

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

Catalog #: EIA-RBP, EIAM-RBP, EIAR-RBP

User Manual
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Caution:
Extraordinarily useful information enclosed



ISO 13485 Certified

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Please read the entire manual carefully before starting your experiment

I. Introduction

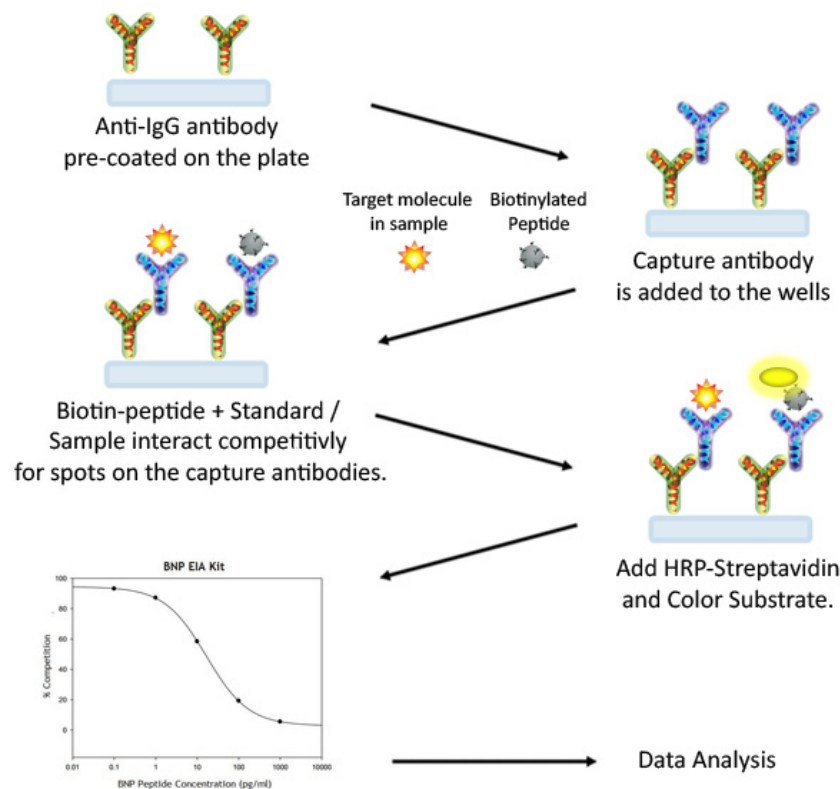
RBP4 (retinol binding protein-4) is an adipocyte-secreted molecule that is elevated in the serum before the development of frank diabetes. In obesity and type 2 diabetes, expression of the GLUT4 glucose transporter is decreased selectively in adipocytes. Adipose-specific Glut4 knockout mice show insulin 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 competitive enzyme immunoassay principle.

In this assay, a biotinylated RBP4 peptide is spiked into the samples and standards. The samples and standards are then added to the plate, where the biotinylated RBP4 peptide competes with endogenous (unlabeled) RBP4 for binding to the anti-RBP4 antibody. After a wash step, any bound biotinylated RBP4 then interacts with horseradish peroxidase (HRP)-streptavidin, which catalyzes a color development reaction. The intensity of the colorimetric signal is directly proportional to the amount of captured biotinylated RBP4 peptide and inversely proportional to the amount of endogenous RBP4 in the 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.

III. How It Works



IV. Storage

The entire kit may be stored at -20°C to -80°C for up to 6 months from the date of shipment. For extended storage, it is recommended to store at -80°C. **Avoid repeated freeze-thaw cycles.** For prepared reagent storage, see table below.

