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Quantikine™ QuicKit™ ELISA

Human CD40 Ligand/TNFSF5 Immunoassay

Catalog Number QK1012
SK1012
PK1012

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

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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Manufactured and Distributed by:

USA R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413
TEL: 800 343 7475   612 379 2956
FAX: 612 656 4400
E-MAIL: info@bio-techne.com

Distributed by:

Europe | Middle East | Africa Bio-Techne Ltd.
19 Barton Lane, Abingdon Science Park
Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info.emea@bio-techne.com

China Bio-Techne China Co., Ltd.
Unit 1901, Tower 3, Raffles City Changning Office,
1193 Changning Road, Shanghai PRC 200051
TEL: +86 (21) 52380373   (400) 821-3475
FAX: +86 (21) 52371001
E-MAIL: info.cn@bio-techne.com

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 T lymphocytes 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).

INTRODUCTION *CONTINUED*

Elevated levels of CD40L 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 suggest that the CD40L-CD40 interaction may play a pathogenic role in the atherosclerotic process and in promoting acute coronary syndromes (34).

The Quantikine™ QuicKit™ Human CD40 Ligand/TNFSF5 Immunoassay is a one-step, 90-minute solid phase ELISA designed to measure human CD40 Ligand levels in cell culture supernates, serum, and platelet-poor plasma. It contains recombinant human CD40 Ligand and antibodies raised against the recombinant protein. Results obtained for naturally occurring human CD40 Ligand showed linear curves that were parallel to the standard curves obtained using the recombinant QuicKit standards. These results indicate that this kit can be used to determine relative mass values for natural human CD40 Ligand.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. An anti-tag antibody has been pre-coated onto a microplate. Standards and samples are pipetted into the wells followed by an antibody cocktail. The antibody cocktail consists of an affinity tag labeled monoclonal capture antibody and an enzyme-linked monoclonal detection antibody, specific for human CD40 Ligand. After washing away any unbound substances, a substrate solution is added to the wells and color develops in proportion to the amount of CD40 Ligand bound. The color development is stopped and the intensity of the color is measured.

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LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with the calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine™ QuicKit™ Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- Ensure reagent addition to plate wells is uninterrupted.
- To ensure accurate results, proper adhesion of the plate sealer during the incubation step is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	CATALOG # QK1012	CATALOG # SK1012	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
QuicKit™ Coated Microplate	899063	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with an anti-tag monoclonal antibody.	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.*
Human CD40 Ligand Standard	899611	2 vials	12 vials	Recombinant human CD40 Ligand in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for the reconstitution volume.*	Use a freshly reconstituted standard for each assay. Discard after use.
Human CD40 Ligand Capture Ab Concentrate	899609	1 vial	6 vials	Lyophilized tagged monoclonal antibody specific for human CD40 Ligand.	May be stored for up to 1 month at 2-8 °C.*
Human CD40 Ligand Detection Ab Concentrate	899610	1 vial	6 vials	400 µL of a monoclonal antibody specific for human CD40 Ligand conjugated to horseradish peroxidase with preservatives.	
Assay Diluent RD1-125	896332	1 vial	6 vials	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5P	895151	1 vial	6 vials	21 mL of a buffered protein base with preservatives. *Use diluted 1:5 in this assay.*	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
TMB ELISA Substrate	642736	1 vial	6 vials	12 mL of a TMB ELISA substrate.	
ELISA Stop Solution	642827	1 vial	6 vials	12 mL of an acid solution.	
Plate Sealers	N/A	4 strips	12 strips	Adhesive strips.	

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

QK1012 contains sufficient materials to run an ELISA on one 96 well plate.
SK1012 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PK1012). Refer to the PharmPak Contents section for specific vial counts.

PHARMPAK CONTENTS

Each PharmPak has enough reagents to assay 50 microplates (96 wells/plate). Although the inserts are the same as those for the single kit inserts, there are minor differences related to the number of reagents and their container sizes.

- Sufficient material is supplied to perform at least 50 standard curves; reuse of each vial may be required. The number of vials, and the number of standard curves obtained per vial will vary with the analyte.
- Wash Buffer 25X Concentrate is bulk packed in 125 mL bottles containing 100 mL.
  Note: *Additional wash buffer is available for purchase* (R&D Systems™, Catalog # WA126).

The reagents provided in this PharmPak are detailed below.

