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I hereby declare that I have carried out this work independently and that the use of other sources is clearly indicated and acknowledged.

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1. Abstract

Epidermal growth factor receptor (EGFR) is a key player in development of many epithelial cancers, which made it a heavily studied drug target. The aim of this thesis was to optimise the transfection protocol with EGFR-EGFP plasmid construct (encoding a green fluorescent protein, EGFP, and EGFR fusion protein) using linear polyethylenimine as the transfection reagent to reach best transfection efficiency with these polyplexes with minimal toxicity. Moreover, T790M mutant of EGFR-EGFP in *E. coli* bacteria was expanded and purified to compare the effects of pharmacological intervention between wild type and mutant EGFR in western blot experiment, which was also optimised in the process. The results revealed the human cell line HEK293T as the most easily transfectable, with transfection efficiencies reaching up to ~ 50% following treatment with 8 µg per well treatment. Moreover, the toxicity of the polyplex transfection was low as there were still ~ 84% live cell after treatment with highest dose. The T790M EGFR-EGFP was expanded in *E.coli* and validated in transfection. This yielded one T790M EGFR-EGFP plasmid clone that behaved as its wild-type counterpart in terms of transfection efficiency. The presence of the mutation was validated in optimised western blot after tyrosine kinase inhibitors treatment. Here, good EGFR activation was achieved with 0.1 ng/mL hEGF stimulation for 10 minutes and reducing the heat treatment time in sample preparation to 5 minutes resulted in better defined protein bands. Furthermore, higher antibody dilutions were sufficient in achieving good signal.

2. Zusammenfassung

Der epidermale Wachstumsfaktor Rezeptor (EGFR) spielt eine Schlüsselrolle bei der Entstehung vieler epithelialer Krebsarten und ist daher ein intensiv untersuchtes Ziel für die Medikamentenentwicklung. Ziel dieser Arbeit war es, ein Transfektionsprotokoll für das EGFR-EGFP-Plasmidkonstrukt (kodierend für ein Fusionsprotein aus Grün fluoreszierendem Protein (EGFP) und EGFR) mit linearem Polyethylenimin als Transfektionsreagens zu optimieren, um eine maximale Transfektionseffizienz mit diesen Polyplexen bei minimaler Toxizität zu erreichen. Darüber hinaus wurde ein EGFR-EGFP Plasmid codierend für die T790M-Mutante von EGFR in E.coli Bakterien expandiert und aufgereinigt, um in weiteren Experimenten nach Transfektion die Auswirkungen pharmakologischer Interventionen zwischen Wildtyp EGFR und mutiertem EGFR im Western-Blot-Experiment zu vergleichen. Ebenso wurde die Western Blot Methode entsprechend optimiert. Die Ergebnisse zeigten, dass die humane Zelllinie HEK293T-Zellen bei Verwendung von 8 µg Plasmid DNA/well Transfektionsraten von bis zu 50 % ergaben. Die Toxizität der Polyplex-Transfektion war niedrig, da nach der Behandlung mit der höchsten Dosis noch etwa 84 % lebende Zellen vorhanden waren. Das T790M EGFR-EGFP Plasmid wurde in E.Coli expandiert und in der Transfektion validiert. Ein T790M EGFR-EGFP-Plasmid Klon wurde isoliert und expandiert, der sich hinsichtlich der Transfektionseffizienz wie sein Wildtyp-Gegenstück verhielt. Das Vorhandensein der Mutation wurde nach der Behandlung mit Tyrosinkinase-Inhibitoren in einem optimierten Western Blot validiert. Hier wurde eine deutliche EGFR-Aktivierung mit 0,1 ng/mL hEGF-Stimulation für 10 Minuten erreicht, und die Reduzierung der Dauer der Hitzebehandlung bei der Probenvorbereitung auf fünf Minuten führte zu besser abgegrenzten Proteinbanden im Western Blot. Außerdem reichten dann höhere Antikörperverdünnungen aus, um ein gutes Signal zu erzielen.

3. Introduction

3.1. Cancer and advancements in gene therapy

Cancer continues to be one of the main burdens on human health. As of 2020, cancer is the leading cause of death, with 10 million deaths recorded that year ¹, overtaking the ischaemic heart disease. Moreover, the number of deaths from cancer is expected to rise by 2.08-fold increase ². Currently, the risk of developing cancer between the age 0-74 stands at ~ 20%, while the risk of dying from cancer in the same age group is ~ 10% ³. In addition to mortality, cancer also represents the largest health burden worldwide. The burden of a disease can be estimated in disability adjusted life years (DALYs). In this estimate, cancer ranked 1st, with 9.2% portion of overall DALYs ². Thus, cancer clearly remains a huge medical challenge, which has a great impact not only on human health and health sector in general, but also on many other socio-economic areas.

As the current pandemic of COVID-19 clearly demonstrated, thorough research is essential to combat such huge biomedical challenges as cancer. However, unlike COVID-19, cancer is not a one single disease, but rather a set of malignancies linked together by common features. Therefore, therapies differ based on what type of cancer is treated, and also often on what kind of mutations the cancerous cells acquire. Nonetheless, all cancers can be characterised by uncontrolled proliferation of cells in tissue, which leads to growth of a tumour. Moreover, the tumour cells can disseminate throughout body and invade other tissues in process known as metastasis ⁴. A landmark review by Hanahan and Weinberg summarised the hallmarks, or common features, that are shared by all cancers (**Figure 1**). These include self-sufficiency in growth signalling, insensitivity to anti-growth signalling, limitless replicative potential, sustained angiogenesis, evasion of apoptosis and tissue invasion and metastasis ⁵. All these features enable cancer cells to grow rapidly, without any control, which leads to clinical manifestation of the disease and death, if untreated. Additionally, further research suggested newer hallmarks of cancer, such as evasion of immune destruction, tumour promoting inflammation, energy metabolism changes and genome instability ⁶.

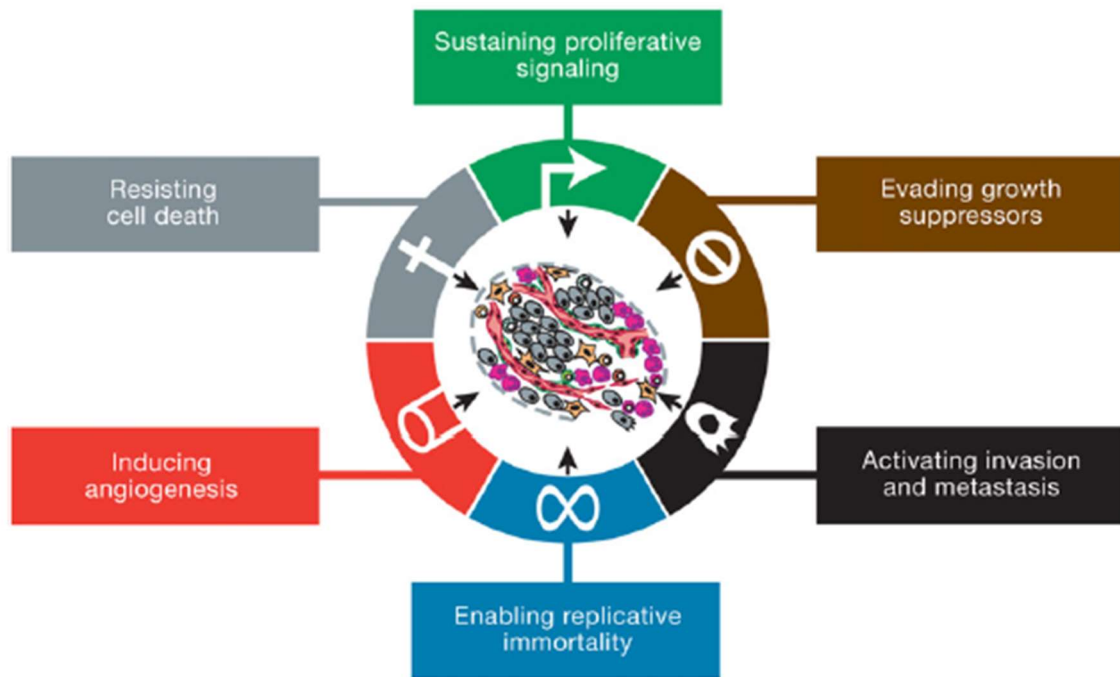


Figure 1. Hallmarks of cancer. Figure summarising 6 original hallmarks of cancer, including sustaining proliferative signalling, evading growth suppression, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death. Figure taken from Hanahan and Weinberg (2011) ⁶.

Having full overview of a disease is of utmost importance in designing effective therapies. Definition of the hallmarks of cancer helped researchers to focus on specific areas of cell machinery, which could be targeted with the promise of inhibiting cancerous growth. Apart from surgical treatment in early stages of the disease, various small molecules or irradiation are employed to kill the tumour cells, with varying degrees of success ⁷. These approaches are generally known as chemotherapy and radiotherapy respectively. However, a newer approach to cancer treatment is currently picking up speed, which is gene therapy.

The idea to employ gene therapy for the treatment of cancer comes from the fact that cancer is caused by genetic factors ⁵. Tumour growth is usually initiated by mutations in so called oncogenes or tumour-suppressor genes (**Figure 2**). Gain-of-function mutations in oncogenes, as well as loss-of-function mutations in tumour-suppressor genes enable the cell to proliferate rapidly, causing cancer ⁸.

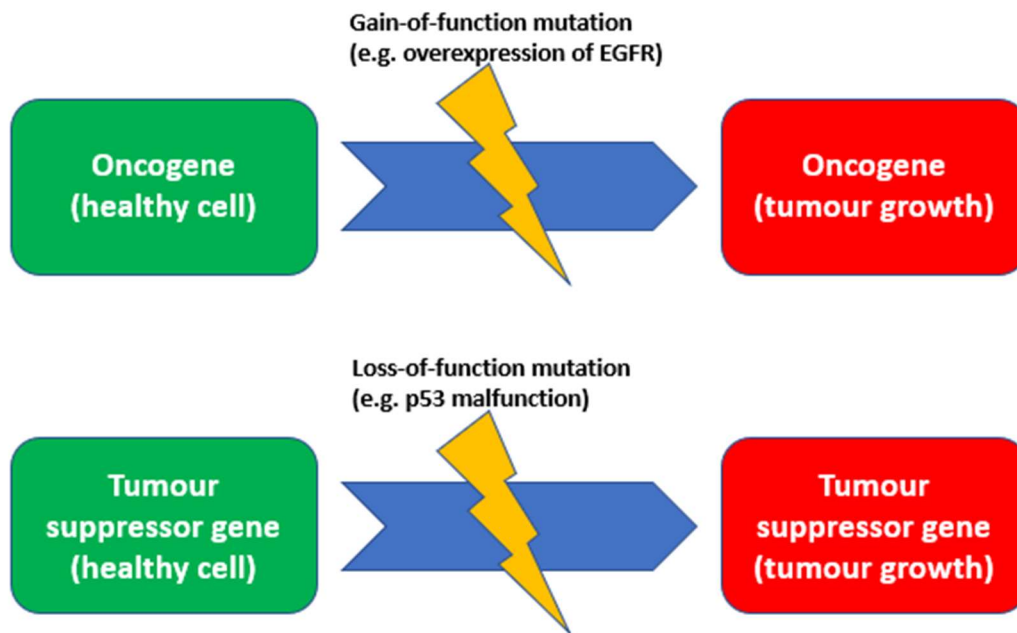


Figure 2. Oncogenes and tumour suppressor genes. Schematic showing the concept of oncogenes and tumour suppressor genes. Gain-of-function mutations and loss-of-function mutations in oncogenes and tumour suppressor genes respectively, lead to tumour growth. Figure drawn by Marian Peteri based on the information from Lee and Muller (2010) ⁸.

Typical example of oncogenes are growth factor receptors, where mutations can lead to overexpression or structural alterations, which in turn lead to responsiveness to low levels of growth factors or spontaneous signalling (i.e. gain of function) ⁵. For example, epidermal growth factor receptor (EGFR or ErbB-1) is overexpressed in 20-30% of human breast cancers ⁸. Elevated levels of EGFR has been found also in head-and-neck cancer, non-small cell lung cancer, ovarian cancer and colon cancer ⁹.

While mutations in oncogenes lead to gain of function (e.g. ability to proliferate in the absence of growth signals), mutations in tumour-suppressor genes disrupt mechanisms that stop the cell from becoming cancerous. An example is gene coding for retinoblastoma protein, which plays essential role in regulation of G1/S-phase progression ⁸. Its loss of function allows the cell to progress to S-phase without regulation, thus leading to malignant growth. Another famous example of tumour-suppressor gene is p53, which is very important in arresting cell division in response to various stress factors ⁸. Mutations of this genes were found in majority of sporadic tumours ⁸.

Of course, these are only few examples of oncogenes and tumour-suppressor genes, and genomic changes can be found behind all of the hallmarks of cancer discussed earlier.

