KAMIYA BIOMEDICAL COMPANY

sCD40L Assay Kit

ELISA for the quantitative detection of human soluble CD40L in cell culture supernatant and serum

Cat. No. KT-004

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

PRODUCT INFORMATION

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PRODUCT

The **K-*ASSAY*®** sCD40L Assay Kit is an enzyme-linked immunosorbent assay for quantitative detection of soluble human CD40 ligand levels in cell culture supernatants, and human serum. **The sCD40L Assay Kit is for research use only. Not for use in diagnostic or therapeutic procedures.**

DESCRIPTION

CD40 belongs to the TNF receptor superfamily. While the biological role of some of the ligand-receptor pairs in this family still remains obscure, CD40 has proven its importance.

A key role of CD40/CD40L interactions in immune activation, particularly in T-cell dependent B cell responses is anticipated. This molecule as well as the other ligands of the family share the property of co-stimulation of T-cell proliferation and are all expressed by activated T-cells.

The programmed cell death has been suggested to be involved in clonal elimination of self-reactive lymphocytes for the normal function of the immune system. Interaction with membrane bound self antigens may eliminate self-reactive nature B cells by apoptosis. Antigen receptor-mediated B cell apoptosis is blocked when a signal is transduced via the CD40 molecule on the B cell surface.

Because CD40L is expressed on activated T helper cells, B cells may escape from apoptosis and are activated when the immune system interacts with foreign antigens, which are normally able to activate T-helper cells. Thus the CD40 - CD40L interaction plays a central role in the various phases of the B cell response to T-dependent antigens.

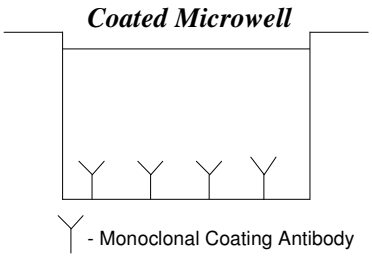
Taken together, B cells can participate in regulating their own destruction. Protection against Fas-dependent apoptosis afforded by immunoglobulin-receptor engagement may constitute a fail-safe mechanism that eliminates bystander B cells activated by CD40L - expressing T cells, but ensures survival of antigen-specific B cells.

CD40L is expressed on the surface of activated CD4+ T cells, basophils, and mast cells. Binding of CD40L to its receptor, CD40, on the surface of B cells stimulates B-cell proliferation, adhesion and differentiation. A soluble isoform of CD40L has been shown to exist in the circulation. This soluble molecule is a homotrimer of a 18kDa protein exhibiting full activity in B cell proliferation and differentiation assays, is able to rescue B cells from apoptosis and binds soluble CD40.

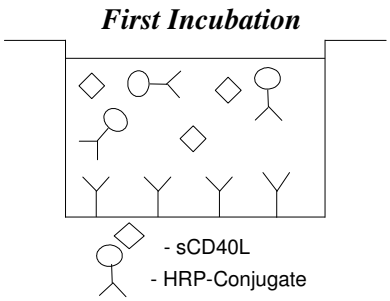
CD40L is discussed in relation to a potential role in supporting B cell tumors and it has been discovered that the molecular defect in the X-linked Hyper-IgM-Syndrome is targeted to the CD40L gene. It is functionally involved in B cell hybridomas and chronic lymphocytic as well as several autoimmune diseases.

PRINCIPLE

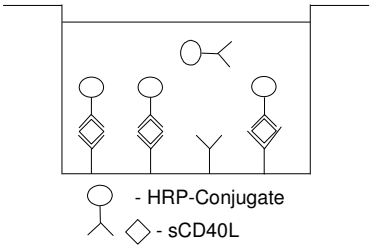
An anti-sCD40L monoclonal coating antibody is adsorbed onto microwells.

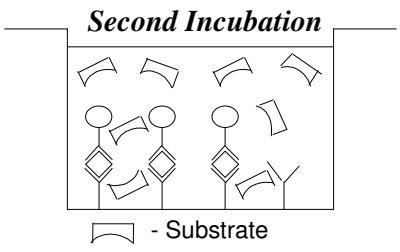


sCD40L present in the sample or calibrator binds to antibodies adsorbed to the microwells; a HRP-conjugated monoclonal anti- sCD40L is added and binds to sCD40L captured by the first antibody.

