Applications of BD Falcon™ and BD BioCoat™ FluoroBlok™ Cell Culture Inserts in Quantitative Cellular Assays

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Overview

Part I: BD FluoroBlok™ Membrane Description

Part II: Assays

Part III: Applications

Appendix: Additional Information
What is the BD FluoroBlok™ Membrane?

- Insert (individual or multiwell)
- Apical chamber
- Pore
- BD FluoroBlok™ membrane
- Basal chamber
- Base plate

Cross section of an insert system – not to scale
What is the BD Falcon™ FluoroBlok™ Insert System?

Light-Tight PET Membrane
• Dyed membrane blocks light from 490-700 nm while allowing for multiplex detection assays

Quantitative Detection
• Use of fluorescence detection

Run Homogeneous Assays in Real Time with Non-Destructive Sample Detection
• Rapid data collection without the need for plate washing or manual cell scraping and counting
• Determine migration or invasion of cells in real time

Choice of Membrane Pore Sizes
• 24-multiwell insert plate and 24 individual inserts are available in 1, 3, and 8 µm pore sizes
• 96-multiwell insert plate are available in 3 and 8 µm pore sizes
What Can You Do With the BD FluoroBlok™ Insert System?

**Cell Migration**
- Haptotaxis, movement in response to a gradient of adhesion sites or ECMs
- Chemotaxis, movement toward a chemical gradient
- Cell motility, Chemokinesis, cell movement

**Cell Invasion**
- Degradation of a physical barrier
- Pore-occluding BD Matrigel™ Matrix-coated BD FluoroBlok™ Insert Systems

**Co-Culture**
- Multiple cell types in close proximity

A variety of cell types on either BD Falcon™ or BD BioCoat™ FluoroBlok™ Insert Systems can be used
How to Determine Your Needs

What should you use?

Choice of membrane pore sizes

- **8 μm**
  - Epithelial cells
  - Fibroblasts
  - Tumor cells
  - Smooth muscle cells
  - Osteoblasts

- **3 μm**
  - Leukocytes
  - Endothelial cells

- **1 μm**
  - Caco-2 cells (permeability)
  - Neuronal cells

In addition to pore size, pore density may also matter. A side-by-side comparison of 3 and 8 μm for your application is recommended.
Do You Need an ECM Coating?

An extracellular matrix (ECM) coating may be needed by your cells. Some cells require ECMs for attachment or signaling. Choice of ECM and coating conditions needs to be optimized.
Things to Think About...

- Insert System Handling
- Detection Instrument
- Testing the System
- Cell Labeling Methods
Insert System Handling

- A repeating pipettor is recommended for the 24-multiwell or individual insert system.
- A multi-channel pipettor is required for the 96-multiwell insert system.
- The 96 square-well plate must be kept level.
Insert System Handling

- Bubbles should be eliminated at all steps
- Chemoattractant should be added to the bottom chamber via the access port
- To minimize bubbles, add to the apical chamber then to the basal chamber

bubble under insert – will influence reading (*i.e.*, cells may not migrate or stain = bad!)
Individual Insert Handling

Individual Inserts (w/flanges) + Companion Plate (w/notches) = Insert Flanges Resting in Notch
Detection Instruments

You will need:

• A fluorescent plate reader with bottom reading capability
  - AND -
  An inverted fluorescent microscope for confirmation and troubleshooting
  - OR -

• A fluorescent imager, *i.e.*, BD Pathway™ Bioimager

*BD Pathway 855 High-Content Bioimager*
Detection Instruments

Plate readers compatible with BD FluoroBlok™ Insert Systems include:

- **BioTek**
  - Synergy (4, 2, HT), FLx800

- **BMG LABTECH**
  - PHERAStr (Plus), OPTIMA, POLARStar/FLUOSTar (Omega, Galaxy)

- **MDS Analytical Technologies (formerly Molecular Devices)**
  - SpectraMax readers (Multimode M5/M5e, M2e; Gemini EM)

- **PerkinElmer**
  - EnVision, Victor series, HTS 7000+

- **Tecan**
  - Safire², GENios, SpectraFluor Plus

- **Thermo LabSystems**
  - Fluoroskan Ascent

...and many more!
How to Test the System

- Acquire 3 µm or 8 µm BD FluoroBlok™ Insert Systems in the format of your choice.
- Label HUVEC (3 µm) or HT-1080 (8 µm) in a TC flask with 5 µg/ml of calcein AM in HBSS [1 hour, 37°C, 5% CO₂]
- Wash the cells, and seed in basal medium
- Add 5% FBS in basal medium as the chemoattractant.
- Incubate for 4 hours at 37°C, 5% CO₂.
- Read the insert system on your plate reader.
- Confirm by observing the cells on the membrane using an inverted fluorescent microscope.

