ELISA Kit for Measuring Human NAMPT/PBEF

CircuLex Human NAMPT/PBEF ELISA Kit

Cat# CY-8111

Intended Use

The MBL Research Product **CircuLex Human NAMPT/PBEF ELISA Kit** is used for the quantitative measurement of human NAMPT/PBEF in serum, plasma, saliva, tear, urine, milk, stool, cell culture supernatant, and cell lysate.

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

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

Storage

- Upon receipt store all components at 4°C.
- Do not expose reagents to excessive light.

Introduction

Nicotinamide phosphoribosyltransferase (Nampt) catalyzes the rate-limiting step in the salvage pathway for nicotinamide adenine dinucleotide (NAD+) biosynthesis (1). Interestingly, Nampt exists not only as an intracellular form (iNampt) but also as an extracellular form (eNampt) in an enzymatically active dimer (2), and eNampt, previously named pre-B cell colony-enhancing factor (PBEF) or visfatin, is produced and secreted by adipocytes, mononuclear cells, hepatocytes, and cardiomyocytes (2-4). This novel cytokine-like 52-kDa protein was shown to be secreted also from peripheral blood lymphocytes and is strongly induced by pokeweed mitogen and cycloheximide, and enhanced the effect of IL-7 and stem cell factor on pre-B cell colony formation (5). Although, its pathophysiological role remains largely unknown, eNampt has been reported to correlate with a number of pathological states, including acute lung injury in sepsis patients (6), rheumatoid arthritis (7), ulcerative colitis (8), and type II diabetes (9). A recent study shows that SIRT1 deacetylate iNampt in adipocytes and enhance its activity and its secretion from adipocytes to blood. The secreted eNampt affect NAD+ biosynthesis, SIRT1 activity, and neural activity in hypothalamus (10). These findings suggest that Nampt could play a critical role in energy metabolism and homeostasis not only in a cell but also at a systemic level.

Principle of the Assay

The MBL Research Product **CircuLex Human NAMPT/PBEF ELISA Kit** employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for human NAMPT/PBEF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any human NAMPT/PBEF present. After washing away any unbound substances, an HRP conjugated antibody specific for human NAMPT/PBEF is added to the wells. Following a wash to remove any unbound antibody HRP conjugate, the remaining conjugate is allowed to react with the substrate H_2O_2-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of human NAMPT/PBEF. A standard curve is constructed by plotting absorbance values versus human NAMPT/PBEF concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

Summary of Procedure

Add 100 µL of diluted samples to the wells
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of HRP conjugated anti-human NAMPT/PBEF antibody
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of Substrate Reagent
↓ Incubate for 10-20 minutes at room temp.
Add 100 µL of Stop Solution
↓
Measure absorbance at 450 nm

Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microplate kit.

Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with anti-human NAMPT/PBEF antibody as a capture antibody.

10X Wash Buffer: One bottle containing 100 mL of 10X buffer containing Tween®-20

Dilution Buffer: One bottle containing 50 mL of 1X buffer; use for reconstitution of Human NAMPT Standard and sample dilution. Ready to use.

Human NAMPT Standard: One vial containing X* ng of lyophilized recombinant human NAMPT/PBEF
***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**
100X HRP conjugated Detection Antibody: One bottle containing 140 µL of HRP (horseradish peroxidase) conjugated anti-human NAMPT/PBEF antibody.

Detection Antibody Dilution Buffer: One bottle containing 12 mL of 1X buffer; use for the dilution of 100X HRP conjugated Detection Antibody.

Substrate Reagent: One bottle containing 20 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle containing 20 mL of 1 N H_2SO_4. Ready to use.

Materials Required but not Provided

- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **(Optional) Microplate washer:** Manual washing is possible but not preferable.
- **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.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1,000 mL graduated cylinder**.
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- **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.**
- Allow all the components to come to room temperature before use.
- All microplate strips that are not immediately required should be returned to the zip-lock pouch, which must be carefully resealed to avoid moisture absorption.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents in this kit may contain preservatives or other chemicals. Care should be taken to avoid direct contact with these reagents.
- 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.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of human origin, and these reagents. In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.
- **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: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution**.

