



CD40, Soluble (human) ELISA Kit

Manufactured by Bender MedSystems.

ALX-850-262-KI01

96 wells (~80 tests)

(Version 6: April 24, 2006)

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For laboratory use only. Not for human or diagnostic use.

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1. INTENDED USE

The sCD40 ELISA is an enzyme-linked immunosorbent assay for quantitative detection of human sCD40 in solutions like supernatants and human body fluids. **The sCD40 ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.**

2. SUMMARY

CD40 is a 50kDa membrane – bound type I glycoprotein expressed by numerous cells, most notably B lymphocytes and monocytes and antigen presenting cells (APC) such as macrophages, dendritic cells and fibroblasts. It is furthermore highly expressed on various malignant cells. The expression of CD40 regulates T–cell–APC interaction and has been shown to be centrally involved in a wide array of inflammatory events. The function of CD40 is very broad.

CD40 belongs to the TNF-receptor family. The ligand for CD40 (CD154) is a 33kDa type II transmembrane protein mainly expressed by activated T-cells, and is a member of the TNF superfamily. CD40/CD40L interactions are essential for T cell – dependent B cell proliferation and differentiation, for activation of antigen presenting cells and for cytokine production on numerous other cells.

It is presently accepted that CD40 plays a critical role in the regulation of immune responses (21,22).

CD40 expression has also been found on non lymphoid cells such as fibroblasts, endothelia, and epithelial cells.

Its critical role in T – cell – dependent humoral immune responses was demonstrated by patients with the hyper – IgM – Syndrome.

CD40 is also involved in the apoptotic pathway of cells (20). While it has been shown that the programmed cell death can be inhibited by the survival signals mediated from the binding of the CD40 receptor to the CD40 Ligand (9,6), very recently a novel proapoptotic mechanism induced by CD40 in carcinoma cells has been described (4). This mechanism is dependent on the endogenous production of cytotoxic cytokines.

The interaction of CD40 and its ligand, CD154 (CD40L) was found to play a crucial role in many aspects of immune response and the development and progress of various diseases.

It was found to be centrally involved in transplant rejection (3,8,19). The interaction of CD40 on synovial fibroblasts and CD40L expressed on activated T lymphocytes is directly involved in the neovascularization in rheumatoid synovitis (2). CD40 expression in thyroid tissue suggests a new pathway of pathogenesis of thyroid diseases (16). Functional expression of CD40 on human melanoma cells mediates T-cell-co-stimulation and tumor cell growth (11).

The interaction of CD40 and CD154 is centrally involved in a wide array of inflammatory events such as multiple sclerosis (5), atherosclerosis (7,10) and asthma – associated airway inflammation (14).

The expression of CD40 on various B cell malignancies such as leukemias, non Hodgkins lymphoma and multiple myeloma has been described (1,12). CD40 was found to regulate cell growth in squamous cell cancer of the head and neck (13). Its expression in hepatocellular carcinomas plays an important role in tumor biology (17).

The expression of CD40 on human lung cancer correlates with metastatic spread and may serve as a prognostic marker and an indicator of advanced disease (15).

An increased CD40 expression on muscle cells of polymyositis and dermatomyositis has been described (18).

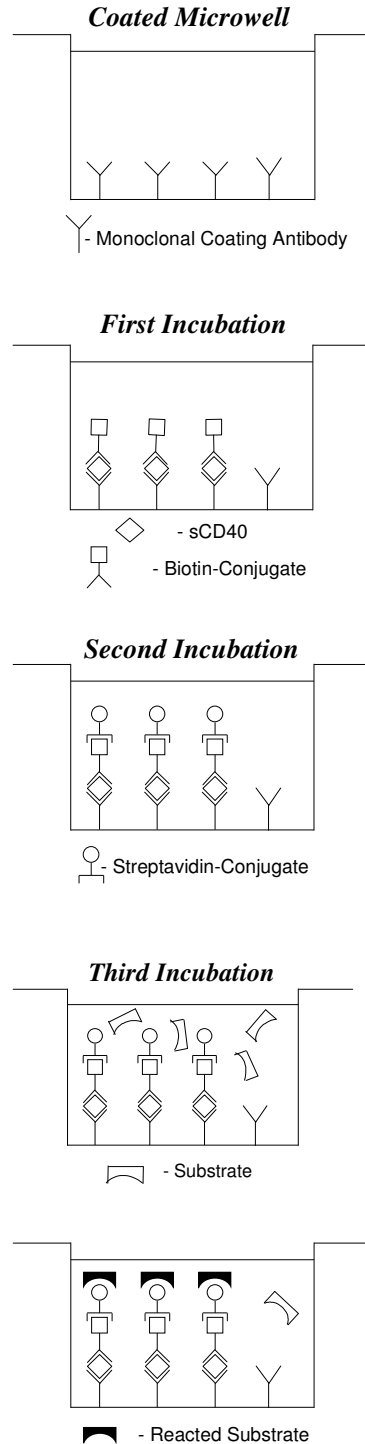
3. PRINCIPLES OF THE TEST

An anti-sCD40 monoclonal coating antibody is adsorbed onto microwells.

sCD40 present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin-conjugated monoclonal anti-sCD40 antibody is added and binds to sCD40 captured by the first antibody.

