



Human CD40 Ligand Immunoassay

Catalog Number: EA800100

For the quantitative determination of human CD40 Ligand concentrations in cell culture supernates, serum, and plasma.

For research use only. Not for use in diagnostic procedures.

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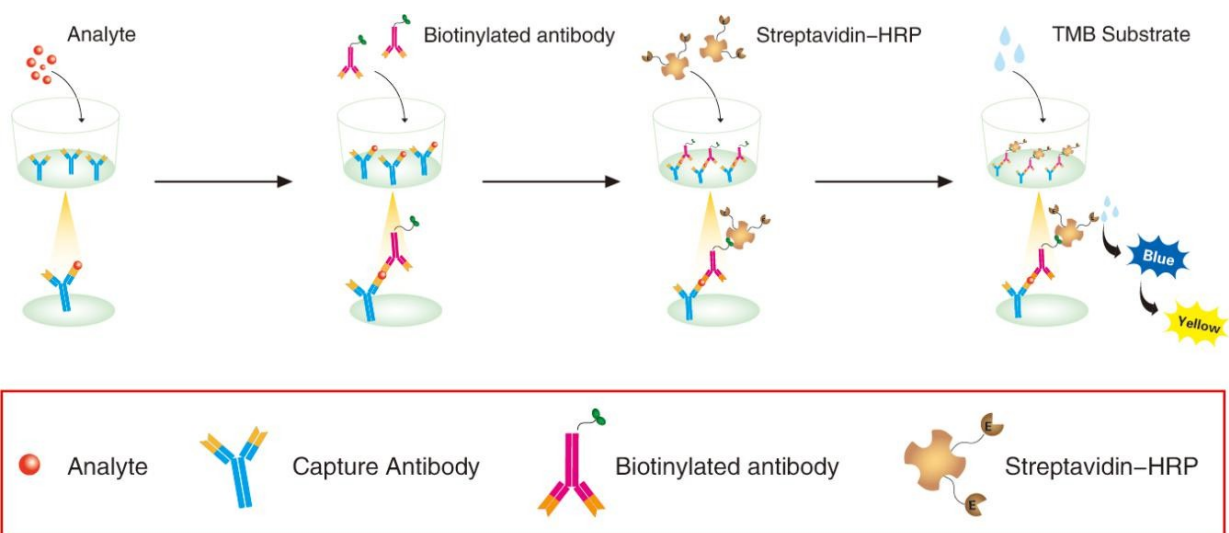
BACKGROUND

CD40 Ligand (CD40L), also known as CD154, gp39, TNFSF5, TRAP (TNF-Related Activation Protein) or TBAM (T-cell B-cell Activating Molecule), is a multifunctional ligand in the TNF superfamily. Interaction between CD40 and CD40L is critical to the control of thymus dependent humoral immunity and cell-mediated immune responses. The major component of the contact-dependent signal leading to B cell activation is CD40L. CD40L stimulates B cell secretion of immunoglobulin isotypes in the presence of cytokines. CD40L is a 39 kDa, 261 amino acid (aa) glycoprotein that can form homotrimers typical of other TNFSF members. Proteolytic cleavage can also produce 15-18 kDa soluble forms of CD40L. Activated T cells and platelets express both a membrane-associated and a soluble form of CD40L (sCD40L). Platelet activation during plasma and serum sample preparation can result in artificially elevated sCD40L levels. Conversely, serum samples stored above 2-8 °C show a progressive loss of the sCD40L signal.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for CD40 Ligand has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any CD40 Ligand present is captured by the coated antibody after incubation. Following extensive washing, a biotin-conjugate antibody specific for CD40 Ligand is added to detect the captured CD40 Ligand protein in sample. For signal development, horseradish peroxidase (HRP)-conjugated Streptavidin is added, followed by tetramethyl-benzidine (TMB) reagent. Following a wash to remove any unbound combination, and enzyme conjugate is added to the wells. Solution containing sulfuric acid is used to stop color development and the color intensity which is proportional to the quantity of bound protein is measurable at 450nm.

Schematic diagram:





1. This ELISA should not be used beyond the expiration data on the kit label.
2. To avoid cross-contamination, use a fresh reagent reservoir and pipette tips for each step.
3. To ensure accurate results, some details, such as technique, plasticware and water sources should be emphasized.
4. A thorough and consistent wash technique is essential for proper assay performance.
5. A standard curve should be generated for each set of samples assayed.
6. It is recommended that all standards and samples be assayed in duplicate.
7. Avoid microbial contamination of reagents and buffers. Buffers containing protein should be made under aseptic conditions and be prepared fresh daily.
8. In order to ensure the accuracy of the results, the standard curve should be made every time.