Therefore, gene therapy is a promising approach in cancer treatment, as it allows us to specifically suppress gain-of-function mutations in oncogenes or to restore function in tumour-suppressor genes. By replacing or suppressing malfunctioning genes via delivery of nucleic acids, gene therapy seeks to overcome hurdles faced by conventional recombinant peptide therapy, such as poor bioavailability, high toxicity or high production costs ¹⁰.

The idea of gene therapy was formulated in the early 1970s ¹¹. It originally focused on simple monogenic diseases, but soon people realised the potential it has in treatment of complex multigenic conditions such as cancer. Several approaches to cancer gene therapy were investigated over the years, ranging from simple replacement of mutated tumour suppressor gene to development of gene-modified tumour cells as cancer vaccines or introduction of genetically engineered immune cells targeting tumours ¹².

Despite initial safety concerns associated with gene therapy, which were exacerbated by the death of Jesse Gelsinger in 1999, there were four gene therapy drugs approved by 2010 (three of these were targeted against cancer) ¹³. After this, the gene therapy field accelerated and by 2017, 2 597 clinical trials were in progress (with 65% of these being targeted against cancer) ¹⁰. As of 2019, 22 gene therapy products were approved for use and majority of these is used for cancer treatment ¹⁰. Although cancer non-related, the success of gene therapy is also highlighted by the recent success of COVID-19 nucleic acid vaccines, which work on the principle of delivery of genetic information coding for the pathogen-associated antigen (spike protein), which can be then recognised by the immune system ¹⁴.

The first gene therapy approved for cancer was Gendicine, which was approved in China in 2003 for treatment of head and neck squamous cell carcinoma ¹⁵. This therapy makes use of adenoviral vector, which delivers p53 gene and restores the function of mutated p53, which then leads to cell apoptosis ¹⁵. Another example of gene therapy targeting p53 deficient cells is Oncorine, which was also approved in China two years later for treatment of head and neck and oesophagus cancer and nasopharyngeal cancer ¹⁶. Oncorine is also adenoviral based therapy, but unlike Gendicine, Oncorine doesn't deliver functional copy of p53 gene. Instead, the adenovirus is engineered in such a way that it is capable of replication only in p53 deficient cells, which then causes lysis of these cells ¹⁶.

Both Gendicine and Oncorine are treatments with in vivo route of administration, meaning that the gene therapy vector is delivered directly to the patient. A different approach is used by chimeric antigen receptor (CAR) T cell therapy, which is an example of ex vivo gene therapy¹⁷. Here, the T cells of the patient are harvested and then they are transfected ex vivo with antigen receptor specific to the tumour cells. The transfected cells are then reintroduced to the patient, where they target the cancer cells¹⁷. Examples of CAR T cell therapy include Kymriah™ which was approved in 2017 for treatment of acute lymphoblastic leukaemia and Yescarta™, also approved in 2017 for the treatment of non-Hodgkin lymphoma¹⁰.

While cancer gene therapy has certainly made a great progress in the last couple of decades, research is still ongoing to increase efficacy and safety. Moreover, decreasing the cost of the gene therapy is also a very important factor in making it more available. It is expected that with development of newer technologies, gene therapy will become safer and cheaper, which could lead to personalisation of cancer treatment on patient to patient basis and greatly improved management of the disease¹⁰.

3.2. EGFR signalling pathways

As already mentioned, one of the prototypical oncogenes found in human genome is EGFR. Sometimes referred to as ErbB-1 or HER-1, EGFR belongs to the ErbB family of receptors¹⁸. All members of this family are receptor tyrosine kinases, which play role in signalling pathways that regulate cellular proliferation¹⁸. As its name suggests, EGFR is activated in response to binding of epidermal growth factor (EGF). However, EGF is not the only ligand activating EGFR, as it is also receptor for other ligands such as tumour growth factor- α , amphiregulin, betacellulin or epiregulin¹⁸. EGFR is a transmembrane receptor and similarly to other ErbB family members, it consists of heavily glycosylated extracellular domain, which is linked by single transmembrane domain to large cytoplasmic domain (**Figure 3**). The ligand binding site is found in the extracellular domain of EGFR. The cytoplasmic domain harbours the kinase activity and also multiple phosphorylation sites¹⁸.

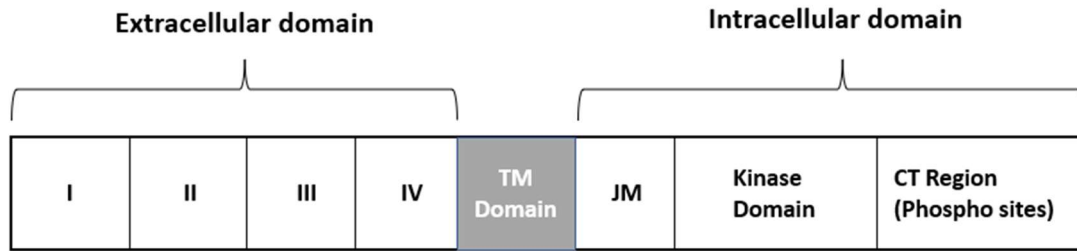


Figure 3. EGFR protein diagram. This diagram shows domains of EGFR. Extracellular domain consists of regions I-IV. Regions I and II bind ligands. Intracellular domain consists of juxta membrane domain (JM) and kinase domain, as well as of CT region which harbours sites for phosphorylation. The extracellular and intracellular domains are separated by single trans-membrane (TM) domain. Figure adapted from Linggi and Carpenter (2006) ¹⁸.

Under basal conditions, EGFR exists in monomeric state in the membrane. However, upon binding of a ligand, EGFR undergoes conformational change, which allows dimerization of two EGFR proteins ¹⁹. Additionally, EGFR can also dimerise with other members of the ErbB family ¹⁸. The dimerization is possible due to exposure of dimerization arm in extracellular domain, which is sequestered by interaction with subdomain IV when no ligand is binding ²⁰. The dimerization is key for activation of EGFR, as it brings the intracellular kinase domains of the two receptors into close proximity, such that transphosphorylation can occur (**Figure 4**) ²¹. Phosphorylation of the EGFR's intracellular sites then enables further propagation of the signal. Moreover, certain degree of signal amplification happens at this stage, as there are multiple phosphorylation sites with which second messenger molecules can interact with ¹⁹.

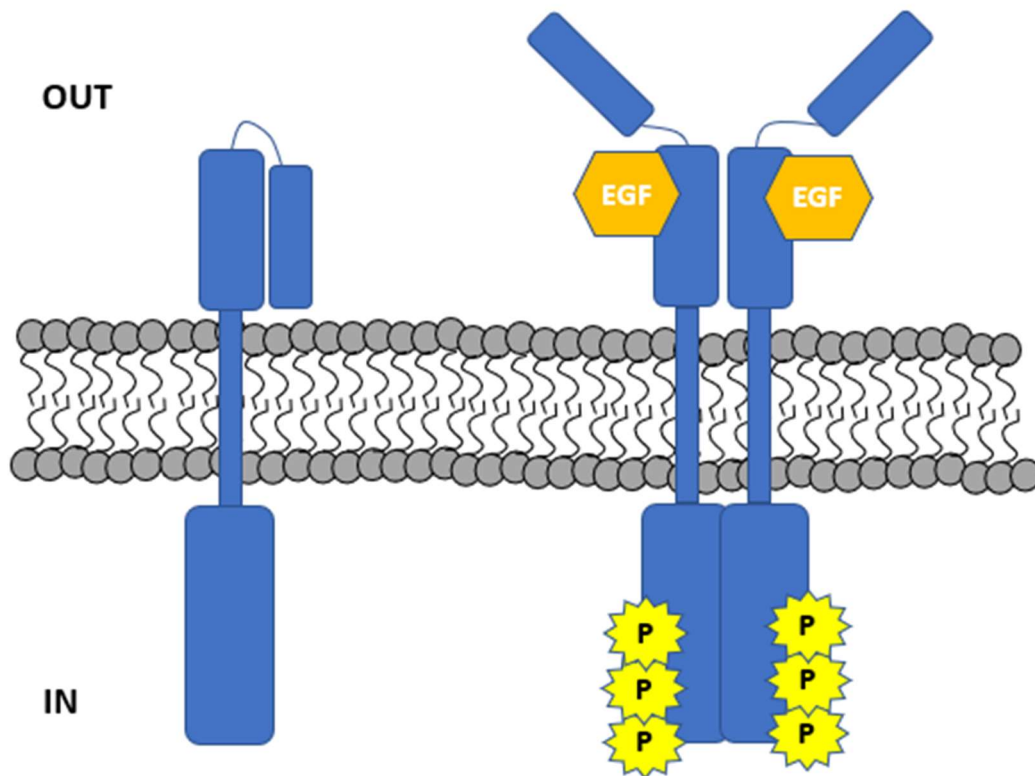


Figure 4. Activation of EGFR. Schematic representation of EGFR activation after EGF binding. Ligand binding causes conformational change in the extracellular domain, which allows association of two EGFR molecules. Close proximity allows transphosphorylation of in the intracellular domain. Figure adapted from Linggi and Carpenter (2006) ¹⁸.

EGFR signals via multiple pathways (**Figure 5**). One example of such pathway is the RAS-RAF-MEK-ERK MAPK pathway. Being perhaps the most important pathway via which EGFR signals, this pathway plays major role in cellular growth, proliferation, differentiation, migration and inhibition of apoptosis ²². Phosphorylated tyrosine residues Y1068 and Y1086 on the EGFR recruit growth factor receptor binding protein 2 (GRB2) via its SH2 domain ²³. GRB2 is then phosphorylated itself and then it propagates the signal further via Ras and Raf proteins ²³. The result of this signalling cascade is expression of genes triggering the before-mentioned processes.

Another major signalling pathway activated by EGFR is the PI3K-Akt-mTOR pathway. which similarly to MAPK pathway is involved in cell proliferation, apoptosis and motility and migration ²⁴. In order to trigger this pathway, EGFR has to dimerise with HER-3 (different member of the ErbB family), as EGFR lacks the sites necessary for interaction with PI3K ²⁴. Although, PI3K can also interact with EGFR indirectly via Gab-1 ²⁴. Active PI3K produces

phosphatidylinositol-3,4,5-triphosphate which is responsible for Akt activation ²⁵. Akt is also important signalling molecule in metabolism regulation ²⁵, pointing to EGFR's role also in this process.

Other examples of EGFR signalling pathways include the PLC- γ 1-PKC pathway or signalling via STAT proteins. The PLC- γ 1-PKC pathway involves interaction of EGFR with phospholipase C- γ (PLC- γ), which when activated, breaks down phosphatidylinositol-4,5-diphosphate to give inositol-1,3,5-triphosphate and 1,2-diacylglycerol ²⁴. Inositol-1,3,5-triphosphate plays important role regulation of intracellular Ca^{2+} release ²⁴. On the other hand, 1,2-diacylglycerol activates protein kinase C (PKC), which then induces further signalling down the MAPK pathway ²⁴. This again leads to promotion of proliferation and survival. Similarly, signalling via STAT (signal transducers and activators of transcription) proteins leads to increase in proliferation and survival as well. Here, the STAT proteins can interact directly with active EGFR via their Src-homology 2 domains, which subsequently leads to STAT activation and dimerization of two STAT molecules ²⁴. The STAT dimers then translocate to the nucleus, where they directly act as transcription factors.

Moreover, EGFR was found to signal directly through internalization and subsequent nuclear translocation. Originally, EGFR internalization was viewed as a desensitization mechanism following activation and signalling ¹⁸. However, new evidence emerged suggesting that EGFR internalization plays role in signalling. Indeed, nuclear localization of EGFR was found to promote cellular proliferation, as it was shown to aid in transcription of important proteins for cell cycle progression, such as cyclin D1 ²⁶.

