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24-Well CacoGoblet Anti-inflammatory Screening User's Manual

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Product Description

CacoGoblet is an *in vitro* cell-based model resulting from the coculture of two human colon cancer cells (Caco-2 and HT-29) with an absorptive and mucus-secreting phenotype, respectively. Cells differentiate for 21-days in 24 Transwell® inserts with semi-porous (0.4 µm) polystyrene (PET) membrane (CORNING Cat#3378) resulting in an apical compartment and a basal compartment that mimic the intestinal lumen and the blood circulation, respectively.

Caco-2/HT-29 cells are considered a relevant cellular model for assessing anti-inflammatory drug testing and can easily be adapted for high-throughput screening of compounds.

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

Intended Use

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

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

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

Principle

Anti-inflammatory capabilities of drugs are carried out with a co-culture of Caco-2 and HT-29 providing an enhanced physiological model of the human gut.
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).

In a standard assay design, an inflammatory response of the model is induced by incubation with a cocktail of cytokines. Once inflammation is triggered, test compounds are exposed to asses their anti-inflammatory capabilities over a period of up to 72 hours. Anti-inflammatory activity is monitored over time using Transepithelial Electrical Resistance (TEER) and Lucifer Yellow paracellular permeability assay, parameters that determine membrane integrity.

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 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 anti-inflammatory assays is Day 21 (Monday) (see Figure 1 for details).

CacoGoblet	Monday	Tuesday	Wednesday	Thursday	Friday
Week 0					**12:00 p.m. (CET) last ordering day**
Week 1	*Pre-Production*	*Start of Production Day 1*			
Week 2	*Day 7*				
Week 3	*Shipment Day 14*	Reception of Plates			*Liquefaction Day 18*
Week 4	*Day 21*	Perform Assay			*Day 25*

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

IMPORTANT NOTE: *TEER evaluation will be carried out on Monday (Day 21) before performing any further processing, including medium replacement.*

Equipment (not included)

- Cell culture laminar flow hood
- CO2 incubator
- Water bath
- Multichannel pipettes
- Automatic multichannel micropipette (recommended)
- Aspiration system
- **24-well format vacuum manifold (Drummond Cat# 3-000-097 recommended)**
- Trans-Epithelial Electrical Resistance (TEER) meter (WPI EVOM series)
- **24-well electrode (WPI EVM-EL-03-03-04 recommended)**
- Fluorometer (Fluoroskan Ascent CF)

***NOTE**: An electrode adaptor may be required depending on the model of the TEER meter you are using. Please refer to WPI for concerns regarding compatibility.*

Consumables

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

Solutions (may be included)

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

- **Caco-2/HT-29 Cell Culture Medium**: Dulbecco's Modified Eagle's Medium - high glucose (4,5 g/L) (CORNING Cat# 10-013-CV) supplemented with (final concentrations):
  - 10 % V/V Fetal Bovine Serum (BIOWEST Cat# DE14-801F)
  - 100 U/mL; 0.1 mg/mL Penicillin-Streptomycin (LONZA Cat# DE17-602F)
  - 1x MEM non-essential amino acid solution (GIBCO Cat# 11140035)
- **Transport Buffer solution**: Hank's 1X Balanced Salt Solutions (HBSS 1x) (HyClone Cat# SH30268)
- **Paracellular permeability compound**: Lucifer Yellow (LY) (SIGMA L0259)
- **Inflammatory inducers:**
  - TNF-α Human (SIGMA Cat# SRP3177)
  - Interferon- Gamma Human (SIGMA Cat# I17001)
  - Interleukin-1β Human (SIGMA Cat# H6291)
  - Lipopolysaccharides from Escherichia Coli (SIGMA Cat# 196322)
- **Recommended anti-inflammatory reference compound**: Baicalein (SIGMA Cat# 196322)