Following incubation unbound biotin-conjugated anti-sCD40 is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-sCD40. Following incubation unbound Streptavidin-HRP 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 sCD40 present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from seven sCD40 standard dilutions and sCD40 sample concentration determined.



4. REAGENTS PROVIDED

- 1 aluminium pouch with a **Microwell Plate coated with Monoclonal Antibody** (murine) to human sCD40
- 1 vial (100 μ l) **Biotin-Conjugate** anti-sCD40 monoclonal (murine) antibody
- 2 vials **sCD40 Standard, lyophilized**, 1000 pg/ml upon reconstitution
- 1 vial (150 μ l) **Streptavidin-HRP**
- 1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)
- 1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 10% BSA)
- 1 vial (12 ml) **Sample Diluent** (buffered protein matrix)
- 1 vial (15 ml) **Substrate Solution**
- 1 vial (12 ml) **Stop Solution** (1M Phosphoric acid)
- 1 vial (0.4 ml each) **Blue-Dye, Green-Dye, Red-Dye**
- 4 adhesive **Plate Covers**

Reagent Labels

5. STORAGE INSTRUCTIONS

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels.

The 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, the reagent is not contaminated by the first handling.

6. SPECIMEN COLLECTION

Cell culture supernatants, human serum, EDTA, or heparinized plasma, amniotic fluid, or other body fluids are suitable for use in the assay. Remove the serum or plasma from the clot or red cells, respectively, as soon as possible after clotting and separation.

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

Samples must be stored frozen at -20°C to avoid loss of bioactive sCD40. If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles. Prior to assay, frozen sera or plasma should be brought to room temperature slowly and mixed gently and properly diluted with Sample Diluent.

For sample stability refer to 13.E.

7. 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 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

8. PRECAUTIONS FOR USE

- 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 solution 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 solution must be at room temperature prior to use.
- 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.

9. PREPARATION OF REAGENTS

Prepare Wash Buffer (reagent A) and Assay Buffer (reagent B) 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 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

B. 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

C. Preparation of sCD40 Standard

Add distilled water to one vial of lyophilized **Standard** as indicated on the label of the standard vial. Mix gently to ensure complete solubilisation.

D. Preparation of Biotin-Conjugate

Dilute the **Biotin-Conjugate** 1:100 just prior to use with **Assay Buffer** (reagent B) in a clean plastic tube. Mix the contents of the tube well.

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

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (ml)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

E. Preparation of Streptavidin-HRP

Make a 1:100 dilution of the concentrated **Streptavidin-HRP** solution as needed according to the following table:

Number of Strips	Streptavidin-HRP (μ l)	Assay Buffer (ml)
1 - 6	60	6
1 - 12	120	12

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

In order to help our customers to avoid any mistakes in pipetting the Bender MedSystems ELISAs, Bender MedSystems 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, Red-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 diluent (1x) according to the test protocol. After addition of **Blue-Dye**, proceed according to the instruction booklet.

5 ml Sample Diluent	20 μ l Blue-Dye
12 ml Sample Diluent	48 μ l Blue-Dye

2. Biotin-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 Biotin-conjugate.

3 ml Assay Buffer	30 μ l Green-Dye
6 ml Assay Buffer	60 μ l Green-Dye
12 ml Assay Buffer	120 μ l Green-Dye

3. Streptavidin-HRP: Before dilution of the concentrated Streptavidin-HRP; add the **Red-Dye** at a dilution of 1:250 (see table below) to the Assay Buffer used for the final Streptavidin-HRP dilution. Proceed after addition of **Red-Dye** according to the instruction booklet, preparation of Streptavidin-HRP.

6 ml Assay Buffer	24 μ l Red-Dye
12 ml Assay Buffer	48 μ l Red-Dye

10. TEST PROTOCOL

- a. Mix all reagents thoroughly without foaming before use.
- b. 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 sCD40 from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
- c. Wash the microwell strips twice with approximately 300 μ l **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.

