

DRG[®] Leptin (Mouse / Rat) ELISA (EIA-4551)

Revised 3 Dec. 2010 rm (Vers. 2.1)

For Research Use Only

*Please use only the valid version of the package insert provided with the kit.***1 INTENDED USE**

The Mouse and Rat Leptin ELISA is a sandwich enzyme immunoassay for measurement of mouse and/or rat leptin.

Features

- **It is intended for research use only**
- The total assay time is less than 3.5 hours
- The kit measures leptin in serum, plasma (EDTA, citrate, heparin) and tissue culture media
- Assay format is 96 wells
- Quality Controls are mouse and rat serum based. No human sera are used
- Standards are recombinant protein based
- Components of the kit are provided ready to use, concentrated or lyophilized

2 STORAGE, EXPIRATION

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

For stability of opened reagents see Chapter 9.

3 INTRODUCTION

Leptin is a protein hormone with important effects in metabolism and regulating body weight. It is a single-chain 16 kDa protein consisting of 146 amino acid residues and encoded by the obese (*ob*) gene.

Leptin is expressed predominantly by adipocytes, small amounts of leptin are also secreted by cells in the epithelium of stomach and in the placenta. Leptin's effect on body weight is mediated through effects on hypothalamic centers, where leptin receptors are highly expressed. Leptin has a dual action, it decreases the appetite and increases energy consumption. A mutations in the *ob* gene of leptin or in the gene of leptin receptor causes hyperphagia, reduced energy expenditure, and severe obesity in the *ob/ob* mice.

Ob gene knockout mice are also characterized by several metabolic abnormalities including hyperglucocorticoidemia, hyperglycaemia, hyperinsulinemia and insulin resistance.

When *ob/ob* mice are treated with injections of leptin, they lose their excess fat and return to normal body weight.

Studies have shown that leptin appears to be a significant regulator of reproductive function. In addition, leptin is involved in bone metabolism and plays a significant role as an immunomodulator.

Areas of investigation:

Energy metabolism and body weight regulation

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In the Mouse and Rat Leptin ELISA, standards, quality controls and samples are incubated in microplate wells pre-coated with anti-mouse leptin antibody. After 60 minutes incubation and washing, biotin labelled polyclonal anti-mouse leptin antibody is added to the wells and incubated with the captured leptin for 60 minutes. After another washing, streptavidin-HRP conjugate is added. After 30 minutes incubation and the last washing step, the remaining conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured. The absorbance is proportional to the concentration of leptin. A standard curve is constructed by plotting absorbance values against concentrations of standards, and concentrations of unknown samples are determined using this standard curve.

5 PRECAUTIONS

- Wear gloves and laboratory coats when handling immunodiagnostic materials
- Do not drink, eat or smoke in the areas where immunodiagnostic materials are being handled
- This kit contains components of animal origin. These materials should be handled as potentially infectious
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents. Stop and/or Substrate Solutions may cause skin/eyes irritation. In case of contact with the Stop Solution and the Substrate Solution wash skin/eyes thoroughly with water and seek medical attention, when necessary
- The materials must not be pipetted by mouth

6 TECHNICAL HINTS

- Reagents with different lot numbers should not be mixed
- Use thoroughly clean glassware
- Use deionized (distilled) water, stored in clean containers
- Avoid any contamination among samples and reagents. For this purpose, disposable tips should be used for each sample and reagent
- Substrate Solution should remain colourless until added to the plate. Keep Substrate Solution protected from light
- Stop Solution should remain colourless until added to the plate. The colour developed in the wells will turn from blue to yellow immediately after the addition of the Stop Solution. Wells that are green in colour indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution
- Dispose of consumable materials and unused contents in accordance with applicable national regulatory requirements

