Amplite™ Colorimetric NADPH Assay Kit

Ordering Information	Storage Conditions	Instrument Platform
Product Number: 15272 (400 assays)	Keep in freezer and avoid light	Absorbance microplate readers

Introduction

Nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate (NADP+) are two important cofactors found in cells. NADH is the reduced form of NAD+. NAD forms NADP with the addition of a phosphate group to the 2' position of the adenyl nucleotide through an ester linkage. The traditional NAD/NADH and NADP/NADPH assays are based on monitoring the changes in NADH or NADPH absorption at 340 nm. The short UV wavelength of NAD/NADH and NADP/NADPH assays makes the traditional methods suffer low sensitivity and high interference. AAT Bioquest's Amplite™ Colorimetric NADPH Assay Kit provides a convenient method for detecting NADPH. The NADPH probe is a chromogenic sensor that has its maximum absorbance at 460 nm upon NADPH reduction. The absorption of the NADPH probe is directly proportional to the concentration of NADPH in the solution. The Amplite™ Colorimetric NADPH Assay Kit provides a sensitive assay to detect as little as 3 µM NADPH in a 100 µL assay volume.

Kit Components

Components	Amount
Component A: NADPH Probe	1 bottle (4 mL)
Component B: Assay Buffer	1 bottle (16 mL)
Component C: NADPH Standard (FW: 833.36)	1 vial (167 µg, lyophilized)
Component D: Lysis Buffer	1 bottle (10 mL)

Assay Protocol for One 96-Well Plate

Brief Summary

Prepare NADPH reaction mixture (50 µL) → Add NADPH standards or test samples (50 µL) → Incubate at RT or 15 minutes to 2 hours → Monitor Absorbance at 460 nm

Note: Thaw one of each kit component at room temperature before starting the experiment.

1. Prepare NADPH stock solution:

Add 200 µL of PBS buffer into the vial of NADPH standard (Component C) to make 1 mM (1 nmol/µL) NADH stock solution. *Note: The unused NADH stock solution should be divided into single use aliquots and stored at -20 ^{o}C.*

2. Prepare NADPH reaction mixture:

Add 1 mL of NADPH Probe (Component A) into 4 mL NADPH Assay Buffer (Component B), and mix well. *Note: 5 mL NADPH reaction mixture is for one 96-well. The unused NADPH reaction mixture should be divided into single use aliquots and stored at -20 ^{o}C.*

3. Prepare serial dilutions of NADPH standard (0 to 100 µM):

3.1 Add 100 µL of NADPH stock solution (from Step 1) into 400 µL PBS buffer (pH 7.4) to generate 200 µM (100 pmol/µL) NADPH standard solution. *Note: Diluted NADPH standard solution is unstable, and should be used within 4 hours.*

3.2 Take 200 µL of 200 µM NADPH standard solution to perform 1:2 serial dilutions to get 100, 50, 25, 12.5, 6.25, 3.13 and 0 µM serial dilutions of NADPH standard.

Add serial dilutions of NADPH standard and NADPH containing test samples into a white/clear bottom 96-well microplate as described in Tables 1 and 2. *Note: Prepare cells or tissue samples as desired. Lysis Buffer (Component D) can be used for lysing the cells for convenience (See appendix for details).*

Table 1 Layout of NADPH standards and test samples in a white/clear bottom 96-well microplate

BL	BL	TS	TS	….	….						
NS1	NS1	….	….	….	….						
NS2	NS2										
NS3	NS3										
NS4	NS4										
NS5	NS5										
NS6	NS6										
NS7	NS7										

Note: NS= NADPH Standards, BL=Blank Control, TS=Test Samples.

Table 2 Reagent composition for each well

NADPH Standard	Blank Control	Test Sample
Serial Dilutions*: 50 µL	PBS: 50 µL	50 µL

**Note: Add the serially diluted NADPH standards from 3.13 µM to200 µM into wells from NS1 to NS7 in duplicate.*

©2013 by AAT Bioquest, Inc. Ordering: sales@aatbio.com; Tel: 800-990-8053; Fax: 408-733-1304

4. Run NADPH assay in supernatants reaction:

4.1 Add 50 µL of NADPH reaction mixture (from Step 2) into each well of NADPH standard, blank control, and test samples (see Step 3.3) to make the total NADPH assay volume of 100 µL/well
Note 1: For a 384-well plate, add 25 µL of sample and 25 µL of NADPH reaction mixture into each well.
Note 2: Prepare cells or tissue samples as desired. Lysis Buffer (Component D) can be used for lysing the cells for convenience (See appendix for details).

4.2 Incubate the reaction at room temperature for 15 minutes to 2 hours, protected from light.

4.3 Monitor the absorbance increase with an absorbance plate reader at 460 nm.

Data Analysis

The absorbance in blank wells (with the PBS buffer only) is used as a control, and is subtracted from the values for those wells with the NADPH reactions. A NADPH standard curve is shown in Figure 1.

