Hochschule für Angewandte Wissenschaften Hamburg
Hamburg University of Applied Sciences

Master Thesis

Life Sciences
Department of Biotechnology

Development of Assay Ready Frozen Cells for a rapid permeability Assay

Annette Schellin
6. October 2016

Hamburg University of Applied Sciences
HAW Hamburg
Faculty of Life Sciences
Department of Biotechnology
Lohbrügger Kirchstraße 65
21033 Hamburg
Germany

In collaboration with:

acCELLerate GmbH
Osterfeldstraße 12-14
22529 Hamburg
Germany

Author: Annette Schellin
Date of Submission: 8. August 2016

1. Examiner: Prof. Dr. Oliver Ullrich
2. Examiner: Dr. Alexander Loa

Abstract

Caco-2 and MDCK are widely used as in vitro-models in the pharmaceutical industry to predict the abortion of new drug candidates in the early stage of drug development. Conventional transport studies in human colon carcinoma cell line Caco-2 are carried out after a culture period of 21 days on a filter support therefore the cells have to be continuous maintained. in parallel to drug screening activities. To save time and cost test compounds can tested already on a 4-day old monolayer. The use of frozen cells has furthermore the advantage, that cells are always ready for seeding into plates. The aim of this thesis is to develop Assay Ready Frozen Cells for a rapid permeability Assay. MDCK as faster growing alternative to Caco-2 cells, show a less tight monolayer, with a maximal TEER value of 30.7 Ω·cm².

Two freezing media are used to cryopreserve cells, the “standard freezing medium” and the “New Freezing Medium Generation 2” (NFM-G2). The freezing and thawing process delays the cell proliferation shown by growth curves in comparison to fresh cells. Caco-2 viability after thawing and cultured is higher in NFM-G2. The marker molecule Texas Red Dextran was used to investigate the monolayer integrity within 4 days. Cells frozen in both media show equivalent results in the permeability assay. Fresh Caco-2 cells, show a permeability value of 0.4%. The permeability value of frozen cell monolayers is 0.8%. Subclones are isolated from parenteral cell line, differ in P-gp expression.

On an optimized protocol for the permeability assay is was able to obtain. The P-gp transporter serves as indicator for the functionality of the monolayer. TEER values in subclones were already high after the second day. The values were in the range of at least 200 Ω·cm². The highest TEER value is 1193.5 Ω·cm². It is measured in one sample of the frozen, parenteral cell line at day 6. P_{app} values which were obtained with continuous cells grown for 20 to 24, were achieved, with frozen cells within 7 days. Subclones are frozen with three freezing devices. In this work Mr. Frosty shows the best results, but differs only marginal from the other devices

The subclone D5 shows a good viability before and after freezing, independent of the freezing medium and device

In the end the results show, that Caco-2 cells can be used for the intestinal permeability assay as frozen cells, if the cryostocks are prepared from cultures of optimal quality and by the use of NFM-G2 in combination with a controlled rate freezer. Sub-cloning changes the properties of the cell lines, indicating that commercially available Caco-2 and MDCK cell lines are heterogeneous cell populations. The results obtained with the isolated subclone D5 are close to the reference values. There is no need to switch to a new clone. Nevertheless, subclone F9 shows no P-gp activity in the calcein AM assay. Here a Caco-2 clone is available which might be interesting if a P-gp activity of Caco-2 cells is unwanted, e.g. as basic cell line for the expression of genetically modified P-gp.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ATCC	American Type Culture Collection
Caco-2	Carcinoma colon, Epithelia cells out of a Human colon adenocarcinoma
Calcein AM	Calcein Acetoxymethylester, non-fluorescence form of calcein
CoolCell®	Freezing device, made of polyethylene foam
Cryomed®	Controlled rate freezer
DMSO	Dimethylsulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
FBS	Fetal bovine serum
Fresh cells	Cells out of a continuous cultivation
Frozen cells	Thawed cells, which had been frozen before
FSC	Forward scatter in flowcytometry
HBSS	Hanks' Balanced Salt solution
HTS	High throughput screening
LC-MS	Liquid chromatography and mass spectrometry
MDCK	Madin-Darby canine kidney, Epithelia cells out of a Canine kidney
MDR1	Multi Drug Resistance transporter 1 gene
Mr. Frosty	Freezing device, isopropanol -filled container
NFM-G2	New Freezing Medium Generation 2, defined freezing medium with freshly added DMSO to a final concentration of 5%
NIH-3T3	Murine fibroblast cells
PCR	Polymerase chain reaction
Pen/Strep	Penicillin/Streptomycin
P-gp	P-glycoprotein, product of the MDR1 gene, P-gp is responsible for the transportation of substances out of the cell.
SSC	Sideward scatter in flowcytometry

Standard freezing medium	Cell specific cell culture medium with freshly added DMSO to a final concentration of 5% freshly added DMSO to a final concentration of 5%
STR	Short tandem repeat
TEER	Trans-epithelial electrical resistance
Transwell®	24-well Insert with a semipermeable polycarbonate membrane

1. Introduction

The discovery and development of one therapeutic agent into a marketable product can take up to 15 years. Current average cost per drug (including the cost of failures) ranges up to $ 2.6 billion (DiMasi, 2014). The costs and the long development process are related to the complexities involved. To develop a marketable therapeutic agent, more than 5000 compounds are screened in advanced. Five compounds, which are deemed as hits or lead compounds of the screened 5000 are then tested in clinical trials. In the end, only one of them reaches the market (Smith, 2000).
Most of the lead compounds fail because of their insufficient absorption, distribution, metabolism and excretion (ADME), which are summarized under the term pharmacokinetics.
Another reason for discontinuation of further development of compounds is unacceptable toxicity in clinical trials. Because of the high failure rate and due to development becoming more expensive, many pharmaceutical companies have established pharmacokinetic and toxicity evaluation in the early screening phase. The early collected data on ADME properties facilitates the selection of candidates, which are most likely suitable as drugs (van Breemen and Li, 2005).

Oral drug administration is the most convenient delivery route considering patient compliance. However, the bioavailability of oral taken drugs is restricted because of the intestine as a functional barrier. Models that predict intestinal drug absorption could help to identify compounds with promising biopharmaceutical properties. Over the last decades, cell culture models (e.g., Caco-2-, MDCK cells) have been developed to predict drug transport from the intestinal lumen into the bloodstream (Volpe, 2008). These models have been widely accepted by pharmaceutical companies and by regulatory authorities as a standard permeability-screening assay for drug intestinal permeability (Mainprize and Grady, 1998). Furthermore, with the aim of the cell-based permeability assay, drugs can be classified according to the Biopharmaceutics Classification System (BCS) (Alsenz and Haenel, 2003).

Conventional transport studies in human colon carcinoma cell line Caco-2 are carried out after a culture period of 21 days on a filter support. The membrane integrity of the monolayer could be determined by the measurement of trans-epithelial electrical resistance (TEER) or the permeability of low marker molecules, such as mannitol, polyethylene glycol (PEG), dextran, inulin or Lucifer yellow (Volpe, 2010). The traditional transport study is time consuming, expensive and has a low throughput (Alsenz and Haenel, 2003).

Due to time constraints and the large number of compounds tested in high throughput screening (HTS), various approaches have been undertaken to accelerate the process or/and to reduce costs. For example, approaches to reduce the growth time from 21 days to 3-5 days include either the use of collagen-coated (Biocoat) filter support and the increase of fetal bovine serum (FBS) concentration in media (Chong et al., 1997) or the replacement of Caco-2 cells with faster growing MDCK cells (Irvine et al., 1999). But also approaches in the opposite direction, the replacement of FBS or lowering the concentration, are undertaken to reduce costs. Approaches to obtain a higher throughput include the automation of permeation studies (Garberg et al., 1999) and the use of 96-well plates instead of 12-well and 24-well plates (Alsenz and Haenel, 2003).

In order to perform *in vitro* permeation studies, cells are maintained in cell culture flasks and seeded from an exponentially growing culture with an appropriate cell density on filter wells. Culture conditions, e.g., cell culture medium, time in culture, cell passage number, use of subclones, initial seeding density and monolayer age, vary from laboratory to laboratory. These variables lead to different results in permeability studies for the same drug (Vople, 2008).

To improve the consistency of assay results, frozen cells can be used instead of cells that are continuously maintained in culture. Using frozen cells, passage effects and batch-to-batch variation are eliminated. All permeability tests for a certain compound can be performed with cells from the same batch. The use of frozen cells has furthermore the advantage that cells are always ready for seeding into plates, which provides more flexibility in assay performance. Also, the maintenance of cells in parallel to drug screening activities is reduced. As a consequence, resources like the cell culture facilities, materials and disposables, which are required to maintain cells in culture are diminished. Finally, the use of frozen cells saves time and costs (Zaman et al., 2007).

2. Theory

2.1 Intestinal Permeability assay

Permeability assays are carried out in a special cell-culture plate that is specifically designed for permeability assays (see figure 2.1). It consists of a normal cell culture plate (e.g., 24-well plate), in which special inserts (e.g., Transwell®) are placed and separate the well into two compartments: the apical and basolateral compartment. The bottom of the Transwell® is a semipermeable polycarbonate membrane, on which epithelia cells can grow. The apical compartment, covered with cells, represents the intestinal lumen, whereas the basolateral compartment serves as a circulation system.

In general, the intestinal permeability assay consists of three phases: cell cultivation, transport experiment and data analysis.

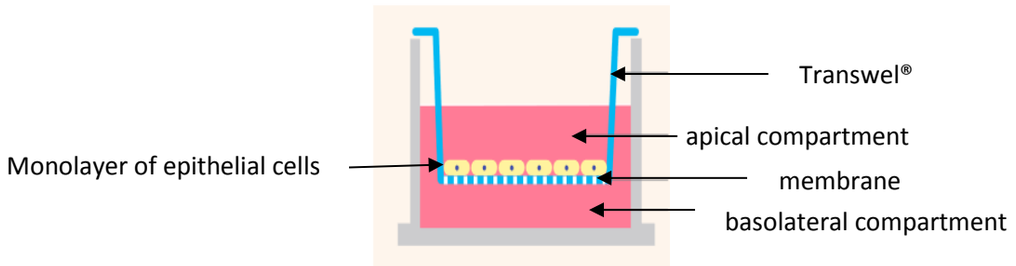


Figure 2.1 Schematic picture of one well in the cell culture plate, where the permeability assay is carried out. Epithelial cells differentiated to a tight monolayer on a semipermeable polycarbonate membrane of the Transwell®-Insert, which is placed in a well of a cell culture plate (e.g., 24-well plate). Transport studies are performed by adding test compounds either to the apical compartment or to the basolateral compartment. The appearance of the compound is measured in the opposite compartment (Corning, 2013)

2.1.1 Cell cultivation

In the first phase, Caco-2 or MDCK cells are growing in cell culture flask until the desired number of cells is reached. Thereafter, the cells are harvested and seeded with appropriate cell density on the semipermeable membrane of the apical compartment. The basolateral compartment is filled with cell culture medium. Depending on cell line and assay protocol, cells are grown up to 21 days to form a tight and fully differentiated monolayer. The filters used as bottom for the Transwell®-Insert are made of an inert material, for example polycarbonate. Normally, a pore size of at least 0.4 µm is used in order to avoid cell migration from the apical chamber to the basolateral chamber. Different plate formats are available. Depending on the application or experimental design, cell culture plates from 6 to 96-well format can be used.

2.1.2 Transport experiment

The second phase of the assay is the transport experiment. To prepare the plates for the transport experiment, cell culture medium is removed from both chambers and replaced by a transport buffer (HBSS containing HEPES). To ensure the integrity of the monolayer, it is recommended to measure the transepithelial electrical resistance (TEER). Typically, TEER values of 500 Ω·cm² indicate adequate monolayer integrity (Minuth et al., 2003). Alternatively, the integrity of the monolayer can be determined with low marker molecules, such as mannitol, polyethylene glycol (PEG), Texas Red Dextran, inulin or Lucifer yellow (Volpe, 2010). These compounds are known to be unable to pass the intact cell layer. In a leaky monolayer, these marker molecules are able to reach the basolateral compartment and can easily be detected.

In a transport experiment, test compounds are added either to the apical chamber or to the basolateral chamber to simulate the absorption or secretion of compounds across the intestinal epithelium, respectively. The appearance of the compound is measured in the opposite chamber after defined time points. Typical concentration of test compounds, depending on the protocol used, are in the range of 10 to 500 µM (van Breemen and Li, 2005).

2.1.3 Data analysis

The final phase is the data analysis. In this phase, the drug concentration in the samples is measured and the apparent permeability coefficient (Papp) is calculated, which describe the rate of drug transport.

$$P_{app} = \frac{\frac{\Delta Q}{\Delta t}}{C_0 \cdot A} \qquad \text{(Eq. 1)}$$

The term $\Delta Q \cdot \Delta t^{-1}$ describes the transport rate ($\mu M \cdot s^{-1}$), C_0 is the initial concentration ($\mu M \cdot mL^{-1}$) in the apical chamber and A is the surface area of the cell monolayer (cm^2). The concentrations of the compound are analyzed by a combination of liquid chromatography and mass spectrometry (LC-MS).

An apparent permeability value (P_{app}) for Lucifer Yellow of ≤ 5 to 6 $nm \cdot s^{-1}$ has been reported to indicate an appropriate monolayer for drug transport studies (Corning, 2007). Furthermore, a limit of 6% permeability from the apical to the basolateral compartment for the marker molecule Texas Red Dextran was used, due to an internal communication with an experienced user.

2.2 Caco-2- and MDCK cells as cell-based *in vitro* models

The small intestine is the primary site of drug absorption for orally taken drugs. It consists of epithelia cells, which form a selective barrier between the gut lumen (apical surface) and the underlying tissue and blood vessels (basal or basolateral surface). To form these surfaces, it is necessary to connect the surface building cells by tight junctions. These junctions block the extracellular space between cells, stick the cells together and prevent that substances pass through the extracellular space. To permeate in the systemic circulation, drugs must pass the barrier. Therefore, they have to enter the cells on the apical side and leave the cells on the basolateral side of the cell layer.

Cell-based models have been developed that show correlation to the drug permeability in human intestine, providing a useful tool for pre-clinical drug screening, and are an alternative to the more expensive and low-throughput *in vivo* methods. The most commonly used cell lines for drug transport studies are the Caco-2 cells and the MDCK cells (Lee, 2014), which are described in more detail in the following sections.

Table 2.1 Cell line characteristics of Caco-2- and MDCK cells

	Caco-2	MDCK
Cell source	Human colon adenocarcinoma	Canine kidney
Cell morphology	Intestinal epithelium	Distal tubule epithelium
Monolayer culture time	3-21 days	3–7 days
TEER values	> 500 Ω·cm²	180 to 250 Ω·cm²
Efflux transporters	P-gp (MDR1)	P-gp (MDR1)

2.2.1 Caco-2

Caco-2 cells were isolated by J. Fogh in 1977 from a colonic tumor in a 72-year-old Caucasian male (Fogh et al., 1977) and show morphological and biochemical similarity to the small intestine. Due to the correlating drug absorption values in humans, Caco-2 cells have become one of the most popular cell culture models for drug absorption (Artursson and Karlsson, 1991, Grès et al., 1998). They were used for the first time as a model in the late 1980s (Pinto et al., 1983). Since then, Caco-2 are widely accepted in the pharmaceutical industry and by regulatory authorities as the standard in vitro model for the prediction of intestinal drug absorption for drug candidates (Mainprize and Grady, 1998).

Caco-2 cells differentiate structurally and functionally into a cell monolayer with distinct apical and basolateral membrane domains, microvillus and tight junctions (Pinto et al., 1989). In addition, several active transport systems

are expressed, including the P glycoprotein (P-gp), which is the product of the MDR gene. This protein is also expressed in the walls of the human small intestine (Hunter et al. 1993). P-gp is responsible for the transportation of substances out of the cell.

For drug transport studies, Caco-2 cells are cultured on a semipermeable polycarbonate membrane (Transwells®), which separate two compartments, the apical- and basolateral compartment from each other (see chapter 2.1). This system should mimic the in vivo situation of the small intestine, where the apical chamber represents the intestinal lumen and the basolateral compartment, the underlying tissue.

Typically, Caco-2 cells are used from exponentially growing cultures and seeded into Transwells®. In the wells, they are maintained for 21 days before usage. TEER values above 500 $\Omega \cdot cm^2$ are characteristic for Caco-2 cells (Minuth et al., 2003). It is described, that this time is required to reach confluence and complete morphological and functional differentiation. To accelerate the process for high throughput screening, Caco-2 cell culture systems and conditions have been modified to allow transport studies after a culture period of 3-7 days (Chong et al., 1997, Lentz, 2000, Alsenz and Haenel, 2003, Olejnik et al., 2003, Olejnik et al., 2008, Uchida et al., 2009). Beside the cell quality, substrate and reagents used for cell growth can also influence the formation of the monolayer. An example for a commercially available cell culture system, suitable for a 3-day procedure, is the Corning® BioCoat™ Intestinal Epithelial Cell Environment. Caco-2 cells prepared with this system, show many morphological features after 3 days, which are also found in traditional 21-day Caco-2 culture system. This includes apical microvilli, tight junction formation and interdigitation of the cell membranes (Woods, 2013).

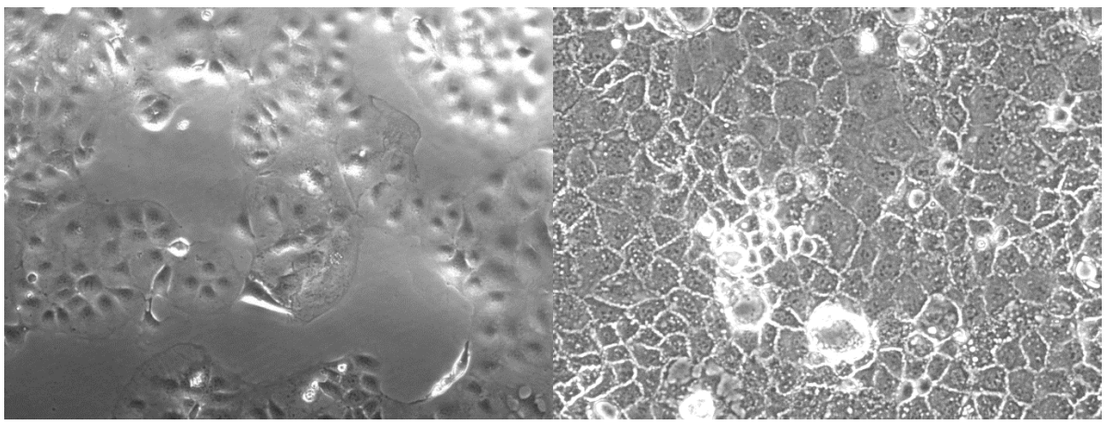


Figure 2.2.1 Caco-2 cells at 200x magnification. (Left) Cells were seeded with a cell density of $1.0 \cdot 10^4$ cells·cm-2, after three day Caco-2 cells are 50% confluent, (right) cells were seeded with a cell density of $4.0 \cdot 10^5$ after 4 days the cells are 100% confluent and Caco-2 cells differentiate into a polarized epithelial monolayer. The formations of domes are clearly visible indicating a tight monolayer (own illustration).

2.2.2 MDCK

Although MDCK (Madin-Darby canine kidney) cells originate from the kidney of an adult female cocker spaniel and, therefore, are not of human origin, they have come more and more into focus as a suitable cell line for in vitro drug absorption. Not only because of its faster growth compared to Caco-2 cells, but also because of its similar permeability results for drug absorption (Irvine et al., 1998). Like Caco-2 cells, MDCK cells differentiate into a polarized epithelial monolayer. Tight junctions between the cells form a barrier and separate the cell surface into an apical and basolateral side, which differ in protein and lipid compositions (Richardson and Simmons, 1979). The apical side exists of brush ordered microvilli and faces the culture medium, whereas the basolateral side is attached to the bottom of the cell culture flask (LouVARD, 1980). For permeability studies, MDCK cells are cultured for 3 to 5 days on polycarbonate membranes (Cho et al., 1989) with TEER values in the range of 180 to 250 $\Omega \cdot cm^2$. Meanwhile, there are two types of MDCK cell lines (MDCK I and MDCK II) available, which differ in morphological and physiological properties (Richardson et al., 1981). MDCK I cells were obtained from low passage of the heterogeneous parental MDCK cells and have high TEER values (4000 $\Omega \cdot cm^2$) whereas MDCK II were isolated from higher passage MDCK cells. The monolayer of MDCK II cells are ‘‘leakier’’ and have lower TEER values (200 $\Omega \cdot cm^2$ 300 $\Omega \cdot cm^2$) (Lee, 2014). However, the MDCK II strain is the most widely used strain (Dukes et al., 2011). To use the MDCK II cells as a tool for the screening of P gp substrates and inhibitors, MDCK II cells have been transfected with the gene of the human Multi Drug Resistance transporter 1 (MDR1). These cells were also used for the study of transport properties of compounds (Horio et al., 1989).

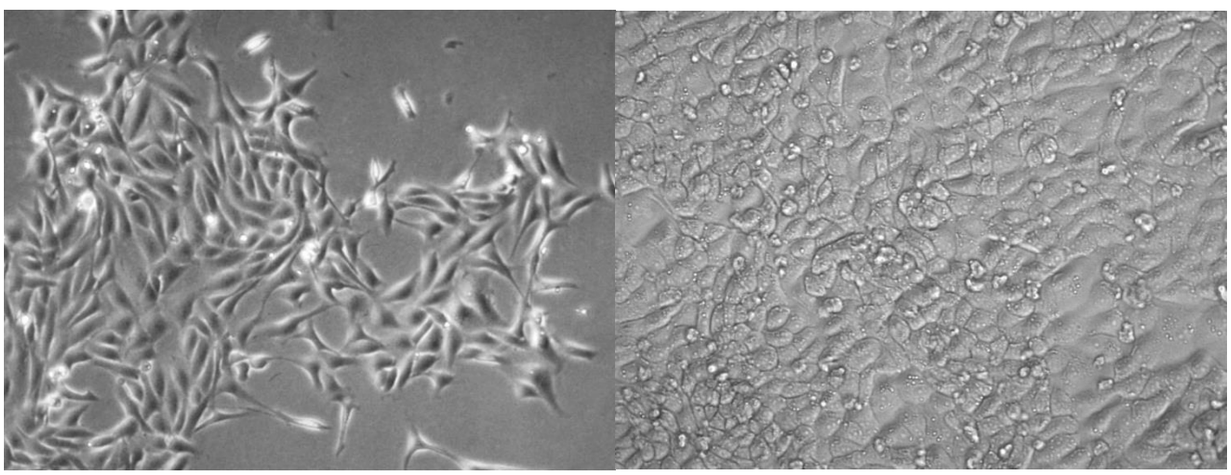


Figure 2.2.2 MDCK (Madin-Darby canine kidney) cells at 200x magnification and different differentiation stages. Cells were seeded with a cell density of $3.0 \cdot 10^3$ cells$\cdot cm^{-2}$. (Left) after 3 days MDCK cells are 50% confluent and have a fibroblastic morphology. (right) At 100% confluence MDCK cells differentiate into a polarized epithelial monolayer. The picture was taken at day 8, where the culture has been already confluent for several days (own illustration).

2.3 Quality control

Credible and reproducible results are crucial for the permeability assay, since it predicts the behavior of the substance in vivo. Contaminations and long-term culture could change the behavior of the cell line and the reliability of results obtained with them. Therefore, it is important to confirm the identity of the cell line one is working with to ensure that the cell line is free from contaminants and to return periodically back to cryopreserved cell stocks.

2.3.1 Cell bank system

A two-step approach, consisting of a master and working cell bank, has been established in the manufacturing of biological products with microorganisms or cell lines. The number of vials in master and working cell bank depends on the needs for the culture in the future and the ability to maintain, freeze and store the required number of vials.
In the first step, the cells are grown to an appropriate density, harvested and frozen to master cell bank containing ten or more vials. A single vial out of the master cell bank is thawed and expanded until enough cells are available to produce a working cell bank. On a research scale, this working cell bank can consist of up to 30 vials. For cell experiments, only vials from the working cell bank are used. When the first working cell bank is used up, the process is repeated by thawing another vial from the original master cell bank for the production of a second working cell bank.

Each working cell bank that is produced, as well as the original master cell bank is tested regarding cell quality control. This includes viability, absence of mycoplasma or microbial contaminants cell line identity and important cell line characteristics (Coecke et al., 2005; Freshney, 2010; Stacey, 2004).

2.3.2 Ensuring absence of microbial and mycoplasma contamination

Whenever a cell line is held in culture, there is always the risk of contamination with another species, like bacteria, mycoplasma, yeast and fungi. Usually this is apparent to the naked eye or to microscopic examination, if the cells are cultured in the absence of antibiotics. However, antibiotics are often used in cell culture work to supress bacterial growth, esspecialy with valuable cell lines, like subclones that are hard to reimburse. Unfortunately the use of antibiotics bears the risk that bacterial contaminations remain undetected. It is also proven that cells cultured under these conditions behave differently than they would in antibiotic free cell culture medium. Sterile working is therefore essential during cell culture maintenance or expansion. Nevertheless, it may happen that contaminations occur even is attention payed on it, but not recognized since the degree of contamination is too small. Therefore, sterility controls are carried out to ensure that the produced cell stock, if master cell bank, working cell bank or custermor production, is free from bacterial and fungial contaminations.

Samples of each newly produced cell stock (approx. 5% of the batch size) are thawed and cultured for up to 4 days in a 25 cm² cell culture flask conaining antibiotic free cell culture medium. Bacterial contaminations can be detected, if the culture medium gets turbid and the color changes yellowish (color of phenol red at low pH, due to more acidic conditions by organic acids as a result of bacterial metabolism). Additionally, each culture is inspected under a microscope, where most bacteria also can be detected. If the color of the medium stays red or orange, the supernatant is clear and no bacteria are visible under the microscope, one can assume that the culture is free of bacteria and fungi.

However, a mycoplasma contamination can not be detected by this method. Mycoplasma are too smal to be detected by an ordinary light microscope. Additionally their metabolism is too slow to show significant pH effects and canges in medium color. Nevertheless, mycoplasma infection can have a number of effects on cell behavioiur including alteration in growth, metabolism, and cell surface modification. To generate reilable experimentel data with cell lines, it is an absolut requirement to detectd mycoplasma contaminated cell lines. The easiest method to detect mycoplasma is the polymerase chain reaction (PCR). It provides the most sensitive and reliable results for the detection of mycoplasmsa. In most cases the 16S rDNA sequence are used as target sequence because this gene containes regions with more ans less conserved sequences (Drexler and Uphoff, 2002; Freshney, 2010).

2.3.3 Authentication of a cell line and ensure absence of Cross-contamination

To contaminate an existing cell line with a second cell line only one cell from a faster growing cell line is needed, to overgrow a slower growing culture. In some cases, only a few passages are needed and the original cell line completely disappeared. Unfortunately, cross-contamination is not a rarity but exist as long as the first cell cultures were established in the 1940s and 1950s.
The transfer of cells can occur, when lab ware (e.g, pipettes) are accidental re-use for different cell lines as well as, if medium and reagents are shared among them. The same can happen if more than one cell line is handled under a flow cabinet, since cells can survive in an aerosol and contaminate an opened cell culture vessel.

Most experiments where impure cell lines were used are rather not the result of a direct cross-contamination by the operator, than a blind trust that cells originating from a commercial cell collections are free of contamination and do not need to be inspected.

In fact, it was proven that various human cell lines, sold by ATCC (American Type Culture Collection) are in reality HeLa cells. Also, the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, German Collection of

Microorganisms and Cell Cultures) reported that 20% of submitted human cancer cell lines are contaminated with other cell lines (MacLeod et al., 1999).

Given these facts, and in terms of the reliability of the results which are obtained with the cell lines, it is essential to determine the cell identity after a new cell line is acquired or a cell stock is frozen for storage. Interspecies cross contamination can be detected with the DNA barcoding method. The mitochondrial cytochrome c oxidase gene is amplified with universal primers, which recognize conserved sequences of thousands of different species. The species can be determined by database research of the sequenced PCR product.

A simple and relatively cheap method to check cross contamination between the same species (established mainly for human cell lines) is to analyze the short tandem repeat (STR) profile. STR are regions on the DNA, composed of short sequence of nucleotides (2-10bp) which repeated many times. The number of repeats is differentiating among individuals. This can be used to distinguish between cell lines and to reveal contaminations. STRs are amplified by polymerase chain reaction (PCR) with the aim of primers with are commercially available. The PCR products are analyzed with a size standard on agarose gel electrophoresis (Masters et al, 2001).

2.4 Standardization to ensure reproducibility

Cells, as a biological system, are generally subject of variations. However, inconstancy can be minimized by controlled cell culture conditions and maintenance. Factors that affect the credibly and reproducibility of results, obtained with MDCK and Caco-2 cells, include i.e. cell culture conditions like quality of serum, cell culture media, additional supplements, temperature, CO_2-concentration, seeding densities, harvest densities, quantity of medium exchange between passages, time between passaging and many more.

2.4.1 Cell culture conditions

Mammalian cells are generally grown under well-established conditions in incubators, where the temperature is typically kept at 37 °C with a controlled humidified gas mixture of 5% CO2 and 95% air.

MDCK and Caco-2 cells consist of inhomogeneous sub-populations. If cultured under varying culture conditions, the subclone growing optimal under the actual conditions will dictate the results. Other conditions will prefer another subclone and the results may be different. This will induce variations of the assay results by changing culture conditions. To achieve optimal cell quality, the composition of the cell culture medium is essential and optimal cells are essential for good experimental reproducibility. To maintain cells, the medium must contain components like pH

buffering compounds, salts, sugars, amino acids and trace elements and a variety of supplements, depending on the culture requirements of the cell type. Consequently, once a medium has been selected, it should not be changed.

In traditional cell culture work, serum is used as natural source of important constituents for cell maintenance and proliferation. This includes growth factors, which promote cell proliferation as well as adhesions factors and antitrypsin activity for cell attachment. Serum can be derived from different animal sources (adult, newborn or fetal sources). However, fetal bovine serum (FBS) has become the standard supplement for cell culture media. Depending on cell type, a ratio of 3-25% is added into the cell culture medium. Serum is a cocktail including low and high molecular weight biomolecules. Albumin and transferrin are known to be the major contribution in serum, whereas nutrients (e.g., amino acids, nucleoside and sugars), proteins, peptides, growth factors hormones, minerals and lipids make up the minor part. The composition differs from animal to animal and so far, it has not been able to identify all the serum components and the exact concentrations. Because of the complexity and the batch-to-batch variation, serum alters the characteristics of certain cells and therefore introduces an unknown variable into the culture system. (Coecke et al., 2005).

In addition, serum from animal sources bear the risk of a potential contamination with microorganism, especially mycoplasma, bovine viruses, and possibly prions that can causes Bovine Spongiform Encephalopathy (BSE). To reduce the risk of such contaminations, suppliers use filtration, irradiation and heat-inactivation, but the presence of the above-mentioned contamination cannot be completely excluded.

Because of the above-mentioned disadvantages, there have been many attempts to find an alternative to serum. Serum free medium often contains supplements from other animal materials (for example, pituitary extracts, chick embryo extracts, bovine milk fractions) and must be tested in the same manner for safety and utility as bovine serum. All biological materials vary in its composition. An alternative is the use of chemically defined media, but this often lags, because not all cell types can be adapted to grow adequately on it and also the results obtained with this media still be inferior to that obtained with serum.

The safety in use of bovine serum has been established over many years and scientists and regulatory agencies accepted the fact that serum is still required for many cell cultures (International Serum Industry Association, 2011).

2.4.2 Handling and maintenance

Another subject that introduces variables into cell culture are subculture intervals and seeding densities. Cells at different phases of growth cycle behave differently with respect to cell proliferation, enzyme activity and expression of specific proteins. Therefore, subculturing should follow a standard regime where routine passage leads to a

standard growth cycle with reproducible behavior and cell yield from a given density (see figure 2.4.2). The number of cells should be recorded after detachment and reseeding to determine the growth rate for each subculture. Strong deviations of growth rates can indicate inconsistency of the culture. MDCK and Caco-2 cells should be subcultured, if roughly 70% to 90% confluency is reached. Especially Caco-2 cells are sensitive to a status of 100% confluence. The reason is that, if full confluence is reached the cells start to differentiate. Full confluence over several passages will lead in the loss of desired characteristics.

