



# **ab203362 – Retinol Binding Protein 4 (RBP4) Rat SimpleStep ELISA<sup>®</sup> Kit**

## Instructions for Use

For the quantitative measurement of Retinol Binding Protein 4 (RBP4) in rat serum, plasma, urine, and cell culture supernatant.

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

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## 1. BACKGROUND

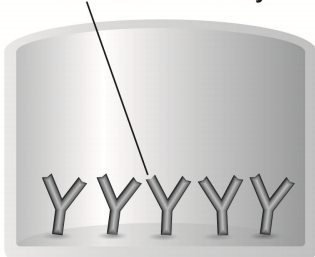
Abcam's Retinol Binding Protein 4 (RBP4) *in vitro* SimpleStep ELISA® (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of RBP4 protein in rat serum, plasma, urine, and cell culture supernatant.

The SimpleStep ELISA® employs an affinity tag labeled capture antibody and a reporter conjugated detector antibody which immunocapture the sample analyte in solution. This entire complex (capture antibody/analyte/detector antibody) is in turn immobilized via immunoaffinity of an anti-tag antibody coating the well. To perform the assay, samples or standards are added to the wells, followed by the antibody mix. After incubation, the wells are washed to remove unbound material. TMB Development Solution is added and during incubation is catalyzed by HRP, generating blue coloration. This reaction is then stopped by addition of Stop Solution completing any color change from blue to yellow. Signal is generated proportionally to the amount of bound analyte and the intensity is measured at 450 nm. Optionally, instead of the endpoint reading, development of TMB can be recorded kinetically at 600 nm.

Retinol-Binding Protein 4 (RBP4) is a small, highly conserved member of the lipocalin superfamily involved in the transportation of retinol (Vitamin A) in blood. Synthesized in hepatocytes and adipocytes, RBP4 is also an adipokine involved in insulin resistance in the AG4KO mouse model. Elevated serum levels of RBP4 are seen in obesity and Type 2 diabetes.

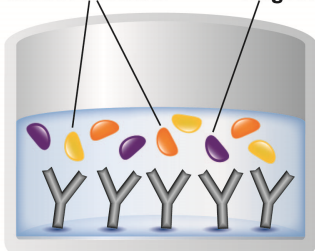
## 2. ASSAY SUMMARY

Immobilization Antibody



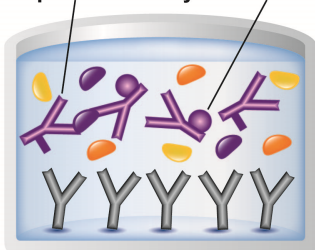
Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare all reagents, samples, and standards as instructed.

Matrix Proteins Target Analyte



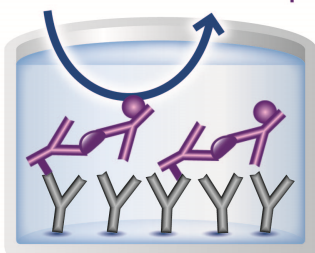
Add standard or sample to appropriate wells.

Capture Antibody Detector Antibody



Add Antibody Cocktail to all wells. Incubate at room temperature.

Substrate Color Development



Aspirate and wash each well. Add TMB Development Solution to each well and incubate. Add Stop Solution at a defined endpoint. Alternatively, record color development kinetically after TMB substrate addition.

## 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 2-8°C immediately upon receipt.**

Refer to list of materials supplied for storage conditions of individual components. Observe the recommended storage conditions for individual prepared components in the Reagent and Standard Preparation sections.

