

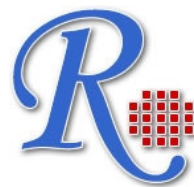
**RayBio<sup>®</sup>**  
**Human/Mouse/Rat Adiponutrin**  
**Enzyme Immunoassay Kit**

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

**User Manual 2.1  
(Revised July 5, 2011)**

**RayBio<sup>®</sup> Adiponutrin Enzyme  
Immunoassay Kit Protocol**

(Cat#: EIA-ADPN-1)



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**RayBiotech, Inc.**

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

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## I. INTRODUCTION

Human Adiponutrin (ADPN) is a 53 kD adipokine protein belong to the PNPLA family of phospholipase A2 enzymes. The 481 amino acid human Adiponutrin is a type II transmembrane protein, consisting of the patatin domain (aa 10 - 179) with enzyme activity and a transmembrane segment (aa 42 – 62). Human and mouse adiponutrins share 61% homology in amino acid sequence.

Adiponutrin is mainly expressed in adipocytes and serves as a triacylglycerol lipase that mediates triacylglycerol hydrolysis in adipocytes. Reports have shown that Adiponutrin plays an important role in the balance of energy usage/storage in adipocytes. Its expression level is enhanced in response to insulin.

Studies by Dr Clement's group have shown that in obese subjects, short- or long-term calorie restriction diets were associated with a reduction in Adiponutrin gene expression. Adiponutrin mRNA level was previously shown to be negatively correlated with fasting glucose plasma levels and associated with insulin sensitivity of non-diabetic obese and non-obese subjects. Reports from the same group have also shown a strong relationship between Adiponutrin expression, insulin sensitivity and glucose metabolism in human adipose tissue.

Other names for Adiponutrin include PNPLA3 (patatin-like phospholipase domain-containing protein 3).

RayBio<sup>®</sup> Adiponutrin EIA kit targets the common sequence of human, mouse, and rat. This kit may be used to detect Adiponutrin expression in all these species with high specificity and sensitivity.

