CELL APPLICATIONS, INC.

Mouse L-Selectin ELISA Kit

Cat. No. CL0504
96-wells

Target Protein Species	Mouse
Range	62.5 pg/ml – 4000 pg/ml
Specificity	No detectable cross-reactivity with any other cytokine.

COMPONENTS

Kit Component	Amount
96-well plate precoated with anti-mouse L-Selectin antibody	1 Plate
Protein Standard: Lyophilized recombinant mouse L-Selectin	2 tubes, 10 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-mouse L-Selectin)	130 µl (100x)
Antibody Diluent Buffer	12ml
Avidin-Biotin-Peroxidase Complex (ABC) Solution	130 µl (100x)
ABC Diluent Buffer	12 ml
Tetramethylbenzidine (TMB) Color Developing Agent	10 ml
TMB Stop Solution	10 ml

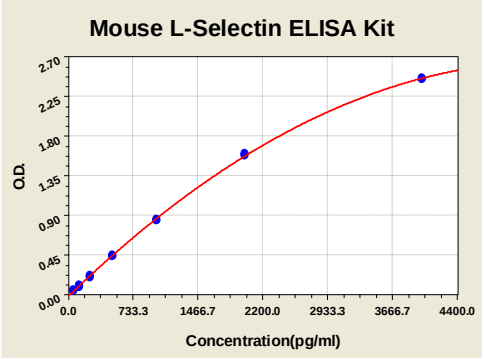
Washing Buffer (not provided): TBS or PBS

0.01M TBS: Add 1.2g Tris, 8.5g NaCl; 450µl of purified acetic acid or 700µl of concentrated hydrochloric acid to 900ml H_2O and adjust pH to 7.2-7.6. Adjust the total volume to 1L.

0.01M PBS: Add 8.5g sodium chloride, 1.4g Na_2HPO_4 and 0.2g NaH_2PO_4 to 900ml distilled H_2O and adjust pH to 7.2-7.6. Adjust the total volume to 1L.

Storage

Store at 4°C. Cell Applications, Inc. recommends using the kit within 6 months of order.



X	pg/ml	0.0	62.5	125	250	500	1000	2000	4000
Y	O.D.450	0.002	0.051	0.104	0.213	0.445	0.852	1.598	2.455

Figure 1: L-Selectin Standard Curve. Using the Mouse L-Selectin ELISA Kit, O.D. data was graphed against L-Selectin protein concentration. The TMB reaction was incubated at 37°C for 23 min.

BACKGROUND

The selectin family of adhesion molecules mediates the initial attachment of leukocytes to venular endothelial cells before their firm adhesion and diapedesis at sites of tissue injury and inflammation. The selectin family consists of three closely related cell-surface molecules with differential expression by leukocytes (L-selectin), platelets (P-selectin), and vascular endothelium (E- and P-selectin). The selectins have characteristic extracellular regions composed of an amino-terminal lectin domain that binds a carbohydrate ligand, an epidermal growth factor-like domain, and two to nine short repeat units homologous to domains found in complement binding proteins. In contrast to most other adhesion molecules, selectin function is restricted to leukocyte interactions with vascular endothelium. Multiple studies indicate that the selectins mediate neutrophil, monocyte, and lymphocyte rolling along the venular wall. The generation of selectin-deficient mice has confirmed these findings and provided further insight into how the overlapping functions of these receptors regulate inflammatory processes. Selectin-directed therapeutic agents are now proven to be effective in blocking many of the pathological effects resulting from leukocyte entry into sites of inflammation.[1]

L-selectin (CD62L) is a cell adhesion molecule consisting of a large, highly glycosylated, extracellular domain, a single spanning transmembrane domain and a small cytoplasmic tail. It is expressed on most leukocytes and is involved in their rolling on inflamed vascular endothelium prior to firm adhesion and transmigration. It is also required for the constitutive trafficking of lymphocytes through secondary lymphoid organs. Like most adhesion molecules, L-selectin function is regulated by a variety of mechanisms including gene transcription, post-translational modifications, association with the actin cytoskeleton, and topographic distribution. In addition, it is rapidly downregulated by proteolytic cleavage near the cell surface by ADAM-17 (TACE) and at least one other “sheddase”. This process of “ectodomain shedding” results in the release of most of the extracellular portion of L-selectin from the cell surface while retaining the cytoplasmic, transmembrane, and eleven amino acids of the extracellular domain on the cell.[2] In addition, The activation of leukocytes by bacterial cell-wall lipopolysaccharide contributes to the pathogenesis of septic shock. It was suggested that in neutrophils, and possibly other leukocytes, L-selectin can act as a low-affinity lipopolysaccharide receptor. Inhibitors of L-selectin may therefore be of therapeutic value in treating this life-threatening condition.[3]

ELISA OVERVIEW

Cell Applications ELISA Kits are based on standard sandwich enzyme-linked immunosorbent assay technology. Freshly prepared standards, samples, and solutions are recommended for best results.

1. Prepare test samples.
2. Prepare a protein standard of the target protein.
3. Add test samples and standards to the pre-coated 96-well plate. Do not wash.
4. Add biotinylated detection antibodies. Wash.
5. Add Avidin-Biotin-Peroxidase Complex (ABC) Solution. Wash.
6. Add Tetramethylbenzidine (TMB) Color Developing Agent, containing HRP substrate.
7. Add TMB Stop Solution
8. Subject the plate to analysis.

