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MANUAL

RBP4 (human) ELISA Kit (Quantitative)

(Penta Plex)

For research use only. Not for diagnostic use.

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1. Intended Use

The RBP4 (human) ELISA Kit (Quantitative) 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 mouse 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. Graham et al. showed that serum RBP4 levels correlated with the magnitude of insulin resistance in subjects with obesity, impaired glucose tolerance, or type 2 diabetes (4). Cho et al. recently showed that plasma RBP4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes (5). 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. Yang, et al.; Nature **436**, 356 (2005)
- (4) Retinol-binding protein 4 and insulin resistance in lean, obese, and diabetic subjects: T.E. Graham, et al.; N. Engl. J. Med. **354**, 2552 (2006)
- (5) Plasma retinol-binding protein-4 concentrations are elevated in human subjects with impaired glucose tolerance and type 2 diabetes: Y.M. Cho, et al.; Diabetes Care **29**, 2457 (2006)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human RBP4 in biological fluids. A monoclonal antibody specific for RBP4 has been precoated onto the 96-well microtiter plate. Standards and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, RBP4 is recognized by the addition of a purified polyclonal antibody specific for RBP4 (Detection Antibody). After removal of excess polyclonal antibody, HRP conjugated anti-rabbit IgG (HRP) is added. Following a final washing, peroxidase activity is quantified using 3,3',5,5'-tetramethylbenzidine (TMB) as substrate. The intensity of the color reaction is measured at 450 nm after acidification and is directly proportional to the concentration of human RBP4 in the samples.

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 for 5 x 96 well Plates

5 plates coated with human RBP4 Antibody	(6 x 16-well strips)	
2 bottles Wash Buffer 10X	(2 x 125 ml)	(Wash Buffer 10X)
1 bottle ELISA Buffer 10X	(125 ml)	(ELISA Buffer 10X)
1 vial Detection Antibody	(120 µl)	(DET)
2 vials HRP 100X (HRP Conjugated anti-rabbit IgG)	(2 x 550 µl)	(HRP 100X)
5 vials human RBP4 Standard (lyophilized)	(5 x 50 ng)	(STD)
2 bottles TMB Substrate Solution	(2 x 30 ml)	(TMB)
2 bottles Stop Solution	(2 x 30 ml)	(STOP)
10 plate sealers (plastic film)		
10 silica Gel Minibags		

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450 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 (for one plate)

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.
- **ELISA Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 20 ml ELISA Buffer 10X + 180 ml water) to obtain ELISA Buffer 1X.
- **Detection Antibody (DET)** has to be diluted to 1:660 in ELISA Buffer 1X (15 µl DET + 10 ml ELISA Buffer 1X).

NOTE: The diluted Detection Antibody is not stable and cannot be stored!

- **HRP 100X (HRP Conjugated anti-rabbit IgG)** has to be diluted to the working concentration by adding 100 µl in 10 ml of ELISA Buffer 1X (1:100).

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

- **Human RBP4 Standard (STD)** has to be reconstituted with 1 ml of deionized water.
 - This reconstitution produces a stock solution of 50 ng/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) (**50 ng/ml**) in ELISA Buffer 1X. A seven-point standard curve using 2-fold serial dilutions in ELISA Buffer 1X is recommended.
- Suggested standard points are:
25 , 12.5 , 6.25 , 3.12, 1.56, 0.78, 0.39 and 0 ng/ml.

Dilute further for the standard curve:

To obtain	Add	Into
25 ng/ml	300 µl of RBP4 (50 ng/ml)	300 µl of ELISA Buffer 1X
12.5 ng/ml	300 µl of RBP4 (25 ng/ml)	300 µl of ELISA Buffer 1X
6.25 ng/ml	300 µl of RBP4 (12.5 ng/ml)	300 µl of ELISA Buffer 1X
3.12 ng/ml	300 µl of RBP4 (6.25 ng/ml)	300 µl of ELISA Buffer 1X
1.56 ng/ml	300 µl of RBP4 (3.12 ng/ml)	300 µl of ELISA Buffer 1X
0.78 ng/ml	300 µl of RBP4 (1.56 ng/ml)	300 µl of ELISA Buffer 1X
0.39 ng/ml	300 µl of RBP4 (0.78 ng/ml)	300 µl of ELISA Buffer 1X
0 ng/ml	300 µl of ELISA Buffer 1X	Empty tube

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 $\leq -20^{\circ}\text{C}$ 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.

Urine : Aseptically collect the urine of the day, voided directly into a sterile container. Assay immediately or aliquot and store at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze/thaw cycles.

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

NOTE: As a starting point, 1/40,000 dilution of serum and 1/100 dilution of urine are recommended! If sample values fall outside the detection range of the assay, a lower or higher dilution may be required!

