RayBio®
Human Visfatin Immunodetection Kit

User Manual
(Revised 031808)

RayBio® Human Visfatin Immunodetection Kit Protocol
(For Western Blot Analysis, 5 tests)

(Cat# RB-08-0003K)

For Obesity and Adipokine Research

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I. **Introduction**

A group from Osaka, Japan identified a protein that is produced in much greater amounts by visceral fat, and it was thus named visfatin in 2005. It's been known for a long time that visceral fat is worse for your health than subcutaneous fat. Visfatin, an adipocytokine that is highly enriched in the visceral fat of both humans and mice whose expression level in plasma increases during the development of obesity. Obesity, which is characterized by an excessive accumulation of adipose tissue in the body, represents one of the greatest public health challenges and has now reached epidemic proportions. Obesity is associated with health problems linked to increased weight-dependent pressure overload on lung, joints and bones. More importantly, obesity represents a risk factor for life-threatening diseases such as cardiovascular diseases, type 2 diabetes and certain forms of cancer.

Visfatin also designated as Pre-B cell-enhancing factor (PBEF) or Nicotinamide hosphoribosyltransferase (Nampt) , belongs to the NAPRTase family of proteins. It is an inflammatory cytokine that plays a requisite role in the delayed neutrophil apoptosis of sepsis. It exerted insulin-mimetic effects in cultured cells and lowered plasma glucose levels in mice. It was found that visfatin binds to and activates the insulin receptor, albeit in a different place than insulin. Despite the slightly altered binding position, visfatin triggers the same cellular responses as insulin, including the induction of glucose uptake in fat and muscle and the suppression of glucose production by the liver. Plasma level of visfatin in patients with type 2 diabetes mellitus was elevated, suggesting that measurement of plasma visfatin provides a relevant tool for understanding metabolic diseases. Furthermore, Visfatin may be involved in enhancing the effect of IL-7 and SCF on the formation of early B-lineage precursor colonies. More recently, several groups have reported the crystal structure of Nampt/PBEF/visfatin and they all show that this protein is a dimeric type II phosphoribosyltransferase enzyme involved in NAD biosynthesis pathway.

Visfatin is a cytoplasmic protein with a molecular weight of 52 kDa composed of 473-amino acid. It has a hydrophobic N terminus, 2 N-glycosylation sites, several putative phosphorylation sites, and 6 cysteines.

Visfatin is expressed primarily in bone marrow, muscle and liver tissue but can also be detected in placenta, lung, kidney and heart tissue.

II. Kit Description

The RayBiotech Visfatin Immunodetection Kit is an in vitro semi-quantitative assay for the detection and identification of Visfatin-containing proteins in Western blot or Dot blot analysis. The kit is a complete kit designed for fast, clean result and maximum convenience when detecting proteins immobilized on nitrocellulose membrane. This kit provides all reagents required for the detection of visfatin-containing proteins by both Western and Dot blot (Immunoblot) analysis including anti-Visfatin specific antibody plus necessary positive, negative and reference controls. Positive and reference control samples react with the probing antibody and show positive signals in the assay.

III. Kit Components

Table 1. List and use guide of Kit Components

Reagents	Description	5 Test Kit	Color Code	Reconstitution and use
Primary antibody: Anti-Visfatin	Affinity purified rabbit antibody in 100µl of 0.1M Tris-glycine, pH 7.4, 0.15M NaCl, without sodium azide	1 vial lyophilized (100µg)	Red	Add 100 µl distilled water, final concentration is 1mg/ml, use for 1 µg/ml
Secondary antibody: Goat Anti-rabbit IgG, HRP-Conjugated	Immunoaffinity purified goat IgG conjugated to horseradish peroxidase in solution	1vial (10 µl)	Yellow	1:5000 dilution with 1× blocking buffer)
MD231 and Hela Cell Lysates	Two Positive controls containing cellular Visfatin	2 vial, each (100 µl, 1µg/µl)	Green	15µl lysate with 5µl of 4 × Loading Buffer, 20µl/lane (**Note: A positive control should be included with every analysis).**
Peptide-conjugated carrier	One Reference control containing peptide derived from C-terminus of Visfatin	1 vial (50 µl, 1µg/µl)	Blue	Ready for use, Suggest to load 10µg/lane
2 × Cell Lysis Buffer	For preparation of cell lysate	1 bottle (10 ml)	N/A	Ready for use.(**See sample preparation in Section V**)

