Caco-2 Permeability Assay

Caco-2 Permeability

Goal

To measure directional Caco-2 permeability of test compounds in cultured Caco-2 monolayer.

Set-up

INSTRUMENTS

- **Tissue culture CO_2 incubator with humidity control**
- **Liquid handler**
- **Orbital shaker**
- **EVOM Epithelial Volt-ohmmeter fitted with planar electrodes (World Precision Instruments, Sarasota, FL) required for measuring transepithelial electrical resistance (TEER)**
- **Bench top centrifuge with 96-well plate adaptor**
- **Caco-2 cells (Human colorectal adenocarcinoma, ATCC #37-HTB, passage 30-45)**
- **Cells seeded onto PET membranes (1 μm pore size, 0.31 cm^2 surface area) inside Falcon HTS multiwell Insert system using 24-well plates (Becton Dickinson plates, Part # 351181, Fisher Scientific, Inc.) at a density of 23,000 cells/well. Cells grown 20-23 days with medium changed every 2-3 days**

REAGENTS

- **Ringers buffer solution (pH 7.4 at 25°C)**
- **Ringers buffer with 1% Methanol**
- **Blk solution: Ringers buffer: Methanol=2:1 (v/v)**

- **100% Methanol including internal standard (IS)**
- **10 mM stock dosing solution in DMSO**
- **100 µM dosing solution in buffer**

Protocol summary

- **Caco-2 permeability: 20-23 day/ Passage 30-45**
- **24-well format transwell: 0.31 cm^2 surface area**
- **Donor conc: 100 µM including 1% DMSO**
- **A: 300 µL pH 7.4/ B: 1200 µL pH 7.4 Ringers buffer**
- **Directionality: A B and B A (N=4)**
- **Donor side sampling: 20 µL at beginning and end (90 min)**
- **Receiver side sampling: 100 µL at 30, 50, 70, and 90 min**
- **Incubation at 50 oscillations per minute, 37 °C, 5%CO_2, 95% humidity**
- **Analysis: LC-UV, LC-MS, or LSC**
- **Output: Peff (cm/sec) = (dX/dt)/(A*Co*60), dX/dt: transported amount (nmole) *versus* time (minute) profile in the receiver chamber; A: surface area (cm^2); and Co: initial donor concentration (µM)**
- **Positive control: Atenolol and propranolol**
- **Membrane integrity: TEER >200 Ωcm^2**
- **Amount required: Approximately 1 mg or 100 µL of 10 mM test compound in DMSO**
- **Instruments: CO_2 incubator with humidity control, liquid handler, epithelial volt-ohmmeter for TEER, Caco-2 cells (ATCC #37-HTB), and 24-well insert plates (PET membranes, 1 µm pore size, 0.31 cm^2 surface area, Becton Dickinson plates, Part # 351181)**
- **Throughput: 6 compounds / 2 Caco-2 plates/1 FTE/ day**

