Quantitative Test Kit for NAD+/NADH

CycLex NAD+/NADH Colorimetric Assay Kit Ver.2

For 100 Assays

Cat# CY-1253V2

Intended Use

The MBL Research Product **CycLex NAD+/NADH Colorimetric Assay Kit Ver.2** provides a convenient method for sensitive measurement of NAD+, NADH, and their ratio in cell extracts. This kit is designed for specific detection of NAD+ and NADH by an enzyme cycling reaction.

Individual users should determine appropriate conditions when using other types of samples.

Applications for this kit include:
1) Measuring NAD+ and NADH concentrations in cell extracts.
2) Measuring NAD+/NADH ratio.
3) Detecting effects of pharmacological agents on cellular level of NAD+ and NADH.

NAD+ and NADH Extraction Solutions, and neutralization Solutions are NOT provided in this kit.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store all components at -70°C.
- Do not expose reagents to excessive light.

Introduction

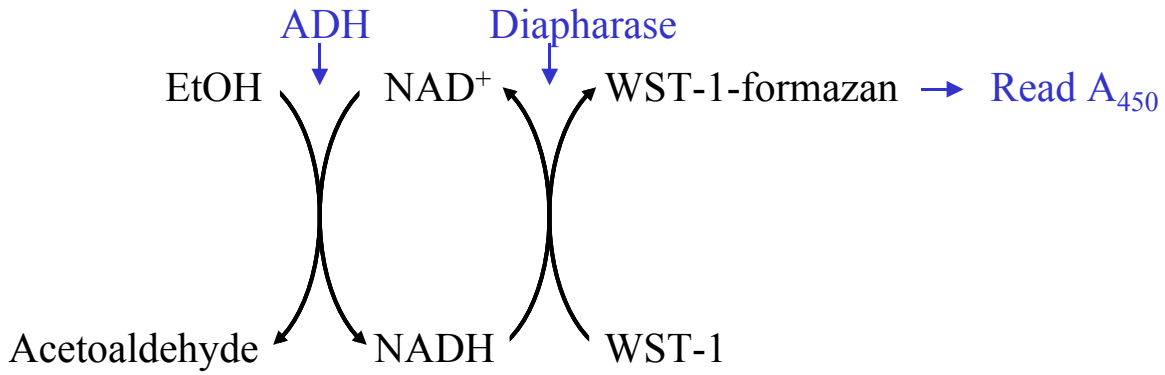
Nicotinamide adenine dinucleotide (NAD^+) as well as nicotinamide adenine dinucleotide phosphate ($NADP^+$) is an important cofactor found in cells. NADH is the reduced form of NAD^+, and NAD^+ is the oxidized form of NADH. It has been reported that NAD^+ metabolism regulates important biological effects including life span. NAD^+, through poly-ADP-ribosyl polymerase (PARP), mono-ADP-ribosyltransferase (ARTs) and recently characterized sirtuin enzymes, exerts potential biological effects. These enzymes modify proteins to regulate their function via ADP-ribosylation or deacetylation in the presence of NAD^+. These enzymes are involved in several pathways including apoptosis, DNA repair, senescence, and endocrine signaling, suggesting that either the enzymes could be an important therapeutical target for cancer, diabetes atherosclerosis, and so on.

The traditional NAD^+/NADH and $NADP^+$/NADPH assays are done by monitoring of NADH or NADPH absorption at 340 nm. This method suffers low sensitivity and high interference since the assay is done in the UV range that requires expensive quartz microplate. The CycLex NAD^+/NADH Colorimetric Assay Kit Ver.2 employs an enzyme cycling reaction, which significantly increases detection sensitivity, and provides a convenient method for sensitive detection of NAD^+, NADH, and their ratio.

Principle of the Assay

The CycLex NAD^+/NADH Colorimetric Assay Kit Ver.2 can measure concentrations of NAD^+ and NADH by an enzyme-coupled reaction using two enzyme, i.e. alcohol dehydrogenase (ADH) and diaphorase, and WST-1 as a chromogenic substrate. Since the reaction is basically not to be stopped, it is necessary to monitor absorbance of WST-1-formazan at 450 nm at regular intervals after the reaction is initiated, and to determine reaction velocity. This simple method should dramatically raise the efficiency of measuring NAD^+ and NADH concentrations. Combined with our optimized extraction methods of NAD^+ and NADH from mammalian cells which is described in this manual, the kit is useful to know aculeate concentrations of NAD^+ and NADH in the cells.

