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CacoGoblet

CacoGoblet 24 (Corning Plate) User's Manual

24-Well Plates (Inserts) with Seeded and Differentiated (21-days) co-culture of Caco-2 and Human Goblet Cells

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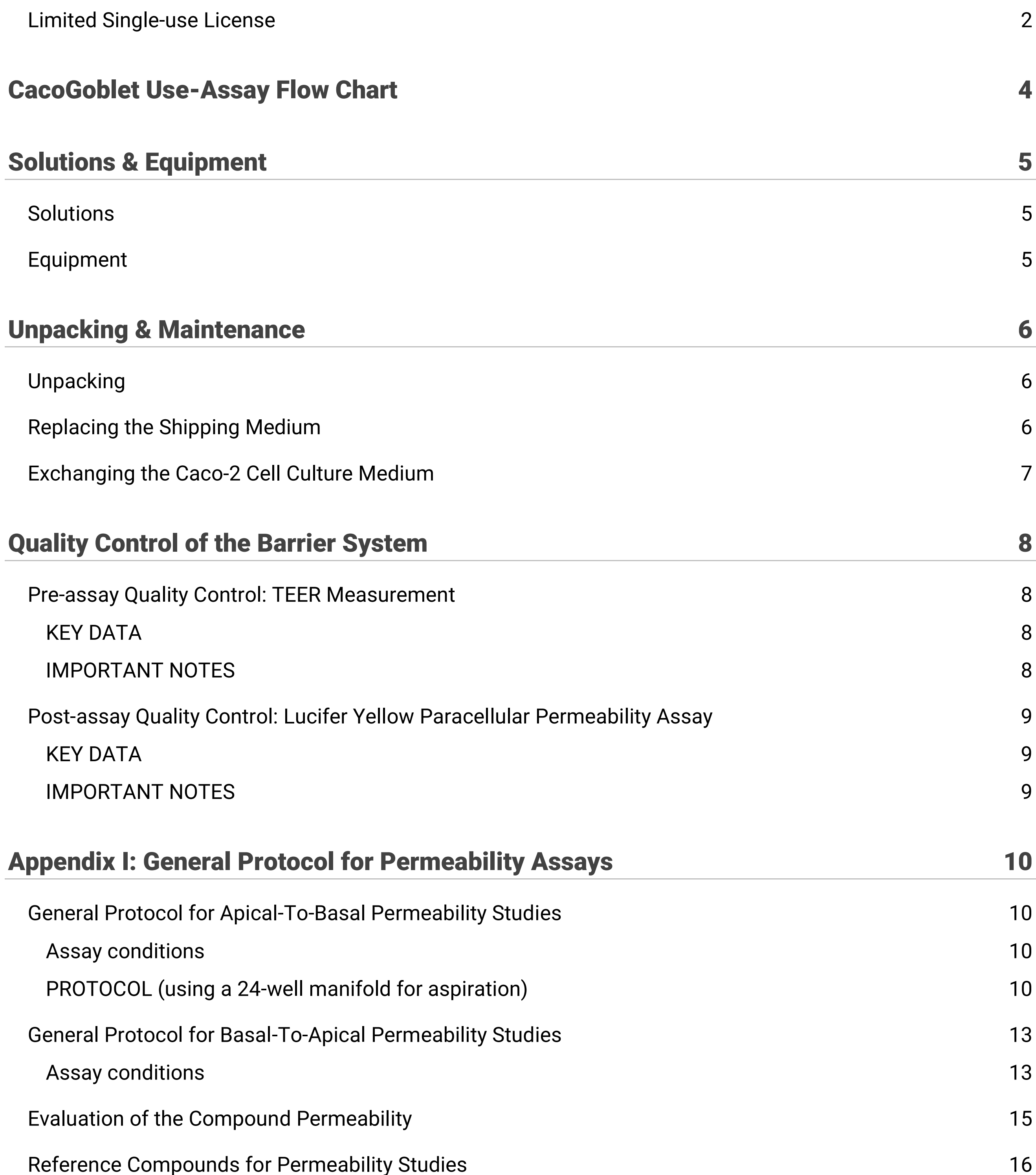

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CacoGoblet Use-Assay Flow Chart