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7 REAGENT SUPPLIED

<i>Kit Components</i>	<i>State</i>	<i>Quantity</i>
Antibody Coated Microtiter Strips	ready to use	96 wells
Biotin Labelled Antibody Conc.(10x)	concentrated	1.3 ml
Streptavidin-HRP Conjugate	ready to use	13 ml
Master Standard MOUSE	lyophilized	1 vial
Master Standard RAT	lyophilized	1 vial
Quality Control MOUSE	lyophilized	2 vials
Quality Control RAT	lyophilized	2 vials
Dilution Buffer	ready to use	2 x 13 ml
Biotin-Ab Diluent	ready to use	13 ml
Wash Solution Conc. (10x)	concentrated	100 ml
Substrate Solution	ready to use	13 ml
Stop Solution	ready to use	13 ml
Instruction for Use + Certificate of Analysis	-	1 pc

8 MATERIAL REQUIRED BUT NOT SUPPLIED

- Deionized (distilled) water
- Test tubes for diluting samples
- Glassware (graduated cylinder and bottle) for Wash Solution (Dilution Buffer)
- Precision pipettes to deliver 5 - 1 000 µl with disposable tips
- Multichannel pipette to deliver 100 µl with disposable tips
- Absorbent material (e.g. paper towels) for blotting the microtitrate plate after washing
- Vortex mixer
- Orbital microplate shaker capable of approximately 300 rpm
- Microplate washer (optional). [Manual washing is possible but not preferable.]
- Microplate reader with 450 ± 10 nm filter, preferably with reference wavelength 630 nm (alternatively another one from the interval 550-650nm)
- Software package facilitating data generation and analysis (optional)

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- All reagents need to be brought to room temperature prior to use
- Always prepare only the appropriate quantity of reagents for your test
- Do not use components after the expiration date marked on their label
- Use MOUSE Leptin Standard to quantify leptin concentration in mouse samples
- Use RAT Leptin Standard to quantify leptin concentration in rat samples

Assay reagents supplied ready to use:

Antibody Coated Microtiter StripsStability and storage:

Return the unused strips to the provided aluminium zip-sealed bag with desiccant and seal carefully. Remaining Microtiter Strips are stable 3 months stored at 2-8°C and protected from the moisture.

Streptavidin-HRP Conjugate**Dilution Buffer****Biotin-Ab Diluent****Substrate Solution****Stop Solution**Stability and storage:

Opened reagents are stable 3 months when stored at 2-8°C.

Assay reagents supplied concentrated or lyophilized:

MOUSE Leptin Standard**Refer to the Certificate of Analysis for current volume of Dilution Buffer needed for reconstitution of standard!!!**

Reconstitute the lyophilized Master Standard with Dilution Buffer just prior to the assay. Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam). The resulting concentration of the mouse leptin in the stock solution is **4 000 pg/ml** (= M-Std. 4 000 pg/ml).

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Prepare set of MOUSE leptin standards using Dilution Buffer as follows:

Volume of standard	Dilution Buffer	Concentration
M-Std. 4 000 pg/ml	-	4 000 pg/ml
250 µl M-Std. 4 000 pg/ml	250 µl	2 000 pg/ml
250 µl M-Std. 2 000 pg/ml	250 µl	1 000 pg/ml
200 µl M-Std. 1 000 pg/ml	300 µl	400 pg/ml
250 µl M-Std. 400 pg/ml	250 µl	200 pg/ml
250 µl M-Std. 200 pg/ml	250 µl	100 pg/ml

Prepared Standards are ready to use, do not dilute them.

RAT Leptin Standard

Refer to the Certificate of Analysis for current volume of Dilution Buffer needed for reconstitution of standard!!!

Reconstitute the lyophilized Master Standard with Dilution Buffer just prior to the assay. Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam). The resulting concentration of the rat leptin in the stock solution is **4 000 pg/ml** (= R-Std. 4 000 pg/ml).

Prepare set of RAT leptin standards using Dilution Buffer as follows:

Volume of standard	Dilution Buffer	Concentration
R-Std. 4 000 pg/ml	-	4 000 pg/ml
250 µl R-Std. 4 000 pg/ml	250 µl	2 000 pg/ml
250 µl R-Std. 2 000 pg/ml	250 µl	1 000 pg/ml
200 µl R-Std. 1 000 pg/ml	300 µl	400 pg/ml
250 µl R-Std. 400 pg/ml	250 µl	200 pg/ml
250 µl R-Std. 200 pg/ml	250 µl	100 pg/ml

Prepared Standards are ready to use, do not dilute them.

