Human CD154 ELISA Kit

User Manual

Catalog # CEK1070

(Version 1.1B)

Sandwich Enzyme-Linked Immunosorbent Assay for Quantitative Detection of Human CD154 Concentrations in Cell Culture Supernatants, Serum, Plasma.

For research use only. Not for diagnostic or therapeutic procedures.

I. INTRODUCTION.....2
II. ASSAY PRINCIPLES.....3
III. KIT COMPONENTS.....4
IV. STORAGE AND STABILITY.....4
V. MATERIALS REQUIRED BUT NOT PROVIDED.....5
VI. HEALTH AND SAFETY PRECAUTIONS.....5
VII. REAGENT PREPARATION.....6
VIII. ASSAY PROCEDURE.....9
IX. ASSAY PROCEDURE SUMMARY.....11
X. TYPICAL DATA.....12
XI. SENSITIVITY.....12
XII. SPECIFICITY.....12
XIII. CROSS REACTIVITY.....13
XIV. REFERENCES.....13
XV. TROUBLESHOOTING GUIDE.....14
XVI. TECHNICAL SUPPORT.....15
XVII. NOTES.....15

I. INTRODUCTION

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 (1-4). Interaction between CD40 and CD40L is critical to the control of thymus-dependent humoral immunity and cell-mediated immune responses (5-10). 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 (1-4, 11, 12). Proteolytic cleavage can also produce 15-18 kDa soluble forms of CD40L (13, 14). Activated T cells and platelets express both a membrane-associated and a soluble form of CD40L (sCD40L) (13, 15, 16). Platelet activation during plasma and serum sample preparation can result in artificially elevated sCD40L levels (17-20). Conversely, serum samples stored above 2-8 °C show a progressive loss of the sCD40L signal (21). sCD40L lacks the transmembrane region and a portion of the extracellular domain but contains the entire TNF- homology region. Both the membrane-bound and soluble forms of CD40L are active (22). The receptor for CD40L is CD40, a member of the TNF receptor superfamily (TNFRSF5). Interaction of CD40L with CD40 not only induces proliferation and isotype switching in B lymphocytes but also mediates a broad variety of other immune and inflammatory responses (5-7). CD40 signaling has been linked with pathogenic processes of chronic inflammatory diseases such as autoimmune diseases, neurodegenerative disorders, graft-versus-host disease, cancer, and atherosclerosis (8). The loss of interaction between CD40 and CD40L can result in impairment of T lymphocyte function, B lymphocyte differentiation, and monocyte function. CD40L is expressed primarily on activated CD4+ T cells; however, vascular endothelial cells, smooth muscle cells, macrophages, basophils, eosinophils, monocytes, dendritic cells, fibroblasts, and mast cells also express CD40L. Cytokine stimulation (e.g. IL-1β, TNF-α, or IFN-γ) can increase surface levels and de novo

synthesis of CD40L in certain cell types (23). Hyper-IgM syndrome (HIGM) is an immunodeficiency characterized by elevated concentrations of serum IgM and the absence of serum IgG, IgA, and IgE. It is caused by mutations within the CD40L gene leading to defective expression on the membrane of activated T lymphocytes (24, 25). B lymphocytes from HIGM patients express functional CD40 and respond normally to wild-type CD40L, but their Tlymphocytes are unable to stimulate CD40 signaling pathways (26, 27). CD40L may play multiple roles in HIV infection (28). It may contribute to viral replication control by inducing HIV-suppressive chemokines, by downregulating monocyte cell surface expression of CCR5 and CD4, and by supporting the production of anti-HIV antibodies and cytotoxic T cells (28-31). It can also promote HIV replication in CD4+ T lymphocytes by activating antigen-presenting cells, subsequently leading to increased CD4+ T cell activation (28). With the onset of AIDS, CD40L-expressing CD4+ T cells become selectively depleted. This loss may explain the similarity between the opportunistic infections characteristic of AIDS and those observed with congenital CD40L deficiency (28). Elevated levels of sCD40L have been observed in sera from patients with systemic lupus erythematosus (SLE), chronic lymphocytic leukemia (CLL), and unstable angina (32-34). A direct relationship has been seen between disease severity and sCD40L in SLE patient sera (32). Aberrant expression of CD40L may thus contribute to autoantibody secretion in SLE through activation of bystander B lymphocytes, including cells that have been exposed to self antigens (32). Prolonged survival of malignant CLL cells may be linked to elevated levels of biologically active sCD40L (33). CD40L can mediate the resistance of CLL cells to apoptosis by Fas Ligand and fludarabine (33). Enhanced levels of both soluble and membrane-bound forms of CD40L in angina patients suggests that the CD40L-CD40 interaction may play a pathogenic role in the atherosclerotic process and in promoting acute coronary syndromes (34).

