User Guide

Mouse Leptin ELISA Kit

96-Well Plate

EZML-82K

Intended Use

This kit is used for the non-radioactive quantification of leptin in mouse sera. Plasma samples may also be used but application to samples of other biological fluids may need validation by the user. One kit is sufficient to measure 37 unknown samples in duplicate.

For Research Use Only. Not for Use in Diagnostic Procedures.

Principles of Assay

This assay is a Sandwich ELISA based, sequentially, on:

- Binding of leptin in the sample by a pre-titered antiserum and immobilization of the resulting complexes in the wells of a microtiter plate
- after washing purified biotinylated detection antibody is allowed to bind to the immobilized leptin
- binding of horseradish peroxidase to the immobilized biotinylated antibodies after free detection antibodies are washed off
- wash away of free enzyme conjugates
- quantification of immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of the substrate 3,3',5,5'-tetramethylbenzidine

The enzyme activity is measured spectrophotometrically by the increased absorbency at 450 nm, corrected from the absorbency at 590 nm, after acidification of formed products. Since the increase in absorbency is directly proportional to the amount of captured leptin in the unknown sample, the latter can be derived by interpolation from a reference curve generated in the same assay with reference standards of known concentrations of mouse leptin.

Reagents Supplied

Each kit is sufficient to run one 96-well plate and contains the following reagents:

Note: Store all reagents at 2-8 °C.

Reagents Supplied	Volume	Quantity	Cat. No.
Rat/Mouse Leptin Microtiter Plate with 2 plate sealers **Note:** Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8 °C.	-	1 plate 2 sealers	EP83
Rat/Mouse Leptin Antiserum	6 mL	-	EAS83
10X HRP Wash Buffer Concentrate 10X concentrate of 50 mM Tris Buffered Saline containing Tween 20®	50 mL	2 bottles	EWB-HRP
Mouse Leptin ELISA Standard Mouse leptin in buffer: 30 ng/mL **Note:** The standard(s) in this kit have been calibrated to an International Reference standard, NIBSC code # 97/626.	0.5 mL	1 vial	E8082-K
Quality Controls 1 and 2	0.5 mL	1 vial each	E6082-K
Rat/Mouse Leptin Matrix Solution	0.5 mL	1 vial	EPS0016
Assay Buffer 0.05 M phosphosaline, pH 7.4, containing 0.025 M EDTA, 0.08% sodium azide, 0.05% Triton™ X-100 and 1% BSA	40 mL	1 vial	EAB-PTR
Rat/Mouse Leptin Detection Antibody Pre-titered biotinylated anti-mouse leptin antibody	12 mL	1 vial	E1083
Enzyme Solution Pre-titered streptavidin-horseradish peroxidase conjugate in buffer	12 mL	1 vial	EHRP-4
Substrate Solution 3,3′,5,5′-tetramethylbenzidine in buffer (Light Sensitive: avoid unnecessary exposure to light)	12 mL	1 vial	ESS-TMB2

Reagents Supplied	Volume	Quantity	Cat. No.
Stop Solution **Caution:** Corrosive Solution 0.3 M HCl		12 mL/vial	ET-TMB
10-pack of Mouse Leptin ELISA kits			EZML-82BK

Storage and Stability

Recommended storage for kit components is 2-8 °C.

All components are shipped and stored at 2-8 °C. Once opened, liquid standards and controls can be stored up to 30 days at 2-8 °C. Refer to expiration dates on all reagents prior to use. Do not mix reagents from different kits unless they have the same lot numbers.

Reagent Precautions

Sodium Azide

Sodium azide has been added to certain 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 explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Hydrochloric Acid

Hydrochloric acid is corrosive and can cause eye and skin burns. It is harmful if swallowed and can cause respiratory and digestive tract burns. Avoid contact with skin and eye. Do not swallow or ingest.

Note: See Full Labels of Hazardous components on next page.

Symbol Definitions

Ingredient	Cat. No.	Label	
Rat Leptin Detection Antibody	E1083		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Mouse Leptin Quality Control 1 & 2	E6082-K		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Mouse Leptin Standards	E8082-K		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Antiserum	EAS83		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Wash Buffer Concentrate	EWB-HRP		**Warning.** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.
Stop Solution	ET-TMB		**Warning.** May be corrosive to metals.