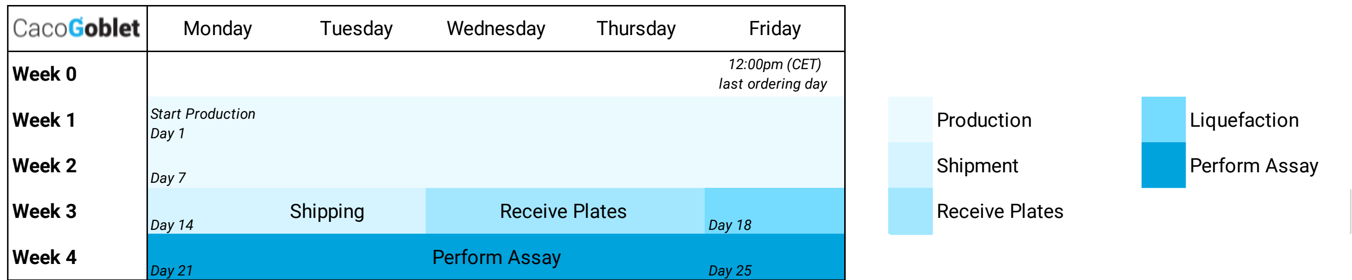

Figure 1 - Assay Flow Chart

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) to measure TEER values and start experiments. If this schedule is followed, no extra medium exchanges are required. Medium exchanges are necessary 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: *We strongly recommend measuring the TEER on Monday, and in case TEER values are low, performing a medium change and waiting until Wednesday to let the cells recover. On Wednesday, reread the TEER and perform the assay accordingly.*

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Solutions & Equipment

The reagents listed below are not provided with the kit and must be made and available for use prior to the delivery of CacoGoblet plates.

Solutions

i. Standard CacoGoblet Cell Culture Medium

- DMEM 4.5g/L glucose (REF. BE12-614F – LONZA)
- 10% FBS (REF. S181B-500 – BIOWEST)
- 1% Glutamine 200 Mm (REF. BE17-605F – LONZA)
- 1% Penicillin (10,000 U/ml) – Streptomycin (10/mg/ml) (REF. DE17-602F – LONZA)
- 1% NEEAA (REF. 11140035 – Life Technologies)

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

ii. Buffer Solution

- HBSS Transport Buffer (1X) + Ca*2+* /Mg*2+* (REF. H8264 – SIGMA)

Note 1: *If you would like to use other references, such as GIBCO – 14025 or HYCLONE – SH30268, please check that the characteristics of the product are similar. Please feel free to contact us with any queries or doubts.*

Note 2: *If you prefer to prepare the solution at your facilities, remember that for transport studies of more than 2 hours, D-glucose should be added at a final concentration of 5mM to the working HBSS buffer.*

Note 3: *If necessary, apical HBSS pH can be adjusted to 6.5 with 1N HCl.*

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).
v. Water bath, 37°C
vi. Aspiration System. It is highly recommended to use REF. 3-000-097 – DRUMMOND manifold for aspiration
vii. Automatic multichannel micropipettor (volume range: 50-1200 µl) (REF. 613-1427 – EPPENDORF)
viii. Sterile culture medium containers (REF. 4870 – CORNING)
ix. TEER measurement equipment (REF. EVOM2 – WPI)

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).*

Unpacking & Maintenance

Unpacking

Upon receipt of the CacoGoblet 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).

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 CacoGoblet plate (one at a 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 CacoGoblet plate and the receiver plate, and leave the lids upwards, next to the plate.
- Carefully lift the apical inserts of the CacoGoblet plate and transfer it onto the receiver plate.
- Remove all liquefied shipping medium from the wells in the basal compartments of the CacoGoblet 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 CacoGoblet 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 CacoGoblet 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 CacoGoblet. Always add the medium against the well's wall, not onto the center directly on the cell monolayer.
- Carefully return the apical inserts onto the basal compartment of the CacoGoblet plate. Replace the lid and place inside the cell culture incubator, set at 37°C, with 5% CO_2 until Monday.

