

Non-Radioisotopic Assay Kit for Measuring PCSK9-cIAP1 interaction *in vitro*

CircuLex PCSK9-cIAP1 *in vitro* Binding Assay Kit

Cat# CY-8152

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Intended Use

The CycLex Research Product **CircuLex PCSK9-cIAP1 *in vitro* Binding Assay Kit** is primarily designed to screening inhibitors of PCSK9-cIAP1 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-cIAP1 interaction *in vitro*. The recombinant human cIAP1 used in this assay kit was designed 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 cIAP1 in a solid-phase assay system such as a conventional ELISA system.

Applications for this kit include:

- 1) Screening inhibitors of PCSK9-cIAP1 interaction *in vitro*.
- 2) Characterization of inhibitor candidates of PCSK9-cIAP1 interaction *in vitro*.
- 3) Screening monoclonal antibody that inhibits PCSK9-cIAP1 interaction *in vitro*.
- 4) Characterization of PCSK9-cIAP1 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.
- Don't 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.

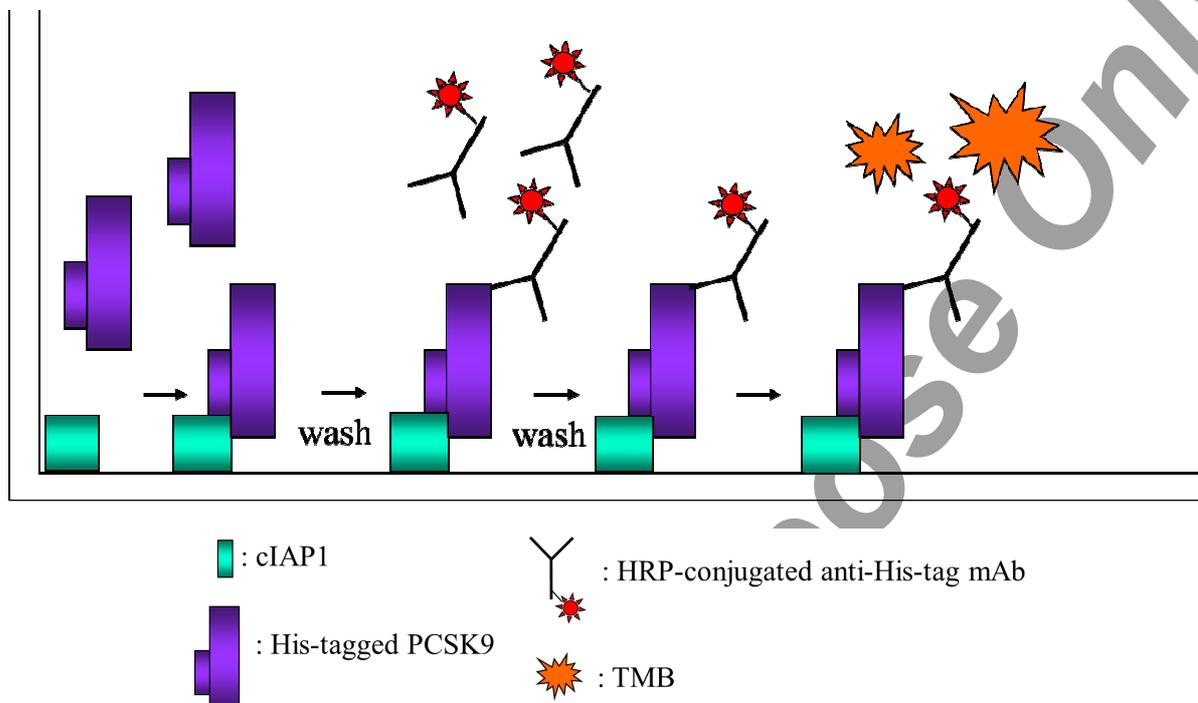
By shotgun proteomic analysis, Weiming Xu et al. identified cellular inhibitor of apoptosis protein-1 (cIAP1) as a binding partner of PCSK9 (9). They revealed that cIAP1 might regulate the activities of PCSK9 by two ways; promoting PCSK9 maturation from the proprotein form to the mature form and leading to ubiquitination of PCSK9 to lysosomal degradation. The following compelling evidences support this conclusion; Knockdown of c-IAP1 impairs PCSK9 processing and autocatalytic cleavage. In c-IAP1 null MEFs, there is a dramatic decrease in secreted mature PCSK9 protein, while in the wild-type MEF cells, proprotein form of the PCSK9 was properly processed and secreted into cell supernatants.

