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LEPTIN ELISA

REF

Catalog No.:E18-073

IVD

INTENDED USE

For the quantitative determination of Leptin in human serum by an enzyme immunoassay method.
For *in vitro* use only.

PRINCIPLE OF THE TEST

The principle of the following enzyme immunoassay test follows a typical two-step capture or 'sandwich' type assay. The assay makes use of two highly specific monoclonal antibodies: A monoclonal antibody specific for leptin is immobilized onto the microwell plate and another monoclonal antibody specific for a different epitope of leptin is conjugated to biotin. During the first step, leptin present in the samples and standards is bound to the immobilized antibody and to the biotinylated antibody, thus forming a sandwich complex. Excess and unbound biotinylated antibody is removed by a washing step. In the second step, streptavidin-HRP is added, which binds specifically to any bound biotinylated antibody. Again, unbound streptavidin-HRP is removed by a washing step. Next, the enzyme substrate is added (TMB), forming a blue coloured product that is directly proportional to the amount of leptin present. The enzymatic reaction is terminated by the addition of the stopping solution, converting the blue colour to a yellow colour. The absorbance is measured on a microtiter plate reader at 450nm. A set of standards is used to plot a standard curve from which the amount of leptin in patient samples and controls can be directly read.

CLINICAL APPLICATIONS

Human Leptin is a 16 kDa, 146 amino acid residue, non-glycosylated polypeptide. Leptin is encoded by the OB gene. Its major source is the adipose tissue, and its circulating concentrations indirectly reflect body fat stores.
Plasma or serum concentrations of leptin are increased in obese humans and strongly correlate with the degree of adiposity as expressed by percentage of body fat or body mass index.
The recently discovered hormone leptin contributes to the regulation of energy balance by informing the brain of the amount of adipose tissue in the body. The brain may then make the appropriate adjustments in either energy intake or expenditure.
Many areas of leptin physiology remain to be investigated. The roles of leptin in metabolism, insulin sensitivity, as a potential therapeutic modality for weight loss as well as involvement in endocrine function are active areas of research. While the future for leptin as a therapeutic agent is not clear, its involvement in many areas of physiology undoubtedly makes this a new hormone which requires extensive study in the future to understand its physiology.

PROCEDURAL CAUTIONS AND WARNINGS

1. This kit is intended for in vitro use only.
2. Practice the following good laboratory practices when handling kit reagents:
   - Do not pipette by mouth.
   - Do not smoke, drink, or eat in areas where specimens or kit reagents are being handled.
   - Wear protective clothing and disposable gloves when handling the specimens and kit reagents.
   - Wash hands thoroughly after performing the test.
   - Avoid contact with eyes; use safety glasses; in case of contact, flush with water immediately and contact a doctor.
3. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
4. Avoid microbial contamination of reagents.
5. A calibrator curve must be established for every run.
6. It is recommended to all customers to prepare their own control materials or serum pools which should be included in every run at a high and low level for assessing the reliability of results.
7. The controls (included in kit) should be included in every run and fall within established confidence limits.
8. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
9. All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
10. Improper procedural techniques, imprecise pipetting, incomplete washing as well asimproper reagent storage may be indicated when assay values for the control do not reflect established ranges.
11. When reading the microplate, the presence of bubbles in the microwells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
12. The substrate solution (TMB) is sensitive to light and should remain colourless if properly stored. Instability or contamination may be indicated by the development of a blue colour, in which case it should not be used.
13. When dispensing the substrate and stopping solution, do not use pipettes in which these liquids will come into contact with any metal parts.
14. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
15. Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiration date printed on the label.
16. Kit reagents must be regarded as hazardous waste and disposed of according to local and/or national regulations.

LIMITATIONS

1. All the reagents within the kit are calibrated for the determination of leptin in human serum. The kit is not calibrated for the determination of leptin in saliva, plasma or other specimens of human or animal origin.
2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
3. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
4. Only assay buffer may be used to dilute any high serum samples. The use of any other reagent may lead to false results.
5. The results obtained with this kit should never be used as the sole basis for a clinical diagnosis. For example, the occurrence of heterophilic antibodies in patients regularly exposed to animals or animal products has the potential of causing interferences in immunological tests. Consequently, the clinical diagnosis should include all aspects of a patient's background including the

frequency of exposure to animals/products if false results are suspected.

SAFETY CAUTIONS AND WARNINGS
POTENTIAL BIOHAZARDOUS MATERIAL

All serum samples should be considered a potential biohazard and handled with the appropriate precautions.

CHEMICAL HAZARDS

Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

SERUM COLLECTION AND STORAGE

Approximately 0.1 ml of serum is required per duplicate determination. Collect 4-5 ml of blood into an appropriately labelled tube and allow it to clot. Centrifuge and carefully remove the serum layer. Store at 4ºC for up to 24 hours or at -10ºC or lower if the analyses are to be done at a later date. Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.