- d. Add 100 μ l of **Sample Diluent** in duplicate to all standard wells. Prepare standard dilutions by pipetting 100 μ l of **sCD40 Standard**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix the contents by repeated aspiration and ejection and transfer 100 μ l to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure five times, creating two rows of sCD40 standard dilutions ranging from 500 to 7.8 pg/ml. Discard 100 μ l of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of sCD40 standard dilutions:

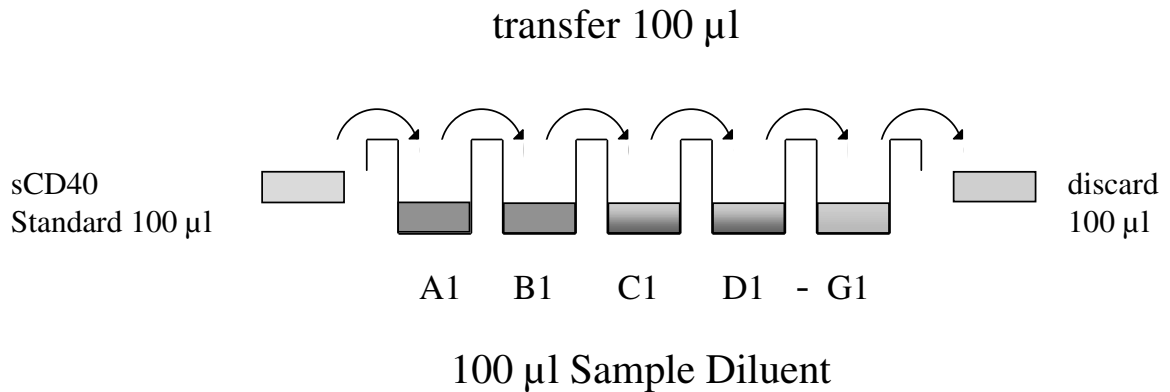


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (500 pg/ml)	Standard 1 (500 pg/ml)	Sample 1	Sample 1
B	Standard 2 (250 pg/ml)	Standard 2 (250 pg/ml)	Sample 2	Sample 2
C	Standard 3 (125 pg/ml)	Standard 3 (125 pg/ml)	Sample 3	Sample 3
D	Standard 4 (63 pg/ml)	Standard 4 (63 pg/ml)	Sample 4	Sample 4
E	Standard 5 (32 pg/ml)	Standard 5 (32 pg/ml)	Sample 5	Sample 5
F	Standard 6 (16 pg/ml)	Standard 6 (16 pg/ml)	Sample 6	Sample 6
G	Standard 7 (8 pg/ml)	Standard 7 (8 pg/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

- e. Add 100 μl of **Sample Diluent** in duplicate to the blank wells.
- f. Add 50 μl of **Sample Diluent** to the sample wells.
- g. Add 50 μl of each **Sample**, in duplicate, to the designated wells.
- h. Prepare **Biotin-Conjugate** (refer to preparation of reagents).
- i. Add 50 μl of diluted **Biotin-Conjugate** to all wells, including the blank wells.
- j. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 2 hours, if available on a rotator set at 100 rpm.
- k. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
- l. Prepare **Streptavidin-HRP** (refer to preparation of reagents).
- m. Add 100 μl of diluted **Streptavidin-HRP** to all wells, including the blank wells.
- n. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 1 hour, if available on a rotator set at 100 rpm.
- o. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.
- p. Pipette 100 μl of **TMB Substrate Solution** to all wells, including the blank wells.

- q. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 minutes. Avoid direct exposure to intense light.

The colour development on the plate should be monitored and the substrate reaction stopped (see point r. 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.

- r. 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.
- s. 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 sCD40 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.