Sample Collection and Storage

Serum: Use a serum separator tube and allow samples to clot for 60 ± 30 minutes. Centrifuge the samples at 4°C for 10 minutes at 1,000 x *g*. Remove serum and assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of serum may also be stored below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using EDTA-2Na as the anticoagulant. If possible, collect the plasma into a mixture of EDTA-2Na and Futhan5 to stabilize the sample against spontaneous *in vitro* complement activation. Immediately centrifuge samples at 4°C for 15 minutes at 1,000 x *g*. Assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of plasma may also be stored below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Note: Heparin and Citrate plasma has not been validated for use in this assay.

Biological media: Remove any particulates by centrifugation and assay immediately or aliquot and store samples below -70°C. Avoid repeated freeze-thaw cycles.

Stool: The following protocol is provided as an example of suitable methods. All steps of the stool extract should be performed at 4°C.

1. Weigh stools and add the extraction buffer (50 mM HEPES-KOH pH7.5, 250 mM NaCl, 0.2% NP-40, 5 mM EDTA, 10% Glycerol) so as to be 1 to 10 mg/mL.

2. Suspend the mixtures well using sonication or pipetting.

3. Transfer the mixtures to microcentrifuge tubes and centrifuge at 15,000 rpm for 10 minutes at 4°C.

4. Collect supernatants to a clean microfuge tube.

5. Assay immediately or store the samples on ice for a few hours before assaying. Aliquots of the samples may also be stored below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Cell lysate: Several extraction methods can be used for measurement cellular proteins. The following protocol is provided as an example of suitable methods. All steps of cell lysate preparation should be performed at 4°C.

1. Harvest and pellet cells by centrifugation using standard methods.

2. Resuspend the cell pellet with the cell lysis buffer (25 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.1 % NP-40, 1 mM EDTA, 0.2 mM PMSF, 1 µg/mL pepstatin, 0.5 µg/mL leupeptin, 0.2 mM DTT).

3. Lyse the resuspended cells using either a Dounce homogenizer, sonication, or three cycles of freezing and thawing.

4. Transfer extracts to microcentrifuge tubes and centrifuge at 15,000 rpm for 10 minutes at 4°C.

5. Aliquot cleared lysate to a clean microfuge tube.

6. Assay immediately or store the samples on ice for a few hours before assaying. Aliquots of the samples may also be stored below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Note-1: The above procedures are intended only as a guideline. The optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.

Note-2: Individual users should determine appropriate conditions when using other types of samples which do not be indicated in this manual.

Detailed Protocol

The MBL Research Product **CircuLex Human NAMPT/PBEF ELISA Kit** is provided with removable strips of wells so the assay can be carried out on separate occasions using only the number of strips required for the particular determination. Since experimental conditions may vary, an aliquot of the Human NAMPT Standard within the kit, should be included in each assay as a calibrator. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solutions

All reagents need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of **10X Wash Buffer**, **HRP conjugated Detection Antibody** and **Human NAMPT Standard**.

1. Prepare a working solution of Wash Buffer by adding **100 mL** of **10X Wash Buffer** to **900 mL** of deionized (distilled) water (**ddH_2O**). Mix well. Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Prepare working solutions of **HRP conjugated Detection Antibody** by 100-fold diluting the **100X HRP conjugated Detection Antibody** with the **Detection Antibody Dilution Buffer**, *e.g.* 10 µL of **100X HRP conjugated Detection Antibody** + 990 µL of Detection Antibody Dilution Buffer and mix well. Prepare the solutions just before use and discard unused one.

3. Reconstitute **Human NAMPT Standard** with X* **mL** of **Dilution Buffer** by gently mixing. After reconstitution, immediately dispense it in small aliquots (e.g. 100 µL) to plastic micro-centrifuge tubes and store below -70°C to avoid non-specific adsorption to glass surface and multiple freeze-thaw cycles. The concentration of the reconstituted Human NAMPT Standard should be **384 ng/mL**, which is referred to as the **Master Standard** of human NAMPT/PBEF.

