

AviBion Human Leptin ELISA Kit

User Manual

REF : LEPT024

RUO  96

Regulatory Status: For research use only (RUO)

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AviBion Human Leptin ELISA

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Revision 3.07



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1. INTENDED USE

Orgenium Laboratories' Human Leptin ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of Leptin in cell culture supernatants, plasma and serum.

2. INTRODUCTION

Human leptin is a 16-kDa (146-amino-acid) protein encoded by the obese (*ob*) gene and secreted from adipocytes (1). Human subjects with mutations in the gene encoding for leptin are morbidly obese and respond to leptin treatment, demonstrating that enhancing or inhibiting leptin's activities *in vivo* may have potential therapeutic benefits. Recent studies with obese and non-obese humans demonstrated a strong positive correlation of serum leptin concentrations with percentage of body fat. While systemic leptin is increased in obesity, adiponectin is reduced (2).

Leptin plays an important role in angiogenesis, autoimmunity, immune function, fertility, and bone formation (3-8). Higher leptin levels are reported in the earlier stages of endometriosis than in more advanced stages (9). In patients with angiographically confirmed coronary atherosclerosis, leptin is a novel predictor of future cardiovascular events independent of other risk factors, including lipid status and CRP (10, 11, 13). Leptin may also play an important role in the pathophysiology of osteoarthritis (OA) (12).

Orgenium Laboratories' human Leptin test is a solid-phase ELISA assay designed to measure the quantitative amount of human Leptin in cell culture supernatants, serum and plasma. This assay employs an antibody specific for human Leptin coated on a 96-well plate. Standards, samples and biotinylated anti-human Leptin are pipetted into the wells and Leptin present in a sample is captured by the antibody immobilized to the wells and by the biotinylated leptin-specific detection antibody. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed. Following this second wash step, TMB substrate solution is added to the wells, resulting in color development proportional to the amount of Leptin bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

3. CONTENTS OF THE KIT

| Test components | Amount/Volume |
|---|---------------|
| <p>96 Well Plate with 12 Strips Break-apart microtiter test strips each with 8 Leptin antibody coated single wells Ready for use</p> | 1 frame |
| <p>Leptin Standard 250 ng/ml Pre-diluted & Stabilized Human-Leptin (see label for stock concentration and dilute as described on page 7) Ready for use.</p> | 1.5 ml |
| <p>Biotinylated Leptin antibody. Ready for use.</p> | 10 ml |
| <p>HRP-Conjugated Avidin. Ready for use.</p> | 12 ml |
| <p>20x Wash Buffer concentrate (sufficient for 1000ml) Dilute 1:20</p> | 50 ml |
| <p>Sample Diluent Ready for use</p> | 100 ml |
| <p>Stop solution 2 N H₂SO₄ Ready for use</p> | 8 ml |
| <p>TMB-Substrate Ready for use</p> | 8 ml |

4. STORAGE AND STABILITY

| Reagent | Storage | Stability |
|---|--|---|
| Antibody coated 96 well plates with 12 strips. Break-apart microtiter test strips each with 8 antibody coated single wells | Store at 2-8°C in closed aluminum pouch with desiccant Strips which are not used must be stored in the re-sealable aluminum pouch in humidity free and airtight conditions | 3 months after opening |
| Leptin Standard Ready for use | Store at 2-8°C | 3 months after opening |
| Biotinylated antibody. Ready for use. | Store at 2-8°C <i>Avoid contamination (Use clean sterile tips)</i> | 3 months after opening |
| HRP-Conjugated Avidin. Ready for use. | Store at 2-8°C <i>Avoid contamination (Use clean sterile tips)</i> | 3 months after opening |
| Sample Diluent | Store at 2-8°C <i>Avoid contamination (use clean sterile tips or pipettes)</i> | 3 months after opening |
| 20x Concentrated Wash Buffer Diluted Wash Buffer | Store at Room Temperature. 1x working dilution <i>Bottles used for the working dilution should be cleaned regularly, discard cloudy solutions</i> | Until expiry date at room temperature 3 working days at room temperature or 2 weeks at +4°C. |
| TMB-Substrate Solution | Ready for use solution at 2-8°C, protected from light! <i>Avoid contamination (Use clean sterile tips)</i> | Until expiry date (written on the bottle). |
| Stop Solution | Store at Room Temperature | Until expiry date at room temperature |