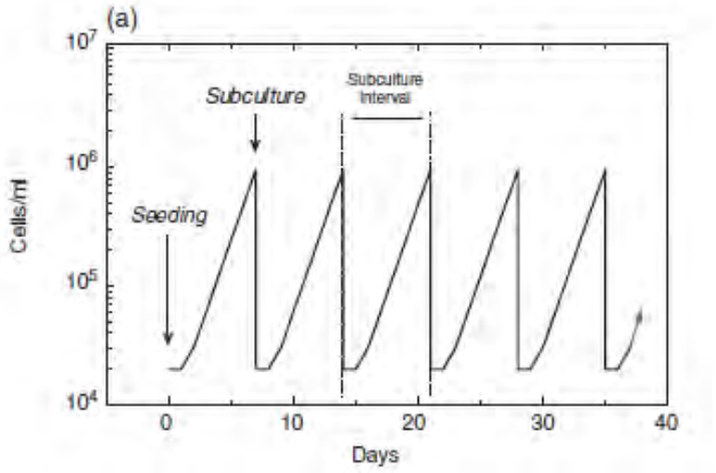


Figure 2.4.2 Subculture intervals. If subculture interval and cell seeding density remains constant; then the same cell density should reach after the same time (peak), provided that the cells are growing correctly. A different behavior indicates a change of the cell (Freshney, 2010)

2.5 Cryopreservation

Maintaining a continuous culture over many months is not only time- and cost intensive, it also bears the risk of accidently losing the culture due to cross-contamination with another cell line or contamination with microorganisms. In addition, during long time culture the cells prone to genetic and phenotypic changes, which may result in the loss of important properties and introducing unwanted variables into long-term experiments. These problems are reduced by freezing the cells down and preserve them for long-term storage in cell banks (master cell bank and working cell bank, see chapter 2.3.1). The reason is that cells kept below -130 °C show no metabolic processes and biological changes are no longer detectable. Therefore, the use of master and working cell banks allows reproducible experiments over a long period of time.

2.5.1 General events during freezing

When water is cooled down slowly and vibration-free, the temperature of the sample is decreased below the "normal" freezing point, without getting solid. This phenomen is also refered under the term "supercooling". The ice formation, beginns around a starting point (the nucleation site) this could be physical disturbances, the vessel surface or "impurities" in the liquid, like salts. The reconstruction of water molecules into the ice crystal structure is an exothermic process and will cause heat release, known under the expression "latent heat of fusion." Because of the previous supercooling, the start of ice formation will cause a sudden rise in temperature, back to the the freezing point of the remaining unfrozen liquid. Some of the water is still in liquid phase. During the following freezing process, heat is released into the cooler environment. As a result, the temperature remains constant. After complete crystallization, the temperature will decrease again. The rise in temperature during the freezing process is stressful for cells. Temperature control can compensate the temperature change during the release of latent heat of fusion and leads to a linear cooling curve that improves the cell recovery (see figure 2.5.4). Temperature compensation can provide by a controlled-rate freezer. Figure 2.5.1 show the type of cooling profile generated within samples during non controlled freezing (Brockbank et al., 2007).

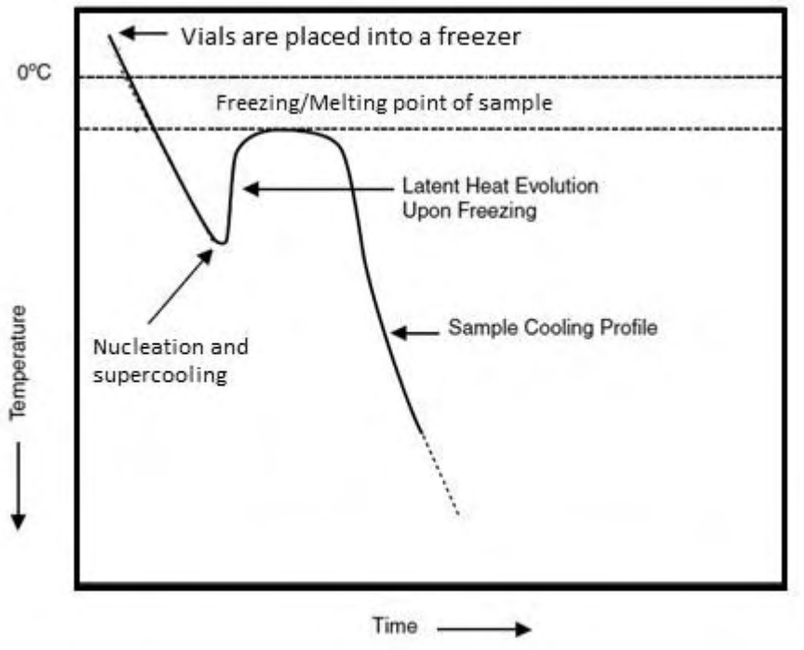


Figure 2.5.1 Freezing Curve within a sample during non controlled freezing, as a result of freezing, supercooling and latent heat evolution. Temperature of the sample is decreased below the normal freezing point, named as supercooling. The ice formation, beginns around the nucleation point. Ice formation is an exothermic reaction and will cause latent heat of fusion, visible as sudden rise in temperature, back to the the freezing point of the remaining unfrozen liquid. During the crystallization process heat is released into the cooler environment. As a result, the temperature remains constant. After complete crystallization the temperature will decreased (changed after Brockbank et al., 2007)

2.5.2 Events during freezing inside the cell

The freezing process in general, has a negative impact on cell survival. During a relatively slow freezing process, ice crystal formation begins in the extracellular environment, resulting in an increase of salt concentration in the extracellular liquid. A concentration gradient is established across the cell membrane. To compensate the imbalance, water leaves the cell by osmosis. The volume of the cell decreases, leading to cellular dehydration and cell shrinkage. Excessive dehydration and shrinkage can cause disruption of the organelles, the cytoskeletal as well as the outer membrane. Which leads at the end to cell death. But also the high salt concentration in the remaining unfrozen liquid leads to pH shifts, membrane damage and protein denaturation.
If the cooling rate is too fast, ice crystals will be formed inside the cell, before parts of the water were able to diffuse out of the cell. These intracellular ice crystals disrupt cellular organelles and membranes and lead to cell death as well.

However, it could be demonstrated that the use of cryoprotectants improves cell recovery if cells are cooled down with a low freezing rate. Unlike freezing cells with a rapid cooling rate, cryoprotectants show hardly any effect (Mazur, 1984).

2.5.3 Cryoptrotectans

A variety of agents show adequate cryoprotection in many biological systems, including dextrans, glycol, starches, sugars and polyvinylpyrrolidone. Also salts, such as magnesium chloride, have been reported to be cryoprotective agents (Karow and Carrier, 1969). Fetal bovine serum (FBS) is also often used in mamallian cryopreservation, it protects due its high protein content (molecular crowding) (Minuth et al., 2003). But the most commonly used cryoprotectans are glycerol and dimethylsulfoxide (DMSO). The mechanisms, how cryprotectans protect slowly frozen cells include, the suppressing of high salt concentrations, the reduction of cell shrinkage, stabilization of lipid membranes, reduction of the frozen fraction and the minimization of intracellular ice formation.

Cryoprotectans are divided into two classes, the intracellular and extracellular cryoprotectans.

Sugars, polyvinylpyrrolidone, hydroxyethyl starch, polyethylene glycols, and dextrans are extracellular cryoprotectants, which do not penetrate the cell, because of their relatively high molecular weight. The primary mechanism of action is the iduction of vitrification (extracellular glass formation). This means that water is too cold to move, and become solid by an increase in viscosity rather than crystallization. In addition these agents protect the cells due to stabilization of proteins and cell membrane. However, high concentrations of non-permeable solutes lead to osmotic cell stress before freezing. This stress is reduced by the use of permeable cryoprotectants. Due to its low

molecular mass, intracellular cryoprotectans are able to penetrate the cell membrane. Ethylene glycol, DMSO and glycerol are intracellular cryoprotectans, provide similar mechanisms of action in the view of cryoprotection. For example, if cells are brought into a medium containing DMSO, glycerol or ethylene glycol, water will leave the cells because of the differences in concentration. At the same time, permeable cryoprotectants will enter the cells, so that the cell retains its initial volume. By the use of permeable cryoprotectants, a part of water in the extracellular and intracellular space is replaced by cryoprotective molecules. Consequently, lower ice formation occurs and a larger part remains unfrozen, thereby the increase of salt concentration during freezing is decreased and the extent of cell shrinkage is limited. (Mazur, 1984)

It also has been reported, that the use of extracellular cryoprotectants alone, do not have protective effects on cells, but often support the effectiveness of permeating agents, which allow the use of a lower concentration of intracellular cryoprotectants (Fahy, 1986; Fahy et al., 1984; Takahashi et al., 1985).

The extent of protection of intracellular cryoprotectants depends on the concentration and the relative permeability of the membrane to water and to the cryoprotectant (Gao and Critser, 2000).

DMSO is usually used in a final concentration of 5 to 15% (v/v), wherelse glycerol is generally used at a final concentration between 5 and 20% (v/v). It is less toxic to cells than DMSO but serves osmotic problems after thawing (Ryan, 2004).

2.5.4 Freezing devices

As already mentioned in the previous chapter, the injury of cells due to freezing can be decreased by the use of a low cooling rate in connection with cryoprotectants. A cooling rate of -1 °C to -3 °C per minute shows the best results for most animal cell cultures (Ryan, 2004).

To generate this low cooling rate, several methods have been developed. These range from a simple and non-expensive Styrofoam box to a cost intensive programmable freezing unit.

The easiest and cheapest method is to use a Styrofoam box, where cell samples are placed in. The walls of the box should have a thickness of approximately 1 cm. The box is then placed into a -80 °C freezer for 2-3 hours and then transferred to the storage temperature of -196 °C. Because Styrofoam boxes differ in size, geometry, density and structure, it is hardly to believe that a uniformly cooling rate is generated. Hence, this method should generally be avoided for applications where a guaranteed high cell viability and a documentation of the freezing process is required (Shu et al., 2010).

To generate a uniform cooling rate, the company Nalgene developed an isopropanol (IPA) -filled container, called “Mr. Frosty.” Just as with the Styrofoam box, Mr. Frosty is placed in a -80 °C freezer.

Due to the isopropanol in the outer chamber, a uniform freezing rate of -1 °C is generated. The disadvantage here is that due vapor condensation, isopropanol is diluted by water. Each freezing-thaw cycle will increase the content of water in the isopropanol and increase the negative effect on the cooling rate. Therefore, Isopropanol in the outer chamber have to be regularly refreshed after a few cycles (5-10 cycles). The inconsistent quality of isopropanol hindering the exact reproducibility and generate costs for fresh isopropanol and waste disposal. Furthermore, the time the isopropanol needs to reach room temperature after freezing, limits the throughput to one run per day.

The company BioCisions offers with their CoolCell® containers an alternative for non-expensive freezing devices. The CoolCell® container is made of polyethylene foam with a solid metal core, which should ensure reproducibly by balances and fine-tunes the freezing profiles. The advantage is that no isopropanol or other fluids are required and that this freezing device can be used again 5-10 minutes after freezing, which enables a higher throughput in comparison to “Mr. Frosty” (BioCision, 2016).

Both methods have the advantage of being very simple and cheap. However, it is limited by the fact that these methods are not able to compensate for the latent heat fusion and produces no recorded documentation to verify the cooling rate and therefore the conditions.

The best way to control the freezing process is the use of an controlled rate freezer. One example is the CryoMed® Freezer (Thermo Scientific), where a large number of vials can be cooled by cold nitrogen vapor. This instrument can be programmed in a way that the samples are cooled down at a cell line-specific temperature profile. To purchase a controlled rate freezer is expensive and each freezing procedure uses considerable amounts of liquid nitrogen, which causes significant costs. However, with the use of controlled rate freezer, the actual temperature profile of the chamber and the samples can be controlled and recorded, resulting in uniformly frozen samples (see figure 2.5.4) (Brockbank et al., 2007; Shu et al., 2010).

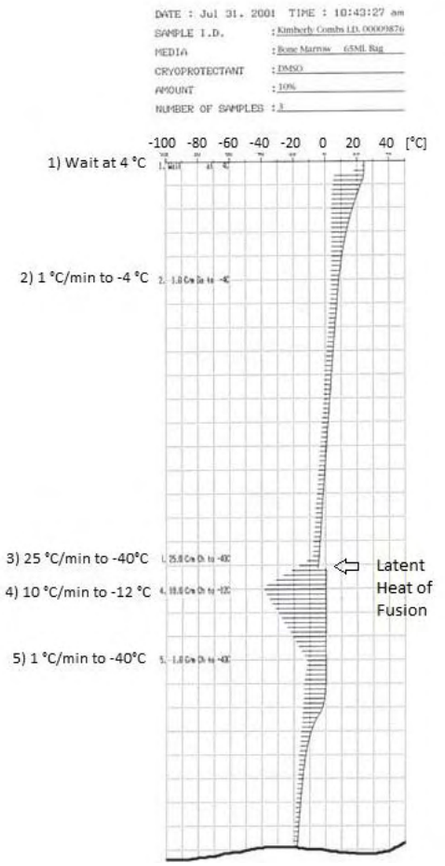


Figure 2.5.4 Example of a temperature profile in a cooling chamber (bars) and sample (solid line) during controlled freezing. The chamber temperature remains at 4 °C until the sample reaches the same temperature (step 1). The freezing process starts at 4°C with a cooling rate of 1 °C min^{-1} (step 2) until the sample temperature reaches -4 °C, where latent heat of fusion occures. In an optimized protocol the controlled rate freezer compensates the temperature change during the release of latent heat of fusion and lead to a more linear cooling curve, which improves cell recovery after thawing (change after Brockbank et al., 2007)

2.6 Limiting dilution cloning

The tumor cell lines MDCK- and Caco-2 consist of heterogeneous subpopulations, with different biochemical and morphological properties. Several laboratories isolate subclones with unique properties. One example is the already mentioned MDCK cell line, where two different strains (MDCK I and MDCK II) were derived from. Both clones show differences in transepithelial electrical resistance (TEER) values. Also subclones from the Caco-2 cell line have been isolated, which expresses high levels of P-gp and are used in kinetic studies (Horie et al., 2003). The P-gp activity in these clones is very stable and show a similar magnitude of expression as in a high P-gp expressing MDCK-MDR1 cell line.

The technique of limited dilution is most widely used to isolate single clones with desired properties or to gain a more homogenous cell line. (Volpe, 2008, Sambuy et al., 2005).

Using this technique, several single clones were isolated out of cell suspension. To start a subcloning by limited dilution the cell suspension is diluted in a way, that after seeding in a 96-well plate ideally only one single cell is seeded per well. In practice no, one, two or more cells are found per well. To increase the efficiency of only one cell per well, the seeding density could be reduced (e.g., 0.4 cells per well). Wells with a single cell are than cultivate until the cells form colonies and proliferate to significant amounts. However, limited dilution is difficult, as very often single cells die because of missing cell -cell contact. In these cell lines, cell-cell contact has to be present for cells starting to divide. The use of cell supernatant of growing cell culture (conditioned medium) from the same cell line may help, because it includes important cell specific factors like hormones, which help to stimulates proliferation (Freshney, 2010).

2.7 Flowcytometer analysis

Flow cytometry is a technique to count and analyze physical and molecular properties of cells in a fluid as it passes through a laser beam. Thereby hydrodynamic focusing plays an important role in passing one cell at a time through a laser beam. Information about the cell size and the structure are obtained by the light diffraction and scattering. The forward scatter (FSC) detects the size and number of cells, whereas the sideward scatter (SSC) observes the granularity of counted cells. For many applications, the cells are fluorescent labeled. Very common are fluorophore-coupled antibodies, which bind directly or indirectly on the cell surface or structures inside the cells. But also fluorescent molecules (e.g., calcein) which have been brought into the cell or GFP-expressing cells could be detected by the system. A schematic picture of how a flow cytometer works is shown in figure 2.7.

The electrons of the fluorophore are excited by monochromatic light of a specific wavelength and are raised to a higher energetic level. For example, the commonly used argon laser sends monochromatic light with a wavelength of 488nm. The energy of the laser lifts the electrons of the fluorophore to a higher energetic level. This level is metastable and therefore, after a short time the electrons fall back to their original energy level. The difference of both energy levels is emitted as light of a specific wavelength. The emitted wavelength is specific for the fluorophore and not identical to the excitatory laser beam. The emitted photons are registered by a photodetector and the resulting signal is proportional to the fluorophore concentration (Luttmann et al., 2009).

A special type of flow cytometry is *flow activated cell sorting* (FACS) which allows besides cell analyzing sorting of labeled cells.

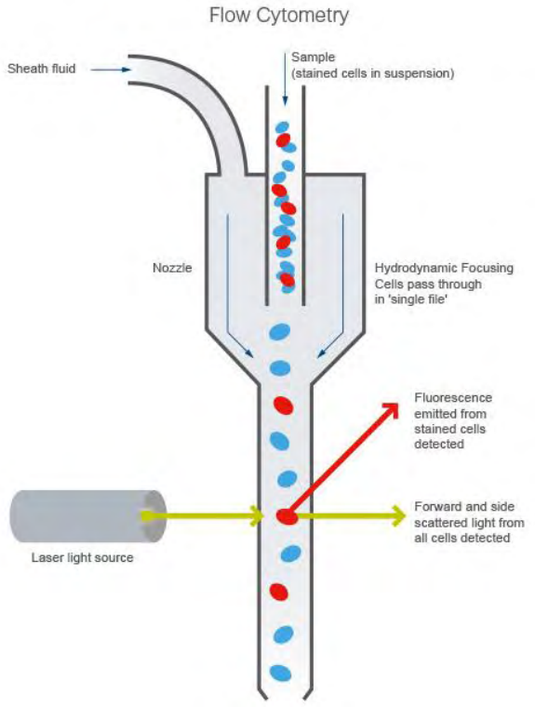


Figure 2.7 Cells that are differentially labeled with a fluorescent reagent are detected by a laser in the flow cytometer. A sample with labeled cell suspension is injected into sheath fluid, before reaching a nozzle. Hydrodynamic focusing causes that cells pass single-file through a laser beam. The fluorescent light emitted and the forward and side scattered light are measured for each cell. Information about the cell size and shape are obtained by the light scattering (Abcam, 2016).

2.7.1 Assessment of P-glycoprotein (P-gp) activity by calcein uptake

In Caco-2 and MDCK cell, several active transport systems are expressed. One of them is the P-glycoprotein (P-gp), which is the product of the MDR (Multi Drug Resistance transporter 1) gene. This Protein is also expressed in the walls of the human small intestine (Hunter et al. 1993).

The primary role of P-gp, as multidrug transporter, is the protection of the cell by actively removing hydrophobic, xenobiotic compounds and toxic metabolites from the cell back into the lumen. This characteristic influences the absorption of drugs. To predict the drug transport in from the intestinal lumen into the bloodstream, it is important that the cell culture model offers the same characteristic. Not only the presence of the P-gp in the membrane, but also the functionality of P-gp is important for a suitable model. To test the functionality of P-gp calcein AM (calcein Acetoxymethylester) can be used.

Calcein AM is a small, non-fluorescent molecule. Due to its hydrophobic properties, it is able to penetrate the cell membrane. As substrate of P-gp, calcein AM is immediately transported out of the cell (figure 2.7.1).

When P-gp is blocked, by an inhibitor (e.g., verapamil), calcein AM reaches unhindered the cytosol of the cell. Here, unspecific esterases cleaves the AM group from calcein. Without the AM group, calcein is no longer a substrate for the P-gp and accumulates in the cell. Now calcein is able to complex calcium ions, resulting in green fluorescence. The activity of P-gp is measured as the difference between the amount of calcein accumulated in the presence and absence of the inhibitor (Homolya et al., 1996).

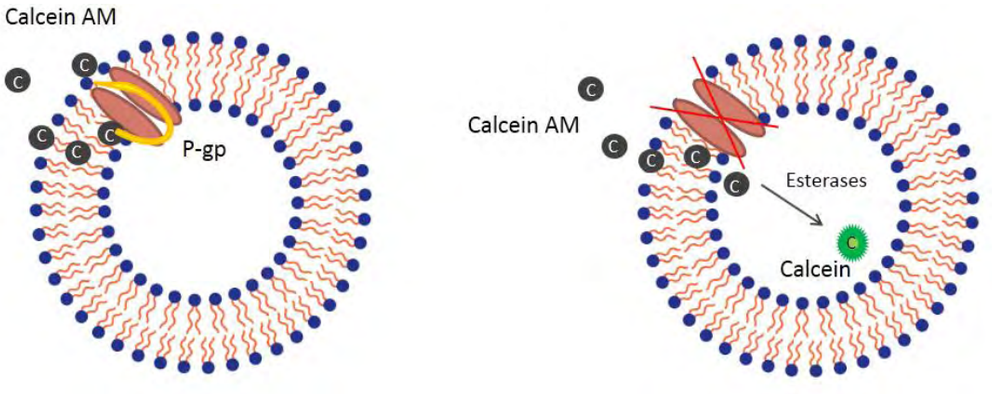


Figure 2.7.1. The basic principle of the calcein uptake. (Left) Calcein AM is a small, non-fluorescent molecule, able to penetrate the cell membrane. As a substrate of P-gp, calcein AM is immediately transported out of the cell. (Right) When P-gp is blocked by an inhibitor (e.g., verapamil), calcein AM is metabolized by endogenous esterases, resulting in green fluorescent form. This form is unable to leave the cells and accumulates inside the cell (changed after Niolip, 2015).

3. Methods

3.1 General procedures

As already mentioned in chapter 2.3.1, it is absolutely necessary to avoid contaminations in cell cultures. Therefore, it is essential to work under aseptic conditions to reduce significantly the risk of contaminations. The elements of aseptic technique are a sterile work area, personal hygiene, sterile reagents and sterile handling.

Personal hygiene is maintained by wearing gloves and a lab coat. This reduces the probability of contamination from bits of skin as well as dirt and dust particles from clothes. Additionally, it is important to wash hands and forearms with antibacterial soap and to use disinfectant before starting the cell culture work.

An aseptic working area is created with the laminar flow hood. Only items that are required for a particular procedure should be placed under the hood to ensure a laminar flow. Before cells are handled in a laminar flow hood, the surface must be wiped with 70% ethanol. The hands and all items that are brought under the hood, with the exception of cell culture flasks, are disinfected with 70% ethanol as well. All solutions and equipment that will come into contact with the cells must be sterile. Therefore, disposable, sterile plastic pipettes and commercial reagents, which undergo strict quality control to ensure their sterility, should be used. If using self-prepared solutions, an appropriate sterilization procedure (e.g., autoclave, 0.2 µm sterile filter) must be used to sterilize the liquids. Culture media cannot be sterilized by autoclaving because of temperature sensitive ingredients.

To reduce contamination while handling, all vessels should be kept closed. The use of antibiotics is risky, because it can mask contaminations and promote their distribution to other cultures. It is only used for cells that will be discarded after use (e.g., assay cells) or to protect valuable cell lines that are hard to reimburse (e.g., no additional vials from cell bank available).

3.2 Cell expansion and maintenance

Material:

Cell lines:

- Adherent cell culture (Caco-2 cells, MDCK cells, NIH-3T3 cells)

Cell culture medium:

For Caco-2 cells: AlphaMEM

- 20% Fetal Bovine Serum
- 4 mmol L^{-1} L-Glutamine
- 1 mmol L^{-1} Sodium Pyruvate
- 1% Non-essential Amino acids

For MDCK cells: DMEM/F12

- 5% Fetal Bovine Serum
- 2 mmol L^{-1} L-Glutamine

For NIH-3T3 cells: DMEM

- 10% Fetal Bovine Serum
- 4 mmol L^{-1} L-Glutamine
- 1 mmol L^{-1} Sodium Pyruvate

Buffer and Solutions:

- Dulbecco's Phosphate Buffered Saline (DPBS)
- Dissociation reagent (Accutase® and TrypLE™ Express)

Devices:

- Water bath
- 37 °C incubator with humidified atmosphere and 5% CO_2
- Aspiration system
- Aspiration pipette
- Pipette aid
- Table centrifuge

Disposables:

- Aspiration pipette (2 mL)
- Serological pipettes (5 mL, 10 mL, 25 mL)
- Reaction tube (1.5 mL)
- Sterile centrifuge tubes (15 mL, 50 mL)
- Cell culture flask (25 cm^2, 75 cm^2 or 175 cm^2)

The increase in cell number as a result of growth is accompanied by a depletion in nutrients and an increase in toxic metabolites. Therefore, cell lines cannot be indefinitely held in culture without subculturing. During subculturing adherent cells are dissociated and seeded with fresh medium to an appropriate cell number in new cell culture vessel. The appropriate seeding density for the cell lines is listed in table 3.2. Fresh medium is added, which provides the cells with nutrients (Schmitz, 2007).

Caco-2 and MDCK cells are adherent cell types. They are cultivated in the culture flask until they reach a confluence of 70% to 90%. At this point, they are detached from the surface of the cell culture flask and seeded into a new vessel.

In the first step, spent medium is removed from the cell culture flask with an aspiration pipette. The cells are washed once with DPBS (3 mL for 25 cm^2, 7 mL for 75 cm^2 or 10 mL for 175 cm^2) before the dissociation reagent is added to the monolayer (1 mL for 25 cm^2, 2 mL for 75 cm^2 or 3 mL for 175 cm^2). As standard dissociation reagent for Caco-2 and MDCK cells TrypLE™ Express is used. However, for the purpose of membrane protein analysis in subsequent flow cytometry, Accutase® is chosen for detachment of cells.

After adding the dissociation reagent, the cell culture flask is incubated at 37 °C and 5% CO_2 for approximately 7 minutes. Gently tapping after the incubation period helps to expedite the cell detachment. Tilting the flask afterwards, allow the cells to drain, before pre-warmed cell culture medium (5 mL for 25 cm^2, 8 mL for 75 cm^2 or 10 mL for 175 cm^2) is added to rinse off detached cells and to stop the enzymatic reaction. Cells are then transferred to a 15-mL or 50-mL centrifuge tube and centrifuged for 5 minutes at room temperature. Centrifugation speed depends on used cell type: For Caco-2 cells 80xg, and for MDCK and NIH 3T3 cells 180xg is used.

After centrifugation, the supernatant is removed and the remaining cell pellet is resuspended in pre-warmed cell culture medium (5 mL, 7 mL or 10 mL). A sample of the cell suspension (approximately 200 µL) is removed for automatic cell counting with the CASY® TT Cell Counter (see chapter 3.5).

The cell suspension is diluted to the appropriate seeding density and transferred in a new culture vessel. Afterwards the cells are returned to the incubator until the next passage.

Table 3.2 Appropriate seeding density of Caco-2, MDCK and NIH-3T3 cells

Next cell expansion in:	2 days	3 days	4 days
Seeding density for Caco-2 cells	$6.0 \cdot 10^4$ cells·cm^{-2}	$3.0 \cdot 10^4$ cells·cm^{-2}	$1.0 \cdot 10^4$ cells·cm^{-2}
Seeding density for MDCK cells	$4.0 \cdot 10^4$ cells·cm^{-2}	$1.0 \cdot 10^4$ cells·cm^{-2}	$3.0 \cdot 10^3$ cells·cm^{-2}
Seeding density for NIH 3T3 cells	$3.0 \cdot 10^4$ cells·cm^{-2}	$1.2 \cdot 10^4$ cells·cm^{-2}	$5.0 \cdot 10^3$ cells·cm^{-2}

3.3 Cryopreservation

The reason for cryopreservation are described in detail in the chapters 2.3.1 and 2.4.1. Long-term cultivation might alter important genotypic and phenotypic properties, leading to variations in results obtaining with the cells. In addition, long-term cultivation increases the risk of accidently loss of culture due to contamination. Cryopreservation of cells supplies a backup for losses due to contamination or accidents. A periodically refreshing of the continuous culture with cells from a cryopreserved cell stocks minimize variations in results due to alteration of long-term cultivation.

Material:

Cell lines:

Detached and counted cells (Caco-2 cells, MDCK cells) in cell culture medium

Cell culture medium:

For Caco-2 cells: AlphaMEM

- 20% Fetal Bovine Serum
- 4 mmol L^{-1} L-Glutamine
- 1 mmol L^{-1} Sodium Pyruvate
- 1% Non-essential Amino acids

For MDCK cells: DMEM/F12

- 5% Fetal Bovine Serum
- 2 mmol L^{-1} L-Glutamine

Buffer and Solutions:

- Dulbecco's Phosphate Buffered Saline (DPBS)
- Dimethylsulfoxide (DMSO)
- New Freezing Medium Generation 2 (NFM-G2)

Devices:

- Water bath

- Aspiration system
- Pipette aid
- Table centrifuge
- Controlled rate freezer or freezing device (isopropanol chamber, polyethylene foam box)
- Freezer (-80°C)
- Liquid nitrogen storage container (-196 °C)

Disposables:

- Aspiration pipette (2 mL)
- Serological pipettes (5 mL, 10 mL, 25 mL)
- Centrifuge tubes (15 mL, 50 mL)
- Cryovials (1.8 mL)

Before cells can be frozen, they have to be detached from the cell culture flask with a dissociation reagent. The procedure of detachment has already been described in the previous section (see chapter 3.2). After cell counting (chapter 3.5), the needed volume of cell suspension is calculated, which leads to a cell concentration of $5 \cdot 10^6$ cells·vial^{-1} (in a final volume of 1.8 mL). The amount of cell suspension is than centrifuged for 5 minutes (for Caco-2 cells: 80xg, and for MDCK cells 180xg) and resuspended in the required volume of freezing medium. Two different freezing media are used in this master thesis: As “standard freezing medium,” cell specific culture medium is used with freshly added DMSO to a final concentration of 5%. As serum free alternative to the standard freezing medium, the “New Freezing Medium Generation 2” (NFM-G2) was developed by Elisabeth Schulze at acCELLerate GmbH, which also containing freshly added DMSO in same concentration.

A volume of 1.8 mL cell suspension in freezing medium is filled into 1.8 mL cryovials. The cryovials are transferred to a controlled rate freezer or a freezing device. The controlled rate freezer decreases the temperature at a rate of 1 °C·min^{-1} to -80 °C. A similar cooling rate can be obtained with the freezing device if it is stored in a -80°C freezer overnight. Once the final temperature is reached, the cryovials are stored in a gas phase of a liquid nitrogen storage tank. The final storage temperature is between -186 °C and -176 °C.

3.4 Thawing Frozen cells

Material:

Cell lines:

Cryovial containing frozen cells (Caco-2 cells, MDCK cells)

Cell culture medium:

For Caco-2 cells: AlphaMEM

- 20% Fetal Bovine Serum
- 4 mmol L^{-1} L-Glutamine
- 1 mmol L^{-1} Sodium Pyruvate
- 1% Non-essential Amino acids

For MDCK cells: DMEM/F12

- 5% Fetal Bovine Serum
- 2 mmol L^{-1} L-Glutamine

Devices:

- Water bath
- Aspiration system
- Pipette aid
- Table centrifuge

Disposables:

- Aspiration pipette (2 mL)
- Serological pipettes (5 mL, 10 mL, 25 mL)
- Centrifuge tubes (15 mL, 50 mL)
- Cryovials (1.8 mL)
- Pipettes (5 mL, 10 mL, 25 mL)
- Reaction tube (1.5 mL)
- Cell culture flask (25 cm^2, 75 cm^2 or 175 cm^2) or cell culture plate (96-well, 24-well, Transwell®)

The thawing process is stressful to cells and should proceed as fast as possible. The cryovial containing the frozen cells is removed from the liquid nitrogen tank and placed for 2 minutes into a 37 °C water bath. To remove the toxic cryoprotectant DMSO from the cells, the cell suspension is diluted in 10 mL pre-warmed medium and centrifuged for 5 minutes at room temperature (for Caco-2 cells: 80xg, and for MDCK cells 180xg). The supernatant is then aspirated and the cell pellet is resuspended again in pre-warmed medium. A sample of approximately 200 µL is taken for automated cell counting with the CASY® TT Cell Counter (chapter 3.5).

3.5 Electronic cell counting

The CASY® Cell Counter measures the cell number and viability (live, dead and total cells) in a sample due to the measurement of resistance causing by the cells when they flow through a capillary.

Cell lines:

Cell suspension which should be measured

Buffer and Solutions:

- CASY® ton

Devices:

- Pipette (100 µL)

Disposables:

- Reaction tube (1.5 mL)
- CASY® cups

A sample of the cell suspension (approximately 200 µL) is removed under sterile conditions for automated cell counting with the CASY® TT Cell Counter. From this sample 70 µL well mixed cell suspension are taken and diluted in 6930 µL (filled in CASY® cups) of an isotonic electrolyte solution (CASY®-Ton). By inverting the CASY® cup three times, the cells were distributed homogeneously in the buffer. The measurement capillary of the CASY® TT Cell Counter is placed in the CASY® cup containing the sample liquid. An aliquot is automatically aspirated and flow through the

capillary, where an electric field is generated via two platinum electrodes. The electrolyte-filled capillary has a defined electrical resistance.

Intact cells can be considered isolators. Each time a cell passes through the capillary, the electrical field changes. The resulting change of the field is proportional to the volume of the cells (or any other particle). In contrast, the membrane of dead cells no longer acts as an electrical barrier. These cells are recorded by the size of their cell nucleus. Each particle (cell) generates one peak in the electrical field. The produced series of signals generate the CASY®-Histogram. In the histogram, the distribution of particle (cell) size is shown. The shown data are the mean of triplicates.

Threshold values are defined in advance for every cell type, so that every cell type has a own counting program. The histogram is divided in debris, dead cells, counted cells and aggregates. The set of these parameters is done by determining the range of dead cells. Therefore, cells are treated with 70% Ethanol for 5 minutes and counted in the CASY® TT. To check whether thresholds for the dead cells were set correctly, living cells are added to the sample and counted again. The first threshold is set at the beginning of the dead cell peak to eliminate small particles (cellular debris). The second threshold is set at the end of the dead cell peak and indicates the beginning of living cell peak. A third threshold can be set either set to infinity or is adjustable and defines the maximum particle sizes, which should be recognized in the measurement. Important parameters derived from the CASY® Histogram are the number of living and dead cells, the viability and aggregation factor. The confidence interval of the measurement is reached once the counts reach between 1,000 and 20,000. For a reliable cell count, the aggregation factor should be below 2.5. Otherwise, the clumping of the sample needs to be reduced and it has to be measured again.

