NAD / NADH Assay Kit

NAD / NADH Assay Kit is a detection kit for the quantification of NAD / NADH content in serum, plasma, urine, tissue extracts, cell lysate, cell culture supernatants.

Catalog number: ARG82283

Package: 96 wells

TABLE OF CONTENTS

MANUFACTURED BY:

Arigo Biolaboratories Corporation

Address: No. 22, Ln. 227, Gongyuan Rd., Hsinchu City 300, Taiwan

Phone: +886 (3) 562 1738

Fax: +886 (3) 561 3008

Email: info@arigobio.com

INTRODUCTION

Nicotinamide adenine dinucleotide (NAD) is a cofactor found in all living cells. The compound is called a dinucleotide because it consists of two nucleotides joined through their phosphate groups. One nucleotide contains an adenine nucleobase and the other nicotinamide. Nicotinamide adenine dinucleotide exists in two forms: an oxidized and reduced form, abbreviated as NAD+ and NADH respectively.

In metabolism, nicotinamide adenine dinucleotide is involved in redox reactions, carrying electrons from one reaction to another. The cofactor is, therefore, found in two forms in cells: NAD+ is an oxidizing agent – it accepts electrons from other molecules and becomes reduced. This reaction forms NADH, which can then be used as a reducing agent to donate electrons. These electron transfer reactions are the main function of NAD. However, it is also used in other cellular processes, most notably a substrate of enzymes that add or remove chemical groups from proteins, in posttranslational modifications. Because of the importance of these functions, the enzymes involved in NAD metabolism are targets for drug discovery.

In organisms, NAD can be synthesized from simple building-blocks (de novo) from the amino acids tryptophan or aspartic acid. In an alternative fashion, more complex components of the coenzymes are taken up from food as niacin. Similar compounds are released by reactions that break down the structure of NAD. These preformed components then pass through a salvage pathway that recycles them back into the active form. [Wikipedia Nicotinamide adenine dinucleotide]

PRINCIPLE OF THE ASSAY

This NAD / NADH Assay Kit is based on an alcohol dehydrogenase cycling reaction, in which the formed NADH reduces a formazan reagent. The kit is used to measure NAD / NADH in urine, serum, plasma, tissue extracts, cell lysate and cell culture supernatants. Samples should be extracted and neutralized with proper protocols and assay buffers. The intensity of the reduced product color, measured at 492 nm. The NAD / NADH concentration in the sample is then determined by comparing the O.D. of samples to the standard.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8°C. Use the kit before expiration date.

Component	Quantity	Storage information
Microplate	96 wells	4°C
NADH Standard	1 vial (Lyophilized)	-20°C, (Protect from light)
NAD+ Standard	1 vial (Lyophilized)	-20°C, (Protect from light)
Assay Buffer A	30 ml (Ready to use)	4°C
Assay Buffer B	30 ml (Ready to use)	4°C
Reaction Buffer	10 ml (Ready to use)	4°C
Enzyme	1 vial (Lyophilized)	-20°C
Substrate	10 ml (Ready to use)	4°C
Reaction Dye A	1 vial (Lyophilized)	4°C, (Protect from light)
Reaction Dye B	1 ml (Ready to use)	4°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 492 nm
- Pipettes and pipette tips
- Deionized or distilled water
- Centrifuge spin at 20000xg

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Upon receipt, Standards and Enzyme should be store at ≤-20°C and protect from light. After reconstruction, Standards, Enzyme and Reaction Dye A should be aliquoted and stored at-20°C to avoid repeated freeze-thaw cycles. Store other components in the kit at 4°C at all time.
- Briefly spin down the reagents before use.
- It is highly recommended that the standards and samples be assayed in at least duplicates.
- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Serum- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g at 2-8°C. Remove serum and assay immediately or aliquot and store samples at ≤ -20°C or below. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g at 2-8°C within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20°C or below. Avoid repeated freeze-thaw cycles.

Cell Culture Supernatants, urine - Remove particulates by centrifugation and aliquot & store samples at ≤ -20°C. Avoid repeated freeze-thaw cycles.

Extract the NAD^+ from liquid samples:

1. Add **50µl** of **samples** into **100µl** of **Assay buffer A** in a centrifuge tube, mix thoroughly.
2. Incubate at **60°C for 20 minutes**.
3. Centrifuge the tube with 4000xg for 10 minutes at 4°C, take the supernatant into a new centrifuge tube.
4. Add **100µl** of **Assay buffer B** into the supernatant, mix thoroughly. Keep it on ice before assay.

Extract the NADH from liquid samples:

1. Add 50µl of samples into 100µl of Assay buffer B in a centrifuge tube, mix thoroughly.
2. Incubate at 60°C for 20 minutes.
3. Centrifuge the tube with 4000xg for 10 minutes at 4°C, take the supernatant into a new centrifuge tube.
4. Add 100µl of Assay buffer A into the supernatant, mix thoroughly. Keep it on ice before assay.

