

# Evaluating the Applicability of Dry Blood Spots for Quantitative Measurement of NAD<sup>+</sup> and NADH in Human Blood

White paper by NADMED Ltd.

This white paper has been produced to review the applicability of dry blood spots in quantitative measurement of NAD<sup>+</sup> and NADH in human blood by NADMED Ltd. Parts of this paper might be published elsewhere and are referred to in this document.

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## INTRODUCTION

Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) is the bioactive form of vitamin B3 that acts as an electron carrier, connecting hundreds of metabolic reactions. It also serves as a substrate for regulatory enzymes that enable cellular metabolism to adapt dynamically to changing conditions. In redox reactions, NAD<sup>+</sup> cycles between its oxidized form (NAD<sup>+</sup>) and reduced form (NADH) as it accepts and donates electrons. In contrast, NAD<sup>+</sup>-dependent enzymatic processes consume NAD<sup>+</sup>, thereby decreasing its cellular concentration.

A growing body of evidence shows that diseases can trigger NAD<sup>+</sup> deficiency even under normal nutritional conditions. Notably, blood NAD<sup>+</sup> levels reflect decreases in peripheral tissues, making blood measurements a useful tool for detecting deficiency. Currently, NAD<sup>+</sup> and NADH levels are measured from venous blood collected in K2 EDTA Vacutainers. Although this procedure is minimally invasive, it requires a laboratory visit and trained personnel. Moreover, if samples cannot be analysed immediately, special temperature-controlled storage and shipment are necessary, which are not always accessible. An alternative is the dry blood spot (DBS) method, which requires only a finger prick to collect a few drops of blood on a paper matrix that dries quickly. The apparent advantage of DBS is that it can be performed at home, and samples can be mailed to a laboratory at ambient temperature without special handling requirements.

Dry blood spot (DBS) sampling is widely used in neonatal screening, allowing detection of a broad range of metabolites and non-metabolite analytes, with over 300 polar compounds commonly measured, including amino acids, acylcarnitines, organic acids, steroid hormones, and

nucleotides. However, the applicability of DBS for quantitative measurement of NAD<sup>+</sup> and NADH in clinical diagnostics remains under investigation. Although recent studies show promising results for measuring NAD<sup>+</sup> from chemically treated DBS cards, this method is not yet clinically validated for real-world routine diagnostic use (1,2). Further validation and standardization are needed before DBS sampling can be widely adopted for NAD<sup>+</sup> and NADH quantification in clinical settings.

## AIM OF THE STUDY

In the presented study, we aimed to validate the suitability of two commonly used DBS cards, namely the Whatman Protein Saver™ 903™ Card and the QIAcard FTA DMPK-B Card, for quantitative measurement of NAD<sup>+</sup> and NADH in human whole blood. The former type has an untreated surface, while the latter is chemically treated to cause immediate cell lysis and protein denaturation, thereby quenching all enzymatic activities, including NAD<sup>+</sup> consumption and degradation. The study was comparative, involving paired measurement of target metabolites in fresh and dried aliquots of the same control human samples.

## EXPERIMENTAL APPROACH

For the extraction and measurement of NAD<sup>+</sup> and NADH concentrations in fresh and dried blood, Q40 NAD<sup>+</sup> & NADH blood kits ([www.nadmed.com](http://www.nadmed.com)) were used. Fresh blood analysis requires 100 microliters (µL) of a sample, which is extracted with 500 µL of hot extraction buffer. After protein precipitation and removal, the extract is divided into two aliquots of 150 µL each. One aliquot is mixed with 100 µL of NAD<sup>+</sup> stabilizer for selective NAD<sup>+</sup> quantification, and the other with 100 µL of NADH stabilizer for NADH measurement. This results in a 10-fold dilution of whole blood for individual metabolite analysis. Quantification is performed using enzymatic cycling assays with colorimetric detection, reporting results in µM concentration units.

Two separate sets of experiments were performed in this study.

1. The first set evaluated the performance of Whatman Protein Saver™ 903™ Cards for short-term storage (24 hours) and extraction of NAD<sup>+</sup> and NADH from dried blood for quantitative measurement. Venous blood

from one healthy volunteer was collected into a 2 mL K2-EDTA Vacutainer and thoroughly mixed. Aliquots of 75  $\mu$ L were applied onto the cards and allowed to dry for 24 hours.

For dried blood samples, the entire blood spot areas were cut out with scissors and extracted with 500  $\mu$ L of hot extraction buffer to ensure quantitative transfer of dried matter equivalent to 75  $\mu$ L of blood, enabling back-calculation of NAD<sup>+</sup> and NADH concentrations in the initial blood sample.

- The second study evaluated the performance of the QIAcard FTA DMPK-B Card for extended storage and for quantitative measurement of NAD<sup>+</sup> and NADH from dried blood. Venous blood from 10 healthy volunteers was collected into 4 mL K2-EDTA Vacutainers, thoroughly mixed, and frozen at -80°C. These samples were used for whole blood NAD<sup>+</sup> and NADH measurements.

