ab119517 – CD40L Mouse ELISA Kit

Instructions for Use

For the quantitative measurement of Mouse CD40L concentrations in cell culture supernatant and serum.

This product is for research use only and is not intended for diagnostic use.

Version 5 Last Updated 11 January 2019

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1. BACKGROUND

Abcam's CD40L Mouse *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for accurate quantitative measurement of Mouse CD40L concentrations in cell culture supernatant and serum.

CD40L specific antibodies have been precoated onto 96-well plates. Standards and test samples are added to the wells along with a biotin-conjugated CD40L detection antibody then incubated at room temperature. Following washing, a Streptavidin-HRP conjugate is added to each well, incubated at room temperature and washed. TMB is added and then catalyzed by HRP to produce a blue product that changes to yellow after the addition of acidic stop solution. The density of yellow coloration is directly proportional to the amount of CD40L captured on the plate.

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/CD40 ligand 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.

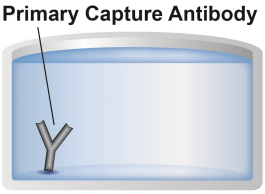
The programmed cell death has been suggested to be involved in clonal elimination of self-reactive lymphocytes for the normal function of the immune system. Interaction with membrane bound self antigens may eliminate self-reactive nature B cells by apoptosis. Antigen-receptor mediated B cell apoptosis 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.

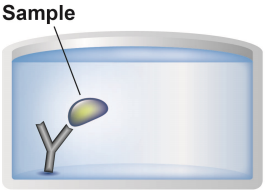
Taken together, B cells can participate in regulating their own destruction. Protection against Fas-dependent apoptosis afforded by immunoglobulin-receptor engagement may constitute a fail-safe mechanism that eliminates bystander B cells activated by CD40L - expressing T cells, but ensures survival of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

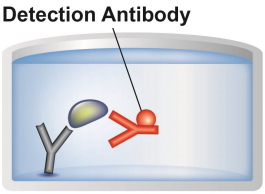
2. ASSAY SUMMARY



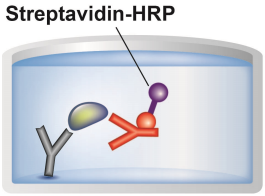
Prepare all reagents, samples and standards as instructed.



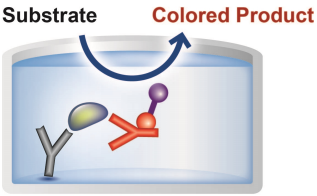
Add standard or sample to each well used.



Add prepared detection antibody to each well. Incubate at room temperature.



Wash and add prepared Streptavidin-HRP conjugate. Incubate at room temperature.



Wash and add TMB Substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at 2-8ºC immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 9 Reagent Preparation.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Microplate coated with monoclonal antibody to mouse CD40L(12 x 8 wells)	96 wells	2-8 ºC
Biotin-Conjugate monoclonal antibody to mouse CD40L	70 µL	2-8 ºC
Streptavidin-HRP	150 µL	2-8 ºC
Mouse CD40L Standard lyophilized (40 ng /mL upon reconstitution)	2 Vials	2-8 ºC
Sample Diluent	12 mL	2-8 ºC
20X Assay Buffer	5 mL	2-8 ºC
20X Wash Buffer Concentrate	50 mL	2-8 ºC
TMB Substrate Solution	15 mL	2-8 ºC
Stop Solution (1M Phosphoric acid)	15 mL	2-8 ºC
Adhesive Films	4 units	2-8 ºC

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- 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)
- Microplate 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

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted

8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers
- Avoid foaming or bubbles when mixing or reconstituting components
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps
- Complete removal of all solutions and buffers during wash steps.
- As exact conditions may vary from assay to assay, a standard curve must be established for every run.
- 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 radio immunotherapy has significantly increased the number of patients with Mouse 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.
- **This kit is sold based on number of tests.** A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18-25°C) prior to use. If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

9.1 1X Wash Buffer

Prepare 1X Wash Buffer by diluting the 20X Wash Buffer Concentrate with distilled or deionized water. To make 500 mL 1X Wash Buffer, combine 25 mL 20X Wash Buffer Concentrate with 475 mL distilled or deionized water. Mix thoroughly and gently to avoid foaming.

Note: The 1X Wash Buffer should be stored at 2-8 ºC and is stable for 30 days.

9.2 1X Assay Buffer

Prepare 1X Assay Buffer by diluting the 20X Assay Buffer Concentrate with distilled or deionized water. To make 50 mL 1X Assay Buffer (1-6 strips), combine 2.5 mL 20X Assay Buffer Concentrate with 47.5 mL distilled or deionized water. To make 100 mL 1X Assay Buffer (1-12 strips), combine 5 mL 20X Assay Buffer Concentrate with 95.0 mL distilled or deionized water. Mix thoroughly and gently to avoid foaming.

