

## Technical Bulletin #418

# Use of the 3-Day BD BioCoat™ HTS Caco-2 Assay System for Compound Permeability Measurements

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## Introduction

Previously, we described the development of a Caco-2 assay system that allows for the formation of a differentiated Caco-2 cell monolayer suitable for compound permeability in 3 days<sup>1</sup>. Comparisons of the 3-day Caco-2 Assay System with traditional 21-day Caco-2 cultures have demonstrated that both culture systems possess comparable Caco-2 differentiation characteristics<sup>2,3</sup>. BD Biosciences recently developed an automation compatible format for the 3-day Caco-2 Assay System<sup>4,5</sup>. Here we report the use of the automation compatible system in compound permeability measurements, and some of the elements of the assay system that must be controlled to ensure optimal system performance.

Many of the differentiation markers of Caco-2 function were assessed in the BD BioCoat™ HTS Caco-2 Assay System. The performance of the system was also tested to assess its ability to measure compound permeabilities, and evaluations of intra- and interassay variation were also performed. The effect of many assay variables on overall system performance will be presented along with key variables to control for optimal system performance. Based upon the data presented, we believe that the 3-day HTS Caco-2 Assay System, when used under controlled conditions, provides researchers with a quick, reliable, easy to use, automatable assay system for use in compound permeability screening.

## Materials and Methods

**Cell Culture:** Caco-2 cells (ATCC) were cultured in DMEM + 20% FBS in BD Falcon™ TC flasks. Cells were grown to different degrees of confluency prior to seeding into the BD BioCoat HTS Caco-2 Assay System (BD Biosciences). Caco-2 Cells were cultured using the BD BioCoat HTS Caco-2 Assay System as per manufacturer's instructions. Briefly, cells were seeded at  $4.65 \times 10^5$  cells/cm<sup>2</sup> in Basal Seeding Media containing Mito+ Serum Extender onto Fibrillar Collagen coated HTS Mutiwell Insert Systems and incubated for 24 hours. Then, media was changed to Entero-STIM Differentiation Media supplemented with Mito+ Serum Extender and cells were incubated for an additional 48 hours. Cells were then rinsed 2-3x with PBS (Cell Grow) and barrier function assessed by Mannitol Permeability assay as described below. Mannitol Permeability data is presented in **Figures 1 and 2**.

**Differentiation Marker Assessment:** Caco-2 cells, cultured as described above, were tested for the presence of various cell differentiation markers. These include Alkaline Phosphatase, Brush Border Peptidases, and P-Glycoprotein activity. These assays were performed as previously described<sup>1,2</sup>. Cells cultured in the BD BioCoat HTS Caco-2 Assay System were compared with cells cultured in individual inserts. The data for these comparisons is presented in **Figures 3 and 4**.

**Mannitol Permeability Assay:** After cells were cultured in the BD BioCoat HTS Caco-2 Assay System, Mannitol Permeability measurements were performed. Media was removed from the cells, and the cell monolayers were washed 2-3x with transport buffer. Immediately after washing, the Insert Plate was placed into a BD Falcon 24-well plate. An appropriate amount of transport buffer was added to the basal side of the insert system, and transport buffer containing 3H-Mannitol was added to the apical side of the insert system. Cells were incubated for an appropriate time at room temperature with 3H-Mannitol. After incubation, samples were taken from the basal side of the insert system and the amount of 3H-Mannitol transported was determined by scintillation counting. A Mannitol Permeability coefficient (P<sub>c</sub>) was then calculated. To determine which variables may affect Mannitol Permeability results, a variety of assay parameters were tested. These variables included: assay time (30 vs. 90 minutes), type of transport buffer (PBS with or without Ca<sub>2+</sub>), Mannitol concentration ( $5 \times 10^5$  cpm/ml vs.  $1 \times 10^6$  cpm/ml), and cell culture conditions (Feeder Tray vs. 24-well plate). The results of these experiments are presented in **Figures 5 and 6**.

**Consistency of Results:** To determine both the intra- and interassay reproducibility of the BD BioCoat HTS Caco-2 Assay System, Mannitol Permeability determinations using the experimentally determined optimal conditions were conducted on several lots of the BD BioCoat HTS Caco-2 Assay System. Average Mannitol P<sub>c</sub> and % CV were determined and reported in **Figures 7 and 8**.