Materials Required (Not Provided)

- Pipettes and pipette tips: 10 µL-20 µL and 20 µL-100 µL
- Multi-channel Pipettes and pipette tips: 0-50 µL and 50-300 µL
- Buffer and Reagent Reservoirs
- Vortex Mixer
- De-ionized Water
- Microtiter Plate Reader capable of reading absorbency at 450 nm and 590 nm
- Orbital Microtiter Plate Shaker
- Absorbent Paper or Cloth

Sample Collection and Storage

Preparation of Serum Samples

1. To prepare serum samples, whole blood is directly drawn into a centrifuge tube that contains no anti-coagulant. Let blood clot at room temperature for 30 min.
2. Promptly centrifuge the clotted blood at 2,000 to 3,000 x *g* for 15 minutes at 4 ±2 °C.
3. Transfer and store serum samples in separate tubes. Date and identify each sample.
4. Use freshly prepared serum or aliquot and store samples at –20 °C for later use. Avoid multiple (> 3) freeze/thaw cycles.

Preparation of Plasma Samples

1. To prepare plasma sample, whole blood should be collected into centrifuge tubes containing enough K3EDTA to achieve a final concentration of 1.735 mg/mL and centrifuge immediately after collection. Observe the same precautions in the preparation of serum samples.
2. If heparin is to be used as anti-coagulant, the effect on the assay outcome at the dose of heparin used should be pre-determined.
3. Avoid using samples with gross hemolysis or lip.

Reagent Preparation

1. Use care in opening the Standard vial.
2. Label seven tubes 1, 2, 3, 4, 5, 6 and 7. Add 0.20 mL Assay Buffer to each of the seven tubes. Prepare serial dilutions by adding 0.20 mL of the 30 ng/mL standard to tube 1, mix well and transfer 0.20 mL of tube 1 to tube 2, mix well and transfer 0.20 mL of tube 2 to tube 3, mix well and transfer 0.20 mL of tube 3 to tube 4, mix well and transfer 0.20 mL of tube 4 to tube 5, mix well and transfer 0.20 mL of tube 5 to tube 6, mix well and transfer 0.20 mL of tube 6 to tube 7 and mix well.

Note: Do not use a Repeater pipette. Change tip for every dilution. Wet tip with standard before dispensing. Unused portions of standard should be stored at ≤ –20 °C. Avoid multiple freeze/thaw cycles.

Tube #	Volume of Assay Buffer to Add	Volume of Standard to Add	Standard Concentration (ng/mL)
Tube 1	-	0.5 mL of 30 ng/mL	30.00
Tube 2	0.20 mL	0.2 mL of Tube 1	15.00
Tube 3	0.20 mL	0.2 mL of Tube 2	7.50
Tube 4	0.20 mL	0.2 mL of Tube 3	3.75
Tube 5	0.20 mL	0.2 mL of Tube 4	1.88
Tube 6	0.20 mL	0.2 mL of Tube 5	0.94
Tube 7	0.20 mL	0.2 mL of Tube 6	0.47
Tube 8	0.20 mL	0.2 mL of Tube 7	0.23

Assay Procedure

Pre-warm all reagents to room temperature immediately before setting up the assay.

1. Dilute the 10X concentrated HRP Wash Buffer 10-fold by mixing the entire contents of both buffer bottles with 900 mL de-ionized or distilled water.
2. Remove the required number of strips from the Microtiter Assay Plate. Unused strips should be resealed in the foil pouch and stored at 2-8 °C. Assemble strips in an empty plate holder and wash each well 3 times with 300 µL of diluted Wash Buffer per wash. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times. Do not let wells dry before proceeding to the next step. If automated machine is used for assay, follow the manufacturer's instructions for all washing steps described in this protocol.
3. Add 30 µL Assay Buffer to Background wells, Standard wells, and QC1 and QC2 wells. Add 40 µL Assay Buffer to sample wells.
4. If samples to be assayed are serum or plasma, add 10 µL Matrix Solution to the Background wells, Standard wells, and QC1 and QC2 wells. If samples are free of significant serum matrix components, add 10 µL Assay Buffer instead.
5. Add 10 µL Assay Buffer to the Background wells and add in duplicates 10 µL Mouse Leptin Standards in the order of ascending concentration to the appropriate wells.
6. Add 10 µL QC1 and 10 µL QC2 to the appropriate wells.
7. Add sequentially 10 µL of the unknown samples in duplicate to the remaining wells.
8. Transfer Antiserum Solution to a reagent reservoir and add 50 µL of this solution to each well with a multi-channel pipette. Cover the plate with plate sealer and incubate at room temperature for 2 hours on an orbital microtiter plate shaker set to rotate at moderate speed, about 400 to 500 rpm.
9. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well.
10. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.
11. Add 100 µL Detection Antibody to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 1 hour on an orbital microtiter plate shaker set to rotate at moderate speed, approximately 400-500 rpm.
12. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well.
13. Wash wells 3 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

14. Add 100 µL Enzyme Solution to each well. Cover plate with sealer and incubate with moderate shaking at room temperature for 30 minutes on the micro-titer plate shaker.

