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OPTIMIZATION OF siRNA TRANSFECTION FOR BREAST AND GLIOMA CANCER CELL CULTURES

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RNA silencing technology and methods based thereupon are important in today's medical biotechnology. Small interfering RNA (siRNA) high-throughput screening has a variety of possibilities in pharmaceutical development, for instance in finding the site of the effect for a certain drug, and confirming its effect and working mechanism. There are various commercial products available for the transfection of siRNA molecules into cell lines of interest. Transfection of primary and stem cells, on the other hand, has been found difficult and careful optimization of the methods and reagents used is always needed.

The purpose of this thesis was to optimize the conditions of siRNA transfection for two cancer stem cell cultures and for three other cancer cell lines that have been found hard-to-transfect. Out of eight different commercially available transfection reagents, there were two possible candidates for use as common chemical transfection reagents, SiLentFect (Bio-Rad) and HiPerFect HTS (Qiagen). For transfection of two cancer stem cell cultures and for one hard-to-transfect cell line, Nucleofection technology was found an efficient method.

The commissioner of this thesis, VTT medical biotechnology, Merja Perälä's research team, has been working with high-throughput screening (HTS) and siRNA technology. The optimized methods will be used in siRNA-high-throughput screenings at VTT.

KEYWORDS:

Transfection, Nucleofection, RNAi, siRNA, Breast Cancer, Glioma, Cancer Stem Cell, Aldehyde dehydrogenase

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siRNA TRANSFEKTION OPTIMOINTI RINTASYÖPÄ- JA GLIOMASOLUVILJELMILLE

RNA-hiljentämiseen pohjautuvat menetelmät ovat tärkeitä työkaluja biolääketieteellisessä tutkimuksessa. siRNA-tehoseulontateknologialla on monia mahdollisuuksia lääkekehityksessä esimerkiksi lääkkeiden vaikutuskohteiden löytämisessä, varmistamisessa ja lääkkeiden vaikutusmekanismien tutkimuksessa. SiRNA-molekyylien transfektioon solulinjoihin on olemassa monia kaupallisia reagensseja. Etenkin primäärisolujen transfektio on usein kuitenkin vaikeaa ja vaatii yksittäisille solutyypeille optimoituja menetelmiä.

Tämän opinnäytetyön tarkoituksena oli optimoida siRNA-transfektio-olosuhteet kahdelle valitulle rinta- ja aivosyöpäprimäärisolulinjalle sekä kolmelle muuten vaikeasti transfektoituvalle solulinjalle. Kahdeksasta kaupallisesta transfektioreagenssista kahden, SiLentFect (Bio-Rad) ja HiPerFect (Qiagen), todettiin toimivan toivotun mukaisesti ja näitä reagensseja voitaisiin käyttää yleisesti transfektioissa. Nukleofektointi todettiin toimivaksi transfektiometodiksi primäärisolulinjoille ja vaikeasti transfektoitavalle solulinjalle. Optimoituja menetelmiä on tarkoitus hyödyntää VTT:n siRNA-tehoseulontaprojekteissa.

Opinnäytetyö tehtiin VTT:n Turun yksikössä, lääkekehityksen biotekniikalla, Merja Perälän tutkimusryhmässä.

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ASIASANAT:

Transfektio, Nukleofektio, RNAi, siRNA, Rintasyöpä, Glioma, Syöpä Kantasolu, Aldehydidehydrogenaasi

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LIST OF ABBREVIATIONS

ALDH	aldehyde dehydrogenase
$ALDH^{br}$	ALDH bright (intracellular marker)
ATP	adenosine triphosphate
CD44	cell-surface glycoprotein
CD133	cell-surface glycoprotein
CK17	cytokeratin, intermediate filament protein
CSC	cancer stem cell
CTG	CellTiter-Glo® -measurement
DEAB	diethylaminobenzaldehyde
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
GFP	green fluorescent protein
HTS	high-throughput screening
NSC	neural stem cell
PBS	phosphate buffered saline
RISC	RNA induced silencing complex
RNAi	RNA interference
SiRNA	short interfering RNA
+ve	positive
-ve	negative

1 AIM OF THE THESIS

This thesis was conducted in Merja Perälä´s group at the Medical Biotechnology department of VTT. I want to thank Merja Perälä for the opportunity to conduct my Bachelor's thesis at VTT, and I am grateful for the advice and guidance received from Saija Haapa-Paananen and the other members of the team.

The aim of this thesis was twofold with the main focus on efficient transfection of two cancer stem cell cultures and three other cell lines with siRNA. The second focus was on detecting the stem cell properties of cultured cancer cells by their specific ALDH enzyme activity. Transfection optimization was performed in order to validate the efficacy of commercially available transfection reagents. It was also possible to discover a competing reagent for the one transfection reagent now mainly used at VTT Turku. ALDH enzyme activity was tested in order to prove its use as a stem-cell marker and as an identifying reagent for cells with stem cell properties.

Merja Perälä's group focuses on high-throughput library screenings, mainly detecting the influence of drug candidates and the effect of small-molecules on breast cancer and prostate cancer cells as well as on glioma cells. In this thesis, breast cancer cells and glioma cells were used.

Today, there are multiple variations of transfection reagents on the market and the manufacturers are being pressurized to develop new sets of formulas and methods all the time. The high-throughput format is still quite a new area of research where transfection reagents and ready-to-use protocols are not always available. For this particular reason optimization of a new reagent is always needed before the actual research work can begin. Each cell culture and cell line has its own characteristics which also determine the requirement for optimization since no general transfection protocol can be adopted. SiLentFect™ (Bio-Rad, Hercules, CA) is a transfection reagent commonly used at VTT Turku at the moment. After the last optimization of transfection reagents,

done in 2004 and 2008 at VTT, the supply of commercial reagents has been extended and developed frequently so it was found beneficial to test new variants of transfection reagents. A Nucleofector™ device was recently purchased to VTT Turku to obtain efficient transfection results for primary cell and cancer stem cell cultures. A second part of the first aim of this thesis was to optimise the electro pulse programs and cell line kits of the Nucleofector™ device. The study of cancer stem cells has drawn a lot of interest in the past few years. For the identification of stem and stem-like cells there are some commercial kits. One of them is the ALDEFLUOR® Assay, in which cells with stem cell properties are fluorescently tagged. The second aim of this thesis was to test this kit for new cancer stem cell lines with a multilabel reader and to visually capture the fluorometric signal.

2 INTRODUCTION

High-throughput screening (HTS) is a set of methods which aims to analyze a large number of samples simultaneously. HTS is especially used in drug and other compound screenings where thousands of potential molecule candidates can be tested without laborious work or unreasonable cost. Large libraries containing thousands of different candidates can be screened in a short time. HTS is based on automated handling of sample volumes from micro- to nanoliters, detecting specific signals and analyzing the data gained. HT screens are carried out in the 384-format and even 1536-format, enabling the use of lower volumes and screening numerous samples per day.

At VTT, multiple large scale HTS screening projects for both compound and siRNA screens have been carried out. Breast cancer and glioma research is one of the main focuses. An example of compound HTS screening accomplished at VTT involved systematic examination of the efficacy of commercial drug-like molecules against prostate cancer cells (Iljin *et al* 2009). High-throughput microRNA (miRNA) transfection, where 319 pre-miRs were transfected, was applied in the study of estrogen receptor signaling in breast cancer cell lines (Leivonen *et al* 2009). HTS screening employed in RNA interference (RNAi), where the influence of four specific genes on cell growth was studied (Vainio *et al* 2011), is an example of one of the recent HTS assays applied at VTT.

3 CANCER AND CANCER CELLS

Cancer is defined as a disease where abnormal cells divide without control with the ability to invade other tissues in the body. To be precise, cancer is a heterogeneous set of diseases and different types of cancers are very different. Typically the organ or the cell type where cancer originates, names the disease (National Cancer Institute).

Cancer is a malignant tumor. Two types of tumors exist: benign and malignant ones. A shared feature is that they are both deformations of tissue, and mainly not dependent on the growth factors of the body. They are unintentional and detrimental to the host. The division into benign and malignant simplifies the understanding of the biology of a cancer (Cooper 2000). Benign tumors are local and slow-growing, and usually do not lead to the patient's death even when left untreated. Cancers, malignant tumors, on the other hand are fast growing and have the tendency of invasion into other parts of the body by metastasis. Cancer cells have lost the cell-to-cell inhibition (Joensuu *et al* 2007). A benign tumor is not a cancer where as a malignant one is and if left untreated, the latter leads to the death of a patient.

There are multiple reasons for the tissue deformation and not only one exact cause for the development of a tumor. Many research methods are used for understanding the development of tissue outgrowth: clinical and epidemiological observations, laboratory animal testing, tissue culturing and molecular biological methods. With laboratory animals, for example, and by cell culturing, detailed information is obtained about the development of a cancer in controlled testing conditions. This type of research gives us detailed information about the influence of certain external factors on the origin of a cancer. Cell and molecular biology based research methods have given for example specific information about the genes affecting tumor development and mutations in DNA, which both have an important pathological significance for abnormal cell growth and for the maturation and progression of a cancer. (Joensuu *et al* 2007)

Carcinogenesis is a two-stage mechanism where a malignant tumor is developed. These two processes are called initiation and promotion. Initiation is a stage where the DNA undergoes a mutation and this way sensitizes the whole cell to be mutated (Joensuu *et al* 2007). In most cases, genes damaged in the initiation stage are so called proto-oncogenes and they are found to play an important role in the development of a cancer. The second stage, promotion, is the actual phase for the tumor development where the target tissue has been shown to overactivate its own cell division. Promotion is a stage of vital damage leading from a tumor to a cancer. After the development of carcinogenesis, the progression phase finalizes the development of a cancer. In progression, the malignant tumor matures and becomes independent of the normal cell growth factors, having the tendency to form metastases (Joensuu *et al* 2007, Visvader 2011). Cancer is usually diagnosed when reaching this progression phase.

3.1 Cancer Stem Cells (CSC)

Normal stem cells are the cells giving rise to all the complex tissues seen in adults. They have the ability to undergo self-renewal over and over again and to generate mature cells for specific tissues through differentiation (Reya *et al* 2001). The self-renewal is crucial for stem cell function. Normal stem cells have a finely planned balance regulation between self-renewal and differentiation.

Understanding the regulation of normal stem cell self-renewal has also been found to be the base for understanding the regulation of cancer cell proliferation, since a malignant tumour can be considered a disease of unregulated self-renewal (Reya *et al* 2001).

The study of cancer stem cells (CSC) got attention when John Dick’s team (Bonnet, Dick 1997) provided evidence that the growth and propagation of leukaemia were driven by a small population of cancer cells having the ability to self-renew unendingly. The cells were designated as cancer stem cells. Since this, CSCs have been identified and isolated from tumours initiating in the breast, the brain, the prostate, the lung etc. (Charafe-Jauffret *et al* 2008).

The thought of stem cell driven cancer provides a whole new way approach to oncogenesis, suggesting new methods can be used for the prevention, detection and for the treatment of cancers, especially those with a metastatic tendency where no therapeutic treatment exists.

In most tissues, stem cells are rare, but the hypothesis exists that normal stem cells undergo an oncogenic transformation resulting in the origination of a cancer (*Figure 1*). There have also been arguments for a transformation of the so called progenitor cells resulting in cancer (Jordan *et al* 2006, Visvader 2011). Progenitor cells are developmentally advanced yet immature cells; the early descendants of stem cells with the ability of differentiation but restricted division (Charafe-Jauffret *et al* 2008; van der Hoogen *et al* 2010).

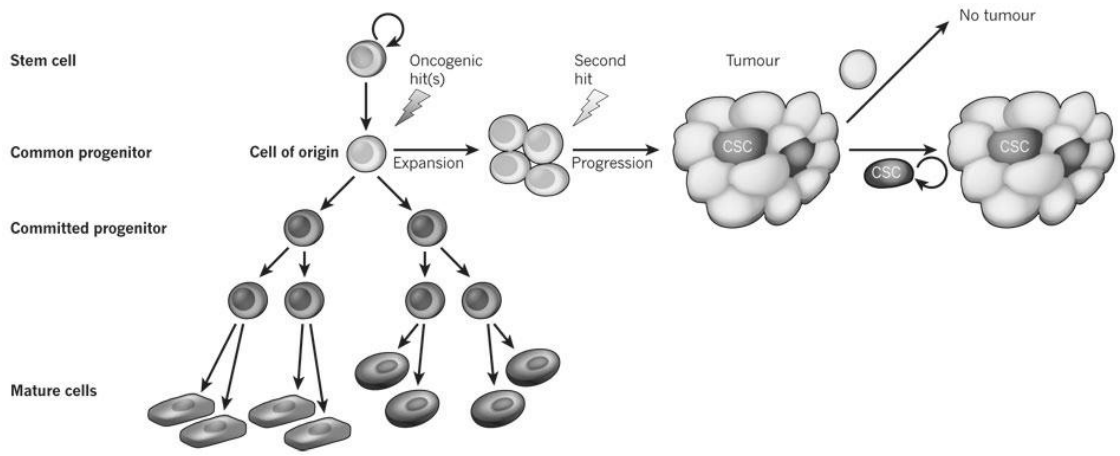


Figure 1. Mutations and Epigenetic changes in stem and progenitor cells.

Normal stem cell are the ones forming differentiated tissues in an adult individual. Before forming the differentiated tissues, stem cells go through a progenitor cell stage. These stem and progenitor cells are vulnerable to oncogenic hits and mutations, that might cause the cells to form a tumour. In here, it is shown a hypothesis of a built-in CSC population which can survive general cancer treatments and the disease might relapse (Visvader 2011).

CSCs and normal stem cells have been shown to share many similarities, including resistance to drugs and treatment with other reagents. Unlike normal stem cells, CSCs have also been found to exist in permanent cancer cell cultures. The hypothesis of a built-in CSC population in malignant tumours has given explanations why some cancers have been able to survive general cancer treatments and the disease has relapsed (Charafe-Jauffret *et al* 2009).

