Quantitative Assay for Nicotinamide Phosphoribosyltransferase Activity

CycLex NAMPT Colorimetric Assay Kit Ver.2

For 100 Assay

Cat# CY-1251V2

Intended Use... 1
Storage.. 1
Introduction...2
Principle of the Assay..................................2
Materials Provided......................................3
Materials Required but not Provided...........3
Precautions...4
Detailed Protocol.......................................5-12
Evaluation of Results.................................. 13
Cautions.. 13
Assay Characteristics.................................. 14
Troubleshooting.. 14
Reagent Stability... 14
Protocol for immunoprecipitation................15-16
Example of Test Results..............................17-21
References... 22

Intended Use

The MBL Research Product **CycLex NAMPT Colorimetric Assay Kit Ver.2** detects nicotinamide phosphoribosyltransferase (NAMPT) activity in recombinant NAMPT or endogenous NAMPT immunoprecipitated from cell lysates. Primarily, the **CycLex NAMPT Colorimetric Assay Kit Ver.2** is designed for the rapid and sensitive evaluation of NAMPT inhibitors or activators using recombinant NAMPT. Since this kit is based on NAD^+ detection system, it is not possible to directly detect NAMPT activity in crude cell lysates in which NAD^+ concentration is relatively high.

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

Applications for this kit include:
1) Measuring activity of NAMPT with immunoprecipitation.
2) Screening inhibitors or activators of NAMPT.
3) Detecting effects of pharmacological agents on NAMPT.

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.

Copy on Web. Just For Reference Purpose Only.

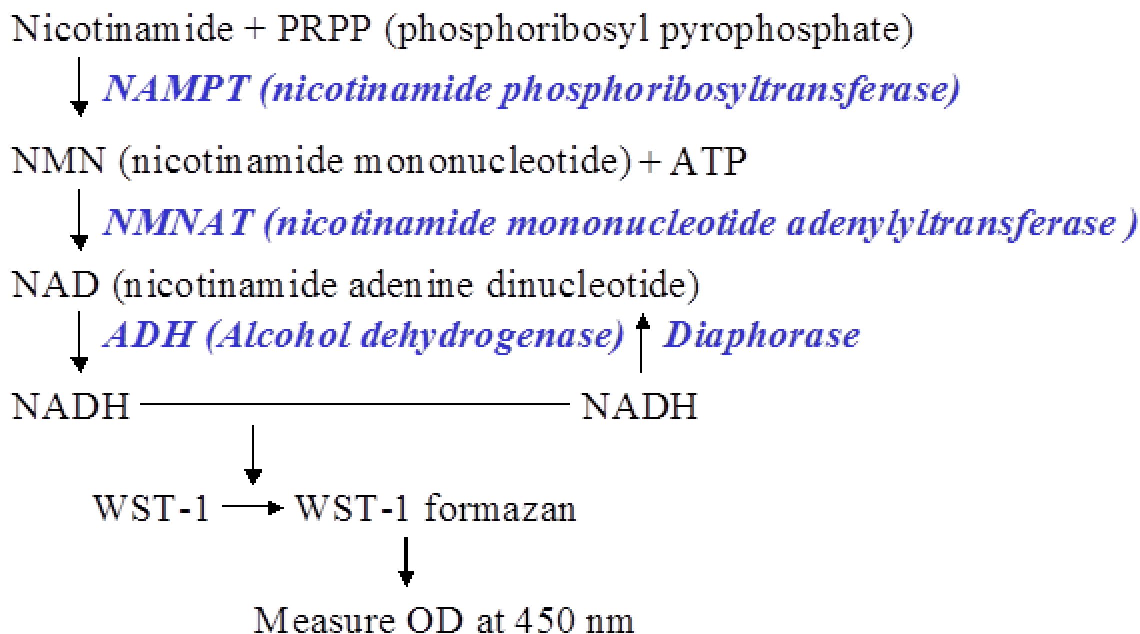
Introduction

Nicotinamide phosphoribosyltransferase (NAMPT), also known as pre-B-cell colony-enhancing factor, is the rate-limiting enzyme that converts nicotinamide to nicotinamide mononucleotide (NMN) from nicotinamide in the salvage pathway of NAD^+ biosynthesis in mammals. Nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) converts NMN to NAD^+. The expression of NAMPT is upregulated during activation of immune cells such as monocytes, macrophages, dendritic cells, T and B cells, as well as in amniotic epithelial cells upon stimulation with several inflammatory cytokines. NAMPT-specific inhibitor, FK866 was found to deplete intracellular NAD content, resulting in apoptotic cell death in many cancer cell lines without any DNA damaging effect. Recently, Nakahata K et al, demonstrated that NAMPT is required to modulate circadian gene expression and circadian oscillation of NAD^+.

Principle of the Assay

Since it is very simple to measure and it can be performed at a low price, the measurement of NAMPT activity in most laboratories is possible if they are equipped with a microtiter plate reader. Considering that the use of fully automatic apparatus to monitor the absorbance has become widespread, NAMPT activity measurement, which could not be made by the conventional method, is now possible with the CycLex NAMPT Colorimetric Assay Kit Ver.2 using the same equipment. This new method of measurement shall dramatically raise the efficiency of inhibitor screening and biochemical analysis of this enzyme.

Measuring Principle of The CycLex NAMPT Colorimetric Assay Kit Ver.2



Copy on Web. Just For Reference Purpose Only.

Materials Provided

Components of the Kit

Components	Quantity	Storage
#1. NAMPT 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. ATP	500 µL x 1	-70°C
#7. NMNAT1*	500 µL x 1	-70°C
#8. Nicotinamide	500 µL x 1	-70°C
#9. PRPP	500 µL x 1	-70°C
#10. NAMPT**	500 µL x 1	-70°C
Instruction manual	1	Room temp.

* Human NMNAT1 (nicotinamide mononucleotide adenylyltransferase 1) expressed in *E. coli*.
** Human NAMPT (nicotinamide phosphoribosyltransferase) expressed in *E. coli*.

