BioVendor Laboratory Medicine, Inc.
Palackeho tr. 56
612 00 Brno, Czech Republic
Phone: +420-5-49124185
Fax: +420-5-49211460
e-mail: info@biovendor.com
http://www.biovendor.com

European Union:

BioVendor GmbH
Im Neuenheimer Feld 583
D-69120 Heidelberg, Germany
Phone.: +49-6221-433 9100
Fax: +49-6221-433 9111
e-mail: roth@biovendor.com

USA, Canada and Mexico:

BioVendor, LLC
1459 Sand Hill Road
Suite 229
Candler, NC 28715, USA
Phone: +1-828-670-7807, +1-800-404-7807
Fax: +1-828-670-7809
e-mail: slee@biovendor.com

Human Leptin ELISA, Clinical Range

Cat. No.: RD191001100

1. Intended Use

The RD191001100 Human Leptin ELISA is a sandwich enzyme immunoassay for the quantitative measurement of human leptin in serum, plasma and tissue culture medium. It is intended for *in vitro* diagnostic use.

Features

- The total assay time is less than four hours.
- The kit measures total serum leptin.
- Quality controls are human serum based. No animal sera are used.
- Components of the kit are ready-to-use (with the exception of Wash Solution and Quality Controls).

2. Summary

Leptin, the product of the *ob* (obese) gene, is a single-chain 16 kDa protein consisting of 146 amino acid residues. Leptin is produced mainly in the adipose tissue, and is considered to play an important role in appetite control, fat metabolism and body weight regulation. It targets the central nervous system, particularly hypothalamus, affecting food intake. The primary effect of leptin appears to be mediated by leptin receptors expressed mainly in the hypothalamus. In humans, leptin levels correlate with body mass index (BMI) and percentage body fat, and are elevated even in obese individuals. Leptin has a dual action; it decreases the appetite and increases energy consumption, causing more fat to be burned. Leptin is secreted in circadian fashion with nocturnal rise in both lean and obese patients.
Mutations of the *ob* gene resulting in leptin deficiency are the cause of obesity in the *ob/ob* mice. Endogeneous leptin can normalize their body weight. In contrast, high levels of leptin in obese human subjects point to an insensitivity to endogeneous leptin.
Other factors in addition to the amount of body fat appear to regulate leptin action: insulin, glucocorticoids, catecholamines and sex hormones. Studies have shown that leptin may be linked to reproductive function.

3. Test Principle

In the BioVendor's Human Leptin ELISA, standards, quality controls and samples of sera are incubated in microtitration wells coated with anti-human leptin antibody. After a thorough wash, anti-human leptin antibody labelled with horseradish peroxidase (HRP) is added to the wells and incubated with the immobilized antibody-leptin complex. Following another washing step, the remaining HRP-conjugated antibody is allowed to react with the substrate tetramethylbenzidine. The reaction is stopped by addition of acidic solution, and absorbance of the resulting yellow product is measured spectrophotometrically at 450 nm. The absorbance is proportional to the concentration of leptin. A standard curve is constructed by plotting absorbance values versus leptin concentrations of standards, and concentrations of unknown samples are determined using this standard curve.

4. Precautions

- For *in vitro* diagnostic use.
- This kit contains components of human origin. These materials were found non-reactive for HBsAg, HCV antibody and for HIV 1/2 antigen and antibody. However, these materials should be handled as potentially infectious, as no tests can guarantee the complete absence of infectious agents.
- Avoid contact with the acidic Stop Solution and Substrate (TMB) Solution, which contains hydrogen peroxide. In case of contact with the Stop Solution and the Substrate Solution wash skin thoroughly with water and seek medical attention, when necessary.

- Wear gloves and laboratory coats when handling immunodiagnostic materials.
- The materials must not be pipetted by mouth.
- Do not drink, eat or smoke in the areas where immunodiagnostic materials are being handled.
- Do not mix different lot numbers of any kit components.