Seeding density
- 24-well: 25,000 - 50,000 cells/well
- 96-well: 10,000 - 20,000 cells/well

Chemoattractant
- a titration of chemoattractant concentration is recommended

Incubation time
- hours - overnight – days, depending on the cell type
Typical Assay Setup

Steps using calcein AM post-label:
• rehydrate (2h)
• aspirate
• seed cells
• add chemoattractant (overnight incubation)
• *stain (incubate 1h)
• read
Cell Labeling Dyes

Any fluorescent dye derived from the fluorescein, rhodamine and cyanine families can all be used with this system

► emission wavelength must be between 490-700nm

Ultraviolet-inducible dyes tend to be incompatible with the BD Falcon™ HTS FluoroBlok™ Insert since they tend to emit light in the blue range.

Multiplexing?
Yes!

For more information on spectra and alternative fluorophore choices, consult the BD FluoroBlok Insert Cross Reference Chart: Technical Bulletin #451

Cell Labeling Methods

Pre-Labeling
  – labeling cells *in vitro* prior to assay

Post-labeling
  – labeling cells on the underside of membrane following migration/invasion

Intrinsically-labeled cells
  – expressing Green Fluorescent Protein or analogs (*e.g.*, RCFP) transfected
Labeling cells *in vitro* prior to assay

**Dyes:** Calcein AM (up to 6 h), CellTracker™ (overnight or longer), DiI (days)

**Advantages:** Homogeneous assay, Kinetic measurement.

**Shortcoming:** Potential alteration of cell function and compound interaction.

**Keys for success:** Optimize labeling time and dye concentration to balance the need for high signal and low side effects. When in doubt, compare with post-labeling.
Tumor Cell Invasion Assay Using Pre-label

- Pre-label cells with fluorophore
- Cells penetrate pores and adhere to the bottom of membrane
- Read on plate reader
Pre-labeling with DiIC$_{12}$(3)

HT-1080

$\leftarrow$ invasion

migration $\rightarrow$

3T3

$\leftarrow$ invasion

migration $\rightarrow$
Labeling cells after migration/invasion

**Dyes:** Wide range of availability calcein AM, SYTO 24, etc.

**Advantages:** Cells are not altered during migration/invasion

**Shortcoming:** End point assay

**Key for success:** Need to select a low background dye if cells fall into the wells so they can be stained without washing.

*Underside of membrane showing SYTO24 (nuclear stain) labeled HT-1080 cells (green) which have migrated. Pores (yellow) are also visible.*
Tumor Cell Invasion Assay Using Post-label

Cells Penetrate Pores and Adhere to the Bottom of Membrane
Label Cells with Fluorophore
Read on plate reader
Intrinsically-Labeled Cells

Cells expressing fluorescent protein, GFP, e.g., RCFP-HT-1080

Advantages: Homogeneous, kinetic measurement. No further manipulations needed.

Limitations: Lack of commercial cell lines, potential alteration of cell function due to transfection, expense. Not all cells can be easily transfected.