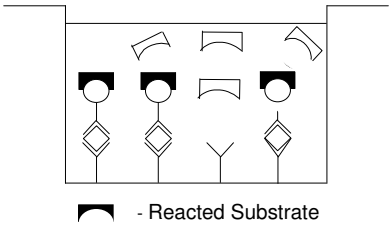


Following incubation unbound enzyme conjugated anti-sCD40L is removed during a wash step and a substrate solution reactive with HRP is added to the wells.





A colored product is formed in proportion to the amount of soluble sCD40L present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A calibration curve is prepared from seven sCD40L calibrator dilutions and sCD40L sample concentration determined.



COMPONENTS

1 aluminum pouche with **a Microwell Plate coated with Monoclonal Antibody** (murine) to human sCD40L

1 vial (0.2 mL) **HRP-Conjugate** anti-sCD40L monoclonal (murine) antibody*

2 vials (20 ng/mL upon reconstitution) **sCD40L Calibrator,** lyophilized*

1 bottle (50 mL) **Wash Buffer Concentrate** 20x *

1 vial (12 mL) **Sample Diluent** (protein matrix)
Please Note: In some cases the Sample Diluent contains insoluble white precipitations, which do not interfere with the test performance. Use according to protocol.)

1 vial (5 mL) **Assay Buffer Concentrate** 20x *

1 vial (7 mL) **Substrate Solution I** (tetramethyl-benzidine)

1 vial (7 mL) **Substrate Solution II** (0.02 % buffered hydrogen peroxide)

1 vial (12 mL) **Stop Solution** (1M Phosphoric acid)

1 vial (0.4 mL each) **Blue-Dye, Green-Dye**

2 **Dilution plates**

2 adhesive **Plate Covers**

* reagents contain preservative

MATERIALS REQUIRED BUT NOT PROVIDED

- 5 mL and 10 mL graduated pipettes
- 10 µL to 1,000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL adjustable multi-channel micropipette with disposable tips
- Multi-channel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multi-channel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or de-ionized water
- Statistical calculator with program to perform linear regression analysis

STORAGE

Store all kit reagents between 2° and 8℃. Immediately after use remaining reagents should be returned to cold storage (2° to 8℃). Expiration date of the kit and reagents is stated on labels.
The expiration date of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

SPECIMEN COLLECTION

Cell culture supernatants and human serum will be suitable for use in the assay. Remove serum from the clot as soon as possible after clotting and separation.
Do not use plasma preparations instead of sera for measurement.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Clinical samples should be kept at 2° to 8℃ and separated rapidly before storing at -20℃ to avoid loss of bioactive sCD40L. Avoid repeated freeze-thaw cycles.

PRECAUTIONS FOR USE

- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Reagents containing thimerosal as preservative may be toxic if ingested.
- Avoid contact of substrate solutions with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test, use disposable pipette tips and/or pipettes.

- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.

- Exposure to acids will inactivate the conjugate

- Glass-distilled water or de-ionized water must be used for reagent preparation.

- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.

- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

PROCEDURES

PREPARATION OF REAGENTS

Except for the HRP-Conjugate, the sCD40L Calibrator, and the TMB Substrate Solution the reagents should be prepared before starting with the test procedure.

A. Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (50 mL) of the **Wash Buffer Concentrate** into a clean 1,000 mL graduated cylinder. Bring final volume to 1,000 mL with glass-distilled or de-ionized water. Mix gently to avoid foaming. The pH of the final solution should be adjusted to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer is stable for 30 days. Wash Buffer may be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

B. Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer Concentrate** (5.0 mL) to 95 mL distilled or de-ionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days. Assay Buffer may be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

C. Preparation of HRP-Conjugate

The HRP-Conjugate must be diluted 1:200 with Assay Buffer just prior to use in a clean plastic test tube.

Please note that the HRP-Conjugate should be used within 30 minutes after dilution. HRP-Conjugate may be prepared as needed according to the following table:

Number of Strips	HRP-Conjugate (mL)	Assay Buffer (mL)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

D. Preparation of sCD40L Calibrator

Reconstitute **sCD40L Calibrator** by addition of distilled water. Reconstitution volume is stated on the label of the calibrator vial. Mix gently to insure complete reconstitution.

