ELISA Kit for Measuring Human Clusterin/Apo-J

CircuLex Human Clusterin/Apo-J ELISA Kit

Cat# CY-8099

Intended Use

The MBL Research Product **CircuLex Human Clusterin/Apo-J ELISA Kit** is used for the quantitative measurement of human Clusterin/Apo-J in serum, plasma, cell culture supernatant, cell lysate, and other biological samples, e.g. tear, milk, saliva.

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

Clusterin, also called apolipoprotein J, sulfated glycoprotein-2, and testosterone-repressed prostate message-2, is a highly conserved secreted heterodimeric glycoprotein constitutively expressed by diverse epithelial cells. Clusterin has been implicated in diverse physiological processes, including lipid transportation (1), complement inhibition (1), tissue remodeling (2), membrane recycling (3), clearance of cellular debris (4), regulation of apoptosis, membrane protection, and promotion of cell-cell interactions (5). Clusterin is induced in injured organs in various disease states, such as Alzheimer's disease, atherosclerosis, myocardial infarction, and multiple forms of acute and chronic renal disease (5, 6). Clusterin has been shown to associate with both normal in vitro aging, namely replicative senescence, as well as with stress induced premature senescence. In vivo, the protein is up-regulated in many severe physiological disturbances that relate to advanced aging, including accumulation in the artery wall during the development of atherosclerosis.

In cancer, clusterin up-regulation has been described in renal cell carcinoma (7), breast carcinoma (8), ovarian cancer (9), anaplastic large cell lymphomas (10), desmoplastic melanoma (11), transitional cell carcinoma of the bladder (12), pancreatic cancer (13), and serous carcinoma and hepatocellular carcinoma (14). However, a number of tumor processes where clusterin is downregulated have also been described such as esophageal squamous cell carcinoma (15), testicular germ cell tumors (16) and prostate cancer (17).

The structure of clusterin has not provided much insight into function. Mammalian clusterins are approximately 80-kDa heterodimers (18, 19) consisting of two 40-kDa chains joined by a unique five-disulfide-bond motif (20). The protein has limited homology to other proteins and lacks clear functional motifs (18). It does contain three putative amphipathic α-helical regions, which could allow it to interact with lipids and hydrophobic regions of other proteins (21).

Principle of the Assay

The MBL Research Product **CircuLex Human Clusterin/Apo-J ELISA Kit** employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for Human Clusterin/Apo-J is pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any Human Clusterin/Apo-J present. After washing away any unbound substances, an HRP conjugated monoclonal antibody specific for Human Clusterin/Apo-J is added to the wells. Following a wash to remove any unbound antibody HRP conjugate, the remaining conjugate is allowed to react with the substrate H_2O_2-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of Human Clusterin/Apo-J. A standard curve is constructed by plotting absorbance values versus Human Clusterin/Apo-J concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

Summary of Procedure

Add 100 µL of diluted samples to the wells
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of HRP conjugated anti-human Clusterin/Apo-J antibody
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of Substrate Reagent
↓ Incubate for 10-20 minutes at room temp.
Add 100 µL of Stop Solution
↓
Measure absorbance at 450 nm

Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microplate 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 anti-Human Clusterin/Apo-J antibody as a capture antibody.

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

Dilution Buffer: One bottle containing 50 mL of 1X buffer; use for reconstitution of Human Clusterin/Apo-J Standard and sample dilution. Ready to use.

Human Clusterin/Apo-J Standard: One vial containing X* of lyophilized recombinant human Clusterin/Apo-J.
***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**
HRP conjugated Detection Antibody: One bottle containing 12 mL of HRP (horseradish peroxidase) conjugated anti-Human Clusterin/Apo-J monoclonal antibody (AS-1B7). Ready to use.

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_2SO_4. Ready to use.