NOTES:

- Before using the kit, quick spin tubes to bring down all solution to the bottom of tube.
- Duplicate assay wells are recommended for both standard and sample testing.
- Do not let the 96-well plate dry, this will lead to inactivation of plate components.
- When diluting samples and reagents, ensure that they are mixed completely and evenly.
- Pre-warm diluted ABC and TMB solutions at 37°C for 30 min before use to avoid variable temperature effects.
- For washes, use TBS or PBS. Do not touch well walls.
- A protein standard is included in the kit. A protein standard detection curve should be generated with each experiment, no more than 2 hours prior to the experiment.
- The user will determine sample dilution fold by estimation of target protein amount in samples.

References

1. Rosen, S.D. et al: Ann. Rev. Immunol. 22:129-56, 2003
2. Smalley, D.M. & Ley, K.: J. Cell. Mol. Med. 9:255-66, 2005
3. Malhotra, R. & Bird, B.I.: Chem. Biol. 4:543-7, 1997

PROTOCOL

I. 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.

II. Preparation of Test Samples

Test Sample Processing

- **Cell culture supernate, tissue lysate or body fluids**: Remove particulates by centrifugation.
- **Serum**: Allow the serum to clot in a serum separator tube (about 2 hours) at room temperature. Centrifuge at approximately 1000 X g for 10 min.
- **Plasma**: Collect plasma using EDTA as an anticoagulant. Centrifuge for 20 min at 2000 x g within 30 min of collection. Analyze immediately or aliquot and store frozen at -20°C. Heparin and citrate are not recommended as the anticoagulant.

Sample Dilution Guideline

Estimate the concentration of the target protein in the sample and select a proper dilution factor such that the diluted target protein concentration falls within the standard curve range. Depending on the sample, several trial dilutions may be necessary. Dilute the sample using the provided diluent buffer, mixing well. Suggested working dilutions of samples are as follows:

Target Protein Concentration Range	Sample Working Dilution	Sample Vol.	Diluent Buffer Vol.
40-400 ng/ml	1:100	1 µl	99 µl
4-40 ng/ml	1:10	10 µl	90 µl
62.5-4000 pg/ml	1:2	50 µl	50 µl
≤62.5 pg/ml	n/a	100µl	n/a

If samples will be assayed within 24 hours, store at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

III. Preparation of Reagents

Reconstitution of the Standard

The standard solutions should be prepared no more than 2 hours prior to the experiment. Two tubes of the standard are included in each kit. Use one tube for each experiment.

1. 20,000pg/ml of mouse L-Selectin standard solution: Add 1 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
2. 4000pg/ml of mouse L-Selectin standard solution: Add 0.2 ml of the above 10ng/ml L-Selectin standard solution into 0.8 ml sample diluent buffer and mix thoroughly.
3. 2000pg/ml→62.5pg/ml of mouse L-Selectin standard solutions: Label 6 Eppendorf tubes with 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, respectively. Aliquot 0.3 ml of the sample diluent buffer into each tube. Add 0.3 ml of the above 4000pg/ml L-Selectin standard solution into 1st tube and mix. Transfer 0.3 ml from 1st tube to 2nd tube and mix. Transfer 0.3 ml from 2nd tube to 3rd tube and mix, and so on.

Preparation of Biotinylated Antibody Working Solution

The solution should be prepared no more than 2 hours prior to the experiment.

1. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
2. Biotinylated antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly.

Preparation of the Avidin-Biotin-Peroxidase Complex (ABC) Working Solution

The solution should be prepared no more than 1 hour prior to the experiment.

1. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
2. Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly.

IV. ELISA

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

1. Aliquot 0.1ml per well of the 4000pg/ml, 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml mouse L-Selectin standard solutions into the precoated 96-well plate. Add 0.1ml of the sample diluent buffer into the control well (Blank 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"** for details. We recommend that each mouse L-Selectin standard solution and each sample is measured in duplicate.
2. Seal the plate with the cover and incubate at 37°C 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 L-Selectin antibody working solution into each well and incubate the plate at 37°C for 60 min.
5. Wash plate 3 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. （Plate Washing Method: 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.）
6. Add 0.1ml of prepared ABC working solution into each well and incubate the plate at 37°C 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. (See Step 5 for plate washing method).
8. Add 90 µl of prepared TMB color developing agent into each well and incubate plate at 37°C in dark for 15-20 min (Note: For reference only, the optimal incubation time should be determined by end user. And the shades of blue can be seen in the wells with the four most concentrated mouse L-Selectin 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.

V. Calculating Protein Concentration

- For all wells, determine O.D.450(Relative):
  O.D.450(Relative) = O.D.450(Reading) – O.D.450(Blank)

- Plot the standard curve:
  Plot O.D.450(Relative) of each standard solution (Y) vs. the respective concentration of the standard solution (X). See **Figure 1** for a typical standard curve.

- The target protein concentration in samples can be interpolated from the standard curve. Multiply the interpolated concentration by the dilution factor to obtain the target protein concentration in the sample.