

Product Information

CompoZr® ADME/Tox Cell Lines Caco-2 MDR1/MRP2 Double Knockout Cell Line 96 Well Assay Ready Plate

Catalog Number **MTOX1005P96**
Store at Room Temperature

TECHNICAL BULLETIN

Product Description

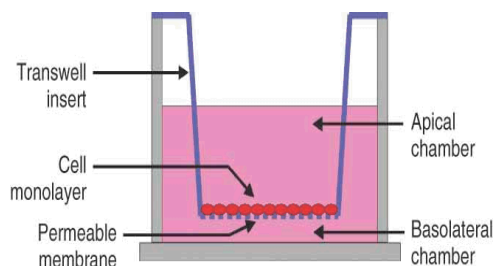
CompoZr® zinc finger nuclease (ZFN) technology is a fast and reliable way to manipulate the genome in a targeted fashion. ZFNs are synthetic proteins engineered to bind DNA at a sequence-specific location and create a double strand break (www.sigma.com/zfn). The cell's natural machinery repairs the break in one of the two ways: non-homologous end joining or homologous recombination. The non-homologous end joining pathway resulted in frame-shift modifications at the desired locus. Single cell knockout clones were isolated and followed for more than twenty passages to establish stable cell lines.

ZFN-mediated gene knockout technology is not limited to diploid targets, allowing the researcher to pursue many of the polyploid cell lines often characteristic of cancer. The colon adenocarcinoma cell line Caco-2 presents unique challenges to knockout technology as this cell line is tetraploid for several targeted genes.¹ Modified cell lines provide the basis for the development of various assays for compound screening. Here, the target gene and corresponding transporter function are eliminated, in contrast to cell lines with normal expression.²

ATP-binding cassette (ABC) transporters are a family of transmembrane proteins that utilize ATP hydrolysis for translocation of substrates across membranes. ABC transporters are known to play a critical role in the development of multidrug resistance. Evaluation of membrane transporter pharmacology in drug disposition and drug-drug interactions (DDI) is critical to the pharmaceutical safety evaluations of new drug entities. Selection of the targeted gene(s) was based on the considerable body of evidence supporting its crucial role in the development of multidrug resistance.³

This product contains MDR1/MRP2 Double Knockout (KO) 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:

MDR1/MRP2 Double Knockout Caco-2 cells
(C2BBE1 sub-clone)
(Cat. No. MTOX1005P96)

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% CO₂ 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 in 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 \cdot cm² 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 CaCl₂
 - 1 ml of 250 mM MgCl₂
 Adjust to pH 7.4
Store up to 4 weeks at 2–8 °C
- Test Compound Stock Solution: Dissolve compound at 200 \times 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 μ l in the apical wells and 250 μ l 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 μ l) or basal (250 μ l) wells, and add Buffer B to the other (basal or apical) wells. Incubate at 37 °C for 2 hours.
 - c. Take 50 μ l 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 (cm²)

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:

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

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 μ l of 0.1 mg/ml Lucifer Yellow Solution to the apical wells and 250 μ l of Buffer B to the basal wells.
 - c. Incubate at 37 °C for 60 minutes.
 - d. Transfer 150 μ l 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).
2. Pratt, J. et al. Use of Zinc Finger Nuclease Technology to Knock Out Efflux Transporters in C2BBe1 Cells. *Current Protocols in Toxicology*, 23.2.1-23.2.22, May (2012).
3. The International Transporter Consortium (2010 White Paper), Membrane transporters in drug development. *Nature Reviews Drug Discovery*, **9**, 215-236 (2010).
4. Chen, W. et al. in *Cell Culture Models of Biological Barriers In-Vitro Test Systems for Drug Absorption 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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