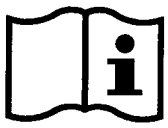
Product information



Userś Manual

MANUAL

CD40L (human) ELISA Kit

[CD154 (human) ELISA Kit]

REF IB99572

Σ 96

Storage: 2-8°C

RUO **For research use only, not for use in diagnostic procedures.**

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1. Intended Use

The CD40L (human) ELISA Kit is to be used for the quantitative determination of human CD40L in cell culture supernatants and serum. This ELISA Kit is for research use only.

2. Introduction

CD40 (also called Tumor Necrosis Factor Receptor Superfamily Member 5, TNFRSF5), a transmembrane type I glycoprotein, belongs to the tumor necrosis factor (TNF) gene superfamily and behaves as a co-stimulatory molecule found on B cells, monocytes, antigen presenting cells, endothelial, smooth muscle cells, fibroblasts and platelets (1, 2). Its ligand, CD40L (also called CD154), a type II transmembrane protein, is a 261 amino acid (aa) glycoprotein that forms homotrimers (3). CD40L is found on activated T cells, B cells, platelets, endothelial, epithelial and smooth muscle cells (3, 4). Proteolytic cleavage can produce a soluble form of CD40L (sCD40L) (4). Platelets are the main source of sCD40L (5).

CD40-CD40L interaction is an essential signal for B cell proliferation, expression of activation markers, immunoglobulin production and isotype switching, formation of B memory cells and germinal centers. It also prevents apoptosis of germinal center B cells (1-4). Defective expression of CD40L in humans leads to an inability to produce isotypes other than IgM (hyper IgM syndrome), and to an absence of germinal centers (2). In dendritic cells, CD40 ligation induces more effective antigen, enhances T-cell stimulatory capacity and induces production of several inflammatory cytokines and chemokines (6). CD40-CD40L interactions are crucially involved in development of autoimmune disease and have also impact on growth regulation of certain carcinomas (7). Elevated levels of sCD40L have been observed in sera from patients with systemic lupus erythematosus (SLE) (8), chronic lymphocytic leukemia (CLL) and unstable angina (1).

3. General References

(1) The Role of Soluble CD40L Ligand in Human Carcinogenesis: A. Angelou, et al.; Anticancer Res. **38**, 3199 (2018)

(2) CD40 as a therapeutic target in Sjögren's syndrome: K. Jobling & W.F. Ng; Expert Rev. Clin. Immunol. **14**, 535 (2018)

(3) Cloning of TRAP, a ligand for CD40 on human T cells: D. Graf, et al.; Eur. J. Immunol. **22**, 3191 (1992)

(4) Heteromultimeric complexes of CD40 ligand are present on the cell surface of human T lymphocytes: Y.M. Hsu, et al.; J. Biol. Chem. **272**, 911 (1997)

(5) LPS stimulation of purified human platelets is partly dependent on plasma soluble CD14 to secrete their main secreted product, soluble-CD40-Ligand: P. Damien, et al.; BMC Immunol. **16**, 3 (2015)

(6) Activation of human dendritic cells through CD40 cross-linking: C. Caux, et al.; J. Exp. Med. **180**, 1263 (1994)

(7) The role of CD40–CD154 interactions in autoimmunity and the benefit of disrupting this pathway: E. Toubi & Y. Shoenfeld; Autoimmunity **37**, 457 (2004)

(8) Elevated levels of soluble CD40 ligand (sCD40L) in serum of patients with systemic autoimmune diseases: A. Goules, et al.; J. Autoimmun. **26**, 165 (2006)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human CD40L in cell culture supernatants and serum. A monoclonal antibody specific for CD40L has been precoated onto the 96-well microtiter plate. Standards (STD) and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, CD40L is recognized by the addition of a biotinylated monoclonal antibody specific for CD40L (DET). After removal of excess biotinylated antibody, streptavidin-peroxidase (STREP-HRP) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450nm after acidification and is directly proportional to the concentration of CD40L in the samples.

