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Table of contents

1	Abstract – English.....	9
2	Zusammenfassung – Deutsch.....	11
3	List of Abbreviations	13
4	Introduction.....	15
4.1	Nucleic acid delivery in gene therapy.....	15
4.2	Nanoparticles as nucleic acid delivery vehicles.....	15
4.2.1	Linear polyethylenimine as transfection agent	17
4.2.2	Lipid-based transfection agents	21
4.3	Nucleic acid based therapeutics.....	22
4.3.1	Deoxyribonucleic acid	22
4.3.2	Ribonucleic acid	28
4.4	Bioluminescence	29
4.4.1	Reporter proteins.....	30
5	Goals of diploma thesis	37
6	Materials and Methods	39
6.1	Equipment.....	39
6.2	Bicinchoninic acid assay (BCA assay)	40
6.2.1	BCA assay including a freezing step.....	42
6.2.2	BCA assay including iodoacetamide	43
6.3	Preparation of buffers.....	44
6.3.1	Preparation of housemade Luciferin Assay Buffer for Firefly luciferase	44
6.3.2	Preparation of commercial Luciferin Assay Buffer for Firefly luciferase	48
6.3.3	Preparation of housemade Coelenterazine Assay Buffer for Gaussia/Lucia luciferase	49
6.3.4	Preparation of commercial Coelenterazine Assay Buffer for Gaussia/Lucia luciferase	50
6.4	Luciferase based reporter gene assays with recombinant luciferase	50
6.4.1	Luciferase based reporter gene assay with recombinant Firefly luciferase	50

6.4.2	Luciferase based reporter gene assay with recombinant Gaussia luciferase	52
6.5	Luminescence measurement.....	53
6.6	Techniques for generation of nanoparticles.....	54
6.6.1	Generation of LPEI based polyplexes by flash pipetting technique	54
6.6.2	Generation of jetPRIME [®] based polyplexes	55
6.6.3	Generation of Lipofectamine [®] 2000 based lipoplexes	56
6.6.4	Generation of Lipofectamine RNAiMAX [®] based lipoplexes	57
6.6.5	Effect of freeze-thawing on transfection efficiency of polyplexes.....	58
6.7	Cell culture techniques	60
6.7.1	Cell seeding into a 96-well plate	60
6.7.2	Transfection of A549 cell line with plasmid DNA via polyplexes and lipoplexes.....	61
6.8	Cell based luciferase reporter gene assays.....	62
6.8.1	Firefly luciferase assay for investigating plasmid delivery.....	64
6.8.2	Firefly luciferase assay for investigating siRNA delivery.....	65
6.8.3	Gaussia luciferase assay.....	66
6.8.4	Lucia luciferase assay	67
6.9	Flow cytometry	68
7	Results.....	70
7.1	Optimization of BCA assay	70
7.1.1	Effect of freezing on cell lysis after treatment with 1X Lysis Buffer	70
7.1.2	Influence of iodoacetamide on BCA assay	71
7.2	Optimization of Firefly luciferase assay	73
7.2.1	Testing housemade versus commercial Luciferin Assay Buffer with recombinant luciferase.....	73
7.2.2	Testing housemade versus commercial Luciferin Assay Buffer with transfected cells.....	75
7.2.3	Aliquot to aliquot and batch to batch variability of housemade Luciferin Assay Buffer across transfection experiments	77
7.2.4	Reproducibility of housemade Luciferin Assay Buffer using recombinant luciferase.....	78
7.2.5	Determination of substrate injection volume	79
7.3	Optimization of coelenterazine based luciferase assays	80

7.3.1	Testing housemade versus commercial Coelenterazine Assay Buffer with transfected cells	80
7.3.2	Aliquot to aliquot and batch to batch variability of housemade Coelenterazine Assay Buffer across transfection experiments.....	83
7.3.3	Reproducibility of housemade Coelenterazine Assay Buffer using recombinant luciferase	85
7.3.4	Signal acquisition settings	85
7.3.5	Kinetics of coelenterazine utilizing luciferases	87
7.3.6	Calculation methods for normalization of luminescence light signals	88
7.4	Applications of optimized assays.....	90
7.4.1	Effect of freeze-thawing on transfection efficiency of polyplexes	90
7.4.2	siRNA knockdown	92
7.4.3	Testing liposome mediated delivery of plasmid DNA and siRNA	95
7.4.4	Dual luciferase assay	98
8	Discussion	102
9	Appendix	111
9.1	Standard Operation Procedures.....	111
9.1.1	Measurement of absorption and luminescence	111
9.1.2	Preparation of HEPES Buffered Glucose (HBG buffer).....	114
9.1.3	Aseptic cell culture techniques	115
9.1.4	Flow cytometry	123
9.2	Adjustments on plate reader and calculation methods relevant for performance of <i>in vitro</i> luciferase assays.....	126
10	References	129

1 Abstract – English

Currently, major aspects of gene therapy research are safety aspects as well as questions concerning efficient delivery and transfection of target cells. Non-viral gene delivery vectors do not bear the risk of insertional mutagenesis or severe immune response from the host, compared to their viral counterparts. Polyplex nanoparticles, consisting of a cationic polymer complexed with nucleic acids, are used for delivery of DNA as well as siRNA *in vivo*. In this diploma thesis, several technical aspects of luciferase based reporter gene assays for nanoparticle mediated nucleic acid delivery into cells were studied. Bioluminescent reporter gene assays, utilizing two different luciferases (intracellular and secreted), were used to monitor efficiency of delivery *in vitro*.

One objective was to optimize housemade buffer systems for both Firefly and Gaussia/Lucia luciferase based reporter gene assays. Housemade and commercial buffers were analyzed for a variety of parameters to test their efficiency for luciferase assay. Luciferases employed for testing the performance of housemade buffer systems included both recombinant luciferase proteins (Firefly or Gaussia luciferase) and *in vitro* transfected cells transiently expressing luciferases (Firefly or Gaussia). Both housemade buffer systems performed as good as their respective (and expensive) commercial counterparts and thus can be reliably used for such assays. In case of housemade buffer for Firefly assay, it was found that housemade substrate, either freshly prepared or stored at -80°C, can reliably replace the expensive commercial substrate. Thus, preparing the substrate in advance and storing aliquots in frozen form is time-saving and guarantees reproducibility.

A further objective was optimizing the BCA assay, which is used for protein normalization of luminescence light signals in Firefly luciferase assay. We found that 30 µl 1X Passive Lysis Buffer was efficient enough to lyse cells within 30 min shaking and additional iodoacetamide is not needed. In conclusion, the amount of protein in the lysate is influenced by many factors, mainly depending on the applied cell lysis buffer.

In other optimization experiments for secreted luciferase based reporters, Gaussia and Lucia luciferase assays were evaluated regarding their coelenterazine substrate. As Gaussia and Lucia luciferases are secreted into the supernatant, normalization of their emitted light signals by total count of live cells (from flow cytometry) was also investigated and compared with the BCA assay based normalization. Both normalization methods were shown to give acceptable results. Flow cytometry has the additional advantage of multiplexing within the same experiment e.g. detecting cytotoxic effects of polyplexes on cells in addition to their transfection ability.

After optimization of several assay parameters, applications of optimized luciferase assays were explored.

In one application, knockdown of Firefly luciferase signal by siRNA delivery was investigated, utilizing a commercial lipofectamine. In a similar second application, cationic liposome mediated siRNA delivery to cells was explored, but knockdown of luciferase expression was not sufficient and further *in vitro* experiments are needed. Furthermore, both assay buffer systems were combined in a dual luciferase assay and co-transfection of Firefly and Gaussia was analyzed confirming their robustness. Employment of these buffer systems for gene delivery investigations was performed to study transfection efficiency of frozen LPEI based polyplexes. Polyplexes stabilized with a cryoprotectant (HP- β -CD) showed slightly higher transfection efficiency *in vitro* than non-stabilized polyplexes. However, cytotoxicity was also higher.

In summary, the present diploma thesis describes the technical optimization of several aspects of luciferase assays used in gene delivery research and explores its robustness by using it for different *in vitro* nucleic acid delivery applications.

2 Zusammenfassung – Deutsch

Wesentliche Fragestellungen der gentherapeutischen Forschung betreffen heutzutage die Sicherheit als auch die Transport- sowie die Transfektionseffizienz in Zielgewebe im Organismus. Nicht-virale Vektoren weisen im Gegensatz zu viralen Vektoren den Vorteil auf, weder eine insertionelle Mutagenese noch eine schwere Immunreaktionen im Empfänger hervorzurufen. Polyplex-Nanopartikel – bestehend aus einem kationischen Polymer komplexiert mit Nukleinsäuren – werden sowohl für den *in vivo* Transport von DNA als auch von siRNA benutzt.

In der vorliegenden Diplomarbeit wurden spezifische Aspekte von Luciferase-basierten Reporter-Gen-Assays für Nanopartikel-vermittelten Transport in Zellen untersucht. Biolumineszenz-basierte Reporter-Gen-Assays, die entweder intrazellulär lokalisierte oder aktiv sezernierte Luciferasen nutzen, wurden eingesetzt um die Effizienz des *in vitro* Transports zu untersuchen.

Eine Aufgabenstellung der Diplomarbeit betraf die Optimierung der hausgemachten Puffersysteme sowohl für Firefly-basierte als auch für Gaussia/Lucia-basierte Reporter-Gen-Assays. Die Aktivität hausgemachter Puffersysteme wurde sowohl mit Hilfe rekombinanter Firefly und Gaussia Luciferasen als auch mit transient *in vitro* exprimierten Luciferasen (von Firefly oder Gaussia) überprüft. Es konnte festgestellt werden, dass hausgemachte und kommerziell erhältliche Substrate eine annähernd gleiche Aktivität aufweisen; hausgemachte Puffer können daher die teuren kommerziellen Puffer ersetzen. Weiters wurde für den Firefly Luciferase Assay festgestellt, dass der hausgemachte Puffer auch bei -80°C gefroren gelagert werden kann ohne dass es dabei zu einer wesentlichen Aktivitätsminderung kommt. Dies ermöglicht es den Puffer im Vorhinein herzustellen und gefroren in Form von Aliquoten zu lagern, was zeitsparend ist und Reproduzierbarkeit garantiert.

Ein weiteres Ziel der Diplomarbeit war die Optimierung des BCA Assays, der für die Normalisierung der Lumineszenz des Firefly Luciferase Assays herangezogen wird. Es wurde festgestellt, dass 30 µl 1X Passive Lysis Buffer für eine Lyseperiode von 30 Minuten zur vollständigen Lyse ausreichen. Der Zusatz

von Iodacetamid beeinflusst den BCA Assay dabei nicht wesentlich. Generell hängt die gemessene Proteinmenge im Lysat wesentlich von der Art des eingesetzten Lysepuffers ab.

Im Zuge weiterer Experimente wurden die Herstellungs- und Lagerungsbedingungen des Substrats Coelenterazine für Gaussia und Lucia optimiert. Da beide Luciferasen in den Überstand sezerniert werden, kann die Lumineszenz auch auf die Zahl lebender Zellen normalisiert und mit dem BCA Assay, der auf die Proteinmenge normalisiert, verglichen werden. Beide Normalisierungsmethoden führten zu akzeptablen Resultaten. Ein Vorteil der Durchflusszytometrie ist, dass nicht nur die Transfektionseffizienz sondern auch zytotoxische Effekte der Polyplexe detektiert werden können.

Nach Optimierung einiger die Luciferase Assays beeinflussender Parameter wurden mögliche Anwendungen dieser Assays untersucht.

In einer Anwendung wurde die Herunterregulation der Genexpression von Firefly Luciferase mittels siRNA untersucht, wobei kommerzielles Lipofectamine als Transfektionsreagens benutzt wurde. Ebenso wurde Liposomen-vermittelter siRNA-Transfer untersucht, wobei dieser allerdings in einer nicht ausreichenden Inhibition der Luciferase-Expression resultierte. Weitere *in-vitro* Experimente sind nötig. In einer anderen Anwendung wurden beide Luciferase Assays in einem dualen Luciferase Assay kombiniert. Dabei wurde eine Co-Transfektion von Firefly und Gaussia Luciferasen durchgeführt und die Robustheit beider Assays gegenüber kleineren Änderungen der Experimentalbedingungen bestätigt.

Weiters wurde die Transfektionseffizienz von stabilisierten LPEI-basierten Polyplexen untersucht, die zuvor bei -80°C eingefroren wurden. Der Zusatz des Kryoprotektivum Hydroxypropylbetacyclodextrin führt zu einer leicht höheren Transfektionseffizienz *in vitro*, es konnten jedoch auch vermehrt zytotoxische Effekte beobachtet werden.

Zusammenfassend beschreibt die vorliegende Diplomarbeit die Optimierung zahlreicher Aspekte von Luciferase Assays in der gentherapeutischen Forschung und untersucht ihre Robustheit im Zuge von Anwendungen des *in vitro* Nukleinsäuretransports.

3 List of Abbreviations

B	Buffer
cCAB	commercial Coelenterazine Assay Buffer
CCLR	Cell Culture Lysis Reagent
cLAB	commercial Luciferin Assay Buffer
CT-15/50	Centrifuge tube 15 ml/50 ml
CTZ	Coelenterazine
DNA	Deoxyribonucleic acid
FBS	Fetal Bovine Serum
g	standard gravity
h	hours
HBG	HEPES-buffered Glucose
HBS	HEPES-buffered Saline
hCAB	housemade Coelenterazine Assay Buffer
HCl	Hydrochloric acid
hLAB	housemade Luciferin Assay Buffer
HP- β -CD	Hydroxypropyl- β -cyclodextrin
kDa	kilodalton
LB	Luciferin Buffer
LF [®] 2000	Lipofectamine [®] 2000 Transfection Reagent
LPEI	Linear polyethylenimine 10 kDa
LF [®] RNAiMAX	Lipofectamine [®] RNAiMAX Reagent
MMCT	Laboratory of Macromolecular Cancer Therapeutics (University of Vienna)
min	minutes
ms	milliseconds
MQ-water	MilliQ-water
NaOH	Sodium hydroxide
Opti-MEM [®]	Opti-MEM [®] Reduced Serum Medium
pDNA	plasmid DNA
PBS	Phosphate Buffered Saline
PLB	Passive Lysis Buffer
RLU	Relative Light Unit
RNA	Ribonucleic acid
rpm	rounds per minute
RT	room temperature
SD	standard deviation
sec	seconds
siRNA	small interfering RNA
UT	Untreated (cells)

4 Introduction

4.1 Nucleic acid delivery in gene therapy

Therapeutics based on nucleic acids have received much attention over the last twenty years¹. Conventionally, solid tumors have been treated by surgery, radiotherapy and chemotherapeutics so far. However, these cancer treatments often neither show desired clinical effects nor completely cure because of dose-limiting toxicity and resistance mechanism. Additionally, severe side effects are of major concern. Nucleic acids represent a promising strategy for a completely new treatment approach. Unlike small molecule based chemotherapeutics, which reach their target cells by passive diffusion and therefore also cause side effects due to unspecific cell interactions, nanoparticles are specifically designed for overcoming extracellular and intracellular biological barriers in order to deliver nucleic acids to tumor cells. However, overcoming these barriers is still a major focus of research and it will take far more time to completely identify all properties of an ideal delivery vector needed for overcoming physiological obstacles. Efficient drug delivery to mammalian cells, combined with simultaneous protection of nucleic acid would permit highly selective treatment of malignant diseases and genetic disorders (e.g. cystic fibrosis)^{2,1}. The purpose of introducing nucleic acids into mammalian cells is altering gene expression either at the transcriptional or at the posttranscriptional level^{1,3}. On the one hand, complementary DNA (cDNA) can function as a pro-drug which becomes a drug by translation into a protein, which can then replace its non-functional pendant. On the other hand, nucleic acids can interfere with complementary mRNA sequence and subsequently lead to a block in translation (RNAi technology)¹.

4.2 Nanoparticles as nucleic acid delivery vehicles

Nanoparticles can be divided into two main groups of nucleic acid delivery vehicles: viral and non-viral vehicles.

Initially, research efforts focused on viral vectors, because transporting nucleic acids to specific cells is within their natural ability¹. They pass through cellular

barriers because they have evolved respective mechanisms by natural selection¹⁰. Though viral vectors show high and selective efficiency in delivering their cargo to cells, they face worrying disadvantages: inflammatory response of host organism, endogenous virus recombination, oncogenic effects (potential for mutations), size limitation for DNA cargo (limited loading capacity), and difficulties in large scale production^{4,5,6}.

Frequently used viral vectors are for instance adenoviral viruses, herpes simplex virus or retro virus (lentivirus). Retroviruses produce a DNA copy from their RNA with reverse transcriptase and the enzyme integrase inserts DNA into the host's genome. Reasonable concerns regarding inserting DNA into an arbitrary position within the host's genome exist, because this can lead to mutation and furthermore triggers cell proliferation by activating pro-oncogenes or by suppressing tumor suppressor genes. Thus, viruses pose risk of cancer initiation itself^{7,5}.

In 1999, Jesse Gelsinger was the first person who died in a clinical study after having received an injection of adenoviral vectors. He died because of an adverse immune response against the adenoviral vector⁸.

Among non-viral methods for gene delivery, the simplest (physical) method is directly injecting naked DNA into skeletal muscles, liver, tumors or other tissues, or administrating DNA intravenously. Disadvantages of intravenous injection are rapid degradation of DNA by nucleases occurring in serum as well as the clearance effect by the mononuclear phagocyte system⁴. Additionally, foreign double stranded nucleic acid activates the innate immune system by interaction with Toll-like-receptors (TLR-3)⁹.

Modified physical methods have been developed, but they require expensive equipment and damage cells nonspecifically¹⁰. For instance, electroporation allows enhanced uptake of locally injected DNA by applying voltage pulses. Pores are formed in the cellular membrane, increasing DNA entry, but also entailing cell death⁷. Particle bombardment as a further method is also known as gene gun because DNA is coated onto gold particles and these are then shot into tissue by a device. DNA directly penetrates into the cytoplasm or even nucleus, circumventing the endosomal system⁴.

Chemical carriers have been proved as versatile non-viral gene delivery vehicles. Bionanomaterial research has gained enormous advances in terms of improving features of chemical non-viral vectors. Its principle is that negatively charged nucleic acid is condensed either by cationic lipids (e.g. 1,2-dioleoyl-3-(trimethylammonium) propane, DOTAP), cationic polymers (e.g. polyethylenimine, PEI), cationic polypeptides (e.g. polylysine, protamine), or cationic polysaccharides (chitosan)^{11,12}. Mainly, polycationic lipids and polycationic polymers have been used for *in vitro* and *in vivo* transfection studies so far⁴⁰.

These synthetic delivery vehicles show the following advantages over viral vectors: simple in use, ease in large-scale production, low cost, higher biosafety, less likelihood causing severe immunogenic reactions, no existence of preformed antibodies, and attachment of moieties, resulting in specific targeting abilities^{13,14,45}. Nevertheless, they render disadvantages, such as limited transfection efficiency after *in vivo* application and therefore low expression levels, and instability in aqueous solutions because they tend to aggregate. Furthermore, they have to be prepared freshly prior to administration and this probably entails batch to batch variability which affects quality and transfection rates in an unfavorable way^{15,45}.

4.2.1 Linear polyethylenimine as transfection agent

Polymer-mediated gene delivery includes polyethylenimine, poly-L-Lysine¹⁶, linear polyamidoamines¹⁷ and PAMAM (Poly(amidoamine)) dendrimers¹⁸ as transfection agents. Their natural cationic feature enables compaction of negatively charged nucleic acids via electrostatic interaction, forming nanoparticles which are referred to as polyplexes (Figure 2). Within the above polymers, polyethylenimine (PEI) is the gold standard transfection agent. It is an organic macromolecule consisting of polymerized subunits of ethylenimine (Figure 1). Its backbone has two carbon atoms and every third atom is amino nitrogen. Two different forms of PEI exist. Branched polyethylenimine (BPEI) has a mixture of primary, secondary and tertiary amines, whereas linear polyethylenimine (LPEI) only possesses secondary amines¹.



LPEI

LPEI

LPEI



Polyion complex
(>40 nm)

Polyion complex
(>40 nm)

size and charge of polyplexes, mainly depend on N/P ratio and less on structure of PEI²⁸.

Mixing order and mixing speed of DNA and polycation represent two more factors affecting physicochemical characteristics of nanoparticles. Polyplexes are usually prepared manually via flash-pipetting. This represents the classical way, but disadvantages such as batch to batch variability, restriction to small volumes and operator-induced variability also have to be considered¹³. In short, small volumes of DNA solution and LPEI solution are rapidly mixed in a tube by pipetting 20 times up and down. Boussif et al.²⁰ reported that adding the LPEI solution to the plasmid solution was more efficient regarding transfection rate than addition vice versa. However, mixing large volumes via flash pipetting is less controllable and leads to heterogeneous batches regarding size (broad polydispersity index) and charge²⁹. Kasper et al.²⁹ and Taschauer et al.¹³ established up-scaled methods for production of large batches of polyplexes.

Incubation time influences particle size. Freshly prepared polyplexes must not be incubated for longer than 20 min at RT, because with increasing incubation time their tendency in forming large aggregates in liquid formulations is elevated^{10,13,29}. Transfection efficiency of polyplexes is influenced by size and charge of polyplexes, amount of plasmid and buffer composition in which polyplexes are generated^{13,22,30}.

Extracellular and intracellular barriers of polyplexes in vivo

An overview of extracellular and intracellular biological barriers faced by nanoparticles is given in Figure 3.

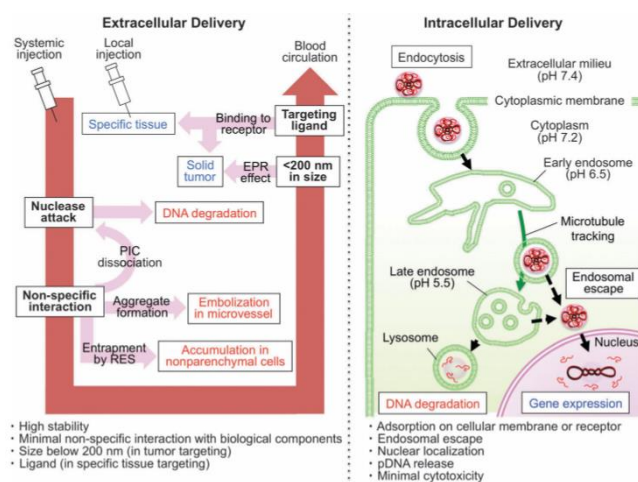


Figure 3: Extracellular and intracellular barriers for nanoparticles; figure from reference ²¹.

To begin with, nanoparticles introduced into the organism face extracellular barriers. Local injection of nanoparticles in muscles or eyes is quite successful because of its easy performance and high transfection rate of solid tumors, but it is restricted to specific tissues. Systemic application is required for treatment of cancer metastases. Nanoparticles must be small enough for transport in blood capillaries and diffusion through tissues ($< 200 \text{ nm}$)²¹.

Normal vasculature consists of tightly packed endothelial cells which prevent diffusion in tissue, whereas endothelial cells of tumor vasculature are only loosely linked, facilitating diffusion into adjacent tumor cells. As tumor tissue lacks lymphatic drainage and in case renal excretion limit of intravenously applied therapeutics has been exceeded, this and also leaky vascular architecture lead to the enhanced permeation and retention effect (EPR) *in vivo*^{31,21}.

However, systemic application has to overcome other obstacles such as nucleases, which degrade DNA, or activation of immune system or, when aggregated, they are removed by clearance effect of RES. Latter means that spleen and liver filter foreign nanoparticles from the bloodstream due to special established structures in its vasculature³².

Aggregation occurs because of non-specific interactions with serum proteins, causing either emboli in microvessels or even leading to dissociation of nanoparticles. These side-effects can be circumvented by conjugating polyethylenglycol (PEG) to the polymer so that its cationic charge is masked. It reduces toxicity and extends half-life in blood circulation due to preventing opsonization and recognition by cells of RES. These modified particles are referred to as 'stealth' polyplexes, also because they are easier accumulated in a solid tumor by passive targeting due to leaky bloodvessels, as described above²¹. Furthermore, PEG can be further functionalized by conjugating a targeting ligand (e.g. antibodies, aptamers, small molecules or peptides) to its distal chain end so that it serves as a linker between cationic polymer and ligand³³. This allows 'active targeting'²¹ and reduces off-target effects, i.e. uptake of therapeutics by non-target cells. For instance, Blessing et al.³⁴ evaluated transfection efficiencies of polyplexes, in which peptides of EGF are conjugated to PEI, enabling a cell-specific targeting of EGF-receptors (EGFR) on tumor cells, which resulted in receptor (EGFR)-mediated cellular uptake. Similar transfection efficiency was also shown by coupling EGF to the PEG linker arm³⁴.

Polyplexes are internalized into endosomes, which protect them from degradation by cytosolic nucleases. Endosomes fuse with lysosomes and polyplexes trigger their release by the proton sponge effect, explained by following mechanisms: Because of its numerous protonable amino groups PEI has a buffering capacity over a broad pH range²⁰. Therefore it buffers protons within the endosome, and subsequently chloride ions as counterions follow. Consequently, this involves the influx of water, resulting in an osmotic swelling and therefore a rupture of the endosome membrane through osmotic pressure³⁵. Nevertheless, this hypothesis is still under discussion³⁶.

The molecular weight of PEI has an impact on how easily its cargo is released from the tight complexation. PEI of low molecular weight condenses DNA less efficiently and leads to less transfection efficiency. Otherwise, even though high molecular weight PEI has stronger condensation ability, it releases DNA less efficiently which then also results in less transfection efficiency. High molecular-weight LPEI is also more cytotoxic. A balance has been found with 22 kDa LPEI^{10,37}. A high positive charge has been found to be highly toxic because of destabilization of membrane integrity, resulting in apoptotic process. Therefore, a low positive charge is desired but this also entails less condensation strength of pDNA which consequently lowers stability of polyplexes²¹.

Trafficking into nucleus is poorly understood³⁰. Cells in S- and M-phase of cell cycle have an elevated transcriptional activity. It is suggested that the nuclear envelope transiently disassembles and polyplexes can enter nucleus more easily^{38,10}. Cells of a high passage number have less metabolic activity. They are not well suited for transfection studies¹⁰.

4.2.2 Lipid-based transfection agents

Lipid-mediated gene delivery was first reported in 1987 by Felgner et al.³⁹. They showed that the cationic synthetic lipid DOTMA N-[1-(2, 3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride forms net positively charged complexes with DNA by ionic interaction and these cationic lipoplexes are taken up by cells within a process called lipofection³⁹. A positively charged polar head group and one or two hydrophobic tails are components of a cationic lipid. Various cationic lipids, i.e. quaternary ammonium detergents, cationic derivatives from cholesterol or

diacylglycerol, and lipid derivatives of polyamines exist⁴. When dispersed in water, they form cationic liposomes. But helper lipids are also often required, e.g. cholesterol or the neutral lipid DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), because they contribute to structural stability⁴⁰. Advantages of cationic liposomes are: biocompatibility, i.e. enzymes can degrade (biodegradable) lipids; functionalization by attaching target-ligands onto PEGylated lipids; co-delivery of nucleic acids, localized in inner aqueous compartment of the liposome, and chemotherapeutics, entrapped in its phospholipid bilayer, is also possible. Cationic liposomes show a higher internalization rate than neutral liposomes due to their positive charge which facilitates interaction with negatively charged proteins on cellular membranes. But the positive charge also leads to interaction with blood compounds^{40,41,42}. Lipofectamine[®] 2000 is a commercially available cationic liposome, capable of successfully transfecting a variety of cell lines, which is used as a positive control for *in vitro* transfection experiments in the present diploma thesis^{42,43}. Cationic lipoplexes are formed by mixing equal volumes of cationic liposomes with siRNA⁴⁴.

4.3 Nucleic acid based therapeutics

Plasmid DNA, antisense oligodeoxynucleotides, small interfering RNA (siRNA) and micro RNA (miRNA) represent possible nucleic acid based drugs for nanoparticle mediated delivery⁴⁵. The following section is focused on plasmid DNA and siRNA.

4.3.1 Deoxyribonucleic acid

4.3.1.1 Cloning technique

Cloning is a standard tool in genetic engineering. In short, a foreign piece of DNA, also known as transgene, is inserted into a cloning vector and latter is transformed into organisms for replication, e.g. bacteria. After isolation and purification of multiplied vector-transgene-constructs, they are introduced into eukaryotic cells by transfection or transduction methods and finally the transgene is expressed into a heterologous recombinant protein. In summary, this technique

enables synthesizing a large variety of genetically engineered vectors via DNA-recombinant technology and producing huge amounts of recombinant proteins in mammalian cells⁴⁶.

4.3.1.2 Cloning vectors

Cloning vectors are DNA pieces from plasmids, viruses or other higher organisms, of which plasmids are the most common used⁴⁷. They can be classified by functionality, e.g. cloning plasmids, expression plasmids or reporter plasmids⁴⁸. In reporter plasmids the cDNA of a reporter protein is introduced; after translation its protein is readily measurable. One application of reporter plasmids is measuring influence of regulatory elements on the reporter gene. In case the expressed reporter protein is a luciferase enzyme, its light intensity directly correlates with activity of regulatory elements. After transformation into bacteria the cloning vector is amplified. The following three elements are basic components of a plasmid (Figure 4)⁴⁸:

- The origin of replication recruits RNA polymerase and initiates transcription, i.e. self-replication of the vector within a bacterium.
- The selection marker is an antibiotic resistance gene and it allows growth of bacteria under antibiotic pressure. Only successfully transformed bacteria are able to grow on media supplemented with antibiotics.
- A promoter drives expression of the transgene in the bacterium.

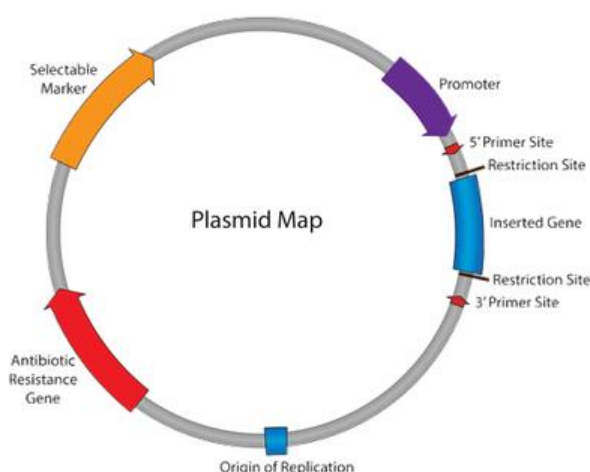


Figure 4: Plasmid map of a cloning vector; figure from reference ⁴⁸.

Escherichia coli is the most commonly used organism for cloning. Various transformation methods exist. After amplification, plasmids are isolated by

plasmid preparation and introduced into other (expression) cell types, e.g. mammalian cells. For expression of a transgene in eukaryote host organisms a mammalian expression cassette is needed, which is composed of the following genetic elements⁴⁸:

- The Multiple Cloning Site (MCS) is characterized by various restriction sites for restriction enzymes so that the transgene can be inserted by ligation or removed by excision⁴⁸. The genetic sequence of the transgene is inserted as complementary DNA (cDNA), a double-stranded DNA produced by reverse transcriptase from mRNA. In principal, cDNA codes for same amino acids as mRNA⁴⁶ but cDNA is often codon-optimized, which means that expression of proteins is adjusted on the host's metabolism⁴⁹.

In expression vectors, the MCS is located downstream from the promotor so that expression of the cloned transgene is driven by this promotor⁴⁸.

- Eukaryotic promoters are either constitutively active or controlled by regulatory elements, i.e. inducible. They initiate transcription and contain binding sites for transcription factors. Enhancers are located widely distant to their related promoters. They increase the transcription rate⁴⁶.

The most common used eukaryotic promotor stems from the human cytomegalovirus (hCMV) because it renders a strong expression in many cell types. Additionally, it shows a constitutive expression profile. It is composed of the immediate early promotor (IEP) and an overlapping enhancer element^{50,51,52}. Because sequence of the enhancer element can be optimized to the host, both a murine (mCMV) and a human (hCMV) form of CMV enhancer exist⁵⁶.

The EF-1 α promotor also contributes to a constitutive expression in mammalian cells⁵².

Moreover, SMAR proteins (scaffold or matrix-attached regions) are found in eukaryotic DNA and are necessary for structural stability of eukaryotic chromatin within the nucleus. SMAR proteins enable nucleus scaffold (matrix) to attach onto eukaryotic DNA, conferring to its topological form. It has been shown that they

form barriers in plasmids between independently regulated domains, i.e. between prokaryotic and eukaryotic expression cassettes⁵³.

In general, a cloning vector equipped with genetic elements for replication within prokaryotes and for expression of transgenes in eukaryotes is also known as shuttle vector^{54,47}.

Generally, influences of enhancer or promotor elements on genes of interest (GOI) can be studied by either replacing the GOI through a reporter gene or by adding, respectively fusing it to the, to the reporter gene itself (Figure 5). In both cases a transgene cell, or even a transgenic organism is produced^{46,55}. For instance, in the present diploma thesis, a vector plasmid containing a reporter gene encoding Firefly luciferase was used and its expression was driven under control of promotor EF1- α and hCMV served as enhancer.

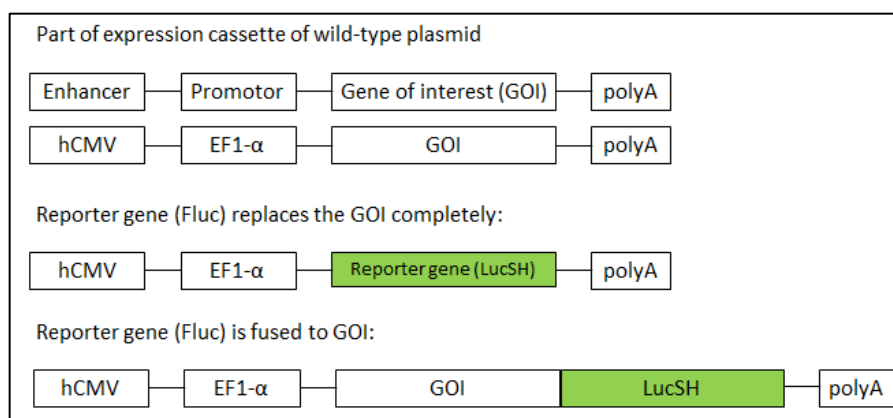


Figure 5: Strategies for cloning. Either a reporter gene (LucSH encoding Firefly luciferase) replaces the GOI or it is fused onto it. Expression of reporter protein or fusion construct is then driven by a promotor (EF1- α) and its expression strength is determined by an enhancer (hCMV).

Administration of nanoparticles often lacks long-term expression of transgene because of two factors associated with occurrence of CpG-dinucleotides in the genome. The term CpG describes dinucleotides composed of 5'-cytosin-phosphate-guanin-3'. In eukaryotic genomes the frequency of CpG-dinucleotides is usually low, whereas the promotor region exhibits a high density of CpG-dinucleotides, also known as CpG-islands. Usually, CpG-islands are unmethylated which correlates with active gene transcription. Methylation of cytosine within CpG-islands by a methyl transferase represses gene transcription (epigenetic silencing). This is the reason for rapid loss of luciferase expression *in vivo* after administration of a plasmid equipped with a eukaryotic promotor. The second factor of this transient transfection, which further shortens transgene

expression, is activation of innate immune system. Prokaryotic and viral DNA is characterized by a higher frequency of CpG-dinucleotides in their genome, compared to eukaryotic DNA. CpG-dinucleotides are not methylated. When administered into a vertebrate, the high frequency of non-methylated CpG-dinucleotides in the DNA is recognized as foreign by Toll-like-receptors (TLR-9). Their activation causes an inflammatory response with high cytokine levels, which in turn inactivates foreign plasmid DNA, conferring to its short transgene expression *in vivo*. Plasmid vectors with a CpG free backbone prevent activation of immune system, contributing to a sustained transgene expression⁵⁶.

Expression strength of a transgene inserted into a CpG depleted vector correlates with methylation level of its enhancer. Hoivik et al.⁵⁷ showed that methylation of CpG-dinucleotids in enhancer elements repressed transgene expression *in vitro*. For example, pCpGfree-promotor-Lucia is a vector suitable for introduction of enhancers via cloning in order to study effects of DNA methylation in this respective enhancer after *in vivo* application. CpG-free non-viral vectors should be used for *in vivo* gene delivery because of reduced inflammatory response as well as rendered sustained transgene expression⁵⁸.

In the present diploma thesis, the plasmid vector pCpG-hCMV-EF1 α -LucSH⁵⁶ was used for transfection experiments (Figure 6). The reporter gene is a fusion gene comprised of Firefly luciferase and Sh ble gene (Luc::Sh), whereby latter confers resistance towards the antibiotic zeocin. polyA describes the late polyadenylation signal under control of simian virus 40 promotor. Transcription is driven by an EF-1 α promotor and enhanced by hCMV enhancer. The plasmid backbone is CpG-reduced. The R6K ori is required for plasmid propagation in E.coli under Zeocin pressure. It depicts the prokaryotic expression cassette. According to Magnusson, the S/MAR-sequences which are located between the bacterial and mammalian expression cassette, stem from IFN β gene and β -globin gene because their sequences are devoid of CpG-dinucleotides⁵⁹.

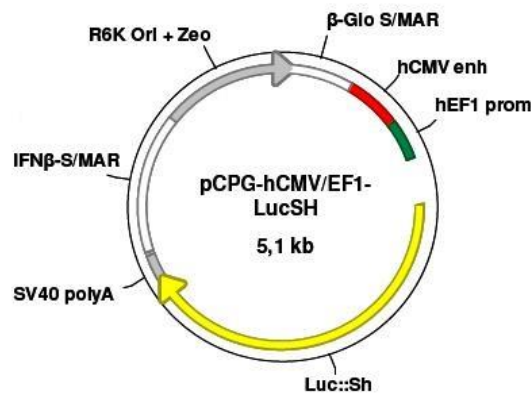


Figure 6: Plasmid map of CpG-free pCpG-hCMV-EF1 α -LucSH; figure from reference ⁵⁹.

The second reporter plasmid used in this work was pCMV-GLuc⁶⁰ (Figure 7). The transgene, a cDNA encoding Gaussia luciferase, was introduced into the CpG-reduced backbone. It is under control of a CMV promoter. It also contains the signal peptide for secretion into the cell culture medium. At MMCT, pCMV-GLuc was propagated in *E. coli* under Ampicillin pressure. Further, it contains a neomycin resistance gene under the control of an SV40 promoter.

