Version 26-04/24

User's Manual

Limostatin (Lst-15) ELISA Kit

REF **DEIA-XYZ94**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

This ELISA is intended for the quantitative determination of the Limostatin in serum or plasma samples.

General Description

Limostatin is a 15-amino acid peptide hormone found in Drosophila melanogaster and is induced by carbohydrate restriction from endocrine cells associated with the gut. It suppresses insulin production and release by insulin-producing cells. Limostatin is important in adaptation to starvation conditions, and represents a mechanism by which insulin is negatively regulated.

Principles of Testing

This ELISA kit is a competitive immunoassay. The antiserum is captured by antibodies coated on a 96-well plate. A constant concentration of Bt-tracer (biotinylated tracer) and varying concentrations of unlabeled standard or sample peptide compete for binding specifically to the antiserum. Captured Bt-tracer is subsequently bound by streptavidin-conjugated horseradish peroxidase (SA-HRP), which produces a soluble colored product after a substrate is added.

The standard is used to make a standard curve in the range specified in the kit's datasheet. Standard curves are S-shaped (on a semi-log plot) but for a few kits they appear to be almost linear over the kit's range. The measuring range is the range of standard concentrations near the middle or near the IC_{50} of the standard curve. Unknown sample concentrations are measured by comparing their absorbance with the standard curve.

Reagents And Materials Provided

1. ELISA buffer concentrate (50ml 20× concentrate)
2. 96-well immunoplate with plate sealer
3. Antiserum (lyophilized powder)
4. Standard (1 mg lyophilized powder)
5. Biotinylated tracer (lyophilized powder)
6. Streptavidin-HRP (100ul 200× concentrate)
7. TMB substrate stock solution (1.5ml)
8. TMB substrate buffer (15ml citrate buffer)
9. Stop solution 2N HCl (15 ml)
10. Datasheet
11. Protocols

The following materials are not included but are recommended for Limostatin (Lst-15) ELISA kits.

Extraction kit (with 50 Sep-columns and buffers A and B)

Buffer A

Buffer B

Sep-Column (200 mg)

Seo-Column adapter

Materials Required But Not Supplied

1. 96-well microtiter plate reader set up to measure 450nm and 650nm
2. 96-well plate washer and shaker (optional)
3. Distilled or deionized water, or comparable quality
4. Curve fitting software (optional)
5. Test tubes, pipettes and various other standard laboratory items

Storage

After you receive the kit, store the lyophilized components and standard diluent at -20°C for up to one year from the kit's assembly date. The remaining components should be stored in the refrigerator (4-8°C) also up to 1 year. Long term storage, improper storage conditions and large temperature fluctuation cycles may cause precipitates in the TMB solution and in the ELISA buffer concentrate. These precipitates should not affect the assay noticeably. Nevertheless, if you observe such precipitates, we recommend to avoid them by allowing them to sink to the bottom.

Specimen Collection And Preparation

1. Sample extraction. Sample extraction is recommended for serum or plasma samples. It may be less important for tissue culture samples. See "Suggested Protocol for Sample Extraction" below for details. The kit may still be used without extraction but this may cause unexpected results due to the possible binding between serum proteins and kit components.
2. Sample concentration. The concentration of the target molecule must be within the measuring range of the kit (around the IC50). If the concentration range of your sample is difficult to estimate, prepare it at different concentrations such that one of the samples should lie within the measuring range.

SUGGESTED PROTOCOL FOR SAMPLE EXTRACTION

We have provided an excess amount of standard that you may use to determine if extraction is required. For example, if you are working with serum, you may spike it with known amounts of standard and check if they are accurately determined by the assay with and without extraction. Extraction eliminates potentially interfering substances, such as albumin. Extraction may also be necessary to concentrate the sample to within the measuring range. As with any purification technique, recovery of the desired substance is likely to be incomplete. Therefore, both optimization and quantification of the extraction procedure are recommended for more accurate determinations. While we cannot provide you with extraction optimization and quantification protocols, we have included enough standard in the kit should you wish to use it for this purpose.