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

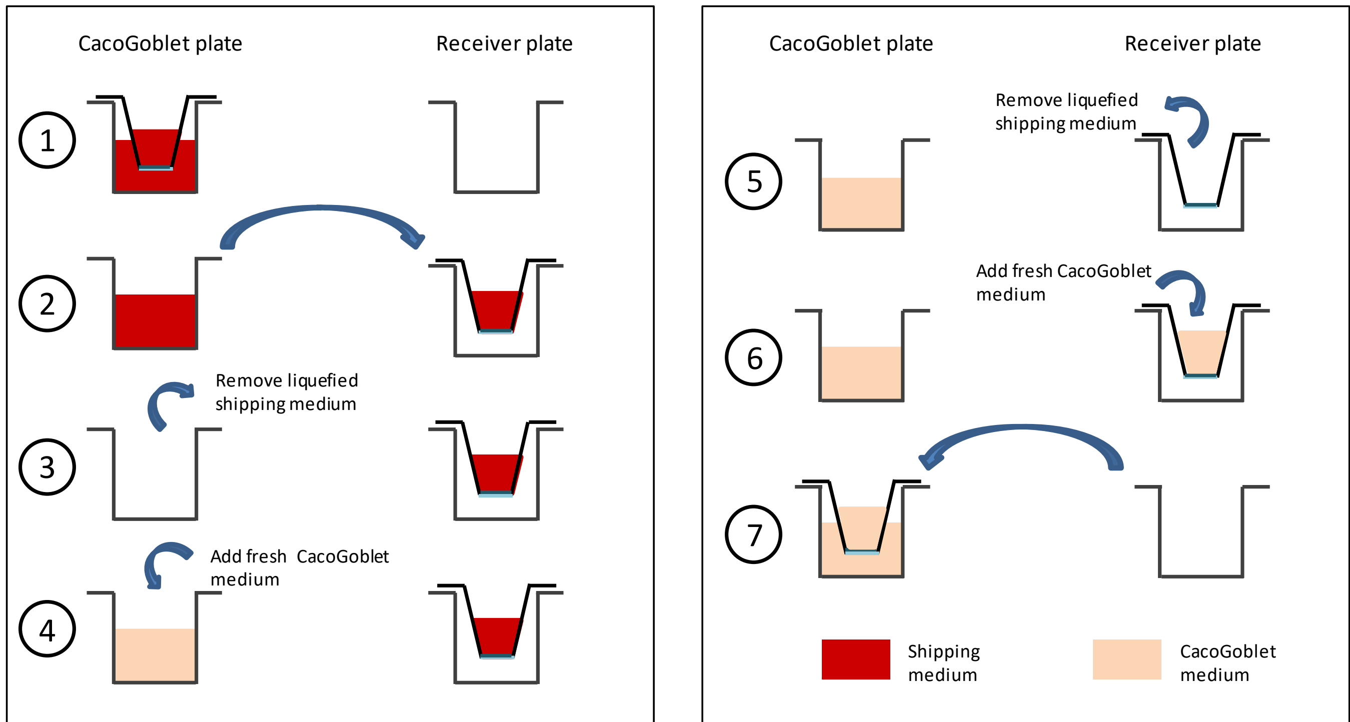

Figure 2 - Scheme for Replacing the Shipping Medium

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 "Replacing the Shipping Medium", pages 6-7.

Quality Control of the Barrier System

Pre-assay Quality Control: TEER Measurement

This section provides general instructions for TEER measurements. It is essential 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:

IMPORTANT NOTE: *It is highly recommended to use the WPI STX 100C electrodes to avoid any cell monolayer damage.*

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

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

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

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

v. A TEER value of 70 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	**70 Ω·cm^2**

IMPORTANT NOTES

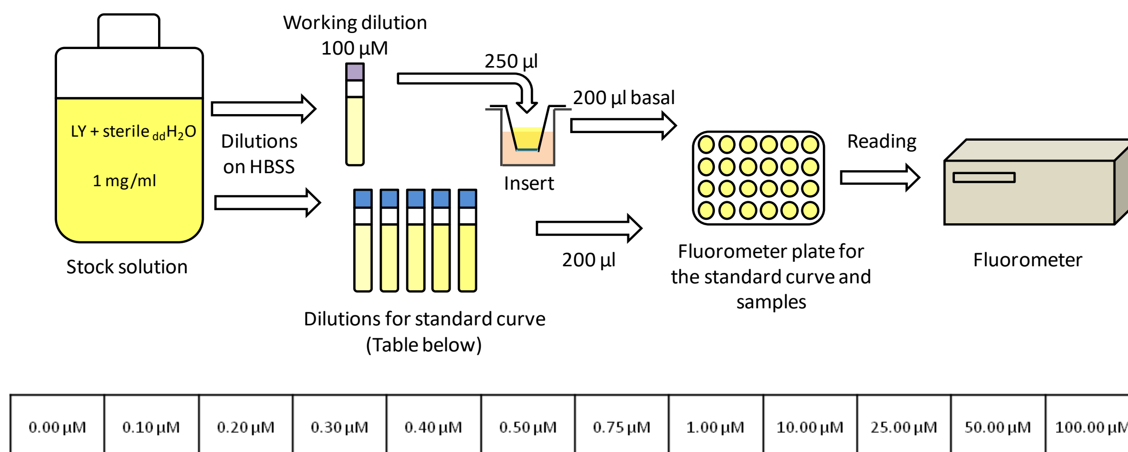
- **Never perform the TEER measurement with the shipping medium.**
- TEER reading should be carried out 48 hours after exchanging medium to verify the monolayer's integrity prior to the permeability assay.
- Do not perform repeated TEER measurements within the same insert.
- For an appropriate TEER measurement, both electrodes must be well submerged within the cell culture medium in both apical and basal compartments.
- See also IMPORTANT NOTES from Pages 4 and 8.