5. ADDITIONAL MATERIALS REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm.
- Precision pipettes (2 μ l to 1 ml volumes).
- Multi-channel pipette (25 μ l to 350 μ l) 12 and 8 channel pipets. Recommended for manual washings and reagent dispensing.
- Adjustable 1-25 ml pipettes for reagent preparation.
- 100 ml and 1 liter graduated cylinders.
- Microplate washer or 12 well Multichannel pipet for washings.
- Absorbent paper.
- Distilled or de-ionized water.
- Log-log graph paper or computer and software for ELISA data analysis.
- Tubes to prepare standard or sample dilutions.
- Timer

6. AMOUNTS OF THE REAGENTS NEEDED TO PERFORM THE TEST

| | Reagents | | | | |
|---|--|--------------------------------|----------------------------------|----------------------------------|---------------------------------|
| No of strips used (with 8 well each) | Biotinylated antibody 50 μ l/well | Avidin-HRP 100 μ l/well | TMB Substrate 50 μ l/well | Stop Solution 25 μ l/well | Wash Buffer 300 μ l/well |
| 1 (8 wells) | 500 μ l | 900 μ l | 500 μ L | 300 μ l | 30 ml |
| 2 (16 wells) | 1 ml | 1.8 ml | 1 ml | 600 μ L | 55 ml |
| 4 (32 wells) | 2 ml | 3.6 ml | 2 ml | 1.2 ml | 110 ml |
| 6 (48 wells) | 3 ml | 5.4 ml | 3 ml | 1.8 ml | 165 ml |
| 8 (64 wells) | 4 ml | 7.2 ml | 4 ml | 2.4 ml | 220 ml |
| 12 (96 wells) | 6 ml | 11 ml | 6 ml | 4 ml | 350 ml |



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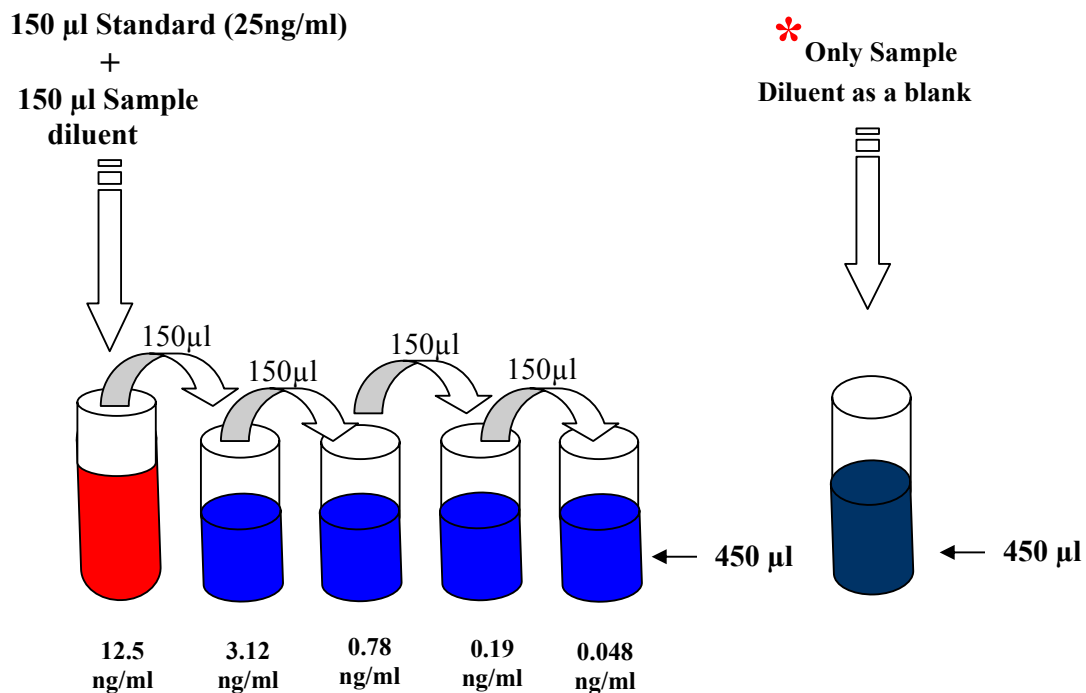
7. REAGENT AND SAMPLE PREPARATION

1. Bring all reagents and samples to room temperature (18-25°C) before use.
2. **Antibody coated plate:** Before opening the foil pouch, determine the number of strips required to test the desired number of samples plus 16 wells needed for running standards and blanks in duplicate. Remove non-used strips from the plate-frame and return them to the foil pouch containing the desiccant for up to 1 month at 2-8°C.

