

**Intended Use**

PCSK9-LDLR in vitro Binding Assay Kit is primarily designed to screening inhibitors of PCSK9-LDLR interaction in vitro. In addition, this kit can be used for the rapid and sensitive evaluation of inhibitor candidates as well as characterization of PCSK9-LDLR interaction in vitro. The recombinant LDLR-EGF-AB domain used in this assay kit for keeping the correct conformation to bind PCSK9, even if immobilized on the microplate surface. This technique allows us measuring efficiently the interaction of recombinant PCSK9 to recombinant LDLR-AB domain in a solid-phase assay system such as a conventional ELISA system.

Applications for this kit include:

- 1) Screening inhibitors of PCSK9-LDLR interaction in vitro.
- 2) Characterization of inhibitor candidates of PCSK9-LDLR interaction in vitro.
- 3) Screening monoclonal antibody that inhibits PCSK9-LDLR interaction in vitro.
- 4) Characterization of PCSK9-LDLR interaction 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.

## **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 LDLC.

## **Principle of the Assay**

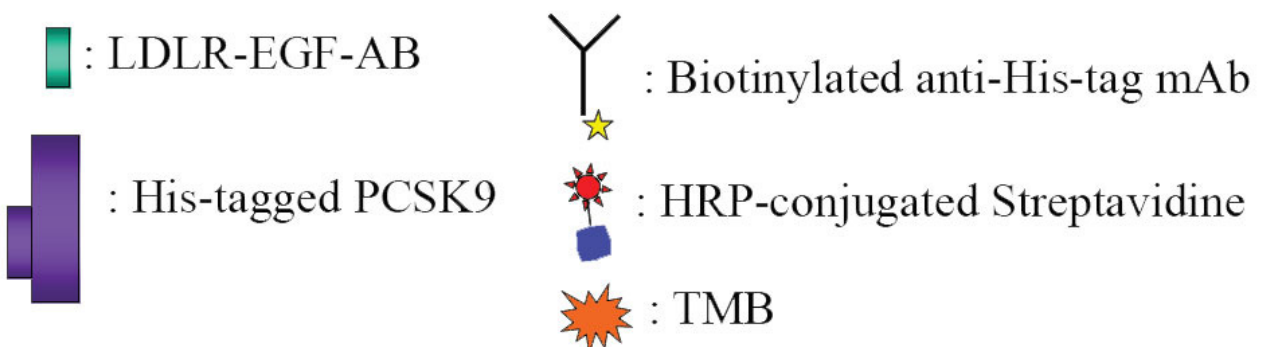
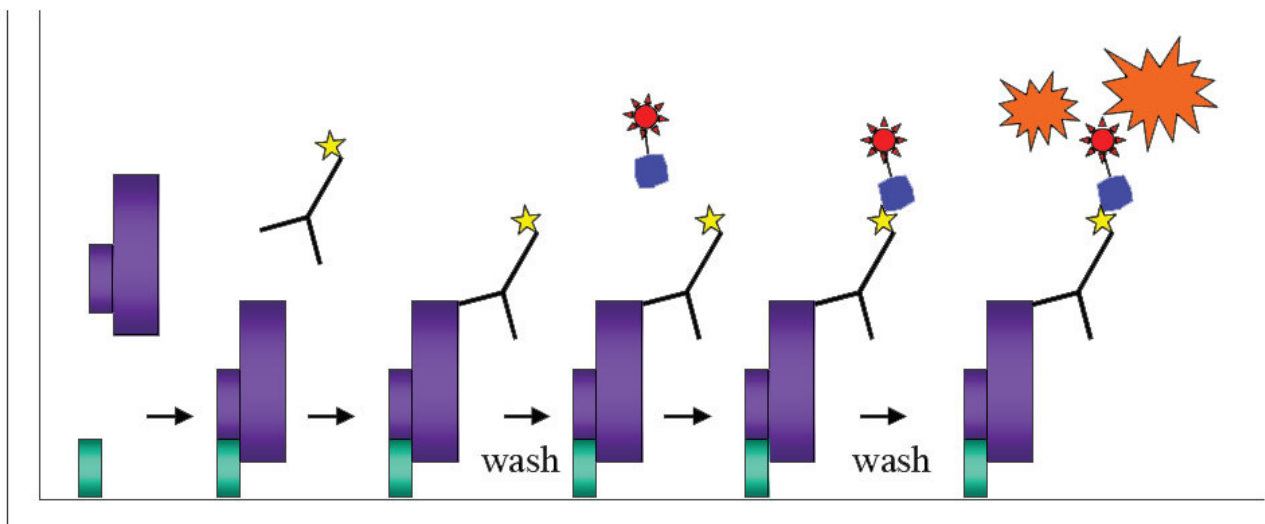
The PCSK9-LDLR in vitro Binding Assay Kit is a semi-quantitative solid phase binding assay between recombinant His-tagged PCSK9 and recombinant LDLR-AB domain. Plates are pre-coated with a recombinant LDLR-AB domain, which contains binding site for PCSK9. Biotinylated anti-His-tag monoclonal antibody specifically reacts with recombinant His-tagged PCSK9 that has been trapped with recombinant LDLR-AB domain immobilized on the microplate surface. The PCSK9-LDLR in vitro Binding Assay Kit may be used to characterize PCSK9-LDLR interaction as well as to screening inhibitors of PCSK9-LDLR interaction in vitro.

