Instant Use of Assay Ready Frozen Caco2 Cells in Permeability Assays

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introduction

Human epithelial cell line Caco2, isolated from a colorectal adenocarcinoma, is the most favored model to investigate epithelial permeability and drug uptake in the intestine. After full differentiation in vitro, the cells form tight junctions and express enzymes and transporter proteins that are characteristic for enterocytes of the small intestine. To measure permeability, Caco2 cells are seeded into trans-well chambers and cultivated for a period of up to three weeks for differentiation and the formation of a closed cell layer. Tight junctions in-between the cells prevent the passage of substances though the intercellular space. Everything needs to be taken up by the cells at the apical side and released again at the basolateral side to pass the cell layer. Tissue specific membrane transporters are expressed in Caco2 cells which either promote or impair the passage of a substance through the cell layer.

When resuscitated from a cryostock, Caco2 cells usually must be cultivated for several passages until they have fully recovered and can be used in a cell based assay. Gentle passaging and optimal culture conditions are key to obtain reproducible results in permeability assays. Debris which Caco2 cells tend to segregate when detached and cryopreserved can obstruct the formation of proper tight junctions. Here we demonstrate the use of assay ready Caco2 cells, which have been prepared and cryopreserved according to an optimized protocol.

preparation of assay ready frozen cells

To prepare Caco2 cells of high quality is critical for the successful performance of a permeability assay. In particular, the level of debris needs to be maintained low because small particles can intercalate in-between the cells and interfere with the formation of a tight cell layer. Unfortunately, cultured Caco2 cells tend to produce elevated levels of debris when not handled properly. For the bulk expansion of Caco2 cells in CellSTACKs (Corning) a carefully controlled process has been developed which limits the time between passages and the manipulating time during passaging. This process results in an overall increase in viability and a consistently low amount of debris during expansion. Caco2 (CLS Cell Lines Service) cells were grown MEMα with 20% FBS and 1% NEAA. At 80% confluence the cells were harvested enzymatically using Accutase and resuspended in a serum free medium formulation containing 5% DMSO for cryoprotection. The cells were automatically dispensed into cryovials at 10 million cells per vial using a XSD-Biofill (FuidX) and frozen in a Cryomed 7452 (Thermo Fisher) at a controlled cooling rate of 1°C per minute. Cryopreserved cells were stored in vapor phase of liquid nitrogen .

Fig. 1: Use of Assay Ready Cells

During cell expansion for quality control of assay ready cells the cells were analyzed in a CASYY TT cell counter, which not only provides information about cell count and viability but also about the grade of cell aggregation and the amount of small particles (debris). With the optimized expansion process the debris can be maintained below 40% over the course of the expansion. The optimized cryopreservation protocol provide assay ready cells which are characterized by a high viability, neglectable lag-phase and low debris after resuscitation of the frozen cells (Fig 2).

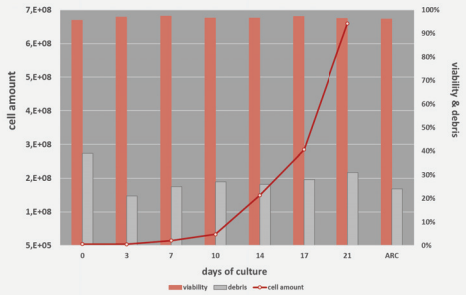
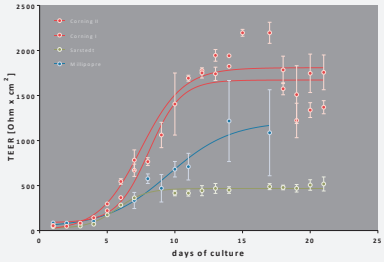


Fig. 2: Expansion of Caco2 cells
Caco2 cells have been expanded in flasks and CellSTACK under careful control of viability and debris to prepare a batch of assay ready cryopreserved cells with high viability (> 95%), high proliferative capacity (>80%) and low level of debris (< 40%).

permeability assay

A vial of assay ready cryopreserved Caco-2 cells were thawed, washed, resuspended in Caco-2 assay medium and directly dispensed into a 24-transwell plate at 100.000 cells/cm² without any prior cultivation or passaging. Over a course of three weeks the cells were cultivated in the trans-wells while the medium was renewed every two days. The permeability of the Caco-2 cell layer was determined every day by measuring the trans epithelial electric resistance TEER (Fig. 3).



