# RayBio®
# Human Retinol Binding Protein 4 (RBP4) Immunodetection Kit

## User Manual

***(Revised 080809)***

**For Western Blotting Analysis, 10 tests**

**(Catalog No. RB-08-0032K)**

## For Obesity and Adipokine Research

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# I. Introduction

Retinol-binding protein 4 (RBP4) is a small transport protein (~ 23 kDa) and belongs to the lipocalin family with diverse functions. It is the specific carrier protein for retinol (vitamin A alcohol) in the serum. It delivers retinol from the liver stores to the peripheral tissues. In human plasma, the RBP-retinol complex interacts with transthyretin which prevents its loss by filtration through the kidney glomeruli. A deficiency of vitamin A blocks secretion of the binding protein posttranslationally and results in defective delivery and supply to the epidermal cells. Recently, RBP4 was known to be one of adipokine secreted by adipocytes and relate to insulin resistance in the AG4KO mouse model. RBP4 may contribute to pathogenesis of type 2 diabetes. Yang et al. demonstrated that serum RBP4 levels are elevated in patients with obesity and type 2 diabetes.

RayBiotech® RBP4 Immunodetection Kit is an *in vitro* semi-quantitative assay for the detection and identification of RBP4-containing proteins in Western blotting or Dot blotting analysis. The kit provides a fast, stable, specific, and sensitive method to detect RBP4 proteins immobilized on nitrocellulose membrane. This kit provides most important validated reagents required for the detection of RBP4 proteins on immunoblots: anti-RBP4 primary antibody, the essential cell lysates, recombinant protein, synthetic polypeptide, etc. In addition, the unique RayBiotech® 2× Cell Lysis Buffer and 1× Blocking Buffer greatly improve the test results. As validated, the kit not only gave target-specific binding signal in both immunogen and standard protein, but also showed clear binding band in selected control samples of cellular lysates.

# II. Kit Components

The reagents provided in the kit can perform at least 10 individual assays. The following table is the description and the brief use instruction of all items listed in the kit.

⚠ **STORAGE:** *Upon arrival, primary antibody, reference control, positive controls-1, 2 and 3 should be kept at -20 $^0$C. All other components should be stored at 4 $^0$C. At the proper storage temperatures, the kit will retain complete activity for up to one year from date of shipment.*

## Table 1. RBP4 Immunodetection Kit Components

| Components | Description | Quantity | Use Guide |
|---|---|---|---|
| **Primary Antibody** Rabbit anti-RBP4 | Affinity purified and lyophilized rabbit polyclonal antibody. | 1 vial 100 µg | Resuspend with 100 µL autoclaved distilled water (1 mg/mL), then dilute to the working concentration of 1 µg/mL using 1× Blocking Buffer. |
| **Secondary Antibody** HRP-conjugated goat anti-rabbit IgG | Immunoaffinity purified and HRP-conjugated goat anti-rabbit IgG, resuspended in 1× PBS. | 1 vial 20 µL | Dilute in 1:5,000 using 1× Blocking Buffer before use. |
| **Reference Control** KLH-conjugated immunogen | The immunogen used to generate rabbit anti-RBP4 antibody. | 1 vial 50 µL (40 ng/µL) | Ready to use. Recommend to load 200 ng/lane |
| **Positive Control-1** Recombinant human RBP4 protein | Purified full-length recombinant protein from *E. coli*. | 1 vial 50 µL (10 ng/µL) | Ready to use. Recommend to load 50 ng/lane |
| **Positive Control-2** HepG2 cell lysate | Positive control lysate expressing RBP4. | 1 vial 150 µL (1 µg/µL) | Mix 5 µL SDS loading buffer with 15 µL lysate prior to loading onto the gel. |
| **Positive Control-3** 3T3-L1 cell lysate | Positive control lysate expressing RBP4. | 1 vial, 150 µL (1 µg/µL) | Mix 5 µL SDS loading buffer with 15 µL lysate prior to loading onto the gel. |
| **2× Cell Lysis Buffer** | For the cell lysate preparation. | 1 bottle (10 mL) | Add 10 mL filtered distilled water and mix well prior to use. |
| **1× Blocking Buffer** | For immunblot blocking and the dilution of primary and secondary antibodies. | 1 bottle (15 g) | Resuspend in 300 mL 1× PBS. |
| **20× Washing Buffer** | For immunoblot washing in each step. | 2 bottles (50 mL each) | Add 950 mL distilled water each bottle before use. |

