MILLIPORE

Protocol Note

Title: **Millicell® 24 - Monolayer Integrity and Drug Transport Guidelines**

Literature no: **PC0016EN00**

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Introduction

The Millicell-24 Cell Culture Plate is a 24-well general purpose device designed to support cell growth, attachment, differentiation, or other desired applications. The procedure described below details how to measure the formation of a differentiated cell monolayer and the rate of drug transport across the cell barrier. All procedures are designed to be carried out in a single device and, if desired, can be performed using automation for cell seeding, cell feeding, washing, and other experimental procedures.

Materials and Reagents

- Millicell-24 Cell Culture Plate (PCF or PET membrane) – Millipore # PSHT010R5, PSRP010R5
- Millicell ERS System – Millipore # MERS 000 01
- Lucifer Yellow, 100 µg/mL concentration – Sigma # L0144
- Hanks Balanced Salt Solution (HBSS) – Fisher # SH3026802 or equivalent
- Radioactive Drug Transport:
    - Wallac/Perkin Elmer 96-well flexible plate, cat. 1450-401, or equivalent
    - Microbeta® Trilux Counter or equivalent
    - Scintillation Cocktail – Fisher # NC9276636 or equivalent

Note: Although the following methods have been optimized for monolayer integrity and cell based drug transport on epithelial cell lines such as Caco-2 or MDCK, they can be applied to any applicable cell system.

Methods

Measurements of Monolayer Integrity

A. Trans-epithelial Electrical Resistance (TEER)

1. At the end of the desired growth period, remove the plates from the incubator and allow them to equilibrate to room temperature (approximately 0.5 hour). Measure the electrical resistance across the monolayer using the Millicell ERS system ohm meter.
2. Position the probe such that one end is immersed in the media inside the filter well and the other is placed through the basolateral access hole into the media in the growth plate. Record the electrical resistance for each well. Take care not to touch the filter during TEER measurements, as it can damage the cell monolayer.

B. Lucifer Yellow (LY) Rejection

1. Using the same methodology as when feeding, rinse the monolayer three times with 400 µL HBSS in the apical wells and 32 mL in the feeder tray.
2. Add 400 µL of LY solution to each well in the filter plate.
3. Add 800 µL HBSS to each well of a 24-well receiver tray.
4. Assemble the filter and 24-well receiver plates and incubate for 1-2 hours at 37° C.
5. Remove the filter plate from the receiver plate and place the receiver plate into a fluorescent plate reader. Determine the LY fluorescence using an excitation wavelength of 485 nm and an emission wavelength of 535 nm.
6. Calculate the percent of LY rejection across the cell monolayer by measuring fluorescence in the receiver plate as compared to an 'equilibrium' standard.

Note: The standard plate should consist of 4 wells with 800 µl HBSS (blank) and 4 wells with 267 µl LY (100 µg/ml) + 533 µl HBSS (equilibrium samples).

Use these values to calculate the LY rejection in the test wells using the following equation.

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

Example: If the measured values for each of these solutions were:
RFU (test) = 2000
RFU (blank) = 1000
RFU (equilibrium) = 300000
Then the percent LY passage would equal:

$$\left[\frac{2000 - 1000}{300000 - 1000}\right] \times 100 = 0.3\%$$

The calculated LY rejection would therefore be: 100% - 0.3% = **99.7%**

Note: Optimizing cell seeding protocol is commonly based on choosing the density that results in the highest average electrical resistance with the least variability (e.g., lowest CV) combined with the lowest LY passage. After the seeding density has been optimized, monolayer integrity can be tested using TEER reading, LY rejection, or transport of a paracellular drug compound such as atenolol or mannitol.

Note: We do not recommend performing LY rejection in tandem with drug transport as it may interfere with radiometric or LC/MS analysis. It is recommended to run LY post drug transport to assess the integrity of the monolayer.