5. Reagents Supplied

- Antibody Coated Microtiter Strips (coated with polyclonal Anti- Leptin Ab), 96 wells, vacuum sealed
- Conjugate Solution (polyclonal Anti-Leptin Antibody, Horseradish Peroxidase Conjugate), 13 ml
- Human Leptin Standards
  (1, 2, 5, 10, 20, 50 ng/ml), 0.35 ml each
- Quality Controls: High and Low, lyophilized from 350 µl each (refer to the Certificate of Analysis for actual Quality Controls values)
- Dilution Buffer, 13 ml
- Wash Solution Concentrate (5x), 100 ml
- Substrate (TMB) Solution, 13 ml
- Stop Solution (0.2 M H_2SO_4), 13 ml

6. Materials Required but Not Supplied

- Test tubes for diluting samples
- Precision pipettes to deliver 50-200 µl
- Multichannel pipette 100 µl
- Microplate reader with 450 ± 10 nm filter
- Orbital microplate shaker capable of approximately 300 rpm
- Software package facilitating data generation and analysis
- Orbital microplate shaker capable of approximately 300 rpm
- Microtitration plate washer (optional)
  [Manual washing is possible but not preferable.]
- Glassware (graduated cylinder and bottle for Wash Solution)
- Deionized (distilled) water

7. Preparation of Reagents

All reagents need to be brought to room temperature prior to the assay.
Assay reagents are supplied ready-to-use, with the exception of Quality Controls (lyophilized) and Wash Solution Concentrate (5x).

Quality Controls
Reconstitute each vial of Quality Control with 0.35 ml of distilled water at least 30 minutes prior to use. Storage conditions for aliquots are – 20°C.

Dilute the reconstituted Quality Controls 1:3 with Dilution Buffer prior to use (e.g. 100 µl Quality Control + 200 µl Dilution Buffer for duplicates).
Do not store the diluted Quality Controls.

Human Leptin Standards
Dilute Standards 1:3 with Dilution Buffer prior to use (e.g. 100 µl standards + 200 µl Dilution Buffer for duplicates).
Do not store the diluted standard solutions.

Wash Solution
Dilute 100 ml of Wash Solution Concentrate with 400 ml deionized (distilled) water.
Stability and storage:
The diluted Wash Solution is stable for 1 month when stored at 2-8°C.

8. Preparation of Samples

Dilute samples 1:3 with Dilution Buffer (e.g. 50 µl sample + 100 µl Dilution Buffer when assaying samples in singlets, or preferably 100 µl sample + 200 µl Dilution Buffer for duplicates).

9. Assay Procedure

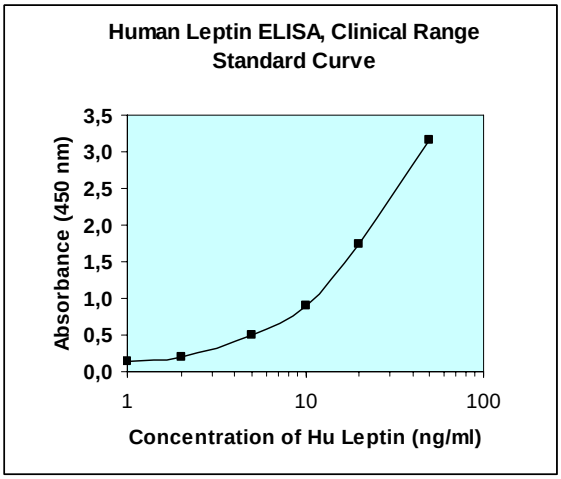
1) Pipet 100 µl of diluted Standards, Quality Controls and samples, preferably in duplicates, into the appropriate wells.
2) Incubate the plate at room temperature (ca. 25°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker.
3) Wash the wells 3-times with Wash Solution (0.35 ml per well).
4) Add 100 µl of Conjugate Solution.
5) Incubate the plate at room temperature (ca. 25°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker.
6) Wash the wells 3-times with Wash Solution (0.35 ml per well).
7) Add 100 µl of Substrate Solution. (Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminium foil is recommended.)
8) Incubate the plate for 10 minutes at room temperature. (The incubation time may be extended [up to 20 minutes] if the reaction temperature is below than 20°C.)
9) Stop the colour development by adding 100 µl of Stop Solution.
10) Determine the absorbance by reading the plate at 450 nm. (The absorbance should be read within 5 minutes following step 9.)

Note: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine leptin concentration of

off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

10. Calculations

Most microtiter plate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the absorbance (Y) of standards versus *log* of the known concentration (X) of standards, using the four-parameter function. Results are reported as concentration of leptin (ng/ml) in samples.
Alternatively, the *logit log* function can be used to linearize the calibration curve (i.e. *logit* of absorbance (Y) is plotted versus *log* of the known concentration (X) of standards).
Standards, Quality Controls and samples are diluted with Dilution Buffer at the same ratio. Therefore, multiplying the respective results by the dilution factor is not necessary.



11. Limits of Assay

Results exceeding 50 ng/ml should be repeated with more diluted sample (e.g. 1:5). Dilution factors need to be taken into consideration in calculating the concentration of leptin.