C_{18} Sep-Column Extraction Method

The following generic protocol is meant to help users with extracting their samples. It should be applicable to

different biological fluids but should not be thought of as an optimized protocol for any particular antigen.

Required Materials

1 SEP-COLUMN containing 200mg of C18

2. Buffer A (BUFF-A): 1% trifluoroacetic acid (TFA, HPLC Grade). (Acidifies plasma sample to remove interfering proteins such as albumin)
3. Buffer B (BUFF-B): 60% acetonitrile (HPLC Grade), 1% TFA, and 39% distilled water. (Elutes peptide from column)
4. You may also consider purchasing Extraction kits, which include SEP-columns and buffers

Withdrawal and Preparation of Plasma

1. Collect blood samples (2-6ml) into a chilled syringe and transfer into a polypropylene tube containing EDTA (1mg/ml of blood) as an anticoagulant and Aprotinin (500KIU/ml of blood) as a protease inhibitor at 4°C. Do not use heparinized tubes as they may interfere with the assay. Vacutainers with EDTA are acceptable.
2. Centrifuge blood at 1,600×g for 15 minutes at 4°C.
3. Collect the top (plasma) layer.
4. Proceed to extraction immediately or freeze at -70°C for later use.

Extraction Procedure

1. Add an equal amount of Buffer A to the plasma.
2. Centrifuge at 6,000×g to 17,000×g for 20 minutes at 4°C.
3. Transfer supernatant to a new tube discarding any pellet that may be present.
4. Equilibrate a SEP-COLUMN by washing with 1 ml Buffer B followed by 3×3ml Buffer A.
5. Load the plasma solution onto the equilibrated SEP-Column.
6. Slowly wash the column with Buffer A (3 ml, twice) and discard the wash. A light vacuum (10 sec/drop) may be applied to the column.
7. Elute the peptide slowly with Buffer B (3 ml, once) and collect eluant in a polypropylene tube. A light vacuum may be applied as in previous step.
8. Freeze-dry eluant to dryness using a dry ice/methanol bath to freeze the sample and a centrifugal concentrator to evaporate it.
9. Dissolve the residue in a suitable volume of ELISA buffer such that the concentration of the substance of interest will fall close to the IC50 (within the measuring range).

Reagent Preparation

Lyophilized kit components should not be re-hydrated until they are needed.

1. Equilibrate unopened kit components to room temperature. Avoid accumulation of moisture, do not open reagents and immunoplate while they are cold.
2. ELISA buffer. Dilute the ELISA buffer concentrate 1 in 20 with water and mix well. Example: mix the 50ml contained in the kit with 950ml of water.
3. Standard. Add 1 ml of standard diluent buffer (working dilution of ELISA buffer) to the vial of lyophilized standard peptide (1 µg) and vortex gently. If samples are to be extracted and re-suspended in ELISA buffer as described below, use ELISA buffer as a diluent. Otherwise, we encourage customers to use their own

diluent such that standards and samples will be treated equally.

4. Standard curve. Make serial dilutions of the standard to cover the range of this kit.
5. Antiserum. Add 5ml (10ml with protocol VII) of ELISA buffer and vortex.
6. Biotinylated tracer. Add 5ml of ELISA buffer to the vial of lyophilized biotinylated peptide and vortex.

Assay Procedure

ELISA buffer and Diluent. Antiserum and Bt-tracer are always reconstituted and used in ELISA buffer. The standards and samples are prepared in "standard diluent" (or diluent). The diluent is also ELISA buffer. If there is no interference with the kit's components, you should use your own diluent for your samples and standards. However, the standard curve should show similar characteristics as the one from the data sheet.

Room Temperature. Reagents, samples, and the plate should be brought to room temperature before use.

Shakers. Shakers (optional) may help lower the experimental variation of duplicates (recommended at 60 rpm).