Principle of the Assay

The CycLex Research Products **CircuLex PCSK9-cIAP1 *in vitro* Binding Assay Kit** is a semi-quantitative solid phase binding assay between recombinant His-tagged PCSK9 and recombinant human cIAP1. Plates are pre-coated with recombinant human cIAP1, which contains binding site for PCSK9. HRP conjugated anti-His-tag monoclonal antibody specifically reacts with recombinant His-tagged PCSK9 that has been trapped with recombinant human cIAP1 immobilized on the microplate surface. The **CircuLex PCSK9-cIAP1 *in vitro* Binding Assay Kit** may be used to characterize PCSK9-cIAP1 interaction as well as to screening inhibitors of PCSK9-cIAP1 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 human cIAP1 immobilized on the wells. After wash the plate, the amount of bound recombinant His-tagged PCSK9 is measured by binding it with HRP conjugated anti-His-tag monoclonal antibody, 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 PCSK that binds to recombinant cIAP1 on the microplate.

For screening inhibitors of PCSK9-cIAP1 interaction *in vitro*, test compounds or inhibitor candidates including monoclonal antibodies are added to appropriate amount of His-tagged PCSK9 in the wells that have been coated with recombinant cIAP1 in a similar fashion as described above, followed by evaluation of inhibitory effect on PCSK9-cIAP1 interaction by measuring the amount of His-tagged PCSK9 on the wells.

Schematic Representation of the CircuLex PCSK9-cIAP1 *in vitro* Binding Assay Kit Principle**Summary of Procedure**

- Add 100 μ L of recombinant His-tagged PCSK9 to the wells
- ↓ Incubate for 2 hrs at room temp.
- Wash the wells
- ↓
- Add 100 μ L of HRP conjugated anti-His-tag monoclonal Antibody.
- ↓ Incubate for 1 hr at room temp.
- Wash the wells
- ↓
- Add 100 μ L of Substrate Reagent
- ↓
- Add 100 μ L of Stop Solution
- ↓
- Measure absorbance at 450 nm

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 coated with recombinant human cIAP1

Recombinant His-tagged PCSK9 wild type: Two vials containing 0.9 µg of lyophilized recombinant His-tagged PCSK9 wild type.

150X HRP-conjugated anti-His-tag monoclonal antibody: One vial containing 100 µL of 150X HRP-conjugated anti-His-tag monoclonal antibody. For long term storage, 150X HRP-conjugated 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.

Reaction Buffer: One bottle containing 50 mL of 1X buffer; used for binding reaction and dilution of Recombinant His-tagged PCSK9 wild type. Ready to use.

Conjugate Dilution Buffer: One bottle containing 15 mL of 1X buffer; used for dilution of HRP-conjugated anti-His-tag monoclonal antibody. Ready to use

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

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.

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**
- **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 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.
- **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

Detailed Protocol

The CycLex Research Product **CircuLex PCSK9-cIAP1 *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 Solutions

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. Reconstitute the **Recombinant His-tagged PCSK9 wild type** with 0.6 mL of **ddH₂O**. After dissolved by gently mixing, immediately dispense in small aliquots (e.g. 200 µL) to micro-centrifuge tubes and store at below -70°C to avoid repeated freezing and thawing. The final concentration of recombinant His-tagged PCSK9 wild type solution should be 1.5 µg/mL, which is referred as a **Master solution of Recombinant His-tagged PCSK9 wild type**.
3. Prepare **His-tagged PCSK9 wild type Solutions** by 2-fold serially diluting the **Master solution** (1.5 µg/mL) to an appropriate concentration (usually 50-0.78 ng/mL only for drawing dose-response curve)* with **Reaction Buffer** (See the section “Standard Assay for Drawing Dose-Response Curve” in page 8) at the time of assay**.
4. Prepare **1X HRP-conjugated anti-His-tag monoclonal antibody** by diluting the **150X HRP-conjugated anti-His-tag monoclonal antibody solution** 150-fold with **Conjugate Dilution Buffer** at the time of assay**.

* For an inhibitor screening assay, the **Master solution** (1.5 µg/mL) should be diluted with **Reaction Buffer** to appropriate concentration, which shows OD value does not exceed plateau range in dose-response curve.

** Prepare appropriate volume for your assay. Discard any unused **Recombinant His-tagged PCSK9 Solutions** and **1X HRP-conjugated anti-His-tag monoclonal antibody** after diluted.