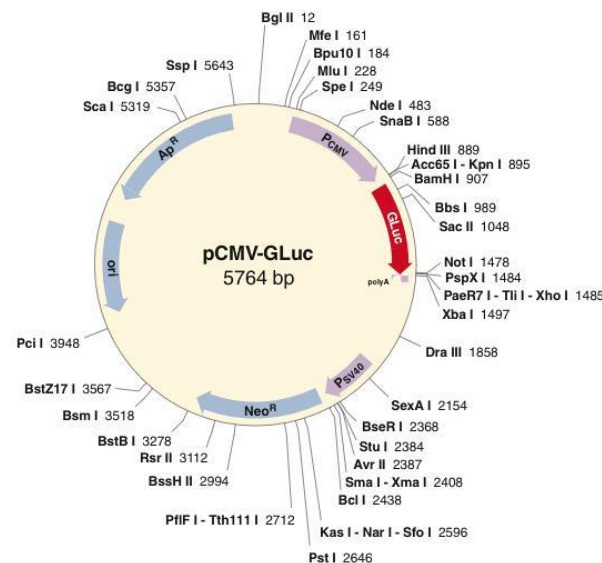


Figure 7: Plasmid map of pCMV-GLuc; figure from reference ⁶⁰.

Lucia luciferase is encoded by a synthetic gene derived from luciferase genes from marine copepods. It is a novel secreted luciferase, only slightly bigger than Gaussia luciferase (23 kDa) and has a 1000 fold stronger bioluminescence activity compared to Firefly or Renilla luciferase. It utilizes coelenterazine as substrate and emits a luminescence of visible blue⁶¹. Purpose of its design aimed

a high and sustained luciferase expression in mammalian cells compared to other luciferases. This is achieved by a CpG-free and codon-optimized cDNA. The plasmid has elements for replication in E.coli and elements for gene expression in mammalian cells, i.e. it has a prokaryotic and mammalian expression cassette (Figure 8). Between both expression cassettes, Matrix attached regions (MAR) are located. The origin of replication (ori), the bacterial promoter (EM2K) and the Zeocin resistance gene belong to the bacterial transcription unit. Multiple cloning site (MCS), EF-1 α promoter, Lucia luciferase gene and polyadenylation signal are components of the eukaryotic expression unit. Within the MCS a CpG-rich enhancer element can be inserted in order to study the effect of methylation of CpG-dinucleotids on Lucia expression⁶².

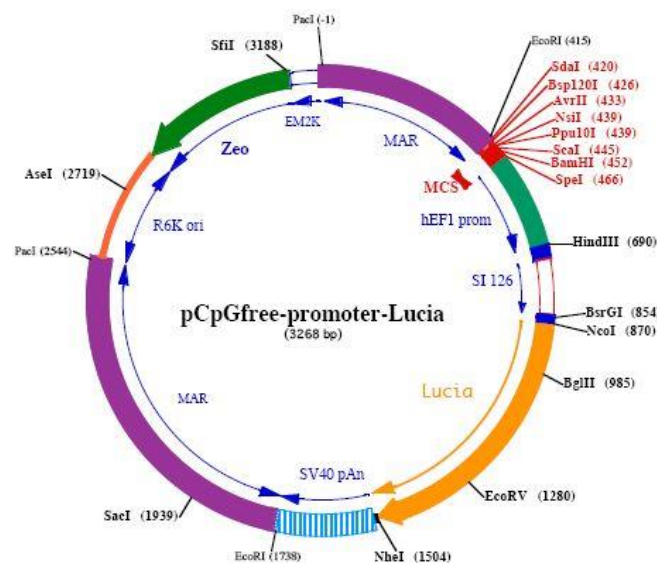


Figure 8: Plasmid map of pCpGfree-promotor-Lucia; figure from Invivogen⁶².

4.3.2 Ribonucleic acid

4.3.2.1 siRNA as nucleic acid compound of nanoparticles

Apart from transfecting host cells with DNA, which leads to expression of a functionally active protein, gene therapy also means influencing the host's genome at the transcriptional level. An antisense oligonucleotide, e.g. double-stranded small interfering RNA (ds siRNA), composed of a typical length of 21-23 base pairs, hybridizes with a complementary region of the host's mRNA and subsequently blocks its translation into a protein by degrading mRNA⁶³. Downregulation of overexpressed endogenous oncogenes or other disease-

causing proteins is a promising tool for further clinical therapies, but research focusing on optimization of delivery vehicles is still needed⁴².

As shown in Figure 9, naturally observed RNA interference is triggered by an external long double stranded RNA, which is hydrolyzed by the endoribonuclease dicer into short RNA duplexes. siRNA is recognized and loaded onto Argonaute protein (AGO2), located inside the RNA-induced silencing complex (RISC). AGO2, binds to the guide (antisense) siRNA strand and cleaves and ejects the passenger (sense) strand. The complementary target mRNA sequence is then bound to antisense strand by Watson-Crick base pairing, sliced by AGO2 and released. RISC is recycled and a new round of mRNA cleavage is set up⁶⁴. RNA interference can also be triggered by introducing chemically synthesized siRNA into the mammalian cells, avoiding Dicer-catalyzed reaction⁶⁵.

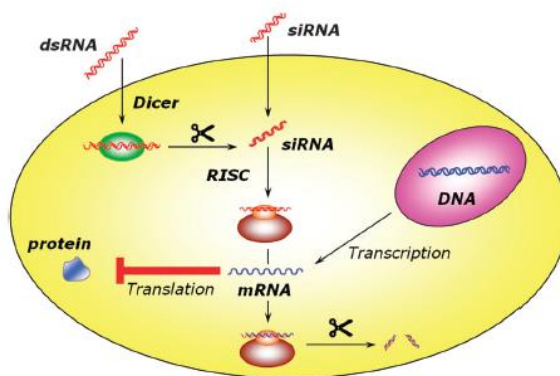


Figure 9: siRNA can be generated by a Dicer-catalysed reaction or it is directly introduced into the mammalian cell; figure from reference ⁶⁵.

Various formulation types for siRNA therapeutics exist⁶⁵. The most studied are non-viral lipid-based delivery vehicles. Similar to pDNA/LPEI polyplexes, also siRNA-liposomes encounter many physiological obstacles when systemically applied. In contrast to polyplexes, lipoplexes fuse with the host's cellular membrane and their action site is the RNAi-machinery which is located in the cytoplasm and not in the nucleus⁴⁴.

4.4 Bioluminescence

Bioluminescence is the occurrence of light produced by a chemical reaction (chemiluminescence) and emitted from a living organism⁶⁶. It has some advantages over other spectroscopic detection techniques, e.g. fluorescence or

absorption. First of all, since no excitation light is required for a bioluminescence signal no unspecific background signal from cellular environment occurs (as autofluorescence in fluorescent measurements). Thus, signal-to-noise ratio is high, leading to a higher detectability of photons. Secondly, the quantum yield of a bioluminescence reaction is much higher than that of a chemiluminescence reaction. Both parameters make bioluminescence measurements highly sensitive due to covering a wide dynamic range of quantitation because high (emitted by nanomoles of luciferase) to weak (emitted by attomoles of luciferases) light intensities can be detected. Hence, bioluminescence imaging is appealing for applications requiring high detection sensitivity, i.e. it is perfect for detecting low concentrations of target analyte or having restricted size volumes⁶⁷.

4.4.1 Reporter proteins

As illustrated in Figure 10, gene expression is regulated by external stimuli, which bind on intracellular receptors or receptors on cell surface, trigger a signal transduction pathway, which phosphorylates transcription factors and, therefore, leads to activation of response elements in the promotor region of a gene⁶⁸.

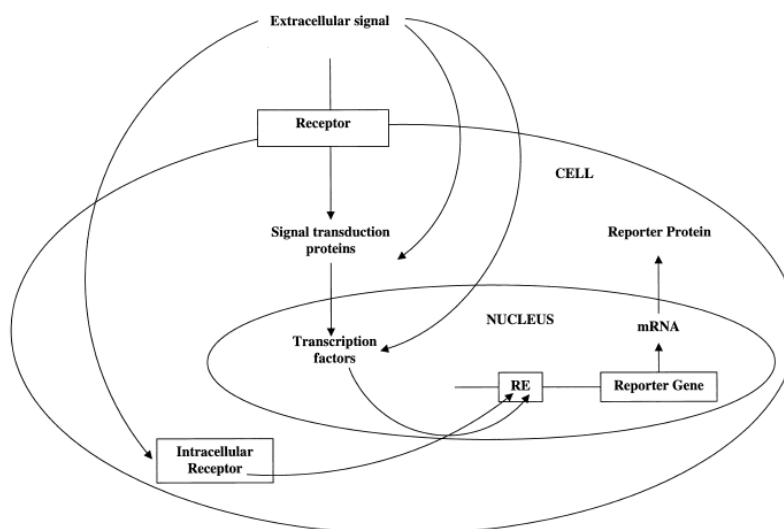


Figure 10: Regulation of gene expression. RE (response element); figure from reference ⁶⁸.

In order to evaluate relationships between signal transduction pathways and their effect on gene expression, promoters or other responsive elements have been fused to reporter genes, e.g. Chloramphenicol-Acetyl-Transferase (CAT, bacterial) , β -Galactosidase (bacterial), Green Fluorescent Protein (GFP, jellyfish), Secreted alkaline phosphatase (SEAP, human) or luciferases (many

organisms)⁶⁸. The promotor region is the binding site for RNA polymerase and transcription factors and therefore regulates transcription of reporter gene in an activating or suppressing way. The amount of expressed reporter proteins directly correlates with transcriptional activity and subsequently indicates the promotor activity (Figure 11). Reporter proteins are easily measurable by different spectroscopic techniques. *In vitro* imaging of reporter proteins uses absorption, fluorescence and bioluminescence as detection methods. For imaging reporter proteins *in vivo*, additional modalities exist, such as Positron emission tomography (PET), Single-photon emission computed tomography (SPECT), Magnetic resonance imaging (MRI), X-ray computed tomography (X-ray CT) and Ultrasound^{69,70}.

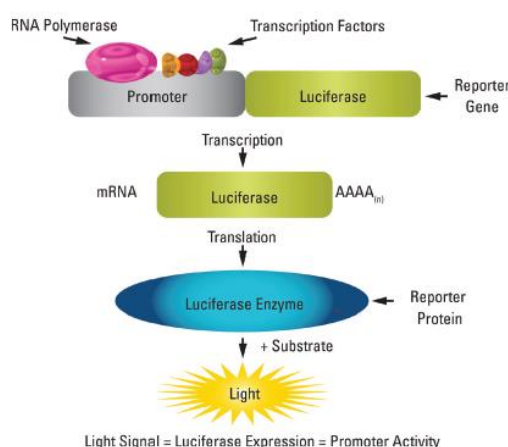


Figure 11: Intensity of bioluminescence signal from luciferase (reporter) enzyme directly correlates to activity of a promotor fused to the reporter gene; figure from reference ⁷¹.

4.4.1.1 Bioluminescence luciferase enzymes

The term luciferase is generic because phylogenic analyses have shown that luciferase enzymes stem from more than thirty evolutionary unrelated organisms⁷². They do not show sequence homology. Preferably, they occur in lower organisms, such as bacteria, fungi, insects (fireflies, click beetles), algae or marine crustaceans⁹⁰. For instance, fireflies possess ventral light organs on posterior segments, which inhabit luciferase enzymes, and trachea supplies oxygen. However, the majority of these bioluminescence organisms live in a marine environment (e.g. dinoflagellates inhabit in sea are perceived by the ocean glow)⁷³. Emission wavelength depends on the habitat of the bioluminescence organism: deep sea to shallow water shows a shift from blue to green and light produced from organisms on land covers a range into red

spectrum⁷⁴. There are three main purposes of bioluminescence naturally emitted by organisms: luring, camouflage or communication during courtship. For instance, some mollusks live in symbiosis with bioluminescence bacteria. Octopuses profit from this during their hunt at night when their ventrally-emitted light matches ambient moonlight so that they are almost invisible for their preys. Another example for bioluminescence in nature is fleeing octopuses, which eject a luminescence ink when vanishing from predators. Apart from symbiosis, there are also some eukaryotic organisms such as fireflies which have their own luciferin-luciferase-emitting system⁷⁵.

Luciferases represent a wide range of enzymes which all catalyze the conversion of a substrate – termed luciferin – into a non-reactive product by oxygenation; concurrently, photons are emitted and light in visible range of the electromagnetic spectrum is recognized^{75,76,77}. Additionally, some luciferases also need co-factors, e.g. ATP or bivalent metal cations (Mg^{2+}) for light production^{78,79}. Luciferase enzymes are either actively secreted from a cell or they remain in cytoplasm after they have run through the transcriptional-translational machinery. Figure 12 characterizes three luciferases which are used for *in vitro* reporter gene assays in this diploma thesis. Some luciferases emit light at a constant level for a longer time period, also known as glow kinetics⁹⁰. A second type of kinetics is characterized by a peak of high intensity immediately after substrate addition, which rapidly decays shortly thereafter. It is known as flash kinetics⁸⁰ (Figure 13).

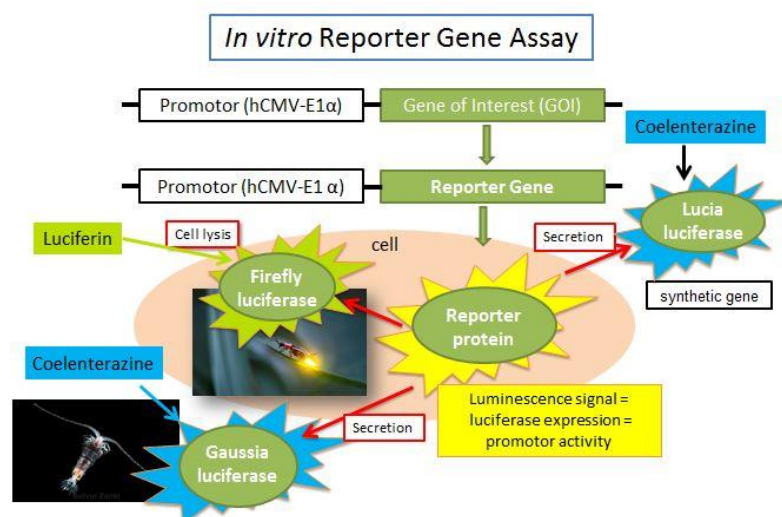


Figure 12: Three different luciferases used in bioluminescence reporter gene assays. Firefly is intracellularly localized and therefore cells need to be lysed. After addition of luciferin Firefly luciferase emits a yellow-greenish light. Both Gaussia and Lucia luciferase are secreted and use the same substrate, coelenterazine. Emitted light has a blue colour. In general, light intensity directly correlates with respective expression strength of bioluminescence proteins.

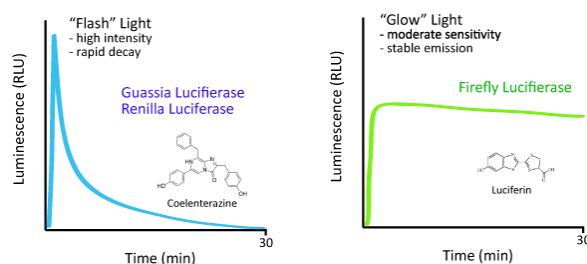


Figure 13: Luciferases differ in kinetics. Firefly luciferase shows a glow kinetics, whereas Guassia luciferase shows a flash kinetics; figure from reference ⁸¹.

Table 1: Features of luciferases. Table adapted from Roda et al. ⁶⁷.

* half-live (Invivogen ⁶¹)

Luciferase protein	Natural occurrence	Size	Secreted	Substrate; Co-factors	Emission peak ($\lambda_{max.}$); visible light colour	Kinetics (half time*)
Firefly luciferase	North American Firefly Photinus pyralis	61 kDa (monomer)	No	D-Luciferin; ATP, Mg^{2+} , O_2	557 nm (yellow-green)	Glow
Gaussia luciferase	Marine copepod Gaussia princeps	20 kDa (monomer)	Yes	Coelenterazine; O_2	480 nm (blue)	Flash
Renilla luciferase	Sea pansy Renilla reniformis	36 kDa (monomer)	No	Coelenterazine; O_2	480 nm (blue)	Flash (30 sec)
Lucia luciferase	Synthetic via recombinant DNA-technology	23 kDa	Yes	Coelenterazine; O_2	480 nm (blue)	Extended flash (5 min)
Firefly luciferase	Luciola italica	61 kDa (monomer)		D-Luciferin; ATP, Mg^{2+} , O_2	566 nm	
Bacterial luciferase	Vibrio, Photobacterium	80 (dimer)		Aliphatic aldehyde; FMNH ₂ , O_2	490 nm	
Photoprotein (Reporter Gene)	Natural occurrence	Size	Secreted	Substrate; other Co-factors	Emission peak ($\lambda_{max.}$)	Kinetics
Aequorin	Jellyfish Aequorea victoria	22 (monomer)		Coelenterazine; Ca^{2+}	469 nm	

Firefly luciferase:

Fireflies, such as Photinus (North American Photinus pyralis) or Luciola, produce Firefly luciferase, which uses D-luciferin as substrate. Firefly luciferase is a monomeric protein with a molecular weight of 61 kDa. It converts D-Luciferin (D-LH₂) into an enzyme-bound luciferyl adenylate (D-LH₂-AMP) by hydrolyzing ATP and releasing pyrophosphate (Figure 14). This adenylation step leads to acidification of C4 so that a proton is released and a carbanion is subsequently produced. A nucleophilic attack of molecular oxygen follows and AMP leaves,

resulting in an unstable four-membered dioxetanone. It breaks up and CO_2 is released. The endproduct is an electronically excited oxyluciferin and by relaxation a photon turns back to the ground level and energy difference is emitted in multiple colours ranging from yellow green to red visible light with an emission peak at 530-640 nm^{82,83,84,85}. Various hypotheses exist according to nature of different light colours⁸⁶. In a side reaction, D-LH₂-AMP is oxidized to dehydroluciferyl adenylate (L-AMP), which inhibits the bioluminescence reaction. L-AMP and oxyluciferin are responsible for the flash-pattern like kinetics of Firefly luciferase⁸². L-Coenzyme A (CoASH) reacts with L-AMP by thiolysis and dehydroluciferyl-CoA (L-CoA) is formed, which is a less inhibitor of luciferase so that it can start a new catalyzation round of luciferin. This is recognized as a boosted signal⁸⁷. It explains its modifying effect on the kinetics profile. Firefly shows a prolonged light emission (glow kinetics) instead of a light peak followed by rapid decay (flash kinetics)^{87,88}.

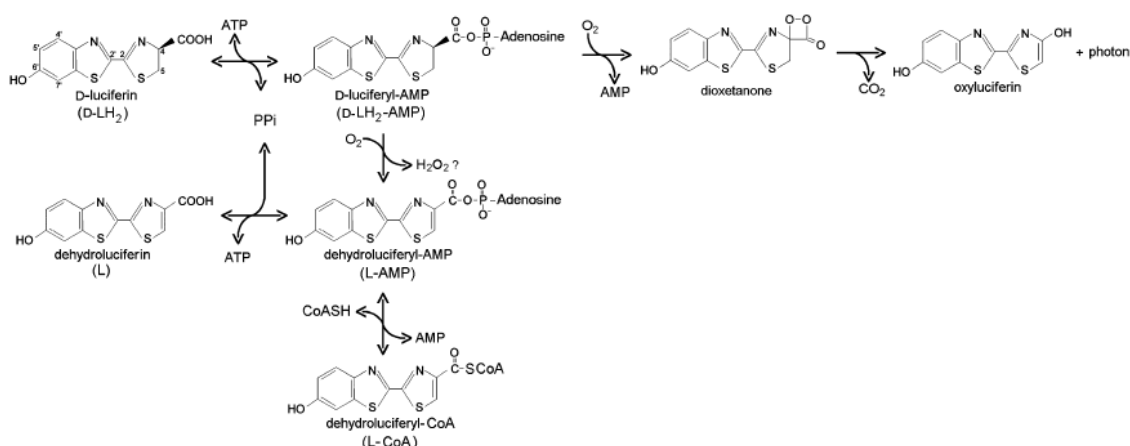


Figure 14: Firefly luciferase catalyzes conversion of D-LH₂ into oxyluciferin. In a side reaction D-LH₂-AMP is oxidized to L-AMP which inhibits the light-emitting reaction. Coenzyme A blocks this inhibitory effect by thiolysis of L-AMP, resulting in a prolonged light emission profile; figure from reference ⁸⁷.

Dyer et al.⁸⁹ prepared a noncommercial assay buffer including Coenzyme A. Its purpose is to diminish the inhibitory effect of dehydroluciferyl adenylate (L-AMP), resulting in a boosted luminescence signal. This is advantageous for high-throughput screenings (HTS)⁸⁹.

Gaussia luciferase:

The marine copepod *Gaussia princeps*, found in temperate and tropical waters worldwide, secretes the smallest luciferase (molecular weight of 19.9 kDa),

Gaussia luciferase. It uses coelenterazine as substrate (Figure 15) and emits a high quantum yield, 200-fold stronger than those of Firefly and Renilla Luciferase. Its activity is stable at varying temperatures and acidic conditions^{90,91}. Because of its marine occurrence conversion of substrate also requires sodium chloride. This must be taken into account when performing *in vitro* Gaussia luciferase assays. Coelenterazine is then mixed with a buffer supplemented with sodium chloride in order to imitate natural conditions⁹³.

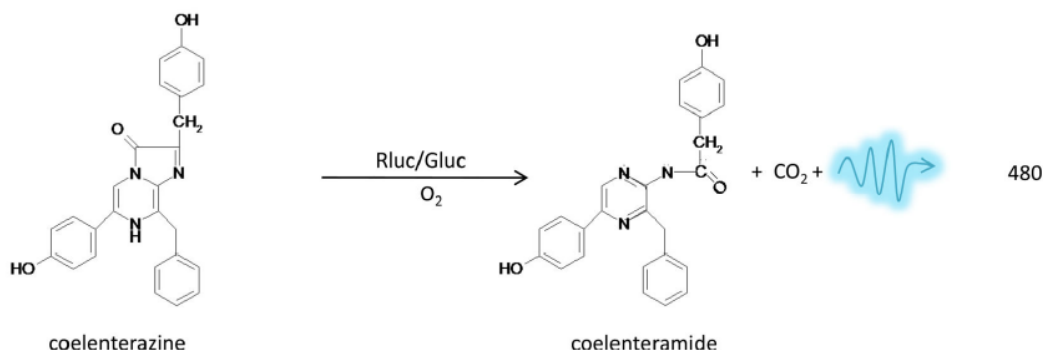


Figure 15: Gaussia (GLuc) and Renilla (RLuc) luciferase catalyze oxidation of coelenterazine to coelenteramide, whereby carbon dioxide is a by-product and blue colour with a typical emission peak at 480 nm is emitted; figure from reference ⁹².

Gaussia luciferase shows a broad emission spectrum including a red light component (wavelength of 480-600 nm), active secretion because of a signal peptide, high quantum yield and no need for ATP⁹⁰. Its strong light intensity contributes to high sensitivity of its respective *in vitro* assay. Tannous et al.⁹⁰ showed that humanized form of Gaussia luciferase *in vitro* yields a higher light output than humanized forms of Firefly or Renilla luciferases, regardless of extra- or intracellular activity. Active secretion allows *ex vivo* real-time monitoring of *in vivo* biological processes. Secretion and diffusion into distant tissues *in vivo* impairs the possibility to directly correlate signal intensity with number of transgenic cells; conversely, secretion enables assessing diffusion parameters in tumor tissue⁹⁰. Gaussia luciferase level in blood and urine is a marker for tumor growth and important for monitoring viral infections and cancer therapies *in vivo*⁹³.

Due to coelenterazine's low solubility in aqueous solvents, it is solubilized in acidified methanol⁹³. Coelenterazine harbors the disadvantageous feature of auto-oxidation into its inactive coelenteramide product⁹⁴. In order to stabilize coelenterazine, it is diluted in a buffer (PBS/5 mM NaCl, pH 7.2) to a working

concentration of 20 μ M and stored for 30 min in the dark⁹³. Within this time period the auto-oxidation reaction equilibrates. In contrast to D-Luciferin, CTZ is costly and is rapidly cleared from bloodstream^{90,95}. Gaussia luciferase possesses a flash kinetics which makes a luminometer with an automated coelenterazine injection by a built-in injector indispensable.

Gaussia's light signal (480 nm) is absorbed by pigmented molecules (hemoglobin) in organisms. Luminescence shifted to longer wavelengths near infrared is more advantageous for in vivo imaging since it remains detectable when emitted from deep tissues^{96,90}.

Lucia luciferase

Lucia luciferase utilizes coelenterazine as substrate and emits a visible blue light (wavelength 465-493 nm)^{61,90}. Its flash signal is of higher intensity than that of Firefly or Renilla luciferase. This enables quantification of small amounts in real-time measurements⁶¹.

5 Goals of diploma thesis

The goal of the present work was development and optimization of luciferase based reporter gene assays for application in nanoparticle mediated nucleic acid delivery.

Successful introduction of nucleic acids into cancer cells via transfection was visualized by measuring activity of reporter proteins via a luciferase reporter gene assay. Three bioluminescence reporter proteins, Firefly, Gaussia and Lucia luciferases, were investigated.

Firefly luciferase emits yellow-green light when converting luciferin into oxyluciferin. The buffer for luciferin is either commercially available or can be housemade as per a protocol from University of Birmingham⁹⁷ Since this housemade substrate is cheaper than the commercial from Promega, the goal was, first, to test whether housemade buffer is equally good as the commercial buffer and, second, whether housemade buffer can be frozen at -80°C in aliquots for long-term storage – without loss in activity. Thus, this would be both money-saving and time-saving. Activity of both substrates was compared measuring light signals emitted from a serial dilution of recombinant Firefly luciferase as well as from Firefly luciferase expressed from lung cancer cells (A549). Within Firefly assay, a sub-goal was to optimize the BCA assay (for protein normalization) in terms of cell lysis and the need for an alkylating reagent such as iodoacetamide.

The secreted luciferases, Gaussia and Lucia, utilize coelenterazine as substrate. Its buffer (housemade Coelenterazine Assay Buffer) is frozen and tested in terms of activity after thawing, compared to the commercial buffer. Experiments are performed with recombinant Gaussia luciferase as well as with cells (A549) which have been transfected with plasmids encoding pCMV-GLuc and pCpGfree-promotor-Lucia. Kinetics of both luciferases are also investigated. For both secreted luciferase enzymes, two normalization methods are possible (protein normalization by BCA assay or live cell number normalization by flow cytometry) and the goal was to investigate whether both methods are comparable.

Breast cancer cells, stably transduced with Firefly luciferase, were treated with liposomes for siRNA delivery. Knockdown of luciferase expression was compared to that induced by the commercially available transfection agent Lipofectamine[®]

RNAiMAX (Invitrogen). Also, delivery of plasmid via liposomes was examined *in vitro*.

A dual luciferase assay was also performed with both optimized Firefly and Gaussia luciferase assays in order to confirm the assay's robustness. Therefore, a subgoal was co-transfection of MDA-MB-231 (PGK-EGFP-Luc) with pCMV-GLuc and Luc-siRNA and measuring a dual light response with both optimized protocols for luciferase reporter assays. In another application, the transfection efficiency of polyplexes stabilized with a cryoprotectant, which prevents aggregation of polyplexes, was also investigated by a Gaussia luciferase assay.

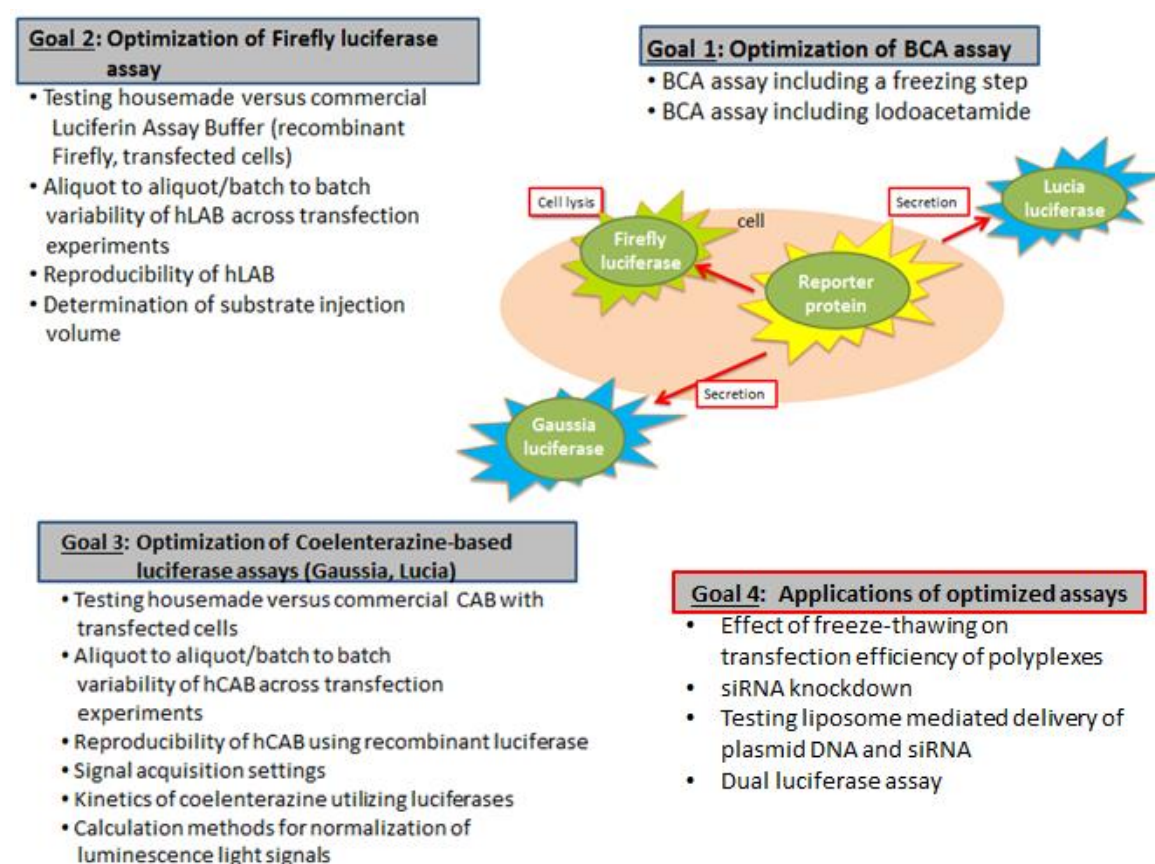


Figure 16: Goals 1-4 of the diploma thesis.

6 Materials and Methods

6.1 Equipment

The following equipments were used for all methods described.

- Cell culture microplate, 96 well, PS, F-bottom, TC, white (Cat. No. 655098) and transparent (Cat. No. 655180) (Cellstar[®], Greiner Bio-One)
- Centrifuge Tube 15 ml (CT-15) (Cat. No. E1415-0200, Starlab)
- Centrifuge Tube 50 ml (CT-50) (Cat. No. E1450-0200, Starlab)
- Cryogenic store vials (Cat. No. 374502, Thermo Scientific)
- Freezer -80°C (REVCO ExF, Thermo Scientific)
- Freezer -150°C (REVCO, Thermo Scientific)
- Hemocytometer (Paul Marienfeld GmbH & Co. KG, Germany)
- Heracell[™] 150i CO₂ Incubator (Thermo Fisher Scientific)
- Heraeus Megafuge 16R Centrifuge (Thermo Fisher Scientific)
- Herasafe[™] KS (NSF) Class II, Type A2 Biological Safety Cabinet (Thermo Fisher Scientific)
- Inverted microscope (AE31 Elite Trinocular, Motic)
- Micropipettes (Eppendorf Research Plus)
- Mr. Frosty Nalgene[®] (Cat. No. 5100-0001, Thermo Scientific)
- MacsQuant[®] Analyzer 10 (Cat.No.130-096-343, Miltenyi Biotec)
- Magnetic stirrer (Cat. No. MR 3001K, Heidolph Instruments)
- Multi-Channel Ultra High-Performance Pipettor, 12-Channel Pipettor, 50 to 300 µl (Cat. No. 89134-758, VWR)
- F1-ClipTip[™] Multichannel Pipette, 12-channel, 10 to 100 µl (Cat. No. 4661170, Thermo Scientific)
- NanoVue[®] Plus Spectrophotometer (GE Healthcare Life Sciences)
- Plate reader (Infinite[®] M200 Pro, Tecan)
- Plate shaker (Eppendorf ThermoMixer[®]C, 05-412-503, Eppendorf)
- pH meter (WTW)
- Syringe filters, cellulose acetate, 0.22 µm (Cat. No. 28145-477, VWR)
- Serological pipettes 5 ml, 10 ml, 25 ml (Sarstedt)
- Water bath (Mettler GmbH + Co. KG)

6.2 Bicinchoninic acid assay (BCA assay)

Background:

Smith et al.⁹⁸ have invented the BCA assay in 1985. BCA is the abbreviation for Bicinchoninic Acid, a copper-chelating agent.

Basically, proteins can be quantified with spectrophotometric methods. Within these, methods based on measuring the absorption of ultraviolet light or visible light are available. Latter methods are called colorimetric protein assay techniques, where a coloured reaction product is either formed by a chromophore, which is the end product of a reaction of a dye with a protein (e.g. Bradford assay), or the visible colour originates from a redox reaction with proteins^{99,100}. The BCA assay belongs to the redox-based reactions with proteins in an alkaline medium.

It is based on the Biuret reaction, where a cupric ion (Cu^{2+}) is reduced to a cuprous ion (Cu^+) by a peptide bound in an alkaline medium, which then forms a purple coloured complex with the peptide. In the BCA assay an additional reaction with bicinchoninic acid occurs: two molecules of bicinchoninic acid form a chelate complex with one cuprous ion. This purple coloured and water-soluble complex shows a characteristic absorption maximum at 562 nm^{98,101,102}.

The BCA assay depends on the structure of the protein, its number of peptide bounds and, most important, on proportion of amino acids. Cystein, Cystin, Tyrosin and Tryptophan are known as responsible amino acids for the reaction¹⁰³, in more detail, their residues play the major role. Thus, the assay is affected by protein to protein variation of samples. Advantages over other protein-determining assays are the ease of handling, its high sensitivity¹⁰⁰ and that it is compatible with up to 5 % detergents¹⁰⁴. For protein determination in the present diploma thesis the BCA assay was used because it is compatible with the Passive Lysis Buffer¹⁰⁵.

Materials:

- PierceTM BCA Protein Assay Kit (Cat. No. 23225, Pierce Biotechnology, Rockford, IL, USA)
- 5X Passive Lysis Buffer (Cat. No. E194A, Promega)
- 5X Cell Culture Lysis Reagent (Cat. No. E153A, Promega)

- MQ-water (Arium[®]pro VF, Sartorius), autoclaved
- Dulbecco's Phosphate Buffered Saline (Cat. No. D8537, Sigma-Aldrich)

Procedure:

5X PLB was thawed for 20 min at RT and diluted with MQ-water to 1X and 2X concentrated buffers. Both were equilibrated at RT for 20 min before usage. In case of 5X CCLR, same dilutions were prepared. According to the manufacturer's protocol¹⁰³, the BCA Working Reagent was made by mixing 50 parts BCA Reagent A with 1 part BCA Reagent B. As soon as both reagents were mixed thoroughly, the solution turned clear and apple green. It is stable for two days at RT.

Samples for the BSA standard curve were mixed in the following order (volumes see Table 2): first MQ-water, then BSA (2 mg/ml) and finally 2X PLB was added. PLB was added carefully, because it easily foams. All tubes were flicked and spun down for homogenous compositions. Because each sample was measured in triplicates a quadruple volume of each was prepared (1x extra volume was considered for pipetting loss).

Table 2: Composition of 1x BSA standard dilutions (25 µl standard/well) giving one standard curve.

Sample ID	MQ-water [µl]	BSA 2mg/ml [µl]	BSA (2mg/ml) [µg]	2X PLB [µl]
8A	-	12.5	25	12.5
7B	3.12	9.38	18.75	12.5
6C	6.25	6.25	12.5	12.5
5D	9.38	3.125	6.25	12.5
4E	10.94	1.56	3.125	12.5
3F	12.19	0.313	0.625	12.5
2G	12.5	---	---	12.5

The transparent 96-well plate with cultured cells was taken out of incubator and cells were checked in inverted microscope. Medium was completely aspirated from each well. Cells were washed with 200 µl prewarmed PBS. For cell lysis 30 µl of 1X PLB was added per well. The 96-well plate was covered and shaken for 30 min at RT on a plate shaker (500 rpm). Meanwhile, 25 µl of each BSA standard dilution was pipetted in respective wells of a second transparent 96-well plate (according to reading out plan). Afterwards, cells were checked in microscope for lysis. Each lysate was then mixed with a micropipette and 20 µl of each well was transferred into the second 96-well plate. Additionally, 5 µl MQ-water was added to each sample in order to get 25 µl total volume per well. For

blank, 20 µl of 1X PLB and 5 µl MQ-water was mixed (in triplicates). 200 µl Working Reagent was added to all wells by reverse pipetting. Because this crucial step needs to be done in a rapid way a multichannel was used. The covered well plate was again briefly shaken for 30 sec (RT, 300 rpm). Then it was incubated for 30 min at 37°C. After cooling down to RT for 9-10 min the absorption was measured at 562 nm (see Appendix, 9.1.1.)

Evaluation:

The absorption of BSA blank (Sample ID: 2G) was subtracted from each absorption value of respective BSA standard samples. The net absorption values of BSA standard samples were then plotted against protein amount [µg]. As shown in the dot diagram (Figure 17) the absorption values were plotted on the y axis, whereas the protein amounts were plotted on the x axis. The (regression) trend line follows an equation and is characterized by the coefficient of determination (R^2). Protein amounts of the unknown samples were calculated by inserting their net absorption values into this equation.

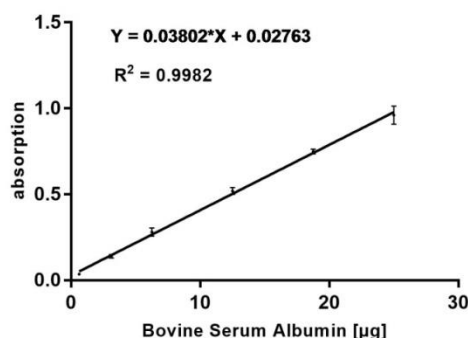


Figure 17: BSA standard curve for a BCA assay. Absorption directly correlates with bovine serum albumin [µg], following an equation.