***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

Prepare Standard Solutions as follows:

Use the **Master Standard** to produce **Std.0 (48 ng/mL)** and make a dilution series (below). Mix each tube thoroughly before the next transfer. **Std.1 (9,600 pg/mL)** serves as the highest standard. The **Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Dilution Buffer	Concentration
Std.0	50 µL of Master Standard (384 ng/mL)	350 µL	48 ng/mL
Std.1	150 µL of Std.0 (48 ng/mL)	600 µL	9,600 pg/mL
Std.2	300 µL of Std. 1 (9,600 pg/mL)	300 µL	4,800 pg/mL
Std.3	300 µL of Std. 2 (4,800 pg/mL)	300 µL	2,400 pg/mL
Std.4	300 µL of Std. 3 (2,400 pg/mL)	300 µL	1,200 pg/mL
Std.5	300 µL of Std. 4 (1,200 pg/mL)	300 µL	600 pg/mL
Std.6	300 µL of Std. 5 (600 pg/mL)	300 µL	300 pg/mL
Std.7	300 µL of Std. 6 (300 pg/mL)	300 µL	150 pg/mL
Blank	-	300 µL	0 pg/mL

Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with Dilution Buffer before dispensing. Discard any unused Standard Solutions after use.

Sample Preparation

Dilute samples with **Dilution Buffer**.

- Serum and plasma may require a 20-fold dilution.
- Cell culture supernatants may require a 2- to 4-fold dilution.
- Cell lysate: 10-30 µg total protein/mL
- Stool: 40-60 µg /mL
- Other biological media require appropriate dilutions.

  e.g. Milk: 20-fold dilution, Urine: 5- to 10-fold dilution, Tear: 10- to 20-fold dilution, Saliva: 20-fold dilution

Note: Do Not Use stored samples of saliva.

Assay Procedure

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.

2. Dilute samples with **Dilution Buffer**. (See "Sample Preparation" above.)

3. Pipette **100 µL** of **Standard Solutions (Std1-Std7, Blank)** and **diluted samples** in duplicates, into the appropriate wells.

4. Incubate the plate **at room temperature (ca.25°C) for 60 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker.

5. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

6. Add **100 µL** of **HRP conjugated Detection Antibody** into each well.

7. Incubate the plate **at room temperature (ca.25°C) for 60 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker.

8. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

9. Add **100 µL** of **Substrate Reagent**. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminum foil is recommended. Return Substrate Reagent to 4°C immediately after the necessary volume is removed

10. Incubate the plate **at room temperature (ca. 25°C) for 10-20 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker. The incubation time may be extended up to 30 minutes if the reaction temperature is below than 20°C.

11. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

12. Measure absorbance in each well using a spectrophotometric microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Note-1: Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspiration or decantation. Invert the plate and blot it against clean paper towels.

Note-2: Reliable standard curves are obtained when either O.D. values do not exceed 0.25 units for the blank (zero concentration), or 2.5 units for the highest standard concentration.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine human NAMPT/PBEF concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

Calculations

Average the duplicate readings for each standard, control and sample, and subtract the optical density of the average zero standard. Plot the optical density versus the concentration of standards and draw the best curve. Most microtiter plate readers perform automatic calculations of analyte concentration. The standard curve fits best to a sigmoidal four-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a four-parameter logistic function.

A standard curve is also to be constructed by plotting the absorbance (Y) versus log of the known concentration (X) of standards, using a cubic function. Alternatively, the logit log function can be used to linearize the standard curve (i.e. logit of optical density (Y) is plotted versus log of the known concentration (X) of standards). To determine the concentration of each sample, first find the optical density 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 concentration.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Measurement Range

The measurement range is 150 pg/mL to 9,600 pg/mL. Any sample reading higher than the highest standard should be diluted with Dilution Buffer in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the human NAMPT/PBEF concentration.

Troubleshooting

1. All samples 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.

2. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate insufficient washing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for washer maintenance.

3. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in the MBL Research Product **CircuLex Human NAMPT/PBEF ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date.

