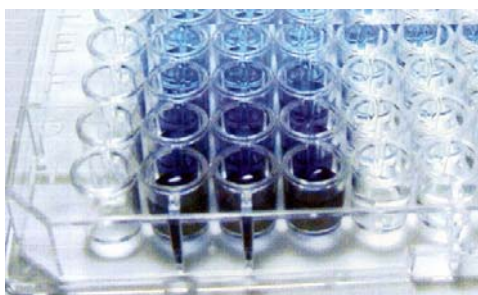


Human Clusterin Competitive ELISA Kit

Cat. No. CC06H2EK



Instruction Manual
Version 1.1.0

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES



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Introduction

Clusterin is a multifunctional protein which has many alternative names; apolipoprotein J (ApoJ), sulfated glycoprotein 2 (SGP2), and complement-associated protein SP-40. Apolipoprotein J is the human analog of the rat protein present in high concentrations in the testis, sulfated glycoprotein-2. It is a 70-kD protein associated with high-density lipoproteins (HDL) in human plasma. Its primary structure was deduced by de Silva et al. (1) using the combined strategies of protein sequencing and cDNA cloning and sequencing. There is a single copy of the APOJ gene in the human and mouse genomes. The protein is synthesized as a 427-amino acid polypeptide that is posttranslationally cleaved at an internal bond between arg205 and ser206. Two subunits, designated alpha (34 to 36 kD), corresponding to residues 1-205, and beta (36 to 39 kD), corresponding to residues 206-427, are associated through disulfide bonds. Studies indicated that the alpha and beta subunits are derived from a common precursor by proteolytic cleavage and that the subunits, while distinct, have limited regions of homology. De Silva et al. (2) found APOJ mRNA (1.9 kb) in all but one tissue examined. Its concentration was relatively high in brain, ovary, testis, and liver, lower in heart, spleen, lung, and breast, and absent in T lymphocytes. Apolipoprotein J is distinct from other known apolipoproteins in molecular weight, subunit structure, and isoelectric point. Clusterin is a major serum protein whose normal concentrations are around 100 ug/ml. Clusterin is a member of the human complement system by directly demonstrating its presence within the S-protein-containing soluble variant of the C5b-9 complex, SC5b-9. It acts as a control mechanism of the complement cascade; specifically, it prevents the binding of a C5b-C7 complex to the membrane of the target cell and in this way inhibits complement-mediated cytolysis. The findings of Kirszbaum et al. (3) document a link between the immune and reproductive systems. For this reason the term clusterin is used for the protein in both human serum and seminal fluid. Clusterin is encoded by a single gene, but there are two distinct forms of clusterin; 1) nuclear form and 2) secretory form. The secretory form exists in both serum and semen. The secretory form undergoes a heavy protein processing including cleavage and glycosylation as described above. The nuclear form is a simple open reading frame initiated from the 2nd initiation codon whereas the secretory form initiates from the 1st initiation codon in the leader peptide. While the secretory form of clusterin is cytoprotective, the nuclear form is apoptotic. The serum levels of the secretory form become increasing due to many cancers (4). Clusterin is related to neuron. There are a growing number of papers regarding the relationship between Alzheimer Disease (AD) and clusterin. Clusterin acts on microglial cells, which are brain macrophage-equivalent (4). Thus, clusterin could be a novel diagnostic marker in many major human diseases like cancers or neurodegenerative diseases.

Assay Principles

This kit is a competitive enzyme-linked immunosorbent assay (ELISA) for quantitative determination of clusterin in human serum, plasma or cell culture supernatants. A polyclonal antibody recognizing native human clusterin reacts with a series of predetermined recombinant human clusterin standard proteins or serum or plasma under competition in the human clusterin-coated plate. Their relative reactivity is plotted with that of the standard proteins.

Kit Components

- 1) Recombinant human clusterin coated 96-well plate, 12X 8-well strips
- 2) 5X Wash concentrate, 100 ml
- 3) 5X Diluent, 50 ml
- 4) Antibody, 12 ml
- 5) 100X Detector, 150 μ l
- 6) Standard, recombinant human clusterin expressed by *E. coli* cells, 1 vial, lyophilized
- 7) QC sample = a positive control of human serum clusterin, 1 vial, lyophilized (For actual concentration of QC sample, see the 'Certificate of analysis' enclosed.)
- 8) Substrate I, 6 ml
- 9) Substrate II, 6 ml
- 10) Stop solution, 12 ml

Reagents Description

Antigen coated 96-well plate, 12X 8-well strips, with absorbed recombinant human clusterin

5X Wash concentrate, buffered detergent solution, supplied as a 5X concentrate

5X Diluent, for sample and reagent dilution

1X antibody, polyclonal antibody against human clusterin

100X detector, HRP conjugated anti-rabbit IgG

Substrate I and II, chromogenic reagents

Stop solution, 1M H₃PO₄

Standard, 5 μ g, recombinant human clusterin

Storage of Reagents

Reagent must be stored at 2-8°C when not in use. Reagents must be brought to room temperature before use. Do not expose reagents to temperature greater than 25°C. Diluted wash solution may be stored at room temperature for up to one month.

