Corning® BioCoat™: Comparison of 3-day System to 21-day Protocol

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Application Note

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Introduction

Caco-2 cells are used as an *in vitro* model for screening drug candidates for their intestinal absorption potential. The traditional protocol of culturing Caco-2 cells requires up to 3 weeks of culture and involves many labor intensive steps. The Corning BioCoat Intestinal Epithelial Differentiation Environment, providing a monolayer of Caco-2 cells ready for compound permeability screening in 3 days, has been reported to be a more convenient and productive system to use to generate compound permeability data[1].

To further characterize the 3-day System and to compare its performance with the 21-day Protocol, several biological measures of Caco-2 cell differentiation were tested. Both the 3-day System and the 21-day Protocol were tested for their ability to generate a monolayer of Caco-2 cells, upregulate Alkaline Phosphatase (AP) activity, express brush border peptidase activity, and show functional P-Glycoprotein. We also report elements of the assay system that must be controlled to ensure optimal system performance.

Evaluations of intra- and interassay variation were 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, 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 Falcon® TC flasks. Cells were grown to different degrees of confluency prior to seeding into the Corning BioCoat HTS Caco-2 Assay System. Caco-2 Cells were cultured using the Corning BioCoat HTS Caco-2 Assay System as per manufacturer's instructions. Briefly, cells were seeded at 4.65 $x10^5$ cells/cm^2 in Basal Seeding Media containing Mito+ Serum Extender onto Fibrillar Collagen coated HTS Mutiwell Permeable Support Systems and incubated for 24 hours. Then, media was changed to Enterocyte Differentiation Medium supplemented with Mito+ Serum Extender and cells were incubated for an additional 48 hours. Cells were then rinsed 2-3x with PBS (Cell Grow).

21-day Protocol

Permeable supports (1.0 µm PET) were coated with type I collagen and dried. Cells were seeded onto the coated permeable supports at 50,000 cells/cm^2 and cultured for 21 days in DMEM + 10% FBS with media changes every other day. After culturing, cells were rinsed 2-3 times with PBS and further analyzed.

Differentiation Markers

To test for markers of differentiation in the 3-day System and the 21-day Protocol, activity of Alkaline Phosphatase (AP), Aminopeptidase A (APA), Aminopeptidase N (APN), and Dipeptidylpeptidase IV (DPP IV) were measured by adding the appropriate enzyme substrates directly to the permeable supports containing the cells and incubating the cells for a given time interval. AP activity was determined using pNPP and the peptidases were assayed by using the 7-amino-4 methylcoumarin derivatives of each enzyme substrate[2,3]. Cells cultured in the Corning BioCoat HTS Caco-2 Assay System were compared with cells cultured in individual permeable supports.

P-Glycoprotein Function

P-Glycoprotein activity was assessed in the Caco-2 systems by placing equal concentrations of vinblastine (10 nm) above and below the cell monolayer. One chamber or the other had a quantity of ^{3}H-Labeled vinblastine added, and the system was incubated for up to 2 hours. Samples were then counted to determine the amount of vinblastine transported from one chamber to the other. In addition, to test for the specificity of transport of the vinblastine, verapamil, a partial antagonist of vinblastine transport was added at 0.1 mm to a group of permeable supports.

Barrier Function Assessment

After cells were cultured as described, 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 Permeable Support Plate was placed into a Falcon 24-well plate. An appropriate amount of transport buffer was added to the basal side of the permeable support system, and transport buffer containing ^{3}H-Mannitol was added to the apical side of the permeable support system. Cells were incubated for an appropriate time at room temperature with ^{3}H-Mannitol. After incubation, samples were taken from the basal side of the permeable support system and the amount of ^{3}H-Mannitol transported was determined by scintillation counting. A Mannitol Permeability coefficient (Pc) 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^2+), Mannitol concentration ($5x10^5$ cpms/ml vs. $1x10^6$ cpms/ml), and cell culture conditions (Feeder Tray vs. 24-well plate). The results of these experiments are presented in **Figures 1 and 2**.