PART	PART #	QUANTITY
QuicKit™ Coated Microplate	899063	50 plates
Human CD40 Ligand Standard	899611	25 vials
Human CD40 Ligand Capture Ab Concentrate	899609	50 vials
Human CD40 Ligand Detection Ab Concentrate	899610	50 vials
Assay Diluent RD1-125	896332	50 vials
Calibrator Diluent RD5P	895151	50 vials
Wash Buffer Concentrate	895126	9 bottles
TMB ELISA Substrate	642736	50 vials
ELISA Stop Solution	642827	50 vials
Plate Sealers	N/A	100 sheets

**If additional standard vials are needed, contact Technical Service at techsupport@bio-techne.com*

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm with the correction wavelength set at 540 nm or 570 nm
- Pipettes and pipette tips
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated microplate washer
- 500 mL graduated cylinder
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for dilution of standards and samples
- Human CD40 Ligand Controls (optional; R&D Systems, Catalog # QC326)

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and 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 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Platelet-poor Plasma - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge at 2-8 °C for 15 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the separated plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Grossly hemolyzed samples are not suitable for use in this assay.
Grossly icteric samples are not suitable for use in this assay.

CD40 Ligand is present in platelet granules and is released upon platelet activation. Therefore, to measure circulating levels of CD40 Ligand, platelet-free plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical Laboratory Standards Institute (CLSI), result in incomplete removal of platelets from blood. This will cause variable and irreproducible results for assays of factors contained in platelets and released by platelet activation.

SAMPLE PREPARATION

Due to high endogenous levels, serum requires a minimum 5-fold dilution. A suggested 5-fold dilution is 40 µL sample + 160 µL Calibrator Diluent RD5P (diluted 1:5)*.

Platelet-poor plasma and cell culture supernates may be tested neat.

Multiple dilutions are recommended for unknown samples.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Human CD40 Ligand Capture Antibody Concentrate - Refer to the vial label for reconstitution volume. Reconstitute the Human CD40 Ligand Capture Ab Concentrate with Assay Diluent RD1-125. This reconstitution produces a 20X Capture Antibody stock. Allow the capture antibody to rest for a minimum of 5 minutes with gentle agitation before diluting. The 20X capture antibody stock can be stored for up to 4 weeks at 2-8 °C once reconstituted.

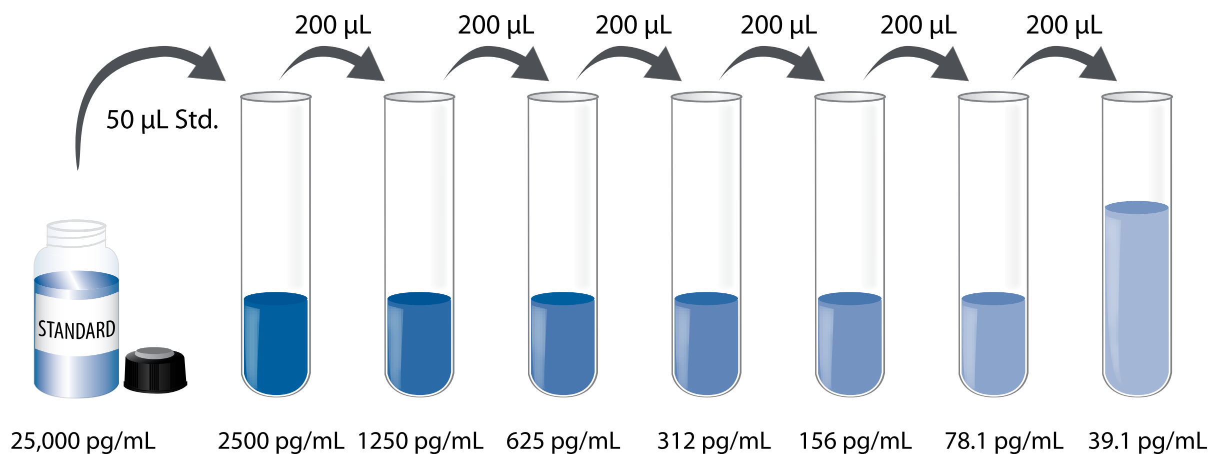
Antibody Cocktail - Dilute the reconstituted Capture Ab stock and the Detection Ab Concentrate 20-fold in Assay Diluent RD1-125. For a full plate, combine 300 µL of the reconstituted Human CD40 Ligand Capture Ab stock and 300 µL of Human CD40 Ligand Detection Ab Concentrate with 5.4 mL of Assay Diluent RD1-125 to create 6 mL of Human CD40 Ligand Antibody Cocktail.

Calibrator Diluent RD5P (diluted 1:5) - Add 2 mL of Calibrator Diluent RD5P to 8 mL of deionized or distilled water to prepare 10 mL of Calibrator Diluent RD5P (diluted 1:5).

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Human CD40 Ligand Standard - **Refer to vial label for reconstitution volume.** Reconstitute the Human CD40 Ligand Standard with deionized or distilled water. This reconstitution produces a stock solution of 25,000 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions. Use the standard stock within 60 minutes.

