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Product Information

CompoZr® ADME/Tox Cell Lines
C2BBe1 MDR1/MRP2 Double Knockout and Wild Type Cell Lines
96 Well Assay Ready Plates

Catalog Number **MTOX1005PC96**
Store at Room Temperature

TECHNICAL BULLETIN

Product Description

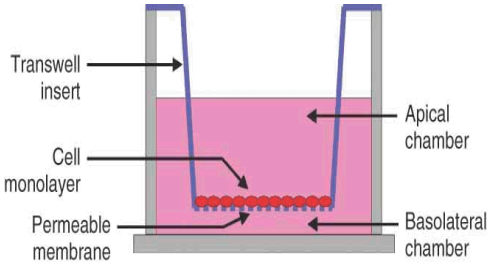
CompoZr® zinc finger nuclease (ZFN) technology is a fast and reliable way to manipulate the genome in a targeted fashion. ZFNs are synthetic proteins engineered to bind DNA at a sequence-specific location and create a double strand break (www.sigma.com/zfn). The cell's natural machinery repairs the break in one of two ways: non-homologous end joining or homologous recombination. The non-homologous end joining pathway resulted in modifications at the desired loci (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 genes 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]

The kit contains MDR1/MRP2 double knockout (KO) and wild type C2BBe1 cells that have been differentiated for 14 days in HTS Multiwell Insert – 96 Well Assay Ready Plates (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 – 96 Well Assay Ready Plate



Components

Each kit is a set of 2 HTS Multiwell Insert – 96 Well Assay Ready Plates:

One plate of MDR1/MRP2 double knockout C2BBe1 cells (Cat. No. MTOX1005P96)

One plate of wild type C2BBe1 cells (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 Material 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
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 plates. 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 of the week the plates were received, remove the HTS Multiwell Insert – 96 well Assay Ready plates (still at room temperature) from the zip lock bags.
2. Unwrap the HTS Multiwell Insert – 96 well Assay Ready plates and carefully remove the Parafilm®.
3. Prepare everything needed to replace the shipping medium with fresh CACO-2 Medium:
   a. cell culture biosafety cabinet
   b. CACO-2 Medium, pre-warmed to 37 °C
   c. aspiration system
   d. standard basal 96 well plates (one for each Assay Ready plate)
   e. sterile containers for culture medium
4. Place the HTS Multiwell Insert – 24 well Assay Ready plates in the cell culture incubator for a minimum of 4 hours to allow the transport medium to liquefy.
5. Prepare the basal plates for changing the medium:
   a. Warm CACO-2 Medium to 37 °C.
   b. In the biosafety cabinet, unwrap one 96 well basal plate for each Assay Ready plate. Open the plates and place the lids by the plates, facing upwards.
   c. Add 250 µl of warm (37 °C) CACO-2 Medium into basal well of new 96 well plate.
   d. Put the lid on each plate and place the plates in the incubator.
   e. Re-warm the CACO-2 Medium by placing it back in the 37 °C bath.

   Notes: Once the shipping medium has softened, replace it with fresh CACO-2 Medium following steps 6–10, 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 cell monolayers.

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 50–75 µl of fresh CACO-2 media.
10. Cover the Assay Ready plate with its lid, then put back into the cell culture incubator.
11. Repeat steps 6–10 for each plate.

    Notes: After the shipping medium has been changed to fresh CACO-2 Medium, the plates 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 with CACO-2 Medium.

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 (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.

   See Appendix for representative TEER data.

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
   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_0 = 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 C2BBe1 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.

   See Appendix for representative Lucifer Yellow data.

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 from the catalog references and the Sigma Life Science Website (www.sigma.com/transporterko).

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.

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Appendix

Genotypic data on the Transporter KO C2BBe1 cell line – The transporter gene knockouts in the C2BBe1 Cells are 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.

MTOX1005P96 – MDR1/MRP2 Transporter Knockout

MDR1 Genotype

Wild Type: 5′-GTCCTGTTCTTGGACtgtcaGCTGCTGTCTGGGCAAAG-3′
Allele 1: 5′-GTCCTGTTCTTGGAC-----GCTGCTGTCTGGGCAAAG-3′ (5 bp deletion)
Allele 2: 5′-GTCCTGTTCTTGGAC-----GCTGCTGTCTGGGCAAAG-3′ (5 bp deletion)
Allele 3: 5′-GTCCTGTTCTTGGA-------CTGCTGTCTGGGCAAAG-3′ (7 bp deletion)
Allele 4: 5′-GTCCTGTTCTTGGA-------CTGCTGTCTGGGCAAAG-3′ (7 bp deletion)

MRP2 Genotype

Wild Type: 5′-GTCTCCCTAGTCCATGATggcagtGAAGAAGAAGACGATGAC-3′
Allele 1: 5′-GTCTCCCTA---------ggcagtGAAGAAGAAGACGATGAC-3′ (9 bp deletion)
Allele 2: 5′-GTCTCCCTA---------ggcagtGAAGAAGAAGACGATGAC-3′ (9 bp deletion)
Allele 3: 5′-GTC------------------------------------GAC-3′ (36 bp deletion)
Allele 4: 5′-GTC------------------------------------GAC-3′ (36 bp deletion)

