

BIOCOAT[®] HTS

Caco-2 Assay System

Catalog No. 354801
(1 plate kit)

Catalog No. 354802
(5 plate kit)

Instructions for Use

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Use restriction for Europe and the United Kingdom – This product may only be used as in *in-vitro* laboratory reagent. This product and its residue must not be allowed to come into contact with ruminating animals or swine.

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BIOCOAT® HTS Caco-2 Assay System

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1.0 INTENDED USE

The BIOCOAT[®] HTS Caco-2 Assay System is an integrated, single-use, automation-friendly cell environment designed to promote the rapid differentiation of Caco-2 cells *in vitro*. Each system contains a fibrillar collagen-coated 24-well insert suitable for robotic screening of prospective pharmaceuticals for oral bioavailability and absorption. For maximum convenience in high throughput screening assays, each system includes a uniquely designed Feeder Tray and automation-friendly lid. BIOCOAT[®] HTS Caco-2 Assay Systems are specially engineered for use with robot systems that accept FALCON[®] 24-well plates. The system may also be used manually with individual or multichannel pipettors, if desired.

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California Proposition 65 Notice

WARNING:	This product contains a chemical known to the state of California to cause cancer, birth defects and/or other reproductive harm.
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Component:	Progesterone, Testosterone, Estradiol, Streptomycin Sulfate
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2.0 SUMMARY

Investigation into the transport of compounds and infectious agents across the intestinal wall is of enormous importance in the development of new pharmaceuticals. The ability to predict the bioavailability of orally administered compounds can be a powerful tool to screen for potential new drug candidates. Until recently, bioavailability data was generated with the use of *in vivo* animal models. However, animal testing is elaborate, expensive and difficult to scale-up for increased throughput.

In search of a simpler, less expensive and higher throughput alternative to animal models, *in vitro* model systems have been developed that use cells of human origin, such as the human colon adenocarcinoma-derived cell line Caco-2^{1,2}. Among the drawbacks associated with *in vitro* systems are requirements for medium supplementation with undefined animal serum and for extended culture periods to obtain the differentiated phenotype of intestinal absorptive cells (enterocytes). In the case of Caco-2 cells, it has been reported that the establishment of mature barrier and transport functions in a serum-containing environment takes between two and four weeks^{2,3}.

These two concerns have been addressed by the development of the BIOCOAT® HTS Caco-2 Assay System. Using this system, a differentiated enterocyte-like monolayer with barrier function can be established within three days in a serum-free environment. The system integrates BIOCOAT® Fibrillar Collagen Cell Culture Inserts (1µm membranes with a thin layer of native type I collagen fibrils) and a defined, serum-free medium supplemented with butyric acid, hormones, growth factors, and other metabolites. Thus the BIOCOAT® HTS Caco-2 Assay System can be used to rapidly create an *in vitro* intestinal model for transport and absorption studies of drugs and natural compounds, as well as for studies involving infectious agents in the intestine.

3.0 MATERIALS PROVIDED WITH THE BIOCOAT HTS CACO-2 ASSAY SYSTEM

Basal Seeding Medium (DMEM-based) for growing Caco-2 cells prior to differentiation (250ml, Part Number 05495).

Entero-STIM™ Medium, a serum-free, fully defined medium containing butyric acid. Butyric acid induces differentiation of intestinal epithelial cells *in vitro* via down-regulation of *c-myc* expression⁴ (250ml - Part Number 05496).

MITO+™ Serum Extender, a concentrated lyophilized formulation of hormones, growth factors and other metabolites required for the maintenance of cells under serum-free conditions (contains EGF, human transferrin, insulin, ECGS, triiodothyronine, hydrocortisone, progesterone, testosterone, estradiol-17B, selenium and *o*-phosphorylethanolamine), that will supplement 500ml of medium (Part Number 356007)

NOTE: The human source material used in the manufacturing of MITO+ Serum Extender was tested and found nonreactive for hepatitis B surface antigen (HBsAG), for antibody to hepatitis C virus (anti-HCV), for antibody to human immunodeficiency virus-1 (anti-HIV-1) and for antibody to human immunodeficiency virus-2 (anti-HIV-2). Regardless of the test data, this product should be handled observing the same Universal Safety Precautions employed when handling any potentially infectious material.

BIOCOAT® HTS Fibrillar Collagen Multiwell™ Insert System(s), each containing 24 PET membranes (1µm pore size) with a uniform thin layer of fibrillar collagen (Cat. No. 354803). The membranes have been treated with rat tail collagen, type I, under conditions that allow *in situ* formation of large collagen fibrils. Each system contains a lid and media Feeder Tray for growing Caco-2 cells simultaneously with just one media change. Depending upon the kit ordered, either one (Cat. No. 354801) or five (Cat. No. 354802) individually wrapped Multiwell Insert Systems are included.