## 5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
10X Rat RBP4 Capture Antibody	600 µL	+2-8°C
10X Rat RBP4 Detector Antibody	600 µL	+2-8°C
Rat RBP4 Lyophilized Recombinant Protein	2 Vials	+2-8°C
Antibody Diluent 4BC	6 mL	+2-8°C
10X Wash Buffer PT	20 mL	+2-8°C
TMB Development Solution	12 mL	+2-8°C
Stop Solution	12 mL	+2-8°C
Sample Diluent NS	50 mL	+2-8°C
Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	+2-8°C
Plate Seal	1	+2-8°C

### 6. MATERIALS REQUIRED, NOT SUPPLIED

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

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- PBS (1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 140 mM NaCl, 2.7 mM KCl, pH 7.4).
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

### 7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

### 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 is necessary to minimize background.
- As a guide, typical ranges of sample concentration for commonly used sample types are shown below in Sample Preparation (section 11).
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples
- Incubate ELISA plates on a plate shaker during all incubation steps.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- **To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.**
- **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 Scientific Support staff with any questions.**

## 9. REAGENT PREPARATION

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells. **The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.**
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

### 9.1 **1X Wash Buffer PT**

Prepare 1X Wash Buffer PT by diluting 10X Wash Buffer PT with deionized water. To make 50 mL 1X Wash Buffer PT combine 5 mL 10X Wash Buffer PT with 45 mL deionized water. Mix thoroughly and gently.

### 9.2 **Antibody Cocktail**

Prepare Antibody Cocktail by diluting the capture and detector antibodies in Antibody Diluent 4BC. To make 3 mL of the Antibody Cocktail combine 300  $\mu$ L 10X Capture Antibody and 300  $\mu$ L 10X Detector Antibody with 2.4 mL Antibody Diluent 4BC. Mix thoroughly and gently.

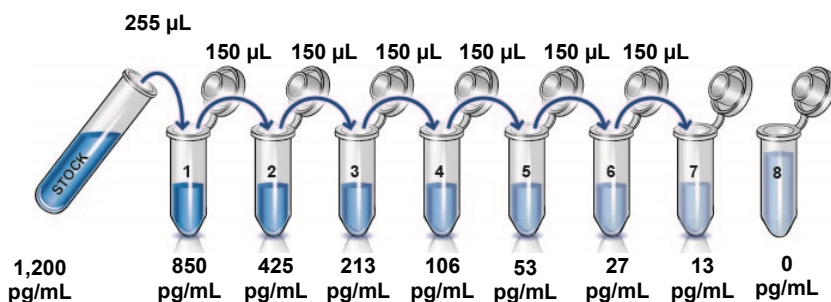


## 10. STANDARD PREPARATION

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of positive controls for every use.

The following section describes the preparation of a standard curve for duplicate measurements (recommended).

- 10.1 **IMPORTANT:** If the protein standard vial has a volume identified on the label, reconstitute the RBP4 standard by adding that volume of Sample Diluent NS indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the RBP4 standard by adding 500  $\mu$ L Sample Diluent NS. Hold at room temperature for 10 minutes and mix gently. This is the 1,200 pg/mL **Stock Standard Solution**.
- 10.2 Label eight tubes, Standards 1–8.
- 10.3 Add 105  $\mu$ L Sample Diluent NS into tube number 1 and 150  $\mu$ L of Sample Diluent NS into numbers 2-8.
- 10.4 Use the Stock Standard to prepare the following dilution series. Standard #8 contains no protein and is the Blank control:



## 11. SAMPLE PREPARATION

TYPICAL SAMPLE DYNAMIC RANGE	
Sample Type	Range
Rat Serum	1:40,000 – 1:640,000
Rat Plasma – Citrate	1:40,000 – 1:640,000
Rat Urine	0.4 – 6%
Cell Culture Supernatant	Varies by type

### 11.1 Plasma

Collect plasma using citrate, EDTA or heparin. Centrifuge samples at 2,000 x g for 10 minutes. Dilute samples into Sample Diluent NS and assay. Store un-diluted plasma samples at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

### 11.2 Serum

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2,000 x g for 10 minutes and collect serum. Dilute samples into Sample Diluent NS and assay. Store un-diluted serum at -20°C or below. Avoid repeated freeze-thaw cycles.

### 11.3 Cell Culture Supernatants

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

### 11.4 Urine

Centrifuge urine at 2,000 x g for 10 minutes to remove debris. Collect supernatants, dilute in Sample Diluent NS

and assay. Store samples at -20°C or below. 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 plate strips should be immediately returned to the foil pouch containing the desiccant pack, resealed and stored at 4°C.
- For each assay performed, a minimum of two wells must be used as the zero control.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Differences in well absorbance or “edge effects” have not been observed with this assay.