Materials Required but not Provided

- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **(Optional) Microplate washer:** 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.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1,000 mL graduated cylinder**.
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**
- 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.
- Avoid contact with Substrate Solution which contains hydrogen peroxide.
- **CAUTION: Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**
- **CAUTION: Stop Solution is a strong acid. Wear disposable gloves and eye protection when handling the solution.**

Sample Collection and Storage

Serum: Use a serum separator tube and allow samples to clot for 60 ± 30 minutes. Centrifuge the samples at 4°C for 10 minutes at 1,000 x *g*. Remove serum and assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of serum may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using EDTA-2Na as the anticoagulant. If possible, collect the plasma into a mixture of EDTA-2Na and Futhan5 to stabilize the sample against spontaneous *in vitro* complement activation. Immediately centrifuge samples at 4°C for 15 minutes at 1,000 x *g*. Assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of plasma may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

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

Cell culture supernatant: Remove any particulates by centrifugation and assay immediately or aliquot and store samples at below -70°C. Avoid repeated freeze-thaw cycles.

Cell lysate:

1. Harvest and pellet cells by centrifugation using standard methods.

2. Resuspend the cell pellet with an appropriate extraction buffer (for example; 20 mM HEPES-KOH, pH 7.5, 250 mM NaCl, 0.1 % NP-40, 2 mM $CaCl_2$, 1 mM EDTA, 0.2 mM PMSF, 1 µg/mL pepstatin, 0.5 µg/mL leupeptin, 0.5 mM DTT) and lyse the resuspended cells using either a Dounce Homogenizer, sonication, or three cycles of freezing and thawing.

3. Transfer extracts to microcentrifuge tubes and centrifuge at 15,000 rpm for 10 minutes at 4°C.

4. Aliquot cleared lysate to a clean microfuge tube.

5. Assay immediately or store the samples on ice for a few hours before assaying. Aliquots of the samples may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Other biological samples: Remove any particulates by centrifugation and assay immediately or aliquot and store samples at below -70°C. Avoid repeated freeze-thaw cycles.

Detailed Protocol

The MBL Research Product **CircuLex Human Clusterin/Apo-J ELISA 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 experimental conditions may vary, an aliquot of the Human Clusterin/Apo-J Standard within the kit should be included in each assay as a calibrator. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solutions

All reagents need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of **10X Wash Buffer** and **Human Clusterin/Apo-J Standard**.

1. Prepare a working solution of Wash Buffer by adding 100 mL of the **10X Wash Buffer** to 900 mL of deionized (distilled) water. Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Reconstitute **Human Clusterin/Apo-J Standard** with X* of **Dilution Buffer** by gently mixing. After reconstitution, immediately dispense it in small aliquots (e.g. 100 µL) to plastic micro-centrifuge tubes and store below -70°C to avoid non-specific adsorption to glass surface and multiple freeze-thaw cycles. The concentration of human Clusterin/Apo-J in vial should be **80 ng/mL**, which is referred to as the **Master Standard** of human Clusterin/Apo-J.

***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

Prepare Standard Solutions as follows:

Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 4,000 pg/mL standard (Std.1) serves as the highest standard. The **Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Dilution Buffer	Concentration
Std.1	50 µL of Master Standard (80 ng/mL)	950 µL	4,000 pg/mL
Std.2	300 µL of Std. 1 (4,000 pg/mL)	300 µL	2,000 pg/mL
Std.3	300 µL of Std. 2 (2,000 pg/mL)	300 µL	1,000 pg/mL
Std.4	300 µL of Std. 3 (1,000 pg/mL)	300 µL	500 pg/mL
Std.5	300 µL of Std. 4 (500 pg/mL)	300 µL	250 pg/mL
Std.6	300 µL of Std. 5 (250 pg/mL)	300 µL	125 pg/mL
Std.7	300 µL of Std. 6 (125 pg/mL)	300 µL	62.5 pg/mL
Blank	-	300 µL	0 pg/mL

Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with Dilution Buffer before dispensing.

Sample Preparation

Dilute samples with **Dilution Buffer**.

- Serum may require 60,000- to 100,000-fold dilution.
- Tears may require 2,000- to 4,000-fold dilution.
- Saliva may require 50- to 100-fold dilution.
- Milk may require 2,000- to 5,000-fold dilution.

Standard Assay Procedure for Human Clusterin/Apo-J

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. Dilute samples with **Dilution Buffer**. (See "Sample Preparation" above.)

3. Pipette **100 µL** of **Standard Solutions (Std1-Std7, Blank)** and **diluted samples** in duplicates, into the appropriate wells.