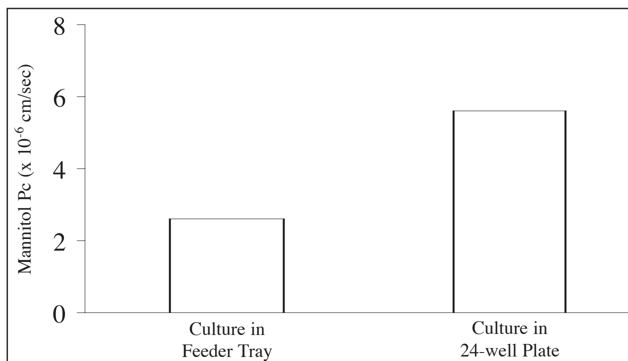


Figure 1. Control of Cell Growth Conditions

Caco-2 cells were used in the Corning 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 1.0 mL of media. After culture, Mannitol Permeability measurements were performed as previously described[2]. Data represents average of n=24 ± CV.

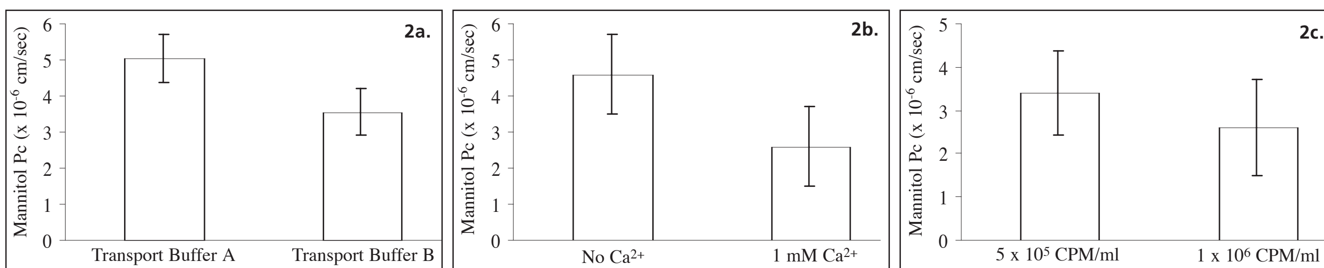


Figure 2. Control of Compound Permeability Assay Conditions

Several compound permeability assay conditions were tested for their effect on permeability measurement. Caco-2 cells were cultured in the Corning BioCoat HTS Caco-2 Assay System according to manufacturer's instruction. After culture, Mannitol Permeability assays were conducted on the cells as previously described[2]. The effect of various transport buffers (panels 2a and 2b), and compound concentrations (2c) 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 Results

To determine both the intra- and interassay reproducibility of the Corning BioCoat HTS Caco-2 Assay System, Mannitol Permeability determinations using the experimentally determined optimal conditions were conducted on several lots of the Corning BioCoat HTS Caco-2 Assay System. Average Mannitol Pc and % CV were determined and reported in **Figures 3 and 4.**

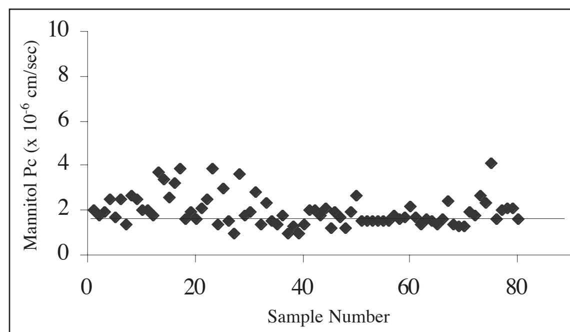


Figure 3. Reproducibility of Corning BioCoat HTS Caco-2 Assay System

Corning 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.

