Leptin (Human)
ELISA Kit Protocol

(Cat. No.: EK-003-12)

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INTRODUCTION AND PROTOCOL OVERVIEW

Leptin is a prtein hormone with imprtant effects in regulating body weight, metabolism and reproductive function. The protein is approximately ~16kDa in mass and encoded by the obese (ob) gene. Leptin is expressed predominantly by adipocytes, which fits with the idea that body weight is sensed as the total mass of fat in the body. Leptin is an important component in the long-term regulation ofbody weight. Its effects on body weight are mediated through the hypothalamic in which Leptin receptors are highly expressed and known to be important in regulating body weight. Recent studies with obese and non-obese humans demopnstrated a strong positive correlation of serum Leptin concentrations with perntage of body fat. Daily injections of recombinant mouse or human Leptin into ob/ob mice (i.e. the obese mutants unable to synthesize Leptin) led to a dramatic reduction in food intake within a few days, and roughly a 50% reduction in body weight within a month. The weight loss resulting from administration of Leptin apprears to results from a conbination pf decreased junfer and food consumption and increased energy expenditure. As expected, injuections of Leptin into db/db mice, which lack the Leptin receptor, had no effect. When Leptin was given to normal mice, they lost weight, and showed profound depletion of adipose tissue. However, the mechanisms by which Leptin excerts its effects on metabolism are largely unknown.

Phoenix Pharmaceutical's Human Leptin ELISA Kit is designed to measure the concentration of Human Lεptin from Human serum/plasma, or conditioned medium.

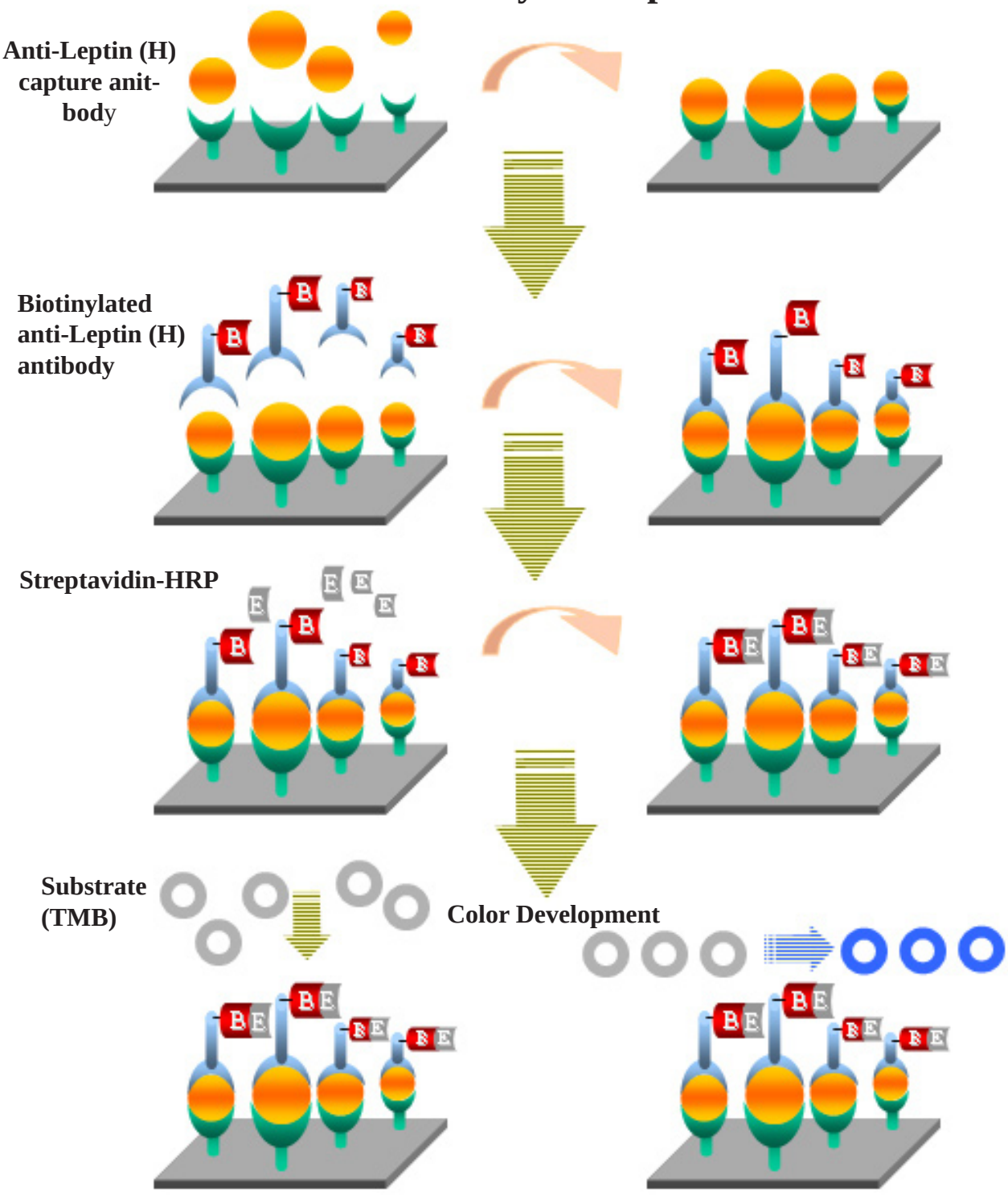
The immunoplate in this kit is pre-coated with Anti-Human Leptin Capture Antibody and the non-specific binding sites are blocked. The Human Leptin in the sample or in the standard solution can bind to the capture antibody immobilized in the wells. After washing procedure, the biotinylated anti-Human Leptin Detection Antibody which can bind to the Human Leptin trapped in the wells is added. The enzyme-substrate reaction is terminated by the addition of a stop solution. The intensity of the color is directly proportional to the amount of Human Leptin in the standard solutions or samples. A standard curve of Human Leptin with known concentration can be established accordingly. The Human Leptin with unknon concentration in samples can be determined by extrapolation to this standard curve.