4.0 MATERIALS REQUIRED BUT NOT SUPPLIED FOR PREPARATION OF THE CACO-2 MONOLAYER

FALCON® 24-well plate, one 24-well plate per each 24-well Multiwell™ Insert System used. Any of the following FALCON® 24-well plates may be used with this system:

Growth Surface	Qty/Pkg	Qty/Case	Cat #
Standard TC	1/Tray	50	353047
Standard TC	6/bag	36	353226
Standard TC	10/RS Tray	60	353935
Primaria™ TC	1/Tray	50	353847
Nontreated surface	1/Tray	50	351147

Caution: Only FALCON 24-well Plates will properly fit the 24-well Multiwell Insert Plate.

FALCON® sterile pipettes: 10ml, 5ml, 2ml 0-200µl & 200-1000µl pipet device
FALCON® sterile centrifuge tubes: 50ml 70% ethanol
Tissue culture hood Trypsin/EDTA solution
Humidified tissue culture incubator, 37°C, 5% CO₂
Caco-2 cells

5.0 MATERIALS REQUIRED BUT NOT SUPPLIED FOR PERMEABILITY STUDIES

Sterile 0-200µl & 200-1000µl pipette tips
Transport buffer
Automated fluid handler or multichannel pipettor (for manual use).

6.0 PRECAUTIONS

Storage:

All components should be stored at 2-8°C. The media should additionally be stored in the dark to avoid possible deterioration by light. **DO NOT FREEZE.**

Reconstitution:

Media supplements should be added under aseptic conditions. Do not filter the supplemented media. The components of BIOCOAT® HTS Caco-2 Assay System have been tested and found negative for the presence of bacteria and fungi.

Use of Supplemented Media:

All cell culture procedures should be performed under strict aseptic conditions. Media as supplied DO NOT CONTAIN ANTIBIOTICS, as antibiotics can interfere with permeability measurements of some compounds.

The Basal Seeding Medium and Entero-STIM™ Medium must be supplemented with MITO+™ Serum Extender prior to use. We recommend that supplemented media be stored at 2-8°C in their original bottles and used within 21 days. Prior to use, pre-incubate as much medium as necessary at 37°C.

Avoid repeated warming/cooling cycles of the stock of supplemented medium, as this may adversely affect its bioactivity.

Use of Caco-2 Cells:

Barrier function may vary with the density of the Caco-2 cells prior to seeding. We recommend that Caco-2 cells be grown at $\geq 250,000$ cells/cm² prior to harvesting for seeding.

7.0 PROCEDURE FOR USE

This system requires an atypical seeding density and media change schedule. Please read these instructions carefully prior to use.

ROBOHINT if desired, many of the following steps may be automated using automated fluid handlers or robotic systems. Due to the large number of systems available, we cannot give specific instructions. However, general hints are noted for each step. For further information, we suggest contacting your equipment vendor.

7.1. Reconstitution of MITO+™ Serum Extender and Media

ROBOHINT Since each Multiwell™ Feeder tray required 35ml of media to submerge all 24 inserts per plate, there will be an excess of both Basal Seeding Medium and Entero0STIM Medium. The excess should be sufficient to account for the dead volume of most automated fluid handling systems and robots. Dispose of all excess media using the same Universal Safety Precautions employed when handling any potentially infectious material.

1. Disinfect the exterior of the vial of MITO+ Serum Extender with 70% ethanol. Reconstitute the lyophilized powder with 500µl of Basal Seeding Medium.
2. Add 250µl of reconstituted MITO+ Serum Extender to the bottle of Basal Seeding Medium
3. Add 250µl of reconstituted MITO+ Serum Extender to the bottle of Entero-STIM™ Medium.

- Both supplemented media are stable at 2-8°C for up to 21 days when stored in their original bottles.

7.2 Seeding of Cells and Cell Growth Phase

ROBOHINT To fill the Feeder Tray automatically, use any of the 24 sampling ports to access the bottom of the tray. The lid may be lifted using either suction or robotic grippers from the side. The lid is non-directional and may be placed back on the Insert Plate in either direction. For best results we recommend careful placement of the lid onto the Insert Plate & Feeder tray to prevent "bouncing".

