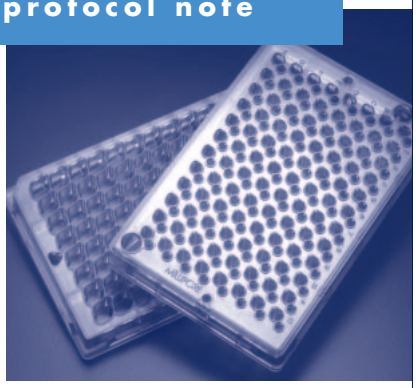
# MultiScreen® Caco-2 Assay System

protocol note



## *Optimization of MDCK cell growth and differentiation for drug transport assay studies using a 96-well assay system*

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### 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 a drug's ability 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. Millipore has developed a 96-well MultiScreen Caco-2 filter plate system (Figure 1), that is automation compatible and allows for the measurement of drug transport in a high throughput format. The MultiScreen Caco-2 assay system 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 MultiScreen Caco-2 plate and suggests an approach for performing a drug absorption evaluation.

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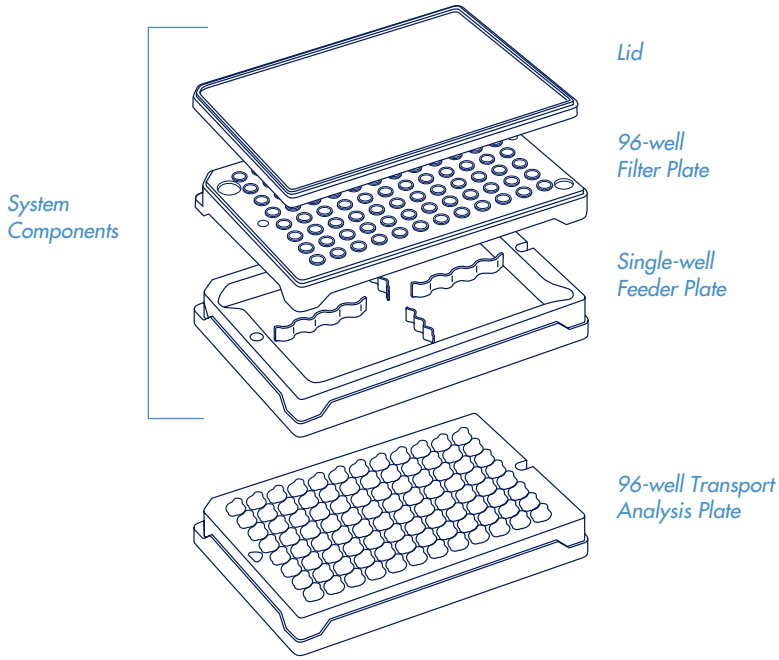



**Figure 1:** *MultiScreen Caco-2 assay system components (cat. MACA C02 B5, with single-well feeder plate) and 96-well transport analysis plate (cat. MACA C0R S5). Not shown, assay system components with 96-well feeder plate (cat. MACA C02 S5).*

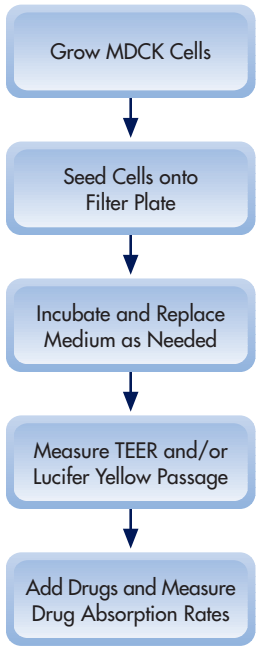



**Figure 2:** *Drug transport study using MultiScreen Caco-2 assay system and MDCK cells.*

## 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.***

### I. Medium, Additives and the Cultivation of MDCK Cells

The following medium, additives and protocol are recommended for the cultivation of the MDCK cell line (cat. CCL-34) purchased from the ATCC® (Manassas, VA). Dulbecco's MEM with high glucose (cat. D5796), non-essential amino acids (NEAA, cat. M7145), HEPES (cat. H0887), penicillin, streptomycin and L-glutamine (cat. G1146), EDTA (cat. E8008) and trypsin/EDTA (cat. T3924) were all purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS, cat. 16000044) was purchased from Invitrogen (Carlsbad, CA).