### Effect of Cell Density Prior to Assay on Cell Barrier Formation

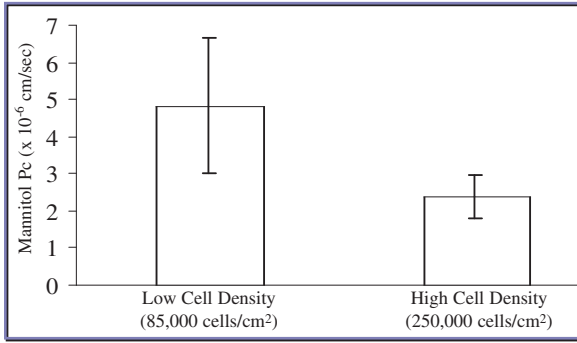
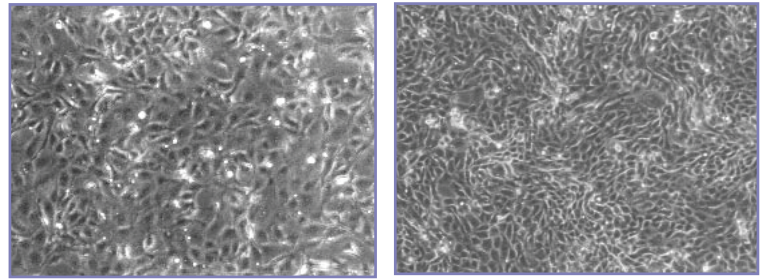


Figure 1. Caco-2 cells were cultured in T-flasks to either low (85,000 cells/cm<sup>2</sup>) or high (250,000 cells/cm<sup>2</sup>) density prior to use in the BD BioCoat™ HTS Caco-2 Assay System. Cells were seeded into the BD BioCoat Caco-2 Assay System and cultured as per manufacturer's instructions. Mannitol Permeability determinations were then performed as previously described<sup>2</sup>. Results are average of n = 12 ± CV.

### Appearance of Caco-2 Cell Cultures at Different Growth States

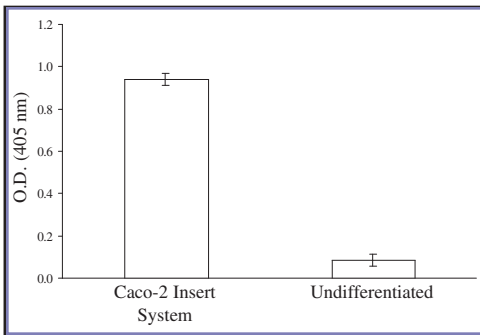


(a) Low Cell Density (85,000 cells/cm<sup>2</sup>)

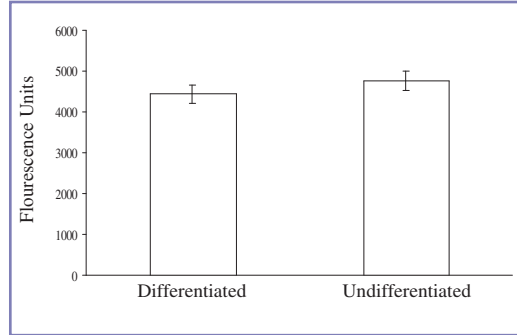
(b) High Cell Density (250,000 cells/cm<sup>2</sup>)

Figure 2. Caco-2 cells were cultured in DMEM + 20% FBS for different lengths of time. The cells in (a) were grown to a density of 85,000/cm<sup>2</sup>. The cells in (b) were grown to a density of 250,000 cells/cm<sup>2</sup>. Note that even at lower densities the Caco-2 cells can completely cover the growth surface of the TC vessel.

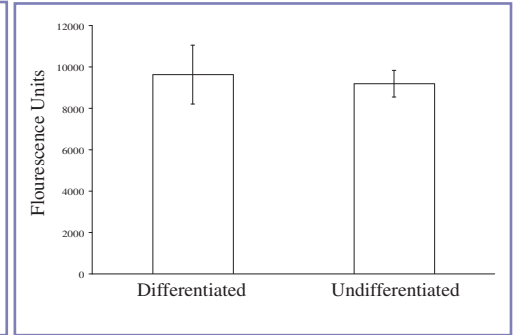
### Alkaline Phosphatase Activity in BD BioCoat Caco-2 Assay System