Blank Wells. Blanks will give you the background to be subtracted from all readings. These should not be confused with the "S0 Standards" which contain no standard peptide and which will yield the highest readings. Blank readings will not influence concentration calculations - thus, they are optional.

LAYOUT

Seven-Point Standard Curve Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	Blk	U1	U1	U9	U9	U17	U17	U25	U25	U33	U33
B	S1	S1	U2	U2	U10	U10	U18	U18	U26	U26	U34	U34
C	S2	S2	U3	U3	U11	U11	U19	U19	U27	U27	U35	U35
D	S3	S3	U4	U4	U12	U12	U20	U20	U28	U28	U36	U36
E	S4	S4	U5	U5	U13	U13	U21	U21	U29	U29	U37	U37
F	S5	S5	U6	U6	U14	U14	U22	U22	U30	U30	U38	U38
G	S6	S6	U7	U7	U15	U15	U23	U23	U31	U31	U39	U39
H	S0	S0	U8	U8	U16	U16	U24	U24	U32	U32	U40	U40

Blk = blank S = standards U = unknown samples

Protocol lll (Std.Ab1hr.Bt)

1. Into each well of the immunoplate add

   50µl standard or sample (in diluent)

   25µl antiserum (in ELISA buffer)

   Add 50µl diluent and 25µl ELISA buffer to blank wells.
2. Incubate at room temperature for 1 hour. Shorter incubation may result in lower sensitivity.
3. Rehydrate the Bt-tracer (in ELISA buffer) and add 25µl per well.
4. Incubate at room temperature for 2 hours.
5. Wash immunoplate 5 times with 300µl per well of ELISA buffer. Be careful not to cross-contaminate between wells in the first wash/dispensing cycle. In each wash cycle empty plate contents with a rapid

flicking motion of the wrist, then gently blot dry the top of plate on paper towels. Dispense 300µl of ELISA buffer into each well and gently shake for at least a few seconds. Thorough washing is essential.

6. Add 100µl per well of streptavidin-HRP. Tap or centrifuge the SA-HRP vial to collect all liquid contents on the bottom of the vial. Dilute 1/200 in ELISA buffer (60µl in 12ml) and vortex gently. Add 100µl to all the wells, including blanks.
7. Incubate at room temperature for 1 hour.
8. Prepare TMB chromogenic solution immediately before use by mixing 20 parts of the TMB substrate buffer (citrate, brought to room temperature) with 1 part TMB - H_2O_2 solution (TMB substrate stock). This dilution should be used within 15 minutes after preparation.
9. Wash immunoplate 5 times (see step 5).
10. Add 100µl per well of TMB solution. Add to all the wells, including blanks.
11. Incubate at room temperature (usually 10 to 30 minutes). You may read the developing blue color at 650nm and use the data for your calculations.
12. Terminate reactions by adding 100µl 2N HCI per well.
13. Read absorbance at 450nm within fifteen minutes.

Quality Control

The kit's IC_{50}, or the shape of the standard curve, may exhibit some variation but this will not affect the kit's accuracy in the measuring range. The kit accurately measures sample peptides if the following conditions are met.

A) Both samples and standards must be measured in the same diluent and under the same conditions (same microtiter plate).

B) The kit's antiserum must not cross-react appreciably with other factors present in the sample. The user may wish to test the cross-reactivity with other peptides.

C) The sample peptides must be identical to the kit's standard. Ideally the kit's synthetic standard mimics the natural peptide perfectly. Sometimes, however, natural peptides exist as families of species related by a common or similar sequence. Also, natural peptides may be modified enzymatically or spontaneously, may exist in complexes, and may assume alternative structural forms. In these cases the kit might not measure the exact concentration of a particular natural peptide species, but it may still be used for relative average measurements.