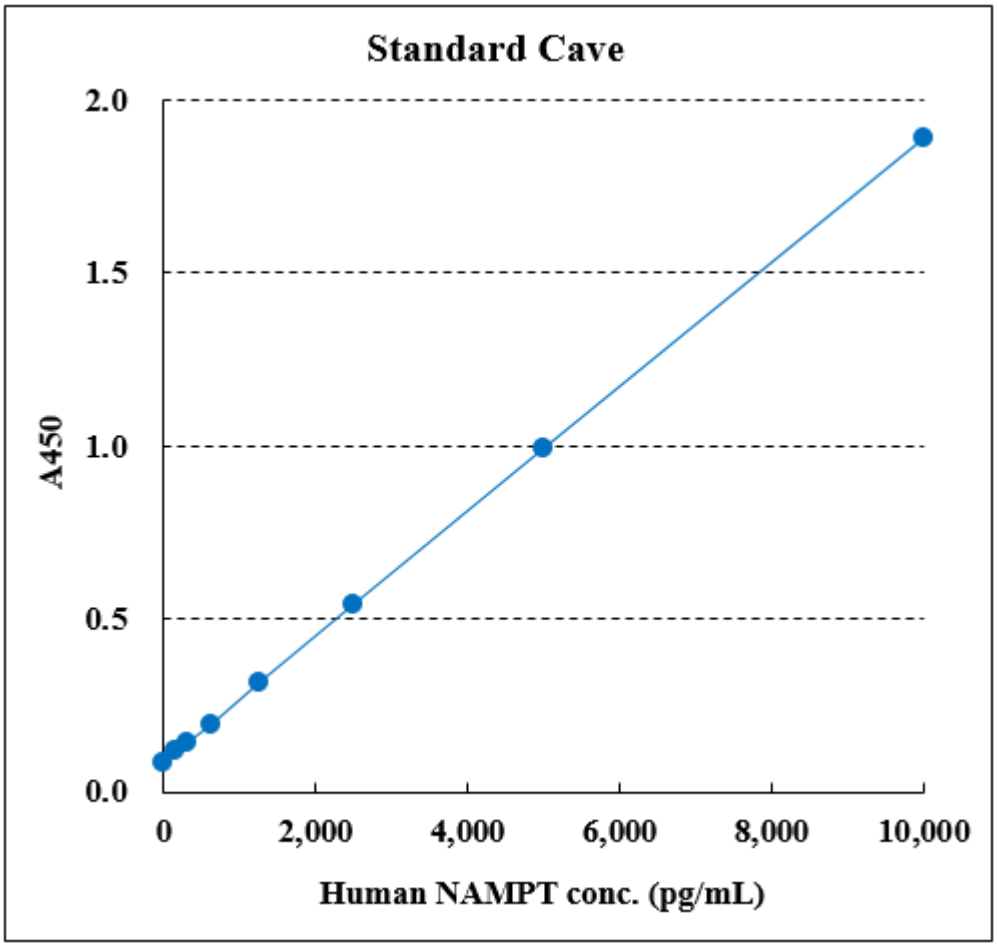
Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of human NAMPT/PBEF giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 60 pg/ml of sample.

* Dilution Buffer was pipetted into blank wells.

Typical standard curve



2. Precision

Intra-assay Precision (Precision within an assay)

Four samples* of known concentration were tested eight times on one plate to assess intra-assay precision.

• Intra-assay (Within-Run, n=8) CV=2.1-9.6 %

*Sample: Serum

Human NAMPT conc. (ng/mL)

	Sample 1	Sample 2	Sample 3	Sample 4
1	25.1	36.1	5.6	2.8
2	23.8	33.8	5.4	2.9
3	23.8	32.7	5.6	2.5
4	24.7	34.8	5.2	2.7
5	24.1	33.3	5.8	2.3
6	23.9	33.9	4.8	2.3
7	24.0	35.2	5.4	2.8
8	23.6	37.2	5.4	2.8
MAX.	**25.1**	**37.2**	**5.8**	**2.9**
MIN.	**23.6**	**32.7**	**4.8**	**2.3**
MEAN	**24.2**	**34.6**	**5.4**	**2.6**
S.D.	**0.52**	**1.50**	**0.28**	**0.25**
C.V.	**2.1%**	**4.3%**	**5.2%**	**9.6%**

Inter-assay Precision (Precision between assays)

Four samples* of known concentration were tested in four separate assays to assess inter-assay precision.

