phosphate Dehydrogenase (G-6-PD):
Plate 1: Two 3mm DBS + 75 µl Elution Buffer -> Mix 30 minutes -> Transfer 15 µl from plate 1 to plate 2
Plate 2: + 75 µl G-6-PD Reagent (glucose-6-phosphate + NADP) + 75 µl G-6-PD Color Reagent (tetrazolium salt) -> Read 1 (550/700 nm) -> Incubate 15 minutes -> Read 2 (550/700 nm) -> Read 3 (405/700 nm)

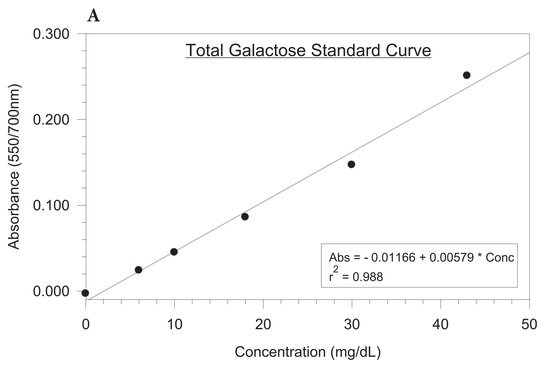
D. Biotinidase:

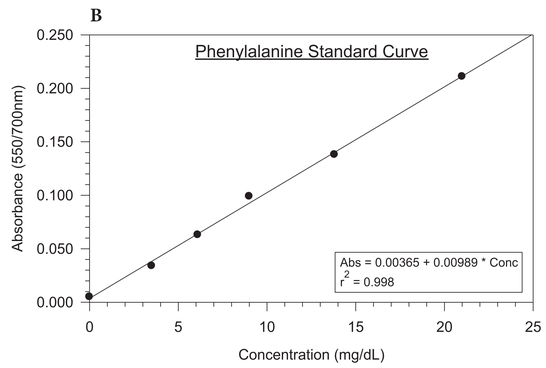
Due to the fact that a precipitate is formed upon stopping the biotinidase reaction with 30% trichloroacetic acid a centrifugation step is required to obtain a clear supernatant that the ChemWell-2 can aspirate a 60 µl aliquot from to complete the reaction. Since our laboratory does not have a microplate centrifuge the DBS extraction was performed in 2 ml microcentrifuge tubes. After the centrifugation step these microcentrifuge tubes were placed in the ChemWell-2 sample rack to complete the assay in a flat bottom microplate.
Sample Microcentrifuge Tube: Two 3mm DBS + 100 µl Substrate Buffer (biotin 4-amidobenzoic acid) -> Mix 10 minutes -> Incubate at 37° C for 18 hours -> + 60 µl Stop Solution (30% trichloroacetic acid) -> 10 minutes centrifugation
Microplate: 60 µl Sample Supernatant + 30 µl Color Reagent 1 (sodium nitrite) -> Incubate 3 minutes -> + 30 µl Color Reagent 2 (ammonium sulfamate) -> Incubate 3 minutes -> + 30 µl Color Reagent 3 (N-(1-Naphthyl)ethylenediamine dihydrochloride) -> Incubate 10 minutes -> Read (550/630 nm).

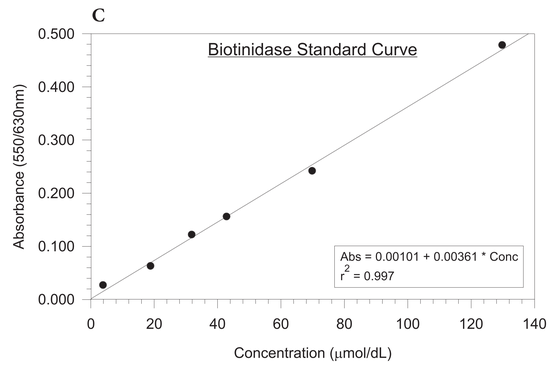
RESULTS:

1. Assay Calibration:

Below are examples of the multipoint calibration curves obtained for Total Galactose (A), Phenylalanine (B) and Biotinidase (C):







D. G-6-PD:

G-6-PD uses the following equation to directly calculate the results in U G-6-PD / g Hb.
Sample Value (in U/g Hb) = (Δ Sample OD550nm/min / Δ Normal Control OD550nm/min) / (Sample OD405nm / Normal Control OD405nm) X Normal Control Concentration Value
The average Δ Normal Control OD550nm/ 15 min and Normal Control OD405nm was 0.244 Abs and 1.813 Abs respectively.