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 "Replacing the Shipping Medium", pages 6-7, 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 fluorometer 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 CacoGoblet barriers present LY Paracellular Flux values of ≤ 1.4% after 1 hour, and/or Papp values of ≤ 3 x 10^{-6} cm/s. These values are considered as reference values for the monolayer integrity.*

KEY DATA

LY Paracellular Flux	**≤ 1.4%**
LY Papp	**≤ 3 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.

Appendix I: General Protocol for Permeability Assays

General Protocol for Apical-To-Basal Permeability Studies

Drug candidates or lead compounds are applied to the apical side of the cell monolayer (upper compartment of the insert), and their apical-to-basal (A-B) transport through the cell barrier is evaluated by sample recovering and compound detection at the basal (lower compartment) over a defined incubation time. A-B permeability of compounds is determined as the coefficient of apparent permeability (Papp in cm/s).

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

Assay conditions

- **i.** Test compound concentration: 50 µM (recommended)
- **ii.** Replicates: 3
- **iii.** Time points: 2 (0 and 1 hours)
- **iv.** 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
- **v.** 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) +
  - Adjust final volume with 21.625 ml of sterile MilliQ water

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 50 mM stock solution of the test compounds, using the proper solvent.
2. Prepare 50 µM working dilutions of test compounds in the HBSS Buffer Working solution (see above).
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 CacoGoblet plate from the incubator and place it in the biosafety cabinet next to the receiver plate.
6. Gently lift the apical inserts of the CacoGoblet plate and transfer them on top of the receiver plate.
7. Remove all cell culture medium from the wells on the basal compartment of the CacoGoblet plate by aspiration with the 24-well manifold.

Note: *Place the manifold perpendicular to the cell monolayer and close to the insert wall.*

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

9. Remove the cell culture medium from the apical inserts of the CacoGoblet plate by aspiration with the manifold.

Note: *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 CacoGoblet 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 CacoGoblet plate (original position).
12. Keep the plate for a minimum of 1 min at room temperature inside the biosafety cabinet.
13. Repeat rinsing the plate (Steps 6 and 12, above) two more times and keep the plate for 30 min at 37° C inside a 5% CO_2 incubator after the last repetition.
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 CacoGoblet plate and transfer them to the top of the receiver plate.
16. Remove the buffer from the wells in the basal compartments of the CacoGoblet plate by aspiration with the 24-well manifold.
17. Fill, column by column, each of the 24 wells of the basal compartment of the CacoGoblet plate with 750 µl of pre-warmed HBSS Buffer Working solution.
18. Remove the buffer from the apical inserts of the CacoGoblet 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 CacoGoblet plate (original position).
22. Cover the CacoGoblet 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 quantifying the test compound, calculate the permeability coefficient (Papp) as indicated in "Evaluation of the Compound Permeability", Page 15, below.