V. Reagents

Component	Size / Description	Storage / Stability After Preparation
RBP4 Microplate (Item A)	96 wells (12 strips x 8 wells) coated with secondary antibody.	1 month at 4°C*
Wash Buffer Concentrate (20X) (Item B)	25 ml of 20X concentrated solution.	1 month at 4°C
Standard RBP4 Peptide (Item C)	2 vials of RBP4 Peptide. 1 vial is enough to run each standard in duplicate.	The first standard: 2-3 days at 4°C Additional dilutions: Do not store
Anti-RBP4 Polyclonal Antibody (Item N)	2 vials of anti-RBP4.	1 month at 4°C
Assay Diluent A (Item D)	30 ml, contains 0.09% sodium azide as preservative. Diluent for standards and serum or plasma.	N/A
Assay Diluent B (Item E)	15 ml of 5X concentrated buffer. Diluent for standards, cell culture media or other sample types, and HRP-Streptavidin.	1 month at 4°C
Biotinylated RBP4 Peptide (Item F)	2 vials of Biotinylated RBP4 Peptide, 1 vial is enough to assay the whole plate.	2-3 days at 4°C
HRP-Streptavidin Concentrate (Item G)	600 µl 400X concentrated HRP-conjugated streptavidin.	Do not store and reuse
Positive Control (Item M)	1 vial of Positive Control.	2-3 days at 4°C
TMB One-Step Substrate Reagent (Item H)	12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffer solution.	N/A
Stop Solution (Item I)	8 ml of 0.2 M sulfuric acid.	N/A

*Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

VI. Additional Materials Required

1. Microplate reader capable of measuring absorbance at 450 nm
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. Plastic wrap

VII. Reagent Preparation

Keep kit reagents on ice during reagent preparation steps.

Note: **Assay Diluent A** should be used for dilution of samples, Item F and Item C when testing **plasma or serum samples**. **1X Assay Diluent B** should be used for dilution of samples, Item F and Item C when testing **cell culture media or other sample types**.

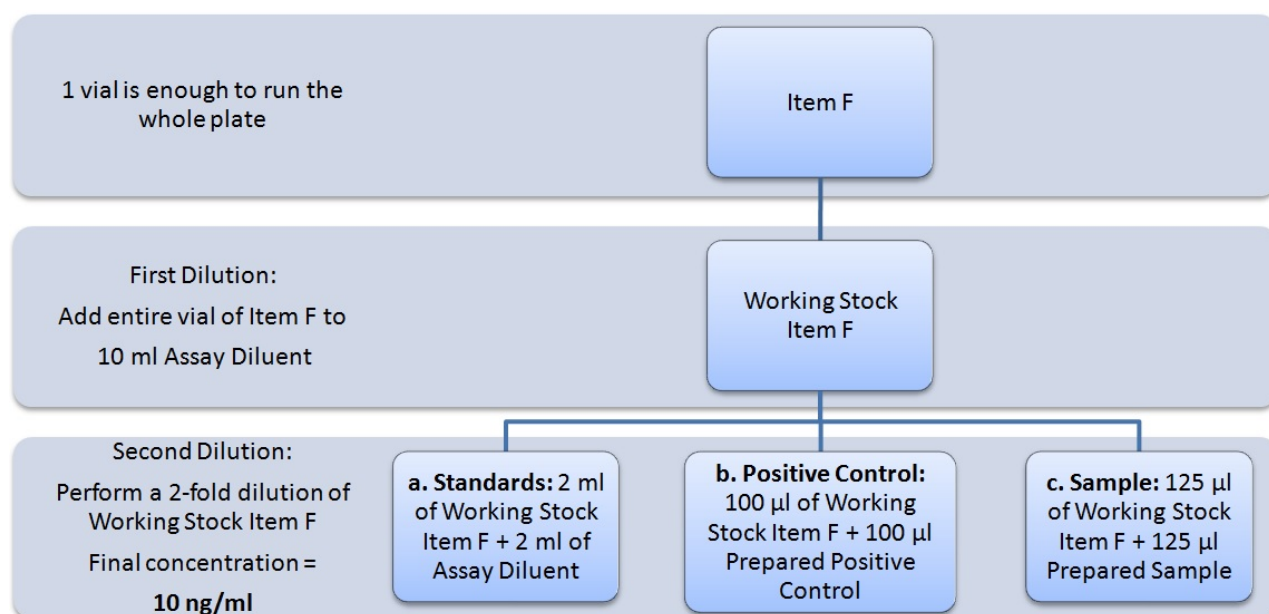
A. Preparation of Plate and Anti-RBP4 Antibody