15. Remove plate sealer and decant solutions from the plate. Tap as before to remove residual solutions in well.

16. Wash wells 6 times with diluted Wash Buffer, 300 µL per well per wash. Decant and tap after each wash to remove residual buffer.

17. Add 100 µL of Substrate solution to each well, cover plate with sealer and shake in the plate shaker for approximately 5 to 20 minutes. Blue color should be formed in wells of leptin standards with intensity proportional to increasing concentrations of leptin.

    Note: Please be aware that the color may develop more quickly or more slowly than the recommended incubation time depending on the localized room temperature. Please visually monitor the color development to optimize the incubation time. One can monitor color development using 370 nm filter, if available on the spectrophotometer. When the absorbance is between 1.2 and 1.8 at 370 nm, the stop solution can be added to terminate the color development.

18. Remove sealer and add 100 µL Stop Solution (**Caution**: Corrosive Solution) and shake plate by hand to ensure complete mixing of solution in all wells. The blue color should turn into yellow after acidification. Wipe the bottom of the ELISA plate before reading absorbance at 450 nm and 590 nm in a plate reader. Plate should be read within 5 minutes to ensure that there are no air bubbles in any well. Record the difference of absorbance units.

	Step 1	Step 2	Step 3	Step 4	Step 5-7	Step 8	Step 9-10	Step 11	Step 12-13	Step 14	Step 15-16	Step 17	Step 18		
Well #	Dilute both bottles of 10X Wash Buffer with 900 mL Deionized Water.	Wash plate 3X with 300 µL Wash Buffer. Remove residual buffer by tapping smartly on absorbent towels.	Assay Buffer	Matrix*	Standards/ Controls/ Samples	Antiserum	Seal, Agitate, Incubate 2 hours at Room Temperature. Wash 3X with 300 µL Wash Buffer.	Detection Antibody	Seal, Agitate, Incubate 1 hour at Room Temperature. Remove residual buffer by tapping smartly on absorbent towels. Wash 3X with 300 µL Wash Buffer.	Enzyme Solution	Seal, Agitate, Incubate 30 minutes at Room Temperature. Wash 6X with 300 µL Wash Buffer.	Substrate	Seal, Agitate, Incubate 5-20 minutes at Room Temperature.	Stop Solution	Shake by hand. Read Absorbance at 450 nm and 590 nm within 5 minutes.
A1, B1			40 µL	10 µL	-	50 µL		100 µL		100 µL		100 µL		100 µL	
C1, D1			30 µL	10 µL	10 µL of 0.23 ng/mL Std	50 µL									
E1, F1			30 µL	10 µL	10 µL of 0.47 ng/mL Std	50 µL									
G1, H1			30 µL	10 µL	10 µL of 0.94 ng/mL Std	50 µL									
A2, B2			30 µL	10 µL	10 µL of 1.88 ng/mL Std	50 µL									
C2, D2			30 µL	10 µL	10 µL of 3.75 ng/mL Std	50 µL									
E2, F2			30 µL	10 µL	10 µL of 7.5 ng/mL Std	50 µL									
G2, H2			30 µL	10 µL	10 µL of 15 ng/mL Std	50 µL									
A3, B3			30 µL	10 µL	10 µL of 30 ng/mL Std	50 µL									
C3, D3			30 µL	10 µL	10 µL of QC 1	50 µL									
E3, F3			30 µL	10 µL	10 µL of QC 2	50 µL									
G3, H3			40 µL	-	10 µL of Sample	50 µL									
A4, B4			40 µL	-	10 µL of Sample	50 µL									

* See Section VIII. Assay Procedure Step 4: If samples are free of significant matrix components, add 10 µL assay buffer instead.

