Measurement of Nicotinamide Adenine Dinucleotide from Dried Blood Spot Cards

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BACKGROUND

- Nicotinamide adenine dinucleotide (NAD+) plays an essential role in many cellular processes. It is consumed by PARPs during free radical-induced DNA damage detection and repair, by Sirtuins during immune response to infection and by other metabolomes crucial to homeostasis during their function. Since all NAD-dependent metabolomes compete for NAD+ from the same intracellular pool of NAD+, intracellular NAD+ depletion has many serious deleterious biological effects, including on cellular bioenergies.
- Studies suggest that NAD+ blood levels might be useful in monitoring patients with mitochondrial disorders, in predicting susceptibility to age-related diseases, and perhaps in predicting severity of COVID-19. Until now, the enzymatic lability of NAD+ has hindered its study in human disease.
- We previously demonstrated that NAD+ could be effectively stabilized on a chemically treated dried blood spot (DBS) card. We have continued the development of this novel method and herein report our progress.

APPROACH

Three separate sets of experiments were performed:

1. A divided sample study to compare whole blood NAD+ concentrations measured using our improved DBS method (see below) to concentrations using a commercially available test offered by NADmed; twelve donors participated in this study;
   - DBS samples were collected via finger stick and allowed to dry for 3 h at 15 – 30 °C.
   - Whole blood samples were collected via venous draw in K_2EDTA tubes, placed at ≤ – 80 °C immediately following collection, and shipped on dry ice for analysis.
2. A comparative stability study of NAD+ in DBS samples stored at two temperatures, at 15 – 30 °C and at ≤ – 20 °C . NAD+ measurements were made through a range of time points and measurements were compared back to the t=0 values;
3. Additional efforts have also been made to refine the chemical treatment of the DBS cards to determine if the stability of NAD+ could be further improved. This refinement lot was prepared with the intent to increase the magnitude of enzyme inactivation when compared to the original lot. A total of 13 enzyme activities were measured from seven patient matched DBS samples collected on both card types.

- DBS samples were prepared as previously described. In brief, this process involved a methanolic extraction in the presence of a heavy labeled internal standard, solvent exchange, and analysis by LC-MS/MS.

CONCLUSION

- The novel DBS method was found to have comparable results to a previously validated whole blood method.
- The chemically treated DBS cards provide acceptable stability to allow for reasonable shipping conditions and potential for sample batching within the laboratory.
- The magnitude of enzyme inactivation was found to be larger in the refinement lot of chemically treated DBS cards compared to the original lot. The effect of this on sample stability will be evaluated in the future.

1. Elhassan, Y.; Philp, A.; Lavery, G., *Journal of the Endocrine Society*, **2017**, *1 (7)*, 816-835.
2. Worley, G.; Pukkila-Worley, R.; Kishnani, P.; Hegde, M., Specific Aims: NAD concentration in blood and severity of coronavirus SARS-CoV-2 Infection. April 23, 2020.

RESULTS

Experiment 1: Methods comparison

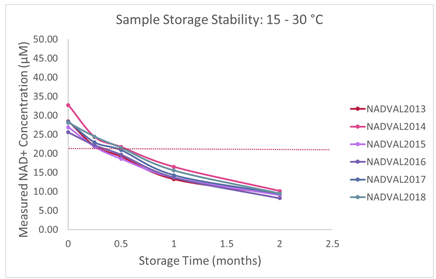
- For the novel dried blood spot method, the average measured NAD+ concentration was 24.81 µM with a range of 19.89 – 29.30 µM.
- The NADmed method resulted in an average of 25.95 µM with a range of 20.39 – 31.23 µM.
- As displayed in table 1, only one subject had a difference in NAD+ concentration > 25 %. The average difference between the two methods was 4.9 %.

Table 1. Comparison of measured NAD+ concentrations using the novel DBS method developed by Revvity Omics and a whole blood-based method developed by NADmed.

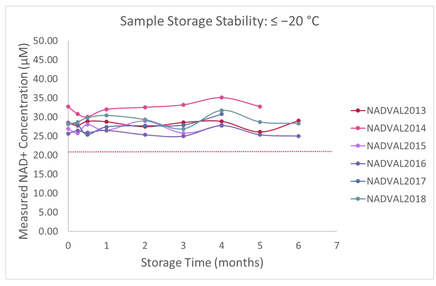
	NAD+ Concentration (µM)		
SAMPLE ID	DBS (PKIG)	Whole Blood (NADmed)	% difference
NADVAL2001	28.46	23.75	16.5
NADVAL2002	23.81	24.06	-1.0
NADVAL2003	20.52	26.52	-29.2
NADVAL2004	25.63	32.13	-25.4
NADVAL2005	23.77	25.02	-5.3
NADVAL2006	26.98	27.40	-1.6
NADVAL2007	19.89	20.36	-2.4
NADVAL2008	23.98	23.70	1.2
NADVAL2009	25.16	27.68	-10.0
NADVAL2010	26.81	26.68	0.5
NADVAL2011	23.43	24.84	-6.0
NADVAL2012	29.30	28.11	4.1

Experiment 2: Sample stability

- Sample stability is a critical factor in determining the potential of this method for routine clinical testing. Measured NAD+ concentration was monitored over 6 months (Figure 1).



