LSBio™ Mouse/Human/Rat GCG / Glucagon Enzyme Immunoassay Kit

Catalog No. LS-F413

User Manual

(Version 3.2 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 Glucagon Enzyme Immunoassay Kit Protocol

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I. INTRODUCTION

Glucagon is a 29-amino acid peptide hormone secreted by the pancreas. Its function is to raise blood glucose levels to opposing the effect of insulin, which lowers blood glucose levels.

Glucagon is synthesized and secreted from alpha cells of the endocrine portion of the pancreas. In rodents, the alpha cells are located in the outer rim of the islet. However, alpha cells in human pancreas are distributed throughout the islet.

Glucagon and insulin are part of a feedback system that keeps blood glucose levels at a stable level. The pancreas releases glucagon when glucose levels fall too low. Glucagon causes the liver to convert stored glycogen into glucose, which is released into the bloodstream. Glucagon raises blood glucose levels. High blood glucose levels stimulate the release of insulin. Insulin allows glucose to be taken up and used by insulin-dependent tissues.

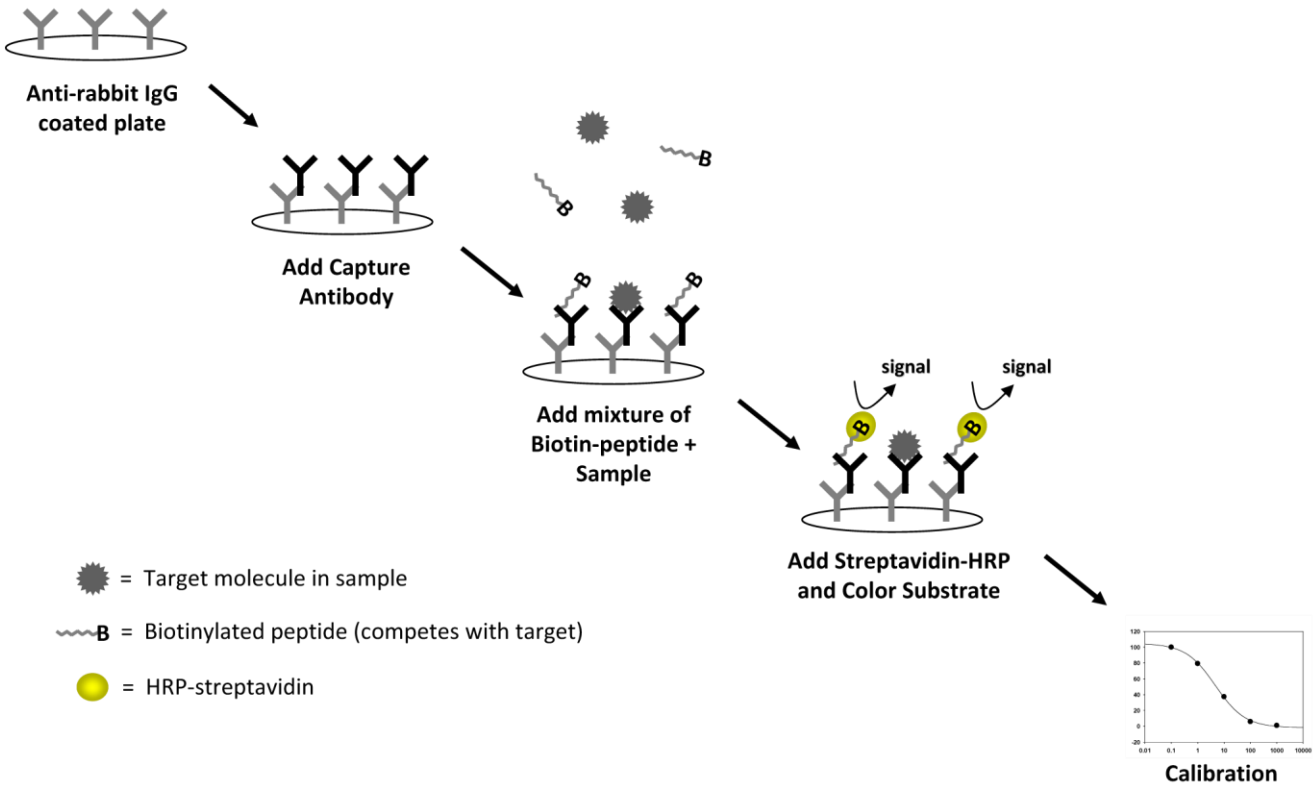
Glucagon has important clinical applications. Abnormally-elevated levels of glucagon may be caused by pancreatic tumors, such as glucagonoma, with symptoms including necrolytic migratory erythema, reduced amino acids, and hyperglycemia. It may occur alone or in the context of multiple endocrine neoplasia type 1 (MENI).