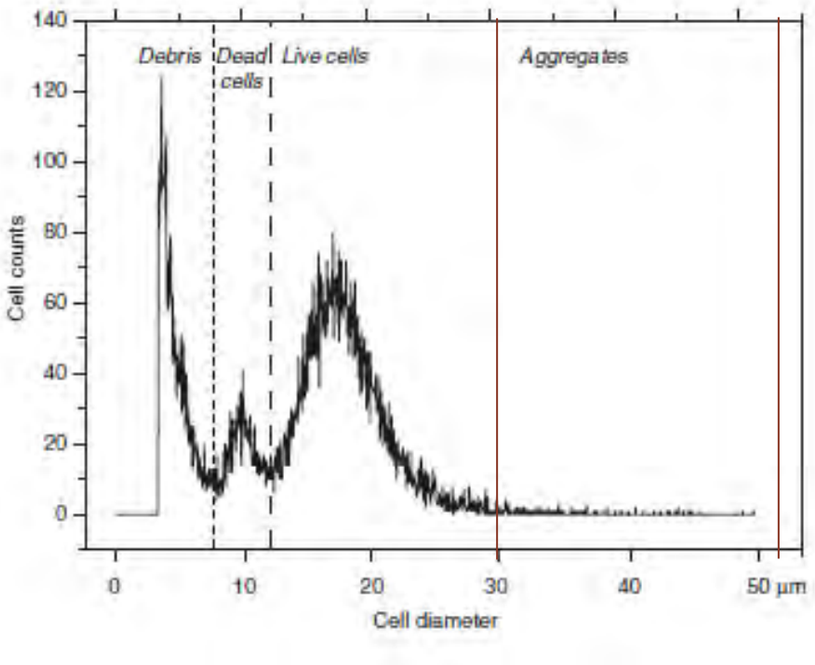


Figure 3.5 Size distribution in CASY® histogram. The peak between zero and the dotted line at 7.6 µM is the debris peak. The dead cells no longer have an intact membrane and are shown by the size of their nucleus. This peak is visible between 7.6 µM and 12.8 µM. This peak is followed by the peak for the living cells followed by cell aggregates (>30µM) (Freshley, 2010).

3.6 24-hour vitality test

Material:

Cell lines:

Cryovial containing frozen cells (Caco-2 cells, MDCK cells)

Cell culture medium:

For Caco-2 cells: AlphaMEM

- 20% Fetal Bovine Serum
- 4 mmol L^{-1} L-Glutamine
- 1 mmol L^{-1} Sodium Pyruvate
- 1% Non-essential Amino acids

For MDCK cells: DMEM/F12

- 5% Fetal Bovine Serum
- 2 mmol L^{-1} L-Glutamine

Buffer and Solutions:

- Dulbecco's Phosphate Buffered Saline (DPBS)
- Dissociation reagent, TrypLE™ Express

Devices:

- Water bath
- 37 °C incubator with humidified atmosphere and 5% CO_2
- Table centrifuge

Disposables:

- Centrifuge tubes (50 mL)
- Pipettes (5 mL, 10 mL, 25 mL)
- Reaction tube (1.5 mL)
- Cell culture flask (25 cm^2)

The 24-hour vitality values are used to evaluate the freezing and thawing process of frozen cells. The cryovial, containing the frozen cells is removed from the liquid nitrogen tank and placed for 2 minutes into a 37 °C water bath. The cell suspension in the cryovial is gentle resuspended with a 2 mL pipette. A sample of approximately 200 µL is taken for automated cell counting with the CASY® TT Cell Counter (chapter 3.5), serves as sample "after thawing". After the determination of cell number, $2.0 \cdot 10^6$ cells in 5 mL cell specific culture medium are seeded into a 25 cm^2-cell culture flask and incubated for 24 hours at 37 °C and 5% CO_2.

At the next day the supernatant is transferred into a 50-mL centrifuge tube. The cell layer is washed once with 500 µL DPBS and collected in the same centrifuge tube. Afterwards 500 µL TrypLE™ Express is added for cell detachment. After adding the dissociation reagent, the cell culture flask is incubated at 37 °C and 5% CO_2 for approximately 7 minutes.

The collected cell culture medium (spent medium and DPBS) is used to rinse off detached cells and to stop the enzymatic reaction. The cell suspension is well mixed by invert the centrifuge tube several times. A sample of approximately 200 µL is taken for automated cell counting with the CASY® TT Cell Counter (chapter 3.5). This is the "after 24 in culture" in culture sample.

3.7 Limited dilution cloning

The tumor cell lines MDCK- and Caco-2 consist of heterogeneous sub-populations. Cell to cell variations cover a wide range of cellular properties, e.g. biochemical and morphological differences. The technique of limited dilution is commonly used to generate homogeneous clones and to isolate clones with desired properties. (Volpe, 2008, Sambuy et al., 2005).

Material:

Cell lines:

- Detached and counted cells (Caco-2 cells, MDCK cells) in cell culture medium

Conditioned and 0,2µM filtered Cell culture medium:

For Caco-2 cells: AlphaMEM

- 20% Fetal Bovine Serum
- 4 mmol L^{-1} L-Glutamine
- 1 mmol L^{-1} Sodium Pyruvate
- 1% Non-essential Amino acids
- 1% Pen/Strep

For MDCK cells: DMEM/F12

- 5% Fetal Bovine Serum
- 2 mmol L^{-1} L-Glutamine
- 1% Pen/Strep

Buffer and Solutions:

- Dissociation reagent (Accutase® and TrypLE™ Express)

Devices:

- Water bath
- 37 °C incubator with humidified atmosphere and 5% CO_2
- Aspiration system
- Aspiration pipette
- Pipette aid

Disposables:

- Aspiration pipette (2 mL)
- Serological pipettes (5 mL, 10 mL, 25 mL)
- Reaction tube (1.5 mL)
- Centrifuge tubes (15 mL, 50 mL)
- 96-well cell culture plate
- 24-well cell culture plate
- 12-well cell culture plate
- 6-well cell culture plate
- Cell culture flask (25 cm^2, 75 cm^2 or 175 cm^2)
- Filter tube, 0,2 µM

To start a subcloning by limited dilution, the cell suspension is diluted to a concentration of 0.4 cells per well. The initial cell suspension is diluted to a final concentration of $2.81 \cdot 10^6$ cells·mL^{-1} in a volume of 20 mL. Afterwards, 200 µL are transferred in each well of a 96-well cell culture plate. Directly after cell seeding, the wells can be microscopically examined. Wells containing a single cell are marked with a pen. The cells are incubated at 37 °C, humidified atmosphere and 5% CO_2. After the fourth day, the medium in the well is aspirated and exchanged with 0.2 µM filtrated supernatant of growing cell culture (conditioned medium) from the same cell line. Medium exchange with conditioned medium occur once a week until the cells in the marked wells reach confluence. Not all single cells were able to proliferate. Cells that have succeeded confluence, were detached and seeded into the next larger cell culture plate. As soon as sufficient cell density was achieved, $1.0 \cdot 10^6$ cells of each subclone were cryopreserved as backup, while the other part serves for further experiments.

3.8 Intestinal permeability assay

Material:

Cell lines:

Detached and counted cells (Caco-2 cells, MDCK cells) in cell culture medium

Cell culture medium:

For Caco-2 cells: AlphaMEM

- 20% Fetal Bovine Serum
- 4 mmol L^{-1} L-Glutamine

- 1 mmol L^{-1} Sodium Pyruvate
- 1% Non-essential Amino acids
- 1% Pen/Strep

For MDCK cells: DMEM/F12

- 5% Fetal Bovine Serum
- 2 mmol L^{-1} L-Glutamine
- 1% Pen/Strep

Buffer and Solutions:

- Dulbecco's Phosphate Buffered Saline (DPBS)
- Sterile Water
- Transport buffer (TB): HBSS, 1mM HEPES pH 7.4
- Receiver solution (RS): HBSS, 1mM HEPES pH 7.4, 0.01 mM Cyclosporin A.
- Donor solution (DS): HBSS, 1mM HEPES pH 7.4, 0.01 mM Cyclosporin A, $1{,}2 \cdot 10^{-3}$ mM Texas Red.
- 5 µM Amitriptyline solved in Transport buffer
- 50 µM Gabapentin solved in Transport buffer

Devices:

- Water bath
- Aspiration system
- Pipette aid
- Electronic pipette
- Table centrifuge
- 37 °C incubator with humidified atmosphere and 5% CO_2
- Micro Plate Reader Safire^2, Software: XFluor4 Safire^2

Disposables:

- Aspiration pipette (2 mL)
- Serological pipettes (5 mL, 10 mL, 25 mL)
- Centrifuge tubes (15 mL, 50 mL)
- Pipette tips (1 mL)
- Reaction tube (1.5 mL, 5 mL)
- 24-well cell culture plate
- Transwell® inserts
- Aluminum foil
- 96-well Plate, flat-black

3.8.1 Seeding cells in Transwell® inserts

Cells from a continuous cultivation are cultivated in cell culture flasks, detached and counted (see chapter 3.2 and 3.5). When use frozen cells, these are thawed, washed and counted (see chapter 3.5) before seeded into Transwell™.

After the determination of cell concentration, the cell suspension is diluted with cell culture medium containing 1% Pen/Strep, in a sterile 1.5-mL reaction tubes to a concentration ranging from $6.0 \cdot 10^4$ cells mL^{-1} to $6.0 \cdot 10^5$ cells mL^{-1} in a volume of 1 mL. These cell concentrations are used to seed $8.0 \cdot 10^4$ cells$\cdot cm^{-2}$ to $8.0 \cdot 10^5$ cells$\cdot cm^{-2}$ in a Transwell™ with area of 0.3 cm^2. This is based on a seeding volume of 400 µL. A filterwell only filled with medium serves as control.

A 24-well cell culture plate with Transwell™ inserts are used for the intestinal permeability assay. Per well 400 µL of cell suspension are filled into the donor compartment of the Transwell™ and the same volume of medium is filled into the receiver compartment. Afterwards, the plate is placed for 72 hours at 37 °C and 5% CO_2. Sterile water in the unused wells limit the evaporation. After the 3th day the initially level in the donor compartment is adjusted with 200 µL medium and cultivated for one more day at 37 °C and 5% CO2.

3.8.2 Assessment of cell monolayer integrity with Texas Red Dextran

Depending on the experiment, but in general after 4 days, the integrity of the monolayer is determined with Texas Red Dextran. Texas Red Dextran is not actively transported through cells and will only diffuse through leaky areas in the monolayer. The amount of dye penetrated through the cell layer can be detected via fluorescence measurement at 590 nm.

In the first experiments (where an aspiration rate of 13.92 $mL \cdot s^{-1}$) is used the monolayer in the apical compartment is washed three times, the basolateral compartment once with 300 µL of the transport buffer before the donor solution (containing Texas Red Dextran or drug) is added in a volume of 300 µL into the apical compartment. The basolateral compartment contains the same volume but with receiver solution (same buffer as donor solution, but without the dye or drug). Because Texas Red Dextran is sensitive to light, the plate is covered with aluminum foil when incubated for 3 hours at 37 °C and 5% CO_2.

3.8.3 Calculation of results

After 3 hours of incubation, the volume of fluid in the basolateral compartment, as well as in the apical compartment is determined with an electronic pipette and collected separately in 1.5-mL reaction tubes. A sample of 50 µL is taken and transferred into a well of a black 96-well plate. The fluorescence is measured at an excitation wavelength of 590 nm and emission wavelength of 635 nm in the Microplate Reader Safire2 (Tecan). A standard series (0 $ng \cdot mL^{-1}$, 1.8 $ng \cdot mL^{-1}$ and 3.6 $ng \cdot mL^{-1}$) of Texas Red Dextran is applied in each experiment to determine the concentration of

Texas Red Dextran in the basolateral compartment as well as in the apical compartment. With the known volume, the mass can be determined (Eq. 2) and a mass balance can be draw set (Eq. 3).

$$m_{Texas\ Red\ Dextran}\,[ng] = \frac{RFU_{donor\ or\ receiver\ compartment} \cdot Volume\ [\mu L]}{correlation\ coefficient\ \left[\frac{RFU\ mL}{ng}\right] \cdot 1000} \qquad \text{(Eq. 2)}$$

$$\%\text{-}Permeability_{Texas\ Red\ Dextran} = \frac{m_{Texas\ Red\ Dextran,receiver\ compartment}}{m_{Texas\ Red\ Dextran,receiver\ +donor\ compartment}} \cdot 100 \quad \text{(Eq. 3)}$$

3.8.4 Drug transport assay

Drugs and drug candidates are ranked according their permeability values through the Caco-2 monolayer in low-, moderate- and high permeability compound. The permeability of drugs through the Caco-2 monolayer is determined with the apparent permeability coefficient (P_{app}) analog to equation 1 in chapter 2.1.3.
The Caco-2 system is characterized in advanced with reference compounds of known permeability behavior. One of the reference compounds is Gabapentin or Amitriptyline hydrochloride. Amitriptyline hydrochloride is known to be highly permeable in Caco-2 cells whereas Gabapentin has low permeability. Depending on the permeability value of the drugs through the Caco-2 monolayer, the unknown drug or drug candidate can be classified and help to predict the potential behavior of the drug in the human intestine.
The permeability of the reference compounds Amitriptyline hydrochloride and Gabapentin are determined, and compared with P_{app} values in the literature to evaluate the functionality of the Caco-2 monolayer.

An initial concentration (C_0) of 5 µM Amitriptyline and 50 µM Gabapentin in volume of 300 µL is added on the apical side of the monolayer. After an incubation period of 2 hours ($t_1 – t_0$ =7200 s) at 37°C, fluids of the basolateral and apical compartment are collected in reaction tubes. The initial drug concentration and the concentration in the basolateral compartment are analyzed by a combination of liquid chromatography and mass spectrometry (LC-MS/MS) at the AescuLabor Hamburg GmbH.

.

3.9 Transepithelial electrical resistance measurement

Material:

Cell lines:

Cells seeded in Transwell™ inserts in an appropriate seeding density

Cell culture medium:

For Caco-2 cells: AlphaMEM

- 20% Fetal Bovine Serum
- 4 mmol L^{-1} L-Glutamine
- 1 mmol L^{-1} Sodium Pyruvate
- 1% Non-essential Amino acids
- 1% Pen/Strep

For MDCK cells: DMEM/F12

- 5% Fetal Bovine Serum
- 2 mmol L^{-1} L-Glutamine
- 1% Pen/Strep

Solutions:

- 70% ethanol

Devices:

- 37 °C incubator with humidified atmosphere and 5% CO_2
- Voltohmmeter

Disposables:

- 24-well cell culture plate
- Transwell® inserts

Transepithelial electrical resistance (TEER) is a method for the assessment of the monolayer integrity by the measurement of ion movement across the para-cellular pathway. The formation of tight junctions between epithelial

cells determine the resistance of the monolayer. The resistance is measured with the voltohmmeter (EVOM^2). TEER measurements are done before the assessment of cell monolayer integrity with Texas Red Dextran (chapter 3.8.3) and drug transport assay (chapter 3.8.4). The preparation of cells is executed as described in chapter 3.8.1.

TEER measurements are performed at room temperature. Therefore, the cells are taken out of the 37°C incubator and placed under the laminar flow hood for at least 30 minutes, before starting the measurements. While cells adjust to room temperature the voltohmmeter is calibrated with a 1000 Ω-test resistor. The electrode is sterilized by immersing it in 70% ethanol for 15 minutes. After the sterilization process the electrode is rinsed with sterile tissue culture medium. The electrodes are immersed, in a way that the shorter electrode is in the Transwell™ insert and the longer one passes the Transwell™ between the outer wall of the well and the Transwell™ insert (see figure 3.9).

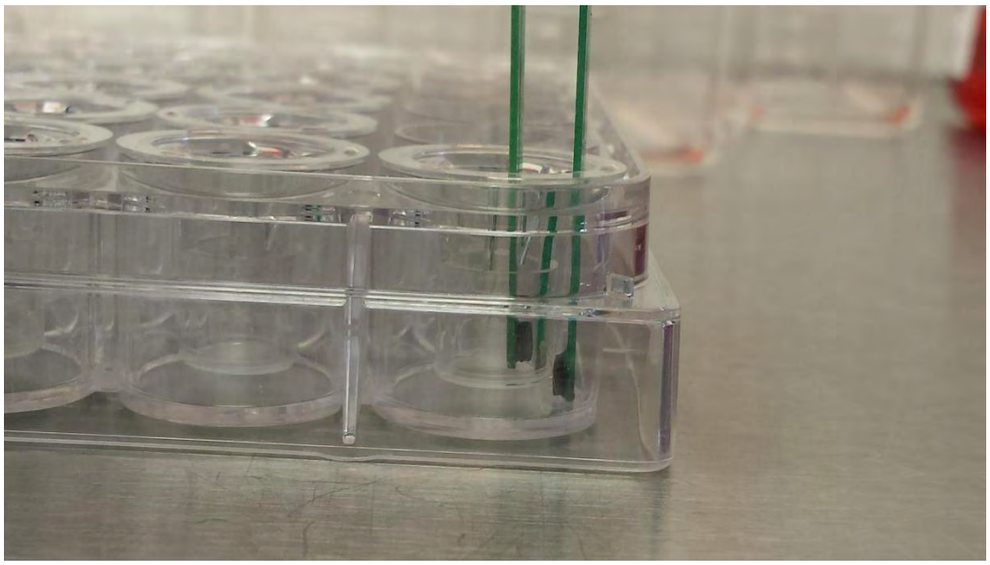


Figure 3.8 positioning of electrode in Transwell™. The lengths of the electrodes are unequal. The electrodes are immersed so that the shorter electrode is in the Transwell™ insert and the longer electrode is inserted between the outer wall culture well and the Transwell™ insert (own illustration).

To ensure that the electrode tips are completely covered, 200 µL cell culture medium (1% Pen/Strep) are added to the apical side of the Transwell™ insert and 600 µL to the basolateral side of the Transwell™. The TEER value is calculated by the following equation:

$$TEER\ [\Omega \cdot cm^2] = Resistance\ [\Omega] \cdot Area\ of\ the\ monolayer\ [cm^2] \quad \text{(Eq. 4)}$$

The blank resistance is measured by immersing them into a Transwell™ containing the same tissue culture medium but without cells growing on it. The blank value is subtracted from the value obtained when measured across the cell

layer. Then the blank corrected value is converted to the TEER value by multiplying it by the surface area of the Transwell™ insert (0.3 cm^2).

3.10 Assessment of P-glycoprotein (P-gp) activity in flow cytometry by calcein AM uptake

Material:

Cell lines:

- $1.0 \cdot 10^6$ cells·mL^{-1} (1 mL) Caco-2 Zellen oder MDCK Zellen
- $1.0 \cdot 10^6$ cells·mL^{-1} (1 mL) NIH-3T3 als negative Kontrolle

Buffer:

- Dulbecco's Phosphate Buffered Saline (DPBS)
- Verapamil
- Calcein AM
- Propidium iodide (10 µg·mL^{-1})
- IsoFlow™ Shealth Fluid
- COULTER CLENZ® Cleaning Agent
-

- Devices:
- Flow cytometer
- 37 °C incubator with humidified atmosphere and 5% CO_2
- Pipette
- Table centrifuge

- Disposables:

- Flow cytometer tubes
- Reaction tube (1.5 mL)

Before the cells are analyzed by flow cytometry, a series of experiments were necessary to set the instrument parameters and gates in the plots for a proper analysis. Once created, the settings serve as a template for the following measurements (figure 3.10). Only the population gate (figure 3.10 A1) needs to be readjusted slightly for a new series of measurements. The first plot (figure 3.10 A1) displays the cell population separated in cell size through

the forward scatter (FSC) and granularity through the sideward scatter (SSC). The flow cytometer acquires as long as a threshold of 5000 events is reached.

Several gates are set in the plots (population, living cells, dead cells, calcein positive) to define certain characteristic of the cells and to include or exclude them for further analysis. In the first plot, the gate is set around the population, which should be taken into account for the next plot.
For calcein positive cells (plot 3.10 A3), only healthy cells are considered. Dead cells are excluded by propidium iodide by plot B2 and A2, respectively. The fluorescence signal of propidium iodide and calcein are displayed in separate plots as histogram (B4 and A4, respectively).

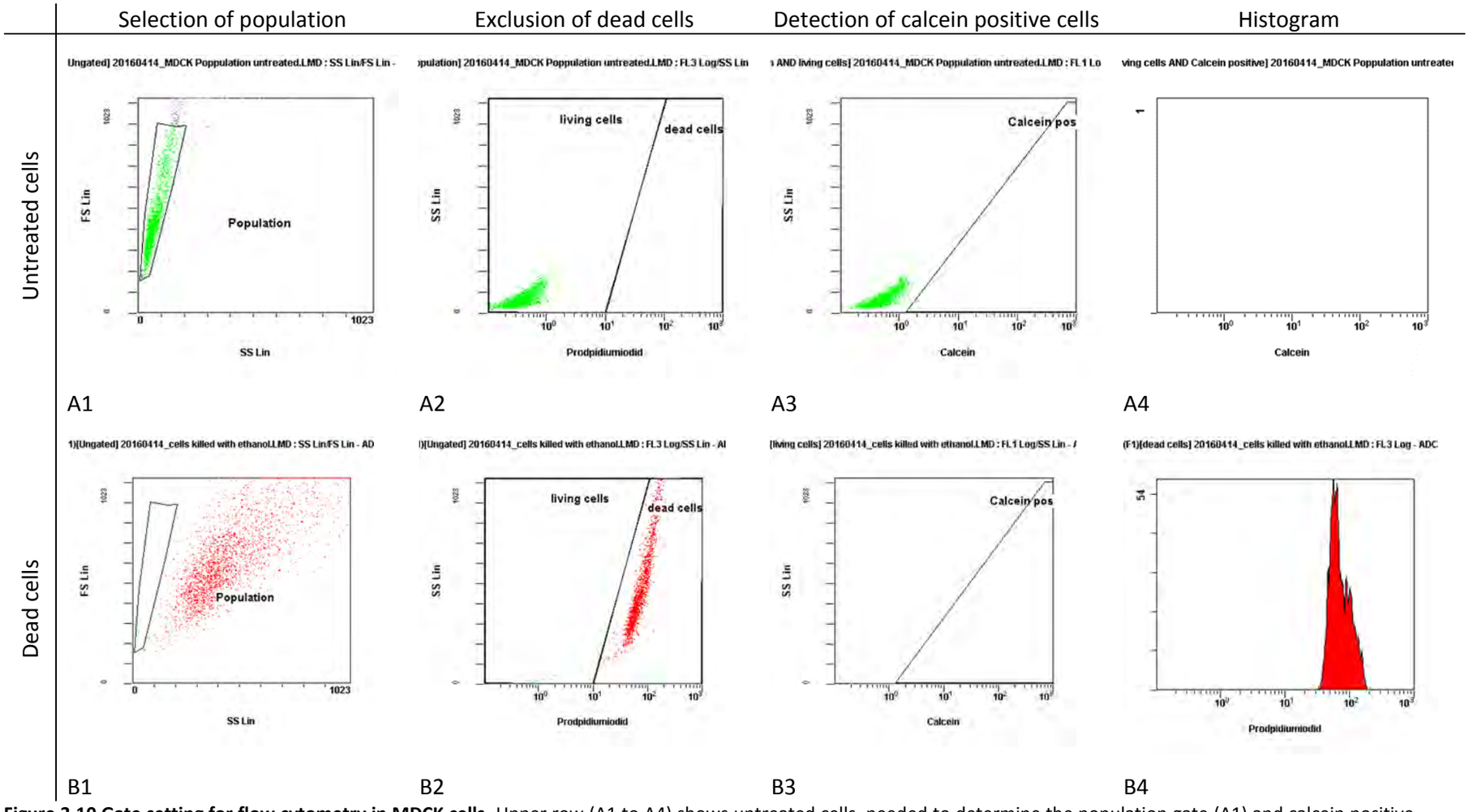


Figure 3.10 Gate setting for flow cytometry in MDCK cells. Upper row (A1 to A4) shows untreated cells, needed to determine the population gate (A1) and calcein positive gate (A3). Bottom row (B1 to B4) show MDCK cells killed with 70% Ethanol for determination of dead cells gate (B2 and A2). First row (A1 and B1) shows forward versus sideward scatter. Propidium iodide indicates dead cells in the second row. For calcein detection only living cells are taken into account (A3). The right figure will show the fluorescence signal in a histogram, in A4 for calcein and in B4 for propidium iodide.

To assess the P-glycoprotein activity in flow cytometry, following samples are required:

- Unstained cells
- Dead cells (treated with 70% ethanol) stained with propidium iodide
- Cells treated with calcein AM and stained with propidium iodide
- Cells treated with verapamil, calcein and stained with propidium iodide

Cells from continuous culture are detached and counted (see chapter 3.2 and 3.5). After cell counting, the cells are adjusted to a cell density of $1.0 \cdot 10^6$ cells$\cdot$mL^{-1} in a volume of 1 mL.

For flow cytometry measurements of calcein AM uptake, Caco-2, MDCK and NIH 3T3 cells are pre-incubated with 100 µM verapamil (final concentration) or with dimethyl sulphoxide (DMSO) for 5 minutes at room temperature, before calcein AM is added.

Calcein AM concentrations ranging from 0.25 µM to 25 pM (final concentration) are added to the cells and incubated for 10 minutes at 37 °C. Non-living cells were detected and gated out by propidium iodide staining. Therefore, shortly before measurement propidium iodide (final concentration 1 µg$\cdot$mL^{-1}) is added, gently mixed and incubated for 1 minute at room temperature (propidium iodide is exited at 488 nm and emitted at 617 nm). Excitation and emission wavelengths for calcein are 493 and 515 nm, respectively. Data acquisition is accomplished with a single-laser flow cytometer set at 488 nm excitation. Calcein-associated fluorescence emission is collected in the FL1 channel (530/30 band-pass filter), and propidium iodide associated fluorescence is collected in the FL3 channel (670 long-pass filter).

4. Results and Discussion

4.1 Assessment of the intestinal permeability assay

4.1.1 Ascertainment of seeding densities in the intestinal permeability assay

The seeding density has an influence on the expression of differentiation-related characteristics, since differentiation process only starts when cells reach confluence. Differentiation-related characteristics includes the formation of tight junctions, as well as the expression of the P-glycoprotein (P-gp) in Caco-2 and MDCK cell monolayers. In the literature, cell densities ranging from $8.0 \cdot 10^4$ cells·cm^{-2} to $8.0 \cdot 10^5$ cells·cm^{-2} are used for the rapid permeability assay.
If the cell density is too low, the cells do not have sufficient time to grow together and close the gaps inbetween. By contrast, a higher seeding density can cause the formation of multilayers, reflecting a disorganization of the cell layers.

To determine the best seeding density for the prospective permeability assays, different cell densities of Caco-2 and MDCK cells are seeded into the Transwells®. In this thesis, four seeding densities are choosen for Caco-2 as well as MDCK cells, covering the range reported in literature: $8.0 \cdot 10^4$ cells·cm^{-2}, $2.0 \cdot 10^5$ cells·cm^{-2}, $4.0 \cdot 10^5$ cells·cm^{-2} and $8.0 \cdot 10^5$ cells·cm^{-2}. The cells are seeded on the filter of the Transwell® in the 24-well plate and grown for 4 days with one feeding step at day three. In addition one Transwell® is used withoutcell ares as control. After 4 days the transport assay is performed as described in the chapter “materials and methods”. Thereby, Texas Red Dextran is indicating the integrity of the cell monolayer for different seeding densities. At least a permeability of Texas Red Dextran less than 6% is needed.

Ascertainment of seeding density for Caco-2 cells

The obtained permeability values for Caco-2 cells are shown in figure 4.1.1.1. In parallel cells were plated with the same seeding density in a 96-well plate, and are microcopicaly obsereved every day (figure 4.1.1.2). In all pictures domes are visible. If cells grow on an impermeable ground, like the bottom of the 96-well plate, they form domes, which are fluid-filled blisters,

generated by transepithelial transport of fluid and solutes, which are trapped between the cell layer and the water-impermeable culture dish (Gstraunthaler, 1988). Due to the optical properties of the filter material used in these experiments, an observation of cells grown on Transwell® is not possible. However, if necessary transpartent filterwells are comercial available an can be serve as alternative.

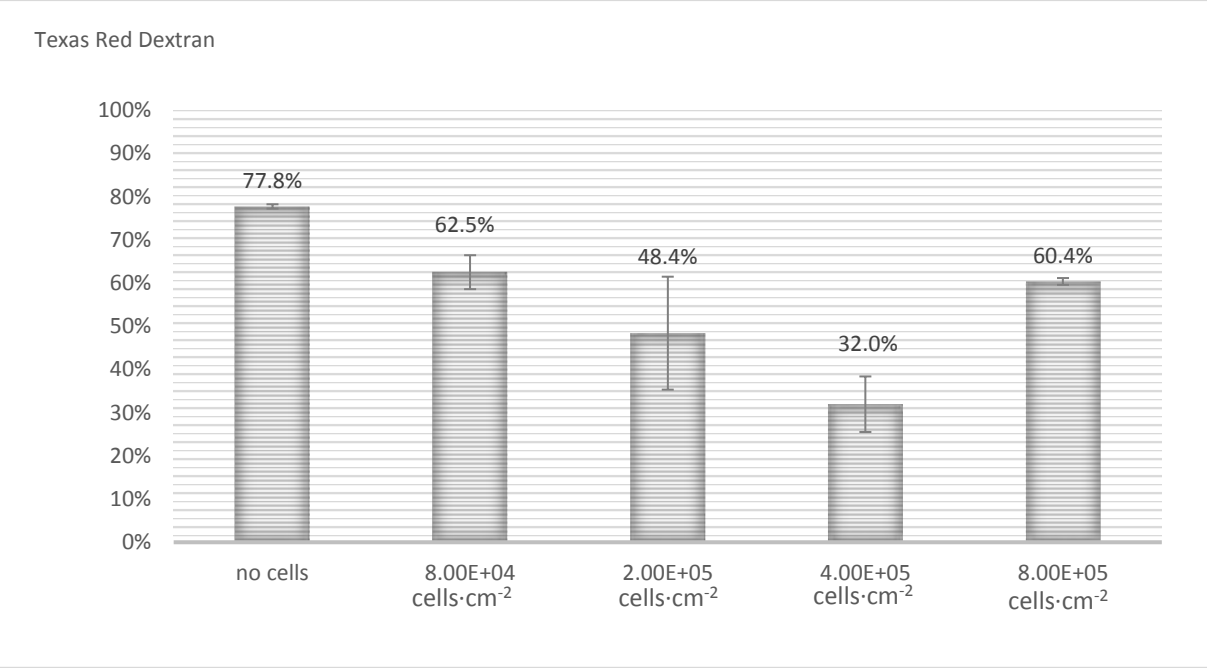


Figure 4.1.1.1 Determination of the Caco-2 monolayer integrity of different seeding densities after 4 days in culture. Texas Red Dextran will diffuse to leaky areas in the monolayer from the apical to the basolateral compartment. Measurement of the Texas Red Dextran after 3 hours at 37 °C in basolateral compartment as % of the total Texas Red Dextran amount applied to the monolayer (mean ±SD, n=2). The obtained permeability values differ from each other. The best permeability value was 32.0% obtained with a seeding density of 4.0E+05 cells·cm^{-2}, otherwise almost 60% (for 8.0·10^4 cells·cm^{-2}, 2.0·10^5 cells·cm^{-2} and 8.0·10^5 cells·cm^{-2}).

The control (no cells) show a Texas Red Dextran permeability of 77.8% (see figure 4.1.1.1).