Materials Required but not Provided

- **Microplate**
- **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**
- **The highest quality deionized (distilled) water (ddH_2O)**
- **500 or 1,000 mL graduated cylinder**
- **Reagent reservoirs**
- **(Optional) NAD^+:** β-Nicotinamide adenine dinucleotide hydrate, available from Sigma, Cat# N7004. Prepare fleshly 10 µM solution in ddH_2O from 1 mM stock solution. Discard any unused 10 µM NAD^+.
- **(Optional) FK866 (APO866):** A specific inhibitor of NAMPT, available from Sigma, Cat# F8557 or Cayman, Cat# 13287. Make 0.4 mM stock solution in DMSO.
- **(Optional) NMN:** β-Nicotinamide mononucleotide, available from Sigma, Cat# N3501. Prepare fleshly 13 mM solution in ddH_2O. Discard any unused solution.

Copy on Web. Just For Reference Purpose Only.

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.

- Avoid freezing-and-thawing cycle of #3. ADH, #4. Diaphorase, #7. NMNAT1, and #10. NAMPT in this kit. There is a possibility that the enzymes may be inactivated. Aliquot to 25-50 µL and store at -70°C

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

- 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.**

Copy on Web. Just For Reference Purpose Only.

Detailed Protocol

The MBL Research Product **CycLex NAMPT Colorimetric Assay Kit Ver.2** can measure the enzyme activity of nicotinamide phosphoribosyltransferase (NAMPT) by an enzyme-coupled reaction as shown in "Principle of the Assay" above. In this method, NAMPT converts nicotinamide to nicotinamide mononucleotide (NMN), subsequently nicotinamide mononucleotide adenylyltransferase 1 (NMNAT1) converts NMN to NAD^+. Resultant NAD^+ can be measured 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 reaction velocity.

The CycLex NAMPT Colorimetric Assay Kit Ver.2 can measure the enzyme activity of NAMPT with two kinds of measuring methods, One-Step Assay Method and Two-Step Assay Method. One-Step Assay Method is accomplished by mixing with four enzymes, i.e. NAMPT, NMNAT1, ADH and diaphorase. Since three coupled reactions are promoted simultaneously with NAMPT enzyme reaction, detection sensitivity of this method is less than that of the Two-Step Assay Method.

Conversely, Two-Step Assay Method is begun by initiating reactions of two enzymes, NAMPT, NMNAT1 within a set time period to produce NAD^+ from nicotinamide and PRPP, then in the second step, followed by adding ADH, diaphorase and WST-1, and the resultant WST-1-formazan is formed by NAD/NADH enzyme cycling reaction.

1. Preparation of Assay Reagents

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

2) For One-Step Assay Method, prepare **One-Step Assay Mixture** (Quantity required: 60 µL/assay). Mix following components and put on ice. Use within 30 minutes after preparation. Discard any unused One-Step Assay Mixture after use.

One-Step Assay Mixture

Components	Volume
#1. NAMPT Assay Buffer (20X)	**5 µL**
#2. WST-1	**5 µL**
#3. ADH	**5 µL**
#4. Diaphorase	**5 µL**
#5. EtOH Solution	**5 µL**
#6. ATP	**5 µL**
#7. NMNAT1	**5 µL**
#8. Nicotinamide	**5 µL**
#9. PRPP	**5 µL**
ddH_2O	**15 µL**
Total	**60 µL**

Or

Copy on Web. Just For Reference Purpose Only.

2') For Two-Step Assay Method, prepare **Two-Step Assay Mixture-I** (Quantity required: 60 µL/assay) and **Two-Step Assay Mixture-II** (Quantity required: 20 µL/assay). Mix following components and put on ice. Use within 30 minutes after preparation. Discard any unused Two-Step Assay Mixture-I and Mixture-II after use.

Two-Step Assay Mixture-I

Components	Volume
#1. NAMPT Assay Buffer (20X)	**5 µL**
#6. ATP	**5 µL**
#7. NMNAT1	**5 µL**
#8. Nicotinamide	**5 µL**
#9. PRPP	**5 µL**
ddH$_2$O	**35 µL**
Total	**60 µL**

Two-Step Assay Mixture-II

Components	Volume
#2. WST-1	**5 µL**
#3. ADH	**5 µL**
#4. Diaphorase	**5 µL**
#5. EtOH Solution	**5 µL**
Total	**20 µL**

Copy on Web. Just For Reference Purpose Only.

2. Assay Methods for Measurement of NAMPT Activity

I. One-Step Assay Method

Assay reagents	Enzyme Sample Assay	No Enzyme Control Assay	Positive Control Assay
ddH_2O	**35 µL**	**35 µL**	**35 µL**
Enzyme Sample*	**5 µL**	**-**	**-**
Buffer of Enzyme Sample*	**-**	**5 µL**	**-**
#10. NAMPT	**-**	**-**	**5 µL**
One-Step Assay Mixture	**60 µL**	**60 µL**	**60 µL**
Total Volume of the Reaction	**100 µL**	**100 µL**	**100 µL**

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

1) Following the table above, add **ddH_2O**, and your **Enzyme Sample**, **Buffer of Enzyme Sample**, or **#10. NAMPT** to each well of the microplate.

2) Initiate the reaction by adding **60 µL** of **One-Step Assay Mixture** to each well and mix thoroughly.

3) Incubate at 30°C and monitor the absorbance at 450 nm for 60 minutes at 5 minute intervals using a microtiter plate reader. Measure and calculate the rate of reaction while the reaction velocity remains constant.