ASSAY CONDITIONS:

Plasma, serum, culture media, tissue homogenate, CSF, urine or any biological fluid can be assayed as long as the level of the sample is high enough for the sistivity of the kit to detect it.

CAUTION: Phoenix Pharmaceuticals gurantees that its products conform to the information contained in this publication. The purchaser must determine the suitability of the product for thier particular needs and establish optimum sample concentrations.

Assay Principle



LIST OF COMPONENTS

Store all components at 4C. DO NOT FREEZE.

1. 20X Assay Buffer concentrate (50ml).....Catalog no. EK-BUF

2. 96 Well Anti-Human Leptin.....Catalog no. EK-Plate-003-12
   Capture Antibody-Coated Plate (1Plate)

3. Human Leptin Standard.....Catalog no. EK-S-003-12
   (100ng/ml)

4. Biotinylated Anti-Human Leptin.....Catalog no.EK-D-003-12
   Detection Antibody (1 vial)

5. Human Leptin.....Catalog no.EK-PC-003-12
   Positive Control (2 vials)

6. Streptavidin-horseradish peroxidase.....Catalog no. EK-SA-HRP
   (SA-HPR) (30ul)

7. Substrate Solution (TMB) (12ml).....Catalog no. EK-SS

8. Stop Solution 2N HCl (15ml).....Catalog no. EK-HCl

9. Acetatae plate sealer (APS) (3 pieces).....Catalog no. EK-APS

10. Assay Diagram (1 sheet)

MATERIAL REQUIRED BUT NOT SUPPLIED

-Micropippettor (s) and disposiable pipette tips
-Multi-channel pipette capable of dispensing 50-100µl
-Solution reservior (recommeded)
-Microtiter plate washer (recommended)
-Orbital plate shaker capable of 300-500rpm (recommended)
-Microtiter plate reader capable of absorbance measurement between 450nm-650nm
-Well-closed containers (15ml tubes or more in capacity)
-Aborbent material for blotting

REAGENT PREPARATION

Note: T***he kit should be equilibrated to room temperature (20-23°C) before opening any vials and starting the assay. It is highly recommended that the solutions be used as soon as possible after rehydration.***

1. **1x Assay buffer:** Dilute the 20x assay buffer concentrate with 950ml of distilled water. This assay buffer will be used to wash the plate and reconstitute all of the other com poundsin this kit. If crystals are observed in the 20x Assay buffer, warm the bottle in a 37°C water bath for approximately 30 minutes or until the crystals disappear. After prepa ration, store **1x** Assay buffer at 4°C.

