


AviBion Human Leptin ELISA Kit

User Manual

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RUO  96

Regulatory Status: For research use only (RUO)

Calibrated against WHO Leptin Reference Reagent 97/594

Please contact Orgenium's customer service representatives for inquiries, feedback or non-conforming products.

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RUO

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AviBion Human Leptin ELISA

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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, serum and breast milk.

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, plasma and breast milk. 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 8000 pg/ml Lyophilized & stabilized recombinant human leptin, add 1ml of Sample Diluent before use</p>	2x1 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
<p>Antibody coated 96 well plates with 12 strips.</p> <p>Break-apart microtiter test strips each with 8 antibody coated single wells</p>	<p>Store at 2-8°C in closed aluminum pouch with desiccant</p> <p>Strips which are not used must be stored in the re-sealable aluminum pouch in humidity free and airtight conditions</p>	3 months after opening
<p>Leptin Standard Lyophilized</p>	Store at 2-8°C	Until date of kit expiry in lyophilized format. At least 3 weeks after dissolving with sample diluent.
<p>Biotinylated antibody. Ready for use.</p>	<p>Store at 2-8°C</p> <p><i>Avoid contamination (Use clean sterile tips)</i></p>	3 months after opening
<p>HRP-Conjugated Avidin. Ready for use.</p>	<p>Store at 2-8°C</p> <p><i>Avoid contamination (Use clean sterile tips)</i></p>	3 months after opening
<p>Sample Diluent</p>	<p>Store at 2-8°C</p> <p><i>Avoid contamination (use clean sterile tips or pipettes)</i></p>	3 months after opening
<p>20x Concentrated Wash Buffer</p> <p>Diluted Wash Buffer</p>	<p>Store at Room Temperature.</p> <p>1x working dilution</p> <p><i>Bottles used for the working dilution should be cleaned regularly, discard cloudy solutions</i></p>	<p>Until expiry date at room temperature</p> <p>3 working days at room temperature or 2 weeks at +4°C.</p>
<p>TMB-Substrate Solution</p>	<p>Ready for use solution at 2-8°C, protected from light!</p> <p><i>Avoid contamination (Use clean sterile tips)</i></p>	Until expiry date (written on the bottle).
<p>Stop Solution</p>	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 µl to 1 ml volumes).
- Multi-channel pipette (25 µl to 350 µl) 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
- Medium or high fat cow's milk for diluting the standard when testing breast milk samples

6. AMOUNTS OF THE REAGENTS NEEDED

No of strips used (with 8 well each)	Reagents				
	Biotinylated antibody 50 µl/well	Avidin-HRP 100µl/well	TMB Substrate 50 µl/well	Stop Solution 25 µl/well	Wash Buffer 300 µl/well
1 (8 wells)	500µl	900 µl	500 µL	300 µl	30 ml
2 (16 wells)	1 ml	1.8 ml	1 ml	600 µL	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

7. REAGENT AND SAMPLE PREPARATION

Caution: TMB (Tetramethylbenzidine) substrate solution and Stop Solution (H_2SO_4) are toxic or corrosive and should be handled with care. Use gloves during handling.

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 3 months at 2-8°C.
3. **Dilution of test standard for testing cell culture supernatants, plasma and serum samples:**