II. GENERAL DESCRIPTION

The Glucagon Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Glucagon 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-Glucagon antibody, both biotinylated Glucagon peptide and peptide standard or targeted peptide in samples interacts competitively with the Glucagon antibody. Uncompeted (bound) biotinylated Glucagon 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 Glucagon peptide in the standard or samples. This is due to the competitive binding to Glucagon antibody between biotinylated Glucagon peptide and peptides in standard or samples. A standard curve of known concentration of Glucagon peptide can be established and the concentration of Glucagon peptide in the samples can be calculated accordingly.

Principle of Competitive EIA



III. REAGENTS

1. Glucagon Microplate (Item A): 96 wells (12 strips x 8 wells) coated with secondary antibody.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml.
3. Lyophilized standard Glucagon peptide (Item C): 2 vials.
4. Lyophilized anti-Glucagon polyclonal antibody (Item N): 2 vials.
5. 1X Assay Diluent E (Item R): 2 vials, 25 ml/vial. Diluent for both standards and samples including serum or plasma, cell culture media or other sample types.
6. Lyophilized biotinylatedGlucagonpeptide (Item F): 2 vials.
7. HRP-Streptavidin concentrate (Item G): 600 µl 200x concentrated HRP-conjugated Streptavidin.
8. Lyophilized positive control (Item M): 1 vial.
9. TMB One-Step Substrate Reagent (Item H): 12 ml of 3, 3', 5, 5'- tetramethylbenzidine (TMB) in buffered solution.
10. Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.
11. Assay Diagram (Item J).
12. User Manual (Item K).

IV. STORAGE

- Standard, Biotinylated Glucagon peptide, and Positive Control should be stored at -20°C after arrival. **Avoid multiple freeze-thaws.**
- The remaining kit components may be stored at 4°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

For sample and positive control dilutions, refer to steps 5, 6, 7 and 9 of Reagent Preparation.

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

2. Briefly centrifuge the Glucagon Antibody vial (Item N) and reconstitute with 5 µl of ddH_2O before use. Add 50 µl of 1x Assay Diluent E into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently.

3. The antibody concentrate should then be diluted 100-fold with 1x Assay Diluent E. This is your anti-Glucagon 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).

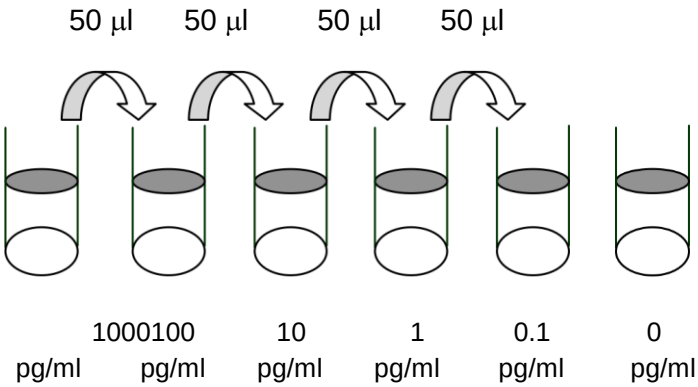
4. Briefly centrifuge the vial of biotinylated Glucagon peptide (Item F) and reconstitute with 20 µl of ddH_2O before use. Add 5 µl of Item F to 5 ml 1X Assay Diluent E. Pipette up and down to mix gently. *The final concentration of biotinylated Glucagon will be 40 pg/ml.* This solution will only be used as the diluent in step 5 of Reagent Preparation.

5. 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 Glucagon 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 Glucagon is 40 pg/ml in all standards.*

   a. Briefly centrifuge the vial of standard Glucagon peptide (Item C) and reconstitute with 10 µl of ddH_2O. In the tube labeled 1000 pg/ml, pipette 8 µl of Item C and 792 µl of 40 pg/ml biotinylated Glucagon solution (prepared in step 4 above). This is your Glucagon stock solution (1000 pg/ml Glucagon, 40 pg/ml biotinylated Glucagon). Mix thoroughly. This solution serves as the first standard.

   b. To make the 100 pg/ml standard, pipette 50 µl of Glucagon 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 Glucagon 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 Glucagon, 40 pg/ml biotinylated Glucagon) serves as the zero standard (or total binding).



6. Prepare a 10-fold dilution of Item F. To do this, add 2 µl of Item F to 18 µl of 1X Assay Diluent E. This solution will be used in steps 7 and 9.

7. Positive Control Preparation: Briefly centrifuge the positive control vial and reconstitute with 100 µl of ddH_2O before use (Item M). To the tube of Item M, add 101 µl 1x Assay Diluent E. Also add 2 µl of 10-fold diluted Item F (prepared in step 6) 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 Glucagon is 40 pg/ml.

8. 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.

9. Sample Preparation: Use 1X Assay Diluent E + biotinylated Glucagon to dilute samples, including serum/plasma, cell culture medium and other sample types.

It is very important to make sure the final concentration of the biotinylated Glucagon is 40 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 6), 185 µl of 1X Assay Diluent E, 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 Glucagon to a final concentration of 40 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.