1. Equilibrate plate to room temperature before opening the sealed pouch.
2. Label removable 8-well strips as appropriate for your experiment.
3. 5X Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water.
4. Briefly centrifuge the anti-RBP4 antibody vial (Item N) Then add 50 μ l of 1X Assay Diluent B to the vial to prepare the antibody concentrate. Pipette up and down to mix gently.
5. 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 Assay Procedure (Section VIII).

Note: The following steps may be done during the antibody incubation procedure (step 2 of Assay Procedure)

B. Preparation of Biotinylated RBP4 (Item F)

5. Briefly centrifuge the vial of Biotinylated RBP4 (Item F) before use.
6. See the image below for proper preparation of Item F. Transfer the entire contents of the Item F vial into a tube containing 10 ml of the appropriate Assay Diluent. This is your Working Stock of Item F. Pipette up and down to mix gently. *The final concentration of biotinylated RBP4 will be **20 ng/ml**.*
 - a. Second Dilution of Item F for Standards: Add 2 ml of Working Stock Item F to 2 ml of the appropriate Assay Diluent. The final concentration of biotinylated RBP4 will be **10 ng/ml**.
 - b. Second Dilution of Item F for Positive Control: Add 100 μ l of Working Stock Item F to 100 μ l of the prepared Positive Control (Item M). (See section D for Positive Control preparation) The final concentration of biotinylated RBP4 will be **10 ng/ml**.
 - c. Second Dilution of Item F for samples: Add 125 μ l of Working Stock Item F to 125 μ l of prepared sample (see section E for sample preparation). This is a 2-fold dilution of your sample. The final concentration of biotinylated RBP4 will be **10 ng/ml**.

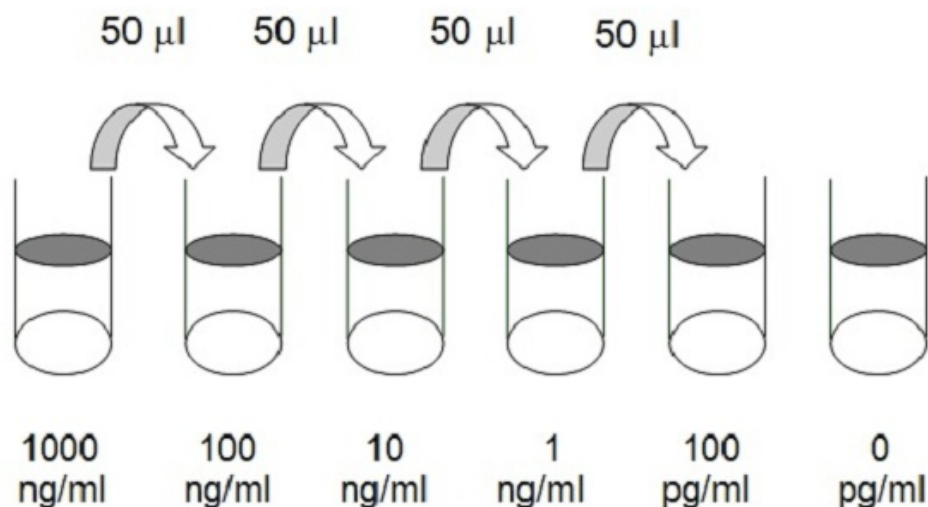


C. Preparation of Standards

- 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 RBP4 Item F working solution (prepared in step 6a) into each tube, except the 1,000 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.

