Rat Leptin

96-Well Plate Assay

Cat. # EZRL-83K

RAT LEPTIN ELISA KIT
96-Well Plate (Cat. # EZRL-83K)

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RAT LEPTIN ELISA KIT
96-Well Plate (Cat. #EZRL-83K)

I. INTENDED USE

This kit is used for the non-radioactive quantification of leptin in rat 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. ***This kit is for research purpose only.***

II. PRINCIPLES OF PROCEDURE

This assay is a Sandwich ELISA based, sequentially, on: 1) binding of leptin in the sample by a pre-titered antiserum and immobilization of the resulting complexes in the wells of a microtiter plate, 2) after washing purified biotinylated detection antibody is allowed to bind to the immobilized leptin, 3) binding of horseradish peroxidase to the immobilized biotinylated antibodies after free detection antibodies are washed off, 4) wash away of free enzyme conjugates, and 5) 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 590nm, 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 rat leptin.

III. REAGENTS SUPPLIED

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

A. Rat/Mouse Leptin ELISA Plate
Coated with pre-titered capture antibodies
Quantity: 1 plate
Preparation: Ready to use.
Note: Unused strips should be resealed in the foil pouch with the desiccant provided and stored at 2-8℃.

B. Adhesive Plate Sealer
Quantity: 2 sheets
Preparation: Ready to use.

C. Rat/Mouse Leptin Antiserum
Pre-titered anti-rodent leptin serum
Quantity: 6 mL
Preparation: Ready to use.

III. REAGENTS SUPPLIED (continued)

D. 10X HRP Wash Buffer Concentrate
10X concentrate of 50 mM Tris Buffered Saline containing Tween-20.
Quantity: Two bottles containing 50 mL each
Preparation: Dilute 1:10 with distilled or de-ionized water.

E. Rat Leptin Standards
Rat leptin in buffer: 0.2, 0.5, 1, 2, 5,10, 20 and 30 ng/mL.
Quantity: 0.5 mL/vial
Preparation: Ready to use.

F. Quality Controls 1 and 2
Various peptides including leptin in QC buffer.
Quantity: 0.5 mL/vial
Preparation: Ready to use.

G. Rat/Mouse Leptin Matrix Solution
Matrix containing 0.08% Sodium Azide
Quantity: 0.5 mL/vial
Preparation: Ready to use.

H. 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.
Quantity: 20 mL/vial
Preparation: Ready to use.

I. Rat/Mouse Leptin Detection Antibody
Pre-titered biotinylated anti-mouse leptin antibody.
Quantity: 12 mL/vial
Preparation: Ready to use.

J. Enzyme Solution
Pre-titered streptavidin-horseradish peroxidase conjugate in buffer.
Quantity: 12 mL/vial
Preparation: Ready to use.

K. Substrate (Light Sensitive: avoid unnecessary exposure to light)
3, 3',5,5'-tetramethylbenzidine in buffer.
Quantity: 12 mL/vial
Preparation: Ready to use.

L. Stop Solution (Caution: Corrosive Solution)
0.3 M HCl
Quantity: 12 mL/vial
Preparation: Ready to use.

IV. STORAGE AND STABILITY

All components of the kit can be stored up to two weeks at 2-8°C. For longer storage (>2 weeks), freeze antiserum, standards, quality controls, and matrix solution at ≤–20°C and avoid repeated freeze and thaw. Unused strips should be resealed in the foil pouch with the desiccant provided and stored 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.

V. REAGENT PRECAUTIONS

A. 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. On disposal, flush with large volume of water to prevent azide build up.

B. 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.

VI. MATERIALS REQUIRED BUT NOT PROVIDED

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

VII. SAMPLE COLLECTION AND STORAGE

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.

VII. SAMPLE COLLECTION AND STORAGE (continued)

5. To prepare plasma sample, whole blood should be collected into centrifuge tubes containing enough K_3EDTA 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.

6. 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.

7. Avoid using samples with gross hemolysis or lipemia.

VIII. 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 Rat 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.

VIII. ASSAY PROCEDURE (continued)

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 10 to 15 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. Read absorbance at 450 nm and 590 nm in a plate reader within 5 minutes and ensure that there is no air bubbles in any well. Record the difference of absorbance units.