10. Briefly centrifuge the HRP-Streptavidin vial (Item G) before use. The HRP-Streptavidin concentrate should be diluted 200-fold with 1X Assay Diluent E.

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-Glucagon antibody (see Reagent Preparation step 3) 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 5), positive control (see Reagent Preparation step 7) and sample (see Reagent Preparation step 9) 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 10) 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-Glucagon antibody to each well. Incubate 1.5 hours at room temperature.

⇩

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

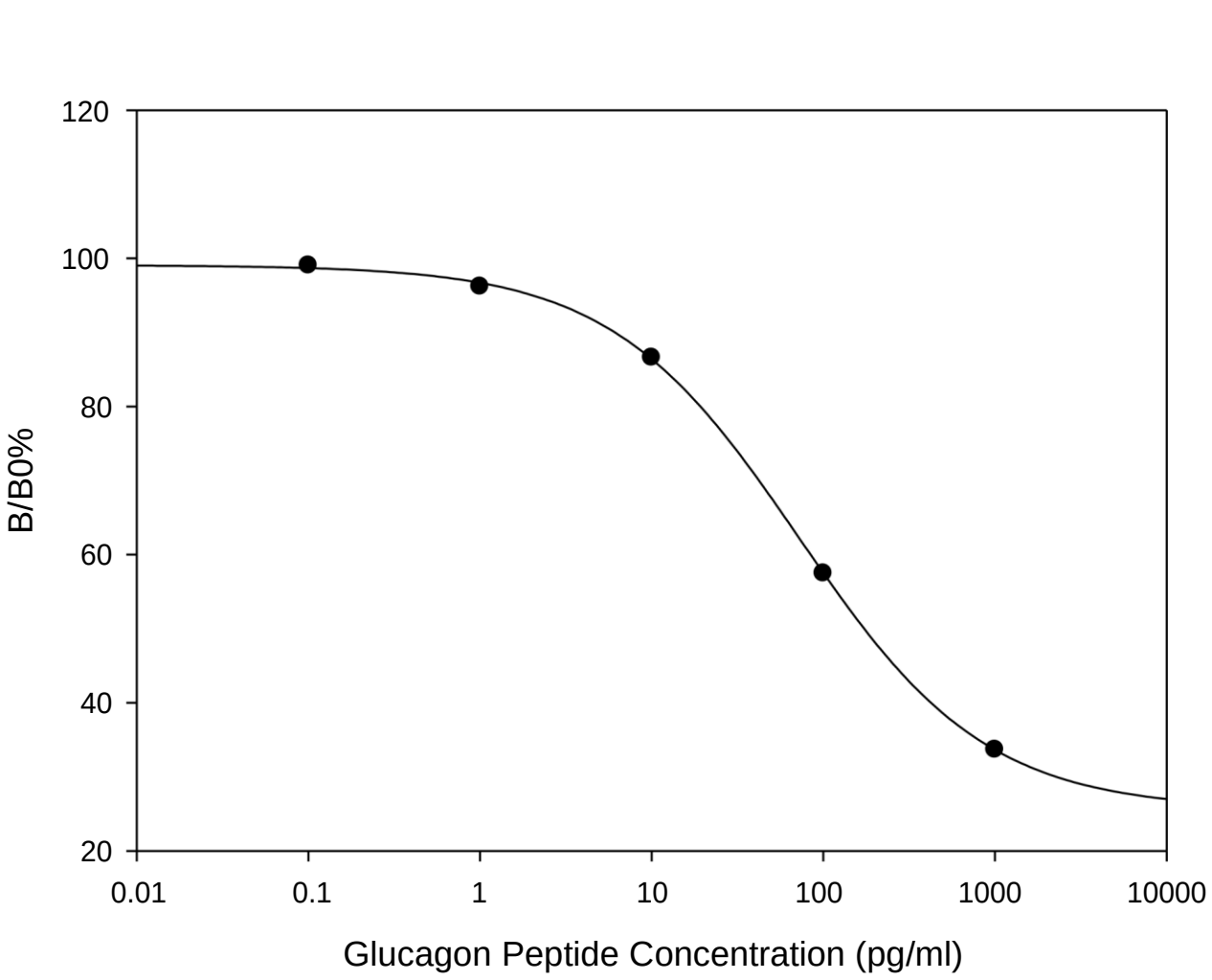
Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis 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 Glucagon is 4.77 pg/ml.

C. DETECTION RANGE

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. Reece J, Campbell N (2002). *Biology*. San Francisco: Benjamin Cummings.
2. Bromer W, Winn L, Behrens O (1957). "The amino acid sequence of glucagon V. Location of amide groups, acid degradation studies and summary of sequential evidence."*J. Am. Chem. Soc.***79** (11): 2807–2810.
3. White CM (May 1999). "A review of potential cardiovascular uses of intravenous glucagon administration". *J Clin Pharmacol***39** (5): 442–7.

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.

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