

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

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

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

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

(Cat#: EIA-ZAG-1)



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RayBiotech, Inc.

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

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I. INTRODUCTION

ZAG is a 41 kDa soluble glycoprotein, which is a homologue of the class 1 major histocompatibility complex (MHC) heavy chain. ZAG was found to stimulate lipolysis through stimulation of adenylyl cyclase in a GTP-dependent process. ZAG produced weight loss when administered to obese mice without a reduction in food and water intake. Loss of body weight was entirely due to loss of adipose tissue with no effect on lean body mass. There was a three-fold increase in oxygen consumption by interscapular brown adipose tissue (BAT), suggesting an increase in thermogenesis. Recent studies have reported that ZAG is not only capable of stimulating lipolysis through activation of HSL, but also increases the utilization of the released NEFA through an increased level of UCPs in BAT and skeletal muscle.

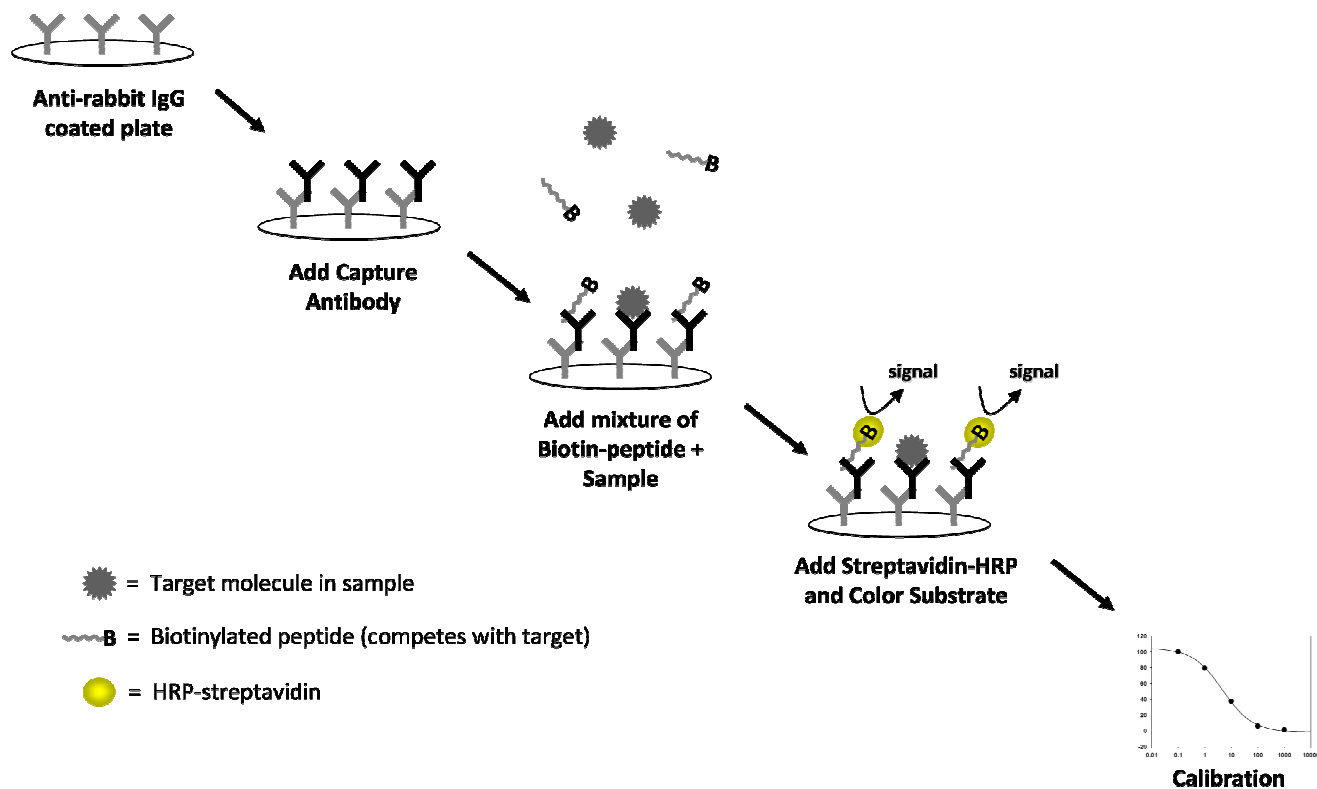
II. GENERAL DESCRIPTION

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

EIA-ZAG-1 detects ZAG 278aa. No other active isoforms have been reported.

