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CacoReady™ 24

24-Well Plates (Inserts) with Seeded and Differentiated (21-days) Caco-2 Cells

USER'S MANUAL *(Corning Plate)*

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I. CacoReady™ USE-ASSAY FLOW CHART

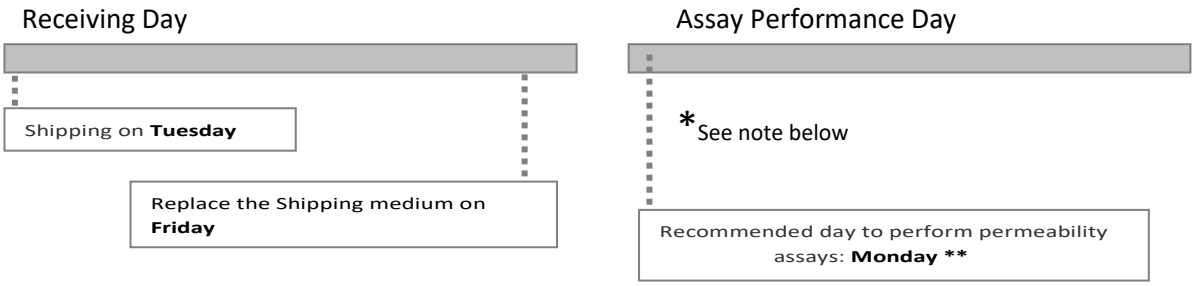

Figure 1. Assay Flow Chart

***Note:** Assays can be performed from Monday to Friday of the week following receipt of the plates. Monday is the recommended day (equivalent to 21 days of barrier differentiation) for measuring TEER values and starting experiments. If this schedule is followed, no extra medium exchanges are required. Medium exchanges are required if the permeability experiments are performed on Tuesday through Thursday. Please consult Table 1 below, for guidelines on exchanging the medium.

DAY OF EXPERIMENT	MEDIUM EXCHANGE
Monday	-
Tuesday	Monday
Wednesday	Monday
Thursday	Monday and Wednesday
Friday	Monday and Wednesday

Table 1. Assay and Medium Exchange Guidelines.

** **IMPORTANT NOTE:** **Due to our previous experience with long-distance shipments and/or extreme temperatures at a destination, we strongly recommend to measure the TEER on Monday and in case TEER values are low, perform a medium change and wait until Wednesday to let the cells recover. On Wednesday, read the TEER again and perform the assay accordingly.**

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II. SOLUTIONS & EQUIPMENT

***Note**: The reagents listed below are not provided with the kit and must be made and available for use prior to delivery of CacoReady™ plates.*

1. Solutions

i. Standard Caco-2 Cell Culture Medium

- DMEM 1g/L glucose (REF. BE12-707F – LONZA)
- 10% FBS (REF. S181B-500 – BIOWEST)
- 1% Glutamine 200 Mm (REF. BE17-605F – LONZA)
- 1% Penecillin (10,000 U/ml) – Streptomycin (10/mg/ml) (REF. DE17-602F – LONZA)

Note: If you would like to use other references, please check that the characteristics of the product are similar. Please feel free to contact us with any queries or doubts.

ii. HBSS (10X) Buffer

- 54.00 mM KCl
- 4.40 mM KH_2PO_4
- 1.37 M NaCl
- 3.30 mM Na_2HPO_4

Adjust to pH ~ 7.4, autoclave and store at 4°C until use.

iii. $CaCl_2 \cdot 2H_2O$ (100mM), autoclave and store at 4°C until use.

iv. $MgCl_2 \cdot 6H_2O$ (50mM), autoclave and store at 4°C until use.

v. Ethanol (70%)

vi. Lucifer Yellow – (REF. L0259 – SIGMA)

vii. The HBSS (1X) + Ca^{2+}/Mg^{2+} Buffer should be prepared from the above stock solution (item ii, above) on the day of experiments as follows:

For 25 ml:
2.5 ml HBSS (10X) +
0.55 ml $MgCl_2 \cdot 6H_2O$ (50 mM) +
0.325 ml $CaCl_2 \cdot 2H_2O$ (100 mM) +
Adjust final volume with 21.625 ml of sterile MilliQ water.

