

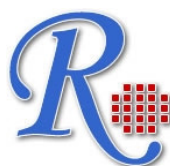
RayBio[®]
Human/Mouse/Rat RBP4
Enzyme Immunoassay Kit

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

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

**RayBio[®] RBP4 Enzyme
Immunoassay Kit Protocol**

(Cat#: EIA-RBP-1)



RayBiotech, Inc.

**We Provide You With Excellent
Protein Array System and Service**

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**RayBio® Human/Mouse/Rat RBP4 Enzyme
Immunoassay Kit Protocol**

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I. INTRODUCTION

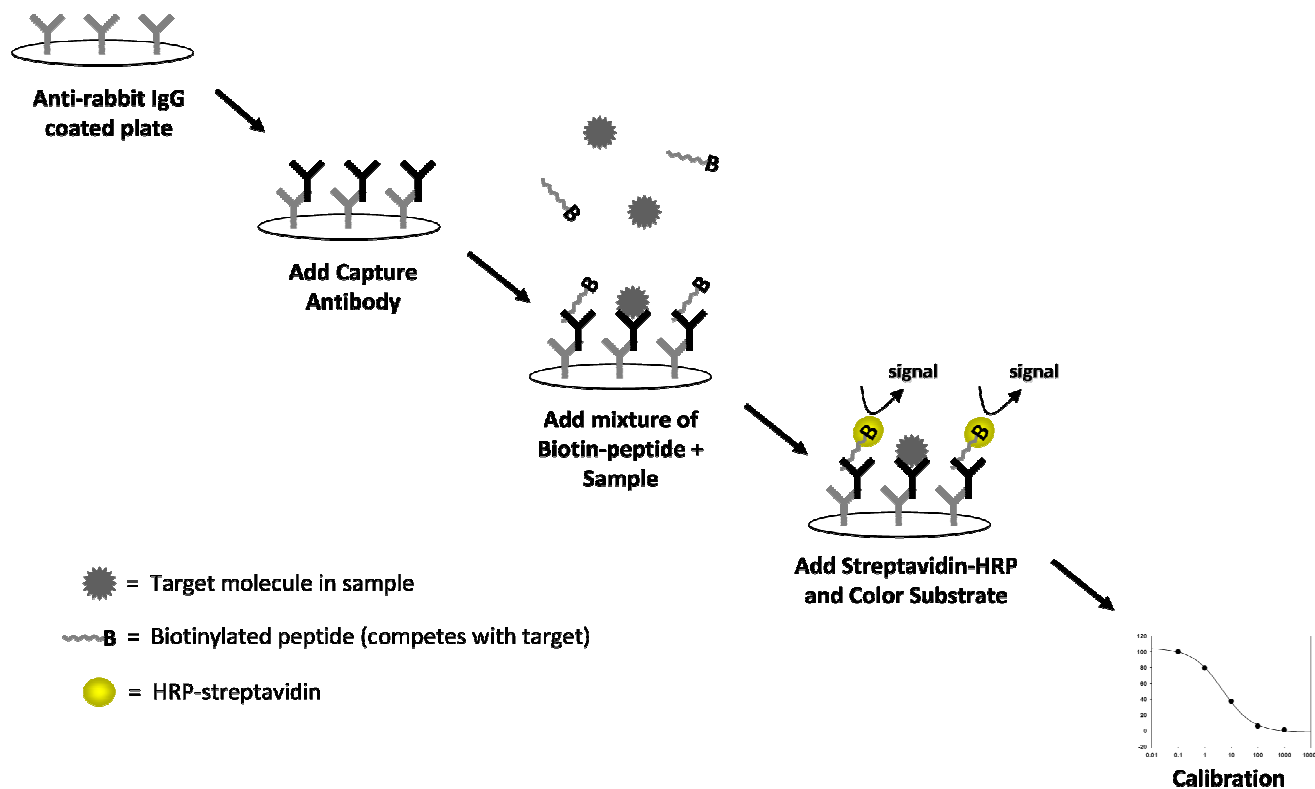
RBP4 (retinol binding protein-4) is an adipocyte-secreted molecule that is elevated in the serum before the development of frank diabetes. In obesity and type 2 diabetes, expression of the GLUT4 glucose transporter is decreased selectively in adipocytes. Adipose-specific Glut4 knockout mice show insulin resistance secondarily in muscle and liver. Recent studies have shown that serum RBP4 levels are elevated in insulin-resistant mice and humans with obesity and type 2 diabetes. RBP4 levels are normalized by rosiglitazone, an insulin-sensitizing drug. Transgenic overexpression of human RBP4 or injection of recombinant RBP4 in normal mice causes insulin resistance. Conversely, genetic deletion of RBP4 enhances insulin sensitivity. Fenretinide, a synthetic retinoid that increases urinary excretion of RBP4, normalizes serum RBP4 levels and improves insulin resistance and glucose intolerance in mice with obesity induced by a high-fat diet. Increasing serum RBP4 induces hepatic expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) and impairs insulin signalling in muscle. Thus, RBP4 is an adipocyte-derived 'signal' that may contribute to the pathogenesis of type 2 diabetes. Lowering RBP4 could be a new strategy for treating type 2 diabetes. Thus, RBP4 appears to identify insulin resistance and associated cardiovascular risk factors in subjects with varied clinical presentations.

