Introduction and Background

A. Overview

The mouse CD30L(CD153) gene is located at 9q33.1 It contains 4 exons and spans more than 17.1 kb.2 CD153 is expressed on the surface of B cells and this expression is upregulated upon CD154 (CD40LG), IL4, and B-cell receptor engagement.3 In these cells, engagement of CD153 by T cell CD30 inhibits immunoglobulin class switch recognition as well as IgG, IgA, and IgE production, suggesting that this 'reverse signaling' modulates the CD154-dependent switching of B cells into the pool producing IgG, IgA, and IgE. Additionally, recombinant mouse CD30L enhanced the proliferation of CD3 -activated T cells, but induced differential responses, including cell death, in several CD30-positive lymphoma-derived cell lines.4 The standard product used in this kit is recombinant mouse CD30L with the molecular mass of 30-45KDa.

B. Test Principle

The mouse CD30L ELISA Kit was based on standard sandwich enzyme-linked immune-sorbent assay technology. Mouse CD30L specific-specific monoclonal antibodies (clone No.82414) were precoated onto 96-well plates. The mouse specific detection polyclonal antibodies were biotinylated. The test samples and biotinylated detection antibodies were added to the wells subsequently and then followed by washing with PBS or TBS buffer. Avidin-Biotin-Peroxidase Complex was added and unbound conjugates were washed away with PBS or TBS buffer. HRP substrate TMB was used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the mouse CD30L amount of sample captured in plate.

C. Notice for Application of Kit

- ✓ This kit has been configured for research use only and is not for diagnostic and clinical use.
- ✓ Before using Kit, spin tubes and bring down all components to bottom of tube.
- ✓ Duplicate well assay was recommended for both standard and sample testing.
- ✓ Don't let 96-well plate dry, dry plate will inactivate active components on plate.
- ✓ In order to avoid marginal effect of plate incubation due to temperature difference (reaction may be stronger in the marginal wells), it is suggested that the diluted ABC and TMB solution will be pre-warmed in 37℃ for 30 min before using.

D. Application

For quantitative detection of mouse CD30L in sera, body fluids, tissue lysates or cell culture supernates.

Material and Method

A. List of component

1. Lyophilized recombinant mouse CD30L standard: 10ng/tube×2.
2. One 96-well plate precoated with anti- mouse CD30L antibody.
3. Sample diluent buffer: 30 ml
4. Biotinylated anti- mouse CD30L antibody : 130µl, dilution 1:100.
5. Antibody diluent buffer: 12ml.
6. Avidin-Biotin-Peroxidase Complex (ABC) : 130µl, dilution 1:100.
7. ABC diluent buffer: 12ml.
8. TMB color developing agent: 10ml.
9. TMB stop solution: 10ml.

B. Additional Required Materials But Not Provided

1. Microplate reader in standard size.
2. Automated plate washer.
3. Adjustable pipettes and pipette tips. Multichannel pipettes are recommended in the condition of large amount of samples in the detection.
4. Clean tubes and Eppendorf tubes.
5. Washing buffer (neutral PBS or TBS).
   Preparation of 0.01M TBS: Add 1.2g Tris, 8.5g Nacl; 450µl of purified acetic acid or 700µl of concentrated hydrochloric acid to 1000ml H2O and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.
   Preparation of 0.01 M PBS: Add 8.5g sodium chloride, 1.4g Na2HPO4 and 0.2g NaH2PO4 to 1000ml distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.

C. Preparation

Plate Washing

Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1~2 minutes. Repeat this process two additional times for a total of THREE washes.

Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the plate onto paper towels or other absorbent material.

Sample Preparation and Storage

Store samples to be assayed within 24 hours at 2-8℃. For long-term storage, aliquot and freeze samples at -20℃.

Avoid repeated freeze-thaw cycles.

- Cell culture supernate: Remove particulates by centrifugation, analyze immediately or aliquot and store at -20℃. For cell lysate, add lysis solution before centrifugation.
- Tissue lysate or body fluids: Remove particulates by centrifuging at approximately 2000 X g for 20 min.
- Serum: Allow the serum to clot in a serum separator tube (about 30 min) at room temperature. Centrifuge at approximately 2000 X g for 20 min. Analyze the serum immediately or aliquot and store frozen at -20℃.