# III. Additional Materials Required

The following equipment and materials are required but not supplied:

- Protein polyacrylamide electrophoresis reagents and apparatus
- Protein transfer reagents and apparatus
- Chemiluminescence (ECL) detection buffer
- Orbital shaker

- Immunoblot incubation containers
- Autoradiography film
- X-ray film developing facilities (or other ECL image capture system), etc.

# IV. Technical Information

## A. Key Reagents

- **Size** Sufficient for at least 10 blot assays per kit.

- **Immunogen** KLH-conjugated synthetic polypeptide derived from *C*-terminus of the human RBP4 was used as an immunogen in rabbit.

- **Primary Antibody**

  **Class** IgG. The anti-RBP4 antibody generated in rabbit was purified from immunized serum by ammonium sulfate precipitation and affinity chromatography.

  **Form** Lyophilized powder (freeze-dried).

  **Working dilutions** Recommended working dilutions are 1:20,000 - 40,000 for ELISA, and 1 µg/mL for Western blotting analysis.

  **Specificity** Recognize human RBP4 protein.

  **Cross reactivity** Recognize mouse and rat RBP4 due to their similar peptide sequences in the area used for generating rabbit anti-human RBP4 polyclonal antibody.

## B. Quality Control Test

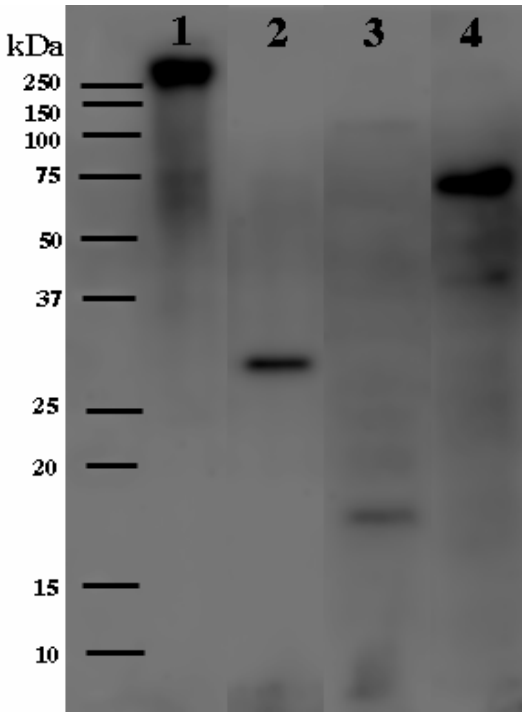
The reagents in this kit have been matched to optimize the range and sensitivity of detection using HepG2 cell and 3T3-L1 cells as a source of RBP4 containing proteins.

- **Molecular weight of RBP4 protein*:*** ~27 kDa

- **Positive controls:** HepG2 cell and 3T3-L1 cell lysates. These controls can be used to normalize the protein transferring, blotting, and ECL detection. They also refer to define RBP4 in the particular assay. If the positive control signals in the detected membranes are similar and stable, it will provide a simple and effective way for normalization.

- **Negative control:** bovine serum albumin (BSA). Normally, it should give a background reading.

- **Performance validated:** RayBio® Mammalian Cell Lysis Buffer, HRP-labeled Secondary Antibody, Blocking Buffer, and Washing Buffer in the kit have been validated to perform as specified.




