Human sCD40L ELISA Kit

Enzyme-linked Immunosorbent Assay for quantitative detection of human CD40L

Catalog Numbers BMS293 and BMS293TEN

Pub. No. MAN0016660 **Rev.** C.0 (32)

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The Human sCD40L ELISA Kit (extra sensitive) is an enzyme-linked immunosorbent assay for the quantitative detection of human CD40L.

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.

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.

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.

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.

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.

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 functional involved in B cell hybridomas and chronic lymphocytic leukemia as well as several autoimmune diseases.

For literature update refer to our website.

Principles of the test

An anti-human CD40L coating antibody is adsorbed onto microwells.

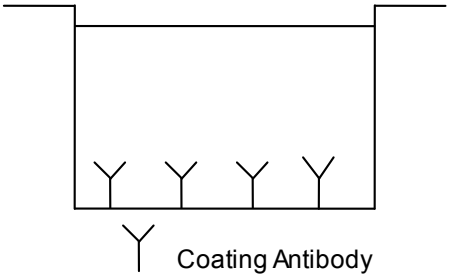

Fig. 1 Coated microwell

Human CD40L present in the sample or standard binds to antibodies adsorbed to the microwells.

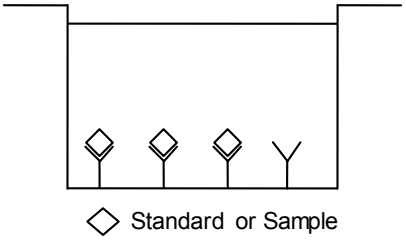

Fig. 2 First incubation

Following incubation unbound biological components are removed during a wash step and a HRP-conjugated anti-human CD40L antibody is added and binds to human CD40L captured by the first antibody.

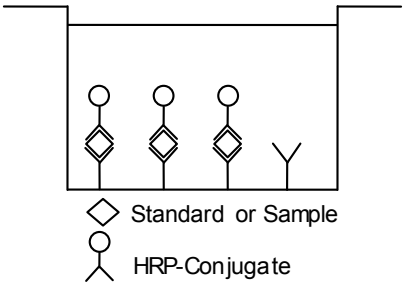

Fig. 3 Second incubation

Following incubation unbound HRP-conjugated anti-human CD40L antibody is removed during a wash step, and substrate solution reactive with HRP is added to the wells.

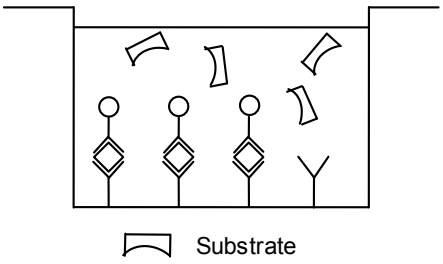

Fig. 4 Third incubation

A colored product is formed in proportion to the amount of human CD40L present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 human CD40L standard dilutions and human CD40L concentration determined.

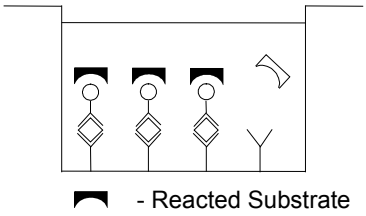

Fig. 5 Stop reaction

Reagents provided

Reagents for human CD40L platinum ELISA (extra sensitive) BMS293 (96 tests)

1 aluminum pouch with a Microwell Plate (12 strips of 8 wells each) coated with monoclonal antibody to human CD40L

1 vial (150 µL) HRP-Conjugate anti-human CD40L monoclonal antibody

2 vials human CD40L Standard lyophilized, 20 ng/mL upon reconstitution

1 vial Control high, lyophilized

1 vial Control low, lyophilized

1 vial (12 mL) Sample Diluent

Note: (In some cases the Sample Diluent contains an insoluble precipitate which does not interfere in any way with the test performance. Use according to protocol.)

1 vial (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween 20, 10% BSA)

1 bottle (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween 20)

1 vial (15 mL) Substrate Solution (tetramethyl-benzidine)

1 vial (15 mL) Stop Solution (1M Phosphoric acid)

4 Adhesive Films

Reagents for human CD40L platinum ELISA (extra sensitive) BMS293TEN (10x96 tests)

10 aluminum pouches with a Microwell Plate (12 strips of 8 wells each) coated with monoclonal antibody to human CD40L

10 vials (150 µL) HRP-Conjugate anti-human CD40L monoclonal antibody

10 vials human CD40L Standard lyophilized, 20 ng/mL upon reconstitution

10 vials Control high, lyophilized

10 vials Control low, lyophilized

10 vials (12 mL) Sample Diluent

Note: (In some cases the Sample Diluent contains an insoluble precipitate which does not interfere in any way with the test performance. Use according to protocol.)

