CULTREX® Instructions

For Research Use Only. Not For Use In Diagnostic Procedures

CultreCoat® 24 well *In Vitro* Vascular Permeability Assay

Reagent kit for investigating vascular permeability.

24 Samples

Catalog #: 3475-024-K

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Table of Contents

I. Quick Reference Procedure for the CultreCoat® 24 well Vascular Permeability Assay (Cat# 3475-024-K):

Read through the complete Instructions for Use prior to using this kit.

Prior to Day 1

1. Culture cells per manufacturer's recommendation; adherent cells should be cultured to no more than 90% confluence. Cell seeding density may vary based on cell type (200,000 cells per well required for HUVECs), so plan accordingly. Cells may be pre-treated during culture if necessary.

Day 1

2. Rehydrate collagen I-coated membranes by adding 100 µl of complete cell culture medium to the upper chamber, and incubate at 37 ^{o}C in CO_2 incubator for 2 hours.
3. Harvest cells.
4. Centrifuge cells at 200 x g for 3 minutes; then count and resuspend at 2 x 10^6 cells/ml in a complete culture medium.
5. Add 100 µl of cells per well to each insert (200,000 cells/well).
6. Add 500 µl of complete culture medium per well to bottom chamber.
7. Incubate chamber at 37 ^{o}C in CO_2 incubator for 72 hours or until a monolayer is formed.

Day 4

8. If serum-starvation is not required, proceed to step 9. If it is needed, then carefully aspirate media from the top and bottom chambers. Quickly add 100 µl of starvation medium (basal culture medium containing 0.5% FBS), and add 500 µl starvation medium to the bottom chamber. Do not allow cells to dry. Incubate for 6 to 24 hours.
9. If not testing compounds, then proceed to step 10. If testing compounds, then carefully aspirate media from the top and bottom chambers. Prepare treatment medium (containing test compounds, with or without serum). Quickly add 100 µl of treatment medium to the inserts, and add 500 µl of treatment medium to the bottom chamber. Do not allow cells to dry. Incubate at 37 ^{o}C in a CO_2 incubator as desired.
10. Prepare FITC-Dextran (see Section VI.2.).
11. Add 300 µl of basal medium to the assay wells.
12. Carefully aspirate media from the top and bottom chambers, and quickly transfer the inserts to the assay wells.
13. Add 100 µl of FITC-Dextran to the each insert, and incubate at 37 ^{o}C in a CO_2 incubator for 5 minutes.
14. To stop the reaction, remove inserts, and read plate at 485 nm excitation, 520 nm emission.

II. Background

The vascular endothelium is the thin monolayer of cells that lines blood vessels and provides a network for the exchange of biological materials such as gases, nutrients, and metabolic waste throughout the body. The endothelium exhibits a selective barrier function between the vessel lumen and the surrounding tissues which controls this exchange. There are many vasoactive cytokines and growth factors that function in regulating the degree of vascular permeability, such as interleukin alpha and beta[1], TNF-alpha[2], IFN gamma[1], hepatocyte growth factor (HGF)[3], and vascular endothelial growth factor (VEGF)[4]. There are also several systemic diseases associated with disruption of vascular permeability, such as cancer[4], diabetes[3], heart disease[3], stroke[5], hypertension[5], and arthritis[6].

Trevigen's **CultreCoat® 24 well Vascular Permeability Assay** accelerates the screening process for signal transduction pathway modulators and compounds that influence vascular permeability, offering a flexible, standardized, high-throughput format for quantitating the degree to which genes or compounds can influence the maintenance of endothelial cell-to-cell adhesion.

This assay employs a simplified Boyden chamber design with a one micron polyethylene terephthalate (PET) membrane coated with collagen I. Ports within the migration chamber (top) allow access to the assay chamber (bottom) without dismantling the device. This design is easier to use, prevents contamination, and is adaptable for robotic high throughput systems. The assay chamber may be directly analyzed in a 24 well plate reader, eliminating transfer steps that introduce additional variability to the assay.