• Inter-assay (Run-to-Run, n=4) CV=5.0-8.1 %

*Sample: Serum

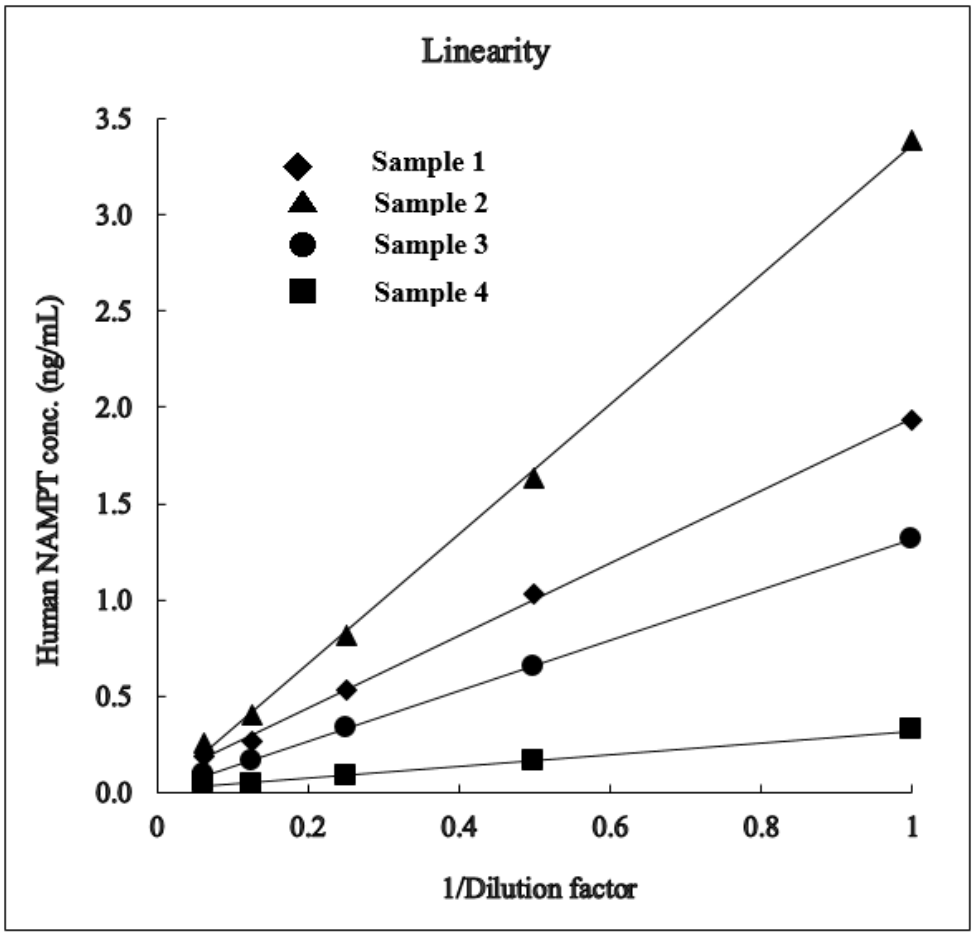
Human NAMPT conc. (ng/mL)

	Sample 1	Sample 2	Sample 3	Sample 4
1	20.3	32.3	10.4	4.0
2	20.4	30.4	10.3	4.4
3	22.2	33.7	11.6	4.6
4	22.2	34.7	11.6	3.9
MAX.	**22.2**	**34.7**	**11.6**	**4.6**
MIN.	**20.3**	**30.4**	**10.3**	**3.9**
MEAN	**21.3**	**32.8**	**11.0**	**4.2**
S.D.	**1.07**	**1.88**	**0.71**	**0.34**
C.V.	**5.0%**	**5.8%**	**6.4%**	**8.1%**

3. Linearity

Four samples* were diluted with Dilution Buffer and assayed after dilution. The neat samples were set to 1.0.

