Drug Transport Assay for Falcon® 24 Multiwell Inserts

Protocol

CORNING

Purpose

This is a method to determine the apical (AP) to basolateral (BL) and BL to AP transport of a test compound in cell monolayers, using a manual (non-robotic) procedure and LC-MS analysis.

Materials

- Caco-2 cells grown in Falcon 24 Multiwell Insert System (Cat. Nos. 351180 and 351181; includes feeder tray), prepared under a separate protocol.
- Receiver plates: Falcon 24-well clear, flat-bottom plates with lids, TC-treated surface, sterile (Cat. No. 353047)
- Falcon 96-well black assay microplates (Cat. No. 353376)
- Falcon serological pipets and 50 mL centrifuge tubes
- Orbital platform shaker
- Hanks Balanced Salt Solution (HBSS) with calcium and magnesium (Thermo Fisher Cat. No. H-8264 or equivalent)
- 1M HEPES Solution (Thermo Fisher Cat. No. H-0887 or equivalent)
- Test compound stock solution prepared to 10 to 20 mM in DMSO
- Internal standard for LC-MS analysis
- Lucifer Yellow (Thermo Fisher Cat. No. L0144 or equivalent)

Solution Preparation

Transport Buffer: HBSS + 10 mM HEPES, pH 7.4

Make a 1:100 dilution of 1M HEPES in HBSS. Bring solution to pH 7.4 using HCl or NaOH. Warm the transport buffer to 37°C prior to preparation of the donor and receiver solutions (below), and before washing the monolayers (see Step 2).

Test Compound Donor Solution

Prepare the test compound in Transport Buffer, at the appropriate concentration (e.g., 10 µM). The final donor solution should contain between 0.5% and 1% DMSO (v/v). **NOTE:** To ensure accuracy of final Test Compound concentrations, prepare dilutions of the Test Compound in DMSO and then transfer into Transport Buffer. This is critical for compounds of limited aqueous solubility.

Lucifer Yellow Donor Solution

Prepare Lucifer Yellow at 300 µM in Transport Buffer. The final donor solution should contain between 0.5% and 1% DMSO (v/v).

Test Compound Receiver Solution

The receiver solution is Transport Buffer with a DMSO content matched to that in the donor solution.

Transport Assay Procedure

The following procedure uses apical volume of 300 µL and BL volume of 1000 µL. **NOTE:** Other assay volumes work well in the assay. The Falcon® 24 Multiwell Insert System protocol also recommends 400 µL AP and 1200 µL BL and 500 µL AP and 1400 µL BL. Other volumes (e.g., 100 µL AP and 600 µL BL) work well in the assay. You may wish to examine different assay volumes during the course of your assay development.

TEER: Measure the resistance (ohms) in the inserts containing culture medium or buffer, using an ohmmeter equipped with probes (e.g., WPI ohmmeter with Chopstick probes). The resistance of a blank insert (no cells) should be determined to subtract as the background value. **NOTE:** In the course of assay development you should establish a minimum TEER value for monolayer acceptance in the assay.

1. **Prepare the transport assay plate and a Lucifer Yellow receiver plate**
   - Add 1000 µL of the test compound donor solution or the receiver solution (depending on the direction of transport) to each well of a 24-well plate. Label the plate accordingly (e.g., "Transport"). Maintain at 37°C.
   - Add 1000 µL receiver solution to each well of a 24-well plate. Label the plate "LY flux". Maintain at 37°C.
2. **Wash the monolayers with Transport Buffer**
   - Prepare a wash plate with 1000 µL Transport Buffer in the wells of a 24-well plate.
   - Transfer the monolayers from the culture medium to the prepared wash plate.
   - In sets of two monolayers, carefully remove and discard the culture medium from the apical side of a monolayer, replacing the volume with transport buffer. Maintain at 37°C until use. **NOTE:** Be careful not to physically damage the monolayers.
3. **Remove Transport Buffer wash and add Test Compound Donor or Receiver Solution**
   - Transfer the monolayers to the "transport" plate prepared in Step 2.
   - In sets of two monolayers, remove the apical Transport Buffer wash and replace with 300 µL Test Compound Donor Solution or Receiver Solution (depending on the direction of transport). Add an extra volume (e.g., 50 µL) to the donor chambers to be removed as t=0 donors from the monolayers.
4. **Collect t=0 minute AP and BL donor samples**

   These will be used to determine initial concentration of the donor in the monolayers at t=0 minutes.
   - Withdraw 50 µL from the donor chamber, AP or BL (depending on the direction of transport), dilute with Receiver Solution (e.g., 1:50) and combine with LC-MS internal standard.
   - Replace the plate lid and incubate the plate with orbital shaking (e.g., 50 rpm) at 37˚C for desired time period (e.g., 90 minutes). **NOTE:** Incubation time is an experimental variable. The effect of incubation time can be examined during assay development.
5. **Collect t=90 minute receiver samples**

   Withdraw 50 µL from the receiver chamber and combine with LC-MS internal standard.
6. **Collect t=90 minute donor samples**

   Withdraw 50 µL from the donor chamber, dilute with receiver solution (e.g., 1:50) and combine with internal standard.
7. **Add Lucifer Yellow to the apical chambers**
   - Remove and discard remaining solution in AP chambers and add 300 µL of 300 µM Lucifer Yellow solution to each of the apical chambers of the monolayers. **NOTE:** An alternative approach is to add an equal volume of 600 µM Lucifer Yellow solution to the remaining volume in the apical chambers.
   - Transfer the plate of inserts to the receiver plate containing Receiver Solution (prepared in Step 1) and place at 37°C with orbital shaking (e.g., 50 rpm) for 30 to 60 minutes.

