HUMAN LEPTIN RIA KIT PROTOCOL
125 TUBES (Cat. # HL-81HK)

Millipore • 6 Research Park Drive • St. Charles, Missouri 63304 USA
Phone: 1-800-MILLIPORE • Fax: 636-441-8050
Techserve_dd@millipore.com • www. millipore.com

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I. INTENDED USE

Leptin is a signaling factor encoded by the obese gene in adipose tissue. Administration of recombinant leptin decreases food intake, increases energy expenditures and promotes weight loss.[1,2] This Human Leptin radioimmunoassay has been developed to quantitate Human Leptin in plasma, serum and tissue culture media.[3] It is a completely homologous assay since the antibody was raised against highly purified Human Leptin and both the standard and tracer are prepared with Human Leptin. ***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 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 calibration or 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 Human Leptin assay utilizes ^{125}I-labeled Human Leptin and a Human Leptin antiserum to determine the level of Leptin 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
0.05M Phosphosaline pH 7.4 containing 0.025M EDTA, 0.08% Sodium Azide, 1% RIA Grade BSA and 0.05% Triton X-100
Quantity: 20 mL/vial
Preparation: Ready to use

B. Human Leptin Antibody
Rabbit anti-Human Leptin Serum in Assay Buffer
Quantity: 13 mL/vial
Preparation: Ready to use

C. ^{125}I-Human Leptin
^{125}I-Human Leptin Label, HPLC purified (specific activity 135 µCi/µg)
Lyophilized for stability. Freshly iodinated label contains <1.5 µCi (<56 kBq) calibrated to the 1st Monday of each month.
Quantity: 13.5 mL/vial upon hydration
Preparation: Contents Lyophilized. Hydrate with entire contents of Label Hydrating Buffer. Allow to sit at room temperature for 30 minutes, with occasional gentle mixing.

III. REAGENTS SUPPLIED (continued)

D. Label Hydrating Buffer
Assay Buffer containing Normal Rabbit IgG as a carrier. Used to hydrate ^{125}I-Human Leptin
Quantity: 13.5 mL/vial
Preparation: Ready to use

E. Human Leptin Standards
Purified Recombinant Human Leptin in Assay Buffer at the following concentrations:
0.5, 1, 2, 5, 10, 20, 50, 100 ng/mL
Quantity: 1 mL/vial
Preparation: Ready to use

F. Quality Controls 1 & 2
Purified Recombinant Human Leptin in Assay Buffer
Quantity: 1 mL/vial
Preparation: Ready to use

G. Precipitating Reagent
Goat anti-Rabbit 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 upon receipt 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.

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 therefrom, 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.

V. REAGENT PRECAUTIONS (continued)

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.

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. Borosilicate glass tubes, 12 x 75 mm. (NOTE: Polypropylene or polystyrene tubes may be used if the investigator finds that the pellet formation is acceptably stable in their system.)
2. 100 µL pipet with disposable tips
3. 100 µL & 1.0 mL repeating dispenser
4. Refrigerated swing bucket centrifuge capable of developing 2,000 - 3,000 xg. (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 maximum of 100 µL per assay tube of serum or plasma can be used, although, 50 µl per assay tube is adequate for most applications. Tissue culture and other media may also be used.

2. Care must be taken when using heparin as an anticoagulant, since an excess will provide falsely high values.[4] Use no more than 10 IU heparin per mL of blood collected.

3. Specimens can be stored at 4°C if they will be tested within 24 hours of collection. For longer storage, specimens should be stored at ≤ -20°C. Avoid multiple (>5) freeze/thaw cycles.

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. Assay Set-Up, Day One

1. Pipet 300 µL of Assay Buffer to the Non-Specific Binding (NSB) tubes (3-4), 200 µL to Reference (Bo) tubes (5-6), and 100 µL to tubes 7 through the end of the assay.
2. Pipet 100 µL of Standards and Quality Controls in duplicate (see flow chart).
3. Pipet 100 µL of each sample in duplicate. (NOTE: Smaller volumes of sample may be used when Leptin concentrations are anticipated to be elevated or when sample size is limited. Additional Assay Buffer should be added to compensate for the difference so that the volume is equivalent to 100 µL, e.g., when using 50 µL of sample, add 50 µL of Assay Buffer). Refer to Section IX for calculation modification.
4. Pipet 100 µL of ^{125}I-Human Leptin to all tubes. Important: For preparation, see Section III, Part C.
5. Pipet 100 µL of Human Leptin antibody to all tubes except Total Count tubes (1-2) and NSB tubes (3-4).
6. Vortex, cover and incubate overnight (20-24 hours) at 4°C.