Standard Assay for Drawing Dose-Response Curve

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. Prepare 2-fold serially diluted **His-tagged PCSK9 wild type Solutions** (150-2.34 ng/mL and Reaction Buffer only).
3. Pipette **100 µL** of the **His-tagged PCSK9 wild type Solutions** (150-2.34 ng/mL and Reaction Buffer only) in duplicates, into the wells. Cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 2 hours, shaking at ca. 300 rpm on an orbital microplate shaker.**
4. Wash 4-times by filling each well with **Wash Buffer** (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer. Remove residual Wash Buffer by gentle tapping or aspiration.
6. Add **100 µL** of **1X HRP-conjugated anti-His-tag monoclonal antibody** into each well, cover with plate sealer or lid, and incubate **at room temperature (ca.25°C) for 1 hour, shaking at ca. 300 rpm on an orbital microplate shaker.**
7. Wash 4-times as same as in step 4.
8. Add 100 µL of **Substrate Reagent** into each well. Avoid exposing the microtiter plate to direct sunlight. Covering the wells with e.g. aluminum foil is recommended. Return Substrate Reagent to 4°C immediately after the necessary volume is removed.
9. Incubate the wells **at room temperature (ca.25°C) for 10-20 minutes, shaking at ca. 300 rpm on an orbital microplate shaker.** (Appropriate incubation time may vary, and incubation time can be extended up to 30 minutes if the reaction temperature is below than 20°C).
10. Add **100 µL** 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 150 ng of His-tagged PCSK9 wild type Solution.

Standard Assay for Inhibitor Screening

Special considerations when screening inhibitors of PCSK9-cIAP1 interaction

In order to estimate the inhibitory effect on PCSK9-cIAP1 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-cIAP1 interaction, the level of A450 is weakened as compared with "Vehicle Control".

For inhibitor screening

Assay Reagents	Test Compound	Vehicle Control
Reaction buffer	85 μ L	85 μ L
20X Test Compound(s)	5 μ L	-
Vehicle for 20X Test Compound	-	5 μ L
10X His-tagged PCSK9 wild type Solution *	10 μ L	10 μ L

* 10x concentrated solution of the His-tagged PCSK9 wild type Solution, which shows OD value does not exceed plateau range in dose-response curve. (Usually 500-750 ng/mL; see below): See the section "I. Preparation of Working Solutions" in page 7.

1. Following the above table, add the "Reaction buffer" and "20X Test compound" or "Vehicle for 20X Test compound" to each well of the microplate.
2. Initiate reaction by adding 10 μ L of "10X His-tagged PCSK9 wild type Solution" to each well and mixing thoroughly. 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. Follow the steps 4-11 of "Standard Assay for Drawing Dose-Response Curve" in page 8.

Note-1: The optimal experimental conditions must be determined by the individual user. Especially, appropriate concentration of 10X His-tagged PCSK9 wild type Solution must be determined by drawing dose-response curve, which shows OD value does not exceed plateau range in the dose response curve.

Note-2: To obtain an accurate inhibition percentage of test compounds, draw a dose-response curve with appropriate serial dilutions of 10X His-tagged PCSK9 wild type Solution in parallel with the inhibition assay of the test compounds.

NOTE: THE ABOVE PROCEDURES ARE INTENDED ONLY AS A GUIDELINE. THE OPTIMAL EXPERIMENTAL CONDITIONS WILL VARY DEPENDING ON THE PARAMETERS BEING INVESTIGATED. NO WARRANTY OR GUARANTEE OF PERFORMANCE USING THESE PROCEDURES IS MADE OR IMPLIED.

Evaluation of Results

To obtain an accurate inhibition percentage of test compounds, it's required to draw and calculate form a dose-response curve with serial dilutions of 10X His-tagged PCSK9 Solution in parallel with the inhibition assay of the test compounds.

1. Average the duplicate readings for each "Test Compound", "Vehicle Control" and "serial dilutions of 10X His-tagged PCSK9 Solution". The absorbance value for "Vehicle Control" 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 four-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a four-parameter logistic function. It is important to make an appropriate mathematical adjustment to accommodate for inhibition percentage.
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).

Note: The CycLex Research Product **CircuLex PCSK9-cIAP1 *in vitro* Binding Assay Kit** has been shown to measure PCSK9-cIAP1 interaction *in vitro*. The assay may be used to screening and characterize inhibitors of PCSK9-cIAP1 interaction *in vitro*.