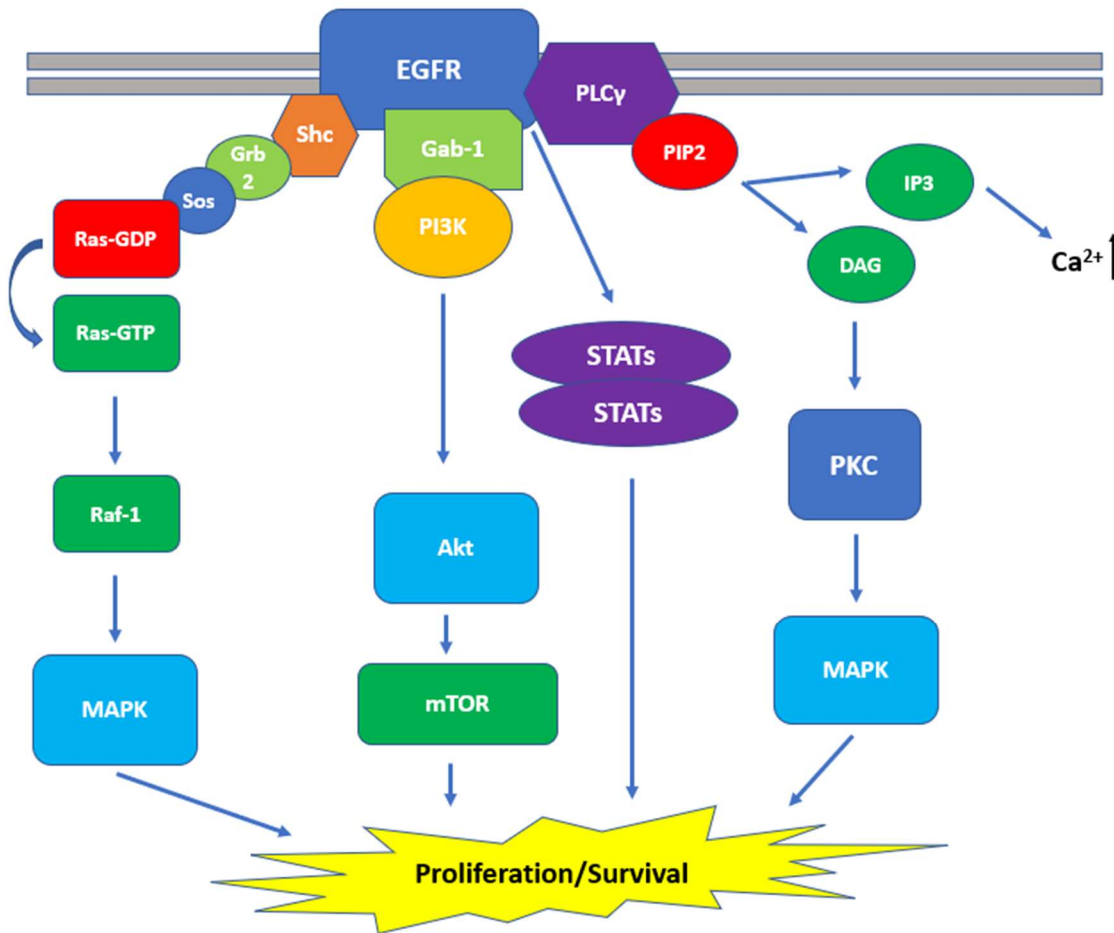


Figure 5. Overview of pathways regulated by EGFR. The most important pathways activated by EGFR are shown. These include, the Ras-Raf-MAPK pathway, PI3K-Akt-mTOR pathway, STAT activation and PLCγ-PKC pathway. Activation of EGFR and subsequent activation of downstream pathways lead to promotion cell proliferation and survival. Figure adapted from Scaltriti and Baselga (2006) ²⁴.

As we can clearly see, the overarching theme of EGFR signalling is enhancement of cell growth and proliferation, as well as increase in survival. It comes as a no surprise that EGFR, together with other ErbB family members, plays absolutely crucial role in embryogenesis and normal development. Indeed, null mutations of EGFR have been found to be lethal in embryonic and perinatal development of mouse models ¹⁹. Also, EGFR is important in development of ductal glands in mammals in adolescence ¹⁹. EGFR or other ErbB family members can be found in all tissues including central nervous system. There, EGFR was found to be regulate the development, as well as phenotypic characteristics of astrocytes ²⁷, pointing towards role in central nervous system development as well.

However, being involved so closely in cell proliferation and survival also means, that any aberrant EGFR signalling can inevitably lead to cancerous growth. The link between cancer

and EGFR was first made, when EGFR was found to be human homologue of avian v-ErbB, oncogene in avian erythroblastosis ²⁸. Later, EGFR was found to be involved in many hallmarks of cancer, including cell proliferation, resistance to apoptosis, angiogenesis and invasion and metastasis. The aberrant EGFR signalling that leads to these malignant processes, is mostly caused by EGFR overexpression. While on healthy cells, the number of EGFR molecules on the cell surface ranges from 40 000 to 100 000, tumour cells overexpressing EGFR can have as many as 2×10^6 EGFR molecules on the surface ²⁹. This large number of EGFR receptors on the cell surface means, that the cell is sensitive to ambient levels of EGF, and thus signalling pathways are triggered even in the absence of proper signal. Although there are cases where EGFR acquires mutations that can lead to spontaneous signalling ³⁰, it is mainly overexpression of this receptor that is found in cancer. Overexpressed EGFR can be found in majority of solid tumours and it plays a substantial role in development of breast cancer, colon cancer, head-and-neck cancer, non-small cell lung cancer, renal cancer or ovarian cancer ²⁹. For instance, as mentioned in the first chapter of this thesis, EGFR is overexpressed in 20-30% cases of all breast cancer ⁸. Additionally, overexpression of EGFR also indicates poor prognosis as well as higher relapse rate in cancer patients ³¹.

Following the realisation that EGFR plays such an important role in many cancers, treatment strategies targeting this receptor emerges shortly afterwards. The fact that EGFR is found on the surface of the cell, as well as clear distinction between cancer cell and healthy cell in terms of EGFR abundance, made EGFR a perfect drug target. Traditional radiotherapy was found to be ineffective in treatment of tumours positive for EGFR overexpression, as radiation stimulates EGFR signalling and thus promotes the growth of the tumour ³². Therefore, targeting EGFR with macromolecules or small molecules is the preferred option. Mainly two approaches have been utilised in EGFR targeting. One of these are monoclonal antibodies (mAbs). Here, antibodies specific for EGFR are delivered to the patient intravenously. Anti-EGFR mAbs then bind to the extracellular domain of EGFR and they block the sites for EGFR dimerization ²⁴. Despite their high specificity and efficacy, mAbs possess some considerable limitations. These include their high cost as well as the inconvenience of repeated intravenous delivery ²⁹. Moreover, the patient's immune system can render repeated use of mAbs ineffective ²⁹. The second most widely used approach to target EGFR positive tumours is the

use of tyrosine kinase inhibitors (TKIs). These will be described in more detail in the next chapter.

3.3. Tyrosine kinase inhibitors and EGFR

Traditional chemotherapy involves taking small molecule drugs that have cytostatic or cytotoxic effects. Usually, these compounds don't have any intrinsic specificity towards tumour cells, which means that healthy cells are also negatively impacted, resulting in array of severe side effects ³³. TKIs, on the other hand, are designed to target a limited number of interaction partners, in this case EGFR. Since the EGFR receptor is largely overexpressed on tumour cells, TKIs target primarily these cells, which in theory should result in better safety profiles of these agents, although clinical use has proven it's not entirely true ³⁴. Nevertheless, similarly to mAbs, TKIs prevent the activation of EGFR very effectively, stopping the downstream signalling. However, while mAbs act on the extracellular side of the protein where they block sites for ligand binding or for dimerization of two receptors, TKIs act on the intracellular site of the protein ⁹. The mechanism of action of TKIs on the intracellular side of EGFR is blocking the binding site of ATP, effectively stopping the kinase activity of the receptor. As a result, the transphosphorylation of dimerised receptors cannot take place, which then prevents further propagation of the signal ⁹.

In comparison to traditional chemotherapeutic agents and to mAbs, TKIs possess several advantages. TKIs maintain reasonably high selectivity, while they also retain easy oral administration, which is one of the main limitations of mAbs ²⁹. Hence, TKIs are usually viewed as the preferred strategy in treatment of EGFR positive tumours. Nevertheless, TKIs have their own limitations. These are mainly connected to resistance that tumours acquire at some point during the therapy duration ⁹. To counteract this resistance, multiple generations of TKIs have been developed (**Figure 6**).

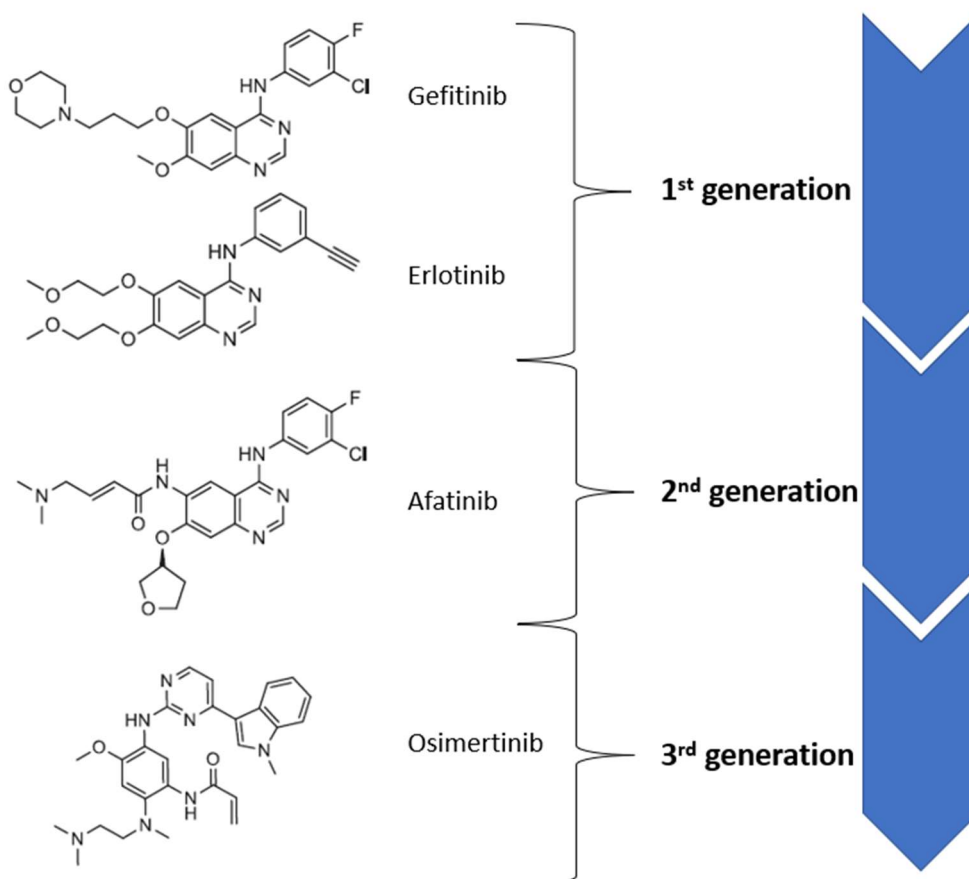


Figure 6. Tyrosine kinase inhibitors examples. Structures of approved examples of tyrosine kinase inhibitors from different generations of tyrosine kinase inhibitors. Chemical structures taken from Ayati, et al. (2020) ⁹.

The first generation of TKIs consists of compounds that are mainly reversible inhibitors of tyrosine kinases. These compounds compete directly with ATP for binding in the ATP binding pocket ⁹. The most prominent example of first generation TKIs is undoubtedly gefitinib, which was approved by the FDA in 2003 for the treatment of non-small cell lung cancer ³⁵. Erlotinib is another first generation TKI, which was approved a year later for the treatment of the same condition ³⁶. Both compounds are quinazoline base derivatives, mainly targeted for EGFR receptors showing activating mutations in the kinase region ⁹. Both gefitinib and erlotinib showed 50 – 80% positive response in patients that harboured L858R and del.19 mutations in the EGFR ³⁷. However, as mentioned their success is limited by occurrence of mutations in EGFR that render these compounds ineffective later on in the therapy. The most prominent mutation causing this resistance is the substitution of threonine for methionine in position 790 (T790M). This mutation increases the affinity of ATP, allowing ATP to outcompete the first generation TKIs for binding ³⁸.

Occurrence of resistance to first generation TKIs prompted development of another class of TKIs, named the second generation of TKIs. Second generation TKIs are derivatives of 4-anilinoquinazoline and 4-anilinoquinoline. These compounds contain acrylamide group which forms covalent interaction with Cys797, blocking the ATP binding pocket⁹. The only compound coming from this class that has been is afatinib, which was approved by the FDA in 2013 to treat lung adenocarcinomas with EGFR mutations³⁹. Unfortunately, compounds from second generation TKIs possess toxicity at low doses, which severely limits their use in clinic⁹.

The third generation of TKIs sought to improve on its predecessors. Similarly to the second generation, compounds in third generation TKIs are irreversible inhibitors⁹. Furthermore, these compounds also form covalent interaction with the Cys797 to block the access to the ATP binding site⁹. However, third generation TKIs are based on aminopyrimidine scaffold⁹. Also, compound belonging to this class are selective for the T790M EGFR mutation⁴⁰. The most prominent example of third generation TKIs is Osimertinib, which was approved for clinics in 2015 for treatment of non-small cell lung cancer positive for T790M EGFR⁴¹. Osimertinib showed response rates of 62% and increased median progression-free survival by 12.3 months⁴². Moreover, the T790M EGFR mutation was observed to disappear in 37% of cases treated with Osimertinib⁴³. Nevertheless, resistance also emerged against Osimertinib and other third generation TKIs. This resistance is mediated via mutation of Cys797, which prevents formation of the covalent interaction⁹.

