



Human RBP4 ELISA Kit

User's Manual

For Research Use Only, Not for use in diagnostic procedures

ELISA Kit for Measuring Human RBP4

CircuLex Human RBP4 ELISA Kit

Cat# CY-8072

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Intended Use

The CycLex Research Product **CircuLex Human RBP4 ELISA Kit** is used for the quantitative measurement of Human Retinol Binding Protein 4 (RBP4) in serum, plasma and other biological media.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store all components at 4°C.
- Don't expose reagents to excessive light.

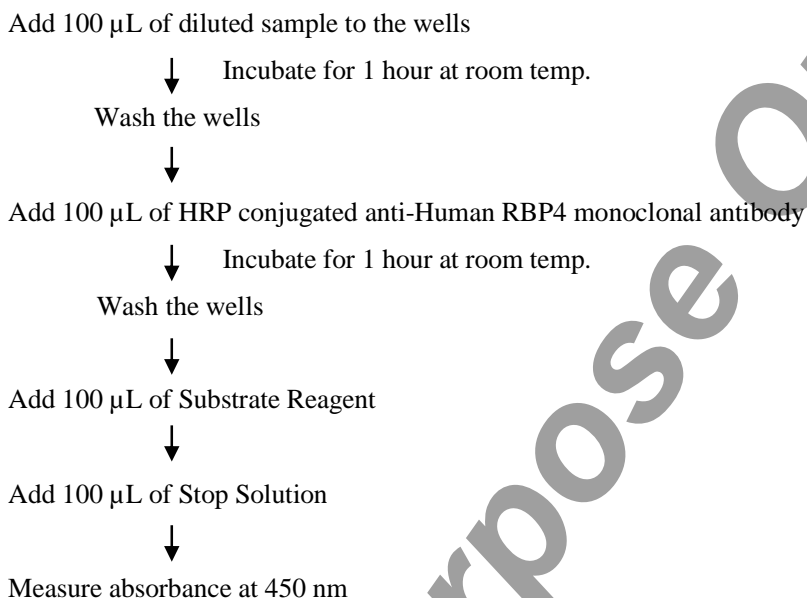
Introduction

Members of the lipocalin family, RBP4, FABP4 and NGAL (Lipocalin 2), which are produced from adipocytes, have recently been reported to play important roles in regulating systemic energy homeostasis, insulin sensitivity, and inflammation in animal models (1-5). Transgenic overexpression of RBP4 or injection of recombinant RBP4 in normal mice causes insulin resistance (1). Conversely, genetic deletion of RBP4 enhances insulin sensitivity. Graham et al. (2006) found that serum RBP4 levels correlated with the magnitude of insulin resistance in human subjects with obesity, impaired glucose tolerance, or type 2 diabetes and in non-obese, non-diabetic subjects with a strong family history of type 2 diabetes (6). Elevated serum RBP4 was associated with components of the metabolic syndrome, including increased body mass index (BMI), waist-to-hip ratio, serum triglyceride levels, and systolic blood pressure and decreased high-density lipoprotein cholesterol levels (6-8). Adipocyte GLUT4 protein and serum RBP4 levels were inversely correlated. Graham et al. concluded that RBP4 is elevated in serum before the development of frank diabetes and appears to identify insulin resistance and associated cardiovascular risk factors in subjects with varied clinical presentations.

Principle of the Assay

The CircuLex Human RBP4 ELISA Kit employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for Human RBP4 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any Human RBP4 present. After washing away any unbound substances, an HRP conjugated monoclonal antibody specific for Human RBP4 is added to the wells. Following a wash to remove any unbound antibody HRP conjugate, the remaining conjugate is allowed to react with the substrate H₂O₂-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of Human RBP4. A standard curve is constructed by plotting absorbance values versus Human RBP4 concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

The CircuLex Human RBP4 ELISA Kit is designed to measure the concentration of Human RBP4 from human serum/plasma, cell lysate of cultured human cell lines and cell culture conditioned medium.

Summary of Procedure

Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microplate kit.

Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with anti-Human RBP4 antibody as a capture antibody.

10X Wash Buffer: One 100 mL bottle of 10X buffer containing 2% Tween®-20

Dilution Buffer: One bottle containing 50 mL of 1X buffer; use for sample dilution. Ready to use.

Human RBP4 Standard (*): Two vials containing each 200 ng of lyophilized Human RBP4.

HRP conjugated Detection Antibody: One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-Human RBP4 monoclonal antibody. Ready to use.

Substrate Reagent: One bottle containing 20 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle containing 20 mL of 1 N H₂SO₄. Ready to use.

(*): It was purified from human urine. Handle as if capable of transmitting infectious agents. This component should be handled at the Bio-safety Level 2 (BSL 2) as recommended for any potentially infectious human body fluids in the CDC / NIH manual "Biosafety in microbiological and Biomedical Laboratories".

Materials Required but not Provided

- **Pipettors:** 2-20 μ L, 20-200 μ L and 200-1000 μ L precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **Microplate washer:** optional (Manual washing is possible but not preferable)
- **Plate reader** capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.
- **Software package facilitating data generation and analysis :**optional
- **500 or 1000 mL graduated cylinder**
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- Allow all the components to come to room temperature before use.
- All microplate strips that are not immediately required should be returned to the zip-lock pouch, which must be carefully resealed to avoid moisture absorption.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents in this kit may contain preservatives or other chemicals. Care should be taken to avoid direct contact with these reagents.
- Do not mouth pipette or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.
- Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of human origin, and these reagents. In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.
- **“Human RBP4 Standard” was purified from human urine. Handle as if capable of transmitting infectious agents. This component should be handled at the Bio-safety Level 2 (BSL 2) as recommended for any potentially infectious human body fluids in the CDC / NIH manual “Biosafety in microbiological and Biomedical Laboratories”.**
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**
- **Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

Sample Collection and Storage

Serum: Use a serum separator tube and allow samples to clot for 60 ± 30 minutes. Centrifuge the samples at 4°C for 10 minutes at 1,000 x g. Remove serum and assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of serum may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using EDTA-Na₂ as the anticoagulant. If possible, collect the plasma into a mixture of EDTA-Na₂ and Futhan5 to stabilize the sample against spontaneous *in vitro* complement activation. Immediately centrifuge samples at 4°C for 15 minutes at 1,000 x g. Assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of plasma may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Note: Citrate plasma has not been validated for use in this assay.

Other biological samples: Remove any particulates by centrifugation and assay immediately or aliquot and store samples at below -70°C. Avoid repeated freeze-thaw cycles.

Detailed Protocol

The CycLex Research Product **CircuLex Human RBP4 ELISA Kit** is provided with removable strips of wells so the assay can be carried out on separate occasions using only the number of strips required for the particular determination. Since experimental conditions may vary, an aliquot of the Human RBP4 Standard within the kit, should be included in each assay as a calibrator. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solutions

All reagents need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of **10X Wash Buffer** and **Human RBP4 Standard**.

1. Prepare a working solution of Wash Buffer by adding 100 mL of the **10X Wash Buffer** to 900 mL of deionized (distilled) water (ddH₂O). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.
2. Reconstitute **Human RBP4 Standard** with **1.0 mL** of ddH₂O. The concentration of the human RBP4 in vial should be **200 ng/mL**, which is referred as a **Master Standard** of human RBP4.

Prepare Standard Solutions as follows:

Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 100 ng/mL standard (Std.1) serves as the highest standard. The **Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Dilution Buffer	Concentration
Std.1	300 µL of Master Standard	300 µL	100 ng/mL
Std.2	300 µL of Std. 1 (100 ng/mL)	300 µL	50 ng/mL
Std.3	300 µL of Std. 2 (50 ng/mL)	300 µL	25 ng/mL
Std.4	300 µL of Std. 3 (25 ng/mL)	300 µL	12.5 ng/mL
Std.5	300 µL of Std. 4 (12.5 ng/mL)	300 µL	6.25 ng/mL
Std.6	300 µL of Std. 5 (6.25 ng/mL)	300 µL	3.13 ng/mL
Std.7	300 µL of Std. 6 (3.13 ng/mL)	300 µL	1.56 ng/mL
Blank	-	300 µL	0 ng/mL

Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with Dilution Buffer before dispensing. Unused portions of Standards should be aliquoted and stored at below -70°C immediately. Avoid multiple freeze and thaw cycles.

