B-Bridge International, Inc.

# Human Leptin ELISA Kit
# User Manual

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See List of Components for Storage Conditions
FOR RESEARCH USE ONLY

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# I. Introduction and Protocol Overview

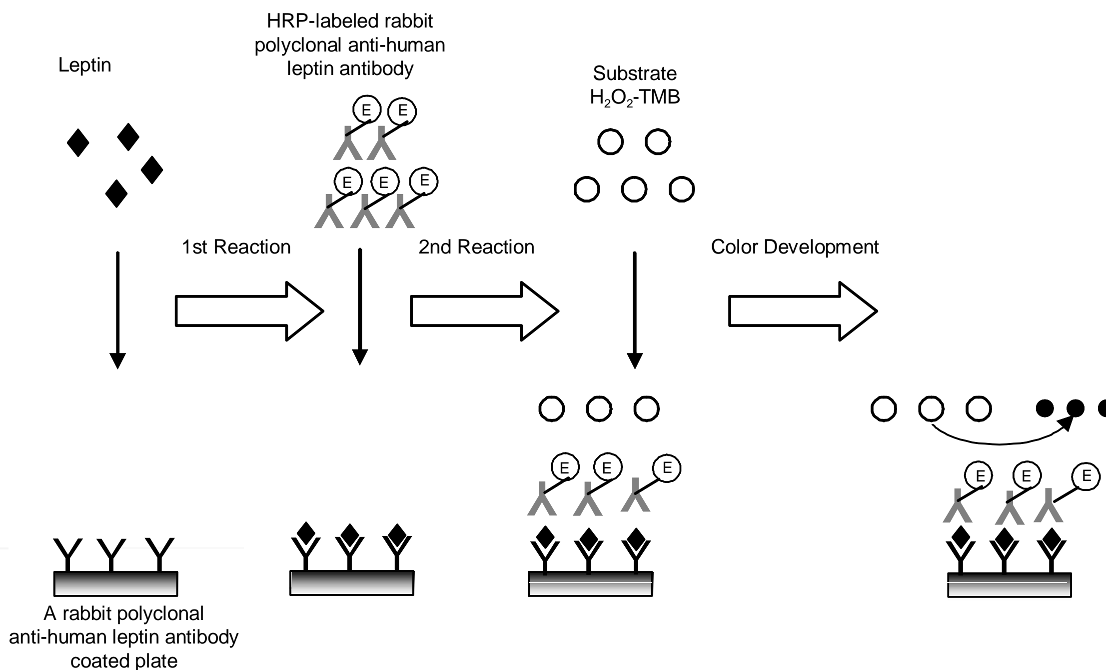
Leptin, the product of the *ob* (obese) gene, is a single-chain 16 kDa protein consisting of 146 amino acid residues. Leptin is produced mainly in adipose tissue, and is considered to play an important role in appetite control, fat metabolism, and body weight regulation. The primary effect of leptin appears to be mediated by leptin receptors expressed mainly in the hypothalamus. In humans, leptin levels correlate with body mass index (BMI) and percentage body fat, and are elevated in obese individuals. Leptin has a dual action; it decreases the appetite and increases energy consumption, causing more fat to be burned. Leptin is secreted in a circadian fashion with a nocturnal rise in both lean and obese patients.

Mutations of the *ob* gene resulting in leptin deficiency are the cause of obesity in the *ob/ob* mice. Endogenous leptin can normalize their body weight. In contrast, high levels of leptin in obese human subjects point to insensitivity to endogenous leptin. In addition to the amount of body fat, other factors that appear to regulate leptin action are insulin, glucocorticoids, catecholamines, and sex hormones. Studies have also shown that leptin may be linked to reproductive function.

The B-Bridge **Human Leptin ELISA Kit** is designed to measure the concentration of human leptin in human serum, plasma, or tissue culture medium.

The principle of the assay is shown in Figure 1. Standards, Quality Controls, and samples are incubated in microtiter wells coated with rabbit polyclonal anti-human leptin antibody. After a thorough wash, rabbit polyclonal anti-human leptin antibody labeled with horseradish peroxidase (HRP) is added to the wells and incubated with the immobilized antibody-leptin complex. Following another washing step, the remaining HRP-conjugated antibody is allowed to react with the substrate tetramethylbenzidine (TMB). The reaction is quenched by the addition of acidic solution, and the absorbance of the resulting product is measured spectrophotometrically at 450 nm. 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.