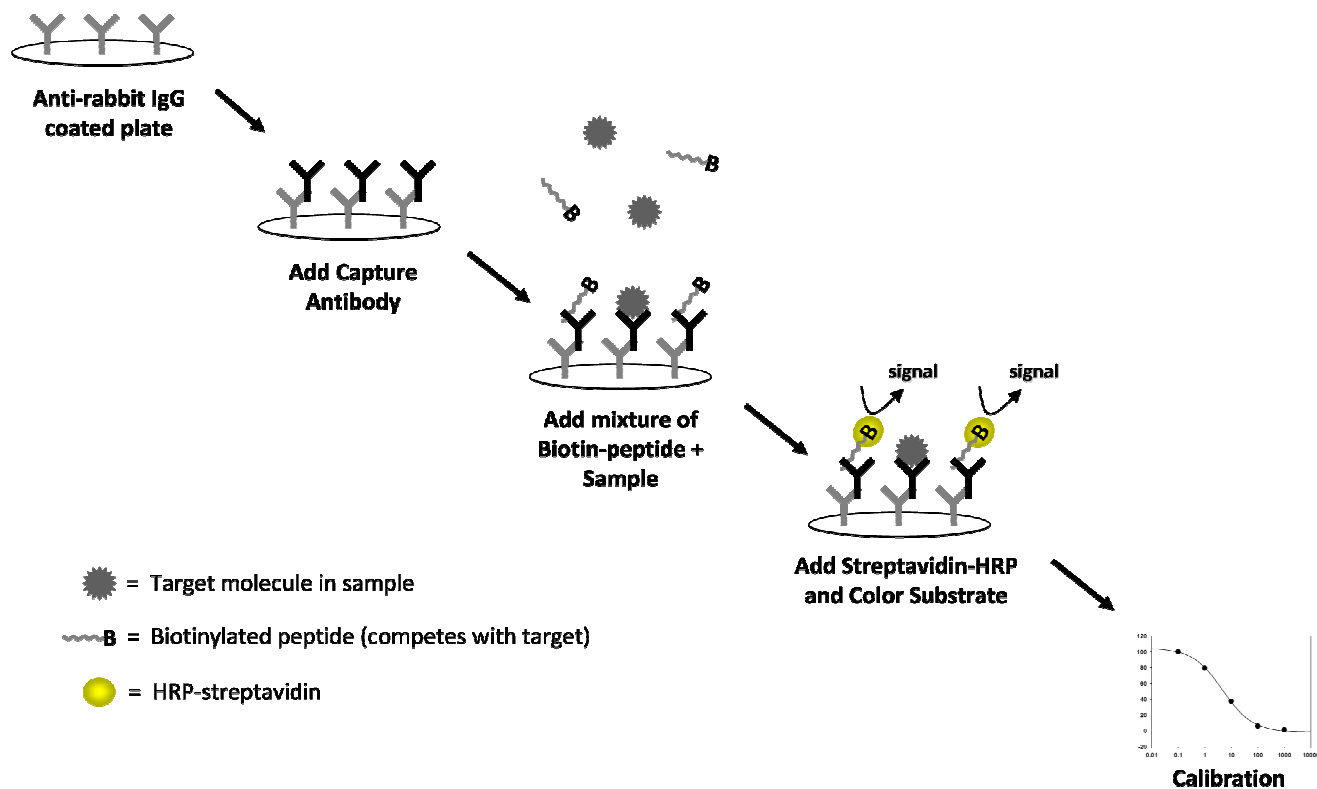
## II. GENERAL DESCRIPTION

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

EIA-ADPN-1 kit detects both long form (481aa) and short form (477aa) Adiponutrin proteins. No other active isoforms have been reported.

# Principle of Competitive EIA



### III. REAGENTS

1. Adiponutrin 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 Adiponutrin Peptide (Item C): 2 vials, 10  $\mu$ l/vial
4. Anti-Adiponutrin polyclonal antibody (Item N): 2 vials, 5  $\mu$ 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 Adiponutrin peptide, (Item F): 2 vials, 20  $\mu$ l/vial
8. HRP-Streptavidin concentrate (Item G): 8  $\mu$ l 40,000x concentrated HRP-conjugated Streptavidin.
9. Positive control (Item M): 1 vial, 100  $\mu$ 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 2 M sulfuric acid.
12. Assay Diagram (Item J).
13. User Manual (Item K)

### IV. STORAGE

- Standard, Biotinylated Adiponutrin 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 450 m.
2. Precision pipettes to deliver 2  $\mu$ 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.

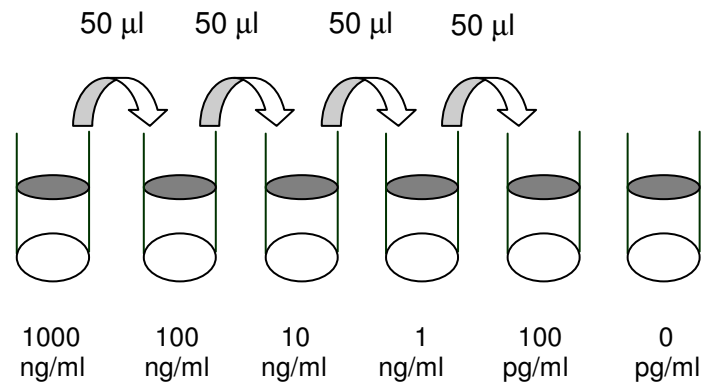
1. Keep kit reagents on ice during reagent preparation steps. Equilibrate plate to room temperature before opening the sealed pouch.
2. Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water.
3. Briefly centrifuge the Anti-Adiponutrin Antibody vial (Item N) before use. Add 50  $\mu$ 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-Adiponutrin 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 Adiponutrin (Item F) before use. Add 5  $\mu$ l of Item F to 5 ml of the appropriate Assay Diluent. Pipette up and down to mix gently. *The final concentration of biotinylated Adiponutrin will be 10 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 ng/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml and 0 pg/ml. Pipette 450  $\mu$ l of biotinylated Adiponutrin 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 Adiponutrin is 10 ng/ml in all standards.*
  - a. Briefly centrifuge the vial of Adiponutrin (Item C). In the tube labeled 1000 ng/ml, pipette 8  $\mu$ l of Item C and 792  $\mu$ l of 10 ng/ml biotinylated Adiponutrin solution (prepared in step 5 above). This is your Adiponutrin stock solution (1000 ng/ml Adiponutrin, 10 ng/ml biotinylated Adiponutrin). Mix thoroughly. This solution serves as the first standard.
  - b. To make the 100 ng/ml standard, pipette 50  $\mu$ l of Adiponutrin stock solution the tube labeled 100 ng/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  $\mu$ l of biotinylated Adiponutrin and 50  $\mu$ l

