Human L-Selectin ELISA Kit

Cat. No. ECM331

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Background Information

Leukocyte-Endothelial Cell Adhesion Molecule-1, L-selectin (LECAM-1, MEL-14, LAM-1, LEU-8, TQ1, LEC.CAM-1, DREG.56) belongs to the selectin family of adhesion molecules [1,2]. Together with ELAM-1 (E-selectin) and GMP-140 (P-selectin), it mediates the initial interactions of leukocytes with endothelial cells [3].

The extracellular part of all selectins consists of an aminoterminal c-type lectin domain, which specifically binds to carbohydrate ligands. This is followed by an EGF-like domain, and in the case of L-selectin, by 2 short consensus repeats similar to the short consensus units in complement regulatory proteins. The transmembrane portion of the molecule is followed by a short cytoplasmic tail.

Selectins guide non-activated polymorphonuclear cells (PMN's) to areas of inflammation by creating initial, loose contacts with the endothelial layer. In particular, L-selectin mediates rolling of PMN's on endothelial cells [4]. The potential ligands of L-selectin carry a negative charge, probably sialic acid and/or sulphate, and may contain mannose and fucose [5,6]. In addition, L-selectin may also interact with E-selectin expressed on cytokine-activated endothelial cells. L-selectin is constitutively expressed on most leukocytes (PMN's, monocytes, lymphocyte subsets) in a seemingly functional form [3,7]. It is required as a receptor for recirculating lymphocytes at peripheral lymph nodes and for the invasion of neutrophils into sites of inflammation [6]. Activated neutrophils shed L-selectin by proteolytic cleavage near the transmembrane region [8]. Lymphocytes and monocytes can also shed L-selectin upon activation although the kinetics are significantly slower. A broad range of activating agents including C5a, fMLP, TNF, GM-CSF and IL-8 can induce shedding of L-selectin by lymphocytes and monocytes [9,10]. The shed form of L-selectin (soluble L-selectin) is functionally active and at high concentrations can inhibit leukocyte attachment to endothelium [11]. The main source for soluble L-selectin in serum appears to be tissue-localized leukocytes.

Determination of circulating L-selectin may provide insights into various pathologies [11,12], including allergy [13], venous thrombosis [14], HIV infection [11], insulin-dependent diabetes mellitus [15], B-cell malignancies [16], neonatal bacterial infection [17] and sepsis [11,18].

Test Principle

An anti-L-selectin monoclonal antibody is adsorbed onto microwells.

Soluble L-selectin present in a sample or standard then binds to antibodies adsorbed to the microwells. A second, HRP-conjugated monoclonal anti-L-selectin antibody is added and binds to L-selectin captured by the first antibody.

Unbound enzyme-conjugated anti-L-selectin is removed with a wash step and HRP substrate solution is added to the wells.

An amount of colored product is formed, proportional to the amount of soluble L-selectin present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from seven L-selectin standard dilutions and the L-selectin sample concentration is determined.

Application

The L-selectin ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of soluble Leukocyte-Endothelial Cell Adhesion Molecule-1 levels in cell culture supernatants, human serum, plasma, amniotic fluid, or other body fluids. **The L-selectin ELISA is for research use only. Not for use in diagnostic or therapeutic procedures.**

Analytical Sensitivity and Detection Limits

Sensitivity:	0.198 ng/mL
Range of Detection:	0.4 - 25 ng/mL
Intra-Assay Variation:	3.7%
Inter-Assay Variation:	4.2%
Recovery:	89% average
Assay Time	135 minutes
Cross reactivity:	no interference from soluble ICAM-1, TNF-α, TNF-β, TNF-R, CD8, IL-2, IL-2R, IL-6, IL-6R, IL-8, IL-10, E-selectin, CD44 and HER-2.