Prepare Calibrator shortly before use. Use immediately after reconstitution. **Do not store reconstituted Calibrator.**

E. TMB Substrate Solution

Using clean pipettes and containers known to be metal free, dispense an equal volume of **Substrate Solution I** into **Substrate Solution II** and swirl gently to mix. The TMB Substrate Solution may develop a yellow tinge over time. This does not seem to affect product performance. A blue color present in the TMB Substrate Solution, however, indicates that it has been contaminated and must be discarded. The TMB Substrate Solution must be used within a few minutes after mixing. Warm to room temperature before use. Avoid direct exposure of TMB reagents to intense light and oxidizing agents during storage or incubation.

Substrate preparation by assay size:

Number of Strips	Substrate Solution I (mL)	Substrate Solution II (mL)
1 - 6	3.0	3.0
1 - 2	6.0	6.0

F. Addition of color-giving reagents: Blue-Dye, Green-Dye

The dye solutions from the stocks provided (***Blue-Dye, Green-Dye***) can be added to the reagents according to the following guidelines:

1. Diluent: Before sample dilution add the ***Blue-Dye*** at a dilution of 1:250 (see table below) to the appropriate diluent (1x) according to the test protocol. After addition of ***Blue-Dye***, proceed according to the instruction booklet.

5 mL Diluent	20 µL ***Blue-Dye***
12 mL Diluent	48 µL ***Blue-Dye***

2. HRP-Conjugate: Before dilution of the concentrated conjugate, add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the Assay Buffer used for the final conjugate dilution. Proceed after addition of ***Green-Dye*** according to the instruction booklet, preparation of HRP-conjugate.

3 mL Assay Buffer	30 µL ***Green-Dye***
6 mL Assay Buffer	60 µL ***Green-Dye***
12 mL Assay Buffer	120 µL ***Green-Dye***

PROTOCOLS

a. Mix all reagents thoroughly without foaming before use.

b. Remove Dilution Plate from the pouch. Add 100 µL of Sample Diluent in duplicate to all calibrator wells of the Dilution Plate. Prepare calibrator dilutions by pipetting 100 µL of solubilized (refer to preparation of reagents) **sCD40L Calibrator**, in duplicate, into well A1 and A2 (see Figure 1 & 2).

Mix the contents by repeated aspiration and ejection, and transfer 100 µL to well B1 and B2, respectively. Continue this procedure five times, creating two rows of sCD40L calibrator dilutions ranging from 10 to 0.16 ng/mL. Discard 100 µL of the contents from the last wells (G1, G2) used.

Figure 1. Preparation of sCD40L calibrator dilutions in Dilution Plate:

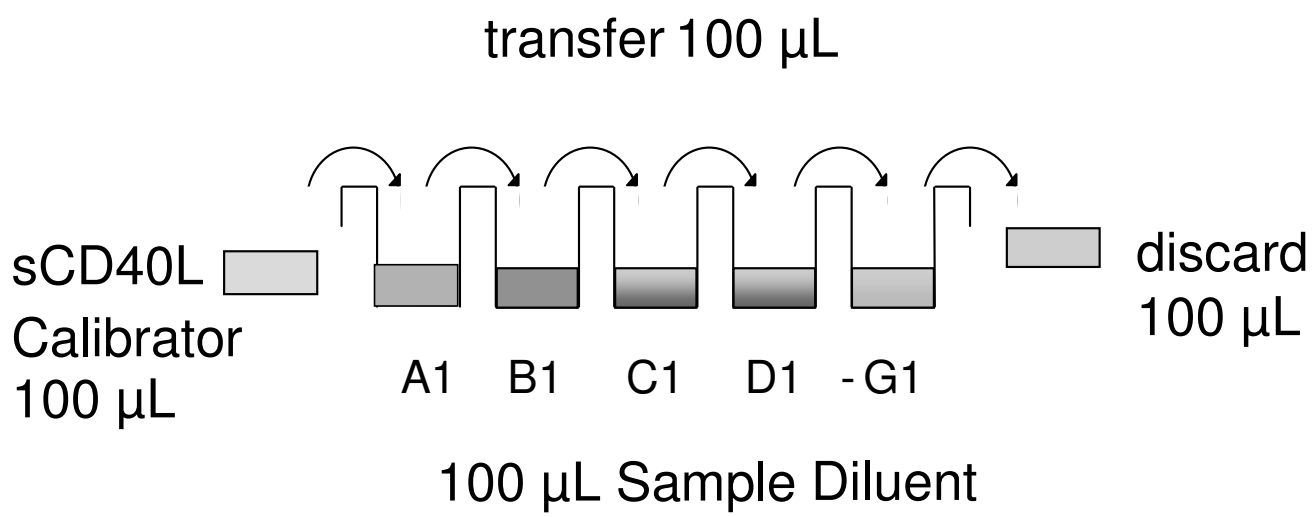


Figure 2. Diagram depicting an example of the arrangement of blanks, calibrators and samples in the Dilution Plate:

	1	2	3	4
A	Calibrator 1 (10 ng/mL)	Calibrator 1 (10 ng/mL)	Sample 1	Sample 1
B	Calibrator 2 (5 ng/mL)	Calibrator 2 (5 ng/mL)	Sample 2	Sample 2
C	Calibrator 3 (2.5 ng/mL)	Calibrator 3 (2.5 ng/mL)	Sample 3	Sample 3
D	Calibrator 4 (1.25 ng/mL)	Calibrator 4 (1.25 ng/mL)	Sample 4	Sample 4
E	Calibrator 5 (0.63 ng/mL)	Calibrator 5 (0.63 ng/mL)	Sample 5	Sample 5
F	Calibrator 6 (0.31 ng/mL)	Calibrator 6 (0.31 ng/mL)	Sample 6	Sample 6
G	Calibrator 7 (0.16 ng/mL)	Calibrator 7 (0.16 ng/mL)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

c. Add 100 µl of **Sample Diluent** in duplicate to the blank wells.

d. Add 80 µl of **Sample Diluent** to all wells designated for samples in the Dilution Plate.

e. Add 20 µl of each **Sample**, in duplicate, to the designated wells.

f. Prepare **HRP-Conjugate**. (Refer to preparation of reagents)

g. Add 100 µl of diluted (1:200) **HRP-Conjugate** to all wells of the Dilution Plate, including the blank wells.

h. Determine the number of Microwell Strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and calibrators. Each sample, calibrator, blank, and optional control sample should be assayed in duplicate. Remove sufficient **Microwell Strips coated with Monoclonal Antibody** (murine) to human sCD40L from holder and store in foil bag with the desiccant provided at 2-8°C sealed tightly.

i. Wash the microwell strips twice with approximately 300 µL **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.

After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

This washing step can alternatively be omitted without influence on the assay results.

j. Transfer 150 µL of the reaction mixture from the Dilution Plate to the Microwell Strips coated with Monoclonal Antibody. Mix the contents of the Dilution Plate by aspiration and ejection before transferring 150 µL to the Microwell Strips coated with Monoclonal Antibody in the same scheme as prepared on the Dilution Plate.

k. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 2 hours, on a rotator set at 100 rpm. If no rotator set is available, the Microwell Plate can alternatively be incubated at 4ºC over night.

l. Prepare TMB Substrate Solution a few minutes prior to use. (Refer to preparation of reagents).

m. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point i. of the test protocol. Proceed immediately to the next step.

n. Pipette 100 µL of mixed **TMB Substrate Solution** to all wells, including the blank wells.

o. Incubate the microwell strips at room temperature (18° to 25°C) for about 15 minutes, if available on a rotator set at 100 rpm. Avoid direct exposure to intense light. The point at which the substrate reaction is stopped is often determined by the ELISA reader being used. Many ELISA readers record absorbance only up to 2.0 O.D. **The O.D. values at the plate must be monitored and the substrate reaction stopped before positive wells are no longer properly recordable.**

p. Stop the enzyme reaction by quickly pipetting 100 µL of **Stop Solution** into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

q. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both, the samples and the sCD40L calibrators.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

CALCULATIONS

- Calculate the average absorbance values for each set of duplicate calibrators and samples. Duplicates should be within 20 percent of the mean.

