Leptin (Mouse/Rat)
ELISA Kit Protocol

(Cat. No.:EK-003-17)

PHOENIX PHARMACEUTICALS, INC.

TABLE OF CONTENT

1. INTENDED USE

The Mouse and Rat Leptin ELISA is a sandwich enzyme immunoassay for the qunatative measurement of mouse and/or rat leptin in serum, plasma, and tissue culture medium. The total assay time is less than four hours. The kit measures total serum leptin. Quality controls are mopuse and rat serum based. No human sera are used. It is intended for in vitro and research only.

2. SUMMARY

Leptin is a protein hormone with important effects in metabolism and regulating body weight. It is a single-chain 16kDa protein consisting of 146 amino acid residues and encoded by the ovese (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 highle expressed. Leptin has a dual action, it decreases the appetite and increases energy consumption. A mutation 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 chracterized by several metavolic abnormalities including hyperglucocortico-idemia, hypergycemia, herinsulinemia and insulin resistance. When ob/ob mice are treated with injections of leptin, the 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 metabolsim and plays a significant role as an immunomodulator.

3. TEST PRINCIPLE

The Mouse and Rat Leptin ELISA, standards, quality controls and samples are incubated in microtitration wells coated with anti-mouse leptin antibody. After a thorough wash, biotin-labelled polyclonal anti-mouse leptin antibody is added to the wells and incubated with the immobilized antibody-leptin complex. After one-hour incubation and a next washing step, streptavidin-horseradish peroxidase conjugate is added and incubated for half an hour.

After the last washing step, the conjugate bound is allowed to react with the substrate (H_2SO_2-tetramethylbenzidine). The reaction is stopped by addition of acidic solution, and absorbance of the resulting yellow product is measure spectrophotometrically at 450nm. 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 use only.
- This kit contains components of animal origin.
- Avoid contact with the acidic Stop Solution and Substrate (TMB) Solution, which contains hydrogen peroxide. Wear gloves and eye protection when handling these reagents. In case of contact with the Stop Solution and the Substrate Solution wash skin thoroughy with water and seek medical attention, when necessary.
- Wear gloves and labratory coats when handling immunodiagnostic materials.
- The materials must no be pipetted by mouth.
- Do not drink, eat or smoke in the areas where immunodiagnostic materials are being handled.
- Reagents with different lot numbers should not be mixed.
- Reagents shoud not be used after the expiration date specified on the kit label.

5. REAGENTS SUPPLIED

- MICROTITER STRIPS (96 wells).....**EK-Plate 003-17**
  coated with Polyclonal Anti-mouse
  Leptin Antibody, vacuum sealed 1 Plate)

- BIOTIN-LABELLED ANTI-MOUSE.....**EK-D-003-17**
  LEPTIN ANTIBODY Concentrate (10x) (1 vial(1.3 ml))

- STREPTAVIDIN-HRP Conjugate.....**EK-HRP-003-17**
  1 vial (13 ml)
- MOUSE LEPTIN MASTER CALIBRATOR.....**EK-SM-003-17**
  (4000pg/ml) (1 vial)

- RAT LEPTIN MASTER CALIBRATOR.....**EK-SR-003-17**
  (4000pg/ml) (1 vial)

- QUALITY CONTROL- Mouse, Lyophilized...**EK-QCM-003-17**
  2 vials

- QUALITY CONTROL- Rat, Lyophilized.....**EK-QCR-003-17**
  2 vials

- DILUTION BUFFER **EK-DB-003-17**
  2 vials (13 ml)

- BIOTIN-Ab DILUENT.....**EK-BAD-003-17**
  1 vial (13 ml)

- WASH SOLUTION CONCENTRATE (10x).....**EK-WS-003-17**
  1 vial (100 ml)

- SUBSTRATE SOLUTION (TMB).....**EK-SS-003-17**
  1 vial (13 ml)

- STOP SOLUTION (0.2M H_2SO_4).....**EK-Stop-003-17**
  1 vial (13 ml)

6. MATERIALS REQUIRED BUT NOT SUPPLIED

- Test tubes for diluting standards and samples
- Precision pipettes to deliver 5-1000 µl
- Mutichannel pipette 100 µl
- Orbital microplate shaker capable of approximately 300rpm
- Software package facilitating data generation and analysis
- Microtitration plate washer (optional) [Manual washing is possible bot 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.
Biotin-Labelled Anitbody: Dilute the Biotin-Labelled Antibody with the Biotin-Ab Diluent 10-fold. Prepare only the volume needed. 100 µl of Biotin-Labelled Antibody + 900 µl of Biotin-Ab Diluent= 1 ml of the diluted Biotin-Labelled Anitbody is sufficient for 1 strip. The diluted Biotin-Labelled Antibody is stable for 1 month when stored at 2-8°C.
Streptavidin-HRP Conjugate: Ready-to-use
Dilution Buffer: Ready-to-use
Biotin-Ab Diluent: Ready-to-use
Wash Solution: Dilute the Wash Solution Concentrate 10-fold with deionized (distilled) water (e.g. 100ml of the Wash Buffer Concentrate + 900 ml of deionized water. Prepare only the volume needed. The diluted Wash Solution is stable up to 6 months when stored at 2-8°C.
Substrate Solution: Ready-to-use
Stop Solution: Ready-to-use

8. PREPARATION OF CALIBRATORS

Use Mouse Leptin Calibrators to quantify leptin concentration in mouse samples, Rat Leptin Calibrators to quantify leptin concentration in rat samples!