***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 % CO_2 humidified atmosphere at 37 °C for **4 hours**, until the **shipping medium** reaches **liquefaction**.
3. Remove one CacoGoblet plate from the incubator and place it inside the laminar flow hood, along with one reservoir plate.
4. Using sterile procedures (**inside the laminar flow hood**), fill a sterile reagent reservoir with 50 mL of pre-warmed (37 °C) Caco-2/HT-29 cell culture medium.
5. Open the CacoGoblet plate and the reservoir plate, and leave their lids upwards, next to the plates.
6. Carefully lift the 24-integrated apical compartments of the CacoGoblet plates and transfer them onto the reservoir plate.
7. Remove all liquefied shipping medium from the basal compartments of the CacoGoblet plate via aspiration with the 24-well manifold.
8. Using a multichannel pipette, dispense **900 µL** of Caco-2/HT-29 cell culture medium from the sterile reservoir, and fill, the **basal compartments** of the CacoGoblet plate, column by column.
9. 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 CacoGoblet plate, taking care not to disrupt the monolayer. Make sure the shipping medium has been removed from all wells. Approximately 50 µL of medium will be left in each well.
10. Using a multichannel pipette, dispense **300 µL** of Caco-2/HT-29 cell culture medium from the sterile reservoir, and fill, the **apical compartments** of the CacoGoblet plate, column by column. Always add the medium against the wall of the well, and not directly onto the cell monolayers.
11. Carefully return the apical inserts onto the basal compartment of the CacoGoblet plate. Replace the lid and place it inside the cell culture incubator, set at 37 °C and 5 % CO_2.
12. Once the shipping medium has been substituted by fresh Caco-2/HT-29 cell culture medium, the plates should be placed inside the incubator until next Monday (Day 21).

NOTE: *Do not discard the reservoir plate, as it will be used in the anti-inflammatory screening 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.

TEER measurement will be carried out before performing any further processing, including the medium replacement and the experiment.

NOTE: *Never perform the TEER measurement with the shipping medium.*

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

1. **Sterilize the electrode** (probe) by submerging both tips in 70 % ethanol for 5 minutes.
2. Equilibrate the electrode (probe) for 5 minutes in Caco-2/HT-29 cell culture medium, **pre-warmed at room temperature**.
3. While the electrode is equilibrating, remove the CacoGoblet plate from the incubator and place it in a laminar flow hood. **Allow the plate to reach room temperature** (approximately 20 minutes), as TEER measurements should be performed under this condition.

4. If using chopstick **electrodes** like the STX2 (WPI EVOM series), place the probe into the insert system, so that the thinner electrode is within the narrowest slit, which corresponds to the basal part of the inserts. The thicker electrode must be placed inside the widest slit, corresponding to the apical part of the inserts. Both electrodes have to be **well submerged** within the cell culture medium of the apical and basal compartments **for a correct evaluation**. Be careful **not to touch the cell monolayers**!

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

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

Acceptance Criterion

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

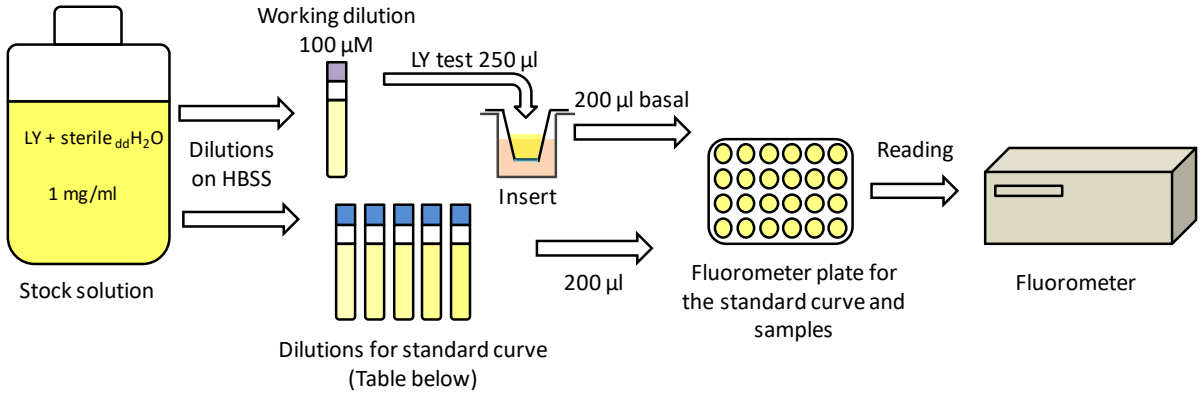
Lucifer Yellow (LY) Paracellular Permeability Assay

