Human CD154 / CD40L ELISA Kit

Instructions for use

Catalogue numbers: 1x96 tests: 650.120.096

For research use only

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Human CD154 / CD40L ELISA KIT

1. Intended use

The CD154 (sCD40L) ELISA is an enzyme-linked immunosorbent assay for quantitative detection of soluble human CD40 Ligand levels in cell culture supernatants, human serum and plasma.

This kit has been configured for research use only. Not suitable for use in therapeutic procedures.

2. Introduction

2.1. Summary

CD40 belongs to the TNF receptor superfamily. While the biological role of some of the ligand-receptor pairs in this family still remains obscure, CD40 has proven its importance (3).

A key role of CD40/CD40ligand interactions in immune activation, particularly in T-cell dependent B cell responses is anticipated. This molecule as well as the other ligands of the family share the property of co-stimulation of T-cell proliferation and are all expressed by activated T-cells.

The programmed cell death has been suggested to be involved in clonal elimination of self-reactive lymphocytes for the normal function of the immune system. Interaction with membrane bound self antigens may eliminate self-reactive nature B cells by apoptosis. Antigen-receptor mediated B cell apoptosis is blocked when a signal is transduced via the CD40 molecule on the B cell surface (10).

Because the ligand of CD40 (CD40L) is expressed on activated T helper cells, B cells may escape from apoptosis and are activated when the immune system interacts with foreign antigens, which are normally able to activate T-helper cells. Thus the CD40 - CD154interaction plays a central role in the various phases of the B cell response to T-dependent antigens (8).

Taken together, B cells can participate in regulating their own destruction. Protection against Fas-dependent apoptosis afforded by immunoglobulin-receptor engagement may constitute a fail-safe mechanism that eliminates bystander B cells activated by CD154- expressing T cells, but ensures survival of antigen-specific B cells (9).

CD40 Ligand is expressed on the surface of activated CD4+ T cells, basophils, and mast cells. Binding of CD154 to its receptor, CD40, on the surface of B cells stimulates B-cell proliferation, adhesion and differentiation. A soluble isoform of CD154has been shown to exist in the circulation. This soluble molecule is a homotrimer of a 18kDa protein exhibiting full activity in B cell proliferation and differentiation assays, is able to rescue B cells from apoptosis and binds soluble CD40 (4, 6).

CD154 is discussed in relation to a potential role in supporting B cell tumors and it has been discovered that the molecular defect in the X-linked Hyper-IgM-Syndrome (1) is targeted to the CD154gene, it is functional involved in B cell hybridomas (5) and chronic lymphocytic leukemia (2) as well as several autoimmune diseases (7).

2.2. Principle of the method

An anti-CD154 monoclonal coating antibody is adsorbed onto microwells. CD154 present in the sample or standard binds to antibodies adsorbed to the microwells; a HRP-conjugated monoclonal anti-CD154 antibody binds to CD154 captured by the first antibody.

Following incubation unbound enzyme conjugated anti-CD154 is removed during a wash step and substrate solution reactive with HRP is added to the wells.

A coloured product is formed in proportion to the amount of soluble CD154 present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from six CD154 standard dilutions and CD154 sample concentration determined.

3. Reagents provided and reconstitution

Reagents (Store@2-8ºC)	**Quantity 1x96 well kit** Cat no. 650.120.096	**Reconstitution**
96-wells precoated microtiter plate	1	Ready-to-use
Plate covers	2	-
Standard 20 ng/ml	2 vials	Reconstitute with the volume of distilled water indicated on the vial.
HRP-Conjugate anti-human sCD154(0.2 ml)	1 vial	Make a 1/100 dilution in Assay Buffer
Assay Buffer Concentrate (5 ml)	1 vial	20X concentrate. Dilute in distilled water
Wash Buffer Concentrate (50 ml)	1 vial	20X concentrate. Dilute in distilled water
Sample Diluent (12 ml)	1 vial	Ready-to-use
Substrate Solution (15 ml)	1 vial	Ready-to-use
Stop Solution (15 ml)	1 vial	Ready-to-use
Dilution plates	2	-

4. Materials required but not provided

- Microtiter plate reader fitted with appropriate filters (450nm required with optional 630nm reference filter)
- Microplate washer or wash bottle
- 10, 50, 100, 200 and 1,000 µl adjustable single channel micropipettes with disposable tips
- 50-300 µl multi-channel micropipette with disposable tips
- Multichannel micropipette reagent reservoirs
- Distilled water
- Vortex mixer
- Orbital Shaker (rotator)
- Miscellaneous laboratory plastic and/or glass, if possible sterile

5. Storage Instructions

Store kit reagents between 2°C and 8°C. Immediately after use reagents should be returned to cold storage (2°C to 8°C). 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.

