Human Leptin ELISA Kit
User Manual

UM-100501
Published 01 August 2004
Catalog# K1005-1

See List of Components for Storage Conditions
FOR RESEARCH USE ONLY

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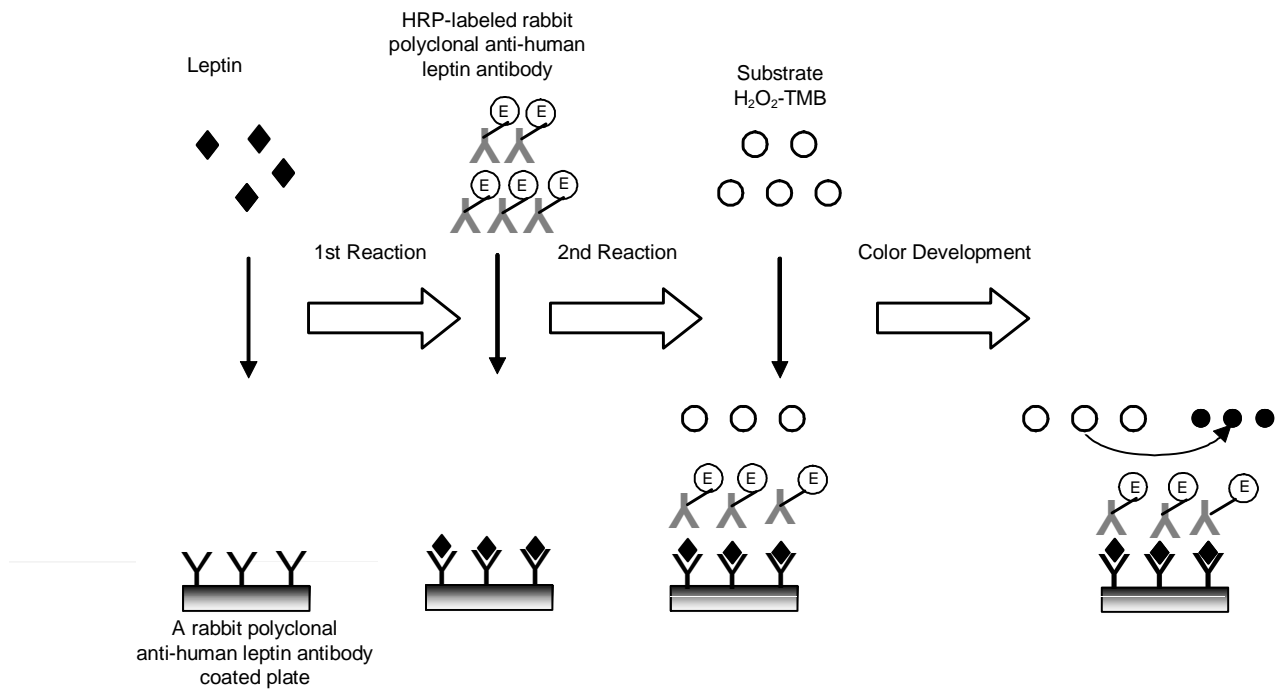
I.   Introduction and Protocol Overview

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 (EDTA, citrate, and heparin), 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 conjugated to 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 2-8⁰C.   DO NOT FREEZE.**

1	**10X WASH SOLUTION**	1 Bottle (100 ml)
2	**SAMPLE DILUENT**	1 Bottle (13 ml)
3	**PRIMARY ANTIBODY-COATED PLATE** One plate holds 12x8-well strips (96 wells), with adsorbed rabbit polyclonal Anti-Human Leptin Antibody. Plate is provided in a resealable foil pouch with desiccant.	1 Plate
4	**HUMAN LEPTIN STANDARD** Recombinant protein, lyophilized	1 Vial
5	**QUALITY CONTROL** High and Low Serum Controls, lyophilized	2 Vials
6	**HRP CONJUGATED SECONDARY ANTIBODY SOLUTION** Rabbit Polyclonal Anti-Leptin Antibody, Horseradish Peroxidase Conjugate	1 Bottle (13 ml)
7	**SUBSTRATE** H_2O_2Tetramethylbenzidine (TMB)	1 Bottle (13 ml)
8	**STOP SOLUTION** (0.2M H_2SO_4)	1 Bottle (13 ml)

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 wavelength at 63 nm)

- 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℃.

    Open 10X Wash Solution is stable for 3 months at 2-8℃ .

2. <u>Human Leptin Standard Solution</u>
    Reconstitute the standard just prior to use.

    Refer to the Certificate of Analysis for volume of Sample Diluent needed to reconstitute the Human Leptin Standard. Let the standard dissolve at least 15 minutes with gentle shaking after adding the volume of Sample Diluent stated on the Certificate of Analysis (avoid making air bubbles). **The reconstituted standard (stock) is 50 ng/ml.**

    Reconstituted standard should be used immediately or aliquot and stored frozen at -20℃ for up to 3 months. Avoid repeated freeze/thaw cycles.