*Sample: Serum



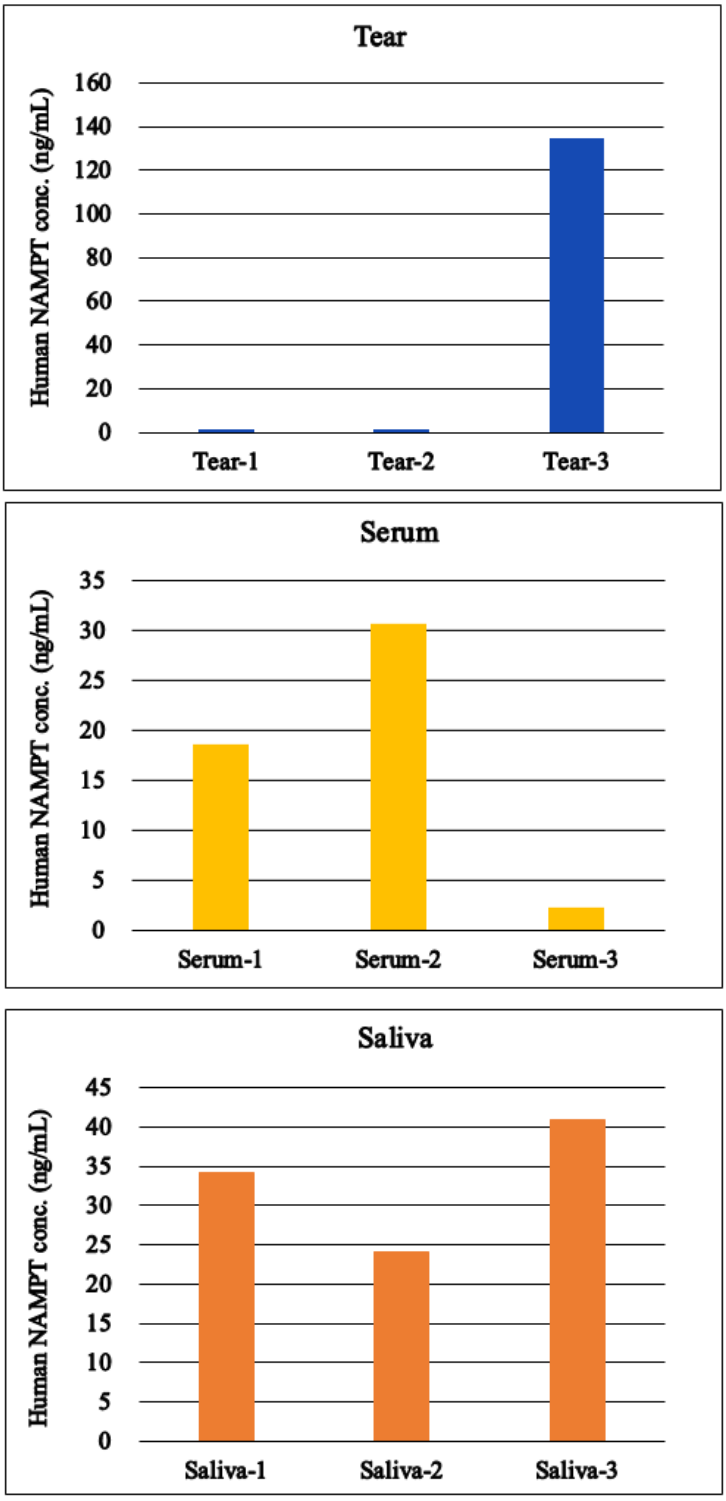
4. Spiking Recover

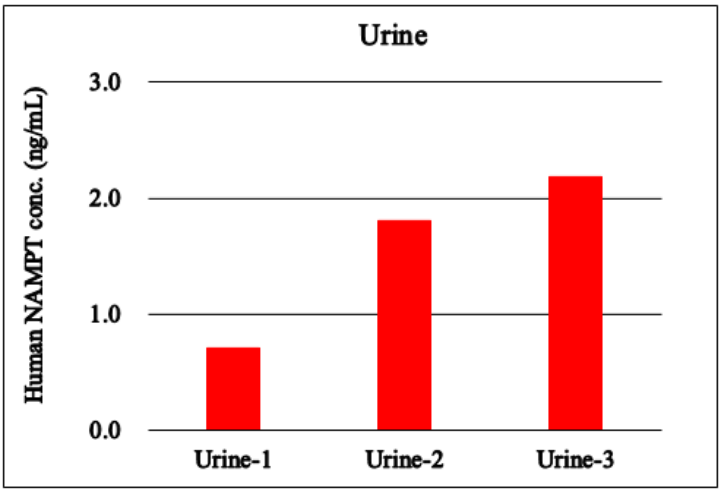
Serum samples were spiked with different amounts of human NAMPT/PBEF and assayed.
The recovery of human NAMPT/PBEF spiked to levels throughout the range of the assay was evaluated.

Sample	Average % Recovery	Range
Serum samples (n=4)	109, 87, 95, 116	

Example of Test Results

Fig.1 Human NAMPT concentration in biological samples including serum, tear, saliva, urine, milk and stool.