Sample Preparation

- Serum and plasma samples require a 2,500-fold dilution.
e.g. First: Make 50-fold dilution (2 µL of sample + 98 µL of Dilution Buffer)
Second: Make 2,500-fold dilution (6 µL of 50-fold diluted sample + 294 µL Dilution Buffer)
Don't store diluted samples.
- Other biological samples require neat, 10- and 100-fold dilution.

Assay Procedure

1. Remove the appropriate number of microtiter wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C.
2. Dilute samples with Dilution Buffer. (See "Sample Preparation" above.)
3. Pipette **100 µL** of **Standard Solutions (Std1-Std7, Blank)** and **diluted samples** in duplicates, into the appropriate wells.
4. Incubate the plate **at room temperature (ca.25°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker.**
5. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.
6. Add **100 µL** of **HRP conjugated Detection Antibody** into each well.
7. Incubate the plate **at room temperature (ca.25°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker.**
8. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.
9. Add **100 µL** of **Substrate Reagent**. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminum foil is recommended. Return Substrate Reagent to 4°C immediately after the necessary volume is removed
10. Incubate the plate **at room temperature (ca. 25°C) for 10-20 minutes, shaking at ca. 300 rpm on an orbital microplate shaker.** The incubation time may be extended up to 30 minutes if the reaction temperature is below than 20°C.
11. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.
12. Measure absorbance in each well using a spectrophotometric microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

Note-1: Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

Note-2: Reliable standard curves are obtained when either O.D. values do not exceed 0.2 units for the blank (zero concentration), or 2.5 units for the highest standard concentration. The plate should be monitored at 5-minute intervals for approximately 30 minutes.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine Human RBP4 concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

Calculations

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density. Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation. To determine the Human RBP4 concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding Human RBP4 concentration. If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

1. The dose-response curve of this assay fits best to a sigmoidal 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 5-parameter logistic function. It is important to make an appropriate mathematical adjustment to accommodate for the dilution factor.
2. Most microtiter plate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the absorbance (Y) of calibrators versus log of the known concentration (X) of calibrators, using the four-parameter function. Alternatively, the logit log function can be used to linearize the calibration curve (i.e. logit of absorbance (Y) is plotted versus log of the known concentration (X) of calibrators).

Measurement Range

The measurement range is 1.56 ng/mL to 100 ng/mL. Any sample reading higher than the highest standard should be diluted with Dilution Buffer in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the human RBP4 concentration.

Troubleshooting

1. The Human RBP4 Standard should be run in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.
2. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate insufficient washing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for washer maintenance.
3. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

Reagent Stability

All of the reagents included in The CycLex Research Product **CircuLex Human RBP4 ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, kit reagents should be stored at 4°C, except the reconstituted Human RBP4 Standard must be stored at below -70°C. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

