LSBio™ Mouse/Human/Rat GHRL / Ghrelin Enzyme Immunoassay Kit

Catalog No. LS-F11

User Manual

(Version 2.2 Revised April 8, 2014)

Please Read the Manual Carefully
Before Starting your Experiment

LSBio
LifeSpan BioSciences, Inc.

For research use only. Not approved for use in humans or for clinical diagnosis.

Human/Mouse/Rat Obestatin Enzyme Immunoassay Kit Protocol

TABLE OF CONTENTS

I. INTRODUCTION

Obestatin is a 24-aa peptide hormone that is produced in the stomach and small intestine of humans and several other mammals. It is encoded by the same gene that also encodes adipokine Ghrelin, which is initially expressed as a preprohormone that is subsequently cleaved into two smaller peptides, Ghrelin and Obestatin. A model has been proposed suggesting a “Yin-Yang” relationship between the two peptides, that Ghrelin and Obestatin have countering effects to each other.

There are reports using Ghrelin knockout mice studies which showed that removing the Ghrelin gene from mice did not significantly reduce their appetite. Additional reports show that Obestatin antagonizes growth hormone secretion and food intake induced by Ghrelin.

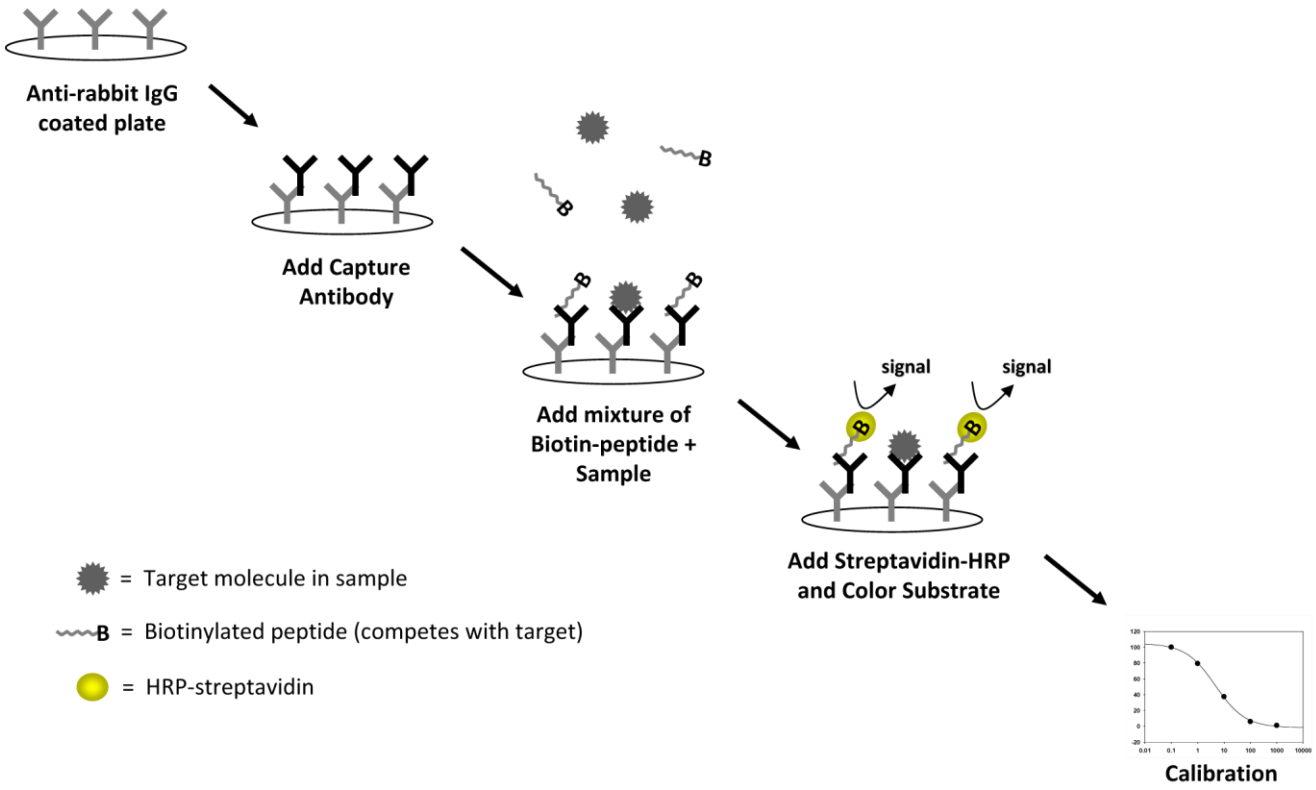
Obestatin has shown some potential clinical applications. Studies on the Obestatin/Ghrelin ratio in the gastrointestinal tract and plasma are associated with metabolic diseases such as irritable bowel syndrome, obesity and type II diabetes mellitus.

