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ab202404 Retinol Binding Protein 4 (RBP4) Mouse SimpleStep ELISA® Kit

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

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

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1. Overview

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 mouse serum, plasma, urine, cell culture supernatant, cell and tissue extracts.

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 substrate 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. Protocol Summary

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

Add standard or sample to appropriate wells.

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

Aspirate and wash each well. Add TMB Substrate 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.

We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.

Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.

Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.

All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store kit at +4°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

5. 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.

6. Materials Supplied

Item	Quantity	Storage Condition
10X Mouse RBP4 Capture Antibody	600 µL	+4°C
10X Mouse RBP4 Detector Antibody	600 µL	+4°C
Mouse RBP4 Lyophilized Recombinant Protein	2 Vials	+4°C
Antibody Diluent 4BC	6 mL	+4°C
10X Wash Buffer PT	20 mL	+4°C
5X Cell Extraction Buffer PTR	10 mL	+4°C
50X Cell Extraction Enhancer Solution	1 mL	+4°C
TMB Development Solution	12 mL	+4°C
Stop Solution	12 mL	+4°C
Sample Diluent NS	50 mL	+4°C
Pre-Coated 96 Well Microplate (12 x 8 well strips)	96 Wells	+4°C
Plate Seal	1	+4°C

7. Materials Required, Not Supplied

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

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Method for determining protein concentration (BCA assay recommended).
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- Plate shaker for all incubation steps.
- Optional: Phenylmethylsulfonyl Fluoride (PMSF) (or other protease inhibitors).

8. Technical Hints

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.

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.

The provided 50X Cell Extraction Enhancer Solution may precipitate when stored at + 4°C. To dissolve, warm briefly at + 37°C and mix gently. The 50X Cell Extraction Enhancer Solution can be stored at room temperature to avoid precipitation.

To avoid high background always add samples or standards to the well before the addition of the antibody cocktail.

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 Cell Extraction Buffer PTR (For cell and tissue extracts only)

Prepare 1X Cell Extraction Buffer PTR by diluting 5X Cell Extraction Buffer PTR to 1X with deionized water. To make 10 mL 1X Cell Extraction Buffer PTR combine 7.8 mL deionized water and 2 mL 5X Cell Extraction Buffer PTR and 200 µL 50X Cell Extraction Enhancer Solution. Mix thoroughly and gently. If required protease inhibitors can be added.

Alternative – Enhancer may be added to 1X Cell Extraction Buffer PTR after extraction of cells or tissue. Refer to note in the Troubleshooting section.

9.2 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.

9.3 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 µL 10X Capture Antibody and 300 µL 10X Detector Antibody with 2.4 mL Antibody Diluent 4BC. Mix thoroughly and gently.

10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- 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 Diluent indicated on the label. Alternatively, if the vial has a mass identified, reconstitute the RBP4 standard by adding 500 µL Diluent. Hold at room temperature for 10 minutes and mix gently. This is the 2,000 pg/mL **stock standard** Solution.

10.2 Label eight tubes, Standards 1– 8

10.3 **For serum, plasma, urine and cell culture supernatant samples measurements**, reconstitute the standard by adding Sample Diluent NS. Add 180 µL Sample Diluent NS into tube number 1 and 150 µL of Sample Diluent NS into numbers 2-8.

For cell and tissue extract samples measurements, reconstitute the standard by adding 1X Cell Extraction Buffer PTR. Add 180 µL 1X Cell Extraction Buffer PTR into tube number 1 and 150 µL of 1X Cell Extraction Buffer PTR 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:

Standard #	Volume to dilute (µL)	Volume Diluent (µL)	Mouse RBP4 (pg/mL)
1	120 µL Stock	180	800
2	150 µL Standard #1	150	400
3	150 µL Standard #2	150	200
4	150 µL Standard #3	150	100
5	150 µL Standard #4	150	50
6	150 µL Standard #5	150	25
7	150 µL Standard #6	150	12.5
8 (Blank)	N/A	150	0

11. Sample Preparation

TYPICAL SAMPLE DYNAMIC RANGE	
Sample Type	Range
Mouse Serum	1:20,000 – 1:640,000
Mouse Plasma - EDTA	1:20,000 – 1:640,000
Mouse Plasma - Heparin	1:20,000 – 1:640,000
Mouse Plasma –Citrate	1:20,000 – 1:640,000
Mouse Urine	1:5 – 1:320
Cell Culture Supernatant	Varies by type
Mouse Liver Homogenate	30 – 1 µg/mL
Mouse Brain Homogenate	200 – 12.5 µg/mL

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.

