MANUAL

AlphaLISA® PCSK9 and LDLR (Human) Binding Kit

Product number: AL3132 C/F

Research Use Only. Not for use in diagnostic procedures.

Product Information

Application: This kit is designed to assess inhibitors of human PCSK9 and human LDLR binding, using a homogeneous AlphaLISA assay (no wash steps). This assay can facilitate the design and development of antibody therapeutics by using competitive binding to human PCSK9/LDLR.

Sensitivity: IC_{50}: 3.21 nM (average, using anti PCSK9 antibody)

Signal to background ratio: 144 using 10 nM PCSK9 and 10 nM LDLR

Kit contents: The kit contains 5 components: anti-6xHis AlphaLISA Acceptor beads, Streptavidin-coated Donor beads, Biotinylated human LDLR, His tagged human PCSK9 and AlphaLISA Immunoassay buffer.

Storage: The proteins in the kit must be stored at -20 ˚C. The other components are stored at 4 ˚C in the dark.

Stability: This kit is stable for at least 6 from the manufacturing date when stored in its original packaging and the recommended storage conditions.

Analyte of Interest

Human proprotein convertase subtilisin/kexin type 9 (PCSK9), also known as FH3, HCHOLA3, NARC1 and PC9, is a crucial player in the regulation of plasma cholesterol homeostasis. PCSK9 binds to low density lipoprotein receptor (LDLR) and promotes PCSK9 lysosomal degradation in the liver, thereby reducing LDL clearance. Studies of PCSK9-LDLR binding have aided in the development of therapeutic anti-PCSK9 antibodies that effectively block this interaction at the cell surface. Therefore, blocking PCSK9 and LDLR binding has been considered a promising therapeutic target against cardiovascular disease.

Description of the AlphaLISA Assay

The AlphaLISA detection of human PCSK9 and LDLR binding uses anti-6xHis AlphaLISA® acceptor beads to capture the His tagged PCSK9 and Streptavidin-coated donor beads to capture the biotinylated LDLR. Donor beads and acceptor beads come into proximity through PCSK9 binding to LDLR. Excitation of the Donor beads provokes the release of singlet oxygen that triggers a cascade of energy transfer reactions in the Acceptor beads, resulting in a sharp peak of light emission at 615 nm (Figure 1).

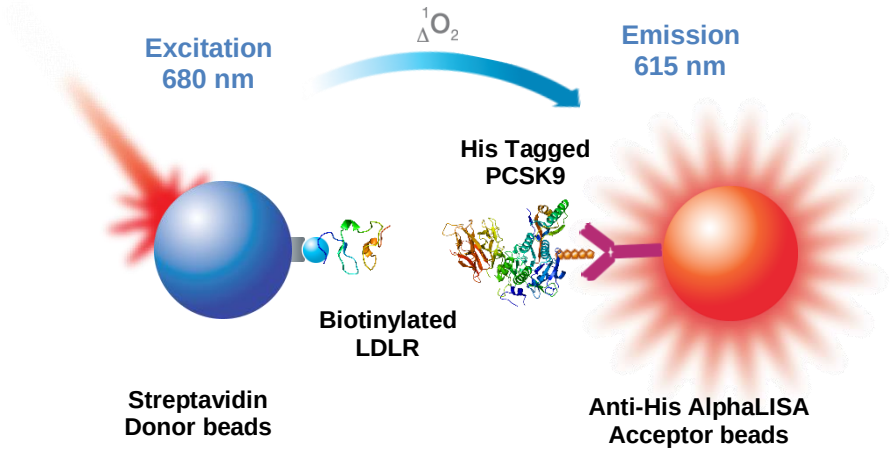


Figure 1. AlphaLISA Assay Principle.

Precautions

- The Alpha Donor beads are light-sensitive. All the other assay reagents can be used under normal light conditions. All Alpha assays using the Donor beads should be performed under subdued laboratory lighting (< 100 lux). Green filters (LEE 090 filters (preferred) or Roscolux filters #389 from Rosco) can be applied to light fixtures.
- All blood components and biological materials should be handled as potentially hazardous. The analyte included in this kit is from a human source.
- Some analytes are present in saliva. Take precautionary measures to avoid contamination of the reagent solutions.

