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24-Well CacoGoblet User's Manual (Individual Transwell Inserts)

MEDTECH INNOVATION ON ADVANCED MEDICINE S.L.U.
Barcelona Science Park – C/ Baldiri Reixac, 10 – 08028 Barcelona (Spain)
Ph: +34 934 037 077 E: reagents@medtechbcn.com W: https://www.medtechbcn.com/

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Product Description

CacoGoblet is an *in vitro* cell-based model resulting from the coculture of two human colon cancer cells (Caco-2 and HT-29) with an absorptive and mucus-secreting phenotype, respectively. Cells differentiate for 21-days in 24 individual Transwell® inserts with semi-porous (0.4 µm) polycarbonate (PC) membrane (CORNING Cat#3413) resulting in an apical compartment and a basal compartment that mimic the intestinal lumen and the blood circulation, respectively.

Caco-2/HT-29 cells are considered a relevant cellular model for assessing drug passive diffusion and/or active transport and can easily be adapted for high-throughput screening of compounds[1].

CacoGoblet is delivered in a 24-well/plate format with a unique Shipping Medium (a gel-like cell culture medium) established by MEDTECH BARCELONA which enables the transport of cells at room temperature and in a ready-to-use format.

Intended Use

This product is mainly indicated for *in vitro* evaluation of:

- drug permeability by passive diffusion and/or active transport through a physiologically relevant barrier
- carrier-mediated transport mechanisms
- drug toxicity
- inflammatory/anti-inflammatory drugs

NOTE: *This cell-based model is intended for scientific research purposes only. Not for human or veterinary use.*

Principle

Passive permeability and outward active transport of drugs are carried out with Caco-2 cells that endogenously express relevant human drug transporters in the apical domain of the plasma membrane and HT-29 cells that produce a relevant mucus barrier.
In the experimental setup, these cells will be differentiated on individual Transwell® inserts to form a tight cell monolayer that prevents media from wicking between the insert (apical compartment) and the plate well (basal compartment). Efflux transporters localized in the apical side will introduce a basolateral-to-apical bias in the distribution of substrate compounds between the two compartments.

In a standard assay design, the reaction is initiated by filling either compartment with the solution containing the test compound. The distribution is assessed over time by withdrawing and analyzing samples from both compartments. After normalization, the speed of translocation is obtained for both directions. The ratio of the two values is a measure of the passive and active transport mechanisms involved in the distribution of the compound.

Handling and experimental procedures are provided below. The manual has been written for users with experience in cell culturing and pharmacological drug discovery *in vitro* testing experiments. For more detailed advice and training opportunities, please contact us at:

reagents@medtechbcn.com

Timeline for Delivery and Experimental Procedures

- Day 1: Start of Production (Seeding of cells)
- Day 14: Pre-shipping Quality Control (TEER and Lucifer Yellow)
- Days 14-15: Package Dispatch (depending on destination)
- Days 16-17: Package Delivery
- Day 18: Replacement of Shipping Medium (liquefaction)
- Day 21-25: Quality Control Experiments and Medium Replacement/Assay Performance (see Table 1)

Packages are dispatched on Mondays/Tuesdays and delivered within 24-48 h to EU countries, 48-72 h to USA, and 48-96 h to Asian countries. For other locations and customized schedules, please contact us at:

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The recommended timing overview for permeability assays is Day 21 (Monday) (see Figure 1 for details).