**a.** Prepare MDCK cell culture medium consisting of:
Dulbecco's MEM with high glucose
8% 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 in a cell culture incubator set at 37 °C, 5% $CO_2$, 95% relative humidity, allowing the cells to achieve 90 –100% confluence before detaching and splitting (step **c**).

## Tips&Techniques

***Converting cells/cm² to cells/mL or cells/well***

**24-Well Filter Plates**
Surface area: 0.3 cm²/well
Volume: 0.4 mL/well

| Cells/cm² | Cells/mL | Cells/well |
|---|---|---|
| 227,200 | 171,000 | 68,200 |

**96-Well Filter Plates (MultiScreen Caco-2 plate)**
Surface area: 0.11 cm²/well
Volume: 0.075 mL/well

| Cells/cm² | Cells/mL | Cells/well |
|---|---|---|
| 227,200 | 333,000 | 25,000 |

**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.

## II. Optimization of MDCK Cell Seeding Density on MultiScreen Caco-2 Assay System

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 MultiScreen Caco-2 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 I, 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 6 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 *Tips & Techniques* on this page, to convert cells/mL to cells/cm² or cells/well).

*Note: 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.*

**d.** Dispense 75 µL of cell dilution into the filter wells of the 96-well MultiScreen Caco-2 plates (Millipore cat. MACA C02 S5 or MACA C02 S2). 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 MultiScreen Caco-2 plate. See Figure 3, on page 4, for an example.

**e.** Dispense 250 µL of MDCK cell culture medium into each of the 96 wells of the receiver plate (MACA C02 S5), or alternatively, place 35 mL in the single-well feeder plate (MACA C02 B5). This may be accomplished by dispensing medium through the basolateral access holes (see Figure 4, on page 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.

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

**g.** Exchanging of medium is recommended once every 2 days. Refer to *Section III, Exchanging the Medium in MultiScreen Caco-2 Assay System*.

*Note: Cells seeded on the MultiScreen Caco-2 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.*

**Figure 3:** MDCK Cell Template for Cell Seeding Density Optimization

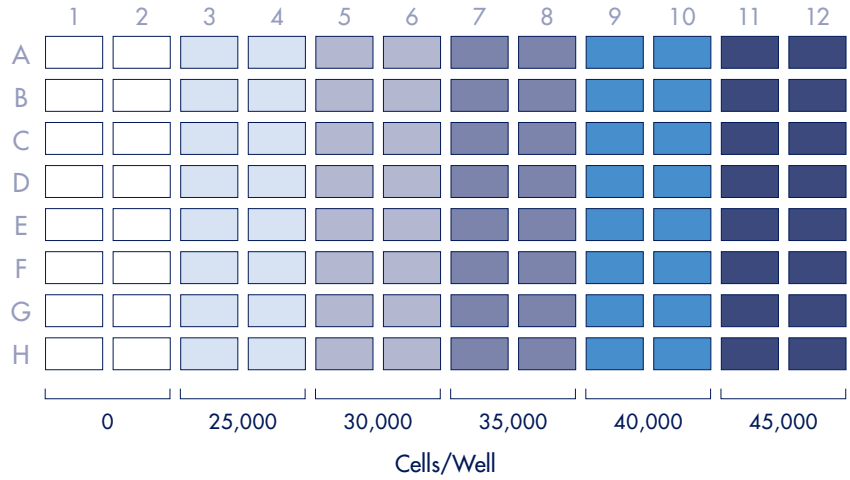
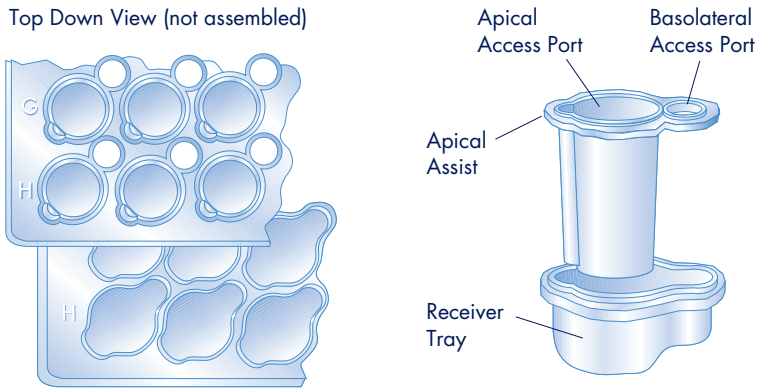