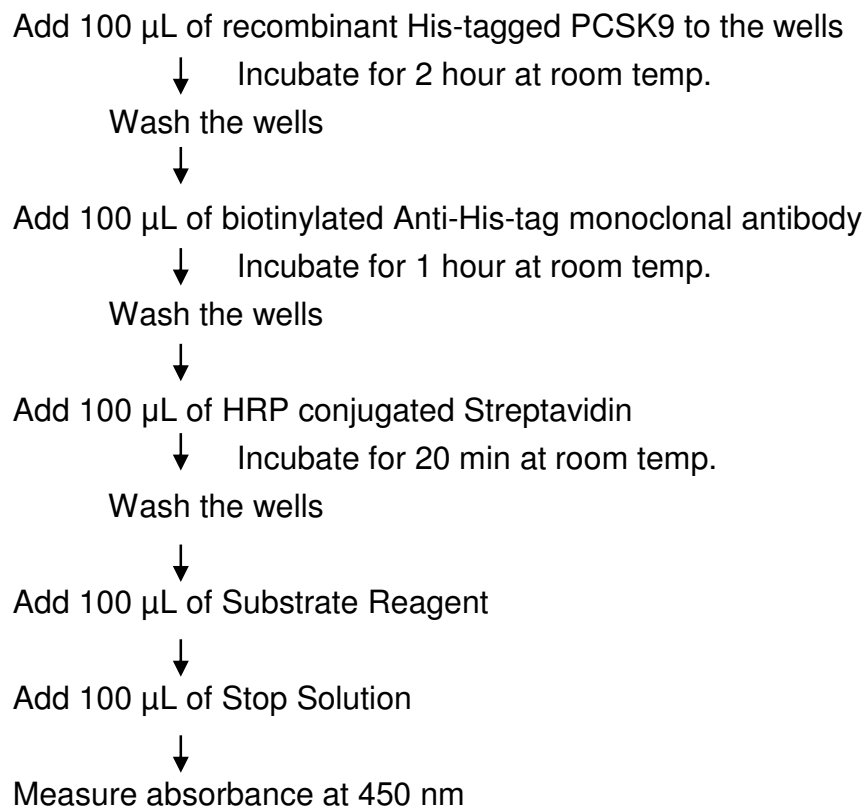
To perform the test, the recombinant His-tagged PCSK9 is diluted in Reaction Buffer, pipetted into the wells and allowed to bind recombinant LDLR-AB domain immobilized on the wells. After wash the plate, the amount of bound recombinant His-tagged PCSK9 is measured by binding it with biotinylated anti-His-tag monoclonal antibody, followed by binding with horseradish peroxidase conjugated Streptavidin, which then catalyzes the

conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stopping reagent). The color is quantitated by spectrophotometry and reflects the relative amount of recombinant His-tagged PCSK9 that binds to recombinant LDLR-AB on the microplate.

For screening inhibitors of PCSK9-LDLR interaction in vitro, test compounds or the inhibitor candidates including monoclonal antibody is added to appropriate amount of His-tagged PCSK9 in the wells that have been coated with recombinant LDLR-AB domain in a similar fashion as described above, followed by evaluation of inhibitory effect on PCSK9-LDLR interaction by measuring the amount of His-tagged PCSK9 on the wells.

### Schematic Representation of the PCSK9-LDLR in vitro Binding Assay Kit Principle



**Summary of Procedure**

## 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.

**(1) 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 Recombinant LDL-EGF-AB domain.

**(2) Recombinant His-tagged PCSK9 wild type:** Lyophilized Recombinant His-tagged PCSK9 wild type. Reconstitute contents of vial with 1.2 mL of H<sub>2</sub>O. Mix gently until dissolved. Final concentration of recombinant His-tagged PCSK9 wild type solution should be 1 µg/mL. Unused 1 µg/mL Recombinant His-tagged PCSK9 wild type solution can be stored in small aliquots (e.g. 200 µL) at below -70°C to avoid repeated freezing and thawing.

**(3) 150X Biotinylated anti-His-tag monoclonal antibody:** One vial containing 100 µL of 150X Biotinylated anti-His-tag monoclonal antibody. For long term storage, unused 150X Biotinylated anti-His-tag monoclonal antibody solution can be stored in small aliquots (e.g. 20 µL) at below -70°C to avoid repeated freezing and thawing.

**(4) Reaction Buffer:** One bottle containing 20 mL of 1X buffer; used for binding reaction and dilution of Recombinant His-tagged PCSK9 wild type. Ready to use.