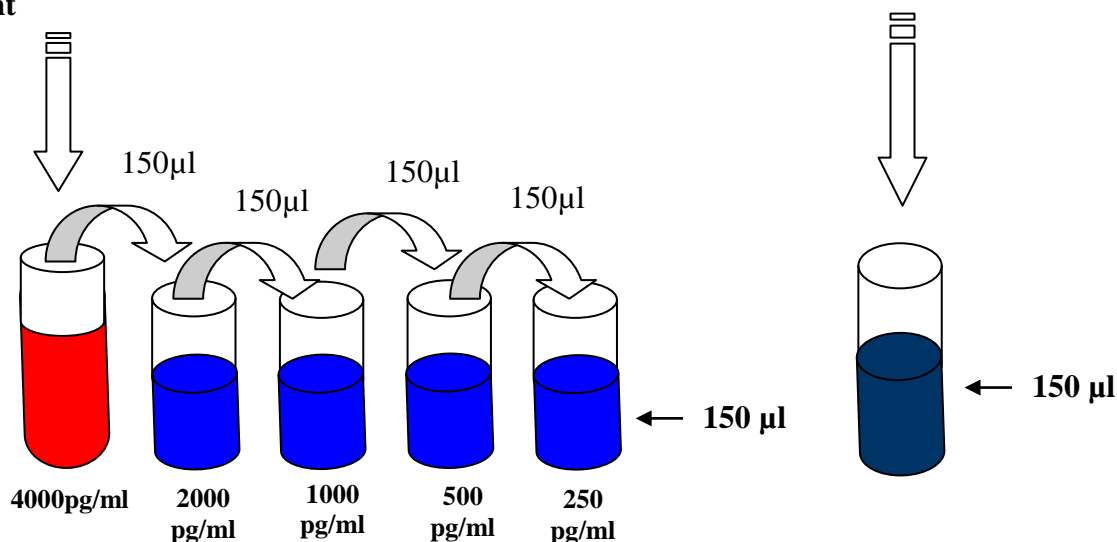
Dissolve the lyophilised Leptin standard with 1 ml of "Sample Diluent". To obtain a standard curve dilute it as follows:

- a) Add 150µl of Leptin standard which contains 8000 pg/ml of Leptin and 150µl of Sample Diluent to the first tube to obtain 4000 pg/ml leptin concentration.
- b) Add 150 µl of dilution buffer to the other 4 dilution tubes. Take 150 µl from the first tube and start 2-fold serial dilutions in dilution tubes as described in the figure below by mixing several times with the pipet in each tube.
- c) Sample Diluent serves as the zero standard (0 ng/ml).

150 µl Standard (8000 pg/ml)

+

**150 µl Sample
diluent**



4. Dilution of test standard for testing breast milk samples:

Dilute the Leptin standard in **medium or high fat cow milk** (not provided with the kit) following the dilution protocol of standard. Simply use “cow milk” instead of sample diluent for diluting the standard.

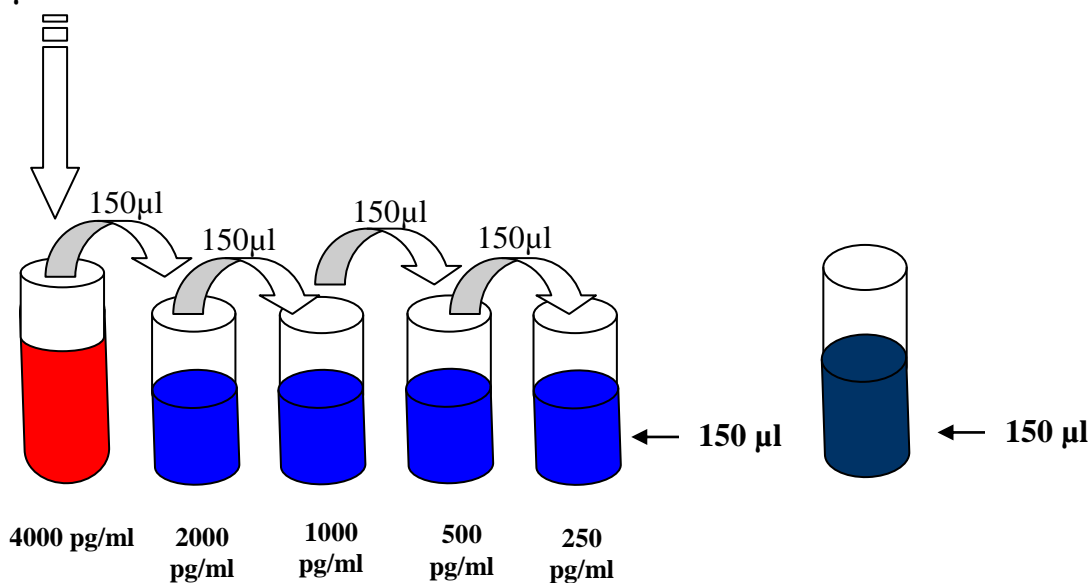
Test standard is ready to use. To obtain a standard curve dilute as follows:

- Add 150 μ l of Leptin standard which contains 8000 pg/ml of Leptin and 150 μ l of cow milk to the first tube to obtain 4000 pg/ml leptin concentration.
- Add 150 μ l of cow milk to other 4 tubes. Take 150 μ l from first tube and start 2-fold serial dilutions in dilution tubes as described in the figure below by mixing several times with the pipet in each tube.
- Cow milk alone serves as the zero standard (0 ng/ml).