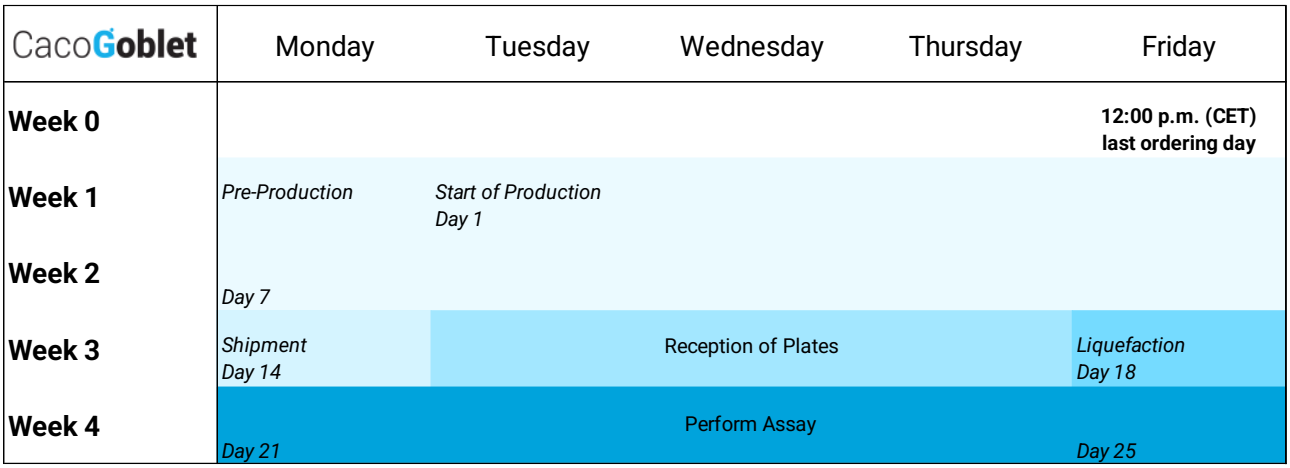


Figure 1. Timeline of manufacturing and operation for CacoGoblet in 24-well format.

In case you choose to conduct the experiments later, TEER measurements and medium replacement must be carried out as follows:

DAY OF EXPERIMENT	TEER MEASUREMENT	MEDIUM REPLACEMENT
Tuesday (Day 22)	Monday (Day 21)	Monday (Day 21)
Wednesday (Day 23)	Monday (Day 21) Wednesday (Day 23)	Monday (Day 21)
Thursday (Day 24)	Monday (Day 21) Wednesday (Day 23)	Monday (Day 21) Wednesday (Day 23)
Friday (25)	Monday (Day 21) Wednesday (Day 23) Friday (25)	Monday (Day 21) Wednesday (Day 23)

Table 1. Recommended day for TEER measurement and medium replacement.

NOTE: *These steps enable the planning of the assay according to the user´s convenience.*

IMPORTANT NOTE: *TEER evaluation will be carried out on Monday (Day 21) before performing any further processing, including medium replacement. Based on our experience with long-distance shipments and/or extreme temperatures at destination, in case TEER values are low, it is recommended to perform a medium change and wait until Wednesday to let the cells recover. On Wednesday, read the TEER again and perform the assay accordingly.*

Equipment (not included)

- Cell culture laminar flow hood
- CO2 incubator
- Water bath
- Multichannel pipettes
- Aspiration system
- Trans-Epithelial Electrical Resistance (TEER) meter (WPI EVOM series)
- **Chopstick electrode for EVOM (STX2)**
- Fluorometer (Fluoroskan Ascent CF)
- Quantitative analytics equipment

Consumables

- **Reservoir plate (Corning Cat# 3524)** ***(not provided)***
- Sterile culture medium containers (i.e., Costar 50 ml, Cat# 4870) *(not provided)*
- 15 and 50 mL conical tubes and 1.5 mL Eppendorf tubes *(not provided)*
- Pipette tips *(not provided)*
- Pasteur pipettes *(not provided)*

Solutions (may be included)

NOTE: *MedTech Barcelona can supply Medium and Transport Buffer if required.*

- **Caco-2/HT-29 Cell Culture Medium**: Dulbecco's Modified Eagle's Medium - high glucose (4.5 g/L) (CORNING Cat# 10-013-CV) supplemented with (final concentrations):
  - 10 % V/V Fetal Bovine Serum (BIOWEST Cat# DE14-801F)
  - 100 U/mL; 0.1 mg/mL Penicillin-Streptomycin (LONZA Cat# DE17-602F)
  - 1x MEM non-essential amino acid solution (GIBCO Cat# 11140035)
- **Transport Buffer solution**: Hank's 1X Balanced Salt Solutions (HBSS 1x) (HyClone Cat# SH30268)
- **Recommended reporter for Low Permeability Substrates**: Atenolol (SIGMA Cat#A7655)
- **Recommended reporter for High Permeability Substrates**: Metoprolol (SIGMA Cat#M5391)
- **Recommended reporter for MDR1 (Pgp) Substrates**: Digoxin (SIGMA Cat# 04599)
- **Recommended reporter for MDR1 (Pgp) Inhibitors**: Verapamil (SIGMA Cat# V4629)