II. ASSAY PRINCIPLES

The Cohesion Bioscience Human CD154 ELISA (Enzyme-Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of Human CD154 in Cell Culture Supernatants, Serum, Plasma. This assay employs an antibody specific for Human CD154 coated on a 96-well plate. Standards and samples are pipetted into the wells and CD154 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-Human CD154 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of CD154 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

III. KIT COMPONENTS

Component	Volume
96-well Plate Coated With Anti-Human CD154 Antibody	8 wells x 12 Strips
Human CD154 Standard	10 ng x 2
Biotin-Labeled Detection Antibody (100X)	120 µl
Streptavidin-HRP (100X)	120 µl
Standard/Sample Diluent	30 ml
Detection Antibody Diluent	12 ml
Streptavidin-HRP Diluent	12 ml
Wash Buffer (20X)	30 ml
TMB Substrate Solution	12 ml
Stop Solution	12 ml
Plate Adhesive Strips	3 Strips
Technical Manual	1 Manual

IV. STORAGE AND STABILITY

All kit components are stable at 2 to 8 °C. Standard (recombinant protein) should be stored at -20 °C or -80 °C (recommended at -80 °C) after reconstitution. Opened Microplate Wells or reagents may be store for up to 1 month at 2 to 8 °C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

Note: the kit can be used within one year if the whole kit is stored at -20 °C. Avoid repeated freeze-thaw cycles.

V. MATERIALS REQUIRED BUT NOT PROVIDED

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Adjustable pipettes and pipette tips to deliver 2 µl to 1 ml volumes.
3. Adjustable 1-25 ml pipettes for reagent preparation.
4. 100 ml and 1 liter graduated cylinders.
5. Absorbent paper.
6. Distilled or deionized water.
7. Computer and software for ELISA data analysis.
8. Tubes to prepare standard or sample dilutions.

VI. HEALTH AND SAFETY PRECAUTIONS

1. Reagents provided in this kit may be harmful if ingested, inhaled or absorbed through the skin. Please carefully review the MSDS for each reagent before conducting the experiment.
2. Stop Solution contains 2 N Sulfuric Acid (H_2SO_4) and is an extremely corrosive agent. Please wear proper eye, hand and face protection when handling this material. When the experiment is finished, be sure to rinse the plate with copious amounts of running water to dilute the Stop Solution prior to disposing the plate.
3. Standard protein and Detection Antibody containing Sodium Azide as a preservative.

VII. REAGENT PREPARATION

1. Sample Preparation

Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

Cell culture supernates: Remove particulates by centrifugation, assay immediately or aliquot and store samples at -20°C.

Serum: Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature. Centrifuge at approximately 1000 X g for 15 minutes. Analyze the serum immediately or aliquot and store samples at -20°C.

Plasma: Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1500 X g within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C.

Cell Lysates: Collect cells and rinse cells with PBS. Homogenize and lyse cells throughly in lysate solution. Centrifuge cell lysates at approximately 10000 X g for 5 minutes to remove debris. Aliquots of the cell lysates were removed and assayed.

Bone Tissue: Extract demineralized bone samples in 4 M Guanidine-HCl and protease inhibitors. Dissolve the final sample in 2 M Guanidine-HCl.

Tissue Homogenates: The preparation of tissue homogenates will vary depending upon tissue type. Rinse tissue with 1X PBS to remove excess blood, homogenized in 20 mL of 1X PBS and stored overnight at ≤ -20 °C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g. The supernate was removed immediately and assayed. Alternatively, aliquot and store samples at ≤ -20 °C.

Note: Some lysis buffer, such as RIPA can not be used. Some components will affect the binding.

Urine: Urinary samples should be cleared by centrifugation and then can be used directly without dilution. Storage at -20°C.

