AssayMax™

Human RBP4 ELISA Kit

Assaypro LLC
3400 Harry S Truman Blvd
St. Charles, MO 63301
T (636) 447-9175
F (636) 395-7419
www.assaypro.com

For any questions regarding troubleshooting or performing the assay, please contact our support team at support@assaypro.com.

Thank you for choosing Assaypro.

Assay Summary

Step 1. Add 50 µl of Standard or Sample per well.
Incubate 2 hours.

Step 2. Wash, then add 50 µl of Biotinylated Antibody per well.
Incubate 1 hour.

Step 3. Wash, then add 50 µl of SP Conjugate per well.
Incubate 30 minutes.

Step 4. Wash, then add 50 µl of Chromogen Substrate per well.
Incubate 12 minutes.

Step 5. Add 50 µl of Stop Solution per well.
Read at 450 nm immediately.

Symbol Key

Consult instructions for use.

Assay Template

	A	B	C	D	E	F	G	H
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								

AssayMax™ Human Retinol-Binding Protein 4 (RBP4) ELISA Kit

Catalog No. ER3005-1

Sample insert for reference use only

Introduction

Serum retinol-binding protein (RBP4), secreted by the liver and adipocytes, is implicated in systemic insulin resistance. RBP4 transports retinol and circulates in the plasma by binding to the larger transthyretin (TTR) homotetramer, forming a protein complex that reduces renal clearance of RBP4. In insulin-resistant ob/ob mice, urinary fractional excretion of RBP4 was reduced, which is consistent with increased retention, while TTR level was elevated (1). RBP4 is encoded by the *RBP4* gene, which maps to chromosome 10q23-q24, and is linked to increased risk for type 2 diabetes in different populations (2-3). Transgenic overexpression of RBP4 or injection of recombinant RBP4 in normal mice causes insulin resistance. Conversely, genetic deletion of RBP4 enhances insulin sensitivity. Increasing serum RBP4 induces hepatic expression of the gluconeogenic enzyme, phosphoenolpyruvate carboxykinase, and impairs insulin signaling in muscle tissue (4). Expression of RBP4 is induced in adipose tissue as a consequence of decreased glucose transporter (GLUT4) expression. Increased human serum RBP4 is associated with insulin resistance, type 2 diabetes, and metabolic syndrome, such as obesity, glucose intolerance, dyslipidemia, and hypertension (5-6). Human plasma RBP4 concentration might be a biomarker of nephropathy and cardiovascular disease in type 2 diabetic subjects (7).

Principle of the Assay

The AssayMax™ Human RBP4 ELISA (Enzyme-Linked Immunosorbent Assay) Kit is designed for detection of RBP4 in human **plasma, serum, milk, urine, saliva, CSF, cell culture, cell lysate, and tissue samples**. This assay employs a quantitative **sandwich enzyme immunoassay** technique that measures human RBP4 in approximately 4 hours. A polyclonal antibody specific for human RBP4 has been pre-coated onto a 96-well microplate with removable strips. RBP4 in standards and samples is sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for human RBP4, which is recognized by a streptavidin-peroxidase (SP) conjugate. All unbound material is 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 product is for **Research Use Only** and is not intended for use in diagnostic procedures.
- Prepare all reagents (diluent buffer, wash buffer, standard, biotinylated antibody, and SP conjugate), as instructed, prior to running the assay.
- Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this insert. However, the user should determine the optimal dilution factor.
- Spin down the SP conjugate vial and the biotinylated antibody vial before opening and using contents.
- The Stop Solution is an acidic solution.
- The kit should not be used beyond the expiration date.

Reagents

- **Human RBP4 Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against human RBP4.
- **Sealing Tapes:** Each kit contains 3 precut, pressure sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Human RBP4 Standard:** Human RBP4 in a buffered protein base (720 ng, lyophilized).
- **Biotinylated Human RBP4 Antibody (40x):** A 40-fold concentrated biotinylated polyclonal antibody against human RBP4 (150 µl).
- **MIX Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x):** A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **SP Conjugate (100x):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate (1x):** A stabilized peroxidase chromogen substrate tetramethylbenzidine (7 ml).
- **Stop Solution (1x):** A 0.5 N hydrochloric acid solution to stop the chromogen substrate reaction (11 ml).