Stability and storage:

Reconstituted Master Standard (4 000 pg/ml) must be used immediately or aliquoted and frozen at -20°C for 3 months. Avoid repeated freeze/thaw cycles.

Do not store the diluted Standard solutions.

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Quality Controls MOUSE / RAT

Refer to the Certificate of Analysis for current volume of Dilution Buffer needed for reconstitution and for current Quality Control concentration!!!

Reconstitute appropriate Quality Control (MOUSE or RAT) with Dilution Buffer just prior to the assay. Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam).

Reconstituted Quality Controls are ready to use, do not dilute them.

Stability and storage:

The reconstituted Quality Controls must be used immediately or aliquoted and frozen at -20°C for 3 months. Avoid repeated freeze/thaw cycles.

Biotin Labelled Antibody Conc. (10x)

Prepare the working Biotin Labelled Antibody solution by adding 1 part Biotin Labelled Antibody Concentrate (10x) with 9 parts Biotin-Ab Diluent.

Example: 100 µl of Biotin Labelled Antibody Concentrate (10x) + 900 µl of Biotin-Ab Diluent for 1 strip (8 wells).

Stability and storage:

Opened Biotin Labelled Antibody Concentrate (10x) is stable 3 months when stored at 2-8°C.

Do not store the diluted Biotin Labelled Antibody solution.

Wash Solution Conc. (10x)

Dilute Wash Solution Concentrate (10x) ten-fold in distilled water to prepare a 1x working solution.

Example: 100 ml of Wash Solution Concentrate (10x)+ 900 ml of distilled water for use of all 96-wells.

Stability and storage:

The diluted Wash Solution is stable 1 month when stored at 2-8°C. Opened Wash Solution Concentrate (10x) is stable 3 months when stored at 2-8°C.

10 PREPARATION OF SAMPLES

The kit measures leptin in serum, plasma (EDTA, citrate, heparin) and tissue culture media.

Samples should be assayed immediately after collection or should be stored at -20°C. Mix thoroughly thawed samples just prior to the assay and avoid repeated freeze/thaw cycles, which may cause erroneous results. Avoid using hemolyzed or lipemic samples.

Dilute samples 20x with the Dilution Buffer just prior to the assay,

e.g. 7 µl of sample + 133 µl of Dilution Buffer for singlets, or preferably 14 µl of sample+ 266 µl of Dilution Buffer for duplicates. **Mix well** (not to foam). Vortex is recommended.

Stability and storage:

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Samples should be stored at -20°, or preferably at -70°C for long-term storage. Avoid repeated freeze/ thaw cycles.

Do not store the diluted samples.

Note: It is recommended to use a precision pipette and a careful technique to perform the dilution in order to get precise results.

Ask for protocol at drg@drg-diagnostics.de if assaying tissue culture medium samples.

11 ASSAY PROCEDURE

1. Pipet **100 µl** of Standards, Quality Controls, Dilution Buffer (=Blank) and diluted samples, preferably in duplicates, into the appropriate wells. See *Figure 1* or *Figure 2* for example of work sheet.
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). After final wash, invert and tap the plate strongly against paper towel.
4. Add **100 µl** of Biotin Labelled Antibody solution into each well.
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). After final wash, invert and tap the plate strongly against paper towel.
7. Add **100 µl** of Streptavidin-HRP Conjugate into each well.
8. Incubate the plate at room temperature (ca. 25°C) for **30 min.**, shaking at ca. 300 rpm on an orbital microplate shaker.
9. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
10. Add **100 µl** of Substrate Solution into each well. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminium foil is recommended.
11. 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. Do not shake the plate during the incubation.
12. Stop the colour development by adding **100 µl** of Stop Solution.
13. Determine the absorbance of each well using a microplate reader set to 450 nm, preferably with the reference wavelength set to 630 nm (acceptable range: 550 – 650 nm). Subtract readings at 630 nm (550 - 650 nm) from the readings at 450 nm. **The absorbance should be read within 5 minutes following step 12.**

Note: If some samples and standard/s have absorbances above the upper limit of your microplate reader, 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 standards and samples. The readings at 405 nm should not replace the readings for samples that were "in range" at 450 nm.