**Figure 4:** Patented Design of the MultiScreen Caco-2 Plate




*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.*

## III. Exchanging the Medium in the MultiScreen Caco-2 Assay System

Long-term cell culture requires exchange of medium to (1) remove waste products that may accumulate and become toxic, and (2) 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). 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 MultiScreen Caco-2 assay system is to avoid damage to the cell monolayer and the filter on which it is supported. In these experiments, a multichannel manifold (Sigma cat. M2656) was used to aspirate the medium, and an electronic multiwell pipettor with speed control at low or medium speed (Matrix Impact® pipettor, cat. 6002) was used to dispense the fresh culture medium.

**b.** The first option for feeding the plates consists of 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, on page 4). 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 µL in the single-well feeder plate. Gently reassemble the filter plate with the receiver plate or the single-well feeder plate.

*Note: 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.*

**c.** The second option is 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, on page 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 (preferably using the recommended multichannel manifold), and carefully aspirate the medium from the filter wells. Take care not to contact the cell monolayer during aspiration.

**d.** Replace the medium in the filter wells as directed in step **b**, and replace the medium in the feeding plate using the basolateral access holes for the receiver plate or the large access holes at either A1 or H12 for the single-well feeding plate.

## IV. 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 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) (Sigma cat. 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 1 hour. Measure the electrical resistance across the monolayer using the Millicell®-ERS system ohm meter (Millipore cat. MERS 000 01) and the STX-100M electrode (World Precision Instruments, Sarasota, FL).

**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 (Invitrogen cat. 14025) using the same method described for *Section III, Exchanging the Medium in the MultiScreen Caco-2 Assay System,* 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.

**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. [Data obtained in this Protocol Note were collected using a Wallac Victor$^{2™}$ plate reader (PerkinElmer, Boston, MA).]

**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:

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

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.

## V. Staining Cell Monolayers with Hema 3® Stain Kit

To visualize cell growth on the filter at the completion of the TEER and lucifer yellow testing, it may be useful to stain the cell monolayers to confirm the integrity and uniformity of the cells. One method for doing this is to use the Hema 3 stain kit (cat. 22122911) available from Fisher Scientific.

**a.** Aspirate the wells to be stained. Add 100 µL of fixative and incubate 1 minute at room temperature.

**b.** Aspirate the fixative and add 100 µL Solution 1. Incubate for 5 minutes at room temperature.

**c.** Aspirate Solution 1 and add 100 µL Solution 2. Incubate for 5 minutes at room temperature.

**d.** Wash the wells extensively with Milli-Q® water, aspirate any residual liquid and allow wells to dry overnight.

**e.** Visualize the wells using a stereomicroscope.

## VI. Performing a Drug Transport Assay using the MultiScreen Caco-2 Assay System

The ultimate goal for using the MultiScreen Caco-2 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. For drug transport experiments using radio-labeled drugs, unlabeled known drugs (10 µM) were spiked with a trace amount of $^{3}H$ or $^{14}C$ radio-labeled drug to monitor the drug transport rate. [Unlabeled caffeine (cat. C8960), ibuprofen (cat. I4883) and propranolol (cat. P08840) were all purchased from Sigma Chemical Company (St. Louis, MO). Radio-labeled caffeine (cat. NET515) and propanolol (cat. NEC412) were both purchased from PerkinElmer (Boston, MA). Radio-labeled ibuprofen (cat. ART392) was purchased from ARC, Inc. (St. Louis, MO).]