8.3. Assay Procedure (Checklist)

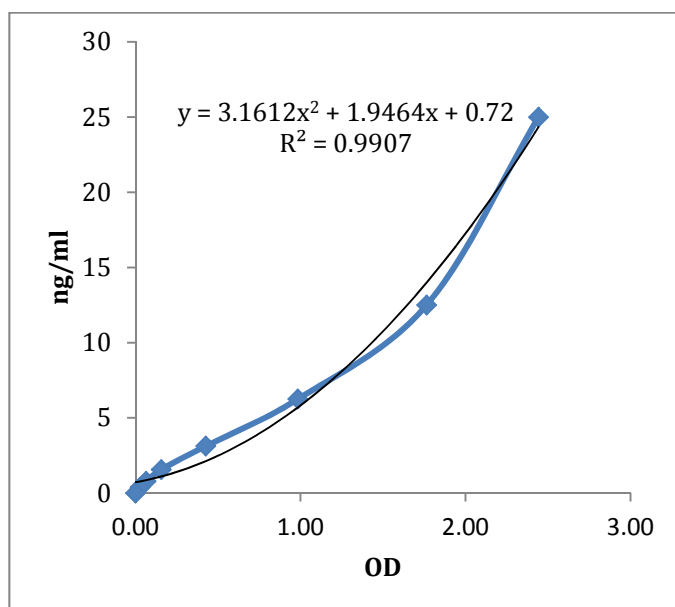
<input type="checkbox"/>	<p>1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C.</p> <p>NOTE: Remaining 16-well strips coated with RBP4 antibody when opened can be stored at 4°C for up to 1 month.</p>
<input type="checkbox"/>	<p>2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 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).</p>
<input type="checkbox"/>	<p>3. Cover the plate with plate sealer and incubate for 1 hour at 37°C.</p>
<input type="checkbox"/>	<p>4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel 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.</p>
<input type="checkbox"/>	<p>5. Add 100 µl to each well of the Detection Antibody (DET). (see 8.1. Preparation and Storage of Reagents).</p>
<input type="checkbox"/>	<p>6. Cover the plate with plate sealer and incubate for 1 hour at 37°C.</p>
<input type="checkbox"/>	<p>7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel 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.</p>
<input type="checkbox"/>	<p>8. Add 100 µl to each well of the diluted HRP (see 8.1. Preparation and Storage of Reagents).</p>
<input type="checkbox"/>	<p>9. Cover the plate with plate sealer and incubate for 1 hour at 37°C.</p>
<input type="checkbox"/>	<p>10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel 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.</p>
<input type="checkbox"/>	<p>11. Add 100 µl to each well of TMB Substrate Solution (TMB).</p>
<input type="checkbox"/>	<p>12. Allow the color reaction to develop at room temperature (RT°C) in the dark for 20 minutes.</p>
<input type="checkbox"/>	<p>13. Stop the reaction by adding 100 µl of Stop Solution (STOP). Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution (STOP) is added.</p>
	<p>! CAUTION: CORROSIVE SOLUTION!</p>
<input type="checkbox"/>	<p>14. Measure the OD at 450 nm in an ELISA reader within 30 minutes.</p>

9. Calculation of Results

- Average the duplicate readings for each standard, control and sample and subtract the average blank value (obtained with the 0 ng/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding RBP4 concentration (ng/ml) on the vertical (Y) axis (see **10. TYPICAL DATA**).
- Calculate the RBP4 concentrations of samples by interpolation of the curve formula as shown above in a form of a quadratic 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:



Standard hRBP4 (ng/ml)	Optical Density (mean)
25	2.44
12.5	1.76
6.25	0.98
3.13	0.43
1.56	0.16
0.78	0.06
0.39	0.03
0	0.00

Figure: Standard curve

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of RBP4 that can be detected by this assay is 380 pg/ml. **NOTE:** *The Limit of detection was measured by adding two standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.39 ng/ml – 25 ng/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, human resistin, human vaspin, human clusterin, human leptin, human IL-33, human GPX3, human progranulin, human FABP4, human ANGPTL3, human ANGPTL4, human ANG1, human ANG2, human Nampt, mouse Nampt.

D. Intra-assay precision:

Six serum samples of known concentrations of human RBP4 were assayed in replicates 8 times to test precision within an assay.

Samples	Means (µg/ml)	SD	CV (%)	n
1	134.56	2.72	2.02	8
2	87.27	3.05	3.50	8
3	89.07	2.18	2.45	8
4	79.11	1.85	2.34	8
5	77.60	1.93	2.49	8
6	46.34	1.66	3.59	8

Six urine samples of known concentrations of human RBP4 were assayed in replicates 9 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
1	309.91	9.36	3.02	9
2	97.60	3.58	3.67	9
3	156.69	9.42	6.01	9
4	113.30	4.90	4.33	9
5	273.93	7.47	2.73	9
6	480.68	20.87	4.34	9

E. Inter-assay precision:

Six serum samples of known concentrations of human RBP4 were assayed in 3 separate assays to test precision between assays.

