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Application Note

## Determination of NADH Concentrations with the Synergy™ 2 Multi-Detection Microplate Reader using Fluorescence or Absorbance

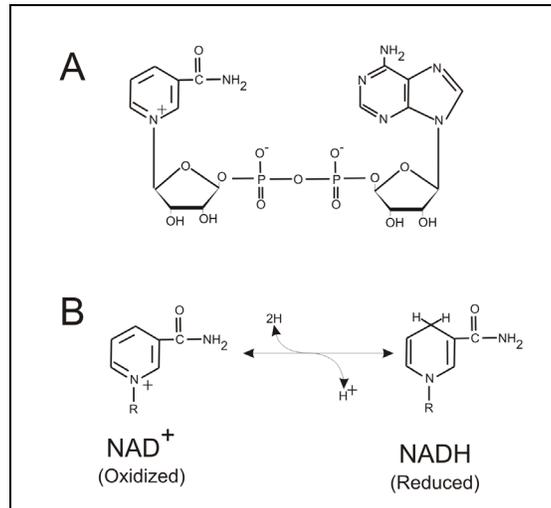
### Do you know what your redox state is?

Many biological assays have as their basis a link to the oxidative status of nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP). Many dehydrogenase enzymes use these coenzymes to transfer hydrogen groups between molecules. Because the reduced forms of these molecules differ from the oxidized forms in their ability to absorb light, it is possible to quantitate reactions based on light absorbance at 340 nm or by the fluorescent emission of light at 445 nm. Here we describe the use of the Synergy™ 2 Multi-Detection Microplate Reader to quantitate NADH using either fluorescence or absorbance modes.

### Introduction

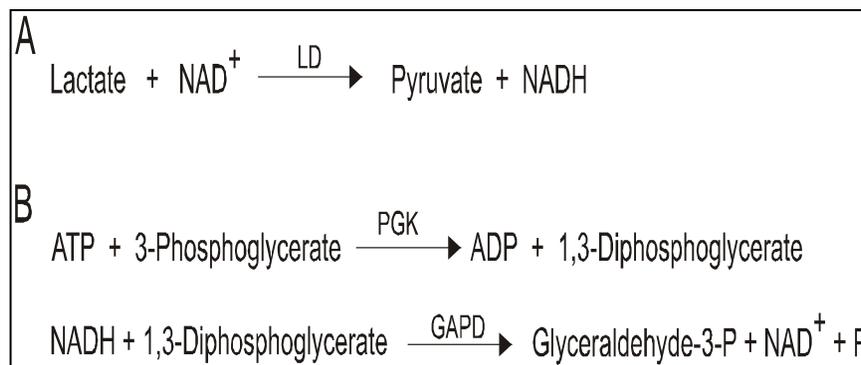
Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) are soluble dinucleotides that can be reversibly reduced by the addition of 2 hydrogen ions. While both molecules act as coenzymes in reversible reactions, they are involved in different types of reactions. NAD is generally used as an acceptor of reducing equivalents in catabolism, particularly glycolysis, the tricarboxylic acid cycle, and  $\beta$ -oxidation of fatty acids, while NADH, is reoxidized by complex I of the electron transport chain or by dehydrogenase enzymes during anaerobic metabolism (1). NADP is characteristically involved with reductive synthesis reactions, such as fatty acid and steroid synthesis (1). As demonstrated in Figure 1, NAD is a multiple ringed structure, which undergoes redox reactions within its nicotinamide ring (2). The closely related NADP molecule is phosphorylated on the 2' position of the adenosine ribose ring. (2).

In terms of quantitation, enzymatic dehydrogenase reactions involving NAD or NADP take advantage of the property of the reduced forms, NADH or NADPH, to absorb light at a wavelength of 340 nm while the oxidized forms do not. Likewise, the reduced forms are capable of fluorescent emission at 445 nm when excited at 340 nm, while the oxidized forms are not (4).



**Figure 1. Structure and redox reaction of nicotinamide adenine dinucleotide (NAD<sup>+</sup>).** Figure (A) depicts the chemical structure of the nicotinamide adenine dinucleotide (NAD<sup>+</sup>) molecule, while (B) demonstrates the bi-directional redox-reaction between NAD and NADH that is catalyzed by dehydrogenase enzymes.