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Note 2: Manual washing: Aspirate wells and pipet 0.35 ml Wash Solution into each well. Aspirate wells and repeat twice. After final wash, invert and tap the plate strongly against paper towel. Make certain that Wash Solution has been removed entirely.

	strip 1+2	strip 3+4	strip 5+6	strip 7+8	strip 9+10	strip 11+12
A	M-Std. 4 000	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
B	M-Std. 2 000	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
C	M-Std. 1 000	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
D	M-Std. 400	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
E	M-Std. 200	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
F	M-Std. 100	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
G	QC MOUSE	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
H	Blank	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40

Figure 1: Example of a work sheet when assaying mouse samples.

	strip 1+2	strip 3+4	strip 5+6	strip 7+8	strip 9+10	strip 11+12
A	R-Std. 4 000	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
B	R-Std. 2 000	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
C	R-Std. 1 000	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
D	R-Std. 400	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
E	R-Std. 200	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
F	R-Std. 100	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
G	QC RAT	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
H	Blank	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40

Figure 2: Example of a work sheet when assaying rat samples.

12 CALCULATIONS

Most microplate readers perform automatic calculations of analyte concentration. The standard curve is constructed by plotting the mean absorbance (Y) of Standards against the known concentration (X) of Standards in logarithmic scale, using the four-parameter algorithm. Results are reported as concentration of leptin (pg/ml) in samples.

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Alternatively, the logit log function can be used to linearize the standard curve, i.e. logit of the mean absorbance (Y) is plotted against log of the known concentration (X) of Standards.

The measured concentration of samples calculated from the standard curve must be multiplied by their respective dilution factor, because samples have been diluted prior to the assay, e.g. 500 pg/ml (from standard curve) x 20 (dilution factor) = 10 000 pg/ml = 10 ng/ml.

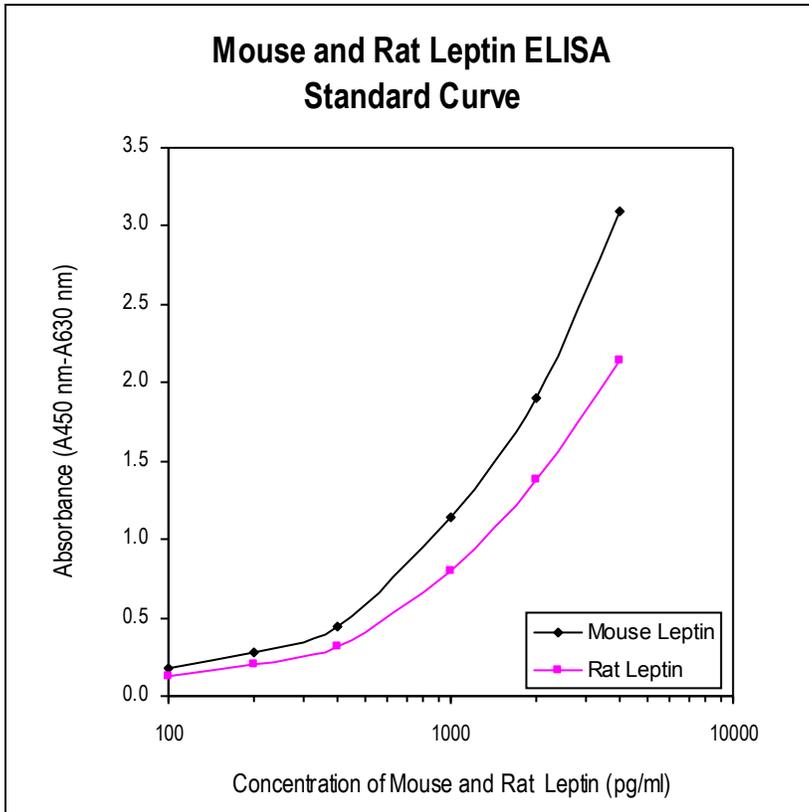


Figure 3: Typical Standard Curve for Mouse and Rat Leptin ELISA.