Kit Content: Reagents and Materials

Kit components	AL3132C*** (500 assay points)	AL3132F*** (5000 assay points)
Anti-6xHis AlphaLISA Acceptor beads stored in PBS, 0.05% Kathon, pH 7.2	20 µL @ 5 mg/mL (1 brown tube, white cap)	200 µL @ 5 mg/mL (1 brown tube, white cap)
Streptavidin (SA)-coated Donor beads stored in 25 mM HEPES, 100 mM NaCl, 0.05% Kathon, pH 7.4	40 µL @ 5 mg/mL (1 brown tube, black cap)	400 µL @ 5 mg/mL (1 brown tube, black cap)
LDLR (Biotinylated) stored in PBS+0.1%BSA*	5 µM, 20 µL (1 tube, clear cap)	5 µM, 200 µL (10 tubes, clear caps)
PCSK9 (His tagged) stored in PBS+0.1%BSA*	5 µM, 20 µL (1 tube, clear cap)	5 µM, 200 µL (10 tubes, clear caps)
AlphaLISA Immunoassay Buffer (10X)**	10 mL, 1 small bottle	100 mL, 1 large bottle

* Unused proteins should be aliquoted and stored at -20°C for further experiments. Avoid multiple freeze-thaw cycles.

** Extra buffer can be ordered separately (cat # AL000C: 10 mL, cat # AL000F: 100 mL).

*** The number of assay points is based on an assay volume of 20 µL in 384 well plates using the kit components at the recommended concentrations.

Sodium azide should **not** be added to the stock reagents. High concentrations of sodium azide (> 0.001 % final in the assay) might decrease the AlphaLISA signal.

Specific additional required reagents and materials:

The following materials are recommended:

Item	Suggested source	Catalog #
TopSeal™-A Adhesive Sealing Film	Revvity Inc.	6050185
AlphaPlate-384, Shallow Well (ProxiPlate)	Revvity Inc.	6008350 6008359
EnVision®-Alpha Reader	Revvity Inc.	-

The following reagents might be required for particular applications:

Item	Supplier	Catalog number
Anti-PCSK9 neutralizing antibody	BPS Bioscience	71207
Anti-LDLR antibody	R&D Systems	AF2148
Human IgG1 control	Athens Research Technology	16-16-090707-1
Goat IgG, control	Jackson Immuno Research	005-000-003
Nonbiotinylated LDLR	BPS Bioscience	71205
Non-his-tagged PCSK9	ACROBiosystems	PC9-H5256

Recommendations

- The volume indicated on each tube is guaranteed for single pipetting. Multiple pipetting of the reagents may reduce the theoretical amount left in the tube. To minimize loss when pipetting beads, it is preferable not to pre-wet the tip.
- Centrifuge all tubes (including lyophilized analyte) before use to improve recovery of content (2000g, 10-15 sec). Re-suspend the beads by vortexing before use. Do not vortex the proteins.
- Use Milli-Q® grade H_2O to dilute 10X AlphaLISA Immunoassay Buffer and to reconstitute the lyophilized proteins.
- When reagents are added to the microplate, make sure the liquids are at the bottom of the well.
- Small volumes may be prone to evaporation. It is recommended to cover microplates with TopSeal™-A Plus Adhesive Sealing Films to reduce evaporation during incubation. Microplates can be read with the TopSeal-A Film.
- The AlphaLISA signal is detected with an EnVision Multilabel Reader equipped with the Alpha option using the AlphaScreen standard settings (e.g. Total Measurement Time: 550 ms, Laser 680 nm Excitation Time: 180 ms, Mirror: D640as, Emission Filter: M570w, Center Wavelength 570 nm, Bandwidth 100 nm, Transmittance 75%).
- AlphaLISA signal will vary with temperature and incubation time. For consistent results, identical incubation times and temperature should be used for each plate.

Competition Assay Procedure

IMPORTANT: PLEASE READ THE RECOMMENDATIONS BELOW BEFORE USE

- The manual described below is an ***example*** for generating one inhibition curve in a 20 µL final assay volume (36 wells, triplicate determinations). If a different number of samples are tested, the volumes of all reagents have to be adjusted accordingly.
- The dilution manual is provided for information only. As needed, the number of replicates or the range of concentrations covered can be modified.

Anti PCSK9 antibody blocking assay manual described as below:

1) Preparation of 1X AlphaLISA Immunoassay Buffer (for 10 mL):
   Add 1 mL of 10X AlphaLISA Immunoassay Buffer to 9 mL H_2O.

