CircuLex™

MBL
A JSR Life Sciences
Company

ELISA Kit for Measuring Mouse and Rat PCSK9

CircuLex Mouse/Rat PCSK9 ELISA Kit

Cat# CY-8078

Intended Use

The MBL Research Product **CircuLex Mouse/Rat PCSK9 ELISA Kit** is used for the quantitative measurement of mouse PCSK9 in serum. This kit is also used for measuring relative amount of rat PCSK9 in serum.

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

Note: The concentration of rat PCSK9 measured by this kit may not be definite because the standard used in this kit is recombinant "mouse" PCSK9. It should be considered as a relative value.

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.
- Do not expose reagents to excessive light.

Introduction

PCSK9 (also known as neural apoptosis-regulated convertase, NARC-1) is a 692-residue extracellular protein expressed primarily in the kidneys, liver and intestines (1) representing the 9th member of the secretory subtilase family. Various genetic observations subsequently mapped PCSK9 as the third gene (along with LDLR and APOB) to cause autosomal dominant hypercholesterolemia (ADH). These studies suggested that gain of function mutations increase plasma levels of LDL-c (2–6), whereas nonsense or missense (loss-of-function) mutations, which interfere with folding or secretion of PCSK9, lead to a reduction of plasma levels of LDL-c and an 88% decrease in the risk of coronary heart disease (CHD) (5). In mice, adenoviral overexpression of PCSK9 results in increased plasma LDL-c level in normal mice but not in LDLR-deficient mice (7). Deletion of PCSK9 causes an increase in level of LDLR protein but not mRNA (8). These findings lead to a hypothesis that PCSK9 exerts its role in cholesterol metabolism through posttranslational down-regulation of LDLR, the receptor responsible for clearing LDL-c from plasma.

Evidence is consistent with the secreted form of PCSK9 binding directly to the LDLR and resulting in degradation of the receptor (9, 10). Zhang et al. (11) localized the binding site of PCSK9 in the LDLR to the first epidermal growth factor-like repeat (EGF-A) of the extracellular domain and showed that PCSK9 binding to this site is required for LDLR degradation. In light of these observations and the fact that PCSK9 in the circulation may cause the degradation of hepatic LDLR in the liver, PCSK9 would seem to be an attractive drug target for lowering LDL-c.
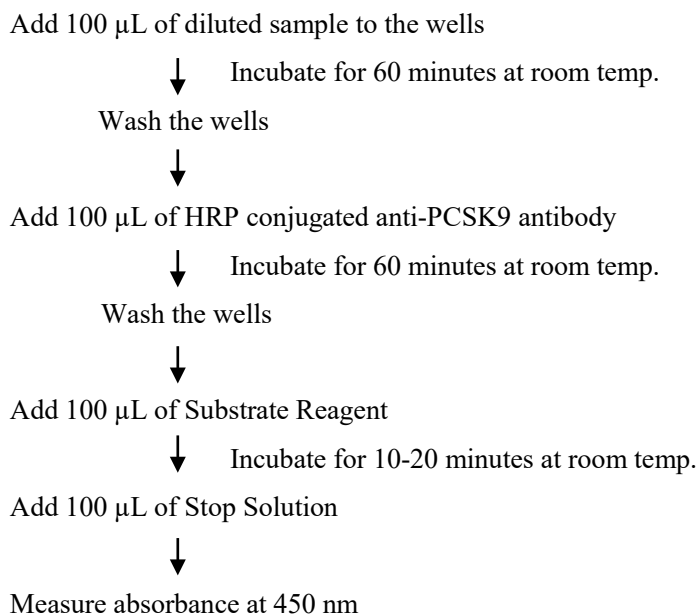
Principle of the Assay

The CircuLex Mouse/Rat PCSK9 ELISA Kit employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for PCSK9 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any PCSK9 present. After washing away any unbound substances, an HRP conjugated antibody specific for PCSK9 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 PCSK9. A standard curve is constructed by plotting absorbance values versus mouse PCSK9 concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

The CircuLex Mouse/Rat PCSK9 ELISA Kit is designed to measure the concentration of mouse and rat PCSK9 in serum samples.