    The volume of Sample Diluent to reconstitute the Standard stock is 1:3, the same dilution factor used to dilute samples and the Quality Controls.

    Prepare a set of standards using Sample Diluent as follows:

Volume of Standard	Sample Diluent	Concentration
Reconstituted Stock	-	50 ng/ml
200 µl of Stock	300 µl	20 ng/ml
250 µl of 20 ng/ml	250 µl	10 ng/ml
250 µl of 10 ng/ml	250 µl	5 ng/ml
200 µl of 5 ng/ml	300 µl	2 ng/ml
250 µl of 2 ng/ml	250 µl	1 ng/ml

    The diluted standards are now ready for use.

3. <u>Quality Controls (High and Low)</u>
    Refer to the Certificate of Analysis for current Quality Control concentration.

    Reconstitute each vial of Quality Control with 350 µl of distilled water at least 30 minutes prior to use. Let dissolve with occasional gentle shaking, avoid making air bubbles.

    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.

    Reconstituted Quality Controls should be used immediately or aliquot and stored frozen at -20℃ for up to 3 months. Avoid repeated freeze/thaw cycles.

Do not store diluted Quality Controls.

4. Unused strip wells in the microtiter plate should be stored in the aluminum pouch with the desiccant.

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 sample preparation.

Samples should be assayed immediately after collection or stored at -20℃ or -70℃ for long term. Avoid repeat freeze/thaw cycles. Do not store diluted samples.

Avoid using hemolyzed or lipemic samples.

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

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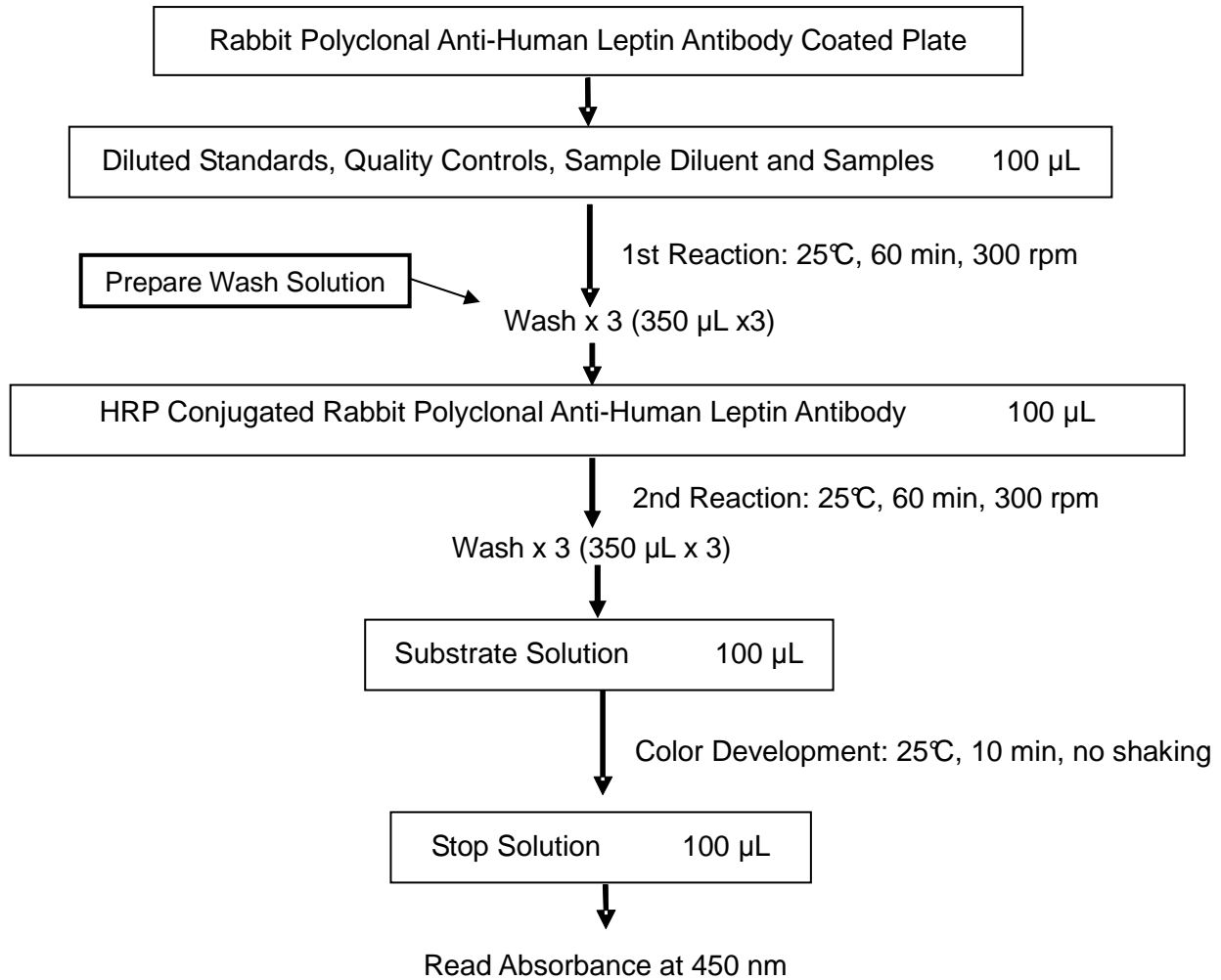   Mix 100 μl of samples with 200 μl of Sample Diluent.

