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Product Information

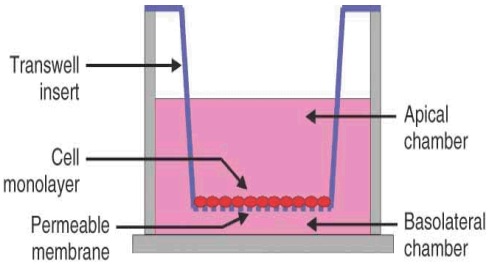
CompoZr® ADME/Tox Cell Lines
Caco-2 Wild Type Cell Line
96 Well Assay Ready Plate

Catalog Number **MTOX1000P96**
Store at Room Temperature

TECHNICAL BULLETIN

Product Description
This product contains Wild Type Caco-2 cells (C2BBe1 sub-clone) that have been differentiated for 14 days in a Millicell Multiwell Insert – 96 Well Assay Ready Plate (see Figure 1). At day 14 the plate is prepared in an exclusive and proprietary shipping medium that is stable at room temperature and allows for up to 4 days of shipping. The plate is shipped overnight for next day delivery.

Figure 1.
Transwell of Millicell Multiwell Insert – 96 Well Assay Ready Plate



Components
This kit contains one Millicell Multiwell Insert – 96 Well Assay Ready Plate:

Wild Type Caco-2 cells (C2BBe1 sub-clone)
(Cat. No. MTOX1000P96)

Cell Line Description
Parental Cell Line: ATCC® Catalog No. CRL-2102™
Note: Please see CRL-2102 product datasheet from ATCC for additional information about the origin of these cell lines. Cytogenetic information is based on initial seed stock at Sigma Life Science. Cytogenetic instability has been reported in the literature for some cell lines.

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Caco-2 Medium: Fetal bovine serum, Catalog No. F4135, at a final concentration of 10% (v/v) in DMEM, Catalog No. D5671, supplemented with L-glutamine, Catalog No. G7513, to a final concentration of 2 mM and penicillin-streptomycin, Catalog No. P4333, at a final concentration of 1% (v/v). This medium is formulated for use with a 5% CO_2 in air atmosphere.

Procedures
Notes: **Receiver tray not included** and must be purchased separately (Catalog Number MACACORS5) for each Assay Ready Plate.

The shipping medium must be changed on the day the plate is received. The unpacking instructions should be followed for each plate that is received.

Unpacking
Upon receipt, open the box and remove the plastic Ziploc® bag containing the Millicell Multiwell Insert – 96 well Assay Ready Plate.

Changing the Shipping Medium
1. On day of receipt, remove the Millicell Multiwell Insert – 96 well Assay Ready Plate (still at room temperature) from the Ziploc bag.
2. Unwrap the Millicell Multiwell Insert – 96 well Assay Ready Plate and carefully pull off the Parafilm®.
3. Place the 96 well Assay Ready Plate in the cell culture incubator for a minimum of 4 hours to allow the transport medium to liquefy.

4. Prepare everything needed to replace the shipping medium with fresh Caco-2 cell culture medium:
   - cell culture biosafety cabinet
   - standard Caco-2 medium, pre-warmed to 37 °C
   - aspiration system
   - 96 well receiver tray (one for each plate received)
   - sterile containers for culture medium
5. Prepare the receiver tray for changing the medium:
   a. Ensure the Caco-2 medium has been warmed to 37 °C
   b. In biosafety cabinet, unwrap one 96 well receiver tray (Catalog Number MACACORS5, must be ordered separately) for each Assay Ready Plate. Place lid next to the plate, facing upwards.
   c. Add 250 µl of warm Caco-2 medium into each basal well.
   d. Place the lid on the plate and place the plate in the incubator.

Notes: Once the shipping medium has liquified, proceed to step 6. The following must be performed using sterile technique in the biosafety cabinet.

Never handle more than one plate at a time while changing the shipping medium.

6. Take one Assay Ready Plate and one receiver tray out of the incubator and place both in biosafety cabinet.
7. In a biosafety cabinet, open the Assay Ready Plate, placing the lid next to the plate, facing upwards. Carefully remove the ThermalSeal® film.
8. Carefully remove the shipping medium from the apical chambers so as to not disturb the cell monolayer.
9. Gently lift the apical (upper) section of the Assay Ready Plate and place it on the new receiver tray.
10. Add 75 µl of fresh Caco-2 medium (warmed to 37 °C) into each apical well of 96 well insert system.
11. Cover the receiver tray with its lid, then put it back into the cell culture incubator.
12. Repeat steps 1–10 for additional plates.

