NAD/NADH Cell-Based Assay Kit

Item No. 600480

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GENERAL INFORMATION

Materials Supplied

This kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity/Size	Storage
600485	WST-1 Developer Reagent	1 vial/600 µl	-20°C
600481	Cell-Based Assay Ethanol Solution	1 vial/250 µl	-20°C
600482	Cell-Based Assay Alcohol Dehydrogenase	1 vial/250 µg	-20°C
600483	Cell-Based Assay NAD^+ Diaphorase	1 vial/1.5 mg	-20°C
600486	NAD^+ Standard	1 vial/70 µg	-20°C
600092	Cell-Based Assay Digitonin Solution	1 vial/250 µl	-20°C
10009322	Cell-Based Assay Buffer Tablet	1 Tablet	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the capacity to measure absorbance at 450 nm
2. A 96-well plate for culturing cells
3. A 96-well clear bottom plate for performing the assay
4. A 96-well V-bottom plate for pelleting cells (non-adherent cells only)
5. A plate centrifuge
6. Adjustable pipettes and a repeating or multichannel pipette
7. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Nicotinamide adenine dinucleotide (NAD) is found in all living cells. It exists in two forms, an oxidized form, NAD^+, and a reduced form, NADH. NAD functions as a cofactor in the vast majority of cellular redox reactions, carrying reducing equivalents from one reaction to another. Therefore, maintaining appropriate levels of NAD is essential for maintaining normal cellular respiratory function. There are two major pathways in NAD biosynthesis. The *de novo* pathway is maintained by the rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT), whereas the salvage pathway recycles degraded NAD products such as nicotinamide. NAD^+ and NAMPT levels decrease significantly in multiple organs during aging.[1] Studies have shown that cytosolic NAD^+ concentrations range from 300 nM in mammalian cells to 2 mM in yeast. Depletion of NAD in cells is a major cause of cell death.[2] The importance of NAD function in modulating cellular redox status and controlling signaling and transcriptional events makes NAD an important cofactor when investigating normal cellular function.

About This Assay

Cayman's NAD/NADH Cell-Based Assay Kit provides a colorimetric method for measuring intracellular NAD^+ and NADH in cultured cells. In this assay, NAD^+ found in cell samples is reduced to NADH by alcohol dehydrogenase during the oxidation of ethanol to acetaldehyde. The newly formed and the existing NADH found in the samples is then oxidized resulting in the reduction of a tetrazolium salt substrate (WST-1) to a highly-colored formazan which absorbs at 450 nm (see reaction diagram below). The amount of formazan produced is proportional to the amount of total NAD in the cell lysate and can be used as an indicator of the total cellular NAD concentration.

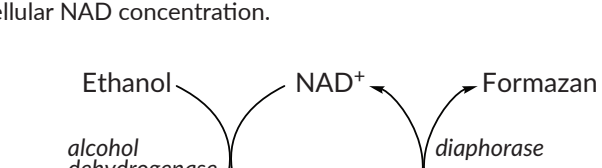


Figure 1. Reaction diagram

PRE-ASSAY PREPARATION

Cell Culture Preparation

1. Seed cells in a 96-well plate at a density of 10^3-10^4 cells/well in 120 µl of culture medium and grow the cells overnight in a CO_2 incubator at 37°C. Be sure to include wells containing culture medium without cells to be used as background controls.
2. The following day, compounds of interest or vehicle controls can be added to cultured cells, at user-determined concentrations. We recommend that each treatment be performed in triplicate.
3. Following treatment with compounds of interest, culture cells in a CO_2 incubator at 37°C for a sufficient period of time to allow for cell adherence to plate (adherent cells) without doubling. This will allow sufficient time for any cells that were disrupted during compound addition to re-attach.

Reagent Preparation

Some of the kit components are in lyophilized or concentrated form and need to be reconstituted or diluted prior to use. Follow the directions carefully to ensure proper volumes of Assay Buffer are used to reconstitute or dilute the vial components.

1. **Assay Buffer Preparation**

   Dissolve the Cell-Based Assay Buffer Tablet (Item No. 10009322) in 100 ml of Ultrapure water. This Assay Buffer is stable for one year at room temperature.

2. **NAD^+ Standard - (Item No. 600486)**

   This vial contains lyophilized NAD^+. Reconstitute the contents of the vial with 1 ml of ultrapure water. The concentration of this solution (the bulk standard) will be 100 µM NAD^+. The reconstituted standard will be stable for 24 hours on ice. If not using all at once, prepare aliquots and store at -20°C for up to three months.

3. **Cell-Based Assay NAD^+ Diaphorase - (Item No. 600483)**

   Reconstitute the contents of the vial with 150 µl Ultrapure water. Any unused reconstituted NAD^+ Diaphorase can be stored at -20°C for two weeks.

4. **Cell-Based Assay Alcohol Dehydrogenase - (Item No. 600482)**

   Reconstitute the contents of the vial with 120 µl Ultrapure water. Any unused reconstituted Alcohol Dehydrogenase can be stored at -80°C for one week.