Note 1: For transport studies of more than 2 hours, D-glucose should be added (at final concentration: 5mM) to the working HBSS buffer.

Alternatively, commercial HBSS Transport Buffer can be purchased from SIGMA (REF. H8264).

Note 2: If necessary, apical HBSS pH can be adjusted to 6.5 with 1N HCl.

2. Equipment

i. Cell culture incubator, 37°C, in a highly humidified atmosphere of 95% air and 5% CO_2

ii. Sterile cell culture cabinet

iii. CO_2 gas tank

iv. Receiver plates (REF.3526 – CORNING). Please note that the receiver should be sterilized and kept sterile for further use.

v. Water bath, 37°C

vi. Aspiration System. It is highly recommended to use the following **manifold** for aspiration: REF. 3-000-097 – DRUMMOND

vii. Automatic multichannel micropipettor (volume range: 50-1200 µl).

viii. Sterile culture medium containers (REF. 4870 – CORNING).

ix. TEER measurement equipment: REF. EVOM2 – WPI

x. Fluorimeter, HPLC-UV/MS, liquid scintillation counter, etc.

Note: ***We highly recommend the use of WPI electrode ref. STX100C (This element is not provided with the TEER measurement equipment and should be purchased separately).***

III. Unpacking and Maintenance

1. Unpacking

Upon receipt of the CacoReady product, retrieve the zip-top bags containing the plates. Open the zip, and leave the bag at a dark location at room temperature until Friday (refer to Table 1, above).

2. Replacing the Shipping Medium

Caution: **Never handle more than one plate at a time while replacing the shipping medium. Re-solidification of the shipping medium may provoke mechanical damage to the cell monolayer.**

i. Retrieve the plates from the bags and remove the Parafilm wrap.

ii. **Incubate** the plates in a 5% CO_2 humidified atmosphere at 37°C **for 4 hours.**

iii. Remove the CacoReady™ plate (one at the time, if you have multiple plates) from the incubator and place inside the laminar flow hood, along with one receiver plate.

iv. Using sterile procedures (inside the laminar flow hood):

- Fill a sterile reagent reservoir with 50 ml of pre-warmed (37°C) Caco-2 culture medium.
- Open the CacoReady™ plate and the receiver plate, and leave the lids upwards, next to the plate.
- Carefully lift the apical inserts of the CacoReady™ plate and transfer onto the receiver plate.
- Remove all liquefied shipping medium from the wells in the basal compartments of the CacoReady™ plate via aspiration with the 24-well manifold.
- Using a multichannel pipette, dispense **900 µl** of the Caco-2 cell culture medium from the sterile reservoir into the wells of the **basal compartment** (column by column) of the CacoReady™ plate.
- Using the aspiration manifold connected to a vacuum (adjust aspiration flux to medium-low), aspirate the liquefied shipping medium from the apical inserts of the

CacoReady™ plate placing the manifold perpendicular to the cell monolayer and close to the insert wall. Check that the shipping medium has been removed from all wells. 70 µl of medium will be left in each well.

- Using a multichannel pipette, dispense **300 µl** of the Caco-2 cell culture medium from the sterile reservoir, and fill, column by column, each of the **apical inserts** of the CacoReady™. Always add the medium against the wall of the well, not onto the center directly on the cell monolayer.
- Carefully return the apical inserts onto the basal compartment of the CacoReady™ plate. Replace the lid and place inside cell culture incubator, set at 37°C, with 5% CO_2 until Monday.

v. Once the shipping medium has been replaced by the fresh Caco-2 cell culture medium, plates should be kept inside the incubator until the following Monday (Day 21).