3. Dilution of test standard:

Test standard is ready to use. To obtain a standard curve dilute as follows:

- a) Add 150 µl of Leptin standard which contains 25 ng/ml of Leptin and 150 µl of Sample Diluent to the first tube to obtain 12.5 ng/ml.
- b) Add 450 µl of dilution buffer to the other 4 tubes. Take 150 µl from first tube and start 4 fold serial dilutions in dilution tubes as described in the figure by mixing several times with the pipet in each tube.
- c) Sample Diluent serves as the zero standard (0 ng/ml).



4. **Sample preparation and dilution:** Sample diluent is used for dilution of all samples (serum/plasma samples and culture supernatants) requiring dilution. Store and dilute all samples in tubes or plates made of material with low binding surface, such as polypropylene. Prior to the assay, frozen samples should be thawed as quickly as possible in tap water (18-25°C). **Do not use 37°C or 56°C water bath for this purpose.**

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 2000 x g for 10 minutes and assay.

Dilute plasma samples 1:8 with **Sample Diluent**. Do not use grossly haemolyzed or lipemic specimens. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2000 x g for 10 minutes. Remove serum and assay. **Dilute serum samples 1:8** with **Sample Diluent**. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

- **Cell Culture Supernatants:** Centrifuge cell culture media at 2000 x g for 10 minutes to remove debris. Collect supernatants and assay without diluting. Store samples at -20°C or below. Avoid repeated freeze-thaw cycles.

Samples with high absorbance values: Samples that exceed the measuring range should be diluted further and measured again. Samples with absorbance values >1.900 can be serially diluted 1:10, 1:50 and 1:100 or further. The dilution factor must be taken in account when calculating the results.

5. **Wash Buffer:** If the 20x concentrated Wash Buffer contains visible crystals, warm it at 37°C and mix gently until dissolved. Dilute 25 ml of Wash Buffer Concentrate with de-ionized or distilled water to yield 500 ml of 1x Wash Buffer.

6. Vortex mix **Biotinylated antibody** solution gently before use.

7. Vortex mix **peroxidase (HRP) labeled avidin** gently before use.

Caution: TMB substrate (Tetramethylbenzidine) and the Stop solution (H₂SO₄) are toxic or corrosive and should be handled with care. Use gloves during handling.

8. TEST PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards. **Dilute Serum & plasma samples 1:8** with **Sample Diluent** (e.g 20µl of sample + 140 µl Sample Diluent) in a test tube. No need to dilute cell culture supernatants.



2. Add 50 µl standard (starting from 12.5 ng/mL), test sample and sample diluent as a blank into the appropriate wells of the strips.

3. Add 50 µl ready for use biotin antibody promptly to each well.

Incubate 1 hour 30 minutes at room temperature.



Wash 5 x with 1x wash buffer

4. Add 100 µl ready for use HRP-Streptavidin solution.

Incubate 30 minutes at room temperature.



Wash 5 x with 1x wash buffer

5. Add 50 µl TMB One-Step Substrate Reagent to each well.

Incubate 15 minutes at room temperature.



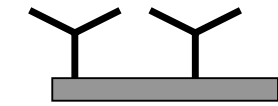
6. Add 25 µl Stop Solution to each well.

Read at 450 nm against *630 nm immediately.

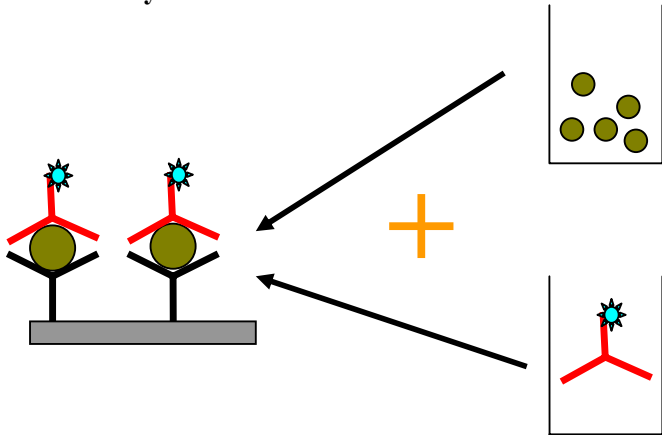
**Correcting for optical imperfections in the microplates by subtracting $A_{630\text{ nm}}$ is recommended, but not an essential procedure.*

