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Application Note

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

Introduction

One of the major roadblocks to the successful development of new drugs lies in understanding and testing the absorption, distribution, metabolism, excretion and toxicity (ADMET) properties of candidate compounds. Absorption is defined as the ability of a drug to cross epithelial and endothelial cell barriers from the point of administration to the site of action. Immortalized cell lines have been used as drug absorption models for many years and aid in the understanding of drug permeability mechanisms.[1] Madin Darby Canine Kidney (MDCK) cells or MDCK cells transfected with human MDR1 cDNA, have been used to measure passive transcellular diffusion and P-glycoprotein mediated efflux, respectively.[2–4]

Absorption studies can be tedious and require expertise in cell culture and assay development. The 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. Data generated using this device can ultimately provide direction to rank order the oral absorption profiles of new candidate drug compounds. The following protocol will provide guidance for the optimization of MDCK cell growth and differentiation on the Millicell® 96-well cell culture plate and suggests an approach for performing a drug absorption evaluation.

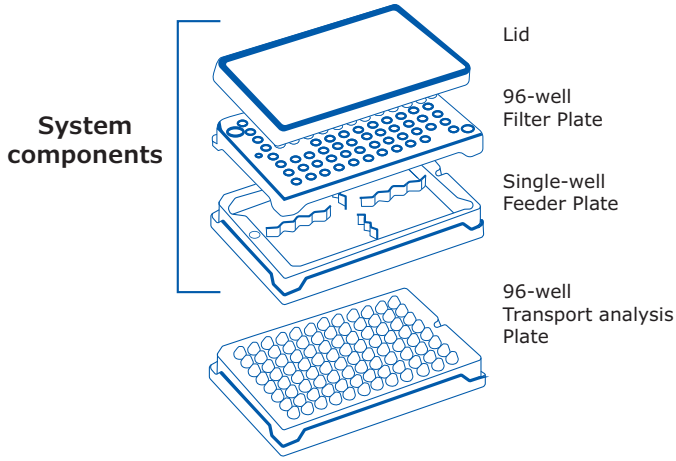

Figure 1. Millicell® 96-well cell culture plate with 0.4 µm PC membrane components (**PSHT004R5**, with single-well feeder plate) and 96-well transport analysis plate (**MACAC0RS5**). Not shown, system components with 96-well feeder plate (**PSHT004S5**).

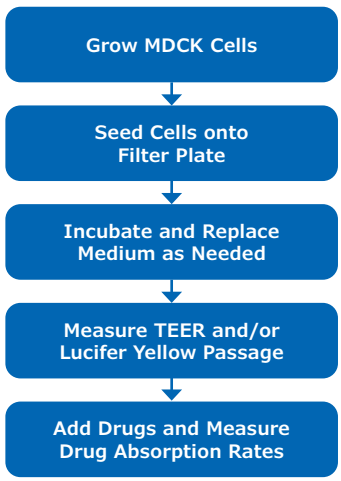

Figure 2. Drug transport study protocol using the Millicell® Caco-2 assay system and MDCK cells

Protocol

The following protocol provides methods for growing an optimized MDCK cell monolayer, evaluating the monolayer, performing a drug transport assay across the cell monolayer, and analyzing the drug transport rate.

1. Medium, Additives and the Cultivation of MDCK Cells

The following medium, additives and protocol are recommended for the cultivation of the MDCK cell line (**84121903–1VL**).

- 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 MDCK cell culture medium consisting of:

- Dulbecco's MEM with high glucose
- 10% FBS
- 1X NEAA
- 10 mm HEPES
- 100 units penicillin
- 0.1 mg/mL streptomycin
- 4 mm L-glutamine

b. Cultivate cells in T-75 flasks (**CLS430641U**) in a cell culture incubator set at 37 °C, 5% CO_2, and 95% relative humidity, allowing the cells to achieve 70–80% confluence before detaching and splitting (step c).

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 10 to 15 minutes or until the cells detach and float. This can be confirmed by periodic visual inspection of the flasks.

d. Add 12 mL of MDCK culture medium to detached cells. Dispense 0.3 mL into 6 new T-75 flasks. Add 14.7 mL of MDCK cell culture medium for a total of 15 mL per flask and replace in 5% CO_2 incubator at 95% relative humidity and 37 °C.