Assay Procedure for Rat Leptin ELISA kit (Cat. # EZRL-83K)

	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 900mL 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 □l Wash Buffer**	Substrate	**Seal, Agitate, Incubate 10-15 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 0.2 ng/mL Standard	50 µL									
E1, F1			30 µL	10 µL	10 µL of 0.5 ng/mL Standard	50 µL									
G1, H1			30 µL	10 µL	10 µL of 1 ng/mL Standard	50 µL									
A2, B2			30 µL	10 µL	10 µL of 2 ng/mL Standard	50 µL									
C2, D2			30 µL	10 µL	10 µL of 5 ng/mL Standard	50 µL									
E2, F2			30 µL	10 µL	10 µL of 10 ng/mL Standard	50 µL									
G2, H2			30 µL	10 µL	10 µL of 20 ng/mL Standard	50 µL									
A3, B3			30 µL	10 µL	10 µL of 30 ng/mL Standard	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.

IX. MICROTITER PLATE ARRANGEMENT

Rat Leptin ELISA

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	2.0 ng/mL	30 ng/mL	Sample 2								
B	Blank	2.0 ng/mL	30 ng/mL	Sample 2								
C	0.2 ng/mL	5.0 ng/mL	QC 1	Etc.								
D	0.2 ng/mL	5.0 ng/mL	QC 1									
E	0.5 ng/mL	10 ng/mL	QC 2									
F	0.5 ng/mL	10 ng/mL	QC 2									
G	1.0 ng/mL	20 ng/mL	Sample 1									
H	1.0 ng/mL	20 ng/mL	Sample 1									

X. CALCULATIONS

Graph a reference curve by plotting the absorbance unit of 450nm, less unit at 590nm, on the Y-axis against the concentrations of rat 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 (e.g., 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.

XI. INTERPRETATION

1. The assay 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.

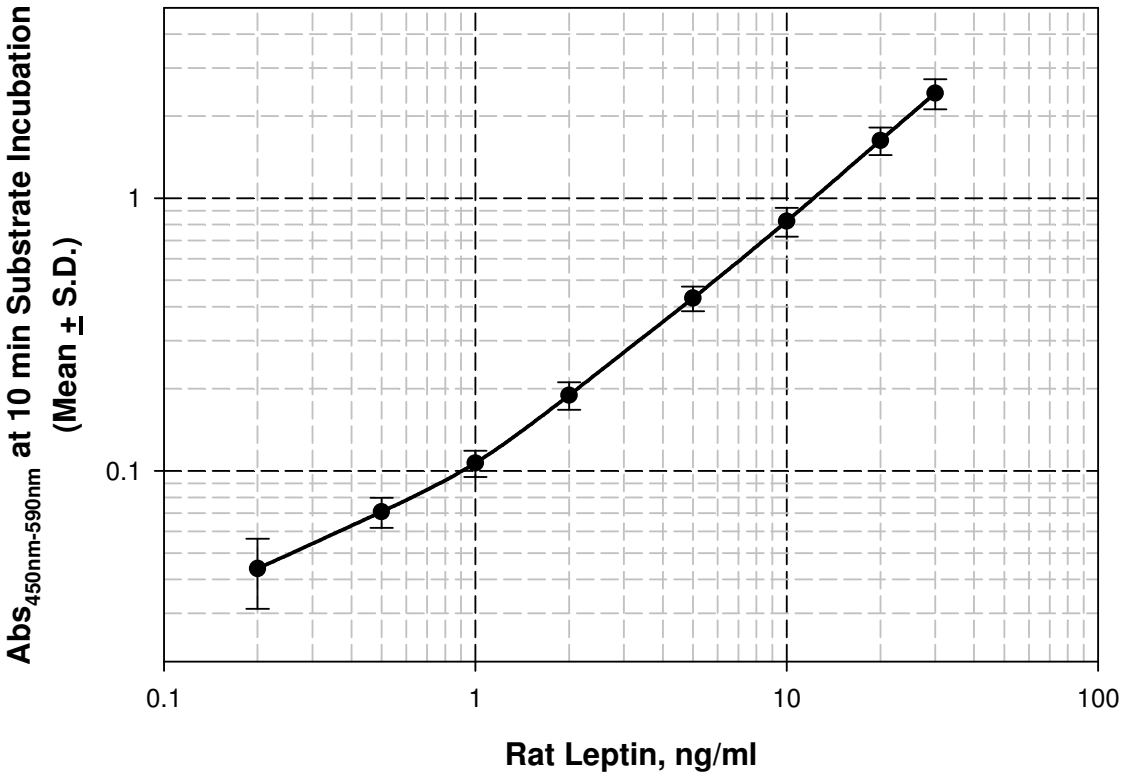
2. If the difference between duplicate results of a sample is >15% CV, repeat the sample.