II. Two-Step Assay Method

1st Reaction: Conversion of nicotinamide to NAD^+

Assay reagents	Enzyme Sample Assay	No Enzyme Control Assay	Positive Control Assay
ddH_2O	**15 µL**	**15 µL**	**15 µL**
Enzyme Sample*	**5 µL**	**-**	**-**
Buffer of Enzyme Sample*	**-**	**5 µL**	**-**
#10. NAMPT	**-**	**-**	**5 µL**
Two-Step Assay Mixture-I	**60 µL**	**60 µL**	**60 µL**
Total Volume of the 1st Reaction	**80 µL**	**80 µL**	**80 µL**

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

1) Following the table above, add **ddH_2O**, and your **Enzyme Sample**, **Buffer of Enzyme Sample**, or **#10. NAMPT** to each well of the microplate.

2) Initiate the 1st reaction by adding **60 µL** of **Two-Step Assay Mixture-I** to each well and mix

Copy on Web. Just For Reference Purpose Only.

thoroughly.

3) Incubate at 30°C for 60 minutes.

2nd Reaction: Measurement of generated NAD^+

4) Add **20 µL** of **Two-Step Assay Mixture-II** to each well of the microplate and mix thoroughly.

5) Incubate at 30°C and monitor the absorbance at 450 nm for 30 minutes at 5 minute intervals using a microtiter plate reader. Measure and calculate the rate of reaction while the reaction velocity remains constant.

3. Special Considerations When Screening Inhibitors

In order to correctly estimate the inhibitory effect on NAMPT enzymatic activity, it is necessary to conduct the control experiments for "Solvent Control Assay" and "Inhibitor Control Assay" at least once for the first experiment, in addition to "Test Compound Assay", as indicated in the following procedure.

I. One-Step Assay Method

Assay reagents	Test Compound Assay	Solvent Control Assay	Inhibitor Control Assay
ddH_2O	**30 µL**	**30 µL**	**30 µL**
#10. NAMPT	**5 µL**	**5 µL**	**5 µL**
20X Test Compound* **Solvent for Test Compound*** **FK866 (0.4 mM)****	**5 µL** - -	- **5 µL** -	- - **5 µL**
One-Step Assay Mixture	**60 µL**	**60 µL**	**60 µL**
Total Volume of the Reaction	**100 µL**	**100 µL**	**100 µL**

* The addition volume can be changed with adjusting the one of ddH_2O.
** Not provided in this kit. See the section "Materials Required but not Provided" above.

1) Following the table above, add **ddH_2O**, **#10. NAMPT**, and your **20X Test Compound**, **Solvent for Test Compound**, or **FK866 (0.4 mM)** to each well of microplate and mix well.

2) Initiate the reaction by adding **60 µL** of **One-Step Assay Mixture** to each well and mix thoroughly.

3) Incubate at 30°C and monitor the absorbance at 450 nm for 60 minutes at 5 minute intervals using a microtiter plate reader. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Copy on Web. Just For Reference Purpose Only.

II. Two-Step Assay Method

1st Reaction: Conversion of Nicotinamide to NAD^+

Assay reagents	Test Compound Assay	Solvent Control Assay	Inhibitor Control Assay
ddH_2O	**10 µL**	**10 µL**	**10 µL**
#10. NAMPT	**5 µL**	**5 µL**	**5 µL**
20X Test Compound* **Solvent for Test Compound*** **FK866 (0.4 mM)****	**5 µL** **-** **-**	**-** **5 µL** **-**	**-** **-** **5 µL**
Two-Step Assay Mixture-I	**60 µL**	**60 µL**	**60 µL**
Total Volume of the 1st Reaction	**80 µL**	**80 µL**	**80 µL**

* The addition volume can be changed with adjusting the one of ddH_2O.
** Not provided in this kit. See the section "Materials Required but not Provided" above.

1) Following the table above, add **ddH_2O**, **#10. NAMPT**, and your **20X Test Compound**, **Solvent for Test Compound**, or **FK866 (0.4 mM)** to each well of the microplate.

2) Initiate the reaction by adding **60 µL** of **Two-Step Assay Mixture-I** to each well and mix thoroughly.

3) Incubate at 30°C for 60 minutes.

2nd Reaction: Measurement of the generated NAD^+

4) Add **20 µL** of **Two-Step Assay Mixture-II** to each well of the microplate and mix thoroughly.

5) Incubate at 30°C and monitor the absorbance at 450 nm for 30 minutes at 5 minute intervals using a microtiter plate reader. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Note: If your Test Compound causes an inhibitory effects on NAMPT enzyme activity, the level of the absorbance at 450 nm of the "Test Compound Assay" is weakened as compared with the one of the "Solvent Control Assay".

Copy on Web. Just For Reference Purpose Only.

4. Control Assay

I. for Inhibitor Screening

The CycLex NAMPT Colorimetric Assay Kit Ver.2 measures the NAMPT enzyme activity by an enzyme-coupled reaction in which four enzymes, i.e. NAMPT, NMNAT1, ADH, and diaphorase, are involved. If test compounds have an inhibitory effect on one of the enzymes, the signal will be reduced. If there is such a possibility, carry out the experiment of the "NMNAT1 activity assay coupled with NAD^+/NADH enzyme cycling reaction" according to the following procedure.
The related MBL Research Product CycLex NMNAT1 Colorimetric Assay Kit Ver2 (Cat# CY-1252V2) can be also used for and will facilitate this experiment.

Assay reagents	Test Compound Assay	Solvent Control Assay
ddH_2O	**60 µL**	**60 µL**
#6. ATP	**5 µL**	**5 µL**
NMN (13 mM)*	**5 µL**	**5 µL**
20X Test Compound** **Solvent for Test Compound****	**5 µL** **-**	**-** **5 µL**
Two-Step Assay Mixture-II	**20 µL**	**20 µL**
Diluted #7. NMNAT1***	**5 µL**	**5 µL**
Total Volume of the Reaction	**100 µL**	**100 µL**

* Not provided in this kit. See the section "Materials Required but not Provided" above.
** The addition volume can be changed with adjusting the one of ddH_2O.
*** Several tens- to a hundred-fold dilution will be required. Dilute the enzyme with 1X NAMPT Assay Buffer.