2. Optimization of MDCK Cell Seeding Density on Millicell® 96-well Cell Culture Plates

MDCK cells are cultured on a membrane support to achieve terminal differentiation. These differentiated monolayers can then be used to estimate drug absorption rates. Cells can be grown to confluence on the Millicell® 96-well cell culture plates for 3 to 7 days, depending on the initial seeding density per well. The user is advised to optimize the seeding density based on the desired time for the culture period. Higher seeding density may result in quicker monolayer formation, lower densities in a slower monolayer formation.

a. Detach MDCK cells from cell culture flask as described in Section 1, step c. using trypsin/EDTA.

b. Resuspend detached cells in a total of 10 mL of MDCK cell culture medium. Count cell suspension to determine cell number per mL.

c. For a 4-day culture of MDCK cells, divide cell suspension into six sterile 15 mL centrifuge tubes. Dilute the cell aliquots with MDCK cell culture medium to different cell concentrations ranging from 333,333 to 600,000 cells/mL (see **Table 1** for examples of conversions from cells/mL to cells/cm^2 or cells/well).

	Well surface area	Well volume	Cells/cm^2	Cells/mL	Cells/ well
24-well filter plate	0.3 cm^2	0.4 mL	227,200	171,000	68,200
96-well filter plate	0.11 cm^2	0.075 mL	227,200	333,000	25,000

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

d. Dispense 75 µL of cell dilution into the filter wells of the Millicell® 96-well cell culture plates with 0.4 µm PC membrane (**PSHT004R5** or **PSHT004S5**). For better optimization, set up several columns for each cell density (minimally 16 wells per seeding concentration). This will provide a clear picture of the performance of the cells at various seeding densities on the Millicell® 96-well cell culture plate. See **Figure 3**, for an example.

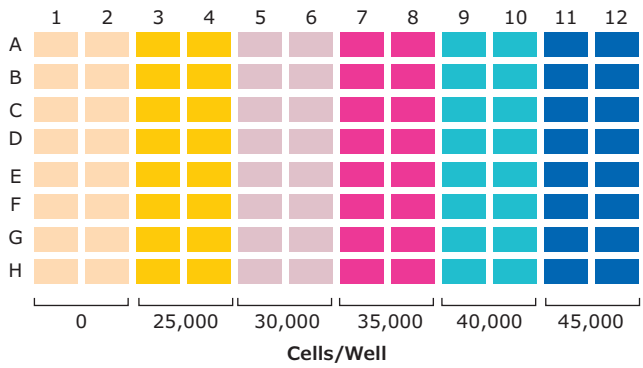

Figure 3. MDCK Cell Template for Cell Seeding Density Optimization

e. Dispense 250 µL of MDCK 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.

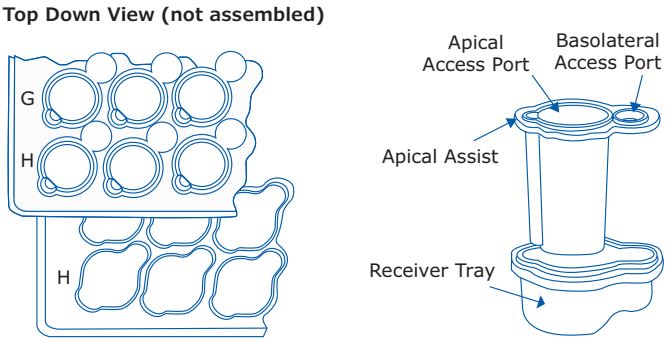

Figure 4. Patented Design of the Millicell® 96-Well Cell Culture Plate: Each well contains an offset apical channel, the apical assist, to guide manual pipette tips. The apical assist channel ends just short of the membrane surface to eliminate the chance of membrane or monolayer disruption while pipetting.

f. Incubate plate for 3 to 7 days at 37 °C, 5% CO_2, 95% relative humidity.

g. Media exchange is recommended once every 2 days. Refer to Section 3.

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

Long-term cell culture requires exchange of medium to remove waste products that may accumulate and become toxic, and to replenish nutrients. 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).

a. The most critical part of removing and replacing the medium in the Millicell® 96-well cell culture plate 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 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.

c. Alternatively, remove 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 aspirate 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. Replace the medium in the basolateral feeding compartments, either 250 µL/well for the 96-well receiver plate or 25 mL in the single-well feeder plate. Gently reassemble the filter plate with the receiver plate or the single-well feeder plate.

4. Evaluating MDCK Cell Seeding Density

Once the MDCK 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.[3]

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, approximately 15–30 minutes. Measure the electrical resistance across the monolayer using the Millicell® ERS 3.0 Digital Voltohmmeter (**MERS03000**) and the 96-well electrode (**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 Section 3, substituting 100 µL HBSS for the 75 µL cell culture medium.

b. Add 75 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 (**MACAC0RS5**).

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® 96-Well Cell Culture Plate

The ultimate goal for using the Millicell® 96-well cell culture plate 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. This experiment utilizes three compounds with high permeabilities.

