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EZ-Set™ ELISA Kit (DIY Antibody Pairs)

Catalog number: EZ0702

For the development of sandwich ELISA kit to measure **Human CD40** concentrations in cell culture supernatants, serum, plasma (heparin, EDTA) , saliva, urine and human milk.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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Human CD40/TNFRSF5 ELISA Kit EZ-Set™ (DIY Antibody Pairs)

Catalog Number: EZ0702

For the development of sandwich ELISA kit to measure Human CD40 in cell culture supernatants, serum, plasma (heparin, EDTA) , saliva, urine and human milk.

This kit contains sufficient materials to run ELISAs on at least five 96 well plates, provided the following conditions are met:
- **The reagents are prepared as described in this package insert.**
- **The assay is run as described in the General ELISA Protocol.**
- **The recommended microplates, buffers, diluents, substrates, and solutions are used.**

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Overview

Size	5 plates/kit
Range	15.6 pg/ml - 1,000 pg/ml
Specificity	Natural and recombinant Human CD40
Immunogen	Expression system for standard: NS0; Immunogen sequence: E21-R193
Cross Reactivity	There is no detectable cross-reactivity with other relevant proteins.
Storage Instructions	Store at 4°C for 6 months, at -20°C for 12 months. Avoid multiple freeze-thaw cycles (Ships with gel ice, can store for up to 3 days in room temperature. Freeze upon receiving.)

Kit Components/Materials Provided

Catalog number	Description	Quantity	Storage of opened/reconstituted material
EZ0702-CA	Rabbit anti- human CD40 polyclonal antibody (Capture Antibody)	500 µl, 0.4 mg/mL (recommended dilution 1:100)	Store undiluted at 4°C for 1 month or at -20°C for 3 months provided this is within the expiration date of the kit.
EZ0702-DA	Biotinylated goat anti- human CD40 polyclonal antibody (Detection Antibody)	500 µl, 5 µg/mL (recommended dilution 1:100)	
AR1103	Avidin-Biotin-Peroxidase Complex (ABC)	500 µl (recommended dilution 1:100)	

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EK0702-ST	Lyophilized recombinant human CD40 standard	10 ng/tube×3	Discard the standard stock solution after 12 hours at 4°C. May be stored at -20°C for 48 hours provided this is within the expiration date of the kit.

Other Materials & Solutions Required But Not Provided

1. Microplate reader in standard size.
2. Automated plate washer.
3. Incubator.
4. Adjustable pipettes and pipette tips. Multichannel pipettes are recommended in the condition of large amount of samples in the detection.
5. Clean tubes and Eppendorf tubes.
6. 96 well microplate (Cat# AR1100)
7. Plate Sealers.
8. Capture Antibody Diluent: PBS.
9. Reagent Diluent: 1% BSA in PBS, pH 7.2-7.4, 0.2 um filtered.
10. Color Developing Reagent: Tetramethylbenzidine (Cat# AR1104)
11. Stop Solution: 2 N H2SO4 (Cat# AR1105)
12. Wash Buffer (PBS and PBS-T).

PBS: 8g NaCl, 0.2g KCl, 1.15g Na2HPO4, 0.2g KH2PO4, adjust the total volume to 1 L with distilled water, pH 7.2-7.4, 0.2 um filtered.

PBS-T: 0.1% Tween? 20 in PBS, pH 7.2-7.4.

*Item 6 – 12 are included in the EZ Set Accessory Kit (EZA001)

Preparation

Bring all reagents to room temperature before use. Working dilutions should be prepared and used immediately.

1. Plate Preparation

1) Dilute the Capture Antibody to the working concentration in 1:100 with Capture Antibody Diluent.(i.e. Add 1 µl anti-Human CD40 Capture Antibody into 99 µl Capture Antibody Diluent.) Immediately coat a 96-well microplate with 100 µl per well of the diluted Capture Antibody. Seal the plate and incubate overnight at 4°C.

2) Remove the cover and discard the liquid in the wells into an appropriate waste receptacle. Invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.

3) Block plates by adding 200 µl of Reagent Diluent to each well. Incubate at room temperature for 2 hours.

4) Aspirate each well and wash with **PBS**, repeating the process two times for a total of three washes. Wash by filling each well with **PBS** (300-350 µl) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining **PBS** by aspirating or by inverting the plate and blotting it against clean paper towels.**(Plate Washing Method)**

2. Reconstitution of Human CD40 standard

1) It is recommended that the standards be prepared no more than 2 hours prior to performing the experiment. Use one 10 ng of lyophilized human CD40 standard for each experiment. Gently spin the vial prior to use. Reconstitute the standard to a stock concentration of 10 ng/ml using 1 ml of Reagent Diluent. Allow the standard to sit for a minimum of 10 minutes with gentle agitation prior to making dilutions.

2) Dilution of Human CD40 Standard

• Number tubes 1 - 8. Final Concentrations to be Tube # 1 - 1,000 pg/ml, # 2 - 500 pg/ml, # 3 - 250 pg/ml, # 4 - 125 pg/ml, # 5 - 62.5 pg/ml, # 6 - 31.2 pg/ml, # 7 - 15.6 pg/ml, # 8 - 0.0 (Blank).