Underside of membrane showing RCFP HT-1080
Specific Applications

- Tumor invasion
- Kinetic assays
- Multiplexing
- Drug screening
- Migration
- Chemotaxis
- Endothelial cell invasion
BD BioCoat™ FluoroBlok™ Tumor Invasion System

BD FluoroBlok™ membrane,
8 µm pore size

Pore occluding
BD Matrigel™ Matrix Layer

BD FluoroBlok™ membrane,
8 µm pore size

Cross section of the insert well.
Drug Solvent Effect on Tumor Cell Invasion

DMSO has no significant effect on Tumor Cell Invasion

![Graph showing the effect of DMSO on tumor cell invasion]

- **RFU**

- **[DMSO]**
  - 2%
  - 1%
  - 0.50%
  - 0.25%
  - 0.13%
  - 0%

- **HT1080**
- **3T3**

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Specific Applications

- Tumor invasion
- Kinetic assays
- Multiplexing
- Drug screening
- Migration
- Chemotaxis
- Endothelial cell invasion
A Kinetic Assay

- HT-1080 and 3T3 pre-labeled with 5 µg/mL DiIC$_{12}(3)$ and plated in a BD BioCoat™ Tumor Invasion System
- Assay was run for 24 hours with readings taken at various time points
- Similar results as post-label (ratio of invasive : non-invasive cells)
- Can determine “point when invasion starts”
Time Course of Tumor Cell Invasion Using DiI Pre-label Cells

kinetic assay

% invasion vs. time (h)

HT-1080
3T3
A Multiplexed Kinetic Assay

- PC3-GFP cells were pre-labeled with SYTO 82, plated in BD FluoroBlok™ Invasion Chambers (self-coated BD Matrigel™ Matrix)
- Real-time analysis of tumor cell invasion with a FLUOstar OPTIMA plate reader enclosed in an AtmosBag (5% CO₂), reader set at 37°C

*BMG Labtech Application Note 144 (12/2006)
A Multiplexed Kinetic Assay

Comparable results were obtained from plates placed in a standard incubator and in a FLUOstar OPTIMA as described.
Specific Applications

- Tumor invasion
- Kinetic assays
- Multiplexing
- Drug screening
- Migration
- Chemotaxis
- Endothelial cell invasion
Inhibition of Invasion and Migration of HT-1080 Cells by Doxycycline When Using Pre-, Post-, Intrinsic-Label

For more information, see Technical Bulletin Nos. 441 and 442.
## Comparison of IC$_{50}$

**Summary of doxycycline IC$_{50}$ (µm)**

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<thead>
<tr>
<th></th>
<th>invasion</th>
<th>migration</th>
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<tr>
<td>Post-label</td>
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<td>45</td>
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<tr>
<td>Pre-label</td>
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<tr>
<td>RCFP</td>
<td>72</td>
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→ Labeling method did not affect IC$_{50}$
Specific Applications

- Tumor invasion
- Kinetic assays
- Multiplexing
- Drug screening
- Migration
- Chemotaxis
- Endothelial cell invasion
Neuronal Motogen Screening


control  motogen

granule cells  interneurons
Neuron migration thru BD Matrigel™ Matrix in response to motogen (BDNF)

Neuronal Motogen Screening

Cells plated on top of insert (A,E)
Migrating neurons (B,C,F,G)
MAP2 stained somata and dendrites (red)
DAPI stained nuclei (blue)

1 μm pores visible with some DAPI stained nuclei (blue) that have migrated thru pores

BD BioCoat™ 96-well Endothelial Cell Migration System

HUVEC Migration
HFN coated vs. Uncoated

Human Fibronectin-coated BD FluoroBlok™ 24- and 96-Multiwell 3 µm pores Insert Systems
- Non-occluded pores
- Optimum endothelial cell migration
Time course of MCP-1 induced chemotaxis in THP-1 and primary monocytes

- Pre-labeled cells in the inserts were incubated with 25 nM MCP-1 in the bottom chamber
- Bottom fluorescence was measured at varying time points
- Data on the graph are means ± SD from a typical experiment (n=4 wells)
FLG 29.1 cell line, human preOC, induced to differentiate by TPA
Underside of 8 µm inserts coated with ECM substrates at 20 µg/mL
Inhibition of migration toward FN by engagement of CD44 by soluble HA
Reversed by BRIC235 mAb blocking CD44-HA interaction
NO-Mediated Migration


eGFP-10T1/2 cells added to apical side; HUVEC seeded on base plate

NOS inhibitor (L-NMMA) reduced migration of mural cell precursors toward HUVEC
Specific Applications

- Tumor invasion
- Kinetic assays
- Multiplexing
- Drug screening
- Migration
- Chemotaxis
- Endothelial cell invasion
Chemotaxis of Calcein AM-Labeled Primary Neutrophils through 3 µm BD Falcon™ FluoroBlok™ Inserts