Since different cell lines may exhibit variation in size, adhesive properties, and proliferation rates, cell seeding densities and culture periods may require some optimization (the current protocol is optimized using human umbilical vein endothelial cells (HUVECs)).

Detection of vascular permeability is quantified using FITC-Dextran. The confluent cell monolayer forms a barrier through cell-cell adhesion mechanisms such as junctions (adherens, tight, and gap) and desmosomes, and this barrier restricts FITC-Dextran to the inserts. Disruption of these cell-cell adhesion mechanisms creates gaps in the intercellular space that allow diffusion of FITC-Dextran into the bottom chamber, so the amount of FITC-Dextran that diffuses is related to the amount of intercellular space, which is representative of vascular permeability.

III. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. The physical, chemical, and toxicological properties of these products may not yet have been fully investigated; therefore, Trevigen recommends the use of gloves, lab coats, and eye protection while using these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products.
3. The CultreCoat® Vascular Permeability Assay contains reagents that may be harmful if swallowed, or come in contact with skin or eyes. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

Material safety data sheets are available on request.

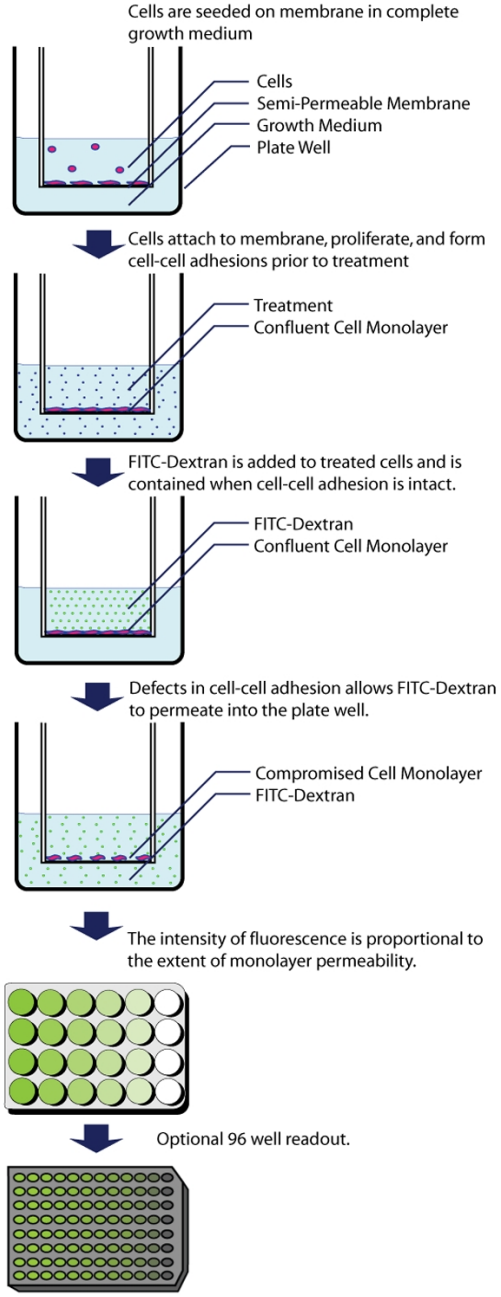


Figure 1. Illustration of protocol for the CultreCoat Vascular Permeability Kit.

IV. Materials Supplied

Component	Quantity	Storage	Catalog#
Vascular Permeability Chamber	each	4 ºC (Desiccated)	3475-024-01
FITC-Dextran	100 µl	4 ºC	3475-096-02

V. Materials/Equipment Required But Not Supplied

Equipment

1. 1 - 20 µl, 20 - 200 µl, and 200 - 1000 µl pipettors
2. 37 ^{o}C CO_2 incubator
3. Laminar Flow Tissue Culture Hood
4. Low speed centrifuge and tubes for cell harvesting
5. Hemocytometer or other means to count cells
6. 50 and 500 ml graduated cylinders
7. -20 ^{o}C and 4 ^{o}C storage
8. Ice bucket
9. Standard light microscope (or inverted)
10. Pipette helper
11. Timer
12. Vortex mixer
13. Fluorescent 24-well plate reader (485 nm excitation, 520 nm emission)
14. Computer and graphing software, such as Microsoft® Excel®