**Figure 1. Assay Principle**



## II. List of Components

- **Store all components at 2-8ºC. DO NOT FREEZE.**

| | | |
|---|---|---|
| 1 | **10X WASH SOLUTION** | 1 Bottle (100 ml) |
| 2 | **SAMPLE DILUENT** | 1 Bottle (13 ml) |
| 3 | **PRIMARY ANTIBODY-COATED PLATE**<br>One plate holds 12x8-well strips (96 wells),<br>with adsorbed rabbit polyclonal Anti-Human Leptin Antibody.<br>Plate is provided in a resealable foil pouch with desiccant. | 1 Plate |
| 4 | **HUMAN LEPTIN STANDARDS**<br>1, 2, 5, 10, 20, 50 ng/ml | 6 Vials (350 µl each) |
| 5 | **QUALITY CONTROL**<br>High and Low, lyophilized | 2 Vials |
| 6 | **HRP CONJUGATED SECONDARY ANTIBODY SOLUTION**<br>Rabbit Polyclonal Anti-Leptin Antibody,<br>Horseradish Peroxidase Conjugate | 1 Bottle (13 ml) |
| 7 | **SUBSTRATE**<br>$H_2O_2$Tetramethylbenzidine (TMB) | 1 Bottle (13 ml) |
| 8 | **STOP SOLUTION**<br>(0.2M $H_2SO_4$) | 1 Bottle (13 ml) |

**MSDS forms are available on our website—please visit www.b-bridge.com**

## III. Additional Materials Required

**The following materials are required, but not supplied:**

- Graduated cylinder
- Micropipettor(s) and disposable pipette tips
- 96-well plate washer
- Paper towels or absorbent paper
- Plate reader capable of measuring absorbance at a wavelength of 450 nm (reference filter at 620-650 nm, optional)
- Orbital microplate shaker capable of approximately 300 rpm
- Tubes for diluting samples
- Deionized water

# IV. Reagent Preparation and Storage

***Allow all the reagents to equilibrate at room temperature (25℃) prior to the start of the reagent preparation.***

1. <u>1X Wash Solution</u>
   Prepare 1X Wash Solution by mixing all of the 10X Wash Solution (100 ml) with 900 ml of deionized water or equivalent. After preparation, store 1X Wash Solution at 2-8℃. The diluted Wash Solution is stable for 1 month at 2-8℃.

2. <u>Human Leptin Standard Solution</u>
   Prepare each Leptin Standard by diluting 1:3 stock Leptin Standard Solution with Sample Diluent (e.g. 100 µl standard with 200 µl Sample Diluent for duplicates). Do not store the diluted standard solutions.

3. <u>Quality Controls</u>
   Reconstitute each vial of Quality Control with 0.35 ml of distilled water at least 30 minutes prior to use. Aliquots can be stored at -20℃.
   Dilute the reconstituted Quality Controls 1:3 with Sample Diluent prior to use in the assay. Add 100 µl of Quality Control to 200 µl Sample Diluent for duplicates. Do not store the diluted Quality Controls.

4. Unused strip wells in the microtiter plate should be stored in the aluminium pouch with the dessicant.

***Note: Do not mix reagents from different kits unless they have the same lot number.***

## V. Sample Preparation

***Allow all the reagents to equilibrate at room temperature (25℃) prior to the start of the sample preparation.***

1. For Assaying Samples in singlets
   Mix 50 µl of samples with 100 µl of Sample Diluent.

2. For Assaying Samples in duplicates
   Mix 100 µl of samples with 200 µl of Sample Diluent.

# VI. Human Leptin ELISA Protocol

**Note:** Allow all reagents to come to room temperature (25°C) prior to the start of the assay and prepare 1X Wash Solution, Quality Controls, Leptin Standards, and Samples as described in the previous sections.

1. Remove Primary Antibody-Coated Plate from its foil pouch. Remove any unneeded strips from the plate frame, reseal them in the foil pouch, and return the foil pouch to 2-8℃. If a 96-well plate washer is used, the plate frame should be completely filled with wells by adding as many null strips as necessary. Identify well position(s) for each sample on a data sheet or plate map.

