Quantikine® ELISA

Mouse/Rat Leptin Immunoassay

Catalog Number MOB00
SMOB00
PMOB00

For the quantitative determination of mouse or rat Leptin concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
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TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
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China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
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INTRODUCTION

Mouse Leptin is a protein product of the mouse *obese* gene (1, 2). Mutant mice that lack functional Leptin have been found to be obese, diabetic, infertile and to have reduced activity, metabolism and body temperature. cDNA clones encoding Leptin have been isolated from human, simian, mouse and rat cells. Mature mouse Leptin shares approximately 96% and 84% amino acid (aa) sequence identity with the rat and human proteins, respectively. Mouse Leptin cDNA encodes a 167 aa residue protein with a 21 aa residue signal sequence that is cleaved to yield the 146 aa residue mature protein. The expression of Leptin mRNA has been shown to be restricted to adipose tissues and placenta (2).

A high-affinity receptor for Leptin (OB-R) with homology to gp130, G-CSF receptor, and LIF receptor has been cloned (3). Multiple isoforms of OB-R, including a long form (OB-R_L) with a large cytoplasmic domain capable of signal transduction, and several receptor isoforms with short cytoplasmic domains (OB-R_S) lacking signal transducing capabilities, have been identified (4-6). An OB-R transcript lacking a transmembrane domain and potentially encoding a soluble form of the receptor has also been described (7). OB-R_L transcripts were reported to be expressed predominantly in regions of the hypothalamus previously thought to be important in body weight regulation. Expression of OB-R_S transcripts have been found in multiple tissues, including the choroid plexus, lung, kidney and primitive hematopoietic cell populations (2). OB-R has been shown to be encoded by the mouse diabetes (*db*) and rat fatty (*fa*) genes (8). Rodents homozygous for the *db* or *fa* mutations have long been known to exhibit an obesity phenotype almost identical to the phenotype of *ob/ob* mice (9).

High Leptin levels (ng/mL) have been detected in mouse, rat and human serum or plasma. Circulating levels of Leptin have been shown to be regulated in response to a variety of stimuli including food intake, insulin, glucocorticoids, cytokines, and reproductive events (2, 10-14). The majority of Leptin in serum was reported to be bound to multiple Leptin-binding proteins (15).

The Quantikine® Mouse/Rat Leptin Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse or rat Leptin in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant mouse Leptin and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant mouse Leptin accurately. Results obtained measuring natural mouse or rat Leptin showed dose-response curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for natural mouse/rat Leptin.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for mouse/rat Leptin has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any Leptin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse/rat Leptin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of Leptin bound in the initial step. The sample values are then read off the standard curve.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with calibrator diluent and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- For best results, pipette reagents and samples into the center of each well.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	CATALOG # MOB00	CATALOG # SMOB00	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse/Rat Leptin Microplate	890548	2 plates	6 plates	96 well polystyrene microplates (12 strips of 8 wells) coated with a polyclonal antibody specific for mouse/rat Leptin.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Mouse/Rat Leptin Standard	890547	3 vials	9 vials	Recombinant mouse Leptin in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new standard and control for each assay. Discard within 8 hours of reconstitution.
Mouse/Rat Leptin Control	890549	3 vials	9 vials	Recombinant mouse Leptin in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
Mouse/Rat Leptin Conjugate	890546	1 vial	3 vials	23 mL/vial of a polyclonal antibody specific for mouse/rat Leptin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895038	1 vial	3 vials	12 mL/vial of a buffered protein base with preservatives.	
Calibrator Diluent RD5-3	895436	2 vials	6 vials	21 mL/vial of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	2 vials	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	3 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	3 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895174	1 vial	3 vials	23 mL/vial of diluted hydrochloric acid.	
Plate Sealers	N/A	8 strips	24 strips	Adhesive strips.	

* Provided this is within the expiration date of the kit.

MOB00 contains sufficient materials to run ELISAs on two 96 well plates.
SMOB00 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PMOB00). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Refer to the literature accompanying your order for specific vial counts.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 1000 mL graduated cylinder.
- **Polypropylene** test tubes for dilution of standards and samples.

