

Product Information

CompoZr® ADME/Tox Cell Lines C2BBE1 MRP4 Knockout Cell Line 24 Well Assay Ready Plate

Catalog Number **MTOX1094P24**
Store at Room Temperature

TECHNICAL BULLETIN

Product Description

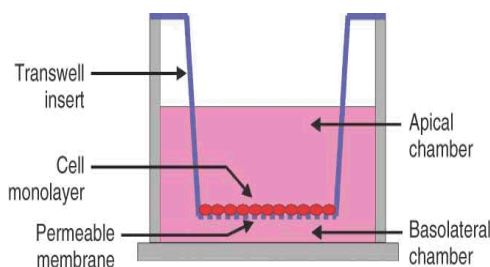
CompoZr® zinc finger nuclease (ZFN) technology is a fast and reliable way to manipulate the genome in a targeted fashion. ZFNs are synthetic proteins engineered to bind DNA at a sequence-specific location and create a double strand break (www.sigma.com/zfn). The cell's natural machinery repairs the break in one of two ways: non-homologous end joining or homologous recombination. The non-homologous end joining pathway resulted in modifications at the desired locus (see Appendix). 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 C2BBE1 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 MRP4 knockout (KO) C2BBE1 cells that have been differentiated for 14 days in a HTS Multiwell Insert – 24 Well Assay Ready Plate (see Figure 1). At day 14 an exclusive and proprietary shipping medium that is stable at room temperature is added to the cells to allow for up to 4 days of shipping.

Figure 1.
Transwell of HTS Multiwell Insert – 24 Well Assay Ready Plate



Components

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

MRP4 knockout C2BBE1 cells
(Cat. No.MTOX1094P24)

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 Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

CACO-2 Medium: Fetal bovine serum, Cat. No. F4135, at a final concentration of 20% (v/v) in DMEM, Cat. No. D5671, supplemented with L-glutamine, Cat. No. G7513, to a final concentration of 2 mM. This medium is formulated for use with a 5% CO₂ in air atmosphere.

Procedures

Unpacking

Note: The shipping medium must be changed on Friday of the week the plate is received. The unpacking instructions should be followed for each plate that is received.

Upon receipt, open the box and remove the plastic Ziploc[®] bag containing the HTS Multiwell Insert – 24 well Assay Ready plate. Leave the Ziploc bag open. The plastic Ziploc bag containing the HTS Multiwell Insert – 24 well Assay Ready plate should be kept at room temperature (15–25 °C) until Friday of the week it is received.

Changing the Shipping Medium

1. On Friday, remove the HTS Multiwell Insert – 24 well Assay Ready plate (still at room temperature).
2. Unwrap the HTS Multiwell Insert – 24 well Assay Ready plate and carefully pull off the Parafilm[®].
3. Prepare everything needed to replace the shipping medium with fresh CACO-2 cell culture medium:
 - a. cell culture biosafety cabinet
 - b. standard CACO-2 medium, pre-warmed to 37 °C
 - c. aspiration system
 - d. standard 24 well plate (one for each plate received)
 - e. sterile containers for culture medium
4. Place the HTS Multiwell Insert – 24 well Assay Ready plate in the cell culture incubator for a minimum of 4 hours to allow the transport medium to liquefy.

5. Prepare the basal plate for changing the medium:
 - a. Ensure CACO-2 medium has been warmed to 37 °C.
 - b. In the biosafety cabinet, unwrap one 24 well basal plate for each Assay Ready plate. Open the plate and place the lid by the plate, facing upwards.
 - c. Add 1,000 µl of warm (37 °C) CACO-2 medium into basal well of new 24 well plate.
 - d. Put the lid on the plate, and place the plate in the incubator.
 - e. Maintain the CACO-2 medium at 37 °C by placing it back in the 37 °C bath.

Notes: Once the shipping medium has liquefied, replace it with fresh CACO-2 culture medium following steps 6–11, which must be performed using sterile technique in the biosafety cabinet.

Never handle more than one plate at a time while changing the shipping medium. Resolidification of the shipping medium could cause mechanical damage to the cellular monolayer.

6. Take one Assay Ready plate and one basal plate out of the incubator and place them both in the biosafety cabinet.
7. Open the Assay Ready plate and the basal plate, placing the lids next to the plates, facing upwards.
8. Gently lift up the apical (upper) section of the Assay Ready plate and place it on the basal plate.
9. Remove all liquefied shipping medium from apical (upper) section and replace with 500 µl of fresh CACO-2 medium.
10. Cover the Assay Ready plate with its lid, then put back into the cell culture incubator.
11. Repeat steps 6–11 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 Monday (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 >1,000 ohms 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

1763	2011	2268	2289	1964	1654	Day 21
2253	2209	2380	2418	2169	2128	
2341	2478	2407	2362	2504	2066	
2473	2352	2069	2607	2049	2367	
2071	1961	1645	2053	1805	1281	Day 23
1842	2098	2051	1912	2078	1247	
2083	2087	2087	2042	2080	1339	
1924	1996	1802	2144	1957	1588	
1355	1451	1474	1503	1430	1259	Day 25
1452	1619	1703	1657	1645	1340	
1506	1592	1763	1779	1635	1446	
1845	1545	1539	1538	1473	1207	

Transwell Assay

This protocol is designed to assess drug transporter functionality in C2BBE1 cells. The experiment must include both the genetically modified C2BBE1 knockout cells and wild type C2BBE1 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: C2BBE1 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

- Aspirate medium from the apical and basal wells and replace with Buffer B (500 μ l in the apical wells and 1,000 μ l in the basal wells). Incubate at 37 °C for 15 minutes.
 - Aspirate all of Buffer B. Depending on the study design, add Test Compound Working Solution to the apical (500 μ l) or basal (1,000 μ l) wells, and add Buffer B to the other (basal or apical) wells. Incubate at 37 °C for 2 hours.
 - Take 250 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).
 - Analyze samples.
 - Following quantitation of test compound, proceed to determination of (P_{app}) value and efflux ratio.
4. Determine P_{app} value and efflux ratio
- 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

- 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 C2BBel 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.

- 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
- Perform Lucifer Yellow Assay
 - After removing samples for sample analysis, aspirate the remaining liquid from the apical and basal wells.
 - Add 500 μ l of 0.1 mg/ml Lucifer Yellow Solution to the apical wells and 1,000 μ l of Buffer B to the basal wells.
 - Incubate at 37 °C for 60 minutes.
 - 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.
- 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 C2BB1 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).

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).

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Appendix

Genotypic data on the Transporter KO C2BBE1 cell line – The transporter gene knockout in the C2BBE1 Cells is a result of ZFN derived nucleotide insertions and/or deletions. The ZFN binding site is in capital letters and the ZFN cut site is in lower case letters. Nucleotide deletions are represented as dashes and nucleotide insertions are in red font.

MTOX1094P24 – MRP4 Transporter Knockout

Wild Type: 5' -ACGTTTCAGTGTgctggGATGA-3'

Allele 1: 5' -ACGTTTCAGTGTgctgg--TGA-3' (2 bp deletion)

Allele 2: 5' -ACGTTTCAGTGTGCTGgctggGATGA-3' (4 bp insertion)

Allele 3: 5' -ACGTTTCAGTGTGCTGgctggGATGA-3' (4 bp insertion)

Allele 4: 5' -ACGTTTCAGTGTgCTGGGctggGATGA-3' (5 bp insertion)