Materials Required but not Supplied

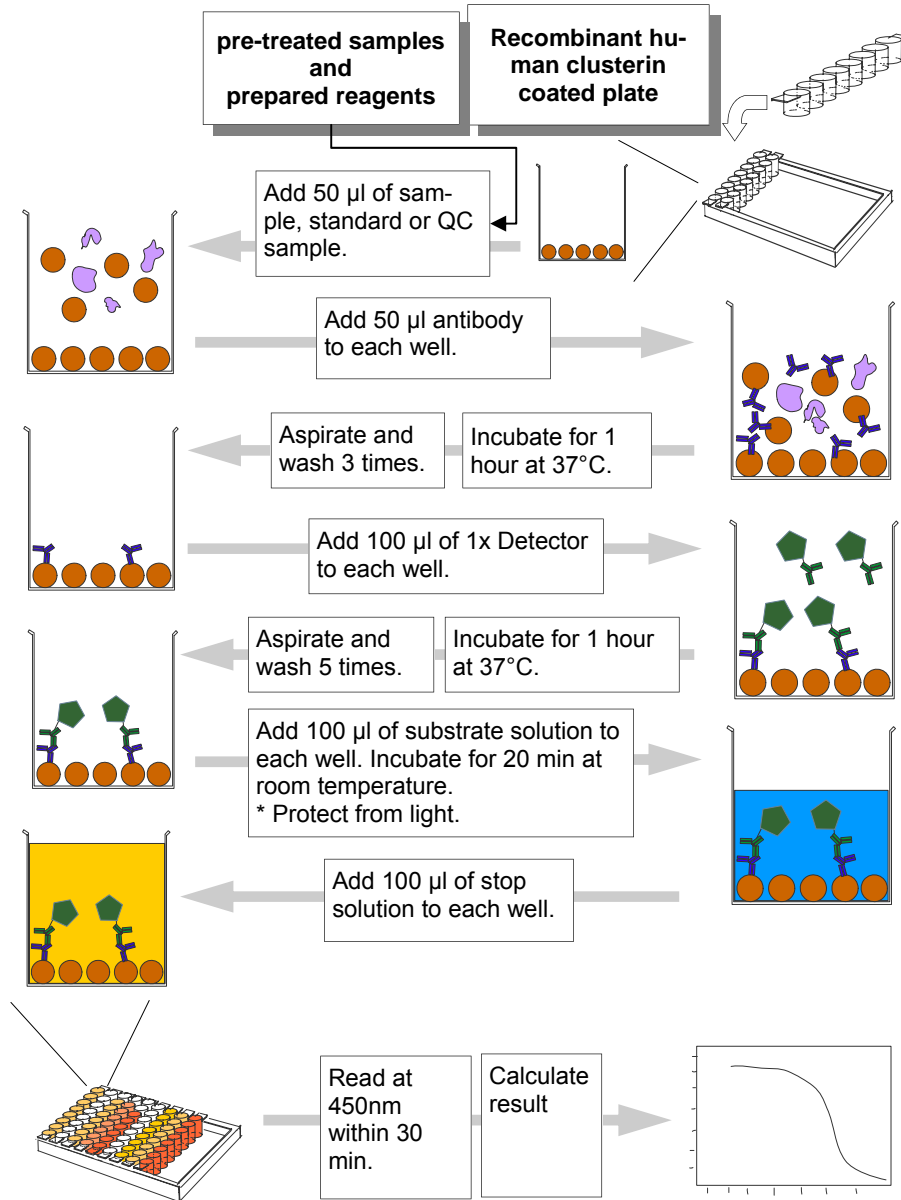
Precision single and multi-channel pipettes.
Disposable pipette tips.
Microtubes or equivalent for preparing dilutions.
Disposable plastic containers for preparing working reagents.
Reagent reservoirs.
Microwell or microstrip plate reader 450 nm
Deionized water

Sample Collection and Storage

Serum Use a serum separator tube. Let samples clot at room temperature for 30 minutes before centrifugation for 20 minutes at 1,000xg. Assay freshly prepared serum or store serum in aliquot at $\leq -20^{\circ}\text{C}$ for later use. Avoid repeated freeze/thaw cycles.

