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Application Note

Optimization of Caco-2 Cell Growth and Differentiation for Drug Transport Assay Studies using Millicell® 96-well Cell Culture Plates

Introduction

Understanding and testing the absorption, distribution, metabolism, excretion and toxicity (ADMET) properties of candidate compounds is critical to successful new drug discovery. The important first step, absorption, is the ability of a drug to cross the target cell membrane barrier from the point of administration to the site of action. This absorption can occur through passive transcellular or paracellular diffusion, active carrier transport or active efflux mechanisms.

Typically, an immortalized cell line is used as a drug absorption model in studies to understand the drug's permeability and absorption mechanisms. A recognized *in vitro* model system for measuring drug absorption uses the Caco-2 cell line, an immortalized, heterogeneous cell line, derived from a human colorectal adenocarcinoma.[1,2]

Our Millicell® 96-well cell culture plate with 0.4 µm polycarbonate membrane (**Figure 1**) is automation compatible and allows for the measurement of drug transport in a high throughput format. The Millicell® 96-well cell culture plate facilitates the use of *in vitro* model cell lines in the measurement of drug absorption rates (**Figure 2**). Data generated using this device can be used to rank order the oral absorption profiles of new candidate drug compounds. The following protocol provides guidance for the optimization of the growth and differentiation of the Caco-2 cell line on the Millicell® 96-well cell culture plate.

Representative drug transport results using an optimized protocol are also presented.

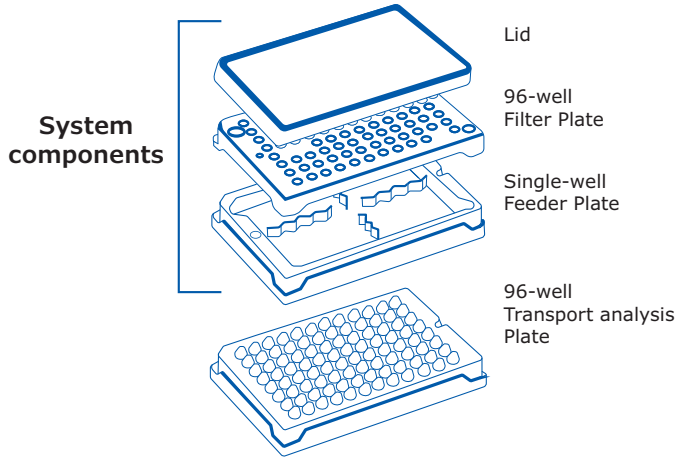

Figure 1. Millicell® 96-well cell culture plate with 0.4 µm PC membrane plate components (**PSHT004R5**, with single-well feeder plate) and 96-well transport analysis plate (**MACAC0RS5**). Not shown, Millicell® 96-well cell culture plate with 0.4 µm PC membrane plate components with 96-well feeder plate (**PSHT004S5**). The 96-well transport analysis plate can also be used for culturing.

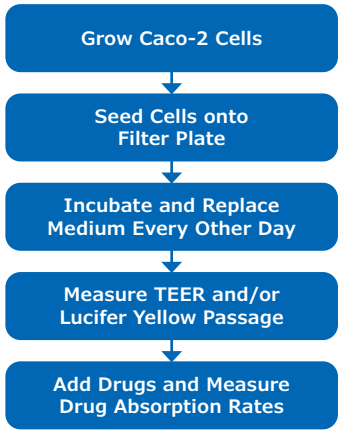

Figure 2. Drug transport study protocol using Millicell® 96-well cell culture plates and Caco-2 cells

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Protocol

1. Medium, Additives and the Cultivation of Caco-2 Cells

The following medium, additives and protocol are recommended for the cultivation of the Caco-2 cell line (**86010202-1VL**).