Notes: After the shipping medium has been changed to fresh Caco-2 medium, the plate should be kept in the incubator until day 21. Transwell assays can be performed on days 21–25.

Culture medium should be replaced every 48–72 hours.

TEER Measurement

Read instructions for proper use of the TEER instrument in addition to these instructions.

1. Sterilize the electrodes (probe): submerge both electrodes in 70% ethanol for 30 minutes.
2. Equilibrate the electrodes (probe) for 30 minutes in Caco-2 Medium.
3. Insert the probe in the Transwell system so the shorter electrode is slightly submerged inside the culture medium of the apical well and the longer arm is placed through the lateral hole of the Transwell, so it is submerged in the medium of the basal well.
4. A TEER value of ≥240 ohms·cm^2 is acceptable (see Appendix for representative TEER data).
   Notes: It may be necessary to adjust X,Y coordinates on the TEER instrument for specific tissue culture plates.

Figure 2.
Representative TEER data

Day 14	ohms	(Raw Data)			
6967	7099	7147	7061	6927	7271
6438	6556	6995	6999	7107	7501
6299	7283	7245	7387	7416	7520
6732	7477	7416	7710	7541	7308

Day 14	ohms*cm2		(Calculated Data)		
766	781	786	777	762	800
708	721	769	770	782	825
693	801	797	813	816	827
741	822	816	848	830	804

Day 21	ohms	(Raw Data)			
6844	6699	6895	6934	6684	4577
6480	6534	6704	6663	6624	7011
6356	6728	6782	6896	6807	7163
6459	6919	7231	7397	7178	6766

Day 21	ohms*cm2		(Calculated Data)		
753	737	758	763	735	503
713	719	737	733	729	771
699	740	746	759	749	788
711	761	795	814	790	744

Transwell Assay

This protocol is designed to assess drug transporter functionality in Caco-2 cells. The experiment must include both the genetically modified Caco-2 knockout cells and wild type Caco-2 cells. Transport is measured in both directions (apical-to-basal and basal-to-apical) across the cell monolayer, enabling an efflux ratio to be determined. It is expected the efflux ratio from the knockout cells will be significantly lower than the ratio from wild type cells. In this study, buffer is taken from the receiver compartment after a designated time point. Compound concentrations in the receiver samples are quantified by LC-MS/MS, and the apparent permeability coefficient (P_{app}) and efflux ratio of the compound across the monolayer are calculated.

1. Materials
   - Assay Ready Plates: Caco-2 knockout and wild type plates
   - Caco-2 Medium
   - Buffer B (see Reagent Preparation)
   - Test compound working solution (see Reagent Preparation)
   - Sample analysis equipment (fluorimeter, HPLC-UV/MS, liquid scintillation counter, etc)

2. Reagent Preparation
   Use ultrapure water or equivalent to prepare reagents and in protocol steps.
   - Buffer B - 500 ml HBSS containing:
     12.5 ml of 1 M D-glucose
     10 ml of 1 M HEPES buffer
     1 ml of 625 mM $CaCl_2$
     1 ml of 250 mM $MgCl_2$
     Adjust to pH 7.4
     Store up to 4 weeks at 2–8 °C

   - Test Compound Stock Solution: Dissolve compound at 200× concentration in DMSO and vortex to mix. If necessary, warm or sonicate to dissolve completely. Store up to 6 months at 2–8 °C.

   - Test Compound Working Solution: Dilute Test Compound Stock Solution 200-fold with HBSS to make a working solution with a final DMSO concentration of 0.5% (v/v). Prepare fresh just before use.

3. Perform Transwell Assay
   a. Aspirate medium from the apical and basal wells and replace with Buffer B (75 µl in the apical wells and 250 µl in the basal wells). Incubate at 37 °C for 15 minutes.
   b. Aspirate all of Buffer B. Depending on the study design, add Test Compound Working Solution to the apical (75 µl) or basal (250 µl) wells, and add Buffer B to the other (basal or apical) wells. Incubate at 37 °C for 2 hours.
   c. Take 50 µl samples from the appropriate wells, depending on the direction of transport (i.e., from the basal well for A-to-B transport or the apical well for B-to-A transport).
   d. Analyze samples.
   e. Following quantitation of test compound, proceed to determination of (P_{app}) value and efflux ratio.