6.2.1 BCA assay including a freezing step

Background:

Repeated cycles of freezing and thawing disrupt cells through ice crystal formation^{106,119}.

Procedure:

All steps were carried out as described in 6.2 including cell lysis. The 96-well plate was wrapped up in aluminum foil and put in -80°C freezer for few min.

Frozen samples were then thawed at RT with aid of hand warmth. Melted lysates were checked for shrunken aggregates which indicated a complete cell lysis.

6.2.2 BCA assay including iodoacetamide

Background:

2-Iodoacetamide is an alkylating reagent which either alkylates the thiol functional group of the amino acid cysteine¹⁰⁷ or that of dithiothreitol (DTT), a reducing agent, contained CCLR¹²³. Alkylation of cysteine residues in proteins is not desired because it belongs to one of the determining components in the BCA assay, reducing Cu^{2+} to Cu^{+} , which subsequently forms a purple complex with two BCA molecules¹⁰⁸. DTT also reduces Cu^{2+} to Cu^{+} because of its thiol group¹⁰⁰. That is the reason why DTT causes a false-positive signal, a high background respectively. This interference reduces the sensitivity of the BCA assay. Thus, excess of iodoacetamide is added in order to block thiol groups of DTT so that a reduction of Cu^{2+} is prevented^{100,109}.

Procedure:

0.1 M iodoacetamide (Cat. No. I1149, Sigma-Aldrich) was prepared by dissolving 1 mg in 54.05 μl MQ-water in a 2 ml Eppendorf tube. It was wrapped up in aluminum foil for light protection and stored at $+4^{\circ}\text{C}$.

All steps as described in 6.2 were carried out including transfer of 25 μl lysate to the second 96-well plate. Light was switched off. 5 μl of 0.1 M iodoacetamide was added to each well and the 96-well plate was incubated for 30 min at 37°C . After this, the BCA was finished as described in 6.2.

6.3 Preparation of buffers

6.3.1 Preparation of housemade Luciferin Assay Buffer for Firefly luciferase

Background:

The substrate luciferin is dissolved in a buffer, which provides essential factors required for the enzymatic conversion into oxyluciferin, such as ATP, Coenzyme A and others. For this housemade produced substrate (consisting of the substrate dissolved in its buffer) different terms exist (Table 3, column 1): Luciferase Assay Reagent (LAR) or housemade Luciferin Assay Buffer (hLAB). hLAB consists of the substrate D-luciferin (L) in a housemade buffer (B). For experiments in this diploma thesis either housemade buffer (B1) alone was frozen or a mixture of luciferin (L2) plus housemade buffer (B2) was frozen (resulting in LB2) (Figure 18).

For comparison regarding activity of Firefly luciferase, B1 and LB2 were thawed. Luciferin was added to B1, resulting in LB1. Both mixtures (LB1 and LB2) were tested as substrates for recombinant Firefly luciferase (Promega) or Firefly luciferase expressed by cells.

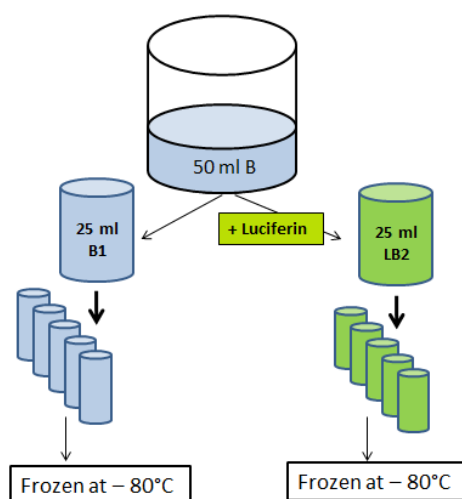


Figure 18: Aliquotion of 50 ml fresh housemade Luciferin Assay Buffer (B) into aliquots of only Buffer (B1) and a mixture of Luciferin Buffer (LB2). Aliquots were frozen at -80°C.

Table 3: Designations of housemade and commercial substrates for Firefly and Gaussia/Lucia luciferases and composition of each.

<u>Substrate (Luciferin + Buffer) for Firefly luciferase:</u>	<u>Substrate (Coelenterazine + Buffer) for Gaussia/Lucia luciferase:</u>
Commercially available (Promega): • LAR II (as named by Promega) • commercial Luciferin Assay Buffer (cLAB) (internal name; term Luciferin indicates that it is the substrate for Firefly luciferase) Housemade produced: • Luciferase Assay Reagent (LAR) (according to protocol from University of Birmingham⁹⁷) • housemade Luciferin Assay Buffer (hLAB) (term Luciferin indicates that it is the substrate for Firefly luciferase)	Commercially available (Promega): • Stop & Glo® (as named by Promega) • commercial Coelenterazine Assay Buffer (cCAB) (term Coelenterazine indicates that it is the substrate for Gaussia/Lucia luciferase) Housemade produced: • housemade Coelenterazine Assay Buffer (hCAB) (term Coelenterazine indicates that it is the substrate for Gaussia/Lucia luciferase)
Compounds: 1) Substrate, which is converted by enzymatic Firefly activity into the product: D-Luciferin (L) → Oxyluciferin 2) Buffer (B): housemade Luciferin Assay Buffer (B) (preparation see 6.3.1.6). <u>For experiments:</u> → <u>Either only Buffer (B1) is frozen:</u> After thawing of B1, L is added, giving the substrate LB1 = housemade Luciferin Assay Buffer (hLAB). → <u>Or mixture of Buffer (B2) and D-Luciferin (L) is frozen (LB2):</u> LB2 = housemade Luciferin Assay Buffer (hLAB) can be directly used as substrate.	Compounds: 1) Substrate, which is converted by enzymatic Gaussia/Lucia activity into the product: Coelenterazine stock (5 mg/ml) → Coelenteramide 2) Buffer (B): Housemade Coelenterazine assay Buffer (B) consists of PBS/5mM NaCl, pH 7.2 (preparation see 6.3.3) → hCAB is frozen, thawed and directly used as substrate.

Chemicals:

- Glycylglycine (Cat. No. G-3915, Sigma-Aldrich)
- Magnesium chloride 6xH₂O (Cat. No. A4425, AppliChem)
- Ethylenediaminetetraacetic acid (Cat. No. E-6758, Sigma-Aldrich)

- Coenzyme A (trilithium salt) (Cat. No. C3019, Sigma-Aldrich)
- L-Dithiothreitol (Cat. No. D9779, Sigma-Aldrich)
- ATP (Cat. No. 10519979001, Roche Diagnostics GmbH, Austria)
- Luciferin powder (Na-salt, Promega)
- Sodium hydroxide (Cat. No. 55881, Sigma-Aldrich)
- Hydrochloric acid (HCl) (Cat. No. 13778-150, Sigma-Aldrich)
- MQ-water (Arium[®]pro VF, Sartorius)

Procedure:

The following five stock solutions for a final volume of 500 ml hLAB were prepared according to the protocol of University of Birmingham⁹⁷.

6.3.1.1 Stock solution #1: 20 ml of 1 M Glycylglycine pH 8.0:

1 M Glycylglycine was always prepared freshly for each batch of hLAB.

2.64 g of Glycylglycine was dissolved in 18 ml MQ-water by mixing on a magnetic stirrer. The pH of the solution was adjusted to 8.0 with 10 M NaOH. MQ-water was added to reach a final volume of 20 ml. The solution was filtered through a 0.45 µm cellulose acetate filter and stored at +4°C.

6.3.1.2 Stock solution #2: 50 ml of 100 mM MgCl₂:

1.0165 g of MgCl₂ was dissolved in MQ-water. The solution was filtered through a 0.45 µm cellulose acetate filter and stored at RT.

6.3.1.3 Stock solution #3: 50 ml of 500 mM EDTA pH 8.0:

7.306 g of EDTA was mixed with 30 ml MQ-water in a Schott-Duran[®] bottle on a magnetic stirrer. Shortly after, 7 ml of 10 M NaOH was added in order to adjust the pH to 8.0. If pH was not adjusted quickly enough the EDTA precipitated, resulting in a solid mixture. The solution was stirred for approximately 1 h. Afterwards, the volume was filled up with MQ-water to 50 ml. The solution was filtered through a 0.45 µm cellulose acetate filter and stored at +4°C.

6.3.1.4 Stock solutions #4: 250 µl Coenzyme A-aliquots:

Usually, 250 µl Coenzyme A aliquots with a concentration of 42.6 mg/ml were prepared by dissolving 100 mg Coenzyme A in 2347 µl MQ-water. These aliquots

of 250 µl each were made in case only a small amount was required i.e. for 50 ml hLAB only 1 aliquot was needed. Here, 2.5 ml Coenzyme A (42.6 mg/ml) for 500 ml was required (see 6.3.1.6).

6.3.1.5 Stock solution #5: 48 ml of 10 mM luciferin:

The preparation was carried out under light protection. 144.3 mg of Luciferin powder was dissolved in 1.41 ml of a freshly prepared 10 M Glycylglycine and 25 ml MQ-water. The pH was adjusted to 8.0 and solution was filled up to 48 ml. The solution was immediately used for preparation of hLAB as described below.

6.3.1.6 Preparation of 500 ml housemade Luciferin Assay Buffer (B):

1. All stock solutions listed above, except 1 M Glycylglycine, were prepared in advance and stored at recommended storage temperatures. 1 M Glycylglycine was prepared freshly.
2. 500 mM EDTA was equilibrated to RT before usage.
3. 2x 2 ml Eppendorf tubes as well as MQ-water were precooled on ice. 10 x 250 µl Coenzyme A-aliquots and 25 ml Luciferin (10 mM; light protection!) were thawed on ice.
4. All stock solutions were mixed well before usage and checked for devoid of precipitates which could form due to inappropriate storage i.e. not suitable temperature.
5. To begin with, 10 ml freshly prepared Glycylglycine (+4°C) and 5 ml MgCl₂ was mixed in a Schott-Duran® bottle. 100 µl EDTA was added. 254 mg of DTT was weighed in a precooled 2 ml Eppendorf tube. 139 mg of ATP was weighed in a second 2 ml Eppendorf tube. Both solids were dissolved in 1 ml MQ-water each and transferred to the already mixed solutions in the Schott-Duran® bottle. Additionally, both Eppendorf tubes were washed with 4 ml MQ-water each and added. Then, 2.5 ml Coenzyme A was added. 472 ml MQ-water (+4°C) was poured to the mixture and the pH was adjusted to 8.2 with 1M NaOH. According to the protocol the pH can range from 8.0 to 8.5. In order to ensure reproducibility, I always adjusted the pH to 8.2. The final volume was reached by filling the solution up to 500 ml with MQ-water. The transparent solution was filtered through a 0.22 µm

cellulose acetate filter. For preparation of hLAB, steps as described below were carried out.

6.3.1.7 Preparation of housemade Luciferin Assay Buffer (hLAB)

250 µl Luciferin (10 mM) was mixed with 5 ml housemade Luciferin Assay Buffer (B), resulting in a greenish yellow solution (hLAB). For upscaling, 25 ml Luciferin (10 mM) was mixed with 500 ml housemade Luciferin Assay Buffer (B). CT-15's were wrapped up in aluminum foil and the housemade Luciferin Assay Buffer (hLAB) was aliquoted in 5 ml-aliquots and stored at -80°C. The buffer must always be prepared freshly and used only once. The final concentrations are enlisted in Table 4.

Table 4: Volumes of stock solutions for hLAB and final concentrations of the injection solution comprised of 5 ml hLAB + 250 µl luciferin.

Chemical	Stock solution	Volume of stock solution used for 500 ml hLAB	Final concentrations of injection solution hLAB (250 µl luciferin 10 mM + 5 ml hLAB (B))
Glycylglycine	1 M Glycylglycine pH 8.0, 20 ml	10 ml	20 mM
Magnesium chloride 6xH ₂ O	100 mM MgCl ₂ , 50 ml	5 ml	1 mM
Ethylenediaminetetraacetic acid	500 mM EDTA pH 8.0, 50 ml	100 µl	0.1 mM
Coenzyme A	250 µl aliquots (42.6 mg/ml)	2.5 ml	0.213 mg/ml
L-Dithiothreitol	---	254.0 mg	3.3 mM
ATP	---	139 mg	0.54 mM
MQ-water	---	up to 500 ml	---
Luciferin powder	10 mM luciferin, 48 ml	250 µl per 5 ml B	0.5 mM

6.3.2 Preparation of commercial Luciferin Assay Buffer for Firefly luciferase

As shown in Table 3, two different terms for the commercially available mixture of luciferin plus buffer exist: LAR II or commercial Luciferin Assay Buffer (cLAB).

Materials:

- Dual-Luciferase[®] Reporter Assay System (Cat.No. E 1960, Promega):
Luciferase Assay Substrate (Cat. No. E151A),
Luciferase Assay Buffer II (Cat. No. E195A)

Procedure:

According to manufacturer's instructions¹¹⁰, the lyophilized Luciferase Assay Substrate was dissolved in 10 ml Luciferase Assay Buffer II. Brief vortexing ensures uniform mixing. It is stored at -80°C and stable for 1 year at -70°C.

6.3.3 Preparation of housemade Coelenterazine Assay Buffer for Gaussia/Lucia luciferase

Materials:

- Coelenterazine (Cat. No. s053, Synchem UG & Co., Germany) stock solution (5 mg/ml; in acidified methanol)
- Dulbecco's Phosphate Buffered Saline (Cat. No. D8537, Sigma-Aldrich)
- Sodium chloride (Cat. No. A2942,5000, Applichem)
- Sodium hydroxide (Cat. No. A6829,1000, Applichem), NaOH dilution

Preparation of PBS/5 mM NaCl, pH 7.2:

29.22 mg of sodium chloride was dissolved in 90 ml PBS and final pH was adjusted to 7.2 with NaOH. PBS was added for a total volume of 100 ml. The solution was sterile filtered (0.22 µm cellulose acetate filter) and stored at RT.

Procedure:

The following steps were carried out in a laminar under light protection, according to the protocol of Tannous⁹³.

A CT-15 was wrapped up in aluminum foil. PBS/5 mM NaCl was filled in with a serological pipette and the appropriate volume of Coelenterazine stock solution (5mg/ml) was added, giving hCAB (20 µM). After mixing well it was incubated at RT for 30 min. Subsequently, it was used as substrate for Gaussia or Lucia luciferase in a luminescence measurement within 2 h. This is because Coelenterazine is prone to auto-oxidation. Consequently, 2 h after preparation

auto-oxidation of CTZ has gone so far that measured light output is significantly less than compared to a fresh CTZ substrate.

Note: As soon as a frozen stock of hCAB (20 μ M) was removed out of -80°C freezer, thawing as well as auto-oxidation started simultaneously at the same time.

6.3.4 Preparation of commercial Coelenterazine Assay Buffer for Gaussia/Lucia luciferase

As shown in Table 3, the commercially available substrate is commercial Coelenterazine Assay Buffer (cCAB) or Stop & Glo[®].

Materials:

- Dual-Luciferase[®] Reporter Assay System (Cat.No. E 1960, Promega):
Stop & Glo[®] Substrate (E640A), Stop & Glo[®] Buffer (E641A)

Procedure:

Stop & Glo[®] Substrate was mixed with Stop & Glo[®] Buffer and vortexed briefly. According to manufacturer's instructions¹¹⁰, Stop & Glo[®] can be stored at -20°C for 15 days without decrease in activity.

6.4 Luciferase based reporter gene assays with recombinant luciferase

6.4.1 Luciferase based reporter gene assay with recombinant Firefly luciferase

Materials:

- QuantiLum[®] Recombinant Luciferase (1mg, Cat. No. E1701, Promega)
- Bovine Serum Albumin (BSA) (Cat. No. A9647, Sigma-Aldrich)
- Cell Culture Lysis Reagent 5X (Cat. No. E153, Promega)
- MQ-water (Arium[®]pro VF, Sartorius)

Procedure:

A solution of BSA in MQ-water with a concentration of 1.25 mg/ml was made. Then, 4 volumes of BSA (1.25 mg/ml) and 1 volume of 5X CCLR were mixed, resulting in 1X CCLR with a BSA concentration of 1 mg/ml. BSA is used to stabilize minimal amounts of recombinant enzymes at extremely diluted concentrations¹¹⁰.

Recombinant Firefly luciferase was reconstituted in 1X CCLR in order to obtain stock B (1 µg/µl). It was stored frozen at -80°C. A 1:10 dilution was prepared, resulting in stock C (100 ng/µl) (Table 5). Dilution 1 was prepared from stock C. Upon this, a 1:10 dilution series was produced. Finally, 10 µl of each dilution was pipetted in wells (triplicates). Dilution 10 was used as a blank. For luminescence measurement steps as described in 6.5 were followed.

Table 5: Serial dilutions of recombinant Firefly luciferase (Promega).

Dilution ID	Volume from stock C (100 ng/µl)	1X CCLR (BSA 1mg/ml)	Final Volume	Concentration	Volume to be used for Luc assay	Amount of enzyme in 10 µl
1	5 µl	45 µl	50 µl	10 ng/µl	10 µl	100 ng
1:10 dilution series of the enzyme from 1						
	Volume taken	1X CCLR (BSA 1mg/ml)	Final Volume	Concentration	Volume to be used for Luc assay	Amount of enzyme in 10 µl
2	5 µl from 1	45 µl	50 µl	1 ng/µl	10 µl	10 ng
3	5 µl from 2	45 µl	50 µl	0.1 ng/µl	10 µl	1 ng
4	5 µl from 3	45 µl	50 µl	0.01 ng/µl	10 µl	0.1 ng
5	5 µl from 4	45 µl	50 µl	0.001 ng/µl = 1 pg/µl	10 µl	0.01 ng
6	5 µl from 5	45 µl	50 µl	0.1 pg/µl	10 µl	0.001 ng = 1 pg
7	5 µl from 6	45 µl	50 µl	0.01 pg/µl	10 µl	0.1 pg
8	5 µl from 7	45 µl	50 µl	0.001 pg/µl = 1 fg/µl	10 µl	0.01 pg
9	5 µl from 8	45 µl	50 µl	0.1 fg/µl	10 µl	0.001 ng = 1 fg
10	-	50 µl	50 µl	0	10 µl	0

6.4.2 Luciferase based reporter gene assay with recombinant Gaussia luciferase

Materials:

- Recombinant Gaussia luciferase (Cat. No. 231, NanoLight™ Technology): reconstituted in TBS (pH 7.8), 1 mg/ml (stock A), frozen at -80°C.
- MQ-water (Arium® pro VF, Sartorius)
- Trizma® base (Cat. No. T1503, Sigma-Aldrich)
- Hydrochloric acid (Cat. No. 30721, Sigma-Aldrich), HCl dilution
- Bovine Serum Albumin (BSA) (Cat. No. A9647, Sigma-Aldrich)
- Sodium chloride (NaCl) (Cat. No. A2942,5000, Applichem)
- Ethylenediaminetetraacetic acid (EDTA) (Cat. No. E-6758, Sigma-Aldrich)

Procedure:

For 100 ml Tris/Cl (1 M, pH 7.8), 12.15 g Trizma® base was dissolved in MQ-water and the pH was adjusted to 7.8 with 5 M HCl.

According to instructions of manufacturer (NanoLight™ Technology)¹¹¹, the dilution buffer for recombinant Gaussia luciferase consists of 25 mM Tris/Cl (pH 7.8), 600 mM NaCl, 1 mM EDTA and 0.05 % (w/w) BSA as stabilizer. For 500 ml dilution buffer 12.5 ml of Tris/Cl (1 M, pH 7.8), 17.53 g of NaCl, 0.149 g of EDTA and 0.25 g of BSA were mixed and filled with MQ-water up to 0.5 L. The solution was stored at +4°C.

A 1:200 dilution of recombinant Gaussia luciferase (1 mg/ml, stock A) was made in order to obtain stock B (5 ng/μl) (Table 6). Starting from this, a 1:10 dilution series of recombinant Gaussia luciferase in dilution buffer was prepared and 20 μl per well were pipetted in wells of a 96-well plate (in triplicates). 20 μl dilution buffer was used as a blank. For luminescence measurement steps as described in 6.5 were followed.

Table 6: Serial dilutions of recombinant Gaussia luciferase (Nanolight™).

Dilution ID	Volume from Stock A (1 µg/µl)	Volume of dilution buffer added	Final Volume	Concentration	Volume to be used for Luc assay	Amount of enzyme in 20 µl
B	5 µl	995 µl	1000 µl	5 ng/µl	20 µl	100 ng
1:10 dilution series of the enzyme from B						
	Volume taken	Dilution buffer [µl]	Final Volume	Concentration	Volume to be used for Luc assay	Amount of enzyme in 10 µl
C	10 µl from B	90 µl	100 µl	0.5 ng/µl	20 µl	10 ng
D	10 µl from C	90 µl	100 µl	0.05 ng/µl	20 µl	1 ng
E	10 µl from D	90 µl	100 µl	0.005 ng/µl = 5 pg/µl	20 µl	0.1 ng
F	10 µl from E	90 µl	100 µl	0.5 pg/µl	20 µl	0.01 ng
G	10 µl from F	90 µl	100 µl	0.05 pg/µl	20 µl	0.001 ng = 1 pg
H	10 µl from G	90 µl	100 µl	0.005 pg/µl = 1 fg/µl	20 µl	0.1 pg
I	10 µl from H	90 µl	100 µl	0.5 fg/µl	20 µl	0.01 pg

6.5 Luminescence measurement

For background information see 9.1.1. Also, considerations for assaying luminescence samples of a 96-well plate are noted.

Procedure:

The plate reader (Tecan) was switched on 30 min prior to actual measurement. After washing it 1x with Ethanol 70 % and 1x with Aqua purificata it was primed first twice with air and then twice with substrate. The 96-well plate was inserted into the plate reader without a cover. Respective wells which should be measured were marked on the template of i-control™–Microplate Reader Software (Tecan). The following adjustments were set up:

- Injection volumes used for Firefly and Gaussia/Lucia see 9.2 (Table 18)
- Speed = 200 µl/sec
- Refill speed = 100 µl/sec
- Wait time = 2 sec
- Integration time = 10 000 msec

Results were displayed as RLU. Remaining substrate, which was still in the system, was removed by doing a backflush. The system was washed 1x with Aqua purificata, 1x with Ethanol 70 % and then primed with Ethanol 70 % for

10 min in order to get rid of any traces of substrate. Finally, it was washed with Aqua purificata again.

6.6 Techniques for generation of nanoparticles

6.6.1 Generation of LPEI based polyplexes by flash pipetting technique

Materials:

- Linear polyethylenimine (10 kDa): received from Mag. Alexander Taschauer, synthesized based on instructions by Rödl et al.²
- pDNA: pCpG-hCMV-EF1 α -LucSH (received from Univ.Prof. Manfred Ogris, Magnusson et al.⁵⁶); pCMV-GLuc (New England Biolabs[®], Germany⁶⁰); pCpGfree-promotor-Lucia (Cat.No. pcpgf-promlc, Invivogen)
- HBG (20 mM HEPES/5 % (w/V) glucose, pH 7.4), preparation see 9.1.2)
- HBS (20 mM HEPES/5 % (w/V) sodium, pH 7.4)
- MQ-water (Arium[®]pro VF, Sartorius), autoclaved

Procedure:

For generation of polyplexes with a defined N/P ratio the following equation was used to calculate the required amount of LPEI needed for a specific amount of pDNA¹³. One well of a 96-well plate was always transfected with 200 ng pDNA.

$$\text{mass (LPEI)} = \frac{\text{mass (pDNA)}}{\text{mass (nucleotide)}} \times \text{mass (subunit LPEI)} \times \frac{N}{P} \text{ratio}$$

mass (nucleotide) = 330 g/mol

average mass (subunit LPEI) = 43 g/mol

For instance, transfecting one well with 200 ng pDNA requires 156.36 ng LPEI in order to generate polyplexes of an N/P 6.0:

$$\text{mass (LPEI)} = \frac{200}{330} \times 43 \times 6.0 = 156.36 \text{ ng}$$

Each transfection was carried out in triplicates. Regarding pipetting loss 1 extra volume was prepared (mass (pDNA) = 800 ng):

$$\text{mass (LPEI)} = \frac{800}{330} \times 43 \times 6.0 = 625.45 \text{ ng}$$

For *in vitro* transfections the pDNA concentration of a polyplex solution was always set to 20 µg/ml. 10 µl was added to 90 µl of basal medium per well (1:10 dilution), resulting in a concentration of 2 µg/ml with 200 ng pDNA per well.

The flash pipetting method for generation of polyplexes requires mixing of equal volumes of pDNA and LPEI. Therefore, a pDNA dilution (40 µg/ml) was prepared in an appropriate buffer (HBG or HBS). LPEI was diluted in the same buffer. Both dilutions were mixed via flash pipetting, resulting in a polyplex solution with a pDNA concentration of 20 µg/ml.

For transfecting 4 wells 40 µl of polyplex solution (20 µg/ml) are required, thus 20 µl of each pDNA and LPEI were prepared. Summing up, 40 µl of a polyplex solution (N/P 6.0) with a pDNA concentration of 20 µg/ml was prepared by mixing 20 µl pDNA dilution (40 µg/ml) with 20 µl LPEI (625.45 ng LPEI). Each well of a 96-well plate was transfected with 200 ng pDNA, 10 µl polyplex solution (40 µg/ml) respectively.

The flash pipetting technique was carried out in the following way: The micropipette was adjusted to 30 µl and 20 µl of LPEI solution was pipetted up, consequently the pipette tip was filled with air. Then, the air was carefully released, the tip was dipped into the pDNA solution and both solutions were mixed by rapidly pipetting 20 times up and down. Formation of air bubbles must be avoided.

6.6.2 Generation of jetPRIME[®] based polyplexes

Background:

jetPRIME[®] is a proprietary polymer-based molecule from Polyplus-Transfection[®]. It is used as a positive control substance for *in vitro* transfections. The exact components are not known. According to manufacturer's protocol¹¹², the ratio of pDNA (µg) to jetPRIME[®] (µl) is 1:2 when performing the transfection in a 96-well plate. Unlike other transfection reagents the transfection efficacy is increased in presence of serum, hence nanoparticles can be added to cells in complete medium¹¹³.

Materials:

- jetPRIME[®] transfection reagent, jetPRIME buffer (Polyplus Transfection[®])

- pDNA

Procedure:

Volumes were prepared for transfection in triplicates (200 ng pDNA/well, 96-well plate). No excess for pipetting loss was included.

1. A dilution of pDNA (600 ng) in jetPRIME[®] buffer was made (28.8 µl total volume).
2. jetPRIME[®] transfection reagent (1,2 µl) was added, the mixture vortexed for 10 sec and finally incubated 10 min at RT.
3. 10 µl of polyplex solution was added per well (see 6.8).

6.6.3 Generation of Lipofectamine[®] 2000 based lipoplexes

Background:

Lipofectamine[®] 2000 is a cationic lipid based formulation and a commercially available transfection agent from Invitrogen. Lipoplexes of pDNA and LF[®] 2000 are used as positive control for generating nanoparticles, similarly to jetPRIME[®] based polyplexes. According to the manufacturer's protocol¹¹⁴, a ratio of DNA [µg] to LF[®] 2000 [µl] of 1:3 was used.

Materials:

- Lipofectamine[®] 2000 (Cat. No. 11668-019, Invitrogen)
- Opti-MEM[®] Reduced Serum Medium (Gibco by Life Technologies)
- pDNA

Procedure:

Volumes were prepared for transfection in triplicates (200 ng pDNA/well). No excess for pipetting loss was included.

1. pDNA solution (600 ng) was diluted in Opti-MEM[®] to a total volume of 15 µl. Also, 1.8 µl LF[®] 2000 was diluted in Opti-MEM[®] up to 15 µl.
2. The pDNA solution was added to LF[®] 2000 solution and both were mixed slowly by pipetting up and down for approximately 7 times. Lipoplexes were incubated for 5 min at RT.
3. 10 µl was added per well (triplicates).

6.6.4 Generation of Lipofectamine RNAiMAX[®] based lipoplexes

Background:

Most steps were carried out according to the manufacturer's protocol¹¹⁸. A forward transfection was performed by seeding cells into a white 96-well plate one day prior to transfection.

Materials:

- Lipofectamine[®] RNAiMAX Reagent (Cat. No. 13778-150, Invitrogen)
- Luc siRNA and negative control siRNA (provided under Innovative Medicines Initiative Joint Undertaking COMPACT)
- MQ-water (Arium[®]pro VF, Sartorius), autoclaved
- Opti-MEM[®] Reduced Serum Medium (Gibco by Life Technologies)

Procedure:

2x 10⁴ cells (MDA-MB-231 (PGK-EGFP-Luc)) per well were seeded in 200 µl Dulbecco's Modified Eagle's Medium - high glucose into a white 96-well plate 24 h prior to transfection. Dilutions of siRNA were made:

1. From Luc siRNA (1 mM) a 100 µM dilution was made with MQ-water. Same was done for negative control siRNA. The concentrations of both dilutions were then measured by UV spectrophotometry and actual molarities were calculated by the following formula:

$$n (siRNA)[\mu mol] = \frac{m (siRNA) [ug]}{MW (siRNA)[ug/\mu mol]}$$

$$MW (siRNA) = 14383.82 \text{ g/mol} = 14383.82 \text{ }\mu\text{g}/\mu\text{mol}$$

2. Afterwards, a 1 µM dilution (1:100) of both in Opti-MEM[®] was made.

In detail, forming lipoplexes was carried out as described:

1. LF[®] RNAiMAX was diluted in Opti-MEM[®] and incubated for 5 min at RT.
2. siRNA was diluted in Opti-MEM[®] and then added to diluted LF[®] RNAiMAX (volume ratio 1:1). It was mixed well and incubated for 5 min at RT.
3. A specific volume of lipoplex solution (containing 1, 5 or 10 pmol siRNA per well) was added directly onto the cells, cultivated in complete medium.
4. Depending on the experiment, cells were incubated for either 48 h or 72 h at 37°C.

6.6.5 Effect of freeze-thawing on transfection efficiency of polyplexes

Background:

The following experiment was carried out in collaboration with Mag. Alexander Taschauer and Jacques Foric. The goal was to investigate long-term stability of stabilized LPEI based polyplexes when frozen at -80°C, compared to frozen non-stabilized as well as to freshly prepared polyplexes. Furthermore, transfection efficiency of polyplexes, generated by flash pipetting versus syringe-pump technique¹³, was compared.

Size and zeta potential (ζ -potential) of stabilized and non-stabilized polyplexes were measured via NTA (Nanoparticle Tracking Analysis) after application of freeze-thaw cycle(s). Also, pCMV-GLuc concentration was determined by UV spectroscopy and polyplexes were characterized by pDNA recovery.

Detailed information about biophysical characterization of polyplexes is described in diploma thesis of Jacques Foric (diploma thesis in preparation).

Biological characterization of polyplexes:

The *in vitro* effect of polyplexes was characterized by its transfection efficiency and toxicity on cells. Samples with indicated treatment (frozen or not, with or without cryoprotectant) were evaluated according to the following scheme:

1. Biological evaluation after one freeze-thaw cycle:
 - a. Transfection efficiency of polyplexes stabilized with cryoprotectant:
 - i. first sample after one week frozen at -80°C
 - ii. second sample after two weeks frozen at -80°C
 - b. Transfection efficiency of polyplexes non-stabilized.
2. Cytotoxicity of only cryoprotectant.
3. Transfection efficiency of freshly prepared polyplexes without applying a freeze-thaw cycle.

Experimental procedure:

Preparation (carried out by Mag. Alexander Taschauer and Jacques Foric):

pCMV-GLuc/LPEI polyplexes (100 µg/ml) of an N/P ratio of 6.0 were generated in HBG buffer (20 mM HEPES, 5 % (w/V) glucose, pH 7.4) by flash pipetting technique. Polyplexes were either diluted with a cryoprotectant or with HBG buffer (1:1). Afterwards, pCMV-GLuc concentration was determined by UV spectroscopy. Finally, polyplex samples were frozen at a constant cooling rate of -1°C/min in Mr. Frosty to -80°C and stored for varying time periods at -80°C.

Since I investigated the transfection efficiency of polyplexes, its procedure is shown in detail as follows:

Materials for transfection studies:

- pCMV-GLuc (New England Bio Labs®⁶⁰)
- Linear polyethylenimine 10 kDa (received from Mag. Alexander Taschauer, synthesized based on instructions by Rödl et al.²)
- HBG buffer (preparation see 9.1.2)
- Cryoprotectant (20 % HP- β-CD/2.4 % glucose in 20 mM HEPES)
- Dapi-hydrochloride (Cat. No. D9542, Sigma-Aldrich)

Procedure:

Day 1:

1x 10⁴ cells per well (A549) were seeded into a transparent 96-well plate in 200 µl complete medium, and incubated overnight at 37°C.

Day 2:

Frozen polyplexes were thawed in Mr. Frosty (+1°C/min) for 4 h. Cells were visually checked for morphology and confluence in inverted microscope. Complete medium was aspirated and 96 µl basal medium was added per well. Fresh polyplexes (100 µg/ml; N/P ratio 6.0) were generated either by flash pipetting or syringe pump technique. 4 µl of each sample (fresh and thawed polyplexes, respectively) was added per well, resulting in a final pCMV-GLuc concentration of 2 µg/ml. Cells were incubated for 4 h at 37°C. Afterwards, polyplexes were diluted by adding 100 µl complete medium per well.

In order to evaluate toxic effects of only cryoprotectant on cells, cryoprotectant solution (20 % HP- β -CD, 2.4 % glucose) was 1:1 diluted in HBG buffer (5 % glucose), resulting in 10 % HP- β -CD and 3.7 % glucose, and cells were treated with various volumes of this mixture (2 μ l, 4 μ l and 8 μ l were added to 98 μ l, 96 μ l and 92 μ l basal medium respectively). 4 μ l of cryoprotectant corresponded to the amount of cryoprotectant contained in samples (0.4 mg HP- β -CD; 0,148 mg glucose).

Day 3:

24 h after addition of polyplexes the supernatant was removed for assaying Gaussia luciferase activity and total count of live cells was determined by flow cytometry (see 6.8.3. b).

6.7 Cell culture techniques

For detailed information about aseptic cell culture techniques see 9.1.3.

6.7.1 Cell seeding into a 96-well plate

Materials:

- Dulbecco's Phosphate Buffered Saline (Cat. No. D8537, Sigma-Aldrich)
- Cell lines, complete medium and detaching reagents:

The lung cancer cell line A549 (ATCC[®] CRM-CCL-185[™]) was purchased from ATCC[®], LGC Standards GmbH (Germany) and cultivated in RPMI-1640 basal medium (Cat. No. R0883, Sigma-Aldrich), supplemented with FBS (Cat. No. F7524, Sigma-Aldrich and Cat. No. 10270-106, Gibco by Life Technologies) and with 200 mM Glutamine (Cat. No. G7513, Sigma-Aldrich). It is a human epithelial cell line from lung tissue and was taken from a 58 years old male Caucasian, suffering from lung adenocarcinoma. The cell line was established in 1972¹¹⁵. Cells were cultured in a flask of 75 cm² area (T-75). 1 ml of TrypLE[™] Express (1X) [+] Phenol Red (Cat.No. 12605010, Gibco by Life Technologies) as detaching reagent was used.

MDA-MB-231 (ATCC[®] CRM-HTB-26[™]) is a breast cancer cell line derived from a mammary adenocarcinoma of a 51 years old female Caucasian. It was obtained from ATCC[®], LGC Standards GmbH (Germany) and cultivated in Dulbecco's

Modified Eagle's Medium – high glucose (Cat. No. D5671, Sigma-Aldrich), supplemented with FBS and 200 mM Glutamine.

Apart from the wild type, MDA-MB-231 cells were also stably transduced with genes for the promotor Phosphoglycerate Kinase (PGK), the Enhanced Green Fluorescent Protein (EGFP) and the gene for Firefly luciferase, termed Luc (carried out by Msc. Julia Mayer). This genetic mutant is known as MDA-MB-231(PGK-EGFP-Luc).

For detaching cells cultured in a T-75 flask 1 ml of Versene was used. It is comprised of PBS and EDTA¹¹⁶. Here, no trypsin was applied because it poses risk to proteolyse membrane proteins¹¹⁷ of MDA-MB-231 cells.

Procedure:

Cells were harvested (see 9.1.3.2) and counted (see 9.1.3.5). The required seeding volume of cell dilution for a density of 1×10^4 cells per well was calculated. First, complete medium was pipetted in each well of a 96-well plate using a multichannel pipette, followed by addition of seeding volume. Prior to this, the cell dilution was mixed well. In total, the volume of each well was 200 μ l.

6.7.2 Transfection of A549 cell line with plasmid DNA via polyplexes and lipoplexes

Materials:

- Dulbecco's Phosphate Buffered Saline (Cat. No. D8537, Sigma-Aldrich)
- RPMI 1640 basal medium (Cat. No. R0883, Sigma-Aldrich)
- Complete medium (preparation see 9.1.3.1)

Procedure:

1. 18-22 h before transfection A549 cells were seeded at a density of 1×10^4 cells per well into a 96-well plate. On day of transfection cells were checked for sufficient confluence (60-80 %) and healthiness.
2. Solutions of LPEI and pDNA in adequate buffers were equilibrated for 20 min at RT.

3. Complete medium was aspirated from each well (cells on well bottom must not be disturbed) and cells were washed once with prewarmed PBS. 90 µl of prewarmed basal medium was added to respective wells.
4. The nanoparticles were generated as described in 6.6.1. 10 µl of nanoparticle solution per well was added so that final pDNA concentration was 2 µg/ml. Afterwards, positive control substances were prepared as described in 6.6.2 and 6.6.3 and added to cells.
5. The 96-well plate was incubated at 37°C for 4 h. Later on, polyplexes were diluted by addition of 100 µl complete medium per well.
6. The 96-well plate was incubated overnight at 37°C.

6.8 Cell based luciferase reporter gene assays

The individual luciferase reporter gene assays follow the protocols of Taschauer et al.¹³.

