Human sCD40L ELISA

650 120 096 **1 x 96 tests**
650 120 192 **2 x 96 tests**

INTENDED USE

The sCD40L ELISA is an enzyme-linked immunosorbent assay for quantitative detection of soluble human CD40 ligand levels in cell culture supernatants, human serum and plasma; **this kit is not suitable for the detection of very low levels of sCD40L in plasma; for this please refer to the human sCD40L high sensitivity ELISA. The sCD40L ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.**

SUMMARY

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.(3)

A key role of CD40/CD40ligand 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 (10).

Because the ligand of CD40 (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 (8).

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 (9).

CD40 Ligand 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 (4, 6).

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 (1) is targeted to the CD40 L gene, it is functional involved in B cell hybridomas (5) and chronic lymphocytic leukemia (2) as well as several autoimmune diseases (7).

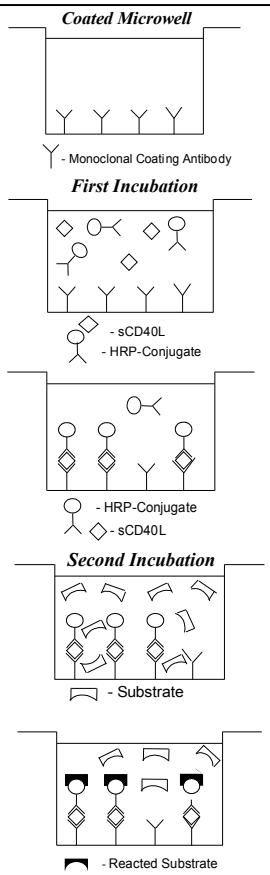
PRINCIPLES OF THE METHOD

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

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

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

A coloured 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 standard curve is prepared from seven sCD40L standard dilutions and sCD40L sample concentration determined.



REAGENTS PROVIDED

REAGENTS (store at 2-8°C)	QTY 1 plate	QTY 2 plates	RECONSTITUTION
96-wells precoated microtiter plate	1	2	Ready-to-use
Plate covers	2	4	
Human sCD40L Standard 20 ng/ml	2 vials	4 vials	Reconstitute with the volume of distilled water indicated on the vial.
Dilution Plates	2	4	(100 µl) Dilute 100 times in Assay buffer
HRP-Conjugate anti-human sCD40L monoclonal antibody	1 vial	2 vials	(0.2ml) Make a 1/100 dilution in Assay Buffer
Assay Buffer Concentrate	1 vial	2 vials	(5 ml) 20X concentrate. Dilute in distilled water
Wash Buffer Concentrate	1 bottle	2 bottles	(50 ml) 20X concentrate. Dilute in distilled water
Sample Diluent	1 bottle	2 bottles	(12 ml)
Substrate Solution (tetrametyl-benzidine)	1 vial	2 vials	(15 ml) Ready- to- use
Stop Solution (1M Phosporic acid)	1 vial	2 vials	(12 ml) Ready-to-use
Blue Dye	1 vial	2 vials	(0.4 ml) Make a 1/250 dilution in the appropriate diluent
Green Dye	1 vial	2 vials	(0.4 ml) Make a 1/100 dilution in the Assay Buffer
Control high	1 vial	2 vials	Add 150 µl distilled water
Control low	1 vial	2 vials	Add 150 µl distilled water

MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes
- 5 µl to 1,000 µl adjustable single channel micropipettes with disposable tips
- 50 µl to 200 µl adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel 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 deionized water
- Statistical calculator with program to perform linear regression analysis

SAFETY

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statements(s) for specific advice.
- 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.
- 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 deionized water must be used for reagent preparation.
- Substrate solutions must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agens. 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.

STORAGE INSTRUCTIONS

Store kit reagents between 2°C and 8°C except controls. Store lyophilized controls at -20°C. Immediately after use reagents should be returned to cold storage (2°C to 8°C), controls to -20°C, respectively. Expiry of the kit and reagents is stated on labels.
Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component ,this reagent is not contaminated by the first handling.

SPECIMEN COLLECTION

Cell culture supernatants, plasma, and human serum will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.
This kit is not suitable for the detection of very low levels of sCD40L in plasma; for this please refer to the human sCD40L high sensitivity ELISA.

