ab195459 –

Mouse Retinol binding protein 4 ELISA Kit (RBP4)

Instructions for Use

For the quantitative measurement of mouse RBP4 in plasma, serum, urine, cell lysates, tissue samples and cell culture supernatants.

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

Version 4e Last Updated 19 September 2023

Table of Contents

1. BACKGROUND

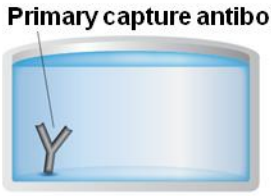
Abcam's Retinol binding protein 4 (RBP4) Mouse *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of RBP4 concentrations in mouse plasma, serum, urine, cell lysates, and cell culture supernatants.

An RBP4 specific antibody has been precoated onto 96-well plates and blocked. Standards or test samples are added to the wells and subsequently an RBP4 specific 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 RBP4 captured in plate.

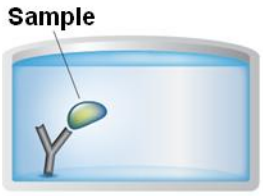
Serum retinol-binding protein (RBP4) is secreted by the liver and adipocytes and is implicated in systemic insulin resistance. RBP4 transports retinol and circulates in the plasma by binding to the larger transthyretin (TTR) homotetramer, forming a protein complex that reduces renal clearance of RBP4. In insulin-resistant ob/ob mice, urinary fractional excretion of RBP4 was reduced, consistent with increased retention; while TTR level is elevated. RBP4 is encoded by the RBP4 gene that maps to chromosome 10q23-q24 linked to increased risk for type 2 diabetes in different populations. 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. Expression of RBP4 is induced in adipose tissue as a consequence of decreased glucose transporter GLUT4 expression. Increased serum RBP4 is associated with insulin resistance, Type II diabetes, and metabolic syndrome such as obesity, glucose intolerance,

dyslipidemia, and hypertension. Plasma RBP4 concentration might be a biomarker of nephropathy and cardiovascular disease in type 2 diabetic subjects.

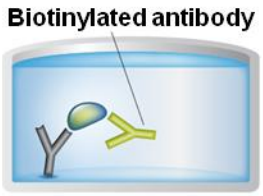
2. ASSAY SUMMARY



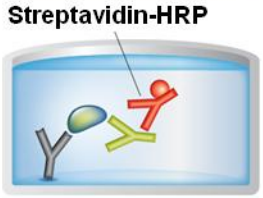
Prepare all reagents, samples and standards as instructed.



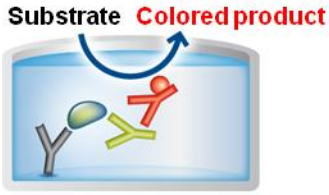
Add standard or sample to each well used. Incubate at room temperature.



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.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at 2-8ºC 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 section 12. Reagent Preparation.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Mouse RBP4 Microplate (12 x 8 well strips)	96 wells	2-8°C
Mouse RBP4 Standard (Lyophilized)	1 vial	2-8°C
10X Diluent N Concentrate	30 mL	2-8°C
Biotinylated Mouse RBP4 Antibody	1 vial	-20°C
100X Streptavidin-Peroxidase Conjugate (SP Conjugate)	80 µL	-20°C
Chromogen Substrate	7 mL	2-8°C
Stop Solution	11 mL	2-8°C
20X Wash Buffer Concentrate	2 x 30 mL	2-8°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.

For Cell lysate sample preparation:

- EDTA
- PBS
- Lysis Buffer (10 mM Tris pH 8.0, 130 mM NaCl, 1% Triton X-100, protease inhibitor cocktail)

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. When diluting the concentrates, 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.

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 2 - 8°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 RBP4 Detector Antibody

9.3.1 The stock Biotinylated RBP4 Antibody must be diluted with 1X Diluent N according to the label concentration to prepare 1X Biotinylated RBP4 Detector Antibody for use in the assay procedure. Observe the label for the "X" concentration on the vial of Biotinylated RBP4 Antibody.

9.3.2 Calculate the necessary volume of 1X Diluent N to dilute the Biotinylated RBP4 Antibody to prepare a 1X Biotinylated RBP4 Detector 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 Detector 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 RBP4 Antibody (variable)

C_F = Final concentration (always = 1X) of 1X Biotinylated RBP4 Detector Antibody solution for the assay procedure

V_T = Total required volume of 1X Biotinylated RBP4 Detector Antibody solution for the assay procedure

V_A = Total volume of (X) stock Biotinylated RBP4 Antibody

V_D = Total volume of 1X Diluent N required to dilute (X) stock Biotinylated RBP4 Antibody to prepare 1X Biotinylated Detector Antibody 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 RBP4 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 = 70X Biotinylated RBP4 Antibody stock

C_F = 1X Biotinylated RBP4 Detector Antibody solution for use in the assay procedure

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

$$(1X/70X) \times 3{,}520\ \mu L = 50.29\ \mu L$$

$$3{,}520\ \mu L - 50.29\ \mu L = 3{,}469.71\ \mu L$$

V_A = 50.29µL total volume of (X) stock Biotinylated RBP4 Antibody required

V_D = 3,469.71 µL total volume of 1X Diluent N required to dilute the 70X stock Biotinylated Antibody to prepare 1X Biotinylated RBP4 Detector Antibody solution for assay procedures