Materials:

- Cells: A549 (ATCC[®] CCL-185[™], LGC Standards GmbH, Germany); MDA-MB-231 (ATCC[®] CRM-HTB-26[™], LGC Standards GmbH, Germany)
- pDNA:
pCpG-hCMV-EF1α-LucSH (received from Univ.Prof. Manfred Ogris, Magnusson et al.⁵⁶); pCMV-GLuc (New England Biolabs[®], Germany⁶⁰); pCpGfree-promotor-Lucia (Cat. No. pcpgf-promlc, Invivogen, USA)
- LPEI 10 kDa, diluted in HBG or HBS (received from Mag. Alexander Taschauer, synthesized based on instructions by Rödl et al.²)
- Lipofectamine[®] 2000 (Cat. No.11668-019, Invitrogen)
- jetPRIME[®] transfection reagent, jetPRIME buffer (Polyplus-Transfection)
- HBG (20mM HEPES, 5 % (w/V) glucose, pH=7.4); preparation see 9.1.2
- Complete medium (see 9.1.3.1); basal medium (A549: RPMI 1640 basal medium (Cat. No. R0883, Sigma-Aldrich); MDA-MB-231: Dulbecco's Modified Eagle's Medium - high glucose (Cat. No. D5671, Sigma-Aldrich))
- Opti-MEM[®] Reduced Serum Medium (Gibco by Life Technologies)
- Luciferase substrate: Firefly (hLAB/cLAB); Gaussia/Lucia (hCAB/cCAB)

Procedure:

Day 1: Seeding 10 000 cells per well

1. A549 lung cancer cells were seeded at a density of 1×10^4 into a white 96-well plate according to the seeding plan, which has been established in advance regarding points to consider for reading out pattern (9.1.1) For each sample as well as for negative controls (untreated cells) and for positive controls triplicates were seeded. Total seeded volume was 200 μ l (complete medium). Cells were incubated at 37°C for approximately 16 h.
2. Solutions of pDNA and LPEI, required for polyplexing the following day, were prepared in PCR tubes in advance and stored at +4°C overnight. For calculation of volumes for polyplexing see 6.6.

Day 2: Generation of nanoparticles and transfection of A549 cells

1. Preparation:
Solutions of pDNA, LF[®] 2000 and jetPRIME[®], which have been prepared the day before, were equilibrated to RT. Plated cells were investigated in microscope for morphology and confluence (60-80 %).
2. Generation of polyplexes/lipoplexes: Steps as described in 6.6.1, 6.6.2 and 6.6.3 were performed.
3. Transfection of A549 with polyplexes/lipoplexes:
Complete medium was aspirated from each well and 90 μ l of warm basal medium was added onto cells. 10 μ l per well of each nanoparticle solution was added. The plate was incubated at 37°C for 4 h.
4. 100 μ l warm complete medium was added per well, resulting in a final volume of 200 μ l.
5. Cells were incubated at 37°C overnight.

Day 3: Luminescence measurement and normalization

RLUs were normalized by two possible methods:

- a. protein amount (for Firefly luciferase)
- b. protein amount or total count of live cells (for Gaussia/Lucia luciferase).

In 9.2 detailed information regarding calculation methods for normalization is provided.

6.8.1 Firefly luciferase assay for investigating plasmid delivery

The Firefly luciferase assay was carried out for investigating delivery of pCpG-hCMV-EF1 α -LucSH via LPEI *in vitro*.

Procedure on day 1 and 2 is described in 6.8. On day 3, luminescence [RLU] was detected by a luminometer and subsequently normalized by protein amount of cells (BCA assay).

Note: Applied volumes conform to volumes of optimized BCA assay.

Preparation:

hLAB/cLAB was thawed at RT in the dark for at least 2 h. 1X and 2X PLB, BCA Working Reagent and standard dilutions of BSA were prepared.

Procedure:

1. Cell lysis:

Complete medium of each well was aspirated. Cells were washed once with 200 μ l PBS so that detached (dead) cells were removed. 30 μ l 1X PLB was added to each well with a micropipette and the 96-well plate was shaken for 30 min on a plate shaker (500 rpm, RT).

2. During lysis of cells 25 μ l of each BSA standard dilution was added per well into a transparent 96-well plate (triplicates). As negative control, 20 μ l 1X PLB per well was used. 5 μ l MQ-water was added for equal volumes (25 μ l).

3. Cell lysis was checked in inverted microscope.

4. Each cell lysate was mixed with a micropipette and 20 μ l of each was transferred into the transparent 96-well plate for conducting the BCA assay.

5. Remaining lysate (10 μ l) was used for assaying Firefly luciferase activity with a luminometer. For this, steps as described in 6.5 were performed.

6.8.2 Firefly luciferase assay for investigating siRNA delivery

Moreover, the optimized Firefly luciferase assay can be used to determine siRNA-induced downregulation of Firefly luciferase. Extent of decrease in light intensity of Firefly luciferase indicates efficiency of siRNA delivery into cells *in vitro*.

Background:

LF[®] RNAiMAX is a commercially available transfection reagent from Invitrogen, capable of transfecting mammalian cells with small ribonucleic acids i.e. siRNA or miRNA. The lipoplexes must be prepared in serum free medium and can be directly added to cells, cultured in complete medium¹¹⁸.

Materials:

- Lipofectamine[®] RNAiMAX (Cat. No. 13778-150, Invitrogen)
- Luc siRNA and negative control siRNA (provided under Innovative Medicines Initiative Joint Undertaking COMPACT)
- Opti-MEM[®] Reduced Serum Medium (Gibco by Life Technologies)

Procedure:

Day 1: Seeding 20 000 cells per well

1. MDA-MB-231 (PGK-EGFP-Luc) cells were seeded at a density of 2×10^4 per well into a white 96-well plate according to seeding plan. Samples (various amounts of siRNA) and negative controls (various amounts of neg. control siRNA) were seeded each in triplicates in a total volume of 200 μ l complete medium per well. Cells were incubated at 37°C overnight (for approximately 16 h).

Day 2: Generation of LF[®] RNAiMAX based lipoplexes of siRNA and transfection of MDA-MB-231 (PGK-EGFP-Luc)

1. Preparation:

Cultured cells were investigated in inverted microscope for morphology and confluence.

Dilutions of 100 μ M Luc siRNA and 100 μ M negative control siRNA in MQ-water were prepared. Actual concentrations of both were checked via UV-spectroscopy. Based on actual molarities (calculation see 6.6.4), 1 μ M

dilutions in Opti-MEM[®] were made of each. Dilutions were stored at -80°C.

Different amounts of siRNA (pmol) and negative control siRNA (pmol) respectively, were prepared. Therefore, volumes of 1 µM siRNA (containing the required amount of siRNA for a triplicate plus 1 reserve well) were mixed with Opti-MEM[®] to a total volume of 25 µl. According to Invitrogen¹¹⁸, ratio siRNA (pmol) to LF[®] RNAiMAX (µl) is 1:0.3 per well (96-well plate). Respective volumes of LF[®] RNAiMAX (n=4) were mixed with Opti-MEM[®] to a total volume of 25 µl.

Finally, equal volumes were mixed for generating lipoplexes.

2. In detail, steps for generation of lipoplexes and transfection of cells were performed as follows:

- a. LF[®] RNAiMAX was diluted in Opti-MEM[®] and incubated for 5 min at RT.
- b. siRNA was diluted in Opti-MEM[®].
- c. Diluted siRNA was added to LF[®] RNAiMAX dilution and after mixing well it was incubated for 5 min at RT.
- d. 12.5 µl per well was added directly to cells in complete medium.
- e. Cells were incubated at 37°C for 48 h or 72 h.

Day 4 or 5: Measuring luminescence and normalization by BCA assay

6.8.3 Gaussia luciferase assay

The Gaussia luciferase assay was carried out for monitoring delivery of pCMV-GLuc by different gene delivery vehicles *in vitro*.

Procedure:

Day 1: Seeding of 10 000 cells per well:

A549 cells were seeded at a density of 1×10^4 into a transparent 96-well plate. Triplicates of samples, positive and negative controls were seeded.

Day 2: Generation of nanoparticles and transfection

Day 3: Measuring luminescence and normalizing RLUs:

Due to secretion into the supernatant two methods for normalizing RLUs exist:

a. Normalization via BCA assay:

1. Preparation: see 6.8.1(substrate: hCAB/cCAB)

2. The supernatant of each well, containing the secreted Gaussia luciferase, was thoroughly mixed and 130 μ l were transferred into either a transparent 96-well plate or Eppendorf tubes and frozen at -80°C . Gaussia activity was measured from 20 μ l supernatant as described below.
 3. Cell lysis:
Remaining complete medium of each well (70 μ l) was aspirated with vacuum pump without disturbing cells on well bottom. Cells were washed once with 200 μ l PBS. 30 μ l 1X PLB was added to each well with a micropipette and the 96-well plate was shaken for 30 min on a plate shaker (500 rpm, RT).
 4. Cell lysis was checked in microscope.
 5. For BCA standard curve, first 25 μ l per well of each BSA standard dilution was added (triplicates) and 5 μ l MQ-water per well was added in order to reach same volumes as for the sample (30 μ l). Only 1X PLB per well (30 μ l) served as blank for evaluation of BCA assay.
 6. The BCA assay was carried out as described in 6.2.
 7. Gaussia luciferase activity was assayed as described below.
- b. Normalization via flow cytometry:
1. Same procedure as described in 6.9 was performed.
 2. Gaussia luciferase activity was assayed as described below.

Measuring luminescence of Gaussia luciferase:

Frozen Gaussia supernatant was thawed and 20 μ l of each sample was transferred into a white 96-well plate. The luminescence was measured after injection of 50 μ l substrate (hCAB or cCAB).

6.8.4 Lucia luciferase assay

Since Lucia luciferase is also a secreted enzyme, the assay is performed according to the Gaussia luciferase assay protocol (6.8.3).

6.9 Flow cytometry

For background information about flow cytometry see 9.1.4.

Materials:

- Dulbecco's Phosphate Buffered Saline (Cat. No. D8537, Sigma-Aldrich)
- Detaching reagent: TrypLE™ Express (1X) [+] Phenol Red (Cat. No. 12605010, Gibco by Life Technologies) for A549, Versene 1:5000 (1X) (Cat. No. 15040-033, Gibco by Life Technologies) for MDA-MB-231
- Dapi-hydrochloride (Cat. No. D9542, Sigma-Aldrich), stock (5mg/ml)

Procedure for counting fluorescence-labeled dead cells:

Day 1:

1. 1×10^4 cells per well were seeded into a transparent 96-well plate. An additional triplicate was seeded for setting appropriate channels and determining autofluorescence of cells.

Day 2:

1. Respective wells were treated with samples (nanoparticles). A triplicate of untreated cells served as negative control.

Day 3:

1. Complete medium was aspirated from each well.
2. Cells were washed with 200 μ l warm PBS.
20 μ l of detaching reagent was added with a multichannel pipette and cells were incubated at 37°C for 5 min.
3. 140 μ l PBS per well was added to samples and the cell suspension was pipetted up and down twice (96-well plate was held tilted). The final volume was 160 μ l per well.
4. Similarly, 180 μ l PBS per well was added to these wells, which were going to be used for measuring autofluorescence of cells and for determining the channel settings. Consequently, wells contained 200 μ l total cell suspension.
5. The 96-well plate was properly placed onto a cool rack (+4°C) and the cover was removed. A light-protected vial, containing a Dapi-HCl solution (5 μ g/ml) was placed in proper position. Light was switched off.

6. Firstly, channels were set accordingly and autofluorescence was measured. No Dapi-HCl was added.
7. 40 μ l of Dapi-HCl (5 μ g/ml) was automatically added to each sample and mixed thoroughly by the injector, resulting in a total volume of 200 μ l per well with a concentration of 1 μ g/ml Dapi-HCl (Dapi-HCl titer was set to 1:5). 150 μ l uptake volume was used for measurement.

For measurement of samples (stained with Dapi-HCl) the following settings were made:

- Rack/F-bottom 96-well plate = MACSPLEX Filter Plate
- Flow Rate = high
- Mode = fast
- Dapi-HCl titer = 1:5

During each measurement the cell population was monitored by a density plot and a histogram.

Evaluation of flow cytometry data:

The data received from flow cytometry were evaluated by FlowJo (FlowJo_V10, FlowJo Enterprise, LLC., Ashland, Oregon, USA). First of all, cells for measuring autofluorescence (untreated and without Dapi-HCl) were displayed in a density plot (x axis: Forward Scatter (FSC), y axis: Sideward Scatter (SSC)). A polygon gate was drawn around the cell population, excluding cell debris, which is characterized by both FSC and SSC close zero. The cell population was then depicted in a separate histogram (x axis: V1 channel for Dapi, y axis: number of events). By setting a vertex the cell population was divided into two regions: Dapi-negative cells constitute the fraction of living cells [%] and Dapi-positive cells represent fraction of dead cells [%]. These gates were applied to all other samples.

Because Dapi-negative cells were counted in 150 μ l sample (uptake) volume, the actual cell number per well was calculated for 200 μ l total volume.

For normalization methods see 7.3.6.

In 9.2 detailed information regarding calculation methods for normalization is provided.

7 Results

7.1 Optimization of BCA assay

7.1.1 Effect of freezing on cell lysis after treatment with 1X Lysis Buffer

Cell lysis is needed to assay intracellular reporter proteins like Firefly luciferase. Lysis of cells is routinely achieved by lysis buffers. However, the lysis can be incomplete in some cell types. Therefore, we included a freezing step because as stated by Thermo Fisher Scientific, a freeze-thaw cycle could promote lysis through ice crystal formation¹¹⁹. Indeed, after freezing cells for approximately 10 min at -80°C morphology of cells was altered. In order to find out if addition of a freeze-thaw cycle to the lysis procedure results in higher protein recovery, a BCA assay with a freezing step was performed and compared to one devoid of a freezing step. Therefore, different cell numbers were seeded into two transparent 96-well plates each and cells were cultured at 37°C for approximately 18 h. As recommended by Promega¹¹⁰, cells with higher resistance to lysis could be treated with an increased volume of 1X PLB (30 μ l). BCA assay was performed as described in 6.2. Figure 19 and Table 7 show, that a freeze-thaw cycle had no relevant influence on cell lysis as measured by BCA assay. Generally, it must be noted that variability in protein amounts [μ g] between separate BCA assays was high.

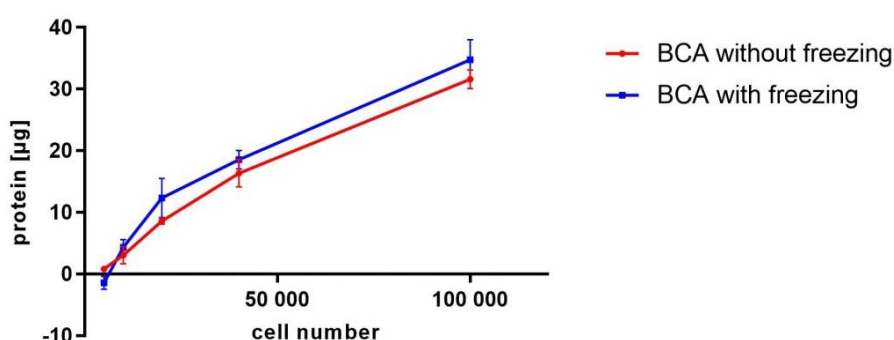


Figure 19: BCA assay based protein amounts of cell lysates when different cell numbers were lysed with 1X PLB with and without freezing. Data are represented as means $\pm$ SD (n=3). The graph is representative of two independent experiments.

Table 7: BCA assay based protein amounts of cell lysates when different cell numbers were lysed with 1X PLB with and without freezing. Data are represented as means $\pm$ SD (n=3).

Cell number	Protein [μ g] (BCA without freezing)		Protein [μ g] (BCA with freezing)	
	Mean	SD	Mean	SD
5 000	0.87	0.390101267	-1.41	0.87360952
10 000	3.06	1.119617883	4.33	1.03279218
20 000	8.59	0.416483005	12.34	2.58315497
40 000	16.36	1.818109547	18.57	1.23708644
100 000	31.59	1.243348024	34.75	2.67564242

In contrast, for cell lysis performed with 1X CCLR, protein amounts [μ g] are observed to be higher when no freezing step was included (Figure 20).

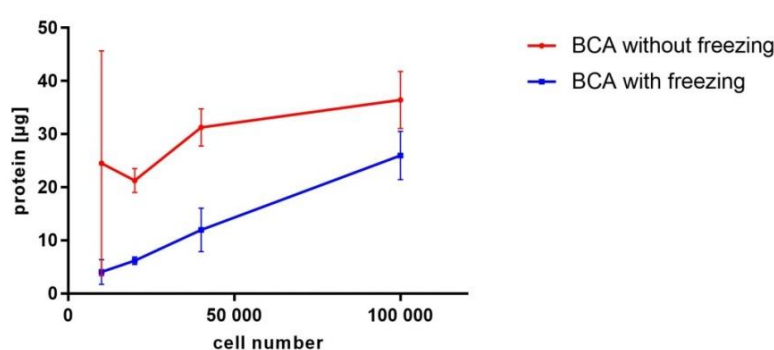


Figure 20: BCA assay based protein amounts of cell lysates when different cell numbers were lysed with 1X CCLR with and without freezing. Results are expressed as means $\pm$ SD (n=3).

7.1.2 Influence of iodoacetamide on BCA assay

Lysis buffer contains DTT which interferes with protein estimation¹²³. Because iodoacetamide can eliminate this interference¹⁰⁰, we investigated the influence of it on BCA assay protein estimation.

5 000, 10 000, 20 000, 40 000 and 100 000 cells (A549) per well were seeded into a transparent 96-well plate. 50 μ l 0.5X PLB was used for lysis and a freezing step was added. Half of the lysate was transferred into a second transparent 96-well plate. A BCA assay with iodoacetamide was performed. With the remaining lysate a BCA assay devoid of iodoacetamide was carried out. Both assays followed steps described in 6.2. Iodoacetamide had no appreciable influence on the BCA assay, because similar protein amounts [μ g] were measured (Figure 21).

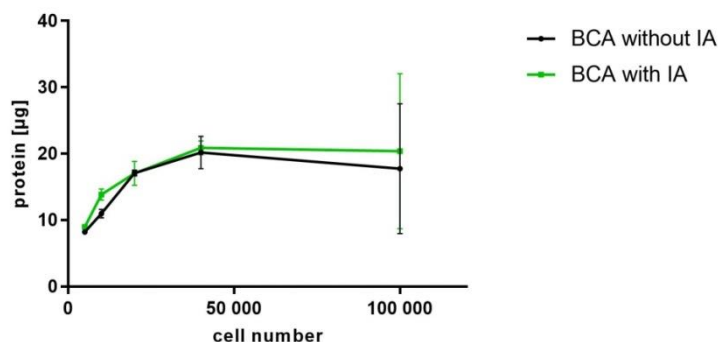


Figure 21: BCA assay based protein amounts of cell lysates when different cell numbers were lysed with 1X PLB with and without iodoacetamide. Results are expressed as means $\pm$ SD (n=3).

In contrast to this, addition of iodoacetamide to 1X CCLR-based lysate lowered protein values (Figure 22). Interestingly, when adding green Working Reagent (containing Cu^{2+}) to the lysate it was observed that colour change from green to purple (Cu^+ -BCA-complex) was very slow. This indicates that iodoacetamide had eliminated DTT contained in 1X CCLR so that less substance interfered with cupric ions in Working Reagent. Thus, also the speed of colour change was decreased. Results were not consistent enough and further investigations are required.

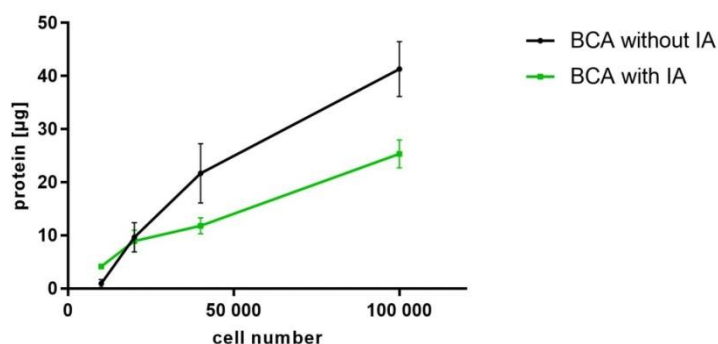


Figure 22: BCA assay based protein amounts of cell lysates when different cell numbers were lysed with 1X CCLR with and without iodoacetamide. Results are expressed as means $\pm$ SD (n=3).

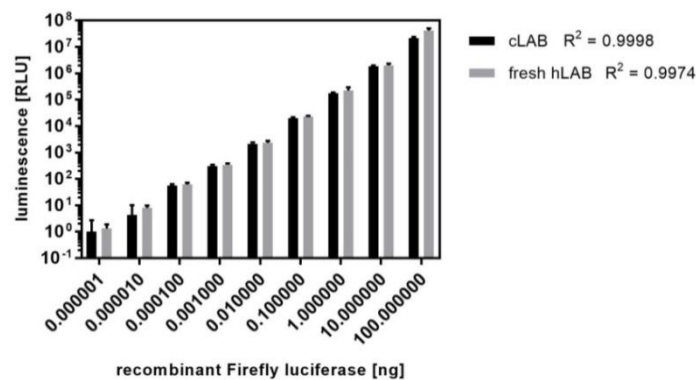
7.2 Optimization of Firefly luciferase assay

7.2.1 Testing housemade versus commercial Luciferin Assay Buffer with recombinant luciferase

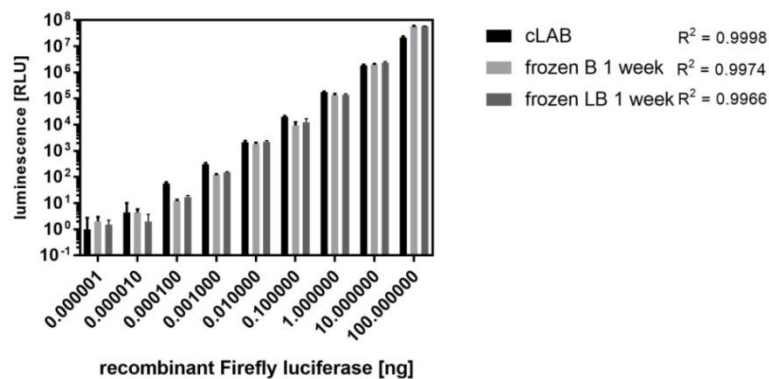
Due to high costs of cLAB the goal was to substitute it by hLAB. Therefore we compared activity of both substrates by measuring light output [RLU] emitted from a serial dilution of recombinant Firefly luciferase, covering a range of 10^{-6} ng to 10^2 ng (eight orders of magnitude). Both substrates showed a similar activity (Figure 23 **a.**).

In addition, it was of high interest, whether hLAB can be frozen at -80°C , thawed and reused as a substrate again – without loss in activity. Thus, activity of frozen-thawed hLAB was investigated compared to fresh hLAB and cLAB. After four weeks frozen, no relevant decrease in activity was seen for frozen hLAB – neither for frozen Buffer (B) (LB1 respectively) nor for frozen mixture of Luciferin and Buffer (LB2). With each kind of substrate used the linear range of the enzyme was shown, displayed as R^2 . Results are shown in Figure 23 **b.-c.** and Figure 24 **a.-b.** As it is more convenient to freeze LB, it was decided to freeze only this kind of substrate for future applications.

a.



b.



c.

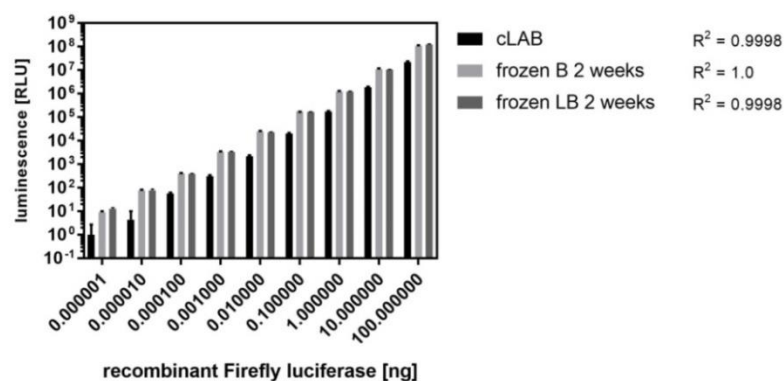
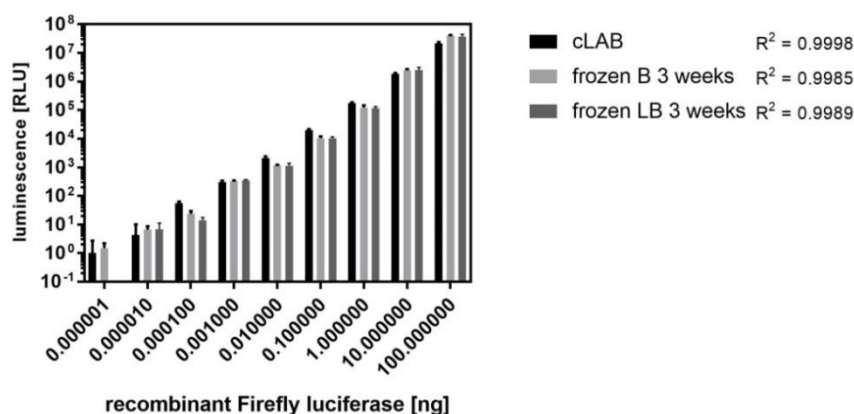


Figure 23: Stability of frozen hLAB over time compared to fresh hLAB and commercial cLAB. Values are represented as means $\pm$ SD (n=3). **a.** cLAB compared to fresh hLAB: no relevant difference was measured. **b.** cLAB compared to B and LB (frozen for 1 week): RLUs are similar. **c.** cLAB compared to B and LB (frozen for 2 weeks): RLUs are similar, but, compared to a., b., d. and e., they are an outlier.

a.



b.

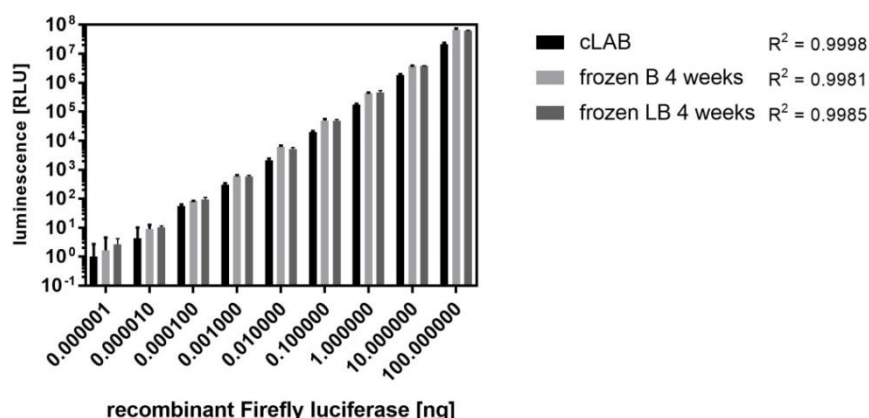


Figure 24: Stability of frozen hLAB over time compared to fresh hLAB and commercial cLAB. Values are represented as means $\pm$ SD (n=3). **a.** cLAB compared to B and LB (frozen for 3 weeks): RLU are similar. **b.** cLAB compared to B and LB (frozen for 4 weeks): RLU are similar.

7.2.2 Testing housemade versus commercial Luciferin Assay Buffer with transfected cells

1×10^4 cells per well were seeded one day prior to transfection into a 96-well plate in 200 μ l complete medium. Cells were transfected with 200 ng pCpG-hCMV-EF1 α -LucSH via LPEI based polyplexes (N/P ratio 7.5). Right after preparation, no incubation time was applied on polyplexes. Untreated cells served as negative control. Nanoparticles consisting of pDNA lipoplexed with LF[®] 2000 and those polyplexed with jetPRIME[®] were used as positive controls. Additionally, LPEI based polyplexes were prepared in HBG buffer and HBS buffer for comparison. Frozen hLAB was used as substrate (100 μ l injection volume per well).

Transfection efficiency of LPEI based nanoparticles was similarly in HBS buffer and HBG buffer and, overall, it was comparable with positive controls (Figure 25). Light intensity of LF[®] based lipoplexes was much lower because of its assumed higher cytotoxic effect on cells. Other cell experiments showed similar results (Figure 26), indicating that frozen hLAB is suitable as a substrate for cell-based Firefly luciferase assays. A reason for lower signal when measuring with cLAB might be the fact that with an increasing number of freeze-thaw cycles activity of the substrate is decreased¹¹⁰.

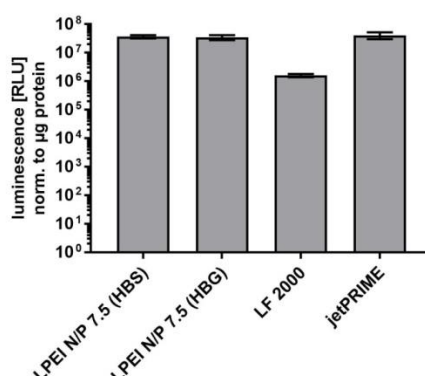


Figure 25: Firefly luciferase expression (luminescence intensity; RLU norm. to µg protein) in A549 cells treated with different polyplexes of pCpG-hCMV-EF1α-LucSH (LPEI, LF[®] 2000 and jetPRIME[®]). Results are expressed as means ± SD (n=3).

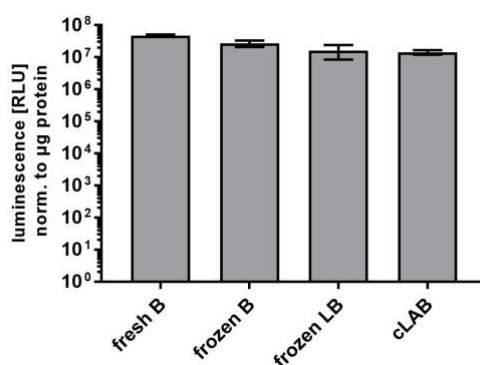


Figure 26: Firefly luciferase expression (luminescence intensity; RLU norm. to µg protein) in A549 cells treated with pCpG-hCMV-EF1α-LucSH/LPEI polyplexes (N/P 7.5) and measured with different substrates, 50 µl injection volume each (fresh and frozen B, frozen LB and commercial substrate).

Furthermore, these cell experiments prove that the Firefly luciferase assay, including generation of nanoparticles, performance of transfection, reading out and normalization, works successfully and is reproducible.

Considering results of Firefly luciferase assays with recombinant luciferase as well as with luciferase expressed after a cell transfection, one can conclude that hLAB can be frozen at -80°C and after thawing up reused as a substrate for Firefly luciferase. The hLAB did not relevantly lose activity after a freezing period of approximately three months. This was investigated by comparing both substrates (hLAB versus cLAB) within a luciferase assay. Figure 27 shows that light output of 30 µl sample was for both substrates nearly equal. This result confirms the assumption that the frozen form of hLAB does not lose activity for even a longer period when stored frozen at -80°C.

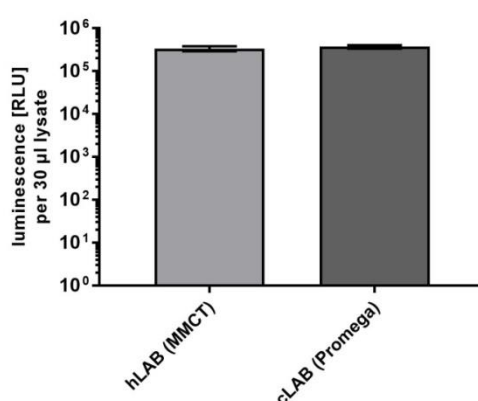


Figure 27: Firefly luciferase expression (luminescence intensity; RLU per 30 µl lysate) assayed with hLAB and with cLAB. RLUs are expressed as means $\pm$ SD (n=12).

7.2.3 Aliquot to aliquot and batch to batch variability of housemade Luciferin Assay Buffer across transfection experiments

One batch of hLAB was prepared and for cell transfection experiments 1,2 and 3 a frozen aliquot of it was used. For cell experiment 4 another batch was used. Figure 28 shows that (not normalized) light intensities [RLU] are similar between same samples of different cell transfections (experiment 1,2 and 3, respectively): RLUs of all cells transfected with pCpG-hCMV-EF1 α -LucSH/LPEI polyplexes (prepared in HBG) are of same orders of magnitude (10^7); RLUs of cells transfected with lipoplexes (pCpG-hCMV-EF1 α -LucSH/LF[®] 2000) are of 10^6 orders of magnitude; jetPRIME[®] based polyplexes showed similar light intensities as LPEI based polyplexes. These results show that cell transfections are reproducible. The transfection process worked reproducibly, irrespective of the hLAB aliquot used and irrespective of sample (LPEI, LF[®] 2000 or jetPRIME[®]).

The batch to batch variability (experiments 1,2,3 versus experiment 4) and aliquot to aliquot variability (experiment 1 versus 2 versus 3) of hLAB was small. Thus, the preparation of hLAB is reproducible.

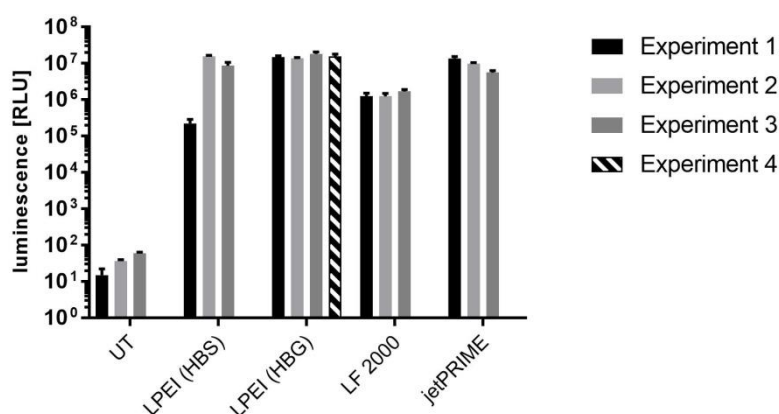


Figure 28: Firefly luciferase expression [RLU per 30 µl lysate] of A549 cells after transfection with pCpG-hCMV-EFα1 polyplexes (N/P ratio 7.5) (LPEI in HBS, LPEI in HBG, LF[®] 2000 and jetPRIME[®]). hLAB of same batch was used for experiments 1-3. For experiment 4 a second batch was used. Differences between transfection efficiencies within samples are low. Values are represented as means ± SD (n=3).

7.2.4 Reproducibility of housemade Luciferin Assay Buffer using recombinant luciferase

A serial dilution series of recombinant Firefly luciferase was assayed with different batches of hLAB. Figure 29 shows RLUs emitted from 1 nanogram recombinant enzyme. In case of batch 2 and batch 3 of hLAB, fresh and frozen were used. No relevant difference in detected signal intensity was seen. RLUs are within same orders of magnitude (10⁵). This shows that prepared batches of hLAB are of reproducible quality regarding their ability of providing optimal conditions for bioluminescence of Firefly luciferase enzyme.

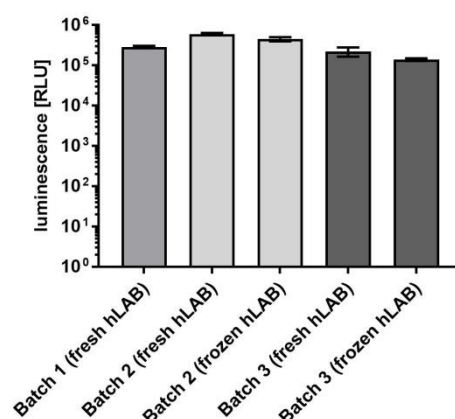


Figure 29: Luminescence [RLU] emitted from 1 ng of recombinant Firefly luciferase (Promega) was assayed with different batches of hLAB (batch 1: fresh hLAB; batch 2: fresh and frozen hLAB; batch 3: fresh and frozen hLAB). Values are represented as means $\pm$ SD (n=3).

7.2.5 Determination of substrate injection volume

In case of commercial buffer, Promega prescribes an injection volume of 100 μ l of cLAB¹¹⁰. However, initial experiments with 50 μ l also gave similar results. Thus, a subgoal was to find the best suited injection volume of the housemade hLAB, which results in RLUs of same orders of magnitude as with cLAB. Therefore, a dilution series of recombinant Firefly luciferase was made and emitted luminescence intensity was measured with 25 μ l, 50 μ l and 100 μ l of frozen hLAB. As positive control, 50 μ l of cLAB was used. 100 μ l of frozen hLAB and 50 μ l of cLAB resulted in higher RLUs than 25 μ l or 50 μ l of frozen hLAB. Results are displayed in Figure 30. 100 μ l of hLAB gave the best results and were used in all further Firefly luciferase assays.

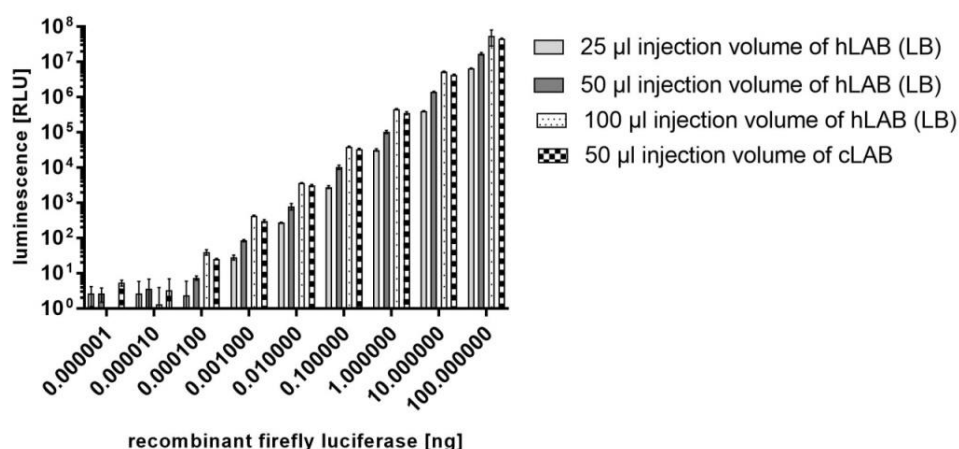


Figure 30: Luminescence [RLU] emitted by different amounts of recombinant Firefly luciferase [ng] (Promega) was assayed with different injection volumes of hLAB (LB) and with 50 μ l of cLAB. Results are expressed as means $\pm$ SD (n=3).

To summarize, the main focus was on optimizing the housemade Luciferin Assay Buffer (hLAB). The results clearly indicate that hLAB is equally good as the commercial Luciferin Assay Buffer (cLAB). Furthermore, experiments with recombinant Firefly luciferase prove that it can be frozen for at least three months and reused, without relevant decrease in activity. Transfection experiments with pCpG-hCMV-EF1 α -LucSH were carried out and light intensities of Firefly luciferase was assayed with different batches of hLAB across luciferase assays showing showing low batch to batch variability. Transfections worked reproducibly because same orders of magnitude of RLUs were detected. Since there was no aliquot to aliquot variability and only low batch to batch variability, it can be concluded that production of hLAB is reproducible.