Plasma Collect plasma using heparin, EDTA, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay freshly prepared plasma or store plasma sample in aliquot at $\leq -20^{\circ}\text{C}$ for later use. Avoid repeated freeze/thaw cycles.

Flow Chart of Assay Procedure

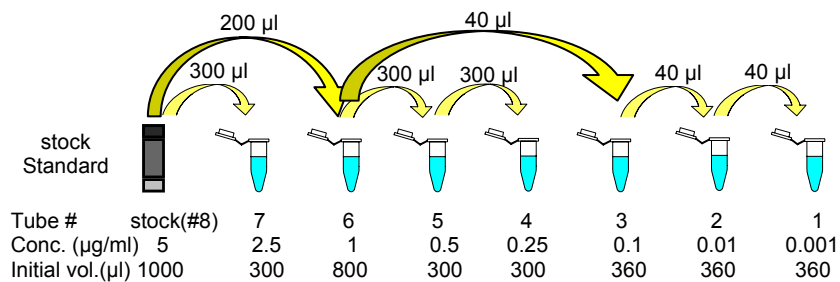


Assay Procedure

1. Preparation of Reagents

- 1) Allow all samples and kit components to equilibrate to room temperature (20-25°C).
- 2) Plan the plate configuration and create a plate map. Calculate the amount of working reagents to use.
It is recommended that Standards and samples be run in duplicate.
- 3) Prepare **1X Wash Solution**. Dilute 5X Wash Concentrate 1:5 with deionized water (1 part 5X Wash Concentrate with 4 parts deionized water). The diluted 1X Wash Solution is stable for one month at room temperature.
- 4) Prepare **1X Diluent**. Dilute 5X Diluent 1:5 with deionized water (1 part 5X Diluent with 4 parts deionized water).
- 5) Prepare **1X Detector**. Dilute 100X Detector 1:100 with 1X Diluent (1 part 100X Detector with 99 parts 1X Diluent). Use the 1X Detector within one hour of preparation.
- 6) Freshly prepare just before use the **Substrate Solution** by adding one part Substrate I to one part Substrate II.
- 7) Prepare working aliquots of the Standard as follows:
When opening the lyophilized Standard, remove cap gently as the lyophilizate may have become dislodged during shipping. Add 1 ml of deionized water to the Standard vial to make a stock Standard concentration of 5 µg/ml (#8). Mix well.
A recommended dilution scheme is as follows:
 - a. Label 7 microcentrifuge tubes #1-7. Add 1X Diluent to the microcentrifuge tubes #1-7 as shown on page 8, respectively.
 - b. Add 300 µl of the stock Standard solution (#8) to tube #7 and mix well. This is Standard tube #7 with a concentration of 2.5 µg/ml.

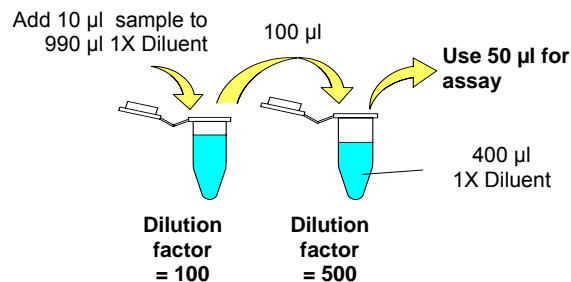
- c. Standard #6 (1 µg/ml) is then prepared by performing a 1:5 dilution of the stock Standard (#8). Standards #5 to #4 are prepared by performing a 1:2 dilution of the preceding Standard solution #6 and #5, respectively and mix well.
- d. Standards #3 are then prepared by performing a 1:10 dilution of #6 Standard solution.
- e. Standard #2 to #1 are prepared by performing a 1:10 dilution of the preceding solution #3 and #2, respectively and mix well.
- f. Use 50 µl of the final diluted Standards (#8 to #1) for ELISA.



8) Reconstitute QC sample in 1 ml of deionized water.