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

Samples should be stored frozen at -20°C, unless they will be assayed the day of collection. Excessive freeze-thaw cycles shoud be avoided. Prior to assay, frozen sera should be brought to room temperature slowly and mixed gently. Do not thaw samples in a 37°C water bath. Do not vortex or sharply agitate samples.

PREPARATION OF REAGENTS

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

1. 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 deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust 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

2. Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer Concentrate** (5.0 ml) to 95 ml distilled or deionized 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

3. Preparation of HRP-Conjugate

Make a 1:100 dilution of the concentrated **HRP Conjugate** with **Assay Buffer** in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (ml)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

4. Preparation of sCD40L Standard

Reconstitute **sCD40L Standard** by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Shake gently to ensure complete reconstitution. Prepare Standard shortly before use. Use immediately after reconstitution. **Do not store reconstituted Standard.**

5.*Controls*

Reconstitute by adding 150µl distilled water to lyophilised controls. Further treat the controls like your samples in the assay. For control range please refer to certificate analysis or vial label. Store reconstituted control aliquoted at -20°C. Avoid repeat freeze and thaw cycles.

6. Addition of colour-giving reagents: Blue-Dye, Green-Dye.

In order to help our customers to avoid any mistakes in pipetting, DIACLONE now offers a new tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colours to each step of the ELISA procedure. This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, 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 Assay Buffer	20 µl ***Blue-Dye***
12 ml Assay Buffer	48 µl ***Blue-Dye***
50 ml Assay Buffer	200 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.

6 ml Assay Buffer	60 µl ***Green-Dye***
12 ml Assay Buffer	120 µl ***Green-Dye***

TEST PROTOCOL

a. Mix all reagents thoroughly without foaming before use.

b. Remove Dilution Plate from the pouche. Add 100 µl of Sample Diluent in duplicate to all standard wells of the Dilution Plate. Prepare standard dilutions by pipetting 100 µl of solubilized (refer to preparation of reagents) **sCD40L Standard**, in duplicate, into well A1 and A2 (see Figure 1 and 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 standard 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 standard dilutions in Dilution Plate:

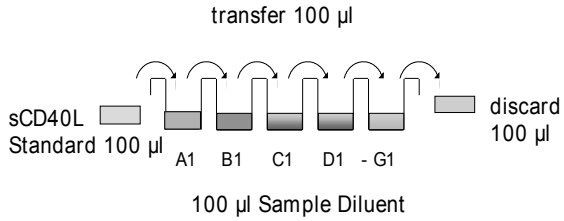


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the dilution plate:

Standard Concentrations — Sample wells — ng/mL

	1	2	3	4	5	6	7	8	9	10	11	12
A	10	10										
B	5	5										
C	2.50	2.50										
D	1.25	1.25										
E	0.63	0.63										
F	0.31	0.31										
G	0.16	0.16										
H	Blank	Blank										

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

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 of the dilution plate

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

g. Add 100 µl of **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 standards. Each sample, standard, blank, and optional control sample should be assayed in duplicate.
Remove extra Microwell Strips coated with Monoclonal Antibody (murine) to human sCD40L from the aluminium pouch. Remove extra microwell strips 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. Allow the wash buffer to sit in the wells for **about 10-15 seconds before aspiration.**
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.

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 transfering 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. 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.

m. Pipette 100 µl of **TMB Substrate Solution** to all wells, including the blank wells.

n. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 minutes, if available on a rotator set at 100 rpm. Avoid direct exposure to intense light.
The colour development on the plate should be monitored and the substrate reaction stopped (see point p. of this protocol) before positive wells are no longer properly recordable.
It is recommended to add the stop solution when the highest standard has developed a dark blue colour. Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as an OD of 0.6 – 0.65 is reached.

o. 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.

p. Read absorbance of each microwell on a spectro-photometer 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 standards.

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

CALCULATION OF RESULTS

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

- Create a standard curve by plotting the mean absorbance for each standard 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 standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding sCD40L concentration.