4. Incubate the plate **at room temperature (ca.25°C) for 60 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker.

5. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

6. Add **100 µL** of **HRP conjugated Detection Antibody** into each well.

7. Incubate the plate **at room temperature (ca.25°C) for 60 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker.

8. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

9. Add **100 µL** of **Substrate Reagent**. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminum foil is recommended. Return Substrate Reagent to 4°C immediately after the necessary volume is removed

10. Incubate the plate **at room temperature (ca.25°C) for 10-20 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker. The incubation time may be extended up to 30 minutes if the reaction temperature is below 20°C.

11. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

12. Measure absorbance in each well using a spectrophotometric microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate 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 standard curves are obtained when either O.D. values do not exceed 0.25 units for the blank (zero concentration), or 3.0 units for the highest standard concentration.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine the concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

Calculations

Average the duplicate readings for each standard, control and sample, and subtract the optical density of the average zero standard. Plot the optical density versus the concentration of standards and draw the best curve. Most microtiter plate readers perform automatic calculations of analyte concentration. The standard curve fits best 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.

A standard curve is also to be constructed by plotting the absorbance (Y) versus log of the known concentration (X) of standards, using a cubic function. Alternatively, the logit log function can be used to linearize the standard curve (i.e. logit of optical density (Y) is plotted versus log of the known concentration (X) of standards). To determine the concentration of each sample, first find the optical density on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding concentration.

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

Measurement Range

The measurement range is 62.5 pg/mL to 4,000 pg/mL. Any sample reading higher than the highest standard should be diluted with Dilution Buffer in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the human Clusterin/Apo-J concentration.

Troubleshooting

1. All samples and controls should be assayed in duplicate, 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 MBL Research Product **CircuLex Human Clusterin/Apo-J ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date.

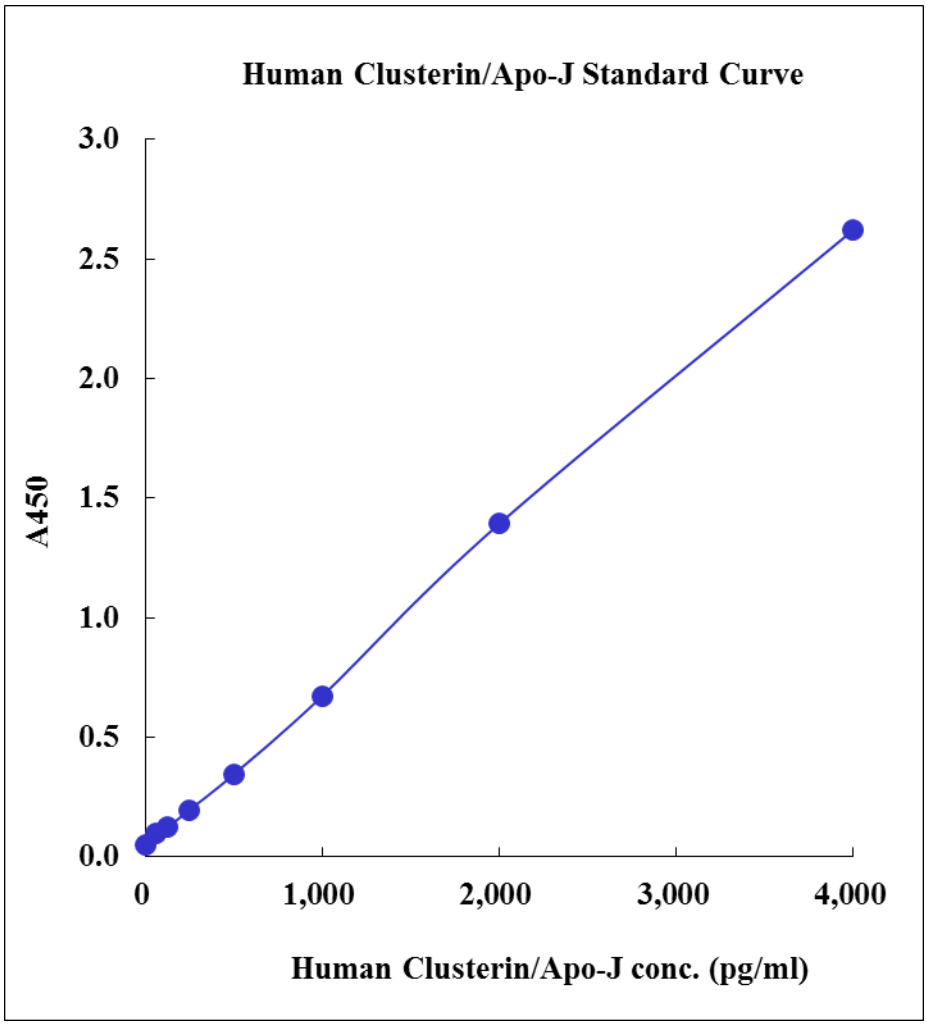
Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of human Clusterin/Apo-J giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 40.0 pg/mL of sample.