6. Specimen collection, processing & storage

Cell culture supernatants, plasma, and human serum will be suitable for use in the assay. Remove serum from the clot or red cells, respectively, as soon as possible after clotting and separation.
Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

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

7. Safety & precautions for use

- Handling of reagents, serum or plasma specimens should be in accordance with local safety procedures, e.g. CDC/NIH Health manual : " Biosafety in Microbiological and Biomedical Laboratories" 1984.
- Laboratory gloves should be worn at all times.
- Avoid any skin contact with Stop Solution and Substrate Solution. In case of contact, wash thoroughly with water.
- Do not eat, drink, smoke or apply cosmetics where kit reagents are used.
- Do not pipette by mouth.
- When not in use, kit components should be stored refrigerated or frozen as indicated on vials or bottles labels.
- All reagents should be warmed to room temperature before use. Lyophilized standards should be discarded after use.
- Once the desired number of strips has been removed, immediately reseal the bag to protect the remaining strips from deterioration.
- Cover or cap all reagents when not in use.
- Do not mix or interchange reagents between different lots.
- Do not use reagents beyond the expiration date of the kit.
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross contamination, for the dispensing of Stop Solution and Substrate Solution, avoid pipettes with metal parts.
- Use a clean plastic container to prepare the washing solution.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells.
- The Substrate Solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the Substrate Solution with metal to prevent colour development. Warning Substrate Solution is toxic avoid direct contact with hands. Dispose off properly.
- If a dark blue colour develops within a few minutes after preparation, this indicates that the Substrate Solution has been contaminated and must be discarded. Read absorbance’s within 1 hour after completion of the assay.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- Follow incubation times described in the assay procedure.
- Dispense the Substrate Solution within 15 min of the washing of the microtitre plate.

8. Assay Preparation

Bring all reagents to room temperature before use

8.1. Assay Design

Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running zeros and standards. Each sample, standard and zero should be tested **in duplicate**. Remove sufficient Microwell Strips for testing from the aluminium pouch immediately prior to use. Return any wells not required for this assay with desiccant to the pouch. Seal tightly and return to 2-8°C storage.

Example plate layout (example shown for a 6 point standard curve)

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

All remaining empty wells can be used to test samples in duplicate

8.2. Preparation of 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** (20X) 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.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer is stable for 30 days.

8.3. Preparation of Assay Buffer

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

Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days.

8.4. Preparation of Standard

Standard vials must be reconstituted with the volume of distilled water shown on the vial immediately prior to use. This reconstitution gives a stock solution of 20 ng/ml of CD154. **Mix the reconstituted standard gently by inversion only**. Serial dilutions of the standard are made directly in the assay plate to provide the concentration range from 10 to 0.31 ng/ml. A fresh standard curve should be produced for each new assay.

- ***Using the dilution plates***, add 100 µl of Sample Diluent to the standard wells A1 and A2 to F1 and F2.
- Immediately after reconstitution add 100 µl of the reconstituted standard to wells A1 and A2, which provides the highest concentration standard at 10 ng/ml.
- Transfer 100 µl from wells A1 and A2 to B1 and B2. Mix the well contents by repeated aspirations and ejections taking care not to scratch the inner surface of the wells.
- Continue this 1:1 dilution using 100 µl from wells B1 and B2 through to wells F1 and F2 providing a serial diluted standard curve ranging from 10 to 0.31 ng/ml.
- Discard 100 µl from the final wells of the standard curve (F1 and F2).

Alternatively these dilutions can be performed in separate clean tubes and immediately transferred directly into the relevant wells.