2. Human CD154 Standard Preparation

Reconstitute the lyophilized Human CD154 Standard by adding 1 ml of Standard/Sample Diluent to make the 10000 pg/ml standard stock solution. Allow solution to sit at room temperature for 5 minutes, then gently vortex to mix completely. Use within one hour of reconstituting. Two tubes of the standard (10 ng per tube) are included in each kit. Use one tube for each experiment.

Perform 2-fold serial dilutions of the top standards to make the standard curve within the range of this assay (156.3 pg/ml - 10000 pg/ml) as below.

Standard/Sample Dilution Buffer serves as the zero standard (0 pg/ml).

Standard	Add	Into
10,000 pg/ml		
5,000 pg/ml	500 µl of the Standard (10,000 pg/ml)	500 µl of the Standard/Sample Diluent
2,500 pg/ml	500 µl of the Standard (5,000 pg/ml)	500 µl of the Standard/Sample Diluent
1,250 pg/ml	500 µl of the Standard (2,500 pg/ml)	500 µl of the Standard/Sample Diluent
625 pg/ml	500 µl of the Standard (1,250 pg/ml)	500 µl of the Standard/Sample Diluent
313 pg/ml	500 µl of the Standard (625 pg/ml)	500 µl of the Standard/Sample Diluent
156 pg/ml	500 µl of the Standard (313 pg/ml)	500 µl of the Standard/Sample Diluent
0 ng/ml	1 ml of the Standard/Sample Diluent	

Note: The standard solutions are best used within 2 hours. The 10000 pg/ml standard solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

3. Biotin-Labeled Detection Antibody Working Solution Preparation

The Biotin-Labeled Detection Antibody should be diluted in 1:100 with the Detection Antibody Diluent and mixed thoroughly. The solution should be prepared no more than 2 hours prior to the experiment.

4. Streptavidin-HRP Working Solution Preparation

The Streptavidin-HRP should be diluted in 1:100 with the Streptavidin-HRP Diluent and mixed thoroughly. The solution should be prepared no more than 1 hour prior to the experiment.

5. Wash Buffer Working Solution Preparation

Pour entire contents (30 ml) of the Wash Buffer Concentrate into a clean 1,000 ml graduated cylinder. Bring final volume to 600 ml with glass-distilled or deionized water (1:20).

VIII. ASSAY PROCEDURE

The Streptavidin-HRP Working Solution and TMB Substrate Solution must be kept warm at 37°C for 30 minutes before use. When diluting samples and reagents, they must be mixed completely and evenly. Standard detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of protein amount in samples.