- Dulbecco's MEM with high glucose (**D5796**)
- Non-essential amino acids (**M7145**)
- HEPES (**H0887**)
- Penicillin, streptomycin, and L-glutamine (**G1146**)
- EDTA (**E8008**)
- Trypsin/EDTA (**T3924**)
- Fetal bovine serum (**ES009**)

a. Prepare Caco-2 cell culture medium consisting of Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium bicarbonate supplemented with:

- 10% Fetal Bovine Serum (FBS)
- 0.1 mg/mL of streptomycin
- 100 units of penicillin
- 10 mM HEPES buffer
- 1X Non-essential Amino Acids (NEAA)

b. Cultivate cells in T-75 flasks (**CLS430641U**) in a cell culture incubator set at 37 °C, 5% CO_2, 95% relative humidity. Allow cells to reach 80–90% confluence before detaching and splitting. Do not allow cells to become over confluent (>90%), as this will impact Millicell® 96-well plates.

c. Rinse cultivated cells in T-75 flasks with 5 mL EDTA. Aspirate off, add 1.5 mL trypsin/EDTA, and incubate at 37 °C for approximately 5 to 10 minutes or until the cells detach and float. This can be confirmed by periodic visual inspection of the flasks.

d. Add 12 mL of Caco-2 cell culture medium to detached cells and pipette up and down to disperse cells. Dispense 2.5 mL into six new T-75 flasks. Add 12.5 mL of Caco-2 cell culture medium for a total of 15 mL per flask and replace in cell culture incubator.

2. Optimization of Caco-2 Cell Seeding Density on Millicell® Caco-2 Assay System

Though Caco-2 cells commercially available for this application originate from immortalized cells, they are very sensitive to their environment. Varied growth and differentiation properties can result in response to culture conditions (such as source and lot of FBS), time in culture (age related to passage number), confluency at time of passage, and other environmental and laboratory factors, which can sub-select for cells with different growth characteristics[3]. For these reasons, optimizing the seeding density for the Caco-2 cells on the Millicell® 96-well cell culture plate is highly recommended.

Laboratories that routinely use 24-well filter plates (**PSHT010R5**) may elect to optimize seeding density for the 96-well filter plate based on the seeding density used for the 24-well plate. This can be accomplished by calculating the number of cells/ cm^2 (see Table 1 for converting cells/mL to cells/cm^2 to cells/well). Select routinely used seeding densities in cells/cm^2, convert to cells/96-well filter area, and follow the methods described in this procedure to evaluate the cells.

	Well surface area	Well volume	Cells/cm^2	Cells/mL	Cells/ well
24-well filter plate	0.3 cm^2	0.4 mL	82,000	61,500	24,600
96-well filter plate	0.11 cm^2	0.075 mL	82,000	120,000	9,000

Table 1. Example conversions from cells/cm^2 to cell/mL or cell/well

a. Detach Caco-2 cells from cell culture flask using trypsin/EDTA as described in step d of Step 1.d.

b. Once the cells are detached and resuspended in Caco-2 cell culture medium, count the cells to determine the cell number/mL.

c. For a 10-day Caco-2 cell culture, divide cell suspension into six sterile, 15 mL centrifuge tubes. Dilute the cell aliquots with Caco-2 cell culture medium into cell concentrations ranging from 125,000 cells/mL to 500,000 cells/mL. These cell concentrations can be used to seed 9,375 cells/well to 37,500 cells/well or 85,000 cells/cm^2 to 341,000 cells/cm^2. This is based on a seeding volume of 75 µL per filter well (see Step d below). See **Table 1** for calculations and **Figure 3**, for an example.