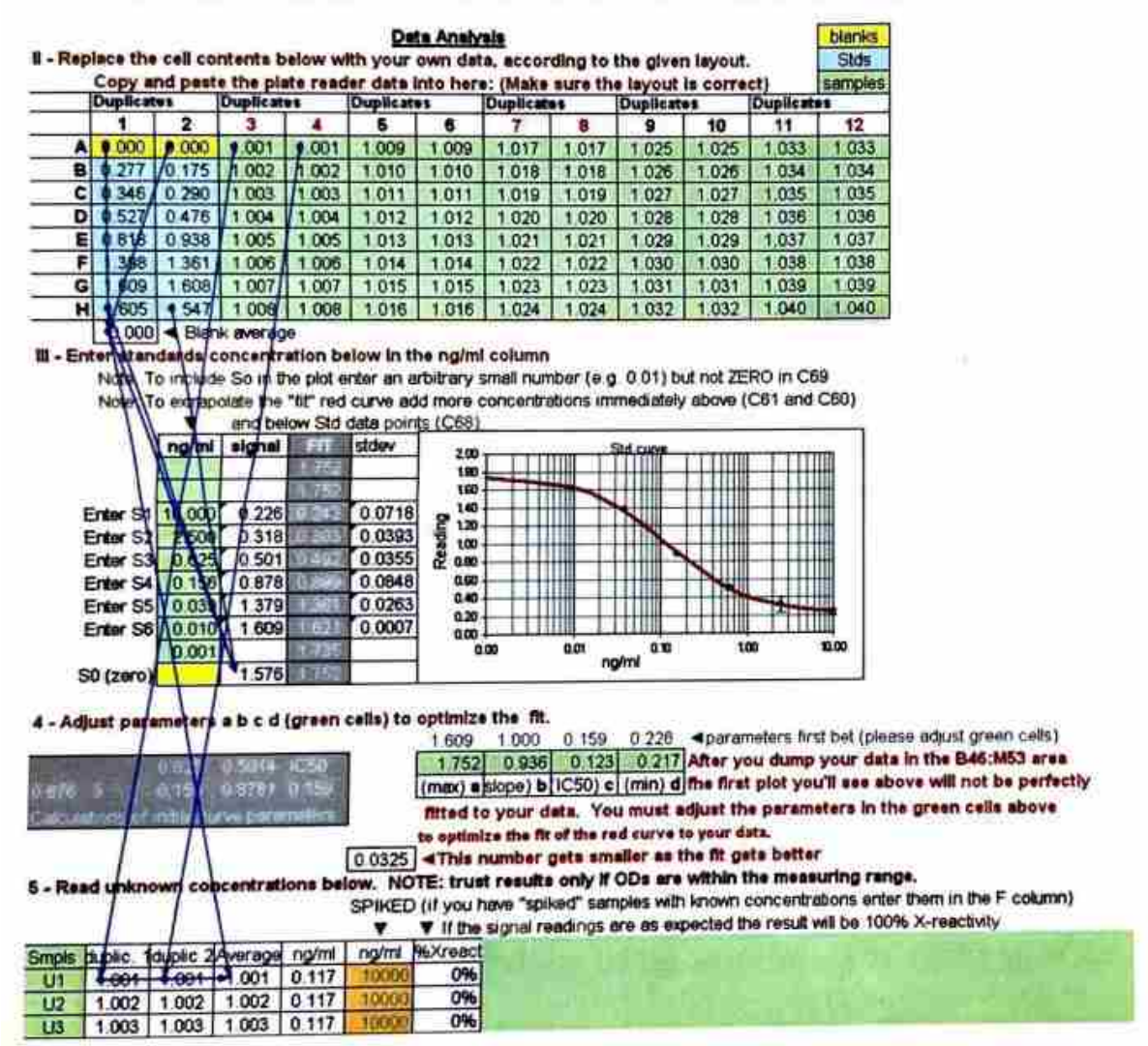
D) Sample extraction. Factors present in serum can bind to the kit components. The effects can vary from negligible to complete obliteration of signal. Therefore, sample extraction may be required prior to using the kits.

