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MedTech BARCELONA by Leitat

96-Well CacoReady User's Manual

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Product Description

CacoReady is an *in vitro* cell-based model built on polarized human colorectal adenocarcinoma-derived cells (Caco-2) which resemble the intestinal epithelial barrier. Cells differentiate for 21-days in 96 Transwell® inserts with semi-porous (1 µm) polystyrene (PET) membrane (CORNING Cat#3392) resulting in an apical compartment and a basal compartment that mimic the intestinal lumen and the blood circulation, respectively.

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

CacoReady is delivered in a 96-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

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.
In the experimental setup, these cells will be differentiated on 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).

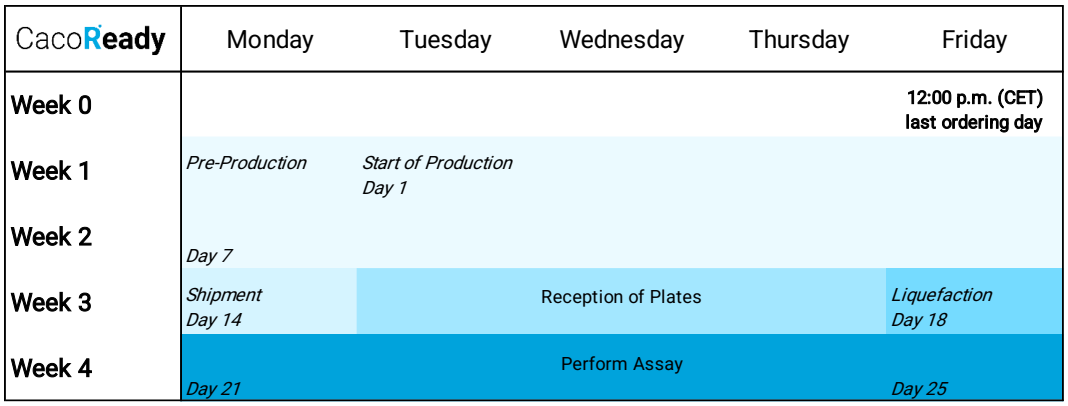


Figure 1. Timeline of manufacturing and operation for CacoReady in 96-well format.

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
- 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.*

- **Caco-2 Cell Culture Medium**: Dulbecco's Modified Eagle's Medium - low glucose (1 g/L) (SIGMA Cat# D5546) supplemented with (final concentrations):
  - 10 % V/V Fetal Bovine Serum (BIOWEST Cat# DE14-801F)
  - 2 mM L-glutamine (LONZA Cat# BE17-605F)
  - 100 U/mL; 0.1 mg/mL Penicillin-Streptomycin (LONZA Cat# DE17-602F)
- **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**: Prazosin (SIGMA Cat# P7791)
- **Recommended reporter for BCRP Inhibitors**: Ko143 (SIGMA Cat# K2144)

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 CacoReady 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 cell culture medium.
5. Open the CacoReady plate and the reservoir plate, and leave their lids upwards, next to the plates.
6. Carefully lift the 96-integrated apical compartments of the CacoReady plates and transfer them onto the reservoir plate.
7. Remove all liquefied shipping medium from the basal compartments of the CacoReady plate via aspiration with the 96-well manifold.
8. Using a multichannel pipette, dispense **250 µL** of Caco2- cell culture medium from the sterile reservoir, and fill, the **basal compartments** of the CacoReady 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 CacoReady 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 Caco-2 cell culture medium from the sterile reservoir, and fill, the **apical compartments** of the CacoReady 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 CacoReady 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 cell culture medium, 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 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 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 cell culture medium, **pre-warmed at room temperature**.
3. While the electrode is equilibrating, remove the CacoReady 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 plates)	**0.14 cm2**
TEER value	**> 500 Ω x cm2**

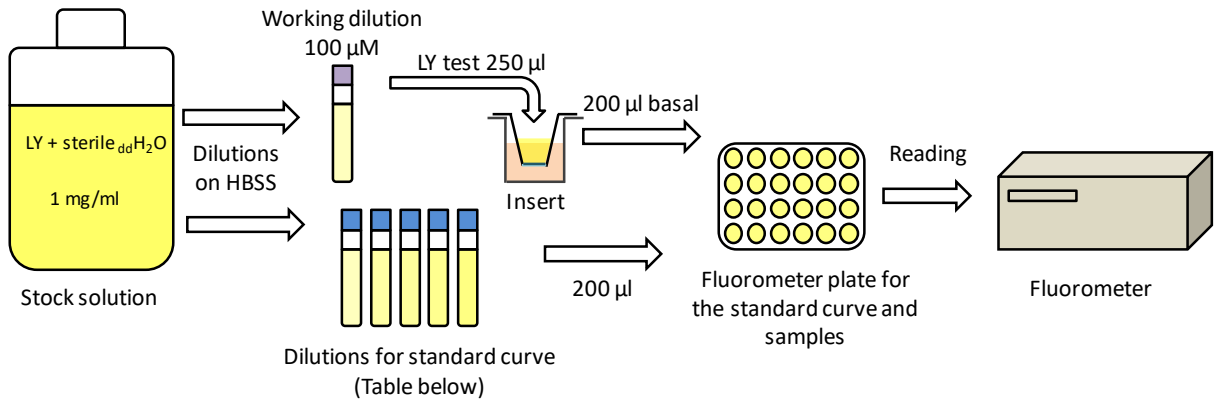
Post-assay Quality Control – Lucifer Yellow (LY) Paracellular Permeability Assay

