Kit for Measuring Binding Activity of Human PCSK9 to LDLR *in vitro*

CircuLex Human PCSK9 Functional Assay Kit

Cat# CY-8153

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Intended Use

The MBL Research Product **CircuLex Human PCSK9 Functional Assay Kit** is primarily designed for the semi-quantitative *in vitro* measurement of functionally active of PCSK9 in serum which is capable of binding to LDL receptor (LDLR). Recombinant LDLR retaining the correct conformation to bind to functionally active PCSK9, even when it's immobilized on the microplate surface, is used in this kit. This kit allows to measure active PCSK9 in a solid-phase assay system based on a conventional ELISA in a semi-quantitative manner.

Applications for this kit include:
1) Semi–quantitative *in vitro* measurement of binding activity of native PCSK9 in serum.
2) Screening and characterization of inhibitor candidates on binding activity of PCSK9 *in vitro*.
3) Functional characterization of binding activity of mutational PCSK9s to LDLR *in vitro*.

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.

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Introduction

PCSK9 (also known as neural apoptosis-regulated convertase, NARC-1) is a 692-residue extracellular protein expressed primarily in the kidney, liver and intestine (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 level 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 MBL Research Product CircuLex Human PCSK9 Functional Assay Kit is primarily designed to measure the binding activity of PCSK9 to LDLR in serum. This kit employs a similar technique of quantitative sandwich enzyme immunoassay. Instead of antibodies, recombinant LDLR EGF-AB domain, which contains the binding site for PCSK9, is pre-coated onto a microplate. Standards and samples are pipetted into the wells and active PCSK9 functionally binds to the immobilized LDLR EGF-AB domain. After washing away any unbound substances, biotinylated anti-human PCSK9 antibody is added to the wells. Following a wash to remove any unbound biotinylated antibody, horseradish peroxidase (HRP) conjugated streptavidin is added to the wells. After wash, the remaining HRP conjugated streptavidin 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 binding activity of PCSK9. A standard curve is constructed by plotting absorbance values versus PCSK9 Binding Activity Standards of calibrators, and binding activity of unknown samples are determined using this standard curve in a semi-quantitative* manner.

* Since this measurement system may be affected by substances in serum, this kit doesn't show dilution linearity completely. For details, see "3.Linearity" in the section "Assay Characteristics" below.