2. Add 100 µl of diluted Standards, Quality Controls, Sample Diluent (blank) and samples to the appropriate number of antibody-coated wells. Every plate must include the standard series to properly correlate the sample readings.

3. Cover plate(s) securely and incubate at room temperature (25℃) for 60 minutes on an orbital microplate shaker at ca. 300 rpm.

4. After incubation, wash the plate(s) 3 times with Wash Solution as follows:
   a. Completely aspirate the liquid from the wells using a plate washer.
   b. Fill each well with 1X Wash Solution (~350 µl/well) and immediately aspirate. Avoid overflow.
   c. Repeat Step 4c two more times for a total of three washes.
   d. Invert the plate(s) and gently tap on a clean absorbent towel.

5. Dispense 100 µl of the Secondary Antibody Solution into each well.

6. Cover plate(s) securely and incubate at room temperature (25℃) for 60 minutes on an orbital microplate shaker at ca. 300 rpm.

7. Repeat the wash procedure described in step 4.

8. Dispense 100 µl of Substrate Solution into each well. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with aluminum foil is recommended.

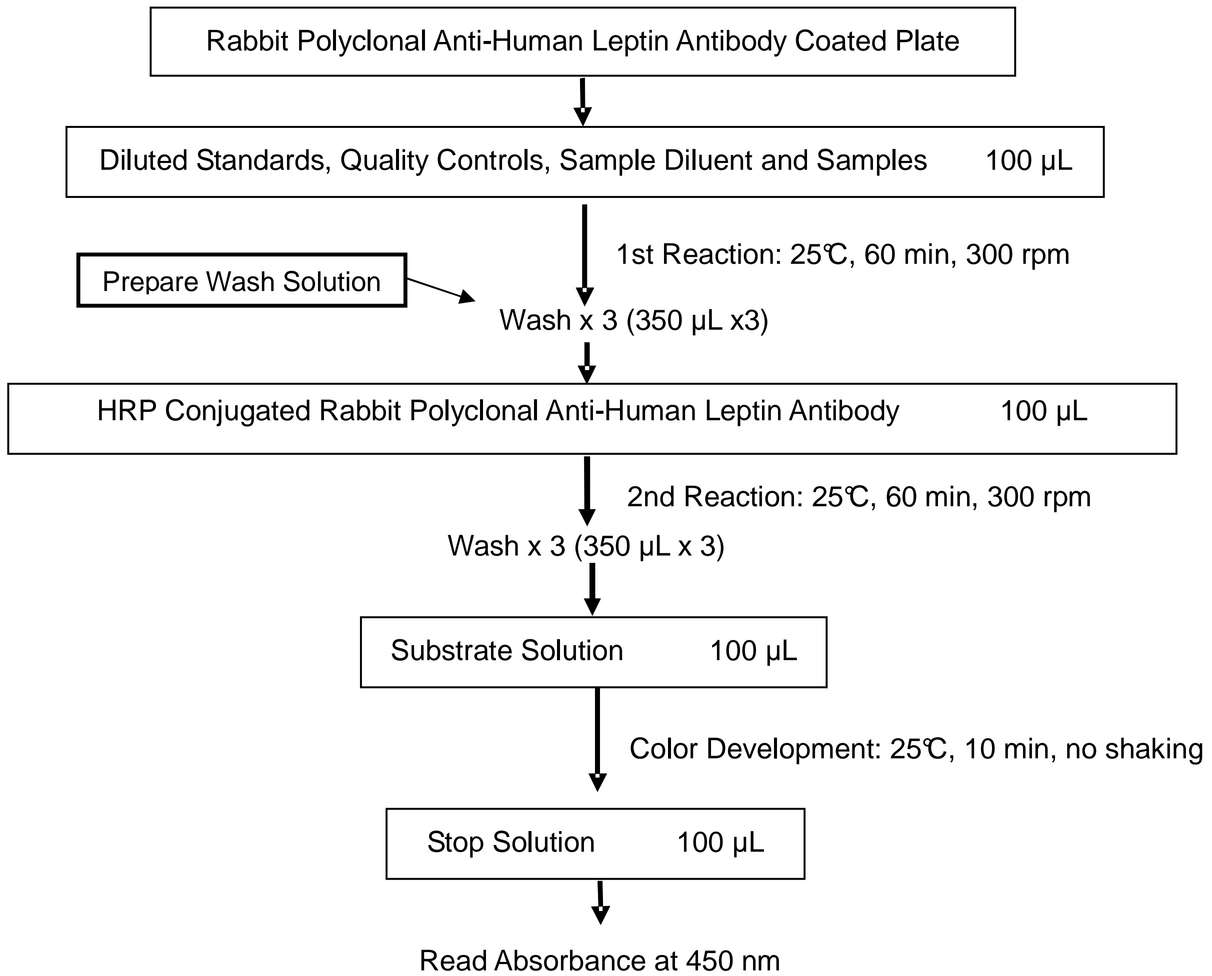
9. Cover plate(s) securely and incubate at room temperature (25℃) for 10 minutes with no shaking. Incubation time may be extended up to 2o minutes, if reaction temperature is below 20℃.