A to B assay

Test compound

Stock solution

Dilution

on assay buffer

Working dilution

10 µM

Legend

Apical compartment 24 Corning plate

Basal compartment 24 Corning plate

Reservoir plate

Aspirate

1 min 1st time
1 min 2nd time
30 min 3rd time

Fill up with buffer

X3

Aspirate

750 µL

275 µL

Fill up with buffer

Fill up with working dilution

0h Apical sample

25 µL

Incubate 2 h at 37º C, 5% CO_2

2h Basal sample

25 µL

2h Apical sample

25 µL

Cell culture medium

Assay buffer

Working dilution

Sample for analysis

Assay conditions

Concentration: 50 µM
Membrane area: 0.33 cm^2
Replicates: 3
Time points: 2 (0 and 2 hours)

Assay volumes
•Apical: 250 µl
•Basal: 750 µl

Sample volume: 25 µl

Quality control parameters

TEER: >70 Ω cm^2

Lucifer Yellow
•Paracellular flux: 1,4%
•Papp : ≤ 3 x 10^{-6} cm/s

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

General Protocol for Basal-To-Apical Permeability Studies

Drug candidates or lead compounds are applied to the basal side of the cell monolayer (lower compartment of the insert), and their basal-to-apical (B-A) transport through the cell barrier is evaluated by sample recovering and compound detection at the apical (upper compartment) over a defined incubation time. B-A permeability of compounds is determined as the coefficient of apparent permeability (Papp in cm/s).

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 A-B protocol above.

Assay conditions

- **i.** Test compound concentration: 50 µM (recommended)
- **ii.** Replicates: 3
- **iii.** Time points: 2 (0 and 1 hours)
- **iv.** 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.)
- **v.** 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) +
  - Adjust final volume with 21.625 ml of sterile MilliQ water