12. Storage, Expiration

Store the kit at 2-8°C. Under these conditions, the kit is stable until the expiration date (see label on the box).

13. Performance Characteristics

Typical analytical data of BioVendor Human Leptin ELISA, Clinical Range are presented in this chapter.
For actual Standard curve and Quality Controls values see the Certificate of Analysis.

A. Sensitivity

The limit of detection (defined as human leptin concentration giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A_{blank} + 3xSD_{blank}) is defined as follows:

- Analytical Limit of Detection is calculated from the real leptin values in wells and is 0.17ng/ml
- Assay Sensitivity takes the dilution of samples into consideration and is calculated according to the formula:

Assay Sensitivity = Analytical Limit of Detection x sample dilution = 0.17ng/ml x 3 = 0.5ng/ml

*Dilution Buffer is pipetted into blank wells.

B. Precision

- Intra-assay (Within-Run) (n=8)

Sample	Mean (ng/ml)	Standard Deviation (ng/ml)	CV (%)
1	3.54	0.27	7.5
2	13.63	0.41	3.0
3	25.44	1.38	5.4
4	25.58	1.68	6.7

- Inter-assay (Run-to-Run) (n=6)

Sample	Mean (ng/ml)	Standard Deviation (ng/ml)	CV (%)
1	5.41	0.50	9.2
2	8.65	0.68	7.8
3	13.9	0.95	6.8
4	25.12	0.81	3.2

C. Spiking Recovery

Serum samples were spiked with different amounts of human leptin and assayed.

Sample	Observed (ng/ml)	Expected (ng/ml)	Recovery O/E (%)
1	7.89	-	-
	10.14	11.69	86.7
	15.12	16.47	91.8
	22.17	24.14	91.8
2	12.95	-	-
	18.26	16.75	109.0
	19.55	21.53	90.8
	27.20	29.20	93.2
3	8.09	-	-
	10.92	12.53	87.2
	14.31	16.43	87.1
	20.37	23.03	88.4
4	13.84	-	-
	16.38	18.28	89.6
	18.08	22.18	81.5
	26.71	28.78	92.8

D. Linearity

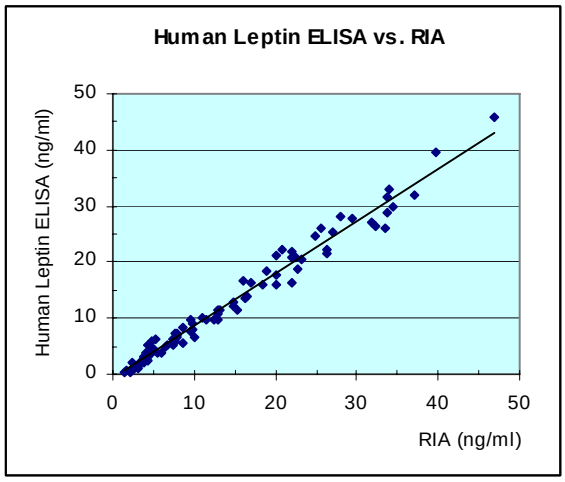
Serum samples were diluted with Dilution Buffer and assayed.

Sample	Dilution	Observed (ng/ml)	Expected (ng/ml)	Recovery O/E (%)
1	-	13.27	-	-
	1:2	7.62	6.64	114.8
	1:4	3.76	3.32	113.3
	1:8	1.58	1.66	95.3
2	-	15.49	-	-
	1:2	8.39	7.75	108.3
	1:4	3.93	3.87	101.5
	1:8	2.31	1.94	119.3
3	-	15.23	-	-
	1:2	7.78	7.62	102.2
	1:4	3.68	3.81	96.7
	1:8	1.84	1.90	96.7
4	-	27.76	-	-
	1:2	13.69	13.88	98.6
	1:4	6.87	6.94	99.0
	1:8	4.10	3.47	118.2

E. Method Comparison

The BioVendor's Human Leptin ELISA was compared to a commercial RIA. Linear regression analysis of the results yielded the following results.

ELISA = 0.94 x RIA - 0.9 r = 0.97



14. Definition of Leptin Calibrator

A recombinant protein is used as the calibrator. The recombinant human leptin is a 16 kDa protein containing 147 amino acid residues.