* Dilution Buffer was pipetted into blank wells.

Typical Standard Curve



2. Precision

Intra-assay Precision (Precision within an assay)

Three samples* of known concentration were tested eight times on one plate to assess intra-assay precision.

• Intra-assay (Within-Run, n=8); CV=2.9-4.5 %

*Sample: human serum

Human Clusterin/Apo-J conc. (µg/mL)

Sample No.	Sample 1	Sample 2	Sample 3
1	43.2	54.4	54.1
2	40.2	50.1	54.6
3	39.2	49.1	52.9
4	39.3	49.6	55.0
5	39.5	49.7	53.1
6	39.2	48.4	52.9
7	39.8	50.8	53.2
8	42.2	54.2	57.6
max.	43.2	54.4	57.6
min.	39.2	48.4	52.9
mean	40.3	50.8	54.2
SD	1.5	2.3	1.6
CV(%)	3.7	4.5	2.9

Inter-assay Precision (Precision between assays)

Three samples* of known concentration were tested in four separate assays to assess inter-assay precision.

• Inter-assay (Run-to-Run, n=4); CV=3.0-5.2 %

*Sample: human serum

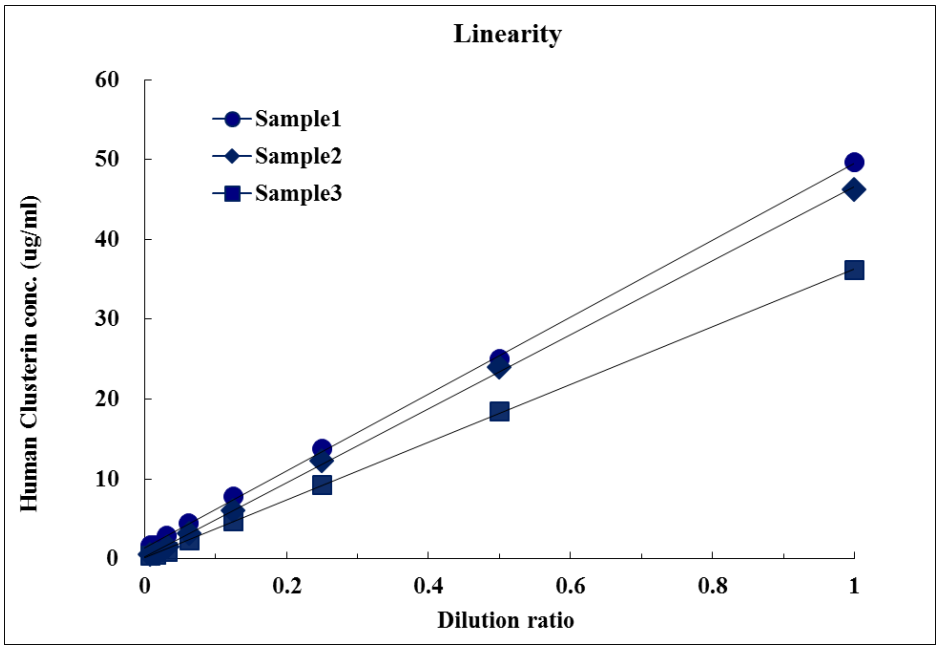
Human Clusterin/Apo-J conc. (µg/ml)

Sample No.	Sample 1	Sample 2	Sample 3
1	37.3	48.4	52.9
2	39.3	48.5	49.4
3	37.0	46.0	53.9
4	34.6	49.2	53.9
max.	39.3	49.2	53.9
min.	34.6	46.0	49.4
mean	37.1	48.0	52.5
SD	1.9	1.4	2.1
CV(%)	5.2	3.0	4.0

3. Linearity

Three samples* were diluted with Dilution Buffer and assayed after dilution. The neat sample was set to 1. Please note that all samples including the neat sample were 32,000-fold diluted as stated in the Assay Procedure. The results are summarized in the figure below.