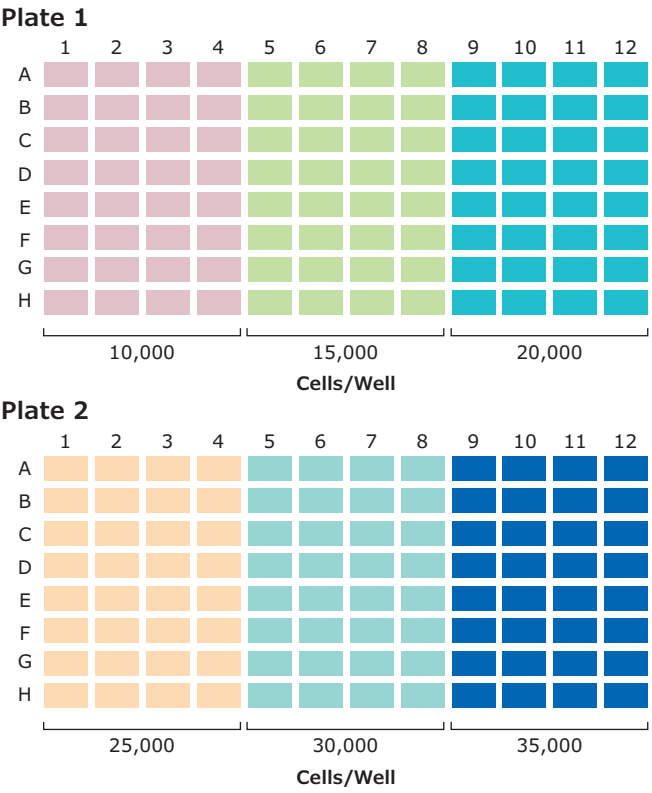


Figure 3. 10-day Caco-2 Cell Seeding Density Template

d. Dispense 75 µL of cell dilution into the filter wells of the Millicell® 96-well cell culture plates (**PSHT004R5** or **PSHT004S5**). To optimize the seeding protocol, it is recommended to set up several columns for each cell density (minimally, 16 wells per seeding concentration). See **Figure 3**, for an example.

e. Dispense 250 µL of Caco-2 cell culture medium into each of the 96 wells of the receiver plate or alternatively place 25–27 mL in the single-well feeder plate. This may be accomplished by dispensing medium through the basolateral access holes (see **Figure 4**) for the receiver plate or the large access holes located at A1 and H12 for the single-well, feeder plate. Alternatively, disassemble the filter plate from the receiver plate or single-well feeder plate. Place the filter plate on a sterile surface in a laminar flow hood and add medium directly to filter plate and receiver plate. Gently reassemble the two components, and place in the cell culture incubator.

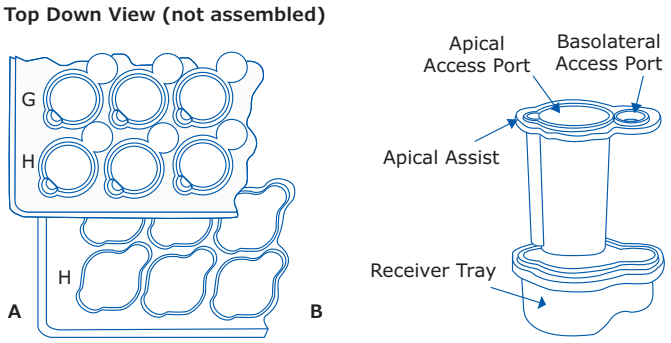
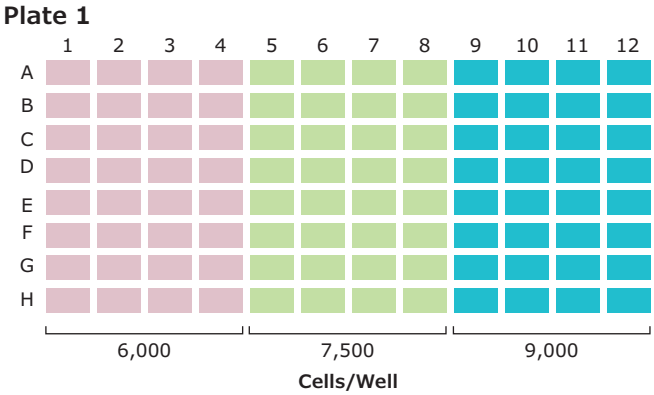


Figure 4. Patented Design of the Millicell® 96-well cell culture plate: **A.** Top down view (not assembled). **B.** Each well contains an offset apical channel, the apical assist, to guide manual pipette tips. The apical assist channel ends just short of the membrane surface to eliminate the chance of membrane or monolayer disruption while pipetting.

f. For a 21-day Caco-2 cell culture, divide cell suspension into 4 to 6 sterile 15 mL centrifuge tubes. Dilute the cell aliquots with Caco-2 cell culture medium into different cell concentrations ranging from 80,000 cells/mL to 167,000 cells/mL. (These cell concentrations can be used to seed 6,000 cells/well to 12,500 cells/well or 55,000 cells/ cm^2 to 114,000 cells/ cm^2. This is based on a seeding volume of 75 µL per filter well. See step g below). See **Table 1** for calculations and **Figure 5** for an example.



