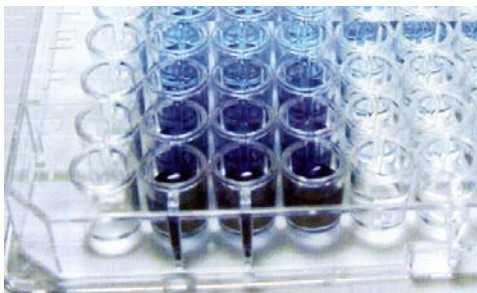


Human RBP4 Sandwich ELISA Kit

Cat. No. R0822EK



Instruction Manual
Version 1.0.0

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES



Table of Contents

Introduction	3
Assay Principles	3
Kit Components	4
Reagent Description	4
Storage of Reagents	5
Materials Required but not Supplied	5
Sample Collection and Storage	5
Flow Chart of Assay Procedure	6
Assay Procedure	7
Performance Characteristics	10
References	13
Plate Layout	14
Troubleshooting Guide	15

Introduction

Retinol binding protein (RBP) 4 is the only specific transport protein for vitamin A in the circulation whose function is to deliver vitamin to target tissues (1). In obesity and type 2 diabetes, expression of Glut4 is significantly impaired in adipocytes. Glucose transport via Glut4 is the rate-limiting step for glucose use by muscle and adipose tissue (2). Yang et al. noted that adipocyte-specific deletion of Gluts led to notable elevation of RBP4 causing systemic insulin resistance, and that reduction of RBP4 improved insulin resistance (3). This identified a novel role of RBP4 in regulating insulin action and RBP4 is recorded as an adipocyte-derived hormone. Thus, measurement of serum or plasma RBP4 is a useful means for understanding of metabolic disorders.

Assay Principles

This kit is an enzyme-linked immunosorbent assay (ELISA) for quantitative determination of RBP4 in human serum, plasma or cell culture supernatants.

A monoclonal antibody specific for human RBP4 has been pre-coated onto 96 well microplate. Standards and samples are pipetted into the wells and any RBP4 present is bound by immobilized antibody. Bound RBP4 is captured by purified anti-human RBP4 polyclonal antibody. HRP conjugated anti-rabbit IgG is added. After washing, a substrate solution is added. The colors develop in proportion to the bounded RBP4 quantity. The color development is stopped and the intensity of color is measured.

Kit Components

- 1) Antibody coated 96-well plate, 12 X 8-well strips
- 2) 5x Wash concentrate, 100 ml
- 3) 5x Diluent, 50 ml
- 4) Secondary antibody, 12 ml
- 5) 100x Detector, 150 µl
- 6) Standard, recombinant human RBP4 expressed by HEK 293 cells, 1 vial, lyophilized
- 7) QC sample = a positive control of human serum RBP4, 1 vial, lyophilized (For actual concentrations of QC sample, see the 'Certificate of analysis' enclosed.)
- 8) Substrate I, 6 ml
- 9) Substrate II, 6 ml
- 10) Stop solution, 12 ml
- 11) Plate sealer, 3 sealers

Reagents Description

Antibody coated 96-well plate, 12 x 8-well strips, with absorbed monoclonal antibody against human RBP4

5x Wash concentrate, buffered detergent solution, supplied as a 5x concentrate

5x Diluent, for sample and reagent dilution

1x Secondary antibody, polyclonal antibody against human RBP4

100x detector, HRP conjugated rabbit IgG

Standard, 50.0 ng, 1 vial each, recombinant human RBP4, lyophilized

QC sample, 1 vial each, a positive control of human serum RBP4

Substrate I and II, chromogenic reagents

Stop solution, 1M H₃PO₄

Storage of Reagents

Reagent must be stored at 2-8°C when not in use. Reagents must be brought to room temperature before use. Do not expose reagents to temperatures greater than 25°C. Diluted wash solution may be stored at room temperature for up to one month.