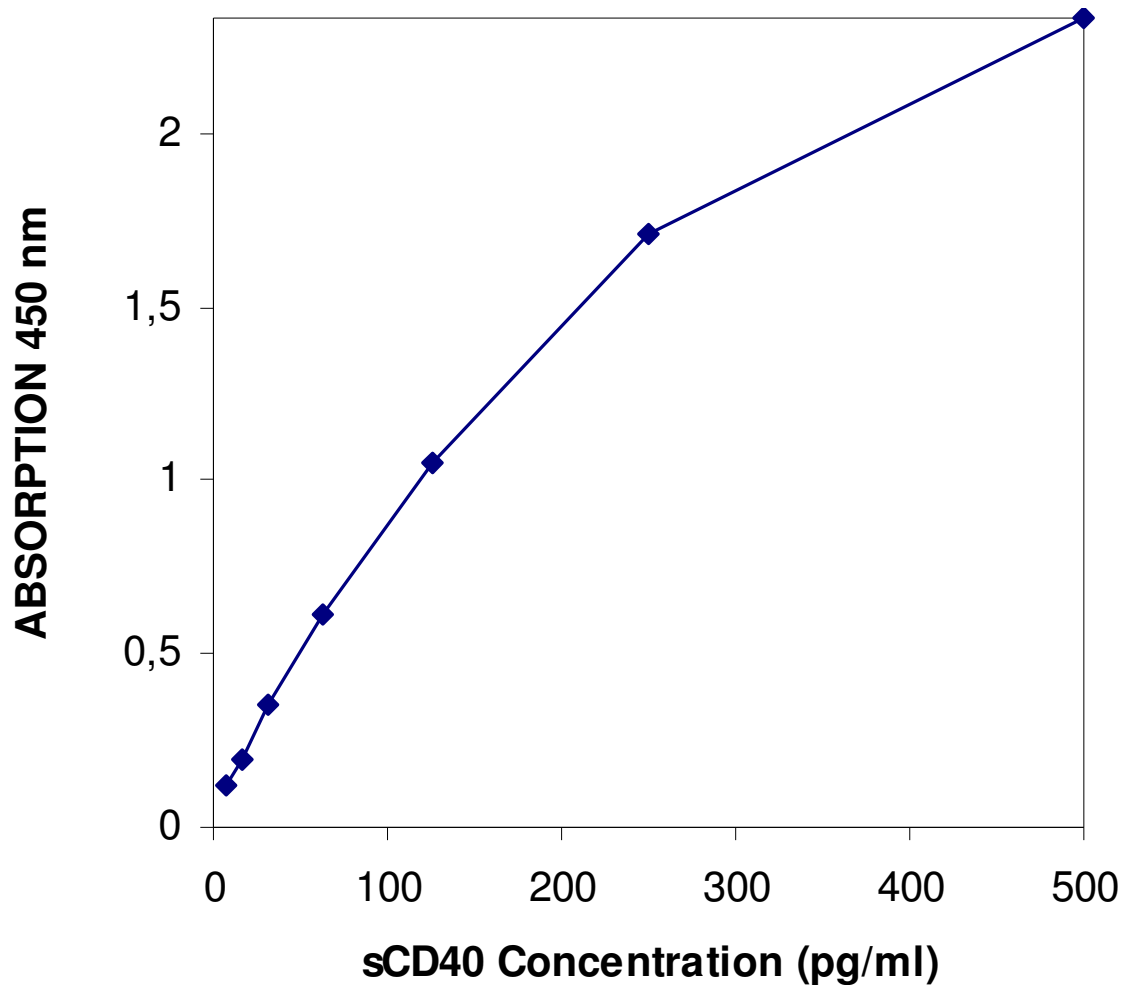
11. CALCULATION OF RESULTS

- 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 sCD40 concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating sCD40 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 sCD40 concentration.
- **For samples which have been diluted according to the instructions given in this manual 1:2, the concentration has to be multiplied by the dilution factor (x2).**

Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low sCD40 levels. Such samples require further dilution of 1:4 - 1:8 with Sample Diluent in order to precisely quantitate the actual sCD40 level.

- It is suggested that each testing facility establishes a control sample of known sCD40 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 sCD40 ELISA. sCD40 was diluted in serial two-fold steps in Sample Diluent, 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 sCD40 ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	sCD40 Concentration (pg/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	500	2.325	2.33	0.3
	500	2.334		
2	250	1.727	1.711	1.4
	250	1.694		
3	125	1.069	1.052	2.4
	125	1.034		
4	63	0.604	0.61	1.4
	63	0.616		
5	32	0.345	0.354	3.6
	32	0.363		
6	16	0.197	0.193	2.9
	16	0.189		
7	8	0.122	0.122	0.0
	8	0.122		
Blank	0	0.024	0.028	
	0	0.032		

12. 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.

13. PERFORMANCE CHARACTERISTICS

A. Sensitivity

The limit of detection of sCD40 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 7.92 pg/ml (mean of 6 independent assays).

B. Reproducibility

a. Intra-assay

Reproducibility within the assay was evaluated in two independent experiments. Each assay was carried out with 4 replicates of 8 serum samples containing different concentrations of sCD40. Two standard curves were run on each plate. Data below show the mean sCD40 concentration and the coefficient of variation for each sample. The overall intra-assay coefficient of variation has been calculated to be 5.5%.