Please consider whether your experimental condition is appropriate by drawing a direct line with the results of "Assay Characteristics" in page 12-16.

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 CycLex Research Product **CircuLex PCSK9-cIAP1 *in vitro* Binding Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date.

Assay Characteristics

Fig.1 Typical dose dependent curve of Recombinant His-tagged PCSK9 wild type (pH 5.0)

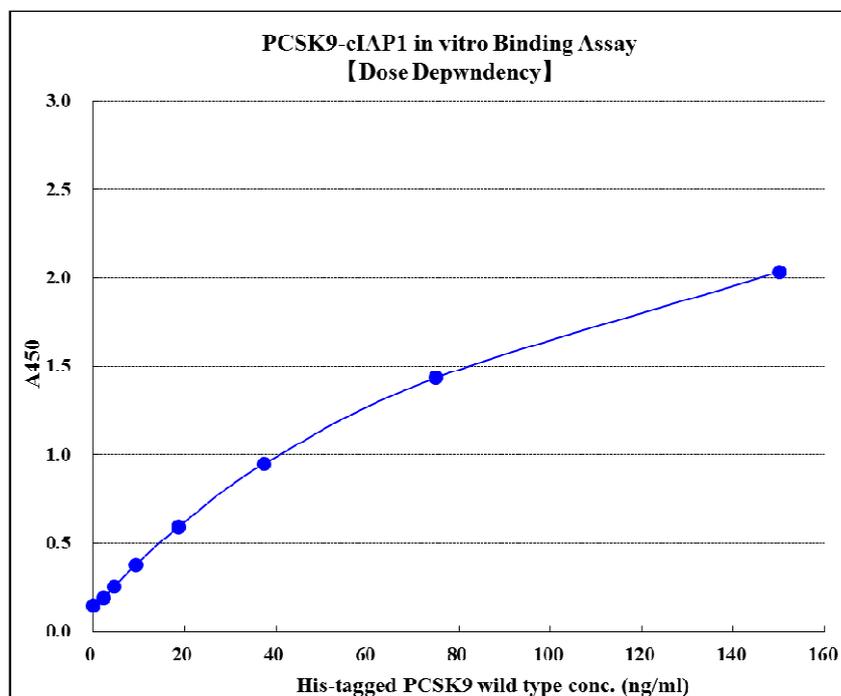


Fig.2 Effect of pH on PCSK9-cIAP1 binding *in vitro* (150 ng/mL His-tagged PCSK9 wild type was used)

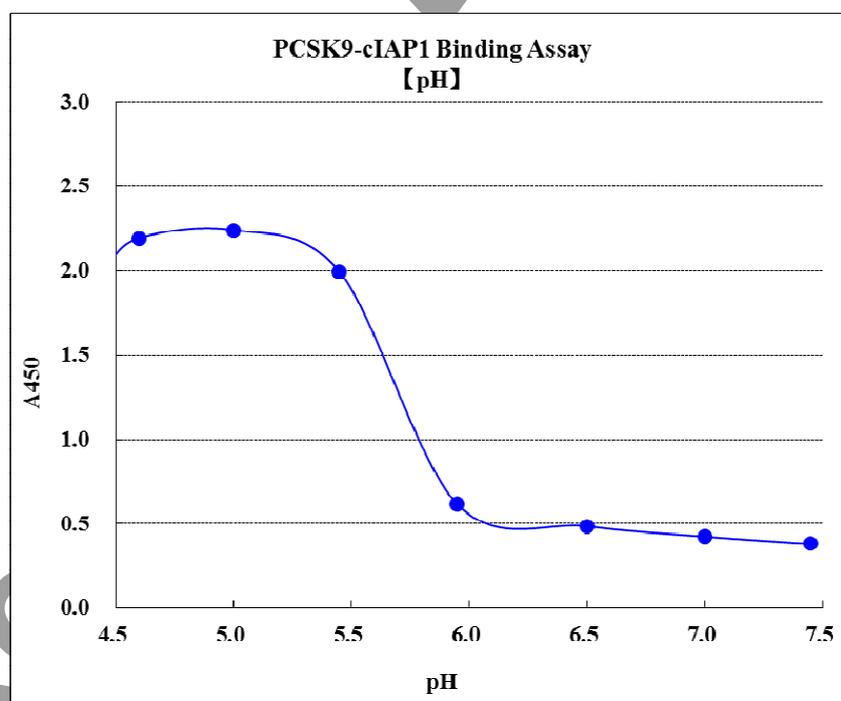


Fig.3 Effect of DMSO on PCSK9-cIAP1 binding *in vitro* (150 ng/mL His-tagged PCSK9 wild type was used)