Storage Condition

- Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.
- Store Standard, SP Conjugate, and Biotinylated Antibody at -20°C.
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8°C.
- Unused microplate wells may be returned to the foil pouch with the desiccant packs and resealed. May be stored for up to 30 days in a vacuum desiccator.

Other Supplies Required

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

Sample Collection, Preparation, and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3000 x *g* for 10 minutes and collect plasma. A 500-fold sample dilution is suggested into MIX Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles (EDTA or Heparin can also be used as an anticoagulant).
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x *g* for 10 minutes and remove serum. A 500-fold sample dilution is suggested into MIX Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Milk:** Collect milk using sample tube. Centrifuge samples at 800 x *g* for 10 minutes. A 2-fold sample dilution is suggested into MIX Diluent or within the range of 1x – 10x; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Urine:** Collect urine using sample pot. Centrifuge samples at 800 x *g* for 10 minutes. The sample is suggested for use at 1x or within the range of 2x – 10x into MIX Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Saliva:** Collect saliva using sample tube. Centrifuge samples at 800 x *g* for 10 minutes. The sample is suggested for use at 1x or within the range of 2x – 10x into MIX Diluent; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **CSF:** Collect cerebrospinal fluid (CSF) using sample pot. Centrifuge samples at 3000 x *g* for 10 minutes. A 2-fold sample dilution is suggested into MIX Diluent or within the range of 1x – 10x; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -80°C for up to 3 months. Avoid repeated freeze-thaw cycles.

- **Cell Culture Supernatant:** Centrifuge cell culture media at 1500 rpm for 10 minutes at 4°C to remove debris and collect supernatant. If necessary, dilute samples into MIX Diluent; user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -80°C. Avoid repeated freeze-thaw cycles.
- **Cell Lysate:** Rinse cell with cold PBS and then scrape the cell into a tube with 5 ml of cold PBS and 0.5 M EDTA. Centrifuge suspension at 1500 rpm for 10 minutes at 4°C and aspirate supernatant. Resuspend pellet in ice-cold Lysis Buffer (PBS, 1% Triton X-100, protease inhibitor cocktail). For every 1 x 10^6 cells, add approximately 100 µl of ice-cold Lysis Buffer. Incubate on ice for 60 minutes. Centrifuge at 13000 rpm for 30 minutes at 4°C and collect supernatant. If necessary, dilute samples into MIX Diluent; user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -80°C. Avoid repeated freeze-thaw cycles.
- **Tissue:** Extract tissue samples with 0.1 M phosphate-buffered saline (pH 7.4) containing 1% Triton X-100 and centrifuge at 14000 x *g* for 20 minutes. Collect the supernatant and measure the protein concentration. If necessary, dilute samples into MIX Diluent; user should determine optimal dilution factor depending on application needs. Store remaining extract at -80°C. Avoid repeated freeze-thaw cycles.

Applicable samples may also include biofluids, cell culture, and tissue homogenates. If necessary, user should determine optimal dilution factor depending on application needs.

Refer to Dilution Guidelines for further instruction.

