Quantikine® ELISA

Mouse Proprotein Convertase 9/PCSK9 Immunoassay

Catalog Number MPC900

For the quantitative determination of mouse Proprotein Convertase 9 (PCSK9) concentrations in cell culture supernates, tissue lysates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

PCSK9 (proprotein convertase subtilisin kexin 9), also called proprotein convertase 9 or NARC-1 (neural apoptosis-regulated convertase 1), is a member of the proteinase K subfamily of subtilisin-related serine endoproteases. Mouse PCSK9 cDNA encodes 694 amino acids, including a signal peptide, a prodomain, and a catalytic domain. PCSK9 is highly expressed in the liver, intestine, and kidney. It is initially synthesized as a soluble 74 kDa precursor protein. In the endoplasmic reticulum, it undergoes autocatalytic intramolecular cleavage to generate a 14 kDa prodomain and a 60 kDa catalytic domain. While within the secretion pathway, the prodomain remains associated and functions as a chaperone for the catalytic domain (1-4). During secretion, a portion of active PCSK9 may undergo additional N-terminal proteolysis by furin or proprotein convertase 5/6A, creating an inactive 53 kDa form (5). This cleavage site is conserved between mouse and human or rat PCSK9, which share 78% or 93% amino acid sequence identity, respectively. While the 60 kDa protein is the major form, its ratio with the 53 kDa forms is variable in humans (5, 6).

The primary physiologic function of PCSK9 is to mediate the degradation of low density lipoprotein receptor (LDLR). Early observations indicated that gain-of-function missense mutations in the human PCSK9 gene can cause an autosomal dominant form of hypercholesterolemia (7, 8). The expression of PCSK9 is also upregulated by the sterol regulatory element binding proteins (SREBPs), a family of transcription factors that are responsible for the upregulation of genes involved in cholesterol and fatty acid metabolism, such as the LDLR gene (9, 10). Further experimental evidence revealed that when the mouse PCSK9 gene is deleted, LDLR expression in hepatocytes is increased. Conversely, PCSK9 over-expression decreases liver LDLR protein expression (11, 12). In humans, genetic analyses have shown that individuals who have nonsense or loss-of-function mutations in the PCSK9 gene have significantly lower plasma LDL cholesterol levels, while in mouse, administration of a PCSK9 neutralizing antibody or antisense oligonucleotides lowers serum cholesterol (1, 13-15). These investigations clearly indicate that PCSK9 plays a key role in reducing the hepatic LDLR levels. Paradoxically, administration of cholesterol-lowering drugs such as statins appear to enhance production of PCSK9 (6).

The underlying mechanism of cholesterol regulation by PCSK9 is as follows: under normal physiologic conditions, the LDLR is internalized at the cell surface and directed to the endosomes in order to be recycled back to the cell surface. PCSK9 binds to the EGF domain of the LDLR and prevents LDLR from being sorted to the endosomes. Instead, the PCSK9/LDLR complex is redistributed to the lysosomes for degradation (16-18). As such, PCSK9 regulates the amount of LDLR in the circulation and hence, modulates cholesterol levels. Serum PCSK9 concentrations have been found to be directly associated with cholesterol levels (19, 20). Since PCSK9 loss-of-function mutations strikingly reduce risk of coronary heart diseases, PCSK9 has become an attractive drug target (1, 21, 22). One approach is to generate small molecules that are able to interfere with PCSK9 autoactivation and its interaction with LDLR. Other approaches aiming to reduce the amount of PCSK9 in the circulation, such as small interfering RNAs (siRNAs), have also shown promise (23, 24).

The Quantikine® Mouse Proprotein Convertase 9/PCSK9 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure mouse PCSK9 in cell culture supernates, tissue lysates, serum, and plasma. It contains NS0-expressed recombinant mouse PCSK9 and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant factor. Results obtained using natural mouse PCSK9 showed linear curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for natural mouse PCSK9.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse PCSK9 has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any PCSK9 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse PCSK9 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of PCSK9 bound in the initial step. The sample values are then read off the standard curve.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse PCSK9 Microplate	893741	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse PCSK9.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Mouse PCSK9 Conjugate	893742	12 mL of a polyclonal antibody specific for mouse PCSK9 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Mouse PCSK9 Standard	893743	Recombinant mouse PCSK9 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Mouse PCSK9 Control	893744	Recombinant mouse PCSK9 in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
Assay Diluent RD1-21	895215	12 mL of a buffered protein solution with preservatives.	
Calibrator Diluent RD5-26 Concentrate	895525	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:4 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895174	23 mL of diluted hydrochloric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- Test tubes for dilution of standards and samples.