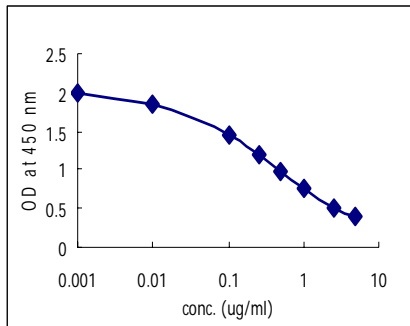
2. Sample dilution

- 1) Dilute samples 1:100 with 1X Diluent (for example, 10 µl sample plus 990 µl 1X Diluent, final 1:100)
 - 2) Dilute the samples (from step 1) 1:5 with 1X Diluent (for example, 100 µl step 1 sample plus 400 µl 1X Diluent, final 1:500)
- * If samples fall the outside range of assay, a lower or higher dilution may be required.
- 3). Use 50 µl of the final diluted sample for ELISA.



3. Experiment procedure

- 1) Remove the appropriate number of microwell strips from the sealed foil pouch.
- 2) Pipette 50 μ l of Standards #1 to #8, the reconstituted QC sample and pre-treated sample into the recombinant protein-coated plate according to the plate configuration. Use a new pipette tip for each Standard or sample.
- 3) Add 50 μ l antibody to each well.
- 4) Incubate at 37°C for 1 hour.
- 5) Remove the solution and wash 3 times with 300 μ l of 1X Wash Solution per well.
- 6) Add 100 μ l 1X Detector to each well.
- 7) Incubate at 37°C for 1 hour.
- 8) Remove the solution and wash 5 times with 300 μ l of 1X Wash Solution per well.
- 9) Using the multi-channel pipette, add 100 μ l of the mixed Substrate Solution to each well.
- 10) Incubate at room temperature for 20 min.
* Protect from light.
- 11) Using the multi-channel pipette, add 100 μ l Stop Solution to each well.
- 12) Read at 450 nm.
- 13) The dose-response curve of this assay fits best to a sigmoidal 4-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4-parameter logistic function.
- 14) A measurable range is typically shown between 0.001 μ g/ml and 5 μ g/ml.



The clusterin concentrations calculated must be multiplied by dilution factor to obtain the concentrations of the undiluted samples (Dilution factor of lyophilized QC sample is 500).

Performance Characteristics

1) Sensitivity

The limit of detection: 1 ng/ml

2) Precision

a. Intra-Assay (precision within an assay)

5 samples were tested 8 times to assess intra-assay precision.

Sample	Mean (µg/ml)	SD (µg/ml)	CV(%)
1	130.61	12.83	9.82
2	61.72	3.13	5.07
3	66.87	4.68	7.02
4	70.35	5.31	7.55
5	35.94	3.26	9.07

b. Inter-Assay (precision between assays)

5 samples were tested 8 times to assess inter-assay precision.

Sample	Mean (µg/ml)	SD (µg/ml)	CV(%)
1	68.33	6.67	9.76
2	51.82	3.47	6.70
3	83.02	8.17	9.84
4	63.92	5.88	9.20
5	49.22	3.53	7.18

3) Specificity

a. No cross reaction with mouse and rat sera

b. Cross Reactivity

Analyte	Max. Conc. (µg/ml)	Cross Reactivity (%)
Human Clusterin	0.1	100
Mouse Clusterin	1	N. R.
Human Adiponectin	1	N. R.
Human Resistin	1	N. R.
Human RBP4	1	N. R.
Human RELM β	1	N. R.
Human FABP4	1	N. R.
Human GPX3	1	N. R.
Human Visfatin	1	N. R.
Human PAI-1	1	N. R.
Human Leptin	1	N. R.
Human IL23	1	N. R.
Human TNF-α	1	N. R.
Human ANGPTL3	1	N. R.
Human ANGPTL4	1	N. R.
Human ANGPTL7	1	N. R.

N. R.: No Cross-reactivity

4) Linearity - Effect of Serum Dilution

Three human serum samples were pre-treated as described in the protocol, resulting in the final dilution of x500(labeled in the table below as dilution: 1)

Sample No.	Serum Dilution	Expected (µg/ml)	Observed (µg/ml)	% Of Expected
1	1	153.77	153.77	100
	1/2	76.885	79.085	103
	1/4	38.443	37.605	98
2	1	165.26	165.26	100
	1/2	82.63	97.128	118
	1/4	41.315	48.633	118
3	1	83.243	83.243	100
	1/2	41.622	45.023	108
	1/4	20.811	22.477	108

$$\% \text{ of expected} = \text{observed} / \text{expected} \times 100\%$$

References

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Troubleshooting Guide

Problem	Possible Cause	Solution
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order
	Washes too stringent	Use an automated plate washer if possible
	Incubation times inadequate	Incubation times should be appropriate for the system.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use
High background	Concentration of detector too high	Use recommended dilution factor
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.
	Technique problem	Proper mixing of reagents and wash steps are critical.

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