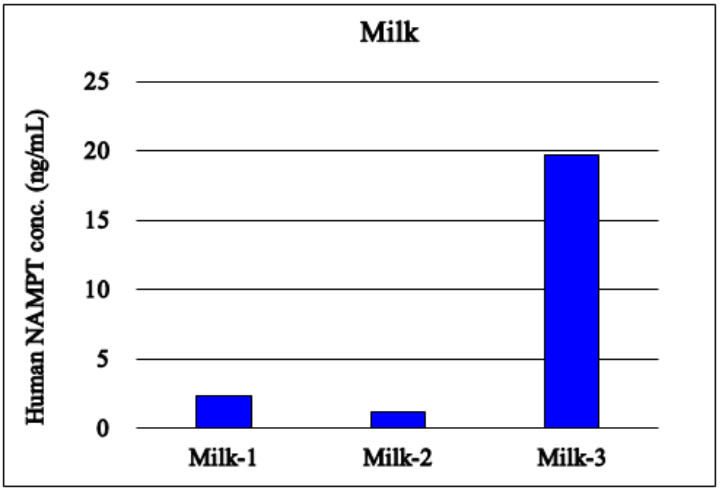


Fig.2 Human NAMPT concentration in cell culture supernatants

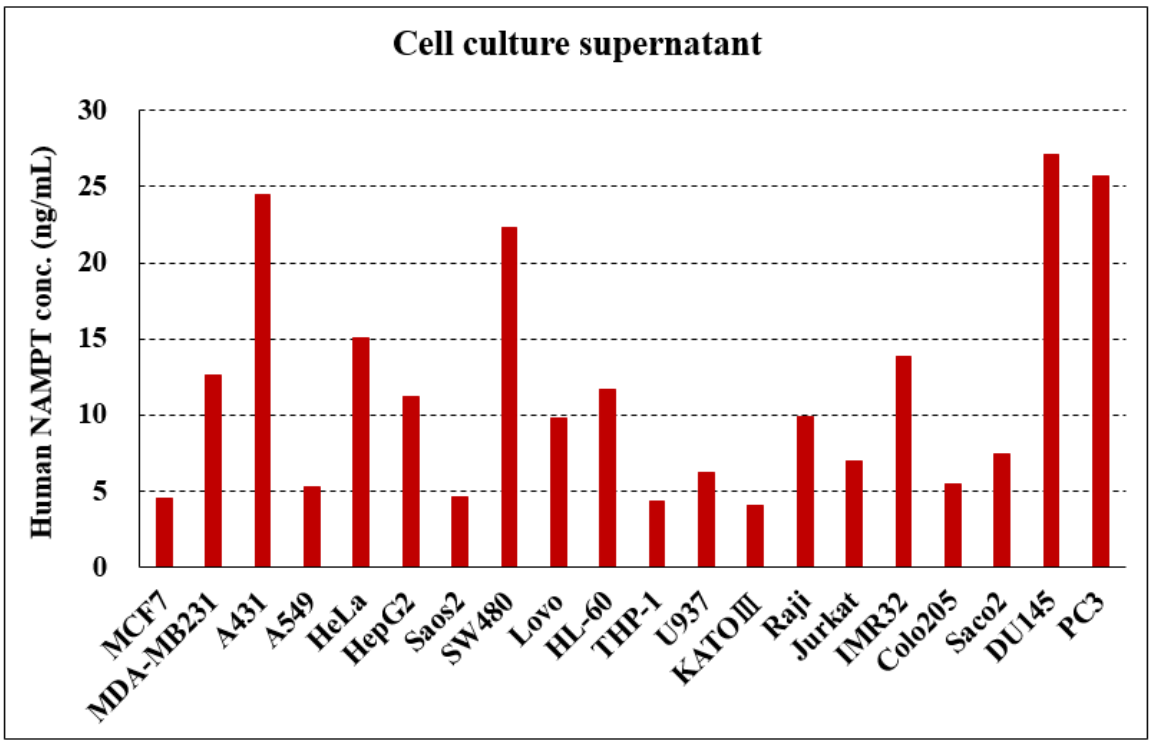
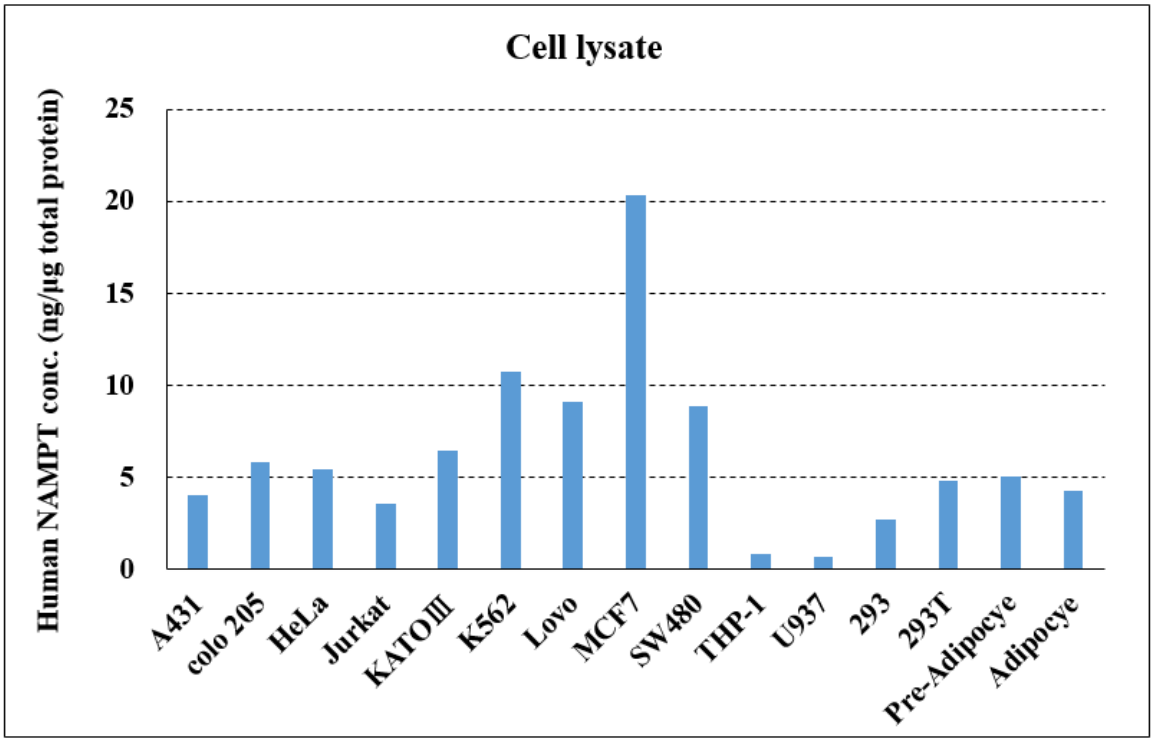