Note: The concentration of rat PCSK9 measured by this kit may not be definite because the standard used in this kit is recombinant "mouse" PCSK9. It should be considered as a relative value.

Summary of Procedure

Add 100 µL of diluted sample to the wells

↓   Incubate for 60 minutes at room temp.

Wash the wells

↓

Add 100 µL of HRP conjugated anti-PCSK9 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-PCSK9 antibody as a capture antibody.

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

Dilution Buffer: One bottle containing 50 mL of 1X buffer; use for reconstitution of Mouse PCSK9 Standard and sample dilution. Ready to use.

Mouse PCSK9 Standard: One vial containing X* ng of lyophilized recombinant mouse PCSK9
*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-PCSK9 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.

CircuLex™

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**

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 the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.

- Wear gloves and eye protection when handling immunodiagnostic materials and samples of mouse and 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.**

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.)

CircuLex™

Detailed Protocol

The MBL Research Product **CircuLex Mouse/Rat PCSK9 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 Mouse PCSK9 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 **Mouse PCSK9 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$_2$O). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Reconstitute **Mouse PCSK9 Standard** with X* **mL** of **Dilution Buffer** by gently mixing. After reconstitution, immediately dispense it in small aliquots (e.g. 100 µL) to plastic micro-centrifuge tubes and store below -70°C to avoid non-specific adsorption to glass surface and multiple freeze-thaw cycles. The concentration of the reconstituted Mouse PCSK9 Standard should be **60 ng/mL**, which is referred to as the **Master Standard** of mouse PCSK9.
   *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 6 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	60 µL of Master Standard	540 µL	6 ng/mL
Std.2	300 µL of Std. 1 (6 ng/mL)	300 µL	3 ng/mL
Std.3	300 µL of Std. 2 (3 ng/mL)	300 µL	1.5 ng/mL
Std.4	300 µL of Std. 3 (1.5 ng/mL)	300 µL	0.75 ng/mL
Std.5	300 µL of Std. 4 (0.75 ng/mL)	300 µL	0.375 ng/mL
Std.6	300 µL of Std. 5 (0.375 ng/mL)	300 µL	0.188 ng/mL
Std.7	300 µL of Std. 6 (0.188 ng/mL)	300 µL	0.094 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.

Sample Preparation

Dilute samples with **Dilution Buffer**.
• Serum samples may require a 100-fold dilution.
   e.g. 3 µL of sample + 297 µL of Dilution Buffer

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. 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 0.25 units for the blank (zero concentration), or 3.0 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 the concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

CircuLex™

Calculations

Average the duplicate readings for each standard, control and sample, and subtract the optical density of the average zero standard. Plot the optical density versus the concentration of standards and draw the best curve. Most microtiter plate readers perform automatic calculations of analyte concentration. The standard curve fits best to a sigmoidal four-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a four-parameter logistic function.

A standard curve is also to be constructed by plotting the absorbance (Y) versus log of the known concentration (X) of standards, using a cubic function. Alternatively, the logit log function can be used to linearize the standard curve (i.e. logit of optical density (Y) is plotted versus log of the known concentration (X) of standards). To determine the concentration of each sample, first find the optical density 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 concentration.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Measurement Range

The measurement range is 0.094 ng/mL to 6 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 concentration of the sample.

Troubleshooting

1. All samples and standards 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 Mouse/Rat PCSK9 ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date.

CircuLex™

Assay Characteristics

1. Sensitivity

Twenty-four assays were evaluated and the minimum detectable dose (MDD) of mouse PCSK9.
The MDD (defined as such a concentration of mouse PCSK9 giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 38.1 pg/ml of sample.

* Dilution Buffer was pipetted into blank wells.