Measuring Principle of the CycLex NAD^+/NADH Colorimetric Assay Kit Ver.2



Materials Provided

Components of the Kit

Components	Quantity	Storage
#1. NAD+/NADH Assay Buffer (20X)	**1 mL x 1**	**-70°C**
#2. WST-1	**500 µL x 1**	**-70°C**
#3. ADH	**500 µL x 1**	**-70°C**
#4. Diaphorase	**500 µL x 1**	**-70°C**
#5. EtOH Solution	**500 µL x 1**	**-70°C**
#6. 400 µM NADH	**200 µL x 1**	**-70°C**
#7. 20X Standard Dilution Buffer	**1 mL x 1**	**-70°C**
Instruction Manual	**1**	**Room temp.**

Materials Required but not Provided

- **Solutions for preparing NAD^+ and NADH extracts from mammalian cells:**
  See the section "Detailed Protocol" below.
  (Soln.1A) NAD^+ Extraction Solution: 0.5 M perchloric acid ($HClO_4$)
  (Soln.1B) NAD^+ Neutralization Solution: 0.55 M K_2CO_3
  (Soln.2A) NADH Extraction Solution: 50 mM NaOH and 1 mM EDTA
  (Soln.2B) NADH Neutralization Solution: 0.3 M potassium phosphate buffer (pH 7.4)

- **Microplate for ELISA**

- **Water bath or heating block**

- **Plate reader:** Capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.

- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.

- **Multi-channel pipette**

- **Microplate shaker**

- **500 or 1,000 mL graduated cylinder**

- **The highest quality deionized (distilled) water (ddH_2O)**

- **Reagent reservoirs**

- **(Optional) 1 N H_2SO_4:** As a Stop Solution.

- **(Optional) FK866 (APO866):** A specific inhibitor of nicotamide phosphoribosyltransferase (NAMPT), causing gradual NAD^+ depletion. Available from Sigma Cat# F8557 or Cayman Cat# 13287.

Precautions

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**

- Thaw all reagents completely in crushed ice before use.

- Keep ADH (alcohol dehydrogenase) and diaphorase in this kit on ice and return them immediately to -70°C after use. There is a possibility that the enzyme activity may be inactivated.

- Avoid mixing of **any reagents containing SH group like DTT or reduced glutathione**, or **alkyl amine** in the samples that will interfere this assay.

- Do not use kit components beyond the indicated kit expiration date.

- Rinse all detergent residue from glassware.

- Use deionized water of the highest quality.

- Do not mix reagents from different kits.

- Do not mouth pipette or ingest any of the reagents.

- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.

- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**

- **CAUTION: NAD^+ Extraction Solution, NADH Extraction Solution, and Stop Solution (1 N H_2SO_4) (Not provided in this kit) are strong acid or alkaline, respectively. Wear disposable gloves and eye protection when handling the solutions.**

Detailed Protocol

The MBL Research Product **CycLex NAD+/NADH Colorimetric Assay Kit Ver.2** can measure NAD^+ and NADH concentrations by enzyme cycling reaction using alcohol dehydrogenase (ADH), diaphorase and WST-1. Since the reaction is not stopped, it is necessary to monitor absorbance of WST-1-formazan at 450 nm at regular intervals after the reaction is initiated, and to determine appropriate reaction time.

1. Preparation of Assay Reagents

1) Place all components on ice to thaw. Use them after thawing and vortexing completely.

2) Prepare Extraction Solutions and Neutralization Solutions, **not provided in this kit**.

(Soln.1A) NAD^+ Extraction Solution: 0.5 M perchloric acid ($HClO_4$)
(Soln.1B) NAD^+ Neutralization Solution: 0.55 M K_2CO_3
(Soln.2A) NADH Extraction Solution: 50 mM NaOH and 1 mM EDTA
(Soln.2B) NADH Neutralization Solution: 0.3 M potassium phosphate buffer (pH 7.4)

3) Prepare a working solution of **Standard Dilution Buffer** by diluting **#7. 20X Standard Dilution Buffer**, 1:20 with deionized (distilled) water (**ddH_2O**). Mix well. Store at 4°C for two weeks or -20°C for long term storage.

NADH Standards as follows:
Dilute **#6. 400 µM NADH** with **Standard Dilution Buffer** to produce the serial NADH Standards (below). Mix each tube thoroughly before the next transfer. The 20,000 nM standard (Std.1) serves as the high standard. The Standard Dilution Buffer serves as the zero standard (Blank).