8.5. Preparation of Samples

Before testing, all samples have to be diluted 1:5 in Sample Diluent :

- ***Using the dilution plates***, add 80 µl of Sample Diluent to the sample wells.
- Add 20 µl of each sample in duplicates to the sample wells.

8.6. Preparation of HRP-Conjugate

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

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

9. Method

We strongly recommend that every vial is mixed thoroughly without foaming prior to use except the standard vial which must be mixed gently by inversion only.

Prepare all reagents as shown in section 8.

Assay Step		Details
1.	Addition	**Prepare Standard curve** as shown in section 8.4 on the Dilution plate Add 100 µl of zero (Sample Diluent) in duplicate to appropriate well.
2.	Addition	**Prepare Samples** as shown in section 8.5 on the Dilution plate.
3.	Addition	Add 100 µl of diluted **HRP-Conjugate** to all wells on the Dilution plate.
4.	Wash	Remove the precoated plate from the pouch removing any un-needed strips and wash the plate as follows: a) Dispense 0.3 ml of **1x washing solution** into each well, leaving for 10-15secs b) Aspirate the contents of each well c) Repeat step a and b d) Use this plate immediately after washing
5.	Transfer	Transfer 150 µl of solution from all wells of the dilution plate into the corresponding wells of the precoated plate.
6.	Incubation	Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for **2 hours** on a rotator set at 400rpm, if no rotator is available please incubate at 4°C overnight.
7.	Wash	Remove the cover from the plate and wash the plate as follows: a) Aspirate the contents of each well b) Dispense 0.3 ml of **1x washing solution** into each well c) Aspirate the contents of each well d) Repeat step a and c a further two times
8.	Addition	Add 100 µl of TMB **Substrate Solution** into all wells
9.	Incubation	Incubate in the dark for **10 minutes*** at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil
10.	Addition	Add 100 µl of **Stop Solution** into all wells
Read the absorbance value of each well (immediately after step 10.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 630 nm as the reference wave length (610 nm to 650 nm is acceptable).		

*****_Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range_

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

10. Data Analysis

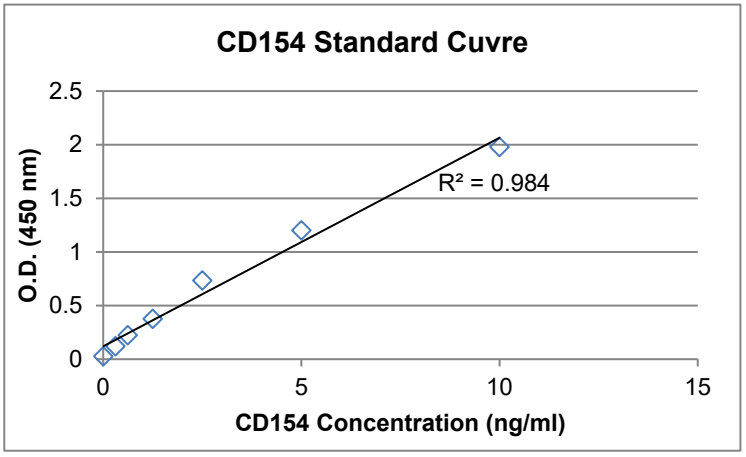
Calculate the average absorbance values for each set of duplicate standards and samples. Ideally duplicates should be within 20% of the mean.

Generate a best fit standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding human CD154standard concentration on the horizontal axis.

The amount of CD154in each sample is determined by extrapolating OD values against CD154standard concentrations using the standard curve**.**

Example of CD154 Standard Curve

Standard	CD154 Conc ng/ml	OD (450nm) Mean	CV%
1	10	1.979	1.3
2	5	1.202	0.9
3	2.5	0.734	4
4	1.25	0.377	3
5	0.62	0.224	0.9
6	0.31	0.122	0
Zero	0	0.028	5.4



Note: curve shown above should not be used to determine results. Every laboratory must produce a standard curve for each set of microwell strips assayed.

For sample serum or plasmas which have been diluted 1:5 according to the protocol, the calculated concentration should be multiplied by the dilution factor (x5).