II. GENERAL DESCRIPTION

The RayBio® RBP4 Enzyme Immunoassay (EIA) Kit is an in vitro quantitative assay for detecting RBP4 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-RBP4 antibody, both biotinylated RBP4 peptide and peptide standard or targeted peptide in samples interacts competitively with the RBP4 antibody. Uncompeted (bound) biotinylated RBP4 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 RBP4 peptide in the standard or samples. This is due to the competitive binding to RBP4 antibody between biotinylated RBP4 peptide and peptides in standard or samples. A standard curve of known concentration of RBP4 peptide can be established and the concentration of RBP4 peptide in the samples can be calculated accordingly.

Principle of Competitive EIA



III. REAGENTS

1. RBP4 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 RBP4 Peptide (Item C): 2 vials, 10 µl/vial
4. Anti-RBP4 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 RBP4 peptide, (Item F): 2 vials, 20 µl/vial
8. HRP-Streptavidin concentrate (Item G): 600µl 400x 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 RBP4 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 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

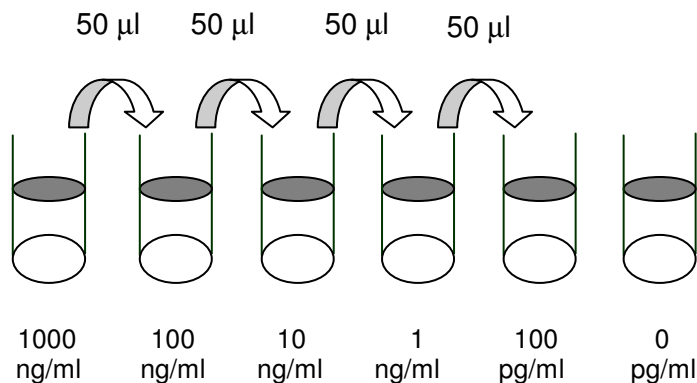
If testing plasma or serum samples, use Assay Diluent A to dilute Item F and Item C. If testing cell culture media or other sample types, use Assay Diluent B to dilute Item F and Item C. For sample and positive control dilutions, refer to steps 6, 7, 8 and 10 of Reagent Preparation.

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-RBP4 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-RBP4 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 RBP4 (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 RBP4 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 µl of biotinylated RBP4 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 RBP4 is 10 ng/ml in all standards.*
 - a. Briefly centrifuge the vial of RBP4 (Item C). In the tube labeled 1000 ng/ml, pipette 8 µl of Item C and 792 µl of 10 ng/ml biotinylated RBP4 solution (prepared in step 5 above). This is your RBP4 stock solution (1000 ng/ml RBP4, 10 ng/ml biotinylated RBP4). Mix thoroughly. This solution serves as the first standard.
 - b. To make the 100 ng/ml standard, pipette 50 µl of RBP4 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 µl of biotinylated RBP4 and 50 µl 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 RBP4, 10 ng/ml biotinylated RBP4) 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 RBP4 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 RBP4 to dilute serum/plasma samples. For cell culture medium and other sample types, use 1X Assay Diluent B + biotinylated RBP4 as the diluent. *It is very important to make sure the final*

concentration of the biotinylated RBP4 is 10 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 RBP4 to a final concentration of 10 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 400-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-RBP4 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 with gentle shaking for 45 minutes 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 µl anti-RBP4 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