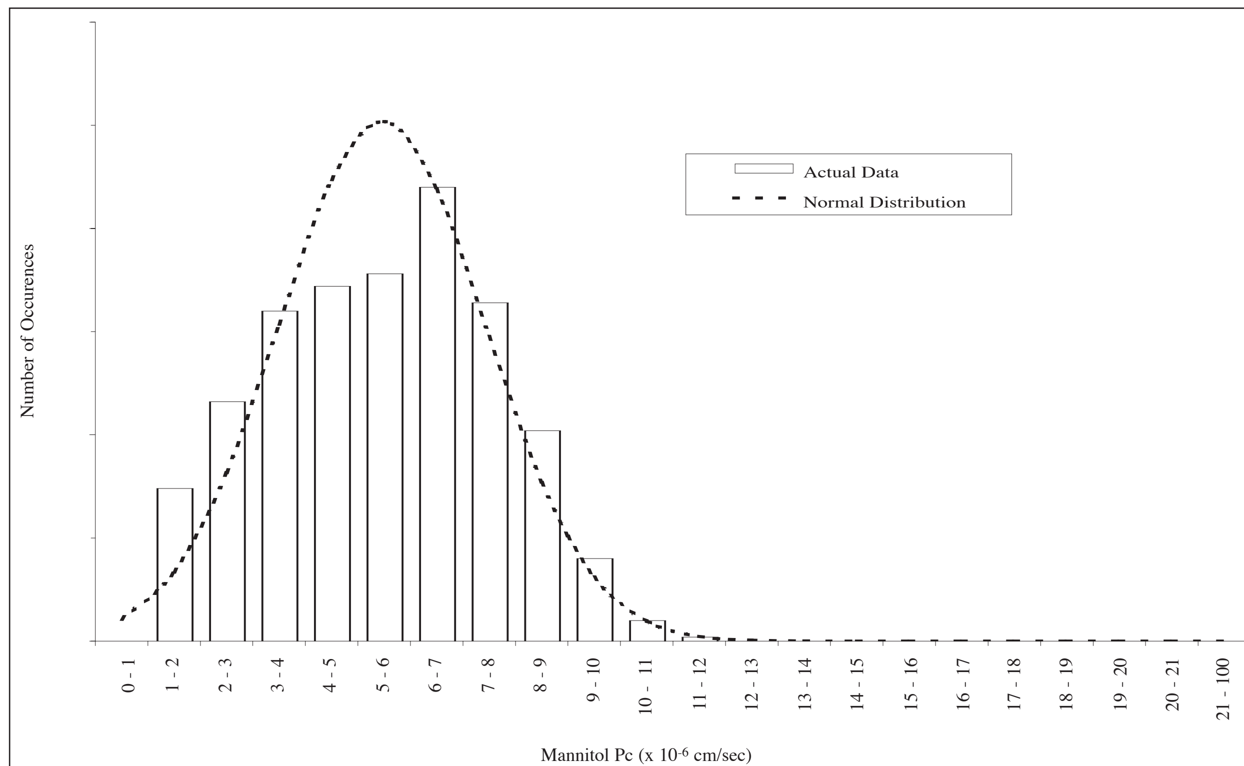


Figure 4. Consistency of Corning BioCoat HTS Caco-2 Assay System

The intra- and interassay performance of the Corning 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.

Results and Conclusions

As seen in **Figures 5a and 5b**, the Mannitol Pc obtained in the 21-day Protocol are lower (2-3 x 10^{-6} cm/sec) than those found in the 3-day Systems tested (4-6 x 10^{-6} cm/sec). By manipulating various components of the 3-day System, it can be seen that each of the components (Corning BioCoat Fibrillar Collagen Cell Culture Permeable Supports and Mito+ Serum Extender) contributes to the overall final cell barrier formation. The data in **Figure 6** shows that both the 3-day System and the 21-day Protocol upregulates AP activity relative to the undifferentiated control. In the 21-day Protocol we see about a 2-fold upregulation of enzyme activity. In the 3-day System with serum, we see a 4-fold upregulation of enzyme activity. While in the 3-day System with Mito+ Serum Extender, that upregulation is about 6-fold. So, all of the systems tested upregulate AP to some level. In fact, the 3-day Systems have even higher levels of upregulation than the 21-day Protocol. As seen in **Figures 7 and 8**, neither APN nor DPP IV showed any upregulation relative to undifferentiated Caco-2 cells in our hands. The 3-day System was almost always at the same enzyme expression level as the 21-day Protocol. Therefore, although we saw no upregulation of the peptidases in the 3-day Corning BioCoat Intestinal Environment, we saw no upregulation in the 21-day Protocol either. Finally, a negative control peptidase (APA) showed no activity in any Caco-2 System tested (data not shown).

