

96-Well PreadyPort^{WT} User's Manual

Limited Single-use License

This is a binding legal agreement (this "License") between you (the "Customer" or "You"), and MEDTECH BARCELONA regarding the enclosed "PreadyPort™ WT" System (this "Product"). Carefully read this License before opening the sealed package. BY OPENING THE SEALED PACKAGE, YOU AGREE TO BE BOUND BY THE TERMS OF THIS LICENSE. If You do not agree to the terms of this License, and You have not opened the sealed package, promptly notify MEDTECH BARCELONA in writing and return the unopened Product and all accompanying items (including all written material and containers) to MEDTECH BARCELONA within five (5) business days.

This License sets forth the Customer's rights to use this Product. The meaning of "Product" hereunder shall include any parts thereof and any documentation made available for use with this Product. Upon receipt of the applicable fee, MEDTECH BARCELONA grants to the Customer the personal, non-exclusive, and non-transferable right to use this Product worldwide. The Customer is solely responsible for the use of this Product by any person.

The Customer acknowledges and agrees that the manufacture, use, sale, or import of this Product may be subject to one or more issued or pending patent applications and the corresponding foreign equivalents owned or controlled by MEDTECH BARCELONA. The Customer acknowledges and agrees that the documentation and components included in the Product are the proprietary and copyrighted material of MEDTECH BARCELONA. The purchase and use of this Product shall be governed under MEDTECH BARCELONA's standard Terms and Conditions, and any other written agreement between the parties, and such terms and agreements are incorporated herein by reference. Your purchase of this Product, subject to this License, conveys to the Customer the non-transferable right to use the purchased amount of the Product and components of the Product in this shipment in research conducted by the Customer; such right shall expire upon completion of the experiments stemming from this shipment.

The Customer shall not detach living cells from the Product and shall not amplify genetic material (DNA, RNA) for purposes other than quantitative or qualitative analysis of proteins or nucleotide sequences.

The Customer shall not sell or otherwise transfer (a) this Product, (b) any components of this Product, or (c) any materials made using this Product or its components. Also, the Customer shall not use this Product or its components for Commercial Purposes other than those defined herein, and especially will not be able to: (1) make use of this Product in manufacturing; (2) make use of this Product for therapeutic, diagnostic or prophylactic purposes; or (3) resale this Product, whether or not such Product is resold for research purposes. For clarification, the Customer may use and transfer information or materials made through the use of this Product to support the Customer's research in any manner the Customer deems necessary or appropriate, and the Customer may provide services to third parties.

If you have any questions or need information on obtaining a license to use this Product or any of its components for purposes beyond the scope of this License, please contact MEDTECH BARCELONA at:

reagents@medtechbcn.com

Table of contents

Product Description	3
Intended Use	3
Principle	3
Timeline for Delivery and Experimental Procedures	4
Equipment (not included)	5
Consumables	5
Solutions (may be included)	5
Handling	6
Replacement of Shipping Medium	6
Quality Control of the Barrier System	7
Pre-assay Quality control – TEER Measurement	7
Post-assay Quality Control – Lucifer Yellow (LY) Paracellular Permeability Assay	8
General Protocol for Transport Assays	9
General Considerations	9
Recommended Reference Compounds	9
Sample Plate Layout	9
Protocol	10
Evaluation of Compound Permeability	12
Apparent Permeability Coefficient (P_{app})	12
Efflux Ratio (ER)	12
Mass Balance	12
Data for Reference Compounds	13
References	13

Product Description

PreadyPort WT is an *in vitro* cell-based model built on differentiated Madin-Darby Canine Kidney II (MDCKII) cells forming a cell monolayer. These cells are transfected with an empty expression vector which contains an antibiotic resistance gene. MDCKII cells are seeded in 96 Transwell® inserts with semiporous (1 µm) polystyrene (PET) membrane (CORNING Cat#3392) that separates an apical and a basal compartment.

PreadyPort is delivered in a 96-well 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.