	Volume of Standard	Standard Dilution Buffer	Concentration
Std.1	10 µL of "#6. 400 µM NADH"	190 µL	20,000 nM
Std.2	100 µL of Std. 1 (20,000 nM)	100 µL	10,000 nM
Std.3	100 µL of Std. 2 (10,000 nM)	100 µL	5,000 nM
Std.4	100 µL of Std. 3 (5,000 nM)	100 µL	2,500 nM
Std.5	100 µL of Std. 4 (2,500 nM)	100 µL	1,250 nM
Std.6	100 µL of Std. 5 (1,250 nM)	100 µL	625 nM
Std.7	100 µL of Std. 6 (625 nM)	100 µL	312.5 nM
Blank	-	100 µL	0 nM

Note-1: Do not use a repeating pipette. Change tips for every dilution. Wet tip with Standard Dilution Buffer before dispensing. Unused portions of NADH Standards should be discarded.

Note-2: Since NAD^+ is converted to NADH in enzyme cycling reaction and relatively labile than NADH, this kit provides only NADH.

4) Prepare **NAD^+/NADH Reaction Mixture** (Quantity required: 60 µL/assay). Mix following components and put in ice water. Use within 30 minutes after preparation. Discard any unused NAD^+/NADH Reaction Mixture after use.

NAD+/NADH Reaction Mixture

Components	Volume
#1. NAD+/NADH Assay Buffer (20X)	**5 µL**
#2. WST-1	**5 µL**
#3. ADH	**5 µL**
#4. Diaphorase	**5 µL**
#5. EtOH Solution	**5 µL**
ddH_2O	**35 µL**
Total	**60 µL**

2. Preparation of Cell Extracts

Numerous extraction methods can be used to isolate NAD+ and NADH. The following protocols for NAD+ and NADH have been shown to work with a number of different mammalian cell lines are provided as examples of suitable methods. If desired, you can employ other methods for extraction of NAD+ and NADH.

Determination of NAD+ and NADH requires two separate samples (acid extract for NAD+ measurement and alkaline extract for NADH measurement), utilizing the character of NAD+: resistant to acidic condition and heat labile, and NADH: resistant to alkaline condition and relatively heat stable.

Note: All extraction and neutralization solutions are not provided in this kit. See the "1. Preparation of Assay Reagents" above.

1) For adherent cells, after washing with PBS, trypsinize, harvest and transfer the cells (1.0-5.0 x 10^6 cells) to microcentrifuge tubes followed by centrifugation at 2,000 rpm for 5 minutes.
Note: Each investigator should optimize the number of cells used per test.

Or

For non-adherent cells, harvest and transfer the cells (1.0-5.0 x 10^6 cells) to microcentrifuge tubes followed by centrifugation at 2,000 rpm for 5 minutes.
Note: Each investigator should optimize the number of cells used per test.

2) Wash the cells twice with cold PBS by centrifugation at 2,000 rpm for 5 minutes.

3) Spin down the cells by microcentrifuge at 10,000 rpm for 1 minute. Remove the supernatant as much as possible by aspiration.

Acid extract for NAD+ measurement

4) Vortex the cell pellet gently. Extract the cells with **100 µL** of **(Soln.1A) NAD+ Extraction Solution** by vortexing 3-4 times for 1 minute each with same time intervals or by homogenization using standard techniques (i.e. sonicate 4 times for 5 seconds each on ice.). Then stand **for 30 minutes on ice.**

5) Add **100 µL** of **(Soln.1B) NAD+ Neutralization Solution** to the acid extract and mix well by vortexing for neutralization.

6) Centrifuge the neutralized cell extract at 15,000 rpm **for 5 minutes at 4°C**. Transfer the supernatant to new microcentrifuge tube. The final pH of the supernatant should be 7.5-8.5. Make sure that the pH is within this range. If not, adjust pH 7.5-8.5 using either **(Soln.1B) NAD+ Neutralization Solution** or **(Soln.2B) NADH Neutralization Solution**.

7) Keep the tube of the cell extract for NAD+ measurement (refer to as an Acid Extract: **"AcE"**) on ice.

8) Measure the protein concentration of "**AcE**", for example using the BCA protein assay, data can be normalized and expressed as pmol NAD+ /mg of protein.

Alkaline extract for NADH measurement

4') Vortex the cell pellet gently. Extract the cells with **100 µL** of **(Soln.2A) NADH Extraction Solution** by vortexing 3-4 times for 1 minute each with same time intervals or by homogenization using standard techniques (i.e. sonicate 4 times for 5 seconds each on ice.). Then incubate **at 60 °C for 30 minutes** to reduce the viscosity of the samples.

5') Add **100 µL** of **(Soln.2B) NADH Neutralization Solution** to the alkaline extract and mix well by vortexing for neutralization, then stand **for at least 5 minutes on ice**.

6') Centrifuge the neutralized cell extract at 15,000 rpm **for 5 minutes at 4°C**. Transfer the supernatant to new microcentrifuge tube. The final pH of the supernatant should be 7.5-8.5. Make sure that the pH is within this range. If not, adjust pH 7.5-8.5 using either **(Soln.1B) NAD+ Neutralization Solution** or **(Soln.2B) NADH Neutralization Solution**.