Immunoblot Blocking Reagent	For membrane blotting and preparing primary and secondary antibodies	1 bottles (powder)	N/A	Add 100 ml 1 × PBS before use
20 × washing buffer	For washing membrane at each step	2 bottles (2×30 ml)	N/A	Add 570 ml distilled water
Protein Markers	For Orientation of membrane blotting and estimating protein size	1 vial (30 µl)	N/A	Ready for use, loading 5ul/lane
Hybond Membrane	For proteins and preparing samples transfer	5 pieces (4×2.5 cm)	N/A	Ready for use
Manual	Workable protocol for kit	1	N/A	Ready for use

Note: Read the enclosed protocol before use.

Upon receipt, primary antibody(red), Peptide-conjugated carrier (Blue) and cell lysates(Green) in RayBio® Human Visfatin immunodetection kit should be kept at -20^0C and all other components in the box should be stored at 4^0C. At both storage temperature the kit will retain complete activity for up to year. The following table gives user clear description and brief instruction for Kit components.

Additional Materials Required but not included as part of kit:

- Orbital shaker, Aluminum foil, Distilled water, Plastic box, Membrane Incubation Containers, Running, transferring and loading buffers
- Kodak AR film and file processor or Chemiluminescence imaging system
- Shaker platform
- Bio-Rad Western device and power
- Running buffer, transferring buffer, methanol
- SDS-PAGE Reagents and Apparatus, Western Transfer Reagents and Apparatus

IV. Technical Information for Kit

A. Key Reagents and their use

Quantity: 5 immunoblot tests per kit.

Immunogen: Synthetic Peptide derived from the C-terminus of the human Visfatin was used as an immunogen after conjugated to KLH.

Antibody Class: Rabbit IgG antibody was purified from rabbit immuned serum by ammonium sulphate precipitation followed by affinity chromatography.

Physical Form of Primary antibody: White lyophilized (freeze-dried) powder.

Dilution and Sensitivity: At working dilution of 1:20,000-40,000, peptide level can be detected up to 3.125ng/ml in ELISA. The kit allows visualization of Visfatin-contained proteins in less than one minute after exposure of the blot to X-ray film. In addition to speed, this kit is sensitive to the low nanomole range.

Storage and Stability: Stable for 6 months at 4°C from date of shipment. **Note:** Upon receipt, take out primary antibody, Peptide-conjugated carrier and Cell Lysates , and store them at -80°C for optimal performance. Store rest in box at 4°C for optimal performance.

Specificity and Species Cross-reactivity: RayBiotech ImmunoDetection Kit contains the most highly specific rabbit antibody available for the detection of the Pro-Visfatin and its posttranslational modification on a membrane. There is no cross-reactivity with the Obestatin, Ghrelin, but recognizes human, mouse and rat Visfatin.

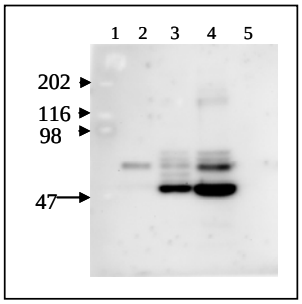
B. Quality Control Testing

The reagents in this kit have been matched to optimize the range and sensitivity of detection using human Hela and mouse MD231 cells as a source of Visfatin containing proteins.
Molecular weight of Visfatin protein: 52kDa

***Positive controls*:** They are MD231 and Hela cell lysates. They can be used to normalize the transferring, blotting and detection steps. It also refers to define visfatin in special assay. If the positive signals from detecting membranes are similar, positive control is a simple and effective way for normalization. **Note:** Other recommend positive cell lysates: HepG2, 3T3-L1 and LPS-treated human peripheral blood leukocyte.

Negative control: Negative control is BSA. Normally, it should only give a background reading.

Performance validated: This Western blot kit also includes our Mammalian Cell Lysis Reagent; an HRP-labeled anti-IgG antibody; blocking buffer; and wash buffer components all validated to perform as specified.



