Human E-Selectin ELISA Kit

Cat. No. ECM330

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

USA & Canada Phone: +1(800) 437-7500 Fax: +1-800-437-7502
www.millipore.com

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Background Information

E-selectin (Endothelial Leukocyte Adhesion Molecule-1, ELAM-1) belongs to the selectin family of adhesion molecules [1,2]. Together with LECAM-1 (L-selectin) and GMP-140 (P-selectin), E-selectin mediates the initial interactions of leukocytes and platelets with endothelial cells [3,4].

Molecular structure: 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 E-selectin, by 6 short consensus repeats. The transmembrane portion of the molecule is followed by a short cytoplasmic tail.

Selectins provide the first, loose contacts of polymorphonuclear cells with the endothelium in areas of inflammation. The binding partner for E-selectin contains sialyl LewisX oligosaccharide or lactosaminoglycans that contain either sialic acid or fucose [5,6]. E-selectin is expressed on cytokine-activated endothelial cells, and promotes the adhesion of leukocytes to the endothelium. This initial binding event is a prerequisite for the activation of the immune cells via inflammatory mediators [7,8]. In contrast to GMP-140, E-selectin is maximally expressed 2-4 hours after cell activation. During the following 24-48 hours E-selectin is shed from the cytoplasmic membrane into the circulation [9]. The circulating form of this selectin attracts neutrophils and activates the β2-integrins in preparing the cells for migration.

Soluble E-selectin levels could provide insights into several pathologies, including allergic reactions [10-12], septic shock [13-15], vascular infection and inflammatory bowel disease [16].

Test Principle

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

The pair of monoclonal antibodies used in this ELISA detects the soluble form of E-Selectin present in serum, plasma, urine, and other biological fluids.

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

Unbound enzyme-conjugated anti-E-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 E-selectin 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 E-selectin standard dilutions and the E-selectin sample concentration is determined.

Application

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

Analytical Sensitivity and Detection Limits

Sensitivity:	0.33 ng/mL
Range of Detection:	0.8 - 50 ng/mL
Intra-Assay Variation:	5.4%
Inter-Assay Variation:	6.0%
Recovery:	86% average
Assay Time:	135 minutes
Crossreactivity:	no interference from sICAM-1, sTNF-R, CD8, IL-2R, IL-6, IL-8, TNF-β, sL-selectin, or sP-selectin.

Kit Components

- 1 aluminum pouch with a Microwell Plate coated with Monoclonal Antibody (murine) to human E-Selectin
- 1 vial (150 µL) HRP-Conjugated Anti-E-Selectin Monoclonal (murine) Antibody
- 2 vials (100 ng/mL each, reconstituted) soluble E-selectin Standard, lyophilized
- 1 vial **Control (high),** lyophilized
- 1 vial **Control (low)**, lyophilized
- 1 bottle (50 mL) Wash Buffer Concentrate 20X (PBS with 1% Tween 20)
- 1 vial (5 mL) Assay Buffer Concentrate 20X (PBS with 1% Tween 20 and 10% BSA)
- 1 bottle (12 mL) Sample Diluent (buffered protein matrix)

- 1 vial (15 mL) Substrate Solution
- 1 vial (15 mL) Stop Solution (1M Phosphoric Acid)
- 1 Microwell Strip Holder
- 2 Adhesive Plate Covers

Materials Not Supplied

- 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.
- 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 E-selectin. If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles.

Sample Stability

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

Freeze-Thaw Stability

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

Preparation of Reagents

All reagents except for HRP-Conjugate (reagent C) 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
1 - 12	50	950

B. Assay Buffer

Mix the contents of the bottle well. Add contents of Assay Buffer Concentrate (5.0 mL) to 95 mL distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days. Assay Buffer may be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	2.5	47.5
1 - 12	5.0	95.0

C. Preparation of HRP-Conjugate

Just prior to use, make a 1:100 dilution with Assay Buffer (reagent B) in a clean plastic tube as needed according to the following table:

Number of Strips	HRP-Conjugate (mL)	Assay Buffer (mL)
1 - 6	0.03	2.97
7 - 12	0.06	5.94

Note: *the HRP-Conjugate should be used within 30 minutes after dilution.*

D. Preparation of E-selectin Standard

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

E. Preparation of Controls

Reconstitute the positive controls by adding 200 uL distilled water. Swirl or mix gently to ensure complete and homogeneous solublization. Treat the controls like a sample in the assay. For control range, please refer to the vial label.Store reconstituted controls aliquots at -20ºC and avoid multiple freeze/thaw cycles.