Principle of Competitive EIA



III. REAGENTS

1. ZAG 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 ZAG Peptide (Item C): 2 vials, 10 µl/vial
4. Anti-ZAG 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 ZAG peptide, (Item F): 2 vials, 20 µl/vial
8. HRP-Streptavidin concentrate (Item G): 8 µl 5,000x 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 2 M sulfuric acid.
12. Assay Diagram (Item J).
13. User Manual (Item K)

IV. STORAGE

- Standard, Biotinylated ZAG 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

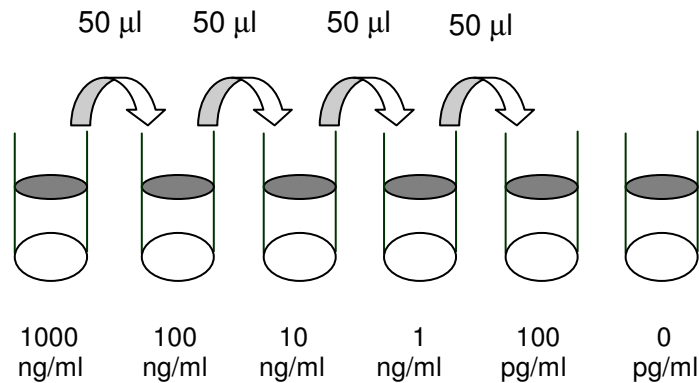
If testing plasma or serum samples, use Assay Diluent A to dilute Item F and Item C. If testing cell culture media or other sample types, use Assay Diluent B to dilute Item F and Item C. For sample and positive control dilutions, refer to steps 6, 7, 8 and 10 of Reagent Preparation.

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-ZAG 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-ZAG 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 ZAG (Item F) before use. Add 5 μ l of Item F to 5 ml of the appropriate Assay Diluent. Pipette up and down to mix gently. *The final concentration of biotinylated ZAG will be 50 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 μ l of biotinylated ZAG 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 ZAG is 50 ng/ml in all standards.*
 - a. Briefly centrifuge the vial of ZAG (Item C). In the tube labeled 1000 ng/ml, pipette 8 μ l of Item C and 792 μ l of 50 ng/ml biotinylated ZAG solution (prepared in step 5 above). This is your ZAG stock solution (1000 ng/ml ZAG, 50 ng/ml biotinylated ZAG). Mix thoroughly. This solution serves as the first standard.
 - b. To make the 100 ng/ml standard, pipette 50 μ l of ZAG 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 μ l of biotinylated ZAG and 50 μ 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 ZAG, 50 ng/ml biotinylated ZAG) 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 ZAG is 50 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 ZAG to dilute serum/plasma samples. For cell culture medium and other sample types, use 1X Assay Diluent B + biotinylated ZAG as the diluent. *It is very important to make sure the final*

concentration of the biotinylated ZAG is 50 ng/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 ZAG to a final concentration of 50 ng/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 5,000-fold with 1X Assay Diluent B.

For example: For a 5000-fold dilution of HRP-Streptavidin solution, briefly spin the vial (Item G) and pipette up and down to mix gently. Add 2 µl of HRP-Streptavidin concentrate into a tube with 198 µl 1X Assay Diluent B to prepare a 100-fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix thoroughly and then pipette 100 µl of prepared 100-fold diluted solution into a tube with 4.9 ml 1x Assay Diluent B to prepare a final 5000-fold diluted HRP-Streptavidin solution.






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 μ l anti-ZAG 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 μ 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 μ 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 μ 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 μ 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 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-ZAG 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