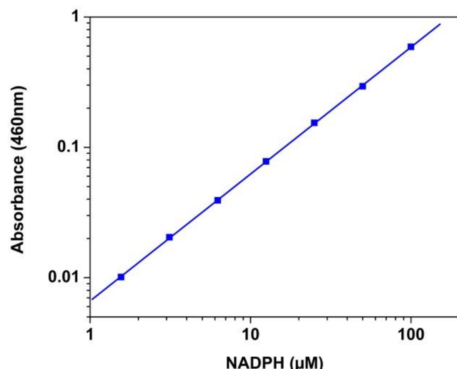

Figure1. NADPH dose response was measured with Amplite™ Colorimetric NADPH Assay Kit in a 96-well white/clear bottom plate using a SpectraMax microplate reader (Molecular devices). As low as 3 µM of NAPDH can be detected with 30min incubation (n=3) with absorbance measurement at 460nm.

Appendix: Test Sample Preparations Using Component D (Lysis Buffer)

1. Plant Cell Samples: Homogenize leave with the lysis buffer at 200 mg/mL, and centrifuge at 2500 rpm for 5-10 minutes, use the supernatant for tests.
2. Bacterial Cell Samples: Collect bacterial cells by centrifugation ((10,000 g, 0°C, 15 min). Use about 100 to 10 million cells/mL lysis buffer, keep the treated solution at room temperature for 15 minutes. Centrifuge at 2500 rpm for 5 minutes, and use the supernatant for tests.
3. Mammalian Cell Samples: Remove medium from plate wells, use about 100 µL lysis buffer per 1-5 million cells (or 50-100 µL/well in a 96-well cell culture plate), and keep the treated solution at room temperature for 15 minutes. Use the cell lysate directly or centrifuge it at 1500 rpm for 5 minutes, use the supernatant for tests.
4. Tissue Samples: Weigh ~20 mg tissue, wash with cold PBS. Homogenize with 400 µl of lysis buffer in a micro-centrifuge tube. Centrifuge at 2500 rpm for 5-10 minutes, use the supernatant for the assay.

References

1. Eugenia Villa-Cuesta, Marissa A. Holmbeckand David M. Rand. Rapamycin increases mitochondrial efficiency by mtDNA-dependent reprogramming of mitochondrial metabolism in Drosophila. *Journal of Cell Science (2014) 127, 2282–2290 doi:10.1242/jcs.142026.*
2. Chao Tong, Alex Morrison, Samantha Mattison, Su Qian, Mark Bryniarski, Bethany Rankin, Jun Wang, D. Paul Thomas, and Ji Li. Impaired SIRT1 nucleocytoplasmic shuttling in the senescent heart during ischemic stress. FASEB J, Nov 2013; 27: 4332 - 4342.
3. Rubin Tan, Jiansha Li, Xiaochun Peng, Liping Zhu, Lei Cai, Tao Wang, Yuan Su, Kaikobad Irani, and Qinghua Hu. GAPDH is critical for superior efficacy of female bone marrow-derived mesenchymal stem cells on pulmonary hypertension**.** Cardiovasc Res, Oct 2013; 100: 19 - 27.
4. Stephen Y. Xue, Valeria Y. Hebert, Danicia M. Hayes, Corie N. Robinson, Mitzi Glover, and Tammy R. Dugas. Nucleoside Reverse Transcriptase Inhibitors Induce a Mitophagy-Associated Endothelial Cytotoxicity That Is Reversed by Coenzyme Q10 Cotreatment**.** Toxicol. Sci**.,** Aug 2013; 134: 323 - 334.
5. Kate J. Roberts, Andrew Cross, Olga Vasieva, Robert J. Moots, and Steven W. Edwards. Inhibition of pre-B cell colony-enhancing factor (PBEF/NAMPT/visfatin) decreases the ability of human neutrophils to generate reactive oxidants but does not impair bacterial killing**.** J. Leukoc. Biol., Sep 2013; 94: 481 - 492.
6. Weijing Cai, Maya Ramdas, Li Zhu, Xue Chen, Gary E. Striker, and Helen Vlassara. Oral advanced glycation endproducts (AGEs) promote insulin resistance and diabetes by depleting the antioxidant defenses AGE receptor-1 and sirtuin 1**.** PNAS, Sep 2012; 109: 15888 - 15893.
7. Yue Qiu, Claus Tittiger, Claude Wicker-Thomas, Gaëlle Le Goff, Sharon Young, Eric Wajnberg, Thierry Fricaux, Nathalie Taquet, Gary J. Blomquist, and René Feyereisen. An insect-specific P450 oxidative decarbonylase for cuticular hydrocarbon biosynthesis. PNAS, Sep 2012; 109: 14858 - 14863.
8. Jaime Uribarri, Weijing Cai, Maya Ramdas, Susan Goodman, Renata Pyzik, Xue Chen, Li Zhu, Gary E. Striker, and Helen Vlassara**.** Restriction of Advanced Glycation End Products Improves Insulin Resistance in Human Type 2 Diabetes: Potential role of AGER1 and SIRT1. Diabetes Care 2011; 34: 1610 - 1616.

©2013 by AAT Bioquest, Inc. **Ordering:** sales@aatbio.com; Tel: 800-990-8053; Fax: 408-733-1304