ELISA Kit for Measuring Rat FABP4/A-FABP

CircuLex Rat FABP4/A-FABP ELISA Kit

Cat# CY-8076

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Intended Use

The MBL Research Product **CircuLex Rat FABP4/A-FABP ELISA Kit** is used for the quantitative measurement of rat FABP4/A-FABP in serum samples.

Individual users should determine appropriate conditions when using other types of samples.

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.

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Introduction

Adipocyte-specific fatty acid–binding protein (A-FABP), also designated aP2 and FABP4, belongs to the fatty acid-binding protein super family whose members have relative molecular masses of ～15, 000, and it is exclusively expressed in differentiated adipocytes (1, 2). FABP4/A-FABP is a predominant cytosolic protein of mature adipocytes, accounting for ～6 % of total cellular proteins. This protein may be an important regulator of systemic insulin sensitivity and lipid and glucose metabolism (1). Mice deficient in FABP4/A-FABP are protected from development of hyperinsulinemia, hyperglycemia, and insulin resistance in the context of both dietary and genetic obesity (3, 4). Adipocytes obtained from FABP4/A-FABP -null mice had markedly reduced efficiency of lipolysis in vivo and in vitro (5, 6) and exhibited a 2- to 3-fold decrease in fatty acid release, suggesting that FABP4/A-FABP mediates efflux of fatty acids in normal physiology (7).

Although the physiological consequences of FABP4/A-FABP deficiency have been predominantly linked to changes in adipocytes, it has reported that the presence of FABP4/A-FABP in macrophages (2, 8) and have shown that FABP4/A-FABP expression can be induced by peroxisome proliferatoractivated receptor gamma (PPAR gamma) agonists (8), by toll-like receptor agonists (9), oxidized LDL (10), and the differentiation of monocytes to macrophages and can be suppressed by treatment with a cholesterol-lowering statin (11). In these cells, FABP4/A-FABP modulates inflammatory cytokine production and cholesterol ester accumulation (12).

In apolipoprotein E-deficient mice, ablation of the FABP4/A-FABP gene conferred remarkable protection against atherosclerosis, which commonly occurs in this rat strain (13, 14). Taken together, these animal studies demonstrate that FABP4/A-FABP, by integrating metabolic and inflammatory pathways, provides a key link between various components of metabolic syndrome. Moreover, Masato Furuhashi, M. et al. (2007) reported that an orally active small-molecule inhibitor of FABP4/A-FABP is an effective therapeutic agent against severe atherosclerosis and type 2 diabetes in mouse models.

Principle of the Assay

The CircuLex Rat FABP4/A-FABP ELISA Kit employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for FABP4/A-FABP has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any FABP4/A-FABP present. After washing away any unbound substances, an HRP conjugated antibody specific for FABP4/A-FABP 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 H_2O_2-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 FABP4/A-FABP. A standard curve is constructed by plotting absorbance values versus FABP4/A-FABP concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

The CircuLex Rat FABP4/A-FABP ELISA Kit is designed to measure the concentration of rat FABP4/A-FABP from rat serum/plasma and other biological media.

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Summary of Procedure

Add 100 µL of diluted samples to the wells
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of HRP conjugated anti-FABP4/A-FABP antibody
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of Substrate Reagent
↓ Incubate for 10-20 minutes at room temp.
Add 100 µL of Stop Solution
↓
Measure absorbance at 450 nm

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-FABP4/A-FABP antibody as a capture antibody.

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

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

Rat FABP4/A-FABP Standard: One vial containing X* ng of lyophilized recombinant rat FABP4/A-FABP
***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

HRP conjugated Detection Antibody: One bottle containing 12 mL of HRP (horseradish peroxidase) conjugated anti-FABP4/A-FABP 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 H_2SO_4. Ready to use.

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Materials Required but not Provided

- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **(Optional) Microplate washer:** 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.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1,000 mL graduated cylinder**.
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

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Precautions and Recommendations

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**
- 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 Substrate Solution which contains hydrogen peroxide.
- Avoid contact with Stop Solution which contains Sulfuric Acid.
- Wear gloves and eye protection when handling immunodiagnostic materials and samples of rat 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.
- **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.**
- **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

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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.

Other biological samples: MBL has not tested.
(*e.g.* Remove any particulates by centrifugation and assay immediately or aliquot and store samples at below -70°C. Avoid repeated freeze-thaw cycles. Individual users should determine appropriate conditions when using other types of samples.)