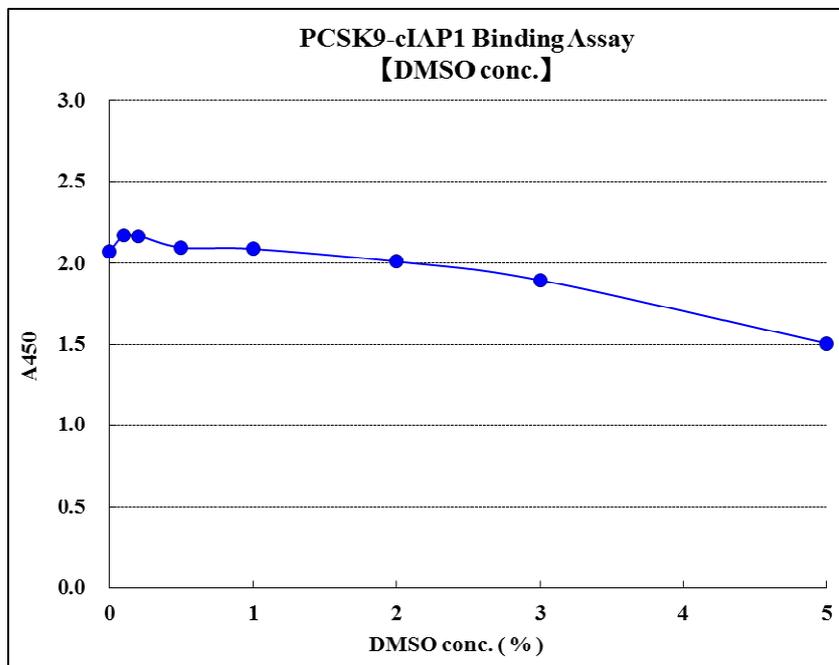


Fig.4 Effect of NaCl on PCSK9-cIAP1 binding *in vitro* (150 ng/mL His-tagged PCSK9 wild type was used)

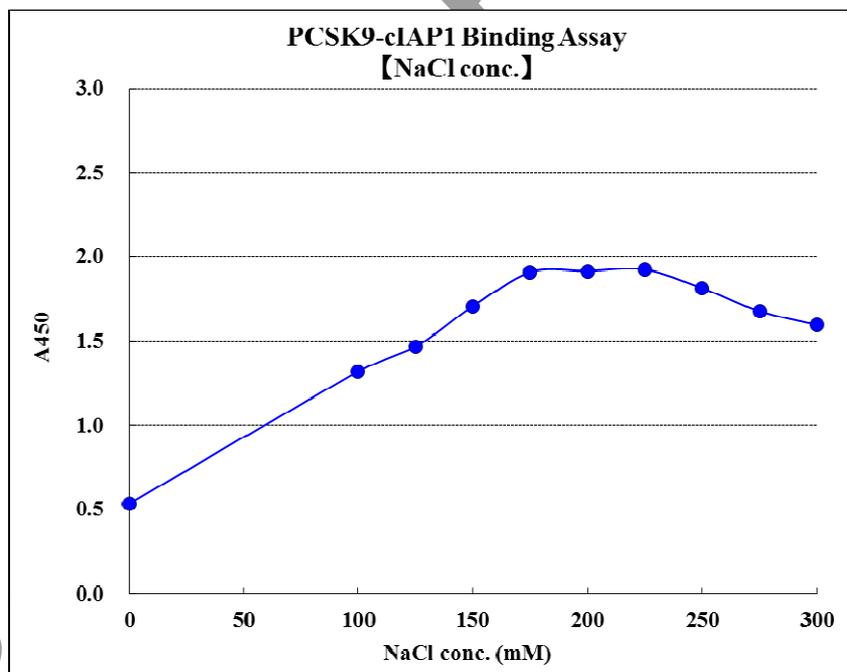


Fig.5 Effect of calcium ion on PCSK9-cIAP1 binding *in vitro* (50 ng/mL His-tagged PCSK9 wild type was used).