*Sample: human serum



3. Spike and Recovery

Recombinant human Clusterin/Apo-J was added to samples at different concentrations.

Sample	Spiked Concentration (pg/ml)	Observed Concentration (pg/ml)	Expected Concentration (pg/ml)	Recovery (%)
Human serum	0	663.74	-	-
	250	862.75	913.74	94.4
	500	1138.33	1163.74	97.8
	1000	1531.04	1663.74	92.0
Human saliva	0	629.26	-	-
	250	797.58	879.26	90.7
	500	1037.62	1129.26	91.9
	1000	1491.21	1629.26	91.5
Human milk	0	1032.99	-	-
	250	1145.58	1282.99	89.3
	500	1315.12	1532.99	85.8
	1000	1702.01	2032.99	83.7
Human tear	0	1420.30	-	-
	250	1643.83	1670.30	98.4
	500	1855.29	1920.30	96.6
	1000	2347.10	2420.30	97.0

Example of Test Results

Fig.1 Human Clusterin/Apo-J concentrations in human sera of 72 healthy volunteers

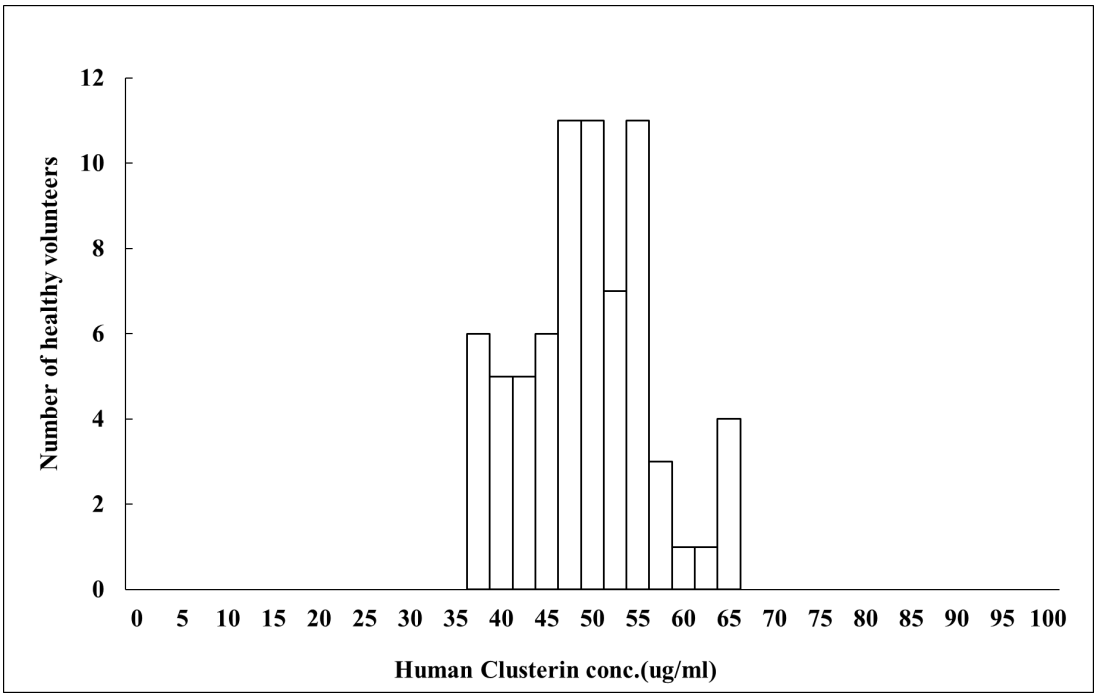


Fig.2 Human Clusterin/Apo-J concentrations in human tears of several volunteers

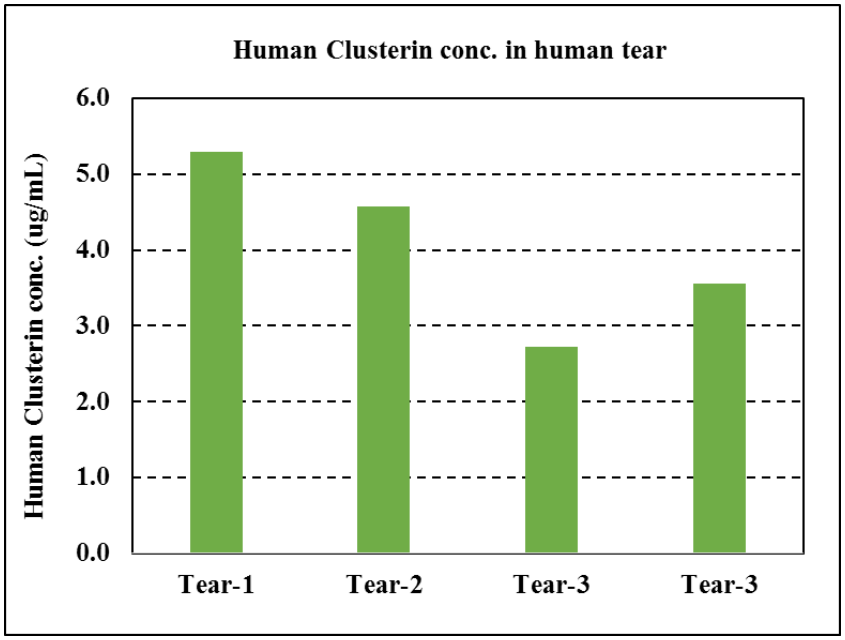


Fig.3 Human Clusterin/Apo-J concentrations in human milk of several volunteers