Typical standard curve

Mouse PCSK9 standard curve
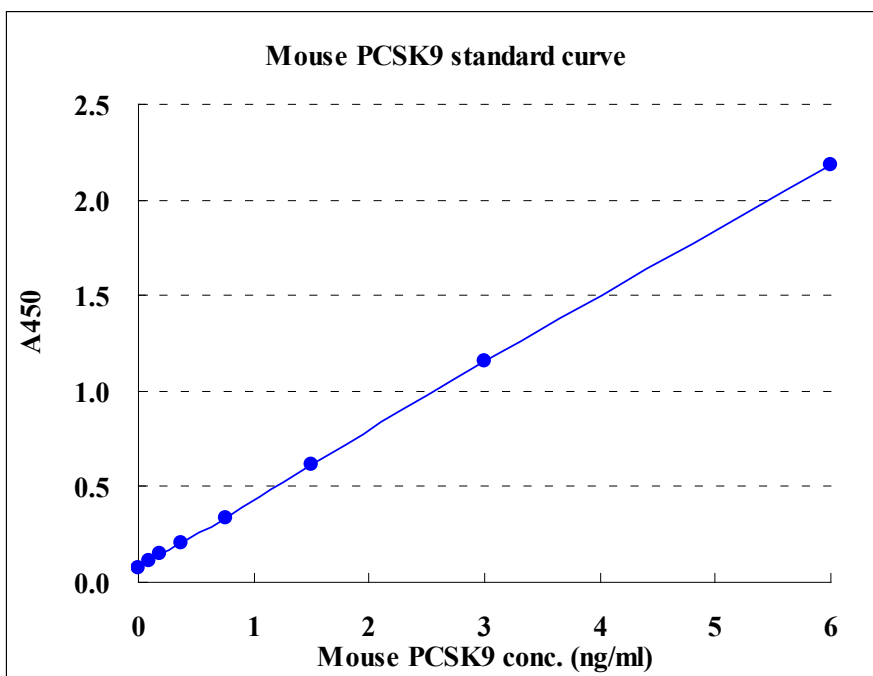
2. Precision

<u>Intra-assay Precision</u> (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=1.45-2.39 %

	Mouse PCSK9 conc. (ng/ml)		
	Serum 1	Serum 2	Serum 3
1	66.4	126.4	193.5
2	63.7	125.9	192.5
3	64.4	125.1	189.9
4	64.8	126.0	189.2
5	64.9	127.6	193.9
6	64.4	126.1	187.3
7	65.3	130.4	196.1
8	65.2	129.6	192.8
9	64.6	126.0	198.8
10	63.1	123.8	191.9
11	63.7	123.4	187.1
12	64.0	126.2	181.3
13	63.9	126.3	184.8
14	66.0	128.9	189.4
15	64.0	127.6	184.4
16	66.0	128.4	190.3
MAX.	66.40	130.40	198.80
MIN.	63.10	123.40	181.30
MEAN	**64.65**	**126.73**	**190.20**
S.D.	**0.94**	**1.93**	**4.55**
C.V.	**1.45%**	**1.52%**	**2.39%**

<u>Inter-assay Precision</u> (Precision between assays)
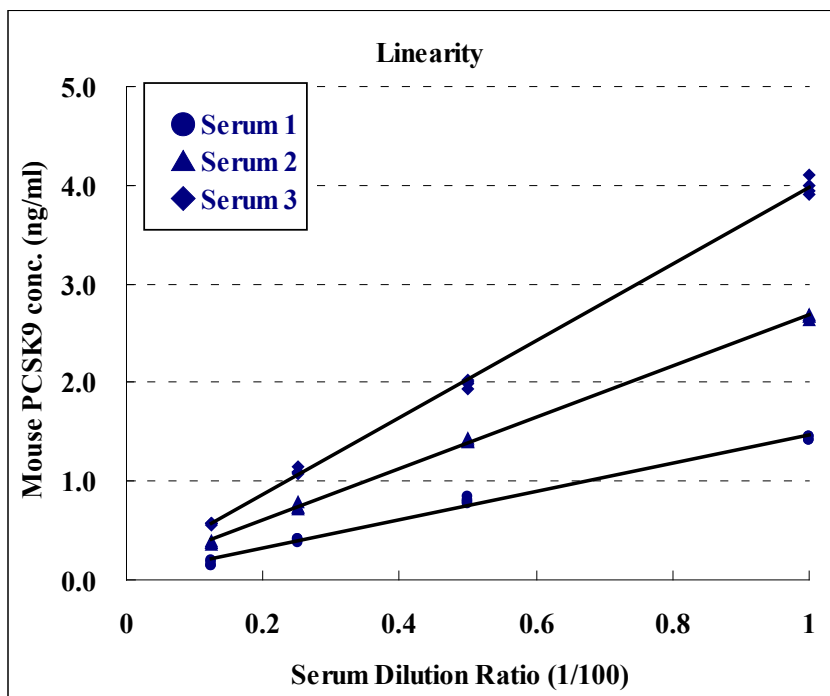   Three samples of known concentration were tested in five separate assays to assess inter-assay precision.