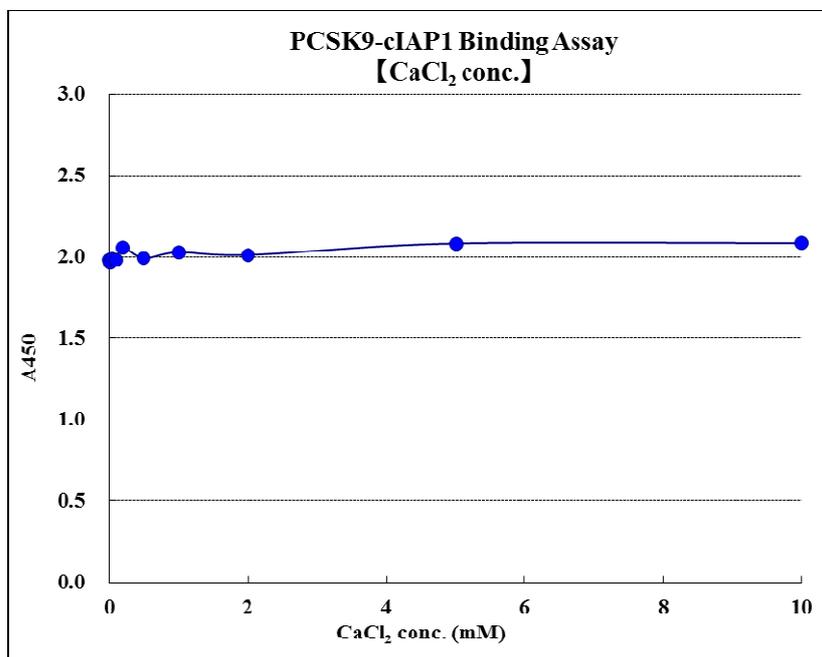


Fig.6 Effect of magnesium ion on PCSK9-cIAP1 binding *in vitro* (150 ng/mL His-tagged PCSK9 wild type was used).

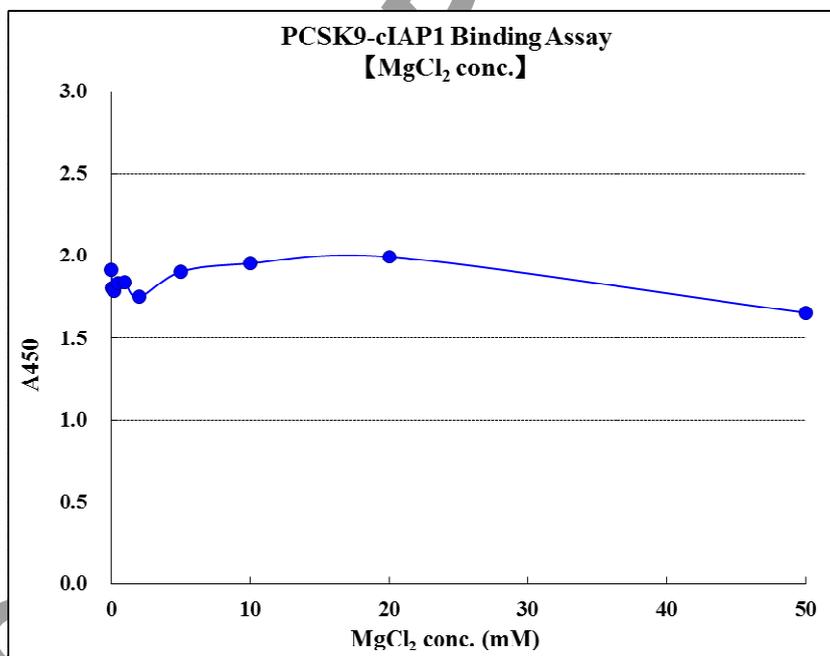


Fig.7 Effect of BSA on PCSK9-cIAP1 binding *in vitro* (150 ng/mL His-tagged PCSK9 wild type was used)