Neural stem cells (NSC) are multipotent cells found in the central nervous system, and found not only in embryos but throughout the life in the adult human and rodent brain. NSC have been found even at the sites of pathology and damaged tissue in the brain. Different from CSC, these NSCs have the ability to form free-floating aggregates *in vitro* (Figure 2)(Corti *et al* 2006). These aggregates are called spheres or neurospheres. Spheres are not a pure population of stem cells because signs of differentiation have been found if spheres are grown in culture growth medium supplemented with serum (Mitrecic *et al* 2009).

In recent years, identification and characterization of CSCs have taken a big leap forward, resulting in better understanding of the link between metastasis, stem cells and cancer survival (van der Hoogen *et al* 2010).

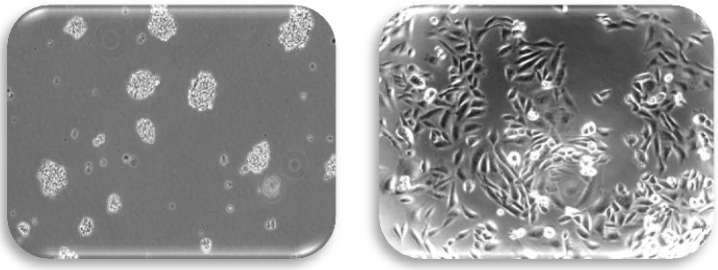


Figure 2. Rat neurosphere (p350) (Invitrogen.com) and breast cancer stem cell (Celprogen) (p16) cultures.

Rat NSCs and breast CSCs are captured with a phase-contrast microscope (Zeiss)(10x).

3.1.1 Stem cell markers

Common for the stem-like cells have been shown to be cell-surface glycoproteins CD44, CD133 and a specific enzyme called aldehyde dehydrogenase (ALDH). The $CD44^+$ and $CD133^+$ cells have been shown to have for example cancer-initiating properties and resistance to chemotherapy (Takaishi *et al* 2009, Ricardo *et al* 2011). $ALDH^+$ cells have shown even more significant resistance to common cancer cell treatments and have been seen to

behave as biologically aggressive cells with the capacity to metastase (van den Hoogen *et al* 2010).

Aldehyde dehydrogenase (ALDH) is an enzyme superfamily responsible for the oxidation of intracellular aldehydes. Enzymes have also been found to have an important role in the development of epithelial homeostasis and in drug resistance (van der Hoogen *et al* 2010).In a number of cancers the ALDH enzyme has been found to be deregulated, especially in stem-like cells and in endothelial progenitor cells as well as in epithelial stem cells. These findings have given rise to an assay of identification and isolation of cells with ALDH overexpression and possible stem-cell properties (Charafe-Jauffret *et al* 2009).The high expression of ALDH in stem and stem-like cells was discovered when research targeted the resistance of cyclophosphamide and other alkylating reagent derivates in primitive hemapoetic stem cells (Sahovic *et al* 1988). It was later found that this resistance was due to the high expression of ALDH enzyme in these cells.

3.1.1.1 Aldefluor® Assay

Aldefluor® Assay (ALDAGEN, STEMCELL technologies) is a protocol developed for identifying cells overexpressing ALDH enzyme 1 (ALDH1). ALDEFLUOR® fluorescent reagent contains a specific ALDH substrate which in viable cells is converted into fluorescent product; non-viable cells are unable to retain this reaction product inside the cell. The ALDH substrate is converted into a fluorescent product by this specific ALDH enzyme. Cells overexpressing ALDH enzyme can so be detected as ALDH bright ($ALDH^{br}$) because of the fluorescence (Stemcell technologies, 2008). $ALDH^{br}$ detection is proportional to the ALDH enzyme present and further proportional to the cancer stem cell properties (Charafe-Jauffret *et al* 2009). The ALDEFLUOR® reagent is known to interact with the ALDH isoform 1A1 and probably also with 3A1 (Stemcell technologies, 2008). Other isoform interactions have not yet been determined.

3.2 Breast cancer cells

The most common cancer among women is breast cancer. The disease occurs almost entirely in women, but also men can have it. About 10% of Finnish women are diagnosed with breast cancer at some point of their lives (Joensuu H *et al* 2007). Prevention of this cancer is not possible yet, but multiple methods have been developed to find tissue deformation early enough. The variations in morphologies and differences in metastatic behaviour and in the response to therapeutic treatments make breast cancers hard to treat.

Nearly all breast cancers diagnosed are found as carcinomas. Epithelial tissue derived malignant tumours are called carcinomas, with the tendency of forming metastases. Adenocarcinomas are derived from the epithelia of glandular tissue; in this case the breast lobules and ducts (American Cancer Society, 2011). The breast cancer cell lines and cell cultures used in this thesis were adenocarcinomas.

3.3 Glioma cells

A glioma is a tumor arising in the central nervous system, and usually as an infiltration to the brain tissue or spinal marrow. The tumor initiates in the glial cells, which are the non-neuronal cells forming the brain and spine supporting tissue. Glioblastoma initiates from the glial of primitive supporting tissue (Joensuu *et al* 2007). A primary brain tumor is not always malignant, but due to the closed position inside the skull and the slow regeneration of the nervous system, the consequences of a tumor in the brain might be more serious than those of a tumor in other parts of the body (IRSA, 2011).

Annually about 650 brain tumors are diagnosed in Finland and 40% of these are gliomas (Joensuu *et al* 2007). Gliomas have the capacity to infiltrate into the brain tissue, which makes them hard to operate surgically. Brain tumors are graded from I to IV, depending on their malignancy, IV being the most aggressive (IRSA). The glioblastomas used in this study were graded IV (Figure 3). Primary brain cancers rarely metastase to other parts of the body, but may

spread inside the brain and spine. In most cases of cancer, the healthy cells of the body are pushed aside by the uncontrolled cancer cell growth. In the case of glioma normal cells are destroyed due to the narrow space inside the skull. Glioblastoma tumours usually contain more than one type of cancer cells, which also makes them hard to treat: while some types of cancer cells are defeated, others continue dividing (IRSA).

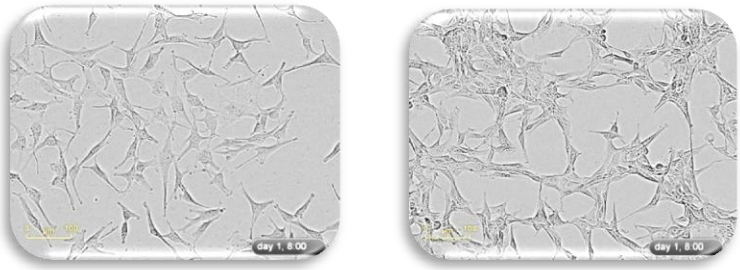


Figure 3.Glioblastoma A172 (p350) and U87 MG (p42) cell cultures.

In this study cell lines were cultured on a plastic tissue culture dish (Corningen). Images of cell cultures were captured with a phase-contrast live-cell imaging instrument, IncuCyte (Essen Bioscience).

4 TRANSFECTION

Transfection is a process of introducing foreign nucleic acids into eukaryotic cells. Numerous techniques can be utilized for the action of transfection, but all together the process has proved to be a powerful and essential tool for *in vitro* applications including studies of gene function and the modulation of gene expression as well as studies of protein production and intracellular signaling pathways (Prathees *et al* 2011). A therapeutic strategy such as tissue engineering and gene therapy also utilizes the technique. As a method, transfection can be carried out via chemical or physical procedures.

The introduction of exogenous molecules, or genetic material like DNA or RNA, by transfection can be either a transient or a stable phenomenon, but for both the main idea is to deliberately modify the genome or the protein production of the targeted cell (Prathees *et al* 2011).

Different transfection methods in principle can be categorized into two main groups, which are viral and non-viral methods. Viral transfection has very high transfection efficiencies, but since the handling and production of these viruses can be difficult, costly, and time consuming, viral transfection is not widely used (Hagemann *et al* 2005).

Several approaches have been developed to overcome the limitations caused by the use of viruses. These so called non-viral methods include both chemical and physical processes. A transfection method combining both chemical and physical techniques is called nucleofection.

4.1 Chemical transfection

The basic idea of chemical transfection is to neutralize the negatively charged exogenous molecule being introduced into the cells. The cell membrane is negatively charged as is the foreign molecule. The chemical reagents coat the molecule with cationic lipids, neutralizing or even creating an overall positive charge (Dean and Gasiorowski 2010). The foreign molecule is endocytosed into the cell.

First generation chemical reagents used for the transfection of cultured mammalian cells were DEAE (diethlyamino ethanol) dextran and calcium phosphate based reagents. Both reagents are still widely used because of their low price and extremely easy use. The varying and low transfection efficiency together with the cytotoxicity of these reagents have limited their use (Dean and Gasiorowski 2011; Hagemann *et al* 2006). Competing chemicals have been on the market since the 1980s.

Chemical transfection requires actively dividing cells (Brunner *et al* 2000). Foreign molecules are endocytosed into the cell where they travel either to the cell nuclei (DNA) or stay in the cytoplasm (RNA, proteins or antisense oligonucleotides) depending on where the site of action is (Watson *et al* 2008).

4.1.1 Liposomal transfection

The most widely used transfection method is the use of cationic liposomes as molecule carriers, a technique called lipofection (Dean and Gasiorowski 2011). Lipofection has been proven to efficiently deliver molecules from small oligonucleotides to entire proteins into the cell. This method also enables the transfection of cells that are too sensitive to calcium phosphate and DEAE based reagents (Felgner *et al* 1995). Both transient and permanent transfections can be obtained with the lipofection method and it can even be applied for *in vivo* transfections.

Cationic lipids in the solution bind to the negatively charged exogenous molecule, together forming positively charged lipid–nucleic acid -complexes.

These complexes are able to bind onto the surface of the negatively charged cell membrane and be endocytosed due to the positive overall charge (da Cruz *et al* 2004). These cationic lipids, liposomes, are synthetic analogues of the cell membrane's phospholipid bilayer.

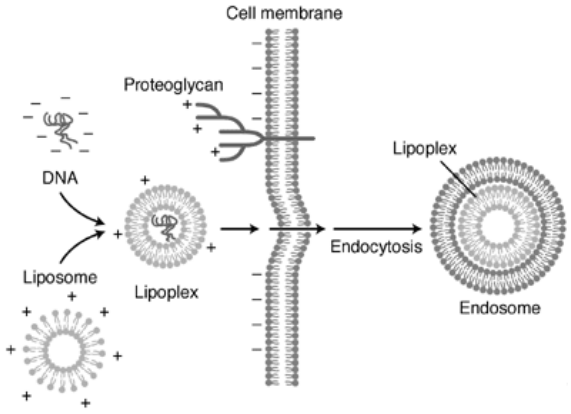


Figure 4. Lipofection.

Exogenous material, DNA, is being introduced into the cell by lipofection . (Expertreviews, Cambridge University Press, 2003)

4.1.2 Non-liposomal transfection

The non-liposomal method is also based on endocytosis. Instead of using cationic lipids, non-liposomal transfection reagent contains polycationic polymers capable of forming micelles. Transfection usually takes place in an aqueous solution which enables the lipophilic heads of the polymer to enclose the exogenous material inside its micelle core with the hydrophobic tails. Non-liposomal reagents are designed for cell lines that are too sensitive for liposome-based transfection.

4.2 Physical transfection

Physical techniques, like microinjection or electroporation, are simply based on a delivery of exogenous material through the membrane by physical force. Introducal can take place either in the cytoplasm or directly in the nucleus.

Microinjection is basically a direct delivery of exogenous nucleic acid into the target cell's nucleus. This particular technique has been used for example for transferring DNA into embryonic stem cells for producing transgenic organisms

(Bockamp *et al* 2002). The process is very effective but laborious and not an appropriate method for studies requiring a large number of transfected cells.

Electroporation is a method based on the use of electrical pulses in order to disrupt the cell membrane. Perturbing causes transient pores on the membrane allowing foreign molecules the passage into the cell (Dean and Gasiorowski 2011). Electroporation is a rather aggressive way of transfection for the animal cells (because of the missing cell wall) and often requires more cells than the chemical transfection procedures.

4.3 Nucleofection

A further alternative for the classic chemical and physical transfections is a method called nucleofection where exogenous material is being introduced into the cell via a method applying both chemical and physical techniques. With the Nucleofector technique, the foreign molecule is bound to proteins containing a nuclear localization signal in a cell–specific–solution. The formed complex is transferred into the cell via small pores on the cell membrane. Pores are formed by specific electrical shocks. As the molecule–protein -complex is being introduced straight into the nucleus of the cell; there is no need of cell division before obtaining the results of the recombinant protein expression. Because of the transfection is independent of cell division, protein expression can be seen already in four hours. (Hagemann et al 2006)

The Nucleofector technology has been seen as a new and first efficient non-viral transfection method for primary and stem cells and hard–to–transfect cell lines, mainly because of the straight nuclear introduction of the exogenous material. Nucleofection has shown better transfection efficiency than common lipofection reagents (Jacobsen *et al* 2006) and higher cell viability after transfection than with electroporation (Maasho *et al* 2004).

In this work Amaxa 4D-Nucleofector™ device, X-unit was used for the nucleofection.

5 RNA INTERFERENCE

RNA interference (RNAi) is an evolutionary conserved mechanism shared in eukaryotic cells. RNAi is a cellular process of RNA silencing resulting in reduced protein expression. RNAi phenomenon is found in fungi, plants, and in animals and it seems to be involved in a variety of regulatory and immune functions. In certain species, RNAi regulated gene activity is a normal defense mechanisms against viruses and the mobilization of transposable genetic elements (Pedraza-Fariña *et al* 2006).