These two physical properties allow investigators to quantitate reactions that directly involve a change in the oxidative state of these coenzymes. An example of such a reaction is outlined in Figure 2A, where the conversion of lactate to pyruvate by the enzyme lactate dehydrogenase requires the conversion of equimolar amounts of NAD<sup>+</sup> to NADH. Reactions of enzymes other than dehydrogenases, which are not directly linked with these coenzymes, can also be measured by coupling their product to a dehydrogenase, which uses that product as a substrate (Figure 2B). When the dehydrogenase is in excess, the rate of appearance or disappearance of NADH may then be used to quantify an enzyme that itself does not use NADH as a substrate.



**Figure 2. Outline of two reactions using NAD<sup>+</sup> as a coenzyme.** Reaction A demonstrates a reaction directly linked with the reduction of the coenzyme NAD<sup>+</sup>. Lactate can be quantitated by formation of NADH as a result of the enzymatic conversion of lactate to pyruvate by the enzyme lactate dehydrogenase. Reaction B depicts a coupled reaction where ATP can be quantitated by a loss of NADH. One of the products of the initial reaction catalyzed by phosphoglycerate phosphokinase (PGK) is utilized as a substrate in a second reaction involving NADH that is catalyzed by glyceraldehyde phosphate dehydrogenase (GAPD).

## Materials and Methods

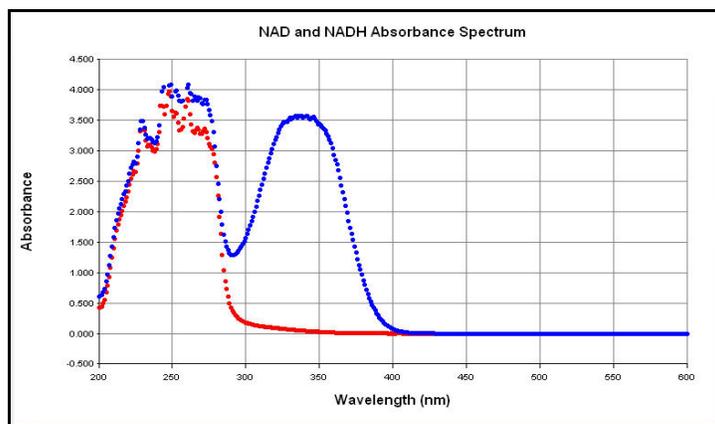
Pre-weighed NADH and NAD<sup>+</sup> vials were purchased from Sigma Chemical Company (St. Louis, MO). Regular and half area 96-well UV transparent microplates, catalogue number 3636 and 3679 respectively, and solid black fluorescence microplates, catalogue number 3915, were purchased from Corning. Stock solutions (1 mg/ml) of each compound were made using TE pH 8.0 (10 mM Tris, 1 mM EDTA) as the buffer. The concentrations of NADH solutions were checked by absorbance at 340 nm using a Shimadzu UV-1700 spectrophotometer.

Further dilutions were then made using TE, pH 8.0 as the diluent and 200  $\mu$ l aliquots of each dilution were dispensed into microplate wells in replicates of 4. Absorbance measurements were made using a Synergy™ 2 Multi-Detection Microplate Reader at 340 nm. Absorbance spectral scans were made using 100 aliquots in half-area UV-transparent microplates from 200 nm to 600 nm in 1-nm increments. The absorbance of TE buffer only was subtracted from the data prior to plotting. The fluorescence of all compounds was determined with the same microplate reader using a 340 nm, 30 nm bandwidth excitation filter and a 440 nm, 40 nm bandwidth emission filter in conjunction with a 400 nm cut off dichroic mirror. The data was collected from the top using the tungsten halogen light source.

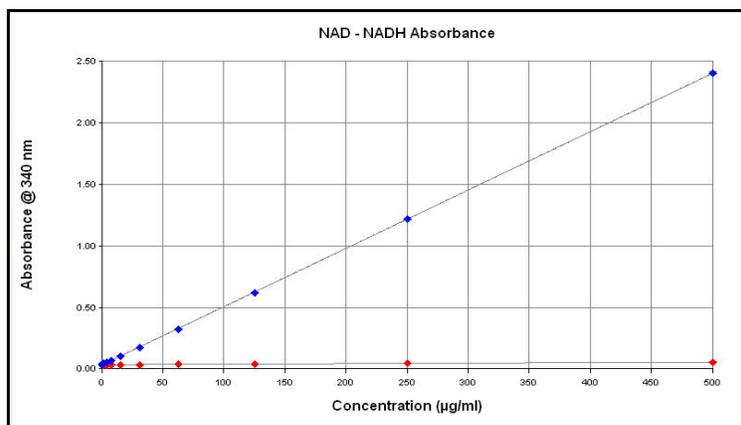
## Results

Spectral scans from 200 nm to 600 nm of NAD<sup>+</sup> and NADH solutions (1 mg/ml) demonstrate the differences between the oxidized and reduced forms of the molecule. NADH in solution produces a significant absorbance peak at 340 nm, while NAD<sup>+</sup> has virtually no absorbance (Figure 3) at this wavelength. This difference is the basis by which numerous assays are monitored. Subsequent experiments used an absorbance, as well as an excitation at 340 nm.