ADDITIONAL REAGENTS REQUIRED

Note: *For sample activation of pregnant mouse serum only. (second trimester through post-partum Day 1)*

- Glacial acetic acid (Reagent Grade A.C.S., 17.4 N)
- HEPES, free acid (Reagent Grade M.W., 238.8)
- Sodium hydroxide (Reagent Grade A.C.S., 10 N)
- Urea (Reagent Grade M.W., 60.06)

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect mouse plasma using EDTA or heparin as an anticoagulant. Collect rat plasma using EDTA as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

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

ACTIVATION REAGENT PREPARATION

To activate Leptin to the immunoreactive form (see Sample Preparation), prepare the following solutions for acid activation and neutralization. The solutions may be stored in polypropylene bottles at room temperature for up to one month. If any precipitation forms, gently heat the solution to 37 °C while mixing. **Caution:** *Wear protective clothing and safety glasses during preparation or use of these reagents.*

2.5 N Acetic Acid/10 M Urea (250 mL) - To 100 mL of deionized water, slowly add 35.9 mL of 17.4 N (Glacial) Acetic Acid. Mix well. Add 150.2 g Urea. Mix well until dissolved. Bring final volume to 250 mL with deionized water.

2.7 N NaOH/1 M HEPES (250 mL) - To 140 mL of deionized water, add 67.5 mL of 10 N NaOH. Mix well. Add 59.5 g HEPES. Mix well. Bring final volume to 250 mL with deionized water.

SAMPLE PREPARATION

Normal mouse serum, plasma, and serum from pregnant mice within the first trimester of pregnancy require a 20-fold dilution into Calibrator Diluent RD5-3. A suggested 20-fold dilution is 20 µL of sample + 380 µL of Calibrator Diluent RD5-3.

Normal rat serum and EDTA plasma require a 10-fold dilution into Calibrator Diluent RD5-3. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent RD5-3.

Pregnant mouse serum from the second trimester of pregnancy through post-partum day 1 require acid/urea activation of mouse Leptin.

Activation protocol for pregnant mouse serum

Note: *Do not acid activate the Mouse/Rat Leptin Standards or Control. The kit standard and control contain immunoreactive mouse Leptin.*

1. To 50 µL serum, add 50 µL of 2.5 N Acetic Acid/10 M Urea.
2. Mix well.
3. Incubate 10 minutes at room temperature.
4. Neutralize the acidified sample by adding 50 µL of 2.7 N NaOH/1 M HEPES.
5. Mix well.
6. Prior to the assay, dilute the activated serum sample 20-fold with Calibrator Diluent RD5-3. A suggested 20-fold dilution is 20 µL of activated sample + 380 µL of Calibrator Diluent RD5-3.

The concentration read off the standard curve must be multiplied by the total dilution factor, 60.

For each new lot of acidification and neutralization reagents, measure the pH of several representative samples after neutralization to ensure that the pH is 7.2-7.6. Adjust the volume and corresponding dilution factor of the neutralization reagent as needed.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

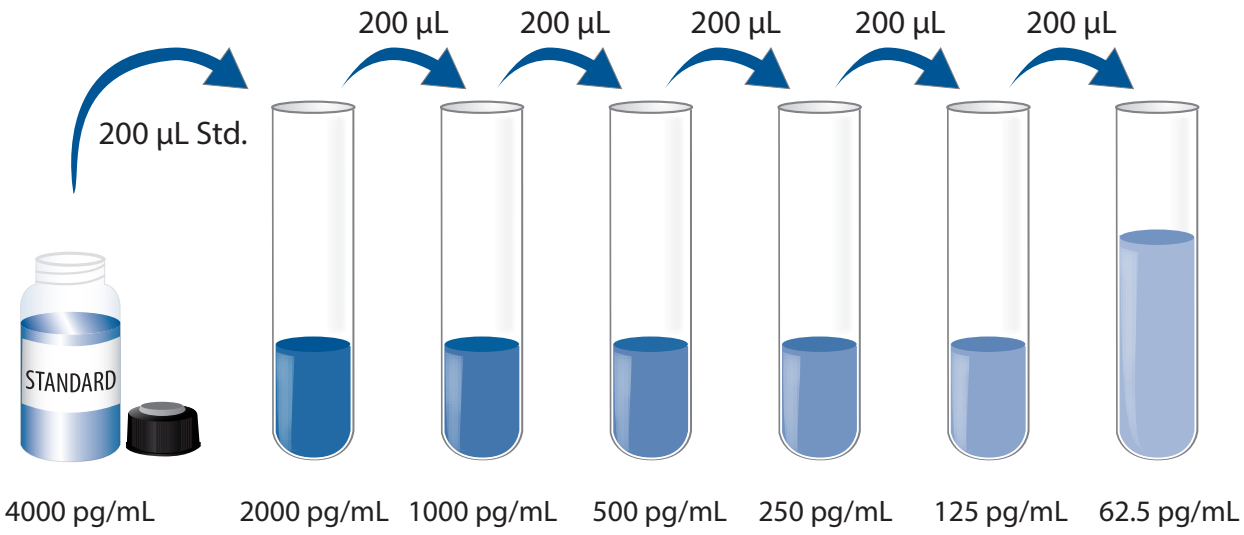
Mouse/Rat Leptin Control - Reconstitute the control with 1.0 mL deionized or distilled water. Mix thoroughly. Assay the control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 20 mL Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