7.3 Optimization of coelenterazine based luciferase assays

7.3.1 Testing housemade versus commercial Coelenterazine Assay Buffer with transfected cells

Cells were transfected with LPEI based nanoparticles of either pCMV-GLuc or pCpGfree-promotor-Lucia. Supernatants containing secreted Gaussia or Lucia luciferase were transferred into separate tubes and stored at -80°C. Prior to a luciferase assay both supernatants were thawed, mixed thoroughly and 20 μ l of each supernatant was pipetted in wells of a white 96-well plate.

Both luciferase activities were measured with different substrates (Figure 31 and Figure 32). Fresh and frozen hCAB worked as good as cCAB. Frozen hCAB worked slightly better than fresh hCAB. Here, additionally a mixture of LAR II and Stop & Glo[®] was tested as substrate, as recommended by Promega^{110,120}. Because of lack of a second injector for the plate reader, used in our lab, we mixed equal volumes of LAR II and Stop & Glo[®]. 100 μ l of this mixture was then injected and indeed, we obtained a signal higher than with Stop & Glo[®] alone, or hCAB respectively.

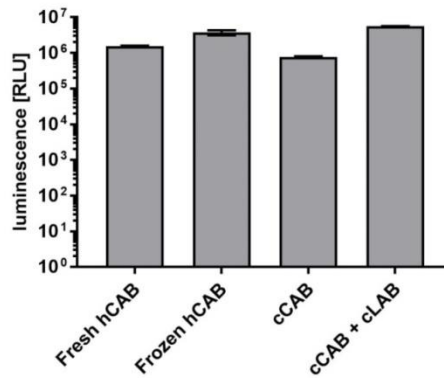


Figure 31: Gaussia luciferase expression (luminescence intensity; [RLU]) of A549 cells, measured with hCAB (fresh and frozen), cCAB and a mixture of cCAB (Stop & Glo[®]) plus cLAB (LAR II). Results are expressed as means $\pm$ SD (n=3).

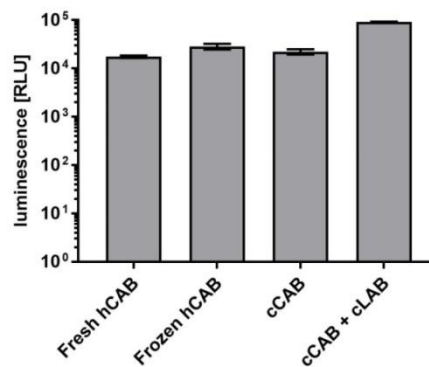


Figure 32: Lucia luciferase expression (luminescence intensity [RLU]) of A549 cells, measured with hCAB (fresh and frozen), cCAB and a mixture of cCAB (Stop & Glo[®]) and cLAB (LAR II). Maximal RLUs are in a order of 10⁴ magnitude. Results are expressed as means $\pm$ SD (n=3).

In conclusion, hCAB leads to RLUs of at least same order of magnitude as obtained with cCAB. Hence, commercial coelenterazine buffer can be replaced by housemade buffer.

Light signal intensity emitted by Lucia luciferase is always less than that of Gaussia luciferase (10⁴ versus 10⁶ RLU). This observation was made across all cell experiments. It is presumed that lack of enhancer in plasmid backbone of pCpGfree-promotor-Lucia causes less signal intensity than pCMV-GLuc, which had been designed for optimal expression in mammalian cells.

Identity of pCpGfree-promotor-Lucia was established by diagnostic restriction digest (DRD) to make sure that the plasmid was correctly replicated by E.coli in

maxi prep. Concentration of isolated pCpGfree-promotor-Lucia after maxi prep was measured by spectrophotometry (235 µg/ml) (Table 8).

Table 8: Concentration, absorption and purity (A 260/280) of dilutions of pCpGfree-promotor-Lucia after a maxiprep.

Sample	1:5 dilution	1:10 dilution
Concentration [ng/µl]	48	23
A 260	1.60	0.70
A 280	—*	0.49
A 260/280	1.846	1.847
Multiplication factor for original concentration [ng/µl]	240	230
Average concentration [ng/µl]	235	

* this value is missing

The restriction map of pCpGfree-promotor-Lucia displaying several restriction sites at a specific palindromic sequence is shown below (Figure 33). For validation of pDNA three restriction enzymes were used (DraI, EcoRI and HindIII). Reaction components for each restriction digest are given in Table 9. From Figure 33 one can see that DraI was expected to cut the plasmid four times (four bands), EcoRI twice (two bands) and HindIII once (linear plasmid). Gel slots were loaded with DNA ladder and samples. Bands were separated within a gel electrophoresis.

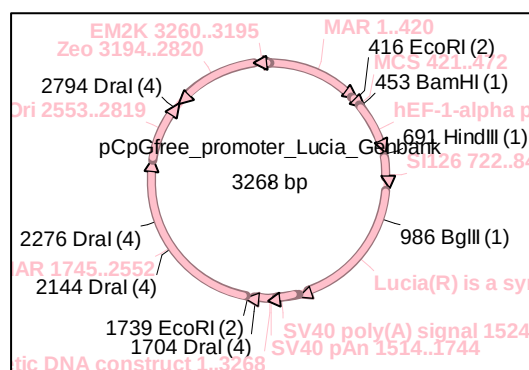


Figure 33: Plasmid map of pCpGfree-promotor-Lucia with restriction sites; map made by sequence from Invivogen⁶².

Table 9: Compounds [µl] of each restriction digest.

Fast Digest Reaction Mix #	#2 (uncut, supercoiled)	#3 (linear)	#4 (three bands)	#5 (four bands)
Reaction component	Volume [µl]			
Nuclease free water	17.0	16.0	15.0	16.0
FD Buffer green	2.0	2.0	2.0	2.0
pDNA (235 µg/ml)	1.0	1.0	1.0	1.0
FD Enzyme 1	0.0	1.0 (HindIII)	1.0 (EcoRI)	1.0 (DraI)
FD Enzyme 2	0.0	0.0	1.0 (HindIII)	0.0
Total Volume	20.0	20.0	20.0	20.0

Sample 1 and 6 in the gel image both represent DNA ladders (Figure 34). They contain a mixture of DNA molecules of defined base lengths. Separated bands of samples (#2-5) were allocated. Sample #2 was the uncut, supercoiled pLucia (~ 3200 bp). Sample #3 was a plasmid single cut by restriction enzyme HindIII so that a linear plasmid resulted. It needs to be discussed why the supercoiled plasmid did not move faster than the linear form. In sample #4 three bands were visible approximately at 2000 bp, at 1000 bp and at 300 bp. This was expected. A quadruple cut of DraI in sample #5 was also recognized: three bands were visible approximately at 2100 bp, at 500 bp and at 440 bp. One weak band was seen at ~ 130 bp. In samples #3, #4 and #5, weak bands at approximately 5000 bp seem to be remnants of supercoiled plasmid. In conclusion, DRD of plasmid Lucia fits the expected band pattern based on its map, thereby validating the plasmid.

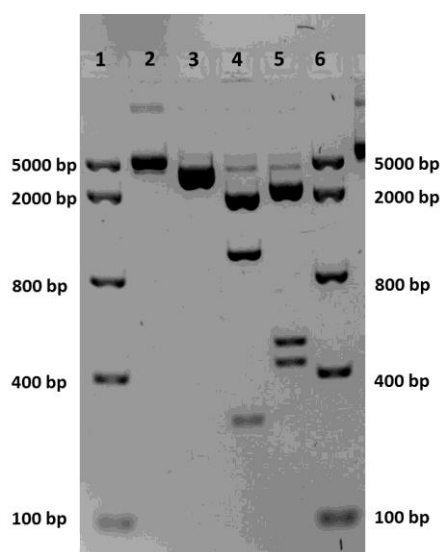


Figure 34: Diagnostic Restriction Digest of pCpGfree-promotor-Lucia (lane 1 and 6: DNA ladder; lane 2: undigested; lane 3: HindIII; lane 4: EcoRI, HindIII; lane 5: DraI).

7.3.2 Aliquot to aliquot and batch to batch variability of housemade Coelenterazine Assay Buffer across transfection experiments

As shown in Figure 35 again, frozen hCAB worked slightly better than fresh hCAB. Additionally, reproducibility of hCAB was checked by comparing aliquots of different batches. A slight difference in light intensity between batch 1 and 2 and batch 1 and 3 was detected (Figure 35). But within the same batch (batch 3), no relevant variability was detected (aliquot 1 and 2).

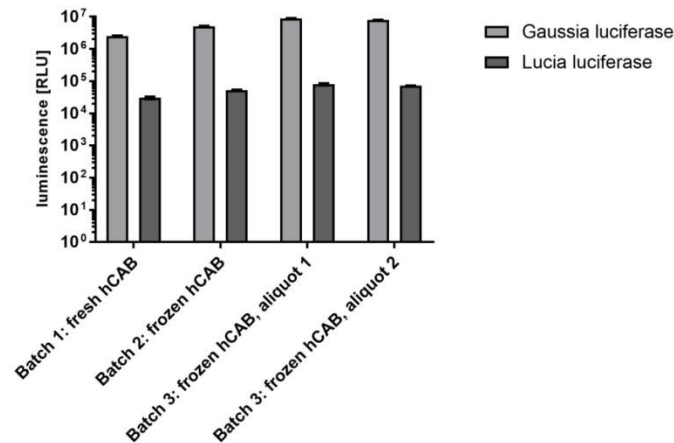


Figure 35: Gaussia luciferase expression (luminescence intensity [RLU]) of A549 cells, measured with batch 1 (fresh hCAB), batch 2 (frozen hCAB) and batch 3 (frozen hCAB, 2 aliquots). Results are expressed as means $\pm$ SD (n=3).

Figure 36 provides an overview of signal intensities emitted from 20 μ l of Gaussia supernatants. Supernatants were collected after cells had been transfected with 200 ng of pCMV-GLuc. Intensity of emitted light does not relevantly differ between experiments, respectively batches (detailed information see figure description). This implicates no relevant batch to batch variability of hCAB. Also, there is no relevant aliquot to aliquot variability. Cell transfections are reproducible.

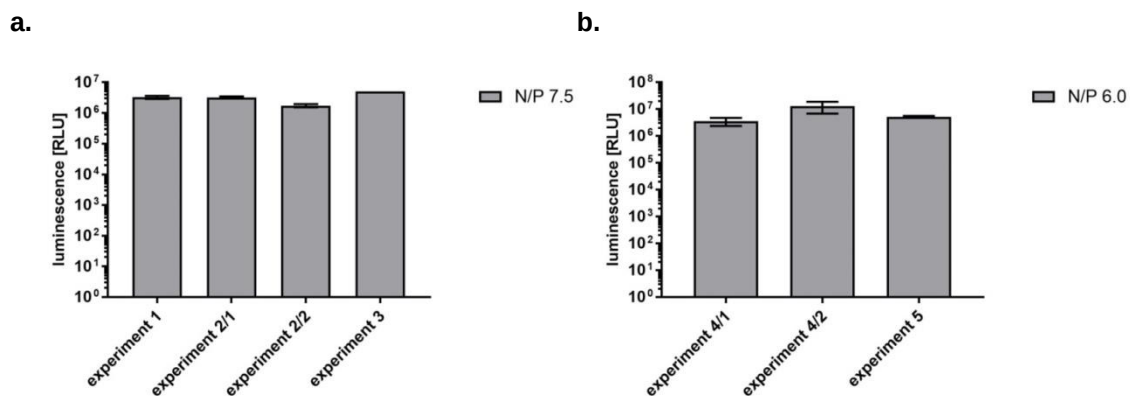


Figure 36: Reproducibility of hCAB. Four batches of hCAB were produced: batch 1 was used for experiment 1, batch 2 for experiment 2 (aliquots 1 and 2), batch 3 for experiment 3, batch 4 for experiment 4 (aliquots 1 and 2) and 5. Variability between aliquots of one batch was analyzed (aliquot to aliquot variability), no relevant variability was found (all RLUs are within same order of magnitude). Variability of two batches was compared (batch to batch variability), without finding variability. **a.** Gaussia luciferase expression after cell treatment with pCMV-GLuc/LPEI polyplexes (N/P 7.5) assayed with hCAB (experiments 1-3; experiment 2 with two replicates). **b.** Gaussia luciferase expression after cell treatment with pCMV-GLuc/LPEI polyplexes (N/P 6.0) assayed with hCAB (experiments 4 and 5 (experiment 4 with two replicates)).

7.3.3 Reproducibility of housemade Coelenterazine Assay Buffer using recombinant luciferase

A Gaussia luciferase assay with recombinant Gaussia luciferase was performed as described in 6.4.2. Gaussia luciferase activity was assayed with aliquots of two different batches of frozen hCAB, as well as with fresh hCAB. Both batches of frozen hCAB resulted in slightly higher RLUs than fresh hCAB (Figure 37). Different aliquots of buffer gave reproducible results.

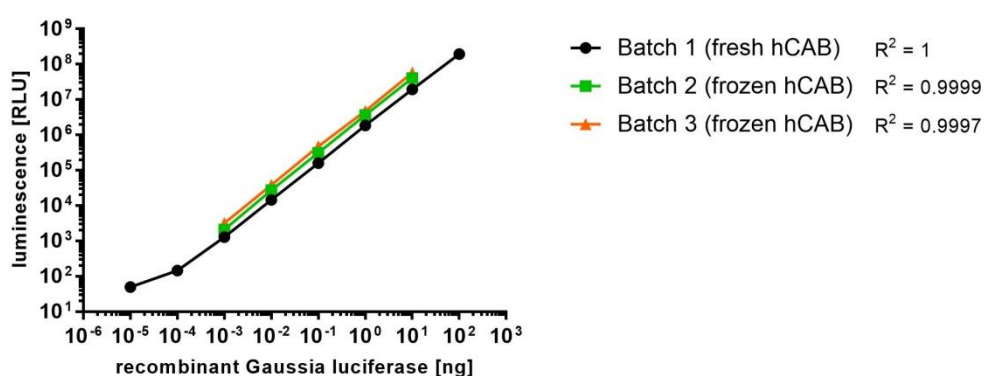


Figure 37: Luminescence [RLU] emitted by different amounts of recombinant Gaussia luciferase [ng] (Nanolight™) was assayed with fresh hCAB (batch 1) and with frozen hCAB (batches 2 and 3). Results are expressed as means $\pm$ SD (n=3).

In detail, for 1 ng of recombinant Gaussia luciferase there was only a small batch to batch variability detectable (Figure 38).

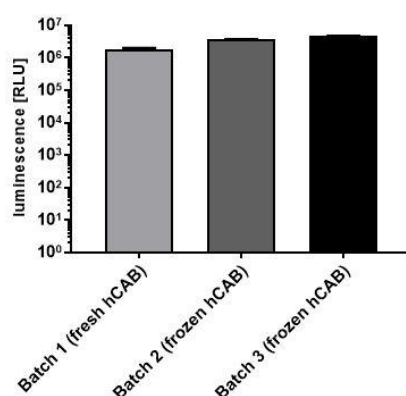


Figure 38: Luminescence [RLU] of 1ng recombinant Gaussia luciferase measured with batches of hCAB (1-3).

7.3.4 Signal acquisition settings

Since light output of different luciferases is measured with a luminometer, this requires also the possibility to adjust several luminometer parameters, such as integration time or wait time. The operator can change these settings manually,

depending on the characteristic kinetics of the luciferase type described in literature (see 4.4.1).

According to Invivogen 50 µl injection volume of substrate (hCAB) was used and the light signal of Lucia luciferase was measured with a 4 second start time and a 0.1 second reading time¹²¹. Therefore, we firstly measured light signal with a wait time of 4 sec and an integration time of 100 ms (Table 10). We explained the extended wait time due to its extended flash kinetics.

Table 10: Different settings for Gaussia and Lucia luciferase adjusted on plate reader (Tecan).

Setting #	Setting description	Wait time [sec]	Integration time [sec]
1	'Lucia setting'	4	0.1
2	'Gaussia setting'	2	10
3	'Wait time (Lucia) + Integration time (Gaussia)'	4	10
4	'Wait time (Gaussia) + Integration time (Lucia)'	2	0.1
5	'Setting used by Dr. Haider Sami'	2	1

In order to check respective settings recommended by various manufacturers, we assayed 20 µl and 5 µl of recombinant Gaussia luciferase with settings enlisted in Table 10. 2 sec wait time and 10 sec integration time were chosen as final settings. The values [RLU] are represented for each setting in Table 11.

Table 11: Settings on plate reader (Tecan) for Gaussia supernatant (20 µl and 5 µl) and respective RLU's assayed by hCAB. Values are represented as means ± SD (n = 3).

Setting (plate reader)					
Wait time	Integration time	20 µl Gaussia supernatant [RLU]	SD	5 µl Gaussia supernatant [RLU]	SD
4 sec	100 msec	2 771 104	60 108	224 698	7 116
2 sec	10 sec	2 986 941	9 3674	220 254	10 970
4 sec	10 sec	3 234 354	1 145829	250 817	11 754
2 sec	100 msec	4 414 827	623 947	221 733	12 514
2 sec	1 sec	3 784 753	565 767	205 860	8 432

Figure 39 shows light intensities obtained by applying respective settings for 20 µl supernatant containing either Gaussia or Lucia luciferase.

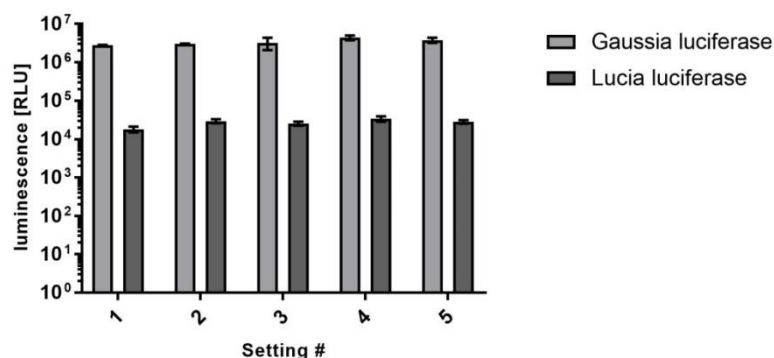


Figure 39: Luminescence [RLU] of 20 μ l supernatant (Gaussia or Lucia luciferase), recorded with different settings on plate reader (Tecan). Setting # is listed in Table 10.

The setting itself does not affect the measurement that much, because similar values were achieved (same orders of magnitude). Thus, the settings used were 10 sec integration time and 2 sec wait time for further luciferase assays. The same settings were used for Lucia luciferase because identical experiments done with Lucia obtained similar results. Final settings for all three luciferases are shown in Table 12.

Table 12: Final settings on plate reader (Tecan) for luciferases.

Luciferase	Wait time [sec.]	Integration time [sec.]
Firefly luciferase	2	10
Gaussia luciferase	2	10
Lucia luciferase	2	10

7.3.5 Kinetics of coelenterazine utilizing luciferases

20 μ l of Gaussia and Lucia supernatants were assayed each with hCAB over time. Signal was detected 2 sec after substrate injection, followed then by 4, 8, 10, 12 and 30 min of signal detection time points. Signal integration was 10 sec for each time point. Gaussia luciferase shows a flash kinetics, visualized by a peak 2 sec after injection and its rapid decay within 4 min. In contrast to this, Lucia luciferase has an extended flash. Its signal, which is generally 1-2 orders of magnitude lower than that of Gaussia remains stable over a prolonged (extended) time period (Figure 40). In summary, we could confirm that Gaussia belongs to a flash-type luciferase and Lucia is characterized by its extended flash, because it's signal intensity only slightly changed over time. Hence, it's light signal is far longer detectable than that of Gaussia luciferase.

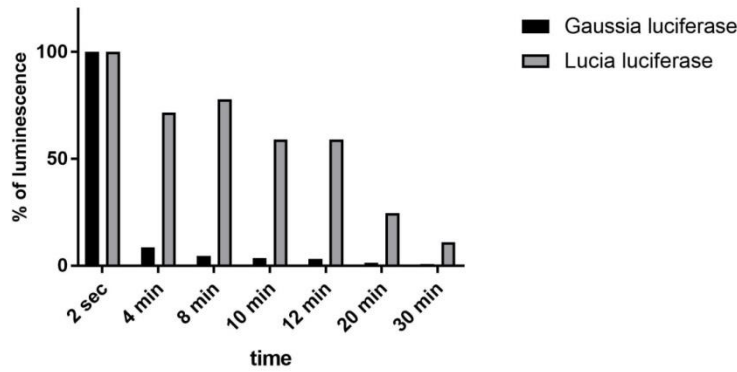


Figure 40: Luminescence [%] of Gaussia and Lucia luciferase in 20 µl supernatant (n=1), recorded over time.

7.3.6 Calculation methods for normalization of luminescence light signals

Relative light units [RLUs] have been normalized either by total protein amount of cells or by total count of live cells so far. Detailed calculation process is shown as follows:

BCA assay:

Firefly luciferase:

$$RLU \text{ per } \mu g \text{ protein} = \frac{RLU (30 \mu l \text{ lysate})}{\text{protein } [\mu g] (30 \mu l \text{ lysate})}$$

Gaussia luciferase:

$$RLU \text{ per } \mu g \text{ protein} = \frac{RLU (200 \mu l \text{ supernatant})}{\text{protein } [\mu g] (30 \mu l \text{ lysate})}$$

Flow cytometry:

Gaussia luciferase:

$$RLU \text{ per } 10\,000 \text{ living cells} = \frac{RLU (200 \mu l \text{ supernatant})}{\text{live cell count}} \times 10\,000$$

However, these normalization methods do not take RLUs from untreated cells (UT) into account. Consequently, it is questionable whether results of different samples in an experiment are comparable. Thus, a normalization factor, based on untreated cells (UT), was included:

BCA assay (UT cells):

Firefly luciferase:

$$RLU \text{ per } \mu g \text{ protein} = \frac{RLU (30 \mu l \text{ lysate})}{\text{protein } [\mu g](30 \mu l \text{ lysate})} \times \text{average protein } [\mu g] \text{ of UT}$$

Gaussia luciferase:

$$RLU \text{ per } \mu g \text{ protein} = \frac{RLU (200 \mu l \text{ supernatant})}{\text{protein } [\mu g](30 \mu l \text{ lysate})} \times \text{average protein } [\mu g] \text{ of UT}$$

Flow cytometry (UT cells):

Gaussia luciferase:

$$RLU = \frac{RLU (200 \mu l \text{ supernatant})}{\text{live cell count}} \times \text{average live cell count of UT}$$

In comparison, slight differences in normalized RLUs are seen (Figure 41). Gaussia activity normalized only by 10 000 living cells showed higher RLUs than by taking also untreated living cells into account. Latter was used as preferable method for normalization Gaussia luciferase activities in the present diploma thesis, because this method suits better to compare RLUs assayed within one luminescence measurement.

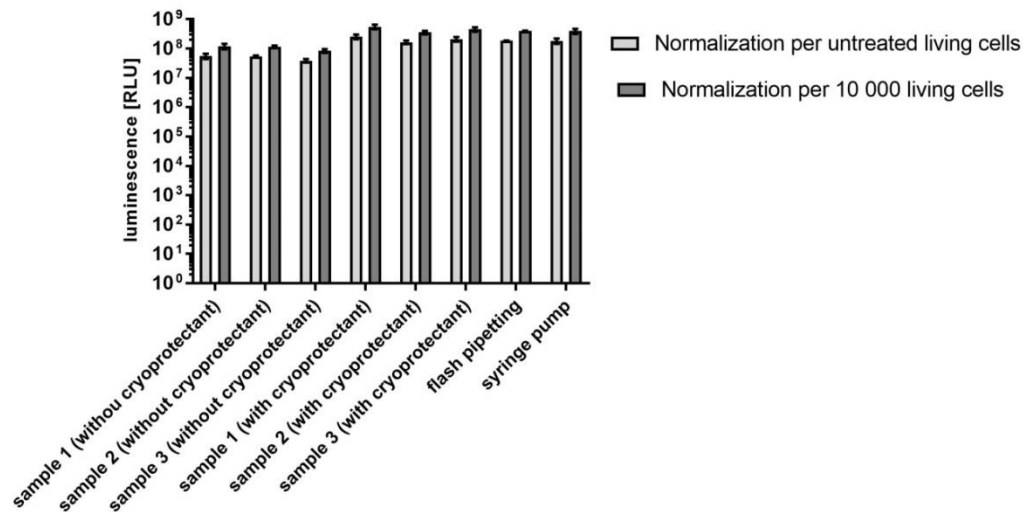


Figure 41: Gaussia luciferase expression (luminescence intensity [RLU]) of A549 cells normalized by flow cytometry. In detail, RLUs are either normalized by 10 000 cells or by taking UT cells into account. Values are taken from experiment shown in the present diploma thesis: see 7.4.1.

7.4 Applications of optimized assays

After optimization of both types of luciferase assays, potential applications were explored:

7.4.1 Effect of freeze-thawing on transfection efficiency of polyplexes

Results regarding biophysical characterization of frozen-thawed stabilized and non-stabilized polyplexes are part of the diploma thesis of Jacques Foric (diploma thesis in preparation). In summary, application of freeze-thaw cycle(s) on non-stabilized polyplexes resulted in precipitation, whereas a cryoprotectant could reduce this. It was also observed that size of non-stabilized polyplexes drastically decreased when applying one freeze-thaw cycle. For stabilized polyplexes slight decrease in size was only seen after applying more than one freeze-thaw cycle.

The biological characterization of polyplexes is presented in the following section. Treating cells only with a cryoprotectant revealed a relevant cytotoxic effect of it *in vitro* (Figure 42). However, it is questionable whether this cytotoxicity, clearly present under *in vitro* conditions, is also relevant *in vivo*, because HP- β -CD is thought to be safe and has been approved by the U.S. Food and Drug administration (FDA) for formulations intended for usage in humans¹²². This means that cytotoxic effects *in vivo* may not be as remarkable as seen *in vitro*.

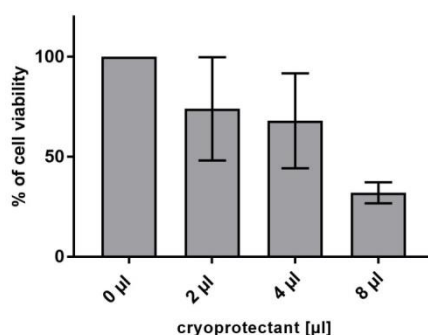


Figure 42: Cell viability [%] of A549 cells after treatment with various volumes [μl] of cryoprotectant. Dead cells were measured by Dapi-staining in flow cytometry.

Figure 43 displays viability of cells [%] after treatment with polyplexes, which have been frozen with or without a cryoprotectant for one week at -80°C.

All 6 samples (sample 1-3 without a cryoprotectant; sample 1-3 with a cryoprotectant) were thawed, added to cells and cytotoxicity was evaluated by Dapi-staining of dead cells in flow cytometry.

Stabilized polyplexes showed a higher cytotoxicity than non-stabilized samples. Freshly prepared polyplexes ('flash pipetting' and 'syringe pump') had a relatively low cytotoxicity compared to frozen stabilized polyplexes. The measurement was repeated with samples frozen for two weeks at -80°C. Similar results regarding cytotoxicity were obtained.

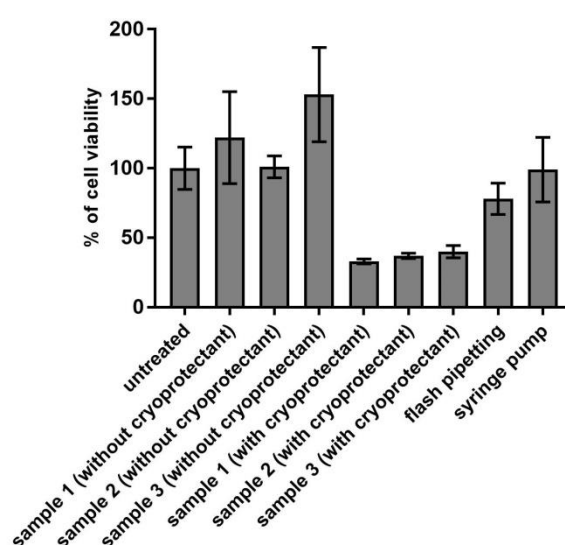


Figure 43: Viability of A549 cells [%] after treatment with polyplexes, which have been frozen with or without a cryoprotectant for one week at -80°C.

Although frozen stabilized polyplexes had a significant cytotoxic effect, their transfection efficiency is about one order of magnitude higher than that of non-stabilized polyplexes (Figure 44). Cryoprotectant stabilizes polyplexes and therefore contributes to a good transfection efficiency of thawed polyplexes.

Non-stabilized polyplexes form aggregates and therefore showed slightly lower transfection efficiency. The reason why they still led to good transfection efficiency (10^7) was that aggregates rapidly sediment on cells due to gravity. Transfection efficiency of polyplexes frozen for two weeks is comparable to that of the first week.

Regardless of preparation technique (flash pipetting, syringe pump), transfection efficiency is similarly. Frozen stabilized polyplexes do not differ in their ability to transfect cells *in vitro* compared to freshly prepared polyplexes.

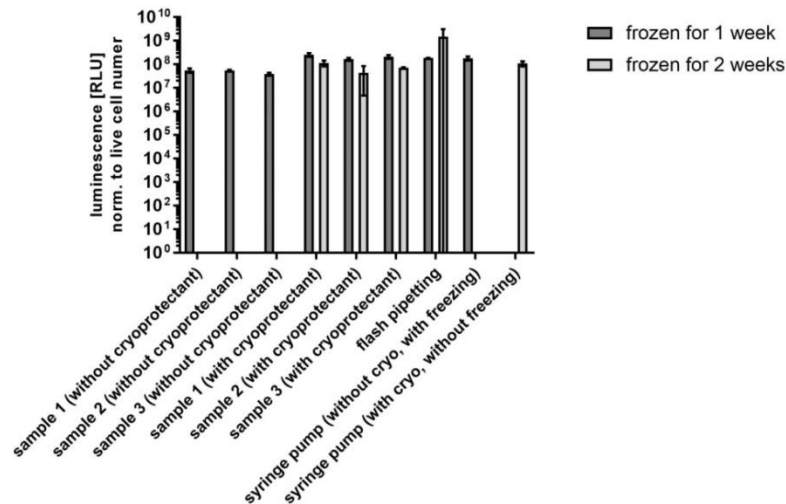


Figure 44: Gaussia luciferase expression (luminescence intensity; [RLU] normalized to live cell number) of A549 cells after treatment with LPEI/pCMV-GLuc polyplexes. Stabilized frozen polyplexes (samples 1-3) show slightly higher transfection efficiency than non-stabilized frozen polyplexes (samples 1-3). Moreover, their transfection efficiency is similar to that of freshly prepared polyplexes (flash pipetting or syringe-pump technique). The freezing length (1 versus 2 weeks) has no relevant influence on stability of polyplexes, as indicated by similar transfection efficiency. Polyplexes prepared by flash pipetting and frozen for 2 weeks are an outlier (high SD).

7.4.2 siRNA knockdown

Light intensity [RLU] of stably transduced MDA-MB-231 (PGK-EGFP-Luc) cells was determined by performing a luciferase assay of not transfected cells. 2 000, 4 000, 6 000, 8 000 and 10 000 cells per well were seeded into a 96-well plate. 48 h later cells were lysed and protein amount was assayed by a BCA assay. All in all, obtained RLUs were lower than RLUs of A549 cells, which had been transiently transfected with Firefly luciferase. This is because cell lines differ in their gene expression. As expected, RLUs increase with cell number (Figure 45).

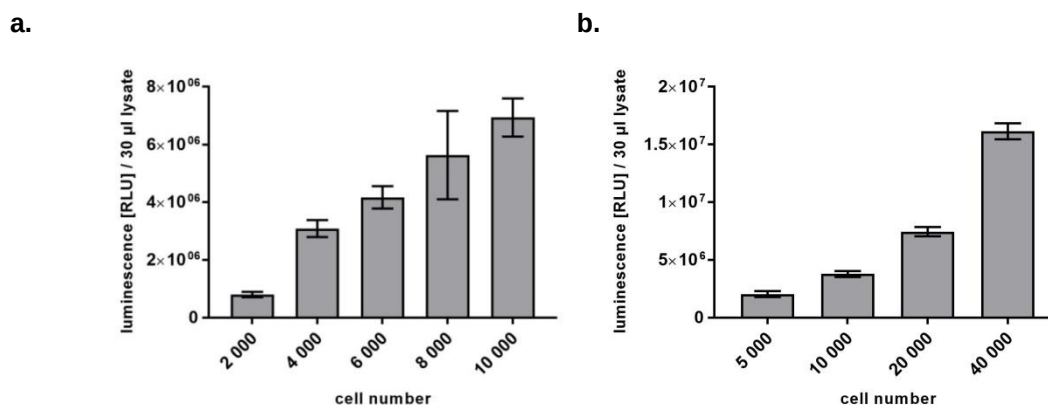


Figure 45: Firefly luciferase expression (luminescence intensity; RLU per 30 µl lysate) of different cell numbers (MDA-MB-231 (PGK-EGFP-Luc)). **a.** Luminescence from 30 µl lysate of 2 000, 4 000, 6 000, 8 000 and 10 000 cells each was measured. **b.** Luminescence from 30 µl lysate of 5 000, 10 000, 20 000 and 40 000 cells each was measured.

Normalization by protein amount gave inconsistent results because BCA assay did not always reflect correct protein amount of a cell. This occurred due to various parameters influencing the assay, e.g. protein transfer with a micropipette from one to another multiwell plate. The experiment was repeated but with different cell numbers (5 000, 10 000, 20 000, 40 000 cells/well).

RLUs (not normalized) of both experiments are displayed in Figure 45 a. and b. By comparing both graphs one can say that there is an inconsistency in emitted RLUs for a defined cell number.

A further experimental goal was to optimize the amount of siRNA in knockdown experiments of constitutive luciferase expression.

siRNA-induced downregulation of luciferase expression within this cell line was not yet tried (optimized). Since effect of siRNA treatment and the following downregulation depends on type of the cell line, the goal of this experiment was to optimize the amount of siRNA needed for MDA-MB-231 (PGK-EGFP-Luc). Moreover, a luciferase assay for siRNA-delivery should be established and optimized. Here, cells were treated with different amounts of siRNA (0.1 pmol, 1 pmol and 5 pmol) and LF[®] RNAiMAX (Invitrogen) was used as delivery vehicle. Treatment lasted either 48 h or 72 h because Invitrogen recommends a treatment of 1-3 days¹¹⁸. A BCA assay was conducted for normalization of RLUs. As negative control mismatched siRNA was used, preventing a knockdown of Luc expression. Untreated cells were used as negative control for constitutive luciferase expression.

In the first experiment 2×10^4 cells per well were seeded into 2x 96-well plates each and transfected with 0.1 pmol, 1 pmol and 5 pmol siRNA and respective amounts of negative control siRNA. Mixing volumes for generating lipoplexes are given in Table 13 and Table 14.

Percentage of downregulation for 1 pmol and 5 pmol siRNA was similar 48 h after treatment (Figure 46). Firefly signal was downregulated to approximately 10 % and after 72 h it was further decreased nearly to zero. Less siRNA (0.1 pmol) led to 46-56 % knockdown for both treatment periods.

A relevant difference in knockdown regarding duration was seen. Treatment for 72 h resulted in a slight greater extent of knockdown (experiment 1).

Cytotoxic effects of lipoplexes between the experiments differ. Therefore, it is difficult to determine whether 1 pmol or 5 pmol of siRNA is best to use in future experiments.

Further investigations regarding cytotoxic effects would be recommended.

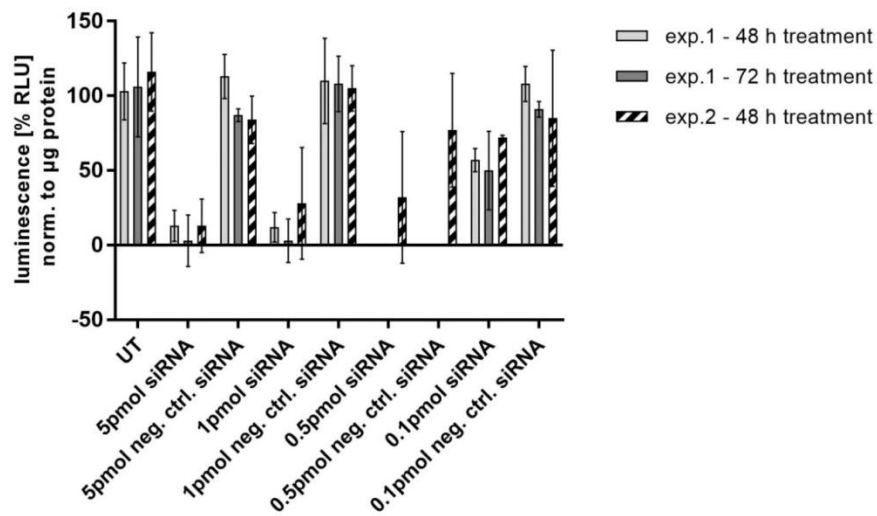
Table 13: Volumes of siRNA stocks and negative control siRNA stocks used for preparing lipoplexes. Underlined values are used for sample preparation.

Sample #	siRNA/neg.ctrl. siRNA per well [pmol]	siRNA/neg.ctrl. siRNA volume (1µM) per well [µl]	Number of samples	Total volume of siRNA/neg.ctrl. siRNA (1 µM) [µl]	Opti-MEM [®] volume [µl] added to fill up to 25 µl	Volume of siRNA/neg.ctrl. siRNA diluted in Opti-MEM [®] [µl]
1	5.0	5.0	4	<u>20.0</u>	<u>5.0</u>	25.0
2	1.0	1.0	4	<u>4.0</u>	<u>21.0</u>	25.0
3	0.1	0.1	4	<u>0.4</u>	<u>24.6</u>	25.0

Table 14: Volumes of Lipofectamine[®] RNAiMAX stocks used for preparing lipoplexes.

Sample #	LF [®] RNAiMAX [µl]	Number of samples	Total volume of LF [®] RNAiMAX [µl]	Opti-MEM [®] volume [µl] added to fill up to 25 µl	Volume of LF [®] RNAiMAX diluted in Opti-MEM [®] [µl]
1	1.5	4	<u>6.0</u>	<u>19.0</u>	25.0
2	0.3	4	<u>1.2</u>	<u>23.8</u>	25.0
3	3.0 (1:10 dilution of LF [®] RNAiMAX)	4	<u>12.0</u>	<u>13.0</u>	25.0

a.



b.