**(5) 150X HRP-conjugated Streptavidin:** One vial containing 100 µL of 150X HRP-conjugated Streptavidin. For long term storage, unused 150X HRP-conjugated Streptavidin solution can be stored in small aliquots (e.g. 20 µL) at below -70°C to avoid repeated freezing and thawing.

**(6) Conjugate Dilution Buffer:** One bottle containing 30 mL of 1X buffer; used for dilution of HRP-conjugated Streptavidin. Ready to use.

- **10X Wash Buffer:** One bottle containing 100 mL of 10X Wash buffer.
- **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<sub>2</sub>SO<sub>4</sub>. Ready to use.

**Materials Required but not Provided**

- **Pipettors:** 2-20  $\mu\text{L}$ , 20-200  $\mu\text{L}$  and 200-1000  $\mu\text{L}$  precision pipettors with disposable tips.
- **Precision repeating pipettor.**
- **Orbital microplate shaker**
- **Wash bottle or multichannel dispenser** for plate washing.
- **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 residues from glassware.
- Use deionized water of the highest quality.
- Do not mix reagents from different kits.
- The buffers and reagents used in this kit contain either sodium Kathon-CG as preservatives. 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.
- **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

## Detailed Protocol

The PCSK9-LDLR in vitro Binding 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 conditions may vary, individual user should optimize the amount of Recombinant His-tagged PCSK9 wild type for inhibitor screening from dose-response curve. Disposable pipette tips and reagent troughs should be used for all transfers to avoid cross-contamination of reagents or samples.

### Preparation of Working Solution

1. Prepare a working solution of Wash Buffer by adding 100 mL of the 10X Wash Buffer (provided) to 900 mL of deionized water. Mix well. Store at 4 °C for two weeks or -20 °C for long-term storage.
2. Prepare Recombinant His-tagged PCSK9 wild type by 2-fold serially diluting the (2)Recombinant His-tagged PCSK9 wild type solution (1 µg/mL) to an appropriate concentration (usually 100-1.56 ng/mL only for drawing dose-response curve) with (4)Reaction Buffer (See section “Standard Assay to Draw Dose-Response Curve” page 9) at the time of assay.

Prepare appropriate volume for your assay. Discard any unused Recombinant His-tagged PCSK9 wild type solution after diluted.

3. Prepare 1X Biotinylated anti-His-tag monoclonal antibody by diluting the (3)150X Biotinylated anti-His-tag monoclonal antibody solution 150-fold with (6)Conjugate Dilution Buffer at the time of assay.

Prepare appropriate volume for your assay. Discard any unused 1X Biotinylated anti-His-tag monoclonal antibody after diluted.

4. Prepare 1X HRP-conjugated Streptavidin by diluting the (5)150X HRP-conjugated Streptavidin solution 150-fold with (6)Conjugate Dilution Buffer at the time of assay.

Prepare appropriate volume for your assay. Discard any unused 1X HRP-conjugated Streptavidin after diluted.



**Standard Assay to Draw Dose-Response Curve**

1. Prepare 2-fold serially diluted Recombinant His-tagged PCSK9 wild type: 100-1.56 ng/mL and Reaction Buffer only. All samples should be assayed in duplicate.
2. Add 100  $\mu$ L of sample per well. Cover with plate sealer or lid, and incubate at room temperature for 2 hours, shaking at ca. 300 rpm on an orbital microplate shaker.
3. Aspirate each well and wash four times with Wash Buffer making sure each well is filled completely. Remove residual Wash Buffer by gentle tapping or aspiration.
4. Pipette 100  $\mu$ L of 1X Biotinylated anti-His-tag monoclonal antibody into each well, cover with plate sealer or lid, and incubate at room temperature for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker.
5. Aspirate and Wash wells four times as same as in step 3.
6. Pipette 100  $\mu$ L of 1X HRP-conjugated Streptavidin into each well, cover with plate sealer or lid, and incubate at room temperature for 20 minutes, shaking at ca. 300 rpm on an orbital microplate shaker.
7. Aspirate and Wash wells five times as same as in step 3.
8. Add 100  $\mu$ L 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 A to 2-8°C immediately after the necessary volume is removed.
9. Incubate the plate for 5-15 minutes at room temperature. (Appropriate incubation time may vary, and incubation time can be extended up to 20 minutes if the reaction temperature is below than 20°C).
10. Add 100  $\mu$ L of Stop Solution to each well in the same order as the previously added Substrate Reagent.
11. Measure absorbance in each well using a spectrophotometric plate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the plate 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 signals are obtained when either O.D. values do not exceed 0.25 units for the blank (Reaction Buffer only), or 3.0 units for the 100 ng of Recombinant His-tagged PCSK9 wild type.

**Note-3:** If the microplate reader is not capable of reading absorbance greater than the absorbance of the 100 ng of Recombinant His-tagged PCSK9 wild type, perform a second reading at 405 nm. A new O.D. values, measured at 405 nm, is used to determine amount of Recombinant His-tagged PCSK9 wild type for off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

## Recommendations

Special considerations when screening inhibitors of PCSK9-LDLR interaction

In order to estimate the inhibitory effect on PCSK9-LDLR interaction in the test compounds correctly, it is necessary to conduct the control experiment of “Vehicle control” at least once for every experiment, in addition to “Test compound”, as indicated in the following table. When test compounds cause an inhibitory effect on PCSK9-LDLR interaction, the level of A450 is weakened as compared with “Vehicle control”.