Prepare a **1 mg/mL (2.187 mM) LY solution** in sterile dd$H_2$0. Make aliquots and store them at -20 °C.

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

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

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



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

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

Acceptance Criterion

LY Paracellular Flux	*≤ 1,4 %*
LY apparent permeability (Papp)	*≤ 3 x 10-6 cm/s*

General Protocol for Anti-inflammatory screening Assays

General Considerations

CacoGoblet is designed for conducting anti-inflammatory *in vitro* assays of established and investigational compounds in order to predict their capabilities for the mitigation of intestinal inflammation.

Recommended inflammatory inducers and Anti-inflammatory reference comnpound

The compounds listed below (also referenced in the "Solutions" section) are recommended for inducing the inflammatory response as well as a reference compound with known anti-inflammatory activity.

- **Inflammatory inducers:**
  - TNF-α Human (SIGMA Cat# SRP3177)
  - Interferon- Gamma Human (SIGMA Cat# I17001)
  - Interleukin-1β Human (SIGMA Cat# H6291)
  - Lipopolysaccharides from Escherichia Coli (SIGMA Cat# 196322)
- **Recommended anti-inflammatory reference compound**: Baicalein (SIGMA Cat# 196322)

The preparation of the inflammation cocktail, including the recommended concentration and the application compartment, is detailed in table 1.

Inducer	Concentration	Application compartment
Human TNF-α	50 µg/mL	Basal
Human Interferon-γ	50 µg/mL	Basal
Human Interleukin-1β	25 µg/mL	Basal
Lipopolysaccharides	1 µg/mL	Apical and Basal

Table 1. Recommended inflammatory cocktail for induce inflammatory response in CacoGoblet.

Sample Plate Layout

The CacoGoblet 24-well format allows evaluating whether a compound presents anti-inflammatory capabilities. Assay is performed in triplicate following the recommended plate layout shown below.

	1	2	3	4	5	6
A	Untreated	Untreated	Untreated	Comp C2_R1	Comp C2_R2	Comp C2_R3
B	Cytokine-treated	Cytokine-treated	Cytokine-treated	Comp C3_R1	Comp C3_R2	Comp C3_R3
C	Baicalein	Baicalein	Baicalein	Comp C4_R1	Comp C4_R2	Comp C4_R3
D	Comp C1_R1	Comp C1_R2	Comp C1_R3	Comp C5_R1	Comp C5_R2	Comp C5_R3

R: replicate
C: concentration
Comp: compound

Figure 3. Recommended sample plate layout to investigate anti-inflammatory activity.
Wells in light purple represents the control conditions for the assay with untreated cells, cytokine-treated cells and cytokines-treated cells with baicalein as a reference anti-inflammatory compound.
Wells in dark purple represents cytokine-treated cells exposed to increasing concetrations of the test compound.

- Recommended anti-inflammatory reference compound: 100 µM Baicalein
- Replicates: 3
- Time points: 0, 24, 48 and 72 h.
- Volumes: *Apical compartment*: 300 µL
  Basal compartment: 900 µL

NOTE: *The anti-inflammatory activity of the compound are assayed at 5 different concentrations. The procedure should be undertaken in biosafety level II containment standards to ensure sterile conditions. All solutions should be pre-warmed at 37 °C to avoid temperature stress.*

Protocol

The following protocol applies for one CacoGoblet plate.