Immunodetection Analysis: Representative blot from a previous lot. Lane 1.protein marker; Lane 2.Reference control; Lane 3-4.Positive control, Hela and MD231 whole cell lysate; Lane 5.Negative control. The membrane blot was probed with anti-Visfatin primary antibody(1μg/ml). Proteins were visualized using a goat anti-rabbit secondary antibody conjugated to HRP and chemiluminescence detection system. Arrows indicate cellular Visfatin from human and mouse cells (52 kDa).

C. Additional Research Applications

This antibody is applied to other assays, Array, ELISA, Immunohistochemistry and Immunocytohistology. Please go corresponding product by checking their availability.

Related products:

1. Visfatin ELISA kit(Cat# ELH-Visfatin-001);
2. Visfatin Kit (IHC,Immunohistochemistry,Cat#RB-08-0003IHC);
3. Visfatin Kit (ICC, Immunocytochemistry, Cat# RB-08-0003ICC);
4. Obesity antibody arrays (Cat# AAH-OBS-1or AAH-OBS-G1).

V. Immunoblotting Analysis Protocol

The following procedure is intended to provide a set of initial conditions for analysis of cell lysates samples by Immunodetection Kit. Further optimization may be required for individual samples or analytes. Follow manufacturer's protocols for specific reagents when applicable.

A. Prior to Immunodetection

1. Prepare the samples for electrophoresis and immunoblotting

Preparation of Cell Lysates

- Collect cells in regular way and Centrifuge suspension at 1,500 rpm for 10 minutes at 4°C and aspirate supernatant.
- Re-suspend pellet in 15 ml PBS and centrifuge at 1,500 rpm for 10 minutes at 4°C and aspirate supernatant. Repeat 2 more times.
- For every 1 × 10^6 cells add approximately 500µl of ice-cold Lysis Buffer and re-suspend pellet, ensuring no clumps remain. Incubate on ice for 60 minutes. During incubation time, transfer contents of each tube to a microcentrifuge tube. Centrifuge at 13,000 rpm for 30 minutes at 4°C.
- Collect supernatant into an appropriately labeled tube and determine protein concentration using the BioRad modification of the Bradford assay.
- Re-suspend the cell lysate is in 2× SDS sample buffer by combining equal volumes of 2X SDS sample buffer and cell lysate. Heat at 95-100 °C for 3-5min.
- Use immediately or aliquot and store at -20°C or -80°C. If storing lysates, warm prior to loading on SDS-PAGE.

Preparation of Tissue Samples (Example for liver tissues from mouse)

- Whole Liver was removed from 10- to 13-d-old mouse and stored at –80°C.
- Liver tissue was slowly thawed and homogenized in ice-cold HSE buffer (10 mM HEPES, 350 mM sucrose, and 5 mM EDTA, pH 7.4) containing protease inhibitors.
- The homogenate is centrifuged at 2000 × *g* for 5 min, and the supernatant is removed and centrifuged at 100,000 × *g* for 1 hour.
- The resulting pellet is resuspended in ice-cold HSE buffer containing protease inhibitors.
- The measuring sample can be store at –80°C until use (For all tissue preparations, protein content is determined using the Bradford assay).
- Use immediately for sample loading or aliquot and store at -20°C or -80°C. If storing lysates, warm prior to loading on SDS-PAGE.

B. Protein Blotting and Membrane Blocking

1. Prepare samples and perform SDS-PAGE following instructions provided with your specific electrophoresis system.
2. Transfer the gel to a nitrocellulose membrane (Hybond) following all instructions provided with your specific blotting device and membrane. Note: The membrane must be pre-soaked with methanol for about 30 second prior to use. Perform Western transfer.
3. After transferring, the membrane twice with Wash Buffer to remove transfer buffer and gel particles.
4. Incubate the membrane in 10 ml of freshly prepared Blocking Buffer, with agitation, for 30-60 minutes at room temperature (or overnight at 4°C).

C. Visfatin antibody probe

1. Incubate the membrane blot in the "**Visfatin Primary Antibody Solution**" for one hour at room temperature (See Table 1 for recommending concentration, 1μg/ml).
2. Again, wash the membrane blot three times with washing buffer.
3. Incubate the membrane blot in the "**Secondary Antibody Solution**" for one hour at room temperature. (See Table 1 for recommending dilution, 1:5000)
4. Wash the membrane blot 3-5 times in washing buffer with constant rocking.