150 μ l Standard (8000 pg/ml)

+
150 μ l cow milk

* Only cow milk
as a blank



5. Sample preparation and dilution:

Sample diluent is used for the dilution of all samples 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 the plasma samples using sodium citrate, EDTA or heparin as an anticoagulant. Centrifuge for 15-20 minutes at 1000 x g within 30 minutes of collection. **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.
 - **Breast Milk samples:** Collect approximately 5 ml of the sample in a sample collection tube by manual expression of breast preferably after 3 hrs of eating. Samples can be stored at +4°C up to 5 working days. For longer storage samples can be stored at -20°C or -80°C. Frozen samples should be thawed at room temperature before analysis. Avoid repeated freeze-thaw cycles. **Dilute breast milk samples 1:2** with **Sample Diluent**. Samples with absorbance values >1.900 can be serially diluted 1:10, 1:20, 1:40 or further.
6. **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.
 7. Vortex mix **Biotinylated antibody** solution gently before use.
 8. Vortex mix **peroxidase (HRP) labeled avidin** gently before use.

8. TEST PROCEDURE SUMMARY FOR CELL CULTURE SUPERNATANTS, SERUM AND PLASMA SAMPLES

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



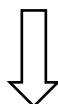
2. Add 50 µl standard (starting from 4000 pg/mL), test sample and sample diluent as a blank into the appropriate wells of the strips.

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 Substrate 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.*

9. TEST PROCEDURE SUMMARY FOR BREAST MILK SAMPLES

1. Prepare all reagents, breast milk samples (1:2 diluted) and standards.



2. Add 50 µl standard (starting from 4000 pg/mL), test sample and cow milk as a blank into the appropriate wells of the strips.

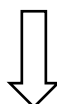
Incubate 1 hour at room temperature.



Wash 5 x with 1x wash buffer

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

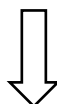
Incubate 30 minutes at room temperature.



Wash 5 x with 1x wash buffer

4. Add 50 µl ready for use HRP-Streptavidin solution.

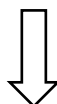
Incubate 30 minutes at room temperature.



Wash 5 x with 1x wash buffer

5. Add 50 µl TMB Substrate to each well.

Incubate 20 minutes at room temperature.

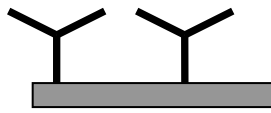


6. Add 25 µl Stop Solution to each well.

Read at 450 nm against *630 nm immediately.

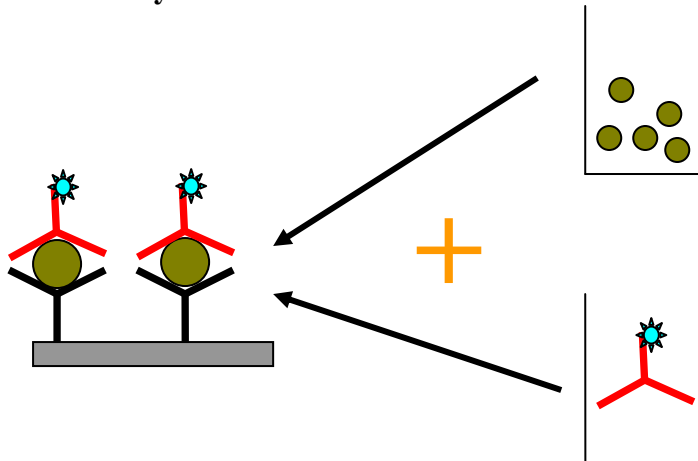
**Correcting for optical imperfections in the microplates by subtracting $A_{630\text{ nm}}$ is recommended, but not an essential procedure.*

