

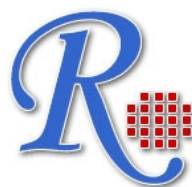
RayBio[®]
Human/Mouse/Rat Proglucagon
Enzyme Immunoassay Kit

**Please Read the Manual Carefully
Before Starting your Experiment**

**User Manual 2.2
(Revised March 15, 2012)**

**RayBio[®] Proglucagon Enzyme
Immunoassay Kit Protocol**

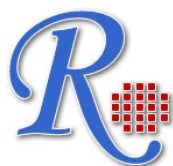
(Cat#: EIA-pGLU-1)



RayBiotech, Inc.

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**RayBio® Human/Mouse/Rat Proglucagon Enzyme
Immunoassay Kit Protocol**

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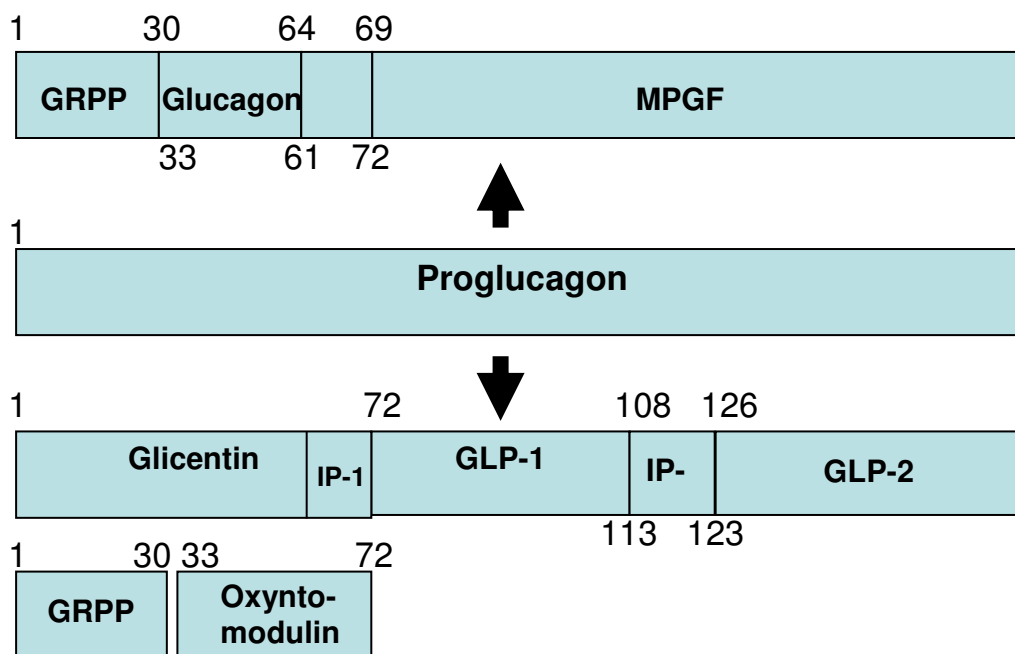
I. INTRODUCTION

Proglucagon is the precursor of glucagon, glucagon-like peptides (GLPs) and several other components. It is expressed in islet α cells, gut enteroendocrine L cells, and in brainstem neurons. Studies of post-translational Proglucagon processing demonstrate that Proglucagon is cleaved in α cells of the pancreas to yield 29-amino-acid glucagon and an unprocessed polypeptide designated 'major Proglucagon fragment' (MPGF). In contrast, Proglucagon is cleaved in gut enteroendocrine L cells to produce Glicentin, Oxyntomodulin, two intervening peptides (IP-1 and IP-2), and two GLPs (GLP-1 and GLP-2).

Glicentin is a 69-amino-acid peptide that contains the sequence of pancreatic glucagon together with amino-terminal and carboxy-terminal extensions (Figure). Administration of exogenous Glicentin stimulates growth of the small bowel in rodents. The actions of Glicentin on gut motility overlap with those of GLP-1 and are inhibited by the GLP-1 receptor (GLP-1R) antagonist exendin (9–39).

Glucagon and the glucagon-like peptides (GLPs) serve important roles in the control of energy ingestion, gastro intestinal motility, nutrient absorption and glucose homeostasis.