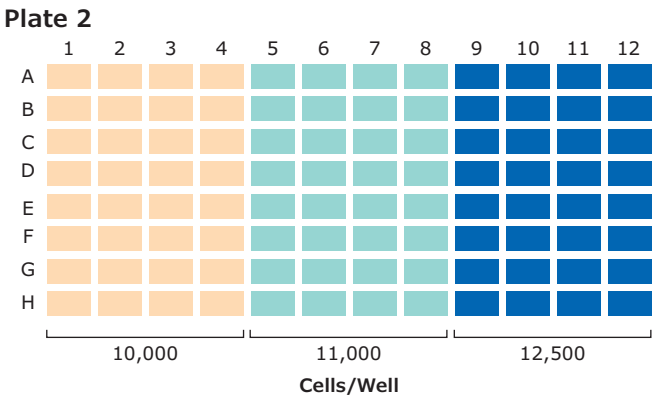


Figure 5. 21-day Caco-2 Cell Seeding Density Template.

g. Dispense 75 µL into the filter well of the Millicell® 96-well cell culture plate. For better optimization, set up several columns for each cell density (minimally 16 wells per seeding concentration).

h. Dispense 250 µL of Caco-2 cell culture medium into each of the 96 wells of the receiver plate, or alternatively, place 25 mL in the single-well, feeder plate as described in Step 2.e.

i. Incubate plates for 10 or 21 days. Replace the medium in the filter well and the growth plate every other day, beginning no sooner than 48 hours after initial plating. Refer to Step 3.

3. Exchanging the Medium in the Millicell® 96-well Cell Culture Plate System

Long-term cell culture requires exchange of medium to remove waste products that may accumulate and become toxic, and to replenish nutrients. Cells grown on the Millicell® 96-well cell culture plates for longer than 3 or 4 days typically need growth medium exchanged to sustain long-term viability of the cells. The Millicell® 96-well cell culture plate assay system has been successfully evaluated for the growth and differentiation of Caco-2 cells from as little as 10 days to as long as 21 days. Medium should be exchanged every 48 to 72 hours beginning no earlier than 48 hours after initial plating. Exchange the medium in the filter well (apical compartment) and receiver plate or single-well feeder plate (basolateral compartment). Alternatively, medium can be exchanged in the basolateral compartment and replenished in the apical compartment (bring the volume back to approximately 75 µL to replace evaporated medium lost during incubation) without removing the medium remaining in the filter well.

a. The most critical part of removing and replacing the medium in the Millicell® 96-well cell culture plate system is to avoid damage to the cell monolayer and the filter on which it is supported. In these experiments, a multichannel manifold (**M2656**) was used to aspirate the medium, and an electronic multi-well pipettor with speed control at low or medium speed was used for dispensing consistency of the fresh culture medium.

b. It is recommended to exchange the medium without disassembling the plate components. Aspirate the volume from the feeder plate directly through the basolateral access holes (see **Figure 4**) or, if using the single-well feeder plate, aspirate through the large access holes located adjacent to either A1 or H12. Guide the tips of the aspirator into the holes and carefully aspirate the medium from the filter wells. Take care not to contact the cell monolayer during aspiration. Replace the medium in the filter wells by gently pipetting 75 µL into the filter well along the apical assist and replace the medium in the feeding plate using the basolateral access holes for the receiver plate (250 µL per well) or the large access holes at either A1 or H12 for the single-well feeding plate (25 mL).

c. Alternatively, exchange media by removing the filter plate (the filter plate can be placed directly on the cell culture hood surface) from the feeding plate (receiver plate or single-well plate) and aspirating the medium from both plates using the multichannel manifold. Be careful to guide the tips of the manifold down the lower left side of the filter wells along the apical assist (see **Figure 4**). Avoid letting the membrane and cell monolayer dry out during this step. Replace the medium by gently pipetting 75 µL into the filter well along the apical assist, using an electronic multichannel pipettor. Replace the medium in the basolateral feeding compartments, either 250 µL/well for the 96-well receiver plate or 35 mL in the single-well feeder plate. Gently reassemble the filter plate with the receiver plate or the single-well feeder plate.