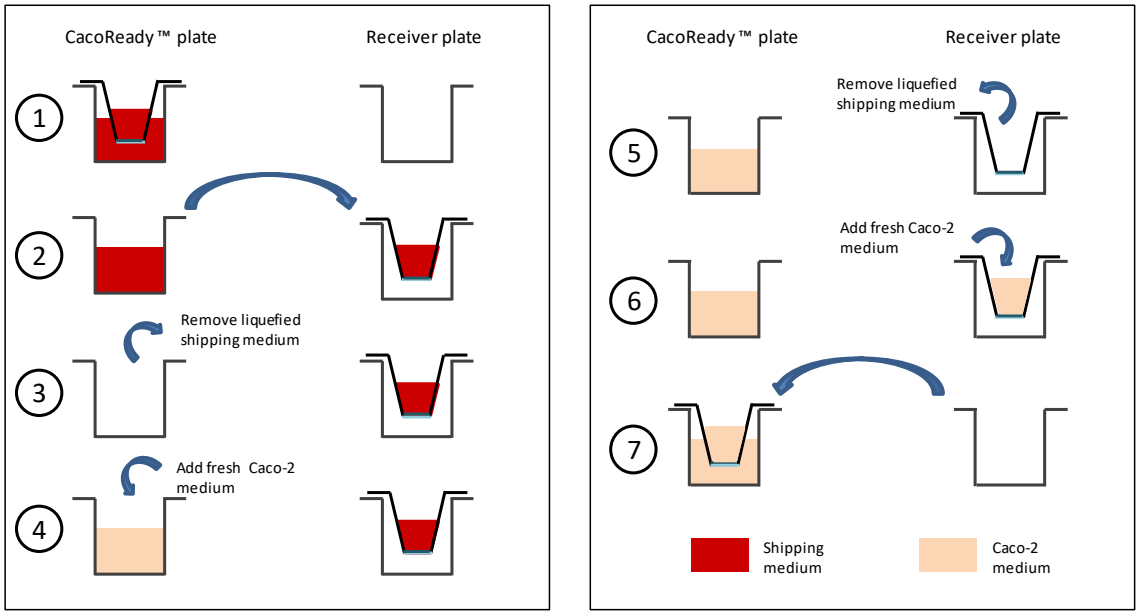

Figure 2. Scheme for Replacing the Shipping Medium

3. Exchanging the Caco-2 Cell Culture Medium

Exchanging the cell culture medium has to be carried out once (at day 21) or twice (at day 21 and day 23) depending on the day of the assay (See table 1, above), following the same procedure described in (Section **III-2**, page 4).

IV. QUALITY CONTROL OF THE BARRIER SYSTEM

1. Pre-assay Quality Control: TEER Measurement

This section provides general instructions for TEER measurements. It is important to carefully read the instructions of your TEER measurement equipment in conjunction with the directions listed below:

On Monday (equivalent to day 21 of cell culture), perform TEER measurement on the plate/plates **before any further processing.**

To proceed with the TEER measurement, follow the steps below:

i. **It is highly recommended to use the WPI STX 100C electrodes, in order to avoid any damage to the cell monolayer.**

ii. Sterilize the electrodes (probe) by submerging both electrodes in ethanol (70%) for 30 minutes.

iii. Equilibrate the electrodes (probe) for 5 minutes in Caco-2 cell culture medium, pre-warmed at room temperature.

iv. At the same time, remove the 24-Insert HTS plate with Caco-2 cells from the incubator (Section **III-iii**, page 4), place in a bio-safety cabinet, and allow it to reach to room temperature (approximately 20 minutes), as TEER measurements need to be performed at room temperature.

v. Place the electrode on top of the apical insert and proceed with the measurement.

vi. A TEER value of 1000 Ω·cm^2 (or higher) indicates that the barrier system is acceptable for an absorption assay (Active membrane area is 0.33 cm^2).

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KEY DATA

Membrane area	**0.33 cm^2**
Minimum TEER value	**1000 Ω·cm^2**

IMPORTANT NOTES

- TEER reading should be carried out 48 hours after exchanging medium, in order to verify the integrity of the monolayer prior to the permeability assay. **Never perform the TEER measurement with the shipping medium.**
- Do not perform repeated TEER measurements within the same insert.
- For an appropriate TEER measurement, both electrodes have to be well submerged within the cell culture medium in both apical and basal compartments.
- Please, see Important Note page 2

2. *Post-assay Quality Control: Lucifer Yellow Paracellular Permeability Assay*

Prepare a Lucifer Yellow (LY) Stock Solution of 1mg/ml in sterile $_{dd}H_2O$. Aliquot (For example 500 µl) and store at –20°C. Pre-warm (37°C) the Working dilutions of LY before use (see Figure 3, below for recommended Working dilutions).