- **Recommended reporter for BCRP Substrates**: Estrone-3-sulfate (E3S) (SIGMA Cat# E9145)

NOTE: *If the specified reagents are not available, other reagents with similar features and specifications can be used.*

Handling

Upon reception, retrieve the zipped bags containing the plates. Open the zip and leave the bag in a dark location at room temperature until Day 18 (refer to Timeline; Figure 1).

Replacement of Shipping Medium

CAUTION: *Never handle more than one plate at a time while changing the shipping medium. Re-solidification of the shipping medium may damage the cell monolayer.*

These **steps** will be **carried out on Day 18** (refer to Timeline; Figure 1). Perform all manipulation under sterile conditions.

1. Retrieve the plates from the bags and remove the parafilm wrap.
2. **Incubate** the plates in a 5 % CO2 humidified atmosphere at 37 °C for **4 hours**, until the **shipping medium** reaches **liquefaction**.
3. Remove one CacoGoblet plate from the incubator and place it inside the laminar flow hood, along with one reservoir plate.
4. Using sterile procedures (**inside the laminar flow hood**), fill a sterile reagent reservoir with 50 mL of pre-warmed (37 °C) Caco-2/HT-29 cell culture medium.
5. Open the CacoGoblet plate and the reservoir plate, and leave their lids upwards, next to the plates.
6. Carefully use **forceps to lift separately** the 24-individual **apical compartments** of the CacoGoblet plates and transfer them onto the reservoir plate.
7. Remove all liquefied shipping medium from the basal compartments of the CacoGoblet plate with a Pasteur pipette.
8. Using the multichannel pipette, dispense **900 µL** of Caco-2/HT-29 cell culture medium from the sterile reservoir, and fill, the **basal compartments** of the CacoGoblet plate, column by column.
9. **Remove** the liquefied **shipping medium from the individual apical inserts** of the CacoGoblet plate with a multichannel pipette, taking care not to disrupt the monolayer. Make sure the shipping medium has been removed from all wells. Approximately 50 µL of medium will be left in each well.
10. Using a multichannel pipette, dispense **300 µL** of Caco-2/HT-29 cell culture medium from the sterile reservoir, and fill, the **apical compartments** of the CacoGoblet plate, column by column. Always add the medium against the wall of the well, and not directly onto the cell monolayers.
11. Carefully **use forceps to return the apical inserts onto the basal compartment** of the CacoGoblet plate. Replace the lid and place it inside the cell culture incubator, set at 37 °C and 5 % CO2.
12. Once the shipping medium has been substituted by fresh Caco-2/HT-29 cell culture medium, the plates should be placed inside the incubator until next Monday (Day 21). **Replacement with a new fresh medium** will be carried out once (Day 21) or twice (Days 21 and 23) depending on the day of the assay (refer to Table 1 for details), following the procedure described above.

NOTE: *Do not discard the reservoir plate, as it will be used in the permeability assay.*

Quality Control of the Barrier System

Pre-assay Quality control – TEER Measurement

This section provides general instructions for TEER evaluation. It is important to read carefully the instructions of the TEER measurement equipment in conjunction with these instructions.

The timeline for TEER evaluation is detailed in Table 1. TEER measurement will be carried out before performing any further processing, including the experiment and the medium replacement.

NOTE: *Never perform the TEER measurement with the shipping medium. Do not repeat TEER measurements in the same well.*

For **TEER evaluation**, follow the steps below:

1. **Sterilize the electrode** (probe) by submerging both tips in 70 % ethanol for 5 minutes.
2. Equilibrate the electrode (probe) for 5 minutes in Caco-2/HT-29 cell culture medium, **pre-warmed at room temperature**.
3. While the electrode is equilibrating, remove the CacoGoblet plate from the incubator and place it in a laminar flow hood. **Allow the plate to reach room temperature** (approximately 20 minutes), as TEER measurements should be performed under this condition.
4. If using chopstick **electrodes** like the STX2 (WPI EVOM series), place the probe into the insert system, so that the thinner electrode is within the narrowest slit, which corresponds to the basal part of the inserts. The thicker electrode must be placed inside the widest slit, corresponding to the apical part of the inserts. Both electrodes have to be **well submerged** within the cell culture medium of the apical and basal compartments **for a correct evaluation**. Be careful **not to touch the cell monolayers**!