For inhibitor screening

Assay Reagents	Test compound For Inhibitor	Vehicle Control
(4)Reaction buffer	85 $\mu$ L	85 $\mu$ L
20X test compound	5 $\mu$ L	-
Vehicle for Inhibitor	-	5 $\mu$ L
Recombinant His tagged PCSK9 wild type solution (1 $\mu$ g/mL)*	10 $\mu$ L	10 $\mu$ L

\* Recombinant His-tagged PCSK9 wild type solution (1  $\mu$ g/mL): See page 5, section “Materials Provided”.

1. Following the above table, add the “(4)Reaction buffer” and “20X test compound” or “Vehicle for Inhibitor” to each well of the microplate. Finally, initiate reaction by adding 10  $\mu$ L of “Recombinant His-tagged PCSK9 wild type solution” to each well and mixing thoroughly at room temperature. Cover with plate sealer or lid, and incubate at room temperature for 2 hours.
2. Follow the Standard Assay steps 3-10, page 9.

**Note: Although we suggest to conduct experiments as outlined in the table above, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user. Especially, appropriate amount of Recombinant His-tagged PCSK9 wild type must be determined by titration of the Recombinant His-tagged PCSK9 wild type and setting the amount, which shows OD value does not exceed plateau range in dose response curve.**

**NOTE: THE ABOVE PROCEDURES ARE INTENDED ONLY AS A GUIDELINE. THE OPTIMAL EXPERIMENTAL CONDITIONS WILL VARY DEPENDING ON THE PARAMETERS BEING INVESTIGATED, AND MUST BE DETERMINED BY THE INDIVIDUAL USER. NO WARRANTY OR GUARANTEE OF PERFORMANCE USING THESE PROCEDURES IS MADE OR IMPLIED.**

### **Evaluation of Results**

1. Average the absorbance values for the Recombinant His-tagged PCSK9 wild type duplicates. The absorbance value for Recombinant His-tagged PCSK9 wild type (100 ng/mL) should be greater than 1.0 with a background less than 0.25 when using Reaction buffer.
2. Inhibition percentage of test compounds should be calculated from the dose-response curve.
3. The dose-response curve of this assay fits 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.

4. 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).
5. For time course analysis, on graph paper, plot the mean absorbance values for each of the time points on the Y-axis versus the time of each reaction (minutes) on the X-axis.

### **Assay Characteristics**

The PCSK9-LDLR in vitro Binding Assay Kit has been shown to measure PCSK9-LDLR interaction in vitro. The assay may be used to screening and characterize inhibitors of PCSK9-LDLR interaction in vitro. Please consider whether your experimental condition is appropriate by drawing a direct line with the results of “Test results” in page 13-17.

### **Troubleshooting**

1. All samples should be run in duplicate, when a standard assay is being performed, 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 PCSK9-LDLR in vitro Binding Assay Kit have been tested for stability. Reagents should not be used beyond the stated expiration date.

### Test Results

Fig.1 Typical dose dependent curve of Recombinant His-tagged PCSK9 wild type (pH 6.5).

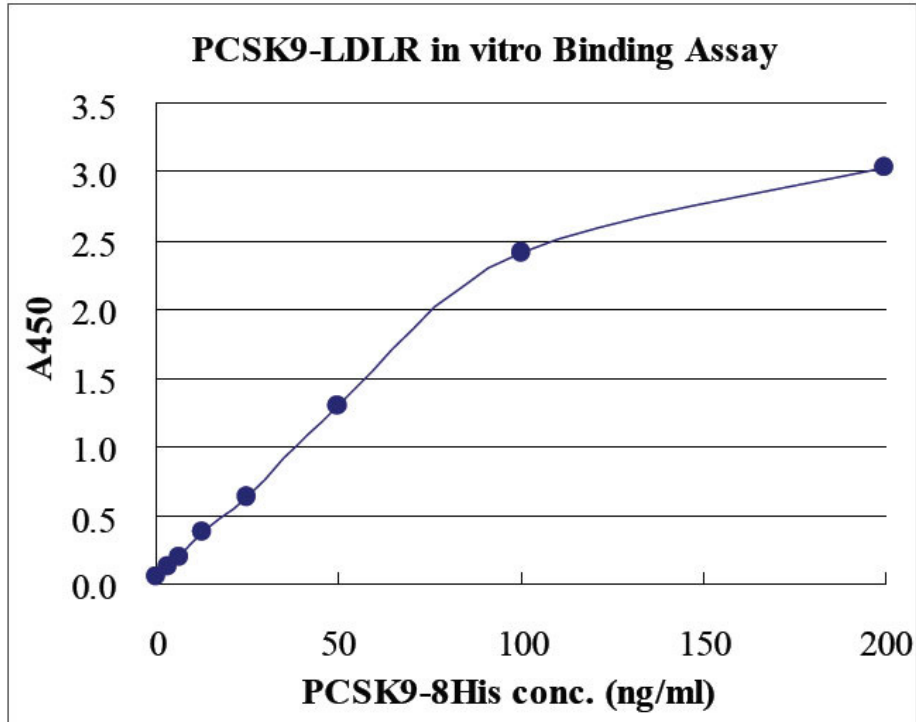


Fig.2 Time course of PCSK9-LDLR binding assay in vitro. (80 ng/mL His-tagged PCSK9 wild type was used. PH 7.2)