The atmospheric pressure forces the fluid from the apical and basolateral compartment to a common level. During the incubation period of three hours, the volume in the apical compartment decreased from initial 300 µL to approximately 100 µL. In contrast the volume of the lower compartment increased by the same volume of 200 µL, from 300 µL to 500 µL. Assuming that Texas Red Dextran distributes homogeneously between the liquids of both compartments, a mass balance lead theoretically to an amount of 83.4% of Texas Red Dextran in the basolateral compartment (600µL =100%; 500µL = 83,4%). The detected value of 77.8% of Texas Red Dextran in the basolateral compartment (Figure 4.1.1.1, no cells) almost matches the theoretically amount of 83.4%. Caco-2 cells, grown on the filter of the Transwell®, will

decrease the permeability. The ratio of Texas Red Dextran passing the filters decreased to 62.5% with a seeding density of $8.0 \cdot 10^4$ cells·cm^{-2} or even to 32.0 % with $4.0 \cdot 10^5$ cells·cm^{-2}. However, the values are much higher than the desired threshold value of 6%. The results indicate that the Texas Red Dextran diffusion is only slightly blocked. The pictures of the monolayers (figure 4.1.1.2) show confluence within 4 days in all samples. As mentioned before, in all images dome formation is visible, indicating tightness of the monolayer. Cells seeded with a cell density of $8.0 \cdot 10^4$ cells·cm^{-2} show a thin monolayer build by cells of larger size in comparison to cells seeded with higher densities. Where the bounderies between the cells are clearly visible, dome formation occurs. In other parts of the monolayer, the cell boundaries are only slightly visible. It might be that in these regions the differentiation is not yet finished. With the lowest seeding density, the permeability of Texas Red Dextran is 62.5%. Almost the same value (60.4%) is reached with the highest seeding density in this experiment ($8.0 \cdot 10^5$ cells·cm^{-2}). A clear monolayer is not really visible, because it is covered by another, darker one. This can be cell aggregates or other cell layers, which leads to an incomplete monolayer, facilitating the diffusion of Texas Red Dextran. Although with a seeding density of $2.0 \cdot 10^5$ cells·cm^{-2}, fewer cells are used and mutilayer formation is visible, explaining the permeability of 48.4%. The best result was obtained with seeding density of $4.0 \cdot 10^5$ cells·cm^{-2} resulting in a permeability of 32.0%. Here a monolayer is cleary visible, although the cells a higher density in comparison to the lowest seeding desity. The desired permeability value of Texas Red Dextran of less than 6% is not seen in this experiment.

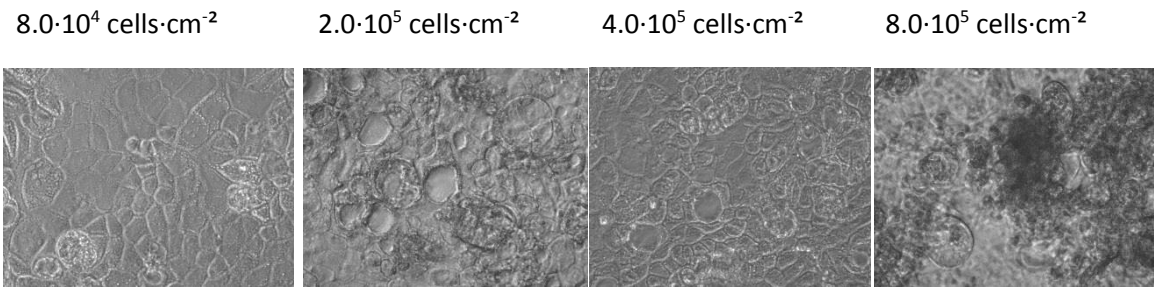


Figure 4.1.1.2 Microscopic picture of Caco-2 cells at different seeding densities in a 96-well Plate in a 200x magnification. Independently from their seeding density, cells reach confluence within 4 days. In all images dome formation is visible, indicating tightness of the monolayer. Cells seeded with a cell density of $8.0 \cdot 10^4$ cells·cm^{-2} show a thin monolayer with larger cell size in comparison to cells seeded at higher densities. At the highest seeding density ($8.0 \cdot 10^5$ cells·cm^{-2}) a clear monolayer is not visible. Here additional cells cover the first layer. These cells can be cell aggregates or other cell layers, which leads to disruption of the monolayer. Although with a seeding density of $2.0 \cdot 10^5$ cells·cm^{-2}, less cells are used, mutilayer formation is visible. At a seeding density of $4.0 \cdot 10^5$ cells·cm^{-2} a monolayer is cleary visible, although the cells are more dense in compare to the lowest seeding desity.

Ascertainment of seeding densities for MDCK cells

The obtained permeability values for MDCK cells are shown in figure 4.1.1.3. The permeability values, obtained with the tested seeding densitis differ only slightly from each other. At the lowest seeding density ($8.0 \cdot 10^4$ cells·cm^{-2}) only 9.0% of the applied Texas Red Dextran diffuses through the cell monolayer. With increasing seeding densities, the permeability remains almost unchanged ($2.0 \cdot 10^5$ cells·cm^{-2} = 10.9%, $4.0 \cdot 10^5$ cells·cm^{-2} and $8.0 \cdot 10^5$ cells·cm^{-2} = 12.2%). The average permeability is 11.1% with a standard deviation of 1.5%.

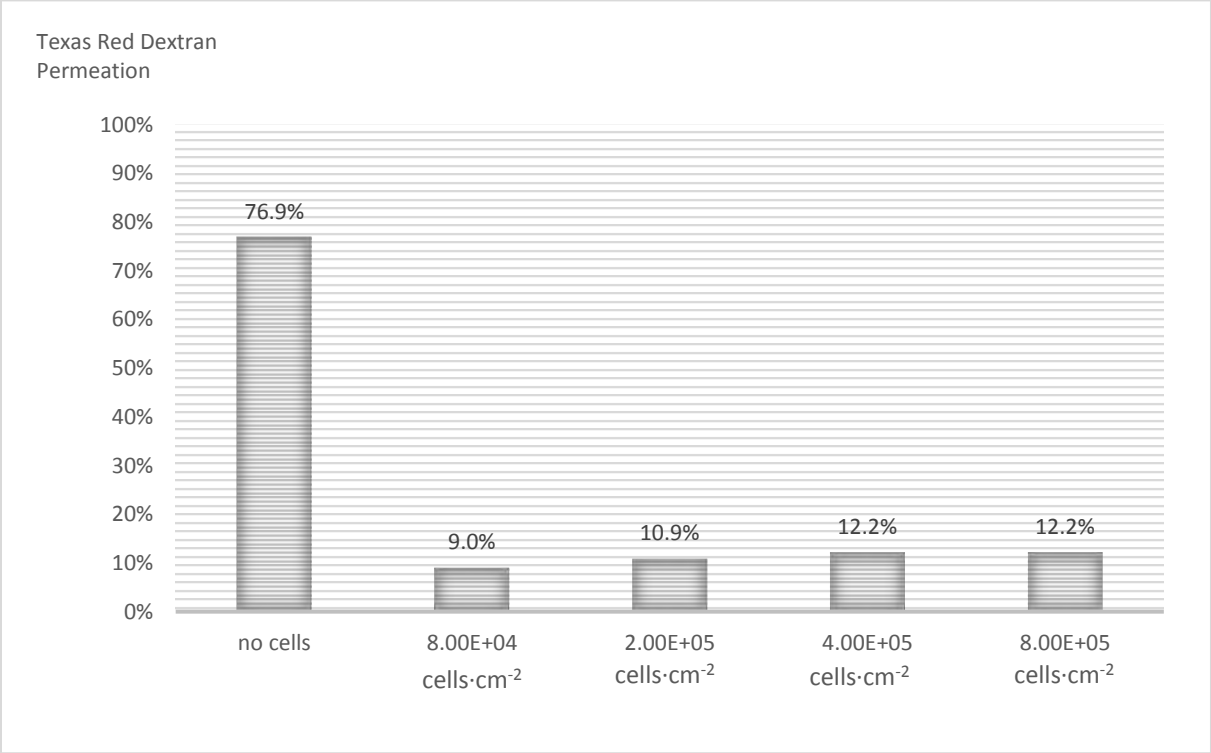


Figure 4.1.1.3 Determination of the MDCK monolayer integrity of different seeding densities after 4 days in culture. Texas Red Dextran will diffuse through leaky areas in the monolayer from the apical to the basolateral compartment. Measurement of the Texas Red Dextran after 3 hours at 37 °C in apical compartment as a % of the total Texas Red Dextran amount applied to the monolayer. The permeability value, obtained in the tested seeding densitis differ not greatly from each other. At the lowest seeding density ($8.0 \cdot 10^4$ cells·cm^{-2}) only 9.0% of the applied Texas Red Dextran diffuse through the cell. With increasing seeding density, the permeability remains almost unchanged ($2.0 \cdot 10^5$ cells·cm^{-2} = 10.9%, $4.0 \cdot 10^5$ cells·cm^{-2} and $8.0 \cdot 10^5$ cells·cm^{-2} = 12. 2%). The average permeability is 11.1% with a standard deviation of ±1.5%.

In comparison to Caco-2 cells, the MDCK show a considerably lower permeability, suggesting that the cell layer of MDCK cells is denser. While the permeability value of Caco-2 is 32.0%

($4.0 \cdot 10^5$ cells·cm^{-2}) in the best case, the seeding density for MDCK cells does not seem to have a decisive effect on their permeability. At the lowest seeding density ($8.0 \cdot 10^4$ cells·cm^{-2}), only 9.0% of the applied Texas Red Dextran diffuse through the cell. With increasing seeding densities, the permeability remains almost unchanged ($2.0 \cdot 10^5$ cells·cm^{-2} = 10.9%, $4.0 \cdot 10^5$ cells·cm^{-2} and $8.0 \cdot 10^5$ cells·cm^{-2} = 12, 2%). It might be the result of a higher proliferation rate of MDCK cells in comparison to Caco-2 cells.

A monolayer has been formed with a few domes, indicating tightness of the cell monolayer. The microscopic pictures taken after 4 days look similar at all densities. A formation of multilayers or aggregates could not be observed microscopically (see figure 4.1.1.4). Because of permeability value of all samples is mostly the same, the lowest seeding density of $8.0 \cdot 10^4$ cells·cm^{-2} is chosen for further experiments. However, a permeability of Texas Red Dextran below 6% also could not be reached with MDCK cells.

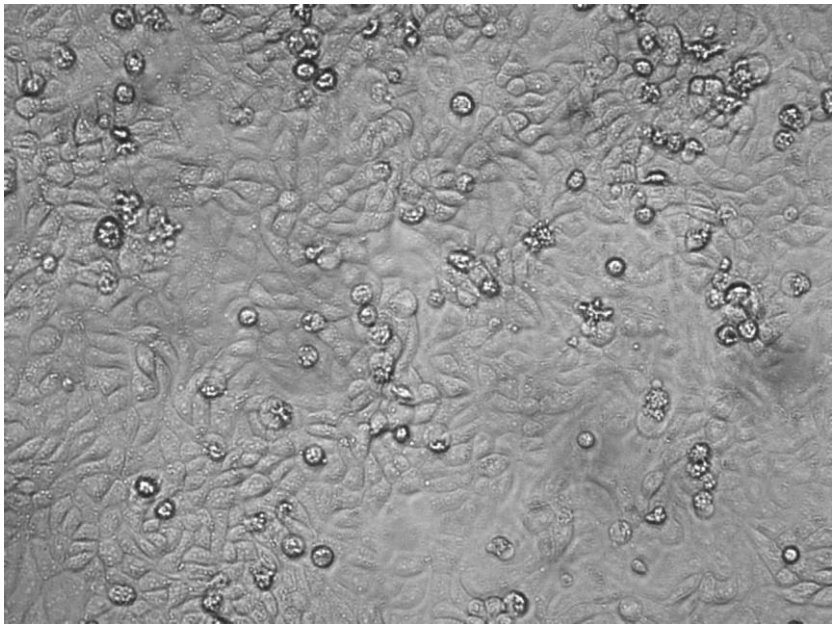


Figure 4.1.1.4 MDCK (Madin-Darby canine kidney) at 200x magnification. Cells were seeded with a cell density of $8.0 \cdot 10^4$ cells·cm^{-2}. After 4 days 100 % confluence has been reached, and the cells have been differentiated into a polarized epithelial monolayer (own illustration).

4.1.2 Does serum influence the monolayer integrity of Caco-2 cells?

As already mentioned in chapter 2.4.1, serum in cell culture medium provides important factors for cell maintenance and proliferation. As a natural product, the composition is not known in detail and also the known compounds vary from batch to batch. The Caco-2 cell culture medium contains a high proportion (20%) of fetal bovine serum, compared to other cell culture media (e.g., serum amount in MDCK cell culture medium is 5%). Therefore, it is

suspected that serum negatively affects the Caco-2 cell behavior and might explain the variation of permeability values between the samples, as well as the higher permeability values in comparison to MDCK cells. Therefore, eight sera of different sources and batches are tested in the permeability assay with Caco-2 cells.

Caco-2 cells are adapted to the new sera in the cell culture medium over three passages, before they are used in the permeability assay. Because of the lowest permeability value of 32.0% (in 4.1.1) $4.0 \cdot 10^4$ cells $\cdot cm^{-2}$, is chosen for this and further intestinal permeability assays. The results of the first experiment with the eight different sera are shown in figure 4.1.2.1. Altogether, the permeability values obtained in this experiment are much lower than in 4.1.1.1. Most of them are around 23%, but there are also values of 14.2% and 16.6%. The best result was obtained with the Serum PAN Biotech P150605 and 2.9% Texas Red Dextran permeability (column 6 in Figure 4.1.2.1). This serum is the same as used in the experiment 4.1. to determine the seeding density. In the first experiment the same seeding density shows a permeability value of 32.0%. To verify the results, the experiment was repeated. In the next experiment the serum PAN Biotech P150605 with a Texas Red Dextran permeability value of 2.9% (column 6 in Figure 4.1.2.1) and SIGMA F7540 Lot: BCBQ789OV with a value of 24.4% were tested again. The results are shown in figure 4.1.2.2. Again, the fluctuations of the results are very large, especially for the serum PAN Biotech P15060, with an average permeability value of 33.7% and a standard deviation of ±34.8%. The serum SIGMA F7540 Lot: BCBQ789OV shows an average permeability of 17.7% with a standard deviation of ±8.3%. It was expected that the use of the same serum will show highly reproducible results. The results indicate that serum, even in high concentrations of 20% (used for Caco-2), is not responsible for the high Texas Red Dextran permeability values in the experiments.

The next assumption is that the aspiration pump used might be too strong, which removes cells during the assay procedure and is causing a leaky monolayer. To confirm this assumption different aspiration rates are tested in the next chapter.

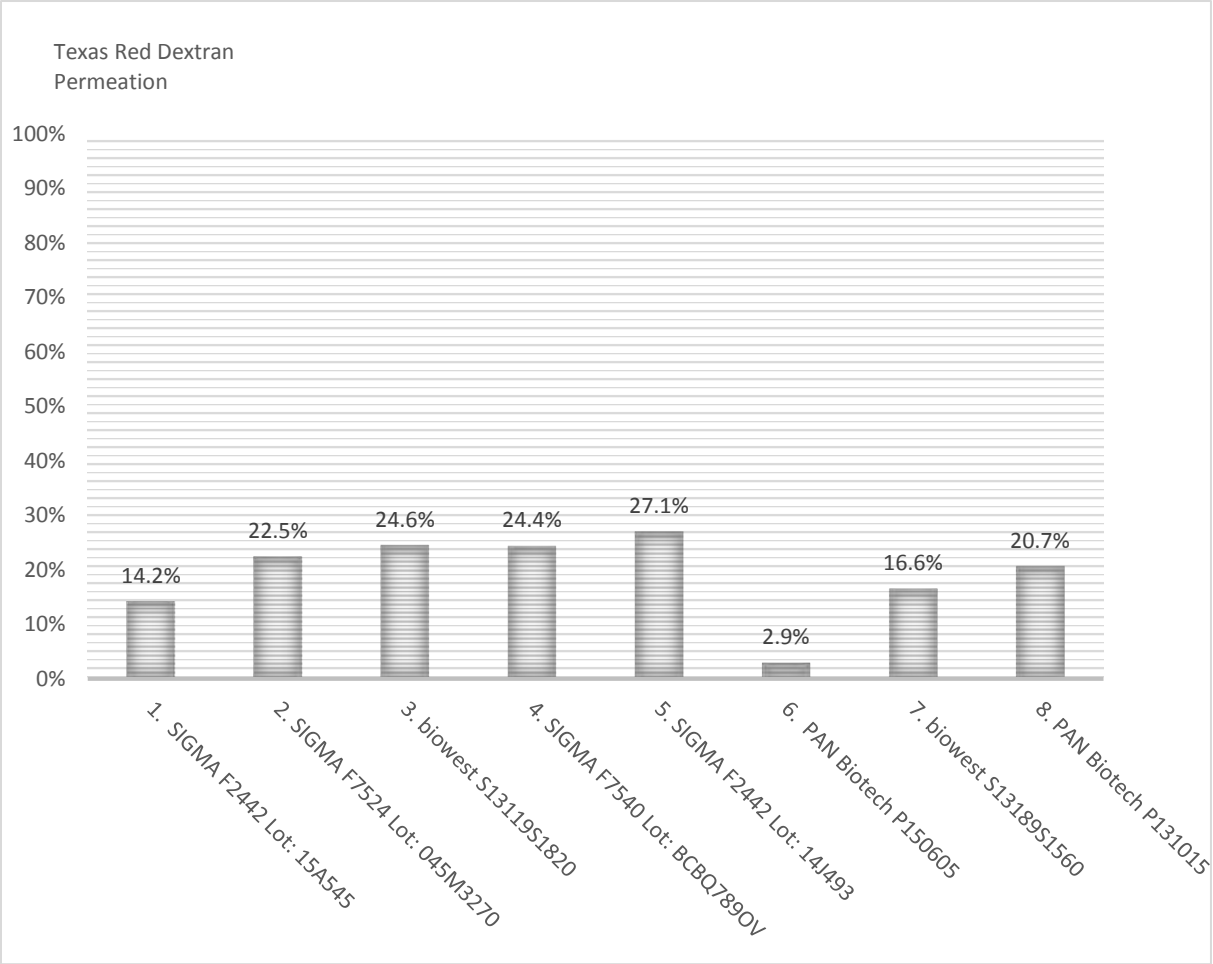


Figure 4.1.2.1. Influence of different eight sera at the Caco-2 monolayer integrity. Seeding density is $4.0 \cdot 10^5$ cells·cm^{-2}. Monolayer integrity was determined after 4 days. Texas Red Dextran will diffuse through leaky areas in the monolayer from the apical to the basolateral compartment. Measurement of the Texas Red Dextran after 3 hours at 37 °C in basolateral compartment as % of the total Texas Red Dextran amount applied to the monolayer. Most of permeability values are around 23%. But there are also values of 14.2% (column 1) and 16.6% (column 7). The best result was obtained with the Serum PAN Biotech P150605 with 2.9% (column 6). This serum is the same one that was used in 4.1.1.1. Serum PAN Biotech P15060 and serum SIGMA F7540 Lot: BCBQ789OV are tested again in figure 4.1.3.2.

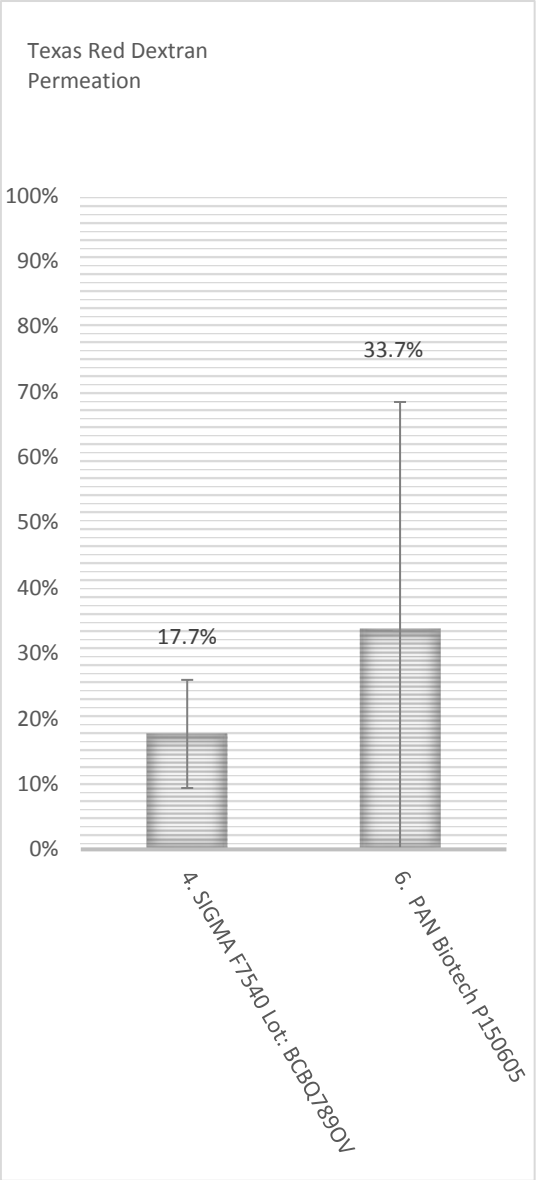


Figure 4.1.2.2. Influence of two different sera on the Caco-2 monolayer integrity. The seeding density is $4.0 \cdot 10^5$ cells·cm^{-2}. Monolayer integrity was determined after 4 days. Texas Red Dextran will diffuse through leaky areas in the monolayer. The dye moves from the apical to the basolateral compartment. Measurement of the Texas Red Dextran content in the basolateral compartment after 3 hours at 37 °C. Calculated as % of the total Texas Red Dextran amount applied to the monolayer. The variations of the results are very high, especially for the serum PAN Biotech P15060, with an average permeability value of 33.7% and a standard deviation of ±34.8%. The serum SIGMA F7540 Lot: BCBQ789OV show an average permeability of 17.7% with a standard deviation of ±8.3%.

4.1.3 Does the aspiration rate influence the monolayer integrity?

The previously shown results lead to the assumption that serum is not responsible for the large variations in the permeability, but rather is caused by something else. A guess was that the pump used for removing liquid from the apical compartment is too strong and damaged the monolayer while performing the assay, causing the variation of permeability values.

Indeed, this experiment was able to demonstrate that the high aspiration rate, used in the beginning, was the reason for the strong variation and the high permeability values. The results are shown in figure 4.1.3.1

The aspiration rate at the vacuum pump in the laboratory cannot be adjusted. However, with a “trick” the aspiration rate was reduced significantly. The “trick” is an additional hole placed in the lid of the withdraw container. The hole is small enough to remain enough negative pressure for proper suction. The extra hole has a diameter of approximately 2 mm. While aspirating fluids with the aspiration pipette, air from outside is aspirated through it in the container, which reduced the vacuum inside and lowered the aspiration rate. The aspiration rates are calculated by determining the time needed to aspirate a defined volume. The measurement was repeated five to six times, and subsequently the mean values are calculated. The results are listed in table 4.1.3.

Table 4.1.3 Determination of high and low aspiration rate. The aspiration rate is calculated by measurement of the time needed for aspirating a defined volume. The experiment was repeated five to six times, and the mean values were calculated.

High aspiration rate:

Volume [mL]	Time [s]	Aspiration rate [mL·s^{-1}]
220	17	12.9
220	16	13.75
220	16	13.75
220	15	14.6
220	15	14.6
Mean:		13.92

Low aspiration rate:

Volume [mL]	Time [s]	Aspiration rate [mL·s^{-1}]
1	18	0.0556
1	13	0.0769
1	13	0.0769
1	13	0.0769
1	15	0.0667
1	20	0.0500
Mean:		0.0672

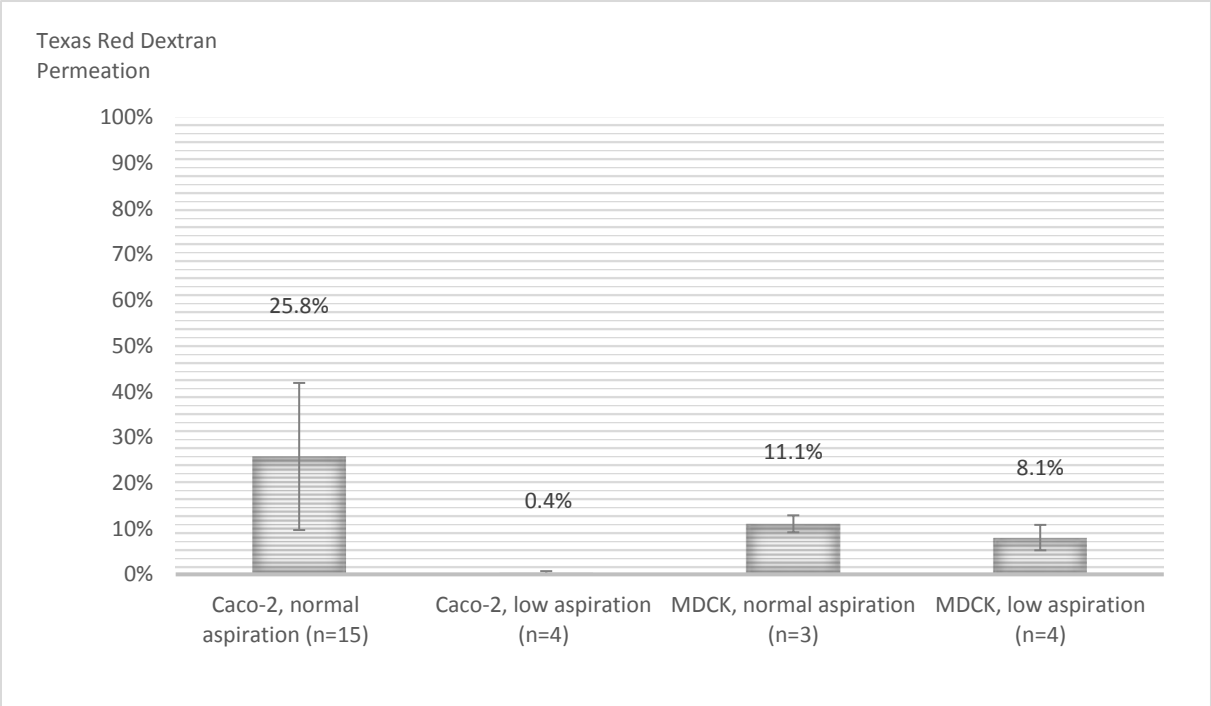


Figure 4.1.3.1 Determination of the Caco-2 and MDCK monolayer integrity at different aspiration rates. Seeding density is $4.0 \cdot 10^5$ cells·cm^{-2} for Caco-2 cells and $8.0 \cdot 10^4$ cells·cm^{-2} for MDCK cells. After 4 days in culture, the high aspiration rate (13.92 mL·s^{-1}) and low aspiration rate (0.0672 mL·s^{-1}) were compared. Texas Red Dextran will diffuse through leaky areas in the monolayer. The dye moves from the apical to the basolateral compartment. Measurement of the Texas Red Dextran after 3 hours of incubation at 37 °C in basolateral compartment. The result is calculated as % of the total Texas Red Dextran amount applied to the monolayer (mean ±SD). The aspiration rate has an impact on the Caco-2 monolayer integrity. The permeability value is reduced to 0.4%. The effect is larger with Caco-2 cells. The permeability value for MDCK cells just decreases slightly from 11.1% to 8.1%.

As shown in figure 4.1.3.1 the aspiration rate has an impact on the monolayer integrity. Especially for Caco-2 cells, the effect is significant. With the high aspiration rate of 13.92 mL·s^{-1} the Texas Red Dextran permeability is 25.8%. The standard deviation is ±16.1%. By contrast, a reduction of the aspiration rate by two-hundredth of one percent the permeability decreases to 0.4%, with a standard deviation of ±0.3%. A strong liquid flow seems to rip out cells from the monolayer. The resulting gaps, increase the diffusion of Texas Red Dextran through the monolayer. The gaps are not visible in microscopic pictures. The monolayers seem to be identical with control samples plated out in parallel.

The desired permeability of less than 6% is achieved in Caco-2 cells by a reduced aspiration rate. MDCK is still above this value, indicating that the leakiness is not a result of mechanical destruction of the monolayer. The permeability value decreased only slightly with a lower aspiration rate (from 11.1% ±1.9% to 8.1% ±2.8%).

To test, if the leakiness of the MDCK monolayer is a result of a slower formation of tight junctions, a new experiment is planned. MDCK were seeded in separate Transwell® compartments. After 4, 5, 8 and 21 days the tightness of the monolayer is tested. As shown in figure 4.1.3.2 the prolonged of time from 4 days to 5, 8 or even 21 days could not improve the tightness of the MDCK monolayer.

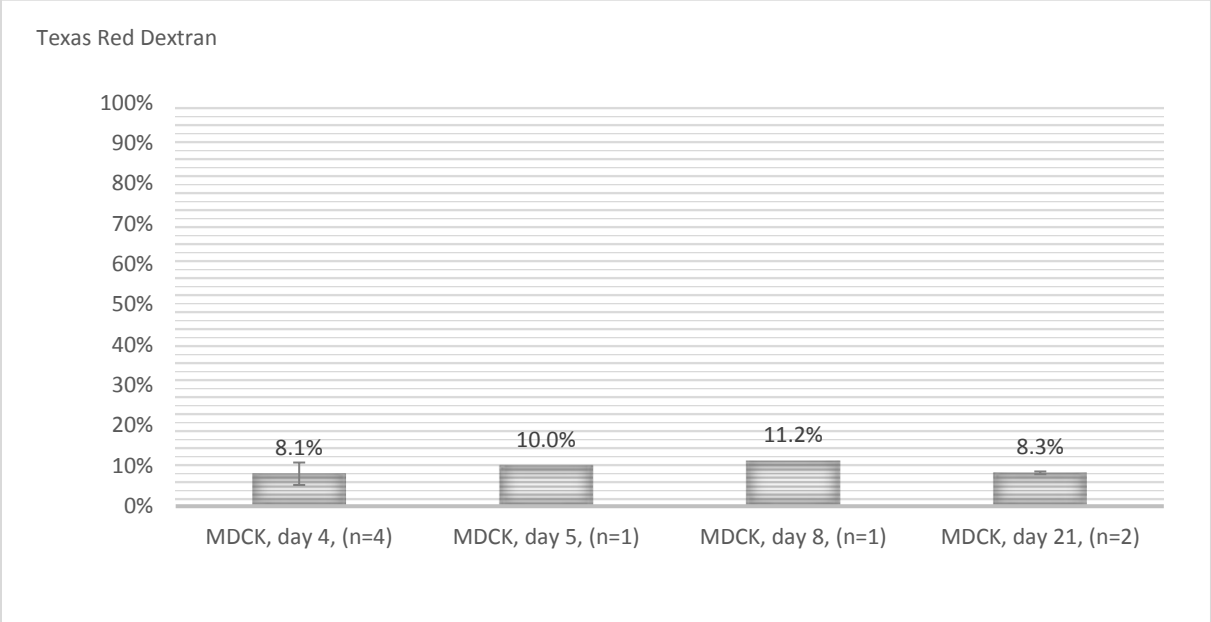


Figure 4.1.3.2 Determination of MDCK monolayer integrity after 4, 5, 8 and 21 days in culture. Use of low aspiration rate 0.0672 mL·s^{-1} for withdrawing fluids. Seeding density is 8.0·10^4 cells·cm^{-2}. The dye moves from the apical to the basolateral compartment. Measurement of the Texas Red Dextran after 3 hours of incubation at 37 °C in basolateral compartment. The result is calculated as % of the total Texas Red Dextran amount applied to the monolayer (mean ±SD).

The results of 4.1.3.1 and 4.1.3.2 indicate that MDCK cells are less sensitive to shear forces caused by the suction than Caco-2 cells. It seems that MDCK cells in general have a leakier monolayer, implying that the tight junctions of MDCK cells consist to great extend out of proteins that increase in junction permeability. One of protein-classes responsible for the barrier tightness are claudines. Different types of claudines exist. For example, claudin-4 is responsible for sealing gaps between cells, whereas claudine-2 forms pores and increases the permeability of the monolayer. One explanation for the high permeability rates of MDCK cells is a higher expression level of claudine-2 in comparison to Caco-2 cells. Therefore, it was planned to detect claudine-2 and claudine-4 by immunofluorescence. Fluorophore labeled antibodies were used to stain the claudines in confluent monolayers of MDCK and Caco-2 cells. A standard protocol was used for the staining of the cells. Unfortunately, no fluorescence was visible in the samples. Therefore, an optimization of the immunofluorescence protocol is

necessary. Regarding the limited period of this thesis, the optimization of the immunofluorescence was put on hold and the work was continued with experiments of higher priority.

Changes in culture- and assay conditions were not able to improve the tightness of the MDCK monolayers. The used MDCK cell line is available at commercial cell banks. Due to the establishment and history of cell lines the material available at commercial sources are quite heterogeneous.

One approach is the isolation of single cells out of the heterogeneous cell line by limited dilution in the hope that one or more of the isolated subclones have the property to form a dense monolayer. The isolated subclones were tested in the permeability assay and in a functional assay, detecting an essential membrane transporter (P-gp). The P-gp activity is demonstrated by the ability to transport calcein AM out of the cell in the presence and absence of an P-gp inhibitor. The detection of P-gp activity in flow cytometric analysis by calcein AM uptake, is first set up in their parenteral cell lines. Afterwards the method was used to characterize the isolated subclones.

4.2 Assessment of P-glycoprotein (P-gp) activity in flow cytometry by calcein AM uptake

To assessed the P-gp activity in Caco-2 and MDCK cells, the assay was executed as described in the publication of Homolya et. al 1996. The working group used a calcein AM concentration of 250 nM and NIH-3T3 murine fibroblast cells as negative control. The results are shown in figure 4.2. If using the suggested concentration of calcein AM most of the cells died. The observed cell death is not a matter of the cell quality used for the assay. The viability of Caco-2, MDCK and NIH 3T3 cells, before 250 nM calcein AM was added was more than 90%. Caco-2 and NIH 3T3 show a remaining viability of 11,10% and 11,32%, respectively. MDCK cells with a remaining viability of approximately 50% seems to be tolerate higher concentrations of calcein AM. Verapamil, as P-gp inhibitor and blocks the calcein AM transported out of the cell, so that endogenous esterases are able to metabolize it in the fluorescence form by cleave the AM unit from calcein. The fluorescence form is trapped inside the cell. Blocking of the P-gp transporter increases the sensitivity of the cells to toxic compounds. Therefore, calcein AM toxicity, as described above, is more potent in verapamil treated cells. (see figure 4.2 bottom row). While in Caco-2 cells the viability remains nearly constant at 10,22% the cell viability in

MDCK cells and in NIH 3T3 cells decreased up to 4,41% and 1,50%, respectively. One possible explanation is that the calcein AM concentration is too high, so that the amount of P-gp transporter are unable to transport it out of the cell. This would also explain why verapamil amplified this effect in MDCK and NIH 3T3 cells. Is the P-gp transporter blocked, more and more calcein AM diffuse inside the cell and lead to more damage.