Mouse/Rat Leptin Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse/Rat Leptin Standard with Calibrator Diluent RD5-3. Do not substitute other diluents. This reconstitution produces a stock solution of 4000 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 200 µL of Calibrator Diluent RD5-3 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Mouse/Rat Leptin Standard (4000 pg/mL) serves as the high standard. Calibrator Diluent RD5-3 serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and control be assayed in duplicate.

1. Prepare reagents, standard curve dilutions, and samples as directed by the previous section.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1W to each well.
4. Add 50 µL of standard, control, or sample* per well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 100 µL of Mouse/Rat Leptin Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

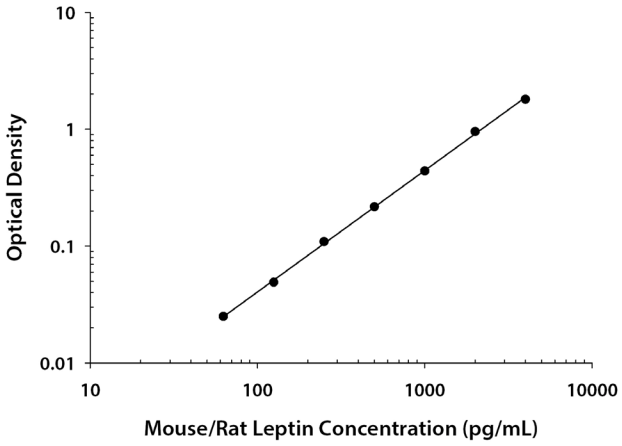
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the mouse/rat Leptin concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.070 0.064	0.067	—
62.5	0.091 0.092	0.092	0.025
125	0.117 0.114	0.116	0.049
250	0.173 0.178	0.176	0.109
500	0.282 0.287	0.284	0.217
1000	0.527 0.484	0.506	0.439
2000	1.035 1.009	1.022	0.955
4000	1.886 1.859	1.872	1.805

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (pg/mL)	182	693	1803	187	654	1736
Standard deviation	7.9	22.6	67.9	14.2	30.5	87.4
CV (%)	4.3	3.3	3.8	7.6	4.7	5.0

RECOVERY

The recovery of mouse/rat Leptin spiked to levels throughout the range of the assay in various matrices was evaluated.

Mouse Samples	Average % Recovery	Range
Cell culture supernates (n=5)	101	85-120%
Serum* (n=18)	108	81-122%
EDTA plasma* (n=4)	92	78-105%
Heparin plasma* (n=4)	95	81-105%

Rat Samples	Average % Recovery	Range
Cell culture supernates (n=4)	114	103-125%
Serum* (n=10)	92	81-115%
EDTA plasma* (n=7)	96	83-114%

*Samples were spiked and then diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

The minimum detectable dose (MDD) of mouse/rat Leptin is typically less than 22 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant mouse Leptin produced at R&D Systems®.

LINEARITY

To assess the linearity of the assay, four or more samples containing and/or spiked with high concentrations of mouse/rat Leptin in each matrix were diluted with calibrator diluent and assayed. Results from typical sample dilutions are shown.