D. Immunodetection of Protein

1. Cover the membrane with a uniform layer of the **ECL Detection Reagent A and B mixture** (Combine 2ml ECL Detection Reagent A and 2ml ECL Detection Reagent B. Mix thoroughly. Incubate for precisely 1 minute at room temperature. Lay the membrane on a transparency sheet, Blot excess water from nitrocellulose membrane with paper towel being careful not to allow blot to dry out.
2. Drain and wick the excess ECL Detection Reagent mixture with a paper towel, making sure that the membrane does not dry out.

3. Cover the membrane with a second transparency sheet being careful to remove all bubbles on the membrane. **Note:** Expose as soon as possible, may be stored in the dark for up to 30 minutes.
4. Place the transparency covered the membrane e in a film cassette, cover entirely with a piece of film and close the cassette securely.
5. Start with a 40 second exposure, remove the film from the cassette and develop. Re-exposure for longer or shorter periods may be necessary depending on intensity of staining.
6. Develop the exposed film for 2 minutes in developer, visualize the reactive bands using X-Ray film or chemiluminescent imaging system.

Summary of Immunoblotting:

Prepare the using solutions

↓

Transfer the electrophoresed proteins to a membrane and Blocking

↓ 3×Washs

Incubate the membrane with primary antibody

↓ 5×Washs

Incubate the membrane with HRP-conjugated secondary antibody

↓ 5×Washs

Detection by ECL and develop image

VI. Troubleshooting Guide

The following tips address most problems encountered during Immuno-blotting and detection:

Table 2. Summary of Troubleshooting

Problem	Cause	Recommendation
No signal	1. Antigen is not recognized by primary antibody. 2. Proteins did not transfer properly to membrane 3. Target protein degradation occurred due to improper storage of blot 4. Substrate had lost activity	Including positive and reference controls in each analysis
Weak signal	1. Not enough protein loaded on the gel 2. The concentration of primary or secondary antibody used was too low 3. Inhibition of secondary antibody conjugate with sodium azide or hemoglobin, SDS, Nonidet P-40, and Triton X-100.	• All buffers must not contain any detergents. Use buffers that we provide in kit • Use recommended dilution of antibody or optimizing their condition based on your test
High background	1. Cross-reactivity between blocking agent and primary antibody 2. Concentration of either primary and/or secondary antibody too high or incubation time too long 3. Too much protein loaded on the gel 4. Insufficient blocking 5. Insufficient washing 6. The level of Tween-20 in blocking buffer was too low 7. Membrane problems: e.g., PVDF membrane was not	• the Washing Buffer containing 0.01% Tween-20 will eliminate the problem • the blocking buffer should used throughout whole membrane blocking and detecting process • the higher the antibody concentration and the longer the incubation time, the greater the non-specific binding. • many short washing steps are better than a few long ones. • Membrane drying during incubation process: care

	wetted thoroughly or dried in processing 8. Transfer buffer been contaminated	should be taken to keep membrane from drying out during incubation.
Smeared Pattern or Distorted Bands	1. Uneven contact between gel and membrane 2. For Tris-Glycine gels, gel not equilibrated in buffer prior to transfer	cassettes used should allow a tight fit, leading to even pressure over the entire surface of the gel and membrane. the gel should be soaked in transfer buffer containing methanol for 15 to 30 minutes before assembling the transfer sandwich.
Incomplete Transfer	1. Incomplete protein transfer 2. Proteins transferred through membrane 3. Inappropriate transfer buffer used 4. Impurities in the transfer buffer:	• this often occurs with high molecular weight proteins, especially when using a methanol-based transfer buffer. • Adding SDS to the transfer buffer and using higher field strengths also improve protein transfer. • this may occur when working with proteins of very low molecular weight. • Optimizing/shortening transfer times • using a double layer of membrane usually enhances retention of small proteins. • the most stable and commonly used buffers are Tris-Glycine based. • Adding SDS to the buffer is one way to make sure all proteins in the gel have a net negative charge and therefore migrate towards the positive electrode. • Fresh buffer should be prepared for each transfer.
		Bubbles create areas of low

Bald Spots	Bubbles between gel and membrane	transfer efficiency. Bubbles should be completely removed when putting together the transfer sandwich.

VII. Selected References

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Notes:

Notes:

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