7') Keep the tube of the cell extract for NADH measurement (refer to as an Alkaline Extract: **"AlE"**) on ice.

8') Measure the protein concentration of "**AlE**",, for example using the BCA protein assay, data can be normalized and expressed as pmol NADH /mg of protein.

Note-1: If necessary, the cell extracts can be stored at -70°C. Avoid multiple freeze/thaw cycles. After thaw the cell extracts, centrifuge at 15,000 rpm for 15 minutes at 4°C again since the cell extracts should be clear of any sediments or particulate matters. However this may result in some loss of NAD+/NADH.

Note-2: Although this protocol has been successfully applied to many mammalian cell lines, users should optimize the cell extraction procedure for their own applications.

Note-3: Although we suggest to conduct experiments as outlined above, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user. Especially, make sure the final preparations of cell extract are **in neutral pH, hopefully, pH 7.5-8.5**.

Important: We do NOT recommend to use sodium carbonate-based buffers as a NAD+/NADH Extraction Solution instead of (Soln.1A) NAD+ Extraction Solution and (Soln.1B) NADH Extraction Solution, respectively, because the extracts prepared by using sodium carbonate-based buffers may give inappropriate NAD+ and NADH concentrations and ratio (See Fig. 6 and 7 in the section "Example of Test Results").

3. NAD+/NADH Assay Procedures

Assay reagents	Standard Reaction	Sample Reaction
ddH_2O	**35 µL**	**35 µL**
NADH Standards (Std.1-Std.7 and Blank) **Test Samples (Cell extracts (AcE and AlE))***	**5 µL** **-**	**-** **5 µL**
Sub-Total Volume	**40 µL**	**40 µL**
NAD+/NADH Reaction Mixture	**60 µL**	**60 µL**
Total Volume of the Reaction	**100 µL**	**100 µL**

* The addition volume can be changed with adjusting the one of ddH_2O.

1) Following the above table, add **ddH_2O**, and **NADH Standards** (Std.1-Std.7 and Blank: Standard Dilution Buffer) or **Test Samples (Cell extracts (AcE and AlE))** to each well of microplate. Next, initiate reaction by adding **60 µL** of **NAD+/NADH Reaction Mixture** to the each well and mix thoroughly. Incubate at room temperature (ca.25°C).

2) Monitor the absorbance at 450 nm for 30 to 90 minutes at 10 minute intervals using microplate reader.

Or

2') Alternatively, after 60 minutes or appropriate time incubation, the reactions can be stopped by adding 50 µL of Stop Solution (1N H_2SO_4, not provided in this kit) into each well and mix well. Take a reading with the absorbance at 450 nm.

Table: Layout of NADH Standards and Test Samples (AcE and AlE) in 96-well microplate

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Blank**	**Blank**	**AcE-1**	**AcE-1**								
B	**Std.7**	**Std.7**	**AlE-1**	**AlE-1**								
C	**Std.6**	**Std.6**	**AcE-2**	**AcE-2**								
D	**Std.5**	**Std.5**	**AlE-2**	**AlE-2**								
E	**Std.4**	**Std.4**	**AcE-3**	**AcE-3**								
F	**Std.3**	**Std.3**	**AlE-3**	**AlE-3**								
G	**Std.2**	**Std.2**	**AcE-4**	**AcE-4**								
H	**Std.1**	**Std.1**	**AlE-4**	**AlE-4**								

AcE: Acid Extract
AlE: Alkaline Extract

Note: The NADH Standards and Test Samples should be run in duplicate.

Evaluation of Results

1. Run reactions as described in the section "Detailed Protocol" above.
2. Subtract A450 at the 0 time from ones of all reaction time points.
3. Plot A450 versus reaction time.
4. Determine the reaction time range in which the increase in A450 is linear.
5. Fix an appropriate reaction time (usually 60 minutes).
6. Take a reading with the absorbance at 450 nm.

Average the duplicate readings for each standard, control, and sample and subtract the average optical density of the zero standard (Blank). Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation. To determine the NAD^+ or NADH concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding NAD^+ or NADH concentration. If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor (See "Fig. 2 Standard curve of NADH concentration" in the section "Example of Test Results" below).

1. The results of unknown samples can be calculated with any computer program having a 5-parameter logistic function. It is important to make an appropriate mathematical adjustment to accommodate for the dilution factor.

2. Most microplate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the absorbance (Y) of calibrators versus log of the known concentration (X) of calibrators, using the four-parameter function.