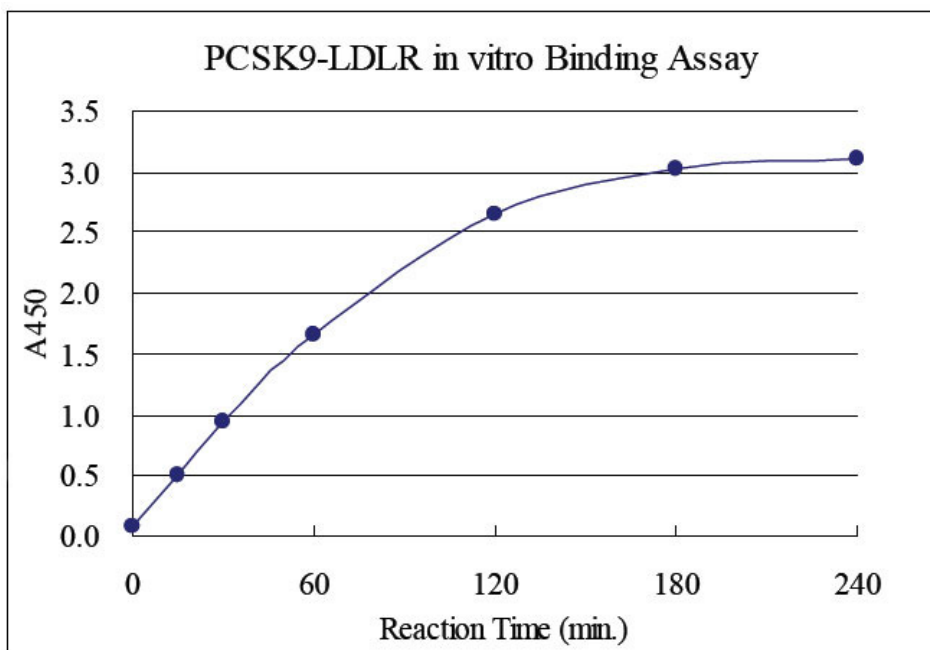


Fig.3 Effect of pH on PCSK9-LDLR binding in vitro. (80 ng/mL His-tagged PCSK9 wild type was used)