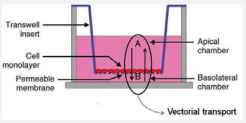
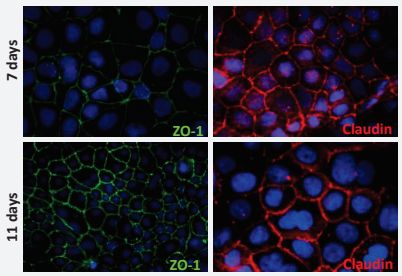


Fig. 3: Permeability assay with assay ready Caco2 cells
Assay Ready Caco2 Cells were instantly seeded instantly into 24-trans-well plates (PCF, 0.4 µM] from three different vendors without prior cultivation or passaging. While cultivated over a course of three weeks TERR values was determined every day.

In cells from a parallel culture the expression of tight junction protein ZO-1 and Claudin 4 were detected by immunofluorescence microcopy (Fig. 4).

Fig. 4: Expression of tight junction proteins
Assay Ready Caco2 Cells were instantly seeded into multi-well plates. After 7 and 11 days of culture the cell were fixed with 4 % paraformaldehyde. Tight junction proteins Claudin 4 & ZO-1 were detected by immunofluorescence labeling using Alexa Fluor 488 (green) and Alexa Fluor 594 (red) coupled monoclonal antibodies (Thermo Fisher Scientific).



In all systems assay ready Caco2 cells from an electrically tight cell layer already after 10 days of culture. The highest TERR values of 1.800 Ω*cm² were observed in trans-well plates from Corning but also in respective products form Sartorius or Millipore sufficiently high values of greater than 500 Ω*cm² could be obtained. Tight junction protein ZO-1 and Claudin 4 were detectable after 7 days of culture and displayed the typical meshwork appearance already after 11 days.

Calcein-AM uptake in Caco2 subclones

Subclones of Caco2 cells were isolated by classical ring cloning and analyzed for their capability to accumulate Calcein-AM within the cells. Permeable Calcein-AM which is converted into a florescent impermeable dye is a substrate of pgp multi-drug-resistance exporter and will accumulate in pgp negative clones only.

In subclone F9 (Fig.5 left) no Calcein florescence could be detected within the cells by FACS analysis (green histogram). After treatment of the cells with Verapamil, a potent inhibitor of pgp, a strong accumulation (red histograms) of the fluorescence dye could be observed, suggesting an expression of pgp in subclone F9. In subclone D5 (Fig. 5 right), a strong Calcein fluorescence could be detected even without a treatment of Verapamil, suggesting the absence of active pgp.

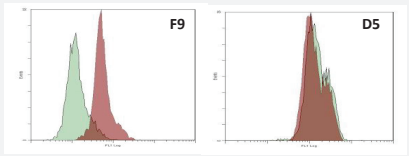


Fig. 5: Accumulation of Calcein-AM
Caco2 subclones F9 and D5 were incubated with Calcein-AM in the absence (green) or presence (red) of Verapamil (100 µM). Accumulation of fluorescence Calcein within the cells was analyzed by FACS (Cytomics FC500)

discussion

A very much controlled production process for the upscaling and cryopreservation of Caco-2 cells has been described to prepare assay ready Caco-2 cells of high viability and functionality. In particular the amount of debris appeared to be a critical culture parameter, which needs to be kept as low as possible. While Caco-2 cells from a normal cryo stock have to be cultivated for at least two week until the cells are of sufficient quality for trans-well experiments, assay ready cells can be used instantly like a reagent. No preparatory cultivation or passaging is required. When seeded directly into trans-well plates the assay ready Caco-2 cells form an electrically tight cell layer already after ten days of culture and display a cell-framing expression of tight junction proteins. Assay ready Caco2 cells cryopreserved at a highly functional state provide a helpful tool for the testing of the uptake of new drug candidates in the intestine.

pgp-expressing and pgp-negative subclones of the Caco2 cells have been isolated. Further investigations are under way to analyse the permeability of reference compounds in the trans-well assays using assay ready cells of the two subclones.

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