Fig.3 Human NAMPT concentration in cell lysates



References

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

2. Revollo JR *et al*. Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. Cell Metab. 6, 363-75 (2007)

3. Imai S *et al*. The importance of NAMPT/NAD/SIRT1 in the systemic regulation of metabolism and ageing. Diabetes Obes Metab. 15, 26-33 (2013)

4. Pillai VB *et al*. Nampt secreted from cardiomyocytes promotes development of cardiac hypertrophy and adverse ventricular remodeling. Am J Physiol Heart Circ Physiol. 304, H415-26 (2013)

5. Samal B *et al*. Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. Mol Cell Biol. 14, 1431-7 (1994)

6. Ye SQ *et al*. Pre-B-cell-colony-enhancing factor is critically involved in thrombin-induced lung endothelial cell barrier dysregulation. Microvasc Res. 70, 142-51 (2005)

7. Nowell MA *et al*. Regulation of pre-B cell colony-enhancing factor by STAT-3-dependent interleukin-6 trans-signaling: implications in the pathogenesis of rheumatoid arthritis. Arthritis Rheum. 54, 2084-95 (2006)

8. Moschen AR *et al*. Visfatin, an adipocytokine with proinflammatory and immunomodulating properties. J Immunol. 178, 1748-58 (2007)

9. Chen M *et al*. Elevated Plasma Level of Visfatin/Pre-B Cell Colony-Enhancing Factor in Patients with Type 2 Diabetes Mellitus. J Clin Endocrinol Metab. 91, 295-9 (2006)

10. Yoon MJ *et al*. SIRT1-Mediated eNAMPT Secretion from Adipose Tissue Regulates Hypothalamic NAD+ and Function in Mice. Cell Metab. 5, 706-17 (2015)

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