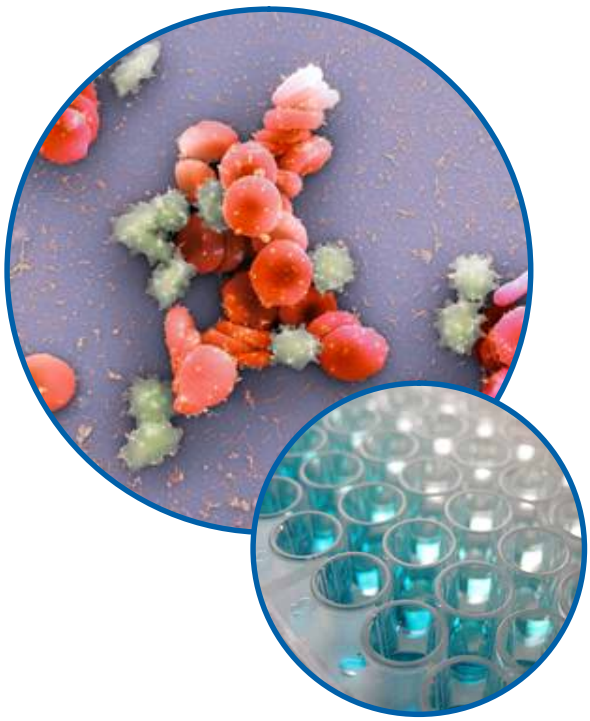


AdipoGen™

MANUAL

RBP4 (human) Competitive ELISA Kit

For research use only. Not for diagnostic use.

Version 2 (14-March-2011)

Cat. No. AG-45A-0010EK-KI01

Table of Contents

1. Intended Use

The RBP4 (human) Competitive ELISA Kit is to be used for the *in vitro* quantitative determination of human RBP4 in serum, plasma, urine and cell culture supernatant. This ELISA Kit is for research use only.

2. 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.

3. General References

(1) Impaired retinol function and vitamin A availability in mice lacking retinol - binding protein: L. Quadro, et al.; EMBO J. 18, 4633 (1999)

(2) Glucose transporters and insulin action - implications for insulin resistance and diabetes mellitus: P.R. Shepherd, et al.; N. Engl. J. Med. 341, 248 (1999)

(3) Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes: Q. Wang, et al.; Nature 436, 356 (2005)

4. Assay Principle

This assay is a competitive Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human RBP4 in biological fluids. A polyclonal antibody recognizing native human RBP4 reacts with a series of predetermined recombinant human RBP4 standard proteins or samples under competition in the human RBP4-coated plate. Their relative reactivity is plotted with that of the standard proteins.

5. Handling & Storage

- Reagent must be stored at 2-8°C when not in use.
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

1 plate coated with human RBP4 Recombinant Protein	(12 x 8-well strips)
1 bottle Wash Buffer 10X	(50 ml)
1 bottle Diluent 5X	(50 ml)
1 bottle Detection Antibody	(12 ml)
1 vial Detector 100X (HRP Conjugated anti-rabbit IgG)	(150 µl)
1 vial human RBP4 Standard (lyophilized)	(5 µg)
1 vial human RBP4 QC sample (lyophilized)	
1 bottle Substrate Solution I (TMB)	(6 ml)
1 bottle Substrate Solution II (Peroxidase)	(6 ml)
1 bottle Stop Solution	(12 ml)
3 plate sealers (plastic film)	

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450 nm, with the correction wavelength set at 540 nm or 570 nm
- Calibrated precision single and multi-channel pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard

8. General ELISA Protocol

8.1. Preparation and Storage of Reagents

NOTE: *Prepare just the appropriate amount of the buffers necessary for the assay.*

- **Wash Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 50 ml Wash Buffer 10X + 450 ml water) to obtain Wash Buffer 1X.
- **Diluent 5X** has to be diluted with deionized water 1:5 before use (e.g. 50 ml Diluent 5X + 200 ml water) to obtain Diluent 1X.
- **Detector 100X (HRP Conjugated anti-rabbit IgG)** has to be diluted to the working concentration by adding 120 µl in 12 ml of Diluent 1X (1:100).