1) Following the table above, add, **ddH_2O**, **#6. ATP**, **NMN (13 mM)**, and your **20X Test compound** or **Solvent for Test compound** to each well of microplate and mix well.

2) Add **20 µL** of **Two-Step Assay Mixture-II**, and finally initiate the reaction by adding **5 µL** of **Diluted #7. NMNAT1** to each well and mix thoroughly.

3) Incubate at 30°C and monitor the absorbance at 450 nm for 60 minutes at 5 minute intervals using microtiter plate reader. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Note: If there is an inhibitory effect of your Test Compound on the "NMNAT1 activity assay coupled with NAD^+/NADH enzyme cycling reaction", the absorbance at 450 nm will not increase in the "Test Compound Assay".

Copy on Web. Just For Reference Purpose Only.

II. for Checking NAD^+ contamination in Immunoprecipitate

Since NAD^+ level in cells is relatively high, around several hundred micromolar concentration, NAD^+ might mix easily with purified NAMPT from various cells or an immunoprecipitate using the specific antibody against NAMPT. Such contaminated NAD^+ in the enzyme sample causes a false positive result by initiating NAD^+/NADH enzyme cycling reaction. If there is such a possibility, carry out the experiment of NAD^+/NADH enzyme cycling reaction in the following procedure.

The related MBL Research Product CycLex NAD^+/NADH Colorimetric Assay Kit Ver.2 (Cat# CY-1253V2) can be also used for and will facilitate this experiment.

Assay reagents	Enzyme Sample Assay	NAD^+ Control Assay
ddH_2O	**75 µL**	**75 µL**
Enzyme Sample **NAD^+ (10 µM)***	**5 µL** **-**	**-** **5 µL**
Two-Step Assay Mixture-II	**20 µL**	**20 µL**
Total Volume of the Reaction	**100 µL**	**100 µL**

* Not provided in this kit. See the section "Materials Required but not Provided" above.

1) Following the table above, add **ddH_2O**, and your **Enzyme Sample** or **NAD^+ (10 µM)** to each well of microplate and mix well.

2) Initiate the reaction by adding **20 µL** of **Two-Step Assay Mixture-II** to each well and mix thoroughly.

3) Incubate at 30°C and monitor the absorbance at 450 nm for 60 minutes at 5 minute intervals using a microtiter plate reader. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Note: If there is NAD^+ contamination in your Enzyme Sample, the level of the absorbance at 450 nm of the "Enzyme Sample Assay" will increase as compared with the one of the "NAD^+ Control Assay".

Copy on Web. Just For Reference Purpose Only.

III. for NAMPT Activity Assay with Immunoprecipitate

In order to measure the activity of NAMPT correctly, it is necessary to conduct the control experiments for "No Enzyme Control Assay" and "No Nicotinamide Control Assay" at least once in addition to "Enzyme Sample Assay" and "Positive Control Assay", as indicated in the following procedure.

Assay reagents	Enzyme Sample Assay	Positive Control Assay	No Enzyme Control Assay	No Nicotinamide Control Assay
ddH_2O	50 µL	50 µL	55 µL	55 µL
#1. NAMPT Assay Buffer (20X)	5 µL	5 µL	5 µL	5 µL
#6. ATP	5 µL	5 µL	5 µL	5 µL
#7. NMNAT1	5 µL	5 µL	5 µL	5 µL
#8. Nicotinamide	5 µL	5 µL	5 µL	-
#9. PRPP	5 µL	5 µL	5 µL	5 µL
Two-Step Assay Mixture-II	20 µL	20 µL	20 µL	20 µL
Enzyme Sample* **#10. NAMPT**	5 µL -	- 5 µL	- -	5 µL -
Total Volume of the Reaction	100 µL	100 µL	100 µL	100 µL

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

1) Following the table above, add all reagents (**ddH_2O** to **Two-Step Assay Mixture-II)** to each well of microplate and mix well.

2) Initiate the reaction by adding **5 µL** of your **Enzyme Sample** or **#10. NAMPT** to each well and mix thoroughly.

3) Incubate at 30°C and monitor the absorbance at 450 nm for 30 minutes at 5 minute intervals using a microtiter plate reader. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Note-1: The difference in the reaction velocity between the "Enzyme Sample Assay" and "No Nicotinamide Control Assay" indicates the actual NAMPT activity.

Note-2: The increasing of the absorbance at 450 nm of the "No Nicotinamide Control Assay", as compared with the one of the "No Enzyme Control Assay", indicates strongly that there is NAD^+ contamination in your Enzyme Sample.

Copy on Web. Just For Reference Purpose Only.

Evaluation of Results

Analysis of Kinetics

Time course curve

1. Run reactions as described in the section **"Detailed Protocol"**.
2. Subtract the absorbance at 450 nm (A450) of the "No Enzyme Control Assay" from the one of the "Enzyme Sample Assay" at all reaction time points.
3. Plot the A450 versus reaction time.
4. Determine the reaction time range in which the increase of the A450 is linear.
5. Calculate activity:

$$\text{Activity (reaction velocity)} = \frac{\text{A450 Variations of Enzyme Sample}}{\text{Reaction time (min.)}}$$

Note: Usually, the linear range is from 20 to 40 minutes. This value is variable depending on reaction conditions and storage/handling of the NAMPT. Decreasing the amount of NAMPT in the assay may help to lengthen the time range.

Cautions

1. Since this kit is based on NAD^+ detection system, it is not possible to detect NAMPT activity in crude cell extract in which NAD^+ concentration is relatively high. **To use an immunoprecipitate as an enzyme sample using the specific antibody against NAMPT is recommended.**

   Note: Anti-Human NAMPT Monoclonal Antibody is available from MBL, Cat# CY-M1035.

2. Contaminated NAD^+ in the enzyme sample causes a false positive result by initiating NAD^+/NADH enzyme cycling reaction. Confirm no NAD^+ in the enzyme sample according to the **"4. Control Assay"** in the section **"Detailed Protocol"** above.