Anti-inflammatory Studies

After the inflammatory induction with the exposure of the inducers, test compound are applied both the apical side (upper compartment of the insert) and the basal side of the cell monolayer (lower compartment of the insert). Anti-inflammatory activity is evaluated by TEER measures and Lucifer Yellow paracellular permeability at different time points. TEER measures are expressed in Ω x cm^2 and transport through the cell barrier of Lucifer Yellow is determined as the coefficient of apparent permeability (Papp) in cm/s.

Preparation

1. **Prepare stock solutions of the inflammatory inducers, reference and tested compounds** in DMSO. It is recommended to keep the percentage of DMSO in the assay solutions below 1%.
2. **Prepare the pre-treatment solutions of unknowns and reference compound** in Dulbecco's Modified Eagle's Medium - high glucose (4,5 g/L) deprived of Fetal Bovine Serum according.
3. **Prepare the inflammatory inducers cocktail** in Dulbecco's Modified Eagle's Medium - high glucose (4,5 g/L) deprived of Fetal Bovine Serum according to table 1.
4. **Prepare working solutions of unknowns and reference compounds** in Dulbecco's Modified Eagle's Medium - high glucose (4,5 g/L) deprived of Fetal Bovine Serum. **Inflammatory inducers** and **anti-inflammatory compounds** are **mixed simultaneously** in the cell medium.
5. Fill a reagent reservoir with pre-warmed (37 °C) Dulbecco's Modified Eagle's Medium - high glucose (4,5 g/L) deprived of Fetal Bovine Serum.
6. Take the reservoir plate into the laminar flow hood.
7. **Remove** one **CacoGoblet plate from the cell incubator** and place it beside the reservoir plate. Both plates should be oriented the same way.

Serum deprivation

8. Open the CacoGoblet plate and the reservoir plate, and leave the lids upwards next to the plates.
9. Carefully lift the 24 **apical inserts** of the **CacoGoblet plate** and **transfer** them to the **reservoir plate**.
10. Using the 24-well manifold, **aspirate the cell culture medium** from the lower compartments of the CacoGoblet plate.
11. Using a multichannel pipette, **fill**, column by column, each of the 24 wells of the **lower compartments** of the CacoGoblet plate with **900 µL** of pre-warmed (37 °C) **cell culture medium deprived of FBS**.
12. Using the 24-well manifold, aspirate the cell culture medium of the apical inserts of the CacoGoblet plate. Place the manifold perpendicular to the cell monolayer and close to the insert wall to avoid disturbing the cell monolayer.
13. Using a multichannel pipette, **fill**, column by column, each of the 24 apical inserts of the **upper compartment** of the CacoGoblet plate with **300 µL** of pre-warmed (37 °C) **cell culture medium deprived of FBS**.
14. Carefully **return** the 24 **apical inserts** onto the wells of the basal compartment of the CacoGoblet plate (**original position**).
15. Incubate the plate for **24 hours** in the **cell incubator** (37 °C, 5 % CO_2).

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

Pre-treatment with Baicalein and tested compounds

16. **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.
17. Carefully lift the 24 **apical inserts** of the **CacoGoblet plate** and **transfer** them to the **reservoir plate**.
18. Using the 24-well manifold, **aspirate the cell culture medium** from the **lower compartment** of the Cacogoblet plate.

