

COMPONENTS

Kit Component	Amount
96-well plate pre-coated with anti-human RBP4 antibody	1 Plate
Protein Standard: Lyophilized recombinant human RBP4	2 tubes, 10 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-human RBP4)	130 µl (100x)
Antibody Diluent Buffer	12ml
Avidin-Biotin-Peroxidase Complex (ABC) Solution	130 µl (100x)
ABC Diluent Buffer	12 ml
Tetramethylbenzidine (TMB) Color Developing Agent	10 ml
TMB Stop Solution	10 ml

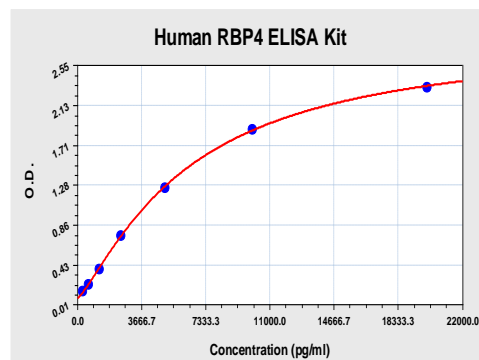
Washing Buffer (not provided): TBS or PBS

0.01M TBS: Add 1.2g Tris, 8.5g NaCl; 450µl of purified acetic acid or 700µl of concentrated hydrochloric acid to 900ml H₂O and adjust pH to 7.2-7.6. Adjust the total volume to 1L.

0.01M PBS: Add 8.5g sodium chloride, 1.4g Na₂HPO₄ and 0.2g NaH₂PO₄ to 900ml distilled H₂O and adjust pH to 7.2-7.6. Adjust the total volume to 1L.

Storage

Store at 4°C. Cell Applications, Inc. recommends using the kit within 6 months of order.



X	pg/ml	0.0	312	625	1250	2500	5000	10000	20000
Y	O.D.450	0.074	0.153	0.231	0.391	0.746	1.251	1.882	2.329

Figure 1: RBP4 Standard Curve. Using the Human RBP4 ELISA Kit, O.D. data was graphed against RBP4 protein concentration. The TMB reaction was incubated at 37°C for 22 min.

BACKGROUND

Retinol binding protein 4 (RBP4) expressed in adipocytes and liver, is an adipokine belonging to the lipocalin family and is involved in the transport of vitamin A/retinol.¹ It delivers retinol from the liver stores to the peripheral tissues. In 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. Apart from its role as a retinol transporter, RBP4 has also been implicated in causing insulin resistance in muscle and liver. By impairing insulin signaling in muscle, RBP4 inhibits glucose uptake and interferes with insulin-mediated suppression of glucose production in the liver, causing blood glucose levels to rise. Conversely, mice lacking the RBP4 gene show increased insulin sensitivity, and normalizing increased RBP4 serum levels improves insulin resistance and glucose intolerance.² Additionally several studies in various populations have reported elevated levels of RBP4 in type 2 diabetes and related metabolic parameters. It was shown that RBP4 modulates the activity of phosphoenolpyruvate carboxyl-kinase (PEPCK) in liver subsequently altering gluconeogenesis. In muscles, RBP4 alters the activation of Phosphoinositide-3-kinase (PI3K) in response to insulin thus decreasing insulin stimulated glucose uptake into muscles. Furthermore, it was demonstrated that Stimulated by Retinoic Acid gene homolog 6 (STRA6) is the high affinity cell surface receptor for RBP4. STRA6 is a multi transmembrane domain protein that mediates the cellular uptake of vitamin A. In accordance with its role as the transmembrane transporter of vitamin A, STRA6 plays a major role in embryonic development. It was demonstrated that genetic variants in STRA6 are significantly associated with type 2 diabetes.³ cAMP pathway may play an important role in maintaining glucose metabolism homeostasis in vivo via upregulation of HMGA1 and RBP4.⁴

References

1. Rask, L. et al: FEBS Lett. 104: 55-8, 1979
2. Yang, Q. et al: Nature 436: 356-62, 2005
3. Nair, A.K. et al: PLoS ONE 5:e11444, 2010
4. Chiefari, E. et al: BMC Biol. 7:24, 2009

ELISA OVERVIEW

Cell Applications ELISA Kits are based on standard sandwich enzyme-linked immunosorbent assay technology. Freshly prepared standards, samples, and solutions are recommended for best results.

1. Prepare test samples.
2. Prepare a protein standard of the target protein.
3. Add test samples and standards to the pre-coated 96-well plate. Do not wash.
4. Add biotinylated detection antibodies. Wash.
5. Add Avidin-Biotin-Peroxidase Complex (ABC) Solution. Wash.
6. Add Tetramethylbenzidine (TMB) Color Developing Agent, containing HRP substrate.
7. Add TMB Stop Solution
8. Subject the plate to analysis.