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).
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 CacoReady 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	**≤ 0.7 %**
LY apparent permeability (Papp)	**≤ 1 x 10-6 cm/s**

General Protocol for Transport Assays

General Considerations

CacoReady 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**: Prazosin (SIGMA Cat# P7791)
- **Recommended reporter for BCRP Inhibitors**: Ko143 (SIGMA Cat# K2144)

Sample Plate Layout

The CacoReady 96-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	Atenolol_R1	Atenolol_R2	Atenolol_R3	Atenolol_R1	Atenolol_R2	Atenolol_R3	Comp 2_R1	Comp 2_R2	Comp 2_R3	Comp 2_R1	Comp 2_R2	Comp 2_R3
B	Metoprolol_R1	Metoprolol_R2	Metoprolol_R3	Metoprolol_R1	Metoprolol_R2	Metoprolol_R3	Comp 2/Inh_R1	Comp 2/Inh_R2	Comp 2/Inh_R3	Comp 2/Inh_R1	Comp 2/Inh_R2	Comp 2/Inh_R3
C	Digoxin_R1	Digoxin_R2	Digoxin_R3	Digoxin_R1	Digoxin_R2	Digoxin_R3	Comp 3_R1	Comp 3_R2	Comp 3_R3	Comp 3_R1	Comp 3_R2	Comp 3_R3
D	Dig/Verap_R1	Dig/Verap_R2	Dig/Verap_R3	Dig/Verap_R1	Dig/Verap_R2	Dig/Verap_R3	Comp 3/Inh_R1	Comp 3/Inh_R2	Comp 3/Inh_R3	Comp 3/Inh_R1	Comp 3/Inh_R2	Comp 3/Inh_R3
E	Prazosin_R1	Prazosin_R2	Prazosin_R3	Prazosin_R1	Prazosin_R2	Prazosin_R3	Comp 4_R1	Comp 4_R2	Comp 4_R3	Comp 4_R1	Comp 4_R2	Comp 4_R3
F	Praz/Ko143_R1	Praz/Ko143_R2	Praz/Ko143_R3	Praz/Ko143_R1	Praz/Ko143_R2	Praz/Ko143_R3	Comp 4/Inh_R1	Comp 4/Inh_R2	Comp 4/Inh_R3	Comp 4/Inh_R1	Comp 4/Inh_R2	Comp 4/Inh_R3
G	Comp 1_R1	Comp 1_R2	Comp 1_R3	Comp 1_R1	Comp 1_R2	Comp 1_R3	Comp 5_R1	Comp 5_R2	Comp 5_R3	Comp 5_R1	Comp 5_R2	Comp 5_R3
H	Comp 1/Inh_R1	Comp 1/Inh_R2	Comp 1/Inh_R3	Comp 1/Inh_R1	Comp 1/Inh_R2	Comp 1/Inh_R3	Comp 5/Inh_R1	Comp 5/Inh_R2	Comp 5/Inh_R3	Comp 5/Inh_R1	Comp 5/Inh_R2	Comp 5/Inh_R3

R = replicate

Figure 3. Recommended sample plate layout to investigate drug intestinal absorption and potential drug-transporter interactions. Dig (digoxina), Verap (verapamil), Praz (prazosin), Comp (compound), Inh (inhibitor).