NOTE: *Other MDCKII cells overexpressing other transporters may be included, if required.*

Intended Use

This product is mainly indicated for assessing:

- drug permeability by passive diffusion through a physiologically relevant barrier
- as controls of drug uptake in MDCKII cells that overexpress the MDR1 (PreadyPort MDR1) and BCRP transporters (PreadyPort BCRP plates)

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

Principle

MDCKII cells are considered to be useful *in vitro* tools for evaluating drug uptake efficiency through the intestinal barrier.

In the experimental setup, these cells will be differentiated on Transwell® inserts to form a cell monolayer that prevents media from wicking between the insert (apical compartment) and the plate well (basal compartment).

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 measurement of passive diffusion and unlikely 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 4: Pre-shipping Quality Control (TEER and Lucifer Yellow)
- Days 4-5: Package Dispatch (depending on destination)
- Days 6-7: Package Delivery
- Day 8: Replacement of Shipping Medium (liquefaction)
- Day 11: Quality Control Experiments and Medium Replacement
- Days 12-15: Assay Performance

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:

reagents@medtechbcn.com

The recommended timing overview for transport experiments is Day 12 (Tuesday) (see Figure 1 for details).

PreadyPort	Monday	Tuesday	Wednesday	Thursday	Friday
Week 0		12:00 p.m. (CET) last ordering day	Pre-Production		Start of Production Day 1
Week 1	Shipment Day 4	Reception of Plates			Liquefaction Day 8
Week 2	Medium Change Day 11	Day 12	Perform Assay		Day 15

Figure 1. Timeline of manufacturing and operation for PreadyPort in 96-well format.

According to the day you choose to conduct the experiments, TEER measurement and medium replacement must be carried out as follows:

DAY OF EXPERIMENT	TEER MEASUREMENT	MEDIUM REPLACEMENT
Tuesday (Day 12)	Monday (Day 11)	Monday (Day 11)
Wednesday (Day 13)	Monday (Day 11) Wednesday (Day 13)	Monday (Day 11)
Thursday (Day 14)	Monday (Day 11) Wednesday (Day 13)	Monday (Day 11) Wednesday (Day 13)
Friday (15)	Monday (Day 11) Wednesday (Day 13) Friday (15)	Monday (Day 11) Wednesday (Day 13)

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 11) 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 (see Table 1 for details).*

Equipment (not included)

- Cell culture laminar flow hood
- CO2 incubator
- Water bath
- Multichannel pipettes
- Automatic multichannel micropipette (recommended)
- Aspiration system
- **96-well format vacuum manifold (Drummond Cat# 3-000-093 recommended)**
- Trans-Epithelial Electrical Resistance (TEER) meter (WPI EVOM series)
- **96-well electrode (WPI STX100C96 recommended)**
- Fluorometer (Fluoroskan Ascent CF)
- Quantitative analytics equipment

Consumables

- Reservoir plate (*provided by MedTech Barcelona*)
- 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*)

Solutions (may be included)

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

- **MDCKII Cell Culture Medium:** Dulbecco's Modified Eagle's Medium (GIBCO Cat# 22320022) 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 BCRP Substrates:** Prazosin (SIGMA Cat# P7791)

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 at a dark location at room temperature until Day 8 (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 8** (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 PreadyPort 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) MDCKII cell culture medium.
5. Open the PreadyPort plate and the reservoir plate, and leave their lids upwards, next to the plates.
6. Carefully lift the 96-integrated apical compartments of the PreadyPort plates and transfer them onto the reservoir plate.
7. Remove all liquefied shipping medium from the basal compartments of the PreadyPort plate via aspiration with the 96-well manifold.
8. Using a multichannel pipette, dispense **250 µL** of MDCKII cell culture medium from the sterile reservoir, and fill, the **basal compartments** of the PreadyPort plate, column by column.
9. Using the aspiration manifold connected to a vacuum pump (adjust aspiration flux to medium-low), aspirate the liquefied shipping medium from the apical integrated inserts of the PreadyPort plate, taking care not to disrupt the monolayer. Make sure the shipping medium has been removed from all wells. Approximately 10 µL of medium will be left in each well.
10. Using a multichannel pipette, dispense **75 µL** of MDCKII cell culture medium from the sterile reservoir, and fill, the **apical compartments** of the PreadyPort plate, column by column. Always add the medium against the wall of the well, and not directly onto the cell monolayers.
11. Carefully return the apical inserts onto the basal compartment of the PreadyPort 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 MDCKII cell culture medium, plates should be placed inside the incubator until next Monday (Day 11). **Replacement with a new fresh medium** will be carried out once (Day 11) or twice (Days 11 and 13) 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 provided by MedTech Barcelona, 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 carefully read 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, medium replacement, or sodium butyrate induction.