2) Preparation of serial dilution of anti-PCSK9 neutralizing antibody in AlphaLISA immunoassay buffer
   a. Dilute anti-PCSK9 Neutralizing antibody to 2 µM (for 2.26 mg/mL stock concentration, add 4 µL of 2.26 mg/mL anti-PCSK9 neutralizing antibody to 26 µL 1X AlphaLISA Immunoassay buffer to make 30 µL of 2 µM working concentration)
   b. Prepare serial dilutions of 4X anti-PCSK9 neutralizing antibody in 1x AlphaLISA Immunoassay Buffer as follows, change tips between each dilution:

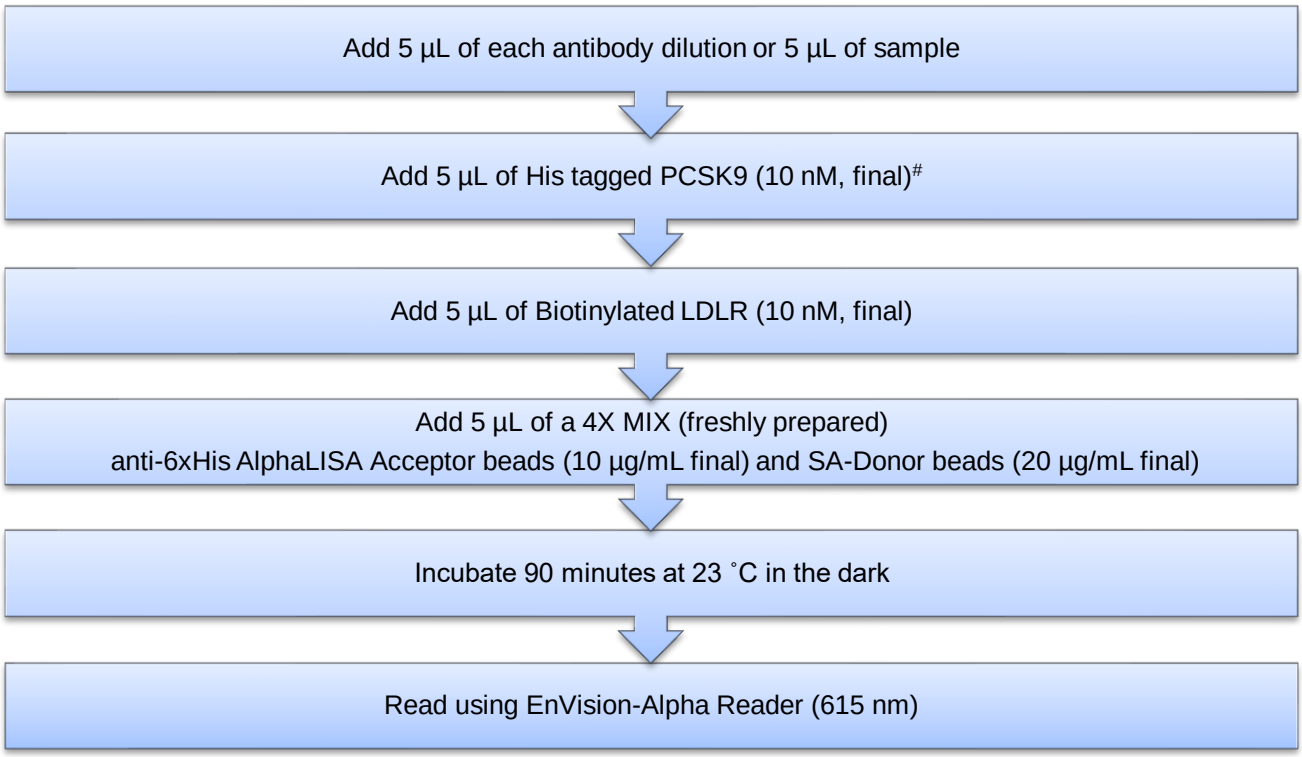
Tube	Volume of Antibody	Volume of 1X buffer	[Ab] (nM) (4X)	[Ab] (nM) (1X)
A	4 L of 2 µM antibody	96	80	20
B	50 µL of tube A	50 µL	40	10
C	50 µL of tube B	50 µL	20	5
D	50 µL of tube C	50 µL	10	2.5
E	50 µL of tube D	50 µL	5	1.25
F	50 µL of tube E	50 µL	2.5	0.625
G	50 µL of tube F	50 µL	1.25	0.3125
H	50 µL of tube G	50 µL	0.625	0.15625
I	50 µL of tube H	50 µL	0.3125	0.07813
J	50 µL of tube I	50 µL	0.15625	0.03906
K	50 µL of tube J	50 µL	0.078125	0.01953
L	0	50 µL	0	0