Structure of Proglucagon-derived Peptides



GRPP: Glicentin-Related Pancreatic Polypeptide
MPGF: Major Proglucagon Fragment
GLP: Glucagon-like Peptide

II. GENERAL DESCRIPTION

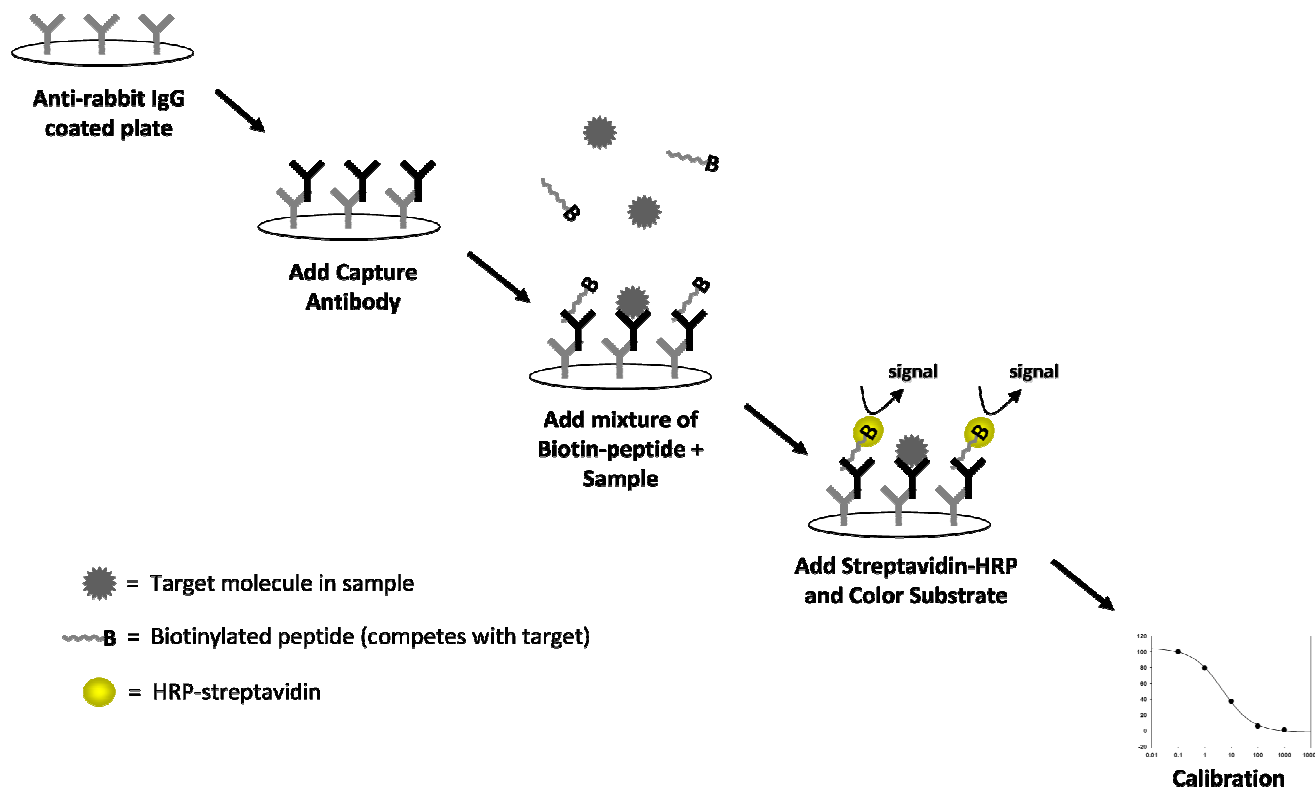
The RayBio® Proglucagon Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting Proglucagon peptide based on the principle of Competitive Enzyme Immunoassay.

The microplate in the kit is pre-coated with anti-rabbit secondary antibody. After a blocking step and incubation of the plate with anti-Proglucagon antibody, both biotinylated Proglucagon peptide and peptide standard or targeted peptide in samples interacts competitively with the Proglucagon antibody. Uncompeted (bound) biotinylated Proglucagon 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 Proglucagon peptide in the standard or samples. This is due to the competitive binding to Proglucagon antibody between biotinylated Proglucagon peptide and peptides in standard or samples. A standard curve of known concentration of Proglucagon peptide can be established and the concentration of Proglucagon peptide in the samples can be calculated accordingly.

RayBio® Proglucagon Enzyme Immunoassay (EIA) Kit is designed to target the N-terminus of the Proglucagon peptide. Thus GRPP, Glicentin, and Proglucagon can all be detected.

Principle of Competitive EIA



III. REAGENTS

1. Proglucagon 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 Proglucagon Peptide (Item C): 2 vials, 10 μ l/vial
4. Anti-Proglucagon polyclonal antibody (Item N): 2 vials, 5 μ l/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 Proglucagon peptide, (Item F): 2 vials, 20 μ l/vial
8. HRP-Streptavidin concentrate (Item G): 600 μ l 100x concentrated HRP-conjugated Streptavidin.
9. Positive control (Item M): 1 vial, 100 μ l
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 Proglucagon 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, RayBiotech warrants 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 μ l 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.