II. GENERAL DESCRIPTION

The Obestatin Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Obestatin peptide based on the principle of Competitive Enzyme Immunoassay.

The microplate in the kit is pre-coated with anti-rabbit secondary antibody. After a blocking step and incubation of the plate with anti-Obestatin antibody, both biotinylated Obestatin peptide and peptide standard or targeted peptide in samples interacts competitively with the Obestatin antibody. Uncompeted (bound) biotinylated Obestatin peptide then interacts with Streptavidin-horseradish peroxidase (SA-HRP) which catalyzes a color development reaction. The intensity of colorimetric signal is directly proportional to the amount of biotinylated peptide-SA-HRP complex and inversely proportional to the amount of Obestatin peptide in the standard or samples. This is due to the competitive binding to Obestatin antibody between biotinylated Obestatin peptide and peptides in standard or samples. A standard curve of known concentration of Obestatin peptide can be established and the concentration of Obestatin peptide in the samples can be calculated accordingly.

Principle of Competitive EIA



III. REAGENTS

1. Obestatin Microplate (Item A): 96 wells (12 strips x 8 wells) coated with secondary antibody.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml
3. Standard Obestatin Peptide (Item C): 2 vials, 10 μl/vial
4. Anti-Obestatin polyclonal antibody (Item N): 2 vials, 5 μl/vial
5. Assay Diluent A (Item D): 30 ml, contains 0.09% sodium azide as preservative. Diluent for standards and serum or plasma samples.
6. Assay Diluent B (Item E): 15 ml of 5x concentrated buffer. Diluent for standards and cell culture media or other sample types.
7. Biotinylated Obestatin peptide, (Item F): 2 vials, 20 μl/vial
8. HRP-Streptavidin concentrate (Item G): 600 μl 1000x concentrated HRP-conjugated Streptavidin.
9. Positive control (Item M): 1 vial, 100 μl
10. TMB One-Step Substrate Reagent (Item H): 12 ml of 3, 3', 5, 5'- tetramethylbenzidine (TMB) in buffered solution.
11. Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.
12. Assay Diagram (Item J).
13. User Manual (Item K)

IV. STORAGE

- Standard, Biotinylated Obestatin peptide, and Positive Control should be stored at -20°C or -80°C (recommended at -80°C) after arrival. **Avoid multiple freeze-thaws.**
- The remaining kit components may be stored at -20°C.
- Opened Microplate Wells and antibody (Item N) may be stored for up to 1 month at 2° to 8°C. Return unused wells to the pouch containing desiccant pack and reseal along entire edge.
- If stored in this manner, Lifespan warranties this kit for 6 months from the date of shipment.

V. ADDITIONAL MATERIALS REQUIRED

1. Microplate reader capable of measuring absorbance at 450nm.
2. Precision pipettes to deliver 2 μl to 1 ml volumes.
3. Adjustable 1-25 ml pipettes for reagent preparation.
4. 100 ml and 1 liter graduated cylinders.
5. Absorbent paper.
6. Distilled or deionized water.
7. SigmaPlot software (or other software which can perform four-parameter logistic regression models)
8. Tubes to prepare standard or sample dilutions.
9. Orbital shaker
10. Aluminum foil
11. Saran Wrap

VI. REAGENT PREPARATION

If testing plasma or serum samples, use Assay Diluent A to dilute Item F and Item C. If testing cell culture media or other sample types, use Assay Diluent B to dilute Item F and Item C. For sample and positive control dilutions, refer to steps 6, 7, 8 and 10 of Reagent Preparation.