TEST PRINCIPLE

Leptin Antibody coated test well



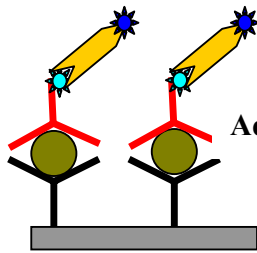
STEP 1



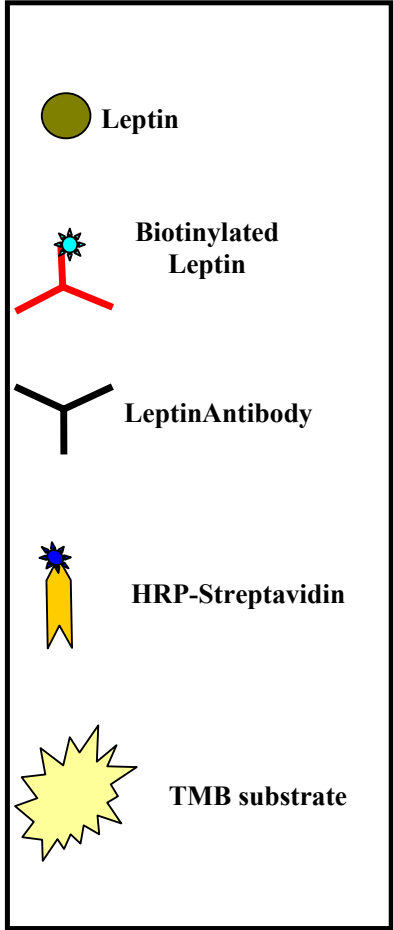
Add 50µL of Leptin containing sample to test well

Add 50 µL of Biotinylated Leptin antibody to test well

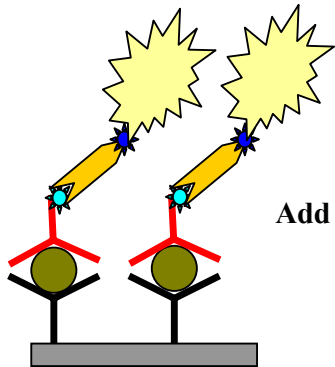
STEP 2



Add 100 µL of HRP-Streptavidin to test well



STEP 3



Add 50 µL of TMB substrate to test well

9. PROCEDURAL NOTES/LAB QUALITY CONTROL

- When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
- Microtiter plates should be allowed to come to room temperature before opening the foil pouches.
- Once the desired number of strips has been removed, immediately reseal the pouch and store at 2 - 8°C to maintain plate integrity. Protect from humidity.
- Samples should be collected in pyrogen/endotoxin-free tubes.
- Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
- When possible, avoid use of badly hemolyzed or lipemic sera. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.
- It is recommended that all standards, controls and samples be run in duplicate.
- Samples that are >500 pg/mL should be diluted with Sample Diluent.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
- Cover or cap all reagents when not in use.
- Do not use reagents after the kit expiration date.
- Read absorbances within 20 minutes of assay completion.
- In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
- All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. *Never* insert absorbent paper directly into the wells.
- Because *TMB Chromogen* is light sensitive, avoid prolonged exposure to light. Also avoid contact between *Stabilized Chromogen* and metal, or color may develop.

10. ASSAY PROCEDURE

1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is recommended that all standards and samples be run at least in duplicate. Leave some wells as a reagent blank (2 to 4 wells).

FIRST STEP: STANDARD, SAMPLES AND BLANK+ BIOTINYLATED-

2. Pipette 50 µl of Sample and 50 µl of each diluted standard starting from 12.5 ng/mL (see page 7) into appropriate wells. Pipette 50 µl of Sample diluent to the wells which will be used as a blank
3. Add 50 µl of Green colored Biotinylated detection antibody to all wells containing standards and samples (total reaction volume is 100 µl). Tap the plate gently by hand to homogenize your mixture.
4. Incubate at room temperature for 1 hr 30 min. without shaking.

SECOND STEP: STREPTAVIDIN-HRP

5. Wash 5 times with 1x Wash Solution (300 µl each).

To wash: Empty plate contents. Use a multi-channel pipette to fill each well with 300 µl of Wash Buffer, then empty plate contents again. Repeat procedure 4 additional times for a total of FIVE washes. Gently blot plate onto paper towels or other absorbent material.

Note: For automated washing, aspirate all wells and wash 5 times with Wash Buffer. Blot plate onto paper towels or other absorbent material. Never let reaction wells dry. Continue to the next step without delay or interruption.

6. Add 100 µl of prepared Streptavidin-HRP solution (Ready to use) to each well. Incubate for 30 minutes at room temperature.

THIRD STEP: TMB SUBSTRATE

7. Wash 5 times with 1x Wash Solution (300 µl each).
8. Add 50 µl of TMB Ready to use Substrate Reagent to each well. Incubate for 20 minutes at room temperature in the dark.