3. The concentration of NAD^+ or NADH in the sample can be calculated, then divide the NAD^+ or NADH concentration by the sample amount (e.g. cell number or extract protein amount) you added into the sample wells. The concentration of NAD^+ or NADH can be expressed in **pmol/10^6 cells** or pmol/mg protein.

NAD^+ concentration (pmol/10^6 cells) = NAD^+ concentration of **AcE** (nM) x 2 x 10^5 /cell number

NADH concentration (pmol/10^6 cells) = NADH concentration of **AlE** (nM) x 2 x 10^5 /cell number

Total volume of **AcE** = 200 µL. Volume of **AcE** for assay = 25 µL.
Total volume of **AlE** = 200 µL. Volume of **AlE** for assay = 25 µL.

$$NAD^+/NADH\ Ratio = \frac{NAD^+ \text{ concentration (pmol/}10^6 \text{ cells)}}{NADH \text{ concentration (pmol/}10^6 \text{ cells)}}$$

Note-1: The absorbance background increases with time, thus it is important to subtract the absorbance value of the zero standard (Blank) wells for each data point.

Note-2: Usually, an appropriate reaction time is from 60 to 90 minutes. This value is variable depending on reaction conditions and preparation of cell extract. Decreasing the amount of cell extract in the assay may help to lengthen the time range.

Cautions

1. Since this kit is based on enzyme cycling reaction, it is not possible to measure NAD^+ and NADH concentrations in conventional crude cell extract without the special extractions for NAD^+ and NADH. Follow the section **"Detailed Protocol"** above.

2. Avoid mixing of **any reagents containing SH group like DTT or reduced glutathione,** or **alkyl amine** in the sample that will interfere this assay.

Assay Characteristics

The MBL Research Product **CycLex NAD^+/NADH Colorimetric Assay Kit Ver.2** has been shown to measure NAD^+ and NADH concentrations or NAD^+/NADH ratio of crude cell extracts using enzyme cycling reaction. The assay shows good linearity of sample response.

Troubleshooting

1. When test samples have not been adjusted to neutral pH, the enzyme cycling reactions might be inhibited, so that NAD^+ and NADH concentrations cannot be measured.

2. All samples, standards and controls should be assayed in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.

3. Poor duplicates indicate inaccurate dispensing. If all instructions in the **Detailed Protocol** are followed accurately, such results may indicate the need for multi-channel pipettor maintenance.

Reagent Stability

All of the reagents included in the MBL Research Product **CycLex NAD^+/NADH Colorimetric Assay Kit Ver.2** have been tested for stability. The kit should not be used beyond the stated expiration date.