PRECAUTIONS

The Stop Solution suggested for use with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

KIT COMPONENTS & STORAGE CONDITIONS

PART	SIZE	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Microwell Plate - antibody coated 96-well Microplate (8 wells ×12 strips)	1 plate	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**
Standard - lyophilized,2000 pg/ml upon reconstitution	2 vials	Aliquot and Store at -20°C** for six months
Concentrated Biotin-Conjugated antibody(100X) - 120 ul/vial	1 vial	Store at 2-8°C **for six months
Concentrated Streptavidin-HRP solution(100X) - 120 ul/vial	1 vial	Store at 2-8°C** for six months
Standard/Sample Diluent - 16 ml/vial	1 bottle	Store at 2-8°C** for six months
Biotin-Conjugate antibody Diluent - 16 ml/vial	1 bottle	Store at 2-8°C** for six months
Streptavidin-HRP Diluent - 16 ml/vial	1 bottle	Store at 2-8°C** for six months
Wash Buffer Concentrate (20x) - 30 ml/vial	1 bottle	Store at 2-8°C** for six months
Substrate Solution - 12 ml/vial	1 bottle	Store at 2-8°C** for six months
Stop Solution - 12 ml/vial	1 bottle	Store at 2-8°C** for six months
Plate Cover Seals	4 pieces	

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

OTHER SUPPLIES REQUIRED BUT NOT SUPPLIED

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Pipettes and pipette tips.
3. Deionized or distilled water.
4. Squirrt bottle, manifold dispenser, or automated microplate washer.
5. 500 mL graduated cylinder.

SPECIMEN COLLECTION & STORAGE

Cell Culture Supernates - Centrifuge cell culture media at 1000×g to remove debris. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

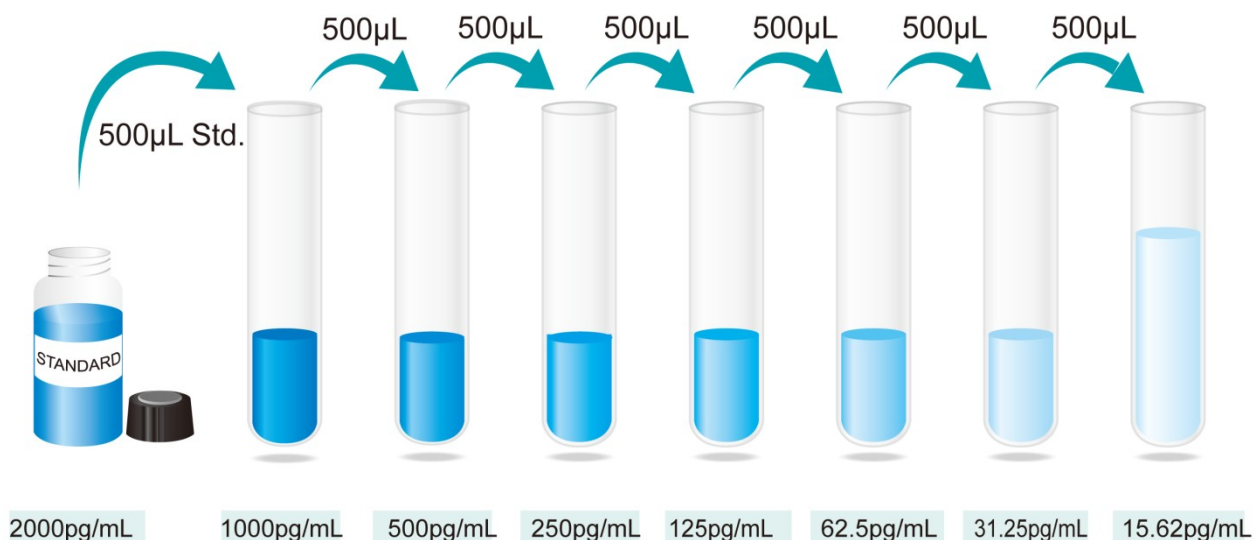
Serum - Use a serum separator tube (SST) and allow samples to clot for 2 hours at room temperature or overnight at 2-8°C. Centrifuge approximately for 15 minutes at 1000×g. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000×g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: The normal human serum or plasma samples are suggested to make a 1:2 dilution.