3. The limit of sensitivity of this assay is 0.04 ng/mL (~2.5 pM) leptin (10 µL sample size).

4. The appropriate range of this assay is 0.2 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.

XII. GRAPH OF TYPICAL REFERENCE CURVE

(n = 9 assays)

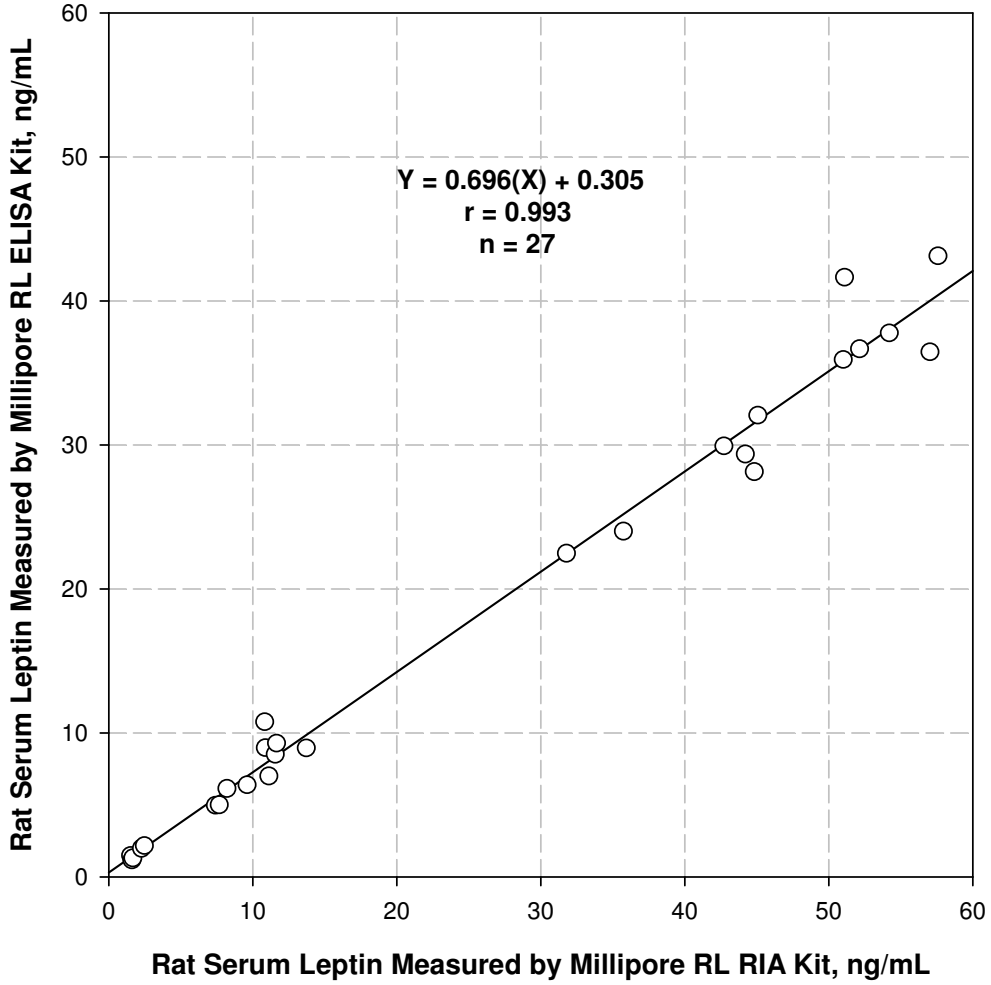


For Demonstration Only - Do not use for calculations

XIII. CORRELATION GRAPH

Rat Leptin Assays:

Correlation of Results by RIA and ELISA Methods



Serum samples from 27 rats were assayed for leptin using both Millipore Rat Leptin RIA Kit (Cat# RL-83K) and Rat Leptin ELISA Kit (Cat# EZRL-83K). Correlation of the two kits are derived by linear regression analysis of paired results from each sample.