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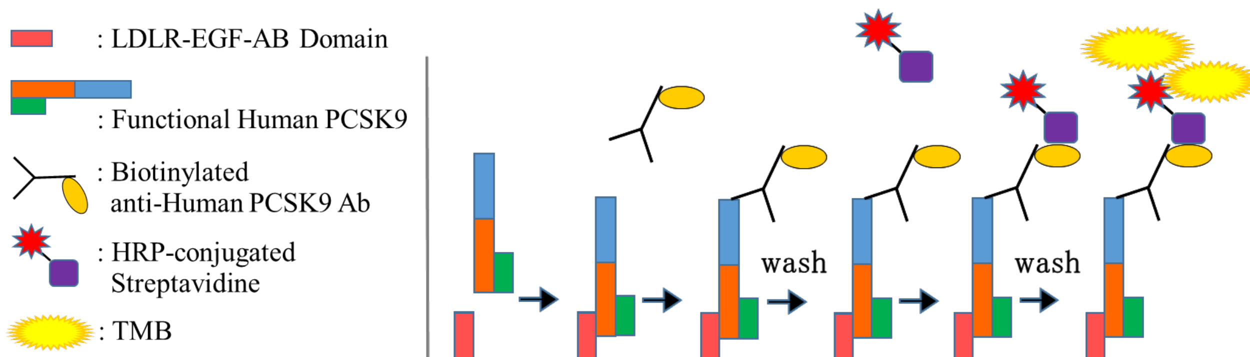
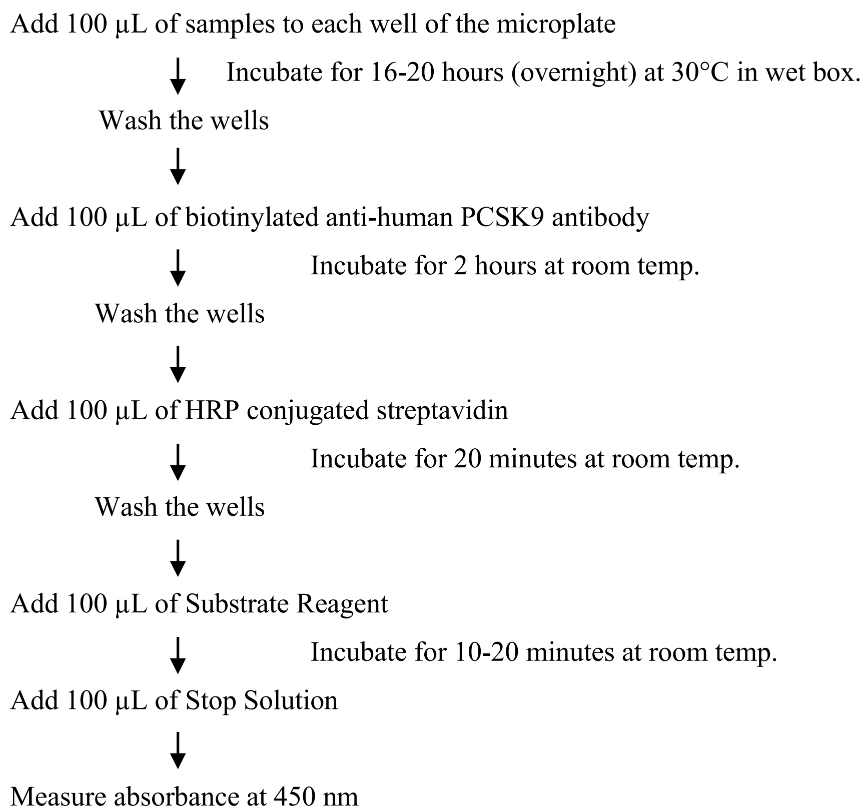
Schematic Representation of the Measuring Principle of This Assay Kit



Summary of Procedure



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Materials Provided

All samples should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microtiter plate 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 pre-coated with recombinant LDLR EGF-AB domain.

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

Sample Dilution Buffer: One bottle containing 50 mL of 1X buffer; used for dilution of samples and standards. Ready to use.

PCSK9 Binding Activity Standard: One vial containing X* mAU (Arbitrary Unit) of binding activity of lyophilized recombinant human PCSK9.
***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

Conjugate Dilution Buffer: One bottle containing 30 mL of 1X buffer; used for dilution of 100X Biotinylated Detection Antibody and 100X HRP conjugated Streptavidin. Ready to use.

100X Biotinylated Detection Antibody: One vial containing 120 µL of 100X biotinylated anti-human PCSK9 antibody.

100X HRP conjugated Streptavidin: One vial containing 120 µL of 100X solution.

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**
- **Wet Box**
- **(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 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.
- **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 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 Human PCSK9 Functional Assay 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 PCSK9 Binding Activity 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**, **100X Biotinylated Detection Antibody, 100X HRP-conjugated Streptavidin**, and **PCSK9 Binding Activity Standard**.

1. Prepare a working solution of **Wash Buffer** by adding **100 mL** of the **10X Wash Buffer** (provided) 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 **PCSK9 Binding Activity Standard** (provided) with X* **µL** of **ddH$_2$O**. After dissolved by gently mixing, immediately dispense 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 **PCSK9 Binding Activity Standard** should be **800 mAU/mL**, which is referred to as the **Master Standard**.

***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. **Std.1 (8 mAU/mL)** serves as the highest standard. **Sample Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Sample Dilution Buffer	Concentration
Std.1	10 µL of Master Standard (800 mAU/mL)	990 µL	8 mAU/mL
Std.2	300 µL of Std. 1 (8 mAU/mL)	300 µL	4 mAU/mL
Std.3	300 µL of Std. 2 (4 mAU/mL)	300 µL	2 mAU/mL
Std.4	300 µL of Std. 3 (2 mAU/mL)	300 µL	1 mAU/mL
Std.5	300 µL of Std. 4 (1 mAU/mL)	300 µL	0.5 mAU/mL
Std.6	300 µL of Std. 5 (0.5 mAU/mL)	300 µL	0.25 mAU/mL
Std.7	300 µL of Std. 6 (0.25 mAU/mL)	300 µL	0.125 mAU/mL
Blank	-	300 µL	0 mAU/mL

Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with Sample Dilution Buffer before dispensing. Discard any unused portion after use.