Dry blood spots were prepared using capillary blood obtained by finger prick. The spots were allowed to dry for 3 hours, then packed into gas-tight plastic bags and stored for one week at ambient temperature, followed by storage at -20°C for a second week. Since the dry blood spots were prepared without volume control, 6 mm-diameter punchers were used to cut out two circles from the dried blood area for each extraction. To enable back-calculation of NAD<sup>+</sup> and NADH concentrations in the initial blood sample based on results from the dried sample, we evaluated the liquid absorbance capacity of the card matrix.

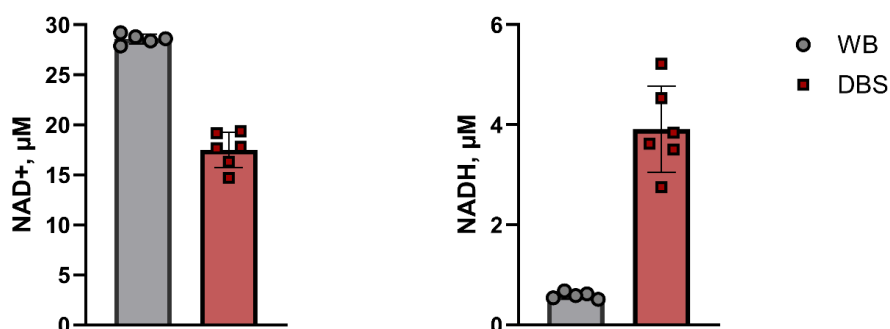
## RESULTS

### Performance evaluation of Whatman Protein Saver™ 903™ Cards

We first analysed recovery of NAD<sup>+</sup> and NADH from Whatman Protein Saver™ 903™ Cards after 24 hours of

storage. We used 75  $\mu$ L blood aliquots to prepare spots with a diameter of approximately 12.5 mm each. For measurement of blood NADs, we used the Q-NADMED Blood NAD<sup>+</sup> and NADH assay kit (IVD\_001), utilizing hot alcohol-based extraction of metabolites, as it has been shown to preserve the stability of both NAD forms in whole-blood extracts (3). The kit design also enables separate measurement of oxidized and reduced NAD forms from the same sample extract. To eluate NAD<sup>+</sup> and NADH from the blood spot, a disk with the absorbed and dried sample was soaked in the extraction solution that represented a mixture of the kit extraction buffer and water (ratio 5:1), to achieve the same final buffer concentration as during the extraction of liquid blood. To facilitate washing of the target metabolites from the paper matrix, a low concentration of non-ionic detergent was added to the extraction solution.

Upon immersion of the paper with a dried blood spot into 500  $\mu$ L of extraction solution, the paper absorbed approximately half of the liquid. This effect made the extraction conditions suboptimal and resulted in only 290–300  $\mu$ L of extract compared to the 550  $\mu$ L extract from the fresh blood. Notably, we were unable to elute all the material from the dry blood spot into the solution, and the paper remained coloured after the extraction. Due to the smaller volume of the extract, we rescaled volumes in the stabilization step to ensure measurement of both metabolites. Measurement of NAD<sup>+</sup> in the dry blood spot extract showed only  $60 \pm 6\%$  (S.D.) recovery compared to the fresh blood sample (Figure 1). Analysis of NADH in the dried spots gave unexpectedly high levels, e.g., 6.6-fold higher than in the fresh blood (Figure 1). This phenomenon can be explained by the extraction of matrix molecules, which interfere with NAD<sup>+</sup> elimination during the NADH stabilization step. Non-complete removal of NAD<sup>+</sup> from the sample results in a higher signal in the NADH assay and makes NADH measurement in dry blood spots unreliable.



**Figure 1.** Comparative analysis of NAD<sup>+</sup> and NADH measurement results in fresh control human blood (n=5 individual extractions) and in dried blood spots (n=6 individual spots) prepared from the same sample using Whatman Protein Saver™ 903™ Cards.

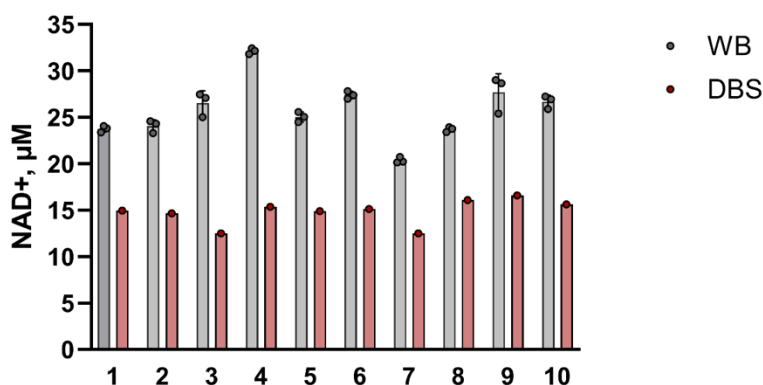
### Performance evaluation of QIAcard FTA DMPK-B Card