- of the prior concentration until 100 pg/ml is reached. Mix each tube thoroughly before the next transfer.
- d. The final tube (0 pg/ml Adiponutrin, 10 ng/ml biotinylated Adiponutrin) 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 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 Adiponutrin is 10 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 Adiponutrin to dilute serum/plasma samples. For cell culture medium and other sample types, use 1X Assay Diluent B + biotinylated Adiponutrin as the diluent. *It is very important to make sure the final concentration of the biotinylated Adiponutrin is 10 ng/ml in every sample.* EXAMPLE: to make a 4-fold dilution of sample, mix together 2.5  $\mu$ l of 10-fold diluted Item F (prepared in step 7), 185  $\mu$ l of appropriate Assay Diluent, and 62.5  $\mu$ l of your sample; mix gently. The total volume is 250  $\mu$ l, 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 Adiponutrin to a final concentration of 10 ng/ml.* EXAMPLE: Add 2.5  $\mu$ l of 10-fold diluted Item F to 247.5  $\mu$ l 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 40,000-fold with 1X Assay Diluent B. *For example: For a 40,000-fold Dilution of HRP-Streptavidin solution, briefly spin the vial (Item G) and pipette up and down to mix gently . Add 2  $\mu$ l of HRP-Streptavidin concentrate into a tube with 398  $\mu$ l 1X Assay Diluent B to prepare a 200-fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix thoroughly and then pipette 100  $\mu$ l of prepared 200-fold diluted solution into a tube with 20 ml 1x Assay Diluent B to prepare a final 40,000-fold diluted HRP-Streptavidin solution.* *Note: Do not use Assay Diluent A for HRP-Streptavidin preparation in Step11.*

## 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  $\mu$ l anti-Adiponutrin 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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  $\mu$ l 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  $\mu$ 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  $\mu$ l anti-Adiponutrin antibody to each well. Incubate 1.5 hours at room temperature or overnight at 4°C.  