### Dipeptidylpeptidase IV Activity in BD BioCoat HTS Caco-2 Assay System



### Aminopeptidase N Activity in BD BioCoat HTS Caco-2 Assay System



### P-Glycoprotein Activity in BD BioCoat HTS Caco-2 Assay System

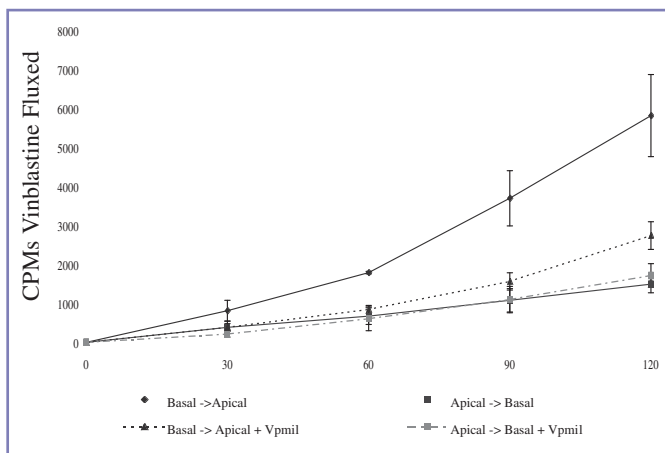


Figure 4. P-glycoprotein activity was assessed in the BD BioCoat HTS Caco 2 Assay System as previously described<sup>2</sup>. Data is average of n=3 for each condition ± S.D.

### Control of Cell Growth Conditions

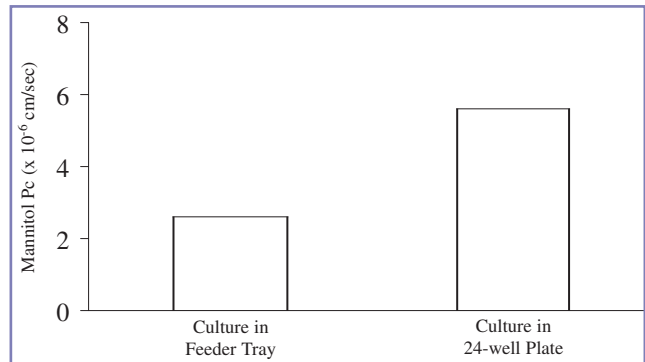


Figure 5. Caco-2 cells were used in the BD BioCoat HTS Caco-2 Assay System as per manufacturer's instructions. Some cells were cultured using a single compartment Feeder Tray (provided with the Assay System), while others were cultured using a 24-well plate containing 1 ml of media. After culture, Mannitol Permeability measurements were performed as previously described<sup>2</sup>. Data represents average of n=24 ± CV.

### Control of Compound Permeability Assay Conditions

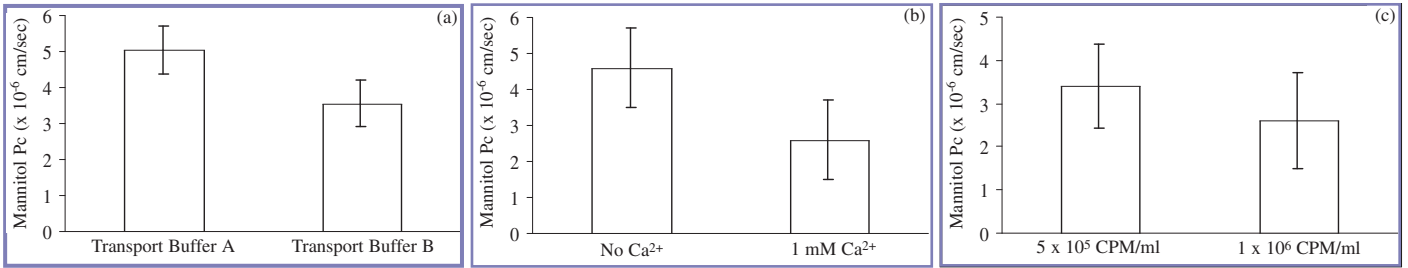


Figure 6. Several compound permeability assay conditions were tested for their effect on permeability measurement. Caco-2 cells were cultured in the BD BioCoat™ HTS Caco-2 Assay System according to manufacturer's instruction. After culture, Mannitol Permeability assays were conducted on the cells as previously described. The effect of various transport buffers (panels a and b), and compound concentrations (c) were tested. Compound permeability measurements performed at either 30 or 90 minutes showed no difference in Mannitol Pc values (data not shown). Data presented is the average of n = 24± CV