The absorbance was determined for NADH and NAD<sup>+</sup> concentrations ranging from 0 to 500  $\mu$ g/ml (Figure 4). Over this range the absorbance increased in a linear fashion with the NADH samples, while there was virtually no increase in absorbance observed with the NAD<sup>+</sup> samples. Using Gen5™ Data Analysis Software (BioTek Instruments, Winooski, VT) a least means squared linear regression analysis can be generated with a coefficient of determination ( $r^2$ ) value of 1.0. The average coefficient of variation (%CV) of the standards was 2%, with the greatest percent variation occurring in the lower NADH concentrations (data not shown). Under appropriate conditions, NADH concentrations as low as 975 ng/ml were found to be statistically different ( $p=0.003$ ) from the TE buffer only (0  $\mu$ g/ml) control.

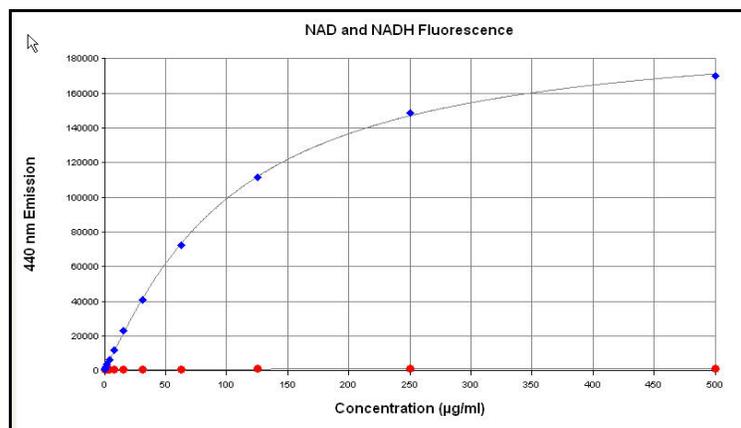


**Figure 3. Spectral Scans of NADH and NAD<sup>+</sup> solutions.** Aliquots (100  $\mu$ l) of NADH (blue dots) and NAD<sup>+</sup> (red dots) solutions (1 mg/ml) were aliquoted into half area-UV transparent plates and a spectral scan from 200 nm to 600 nm in 1 nm increments performed. Data was plotted using Gen5™ Data Analysis Software.



**Figure 4. NADH and NAD<sup>+</sup> concentration curve measured using absorbance at 340 nm.** Serial dilutions of NADH and NAD<sup>+</sup> ranging from 0-500 µg/ml were made using TE pH 8.0 aqueous buffer as the diluent. The absorbance was determined using a Synergy™ 2 Multi-Detection Microplate Reader in absorbance mode. Gen5™ Data Analysis Software was used for reader control and data capture, as well as linear regression analysis of the data.

When the fluorescence of NADH was determined for concentrations ranging from 0 to 500 µg/ml a hyperbolic response was observed (Figure 5) while the oxidized form, NAD, does not exhibit any fluorescent emission at 440 nm when excited by 340 nm light. High concentrations of NADH tend to absorb a significant amount of the excitatory light. As a result of incomplete light saturation of the NADH at the excitation wavelength of 340 nm, a quenching of NADH fluorescence is observed at higher NADH concentrations.