  NOTE: *The diluted Detector is used within one hour of preparation.*

- **Substrate Solution I and II** have to be mixed together in equal volumes within 15 minutes of use.

  NOTE: *Freshly prepare just before use the Substrate Solution and protect from light!*

- **Human RBP4 Standard (STD)** has to be reconstituted with 1 ml of deionized water.

  - This reconstitution produces a stock solution of 5 µg/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

  NOTE: *The reconstituted standard is aliquoted and stored at -20°C.*

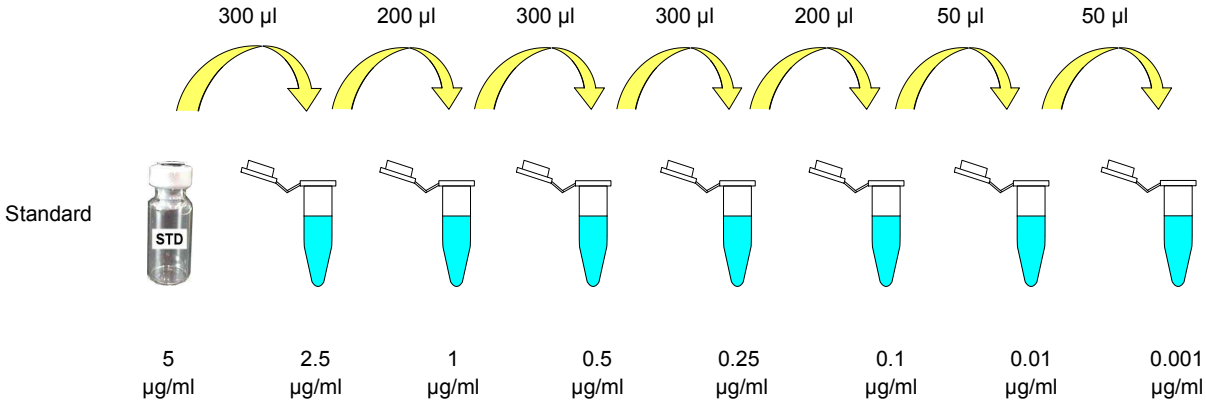
  - Dilute the standard protein concentrate (STD) (**5 µg/ml**) in Diluent 1X. A seven-point standard curve in Diluent 1X is recommended.
  - Suggested standard points are:
    5 , 2.5 , 1 , 0.5 , 0.25 , 0.1 , 0.01 and 0.001 µg/ml.

- **Human RBP4 QC sample** has to be reconstituted with 1 ml of deionized water.

  - Refer to the Certificate of Analysis for current QC sample concentration. Mix the QC sample to ensure complete reconstitution and allow the QC sample to sit for a minimum of 15 minutes. The reconstituted QC sample is ready to use, do not dilute it.

Dilute further for the standard curve:

To obtain	Add	Into
5 µg/ml	-	-
2.5 µg/ml	300 µl of RBP4 (5 µg/ml)	300 µl of Diluent 1X
1 µg/ml	200 µl of RBP4 (2.5 µg/ml)	300 µl of Diluent 1X
0.5 µg/ml	300 µl of RBP4 (1 µg/ml)	300 µl of Diluent 1X
0.25 µg/ml	300 µl of RBP4 (0.5 µg/ml)	300 µl of Diluent 1X
0.1 µg/ml	200 µl of RBP4 (0.25 µg/ml)	300 µl of Diluent 1X
0.01 µg/ml	50 µl of RBP4 (0.1 µg/ml)	450 µl of Diluent 1X
0.001 µg/ml	50 µl of RBP4 (0.01 µg/ml)	450 µl of Diluent 1X



8.2. Sample Collection, Storage and Dilution

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 ≤ -20°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 ≤ -20°C for later use. Avoid repeated freeze/ thaw cycles.

Urine : Aseptically collect the urine of the day, voided directly into a sterile container. Assay immediately or aliquot and store at ≤ -20°C. Avoid repeated freeze/thaw cycles.