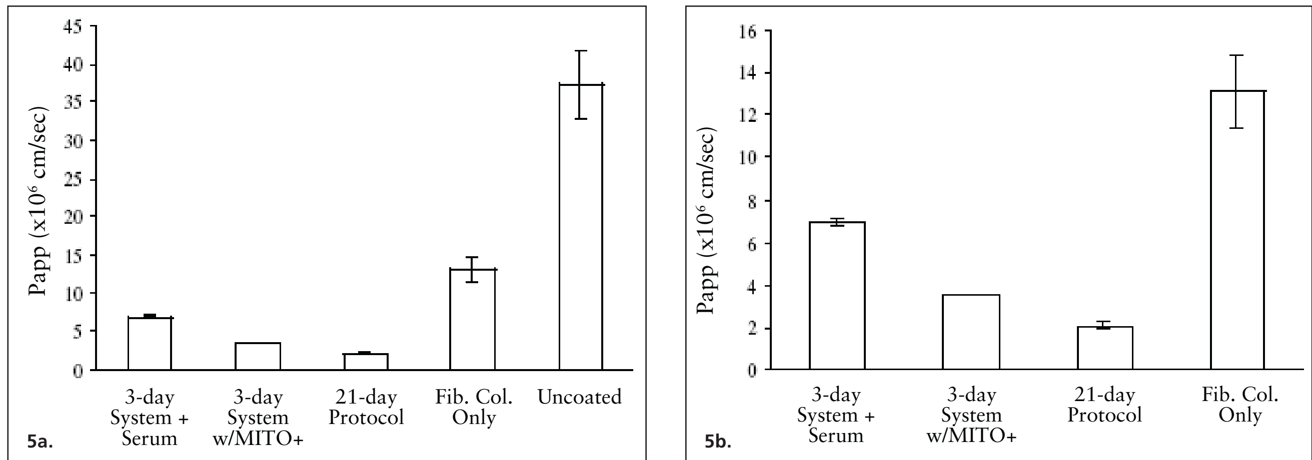
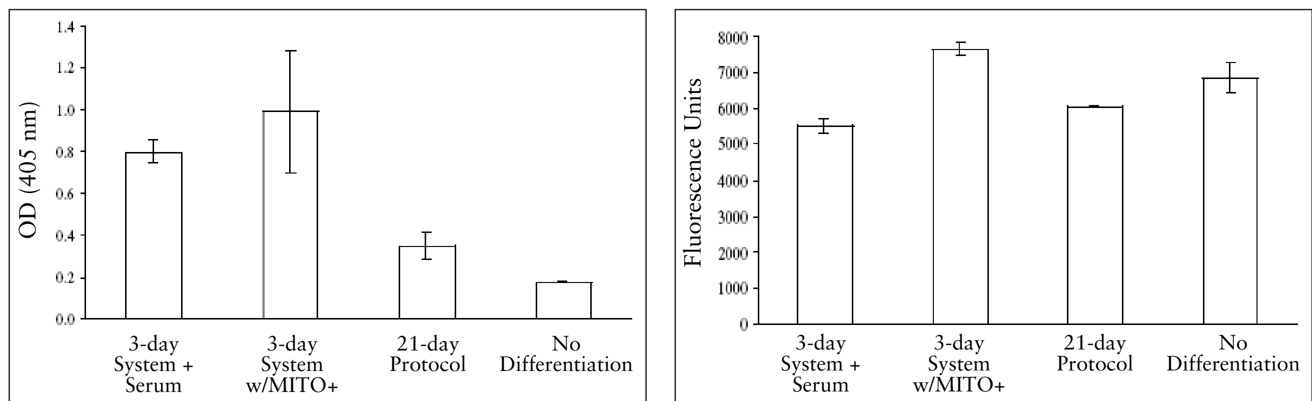


Figure 5. Mannitol Permeability Coefficients of Caco-2 Cells

"Fibrillar Collagen Only" samples did not use the differentiation media containing butyrate, but used normal growth media instead (DMEM + 10% FBS) for 3 days on Corning BioCoat Fibrillar Collagen Cell Culture Permeable Supports. Uncoated samples were grown for 3 days with no coating and normal growth media. Other culture conditions were as described above. Data shown is mean of n=3.