3. Prepare **1X Biotinylated Detection Antibody** by diluting the **100X Biotinylated Detection Antibody** (provided) 100-fold with **Conjugate Dilution Buffer** at the time of use and discard any unused portion after use.

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4. Prepare **1X HRP conjugated Streptavidin** by diluting the **100X HRP-conjugated Streptavidin** (provided) 100-fold with **Conjugate Dilution Buffer** at the time of use and discard any unused portion after use.

Sample Preparation

Dilute samples with **Sample Dilution Buffer**.
• Serum samples may require **100-fold** dilution.

Assay Procedure

1. Remove the appropriate number of strips from the foil pouch and place them into the strip holder. Return any unused strips to the foil pouch, refold, seal with tape and store at 4°C.

2. Dilute samples with **Sample Dilution Buffer**. (See "Sample Preparation" above.)

3. Pipette **100 µL** of **Standard Solutions (Std1-Std7, Blank)** and **diluted samples** in duplicates, into each well.

4. Incubate the plate **at 30°C in Wet Box for 16-20 hours (O/N)** at rest.

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 **1X Biotinylated Detection Antibody** to each well.

7. Incubate the plate **at room temperature (ca.25°C) for 2 hours**, 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 **1X HRP conjugated Streptavidin** to each well.

10. Incubate the plate **at room temperature (ca.25°C) for 20 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker.

11. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

12. Add **100 µL** of **Substrate Reagent** to each well. 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

13. 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.

14. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate

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Reagent.

15. 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 aspiration or decantation. 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 binding activity 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 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 0.125 mAU/mL to 8 mAU/mL. Any sample reading higher than the highest standard should be diluted with Sample Dilution Buffer in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the binding activity of PCSK9.

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 Human PCSK9 Functional Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date.

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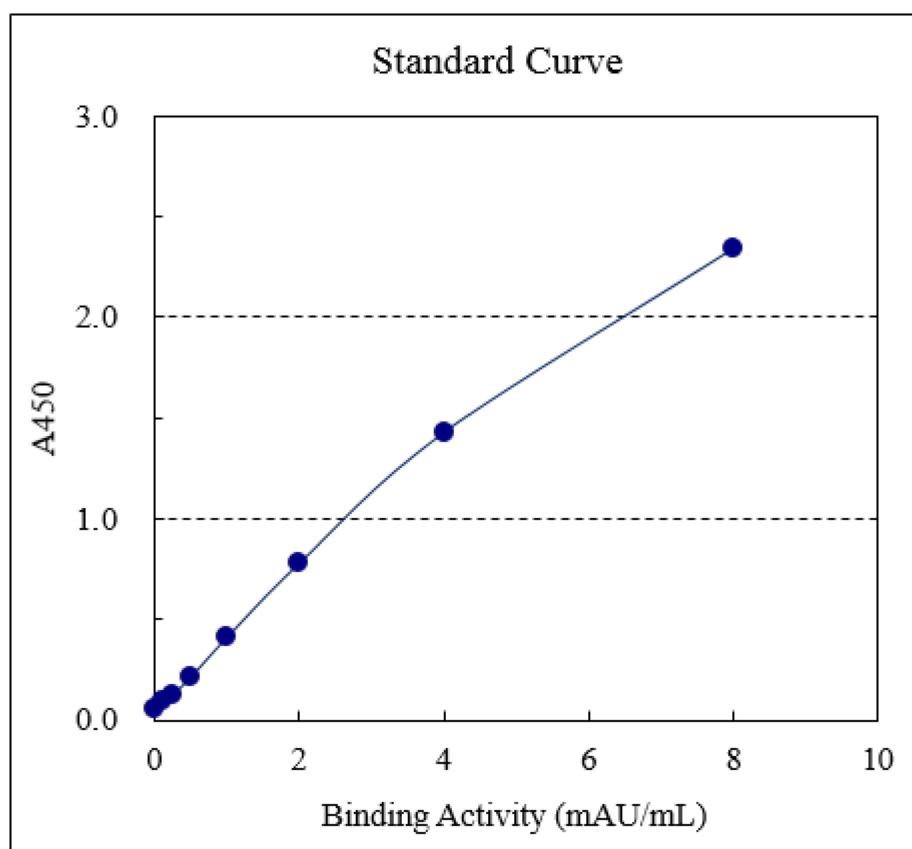
Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a binding activity of human 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 0.042 mAU/mL of sample.