If using cell lysate samples, the following is also required:

- 1% v/v Triton™ X-100 in PBS.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Tissue Lysates - Cells must be lysed as directed in the Sample Values section before assaying.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

SAMPLE PREPARATION

Tissue lysate samples may require dilution. A suggested 10-fold dilution is 40 µL of sample + 360 µL of Calibrator Diluent RD5-26 (diluted 1:4)*.

Mouse serum and plasma samples require a 200-fold dilution. A suggested 200-fold dilution can be achieved by adding 20 µL of sample to 180 µL of Calibrator Diluent RD5-26 (diluted 1:4)*. Complete the 200-fold dilution by adding 20 µL of the diluted sample to 380 µL of Calibrator Diluent RD5-26 (diluted 1:4)*.

*See Reagent Preparation section

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Mouse PCSK9 Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

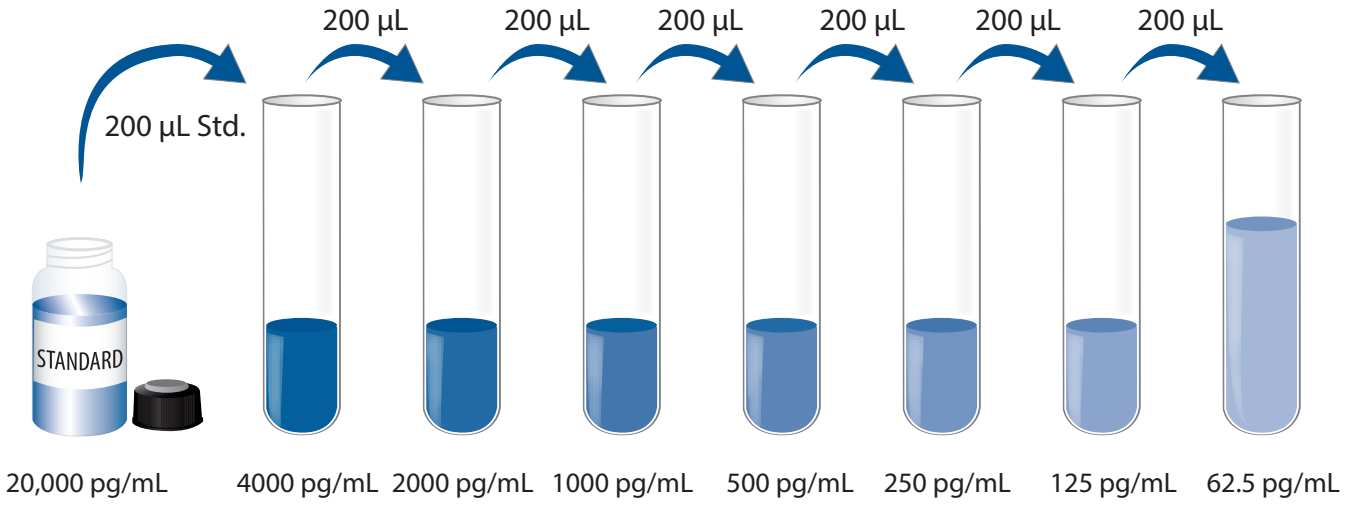
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-26 (diluted 1:4) - Add 20 mL of Calibrator Diluent RD5-26 Concentrate to 60 mL of deionized water to prepare 80 mL of Calibrator Diluent RD5-26 (diluted 1:4).

Mouse PCSK9 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse PCSK9 Standard with Calibrator Diluent RD5-26 (diluted 1:4). Do not substitute other diluents. This reconstitution produces a stock solution of 20,000 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 800 µL of Calibrator Diluent RD5-26 (diluted 1:4) into the 4000 pg/mL tube. Pipette 200 µL into each of the remaining tubes. Use the stock solutions to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 4000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-26 (diluted 1:4) serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, control, and samples be assayed in duplicate.

1. Prepare all reagents, standard dilutions, control, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1-21 to each well.
4. Add 50 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. 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.
6. Add 100 µL of Mouse PCSK9 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation.