Example of Test Results

Fig.1 Typical time course curve of NADH Standards

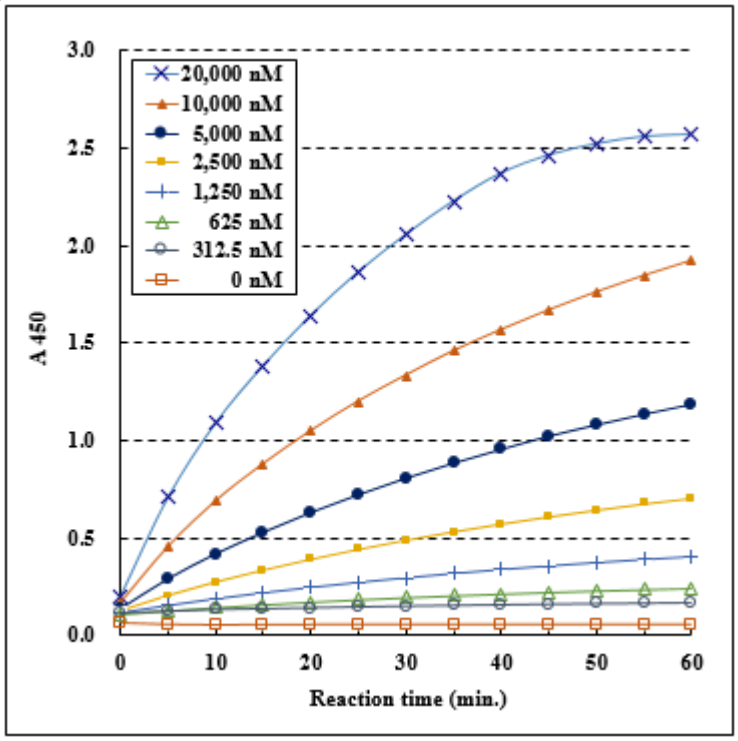


Fig.2 Typical standard curve of NADH concentration

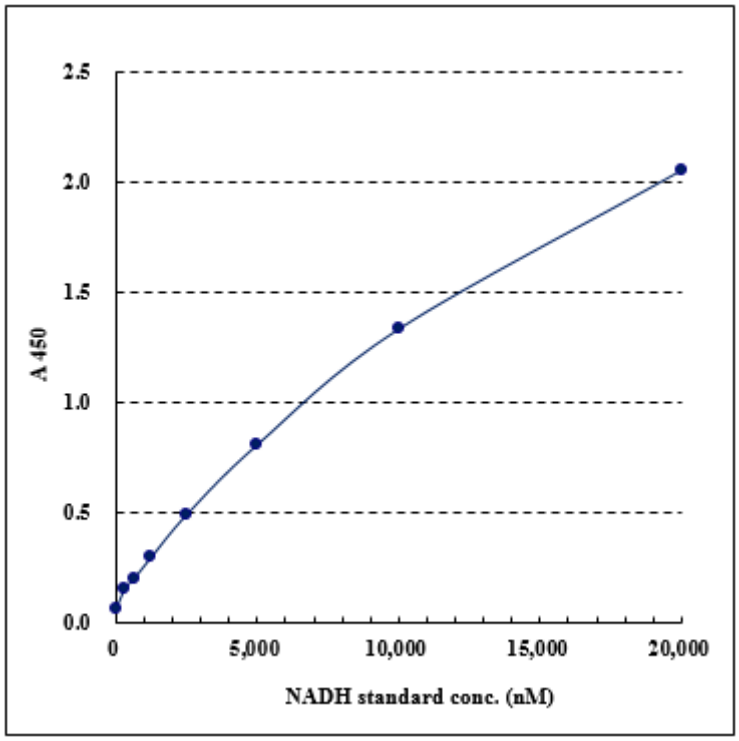


Fig.3 Specific detection and measurement of NADH, not NADPH (As low as 312.5 nM of NADH can be detected with 30 minutes incubation time (n=2), there is no response to NADPH.)