2. **Biotinylated anti Leptin Detection Antibody**: Rehydrate biotinylaerd anti-Human Leptin detection antibody with 100µl of **1x** assay buffer, vortex (centrifuge the tube to dislodge powder from the cap or walls). Dilute biotinylated Leptin Detection Antibody to 1:160 and mix thoroughly before use.

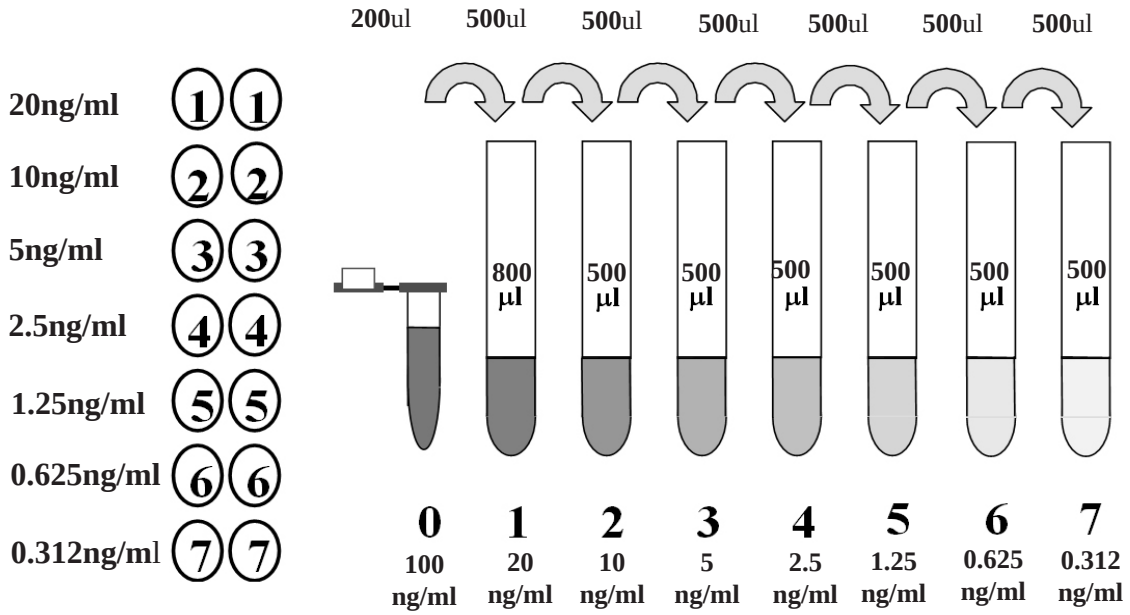
3. **Streptavidin-Horseradish Peroxidase (SA-HRP):** Centrifuge the HRP vial (30µl) pro- vided in this kit (3,000-5,000 rpm, 5 seconds) and dilute SA-HRP with **1x** assay buffer to 1:2000 before use. Vortex thoroughly.

4. **Human Leptin Positive Control:** Rehydrate Human Leptin Positive Control with 250µl of **1x** assay buffer (centrifuge the tube to dislodge powder from cap or walls). Vortex thoroughly.