2. Assay Results:

DBS samples with known concentration levels spanning the reportable range of each assay were run 4 times each in 4 separate assay runs with recalibration for each run. The assay results are summarized in the tables below:

Total Galactose Results (mg/dL)

Sample	Run	Replicate 1	2	3	4	Mean	S.D.	% C.V.	Mean Recovery (%)
10.0 mg/dL	1	9.7	9.4	8.5	8.2	8.95	0.7141	8.0	89.5
	2	7.9	10.1	10.8	9.7	9.63	1.2366	12.8	96.3
	3	8.9	9.6	9.3	10.7	9.63	0.7719	8.0	96.3
	4	10.0	10.1	8.7	10.0	9.70	0.6683	6.9	97.0
					Overall:	9.48	0.8458	8.9	94.8
18.0 mg/dL	1	16.7	16.5	16.4	16.4	16.50	0.1414	0.9	91.7
	2	16.9	17.1	16.0	15.0	16.25	0.9609	5.9	90.3
	3	17.1	15.2	17.4	17.1	16.70	1.0100	6.0	92.8
	4	18.2	17.4	18.1	18.2	17.98	0.3862	2.1	99.9
					Overall:	16.86	0.9458	5.6	93.6
30.0 mg/dL	1	28.2	27.6	26.6	26.6	27.25	0.7895	2.9	90.8
	2	25.3	26.6	29.0	26.3	26.80	1.5684	5.9	89.3
	3	28.2	26.3	26.0	26.3	26.70	1.0100	3.8	89.0
	4	29.6	27.6	30.2	32.2	29.90	1.8938	6.3	99.7
					Overall:	27.66	1.8341	6.6	92.2

Glucose-6-Phosphate Dehydrogenase (G-6-PD) Results (U/g Hb)

Sample	Run	Replicate 1	2	3	4	5	Mean	S.D.	% C.V.	Mean Recovery (%)
9.7 U/g Hb	1	9.7	9.6	9.1	9.4	10.2	9.60	0.4062	4.2	99.0
	2	9.7	9.5	10.0	10.2	10.0	9.88	0.2775	2.8	101.9
	3	9.7	9.9	9.6	9.6	9.4	9.64	0.1817	1.9	99.4
	4	9.7	9.9	9.3	9.9	9.4	9.64	0.2793	2.9	99.4
						Overall:	9.69	0.2954	3.0	99.9
5.24 U/g Hb	1	5.7	5.9	5.7	5.6	5.5	5.68	0.1483	2.6	108.4
	2	5.7	5.6	5.6	5.8	5.7	5.68	0.0837	1.5	108.4
	3	5.5	5.9	5.3	5.5	5.5	5.54	0.2191	4.0	105.7
	4	5.4	5.5	5.6	5.7	6.0	5.64	0.2302	4.1	107.6
						Overall:	5.64	0.1755	3.1	107.5
< 1.5 U/g Hb	1	0.4	0.8	0.4	0.9	0.9	0.68	0.2588	31.8	---
	2	0.9	0.8	0.9	1.3	1.2	1.02	0.2168	21.3	---
	3	1.0	0.6	0.5	0.1	0.6	0.56	0.3209	57.3	---
	4	0.7	0.6	1.0	0.5	1.1	0.78	0.2588	33.2	---
						Overall:	0.76	0.2998	39.5	---

Phenylalanine Results (mg/dL)

