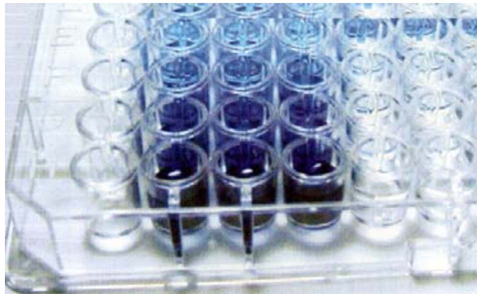
Dual Mouse/Rat intracellular Nampt (visfatin) ELISA

Cat. No. N0812EK



Instruction Manual
Version 2.0.0

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

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Introduction

Nampt, nicotinamide phosphoribosyl-transferase, is the rate-limiting enzyme of the mammalian NAD, nicotinamide adenine dinucleotide, biosynthesis pathway from nicotinamide to NMN, nicotinamide mononucleotide (1), which is further converted to NAD by nicotinamide/nicotinic acid mononucleotide adenyltransferase abbreviated Nmnat. Nampt was originally identified in *Haemophilus ducreyi* (2), and was then found to have a significant homology to the mammalian pre-B cell colony-enhancing factor (PBEF) (3) or visfatin (4), which is also called extracellular Nampt (eNampt) (5) due to the fact that it is found as a circulating form in human serum and secreted from differentiated adipocytes. Although visfatin was originally reported as an insulin-mimicking hormone by its capability of binding to and activating the insulin receptor, subsequent studies do not support this observation (5, 6). However, Revollo et al. observed that haplodeficiency and chemical inhibition of Nampt caused defects in NAD biosynthesis and glucose-stimulated insulin secretion in pancreatic islets in vivo and in vitro and plasma visfatin and NMN levels were reduced in Nampt heterozygous females (5). This study proposed a model that NMN exists in high amounts in plasma, presumably derived from nicotinamide with help of eNampt. This circulating NMN and nicotinamide are uptaken by beta cells via unknown transport mechanism (s) are converted to NAD by Nmnat and intracellular Nampt (hereinafter abbreviated iNampt), respectively, concluding that Nampt-mediated systemic NAD biosynthesis is critical for insulin secretion presumably via a NAD-dependent histonedeacetylase, Sirt1. Since it has been shown that NAD(+) levels in mitochondria remain at physiological levels following genotoxic stress and can maintain cell viability even when nuclear and cytoplasmic pools of NAD(+) are depleted (7), the NAD(+) biosynthetic enzyme Nampt plays a critical role in enhancing life span and protecting against oxidative cell damage. Therefore, measurement of iNampt located at different cell compartments like cytoplasm, motochondria, and nucleus may be able to give us some biological clue on the intracellular functions of Nampt.

Assay Principles

The dual mouse/rat iNampt (visfatin) ELISA is to be used for quantitative determination of iNampt in mouse or rat cell lysates.

This kit is an enzyme-linked immunosorbent assay (ELISA). A monoclonal antibody specific for mouse iNampt has been pre-coated onto 96 well microplate. Standards and samples are pipetted into the wells and any iNampt present is bound by immobilized antibody. Bound iNampt is captured by anti-mouse Nampt (visfatin) polyclonal antibody. HRP conjugated anti-IgG is added. After washing, a substrate solution is added. The colors develop in proportion to the bounded iNampt quantity. The color development is stopped and the intensity of color is measured.

Kit Components

1) Antibody coated 96-well plate, 12X 8-well strips
2) 5X Wash concentrate, 100 ml
3) 5X Diluent, 50 ml
4) 10X Lysis buffer, 12 ml
5) Secondary antibody, 12 ml
6) 100X Detector, 150 µl
7) Standard, recombinant mouse Nampt (visfatin) expressed by HEK 293 cells, 1 vial, lyophilized
8) QC sample = a positive control of recombinant mouse Nampt (visfatin) protein, 1 vial, lyophilized (For actual concentration of QC sample, see the 'Certificate of analysis' enclosed.)
9) Substrate, 12 ml
10) Stop solution, 12 ml

Reagents Description

Antibody coated 96-well plate, 12X 8-well strips, with absorbed monoclonal antibody against recombinant mouse Nampt (visfatin)

5X Wash concentrate, buffered detergent solution, supplied as a 5X concentrate
5X Diluent, for sample and reagent dilution
10X Lysis buffer
1X Secondary antibody, polyclonal antibody against recombinant mouse Nampt (visfatin)
100X detector, HRP conjugated anti-IgG
Standard, 64.0 ng, recombinant mouse Nampt (visfatin)
QC sample, recombinant mouse Nampt (visfatin)
Substrate solution, chromogenic reagents
Stop solution, 1M H_3PO_4

Storage of Reagents

Reagents must be stored at 2-8°C when not in use. Reagents must be brought to room temperature before use. Do not expose reagents to temperatures greater than 25°C. Diluted wash solution may be stored at room temperature for up to one month.