Positive Sample	Experiment	sCD40 Concentration (pg/ml)	Coefficient of Variation (%)
1	1	221.2	8.7
	2	224.9	2.0
2	1	105.2	3.0
	2	133.8	8.7
3	1	272.8	4.6
	2	269.6	0.7
4	1	223.2	0.5
	2	222.8	7.9
5	1	51.6	11.1
	2	58.3	12.5
6	1	153.3	13.0
	2	138.8	8.3
7	1	1630.1	3.3
	2	1308.8	0.4
8	1	742.3	0.4
	2	682.5	2.9

b. Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in two independent experiments. Each assay was carried out with 4 replicates of 8 serum samples containing different concentrations of sCD40. Two standard curves were run on each plate. Data below show the mean sCD40 concentration and the coefficient of variation calculated on 8 determinations of each sample. The overall inter-assay coefficient of variation has been calculated to be 7%.

Sample	sCD40 Concentration (pg/ml)	Coefficient of Variation (%)
1	223.0	1.2
2	119.5	16.9
3	271.2	0.8
4	223.0	0.1
5	54.9	8.7
6	149.1	7.0
7	1469.4	15.5
8	712.4	5.9

C. Spike Recovery

The spike recovery was evaluated by spiking of recombinant sCD40 into four different sera. Recoveries were determined in three independent experiments with 6 replicates each. The amount of endogenous sCD40 in unspiked serum was subtracted from the spike values. Recoveries ranged from 81 to 110% with an overall mean recovery of 94%.

D. Dilution Parallelism

Four serum samples with different levels of sCD40 were assayed at three serial two-fold dilutions with 4 replicates each. In the table below the per cent recovery of expected values is listed. Recoveries ranged from 82% to 104% with an overall mean recovery of 94 %.

Sample	Dilution	sCD40 Concentration (pg/ml)		
		Expected Value	Observed Value	% Recovery of Exp. Value
1	1:2	-	121.4	-
	1:4	60.7	62.9	104
	1:8	30.3	31.4	104
2	1:2	-	88.0	-
	1:4	44.0	43.2	98
	1:8	22.0	21.4	97
3	1:2	-	419.9	-
	1:4	209.9	197.2	94
	1:8	105.0	85.7	82
4	1:2	-	282.0	-
	1:4	141.0	130.7	93
	1:8	70.5	58.8	83

E. Sample Stability

a. Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -20°C and thawed up to 5 times, and sCD40 levels determined. There was no significant loss of sCD40 by freezing and thawing up to 5 times.

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 sCD40 level determined after 24 h. There was no loss of sCD40 immunoreactivity during storage at above conditions.

F. Comparison of Serum and Plasma

From eight individuals, serum as well as EDTA and citrate, and heparin plasma obtained at the same time point were evaluated. sCD40 concentrations were not significantly different and therefore all these body fluids are suitable for the assay. It is nevertheless highly recommended to assure the uniformity of blood preparations.

G. Specificity

The interference of circulating factors of the immune systems was evaluated by spiking these proteins at physiologically relevant concentrations into a serum sample. There was no detectable cross reactivity.

H. Expected Values

sCD40 was measured in serum of 8 healthy donors. The levels detected were 53.3 to 146.9 pg/ml with a mean of 102.1 pg/ml and a standard deviation of 37.1 pg/ml.

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15. ORDERING INFORMATION

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16. 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. Standard Reconstitute sCD40 Standard by addition of distilled water as stated on vial label.

D. Biotin-Conjugate Make 1:100 dilution according to the table.

	Number of Strips	Biotin-Conjugate (ml)	Assay Buffer (ml)
	1 - 6	0.03	2.97
	1 - 12	0.06	5.94

E. Streptavidin-HRP	Number of Strips	Streptavidin-HRP (μ l)	Assay Buffer (ml)
	1 - 6	60	6.0
	1 - 12	120	12.0

17. TEST PROTOCOL SUMMARY

- Wash microwell strips twice with **Wash Buffer**
- Add 100 μl **Sample Diluent**, in duplicate, to standard wells
- Pipette 100 μl **sCD40 Standard** into the first wells and create standard dilutions ranging from 500 to 7.8 pg/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
- Add 50 μl **Sample Diluent** to sample wells
- Add 50 μl **Sample**, in duplicate, to designated wells
- Prepare **Biotin-Conjugate**
- Add 50 μl of diluted **Biotin-Conjugate** to all wells
- Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C)
- Prepare **Streptavidin-HRP**
- Empty and wash microwell strips 3 times with **Wash Buffer**
- Add 100 μl diluted **Streptavidin-HRP** to all wells
- Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C)
- 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 10 to 20 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: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low sCD40 levels. Such samples require further dilution of 1:4 - 1:8 with Sample Diluent in order to precisely quantitate the actual sCD40 level.