Innovative RESEARCH Results Driven Research

Retinol-Binding Protein 4 (RBP 4) ELISA Kit

Introduction

Serum retinol-binding protein (RBP 4) is secreted by liver and adipocytes and is implicated in systemic insulin resistance. RBP 4 transports retinol and and circulates in the plasma by binding to the larger transthyretin (TTR) homotetramer, forming a protein complex that reduces renal clearance of RBP 4. In insulin-resistant ob/ob mice, urinary fractional excretion of RBP 4 was reduced, consistent with increased retention; while TTR level is elevated (1). RBP 4 is encoded by the *RBP 4* gene that maps to chromosome 10q23-q24 linked to increased risk for type 2 diabetes in different populations (2, 3). Transgenic overexpression of human RBP 4 or injection of recombinant RBP 4 in normal mice causes insulin resistance. Conversely, genetic deletion of RBP 4 enhances insulin sensitivity. Increasing serum RBP 4 induces hepatic expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase and impairs insulin signalling in muscle (4). Expression of RBP 4 is induced in adipose tissue as a consequence of decreased glucose transporter GLUT4 expression. Increased human serum RBP 4 is associated with insulin resistance, Type II diabetes, and metabolic syndrome such as obesity, glucose intolerance, dyslipidemia, and hypertension (5, 6). Human plasma RBP 4 concentration might be a biomarker of nephropathy and cardiovascular disease in type 2 diabetic subjects (7).

Principal of the Assay

The Human RBP 4 ELISA kit is designed for detection of human RBP 4 in urine, plasma, serum, milk, saliva, and cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay that measures RBP 4 in 4 hours. A polyclonal antibody specific for RBP 4 has been pre-coated onto a microplate. RBP 4 in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for RBP 4, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- This kit is for research use only.
- The kit should not be used beyond the expiration date.
- The Stop Solution is an acid solution.

Reagents

- **RBP 4 Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against human RBP 4.
- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes, which can be cut to fit the format of the individual assay.
- **RBP 4 Standard:** Human RBP 4 in a buffered protein base (1.6 µg, lyophilized).
- **Biotinylated RBP 4 Antibody (50x):** A 50-fold concentrated biotinylated polyclonal antibody against RBP 4 (140 µl).
- **EIA Diluent Concentrate (10x)**: A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate**: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution**: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store kit at 2-8^{0}C or -20^{0}C upon arrival up to the expiration date.
- Opened EIA Diluent may be stored for up to 1 month at 2-8^{0}C. Store reconstituted reagents at -20^{0}C or below.
- Opened unused strip wells may return to the foil pouch with the desiccant pack, reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm.
- Pipettes (1-20 µl, 20-200 µl, 200-1000µl and multiple channel).
- Deionized or distilled reagent grade water.

Sample Collection and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 2000 x g for 10 minutes and assay. Dilute samples 1:1000 into EIA Diluent. The undiluted samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles. (EDTA or Heparin can also be used as anticoagulant.)
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2000 x g for 10 minutes. Remove serum and assay. Dilute samples 1:1000 into EIA Diluent. The undiluted samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatants:** Centrifuge cell culture media at 2000 x g for 10 minutes to remove debris. Collect supernatants and assay. Store samples at -20^{0}C or below. Avoid repeated freeze-thaw cycles.
- **Urine:** Collect urine using sample pot. Centrifuge samples at 600 x g for 10 minutes and assay. Store samples at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Saliva:** Collect saliva using sample tube. Centrifuge samples at 600 x g for 10 minutes and assay. Store samples at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

- **Milk:** Collect milk using sample tube. Centrifuge samples at 600 x g for 10 minutes. Dilute samples 1:2 into EIA Diluent. Store samples at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.
- **EIA Diluent Concentrate (10x)**: Dilute the EIA Diluent Concentrate 1:10 with reagent grade water. Store for up to 1 month at 2 - 8^{0}C.
- **RBP 4 Standard:** Reconstitute the 1.6 µg of human RBP 4 Standard with 4 ml of EIA Diluent to generate a standard solution of 400 ng/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting the standard solution (400 ng/ml) 1:2 with EIA Diluent to produce 200, 100, 50, 25, 12.5 and 6.25 ng/ml. EIA Diluent serves as the zero standard (0 ng /ml). Any remaining solution should be frozen at -20^{0}C.