Microtiter Plate Arrangement

Mouse Leptin ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	1.88 ng/mL	30 ng/mL	Sample 2								
B	Blank	1.88 ng/mL	30 ng/mL	Sample 2								
C	0.23 ng/mL	3.75 ng/mL	QC1	Etc.								
D	0.23 ng/mL	3.75 ng/mL	QC!	Etc.								
E	0.47 ng/mL	7.5 ng/mL	QC2									
F	0.47 ng/mL	7.5 ng/mL	QC2									
G	0.94 ng/mL	15 ng/mL	Sample 1									
H	0.94 ng/mL	15 ng/mL	Sample 1									

Calculations

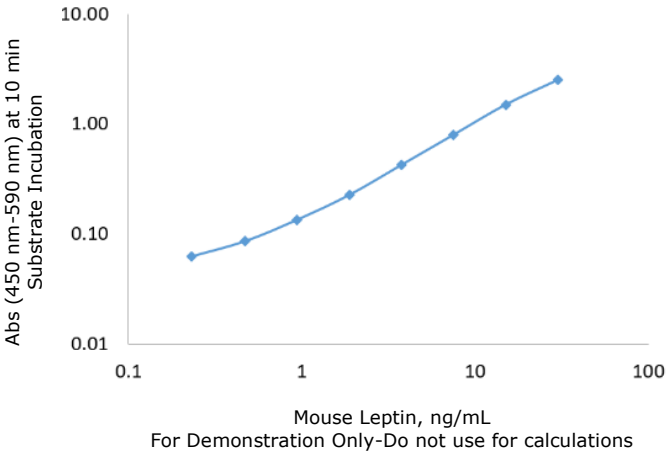
Graph a reference curve by plotting the absorbance unit of 450 nm, less unit at 590 nm, on the Y-axis against the concentrations of mouse leptin standard on the X-axis. The dose-response curve of this assay fits best to a sigmoidal 4- or 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4- or 5-parameter logistic function.

Note: When sample volumes assayed differ from 10 µL, an appropriate mathematical adjustment must be made to accommodate for the dilution factor (For example, if 5 µL of sample is used, then calculated data must be multiplied by 2). When sample volume assayed is less than 10 µL, compensate the volume deficit with either matrix solution or assay buffer, whichever is appropriate.

Interpretations

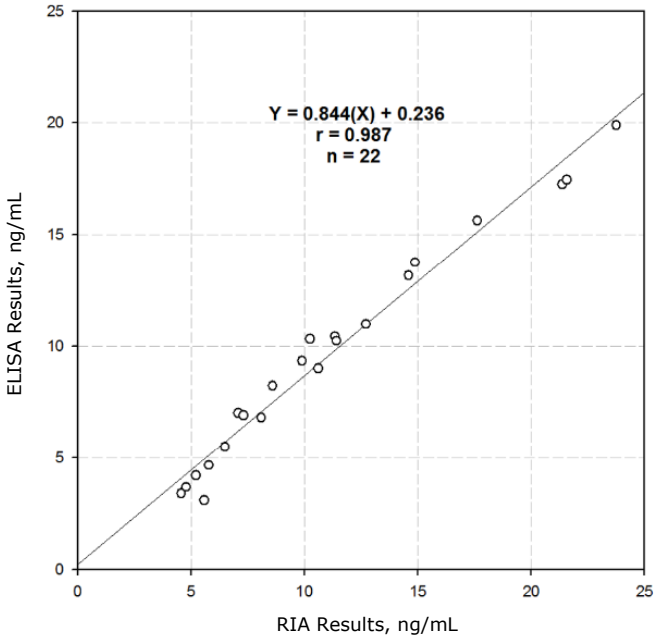
- If the difference between duplicate results of a sample is > 15% CV, repeat the sample.
- The limit of sensitivity of this assay is 0.05 ng/mL (~3.13 pM) leptin (10 µL sample size).
- The appropriate range of this assay is 0.23 ng/mL to 30 ng/mL leptin (10 µL sample size). Any result greater than 30 ng/mL in a 10 µL sample assayed should be repeated on dilution using either matrix solution or assay buffer, whichever is appropriate, as diluent until it falls within range.

Graph of Typical Reference Curve



Mouse Leptin Assays

Correlation of results by RIA and ELISA Methods



Serum samples from 22 mice were assayed for Leptin using Mouse RIA Kit (Cat. No. ML-82K and Mouse Leptin Kit ELISA Kit (Cat. No. EZML-82K). Correlation of the two kits are derived by linear regression analysis of paired results from each sample.