Kit Components

- 1 aluminum pouch containing a plate coated **with a Monoclonal Antibody** (murine) to human L-Selectin coated plate
- 1 vial (6 mL) **HRP-Conjugated** Anti-L-Selectin Monoclonal (murine) Antibody, ready to use
- 2 vials (50 ng each) **soluble L-Selectin Standard**, lyophilized*
- 1 bottle (50 mL) **Wash Buffer Concentrate** 20X (PBS with 1% Tween 20)*
- 1 bottle (50 mL) **Sample Diluent** (buffered protein matrix)
- 1 vial (15 mL) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (12 mL) **Stop Solution** (1M Phosphoric Acid)
- 2 **Adhesive Plate Covers**

* Reagents containing 0.01% thimerosal as preservative

Materials Required But Not Provided

- 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)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wavelength)
- Glass-distilled or deionized water
- Statistical calculator with program to perform linear regression analysis.

Precautions

- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Some reagents contain thimerosal as preservative, which is highly toxic by inhalation, ingestion, or contact with skin. Thimerosal is a possible mutagen and should be handled accordingly.
- Avoid contact of substrate solutions with oxidizing agents and metal.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.
- Exposure to acids will inactivate the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solutions must be at room temperature prior to use.

Sample Collection

Cell culture supernatants, human serum, EDTA-treated or heparinized plasma or other biological samples will be suitable for use in the assay. Remove serum or plasma 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 frozen at -20°C to avoid loss of bioactive L-selectin. If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Prior to assay, frozen sera or plasma should be brought to room temperature slowly and gently mixed by hand. Avoid repeated freeze-thaw cycles.

Sample Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the L-selectin level determined after 24 hours. There was no significant loss of L-selectin immunoreactivity during storage.

Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored frozen at -20°C, thawed repeatedly, and L-selectin levels determined. There was no significant loss of L-selectin after 5 cycles of freezing and thawing.

Preparation of Reagents

All reagents except for the L-selectin Standard (reagent B) should be prepared in advance before starting the test procedure.

A. 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** 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. The pH of the final solution should be 7.4.

Transfer to a clean wash bottle and store between 2° and 25°C. Please note that the Wash Buffer is stable for 30 days. Wash Buffer may be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	25	475
7 - 12	50	950

B. Preparation of sL- selectin Standard

The lyophilized standard must be solubilized by addition of distilled water. Reconstitution volume is stated on the vial label. Swirl or mix gently to insure complete solubility Allow the standard to reconstitute for 10-30 minutes. Mix well prior to use.

C. Stop Solution (1M Phosphoric Acid)

Stop Solution is provided ready to use.

Rubber or disposable gloves and protective glasses should be worn while handling Phosphoric Acid.

Storage of Kit Components

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiration date of the kit and reagents is stated on labels.

Assay Instructions

a. Mix all reagents thoroughly before use. Avoid foaming.

b. Dilute serum or plasma samples 1:100 with **Sample Diluent** according to one of the following dilution schemes:
   I. 5 µL Sample + 495 µL Sample Diluent, alternatively,
   II. 10 µL Sample + 90 µL Sample Diluent, take 50 µL of this 1:10 diluted sample and add 450 µL Sample Diluent.

c. Determine the number of Microwell Strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank, and optional control sample should be assayed in duplicate. Remove sufficient **Microwell Strips coated with Monoclonal Antibody** (murine) to human L-selectin from their aluminum pouches immediately prior to use. Load them into the 96 microwell strip holder making sure to place the first microwell strip into row 1.

d. Wash the microwell strips twice with approximately 300 - 400 µL **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.

   After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

e. Add 100 µL of **Sample Diluent** in duplicate to all standard wells. Prepare standard dilutions by pipetting 100 µL of **soluble L-selectin Standard,** in duplicate, into wells A1 and A2. Mix the contents by repeated aspiration and ejection, and transfer 100 µL to wells B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Repeat this procedure five times, creating two rows of L-selectin Standard dilutions ranging from 25 to 0.4 ng/mL. Discard 100 µL of the contents from the last microwells used (G1, G2).

f. Add 100 µL of **Sample Diluent** in duplicate to the blank wells.

g. Add 50 µL of **Sample Diluent** to all wells designated for samples.