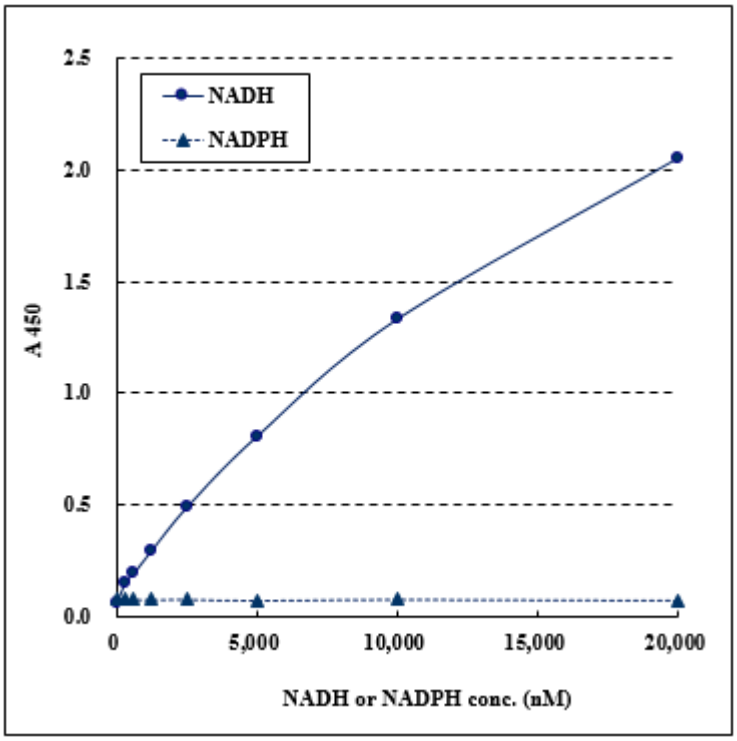


Fig.4 NAD+ and NADH concentrations in cell extracts of SW480 and Jurkat cells

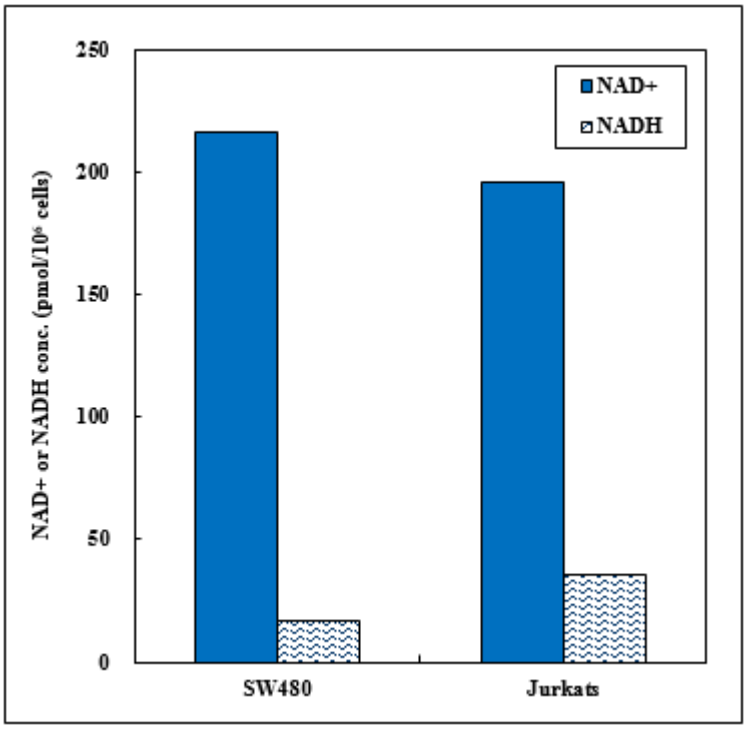


Fig.5 NAD+ and NADH concentrations in cell extracts of Jurkat cells treated with NAMPT specific inhibitor FK866 at indicated concentrations

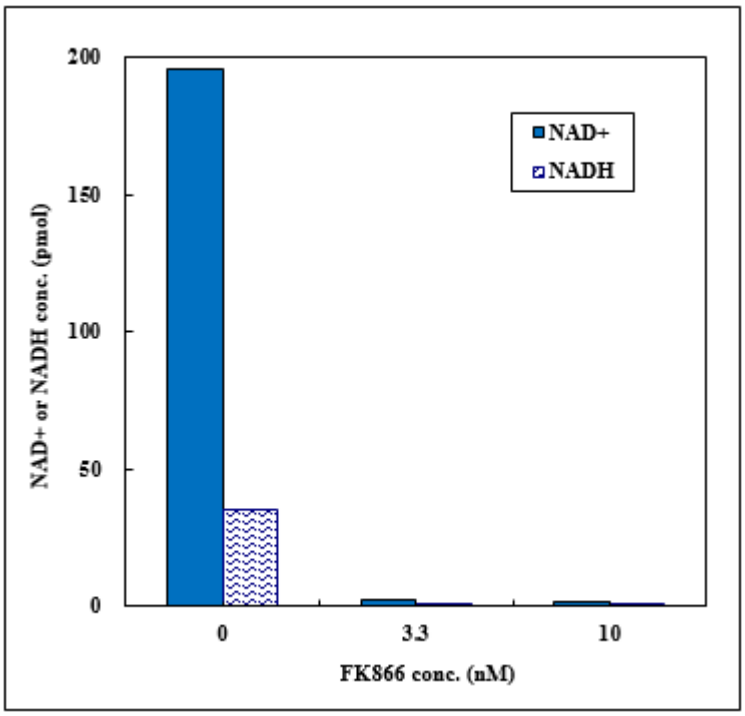


Fig.6 Comparisons of NAD+ and NADH concentrations in SW480 cell extracts made by each extraction method in and measured by CycLex NAD+/NADH Colorimetric Assay Kit and Company B's "NAD/NADH Quantitation Kit"