Note: The 1X Assay Buffer should be stored at 2-8 ºC and is stable for 30 days.

9.3 1X Biotin Conjugated Antibody

To prepare the Biotin Conjugated Antibody, dilute the anti-mouse CD40L monoclonal antibody 100-fold with 1X Assay Buffer. Use the following table as a guide to prepare as much 1X Biotin Conjugated Antibody as needed by adding the required volume (µL) of the Biotin Conjugated Antibody to the required volume (mL) of 1X Assay Buffer. Mix gently and thoroughly.

Number of strips	Volume of Biotin-Conjugated CD40L antibody (µL)	1X Assay Buffer (mL)
1 - 6	30	2.97
7 - 12	60	5.94

Note: The 1X Biotin-Conjugated Antibody should be used within 30 minutes after dilution.

9.4 1X Streptavidin-HRP Conjugate

To prepare the Streptavidin-HRP Conjugate, dilute the anti-Streptavidin-HRP Conjugate 100-fold with 1X Assay Buffer. Use the following table as a guide to prepare as much 1X Streptavidin-HRP Conjugate as needed by adding the required volume (µL) of the Streptavidin-HRP Conjugate to the required volume (mL) of 1X Assay Buffer. Mix gently and thoroughly.

Number of strips	Volume of Streptavidin-HRP (µL)	1X Assay Buffer (mL)
1 - 6	60	5.94
7 - 12	120	11.88

Note: The 1X Streptavidin-HRP should be used within 30 minutes after dilution.

- All other solutions are supplied ready to use

10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use.

10.1 Prepare a 40 ng/mL **Stock Standard** by reconstituting one vial of the Mouse CD40L standard with the volume of distilled water stated on the label. Hold at room temperature for 10-30 minutes. The 40 ng/mL **Stock Standard** cannot be stored for later use.

10.2 Label eight tubes with numbers 1 - 8.

10.3 Add 225 µL sample diluent to all tubes.

10.4 Prepare a 20 ng/mL **Standard 1** by adding 225 µL of the 40 ng/mL Stock Standard to tube 1. Mix thoroughly and gently.

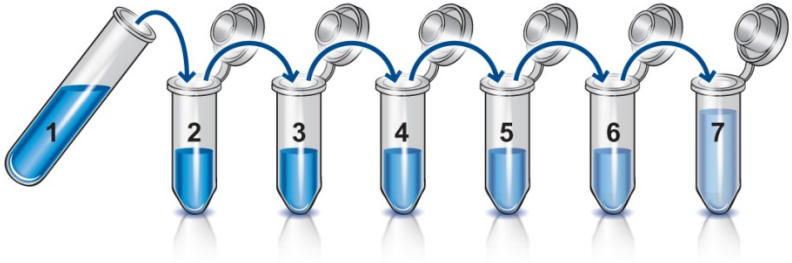
10.5 Prepare **Standard 2** by transferring 225 µL from Standard 1 to tube 2. Mix thoroughly and gently.

10.6 Prepare **Standard 3** by transferring 225 µL from Standard 2 to tube 3. Mix thoroughly and gently.

10.7 Using the table below as a guide, repeat for tubes number 4 through to 7.

10.8 **Standard 8** contains no protein and is the Blank control

Standard	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Stock	225	225	40.00	20.00
2	Standard 1	225	225	20.00	10.00
3	Standard 2	225	225	10.00	5.00
4	Standard 3	225	225	5.00	2.50
5	Standard 4	225	225	2.50	1.25
6	Standard 5	225	225	1.25	063
7	Standard 6	225	225	0.63	0.31
8	None	-	225	-	0



11. SAMPLE COLLECTION AND STORAGE

- Cell culture supernatant and serum were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum 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 specimens.

- Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive Mouse CD40L. 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, the frozen sample should be brought to room temperature slowly and mixed gently and properly diluted with Sample Diluent.

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

12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use
- Unused well strips should be returned to the plate packet and stored at 2-8°C
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

13.1. Prepare all reagents, working standards, and samples as directed in the previous sections. Determine the number of microplate strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards.

13.2. Wash the microplate twice with approximately 400 µL 1X Wash Buffer per well with thorough aspiration of microplate contents between washes. Allow the 1X Wash Buffer to remain in the wells for about 10 - 15 seconds before aspiration. Take care not to scratch the surface of the microplate.

13.3. After the last wash step, empty wells and tap microplate on absorbent pad or paper towel to remove excess 1X Wash Buffer. Use the microplate strips immediately after washing. Alternatively the microplate strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

13.4. Add 100 µL of prepared standards (including the standard blank control) to the appropriate wells.

13.5. Add 50 µL of Sample Diluent to all sample wells.

13.6. Add 50 µL of sample to appropriate wells.

13.7. Add 50 µL of 1X Biotin Conjugated Antibody to all wells.

13.8. Cover with adhesive film and incubate at room temperature (18° to 25°C) for 2 hours (microplate can be incubated on a shaker set at 400 rpm).

