

Rat Visfatin RIA KIT
125 TUBES (Cat. # RVFTN52HK)

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I. INTENDED USE

Visfatin is an approximately 50 kilodalton protein reported to have variable roles in energy regulation and fat metabolism. Also known as PBEF or Nampt, Visfatin is expressed in visceral adipose tissue, but can also be detected in serum or plasma. Originally reported to be an insulin-mimetic, Visfatin has since been identified as a key enzyme in NAD biosynthesis, and is an important biomarker for conditions associated with insulin resistance and obesity.

Millipore's Rat Visfatin Radioimmunoassay (RIA) Kit utilizes an antibody which recognizes the rat form of Visfatin. Assay sensitivity of less than 1 ng/mL can be achieved when using a 100 µl serum or plasma sample in a two-day, disequilibrium assay. ***This kit is for research purposes only.***

II. PRINCIPLES OF PROCEDURE

In radioimmunoassay, a fixed concentration of labeled tracer antigen is incubated with a constant dilution of antiserum such that the concentration of antigen binding sites on the antibody is limited, for example, only 40%-50% of the total tracer concentration may be bound by antibody. If unlabeled antigen is added to this system, there is competition between labeled tracer and unlabeled antigen for the limited and constant number of binding sites on the antibody. Thus, the amount of tracer bound to antibody will decrease as the concentration of unlabeled antigen increases. This can be measured after separating antibody-bound from free tracer and counting one or the other, or both fractions. A standard curve is set up with increasing concentrations of standard unlabeled antigen and from this curve the amount of antigen in unknown samples can be calculated. Thus, the four basic necessities for a radioimmunoassay system are: a specific antiserum to the antigen to be measured, the availability of a radioactive labeled form of the antigen, a method whereby antibody-bound tracer can be separated from the unbound tracer, and finally, an instrument to count radioactivity.

The Millipore Rat Visfatin assay utilizes ¹²⁵I-labeled Rat Visfatin and a Rat Visfatin antiserum to determine the level of Visfatin in serum, plasma or tissue culture media by the double antibody/PEG technique.

III. REAGENTS SUPPLIED

Each kit is sufficient to run 125 tubes and contains the following reagents.

A. Assay Buffer

Buffer containing BSA, Triton X-100 and 0.08% sodium azide

Quantity: 40 mL/vial, 1 bottle

Preparation: Ready to use

B. Rat Visfatin Antibody

Guinea Pig anti-Rat Visfatin Serum in Assay Buffer

Quantity: 13 mL/vial

Preparation: Ready to use

C. ¹²⁵I-Visfatin

¹²⁵I-Visfatin Label (<1.5 μCi, <56 kBq)

Lyophilized for stability. Freshly iodinated label contains <1.5 μCi, (56 kBq), calibrated to the 3rd Monday of each month.

Quantity: 13.5 mL/vial upon hydration

Preparation: Contents Lyophilized. Hydrate with 13.5 mL of Assay Buffer.

Allow to set at room temperature for 30 minutes, with occasional mixing.

D. Rat Visfatin Standard

Recombinant Rat Visfatin in Assay Buffer.

Lyophilized for stability

Quantity: 1 mL/vial upon hydration

Preparation: Contents Lyophilized. Hydrate with 1 mL distilled or deionized water. The actual concentration of Rat Visfatin present in the vial will be lot-dependent. Refer to the analysis sheet for the exact concentration in a specific lot.

E. Rat Visfatin Quality Controls 1 & 2

Recombinant Rat Visfatin in Assay Buffer.

Lyophilized for stability.

Quantity: 1 mL/vial upon hydration

Preparation: Contents Lyophilized. Hydrate with 1 mL distilled or deionized water.

F. Guinea Pig Carrier

Normal Guinea Pig Serum

Quantity: 2 mL/vial

Preparation: Ready to use

G. Precipitating Reagent

Goat anti-Guinea Pig IgG Serum, 3% PEG and 0.05% Triton X-100 in 0.05M Phosphosaline, 0.025M EDTA, 0.08% Sodium Azide

Quantity: 130 mL/vial

Preparation: Ready to use; chill to 4°C.