Mouse Leptin Calibrators

- **Preparation of Mouse Leptin Calibrator of 4000 pg/ml (M-Cal 4000pg/ml):** Add 1050 µl of DIlution buffer to the Mouse Leptin Master Calibrator vial and mix gently.
- **Preparation of all concentrations of mouse leptin calibrators:** DIlute the M-Cal 4000pg/ml with the Dilution Buffer as described below:

Volume of calibrator	Dilution Buffer	Concentration
M-Cal. 4000pg/ml	----	4000pg/ml
250µl M-Cal.4000pg/ml	250µl	2000pg/ml
250µl M-Cal.2000pg/ml	250µl	1000pg/ml
250µl M-Cal.1000pg/ml	250µl	500pg/ml
250µl M-Cal.500pg/ml	250µl	250pg/ml
250µl M-Cal.250pg/ml	250µl	125pg/ml
250µl M-Cal.125pg/ml	250µl	62.5pg.ml

Rat Leptin Calibrators

- **Preparation of the Rat Leptin Calibratorof 4000pg/ml (R-Cal 4000pg/ml):** Add 950µl Dilution Buffer to the Rat Leptin Master Calibrator vial and mix gently.
- **Preparation of all concentration of rat leptin calibrators:** Dilute the R-Cal 4000pg/ml with the Dilution Buffer as described below:

Volume of calibrator	Dilution Buffer	Concentration
R-Cal. 4000pg/ml	----	4000pg/ml
250µl R-Cal.4000pg/ml	250µl	2000pg/ml
250µl R-Cal.2000pg/ml	250µl	1000pg/ml
250µl R-Cal.1000pg/ml	250µl	500pg/ml
250µl R-Cal.500pg/ml	250µl	250pg/ml
250µl R-Cal.250pg/ml	250µl	125pg/ml
250µl R-Cal.125pg/ml	250µl	62.5pg.ml

Stability and Storage

250µl of each calibrator is sufficient to run the calibration curve in suplicates. Diluted calibrators can be aliquoted and stored at -20°*C* until next use (up to 6 months). Avoid repeating freezing/thawing cycles.

9. PREPARATION QUALITY CONTROLS

Reconstitution of Quality Controls: Add 250µl of the Dilution Buffer to the vial containing a lyophilized Quality Control (Mouse or Rat), let it dissolve for at least 15 minutes and mix thoroughly.
Stability and storage: The reconstituted control serum has to be used immediately or to be stored frozen. Avoid repeating.

10. PREPARATION OF SAMPLES

Dilute mouse or rat samples 20-fold with Dilution Buffer, e.g. 7µl sample +133µl Dilution Buffer when assaying sampls in singlets, or preferably 14µl sample + 266µl Dilution Buffer for duplicates. If expected concentration of leptin are very low, dilute samples only 1:10 with Dilution Buffer.
Stability and storage of samples:
Undiluted Serum samples should be stored frozen at -20°C.
Avoid repeating freezing/thawing to a maximum of 1 cycle.

11. ASSAY PROCEDURE

1. Pipet 100µl of Standards, Quality Control and diluted samples, preferably in duplicates, into the appropriate wells.
2. Incubate the plate at room temperature (ca. 20-28°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker.
3. Wash the wells 3-times with Wash Solution (350µl per well).
4. Add 100µl of the diluted Biotin-Labelled Anti-mouse Leptin Antibody Solution into each well.
5. Incubate the plate at room temperature (ca. 20-28°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker.
6. Wash the wells 3-times with Wash Solution (350µl oer well).
7. Add 100µl of Streptavidin-HRP Conjugate solution.
8. Incubate the plate for 30 minutes, shaking at ca. 300 rpm on an orbital microplate shaker at room temperature.
9. Wash the wells 3-times with Wash Solution (350µl oer well).
10. Add 100µl of Substrate Solution. (Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminum foil is recommended).

11. Incubate the plate to 10 minutes at room temperature. (The incubation time may be extended [up to 20 minutes] it the reac tion temperature is below 20°C).
12. Stop the color development completely by adding 100µl of Stop Solution.
13. Determine the absorbance by reading the plate at 450 nm (op tionally, to measure in dual wavelength mode 620-650nm filter can be used to measure the reference absorbance. The absor bance should be read within 5 minutes following step 12).

