Application Note

Improved Assays for Quantification of *In Vitro* Vascular Permeability

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ABSTRACT

The endothelial cell lining of the body's vasculature provides a semi-permeable barrier between the blood and tissue compartments, maintaining a diffusion interface that is critical for proper physiological performance and homeostasis. A variety of vasoactive cytokines, growth factors and signal modulators can induce alterations in endothelial cell junction complexes to regulate passage of ions, nutrients, therapeutic agents and macromolecules. Disruptions in appropriate vascular permeability are associated with a broad range of pathological disease states, including diabetes, hypertension, stroke and cancer. An *in vitro* assay for quantification of endothelial monolayer integrity is a valuable tool for the efficient investigation of compounds that can either stimulate or inhibit vascular permeability. Here, we demonstrate the capabilities of two new Millipore permeability assay kits, in pre-coated 24-well and 96-well formats, which have been optimized for ease of use and extensively validated in multiple experimental systems.

INTRODUCTION

A fundamental requirement for the physiological performance of organs is the formation of diffusion barriers that separate and maintain compartments of different structure. The endothelial cell lining of the internal vasculature defines a semi-permeable barrier between the blood and the interstitial spaces of the body. This barrier is composed of intercellular adherens, tight, and gap junction complexes, as well as desmosomes[1]. Junction substructure components such as connexins, integrins, cadherins, catenins, occludins, desmoplakins, selectins, and platelet endothelial cell adhesion molecule-1 (PECAM-1) all act as interface regulators for paracellular permeability of ions, nutrients, therapeutic agents, and macromolecules[2,3]. Endothelial cell adhesive characteristics provide strength and stability for neighboring cells and the cellular cytoskeleton by interacting with actin and myosin contractile filaments[4,5]. Junctional molecules also influence cell signaling and trigger responses that are translated into cell morphology changes and physiological angiogenesis[6,7].

A multitude of vasoactive cytokines, growth factors, and signaling molecules modulate expression and function of endothelial cell substructural components to control permeability. Vascular endothelial growth factor (VEGF), interleukin-1 alpha and beta (IL-1α and IL-1β), tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) have been shown to increase endothelial monolayer permeability[8-11]. Thrombin also increases vascular permeability by stimulating reorganization of the actin cytoskeleton[12]. Lipopolysaccharide (LPS) induces

junction barrier loss and cell detachment by activating protein tyrosine kinases (PTKs) and caspase cleavage reactions[13].
In contrast, junctional adhesion molecule (JAM) decreases permeability by initiating cell adhesion[14] and angiopoietin-1 (Ang-1) can protect endothelial barrier function through regulation of junctional complexes[15,16].

Disruptions of the barrier integrity are manifested as microvascular hyperpermeability, which is associated with many systemic disease states. Pathological angiogenic disease states include heart disease, diabetes, cancer, stroke, hypertension, arthritis, and Alzheimer's disease[1,17,18]. Increases in tissue permeability may be caused by weak, hemorrhaging vessels that become edematous, and permeability increases intensify with irregular fluid flow through the vessels[17]. Expanding the knowledge of endothelial junction behavior and the agents that influence that behavior may lead to new therapies for controlling endothelial permeability.

An essential foundation for any *in vitro* permeability study is an intact, confluent cell monolayer. Endothelial cell monolayers cultured on semi-permeable membranes have been shown to form adherent and tight junctions[19]. Millipore's *in vitro* vascular permeability assay kits provide an efficient model system for the formation of endothelial monolayers on semi-permeable membranes, which enables the evaluation of chemical or drug compound effects on endothelial cell adsorption, transport, and permeability. These permeability assay kits are available in both a standard 24-well format as well as a 96-well format for higher throughput experimentation. Both new kits improve ease of use by providing pre-coated substrates for rapid initiation of endothelial monolayers. Passage of fluorescent molecules through the monolayer and membrane is quantified with a fluorescent plate reader to determine the degree of permeability. In addition, monolayers on the membranes may be stained for convenient cell visualization. The manual for the kits contain troubleshooting suggestions and extensive validation in multiple experimental systems (e.g., time-courses, stimulation and inhibition).