Tissue samples-

Extract the NAD^+ from Tissue samples:

1. Weigh out 0.05g of tissue and homogenize it with 100µl of Assay buffer A on ice.
2. Incubate at 60°C for 20 minutes.
3. Centrifuge the tube with 4000xg for 10 minutes at 4°C, take the supernatant into a new centrifuge tube.
4. Add 100µl of Assay buffer B into the supernatant, mix thoroughly. Keep it on ice before assay.

Extract the NADH from Tissue samples:

1. Weigh out 0.05g of tissue and homogenize it with 100µl of Assay buffer B on ice.
2. Incubate at 60°C for 20 minutes.
3. Centrifuge the tube with 4000xg for 10 minutes at 4°C, take the supernatant into a new centrifuge tube.
4. Add 100µl of Assay buffer A into the supernatant, mix thoroughly. Keep it on ice before assay.

Cell samples-

Extract the NAD^+ from cell samples:

1. Collect cells into centrifuge tubes, wash the cells once with PBS and discard the supernatant after centrifugation.
2. For 1 $X10^6$ cells (100 X 10^4 cells), add **100µl** of **Assay buffer A** resuspend and sonicate (with power 20%, sonication 2s, intervention 1s, repeat 30 times) cells on ice.
3. Incubate at **60°C for 20 minutes**.
4. Centrifuge the tube with **8000xg** for 10 minutes at 4°C, take the supernatant into a new centrifuge tube.
5. Add **100µl** of **Assay buffer B** into the supernatant, mix thoroughly. Keep it on ice before assay.

Extract the NADH from cell samples:

1. Collect cells into centrifuge tubes, wash the cells once with PBS and discard the supernatant after centrifugation.
2. For 1 $X10^6$ cells (100 X 10^4 cells), add **100µl** of **Assay buffer B** resuspend and sonicate (with power 20%, sonication 2s, intervention 1s, repeat 30 times) cells on ice.
3. Incubate at **60°C for 20 minutes**.
4. Centrifuge the tube with **8000xg** for 10 minutes at 4°C, take the supernatant into a new centrifuge tube.
5. Add **100µl** of **Assay buffer A** into the supernatant, mix thoroughly. Keep it on ice before assay.

REAGENT PREPARATION

- **Reaction Dye A:** Reconstitute the Reaction Dye A with **1 ml** of **distilled water.** Allow the Reaction Dye to sit for few minutes with gentle agitation to make sure the Reaction Dye A is dissolved completely before use. Aliquot & store the reconstituted Reaction Dye at -20°C. Avoid repeated freeze-thaw cycles.
- **Enzyme:** Reconstitute the Enzyme with **1 ml** of **Reaction Buffer.** Allow the Enzyme to sit for few minutes with gentle agitation to make sure the Enzyme is dissolved completely before use. Aliquot & store the reconstituted Enzyme at ≤ -20°C (-80°C is recommended). Avoid repeated freeze-thaw cycles.
- **NADH Standard:** Reconstitute the NADH Standard with **1 ml** of **distilled water**. Allow the NADH Standard to sit for few minutes with gentle agitation to make sure the NADH Standard is dissolved completely before use. Aliquot & store the reconstituted NADH Standard stock at -20°C for up to a month. Avoid repeated freeze-thaw cycles.

  Dilute the reconstituted NADH Standard stock **40X** with **distilled water** to yield a working standard concentration of **50 µmol/L**. (e.g. add 25 µl of reconstituted NADH Standard stock with 975 µl of distilled water)
- **NAD^+ Standard:** Reconstitute the NAD^+ Standard with **1 ml** of **distilled water**. Allow the NAD^+ Standard to sit for few minutes with gentle agitation to make sure the NAD^+ Standard is dissolved completely before use. Aliquot & store the reconstituted NAD^+ Standard stock at -20°C for up to a month. Avoid repeated freeze-thaw cycles.

  Dilute the reconstituted NAD^+ Standard stock at **40X** with **distilled water**

to yield a working standard concentration of **50 µmol/L.** (e.g. add 25 µl of reconstituted NAD^+ Standard stock with 975 µl of distilled water)

- **Sample**: If the initial assay found samples contain NAD / NADH Assay Kit higher than the standard, the samples can be diluted with distilled water and then re-assay the samples. For the calculation of the concentrations this dilution factor has to be taken into account.
  (It is recommended to do pre-test to determine the suitable dilution factor).

ASSAY PROCEDURE

Standards and samples should be assayed in at least duplicates.

1. Add **20 µl** per well of **samples** and **standard working solution** in duplicates into the appropriate well of the 96-well plate.
2. Add **20 µl** per well of **distilled water** in duplicates as **blank**
3. Add **70 µl** of **Reaction Buffer** in each well.
4. Add **10 µl** of **Enzyme** in each well.
5. Add **80 µl** of **Substrate** in each well.
6. Add **10 µl** of **Reaction Dye A** in each well.
7. Add **10 µl** of **Reaction Dye B** in each well.
8. Mix thoroughly, incubate the tubes at **RT for 10 min** in dark.
9. Read the absorbance measured at **492 nm** immediately.

