ab108897 – Retinol binding protein 4 (RBP4) Human ELISA Kit

Instructions for Use

For the quantitative measurement of Human Retinol binding protein 4 (RBP4) in plasma, serum, saliva, urine, milk, cerebrospinal fluid and cell culture supernatants.

This product is for research use only and is not intended for diagnostic use.

Version 5 Last Updated 11 January 2019

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1. BACKGROUND

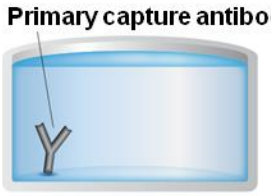
Abcam's Retinol binding protein 4 (RBP4) Human *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of Retinol binding protein 4 concentrations in plasma, serum, saliva, urine, milk, cerebrospinal fluid and cell culture supernatants.

A Retinol binding protein 4 specific antibody has been precoated onto 96-well plates and blocked. Standards or test samples are added to the wells and subsequently a Retinol binding protein 4specific biotinylated detection antibody is added and then followed by washing with wash buffer. Streptavidin-Peroxidase Conjugate is added and unbound conjugates are washed away with wash buffer. TMB is then used to visualize Streptavidin-Peroxidase enzymatic reaction. TMB is catalyzed by Streptavidin-Peroxidase to produce a blue color product that changes into yellow after adding acidic stop solution. The density of yellow coloration is directly proportional to the amount of Retinol binding protein 4 captured in plate.

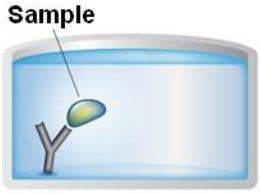
Serum retinol-binding protein (RBP 4) is secreted by liver and adipocytes and is implicated in systemic insulin resistance. RBP 4 transports retinol and circulates in the plasma by binding to the larger transthyretin (TTR) homotetramer, forming a protein complex that reduces renal clearance of RBP 4. In insulin-resistant ob/ob mice, urinary fractional excretion of RBP 4 was reduced, consistent with increased retention; while TTR level is elevated. 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. 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. 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. Human plasma RBP 4 concentration might be a biomarker of nephropathy and cardiovascular disease in type 2 diabetic subjects.

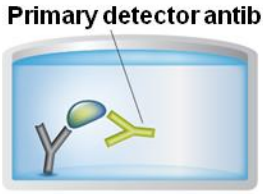
2. ASSAY SUMMARY



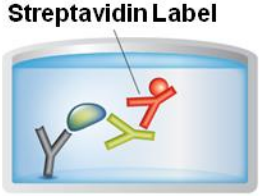
Prepare all reagents, samples and standards as instructed.



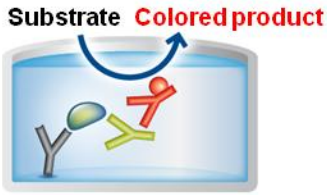
Add standard or sample to each well used. Incubate at room temperature.



Wash and add prepared biotin antibody to each well. Incubate at room temperature.



Wash and add prepared Streptavidin-Peroxidase Conjugate. Incubate at room temperature.



Add Chromogen Substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at 4°C immediately upon receipt, apart from the SP Conjugate & Biotinylated Antibody, which should be stored at -20°C.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in sections 9 & 10.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Retinol binding protein 4 Microplate (12 x 8 well strips)	96 wells	4°C
Retinol binding protein 4 Standard	1 vial	4°C
10X Diluent N Concentrate	30 mL	4°C
Biotinylated Human Retinol binding protein 4 Antibody	1 vial	-20°C
100X Streptavidin-Peroxidase Conjugate (SP Conjugate)	80 µL	-20°C
Chromogen Substrate	8 mL	4°C
Stop Solution	12 mL	4°C
20X Wash Buffer Concentrate	2 x 30 mL	4°C
Sealing Tapes	3	N/A

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- 1 Microplate reader capable of measuring absorbance at 450 nm.
- Precision pipettes to deliver 1 µL to 1 mL volumes.
- Adjustable 1-25 mL pipettes for reagent preparation.
- 100 mL and 1 liter graduated cylinders.
- Absorbent paper.
- Distilled or deionized water.
- Log-log graph paper or computer and software for ELISA data analysis.
- 8 tubes to prepare standard or sample dilutions.

7. LIMITATIONS

- Do not mix or substitute reagents or materials from other kit lots or vendors.

