MILLIPORE

Protocol Note

Literature no: **PC0015EN00**

Title: **Millicell®-24 Cell Culture Plate Seeding and Feeding Guidelines**

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Introduction

The Millicell-24 Cell Culture Plate is a 24-well device designed to support cell attachment, growth and differentiation for many applications. Optimizing cell seeding protocols and densities is extremely important. To ensure good cell attachment and growth, testing a range of seeding densities is recommended. The following guidelines are designed to be carried out in a single device and can be performed using automation. The Millicell-24 Cell Culture Plate is available with a 0.4 µm polycarbonate (PCF) membrane or a 1.0 µm polyester (PET) membrane which allows the cells to be visualized during the seeding and growth stages.

Materials and Reagents

- Millicell-24 Cell Culture Plate (PCF or PET membrane)
- Tissue Culture flasks
- Millipore Stericup® Vacuum Filter Unit
- 0.02% EDTA
- 1x Trypsin/EDTA (Sigma T3924 or equivalent)
- Cell culture media
- Sterile 1 mL pipet tips, pipettors, microfuge/centrifuge tubes and basins
- Haemocytometer or other cell counter

Note: Although the following methods have been optimized for seeding and feeding adherent, epithelial cell lines such as Caco-2 and MDCK, they contain the basic principles for handling the plate using any method or cell line.

Methods

Optimization of Seeding Density

1. Expand and cultivate cells in T-75 flasks in a cell culture incubator set at 37^{o} C, 5-6% CO_2 and 95% relative humidity. Allow cells to reach 80-90% confluence before detaching and passing. Do not allow cells to become over confluent (>90%) as this will impact subsequent monolayer formation on the Millicell-24 cell culture plates.

Note: The number of cell passages can be a factor in the formation of an optimal monolayer. It is therefore recommended that Caco-2 cells be subjected to no more than 20 passages before a new line is established. Similarly, MDCK cells should be subjected to no more than 40 passages.

2. Aspirate the media, rinse the cultivated cells in T-75 flasks with 5 mL EDTA and incubate for 3-5 minutes. After aspiration of the EDTA, and add 1.5 mL of the trypsin/EDTA solution. Incubate at 37^{o} C for approximately 5 to 15 minutes or until the cells detach and float. This can be confirmed by periodic visual inspection of the flasks.
3. Once the cells are detached, add fresh cell culture medium and mix until all cell clumps are dispersed. Count the cells using a haemocytometer or other cell counter to determine the cell number and pass cells accordingly to maintain stock of cell line. Mix cells frequently to ensure accurate counts.

Note: To optimize the seeding density of a cell line, it is recommended that a range of cell concentrations across the plates be used in replicates of 6 or 8 (see Table 1a and 1b).

4. In sterile centrifuge tubes, dilute the cell solution with medium to enable the plating of a range of seeding densities. A good starting point is calculated by multiplying your present cell density by the fold increase or decrease in surface area.

   Example: If your seeding density for the 12 mm Millicell inserts (surface area = 0.3 cm^2) is 60,000 cells/well then you would multiply 60,000 by 2.3 to calculate your seeding density for the Millicell 24 (surface area = 0.7 cm^2).

   0.7 cm^2/0.3 cm^2 = 2.3 60,000 cells/well x 2.3 = 138,000 cell/well

   A suitable range might therefore encompass 120,000 – 160,000 cell/well, depending on individual cell lines used. **Table 1** lists Millipore's optimized densities (in terms of three different units) for seeding 3 day MDCK and 21 day Caco-2 cell monolayers and includes surface area measurements for Millicell and MultiScreen® Caco-2 products.

Note: If starting without any prior platform, it is suggested to initially bracket a range of seeding densities around our values listed in Table 1.

Note: Achieving a uniform cell suspension when plating the cells will promote a more consistent monolayer across the 24 wells. This may be particularly difficult when seeding multiple plates. Frequent mixing is recommended to minimize the risk of cells settling to the bottom, resulting in an inaccurate distribution of cells across the wells or plates.

5. Pipette 400 μL of cells at each seeding density into the appropriate filter wells of the Millicell-24 Cell Culture Plates **(Tables 2a and 2b).** Pipette 32 mL of cell culture medium into the single well feeder plate via the large access hole located at the lower right of the plate. Alternatively, disassemble the filter plate from the feeder plate. Place the filter plate on a sterile surface in a laminar flow hood and add medium directly to the feeder plate. Gently reassemble the two components and place in the cell culture incubator.

Note: Cells seeded onto the Millicell-24 Cell Culture Plate should be placed in an incubator that provides adequate humidity control. A cell culture incubator with electronic humidity control is recommended -- if this is not possible, plates should be placed in an incubator that will not be opened frequently.

Feeding

Note: Replacing the growth medium every other day is recommended for optimal cell growth. The most critical part of removing and replacing the medium in the Millicell-24 Cell Culture Plate is to avoid damaging the monolayer of cells and the filter on which they are supported. This can be accomplished using a sterile 1 mL pipette tip and utilizing the apical assist feature.

1. Aspirate the medium from the feeder plate (basolateral) using a sterile 1 mL pipette tip via the large access hole. Alternatively, the filter plate can be removed from the feeder plate, then set on its "feet" to stand while medium is aspirated from the feeder tray.
2. Aspirate the medium from the filter wells using the apical assist feature. Use care not to contact the filter inside the wells when removing or adding medium. Add back 400 µL growth medium to the filter wells (at the apical assist) before adding back the 32 mL of medium to the feeder plate (basolateral).

Note: Left handed users may be more comfortable if the plate is rotated 180^{o} so that the apical assist is on the right. This facilitates pipetting with the left hand.

Note: If setting the filter down on the hood surface using the "feet", it is recommended to let the whole assembly sit in the hood for 15 seconds after removing it from the incubator. Disassembly of the plate after 15 seconds will minimize the potential for droplet formation on the underside of the membrane, which may contact the hood surface.

Note: Evaluation of seeding densities is usually performed by testing the integrity of the monolayer with trans-epithelial electrical resistance (TEER) measurements, by evaluation of the transport of Lucifer yellow (LY) or by using standard drug compounds.

Table 1

Millicell-24 Cell Culture Plate – Cell Seeding Density Unit Conversions			
	Cells per cm^2	Cells per mL (0.4 mL)	Cells per well
21 day Caco-2 Seeding Density	**85,700**	**150,000**	**60,000**
3 day MDCK Seeding Density	**714,000**	**1,250,000**	**500,000**

Surface areas:
Millicell-24 Cell Culture Plate = 0.7 cm^2
Millicell 12 mm Cell Culture Inserts = 0.3 cm^2
Millicell 30 mm Cell Culture Inserts = 4.5 cm^2
MultiScreen Caco-2 Assembly = 0.11 cm^2

Table 2a
Millicell-24 Cell Culture Plate Template – Range of three cell seeding densities.

	1	2	3	4	5	6
A	Seeding density #1		Seeding density #2		Seeding density #3	
B						
C						
D						

Table 2b

Millicell-24 Cell Culture Plate Template – Range of four cell seeding densities.

	1	2	3	4	5	6
A	Seeding density #1					
B	Seeding density #2					
C	Seeding density #3					
D	Seeding density #4					

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