Although, some success has been achieved with TKIs, the research in this field is still ongoing, as many challenges remain unresolved. First and foremost, the occurrence of resistance to Osimertinib and other third generation TKIs has prompted the development of fourth generation TKIs, which seek to inhibit EGFR allosterically⁹. This approach is hoped to overcome the resistance mutations appearing at the ATP binding site. Additionally, combinatorial therapy was proposed to hit the EGFR more effectively²⁹. Combination of two TKIs in one formulation, or combination of one TKI targeted for EGFR with another drug targeting downstream component of the EGFR signalling pathways could potentially inhibit development of resistance mutations and improve the potency of the treatment⁴⁴. Similarly, combination with mAb treatment could improve the efficacy of the therapy as well²⁹.

Another area of interest in TKI research is development of nanomedicine formulations. Nanoparticles are heavily utilised in gene therapy, which requires precise targeting and delivery of large molecules. Nanoparticle TKI formulation could provide targeted local delivery of high dose of drug, which would otherwise be toxic systemically, albeit at the cost of intravenous delivery ⁴⁴. Different nanocarrier types were investigated for the usage of TKI delivery. These include for instance liposomes, which are used extensively in cancer drug delivery thanks to their high drug loading capacity, as well as low immunogenicity and good tolerability ⁴⁴. One example of liposomal formulation loaded with TKI is system designed by Morton, et al., which combines erlotinib with doxorubicin in one liposomal nanoparticle ⁴⁵. Moreover, this formulation releases the drug in desired sequence, with erlotinib being released first. This then enhances the cytotoxicity of doxorubicin. This example clearly demonstrates the broadening of options in design when utilising nanoparticles and shows a great promise to the future.

3.4. Studying EGFR

As previous sections demonstrated, due to its central role in cancer, EGFR is extensively studied protein. Since its discovery, a vast amount of information has been gathered about its structure, function and involvement in disease, which eventually lead to development of targeted therapies. This advancement in understanding of EGFR biology was possible thanks to a plethora of biochemistry and molecular biology techniques that were largely developed in the last century.

Studying EGFR *in vitro* in cell lines is widely used approach due to its convenience. Depending on the readout, it is sometimes necessary to overexpress recombinant protein in a cell. One way how to achieve EGFR overexpression is transfection with plasmid coding for EGFR. The recombinant plasmid technology and molecular cloning allowed us to design plasmid vectors that can be introduced to cells, which then express the genes encoded by these vectors. Plasmid vectors are also the basis of gene therapy. Plasmids are extrachromosomal circular pieces of DNA originating in bacteria, which are used for exchange of genetic information between bacterial cells ⁴⁶. They usually harbour genes promoting survival in certain conditions, such as antibiotic resistance genes ⁴⁶. The discovery of plasmids and later the discovery of restriction enzymes and ligases enabled researcher to place genes of interest into

plasmid vectors and thus express genes in cells that originally don't possess these genes. A plasmid vector typically includes origin of replication and antibiotic resistance gene, which allow the plasmid to be amplified in bacteria and allow the bacteria to grow in presence of the antibiotic, making it easy for researchers to determine which bacteria carry the plasmid ⁴⁶. Typical plasmid vector also has multiple cloning site, which carries multiple restriction enzymes sites. Here gene of interest can be inserted. The gene of interest in places under promoter, which triggers the expression of the gene of interest once the plasmid is taken up by the cell that recognises this promoter ⁴⁶. Finally plasmid can also carry selectable marker, which marks out cells that have taken up the plasmid. This can be for example fluorescent protein that gives fluorescent signal ⁴⁶. It is also possible to create protein fusions. For example EGFR has been fused to green fluorescent protein (EGFP) (**Figure 7**), which allows direct assessment of transfection efficiency, but also allows visualisation and localisation of EGFR through observation of the green fluorescent signal ⁴⁷. Therefore, researcher can for example follow internalisation or clustering of EGFR in response to different stimuli.

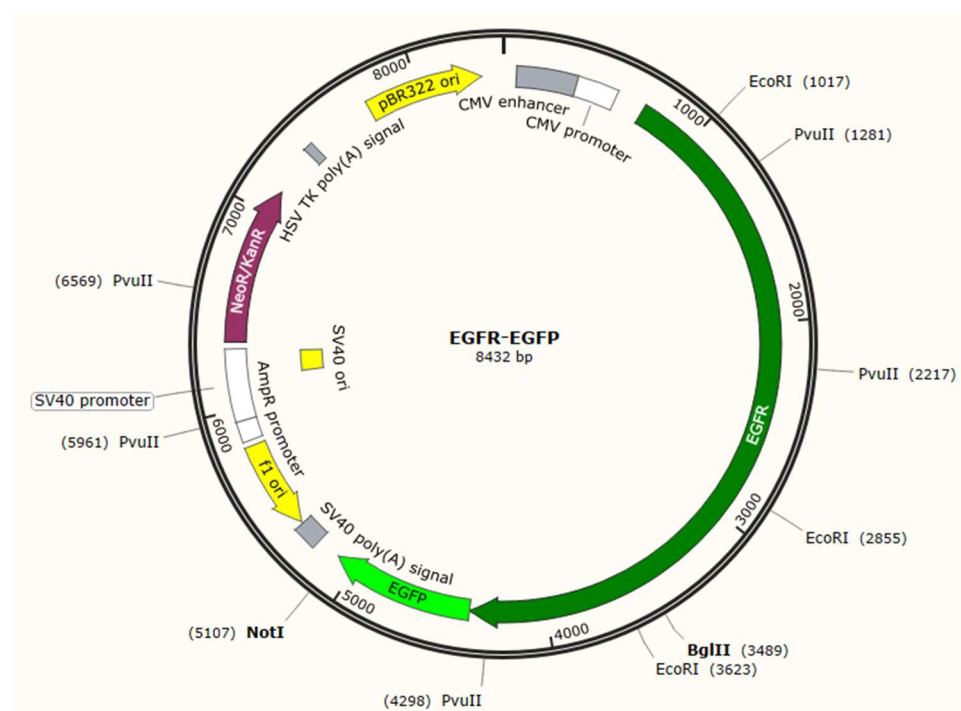


Figure 7. Plasmid coding for EGFR-EGFP fusion protein. This figure shows plasmid map of EGFR-EGFP plasmid. The plasmid carried EGFR-EGFP fusion construct (shown in green) in its multiple cloning site, which is placed under the control of CMV promoter and CMV enhancer. The plasmid also contains multiple origins or replications (shown in yellow), which allow the plasmid to be replicated in bacterial hosts. Additionally, plasmid also contains antibiotic resistance gene (purple), which is placed under control of bacterial promoter. Restriction enzymes sites are also shown for multiple restriction enzymes. The plasmid map is shown in SnapGene Viewer and the plasmid comes from Carter and Sorkin (1998) ⁴⁷.

To overexpress a protein of interest, such as EGFR, the plasmid has to be taken up by a cell of interest in process known as transfection. The main challenge here is the delivery of large negatively charged plasmid DNA through largely negatively charged plasma membrane. To circumvent this issue, viruses have been utilised as delivery vectors ⁴⁸. The main advantage of virus vectors is their high transfection efficiency. However, there are certain important considerations. In gene therapy context, the main limitation of virus vectors is their high immunogenicity and potential to trigger inflammatory response, which limits their repeated use ⁴⁸. In research setting, one has to consider stricter safety measures that are needed when working with viruses.

Alternative transfection technology to viruses are polyplex nanoparticles, or polyplexes. The building block of polyplexes are cationic polymers, such as polyethylenimine (PEI). These positively charged polymers are capable of spontaneously forming complexes with negatively charged plasmid DNA, where the positive charge of the polymer is on the outside of the polyplex particle ⁴⁹. Hence, the polyplex can bind the negatively charged plasma membrane of the cell, which then takes up the nanoparticle by endocytosis ⁴⁹. The cationic polymer coating also protects the DNA cargo from degradation by nucleases and thus stabilises the DNA. It also helps to facilitate the escape from endosomes, by proton sponge effect ⁴⁹. Moreover, the outer coating of polyplexes can be modified by different targeting molecules, so only specific cells in certain tissue are transfected, which is a major advantage in gene therapy setting ⁴⁹.

Once the cells overexpress EGFR, different biochemical assays can be used to follow EGFR's function. One such example is western blot, which allows detection of expressed proteins via interaction with antibodies specific against epitopes of protein of interest ⁵⁰. Western blot is useful in assessing protein expression levels and so it can be used to detect successful transfection. Moreover, as EGFR is a kinase that also has phosphorylation sites, antibodies specific only to phosphorylated EGFR can be made ⁵¹. Thus it is possible to follow activation of EGFR in response to stimulation. This technique is also useful in following the efficacy of TKIs, as it allows us to see inhibition through depletion of phosphorylated EGFR ⁵¹.

4. Aims of the work

This master's thesis focused on *in vitro* transfection with EGFR-EGFP plasmids, which allows to study EGFR's biology. The main objective was to optimise the *in vitro* transfection protocol, which would lead to best outcome in terms of transfection efficiency and toxicity. To do this, the first aim was to expand and validate wild-type (WT) EGFR-EGFP plasmid clone, which was subsequently used in main optimisation in 96-well plate format with fluorescence microscopy and resazurin assay as readouts. Once the optimisation was done, the next aim was to upscale the optimised parameters to 6-well plate format and also to gain information on which transfection parameters influence the outcome of the transfection by employing flow cytometry and DAPI staining as live-dead assay.

Moreover, the thesis focused on expansion and validation of T790M mutant version of EGFR-EGFP generated by site-directed mutagenesis and subsequent heat-shock transformation, so that the plasmid could be utilised in optimised transfection protocol and then differences in response towards TKIs between WT and T790M EGFR could be observed in western blot experiment. Identification of working T790M EGFR-EGFP plasmid was the main objective of this part, along with optimisation of the western blot parameters, which included TKI treatments with gefitinib and osimertinib, as well as hEGF stimulation steps.

Finally, the thesis validated the information gained by expansion of the identified working EGFR-EGFP clones (both WT and T790M) and subsequent use of these clones in transfection experiments using the optimised parameters. The main aim was to demonstrate reproducibility in the functionality outcome by flow cytometry for transfection success, resazurin assay for viability profiling, and western blot experiments for studying effect of gefitinib and osimertinib on phospho-EGFR within cells transfected with WT and T790M EGFR-EGFP clones.

5. Materials

The following section lists all materials used in this work. The source of all materials in given and its use in bacterial culture or mammalian cell culture is indicated.

5.1 Chemicals and Kits

This part lists all chemicals and kits used (**Table 1**).

Table 1. Chemicals and kits used in this work.

Chemical/Kit	Manufacturer/Source	Use
Tryptone	Sigma-Aldrich	Bacterial culture
Yeast extract	Sigma-Aldrich	Bacterial culture
NaCl	AppliChem/Roth	Bacterial culture/Buffers
Agarose (DNA grade)	Electran®	Bacterial culture/Gel electrophoresis
Ampicillin	Sigma-Aldrich	Bacterial culture
Kanamycin	Sigma-Aldrich	Bacterial culture
Zeocin	Thermo Scientific™	Bacterial culture
Glycerol	Sigma-Aldrich/Roth	Glycerol stock/3X SDS sample buffer
Ethanol (99%)	Fischer Chemical	Plasmid extraction
Isopropanol	AppliChem	Maxiprep kit prep
MilliQ Water	Sartorius Arium® Pro	Various
GeneJET Plasmid Maxiprep Kit	Thermo Scientific™	Plasmid extraction
Ethidium Bromide	Sigma-Aldrich	Gel electrophoresis
NaOH	AppliChem	20X SB buffer
Boric acid	Sigma-Aldrich	20X SB buffer
Gibco TrypLE	Thermo Scientific™	Cell culture – trypsinisation
FCS		Cell culture
Penicillin/Streptomycin		Cell culture

L-glutamine		Cell culture
LPEI	MMCT, University of Vienna	Polyplex transfections
almarBlue™ HS Cell Viability Reagent	Invitrogen™	Resazurin assay
Resazurin, sodium salt, pure, certified	Acros Organics	Resazurin assay
Formaldehyde		Cell fixation
HEPES	AppliChem	HBG buffer
Glucose	Merck	HBG buffer
DAPI		DAPI staining
hEGF	Peprotech	EGFR activation
Complete® 25X	Roche	Lysis buffer
PMSF	Fluka	Lysis buffer
NaF	Fluka	Lysis buffer
Na ₃ VO ₄	Sigma-Aldrich	Lysis buffer
β-mercaptho-ethanol	Sigma-Aldrich	1X SDS sample buffer
RotiQuant bradford reagent	Roth	Bradford assay
BSA	Roth	Blocking buffer/Antibody dilutions
Luminol	Sigma-Aldrich	ECL solution
Coumaric acid	Sigma-Aldrich	ECL solution
Hydrogen peroxide	Roth	ECL solution
Rotiphorese® Gel 30 (37.5:1)	Roth	SDS-PAGE
SDS	Roth	SDS-PAGE
TEMED	Roth	SDS-PAGE
APS	Sigma-Aldrich	SDS-PAGE
1.5 M Tris-HCl (pH 8.8)	Roth	SDS-PAGE
1.5 M Tris-HCl (pH 6.8)	Roth	SDS-PAGE
Nonidet P40	Sigma-Aldrich	RIPA buffer
Sodium Deoxycholate	Sigma-Aldrich	RIPA buffer

Sodium Azide	Sigma-Aldrich	RIPA buffer
Bromophenol blue	Sigma-Aldrich	3X SDS sample buffer
Tris-base	Roth	Various buffers
Glycine	Roth	Various buffers
Methanol	VWR	1X blotting buffer
Tween 20	Roth	TBST buffer
Amersham™ Protran® Premium Western-blotting membrane	Sigma-Aldrich	Western blotting
DMSO	Gibco	Various
Gefitinib		TKI treatment
Osimertinib		TKI treatment

5.2. Buffers

All buffers used and their composition is listed here (**Table 2**).