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Detailed Protocol

The MBL Research Product **CircuLex Rat FABP4/A-FABP 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 rat FABP4/A-FABP 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 **Rat FABP4/A-FABP 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 (ddH_2O). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Reconstitute **Rat FABP4/A-FABP Standard** with X* **mL** of **ddH_2O**. The concentration of the rat FABP4/A-FABP in vial should be **40 ng/mL**, which is referred to as a **Master Standard** of rat FABP4/A-FABP.
   ***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

   Prepare Standard Solutions as follows:
   Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 10 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	150 µL of Master Standard	450 µL	10 ng/mL
Std.2	300 µL of Std. 1 (10 ng/mL)	300 µL	5 ng/mL
Std.3	300 µL of Std. 2 (5 ng/mL)	300 µL	2.5 ng/mL
Std.4	300 µL of Std. 3 (2.5 ng/mL)	300 µL	1.25 ng/mL
Std.5	300 µL of Std. 4 (1.25 ng/mL)	300 µL	0.63 ng/mL
Std.6	300 µL of Std. 5 (0.63 ng/mL)	300 µL	0.313 ng/mL
Std.7	300 µL of Std. 6 (0.313 ng/mL)	300 µL	0.156 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

Dilute samples with **Dilution Buffer**.
- Serum and plasma samples may require a 100-fold dilution.
  e.g. 3 µL of sample + 297 µL of Dilution Buffer)
- Other biological samples may require 10- and 100- and 400- fold dilution or appropriate dilution.

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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 60 minutes**, 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 60 minutes**, 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. 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.

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 FABP4/A-FABP concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

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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 rat FABP4/A-FABP 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 rat FABP4/A-FABP 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 0.156 ng/mL to 10 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 rat FABP4/A-FABP concentration.

Troubleshooting

1. All samples and controls should be assayed 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 MBL Research Product **CircuLex Rat FABP4/A-FABP 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 FABP4/A-FABP 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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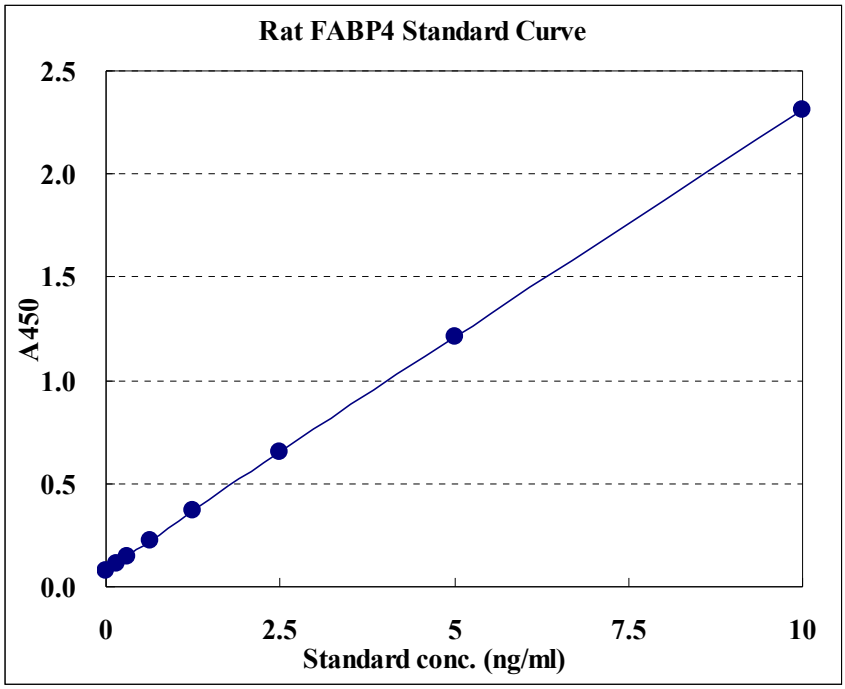
Assay Characteristics

1. Sensitivity

Twenty-four assays were evaluated and the minimum detectable dose (MDD) of rat FABP4/A-FABP. The MDD (defined as such a concentration of rat FABP4/A-FABP giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 92.5 pg/ml of sample.

* Dilution Buffer was pipetted into blank wells.

Typical standard curve



2. Specificity

The antibodies in the CircuLex Rat FABP4/A-FABP ELISA Kit react with rat FABP4/A-FABP and with detectable cross-reactivities to mouse FABP4/A-FABP. Cross-reactivities to other FABP family member have not been checked yet.

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3. Precision

Intra-assay Precision (Precision within an assay)

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

• Intra-assay (Within-Run, n=16), CV=2.5-4.0 %

rat FABP4 conc. (ng/ml)

	Serum 1	Serum 2	Serum 3
1	339.00	186.85	80.95
2	320.55	189.25	80.35
3	318.95	190.80	79.10
4	321.20	194.20	80.75
5	320.30	194.40	81.60
6	333.75	181.35	82.45
7	322.55	197.20	80.55
8	339.00	197.00	83.70
9	342.95	177.80	76.15
10	319.40	176.20	77.40
11	329.40	175.80	78.05
12	318.95	179.55	78.25
13	341.55	180.35	79.50
14	322.55	179.75	78.25
15	325.05	182.10	78.45
16	321.20	185.10	77.85
MAX.	342.95	197.20	83.70
MIN.	318.95	175.80	76.15
MEAN	**327.27**	**185.48**	**79.58**
S.D.	**8.90**	**7.45**	**2.02**
C.V.	**2.7%**	**4.0%**	**2.5%**