2 vials (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween 20, 10% BSA)

4 bottles (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween 20)

10 vials (15 mL) Substrate Solution (tetramethyl-benzidine)

1 vial (100 mL) Stop Solution (1M Phosphoric acid)

20 Adhesive Films

Storage instructions – ELISA kit

Store kit reagents between 2° and 8°C except controls. Store lyophilized controls at -20°C.

Immediately after use remaining reagents should be returned to cold storage (2° 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.

The standard has to be used immediately after reconstitution and cannot be stored.

Sample collection and storage instructions

Cell culture supernatant, serum, and plasma (EDTA, citrate, heparin) were tested with this assay. Other body fluids might be suitable for use in the assay. Remove serum or plasma from the clot or cells 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 samples.

Samples should be aliquoted and must be stored frozen at –20°C to avoid loss of bioactive human CD40L. If samples are to be run within 24 hours, they may be stored at 2–8°C (refer to “Sample stability“ on page 6).

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

Materials required but not provided

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1000 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 regression analysis

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 statement(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 pipet 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 samples.
- 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.
- To avoid microbial contamination or cross-contamination of reagents or samples that may invalidate the test, use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acid inactivates 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 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°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.

Preparation of reagents

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

Wash buffer (1x)

1. Pour entire contents (50 mL) of the Wash Buffer Concentrate (20x) into a clean 1000 mL graduated cylinder. Bring to final volume of 1000 mL with glass-distilled or deionized water.
2. Mix gently to avoid foaming.
3. Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.
4. Wash Buffer (1x) may also 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

Assay buffer (1x)

1. Pour the 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.
2. Store at 2° to 8°C. Please note that the Assay Buffer (1x) is stable for 30 days.
3. Assay Buffer (1x) may also 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

HRP-Conjugate

Note: The HRP-Conjugate should be used within 30 minutes after dilution.

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

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

Human CD40L standard

1. Reconstitute human CD40L standard by addition of distilled water.
2. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 20 ng/mL).
3. Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.
4. Further dilute standard 1:2 by adding the same volume Sample Diluent as the reconstitution volume to the reconstituted standard (concentration of prepared standard = 10 ng/mL).
5. After usage remaining standard cannot be stored and has to be discarded.
6. Standard dilutions can be prepared directly on the microwell plate (see “Test protocol“ on page 4) or alternatively in tubes (see “External standard dilution“ on page 3).

External standard dilution

1. Label 7 tubes, one for each standard point: S1, S2, S3, S4, S5, S6, S7
2. Then prepare 1:2 serial dilutions for the standard curve as follows: Pipette 225 µL of Sample Diluent into each tube.
3. Pipette 225 µL of diluted standard (concentration = 10 ng/mL) into the first tube, labeled S1, and mix (concentration of Standard 1 = 5 ng/mL).
4. Pipette 225 µL of this dilution into the second tube, labeled S2, and mix thoroughly before the next transfer.
5. Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 6).

   Sample Diluent serves as blank.

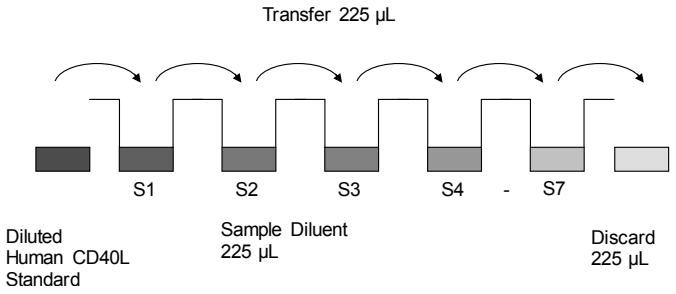

Fig. 6 Dilute standards - tubes

Controls

Reconstitute lyophilized controls by addition of distilled water (10-30 minutes). Reconstitution volume is stated on the label of the control vial. Swirl or mix gently to ensure complete and homogeneous solubilization. Further treat the controls like your samples in the assay. For control range please refer to certificate of analysis. Store reconstituted controls aliquoted at -20°C.

Avoid repeated freeze and thaw cycles.

Test protocol

1. 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 from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
2. 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 surface of the microwells.