   NOTE: *Watch out to set the electrode in the right position.*

5. Record the resistance readout in ohms (Ω) for each well. **TEER value is the result of multiplying the resistance value by the cell growth area (cm^2)**.

Acceptance Criterion

Active membrane surface (Corning plates)	**0.33 cm^2**
TEER value	**> 70 Ω x cm^2**

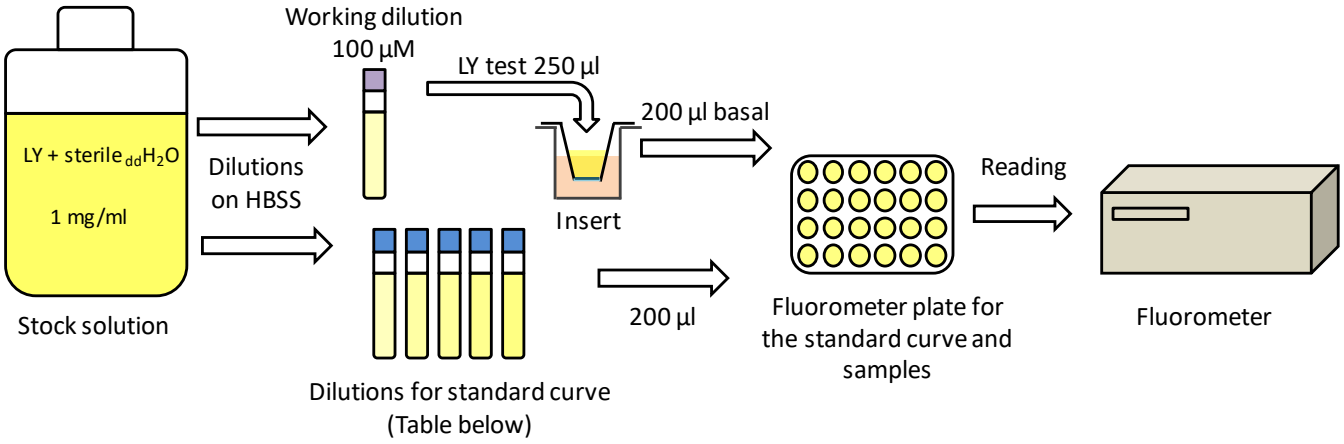
Post-assay Quality Control – Lucifer Yellow (LY) Paracellular Permeability Assay

Prepare a **1 mg/mL (2.187 mM) LY solution** in sterile ddH_20. Make aliquots (e.g., 500 µL) and store them at -20 °C.

Dilute LY stock solution in transport assay buffer to a **100 µM final concentration**. Working dilution will be used to prepare the calibration curve and for the LY test (see Figure 2 for details).

To proceed with the LY permeability assay, follow the steps below:

1. Prewarm the 100 µM working LY solution at 37 °C covered with foil to protect it from light.
2. **Prepare the calibration curve** by making serial 1:2 dilutions of the working solution (see Figure 2).
3. **Rinse both** the apical and the basal **compartments** gently with transport assay buffer following the procedure described in "Replacement of Shipping Medium" (steps 4-11). Instead, use a volume of **250 µL and 750 µL** for the apical and basal compartments, respectively.
4. Remove the transport assay buffer from the apical and basal compartments following the same procedure.
5. Add **250 µL of 100 µM LY** working dilution into the **apical compartment**.
6. Add **750 µL of transport assay buffer** to the **basal compartment**.
7. **Incubate** the CacoGoblet plate, protected from light, in the cell incubator (at 37 °C and 5 % CO_2) for **1 h**.
8. **Take 200 µL** from the **basal compartment and** from the **calibration curve,** and load them into an empty 96-well plate for fluorescence-based assays. Mix well and avoid bubble formation when getting samples and standards!
9. **Read the fluorescence** intensity in a fluorometer at **485/527** excitation/emission wavelengths.