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=1.38-4.45 %

rat FABP4 conc. (ng/ml)

	Serum 1	Serum 2	Serum 3
1	645.7	367.7	158.7
2	665.4	343.2	149.9
3	656.4	408.4	167.7
4	656.2	352.6	152.6
5	643.2	342.0	160.8
MAX.	665.4	408.4	167.7
MIN.	643.2	342.0	149.9
MEAN	**653.4**	**362.8**	**158.0**
S.D.	**9.01**	**27.48**	**7.02**
C.V.	**1.38%**	**7.58%**	**4.45%**

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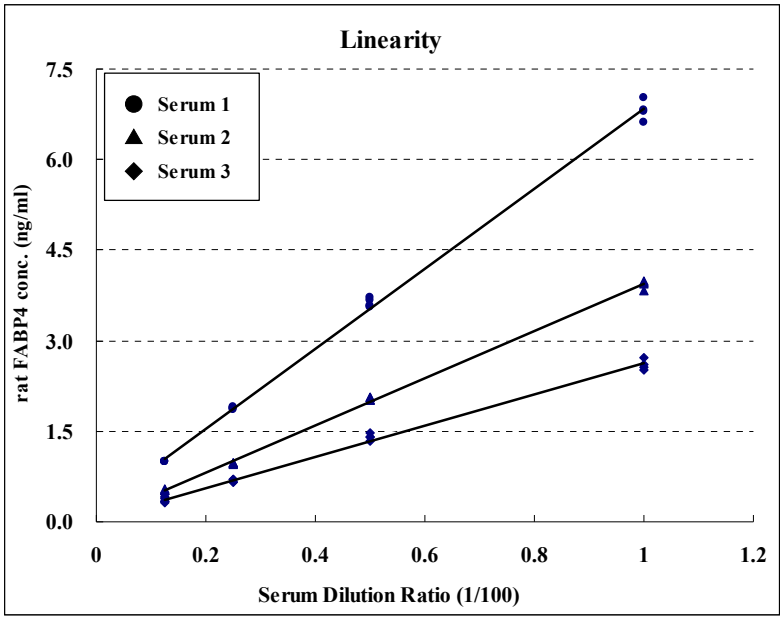
4. Linearity

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of rat FABP4/A-FABP were serially diluted with the Dilution Buffer to produce samples with values within the dynamic range of the assay.



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Example of Test Results

Fig.1 Concentrations of rat FABP4/A-FABP

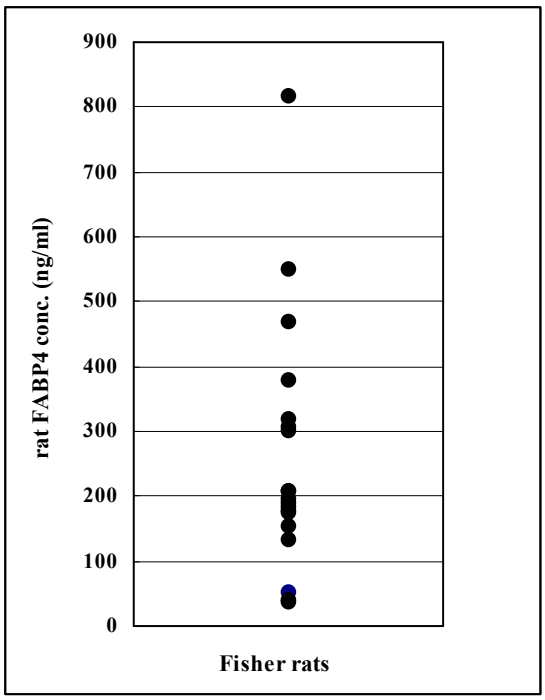
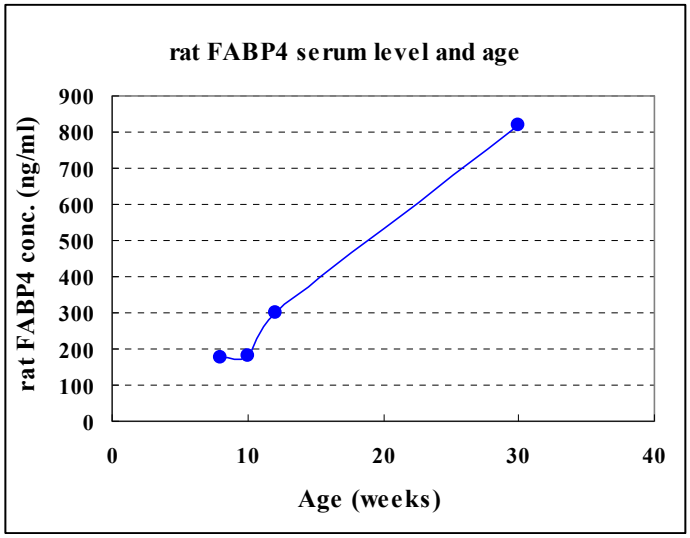


Fig.2 Rat FABP4/A-FABP serum level and age



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