Reagent Preparation and Storage

1. Reconstitution of the mouse CD30L standard: CD30L standard solution should be prepared no more than 2 hours prior to the experiment. Two tubes of CD30L standard (10ng per tube) are included in each kit. Use one tube for each experiment.
    a. 10,000pg/ml of mouse CD30L standard solution: Add 1 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
    b. 1000pg/ml of mouse CD30L standard solution: Add 0.1 ml of the above 10ng/ml CD30L standard solution into 0.9 ml sample diluent buffer and mix thoroughly.
    c. 500pg/ml→15.6pg/ml of mouse CD30L standard solutions: Label 6 Eppendorf tubes with 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml, 15.6pg/ml, respectively. Aliquot 0.3 ml of the sample diluent buffer into each tube. Add 0.3 ml of the above 10, 000pg/ml CD30L standard solution into 1st tube to 2nd tube and mix. Transfer 0.3 ml from 2nd tube to 3rd tube and mix, and so on.

    Note: The standard solutions are best used within 2 hours. The 10 ng/ml standard solution may be stored at 4℃ for up to 12 hours, or at -20℃ for up to 48 hours. Avoid repeated freeze-thaw cycles.
2. Prepamouseion of biotinylated anti-mouse CD30L antibody working solution: The solution should be prepared no more than 2 hours prior to the experiment.
    a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
    b. Biotinylated anti-mouse CD30L antibody should be diluted in 1:99 with the antibody diluent buffer and mixed thoroughly.
3. Prepamouseion of Avidin-Biotin-Peroxidase Complex (ABC) working solution: The solution should be prepared no more than 1 hour prior to the experiment.
    a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
    b. Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:99 with the ABC dilution buffer and mixed thoroughly.

Sample Dilution Guideline

The user needs to estimate the concentration of the target protein in the sample and select a proper dilution factor so that the diluted target protein concentration falls near the middle of the linear regime in the standard

curve. Dilute the sample using the provided diluent buffer. The following is a guideline for sample dilution. Several trials may be necessary in practice. The sample must be well mixed with the diluents buffer.

- High target protein concentration (10-100ng/ml). The working dilution is 1:100. i.e. Add 1 µl sample into 99 µl sample diluent buffer.
- Medium target protein concentration (1-10ng/ml). The working dilution is 1:10. i.e. Add 10 µl sample into 90 µl sample diluent buffer.
- Low target protein concentration (15.6-1000pg/ml). The working dilution is 1:2. i.e. Add 50 µl sample to 50 µl sample diluent buffer.
- Very Low target protein concentration (≤15.6pg/ml). No dilution necessary, or the working dilution is 1:2.

D. Stability and storage

Store at 4℃ for frequent use, at -20℃ for infrequent use. Avoid multiple freeze-thaw cycles (Shipped with wet ice.)

E. Expiration

Four months at 4℃ and eight months at -20℃.

F. Protocol

The ABC working solution and TMB color developing agent must be kept warm at 37℃ for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. Standard CD30L detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of CD30L amount in samples.

1. Aliquot 0.1ml per well of the 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml, 15.6pg/ml mouse CD30L standard solutions into the precoated 96-well plate. Add 0.1ml of the sample diluent buffer into the control well (Zero well). Add 0.1ml of each properly diluted sample of mouse sera, plasma, body fluids, tissue lysates or cell culture supernatants to each empty well. See “Sample Dilution Guideline” above for details. We recommend that each mouse CD30L standard solution and each sample is measured in duplicate.
2. Seal the plate with the cover and incubate at 37℃ for 90 min.
3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 0.1ml of biotinylated anti-mouse CD30L antibody working solution into each well and incubate the plate at 37℃ for 60 min.
5. Wash the plate three times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
6. Add 0.1ml of prepared ABC working solution into each well and incubate the plate at 37℃ for 30 min.

7. Wash plate 5 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material.
8. Add 90 µl of prepared TMB color developing agent into each well and incubate plate at 37℃ for 10-15 min (shades of blue can be seen in the wells with the four most concentrated mouse Survivin standard solutions; the other wells show no obvious color).
9. Add 0.1ml of prepared TMB stop solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution.