3. Duplicate measurement is strongly recommended for accurate measurement.

4. Although we suggest to conduct experiments as outlined in the section **"Protocol for Immunoprecipitation"** below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by individual users.

Copy on Web. Just For Reference Purpose Only.

Assay Characteristics

The MBL Research Product **CycLex NAMPT Colorimetric Assay Kit Ver.2** has been shown to detect the activity of nicotinamide phosphoribosyltransferase in recombinant NAMPT or an immunoprecipitate using the specific antibody against NAMPT. The assay shows good linearity of sample response. The assay may be used to follow the purification of NAMPT.

Troubleshooting

1. When test chemicals have an inhibitory effect on NMNAT1, ADH or diaphorase, precise inhibitory effect on NAMPT enzyme activity 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** were followed accurately, such results indicate a need for multi-channel pipettor maintenance.

Reagent Stability

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

Copy on Web. Just For Reference Purpose Only.

Protocol for Immunoprecipitation

Immunoprecipitation Followed by Measuring NAMPT Activity Protocol

Note: 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.

Solutions and Reagents

Cell Lysis Buffer: 20 mM Tris (pH 8.0), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM DTT and protease inhibitor cocktail.

1X NAMPT Assay Buffer: Dilute "#1. NAMPT Assay Buffer (20X)" 20-fold with ddH_2O.

Protein A agarose beads: Add 5 mL of 1X PBS to 1.5 g of Protein A agarose beads. Shake 2 hours at 4°C and spin down. Wash the pellet twice with PBS. Resuspend beads in 1 volume of PBS. (Can be stored for 2 weeks at 4°C)

Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing test compound for desired time.

2. To harvest cells under non-denaturing conditions, remove media and rinse cells once with ice-cold PBS.

3. Remove PBS and add 0.5 mL of ice-cold Cell Lysis Buffer to each plate (10 cm dish) and incubate the plate on ice for 5 minutes.

4. Scrape cells off the plate and transfer to microcentrifuge tubes. Keep on ice.

5. Sonicate 4 times for 5 seconds each on ice.

6. Microcentrifuge for 10 minutes at 4°C, and transfer the supernatant to a new tube. The supernatant is the cell lysate. If necessary, the lysate can be stored at -70°C.

Immunoprecipitation

1. Take 250 µL of cell lysate and add protein A agarose beads (40 µL of 50% bead slurry). Incubate with gentle rocking for 1-3 hours at 4°C for pre-clearance

2. Microcentrifuge for 30 seconds at 4°C. Take the supernatant and transfer to a new tube.

3. Add 1-2 µg of anti-NAMPT antibody* which can be used for immunoprecipitation, and incubate with gentle rocking for 2 hours or overnight at 4°C.
   * Anti-Human NAMPT Monoclonal Antibody (MBL, Cat# CY-M1035) is recommended.

4. Add protein A agarose beads (20 µL of 50% bead slurry). Incubate with gentle rocking for 1-3 hours at 4°C.

Copy on Web. Just For Reference Purpose Only.

5. Microcentrifuge for 10 seconds at 4°C. Wash the beads twice with 500 µL of ice-cold Cell Lysis Buffer, subsequently twice with 500 µL of ice-cold 1X NAMPT Assay Buffer. Keep on ice during washes.

6. Resuspend the beads with 20 µL of ice-cold 1X NAMPT Assay Buffer and measure NAMPT activity according to the "Detailed Protocol" above.

Copy on Web. Just For Reference Purpose Only.

Example of Test Results

Fig.1 Dose dependency curve of recombinant NAMPT activity using One-Step Assay Method

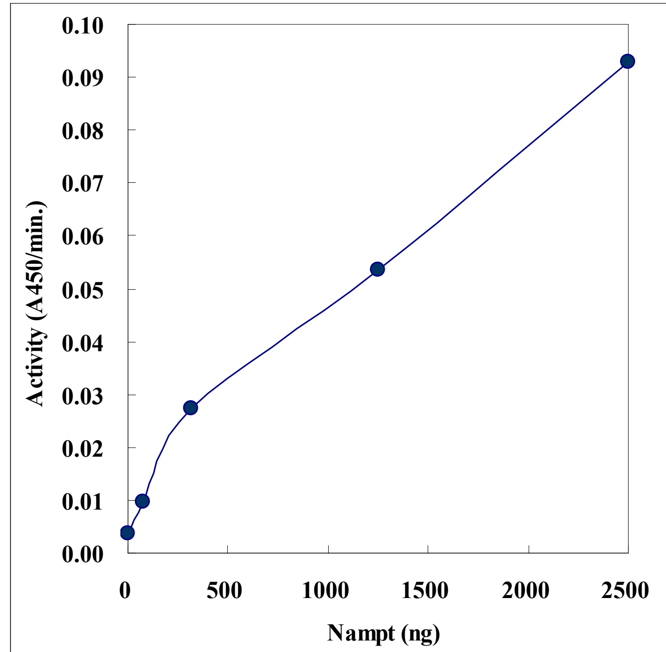
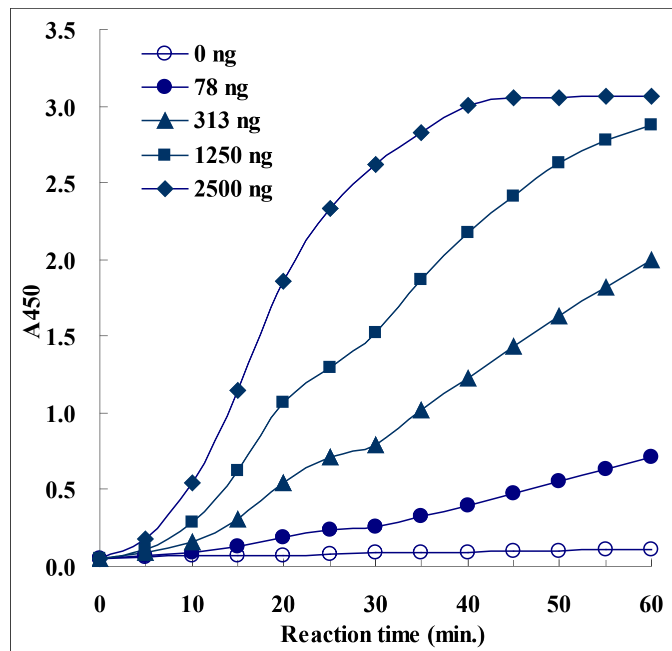


Fig.2 Time course of NAMPT activity in recombinant NAMPT using One-Step Assay Method



Copy on Web. Just For Reference Purpose Only.