4. Evaluating Caco-2 Cell Seeding Density

Once the Caco-2 cells have been in culture for the desired length of time, verify the integrity of the cell monolayer by measuring the transepithelial electrical resistance (TEER) for every well. This is a non-invasive method for determining monolayer integrity and can be used prior to the addition of test drug samples. Wells which have the appropriate electrical resistance can then be used as test wells for the transport studies. Another method for verifying monolayer integrity utilizes the fluorescent dye, lucifer yellow (LY) (**L0144**). After the completion of the drug transport experiment, LY can be added to each well for %LY passage studies. Alternatively, a control population of cells within a plate can be selected for addition of the dye to monitor monolayer integrity during drug transport experiments.[4]

Transepithelial Electrical Resistance Measurement

a. At the end of the desired growth period, remove the plates from the incubator and allow them to equilibrate to room temperature 15–30 min. Measure the electrical resistance across the monolayer using the Millicell® ERS 3.0 Digital Voltohmmeter (**MERS03000**) with the 96-well probe (**MERS0396P**).

b. Position the probe such that one end is immersed in the medium inside the filter well and the other is placed through the basolateral access hole into the medium in the growth plate. The probe should sit flat on the plate when positioned correctly with the thin collared electrode in the basolateral access hole. Record the electrical resistance for each well.

Lucifer Yellow Passage Test

a. Rinse the monolayer three times with 100 µL HBSS (**H8264**) using the same method described for Step 3, substituting 100 µL HBSS for the 75 µL cell culture medium.

b. Add 100 µL of lucifer yellow at a concentration of 100 µg/mL to each well in the filter plate after transport studies or to selected wells in a plate prior to transport studies.

c. Add 250 µL HBSS to the basolateral compartments of a 96-well transport analysis plate.

d. Assemble the plate components and incubate for 1 hour at 37 °C.

e. Remove the filter plate from the transport analysis plate and place into a fluorescent plate reader.

f. Determine the fluorescent absorbance using wavelengths of 485 nm excitation and 535 nm emission.

g. Calculate the percent of lucifer yellow passage across the cell monolayer into the transport analysis plate. Use the relative fluorescence measured for an equilibrium dilution of lucifer yellow in a separate analysis plate for reference. Following is the procedure:

Calculation of Percent Lucifer Yellow Passage

Measure the relative fluorescence units (RFU) in 250 µL of a 23 µg/mL solution of lucifer yellow. This is the equilibrium dilution of the starting material. If the RFU measured in the basolateral compartment is equal to the RFU measured in this sample, 100% passage of lucifer yellow occurred:

$$\frac{(100\ \mu g/mL)\ (0.075\ mL)}{0.075\ mL + 0.250\ mL} = 23\ \mu g/mL = RFU\ (equilibrium)$$

In addition, determine the RFU for HBSS alone (blank). Then use these values to calculate the lucifer yellow passage in the test wells using the following equation:

$$\%\ \text{Lucifer Yellow Passage} = \left[\frac{RFU\ (test) - RFU\ (blank)}{RFU\ (equilibrium) - RFU\ (blank)}\right] \times 100$$

For example, if the measured values for each of these solutions equaled RFU (test) = 2000, RFU (blank) = 1000, and RFU (equilibrium) = 300,000, then the percent lucifer yellow passage would equal:

$$\left[\frac{2000-1000}{300{,}000-1000}\right] \times 100 = 0.3\%$$

Choose the seeding density that provides the highest average electrical resistance with the least variability (lowest CV) and lowest lucifer yellow passage.

5. Performing a Drug Transport Assay using the Millicell® Caco-2 Assay System

The ultimate goal for using the Millicell® 96-well cell culture plate assay system is to perform a drug transport assay. This section provides a guide for the volumes to be tested in the device. In addition, washing the monolayer prior to the addition of test compounds is recommended.