Serum, Plasma, Urine or **Cell Culture Supernatant** have to be diluted in Diluent 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1/100 dilution of serum or plasma is recommended! If samples fall the outside range of assay, a lower or higher dilution may be required!*

8.3. Assay Procedure (Checklist)

☐	1. Determine the number of 8-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C. ***NOTE:*** *Remaining 8-well strips coated with RBP4 protein when opened can be stored at 4°C for up to 1 month*.
☐	2. Add 50 µl of the different standards and reconstituted QC sample into the appropriate wells in duplicate! At the same time, add 50 µl of diluted serum, plasma, urine or cell culture supernatant samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2. Preparation of Samples).**
☐	3. Add 50 µl to each well of the Detection Antibody and tap gently on the side of the plate to mix.
☐	4. Cover the plate with plate sealer and incubate for **1 hour at 37°C.**
☐	5. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multi-channel pipette or auto-washer. Repeat the process for a total of three washes. After the last wash, complete removal of liquid is essential for good performance.
☐	6. Add 100 µl to each well of the diluted Detector **(see 8.1. Preparation and Storage of Reagents)**.
☐	7. Cover the plate with plate sealer and incubate for **1 hour at 37°C.**
☐	8. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multi-channel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	9. Add 100 µl to each well of mixed substrate solution.
☐	10. Allow the color reaction to develop **at room temperature (RT°C) in the dark for 20 minutes**.
☐	11. Stop the reaction by adding 100 µl of Stop Solution. Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.
	! CAUTION: CORROSIVE SOLUTION!
☐	12. Measure the OD at 450 nm in an ELISA reader within 30 minutes.

9. Calculation of Results

- Average the duplicate readings for each standard, QC and sample.
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the vertical (Y) axis vs. the corresponding RBP4 concentration (µg/ml) on the horizontal (X) axis (see **10.** TYPICAL DATA).
- Calculate the RBP4 concentrations of samples by interpolation of the regression curve formula as shown above in a form of a 4-parameter logistic equation.
- If the test samples were diluted, multiply the interpolated values by the dilution factor to calculate the concentration of human RBP4 in the samples.

10. Typical Data

The following data are obtained using the different concentrations of standard as described in this protocol:

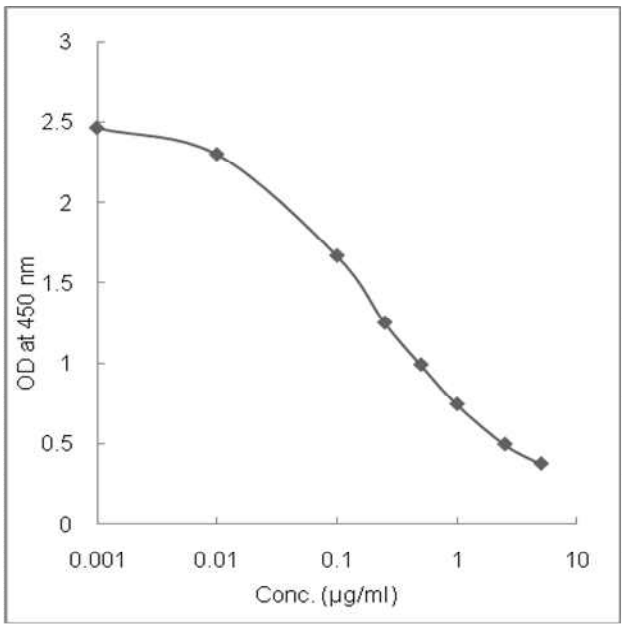


Figure: Standard curve

Standard hRBP4 (µg/ml)	Optical Density (mean)
5	0.377
2.5	0.497
1	0.745
0.5	0.991
0.25	1.252
0.1	1.669
0.01	2.300
0.001	2.464

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of RBP4 that can be detected by this assay is 1 ng/ml. ***NOTE:*** *The Limit of detection was measured by adding two standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.001 µg/ml – 5 µg/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human RBP4. It does not cross-react with mouse RBP4, rat RBP4, human adiponectin, rat adiponectin, human resistin, human vaspin, human clusterin, human leptin, human IL-23, human IL-33, human GPX3, human Nampt, human ANG1, human ANG2, human ANGPTL3, human ANGPTL4, human ANGPTL6, human FABP4, human RELM-β, rat RELM-α, mouse Nampt, human PAI-1.