1. Keep kit reagents on ice during reagent preparation steps. Equilibrate plate to room temperature before opening the sealed pouch.

2. Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water.

3. Briefly centrifuge the Anti-Obestatin Antibody vial (Item N) before use. Add 50 µl of 1x Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently.

4. The antibody concentrate should then be diluted 100-fold with 1x Assay Diluent B. This is your anti-Obestatin antibody working solution, which will be used in step 2 of the Assay Procedure.

NOTE: the following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure).

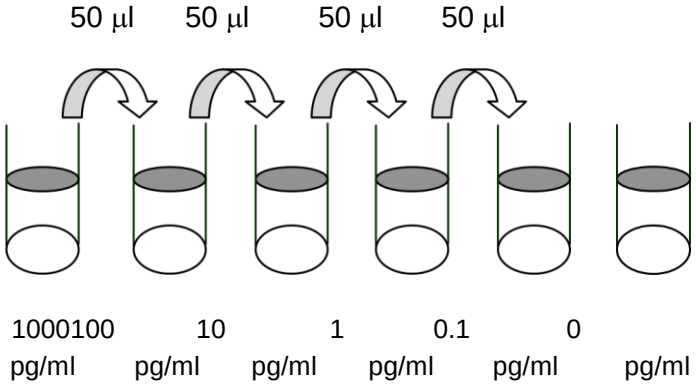
5. Briefly centrifuge the vial of Biotinylated Obestatin (Item F) before use. Add 5 µl of Item F to 5 ml of the appropriate Assay Diluent. Pipette up and down to mix gently. *The final concentration of biotinylated Obestatin will be 10 pg/ml.* This solution will only be used as the diluent in step 6 of Reagent Preparation.

6. Preparation of Standards: Label 6 microtubes with the following concentrations: 1000 pg/ml, 100 pg/ml, 10 pg/ml, 1 pg/ml, 0.1 pg/ml and 0 pg/ml. Pipette 450 µl of biotinylated Obestatin solution into each tube, except for the 1000 pg/ml (leave this one empty). *It is very important to make sure the concentration of biotinylated Obestatin is 10 pg/ml in all standards.*

a. Briefly centrifuge the vial of Obestatin (Item C). In the tube labeled 1000 pg/ml, pipette 8 µl of Item C and 792 µl of 10 pg/ml biotinylated Obestatin solution (prepared in step 5 above). This is your Obestatin stock solution (1000 pg/ml Obestatin, 10 pg/ml biotinylated Obestatin). Mix thoroughly. This solution serves as the first standard.

b. To make the 100 pg/ml standard, pipette 50 µl of Obestatin stock solution the tube labeled 100 pg/ml. Mix thoroughly. c. Repeat this step with each successive concentration, preparing a dilution series as shown in the illustration below. Each time, use 450 µl of biotinylated Obestatin and 50 µl of the prior concentration until 0.1 pg/ml is reached. Mix each tube thoroughly before the next transfer.

d. The final tube (0 pg/ml Obestatin, 10 pg/ml biotinylated Obestatin) serves as the zero standard (or total binding).



7. Prepare a 10-fold dilution of Item F. To do this, add 2 µl of Item F to 18 µl of the appropriate Assay Diluent. This solution will be used in steps 8 and 10.

8. Positive Control Preparation: briefly centrifuge the positive control vial (Item M). To the tube of Item M, add 101 µl 1x Assay Diluent B. Also add 2 µl of 10-fold diluted Item F (prepared in step 7) to the tube. This is a 2-fold dilution of the positive control. Mix thoroughly. The positive control is a cell culture medium sample that is meant to be a system control (to verify that the detection & kit components are working). The resulting OD will not be used in any calculations; if no positive competition is observed please contact Lifespan Technical Support. It may be diluted further if desired, but be sure the final concentration of biotinylated Obestatin is 10 pg/ml.