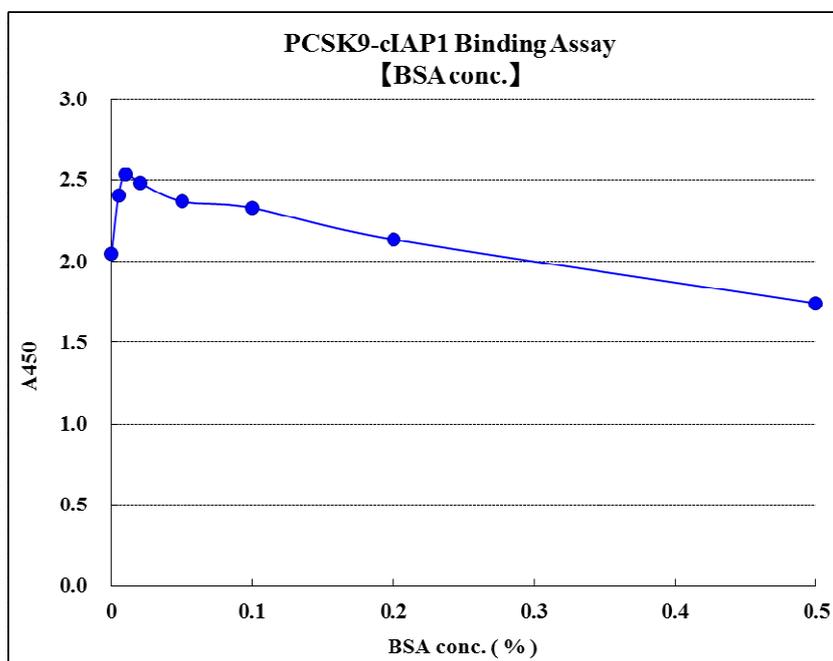


Fig.8 Effect of EDTA on PCSK9-cIAP1 binding *in vitro* (150 ng/mL His-tagged PCSK9 wild type was used)

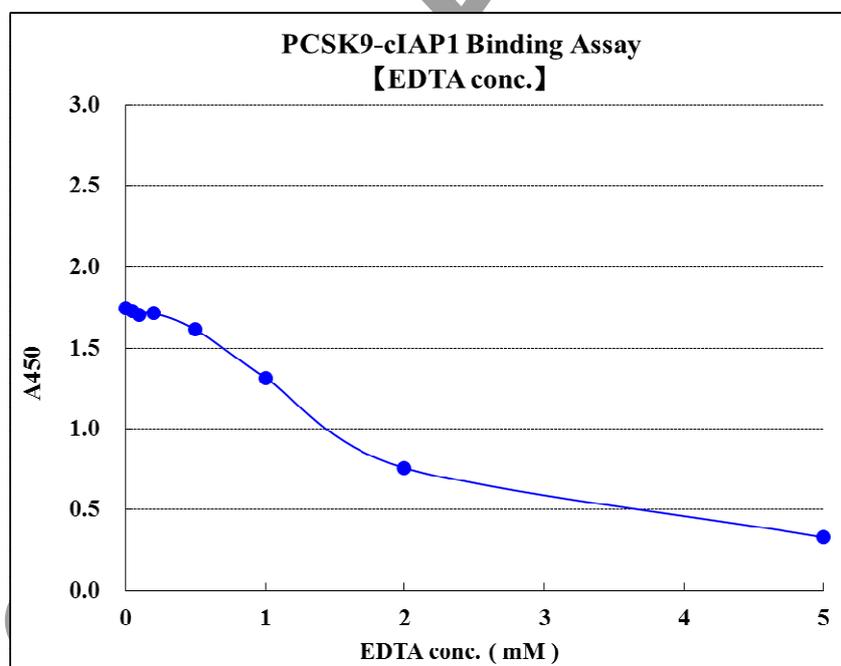


Fig.9 Effect of EGTA on PCSK9-cIAP1 binding *in vitro* (150 ng/mL His-tagged PCSK9 wild type was used)