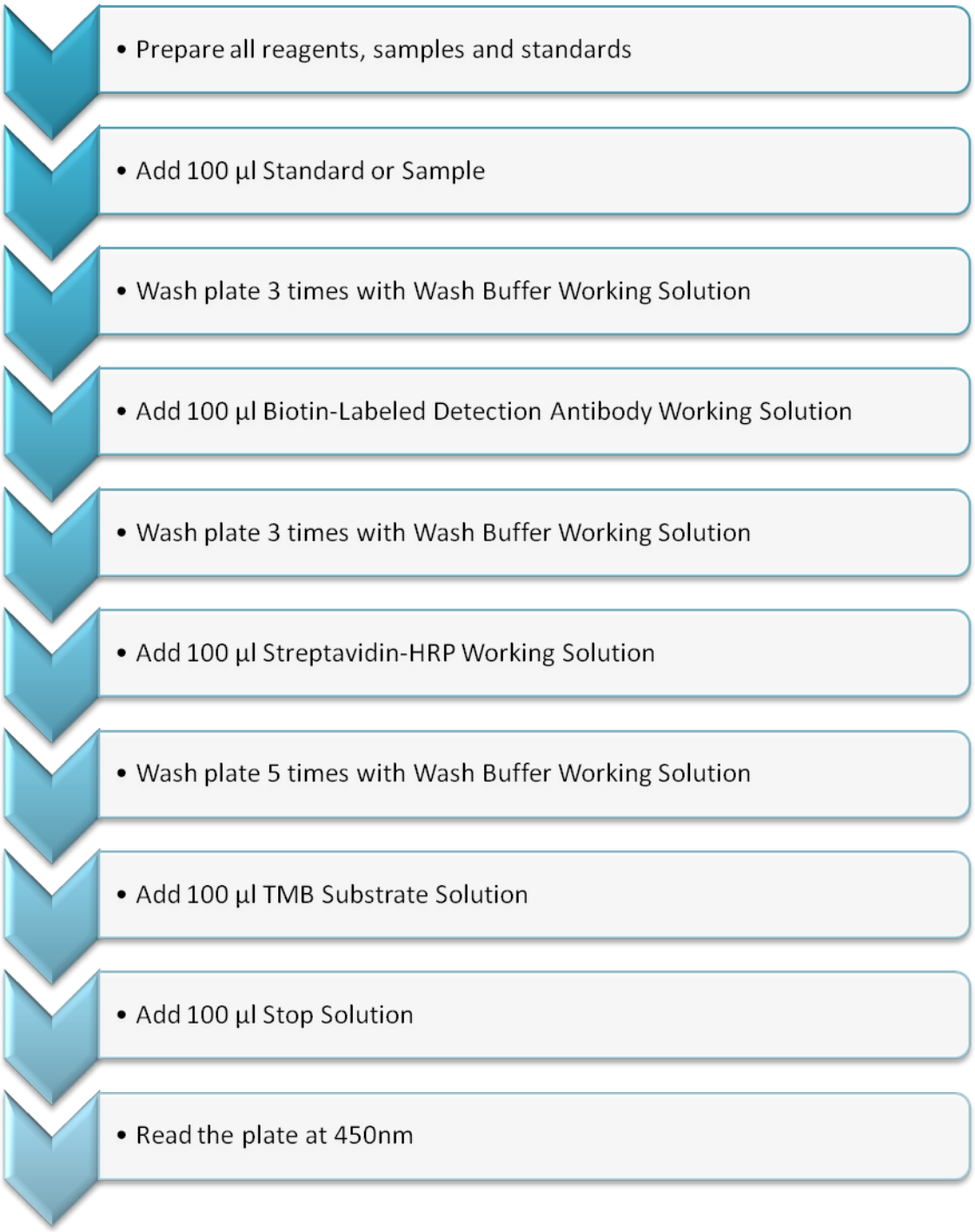
1. Add 100 µl of each standard and sample into appropriate wells.
2. Cover well and incubate for 90 minutes at room temperature or over night at 4°C with gentle shaking.
3. Remove the cover, discard the solution and wash plate 3 times with Wash Buffer Working Solution, and each time let Wash Buffer Working Solution stay in the wells for 1 - 2 minutes. Blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 100 µl of Biotin-Labeled Detection Antibody Working Solution into each well and incubate the plate at 37°C for 60 minutes.
5. Wash plate 3 times with Wash Buffer Working Solution, and each time let Wash Buffer Working Solution stay in the wells for 1 - 2 minutes. Discard the Wash Buffer Working Solution and blot the plate onto paper towels or other absorbent material.
6. Add 100 µl of Streptavidin-HRP Working Solution into each well and incubate the plate at 37°C for 45 minutes.
7. Wash plate 5 times with Wash Buffer Working Solution, and each time let wash buffer stay in the wells for 1 - 2 minutes. Discard the wash buffer and blot the plate onto paper towels or other absorbent material.
8. Add 100 µl of TMB Substrate Solution into each well and incubate plate at 37°C in dark for 10-20 minutes.
9. Add 100 µl of Stop Solution into each well. The color changes into yellow immediately.

10. Read the O.D. absorbance at 450nm in a microplate reader within 30 minutes after adding the Stop Solution.

For calculation, (the relative O.D.450) = (the O.D.450 of each well) - (the O.D.450 of Zero well). The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The concentration of the samples can be interpolated from the standard curve.