9. If Item B (20X Wash Concentrate) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.

10. Sample Preparation: Use Assay Diluent A + biotinylated Obestatin to dilute serum/plasma samples. For cell culture medium and other sample types, use 1X Assay Diluent B + biotinylated Obestatin as the diluent. *It is very important to make sure the final concentration of the biotinylated Obestatin is 10 pg/ml in every sample.* EXAMPLE: to make a 4-fold dilution of sample, mix together 2.5 µl of 10-fold diluted Item F (prepared in step 7), 185 µl of appropriate Assay Diluent, and 62.5 µl of your sample; mix gently. The total volume is 250 µl, enough for duplicate wells on the microplate.

    Do not use Item F diluent from Step 5 for sample preparation. If you plan to use undiluted samples, you must still add biotinylated Obestatin to a final concentration of 10 pg/ml. EXAMPLE: Add 2.5 µl of 10-fold diluted Item F to 247.5 µl of sample. NOTE: Optimal sample dilution factors should be determined empirically, however you may contact technical support (888-494-8555; techsupport@raybiotech.com) to obtain recommended dilution ranges for serum or plasma.

11. Briefly centrifuge the HRP-Streptavidin vial (Item G) before use. The HRP-Streptavidin concentrate should be diluted 1000-fold with 1X Assay Diluent B.

    Note: Do not use Assay Diluent A for HRP-Streptavidin preparation in Step 11.

VII. ASSAY PROCEDURE:

1. Keep kit reagents on ice during reagent preparation steps. It is recommended that all standards and samples be run at least in duplicate.

2. Add 100 µl anti-Obestatin antibody (see Reagent Preparation step 4) to each well. Incubate for 1.5 hours at room

temperature with gentle shaking (1-2 cycles/sec). You may also incubate overnight at 4 degrees C.

3. Discard the solution and wash wells 4 times with 1x Wash Buffer (200-300 µl each), Washing may be done with a multichannel pipette or an automated plate washer. Complete removal of liquid at each step is essential to good assay performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 100 µl of each standard (see Reagent Preparation step 6), positive control (see Reagent Preparation step 8) and sample (see Reagent Preparation step 10) into appropriate wells. Be sure to include a blank well (Assay Diluent only). Cover wells and incubate for 2.5 hours at room temperature with gentle shaking (1-2 cycles/sec) or overnight at 4°C.

5. Discard the solution and wash 4 times as directed in Step 3.

6. Add 100 µl of prepared HRP-Streptavidin solution (see Reagent Preparation step 11) to each well. Incubate for 45 minutes with gentle shaking at room temperature. It is recommended that incubation time should not be shorter or longer than 45 minutes.

7. Discard the solution and wash 4 times as directed in Step 3. 8.
Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec).

9. Add 50 µl of Stop Solution (Item I) to each well. Read absorbances at 450 nm immediately.

VIII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.