10. TEST PRINCIPLE



Leptin Antibody coated test well

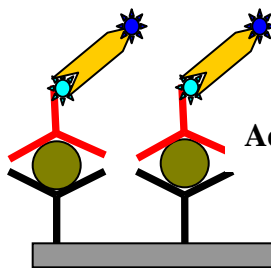
STEP 1



Add 50 μ L of Leptin containing sample to test well

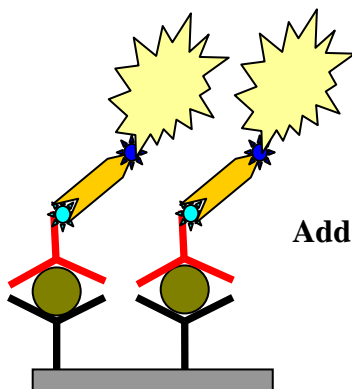
Add 50 μ L of Biotinylated Leptin antibody to test well

STEP 2

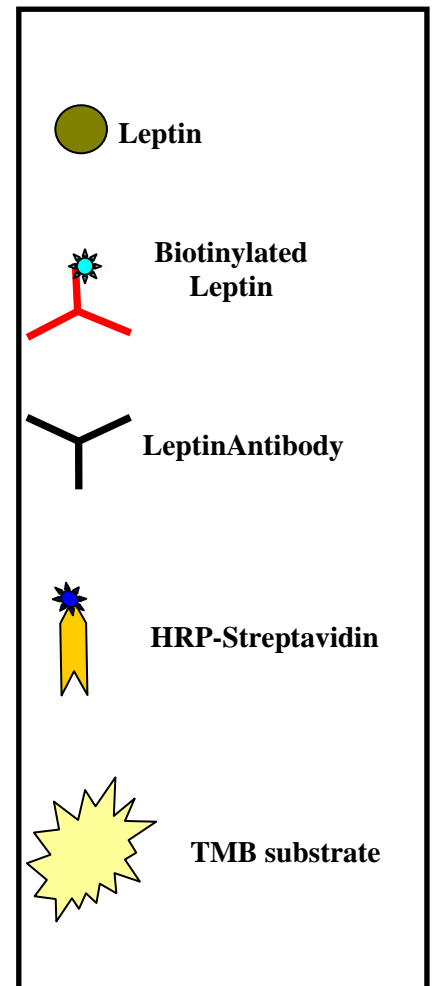


Add 100 μ L of HRP-Streptavidin to test well

STEP 3



Add 50 μ L of TMB substrate to test well



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11. 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 >4000 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 substrate solution is light sensitive, avoid prolonged exposure to light. Also avoid contact between TMB substrate solution and metal, or color may develop.

12. ASSAY PROCEDURE FOR CELL CULTURE SUPERNATANTS, SERUM AND PLASMA SAMPLES

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.

Running all standards and samples at least in duplicate is highly recommended. Leave some wells as a reagent blank (2-4 wells).

FIRST STEP: STANDARD, SAMPLES AND BLANK+ BIOTINYLATED ANTIBODY

2. Pipette 50 µl of Sample and 50 µl of each diluted standard starting from 4000 pg/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 manually: Empty plate contents. Use a multi-channel pipette to fill each well with 300 µl of diluted 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. Never let reaction wells dry. Continue to the next step without delay or interruption.

For automated washing: Aspirate all wells and wash 5 times with 300 µl diluted 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.

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.

13. ASSAY PROCEDURE FOR BREAST MILK SAMPLES

1. Bring all reagents and breast milk samples to room temperature (18 - 25°C) before use. Dilute samples 1:2 with Sample Diluent. Running all standards and samples at least in duplicate is highly recommended. Leave some wells as a reagent blank (2-4 wells).

FIRST STEP: STANDARD, SAMPLES AND BLANK

2. Pipette 50 µl of 1:2 diluted breast milk samples and 50 µl of diluted standard starting from 4000 pg/ml (see page 8) into appropriate wells. Pipette 50 µl of cow milk to the wells which will be used as a blank.