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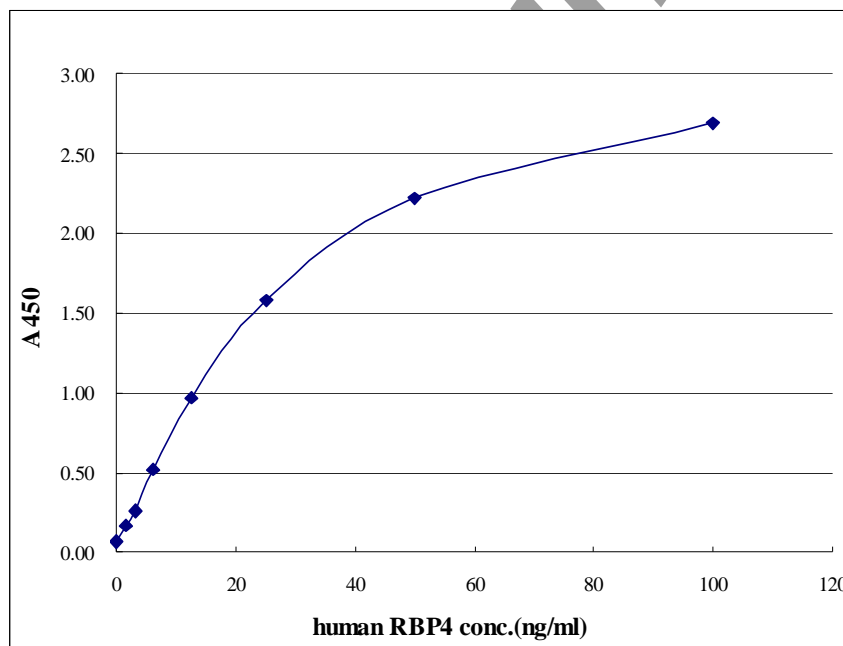
Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of Human RBP4 giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3*SD blank) is better than 1.08 ng/mL of sample.

* Dilution Buffer is pipetted into blank wells.

Typical standard curve in RBP4 ELISA Kit



2. Specificity

The antibodies in the CircuLex Human RBP4 ELISA Kit are highly specific of Human RBP4. However cross-reactivities to other RBP family members and to other species RBP4 have not checked yet.

3. Precision

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

- Intra-assay (Within-Run, n=16) CV = 2.4-3.7 %

human RBP4 conc. (ug/ml)			
	Serum 1	Serum 2	Serum 3
1	20.88	12.59	7.88
2	18.94	12.11	7.41
3	19.95	12.64	7.50
4	19.29	12.29	7.20
5	20.66	12.11	6.99
6	19.99	12.09	7.29
7	19.05	11.85	7.85
8	19.15	12.26	7.94
9	19.11	12.24	7.36
10	20.24	12.85	7.55
11	20.49	12.74	7.53
12	19.88	12.74	7.61
13	19.85	12.53	7.48
14	19.56	12.38	7.34
15	19.39	11.98	7.29
16	19.99	12.26	7.91
MAX.	20.88	12.85	7.94
MIN.	18.94	11.85	6.99
MEAN	19.78	12.35	7.51
S.D.	0.60	0.30	0.28
C.V.	3.0%	2.4%	3.7%

Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in five separate assays to assess inter-assay precision.

- Inter-assay (Run-to-Run, n=5) CV = 4.9-8.9 %

Human RBP4 conc. (ug/ml)			
	Serum 1	Serum 2	Serum 3
1	47.0	29.1	16.0
2	50.0	27.5	15.9
3	44.0	24.1	15.3
4	50.3	29.3	15.4
5	43.2	24.9	14.1
MAX.	50.3	29.3	16.0
MIN.	43.2	24.1	14.1
MEAN	46.9	27.0	15.4
S.D.	3.285	2.398	0.751
C.V.	7.01%	8.89%	4.89%

4. Spiking Recover

Serum samples were spiked with different amounts of Human RBP4 and assayed. The recovery of Human RBP4 spiked to levels throughout the range of the assay was evaluated.

Sample Average % Recovery Range: 88-114 %

Serum 1

	None	+ 45 ug/ml	+ 15 ug/ml	+ 7.5 ug/ml
Average (ug/ml)	31.33	76.89	46.84	38.87
Recovery rate (%)	-	101.24	103.38	100.50

