KAMIYA BIOMEDICAL COMPANY

Human sCD40 ELISA

For the quantitative determination of human soluble CD40 in cell culture supernatant, plasma, amniotic fluid, and serum

Cat. No. KT-003

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

Product Information

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PRODUCT
The **K-*ASSAY*®** Human sCD40 ELISA is an enzyme-linked immunosorbent assay for the quantitative determination of human sCD40 in cell culture supernatant, plasma, amniotic fluid, and serum. **The Human sCD40 ELISA is for research use only. Not for use in diagnostic procedures.**

DESCRIPTION
CD40 is a 50 kDa 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 33 kDa 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.

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. 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, very recently a novel proapoptotic mechanism induced by CD40 in carcinoma cells has been described. 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. The interaction of CD40 on synovial fibroblasts and CD40L expressed on activated T lymphocytes is directly involved in the neovascularization in rheumatoid synovitis. CD40 expression in thyroid tissue suggests a new pathway of pathogenesis of thyroid diseases. Functional expression of CD40 on human melanoma cells mediates T-cell co-stimulation and tumor cell growth.

The interaction of CD40 and CD154 is centrally involved in a wide array of inflammatory events such as multiple sclerosis, atherosclerosis and asthma-associated airway inflammation.

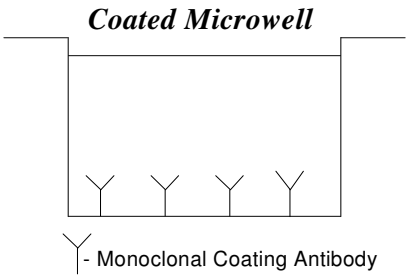
The expression of CD40 on various B cell malignancies such as leukemias, non-Hodgkin's lymphoma and multiple myeloma has been described. CD40 was found to regulate cell growth in squamous cell cancer of the head and neck. Its expression in hepatocellular carcinomas plays an important role in tumor biology.

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.

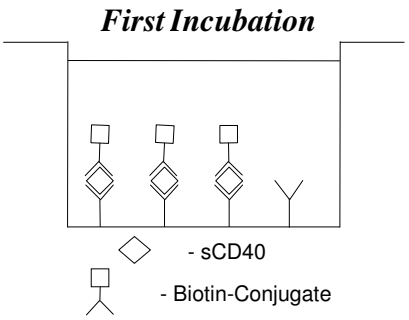
An increased CD40 expression on muscle cells of polymyositis and dermatomyositis has been described.

PRINCIPLE

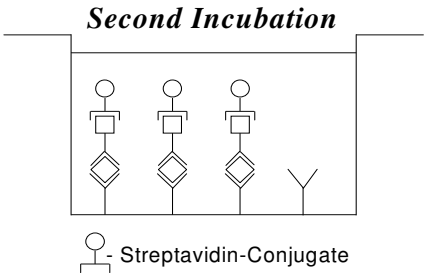
An anti-sCD40 monoclonal coating antibody is adsorbed onto microwells.



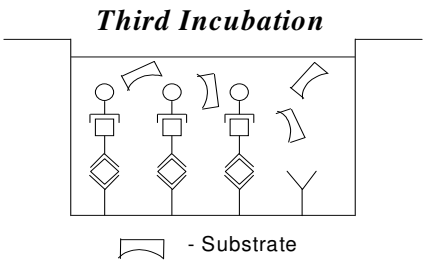
sCD40 present in the sample or calibrator binds to antibody adsorbed to the microwells; a biotin-conjugated monoclonal anti-sCD40 antibody is added and binds to sCD40 captured by the first antibody.

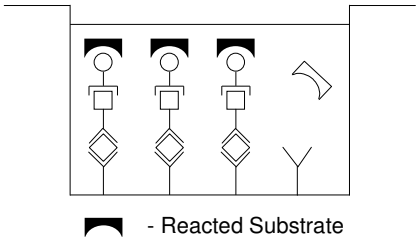


Following incubation unbound biotin-conjugated anti-sCD40 is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-sCD40. Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.



A colored 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 450 nm. A calibration curve is prepared from seven sCD40 calibrator dilutions and sCD40 sample concentration determined.





COMPONENTS

- 1 aluminum pouch with **Microwell Plate** (12 x 8-well strips) coated with monoclonal antibody (mouse) to human sCD40
- 1 vial (100 µL) **Biotin-Conjugate anti-sCD40** monoclonal (mouse) antibody
- 1 vial (150 µL) **Streptavidin-HRP**
- 2 vials **sCD40 Calibrator,** lyophilized; 1,000 pg/mL upon reconstitution
- 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 protein stabilizer)
- 1 vial (12 mL) **Sample Diluent**
- 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**

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 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)
- Microplate reader capable of reading at 450 nm (620 nm as optional reference wavelength)
- Distilled or de-ionized water
- Statistical calculator with program to perform linear regression analysis.