8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps.
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. REAGENT PREPARATION

Equilibrate all reagents to room temperature (18-25°C) prior to use. Prepare fresh reagents immediately prior to use. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

9.1 **1X Diluent N**

Dilute the 10X Diluent N Concentrate 1:10 with reagent grade water. Mix gently and thoroughly. *Store for up to 1 month at 4°C.*

9.2 **1X Wash Buffer**

Dilute the 20X Wash Buffer Concentrate 1:20 with reagent grade water. Mix gently and thoroughly.

9.3 **1X Biotinylated Retinol binding protein 4 Detector Antibody**

9.3.1 The stock Biotinylated Retinol binding protein 4 Antibody must be diluted with 1X Diluent N according to the label concentration to prepare 1X Biotinylated Retinol binding protein 4 Antibody for use in the assay procedure. Observe the label for the "X" concentration on the vial of Biotinylated Retinol binding protein 4 Antibody.

9.3.2 Calculate the necessary amount of 1X Diluent N to dilute the Biotinylated Retinol binding protein 4 Antibody to prepare a 1X Biotinylated Retinol binding protein 4 Antibody solution for use in the assay procedure according to how many wells you wish to use and the following calculation:

Number of Wells Strips	Number of Wells	(V_T) Total Volume of 1X Biotinylated Antibody (µL)
4	32	1,760
6	48	2,640
8	64	3,520
10	80	4,400
12	96	5,280

Any remaining solution should be frozen at -20°C.

Where:

C_S = Starting concentration (X) of stock Biotinylated Retinol binding protein 4 Antibody (variable)

C_F = Final concentration (always = 1X) of 1X Biotinylated Retinol binding protein 4 Antibody solution for the assay procedure

V_T = Total required volume of 1X Biotinylated Retinol binding protein 4 Antibody solution for the assay procedure

V_A = Total volume of (X) stock Biotinylated Retinol binding protein 4 Antibody

V_D = Total volume of 1X Diluent N required to dilute (X) stock Biotinylated Retinol binding protein 4 Antibody to prepare 1X Biotinylated Retinol binding protein 4 solution for assay procedures

Calculate the volume of (X) stock Biotinylated Antibody required for the given number of desired wells:

$$(C_F / C_S) \times V_T = V_A$$

Calculate the final volume of 1X Diluent N required to prepare the 1X Biotinylated Retinol binding protein 4 Antibody:

$$V_T - V_A = V_D$$

Example:

NOTE: This example is for demonstration purposes only. Please remember to check your antibody vial for the actual concentration of antibody provided.

C_S = 50X Biotinylated Retinol binding protein 4 Antibody stock

C_F = 1X Biotinylated Retinol binding protein 4 Antibody solution for use in the assay procedure

V_T = 3,520 µL (8 well strips or 64 wells)

$$(1X/50X) \times 3{,}520\ \mu L = 70.4\ \mu L$$

$$3{,}520\ \mu L - 70.4\ \mu L = 3{,}449.6\ \mu L$$

V_A = 70.4 µL total volume of (X) stock Biotinylated Retinol binding protein 4 Antibody required

V_D = 3,449.6 µL total volume of 1X Diluent N required to dilute the Retinol binding protein 4 50X stock Biotinylated Antibody to prepare 1X Biotinylated Antibody solution for assay procedures

9.3.3 First spin the Biotinylated Retinol binding protein 4 Antibody vial to collect the contents at the bottom.

9.3.4 Add calculated amount V_A of stock Biotinylated Retinol binding protein 4 Antibody to the calculated amount V_D of 1X Diluent N. Mix gently and thoroughly.

9.4 **1X SP Conjugate**

Spin down the 100X Streptavidin-Peroxidase Conjugate (SP Conjugate) briefly and dilute the desired amount of the conjugate 1:100 with 1X Diluent N.

Any remaining solution should be frozen at -20°C.

10. STANDARD PREPARATIONS

- **Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use.**
- **Any remaining standard should be stored at -20°C after reconstitution and used within 30 days.**
- **This procedure prepares sufficient standard dilutions for duplicate wells.**

10.1 Reconstitution of the Retinol binding protein 4 Standard vial to prepare the 400 ng/mL Retinol binding protein 4 **Standard #1**:

10.1.1 First consult the Retinol binding protein 4 Standard vial to determine the mass of protein in the vial.

10.1.2 Calculate the appropriate volume of 1X Diluent N to add when resuspending the Retinol binding protein 4 Standard vial to produce a 400 ng/mL Retinol binding protein 4 **Standard #1** by using the following equation:

C_S = Starting mass of Retinol binding protein 4 Standard (see vial label) (ng)

C_F = 400 ng/mL Retinol binding protein 4 **Standard #1** final required concentration

V_D = Required volume of 1X Diluent N for reconstitution (µL)

Calculate total required volume 1X Diluent N for resuspension:

$$(C_S / C_F) \times 1{,}000 = V_D$$

Example:

NOTE: This example is for demonstration purposes only. Please remember to check your standard vial for the actual amount of standard provided.