Fig.3 Effect of FK866 on recombinant NAMPT activity

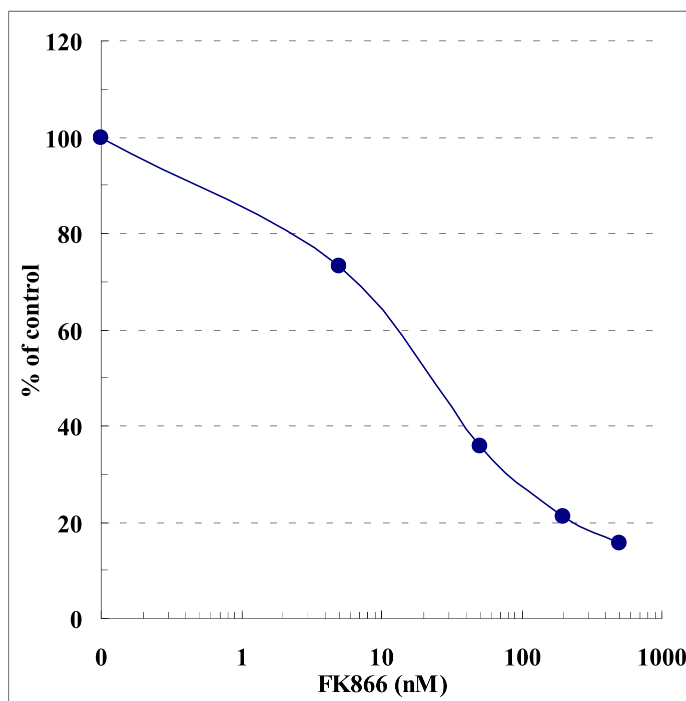
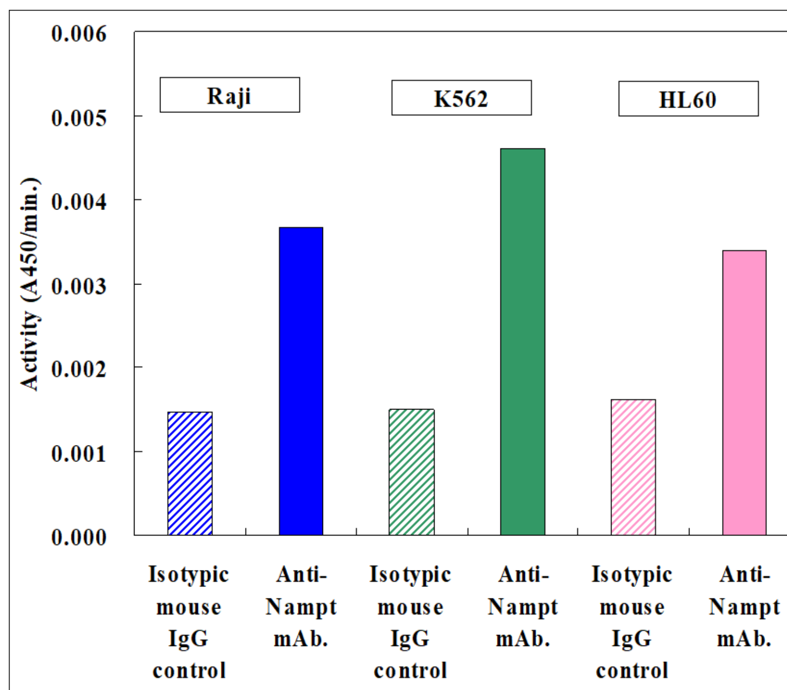


Fig.4 Measurement of endogenous NAMPT activity in immunoprecipitates using Anti-Human NAMPT Monoclonal Antibody (MBL, Cat# CY-M1035) from cell lysates



Copy on Web. Just For Reference Purpose Only.

Fig.5 Measurement of NAMPT activity in immunoprecipitates from lysates of 293T cells transfected with NAMPT expression plasmid DNA