• Inter-assay (Run-to-Run, n=5) CV=2.78-4.72 %

	Mouse PCSK9 conc. (ng/ml)		
	Serum 1	Serum 2	Serum 3
1	129.1	252.5	380.8
2	143.7	266.6	398.6
3	136.1	263.3	410.8
4	131.6	250.3	376.7
5	142.5	262.6	412.9
MAX.	143.7	266.6	412.9
MIN.	129.1	250.3	376.7
MEAN	**136.6**	**259.1**	**395.9**
S.D.	**6.452**	**7.190**	**16.699**
C.V.	**4.72%**	**2.78%**	**4.22%**

CircuLex™

MBL
A JSR Life Sciences
Company

3. Linearity

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of mouse PCSK9 were serially diluted with the Dilution Buffer to produce samples with values within the dynamic range of the assay.

Mouse/Rat PCSK9 ELISA Kit
User's Manual
For Research Use Only, Not for use in diagnostic procedures

CircuLex™

MBL
A JSR Life Sciences
Company

4. Rat PCSK9 Measurement

This kit can be used for measuring relative amount of rat PCSK9, because the anti-PCSK9 antibody used in this kit has strong reactivity to rat PCSK9 also. However the concentration of rat PCSK9 measured by this kit may not be definite because the standard used in this kit is recombinant "mouse" PCSK9. It should be considered as a relative value.
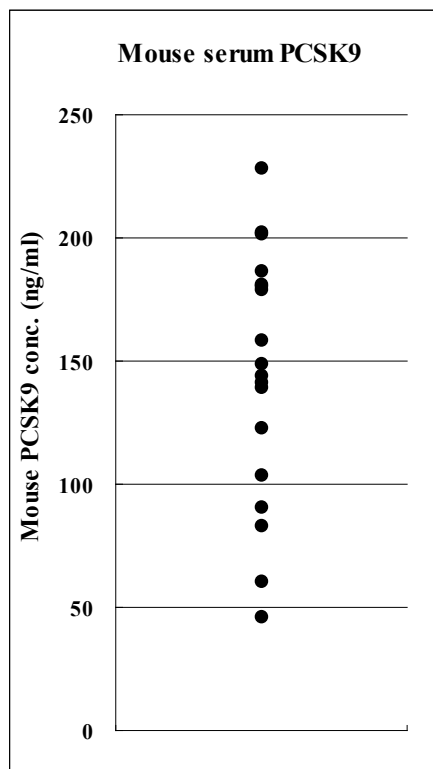
   In addition, the anti-PCSK9 antibody used in this kit shows weak cross-reactivity to human PCSK9 (<10 %).