Materials Required but not Supplied

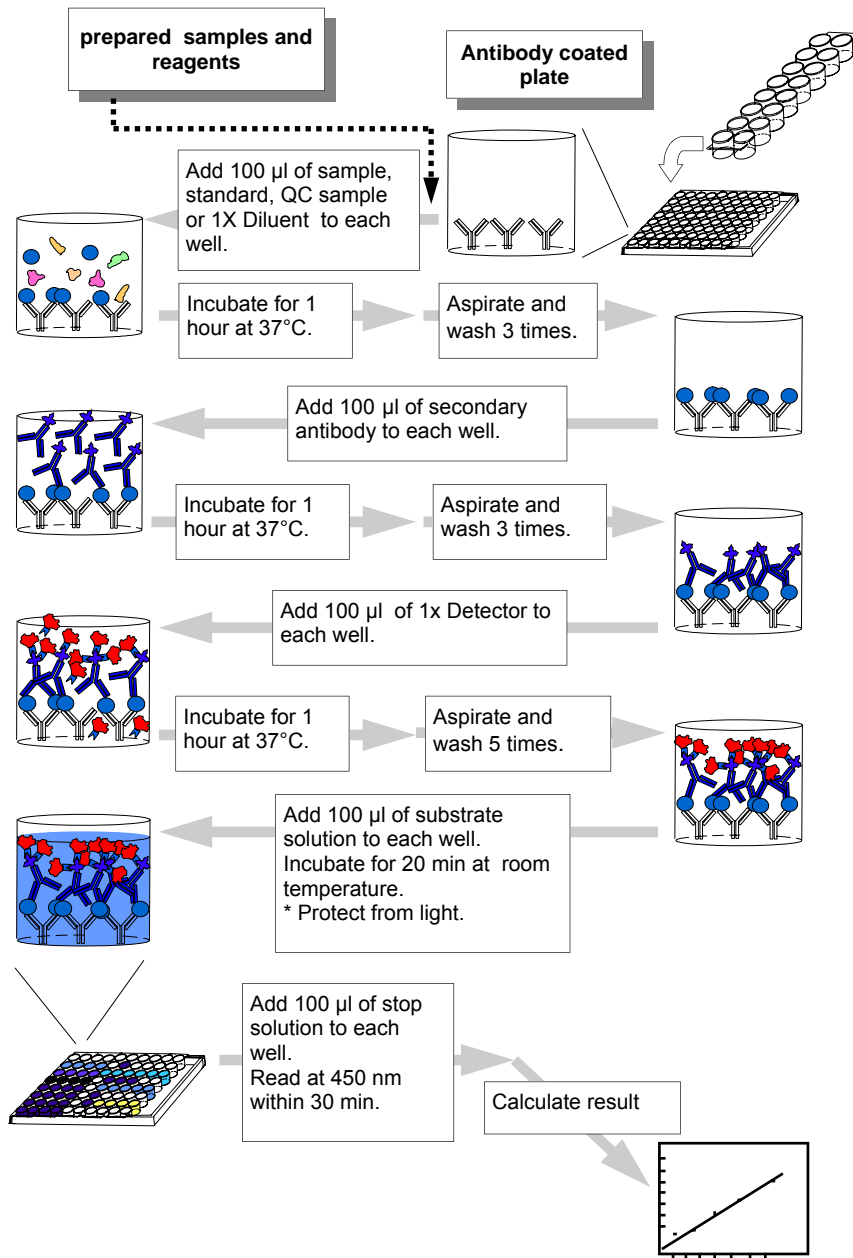
- Precision single and multi-channel pipettes
- Disposable pipette tips
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working reagents
- Reagent reservoirs
- Microwell or microstrip plate reader 450 nm
- Deionized water

Sample Collection and Storage

Serum Use a serum separator tube. Let samples clot at room temperature for 30 minutes before centrifugation for 20 minutes at 1,000xg. Assay freshly prepared serum or store serum in aliquot at $\leq -20^{\circ}\text{C}$ for later use. Avoid repeated freeze/thaw cycles.

Plasma Collect plasma using heparin, EDTA, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay freshly prepared plasma or store plasma sample in aliquot at $\leq -20^{\circ}\text{C}$ for later use. Avoid repeated freeze/thaw cycles.

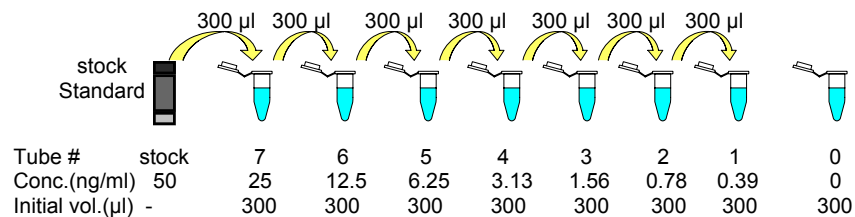
Flow Chart of Assay Procedure



Assay Procedure

1. Preparation of Reagents

- 1) Allow all samples and kit components to equilibrate to room temperature (20-25°C).
- 2) Plan the plate configuration and create a plate map. Calculate the amount of working reagents to use.
It is recommended that Standards and samples be run in duplicate.
- 3) Prepare **1X Wash Solution**. Dilute 5X Wash Concentrate 1:5 with deionized water (1 part 5X Wash Concentrate with 4 parts deionized water). The diluted 1X Wash Solution is stable for one month at room temperature.
- 4) Prepare **1X Diluent**. Dilute 5X Diluent 1:5 with deionized water (1 part 5X Diluent with 4 parts deionized water).
- 5) Prepare **1X Detector**. Dilute 100X Detector 1:100 with 1X Diluent (1 part 100X Detector with 99 parts 1X Diluent). Use the 1X Detector within one hour of preparation.
- 6) Freshly prepare just before use the **Substrate Solution** by adding one part Substrate I to one part Substrate II.
- 7) Prepare working aliquots of the **Standard** as follows :
When opening the lyophilized Standard, remove cap gently as the lyophilizate may have become dislodged during shipping. Add 1 ml of deionized water to the Standard vial to make a stock concentration of 50 ng/ml. Mix well.