- Create a calibration curve by plotting the mean absorbance for each calibrator concentration on the ordinate against the sCD40L concentration on the abscissa. Draw a best fit curve through the points of the graph.

- To determine the concentration of circulating sCD40L for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the calibration curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding sCD40L concentration.

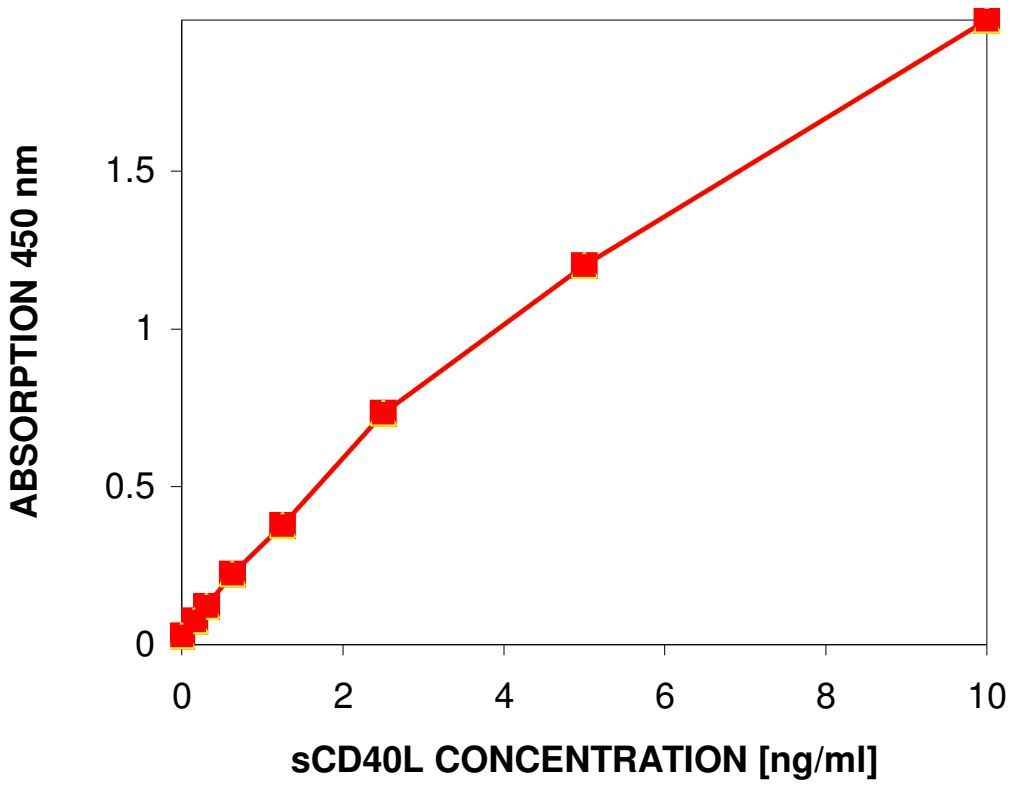
- **For samples which have been diluted 1:5 prior to testing, the concentration read from the calibration curve must be multiplied by the dilution factor (x 5).**

- **Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low sCD40L levels. Such samples require further dilution of 1:10 - 1:20 with Sample Diluent in order to precisely quantitative the actual sCD40L level.**

- It is suggested that each testing facility establishes a control sample of known sCD40L concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.

- A representative calibration curve is shown in Figure 3. This curve cannot be used to derive test results. Every laboratory must prepare a calibration curve for each group of microwell strips assayed.

Figure 3. Representative calibration curve for sCD40L ELISA. sCD40L was diluted in serial two-fold steps in Assay Buffer, symbols represent the mean of three parallel titrations. Do not use this calibration curve to derive test results. **A calibration curve must be run for each group of microwell strips assayed.**



Typical data using the sCD40L ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Calibrator	SCD40L Concentration (ng/mL)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	10	1.997	1.979	1.3
	10	1.960		
2	5	1.194	1.202	0.9
	5	1.210		
3	2.5	0.713	0.734	4.0
	2.5	0.755		
4	1.25	0.385	0.377	3.0
	1.25	0.369		
5	0.63	0.225	0.224	0.9
	0.63	0.222		
6	0.31	0.122	0.122	0
	0.31	0.122		
7	0.16	0.076	0.075	0.5
	0.16	0.074		
Blank	0.00	0.029	0.028	
	0.00	0.026		

LIMITATIONS

- Since exact conditions may vary from assay to assay, a calibration curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

- The use of immunotherapy has significantly increased the number of patients with human anti-mouse IgG antibody (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analyzed in such assays when murine immunoplobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the Sample Diluent.