Standard Point	Dilution	[RBP 4] (ng/ml)
P1	1 part Standard (400 ng/ml)	400.00
P2	1 part P1 + 1 part EIA Diluent	200.00
P3	1 part P2 + 1 part EIA Diluent	100.00
P4	1 part P3 + 1 part EIA Diluent	50.00
P5	1 part P4 + 1 part EIA Diluent	25.00
P6	1 part P5 + 1 part EIA Diluent	12.50
P7	1 part P6 + 1 part EIA Diluent	6.25
P8	EIA Diluent	0.00

- **Biotinylated RBP 4 Antibody (50x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:50 with EIA Diluent. Any remaining solution should be frozen at -20^{0}C.
- **Wash Buffer Concentrate (20x):** Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with EIA Diluent. Any remaining solution should be frozen at -20^{0}C.

Assay Procedure

- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30^{0}C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Standard or sample per well. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
- Add 50 µl of Biotinylated RBP 4 Antibody to each well and incubate for one hour.
- Wash a microplate as described above.

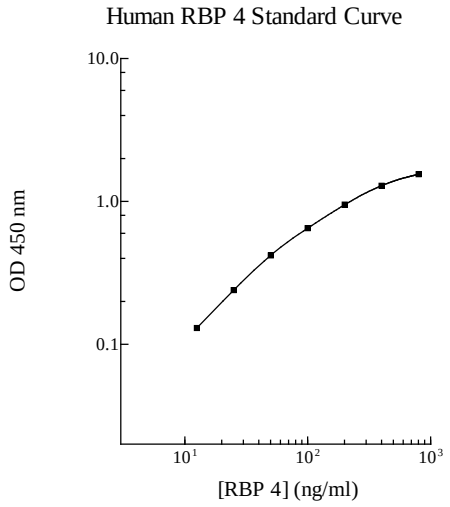
- Add 50 µl of Streptavidin-Peroxidase Conjugate per well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash a microplate as described above.
- Add 50 µl of Chromogen Substrate per well and incubate for about 10 minutes or till the optimal blue color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 µl of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a Standard Curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.



Performance Characteristics

- The minimum detectable dose of RBP 4 is typically 6 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 4.5 % and 7.0% respectively.

Linearity

	Average Percentage of Expected Value	
Sample Dilution	**Plasma**	**Serum**
1:1000	92%	91%
1:2000	109%	110%

	Average Percentage of Expected Value		
Sample Dilution	**Urine**	**Saliva**	**Milk**
No dilution	98%	95%	96%
1:2	97%	94%	97%

Recovery

Standard Added Value	10 – 100 ng/ml
Recovery %	83-109 %
Average Recovery %	99.5 %

Cross-Reactivity

Species	% Cross Reactivity
Beagle	<1
Bovine	None
Monkey	< 10
Mouse	<2
Rat	None
Swine	None
Rabbit	None

- 10% FBS in culture media will not affect the assay.

References

(1) Mody N *et al.* (2008) *Am. J. Physiol Endocrinol Metab*. 294(4): E785-793
(2) Meigs JB *et al.* (2002) *Diabetes* 51:833–840
(3) Duggirala R *et al.* (1998) *Diabetes* 47 (Suppl. 1): A170
(4) Yang Q *et al.* (2005) Nature 436 (7049): 356-362
(5) Graham T.E. *et al.* (2006) *N. Engl. J. Med.* 354:2552-2563
(6) McTernan PG *et al.* (2007*) J. Clin. Endocrinol. Metab*. 92:2430 –2432
(7) Cabre A *et al.* (2007) J. Intern Med. 262(4): 496-503

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