3) Preparation of 4X His tagged PCSK9 (40 nM):
   a. Add 2 µL of 5 µM PCSK9 to 248 µL 1X AlphaLISA Immunoassay buffer.
   b. Prepare just before use.

4) Preparation of 4X biotinylated LDLR (40 nM):
   a. Add 2 µL of 5 µM LDLR to 248 µL 1X AlphaLISA Immunoassay buffer.
   b. Prepare just before use.

5) Preparation of the mix of 4X Anti-6xHis AlphaLISA Acceptor beads (40 µg/mL) and 4X Streptavidin (SA) Donor beads (80 µg/mL):
   a. Keep the beads under subdued laboratory lighting.
   b. Add 2 µL of 5 mg/mL Anti-6xHis AlphaLISA Acceptor beads and 4 L of 5 mg/mL SA-Donor beads to 244 µL of 1X AlphaLISA Immunoassay Buffer.
   c. Prepare just before use.

6) In a ProxiPlate (384 wells):

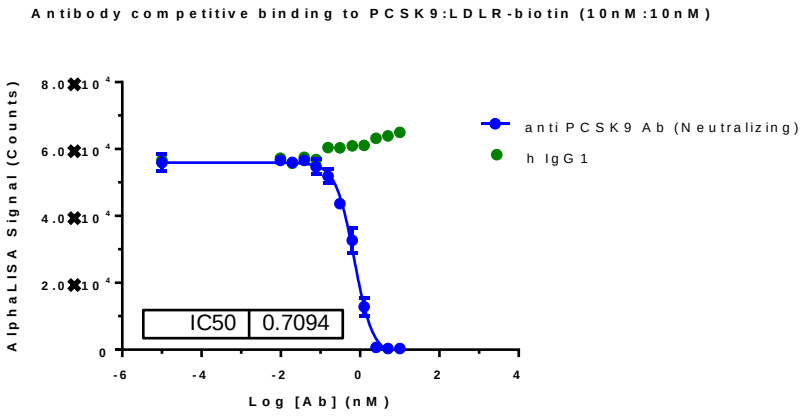


Read Settings: AlphaLISA signal is detected using an EnVision Multilabel Reader equipped with the Alpha option using the following settings: Total Measurement Time: 550 ms, Laser: 680 nm, Excitation Time: 180 ms, Mirror: 640as (Barcode# 444), Emission Filter: Wavelength 570nm, bandwidth: 100nm, Transmittance 75%, (Barcode# 244).

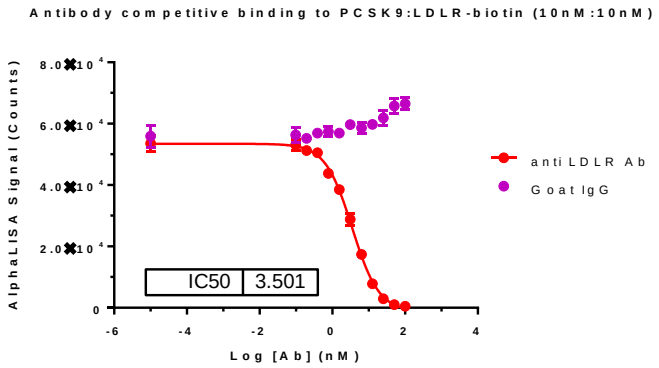
If screening anti-LDLR antibodies, add LDLR first, then add PCSK9.