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 MDCKII cell culture medium, **pre-warmed at room temperature**.
3. While the electrode is equilibrating, remove the PreadyPort 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.

NOTE: *It is highly recommended to use the WPI STX 100C96 electrode to prevent cell damage.
Watch out to set the electrode in the right position.*

4. Place the electrode onto the well and 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 and Falcon plates)	0.14 cm^2
TEER value	> 75 $\Omega \times \text{cm}^2$

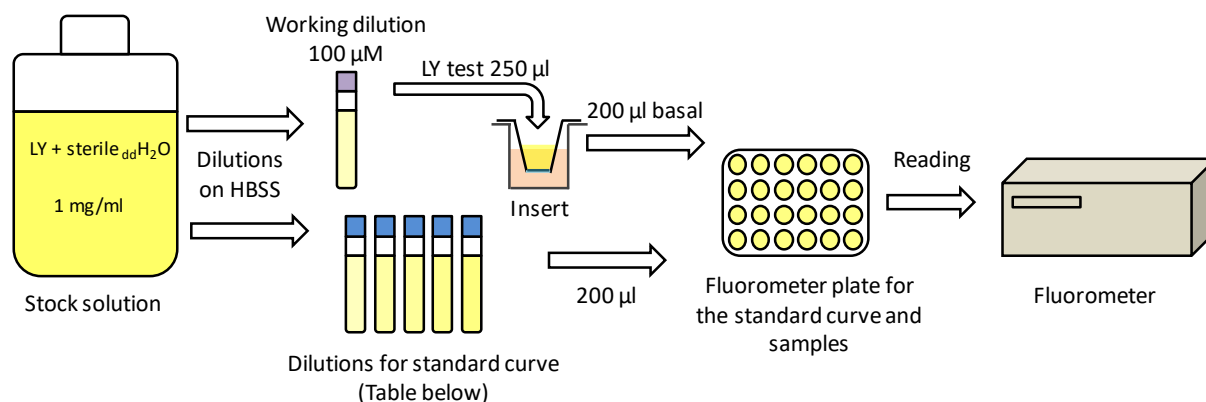
Post-assay Quality Control – Lucifer Yellow (LY) Paracellular Permeability Assay

Prepare a **1 mg/mL (2.187 mM) LY solution** in sterile ddH₂O. 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).
4. Remove the transport assay buffer from the apical and basal compartments following the same procedure.
5. Add **75 µL of 100 µM LY** working dilution into the **apical compartment**.
6. Add **250 µL of transport assay buffer** to the **basal compartment**.
7. **Incubate** the PreadyPort plate, protected from light, in the cell incubator (at 37 °C and 5 % CO₂) 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	≤ 2 %
LY apparent permeability (Papp)	≤ 3 x 10 ⁻⁶ cm/s

General Protocol for Transport Assays

General Considerations

PreadyPort WT is designed for conducting permeability *in vitro* assays of established and investigational compounds in order to predict drug permeation across a pharmacological barrier.

Cells may be used as controls of drug uptake in MDCKII cells that overexpress the MDR1(PreadyPort MDR1) and BCRP transporters (PreadyPort BCRP plates).

Recommended Reference Compounds

The compounds listed below (also referenced in the "Solutions" section) are recommended for the assay as reference substrates.

