MULTI-SPECIES LEPTIN RIA KIT

250 TUBES (Cat. # XL-85K)

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I. INTENDED USE

Millipore's Multi-Species Leptin Radioimmunoassay (RIA) Kit has been developed to quantitate leptin in plasma or serum from many animals. The antibody used in this kit was raised against Human Leptin but displays broad crossreactivity to leptin molecules of many, but not all, species. It is recommended that investigators determine the suitability of this assay for the analysis of leptin in the species of interest. ***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 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 Multi-Species Leptin assay utilizes ^{125}I-labeled Human Leptin and a Multi-Species 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 250 tubes and contains the following reagents.

A. Assay Buffer

0.05M Phosphosaline, pH 7.4, containing 0.025M EDTA, 0.08% Sodium Azide, 0.05% Triton X-100, and 1% RIA Grade BSA
Quantity: 40 mL/vial
Preparation: Ready to use

B. Antiserum

Guinea Pig anti-Multi-Species Leptin Antibody in Assay Buffer
Quantity: 26 mL/vial
Preparation: Ready to use

C. ^{125}I-Human Leptin

^{125}I-Human Leptin Label, HPLC purified (specific activity 135 □Ci/□g).
Lyophilized for stability. Freshly iodinated label contains <3 µCi, (<111 kBq) calibrated to the 1st Monday of each month.
Quantity: 27 mL/vial upon hydration
Preparation: Contents Lyophilized. Hydrate with entire contents of Label Hydrating Buffer. Allow to set at room temperature for 30 minutes, with occasional gentle mixing.

III. REAGENTS SUPPLIED (continued)

D. Label Hydrating Buffer

Assay Buffer containing Normal Guinea Pig IgG as a carrier. Used to hydrate ^{125}I-Human Leptin
Quantity: 27 mL/vial
Preparation: Ready to use

E. Standards

Purified Recombinant Human Leptin in Assay Buffer at the following concentrations:
1, 2, 5, 10, 20, 50 ng/mL.
Since the Multi Species Leptin antibody was raised against Human Leptin, Human Leptin standards are used in this assay. The crossreactivity of this antibody to leptin molecules of many other species is unknown. Therefore, it is recommended that investigators use ng/mL Human Equivalent (ng/mL HE) as the unit of measure.
Quantity: 1mL/vial
Preparation: Ready to use

F. Quality Controls 1 & 2

Purified Recombinant Human Leptin in Assay Buffer
Quantity: 1 mL each
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: 260 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.

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.

V. REAGENT PRECAUTIONS (continued)

3. Monitor hands, shoes, and clothing and immediate area surrounding the work station 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.

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 are 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 the Reference (B_O) 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 Assay Procedure 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 must be added to compensate for the difference so the volume is equivalent to 100 µl, e.g., when using 50 µL sample, add 50 µL Assay Buffer.)
4. Pipet 100 µL of Multi Species Leptin 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.

B. Day Two

6. Pipet 100 µL of ^{125}I-Human Leptin to all tubes.
7. Vortex, cover, and incubate overnight (20-24 hours) at 4°C.

C. Day Three

8. Add 1.0 mL of cold (4°C) Precipitating Reagent to all tubes except Total Count tubes (1-2).
9. Vortex and incubate 20 minutes at 4°C.
10. Centrifuge, 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 firmer, more stable pellet (e.g., 40 minutes). A swing-bucket rotor is recommended. Conversion of rpm to xg:

    $xg = (1.11 \times 10^{-5})\ (r)\ (rpm)^2$

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

    rpm = rotational velocity of the rotor

11. Immediately decant the supernate from all tubes except Total Count tubes (1-2), drain tubes for 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.
12. Count all tubes in a gamma counter for 1 minute. Calculate the ng/mL HE of leptin in unknown samples using automated data reduction procedures (see the following "Calculations" section).

Assay Procedure Flow Chart

Day One					Day Two		Day Three	
Set-up	Step 1	Step 2&3	Step 4	Step 5	Step 6	Step 7	Step 8	Steps 9-12
Tube Number	Add Assay Buffer	Add Standard/QC Sample	Add Multi-Species Leptin Antibody	Vortex, Cover, and Incubate 20-24 hrs at 4°C	Add I-125 Human Leptin Tracer	Vortex, Cover and Incubate 20-24 hrs at 4°C	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		1.0 mL	
5,6	200 µl	-	100 µl		100 µl		1.0 mL	
7,8	100 µl	100 µl of 1 ng/mL HE*	100 µl		100 µl		1.0 mL	
9,10	100 µl	100 µl of 2 ng/mL HE	100 µl		100 µl		1.0 mL	
11,12	100 µl	100 µl of 5 ng/mL HE	100 µl		100 µl		1.0 mL	
13,14	100 µl	100 µl of 10 ng/mL HE	100 µl		100 µl		1.0 mL	
15,16	100 µl	100 µl of 20 ng/mL HE	100 µl		100 µl		1.0 mL	
17,18	100 µl	100 µl of 50 ng/mL HE	100 µl		100 µl		1.0 mL	
19,20	100 µl	100 µl of QC1	100 µl		100 µl		1.0 mL	
21,22	100 µl	100 µl of QC2	100 µl		100 µl		1.0 mL	
23-n	100 µl	100 µl of unknown	100 µl		100 µl		1.0 mL	

***HE = Human Equivalent**

IX. CALCULATIONS

Automated

The calculations for 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. 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.

Millipore recommends the use of ng/mL HE as the unit of measure.

