Astarte Biologics, LLC

コスモ・バイオ株式会社

HUVEC/E-Selectin ELISA Kit Cat#1016

ELISA Assay Kit to measure human E-Selectin on Human Umbilical Vein Endothelial Cells (HUVEC).

Introduction

HUVEC are derived from normal human umbilical cords and are used to investigate endothelial physiology such as vasculogenesis and pathologic conditions such as atherosclerosis and inflammation. They are also used for high throughput screening for drug discovery.

HUVEC respond to endotoxin and cytokine stimulation by expressing cell adhesion molecules such as VCAM, ICAM and Selectins. Adhesion molecules mediate the attachment of Neutrophils and polymorphonuclear leukocytes (PMNLs) to the vascular endothelium and mitigate their extravasation to sites of inflammation in the connective tissue. E-Selectin (Endothelial Leukocyte Adhesion Molecule-1) is also called ELAM-1 or CD62E and is a 115 kDa, type-I transmembrane glycoprotein expressed only on activated endothelial cells. The expression of E-Selectin is maximally reached after approximately 6 hours of stimulation with proinflammatory cytokines such as TNF-α and IL-1B and *E.coli* LPS. The extracellular portion of E-Selectin contains six-complement-like repeats, a calcium-dependent C2-type lectin domain, and an epidermal growth factor domain. E-Selectin binds sialyl Lewis X.

Principle of the Assay

This assay measures membrane-bound E-Selectin expressed on stimulated HUVEC using enzyme-linked immunosorbent assay (ELISA). After stimulation of HUVEC (with *E.coli* LPS, for example), the cells are fixed and incubated with a specific mouse monoclonal primary antibody to human membrane-bound E-Selectin. The cells are then incubated with an anti-mouse IgG antibody conjugated with peroxidase to detect the primary Ab. A chromogen substrate solution is added to the wells and color develops in proportion to the amount of membrane-bound E-Selectin present on the cells. As the E-Selectin is membrane-bound, a protein concentration standard cannot be performed. Rather, this assay measures test sample stimulation HUVEC E-Selectin expression relative to positive and negative controls.

Reagents Included

A. Stimulation medium (1X) - 20ml. Contains 5% human serum as a source of soluable CD14**.**
B. *E.coli* LPS - 20 µl (1mg/ml). Positive control.
C. Cell Fixative (16X) – 0.65ml (8%). Dilute 1:16 in 1XPBS.
D. Diluent/Wash Buffer (10X) – 50ml. Dilute 1:10 with dH_2O**.**
E. Antibody Dilution Buffer (1X) – 25ml. Dilution buffer for anti-E-Selectin and secondary Abs.
F. Anti-E-Selectin Ab. Lyophylized. Dilute one vial in 10.0 ml 1X Antibody Dilution Buffer (E).
G. Secondary Ab. Lyophylized. Anti-mouse IgG, peroxidase conjugated. Dilute one vial in 10.0 ml 1X Antibody Dilution Buffer (E).
H. TMB (1X) - 10ml. Chromogen substrate for peroxidase detection.
I. Stop Solution (1X) – 5ml. Diluted sulfuric acid to stop the color reaction.

Reagents Not Included But Required

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HUVEC are not included in this assay kit. HUVEC need to be grown in standard, 96-well, sterile, tissue culture plates pre-coated with a sterile, endotoxin-free extracellular matrix such as native type I collagen or gelatin. HUVEC density should be around 1.0 to 1.5X10^4 cells/well.

HUVEC and media can be purchased through Astarte Biologics, LLC as follows:

Product	Catalog Number
Vial of 5 X10^6 cells	1010
96-well microtiter plate	1011
T-25 Flask (confluent)	1012
T-75 Flask (confluent)	1013
Media, 500ml	1014
Media, 100ml	1015
E-Selectin kit only	1016
E-Selectin kit with HUVEC	1017

PRECAUTIONS

The Stimulation Media (Solution A) contains human serum. This serum was tested and was found to be non-reactive for HIV and Hepatitis B surface antigen. As no testing can offer complete assurance of freedom from infectious agents, this reagent should be handled as if capable of transmitting disease.

The following agents are hazardous, wear appropriate eye, hand, face, and clothing protection:

Solution I (dilute sulfuric acid).
Solution C (fixative)
Solution H (TMB)

HUVEC are extremely sensitive to endotoxin (even at picogram levels) so care must be taken to ensure that any reagents incubated with **live cells** such as media, samples and controls are **endotoxin free**.