11.5 Preparation of extracts from cell pellets:

11.5.1 Collect non-adherent cells by centrifugation or scrape to collect adherent cells from the culture flask. Typical centrifugation conditions for cells are 500 x g for 5 minutes at 4°C.

11.5.2 Rinse cells twice with PBS.

11.5.3 Solubilize pellet at 2x107 cell/mL in chilled 1X Cell Extraction Buffer PTR.

11.5.4 Incubate on ice for 20 minutes.

11.5.5 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.5.6 Transfer the supernatants into clean tubes and discard the pellets.

11.5.7 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.5.8 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

11.6 Preparation of extracts from adherent cells by direct lysis (alternative protocol)

11.6.1 Remove growth media and rinse adherent cells 2 times in PBS.

11.6.2 Solubilize the cells by addition of chilled 1X Cell Extraction Buffer PTR directly to the plate (use 750 µL - 1.5 mL 1X Cell Extraction Buffer PTR per confluent 15 cm diameter plate).

11.6.3 Scrape the cells into a microfuge tube and incubate the lysate on ice for 15 minutes.

11.6.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.6.5 Transfer the supernatants into clean tubes and discard the pellets.

11.6.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.6.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

11.7 Preparation of extracts from tissue homogenates

11.7.1 Tissue lysates are typically prepared by homogenization of tissue that is first minced and thoroughly rinsed in PBS to remove blood (dounce homogenizer recommended).

11.7.2 Homogenize 100 to 200 mg of wet tissue in 500 µL – 1 mL of chilled 1X Cell Extraction Buffer PTR. For lower amounts of tissue adjust volumes accordingly.

11.7.3 Incubate on ice for 20 minutes.

11.7.4 Centrifuge at 18,000 x g for 20 minutes at 4°C.

11.7.5 Transfer the supernatants into clean tubes and discard the pellets.

11.7.6 Assay samples immediately or aliquot and store at -80°C. The sample protein concentration in the extract may be quantified using a protein assay.

11.7.7 Dilute samples to desired concentration in 1X Cell Extraction Buffer PTR.

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.

We recommend that you 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 samples and standards 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.

Add 100 µL of TMB Substrate 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 minutes
Interval:	20 sec - 1 minute
Shaking:	Shake between readings

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

13.9 Analyze the data as described below.

14. Calculations

Subtract average of the zero standard absorbance measurement from all readings.

Determine the average of the duplicate readings of the standards and plot against their concentrations.

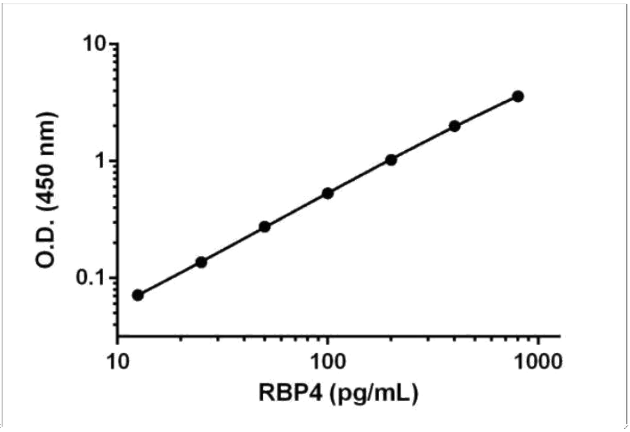
Draw the best smooth curve through these points to construct a standard curve.

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 fit (e.g. linear, semi-log, log/log, 4 parameter logistic).