- Prelabeled for 15 minutes in flask
- Cells migrate through and then fall from insert and collect beneath insert
Chemotaxis: Undifferentiated and Transfected
HL-60 cells using 3 µm BD FluoroBlok™ Inserts

**Cells:** Transfected HL-60 cells (human promyelocytic cells, neutrophils)

**Receptor:** FPRL2, G protein-coupled N-formyl peptide receptor homologue

**Chemoattractant:** Synthetic hexapeptide, WKYMVm

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Human MSC Chemotaxis

Human MSCs
• pre-labeled with CellTracker Green
• working [fMLP] established

Human MSCs from different donors
• pre-labeled with CellTracker Green
• 100 nM fMLP as chemoattractant

Specific Applications

- Tumor invasion
- Kinetic assays
- Multiplexing
- Drug screening
- Migration
- Chemotaxis
- Endothelial cell invasion
Effect of MMP inhibitor 1'10' Phenanthroline on HMVEC Invasion

Fluorescent Units

Control
VEGF(4ng/ml)
0.1ug/ml
1ug/ml
10ug/ml
20ug/ml

VEGF(4ng/ml)+ 1'10' Phenanthroline

BD Matrigel™ Matrix-coated
BD FluoroBlok™ 24-Multiwell
3 µm pore size Insert System
Optimized for endothelial cell invasion
Effects of Eph B4 Receptor Activation
Primary Human Microvascular Endothelial Cells

Eph: family of 14 receptor tyrosine kinases with a role in vascular growth and remodeling

Stimulation of Eph B4 with 50nM ephrin B2/Fc results in 63% increase in migration event

Conclusions

- Use for a variety of applications
- Save time and labor
- Achieve quantitative results
Contact Us

Technical Support

In the U.S.
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  tel: 877.232.8995

Outside the U.S.
  e-mail: help.biosciences@bd.com
  Contact your local distributor or nearest BD Biosciences office

Visit bdbiosciences.com
Appendix

Useful information not presented
### Products

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<tr>
<th>Product Description</th>
<th>Size</th>
<th>Code Combination</th>
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<td></td>
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<tr>
<td>24 MW</td>
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<td>96 MW</td>
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<tr>
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<tr>
<td>96w feeder tray + lid</td>
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<tr>
<td>24 MW w/feeder tray (1 μm)</td>
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Products

Falcon (uncoated)

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*When using individual cell culture inserts, both the inserts and companion plates must be purchased.
BioCoat

**Tumor Invasion System** (8 μm FB, Matrigel coated)

- 24 multiwell: 354165, 354166
- 96 multiwell: 354167, 354168
Products

BioCoat

**Angiogenesis System (3 μm FB)**
Endothelial Cell Invasion (Matrigel coated)
- 24 MW 354141, 354142

Endothelial Cell Migration (human fibronectin coated)
- 24 individual 354597
- 24 MW 354143, 354144
- 96 MW 354147, 354148
Products

Misc

Fluorescent Dyes
  calcein-AM 354216, 354217
  DiIC$_{12}(3)$ 354218

  cell recovery solution 354253
BD BioCoat™ Tumor Invasion System

Combined Benefits of the FluoroBlok Inserts and Matrigel™
   Save time and labor in invasion assays using an authentic Basement Membrane Model.

Consistent and Uniform Matrix-
   Wells are evenly coated with Matrigel by a proprietary coating process*
   Only truly invasive cells move through membrane

Rigorous QC Testing-
   Each lot is tested for its ability to allow invasion of invasive cells while blocking non-invasive cells
BD BioCoat™ 24-well Tumor Invasion System

Inter- and Intra-lot Reproducibility

Inter-assay Reproducibility

3T3 and HT-1080 Fluorescent Units (FU)
BD BioCoat 24-well Tumor Invasion System

Z' Factor Between invasive HT-1080 and Non-invasive 3T3 Cells

Average Z' = 0.70
BD BioCoat 96-well Tumor Invasion System

Mean Percent Invasion

% invasion

replicate #
Z' Factor between Invasive HT-1080 and Non-invasive 3T3 Cells

Average Z' = 0.66
Criteria for a Robust Migration/Invasion Assay on the BD FluoroBlok™ Insert system