h. Add 50 µL of each 1:100-diluted **Sample**, in duplicate, to the designated wells and mix contents.

i. Prepare **HRP Conjugate**. (Refer to preparation of reagents)

j. Add 50 µL of HRP **Conjugate** to all wells, including the blank wells.

k. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 2 hours, preferably on a rotator set at 100 rpm.

l. Remove Plate Cover and empty wells. Wash microwell strips 3 times according to point d of the test protocol. Proceed immediately to the next step.

m. Pipette 100 µL of mixed **TMB Substrate Solution** to all wells, including the blank wells.

n. Incubate the microwell strips at room temperature (18° to 25°C) for approximately 10 minutes, preferably on a rotator set at 100 rpm. Avoid direct exposure to intense light. The point at which the substrate reaction is stopped is often determined by the ELISA reader being used. Many ELISA readers record absorbance only up to 2.0 O.D. **Therefore the color development within individual microwells must be watched by the person running the assay and the substrate reaction stopped before positive wells are no longer properly recordable.**

o. Stop the enzyme reaction by quickly pipetting 100 µL of **Stop Solution** into each well, including the blank wells. It is important that the Phosphoric Acid is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Phosphoric Acid is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.

p. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wavelength (optionally 620 nm as the reference wavelength; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the L-selectin standards.

Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 percent of the mean.

- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the soluble L-selectin concentration on the abscissa. Draw a best fit curve through the points of the graph.

- To determine the concentration of circulating L-selectin for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding L-selectin concentration.

- **For samples, which have been diluted 1:200, the concentration read from the standard curve must be multiplied by the dilution factor (x200).**

  Note: *Calculation of samples with a concentration. Exceeding standard 1 may result in incorrect, low L-selectin levels. Such samples require further dilution with Sample Diluent in order to precisely quantitate the actual L-selectin level.*

It is suggested to run a control sample of known L-selectin concentration with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.

Typical data using the soluble L-selectin ELISA
Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	L-selectin Conc. (ng/mL)	O. D. (450 nm)	O. D. Mean	C. V. (%)
1	25	2.273	2.228	2.0
	25	2.183		
2	12.5	1.207	1.189	1.5
	12.5	1.172		
3	6.3	0.489	0.465	0.8
	6.3	0.440		
4	3.2	0.174	0.166	4.9
	3.2	0.158		
5	1.6	0.081	0.078	2.9
	1.6	0.076		
6	0.8	0.039	0.038	2.0
	0.8	0.037		
7	0.4	0.024	0.024	0
	0.4	0.024		
Blank	0	0.015	0.015	3.4
	0	0.014		

Technical Hints

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.

- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.

- 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. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

- Any human anti-mouse IgG antibody (HAMA) present may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. HAMA interference can be reduced by adding murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) to the Sample Diluent.

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TEST PROTOCOL SUMMARY

1. Dilute samples in **Sample Diluent**.
2. Wash microwell strips twice with **Wash Buffer**.
3. Add 100 µL **Sample Diluent**, in duplicate, to all standard wells.
4. Pipette 100 µL solubilized **L-selectin Standard** in duplicate into the first standard wells and create standard dilutions ranging from 25 to 0.4 ng/mL by transferring 100 µL from well to well; Discard 100 µL from the last wells. Add 100 µL **Sample Diluent**, in duplicate, to the blank wells.
5. Add 50 µL **Sample Diluent**, in duplicate, to sample wells.
6. Add 50 µL diluted Sample, in duplicate, to designated wells.
7. Prepare **HRP-Conjugate**.
8. Add 50 µL diluted **HRP-Conjugate** to all wells.
9. Cover microwell strips and incubate 2 hours at room temperature (18º to 25ºC).
10. Empty and wash microwell strips 3 times with Wash Buffer.
11. Add 100 µL of **TMB Substrate Solution** to all wells including blank wells.
12. Incubate the microwell strips for approximately 15 minutes at room temperature (18º to 25ºC).
13. Add 100 µL **Stop Solution** to all wells including blank wells.
14. Blank microwell reader and measure color intensity at 450 nm.