13.9. Remove adhesive film and empty wells. Wash microplate strips 4 times according to step 13.2. Proceed immediately to step 13.10.

13.10. Add 100 µL of 1X Streptavidin-HRP to all wells, including the blank wells.

13.11. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour (microplate can be incubated on a shaker set at 100 rpm).

13.12. Remove adhesive film and empty wells. Wash microplate strips 4 times according to step 13.2. Proceed immediately to the next step.

13.13. Pipette 100 µL of TMB Substrate Solution to all wells.

13.14. Incubate the microplate strips at room temperature (18 to 25°C) for 10 minutes. Avoid direct exposure to intense light.

Note: The color development on the plate should be monitored and the substrate reaction stopped (see step 13.15) before the signal in the positive wells becomes saturated. Determination of the ideal time period for color development should 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.

13.15. Stop the enzyme reaction by adding 100 µL of Stop Solution into each well.

Note: It is important that the Stop Solution is mixed quickly and uniformly throughout the microplate to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microplate strips are stored at 2 - 8°C in the dark.

13.16. Read absorbance of each microplate on a spectrophotometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader

using the blank wells. Determine 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.

14. CALCULATIONS

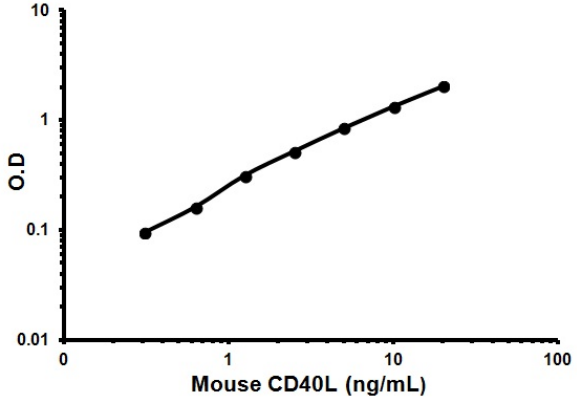
Average the duplicate readings for each standard, sample and control blank. Subtract the no protein control blank from all mean readings. Plot the mean standard readings against their concentrations and draw the best smooth curve through these points to construct a standard curve. Most plate reader software or graphing software can plot these values and curve fit. A five parameter algorithm (5PL) usually provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 5-parameter logistic). Extrapolate protein concentrations for unknown and control samples from the standard curve plotted. Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

If instructions in this protocol have been followed samples have been diluted 1:2, as stated in Step 13.6, the concentration read from the standard curve must be multiplied by the dilution factor (x 2) to obtain an accurate value, in addition to any initial sample dilution factor.

Calculation of samples with a concentration exceeding standard 1 will result in incorrect, low Mouse CD40L levels. Such samples require further external predilution according to expected Mouse CD40L values with Sample Diluent in order to precisely quantitate the actual Mouse CD40L level.

15. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Standard Curve Measurements			
Conc.	**O.D. 450 nm**		**Mean**
(ng/mL)	**1**	**2**	**O.D.**
0	0.024	0.023	0.024
0.31	0.094	0.095	0.095
0.63	0.159	0.165	0.162
1.25	0.316	0.309	0.313
2.50	0.514	0.525	0.520
5.00	0.830	0.861	0.846
10.00	1.363	1.303	1.333
20.00	2.054	2.009	2.032

Figure 1. Example of a Mouse CD40L standard protein curve

16. TYPICAL SAMPLE VALUES

SENSITIVITY –

The limit of detection for CD40L defined as the analyte concentration resulting in an absorption significantly higher than the absorption of the dilution medium (mean plus two standard deviations) was determined to be 0.14 ng/mL (mean of 6 independent assays).

RECOVERY –

Spiked samples were prepared by adding four different levels of recombinant CD40L into serum. Recoveries were determined in 3 independent experiments with 8 replicates each. The unspiked serum was used as blank in these experiments. The overall mean recovery was 94%.

LINEARITY OF DILUTION –

A serum sample was assayed at four two-fold dilutions covering the working range of the standard curve. The overall mean recovery was 96%. Recoveries were shown to depend on the serum used.

PRECISION –

Intra- and Inter-assay reproducibility was determined by measuring samples containing different concentrations of Human CD137.

	Intra-Assay	Inter-Assay
n=	8	8
%CV	6.5	11.1

17. ASSAY SPECIFICITY

The assay detects both endogenous and recombinant Mouse CD40L. The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a CD40L positive serum. There was no detectable cross reactivity with any of the tested proteins.

18. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed.

19. NOTES

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