0 µM BLANK	*0.048 µM*	*0.097 µM*	*0.195 µM*	*0.390 µM*	*0.781 µM*	*1.562 µM*	*3.125 µM*	*6.25 µM*	*12.5 µM*	*25 µM*	*50 µM*	*100 µM*

Figure 2. General procedure for LY permeability assay and recommended concentrations for the calibration curve.

Acceptance Criterion

LY Paracellular Flux	**≤ 1.4 %**
LY apparent permeability (P_{app})	**≤ 3 x 10^{-6} cm/s**

General Protocol for Transport Assays

General Considerations

CacoGoblet is designed for conducting permeability *in vitro* assays of established and investigational compounds in order to predict their absorption and interaction with membrane-associated proteins (transporters: MDR1 (Pgp) and BCRP).

Recommended Reference Compounds

The compounds listed below (also referenced in the "Solutions" section) are recommended for testing as a reference for low- and high-permeability compounds and for substrates of the MDR1 (Pgp) transport protein.

- **Recommended reporter for Low Permeability Substrates**: Atenolol (SIGMA Cat#A7655)
- **Recommended reporter for High Permeability Substrates**: Metoprolol (SIGMA Cat#M5391)
- **Recommended reporter for MDR1 (Pgp) Substrates**: Digoxin (SIGMA Cat# 04599)
- **Recommended reporter for MDR1 (Pgp) Inhibitors**: Verapamil (SIGMA Cat# V4629)
- **Recommended reporter for BCRP Substrates**: Estrone-3-sulfate (E3S) (SIGMA Cat# E9145)

Sample Plate Layout

The CacoGoblet 24-well format allows evaluating the permeability of 1 compound in triplicate in the A-B/B-A directions following the recommended plate layout shown below.

	A-B			B-A		
	1	2	3	4	5	6
A	At/Met_R1	At/Met_R2	At/Met_R3	At/Met_R1	At/Met_R2	At/Met_R3
B	Dig_R1 or E3S_R1	Dig_R2 or E3S_R2	Dig_R3 or E3S_R3	Dig_R1 or E3S_R1	Dig_R2 or E3S_R2	Dig_R3 or E3S_R3
C	Comp 1_R1	Comp 1_R2	Comp 1_R3	Comp 1_R1	Comp 1_R2	Comp 1_R3
D	Comp 1/Inh_R1	Comp 1/Inh_R2	Comp 1/Inh_R3	Comp 1/Inh_R1	Comp 1/Inh_R2	Comp 1/Inh_R3

R = replicate

Figure 3. Recommended sample plate layout to investigate drug intestinal absorption and potential drug-transporter interactions At (atenolol), Met (metoprolol), Dig (digoxina), E3S (estrone-3-sulfate), Comp (compound), Inh (inhibitor).

- Initial concentration suggested for unknowns: 10 µM
- Replicates: 3
- Time points: 0 and 2 h
- Volumes: *Apical compartment*: 250 µL
  Basal compartment: 750 µL

NOTE: *The procedure should be undertaken in biosafety level II containment standards to ensure sterile conditions. Assay transport buffer solution should be pre-warmed to 37 °C to avoid temperature stress. Do not use LY and tested compounds concomitantly in the same well. LY may interfere with certain substances, resulting in false data.*

Protocol

*The **general procedure for permeability assays** in the A-B/B-A directions is described below:*

Apical-to-Basal Studies

Test compounds are applied to the apical side of the cell monolayer (upper compartment of the insert), and the **apical-to-basal (A-B)** transport through the cell barrier is evaluated by sample recovery and test compound detection in the basal (lower) compartment over a defined incubation period. A-B permeability of test compounds is determined as the coefficient of apparent permeability (P_{app}) in cm/s.