As a phenomenon, RNAi is triggered by a double-stranded RNA (dsRNA). This dsRNA suppresses the expression of a target protein by stimulating the sequence specific degradation of messenger RNA (mRNA). Messenger RNA is a mobile, single-stranded copy of a gene coding for the production of its specific protein. This copying is called transcription. Translation is the next step, where the mRNA is converted to its functional protein form (Joensuu *et al* 2007). RNAi occurs between the transcription and translation. RNAi mechanism involves a two-step process where ribonuclease, specifically called Dicer, first cleaves the dsRNA into small interfering RNA (siRNA) molecules. The small molecules generated are about 20 nucleotides long. Second, these siRNA's are incorporated into an RNAi targeting complex called RISC (RNA-induced silencing complex) that results in a base-pair-dependent mRNA cleavage and decreased protein expression (*Figure 5*). Since the impact is targeted towards mRNA instead of the DNA, the blockage of protein synthesis is reversible (Pedraza-Fariña *et al* 2006).

The phenomenon of RNAi was first discovered in plants at the start of the nineties, but theory for the observations was provided in 1998 when dsRNA was found to be the one inhibiting the expression of its homologous RNA. The observation was first found in a nematode worm, *Caenorhabditis elegans* (Fire *et al* 1998). Findings of the long dsRNA molecule, which induced nonspecific knockdown of a gene, expanded the experimental use of RNAi to mammalian cell associated research. It was a couple of years later when short dsRNA molecules were found to downregulate specific genes (Krueger *et al* 2007).

Today, RNAi is considered as an important tool for the study of gene expression and a tool for the analysis of molecular mechanisms of various diseases. Degradation of the mRNA results in the interference of the protein production and further to the function of the whole cell. The phenomenon is, in other words, used for studying the influence of certain protein on the cell function. In future, RNAi could enable the usage of dsRNA as a therapeutic molecule.

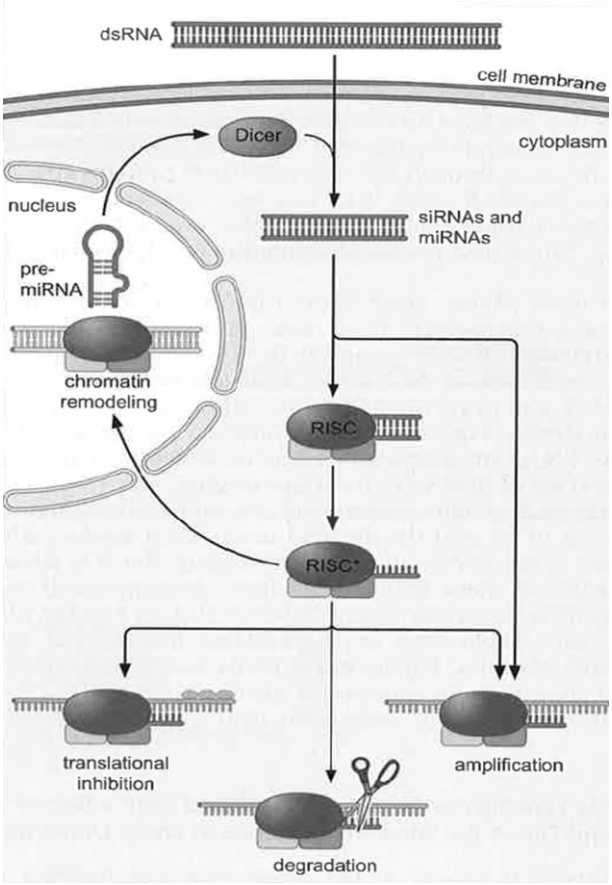


Figure 5. RNA interference.

The phenomenon of RNAi occurring in mammalian cells. Endogenous microRNAs (miRNA) are exported from the nucleus to the cytoplasm. Small interfering RNAs (siRNA) being integrated into the RNA induced silencing complex (RISC) results to the degradation of messenger RNA (mRNA) and reduced protein expression. (Watson et al 2008, pp 642)

5.1 RNAi and Cancer

RNAi has already made its way as a new tool in the analysis of molecular mechanisms for various diseases. Cancer is no exception. RNAi has made it possible to functionally identify genes that are involved in cancer initiation and new cancer-relevant genes have been found.

The use of RNAi has encouraged the development of a new technology for gene therapy applications in the treatment of cancers. Gene specific silencing has allowed systematic screens for new drugs, and the effect of already existing drugs could have been enhanced. RNAi has enabled silencing to occur with high specificity and more efficiently than with any other technique before (Thakur 2003).

Instead of transfecting big dsRNA molecules in to the cells, chemically engineered siRNA's enable targeting the specific genes. This has resulted in identification of proteins that are crucial for cell viability (Manoharan 2004; Krueger *et al* 2007).

Today, there are lots of hopes and promises for using siRNAs as drugs directed directly and only into the cancer cells. RNAi protein silencing has been used and shown good promises in cell cultures and in animal models, which encourage siRNA based reagents for clinical usage to treat cancer as well as other diseases. SiRNA can be transfected directly into the cells or organs, but stability in the blood stream, the duration of the effect and the delivery techniques are still quite big questions before RNAi-based cancer therapy can be used (Ozpolat *et al* 2009).

6 MATERIALS AND METHODS

6.1 Stem Cells and Cancer Cell Cultures

Breast Epithelial Cell Culture and Breast Cancer Stem Cells were purchased from the Celprogen (St San Pedro, CA). Glioma Cancer Stem Cell Culture (CSC 1904) was obtained from Helsinki University (Sariola/ Laura Kerosuo) and brain glioblastomas A-172 and U-87 MG from the ECACC (European Collection of Cell Cultures). Breast adenocarcinoma cell culture MCF 7 was purchased from the ICLC (Interlab Cell Line Collection), ductal breast carcinoma BT-474 and breast mammary gland adenocarcinoma (metastatic site) SK-BR-3 from the LGC Standards (Laboratory of Government Chemist). Prostate, lymph node carcinoma LNCaP, which was used as a control, was obtained from the ATCC (American Type Culture Collection).

Cell Line Characteristics

Epithelial Breast Stem Cells are Nestin, estrogen receptor (ER) and progesterone receptor (PR) positive. Breast Cancer Stem Cells are HER2 receptor positive but ER and PR negative (double negative). (Certificate of Analysis, Celprogen; Sharma *et al,* Celprogen)

Thawing the Cells

All the cells and cell lines were thawed by warming the frozen ampoules in 37 °C for 1-2 minutes. Vials were submerged only partially and swirled constantly in the water bath. Thawed cells were immediately transferred into a 15 ml sterile centrifuge tube with 5 ml of fresh prewarmed growth media. Cells were centrifuged (Eppendorf AG, Hamburg, Germany) to a soft pellet at 100 x g for 5 minutes. Supernatant was removed and cells were resuspended into 10 ml of fresh prewarmed media. Cells were cultured on an Ø 10 cm tissue culturing dish (Corning) or pre-coated T75 flasks (Celprogen cells; Celprogen flasks). All complete growth media - receipts are listed in **Appendix 1.**

Cell Seeding and Subculturing

Cells were subcultured after reaching 70-80% confluency. Cell monolayers were washed with 1 x PBS (Dulbecco's Phosphate Buffered Saline, Sigma-Aldrich, St. Louis, MO) and trypsinised with 1 x trypsin-EDTA (ethylene-diamine-tetraacetic acid, stock 10 x, Sigma-Aldrich, St. Louis, MO) diluted with PBS for 1x concentration. Trypsinized cells were subcultured at a ratio of 1:2 to 1:10, depending on the next assay the cells were used for. Cells were incubated at 37 °C and 5% CO_2 cell incubator (HERAcell 240i, Thermo Scientific). Media for the Celprogen cells was changed every 24 hours, 2-3 times per week for the other cell cultures. CSC 1904 cells were cultured on ultra-low attachment plates (Corning, NY).

Freezing media for the different cell cultures was the growth medium (listed in **Appendix 1**) supplemented with 10% of DMSO (Dimethyl sulfoxide, Sigma-Aldrich)**.** Cells were first trypsinised as usual and counted by using the Bürker cell counting chamber. 2-5 million cells were counted for 1 ml of freezing media into one freezing ampoule (Nalgene, Thermo Scientific, Rochester, NY). Sufficient number of cells was centrifuged at 100 x g for 5 minutes. Obtained cell pellet was resuspended to the precooled freezing media and transferred into -80°C freezer for at least 24 hours before removing the ampoules into -150 °C freezer for long term storage.

6.2 High Throughput (HT) Transfection

Reverse transfection protocol was used since this technique is functional for HTS screenings. Briefly, siRNAs were pipetted into the wells of 384-well plates and covered by the transfection reagents. After one hour incubation at room temperature (RT), cells were cultured on top of the siRNA-reagent -complexes. After 72 hour incubation transfection efficiency was determined by measuring the effect of cell death control to the effect of cell proliferation with CellTiter-Glo® (CTG)-assay. In order to maintain the transfection conditions stable, cells were passaged 2-3 days prior transfection and never exceeding 85% confluency.

6.2.1 Transfection Reagents

Eight different commercial transfection reagents were tested. Reagents were chosen for their ready-to-use 384-protocols, or because of the expectations for efficient primary and stem cells transfection or just because of the economic point-of-view. The efficiency and toxicity values of transfections were compared with the results from siLentFect™ transfection. Reagents tested were HiperFect HTS (Qiagen), INTERFERin-HTS (Polyplus), Lipofectamine™ (Life Technologies), X-tremeGENE siRNA (Roche), RNAi MAX (Invitrogen), DharmaFect1 (Thermo Scientific), TrueFect-Lipo™ (United Biosystems) and PromoFectin (PromoKine). The latter reagent was non-lipid based. All transfection reagents and volume ranges per transfection recommended by the manufacturer are listed below (*Table 1*.). Two new batches of siLentFect™ were also tested.

Table 1. Commercial transfection reagents tested with six different cell cultures.

Reagent	Manufacturer	Recommended volume range for siRNA transfection (µl) in 384-format
siLentFect	Bio-Rad	0,013 – 0,1
INTERFERin™-HTS	Polyplus	0,05 – 0,1
True-Fect Lipo	United Biosystems	0,09 – 0,18
HiPerFect	Qiagen	0,01 – 0,3
x-tremeGENE	Roche	0,03 – 1,0
PromoFectin	PromoKine	0,013 – 0,13
RNAi MAX	Invitrogen	0,025 – 0,075
DharmaFect 1	Thermo Scientific	0,013 – 0,13

Scrambled target sequence was used as a negative siRNA control (siNEG) and cell death siRNA as a positive control. AllStars negative control (Qiagen) is a nonsilencing siRNA where non-specific effects are controlled. AllStars cell death control siRNA (Qiagen) targets universally expressed human genes, which are essential for cell survival. With cell death, the transfection efficiency can be quickly estimated. Other siRNA's used were siPLK, siKIF, siERBB and siSTARD (Qiagen) (*Table 2.*).

Table 2. SiRNA's used in the transfections

siRNA	Target	Sequence
PLK1_7	polo-like kinase 1_7 (Drosophila)	5' CGC GGG CAA GAT TGT GCC TAA 3'
KIF11_7	kinesin family member 11_7	5' GCC GAT AAG ATA GAA GAT CAA 3'
ERBB2_15	receptor tyrosine-protein kinase erbB2 precursor	5' CAC GTT TGA GTC CAT GCC CAA 3'
STARD3_2	stAr-related lipid transfer protein	5' CAC CTT TGT CTG GAT TCT TAA 3'

6.2.2 Transfection protocol

The first sets of transfections were carried out by following the manufacturers' recommended volume ranges for the reagent. Each reagent was first tested with 3 different volumes: median from the recommended, one below the median, and one above.

5 µl of 165nM siRNA was pipetted into the wells of 384-well plate (Corning). Each siRNA was pipetted as four replicates (quadruplicates). Transfection reagents were first diluted with Opti-MEM® I (Gibco®, Invitrogen, Paisley, UK) or with filtered nuclease free water (Ambion), depending on whether the reagent was lipid or non-lipid based. Reagent dilutions were mixed by pipetting and let to incubate in room temperature (RT) for 10-15mins. 10 µl of the reagent dilution was pipetted on top of the siRNA and centrifuged at 100 x g for 1 min. Reagent - siRNA complex was incubated at RT for 60 mins. While incubating, cells were detached with 1 x trypsin-EDTA and trypsinization was inactivated with prewarmed growth media. Cell suspension was centrifuged at 100 x g for 5 mins, supernatant removed and the cell pellet resuspended into 5 ml of growth media. Cell number was determined with Bürker Chamber. Depending on the cell line, 1500 – 2000 cells / 35 µl media / well were recovered. After the incubation, cells were pipetted on top of the siRNA – reagent complexes and centrifuged at 100 x g for 1 min. Transfection was incubated in 37°C, 5% CO_2 cell incubator for 72 hours.

6.2.3 CellTiter-Glo® (CTG) –measurement

CTG® -measurement is a luminescent cell viability assay, where the number of viable cells in the sample is determined. Active, viable cells use ATP as their energy supplier between anabolic and catabolic reactions. For this reason the presence of ATP can be used as an indicator of metabolically active cells (solunetti, 2012). Addition of CTG® reagent results in cell lysis, releasing the ATP and enzyme called luciferase to interact with the compounds in the CTG® reagent forming a luminescence signal. The signal detected is proportional to the concentration of ATP present and the amount of ATP is proportional to the number of viable cells present (Promega, information sheet 08/2011). After 72 hour incubation of the siRNA treated cells, the cell viability was determined by CTG® and the luminescence detected by multilabel reader. CTG® reagent was first let to thaw to room temperature. For the 384-well plate 25 µl of this reagent was pipetted per well, without removing the cell culture media. For induction, the cell lysis sample plate was left on the shaker for 30 mins at RT with speed of 175 rpm (Heidolph Unimax 1010). After mixing, the luminescence was recorded with a multilabel reader (Wallac Envision, PerkinElmer).