Reagents

1. Cell Harvesting Buffer; EDTA, trypsin, or other cell detachment buffer.
2. Complete Culture Growth Medium, as recommended by cell supplier.
3. Basal Medium, Complete Culture Growth Medium without serum and other supplements.
4. Chemoattractants or pharmacological agents for addition to culture medium.
5. Starvation medium: basal medium with 0.5% FBS.
6. Sterile PBS or HBSS to wash cells.
7. Distilled, deionized water
8. Trypan blue or equivalent viability stain

Disposables

1. Cell culture flask, 25 cm^2 or 75 cm^2
2. 50 ml tubes
3. 1 - 200 µl and 200 - 1000 µl pipette tips
4. 1, 5, and 10 ml serological pipettes
5. Gloves
6. Black 96 well plate (optional)

VI. Reagent Preparation

1. Vascular Permeability Chamber

Rehydrate collagen I-coated chambers using 100 µl of warm (37ºC) Complete Culture Medium for 2 hours at 37 ºC prior to starting assay.

2. FITC-Dextran

Dilute reagent 1:100 using Complete Culture Medium, and add 100 µl in the insert for each sample (except background control); dilution may be optimized if needed. For optimal stability, dilute only as needed.

VII. Assay Protocol

These procedures should be performed in a biological hood utilizing aseptic technique to prevent contamination.

A. Vascular Endothelial Cell Culture

Subject cells may be prepared for investigation as desired. The following procedure has been designed using HUVECs. Other cell lines/types may require optimization of seeding concentrations and incubation periods for best results.

1. Cells should be passaged 2 or 3 times prior to use in the assay, and adherent cells need to be no greater than 90% confluent. Each chamber requires approximately 200,000 cells (may vary by cell type), and a 25 cm^2 or 75 cm^2 flask will yield approximately 1 x 10^6 or 3 x 10^6 cells, respectively. Plan to have enough cells to accommodate all samples; insufficient cell densities will adversely affect the assay.
2. Prior to harvest, visually inspect cells, and record cell health, relative number, and morphology.
3. Cells should not be used if they are fully confluent, appear unhealthy, or exhibit impaired proliferation, and primary cell lines should not be used beyond passage 10.

B. Preparation of Vascular Monolayer

Note: Warm all cell culture medium to 37°C prior to use.

4. To evaluate vascular permeability, controls need to be set up for background (no FITC-Dextran), 100% Permeability (no cells), and no treatment. Each control and experimental condition should be evaluated in replicates of four or more wells for optimal statistical significance.
5. Rehydrate the collagen I-coated inserts by adding 100 µl of complete culture medium to each well, and incubate at 37 ºC in a CO_2 incubator for two hours.
6. Harvest Cells.
   A. Wash cells two times with sterile PBS or HBSS. Use 5 ml per wash for a 25 cm^2 flask and 10 ml per wash for a 75 cm^2 flask.
   B. For 25 cm^2 or 75 cm^2 flask, add 1 ml or 2 ml, respectively, of Cell Harvesting Buffer (see *Materials/Equipment Required But Not Supplied*), and incubate at 37˚C for 5 to 15 minutes (until cells have dissociated from bottom of flask).
   C. Transfer cells to a 15 ml conical tube, and add 5 ml of Complete Culture Medium (see *Materials/Equipment Required but Not Supplied*).
   D. Centrifuge cells at 200 x *g* for 3 minutes to pellet, remove quenching medium, and resuspend cells in 2 ml of Complete Culture Medium. Cells may need to be gently pipetted up and down using a serological pipette to break up clumps.
   E. Count cells, and dilute to 2 x 10^6 cells per ml in Complete Culture Medium.
7. Add 100 µl of cells per hydrated insert (do not add cells to control wells), and add 500 µl Complete Culture Medium to bottom chambers. Incubate at 37˚C in a CO_2 incubator for 72 hours or until a confluent cell monolayer is formed.