Table 2. Information on buffers used in this work.

Buffer	Composition	Manufacturer/Source	Use
20X SB buffer	0.8% NaOH, 4.8% Boric acid, pH ~ 8.0	MMCT, University of Vienna, see sources of individual components in section 5.1.	Gel electrophoresis
1X SB buffer	5% 20X SB buffer, 95% MilliQ water	MMCT, University of Vienna	Gel electrophoresis
PBS	Dulbecco's Phosphate Buffered Saline Modified, without calcium chloride and magnesium chloride, liquid, sterile-filtered, suitable for cell culture	Sigma-Aldrich	Various cell culture applications

HBG buffer	20 mM HEPES, 5% Glucose, pH = 7.4, sterile	MMCT, University of Vienna, see sources of individual components in section 5.1.	Polyplex transfections
1X TE buffer	Tris-EDTA, pH 8.0	Fisher Bioreagents	Elution of plasmid from filter paper
Lysis buffer	1410 μ L RIPA buffer, 60 μ L Complete® 25X stock, 15 μ L PMSF stock (100 mM), 7.5 μ L NaF, 7.5 μ L Na ₂ VO ₃	Prof. Dirsch work group, University of Vienna, see sources of individual components in section 5.1. and section 5.2.	Cell lysis
RIPA buffer	29.22 mg/mL NaCl, 50 mM Tris-HCl (pH 6.8), 10 mg/mL Nonidet P40, 5 mg/mL Sodium Deoxycholate, 1 mg/mL SDS, 0.5 mg/mL Sodium Azide, In ddH ₂ O	Prof. Dirsch work group, University of Vienna, see sources of individual components in section 5.1	Lysis buffer
3X SDS sample buffer	0.1 M Tris-HCl, 35 mg/mL SDS, 18% Glycerol, 90 μ g/mL Bromophenol blue In ddH ₂ O	Prof. Dirsch work group, University of Vienna, see sources of individual components in section 5.1.	1X sample buffer
1X sample buffer	85% 3X SDS sample buffer,	Prof. Dirsch work group, University of Vienna, see sources of individual	Protein sample preparation for SDS-PAGE

	15% β -mercaptho-ethanol	components in section 5.1. and section 5.2.	
Electrophoresis buffer 10X	30 mg/mL Tris-base, 144 mg/mL Glycine, 10 mg/mL SDS, In ddH ₂ O	Prof. Dirsch work group, University of Vienna, see sources of individual components in section 5.1.	Electrophoresis buffer 1X
Electrophoresis buffer 1X	10% Electrophoresis buffer 10X, 90% ddH ₂ O	Prof. Dirsch work group, University of Vienna, see sources of individual components in section 5.1. and section 5.2.	SDS-PAGE
Blotting buffer 5X	15.169 mg/mL Tris-base, 72.9 mg/mL Glycine, In ddH ₂ O	Prof. Dirsch work group, University of Vienna, see sources of individual components in section 5.1.	Blotting buffer 1X
Blotting buffer 1X	20% Blotting buffer 5X, 20% Methanol, 80% ddH ₂ O	Prof. Dirsch work group, University of Vienna, see sources of individual components in section 5.1. and section 5.2.	Western blotting
TBST buffer 10X (pH 8.0)	30 mg/mL Tris-base, 111 mg/mL NaCl, 1% Tween 20, In ddH ₂ O	Prof. Dirsch work group, University of Vienna, see sources of individual components in section 5.1.	1X TBST
1X TBST	10% TBST buffer 10X 90% ddH ₂ O	Prof. Dirsch work group, University of Vienna, see sources of individual	Western blotting

		components in section 5.1. and section 5.2.	
Blocking buffer	5% BSA in 1X TBST	Prof. Dirsch work group, University of Vienna, see sources of individual components in section 5.1.	Western blotting
ECL solution	10% ECL buffer, 0.03% H ₂ O ₂ , 0.22% Coumaric acid, 0.5% Luminol, 90% ddH ₂ O	Prof. Dirsch work group, University of Vienna, see sources of individual components in section 5.1. and section 5.2.	Chemiluminescence solution
ECL buffer	121.44 mg/mL Tris-base in ddH ₂ O	Prof. Dirsch work group, University of Vienna, see sources of individual components in section 5.1.	ECL solution

5.3. Enzymes, buffers and ladders

Restriction enzymes, buffers and ladders used for diagnostic restriction digest are listed here (Table 3).

Table 3. Information on restriction enzymes, buffers and molecular ladders used during diagnostic restriction digests.

Enzyme/Buffer/Ladder	Manufacturer	Reference number
10X FastDigest™ Green Buffer	Thermo Scientific™	B72
FastDigest™ BglII	Thermo Scientific™	FD0083
FastDigest™ EcoRI	Thermo Scientific™	FD0274
FastDigest™ PvuII	Thermo Scientific™	FD0634
FastDigest™ NotI	Thermo Scientific™	FD0593
FastDigest™ BamHI	Thermo Scientific™	FD0054

FastDigest™ Sall	Thermo Scientific™	FD0644
FastDigest™ HindIII	Thermo Scientific™	FD0504
6X DNA Gel Loading Dye	Thermo Scientific™	R0611
FastRuler™ Middle Range DNA Ladder	Thermo Scientific™	SM1113

5.4. Culture media

Culture media used in bacterial culture, as well as mammalian cell culture are shown in this section (**Table 4**). Composition of complete media is shown. The manufacturer column and reference number indicates source of basal media used on their own or as a component of complete media. Use indicates for which cell line/bacteria culture application the medium was used.

Table 4. Information on culture media used in this work.

Medium	Composition	Manufacturer/Source	Use
LB Agar Medium	1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agarose	See manufacutrers for each respective component	Solid bacterial culture on plate
LB Agar Medium with Ampicillin	1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agarose, 100 µg/mL ampicillin	See manufacutrers for each respective component	Solid bacterial culture on plate
LB Agar Medium with Kanamycin	1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agarose, 50 µg/mL kanamycin	See manufacutrers for each respective component	Solid bacterial culture on plate

LB Agar Medium with Zeocin	1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agarose, 25 µg/mL kanamycin	See manufacutrers for each respective component	Solid bacterial culture on plate
LB Medium	1% tryptone, 0.5% yeast extract, 0.5% NaCl	See manufacutrers for each respective component	Liquid bacterial culture
LB Medium with Ampicillin	1% tryptone, 0.5% yeast extract, 0.5% NaCl, 100 µg/mL ampicillin	See manufacutrers for each respective component	Liquid bacterial culture
LB Medium with Kanamycin	1% tryptone, 0.5% yeast extract, 0.5% NaCl, 50 µg/mL kanamycin	See manufacutrers for each respective component	Liquid bacterial culture
SOC Outgrowth Medium	2% vegetable peptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl ₂ , 10 mM MgSO ₄ , 20 mM glucose	New England Biolabs® Inc.	Outgrowth in bacterial transformation
DMEM High Glucose Complete Medium	DMEM High Glucose + 10% FCS + 1% penicillin/streptomycin + 1% L-glutamine	See manufacutrers for each respective component	Cell culture: HEK293 HEK293T Hela
DMEM High Glucose Basal Medium	DMEM High Glucose	Sigma-Aldrich	Cell culture: HEK293 HEK293T HeLa

RPMI 1640 Complete Medium	RPMI 1640 + 10% FCS + 1% penicillin/streptomycin + 1% L-glutamine	See manufacutrers for each respective component	Cell culture: A549
RPMI 1640 Basal Medium	RPMI 1640	Sigma-Aldrich	Cell culture: A549

5.5. Cell culture plates

This section presents cells culture plates used in mammalian cell culture experiments (**Table 5**).

Table 5. Information on cell culture plates used in this work.

Plate	Manufacturer	Reference number	Use
CELLSTAR 96-well, cell culture-treated, flat-bottom microplate (white)	Greiner Bio-One	655098	Transfection experiments
6-well CytoOne® Plate, TC-treated	Starlab	CC7682-7506	Transfection experiments
CELLSTAR 96-well, cell culture-treated, flat-bottom microplate (clear)	Greiner Bio-One	655180	Resazurin readout

5.6. Cell lines and strains

This section lists the cell lines and bacterial strains used in this work (**Table 6 and 7**).

Table 6. Information on cell lines used in this work

Cell type	Genotype	Background info
A549	WT	ATCC® CCL-185™

HEK293	WT	Gift from Department of Pharmacognosy, University of Vienna
HEK293T	WT	Gift from Prof. Martina Anton, Experimentelle Onkologie und Therapieforschung Klinikum rechts der Isar der TUM
HeLa	WT	ATCC® CCL-2™

Table 7. Information on bacterial strains used in this work.

<i>E. coli</i> strain	Genotype	Plasmid	Source
DH5α	fhuA2 lac(del)U169 phoA glnV44 Φ80' lac- Z(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	EGFR-EGFP	MMCT, University of Vienna
NEB® 5-alpha Competent <i>E. coli</i> (High Efficiency)	fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	T790M EGFR-EGFP (NE/DP), Ext-Gluc	New England Biolabs® Inc.
stbl3 Competent cells	<i>F</i> – <i>mcrB mrr hsdS20</i> (<i>rB</i> –, <i>mB</i> –) <i>recA13 supE44 ara-14 galK2 lacY1 proA2 rpsL20 (StrR) xyl-5 λ</i> – <i>leu mtl-1</i>	Ext-Gluc	Addgene

5.7. Plasmids

Following plasmids were used in this work (**Table 8**).

Table 8. Information on plasmids used in this work.

Plasmid	Vector backbone	Characteristics	Source
EGFR-EGFP	pEGFP-N1	Human EGFR fused with EGFP, CMV promoter, pUC origin, kanamycin resistance	EGFR-GFP was a gift from Alexander Sorkin (Addgene plasmid # 32751 ; http://n2t.net/addgene:32751 ; RRID:Addgene_32751); further expanded by Manuel Blaschek.
T790M EGFR-EGFP (NE)	pEGFP-N1	Human EGFR fused with EGFP, CMV promoter, pUC origin, kanamycin resistance, T790M mutation in EGFR	Site directed mutagenesis (SDM) of EGFR-EGFP plasmid (addgene plasmid 32751, further expanded in this work). The SDM was done by Dominik Peischl using commercially designed primers (New England Biolabs).
T790M EGFR-EGFP (DP)	pEGFP-N1	Human EGFR fused with EGFP, CMV promoter, pUC origin, kanamycin resistance, T790M mutation in EGFR	Site directed mutagenesis (SDM) of EGFR-EGFP plasmid (addgene plasmid 32751, further expanded in this work). The SDM was done by Dominik Peischl using custom designed primers by Dominik Peischl.
Ext-Gluc	#88-pCDH-EF1a-extGLuc-T2A-copGFP	Ext-Gluc plasmid for lentiviral transfection with	Prof Irmela Jeremias (PMID: 23300782)

		ampicillin resistance, pUC origin of replication	
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5.8. Antibodies and protein molecular weight markers

This section lists antibodies and protein molecular weight markers used for detection of proteins in Western blotting (**Table 9**).

Table 9. Information on antibodies used in western blot experiments.

Antibody	Manufacturer	Reference number	Use
Precision Plus Protein™ All Blue Prestained Protein Standards	Bio-Rad	#1610373	Molecular weight ladder
Mouse anti-EGFR monoclonal antibody, clone LA22	Merck	05-104	Primary antibody for EGFR detection
Rabbit anti-pEGFR (T1068) polyclonal antibody	Invitrogen	44-788G	Primary antibody for phosphorylated EGFR detection
Rabbit anti- α/β -tubulin polyclonal antibody	CST	#2148	Primary antibody for tubulin detection
Goat anti-mouse IgG (H+L) DyLight 488 linked secondary antibody	Invitrogen	35502	Secondary antibody for EGFR detection

Goat anti-rabbit IgG (H+L) highly cross-absorbed secondary antibody, Alexa Fluor™ Plus 647 linked	Invitrogen	32733	Secondary antibody for phosphorylated EGFR detection
Goat anti-rabbit IgG HRP linked secondary antibody	CST	#7074	Secondary antibody for pEGFR and tubulin detection

5.9. Devices

This list shows all laboratory devices used in this work (**Table 10**).