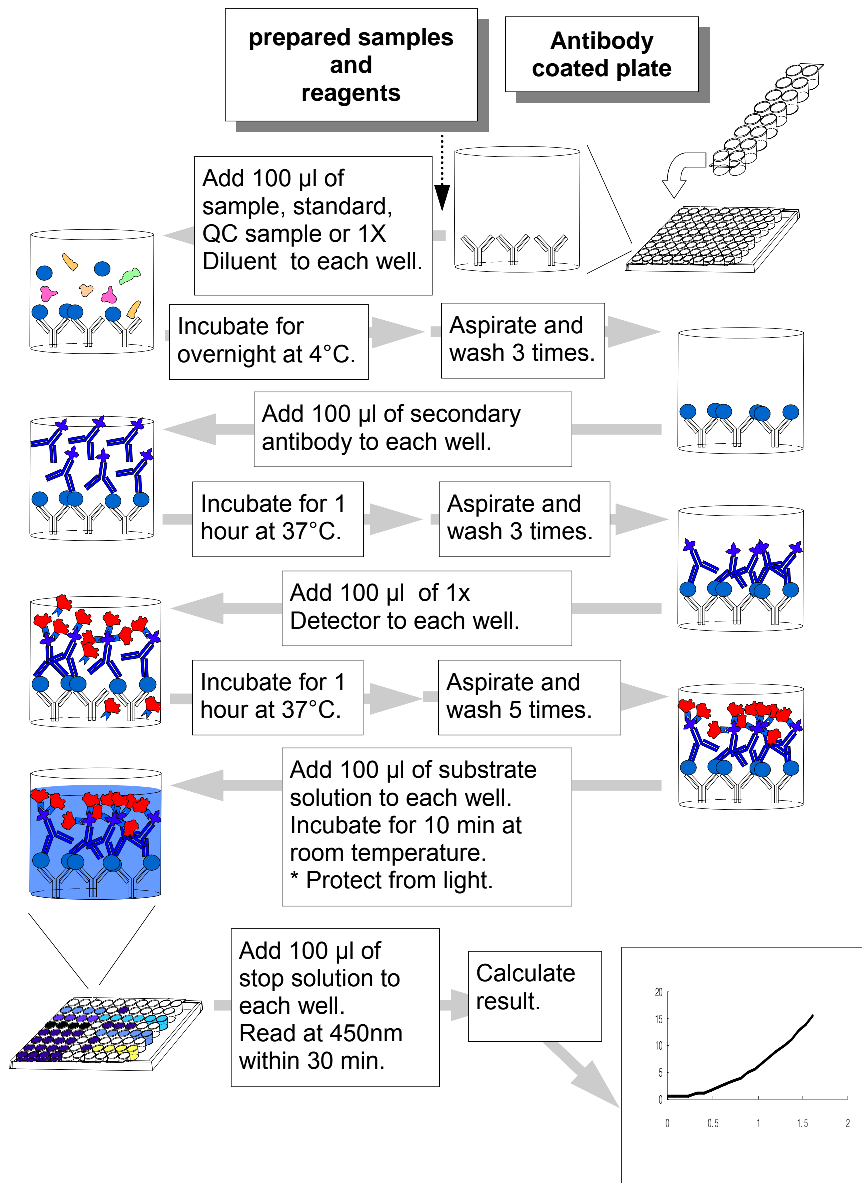
Materials Required but not Supplied

Precision single and multi-channel pipettes.
Disposable pipette tips.
Microtubes or equivalent for preparing dilutions.
Disposable plastic containers for preparing working reagents.
Reagent reservoirs.
Microwell or microstrip plate reader 450 nm
Deionized water
Phenyl methylsulfonyl fluoride (PMSF)

Sample Collection and Storage

Lysate Grow cell until 90% confluency. Scrap cells off the plate and transfer to an appropriate tube. Keep on ice and microcentrifuge at 1,200 rpm for 5 minutes at 4 ℃. Remove supernatant, rinse cells once with ice-cold PBS. Remove PBS and add 200 µl ice-cold 1x lysis buffer supplemented with 1 mM phenyl methylsulfonyl fluoride (PMSF) to ten million cells of interest and incubate on ice for 30 minutes. Microcentrifuge at 12,000 rpm for 5 minutes at 4 ℃ and transfer the supernatant to a new tube. The supernatant is the cell lysate. Use freshly prepared cell lysate samples.