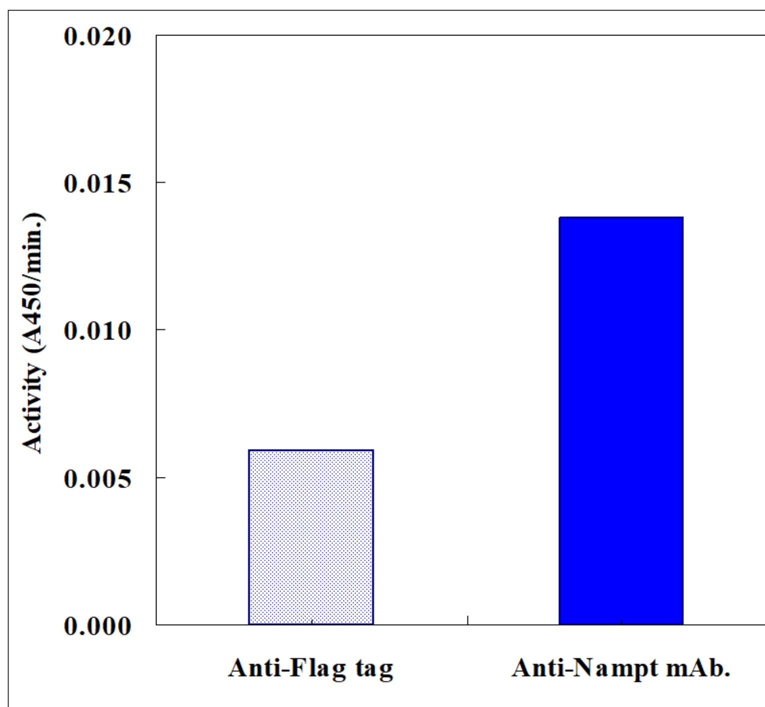
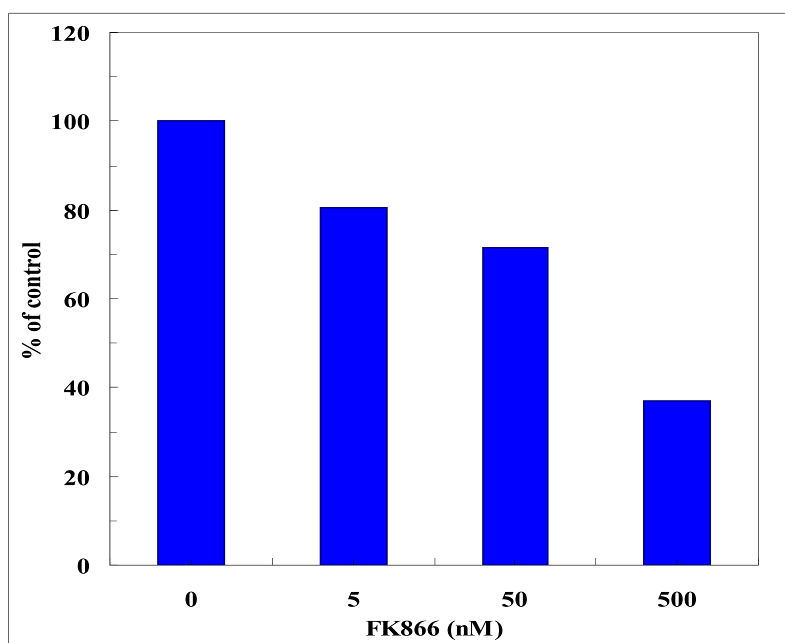


Fig.6 Effect of FK866 on NAMPT activity in an immunoprecipitate from a lysate of Raji cells with Anti-Human NAMPT Monoclonal Antibody (MBL, Cat# CY-M1035)



Copy on Web. Just For Reference Purpose Only.

Fig.7 Effect of FK866 on NAMPT activity in the immunoprecipitate using anti-Flag antibody from cell lysates of 293T cells transfected with Flag-NAMPT expression plasmid DNA

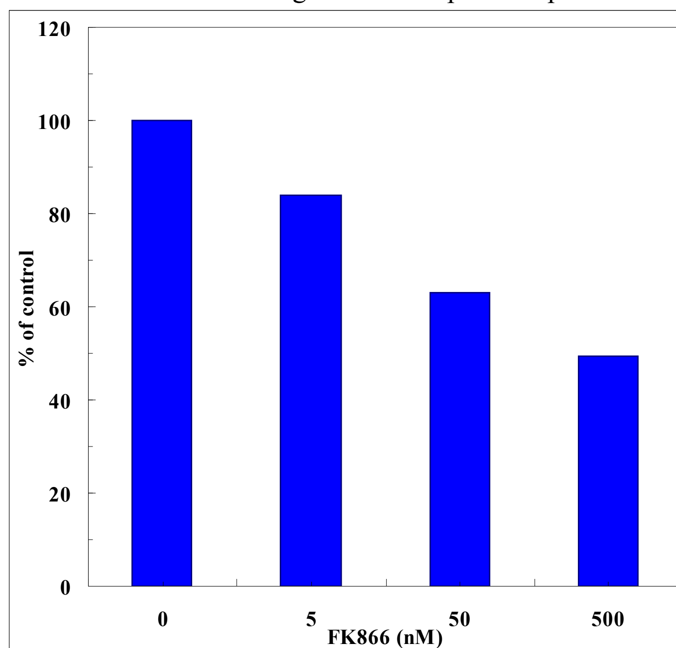
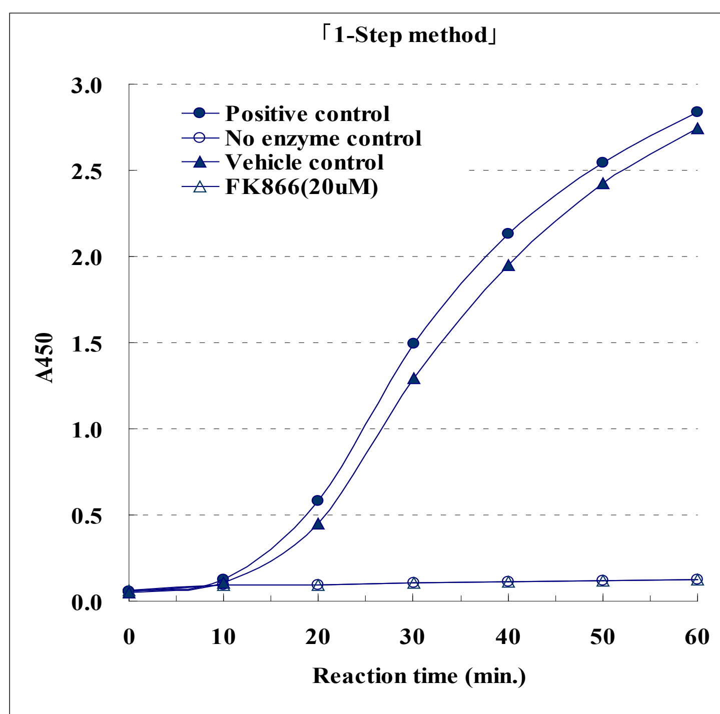


Fig.8 Typical data of One-Step Assay Method according to "3. Special Considerations When Screening Inhibitors" in the section "Detailed Protocol" above



Copy on Web. Just For Reference Purpose Only.