STORAGE

Store kit reagents at 4℃ as indicated. Immediately after use remaining reagents should be returned to cold storage (4℃). 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, 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 should be aliquoted and must be stored frozen at -20℃ to avoid loss of bioactive sCD40. If samples are to be run within 24 hours, they may be stored at 4℃. Avoid repeated freeze-thaw cycles. Prior to assay, frozen samples should be brought to room temperature slowly and mixed gently. (for sample stability see page 12)

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.
- 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 samples.
- 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 assay, 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.
- Distilled water or de-ionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose samples 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℃.
- 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

Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure. If crystals have formed in the **Buffer Concentrates**, warm them gently until they have completely dissolved.

A. Wash Buffer (1X)

Pour entire contents (50 mL) of the **Wash Buffer Concentrate** (20X) into a clean 1,000 mL graduated cylinder. Bring final volume to 1,000 mL with distilled or de-ionized 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℃. Please note that the Wash Buffer (1X) is stable for 30 days. Wash Buffer (1X) may be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20X) (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

B. Assay Buffer (1X)
Pour entire contents (5 mL) of the **Assay Buffer Concentrate** (20X) into a clean 100 mL graduated cylinder. Bring to final volume of 100 mL with distilled water. Mix gently to avoid foaming.

Store at 4°C. Please note that the Assay Buffer (1X) is stable for 30 days. Assay Buffer (1X) may be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20X) (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

C. Preparation of sCD40 Calibrator
Reconstitute **sCD40 Calibrator** by addition of distilled water. Reconstitution volume is stated on the label of the Calibrator vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted calibrator = 1,000 pg/mL). Allow the reconstituted calibrator to sit for exactly 10 minutes. Mix well prior to making dilutions.

After usage remaining calibrator cannot be stored and has to be discarded.

D. Preparation of Biotin-Conjugate
Please note that the **Biotin-Conjugate** should be used within 30 minutes after dilution.

Dilute the **Biotin-Conjugate** 1:100 just prior to use with Assay Buffer (1X) in a clean plastic tube. Mix the contents of the tube well. The **Biotin-Conjugate** may be prepared as needed according to the following table:

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

E. Preparation of Streptavidin-HRP
Please note that the **Streptavidin-HRP** should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated **Streptavidin-HRP** solution with Assay Buffer (1X) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer (1X) (mL)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

F. Addition of color-giving reagents: Blue Dye, Green Dye, Red Dye
In order to help our customers to avoid any mistakes in pipetting the **K-*ASSAY*®** Human sCD40 ELISA, we now offer a new tool that helps to monitor the addition of even very small volumes of a solution to the reaction well by giving distinctive colors to each step of the ELISA procedure.

This procedure is optional, does not in any way interfere with the assay results, and is designed to help the customer with the performance of the assay, but can also be omitted, just following the package insert.

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 calibrator and 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 package insert.

5 mL Sample Diluent	20 µL ***Blue Dye***
12 mL Sample Diluent	48 µL ***Blue Dye***
50 mL Sample Diluent	200 µL ***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 (1X) used for the final conjugate dilution. Proceed after addition of ***Green Dye*** according to the package insert, preparation of Biotin-Conjugate.

3 mL Assay Buffer (1X)	30 µL ***Green Dye***
6 mL Assay Buffer (1X)	60 µL ***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 (1X) used for the final Streptavidin-HRP dilution. Proceed after addition of ***Red Dye*** according to the package insert, preparation of Streptavidin-HRP.

6 mL Assay Buffer (1X)	24 µL ***Red Dye***
12 mL Assay Buffer (1X)	48 µL ***Red Dye***

ASSAY PROTOCOLS

a. Mix all reagents thoroughly without foaming before use.

b. Determine the number of Microwell Strips required to assay 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 extra Microwell Strips from holder and store in the foil bag sealed tightly with the provided desiccant at 4°C.

c. Wash the Microwell Strips twice with approximately 400 µL **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10-15 seconds before aspiration. Take care not to scratch the inner 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 µL of **Sample Diluent** in duplicate to all calibrator wells. Prepare calibrator dilutions by pipetting 100 µL of **sCD40 Calibrator**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix the contents by repeated aspiration and ejection (concentration of Calibrator 1 = 500 pg/mL) and transfer 100 µL to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of sCD40 calibrator dilutions ranging from 500 to 7.8 pg/mL. Discard 100 µL of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of sCD40 Calibrator dilutions:

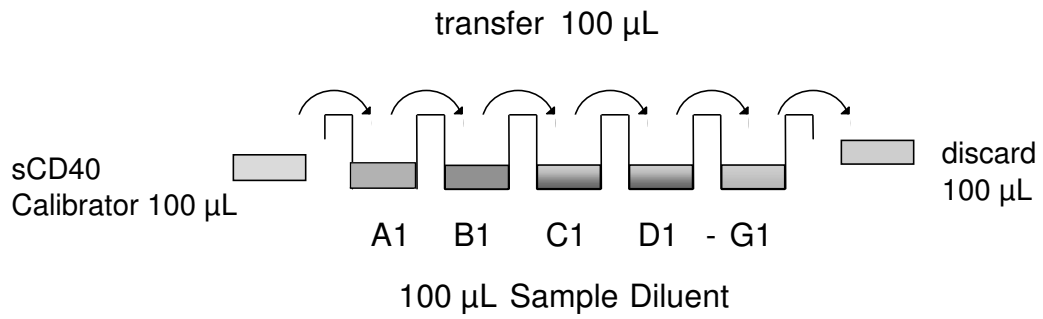


Figure 2. Diagram depicting an example of the arrangement of blanks, calibrators and samples in the Microwell Strips:

	1	2	3	4
A	Calibrator 1 (500.0 pg/mL)	Calibrator 1 (500.0 pg/mL)	Sample 1	Sample 1
B	Calibrator 2 (250.0 pg/mL)	Calibrator 2 (250.0 pg/mL)	Sample 2	Sample 2
C	Calibrator 3 (125.0 pg/mL)	Calibrator 3 (125.0 pg/mL)	Sample 3	Sample 3
D	Calibrator 4 (62.5 pg/mL)	Calibrator 4 (62.5 pg/mL)	Sample 4	Sample 4
E	Calibrator 5 (31.3 pg/mL)	Calibrator 5 (31.3 pg/mL)	Sample 5	Sample 5
F	Calibrator 6 (15.6 pg/mL)	Calibrator 6 (15.6 pg/mL)	Sample 6	Sample 6
G	Calibrator 7 (7.8 pg/mL)	Calibrator 7 (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. Prepare **Streptavidin-HRP** (Refer to PREPARATION OF REAGENTS).

l. Remove Plate Cover and empty wells. Wash Microwell Strips 3 times according to point c. of the Assay Protocols. Proceed immediately to the next step.

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 Assay Protocols. 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 color 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. Determination of the ideal time period for color development has to be done individually for each assay.** It is recommended to add the stop solution when the highest calibrator has developed a dark blue color. Alternatively, the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Calibrator 1 has reached an OD of 0.6 – 0.65.

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 4℃ in the dark.

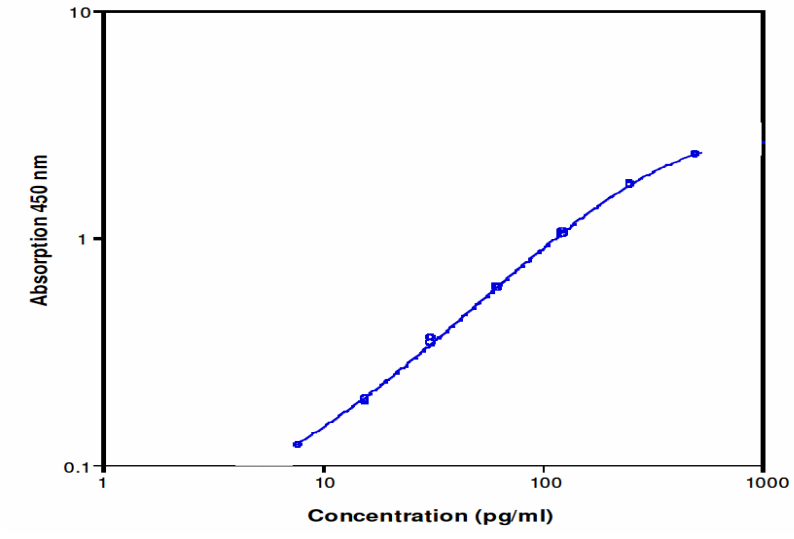
s. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wavelength (optionally 620 nm as the reference wavelength; 610 nm to 650 nm is acceptable). Blank the microplate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the sCD40 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 mean 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 sCD40 concentration on the abscissa. Draw a best-fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- 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 calibration 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 (1:2) according to the instructions given in this package insert, the concentration read from the calibration curve must be multiplied by the dilution factor (x2).**
- **Calculation of samples with a concentration exceeding Calibrator 1 may result in incorrect, low human sCD40 levels. Such samples require further external predilution according to expected human sCD40 values with Sample Diluent in order to precisely quantitate the actual human 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 calibration curve is shown in Figure 3. This curve cannot be used to derive assay results. Every laboratory must prepare a calibration curve for each assay run.