- Briefly centrifuge the vial of RBP4 Standard (Item C). Pipette 8 μ l of Item C and 792 μ l of 10 ng/ml biotinylated RBP4 working solution (prepared in step 6a) into the tube labeled 1000 ng/ml. Mix thoroughly. This solution serves as the first standard (1,000 ng/ml RBP4 standard, 10 ng/ml biotinylated RBP4).
- To make the 100 ng/ml standard, pipette 50 μ l of the 1000 ng/ml RBP4 standard into the tube labeled 100 ng/ml. Mix thoroughly.
- 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 RBP4 and 50 μ l of the prior concentration until the 100 pg/ml is reached. Mix each tube thoroughly before the next transfer.



D. Positive Control Preparation

11. Briefly centrifuge the Positive Control vial (Item M).
12. Refer to step 6b. This is a 2-fold dilution of the Positive Control. The final concentration of biotinylated RBP4 should still be 10 ng/ml.

The Positive Control is a cell culture media sample that serves as a system control to verify that the kit components are working. The resulting OD will not be used in any calculations; if no positive competition is observed please contact RayBiotech Technical Support. The Positive Control may be diluted further if desired, but be sure the final concentration of biotinylated RBP4 is 10 ng/ml.

E. Sample Preparation

13. If you wish to perform a 2-fold dilution of your sample, proceed to step 6c. If you wish to perform a higher dilution of your sample, dilute your sample with the appropriate Assay Diluent before performing step 6c.
EXAMPLE (to make a 4-fold dilution of sample):
 - a. Dilute sample 2-fold (62.5 μ l of sample + 62.5 μ l of the appropriate Assay Diluent.).
 - b. Perform step 6c (125 μ l of working solution Item F + 125 μ l of sample prepared above).

The total volume is 250 μ l, enough for duplicate wells on the microplate.

It is very important to make sure the final concentration of the biotinylated RBP4 is **10 ng/ml**.

Note: Optimal sample dilution factors should be determined empirically, however you may reference below for recommended dilution factors for serum: Human=4X
Mouse=2X .

You may also contact technical support (888-494-8555; techsupport@raybiotech.com) to obtain additional recommended dilution factors for serum.

F. Preparation of Wash Buffer and HRP

14. If Item B (20X Wash Concentrate) contains visible crystals, warm to room temperature and mix gently until dissolved.
15. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.
16. Briefly centrifuge the HRP-Streptavidin vial (Item G) before use.
17. Dilute the HRP-Streptavidin concentrate 400-fold with 1X Assay Diluent B.

*Note: do **not** use Assay Diluent A for HRP-Streptavidin preparation in step 17*

VIII. 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 of Anti-RBP4 Antibody (Item N) (See Reagent Preparation step 3) to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1-2 cycle/sec). You may also incubate overnight at 4°C.
3. Discard the solution and wash wells 4 times with 1X Wash Solution 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 Section C), Positive Control (see Reagent Preparation Section D) and sample (see Reagent Preparation Section E) in 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) overnight or 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 7) to each well. Incubate for 45 minutes at room temperature with gentle

shaking. 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 at 450 nm immediately.

IX. Assay Procedure Summary

1. Prepare all reagents, samples and standards as instructed.
2. Add 100 μ l anti-RBP4 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.

X. Calculation of Results

Calculate the mean absorbance for each set of duplicate stands, 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.

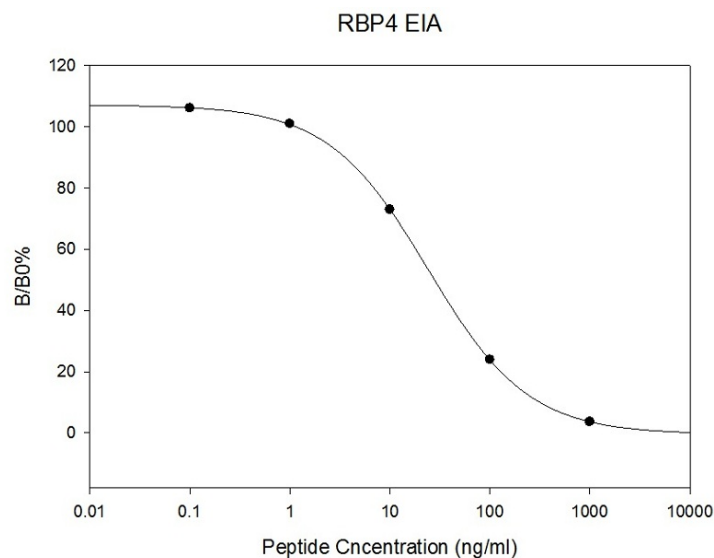
Percentage absorbance = $(B - \text{blank OD}) / (B_0 - \text{blank OD})$ where

B = OD of sample or standard and

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 concentrations of RBP4 is 460 pg/ml.

