3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

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

Catalog Number **MTOX1005P24**
Store at Room Temperature

TECHNICAL BULLETIN

Product Description

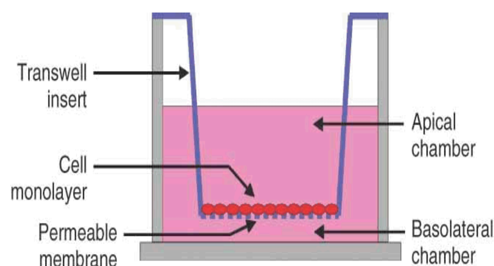
CompoZr® zinc finger nuclease (ZFN) technology is a fast and reliable way to manipulate the genome in a targeted fashion. ZFNs are synthetic proteins engineered to bind DNA at a sequence-specific location and create a double strand break (www.sigma.com/zfn). The cell's natural machinery repairs the break 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 – 24 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 – 24 Well Assay Ready Plate



Components

This kit contains one Millicell Multiwell Insert – 24 Well Assay Ready Plate:

MDR1/MRP2 Double Knockout Caco-2 cells
(C2BBe1 sub-clone)
(Cat. No.MTOX1005P24)

Cell Line Description

Parental Cell Line: ATCC® Cat. 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 SPMW010R5) 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 – 24 well Assay Ready Plate.

Changing the Shipping Medium
1. On day of receipt, remove the Millicell Multiwell Insert – 24 well Assay Ready Plate (still at room temperature) from the Ziploc bag.
2. Unwrap the Millicell Multiwell Insert – 24 well Assay Ready Plate and carefully pull off the Parafilm®.
3. Place the 24 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
   - 24 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 24 well receiver tray (Catalog Number PSMW010R5, must be ordered separately) for each Assay Ready Plate. Place lid next to the plate, facing upwards.
   c. Add 800 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 400 μl of fresh Caco-2 medium (warmed to 37 °C) into each apical well of 24 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. Note: 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)					
1215	1203	1198	1167	1052	1135
1258	1256	1192	1163	1121	1153
1252	1193	1211	1221	1130	1228
1418	1228	1227	1164	1172	1285
Day 14 Ohms*cm2 (Calculated Data)					
851	842	839	817	736	794
881	879	834	814	785	807
876	835	848	855	791	859
993	859	859	815	820	900
Day 21 Ohms (Raw Data)					
918	943	922	915	746	724
872	882	807	771	705	711
867	827	754	557	802	829
763	678	611	730	620	958
Day 21 Ohms*cm2 (Calculated Data)					
643	660	646	641	522	507
610	618	565	539	494	497
607	579	528	390	561	580
534	475	427	511	434	671

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 of 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 (500 µl in the apical wells and 1,000 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 (500 µl) or basal (1,000 µl) wells, and add Buffer B to the other (basal or apical) wells. Incubate at 37 °C for 2 hours.
    c. Take 250 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:

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

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, Cat. 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 500 µl of 0.1 mg/ml Lucifer Yellow Solution to the apical wells and 1,000 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

0.037	0.017	0.013	0.009	0.014	0.056	Day 21
0.017	0.009	0.012	0.024	0.011	4.204	
0.009	0.016	0.008	0.009	0.018	0.012	
0.201	0.154	0.158	1.020	1.092	0.996	
0.021	0.023	0.029	0.027	0.029	0.027	Day 23
0.039	0.021	0.018	0.021	0.030	0.037	
0.016	0.038	0.046	0.029	0.016	0.036	
0.130	0.139	0.110	1.345	1.377	1.394	
0.238	0.094	0.074	0.094	0.106	0.183	Day 25
0.056	0.055	0.043	0.050	0.066	0.110	
0.250	0.117	0.096	0.058	0.044	0.066	
0.099	0.189	0.089	0.961	0.847	0.789	

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 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).

Please see the Label License Agreement (LLA) for further details regarding the use of this product, the LLA is available on our website at (www.sigma.com).

These cells are distributed for research purposes only. Sigma Life Science requires that individuals contemplating commercial use of any cell line first contact us to negotiate an agreement. Third party distribution of this cell line is prohibited.

CompoZr is a registered trademark of Sigma-Aldrich Co. LLC.
ATCC is a registered trademark of American Type Culture Collection.
CRL-2102 is a trademark of American Type Culture Collection.
Parafilm is a registered trademark of Bemis Company, Inc.
Ziploc is a registered trademark of S.C. Johnson & Son, Inc.
ThermalSeal Film is a registered trademark of Excel Scientific, Inc.

MD,KJ,MAM 05/15-1

©2015 Sigma-Aldrich Co. LLC. All rights reserved. SIGMA-ALDRICH is a trademark of Sigma-Aldrich Co. LLC, registered i the US and other countries. Sigma brand products are sold through Sigma-Aldrich, Inc. Purchaser must determine the suitability of the product(s) for their particular use. Additional terms and conditions may apply. Please see product information on the Sigma-Aldrich website at www.sigmaaldrich.com and/or on the reverse side of the invoice or packing slip.