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

i) Following the procedure described in Section **III-2**, page 4, gently rinse both the apical and the basal compartments of the 24-insert HTS Plate with HBSS Buffer Working solution (250 µl apical / 750 µl basal).

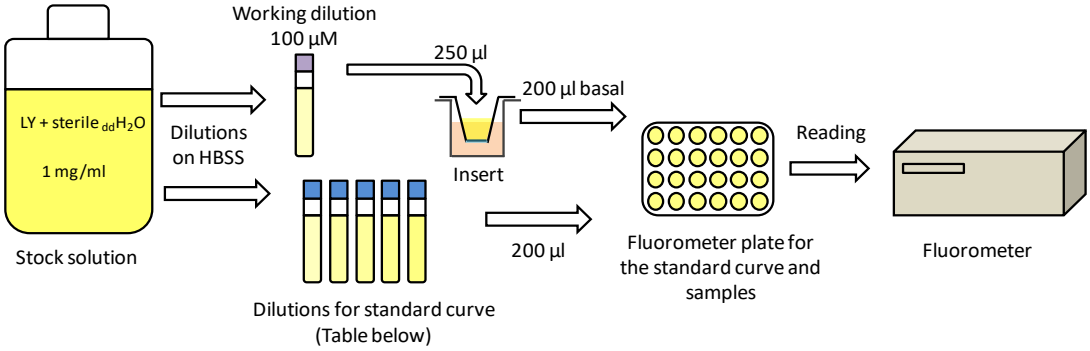
ii) Add 250 µl of LY Working dilutions, as recommended in Figure 3 below, into the wells on the wells in the apical compartment.

iii) Add 750 µl of HBSS Buffer Working solution onto the basal compartment.

iv) Incubate the 24-Insert HTS plate protected from light in a 37°C incubator for 1 hour.

v) Recover 200 µl from each well in the basal compartment and dispense onto corresponding wells on an empty flourometer plate, (mix well and avoid bubble formation when dispensing each aliquot).

vi) Read the fluorescence intensity directly in a fluorometer, using a **485 nm excitation** filter and a **527 nm emission** filter.



0.00 µM	0.10 µM	0.20 µM	0.30 µM	0.40 µM	0.50 µM	0.75 µM	1.00 µM	10.00 µM	25.00 µM	50.00 µM	100.00 µM

Figure 3. General Procedure and Recommended Concentrations for Lucifer Yellow Standard Curve

Note: Please note that under standard conditions (i.e., in HBSS Buffer), 21-day CacoReady™ barriers present LY Paracellular Flux values of ≤ 0.7% after 1 hour, and/or Papp values of ≤ 1 x 10^{-6} cm/s. These values are considered as reference values for the monolayer integrity.

KEY DATA

LY Paracellular Flux	**0.7%**
LY Papp	**≤ 1 x 10^{-6} cm/s**

IMPORTANT NOTES:

- **Perform the LY Assay after the permeability assay.**
- It is highly recommended to use some of the inserts only for testing LY as a quality control test of the monolayer status.

V. APPENDIX I: GENERAL PROTOCOL FOR PERMEABILITY ASSAYS

1. *General Protocol for Apical-To-Basal Permeability Studies*

The protocol below describes a suggested procedural guide to perform A-B permeability assays.

i. Assay conditions

- Test compound concentration: 10 µM (recommended)
- Replicates:3
- Time points: 2 (0 and 2 hours)
- Assay volumes:
    - Apical: 250 µl of the test compound at working dilution (275 µl is added to the basal compartment and 25 µl is recovered at time 0 hr.)
    - Basal: 750 µl of HBSS Buffer Working solution
- HBSS Buffer Working solution for 25 ml:
    - 2.5 ml HBSS (10X) +
    - 0.55 ml $MgCl_2$, $6H_2O$ (50 mM) +
    - 0.325 ml $CaCl_2$, $2H_2O$ (100 mM) +
    - 0.125 ml D-glucose (500 mM)

ii. PROTOCOL (using a 24-well manifold for aspiration)

***CAUTION**: Do not add the Lucifer yellow reagent together with the test compounds. The Lucifer Yellow could interfere with certain substances producing false results.*

1- Prepare a 10 mM stock solution of the test compounds, using the proper solvent.