**Lane 1** Reference Control
200 ng of KLH-conjugated RBP4 immunogen
> 250 kDa

**Lane 2** Positive Control-1
50 ng of the full-length recombinant human RBP4 ~27 kDa, including 3 kDa derived from expression vector

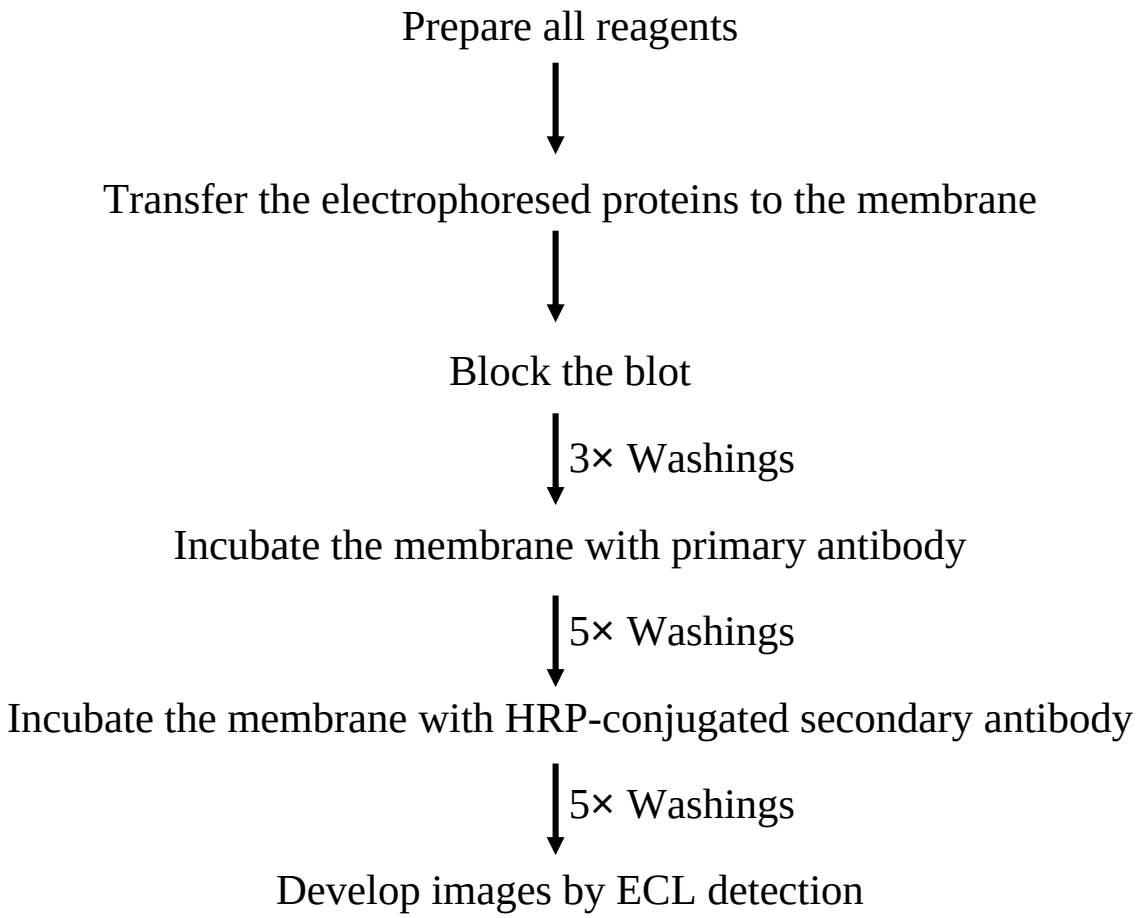
**Lane 3** Positive Control-2
15 µg of HepG2 cell lysate, shown the mature RBP4 ~17 kDa

**Lane 4** Positive Control-3
15 µg of 3T3-L1 lysate, shown the recognized band, ~70 kDa

**Fig. 1 RBP4 immunodetection analysis.** The proteins were separated by a 12% SDS-PAGE and transferred to nitrocellulose membranes. After blocked with 1× Blocking Buffer, the blot was probed with rabbit anti-RBP4 primary antibody (1 µg/mL). The target protein bands were visualized using ECL reagents after incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:5,000 dilution). The Precision Plus Protein Standards (BioRad, Catalog # 161-0373) was shown on the left (*kDa*). No any signal was detected with BSA under the identical condition (*not shown*).