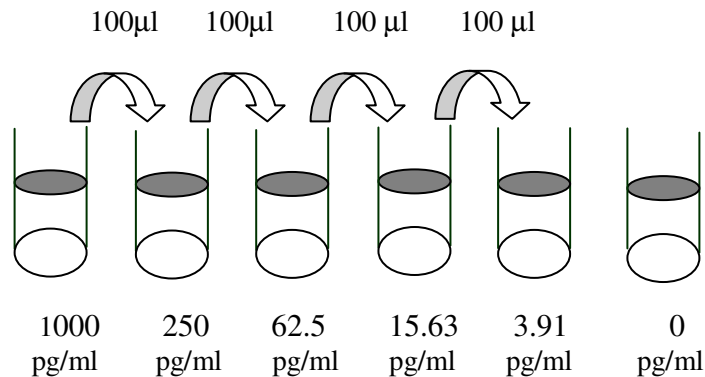
1. Keep kit reagents on ice during reagent preparation steps. Equilibrate plate to room temperature before opening the sealed pouch.
2. Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water.
3. Briefly centrifuge the Anti-Proglucagon Antibody vial (Item N) before use. Add 50 μ l 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-Proglucagon 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 Proglucagon (Item F) before use. Add 10 µl of biotinylated Proglucagon (Item F) to 5 ml of Assay Diluent A. Pipette up and down to mix gently. *The final concentration of biotinylated Proglucagon will be 40 ng/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, 250 pg/ml, 62.5 pg/ml, 15.63 pg/ml, 3.91 pg/ml and 0 pg/ml. Pipette 300 µl of biotinylated Proglucagon solution into each tube, except for the 1000 ng/ml (leave this one empty). *It is very important to make sure the concentration of biotinylated Proglucagon is 40 ng/ml in all standards.*
 - a. Briefly centrifuge the vial of Proglucagon (Item C). In the tube labeled 1000 ng/ml, pipette 8 µl of Item C and 792 µl of 40 ng/ml biotinylated Proglucagon solution (prepared in step 5 above). This is your Proglucagon stock solution (1000 ng/ml Proglucagon, 40 ng/ml biotinylated Proglucagon). Mix thoroughly. This solution serves as the first standard.
 - b. To make the 250 pg/ml standard, pipette 100 µl of Proglucagon stock solution the tube labeled 250 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 300 µl of biotinylated Proglucagon and 100

- μ l of the prior concentration until 3.91 pg/ml is reached. Mix each tube thoroughly before the next transfer.
- d. The final tube (0 pg/ml Proglucagon, 40 ng/ml biotinylated Proglucagon) serves as the zero standard (or total binding).



7. Prepare a 10-fold dilution of Item F. To do this, add 2 μ l of Item F to 18 μ l 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 μ l 1x Assay Diluent B. Also add 4 μ l 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 with an expected signal between 10% and 30% of total binding (70-90% competition) if diluted as described above. It may be diluted further if desired, but be sure the final concentration of biotinylated Proglucagon is 40 ng/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 Proglucagon to dilute serum/plasma samples. For cell culture medium and other sample types, use 1X Assay Diluent B + biotinylated Proglucagon as the diluent. *It is very important to make sure the final concentration of the biotinylated Proglucagon is 40 ng/ml in every sample.* EXAMPLE: to make a 4-fold dilution of sample, mix together 5 µl of 10-fold diluted Item F (prepared in step 7), 182.5 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 Proglucagon to a final concentration of 40 ng/ml. EXAMPLE: Add 5 µl of 10-fold diluted Item F to 245 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 100-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-Proglucagon 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 gently 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 μ l 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 μ l anti-Proglucagon antibody to each well. Incubate 1.5 hours at room temperature or overnight at 4°C.