**definition:**
- Positive: Cells with chemoattractant (FBS)
- Negative: Cells without chemoattractant
- Blank:
  - Pre-label: zero time point is blank
  - Post-label: unlabeled cells is blank

**assay criteria:**
- %CV
- Dynamic range
- \[ Z' = 1 - \frac{3\times SD_{POS} + 3\times SD_{NEG}}{mean_{POS} - mean_{NEG}} \]*
  
  >=0.5 is preferred

*Zhang, et. al., (1999).*
Inhibition of Invasion & Migration by Paclitaxel

HT-1080

Inhibition of Invasion/Migration
HT1080 Cells by Paclitaxel

Activity
Invasion
Migration

Invasion IC50 = 18.41
Migration IC50 = 49.7

PC-3

Inhibition of Invasion/Migration of PC-3 cells by Paclitaxel

Activity
Invasion
Migration

IC50 = 16.56
IC50 = 35

MDA-MB-231

Inhibition Of Invasion/Migration of MDA-MB-231 Cells By Paclitaxel

Activity
Invasion
Migration

Invasion IC50 = 30.65
Migration IC50 = 37.65

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Inhibition of Invasion & Migration by Doxycycline

HT-1080

Inhibition of Invasion/Migration
HT1080 Cells by Doxycycline

Activity
Invasion
Migration

Invasion IC50 = 68.57
Migration IC50 = 66.84

PC-3

Inhibition of Invasion/Migration
PC3 Cells by Doxycycline

Activity
Invasion
Migration

Invasion IC50 = 38.84
Migration IC50 = 93.96

MDA-MB-231

Inhibition of Invasion/Migration
MDA-MB-231 Cells by Doxycycline

Activity
Invasion
Migration

Invasion IC50 = 89.56
Migration IC50 = 76.76
Inhibition of Invasion & Migration by 1’10’ Phenanthroline

HT-1080

PC-3

MDA-MB-231

Inhibition of Invasion/Migration
HT1080 Cells by 1’10’ Phenanthroline

Inhibition of Invasion/Migration
PC-3 Cells by 1’10’ Phenanthroline

Inhibition of Invasion/Migration
MDA-MB-231 Cells by 1’10’ Phenanthroline

IC50 values:
- HT-1080: 17.17
- PC-3: 4.05
- MDA-MB-231: 3.51

Graphs showing the inhibition of invasion and migration for each cell line with different concentrations of 1’10’ Phenanthroline.
Detection instrument

Plate readers that are not 100% compatible with the BD FluoroBlok™ Insert Systems:

MDS FlexStation II
   can only be used if the carrier device is removed
   24-Multiwell format only

MDS Analyst HT and Analyst GT
   are compatible with the 96-Multiwell format
   not the 24-Multiwell format, as the door is too short

PerkinElmer Fusion
   beam size is too large for 24-well plates
   autofluorescence
Confirm the plate map supplied with the reader or input the correct coordinates and well diameter into the plate map.

Make sure the correct diameter is used: 6.5 mm for 24-well, and 3.2 mm for 96-well.

Single or multiple reads per well?

For 24-multiwell, multiple reads are generally available. Single read focuses on center of the membrane, and multiple reads follow a reader-specific pattern, which gives a within-well average.

Overall CV improves with multiple reads, however it will take longer to read a plate.

Plate reader set-up guide

Specific Applications

Multiplexing
CD155 (Polio Virus Receptor) plays a key role in cell motility during tumor cell invasion and migration

Two color invasion/migration
HT1080 labeled w/CellTracker Orange –FITC

Inhibition of CD155 by D171(MAb) decreased invasion through Matrigel of HT1080 cells

Chemotaxis Study: To Determine Therapeutic Potential of SDF-1 in Hematopoietic Stem Cell Mobilization

Cells: SUP-T1, Lymphoblastic Leukemia Cell Line
Receptor: SDF-1 analogs (CXC chemokine stromal cell-derived factor-1)
Chemoattractant: Peptide agonist


Labeled with Vybrant® CFDA SE
3 µm BD FluoroBlok™ Inserts