Preparation

1. **Prepare stock solutions of reference and tested compounds** in transport buffer. In case of poorly water-soluble compounds, DMSO may be used as a solvent. If so, it is recommended to keep the percentage of DMSO in the assay buffer below 1 %.
2. **Prepare working solutions of unknowns and reference compounds** in transport buffer. Substrates and inhibitors are mixed simultaneously in the working solution when both compounds are concomitantly assayed.
3. Fill a reagent reservoir with pre-warmed (37 °C) transport buffer.
4. Take the reservoir plate into the laminar flow hood.
5. **Remove** one **CacoGoblet plate from the cell incubator** and place it beside the reservoir plate. Both plates should be oriented the same way.

Washing Steps

6. Open the CacoGoblet plate and the reservoir plate, and leave the lids upwards next to the plates.
7. Carefully **use forceps to lift separately the** 24 **apical inserts** of the **CacoGoblet plate** and **transfer** them to the **reservoir plate**.
8. Using a Pasteur pipette, **aspirate the cell culture medium** from the lower compartments of the CacoGoblet plate.
9. Using a multichannel pipette, **fill**, column by column, each of the 24 wells of the **lower compartments** of the CacoGoblet plate with **750 µL** of pre-warmed (37 °C) **transport buffer**.
10. Using a multichannel pipette, **remove the cell culture medium of the apical inserts** of the CacoGoblet plate, taking care not to disrupt the monolayer.
11. Using a multichannel pipette, **fill**, column by column, each of the 24 apical inserts of the **upper compartment** of the CacoGoblet plate with **250 µL** of pre-warmed (37 °C) **transport buffer**. Always add the medium against the wall of the well, and not directly onto the cell monolayers.
12. Carefully **use forceps to return** the 24 **apical inserts** onto the wells of the basal compartment of the CacoGoblet plate (**original position**).
13. Incubate the plate for **1 minute at room temperature** inside the laminar flow hood.
14. **Repeat steps 6 to 12 twice**. The first time, incubate the plate as performed in step 13. The **second time**, **incubate** the plate for **30 minutes** in the **cell incubator** (37 °C, 5 % CO_2).

Transport Assay

15. **Take the plate** from the incubator, return it to the laminar flow hood and place it next to the reservoir plate. Both plates should be oriented in the same way.
16. Carefully **use forceps to lift separately the 24 apical inserts** of the **CacoGoblet plate** and **transfer** them to the **reservoir plate**.
17. Using a Pasteur pipette, **aspirate the transport buffer** from the **lower compartment** of the CacoGoblet plate.
18. Using a multichannel pipette, **fill**, column by column, each of the 24 wells of the lower compartments of the CacoGoblet plate with **750 µL** of pre-warmed (37 °C) **transport buffer**.

19. Using a multichannel pipette, **remove the transport buffer of the apical inserts** of the CacoGoblet plate, taking care not to disrupt the monolayer.
20. Add **275 µL of working solutions** (see sample layout in Figure 3 for details) to the 24 **apical inserts** of the CacoGoblet plate. Immediately after (0 hours), **recover 25 µL (t0)** and keep them at -20 ºC until further analysis is performed.
21. Carefully **use forceps to return the 24 apical inserts onto** the wells of the basal compartment of the CacoGoblet plate (**original position**) and **leave** the plate in the **cell incubator** (37 °C, 5 % CO2) for **2 hours**. Shorter or longer periods of incubation may be required for very high or low permeability compounds.

Sample Collection

22. **Take the plate** from the incubator, return it to the laminar flow hood and place it next to the reservoir plate. **Transfer with forceps** the **apical inserts** onto the **reservoir plate**.
23. Recover **25 µL** from the **apical inserts (t2h apical)** and the **lower compartments (t2h basal),** and keep them at -20 °C until further analysis is performed.
24. **Analyze all samples** using mass spectrometry according to your analytical procedures for tested and reference compounds.
25. **Calculate the compound apparent permeability (P_{app})** coefficient as indicated in the following section ("Evaluation of Compound Permeability").

Basal-to-Apical Studies

Test compounds are applied to the basal side of the cell monolayer (lower compartment of the insert), and the **basal-to-apical (B-A)** transport through the cell barrier is evaluated by sample recovery and test compound detection in the apical (upper) compartment over a defined incubation period. B-A permeability of test compounds is determined as the coefficient of apparent permeability (P_{app}) in cm/s.