10. Dispense 100 µl of Stop Solution into each well. The plate should be read immediately.

11. Read the plate at 450 nm using a plate reader. If using a dual filter instrument, the recommended reference wavelength is 620-650 nm.

**Note**: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, the standard curve constructed using the values measured at 405 nm can be used to determine leptin concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

**Figure 2. Flow Chart of Assay Procedure**



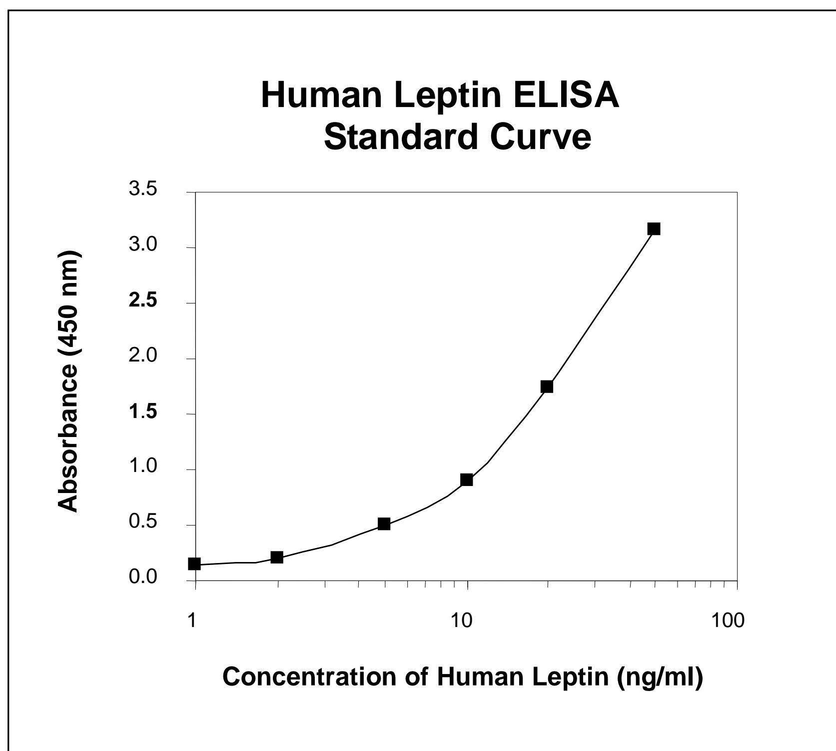
# VII. Calculation of Results

1. Subtract the mean absorbance value of the 0 ng/ml blank from each mean absorbance value of the standard series and samples tested (Net Absorbance).

2. Plot the log of known concentrations of each standard and the calculated Net Absorbances on the X-axis and Y-axis, respectively. Fit an appropriate regression curve on the plots.

3. Determine the leptin concentrations of the samples by interpolation of the regression curve.

**Figure 3. Typical Standard Curve**



## VIII. Troubleshooting Guide

1. **Lack of signal or 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 25°C.
   - Plate reader did not perform well.

2. **High signal and background in all wells**
   Possible explanations:

   - Improper or inadequate washing; be certain that all wash volumes and repetitions were correct.
   - Decrease color development time.
   - The plate should be read within 5 minutes of stopping the color development

3. **High background in sample wells only**
   Possible explanations:

   - Sample concentration was too high.

4. **Weak signal in sample wells only**
   Possible explanations:

   - Sample concentration was too low.

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