C. Vascular Permeability Assay

Note: Warm all cell culture medium to 37°C prior to use.

8. If serum-starvation is not required, proceed to step 9. If it is needed, then carefully aspirate media from the top and bottom chambers. Quickly add 100 µl of starvation medium (basal culture medium containing 0.5% FBS), and add 500 µl starvation medium to the bottom chamber. Incubate for 6 to 24 hours.
9. If not testing compounds, then proceed to step 10. If testing compounds, then carefully aspirate media from the top and bottom chambers. Prepare treatment medium (containing test compounds, with or without serum). Quickly add 100 µl of treatment medium, and add 500 µl of treatment medium to the bottom chamber. Incubate at 37 ºC in CO_2 incubator as desired.
10. Prepare FITC-Dextran (see Section VI.2.).
11. Add 300 µl of basal medium to the assay chamber.
12. Carefully aspirate media from the top and bottom chambers, and transfer the inserts to the assay wells.
13. Add 100 µl of FITC-Dextran to each insert, and incubate at 37˚C in CO_2 incubator for 5 minutes.
14. To stop the reaction, remove inserts, and read plate at 485 nm excitation, 520 nm emission.
15. Optional reading in a black 96 well plate:
    A. Pipe the contents of each well up and down to create a homogeneous solution.
    B. Transfer 100 ul from each well of the 24 well plate to a different well in a black 96 well plate.
    C. Read plate at 485 nm excitation, 520 nm emission.

VIII. Data Analysis

1. Each condition should be evaluated in replicates of four or more data points for optimal statistical significance.
2. For assay samples, first average all wells for each condition (Table 2).
3. Next, subtract background from averages (Table 3).
4. Determine percent vascular permeability by dividing the relative fluorescent units (RFU) for each sample by the maximum (no cell) RFU (Table 4).
5. Graph the vascular permeability with standard deviations (Figure 1).

Table 1. Raw data (RFU):

Condition	Well 1	Well 2	Medium
Background	8997	8734	EGM2 w/o FITC-dextran
No cells	55733	57525	EGM2
200,000 cells	10254	10443	EGM2

Table 2. Summarize data (RFU):

Condition	Average	Std Dev	Medium
Background	8866	186	EGM2 w/o FITC-dextran
No cells	56629	1267	EGM2
200,000 cells	10349	134	EGM2

Table 3. Subtract background (RFU):

Condition	Average	Std Dev	Medium
No cells	47764	1267	EGM2
200,000 cells	1483	134	EGM2

Table 4. Determine percent vascular permeability:

Condition	Average	Std Dev	Medium
No cells	100%	3%	EGM2
200,000 cells	3%	0%	EGM2

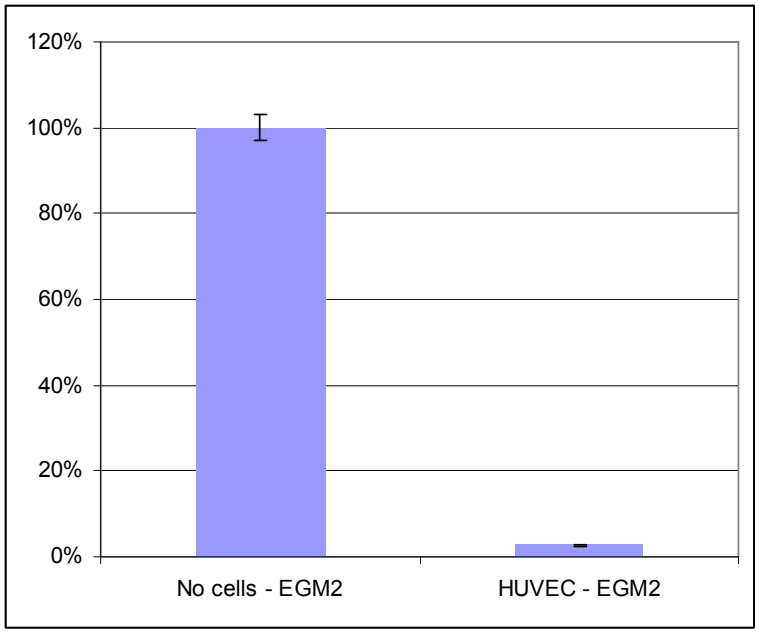


Figure 2. HUVEC Vascular Permeability. HUVECs were seeded at 200,000 cells/well and cultured for 72 hours at 37°C in a CO_2 incubator. Cells were evaluated for vascular permeability using the Cultrex® 24 well *In Vitro* Vascular Permeability Assay.

IX. Troubleshooting

Problem	Cause	Solution
No Fluorescence in the no cell control	FITC-Dextran reagent was not added.	Add FITC-Dextran to the inserts.
	The instrument is incorrectly configured.	Make sure plate reader reads from top if using black plate. Gain may need to be adjusted for optimal fluorescence range.

Problem	Cause	Solution
High Background	Medium additives may contribute to background fluorescence	Compare autofluorescence of Complete Culture Medium to Basal Medium
High Fluorescence in no treatment control	Insufficient cell density (no confluent monolayer)	Increase seeding density or extend cell culture period on insert.
	Insufficient cell health	Restart assay with healthy cell culture.

X. References

1. Martin, S.S., *IL-1 and IFN-gamma increase vascular permeability.* Immunology, 1988. **64**(2): p. 301-305.
2. Edamitsu, S., et al., *Role of TNF[alpha], IL-1, and IL-1ra in the Mediation of Leukocyte Infiltration and Increased Vascular Permeability in Rabbits with LPS-Induced Pleurisy.* Clinical Immunology and Immunopathology, 1995. **75**(1): p. 68-74.
3. Emani, S., et al., *Increased vascular permeability after cardiopulmonary bypass in patients with diabetes is associated with increased expression of vascular endothelial growth factor and hepatocyte growth factor.* The Journal of Thoracic and Cardiovascular Surgery, 2009. **138**(1): p. 185-191.
4. Cornali, E.E., *Vascular endothelial growth factor regulates angiogenesis and vascular permeability in Kaposi's sarcoma.* The American Journal of Pathology, 1996. **149**(6): p. 1851-1869.
5. Lee, J.-M., et al., Vascular Permeability Precedes Spontaneous Intracerebral Hemorrhage in Stroke-Prone Spontaneously Hypertensive Rats. Stroke, 2007. **38**(12): p. 3289-3291.
6. Huang, M., et al., *Mast cell deficient W/W(v) mice lack stress-induced increase in serum IL-6 levels, as well as in peripheral CRH and vascular permeability, a model of rheumatoid arthritis.* International Journal of Immunopathology and Pharmacology, 2002. **15**(3): p. 249-254.
7. Peisheng Hu, P., et al., *Generation of low-toxicity interleukin-2 fusion proteins devoid of vasopermeability activity.* Blood, 2003, **101**: p. 4853-4861.

XI. Related products available from Trevigen.

Catalog#	Description	Size
3475-096-K	CultreCoat® 96 well *In Vitro* Vascular Permeability Assay	96 samples
3480-024-K	CultreCoat® 24 Well BME-Coated Cell Invasion Assay	24 inserts
3481-096-K	CultreCoat® 96 Well Low BME Cell Invasion Assay	96 samples
3482-096-K	CultreCoat® 96 Well Medium BME Cell Invasion Assay	96 samples
3483-096-K	CultreCoat® 96 Well High BME Cell Invasion Assay	96 samples
3484-096-K	CultreCoat® 96 Well BME Cell Invasion Optimization Assay	96 samples
3455-024-K	Cultrex® 24 Well BME Cell Invasion Assay	24 inserts
3465-096-K	Cultrex® 96 Well Cell Migration Assay	96 samples
3465-024-K	Cultrex® 24 Well Cell Migration Assay	12 samples
3456-096-K	Cultrex® 96 Well Laminin I Cell Invasion Assay	96 samples
3457-096-K	Cultrex® Collagen I Cell Invasion Assay	96 samples
3458-096-K	Cultrex® Collagen IV Cell Invasion Assay	96 samples
3471-096-K	In vitro Angiogenesis Assay Endothelial Cell Invasion	96 samples