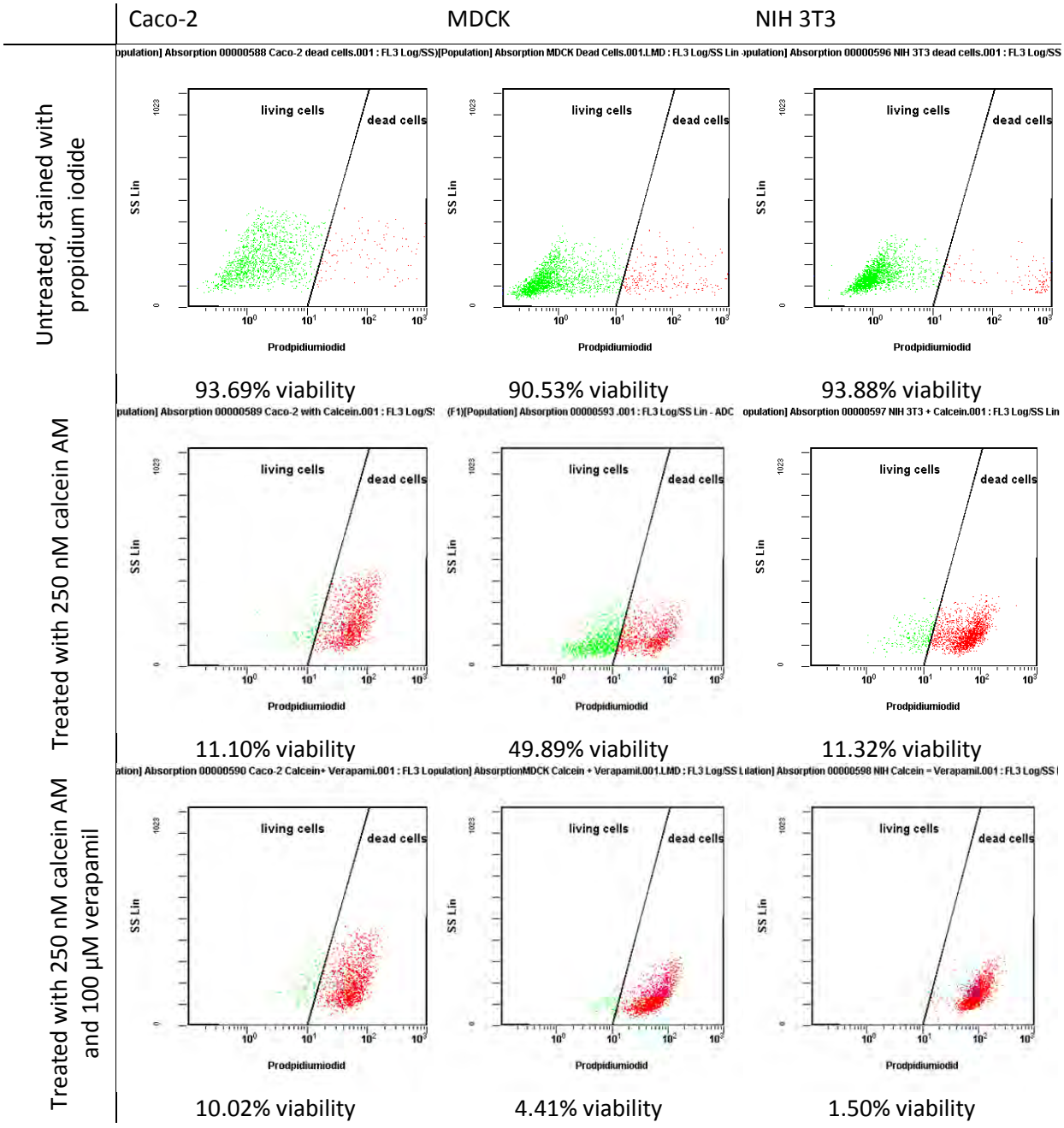


Figure 4.2.1 Influence of 250 nM calcein on cell viability of Caco-2 (left column), MDCK (middle column) and NIH 3T3 (right column). Dead cells were detected with 1 µg·mL^{-1} propidium iodide (red color), with filter FL3 of the flow cytometer. Viable cells remain unstained (green). Upper row shows untreated cells. The middle row show cell viability in the presence of 250 nM calcein AM without an inhibitor. The bottom row show cell viability in the presence of 100 µM verapamil and 250 nM calcein AM. Calcein AM addition reduces viability of Caco-2 cells from 93.68% to 11.10%, viability of MDCK cells from 90.53% to 49.89% and NIH-3T3 from 93.88% to 11.32%, respectively. Verapamil reinforces the effect. Here the cell viability decreases down to 10.22% in Caco-2 cells 4.41% in MDCK cells and 1.50% in NIH-3T3 cells.

As mentioned above, MDCK cells seem to be more resistant. Calcein toxicity occurs less than in Caco-2 and NIH-3T3 cells, but is similar, if the P-gp transporter is blocked. To exclude influences of toxic effects of calcein AM, concentration which are not harmful to cell viability needed to be determined for further experiments.

4.2.1 Calcein AM titration

Since 250 nM calcein AM shows a significantly reduced viability, it is important to find the maximum calcine AM concentration without toxic effects to the cells. Therefore, a dose-response-experiment is performed by calcein AM titration with MDCK cells. In a serial dilution the calcein AM concentration is decreased by the factor of 10 in each step. Staring with 250 nM the dilution series contains tested concentrations of 25 nM, 2.5 nM and 250 pM. The effect of the different calcein AM concentrations on the cell viability in the presence and the absence of verapamil is shown in figure 4.2.1.1. The toxic effect of 250 nM calcein AM noticed in the first experiment (4.2.) could be reproduced a second time. 250 nM calcein AM reduces cell viability of MDCK cells from 99.69% to 53,99%. Addition of verapamil intensify the effect and decreases cell viability to 0.09% viability. However, a concentration of 25 nM calcein AM and less show no toxic effects on cell viability, even if verapamil is used. The cell viability is in all cases above 99%.

Nevertheless, for the P-gp screening of the subclones this concentration is still too high. The amount of calcein entering the cells seem to be too much for the P-gp transporter. The fluorescent form is still detectable inside the cell, even verapamil is not present. The expressed P-gp transporter is unable to transport all calcein AM out of the cell before the enzymes cleaves the AM group. The reduction of calcein AM to a concentration of 2.5 nM improves the results (figure 4.2.1.2). At this concentration the effect of verapamil is visible. However, a background signal of 5.08% of calcein is still present at the reduced calcein AM concentration. An additional experiment shows best results with 1 nM calcein AM. Therefore, a calcein AM concentration of 1 nM is chosen and also tested in the other cell lines. The results are shown in Figure 4.2.1.3 Using this calcein AM concentration no cell death is detectable. The viability of all cell lines before and after the addition of calcein AM and verapamil was more than 92% (92% in NIH-3T3m 96% in MDCK and 99% in Caco-2).

In the experiment, MDCK cells show the highest P-gp activity in comparison to the other cell lines (82.85%). Unexpected is the results of the NIH-3T3. NIH-3T3 as control cell type, should not express the P-gp transporter inside the membrane (Homolya et al., 1996), so that calcein AM, passes unhindered the cell membrane, get metabolize and causing a significant fluorescence signal. In contrast the fluorescence signal actually measured is quite low (15.62%). The use of verapamil should not show an effect. In contrast to the expected behavior, the fluorescence signal inside the cell increase up to 66.30%, after verapamil addition, leading to an overall P-gp activity of 50.63% of NIH-3T3 cells. The results demonstrate that NIH 3T3 cells are not suitable as negative control for further experiments.

Caco-2 cells show the worst result in the P-gp activity test. Only 12.51% of the cells are P-gp positive. Calcein AM already accumulates in the absence of the P-gp inhibitor. 47.51% of the cells express no or very low amounts of P-gp. Therefore, it is not surprising, that an addition of verapamil shows no effect and the fluorescence signal increases only marginal to a value of 60.02%. Caco-2 does not express P-gp in sufficient concentrations. Needed is a P-gp activity comparable to the activity of MDCK cells. Therefore, it was also tried with Caco-2 cells to improve the cellular properties by subcloning. The aim for Caco-2 cells is to isolate clones which express a higher levels of functional P-gp in the membranes. Since MDCK cells are used as alternative to Caco-2, it is necessary to achieve at least an equivalent activity in Caco-2 cells. Another interesting approach for pharmacological applications would be to isolate also MDCK or Caco-2 cells which does not express P-gp. Therefore, it was also screened for P-gp negative cells.

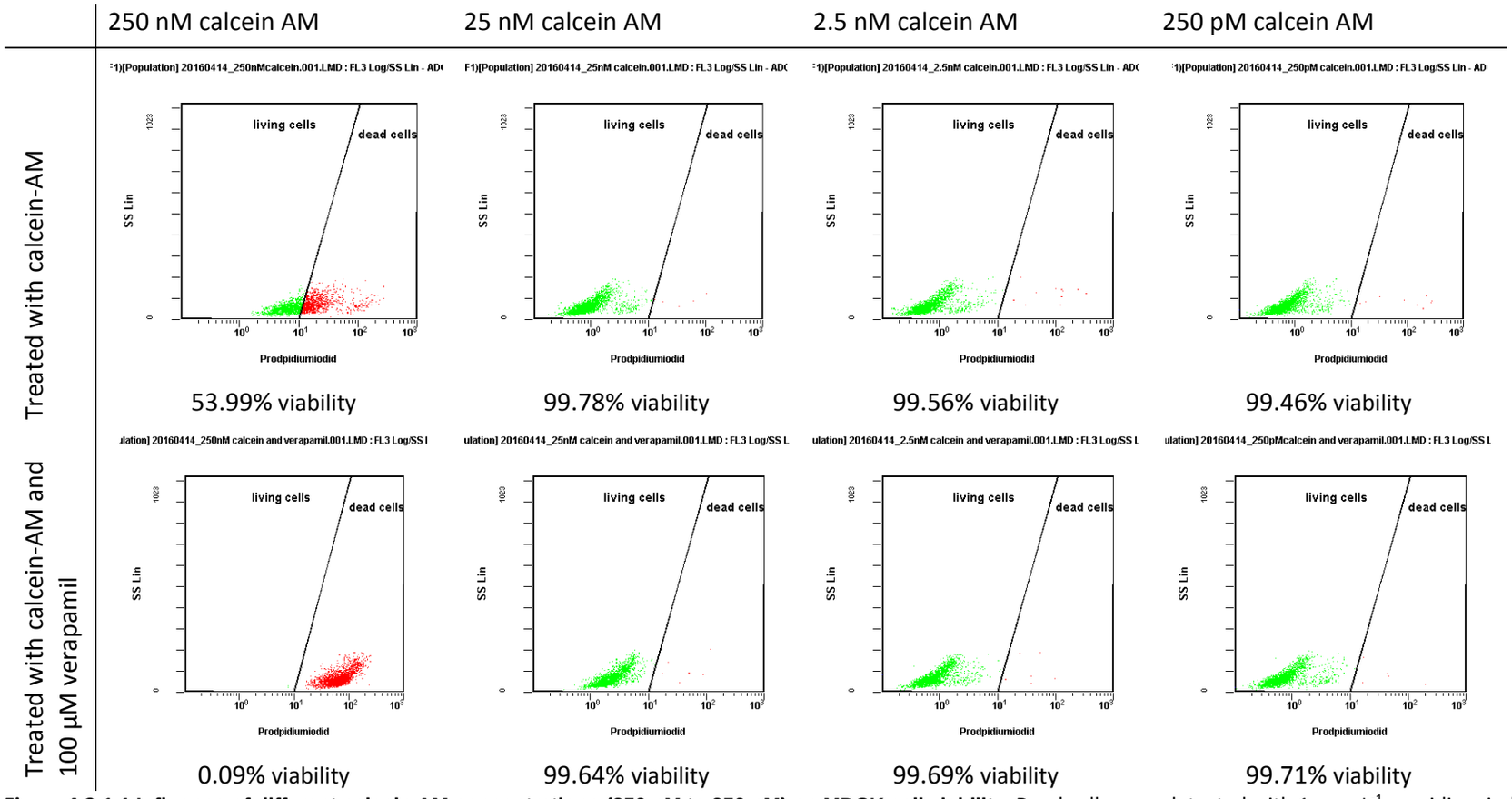


Figure 4.2.1.1 Influence of different calcein AM concentrations (250 pM to 250 nM) on MDCK cell viability. Dead cells were detected with 1 µg·mL^{-1} propidium iodide and displayed in red color, detectable with FL3 of the flow cytometer. Viable cells remain unstained and are stained in green. Upper row show cell viability in the presence of 250 nM calcein AM without an inhibitor. The bottom row show cell viability in the presence of 100 µM verapamil and 250 nM calcein. A concentration of 250 nM calcein AM show a cell viability of 53,99%. If verapamil is used, cell viability is decreased down to 0,09%. Calcein AM concentrations of 25 nM calcein AM and below shows a cell viability of >99%.

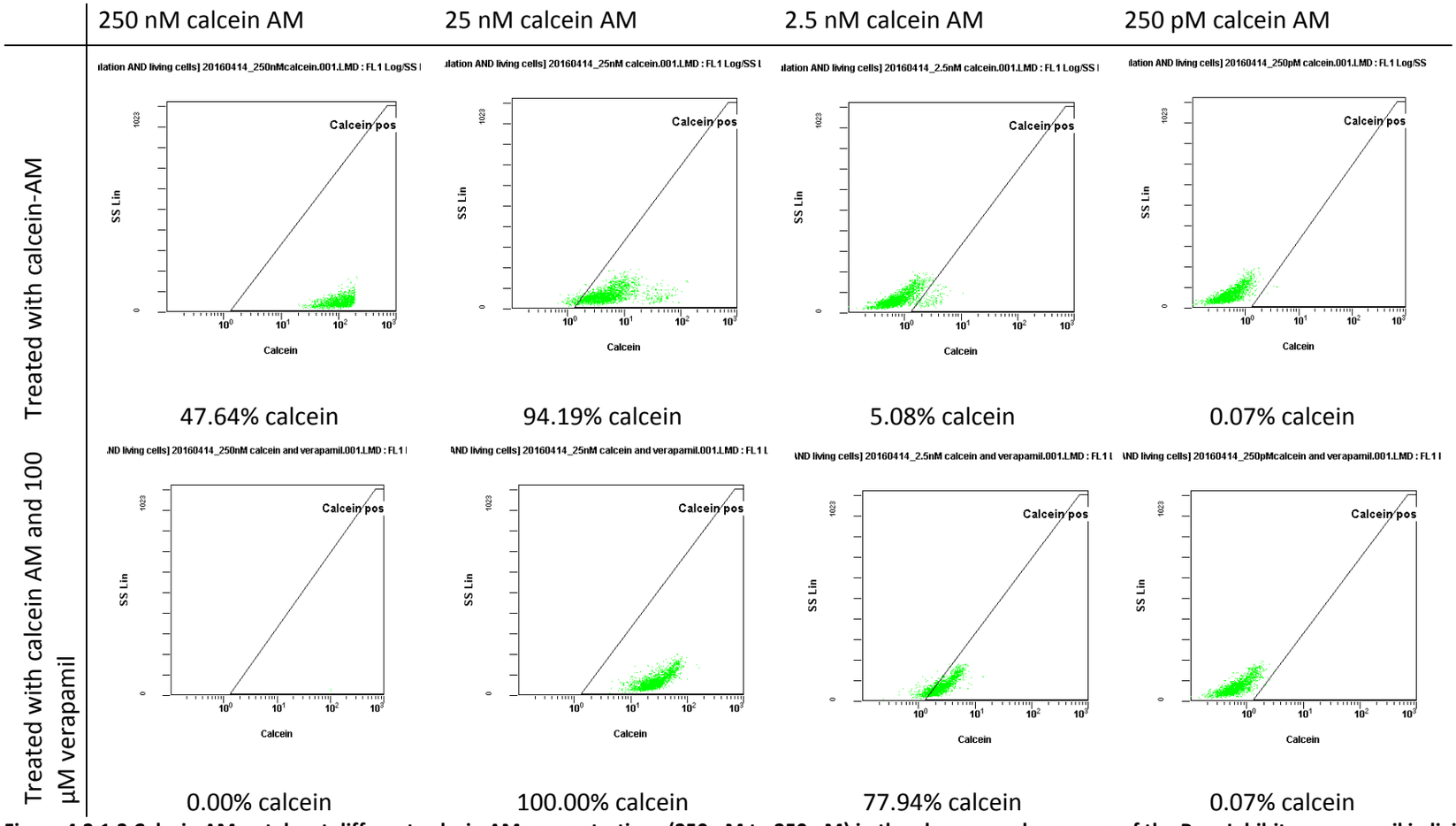


Figure 4.2.1.2 Calcein AM uptake at different calcein AM concentrations (250 pM to 250 nM) in the absence and presence of the P-gp Inhibitor verapamil in living MDCK cells. Dead cells are gated out (see figure 4.2.2). Upper row show MDCK cells treated with different concentrations of calcein AM without an inhibitor. The bottom row shows MDCK cells treated with different calcein AM concentrations in the presence of 100 µM verapamil. No calcein signal is detectable in MDCK cells treated with 250 nM calcein AM and verapamil. This can be explained by the toxic effect of calcein AM (see figure 4.2.2). The results were excluded from further analysis. Calcein AM concentrations below 25 nM, cells are not toxic, (figure 4.2.2) but the concentration is too high for the P-gp transporter. The amount of expressed P-gp transporter is unable to compensate the transporter independent import of calcein AM. A calcein concentration of 250 pM is too low for detection, (no difference between calcein alone, and calcein with verapamil). At a 2.5 nM calcein concentration it is easy to distinguish between calcein AM alone and calcein AM in the presence of verapamil. The background signal of 5.08% is tolerable.

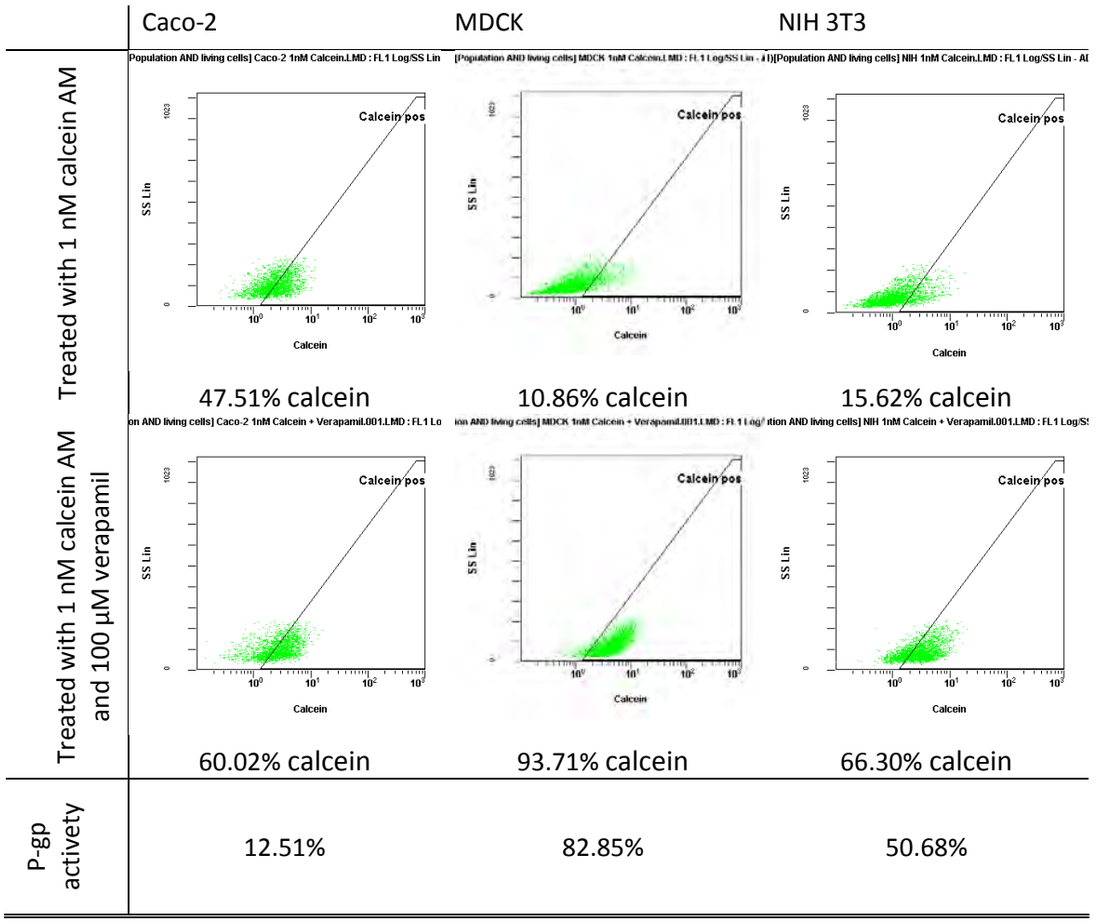


Figure 4.2.1.3 Determination of P-gp activity with 1 nM calcein on Caco-2, MDCK- and NIH 3T3 by quantification the amount of calcein containing cells in the presence or absence of the P-gp inhibitor verapamil. Upper row shows the fluorescent signal of cleaved calcein AM inside the cell without an P-gp inhibitor. Without an inhibitor, a functional P-gp transporter is able to export calcein AM. The lower row shows the fluorescent signal of cleaved calcein AM inside the cell in the presence of 100 µM verapamil. Verapamil blocks the P-gp transporter and calcein AM is trapped inside the cell, where estereases cleave it into the fluorescent form. The P-gp activity is measured as difference between the amount of calcein accumulated in the presence and absence of the inhibitor (bottom row). MDCK cells show with 82.85% the highest P-gp activity in comparison to the other cell lines. Unexpected is the behavior of the NIH-3T3 cells. NIH-3T3 should not express the P-gp transporter (Homolya, 1996) and therefore, the presence of verapamil should not have any impact on calcein accumulation.

4.3 Assessment of P-glycoprotein (P-gp) activity in flow cytometry by calcein AM uptake for subclone screening

Since a desired permeability of Texas Red Dextran, less than 6% could not be achieved in MDCK cells (from 11.1% ±1.9% to 8.1% ±2.8%) and since the functionality of P-gp in Caco-2 cells (12.51%) is by far below the expected value, limited dilution is used to generate subclones, which combine low permeability and good P-gp expression.

A cell suspension was diluted and seeded with a theoretical cell density of 0.4 cells per well in three to four 96-well plates. Using this seeding density a range from no up to four cells per well were detectable in the wells of the assay plate. Finally, it was possible to isolate 31 Caco-2- and 47 MDCK single-cell clones. During cell expansion some of them were lost. In the end 15 Caco-2- and 7 MDCK subclones were saved as cryopreserved cell stocks. In total 22 subclones were screened for their P-gp activity. The results are shown in the table 4.3.1 and 4.3.2. The acquired P-gp activity values of the Caco-2 subclones shows high variabilities. These results demonstrate the heterogeneity of the parenteral Caco-2 cell line. The P-gp activity of the parenteral Caco-2 cell line was 12.51%. The best value shows the Caco-2 subclone D5 with 98.97%. The lowest value wat obtained in subclone C7. Similar values were detected in subclone D3 (6.7%), C3 (7.19%) and 7.86% in subclone F9. The subclones D5 andF9 were tested in the intestinal permeability assay if they kept still their monolayer integrity.

The variations between the MDCK subclones are not as large as between the Caco-2 subclones. The subclone C9 shows the highest P-gp activity (84.56%), B2 the lowest. Since each MDCK subclone shows more than 60% (except subclone B2) active cells they are all tested in the intestinal permeability assay.

Table 4.3.1 Determination of P-gp activity with 1 nM Calcein on Caco-2- subclones by quantification the amount of calcein containing cells in the presence or absence of the P-gp inhibitor verapamil. Upper row shows the percentage of calcein AM containing cells in the absence of a P-gp inhibitor. The middle row shows the percentage of calcein containing cells in the presence of 100 µM verapamil. The percentage of P-gp positive cells is measured as the difference between the calcein positive cells in the presence and absence of the inhibitor. The results are listed in the bottom row. The subclone D5 shows the highest percentage of P-gp positive cells. C7, D3, C3 and F9 show the lowest percentage(n=1).

	B5	B6	B7	B9	B10
Remaining calcein inside the cell (basal value) [%]	89.59	39.94	15.65	18.76	56.31
Remaining calcein after P-gp inhibition [%]	96.93	75.48	67.04	63.36	98.58
Active out transport of 1nM Calcein AM [%]	7.34	35.54	51.39	44.6	42.27
	C3	C7	D3	D5	D6
Remaining calcein inside the cell (basal value) [%]	85.93	92.61	92.39	0.27	78.5
Remaining calcein after P-gp inhibition [%]	93.12	96.8	99.09	99.24	95.67
Active out transport of 1nM Calcein [%]	7.19	4.19	6.7	98.97	17.17

	D8	D11	E5	F2	F9
Remaining calcein inside the cell (basal value) [%]	17.11	42.69	5.59	41.66	87.22
Remaining calcein after P-gp inhibition [%]	89.54	92.57	66.96	83.97	95.08
Active out transport of 1nM Calcein [%]	72.43	49.88	61.37	42.31	7.86

Table 4.3.2 Determination of P-gp activity with 1 nM Calcein on MDCK subclones by quantification the amount of calcein containing cells in the presence or absence of the P-gp inhibitor verapamil. Upper row shows the percentage of calcein AM containing cells in the absence of a P-gp inhibitor. The middle row shows the percentage of calcein containing cells in the presence of 100 µM verapamil. The percentage of P-gp positive cells is measured as the difference between the calcein positive cells in the presence and absence of the inhibitor. The results are listed in the bottom row. **All Subclones show an effect in the presence of verapamil.** The Subclone C9 show the highest P-gp activity with 84.56%, B2 the lowest with 47.34% (n=1).

	B2	C9	C10	B3
Remaining calcein inside the cell (basal value) [%]	52.6	15.42	4.24	32.02
Remaining calcein after P-gp inhibition [%]	99.94	99.98	68.89	99.98
Active out transport of 1nM Calcein [%]	47.34	84.56	64.65	67.96

	B5	B4	B9
Remaining calcein inside the cell (basal value) [%]	36.54	32.77	28.59
Remaining calcein after P-gp inhibition [%]	99.9	98.3	98.28
Active out transport of 1nM Calcein [%]	63.36	65.53	69.69

	B2	B3	B4	B5
Remaining calcein inside the cell (basal value) [%]	52.6	32.02	32.77	36.54
Remaining calcein after P-gp inhibition [%]	99.94	99.98	98.3	99.9
Active out transport of 1nM Calcein [%]	47.34	67.96	65.53	63.36

	D4	C9	C10
Remaining calcein inside the cell (basal value) [%]	28.59	15.42	4.24
Remaining calcein after P-gp inhibition [%]	98.28	99.98	68.89
Active out transport of 1nM Calcein [%]	69.69	84.56	64.65

4.4 Subclones in the intestinal permeability assay

With optimized conditions, it was possible to generate the lowest Texas Red Dextran permeability of 8.1% ±2.8% in MDCK cells (4.1.3.1). Unfortunately, a Texas Red Dextran permeability of 6% in MDCK cells was impossible to generate. Therefore, subclones, generated by limited dilution from the parental cell line, should help to overcome the issue of a leaky monolayer formation.

With the assessment of P-gp activity in chapter 4.3, it is evident, that all MDCK subclones express P-gp inside the cell membrane. The addition of a P-gp inhibitor shows an accumulation of fluorescent calcein. Therefore, all subclones were tested in the permeability assay. The results of the intestinal permeability assay are shown in figure 4.4.1. Remarkable is MDCK subclone D4 with a permeability value of 72.6%, indicating that no intact cell layer is build. The permeability value, obtained in the other subclones are ranging from 6.4% to 11.11%. The MDCK subclone C10, with a permeability value of 6.4% comes close to the specification limit of 6% Texas Red Dextran permeability.

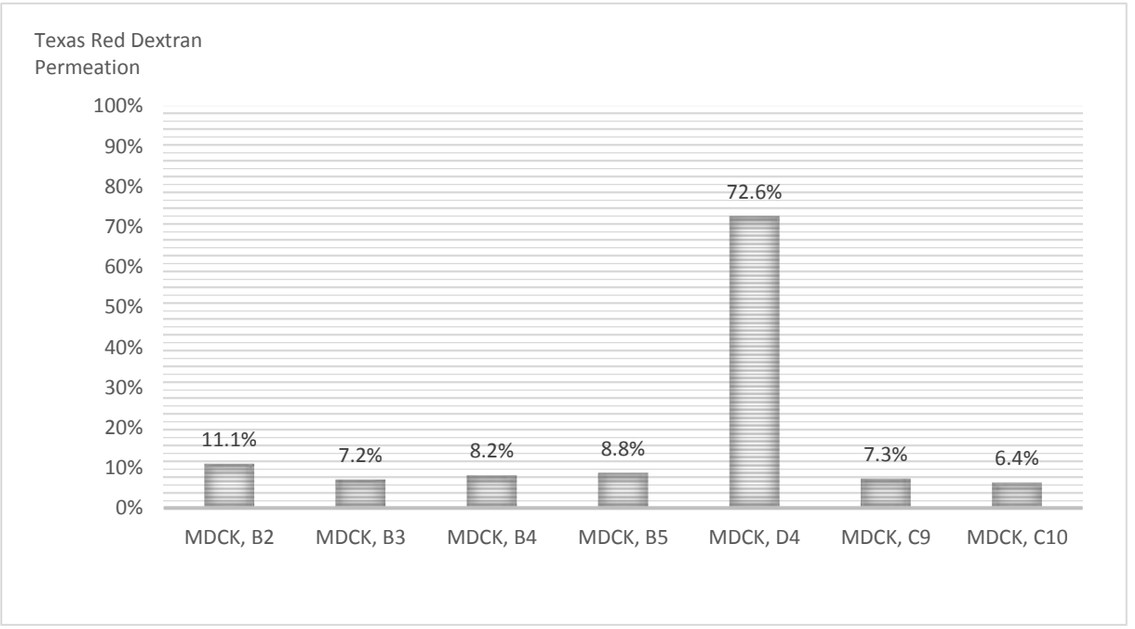
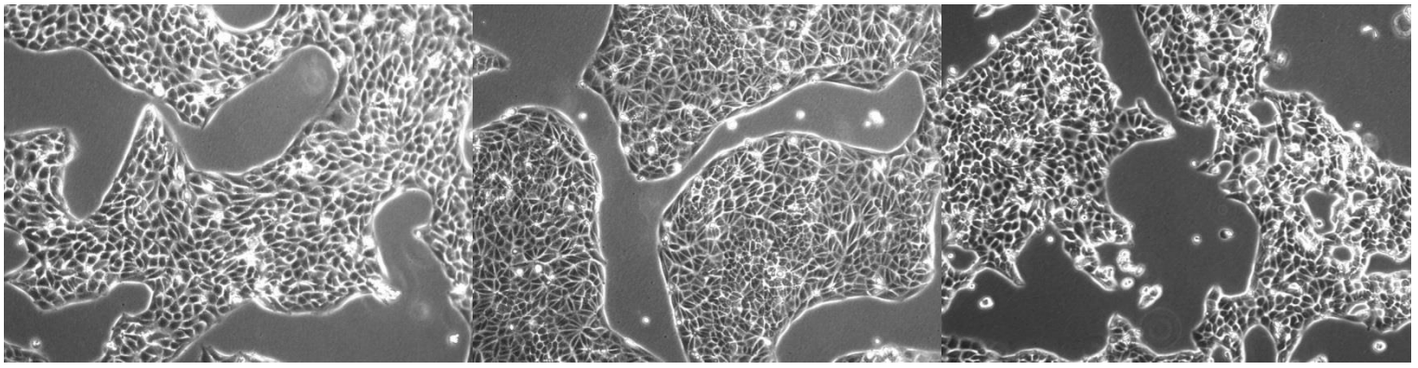


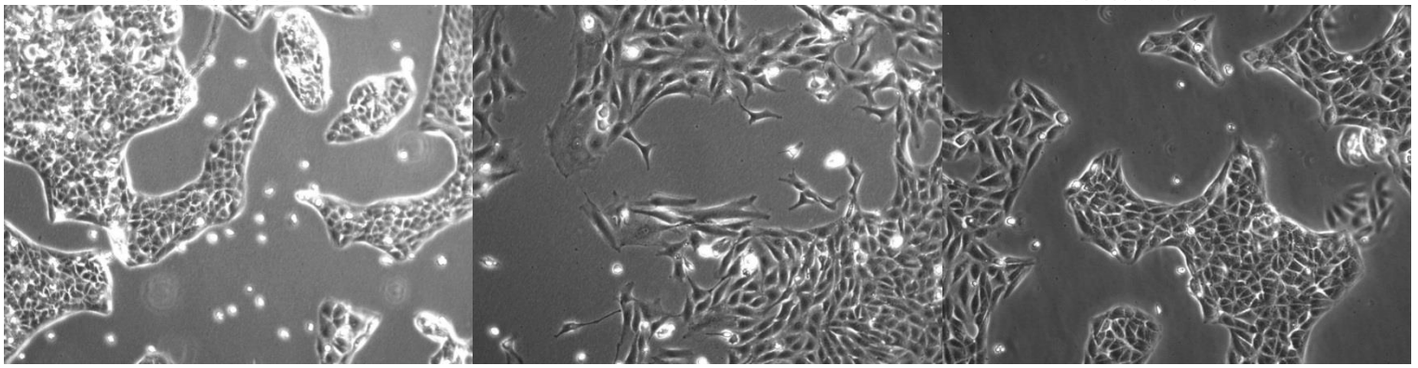
Figure 4.4.1 Determination of the cell monolayer integrity of different MDCK subclones after 4 days in culture. Seeding density is $8.0 \cdot 10^4$ cells·cm^{-2}. Texas Red Dextran will diffuse through gaps in the monolayer from the apical to the basolateral compartment. Measurement of the Texas Red Dextran after 3 hours at 37 °C in apical compartment as % of the total Texas Red Dextran amount applied to the monolayer. Remarkable is the MDCK subclone D4 with a permeability value of 72.6%. The permeability value, obtained in the other subclones are ranging from 6.4% to 11.11%. The MDCK subclone C10, comes close to the specification limit of 6.0%Texas Red Dextran permeability.