SERUM SAMPLE PRE-TREATMENT

Preparation: Dilute each serum sample 1:10 in assay buffer for use. Example: Add 20 µL of serum sample to 180 µL of assay buffer in a clean disposable glass test tube.
Diluted Stability: Tightly capped diluted serum samples are stable at 4ºC for 7 days.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

1. Precision pipette to deliver 20-200 µL
2. Disposable pipette tips
3. Disposable glass test tubes
4. Distilled or deionized water
5. Plate shaker
6. Microtiter plate washer (recommended)
7. Microwell plate reader with a filter set at 450nm and an upper OD limit of 3.0 or greater

REAGENTS PROVIDED

1. Anti-Leptin Monoclonal Antibody Coated Microwell Plate-Break Apart Wells
Contents: One 96 well (12x8) monoclonal antibody-coated microwell plate in a resealable pouch with desiccant.
Storage: Refrigerate at 2-8ºC
Stability: 12 months or as indicated on label.

2. Monoclonal Anti-Leptin-Biotin Conjugate
Contents: Monoclonal anti-leptin antibody conjugated to biotin in a protein-based buffer with a non-mercury preservative.
Volume: 10 mL/bottle
Storage: Refrigerate at 2-8ºC
Stability: As indicated on label.

3. Streptavidin-HRP Conjugate Concentrate
Contents: Streptavidn conjugated to horseradish peroxidase in a protein-based buffer with a non-mercury preservative.
Volume: 0.4 mL/bottle
Storage: Refrigerate at 2-8ºC
Stability: As indicated on label.
Preparation: Dilute 1:50 in assay buffer before use (eg. 40 µL of concentrate in 2 mL of assay buffer). If the whole plate is to be used dilute 240 µL of concentrate in 12 mL of assay buffer. Discard any that is left over.

4. Leptin Calibrators
Contents: Six bottles containing leptin in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of leptin. Listed below are approximate concentrations, please refer to bottle labels for exact concentrations.

Calibrator	Concentration	Volume/Bottle
Calibrator A	0 ng/mL	0.5 m:
Calibrator B	1 ng/mL	0.5 mL
Calibrator C	5 ng/mL	0.5 mL
Calibrator D	10 ng/mL	0.5 mL
Calibrator E	20 ng/mL	0.5 mL
Calibrator F	50 ng/mL	0.5 mL
Calibrator G	100 ng/mL	0.5 mL

Storage: Refrigerate at 2-8ºC
Stability: Unopened at 2-8ºC until expiration date on label.
Diluted Stability: Tightly capped diluted calibrators are stable at 4ºC for 7 days.
Preparation: Dilute each calibrator 1:10 in assay buffer for use. Example: Add 20 µL of calibrator to 180 µL of assay buffer in a clean disposable glass test tube.

5. Control
Contents: One bottle containing leptin in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of leptin. Refer to bottle label for expected value and acceptable range.
Volume: 0.5 mL/bottle
Storage: Refrigerate at 2-8ºC
Stability: Unopened at 2-8ºC until expiration date on label.
Diluted Stability: Tightly capped diluted control is stable at 4ºC for 7 days.
Preparation: Dilute the control 1:10 in assay buffer for use. Example: Add 20 µL of the control to 180 µL of assay buffer in a clean disposable glass test tube.

6. Wash Buffer Concentrate
Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume: 50 mL/bottle
Storage: Refrigerate at 2-8ºC
Stability: As indicated on label.
Preparation: Dilute 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 ml of the wash buffer concentrate in 450 ml of water.

7. Assay Buffer
Contents: One bottle containing a protein-based buffer with a non-mercury preservative.
Volume: 50 mL/bottle
Storage: Refrigerate at 2-8ºC
Opened bottle is stable at 2-8ºC until expiration date on label,

8. TMB Substrate
Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
Volume: 16 mL/bottle
Storage: Refrigerate at 2-8ºC
Stability: As indicated on label.

9. Stopping Solution
Contents: One bottle containing 1M sulfuric acid.
Volume: 6 mL/bottle
Storage: Refrigerate at 2-8ºC
Stability: As indicated on label.

ASSAY PROCEDURE

Pretreatment: Dilute calibrators, control and serum samples 1:10 in assay buffer.

All reagents must reach room temperature before use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. Prepare working solutions of the streptavidin- HP conjugate and wash buffer.

2. Pipette 20 µL of each diluted calibrator, control and serum sample into correspondingly labelledwells in duplicate.

3. Pipette 80 µL of the monoclonal anti-leptin-biotin conjugate into each well.

4. Incubate on a plate shaker (approximately 200 rpm) for 1 hour at room temperature.

5. Wash the wells 3 times with 300 µL of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry (The use of a washer is highly recommended).