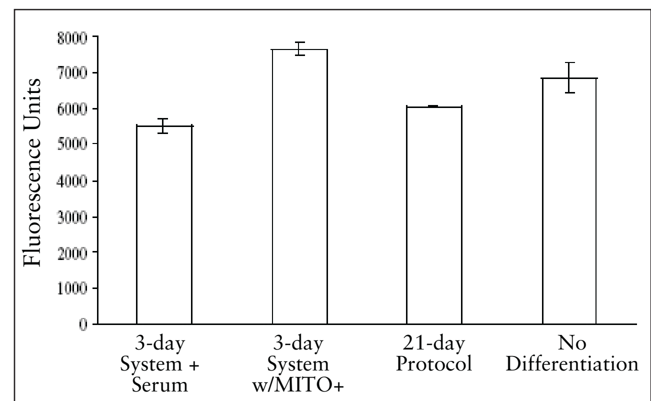


Figure 6: Alkaline Phosphatase Activity in Caco-2 Cells

AP activity was assessed in Caco-2 cells grown in various culture conditions. The 3-day System contains Corning BioCoat Fibrillar Collagen Cell Culture Permeable Supports and butyrate containing differentiation media, supplemented with either 10% FBS or Mito+ Serum Extender as indicated. The 21-day Protocol utilizes amorphous collagen coated permeable supports and normal growth media (DMEM + 10% FBS). The no differentiation system utilizes uncoated permeable supports and normal growth media and cells are cultured for 3 days. AP activity was measured using intact cells grown on permeable supports. pNPP was used as enzyme substrate and endpoint measurements were made at 20 minutes at 37°C. Data shown is mean of n=4.

Figure 7: Aminopeptidase N Activity in Caco-2 Cells

APN activity was assessed in Caco-2 cells grown in various culture conditions. APN activity was measured using intact cells on permeable supports. APN activity was measured using 200 µM Ala-7-amino-4-methylcoumarin as substrate. Activity was measured by detecting the fluorescence of the liberated 7-amino-4-methylcoumarin (excitation: 370 nm emission: 442 nm). Reactions were carried out for 60 minutes at 37°C. Data shown is mean of n=4.

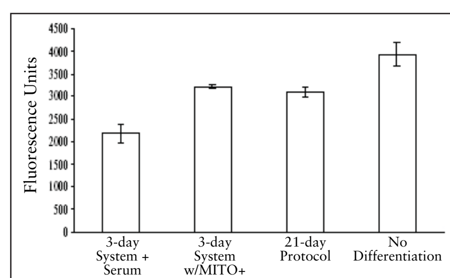


Figure 8: Dipeptidyl Peptidase IV Activity in Caco-2 Cells

DPP IV activity was assessed in Caco-2 cells grown in various culture conditions. DPP IV activity was measured using intact cells on permeable supports. DPP IV activity was measured using 200 µM Gly-Pro-7-amino-4-methylcoumarin as substrate. Activity was measured by the fluorescence of the liberated 7-amino-4-methylcoumarin product (excitation: 370 nm emission: 442 nm). Reactions were carried out for 60 minutes at 37°C. Data shown is mean of n=4.

Cell Culture Conditions

The Corning 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 Corning BioCoat HTS Caco-2 Assay System it is necessary to control many of the variables examined here. **Figures 9 and 10** demonstrate the importance of controlling the growth state of the Caco-2 cells prior to use in the Corning BioCoat HTS Caco-2 Assay System. When Caco-2 cells grown to a density of 85,000 cells/cm² in TC flasks prior to use with the Corning 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/cm² (Avg. Mannitol Pc=2-4, CV>20%) (**Figure 9**). 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 10**). By allowing additional culture time, a higher Caco-2 cell density can be achieved. At higher cell densities, the Corning BioCoat HTS Caco-2 Assay System provides a better barrier for compound permeability assessment. Allowing Caco-2 cells to achieve a higher cell density and become contact inhibited, many of the proliferative genes and cellular proliferation signals are downregulated. Therefore, when the non-proliferating Caco-2 cells are exposed to the cell differentiating agents of the Corning 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.