C. Detection Range

0.1-1,000 ng/ml

D. Reproducibility

Intra-Assay: CV<10%

Inter-Assay: CV<15%

E. Assay Diagram

Recommended Plate Layout:

Blank	Blank	SA1	SA1	SA9	SA9	SA17	SA17	SA25	SA25	SA33	SA33
Total Binding	Total Binding	SA2	SA2	SA10	SA10	SA18	SA18	SA26	SA26	SA34	SA34
Standard1	Standard1	SA3	SA3	SA11	SA11	SA19	SA19	SA27	SA27	SA35	SA35
Standard2	Standard2	SA4	SA4	SA12	SA12	SA20	SA20	SA28	SA28	SA36	SA36
Standard3	Standard3	SA5	SA5	SA13	SA13	SA21	SA21	SA29	SA29	SA37	SA37
Standard4	Standard4	SA6	SA6	SA14	SA14	SA22	SA22	SA30	SA30	SA38	SA38
Standard5	Standard5	SA7	SA7	SA15	SA15	SA23	SA23	SA31	SA31	SA39	SA39
Pos Control	Pos Control	SA8	SA8	SA16	SA16	SA24	SA24	SA32	SA32	SA40	SA40

Key:

Blank = Buffer Only

Total Binding = Biotin- RBP4 only

Standard 1 = 1000 ng/ml

Standard 2 = 100 ng/ml

Standard 3 = 10 ng/ml

Standard 4 = 1 ng/ml

Standard 5 = 100 pg/ml

Pos Control = Biotin with Item M

XI. Specificity

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

Cross Reactivity: This EIA kit shows no cross-reactivity with any of the cytokines tested: Ghrelin, Nesfatin, Angiotensin II, NPY and APC.

XIV. Publications Citing This Product

1. Anna P., Lech C., Jerzy J., Robert F. Assessment of serum IGF-1 and adipokines related to metabolic dysfunction in HIV-infected adults. Cytokine 64 (2013) 97-102

Species: Human

Sample Type: Serum

XIII. Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	<ul style="list-style-type: none"> • Inaccurate pipetting • Improper standard dilution 	<ul style="list-style-type: none"> • Check pipettes • Briefly centrifuge Item C and dissolve the powder thoroughly by gently mixing
Low signal	<ul style="list-style-type: none"> • Improper preparation of standard and/or biotinylated antibody • Too brief incubation times • Inadequate reagent volumes or improper dilution 	<ul style="list-style-type: none"> • Briefly spin down vials before opening. Dissolve the powder thoroughly. • Ensure sufficient incubation time; assay procedure step 2 may be done overnight • Check pipettes and ensure correct preparation
Large CV	<ul style="list-style-type: none"> • Inaccurate pipetting • Air bubbles in wells 	<ul style="list-style-type: none"> • Check pipettes • Remove bubbles in wells
High background	<ul style="list-style-type: none"> • Plate is insufficiently washed • Contaminated wash buffer 	<ul style="list-style-type: none"> • Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed. • Make fresh wash buffer
Low sensitivity	<ul style="list-style-type: none"> • Improper storage of the ELISA kit • Stop solution 	<ul style="list-style-type: none"> • Follow storage recommendations in sections IV and V. Keep substrate solution protected from light. • Add stop solution to each well before reading plate

RayBio[®] ELISA Kits

Over 2,000 ELISA kits available, visit www.RayBiotech.com/ELISA-Kits.html for details.

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