6.2.4 Optimization

Optimization of the transfection reagent volume was done according to the results gained from the CTG® measurements. The effects of siPLK, siKIF, siERBB2, and siSTAD3 and cell death were compared to the siNEG in order to determine the transfection efficiencies. Overall cell viability was controlled by samples of “cells only”.

6.3 Nucleofection

The Nucleofector technique developed by Amaxa is based on the variation between the electrical pulse programs and the cell-type specific solution. The manufacturer provides optimization protocols for both cell lines and primary cells. Here, transfections were done with 4D- Nucleofector® X unit (Amaxa, Lonza Cologne, GmbH) for cells in suspension. For the cell lines, there were 3

different Nucleofector® X solutions (Cell Line Kit SE, SF, and SG) and five for primary cells (Cell Line Kit P1 to P5). Here, Cell Line Kit SF and SG were used for the BT 474 cell line and Cell Line Kit P3 for cancer stem cells and breast epithelial cell culture. Cell-specific solutions were suggested by a representative from Lonza. The electrical parameters of the nucleofector device were optimized for each cell culture. The correct electric pulse for each sample was chosen by first transfecting pmaxGFP® vector (Amaxa, Lonza Cologne, AG) into the cells. Analysis of the transfection efficiency and cell viability determined which programs to choose for following siRNA transfection. Optimization and siRNA transfection protocols are described next. All nucleofections were carried out in a 16-well Nucleocuvette® Strip.

6.3.1 Optimization – Nucleofection of pmaxGFP® Vector

Cells were subcultured 2-3 days before the nucleofection, depending on the cell culture. The slow growing cell line, BT 474 for example, was passaged 3 days prior. Cells were let to grow to about 80% confluency. Next, cells were trypsinized with 1x Trypsin-EDTA and suspended with cell growth culture media. 5 x 10^5 cells per well were centrifuged at 90 x g for 10mins in room temperature, and cell pellet resuspended with 360 µl of Nucleofector® Solution where supplement (supplement 1, Amaxa, Cologne, AG) and 5 µl of pmaxGFP® vector was added before the use. 150 µl of Supplement™ 1 was mixed with 675 µl of the Nucleofector® solution. Cell line kit P3 was used for the stem cancer and epithelial cells, cell line kits SF and SG for the BT 474. 20 µl of the cell – solution mix was pipetted into each of the 16 wells on the strip. Amaxa 4D-Nucleofector® Optimization Protocol was followed when setting up the nucleofector programs (Optimization Protocol for Primary Mammalian Neurons and Protocol for Primary Breast Cancer Cells and Protocol for Cell Lines, Amaxa). Nucleocuvette® was placed into the X-unit of the device and ran through selected programs. Subsequently, 80 µl of prewarmed low-calcium RPMI-1640 (Sigma-Aldrich) was pipetted into the wells and incubated for 5 mins at room temperature before plating the cells on 12-well culture plates (Corning)

with 1 ml of prewarmed cell growth media. Cells were incubated in 37°C, 5% CO_2 IncuCyte FLR (Essen Instrument) for 24 hours.

6.3.2 Cell Viability and Transfection Efficiency

After 24 hours of incubation cells were trypsinized, centrifuged briefly and resuspended with 20 µl of PBS diluted, 0.4 % trypan blue solution (Sigma-Aldrich), dilution 1:1. Cell viability and the GFP+ (green fluorescent protein) cell number were determined with Cellometer™ (Nexcelom, Bioscience). According to the results and optimization guideline of nucleofection conditions (Figure 13.) programs for siRNA transfections were chosen.

6.3.3 Nucleofection of siRNA

SiRNA's used in nucleofection are listed previously, see table 2 page 25.

Cells were first harvested by trypsinization and the required cell number, $5x10^5$ per well, was centrifuged. Cell pellet was resuspended with 360 µl of the cell specific Nucleofector® solution. 90 µl of the cell suspension was pipetted into four eppendorf tubes. 100 nM of each of the four siRNA was added into eppendorfs. P3 Nucleofector® solution was used for the stem cancer and epithelial cells, and SF Nucleofector® solution for the BT 474 cell line. 20 µl of the cell – siRNA mix was pipetted into the 16 wells of the cuvette and ran through the optimized Nucleofector® program. 80 µl of the low-calcium cell culture medium RPMI-1640 was added into the wells and let to incubate for 5 minutes in room temperature. Subsequently, cells were plated on a 12-well plate and incubated in 37°C, 5% CO_2 phase-contrast IncuCyte (Essen Instrument) for 72 hours.

6.4 Aldefluor® Assay

Aldefluor® Assay was started by activating the specific ALDEFLUOR® substrate. Activation was done by following the product information sheet provided with the ALDEFLUOR® kit. Aldefluor stained cells have earlier been detected by a fluorescence-activated cell sorting device (FACS) that is a specialized flow cytometer (Ketola *et al* 2011; Charafe-Jauffret *et al* 2009). In here, aldefluor stained cells were detected by a multilable reader and fluorescent microscopy.

6.4.1 Determination of ALDH activity

384-well Format, Adherent Cells

Aldefluor® assay was first performed by using a previously established manufacturer's protocol, downscaled to 384-well format (Ketola *et al* 2010). ALDH activity determination was done with breast cancer stem cells, breast epithelial cells and LNCaP prostate carcinoma cells. Briefly, 2,000 cells per well plated with 35 µl of media in 384-well plates with replicates of 8 and incubated overnight in 37°C. Salinomycin (Sigma-Aldrich) and cyclopamine (Sigma-Aldrich) were added in 15 µl to final concentration of 1 µmol/L in 50 µl. DMSO (Sigma-Aldrich) was added as a control to a final concentration of 1 ‰. Samples were incubated for 48 hours in 37°C. After incubation, medium was removed and cells washed with 20 µl of PBS. 10 µl of Aldefluor or Aldefluor with DEAB (ALDH inhibitor diethlyaminobenzaldehyde) was pipetted onto the cells and incubated at 37°C for 30 minutes. Solutions were removed, cells washed with 20 µl of PBS and 20 µl of assay buffer was added into each well. The fluorometric signal was determined.

Cell In Suspension

Second set of ALDH activity determination was done with breast cancer stem cells and SK-BR-3 breast carcinoma cells. Cells were grown to about 70% confluency before harvesting by trypsinization. 1 x 10^6 cells/ml were centrifuged at 200 x g for 7 mins. Supernatant was removed.

2,5 µl of activated ALDEFLUOR® substrate was added to 500 µl of assay buffer. 250 µl of this solution was placed into another eppendorf called “control”, where 5 µl of DEAB was added. Obtained cell pellets were resuspended into ALDH positive solution and negative control solution and incubated in 37°C water bath for 30 minutes. Aldefluor substrate solution was removed by centrifuging at 300 x g for 5 minutes at 4°C. Supernatant was removed and pellets resuspended with 125 µl of ice cold assay buffer. Samples were placed and kept on ice. From 20 through to 60 µl of sample was pipetted to a 384 well plate (black, Corning) for fluorometric signal determination.

6.4.2 Multilabel Reader and Fluorescence Microscope

The fluorometric signal was determined with Envision 2100 Multilabel Reader. Program used for the signal detection was with a scanning program (excitation 485 nm, emission 535 nm). In between of the detection programs, sample plate was placed on ice in order to prevent the efflux and thus saving the fluorescence stain in the cells.

20 µl of the sample was pipetted onto a glass microscopic slide. Samples were visualized by a fluorescent microscope (eCFP and eGFP filters)(Zeiss) for further demonstrating the ALDH expression.

7 RESULTS

Good transfection results are never unambiguous; transfection efficacy and the cytotoxic effect need to be considered. The raw results for transfection efficacy were gained by comparing siRNA transfection samples against cells only. The cytotoxicity of the transfection reagents was observed with the siNEG control. Other factors influencing the selection of the best transfection reagent were the simplicity of the protocol, the volume of the reagent needed for the experiment, and the financial point-of-view.

7.1 Chemical Transfection

After 72-hour incubation, the effect of siRNA transfection was determined by changes in cell proliferation. The results were achieved by CTG assay and an Envision Multilabel reader. The toxicity of the reagent and the transfection efficiencies were determined by the cell viability. All the cells were transfected with eight different commercial reagents (table 1). Here, only the best transfection results are shown.

MCF-7 cell culture

An MCF-7 cell culture was used as a starting control for the transfection reagent testing. MCF-7 has previously shown to be easy to transfect with SiLentFect. HiPerFect, with a volume of 0.05 μl per well (total volume 50 μl), showed similar efficacy results as achieved with SiLentFect, the volume being 0.09 μl per well (Figure 6). SiKIF transfection was slightly more efficient with HiPerFect, but the SiLentFect siPLK and cell death siRNA values were better (Table 3) and this reagent showed lower reagent toxicity (Figure 7). The most efficient reagent however was a novel TrueFect-Lipo reagent that has not previously been used at VTT for HT transfections.

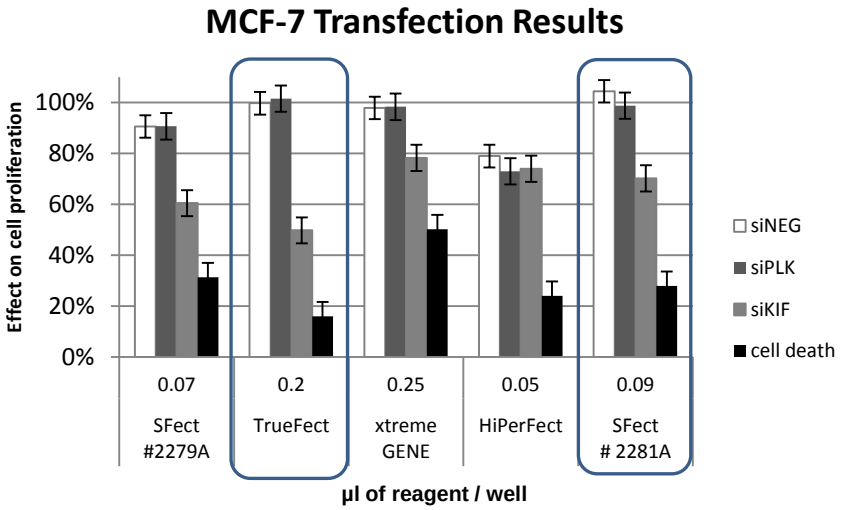


Figure 6. MCF-7 transfection results.

MCF-7 cells were transfected with eight different commercial transfection reagents. The five best transfection values were obtained with SiLentFect, TrueFect, x-TremeGene and HiPerFect HTS. From these five reagents, True-Fect shows to be the most efficient reagent for transfecting MCF-7 cells with over 80% transfection efficacy. Relatively good transfection results, over 70%, were obtained with SilentFect (#2281A). Effect on cell proliferation is shown relative to cells only (100%).

Table 3. MCF-7 transfection results with the highest transfection efficiencies. Transfection efficiency is shown relative to negative control (siNEG).

siRNA	TrueFect-Lipo	SiLentFect #2281A
	[0.02 µl]	[0.09 µl]
siNEG	100 %	100 %
siPLK	102 %	95 %
siKIF	50 %	67 %
Cell Death	16 %	27 %

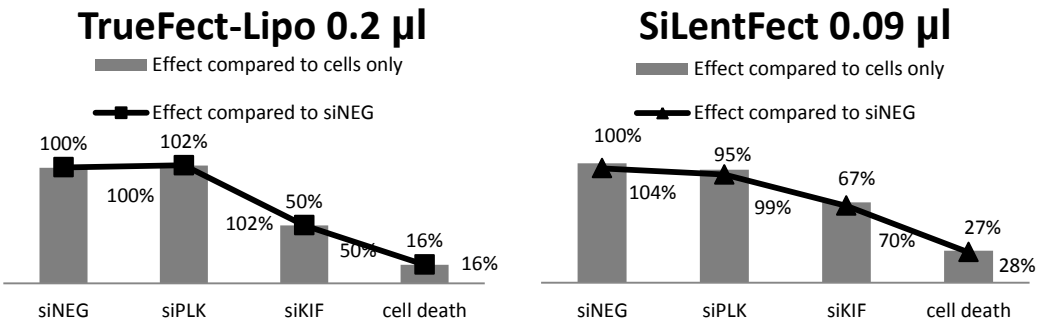


Figure 7. MCF-7 transfection results with HiPerFect and SiLentFect

The best two transfection results were achieved with TrueFect-Lipo (0.2 µl) and SiLentFect #2281A (0.09µl). The efficacy of the transfections (shown as the effect compared to siNEG) and cytotoxicy (shown as the effect of siNEG compared to cells only) of the reagents are compared with each other. Better transfection efficacy was obtained with TrueFect-Lipo (84%), but neither of the reagents showed high cytotoxicity.

BT-474 cell culture

Transfection reagent TrueFect-Lipo showed also the best efficiency for BT-474 transfection (Figure 8). With a volume of 0.08 µl per well, approximately 30% transfection efficacy was obtained with TrueFect-Lipo. SiRNA cell death did not show high specificity, since the effect on the cell proliferation was similar between siPLK, siKIF and siRNA cell death transfected cells (Table 4). BT-474 cells are considered difficult to transfect, with sensitivity to toxic effects of the transfection reagents (Figure 9).

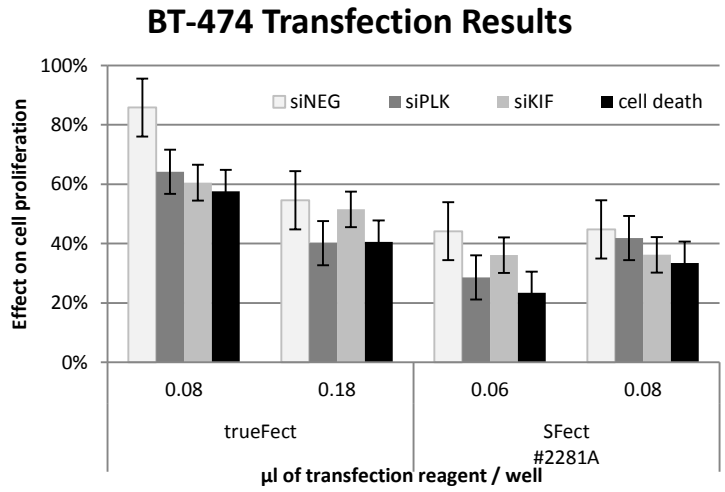


Figure 8. BT-474 transfection results.