All **steps are identical** to those described for the apical-to-basal studies, **except for the volumes added in steps 18 and 20**:

Step 18. Using a multichannel pipette, fill, column by column, each of the 24 wells of the **upper compartment** of the CacoGoblet plate with **250 µL** of pre-warmed (37 °C) **transport buffer**.

Step 20. Add **775 µL of working solutions** (see sample layout in Figure 3 for details) to the **basal compartments** of the CacoGoblet plate. Immediately after (0 hours), **recover 25 µL (t0)** and keep them at -20 °C until further analysis is performed.

NOTE: *At the end of the transport assay (A-B/B-A directions) perform the post-assay quality control as indicated in the section "Quality control of the barrier system".*

Evaluation of Compound Permeability

Apparent Permeability Coefficient (P_{app})

The transport efficiency of test substances and reference compounds is evaluated in each sample through P_{app} calculation, which is defined as follows:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

where P_{app} represents the coefficient of apparent permeability (in cm/s), which corresponds to the proportion of test compound that crosses the barrier at each time point (dQ/dt in nmol/s), divided by the product of the crossed area (A in cm^2) by the initial concentration of test compound (C_0 in nmol/ml) applied to the apical (A-B) or basal (B-A) compartments.

Considerations for calculations:

- When plotting Q versus time, consider the amount of material lost in previous stages
- A single time point can only be used in the linear range. Otherwise, the P_{app} value will be an underestimation of the real value. Sampling compounds with unknown behavior at a single time point is not recommended.

Efflux Ratio (ER)

The efflux ratio is a general measure of the involvement of active processes in compound permeability. This value results from dividing the compound P_{app} in the B-A direction by the P_{app} in the A-B direction.

$$ER = \frac{P_{app\ B\text{-}A}}{P_{app\ A\text{-}B}}$$

Mass Balance

Material balance calculation for each compound is determined as follows:

$$\text{Mass Balance (\%)} = \left[\frac{((Cap@time(i) \times Vap) + (Cbs@time(i) \times Vbs))}{Cinit@time0 \times V} \right] \times 100$$

Where

- Cap@time(i) and Cbs@time(i) correspond to test compound concentrations in pmols/mL at time (i) in the apical and basal compartments, respectively.
- Vap and Vbs are apical and basal volumes in mL.
- Cinit@time0 is the initial concentration of the test compound in pmols/mL at t0.
- V is the volume of either the apical compartment (A-B direction) or the basal compartment (B-A direction).

Data for Reference Compounds

Normal values and ranges for reference substances (according to MEDTECH BARCELONA's internal data) are detailed below:

- TEER values > 70 Ω x cm^2 and LY paracellular flux values ≤ 1.4 % are strong indicators of cell barrier integrity.
- Substrates of membrane protein transporters must have an efflux ratio greater than 2.
- In the presence of transporter inhibitors, the substrate's efflux ratio must decrease significantly (> 50 %).
- Material balance (mass balance) range must be between 80 % - 120 %.

CacoGoblet 24						
Substrate	**Inhibitors**	**Permeability range**	**Concentration (µM)**	**Papp (x 10^{-6} cm/s)**		**ER**
				A-B	**B-A**	
Atenolol	---	Low	10	4.5 ± 0.8	---	---
Metoprolol	---	High	10	22.51 ± 0.44	10.13 ± 1.27	0.45
Digoxin	---	Pgp substrate	10	0.45 ± 0.20	2.78 ± 0.54	6.19
Digoxin	Verapamil		10	0.95 ± 0.27	1.99 ± 0.78	2.09
Estrone-3-sulfate	---	BCRP substrate	10	0.95 ± 0.02	5.59 ± 0.53	5.86

Table 2. Apparent permeability (Papp) coefficient values and efflux ratio of reference compounds across CacoGoblet.

References

[1] Food and Drug Administration (FDA) (2020). *In Vitro Drug Interaction Studies — Cytochrome P450 Enzyme- and Transporter-Mediated Drug Interactions Guidance for Industry.* U.S. Department of Health and Human Services, Center for Drug Evaluation and Research (CDER). https://www.fda.gov/regulatory-information/search-fda-guidance-documents/vitro-drug-interaction-studies-cytochrome-p450-enzyme-and-transporter-mediated-drug-interactions