Pipette 450 µL of Calibrator Diluent RD5P (diluted 1:5) into the 2500 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 2500 pg/mL standard serves as the high standard. Calibrator Diluent RD5P (diluted 1:5) serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

1. Prepare all reagents, samples, and working standards as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of standard, control, or sample* per well. A plate layout is provided to record standards and samples assayed.
4. Add 50 µL Antibody Cocktail to each well. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Aspirate each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. 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.
6. Add 100 µL of TMB ELISA Substrate solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
7. Add 100 µL of ELISA Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
8. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation Section.

CALCULATION OF RESULTS

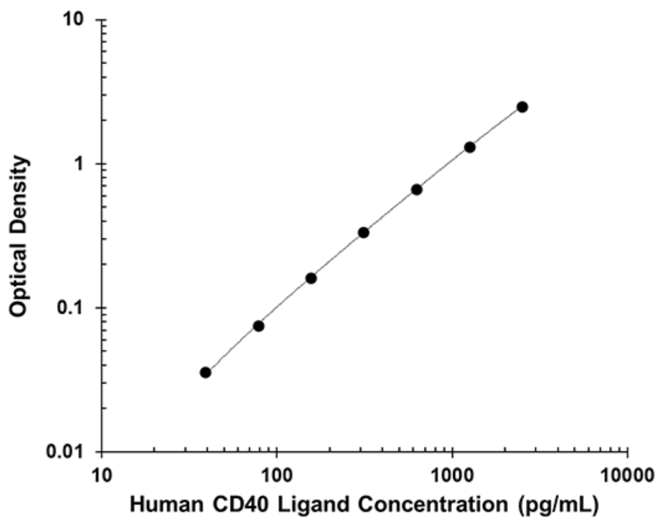
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create 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. 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.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.016 0.017	0.017	—
39.1	0.053 0.053	0.053	0.036
78.1	0.091 0.092	0.092	0.075
156	0.172 0.183	0.178	0.161
312	0.348 0.356	0.352	0.335
625	0.680 0.692	0.686	0.669
1250	1.304 1.351	1.328	1.311
2500	2.493 2.543	2.518	2.501

PRECISION

Intra-Assay Precision (Precision within an assay)
Two samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays)
Two samples of known concentration were tested in ten separate assays to assess inter-assay precision. Assays were performed by at least five technicians.

	Intra-Assay Precision		Inter-Assay Precision	
Sample	**1**	**2**	**1**	**2**
n	20	20	10	10
Mean (pg/mL)	211	1277	217	1370
Standard deviation	8.46	31.0	20.3	99.2
CV (%)	4.0	2.4	9.4	7.2

RECOVERY

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

Sample Type	Average % Recovery	Range %
Cell culture media (n=2)	117	112-126
Serum (n=2)	94	85-102
PP-EDTA plasma (n=2)	101	90-118
PP-heparin plasma (n=2)	102	87-126

SENSITIVITY

Thirteen assays were evaluated and the minimum detectable dose (MDD) of CD40 Ligand ranged from 1.15-4.76 pg/mL. The mean MDD was 2.43 pg/mL.

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

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LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of CD40 Ligand were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=2)	Serum (n=2)	PP-EDTA plasma (n=2)	PP-heparin plasma (n=2)
1:2	Average % of Expected	93	99	98	99
	Range (%)	92-94	96-102	98-99	98-100
1:4	Average % of Expected	88	95	96	98
	Range (%)	88-89	92-99	96-96	95-101
1:8	Average % of Expected	86	93	96	101
	Range (%)	85-86	90-97	95-96	97-105
1:16	Average % of Expected	89	91	97	100
	Range (%)	88-89	90-92	96-98	96-104

CALIBRATION

This immunoassay is calibrated against a highly purified recombinant human CD40 Ligand produced at R&D Systems™.

SAMPLE VALUES

Serum/plasma - Samples from apparently healthy volunteers were evaluated for the presence of CD40 Ligand in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=10)	4160	100	2171-6027
PP-EDTA plasma (n=10)	199	100	42.3-475
PP-heparin plasma (n=10)	194	80	ND-451

ND=Non-Detectable

Cell Culture Supernates - N1186 cells were cultured in RPMI supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. The culture was further supplemented with 10 ng/mL recombinant human IL-2 (Catalog # 202-IL or BT-002) and maintained until reaching a cell density between 1-2 x 10^6 cells/mL. Cells were either left unstimulated or stimulated for 24 hours with 500 ng/mL ionomycin calcium salt and 10 ng/mL PMA. An aliquot of the cell culture supernate was removed and assayed for human CD40 Ligand.

Condition	pg/mL
Unstimulated	213
Stimulated	555

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SPECIFICITY

This assay recognizes natural and recombinant human CD40 Ligand.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5P (diluted 1:5) and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a low level recombinant human CD40 Ligand control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

TNF-α

CD28/Fc

CD40/Fc

Other recombinants:

marmoset CD40 Ligand

mouse CD40 Ligand

rat CD40 Ligand

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

	A	B	C	D	E	F	G	H
12								
11								
10								
9								
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5								
4								
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2								
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NOTES

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