VI.   Human Leptin ELISA Protocol

Note: Allow all reagents to come to room temperature (25℃) 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 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 4b 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 HRP Conjugated 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 20 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 630 nm (acceptable range is 550-650 nm). Subtract readings at 630 nm from 450 nm. The absorbance should be read within 5 minutes following step 10.

Note: If some samples and standards have an absorbance above the upper limit of your microplate reader, perform a second reading at 405 nm. A new standard curve constructed from 405 nm values is used to determine leptin concentrations for off-scale standards and samples. The readings for 405 nm do not replace the readings of samples and standards that were in-range at 450 nm.

Figure 2.   Flow Chart of Assay Procedure

| Rabbit Polyclonal Anti-Human Leptin Antibody Coated Plate |

↓

| Diluted Standards, Quality Controls, Sample Diluent and Samples   100 µL |

1st Reaction: 25℃, 60 min, 300 rpm

| Prepare Wash Solution | →

↓

Wash x 3 (350 µL x3)

↓

| HRP Conjugated Rabbit Polyclonal Anti-Human Leptin Antibody   100 µL |

↓   2nd Reaction: 25℃, 60 min, 300 rpm

Wash x 3 (350 µL x 3)

↓

| Substrate Solution   100 µL |

↓   Color Development: 25℃, 10 min, no shaking

| Stop Solution   100 µL |

↓

Read Absorbance at 450 nm

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.

The Human Leptin Standards were diluted 1:3 during reconstitution with the specified volume of Sample Diluent and the Samples and Quality Controls were diluted 1:3 prior to analysis. Therefore a 1:3 dilution factor does not have to be accounted for.

Figure 3.   Typical Standard Curve

Human Leptin ELISA Standard Curve

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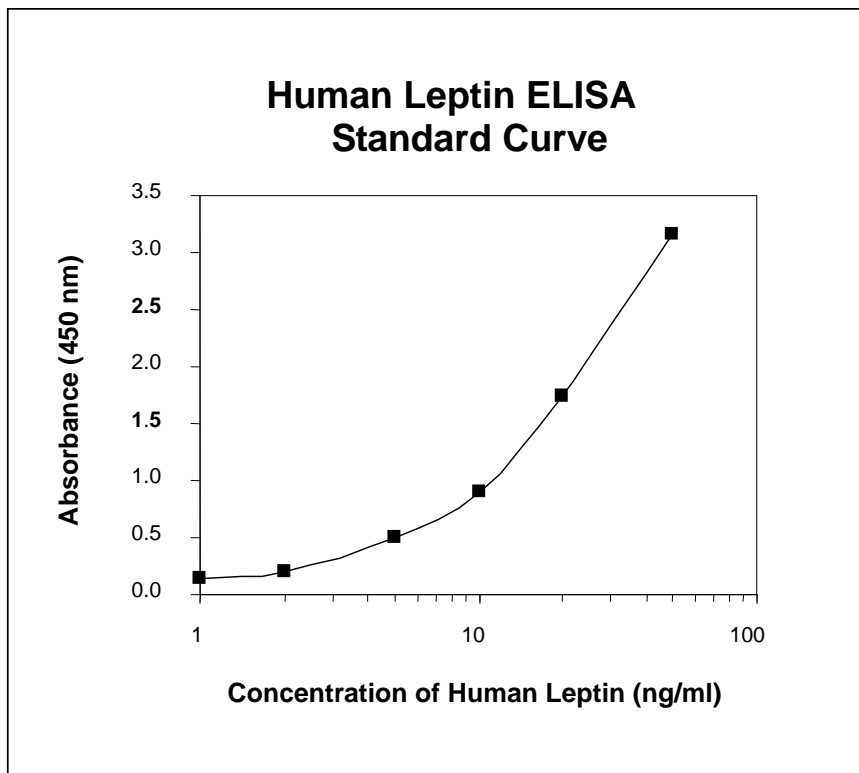
VIII. Troubleshooting Guide

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.
   - Overdevelopment. 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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