- 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 BCRP Substrates: Prazosin (SIGMA Cat# P7791)

Sample Plate Layout

The PreadyPort 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			A-B			B-A		
	1	2	3	4	5	6	7	8	9	10	11	12
A	Ref Subst 1_R1	Ref Subst 1_R2	Ref Subst 1_R3	Ref Subst 1_R1	Ref Subst 1_R2	Ref Subst 1_R3	Comp 5_R1	Comp 5_R2	Comp 5_R3	Comp 5_R1	Comp 5_R2	Comp 5_R3
B	Ref Subst 2_R1	Ref Subst 2_R2	Ref Subst 2_R3	Ref Subst 2_R1	Ref Subst 2_R2	Ref Subst 2_R3	Comp 6_R1	Comp 6_R2	Comp 6_R3	Comp 6_R1	Comp 6_R2	Comp 6_R3
C	Ref Subst 3_R1	Ref Subst 3_R2	Ref Subst 3_R3	Ref Subst 3_R1	Ref Subst 3_R2	Ref Subst 3_R3	Comp 7_R1	Comp 7_R2	Comp 7_R3	Comp 7_R1	Comp 7_R2	Comp 7_R3
D	Ref Subst 4_R1	Ref Subst 4_R2	Ref Subst 4_R3	Ref Subst 4_R1	Ref Subst 4_R2	Ref Subst 4_R3	Comp 8_R1	Comp 8_R2	Comp 8_R3	Comp 8_R1	Comp 8_R2	Comp 8_R3
E	Comp 1_R1	Comp 1_R2	Comp 1_R3	Comp 1_R1	Comp 1_R2	Comp 1_R3	Comp 9_R1	Comp 9_R2	Comp 9_R3	Comp 9_R1	Comp 9_R2	Comp 9_R3
F	Comp 2_R1	Comp 2_R2	Comp 2_R3	Comp 2_R1	Comp 2_R2	Comp 2_R3	Comp 10_R1	Comp 10_R2	Comp 10_R3	Comp 10_R1	Comp 10_R2	Comp 10_R3
G	Comp 3_R1	Comp 3_R2	Comp 3_R3	Comp 3_R1	Comp 3_R2	Comp 3_R3	Comp 11_R1	Comp 11_R2	Comp 11_R3	Comp 11_R1	Comp 11_R2	Comp 11_R3
H	Comp 4_R1	Comp 4_R2	Comp 4_R3	Comp 4_R1	Comp 4_R2	Comp 4_R3	Comp 12_R1	Comp 12_R2	Comp 12_R3	Comp 12_R1	Comp 12_R2	Comp 12_R3

R = replicate

Ref Subst = reference substrate

Figure 3. Recommended sample plate layout to evaluate drug permeability.

- Initial concentration suggested for unknowns: 10 μ M
- Replicates: 3
- Time points: 0 and 2 h
- Volumes: *Apical compartment*: 75 μ L
Basal compartment: 250 μ L

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

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 PreadyPort plate from the cell incubator** and place it beside the reservoir plate. Both plates should be oriented the same way.

Washing Steps

1. Open the PreadyPort plate and the reservoir plate, and leave the lids upwards next to the plates.
2. Carefully lift the 96 **apical inserts** of the **PreadyPort plate** and **transfer** them to the **reservoir plate**.
3. Using the 96-well manifold, **aspirate the cell culture medium** from the lower compartments of the PreadyPort plate.
4. Using a multichannel pipette, **fill**, column by column, each of the 96 wells of the **lower compartments** of the PreadyPort plate with **250 µL** of pre-warmed (37 °C) **transport buffer**.
5. Using the 96-well manifold, aspirate the cell culture medium of the apical inserts of the PreadyPort plate. Place the manifold perpendicular to the cell monolayer and close to the insert wall to avoid disturbing the cell monolayer.
6. Using a multichannel pipette, **fill**, column by column, each of the 96 apical inserts of the **upper compartment** of the PreadyPort plate with **75 µL** of pre-warmed (37 °C) **transport buffer**.
7. Carefully **return** the 96 **apical inserts** onto the wells of the basal compartment of the PreadyPort plate (**original position**).
8. Incubate the plate for **1 minute at room temperature** inside the laminar flow hood.
9. **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 % CO₂).