(A)



(B)

Figure 1. Measured NAD+ concentrations from six samples stored at different storage temperatures (A) 15 – 30 °C and (B) ≤ – 20 °C.

- The dotted line in each graph represents a loss of 25 % of NAD+ compared to the initial result.
- For the samples stored at 15 – 30 °C, loss of NAD+ was observed throughout the study. This loss was found to be beyond 25 % at the 14-day timepoint. With this coated DBS, samples are stable for 7 days at this temperature range.

Note: due to the amount of loss this study was concluded after 2 months.

- Measured NAD+ concentrations were found to be stable when samples were stored at ≤ – 20 °C.

Note: several samples did not contain enough blood to complete the entire study. Three out of the six were evaluated across the full 6 months.

Experiment 3: Refinement of chemical coating

- Table 2 displays the measured enzyme activities for each sample from each card type. The ability of a coating to reduce enzymatic reactions was evaluated by comparing these measured activity values to the established normal range and abnormal cutoff for each enzyme.
- Both card types were found to be successful in comprehensively reducing enzyme activity. The refinement lot did show some improvement as it had a larger percentage of measured enzyme activities below the abnormal cutoff than the original lot.

Table 2. Measured enzyme activities for 13 different lysosomal enzymes. DBS samples were collected on two different lots of coated DBS cards from seven individuals. The legend below provides detail related to the color coating system.

Indicator	% of measurements Coated Card 1	Refinement Lot 1
below normal mean	39.6	37.4
below abnormal cutoff	59.3	62.6
at or above normal mean	1.1	0

		SAMPLE ID						
	Enzyme Code	NADVAL2019	NADVAL2020	NADVAL2021	NADVAL2023	NADVAL2026	NADVAL2028	NADVAL2030
Coated Card 1 (Enzyme activities in units of µM/h)	ABG	2.555	2.826	1.829	3.406	1.329	3.755	4.452
	ASM	2.504	2.49	2.744	1.764	1.829	3.731	3.803
	GAA	3.112	3.217	4.227	2.903	1.719	6.027	2.52
	GALC	0.542	0.208	0.639	0.344	0.518	1.897	0.416
	GLA	0.511	0.29	0.615	0.302	0.314	0.511	0.465
	IDUA	3.165	2.78	4.253	3.898	1.604	8.623	6.46
	ARSB	1.68	1.09	2.01	1.14	1.15	2.9	1.68
	bGAL	1.33	0.925	1.01	1.25	0.898	1.5	3.09
	GALNS	1.03	0.437	0.826	0.422	0.294	0.535	0.842
	GUSB	3.47	2.47	4.44	3.36	2.43	5.24	4.18
	IDZS	3.43	2.16	5.11	2.46	3.26	2.87	2.58
	NAGLU	1.26	1.12	1.99	2.3	1.13	1.86	1.99
	TPP1	5.2	5.2	6.55	3.74	4.35	4.76	5.7
Coating Refinement Lot 1 (Enzyme activities in units of µM/h)	ABG	0.329	1.819	2.061	7.045	0.89	5.439	5.535
	ASM	0.145	0.494	0.944	1.597	0.668	1.989	1.575
	GAA	0.174	0.639	1.37	3.275	0.499	2.311	0.801
	GALC	0.232	0.319	1.282	1.065	0.871	2.245	0.556
	GLA	0.046	0.186	0.486	1.788	0.159	1.01	0.554
	IDUA	0.247	0.871	2.569	4.319	0.82	5.407	3.165
	ARSB	1.98	1.43	3.04	1.67	1.81	3.59	2.24
	bGAL	2.31	3	5.63	4.61	4.19	4.58	4.99
	GALNS	0.922	0.536	0.7	0.568	0.294	0.568	1.1
	GUSB	1.33	2.8	6.03	4.52	2.76	6.06	4.49
	IDZS	0.0168	0.0936	0.322	0.886	0.231	0.818	0.364
	NAGLU	0.031	0.314	0.962	1.71	0.409	1.17	0.807
	TPP1	1.56	4.73	6.62	4.66	5.41	5.86	6.36