D. Intra-assay precision:

Five samples of known concentrations of human RBP4 were assayed in replicates 4 times to test precision within an assay.

Samples	Means (µg/ml)	SD	CV (%)	n
1	16.42	0.43	2.64	4
2	19.14	0.70	3.63	4
3	22.29	1.01	4.51	4
4	25.31	1.34	5.31	4
5	45.76	4.22	9.22	4

E. Inter-assay precision:

Five samples of known concentrations of human RBP4 were assayed in 4 separate assays to test precision between assays.

Samples	Means (µg/ml)	SD	CV (%)	n
1	15.39	0.54	3.48	4
2	21.48	1.54	7.15	4
3	24.98	1.62	6.50	4
4	26.89	2.31	8.58	4
5	31.88	3.28	10.27	4

F. Linearity:

Different human serum samples containing RBP4 were diluted several fold (1/100 to 1/800) and the measured recoveries ranged from 81% to 110%.

Samples	Sample Dilution	Expected (µg/ml)	Observed (µg/ml)	% of Expected
1	1 : 100	14.59	14.59	100
	1 : 200	7.29	7.95	109
	1 : 400	3.65	4.01	110
	1 : 800	1.82	1.95	107
2	1 : 100	23.09	23.09	100
	1 : 200	11.55	9.81	85
	1 : 400	5.77	4.71	82
	1 : 800	2.89	2.33	81
3	1 : 100	31.03	31.03	100
	1 : 200	15.52	13.55	87
	1 : 400	7.76	6.69	86
	1 : 800	3.88	3.26	84

G. Comparison of serum samples with plasma samples:

Different human serum samples containing RBP4 were compared with plasma samples.

Samples	Serum (µg/ml)	Plasma (µg/ml)		
		Citrate	EDTA	Heparin
1	19.94	15.05	22.04	19.81
2	25.91	18.95	22.30	28.42

H. Expected values:

RBP4 levels range in plasma and serum from **10 to > 70 µg/ml** (from healthy donors).