- Mannitol (**M9546**)
- Digoxin (**D6003**)
- Propranolol (**P0884**)
- Testosterone (**T1500**)
- Methotrexate (**M8407**)

a. When the 10-day or 21-day Caco-2 cultures have reached confluence and are differentiated, they are ready to be used for transport studies. Remove the Millicell® 96-well cell culture plate with Caco-2 cultures from the incubator and determine the electrical resistance for each well (as described in Step 4). Next, wash the monolayer, exchanging the volume three times using sterile HBSS, pH 7.4. The method for the addition and removal of the wash buffer is similar to exchanging medium (Step 3) except that the volume in the filter well may be increased to 100 µL/well.

b. Transfer the filter plate to a 96-well transport analysis plate after washing is complete.

c. To determine the rate of drug transport in the apical to basolateral direction, add 75 µL of the test compounds to the filter well. Drug concentrations typically ranging from 10 µM to 200 µM and diluted in HBSS, pH 7.4 or in an alternative buffer of desired pH, may be used. Fill the wells in the transport analysis plate with 250 µL buffer.

d. To determine transport rates in the basolateral to apical direction, add 250 µL of the test compounds to the transport analysis plate wells. Fill the filter wells (apical compartment) with 75 µL of the buffer.

e. Incubate at 37 °C with or without shaking at 60 rpm on a rotary shaker. Typical incubation times are 1 to 2 hours. The data in this protocol note were generated using shaking for 1 hour.

f. At the end of the transport period, disassemble the plates or remove a fixed volume (typically 50 µL) directly from the apical and basolateral wells (using the basolateral access holes) and transfer to a fresh transport analysis plate for LC/MS analysis.

6. Calculating Drug Transport Rates

The apparent permeability (P_{app}), in units of centimeter per second, can be calculated for Caco-2 drug transport assays using the following equation:[1]

$$P_{app} = \left[\frac{V_A}{Area \times time}\right] \times \left[\frac{[drug]_{acceptor}}{[drug]_{(initial,\ donor)}}\right]$$

Where V_A is the volume (in mL) in the acceptor well, Area is the surface area of the membrane (0.11 cm^2 for Millicell® 96-well plate), time is the total transport time in seconds and [drug] is drug concentration.

RESULTS AND DISCUSSION

Optimal Seeding Density for Caco-2 Cells

The optimal seeding density was determined for a 10- and 21-day culture of Caco-2 cells using the method described above. Six different seeding densities were tested, with 32 wells tested per seeding density (see **Figures 3** and **5**). The electrical resistance and lucifer yellow passage were determined for the 10- and 21-day cultures of Caco-2 cells (see **Figure 6**).

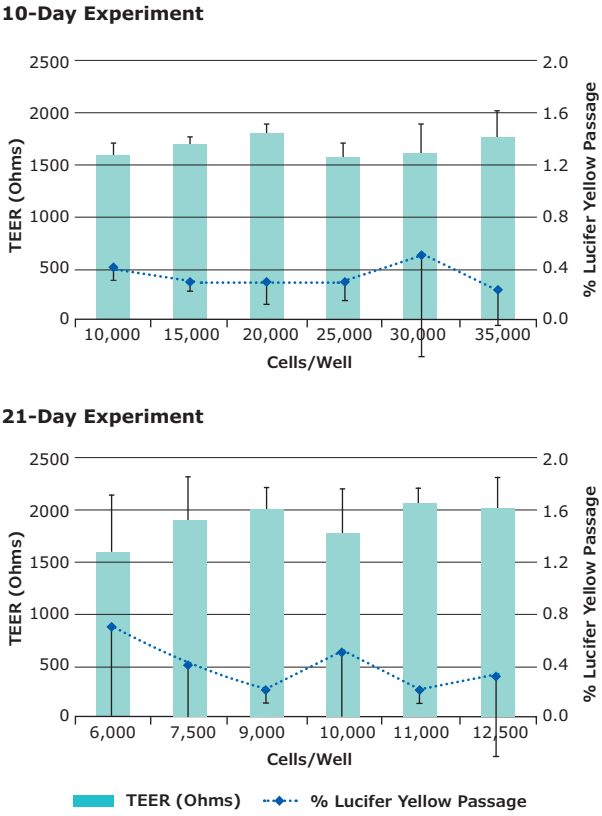


Figure 6. TEER and Lucifer Yellow Passage Results for 10- and 21-day Caco-2 Optimization Experiments