* Sample Dilution Buffer was pipetted into blank wells.

Typical Standard Curve



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2. Precision

Intra-assay Precision (Precision within an assay)

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

• Intra-assay (Within-Run, n=16) CV=4.6-5.5 %

*Sample: Human serum

Binding Activity (mAU/mL)

	Sample 1	Sample 2	Sample 3
1	345.4	222.8	159.7
2	343.6	231.8	161.4
3	342.3	234.6	172.3
4	341.9	243.3	179.1
5	343.2	248.8	182.5
6	321.8	222.8	185.5
7	300.0	221.3	176.4
8	314.1	235.6	174.4
9	342.5	227.3	172.7
10	332.0	226.5	167.6
11	341.8	246.9	175.8
12	341.4	248.9	180.7
13	334.2	254.4	187.8
14	323.0	249.6	184.4
15	302.5	258.8	175.4
16	322.0	258.0	166.1
MAX.	**345.4**	**258.8**	**187.8**
MIN.	**300.0**	**221.3**	**159.7**
MEAN	**330.7**	**239.5**	**175.1**
S.D.	**15.1**	**13.1**	**8.3**
C.V.	**4.6%**	**5.5%**	**4.7%**

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Inter-assay Precision (Precision between assays)

Three samples* were tested in five separate assays to assess inter-assay precision.

• Inter-assay (Run-to-Run, n=5) CV=8.8-10.2 %

*Sample: Human serum

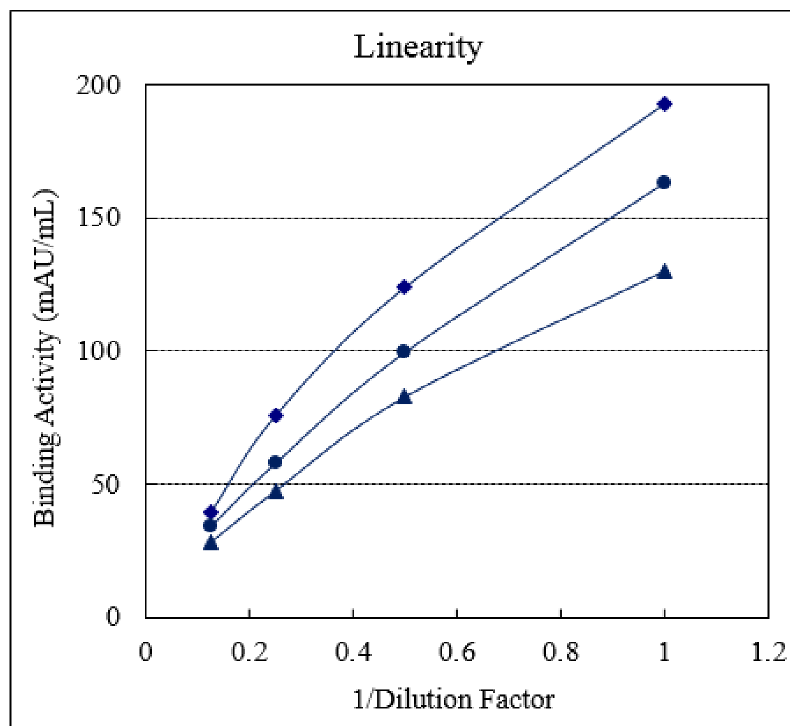
Binding Activity (mAU/mL)