Interpolate protein concentrations for unknown samples from the equation of the line of the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

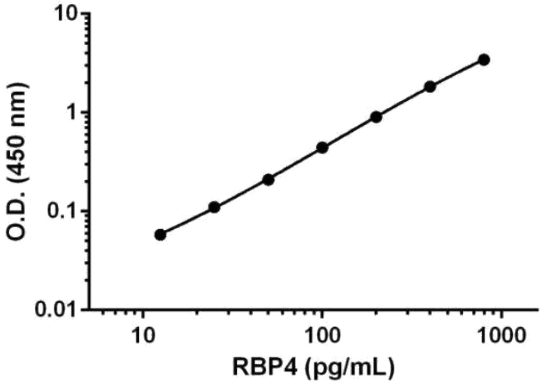
15. Typical Data

Typical standard curve – data provided **for demonstration purposes only.** A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Conc.	O.D. 450 nm		Mean
(ng/mL)	1	2	O.D.
0	0.055	0.053	0.054
12.5	0.125	0.127	0.126
25	0.195	0.188	0.191
50	0.325	0.334	0.329
100	0.572	0.602	0.587
200	1.121	1.047	1.084
400	2.072	2.033	2.052
800	3.695	3.589	3.642

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 +/- SD) are graphed.



Standard Curve Measurements			
Conc.	O.D. 450 nm		Mean
(ng/mL)	1	2	O.D.
0	0.063	0.058	0.060
12.5	0.174	0.116	0.145
25	0.177	0.165	0.171
50	0.285	0.255	0.270
100	0.512	0.492	0.502
200	1.002	0.925	0.963
400	1.967	1.836	1.901
800	3.574	3.443	3.508

Figure 2: Example of RBP4 standard curve prepared in 1X Cell Extraction Buffer PTR. The RBP4 standard curve was prepared as described in Section 10. Raw data values are shown in the table. Background-subtracted data values (mean +/- SD) are graphed.

16. Typical Sample Values

SENSITIVITY –

The calculated minimal detectable dose (MDD) is determined by calculating the mean of zero standard replicates and adding 2 standard deviations then extrapolating the corresponding concentrations. The MDD is dependent on the Sample Diluent buffer used:

Sample Diluent Buffer	n=	Minimal Detectable Dose
Sample Diluent NS	25	3.3 pg/mL
1X Cell Extraction Buffer PTR	25	1.6 pg/mL

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 (%)
0.001% Mouse Serum	101	96 – 106
0.001% Mouse Plasma - EDTA	99	94 – 103
0.001% Mouse Plasma - Heparin	94	91 – 96
0.001% Mouse Plasma – Citrate	99	92 – 104
2% Mouse Urine	98	94 – 104
0.5% Cell Culture Supernatant	105	97 – 112
10 µg/mL Mouse Liver	112	104 – 130

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 the following biological samples in a 2-fold dilution series. Sample dilutions are made in Sample Diluent NS.

Dilution Factor	Interpolated value	0.005% Mouse Serum	0.005% Mouse Plasma (Citrate)	0.005% Mouse Plasma (EDTA)	0.005% Mouse Plasma (Heparin)
Undiluted	pg/mL	687	595	611	585
	% Expected value	**100**	**100**	**100**	**100**
2	pg/mL	308	261	325	322
	% Expected value	**90**	**88**	**106**	**110**
4	pg/mL	166	140	144	160
	% Expected value	**97**	**94**	**94**	**109**
8	pg/mL	86	87	80	89
	% Expected value	**100**	**116**	**104**	**121**
16	pg/mL	41	40	37	38
	% Expected value	**95**	**106**	**95**	**104**

Dilution Factor	Interpolated value	20% Mouse Urine	2.5% Mouse Lung Supernatant
Undiluted	pg/mL	567	547
	% Expected	**100**	**100**
2	pg/mL	302	274
	% Expected	**107**	**100**
4	pg/mL	150	143
	% Expected	**106**	**104**
8	pg/mL	79	75
	% Expected	**111**	**109**
16	pg/mL	35	33
	% Expected	**98**	**96**

Native RBP4 was measured in the following biological samples in a 2-fold dilution series. Sample dilutions are made in Cell Extraction Buffer PTR.