TEST PRINCIPLE

The Millipore *in vitro* vascular permeability assays are performed in 24- or 96-well receiver plates with hanging cell culture inserts. The inserts contain 1 µm pores within a transparent polyethylene terephthalate (PET) membrane. Each insert has been pre-coated with an optimized concentration of type I collagen purified from rat tail. The membranes have a high pore density that permits apical and basolateral access of cells to media and permeability molecules of interest.

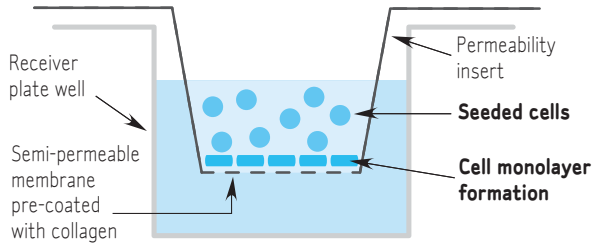
Endothelial cells are first seeded onto the collagen-coated inserts. An endothelial monolayer forms in several days, which occludes the membrane pores. The cell monolayer is then treated with cytokines, growth factors, or other compounds of interest. After treatment, a high molecular weight FITC-dextran is added on top of the cells, and the fluorescent molecules then pass through the endothelial cell monolayer at a rate proportional to the monolayer's permeability. The extent of permeability can be determined by measuring the fluorescence of the receiver plate well solution (Figure 1).

MATERIALS AND METHODS

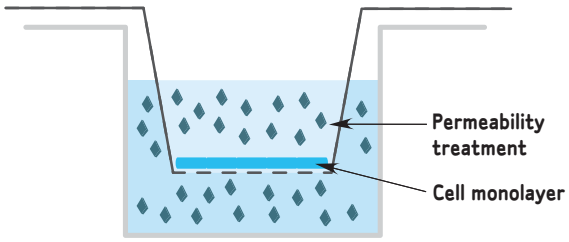
Cell Culture

EndoGRO™ human umbilical vein endothelial cells (HUVEC) were grown in EndoGRO-LS Complete Medium in T75 tissue culture flasks until 80-90% confluence. HUVEC were utilized at or before passage 5 after thaw of original stock for best assay performance. Cells were detached using 0.05% trypsin-EDTA, pelleted, then resuspended in growth medium to a concentration of 0.5 - 1 x 10^6 cells/mL. Dry collagen pre-coated porous inserts were rehydrated for 15 minutes in growth medium prior to cell seeding. HUVEC were seeded in 200 µL or 100 µL of growth medium per insert, for the 24-well and 96-well

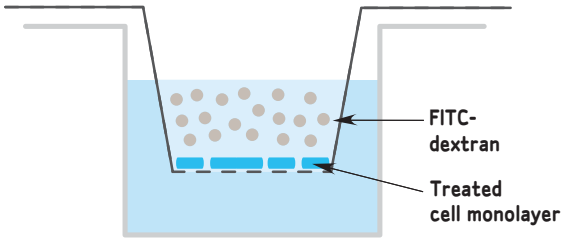
1. Endothelial cells are seeded into the inserts and cultured until complete monolayer formation occurs



2. Cells are treated with permeability modulator of choice.



3. The permeability treatment is removed, then FITC-dextran is added to the semi-permeable insert coated with an endothelial monolayer.



4. FITC-dextran permeates the treated cell monolayer into the plate well. The resulting fluorescence in the plate well is measured and used as an indicator of the extent of monolayer permeability.