Samples	Means (µg/ml)	SD	CV (%)	n
1	136.17	5.44	3.99	3
2	86.27	4.08	4.73	3
3	87.29	5.18	5.94	3
4	79.51	3.29	4.14	3
5	80.12	4.28	5.34	3
6	46.01	2.69	5.85	3

Six urine samples of known concentrations of human RBP4 were assayed in 3 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
1	299.52	18.55	6.19	3
2	96.51	9.80	10.16	3
3	151.36	12.49	8.25	3
4	107.94	8.31	7.70	3
5	261.44	15.34	5.87	3
6	463.20	29.32	6.33	3

F. Recovery:

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

Samples	Average recovery (%)	Range (%)	
Serum	1	99.81	95-105
	2	100.08	95-105
	3	97.97	95-105
	4	91.76	90-100
Urine	1	106.71	100-110
	2	100.14	95-105
	3	100.40	95-105
	4	95.10	95-105

G. Linearity:

Different human serum samples containing RBP4 were diluted several fold (1/40,000 to 1/60,000) and the measured recoveries ranged from 92% to 105%.

Samples	Sample Dilution	Expected (µg/ml)	Observed (µg/ml)	% of Expected
1	1 : 40,000	73.98	73.98	100
	1 : 50,000	59.18	56.74	95.87
	1 : 60,000	49.32	45.51	92.27
2	1 : 40,000	43.10	43.10	100
	1 : 50,000	34.48	34.62	100.40
	1 : 60,000	28.73	29.70	103.38
3	1 : 40,000	49.87	49.87	100
	1 : 50,000	39.90	40.47	101.42
	1 : 60,000	33.25	33.49	100.72

Different human urine samples containing RBP4 were diluted several fold (1/100 to 1/200) and the measured recoveries ranged from 97% to 107%.

Samples	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
1	1 : 100	127.96	127.96	100
	1 : 200	63.98	66.69	104.23
2	1 : 100	141.01	141.01	100
	1 : 200	70.51	72.33	102.59
3	1 : 100	95.52	95.52	100
	1 : 200	47.76	50.74	106.24

H. Expected values:

RBP4 levels range in plasma and serum from **20 to > 200 µg/ml** (from healthy donors).

RBP4 levels range in urine from **40 to > 800 ng/ml** (from healthy donors).

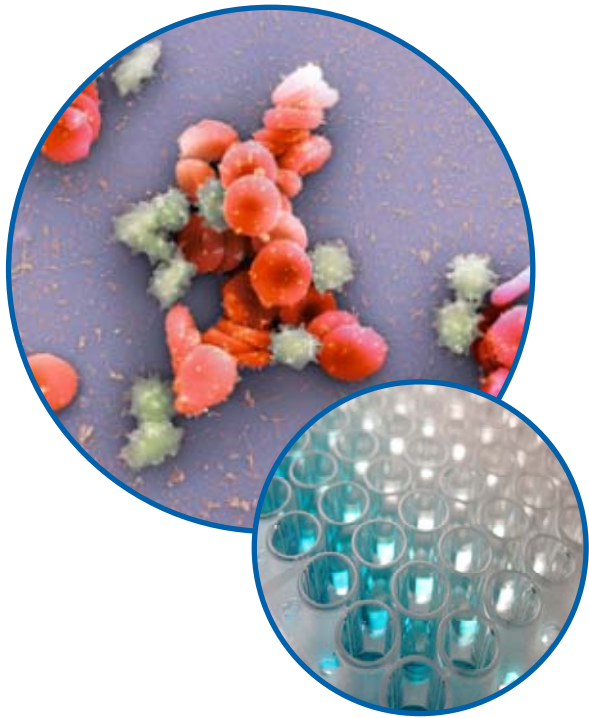
12. Technical Hints and Limitations

- It is recommended that all standards, controls 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 16-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Substrate Solution (TMB) protected from light.
- The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution (STOP) 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 HRP 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. Notes



Product Specific References:

1. M. Blueher, et al.; Diabetes Care **35**, 342 (2012)
2. S. Akbarzadeh, et al.; Cytokine **58**, 295 (2012)
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4. M. Mansouri, et al.; J. Diabet. Metab. Dis. **11**, 2 (2012)
5. H.Y. Choi, et al.; Diabetes Care **36**, 3321 (2013)
6. Z. Goktas, et al.; Mediators Inflamm. **2013**, 861496 (2013)
7. H. Ortega-Senovilla, et al.; PLoS One **8**, e81201 (2013)
8. Y. Liu, et al.; Diabetol. Metab. Syndr. **6**, 25 (2014)

For more References please visit www.adipogen.com!

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