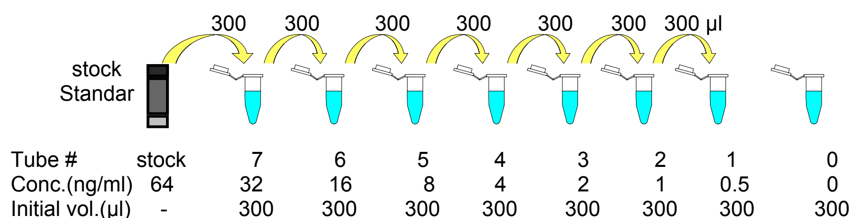
Flow Chart of Assay Procedure



Assay Procedure

1. Preparation of Reagents

1) Allow all samples and kit components to equilibrate to room temperature (20-25°C).
2) Plan the plate configuration and create a plate map. Calculate the amount of working reagents to use. It is recommended that standards and samples be run in duplicate.
3) Prepare **1X lysis buffer**. Dilute 10X Diluent 1:10 with deionized water (1 part 10X Diluent with 9 parts deionized water). Add 1 mM phenyl methylsulfonyl fluoride (PMSF) immediately before use.
4) Prepare **1X Wash Solution**. Dilute 5X Wash Concentrate 1:5 with deionized water (1 part 5X Wash Concentrate with 4 parts deionized water). The diluted 1X Wash Solution is stable for one month at room temperature.
5) Prepare **1X Diluent**. Dilute 5X Diluent 1:5 with deionized water (1 part 5X Diluent with 4 parts deionized water).
6) Prepare **1X Detector**. Dilute 100X Detector 1:100 with 1X Diluent (1 part 100X Detector with 99 parts 1X Diluent). Use the 1X Detector within one hour of preparation.
7) Warm **Substrate Solution** to room temperature before use.
8) Prepare working aliquots of the Standard as follows :
   When opening the lyophilized Standard, remove cap gently as the lyophilizate may have become dislodged during shipping.



Add 1 ml of deionized water to the Standard vial to make a stock
concentration of **64 ng/ml.** Mix well.

A recommended dilution scheme is as follows :

a. Label 8 microcentrifuge tubes #0-7. Add 300 µl of the 1X Diluent to the microcentrifuge tubes #0-7, respectively.

b. Add 300 µl of the stock Standard solution to tube #7 and vortex. This is Standard tube #7 with a concentration of 32 ng/ml.

c. Standards #6 to #1 are then prepared by performing a 1:2 dilution of the preceding standard. Do not add any standard to the tube #0.

9) Reconstitute QC sample in 1 ml of deionized water. Mix well.

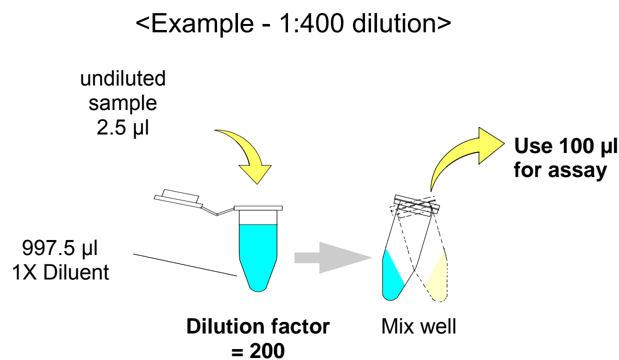
2. Sample Preparation

1) Dilute samples between 1:10 and 1:1000 and mix well.

* If samples fall the outside range of assay, a lower or higher dilution may be required.

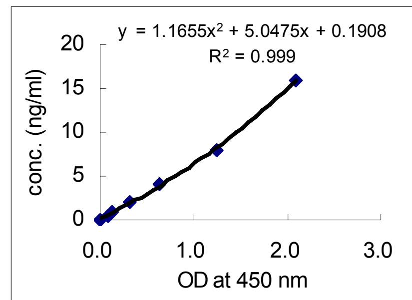
2) Use 100 µl of the final diluted sample for ELISA.