PERFORMANCE CHARACTERISTICS

A. Sensitivity

The limit of detection of sCD40L defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus three standard deviations) was determined to be 0.095 ng/mL (mean of 6 independent assays).

B. Reproducibility

a. Intra-assay

Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of sCD40L. Two calibration curves were run on each plate. Data below show the mean sCD40L concentration and the coefficient of variation for each sample. The overall Intra-assay coefficient of variation has been calculated to be 4.0%.

Positive Sample	Experiment	sCD40L Concentration (ng/mL)	Coefficient of Variation (%)
1	1	14.8	1.6
	2	15.8	3.1
	3	15.0	1.4
2	1	12.6	0.5
	2	13.9	1.7
	3	13.3	5.8
3	1	10.9	1.3
	2	12.8	5.9
	3	11.8	3.9
4	1	10.2	2.2
	2	11.9	0.7
	3	11.1	3.4
5	1	7.3	0.8
	2	8.0	4.6
	3	7.4	5.1
6	1	6.6	5.9
	2	7.4	4.6
	3	7.0	8.3
7	1	4.9	5.9
	2	5.5	3.0
	3	4.7	2.6
8	1	2.9	13.6
	2	3.5	4.5
	3	2.9	4.8

b. Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in three independent experiments by two technicians. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of sCD40L. Two calibration curves were run on each plate. Data below show the mean sCD40L concentration and the coefficient of variation calculated on 18 determinations of each sample. The overall inter-assay coefficient of variation has been calculated to be 6.8%.

Sample	sCD40L Concentration (ng/mL)	Coefficient of Variation (%)
1	15.2	3.4
2	13.3	5.0
3	11.8	8.2
4	11.1	7.7
5	7.6	5.3
6	7.0	6.0
7	5.1	8.5
8	3.1	10.1

C. Spike Recovery

The spike recovery was evaluated by spiking four levels of sCD40L into normal human sera. As shown below recoveries were determined in four independent experiments with 6 replicates each. The amount of endogenous sCD40L in unspiked serum was substracted from the spike values. Recoveries ranged from 78 to 112% with an overall mean recovery of 91%.

Experiment 1

sCD40L Base Level (ng/mL)	Recovery (%) sCD40L Spike			
1.3	35 ng	15 ng	5 ng	2 ng
	89	89	97	91

Experiment 2

sCD40L Base Level (ng/mL)	Recovery (%) sCD40L Spike			
1.8	35 ng	15 ng	5 ng	2 ng
	88	93	99	101

Experiment 3

sCD40L Base Level (ng/mL)	Recovery (%) sCD40L Spike			
3.2	35 ng	15 ng	5 ng	2 ng
	78	89	88	112

Experiment 4

sCD40L Base Level (ng/mL)	Recovery (%) sCD40L Spike			
0.7	35 ng	15 ng	5 ng	2 ng
	79	101	81	84

D. Dilution Parallelism

Four serum samples with different levels of sCD40L were assayed at four serial two-fold dilutions (1:5 - 1:40) with 4 replicates each. In the table below the per cent recovery of expected values is listed. Recoveries ranged from 100.3% to 112.8% with an overall mean recovery of 105%.

		sCD40L Concentration (ng/mL)		
Sample	Dilution	Expected Value	Observed Value	% Recovery of Exp. Value
1	1:5	--	14.5	--
	1:10	7.3	7.3	101.1
	1:20	3.6	3.7	102.5
	1:40	1.8	1.8	100.3
2	1:5	--	12.5	--
	1:10	6.3	6.7	106.1
	1:20	3.1	3.2	101.8
	1:40	1.6	1.8	111.6
3	1:5	--	11.1	--
	1:10	5.5	6.0	107.6
	1:20	2.8	2.9	105.6
	1:40	1.4	1.4	102.9
4	1:5	--	10.5	--
	1:10	5.3	5.5	103.8
	1:20	2.6	2.7	103.7
	1:40	1.3	1.5	112.8

E. Sample Stability

a. Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -70℃ and thawed up to 5 times, and sCD40L levels determined. As shown in the table below, there was no significant loss of sCD40L by freezing and thawing.