One explanation, why MDCK subclone D4 behaves so different is an injured monolayer caused by incautious handeling. To verify the results of subclone D4, the experiment needs to be repeated. While subclone B2, B3, B4, B5 and C9 grow in compact colonies, subclone D4 and C10 and the parenteral cell line grow more disturbed and star-shaped. Because of the different behaviour and different appearance of the colonies, the question

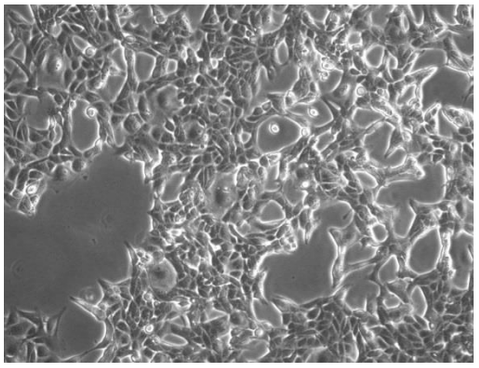
arises, if these subclones were derived from a different cell types. As mentioned in the theory part up to 20% of cross-contaminated cell lines are described in test series of cell banks (e.g ATCC). Human cell lines can be identified by STR analysis. Unfortunately, MDCK cells are of non-human origin. Therefore, the curious subclones were tested by species PCR. The results confirm that all subclones orginate from dog. Additionally there is no evidence that the parenteral cell line contains contaminations of other species.

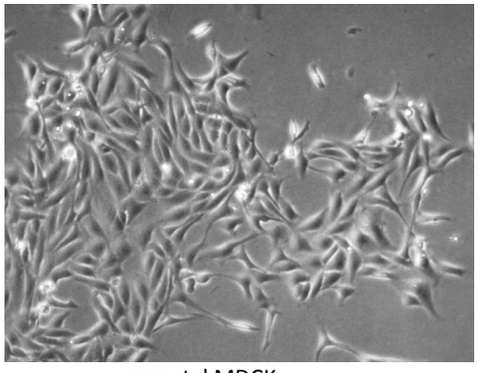


MDCK subclone B2 MDCK subclone B3 MDCK subclone B4



MDCK subclone B5 MDCK subclone D4 MDCK subclone C9





MDCK subclone C10 parental MDCK

Figure 4.4.2 Morphological differences between MDCK subclones and the parental MDCK cell line. Microscopic image in a 200x magnification. Subclones B2, B3, B4, B5 and D4 were thawed out of a frozen stock. C9, C10 and the parental MDCK were taken from continuous culture. Seeding density is $3.0 \cdot 10^3$ cells$\cdot$cm^{-2} in all cell lines. Pictures of subclones B2, B3, B4, B5 and D4 were taken after 6 days, of C9 and C10 after 4 days and the parental MDCK cells after 3 days

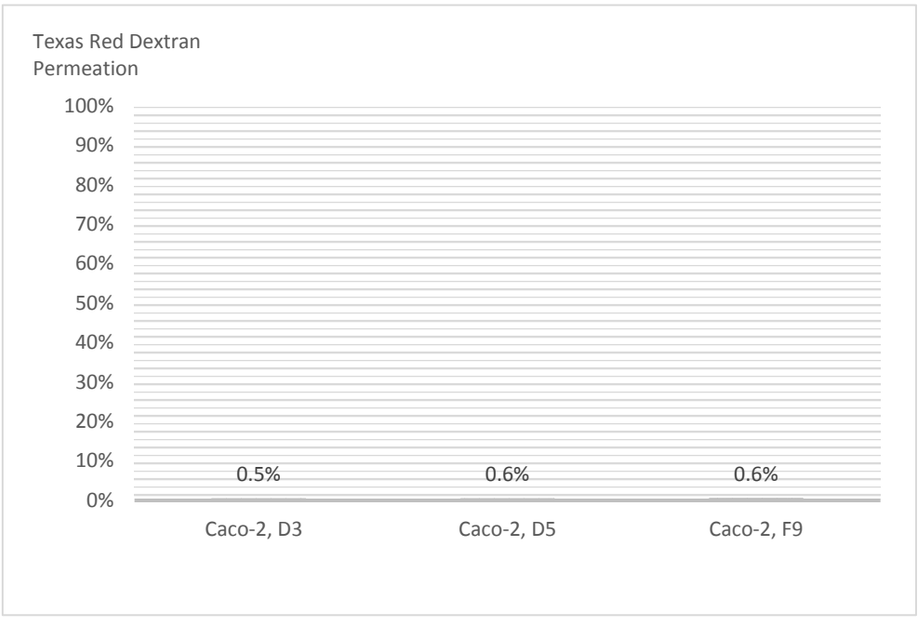


Figure 4.4.3 Determination of the cell monolayer integrity of different Caco-2 subclones after 4 days in culture. Seeding density is $4.0 \cdot 10^5$ cells·cm^{-2}. Texas Red Dextran will diffuse to leaky areas in the monolayer from the apical to the basolateral compartment. Measurement of the Texas Red Dextran after 3 hours at 37 °C in apical compartment as % of the total Texas Red Dextran amount applied to the monolayer. Noticeable is the Caco-2 subclone E5 with a permeability value of 4.9%. The permeability value, obtained in the other subclones are ranging from 0.5% to 0.6%

The results of Caco-2 cells tested in the assay do not show any differences. The permeability values after days are excellent and almost the same in all three subclones. Further permeability experiments with frozen cells were carried out by using subclones D5 (high P-gp activity) and F9 (low P-gp activity).

4.5 Comparison of frozen and “fresh” cells

So far all experiments are performed with “fresh” cells (cells from continuous culture). These cells were detached, counted and seeded into Transwells® or used in the flow cytometric assay for P-gp assessment by calcein AM uptake. The assay was established with fresh Caco-2 cells. The next step in this thesis is to prove, if frozen cells behave similar to fresh cells and can be used as alternative. Therefore, the viability and the growth behavior of cryopreserved cells (“frozen”) are compared with fresh cells.

There are two freezing media used in the laboratory of the acCELLerate GmbH for preparing cryopreserved cell stocks of cells. The standard freezing medium, contains cell specific culture medium and freshly added DMSO at a final concentration of 5%. A serum free alternative to the standard freezing medium is the “New Freezing Medium Generation 2” (NFM-G2). The composition is developed at acCELLerate and contains several cryoprotective additives. The cryoprotective properties of NFM-G2 are achieved by a C6 sugar and a protein complex diluted in a physiological buffer. However, 5% freshly added DMSO is also necessary in NFM-G2.

4.5.1 Comparison of viability and growth behavior with “fresh” and frozen cells

MDCK and Caco-2 cells are frozen in two different freezing media, named as “standard freezing medium” and NFM-G2. The viability of the cells and the ability to proliferate within the first 96 hours are compared. Cells from continuous culture are named as “fresh cells” in this thesis. The cells are thawed as described in the chapter 3.4 and seeded into a 24-well cell culture plate. Cell density for MDCK cells is $3.0 \cdot 10^3$ cells$\cdot$cm^{-2} and $1.0 \cdot 10^4$ cells$\cdot$cm^{-2} for Caco-2 cells. Cells are detached and counted as described in chapter 3.2 and 3.5.

The results of the MDCK cells are shown in figure 4.5.1.1 and table 4.5.1.1

The graph shows the total living cell number of fresh cells and cells which are frozen in NFM-G2 or standard freezing medium as bars. The lines are the associated fitting curves. The growth curves of all three samples are similar within the first 48 hours. 72 hours after seeding, the cell number of fresh cells and cells, frozen in standard freezing medium are similar with $3.80 \cdot 10^4$ cells and $3.32 \cdot 10^4$ cells, respectively. Whereas the cell number of cells frozen in NFM-G2 decreased from 48 hours to 72 hours (from $1.74 \cdot 10^4$ cells to $1.48 \cdot 10^4$ cells). At the end (96 h) the frozen cells have an equivalent cell number ($1.19 \cdot 10^5$ cells in standard medium and $1.74 \cdot 10^5$ cells in NFM-G2). The fitting curve of the standard medium is close to the curve of fresh cells. The cell number in the culture started with fresh cells is at the end the highest cell number ($1{,}77 \cdot 10^5$ cells, followed by both frozen cells ($1{,}19 \cdot 10^5$ cells for standard and $1{,}16 \cdot 10^5$ cells for NFM-G2).

Another important aspect is the viability of the cells, which is listed in table 4.5.1.1.

Although the freezing and thawing is a stressful process for cells, the viability of frozen cells (with 98.02% in cells frozen in standard medium and 97.02% in cells frozen in NFM-G2 is comparable to the viability of fresh cells (97.37%). Indicating that MDCK cells are less sensitive. During the experiment, the cell viability in all samples remains between 93.06% and 98.02%. Variations from one to another day are in the range of normal assay variation. Fresh cells have a faster growth in comparison to frozen cells, indicating that the freezing and thawing process delay the cell proliferation. Both freezing media can be used for cryopreservation of cells.

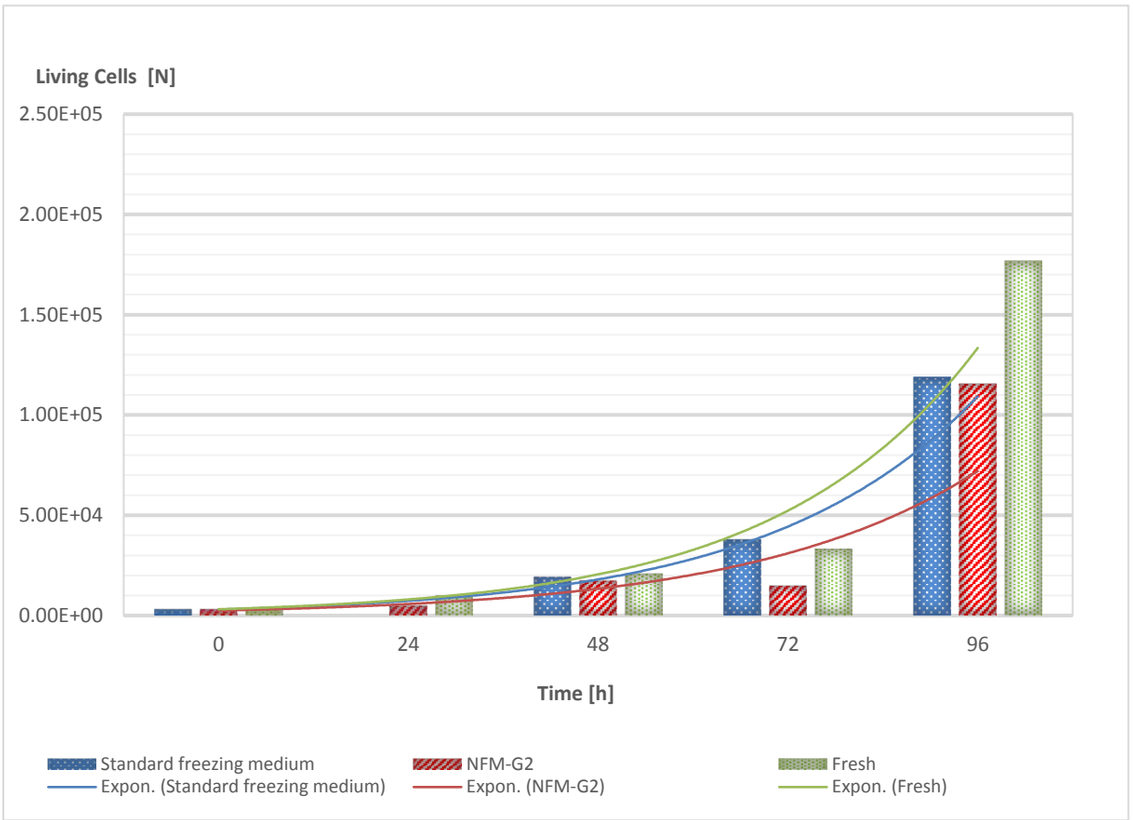


Figure 4.5.1.1 Comparison of growth between MDCK cells frozen in standard freezing medium and NFM-G2 and cells out of a continuous cultivation (fresh cells). MDCK cell are seeded within a cell density of $3.0 \cdot 10^3$ cells$\cdot$cm^{-2} in a 24-well plate and cultured up to 96 hours. Cells are detached every 24 hours and counted. Shown are the total number of living cells of fresh cells and cells which are frozen in NFM-G2 and standard freezing medium, as well as the associated fitting

Table 4.5.1.1 Number of living cells and viability of MDCK cells frozen in standard freezing medium and NFM-G2 and cells out of a continuous cultivation (fresh cells). MDCK cell are seeded within a cell density of $3.0 \cdot 10^3$ cells·cm^{-2} in a 24-well plate and cultured up to 96 hours. Cells are detached every 24 hours and counted.

	Living cells [N]			Viability [%]		
Time [h]	Std. Medium	NFM-G2	Fresh	Std. Medium	NFM-G2	Fresh
0	3.00E+03	3.00E+03	3.00E+03	98.02	97.95	97.37
24		4.81E+03	9.87E+03	97.70	94.29	96.36
48	1.92E+04	1.74E+04	2.09E+04	97.38	96.84	97.28
72	3.80E+04	1.48E+04	3.32E+04	97.65	93.06	94.53
96	1.19E+05	1.16E+05	1.77E+05	96.98	96.30	96.34

The results of the Caco-2 cells are shown in figure 4.5.1.2. The associated values of cell number and viability are listed in table 4.5.1.2. The growth curves of fresh and frozen Caco-2 cells are almost identical within the first 24 hours. 48 hours after seeding, the cell number of cells, frozen in standard freezing medium and fresh cells is similar ($5.31 \cdot 10^4$ cells in standard freezing medium and $6.27 \cdot 10^4$ cells in fresh cells), but the cell number of cells frozen in NFM-G2 only increased slightly (from $2.18 \cdot 10^4$ cells to $2.94 \cdot 10^4$ cells). The results of the next cell count indicate, that this value is an outlier. 72 and 96 hours after seeding the cell number of both frozen cell samples increases in the same way. The fitting curve of the both freezing media are similar. The slope of the fitting curve of the fresh cell is already after 72 hours far above of fitting curve of the both freezing media. The total cell number of fresh cells after 72 hours is $1.47 \cdot 10^5$ cells. Frozen cells reach this value 24 hours later (after 96 hours). The cell number obtained in standard freezing medium is $1.38 \cdot 10^5$ cells and in NFM-G2 $1.49 \cdot 10^5$ cells after 96 hours.

In general, the viability in all frozen Caco-2 cell samples increased within the time of the experiment (table 4.1.2). The viability of both frozen cell samples drops within the first 24 hours. Especially with the standard freezing medium the cells show a significant loss in viability within the first 24 hours, but viability recovers 48 hours after seeding. The viability of cells frozen in standard freezing medium decreases from 83.92% to 82.53%, but reach after 96 hours a viability of 91.85%. Cells frozen in NFM-G2 start with a viability of 90.42% and end up with 94.25%. Fresh cells show a viability between 88.05% and 93.29%.

In comparison to MDCK cells the Caco-2 cells are more sensitive to the freezing procedure and show in general a lower cell viability. The viability of MDCK cells is above 97% after thawing, whereas the viability in Caco-2 cells is between 83.92% and 90,42%. Also Caco-2 cells show that the freezing and thawing process delays the cell proliferation. The growth of cells in both freezing media are similar. In the view of viability, the NFM-G2 shows a better performance.

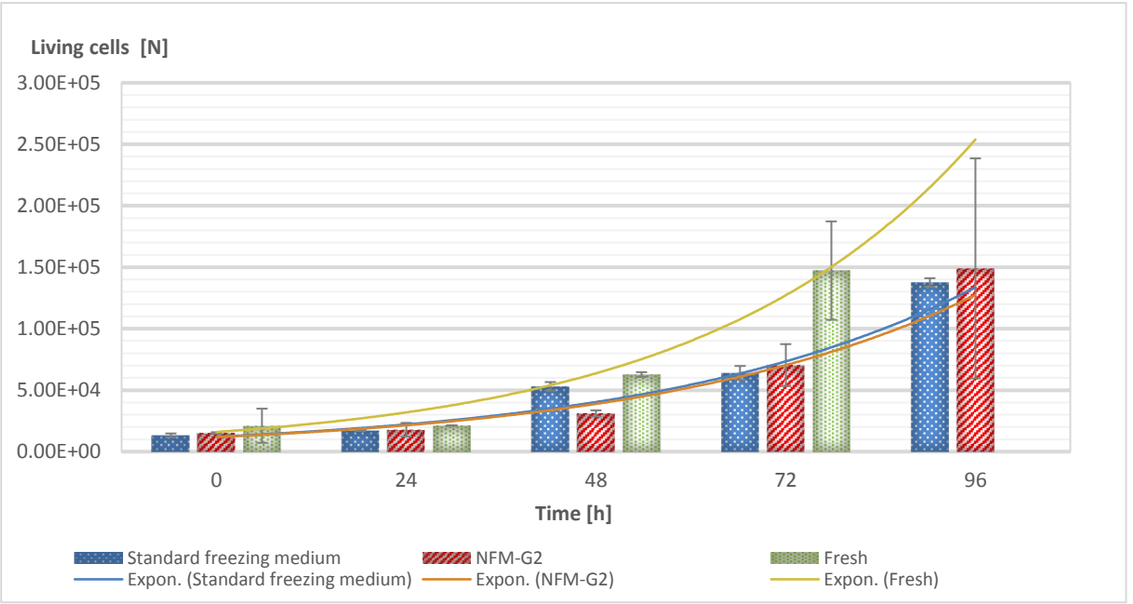


Figure 4.5.1.2 Comparison of growth between Caco-2 cells frozen in standard freezing medium and NFM-G2 and cells out of a continuous cultivation (fresh cells). Caco-2 cells are seeded at a cell density of $3.0 \cdot 10^3$ cells·cm^{-2} in a 24-well plate and cultured for 96 hours. Cells are detached every 24 hours and counted. The graph shows the total number of living cells, as well as the associated fitting curves (mean ±SD, n=2).

Table 4.5.1.2 Number of living cells and viability of Caco-2 cells frozen in standard freezing medium and NFM-G2 and cells out of a continuous cultivation (fresh cells). MDCK cell are seeded within a cell density of $3.0 \cdot 10^3$ cells·cm^{-2} in a 24-well plate and cultured up to 96 hours. Cells are detached every 24 hours and counted (mean ±SD, n=2).

	Living cells [N]					
	Std. Medium		NFM-G2		Fresh cells	
Time [h]	mean	Std. Dev.	mean	Std. Dev.	mean	Std. Dev.
0	1.33E+04	1.54E+03	1.52E+04	5.94E+02	2.11E+04	1.39E+04
24	1.72E+04	7.78E+01	1.78E+04	5.57E+03	2.12E+04	0.00E+00
48	5.31E+04	3.46E+03	3.11E+04	2.50E+03	6.27E+04	1.94E+03
72	6.40E+04	5.66E+03	7.04E+04	1.70E+04	1.47E+05	4.00E+04
96	1.38E+05	3.51E+03	1.49E+05	8.95E+04		

	Viability [%]					
	Std. Medium		NFM-G2		Fresh cells	
Time [h]	mean	Std. Dev.	mean	Std. Dev.	mean	Std. Dev.
0	83.92	0.948	90.42	0.926	88.05	10.73
24	82.53	2.001	88.46	1.492	91.61	2.81
48	92.37	1.223	89.94	1.626	91.20	0.56
72	92.23	0.354	93.92	0.799	93.29	0.59
96	91.85	1.655	94.25	0.191		

4.6 Comparison of the intestinal permeability assay with “fresh” and frozen cells

The previous experiments show, that frozen cells came close to the performance of fresh cells, with respect to the viability and lack of a lag phase in their growth. Although the frozen cells are stressed by freezing and thawing, both cell types show good proliferation, independent of the used freezing medium. The viability of MDCK cells after thawing is without exception high, whereas Caco-2 cells are slightly affected. MDCK cells, frozen in standard freezing medium shows slightly better results. In Caco-2 cells a higher viability is observed, if cells are frozen inNFM-G2.

Viability and proliferation of frozen cells are suitable for the permeability assay. Therefore, the next step is to test, if the freezing process influences the permeability of MDCK and Caco-2 monolayers after 4 days of incubation. Cells frozen in both freezing media were compared with fresh cells. The result is shown in 4.6.1.

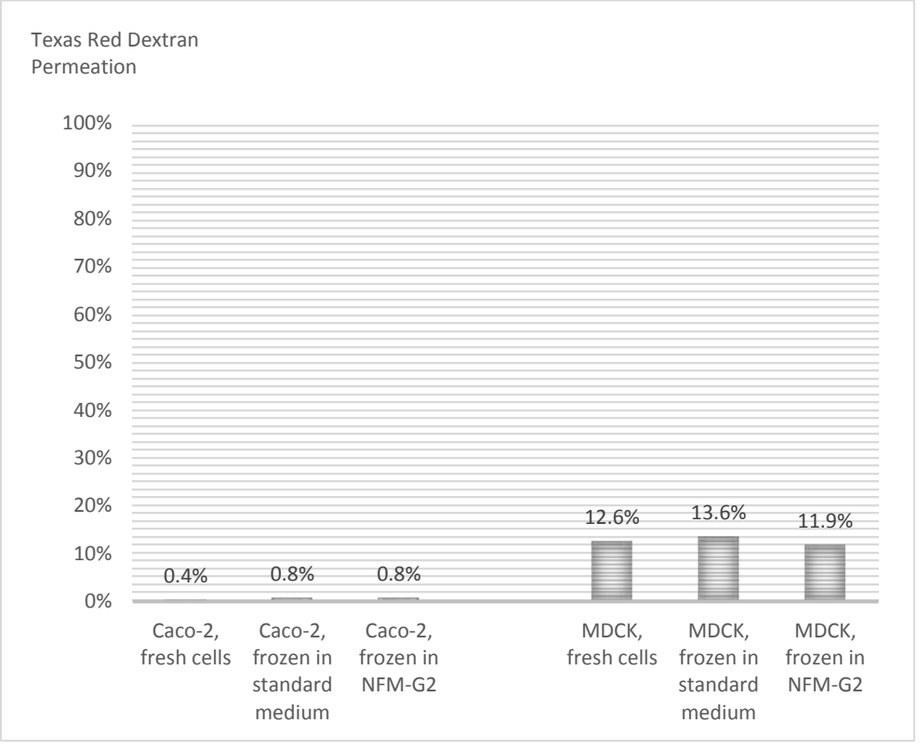


Figure 4.6.1 Determination of the cell monolayer integrity of frozen and fresh Caco-2 and MDCK cells after 4 days of incubation. Cells are frozen in standard freezing medium and NFM-G2. Seeding density in all samples is $4.0 \cdot 10^5$ cells·cm^{-2} for Caco-2 cells and $8.0 \cdot 10^4$ cells·cm^{-2} for MDCK cells. Texas Red Dextran will diffuse through gaps in the monolayer. The dye moves from the apical to the basolateral compartment. Measurement of the Texas Red Dextran after 3 hours of incubation at 37 °C in basolateral compartment. The result is calculated as % of the total Texas Red Dextran amount applied to the monolayer. Frozen cells are equivalent to fresh cells in the view of permeability value of Texas Red Dextran. MDCK do not meet the prerequisites of Texas Red Dextran Permeability less than 6%.

In figure 4.6.1 fresh and frozen Caco-2 and MDCK cells are shown. Fresh Caco-2 cells, show a permeability value of 0.4%. The permeability value of frozen cell monolayers is twice the permeability of the fresh cells. However, the

permeability values are very low and far away from the threshold of 6% permeability. Frozen Caco-2 cells, prepared with standard freezing medium or NFM-G2 can be used in the permeability assay. With MDCK cells the differences between frozen and fresh cells are small. The lowest value was obtained with MDCK cells, frozen in NFM-G2 (11.9%), followed by fresh cells with a Texas Red Dextran permeability of 12.6% and finally with 13.6%, MDCK cells, frozen in standard freezing medium. However, the differences are too small and can be neglected. Frozen cells are comparable to fresh cells in the view of permeability value of Texas Red Dextran. As already shown in previous experiments MDCK do not meet the specification.

4.7 TEER Measurement

Frozen cells and cells out of a continuous culture, named as “fresh cells” were seeded into a Transwell® and cultured for up to 21 days. On day 4, 8 and 21. The monolayer integrity was measured with Texas Red Dextran to assess a correlation between TEER value and Texas Red Dextran permeability (see table 4.8).

Looking first on frozen cells, the TEER values starts between 1.7 Ω·cm² (parenteral cell line) and 6.3 Ω·cm² (subclone B5). While the TEER value of the frozen, parenteral cell line and the frozen subclones C9 and C10 increases, the TEER value of subclone B5 stays almost constant over the first 4 days. This is indicating that subclone B5 needs more time for adaptation to the culture conditions, resulting in a 4-day lag phase. On day 4 all frozen cell lines reach almost the same TEER value. The TEER values were between 6.4 Ω·cm² (obtained in subclone B5) and 8.1 Ω·cm² (in subclone C10). The permeability assay, which was executed with the parenteral cell line, shows a value of 11.7% at a TEER value of 6.8 Ω·cm².

The permeability assay was also done in fresh cells. Although, the TEER values are higher (between 13.9 Ω·cm² in subclone C9 and 17.7 Ω·cm² in parenteral cell line) the permeability values of the fresh cells were significantly higher (8.6% and 19.3%). The expectation was, the higher the TEER value, the lower the permeability of Texas Red Dextran.

TEER values of the frozen subclones C9 and C10 rises step-by-step until the 6th day (11.6 Ω·cm² in subclone C9 and 12.2 Ω·cm² in subclone C10). The TEER value of subclone B5, rises until day 11 and reaches a value of 18.0 Ω·cm². The frozen, parenteral cell line stays constant until day 18. On day 18 the TEER value is 6.5 Ω·cm². After the stepwise increase, the TEER value of the sub--clones stay almost constant or decreases. For example, after the 6th day the TEER value of subclone C10 decreases until day 11. On this day a TEER value of 7.4 Ω·cm² is obtained. The TEER stays almost unchanged until the end on day 18 (6.8 Ω·cm²). S Also the TEER value of subclone B5 stays almost constant for the next 3 days (18.0 Ω·cm², 16.8 Ω·cm², 17.0 Ω·cm²) and decreases on day 14 to a value of 13.8 Ω·cm². The TEER value of subclone C9, increases only slightly up to day 14 with a TEER value of 14.0 Ω·cm².

Starting on day 19, the TEER values in all subclones and parenteral cell line increases in the same magnitude (an average increase of 11 Ω·cm²). The jump of all TEER values between day 18 and 19 might be connected to the formation of tight junctions and the reason, why many protocols recommend 21 days of culture before performing the permeability assay.

However, on day 21 the permeability was measured again, 8.25% Texas Red Dextran diffuse at a TEER of 16.3 Ω·cm².

In total the TEER values of MDCK cells are very low and changes are marginal. The results correspond with the high Texas Red Dextran permeability values, not meeting the specification limits.

In general, fresh cells show a higher TEER value than frozen cells. The highest TEER value of 30.7 Ω·cm² is obtained with fresh subclone C10 on day 7. But a TEER value of 500 Ω·cm², which describes a tight monolayer in literature, is far away and is not reached with MDCK cells. Therefore, MDCK cells are not suitable for the intestinal permeability assay.

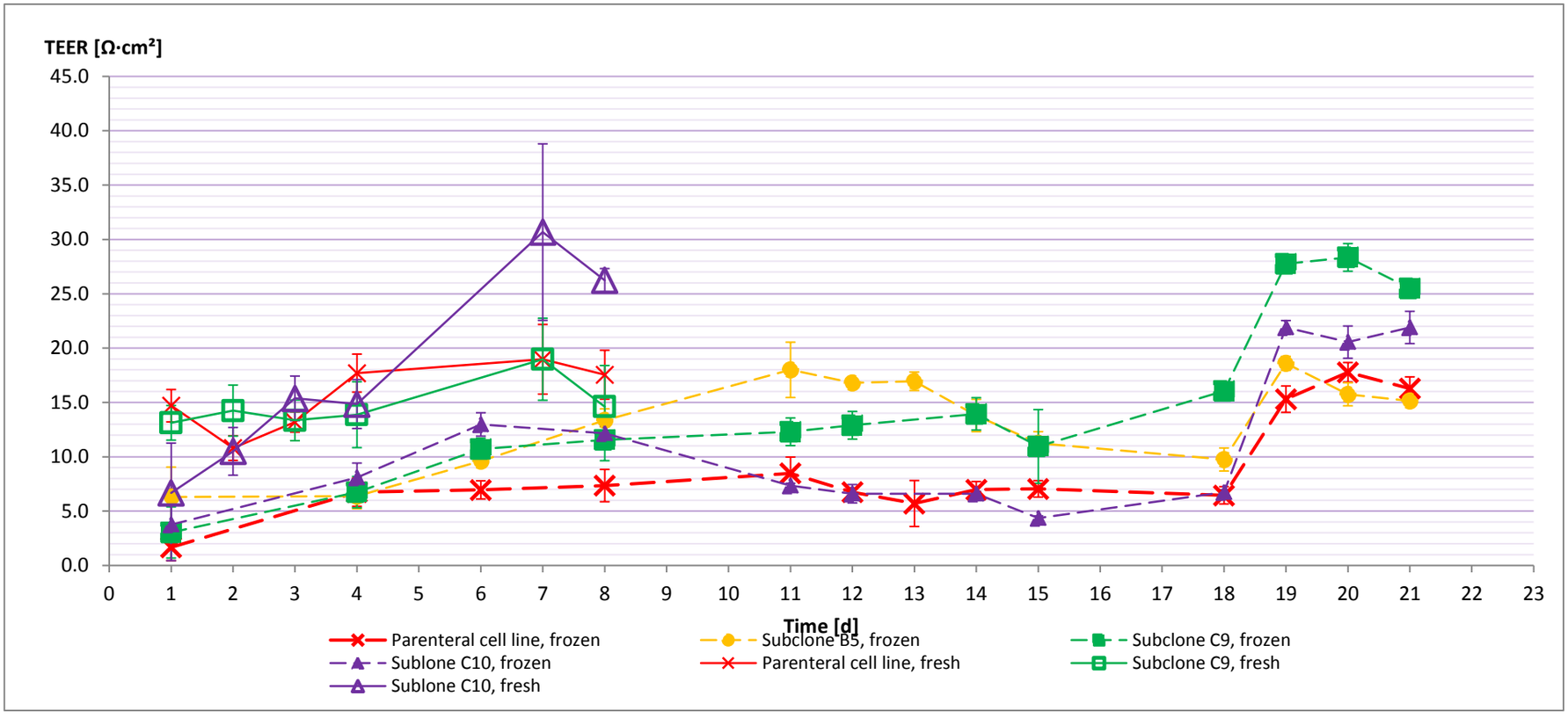


Figure 4.7.1 Transepithelial electrical resistance of cell monolayer of MDCK subclones and parenteral cell line as a function of culture time. Dotted line are cells, which were frozen in cell culture medium containing 5% DMSO and seeded immediately after thawing into a Transwell®. Seeding density is $8.0 \cdot 10^4$ cells·cm^{-2}. Whereas the solid line are cells out of continuous cell culture, named as “fresh cells”, to highlight the difference. Frozen cells were cultured up to 21 days and fresh cells up to 8 days. In general, fresh cells show a higher TEER value than frozen cells. The highest TEER value of 30.7 Ω·cm² is obtained with fresh subclone C10 on day 7. But 500 Ω·cm², described in literature as tight monolayer, could not be reached in MDCK cells (mean ±SD, n=2).