SAMPLE PREPARATION

HUVEC are extremely sensitive to endotoxin (even at picogram levels) so care must be taken to ensure that any reagents incubated with **live cells** such as media, samples and controls are **endotoxin free**.

Dilute all test samples in Stimulation Media (**A**) or first in endotoxin-free water (not supplied).

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Caution: HUVEC are extremely sensitive to endotoxin (even at picogram levels) so care must be taken to ensure that any reagents incubated with **live cells** such as media, samples and controls are **endotoxin free**

Solution B. *E.coli* LPS - 20µl (1mg/ml). Add 1.0 µl of positive control (**B;** 1mg/ml) to 999.0 µl of Stimulation Media (**A**). This solution is now 1000 ng/ml. Take l of this solution and add it to 900.0 µl of Stimulation Media (**A**). This solution is now 100 ng/ml.

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Solution C. Cell Fixative (16X) – Dilute contents of vial (0.65 ml) into 9.35 ml 1X Solution D.
Solution D. Dliuent/Wash Buffer (10X) – 50ml. Dilute 1 part in 9 parts of dH_2O to make 1X**.**
Solution F. Anti-E-Selectin Ab. Lyophylized. Dilute the contents of the vial in 10.0 ml Antibody Dilution Buffer (**E**).
Solution G. Secondary Ab. Lyophylized. Anti-mouse IgG, peroxidase conjugated. Dilute the contents of the vial in 10.0 ml Antibody Dilution Buffer (**E**).

Assay Procedure

The following protocol is based on HUVEC grown at 1.0 to 1.5X10^4 cells/well in standard 96-well tissue culture plates pre-coated with collagen or gelatin.

It is recommended that test samples and positive and negative controls be run in duplicate or triplicate.

1. Stimulation of HUVEC. Add 100.0 µl of **test samples, positive control** (**B;** 100 ng/ml) and Stimulation Media (**A**) alone as the **negative control** to wells. Incubate 2 to 6 hours in standard tissue culture 37^{o}C, CO_2 incubator.
2. Remove Stimulation Media. Turn the plate upside down on absorbent material and immediately proceed to step 3. **Do not allow cells to dry out**.
3. Fix Cells. Add 100 µl of 1X Solution C to all wells and place plate at 4^{0}C for 10 minutes.
4. Wash 3 times with 1 X Solution D using a gentle stream.
5. Primary Ab. Add 100 µl of anti-E-Selectin Ab to all wells and incubate for 2 hours at room temperature. **Best results (i.e. higher OD values) are achieved by incubating overnight at 4^{0}C**.
6. Wash 3 times with 1 X Solution D using a gentle stream**.**
7. Secondary Ab. Add 100 µl of anti-mouse IgG peroxidase conjugated to all wells and incubate for 2 hours at room temperature.
8. Wash 3 times with 1 X Solution D using a gentle stream**.**
9. **Chromogen.** Add 100 µl of TMB (**H**) to all wells and incubate 30 minutes at room temperature.
10. Stop reaction. Add 50 µl of Stop solution (**I**) to each well.
11. Read OD value at 450 nm (A 650 nm filter can be used as a reference).

Calculation of Results

1. Average the duplicate or triplicate OD values for test samples and positive control.
2. Subtract the negative control OD values from the averaged OD values in step 1.
3. Plot the results and standard errors using a graphics program.

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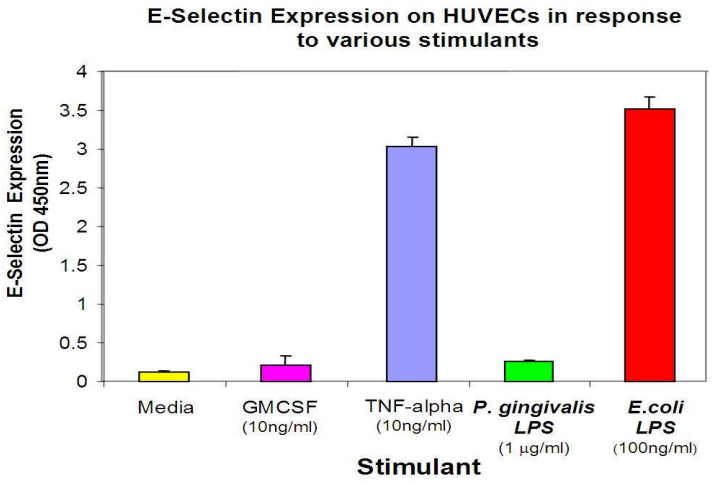
Figure 3. A typical experiment with 2 positive and 2 negative stimulants



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