No. of Freeze Thaw Cycles	sCD40L Concentration (ng/mL)	Serum Recovery (%)	sCD40L Concentration (ng/mL)	Spike Recovery (%)
0	5.6	100	6.0	100
1	5.6	100	5.9	99
3	5.4	97	5.9	98
5	5.3	95	6.0	100

b. Storage Stability
Aliquots of a serum sample (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the sCD40L level determined after 24 h. As shown in the table below, there was significant loss of sCD40L immunoreactivity during storage at RT and 37ºC.

Storage Temperature	sCD40L Serum Concentration (ng/mL)	Recovery (%)	sCD40L Spike Concentration (ng/mL)	Recovery (%)
- 20°C	5.7	100	5.8	100
2 - 8°C	4.7	82	5.6	96
RT	0.7	13	3.3	57
37°C	0.0	0	0.2	4

F. Comparison of Serum and Plasma
From eight individuals, serum as well as EDTA plasma, citrate plasma, and heparin plasma obtained at the same time point were evaluated. It clearly turned out that plasma preparations give results that do not correlate with the respective serum data. Thus plasma preparations are not suitable for the assay.

G. Expected Values
A panel of 40 sera from healthy blood donors (males and females) was tested for sCD40L. The detected sCD40L levels ranged between 0.03 and 3.98 ng/mL with a mean level of 2.13 ng/mL and a standard deviation of $\pm$1.0 ng/mL.

REAGENT PREPARATION SUMMARY

A. Wash Buffer
Add **Wash Buffer Concentrate** 20 x (50 mL) to 950 mL distilled water

B. Assay Buffer

Number of Strips	Assay Buffer Concentr. (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

C. HRP-Conjugate

Number of Strips	HRP-Conjugate (mL)	Assay Buffer (mL)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

D. Calibrator
Reconstitute **sCD40L Calibrator** by addition of distilled water. Reconstitution volume is stated on the label of the calibrator vial

E. TMB Substrate Solution

Number of Strips	Substrate Solution I (mL)	Substrate Solution II (mL)
1 - 6	3.0	3.0
1 - 12	6.0	6.0

TEST PROTOCOL SUMMARY

- Remove Dilution Plate from pouch
- Add 100 µL **Sample Diluent**, in duplicate, to calibrator wells of Dilution Plate
- Pipette 100 µL solubilized **sCD40L Calibrator** into the first wells and create calibrator dilutions ranging from 10 to 0.16 ng/mL by transferring 100 µL from well to well. Discard 100 µL from the last wells
- Add 100 µL **Sample Diluent**, in duplicate, to the blank wells of Dilution Plate
- Add 80 µL **Sample Diluent** to the sample wells of Dilution Plate
- Add 20 µL **Sample**, in duplicate, to designated wells
- Prepare **HRP-Conjugate**
- Add 100 µL of diluted **HRP-Conjugate** to all wells of Dilution Plate
- Wash microwell strips twice with **Wash Buffer.** Transfer 150 µL of reaction mixture from Dilution Plate to Microwell strips. Mix before transferring
- Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C) on a rotator set
- Prepare **TMB Substrate Solution** few minutes prior to use
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µL of mixed **TMB Substrate Solution** to all wells including blank wells
- Incubate the microwell strips for about 15 minutes at room temperature (18°to 25°C)
- Add 100 µL **Stop Solution** to all wells including blank wells
- Blank microwell reader and measure color intensity at 450 nm

Note: For samples which have been diluted 1:5, the concentration read from the calibration curve must be multiplied by the dilution factor (x 5). Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low sCD40L levels. Such samples require further dilution of 1:10 - 1:20 with Sample Diluent in order to precisely quantitative the actual sCD40L level.

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

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