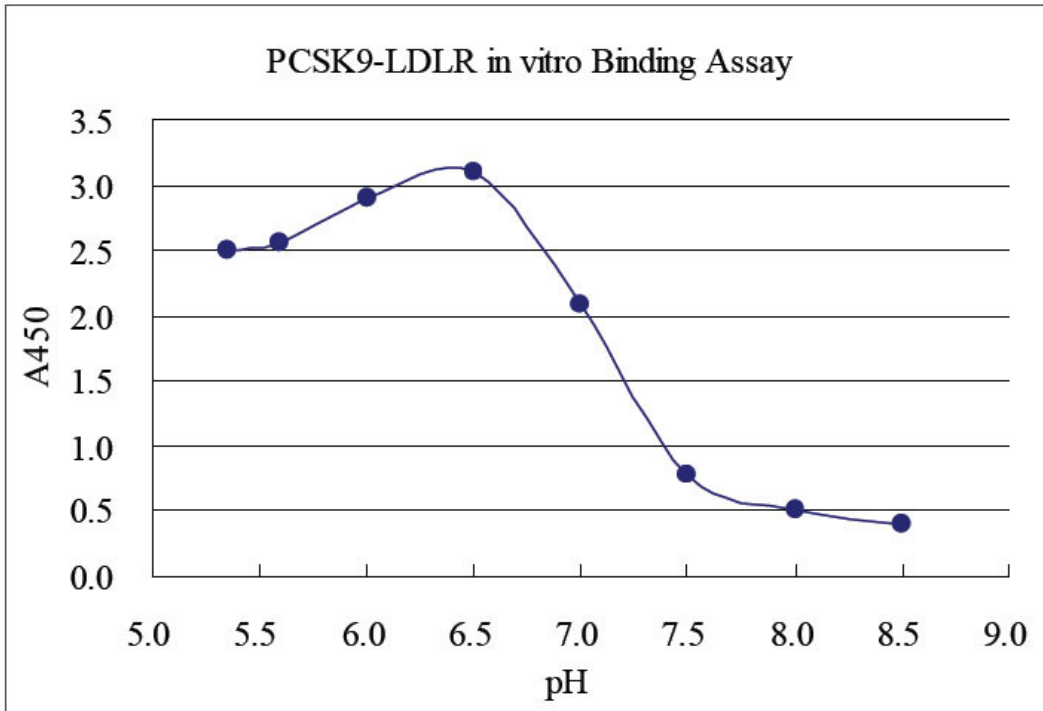


Fig.4 Effect of DMSO on PCSK9-LDLR binding in vitro

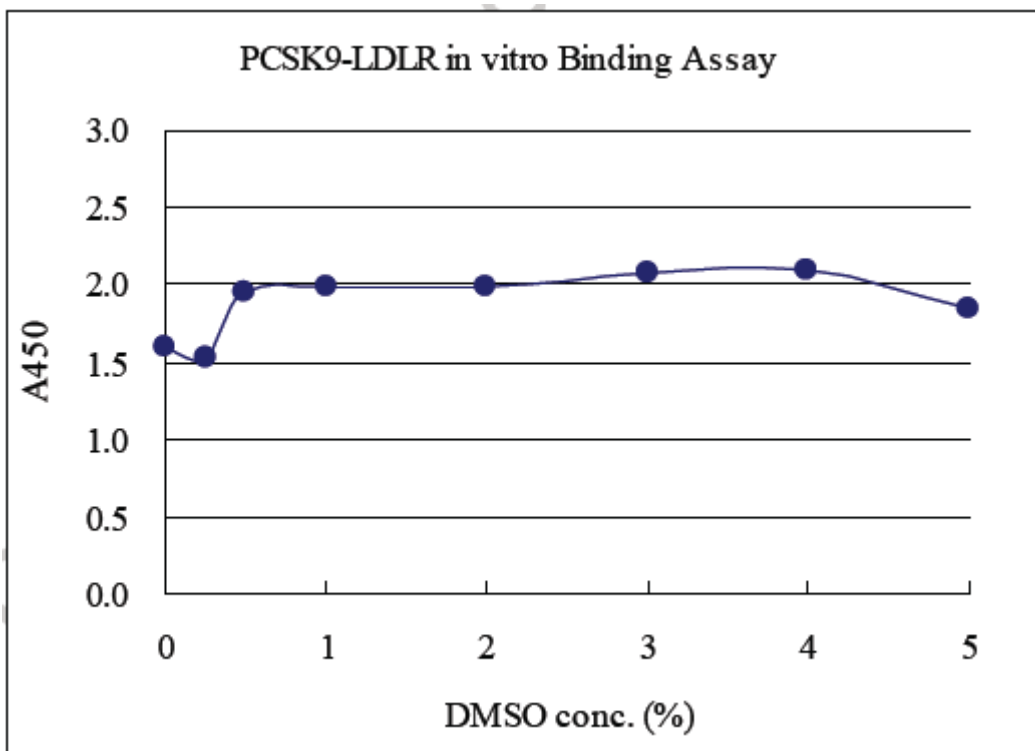


Fig.5 Effect of calcium ion on PCSK9-LDLR binding in vitro (40 ng/mL His-tagged PCSK9 wild type was used).