Incubate 1 hr at room temperature without shaking.

SECOND STEP: BIOTINYLATED ANTIBODY

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

To wash manually: Empty plate contents. Use a multi-channel pipette to fill each well with 300 µl of diluted 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. Never let reaction wells dry. Continue to the next step without delay or interruption.

For automated washing: Aspirate all wells and wash 5 times with 300 µl diluted 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.

4. Promptly add 50 µl of green colored Biotinylated Leptin detection antibody to all wells. Tap the plate gently by hand to homogenize your mixture. Avoid touching the reaction wells with the pipette tip.

Incubate 30 minutes at room temperature without shaking.

THIRD STEP: STREPTAVIDIN-HRP

5. Wash 5 times as described in Step 1.
6. Add 50 µl of prepared HRP-conjugated avidin solution (ready to use) to each well.

Incubate 30 minutes at room temperature without shaking.

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FOURTH 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.

FIFTH 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.

SIXTH 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.

14. CALCULATION OF RESULTS

The standard curve is used for determining the amount of leptin in a sample. The standard curve must be determined individually for each experiment. For an example of a typical standard curve obtained using Orgenium's AviBion Leptin ELISA kit, please refer to the Quality Control Certificate accompanying this kit.

Correct the absorbance values of all standards by subtracting from them the O.D. value of the reagent blank (BI = only sample diluent or cow milk). Calculate the mean absorbance value for each standard from the duplicates.

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.

Multiply the concentration results with the dilution factor used;

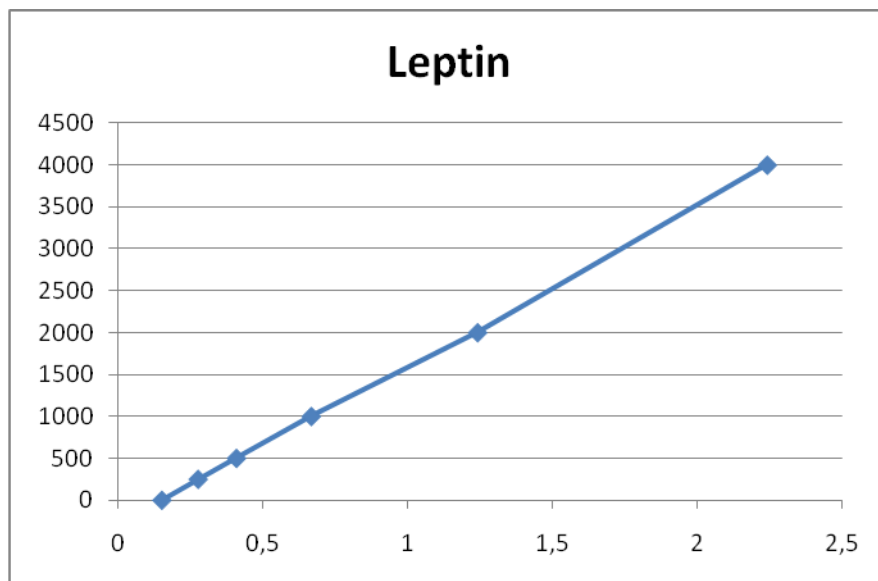
- Plasma and serum samples: Dilution factor = 8
- Breast Milk samples: Dilution factor = 2

Note: If samples generate values higher than the highest standard, dilute the samples with sample diluent and repeat the assay. When calculating results, this additional dilution factor must be taken into account

15. TYPICAL DATA

The following standard curve is obtained for the various Leptin standards over the range of 0 to 4000 pg/ml

Please note: The curve is provided for illustration only. A standard curve should be generated each time the assay is performed. Do not use this standard curve in your calculations.



16. TEST PERFORMANCE

	Leptin
Assay range	0 - 4000 pg/mL.
Standard curve points	4000, 2000, 1000, 500, 250 and 0 pg/ml
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 leptin
Sensitivity	<250 pg/ml.

17. REFERENCES

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18. 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.

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