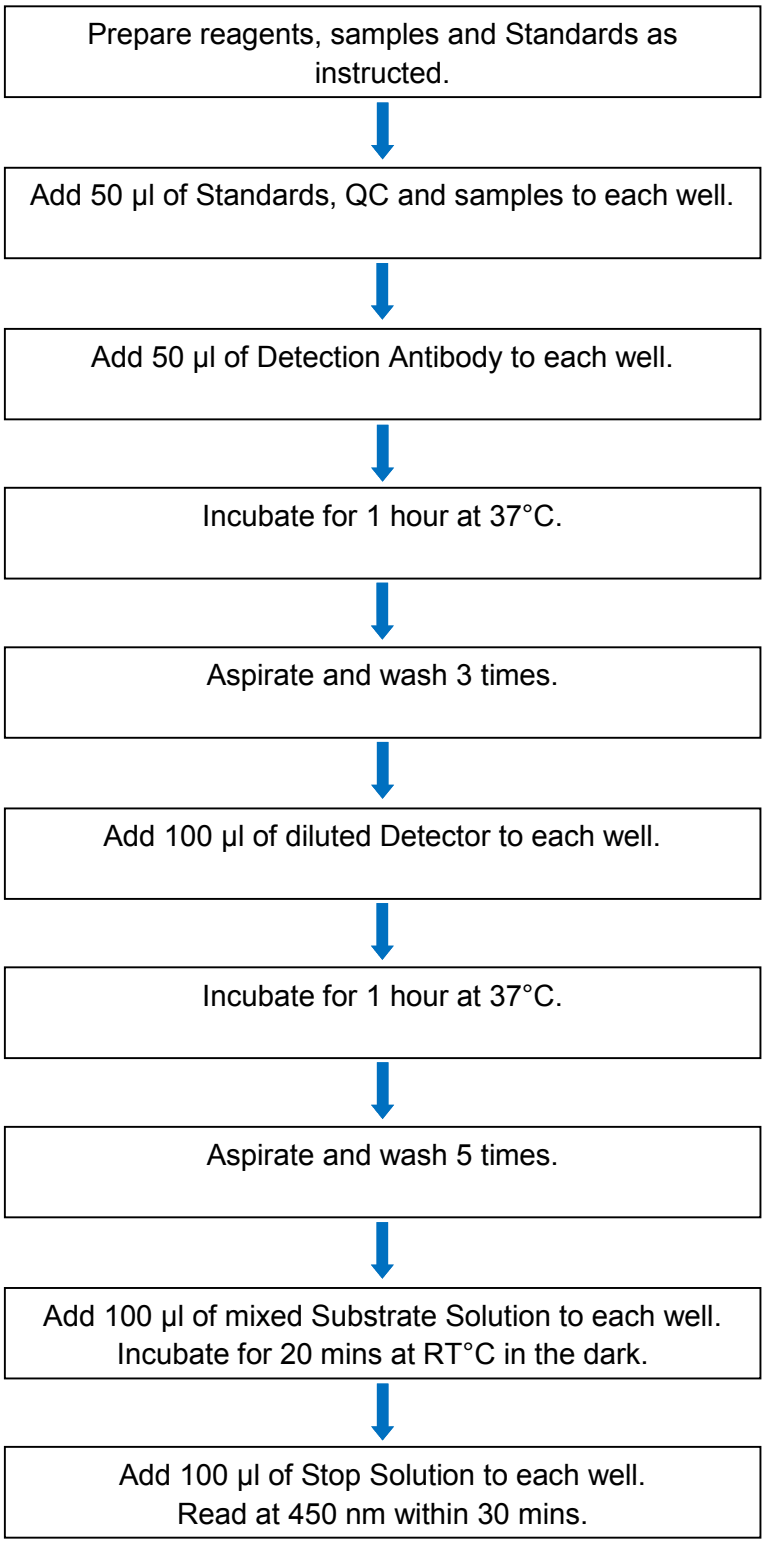
12. Technical Hints and Limitations

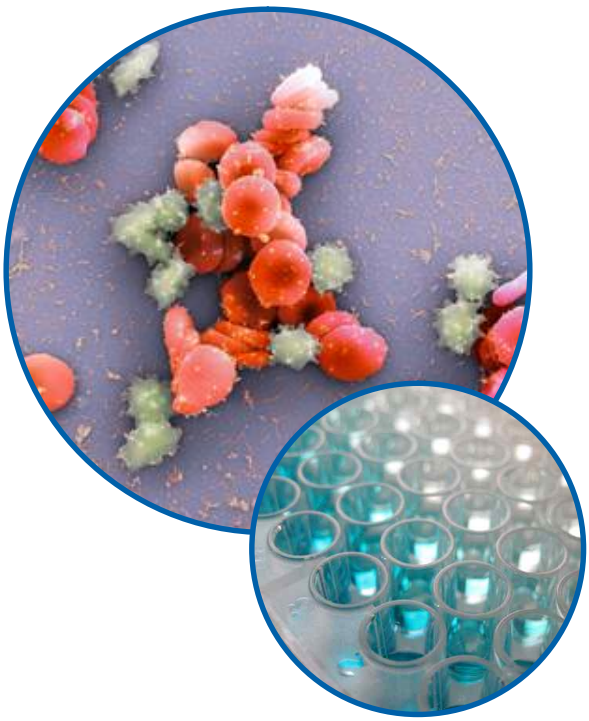
- It is recommended that all standards, QC sample and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100 µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 8-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep Substrate Solution protected from light.
- The Stop Solution consists of phosphoric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

13. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
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 followed as indicated in the manual.
	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.

14. Assay Flow Chart





Product Specific References:

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For more References please visit www.adipogen.com!

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