• To generate standard # 1, add 100 µl of the reconstituted standard stock solution of 10 ng/ml and 900 µl of Reagent Diluent to tube # 1 for a final

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volume of 1,000 µl. Mix thoroughly.
• Add 300 µl of Reagent Diluent to tubes # 2 - 7.
• To generate standard # 2, add 300 µl of standard # 1 from tube # 1 to tube # 2 for a final volume of 600 µl. Mix thoroughly.
• To generate standard # 3, add 300 µl of standard # 2 from tube # 2 to tube # 3 for a final volume of 600 µl. Mix thoroughly.
• Continue the serial dilution for tube # 4 - 7.
• Tube # 8 is a blank standard to be used with every experiment.

3. Preparation of Rabbit anti- human CD40 polyclonal antibody working solution
1) Each vial contains 500 µl of Rabbit anti- human CD40 polyclonal antibody.
2) Rabbit anti- human CD40 polyclonal antibody should be diluted in 1:100 with Capture Antibody Diluent and mixed thoroughly. (i.e. Add 1 µl Rabbit anti- human CD40 polyclonal antibody to 99 µl Capture Antibody Diluent.)

4. Preparation of Biotinylated goat anti- human CD40 polyclonal antibody working solution
1) Each vial contains 500 µl of Biotinylated goat anti- human CD40 polyclonal antibody.
2) Biotinylated goat anti- human CD40 polyclonal antibody should be diluted in 1:100 with Reagent Diluent and mixed thoroughly. (i.e. Add 1 µl Biotinylated goat anti- human CD40 polyclonal antibody to 99 µl Reagent Diluent.)

5. Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution
1) Each vial contains 500 µl of Avidin-Biotin-Peroxidase Complex (ABC).
2) Avidin-Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with Reagent Diluent and mixed thoroughly. (i.e. Add 1 µl ABC to 99 µl Reagent Diluent.)

Assay Protocol

It is recommended that all reagents and materials be equilibrated to room temperature (18-25°C) prior to the experiment (see Preparation Before The Experiment, if you have missed this information).

1. Prepare all reagents and working standards as directed previously.
2. Remove excess microplate strips from the plate frame and seal and store them in the original packaging.
3. Add 100 µl of the standard, samples, or control per well. At least two replicates of each standard, sample, or control is recommended.
4. Cover with the plate sealer provided and incubate for 120 minutes at room temperature (or 90 min. at 37 °C).
5. Remove the cover and discard the liquid in the wells into an appropriate waste receptacle. Invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.
6. Add 100 µl of the prepared 1x Biotinylated goat anti- human CD40 polyclonal antibody to each well.
7. Cover with a plate sealer and incubate for 90 minutes at room temperature (or 60 minutes at 37°C).
8. Wash the plate 3 times with **PBS**:
 a. Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.
 b. Add 300 µl of **PBS** to each assay well. (For cleaner background incubate for 60 seconds between each wash).
 c. Repeat steps a-b 2 additional times.
 d. Discard the wash buffer in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid.
9. Add 100 µl of the prepared 1x Avidin-Biotin-Peroxidase Complex into each well and incubate for 40 minutes at RT (or 30 minutes at 37°C).
10. Wash the plate 5 times with **PBS-T**:
 a. Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.
 b. Add 300 µl of **PBS-T** to each assay well. (For cleaner background incubate for 60 seconds between each wash).

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c. Repeat steps a-b 4 additional times.
d. Discard the wash buffer in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid.
11. Add 90 µl of Color Developing Reagent to each well and incubate in the dark for 30 minutes at RT (or 25-30 minutes at 37°C). (The optimal incubation time must be empirically determined. A guideline to look for is blue shading the top four standard wells, while the remaining standards remain clear.)
12. Add 100 µl of Stop Solution to each well. The color should immediately change to yellow.
13. Within 30 minutes of stopping the reaction, the O.D. absorbance should be read with a microplate reader at 450nm.

Data Analysis

Average the duplicate readings for each standard, sample, and control. Subtract the average zero standard O.D. reading.

It is recommended that a standard curve be created using computer software to generate a four-parameter logistic (4-PL) curve-fit. A free program capable of generating a four-parameter logistic (4-PL) curve-fit can be found online at: www.myassays.com/four-parameter-logistic-curve.assay.

Alternatively, plot the mean absorbance for each standard against the concentration. The measured concentration in the sample can be interpolated by using linear regression of each average relative O.D. against the standard curve generated using curve fitting software. This will generate an adequate but less precise fit of the data.

For diluted samples, the concentration reading from the standard curve must be multiplied by the dilution factor.

Background on CD40

CD40 is a cell surface receptor that is expressed on the surface of all mature B cells, most mature B-cell malignancies, and some early B-cell acute lymphocytic leukemias, but is not expressed on plasma cells. The CD40 gene is localized to the long arm of chromosome 20, bands q12-q13.2. The protein encoded by this gene is a member of the TNF-receptor superfamily. This receptor has been found to be essential in mediating a broad variety of immune and inflammatory responses including T cell-dependent immunoglobulin class switching, memory B cell development, and germinal center formation.

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