F. Stop Solution (1M Phosphoric Acid)

Rubber or disposable gloves and protective glasses should be worn while handling phosphoric acid.

Phosphoric acid stop solution is provided at a ready-to-use concentration. No further preparation is required.

Storage of Kit Components

Store kit reagents between 2° and 8°C. Store the lyophilized control at -20°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C) with the exception of reconstituted control (-20ºC**)**. Expiration date of the kit and reagents is stated on the kit box.

Assay Instructions

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

b. 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 controls should be assayed in duplicate. Remove sufficient Microwell Strips coated with Monoclonal Antibody to human E-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.

c. Wash the microwell strips twice with approximately 300 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.

d. Add 100 µL of Sample Diluent in duplicate to all standard wells. Prepare standard dilutions by pipetting 100 µL of soluble E-selectin Standard**,** in duplicate, into wells A1 and A2. Transfer 100 µL to wells B1 and B2, respectively. Mix the contents by repeated aspiration and ejection, and transfer 100 µL to wells C1 and C2, respectively. Take care not to scratch the inner surface of the microwells. Repeat this procedure, creating two rows of E-selectin Standard dilutions ranging from 50 to 0.8 ng/mL. Discard 100 µL of the contents from the last microwells (G1, G2) used.

e. Add 100 µL of Sample Diluent to all blank wells.

f. Add 80 µL of Sample Diluent to all sample wells.

g. Add 20 µL of each Sample, in duplicate, to the designated wells and mix.

h. Prepare the HRP Conjugate. (Refer to preparation of reagents)

i. Add 50 µL of diluted (1:100) HRP Conjugate to each well, including the blank wells.

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

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

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

m. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 minutes, if available 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 color development within individual microwells must be monitored and the substrate reaction stopped before positive wells are no longer properly recordable.

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

o. 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 E-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 E-selectin concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating E-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 E-selectin concentration.
- For samples, which have been diluted 1:5, the concentration read from the standard curve must be multiplied by the dilution factor (x5).

  Note: *Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low E-selectin levels. Such samples require further dilution with Sample Diluent in order to precisely quantitate the actual E-selectin level.*

It is suggested to run a control sample of known E-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 E-selectin ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

Standard	E-selectin Concentration (ng/mL)	O. D. (450 nm)	O. D. Mean	C. V. (%)
1	50	2.045	1.991	3.9
	50	1.936		
2	25	1.018	0.969	7.3
	25	0.919		
3	12.5	0.471	0.456	4.7
	12.5	0.441		
4	6.3	0.230	0.222	5.6
	6.3	0.213		
5	3.1	0.109	0.106	4.3
	3.1	0.103		
6	1.6	0.058	0.056	6.0
	1.6	0.054		
7	0.8	0.032	0.034	10.9
	0.8	0.036		
Blank	0	0.008	0.008	0.0
	0	0.008		

Limitations

- 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. Wash microwell strips twice with Wash Buffer.
2. Add 100 µL Sample Diluent, in duplicate, to all standard wells except the first wells (A1 and A2).
3. Pipette 100 µL solublized E-selectin Standard in duplicate into the first standard wells and create standard dilutions ranging from 50 to 0.8 ng/mL by transferring 100 µL from well to well; Discard 100 µL from the last wells.
4. Add 100µL Sample Diluent to the blank wells.
5. Add 80 µL Sample Diluent, to sample wells.
6. Add 20 µL 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 25º C).
10. Empty and wash microwell strips 3 times with Wash Buffer.
11. Add 100 µL of mixed TMB Substrate Solution to all wells including blank wells.
12. Incubate the microwell strips for approximately 15 minutes at room temperature (18º to 25º C).
13. Add 100 µL 1M Phosphoric Acid Stop Solution to all wells including blank wells.
14. Blank microwell reader and measure color intensity at 450 nm.

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