The highest mean electrical resistance (1700 and 1801 Ω) with the lowest variability (4.4 and 5.9% CV) for the 10-day culture was observed at 15,000 and 20,000 cells/well. The lucifer yellow passage was lowest (0.3%) with the lowest variability at these same dilutions. Therefore, seeding Millicell® 96-well cell culture plates with Caco-2 cells for a 10-day culture would likely be successful starting with either 15,000 or 20,000 cells/well (which equals 200,000 to 267,000 cells/mL or 136,000 to 180,000 cells/cm^2).

The highest mean electrical resistance (1999 and 2034 Ω) with the lowest variability (10 and 7.4% CV) for the 21-day culture was observed at 9,000 and 11,000 cells/well. The lucifer yellow passage was lowest (0.2%) with the lowest variability at these same dilutions. Therefore, seeding Millicell® 96-well cell culture plates with Caco-2 cells for a 21-day culture would likely be successful starting with 9,000 to 11,000 cells/well (which equals 120,000 to 147,000 cells/mL or 82,000 to 100,000 cells/cm^2).

Drug Transport Study Results

Caco-2 cells were cultivated for 10- or 21-days in the Millicell® 96-well cell culture plate using optimal seeding densities (20,000 and 9,000 cells/well, respectively), and a drug transport assay was performed as in Step 6. Drugs (mannitol, digoxin, propranolol, testosterone and methotrexate) at 10 µM were added either apically, in HBSS supplemented with 10 mM MES, pH 6.8 or basolaterally, in HBSS at pH 7.4 and incubated for 1 hour at 37 °C, shaking at 60 rpm. The TEER was measured prior to the start of the drug transport assay, and lucifer yellow passage was evaluated on selected wells within the plate during the drug transport assay. The results demonstrate that the monolayers were well formed, the TEER values were higher at 21 than 10 days (as expected), the lucifer yellow passage was less than 1%, and the drugs tested gave transport rates which provided appropriate classification into high and low permeability (see **Table 2**). In addition, digoxin is appropriately categorized as an active efflux drug since the digoxin transport in the basolateral to apical direction was shown to be 22 times faster than in the apical to basolateral direction after 10 days in culture. By 21 days, this difference increased to 60 times faster (data not shown).

	TEER Ω·cm^2	% LY Passage	Drug Transport P_{app} (10^{-6} cm/s)*				
			Mannitol	Propranolol	Digoxin	Testosterone	Methotrexate
10 day	158	0.2	1.05 ±0.11	9.2 ±1.0	1.1 ±0.1	34.5 ±2.3	0.6 ±0.08
21 day	239	0.1	1.61 ±0.24	8.9 ±1.8	0.36 ±0.09	33.4 ±3.3	0.9 ±0.16
Permeability			Low[1]	High[1]	Low[6]	High[1]	Low[7]

***Note:** The data shown are selected from three plates tested on the same day with a representative average from one plate shown. The standard deviation is calculated from the 12 replicates per drug.

Table 2. Performance of 10- and 21-day Caco-2 Cell Cultures at Optimized Seeding Densities on Millicell® Caco-2 Plates

The FDA Biopharmaceutical Classification system describes the use of the Caco-2 cell model for drug absorption measurements. The system characterizes new drug compounds as either low or high permeability. The drug transport rates measured here demonstrate that the Millicell® 96-well cell culture plate can be used to determine whether a drug is highly permeable by assigning low permeability to compounds with apparent permeability rates less than 2 x 10^{-6} cm/s.