C_S = 1,000 ng of Retinol binding protein 4 Standard in vial

C_F = 400 ng/mL Retinol binding protein 4 **Standard #1** final concentration

V_D = Required volume of 1X Diluent N for reconstitution

(1,000 ng / 400 ng/mL) x 1,000 = 2,500 µL

10.1.3 First briefly spin the Retinol binding protein 4 Standard Vial to collect the contents on the bottom of the tube.

10.1.4 Reconstitute the Retinol binding protein 4 Standard vial by adding the appropriate calculated amount V_D of 1X Diluent N to the vial to generate the 400 ng/mL Retinol binding protein 4 **Standard #1**. Mix gently and thoroughly.

10.2 Allow the reconstituted 400 ng/mL Retinol binding protein 4 **Standard #1** to sit for 10 minutes with gentle agitation prior to making subsequent dilutions

10.3 Label seven tubes #2 – 8.

10.4 Add 120 µL of 1X Diluent N to tube #2 –8.

10.5 To prepare **Standard #2**, add 120 µL of the **Standard #1** into tube #2 and mix gently.

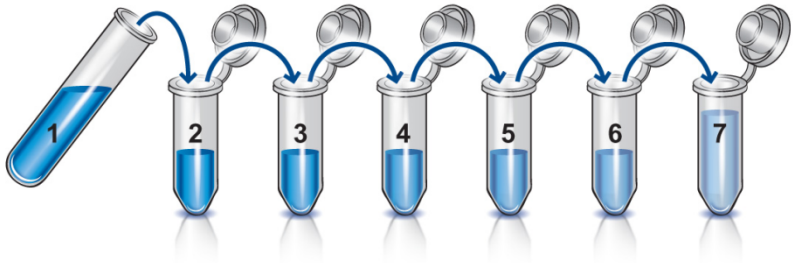
10.6 To prepare **Standard #3**, add 120 µL of the **Standard #2** into tube #3 and mix gently.

10.7 Using the table below as a guide, prepare subsequent serial dilutions.

10.8 1X Diluent N serves as the zero standard, 0 ng/mL (tube #8).

Standard Dilution Preparation Table

Standard #	Volume to Dilute (µL)	Volume Diluent N (µL)	Total Volume (µL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Step 10.1				400.0
2	120	120	240	400.0	200.0
3	120	120	240	200.0	100.0
4	120	120	240	100.0	50.00
5	120	120	240	50.00	25.00
6	120	120	240	25.00	12.50
7	120	120	240	12.50	6.250
8	-	120	120	-	0



11. SAMPLE PREPARATION

11.1 Plasma

Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 2,000 x *g* for 10 minutes. Dilute samples 1:500 into 1X Diluent N (or within the range 1:100 and 1:1000) and assay. 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 anticoagulant.)

11.2 Cell Culture Supernatants

Centrifuge cell culture media at 2,000 x *g* for 10 minutes to remove debris. Collect supernatants and assay. Store samples at -20°C or below. Avoid repeated freeze-thaw cycles.

11.3 Serum

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x *g* for 10 minutes and remove serum. Dilute samples 1:500 into 1X Diluent N (or within the range 1:100 and 1:1000) and assay. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.4 Saliva

Collect saliva using sample tube. Centrifuge samples at 600 x *g* for 10 minutes and assay. Store samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.5 Milk

Collect milk using sample tube. Centrifuge samples at 600 x *g* for 10 minutes. Dilute samples 1:2 into 1X Diluent N and assay. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.6 **Urine**

Collect urine using sample pot. Centrifuge samples at 600 x *g* for 10 minutes and assay. Store samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.7 **Cerebrospinal Fluid**

Collect cerebrospinal fluid using sample pot. Centrifuge samples at 3,000 x *g* for 10 minutes. Dilute samples 1:2 into 1X Diluent N and assay. The undiluted samples can be stored at -80°C for up to 3 months. Avoid repeated freeze-thaw cycles.

12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused well plate strips should be returned to the plate packet and stored at 4°C.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Well effects have not been observed with this assay. Contents of each well can be recorded on the template sheet included in the Resources section.

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature (18 - 25°C) prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

13.1 Prepare all reagents, working standards and samples as instructed. Equilibrate reagents to room temperature before use. The assay is performed at room temperature (18-25°C).