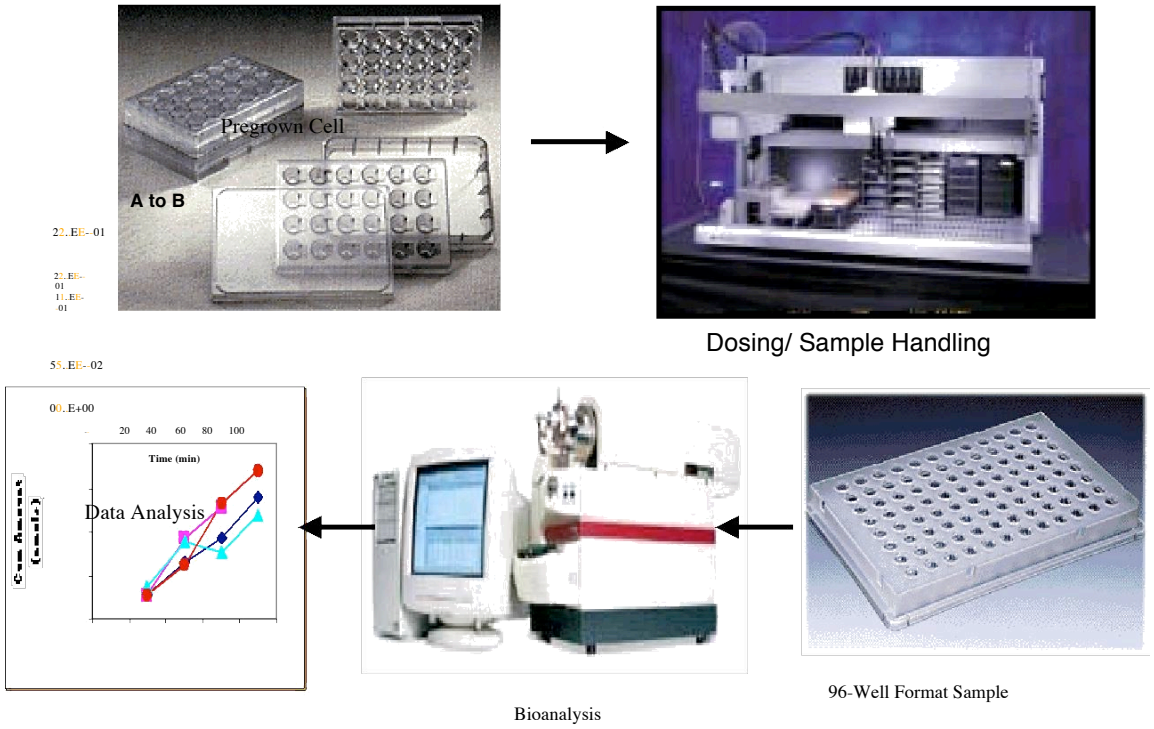

Figure 31. The Caco-2 Permeability Assay Procedure

Preparation

Table 24. Preparation of Ringers with Glucose (Isotonic = 290 mOsm/kg), pH 7.4

Chemical	Molecular Wt	Concentration	Mass(g) for 1L	Mass(g) for 2L	Mass(g) for 4L
$Ca\ SO_4\ 2H_2O$	172.2	1.25 mM	0.2152	0.4305	0.861
$MgSO_4\ 7H_2O$	246.5	1.1 mM	0.2712	0.5423	1.0846
KCl	74.55	5 mM	0.3728	0.7455	1.491
Na_2HPO_4	142.0	1.15 mM	0.1633	0.3266	0.6532
$NaH_2PO_4\ H_2O$	138.0	0.3 mM	0.0414	0.0828	0.1656
$NaHCO_3$	84.01	25 mM	2.100	4.200	8.401
Glucose($C_6H_{12}O_6$)	180.2	25 mM	4.505	9.01	18.02
NaCl	58.44	110 mM	6.428	12.86	25.71

PREPARATION OF 4 L SOLUTION

1. **To 3.5 L distilled water, add Calcium Sulfate and Magnesium Sulfate.**

   Note: Add Calcium Sulfate and Magnesium Sulfate first due to low solubility and add the remaining ingredients in the order listed in Table 1.

2. **Adjust the final volume of the solution to 4 L with distilled water, with continuous stirring.**

3. **Adjust final solution to a pH of 7.4 using 1N HCl or 1N NaOH.**

4. **Make the buffer iso-osmotic using NaCl. Measure tonicity of the solution using a tonometer. Given that an isotonic solution is equivalent to 0.9% NaCl (290 mOsm/L),** ***Y= {(*****290-*****x)/290} x 9mg x 4000 mL,*** **where y = NaCl required (in mg) to make the solution isotonic and x = observed tonicity of solution (reported as mOsm/L).**

PREPARATION OF DOSING SOLUTION IN 15 ML PP TUBE

1. **100 µM dosing solution in RG: 140 µL 10 mM stock + (14 mL – 140 µL) RG**

PREPARATION OF CALIBRATION IN 96 SHALLOW WELL

1. **Prepare 10 µM standard: 100 µL of 100 µM dosing solution + 0.9 mL Ringers with 1% Methanol.**

2. **Prepare analytical standard solutions 10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01, and 0 µM. (See Table 26)**

Table 25. Preparation of analytical calibration in 96 shallow well

	1	2	3	4	5	6	7	8	9	10	11	12
	0	20 µL of 0.1 µM	20 µL of 0.2 µM	20 µL of 0.5 µM	20 µL of 1 µM	20 µL of 2 µM	20 µL of 5 µM	20 µL of 10 µM	40 µL of 10 µM	100 µL of 10 µM	200 µL of 10 µM	Source solution
	180 µL	180 µL	180 µL	180 µL	180 µL	180 µL	180 µL	180 µL	160 µL	100 µL	0	1% MeOH in buffer
Comp 1	Blk	0.01 µM	0.02 µM	0.05 µM	0.1 µM	0.2 µM	0.5 µM	1 µM	2 µM	5 µM	10 µM	
Comp 2												
Comp 3												

Transport Studies

DOSING AND SAMPLING

1. **Equilibrate both sides of the monolayers for 10 minutes with prewarmed (37°C) drug-free Ringers buffer (300 µL apical side, 1,200 µL basolateral side) supplemented with glucose (25 mM).**

2. **Measure TEER under 37°C water bath conditions.**

   Note: The TEER value serves as a quality control check for monolayer integrity. At 21 days post-seeding, each Caco-2 cell monolayer should have a TEER value of greater than or equal to 200Ω x cm^2 and those not meeting this criteria are not suitable for permeability evaluations.