2- Prepare 10 µM working dilutions of test compounds in HBSS Buffer Working solution (see above, Section V-1-i).

3- Fill a sterile container for cell culture medium with 50 ml of pre-warmed (37°C) HBSS Buffer Working solution.

4- Unwrap one "receiver plate" under the biosafety cabinet.

5- Take 1 CacoReady™ plate from the incubator and place it in the biosafety cabinet next to the receiver plate.

6- Gently lift the apical inserts of the CacoReady™ plate and transfer them on top of the "receiver plate".

7- Remove all Caco-2 medium from the wells on the basal compartment of the CacoReady™ plate by aspiration with the 24-well manifold.

8- Fill, column by column, each of the 24 wells of the basal compartments of the CacoReady™ plate with 750 µl of pre-warmed HBSS Buffer Working solution.

9- Remove the Caco-2 medium from the apical inserts of the CacoReady™ plate by aspiration with the manifold. Place the manifold perpendicular to the cell monolayer and close to the insert wall.

10-Fill, column by column, each of the 24 apical inserts of the CacoReady™ plate with 250 µl of pre-warmed HBSS Buffer Working solution.

11-Return the apical inserts onto the wells of the basal compartment of the CacoReady™ plate (original position).

12-Keep the plate for minimum of 1 min at room temperature inside the biosafety cabinet.

13-Repeat rinsing the plate (Steps 8 and 9, of Section V-1, ii, above) two more times and keep the plate for 30 min at 37° C inside a 5% CO_2 incubator.

14-Take the plate from the incubator, return it to the biosafety cabinet and place it next to the receiver plate.

15-Gently lift the apical inserts of the CacoReady™ plate and transfer them to the top of the "receiver plate".

16-Remove the buffer from the wells in the basal compartments of the CacoReady™ plate by aspiration with the 24-well manifold.

17- Fill, column by column, each of the 24 wells of the basal compartment of the CacoReady™ plate with 750 µl of pre-warmed HBSS Buffer Working solution.

18-Remove the buffer from the apical inserts of the CacoReady™ plate by aspiration with the manifold.

19-Fill, column by column, each of the 24 apical inserts with 275 µl of the test compound working dilution.

20-Recover 25 µl from the apical inserts (**0 hr. Apical samples)** and keep them at -20°C until analysis.

21-Return the apical inserts onto the wells of the basal compartment of the CacoReady™ plate (original position).

22- Cover the CacoReady™ plate with its lid, and place it inside the 5% CO_2 incubator, at 37°C, for 2 hours.

23-Take the plate from the incubator, return it to the biosafety cabinet and place it next to the receiver plate. Transfer the apical inserts onto the receiver plate.

24-Recover 25 µl from the apical inserts (**2 hr. Apical samples**), and keep them at -20°C until analysis.

25- Recover samples of 25 µl from the wells of the basal compartment (**2 hr. Basal samples**), and keep them at -20°C until analysis.

26- Proceed to sample analysis (0 h and 2 h samples, above).

27- After quantification of the test compound, proceed to calculate permeability coefficient (Papp) as indicated in "Evaluation of the Compound Permeability" Section V-3, below.