Summary of NAD^+ Assay Procedure

Reagent	Sample	NAD^+ Standard	Blank
Sample	20 µl	-	-
Standard	-	20 µl	-
Distilled water	-	-	20 µl
Reaction Buffer	70 µl	70 µl	70 µl
Enzyme	10 µl	10 µl	10 µl
Substrate	80 µl	80 µl	80 µl
Reaction Dye A	10 µl	10 µl	10 µl
Reaction Dye B	10 µl	10 µl	10 µl
Mix thoroughly, incubate the plate at **RT** for **10 min** in dark.			
Read at 492 nm.			

Summary of NADH Assay Procedure

Reagent	Sample	NADH Standard	Blank
Sample	20 µl	-	-
Standard	-	20 µl	-
Distilled water	-	-	20 µl
Reaction Buffer	70 µl	70 µl	70 µl
Enzyme	10 µl	10 µl	10 µl
Substrate	80 µl	80 µl	80 µl
Reaction Dye A	10 µl	10 µl	10 µl
Reaction Dye B	10 µl	10 µl	10 µl
Mix thoroughly, incubate the plate at **RT** for **10 min** in dark.			
Read at 492 nm.			

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of samples, standards and blank.

2. Calculation:

A. Definition:

$C_{Protein}$: the protein concentration, mg/ml.

$C_{Standard}$: the protein concentration, 50 µmol/L = 0.05 µmol/ml

W: the weight of sample, g.

N: the quantity of cell or bacteria, $N \times 10^{4.}$ (If 2.5 X 10^6 cells were used, N=250)

$V_{Standard}$: the volume of the standard, 0.02 ml.

V_{Sample}: the total volume of sample, 0.02 ml.

V_{Assay}: the volume of Assay buffer A and Assay buffer B in sample preparation, 0.2 ml.

n: dilution factor = 5

B. Formula of NAD^+: (OD from NAD^+ assay)

a). According to the volume of serum, plasma (or other liquid) samples

NAD^+ (µmol/ml) = n X [($C_{Standard}$ X $V_{satadard}$) X ($OD_{Sample} - OD_{Blank}$) / [V_{Sample} X ($OD_{Standard} - OD_{Blank}$)]

$= 0.25 \times [(OD_{Sample} - OD_{Blank}) / (OD_{Standard} - OD_{Blank})]$

b). According to the weight of sample

NAD^+ (µmol/g) = [($C_{Standard}$ X $V_{satadard}$) X ($OD_{Sample} - OD_{Blank}$) / [(W X (V_{Sample} / V_{assay})) X ($OD_{Standard} - OD_{Blank}$)]

$= 0.01 \times (OD_{Sample} - OD_{Blank}) / [(OD_{Standard} - OD_{Blank}) \text{ X W}]$

c). According to the quantity of cells

NAD^+ (μmol/10^4 cell) = [($C_{Standard}$ X $V_{satadard}$) X (OD_{Sample} – OD_{Blank}) / [(N X (V_{Sample} / V_{assay})) X ($OD_{Standard}$ - OD_{Blank})]

= 0.01 × (OD_{Sample} – OD_{Blank}) / [($OD_{Standard}$ - OD_{Blank}) X N]

C. Formula of NADH: (OD from NADH assay)

a). According to the volume of serum, plasma (or other liquid) samples

NADH (μmol/ml) = n X [($C_{Standard}$ X $V_{satadard}$) X (OD_{Sample} – OD_{Blank}) / [V_{Sample} X ($OD_{Standard}$ - OD_{Blank})]

= 0.25 × [(OD_{Sample} – OD_{Blank}) / ($OD_{Standard}$ - OD_{Blank})]

b). According to the weight of sample

NADH (μmol/g) = [($C_{Standard}$ X $V_{satadard}$) X (OD_{Sample} – OD_{Blank}) / [(W X (V_{Sample} / V_{assay})) X ($OD_{Standard}$ - OD_{Blank})]

= 0.01 × (OD_{Sample} – OD_{Blank}) / [($OD_{Standard}$ - OD_{Blank}) X W]

c). According to the quantity of cells

NADH (μmol/10^4 cell) = [($C_{Standard}$ X $V_{satadard}$) X (OD_{Sample} – OD_{Blank}) / [(N X (V_{Sample} / V_{assay})) X ($OD_{Standard}$ - OD_{Blank})]

= 0.01 × (OD_{Sample} – OD_{Blank}) / [($OD_{Standard}$ - OD_{Blank}) X N]

3. Detection range:

The detection range is from 0.1 μmol/L to 50 μmol/L