IV. STORAGE AND STABILITY

Refrigerate all reagents between 2 and 8 °C for short-term storage. For prolonged storage (>2 weeks), freeze at ≤ -20 °C. Avoid multiple (>5) freeze/thaw cycles. Refer to date on bottle for expiration when stored at ≤ -20 °C. Do not mix reagents from different kits unless they have the same lot number. Store remaining hydrated Standard, Quality Controls and Tracer at -20 °C.

V. REAGENT PRECAUTIONS

A. Radioactive Materials

This radioactive material may be received, acquired, possessed and used only by research personnel or clinical laboratories for in vitro research tests not involving internal or external administration of the material, or the radiation there from to human beings or animals. Its receipt, acquisition, possession, use and transfer are subject to the regulations of the U. S. Nuclear Regulatory Commission (NRC) or of a State with which the Commission has entered into an agreement for the exercise of regulatory authority.

The following are suggested general rules for the safe use of radioactive material. The customer's Radiation Safety Officer is ultimately responsible for the safe handling and use of radioactive material.

1. Wear appropriate personal devices at all times while in areas where radioactive materials are used or stored.
2. Wear laboratory coats, disposable gloves, and other protective clothing at all times.
3. Monitor hands, shoes, and clothing and immediate area surrounding the workstation for contamination after each procedure and before leaving the area.
4. Do not eat, drink, or smoke in any area where radioactive materials are stored or used.
5. Never pipette radioactive material by mouth.
6. Dispose of radioactive waste in accordance with NRC rules and regulations.
7. Avoid contaminating objects such as telephones, light switches, doorknobs, etc.
8. Use absorbent pads for containing and easily disposing of small amounts of contamination.
9. Wipe up all spills immediately and thoroughly and dispose of the contaminated materials as radioactive waste. Inform Radiation Safety Officer.

V. REAGENT PRECAUTIONS (continued)

B. Sodium Azide

Sodium Azide has been added to all reagents as a preservative at a concentration of 0.08%. Although it is at a minimum concentration, Sodium Azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

1. Plastic polystyrene tubes, 12 x 75 mm.
2. 20 μ L and 100 μ L pipettes with disposable tips
3. 10 μ L, 100 μ L & 1.0 mL repeating dispenser
4. Refrigerated swing bucket centrifuge capable of developing 2,000 – 3,000xg. (Use of fixed-angle buckets is not recommended.)
5. Absorbent paper
6. Vortex mixer
7. Refrigerator
8. Gamma Counter

VII. SPECIMEN COLLECTION AND STORAGE

1. A minimum of 100 μ L per assay tube of serum or plasma should be used. Tissue culture and other media may also be used but the volume required for the assay will vary on incubation conditions, cell type, and cell concentration.
2. Care must be taken when using heparin as an anticoagulant, since excess will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.
3. For longer storage, specimens should be aliquot and stored at $\leq -20^{\circ}\text{C}$ or below. Multiple freeze/thaw cycles should be avoided.
4. Avoid using samples with gross hemolysis or lipemia.

VIII. ASSAY PROCEDURE

For optimal results, accurate pipetting and adherence to the protocol are recommended.

A. Rat Visfatin Standard Preparation

Use care in opening the lyophilized Standard vial. Using a pipette reconstitute the Rat Visfatin Standard with 1 mL distilled or deionized water to give the concentration described on the analysis sheet. Swirl gently and let stand for five minutes or until completely dissolved then mix well.

Label six tubes 1, 2, 3, 4, 5, and 6. Add 0.5 mL Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 0.5 mL of the reconstituted standard to tube 1, mix well and transfer 0.5 mL of tube 1 to tube 2, mix well and transfer 0.5 mL of tube 2 to tube 3, mix well and transfer 0.5 mL of tube 3 to tube 4, mix well and transfer 0.5 mL of tube 4 to tube 5, mix well and transfer 0.5 mL of tube 5 to tube 6.

Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with Standard before dispensing. Unused portions of standard should be stored at $\leq -20^{\circ}\text{C}$. Avoid multiple freeze/thaw cycles.