19. Using a pipette, **fill**, the corresponding lower compartments of the CacoGoblet plate with **900 µL** of pre-warmed (37 °C) **FBS deprived cell culture medium with Baicalein or the tested compounds**. Check figure 3 and figure 4 for more details.
20. Using the 24-well manifold, **aspirate** the **cell culture medium** of the **apical inserts** of the CacoGoblet plate. Place the manifold perpendicular to the cell monolayer and close to the insert wall to avoid disturbing the cell monolayer.
21. Add **300 µL of FBS deprived cell culture medium with Baicalein or the tested compounds** to the corresponding **apical inserts** of the CacoGoblet plate. Check figure 3 and figure 4 for more details.
22. Carefully **return** the 24 **apical inserts** onto the wells of the basal compartment of the CacoGoblet plate (**original position**) and **leave** the plate in the **cell incubator** (37 °C, 5 % CO_2) for **3 hours**.

Inflammatory induction

23. **Repeat steps 16 to 20 once** applying the **inflammatory inducers-cocktail** prepared with **FBS deprived cell culture médium**. Check figure 3 and figure 4 for more details.
24. Carefully **return** the 24 **apical inserts** onto the wells of the basal compartment of the CacoGoblet plate (**original position**) and leave the plate in the **cell incubator** (37 °C, 5 % CO_2) up to 72 hours.

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

	FBS Deprivation	Anti-inflammatory Pre-treatment	Cytokine-Induction
Untreated	Yes	No	No
Cytokine-treated	Yes	No	Yes (without anti-inflammatory)
Baicalein	Yes	Yes	Yes (with anti-inflammatory)
Compound	Yes	Yes	Yes (with anti-inflammatory)

Figure 4. Steps performed for each exptimental conditions during the anti-inflammatory screening protocol.

TEER measures and Lucifer Yellow Assay

25. **Take the plate** from the incubator, return it to the laminar flow Hood.
26. Proceed with **TEER measurements** following the instructions detailed in the **Pre-assay Quality control – TEER Measurement** section.
27. Proceed with Lucifer Yellow (LY) assay following the instructions detailed in **the Lucifer Yellow (LY) Paracellular Permeability Assay** section.
28. **Calculate the LY apparent permeability (P_{app})** coefficient as indicated in the following section ("Evaluation of Lucifer Yellow Permeability").

IMPORTANT NOTE: *TEER measurements can be performed in the same well up to 72 hours of incubation. Before performing the LY permeability assay, samples can be collected for quantification of IL-8 pro-inflammatory cytokine release by ELISA. Consider that once the LY permeability assay has been performed, the well will be discarded.*

Evaluation of Lucifer Yellow (LY) 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 cm2) by the initial concentration of test compound (C0 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.

Data for Reference Compound

MedTech Barcelona's internal data of TEER and Lucifer Yellow Apparent Permeability (LY-P_{app}) up to 72h of incubation of cytokine-induced cells in the absence/presence of 100µM baicalein.

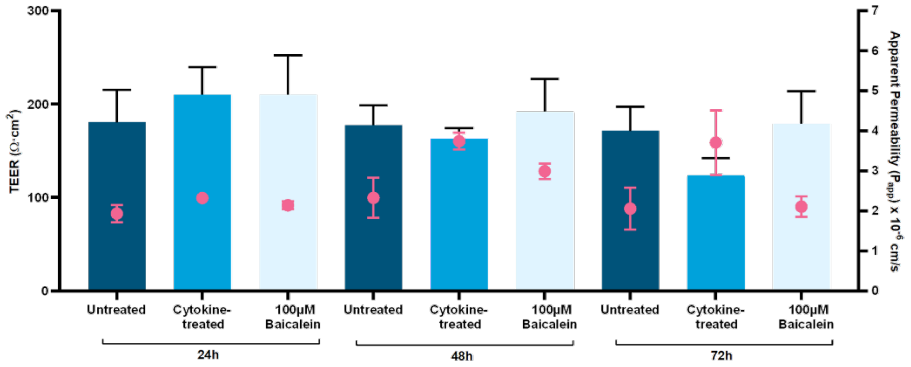


Figure 5. Reference data of TEER (bars) and LY P_{app} (dots) after 72h cytokine treatment ±100 µM baicalein.

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