- Caffeine (**C8960**)
- Ibuprofen (**I4883**)
- Propranolol (**P0840**)

a. When the cells have reached confluence and are differentiated, they are ready to be used for transport studies. Remove the Millicell® 96-well cell culture plate from the incubator and determine the electrical resistance for each well (as described in Section 4). Next, wash the monolayer by 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 (see Section 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 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.

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 MDCK 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 cm² for Millicell® 96-well cell culture plate), time is the total transport time in seconds, and [drug] is the drug concentration.

Results and Discussion

Optimal Seeding Density for MDCK Cells

The optimal seeding density was determined for a 4-day culture of MDCK cells using the method described in this Protocol Note. **Figure 3** illustrates the template used for cell dilutions; 16 wells each of 5 dilutions were cultured on two, identical, 96-well plates. After 4 days in culture, the electrical resistance (TEER) and lucifer yellow (LY) passage were determined as described. The results of this evaluation are shown in **Figure 5**. The electrical resistance of MDCK cell monolayers at the densities chosen ranged from 991 to 1055 Ω.

The percent passage of lucifer yellow ranged from 0.2 to 0.3%. Although all of the cell densities measured resulted in monolayers with acceptable electrical resistance and low lucifer yellow passage, an MDCK cell density of 35,000 cells/well (which is equal to 467,000 cells/mL or 318,000 cells/cm^2) was chosen for these experiments.

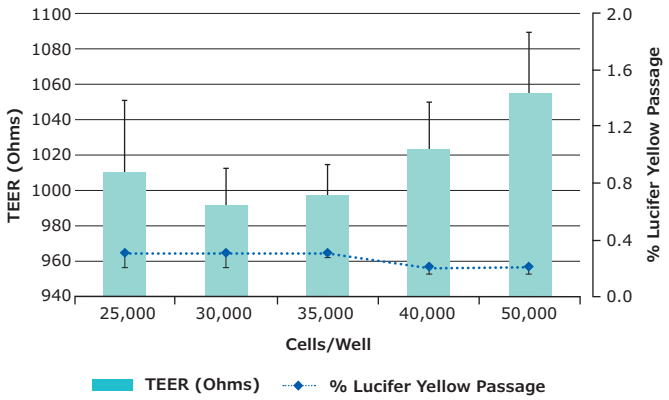

Figure 5. TEER and Lucifer Yellow Passage Results for 4-day MDCK Cell Optimization Experiment

Drug Transport Study Results

A drug transport experiment was performed with 4-day MDCK cultures to determine apparent permeability of known drugs at the optimized seeding density of 35,000 cells/well. The TEER value measured in this experiment was 996 ±19 Ω and the % lucifer yellow passage was 0.3 ±0.03. Caffeine, ibuprofen and propranolol were chosen as the test compounds. Drug transport rate results in **Table 2** are acceptable for these highly permeable compounds. In addition, the TEER and % lucifer yellow passage are in the range predicted from the optimization experiment.

MDCK cells are a viable alternative to Caco-2 cells as an *in vitro* model system to evaluate potential drug candidates. The drug transport rates measured using this model system can help determine the probability of the drug being absorbed orally. The Millicell® 96-well cell culture plate is ideal for culturing MDCK cells and performing these drug transport analyses.

Drug	Papp (10–6 cm/s)	Expected Permeability
Caffeine	50.3 ±9.7	High
Ibuprofen	19.8 ±0.7	High
Propranolol	10.5 ±1.2	High

Table 2. Drug Transport Results for 4-day MDCK Cell Culture

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 to 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 6** (**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.

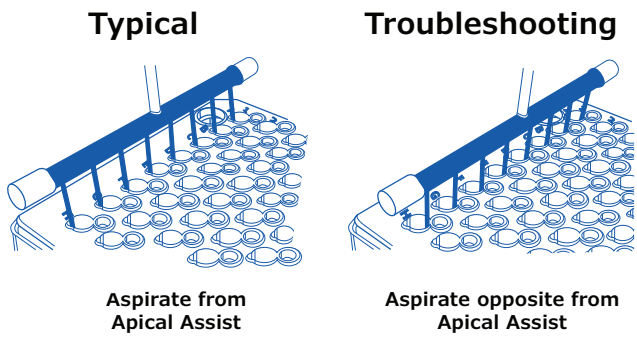

Figure 6: Manifold placement near apical assist for maximum media/buffer removal (**left**). Manifold placement opposite apical assist for reduced media/buffer removal (**right**)

Related Products

- **PSHT004**
- **PSHT004S5**
- **MACAC0RS5**
- **MERS03000**
- **MERS0396P**
- **84121903–1VL**

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Related Application and Protocol Notes

Application Note: Optimization of Caco-2 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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