Standard Concentration ng/mL	Volume	Volume of Standard to Add
X (Refer to analysis sheet for exact concentration)	1.0 mL	

Tube #	Standard Concentration ng/mL	Volume of Assay Buffer to Add	Volume of Standard to Add
1	X/2	0.5 mL	0.5 mL of Original Standard
2	X/4	0.5 mL	0.5 mL of Tube 1
3	X/8	0.5 mL	0.5 mL of Tube 2
4	X/16	0.5 mL	0.5 mL of Tube 3
5	X/32	0.5 mL	0.5 mL of Tube 4
6	X/64	0.5 mL	0.5 mL of Tube 5

VIII. ASSAY PROCEDURE (continued)

B. Rat Visfatin Quality Control 1 and 2 Preparation

Reconstitute Rat Visfatin Quality Control 1 and Quality Control 2 with 1 mL distilled or deionized water. Swirl gently and let stand for five minutes or until completely dissolved then mix well.

Note: For Quality Control 1 and 2 ranges, refer to Analysis Sheet. Unused portions of Quality Controls should be stored at $\leq -20^{\circ}\text{C}$. Avoid multiple freeze/thaw cycles.

The assay should be run using plastic polystyrene tubes. Use of glass tubes will result in high nonspecific binding of tracer.

Day One

1. Pipette 300 μL of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4). Pipette 200 μL of Assay Buffer in the Reference (B_0) tubes (5-6). Pipette 100 μL of Assay Buffer to the Standard tubes (7-20) and Control tubes (21-24). Pipette 100 μL of Assay Buffer in sample tubes 25 through the end of the assay.
2. Pipette 100 μL of each Standard (tubes 7-20) and Quality Controls (tubes 21-24).
3. Pipette 100 μL of each sample in duplicate.
4. Pipette 100 μL of Rat Visfatin Antibody to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).
5. Vortex, cover, and incubate overnight (20-24 hours) at 4°C .

Day Two

6. Hydrate the ^{125}I -Visfatin tracer with 13.5 mL of Assay Buffer and gently mix. Pipette 100 μL of ^{125}I -Visfatin to all tubes.
7. Vortex, cover and incubate overnight (22-24 hours) at 4°C .

VIII. ASSAY PROCEDURE (continued)

Day Three

8. Add 10 μ L of Guinea Pig Carrier to all tubes except Total Counts tubes (1-2).
9. Add 1.0 mL of cold (4 °C) Precipitating Reagent to all tubes except Total Count tubes (1-2).
10. Vortex and incubate 20 minutes at 4 °C.
11. Centrifuge, at 4 °C, for 20 minutes at 2,000-3,000xg. Note: If less than 2,000xg is used, the time of centrifugation must be increased to obtain a firm pellet (e.g. 40 minutes). Multiple centrifuge runs within an assay must be consistent.
Conversion of rpm to xg:
$$\text{xg} = (1.12 \times 10^{-5}) \text{ @ } (\text{rpm})^2$$
$$r = \text{radial distance in cm (from axis of rotation to the bottom of the tube)}$$
$$\text{rpm} = \text{revolutions per minute}$$
12. Immediately decant supernatant from all centrifuged tubes except Total Count tubes (1-2). Drain tubes for 10-20 seconds (be consistent between racks), blot excess liquid from lip of tubes and count pellet using the gamma counter according to the manufacturer's instructions.

Note: Avoid shaking or tapping tubes excessively as pellets may dislodge.