4. Determine P_{app} value and efflux ratio
   a. Calculate the permeability coefficient as follows;

$$P_{app} = \frac{1}{A \times C_o} \times \frac{dM_r}{dt}$$

   A = area (cm^2)
   C_o = mass of compound initially in the donor compartment
   dM_r/dt = the rate of drug permeation across the cells

   b. Calculate the efflux ratio (ER) as the ratio of P_{app} determined in the A-to-B direction to P_{app} determined in the B-to-A direction:

$$ER = P_{app,\ B\text{-}to\text{-}A}/P_{app,\ A\text{-}to\text{-}B}$$

Measurement of Cell Monolayer Integrity using Lucifer Yellow

Evaluation of permeability characteristics of Caco-2 cells can be performed by measuring passive passage of different molecules across the monolayer. Small hydrophilic compounds cross the monolayer mainly via the paracellular space, such as through the tight junctions, and can be considered markers of passage by this route. Lucifer Yellow is one such marker that is easily detectable. It is used to check the barrier integrity and to determine whether the working concentration of a test compound disturbs the integrity of the monolayer. In this protocol, the Lucifer Yellow assay is performed after the Transwell assay.

1. Materials
   - Transwell assay plates
   - Buffer B
   - 0.1 mg/ml Lucifer Yellow Solution - (Lucifer Yellow CH dipotassium salt, Catalog No. L0144) in Buffer B
   - 96 well plate
   - Fluorescence multiwell plate reader

2. Perform Lucifer Yellow Assay
   a. After removing samples for sample analysis, aspirate the remaining liquid from the apical and basal wells.
   b. Add 75 µl of 0.1 mg/ml Lucifer Yellow Solution to the apical wells and 250 µl of Buffer B to the basal wells.
   c. Incubate at 37 °C for 60 minutes.
   d. Transfer 150 µl from the basal wells to a 96 well plate and read in a spectrofluorometer with excitation at 485 nm and emission at 535 nm. Also measure fluorescence for Buffer B (blank) and 0.1 mg/ml Lucifer Yellow Solution.

3. Calculate the percent permeability from the fluorescence values as follows:

$$\% \text{ permeability} = \frac{\text{sample} - \text{blank}}{\text{Lucifer Yellow} - \text{blank}} \times 100$$

A permeability of <3% is acceptable.

Figure 3.
Representative Lucifer Yellow data.

0.05	0.06	0.05	7.26	0.18	6.34	Day 21
0.05	0.05	0.07	1.49	0.05	0.04	
0.07	0.04	0.04	2.70	0.08	0.04	
0.12	0.16	0.18	3.84	0.38	0.32	
0.05	0.05	0.04	0.08	0.05	0.06	
0.06	0.05	0.05	4.78	0.16	0.08	
0.07	0.04	0.06	0.05	0.08	0.05	
0.07	0.03	0.06	0.07	0.04	0.05	
0.15	0.10	0.14	0.18	1.39	0.27	Day 23
0.15	0.10	0.13	0.13	0.16	0.18	
0.14	0.11	0.12	0.12	0.14	0.18	
0.21	0.17	0.14	0.16	0.18	0.38	
0.17	0.13	0.15	0.12	0.21	0.22	
0.16	0.21	0.21	0.17	0.21	0.37	
0.44	0.15	0.15	0.14	0.13	0.11	
0.16	0.15	0.19	0.14	0.11	0.14	
0.08	0.05	40.23	0.68	0.12	0.05	Day 25
0.09	0.08	0.08	0.08	0.07	0.08	
0.11	0.08	0.14	0.11	0.13	0.07	
0.21	0.12	0.18	0.23	0.46	0.26	
0.07	0.06	0.08	0.07	0.10	0.24	
0.11	0.13	0.08	0.20	0.20	1.44	
0.11	0.04	0.07	0.06	0.87	0.73	
0.12	0.08	0.03	0.06	0.17	0.05	

References

1. Peterson, M.D., and Mooseker, M.S., Characterization of the enterocyte-like brush border cytoskeleton of the C2BB3 clones of the human intestinal cell line, Caco-2. J. Cell Sci., **102**, 581-600 (1992).
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4. Chen, W. et al. in Cell Culture Models of Biological Barriers In-Vitro Test Systems for Drug Absorptoin and Delivery. (Lehr, C-M., ed.), Taylor & Francis, (New York, NY: 2002) pp. 143-163.

Additional product and technical information can be obtained by searching for the catalog number at the following web page (www.sigma.com).

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