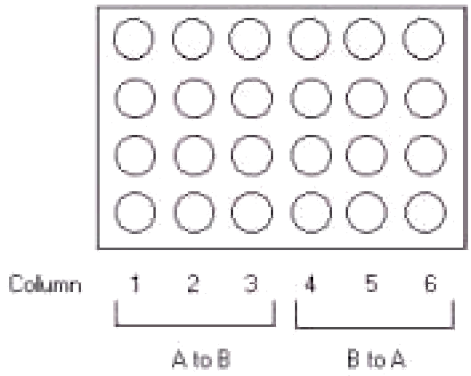

Figure 32. Representative 24-well Caco-2 Plate

3. **When studying A to B transport: Fill basolateral side with 1,200 µL of Ringers buffer. Initiate transport experiments by transferring test drug dosing solution (320µL) to apical side.**

4. **When studying B to A transport: Fill apical side with 300 µL of Ringers buffer. Initiate transport experiments by transferring test drug dosing solution (1,220 µL) to basolateral side. Transport studies for each direction (A to B, B to A) are performed in quadruplicate for each test drug.**

5. **Start timer after dosing last donor well.**

6. **Remove 20 µL aliquots from the donor wells at 0 minutes (D_o) and transfer these aliquots to the donor site of the 96-well plate containing 180 µL buffer with 1% Methanol. This step effectively dilutes the D_0 ten times.**

7. **Initiate transport studies by placing plate on orbital shaker maintained inside a prewarmed (37°C) and humidified (5% CO_2) incubator. Studies are performed under stirring conditions at 50 oscillations per minute.**

8. **Remove 100 µL aliquots from the receiver side of the monolayer at 30, 50, 70, and 90 minutes postdosing and transfer these aliquots to the corresponding 96-well sample plate (See Table 26). Replace with an equivalent volume of prewarmed buffer.**

9. **Remove 20 µL aliquots from the donor side of the monolayer at 90 minutes postdosing (D_f) and transfer these aliquots to a donor site of a 96-well plate containing 180 µL Ringers buffer with 1% Methanol. This step effectively dilutes the D_f ten times.**

10. **Replace both sides of monolayer with fresh, drug-free, prewarmed Ringers buffer (300 µL apical side, 1,200 µL basolateral side) and equilibrate for 10 minutes.**

11. **Measure TEER under 37°C water bath conditions.**

SAMPLE HANDLING

The following steps refer to 96-well analytical plate for Caco-2, Table 26.

1. **Transfer 20 µL of diluted D_0 and D_f to corresponding 96-well sample plate with each well containing 80 µL buffer with 1% Methanol. This step effectively dilutes the samples five times further. Therefore, donor samples are diluted 50 times from their initial concentration.**
2. **Transfer 100 µL of analytical calibration (from 0 to 10 µM) to the sample plate row 1.**
3. **Add 50 µL Methanol including IS to all sample wells and mix (standards, samples, and D_0 and D_f).**
4. **Transfer 150 µL of Blk solution to the analytical plate row 2.**
5. **Seal the analytical plate with adhesive sealing film and store samples with label at -80°C for LC-UV or LC-MS analysis.**
6. **Analyze 20 µL aliquots of the individual permeability samples and the standards using a suitable analytical instrument.**
7. **Peff = (dX/dt)/(A x C_0 x 60), where P_{eff} is the effective permeability in cm/sec, X = mass transported, A is the surface area (cm^2) available for transport, C_0 is the initial donor drug concentration (µM), and dX/dt is the slope of the best fit line through the transported amount (nmole) versus time (min) profile in the receiver chamber.**

Table 26. Analytical Plate for Caco-2 (96-well plate)

0	0.01µM	0.02µM	0.05µM	0.1µM	0.2µM	0.5µM	1µM	2µM	5µM	10µM	
Blk	Blk	Blk	Blk	**A to B**			**B to A**	Blk	Blk	Blk	Blk
1-30	2-30	3-30	4-30					5-30	6-30	7-30	8-30
1-50	2-50	3-50	4-50					5-50	6-50	7-50	8-50
1-70	2-70	3-70	4-70					5-70	6-70	7-70	8-70
1-90	2-90	3-90	4-90					5-90	6-90	7-90	8-90
1-Do	2-Do	3-Do	4-Do					5-Do	6-Do	7-Do	8-Do
1-Df	2-Df	3-Df	4-Df					5-Df	6-Df	7-Df	8-Df

POSITIVE CONTROL DATA

Mean data in Table 27 represent the mean value from 12 separate inter-day experiments.

Table 27. P_{eff} (x E-6 cm/sec) in pH 7.4 Caco-2

	A B	B A
Atenolol		
Mean	1.08	2.29
Range	0.69 – 1.80	1.69 – 2.68
Propranolol		
Mean	28.53	20.91
Range	18.50 – 36.80	16.30 – 31.40