Assay Procedure Flow Chart

Day 1					Day 2		Day 3		
Set-up	Step 1	Step 2-3	Step 4	Step 5	Step 6	Step 7	Step 8	Step 9	Steps 10-12
Tube Number	Add Assay Buffer	Add Standard/QC Sample	Add Rat Visfatin Antibody	Vortex, Cover, and Incubate 20-24 hrs at 4 °C	Add ¹²⁵ I-Visfatin Tracer	Vortex, Cover and Incubate 22-24 hrs at 4 °C	Add Guinea Pig Carrier	Add Precipitating Reagent	Incubate 20 min. at 4 °C, Centrifuge at 4 °C for 20 min Decant and Count
1,2	--	--	--		100 µL		--	--	
3,4	300 µL	--	--		100 µL		10 µL	1.0 mL	
5,6	200 µL	--	100 µL		100 µL		10 µL	1.0 mL	
7,8	100 µL	100 µL of tube 6	100 µL		100 µL		10 µL	1.0 mL	
9,10	100 µL	100 µL of tube 5	100 µL		100 µL		10 µL	1.0 mL	
11,12	100 µL	100 µL of tube 4	100 µL		100 µL		10 µL	1.0 mL	
13,14	100 µL	100 µL of tube 3	100 µL		100 µL		10 µL	1.0 mL	
15,16	100 µL	100 µL of tube 2	100 µL		100 µL		10 µL	1.0 mL	
17,18	100 µL	100 µL of tube 1	100 µL		100 µL		10 µL	1.0 mL	
19,20	100 µL	100 µL of Original Standard	100 µL		100 µL		10 µL	1.0 mL	
21,22	100 µL	100 µL of QC 1	100 µL		100 µL		10 µL	1.0 mL	
23,24	100 µL	100 µL of QC 2	100 µL		100 µL		10 µL	1.0 mL	
25,n	100 µL	100 µL of unknown	100 µL	100 µL	10 µL	1.0 mL			

IX. CALCULATIONS

A. Explanation

The calculations for Rat Visfatin can be automatically performed by most gamma counters possessing data reduction capabilities or by independent treatment of the raw data using a commercially available software package. Choose weighted 4-parameter or weighted log/logit for the mathematical treatment of the data. [NOTE: Be certain the procedure used subtracts the NSB counts from each average count, except Total Counts, prior to final data reduction.]

B. Manual Calculation

1. Average duplicate counts for Total Count tubes (1-2), NSB tubes (3-4), Total Binding tubes (reference, B_0), (5-6), and all duplicate tubes for standards and samples to the end of the assay.
2. Subtract the average NSB counts from each average count (except for Total Counts). These counts are used in the following calculations.
3. Calculate the percentage of tracer bound.
 $(\text{Total Binding Counts} / \text{Total Counts}) \times 100$
This should be 25-45%.
4. Calculate the percentage of total binding (%B/ B_0) for each standard and sample
 $\%B/B_0 = (\text{Sample or Standard} / \text{Total Binding}) \times 100$
5. Plot the % B/ B_0 for each standard on the y-axis and the known concentration of the standard on the x-axis using log-log graph paper.
6. Construct the reference curve by joining the points with a smooth curve.
7. Determine the ng/mL of Rat Visfatin in the unknown samples and controls by interpolation of the reference curve.

X. INTERPRETATION

A. Acceptance Criteria

1. The run will be considered accepted when all Quality Control values fall within the calculated Quality Control Range; if any QC's fall outside the control range, review results with the supervisor.
2. If the difference between duplicate results of a sample is >10% CV, repeat the sample.

XI. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Rat Visfatin that can be measured by this assay is 0.78 ng/mL when using a 100µl sample size.

B. Performance

The following parameters of assay performance are expressed as Mean \pm Standard Deviation from 6 assays run on different days.

$$ED_{80} = 2.7 \pm 0.24 \text{ ng/mL}$$

$$ED_{50} = 8.2 \pm 0.71 \text{ ng/mL}$$

$$ED_{20} = 26.5 \pm 2.28 \text{ ng/mL}$$

C. Specificity

The specificity (also known as selectivity) of an analytical test is its ability to selectively measure the analyte in the presence of other like components in the sample matrix.

Cross reactivity of different analytes in Rat Visfatin RIA

Analyte	% Cross-Reactivity
Rat Insulin (200 µU/mL)	ND
Glucagon (400 pg/mL)	ND
Rat Leptin (100 ng/mL)	ND
Rat C-Peptide (2.5 ng/mL)	ND
Mouse Visfatin	6%
Human Visfatin	0.1%

ND - Not detectable up to the concentration shown in parenthesis.