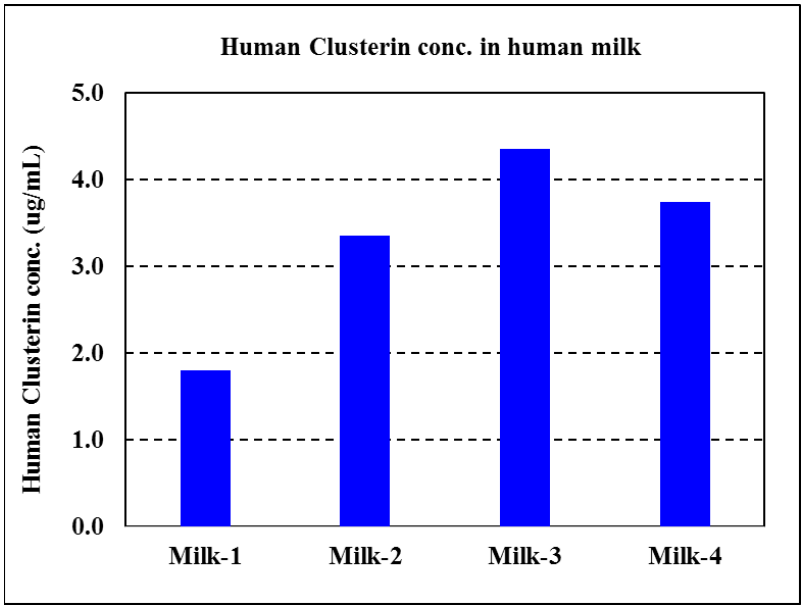


Fig.4 Human Clusterin/Apo-J concentrations in human saliva of several volunteers

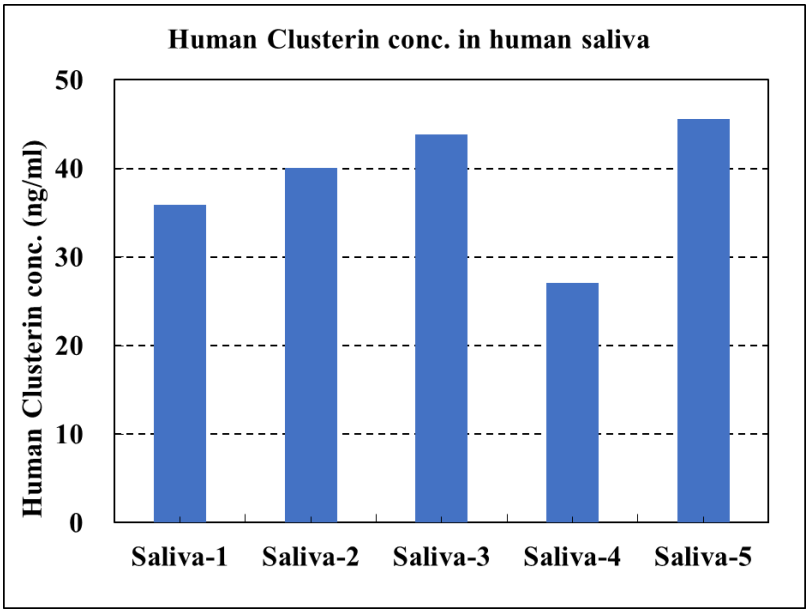


Fig.5 Concentrations of human Clusterin/Apo-J in cell lysates of three cell lines

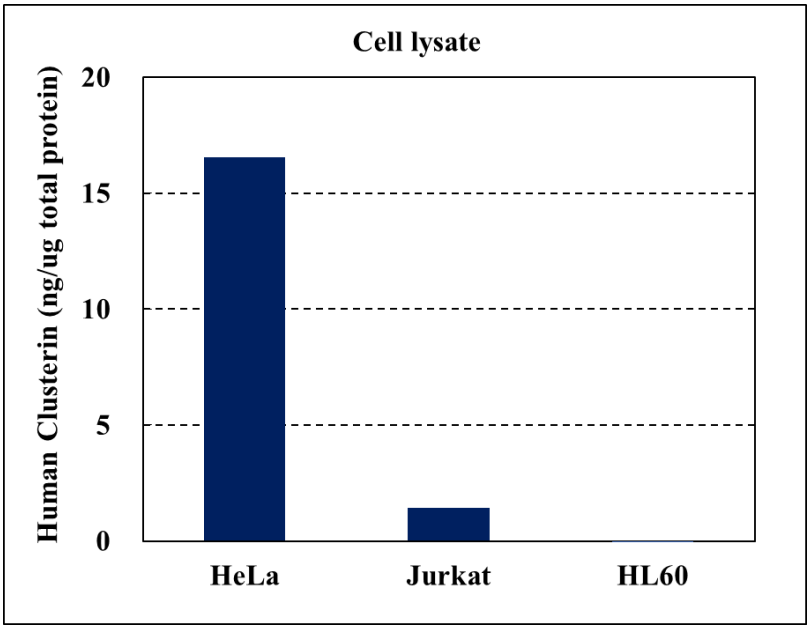
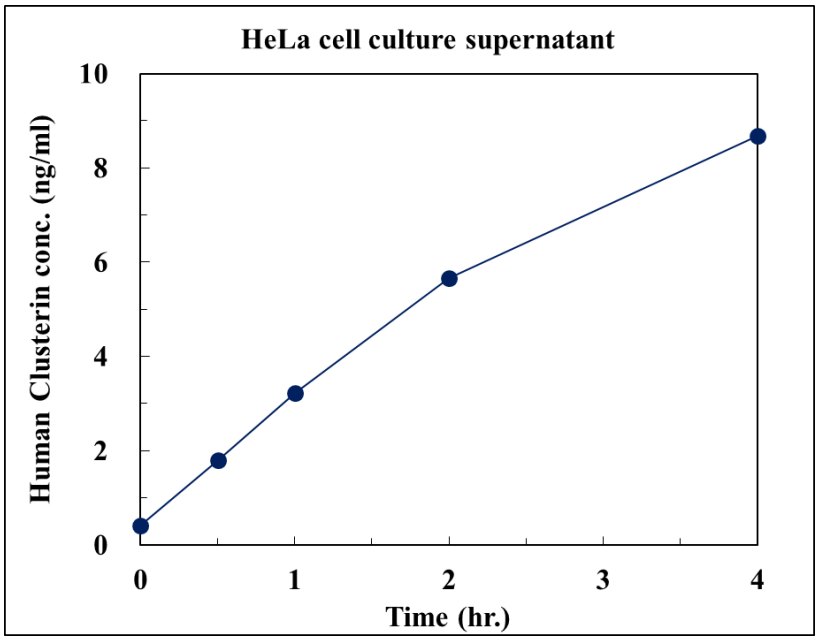


Fig.6 Concentrations of human Clusterin/Apo-J in HeLa cell culture supernatant after replaced with fresh media



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