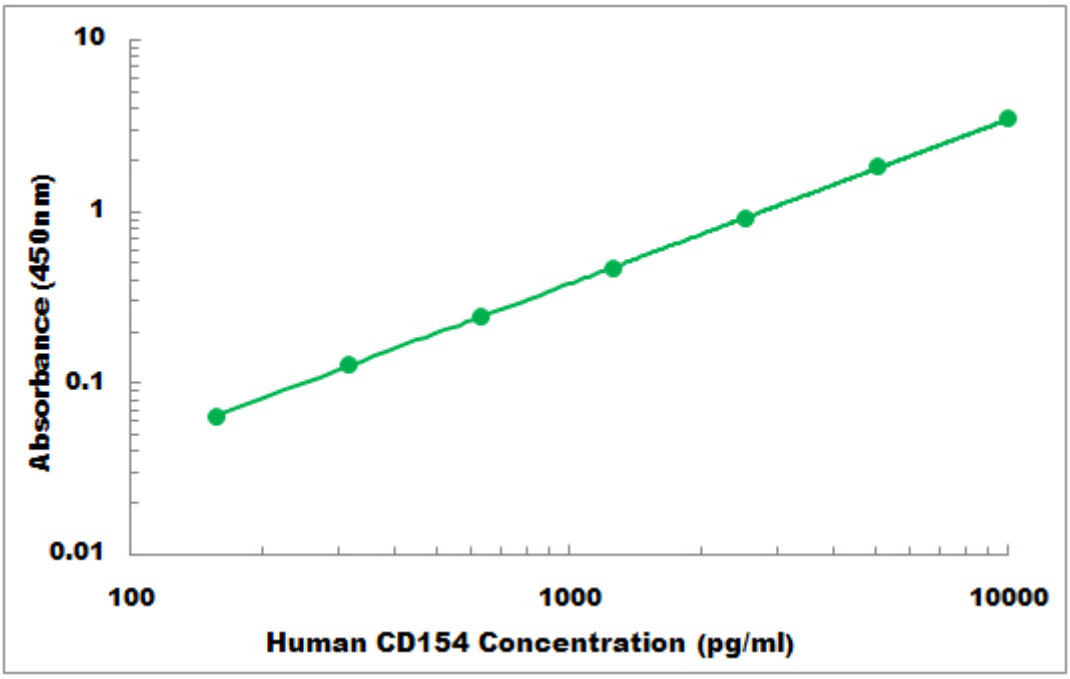
Note: If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

IX. ASSAY PROCEDURE SUMMARY



X. TYPICAL DATA

The standard curve is for demonstration only. A standard curve must be run with each assay.



XI. SENSITIVITY

The minimum detectable dose of Human CD154 is typically less than 40 pg/ml.

XII. SPECIFICITY

The Human CD154 ELISA Kit allows for the detection and quantification of endogenous levels of natural and/or recombinant Human CD154 proteins within the range of 156.3 pg/ml - 10000 pg/ml.

XIII. CROSS REACTIVITY

No detectable cross-reactivity with other relevant proteins.

XIV. REFERENCES

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XV. TROUBLESHOOTING GUIDE

Problem	Possible Cause	Solution
High signal and background in all wells	• Insufficient washing	• Increase number of washes • Increase time of soaking between in wash
	• Too much Streptavidin-HRP	• Check dilution, titration
	• Incubation time too long	• Reduce incubation time
	• Development time too long	• Decrease the incubation time before the stop solution is added
No signal	• Reagent added in incorrect order, or incorrectly prepared	• Review protocol
	• Standard has gone bad (If there is a signal in the sample wells)	• Check the condition of stored standard
	• Assay was conducted from an incorrect starting point	• Reagents allows to come to 20 - 30 °C before performing assay
Too much signal-whole plate turned uniformly blue	• Insufficient washing-unbound Streptavidin-HRP remaining	• Increase number of washes Carefully
	• Too much Streptavidin-HRP	• Check dilution
	• Plate sealer or reservoir reused, resulting in presence of residual Streptavidin-HRP	• Use fresh plate sealer and reagent reservoir for each step
Standard curve achieved but poor discrimination between point	• Plate not developed long enough	• Increase substrate solution incubation time
	• Improper calculation of standard curve dilution	• Check dilution, make new standard curve
No signal when a signal is expected, but standard curve looks fine	• Sample matrix is masking detection	• More diluted sample Recommended
Samples are reading too high, but standard curve is fine	• Samples contain protein levels above assay range	• Dilute samples and run Again
Edge effect	• Uneven temperature around work surface	• Avoid incubating plate in areas where environmental conditions vary • Use plate sealer

XVI. TECHNICAL SUPPORT

For troubleshooting, information or assistance, please go online to www.cohesionbio.com or contact us at techsupport@cohesionbio.com

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XVII. NOTES