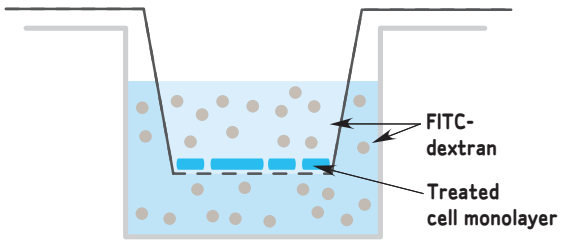


Figure 1.
Vascular permeability assay test principle.

formats, respectively. 500 µL or 250 µL of growth medium (for 24-well or 96-well assays, respectively) were added to each receiver plate well. HUVEC were cultured in a 37 °C / 5% CO_2 tissue culture incubator for 72 hours to allow for monolayer formation.

Permeability Treatment and Testing

Following endothelial monolayer formation, growth medium was carefully removed from each insert and receiver plate well (so as not to touch or disturb the monolayer) and replaced with medium containing the vascular permeability modulator of interest. Samples were returned to 37 °C incubation for the appropriate duration for the treatment of interest (e.g., 2 to 24 hours).

Upon completion of permeability treatment, treatment medium was carefully removed from each insert and receiver plate well. A high molecular weight FITC-dextran solution was created by 1:40 dilution in growth medium and added at 150 µL or 75 µL per insert (for 24-well or 96-well formats, respectively). Growth medium was added to each receiver plate well at 500 µL (for 24-well) or 250 µL (for 96-well). The FITC-dextran was allowed to permeate the monolayers for 20 minutes at room temperature, protected from light. Permeation was stopped by removing the inserts from the receiver wells. The medium in the receiver wells was then thoroughly mixed and 100 µL was removed from each well to a black 96-well opaque plate for fluorescence measurement. Permeability was quantified on a VICTOR²™ 1420 Multilabel Counter (PerkinElmer Life Sciences) via fluorescence at 485 nm excitation / 535 nm emission wavelengths (1 second fluorescent count time).

Monolayer Staining

After completion of FITC-dextran permeability testing, the endothelial monolayer was stained for brightfield imaging with cell stain provided in the kit. 100 µL (for 24-well) or 50 µL (for 96-well) of cell stain was added to each insert and incubated for 20 minutes at room temperature. Cell stain was carefully removed, and the inserts and receiver wells were rinsed twice with