### Consistency of BD BioCoat HTS Caco-2 Assay System

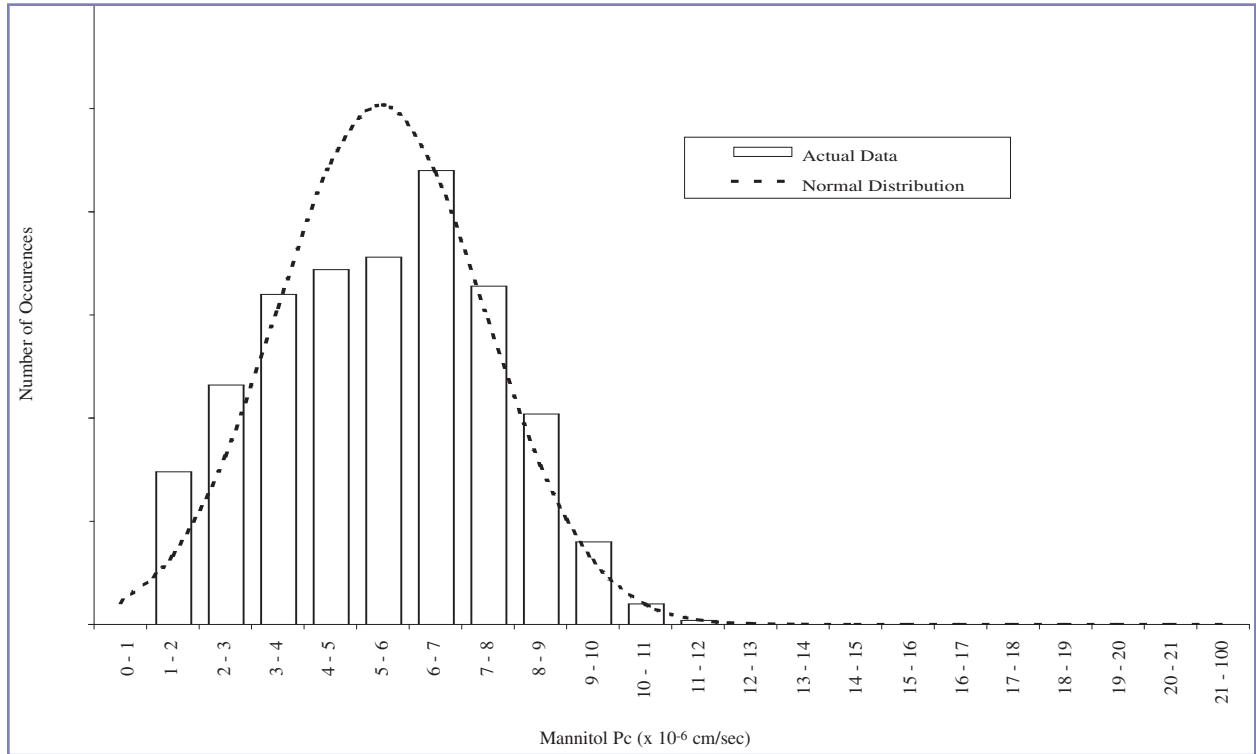


Figure 7. The intra- and interassay performance of the BD BioCoat HTS Caco-2 Assay System was assessed by performing Mannitol Permeability measurements at different times on Assay Systems from 3 different lots, using 26 different plates (619 wells) and 5 separate operators. Data presented is the distribution of Mannitol Permeability results of all 619 wells tested.

### Reproducibility of BD BioCoat HTS Caco-2 Assay System

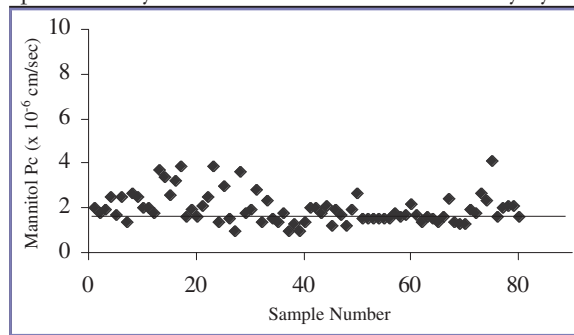


Figure 8. BD BioCoat HTS Caco-2 Assay System reproducibility was assessed by performing Mannitol Permeability measurements on several plates of a single lot of the Assay System. Data presented represents all data points from plates tested under experimentally determined optimal conditions.