## 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 directed in the previous sections.

13.2 Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal and return to 4°C storage.

13.3 Add 50 µL of all sample or standard to appropriate wells.

13.4 Add 50 µL of the Antibody Cocktail to each well.

13.5 Seal the plate and incubate for 1 hour at room temperature on a plate shaker set to 400 rpm.

13.6 Wash each well with 3 x 350 µL 1X Wash Buffer PT. Wash by aspirating or decanting from wells then dispensing 350 µL 1X Wash Buffer PT into each well. Complete removal of liquid at each step is essential for good performance. After the last wash invert the plate and blot it against clean paper towels to remove excess liquid.

13.7 Add 100 µL of TMB Development Solution to each well and incubate for 10 minutes in the dark on a plate shaker set to 400 rpm.

*Given variability in laboratory environmental conditions, optimal incubation time may vary between 5 and 20 minutes.*

*Note: The addition of Stop Solution will change the color from blue to yellow and enhance the signal intensity about 3X. To avoid signal saturation, proceed to the next step before the high concentration of the standard reaches a blue color of O.D.600 equal to 1.0.*

13.8 Add 100 µL of Stop Solution to each well. Shake plate on a plate shaker for 1 minute to mix. Record the OD at 450 nm. This is an endpoint reading.

*Alternative to 13.7 – 13.8: Instead of the endpoint reading at 450 nm, record the development of TMB Substrate kinetically. Immediately after addition of TMB Development Solution begin recording the blue color development with elapsed time in the microplate reader prepared with the following settings:*

Mode:	Kinetic
Wavelength:	600 nm
Time:	up to 20 min
Interval:	20 sec - 1 min
Shaking:	Shake between readings

*Note that an endpoint reading can also be recorded at the completion of the kinetic read by adding 100  $\mu$ L Stop Solution to each well and recording the OD at 450 nm.*

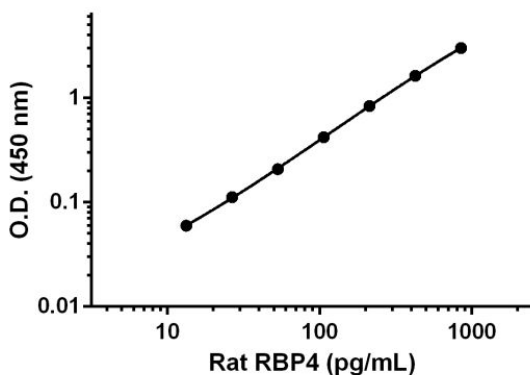
13.9 Analyze the data as described below.

### 14. CALCULATIONS

Subtract the average zero standard absorbance value from all readings. Average the duplicate absorbance measurements of the positive control dilutions and plot against their concentrations. Draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A four parameter algorithm (4PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Interpolate protein concentrations for unknown samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration by the appropriate 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.



Standard Curve Measurements			
Conc. (pg/mL)	O.D. 450 nm		Mean O.D.
	1	2	
0	0.092	0.086	0.089
13.3	0.114	0.114	0.114
26.6	0.167	0.162	0.165
53.1	0.253	0.260	0.256
106	0.459	0.461	0.460
213	0.840	0.819	0.829
425	1.668	1.621	1.64
850	3.051	2.874	2.96

**Figure 1.** Example of RBP4 standard curve prepared in Sample Diluent NS. The RBP4 standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean  $\pm$  SD) are graphed.

## 16. TYPICAL SAMPLE VALUES

### SENSITIVITY –

The calculated minimal detectable dose (MDD) is 3.07 pg/mL in Sample Diluent NS. The MDD was determined by calculating the mean of zero standard replicates (n=25) and adding 2 standard deviations then extrapolating the corresponding concentration.

### RECOVERY –

Three concentrations of RBP4 were spiked in duplicate to the indicated biological matrix to evaluate signal recovery in the working range of the assay.