Accessories:

Catalog#	Description	Size
3415-001-02	Cultrex® Human BME, PathClear®	1 mg
3420-001-01	Cultrex® Human FIbronectin, PathClear®	1 mg
3421-001-01	Cultrex® Human Vitronectin, PathClear®	50 µg
3400-010-01	Cultrex® Mouse Laminin I	1 mg
3440-100-01	Cultrex® Rat Collagen I	100 mg
3442-050-01	Cultrex® Bovine Collagen I	50 mg
3410-010-01	Cultrex® Mouse Collagen IV	1 mg
3430-005-02	Cultrex® BME with phenol red, PathClear®	5 ml
3431-005-02	Cultrex® BME with phenol red, reduced growth factor PathClear®	5 ml
3432-005-02	Cultrex® BME no phenol red, PathClear®	5 ml
3433-005-02	Cultrex® BME no phenol red, reduced growth factor PathClear®	5 ml
3430-005-01	Cultrex® BME with Phenol Red	5 ml
3432-005-01	Cultrex® BME; no Phenol Red	5 ml
3431-005-01	Cultrex® BME with Phenol Red; Reduced Growth Factors	5 ml
3433-005-01	Cultrex® BME no Phenol Red; Reduced Growth Factors	5 ml
3416-001-01	Cultrex® Bovine Fibronectin	1 mg
3417-001-01	Cultrex® Bovine Vitronectin	50 µg
3438-100-01	Cultrex® Poly-L-Lysine	100 ml
3439-100-01	Cultrex® Poly-D-Lysine	100 ml
3439-100-01	Cultrex® Cell Recovery Solution	100 ml
3437-100-K	Cultrex® Cell Staining Kit	100 ml
3450-048-05	CellSperse™	15 ml

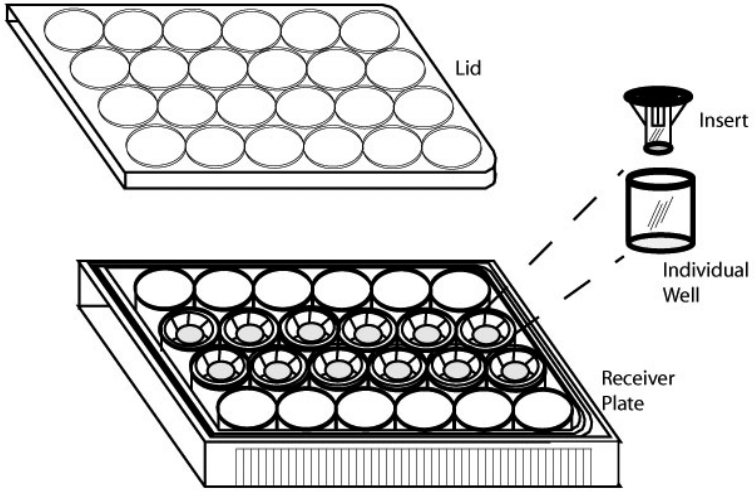
XII. Appendix

Appendix A. Reagent and Buffer Composition

1. Vascular Permeability Chamber

24 well Boyden Chamber, 0.45 µm PET membrane pre-coated with collagen I.

Vascular Permeability Chamber



2. FITC-Dextran

FITC-labeled dextran. For optimal stability, dilute as needed.

Appendix B. Suggested sample configuration

	1	2	3	4	5	6
A	Background (No Cells/No FITC-Dextran)			Condition 2		
B	Maximum Permeabilty (no cells)			Condition 3		
C	Minimum Permeability (no treatment)			Condition 4		
D	Condition 1			Condition 5		

The product accompanying this document is intended for research use only and is not intended for diagnostic purposes or for use in humans.

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