Table 10. Information on devices employed in this work.

Device	Manufacturer	Use
WTB Incubator	Binder	Solid bacterial culture
MaxQ™ 4450 Benchtop Orbital Shaker	Thermo Scientific™	Liquid bacterial culture
GeneQuant 1300 Spectrophotometer	Biochrom	OD600 measurement
NanoVue Plus Spectrophotometer	Biochrom	pDNA quantification
Megaforce™ 16R Centrifuge	Thermo Scientific™	All centrifugation purposes
Microcentrifuge	Thermo Scientific™	All centrifugation purposes
TC412 Thermal Cycler	Techne	Diagnostic restriction digest incubation
PowerPac 200 Electrophoresis Power Supply	Bio-Rad	Electrophoresis
Molecular Imager® Gel Doc™ XR System	Bio-Rad	Ethidium bromide imaging
IXplore Standard Compound Microscope System	Olympus	FLM microscopy

Neubauer-improved Counting Chamber	Marienfeld	Cell counting
Heracell™ 150i CO ₂ Incubator	Thermo Scientific™	Cell culture incubator
Infinite® 200 Pro	Tecan	Resazurin fluorescence readout
MACSQuant® Analyser	Miltenyi Biotec	Flow cytometry
Bandelin Sonoplus	BANDELIN Electronic GmbH	Sonication of lysates
Sunrise™	Tecan	Bradford assay plate reader
ImageQuant™ 800 Western blot imaging system	Amersham	Western blot readout

5.10. Software

Software used for analysis and handling of data is listed here (**Table 11**).

Table 11. Information on software used for completion of this work.

Software	Version	Developer	Use
Snap Gene	3.1.4	Dotmatics	Restriction digest simulations
cellSens Imaging Software	2.2 (build 17989)	Olympus	FLM imaging
FlowJo	V10	FlowJo, LLC	Flow cytometry analysis
R Studio	1.1.383	R Studio	Statistical analysis
Microsoft Office Package	2016	Microsoft	Data analysis and figure preparation

6. Methods

This chapter summarises all methodologies employed throughout this work.

6.1. EGFR-EGFP plasmid expansion

6.1.1. Streaking from glycerol stock

A glycerol stock of DH5 α strain carrying EGFR-EGFP plasmid was taken from its -80°C storage and using inoculation loop LB agar plates were streaked. LB agar with kanamycin plate was used as a selection plate, while LB agar only plate was used as a positive control for growth and LB agar with ampicillin or LB agar with zeocin plate was used as a negative control. The plates were incubated overnight (20 – 24 hours) at 37°C.

6.1.2. Liquid culture

Following the solid culture, single colonies from the selection plate were picked to inoculate two liquid pre-cultures. The liquid pre-culture contained 5 mL LB medium with 50 μ g/mL of appropriate antibiotic (kanamycin in this case). The liquid pre-cultures were incubated for 5.5 to 6 hours at 37°C while shaking at 150 rpm. Additionally, control pre-cultures were set-up. These included contamination control (5 mL LB medium only and 5 mL LB medium with 50 μ g/mL kanamycin without inoculation) and positive control (5 mL LB medium with inoculation).

After the incubation period, liquid overnight cultures were inoculated from the main pre-culture (pre-culture with best growth was selected based on either visual inspection or OD600 measurement). The overnight culture contained 250 mL LB medium with 50-100 μ g/mL kanamycin. The cultures were inoculated by addition of 250 μ L of the liquid pre-culture. The overnight culture was incubated for 16 to 17 hours at 37°C while shaking at 150 rpm. These cultures were used for plasmid isolation by Maxiprep.

In case of giga prep expansion, parallelly to overnight culture used for maxiprep extraction, 4 overnight cultures were set up, which were all inoculated from the same pre-culture as the parallel maxiprep culture. One giga prep overnight culture flask contained 350 mL LB medium with 50 μ g/mL kanamycin, resulting in total of 1.4 L of culture when combined. Each flask was inoculated with 350 μ L of pre-culture. The giga prep overnight cultures were incubated for 17

to 17.5 hours at 37°C, while shaking at 150 rpm. The giga prep overnight cultures were pelleted after this time period by centrifugation at 4 327g for 10 min at 4°C. The pellet were stored at -20°C. Plasmid extraction from the giga prep cultures wasn't carried out in this work.

6.1.3. Glycerol stock preparation

From each Maxiprep overnight culture glycerol stock was prepared, for the possibility to culture these bacteria in the future. A sample from the overnight culture (500 µL) was mixed with 50% glycerol in MilliQ water (500 µL) in 1:1 ratio. This mixture was then stored at -80°C till further use.

6.1.4. Plasmid extraction

Optical density at $\lambda = 600$ nm (OD600) of each Maxiprep overnight culture was determined using GeneQuant 1300 Spectrophotometer. Based on the obtained OD600, maximal volume of the overnight culture to be processed in plasmid extraction via GeneJET Plasmid Maxiprep kit was calculated, as follows:

$$\text{Maximum culture volume (mL)} = 750/\text{OD600}$$

The calculated volume of the overnight culture was then pelleted by centrifugation at 4 327 g for 10 min at 4°C. The supernatant was discarded and the pellet was either stored at -20°C for further use or processed immediately by the GeneJET Plasmid Maxiprep kit. Plasmid DNA was extracted from the pellet using the GeneJET Plasmid Maxiprep kit as per manufacturer's instructions in two step elution. In the first elution step, plasmid DNA was eluted with 0.7 mL elution buffer, while in the second step, plasmid DNA was eluted with 0.5 mL elution buffer. The plasmid DNA was then quantified using NanoVue Plus Spectrophotometer and stocks were stored at 4°C and -20°C for short term and long term storage respectively.

6.1.5. Diagnostic restriction digest and plasmid validation

The extracted EGFR-EGFP plasmid was validated via diagnostic restriction digest (DRD). The reaction was set-up using 2 µL of 10X FastDigest™ Green Buffer and 1 µL FastDigest™ restriction enzymes. The total amount of plasmid DNA in the restriction reaction was 250 ng. The total volume of the reaction was 20 µL and to reach this volume MilliQ water was used. For the EGFR-EGFP plasmid, following restriction reactions were performed:

- uncut control (no restriction enzyme added)
- reaction with BglII
- reaction with EcoRI
- reaction with PvuII
- reaction with combination of EcoRI and NotI

The reaction mix was incubated in thermocycler for 90 min at 37°C, with 10 min inactivation step at 80°C to inactivate the restriction enzymes. The resultant DRD fragments were separated using 1% agarose gel (with ethidium bromide) electrophoresis at 90V for 90 min. Subsequently, the fragment pattern on the gel was visualised using Molecular Imager® Gel Doc™ XR System with different exposure times ranging from 0.1 s to 3 s. The observed fragment pattern was then compared to simulation of DRD and agarose gel electrophoresis carried out in SnapGene Viewer. Once the fragment pattern matched the plasmid was approved to be used in further applications.

6.2. T790M EGFR-EGFP transformation and expansion

6.2.1. Heat-shock transformation

The T790M EGFR-EGFP plasmid was derived from the EGFR-EGFP plasmid expanded in this work by site directed mutagenesis (SDM), which was performed by Dominik Peischl (master's thesis by Dominik Peischl). The SDM was carried out using two different primer sets (here designated as NE and DP, for more information refer to Dominik Peischl thesis), and thus two T790M EGFR-EGFP plasmids were produced. To expand the product of the SDM, competent bacteria cells were transformed by heat-shock transformation and then cultured to obtain high yield of the T790M EGFR-EGFP plasmids.

To carry out the transformation, 5 uL of the NE or DP T790M EGFR-EGFP plasmid was added to 50 uL of the competent cells (from Q5® Site-Directed Mutagenesis kit). These cells were then incubated with the plasmid on ice for 30 min, after which they were subjected to heat-shock at 42°C for 30 s. The heat-shock was followed by brief 5 min incubation on ice. Subsequently, 950 uL of SOC medium (warmed-up to 37°C) was added and the bacteria were cultured while shaking (250 rpm) for 1 hour at 37°C in the outgrowth step.

Following the outgrowth step, 100 μ L or 700 μ L of the bacteria in SOC medium was spread on pre-warmed (37°C) kanamycin selection plate to culture transformed bacteria. Similarly, 100 μ L of the bacteria were spread also on pre-warmed LB only plates or ampicillin/zeocin plates to act as a positive and negative control respectively. The plates were incubated overnight (20 - 24 hours) at 37°C.

6.2.2. Liquid culture

The pre-culture incubation time ranged from 5 to 6 hours, while the overnight culture incubation time ranged from 16 to 17 hours. For the giga prep expansion the incubation time of overnight cultures was 17.5 hours. For more details see section 6.1.2.

6.2.3. Glycerol stock preparation

See section 6.1.3.

6.2.4. Plasmid extraction

See section 6.1.4.

6.2.5. Diagnostic restriction digest and plasmid validation

The T790M EGFR-EGFP plasmid was validated as presented in section 6.1.5. Additionally, the presence of the T790M mutation introduced by the SDM was validated by exon 20 PCR and subsequent DRD. This was performed by Dominik Peischl. For more information see his thesis. Successfully validated plasmids were then used in further applications.

6.3. EGFR-EGFP transfections in 96-well format

6.3.1. Cell culture and seeding for experiments

For the EGFR-EGFP transfection experiments in 96-well plate format, four different cell lines were examined. HEK293, HEK293T and HeLa cells were cultured in DMEM High Glucose medium (with 10% FCS, 1% P/S and 1% L-glu), while A549 cells were cultured in RPMI 1640 medium (with 10% FCS, 1% P/S and 1% L-glu). The cells were cultured in T75 flasks and were kept at 37°C in 5% CO₂ atmosphere. Upon reaching ~ 85-95% confluency, the cells were split and cultured further or also seeded for experiments. The seeding density for the 96-well plate format experiments was usually 10 000 cells per well (each well contained 200 μ L of the

respective medium). For HEK293T cell line and A549 cell line, seeding densities of 15 000 cells per well and 20 000 cells per well were tried in addition to 10 000 cells per well. HEK293 cells were seeded between passages p29 and p38, HEK293T between passages p22 and p24, HeLa at passage p104 and A549 between passages p87 and p89. The seeded cells were left incubating for ~ 24 hours at 37°C in 5% CO₂ before any treatments.

6.3.2. Polyplex transfection

The next day after seeding, cells were transfected with EGFR-EGFP plasmid using polyplex transfection. Polyplexes were prepared mixing the appropriate amounts of plasmid DNA with LPEI10k in HBG buffer (37°C). The ratio of LPEI10k to EGFR-EGFP plasmid DNA during polyplex preparation was 9:1. The polyplexing concentrations (i.e. the final concentration of plasmid in prepared polyplexes) tried were 20 µg/mL and 40 µg/mL.

The cells were treated with the polyplexes in basal medium. Therefore, the respective complete medium for cell line in question was replaced by respective basal medium. To this, appropriate amount of prepared polyplexes was added to result in either 200 ng or 400 ng plasmid DNA dose. The cells were left incubating for 4 hours at 37°C, 5% CO₂, before adding appropriate amount of complete medium, resulting in final FCS concentration of 5%. After this addition, the cells were incubated for further 20 hours, resulting in total polyplex incubation time of 24 hours.

6.3.3. Resazurin assay

The cell viability following the polyplex treatment was established via resazurin assay. The cells were incubated in presence of 10 ng/mL of resazurin (either almarBlue™ HS Cell Viability Reagent or house made resazurin solution was used) for 1 hour at 37°C, 5% CO₂. Subsequently, 50 – 80 µL of the supernatant was transferred to clear 96-well plate. The fluorescence intensity at wavelength of 590 nm was then measured by Infinite® M200 PRO plate reader, with excitation wavelength of 560 nm.

6.3.4. Fixation of cells and DAPI staining

The cells were fixed by incubation in 4% formaldehyde in HBS buffer. The medium was removed from the wells before adding the formaldehyde. The incubation was done at room temperature and lasted 45 min. Following this the wells were washed with PBS and then 50

uL of PBS (minimal PBS) was added to each well. The plate could then be stored at 4°C until further use.

Following the fixation, the nucleuses of the cells could be stained with DAPI. For this 2 µg/mL of DAPI in PBS was added to the wells. The cells were incubated with DAPI for 10 min at room temperature. The wells were then washed with PBS and minimal PBS was added for storage.