NOTES:

- Before using the kit, quick spin tubes to bring down all solution to the bottom of tube.
- Duplicate assay wells are recommended for both standard and sample testing.
- Do not let the 96-well plate dry, this will lead to inactivation of plate components.
- When diluting samples and reagents, ensure that they are mixed completely and evenly.
- Pre-warm diluted ABC and TMB solutions at 37°C for 30 min before use to avoid variable temperature effects.
- For washes, use TBS or PBS. Do not touch well walls.
- A protein standard is included in the kit. A protein standard detection curve should be generated with each experiment, no more than 2 hours prior to the experiment.
- The user will determine sample dilution fold by estimation of target protein amount in samples.

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PROTOCOL

I. Plate Washing

Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1–2 minutes. Repeat this process two additional times for a total of three washes.

II. Preparation of Test Samples

Test Sample Processing

- **Cell culture supernate, tissue lysate or body fluids:** Remove particulates by centrifugation.
- **Serum:** Allow the serum to clot in a serum separator tube (about 2 hours) at room temperature. Centrifuge at approximately 1000 X g for 10 min.
- **Plasma:** Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 min at 1000 x g within 30 min of collection. Analyze immediately or aliquot and store frozen at -20°C.

Sample Dilution Guideline

Estimate the concentration of the target protein in the sample and select a proper dilution factor such that the diluted target protein concentration falls within the standard curve range. Depending on the sample, several trial dilutions may be necessary. Dilute the sample using the provided diluent buffer, mixing well. Suggested working dilutions of samples are as follows:

Target Protein Concentration Range	Sample Working Dilution	Sample Vol.	Diluent Buffer Vol.
200-2,000 ng/ml	1:100	1 µl	99 µl
20-200 ng/ml	1:10	10 µl	90 µl
312-20,000 pg/ml	1:2	50 µl	50 µl
≤312 pg/ml	n/a	100µl	n/a

If samples will be assayed within 24 hours, store at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

III. Preparation of Reagents

Reconstitution of the Standard

The standard solutions should be prepared no more than 2 hours prior to the experiment. Two tubes of the standard are included in each kit. Use one tube for each experiment.

1. 20,000pg/ml of human RBP4 standard solution: Add 1 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
2. 10,000pg/ml→312pg/ml of human RBP4 standard solutions: Label 6 Eppendorf tubes with 10,000pg/ml, 5000pg/ml, 2500pg/ml, 1250pg/ml, 625pg/ml, 312pg/ml, respectively. Aliquot 0.3 ml of the sample diluent buffer into each tube. Add 0.3 ml of the above 20,000pg/ml RBP4 standard solution into 1st tube and mix. Transfer 0.3 ml from 1st tube to 2nd tube and mix. Transfer 0.3 ml from 2nd tube to 3rd tube and mix, and so on.

Preparation of Biotinylated Antibody Working Solution

The solution should be prepared no more than 2 hours prior to the experiment.

1. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
2. Biotinylated antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly.

Preparation of the Avidin-Biotin-Peroxidase Complex (ABC) Working Solution

The solution should be prepared no more than 1 hour prior to the experiment.

1. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
2. Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly.

IV. ELISA

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. A standard detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of target protein amount in samples.

1. Aliquot 0.1ml per well of the 20,000pg/ml, 10,000pg/ml, 5000pg/ml, 2500pg/ml, 1250pg/ml, 625pg/ml, 312pg/ml human RBP4 standard solutions into the precoated 96-well plate. Add 0.1ml of the sample diluent buffer into the control well (Blank well). Add 0.1ml of each properly diluted sample of mouse sera, plasma, body fluids, tissue lysates or cell culture supernatants to each empty well. See "Sample Dilution Guideline" for details. We recommend that each human RBP4 standard solution and each sample is measured in duplicate.
2. Seal the plate with the cover and incubate at 37°C for 90 min.
3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 0.1ml of biotinylated anti- human RBP4 antibody working solution into each well and incubate the plate at 37°C for 60 min.
5. Wash the plate three times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
6. Add 0.1ml of prepared ABC working solution into each well and incubate the plate at 37°C for 30 min.
7. Wash plate 5 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
8. Add 90 µl of prepared TMB color developing agent into each well and incubate plate at 37°C for 20-25 min (shades of blue can be seen in the wells with the four most concentrated human RBP4 standard solutions; the other wells show no obvious color).
9. Add 0.1ml of prepared TMB stop solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution.

V. Calculating Protein Concentration

- For all wells, determine O.D.450(Relative):
 $O.D.450(Relative) = O.D.450(Reading) - O.D.450(Blank)$
- Plot the standard curve:
Plot O.D.450(Relative) of each standard solution (Y) vs. the respective concentration of the standard solution (X). See **Figure 1** for a typical standard curve.
- The target protein concentration in samples can be interpolated from the standard curve. Multiply the interpolated concentration by the dilution factor to obtain the target protein concentration in the sample.

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