BT-474 cells were transfected with eight different commercial transfection reagents. Of these reagents, only TrueFect and siLentFect showed even a slight efficacy. Other reagents were either cytotoxic or showed no transfection effect at all. Effect on cell proliferation is shown relative to cells only (100%).

Table 4. BT-474 transfection results with the highest transfection efficiencies.

Transfection efficacy is shown relative to negative control (siNEG). In case of SiLentFect the strong toxic effects should be noted.

siRNA	TrueFect-Lipo [0.08 µl]	SiLentFect #2281A [0.06 µl]
siNEG	100 %	100 %
siPLK	75 %	65 %
siKIF	71 %	82 %
Cell Death	67 %	53 %

The transfection of BT-474 cells was concluded as non-efficient, mainly because of strong cytotoxic effect of all tested reagents.

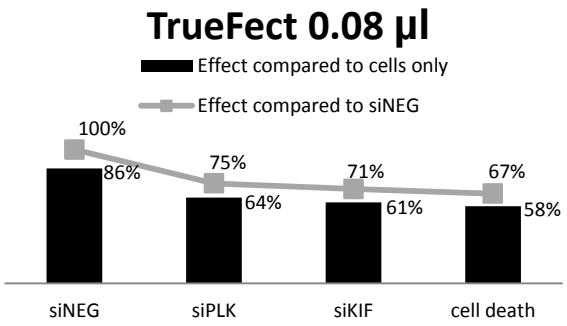


Figure 9. BT-474 transfection results with TrueFect

TrueFect-Lipo with a volume of 0.08 µl showed the best cell survival and transfection efficacy, approximately 30%, for the BT-474 cell culture. The efficacy of the transfections (shown as the effect compared to siNEG) and cytotoxicy (shown as the effect of siNEG compared to cells only) of the reagents are compared with each other.

Breast Epithelial Cell Culture

In small concentrations, 0.02 to 0.04 μl, SiLentFect #2281A and HiPerFect showed the best transfection efficacy for the breast epithelial cells with approximately 30% of transfected cells (Figure 10). SiRNA cell death was seen to have no targeted effect, while siKIF showed more cell growth inhibiting factor (Table 5). Both of these reagents showed cytotoxicity, even at small concentrations (Figure 11).

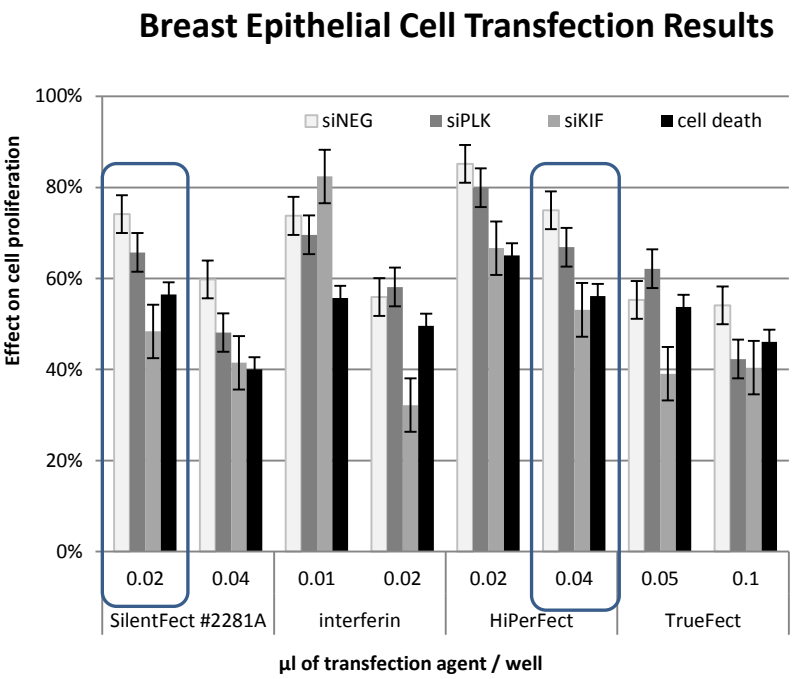


Figure 10. Breast epithelial cell transfection results

Breast epithelial cells were transfected with eight different commercial transfection reagents. Of these eight reagents, siLentFect (#2281A) and HiPerFect showed some efficacy at small concentrations, approximately 30 % transfected cells. Higher concentrations of reagents were cytotoxic for the epithelial cells. Cell proliferation is relative to cells only (100%).

Table 5. Breast Epithelial Cell transfection results with the highest transfection efficiencies.

Transfection efficacy is shown relative to negative control (siNEG).

siRNA	siLentFect #2281A [0.02 µl]	HiPerFect [0.04 µl]
siNEG	100 %	100 %
siPLK	89 %	89 %
siKIF	65 %	71 %
Cell Death	76 %	75 %

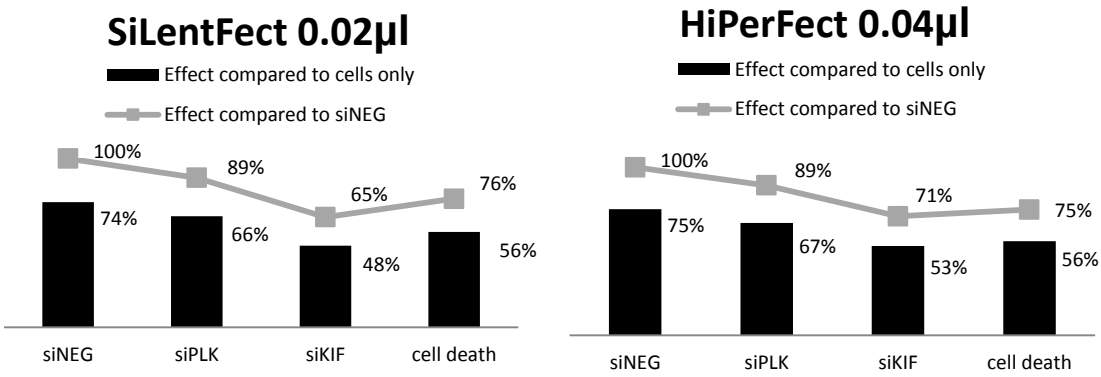


Figure 11. Breast epithelial cell transfection results with SiLentFect and HiPerFect

Breast Epithelial cells showed sensitivity against the transfection reagents, even with small concentrations cell viability was shown to be less than 75 %. The efficacy of the transfections (shown as the effect compared to siNEG) and cytotoxicy (shown as the effect of siNEG compared to cells only) of the reagents are compared with each other.

Breast cancer stem cells

The transfection of Breast CSCs showed promising results with SiLentFect #2281A and HiPerFect where more than 50% transfection efficacy was obtained (Figure 12, Table 6). SiLentFect showed some cytotoxicity, but showed good transfection efficacy even with small concentrations (Figure 13).

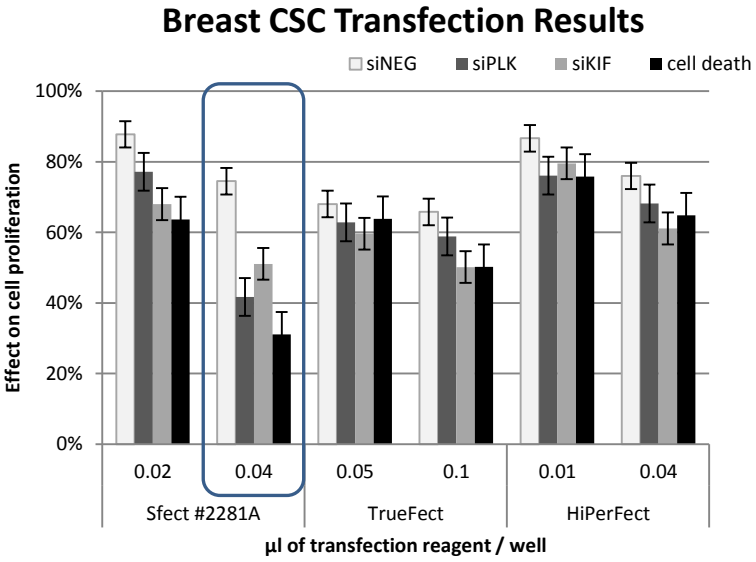


Figure 12. Breast CSC transfection results.

Breast Cancer Stem Cells were transfected with eight different commercial transfection reagents. From these reagents SiLentFect #2281A showed the best transfection efficiency. HiPerFect also showed high cell viability and but not good transfection results. Effect on cell proliferation is relative to cells only (100%).

Table 6. Breast CSC transfection results with the highest transfection efficiencies.

The transfection efficiency is shown relative to negative control (siNEG).

siRNA	siLentFect #2281A [0.02 µl]	siLentFect #2281A [0.04 µl]	TrueFect [0.1 µl]
siNEG	100 %	100 %	100 %
siPLK	88 %	56 %	89 %
siKIF	77 %	69 %	76 %
Cell Death	73 %	42 %	76 %

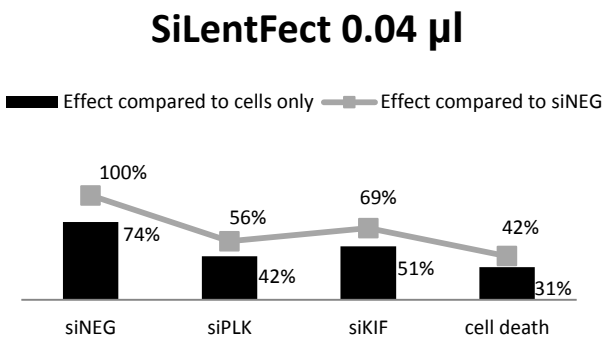


Figure 13. Breast CSC transfection results with SiLentFect

SiLentFect #2281A, with a volume of 0.04 µl, showed the best cell survival and transfection efficiency, approximately 60%, for the breast CSC cell culture. The efficacy of the transfections (shown as the effect compared to siNEG) and cytotoxicy (shown as the effect of siNEG compared to cells only) of the reagents are compared with each other.

U-87 MG cell culture

Transfection of U-87 MG was shown to be most efficient with SiLentFect #2281A and x-tremeGENE (Figure 14.) reagents. The highest transfection efficacy was achieved with SiLentFect, with over 80% transfected cells (Table 6). High transfection efficacy was achieved with both of these reagents without strong cytotoxicity (Figure 15). SiPLK was not used when testing SiLentFect #2281A reagent.

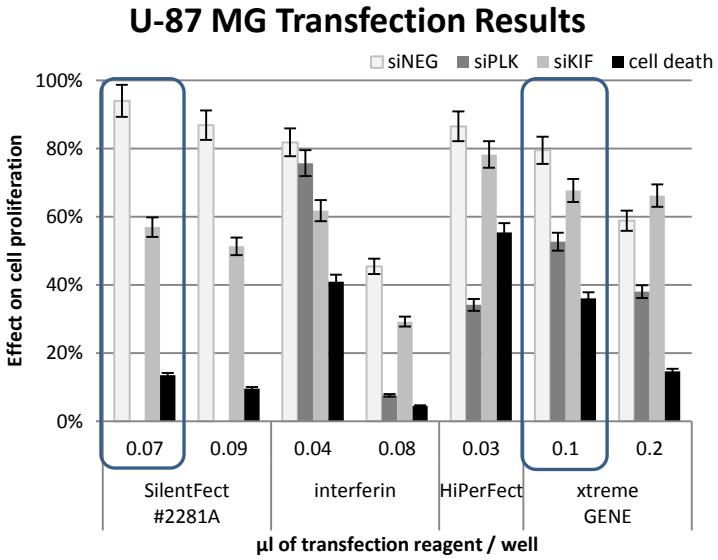


Figure 14. U-87 MG transfection results.

U-87 MG cells were transfected with eight different commercial transfection reagents. Some of these reagents were only used for transfecting siKIF, siNEG and siRNA cell death. The best transfection efficiency was obtained with SiLentFect #2281A with over 80% transfected cells and x-tremeGENE with approximately 50 % transfected cells. Effect on cell proliferation is relative to cells only (100%).

Table 7. U-87 MG transfection results with highest transfection efficiencies.

The transfection efficiency is shown relative to negative control (siNEG).

siRNA	siLentFect #2281A [0.07 µl]	Interferin [0.04 µl]	x-tremeGENE [0.1 µl]
siNEG	100 %	100 %	100 %
siPLK	n/a %	93 %	66 %
siKIF	61 %	76 %	85 %
Cell Death	14 %	50 %	45 %

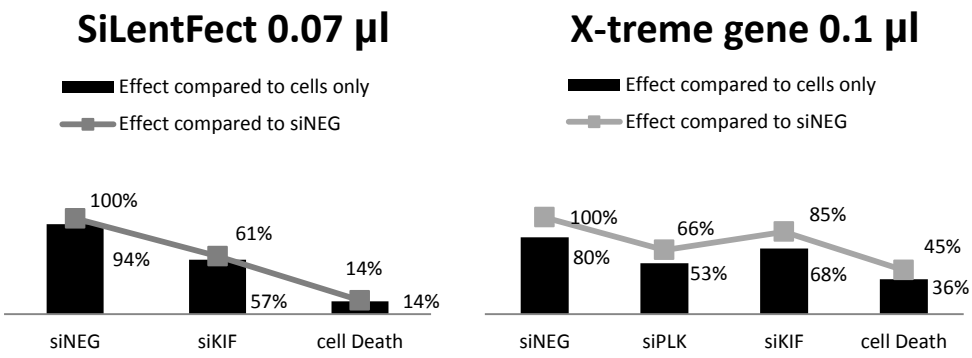


Figure 15. U-87 MG transfection results with SiLentFect and X-tremeGENE

SiLentFect, with a volume of 0.07µl, showed the highest transfection efficacy than other reagents. With SiLentFect more the 85 % of the cells were transfected, and cell viability was more than 90 %. Transfection efficacy with X-tremeGENE was also promising with more than 60 % transfected cells. The efficacy of the transfections (shown as the effect compared to siNEG) and cytotoxicy (shown as the effect of siNEG compared to cells only) of the reagents are compared with each other.