5. Handling & Storage

- Reagent must be stored at 2-8°C when not in use
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

• 1 vial human CD40L Standard (lyophilized)	(100 ng)	(STD)
• 1 vial Detection Antibody	(30 µl)	(DET)
• 1 vial HRP Labeled Streptavidin (lyophilized)	(2 µg)	(STREP-HRP)
• 2 bottles Wash Buffer 10X	(2 x 30 ml)	(Wash Buffer 10X)
• 2 bottles ELISA Buffer 10X	(2 x 30 ml)	(ELISA Buffer 10X)
• 1 bottle TMB Substrate Solution	(12 ml)	(TMB)
• 1 bottle Stop Solution	(12 ml)	(STOP)
• 1 plate coated with CD40L Antibody	(6 x 16-well strips)	
• 2 plate covers (plastic film)		
• 2 silica gel minibags		

7. Materials Required but *Not* Supplied

- Microtiterplate reader at 450nm
- Calibrated precision pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard

8. General ELISA Protocol

8.1. Preparation and Storage of Reagents

NOTE: *Prepare just the appropriate amount of the buffers necessary for the assay.*

- **Wash Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 30 ml Wash Buffer 10X + 270 ml water) to obtain Wash Buffer 1X.
- **ELISA Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 10 ml ELISA Buffer 10X + 90 ml water) to obtain ELISA Buffer 1X.
- **Detection Antibody (DET)** has to be diluted to 1:500 in ELISA Buffer 1X (20 µl DET + 10 ml ELISA Buffer 1X).

  NOTE: *The diluted Detection Antibody is not stable and cannot be stored!*

- **HRP Labeled Streptavidin (STREP-HRP)** has to be reconstituted with 100 µl of ELISA Buffer 1X.

  - After reconstitution of STREP-HRP, prepare aliquots and store them at -20°C. **Avoid freeze/thaw cycles**.
  - Dilute the reconstituted STREP-HRP to the working concentration by adding 50 µl in 10 ml of ELISA Buffer 1X (1:200).

  NOTE: *The diluted STREP-HRP is not stable and cannot be stored!*

- **Human CD40L Standard (STD)** has to be reconstituted with 100 µl of ELISA Buffer 1X.

  - This reconstitution produces a stock solution of 1 µg/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes **at room temperature.** Mix well prior to making dilutions.

  NOTE: *The reconstituted standard is aliquoted and stored at -20°C!*

  - Dilute the standard protein concentrate (STD) (**1 µg/ml**) in ELISA Buffer 1X. A seven-point standard curve using 2-fold serial dilutions in ELISA Buffer 1X is recommended.

  - Suggested standard points are:
    2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0 ng/ml.

Start with the dilution of the concentrate (STD):

To obtain	Add	Into
10 ng/ml	10µl of CD40L (STD) (1 µg/ml)	990 µl of ELISA Buffer 1X

Dilute further for the standard curve:

To obtain	Add	Into
2ng /ml	200 µl of CD40L (10 ng/ml)	800 µl of ELISA Buffer 1X
1 ng/ml	300 µl of CD40L (2 ng/ml)	300 µl of ELISA Buffer 1X
0.5 ng/ml	300 µl of CD40L (1 ng/ml)	300 µl of ELISA Buffer 1X
0.250 ng/ml	300 µl of CD40L (0.5 ng/ml)	300 µl of ELISA Buffer 1X
0.125 ng/ml	300 µl of CD40L (0.25 ng/ml)	300 µl of ELISA Buffer 1X
0.0625 ng/ml	300 µl of CD40L (0.125 ng/ml)	300 µl of ELISA Buffer 1X
0.03125 ng/ml	300 µl of CD40L (0.0625 ng/ml)	300 µl of ELISA Buffer 1X
0 ng/ml	300 µl of ELISA Buffer 1X	Empty tube

8.2. Sample collection, storage and dilution

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 ≤ -20°C for later use. Avoid repeated freeze/thaw cycles.

Serum, and Cell Culture Supernatant have to be diluted in ELISA Buffer 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1/2 - 1/4 dilutions of serum of healthy patients are recommended! For sera of patients with cancers, 1/100 - 1/200 dilutions of sera are recommended. If sample values fall outside the detection range of the assay, a lower or higher dilution may be required!*

This kit has not been tested with plasma. But CD40 Ligand is expressed in platelet and is released as soluble form upon platelet activation. To measure circulating levels of CD40L in plasma, platelet-free plasma should be used.