5. **Permeabilization Buffer**

   To prepare the Permeabilization Buffer, add 200 µl of the Cell-Based Assay Digitonin Solution (Item No. 600092) to 19.8 ml of reconstituted Assay Buffer.

6. **Reaction Solution**

NOTE: Prepare the Reaction Solution immediately before use in the assay. If you are not using a whole 96-well plate, prepare the amount of Reaction Solution required in proportion to the following. Any unused Reaction Solution should be discarded.

To make 10 ml of Reaction Solution sufficient for use on one 96-well plate, add each of the following to 9.5 ml of the Assay Buffer:

200 µl of WST-1 Developer Reagent

100 µl of Cell-Based Assay Ethanol Solution (Item No. 600481)

100 µl of reconstituted Cell-Based Assay Alcohol Dehydrogenase Solution

100 µl of reconstituted Cell-Based NAD$^+$ Assay Diaphorase Solution

ASSAY PROTOCOL

Preparation of Assay-Specific Reagents

Cell-Based Assay NAD$^+$ Standard

To prepare the standard for use in the NAD/NADH Assay, obtain eight clean test tubes and label them A through H. Aliquot 5 ml of Permeabilization Buffer into tube A and 250 µl into tubes B-H. Transfer 50 µl of the NAD$^+$ Standard (Item No. 600486) into tube A and mix thoroughly. The NAD$^+$ concentration of this standard, the first point on the standard curve, is 1,000 nM. Serially dilute the standard by transferring 250 µl from tube A into tube B; mix thoroughly. Next, transfer 250 µl from tube B into tube C; mix thoroughly. Repeat this procedure for tubes E-G. Do not add any standard to tube H. This tube will be your blank.

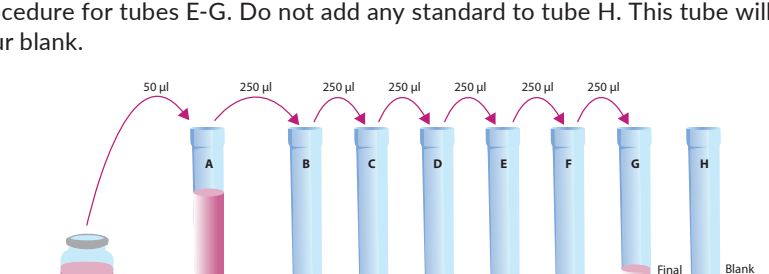


Figure 2. Preparation of the NAD$^+$ Standards. *Buffer in the image above refers to the *Permeabilization Buffer*.

Performing the Assay

1. **For adherent cells** – remove culture medium and proceed to step 3.
2. **For non-adherent cells** - transfer cells to a 96-well v-bottom plate and spin at 500 x g. Proceed to step 3.
3. Wash cells with 120 µl of Assay Buffer to remove culture medium and proceed to step 4.
4. Centrifuge the 96-well plate at 500 x g for five minutes.
5. Aspirate the Assay Buffer, being careful not to disturb the cell monolayer (adherent) or pellet (non-adherent).
6. Add 110 µl of Permeabilization Buffer to each well.
7. Incubate with gentle shaking on an orbital shaker for 30 minutes at room temperature.
8. Centrifuge the plate at 1,000 x g for 10 minutes at room temperature.
9. On a new clear bottom 96-well plate, transfer 100 µl of each standard into appropriate wells. We recommend that the standards be run in duplicate.
10. Transfer 100 µl of the supernatant from each cell culture well to corresponding wells of the new plate.
11. Add 100 µl of Reaction Solution to each well of the clear plate using a repeating or multichannel pipette.
12. Incubate the plate with gentle shaking on an orbital shaker for 90 minutes at room temperature.
13. Measure the absorbance of each sample using a microplate reader at a wavelength of 450 nm.

ANALYSIS

Calculations

1. Calculate the average absorbance of each standard.
2. Subtract the absorbance values of the blank from itself and all other standards and samples. This is the corrected absorbance.
3. Plot the corrected absorbance values (from step 2 above) of each standard as a function of the final concentration of NAD. See Figure 3, on page 14, for a typical standard curve.
4. Calculate the corrected absorbance of each sample by subtracting the absorbance value of the background wells (wells containing culture medium without cells) from each sample.
5. Calculate the NAD concentration of the samples using the corrected absorbance of each sample and the equation below.

$$\text{Total Cellular NAD (nM)} = \left[\frac{\text{Corrected absorbance - (y-intercept)}}{\text{Slope}}\right]$$

Performance Characteristics

The standard curve presented here is an example of the data typically produced with the assay. However, your results will not be identical to these. You must run a new standard curve for each experiment.