11. Assay limitations

Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

The influence of various drugs on end results has not been investigated. Bacterial or fungal contamination and laboratory cross-contamination may also cause irregular results.

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 Washing Buffer, fill with Washing Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

As with most biological assays conditions may vary from assay to assay therefore **a fresh standard curve must be prepared and run for every assay.**

12. Performance Characteristics

12.1. Sensitivity

The limit of detection of CD154 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 **0.06 ng/ml** (mean of 6 independent assays).

12.2. Specificity

The assay detects both natural and recombinant human CD154.
The cross reactivity and interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human CD154 positive sample.
There was no cross reactivity or interference.

12.3. Precision

Intra-assay
Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of CD154. Two standard curves were run on each plate. Data below show the mean CD154 concentration and the coefficient of variation for each sample. The overall intra-assay coefficient of variation has been calculated to be 4.0 %.

Positive Sample	Experiment	CD154 Concentration (ng/ml)	Coefficient of Variation (%)
1	1	14.8	1.6
	2	15.8	3.1
	3	15.0	1.4
2	1	12.6	0.5
	2	13.9	1.7
	3	13.3	5.8
3	1	10.9	1.3
	2	12.8	5.9
	3	11.8	3.9
4	1	10.2	2.2
	2	11.9	0.7
	3	11.1	3.4
5	1	7.3	0.8
	2	8.0	4.6
	3	7.4	5.1
6	1	6.6	5.9
	2	7.4	4.6
	3	7.0	8.3
7	1	4.9	5.9
	2	5.5	3.0
	3	4.7	2.6
8	1	2.9	13.6
	2	3.5	4.5
	3	2.9	4.8

Inter-assay
Assay to assay reproducibility within one laboratory was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 samples containing different concentrations of CD154. Two standard curves were run on each plate. Data below show the mean CD154 concentration and the coefficient of variation calculated on 18 determinations of each sample. The overall inter-assay coefficient of variation has been calculated to be 6.8 %.

Sample	CD154 Concentration (ng/ml)	Coefficient of Variation (%)
1	15.2	3.4
2	13.3	5.0
3	11.8	8.2
4	11.1	7.7
5	7.6	5.3
6	7.0	6.0
7	5.1	8.5
8	3.1	10.1

12.4. Dilution Parallelism

Four serum samples with different levels of CD154 were assayed at four serial two-fold dilutions (1:5-1:40) with 4 replicates each. Recoveries ranged from 100% to 113% **with an overall mean recovery of 105%.**

12.5. Spike Recovery

The spike recovery was evaluated by spiking four different levels of CD154 into normal human serum. Recoveries were determined in four independent experiments with 6 replicates each. The amount of endogenous CD154 in unspiked serum was subtracted from the spike values. Recoveries ranged from 78 to 112 % with an **overall mean recovery of 91 %.**

12.6. Stability

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 CD154 level determined after 24h. There was significant loss of CD154 immunoreactivity during storage at RT (57% immunoreactivity) and 37ºC (4% immunoreactivity).

Freeze-thaw Stability
Aliquots of serum samples (unspiked or spiked) were stored at -70°C and thawed up to 5 times, and CD154 levels determined. There was no significant loss of CD154 by freezing and thawing.

12.7. Normal Serum Values

A panel of 40 sera from healthy blood donors (males and females) was tested for CD154. The detected CD154 levels ranged between 0.03 and 3.98 ng/ml with a mean level of 2.13 ng/ml and a standard deviation of $\pm$1.0 ng/ml.

12.8. Comparison of Serum and Plasma

Serum as well as EDTA, citrate and heparin plasma obtained from 8 individuals at the same time point were evaluated. It clearly turned out that plasma preparations give results that do not correlate with the respective serum data.

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14. Assay Summary

Total procedure length : 2h10mn

Add diluted sample, diluted standard, blank and diluted HRP-conjugate to pre-coated plate

↓

Incubate 2 hours at room temperature

↓

Wash three times

↓

Add 100 µl of ready-to-use Substrate Solution
Protect from light. Let the color develop for 10 min.

↓

Add 100 µl Stop Solution

↓

Read Absorbance at 450 nm

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