B. Day Two

7. Add 1.0 mL of cold (4°C) Precipitating Reagent to all tubes (except Total Count tubes).
8. Vortex and incubate 20 minutes at 4°C.
9. Centrifuge at 4°C all tubes [except Total Count tubes (1-2)] for 20 minutes at 2,000-3,000 xg. NOTE: If less than 2,000 xg is used or if slipped pellets have been observed in previous runs, 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:

    $xg = (1.12 \times 10^{-5})\ (r)\ (rpm)^2$

    r = radial distance in cm (from axis of rotation to the bottom of the tube)

    rpm = rotational velocity of the rotor
10. Immediately decant the supernate of all tubes except Total Count tubes (1-2), drain tubes for at least 15-60 seconds (be consistent between racks), and blot excess liquid from lip of tubes. NOTE: Invert tubes only one time. Pellets are fragile and slipping may occur.
11. Count all tubes in a gamma counter for 1 minute. Calculate the ng/mL of Human Leptin in unknown samples using automated data reduction procedures.

Assay Flow Chart

	Day One					Day Two		
	Step 1	Step 2-3	Step 4	Step 5	Step 6	Step 7	Step 8	Step 9-11
Tube #	Add Assay Buffer	Add Standard / QC Sample	Add ^{125}I-Leptin Tracer	Add Leptin Antibody	Vortex, Cover, and Incubate 20-24 hrs at 4°C	Add Precipitating Reagent	Vortex, and Incubate 20 min. at 4°C	Centrifuge at 4°C for 20 min., Decant, and Count pellets
1,2	----	----	100 µl	----		----		
3,4	300 µl	----	100 µl	----		1.0 mL		
5,6	200 µl	----	100 µl	100 µl		1.0 mL		
7,8	100 µl	100 µl of 0.5 ng/mL	100 µl	100 µl		1.0 mL		
9,10	100 µl	100 µl of 1 ng/mL	100 µl	100 µl		1.0 mL		
11,12	100 µl	100 µl of 2 ng/mL	100 µl	100 µl		1.0 mL		
13,14	100 µl	100 µl of 5 ng/mL	100 µl	100 µl		1.0 mL		
15,16	100 µl	100 µl of 10 ng/mL	100 µl	100 µl		1.0 mL		
17,18	100 µl	100 µl of 20 ng/mL	100 µl	100 µl		1.0 mL		
19,20	100 µl	100 µl of 50 ng/mL	100 µl	100 µl		1.0 mL		
21,22	100 µl	100 µl of 100 ng/mL	100 µl	100 µl		1.0 mL		
23,24	100 µl	100 µl of QC 1	100 µl	100 µl		1.0 mL		
25,26	100 µl	100 µl of QC 2	100 µl	100 µl		1.0 mL		
27,28	100 µl	100 µl of unknown	100 µl	100 µl		1.0 mL		
29-n	100 µl	100 µl of unknown	100 µl	100 µl		1.0 mL		

IX. CALCULATIONS

A. Explanation

The calculations for Human Leptin 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.[5] 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, Bo) (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
   (Total Binding Counts/Total Counts) X 100
   This should be 35-50%.

4. Calculate the percentage of total binding (%B/Bo) for each standard and sample:
   %B/Bo = (Sample or Standard/Total Binding) X 100

5. Plot the % B/Bo 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 Human Leptin in the unknown samples and controls by interpolation of the reference curve.

   [NOTE: When sample volumes assayed differ from 100 µl, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (e.g. if 50 µL of sample is used, then calculated data must be multiplied by 2).]

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.

3. The limit of sensitivity for the Human Leptin assay is 0.5 ng/mL (100 µL sample size).

4. The limit of linearity for the Human Leptin assay is 100 ng/mL (100 µL sample size). Any result greater than 100 ng/mL should be repeated on dilution using Assay Buffer as a diluent.

XI. NORMAL FASTING RANGE[3]

Leptin levels are directly correlated with degree of adiposity.
Mean Leptin Values (BMI ranges 18-25):
- Lean Men 3.8 ± 1.8 µg/L
- Lean Women 7.4 ± 3.7 µg/L

Levels rise approximately 2.5 times faster in women per unit BMI as compared to men.

XII. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of Leptin that can be detected by this assay is 0.5 ng/mL when using a 100 µL sample size.

B. Performance

The following parameters of assay performance are expressed as Mean ± Standard Deviation.