⇩

2. Add 100 μl anti- Obestatin antibody to each well. Incubate 1.5 hours at room temperature or overnight at 4°C.

⇩

3. Add 100 μl standard or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.

⇩

4. Add 100 μl prepared streptavidin solution. Incubate 45 minutes at room temperature.

⇩

5. Add 100 μl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.

⇩

6. Add 50 μl Stop Solution to each well. Read at 450 nm immediately

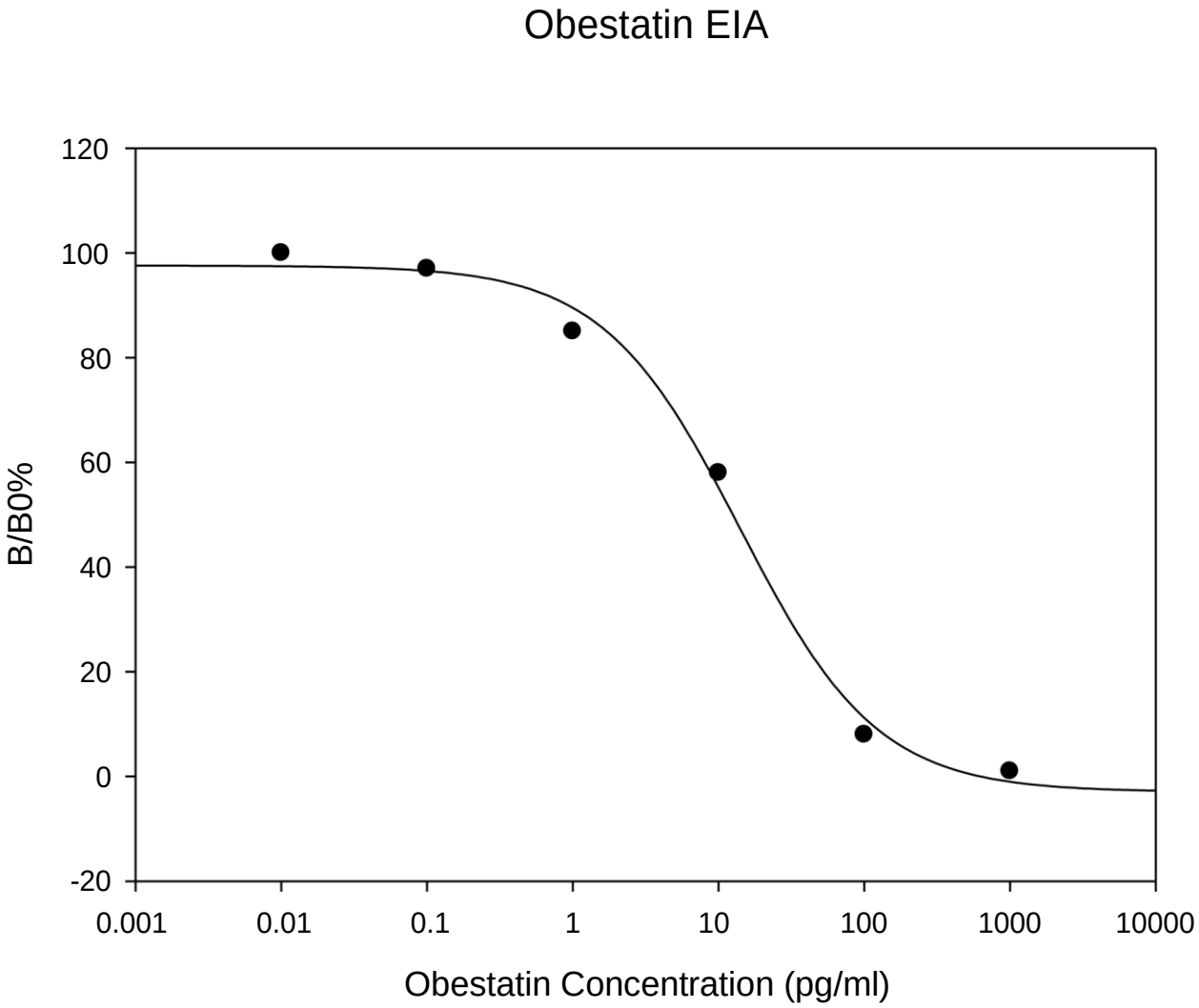
IX. CALCULATION OF RESULTS

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis and percentage of absorbance (see calculation below) on the y-axis. Draw the best-fit curve through the standard points.

Percentage absorbance = (B – blank OD)/(B_o – blank OD) where
B = OD of sample or standard and
B_o = OD of zero standard (total binding)

A. TYPICAL DATA

These standard curves are for demonstration only. A standard curve must be run with each assay.



B. SENSITIVITY

The minimum detectable concentration of Obestatin is 0.1pg/ml.

C. DETECTION RANGE

0.1-1,000 pg/ml

D. REPRODUCIBILITY

Intra-Assay: CV<10%

Inter-Assay: CV<15%

X. SPECIFICITY

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested: Ghrelin, Nesfatin, Angiotensin II, NPY and APC.

XI. REFERENCES

1. Zhang JV, Ren PG, Avsian-Kretchmer O, Luo CW, Rauch R, Klein C, Hsueh AJ (2005). "Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake". *Science* 310 (5750): 996–9.

2. Gourcerol G, St-Pierre DH, Taché Y (2007). "Lack of obestatin effects on food intake: should obestatin be renamed ghrelin-associated peptide (GAP)?". *Regul. Pept.* 141 (1–3): 1–7.

3. Hassouna R, Zizzari P, Tolle V (2010). "The ghrelin/obestatin balance in the physiological and pathological control of growth hormone secretion, body composition and food intake". *J. Neuroendocrinol.* 22 (7): 793–804.