Next, we examined the recovery of NAD<sup>+</sup> and NADH from dry blood spots prepared on the QIAcard FTA DMPK-B Card using the same elution protocol and measurement assays. This cellulose matrix is chemically treated and should inhibit any NAD<sup>+</sup>-dependent enzymatic activity in the blood sample upon application, thereby preserving NAD<sup>+</sup> content. In this experiment, collection conditions mimicked in-home collection, in which drops of blood from a finger prick are applied to the paper and dried. It is known that blood applied to DBS paper does not distribute uniformly within the matrix but tends to form a gradient of cell concentration, peaking at the sample contact spot. To ensure reliable analysis, it is advised to use several punched circles fully saturated with blood. The size of the dry blood spots allowed us to punch two 6 mm-diameter discs.

To empirically estimate the volume of whole blood fully absorbed by a 6 mm-diameter area, we punched 6 mm discs from the QIAcard FTA DMPK-B Card. We tested their absorbance capacity by titrating blood volumes until the volume uniformly filled the entire matrix piece. This experiment showed that the 6 mm-diameter circle disc of the QIAcard FTA DMPK-B Card absorbs approximately

14  $\mu$ L of whole blood. The estimate was verified using a complementary approach, in which weighed empty dry 6 mm circles were compared with 6 mm wet circles punched after blood application. For volume calculation, we considered the blood density of 1.055 g/mL. This approach was suboptimal due to difficulties in precisely cutting the wet card. In this setup, a 6 mm circle absorbed about 11  $\mu$ L of blood. For the back-calculation of NAD<sup>+</sup> measured from the dried blood spot sample, we determined that one 6 mm circle absorbs 14  $\mu$ L of blood. Altogether, for extraction from two 6 mm circles, we used material equivalent to approximately 28  $\mu$ L of blood, which is 3-fold lower than the volume of the liquid blood sample used for routine NAD<sup>+</sup> and NADH measurement.

Measurement of NAD<sup>+</sup> in the eluate from two 6 mm dry blood spot discs showed lower NAD<sup>+</sup> concentrations than those measured directly from whole blood. Concentration of NAD<sup>+</sup> measured from dried blood was  $58 \pm 6.5\%$  (S.D.) of the whole blood (Figure 2). Similarly to the Whatman Protein Saver™ 903™ Card evaluation study, NADH measurement from the dried sample of the QIAcard FTA DMPK-B Card showed artificially high concentrations, e.g., 3.5–4-fold higher than in whole blood (not shown).



**Figure 2.** Comparative analysis of NAD<sup>+</sup> measurement results in frozen whole blood samples from 10 healthy individuals (n=3 extractions per sample) and in dried blood spots (one extraction per spot) prepared from the same sample using QIAcard FTA DMPK-B Cards.

### DISCUSSION

Applicability of the QIAcard FTA DMPK-B card for NAD<sup>+</sup> measurement in dried blood spot samples was previously evaluated by Matsuyama et al. (1), who reported that approximately 85% of NAD<sup>+</sup> in the blood sample was preserved in the dried spot. However, we were unable to replicate this result, as our measured recovery was  $58 \pm 6\%$ . A plausible explanation for this discrepancy lies in the methodology used to estimate the blood volume absorbed by the card material, which is crucial for accurate quantification of metabolites per unit volume of the original liquid sample. In the earlier study, the authors

applied a known volume of 5  $\mu$ L of blood onto the card and extracted the entire area of the resulting dried blood spot. We used a similar approach when working with the Whatman Protein Saver™ 903™ card. However, dried blood spot sampling is often intended for home collection, where users cannot precisely control the volume of blood applied. Consequently, metabolic analyses typically rely on punching out discs from the dried blood spot for elution and measurement. Because blood spreads unevenly within the cellulose matrix, multiple discs must be punched and combined to achieve reliable metabolite extraction.

Given the inherently small volumes of blood absorbed by these cards, minor variations in absorbed volume can substantially influence the concentration estimates derived from dried blood spots. To date, no universally reliable method exists to accurately determine the actual volume of blood retained in each punched disc of a dried blood spot. This limitation likely contributes to the variability observed between studies measuring NAD<sup>+</sup> recovery from dried blood spot samples using QIAcard FTA DMPK-B cards.

Additionally, applying multiple blood drops to the same spot risks exceeding the card's chemical treatment capacity to fully denature proteins and inhibit NAD<sup>+</sup> degradation during sample drying. This saturation effect may contribute to the underestimation of NAD<sup>+</sup> levels in dried blood spot samples.

## CONCLUSIONS

Based on our preliminary results, the current state of NAD<sup>+</sup> measurement from dried blood spot samples is not yet reliable enough for diagnostic purposes, due to a high risk for underestimation of the levels. Technical challenges, including variability in blood volume absorbed by punched discs and incomplete chemical stabilization under repeated blood application to the same area on the paper, limit the accuracy and clinical utility of this method at present.

## REFERENCES

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