HUMAN LEPTIN STANDARD PREPARATION

1. Rehydrate recombinant Leptin standard with 1ml **1x** assay buffer, vortex. Allow the solution to sit at least 10 moinutes at toom temperature (20-23°C) to completely dis solve in solution. Vortex and centrifuge before use. The concentration of this stock solu tion is 8ng/ml.
2. Prepare Human Leptin standard solutions as follows:

Standard No.	Std. volume	Assay Buffer	Concentrations
Stock	Powder	1000µl	100ng/ml
#1	200µl of stock	800µl	20ng/ml
#2	500µl of #1	500µl	10ng/ml
#3	500µl of #2	500µl	5ng/ml
#4	500µl of #3	500µl	2.5ng/ml
#5	500µl of #4	500µl	1.25ng/ml
#6	500µl of #5	500µl	0.625ng/ml
#7	500ul of #6	500ul	0.312ng/ml



HUMAN LEPTIN ELISA PROTOCOL

1. Thoroughly read this protocol before performing an assay.
2. Remove Capture Antibody-Coated Plate from its zip-lock foil pouch. Remove any un-eeded strips from the plate frame, reseal them in the foil pouch, and return the foil pouch to 4°C.
3. Wash each well with 300µl of **1x** assay buffer. Allow to sit for at least 5 minutes. Discard the buffer, invert and blot dry plate. Do not let wells dry before proceeding to the next step.
4. Leave wells A-1 and A-2 empty as **Blank**.

5. Add 100µl of the prepared Human Leptin Standard solutions from #7 to #1 (reverse order of serial dilution) in duplicate to wells B-1 and B-2 to H-1 and H-2, respec tively.
6. Add 100µl of Human Leptin positive control solution in duplicate.
7. Add 100µl diluted samples in duplicate into their designated wells.
8. Seal the immunoplate with acetate plate sealer (APS). Incubate for 2 hours at toom temperature (20-23°C) on a plate shaker (300-400rpm).
9. Before washing the plate, remove the plate sealer carefully. Completely discard the liq uid from wells. Wash each well with 300-350µl assay buffer four times. At the end of the wash, discard the buffer, invert the plate and tap on a clean absorbent towel.
10. Add 100µl biotinluated anti-Leptin Detection Antibody into each well **except the Blank** well. Reseal the immunplate with plate sealer and incubate for 2 hours at room temperature (20-23°C) on a plate shaker (300-400 rpm).
11. Wash 4 times with the **1x** assay buffer as described in step 9.
12. Add 100µl SA-HRP solution into each well. Reseal the immunplate with plate sealer and incubate the plate for 30 minutes at toom temperature (20-23°C) on a plate shaker (300-400 rpm).
13. Wash 4 times with the **1x** assay buffer as described in step 9.
14. Add 100µl substrate soution (TMB) provided in this kit into each well. Reseal the plate with plate sealer to protect from light and incubate the plate for 20-30 minutes at room temperature (20-23°C) on a plate shaker (300-400 rpm).
15. Add 100µl Stop Solution (2N Hydrochloric Acid) into each well to stop the reaction. The color in the well should change from blue to yellow. If the color change does not appear to be unifrom, gently tap the plate to ensure through mixing. Go to the next step within 20 minutes.
16. Read the absorbance O.D. at 450 nm using a Microtiter Plate Reader.

ADDITIONAL RECOMMENDED PROCEDURAL NOTES:

- Reagents of different lot numbers should not be mixed.
- Recheck the reagent labels when loading the plate to ensure that everything is added correctly.
- Unused microplate strips should be placed back in the foil pouch with a dessicant and stored at 4°C. Do not allow moisture to enter the wells.
- When handling the plate, avoid touching the bottom.
- Manual washing may cause high duplicate coefficient variations. To reduce this factor, liquid from the plate should be removed by inverting and blotting the plate on an absorbent material.
- If the room temperature is not within the suggested range (20-23°C), variations in results may occur.
- The same reservior for the reagents may be reused if the reservoir is washed well with distilled water before each use.
- Each laboratory must determine the appropirate dilution factors for the samples to be measured to ensure that the samples are within the dynamic range of the standard curve.
- High levels of interfering proteins may cause variations within the sample results; therefore, it is imperative to select the appropriate sample preparation procedure to obtain the optimal results.
- Each time a new tip is used , make sure the tip is secure and free of air bubbles. For better intra-assay variation, aspirate and expel a reagent or sample back into the container a few times prior to loading.
- Avoid submerging the whole tip into reagents because droplets can accumulate at the end of the tip causing an excess of reagent to be loaded into the well. This can lead to poor results.
- A muti-channel pipette is **NOT** recommended to load the biotinylated detection antibody or standard because vbariations in results may occur.
- For optimal results, an orbital plate shaker capable of 300-500 rpm is recommended for all incubations.
- Modification of the existing protocol (i.e. standard dilutions, pipetting technique, washing technique, incubation time or temperature, storage cnditions, and kit expiration) may affect the sensitivity and specificity of the test.