CALCULATION OF RESULTS

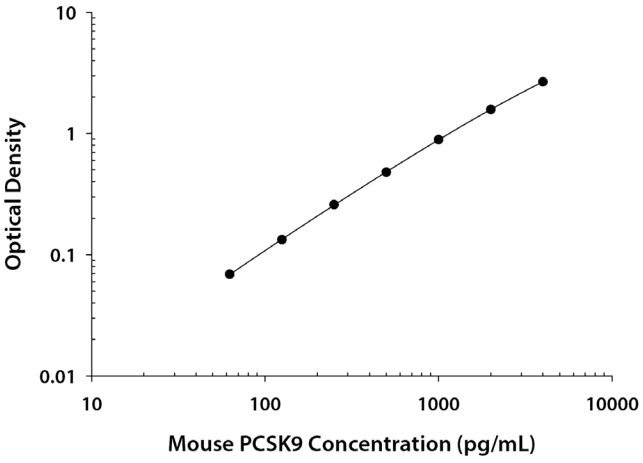
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the mouse PCSK9 concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

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

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.016 0.017	0.017	—
62.5	0.085 0.086	0.086	0.069
125	0.149 0.150	0.150	0.133
250	0.275 0.277	0.276	0.259
500	0.494 0.497	0.496	0.479
1000	0.906 0.909	0.908	0.891
2000	1.584 1.607	1.596	1.579
4000	2.663 2.706	2.685	2.668

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (pg/mL)	181	427	1427	213	469	1511
Standard deviation	10.3	32.1	44.1	18.9	25.9	102
CV (%)	5.7	7.5	3.1	8.9	5.5	6.8

RECOVERY

The recovery of mouse PCSK9 spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates (n=6)	102	94-115%
Tissue lysates* (n=5)	103	91-117%

*Samples were diluted prior to assay as directed in the Sample Preparation section.

LINEARITY

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

		Cell culture supernates (n=6)	Tissue lysates (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	98	101	100	98	97
	Range (%)	94-101	99-103	97-104	93-101	91-104
1:4	Average % of Expected	97	100	94	99	94
	Range (%)	91-100	90-104	93-95	99-104	87-99
1:8	Average % of Expected	97	101	94	101	92
	Range (%)	90-101	92-106	91-95	95-107	88-96
1:16	Average % of Expected	102	100	90	97	93
	Range (%)	93-106	90-107	85-94	92-101	91-95

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

Forty-five assays were evaluated and the minimum detectable dose (MDD) of mouse PCSK9 was less than 21.9 pg/mL. The mean MDD was 5.32 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant mouse PCSK9 produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of mouse PCSK9 in this assay.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=20)	343	153-649	163
EDTA plasma (n=20)	337	166-612	118
Heparin plasma (n=20)	324	141-565	100

Cell Culture Supernates - Organs from 2-3 mice were chopped into 1-2 mm pieces and cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin sulfate. The cell culture supernates were unstimulated or stimulated with 1.0 mg/mL of lipopolysaccharide for 1 or 2 days. An aliquot of the cell culture supernate was removed and assayed for levels of mouse PCSK9. Cell culture supernates from mouse brain, heart, lung, and spleen tissue measured below the low standard, 62.5 pg/mL. Cell culture supernates from liver and kidney tissue measured as follows.

Tissue Type	(pg/mL)
Kidney (unstimulated for 1 day)	140
Liver (unstimulated for 2 days)	889
Liver (stimulated with 1.0 mg/mL LPS for 2 days)	899

Tissue Lysates - Organs from 2-3 mice were rinsed with PBS to remove excess blood, chopped into 1-2 mm pieces, homogenized with a tissue homogenizer, and 1% v/v Triton™ X-100 was added. An aliquot of each tissue lysate was removed and assayed for levels of mouse PCSK9.

Tissue Type	(pg/mL)
Brain	1741
Heart	1603
Kidney	8953
Liver	36,350
Lung	3898
Spleen	1800

SPECIFICITY

This assay recognizes natural and recombinant mouse PCSK9.

Mouse factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Human factors listed below were prepared at 500 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following mouse factors (at 50 ng/mL) and human factors (at 500 ng/mL) in a mid-range mouse PCSK9 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:	Recombinant human:
LDLR	Furin
VLDLR	PCSK1
	PCSK7
	PCSK9

This assay detects 60 kDa, 53 kDa, and LDLR-complexed recombinant mouse PCSK9.

Rat serum was tested and found to be non-detectable in this assay.

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