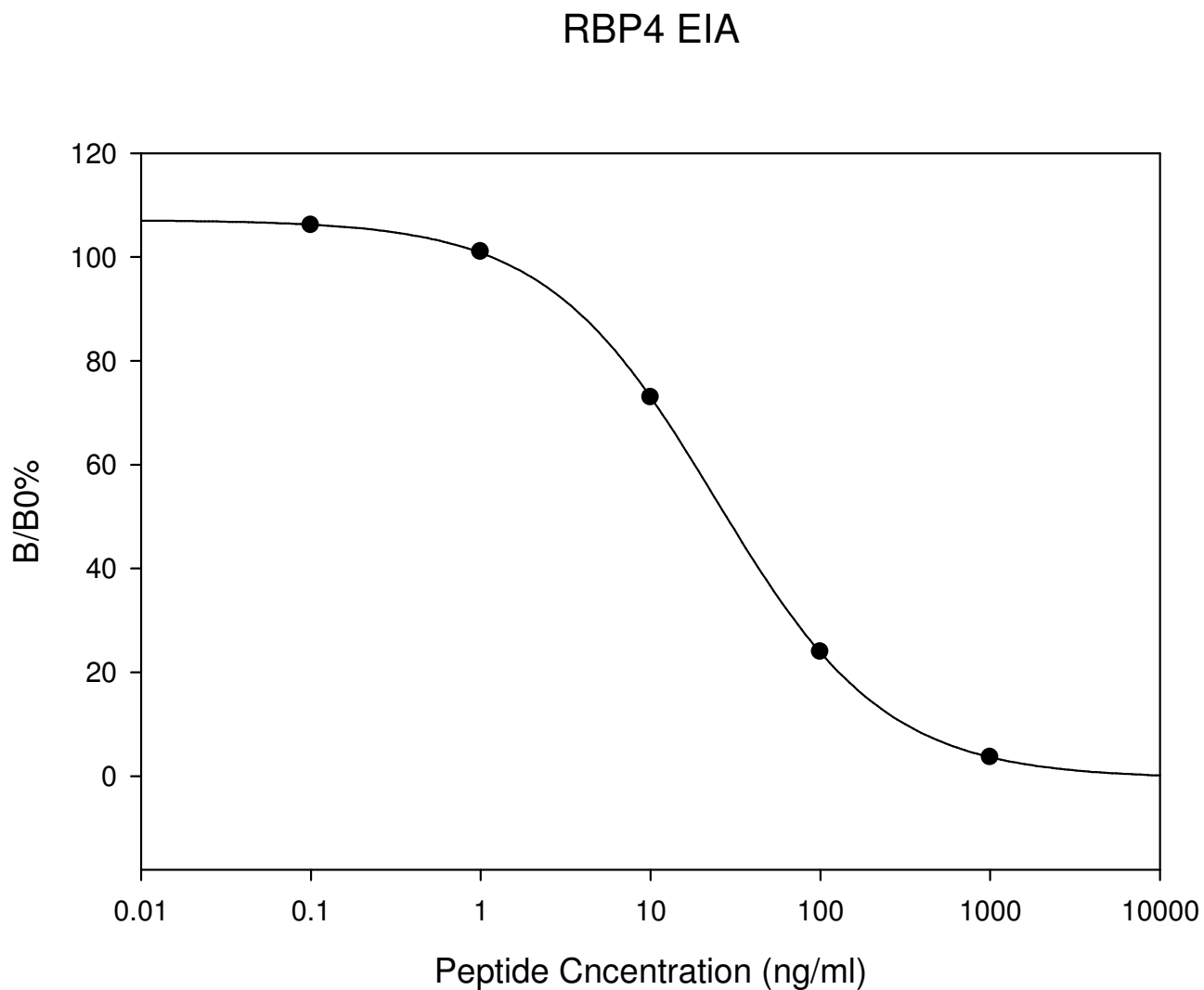
IX. CALCULATION OF RESULTS

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 = $(B - \text{blank OD}) / (B_0 - \text{blank OD})$ 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.



B. SENSITIVITY

The minimum detectable concentration of RBP4 is 460 pg/ml.

C. DETECTION RANGE

0.1-1000 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.

This kit detects RBP4 (183aa) and all other active forms including RBP4-182,181,179,176.

XI. REFERENCES

1. Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, Kahn BB. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature. 2005 Jul 21; 436(7049):356-62.

XII. TROUBLESHOOTING GUIDE

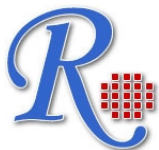
Problem	Cause	Solution
1. Poor standard curve	1. Inaccurate pipetting 2. Improper standard dilution	1. Check pipettes 2. Ensure briefly spin the vial of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	1. Too brief incubation times 2. Inadequate reagent volumes or improper dilution	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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