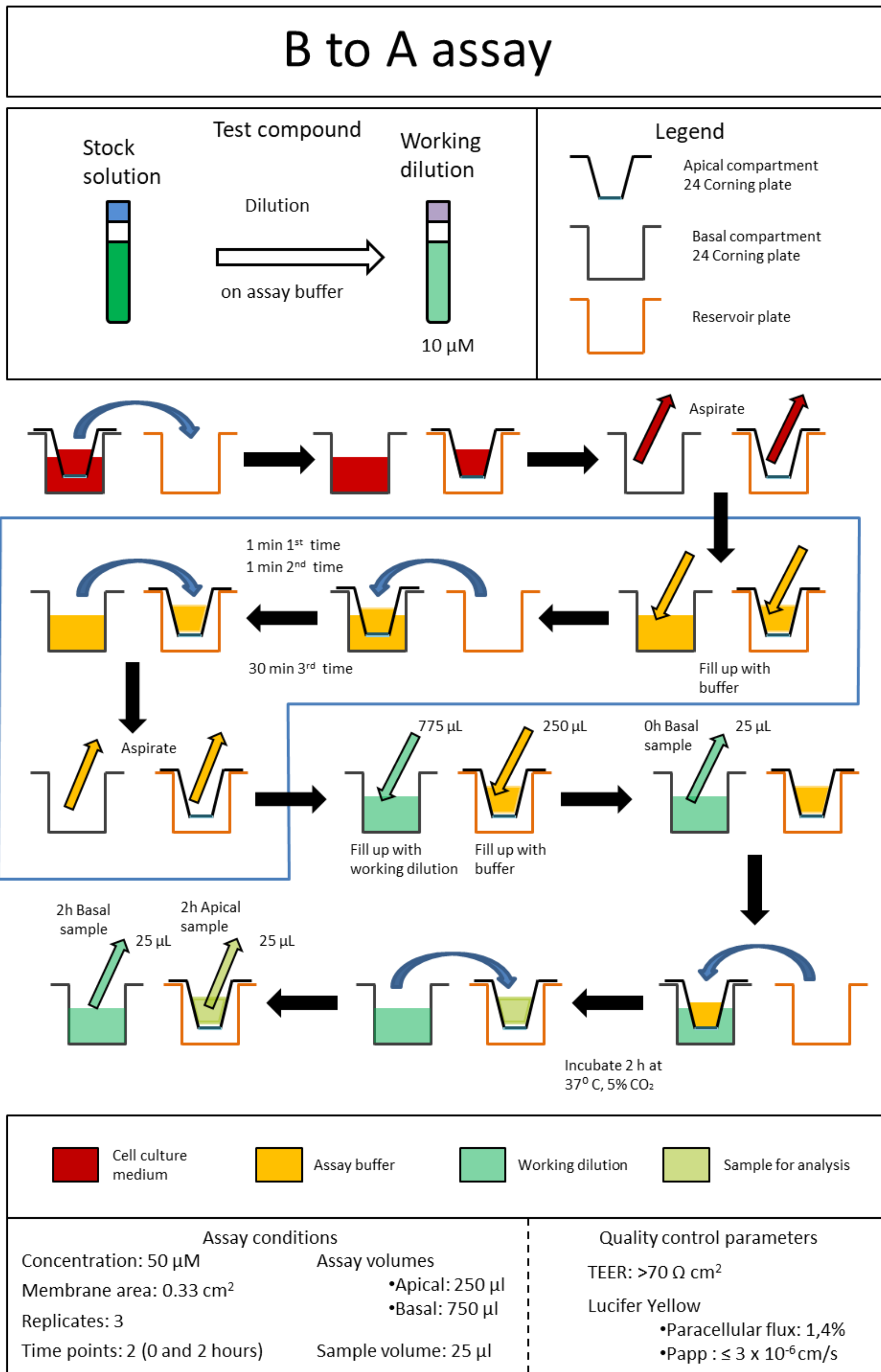


Figure 5 - Diagram Showing Steps involved in the Basal to Apical Permeability Assay Protocol

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 the 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 the 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'

Reference Compounds for Permeability Studies

Functionality of co-culture Caco-2 and HT-29 barrier is evaluated by permeability assays of different compounds. Results are expressed as the average of Papp values.

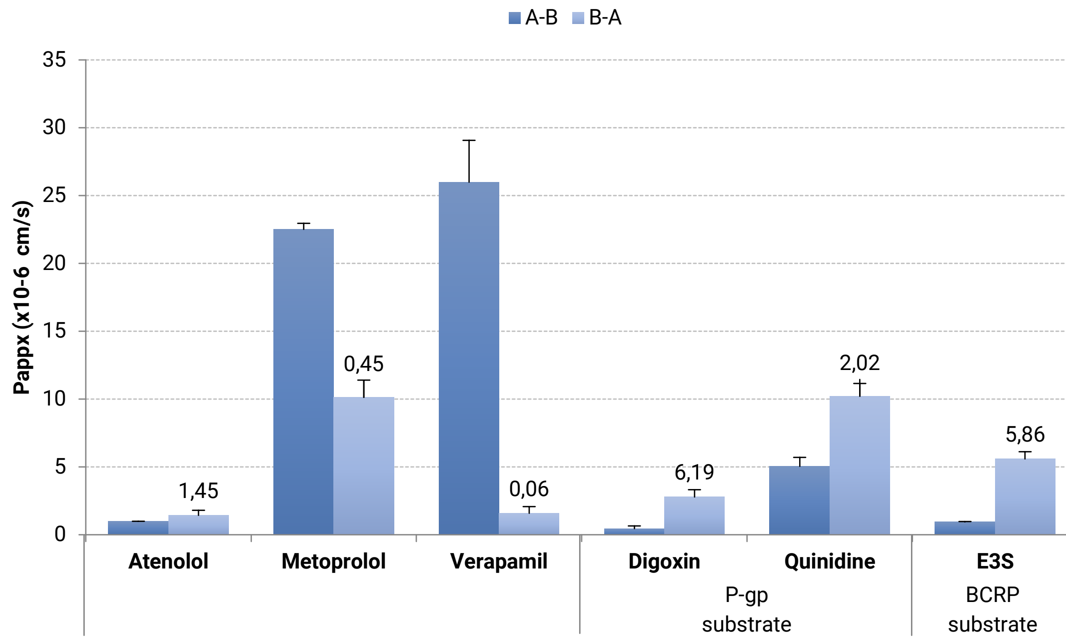


Figure 6 - Papp Values for Reference Compounds

Test compound	Concentration (µM)	APICAL BASAL		BASAL APICAL		Permeability	Efflux Ratio
		Papp x 10^{-6} (cm/s)	SD	Papp x 10^{-6} (cm/s)	SD		
Atenolol	10	0.99	0.01	1.43	0.37	Low	1.45
Metoprolol	10	22.51	0.44	10.13	1.27	High	0.45
Verapamil	10	25.99	3.09	1.58	0.49	High	0.06
Digoxin	10	0.45	0.20	2.78	0.54	Low	6.19
Quinidine	10	5.04	0.66	10.21	0.94	Medium	2.02
Estrone 3-Sulfate (E3S)	10	0.95	0.02	5.59	0.53	Low	5.86

Table 3 - Efflux Ratio Values for Reference Compounds