SUMMARY OF ASSAY PROTOCOL

Add 100µl/well of Human Leptin Standard, Positive Control, or diluted Samples

Incubate at romm temperature (20-23°C) for 2 hours

Wash immunoplate 4 times with 300-350µl/well of **1x** assay buffer

Add 100µl/well Biotinylated anti- human Leptin Detection Antibody

Incubate at romm temperature (20-23°C) for 2 hours

Wash immunoplate 4 times with 300-350µl/well of **1x** assay buffer

Add 100µl/well of SA-HRP solution

Incubate at romm temperature (20-23°C) for 30 minutes

Wash immunoplate 4 times with 300-350µl/well of **1x** assay buffer

Add 100µl/well of substrate solution (TMB)

Incubate at romm temperature (20-23°C) for 20-30 minutes

Terminate the reaction with 100µl/well of Stop Solution

Read absorbance O.D. at 450nm and calculate results

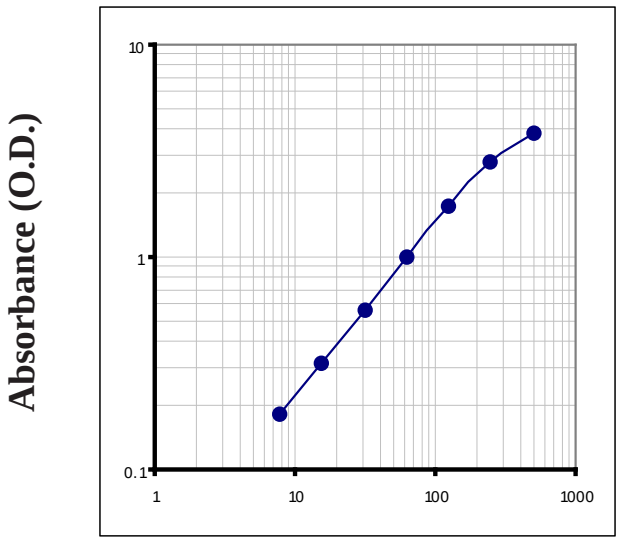
CALCULATION OF RESULTS

Plot the standard curve on log-log graph paper. Known concentrations of Human Leptin Standard and its corresponding reading is plotted on the log scale (x-axis) and the log scale (y-axis) respectively. The standard curve shows a correlated relationship between Human 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 Human Leptin in sample is determined by plotting the sample'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 in the unknown sample.

Refer to QC Data sheet for acceptable values of the Positive Control.

Leptin (Human) Standard Curve



STORAGE

1. Store the kit at 4°C upon receipt. The kit should be equilibrated to toom temperature (20-23°C) before the assay.
2. Store **1x** assay buffer at 4°C.
3. Remove any unneeded strips from Human Leptin Antibody-Coated plate, reseal them in zip-lock foil pouch and keep at 4°C.
4. Keep rehydrated solution of Human Leptin Standard, Bioitnylated Anti-human Leptin Detection Antibody and SA-HRP at 4°C. Prepare only the required amount.

NOTE:

1. It is recommended that the solutions be used on the same day of rehydration.
2. Unextracted serum samples of normal subjects are to be diluted with **1x** assay buffer.
3. After adding stop buffer, read the plate within 20 minutes.

REFERENCES

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4. Pelleymounter MA, Cullen MJ, Baker MB, etc: Effects of the obese gene product on body weight regulation in ob/ob mice. Science 269:540, 1995.
5. Zhang Y, Proenca R, Maffei M, etc: Positional cloning of the mouse obese gene and its human homologue. Nature 372:425, 1994.

ASSAY DIAGRAM