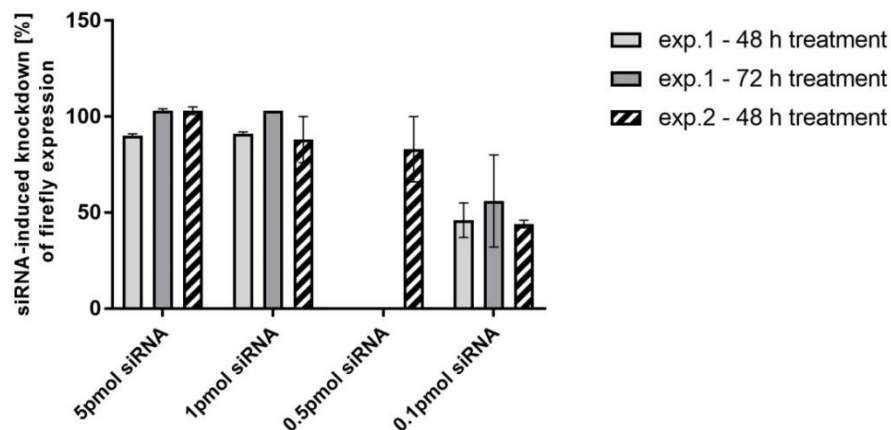


Figure 46: siRNA-induced downregulation of Firefly luciferase expression in MDA-MB-231 (PGK-GFP-Luc). **a.** Remaining luminescence [% RLU normalized to µg protein] after treatment with 5 pmol, 1 pmol, 0.5 pmol, 0.1 pmol siRNA for 48h or 72h. **b.** Knockdown [%] of 5, 1, 0.5 and 0.1 pmol siRNA after 48 h or 72 h treatment.

7.4.3 Testing liposome mediated delivery of plasmid DNA and siRNA

The experiment was divided into two sub-experiments (1 and 2) (Figure 47).

In experiment 1 the ability of liposomes delivering plasmid DNA (pCMV-GLuc) into A549 cells was tested and pCMV-GLuc/LPEI polyplexes served as positive controls.

Experiment 2 tested siRNA delivery by liposomes into MDA-MB-231 (PGK-EGFP-Luc) and lipoplexes made of siRNA/LF[®] RNAiMAX served as positive control.

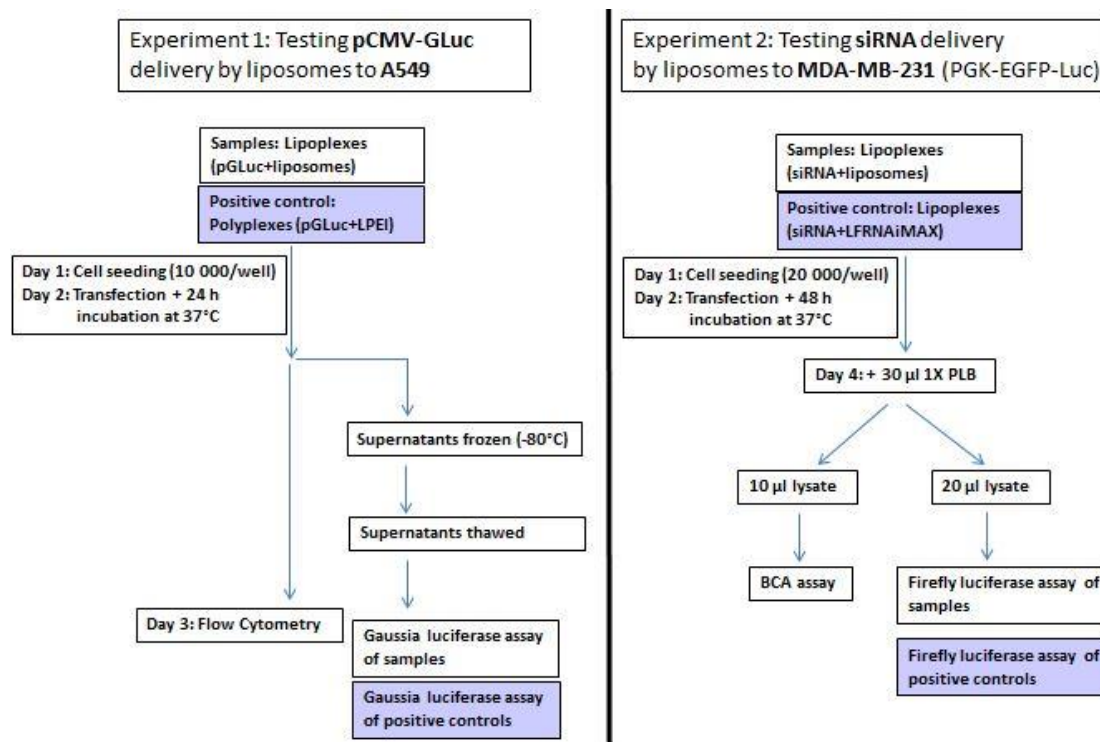


Figure 47: Schedule of experiment with liposomes, divided into two sub-experiments. pGLuc = pCMV-GLuc.

Both experiments were set up on same day.

For experiment 1, 1×10^4 cells (A549) per well were seeded into a transparent 96-well plate and incubated overnight so that a cell density of approximately 70 % was reached next day. Cells were transfected with 200 ng pCMV-GLuc per well (in triplicates). For pCMV-GLuc delivery via LPEI (positive control), polyplexes of N/P ratio 6 were generated in HBS and HBG and 10 μ l of each polyplex solution was added to cells in 90 μ l basal medium (without applying an incubation time). Remaining steps are performed as described in 6.7.2.

For siRNA based gene delivery via cationic liposomes, lipoplexes of N/P ratio 6 and 12 were generated in HBS. 8 μ l of lipoplex solution was added to cells in 92 μ l basal medium. As for positive control, after incubation at 37°C for 4 h, 100 μ l of complete medium was added to each well and cells were incubated overnight at 37°C.

The following day, supernatant was removed and total count of live cells was obtained by performing a flow cytometry (6.9). Gaussia luciferase activity was assayed from 20 μ l supernatant with frozen hCAB.

Delivery of pCMV-GLuc by liposomes has not worked as judged by the height of detected luciferase signal which is equally to that of negative control (only

cationic liposomes) or untreated cells (Figure 48). Of great importance is that delivery of Gaussia plasmid by LPEI has worked, which indicates correct handling of all experimental steps. Also importantly, housemade Assay Buffer (hCAB) has worked.

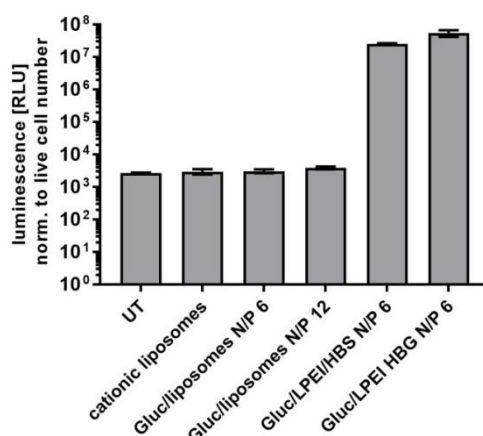


Figure 48: Gaussia luciferase expression (luminescence intensity [RLU] normalized to live cell number) of A549 cells after transfection by different vehicles (cationic liposomes or LPEI).

In experiment 2, 2×10^4 cells (MDA-MB-231) per well were seeded into a white 96-well plate. Cell transfection with siRNA/LF[®] RNAiMAX lipoplexes (5 pmol siRNA/well) served as positive control and was done on the following day. Unlike described in 6.6.4, a 10 μ M dilution from a 1 mM siRNA/negative control siRNA stock was prepared. Concentration was determined by UV spectroscopy (siRNA 8 μ M and negative control siRNA 7.3 μ M). A second dilution (1 μ M) of each was prepared in Opti-MEM[®] and used for generation of lipoplexes (see 6.6.4).

Lipoplexes of siRNA/cationic lipids and negative control siRNA/cationic lipids were prepared. As done in experiment 1, two N/P ratios (N/P 6 and 12) were tested. 20 μ l of lipoplex solution was diluted in HBS (1:10) and 20 μ l was then added to cells in 80 μ l basal medium. Cells were incubated at 37°C for 4 h. 100 μ l complete medium was added after 4 h and cells were incubated at 37°C for 48 h. On day 4 protein amount was estimated by BCA assay and extent of downregulated Firefly luciferase signal was determined by a luciferase assay (see 6.8.2).

Unfortunately, light output was not downregulated by liposome-mediated siRNA delivery (Figure 49). However, LF[®] RNAiMAX mediated siRNA delivery resulted in a downregulation of signal to approximately 4 %. Negative control lipoplexes

did not show any decreased light signal, as expected. This experiment also proves that hLAB has been produced correctly.

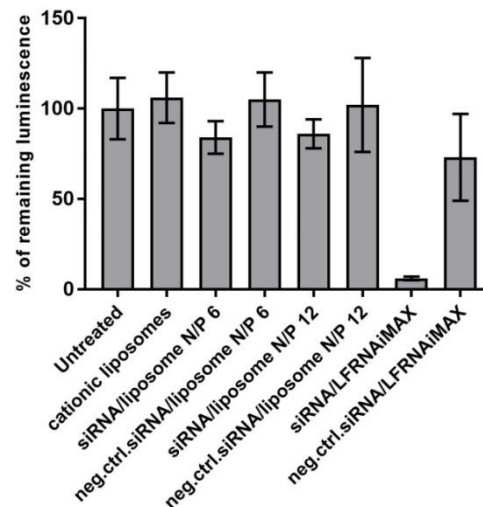


Figure 49: Remaining luminescence [% RLU] after siRNA-induced knockdown. Downregulation via liposome-mediated siRNA delivery only slightly worked (~ 10 % downregulation), whereas the positive control LF[®] RNAiMAX successfully led to a downregulation of 94 %.

7.4.4 Dual luciferase assay

MDA-MB-231 (PGK-EGFP-Luc) cells have been stably transduced with the fusion gene for the fluorescent protein EGFP and the Firefly luciferase (Luc), driven by the promotor Phosphoglycerate kinase. The goal was a co-transfection of these cells with pCMV-GLuc and Luc siRNA, mediated by LPEI. A dual luciferase assay should enable us on the one hand to detect a downregulation of Firefly luciferase signal due to siRNA delivery and on the other hand to detect a Gaussia luciferase signal as a result of pCMV-GLuc delivery. Both, double-stranded plasmid DNA and double-stranded RNA were complexed with LPEI to an N/P ratio of 6. As positive control, lipoplexes of LF[®] 2000 complexed with pCMV-GLuc and Luc siRNA were also generated.

1x 10⁴ cells (MDA-MB-231) per well were seeded into a white 96-well plate. The following polyplex solutions were prepared (Table 15) and expected effects on protein expression are listed.

Table 15: Expected luminescence signals after cell treatment with polyplexes containing Luc siRNA and for pCMV-GLuc.

Treatment of MDA-MB-231 (PGK-EGFP-Luc)	Firefly luciferase signal (expected)	Gaussia luciferase signal (expected)
Untreated cells	High	Zero
pCMV-GLuc /Luc siRNA/LF [®] 2000	Zero	High
pCMV-GLuc /neg.control Luc siRNA/LF [®] 2000	High	High
pCMV-GLuc /LF [®] 2000	High	High
pCMV-GLuc /Luc siRNA/LPEI	Zero	High
pCMV-GLuc /LPEI	High	High

From siRNA/negative control siRNA (1 mM stock each) a 1 μ M dilution in MQ-water was prepared. Since molecular weight of siRNA is 14383.82 g/mol, 1 μ l of 1 μ M dilution contains 1 pmol siRNA/neg. control siRNA. Per 200 ng pCMV-GLuc 1 pmol siRNA/negative control siRNA was co-transfected. LPEI amount required for N/P ratio of 6 was calculated by taking 200 ng pCMV-GLuc as well as 14.38 ng siRNA/neg. control siRNA into account. Thus, LPEI [ng] for one sample was calculated as follows:

$$mass(LPEI) = \frac{214.38 \text{ ng}}{330} \times 43 \times 6 = 167.60 \text{ ng}$$

Cells were transfected in triplicates and the detailed composition of each nanoparticle solution is shown in Table 16.

Table 16: Composition of nanoparticles consisting of LPEI, pDNA and siRNA.

Total volume [μ l]	pDNA conc. [μ g/ml]	pDNA [ng]	pDNA solution for complexation	LF 2000 solution for complexation	Lipoplex solution per well [μ l]
pCMV-GLuc/Luc siRNA/LF[®] 2000 (for negative control siRNA respectively): volumes for 3 wells					
30	20	600	7.11 μ l Opti-MEM [®] + 3 μ l siRNA/neg.ctrl siRNA (1 μ M) + 4.89 μ l pCMV-GLuc	13.20 μ l Opti-MEM [®] + 1.8 μ l LF [®] 2000	10
pCMV-GLuc/ LF[®] 2000: volumes for 3 wells					
30	20	600	10.11 μ l Opti-MEM [®] + 4.89 μ l pCMV-GLuc	13.20 μ l Opti-MEM [®] + 1.80 μ l LF [®] 2000	10
pCMV-GLuc/Luc siRNA/LPEI (for negative control siRNA respectively): volumes for 4 wells (+ 1 extra for pipetting loss)					
40	20	800	9.47 μ l HBG + 6.53 μ l pCMV-GLuc + 4 μ l siRNA	15.26 μ l HBG + 4.74 μ l LPEI	10
pCMV-GLuc/LPEI: volumes for 4 wells (+ 1 extra for pipetting loss)					
40	20	800	13.47 μ l HBG + 6.53 μ l pCMV-GLuc	15.58 μ l HBG + 4.42 μ l LPEI	10

LF[®] 2000 based lipoplexes were incubated for 5 min. LPEI based polyplexes were added to cells immediately after preparation (no incubation time). After 4 h incubation at 37°C 100 µl complete medium was added to each well and 24 h after treatment the Gaussia supernatant was removed and frozen at -80°C. Cells were lysed and BCA assay and Firefly luciferase assay were performed on the same day.

The main goal of this experiment was to employ the optimized Firefly luciferase assay and Gaussia luciferase assay as a dual luciferase assay in the same experiment. Towards this, transfection ability of LPEI for plasmid DNA (pCMV-GLuc) and siRNA (against firefly luciferase) was studied in MDA-MB-231 cells stably expressing PGK-EGFP-Luc.

Transfection of pCMV-GLuc via LPEI and LF[®] 2000 has worked well because Gaussia light signal has been detected, irrespective of simultaneous co-transfection (Figure 50). Similarly, Firefly luciferase signal can also be observed (Figure 51), indicating that these two assays can be combined into a Dual assay within an experiment. However, downregulation of Firefly luciferase gene expression by siRNA was difficult to assess because of inherent toxicity of nanoparticles which also leads to erroneous protein normalization. Consequently, a decrease in Firefly signal can result from both, a successful downregulation or from cell death. This experiment shows again that BCA assay of protein determination suffers from reproducibility problems due to inconsistency in obtained protein values of cell lysates.

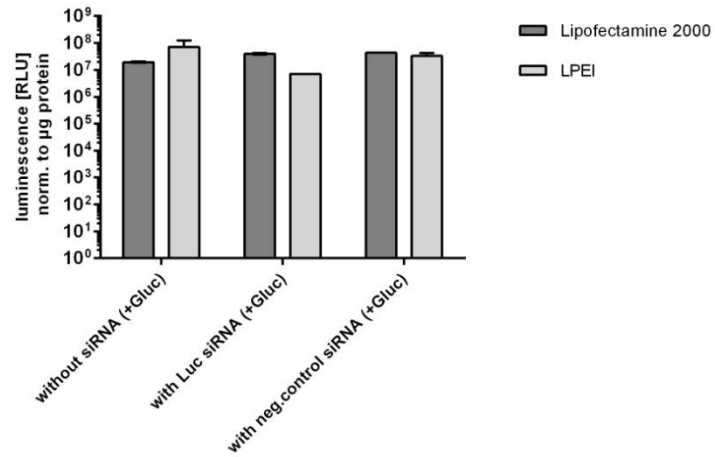


Figure 50: Gaussia luciferase expression (luminescence intensity [RLU] normalized to μg protein) of A549 cells after delivery by LPEI (pCMV-GLuc/LPEI) or LF[®] 2000 (pCMV-GLuc/LF[®] 2000) or with simultaneous co-transfection with siRNA/neg.control siRNA.

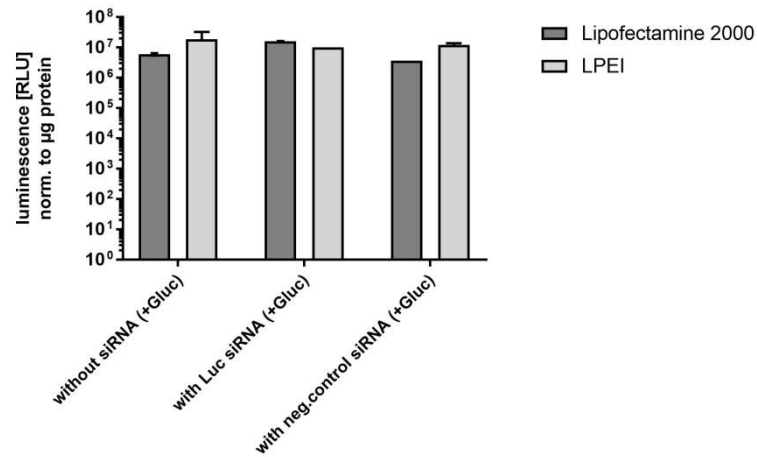


Figure 51: Firefly luciferase expression (luminescence intensity [RLU] normalized to μg protein) of MDA-MB-231 (PGK-EGFP-Luc) cells after delivery by LPEI (pCMV-GLuc/LPEI) or LF[®] 2000 (pCMV-GLuc/LF[®] 2000) or with simultaneous co-transfection with siRNA/neg.control siRNA.

8 Discussion

The present diploma thesis aimed at optimizing various aspects of specific laboratory techniques used in gene therapy.

Optimization of BCA assay

We found that thorough lysis of cells is of paramount importance in determination of cellular protein content. Promega recommends treating cells cultured in a 96-well plate with 20 µl of 1X Passive Lysis Buffer for 15 min in order to lyse them properly. Though effectiveness of cell lysis depends on the cell type and lysis process should be individually optimized for each cell line, Promega found that in most cell lines luciferase was sufficiently solubilized after 15 min treatment. Also, Promega could not find differences between passive procedures (lysis with detergents) and active procedures (mechanical lysis such as freezing or scraping cells) for achieving optimal cell lysis¹¹⁰.

Lysis of cells was not optimized yet and therefore an additional freezing step was added right after treating with cell lysis buffer. Successful lysis was only assessed by checking cell morphology in microscope so far. Promega describes lysed cells as follows: either cells have lost their typical morphological shape because of dissolved cell structure, or cell silhouettes are still recognizable, or accumulated cell debris is floating¹¹⁰. In our case, cell aggregates on the bottom in the middle of the cultured well indicated a successful cell lysis. Inefficient cell lysis was indicated by the appearance of not aggregated, well-shaped cells, spread over the whole well.

Promega states that overgrown cells or some cell types are more resistant towards commonly applied lysis conditions. Therefore our observation of not successfully lysed cells might be due to insufficient treatment. As recommended by Promega, we increased volume of lysis buffer and extended lysis period¹¹⁰.

Two different proprietary lysis buffers have been compared and cell lysis was assessed by the amount of protein found in cell lysate. Treating cells first with 1X Passive Lysis Buffer (PLB) and applying a freezing step right afterwards did not lead to an increased protein amount. In contrast to this, using 1X Cell Culture Lysis Reagent (CCLR) without freezing led to higher protein amounts, corresponding to a more complete lysis.

However, it is important to point out, that the BCA assay is influenced by many factors, which lead to high background. For example, dithiothreitol (DTT) is a compound of CCLR¹²³. It poses the risk of causing false positive protein values, resulting in a loss of sensitivity of BCA assay. As a strategy to overcome this hurdle we included iodoacetamide, which alkylates its thiol groups and therefore blocks its reducing potential. Hence, thiol groups are not able to reduce Cu^{2+} to Cu^+ which subsequently makes complexation with BCA molecules impossible¹⁰⁹. As a result, the intensity of purple background colour should be reduced. Indeed, this was found when performing BCA assays in presence of iodoacetamide: When cells had been lysed with PLB, addition of iodoacetamide to the lysate did not influence the protein outcome. Conversely, iodoacetamide affected the BCA assay based on CCLR-lysates. Lower absorption values and protein values respectively, were obtained. This resulted from iodoacetamide, which has alkylated DTT in CCLR and thus the interfering background activity was minimized. It was also observed that the speed of colour change from green to purple was influenced by presence of iodoacetamide. Addition of green coloured Working Reagent to a CCLR based lysate devoid of iodoacetamide resulted in a rapid colour change to purple. Conversely, in presence of iodoacetamide this colour change was very slow. These observations indicate that iodoacetamide has inactivated interfering substances, which resulted in a less rapid purple colour development.

Summing up, the BCA protocol was updated in respect to the following points:

- 30 μl 1X Lysis Buffer for cell lysis. In contrast to 50 μl 0.5X Lysis Buffer, which had been applied so far, a higher concentrated buffer was found to suit better. But, a lower volume has to be used. Otherwise, it would affect cell viability adversely.
- 30 min treatment with 1X Lysis Buffer on a plate shaker (500 rpm). It has been observed that prolongation of the originally used 20 min up to 30 min can improve cell lysis, which was confirmed by occurrence of more cell aggregates in microscope.
- No freezing step after detergent-mediated cell lysis. Freezing did not significantly increase the amount of protein in the lysate in case of 1X PLB,

which was used in this diploma thesis. Moreover, freeze-thaw cycles might damage luciferase enzymes¹¹⁰.

- Iodoacetamide is not needed for a BCA assay which quantifies protein amount in a 1X PLB-based lysate. Unlike this, 1X CCLR needs iodoacetamide because of presence of interfering substances.

Promega dissuades using 1X CCLR for performing protein assays because of high amounts of DTT contained¹²³. Moreover, there are few points which indicate that 1X PLB is preferably suited as lysis reagent, particularly when performing a luciferase assay:

- First of all, it was observed that 1X CCLR foamed during pipetting. Onward, a pipetting loss was inevitable.
- Second, Promega urgently recommends lysing cells with PLB and not with CCLR as the latter would inhibit activity of Renilla luciferase and would increase coelenterazine's autoluminescence during a Promega's dual luciferase assay¹²³.
- And last but not least, skipping the addition of iodoacetamide shortens the time needed to perform the BCA assay.

Few weaknesses of the BCA assay have been noted:

- First, the linearity of BCA assay with respect to cell number revealed that cell numbers ranging from 10 000 up to 40 000 cells are within the linear range, whereas 100 000 cells are not.
- Next, amounts of protein obtained from the same cell numbers differ between separate experiments, resulting in inconsistency of protein measurements. A reason might be that transfer of lysate between multiwell plates harbors the risk of incomplete transfer of proteins. Though lysate is mixed thoroughly, cell debris can also be transferred. This could influence absorption measurement.
- Furthermore, a critical point might be the time period of cooling down after incubating the lysate with Working Reagent for 30 min. The kit prescribes to cool down the plate to RT. I have left the plate for 9-10 min at RT in all BCA assays. Nevertheless, temperature influence has not been investigated but might be a relevant influencing factor on measured protein quantity.

Optimization of Firefly luciferase assay

The commercial assay buffer for Firefly luciferase from Promega is quite costly and its exact composition in terms of components and amounts is undisclosed. However, several protocols for production of noncommercial buffers exist, such as one described by Dyer et al.⁸⁹. In the present diploma thesis the hLAB was produced following an in-house optimized protocol as suggested by University of Birmingham⁹⁷. For a successful preparation of hLAB it is crucial to use freshly prepared 1M Glycylglycine.

Experiments with recombinant Firefly luciferase as well as with luciferase expressed by cells showed that the activity of hLAB was as good as of cLAB (Promega). However, storage of fresh hLAB for longer than two days at +4°C might result in reduced RLUs. Its stability for long-term storage at -80°C was also analyzed, because Promega points out that repeated freeze-thaw cycles of cLAB (LAR II) decrease its activity¹¹⁰. We found that storage of either only the Buffer (B) or the mixture of Luciferin and Buffer (LB) for up to three months at -80°C did not relevantly decrease activity, compared to cLAB. Due to convenience it was decided to freeze LB for future applications. Storing frozen aliquots of hLAB is time-saving as preparation time for the buffer is concerned, and moreover, it is also cost-saving because it is not necessary to buy cLAB from commercial sources.

It was shown that 100 µl injection volume of hLAB is required in order to achieve the same orders of signal intensity as with 50 µl of cLAB. 100 µl of hLAB resulted in higher RLUs than using only 25 µl or 50 µl.

Across several cell transfection experiments one can say that transfections are reproducible and different (frozen) batches or different frozen aliquots of hLAB showed similar activities. In conclusion, luciferase assays are reproducible using hLAB. Also, there is only low batch to batch and no aliquot to aliquot variability of hLAB.

One nanogram of recombinant Firefly luciferase was assayed with different batches of hLAB and same orders of magnitude were achieved (10^5 RLU). This proves reproducibility of hLAB.

According to Navarro et al.¹²⁴, two nanograms of recombinant luciferase correspond to 10^7 light units. In the present diploma thesis, 1 nanogram of recombinant luciferase (Promega) corresponds to 10^5 light units.

Optimization of coelenterazine based luciferase assays

Housemade Coelenterazine Assay Buffer (hCAB) can be frozen at -80°C and reused after thawing up, without loss in activity compared to commercial CAB (cCAB). Only 50 μl of Stop & Glo[®] was used instead of recommended 100 μl by Promega¹¹⁰ because injection volume had been optimized previously in this department. Interestingly, frozen hCAB performed even better than fresh hCAB. This was shown with recombinant luciferase as well as with luciferase expressed by transfected cells. Some batch to batch variability was also found. No clear aliquot to aliquot variability was seen. The question arises whether hCAB should be frozen for prospective applications at all – or simply prepared fresh. Preparation of hCAB is simple; one must only dissolve coelenterazine stock (5 mg/ml) in PBS supplemented with 5 mM sodium chloride (pH 7.2). Incubation of CTZ in the dark is highly important in order to let auto-oxidation of coelenterazine equilibrate. It is also recommended to degas the PBS before usage in order to minimize its content of dissolved oxygen¹²⁵. However, this was not done in this diploma thesis, but should be included in further optimization studies.

In the Dual Luciferase Assay from Promega Firefly and Renilla light signals are consecutively detected by dispensing first 100 μl LAR II in order to measure Firefly luciferase activity, followed by an injection of 100 μl Stop & Glo[®] for quenching Firefly activity and measuring Renilla activity¹¹⁰. For assaying only Renilla activity, Promega still recommends combining both substrates^{110,120}. We implemented this recommendation by assaying Gaussia supernatant with 100 μl of a mixture of LAR II and Stop & Glo[®] and got higher RLUs than using Stop & Glo[®] alone. This indicates that Gaussia activity should be measured with a mixture of both commercial substrates in further measurements. The reason for higher signals implicates that apart from luciferin and CTZ the commercial substrates also contain some additional agents which might contribute to a boosted light signal, when combined. Same observations were made with Lucia supernatant.

As final settings for the plate reader, a 2 sec wait time and a 10 sec integration time was found for luciferases with flash kinetics. These settings have also been recommended by Promega¹¹⁰, Tecan¹²⁶ and MGM Instruments¹²⁷. New England Biolabs recommends an integration time of 2-10 sec but a longer wait time¹²⁸.

The flash kinetics of Gaussia luciferase and Lucia's extended flash were successfully shown (Figure 40). Results also correspond to those found in literature. Binkowski¹²⁹ showed that signal intensity of Gaussia's flash has dropped to approximately 10% after 20 min, 1200 sec respectively (Figure 52). This is in line with Figure 40, where signal has almost dropped down to 0% after 20 min.

Other figures from literature (Tannous et al⁹⁰, Figure 53 and Invivogen, Figure 54 and Figure 55) display similar light intensities as measured in this diploma thesis after 10 min, 600 sec respectively. All in all, kinetics of Gaussia was confirmed by our results.

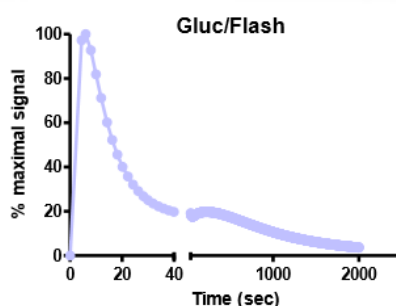


Figure 52: Flash signal emitted by Gaussia luciferase; figure from reference ¹²⁹.

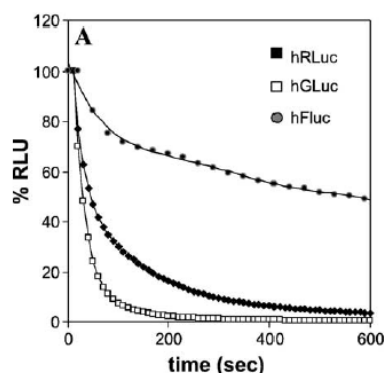


Figure 53: Light signals emitted from humanized forms of Renilla (hRLuc), Gaussia (hGLuc) and Firefly (hFLuc) luciferase; figure from reference ⁹⁰.

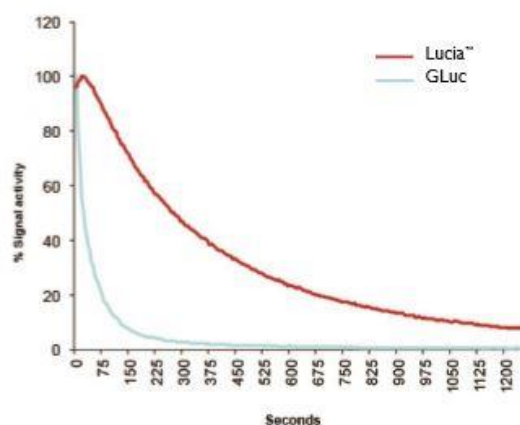


Figure 54: Kinetics of Gaussia and Lucia luciferase; figure from reference ⁶¹.

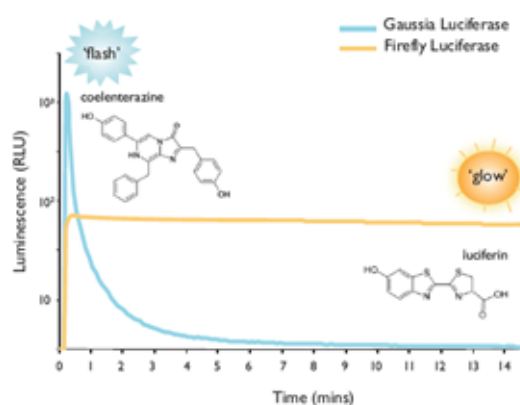


Figure 55: Kinetics of Gaussia and Firefly luciferase; figure from reference ⁶¹

Lucia plasmid was validated by doing a diagnostic restriction digest and gel electrophoresis revealed that transfected E.coli contained correctly amplified Lucia plasmid.

The transfection with LPEI based polyplexes as well as with positive control substances has worked successfully. Nevertheless, signal intensity was lower than that of Gaussia luciferase. But both signal intensities cannot be compared because both plasmid backbones have completely different promoter-enhancer combinations. pCMV-GLuc is optimized for expression in mammalian cells, whereas pCpGfree-promotor-Lucia is not yet optimized, therefore shows only a basal expression of Lucia luciferase. This might be a reason for its weak light signal across all transfection experiments in the present diploma thesis.

It has to be remarked that in transfections conducted by Invivogen (plasmid: pSELECT-Lucia-zeo; cell lines: HEK293, B16, RAW 264.7), supernatants with

secreted Lucia were collected after 48 h and not already after 24 h as done in transfection experiments of this diploma thesis⁶¹. Maybe higher light signals can be measured if the supernatant is collected after 48 h treatment.

However, we reached successfully our main goal in showing that the substrate is usable for Lucia luciferase.

Effect of freeze-thawing on transfection efficiency of polyplexes

LPEI based polyplexes are not stable in aqueous solution because they tend to aggregate, thus preparing fresh polyplexes prior to each administration *in vivo* is of great importance. Nevertheless, preparing fresh polyplexes manually via flash pipetting results in batch to batch variability and therefore different product qualities of polyplex formulations are of major concern because it also affects *in vitro* and *in vivo* gene delivery^{15,130,134}.

Although Taschauer et al.¹³ has shown that an up-scaled synthesis of polyplexes is possible via syringe-pump technique, freezing of polyplexes would be an additional advantage. Long-term storage at -80°C would be advantageous for shipping polyplexes between laboratories or preparing huge batches in advance for animal experiments. Freezing without a cryoprotectant leads to aggregation of polyplexes, resulting in reduced *in vitro* transfection efficiency^{134,131,132,133}.

Kasper et al. reported that lyophilization is a very effective method to keep freeze-dried polyplexes for long-term storage without loss of stability. The cryoprotectant HP- β -CD in combination with sucrose at isotonic concentrations was used to preserve size of polyplexes and therefore also their transfection efficiency¹³⁴.

In the present diploma thesis, polyplexes stabilized with HP- β -CD and non-stabilized polyplexes were frozen-thawed. Analysis of size and ζ -potential was part of another thesis and *in vitro* transfection experiments were performed in the present diploma work. They revealed that frozen stabilized polyplexes have a slightly higher transfection rate compared to non-stabilized ones and, moreover, their efficiency is equally well compared to freshly prepared polyplexes. However, the cryoprotectant seems to be quite toxic *in vitro*. On the other hand, the stabilizing effect on polyplexes outweighs its cytotoxicity and contributes to their higher transfection efficiency.

siRNA knockdown

For investigating delivery efficiency of siRNA into cells via various transfer vehicles a straightforward and well-established assay is needed. For research at MMCT, the already established protocol for Firefly luciferase assay (see 6.8.1) was used to further optimize it for siRNA delivery (see 6.8.2). Therefore, Invitrogen's protocol¹¹⁸ was used for conducting a transfection with LF[®] RNAiMAX. It worked successfully and best knockdown of luciferase expression was seen for 1 pmol and 5 pmol siRNA, both inducing a downregulation of similar extent. Cytotoxic effects must be further analyzed.

9 Appendix

9.1 Standard Operation Procedures

The following SOPs were used in the present diploma thesis. Equipment as listed in 6.1 was used.

9.1.1 Measurement of absorption and luminescence

Background:

Nowadays, most research institutions use a multipurpose plate reader, which enables scientists applying various measurement techniques, e.g. absorption, luminescence or fluorescence.

A luminometer is a device for measuring luminescence light and it consists of a chamber for the sample, a detection unit and a processor for the light signal¹³⁵.

A single tube or a well plate is placed into a sealed sample chamber. Ambient light is excluded otherwise it would interfere with emitted light. Therefore the chamber is sealed¹³⁵. Also, according to Tecan¹²⁶, exposure of a white well plate to laboratory light prior to a luminescence measurement must be avoided because it might lead to phosphorescence of white plate material during the measurement in the dark sample chamber. This would result in a false too high luminescence signal. On the other side, Tecan states that the use of white (opaque) well plates for luminescence measurements is important because of boosting the signal¹²⁶. Additionally, there should be no crosstalk or spill over in-between two (neighbor) luminescence samples placed in adjacent wells.

The sample chamber is as closely as possible near the detector in order to facilitate a maximum of measured signal¹³⁵.

The luminescence signal is usually detected by a photomultiplier tube (PMT)¹³⁷. It enables amplification of signals of weak intensity. A photon strikes an electron out of a cathode, which is subsequently accelerated to a dynode because of electric field. There it causes ejection of several electrons and these secondary electrons are then accelerated to the second dynode, which is held at a slightly lower potential. Here, they cause again knock out of electrons. Hence, one electron triggers an amplified cascade and finally electrons hit the anode¹³⁶.

Either the photons are counted (photons per sec) with a PMT or a current as described above is measured by a PMT. When photons are processed as current, the signal is displayed as arbitrary light units, also known as Relative Light Units (RLU)¹³⁷.

As shown in Figure 56, the fiber bundle collects emitted photons and guides them through a filterwheel towards the detection unit of a photomultiplier tube (PMT). Wells which should be measured are marked on a corresponding template of a 96-well plate in the software (i-control™–Microplate Reader Software, Tecan) and the z-position of the fiber bundle is then adjusted accordingly. This is important regarding the fact that luminescence light can be refracted at the liquid surface of the sample. Therefore, the z-adjustment is advantageous because it provides a maximized signal to noise ratio¹³⁸.

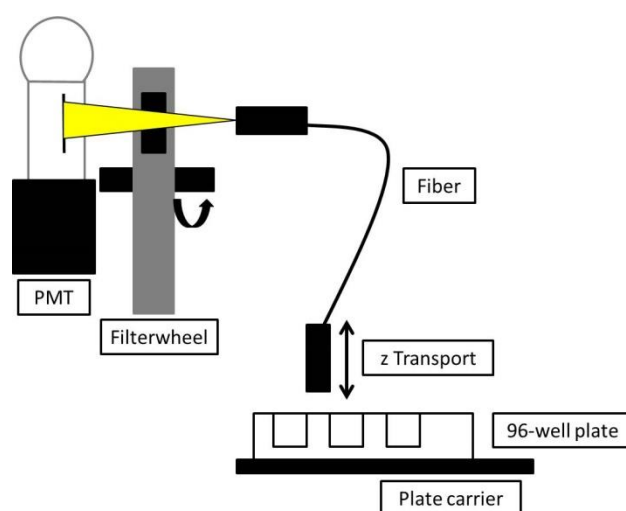


Figure 56: Design of Tecan® Infinite 200 PRO. Adapted from Tecan Austria GmbH¹³⁸. PMT: Photomultiplier.

The measurement can be disturbed by phosphorescence of white material of the well plates. It causes a noise – a background signal – however, this is quite low compared to other measurement techniques. It owns a superior sensitivity, for instance it is 1000-fold more sensitive than fluorescent measurement. A second advantage is that it has a wide dynamic range in quantitation. Samples, which contain luciferase in concentrations ranging from low to decades of it, can be measured without need of a dilution¹³⁷.

Wait time starts with injection of substrate and ends with start of photon detection¹²⁷. It is also known as preread delay¹¹⁰, delay time¹²⁷ or start time¹²¹

time. Thereafter, integration time¹²⁶, measurement period¹¹⁰ or reading time¹²¹ respectively, starts. It lasts as long as photons are detected¹²⁶.