   After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.
3. Standard dilution on the microwell plate (Alternatively the standard dilution can be prepared in tubes - see “External standard dilution“ on page 3):

   Add 100 µL of Sample Diluent in duplicate to all standard wells. Pipette 100 µL of prepared standard (see “External standard dilution“ on page 3, concentration = 10.00 ng/mL), in duplicate, into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1 S1 = 5.00 ng/mL), and transfer 100 µL to wells B1 and B2, respectively (see Figure 7). Take care not to scratch the inner surface of the microwells.

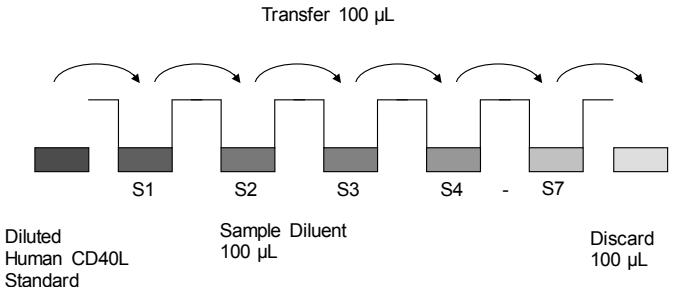

Fig. 7 Dilute standards - microwell plate

Table 1 Example of the arrangement of blanks, standards and samples in the microwell strips.

	1	2	3	4
A	Standard 1 5.00 ng/mL	Standard 1 5.00 ng/mL	Sample 1	Sample 1
B	Standard 2 2.50 ng/mL	Standard 2 2.50 ng/mL	Sample 2	Sample 2
C	Standard 3 1.25 ng/mL	Standard 3 1.25 ng/mL	Sample 3	Sample 3
D	Standard 4 0.63 ng/mL	Standard 4 0.63 ng/mL	Sample 4	Sample 4
E	Standard 5 0.31 ng/mL	Standard 5 (0.31 ng/mL)	Sample 5	Sample 5
F	Standard 6 0.16 ng/mL	Standard 6 0.16 ng/mL	Sample 6	Sample 6
G	Standard 7 0.08 ng/mL	Standard 7 0.08 ng/mL	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

   Continue this procedure 5 times, creating two rows of human CD40L standard dilutions, ranging from 5.00 to 0.08 ng/mL. Discard 100 µL of the contents from the last microwells (G1, G2) used.

   In case of an external standard dilution (see “External standard dilution“ on page 3), pipette 100 µL of these standard dilutions (S1 - S7) in the standard wells according to Table 1.
4. Add 100 µL of Sample Diluent in duplicate to the blank wells.
5. Add 80 µL of Sample Diluent to the sample wells designated for serum and control samples.
6. Add 50 µL of Sample Diluent to the sample wells designated for plasma samples.
7. Add 20 µL of each serum and control sample in duplicate to the designated sample wells.
8. Add 50 µL of each plasma sample in duplicate to the designated sample wells.
9. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours on a microplate shaker.
10. Prepare HRP-Conjugate (see “HRP-Conjugate“ on page 3).
11. Remove adhesive film and empty wells. Wash microwell strips 3 times according to point 2. of the test protocol. Proceed immediately to the next step.
12. Add 100 µL of HRP-Conjugate to all wells.
13. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 1 hours on a microplate shaker.
14. Remove adhesive film and empty wells. Wash microwell strips 3 times according to point 2. of the test protocol. Proceed immediately to the next step.
15. Pipette 100 µL of TMB Substrate Solution to all wells.
16. 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 next point 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 standard 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 Standard 1 has reached an OD of 0.9 – 0.95.
17. Stop the enzyme reaction by quickly pipetting 100 µL of Stop Solution into each well. 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.
18. 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 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 percent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human CD40L 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 human CD40L 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 human CD40L concentration.
- If instructions in this protocol have been followed, serum and control samples have been diluted 1:5 (20 µL sample + 80 µL Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 5). Plasma samples have been diluted 1:2 (50 µL sample + 50 µL Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 2).