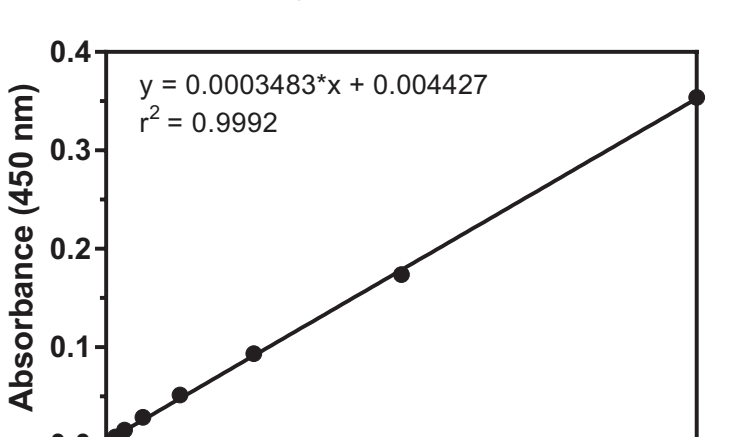


Figure 3. NAD Standard Curve

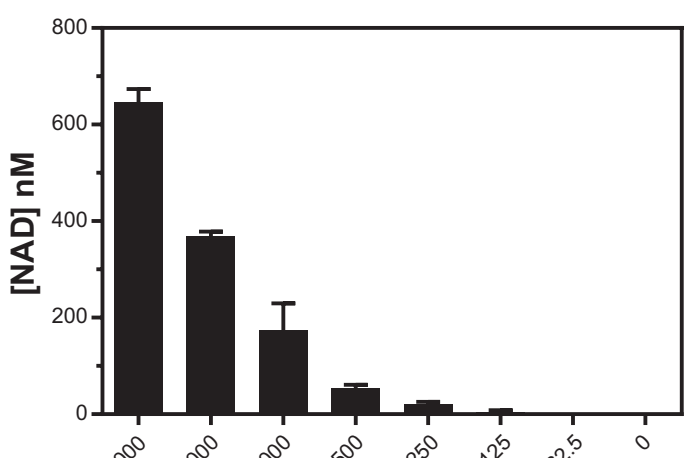


Figure 4. Relationship between cell density and total cellular NAD concentration. HepG2 cells were plated in 96-well tissue culture treated plates at the indicated density. Cells were cultured overnight in DMEM supplemented with 10% FBS at 37°C in a CO_2 incubator (5%) prior to the start of this assay. Total NAD was determined using the protocol found on page 12. Data are presented as means ± standard deviation (n=8).

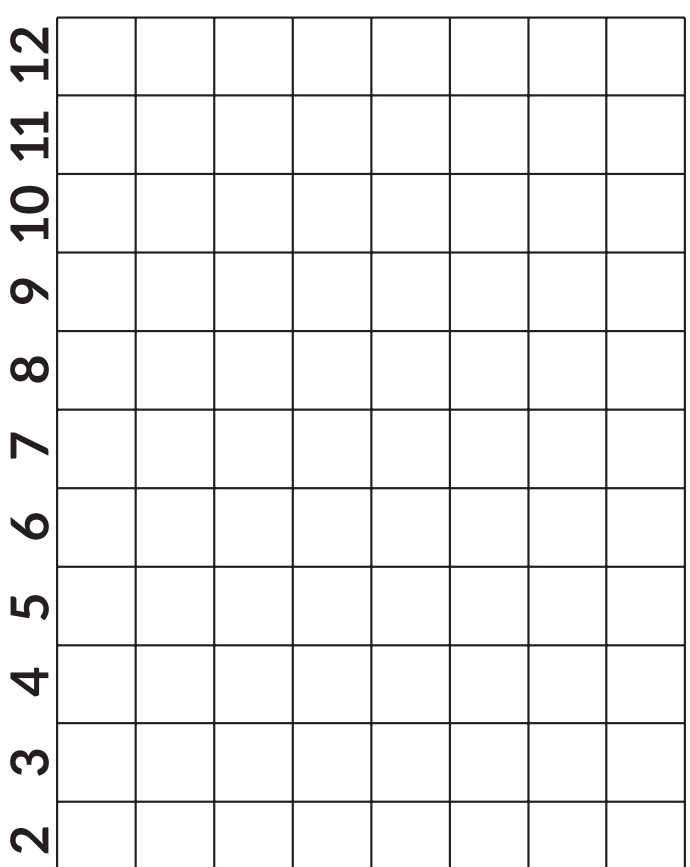
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No signal detected above background in the sample wells	Sample was too dilute	Increase cell density when seeding the cells
Signal from sample wells too low	Low level of NAD in experimental cells	A. Use a different cell line B. Increase incubation time to 2-3 hours after addition of reaction solution

References

1. Yoshino, J., Mills, K.F., Yoon, M.J., *et al*. Nicotinamide mononucleotide, a key NAD^+ intermediate, treats the pathophysiology of diet- and age-induced diabetes in mice. *Cell Metab.* **14**, 528-536 (2011).
2. Yang, H., Yang, T., Baur, J.A., *et al*. Nutrient-sensitive mitochondrial NAD^+ levels dictate cell survival. *Cell* **130**, 1095-1107 (2007).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

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