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13 PERFORMANCE CHARACTERISTICS

Typical analytical data of Mouse/Rat Leptin ELISA are presented in this chapter

13.1 Sensitivity

Limit of Detection (LOD), defined as concentration of analyte giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: $A_{\text{blank}} + 3 \times \text{SD}_{\text{blank}}$, is calculated from the real leptin values in wells and is 30 pg/ml for mouse leptin and 50 pg/ml for rat leptin.

*Dilution Buffer is pipetted into blank wells.

13.2 Limit of assay

Results exceeding leptin level of 4 000 pg/ml should be repeated with more diluted samples (e.g 40x). Dilution factor needs to be taken into consideration in calculating the leptin concentration.

13.3 Specificity

Sera of several mammalian species were measured in the assay. See results below.

For details please contact us at drg@drg-diagnostics.de.

Mammalian serum sample	Observed cross reactivity
Bovine	no
Cat	no
Dog	no
Goat	no
Hamster	no
Horse	no
Human	yes
Monkey	no
Mouse	yes
Pig	no
Rabbit	no
Rat	yes

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Presented results are multiplied by respective dilution factor

13.4 Precision

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

Sample	Mean (ng/ml)	SD (ng/ml)	CV (%)
1 mouse	12.31	0.25	2.0
2 mouse	31.48	0.91	2.9
3 rat	9.74	0.18	1.8
4 rat	39.96	0.75	1.9

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

Sample	Mean (ng/ml)	SD (ng/ml)	CV (%)
1 mouse	21.32	0.48	2.3
2 rat	17.13	0.76	4.4

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13.5 Spiking Recovery

Serum samples were spiked with different amounts of mouse or rat leptin and assayed.

Sample	Observed (ng/ml)	Expected (ng/ml)	Recovery O/E (%)
1 mouse	12.02	-	-
	15.19	16.02	94.8
	18.46	20.02	92.2
	25.90	28.02	92.4
2 mouse	19.56	-	-
	21.86	23.56	92.8
	24.04	27.56	87.2
	32.03	35.56	90.8
3 rat	9.32	-	-
	13.33	13.32	100.1
	16.29	17.32	94.1
	25.47	25.32	100.6
4 rat	19.61	-	-
	22.41	23.61	94.9
	25.25	27.61	95.1
	34.83	35.61	97.8

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13.6 Linearity

Serum samples were serially diluted with Dilution Buffer and assayed.

Sample	Dilution	Observed (ng/ml)	Expected (ng/ml)	Recovery O/E (%)
1 mouse	-	34.77	-	-
	2x	16.78	17.39	96.5
	4x	8.35	8.69	96.0
	8x	4.06	4.35	93.4
2 mouse	-	23.44	-	-
	2x	11.69	11.72	99.7
	4x	5.85	5.86	99.8
	8x	2.77	2.93	94.6
3 rat	-	29.67	-	-
	2x	14.53	14.83	98.0
	4x	7.11	7.42	95.8
	8x	3.91	3.71	105.5
4 rat	-	40.78	-	-
	2x	19.86	20.39	97.4
	4x	9.84	10.19	96.6
	8x	5.23	5.10	102.7

14 DEFINITION OF THE STANDARD

Mouse and Rat leptin standards are recombinant *E.coli* expressed proteins.

Mouse leptin standard concentration was determined using the international standard: Leptin, mouse rDNA-derived 1st International Standard NIBSC code 97/626, Version 02, dated April 19 2004.

No rat leptin international standard is available.

15 METHOD COMPARISON

The Mouse and Rat Leptin ELISA was not compared to the other commercial immunoassays.

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16 TROUBLESHOOTING AND FAQs

• **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
- Improper wavelength when reading absorbance

• **High signal and background in all wells**

- Possible explanations:
- Improper or inadequate washing
- Overdeveloping; incubation time with Substrate Solution should be decreased before addition of Stop Solution
- Incubation temperature over 30°C

• **High coefficient of variation (CV)**

- Possible explanation:
- Improper or inadequate washing
- Improper mixing Standards, Quality Controls or samples

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17 REFERENCES /LITERATURE

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18 ASSAY PROCEDURE SUMMARY