## Results/Conclusions

**Cell Culture Conditions:** The BD BioCoat™ HTS Caco-2 Assay System offers a more efficient, easier to use, practical alternative to the traditional 21-day Caco-2 assay system. To achieve optimal performance with the BD BioCoat HTS Caco-2 Assay System it is necessary to control many of the variables examined here. Figures 1 and 2 demonstrate the importance of controlling the growth state of the Caco-2 cells prior to use in the BD BioCoat HTS Caco-2 Assay System. When Caco-2 cells grown to a density of 85,000 cells/cm<sup>2</sup> in TC flasks prior to use with the BD BioCoat HTS Caco-2 Assay System, they did not form as complete a barrier (Avg. Mannitol Pc=6-8, CV>30%) as compared to cells which were grown to a density of 250,000 cells/cm<sup>2</sup> (Avg. Mannitol Pc=2-4, CV>20%) (Figure 1). It is important to note that even at the lower cell density, the Caco-2 cells almost completely cover the growth surface of the flask (Figure 2). By allowing additional culture time, a higher Caco-2 cell density can be achieved. At higher cell densities, the BD BioCoat HTS Caco-2 Assay System provides a better barrier for compound permeability assessment. We speculate that by allowing Caco-2 cells to achieve a higher cell density and become contact inhibited, that many of the proliferative genes and cellular proliferation signals are downregulated. So, when the non-proliferating Caco-2 cells are exposed to the cell differentiating agents of the BD BioCoat HTS Caco-2 Assay System, the ability of the system to differentiate the Caco-2 cells is enhanced. However, if cells are in a more proliferative state, they may require more time to first downregulate proliferative signals, and then initiate cellular differentiation.

Figures 3 and 4 demonstrate the presence and comparable expression of various Caco-2 cell differentiation markers in either the BD BioCoat HTS Caco-2 Assay System, or in an individual insert environment. Both Alkaline Phosphatase and Brush Border Peptidases are expressed to similar degrees. (Figure 3) as was seen with 21-day Caco-2 cultures<sup>2</sup>. P-Glycoprotein activity in both systems is also very similar (Figure 4) to the 21-day system<sup>2</sup>. These data seem to indicate that any differences between the individual insert system and the HTS-Multiwell platform have no effect on overall Caco-2 cell differentiation.

**Mannitol Permeability Assay Variables:** Figures 5 and 6 illustrate several factors that are important to control when performing Mannitol Permeability measurements in a Caco-2 Assay System. By varying assay time, Mannitol concentration, transport buffer, and cell culture system, we were able to identify an optimal set of conditions for performing compound permeability measurements. We determined that assay time (30 minutes vs. 90 minutes) had little effect on Mannitol Pc values (data not shown). However, when transport buffer with and without Ca<sup>2+</sup> were compared, it was determined that the presence of Ca<sup>2+</sup> in the transport buffer led to better barrier. This may be due to the requirement by many of the proteins responsible for cell/cell junctional complexes for Ca<sup>2+</sup> ions to assure proper function. Lack of Ca<sup>2+</sup> ions in the

buffer could lead to weakening or dissociation of those cell/cell interactions responsible for barrier formation. The data shown in Figure 6 also demonstrates that different transport buffers can have effects on barrier function. Finally, we found that Caco-2 cells cultured in the BD BioCoat HTS Caco-2 Assay System using the unique Feeder Tray formed better barrier than those grown in individual wells of a 24-well plate. This may be due to the Feeder Tray having more media per insert relative to a 24-well plate and that when cells are in a Feeder Tray they are all exposed to common media and are not compartmentalized as in the 24-well plate growth condition.

**Assay Reproducibility:** The data in Figures 7 and 8 demonstrate the overall assay reproducibility performance of the BD BioCoat HTS Caco-2 Assay System. Using our experimentally determined optimal assay conditions, it can be seen that with at least 3 different lots of the BD BioCoat HTS Caco-2 Assay System, the results are very reproducible. Average Mannitol Pc values are very consistent when examined either well to well, plate to plate, or lot to lot. Overall % CV in any lot is also very consistent. By looking at both the distribution of Mannitol Pc results and the scatter diagram of actual data points of actual lots tested, it can be seen that not only is the overall performance of the system very good, but within any given plate or any given lot the scatter about the overall mean Mannitol Pc values are excellent. These data, taken together, demonstrate the overall excellent intra- and intersassay reproducibility and consistency of the BD BioCoat HTS Caco-2 Assay System. By further monitoring the performance of the BD BioCoat HTS Caco-2 Assay System, we expect to maintain similar performance with this system.

**Summary:** We have identified many important assay variables to consider when using the BD BioCoat HTS Caco-2 Assay System to achieve optimal performance. Our results indicate that for best performance, several experimental variables should be controlled. Specifically, it is necessary to carefully control the growth state of Caco-2 cells prior to use in the system. When performing compound permeability assays, factors such as transport buffer, compound concentration, and cell growth conditions need to be controlled. By experimentally determining and controlling our assay conditions, we have found that the BD BioCoat HTS Caco-2 Assay System is capable of providing an efficient and consistent assay system for compound permeability measurements.

## References

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