# V. Immunodetection Protocol

The whole procedure to perform Western blotting using RayBiotech® RBP4 Immunodetection Kit is summarized as following:




## A. Sample Preparation (for reference only)

1) Collect cells in regular way and centrifuge cell suspension at 1,500 rpm for 10 minutes at 4 °C. Aspirate the supernatant without touching the cell pellets.
2) Resuspend pellets with 15 mL 1× PBS and centrifuge at 1,500 rpm for 10 minutes at 4 °C. Carefully aspirate supernatant.
3) Repeat Step 2 to wash the cells completely once or twice.
4) Add approximate amount of ice-cold 1× Cell Lysis Buffer with protease inhibitor cocktails to resuspend cell pellets well.
5) Incubate the cell suspensions on ice for 1 hour.
6) Transfer the cell suspensions to new microcentrifuge tubes.
7) Centrifuge at 13,000 rpm for 30 minutes at 4 °C.
8) Transfer the supernatants into new tubes.
9) Determine the protein concentrations using BioRad Protein Assay Reagent or other commercial protein assay kits with BSA as a standard.

10) For protein polyacrylamide electrophoresis, mix the cell lysates with the equal volume of 5× SDS loading buffer. Heat at 95-100 °C for 3-5 minutes.
11) Use immediately or aliquot at -20 °C or -80 °C freezer. The frozen lysates need to be heated again before SDS-PAGE analysis.

⚠ *Outlined below (parts B-D) is the instruction for one blot assay. Please scale up the reagent amount when multiple blots are applied.*

## B. Protein Blotting and Membrane Blocking

1) Following the instructions of protein electrophoresis, separate the proteins using an appropriate concentration of polyacrylamide gel.
2) Equilibrate the nitrocellulose membrane with the transfer buffer for at least 5 minutes.
3) Transfer the separated proteins from the gel onto membrane according to the manufacture's instructions of immunoblotting device and membrane.
4) Briefly wash the blot with 20 mL 1× Washing Buffer to remove any gel pieces.
5) Block the blot with 10 mL of freshly prepared 1× Blocking Buffer with gentle agitation for 1 hour at room temperature (or overnight at 4 °C).

## C. Antibody Incubation

1) Dilute the primary antibody (rabbit anti-RBP4 antibody) to the recommended concentration of 1 μg/mL using 1× Blocking Buffer, and incubate the membrane blot with 5~10 mL of diluted antibody for 1 hour at room temperature.
2) Wash the membrane blot with 20 mL 1× Washing Buffer for 5 times.
3) Dilute the secondary antibody (HRP-conjugated anti-rabbit IgG) to 1:5,000 using 1× Blocking Buffer, and incubate the membrane blot with 5~10 mL of diluted antibody for 1 hour at room temperature.
4) Wash as detailed in Step 2.

## D. ECL Detection

1) Cut one sheet of plastic SaranWrap™ and put it on even surface.
2) Pipette 2 mL ECL Detection Reagent A and 2 mL ECL Detection Reagent B onto the plastic wrap according to the manufacture's instruction.
3) Hold one corner of blot against Kimwipes and drain the excess washing buffer from the blot.
4) Place the blot on the mixed ECL Detection Reagent and incubate for 1 minute at room temperature (**Note**: *keep protein side down and avoid any air bubbles between the blots and detection reagent*).
5) Drain off the excess ECL Detection Reagent.
6) Wrap the blot with new SaranWrap™ sheet and remove any bubbles on the blot.
7) Keep the protein side up and immediately capture the images using Chemiluminescence Image system, or place the blot in an X-ray film cassette and expose to the films using X-ray film developing facilities (**Note**: *the exposure time varies from 30 seconds to 1 hour, depending on the signal density*).