13.2 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.

13.3 Add 50 µL of Retinol Sbinding protein 4 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 two hours. Start the timer after the last sample addition.

13.4 Wash five times with 200 µL of 1X Wash Buffer manually. Invert the plate each time and decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 µL of 1X Wash Buffer and then invert the plate, decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid.

13.5 Add 50 µL of 1X Biotinylated Retinol binding protein 4 Antibody to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed and incubate for one hour.

13.6 Wash microplate as described above.

13.7 Add 50 µL of 1X SP Conjugate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed, Cover wells with sealing tape and

incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.

13.8 Wash microplate as described above.

13.9 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 for 15 minutes or till the optimal blue colour density develops.

13.10 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.

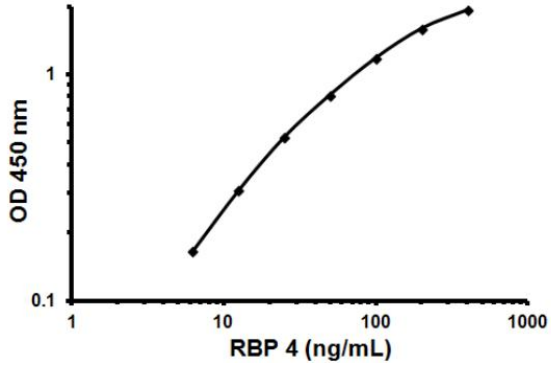
13.11 Read the absorbance on a microplate reader at a wavelength of 450 nm immediately. If wavelength correction is available, subtract readings at 570 nm from those at 450°nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

14. CALCULATIONS

Calculate the mean value of the 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.

15. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.



16. TYPICAL SAMPLE VALUES

SENSITIVITY –

The minimum detectable dose of Retinol binding protein 4 is typically 3.4 ng/mL.

RECOVERY –

Standard Added Value: 12.5 – 100 ng/mL

Recovery %: 87 – 110.

Average Recovery %: 95

LINEARITY OF DILUTION –

Plasma Dilution	Average % Expected Value
1:500	86
1:1,000	97
1:2,000	106

Serum Dilution	Average % Expected Value
1:500	87
1:1,000	96
1:2,000	107

Saliva Dilution	Average % Expected Value
No Dilution	88
1:2	94

Urine Dilution	Average % Expected Value
No Dilution	92
1:2	97

Milk Dilution	Average % Expected Value
No Dilution	91
1:2	97

PRECISION –

	Intra-Assay	Inter-Assay
% CV	4.8	9.0

17. ASSAY SPECIFICITY

Species	% Cross Reactivity
Beagle	None
Bovine	None
Monkey	None
Mouse	None
Rat	None
Swine	None
Rabbit	None

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

18. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Improper standard dilution	Confirm dilutions made correctly
	Standard improperly reconstituted (if applicable)	Briefly spin vial before opening; thoroughly resuspend powder (if applicable)
	Standard degraded	Store sample as recommended
	Curve doesn't fit scale	Try plotting using different scale
Low signal	Incubation time too short	Try overnight incubation at 4ºC
	Target present below detection limits of assay	Decrease dilution factor; concentrate samples
	Precipitate can form in wells upon substrate addition when concentration of target is too high	Increase dilution factor of sample
	Using incompatible sample type (e.g. serum vs. cell extract)	Detection may be reduced or absent in untested sample types
	Sample prepared incorrectly	Ensure proper sample preparation/dilution
Large CV	Bubbles in wells	Ensure no bubbles present prior to reading plate
	All wells not washed equally/thoroughly	Check that all ports of plate washer are unobstructed wash wells as recommended
	Incomplete reagent mixing	Ensure all reagents/master mixes are mixed thoroughly
	Inconsistent pipetting	Use calibrated pipettes and ensure accurate pipetting
	Inconsistent sample preparation or storage	Ensure consistent sample preparation and optimal sample storage conditions (eg. minimize freeze/thaws cycles)

Problem	Cause	Solution
High background/ Low sensitivity	Wells are insufficiently washed	Wash wells as per protocol recommendations
	Contaminated wash buffer	Make fresh wash buffer
	Waiting too long to read plate after adding STOP solution	Read plate immediately after adding STOP solution
	Improper storage of ELISA kit	Store all reagents as recommended. Please note all reagents may not have identical storage requirements.
	Using incompatible sample type (e.g. Serum vs. cell extract)	Detection may be reduced or absent in untested sample types

19. NOTES

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