NOTE: Use low-medium suction power to avoid disrupting the cell monolayer.

Transport Assay

10. **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.
11. Carefully lift the 96 **apical inserts** of the **PreadyPort plate** and **transfer** them to the **reservoir plate**.
12. Using the 96-well manifold, **aspirate the transport buffer** from the **lower compartment** of the PreadyPort plate.

13. Using a multichannel pipette, **fill**, column by column, each of the 96 wells of the lower compartments of the PreadyPort plate with **250 µL** of pre-warmed (37 °C) **transport buffer**.
14. Using the 96-well manifold, **aspirate** the **transport buffer** of the **apical inserts** of the PreadyPort plate. Place the manifold perpendicular to the cell monolayer and close to the insert wall to avoid disturbing the cell monolayer.
15. Add **100 µL of working solutions** (see sample layout in Figure 3 for details) to the 96 **apical inserts** of the PreadyPort plate. Immediately after (0 hours), **recover 25 µL (t0)** and keep them at -20 °C until further analysis is performed.
16. Carefully **return** the 96 **apical inserts** onto the wells of the basal compartment of the PreadyPort 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.

NOTE: Use low-medium suction power to avoid disrupting the cell monolayer.

Sample Collection

17. **Take the plate** from the incubator, return it to the laminar flow hood and place it next to the reservoir plate. **Transfer the apical inserts** onto the **reservoir plate**.
18. 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.
19. **Analyze all samples** using mass spectrometry according to your analytical procedures for tested and reference compounds.
20. **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 96 wells of the **upper compartment** of the PreadyPort plate with **75 µL** of pre-warmed (37 °C) **transport buffer**.

Step 20. Add **275 µL of working solutions** (see sample layout in Figure 3 for details) to the **basal compartments** of the PreadyPort 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.

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

Mass Balance

Material balance calculation for each compound is determined as follows:

$$\text{Mass Balance (\%)} = \left[\frac{((Cap@time(i) \times V_{ap}) + (Cbs@time(i) \times V_{bs}))}{C_{init@time0} \times V} \right] \times 100$$

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.
- V_{ap} and V_{bs} are apical and basal volumes in mL.
- $C_{init@time0}$ is the initial concentration of the test compound in pmols/mL at t_0 .
- 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 FDA guidelines¹ and MEDTECH BARCELONA's internal data) are detailed below:

- TEER values $> 75 \Omega \times \text{cm}^2$ and LY paracellular flux values $\leq 2 \%$ are strong indicators of cell barrier integrity.
- Efflux ratios must be < 2 for passive diffusion and greater than 2 for transporter-mediated uptake.
- Material balance (mass balance) range must be between 80 % - 120 %.

PreadyPort WT				
Substrate	Concentration (μM)	Papp ($\times 10^{-6} \text{ cm/s}$)		ER
		A-B	B-A	
Atenolol	10	0.23 ± 0.55	0.11 ± 0.10	0.48
Metoprolol	10	27.75 ± 0.33	25.41 ± 7.42	0.92
Digoxin	10	2.23 ± 0.70	1.57 ± 0.24	0.73 ± 0.14
Quinidine	1	10.87 ± 0.22	11.37 ± 0.73	1.05 ± 0.06
Prazosin	1	17.37 ± 9.56	9.89 ± 3.03	0.67 ± 0.29
Dantrolene	1	15.15 ± 2.43	6.50 ± 1.17	0.43 ± 0.08

Table 2. Reference values for compounds either absorbed by passive diffusion (atenolol and metoprolol) or by active transport (digoxin, quinidine, prazosin and dantrolene) when incubated in MDCKII cells (PreadyPort WT). The data for atenolol and metoprolol are the result of a single experiment. Results for digoxin, quinidine, prazosin and dantrolene are the mean of 3 independent experiments.

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>