Table 4.7.1 Resistance measurement of MDCK cell monolayer of MDCK subclones and the parenteral cell line. Transepithelial electrical resistance were calculated by multiply the resistance with the area of the monolayer (0.3 cm²). The graphic illustration is shown in figure 4.8.1. Cells were frozen in cell culture medium containing 5% DMSO and seeded immediately after thawing into a Transwell®. Fresh cells are derived out of continuous cell culture. Seeding density is $8.0 \cdot 10^4$ cells·cm^{-2}. Frozen cells were cultured up to 21 days and fresh cells up to 8 days (mean ±SD, n=2).

	Parenteral cell line, frozen				Subclone B5, frozen				Subclone C9, frozen				Subclone C10, frozen			
Day	Resistance [Ω]	TEER [Ω ·cm²]	SD	Texas Red Dextran Permeability [%]	Resistance [Ω]	TEER [Ω ·cm²]	SD	Texas Red Dextran Permeability [%]	Resistance [Ω]	TEER [Ω ·cm²]	SD	Texas Red Dextran Permeability [%]	Resistance [Ω]	TEER [Ω·cm²]	SD	Texas Red Dextran Permeability [%]
1	5.6	1.7	1.2		21.0	6.3	2.7		10.1	3.0	2.4		12.5	3.8	3.3	
4	22.5	6.8	1.3	11.70	21.3	6.4	1.2		22.5	6.8	1.4		27.0	8.1	1.3	
6	23.2	7.0	0.8		32.0	9.6	0.5		35.8	10.7	1.0		43.3	13.0	1.1	
8	24.5	7.4	1.5	11.20	44.5	13.4	1.1	15.4	38.5	11.6	1.9	15.6	40.5	12.2	0.6	14.6
11	28.25	8.5	1.5		60	18.0	2.5		41	12.3	1.3		24.5	7.4	0.2	
12	22.5	6.8	0.4		56	16.8	0.4		43	12.9	1.3		22	6.6	0.8	
13	19	5.7	2.1		56.5	17.0	0.8									
14	23.25	7.0	0.8		46	13.8	1.5		46.5	14.0	3.4		22	6.6	0.2	
15	23.5	7.1	0.8		37.5	11.3	1.1		36.5	11.0	0.2		14.5	4.4	0.2	
18	21.5	6.5	0.8		32.5	9.8	1.1		53.5	16.1	0.6		22.5	6.8	0.6	
19	51	15.3	1.2		62	18.6	0.6		92.5	27.8	1.3		73	21.9	1.5	
20	59.25	17.8	0.9		52.5	15.8	1.1		94.5	28.4	0.2		68.5	20.6	1.5	
21	54.25	16.3	1.1	8.3	50.5	15.2	0.2	8.5	85	25.5	0.2	7.9	73	21.9	0.2	8.5
	Parenteral cell line, fresh								Subclone C9, fresh				Subclone C10, fresh			
1	49.0	14.7	1.5						43.8	13.1	1.6		22.3	6.7	4.6	
2	36.0	10.8	1.1						47.5	14.3	2.3		35.0	10.5	2.2	
3	44.0	13.2	0.9						44.5	13.4	1.9		51.3	15.4	2.1	
4	59.0	17.7	1.7	8.6					46.3	13.9	3.0	19.3	49.5	14.9	2.3	15.2
7	63.3	19.0	3.2						63.3	19.0	3.8		102.3	30.7	8.1	
8	58.5	17.6	2.3						48.8	14.6	3.8		87.5	26.3	1.1	

Frozen Caco-2 cells and cells out of a continuous culture, named as “fresh cells” were seeded into a Transwell® and maintained for up to 19 days. On day 2, 4 and 11 the monolayer integrity was measured with Texas Red Dextran to assess a correlation between TEER value and Texas Red Dextran permeability (see table 4.8).

In comparison to the MDCK cells the transepithelial resistance of Caco-2 is higher. Already after 24 hours the TEER value of the Caco-2 cells is higher than the maximum value of MDCK cells (30.7 Ω·cm²). After the second day all subclones are in the range of at least 200 Ω·cm². The highest TEER value is 1193.5 Ω·cm². It is measured in the well of the frozen, parenteral cell line on day 6. In general, the TEER values of this cell line are much higher than the values of the other Caco-2 cell lines.

The TEER course of the parenteral, frozen cell line is ascending continously, until it reaches its maximum of 1193.5 Ω·cm² on day 6. Thenceforth, the TEER value decreases continuously until day 18. On day 18 the TEER value reaches 384.3 Ω·cm² where is remains and remains for another day.

In contrast, the slope of the other samples is lower and they stay below such high values.
The fresh parenteral cell line starts at 68.5 Ω·cm² and reaches a value of 315 Ω·cm² within 3 days. From henceforward the TEER course stays constant until the end. At the end, on day 8 a TEER value of 332.7 Ω·cm² is reached.
The course of frozen subclones D5 and F9 are similar. The TEER values of frozen subclone D5 and F9 start at 6.8 Ω·cm² and 69.8 Ω·cm², respectively. On day 7 the course of D5 stops increasing. The TEER slightly decreases from 277.2 Ω·cm² to 201.9 Ω·cm² while F9 still increases from 373.0 Ω·cm² to 399.5 Ω·cm².

The TEER of fresh subclone D5 starts at 58.7 Ω·cm². It increases steeply, from one day to another and reaches a value of 172.2 Ω·cm². At this point the TEER increases only linear to day 4, where it reaches a value of 222.4 Ω·cm². From here it increases again steeply to a final value of 701.9 Ω·cm² on day 8.
The course of the fresh subclone F9 is also steeply increasing from the first to the second day, from 94.5 Ω·cm² to 225.2 Ω·cm². After that the slope increases only slightly until day 7, where the TEER value is 594.9 Ω·cm². From day 7 to day 8 the TEER value increases again steeply to da final value of 701.9 Ω·cm².

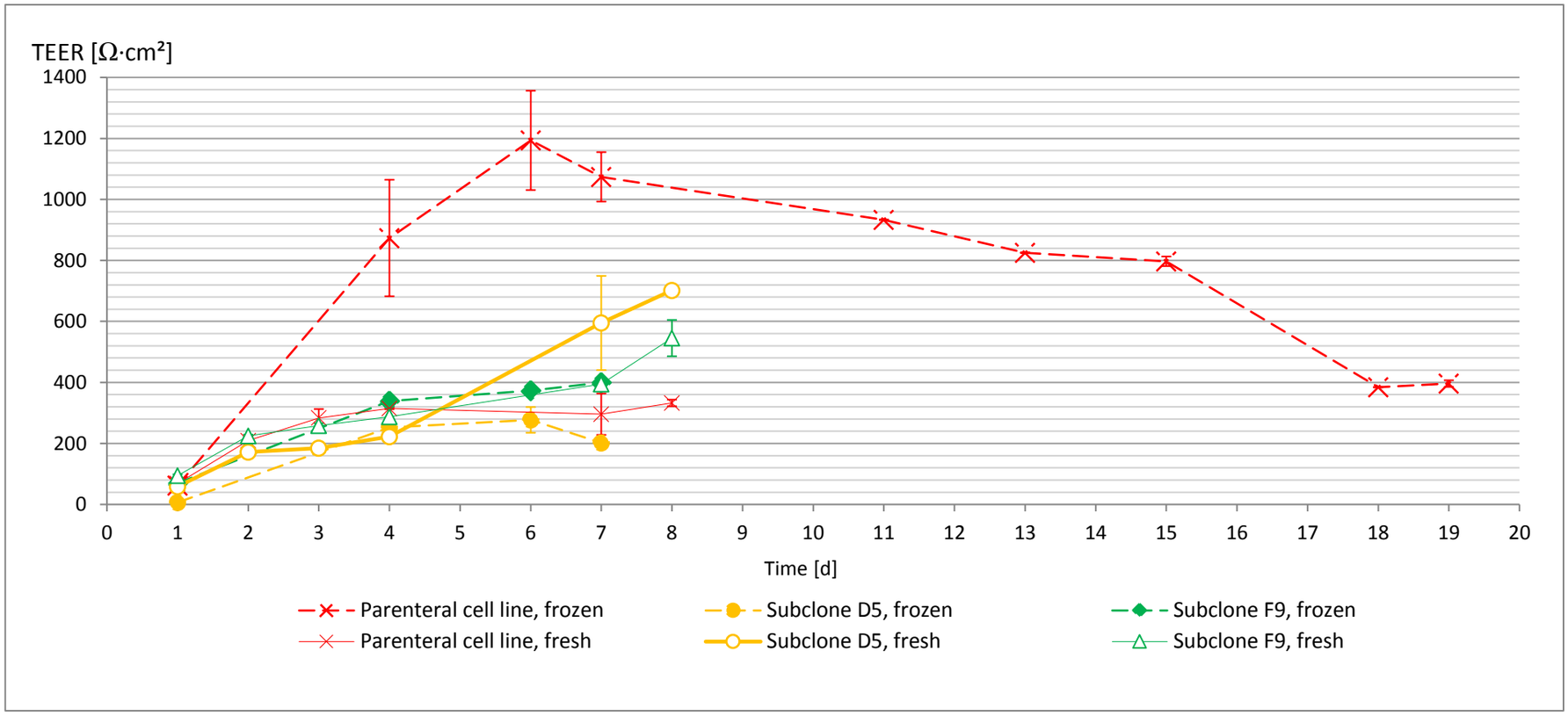


Figure 4.7.2 Transepithelial electrical resistance of cell monolayer of Caco-2 subclones and parenteral cell line as a function of culture time. Dotted line are cells, which were frozen in cell culture medium containing 5% DMSO and seeded immediately after thawing into a Transwell®. Seeding density is $4.0 \cdot 10^5$ cells·cm^{-2}. Whereas the solid line are cells out of continuous cell culture, named as "fresh cells", to highlight the difference. Frozen cells were cultured up to 19 days and fresh cells up to 8 days. Except the parenteral frozen cell line, all other behave almost equal. The highest TEER value of 3978.4 Ω·cm² is obtained with fresh subclone C10 on day 6. A TEER value of 500 Ω·cm², described in literature as tight monolayer (mean ±SD, n=2).

Table 4.7.2 Resistance measurement of Caco-2 cell monolayer of Caco-2 subclones and the parenteral cell line. Transepithelial electrical resistance were calculated by multiply the resistance with the area of the monolayer (0.3 cm^2). The graphic illustration is shown in figure 4.8.2. Cells were frozen in cell culture medium containing 5% DMSO and seeded immediately after thawing into a Transwell®. Fresh cells are derived out of continuous cell culture. Seeding density is $4.0 \cdot 10^5$ cells·cm^{-2}. Frozen cells were cultured up to 19 days and fresh cells up to 7 days (mean ±SD, n=2)

	Parenteral cell line, frozen				Subclone D5, frozen				Subclone F9, frozen			
Day	Resistance [Ω]	TEER [Ω ·cm^2]	SD	Texas Red Dextran Permeability [%]	Resistance [Ω]	TEER [Ω ·cm^2]	SD	Texas Red Dextran Permeability [%]	Resistance [Ω]	TEER [Ω ·cm^2]	SD	Texas Red Dextran Permeability [%]
1	207.7	62.3	4.7		22.0	6.6	1.5		232.5	69.8	1.7	
4	2911.5	873.5	190.9	0.1	842.5	252.8	12.9		1128.8	338.6	2.0	
6	3978.4	1193.5	162.8		924.0	277.2	42.1		1243.2	373.0	10.0	
7	3580.0	1074.0	80.9		673	201.9	7.64		1331.5	399.5	5.30	
11	3110.5	933.2	1.1	0.9								
13	2750.5	825.2	0.8									
15	2657	797.1	0.8									
18	1281	384.3	10.8									
19	1321	396.3	5.3									
	Parenteral cell line, fresh				Subclone D5, fresh				Subclone F9, fresh			
1	228.3	68.5	2.5		195.8	58.7	1.7		315.0	94.5	4.2	
2	698.5	209.6	4.6	0.7	574.0	172.2	3.3	0.9	750.8	225.2	3.9	0.4
3	944.8	283.4	29.4		616.3	184.9	2.5		862.5	258.8	7.4	
4	1050.8	315.2	2.8	0.3	741.3	222.4	9.3	0.6	957.0	287.1	34.3	0.4
7	986.5	296.0	68.0		1983.0	594.9	154.4		1315.0	394.5	11.6	
8	1109.0	332.7	10.8		2339.8	701.9	6.1		1817.8	545.3	59.6	

.

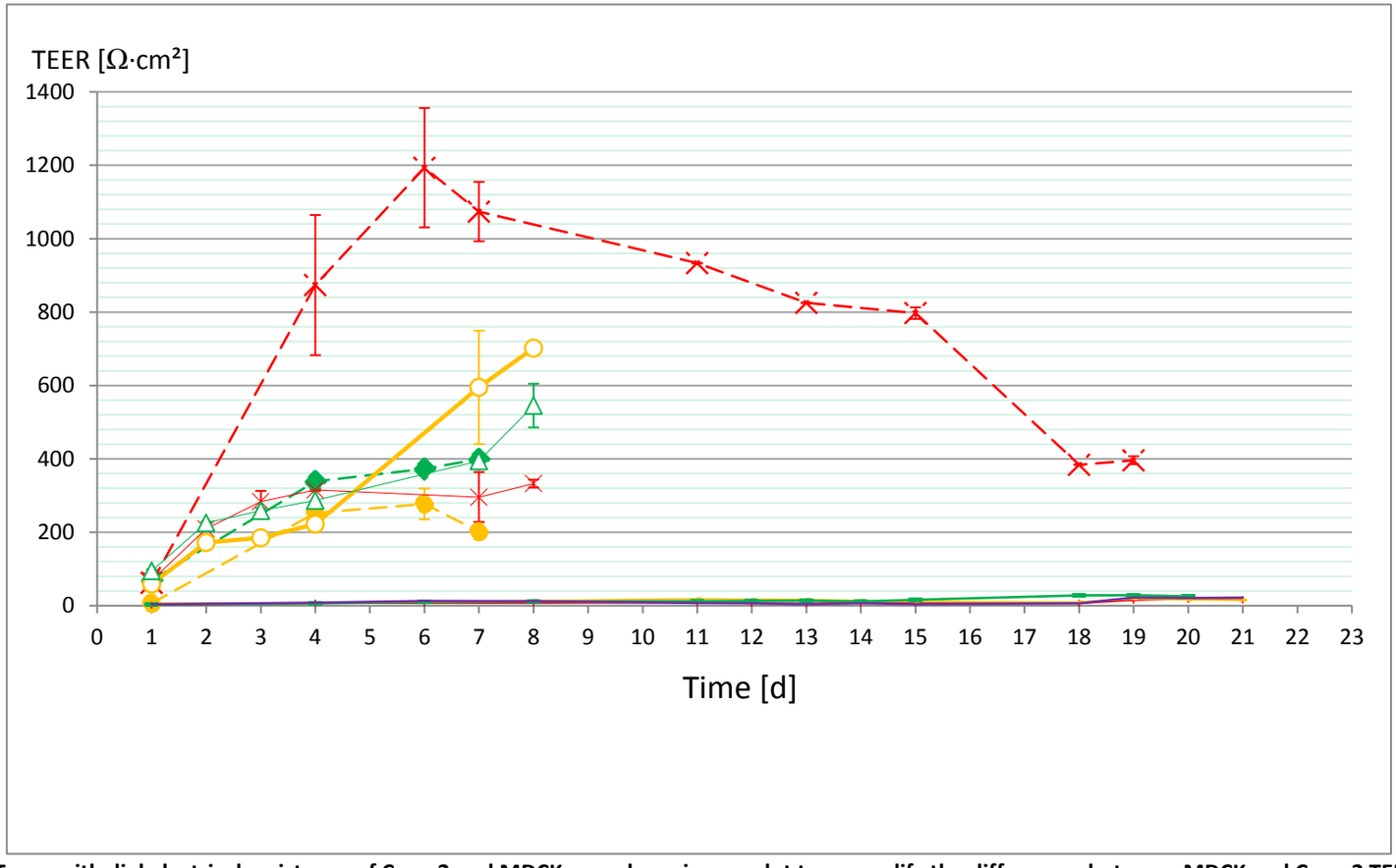


Figure 4.7.3 Transepithelial electrical resistance of Caco-2 and MDCK monolayer in one plot to exemplify the differences between MDCK and Caco-2 TEER values. The TEER values obtained in MDCK cells are so small that they run in parallel to the x-axis.

The curves of Caco-2 and MDCK cells from figure 4.7.1 and figure 4.7.2 are merged in one plot to demonstrate the differences between MDCK and Caco-2 with respect to their TEER and therefore their monolayer integrity. The differences that have been observed between the single MDCK subclones in chapter 4.7 are no longer recognized by the selected scale of the y-axis. The TEER values obtained in MDCK cells are so small that they run in parallel to the x-axis. The permeability values of Texas Red Dextran in MDCK cells were between 7.9% and 19.3%. The associated TEER values, of the obtained permeability values were 25.5 Ω·cm² and 13.9 Ω·cm², respectively. A Texas Red Dextran permeability less than 6% Texas Red Dextran could not be achieved in MDCK cells. Apparently TEER values less than 25.5 Ω·cm², indicating a less tight monolayer. Therefore, MDCK cells are not suitable for an intestinal permeability assay.

4.8 Relationship between Texas Red Dextran vs. TEER

To investigate the relationship between Texas Red Dextran permeability and the TEER across the monolayer and to find the TEER value where 6% or less Texas Red Dextran penetrates through the membrane, the permeability values of 20 experiments with the associated TEER values were illustrate in a diagram. A fitted curve through the data points describe the mathematical relationship. As seen in the diagram the relationship between TEER and Texas Red Dextran permeability is an exponential decay.

With the rearranged mathematical formula (Eq. 5), obtained from the data points the TEER value can be determined, where 6% Texas Red Dextran would be achieved.

$$TEER\ [\Omega \cdot \mathrm{cm}^2] = \left(\frac{Texas\ Red\ Dextran\ Permeability\ [\%]}{141.66 \left[\frac{\%}{\Omega \cdot \mathrm{cm}^2} \right]} \right)^{-\left(\frac{1}{0.979} \right)} \quad \text{(Eq. 5)}$$

Using this formula, a TEER of 25.3 Ω·cm² would lead to a permeability of 6%. In praxis with a TEER of 25.5 Ω·cm² a permeability of 7.9% could be reached. This value comes close to the mathematical value. But in praxis also a TEER of 11.7 Ω·cm² lead to a permeability of 6.6%, indicating a high fluctuation in the measurement range between 7.4 Ω·cm² and 25.5 Ω·cm². To be sure a permeability value less than 6% is achieved, the TEER value should be far above 24.5 Ω·cm². From 172.2 Ω·cm² on, the permeability values drop below 1% and the variations are small, indicating a threshold where the transport is independent from Texas Red Dextran and comes close to the threshold value observed in other publications. Madara (1998) explain the nonlinearity between the transport of marker molecule mannitol and insulin and the electrical resistance by the fact, that the transport of mannitol depends on the sum of

transport across all junctional pathways, whereas the electrical resistance dependent on areas with the lowest electrical resistance between single cells. Therefore, large changes in permeability will be determined in a leaky monolayer, whereas in tight monolayer changes in permeability are not so large (Gaillard and de Boer, 2000). Madara (1998) described threshold values of 200 Ω·cm² in epithelial cells, where the transport of mannitol and insulin was almost independent on the absolute value of transepithelial electrical resistance.

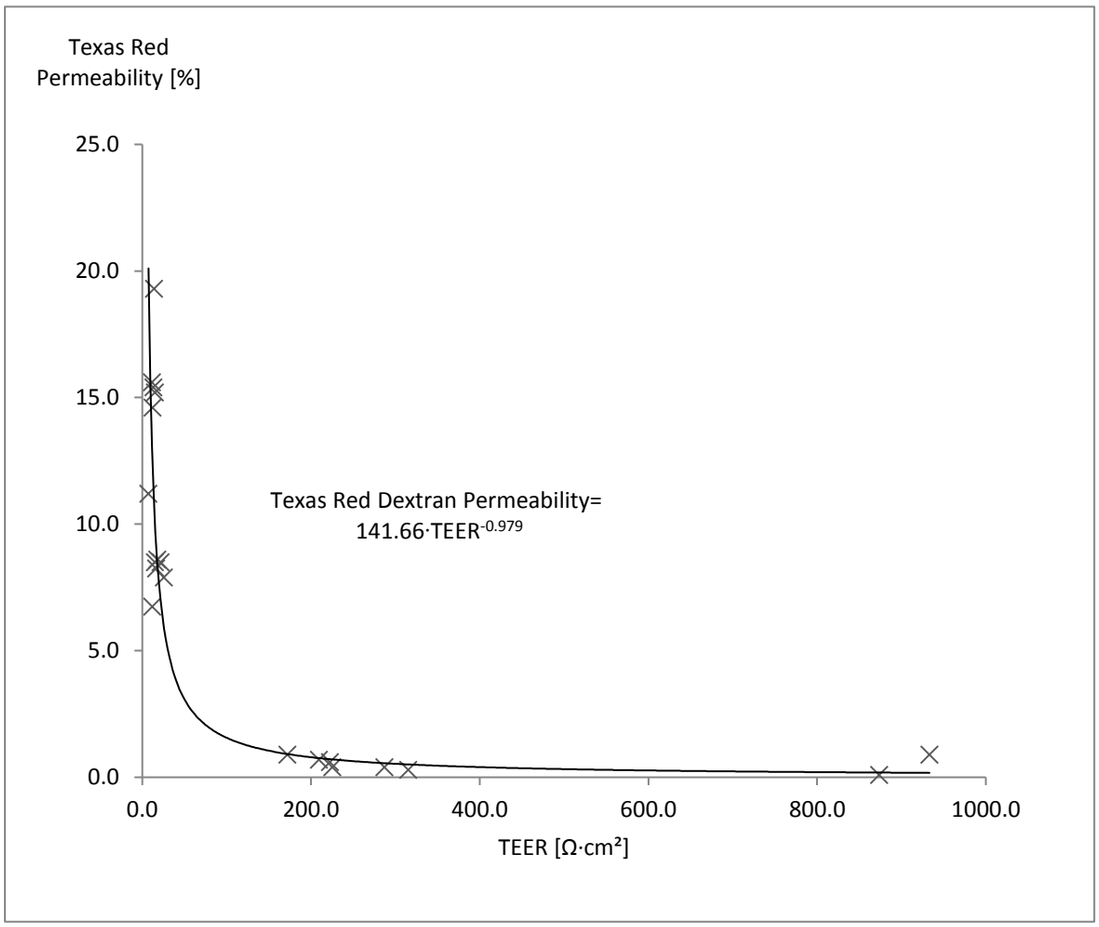


Figure 4.8 Nonlinear relationship between Texas Red Dextran Permeability and transepithelial resistance. At low TEER values (<172.2 Ω·cm²) large changes in Texas Red Dextran permeability are visible. At high TEER values (>172.2 Ω·cm²) the changes of Texas Red Dextran permeability are very modest.

Table 4.8 Empirical Relationship between Texas Red Dextran Permeability and transepithelial resistance

TEER [Ω·cm²]	Texas Red Permeability [%]
7.4	11.2
11.6	15.6
11.7	6.8
12.2	14.6
13.4	15.4
13.9	19.3
14.9	15.2
15.2	8.5
16.3	8.3
17.7	8.6
21.9	8.5
25.5	7.9
172.2	0.9
209.6	0.7
222.4	0.6
225.2	0.4
287.1	0.4
315.2	0.3
873.5	0.1
933.2	0.9

4.9 Drug transport in Caco-2

Dependent on the permeability value of the drugs they are classified in high, moderate or low permeable. The classification helps to predict the potential behavior of the drug in the human intestine. The company Cerep ranked the compounds in their commercially available Caco-2 permeability assay according following permeability values: Compounds with a P_{app} value < 2.0 10^{-6} cm·s^{-1} are classified as low permeable, and compounds >2.0·10^{-5} cm·s^{-1} are classified as high permeable (Cerep, 2013).

In this experiment amitriptyline hydrochloride and gabapentin are used as reference compounds to evaluate the functionality of the parenteral Caco-2 monolayer, as well as the monolayer of the subclones D5 and F9.

Table 4.9 Obtained P_{app} values of Amitriptyline hydrochloride and Gabapentin in Parenteral Cell line and in Caco-2 Subclones D5 and F9.

	Amitriptyline hydrochloride			Gabapentin		
	Parenteral Caco-2	Caco-2 subclone D5	Caco-2 subclone F9	Parenteral Caco-2	Caco-2 subclone D5	Caco-2 subclone F9
P_{app} [cm·s^{-1}]	2.6·10^{-5}	3.2·10^{-5}	7.0·10^{-5}	2.5·10^{-7}	1.4·10^{-6}	2.3·10^{-6}

Amitriptyline is known to be highly permeable in Caco-2 cells whereas gabapentin is low permeable. The permeability of the reference compounds amitriptyline hydrochloride and gabapentin are determined, and compared with P_{app} values in the literature to evaluate the functionality of the Caco-2 monolayer. For amitriptyline the working group, Faassen et al., 2003 determined a permeability value of 2.10·10^{-5} cm·s^{-1} and 1.73·10^{-5} cm·s^{-1} with a Caco-2 monolayer cultured on a filter well for 22-24 days. The company cyprotex identified a permeability value of 5.0·10^{-5} cm·s^{-1} on their 20 days old culture. For gabapentin the same company indicate a P_{app} value of approximately 3.0·10^{-7} cm·s^{-1}.

The used cell lines were frozen in standard freezing medium, thawed and seeded with a seeding density of 4.0·10^{5} cells·cm^{-2} on the apical side of the Transwell™ insert and cultured up to 7 days. The TEER values before drug application on the apical side of the monolayer, are 1074.0 Ω·cm² in the parenteral cell line, 201.9 Ω·cm² in subclone D5 and 399.5 Ω·cm² in subclone F9 (see table 4.7.2). The obtained P_{app} values are listed in table 4.9.

The permeability values between subclones and parenteral side differ slightly. However, the obtained P_{app} values correspond to the values reported by the company Cerep.

The P_{app} values of amitriptyline hydrochloride in Caco-2 cells are 2.6·10^{-5} cm·s^{-1} or higher, indicating amitriptyline as highly permeable compound. And also for gabapentin the cells showed a permeability of approximately 2.0·10^{-6} cm·s^{-1}, characterizes gabapentin as low preamble.

The company Cyprotex specify a permeability of 3.0·10^{-7} cm·s^{-1} for gabapentin.

With a P_{app} value of $2.5 \cdot 10^{-7}$ $cm \cdot s^{-1}$, the parenteral cell line shows a similar result. P_{app} values for amitriptyline hydrochloride in literature range from $1.73 \cdot 10^{-5}$ $cm \cdot s^{-1}$ to $5.0 \cdot 10^{-5}$ $cm \cdot s^{-1}$. And again the parenteral cell line and the subclone D5 with P_{app} values of $2.6 \cdot 10^{-5} cm \cdot s^{-1}$ and $3.2 \cdot 10^{-5}$ $cm \cdot s^{-1}$, respectively show similar values.

P_{app} values which were obtained with continuously grown cells for a period of 20 to 24 days, were achieved, with frozen cells after 7 days of cultivation. The P_{app} values of the parenteral cell line were close to the literature. This result is not surprising, because the parental cell line stem from a commercial source and the cell pool should be similar to the pool used in other groups. The subclone D5 shows slightly changed values. Here the divergent behavior of the more homogeneous subclone is responsible for the shift. Subclone F9 differs significantly from D5 in previous experiments and this behavior also reflects in the P_{app} values obtained for the both drugs.

4.10 Freezing Medium and Freezing Devices in Subclones.

In previous experiments MDCK cells were sorted out, because they do not meet the specification. However, this experiment is focused on the subclones D5 and F9. Both subclones are interesting for intestinal permeability, because one express P-gp (subclone D5) and for the other (subclone F9) P-gp expression was not detectable. Both subclones show good performance in the view of monolayer integrity (investigate with Texas Red Dextran permeability assay and TEER measurement). It was also possible to obtain similar Papp values for the reference compounds, with frozen subclone D5.

Because freezing is a stressful procedure for cells, the process should be as gentle as possible to generate a high cell vitality after thawing. As mentioned in the theory part, cryoprotectants and freezing devices have an influence on the freezing process. Therefore, subclones are frozen with three different freezing devices (Mr. Frosty, Cryomed® and CoolCell®) in combination with two freezing media (standard freezing medium and NFM-G2) to proof which combination “is the best”. Criteria for evaluating the freezing process, are the viability, the amount of debris in comparison to the total number of living cells (cell/debris ratio) and the aggregation factor. The viability should be above 90%. The counts for debris should be below the counts for living cells. This means that the cell/debris ratio should be below 1.0. Cell aggregation should be as small as possible, but not exceed an aggregation factor of 2.5.

These specifications are determined before cells are frozen, immediately after thawing and after 24 hours in culture.

Looking first on the cell viability of both subclones, a viability above 90% is desirable. The cell viability before freezing is for both subclones are above 92%. The subclones were frozen either with the freezing devices Mr. Frosty, Cryomed® or CoolCell®. Immediately after thawing and before seeding into a cell culture flask, the viability is measured again.

In every sample the viability decreases slightly. Strong effects are visible in the samples of subclone F9. Heree the viability of all samples except the sample “NFM-G2, Cryomed®” and “NFM-G2, CoolCell®” fall below the 90% threshold. After 24 hours in culture all samples of subclone F9 show a viability below 90%. Samples of subclone D5 all show a sufficient vaibility, only the sample, frozen in standard freezing medium with the Cryomed®, misses the threshold with a viability of 86.36% after 24 hours in culture.

Of course viability at the day of harvest affects the output. For example, the cell viability of subclone D5 is approximately 97% before cryopreservation in almost all samples. The samples of subclone F9 show already a lower cell viability (92.9% in NFM-G2 and 94.99% in standard freezing medium) before they are frozen. Based on these observations, cells should be frozen with a viability of at least 97%.

The cell/debris ratio is calculated by the amount of debris divided by the number of viable cells. The amount of debris should be less than the amount of living cells, means that the debris ratio should be less than 1.0. (An extremely high level of debris can also indicate a bacterial contamination.)

The debris ratio before the freezing process fluctuates in the subclone F9 between 0.3 and 0.45. For subclone D5 the range is between 0.08 and 0.09. The cell/debris ratio increases in all subclones after thawing. Indicating that cells suffer under the freezing and thawing process. Most debris are visible in the sample “Cryomed®, D5, standard freezing medium”. The lowest debris ratio is observed within the sample, frozen with freezing device Mr. Frosty in NFM-G2 and standard freezing medium, respectively. After thawing the debris ratios are remarkably high in subclone F9. The cell/debris ratio is above 1. Microscopic observation shows no bacterial contamination.

The aggregation factor keeps constant in all three phases (before freezing, after thawing and cultured for 24 hours) under 2.5 (data not shown).

In table 4.10 the viability, cell/debris ratio and aggregation factor after 24 hours in culture are listed. Subclones frozen into standard freezing medium and NFM-G2 with Mr. Frosty, Cryomed® and CoolCell®are shown.

Combinations of device and freezing medium, which fulfill the specifications are marked grey.

By using the established parameter, the subclone F9 shows poor viability and a high cell debris ratio. This correlates with the cell conditions before freezing. The cell viability was in comparison to subclone D5 (97%) lower (92.9% in NFM-G2 and 94.99% in standard freezing medium). The low viability of subclone F9 is a characteristic of the subclone itself and difficult to change. Even under optimal conditions during cultivation and maintenance, the average viability is 90.52%±4%. Subclone D5 almost meet the defined criteria. It was able to fulfill the specifications with both freezing media. A difference between both freezing media cannot be seen. Almost all freezing devices can be used.

Unexpected were the results of the freezing device Cryomed®, which works with NFM-G2 but not with standard freezing medium. Theoretically the freezing device Cryomed® should perform the best, because it is supposed to compensate the latent heat fusion, which arise during freezing, by controlling the cooling rate.

In this experiment the non-expensive alternative Mr. Frosty shows the best results but differs only marginal from the other devices.

NFM-G2 is in contrast to the standard freezing medium, a defined medium, without Serum, which also reduces the costs for the freezing process.

Table 4.10 Results after cells 24 hours in culture of viability, cell/debris ratio and aggregation factor are listed of subclones frozen with three different freezing devices (Mr. Frosty, Cryomed® and CoolCell®) into two freezing media (standard freezing medium and NFM-G2). Combination of device and freezing medium which fulfill the requirements are marked grey, criteria which are not fulfilled remained white.