REAGENTS PREPARATION

1. **Temperature returning** - Bring all kit components and specimen to room temperature (20-25°C) before use.
2. **Wash Buffer** - Dilute 30mL of Wash Buffer Concentrate with 570mL of deionized or distilled water to prepare 600mL of Wash Buffer. If crystals have formed in the concentrate Wash Buffer, warm to room temperature and mix gently until the crystals have completely dissolved.
3. **Standard/Specimen** - Reconstitute the Standard with 1.0mL of Standard/Sample Diluent. This reconstitution produces a stock solution of 2000pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. Pipette 500µL of Standard/Sample Diluent into 1000pg/ml tube and the remaining tubes. Use the stock solution of 2000pg/mL to produce a 2-fold dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 1000 pg/mL standard serves as the high standard. The Standard/Sample Diluent serves as the zero standard (0 pg/mL).



Preparation of CD40 Ligand standard dilutions

***If you do not run out of re-melting standard, store it at -20°C. Diluted standard shall not be reused.**

- 4. Working solution of Biotin-Conjugate anti-human CD40 Ligand antibody:** Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with the Biotin-Conjugate antibody Diluent in a clean plastic tube.

***The working solution should be used within one day after dilution.**

- 5. Working solution of Streptavidin-HRP:** Make a 1:100 dilution of the concentrated Streptavidin-HRP solution with the Streptavidin-HRP Diluent in a clean plastic tube.

***The working solution should be used within one day after dilution.**

ASSAY PROCEDURE

Prepare all reagents and standards as directed. Wash the plate 3 times before assay.



Add 100µl standard or samples to each well, incubate 90 minutes, 37°C.



Aspirate and wash 4 times

Add 100µl working solution of Biotin-Conjugate anti-human CD40 Ligand antibody to each well, incubate 60 minutes, 37°C.

↓ Aspirate and wash 4 times

Add 100µl working solution of Streptavidin-HRP to each well, incubate 30 minutes, 37°C.

↓ Aspirate and wash 5 times

Add 100µl Substrate solution to each well, incubate 15 minutes, 37°C. Protect from light.

↓

Add 50µl Stop solution to each well. Read at 450nm within 5 minutes.
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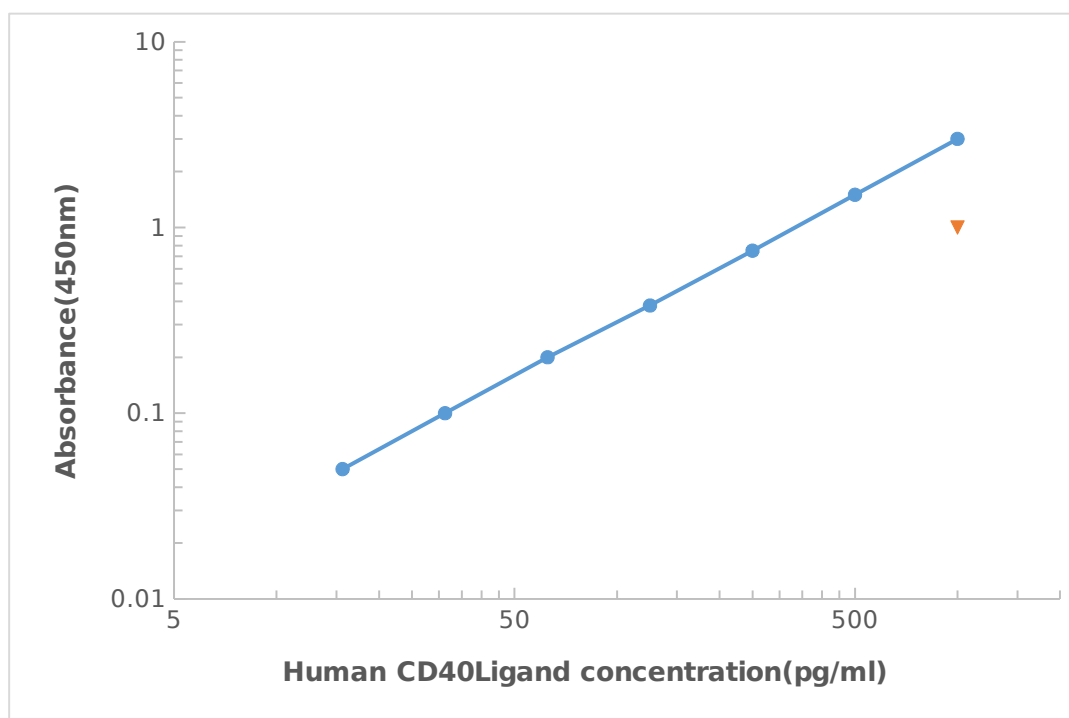
CALCULATION OF RESULTS