6.3.5. Fluorescence microscopy

Images of the cells were taken using fluorescence microscopy. The green fluorescent signal of the EGFR-EGFP fusion protein was detected using the FITC channel of the microscope. The DAPI stain was visualised using the DAPI channel of the microscope. Images were taken at 10X, 20X and 40X magnification, with three different field views per each well at 40X magnification. In addition to FITC and DAPI channel images, phase contrast images were taken as well. The exposure times in FITC and DAPI channels varied in order to obtain best signal-to-noise ratio.

6.4. EGFR-EGFP transfections in 6-well format

6.4.1. Cell culture and seeding for experiments

Based on optimisation carried out in 96-well plate format, HEK293T cells were cultured as described in section 6.3.1. for experiments in 6-well plate. The cells were seeded at density of 415 000 cells per well. The seeding density was established by adjusting the 15 000 cells per well seeding in 96-well plate to 6-well plate format by linear relationship between the seeding density and surface area of the well. The cells used for the experiments were between passages p29 to p65. After seeding, the cells were incubated for 24 hours at 37°C, 5% CO₂, before further treatments.

6.4.2. Polyplex transfection

Approximately 24 hours post seeding, the cells were transfected using polyplex transfection as described in section 6.4.2. For the 6-well plate format experiments the cells were transfected either with EGFR-EGFP plasmid or with T790M EGFR-EGFP plasmid. The dosage was upscaled for the 6-well plate to 4 µg or 8 µg (40 µg/mL or 80 µg/mL polyplexing concentration respectively). Four hours after transfection, complete medium was added to result in 5% or 1% final FCS concentration.

6.4.3. TKI treatment

In some experiments transfected cells were treated with gefitinib or Osimertinib. The drugs were added to the cells 4 hours post transfection. The TKIs were diluted in 2% FCS containing medium, which was added to the cells. The resulting FCS concentration was 1%. Final TKIs concentrations examined were 0.1 μ M and 1 μ M for both gefitinib and Osimertinib. As a negative control, DMSO was added to the cells in volume corresponding to the added TKIs, as the TKI stock solutions were dissolved in DMSO.

6.4.4. Resazurin assay

For the details see section 6.3.3.

6.4.5. Flow cytometry

For flow cytometry, cells were harvested from the 6-well plate. The medium was removed and the cells were incubated with 300 μ L TrypLE for 5 min at 37°C. TrypLE was neutralised with complete DMEM high glucose medium and the contents of the wells were collected in separate CT-15 tubes. These were centrifuged at 200 g for 5 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 200 μ L PBS.

The cell suspension was diluted in 1:5 ratio in PBS to prepare sample for measurement. For detection of dead cells, 5 μ g/mL DAPI in PBS was added to the sample resulting in final 1 μ g/mL DAPI concentration. The uptake volume for the measurement was adjusted to reach ~ 50 000 to 100 000 cells per measurement. The DAPI fluorescence was detected in the VioBlue channel, and the EGFP fluorescence was detected in the FITC channel.

The data were analysed in the FlowJo V10 software (**Figure 8**). First the population of events was visualised on SSC-A/FSC-A plot. Here gating was applied to select population of HEK293T cells. Following this, single cells were selected using gating on FSC-A/FSC-H plot. Using only single cell HEK293T population, gate was applied to select only live cells. This was done by plotting the population on VioBlue-A/FSC-A plot. Using sample without DAPI stain, the gate was set up to include all cells without DAPI signal. This gate was then used on all samples to differentiate between live and dead cells. The live cells were then plotted as a histogram of FITC-A and gate was set-up so cells positive for EGFR-EGFP transfection were distinguished

from cells negative for EGFR-EGFP signal. Diagonal gating was also tried to distinguish between cell population emitting genuine EGFP signal (**Figure 9**).

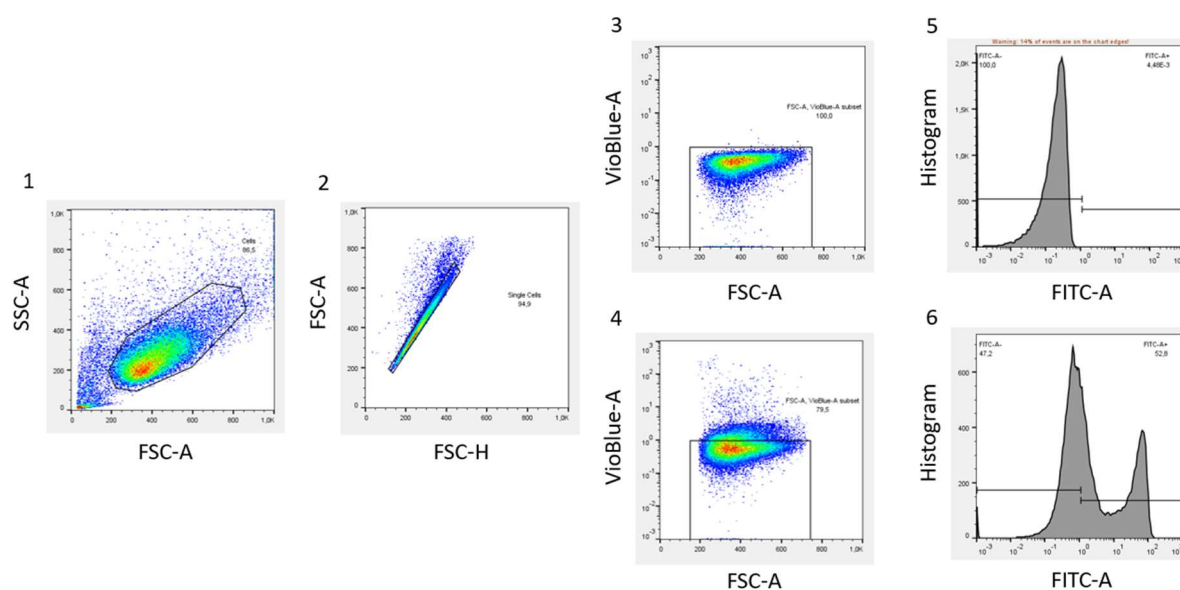


Figure 8. FlowJo analysis workflow and gating strategy. This figure overviews the analysis of flow cytometry data in FlowJo V10 analysis software. **1)** Gating applied to select HEK293T cell population. **2)** Gating applied to select only single cells. **3)** Gating applied on untransfected single HEK293T cells without DAPI staining to select cell negative for DAPI signal (i.e. live cells). **4)** The same gate as in image 3 applied on transfected single HEK293T cells stained with DAPI. **5)** Gating applied on untransfected single live HEK293T cells to differentiate cells negative and positive for EGFP signal in FICT spectrum. **6)** The same gate as in image 6 applied on transfected single live HEK293T cells.

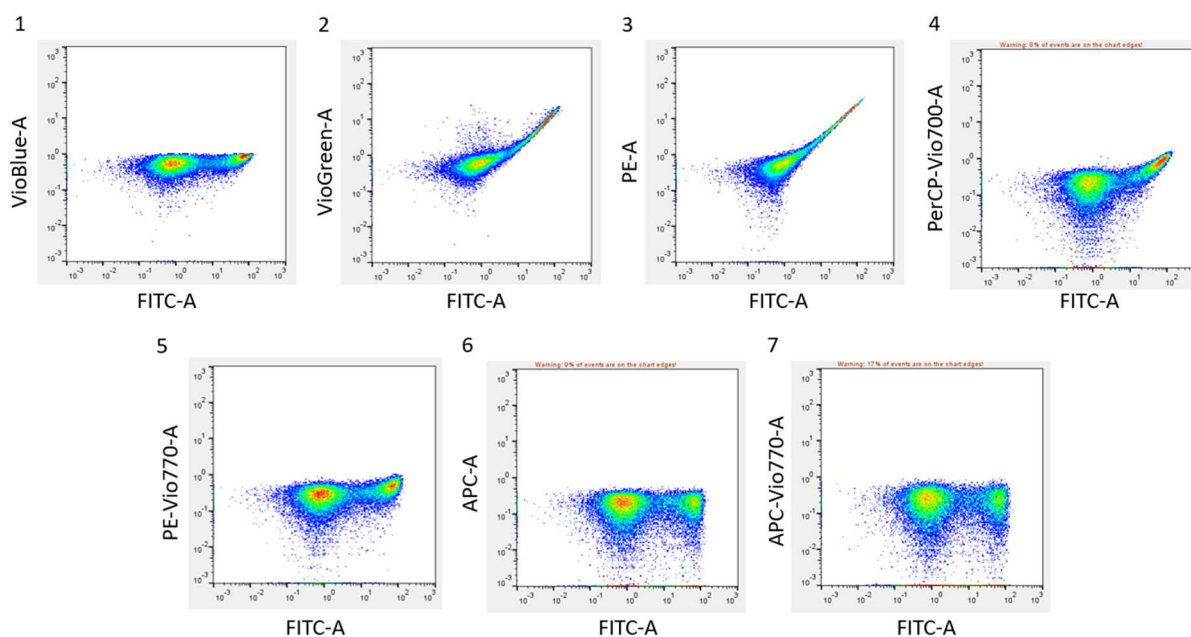


Figure 9. Bleed of EGFR signal into different channels of flow cytometer. In this figure population of transfected single live HEK293T cells is plotted by their signal in different channels of flow cytometer (y-axis) against their EGFP signal in FITC channel (x-axis) to visualise bleed of the EGFP signal to different channels. **1)** VioBlue against FITC. **2)** VioGreen against FITC. **3)** PE against FITC. **4)** PerCP-Vio700 against FITC. **5)** PE-Vio770 against FITC. **6)** APC against FITC. **7)** APC-Vio770 against FITC.

6.4.6. hEGF stimulation and cell lysis

In transfection experiments with western blot readout, cells were stimulated with hEGF to induce phosphorylation of EGFR. The hEGF stimulation was performed 24 hours post transfection with either WT EGFR-EGFP or T790M EGFR-EGFP. The medium was removed from the wells and the cells were washed once with basal DMEM high glucose medium. Subsequently 1 mL of 1 ng/mL or 0.1 ng/mL hEGF in basal DMEM high glucose medium was added. The cells were incubated in the presence of hEGF for 10 or 30 min at 37°C. After the incubation period, the cells were lysed to extract protein samples.

The medium in the wells was discarded and the cells were washed once with ice cold PBS. The cells were then incubated in 100 μ L lysis buffer per well on ice for 10 minutes. The lysates were then collected into separate microcentrifuge tubes and they were subjected to sonication for 10 seconds. Following sonication, the lysates were centrifuged at 13 000 g for 20 min at 4°C. The supernatant containing proteins was then utilised for Bradford assay and SDS-PAGE.

6.4.7. Bradford assay

For the Bradford assay, 5 μ L of the supernatant from cell lysis was added to 45 μ L of ddH₂O, resulting in 1:10 dilution. Subsequently, 96-well plate was loaded in triplicates for each sample with 5 μ L 1:10 sample dilution + 10 μ L ddH₂O + 190 μ L 1X RotiQuant Bradford reagent. The plate was shook for 30 seconds to mix all components, and then it was left incubating for 5 min at room temperature. Then, the absorbance at 595 nm was read by SUNRISE plate reader. The protein concentration was calculated from absorbance using BSA standard curve, which was established by reading of absorbance at 595 nm of samples with known concentration. For the construction of the BSA standard curve 10 μ L BSA samples of 50, 100, 150, 200, 300, 400 and 500 μ g/mL were loaded to 96-well plate, to which 5 μ L of 1:10 dilution of lysis buffer and 190 μ L of 1X Rotiquant were added. The components were mixed by shaking for 30 seconds and the plate was incubated at room temperature for 5 min.

6.4.8. SDS-PAGE and Western blot

For the SDS-PAGE and subsequent western blotting, 80 – 90 μ L of the supernatant produced from cell lysis was mixed with 40 – 45 μ L of 3X SDS sample buffer with 15% β -mercapto-ethanol. The protein sample was then heated up to 95°C for duration of 5 to 10 minutes. The samples were then stored at -20°C until further use.

To separate the proteins on a gel, 7.5% polyacrylamide separation gel was prepared with stacking gel on top for protein loading. Based on the results of the Bradford assay, the gel was loaded generally with 20 μ g of protein (although 15 μ g loading amount was also tested) and with 5 μ L of Precision Plus Protein™ All Blue Prestained Protein Standards. Submerged in the Electrophoresis buffer, the gel was run at constant voltage (150 V) until the proteins reached the bottom of the gel.

Subsequently, the proteins separated on the polyacrylamide gel were transferred to nitrocellulose membrane by western blotting. The transfer was made under constant voltage (100 V) for 90 minutes in 1X blotting buffer. Following the transfer, the nitrocellulose membrane was blocked by overnight incubation (at 4°C) in blocking buffer (5% BSA in 1X TBST) on shaking platform.