- Calculation of samples with a concentration exceeding standard 1 may result in incorrect human CD40L levels. Such samples require further external predilution according to expected human CD40L values with Sample Diluent in order to precisely quantitate the actual human CD40L level.
- It is suggested that each testing facility establishes a control sample of known human CD40L concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 8. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

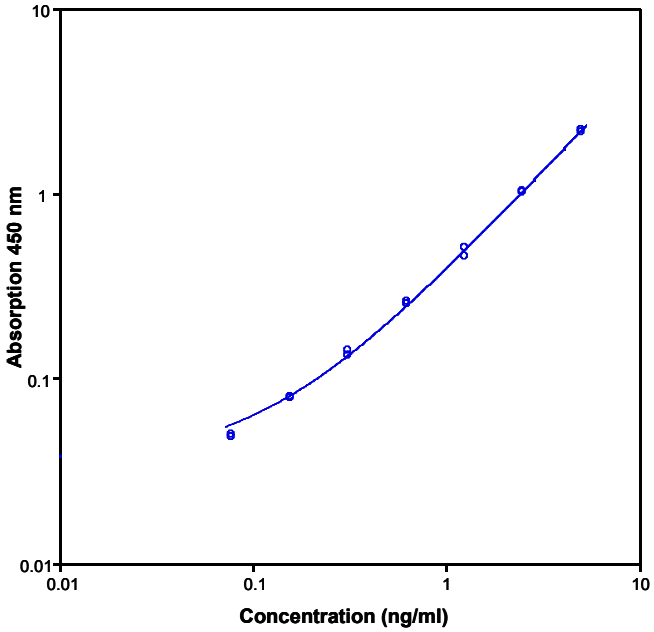

Fig. 8 Representative standard curve for human CD40L Platinum ELISA (extra sensitive). Human CD40L was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

Table 2 Typical data using the human CD40L platinum ELISA (extra sensitive)

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Human CD40L Concentration (ng/mL)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	5.00	2.189 2.136	2.163	1.2
2	2.50	1.013 1.021	1.017	0.4
3	1.25	0.508 0.458	0.483	5.2
4	0.63	0.260 0.254	0.257	1.2
5	0.31	0.141 0.134	0.138	2.5
6	0.16	0.080 0.078	0.079	1.3
7	0.08	0.050 0.048	0.049	2.0
Blank	0	0.022 0.016	0.019	15.8

The OD values of the standard 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 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. Empty wells completely before dispensing fresh wash solution, 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 radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibodies (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 immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the sample.

Performance characteristics

Sensitivity

The limit of detection of human CD40L defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be less than 0.01 ng/mL (mean of 6 independent assays).

Reproducibility

Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human CD40L. 2 standard curves were run on each plate. Data below show the mean human CD40L concentration and the coefficient of variation for each

sample (see Table 3). The calculated overall intra-assay coefficient of variation was 5.5%.

Table 3 The mean human CD40L concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human CD40L Concentration (ng/mL)	Coefficient of Variation (%)
1	1	28.4	9.5
	2	25.8	9.3
	3	23.3	5.2
2	1	22.8	4.8
	2	20.7	5.4
	3	21.5	3.0
3	1	15.7	6.9
	2	12.2	8.7
	3	13.1	6.1
4	1	10.1	4.7
	2	8.8	4.9
	3	8.8	4.0
5	1	5.1	5.1
	2	4.2	8.4
	3	4.5	8.9
6	1	12.9	3.1
	2	12.6	5.1
	3	10.5	2.2
7	1	9.9	1.3
	2	9.5	5.1
	3	10.1	6.3
8	1	6.2	5.2
	2	6.5	7.0
	3	6.7	1.5

Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human CD40L. 2 standard curves were run on each plate. Data below show the mean human CD40L concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 6.6%.

Table 4 The mean human CD40L concentration and the coefficient of variation of each sample

Sample	Mean Human CD40L Concentration (ng/mL)	Coefficient of Variation (%)
1	25.8	8.1
2	21.7	3.8
3	13.7	10.5
4	9.2	6.8
5	4.6	8.7
6	12.0	8.8
7	9.8	2.7
8	6.5	3.3

Spike recovery

The spike recovery was evaluated by spiking 4 levels of human CD40L into serum and plasma. Recoveries were determined in 3 independent experiments with 6 replicates each. The unspiked serum and plasma was used as blank in these experiments. The recovery ranged from 87–112% with an overall mean recovery of 99%.

Dilution parallelism

Serum samples with different levels of human CD40L were analyzed at serial 2-fold dilutions with 4 replicates each.

The recovery ranged from 84–114% with an overall recovery of 97%.