A-172 cell culture

Transfection of the A-172 cells showed good results with the HiPerFect and SiLentFect #2281A reagents, with approximately 90 % transfection efficacy (Figure 16 and Table 8).

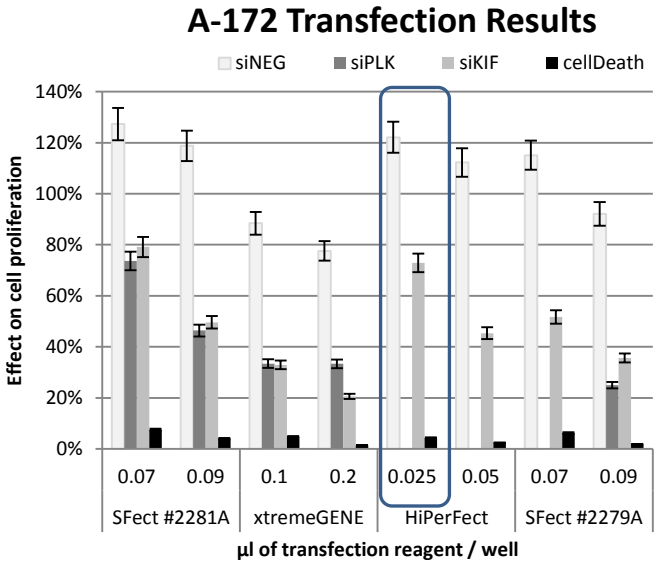


Figure 16. A-172 transfection results

A-174 cells were transfected with the eight different commercial transfection reagents. Some of the reagents were not used for transfecting siPLK. The best transfection efficacy was achieved with HiPerFect. SiLentFect #2281A, with a volume of 0.07 µl, was also suggesting good transfection results with better cell viability than with HiPerFect. Cell proliferation is relative to cells only (100%).

Table 8. A-172 transfection results with highest transfection efficiencies.

The transfection efficiency is shown relative to negative control (siNEG).

siRNA	siLentFect #2281A [0.07µl]	HiPerFect [0.025 µl]	SiLentFect #2279A [0.07µl]
siNEG	100 %	100 %	100 %
siPLK	58 %	n/a %	n/a %
siKIF	62 %	60 %	45 %
Cell Death	6 %	3 %	5 %

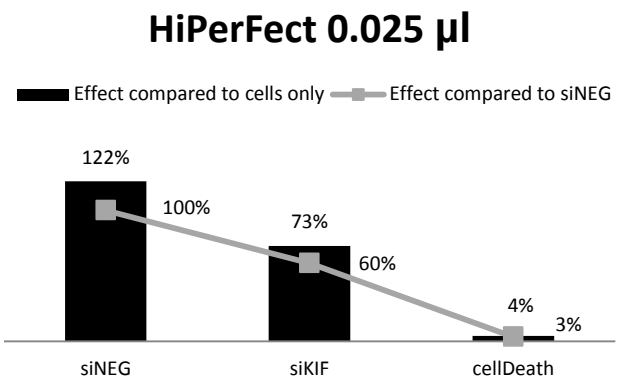


Figure 17. A-172 transfection results with HiPerFect

HiPerFect showed the best transfection results with a volume of 0.025 µl. SiNEG showed better cell survival than the cells only, which may have given unreliable results on the expense of efficacy percentage. The efficacy of the transfections (shown as the effect compared to siNEG) and cytotoxicy (shown as the effect of siNEG compared to cells only) of the reagents are compared with each other

Glioma CSC 1904 cells

Transfection of the glioma CSC 1904 cells was shown to be non-efficient. Reagents showed to be toxic with higher concentrations, and non-effective when higher cell viability was achieved (Figure 18)(Table 9). TrueFect-Lipo showed an effect but also high cytotoxicity with 0,1 µl per well. Further test between 0,06 and 0,1µl may still be beneficial.

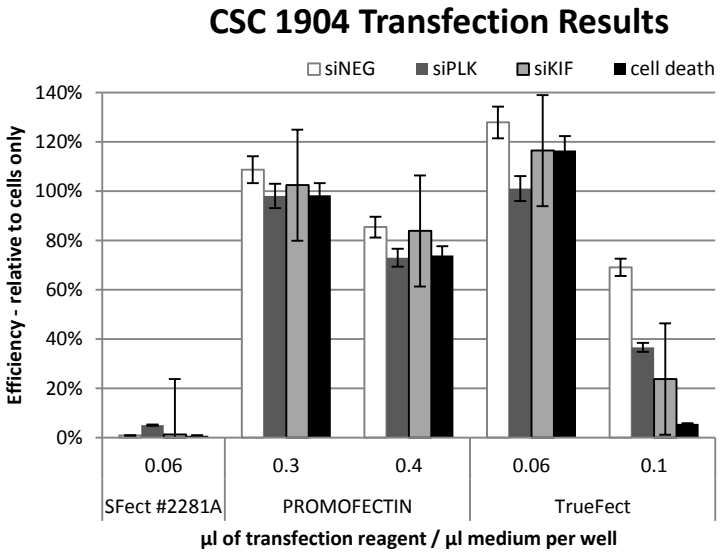


Figure 18. Glioma CSC 1904 transfection results

Glioma CSC 1904 cells were transfected with the eight different commercial transfection reagents. All the reagents showed either no effect or cytotoxicity values were too high, except for TrueFect-Lipo where further testing may still be beneficial. Cell proliferation is relative to cells only (100%).

Table 9. CSC 1904 transfection results.

The transfection efficiency is shown relative to negative control (siNEG).

siRNA	siLentFect #2281A [0.06/µl]	PromoFectin [0.4/ µl]	TrueFect [0.1/µl]
siNEG	100 %	100 %	100 %
siPLK	53 %	86 %	53 %
siKIF	34 %	98 %	34 %
Cell Death	8 %	87 %	8 %

7.2 Nucleofection

According to the results gained from the pmaxGFP™ nucleofection (Tables 10 to 13), two to four programs were selected for the second nucleofection (siRNA transfection). Nucleofection efficacy was determined by the cell viability and the number of cells with green fluorescence protein (GFP-positive) in the optimization step. By following the “Optimization of Nucleofection™ Conditions” (Figure 19.) the Nucleofector programs were reselected for gaining either an increase in the cell viability or in the transfection efficiency.

← Increasing viability				Best program(s)	Increasing efficiency →			
CA-113	CA-123	CA-132	CA-139	**CA-137**	CA-158	CA-189	CA-167	CA-201
CL-138	CM-113	CM-119	CM-132	**CM-138**	CM-134	CM-167	CU-138	DG-138
CL-135	CL-137	CM-134	CM-135	**CM-137**	CM-158	CM-189	CU-137	DG-137
CL-120	CL-150	CM-116	CM-120	**CM-150**	CM-198	CM-156	CU-150	DG-150
CY-100	DA-100	DH-100	DI-100	**DN-100**	DP-100	EH-100	ER-100	FA-100
DG-138	DS-113	DS-119	DS-132	**DS-138**	DS-134	DS-167	DT-138	ED-138
DG-135	DG-137	DS-134	DS-135	**DS-137**	DS-158	DS-189	DT-137	ED-137
DG-130	DS-128	DS-107	DS-118	**DS-130**	DS-155	DS-139	DT-130	ED-130
DG-120	DG-150	DS-116	DS-120	**DS-150**	DS-198	DS-156	DT-150	ED-150
DG-120	DS-106	DS-103	DS-111	**DS-120**	DS-141	DS-142	DT-120	ED-120
DG-113	DS-112	DS-109	DS-104	**EH-100**	DS-123	DS-118	DT-113	ED-113
CM-100	CV-100	DG-100	DR-100	**EO-100**	DT-100	DU-100	ED-100	EN-100
DZ-135	DZ-137	EH-134	EH-135	**EN-138**	EH-158	EH-189	ER-137	FA-137
DZ-120	DZ-150	EH-116	EH-120	**EN-150**	EH-198	EH-156	ER-150	FA-150
DZ-113	EH-112	EH-109	EH-104	**EW-113**	EH-123	EH-118	ER-113	FA-113

Figure 19. Optimization of Nucleofection™ Conditions

A short guideline for the program optimization when an increase of the transfection efficiency or cell viability is needed (Lonza, Amaxa). 15 programs in the middle-section (Best program(s)) are set as default programs in optimization. According to the results of each program, the viability can be increased by selecting a pulse program from the left hand side or right hand side in order to increase the efficiency.

Glioma CSC 1904

Programs selected for the siRNA nucleofection of CSC 1904 cell culture were CA 137, CA 158, EN 138, and EH 118, since the best efficacy and cell viability were achieved with these programs (Table 10). After the 3-day-incubation, nucleofection efficacy was determined by the confluency results gained from the phase-contrast IncuCyte. Program EN 138 with the Cell Line Kit P3 gave the best transfection results, with up to 50% efficacy (Figure 20). SiRNA transfection can be seen as a transient, since after 60 hour-incubation the cell growth seems to emerge even with siRNA cell death nucleofected cells; the cell growth with siNEG and siPLK seem to reach the confluency after 70-hour-incubation, which is seen as stabilized cell number.

Table 10. Results of CSC 1904 pmaxGFP™ nucleofection.

Results gained from the 16-well program format. Highest efficiencies with best cell viabilities were CA 137, CA 158, EN 138 and EH 118.

Program ID	Cell Viability	GFP –positive cells	
CONTROL	65.5 %	0.0 %	
CA 137	92.9 %	31.2 %	←
CA 158	89.2 %	51.0 %	←
CA 167	93.1 %	17.1 %	
DS 130	96.8 %	13.4 %	
DS 139	75.0 %	16.8 %	
DT 139	95.5 %	20.2 %	
ED 130	98.9 %	18.1 %	
EN 138	88.5 %	49.3 %	←
EH 189	64.6 %	6.5 %	
EW 113	73.3 %	13.4 %	
EH 118	90.8 %	47.3 %	←
FF 113	78.9 %	19.1 %	

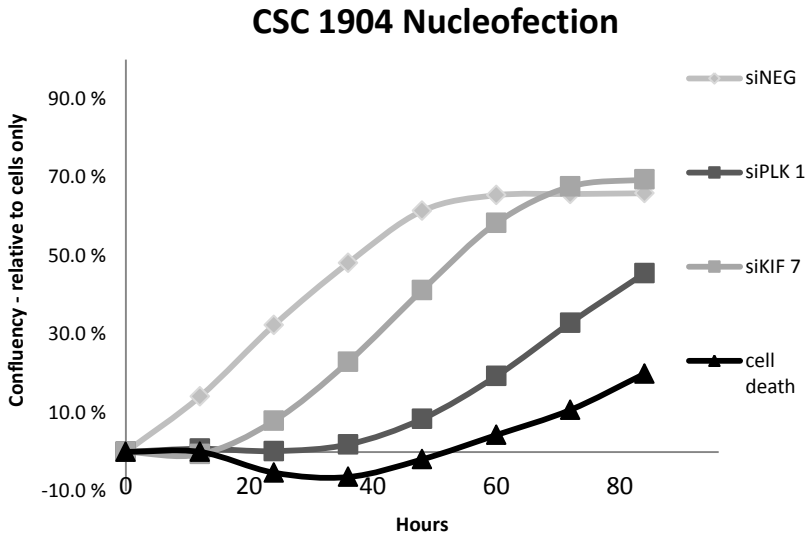


Figure 20. Nucleofection of CSC 1904 with EN 138 program and Cell Line Kit P3.

CSC 1904 nucleofection results after 3-day-incubation.Confluency values are scaled to 0% confluency as a starting point.

Breast epithelial and CSC

PmaxGFP™ nucleofection results were obtained from the FLR IncuCyte detections; cell viability from the confluency and GFP-positive cell number from the fluorescence mean. For the epithelial cells best programs were DS 138 and En 104 with both more the 96 % cell viability and about 20 % transfection efficacy (Table 11). These two programs were selected for the siRNA transfection, where DS 138 showed better results (Figure 22). Nucleofection efficacy was determined by the confluency values achieved by phase-contrast IncuCyte. SiRNA showed to be more stable for epithelial cells than for glioma CSCs (Figure 20); even after 80-hour-incubation the siRNA cell death nucleofected cells were with confluency less than 10 %.

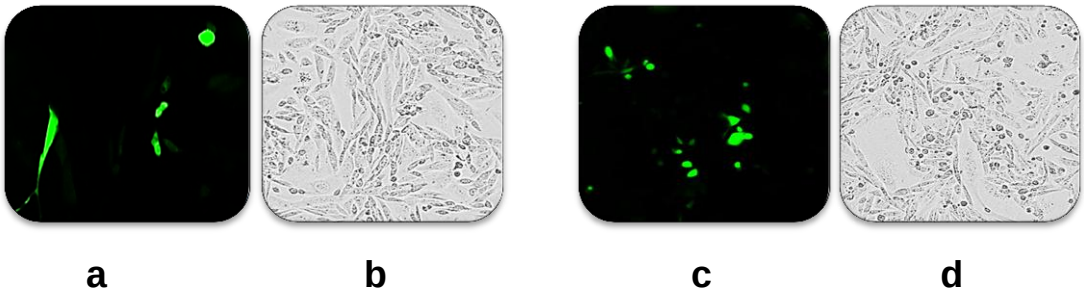


Figure 21. *pmax™GFP nucleofected breast epithelial cells and CSCs.*

Cells at 70 hour time point at the IncuCyte FLR (Essen Instrument). a) breast epithelial cells pictured with fluorescent filter, b) breast epithelial cells from the same spot with the phase contrast. Picture c) Breast CSCs with fluorescent filter, and d) Breast CSC with phase contrast. Bright GFP-fluorescence showed good nucleofection result.