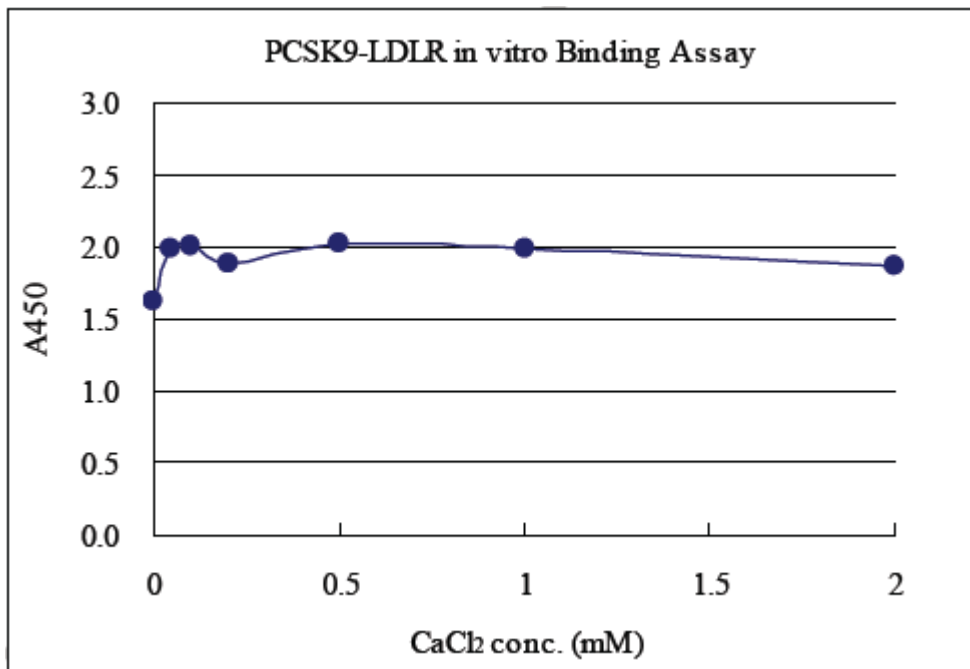
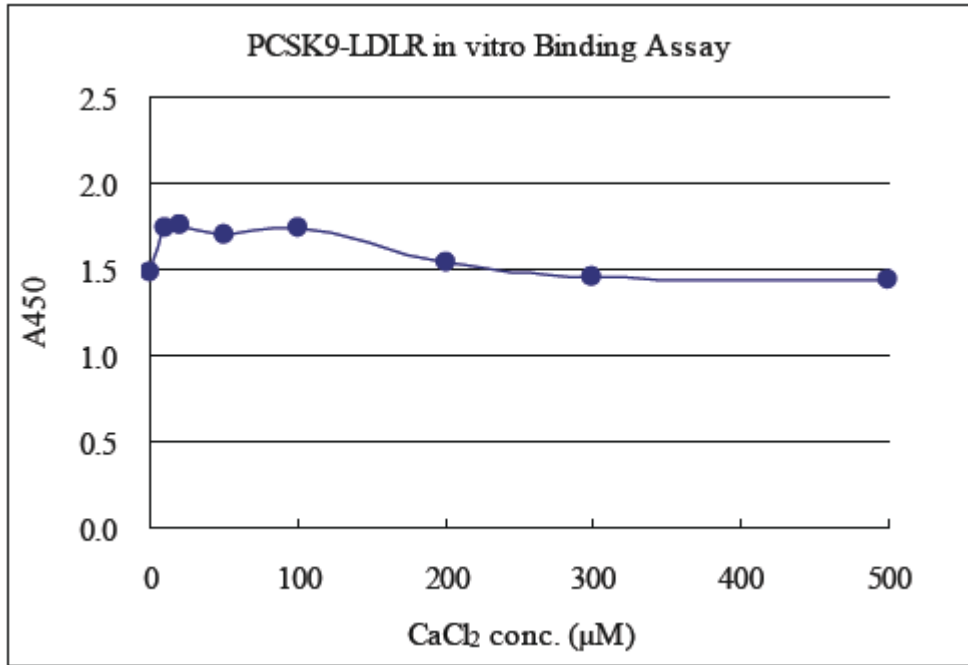


Fig.6 Effect of NaCl on PCSK9-LDLR binding in vitro (40 ng/mL His-tagged PCSK9 wild type was used).

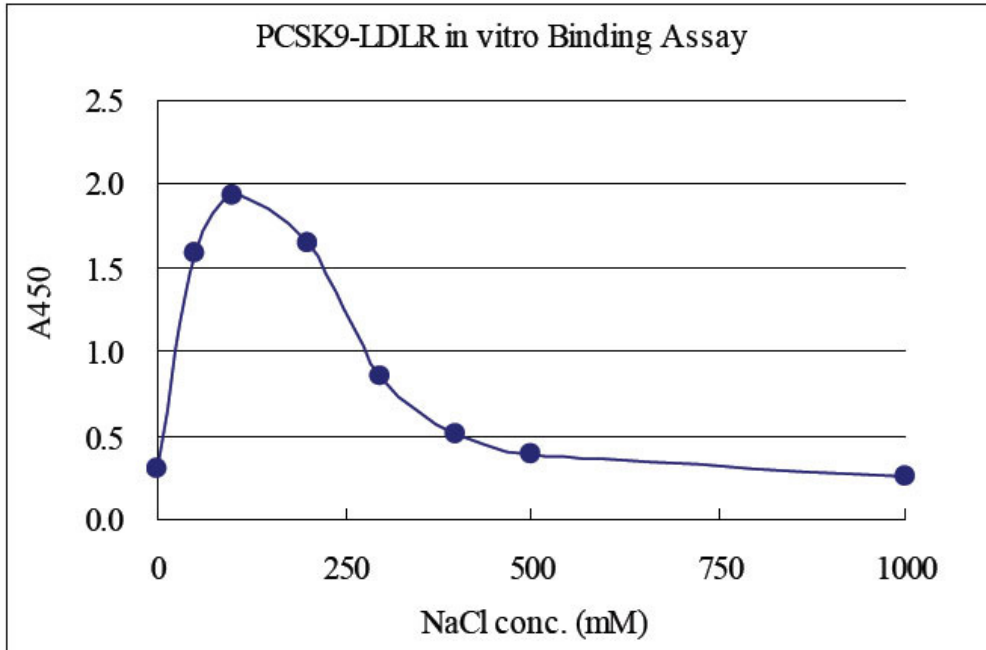


Fig.7 Effect of BSA on PCSK9-LDLR binding in vitro (40 ng/mL His-tagged PCSK9 wild type was used).