6. Pipette 100 µL of streptavidin-HRP conjugate into each well.

7. Incubate on a plate shaker (approximately 200 rpm) for 30 minutes at room temperature.

8. Wash the wells again in the same manner as step 5.

9. Pipette 100 µL of TMB substrate into each well at timed intervals.

10. Incubate on a plate shaker for 10-15 minutes at room temperature.

11. Pipette 50 µL of stopping solution into each well at the same timed intervals as in step 9.

12. Read the plate on a microwell plate reader at 450nm within 20 minutes after addition of the stopping solution.

CALCULATIONS

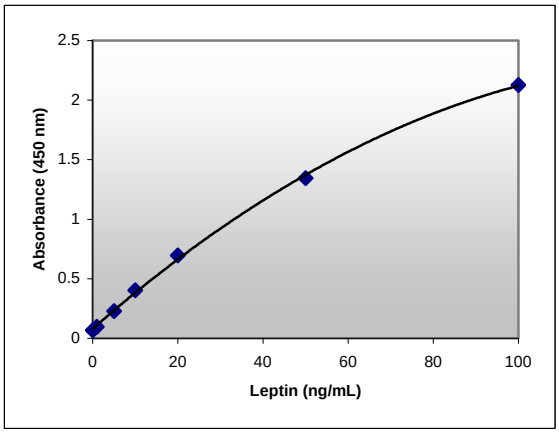
1. Calculate the mean optical density of each calibrator duplicate.
2. Draw a calibrator curve on semi-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter curve is recommended.
3. Calculate the mean optical density of each unknown duplicate.
4. Read the values of the unknowns directly off the calibrator curve.
5. If a sample reads more than 100 ng/mL then dilute it with assay buffer at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor. For example, if diluting a sample (which is already 1:10 diluted) 1:8 then the obtained results must be multiplied by 8.

TYPICAL TABULATED DATA

Calibrator	OD 1	OD 2	Mean OD	Value (ng/mL)
A	0.069	0.064	0.067	0
B	0.098	0.095	0.097	1
C	0.234	0.223	0.229	5
D	0.396	0.412	0.404	10
E	0.691	0.698	0.695	20
F	1.339	1.352	1.346	50
G	2.106	2.145	2.126	100
Unknown	0.224	0.226	0.225	4.56

TYPICAL CALIBRATOR CURVE

Sample curve only. **Do not** use to calculate results.



PERFORMANCE CHARACTERISTICS

SENSITIVITY

The limit of detection (LoD) for Leptin is 0.35 ng/mL, as determined by use of a NCCLS protocol and with proportions of false positives (α) less than 5% and false negatives (β) less than 5%; based on 82 blank determinations; LoB=0.02 ng/mL.

SPECIFICITY

The following substances were tested at 1000 ng/mL and exhibited no cross-reactivity:
Mouse Leptin, TNF-α, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12, IL-16, GM-CSF, CSF and EGF.

INTRA-ASSAY PRECISION

Four serum samples were assayed twenty times each on the same calibrator curve. The results (in ng/mL) are tabulated below:

Sample	Mean	SD	CV%
1	2.45	0.09	3.7
2	7.94	0.34	4.3
3	11.67	0.64	5.5
4	27.51	1.37	5.0

INTER-ASSAY PRECISION

Four samples were assayed ten times over a period of ten days. Standards and samples were freshly diluted 1:10 prior to every run. The results (in ng/mL) are tabulated below:

Sample	Mean	SD	CV%
1	2.71	0.16	5.9
2	8.24	0.48	5.8
3	12.01	0.82	6.8
4	24.98	1.45	5.8

RECOVERY

Spiked samples were prepared by adding defined amounts of leptin to three patient serum samples. The results (in ng/mL) are tabulated below:

Sample	Observed	Expected	%Recovery
1	3.89	-	-
Unspiked	6.28	6.95	90.4
	10.98	11.95	91.9
	25.43	26.95	94.4
2	7.89	-	-
Unspiked	8.82	8.95	98.5
	15.03	13.95	107.7
	30.32	28.95	104.7
3	11.61	-	-
Unspiked	15.71	15.81	99.4
	25.42	24.41	104.1
	41.18	41.07	100.3

LINEARITY

Three patient serum samples were serially diluted with leptin assay buffer. The results (in ng/mL) are tabulated below:

Sample	Observed	Expected	% Recovery
1	3.03	-	-
1:2	1.42	1.52	93.4
1:4	0.71	0.76	93.4
1:8	0.35	0.38	92.1
2	11.27	-	-
1:2	5.93	5.64	105.1
1:4	3.05	2.82	108.2
1:8	1.35	1.41	95.7
3	27.91	-	-
1:2	14.91	13.96	106.8
1:4	6.74	6.98	96.6
1:8	3.29	3.49	94.3

COMPARATIVE STUDY

The Immunospec's Leptin ELISA was compared against a leading competitor's Leptin EIA kit (Kit X).
Thirty-eight serum samples ranging from 1.05-75.62 ng/mL were assayed with both kits, yielding the following results:
Regression: Kit X=0.9644 (Immunospec) + 1.5489
r=0.98
Kit X Mean: 21.13
Immunospec Mean: 20.30

EXPECTED NORMAL VALUES

As for all clinical assays each laboratory should collect data and establish their own range of expected normal values.

Group	Mean (ng/mL)	Range (ng/mL)
Lean Women	7.4	3.7-11.1
Lean Men	3.8	2.0-5.6

Leptin values are approximately 2.5 times higher in women than men per unit BMI.

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