Figure 3. Representative calibration curve for sCD40 ELISA.
sCD40 was diluted in serial 2-fold steps in Sample Diluent. Do not use this calibration curve to derive assay results. A calibration curve must be run for each group of microwell strips assayed.



Typical data using the Human sCD40 ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Calibrator	sCD40 Concentration (pg/mL)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	500.0	2.325	2.330	0.3
	500.0	2.334		
2	250.0	1.727	1.711	1.4
	250.0	1.694		
3	125.0	1.069	1.052	2.4
	125.0	1.034		
4	62.5	0.604	0.610	1.4
	62.5	0.616		
5	31.3	0.345	0.354	3.6
	31.3	0.363		
6	15.6	0.197	0.193	2.9
	15.6	0.189		
7	7.8	0.122	0.122	0.0
	7.8	0.122		
Blank	0	0.024	0.028	
	0	0.032		

The OD values of the calibration curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus color intensity. Values measured are still valid.

LIMITATIONS

- Since exact conditions may vary from assay to assay, a calibration curve must be established for every assay run.
- Bacterial or fungal contamination of either 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. Empty wells completely 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 mouse monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to mouse immunoglobulins can still be analyzed in such assays when mouse immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

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 2 standard deviations) was determined to be 1.3 pg/mL (mean of 6 independent assays).

B. Reproducibility

a. Intra-assay

Reproducibility within the assay was evaluated in 2 independent experiments. Each assay was carried out with 4 replicates of 8 serum samples containing different concentrations of sCD40. Two calibration curves were run on each plate. Data below shows 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%.

Sample	Experiment	Mean 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 2 independent experiments. Each assay was carried out with 4 replicates of 8 serum samples containing different concentrations of sCD40. Two calibration curves were run on each plate. Data below shows 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 7.0%.

Sample	Mean 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 4 levels of sCD40 into pooled normal serum samples. Recoveries were determined in 3 independent experiments with 6 replicates each. The amount of endogenous sCD40 in unspiked serum was subtracted from the spike values. The recovery ranged from 81% to 110% with an overall mean recovery of 94%.

D. Dilution Parallelism

Four serum samples with different levels of sCD40 were analyzed at serial 2 fold dilutions with 4 replicates each. In the table below the percent 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 frozen at -20°C and thawed up to 5 times, and sCD40 levels determined. There was no significant loss of sCD40 immunoreactivity detected by freezing and thawing.

b. Storage Stability

Aliquots of a serum sample (spiked or unspiked) were stored at -20°C, 4°C, room temperature and at 37°C, and the sCD40 level determined after 24 hours. There was no significant loss of sCD40 immunoreactivity detected during storage at above conditions.

F. Comparison of Serum and Plasma

From 8 individuals, serum as well as EDTA, 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 system was evaluated by spiking these proteins at physiologically relevant concentrations into a serum sample. There was no detectable cross reactivity.

REAGENT PREPARATION SUMMARY

A. Wash Buffer (1X) Add **Wash Buffer Concentrate** 20X (50 mL) to 950 mL distilled water.

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

B. Assay Buffer (1X) Add **Assay Buffer Concentrate** 20X (5 mL) to 95 mL distilled water.

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

C. Calibrator

Reconstitute **sCD40 Calibrator** by addition of distilled water as stated on vial label.

D. Biotin-Conjugate

Make a 1:100 dilution of **Biotin-Conjugate** in Assay Buffer (1X):

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

E. Streptavidin-HRP

Make a 1:100 dilution of **Streptavidin-HRP** in Assay Buffer (1X):

Number of Strips	Streptavidin-HRP (mL)	Assay Buffer(1X) (mL)
1 - 6	0.06	5.94
1 - 12	0.12	11.88

ASSAY PROTOCOL SUMMARY

- Determine the number of microwell strips required.
- Wash Microwell Strips twice with **Wash Buffer**.
- Add 100 µL **Sample Diluent**, in duplicate, to all calibrator wells.
- Pipette 100 µL reconstituted **sCD40 Calibrator** into the first wells and create calibrator dilutions 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.
- Prepare **Streptavidin-HRP**.
- Empty and wash Microwell Strips 3 times with **Wash Buffer**.
- Add 100 µL of diluted **Streptavidin-HRP** to all wells.
- Cover Microwell Strips and incubate 1 hour at room temperature.
- 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 minutes at room temperature (18° to 25°C).
- Add 100 µL **Stop Solution** to all wells including blank wells.
- Blank microplate reader and measure color intensity at 450 nm.

Note: If instructions in this protocol have been followed, samples have been diluted 1:2 (50 µL sample + 50 µL Sample Diluent), the concentration read from the calibration curve must be multiplied by the dilution factor (x2).

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