For calculation, (the relative O.D.450) = (the O.D.450 of each well) – (the O.D.450 of Zero well). The standard curve can be plotted as the relative O.D.450 of each standard solution (Y) vs. the respective concentration of the standard solution (X). The mouse EGF concentration of the samples can be interpolated from the standard curve.

Note: if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

Summary

1. Add samples and standards and incubate the plate at 37℃ for 90 min. Do not wash.
2. Add biotinylated antibodies and incubate the plate at 37℃ for 60 min. Wash plate 3 times with 0.01M TBS.
3. Add ABC working solution and incubate the plate at 37℃ for 30 min. Wash plate 5 times with 0.01M TBS.
4. Add TMB color developing agent and incubate the plate at 37℃ for 20-25 min.
5. Add TMB stop solution and read.

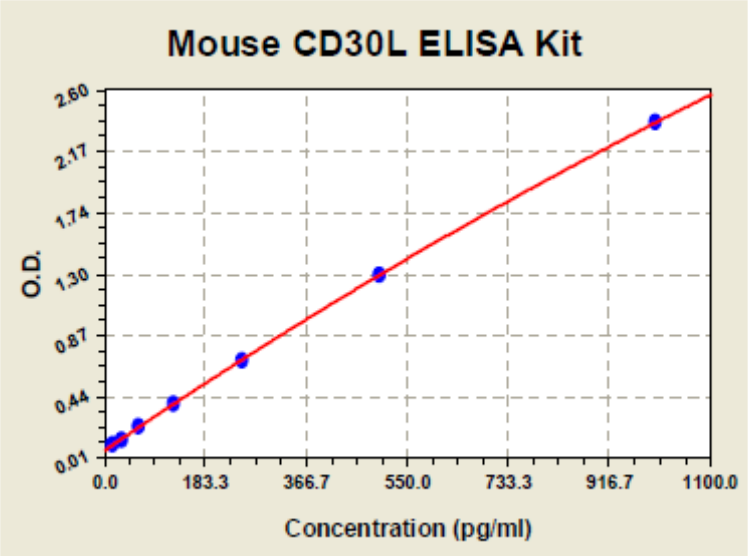
G. Performance Characteristics

Typical result

Typical Data Obtained from Mouse CD30L

Concentration (pg/ml)	0.0	15.6	31.3	62.5	125	250	500	1000
O.D.	0.064	0.102	0.143	0.232	0.393	0.699	1.301	2.369

(TMB reaction incubate at 37℃ for 16 min)



This standard curve was generated for demonstration purpose only. A standard curve must be run with each assay.

Range

15.6pg/ml-1000pg/ml

Sensitivity

< 1 pg/ml

Specificity

No detectable cross-reactivity with any other cytokine.

Reference:

1. Smith, C. A.; Gruss, H.-J.; Davis, T.; Anderson, D.; Farrah, T.; Baker, E.; Sutherland, G. R.; Brannan, C. I.; Copeland, N. G.; Jenkins, N. A.; Grabstein, K. H.; Gliniak, B.; and 9 others : CD30 antigen, a marker for Hodgkin's lymphoma, is a receptor whose ligand defines an emerging family of cytokines with homology to TNF. Cell 73: 1349-1360, 1993.
2. Croager, E. J.; Abraham, L. J. : Characterisation of the mouse CD30 ligand gene structure. Biochim. Biophys. Acta 1353: 231-235, 1997.
3. Cerutti, A.; Schaffer, A.; Goodwin, R. G.; Shah, S.; Zan, H.; Ely, S.; Casali, P. : Engagement of CD153 (CD30 ligand) by CD30-positive T cells inhibits class switch DNA recombination and antibody production in mouse IgD-positive IgM-positive B cells. J. Immun. 165: 786-794, 2000.
4. Smith, C. A.; Gruss, H.-J.; Davis, T.; Anderson, D.; Farrah, T.; Baker, E.; Sutherland, G. R.; Brannan, C. I.; Copeland, N. G.; Jenkins, N. A.; Grabstein, K. H.; Gliniak, B.; and 9 others : CD30 antigen, a marker for Hodgkin's lymphoma, is a receptor whose ligand defines an emerging family of cytokines with homology

to TNF. Cell 73: 1349-1360, 1993