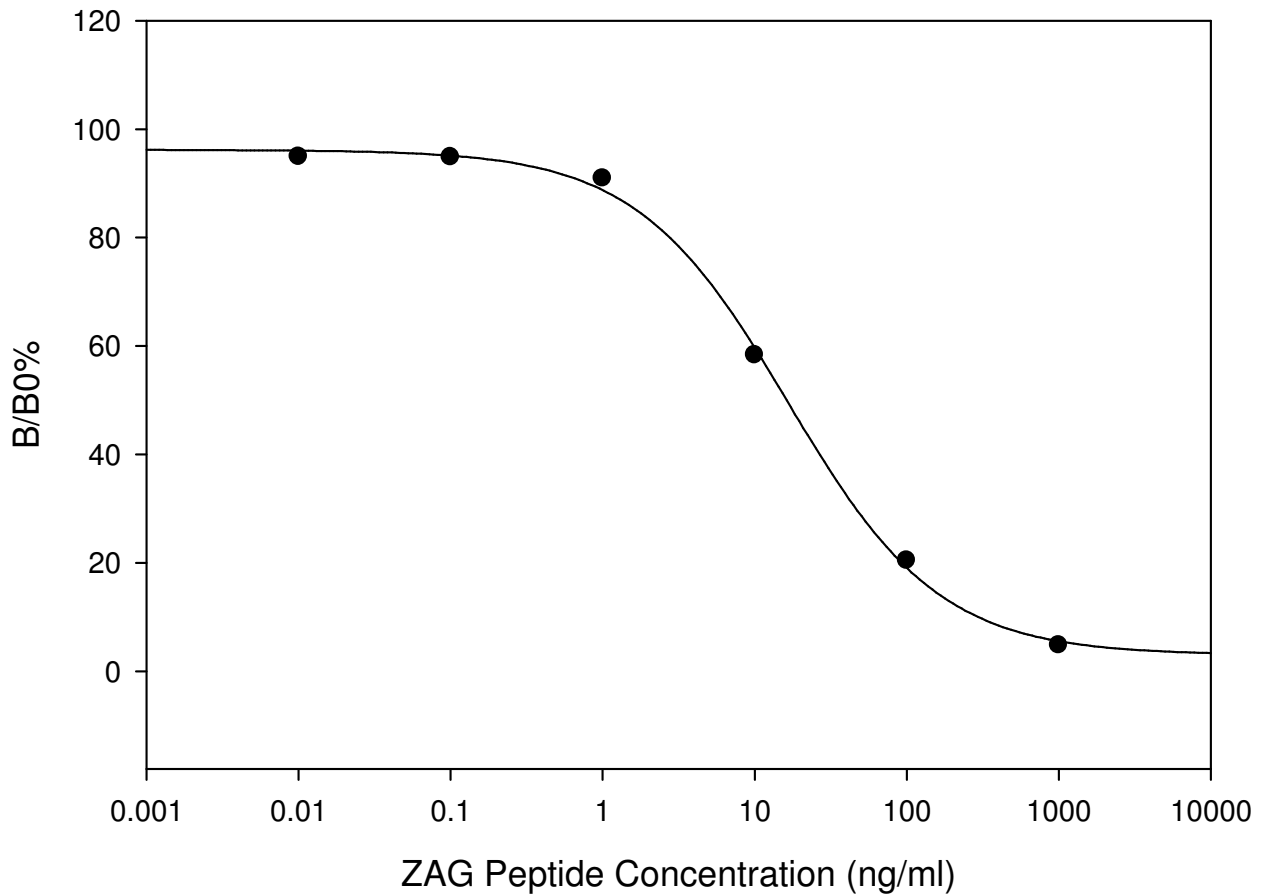
Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis 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
B₀ = 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.

ZAG EIA



B. SENSITIVITY

The minimum detectable concentration of ZAG 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, ZAG, NPY and APC.

XI. REFERENCES

1. Marrades MP, Martínez JA, Moreno-Aliaga MJ (2008). "ZAG, a lipid mobilizing adipokine, is downregulated in human obesity". *J Physiol Biochem.* 64(1):61-6.

XII. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Poor standard curve	<ol style="list-style-type: none"> 1. Inaccurate pipetting 2. Improper standard dilution 	<ol style="list-style-type: none"> 1. Check pipettes 2. Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	<ol style="list-style-type: none"> 1. Too brief incubation times 2. Inadequate reagent volumes or improper dilution 	<ol style="list-style-type: none"> 1. Ensure sufficient incubation time; assay procedure step 2 change to over night 2. Check pipettes and ensure correct preparation
3. Large CV	<ol style="list-style-type: none"> 1. Inaccurate pipetting 	<ol style="list-style-type: none"> 1. Check pipettes
4. High background	<ol style="list-style-type: none"> 1. Plate is insufficiently washed 2. Contaminated wash buffer 	<ol style="list-style-type: none"> 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	<ol style="list-style-type: none"> 1. Improper storage of the EIA kit 2. Stop solution 	<ol style="list-style-type: none"> 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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