For both time periods manufacturers recommend different lengths (Table 17).

Table 17: Recommended lengths of time periods for measuring luminescence on a plate reader.

	Wait time	Integration time
Promega (for Firefly luciferase) ¹²⁰	2 sec	10 sec
Tecan ¹²⁶	---	Flash luminescence: 1-20 sec Glow luminescence: 100 ms -1 sec
Tecan and Promega ¹³⁹	2 sec	10 sec

Procedure for measuring luminescence (see 6.5).

Procedure for measuring absorbance (BCA assay):

The plate reader was switched on 30 min prior to actual measurement. The well plate with samples and BSA standard was put without cover in the plate reader (Infinite® M200 PRO, Tecan). Respective wells were marked in the software program (i-control™–Microplate Reader Software, Tecan) and absorption was measured with following settings:

- shaking (linear) duration: 5 sec
- shaking (linear) amplitude: 1 mm
- wavelength: 562 nm
- bandwidth: 9 nm
- number of flashes: 25
- settle time = 0 msec

Considerations for assaying luminescence samples of a 96-well plate:

1. Luminescence of wells without samples (blanks) is measured first, followed by those wells containing samples with an expected low signal. Next, samples with higher and lastly, those with highest expected signal are measured. Wells containing positive control are measured last. The reason for this reading-out pattern is that luminescence emitted from sample A is still there while next sample B is measured (photons from well

A beam into well B); thus, photons from A are affecting detected light intensity from B. Since reading direction of a plate reader cannot be controlled (it follows an algorithm depending on marked wells of template in software) but anticipated by experience, the reading pattern requires planning. Therefore, an individual reading out plan is established for each experiment. This needs to be considered already when seeding cells into a 96-well plate.

2. A sample is read out in triplicates and replicates are positioned in adjacent wells. Between two different samples at least two empty wells should be measured in-between. Optionally, one row can be left free between two samples; this slightly reduces beaming in adjacent wells and therefore contributes to a slight lower detected signal.
3. For a Firefly luciferase assay cells are seeded into a white 96-well plate. That means seeding plate and reading out plate are the same.

9.1.2 Preparation of HEPES Buffered Glucose (HBG buffer)

Background:

HBG buffer consists of 20 mM HEPES [N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)] and 5 % (w/V) glucose (pH 7.4). It is stable for 6 months at +4°C.

Materials:

- HEPES (Cat. No. A1069, AppliChem)
- D(+)-Glucose (Cat. No. 108.371.000, Merck)
- MQ-water (Arium[®]pro VF, Sartorius), autoclaved
- Sodium hydroxide (Applichem, A6829,1000), NaOH dilution
- Syringe filters, cellulose acetate, 0.22 µm (Cat. No. 28145-477, VWR)

Procedure:

10 g of glucose and 0.9532 g of HEPES were dissolved in 200 ml MQ-water (+4°C). The initial pH of approximately 6.0 was adjusted to pH 7.4 using 1M NaOH. The buffer was sterile filtered through a 0.22 µm cellulose acetate filter and stored at +4°C.

9.1.3 Aseptic cell culture techniques

The term cell culture is used for maintaining mammalian cells under *in vitro* conditions¹⁴³. Aseptic working techniques are required for each distinct working step in a cell culture laboratory; therefore, they are a crucial precondition for the successful performance of any cell experiment.

Biologic material always poses the risk of being a source of biological hazards such as prions, viruses or other pathogens. Thus, everyone working in a cell culture laboratory must be protected against an infection by wearing proper clothing e.g. lab coats and gloves. On the other hand, contaminations of cells with microorganisms from environment, e.g. bacteria, fungi, viruses and mycoplasma, must be strictly avoided. Otherwise, microorganisms outgrow cells and lead to cell death^{140,141}.

9.1.3.1 Preparation of cell culture media

Background:

A cell culture medium is usually a mixture of nutrients, inorganic salts and serum in water. Additionally, a pH indicator allows monitoring pH shifts. Fetal calf serum (FCS) or fetal bovine serum (FBS) is essential, since it contains nutrients and hormone-growth factors. Though cells can produce these on their own, an additional supply guarantees optimal growth. However, serum might also harbor infectious agents¹⁴¹.

Generally, medium obtained from the manufacturer was referred to as basal medium or serum-free medium in our lab. Medium supplemented with compounds such as FBS, Glutamine, antibiotics or others is known as complete growth medium^{141,140}. Glutamine is needed because it might be a limiting factor during cell growth¹⁴⁰.

Materials:

- A549 (ATCC® CCL-185™, LGC Standards GmbH, Germany)
- RPMI 1640 basal medium (Cat. No. R0883, Sigma-Aldrich)
- Dulbecco's Modified Eagle's Medium - high glucose (Cat. No. D5671, Sigma-Aldrich)
- Dulbecco's Phosphate Buffered Saline (Cat. No. D8537, Sigma-Aldrich)

- Fetal Bovine Serum (Cat. No. F7524, Sigma-Aldrich; Cat. No. 10270-106, Gibco by Life Technologies)
- Glutamine 200 mM (Cat. No. G7513, Sigma-Aldrich)

Method:

FBS was thawed overnight at +4°C rather than warmed rapidly up at 37°C in a water bath, as recommended by Gibco¹⁴². Before usage, FBS was passed through a cellulose acetate filter (0.22 µm) and prewarmed at 37°C. Frozen Glutamine 200 mM aliquot was thawed at 37°C in water bath. Since it is not very stable it should be used up within 2 months and it can only be frozen twice.

Preparation of cell culture medium for A549 cell line:

The following steps were carried out in a sterile laminar air flow. Starting from 500 ml of RPMI 1640 basal medium, 50 ml was pipetted into a sterile CT-50 and stored at +4°C. Then, 50 ml prewarmed FBS was mixed gently with a serological pipette and added to the remaining 450 ml basal medium, resulting in a final concentration of 10 % FBS. Since FBS tends to form air bubbles when mixed care must be taken avoiding this. Because thawed Glutamine 200 mM forms a white precipitate, which settles down on the bottom of the CT-15, it was dissolved by pipetting the solution up and down until it got transparent. 5 ml was added to the medium. Finally, the medium was mixed thoroughly using a 50 ml serological pipette, aliquoted in 50 ml aliquots and stored at +4°C.

Preparation of Cell Culture Medium for MDA-MB-231 cell line:

All steps were same as described above except that Dulbecco's Modified Eagle's Medium - high glucose was supplemented with 10 ml Glutamine 200 mM instead of 5 ml.

9.1.3.2 Cell passaging

Background:

Mammalian cells in cell culture:

Mammalian cells are either available as a primary or a continuous cell line¹⁴¹.

A cell line is referred to as a population of cells derived from a single cell containing the same genetic profile¹⁴³. Primary cells are isolated from mammalian tissue. Either they are taken directly as an explant from the tissue or they are separated by enzymatic digestion or another separation method. The source of tissue specifies the cell type i.e. the endothelium of a blood vessel provides an endothelial primary cell line¹⁴¹.

Usually, primary cell lines enter stage of senescence and subsequently die out. They have only a limited life span, which means that the cell divisions are finite. But sometimes primary cells undergo genetic alterations which allow them to grow *in vitro* for a long time. This is known as *in vitro* transformation and might also occur after treatment with carcinogens or exposure to viruses, e.g. monkey simian virus 40. Because they have gained a different genetic profile, cell characteristics differ from those under *in vivo* conditions. On the other hand, an enormous advantage is that transformed cells are easier to handle than primary cells because they are immortalized, i.e. having an infinite life span¹⁴¹.

Cells need to be cultured under optimum conditions. Two factors play an important role regarding nutrient depletion in medium: medium exchange and cell subculturing¹⁴¹.

Cells need to be fed regularly. Thereby, old medium is replaced by fresh medium in order to guarantee an optimal supply with nutrients and growth factors¹⁴⁰. On the other side, cells growing at a too high density must be splitted which means that an aliquot is transferred into a new flask with new medium. This procedure is referred to as passaging or subculturing and the applied method depends on the growth behavior of cells¹⁴¹. Cells grow either adherent or in suspension^{141,140}.

For adherent cell types, covering the special treated surface of the flask bottom as a monolayer, passaging is also referred to as trypsinization because trypsin catalyzes the enzymatic detaching of adherent cells. It is important not to expose cells for too long to trypsin as this can damage receptors on cell surface¹⁴⁰.

In contrast, suspension cells are single cells or cell clumps which grow in suspension. They are subcultured by diluting a cell aliquot in new medium¹⁴⁴.

After each trypsinization and reseeding, cells go through a characteristic growth pattern, referred to as growth cycle¹⁴⁴.

Initially, cells are in the latent phase (lag phase). Here they do not grow, but show a high cellular metabolism because of recovering from prior detaching process.

The precise duration of the lag phase depends on cell viability and seeding density and takes usually between 12 h and 24 h^{141,144}.

Then cells enter log phase, in which cell number increases exponentially under optimum conditions¹⁴¹. Each cell line shows a characteristic doubling time¹⁴⁴, e.g. A549 lung carcinoma cells double every 22 h¹¹⁵. In microscope the surface area which is occupied by growing cells (cell density) correlates with the confluence stage¹⁴⁵. For instance, at the end of the log phase cells cover nearly the whole surface of the flask bottom and this implies – obviously from a subjective assessment – a confluence of 80-90 %. This confluence stage offers the best moment for subculturing because cells are still growing but have not reached the stationary phase yet, where cell dividing stops¹⁴⁴. In the stationary phase, where the cell density and therefore the confluence stage are both very high, cells do not grow anymore which is visualized by a plateau (Figure 57). Reasons are depletion of nutrients in medium or accumulation of toxic metabolites released into the medium. Cells begin to die when remaining far longer in this stage, resulting in a decline of growth curves¹⁴¹.

Subculturing is a crucial step to guarantee that cells stay viable and healthy, otherwise several features of the cells change because of altered cell metabolism, e.g. cell shape and receptors on cell surface. This can negatively affect cell experiments¹⁴⁴.

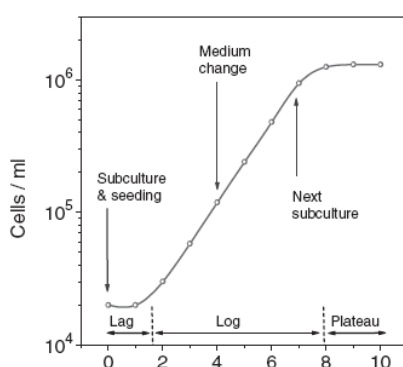


Figure 57: Growth curve of cell lines. Cell density [cells/ml] changes over time (x axis); figure from reference¹⁴⁴.

Split ratios:

The split ratio determines the seeding density which should be chosen in a way that facilitates optimal growth of cells¹⁴⁴. For example, a split ratio of 1:10 means that – based on 1 ml cell suspension – the tenth part, 100 µl, is diluted in fresh

medium to a final volume of 1000 μ l. If cells are seeded too sparse it takes longer till confluence is reached. One should keep in mind that seeding at a too low density, or a high split ratio respectively, poses the risk of cell dying. Seeding at a high density, low split ratio respectively, implies that confluence is reached (too) soon. Both used cell lines vary in split ratios because it is a characteristic feature of each and depends on their growth behavior. Usually, for A549 a split ratio of 1:10 or 1:15 was used and for MDA-MB-231 the split ratio varied between 1:4 and 1:6. Applying these split ratios, cells could be passaged every third or fourth day.

Materials:

- RPMI 1640 basal medium (Cat. No. R0883, Sigma-Aldrich)
- Complete medium
- Dulbecco's Phosphate Buffered Saline (Cat. No. D8537, Sigma-Aldrich)
- Detaching Reagent

Procedure for cells cultured in a flask of 75 cm² (T-75):

A T-75 flask was filled with 12-13 ml complete medium and put in the incubator for prewarming the medium. Aliquots of complete medium and PBS were warmed in water bath (37°C).

The confluence stage of growing cells was assessed by observing cells in inverted microscope (magnification 4x). Old medium was then aspirated by vacuum pump. Cells were washed with 5 ml PBS. For this, PBS was pipetted in one corner of the flask and then swirled over the whole cell monolayer by tilting the flask so that dead cells and traces of medium were washed off. Finally, it was aspirated and discarded. 1 ml of the appropriate detaching reagent was added directly onto the cell monolayer and the flask was incubated at 37°C for 4-5 minutes. Complete cell detaching was checked in microscope. If so, cells were spherical and floating. If some were still sticking at the flask bottom their release was enhanced by tapping the flask with palm of hands. Subsequently, the detaching process was stopped by diluting the detaching reagent. For this, 4 ml of complete medium were added directly onto the flask bottom applying some pressure in order to dislodge all cells from the bottom. This step is called flushing cells. The cell suspension was pipetted up and cell flush was repeated once more

with 3 ml complete medium. Cell suspension was collected after each flush in a CT-15, containing finally 8 ml cell suspension in total. Cells were centrifuged at 1200 rpm for 5 min. The supernatant was removed and the cell pellet resuspended in 1 ml complete medium. According to the split ratio a proper volume of cell suspension was transferred into the prewarmed complete medium of the new T-75 flask. The flask was put in the incubator.

For a flask of 25 cm² area (T-25) indicated volumes were halved.

9.1.3.3 Cell freezing

Background:

Cells are frozen in a mixture of 10 % Dimethyl sulfoxide (DMSO; Cat. No. D5879, Sigma-Aldrich) in FBS, also referred to as cryomedium. The cryopreservative agent can be Dimethyl sulfoxide or Glycerol, depending on cell type. It inhibits formation of large ice crystals inside the cell, which badly affects cell viability and leads to cell death. However, DMSO as a liquid is toxic for cells¹⁴⁰. Besides, it lowers freezing point of complete medium¹⁴¹.

Ideally, cells should be harvested and frozen in log phase because in that stage they have the greatest potential to recover when thawed. Harvested cells are put into a cryovial, which is labeled with cell line, date of harvesting and passage number. Cryovials are made of polypropylene, which withstands low temperatures down to -190°C¹⁴¹. The cryovial is put in a cryo freezing container, termed Mr.Frosty, which is half filled with isopropyl alcohol. Mr. Frosty is stored at -80°C for approximately 4 h. It ensures a constant cooling rate of 1°C per min from RT to -80°C. This slowly freezing process allows water to move outside of the cell¹⁴⁶. Thereafter, the cryovial is transferred into the gas phase of liquid nitrogen storage (-150 °C). Liquid nitrogen boils at -196°C. It is irrelevant whether cells are stored in the gas phase of liquid nitrogen or directly in liquid nitrogen because in both cases their metabolism stops¹⁴⁷. Purpose of freezing stocks at -150°C is that cells can be stored for long-term. This is advantageous regarding a contamination of actual maintained cells, since then the cells from original stock can be thawed. Also, if cells have reached a high passage number, one can thaw up new cells of a low passage from the same stock¹⁴¹.

Procedure:

If cell number is disregarded for freezing it is standard to freeze the content of one T-75 in one sterile cryovial.

Appropriate number of cryovials was labeled. Each label must provide the following details: name of cell line, passage number, date, initials of operator. Mr. Frosty was put at least 4 h prior to freezing at RT. Cells were harvested following the protocol of cell passaging including the centrifugation step (see 9.1.3.2) so that a cell pellet was obtained. The following steps were carried out as rapidly as possible because of toxic liquid cryomedium: The cell pellet was resuspended in 1 ml cryomedium and the suspension was transferred into a cryovial, screwed tightly shut and placed in Mr. Frosty, which was immediately put at -80°C overnight. The following day the cryovial was transferred from Mr. Frosty into the gas phase of liquid nitrogen storage (-150°C).

9.1.3.4 Cell thawing

Background:

Frozen cell stocks are stored at -150°C in the gas phase of liquid nitrogen. When removing a cryovial appropriate protection of skin must be ensured, because skin contact can entail severe burn. For melting rough ice crystals, the cryovial with frozen cells is held into a 37°C tempered water bath. Afterwards, liquid medium containing DMSO is rapidly removed because of its cytotoxicity^{144,141}.

Procedure:

A new flask was filled with complete medium and warmed up to 37°C. An aliquot of 7 ml complete medium in a CT-15 was heated up to 37°C in water bath.

The cryovial was taken out of -150°C and its bottom half was immediately dipped into the water bath (37°C) and moved slowly forth and back. When only a small ice clump was left the tube was disinfected with Ethanol 70 %, wiped dry and put in laminar. The thawed cell suspension was added to the 7 ml warm medium and the cryovial was washed twice with 1 ml complete medium. Hence, the CT-15 contained 10 ml and this cell suspension was then centrifuged at 100 g for 10 min with an acceleration and deceleration rate of 6. The supernatant was removed and the cell pellet was resuspended in 1 ml complete medium and added to

prewarmed complete medium in the T-75 flask. The CT-15 was washed once with 1 ml complete medium, which also was added to the flask. Cells were incubated overnight and checked the following day. Medium was exchanged.

9.1.3.5 Cell counting

Background:

The most common method for cell quantification is counting with a hemocytometer or Neubauer chamber (Celeromics)¹⁴⁸. It represents a thickened glass slide in which two counting grids are engraved in a central support. Two external supports of the slide are moistened with water and a coverslip is placed on it¹⁴⁹. By this, a counting chamber as shown in Figure 58 is formed.

Each counting grid consists of nine subgrids, respectively squares (Figure 58). Each square has an area of 1 mm² and a depth of 0.1 mm. Hence, each square contains a volume of 1x 10⁻⁴ ml, respectively 0.1 µl¹⁴¹. The grid facilitates counting because it enables following a particular and repeatable counting order. First, the middle square was counted, followed by the left upper square, right upper square, left lower square and finally right lower square. In avoidance of counting cells twice, only these, which are touching top and left line of a square are counted, and not these on the right or bottom line.

In practice, after the hemocytometer is loaded with the cell suspension five squares are counted and the average cell number of one square, respectively 0.1 µl, is calculated. This number is multiplied by the factor 10 000 in order to obtain cell number for 1 ml. In case a dilution was made, cell number was multiplied by the dilution factor. For calculating the cell number per ml cell suspension the following formula was used¹⁴¹:

$$\text{Cell number/ml} = \frac{\text{average cell number of all counts}}{\text{number of counted squares}} \times 10\,000 \times \text{dilution factor}$$

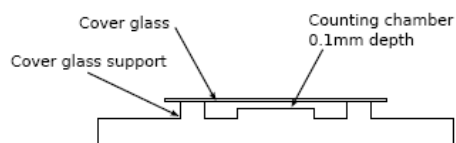


Figure 58: Hemocytometer; figure from ref.¹⁵⁰.

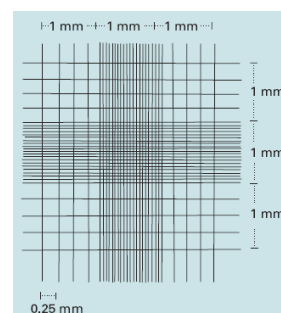


Figure 59: Counting grid; figure from ref.¹⁴¹.

Procedure:

Hemocytometer and coverslip were cleaned with distilled water, disinfected with Ethanol 70 %, wiped dry with a tissue and put in the laminar. Both external supports were moistened with MQ-water. The coverslip was placed on the chamber and appearance of Newton rings indicated its correct position. For harvesting cells all steps as described in 9.1.3.2 were carried out, until 1 ml cell suspension was obtained. From this, 1:10 and 1:20 dilutions were made. The chamber was loaded with 10 μ l cell suspension. The chamber grid was focused in microscope (magnification 10x) and cells were counted with a cell counter. The counting was repeated and average cell number was used for calculating the seeding volume.

9.1.4 Flow cytometry

Background:

The ability of flow cytometry to provide information about multiple physical properties of a large number of individual particles (mammalian cells, organelles, nanoparticles, microparticles, bacteria, yeast, protozoa) within a short time period makes it a valuable tool for research in biochemistry and related fields¹⁵².

Only particles with sizes ranging from 0.2-150 μ m can be measured¹⁵¹.

A disadvantage might be that only single cells can be analyzed. Thus, it does not provide information about tissue structure or cell-cell-interactions. Preparing a cell suspension of the sample is a precondition for performing flow cytometry¹⁵².

In principal, single cells are hit by a laser beam and based on scattered light three parameters can be analyzed: size and granularity of cells as well as fluorescence in case specific cell structures are labeled by a fluorochrome. The

sample is injected into a sheath fluid by air pressure. Alternatively, the cell suspension can also be sucked in by applying vacuum. Sheath fluid consists of water or buffer with the sample stream localized in its center. Both fluids flow continuously, without mixing, through the flow cell and closely to the measuring point, where the laser passes the flow cell and is scattered by one single cell, the diameter narrows so that cells get separated and the laser beam hits a single cell. By varying the pressure of sample fluid the flow rate can be adjusted to high, middle or low. The laser provides monochromatic light¹⁵². The scattered light is measured at two angles. Forward scatter is recorded by a forward scatter detector in longitudinal direction of laser beam and it is proportional to the size of cell. The sideward scatter detector registers scattered light that is deflected at right angles to the laser. It reveals information about cell granularity and with increasing density of intracellular organelles the sideward scatter rises proportionally. The laser excites fluorochromes and emitted wavelength is detected by fluorescence detectors, also arranged at right angle to the laser beam. Often, flow cytometers are equipped with a great variety of lasers and detectors¹⁵¹.

The optical system of a flow cytometer is depicted in Figure 60. The laser passes first the focusing lens and right behind the flow cell a blocker (panel/aperture) blocks it and light, which is scattered in small angles, is recorded by the forward scatter detector. A second collecting lens at right angles to the laser guides light to a series of dichroic mirrors. Combined with barrier filters they filter specific wavelengths and these are registered subsequently by photomultipliers. This gives possibility for measuring specific fluorescent light, emitted by a fluorophore. A dichroic mirror is only permeable for light covering a specific range of wavelengths e.g. 200-500 nm. Other wavelengths are excluded. Selected light passes then a barrier filter which selects a specific wavelength ($\lambda_{\max}$)¹⁵².

This light beam is converted by a photomultiplier tube (PMT) into analog voltage pulses. They are transformed into digital information by an Analog-to-Digital-Converter (ADC). Photodiodes measure intensity of forward scattered light and PMTs measure intensity of sideward scattered light and fluorescence. Therefore a weak fluorescent signal can be amplified¹⁵¹.

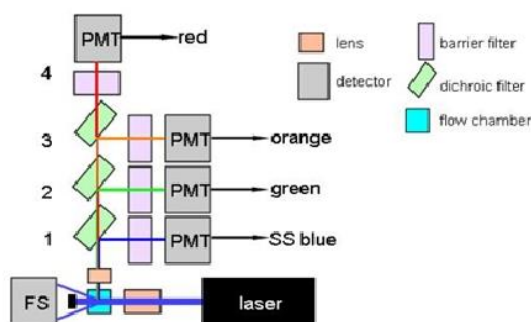


Figure 60: Optical system of a flow cytometer; figure from reference ¹⁵².

Flow cytometry is also applied for sorting out cells from a cell pool because often one wants to collect cells owning specific features for further analysis. In order to separate cells and collect a specific fraction of them, each droplet harboring a single cell gets a charge and, based on charge of individual droplets, cells are separated in respective containers¹⁵³. For this application the acronym FACS (Fluorescence activated cell sorting) has been established. FACS® is also a registered trademark of Becton, Dickinson and Company¹⁵³. However, it is often used as synonym for general performance of a flow cytometry measurement¹⁵¹.

DAPI (4',6-Diamidin-2-phenylindol) penetrates only in dead cells and binds to the minor groove of double-stranded deoxyribonucleic acid. Thus, it is widely used as a specific fluorescence dye for staining dead cells and for cell cycle analyses. Its maximum excitation wavelength is at 358 nm and its maximum emission wavelength is at 461 nm¹⁵⁴.

Acquired data can be presented in a histogram or in a two parameter correlated plot. Latter is either a dot plot (each count (cell) is displayed by a dot) or a density plot (density of cells is highlighted in different colours)¹⁵².

Various software programs for data analysis exist, for experiments in this diploma thesis FlowJo-V10 (FlowJo, LLC. Ashland, Oregon, USA) was used. In principle, a gate is drawn around a population of interest (cell gating) and features of these selected cells are then displayed in a separate graph. Based on this, further analysis can be performed. This strategy enables one to evaluate multiple data in an easy way¹⁵².

Procedure of flow cytometry is described in 6.9.

9.2 Adjustments on plate reader and calculation methods relevant for performance of *in vitro* luciferase assays

Table 18, Table 19 and Table 20 give an overview about calculation methods:

Table 18: Adjustments on plate reader (Tecan).

Parameter	Firefly luciferase assay	Gaussia/Lucia luciferase assay
Substrate	Luciferin	Coelenterazine
Substrate + buffer	100 µl housemade Luciferin Assay Buffer, 50 µl commercial Luciferin Assay Buffer	50 µl housemade Coelenterazine Assay Buffer (20 µM), 50 µl commercial Coelenterazine Assay Buffer
Wait time	2 sec	2 sec
Integration time	10 sec	10 sec
Attenuation	Automatic	Automatic
Kinetics	Glow	Flash/extended flash

Table 19: Volumes [µl] for luciferase reporter gene assays in combination with BCA normalization. * 96-well plate.

	Firefly luciferase assay	Gaussia/Lucia luciferase assay
BCA assay – experimentally used volumes		
Sample volume after cell lysis	(white*) 30 µl lysate	(transparent*) 30 µl lysate
→ volume for BCA assay	(transparent*) 20 µl lysate + 5 µl MQ-water	(transparent*) 30 µl lysate
→ volume for luciferase assay	(white*) 10 µl lysate	(white*) 20 µl supernatant
BSA standard volume	(transparent*) 25 µl	(transparent*) 25 µl + 5 µl MQ-water
Evaluation of BCA normalization		
Protein [µg] in 30 µl lysate	Protein [µg] in 20 µl lysate x 1.5	Protein [µg] in 30 µl lysate
Signal intensity [RLU]	RLU of 30 µl lysate: Signal intensity [RLU] of 10 µl lysate x 3	RLU of 200 µl supernatant: Signal intensity [RLU] of 20 µl supernatant x 10
Normalization of RLU	$RLU \text{ per } \mu g \text{ protein} = \frac{RLU (30 \mu l \text{ lysate})}{\text{protein } [\mu g] (30 \mu l \text{ lysate})}$ Taking untreated cells into account: $RLU \text{ per } \mu g \text{ protein} = \frac{RLU (30 \mu l \text{ lysate})}{\text{protein } [\mu g] (30 \mu l \text{ lysate})} \times \text{av. protein } [\mu g] \text{ of UT}$	$RLU \text{ per } \mu g \text{ protein} = \frac{RLU (200 \mu l \text{ supernatant})}{\text{protein } [\mu g] (30 \mu l \text{ lysate})}$ Taking untreated cells into account: $RLU \text{ per } \mu g \text{ protein} = \frac{RLU (200 \mu l \text{ supernatant})}{\text{protein } [\mu g] (30 \mu l \text{ lysate})} \times \text{av. protein } [\mu g] \text{ of UT}$

Table 20: Volumes [μl] for luciferase reporter gene assays in combination with flow cytometry (FCM) normalization. * 96-well plate.

	Firefly luciferase assay	Gaussia/Lucia luciferase assay
Flow cytometry – experimentally used volumes		
Sample volume for FCM	---	(transparent*) Treated cells: 160 μl (= 20 μl trypsin + 140 μl PBS) cell suspension + 40 μl Dapi-HCl (5 μg/ml) = 200 μl (1 μg/ml Dapi-HCl) → 150 μl analyzed Untreated cells: 200 μl cell suspension (= 20 μl trypsin + 180 μl PBS) → 150 μl analyzed
Sample volume for luciferase assay	---	(transparent*) 20 μl supernatant (containing secreted luciferase)
Evaluation of Flow cytometry normalization		
Total count of live cells	---	FlowJo®: (Detected count of Dapi-negative cells in 150 μl)/150) x 200 = Total count of live cells in 200 μl
Signal intensity [RLU] of 200 μl supernatant	---	Signal intensity [RLU] of 20 μl supernatant x 10
Normalization of RLU	---	<i>RLU per 10 000 live cells (10 000 seeded cells)</i> $= \frac{RLU (200 \mu l \text{ supernatant})}{live \text{ cell count}} \times 10\,000$ Taking untreated cells into account: RLU $= \frac{RLU (200 \mu l \text{ supernatant})}{live \text{ cell count}} \times av. \text{ live cell count of UT}$

10 References

- ¹ Schaffert D, Ogris M. Nucleic acid carrier systems based on polyethylenimine conjugates for the treatment of metastatic tumors. *Curr Med Chem*. 2013;20(28):3456-70.
- ² Rödl W, Schaffert D, Wagner E, Ogris M. Synthesis of polyethylenimine-based nanocarriers for systemic tumor targeting of nucleic acids. *Methods Mol Biol*. 2013;948:105-20. DOI: 10.1007/978-1-62703-140-0_8.
- ³ Opalinska JB, Gewirtz AM. Nucleic-acid therapeutics: basic principles and recent applications. *Nat Rev Drug Discov*. 2002 Jul;1(7):503-14.
- ⁴ Niidome T, Huang L. Gene therapy progress and prospects: nonviral vectors. *Gene Ther*. 2002 Dec;9(24):1647-52.
- ⁵ El-Anead A. An overview of current delivery systems in cancer gene therapy. *J Control Release*. 2004 Jan 8;94(1):1-14.
- ⁶ Bouard D, Alazard-Dany N, Cosset F-L. Viral vectors: from virology to transgene expression. *Br J Pharmacol*. 2009 May; 157(2): 153–165. DOI: 10.1038/bjp.2008.349
- ⁷ Wikipedia contributors. Vectors in gene therapy [Internet]. Wikipedia, The Free Encyclopedia; 2016 May 28 [cited 2016 Aug 6]. Available from: https://en.wikipedia.org/w/index.php?title=Vectors_in_gene_therapy&oldid=722508626
- ⁸ Raper SE, Chirmule N, Lee FS., Wivel NA. Bagg A, Gao GP, Wilson JM, Batshaw ML. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab*. 2003 Sep-Oct;80(1-2):148-58.
- ⁹ Estornes Y, Toscano F, Virard F, Jacquemin G, Pierrot A, Vanbervliet B, Bonnin M, Lalaoui N, Mercier-Gouy P, Pachéco Y, Salaun B, Renno T, Micheau O, Lebecque S. dsRNA induces apoptosis through an atypical death complex associating TLR3 to caspase-8. *Cell Death Differ*. 2012 Sep;19(9):1482-94. DOI: 10.1038/cdd.2012.22.
- ¹⁰ Hsu CY, Uludağ H. A simple and rapid nonviral approach to efficiently transfect primary tissue-derived cells using polyethylenimine. *Nat Protoc*. 2012 Apr 19;7(5):935-45. DOI: 10.1038/nprot.2012.038.
- ¹¹ Zhang Y, Satterlee A, Huang L. *In vivo* gene delivery by nonviral vectors: overcoming hurdles? *Mol Ther*. 2012 Jul;20(7):1298-304. DOI: 10.1038/mt.2012.79.
- ¹² Wolff JA, Rozema DB. Breaking the bonds: non-viral vectors become chemically dynamic. *Mol Ther*. 2008 Jan;16(1):8-15.
- ¹³ Taschauer A, Geyer A, Gehrig S, Maier J, Sami H, Ogris M. Up-Scaled Synthesis and Characterization of Nonviral Gene Delivery Particles for Transient *In vitro* and *In vivo* Transgene Expression. *Hum Gene Ther Methods*. 2016 Jun;27(3):87-97. DOI: 10.1089/hgtb.2016.027.
- ¹⁴ Vetter A, Viridi KS, Espenlaub S, Rödl W, Wagner E, Holm PS, Scheu C, Kreppel F, Spitzweg C, Ogris M. Adenoviral vectors coated with PAMAM dendrimer conjugates allow CAR independent virus uptake and targeting to the EGF receptor. *Mol Pharm*. 2013 Feb 4;10(2):606-18. DOI: 10.1021/mp300366f.
- ¹⁵ Talsma H, Cherng J, Lehrmann H, Kursu M, Ogris M, Hennink WE, Cotten M, Wagner E. Stabilization of gene delivery systems by freeze-drying. *Int J Pharm*. 1997 Nov 28;157(2):233-238.

- ¹⁶ Zhou XH, Klibanov AL, Huang L. Lipophilic polylysines mediate efficient DNA transfection in mammalian cells. *Biochim Biophys Acta*. 1991 May 31;1065(1):8-14.
- ¹⁷ Lee CC, Liu Y, Reineke TM. General structure-activity relationship for poly(glycoamidoamine)s: the effect of amine density on cytotoxicity and DNA delivery efficiency. *Bioconjug Chem*. 2008 Feb;19(2):428-40. DOI: 10.1021/bc7001659.
- ¹⁸ Haensler J, Szoka FC Jr. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjug Chem*. 1993 Sep-Oct;4(5):372-9.
- ¹⁹ Jones GD, Langsjoen A, Neumann SMMC, Zomlefer J. The polymerization of ethylenimine. *Org. Chem*. 1944; 09(2):125–147. DOI: 10.1021/jo01184a002.
- ²⁰ Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc Natl Acad Sci U S A*. 1995 Aug 1;92(16):7297-301.
- ²¹ Miyata K, Nishiyama N, Kataoka K. Rational design of smart supramolecular assemblies for gene delivery: chemical challenges in the creation of artificial viruses. *Chem Soc Rev*. 2012 Apr 7;41(7):2562-74. DOI: 10.1039/c1cs15258k.
- ²² Boeckle S, von Gersdorff K, van der Piepen S, Culmsee C, Wagner E, Ogris M. Purification of polyethylenimine polyplexes highlights the role of free polycations in gene transfer. *J Gene Med*. 2004 Oct;6(10):1102-11. DOI: 10.1002/jgm.598.
- ²³ Rejman J, Bragonzi A, Conese M. Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes. *Mol Ther*. 2005 Sep;12(3):468-74.
- ²⁴ Behr JP, Demeneix B, Loeffler JP, Perez-Mutul J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc Natl Acad Sci U S A*. 1989 Sep;86(18):6982-6.
- ²⁵ Mislick KA, Baldeschwieler JD. Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc Natl Acad Sci U S A*. 1996 Oct 29;93(22):12349-54.
- ²⁶ Kircheis R, Wightman L, Wagner E. Design and gene delivery activity of modified polyethylenimines. *Adv Drug Deliv Rev*. 2001 Dec 31;53(3):341-58.
- ²⁷ Kircheis R, Schüller S, Brunner S, Ogris M, Heider KH, Zauner W, Wagner E. Polycation-based DNA complexes for tumor-targeted gene delivery *in vivo*. *J Gene Med*. 1999 Mar-Apr;1(2):111-20. DOI: 10.1002/(SICI)1521-2254(199903/04)1:2<111::AID-JGM22>3.0.CO;2-Y.
- ²⁸ Choosakoonkriang S, Lobo BA, Koe GS, Koe JG, Middaugh CR. Biophysical characterization of PEI/DNA complexes. *J Pharm Sci*. 2003 Aug;92(8):1710-22.
- ²⁹ Kasper JC, Schaffert D, Ogris M, Wagner E, Friess W. **(b)** The establishment of an up-scaled micro-mixer method allows the standardized and reproducible preparation of well-defined plasmid/LPEI polyplexes. *Eur J Pharm Biopharm*. 2011 Jan;77(1):182-5. DOI: 10.1016/j.ejpb.2010.11.012.
- ³⁰ Di Gioia S, Conese M. Polyethylenimine-mediated gene delivery to the lung and therapeutic applications. *Drug Des Devel Ther*. 2009 Feb 6;2:163-88.
- ³¹ Maeda H. Macromolecular therapeutics in cancer treatment: the EPR effect and beyond. *J Control Release*. 2012 Dec 10;164(2):138-44. DOI: 10.1016/j.jconrel.2012.04.038.