XIV. ASSAY CHARACTERISTICS

A. Sensitivity

The lowest level of rat leptin that can be detected by this assay is 0.04 ng/mL using a 10 µL sample size.

B. 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.

Rat Leptin	100%
Mouse Leptin	143%
Human Leptin	15%
Porcine Leptin	< 0.1%
Ovine Leptin	< 0.1%
Chicken Leptin	< 0.1%
Rat Insulin	0%
Rat C-peptide	0%
Human Proinsulin	0%
Porcine Proinsulin	0%
Bovine Proinsulin	0%
Glucagon	0%
Human Ghrelin	0%

C. Precision

Sample Number	Mean Leptin Level (ng/mL)	Assay Variation (CV)	
		Intra-assay	Inter-assay
1	1.26	2.49 %	3.93 %
2	5.19	1.88 %	3.31 %
3	16.56	2.13 %	2.95 %

The assay variations of Millipore Rat Leptin ELISA kit were studied on three rat 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.

XIV. ASSAY CHARACTERISTICS (continued)

D. Recovery

Spike and Recovery of Rat Leptin in Rat Serum

Serum Sample #	Rat Leptin		Recovery (%) of Spiked Insulin
	Added (ng/mL)	Observed (ng/mL)	
Rat Serum # 1	0	5.00	--
	0.5	5.61	122
	2.0	7.29	115
	10.0	16.17	112
Rat Serum # 2	0	5.85	--
	0.5	6.36	102
	2.0	8.20	118
	10.0	16.93	111

Rat leptin at indicated levels was added to two rat 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 112%, 117%, and 112%, respectively.

E. Linearity

Effect of Serum Dilution

Serum Sample #	Dilution Factor	Leptin Level		
		Observed (ng/mL)	Expected (ng/mL)	% Of Expected
Rat Serum # 1	--	21.18	21.18	100
	2x	19.56		92
	5x	19.20		91
	10x	18.40		87
	20x	19.20		91
Rat Serum # 2	--	21.39	21.39	100
	2x	20.92		98
	5x	21.00		98
	10x	21.10		99
	20x	20.80		97

Two rat 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.

XV. QUALITY CONTROLS

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/bmia.

XVI. TROUBLESHOOTING GUIDE

1. 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.
2. Throughout the assay the operator should adhere strictly to the procedures with good laboratory practice.
3. 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.
4. Avoid cross contamination of any reagents or samples to be used in the assay.
5. Make sure that all reagents and samples are added to the bottom of each well.
6. 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.
7. Remove any air bubble formed in the well after acidification of substrate solution because bubbles interfere with spectrophotometric readings.
8. 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.
9. High absorbance in background or blank wells could be due to 1) cross well contamination by standard solution or sample and 2) inadequate washing of wells with wash buffer.

XVII. REPLACEMENT REAGENTS

Reagents	**Cat#**
Rat/Mouse Leptin ELISA Plates	EP83
Rat/Mouse Leptin Antiserum	EAS83
10X HRP Wash Buffer Concentrate (50 mL)	EWB-HRP
Rat Leptin Standards	E8083-K
Quality Controls 1 and 2	E6083-K
Rat/Mouse Leptin Matrix Solution	EPS0016
Assay Buffer	AB-PTRHK
Rat/Mouse Leptin Detection Antibody	E1083
Enzyme Solution	EHRP-4
Substrate	ESS-TMB2
Stop Solution	ET-TMB

XVIII. 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

TELEPHONE ORDERS:
Toll Free US (800) MILLIPORE
FAX ORDERS: (636) 441-8050
MAIL ORDERS: Millipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

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Material safety data sheets for Millipore products may be ordered by fax or phone. See Section A above for details on ordering.