Serum 2

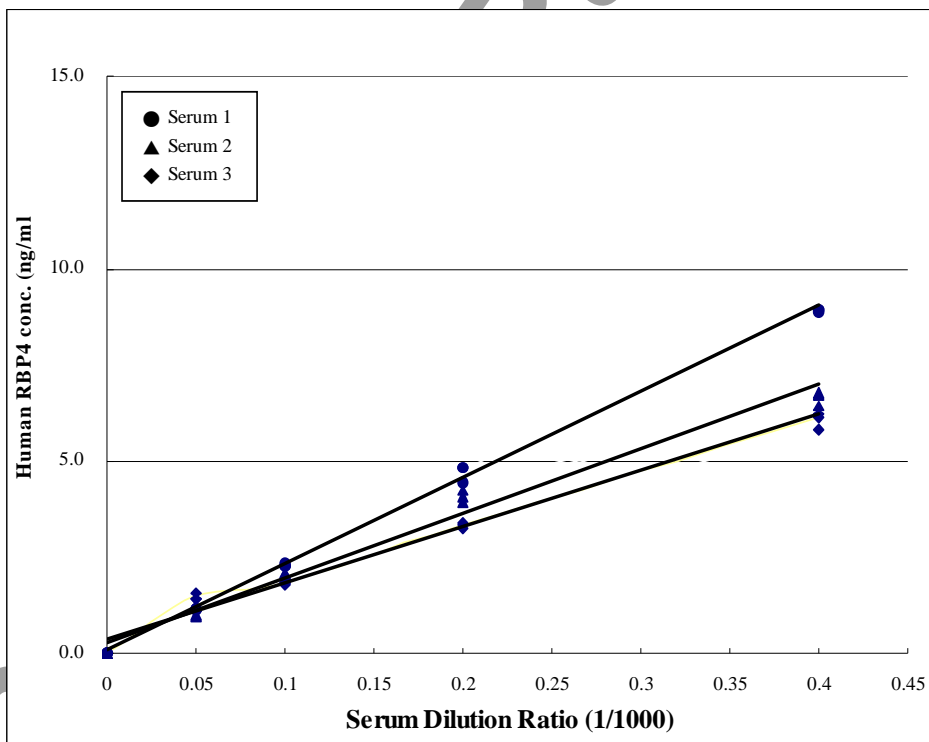
	None	+ 45 ug/ml	+ 15 ug/ml	+ 7.5 ug/ml
Average (ug/ml)	26.74	68.46	40.65	33.34
Recovery rate (%)	-	92.71	92.71	88.00

Serum 3

	None	+ 45 ug/ml	+ 15 ug/ml	+ 7.5 ug/ml
Average (ug/ml)	15.45	62.34	32.29	24.00
Recovery rate (%)	-	104.19	112.25	114.00

5. Linearity

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Human RBP4 were serially diluted with the Dilution Buffer to produce samples with values within the dynamic range of the assay.