Concentrations (relative values for Mouse PCSK9 Standard) of rat PCSK9 in Wister rat serum (n=19)
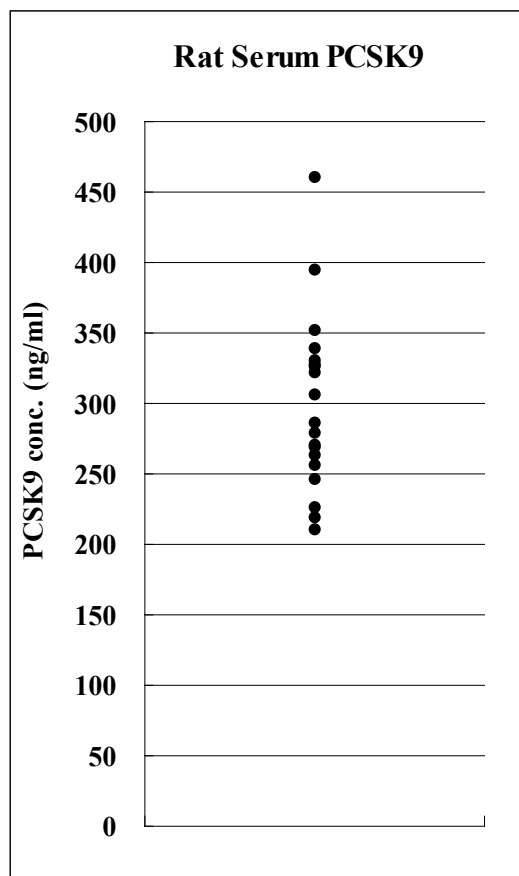
Example of Test Results

Fig.1 Concentrations of mouse PCSK9 in Balb/c mice (n=20)

Mouse serum PCSK9

References

1. Seidah NG *et al*. The secretory proprotein convertase neural apoptosis-regulated convertase 1 (NARC-1): liver regeneration and neuronal differentiation. Proc Natl Acad Sci U S A. 2003 Feb 4;100(3):928-33.

2. Abifadel M *et al*. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet. 2003 Jun;34(2):154-6.

3. Leren TP. Mutations in the PCSK9 gene in Norwegian subjects with autosomal dominant hypercholesterolemia. Clin Genet. 2004 May;65(5):419-22.

4. Allard D *et al*. Novel mutations of the PCSK9 gene cause variable phenotype of autosomal dominant hypercholesterolemia. Hum Mutat. 2005 Nov;26(5):497.

5. Cohen JC *et al*. Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med. 2006 Mar 23;354(12):1264-72.

6. Berge KE *et al*. Missense mutations in the PCSK9 gene are associated with hypocholesterolemia and possibly increased response to statin therapy. Arterioscler Thromb Vasc Biol. 2006 May;26(5):1094-100.

7. Maxwell KN and Breslow JL. Adenoviral-mediated expression of Pcsk9 in mice results in a low-density lipoprotein receptor knockout phenotype. Proc Natl Acad Sci U S A. 2004 May 4;101(18):7100-5.

8. Rashid S *et al*. Decreased plasma cholesterol and hypersensitivity to statins in mice lacking Pcsk9. Proc Natl Acad Sci U S A. 2005 Apr 12;102(15):5374-9.

9. Lagace TA *et al*. Secreted PCSK9 decreases the number of LDL receptors in hepatocytes and in livers of parabiotic mice. J Clin Invest. 2006 Nov;116(11):2995-3005.

10. Cameron J *et al*. Effect of mutations in the PCSK9 gene on the cell surface LDL receptors. Hum Mol Genet. 2006 May 1;15(9):1551-8. Epub 2006 Mar 28.

11. Zhang DW *et al*. Binding of proprotein convertase subtilisin/kexin type 9 to epidermal growth factor-like repeat A of low density lipoprotein receptor decreases receptor recycling and increases degradation. J Biol Chem. 2007 Jun 22;282(25):18602-12.

For more information, please visit our web site**.**
https://ruo.mbl.co.jp/

MANUFACTURED BY

MEDICAL & BIOLOGICAL LABORATORIES CO., LTD.
URL: https://ruo.mbl.co.jp
E-mail: support@mbl.co.jp

CycLex/CircuLex products are supplied for research use only. CycLex/CircuLex products and components thereof may not be resold, modified for resale, or used to manufacture commercial products without prior written approval from MBL. To inquire about licensing for such commercial use, please contact us via email.