- Initial concentration suggested for unknowns: 10 µM
- Replicates: 3
- Time points: 0 and 2 h
- Volumes: *Apical compartment*: 75 µL
  Basal compartment: 250 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 represented in **Annex I**.*

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. Unwrap the receiver plate in the laminar flow hood.
5. **Remove** one **CacoReady 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 CacoReady plate and the reservoir plate, and leave the lids upwards next to the plates.
7. Carefully lift the 96 **apical inserts** of the **CacoReady plate** and **transfer** them to the **reservoir plate**.
8. Using the 96-well manifold, **aspirate the cell culture medium** from the lower compartments of the CacoReady plate.
9. Using a multichannel pipette, **fill**, column by column, each of the 96 wells of the **lower compartments** of the CacoReady plate with **250 µL** of pre-warmed (37 °C) **transport buffer**.
10. Using the 96-well manifold, aspirate the cell culture medium of the apical inserts of the CacoReady plate. Place the manifold perpendicular to the cell monolayer and close to the insert wall to avoid disturbing the cell monolayer.
11. Using a multichannel pipette, **fill**, column by column, each of the 96 apical inserts of the **upper compartment** of the CacoReady plate with **75 µL** of pre-warmed (37 °C) **transport buffer**.
12. Carefully **return** the 96 **apical inserts** onto the wells of the basal compartment of the CacoReady 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).

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

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 lift the 96 **apical inserts** of the **CacoReady plate** and **transfer** them to the **reservoir plate**.

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

22. **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**.
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 96 wells of the **upper compartment** of the CacoReady 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 CacoReady 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 FDA guidelines[1] and MEDTECH BARCELONA's internal data) are detailed below:

- TEER values ≥ 500 Ω x cm^2 and LY paracellular flux values < 0.7 % 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 %.

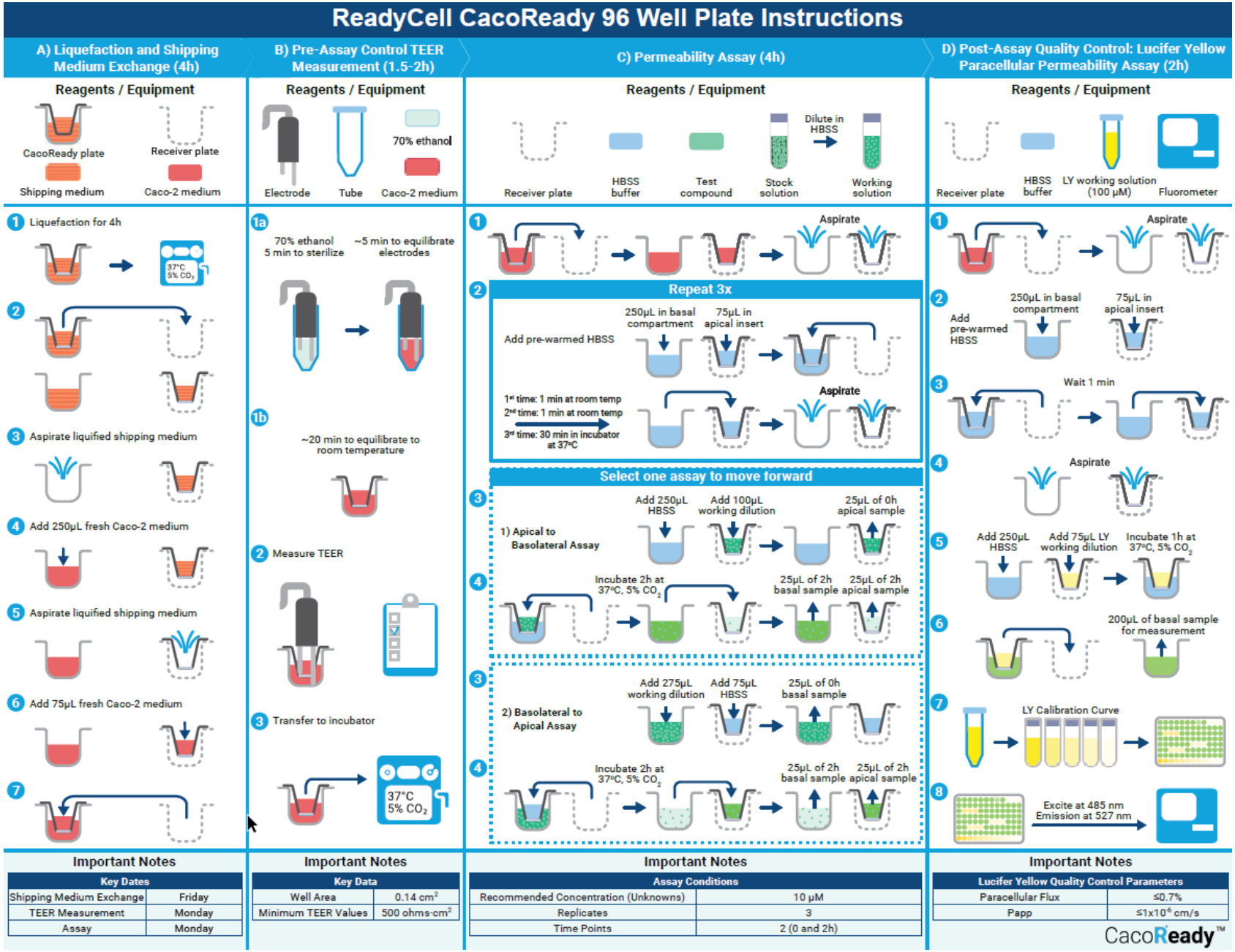
CacoReady 96						
Substrate	Inhibitor	Permeability range	Concentration (µM)	Papp (x 10^{-6} cm/s)		ER
				A-B	B-A	
Atenolol	---	Low	10	0.29 ± 0.03	0.10 ± 0.01	0.33
Nadolol	---		10	0.34 ± 0.02	0.11 ± 0.01	0.33
Metformin	---		10	0.91 ± 0.65	0.07 ± 0.04	0.08
Pindolol	---	Medium	10	5.93 ± 0.92	9.59 ± 0.79	1.62
Metoprolol	---	High	10	30.07 ± 4.58	13.81 ± 2.14	0.46
Caffeine	---		10	27.46 ± 2.20	14.90 ± 1.33	0,54
Digoxin	---	Pgp substrate	10	0.77 ± 0.04	9.12± 1.26	11.88
Digoxin	Cyclosporin A		10:10	0.76 ± 0.05	3.92 ± 0.76	5.16
Talinolol	---		10	0.14 ± 0.09	2.26 ± 0.51	15,82
Talinolol	Verapamil		10:50	6.23 ± 2.54	0.91 ± 0.68	0.15
Estrone-3-sulfate	---	BCRP substrate	10	0.51 ± 0.19	9.70 ± 1.71	19.17
Prazosin	---		10	2.14 ± 0.16	17.18 ± 1.18	8.01