Table 11. Results of Breast Epithelial cell pmaxGFP™ nucleofection.

Highest transfection efficiency with best cell viability was achieved with DS 138 and En 104 programs and Cell Line Kit P3.

Program ID	Cell Viability	GFP –positive cells
CONTROL	96.7 %	7.4 %
CM 138	93.7 %	13.1 %
CM 150	96.3 %	14.2 %
DS 138	97.1 %	22.6 %
DS 120	96.4 %	11.8 %
EO 100	95.7 %	7.2 %
EL 100	97.0 %	9.0 %
EN 104	96.0 %	19.7 %

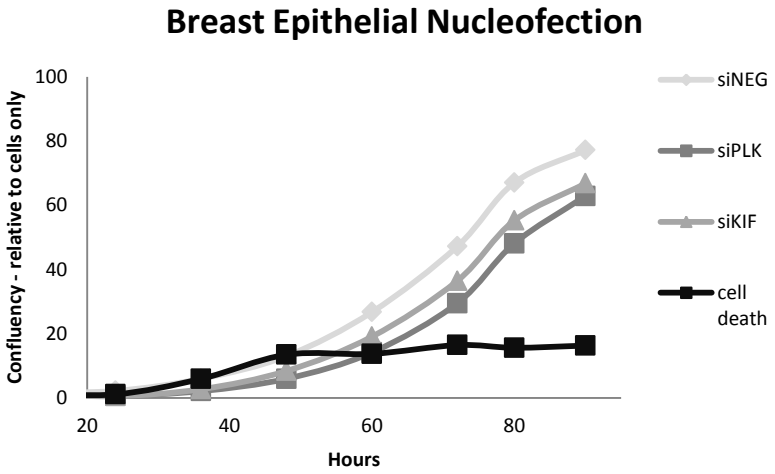


Figure 22. Nucleofection of Breast Epithelial cells.

Breast Epithelial cell nucleofection results after the 3-day-incubation. The best nucleofection efficacy values, more than 50%, were obtained with the nucleofector program DS 138 and the primary cell line kit P3. Confluency value is relative to 0% confluency as a starting point.

Breast CSC Nucleofector showed the best results with programs CM 138 and DS 138. DS 138 cell mortality was higher than with most of the programs, but efficiency was above the average.

Table 12. Results of Breast CSC pmaxGFP™ nucleofection

Program ID	Cell Viability	GFP –positive cells
CONTROL	96.6	6.3
CM 138	84.6	15.7
CM 150	93.4	13.9
DS 138	88.2	20.0
DS 120	96.1	12.1
EO 100	97.2	7.0
EL 100	93.9	9.2
EN 104	75.3	28.7

Programs selected for the siRNA nucleofection of breast CSC culture were CM 138 and DS 138. After the 3-day-incubation, nucleofection efficiency was determined by the confluency results gained from the phase-contrast IncuCyte.

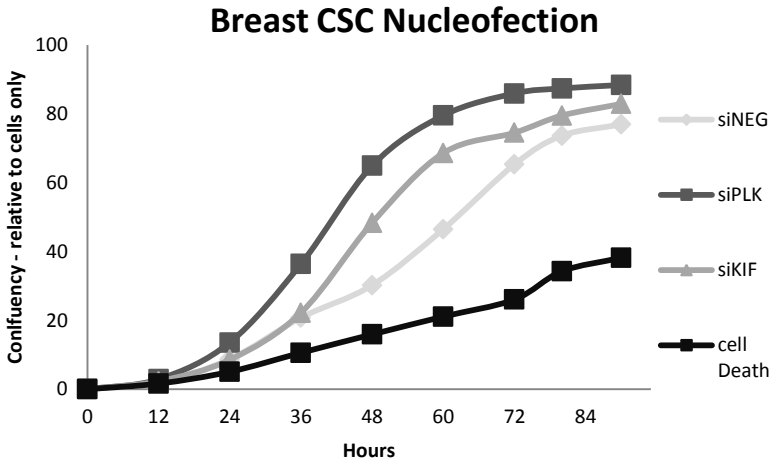


Figure 23. Nucleofection of Breast CSC.

Breast CSC nucleofection results after the 3-day-incubation. The best nucleofection efficacy was achieved with the Nucleofector program DS 138 with approximately 50% efficiency. Primary cell line kit P3 was used. The confluency value is relative to 0% confluency as a starting point.

BT-474 cell culture

PmaxGFP™ nucleofection was carried out with the Cell Line Kit SF, and the siRNA nucleofection with the Cell Line Kit SG. Both of these solution kits were suggested by the representative from Lonza. Optimization results gave promising results, with almost 60 % of efficacy and only 10 % mortality (Table 13) with EN 138 program. Viability and GFP-positive cell number were determined with 0.4% trypan-blue and CelloMeter. The conditions of the pulse program were optimized by following the Lonza guideline (Figure 19 and 24) for achieving higher efficacy, even probable cost of viability. SiRNA nucleofection was carried out with four different programs: ED 138, ED 113, ER 137 and DD 137. Latter pulse program was suggested by the demonstrator from Lonza. After the 3-day-incubation, the efficacies of the nucleofections were determined by the confluency results gained from a phase-contrast IncuCyte. Results showed poor cell viability, less than 60 %, and an approximate of 30 % as the nucleofection efficacy (Figure 25). After a further, microscopic visualization, the siRNA cell death nucleofected cells were shown to be ruptured, and a clear effect on cell proliferation was showed (Figure 26). The relatively high conluency value obtained for these “cell death” cells, by the IncuCyte, was prooven as a missleading value. The highest cell viability and nucleofection efficacy were achieved with ED 137 pulse program.

Table 13. Results of BT-474 pmaxGFP™ nucleofection

Programs that showed most promising reasults for the nucleofection of BT 474 cells were CD 137, DS 138, EH 100, and EN 138. From these four programs four further programs were selected for increasing the transfection efficiency. Programs selected for the sirRNA transfection were ED 138, ED 113, ER 137, and DD 137 (suggested by the representative from Lonza).

Program ID	Cell Viability	GFP –positive cells
CONTROL	99.3 %	0.5 %
CA 137	97.6 %	30.7 %
CM 138	96.3 %	29.0 %
CM 137	76.5 %	45.5 %
CM 150	99.2 %	32.3 %
DN 100	99.0 %	20.0 %
DS 138	92.9 %	43.1 %
DS 137	95.9 %	24.5 %
DS 130	95.5 %	34.0 %
DS 150	98.3 %	33.5 %
DS 120	94.3 %	24.3 %
EH 100	99.3 %	39.4 %
EO 100	95.1 %	7.1 %
EN 138	90.5 %	58.6 %
EN 150	92.0 %	33.4 %
EW 113	88.3 %	29.9 %

Increasing viability				Best program(s)	Increasing efficiency			
CA-113	CA-123	CA-132	CA-139	CA-137	CA-158	CA-189	CA-167	CA-201
CL-138	CM-113	CM-119	CM-132	CM-138	CM-134	CM-167	CU-138	DG-138
CL-135	CL-137	CM-134	CM-135	CM-137	CM-158	CM-189	CU-137	DG-137
CL-120	CL-150	CM-116	CM-120	CM-150	CM-198	CM-156	CU-150	DG-150
CY-100	DA-100	DH-100	DI-100	DN-100	DP-100	EH-100	ER-100	FA-100
DG-138	DS-113	DS-119	DS-132	DS-138	DS-134	DS-167	DT-138	ED-138
DG-135	DG-137	DS-134	DS-135	DS-137	DS-158	DS-189	DT-137	ED-137
DG-130	DS-128	DS-107	DS-118	DS-130	DS-155	DS-139	DT-130	ED-130
DG-120	DG-150	DS-116	DS-120	DS-150	DS-198	DS-156	DT-150	ED-150
DG-120	DS-106	DS-103	DS-111	DS-120	DS-141	DS-142	DT-120	ED-120
DG-113	DS-112	DS-109	DS-104	EH-100	DS-123	DS-118	DT-113	ED-113
CM-100	CV-100	DG-100	DR-100	EO-100	DT-100	DU-100	ED-100	EN-100
DZ-135	DZ-137	EH-134	EH-135	EN-138	EH-158	EH-189	ER-137	FA-137
DZ-120	DZ-150	EH-116	EH-120	EN-150	EH-198	EH-156	ER-150	FA-150
DZ-113	EH-112	EH-109	EH-104	EW-113	EH-123	EH-118	ER-113	FA-113

Figure 24. Optimization of Nucleofection™ Conditions

A short guideline for the program optimization that was followed for achieving higher efficiency. From the default optimization programs ("Best programs") DS 138, EH 100, EN 138 and EW 113 showed the best results. From these four, pulses with "increasing efficiency" were selected. Program ER 137 was not obtained from these guidelines; program was suggested by a representative.

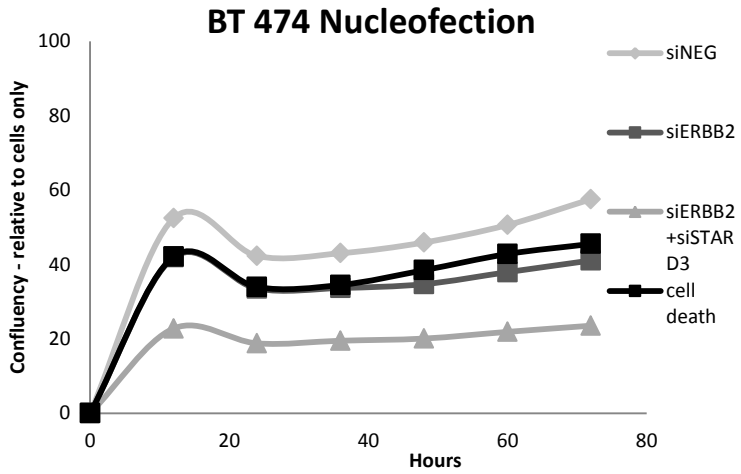


Figure 25. Nucleofection of BT-474.

BT-474 nucleofection results after the 3-day-incubation. In the figure 15. only the highest transfection efficiency and cell viability are shown. Here, the confluency value is relative to 0% confluency as a starting point. Nucleofector program ED 137 and Cell Line Kit SG were used.

Figure 26. *Nucleofected BT-474 cells.*

Image of the cells, captured at the time point after 70-hour-incubation. A) the nucleofection of siNEG and b) the nucleofection of cell death. Captured with the phase-contrast IncuCyte. Nucleofector program ED 113 was used with the Cell line Kit SG.

7.3 ALDH activity

Fluorescent, ALDH overexpressing cells were first detected by Envision multilabel scanning program and visualized by fluorescent microscope. ALDH activity has been successfully detected with FACS, flow cytometry. Multilabel reader has been successfully used for identifying $ALDH^{br}$ in prostate cancer cells in 384-format (Ketola *et al* 2011). This 384-HT-reader based format was being tested and seen if microscopic visualization could be used for the detection.

384-well format – Adherent Cells

Fluorometric results gained with the Envision Multilabel reader showed variable results (Table 14.) According to the results, cyclopamine increased the $ALDH^{br}$ of the breast CSC's when added in high concentrations. Salinomycin treated LNCaP cells showed the same effect (Figure 27). Cells with stem-cell properties have been found to have decreased $ALDH^{br}$ value when treated with salinomycin and cyclopamine (Ketola *et al* 2011; Bar *et al* 2008). Both of these drugs were diluted in DMSO, so that DMSO treated cells were used as an appropriate control. ALDH –ve samples, where DEAB was used as an inhibitor of ALDH, showed only a slight inhibition (in LNCaP cells) and a slight increase in $ALDH^{br}$ (in breast CSC).

Table 14. Results of ALDEFLUOR® Assay for Adherent Cells.

Detection values achieved with Envision multilable reader. Measurements gave inconsistent values; no accurate conclusions could be made.

Chemical Treatment	LNCaP	Breast CSC
ALDH +ve	283 183	273 085
DMSO	179 694	108 621
salinomycin 1 µM	302 271	203 564
cyclopamine 5 µM	225 658	266 478
cyclopamine 10 µM	171 083	310 357
ALDH –ve (DEAB)	256 628	279 774

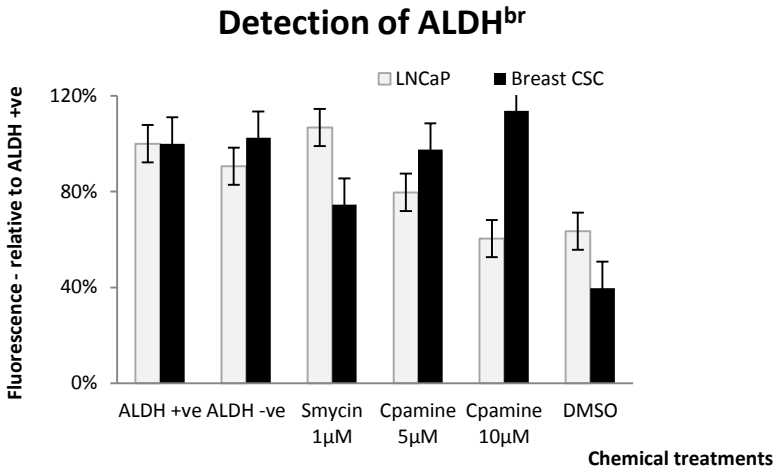


Figure 27. ALDH^{br} detection of LNCaP and Breast CSC cell cultures.