C. Cell Based Drug Transport

1. After the desired cell growth period, remove the Millicell-24 well plate from the incubator and determine the electrical resistance for each well (as described above). Wash the monolayer exchanging the volume three times using sterile HBSS, pH 7.4. After washing, remove the buffer from the filter plate and feeder tray.
2. Transfer the filter plate to a 24-well transport analysis plate.
3. To determine the rate of drug transport in the apical to basolateral direction, add 400 μL of the test compounds to the filter well. Drug concentrations typically ranging from 10 μM to 200 μM may be used (HBSS, pH 7.4 or in an alternative buffer of desired pH). Fill the wells of the 24-well receiver plate with 800 μL buffer. **(Table 1)**
4. Add 800 μL of the compounds to the wells of the 24-well receiver plate to measure transport rates in the basolateral to apical direction. Fill the filter wells (apical compartment) with 400 μL of buffer. **(Table 1)**
5. Combine the filter and receiver plates once all drugs and/or buffer has been added. Begin timing the experiment.
6. Incubate at 37°C shaking at 60 rpm on a rotary shaker. Typical incubation times are 1 to 2 hours. We recommend a 2 hour incubation for optimal results.
7. At the end of the incubation, disassemble the plates or remove a fixed volume (typically 50-100 μL) directly from the apical and basolateral wells (using the basolateral access holes) and transfer to a clean plate for LC/MS analysis.
8. For radiolabeled drug evaluation, remove 25 μL or applicable volume from each compartment and transfer to a plate containing 100 μL of scintillation fluid, mix and determine the radioactivity per sample using a Multiwell Plate Scintillation Reader such as the Trilux from Perkin Elmer. Add 25 μL of your initial drug to 100 μL of scintillation fluid to obtain your standard counts.

Table 1- Cell Drug Transport Template (Four Compounds - Three Replicates)

Filter Plate (Apical) Template

400μL Drug 1	400μL Drug 1	400μL Drug 1	400μL Drug 2	400μL Drug 2	400μL Drug 2
400μL HBSS	400μL HBSS	400μL HBSS	400μL HBSS	400μL HBSS	400μL HBSS
400μL Drug 3	400μL Drug 3	400μL Drug 3	400μL Drug 3	400μL Drug 3	400μL Drug 3
400μL HBSS	400μL HBSS	400μL HBSS	400μL HBSS	400μL HBSS	400μL HBSS

Receiver Plate (Basolateral) Template

800μL HBSS	800μL HBSS	800μL HBSS	800μL HBSS	800μL HBSS	800μL HBSS
800μL Drug 1	800μL Drug 1	800μL Drug 1	800μL Drug 2	800μL Drug 2	800μL Drug 2
800μL HBSS	800μL HBSS	800μL HBSS	800μL HBSS	800μL HBSS	800μL HBSS
800μL Drug 3	800μL Drug 3	800μL Drug 3	800μL Drug 4	800μL Drug 4	800μL Drug 4

Calculating drug transport rates

The apparent permeability, in units of centimeter per second, can be calculated for Millicell-24 Cell Culture Plate drug transport assays using the following equation.

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

Where V_A is the volume in the acceptor well, *area* is the surface area of the membrane (0.7cm^2 for Millicell-24 Cell Culture Plates), and *time* is the total transport time in seconds. For radiolabeled drug transport experiments the CPM units obtained from the Trilux Multiwell Plate Scintillation Counter are used directly for the drug acceptor and initial concentrations such that the formula becomes:

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

Note: Caco-2 or MDCK monolayer differentiation is evaluated by the transport of compounds that are effluxed such as digoxin and vinblastine. The (B to A)/(A to B) ratios are good measurements of expression and localization of P-glycoprotein (P-gp) to the apical plasma membrane. Optimization of seeding densities may also be assessed by monolayer differentiation.

Note: The growth, integrity and differentiation of the cell monolayers need to be carefully monitored when optimizing the assay for use in a drug transport analysis. Many factors may contribute to the assay variability. Note that the cell passage number and culture medium can influence how the cells perform on the Millicell-24 Cell Culture Plate. These factors may cause a shift in the behavior, both tight junction formation and polarized expression of membrane proteins as the cell passage number increases. How this will ultimately affect the measurement of drug transport rates needs to be carefully considered in the experimental design.

References

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