15. Troubleshooting and FAQs

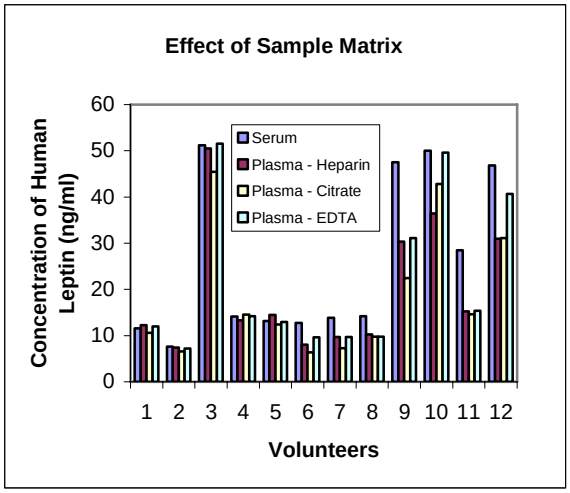
1/ Weak signal in all wells
Possible explanations:
· Omission of a reagent or a step
· Improper preparation or storage of a reagent
· Assay performed before reagents were allowed to come to room temperature

2/ High signal and background in all wells
Possible explanations:
· Improper or inadequate washing
· Overdeveloping; incubation time should be decreased before addition of Stop Solution

3/ High coefficient of variation (CV)
Possible explanation:
· Improper or inadequate washing

4/ Effect of sample matrix (serum/plasma)
Samples from 12 patients were taken and treated by different methods, results shown below:

Volunteer No.	Serum (ng/ml)	Plasma (ng/ml)		
		Heparin	Citrate	EDTA
1	11,56	12,24	10,63	12,01
2	7,59	7,39	6,61	7,23
3	51,18	50,5	45,46	51,58
4	14,13	13,27	14,52	14,21
5	13,16	14,47	12,42	12,96
6	12,73	8,02	6,35	9,63
7	13,86	9,73	7,27	9,68
8	14,22	10,22	9,75	9,79
9	47,52	30,32	22,45	31,12
10	50	36,45	42,79	49,6
11	28,51	15,27	14,64	15,4
12	46,81	30,95	31,13	40,7



Mean values of human leptin serum, heparin plasma, citrate plasma and EDTA plasma:

Sample (n=12)	Mean (ng/ml)	Plasma / Serum (%)
Serum	25,9	-
Plasma – Heparin	19,9	76,7
Plasma – Citrate	18,7	72,0
Plasma - EDTA	22,0	84,8

5/ Effect of freezing/thawing on the concentration of Human Leptin in samples

No decline was observed in concentration of human Leptin in serum and plasma samples after repeated (5x) freezing/thawing cycles.

Sample	Number of freezing/ thawing cycles	Serum (ng/ ml)	Plasma (ng/ ml)		
			Heparin	Citrate	EDTA
1	1x	7,4	7,1	6,61	6,4
	3x	10,2	7,2	6,3	8,5
	5x	12,8	7,5	6,3	9,4
2	1x	9,4	14,0	13,6	13,5
	3x	13,34	14,2	12,3	10,7
	5x	13,9	11,9	11,0	11,8

6/ Stability of samples at 4°C

Samples should be stored at –20°C. However, no decline was observed in concentration of leptin in serum and plasma samples when stored at 4°C for 1 week. To avoid microbial contamination, add NaN_3 to a final concentration 0,1% to the samples.

Sample	Incubation: Temperature Period	Serum (ng/ ml)	Plasma (ng/ ml)		
			Heparin	Citrate	EDTA
1	-20˚C	26,9	33,52	27,4	45,4
	4˚C, 7 day	28,0	33,2	27,8	44,3
	4˚C, 14 days	26,0	30,1	34,1	40,3
2	-20˚C	32,2	41,3	45,4	42,9
	4˚C, 7 day	35,5	45,4	41,2	39,8
	4˚C, 14 days	33,8	37,9	34,8	32,8

7/ Why are calibrator solutions diluted 1:3 prior to use in ELISA and what does it mean for calculation of results?

The dilution buffer suppresses the matrix effect of samples. It is a common practice to use the same dilution for samples and calibrators because of simple calculation of results: concentrations of samples can be read directly off the curve without considering a dilution factor. Thus the same dilution 1:3 is used for calibrators, controls and serum/plasma samples in Human Leptin ELISA.

Samples exceeding human leptin level of 50 ng/ml should be measured at higher degree of dilution (e.g. 1:9) and dilution factors need to be taken into consideration when calculating human leptin concentrations then (the dilution factor is 3 in this case).