Typical competitive binding Data:

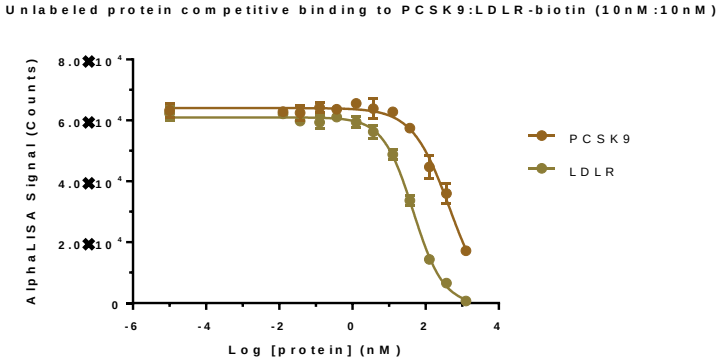
(A)



(B)



(C)



	PCSK9	LDLR
IC50	448.1	45.79

Figure 2. Competitive Binding: (A) anti-PCSK9 antibody blocking PCSK9/LDLR binding with IC_{50} = 0.71 nM. Human IgG1 was measured as a negative control. The IgG1 has processed on a zeba column (ThermoFisher, Cat. no. 89882) for a buffer exchange with PBS before testing to remove NaN_3. The concentration of IgG1 was measured with NanoDrop (E 1%=13.6). (B) anti-LDLR antibody blocking PCSK9/LDLR binding with IC_{50} = 3.5 nM. Goat IgG was measured as a negative control. (C) Non-his-tagged PCSK9 and nonbiotinylated LDLR competitive binding to PCSK9/LDLR binding. The IC_{50} were 448 nM and 45.8 nM respectively. All IC_{50} values were calculated by using nonlinear regression fitting with GraphPad Prism 7.

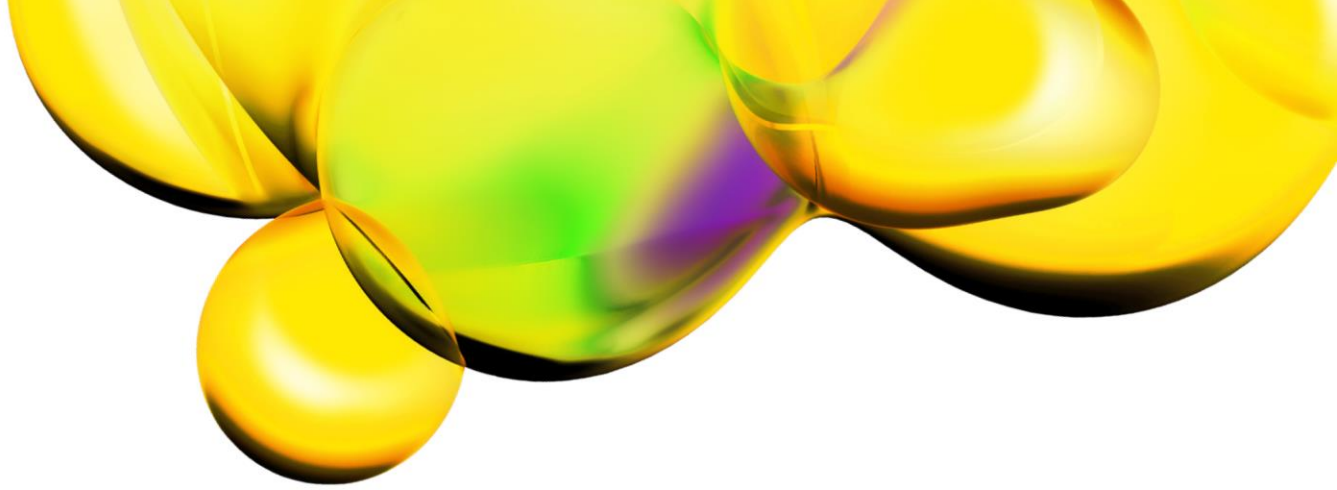
Troubleshooting Guide

You will find below recommendations for common situations that you might encounter with your AlphaLISA binding assay. If further assistance is needed, do not hesitate to contact our technical support team for assistance.

Issue	Recommendations and Comments
High background signal	• Buffer is not freshly made. Make new. • Incubation time is longer than recommended range.
Low AlphaLISA signal	• Optimize EnVision with Plate format.
High variation between replicates or low Z' values	• Make sure that reagents are at the bottom of the well by tapping or swirling the plate gently on a smooth surface after each addition.

You will find detailed recommendations for common situations you might encounter with your AlphaLISA Assay kit at: www.revvity.com

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.



www.revvity.com

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Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA
www.revvity.com

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