8.3. Assay Procedure (Checklist)

☐	1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips are left in the bag with 2 silica gel minibags and stored at 4°C. **NOTE:** *Remaining 16-well strips coated with CD40L antibody when opened can be stored in the presence of 2 silica gel minibags at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted plasma, serum or cell culture supernatant samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2 Preparation of Samples).**
☐	3. Cover the plate with plastic film and incubate for **2 hours at Room Temperature.**
☐	4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	5. Add 100 µl to each well of the diluted Detection Antibody **(DET) (see 8.1 Preparation and Storage of Reagents)**.
☐	6. Cover the plate with plastic film and incubate for **1 hour at Room Temperature.**
☐	7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	8. Add 100 µl to each well of the diluted HRP Labeled Streptavidin **(STREP-HRP) (see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plastic film and incubate for **30 min at Room Temperature**.
☐	10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	11. Add 100 µl to each well of TMB substrate solution **(TMB)**.
☐	12. Allow the color reaction to develop **at Room Temperature in the dark for 15-20 minutes**. Do not cover the plate.
☐	13. Stop the reaction by adding 100 µl of Stop Solution (**STOP**). Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution (**STOP**) is added.
	! CAUTION: CORROSIVE SOLUTION !
☐	14. Measure the OD at 450 nm in an ELISA reader.

9. Calculation of Results

- Average the duplicate readings for each standard and sample and subtract the average blank value (obtained with the 0 ng/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding CD40L concentration (ng/ml) on the vertical axis (see **10.** TYPICAL DATA).
- Calculate the CD40L concentrations of samples by interpolation of the regression curve formula in a form of a quadratic equation.
- If the test sample was diluted, multiply the interpolated value by the dilution factor to calculate the concentration of human CD40L in the sample.

10. Typical Data

The following data are obtained using the different concentrations of standard as described in this protocol:

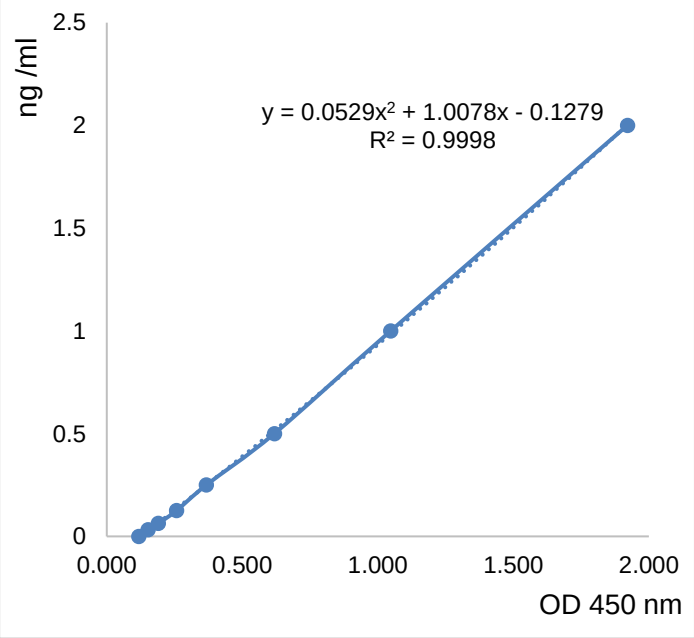

Figure: Standard curve

Standard CD40L (ng/ml)	Optical Density (mean)
2	1.921
1	1.048
0.5	0.618
0.25	0.367
0.125	0.257
0.0625	0.190
0.03125	0.152
0	0.118

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of human CD40L that can be detected by this assay is **20 pg/ml**.

NOTE: *The Limit of detection was measured by adding three standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.03125 ng/ml – 2 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human CD40L [CD254].

D. Intra-assay precision:

Four samples of known concentrations of human CD40L were assayed in replicates 6 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
A1	1.499	0.049	3.25	6
A2	0.245	0.009	3.84	6
A3	0.121	0.003	2.68	6
A4	0.890	0.047	5.29	6

E. Inter-assay precision:

Four samples of known concentrations of human CD40L were assayed in 5 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
B1	4.925	0.052	1.06	5
B2	0.500	0.011	2.27	5
B3	12.716	0.958	7.53	5
B4	0.062	0.002	2.62	5

F. Recovery:

When samples are spiked with known concentrations of human CD40L, the recovery averages range from 96% to 120%.

G. Linearity:

Different samples (sera and plasma) containing human CD40L were diluted several fold (1/2 or 1/4 for healthy patients; 1/400 or 1/800 for patients for cancers) and the measured recoveries ranged from 89% to 112%.

H. Expected values:

Human CD40L levels range in serum from **not detectable to >15ng/ml.**

12. Technical Hints and Limitations

- It is recommended that all standards and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 16-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Solution protected from light.
- The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

13. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
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 followed as indicated in the manual.
	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 STREP-HRP 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.

14. Notes