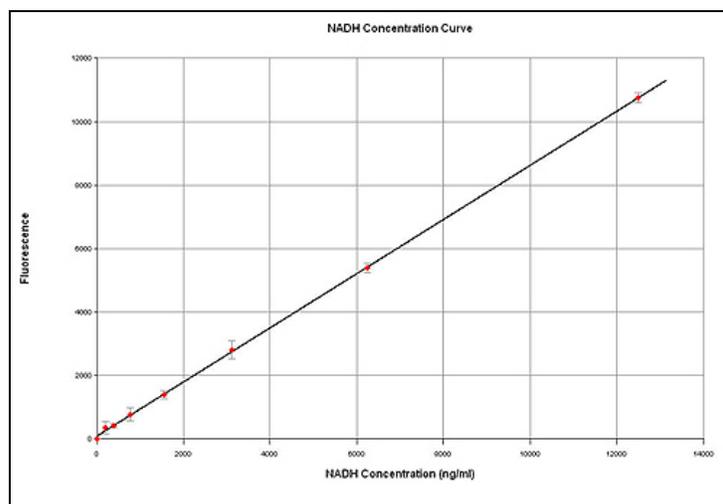


**Figure 5. NADH concentration curve measured using fluorescence.** Serial dilutions of NADH and NAD<sup>+</sup> ranging from 0-500 µg/ml were made using TE pH 8.0 aqueous buffer as the diluent. The fluorescence was determined using a Synergy™ 2. Gen5™ Data Analysis Software was used for reader control and data capture, as well as a 4-parameter logistic fit best fit of the data.

Nevertheless, a very good correlation between concentration and fluorescence is observed using a 4-parameter logistic fit of the data. Unknown concentrations can safely be determined in this range with a high degree of confidence as the coefficient of determination ( $r^2$ ) value was calculated to be 0.999.

When lower NADH concentrations are measured (0-12.5 µg/ml) a linear response is seen (Figure 6). Linear regression analysis of these data suggests that at low concentrations a straight-line function best describes the relationship between NADH concentration and

fluorescence. Under appropriate sensitivity settings, NADH concentrations as low as 37 ng/ml were found to be statistically different ( $p=0.003$ ) from the TE only ( $0 \mu\text{g/ml}$ ) control (Figure 6). Taking into consideration the sample volume in a well, this detection limit represents the determination of as little as  $1.03 \times 10^{-11}$  moles of NADH.



**Figure 6. Linearity of low NADH concentrations.** Dilutions of NADH ranging from 0-12.5  $\mu\text{g/ml}$  were made and their fluorescence determined. Gen5™ Data Analysis Software was used for reader control and data capture, as well as sample blanking and linear regression analysis.

## Discussion

Traditionally, enzymatic reactions involving NADH have been quantitated using changes in absorbance at 340 nm. The reduced form of the coenzymes also has the characteristic of being fluorescent when excited with 340 nm light. In this application note we have described how both methods of quantitating NADH in solution can be performed with the same instrument.

Not surprisingly, detection of low concentrations of NADH is more successful when fluorescence is utilized. The ability of small amounts of light signal to be amplified by the photomultiplier enables their detection against a low background. When using absorbance to measure low levels of the compounds, the opposite is true; very little light signal is being absorbed against a high background. In terms of detection limits, the use of fluorescence to determine NADH and NADPH concentrations translates to approximately an order of magnitude greater sensitivity than absorbance.

In this application note we describe the use of the Synergy™ 2 Multi-Detection Microplate Reader to monitor changes in NADH levels using either absorbance or fluorescence modes. Although we have only demonstrated the capability of measuring different dilutions of NADH, the phosphorylated relative NADPH demonstrates very similar characteristics. These measurements can be utilized to quantitate many different enzymatic assays that utilize these coenzymes for hydrogen ion transfer provided that there is a known molar relationship between the analyte in question and the generation or destruction of NADH or NADPH.

Some assays use the reaction kinetics of the increase or decrease in the amounts of the reduced form of these coenzymes rather than an endpoint determination. Although we have not demonstrated the use of kinetic reactions in this application note, the Synergy™ 2 in conjunction

with Gen5™ Data Analysis Software is capable of making the kinetic determinations (either absorbance or fluorescence) and performing all of the necessary reaction rate calculations (5).

## References

- (1) Stryer, L. (1998) Biochemistry 3<sup>rd</sup> ed., W,H. Freeman and Co., New York, NY.
- (2) Kendrew, J and E. Lawrence, eds. (1994) The Encyclopedia of Molecular Biology, Blackwell Science Ltd, Cambridge, MA.
- (3) Martin, D., P. Mayes, V. Rodwell Eds. (1981) Harper's Review of Biochemistry 18<sup>th</sup> ed. Lange Medical Publications, Los Altos CA.
- (4) Rost, F.W.D. (1995) Fluorescence Microscopy vol II. Cambridge University Press, New York, NY.
- (5) Held. P. (1997) "Kinetic Analysis of  $\beta$ -Galactosidase Activity using the PowerWave 200 in Conjunction with KC4 Data Reduction Software", BioTek Instruments application note.

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