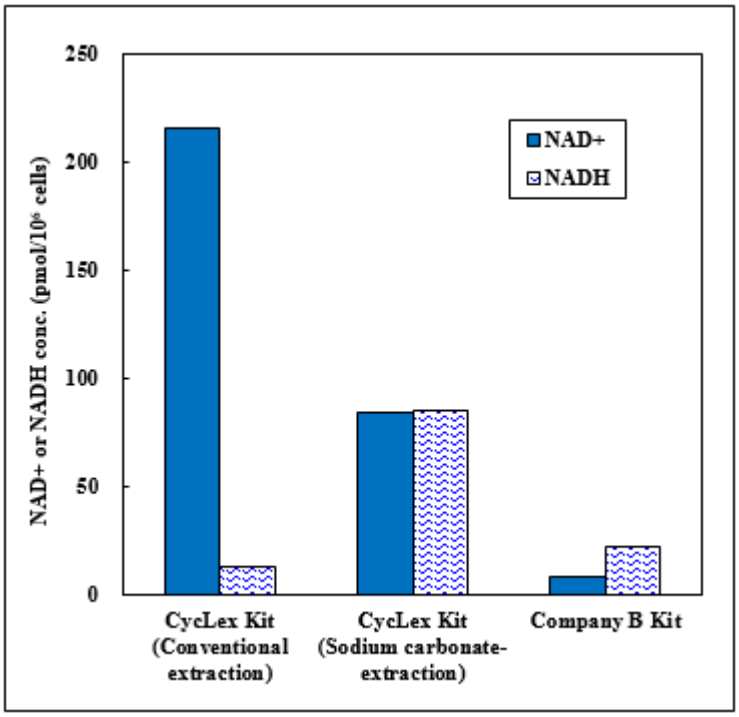
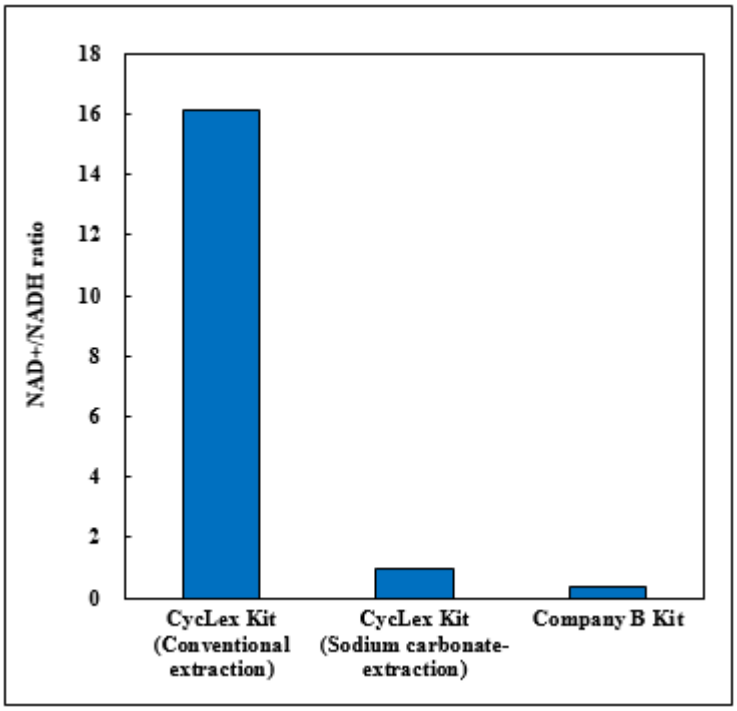


Fig.7 Comparisons of NAD+/NADH ratio in SW480 cell extracts made by each extraction method in and measured by CycLex NAD+/NADH Colorimetric Assay Kit and Company B's "NAD/NADH Quantitation Kit"



References

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Related Products

Kit
*** CycLex Cellular Histone Acetylation Assay Kit: Cat# CY-1140**
*** CycLex HDACs Deacetylase Fluorometric Assay Kit Ver.2: Cat# CY-1150V2**
*** CycLex SIRT1/Sir2 Deacetylase Fluorometric Assay Kit Ver.2: Cat# CY-1151V2**
*** CycLex SIRT2 Deacetylase Fluorometric Assay Kit Ver.2: Cat# CY-1152V2**
*** CycLex SIRT3 Deacetylase Fluorometric Assay Kit Ver.2: Cat# CY-1153V2**
*** CycLex SIRT5 Deacetylase Fluorometric Assay Kit: Cat# CY-1155**
*** CycLex SIRT6 Deacetylase Fluorometric Assay Kit Ver.2: Cat# CY-1156V2**
*** CycLex HDAC8 Deacetylase Fluorometric Assay Kit Ver.2: Cat# CY-1158V2**
*** CycLex NAMPT Colorimetric Assay Kit Ver.2: CY-1251V2**
*** CycLex NMNAT1 Colorimetric Assay Kit Ver.2: CY-1252V2**
*** CycLex NAD+/NADH Colorimetric Assay Kit Ver.2: CY-1253V2**

Enzyme
*** NAD+-Dependent Deacetylase SIRT1: Cat# CY-E1151**
*** NAD+-Dependent Deacetylase SIRT2: Cat# CY-E1152**
*** NAD+-Dependent Deacetylase SIRT3: Cat# CY-E1153**
*** NAD+-Dependent Deacetylase SIRT5: Cat# CY-E1155**
*** NAD+-Dependent Deacetylase SIRT6: Cat# CY-E1156**
*** NAMPT (Nicotinamide Phosphoribosyltransferase): Cat# CY-E1251**
*** NMNAT1 (Nicotinamide Mononucleotide Adenylyltransferase 1): Cat# CY-E1252**

Antibody
*** Anti-Acetylated Histone/p53-K382 Mouse Monoclonal Antibody: Cat# CY-M1029**
*** Anti-Human NAMPT Mouse Monoclonal Antibody: Cat# CY-M1035**
*** Anti-Histone Deacetylase 1 (HDAC1) Rabbit Polyclonal Antibody: Cat# CY-P1011**
*** Anti-Histone Deacetylase 2 (HDAC2) Rabbit Polyclonal Antibody: Cat# CY-P1012**
*** Anti-Human SIRT1 Rabbit Polyclonal Antibody: Cat# CY-P1016**

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