1. The standard curve is used to determine the amount of specimens.
2. First, average the duplicate readings for each standard, control, and sample. All O.D. values are subtracted by the mean value of blank control before result interpretation.
3. Construct 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.
4. The data may be linearized by plotting the log of the CD40 Ligand 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.
5. This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Typical data using the CD40 Ligand ELISA

Standard (pg/ml)	OD.	OD.	Average	Corrected
0	0.100	0.095	0.098	---
15.625	0.145	0.144	0.145	0.047
31.25	0.185	0.188	0.187	0.089

62.5	0.276	0.272	0.274	0.177
125	0.461	0.460	0.461	0.363
250	0.811	0.812	0.812	0.714
500	1.493	1.497	1.495	1.398
1000	2.491	2.493	2.492	2.395



Representative standard curve for CD40 Ligand ELISA.

Performance Characteristics

SENSITIVITY: The minimum detectable dose was 8pg/mL.

SPECIFICITY: This assay recognizes both natural and recombinant human CD40 Ligand. The



factors listed below were prepared at 100ng/ml in Standard /Sample Diluent and assayed for cross-reactivity and no significant cross-reactivity or interference was observed.

Factors assayed for cross-reactivity

Recombinant human	Recombinant mouse	Recombinant porcine
ANG	CD40 Ligand	IL-1 α
BDNF	CTLA-4	IL-1 β
CD4	Fas	IL-2
CD40	Fas Ligand	IL-4
CNTF	IFN- γ	IL-6
GDNF	IL-1 α	IL-8
IL-2	IL-1 β	IL-10
TNF- α	Leptin	TNF- α
TNF- β	LIF	
Tpo	TRANCE	

REPEATABILITY: The coefficient of variation of both intra-assay and inter-assay were less than 10%.

RECOVERY : The recovery of CD40 Ligand spiked to three different levels in four samples throughout the range of the assay in various matrices was evaluated.

Recovery of CD40 Ligand in two matrices

Sample Type	Average % of Expected Range (%)	Range (%)
Citrate plasma	95	87-103
Cell culture supernatants	97	89-106

LINEARITY: To assess the linearity of the assay, three samples were spiked with high concentrations of CD40 Ligand in various matrices and diluted with the appropriate Sample Diluent to produce samples with values within the dynamic range of the assay. (The plasma samples were initially diluted 1:1)

Dilution ratio	Recovery (%)	Citrate plasma	Cell culture supernatants
1:2	Average% of Expected	97	103
	Range (%)	88-102	93-114
1:4	Average% of Expected	99	105
	Range (%)	92-109	96-114
1:8	Average% of Expected	94	109
	Range (%)	82-103	101-118
1:16	Average% of Expected	95	107
	Range (%)	85-112	98-119

REFERENCES

- Grewal, I.S. and R.A. Flavell (1998) Annu. Rev. Immunol. 16:111.



2. Schonbeck, U. and P. Libby (2001) *Cell Mol. Life Sci.* 58:4.
3. van Kooten, C. and J. Banchereau (2000) *J. Leukoc. Biol.* 67:2.
4. Armitage, R.J. et al. (1993) *Semin. Immunol.* 5:401.
5. Peitsch, M.C. and C.V. Jongeneel (1993) *Int. Immunol.* 5:233.
6. Pietravalle, F. et al. (1996) *J. Biol. Chem.* 271:5965.
7. Graf, D. et al. (1995) *Eur. J. Immunol.* 25:1749.
8. Hsu, Y.M. et al. (1997) *J. Biol. Chem.* 272:911.
9. Henn, V. et al. (2001) *Blood* 98:1047.
10. Henn, V. et al. (1998) *Nature* 391:591.
11. Weber, M. et al. (2006) *Clin. Chem.* 52:888.
12. Khan, S.Y. et al. (2006) *Blood* 108:2455.
13. Varo, N. et al. (2006) *Clin. Sci.* 111:341.
14. Cognasse, F. et al. (2006) *Transfusion* 46:1184.
15. Lengelle, J. et al. (2008) *Cytokine* 44:275.
16. Mazzei, G.J. et al. (1995) *J. Biol. Chem.* 270:7025.