4. Sjölund K, Ekman R, Wierup N (2010). "Covariation of plasma ghrelin and motilin in irritable bowel syndrome". *Peptides* 31 (6): 1109–12.

5. Qi X, Li L, Yang G, Liu J, Li K, Tang Y, Liou H, Boden G (2007). "Circulating obestatin levels in normal subjects and in patients with impaired glucose regulation and type 2 diabetes mellitus". *Clin. Endocrinol. (Oxf)* 66 (4): 593–7.

6. Harsch IA, Koebnick C, Tasi AM, Hahn EG, Konturek PC (2009). "Ghrelin and obestatin levels in type 2 diabetic patients with and without delayed gastric emptying". *Dig. Dis. Sci.* 54 (10): 2161–6.

XII. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Poor standard curve	1. Inaccurate pipetting	1. Check pipettes
	2. Improper standard dilution	2. Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	1.Too brief incubation times	1. Ensure sufficient incubation time; assay procedure step 2 change to over night
	2. Inadequate reagent volumes or improper dilution	2. Check pipettes and ensure correct preparation
3. Large CV	1. Inaccurate pipetting	1. Check pipettes
4. High background	1. Plate is insufficiently washed	1. Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	2. Contaminated wash buffer	2. Make fresh wash buffer
5. Low sensitivity	1. Improper storage of the EIA kit	1. Store your standard at ≤ -20°C after receipt of the kit.
	2. Stop solution	2. Stop solution should be added to each well before measure

Important Note: During shipment, small volumes of product will occasionally become entrapped in the seal of the product vial. We recommend briefly centrifuging the vial to dislodge any liquid in the container's cap prior to opening.

Warning: This reagent may contain sodium azide and sulfuric acid. The chemical, physical, and toxicological properties of these materials have not been thoroughly investigated. Standard Laboratory Practices should be followed. Avoid skin and eye contact, inhalation, and ingestion.

Sodium azide forms hydrazoic acid under acidic conditions and may react with lead or copper plumbing to form highly explosive metal azides. On disposal, flush with large volumes of water to prevent accumulation.

Returns, Refunds, Cancelations: Any problems with LifeSpan products must be reported to LifeSpan within 10 days of product receipt. The customer must obtain written authorization from LifeSpan before returning items. To request that goods be returned, please contact LifeSpan Technical Support. If an error by LifeSpan BioSciences results in shipment of an incorrect order, LifeSpan will, at its option, either ship a replacement order at no charge, or credit the customer's account for the original product shipped in error. Returns and cancelations may be subject to a 30% restocking fee.

Conditions & Warranty: All LifeSpan products are intended for Research Use Only and are not for use in human therapeutic or diagnostic applications. The information supplied with each product is believed to be accurate, but no warranty or guarantee is offered for the products, because the ultimate conditions of use are beyond LifeSpan's control. The information supplied with each product is not to be construed as a recommendation to use this product in violation of any patent, and LifeSpan will not be held responsible for any infringement or other violation that may occur with the use of its products. Under no event will LifeSpan be responsible for any loss of profit or indirect consequential damage, including, but not limited to, personal injuries resulting from use of these products. LifeSpan's liability to any user of Products for damages that do not result from any fault of the user, will be limited to replacement of the Product(s) only, and in no event shall LifeSpan's liability exceed the actual price received by LifeSpan for the Product(s) at issue. LifeSpan shall not be liable for any indirect, special, incidental or consequential damages. LIFESPAN FURTHER DISCLAIMS ANY AND ALL EXPRESS AND IMPLIED OR STATUTORY WARRANTIES WITH RESPECT TO THE PRODUCTS, INCLUDING BUT NOT LIMITED TO ANY IMPLIED WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE. LifeSpan disclaims any and all responsibility for any injury or damage which may be caused by the fault of the user.

For research use only. Not approved for use in humans or for clinical diagnosis.

LSBio
LifeSpan BioSciences, Inc.

2401 Fourth Avenue Suite 900 Seattle, WA 98121
Tel: 206.374.1102
Fax: 206.577.4565

Technical.Support@LSBio.com