Note 1: *If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standar, perform a second reading at 405nm. A new standard curve, constructed using the calues measure at 405nm, is used to determine Leptin concentration of off-scale samples. The readings at 405nm should not replace the on-scale readings at 450nm.*

Note 2: *Manual Washing: Aspirate wells and pipet 350µl Wash Solution into each well. Aspirate wells and repeat twice. After the final wash, invert and tap the plate strongly against absorbent paper or paper towel. Make certain that Wash Solution has been removed entirely.*

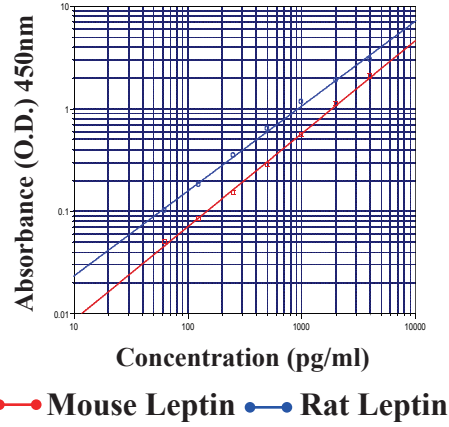
12. CALCULATIONS

Plot the standard curve on log-log graph paper. Known concentrations of Rat and Mouse Calibrators and its corresponding O.D. reading is plotted on the log scale (X-axis) and the log scale (Y-axis) respectively. The standard curve shows a correlated relationship between Mouse/Rat Leptin concentrations and the corresponding O.D. absorbance. As the standard concentration increases, the intensity of the blue color increases, and in turn the O.D. absorbance increases.

The concentration of Rat and Mouse Leptin in a sample is determined by plotting the sampl's O.D. on the Y-axis, then drawing a horizontal line to intersect with the standard curve. A vertical line dropped from this point will intersect the X-axis at a coordinate corresponding to the Rat and Mouse Leptin concentration in the unknown sample.

Refer to QC data sheet for acceptable values of the positive control.

Figure 1: Typical Rat/Mouse Leptin Standard Curve



13. LIMITS OF THE ASSAY

Results exceeding 4000pg/ml should be repeated with more dilued sample (e.g. 1:40).

14. STORAGE, EXPIRATION

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

15. SUMMARY OF ASSAY PROTOCOL

Prepare Calibrators, QC and Diluted Samples (1:20)
↓
Add 100µl per well of Calibrators, QC and Samples
↓
Incubate at room temperature for 1 hour
↓
Wash immunoplate 4 times with 300-350µl/well of Wash Solution
↓
Add 100µl per well of diluted Biotin-Labelled Antibody
↓
Incubate at room temperature for 1 hour
↓
Wash immunoplate 4 times with 300-350µl/well of Wash Solution
↓
Add 100µl/well of Streptavidin-HRP Conjugate
↓
Incubate at room temperature for 30 minutes
↓
Wash immunoplate 4 times with 300-350µl/well of Wash Solution
↓
Add 100µl/well of Substrate Solution (TMB)
↓
Incubate at room temperature for 10 minutes avoiding the expsure of the microplate from light
↓
Terminate reaction with 100µl/well of Stop Solution
↓
Read absorbance at 450nm and calculate results

16. REFERENCES

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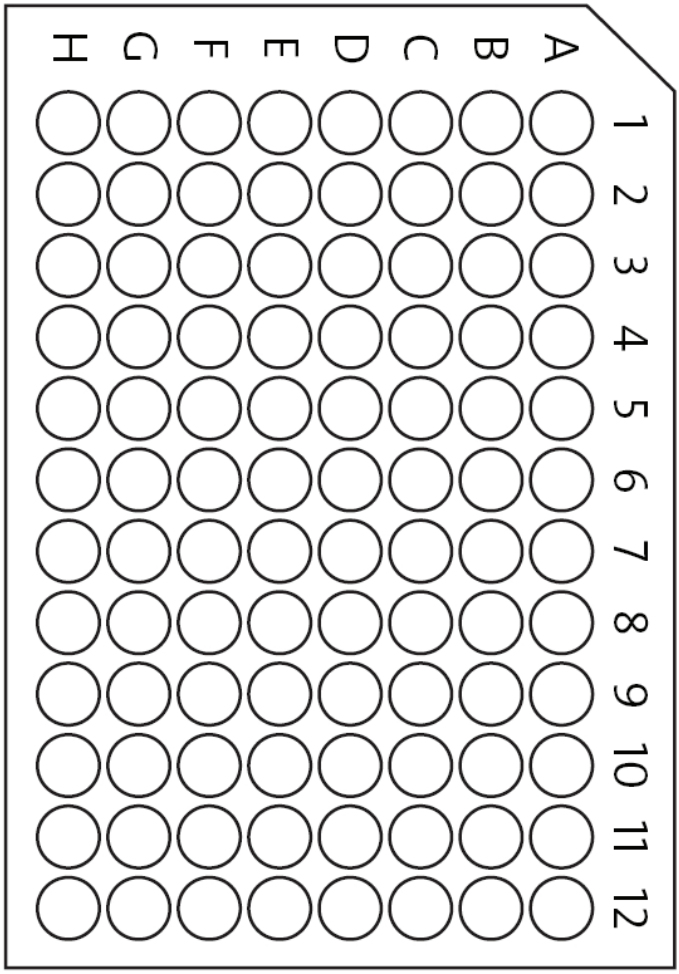
ASSAY DIAGRAM



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