Amplite™ Colorimetric NAD/NADH Ratio Assay Kit **Yellow or Blue Color**

Ordering Information	*Storage Conditions*	*Instrument Platform*
Product Number: 15273 (250 assays)	Keep in freezer. Avoid exposure to light.	Absorbance microplate readers

Introduction

Nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) are two important coenzymes found in cells. NAD forms NADP with the addition of a phosphate group to the 2' position of the adenyl nucleotide through an ester linkage. NADH (NADPH) is the reduced form of NAD (NADP), and NAD (NADP) is the oxidized form of NADH (NADPH). NAD or NADP functions as a cofactor in redox reaction, transferring electrons in cellular reaction. The balance between the oxidized and reduced forms is NAD/NADH (NADP/NADPH) ratio. This ratio is an important component to indicate the redox state of a cell, and it is a measurement that reflects both the metabolic activities and the health of cells. In healthy mammalian tissues, estimates of the ratio between free NAD^+ and NADH can be as high as 700. In contrast, the NADP/NADPH ratio is normally about 0.005, so NADPH is the dominant form of this coenzyme.

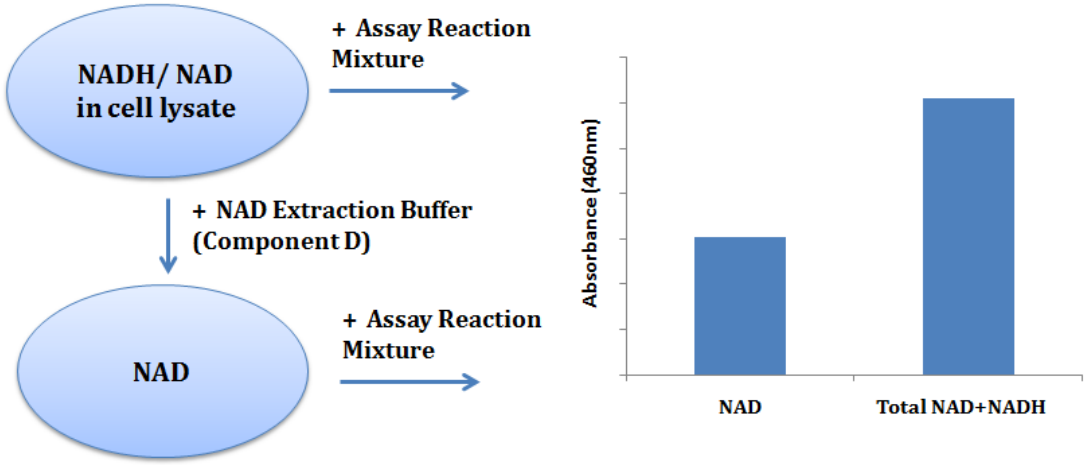

Figure 1. NAD/NADH Ratio Assay Principle

This Amplite™ Colorimetric NAD/NADH Ratio Assay Kit provides a colorimetric method for measuring intracellular total NAD/NADH amount and NAD/NADH ratio in culture cells. In the assay, NAD in the lysate can be extracted with NAD extraction solution and converted to NADH through enzyme reaction, and then recognized by the NADH probe to give a yellow-color dye after reaction, which has the absorbance at 460nm. The amount of the dye generated is directly proportional to the concentration of NAD or NADH in the cell lysate and can be used as an indicator of the cellular NAD/NADH concentration.

Kit Components

Components	Amount
Component A: NAD/NADH Recycling Enzyme Mixture	2 bottles (lyophilized powder)
Component B-I: NADH Probe	1 bottle (4 mL)
Component B-II: NADH Probe Buffer	1 bottle (16 mL)
Component C: NADH Standard (FW: 709)	1 vial (142 µg)
Component D: NAD Extraction Solution	1 bottle (10 mL)
Component E: Neutralization Solution	1 bottle (10 mL)
Component F: Extraction Control Solution	1 bottle (10 mL)
Component G: Lysis Buffer	1 bottle (10 mL)

Assay Protocol for One 96-Well Plate

Brief Summary

Prepare 25 µL of NADH standards and/or test samples → Add 25 µL of NAD Extraction Solution → Incubate at room temperature for 15 minutes → Add 25 µL of Neutralization Solution → Add 75 µL of NAD/NADH reaction mixture → Incubate at RT for 15 minutes to 2 hours → Monitor Absorbance at 460 nm

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

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Technical Support: support@aatbio.com; Tel: 408-733-1055

1. Prepare NADH stock solution:

Add 200 µL of PBS buffer into the vial of NADH standard (Component C) to have 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°C.

2. Prepare NAD/NADH reaction mixture:

2.1 Add 8 mL of NADH Probe buffer (Component B-II) to the bottle of NAD/NADH Recycling Enzyme Mixture (Component A), and mix well.

2.2 Add 2 mL NADH Probe (Component B-I) into above bottle (from Step 2.1) and mix well.
Note: This NAD/NADH reaction mixture is enough for 125~200 assays. The unused NAD/NADH reaction mixture should be divided into single use aliquots and stored at -20°C.

3. Prepare serially diluted NADH standards (0 to 10 µM):

3.1 Add 10 µL of 1 mM NADH stock solution (from Step 1) into 990 µL PBS buffer (pH 7.4) to generate 10 µM (10 pmols/µL) NADH standard solution.
Note: Diluted NADH standard solution is unstable, and should be used within 4 hours.

3.2 Take 200 µL of 10 µM NADH standard solution (from Step 3.1) to perform 1:2 serial dilutions to get 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078 and 0 µM serially diluted NADH standards.

4. Run Total NAD/NADH Assay (total 400 assays/kit):

4.1 Add serial dilutions of NADH standard and NAD/NADH 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 NAD/NADH Lysis Buffer (Component G) can be used for lysing the cells (See appendix for details).