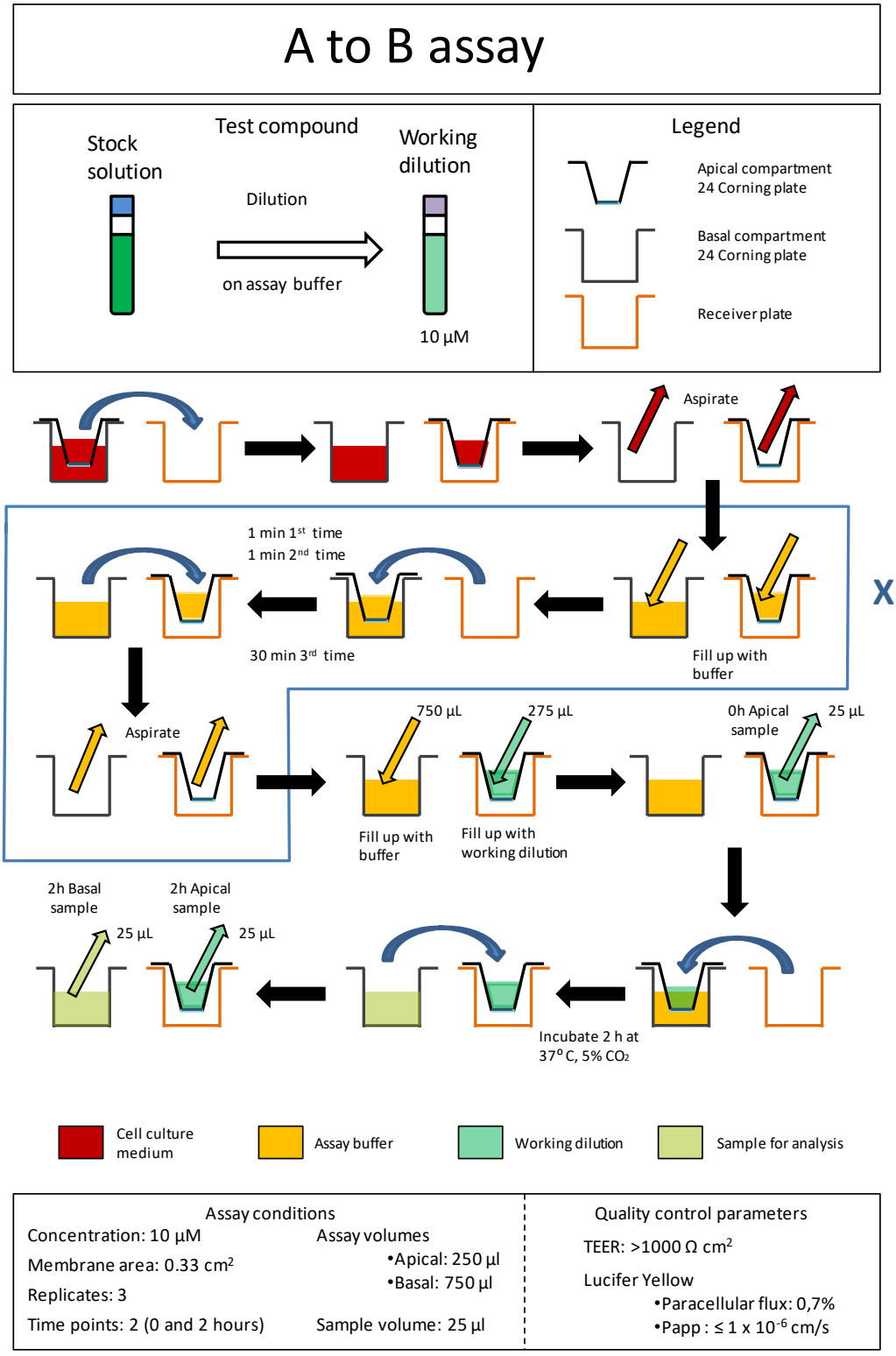


Figure 4. Diagram Showing the Steps involved in the Apical to Basal Permeability Assay Protocol

2. *General Protocol for Basal-To-Apical Permeability Studies*

The B-A permeability assay protocol remains essentially the same as the A-B protocol described above, except for the fact that the test compound is added to the basal compartment, acting as a donor side this time (See Figure 5). In this case, 775 µl of the test compound is added to the basal compartment and 25 µl is recovered at time 0 (0 hr. basal sample) while 250 µl of the HBSS Buffer Working solution is added to the apical compartment. All other steps are identical as in the AB protocol, above.

- Test compound concentration: 10 µM (recommended)
- Replicates: 3
- Time points: 2 (0 and 2 hours)
- Assay volumes:
    - Apical: 250 µl of HBSS Buffer Working solution.
    - Basal: 750 µl of the test compound at working dilution (775 µl is added to the basal compartment and 25 µl is recovered at time 0 hr.)
- HBSS Buffer Working solution for 25 ml:
    - 2.5 ml HBSS (10X) +
    - 0.55 ml $MgCl_2$, $6H_2O$ (50 mM) +
    - 0.325 ml $CaCl_2$, $2H_2O$ (100 mM) +
    - 0.125 ml D-glucose (500 mM)