Assay Characteristics

Sensitivity

The lowest level of mouse leptin that can be detected by this assay is 0.05 ng/mL using a 10 µL sample size.

Specificity

The specificity (also known as selectivity) of the analytical test is its ability to selectively measure the analytes in the presence of other like components in the sample matrix.

Mouse Leptin	100%
Rat Leptin	70%
Human Leptin	9%
Porcine Leptin	< 0.05%
Ovine Leptin	< 0.05%
Chicken Leptin	< 0.05%
Rat Insulin	0%
Rat C-peptide	0%
Human Proinsulin	0%
Bovine Proinsulin	0%
Porcine Proinsulin	0%
Glucagon	0%
Human Ghrelin	0%

Precision

Sample	Mean Leptin Level (ng/mL)	Assay Variation (CV)	
		Intra-assay (%)	Inter-assay (%)
1	1.66	1.06	4.59
2	5.78	1.64	3.96
3	17.60	1.76	3.01

The assay variations of Mouse Leptin ELISA kit were studied on three mouse serum samples with varying concentrations of spiked analyte. The intra-assay variations are calculated from eight duplicate determinations in an assay. The inter-assay variations are calculated from results of 6 separate assays with duplicate samples in each assay.

Spike Recovery of Mouse Leptin in Mouse Serum

Serum Sample	Mouse Leptin		Recovery (%) of Spiked Insulin
	Added (ng/mL)	Observed (ng/mL)	
Mouse Serum 1	0	1.33	-
	0.5	1.83	100
	2.0	3.14	91
	10.0	10.04	87
Mouse Serum 2	0	1.76	-
	0.5	2.24	96
	2.0	3.49	87
	10.0	10.48	87

Mouse leptin at indicated levels was added to two pooled mouse serum samples and the resulting leptin content of each sample was assayed by ELISA. The % of recovery = [(observed leptin level after spike - observed leptin level before spike) / spiked level of leptin] x 100%. Mean recovery rate at spiked leptin level of 0.5, 2, and 10 ng/mL is 98%, 89%, and 87%, respectively.

Linearity of Sample Dilution

Serum Sample	Dilution Factor	Observed (ng/mL)	Expected (ng/mL)	% of Expected
1	-	16.43	16.43	100
	2x	16.42		100
	5x	16.60		101
	10x	17.50		107
	20x	17.20		105
2	-	16.44	16.44	100
	2x	16.56		101
	5x	17.60		107
	10x	17.70		108
	20x	18.20		111

Two leptin-spiked pooled mouse serum samples are diluted each with matrix solution to various degrees as indicated and assayed for leptin levels along with neat samples of each serum. Measured leptin levels are corrected for dilution factors and reported as observed leptin level.

Quality Controls

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert, or available at our website SigmaAldrich.com.

Troubleshooting

- To obtain reliable and reproducible results the operator should carefully read this manual and fully understand all aspects of each assay step before attempting to run the assay.
- Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
- Have all necessary reagents and equipment ready on hand before starting. Once the assay has been started all steps should be completed with precise timing and without interruption.
- Avoid cross contamination of any reagents or samples to be used in the assay.
- Make sure all reagents and samples are added to the bottom of each well.
- Careful and complete mixing of solutions in the well is critical. Poor assay precision will result from incomplete mixing or cross well contamination due to inappropriate mixing.
- Remove any air bubbles formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
- Do not let the absorbance reading of the highest standard fall beyond the limit of your microtiterplate reader's capacity. Adjust the length of substrate incubation time accordingly.
- High absorbance in background or blank wells could be due to:
    - cross well contamination by standard solution or sample and,
    - inadequate washing of wells with wash buffer.

Product Ordering

Products are available for online ordering at SigmaAldrich.com.

Replacement Reagents

Reagents	Catalogue Number
Rat/Mouse Leptin Microtiter Plate	EP83
Rat/Mouse Leptin Antiserum	EAS83
10X HRP Wash Buffer Concentrate	EWB-HRP
Mouse Leptin ELISA Standards	E8082-K
Quality Controls 1 and 2	E6082-K
Rat/Mouse Leptin Matrix Solution	EPS0016
Assay Buffer	EAB-PTR
Rat/Mouse Leptin Detection Antibody	E1083
Enzyme Solution	EHRP-4
Substrate	ESS-TMB2
Stop Solution	ET-TMB
Rat/Mouse Leptin Microtiter Plate	EP83
10-pack of Mouse Leptin ELISA kits	EZML-82BK

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