ED_{80} = 1.5 ng/mL
ED_{50} = 7.1 ng/mL
ED_{20} = 36.4 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.

Human Leptin	100%
Rat Leptin	<0.2%
Mouse Leptin	<0.2%
Human Insulin	*
Human Proinsulin	*
Rat Insulin	*
Human C-Peptide	*
Glucagon	*
IGF-1	*

*not detectable

D. Precision

Within and Between Assay Variation

Sample No.	Mean ng/mL	Within % CV	Between % CV
1	4.9	8.3	6.2
2	7.2	4.6	5.0
3	10.4	3.9	4.7
4	15.7	4.7	3.0
5	25.6	3.4	3.6

Within and between assay variation was performed on five human serum samples containing varying concentrations of Human Leptin. Data (mean and % CV) shown are from five duplicate determinations of each serum sample in five separate assays.

XII. ASSAY CHARACTERISTICS (continued)

E. Recovery

Spike & Recovery of Leptin in Human Serum

Sample No.	Leptin Added ng/mL	Observed ng/mL	Expected ng/mL	% Recovery
1	0	4.9	-	-
2	2	7.2	6.9	104
3	5	10.4	9.9	105
4	10	15.7	14.9	105
5	20	25.6	24.9	103

Varying concentrations of Human Leptin were added to five human serum samples and the Leptin content was determined by RIA. Mean of the observed levels from five duplicate determinations in five separate assays are shown. Percent recovery was calculated on the observed vs. expected.

F. Linearity

Effect of Serum Dilution

Sample No.	Volume Sampled	Observed ng/mL	Expected ng/mL	% Of Expected
1	100 µl	45.7	45.7	100
	75 µl	45.3		99
	50 µl	45.6		100
	25 µl	46.1		101
2	100 µl	31.2	31.2	100
	75 µl	31.2		100
	50 µl	31.3		100
	25 µl	31.0		99
3	100 µl	13.8	13.8	100
	75 µl	13.1		95
	50 µl	12.5		91
	25 µl	12.1		88
4	100 µl	9.1	9.1	100
	75 µl	8.6		95
	50 µl	8.7		96
	25 µl	8.4		92

Aliquots of pooled human serum containing varying concentrations of Leptin were analyzed in the volumes indicated. Dilution factors of 1, 1.33, 2 and 4 representing 100 µL, 75 µL, 50 µL and 25 µL, respectively, were applied in calculating observed concentrations. Mean Leptin levels and percent of expected for five separate assays are shown.

XII. ASSAY CHARACTERISTICS (continued)

G. Example of Assay Results

This data is presented as an example only and should not be used in lieu of a standard curve prepared with each assay.

Tube #	ID	CPM	Ave CPM	Ave Net CPM	% B/Bo	ng/mL
1	Totals	17236				
2	″	16675	16956			
3	NSB	445				
4	″	487	466			
5	Bo	7777				
6	″	7716	7747	7281		
Standards						
7	0.5 ng/mL	7087				
8		7109	7098	6632	91.1	
9	1 ng/mL	6678				
10		6450	6564	6098	83.8	
11	2 ng/mL	5817				
12		5894	5856	5390	74.0	
13	5 ng/mL	4494				
14		4371	4433	3967	54.5	
15	10 ng/mL	3339				
16		3290	3315	2849	39.1	
17	20 ng/mL	2501				
18		2381	2441	1975	27.1	
19	50 ng/mL	1543				
20		1610	1577	1111	15.3	
21	100 ng/mL	1027				
22		1039	1033	567	7.8	
Controls/Unknown						
23	QC 1	5733				
24		5576	5655	5189	71.3	2.2
25	QC 2	2962				
26		2936	2949	2483	34.1	13.5
27-n	Unknown					

XIII. QUALITY CONTROLS

Good Laboratory Practice (GLP) requires that Quality Control (QC) 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/bmia.

Recommended batch analysis decision using two controls (Westgard Rules):[6]

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

XIV. REPLACEMENT REAGENTS

Reagents	Cat #
^{125}I-Human Leptin (<1.5 µCi, <56 kBq)	9081-HK
Label Hydrating Buffer (13.5 mL)	LHB-81HK
Human Leptin Standards (1 mL each)	8081-K
Human Leptin Antibody (13 mL)	1081-HK
Precipitating Reagent (130 mL)	PR-81HK
Quality Control 1 & 2 (1 mL each)	6081-K
Assay Buffer (20 mL)	AB-PTRHK

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 1-800-MILLIPORE

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.

XVI. REFERENCES

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