Dilution Factor	Interpolated value	30 µg/mL Mouse Liver Extract
Undiluted	pg/mL	601
	% Expected value	**100**
2	pg/mL	299
	% Expected value	**100**
4	pg/mL	159
	% Expected value	**106**
8	pg/mL	79.8
	% Expected value	**106**
16	pg/mL	40.3
	% Expected value	**107**

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 (%)	4.5	3.8

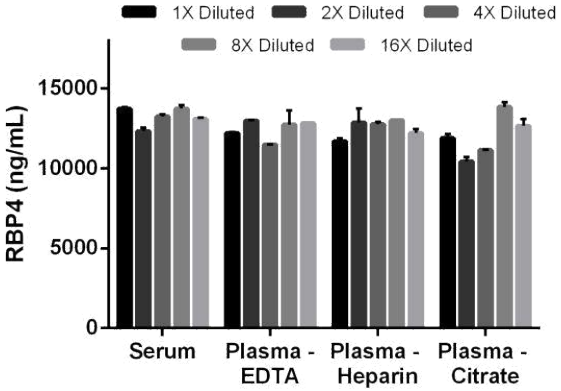


Figure 3. Titration of mouse serum and plasma within the working range of the assay. Background-subtracted data values (mean +/- SD, n = 2) are graphed.

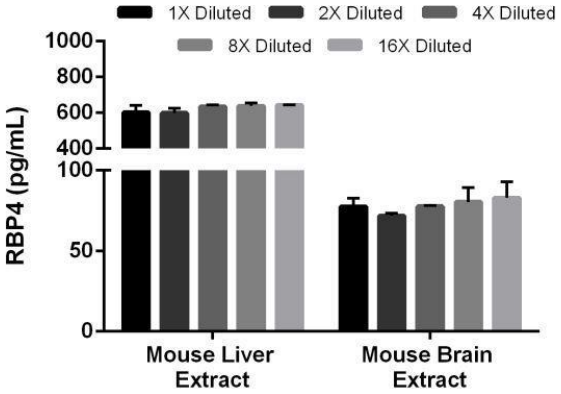


Figure 4. Titration of mouse liver extract and mouse brain extract within the working range of the assay. Interpolated values of RBP4 are plotted for the indicated tissue extracts based on an extract load of 30 µg/mL. Background-subtracted data values (mean +/- SD, n = 2) are graphed.

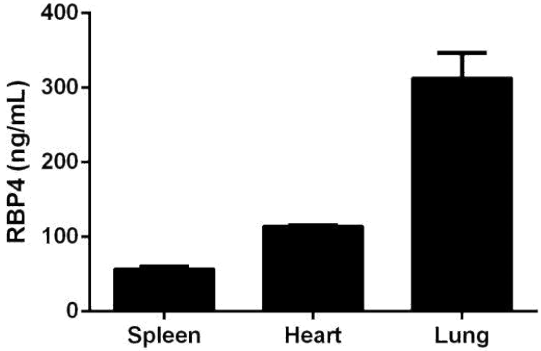


Figure 5. Quantitation of RBP4 expression in different tissue culture supernatants. Background-subtracted data values (mean +/- SD, n = 2) are graphed

17. Assay Specificity

This kit recognizes both native and recombinant mouse RBP4 protein in serum, plasma, urine, supernatant cell and tissue extract samples only.

CROSS REACTIVTY

The following proteins were prepared at 5,000 and 500 pg/mL and assayed for cross reactivity. No cross-reactivity was observed.
Human RBP4
Mouse Lipocalin2

Retinol was also tested at 5,000 and 6.5 pg/mL and assayed for cross reactivity. No cross-reactivity was observed.

INTERFERENCE

The following proteins were prepared at 5,000 and 500 pg/mL and assayed for assay interference in the presence of 500 pg/mL mouse RBP4. No interference was observed. Human RBP4

Mouse Lipocalin2
Retinol was also tested at 5,000 and 6.5 pg/mL and assayed for assay interference in the presence of 500 pg/mL mouse RBP4. No interference was observed.

18. Species Reactivity

This kit recognizes mouse RBP4 protein.

Other species reactivity was determined by measuring 0.005% serum samples of various species, interpolating the protein concentrations from the mouse standard curve, and expressing the interpolated concentrations as a percentage of the protein concentration in mouse 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	Reason	Solution
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 hours 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 substrate 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.
Difficulty pipetting lysate; viscous lysate.	Genomic DNA solubilized	Prepare 1X Cell Extraction Buffer PTR (without enhancer). Add enhancer to lysate after extraction.

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)