A recommended dilution scheme is as follows :

- A) Label 8 microcentrifuge tubes #0-7. Add 300 μ l of the 1X Diluent to the microcentrifuge tubes # 0-7.
- B) Add 300 μ l of the stock Standard solution to tube # 7 and vortex. This is Standard tube # 7 with a concentration of 25 ng/ml.
- C) Standards # 6 to # 1 are then prepared by performing a 1:2 dilution of the preceding standard. Do not add any standard to the tube # 0.

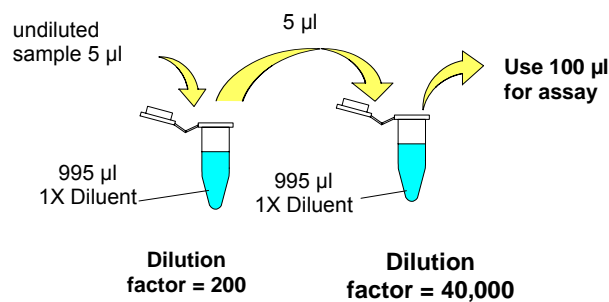
8) Reconstitute **QC sample** in 1 ml of deionized water. Mix well.

2. Sample Preparation

- 1) Dilute samples 1:200 with 1X Diluent (for example, 5 μ l sample plus 995 μ l 1X Diluent, dilution factor=200) and mix well.
- 2) Dilute the step 1) sample 1:200 with 1X Diluent (example, 5 μ l of the step 1) sample plus 995 μ l 1X Diluent, final dilution factor=40,000).

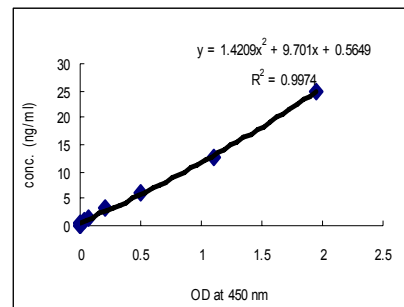
* If samples fall the outside range of assay, a lower or higher dilution may be required.

3) Use 100 μ l of the final diluted sample for ELISA.



3. Experiment procedure

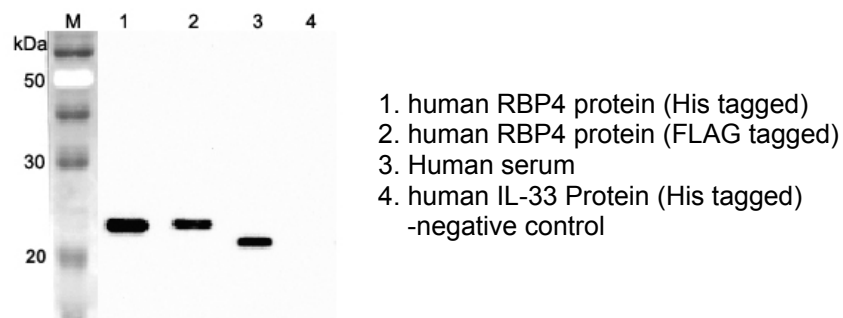
- 1) Remove the appropriate number of microwell strips from the sealed foil pouch.
- 2) Pipette 100 μ l of standard # 0 to # 7, the reconstituted QC sample and diluted sample into the antibody-coated plate according to the plate configuration. Use a new pipette tip for each standard or sample.
- 3) Incubate at 37°C for 1 hour.
- 4) Remove the solution and wash 3 times with 300 μ l of 1X Wash Solution to each well.
- 5) Add 100 μ l Secondary Antibody to each well.
- 6) Incubate at 37°C for 1 hour.
- 7) Remove the solution and wash 3 times with 300 μ l of 1X Wash Solution to each well.
- 8) Add 100 μ l 1X Detector to each well.
- 9) Incubate at 37°C for 1 hour.
- 10) Remove the solution and wash 5 times with 300 μ l of 1X Wash Solution to each well.
- 11) Add 100 μ l of the Substrate Solution to each well.
- 12) Incubate at room temperature for 20 min.
* Protect from light.
- 13) Using the multi-channel pipette, add 100 μ l Stop Solution to each well.
- 14) Read at 450 nm.
- 15) Subtract the absorbance of the blank from the readings for each standard and sample.
- 16) Construct a standard curve by plotting the known concentrations (Y) of standard versus the absorbances (X) of standard. A measurable range is typically shown between 0.39 ng/ml and 25 ng/ml.
- 17) Calculate the RBP4 concentrations of samples by interpolation of the regression curve formula as shown above in a form of a quadratic equation
- 18) The RBP4 concentrations calculated must be multiplied by dilution factor [see 2. **Sample Preparation**] to obtain the concentrations of the undiluted samples.