**a.** When the cells have reached confluence and are differentiated, they are ready to be used for transport studies. Remove the MultiScreen Caco-2 plate from the incubator and determine the electrical resistance for each well (as described in *Section IV, Evaluating MDCK Cell Seeding Density*). 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 III, Exchanging the Medium in MultiScreen Caco-2 Assay System*) 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.

**g.** For radio-labeled drug evaluation, remove 20 µL from each compartment and transfer to a plate (Wallac/PerkinElmer 96-well flexible plate, cat. 1450-401) containing 100 µL scintillation fluid. Mix and determine the radioactivity per sample using a multiwell plate scintillation reader such as the Wallac 1450 MicroBeta® Plus Scintillation Counter or the MicroBeta Trilux Multiwell Plate Scintillation Counter from PerkinElmer (Boston, MA).

## VII. 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]

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

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 MultiScreen Caco-2 plate), and **time** is the total transport time in seconds. For radio-labeled drug transport experiments the CPM units obtained from the Microbeta Trilux Multiwell Plate Scintillation Counter are used directly for the drug acceptor and initial concentrations such that the formula becomes:

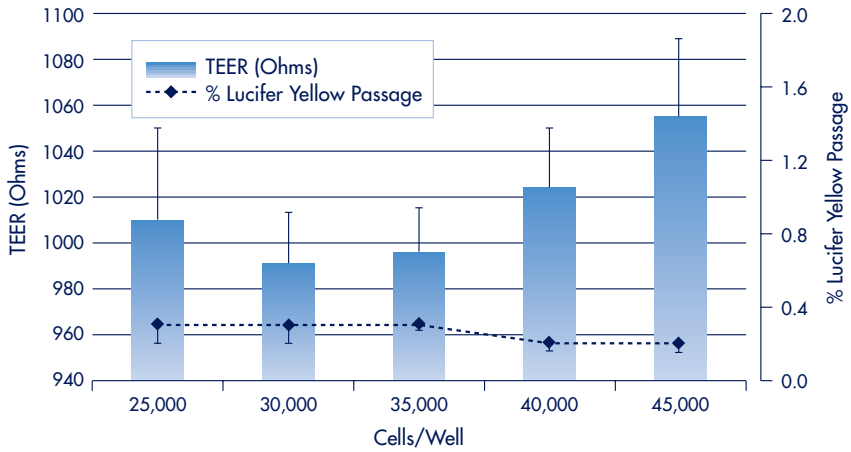
$$P_{app} = \left( \frac{V_A}{\text{Area x time}} \right) x \left( \frac{\text{CPM}_{\text{acceptor}}}{\text{CPM}_{\text{initial,donor}}} \right)$$

## 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, on page 4, 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 ohms. 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 the rates of radio-labeled known drugs at the optimized seeding density of 35,000 cells/well. The TEER value measured in this experiment was 996 ± 19 ohms and the % lucifer yellow passage was 0.3 ± 0.03. Caffeine, ibuprofen and propanolol were chosen as the test compounds. Drug transport rate results in Table 1 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 MultiScreen Caco-2 assay system is ideal for culturing MDCK cells and performing these drug transport analyses.

**Table 1:** Drug Transport Results for 4-day MDCK Cell Culture

| Drug | $P_{app}$ ($10^{-6}$ cm/s) | Expected Permeability |
|---|---|---|
| Caffeine | 50.3 ± 9.7 | High |
| Ibuprofen | 19.8 ± 0.7 | High |
| Propranolol | 10.5 ± 1.2 | High |

## References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## Related Application and Protocol Notes

PC1060EN00: Optimization of Caco-2 cell growth and differentiation for drug transport assay studies using a 96-well assay system

AN1727EN00: Drug transport assays in a 96-well system: reproducibility and correlation to human absorption

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