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

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

Annex I


ReadyCell CacoReady 96 Well Plate Instructions
A) Liquefaction and Shipping Medium Exchange (4h)
Reagents / Equipment
CacoReady plate
Receiver plate
Shipping medium
Caco-2 medium
1 Liquefaction for 4h
37°C 5% CO2
2
3 Aspirate liquified shipping medium
4 Add 250µL fresh Caco-2 medium
5 Aspirate liquified shipping medium
6 Add 75µL fresh Caco-2 medium
7
B) Pre-Assay Control TEER Measurement (1.5-2h)
Reagents / Equipment
Electrode
Tube
70% ethanol
Caco-2 medium
1a
70% ethanol 5 min to sterilize
~5 min to equilibrate electrodes
1b
~20 min to equilibrate to room temperature
2 Measure TEER
3 Transfer to incubator
37°C 5% CO2
C) Permeability Assay (4h)
Reagents / Equipment
Receiver plate
HBSS buffer
Test compound
Stock solution
Dilute in HBSS
Working solution
1
Aspirate
2
Repeat 3x
Add pre-warmed HBSS
250µL in basal compartment
75µL in apical insert
Aspirate
1st time: 1 min at room temp
2nd time: 1 min at room temp
3rd time: 30 min in incubator at 37°C
Select one assay to move forward
3
1) Apical to Basolateral Assay
Add 250µL HBSS
Add 100µL working dilution
25µL of 0h apical sample
4
Incubate 2h at 37°C, 5% CO2
25µL of 2h basal sample
25µL of 2h apical sample
3
2) Basolateral to Apical Assay
Add 275µL working dilution
Add 75µL HBSS
25µL of 0h basal sample
4
Incubate 2h at 37°C, 5% CO2
25µL of 2h basal sample
25µL of 2h apical sample
D) Post-Assay Quality Control: Lucifer Yellow Paracellular Permeability Assay (2h)
Reagents / Equipment
Receiver plate
HBSS buffer
LY working solution (100 µM)
Fluorometer
1
Aspirate
2
Add pre-warmed HBSS
250µL in basal compartment
75µL in apical insert
3
Wait 1 min
4
Aspirate
5
Add 250µL HBSS
Add 75µL LY working dilution
Incubate 1h at 37°C, 5% CO2
6
200µL of basal sample for measurement
7
LY Calibration Curve
8
Excite at 485 nm
Emission at 527 nm
Important Notes
Key Dates
Shipping Medium Exchange | Friday
TEER Measurement | Monday
Assay | Monday
Important Notes
Key Data
Well Area | 0.14 cm2
Minimum TEER Values | 500 ohms·cm2
Important Notes
Assay Conditions
Recommended Concentration (Unknowns) | 10 µM
Replicates | 3
Time Points | 2 (0 and 2h)
Important Notes
Lucifer Yellow Quality Control Parameters
Paracellular Flux | ≤0.7%
Papp | ≤1x10-6 cm/s
CacoReady™