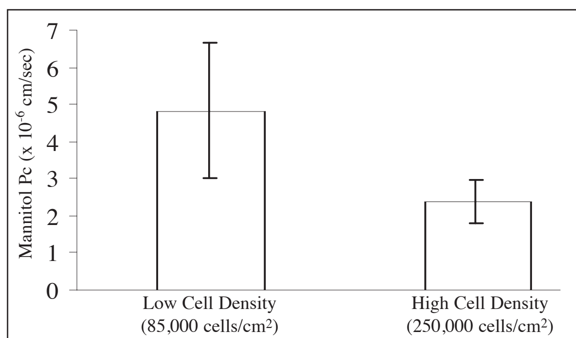
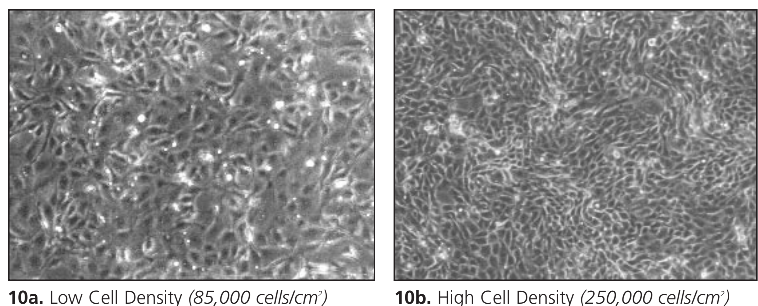


Figure 9. Effect of Cell Density Prior to Assay on Cell Barrier Formation

Caco-2 cells were cultured in T-flasks to either low (85,000 cells/cm²) or high (250,000 cells/cm²) density prior to use in the Corning BioCoat HTS Caco-2 Assay System. Cells were seeded into the Corning BioCoat Caco-2 Assay System and cultured as per manufacturer's instructions. Mannitol Permeability determinations were then performed as previously described[2]. Results are average of n=12±CV.



10a. Low Cell Density *(85,000 cells/cm²)* **10b.** High Cell Density *(250,000 cells/cm²)*

Figure 10. Appearance of Caco-2 Cell Cultures at Different Growth States

Caco-2 cells were cultured in DMEM + 20% FBS for different lengths of time. The cells in (10a) were grown to a density of 85,000/cm². The cells in (10b) were grown to a density of 250,000 cells/cm². Note that even at lower densities the Caco-2 cells can completely cover the growth surface of the TC vessel.

Differentiation Markers

Figures 11 and 12 demonstrate the presence and comparable expression of various Caco-2 cell differentiation markers in either the Corning BioCoat HTS Caco-2 Assay System, or in an individual permeable support environment. Both Alkaline Phosphatase and Brush Border Peptidases are expressed to similar degrees. (**Figure 11**) as was seen with 21-day Caco-2 cultures[2]. P-Glycoprotein activity in both systems is also very similar (**Figure 12**) to the 21-day protocol[2]. These data seem to indicate that any differences between the individual permeable support system and the HTS-Multiwell platform have no effect on overall Caco-2 cell differentiation.

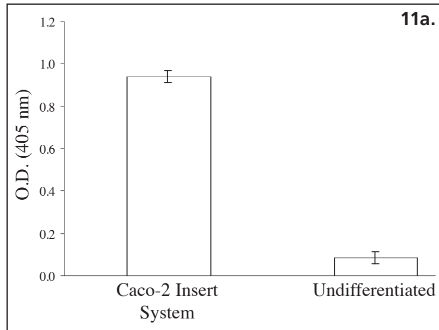


Figure 11a. Alkaline Phosphatase Activity in Corning BioCoat Caco-2 Assay System

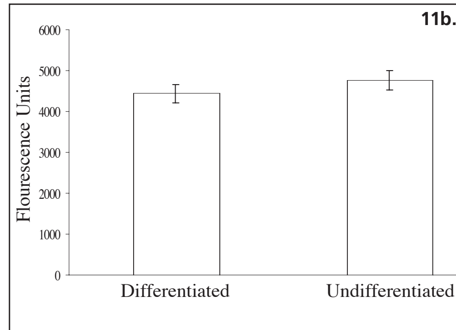


Figure 11b. Dipeptidyl peptidase IV Activity in Corning BioCoat HTS Caco-2 Assay System