- Remove Caco-2 cells from the culture flask using Trypsin/EDTA solution. For best results, we recommend that Caco-2 cells should be $\geq 250,000$ cells/cm² prior to harvesting by trypsinization. After Trypsin/EDTA has been removed or neutralized, re-suspend cells in pre-warmed (37°C) Basal Seeding Medium supplemented with MITO+ Serum Extender to a density of 4×10^5 cells/ml.
- Add 500µl of the cell suspension from step 7.2.1 to each insert. (This will result in a seed density of 200,000 cells per insert or 6.6×10^5 cells/cm²).
- Add 35ml pre-warmed (37°C) Basal Seeding Medium supplemented with MITO+ Serum Extender (Step 7.1.2) to each Multiwell™ Feeder Tray.
- Incubate at 37°C, 5% CO₂, and 100% humidity for 20-24 hours.

7.3 Addition of Entero-STIM™ Medium, Cell Differentiation Phase.

ROBOHINT To aspirate the Feeder Tray automatically, use any of the 24 sampling ports to access the bottom of the Tray. The 24 wells of the insert plate are indexed to a FALCON 24-well format. Note that the center of each insert is offset slightly from the corresponding 24-well plate to facilitate placement of the sampling ports.

- Carefully remove the Basal Seeding Medium, after completion of step 7.2.4, both from the plate well (basal aspect) and the interior of each insert plate (apical aspect).

CAUTION: Excessive aspiration force may cause damage to the fibrillar collagen coating and/or the cell monolayer, which could result in erroneous permeability measurements.

- Add 500µl pre-warmed (37°C) Entero-STIM™ Medium supplemented with MITO+™ Serum Extender to the interior of each insert. Then, add 35ml pre-warmed (37°C) Entero-STIM Medium supplemented with MITO+ Serum Extender (Step 7.1.3) to the Multiwell™ Feeder Tray.
- Incubate at 37°C, 5% CO₂, 100% humidity for 44-48 hours. It has been determined by mannitol diffusion that Caco-2 cells grown in this fashion are differentiated within this time-frame.^{5,6,7}

8.0 RECOMMENDED PROCEDURES FOR PERMEABILITY STUDIES

NOTE: This portion of the procedure need not be conducted aseptically.

For permeability studies, cell monolayers grown on the BIOCOAT[®] HTS Caco-2 Assay System should be placed onto a FALCON[®] 24-well plate for analysis of compound permeability.

Although the lid and Feeder Tray are non-directional, the insert plate is designed to be placed on FALCON[®] 24-well plates to prevent cross contamination of wells. To properly align the Multiwell[™] Insert Plate with any FALCON[®] 24-well plate, make sure the FALCON[®] logos on the top of both pieces face the same direction. As a further check, the sampling ports at the ends of each Multiwell Insert Plate well should be on the same side as the 2 corner notches on the side of any FALCON[®] 24-well plate.

ROBOHINT Using the Multiwell Insert System for Permeability Studies:

1. If desired, the insert plate plus lid may be lifted together with robotic grippers. Under certain handling conditions, media may drip slightly from the Multiwell Insert Plate during this transition. Prior to use, we recommend a thorough "dry-run" of the BIOCOAT[™] HTS Caco-2 Assay system to minimize these effects and other possible problems prior to use. Use Universal Safety Precautions for any decontamination of media during use of this or any other product.
2. When used with a companion FALCON 24-well plate, sampling of wells beneath each membrane may be done with standard 200 or 100 μ l pipet tips or automated fluid handler tips. Top sample ports are indexed in a standard FALCON 24-well grid.

8.1 Apical/Basal Permeability

1. Remove the BIOCOAT[®] HTS Multiwell Insert Plate from its Feeder Tray and place directly on a FALCON 24-well plate. (Note: This plate is not supplied. See MATERIALS section for ordering information).
2. Gently remove Entero-STIM Medium from each insert. Wash gently with transport buffer, if desired.
3. Gently add 300 μ l of test material dissolved in transport buffer to the inside of each insert. (Alternate volumes may be used - See Table 1).
4. Add 1000 μ l of transport buffer to each well of the FALCON[®] 24-well plate. (Alternate volumes may be used - See Table 1).

TABLE 1: Recommended Volume Pairings

Volume in well (apical side)	Corresponding volume in plate well (basal side)
300µl	1000µl
400µl	1200µl
500µl	1400µl

NOTE: Addition of a volume >500µl may cause test material to overflow into the lower chamber.

5. Incubate at appropriate temperature with optional shaking (typically 50 rpm). Shaking will minimize unstirred water layer effects which can affect compound permeability measurements. The assay temperature, length of incubation time, and shaking parameters, should be experimentally determined by each user for their particular compounds.
6. Determine the concentration of test material in the lower chamber by removing aliquots through each sampling port. Sample ports are designed to accommodate 200µl and 1000µl pipette tips. For recommendations on liquid scintillation counting, see enclosed TROUBLESHOOTING HINTS Question 5. (See SA-023 enclosed with kit).