Example of Test Results

Fig.1 Human RBP4 level in 36 healthy volunteers

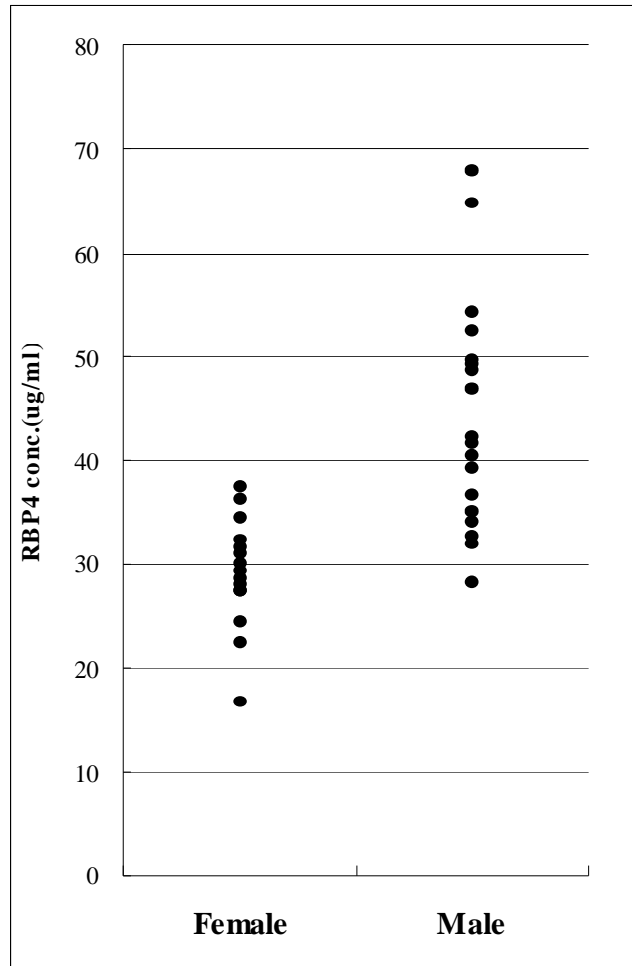
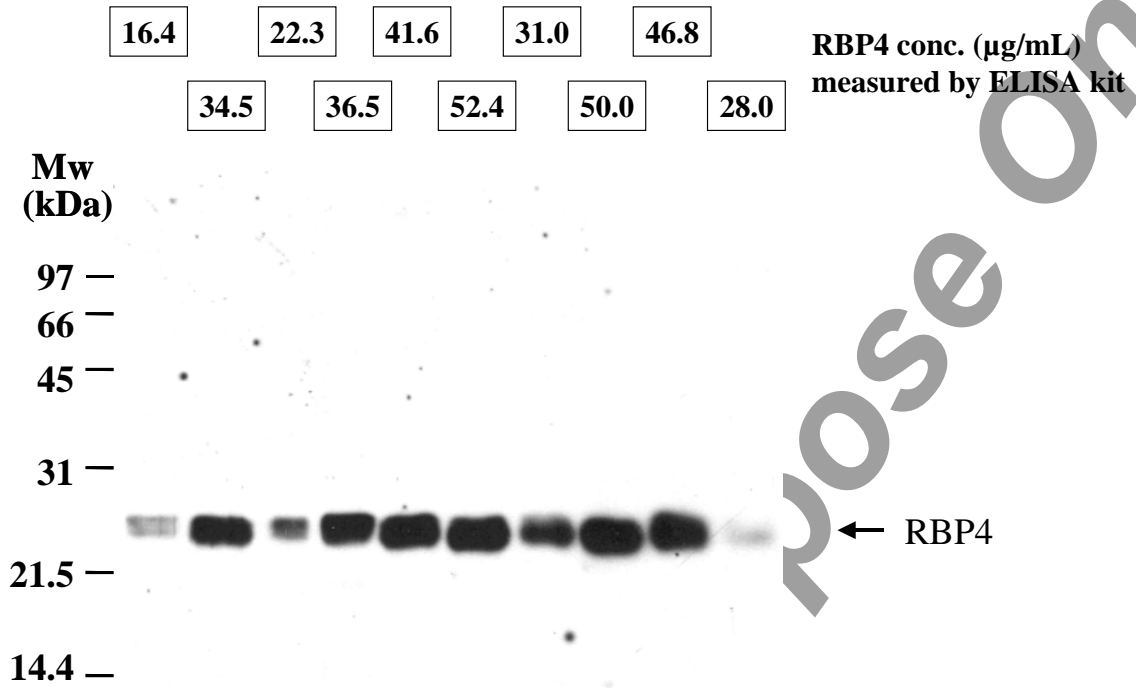


Fig.2 Comparison of Serum RBP4 level measured by ELISA & WB



References

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Related Products

- *CircuLex Rat Adiponectin ELISA Kit: Cat# CY-8049
- *CircuLex Human Adiponectin ELISA Kit: Cat# CY-8050
- *CircuLex Mouse Adiponectin ELISA Kit: Cat# CY-8051
- *CircuLex CML/N^ε-(carboxymethyl)lysine ELISA Kit: Cat# CY-8066
- *CircuLex Mouse Visfatin/PBEF ELISA Kit: Cat# CY-8065

PRODUCED BY

CycLex Co., Ltd.
1063-103 Terasawaoka
Ina, Nagano 396-0002
Japan
Fax: +81-265-76-7618
e-mail: info@cyclex.co.jp
URL: <http://www.cyclex.co.jp>

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