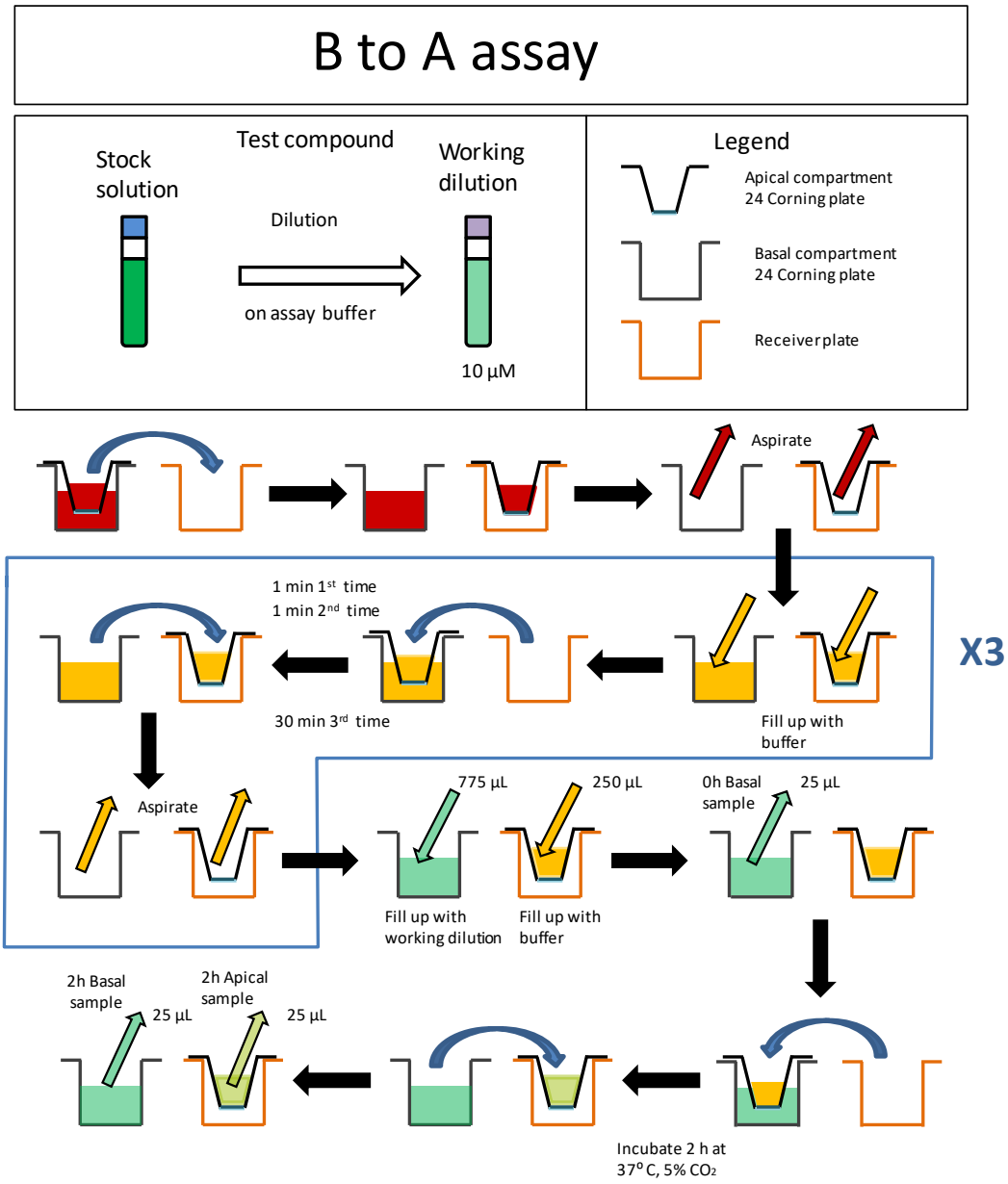


Figure 5. Diagram Showing Steps involved in the Basal to Apical Permeability Assay Protocol

3. Evaluation of the Compound Permeability.

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

Where:

dQ/dt: amount of product present in basal (A-B) or apical (B-A) compartment as a function of time (nmol/s).
A: area of transwell (cm^2).
C_0: initial concentration of product applied in apical (A-B) or basal (B-A) compartment (nmol/ml).