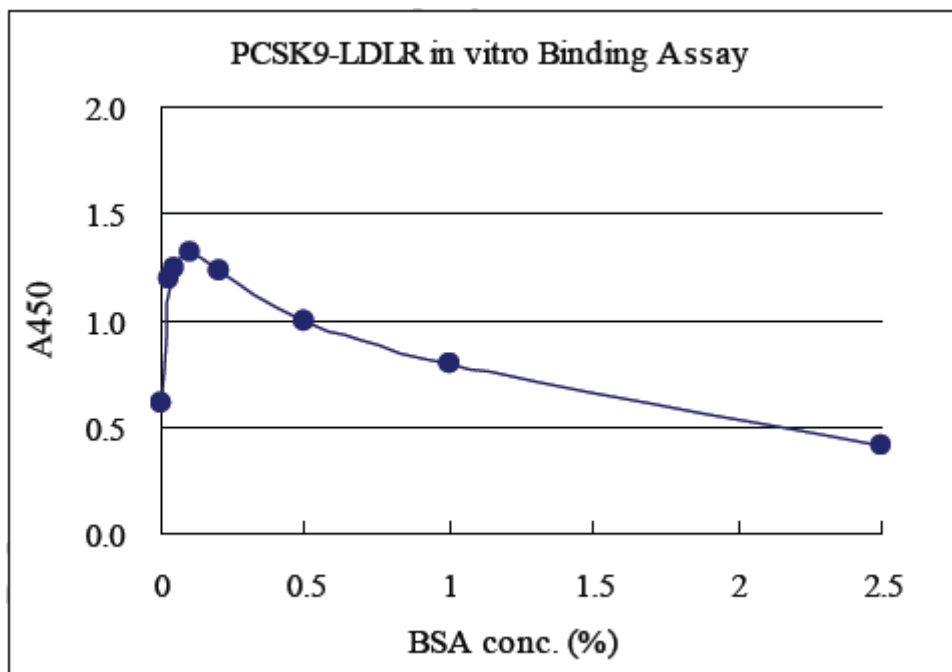
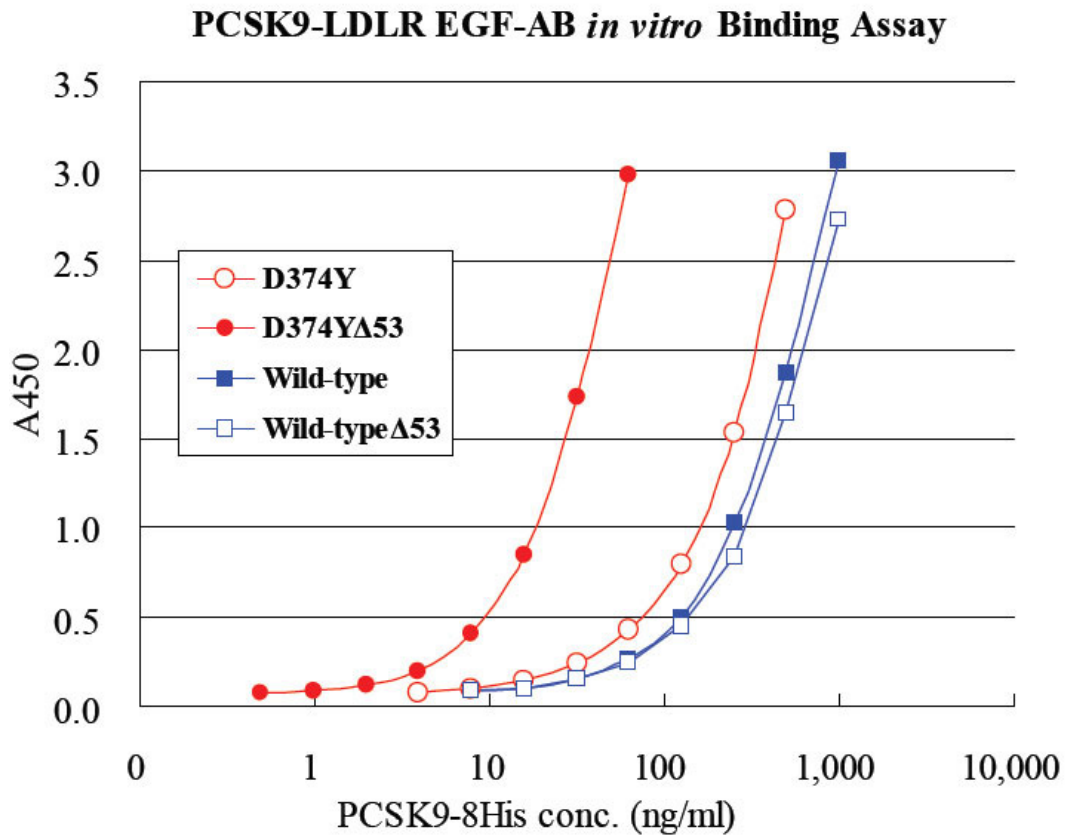




Fig. 8 Binding capability of several recombinant PCSK9s to EGF-AB domain of LDL receptor. In this experiment, following recombinant PCSK9 were used; PCSK9 Wild Type in culture medium, PCSK9 D374Y in culture medium, PCSK9 Wild Type/ $\Delta$ 53 in culture medium, PCSK9 D374Y/ $\Delta$ 53 in culture medium.



**References**

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