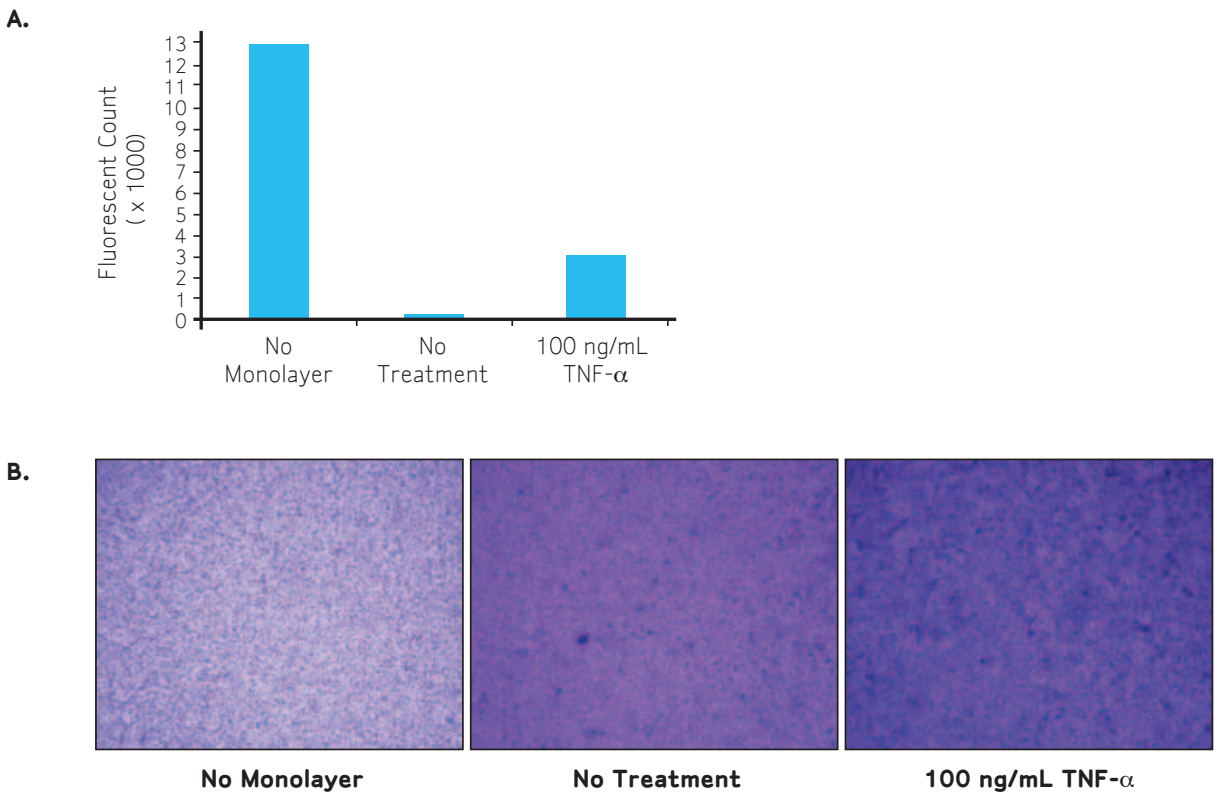


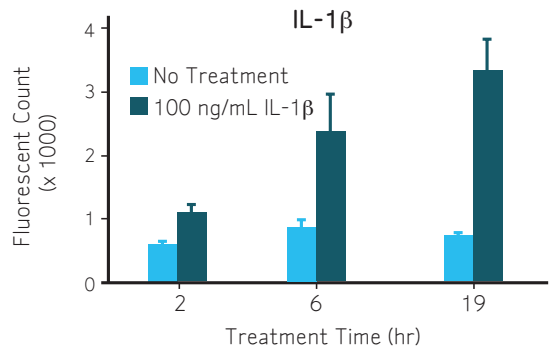
Figure 2.
Example staining and permeability analysis of HUVEC monolayers.
(A) The "No Monolayer" sample demonstrated high permeability in the absence of an occlusive endothelial cell monolayer. The "No Treatment" sample displayed low FITC-dextran permeability. Following TNF-α treatment, fluorescent count was significantly increased compared to the "No Treatment" sample. (B) Consistent with the fluorescence measurements demonstrating low permeability, the "No Treatment" sample exhibited a visually confluent monolayer. Similarly, the increase in permeability quantified following TNF-α treatment was supported by evidence of a visibly disrupted HUVEC monolayer.

Dulbecco's phosphate-buffered saline (DPBS). HUVEC monolayers were imaged on an inverted microscope at 5X objective magnification.

RESULTS AND DISCUSSION

Using Millipore's *in vitro* vascular permeability assays, we demonstrate the ability to visualize and quantify the integrity of a HUVEC monolayer under several treatment conditions. Figure 2 depicts example data from a 96-well format assay. HUVEC at passage 4 were seeded into 96-well inserts at 50,000 cells per insert and cultured for 72 hours in growth medium ("No Monolayer" negative control cultured in growth medium only). Following this culture period, monolayers underwent "No Treatment" (growth medium only) or treatment with 100 ng/mL TNF-α in growth medium for 23 hours.

Inserts upon which HUVEC were not seeded (No Monolayer) showed minimal staining upon brightfield microscopy and correspondingly high permeability (high FITC fluorescent count) in the absence of any occlusive cell monolayer. Samples upon which endothelial monolayers were allowed to grow (No Treatment) showed homogeneous cell staining and low FITC-dextran permeability (essentially a "positive control" for proper HUVEC growth, as monolayer formation may be compromised by poor cell health, high passage number, etc.). Following overnight treatment with a molecule known to increase vascular permeability (100 ng/mL TNF-α), fluorescent count markedly increased over the No Treatment control (>10-fold) and subsequent staining and imaging