Manual

1. Average duplicate counts for Total Counts tubes (1-2), NSB tubes (3-4), Maximum Binding tubes (Reference, B_0) (5-6), and all remaining tubes.

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
   (Maximum Binding Counts/Total Counts) X 100
   This should be 35-50%.

4. Calculate the percentage of maximum binding (%B/B_0) for each standard and sample
   %B/B_0 = (Sample or Standard/Maximum Binding) X 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 HE of leptin in 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

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 Multi Species Leptin assay is 1.0 ng/mL HE (100 µL sample size).

4. The limit of linearity for the Multi Species Leptin assay is 50 ng/mL HE (100 µL sample size). Any result greater than 50 ng/mL HE should be repeated on dilution using Assay Buffer as a diluent.

XI. NORMAL FASTING RANGE

Normal ranges must be established in each laboratory for the species being studied.

XII. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of leptin that can be detected by this assay is 1.0 ng/mL (Human Equivalent) when using a 100 µL sample size.

B. Performance

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

ED_{80} = 3.0 ± 0.28 ng/mL HE*
ED_{50} = 8.1 ± 0.60 ng/mL HE
ED_{20} = 24.4 ± 2.00 ng/mL HE

* HE = Human Equivalent

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 %
Porcine Leptin	67 %
Rat Leptin	61 %
Mouse Leptin	73 %
Canine Leptin	3 %
Human Insulin	*
Human Proinsulin	*
Human C-Peptide	*
Rat Insulin	*
Glucagon	*
IGF-1	*

* Not Detectable

D. Precision

Within and Between Assay Variation

Sample No.	Mean ng/mL HE*	Within % CV	Between % CV
1	4	3.4	8.7
2	7	3.1	7.8
3	10	2.8	8.0
4	32	3.6	6.5

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

XII. ASSAY CHARACTERISTICS (continued)

E. Recovery

Spike & Recovery of Leptin in Human Serum

Sample No.	Leptin Added ng/mL	Observed ng/mL HE*	Expected ng/mL HE*	% Recovery
1	0	4	--	--
2	2	5	6	83
3	5	8	9	89
4	10	13	14	93
5	20	25	24	104

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 four separate assays are shown. Percent recovery was calculated on the observed vs. expected. (*Human Equivalent)

F. Linearity

Effect of Serum Dilution

Sample No.	Volume Sampled	Observed ng/mL HE*	Expected ng/mL HE*	% Of Expected
1	100 µl	4.31	4.31	100
	75 µl	4.37		101
	50 µl	4.29		100
	25 µl	4.95		115
2	100 µl	7.28	7.28	100
	75 µl	7.40		102
	50 µl	7.48		103
	25 µl	7.70		106
3	100 µl	10.13	10.13	100
	75 µl	9.51		94
	50 µl	10.12		100
	25 µl	10.07		99
4	100 µl	32.02	32.02	100
	75 µl	32.15		100
	50 µl	32.01		100
	25 µl	31.85		99

Aliquots of pooled human serum containing varying concentrations of leptin were analyzed in the volumes indicated. Dilution factors of 1.0, 1.33, 2.0, and 4.0 representing 100 µl, 75 µl, 50 µl, and 25 µl, respectively, were applied in calculating observed concentrations. The mean leptin levels and percent of expected for four separate assays are shown. (* Human Equivalent)

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	13201				
2	″	13062	13132			
3	NSB	422				
4	″	377	400			
5	Bo	4704				
6	″	4613	4659	4259		
Standards						
7	1 ng/mL	4458				
8		4339	4399	3999	93.9	
9	2 ng/mL	4027				
10		3924	3976	3576	84.0	
11	5 ng/mL	2844				
12		2835	2840	2440	57.3	
13	10 ng/mL	1937				
14		1889	1913	1513	35.5	
15	20 ng/mL	1268				
16		1264	1266	866	20.3	
17	50 ng/mL	878				
18		817	848	448	10.5	
Controls/Unknown						
19	QC 1	3294				
20		3453	3374	2974	69.8	3
21	QC 2	1190				
22		1234	1212	812	19.1	22
23-n	Unknown					

XIII. QUALITY CONTROLS

Good Laboratory Practice (GLP) 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 Rules)[4]:

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. RECOMMENDED SPECIES FOR ANALYSIS

Pig	Sheep
Cat	Bat
Cow	Ground Squirrel
Horse	

Others to be determined

XV. REPLACEMENT REAGENTS

REAGENTS	**Cat #**
^{125}I-Human Leptin (<3 uCi, 111 kBq)	9081
Label Hydrating Buffer (27mL)	LHB-P
Human Leptin Standards (1 mL each)	8081-K
Guinea Pig anti-Leptin Antibody (26 mL)	1085-K
Precipitating Reagent (260 mL)	PR-UV
QC 1&2 (1 mL each)	6081-K
Assay Buffer (40 mL)	AB-PTR

XVI. 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.

XVII. REFERENCES

1. Ma, Zhongmin, et. al. Radioimmunoassay of leptin in human plasma. *Clinical Chemistry.* 42:942-946, June, 1996.

2. Maffei, M., et. al. Leptin levels in human and rodent: Measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nature Med.* Vol. 1, 11:1155-1161, 1995.

4. Pelleymounter, M.A., et. al. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science.* 269:540-543, 1995.

4. Westgard, J.O., et. al. A multi-rule Shewhart chart for quality control in clinical chemistry. *Clin. Chem.* 27:493-501, 1981.