Sample	Run	Replicate 1	2	3	4	Mean	S.D.	% C.V.	Mean Recovery (%)
3.6 mg/dL	1	3.3	3.9	3.6	3.6	3.60	0.2449	6.8	100.0
	2	3.0	3.9	3.9	3.5	3.58	0.4272	11.9	99.3
	3	3.3	4.0	3.8	3.9	3.75	0.3109	8.3	104.2
	4	3.1	3.2	4.1	3.8	3.55	0.4796	13.5	98.6
					Overall:	3.62	0.3468	9.6	100.5
6.3 mg/dL	1	6.1	8.1	6.9	6.9	7.00	0.8246	11.8	111.1
	2	6.8	6.3	7.2	7.5	6.95	0.5196	7.5	110.3
	3	6.0	7.0	6.4	5.5	6.23	0.6344	10.2	98.8
	4	7.1	6.0	6.7	7.8	6.90	0.7528	10.9	109.5
					Overall:	6.77	0.7002	10.3	107.4
9.5 mg/dL	1	11.2	11.1	12.2	11.3	11.45	0.5066	4.4	120.5
	2	11.6	10.5	10.5	11.7	11.08	0.6652	6.0	116.6
	3	11.6	11.3	10.8	10.3	11.00	0.5715	5.2	115.8
	4	11.4	11.3	11.6	11.2	11.38	0.1708	1.5	119.7
					Overall:	11.23	0.5000	4.5	118.2
15.4 mg/dL	1	17.1	17.7	18.6	17.7	17.78	0.6185	3.5	115.4
	2	16.9	16.5	15.3	15.1	15.95	0.8851	5.5	103.6
	3	15.1	15.9	15.2	15.2	15.35	0.3697	2.4	99.7
	4	19.7	16.1	16.9	17.1	17.45	1.5610	8.9	113.3
					Overall:	16.63	1.3553	8.1	108.0

Biotinidase Results (µmol/dL)

Sample	Run	Replicate 1	2	3	4	Mean	S.D.	% C.V.	Mean Recovery (%)
20.0 µmol/dL	1	18.3	18.3	18.8	19.9	18.83	0.7544	4.0	94.1
	2	18.7	17.9	19.5	15.9	18.00	1.5449	8.6	90.0
	3	17.4	16.9	17.4	19.6	17.83	1.2066	6.8	89.1
	4	22.2	21.9	21.3	21.3	21.68	0.4500	2.1	108.4
					Overall:	19.08	1.8620	9.8	95.4
36.0 µmol/dL	1	34.3	32.1	32.4	32.4	32.80	1.0100	3.1	91.1
	2	32.5	34.1	29.2	32.2	32.00	2.0445	6.4	88.9
	3	31.9	33.0	32.8	29.3	31.75	1.7020	5.4	88.2
	4	37.3	37.0	35.8	36.4	36.63	0.6652	1.8	101.7
					Overall:	33.29	2.4112	7.2	92.5
54.0 µmol/dL	1	44.3	44.3	49.0	49.3	46.73	2.8028	6.0	86.5
	2	45.7	48.2	48.7	45.1	46.93	1.7896	3.8	86.9
	3	44.8	49.4	43.2	47.0	46.10	2.6957	5.8	85.4
	4	50.3	52.1	49.7	50.9	50.75	1.0247	2.0	94.0
					Overall:	47.63	2.7287	5.7	88.2
82.0 µmol/dL	1	78.0	77.5	67.5	76.1	74.78	4.9162	6.6	91.2
	2	76.3	74.0	74.9	65.2	72.60	5.0233	6.9	88.5
	3	69.5	66.1	69.5	75.5	70.15	3.9102	5.6	85.5
	4	72.8	74.1	78.1	79.3	76.08	3.1160	4.1	92.8
					Overall:	73.40	4.5033	6.1	89.5

CONCLUSION:

This study shows that in addition to processing neonatal screening assays using ELISA methods for such conditions as hypothyroidism (TSH), congenital adrenal hyperplasia (17-alpha-hydroxyprogesterone) and cystic fibrosis (immunoreactive trypsinogen) some automated ELISA processors such as the Awareness Technology ChemWell-2 can successfully process neonatal enzymatic assays as well. In this study assays for galactosemia (total galactose), phenylketonuria (phenylalanine), glucose-6-phosphate dehydrogenase deficiency and biotinidase deficiency were shown to yield precise and accurate results from dried blood spot extracted samples.