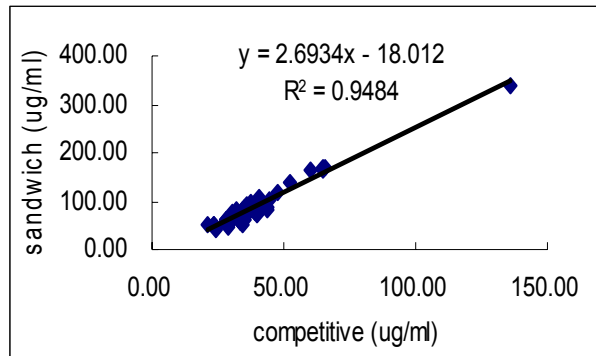
Performance Characteristics

1) Sensitivity : 380 pg/ml

2) Western blot analysis using the capture antibody



3) Correlation between sandwich and competitive ELISA system



4) Precision

a. Intra-Assay (precision within an assay)

6 samples were tested 8 times to assess intra-assay precision.

Sample	Mean (ug/ml)	SD (ug/ml)	CV (%)
1	168.95	6.26	3.70
2	178.48	3.10	1.74
3	127.35	2.42	1.90
4	141.84	3.51	2.47
5	66.84	2.30	3.44
6	77.69	2.76	3.56

b. Inter-Assay (precision between assays)

6 samples were tested 8 times to assess inter-assay precision.

Sample	Mean (ug/ml)	SD (ug/ml)	CV (%)
1	194.70	14.40	7.40
2	192.44	15.50	8.05
3	141.17	11.56	8.19
4	154.52	9.18	5.94
5	89.82	7.93	8.83
6	73.29	5.19	7.09

5) Recovery

The recovery of RBP4 spiked to three different levels in four different human serum samples throughout the range of assay was evaluated.

Sample No.	Average recovery (%)	Range (%)
1	100.31	95-105
2	104.35	100-110
3	109.68	105-115
4	103.72	95-105

6) Specificity

- a. No cross reaction with mouse and rat sera
- b. Cross Reactivity

Analyte	Max. Conc. (ng/ml)	Cross Reactivity (%)
Human RBP4	10	100
Mouse RBP4	100	N. R.
Rat RBP4	100	N. R.
Human adiponectin	100	N. R.
Human Resistin	100	N. R.
Human Vaspin	100	N. R.
Human Clusterin	100	N. R.
Human Leptin	100	N. R.
Human IL-33	100	N. R.
Human GPX3	100	N. R.
Human Progranulin	100	N. R.
Human FABP4	100	N. R.
Human ANGPTL3	100	N. R.
Human ANGPTL4	100	N. R.
Human ANG1	100	N. R.
Human ANG2	100	N. R.
Human Visfatin	100	N. R.
Mouse Visfatin	100	N. R.

N. R. : No Cross-reactivity

7) Linearity - Effect of Serum Dilution

To assess the linearity of the assay, three serum samples were first diluted as indicated below prior to sample preparation as described in the protocol.

Sample No.	Serum Dilution	Expected (ug/ml)	Observed (ug/ml)	% Of Expected
1	1:40000	238.79	238.79	100
	1:50000	191.03	184.07	96.35
	1:60000	159.19	144.58	90.82
2	1:40000	47.50	47.50	100
	1:50000	38.00	36.81	96.87
	1:60000	31.67	28.98	91.51
3	1:40000	39.05	39.05	100
	1:50000	31.24	31.37	100.42
	1:60000	26.03	25.02	96.12

% of expected = observed / expected x 100%

References

1. Quadro L, Blaner WS et al. 1999 Impaired retinol function and vitamin A availability in mice lacking retinol - binding protein. *EMBO J.* 18:4633-44.
2. Shepherd PR, Kahn BB. 1999 Glucose transporters and insulin action - implications for insulin resistance and diabetes mellitus. *N Engl J Med.* 341:248-57.
3. Yang Q, Graham TE et al. 2005 Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* 436:356-62.

Troubleshooting Guide

Problem	Possible Cause	Solution
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be appropriate for the system.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of detector too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.
	Technique problem	Proper mixing of reagents and wash steps are critical.

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