	Subclone F9						Subclone D5					
	NFM-G2			Std. Medium			NFM-G2			Std. Medium		
	Mr. Frosty	Cryomed	Cool Cell	Mr. Frosty	Cryomed	Cool Cell	Mr. Frosty	Cryomed	Cool Cell	Mr. Frosty	Cryomed	Cool Cell
Viability [%]	84.96	85.11	78.65	81.57	81.50	79.25	94.58	93.64	90.72	95.76	86.36	95.16
Cell/Debris	1.39	1.32	1.99	1.37	1.65	2.04	0.39	0.60	0.65	0.44	1.21	0.58
Aggregation factor	1.291	1.190	1.062	1.193	1.130	1.089	1.232	1.240	1.361	1.399	1.149	1.237

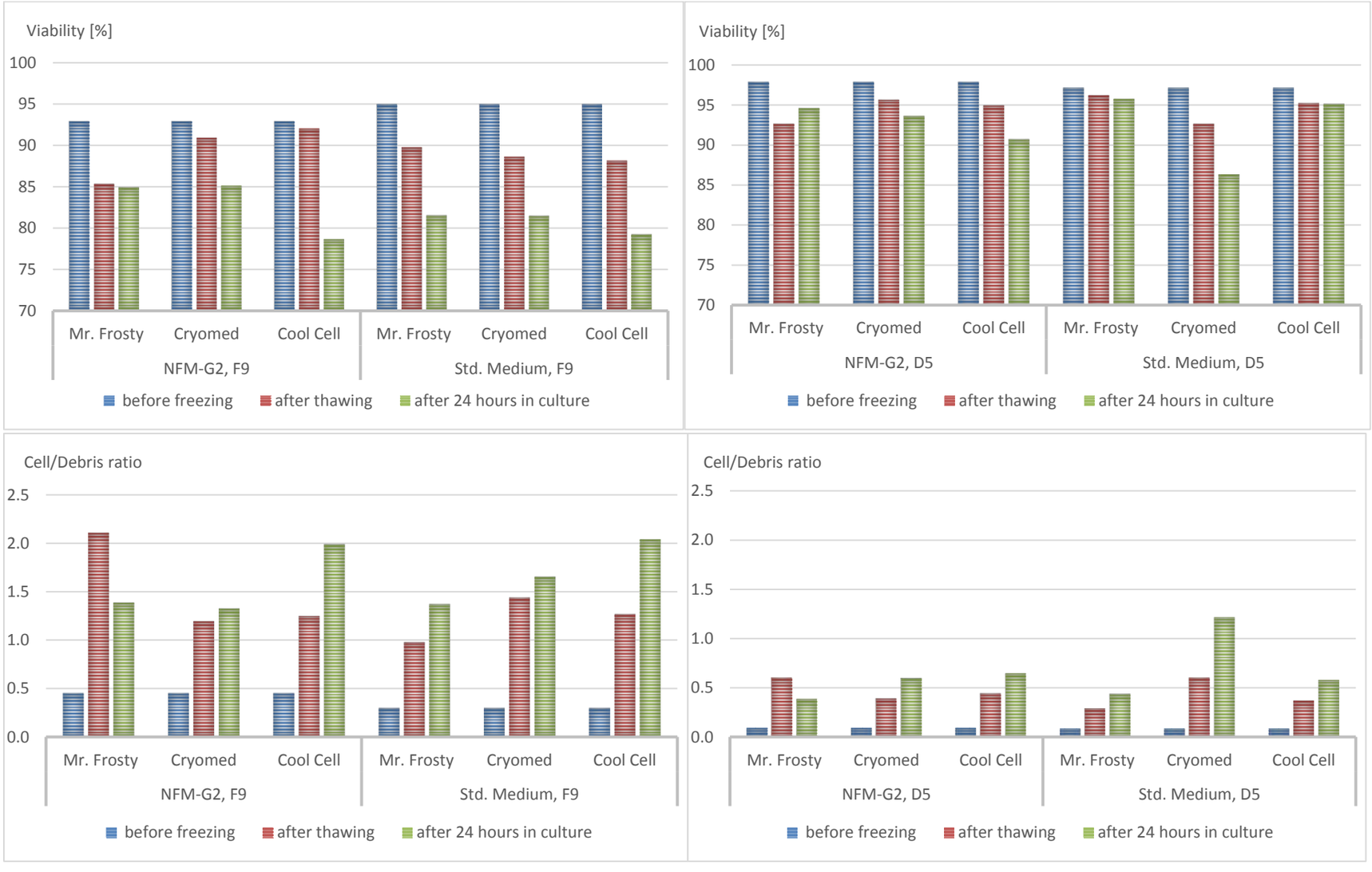


Figure 4.10 Subclones F9 and D5 are frozen with three different freezing devices (Mr. Frosty, Cryomed® and CoolCell®) into two freezing media (standard freezing medium and NFM-G2). Viability and cell/debris ratio before freezing (blue), after thawing (red) and after 24 hours in culture (green) are evaluated. The viability should be above 90%. The amount of debris should be less than 1.0. Strong affected are the samples of subclone F9, where all samples fall below the 90% boundary. In subclone D5 all samples show a sufficient vitality, only the sample, which are frozen in standard freezing medium with the Cryomed® falls to a viability of 86.36% after 24 hours in culture. The cell/debris ratio increased in all subclones after thawing. After thawing the debris ratios are remarkably high in subclone F9. Debris ratio are above 1.

5. Summary

The aim of this thesis is to develop Assay Ready Frozen Cells for a rapid permeability Assay.

Approaches in which the substance can be tested after 4 days are already described in literature. That is the starting point of this thesis. For the permeability assay MDCK and Caco-2 cells are used. The permeability assay is evaluated in advanced with fresh cells. To determine the best seeding density for the prospective permeability assays, different cell densities were tested at the beginnng. The lowest permeability values for the marker molecule Texas Red Dextran were obtained with a seeding density of $4.0 \cdot 10^5$ cells·cm^{-2} in Caco-2 and $8.0 \cdot 10^4$ cells·cm^{-2} in MDCK cells. Further optimization regarding the permeability assay were necessary to reduce the permeability and high variations. A prolonged seeding time in MDCK up to 21 days or the use of a specific serum were not able to improve the results. An important step was the demonstration, that a high aspiration rate causing strong variations and high permeability values in Caco-2 cells. Lowering the aspiration rate from 13.92 mL·s^{-1} to 0.0672 mL·s^{-1} lead to a reduction of Texas Red Dextran permeability from 25.8% ±16.1% to 0.4%±0.3%. MDCK cells are less sensitive to shear forces caused by the suction than Caco-2 cells. The permeability in MDCK cells was reduced only slightly (from 11.1% ±1.9% to 8.1% ±2.8%). Also changes in culture- and assay conditions, like a prolonged incubation time from 4 days to 5, 8 or even 21 days, had no effect on the permeability. No clone of 14 isolated subclones of MDCK was able to form a dense monolayer and fulfill the prerequisite to allow a Texas Red Dextran less than 6%. The highest TEER values of 30.7 Ω·cm². It was obtained in the MDCK subclone C10 on day 7. The results correspond with the high Texas Red Dextran permeability values, not meeting the specification limits. Therefore, MDCK cells are not suitable for the intestinal permeability assay.

But not only a tight, but also a functional monolayer is important for the permeability assay. The P-gp transporter influences the drug permeability a serves as indicator for the functionality of the monolayer. The Calcein AM assay was established for subclone screening based on the ability to express the P-gp transporter. Next to the parenteral Caco-2 cell line, which show a P-gp activity of only 12%, two additional subclones, subclone D5 with a high- (98.97%) and subclone F9 with a low P-gp activity (7.86%) were chosen to evaluate them for the permeability assays. TEER values in subclones were already high after the second day. The values were in the range of at least 200 Ω·cm². The highest TEER value is 1193.5 Ω·cm². It is measured in one sample of the frozen, parenteral cell line at day 6.

In this thesis amitriptyline hydrochloride and gabapentin are used as reference compounds to evaluate the functionality of the parenteral Caco-2 monolayer, as well as the monolayer of the subclones D5 and F9. P_{app} values which were obtained with continuous cells grown for 20 to 24, were achieved, with frozen cells within 7 days. Best accordance to literature values shows the parenteral cell line, followed by subclone D5. This result is not surprising because the parental cell line is close to the cellular material used in other groups.

Two freezing media are used to cryopreserve cells, the “standard freezing medium” and the “New Freezing Medium Generation 2” (NFM-G2). Both media show equivalent results.

The freezing and thawing process delays the cell proliferation shown by growth curves in comparison to fresh cells. The growth of cells cryopreserved in both freezing media, is similar after thawing. In the view of viability, the NFM-G2 shows a better performance in Caco-2. Fresh Caco-2 cells, show a permeability value of 0.4%. The permeability value of frozen cell monolayers is 0.8%. However, the permeability values are very low and far away from the threshold of 6% permeability.

Subclones are frozen with three different freezing devices (Mr. Frosty, Cryomed® and CoolCell®) with two different freezing media (standard freezing medium and NFM-G2) to proof which combination “is the best”. Again both media show similar results. Subclone F9 show a general a lower viability, which affects the quality of frozen cells negatively. The subclone D5 shows a good viability before and after freezing, independent of the freezing medium and device. In this work Mr. Frosty shows the best results, but differs only marginal from the other devices. Almost all freezing devices can be used. Unexpected is the result of the freezing device Cryomed®, which works with NFM-G2 but not with standard freezing medium. NFM-G2 is in contrast to the standard freezing medium, a defined medium, without Serum, which also reduced costs.

In the end the results show, that Caco-2 cells can be used for the intestinal permeability assay as frozen cells, if the cryostocks are prepared from cultures of optimal quality and by the use of NFM-G2 in combination with a controlled rate freezer. Sub-cloning changes the properties of the cell lines, indicating that commercially available Caco-2 and MDCK cell lines are heterogeneous cell populations. The results obtained with the isolated subclone D5 are close to the reference values. There is no need to switch to a new clone. Nevertheless, subclone F9 shows no P-gp activity in the calcein AM assay. Here a Caco-2 clone is available which might be interesting if a P-gp activity of Caco-2 cells is unwanted, e.g. as basic cell line for the expression of genetically modified P-gp.

6. References

6.1 Literature

Accutase (2016). Accutase® Cell Detachment Solution, available: http://www.accutase.com/accutase.html [accessed: 27. August 2016]

Alsenz, J., and Haenel, E. (2003). Development of a 7-day, 96-well Caco-2 permeability assay with high-throughput direct UV compound analysis. *Pharmaceutical research*, *20*(12), 1961-1969.

Artursson, P., and Karlsson, J. (1991). Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochemical and biophysical research communications*, 175(3), 880-885.

BioCision (2016). Cell Cryopreservation, available: http://www.biocision.com/applications/cell-cryopreservation [accessed: 27. August 2016]

Brockbank K. G. M., Covault J. C., Taylor M. J. (2007). Cryopreservation Guide. Thermo Fisher Scientific. Available: https://www.thermofisher.co.nz/Uploads/file/Scientific/Applications/Equipment-Furniture/Cryopreservation-Guide.PDF [accessed: 27. August 2016]

Cerep (2013). In vitro drug a absorbtion, available: http://www.cerep.fr/cerep/users/pages/Downloads/Documents/Marketing/Pharmacology%20&%20ADME/Application%20notes/2013/In%20vitro%20drug%20absorption.pdf [accessed: 6. October 2016]

Cyprotex (2016) Caco-2 Permeability, available: http://www.cyprotex.com/product_sheets/Cyprotex_Caco-2_Product_Sheet.pdf [accessed: 6. October 2016]

Cho, M. J., Thompson, D. P., Cramer, C. T., Vidmar, T. J., and Scieszka, J. F. (1989). The Madin Darby canine kidney (MDCK) epithelial cell monolayer as a model cellular transport barrier. *Pharmaceutical research*, 6(1), 71-77.

Chong, S., Dando, S. A., and Morrison, R. A. (1997). Evaluation of Biocoat® intestinal epithelium differentiation environment (3-day cultured Caco-2 cells) as an absorption screening model with improved productivity. *Pharmaceutical research,* 14(12), 1835-1837.

Coecke, S., Balls, M., Bowe, G., Davis, J., Gstraunthaler, G., Hartung, T., Hay, R., Merten O., Schlechtman, L., Staceys, G. and Stokes, W. (2007). Guidance on Good Cell Culture Practice. *In Cell Technology for Cell Products* (313-315).

Corning (2007) HTS ® -96 Permeable Support Protocols for Drug Transport: Application Note, available: http://www.level.com.tw/html/ezcatfiles/vipweb20/img/img/34961/2-10t_HTS_®_96_Protocols_Drug_Transport_CLS-AN-058.pdf [accessed: 27. August 2016]

Corning (2013) Transwell® Permeable Supports Selection and Use Guide, available: http://csmedia2.corning.com/LifeSciences/Media/pdf/transwell_guide.pdf [accessed: 27. August 2016]

Dimasi J. A., (2014). Innovation in the Pharmaceutical Industry: New Estimates of R&D Costs. R&D Cost Study Briefing; November 18, 2014. *Tufts Center for the Study of Drug Development (TCSDD),5,* available: http://csdd.tufts.edu/files/uploads/Tufts_CSDD_briefing_on_RD_cost_study_-_Nov_18,_2014. [accessed: 27. August 2016]

Drexler, H. G., and Uphoff, C. C. (2002). Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention. Cytotechnology, 39(2), 75-90.

Dukes, J. D., Whitley, P., and Chalmers, A. D. (2011). The MDCK variety pack: choosing the right strain. *BMC cell biology*, 12(1), 1.

Faassen, F., Vogel, G., Spanings, H., and Vromans, H. (2003). Caco-2 permeability, P-glycoprotein transport ratios and brain penetration of heterocyclic drugs. *International journal of pharmaceutics*, *263*(1), 113-122.

Fahy, G. M., MacFarlane, D. R., Angell, C. A., and Meryman, H. T. (1984). Vitrification as an approach to cryopreservation. Cryobiology, 21(4), 407-426.

Fahy, G. M. (1986). The relevance of cryoprotectant "toxicity" to cryobiology. Cryobiology, 23(1), 1-13.

Fogh, J., Fogh, J. M., and Orfeo, T. (1977). One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice. Journal of the National Cancer Institute, 59(1), 221-226.

Freshney R. I. (2010) Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications, 6th Edition, New Jersey: Wiley-Blackwell

Gaillard, P. J., and de Boer, A. G. (2000). Relationship between permeability status of the blood–brain barrier and in vitro permeability coefficient of a drug. European journal of pharmaceutical sciences, 12(2), 95-102.

Gao, D., and Critser, J. K. (2000). Mechanisms of cryoinjury in living cells. *ILAR journal*, *41*(4), 187-196.

Garberg, P., Eriksson, P., Schipper, N., and Sjöström, B. (1999). Automated absorption assessment Using Caco-2 cells cultured on both sides of polycarbonate membranes. *Pharmaceutical research*, 16(3), 441-445.

Grès, M. C., Julian, B., Bourrié, M., Meunier, V., Roques, C., Berger, M., Boulenc X., Berger Y. and Fabre, G. (1998). Correlation between oral drug absorption in humans, and apparent drug permeability in TC-7 cells, a human epithelial intestinal cell line: comparison with the parental Caco-2 cell line. *Pharmaceutical research,* 15(5), 726-733.

Gstraunthaler, G. J. A. (1988). Epithelial cells in tissue culture. Kidney and Blood Pressure Research, 11(1-2), 1-42.

Hunter, J., Jepson, M. A., Tsuruo, T., Simmons, N. L., and Hirst, B. H. (1993). Functional expression of P-glycoprotein in apical membranes of human intestinal Caco-2 cells. Kinetics of vinblastine secretion and interaction with modulators. *Journal of Biological Chemistry*, 268(20), 14991-14997.

Homolya, L., Hollo, M., Müller, M., Mechetner, E. B., and Sarkadi, B. (1996). A new method for a quantitative assessment of P-glycoprotein-related multidrug resistance in tumour cells. *British journal of cance*r, 73(7), 849.

Horie, K., Tang, F., and Borchardt, R. T. (2003). Isolation and characterization of Caco-2 subclones expressing high levels of multidrug resistance protein efflux transporter. Pharmaceutical research, 20(2), 161-168.

Horio, M., Chin, K. V., Currier, S. J., Goldenberg, S., Williams, C., Pastan, I., Gottesman M. M. and Handler, J. (1989). Transepithelial transport of drugs by the multidrug transporter in cultured Madin-Darby canine kidney cell epithelia. *Journal of Biological Chemistry*, 264(25), 14880-14884.

International Serum Industry Association (2011), frequently Asked Questions 20110511 available: http://www.serumindustry.org/documents/sera20110511_000.pdf [accessed: 27. August 2016]

Irvine, J. D., Takahashi, L., Lockhart, K., Cheong, J., Tolan, J. W., Selick, H. E., and Grove, J. R. (1999). MDCK (Madin–Darby canine kidney) cells: a tool for membrane permeability screening. *Journal of pharmaceutical sciences,* 88(1), 28-33.

Karow, A. M., and Carrier, O. (1969). Effects of cryoprotectant compounds on mammalian heart muscle. Surgery, gynecology & obstetrics, 128(3), 571-583.

Lee, M. K. K. (2014). Drug Development in Cell Culture: Crosstalk from the Industrial Prospects. *Journal of Bioequivalence & Bioavailability*, 6(3) 96-114.

Lentz, K. A., Hayashi, J., Lucisano, L. J., and Polli, J. E. (2000). Development of a more rapid, reduced serum culture system for Caco-2 monolayers and application to the biopharmaceutics classification system. *International journal of pharmaceutics*, *200*(1), 41-51.

LouVARD, D. (1980). Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. *Proceedings of the National Academy of Sciences*, 77(7), 4132-4136.

Luttmann, W., Bratke, K., Küpper, M., and Myrtek, D. (2009). Der Experimentator: Immunologie, 3th Edition, Heidelberg, Springer-Verlag.

Madara, J. L. (1998). Regulation of the movement of solutes across tight junctions. Annual review of physiology, 60(1), 143-159.

Mainprize, T., and Grady, L. T. (1998). Standardization of an in vitro method of drug absorption. In *Pharmacopeial Forum*, 24, 6015-6023.

MacLeod, R. A., Dirks, W. G., Matsuo, Y., Kaufmann, M., Milch, H., and Drexler, H. G. (1999). Widespread intraspecies cross-contamination of human tumor cell lines arising at source. *International Journal of Cancer*, 83(4), 555-563.

Masters, J. R., Thomson, J. A., Daly-Burns, B., Reid, Y. A., Dirks, W. G., Packer, P., Toji, L. H., Ohno, T. Tanable, H., Arlett, C. F., Kelland, L. R., Harrison, M., Virmani, A., Ward, T. H., Ayres L. L. and Debenham P. G. (2001). Short tandem repeat profiling provides an international reference standard for human cell lines. Proceedings of the National Academy of Sciences, 98(14), 8012-8017.

Mazur, P. (1984). Freezing of living cells: mechanisms and implications. *American Journal of Physiology-Cell Physiology*, 247(3), C125-C142.

Minuth, W. W., Strehl, R., and Schumacher, K. (2003). Zukunftstechnologie Tissue Engineering: Von der Zellbiologie zum künstlichen Gewebe. John Wiley & Sons.

Olejnik, A., Lewandowska, M., Grajek, W., and Czaczyk, K. (2003). New rapid method of Caco-2 cell differentiation. Methodology of the Novel Food Evaluation. *Polish Journal of Food and Nutrition Sciences*, 12(53), 60-64.

Olejnik, A., Marecik, R., Skrzypczak, M., Czaczyk, K., and Grajek, W. (2008). Application of rapid Caco-2 cell culture system in the studies on the bacterial adhesion and transepithelial transport. *Polish Journal of Food and Nutrition Sciences*, 58(3).

Pinto, M. (1983). Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell*, 47, 323-330.

Richardson, J. C. W., Scalera, V., and Simmons, N. L. (1981). Identification of two strains of MDCK cells which resemble separate nephron tubule segments. *Biochimica et Biophysica* Acta (BBA)-General Subjects, 673, 26-36.

Richardson, J. C. W., and Simmons, N. L. (1979). Demonstration of protein asymmetries in the plasma membrane of cultured renal (MDCK) epithelial cells by lactoperoxidase-mediated iodination. *FEBS letters*, 105(2), 201-204.

Ryan J. (2004) General Guide for Cryogenically Storing Animal Cell Cultures: Technical Bulletin, Corning, available: https://www.corning.com/media/worldwide/cls/documents/t_cryoanimalcc.pd f [accessed: 27. August 2016]

Sambuy, Y., De Angelis, I., Ranaldi, G., Scarino, M. L., Stammati, A., and Zucco, F. (2005). The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell biology and toxicology*, 21(1), 1-26.

Schmitz, (2007). *Der Experimentator: Zellkultur*. München, Elsevier, 130

Shu, Z., Kang, X., Chen, H., Zhou, X., Purtteman, J., Yadock, D. and Gao, D. (2010). Development of a reliable low-cost controlled cooling rate instrument for the cryopreservation of hematopoietic stem cells. Cytotherapy, 12(2), 161-169.

Smith P. L. (2000) 'The Role of Permeability Studies in Preclinical Evaluation' in Dressman J. B. and Lennernäs H. *Oral drug absorption: Prediction and assessment*. Vol. 106, New York and Basel: Marcel Dekker AG, 119

Stacey, G. N. (2004). 'Fundamental Issues for Cell-Line Banks in Biotechnology and Regulatory Affairs' in B. Fuller, E. E. Benson, and N. Lane, *Life in the frozen state* (volume?) Boca Raton, CRC Press LLC, 37-452.

Takahashi, T., Inada, S., Pommier, C. G., O'Shea, J. J., and Brown, E. J. (1985). Osmotic stress and the freeze-thaw cycle cause shedding of Fc and C3b receptors by human polymorphonuclear leukocytes. *The Journal of Immunology*, 134(6), 4062-4068.

Uchida, M., Fukazawa, T., Yamazaki, Y., Hashimoto, H., and Miyamoto, Y. (2009). A modified fast (4 day) 96-well plate Caco-2 permeability assay. Journal of pharmacological and toxicological methods, 59(1), 39-43.

Van Breemen, R. B., and Li, Y. (2005). Caco-2 cell permeability assays to measure drug absorption. *Expert opinion on drug metabolism & toxicology*, *1*(2), 175-185.

Volpe, D. A. (2008). Variability in Caco-2 and MDCK cell-based intestinal permeability assays. *Journal of pharmaceutical sciences*, 97(2), 712-725.

Volpe, D. A. (2010). Application of method suitability for drug permeability classification. *The AAPS journal,* 12(4), 670-678.

Woods, W. J. (2013). Morphological Comparison of Caco-2 Cells in the Corning® BioCoat™ Intestinal Epithelial Cell Environment and the Traditional 21-day Caco-2 Culture System. Technical Bulletin #426, Corning, available: https://www.corning.com/media/worldwide/cls/documents/an_DL_073_Morphological_Comparison_of_Caco-2_Cells_Corning_BioCoat_Intestinal.pdf [accessed: 27. August 2016]

Zaman, G. J., de Roos, J. A., Blomenröhr, M., van Koppen, C. J., and Oosterom, J. (2007). Cryopreserved cells facilitate cell-based drug discovery. *Drug discovery toda*y, 12(13), 521-526.

6.2 Illustrations

Abcam (2016) Overview of the flow cytometer [image online], available: http://www.abcam.com/protocols/introduction-to-flow-cytometry [accessed 27. August 2016]

Corning (2013). (no title) Transwell® Permeable Supports Selection and Use Guide, 7, available: http://csmedia2.corning.com/LifeSciences/Media/pdf/transwell_guide.pdf [accessed: 27. August 2016]

Brockbank K. G. M., Covault J. C., Taylor M. J. (2007) *Figure 1. Freezing graph shows heat being released from the sample.* Cryopreservation Guide. Thermo Fisher Scientific,5, available: https://www.thermofisher.co.nz/Uploads/file/Scientific/Applications/Equipment-Furniture/Cryopreservation-Guide.PDF [accessed: 27. August 2016]

Brockbank K. G. M., Covault J. C., Taylor M. J. (2007) *Figure 2. Schematic cooling curves showing the exothermic latent heat evolution of a sample after nucleation.* Cryopreservation Guide. Thermo Fisher Scientific,6, available: https://www.thermofisher.co.nz/Uploads/file/Scientific/Applications/Equipment-Furniture/Cryopreservation-Guide.PDF [accessed: 27. August 2016]

Freshney R. I. (2010). ***Fig. 12.4. Serial Subculture*** Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications, 6th Edition, New Jersey: Wiley-Blackwell,201, image

Niolip (2015) liposome_2b [image online], available: http://www.niolip.com/liposomes/ [accessed 27. August 2016]

7. List of Materials

7.1 Used Cell line

Name	Manufacturer	Article No.
Caco-2	Cell lines service	300137
MDCK (NMBL-2)	Cell lines service	662280
NIH 3T3	Cell lines service	400101

7.2 Used devices

Item	Device name	Supplier

A		
Autoclave	VX 150 0507	Systec
C		
Cell Counter	Model TT Cell Counter	Schärfe Systems
CO_2 Incubator, 37°C	Reach-In CO_2 Incubator 3951	Thermo Scientific
Controlled rate freezer	CryoMed® Freezer	Thermo Electron Corporation
D		
Dishwasher	PG8535	Miele Professional
E		
Electronic pipette, 1000µL	Xplorer	Eppendorf
F		
Flow cytometer	Cytomics FC 500	Beckman Coulter
Freezer, -20°C		BOMANN
Freezer, -70°C	U410 Premium	
Freezing Device, Isopropanol chamber	Mr. Frosty	Nalgene
Freezing device, polyethylene foam box	CoolCell® FTS 30	Biocision
Fridge, 4°C		Gorenje
L		
Laminar flow hood, class 2	HeraSafe HS18	Heraeus
Liquid nitrogen storage container (-196 °C)	Labotech Isothermal V-1500	Custom BioGenic System
M		
Micro Plate Reader Safire^2, Software: XFluor4 Safire^2	Safire^2	Tecan
Microscope	CKX41	Olympus
Microscope camera	MikroCamLab	Bresser
P		
Pipette aid	Matrix CellMate II	Thermo Scientific
Pipette, 200 µL, 1000 µL	Eppendorf research	Eppendorf
Pipette, 8-Channel-, 200 µL, 1000 µL	Eppendorf Xplorer	Eppendorf
T		
Table centrifuge for 15 mL und 50 mL centrifuge tubes	Hereaus Biofuge Stratos	Thermo Scientific

V		
Vacuum pump, for aspiration	KNF Neuberger LABOPORT	Neolab
Voltohmmeter	EVOM²	World Precision Instruments
W		
Water bath, 37 °C	Typ 1003	Gesellschaft für Labortechnik

7.3 Disposables

Article	Supplier	Article No.
6-well cell culture plate	Sarstedt	83.3920
12-well cell culture plate	Sarstedt	83.3921
24-well cell culture plate	Sarstedt	83.3922300
96-well Cell culture plate	Eppendorf	0030 730.119
96-well Plate, flat-black	Greiner	655077
A		
Aspirations pipettes, 2 mL	Sarstedt	86.1252.001
C		
Casy® cup, for cell counting	Ominlab	6347574
Cell culture flask, 25 cm^2	Sarstedt	83.3910.002
Cell culture flask, 75 cm^2		83.3911.002
Cell culture flask, 175 cm^2		83.3912.002
Centrifuge tube, 15mL	Sarstedt	62.554.502
Centrifuge tube, 50mL		62.547.254
Cryovials, Nunc (1.8 mL)	Thermo Scientific	377267
F		
Filter tube, Steriflip®, 0.2µM, 50 mL	Millipore	SCGP00525
Flow cytometer tubes,	Lab Equip	2523749
G		
gloves, disposable, Nitrile	Kimtech	90627
P		
Pipette tips, 1000µL	Sarstedt	70.1186.200
Pipette tips, 200µL	Sarstedt	70.760.502
Pipette tips, 10µL	Corning	4125

R		
Reaction tubes, 1.5 mL	SafeSed	72.706.400
Reaction tubes, 2 mL	Sarstedt	72.695.500
Reaction tubes, 5 mL	Eppendorf	0030119401
Reagent reservoir, 60 mL, for 8-Channel-Pipette	Brand	5428123
S		
Serological pipette, 2mL	Sarstedt	86.1252.001
Serological pipette, 5mL	Sarstedt	86.1253.001
Serological pipette, 10mL	Sarstedt	86.1254.001
Serological pipette, 25mL		86.1685.001
Serological pipette, 50mL		86.1689.001
T		
Transwell® inserts	Corning	#3413

7.4 Chemicals

7.4.1 Defined Substances

Article	Supplier	Article No.
A		
Amitriptyline hydrochloride	Sigma Aldrich	A8404-10G
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D4540-100mL
C		
Calcein AM	Sigma	5549620x50ug
Cyclosporin A,	Sigma	30024-25M
Calcium Chloride 2-hydrate	AppliChem	A4689,0250
E		
Ethanol, 96%	VWR Chemicals	84836.360
G		
Gabapentin	Sigma Aldrich	PHR1049-1G
Galactose, D (+)	AppliChem	A4073,0500
Gelatin, from porcine skin	Sigma Aldrich	G1890-100G
H		

HEPES	AppliChem	A3268,0500
M		
Magnesium chlorid hexahydrat	Sigma Aldrich	M2393-500G
P		
Propidium iodide	Sigma Aldrich	P4170-10MG
S		
Sodium hydrogen carbonate	AppliChem	A0384,0500
V		
Verapamil	Sigma Aldrich	V4629-1G

7.4.2 Buffer and Solutions

Article	Concentration	Supplier	Article No.
Accutase® Solution		Sigma Aldrich	A6964-100ML
Alpha MEM Eagle		PAN Biotech	P04-21050
Casy® Clean		Omni Life Science	5651786
Casy® Ton		Omnilab	6347573
COULTER CLENZ® Cleaning Agent		Beckman Coulter	8448222
DMEM (Dulbecco's Modified Eagle's medium)		Sigma Aldrich	D5671-500ML
DMEM/F12		Sigma Aldrich	D6434-500ML
Dulbecco’s Phosphate Buffered Saline (DPBS)	1x	Sigma-Aldrich	D8537
FBS Good, Filtrated Bovine Serum		PAN Biotech	P40-37500
Fetal Bovine Serum		Sigma Aldrich	F7529
Fetal Bovine Serum		Sigma Aldrich	7524
Fetal Bovine Serum, 045M3270		Sigma Aldrich	7524
Fetal Bovine Serum, BCBQ7890V		Sigma Aldrich	7524

Fetal Bovine Serum, Lot: 14J493		Sigma Aldrich	F224-500ML
Fetal Bovine Serum, Lot: 15A545		Sigma Aldrich	F224
Fetal Bovine Serum, S1560-050		biowest	
Fetal Bovine Serum, S1820-050		biowest	
Hanks' Balanced Salt solution (HBSS)	1x	Sigma Aldrich	H6648
HEPES Buffer Solution	1 mol L^{-1}		
IsoFlow™ Shealth Fluid		Beckman Coulter	8546859
L-Glutamine	200 mmol L^{-1}	Sigma-Aldrich	G7513
Non Essential Amino Acid Solution (MEM NEAA)	100x	PAN Biotech	P08-32100
Penicillin/Streptomycin	10,000 Units Penicillin/mL 10 mg Streptomycin/mL	PAN Biotech	P06-07100
Pluronic F-68	10 %	PAN Biotech	P08-02100
Sodium Pyruvat	100 mmol L^{-1}	Sigma-Aldrich	S8636
Texas Red Dextran		ThermoFisher	D3328
TrypLE Express	1x	Thermo Fisher Scientific	
Wasser, sterile-filtrated		Sigma-Aldrich	RNBF0221

7.5 Preparation of Reagents

Amitryptyline hydrochloride	MW (Amitriptylin Hydrochloride): 313.86 g/mol Worksolution: 30 mM For Experiment add 5 µL from worksolution to transport buffer (final concentration: 5 µM)
Gabapentin	MW(Gabapentin): 171,24 g/mol Worksolution: 300 mM

	For Experiment add 50 µL from worksolution to transport buffer (final concentration: 50 µM)
Verapamil	(MW) Verapamil = 491,06 g/mol Worksolution: 10 mM Final concentration in solution: 100 µM Solve 49,106 mg in 10 mL water Aliquoted in 10x1 mL reaction tubes Storage at 4°C Add 10 µL in 1 mL cell solution with 2,0E+05 cells/mL for 5 min, before incubation with Calcein-AM
Propidium iodide	Worksolution: 10 µg/mL Final concentration in solution: 1µg/mL Solve 0,5 mg in 50 mL PBS and aliquot in 20x1,5 mL reaction tube (brown) Storage at 4°C Add 100 µL in 1 mL cellsolution
Calcein AM	(MW) Calcein AM = 994,86 g/mol Worksolution: 10 µM Endconcentration in Solution: 0,25µM Solve 50 µg in 5 mL DMSO Aliquoted in 5x1 mL reaction tube (brown) Storage at -20°C

	Add 25 µL in 1 mL cells solution with 2,0E+05 cells/mL
Donor Solution	2 mL Transport buffer + 2 µL 10mM Cyclosporin A + 1,6 µL 1.5 mM Texas Red
Receiver Solution	(2 mL Transpor buffer + 2 µL 10mM Cyclosporin A)
Transportbuffer	1 L HBSS +10 mL 1M HEPES

STATUTORY DECLARATION

I declare that I have authored this thesis independently, that I have not used other than the declared sources / resources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

…… ……..

date (signature)