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

4. Add 100  $\mu$ l prepared streptavidin solution. Incubate 45 minutes at room temperature.  

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

6. Add 50  $\mu$ l Stop Solution to each well. Read at 450 nm immediately

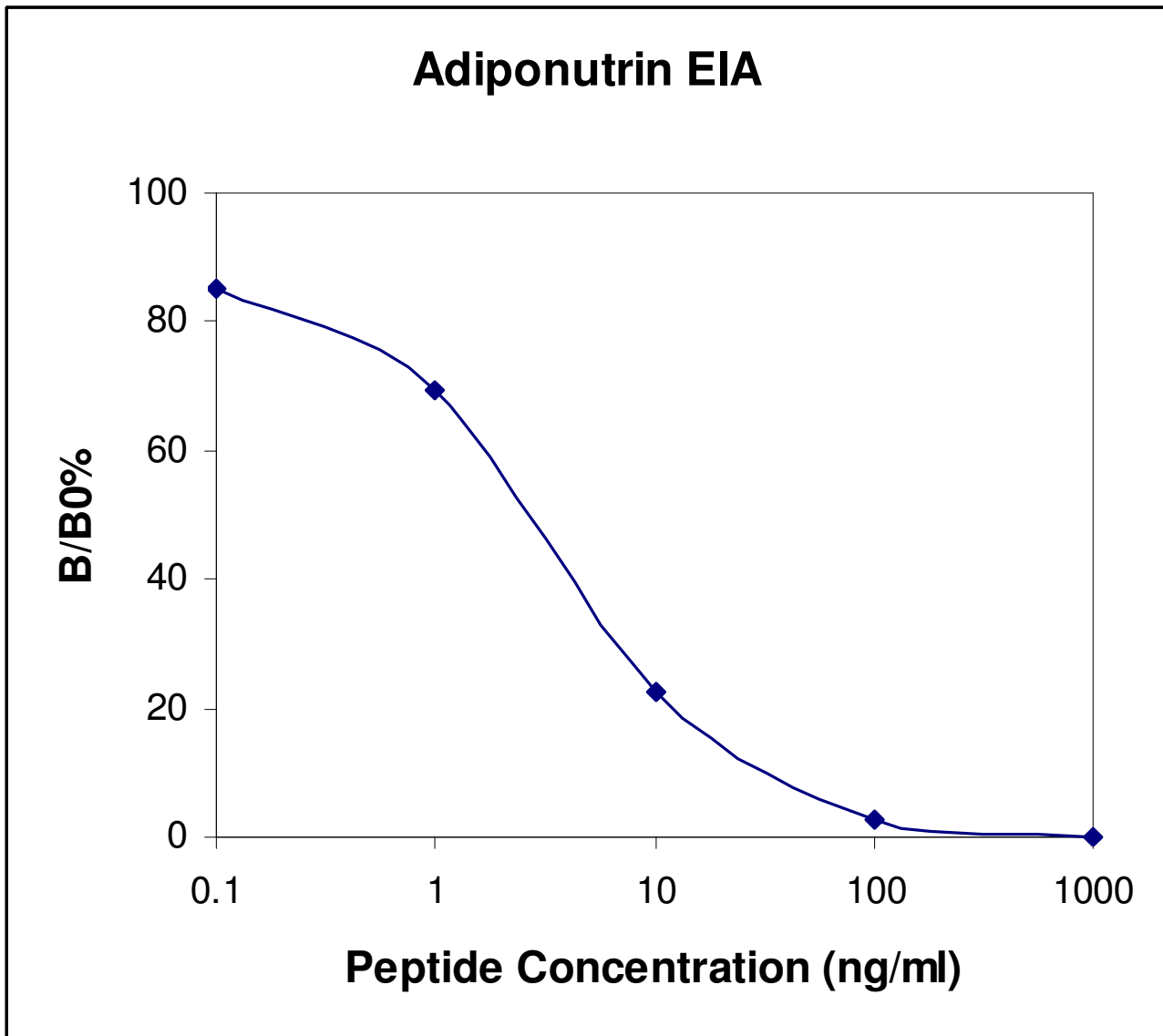
## **IX. CALCULATION OF RESULTS**

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<sub>0</sub> = 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 Adiponutrin is 21 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, Angiotensin II, NPY and APC.

## XI. REFERENCES

1. Expression, regulation, and triglyceride hydrolase activity of Adiponutrin family members. Lake AC, Sun Y, Li JL, *et al.* *J. Lipid Res.* 2006;46 (11): 2477–87.
2. Adiponutrin gene is regulated by insulin and glucose in human adipose tissue. (2006) Moldes M, Beauregard G, Faraj M, Peretti N, Ducluzeau PH, Laville M, Rabasa-Lhoret R, Vidal H, Clément K. *Eur J Endocrinol.* 2006; 155(3):461-8.
3. Adiponutrin: A new gene regulated by energy balance in human adipose tissue. Liu YM, Moldes M, Bastard JP, Bruckert E, Viguerie N, Hainque B, Basdevant A, Langin D, Pairault J, Clément K. *J Clin Endocrinol Metab.* 2004; 89(6):2684-9.

## XII. TROUBLESHOOTING GUIDE

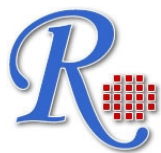
<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
1. Poor standard curve	<ol style="list-style-type: none"> <li>1. Inaccurate pipetting</li> <li>2. Improper standard dilution</li> </ol>	<ol style="list-style-type: none"> <li>1. Check pipettes</li> <li>2. Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.</li> </ol>
2. Low signal	<ol style="list-style-type: none"> <li>1. Too brief incubation times</li> <li>2. Inadequate reagent volumes or improper dilution</li> </ol>	<ol style="list-style-type: none"> <li>1. Ensure sufficient incubation time; assay procedure step 2 change to over night</li> <li>2. Check pipettes and ensure correct preparation</li> </ol>
3. Large CV	<ol style="list-style-type: none"> <li>1. Inaccurate pipetting</li> </ol>	<ol style="list-style-type: none"> <li>1. Check pipettes</li> </ol>
4. High background	<ol style="list-style-type: none"> <li>1. Plate is insufficiently washed</li> <li>2. Contaminated wash buffer</li> </ol>	<ol style="list-style-type: none"> <li>1. Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.</li> <li>2. Make fresh wash buffer</li> </ol>
5. Low sensitivity	<ol style="list-style-type: none"> <li>1. Improper storage of the EIA kit</li> <li>2. Stop solution</li> </ol>	<ol style="list-style-type: none"> <li>1. Store your standard at <math>\leq -20^{\circ}\text{C}</math> after receipt of the kit.</li> <li>2. Stop solution should be added to each well before measure</li> </ol>

RayBio® EIA kits:

If you are interested in other EIA kits, please visit [www.raybiotech.com](http://www.raybiotech.com) for details.

**Notes:**

This product is for research use only.



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3607 Parkway Lane, Suite 200  
Norcross, GA 30092  
Tel: 770-729-2992, 1-888-494-8555  
Fax: 770-206-2393  
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