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



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



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



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

IX. CALCULATION OF RESULTS

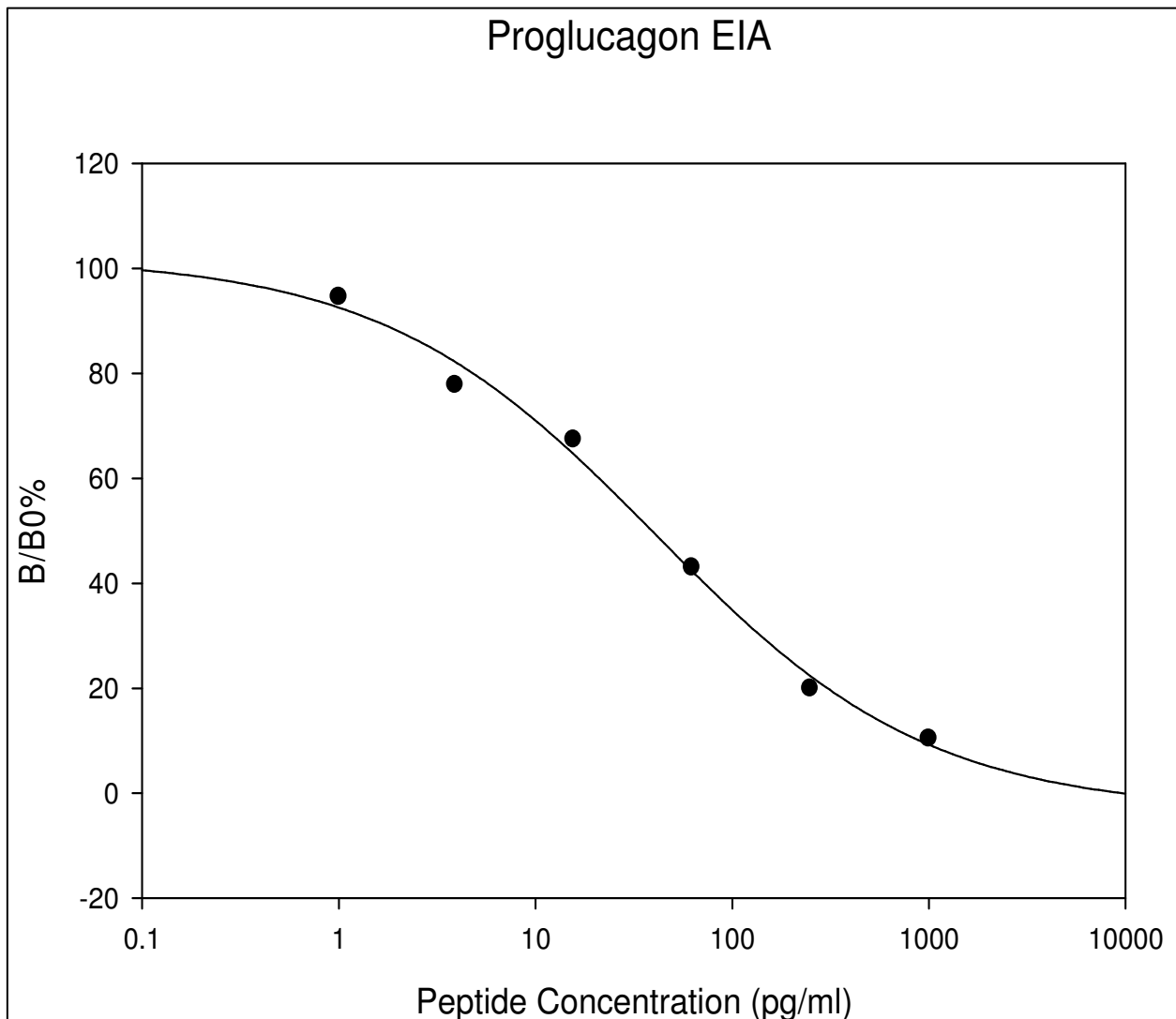
Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis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

calculation below) on the y-axis. Draw the best-fit straight line through the standard points.

Percentage absorbance = $(B - \text{blank OD}) / (B_0 - \text{blank OD})$ where
B = OD of sample or standard and
 B_0 = 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 Proglucagon is 2.52 pg/ml.

C. DETECTION RANGE

0.1-1,000 ng/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, Proglucagon, NPY and APC.

XI. REFERENCES

1. Drucker DJ. (2001). "Glucagon-like peptide 2". *J Clin Endocrinol Metab* 86: 1759-1764.
1. Dowling RH. (2003). "Glucagon-like peptide 2 and intestinal adaption: an historical and clinical perspective". *J Nutr.* 133: 3703-3707.

XII. TROUBLESHOOTING GUIDE

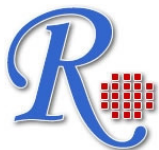
Problem	Cause	Solution
1. Poor standard curve	1. Inaccurate pipetting 2. Improper standard dilution	1. Check pipettes 2. Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	1. Too brief incubation times 2. Inadequate reagent volumes or improper dilution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

RayBio® EIA kits:

If you are interested in other EIA kits, please visit www.raybiotech.com for details.

Notes:

This product is for research use only.



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