	Sample 1	Sample 2	Sample 3
1	228.7	165.5	116.9
2	253.6	190.0	137.2
3	200.5	158.4	119.8
4	237.8	194.3	149.6
5	218.1	176.1	133.9
MAX.	**253.6**	**194.3**	**149.6**
MIN.	**200.5**	**158.4**	**116.9**
MEAN	**227.7**	**176.9**	**131.5**
S.D.	**20.0**	**15.4**	**13.4**
C.V.	**8.8%**	**8.7%**	**10.2%**

3. Linearity

Three samples* were diluted with Sample Dilution Buffer and assayed after dilution. The neat sample was set to 1.0.

*Sample: Human serum



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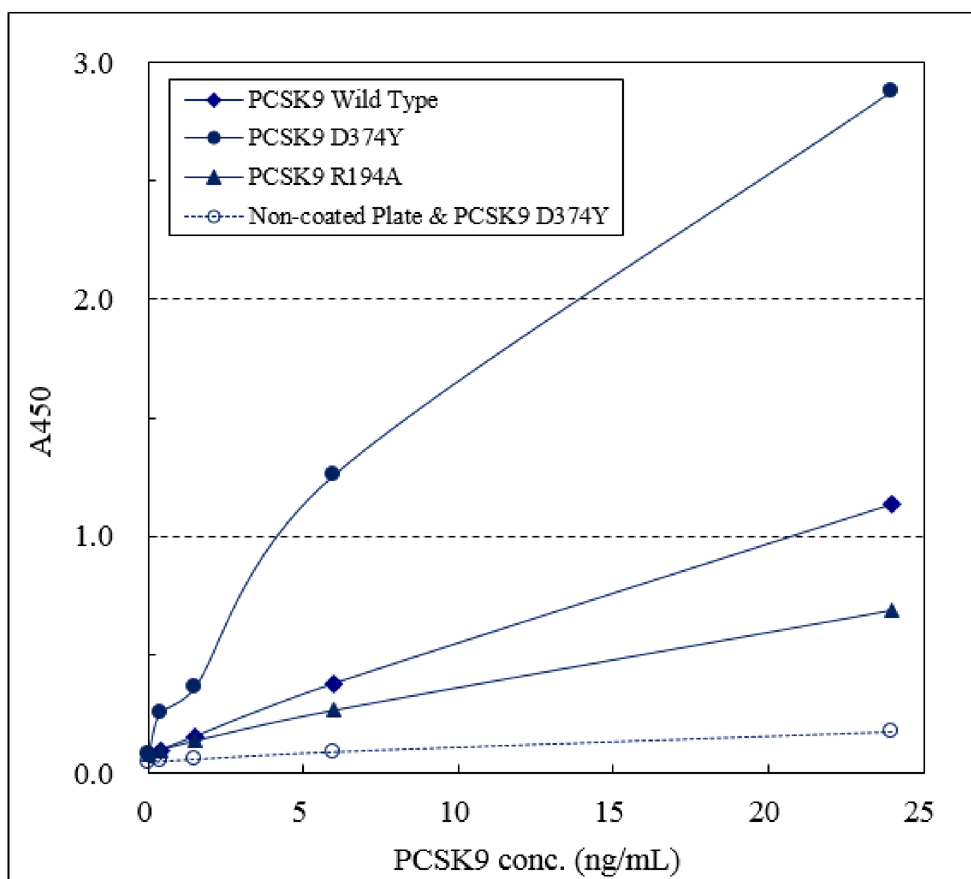
4. Reactivity

Binding activities of three types of recombinant PCSK9 proteins* were measured with this kit. All assay procedures were performed according to the section "Detailed Protocol" above, except that the incubation time at the step 4 of "Assay Procedure" was shortened to "for 2 hours".

* For the detail information of these proteins, see the section "Related Products" below).



Note: Binding activity of 1 mAU is approximately equal to the one of 1 ng of recombinant PCSK9 Wild Type (Cat# CY-R2330).

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