8.2 Basal/Apical Permeability

1. Remove the BIOCOAT® HTS Multiwell™ Insert Plate from the Feeder Tray and place directly on a FALCON® 24-well plate. Cells may be gently washed with transport buffer, if desired. (NOTE: This plate is not supplied. See MATERIALS section for ordering information).
2. Gently remove Entero-STIM™ Medium from each insert. Wash gently with transport buffer, if desired.
3. Gently add 300µl of transport buffer to the inside of each insert. (Alternate volumes may be used - See Table 1).
4. Add 1000µl/well of test material dissolved in transport buffer to each well of the FALCON 24-well plate. Alternate volumes may be used - See Table 1. (NOTE: This plate is not supplied. See MATERIALS section for ordering information).
5. Incubate with optional shaking at ~50 rpm (if desired) for a predetermined period of time at appropriate temperatures (see Step 8.1.5).
6. Determine the concentration of test material in the upper chamber by removing and testing aliquots.

9.0 TYPICAL RESULTS

The BIOCOAT[®] HTS Caco-2 Automated Assay System will provide the following results when used according to instructions.

- a. Confluent monolayer of cells is observed within 24 hours of seeding.
- b. Barrier function as measured by mannitol permeabilities is established 48 hours after adding supplemented Entero-STIM[™] Medium. Mannitol Papp $\sim 4 \times 10^{-6}$ cm/sec typically result when the assay is performed at room temperature, with PBS as transport buffer. (NOTE: effective growth area per well is 0.31 cm²).

For more information regarding the characterization of the BIOCOAT HTS Caco-2 Automated Assay System, contact our Technical Service Department (See Section 13.0).

10.0 ALTERNATE PROCEDURES

NOTE: We recommend that any modification to the suggested protocol be validated by the user prior to use.

1. Extending the cell growth phase to accommodate working schedules:

Once the supplemented Entero-STIM Medium is added, differentiation begins and the monolayers should be used 44-48 hours later. After that, the monolayers may become leakier.

If some variation to the 3 day schedule is desired, some of the steps in the growth of the monolayer may be adjusted.

Step 7.2 (cell growth) timeframe of 20-24 hours in the Procedure for Use is not critical. Cells can be grown in the MITO+[™] Serum Extender - supplemented Basal Seeding Medium for up to 3 days, as long as the medium does not get depleted (pH shift) during the growth phase. It is, therefore, possible to start cell seeding on Friday afternoon and change to supplemented Entero-STIM Medium on Monday morning, leading to monolayers 48 hours later on the following Wednesday. In some cases, medium may be depleted before 3 days in which case a media change would be required. If desired, additional Basal Seeding Medium and MITO+ Serum Extender may be purchased separately as Cat. No. 355058.

2. Creating Longer Lasting Differentiated Cell Monolayers

If longer lasting monolayers are desired, the following modifications to the protocol will prolong the life of the monolayer by several days. The modification calls for diluting the Entero-STIM Medium 1:10 with additional Basal Seeding Medium (Cat. No. 355257) and using the resulting solution to achieve differentiation. (Both products should still be supplemented with MITO+ Serum Extender prior to use). The advantage of this modification is that it provides a longer lasting monolayer. The disadvantage of this modification is that some differentiation marker & transport function enzyme activities (*e.g.*, alkaline phosphatase, P-Glycoprotein) may not be “upregulated” or active to the same extent as in the typical protocol. It is also possible to maintain monolayers in Entero-STIM™ Medium by changing the medium every 24 hours.

11.0 STABILITY

The components of the BIOCOAT® HTS Caco-2 Assay System are stable for at least 3 months from the date of shipment when stored at 2-8°C. Upon supplementation with MITO+™ Serum Extender, both the Basal Seeding Medium and Entero-STIM Medium are stable for up to 21 days when stored in their original containers at 2-8°C under subdued lighting conditions.

12.0 RELATED PRODUCTS AVAILABLE

55058 Intestinal Differentiation Media Pack (1 pack)
355257 Basal Seeding Medium, 500ml
355357 Entero-STIM™ Enterocyte Differentiation Medium, 500ml

13.0 TECHNICAL SERVICES

For technical information, please call our Technical Service Department at (800) 343-2035, or e-mail us at: mail@bdl.bd.com.

Visit our Worldwide Web site at <http://www.bd.com/labware/> for further Technical Information.

14.0 REFERENCES

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15.0 ORDERING INFORMATION

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