16. References

A. References to Leptin

- Auwerx J. and Staels B.: Leptin (Review article). *The Lancet* 13, 737 (1998)
- Blum W.F., Englaro P., Hanitsch S. et al.: Plasma leptin levels in healthy children and adolescents: Dependence on body mass index, body fat mass, gender, pubertal stage, and testosterone. *J. Clin. Endocrinol. Metab.* 82, 2904 (1997)
- Cohen B., Novick D. and Rubinstein M.: Modulation of insulin activities by leptin. *Science* 274, 1185 (1996)
- Considine R.V., Sinha M.K., Heiman M.L., Kriaciunas A., Stephens T.W., Nyce M.R., Ohannesian J.P., Marco C.C., McKee L.J., Bauer T.L. and Caro J.F.: Serum immunoreactive leptin concentrations in normal-weight and obese humans. *N. Engl. J. Med.* 334, 292-295 (1996)
- Halaas J.L., Gajiwala K.S., Maffei M., Cohen S.L., Chait B.T., Rabinowitz D., Lallone R.L., Burley S.K. and Friedman J.M.: Weight-reducing effects of the plasma protein encoded by the *obese* gene. *Science* 269, 543-546 (1995)
- Harigaya A., Nagashima K., Nako Y. and Marikawa A.: Relationship between concentration of serum leptin and fetal growth. *J. Clin. Endocrinol. Metab.* 82, 3281 (1997)
- Lönnqvist F., Arner P., Nordfors L. and Shalling M.: Overexpression of the *obese* (*ob*) gene in adipose tissue of human subjects. *Nature Med.* 1, 950-953 (1995)
- Maffei M., Halaas J., Ravussin E., Pratley R.E., Lee G.H., Zhang Y., Fei H., Kim S., Lallone R., Ranganathan S., Kern P.A. and Friedman J.M.: Leptin levels in human and rodent: measurement of plasma leptin and *ob* RNA in obese and weight-reduced subjects. *Nature Med.* 1, 1155-1161 (1995)
- Pelleymounter M.A., Cullen M.J., Baker M.B., Hecht R., Winters D., Boone T. and Collins F.: Effects of

the *obese* gene product on body weight regulation in *ob/ob* mice. *Science* 269, 540-543 (1995)
- Spiegelman B.M., Flier J.S.: Adipogenesis and obesity: Rounding out the big picture, *Cell* 87, 377-389 (1996)
- Tartaglia L.A.: The leptin receptor, *J. Biol. Chem.* 272, 6093-6096 (1997)
- Tritos N.A., Mantzoros C.S.: Leptin: its role in obesity and beyond, *Diabetologia* 40, 1371-1379 (1997)
- Zhang F., Basinski M. B., Beals J.M., Briggs S.L., Churgay L.M., Clawson D.K., DiMarchi R.D., Furman T.C., Hale J.E., Hsiung H.M., Schoner B.E., Smith D.P., Zhang X.Y., Wery J.P., and Schevitz R.W.: Crystal structure of the *obese* protein leptin-E100, *Nature* 387, 206-209 (1997)
- Zhang Y., Proenca R., Maffei M., Barone M., Leopold L., Friedman J.M.: Positional cloning of the mouse *obese* gene and its human homologue. *Nature* 372, 425-432 (1994)

B. References to this product

- Lichnovska R, Gwozdziewiczova S, Chlup R, Hrebicek J.: Serum Leptin in the Development of Insulin Resistance and Other Disorders in the Metabolic Syndrome. *Biomed. Papers* 149 (1): 119-126 (2005)
- Meier U, and Gressner A.M.: Endocrine Regulation of Energy Metabolism: Review of Pathobiochemical and Clinical Chemical Aspects of Leptin, Ghrelin, Adiponectin, and Resistin. *Clinical Chemistry* 50(9): 1511-1525 (2004)
- Lichnovska R, Gwozdziewiczova S, Hrebicek J.: Gender differences in factors influencing insulin resistance in elderly hyperlipemic non-diabetic subjects. *Cardiovasc Diabetol* Oct 14;1(1):4 (2002)
- Adam JA, Menheere PP, van Dielen FM, Soeters PB, Buurman WA, Greve JW: Decreased plasma orexin-A levels in obese individuals. *Int J Obes Relat Metab Disord* 26(2):274-6 (2002)
- Haluzik M, Sindelka G, Widimsky J Jr, Prazny M, Zelinka T, Skrha J.: Serum leptin levels in patients with primary hyperaldosteronism before and after treatment: relationships to insulin sensitivity. *J Hum Hypertens* 16(1):41-5 (2002)

For more references on this product
see our webpages at www.biovendor.comT

Assay Procedure Summary