The Millicell® 96-well cell culture plate is an excellent high throughput tool to screen compounds for intestinal transport properties. It provides a robust and reproducible format for conducting automated, high-throughput studies. Once optimized, growing cells in the Millicell® 96-well cell culture plate provides an opportunity to measure drug transport rates of known and unknown com pounds in a controlled and consistent manner. Other polarized epithelial cell model systems can be cultured in this device and used for discovery screening and ADME applications (see *Optimization of MDCK cell growth and differentiation for drug transport assay studies using a 96-well assay system*).

Tips & Tricks

- Achieving a uniform cell suspension when initially plating the cells will promote a more consistent monolayer across the 96 wells. This may be particularly difficult when seeding multiple plates. Frequent mixing is recommended to minimize the risk of large clumps of cells settling to the bottom of the tube, which could result in an inaccurate distribution of cells across the wells or plates.
- Cells seeded on the Millicell® 96-well cell culture plate should be placed in an incubator that provides adequate humidity control. If a significant difference is observed in the performance of the wells on the perimeter of the plate relative to the interior, this indicates that the culturing environment is not adequately humidified. A cell culture incubator with electronic humidity control is recommended. If this is not possible, place the plates in an incubator that is opened infrequently.
- For left-handed users, it may be more comfortable to rotate the plate 180° so that the apical assist is in the upper right corner.
- It is important to prevent membrane and cellular monolayer from drying out during media or buffer exchange. If unexpectedly high levels of well failures occur - as judged by low TEER values or high LY pass through - compared user's past experiences, it is recommended to keep the duration of separation short (<1 min) when filter plates are separated from feeder plates or simply exchange the medium without disassembling the plate components. In addition, it may be beneficial to place the vacuum manifold in opposite position to the apical assist port during aspiration (see **Figure 7** (right) below). This will allow the manifold to sit slightly higher when pressed down during aspiration, leaving a higher volume of liquid within wells, preventing cellular monolayer from drying out.

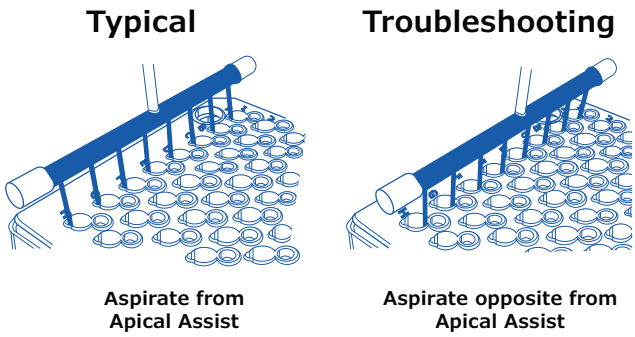


Figure 7: Manifold placement near apical assist for maximum media/buffer removal (**left**). Manifold placement opposite apical assist for reduced media/buffer removal (**right**)

- Caco-2 Cell Differentiation
  - If no significant expression and localization of P-glycoprotein (P) to the apical plasma membrane is observed after 10 days in culture of Caco-2 cells, be certain that the seeding density chosen is appropriate. Typically, to achieve good differentiation of the Caco-2 cells in 10 days, it is important to start with a sub-confluent flask of cells and to seed the wells at a higher density than would normally be used for a 21-day culture of Caco-2 cells.
  - The growth and differentiation of the Caco-2 cell lines need to be carefully monitored when optimizing the assay for use in a drug transport analysis. Many factors may contribute to the variability in the cell behavior. Cell passage number, culture medium, and seeding density can all influence how the cells perform on the Millicell® 96-well cell culture plates. Both tight junction formation and polarized expression of membrane proteins can exhibit changes as the cell passage number increases. How this will ultimately affect the rate measured in the drug transport analysis needs to be understood in each laboratory. As a rule, do not passage the Caco-2 cells more than 25 or 30 times before thawing a new vial.[5]

Related Products

- **PSHT004**
- **PSHT004S5**
- **MACAC0RS5**
- **MERS03000**
- **MERS0396P**
- **86010202-1VL**

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Related Application and Protocol Notes

Application Note: Optimization of MDCK Cell Growth and Differentiation for Drug Transport Assay Studies using Millicell® 96-well Cell Culture Plates.

Technical Article: Drug transport assays in a 96-well system – reproducibility and correlation to human absorption

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