XI. ASSAY CHARACTERISTICS (continued)

D. Precision

Intra- and Inter-Assay Variation:

Sample no.	Mean ng/mL	Intra-Assay %CV	Inter-Assay %CV
1	4.26	4.6	10.5
2	17.83	2.8	9.0

Intra- and inter-assay variations were performed on two samples containing low and high concentrations of Rat Visfatin. Data (mean and %CV) shown are from one assay with ten duplicate determinations of each sample for intra-assay precision. For inter-assay precision, data are generated using six separate assays run for the two high and low samples in duplicate.

E. Spike and Recovery

Rat Visfatin ng/mL	% Expected
3.13	72.8 ± 1.7
12.5	71.4 ± 2.9
25	72.8 ± 6.9

Six different rat serum samples were spiked with different amounts of exogenous Rat Visfatin. These spiked samples were assayed by Rat Visfatin RIA. Expected values are the basal levels plus the spiked amount (3.13, 12.5, 25 ng/mL) of Rat Visfatin. The % Expected is observed value divided by expected value X 100 (Mean ± SD).

XI. ASSAY CHARACTERISTICS (continued)

F. Linearity and Dilution

Sample No.	Spiked Rat Serum % Expected
25µl	115.3 ± 11.4
50µl	102.4 ± 11.1
100µl	100.0 ± 0.0

Ten different rat serum samples spiked with exogenous Rat Visfatin were assayed by Rat Visfatin RIA at 25, 50 and 100 µl after adding the remainder of 100 µl sample volume with assay buffer. % Expected values (mean ± SD) are 1/4, 1/2 and 1/1 of the 100 µl sample value.

XII. QUALITY CONTROLS

Good laboratory practice requires that quality control specimens be run with each standard curve to check the assay performance. Two levels of controls are provided for this purpose. These and any other control materials should be assayed repeatedly to establish mean values and acceptable ranges. Each individual laboratory is responsible for defining their system for quality control decisions and is also responsible for making this system a written part of their laboratory manual. The ranges for Quality Control 1 and 2 are provided on the card insert or can be located at the Millipore website www.millipore.com.

Recommended batch analysis decision using two controls (Westgard Rules⁴):

1. When both controls are within ± 2 SD.
Decision: Approve batch and release analyte results.
2. When one control is outside ± 2 SD and the second control is within ± 2 SD.
Decision: Hold results, check with supervisor. If no obvious source of error is identified by the below mentioned check of systems, the supervisor may decide to release the results.

Technician check of systems:

1. Check for calculation errors
2. Repeat standards and controls
3. Check reagent solutions
4. Check instrument

XIII. REFERENCES

1. Stephens JM, Vidal-Puig AJ. An update on visfatin/pre-B cell colony-enhancing factor, an ubiquitously expressed, illusive cytokine that is regulated in obesity. *Curr Opin Lipidol.* 2006 Apr;17(2):128-31.
2. Revollo JR, et al. Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metab.* 2007 Nov; 6(5):363-75.

XIV. REPLACEMENT REAGENTS

Reagent	Cat #
¹²⁵ I-Visfatin (<1.5 μCi, 56 kBq)	9052HK
Guinea Pig Carrier	GPC-HK
Rat Visfatin Standard	8052K
Rat Visfatin Antibody (13 mL)	1052HK
Precipitating Reagent (130 mL)	PR-UVHK
Rat Visfatin Quality Control 1&2 (1 mL each)	6052K
Assay Buffer (40 mL)	AB-PTR

XV. ORDERING INFORMATION

A. To place an order:

For USA Customers:

Please provide the following information to our customer service department to expedite your telephone, fax or mail order:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity and product size

NOTE: Appropriate license from NRC (or equivalent) must be on file at Millipore before radioactive orders can be shipped.

TELEPHONE ORDERS:

Toll Free US (866) 441-8400
(636) 441-8400

FAX ORDERS: (636) 441-8050

MAIL ORDERS: Millipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:

To best serve our international customers, it is Millipore's policy to sell our products through a network of distributors. To place an order or to obtain additional information about Millipore products, please contact your local distributor.

B. Conditions of Sale

All products are for research or manufacturing use only. They are not intended for use in clinical diagnosis or for administration to humans or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)

Material safety data sheets for Millipore products may be ordered by fax or phone. See Section A above for details on ordering.