Sample	Dilution	Human CD40L Concentration (ng/mL)		Recovery of expected concentration (%)
		Expected value	Observed value	
1	1:5	–	25.2	–
	1:10	12.6	14.3	114
	1:20	7.2	6.7	94
	1:40	3.4	3.5	104
2	1:5	–	23.5	–
	1:10	11.7	12.0	103
	1:20	6.0	5.1	85
	1:40	2.5	2.3	92
3	1:5	–	27.5	–
	1:10	13.7	13.5	98
	1:20	6.7	7.3	109
	1:40	3.7	3.6	97
4	1:5	–	28.1	–
	1:10	14.1	11.7	84
	1:20	5.9	5.5	94
	1:40	2.8	2.6	93

Sample stability

Freeze-Thaw stability

Aliquots of serum samples (unspiked or spiked) were stored at -20°C and thawed 5 times, and the human CD40L levels determined. There was no significant loss of human CD40L immunoreactivity detected by freezing and thawing.

Storage stability

Aliquots of serum samples (spiked or unspiked) were stored at –20°C, 2–8°C, room temperature, and at 37°C, and the human CD40L level determined after 24 hours.

A significant loss of human CD40L immunoreactivity was detected during storage at room temperature (57% immunoreactivity) and at 37°C (4% immunoreactivity) after 24 hhours.

Comparison of serum and plasma

From two individuals, serum as well as EDTA and citrate plasma samples, obtained at the same time point, were evaluated. Human CD40L concentrations were significantly different, but if the uniformity of blood preparations is assured, all these body fluids are suitable for the assay.

Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human CD40L positive serum. No cross-reactivity was detected.

Expected values

Panels of 40 serum as well as EDTA, citrate, and heparin plasma samples from randomly selected apparently healthy donors (males and females) were tested for human CD40L. The levels measured may vary with the sample collection used. Elevated human CD40L levels depend on the type of immunological disorder. For detected human CD40L levels see Table 5.

Table 5 Expected values.

Sample matrix	Number of samples evaluated	Range (ng/mL)	% detectable	Mean of detectable (ng/mL)
Serum	40	nd [1]–29.1	32.5	4.2
Plasma (EDTA)	40	nd [1]–10.6	72.5	2.9
Plasma (citrate)	40	nd [1]–7.5	80	2.0
Plasma (heparin)	40	nd [1]–7.5	77.5	1.8

[1] nd = nondetectable, samples measured below the lowest standard point are considered to be nondetectable.

Reagent preparation summary

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

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

HRP-Conjugate

Make a 1:100 dilution of HRP-Conjugate in Assay Buffer (1x):

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

Human CD40L standard

Reconstitute lyophilized human CD40L standard with distilled water. (Reconstitution volume is stated on the label of the standard vial.) Dilute reconstituted standard 1:2 by adding the same volume as the reconstitution volume of Sample Diluent to the reconstituted standard.

Controls

Reconstitute lyophilized controls by addition of distilled water (10-30 minutes). Reconstitution volume is stated on the label of the control vial.

Test protocol summary

1. Determine the number of microwell strips required.
2. Wash microwell strips twice with Wash Buffer.
3. Standard dilution on the microwell plate: Add 100 µL Sample Diluent, in duplicate, to all standard wells. Pipette 100 µL prepared standard into the first wells and create standard dilutions by transferring 100 µL from well to well. Discard 100 µL from the last wells.

   Alternatively external standard dilution in tubes (see "External standard dilution" on page 3): Pipette 100 µL of these standard dilutions in the microwells strips.
4. Add 100 µL Sample Diluent, in duplicate, to the blank wells.
5. Add 80 µL Sample Diluent to all wells designated for serum and control samples.

   Add 50 µL Sample Diluent to all wells designated for plasma samples.
6. Add 20 µL of each serum and control sample in duplicate to designated sample wells.

   Add 50 µL of each plasma sample in duplicate to designated sample wells.
7. Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C).
8. Prepare HRP-Conjugate.
9. Empty and wash microwell strips 3 times with Wash Buffer.
10. Add 100 µL HRP-Conjugate to all wells.
11. Cover microwell strips and incubate 1 hour at room temperature (18° to 25°C).
12. Empty and wash microwell strips 3 times with Wash Buffer.
13. Add 100 µL of TMB Substrate Solution to all wells.
14. Incubate the microwell strips for about 10 minutes at room temperature (18°to 25°C).
15. Add 100 µL Stop Solution to all wells.
16. Blank microwell reader and measure color intensity at 450 nm.

Note: If instructions in this protocol have been followed, serum and control samples have been diluted 1:5 (20 µL sample + 80 µL Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 5).

Plasma samples have been diluted 1:2 (50 µL sample + 50 µL Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x 2).

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