Calculation

Plot data and calculate results. We recommend that you use curve fitting software for your data analysis. Plate readers often include such software packages. This is, however, not essential and you may opt to plot manually on semi-log paper. You can also use a spreadsheet program. Should you need help with the latter method we recommend the following procedure.

Set up a spreadsheet as shown below (note that the values on the spreadsheet are merely illustrative and

are not necessarily typical for this particular kit). If you contact us, we will be happy to send you the actual working Excel spreadsheet shown below.



Set up an 8×12 area to match the layout of the plate and copy the plate reader data in it. Calculate the average of the blanks on another cell as indicated by the arrows starting from cells A 1 and A2.

Enter the concentration of the standards (see under ng/ml in figure). Calculate the average of the ODs of the standards and subtract the background (blank) as indicated by the arrows for the last standard (cells H1 and H2).

Make a standard curve by plotting the OD readings (minus the blank average) against the standard concentrations in ng/ml.

Use the equation shown below to calculate the values on the "FIT" column and plot a smooth line of FIT values versus standard concentrations. Then change the parameters a(max), b(slope), c(IC_{50}), and d(min), until you are satisfied that fit is good.

$y = [(a-d) / (1 + (x/c)^b)] + d$

Next, calculate the average of your sample readings and subtract the blank average (see arrows starting from A3 and A4, and the arrows leading to "Average")

Finally, you may isolate x in the equation above to calculate the concentrations in ng/ml for all your samples:

$x = c\ [(y-a) / (d-y)\ )]^{1/b}$

Caution: when you calculate sample concentrations using the "reverse" equation if y = d or y > a or y < d, the reading is out of range and the calculation will yield an error or a meaningless negative concentration.

Detection Range

0-25ng/ml

Sensitivity

IC_{50}: 0.3ng/ml

Specificity

Cross-Reactivity:

Limostatin (Lst-15) (Drosophila): 100%

Precautions

1. The physical and chemical properties of the reagents contained in this kit have been tested individually. Reagents do not contain ingredients which have been determined to be health hazards and which comprise greater than 1% of the mixture or which could be released from the mixture in concentrations that would exceed OSHA permissible exposure limits.
2. Hazardous Ingredients: The lyophilized standard, antiserum and biotinylated tracer contain thimerosal. The ELISA buffer concentrate contains Tris and thimerosal. The buffer is in liquid form. The SA-HRP contains 0.01% methylisothiazolone, 0.01% bromonitrodioxane, and 10ppm Proclin 300 as a preservative.
3. Physical and Chemical Data: Components are stable in closed containers under normal temperatures and pressures. No hazardous polymerization is known.
4. Fire and Explosion Data: Components are non-combustible with negligible fire hazard when exposed to heat or flame. Fire fighting media should be appropriate to burning material.
5. Health Hazards: Components may be harmful by inhalation, ingestion, or skin absorption and may cause skin irritation or eye irritation. In case of eye contact, flush eye with water and contact a physician. In case of skin contact, wash skin with soap and water.
6. Reactivity Data: Components are stable in closed containers under normal temperatures and pressures.
7. Spill and Disposal Procedures: For spills, ventilate area and wash spill site. For disposal, please dispose in accordance with local regulations.
8. Handling and Storage Information: Safety glasses, gloves, and a full-length lab coat should be worn to prevent unnecessary contact.

The above information is believed to be correct but does not purport to be all-inclusive and shall be used only as a guide. It is the user's responsibility to determine the suitability of this information for the adoption of safety precautions as may be necessary. Creative Diagnostics shall not be held liable for any damage resulting from the handling or use of the above product.

References

1) T. Porstmann and S.T. Kiessig Enzyme immunoassay techniques. An Overview. J. lmmunol. Methods 150, 5-21 (1992)

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3) E. Bucht et al. A rapid extraction method for serum calcitonin. Clin. Chim. Acta, 195, 115-124 (1991)