FOURTH STEP: STOP REACTION

9. Add 25 µl of Stop Solution to each well. Read at 450 nm within 15 minutes.

Correcting for optical imperfections in the microplates by subtracting $A_{630\text{ nm}}$ is recommended, but not an essential procedure.



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FIFTH STEP: READING AND CALCULATION

10. Calculate the mean of reagent blank absorbance values and subtract it from all test well values (standard and test samples). Mean reagent blank value should be less than 0.200

11. Calculate your results against standard.

11. CALCULATION OF RESULTS

The standard curve must be determined individually for each experiment. Correct each absorbance value of all standards by subtracting from it the O.D. value of the reagent blank (Bl = only sample diluent). Calculate the mean absorbance value for each standard from the duplicates.

The standard curve is used to determine the amount of leptin in an unknown sample. The standard curve is generated by plotting the average O.D. (450 nm) obtained for each of the standard concentrations on the vertical (Y) axis versus the corresponding leptin concentration (ng/mL) on the horizontal (X) axis.

Construct the standard curve using graph paper or statistical software.

If samples generate values higher than the highest standard, dilute the samples with sample diluent and repeat the assay. Note that the concentration read from the standard curve must be multiplied by the dilution factor.

Multiply the plasma or serum value by the dilution factor of 8.

12. TYPICAL DATA

The following data were obtained for the various Leptin standards over the range of 0 to 12.5 ng/mL.

Please note: This results are an example only. A standard curve should be generated each time the assay is performed. Do not use these values in your calculations.

| Standard | Mean Optical Density (450 nm) |
|--------------------------|-------------------------------|
| 12.5 ng/mL | 2.657 |
| 3.12 ng/mL | 1.493 |
| 0.78 ng/mL | 0.518 |
| 0.19 ng/mL | 0.130 |
| 0.048 ng/mL | 0.095 |
| 0 pg/mL (Sample Diluent) | 0.086 |

****Mean sample diluent value should be less than 0.200***

13. TEST PERFORMANCE

| | Leptin |
|--|--|
| Assay range | 0 -12.5 ng/mL. |
| Standard curve points | 12.5, 3.12, 0.78, 0.19, 0.048 ng/ml |
| Expected values in healthy adults | Males: 3.83±1.79 Females: 7.36±3.73 |
| Intra-Assay-Precision | ≤9% |
| Inter-Assay-Precision | ≤12% |
| Inter-Lot-Precision | ≤12% |
| Cross-Reactivity | No cross-reactivity was observed with the following recombinant human proteins: Adiponectin, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, TNF α, TARC |
| Interferences | No interferences to bilirubin up to 0.3 mg/mL, haemoglobin up to 8.0 mg/mL and triglycerides up to 5.0 mg/mL |
| Specificity | Recognizes both natural and recombinant human IL-1α. |
| Sensitivity | <1 ng/ml. |

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15. TROUBLESHOOTING

| Problem | Cause | Solution |
|----------------------------|--|--|
| Poor standard Curve | <ol style="list-style-type: none"> 1. Inaccurate pipetting or pipetting error 2. Improper standard dilution | <p>Check pipettes and calibrate regularly.</p> <p>Vortex the stock before use and dilute carefully in an eppendorf tube.</p> |
| Low signal | <ol style="list-style-type: none"> 1. Shorter incubation than recommended 2. Inadequate reagent volumes or improper dilution or pipetting error | <p>Ensure sufficient incubation time;</p> <p>Check pipettes and ensure correct performance.</p> |
| Large CV | Inaccurate pipetting and drying of wells during test procedure. | <p>Check pipettes</p> <p>Fill the wells promptly with wash buffer and reagents.</p> |
| High background | <ol style="list-style-type: none"> 1. Plate is insufficiently washed 2. Contaminated wash Buffer 3. Wash buffer volume is less than advised | <p>Use multichannel pipet for washings. If using a plate washer, check that all ports are unobstructed and clean.</p> <p>Make a fresh wash buffer</p> <p>Use 300µl per well</p> <p>Use multichannel pipet during the test.</p> |

LIABILITY

This kit is intended for research use only by personnel trained and qualified to carry out diagnostic or research activities.

If the recipient of this kit passes it on in any way to a third party, this instruction must be enclosed, and said recipient shall at own risk secure in favor of Orgenium Laboratories all limitations of liability herein.

Orgenium Laboratories shall not be responsible for any damages or losses due to using the kit in any way other than as expressly stated in these Instructions.

The liability of Orgenium Laboratories shall in no event exceed the commercial value of the kit.

Orgenium Laboratories shall under no circumstances be liable for indirect, special or consequential damages, including but not limited to loss of profit.