Guidelines for Dilutions of 100-fold or Greater (*for reference only; please follow the insert for specific dilution suggested)*	
100x	**10000x**
A) 4 µl sample : 396 µl buffer (100x) = 100-fold dilution *Assuming the needed volume is less than or equal to 400 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) = 10000-fold dilution *Assuming the needed volume is less than or equal to 400 µl.*
1000x	**100000x**
A) 4 µl sample : 396 µl buffer (100x) B) 24 µl of A : 216 µl buffer (10x) = 1000-fold dilution *Assuming the needed volume is less than or equal to 240 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) C) 24 µl of B : 216 µl buffer (10x) = 100000-fold dilution *Assuming the needed volume is less than or equal to 240 µl.*

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **MIX Diluent Concentrate (10x):** Dilute the MIX Diluent Concentrate 10-fold with reagent grade water to produce a 1x solution. When diluting the concentrate, make sure to rinse the bottle thoroughly to extract any precipitates left in the bottle. Mix the 1x solution gently until the crystals have completely dissolved. Store for up to 30 days at 2-8°C.
- **Human RBP4 Standard:** Reconstitute the Human RBP4 Standard (720 ng) with 0.9 ml of MIX Diluent to generate an 800 ng/ml standard stock solution. Allow the vial to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting from the standard stock solution (800 ng/ml) 2-fold with equal volume of MIX Diluent to produce 400, 200, 100, 50, 25, and 12.5 ng/ml solutions. MIX Diluent serves as the zero standard (0 ng/ml). Aliquot remaining stock solution to limit repeated freeze-thaw cycles. This solution should be stored at -20°C and **used within 30 days**.

Standard Point	Dilution	[RBP4] (ng/ml)
P1	1 part Standard (800 ng/ml)	800
P2	1 part P1 + 1 part MIX Diluent	400
P3	1 part P2 + 1 part MIX Diluent	200
P4	1 part P3 + 1 part MIX Diluent	100
P5	1 part P4 + 1 part MIX Diluent	50
P6	1 part P5 + 1 part MIX Diluent	25
P7	1 part P6 + 1 part MIX Diluent	12.5
P8	MIX Diluent	0.0

- **Biotinylated Human RBP4 Antibody (40x):** Spin down the antibody briefly and dilute the desired amount of the antibody 40-fold with MIX Diluent to produce a 1x solution. The undiluted antibody should be stored at -20°C.
- **Wash Buffer Concentrate (20x):** Dilute the Wash Buffer Concentrate 20-fold with reagent grade water to produce a 1x solution. When diluting the concentrate, make sure to rinse the bottle thoroughly to extract any precipitates left in the bottle. Mix the 1x solution gently until the crystals have completely dissolved.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 100-fold with MIX Diluent to produce a 1x solution. The undiluted conjugate should be stored at -20°C.

Assay Procedure

- Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-25°C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccants inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Human RBP4 Standard or sample to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 2 hours. Start the timer after the last addition.
- Wash the microplate manually or automatically using a microplate washer. Invert the plate and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If washing manually, wash five times with 200 µl of Wash Buffer per well. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a microplate washer, wash six times with 300 µl of Wash Buffer per well; invert the plate and hit 4-5 times on absorbent material to completely remove the liquid.
- Add 50 µl of Biotinylated Human RBP4 Antibody to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 1 hour.
- Wash the microplate as described above.
- Add 50 µl of SP Conjugate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 µl of Chromogen Substrate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Incubate in ambient light for 12 minutes or until the optimal blue color density develops.
- Add 50 µl of Stop Solution to each well. The color will change from blue to yellow. Gently tap plate to ensure thorough mixing. Break any bubbles that may have formed.
- 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 (OD) 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.

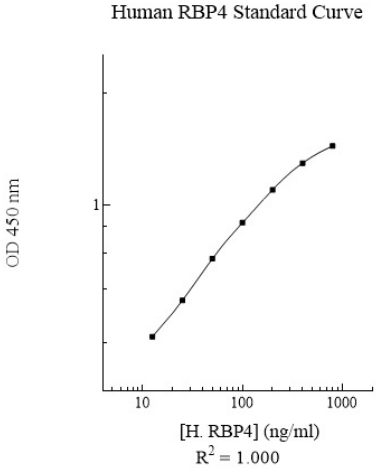
Typical Data

- The typical data is provided for reference only. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences.