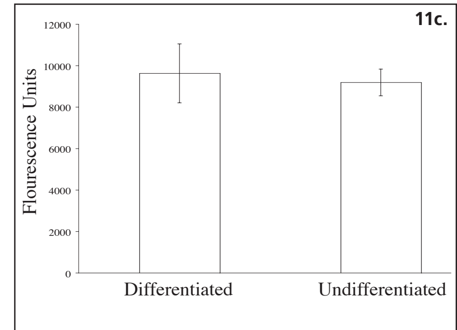


Figure 11c. Aminopeptidase N Activity in Corning BioCoat HTS Caco-2 Assay System

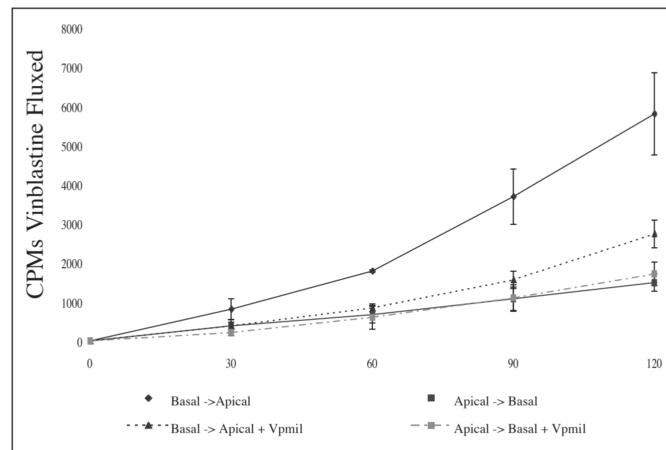


Figure 12. P-Glycoprotein Activity in Corning BioCoat HTS Caco-2 Assay System

P-glycoprotein activity was assessed in the Corning BioCoat HTS Caco 2 Assay System as previously described[2]. Data is average of n=3 for each condition ± S.D.

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, an optimal set of conditions for performing compound permeability measurements was identified. 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^2+ were compared, it was determined that the presence of Ca^2+ 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^2+ ions to assure proper function. Lack of Ca^2+ 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 affects on barrier function. Finally, we found that Caco-2 cells cultured in the Corning BioCoat HTS Caco-2 Assay System using the 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 permeable support 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 Corning 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 Corning BioCoat HTS Caco-2 Assay System, the results are reproducible. Average Mannitol Pc values are consistent when examined either well to well, plate to plate, or lot to lot. Overall % CV in any lot is also 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 good, but within any given plate or any given lot the scatter about the overall mean Mannitol Pc values are reproducible. These data, taken together, demonstrate the overall intra- and interassay reproducibility and consistency of the Corning BioCoat HTS Caco-2 Assay System.

Summary

To characterize and compare the Corning BioCoat 3-day System with the standard 21-day Protocol, several important Caco-2 differentiation characteristics have been examined. The 3-day System supports similar levels of differentiation marker enzyme activity, has properly functioning, appropriate P-Glycoprotein activity, and forms a monolayer with barrier function sufficient for screening of compounds for bioavailability. Therefore, offering a more productive alternative to the 21-day Protocol to generate the data needed for compound permeability assessments, while retaining many of the performance characteristics of the standard method. A 5-day modification of the 3-day Caco-2 System has been published by Yamashita[5].

Important assay variables have been identified when using the Corning BioCoat HTS Caco-2 Assay System to achieve optimal performance. Results indicate 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 assay conditions, the Corning BioCoat HTS Caco-2 Assay System is capable of providing an efficient and consistent assay system for compound permeability measurements.

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