# VI. Troubleshooting Guide

The following tips address most problems encountered during immunoblotting and ECL detection:

| Problems | Possible Causes | Solutions |
|---|---|---|
| **No signal** | 1. Antigen was not recognized by primary antibody.<br>2. Proteins didn't transfer properly to membrane.<br>3. Target protein degradation occurred due to improper storage of immunoblots.<br>4. ECL substrates had lost activity or were contaminated. | 1. Including positive and reference controls in each analysis.<br>2. Stain the blots to visualize protein transfer effect.<br>3. Store the immunoblots at -20 $^0$C freezer preventing the degradation of target proteins.<br>4. Use fresh ECL reagents. |
| **Weak signal** | 1. Protein amount loaded was not sufficient.<br>2. The concentration of primary or secondary antibodies used was too low.<br>3. Inhibition of secondary antibody with sodium azide or hemoglobin, SDS, Nonidet P-40, Triton X-100, etc. | 1. Load more protein.<br>2. Use the recommended antibody dilution.<br>3. Avoid any detergents in the buffer when storing and diluting antibody. Use the buffers provided in the kit. |
| **High background** | 1. Crossreaction between the blocking agent and the primary antibody.<br>2. Concentration of either primary and/or secondary antibody was too high.<br>3. Protein was overloaded on the gel.<br>4. Insufficient blocking.<br>5. Insufficient washing.<br>6. The level of Tween-20 in blocking buffer was too low for the application.<br>7. Membrane was not equilibrated well with transfer buffer, or dried in the following procedures.<br>8. Transfer buffer had been contaminated. | 1. Usually 0.01% Tween-20 in 1× Washing Buffer greatly eliminates this crossreaction.<br>2. Decrease antibody concentration and/or reduce incubation time.<br>3. Reduce the protein amount loaded.<br>4. 1× Blocking Buffer should be used throughout membrane blocking and antibody dilution.<br>5. Wash the blots completely.<br>6. Increase Tween-20 concentration.<br>7. Equilibrate membrane completely and prevent blots from drying any steps.<br>8. Use fresh transfer buffer. |
| **Smeared pattern or distorted bands** | 1. Uneven contact between gel and membrane during transfer.<br>2. Gel was not equilibrated in the transfer buffer prior to transfer if Tris-Glycine buffer used. | 1. Match the gel and membrane tightly and build an even pressure over the entire surfaces of the gel and blot.<br>2. The gel should be soaked in transfer buffer containing methanol for 10 to 20 minutes prior to transfer. |
| **Incomplete transfer** | 1. Proteins transferred through membrane.<br>2. Inappropriate transfer buffer used.<br>3. Impurities in the transfer buffer. | 1. This may occur the target proteins are small molecules. Using a double layer of membrane usually enhances retention of small proteins.<br>2. The most stable and commonly buffer used is Tris-Glycine based. Adding SDS into transfer buffer and using high field strengths buffer may improve protein transfer.<br>3. Fresh buffer should be prepared for each transfer. |
| **Bald spots** | Air bubbles between gel and membrane | Bubbles lead to low transfer efficiency. Avoid any air bubbles in assembly protein transfer sandwich. |

# VII. Other RBP4 Related Products

- Recombiant human RBP4.....Catalog No. RB-08-0016P
- Human RBP4 western blotting control.....Catalog No. RB-08-0016-WBC
- Goat anti-RBP4 antibody..... Catalog No. ER-14-1200, ER-14-1201
- Obesity antibody arrays…….. Catalog No. AAH-ADI-G1-8

# VIII. References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6. Lewis, J., et al. (2008) Plasma retinol-binding protein is unlikely to be a useful marker of insulin resistance. *Diabetes Res. Clin. Prac*t. 80, 13-15.

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