8. **Lucifer Yellow Flux Determination:**
   - Prepare a well in a 96-well black assay microplate, containing 300 µL of the 300 µM Lucifer Yellow solution.
   - Add 300 µL of (blank) receiver solution to one well (plate blank).
   - Following incubation, transfer 300 µL from the receiver chamber of each monolayer to a well in the 96-well black assay microplate.
   - Prepare a standard curve of lucifer yellow, by supplementing transport buffer with a range of Lucifer Yellow concentrations (e.g., 0, 0.012, 0.037, 0.11, 0.33, 1.0 µM).
   - Scan the plate on a fluorescence reader (excitation/emission filter wavelength of 485 nm/538 nm) and calculate the concentration of Lucifer Yellow present using the standard curve.
9. **Quantify the concentration of Test Compound in the various Donor and Receiver Samples:**
   - Analyze the various donor and receiver samples for the amount of Test Substance by LC-MS using an appropriate analytical method and standard curves. **NOTE:** Techniques for accurate LC-MS quantitation are outside the scope of this assay protocol.

Calculations

Lucifer Yellow Flux (% LY Flux)

$$\% \text{ LY Flux} = 100 \times ((LY_{BL} \times Vol_{BL}) / (LY_{AP} \times Vol_{AP}))$$

Where:

LY_{BL} is the concentration of lucifer yellow in the basolateral receiver chamber [µM]

Vol_{AP} is the volume in the apical receiver chamber [cm^3]

LY_{AP} is the concentration of lucifer yellow added to the apical donor chamber [µM]

Vol_{BL} is the volume in the basolateral donor chamber [cm^3]

NOTE: Lucifer Yellow flux range for intact cell monolayers is typically 0.3% to 2%. A flux value >2% suggests that the monolayer was likely compromised during the assay and the results are suspect.

Transepithelial Electrical Resistance (TEER)

$$TEER = (R_m - R_b) \times A$$

Where:

TEER is the transepithelial electrical resistance [Wcm^2]

R_m is the resistance reading obtained for the cell monolayer [W]

R_b is the resistance reading obtained for the blank insert (without cells) [W]

A is the surface area of insert filter membrane [cm^2]

NOTE: In the course of assay development, establish a minimum TEER value (e.g., 200 Wcm^2) for monolayer acceptance.

Apparent Permeability (Papp)

$$P_{app} \text{ [cm/sec]} = (Flux \times V_d) / (t \times A) = dQ/dt \times 1/(A \times C_d)^{[1]}$$

Where:

Flux is the fraction of the donated amount recovered in the receiver chamber

V_d is the volume in the donor chamber [cm^3]

C_d is the initial concentration in the donor solution [mM]

A is the surface area of insert filter membrane [cm^2]

t is the incubation time [sec]

dQ/dt is the amount of drug transported within a given time period [pmol/sec]

Efflux Ratio (ER)

$ER = P_{app\ B\text{-}A} / P_{app\ A\text{-}B}$ [2]

Where:

$P_{app\ B\text{-}A}$ is the P_{app} value measured in the B to A direction

$P_{app\ A\text{-}B}$ is the P_{app} value measured in the A to B direction

NOTE: Efflux ratios greater than 2 (or 3) are generally considered to be evidence for transport. Follow-up studies using inhibitors of drug transporters (e.g., ketoconazole) can be performed to develop further evidence.

Mass Balance

Mass balance will be calculated as % recovery of test article from the donor and receiver chambers.

$\text{Mass balance} = 100 \times ((V_r \times C_r^{final}) + (V_d \times C_d^{final}))/(V_d \times C_0)$

Where:

C_0 is the initial concentration in the donor solution [nmol/cm³]

V_r is the volume of the receiver compartment [cm³]

V_d is the volume of the donor compartment [cm³]

C_r^{final} is the mean receiver concentration at the end of the incubation [nmol/cm³]

C_d^{final} is the mean donor concentration at the end of the incubation [nmol/cm³]

NOTE: If mass balance is poor (for example, <65% or >135%), assay results may be suspect. Poor mass balance could be due to poor aqueous solubility of the Test Compound and/or sequestration of the Test Compound in the cell monolayer or on the plastic surfaces of the consumables.

References

1. Polli JW, et al. (2001). Rational use of in vitro P-glycoprotein assays in drug discovery. J Pharmacol Exp Ther 299:620.
2. U.S. FDA/CDER, Drug Interaction Studies - Study Design, Data Analysis, and Implications for Dosing and Labeling, DRAFT GUIDANCE, September 2006.

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