Standard Point	ng/ml	OD	Average OD
P1	800	1.743 1.713	1.728
P2	400	1.437 1.513	1.475
P3	200	1.130 1.176	1.153
P4	100	0.866 0.834	0.850
P5	50	0.630 0.590	0.610
P6	25	0.434 0.396	0.415
P7	12.5	0.308 0.286	0.297
P8	0.0	0.203 0.193	0.198
Sample: Pooled Normal Sodium Citrate Plasma (500x)		0.667 0.625	0.646
Sample: Pooled Normal Serum (500x)		0.673 0.627	0.650

Standard Curve

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



Reference Value

- Normal human RBP4 plasma and serum levels range from 20 – 35 µg/ml.
- Plasma and serum samples from healthy adults were tested (n=40). On average, human RBP4 level was 29.9 µg/ml.

Sample	n	Average Value (µg/ml)
Pooled Normal Plasma	10	29.9
Normal Plasma	20	29.5
Pooled Normal Serum	10	30.3

Performance Characteristics

- The minimum detectable dose of human RBP4 as calculated by 2SD from the mean of a zero standard was established to be 8.3 ng/ml.
- Intra-assay precision was determined by testing three plasma samples twenty times in one assay.
- Inter-assay precision was determined by testing three plasma samples in twenty assays.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
CV (%)	3.3%	5.3%	2.9%	8.5%	9.4%	7.7%
Average CV (%)	3.8%			8.5%		

Recovery

Standard Added Value	25 – 200 ng/ml
Recovery %	90 – 113%
Average Recovery %	97%

Cross-Reactivity

Species	**Cross-Reactivity (%)**
Canine	2%
Bovine	None
Equine	None
Monkey	20%
Mouse	5%
Rat	None
Swine	None
Rabbit	None
Protein	**Cross-Reactivity (%)**
RBP	100%
RBP2	None
RBP7	None

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

Troubleshooting

Issue	**Causes**	**Course of Action**
Low Precision	Use of improper components	• Check the expiration date listed before use. • Do not interchange components from different lots.
	Improper wash step	• Check that the correct wash buffer is being used. • Check that all wells are empty after aspiration. • Check that the microplate washer is dispensing properly. • If washing by pipette, check for proper pipetting technique.
	Splashing of reagents while loading wells	• Pipette properly in a controlled and careful manner.
	Inconsistent volumes loaded into wells	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	• Thoroughly agitate the lyophilized components after reconstitution. • Thoroughly mix dilutions.
	Improperly sealed microplate	• Check the microplate pouch for proper sealing. • Check that the microplate pouch has no punctures. • Check that three desiccants are inside the microplate pouch prior to sealing.

Unexpectedly Low or High Signal Intensity	Microplate was left unattended between steps	• Each step of the procedure should be performed uninterrupted.
	Omission of step	• Consult the provided procedure for complete list of steps.
	Steps performed in incorrect order	• Consult the provided procedure for the correct order.
	Insufficient amount of reagents added to wells	• Check pipette calibration. • Check pipette for proper performance.
	Wash step was skipped	• Consult the provided procedure for all wash steps.
	Improper wash buffer	• Check that the correct wash buffer is being used.
	Improper reagent preparation	• Consult reagent preparation section for the correct dilutions of all reagents.
	Insufficient or prolonged incubation periods	• Consult the provided procedure for correct incubation time.
Deficient Standard Curve Fit	Non-optimal sample dilution	• Sandwich ELISA: If samples generate OD values higher than the highest standard point (P1), dilute samples further and repeat the assay. • Competitive ELISA: If samples generate OD values lower than the highest standard point (P1), dilute samples further and repeat the assay. • User should determine the optimal dilution factor for samples.
	Contamination of reagents	• A new tip must be used for each addition of different samples or reagents during the assay procedure.
	Contents of wells evaporate	• Verify that the sealing film is firmly in place before placing the assay in the incubator or at room temperature.
	Improper pipetting	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	• Thoroughly agitate the lyophilized components after reconstitution. • Thoroughly mix dilutions.

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 TE *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.

Version 4.3R1