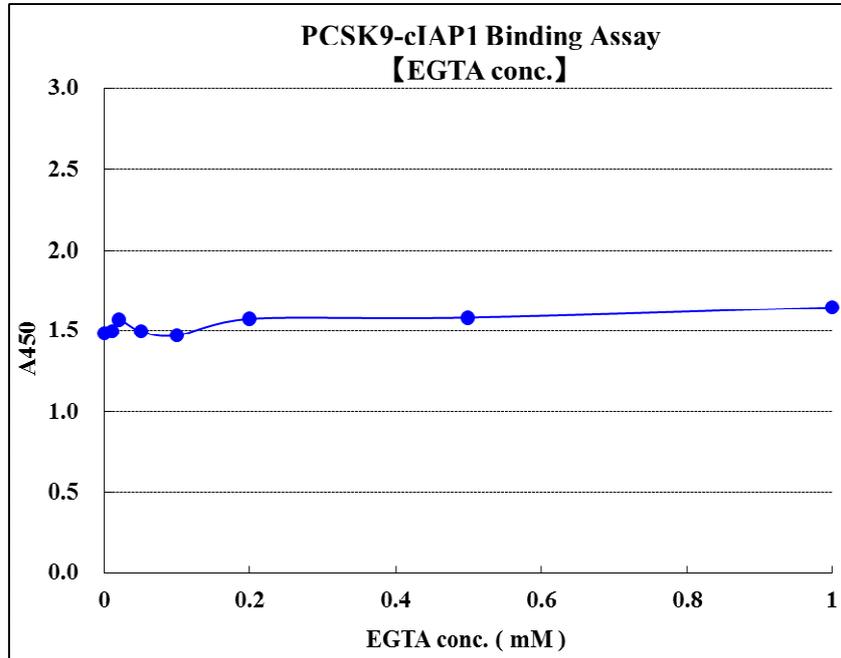
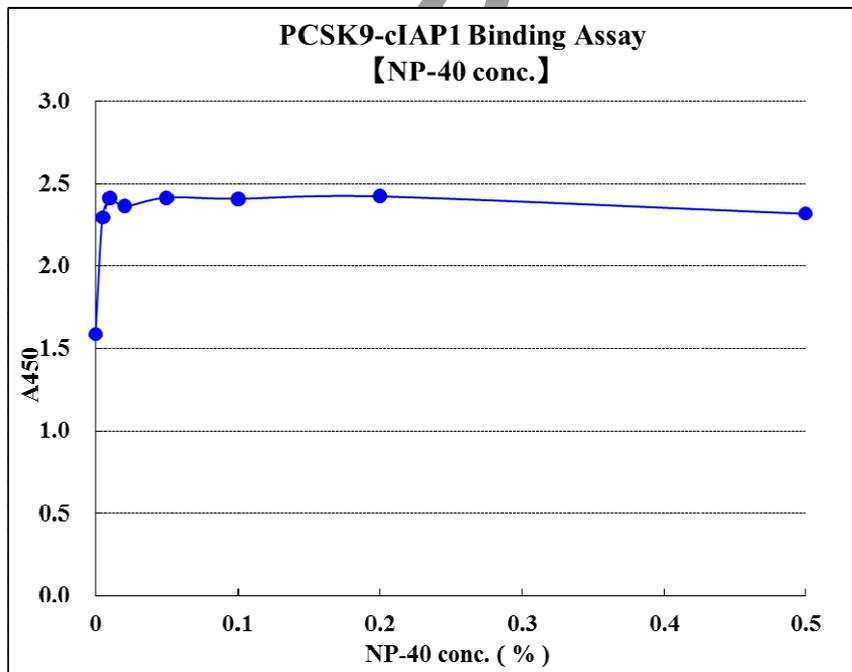


Fig.10 Effect of NP-40 on PCSK9-cIAP1 binding *in vitro* (150 ng/mL His-tagged PCSK9 wild type was used)



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Related Products

- * CircuLex PCSK9-LDLR *in vitro* Binding Assay Kit: Cat# CY-8150
- * CircuLex PCSK9-cIAP1 *in vitro* Binding Assay Kit: Cat# CY-8152
- * CircuLex Mouse/Rat PCSK9 ELISA Kit: Cat# CY-8078
- * CircuLex Human PCSK9 ELISA Kit: Cat# CY-8079
- * Anti-Human PCSK9 prodomain monoclonal antibody (KS-3C8): Cat# CY-M1032
- * Anti-Human PCSK9 monoclonal antibody (KS-4H12): Cat# CY-M1033
- * PCSK9 Wild Type in culture medium: Cat# CY-R2310
- * PCSK9 Δ (33-53) / Wild Type in culture medium: Cat# CY-R2320
- * PCSK9 D374Y in culture medium: Cat# CY-R2311
- * PCSK9 Δ (33-53) / D374Y in culture medium: Cat# CY-R2321
- * PCSK9 Wild Type/ Δ 53 in culture medium: Cat# CY-R2320
- * PCSK9 Wild Type: Cat# CY-R2330
- * PCSK9 D374Y: Cat# CY-R2331
- * PCSK9 R194A: Cat# CY-R2333
- * LDLR EGF-AB domain: Cat# CY-R2340
- * LDLR EGF-AB domain, Myc-tagged: Cat# CY-R2341
- * LDLR EGF-AB domain H306Y: Cat# CY-R2342
- * LDLR EGF-AB domain H306Y, Myc-tagged: Cat# CY-R2343

PRODUCED BY

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

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