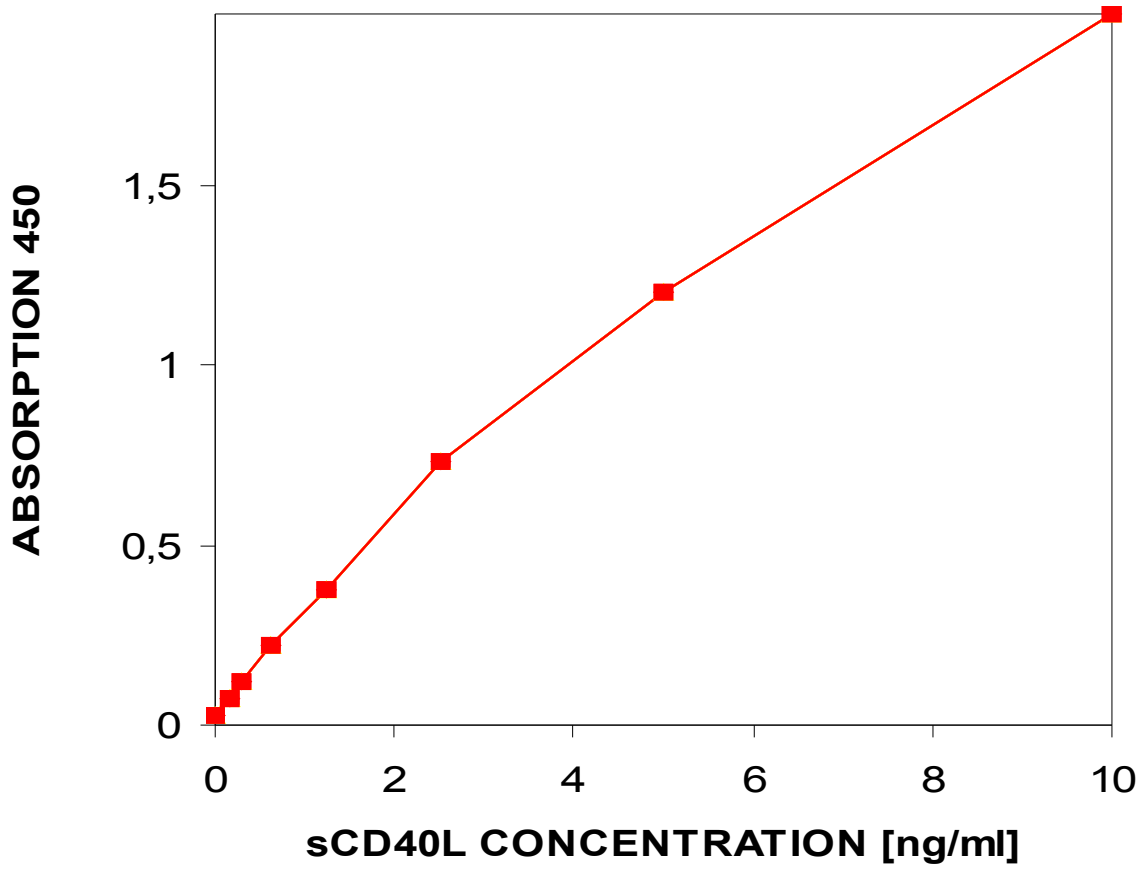
- **For samples which have been diluted according the instructions given in this manual 1:5 the concentration read from the standard curve must be multiplied by the dilution factor (x5).**

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

Figure 3. Representative standard 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 standard curve to derive test results. A standard 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

Standard	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	0.029	0.028	
	0	0.026		

LIMITATIONS

- Since exact conditions may vary from assay to assay, a standard 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 analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the Sample Diluent.

PERFORMANCE CHARACTERISTICS

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 two standard deviations) was determined to be 0.06 ng/ml (mean of 6 independent assays).

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 standard 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 three technicians. Each assay was carried out with 6 replicates of 8 samples containing different concentrations of sCD40L. Two standard 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

Spike Recovery

The spike recovery was evaluated by spiking four different levels of sCD40L into normal human serum. Recoveries were determined in four independent experiments with 6 replicates each. The amount of endogenous sCD40L in unspiked serum was subtracted 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

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%.

Sample	Dilution	sCD40L Concentration (ng/ml) 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

Sample Stability

a. Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -70°C 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

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.

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.

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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	0.06	5.94
1 - 12	0.12	11.88

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

E. Controls Add 150µl distilled water to lyophilized controls

TEST PROTOCOL SUMMARY

- Remove Dilution Plate from pouche
- Add 100 µl **Sample Diluent**, in duplicate, to standard wells of Dilution Plate
- Pipette 100 µl solubilized **sCD40L Standard** into the first wells and create standard 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
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 µl of **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 colour intensity at 450 nm

Note: For samples which have been diluted according to the instructions given in this manual 1:5, the concentration read from the standard curve must be multiplied by the dilution factor (x 5).