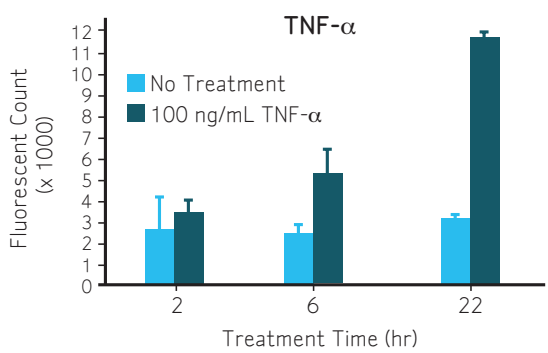
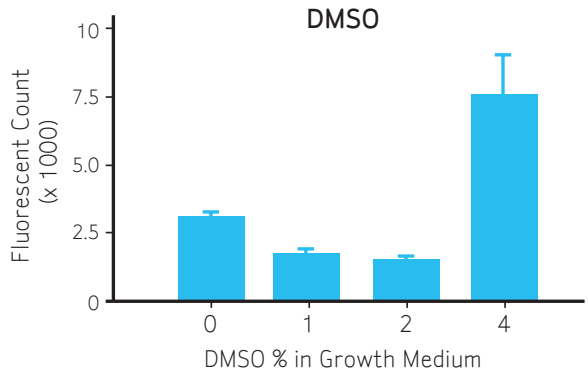


Figure 3.
Time-courses for stimulation of vascular permeability by IL-1β and TNF-α.
HUVEC were seeded into 24-well inserts at 200,000 (for IL-1β, passage 4) or 100,000 (for TNF-α, passage 3) cells per insert and cultured for 72 hours in growth medium. Following this culture period, monolayers underwent "No Treatment" (growth medium only) or treatment with 100 ng/mL IL-1β (left panel) or TNF-α (right panel) in growth medium for a range of durations. Duplicate samples demonstrated low permeability for non-treated samples, and time-dependent increases in permeability following IL-1β or TNF-α treatment. Bars represent mean + standard error of the mean (SEM).



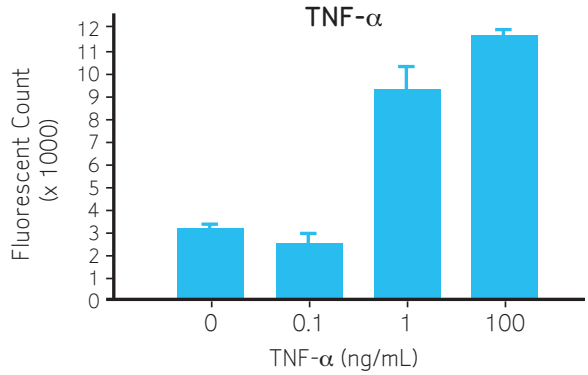


Figure 4.
Dose responses to DMSO and TNF-α in the vascular permeability assay.
HUVEC at passage 3 were seeded into 24-well inserts at 100,000 cells per insert and cultured for 72 hours in growth medium. Following this culture period, monolayers underwent treatment with DMSO (left panel) or TNF-α (right panel) in growth medium at a range of concentrations for 22 hours. Duplicate samples demonstrated that DMSO did not detrimentally affect monolayer integrity until a concentration of 4% DMSO in growth medium. Dose-dependent increases in permeability were observed for increasing TNF-α treatment concentrations. Bars are mean + SEM.