Sample Type	Average % Recovery	Range (%)
0.000156% Rat Serum	113	107-116
0.000156% Rat Plasma – Citrate	100	91-106
0.8% Rat Urine	104	103-105
25% Cell Culture Supernatant	116	109-126



## LINEARITY OF DILUTION –

Linearity of dilution is determined based on interpolated values from the standard curve. Linearity of dilution defines a sample concentration interval in which interpolated target concentrations are directly proportional to sample dilution.

Native RBP4 was measured in serum, plasma and urine samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

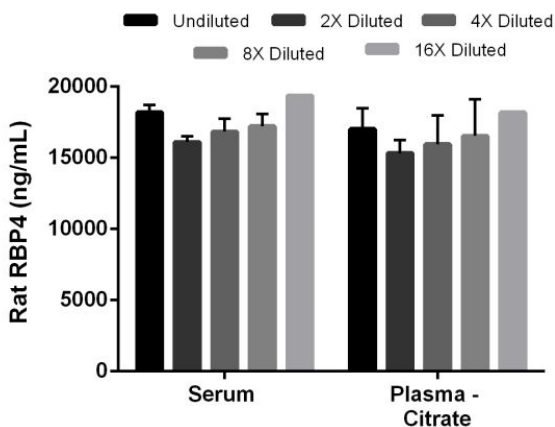
Recombinant RBP4 was spiked into RAW supernatant samples and measured in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	0.0025% Rat Serum	0.0025% Rat Plasma (Citrate)	6% Rat Urine	50% RAW Supernatant
Undiluted	pg/mL	425.8	455	598.3	538.6
	% Expected value	100	100	100	100
2	pg/mL	191.6	201.3	291.5	269.2
	% Expected value	90	89	97	100
4	pg/mL	99.8	105.3	139.5	142.8
	% Expected value	94	93	93	106
8	pg/mL	51.7	53.8	73.2	77.2
	% Expected value	97	95	98	115
16	pg/mL	28.4	30.3	35.3	3.6
	% Expected value	107	106	94	112

## PRECISION –

Mean coefficient of variations of interpolated values from 3 concentrations of RBP4 within the working range of the assay.

	Intra- Assay	Inter- Assay
n=	5	3
CV (%)	3.3	2.7



**Figure 2.** Interpolated concentrations of RBP4 in rat plasma (citrate) and serum. The concentrations of RBP4 were measured in duplicate and interpolated from the RBP4 standard curve and corrected for sample dilution. The interpolated dilution factor corrected values are plotted (mean  $\pm$  SD, n=2).

### **17. ASSAY SPECIFICITY**

This kit recognizes both native and recombinant rat RBP4 protein in serum, plasma, urine, and cell culture supernatant samples only.

#### **CROSS REACTIVITY**

Retinol was tested at 250 and 6.5 pg/mL and assayed for cross reactivity. No cross-reactivity was observed.

#### **INTERFERENCE**

Retinol was tested at 250 and 6.5 pg/mL and assayed for assay interference in the presence of 250 pg/mL rat RBP4. No interference was observed.

### **18. SPECIES REACTIVITY**

This kit recognizes rat RBP4 protein.

Other species reactivity was determined by measuring 0.005% serum samples of various species, interpolating the protein concentrations from the rat standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in rat serum assayed at the same dilution.

Reactivity < 1% was determined for the following species:

- Human
- Sheep
- Rabbit
- Dog
- Goat
- Pig
- Cow

Please contact our Technical Support team for more information.

## 19. TROUBLESHOOTING

Problem	Cause	Solution
Difficulty pipetting lysate; viscous lysate.	Genomic DNA solubilized	Prepare 1X Cell Extraction Buffer PTR (without enhancer). Add enhancer to lysate after extraction.
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard 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; increase to 2 or 3 hour standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
	Incubation times with TMB too brief	Ensure sufficient incubation time until blue color develops prior addition of Stop solution
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 your reconstituted standards at -80°C, all other assay components 4°C. Keep TMB Development Solution protected from light.
Precipitate in Diluent	Precipitation and/or coagulation of components within the Diluent.	Precipitate can be removed by gently warming the Diluent to 37°C.

### 20. NOTES





## Technical Support

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