Detection values are relative to ALDH+ve cells. Salinomycin and cyclopamine were added as known ALDH^{br} decreasing drugs. LNCaP cells seem to behave as expected in case of cyclopamine and DMSO treated cells. Breast CSC's seemed to behave as expected with salinomycin and DMSO. Because of the variation, any conclusions could not be made.

Visualization of the ALDEFLUOR® stained cells on 384-well plate was done with the fluorescent microscope, cGFP and eGFP filters. However, there was fluorescent signal only detected with LNCaP cells (Figure 28).

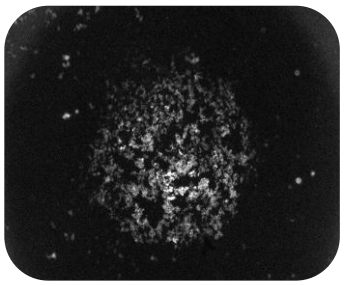


Figure 28. *ALDEFLUOR® stained LNCaP cells*

Aldefluor® treated LNCaP cells, cultured on a 384-well plate (black, Corning). Cells captured with a fluorescence microscope (5x) with eGFP filter.

Cells in Suspension

ALDEFLUOR® treated cells were suspended and pipetted into the wells of 384-well plate and the fluorometric signal was detected with Envision multilabel reader. Detection values were set against with the background. ALDH inhibitor DEAB systematically showed higher ALDH^{br} (Figure 29). DEAB was used as a negative control, so no conclusions could be made. The only observation was on the decrease of fluorometric signal during the time course. Highest fluorometric signal was detected straight after the ALDEFLUOR® treatment. After 12 hours since the treatment the signal showed stabilization for as long as 72 hours since the treatment. Sample plate was kept on ice or in +4°C.

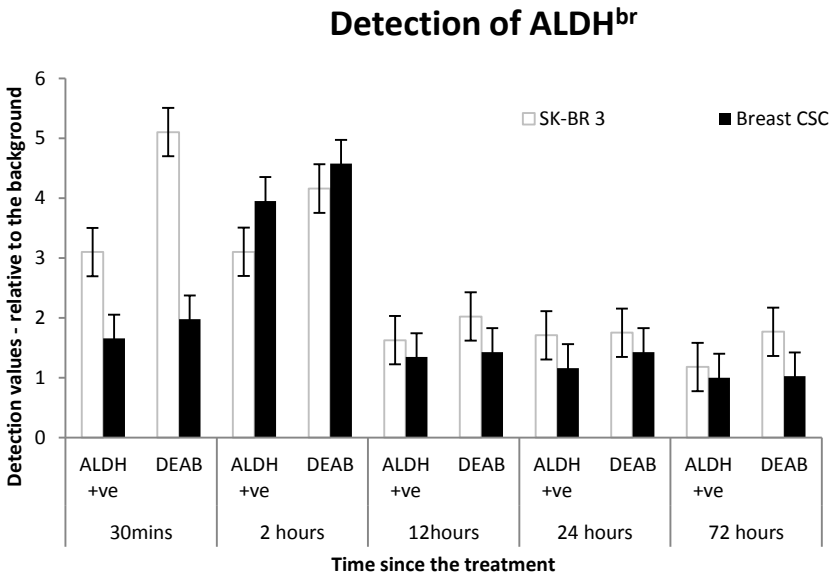
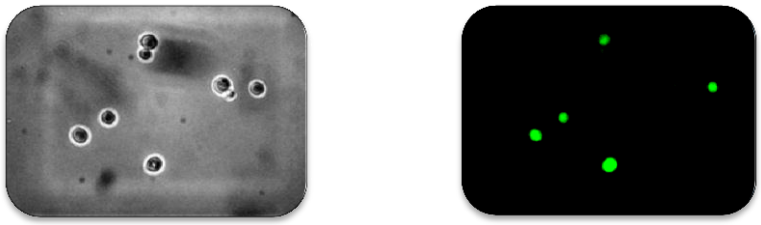


Figure 29. ALDH^{br} detection of SK-BR 3 and Breast CSC cell cultures.

Detection values are relative to the background (empty wells).First detection was applied after 30minutes of the ALDEFLUOR® substrate treatment. After every detection, sample plate was placed on ice or +4°C. ALDH inhibitor was showing no effect or even increased ALDH^{br}. Fluorometric signal was shown to degrease already between 30mins and 2hours.

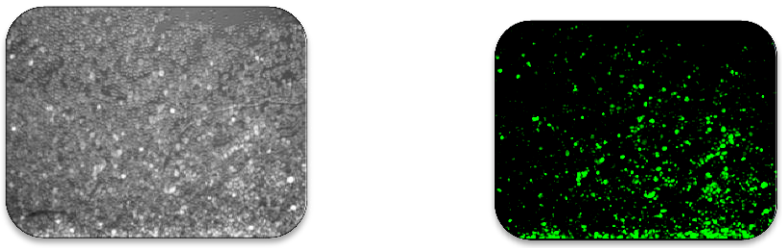
Visualization of the ALDEFLUOR® stained cells was done with the fluorescent microscope, cGFP and eGFP filters (Figures 30 and 31). Suspension cells were pipetted onto a glass microscopic slide. ALDEFLUOR® stained cells were assumptioned to be successfully visualized (Figure 32)(Stemcell-technology ALDFELUOR®-protocol).



a b

Figure 30 *ALDEFLUOR® treated SK-BR 3 cells.*

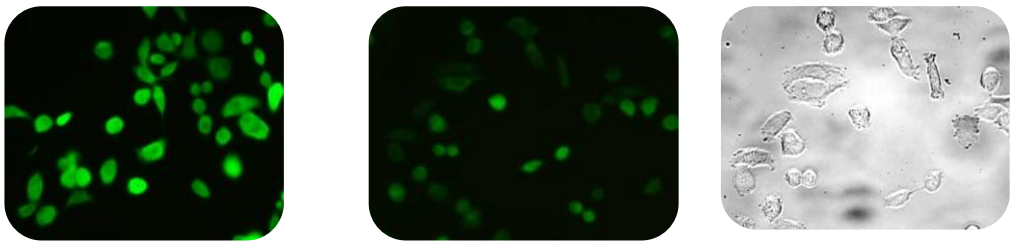
Aldefluor® treated SK-BR 3 cells captured with fluorescence microscope (40x) on a glass microscopic slide. A) Cells visualized with phase-contrast filter and b) with eGFP filter



a b

Figure 31. *ALDEFLUOR® treated breast CSC cells,*

Aldefluor® treated breast CSC's captured with fluorescence microscope (10x) on a glass microscopic slide. A) cells visualized with phase-contrast filter and b) with eGFP filter.



a b c

Figure 32. *ALDEFLUOR® stained SK-BR 3 cells*

Fluorescent and phase images of ALDEFLUOR® stained cells a) "SKBR3 is brightly stained with ALDEFLUOR® due to high expression of ALDH (expression indicated by flow cytometry)" b) " DEAB control of SKBR3 cells show dim fluorescence" and c) "Brighfield images of SKBR3 monolayers" (Stemcell Technologies, "Identification of ALDH-expressing cancer stem cells" –technical bulletin)

8 DISCUSSION

Chemical Transfection and Nucleofector technology

When applying a chemical transfection method for HTS format there are a number of requirements for the protocol and the assay development requires standardization as far as possible. For this reason the concentration of siRNA, the cell number and the volume of the medium were kept constant during all the experiments. Optimization in this case comprised trial and error experiments with the concentration of the reagent before finding the right volume for each cell line and culture. Transfection conditions always vary between cell lines, especially between hard-to-transfect cell lines and cancer stem cell cultures. The variation is due to the characteristics of the cells and the conditions require optimization before the actual research work. Other than the technical factors mentioned above, the condition of the cell is a crucial factor which also affects the transfection result. Eight different commercial transfection reagents were tested for two cancer stem cell cultures and five other cell cultures. Since no single transfection reagent or delivery method can be applied for all types of cells, each of these reagents was tested with every culture. Depending on the reagent, the efficiency and the cytotoxicity of the transfection may vary greatly. SiLentFect (Bio-Rad) is now commonly used at VTT and achieves moderate results with normal cell lines and low results with stem cell cultures. The optimization results of this study were compared with the results obtained with SiLentFect. The transfection reagents used in this study were selected because of different reasons; some of reagents were selected because of the manufacturer had a ready protocol suiting HTS-format, or because of the promises as an effective and non-toxic reagent for stem and primary cells, or purely because of the financial point-of-view. From these eight reagents, the highest transfection efficiency values (60-80%) for the MCF-7, A-172 and U-87 MG cell lines with about 60-90 % cell viability were obtained with SiLentFect #2281A and volumes of 0.07 to 0.09 µl per well. HiPerFect (Qiagen) showed promising results also at small concentrations, from only 0.03 to 0.05 µl per well. A mortality rate of only 10-20 % was exhibited with 70-80 % transfection

efficiency. The HiPerFect HTS reagent is targeted for siRNA and miRNA transfections in 384-format and is provided with a ready-to-use reverse transfection protocol. TrueFect-Lipo transfection reagent gave also promising results, and in some cases even better transfection efficacy values than other reagents. But TrueFect-Lipo was found as non-efficient by the financial point-of-view.

The transfection of cancer stem cells, epithelial cell culture and BT-474 was proven efficient with the Nucleofector® device. With the Cell Line Kit P3 more than 50 % nucleofection efficacy was obtained for these cells while chemical transfection was approximately 50 %. Bigger difference was seen on the mortality rate, as chemical transfection showed more cytotoxic effects than the Nucleofector transfection. With the chemical transfection of the BT-474 cell culture, the transfection efficacy was 55% with 20% mortality, pmaxGFP ® vector nucleofection of these same cells gave promising suggestions with 60 % efficacy and 10% mortality. However, siRNA nucleofection was concluded with a different Cell Line Kit than the optimization, and the siRNA nucleofection efficacy was only 30%. The pmaxGFP® vector nucleofection was concluded with the Cell Line Kit SF, which suggests higher efficacy for BT-474 cell culture nucleofection than the Cell Line Kit SG. Pulse program EN 138 was found to be optimal for BT-474 cells. Nucleofection technology was shown to be a useful and powerful tool for primary and stem cells and other hard-to-transfect cells. The nucleofector™ 16-model program was found useful for medium-throughput transfections. Now also a 384-well version is available but at a very high cost.

ALDH activity

The overexpression of aldehyde dehydrogenase has been identified as a marker for cells with stem-cell properties (Charafe-Jauffret *et al* 2009). The ALDEFLUOR™ Kit (STEMCELL technology) has enabled the identification and isolation of these stem-like cells from cancer cell cultures. The protocol of the kit is developed for cells in suspension and detection of the aldefluor fluorescence ($ALDH^{br}$) is intended to be carried out by fluorescence-activated cell sorting (FACS) flow cytometry. The protocol was further developed for HT-based,

384-format, adherent prostate cell cultures (Ketola *et al* 2011) with the detection by Envision Multilabel reader. Here the aim was also to develop the assay further for breast cancer stem cells. $ALDH^{br}$ detection results obtained from this work were, however, inconsistent and visualization suggested cell rupturing caused by the ALDEFLUOR® assay treatment.

The $ALDH^{br}$ of dead and ruptured cells has been shown to have decreased (Figure 32). Cells were cultured on a black 384-well plate in order to achieve advantageous results by multilabel reading, which for its part may have caused only faint or no fluorescence signal visualization with a microscope even though Envision detection suggested otherwise. The 384-format protocol for adherent cell cultures was aimed to be tested and extended for an automated technique. SK-BR 3 cells were known to be high in ALDH1A1 expression (Charafe-Jaffraut *et al* 2009), wherefore they were used as a positive control in the $ALDH^{br}$ determination of breast cancer stem cells. Cells were suspended for the assay, and plated on a black 384-well plate for Envision detection. The fluorescent stain was successfully visualized (Figures 30 and 31), and Envision multilabel reader values suggested high $ALDH^{br}$. However, the results could not be reported to be successful since the negative control, DEAB, showed an increase in the detection values. The increase could be explained by the high overexpression of aldefluor hydrogenase when the recommended DEAB concentration is not sufficient for inhibiting the formation of the fluorescence substrate. In this case, an increase by the DEAB concentration could give reliable results. To further test this assumption, a re-run of the experiment is needed. The ALDH activity testing done in this thesis gives some suggestions and assumptions, but scientific evidence or technical improvement was not achieved.

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Complete Cell Growth Media

MCF-7 cell growth medium

Dulbecco's Modified Eagle Medium (DMEM, 1000 mg/l glucose) supplemented with 2mM Glutamine, 1 % Penicillin/Streptomycin and 10 % Fetal Bovine Serum (FBS)

BT-474 cell growth medium

Dulbecco's Modified Eagle Medium (DMEM, 4500 mg/l glucose) supplemented with 1mM Sodium Guryvate, 4mM Glutamine, 0.01 mg/l Insulin, 1% Penicillin/Streptomycin, and 10 % FBS

SK-BR 3 cell growth medium

McCoy's 5A Modified Medium supplemented with 1,5 mM Glutamine, 1 % Penicillin/Streptomycin, and 10 % FBS

Glioma CSC 1904 cell growth medium

Dulbecco's Modified Eagle Medium (DMEM, 4500 mg/l glucose) and Ham's F-12 Glutamax supplemented with 2mM L-Glutamine, 1% Penicillin/Streptomycin, and B27 Neuromix (50x), N2 Supplement (100x), and basic Fibroglast Browth Factor (bFGF, human recombinant), and Epidermal Growth Facotr (EGF)

A 172 cell growth medium

Dulbecco's Modified Eagle Medium (DMEM, 4500 mg/l glucose) supplemented with 1% Penicillin/Streptomycin, and 10 % FBS.

U-87 MG cell growth medium

Eagle's Minimum Essential Medium supplemented with 10 % FBS