- ³² Shen H, Sun T, Ferrari M. Nanovector Delivery of siRNA for Cancer Therapy. *Cancer gene therapy*. 2012;19(6):10.1038/cgt.2012.22. DOI:10.1038/cgt.2012.22.
- ³³ Lee M, Kim SW. Polyethylene glycol-conjugated copolymers for plasmid DNA delivery. *Pharm Res*. 2005 Jan;22(1):1-10.
- ³⁴ Blessing T, Kursa M, Holzhauser R, Kircheis R, Wagner E. Different strategies for formation of pegylated EGF-conjugated PEI/DNA complexes for targeted gene delivery. *Bioconjug Chem*. 2001 Jul-Aug;12(4):529-37.
- ³⁵ Behr JP. The Proton Sponge: a Trick to Enter Cells the Viruses Did Not Exploit. *Chimia*.1997; 51:34-36.
- ³⁶ Benjaminsen RV, Matthebjerg MA, Henriksen JR, Moghimi SM, Andresen TL. The possible "proton sponge " effect of polyethylenimine (PEI) does not include change in lysosomal pH. *Mol Ther*. 2013 Jan;21(1):149-57. DOI: 10.1038/mt.2012.185.
- ³⁷ Wightman L, Kircheis R, Rössler V, Carotta S, Ruzicka R, Kursa M, Wagner, E. Different behavior of branched and linear polyethylenimine for gene delivery *in vitro* and *in vivo*. *J. Gene Med*. 2001; 3: 362–372. DOI:10.1002/jgm.187
- ³⁸ Brunner S, Sauer T, Carotta S, Cotten M, Saltik M, Wagner E. Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. *Gene Ther*. 2000 Mar;7(5):401-7. DOI: 10.1038/sj.gt.3301102.
- ³⁹ Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A*. 1987 Nov;84(21):7413-7.
- ⁴⁰ Khalil IA, Kogure K, Akita H, Harashima H. Uptake pathways and subsequent intracellular trafficking in nonviral gene delivery. *Pharmacol Rev*. 2006 Mar;58(1):32-45. DOI: 10.1124/pr.58.1.8.
- ⁴¹ Kozielskia KL, Ruia Y, Greena JJ. Non-viral nucleic acid containing nanoparticles as cancer therapeutics. *Expert Opin Drug Deliv*. 2016 Oct; 13(10): 1475–1487.
- ⁴² Gao Y, Liu XL, Li XR. Research progress on siRNA delivery with nonviral carriers. *Int J Nanomedicine*. 2011;6:1017-25. DOI: 10.2147/IJN.S17040.
- ⁴³ Dalby B, Cates S, Harris A, Ohki EC, Tilkins ML, Price PJ, Ciccarone VC. Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications. *Methods*. 2004 Jun;33(2):95-103. DOI: 10.1016/j.ymeth.2003.11.023.
- ⁴⁴ Buyens K, De Smedt SC, Braeckmans K, Demeester J, Peeters L, van Grunsven LA, de Mollerat du Jeu X, Sawant R, Torchilin V, Farkasova K, Ogris M, Sanders NN. Liposome based systems for systemic siRNA delivery: stability in blood sets the requirements for optimal carrier design. *J Control Release*. 2012 Mar 28;158(3):362-70. DOI: 10.1016/j.jconrel.2011.10.009.
- ⁴⁵ Ramamoorth M, Narvekar A. Non Viral Vectors in Gene Therapy- An Overview. *J Clin Diagn Res*. 2015 Jan; 9(1): GE01–GE06. DOI: 10.7860/JCDR/2015/10443.5394
- ⁴⁶ Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Isolating, Cloning, and Sequencing DNA [Internet]. In: *Molecular Biology of the Cell*. 4th edition. New York: Garland Science; 2002 [cited 2016 Dec 20]. Available from: <https://www.ncbi.nlm.nih.gov/books/NBK26837/>

- ⁴⁷ Wikipedia contributors. Cloning vector [Internet]. Wikipedia, The Free Encyclopedia; 2016 Jul 7 [cited 2016 Aug 7]. Available from: https://en.wikipedia.org/w/index.php?title=Cloning_vector&oldid=728772805
- ⁴⁸ Addgene. What is a Plasmid [Internet]. Cambridge, MA, USA: Addgene; [cited 2016 Aug 19]. Available from: <https://www.addgene.org/mol-bio-reference/plasmid-background/>
- ⁴⁹ Nagata T, Uchijima M, Yoshida A, Kawashima M, Koide Y. Codon optimization effect on translational efficiency of DNA vaccine in mammalian cells: analysis of plasmid DNA encoding a CTL epitope derived from microorganisms. *Biochem Biophys Res Commun*. 1999 Aug 2;261(2):445-51. DOI: 10.1006/bbrc.1999.1050.
- ⁵⁰ Isomura H, Stinski MF. The human cytomegalovirus major immediate-early enhancer determines the efficiency of immediate-early gene transcription and viral replication in permissive cells at low multiplicity of infection. *J Virol*. 2003 Mar;77(6):3602-14. DOI: 10.1128/JVI.77.6.3602-3614.2003.
- ⁵¹ Boshart M, Weber F, Jahn G, Dorsch-Häsler K, Fleckenstein B, Schaffner W. A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell*. 1985 Jun;41(2):521-30. DOI: [http://dx.doi.org/10.1016/S0092-8674\(85\)80025-8](http://dx.doi.org/10.1016/S0092-8674(85)80025-8).
- ⁵² Calado SM, Oliveira AV, Machado S, Haase R, Silva GA. Sustained gene expression in the retina by improved episomal vectors. *Tissue Eng Part A*. 2014 Oct;20(19-20):2692-8. DOI: 10.1089/ten.TEA.2013.0672.
- ⁵³ Bode J, Stengert-Iber M, Kay V, Schlake T, Dietz-Pfeilstetter A. Scaffold/matrix-attached regions: topological switches with multiple regulatory functions. *Crit Rev Eukaryot Gene Expr*. 1996;6(2-3):115-38.
- ⁵⁴ Wikipedia contributors. Shuttle vector [Internet]. Wikipedia, The Free Encyclopedia; 2016 Jul 31 [cited 2016 Aug 7]. Available from: https://en.wikipedia.org/w/index.php?title=Shuttle_vector&oldid=732410754
- ⁵⁵ Wikipedia contributors. Transgene [Internet]. Wikipedia, The Free Encyclopedia; 2016 Jul 26 [cited 2016 Aug 7]. Available from: <https://en.wikipedia.org/w/index.php?title=Transgene&oldid=731650884>
- ⁵⁶ Magnusson T, Haase R, Schleef M, Wagner E, Ogris M. Sustained, high transgene expression in liver with plasmid vectors using optimized promoter-enhancer combinations. *J Gene Med*. 2011 Jul;13(7-8):382-91. DOI: 10.1002/jgm.1585.
- ⁵⁷ Hoivik EA, Bjanesoy TE, Mai O, Okamoto S, Minokoshi Y, Shima Y, Morohashi K, Boehm U, Bakke M. DNA methylation of intronic enhancers directs tissue-specific expression of steroidogenic factor 1/adrenal 4 binding protein (SF-1/Ad4BP). *Endocrinology*. 2011 May;152(5):2100-12. DOI: 10.1210/en.2010-1305.
- ⁵⁸ Yew NS, Zhao H, Przybylska M, Wu IH, Tousignant JD, Scheule RK, Cheng SH. CpG-depleted plasmid DNA vectors with enhanced safety and long-term gene expression *in vivo*. *Mol Ther*. 2002 Jun;5(6):731-8. DOI: 10.1006/mthe.2002.0598.
- ⁵⁹ Magnusson T. Optimised plasmids for sustained transgene expression *in vivo* [dissertation]. [Munich]: Ludwig-Maximilians-University Munich; 2010. Available from: https://edoc.ub.uni-muenchen.de/12325/1/Magnusson_Terese.pdf
- ⁶⁰ EcoliWiki. pCMV-GLuc [Internet]. EcoliWiki; last modified 2011 Aug 12 [cited 2016 Aug 25]. Available from: <http://ecoliwiki.net/colipedia/index.php/pCMV-GLuc>

- ⁶¹ InvivoGen. InvivoGen Insight Newsletters [Internet]. San Diego, California, USA: InvivoGen; 2016 [cited 2016 Aug 25]. Available from: <http://www.invivogen.com/newsletters.php>
Access to: Invivogen. Introducing Lucia™: a new secreted luciferase [Internet]. San Diego, California, USA: Invivogen; 2012 Jun [cited 2016 Aug 25]. Available from: http://www.invivogen.com/docs/Insight_201207.pdf
- ⁶² InvivoGen. pCpGfree-promoter [Internet]. San Diego, California, USA: InvivoGen; 2016 [cited 2016 Aug 25]. Available from: <http://www.invivogen.com/pcpgfree-promoter> Access to: pCpGfree-promoter-Lucia. A Lucia luciferase reporter plasmid without an enhancer and devoid of CpG dinucleotide. Version # 16C09-MMv02 Cat# pcpgf-promlc [Internet]. San Diego, California, USA: Invivogen; 2016 [cited 2016 Aug 25]. Available from: http://www.invivogen.com/PDF/pCpGfree_promoter_Lucia_TDS.pdf
- ⁶³ Rana TM. Illuminating the silence: understanding the structure and function of small RNAs. *Nat Rev Mol Cell Biol.* 2007 Jan;8(1):23-36.
- ⁶⁴ Gavrillov K, Saltzman WM. Therapeutic siRNA: principles, challenges, and strategies. *Yale J Biol Med.* 2012 Jun;85(2):187-200.
- ⁶⁵ Gallas A, Alexander C, Davies MC, Puri S, Allen S. Chemistry and formulations for siRNA therapeutics. *Chem Soc Rev.* 2013 Oct 21;42(20):7983-97. DOI: 10.1039/c3cs35520a.
- ⁶⁶ Navizet I, Liu YJ, Ferré N, Roca-Sanjuán D, Lindh R. The chemistry of bioluminescence: an analysis of chemical functionalities. *Chemphyschem.* 2011 Dec 9;12(17):3064-76. DOI: 10.1002/cphc.201100504.
- ⁶⁷ Roda A, Guardigli M, Michelini E, Mirasoli M. Bioluminescence in analytical chemistry and *in vivo* imaging. *Trends in Anal Chem.* 2009 Mar;28(3):307–322. DOI:10.1016/j.trac.2008.11.015.
- ⁶⁸ Naylor LH. Reporter gene technology: the future looks bright. *Biochem Pharmacol.* 1999 Sep 1;58(5):749-57.
- ⁶⁹ Youn H, Chung JK. Reporter gene imaging. *AJR Am J Roentgenol.* 2013 Aug;201(2):W206-14. DOI: 10.2214/AJR.13.10555.
- ⁷⁰ Shaikh F, Jacob A, Van gestel F, Yaghoubi S. Molecular Imaging in Genetic Medicine. *Cureus.* 2016 Apr 11;8(4): e565. DOI: 10.7759/cureus.565.
- ⁷¹ Thermo Fisher Scientific. Luciferase Reporters [Internet]. 2015 [cited 2016 Aug 25]. Available from: <https://www.thermofisher.com/at/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/luciferase-reporters.html>
- ⁷² Wilson T, Hastings JW. Bioluminescence. *Annu Rev Cell Dev Biol.* 1998;14:197-230. DOI: 10.1146/annurev.cellbio.14.1.197.
- ⁷³ Hastings JW. Chapter 52 – Bioluminescence. In: Sperelakis N., editor. *Cell Physiology Source Book* (Fourth Edition). San Diego: Academic Press; 2012. p. 925-947. Available from: <http://dx.doi.org/10.1016/B978-0-12-387738-3.00052-4>.
- ⁷⁴ Kahlke T., Umbers KDL. Bioluminescence. *Current Biology.* 2016 Apr 25;26(8): R313-R314. <http://dx.doi.org/10.1016/j.cub.2016.01.007>.
- ⁷⁵ Greer LF 3rd, Szalay AA. Imaging of light emission from the expression of luciferases in living cells and organisms: a review. *Luminescence.* 2002 Jan-Feb;17(1):43-74. DOI: 10.1002/bio.676.
- ⁷⁶ Gates BJ, DeLuca M. The production of oxyluciferin during the firefly luciferase light reaction. *Arch Biochem Biophys.* 1975 Aug;169(2):616-21.

- ⁷⁷ Bowie LJ, Irwin R, Loken M, De Luca M, Brand L. Excited-state proton transfer and the mechanism of action of firefly luciferase. *Biochemistry*. 1973 May 8;12(10):1852-7. DOI: 10.1021/bi00734a002.
- ⁷⁸ Lambert N, Idahl LA. Regulatory effects of ATP and luciferin on firefly luciferase activity. *Biochem J*. 1995 Feb 1;305 (Pt 3):929-33. DOI: 10.1042/bj3050929.
- ⁷⁹ McELROY WD. Properties of the reaction utilizing adenosinetriphosphate for bioluminescence. *J Biol Chem*. 1951 Aug;191(2):547-57.
- ⁸⁰ Bhaumik S, Gambhir SS. Optical imaging of Renilla luciferase reporter gene expression in living mice. *Proc Natl Acad Sci U S A*. 2002 Jan 8;99(1):377-82. DOI: 10.1073/pnas.012611099.
- ⁸¹ BioSettia. Firefly Luciferase (FLuc)-IRES-Bsd Lentivirus: About Luciferase Lentivirus [Internet]. San Diego, California USA: BioSettia; c2011 [cited 2016 Aug 27]. Available from: <http://biosettia.com/products-page/lentiviral-cell-labeling-and-imaging/luciferase-lentivirus/fluc-ires-bsd-lentivirus/>
- ⁸² Marques SM, Esteves da Silva JC. Firefly bioluminescence: a mechanistic approach of luciferase catalyzed reactions. *IUBMB Life*. 2009 Jan;61(1):6-17. DOI: 10.1002/iub.134.
- ⁸³ Dukhovich A, Sillero A, Sillero MA. Time course of luciferyl adenylate synthesis in the firefly luciferase reaction. *FEBS Lett*. 1996 Oct 21;395(2-3):188-90.
- ⁸⁴ Viviani VR. The origin, diversity, and structure function relationships of insect luciferases. *Cell Mol Life Sci*. 2002 Nov;59(11):1833-50. DOI: 10.1007/PL00012509.
- ⁸⁵ Suzuki N., Goto T. Studies on firefly bioluminescence—II: Identification of oxyluciferin as a product in the bioluminescence of firefly lanterns and in the chemiluminescence of firefly luciferin. *Tetrahedron*. 1972 Aug; 28(15): 4075-4082. [http://dx.doi.org/10.1016/S0040-4020\(01\)93638-5](http://dx.doi.org/10.1016/S0040-4020(01)93638-5).
- ⁸⁶ Hosseinkhani S. Molecular enigma of multicolor bioluminescence of firefly luciferase. *Cell Mol Life Sci*. 2011 Apr;68(7):1167-82. DOI: 10.1007/s00018-010-0607-0.
- ⁸⁷ Fraga H, Fernandes D, Fontes R, Esteves da Silva JC. Coenzyme A affects firefly luciferase luminescence because it acts as a substrate and not as an allosteric effector. *FEBS J*. 2005 Oct;272(20):5206-16. DOI: 10.1111/j.1742-4658.2005.04895.x.
- ⁸⁸ Fraga H, Esteves da Silva JC, Fontes R. Identification of luciferyl adenylate and luciferyl coenzyme a synthesized by firefly luciferase. *Chembiochem*. 2004 Jan 3;5(1):110-5.
- ⁸⁹ Dyer BW, Ferrer FA, Klinedinst DK, Rodriguez R. A noncommercial dual luciferase enzyme assay system for reporter gene analysis. *Anal Biochem*. 2000 Jun 15;282(1):158-61. DOI: 10.1006/abio.2000.4605.
- ⁹⁰ Tannous BA, Kim DE, Fernandez JL, Weissleder R, Breakefield XO. Codon-optimized Gaussia luciferase cDNA for mammalian gene expression in culture and *in vivo*. *Mol Ther*. 2005 Mar;11(3):435-43. DOI: 10.1016/j.ymthe.2004.10.016.
- ⁹¹ Szent-Gyorgyi C, Ballou BT, Dagnal E, Bryan B. Cloning and characterization of new bioluminescent proteins. *Proc. SPIE 3600. Biomedical Imaging: Reporters, Dyes, and Instrumentation*, 4 (July 2, 1999); DOI:10.1117/12.351015.
- ⁹² Badr CE, Tannous BA. Bioluminescence imaging: progress and applications. *Trends Biotechnol*. 2011 Dec;29(12):624-33. DOI: 10.1016/j.tibtech.2011.06.010.

- ⁹³ Tannous BA. Gaussia luciferase reporter assay for monitoring biological processes in culture and *in vivo*. *Nat Protoc*. 2009;4(4):582-91. DOI: 10.1038/nprot.2009.28.
- ⁹⁴ Shimomura O, Kishi Y, Inouye S. The relative rate of aequorin regeneration from apoaequorin and coelenterazine analogues. *Biochem J*. 1993 Dec 15;296 (Pt 3):549-51.
- ⁹⁵ Zhao H, Doyle TC, Wong RJ, Cao Y, Stevenson DK, Piwnica-Worms D, Contag CH. Characterization of coelenterazine analogs for measurements of Renilla luciferase activity in live cells and living animals. *Mol Imaging*. 2004 Jan;3(1):43-54. DOI: 10.1162/153535004773861714.
- ⁹⁶ Rice BW, Cable MD, Nelson MB. In vivo imaging of light-emitting probes. *J Biomed Opt*. 2001 Oct;6(4):432-40. DOI: 10.1117/1.1413210.
- ⁹⁷ University of Birmingham. Standard Protocols: Luciferase Assay [Internet]. Phil Dash: [unknown date], [cited 2016 Dec 20]. Available from: http://www.protocol-online.org/cgi-bin/prot/view_cache.cgi?ID=2507.
- ⁹⁸ Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. *Anal Biochem*. 1985 Oct;150(1):76-85.
- ⁹⁹ Sözgen K, Cekic SD, Tütem E, Apak R. Spectrophotometric total protein assay with copper(II)-neocuproine reagent in alkaline medium. *Talanta*. 2006 Feb 28;68(5):1601-9. DOI: 10.1016/j.talanta.2005.08.043.
- ¹⁰⁰ Sapan CV, Lundblad RL, Price NC. Colorimetric protein assay techniques. *Biotechnol Appl Biochem*. 1999 Apr;29 (Pt 2):99-108.
- ¹⁰¹ Huang T, Long M, Huo B. Competitive Binding to Cuprous Ions of Protein and BCA in the Bicinchoninic Acid Protein Assay. *Open Biomed Eng J*. 2010;4:271-8. DOI: 10.2174/1874120701004010271.
- ¹⁰² Gornall AG, Bardawill CJ, David MM. Determination of serum proteins by means of the biuret reaction. *J Biol Chem*. 1949 Feb;177(2):751-66.
- ¹⁰³ Thermo Fisher Scientific. Pierce™ BCA Protein Assay Kit [Internet]. Rockford, IL 61105. USA: Thermo Scientific; 2016 [cited 2016 Aug 17]. Available from: <https://www.thermofisher.com/order/catalog/product/23225>
Access to: Pierce Biotechnology. Instructions: Pierce™ BCA Protein Assay Kit [Internet]. Rockford, IL 61105. USA: Thermo Scientific. [Cited 2016 Aug 17] Available from: https://tools.thermofisher.com/content/sfs/manuals/MAN0011430_Pierce_BCA_Protein_Asy_UG.pdf
- ¹⁰⁴ Thermo Fisher Scientific. Chemistry of Protein Assays [Internet]. Thermo Fisher Scientific Inc; 2016 [cited 2016 Aug 17]. Available from: <https://www.thermofisher.com/at/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/chemistry-protein-assays.html>
Access to: Thermo Scientific Fisher. Thermo Scientific Pierce Protein Assay Technical Handbook. Version 2 [Internet]. Thermo Fisher Scientific Inc; 2010 [cited 2016 Aug 17]. Available from: <https://tools.thermofisher.com/content/sfs/brochures/1602063-Protein-Assay-Handbook.pdf>
- ¹⁰⁵ Promega. Compatibility of Promega Lysis Buffer with Reporter and Protein Assays, Technical Reference. PART #GE637 [Internet]. Madison, WI, USA: Promega Corporation; 2010 [cited 2017 Jan 28]. Available from: <https://at.promega.com/~media/files/resources/technical%20references/lysis%20buffer%20compatibility%20with%20reporter%20and%20protein%20assays.pdf>

- ¹⁰⁶ Bodzon-Kulakowska A, Bierzynska-Krzysik A, Dylag T, Drabik A, Suder P, Noga M, Jarzebinska J, Silberring J. Methods for samples preparation in proteomic research. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2007 Apr 15;849(1-2):1-31. DOI: 10.1016/j.jchromb.2006.10.040.
- ¹⁰⁷ Thermo Scientific. Pierce™ Iodoacetamide, Single-Use. Thermo Fisher Scientific Inc; 2017 [cited 2017 Jan 26]. Available from: <https://www.thermofisher.com/order/catalog/product/90034> Access to: Thermo Scientific. Iodoacetamide, Single-Use: Numer 90034 [Internet]. Rockford, IL 61105 USA: Pierce Biotechnology; 2009 [cited 2017 Jan 26]. Available from: https://tools.thermofisher.com/content/sfs/manuals/MAN0011661_Iodoacetamide_SggleUse_UG.pdf
- ¹⁰⁸ Wiechelmann KJ, Braun RD, Fitzpatrick JD. Investigation of the bicinchoninic acid protein assay: identification of the groups responsible for color formation. *Anal Biochem.* 1988 Nov 15;175(1):231-7.
- ¹⁰⁹ Hill HD, Straka JG. Protein determination using bicinchoninic acid in the presence of sulfhydryl reagents. *Anal Biochem.* 1988 Apr;170(1):203-8. DOI:10.1016/0003-2697(88)90109-1
- ¹¹⁰ Promega. Dual-Luciferase® Reporter Assay System Technical Manual: Instructions for Use of Product(s) E1910, E1960 Literature # TM040 [Internet]. Madison, WI, USA: Promega Corporation; 2016 [cited 2016 Aug 25]. Available from: <https://at.promega.com/resources/protocols/technical-manuals/0/dual-luciferase-reporter-assay-system-protocol/> Access to: Promega. Dual-Luciferase® Reporter Assay System: Instructions for use of Products E1910, E1960, Literature # TM040 [Internet]. Madison, WI, USA: Promega Corporation; 2016, revised 06/2015 [cited 2016 Aug 9]. Available from: <http://at.promega.com/-/media/files/resources/protocols/technical-manuals/0/dual-luciferase-reporter-assay-system-protocol.pdf>
- ¹¹¹ Nanolight™ Technology. CAT# 321: Gaussia-Luciferase Protein, Certificate Analysis [Internet]. Nanolight™ Technology (division of Prolume Ltd., Arizona, USA); [cited 2017 Jan 30]. Available from: <http://nanolight.com/Uploads/file/NanoFuels/321%20Gaussia%20Luciferase%20/%23321%20Glu c%20Lot%23G2213.pdf>
- ¹¹² Polyplus-transfection. jetPRIME® Versatile DNA/siRNA transfection reagent [Internet]. Illkirch, France: Polyplus-Transfection; 2016 [cited 2016 Nov 27]. Available from: <http://www.polyplus-transfection.com/products/jetprime/> Access to: Polyplus-transfection. jetPRIME® in vitro DNA & siRNA transfection reagent Protocol, CPT114 vK [Internet]. Illkirch, France: Polyplus-Transfection; [cited 2016 Nov 27]. Available from: http://www.polyplus-transfection.com/wp-content/uploads/2015/08/CPT_114_jetPRIME_vK.pdf
- ¹¹³ polyplus-transfection.com. FAQ [Internet]. Illkirch, France: Polyplus-Transfection; 2016 [cited 2016 Aug 9] Available from: <http://www.polyplus-transfection.com/resources/faq/>
- ¹¹⁴ Thermo Fisher Scientific. Lipofectamine® 2000: Protocols [Internet]. Thermo Fisher Scientific Inc; 2006 Jul 11 [cited 2016 Aug 17]. Available from: <https://www.thermofisher.com/at/en/home/references/protocols/cell-culture/transfection-protocol/lipofectamine-2000.html>
- ¹¹⁵ ATCC®. A549 (ATCC® CCL-185™) [Internet]. ATCC; 2016 [cited 2016 Dec 06]. Available from: https://www.lgcstandards-atcc.org/Products/All/CCL-185.aspx?geo_country=at
- ¹¹⁶ Thermo Fisher Scientific. Versene Solution [Internet]. Thermo Fisher Scientific Inc.; 2015 [cited 2016 Aug 9]. Available from: <https://www.thermofisher.com/order/catalog/product/15040066>

¹¹⁷ Lillico DM, Pemberton JG, Stafford JL. Trypsin differentially modulates the surface expression and function of channel catfish leukocyte. *Dev Comp Immunol*. 2016 Dec;65:231-44. DOI: 10.1016/j.dci.2016.07.014.

¹¹⁸ Thermo Fisher Scientific. Lipofectamine® RNAiMAX Transfection Reagent - Delivery of siRNA [Internet]. Thermo Fisher Scientific Inc; 2015 [cited 2016 Aug 18] Available from: <https://www.thermofisher.com/at/en/home/brands/product-brand/lipofectamine/lipofectamine-rnaimx.html>

Access to: Invitrogen by Life technologies. Lipofectamine® RNAiMAX, Protocol Pub. No. MAN0007825 Rev.1.0 [Internet]: Thermo Fisher Scientific Inc.; 2015 [cited 2016 Aug 18]. Available from:

<https://www.thermofisher.com/content/dam/LifeTech/migration/files/cell-culture/pdfs.par.72509.file.dat/lipofectamine%20rnaimax%20protocol%20v2.0.pdf>

¹¹⁹ Thermo Fisher Scientific. Traditional Methods of Cell Lysis. [Internet]: Thermo Fisher Scientific Inc.; 2015 [cited 2016 Aug 19]. Available from: <https://www.thermofisher.com/at/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/traditional-methods-cell-lysis.html>

¹²⁰ Promega. Dual-Luciferase® Reporter Assay System Technical Manual: Instructions for Use of Product(s) E1910, E1960 Literature # TM040. 2016 [Internet]. Madison, WI, USA: Promega Corporation. 2016 [cited 2016 Aug 25]. Available from:

<https://at.promega.com/resources/protocols/technical-manuals/0/dual-luciferase-reporter-assay-system-protocol/>

Access to: Promega. Dual-Luciferase® Reporter Assay and Dual-Luciferase® Reporter 1000 Assay Systems: Instructions for use of products E1910, E1960 AND E1980. Quick Protocol [Internet]. Madison, WI, USA: Promega GmbH; 2003, revised 3/09 [cited 2016 Aug 25]. Available from: <http://at.promega.com/-/media/files/resources/protcards/dual-luciferase-reporter-assay-and-dual-luciferase-reporter-1000-assay-systems-quick-protocol.pdf>

¹²¹ InvivoGen. Recombinant Lucia Luciferase Protein [Internet]. San Diego, California, USA: Invivogen; 2016 [cited 2016 Aug 25]. Available from: <http://www.invivogen.com/recombinant-lucia>
Access to: Invivogen. Recombinant Lucia™ Protein: A coelenterazine luciferase encoded by a synthetic gene Catalog # rec-lucia; Version #13A21-MM [Internet]. San Diego, California, USA: Invivogen; 2016 [cited 2016 Aug 25]. Available from:

http://www.invivogen.com/PDF/Recombinant_Lucia_TDS.pdf

¹²² European Medicines Agency. Background review for cyclodextrins used as excipients. In the context of the revision of the guideline on 'Excipients in the label and package leaflet of medicinal products for human use' (CPMP/463/00 Rev. 1). London: Committee for Human Medicinal Products (CHMP); 2014 Nov 20. Available from:

http://www.ema.europa.eu/docs/en_GB/document_library/Report/2014/12/WC500177936.pdf

¹²³ Promega. Luciferase Assay System Technical Bulletin: Instructions for Use of Product(s) E1483, E1500, E1501, E1531, E4530, E4030, E4550; Literature # TB281 [Internet]. Madison, WI, USA: Promega GmbH; 2016 [cited 2016 Aug 9]. Available from: <https://at.promega.com/resources/protocols/technical-bulletins/0/luciferase-assay-system-protocol/>

Access to: Dual-Luciferase® Reporter Assay System: Instructions for use of Products E1483, E1500, E1501, E1531, E4030, E4530 and E4550 [Internet]. Madison, WI, USA: Promega Corporation; 2016, Revised 8/15. [cited 2016 Aug 9]. Available from:

<https://at.promega.com/-/media/files/resources/protocols/technical-bulletins/0/luciferase-assay-system-protocol.pdf>

¹²⁴ Navarro G, Maiwald G, Haase R, Rogach AL, Wagner E, de Ilarduya CT, Ogris M. Low generation PAMAM dendrimer and CpG free plasmids allow targeted and extended transgene

expression in tumors after systemic delivery. J Control Release. 2010 Aug 17;146(1):99-105. DOI: 10.1016/j.jconrel.2010.04.030.

¹²⁵ NanoLight™ Technology. Cat # 303 Coelenterazine native [Internet]. NanoLight Technologies division of Prolume Ltd, USA; 2011 [cited 2016 Nov 30]. Available from: http://nanolight.com/CoelenterazineCyan/113_Native_Coelenterazine.html
Access to: NanoLight™ Technology. How to use “native-CTZ” Guide [Internet]. NanoLight Technologies division of Prolume Ltd, USA; 2011 [cited 2016 Nov 30]. Available from: http://nanolight.com/Uploads/file/NanoFuels/303%20CTZ%20native%20correct%20files/303%20NF-CTZ-FB%20HTU%2002-12_%20pdf.pdf

¹²⁶ Tecan. Infinite® 200 – Download [Internet]. Switzerland: Tecan Trading AG; 2016 [cited 2016 Aug 9]. Available from: http://lifesciences.tecan.com/products/reader_and_washer/microplate_readers/infinite_200_pro/download
Access to: Tecan. Infinite® 200 PRO – luminescence sensitivity: Optimizing the luminescence sensitivity in the Infinite 200 PRO multimode reader series. Technical Note [Internet]. Switzerland: Tecan Trading AG; 2010 Jul 13 [cited 2016 Aug 9].

¹²⁷ MGM Instruments. Application Note: USING THE PROMEGA DUAL-LUCIFERASE REPORTER ASSAY SYSTEM WITH THE OPTOCOMP™ II LUMINOMETER. MGM Document no. 8900-4000-00 [Internet]. MGM Instruments, Inc.; Revision A 2001 Apr [cited 2016 Aug 25]. Available from: <http://www.mgminstruments.com/PDF/DLR%20Application%20Note.pdf>

¹²⁸ New England Biolabs. BioLux Gaussia luc assay kit [Internet]. Frankfurt am Main, Germany: New England Biolabs GmbH; 2016 [cited 2016 Aug 09]. Available from: <https://www.neb.com/products/e3300-biolux-gaussia-luciferase-assay-kit#tabselect2> Access to: New England Biolabs, Inc. Reporter Systems: BioLux® Gaussia Luciferase Assay Kit, Instruction Manual, Version 2.0 [Internet]. Frankfurt am Main, Germany: New England Biolabs GmbH; 2014 Nov [cited 2016 Aug 9]. Available from: <https://www.neb.com/~media/Catalog/All-Products/946EA4128B77477681AA2A267CD51FD8/Datacards%20or%20Manuals/manualE3300.pdf>

¹²⁹ Binkowski BF. NanoLuc™: A Smaller, Brighter and More Versatile Luciferase Reporter [Internet]. Madison, WI, USA: Promega Corporation; 2017 [cited 2017 Jan 29]. Available from: <https://at.promega.com/resources/webinars/worldwide/archive/nanoluc-a-smaller-brighter-and-more-versatile-luciferase-reporter/>
Access to: Binkowski BF. NanoLuc™ Luciferase: A Smaller, Brighter & More Versatile Luciferase Reporter: Promega Corporation [Internet]. 2012 Jun. Available from: <https://at.promega.com/~media/files/promega%20worldwide/north%20america/promega%20us/webinars%20and%20events/nanoluc%20webinar%20presentation2%20062012.pdf?la=de-de>

¹³⁰ Anchordoquy TJ, Koe GS. Physical stability of nonviral plasmid-based therapeutics. J Pharm Sci. 2000 Mar;89(3):289-96. DOI: 10.1002/(SICI)1520-6017(200003)89:3<289::AID-JPS1>3.0.CO;2-N.

¹³¹ Cherg JY, vd Wetering P, Talsma H, Crommelin DJ, Hennink WE. Stabilization of polymer-based gene delivery systems. Int J Pharm. 1999 Jun 10;183(1):25-8.

¹³² Hinrichs WL, Manceñido FA, Sanders NN, Braeckmans K, De Smedt SC, Demeester J, Frijlink HW. The choice of a suitable oligosaccharide to prevent aggregation of PEGylated nanoparticles during freeze thawing and freeze drying. Int J Pharm. 2006 Mar 27;311(1-2):237-44.

¹³³ Anchordoquy TJ, Girouard LG, Carpenter JF, Kroll DJ. Stability of lipid/DNA complexes during agitation and freeze-thawing. J Pharm Sci. 1998 Sep;87(9):1046-51.

¹³⁴ Kasper JC, Schaffert D, Ogris M, Wagner E, Friess W. (a) Development of a lyophilized plasmid/LPEI polyplex formulation with long-term stability--A step closer from promising

technology to application. J Control Release. 2011 May 10;151(3):246-55. DOI: 10.1016/j.jconrel.2011.01.003.

¹³⁵ Stanlex J. Essentials of Immunology & Serology. In: Eserti CL, Scelsi DM, editors [Internet]. USA: Delmar, a division of Thomson Learning, Inc.; 2002 [cited 2016 Jun 12]. p. 137. Available from: <https://books.google.at/books?id=nuCT5RVToN4C&pg=PA137&lpg=PA137&dq=photomultiplier+luminometer&source=bl&ots=SIQwnMg5zy&sig=dAnlF8nKYFjx3hzBi9RxVJbSCHc&hl=de&sa=X&ved=0ahUKEwj0i-3QggPNAhXEBBoKHfmRAbkQ6AEIXjAH#v=onepage&q=photomultiplier%20luminometer&f=false>

¹³⁶ Wikipedia contributors. Photomultiplier [Internet]. Wikipedia, The Free Encyclopedia; 2016 Aug 17 [cited 2016 Aug 17]. Available from: <https://en.wikipedia.org/w/index.php?title=Photomultiplier&oldid=734907759>

¹³⁷ Rana SVS. Biotechnique: Theory and Practice [Internet]. Meerut-250 002, India: Rastogi Publications; 2008 [cited Aug 9]. p.109-112. Available from: https://books.google.at/books?id=ggJZGLkCqN4C&pg=PA110&lpg=PA110&dq=rana+luminometer&source=bl&ots=9JmbRagPwi&sig=IKR43c-yKrJP_OoQRenloQ6r-Wl&hl=de&sa=X&ved=0ahUKEwjKt62i66XNAhVLLsAKHS8JDgAQ6AEILjAA#v=onepage&q=rana%20luminometer&f=false

¹³⁸ Tecan Austria GmbH. Instructions for use for Infinite 200® PRO. No. 30052730 Rev. Nor. 1.0. Austria; 2010 Oct.

¹³⁹ Tecan. Dual-Luciferase® Reporter (DLR™) Assay System: Implementation on Tecan's Infinite® F500 Multimode Reader [Internet]. Switzerland: Tecan Trading AG; 2008 Oct [cited Dec 20]. Available from: http://ww3.tecan.com/platform/content/element/6940/tn_infinitef500_dlr_395676_v1.0.pdf

¹⁴⁰ Meleady P, O'Connor R. General Procedures for Cell Culture. In: Celis JE., editor. 3rd ed. Cell Biology. A Laboratory Handbook. Volume 1. USA: Elsevier Academic Press; 2006 [cited 2016 Dec 7]. Available from: <https://books.google.at/books?id=02mLqzXwStUC&pg=PA5&lpg=PA5&dq=o+connor+general+procedures+for+cell+culture+techniques&source=bl&ots=75fSbAjNPL&sig=6AsWyccdGIFjmtsdzPz aeTtaH6Y&hl=de&sa=X&ved=0ahUKEwi4so-2xOHQAhXB6xQKHQ65AbUQ6AEIHTAA#v=onepage&q=o%20connor%20general%20procedures%20for%20cell%20culture%20techniques&f=false>

¹⁴¹ Baydoun AR. Cell culture techniques. In: Wilson K, Walker J, editors. 7th ed. Principles and Techniques of Biochemistry and Molecular Biology. New York: Cambridge University Press; 2010.p. 38-72.

¹⁴² Invitrogen Cooperation. GIBCO® serum - it passes the test [Internet]. Invitrogen Cooperation; 2007 [cited 2017 Jan 28]. Available from: https://tools.thermofisher.com/downloads/B-066802-Sera_Bro.pdf

¹⁴³ Wikipedia contributors. Cell culture [Internet]. Wikipedia, The Free Encyclopedia; 2016 Jul 31 [cited 2016 Aug 25]. Available from: https://en.wikipedia.org/w/index.php?title=Cell_culture&oldid=732324503

¹⁴⁴ Freshney RI. Basic principles of Cell Culture. In: Vunjak-Novakovic G, Freshney RI, editors. Culture of Cells for Tissue Engineering. Hoboken, NJ, USA: John Wiley & Sons, Inc; 2006 [cited 2016 Jun 13]. p. 3-21. Available from: <http://histologia.ugr.es/pdf/0471629359.pdf>

¹⁴⁵ Wikipedia contributors. Confluency [Internet]. Wikipedia, The Free Encyclopedia; 2016 Nov 26 [cited 2017 Jan 28]. Available from: <https://en.wikipedia.org/w/index.php?title=Confluency&oldid=751539503>.

- ¹⁴⁶ Wolf JB (UMBC, An Honors University in Maryland). Tissue Culture Methods [Internet]. Baltimore, MD, U.S: University of Maryland, Baltimore County, Baltimore, MD 21250; Department of Biological Sciences; [updated 2010 Feb 3]. Available from: <http://userpages.umbc.edu/~jwolf/method5.htm>
- ¹⁴⁷ Schrödel A. Kryokonservierung von eukaryotischen Zellkulturzellen [Cryoconservation of eucaryotic cell culture cells]. Biologie in unserer Zeit [Internet]. 2009 Oct 15; 39(5):308. German DOI:10.1002/biuz.200990072.
- ¹⁴⁸ Celeromics. Cell Counting with Neubauer Chamber: Basic Hemocytometer Usage [Internet]. Celeromics: Spain; 2017 [cited 2016 Dec 7]. Available from: <http://www.celeromics.com/en/resources/Technical%20Notes/cover-cell-glass.php>. Access to: Bastidas O. Cell Counting with Neubauer Chamber: Basic Hemocytometer Usage. Celeromics; [cited 2016 Dec 7]. Available from: <http://www.celeromics.com/en/resources/docs/Articles/Cell-counting-Neubauer-chamber.pdf>
- ¹⁴⁹ Marienfeld-Superior. Manuals [Internet]. Lauda-Königshofen, Germany: Paul Marienfeld GmbH & Co. KG; 2017 [cited 2017 Jan 28]. Available from: <http://www.marienfeld-superior.com/index.php/manuals.html>. Access to: Marienfeld-Superior. Counting Chamber (Hemacytometer). Lauda-Königshofen, Germany: Paul Marienfeld GmbH & Co. KG; 2017 [cited 2017 Jan 28].
- ¹⁵⁰ Hemocytometer [Internet]. Wikipedia, The Free Encyclopedia; 2016 Jul 22, 03:58 UTC [cited 2016 Aug 9]. Available from: <https://en.wikipedia.org/w/index.php?title=Hemocytometer&oldid=730982295>
- ¹⁵¹ Gompf A. Grundlagen der Durchflusszytometrie. Sack U (Hrsg.). Zelluläre Diagnostik. Berlin: Walter de Gruyter GmbH & Co KG; 2016: p.1-8. Available from: https://books.google.at/books?id=KPg-CwAAQBAJ&pg=PA1&dq=Gompf+A.+Grundlagen+der+Durchflusszytometrie.&hl=de&source=gbs_toc_r&cad=4#v=onepage&q=Gompf%20A.%20Grundlagen%20der%20Durchflusszytometrie.&=false
- ¹⁵² Ormerod MG. Flow Cytometry - A Basic Introduction [Internet]. [unknown publisher]; 2008: Chapter 4: Data Analysis. Available from: <http://flowbook.denovosoftware.com/>
- ¹⁵³ Wikipedia contributors. Flow cytometry [Internet]. Wikipedia, The Free Encyclopedia; 2017 Jan 22 [cited 2017 Jan 28]. Available from: https://en.wikipedia.org/w/index.php?title=Flow_cytometry&oldid=761314612.
- ¹⁵⁴ Wikipedia contributors. DAPI [Internet]: Wikipedia, The Free Encyclopedia; [revised 2016 May 31 [cited 2016 Jun 11]. Available from: <https://en.wikipedia.org/w/index.php?title=DAPI&oldid=723051178>