<Example - 1:400 dilution>



3. Experiment procedure

1) Remove the appropriate number of microwell strips from the sealed foil pouch.
2) Pipette 100 µl of standards #0 to #7, the reconstituted QC sample and diluted sample into the antibody-coated plate according to the plate configuration. Use a new pipette tip for each standard or sample.
3) Incubate at 4°C for overnight.
4) Remove the solution and wash 3 times with 300 µl of 1X Wash Solution to each well.
5) Add 100 µl Secondary Antibody to each well.
6) Incubate at 37°C for 1 hour.
7) Remove the solution and wash 3 times with 300 µl of 1X Wash Solution to each well.
8) Add 100 µl 1X Detector to each well.
9) Incubate at 37°C for 1 hour.
10) Remove the solution and wash 5 times with 300 µl of 1X Wash Solution to each well.
11) Add 100 µl of the Substrate Solution to each well.
12) Incubate at room temperature for 10 min.
    * Protect from light.
13) Using the multi-channel pipette, add 100 µl Stop Solution to each well.



14) Read at 450 nm.
15) Subtract the absorbance of the blank from the readings for each standard and sample.
16) Construct a standard curve by plotting the known concentrations (Y) of standard versus the absorbance (X) of standard. A measurable range is typically shown between 0.5 ng/ml and 32 ng/ml.
17) Calculate iNampt concentrations of samples by interpolation of the regression curve formula as shown above in a form of a quadratic equation.
18) The iNampt concentrations calculated must be multiplied by dilution factor [see **2. Sample Preparation**] to obtain the concentrations of the undiluted samples.

Performance Characteristics

1) Sensitivity : 50 pg/ml

2) Precision

a. Intra-Assay (precision within an assay)

2 samples was tested 10 times to assess intra-assay precision.

Sample	Mean (ug/ml)	SD (ug/ml)	CV (%)
Mouse sample	1.04	0.04	3.50
Rat sample	5.89	0.13	2.25

b. Inter-Assay (precision between assays)

2 samples was tested 3 times to assess inter-assay precision.

Sample	Mean (ug/ml)	SD (ug/ml)	CV (%)
Mouse sample	0.99	0.07	6.80
Rat sample	3.72	0.29	5.12

***The mouse cell lysate sample was prepared from mouse 3T3L1 cells. 1:200 dilution was made.**

***The rat cell lysate sample was prepared from rat hepatoma cells. 1:400 dilution was made.**

3) Specificity

- Cross Reactivity

Cross reactivity of a given analyte at 10 ng/ml or 100 ng/ml was calculated as an optical density in relative to 10 ng/ml of recombinant mouse visfatin.

Analyte (recombinant proteins)	Max. Conc. (ng/ml)	Cross Reactivity (%)
Mouse Nampt (visfatin)	10	100
Rat Nampt (visfatin)	10	100
Human Nampt (visfatin)	100	N. R.
Mouse Adiponectin	100	N. R.
Mouse Resistin	100	N. R.
Mouse Vaspin	100	N. R.
Mouse RBP4	100	N. R.
Mouse GPX3	100	N. R.
Mouse Progranulin	100	N. R.
Mouse IL-33	100	N. R.
Mouse Clusterin	100	N. R.
Mouse ANGPTL3	100	N. R.
Mouse ANGPTL4	100	N. R.
Human AGF	100	N. R.
Mouse RBP4	100	N. R.
Mouse Leptin	100	N. R.
Mouse TNF-α	100	N. R.
Human adiponectin	100	N. R.

N. R. : No Cross-reactivity

4) Recovery

The recovery of recombinant mouse Nampt (visfatin) spiked to three different levels in two samples throughout the range of assay was evaluated.

Sample No.	Average recovery (%)	Range (%)
Mouse sample	100.81	95-105
Rat sample	104.25	95-105

5) Linearity - Effect of Lysate Dilution

To assess the linearity of the assay, two lysate samples was first diluted as indicated below prior to sample preparation as described in the protocol.

Sample No.	Serum Dilution	Expected (ug/ml)	Observed (ug/ml)	% Of Expected
Mouse sample	1:200	1.50	1.50	100
	1:400	0.75	0.78	104.05
	1:800	0.37	0.41	109.54
Rat sample	1:400	0.61	0.61	100
	1:800	0.31	0.32	103.23
	1:1600	0.15	0.17	108.72

% of expected = observed / expected x 100%

References

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	1	2	3	4	5	6	7	8	9	1	1	1
A												
B												
C												
D												
E												
F												
G												
H												

Troubleshooting Guide

Problem	Possible Cause	Solution
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be appropriate for the system.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of detector too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.
	Technique problem	Proper mixing of reagents and wash steps are critical.

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