Table 1. Layout of NADH 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= NADH Standards, BL=Blank Control, TS=Test Samples.

Table 2. Reagent composition for each well

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

**Note: Add the serially diluted NADH standards from 0.078 µM to 5 µM into wells from NS1 to NS7 in duplicate. High concentration of NADH (e.g., >100 µM, final concentration) will cause saturated signal and make the calibration curve non-linear.*

4.2 Add 50 µL of NAD/NADH reaction mixture (from Step 2.2) into each well of NADH standard, blank control, and test samples (from Step 4.1) to make the total NAD/NADH assay volume of 100 µL/well.

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

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

5. Run NAD/NADH Ratio Assay (total 250 assays/kit):

5.1 Add serially diluted NADH standards and/or NAD/NADH containing test samples into a white/clear 96-well microplate as described in Tables 3 and 4.
Note: Prepare cells or tissue samples as desired. Lysis Buffer (Component G) can be used for lysing the cells for convenience (See appendix for details).

Table 3. Layout of NADH standards and test samples in a white/clear 96-well microplate

BL	BL	TS	TS	TS (NAD)	TS (NAD)						
NS1	NS1	….	….	….	….						
NS2	NS2										
NS3	NS3										
NS4	NS4										

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NS5	NS5										
NS6	NS6										
NS7	NS7										

*Note: NS= NAD/NADH Standards; BL=Blank Control; TS=Test Samples; TS (NAD) = Test Samples treated with **NAD Extraction Solution (Component D)** for 10 to 15 minutes, then neutralized by **Neutralization Solution (Component E).***

Table 4. Reagent compositions for each well

NADH Standard	**Blank Control**	**Test Sample (NAD/NADH)**	**Test Sample (NAD Extract)**
Serial Dilutions*: 25 µL	PBS: 25 µL	Test Sample: 25 µL	Test Sample: 25 µL
Component F: 25 µL	Component F: 25 µL	Component F: 25 µL	Component D: 25 µL
Incubate at room temperature for 10 to 15 minutes			
Component F: 25 µL	Component F: 25 µL	Component F: 25 µL	Component E: 25 µL
Total: 75 µL	Total: 75 µL	Total: 75 µL	Total: 75 µL

**Note: Add the serially diluted NADH standards from 0.078 µM to5 µM into wells from NS1 to NS7 in duplicate. High concentration of NADH (e.g., > 100 µM, final concentration) will cause saturated signal and make the calibration curve non-linear.*

5.2 **For NAD Extraction (NAD amount):** Add 25 µL of NAD Extraction Solution (Component D) into the wells of NAD/NADH containing test samples. Incubate at room temperature for 10 to 15 minutes, then add 25 µL of NADH Neutralization Solution (Component E) to neutralize the NAD extracts as described in Tables 3 & 4.

For Total NAD and NADH (Total amount): Add 25 µL of NAD/NADH Control Solution (Component F) into the wells of NADH standards and NAD/NADH containing test samples. Incubate at room temperature for 10 to 15 minutes, and then add 25 µL of Extraction Control Solution (Component F) as described in Tables 3 and 4.
Note: Prepare cells or tissue samples as desired. Lysis Buffer (Component G) can be used for lysing the cells (See appendix for details).

5.3 Add 75 µL of NADH reaction mixture (from Step 2.2) into each well of NADH standard, blank control, and test samples (NAD/NADH, and NAD Extract) (from Step 5.1) to make the total NADH assay volume of 150 µL/well.

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

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

Data Analysis

The absorbance in blank wells (PBS buffer only) is used as a control, and is subtracted from the values for those wells with the NADH reactions.

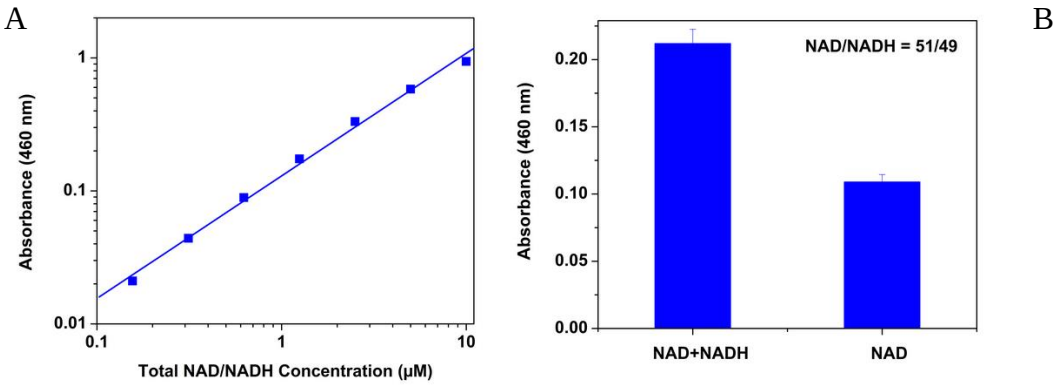

Figure 1. Amplite™ Colorimetric NAD/NADH Ratio Assay Kit is used to measure total NAD/NADH amount and NAD/NADH ratio in a white/clear 96-well microplate using a SpectraMax microplate reader (Molecular devices).
A- Total NADH and NAD dose response: As low as 0.1 µM of Total NADH can be detected with 1 hour incubation.
B- NAD/NADH ratio: Equal amount of NAD and NADH mixture was treated with or without NAD extraction solution for 15 minutes, and then neutralized with extraction solution at room temperature. The signal was read at 460 nm. NAD/NADH molar ratio is calculated based on the absorbance shown in the figure 1B.

Appendix: Test Sample Preparations Using Component G (NAD/NADH 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, And**r**ew 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.