9.3.3 First spin the Biotinylated RBP4 Antibody vial to collect the contents at the bottom.

9.3.4 Add calculated amount V_A of stock Biotinylated RBP4 Antibody to the calculated amount V_D of 1X Assay 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.**
- **Avoid repeated freeze-thaw cycles.**
- **This procedure prepares sufficient standard dilutions for duplicate wells.**

10.1 Reconstitution of the stock RBP4 Standard vial to prepare a 40 ng/mL RBP4 **Standard #1**:

10.1.1 First consult the RBP4 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 RBP4 Standard vial to produce a 40 ng/mL RBP4 Standard stock by using the following equation:

C_S = Starting mass of RBP4 Standard stock (see vial label) (ng)
C_F = 40 ng/mL RBP4 **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 = 60 ng of RBP4 Standard in vial

C_F = 40 ng/mL RBP4 **Standard #1** final concentration

V_D = Required volume of 1X Diluent N for reconstitution

(60 ng / 40 ng/mL) x 1,000 = 1500 µL

10.1.3 First briefly centrifuge the RBP4 Standard Vial to collect the contents on the bottom of the tube.

10.1.4 Reconstitute the RBP4 Standard vial by adding the appropriate calculated amount V_D of 1X Diluent N to the vial to generate the 40 ng/mL RBP4 **Standard #1**. Mix gently and thoroughly.

10.2 Allow the reconstituted 40 ng/mL RBP4 **Standard #1** to sit for 10 minutes with gentle agitation prior to making subsequent dilutions

10.3 Label six tubes #2 – 7.

10.4 Add 120 µL of 1X Diluent N to tubes #2 – 7.

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 blank control, 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				40
2	120	120	240	40.00	20
3	120	120	240	20.00	10
4	120	120	240	10.000	5
5	120	120	240	5.000	2.5
6	120	120	240	2.500	1.25
8	120	120	240	1.250	0.625
8 (Blank)	-	120	120	-	0

11. SAMPLE PREPARATION

11.1 Cell culture supernatants

Centrifuge cell culture media at 1500 rpm for 10 minutes at 4°C to remove debris and collect supernatants. If necessary, dilute samples into 1X Diluent N; user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -80°C. Avoid repeated freeze-thaw cycles.

11.2 Cell Lysates

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 1X Diluent N; user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -80°C. Avoid repeated freeze-thaw cycles.

11.3 Serum

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3,000 x *g* for 10 minutes and remove serum. Dilute samples 1:8,000 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.

11.4 Plasma

Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3,000 x *g* for 10 minutes. Dilute samples 1:8,000 into 1X Diluent N. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

11.5 **Urine**

Collect urine using sample pot. Centrifuge samples at 800 x g for 10 minutes. A 10-fold sample dilution is suggested into Diluent N or within the range of 1x – 100x; 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.

11.6 **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.

12. PLATE PREPARATION

- The 96 well plate strips included with this kit is 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 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 (20-30°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 Mouse RBP4 Standard or sample per well. 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 RBP4 Detector Antibody to each well and incubate for one hour.

13.6 Wash microplate as described above.

13.7 Add 50 µL of 1X SP Conjugate to each well 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 per well and incubate in ambient light for 30 minutes or until the optimal blue color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.

13.10 Add 50 µL of Stop Solution to each well. The color will change from blue to yellow.

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.

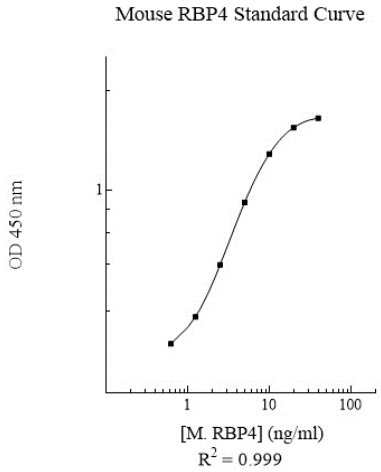


Figure 1. Example of RBP4 standard curve prepared as described in Section 10.

16. TYPICAL SAMPLE VALUES

SENSITIVITY -

The minimum detectable dose of mouse RBP4 is typically ~ 0.44 ng/mL.

RECOVERY –

Standard Added Value: 1.25 – 20 ng/mL

Recovery %: 88 – 116

Average Recovery %: 97

LINEARITY OF DILUTION -

Plasma Dilution	Average % Expected Value
1:4,000	90
1:8,000	97
1:16,000	105

Serum Dilution	Average % Expected Value
1:4,000	90
1:8,000	99
1:16,000	106

PRECISION –

	Intra-Assay	Inter-Assay
% CV	3.2	9.2

17. ASSAY SPECIFICITY

Species	% Cross Reactivity
Beagle	None
Bovine	None
Equine	None
Monkey	None
Rat	5
Swine	None
Human	None
Rabbit	None

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

18. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store the reconstituted protein at -80°C, all other assay components 4°C. Keep substrate solution protected from light.

19. NOTES

Technical Support

For all technical or commercial enquiries please go to:
www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)

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