Mouse Samples	Dilution	Observed (pg/mL)	Expected (pg/mL)	$\frac{\text{Observed}}{\text{Expected}} \times 100$
Cell culture supernates (fat cell CM)	Neat	2382	———	———
	1:2	1226	1191	103
	1:4	560	596	94
	1:8	289	298	97
	1:16	144	149	97
Serum* (normal mouse)	Neat	2233	———	———
	1:2	1168	1116	105
	1:4	604	558	108
	1:8	303	279	109
	1:16	152	140	109
Serum* (day 19 pregnant)	Neat	3300	———	———
	1:2	1708	1650	104
	1:4	942	825	114
	1:8	441	412	107
	1:16	204	206	99
EDTA plasma* (normal mouse)	Neat	727	———	———
	1:2	368	364	101
	1:4	173	182	95
	1:8	81	91	89
Heparin plasma* (normal mouse)	Neat	968	———	———
	1:2	476	484	98
	1:4	220	242	91
	1:8	110	121	91

LINEARITY *CONTINUED*

Rat Samples	Dilution	Observed (pg/mL)	Expected (pg/mL)	$\frac{\text{Observed}}{\text{Expected}} \times 100$
Cell culture supernates	Spiked	1921	———	———
	1:2	818	960	85
	1:4	441	480	92
	1:8	233	240	97
	1:16	122	120	102
Serum* (normal rat)	Neat	3005	———	———
	1:4	774	751	103
	1:8	395	376	105
	1:16	193	188	103
EDTA plasma* (normal rat)	Neat	1477	———	———
	1:4	376	369	102
	1:8	183	185	99
	1:16	90	92	98

*The observed neat value (pg/mL) represents the assay value prior to multiplying the normal mouse serum/plasma, pregnant mouse serum, and rat serum/plasma values by 20, 60, and 2, respectively.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of mouse/rat Leptin in this assay.

Sample		Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Mouse serum (NSA mice, 6 weeks)	males (n=5)	1.5	ND-3.2	1.2
	females (n=6)	8.7	4.6-16.3	4.5
Mouse serum (Pregnant mice, NSA)	day 4 (n=7)	4.6	3.0-6.7	1.8
	day 13 (n=6)	14.2	5.6-20.2	5.6
	day 19 (n=4)	50.9	43.5-57.6	6.4
Mouse serum (Females, post-partum)	day 1 (n=2)	74	70-78	5.3
	day 5 (n=2)	1.7	1.4-2.0	0.5
Mouse serum (OB/OB mice)	(n=1)	ND	———	———
Mouse serum (CD-1 females)	(n=7)	6.5	1.6-15.4	5.1
Mouse serum (Retired breeders)	(n=40)	7.5	2.1-18.8	3.9
Mouse EDTA plasma (Retired breeders)	(n=10)	13.4	4.0-32	9.7
Mouse heparin plasma (Retired breeders)	(n=10)	13.8	8.0-23	5.2
Rat serum	male (n=9)	2.4	1.3-3.1	0.6
	female (n=10)	4.0	2.9-6.7	1.1
Rat EDTA plasma	male (n=9)	1.9	0.7-2.7	0.8
	female (n=9)	3.8	2.2-5.7	1.2

ND=Non-detectable

Cell Culture Supernates - Abdominal fat tissue (6 retired breeders, NSA mice) was placed into 25 mL of RPMI supplemented with 10% heat-inactivated fetal bovine serum and underwent 3 freeze/thaw cycles. The preparation was centrifuged at 3000 x g for 30 minutes, and the fat was discarded from the surface. The remaining media was sterile filtered, assayed for mouse Leptin, and measured 1.1 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant mouse and rat Leptin.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range mouse Leptin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

C10
G-CSF
GM-CSF
IFN-γ
IL-1α
IL-1β
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-10 R
IL-12
IL-13
JE/MCP-1
LIF
M-CSF
MIP-1α
MIP-1β
MIP-2
SCF
TNF-α
Tpo R
VEGF

Recombinant human:

Leptin R

Recombinant human Leptin cross-reacts approximately 0.24% in this assay.

Recombinant mouse Leptin R intereferes in this assay at concentrations > 2000 pg/mL.

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