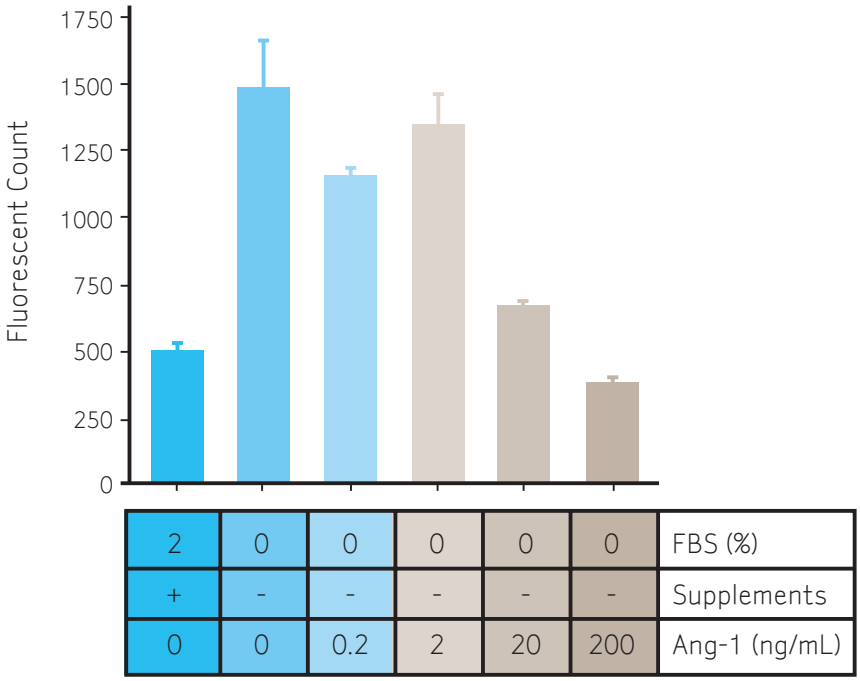


Figure 5.

Dose responsive inhibition of vascular permeability by Ang-1.
HUVEC at passage 1 were seeded into 96-well inserts at 50,000 cells per insert and cultured for 72 hours in growth medium. Following this culture period, monolayers were incubated for 19 hours in growth medium, basal medium alone or in basal medium with increasing concentrations of Ang-1. Monolayers in basal medium alone exhibited increased permeability over those cultured in growth medium containing FBS and supplements. The increased permeability caused by serum/growth factor withdrawal was completely reversed by Ang-1 in a dose-dependent fashion. Conditions were analyzed in triplicate. Bars are mean + SEM.

revealed discontinuities in the monolayer. Similar results are routinely observed in a 24-well format assay (data not shown).

Stimulation time-courses and dose responses

In Figure 3, HUVEC monolayers in a 24-well format assay demonstrated consistently low permeability under No Treatment conditions, following evaluation at 2, 6 and 19-22 hour time-points. At the same time-points, monolayers that had been treated with known stimulators of vascular permeability (100 ng/mL IL-1β or TNF-α) displayed increasing FITC fluorescent count (increasing permeability) with increasing treatment time.

Figure 4 shows increases in HUVEC monolayer permeability following treatment with either DMSO or TNF-α (using a 24-well format assay). DMSO is a common solvent for chemical compounds, but also has known cellular toxicity. Following overnight treatment with final concentrations of 1 or 2% DMSO, HUVEC monolayer integrity (low permeability) was not shown to be adversely affected compared to control (0%) conditions. However, with 4% DMSO in the treatment growth medium, monolayer integrity became significantly compromised (high permeability). Overnight treatment with 0.1, 1 or 100 ng/mL TNF-α induced dose-dependent increases in FITC permeation with increasing cytokine concentration.

Inhibition dose response

In Figure 5, HUVEC monolayers were formed in a 96-well format assay. Basal medium (medium lacking fetal bovine serum (FBS) and essential nutrient supplements included in growth medium) is known to adversely affect endothelial cell health and monolayer integrity. Angiopoietin-1 (Ang-1), a molecule

recognized to be critical in vascular maintenance, was utilized to inhibit the increases in monolayer permeability brought on by serum/growth factor depletion. Overnight addition of 0.2-200 ng/mL Ang-1 demonstrated dose-dependent reversal of basal medium-induced permeability.

CONCLUSION

Millipore's *in vitro* vascular permeability assay kits offer an efficient, convenient model system for monitoring endothelial monolayer junction behavior and characterizing compounds that may influence this critical physiological barrier. In addition, these kits enable the quantitative analysis of time- and dose-dependent stimulation or inhibition of permeability in a higher throughput format.

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Related Products

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In Vitro Vascular Permeability Assay (96-Well)	ECM642
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	SCME003
	SCME004
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