Representative TEER data

8986	9087	8875	8752	8961	8823	8831	8429	8250	8419	8792	9243	**Day 21**
9100	8315	8105	8087	8121	8113	7917	7906	7823	7663	8286	8863	
8790	8376	8215	8145	8291	8312	8236	7897	8111	7861	8289	9076	
8783	8222	8081	8015	7795	8043	7938	7813	7757	7897	7928	8732	
8755	8162	8254	7975	8020	7995	8171	8071	8117	7911	8203	9507	
8840	8521	8386	8384	8738	8747	8520	8426	8488	8441	8271	9138	
8933	8593	8656	8754	8794	8677	8668	8623	8833	8842	8523	9357	
10116	9186	9175	9312	9416	9434	9415	9281	9430	9468	9525	9669	
8792	9280	8809	9009	8754	8599	8516	8419	8447	8644	8648	9110	**Day 23**
9130	8314	8040	8249	7928	7926	7897	7885	7793	7585	7654	8256	
8821	8863	8386	8755	8301	8336	8221	8090	8077	7790	7630	9328	
9081	8447	8610	8436	8523	8164	8256	8255	8132	7752	7582	8212	
8751	8273	8312	8105	7798	7645	7937	8052	8038	7954	7816	8657	
8875	8387	8450	8483	7930	8097	8076	8352	8095	7890	7717	8229	
8756	7731	8532	8511	8507	8134	8291	7980	8339	8132	8357	8629	
9296	9014	8630	8588	8975	8618	8920	8848	9020	8831	8622	8624	
8773	8496	8304	7687	8110	7995	8063	7589	7551	7658	7800	8180	**Day 25**
8335	6885	6842	6890	7007	6912	6965	6773	6604	6476	6757	7569	
7768	7095	7083	6653	6583	6514	6721	6724	6497	6705	6570	7852	
7728	7049	6832	6815	6543	6625	6606	6628	6376	6561	6544	7377	
7853	7021	6911	6867	7119	6769	6783	6740	6867	6397	6717	7506	
7649	6238	7265	7221	7133	6813	6995	6772	6534	6505	6813	7757	
7986	6593	7037	7027	6785	6546	6916	6662	6194	6822	6834	7824	
8085	7421	7791	7609	7888	7983	7847	7367	7688	7531	7974	10125	

Representative Lucifer Yellow Data

1.14	0.07	0.07	0.04	0.04	0.04	0.05	0.06	0.05	7.26	0.18	6.34	**Day 21**
0.10	0.05	0.04	0.06	0.05	0.06	0.05	0.05	0.07	1.49	0.05	0.04	
0.07	0.04	0.06	0.03	0.03	0.06	0.07	0.04	0.04	2.70	0.08	0.04	
0.06	0.04	0.04	0.04	0.04	0.12	0.12	0.16	0.18	3.84	0.38	0.32	
0.06	0.04	0.05	0.05	0.04	0.08	0.05	0.05	0.04	0.08	0.05	0.06	
0.05	0.05	0.06	0.06	0.05	1.17	0.06	0.05	0.05	4.78	0.16	0.08	
0.05	0.04	0.05	0.05	0.06	0.54	0.07	0.04	0.06	0.05	0.08	0.05	
0.05	0.03	0.05	0.05	0.03	0.80	0.07	0.03	0.06	0.07	0.04	0.05	
0.11	0.11	0.09	0.15	0.13	0.12	0.15	0.10	0.14	0.18	1.39	0.27	**Day 23**
0.13	0.13	0.09	0.13	0.11	0.11	0.15	0.10	0.13	0.13	0.16	0.18	
0.13	3.27	0.14	0.15	0.12	0.12	0.14	0.11	0.12	0.12	0.14	0.18	
0.18	1.50	0.20	0.19	0.20	0.14	0.21	0.17	0.14	0.16	0.18	0.38	
0.09	0.10	0.09	0.13	0.13	0.11	0.17	0.13	0.15	0.12	0.21	0.22	
0.11	5.41	3.40	0.23	0.13	0.11	0.16	0.21	0.21	0.17	0.21	0.37	
0.11	1.42	3.86	0.24	0.11	0.11	0.44	0.15	0.15	0.14	0.13	0.11	
0.13	1.63	1.98	0.23	0.11	0.12	0.16	0.15	0.19	0.14	0.11	0.14	
0.05	0.05	0.05	0.03	0.03	0.03	0.08	0.05	40.23	0.68	0.12	0.05	**Day 25**
0.04	0.05	0.03	0.03	0.05	0.03	0.09	0.08	0.08	0.08	0.07	0.08	
0.04	0.05	0.03	0.03	0.03	0.03	0.11	0.08	0.14	0.11	0.13	0.07	
0.05	0.08	0.09	0.09	0.11	0.11	0.21	0.12	0.18	0.23	0.46	0.26	
0.04	0.04	0.02	0.04	0.03	0.02	0.07	0.06	0.08	0.07	0.10	0.24	
0.01	0.03	0.05	0.03	0.03	0.03	0.11	0.13	0.08	0.20	0.20	1.44	
0.03	0.03	0.03	0.02	0.02	0.03	0.11	0.04	0.07	0.06	0.87	0.73	
0.04	0.14	0.57	0.06	0.04	0.06	0.12	0.08	0.03	0.06	0.17	0.05	