Fig.9 Typical data of Two-Step Assay Method according to "3. Special Considerations When Screening Inhibitors" in the section "Detailed Protocol" above

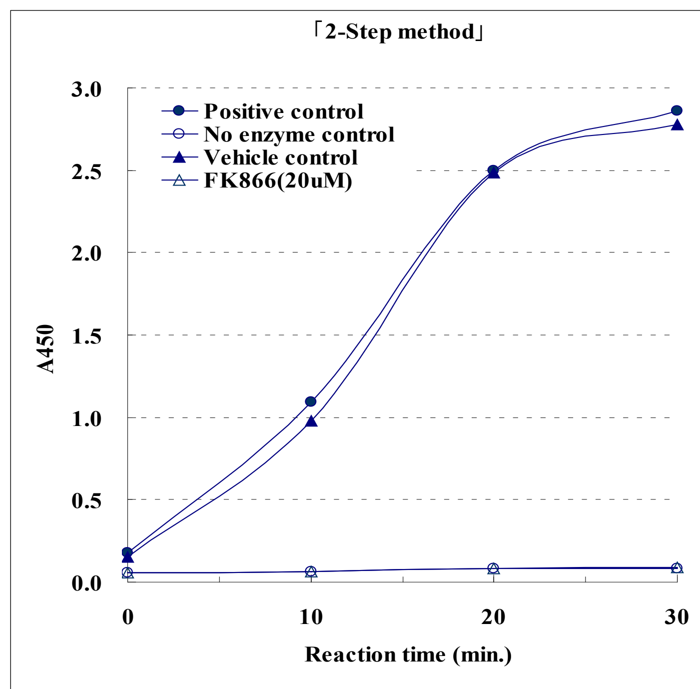
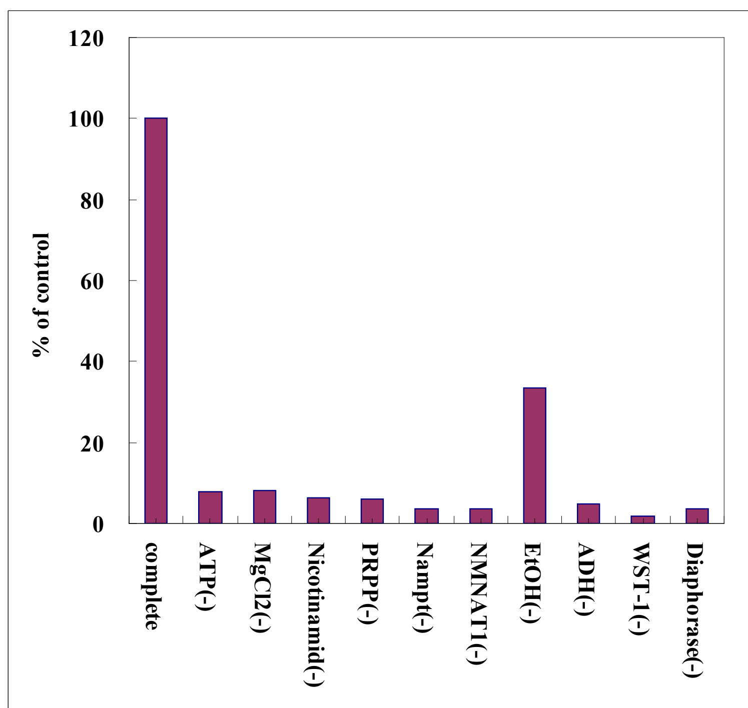


Fig.10 Requirement of each assay component for measurement of NAMPT activity



Copy on Web. Just For Reference Purpose Only.

References

1. Revollo JR *et al*. The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. J Biol Chem. 2004 Dec 3;279(49):50754-63.

2. Rongvaux A *et al*. Pre-B-cell colony-enhancing factor, whose expression is up-regulated in activated lymphocytes, is a nicotinamide phosphoribosyltransferase, a cytosolic enzyme involved in NAD biosynthesis. Eur J Immunol. 2002 Nov;32(11):3225-34.

3. Iqbal J and Zaidi M. TNF regulates cellular NAD^+ metabolism in primary macrophages. Biochem Biophys Res Commun. 2006 Apr 21;342(4):1312-8.

4. Nau GJ *et al*. Human macrophage activation programs induced by bacterial pathogens. Proc Natl Acad Sci U S A. 2002 Feb 5;99(3):1503-8.

5. Huang Q *et al*. The plasticity of dendritic cell responses to pathogens and their components. Science. 2001 Oct 26;294(5543):870-5.

6. Ognjanovic S *et al*. Genomic organization of the gene coding for human pre-B-cell colony enhancing factor and expression in human fetal membranes. J Mol Endocrinol. 2001 Apr;26(2):107-17.

7. Hasmann M and Schemainda I. FK866, a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, represents a novel mechanism for induction of tumor cell apoptosis. Cancer Res. 2003 Nov 1;63(21):7436-42.

8. Ramsey KM *et al*. Circadian clock feedback cycle through NAMPT-mediated NAD^+ biosynthesis. Science. 2009 May 1;324(5927):651-4.

9. Nakahata Y *et al*. Circadian control of the NAD^+ salvage pathway by CLOCK-SIRT1. Science. 2009 May 1;324(5927):654-7.

For more information, please visit our web site.
https://ruo.mbl.co.jp/

MANUFACTURED BY

A JSR Life Sciences Company
MEDICAL & BIOLOGICAL LABORATORIES CO., LTD.
URL: https://ruo.mbl.co.jp
E-mail: support@mbl.co.jp

CycLex/CircuLex products are supplied for research use only. CycLex/CircuLex products and components thereof may not be resold, modified for resale, or used to manufacture commercial products without prior written approval from MBL. To inquire about licensing for such commercial use, please contact us via email.

Copy on Web. Just For Reference Purpose Only.