Based on *in vitro/in vivo* correlation studies (see Table 2, below), Papp values obtained from Caco-2 barrier studies are used to define and predict oral/intestinal, brain bioavailability or toxicity of the tested compounds.

In vitro Papp Values	Range of Predicted *in vivo* Absorption
Papp $\leq 10^{-6}$ cm/s	Low (0-20%)
10^{-6} cm/s < Papp $\leq 10 \times 10^{-6}$ cm/s	Medium (20-70%)
Papp > 10×10^{-6} cm/s	High (70-100%)

Table 2. Prediction of 'in vivo' Oral Absorption in Humans Based on 'in vitro'. Papp Values Obtained from Caco-2 Barrier Studies

4. Reference Compounds for Permeability Studies

Different markers can be used to check if a Caco-2 barrier (21-day) is acceptable for transport studies. Data related to experimental conditions and standard Papp values for different markers are provided below, and should be considered by end users as guidelines to confirm the CacoReady™ barrier quality and performance.

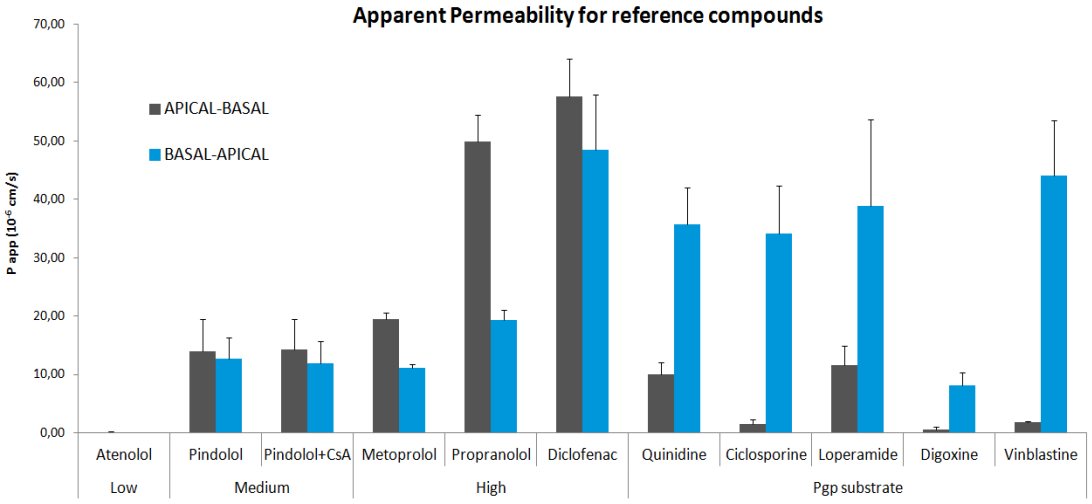


Figure 6. Papp Values for Reference Compounds

Reference Compound	Permeability Range	Concentration (µM)	APICAL-BASAL		BASAL-APICAL		Efflux Ratio
			Papp (10^{-6} cm/s)	Standard Deviation	Papp (10^{-6} cm/s)	Standard Deviation	
Atenolol	Low	10.00	0,15	0,02	0,07	0,01	0.51
Pindolol	Medium	1.00	14,00	5,40	12,70	3,60	0,90
Pindolol+CsA		1.00	14,20	5,30	11,90	3,80	0,80
Metoprolol	High	10.00	19,42	1,18	11,14	0,69	0.57
Propranolol		10.00	49,87	4,65	19,35	1,65	0,39
Diclofenac		10.00	57,59	6,44	48,52	9,40	0,84
Quinidine	Pgp substrate	10.00	10,00	2,01	35,69	6,36	3,57
Ciclosporine		10.00	1,57	0,71	34,14	8,18	21,79
Loperamide		10.00	11,66	3,22	38,86	14,84	3,13
Digoxine		10.00	0,54	0,47	8,06	2,32	14.80
Vinblastine		10.00	1,86	0,18	44,07	9,52	23,72

Table 3. Experimental Conditions and Papp Values for Reference Compounds.

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