The following day, the blocking buffer was discarded and the membrane was washed 3x in 1X TBST (1 wash is 10 min in 1X TBST on shaking platform). Then the membrane was cut into two halves just above the 75 kDa mark. The upper part of the membrane was incubated with mouse anti-EGFR monoclonal antibody and rabbit anti-pEGFR polyclonal antibody, and the lower part of the membrane was incubated with rabbit anti- α/β -tubulin polyclonal antibody for 2 hours at room temperature. The anti-EGFR and anti-pEGFR antibodies were prepared as either 1:1000 or 1:2000 dilutions in 5% BSA in TBST, and the anti- α/β -tubulin antibody was prepared as 1:1000 dilution in 5% BSA in TBST.

After the incubation period, the membranes were again washed 3x in 1X TBST and then they were incubated with secondary antibodies for 1 hour at room temperature. EGFR was detected with goat anti-mouse IgG (H+L) DyLight 488 linked secondary antibody (1:5000 or 1:10 000 dilution in 5% BSA in TBST), while pEGFR was detected with goat anti-rabbit IgG (H+L) highly cross-absorbed secondary antibody, Alexa Fluor™ Plus 647 linked (1:10 000 or 1:20 000 dilution in 5% BSA in TBST). The α/β -tubulin was detected using goat anti-rabbit IgG HRP linked secondary antibody (1:2000 dilution in 5% BSA in TBST). Following the incubation, the membranes were again washed 3x in TBST.

The membranes were then imaged on ImageQuant™ 800 Western blot imaging system. The EGFR and pEGFR were detected using fluorescence, channels Cy2 and Cy5 respectively. α/β -tubulin was detected using chemiluminescence. For the chemiluminescence readout, the membrane was incubated for 1 minute prior to imaging in ECL solution (9 mL ddH₂O, 1 mL ECL stock, 50 μ L luminol, 22 μ L coumaric acid and 3 μ L H₂O₂) and protected from light.

6.5. Statistical analysis

Experiments with $n = 3$ or more independent replicates were analysed for statistical significance. Data with one dependent variable and one independent variable were analysed by one-way ANOVA followed by Tukey's multiple comparison test. Data with one dependent variable and two independent variables were analysed by two-way ANOVA followed by Tukey's multiple comparison test. Differences in data were proclaimed statistically significant if the p-value was less than 0.05. All statistical tests were carried out in R studio version 1.1.383.

7. Results and discussion

7.1. Optimisation of *in vitro* transfection with EGFR-EGFP plasmid

The main aim of this master's thesis was to optimise the transfection protocol with EGFR-EGFP plasmid, so that highest possible transfection efficiency with least toxicity could be achieved. Achieving the best transfection results with least toxicity is of high importance, as this will enhance the experiments for allowing to visualise EGFR receptor as well as the effects of pharmacological targeting of this receptor.

First of all, this chapter summarises the expansion of EGFR-EGFP WT plasmid clones, as well as their validation, which were used throughout this thesis in later transfections. This chapter also provides results of screening of transfection parameters in 96-well plate format and the subsequent upscaling of selected parameters to 6-well plate format. Furthermore, the question of importance of polyplexing concentration on transfection efficiency was investigated.

7.1.1. Expansion and validation of EGFR-EGFP plasmid

In order to obtain sufficient amount of EGFR-EGFP plasmid DNA for *in vitro* transfections, expansion of the plasmid was carried out with maxiprep isolation. The WT EGFR-EGFP plasmid was expanded from stock prepared in the work of Manuel Blaschek⁵² (denoted as clone 3 – stored in -80°C storage of MMCT group, position 5C/B-IX/32), which is expanded from the original EGFR-EGFP plasmid by Carter and Sorkin⁴⁷. The DH5α *E. coli* cells harbouring the plasmid were streaked on agar plates containing kanamycin to select only cells containing the plasmid. Colonies growing on the agar plates were then picked for pre-culture. Pre-cultures 1 and 3 had the highest visible growth after 5.5 incubation time at 37°C. Therefore, these pre-cultures were selected to inoculate overnight (O/N) cultures.

In total three O/N cultures were inoculated (**Table 12**). One O/N culture was inoculated from pre-culture 1 (N1), and the other two cultures were inoculated from pre-culture 3 (N3 and H3). The difference between N3 and H3 O/N culture was in the concentration of kanamycin, where N3 (normal 3) contained standard 50 µg/mL kanamycin, while H3 (high 3) contained high 100 µg/mL kanamycin. The antibiotic concentration was raised in order to minimise

chance of contamination in the culture by bacteria that doesn't carry the plasmid, as there was a degree of uncertainty in the culture process introduced by growth on ampicillin agar plates (data not shown). After total of 21.5 hour culture time (pre-culture time and O/N culture time combined) the OD600 values of the three O/N cultures was in the range of 8.075 – 8.535 (**Table 12**). These OD600 values showed a good growth of the bacteria in all three O/N cultures, and it was possible to prepare two pellets for maxiprep isolation per each O/N culture (**Table 13**).

Table 12. Summary of EGFR-EGFP cultures. This table summarises the cultures in EGFR-EGFP plasmid expansion run. Each culture has its culture ID assigned and the OD600 measurement value after O/N culture is shown, as well as the total duration of pre-culture and O/N culture.

Culture ID	OD600	Pre-culture time (hrs)	O/N culture time (hrs)
N1	8.535	5.5	16
N3	8.2	5.5	16
H3	8.075	5.5	16

The pellets from the O/N cultures were processed by GeneJET Plasmid Maxiprep Kit. The plasmid was eluted in 1.2 mL of elution buffer provided in the kit and the concentrations obtained are shown in **Table 13**. The concentration were reasonably high for all pellets processed, resulting in good yields of the plasmid DNA which ranged from 679.2 µg to 760.2 µg per pellet and from 1.373 to 1.516 mg per O/N culture. These yields are not to be completely unexpected as EGFR-EGFP is high copy plasmid ⁴⁷, meaning that one bacterium cell contains multiple copies of the plasmid increasing the overall yield. Moreover, the A260/A280 ratio indicated good purity of the extracted plasmid DNA, as the values generally moved ~ 1.85 – 1.86. The A260/A230 ratio was little higher than generally accepted 2.0 – 2.2 range, which can indicate problematic blank measurement ⁵³. Nevertheless, the yields together with purity ratios didn't indicate a major problem that would render the plasmid DNA unsuitable for further applications.

Table 13. Overview of EGFR-EGFP plasmid yields. This table summarises the yields of plasmids obtained from cultures in Table 12. From each culture, two pellets were obtained, which were processed by Maxiprep kit to purify the plasmid DNA. The plasmid ID refers to culture ID and pellet number. Concentration of the plasmid after elution is shown, as well as the total yield. The purity parameters, A260/A280 and A260/A230, are shown as well.

Plasmid ID	Concentration (µg/mL)	Yield (µg)	A260/A280	A260/A230
N1 P1	566	679.2	1.8635	2.255
N1 P2	578.5	694.2	1.854	2.305
N3 P1	607	728.4	1.862	2.2995
N3 P2	623	747.6	1.857	2.283
H3 P1	633.5	760.2	1.8575	2.283
H3 P2	630	756	1.8585	2.287

The final step of this EGFR-EGFP expansion was to validate the identity of the obtained plasmids. This was done by DRD using BglII, EcoRI, PvuII and EcoRI + NotI restriction enzymes (**Figure 10**). The resulting fragment pattern of the DRD (**Figure 10b**) was compared to simulation of the DRD in Snap Gene software (**Figure 10a**). Moreover, the EGFR-EGFP plasmid clone (clone 3) from which plasmid clones in this work are expanded from, and which was already validated by its use in *in vitro* transfections was processed by the same DRD, so it was possible to compare directly also with functionally working plasmid. As can be seen in **Figure 10** the fragment pattern of the newly expanded EGFR-EGFP plasmids (H3 P2 and N3 P1) matched both the simulation in Snap Gene and also the fragment pattern of clone 3. Therefore, the identity of the expanded plasmids was confirmed and the plasmids could be used in further applications. With very high exposure times (1.5 – 3 sec) the image of the agarose gel showed some additional bands in EcoRI restriction reaction, however this was also visible with the clone 3 control plasmid, indicating that the additional bands appeared as a result of star activity of the restriction enzyme, which can occur with longer restriction reaction times⁵⁴, such as 90 min used in this work.

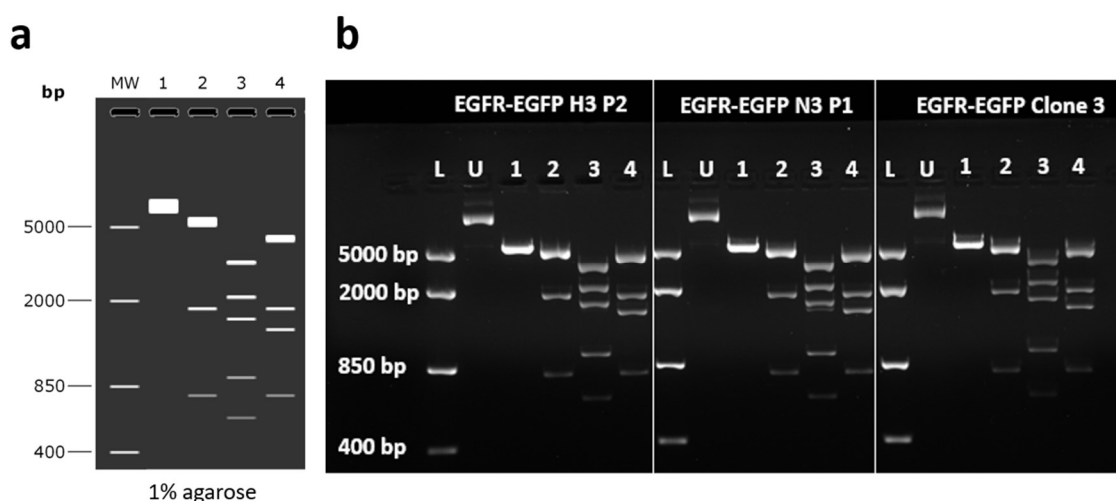


Figure 10. Validation of expanded EGFR-EGFP plasmid by diagnostic restriction digest. a) Predicted fragment pattern by simulation of diagnostic restriction digest in Snap Gene. b) Diagnostic restriction digest of expanded EGFR-EGFP (clones H3 P2 and N3 P1) and the EGFR-EGFP clone 3, from which the plasmid were expanded. Labels: MW = molecular weight, L = molecular ladder, U = uncut, 1 = reaction with BglII, 2 = reaction with EcoRI, 3 = reaction with PvuII, 4 = reaction with combination of EcoRI and NotI.

7.1.2. Optimisation of EGFR-EGFP *in vitro* transfection parameters in 96-well plate format

Once the WT EGFR-EGFP plasmid was expanded, the next objective was to utilise this plasmid in *in vitro* transfections. The initial run of transfections were carried out in 96-well plate format, so it was easier to screen multiple parameters in the optimisation efforts. Some parameters, for instance HBG buffer used in polyplex preparation instead of HBS buffer leads to better transfection efficiency, were optimised in previous work⁵². In this work, the main focus of optimisation was taken on cell line used, polyplexing concentration (i.e. the concentration of polyplexes formed after addition of cationic LPEI to anionic plasmid DNA), dose of plasmid DNA complexed in polyplexes added to cells and seeding density of cells. The output of the screening in term of data produced were fluorescent light microscopy (FLM) images, which were used to qualitatively assess the transfection efficiency, and resazurin assay data, which were used to assess cell viability after transfection, providing information on transfection toxicity.

In the first round of optimisation (**Figure 11**), transfection efficiency of HEK293 cells was compared to that of HeLa cells. Both cell types were seeded at density of 10 000 cells per well. The cells were transfected with polyplexes prepared at 20 µg/mL polyplexing concentration, with doses of plasmid DNA of 200 ng or 400 ng per well. The EGFR-EGFP plasmid clones used

in this transfection were clone 3 (from which clones in previous section are expanded) and clone 1 (original clone from which clone 3 was expanded).

The results (**Figure 11**) showed a clear difference between 200 ng and 400 ng dose in HEK293 transfection, however with HeLa cells there didn't seem to be the same difference in transfection efficiency between the two doses. When comparing HEK293 cells with HeLa cells, HeLa cells appeared to transfect better than HEK293 cells in terms of number of green cells, however the signal in HEK293 cells was stronger as lower exposure times were necessary in FLM imaging. With HeLa cells the higher exposure time resulted in higher background signal in the images. The reason behind the stronger signal in HEK293 cells could be caused by the fact that HEK293 cells grow in clusters and also that their size compared to HeLa cells is smaller. This could result in higher localised EGFR-EGFP presence causing stronger local signal, whereas the EGFR-EGFP signal is more spread out in space in the HeLa cells. As expected there wasn't any clear difference between plasmid clones, which is of high importance for reproducibility of the results.

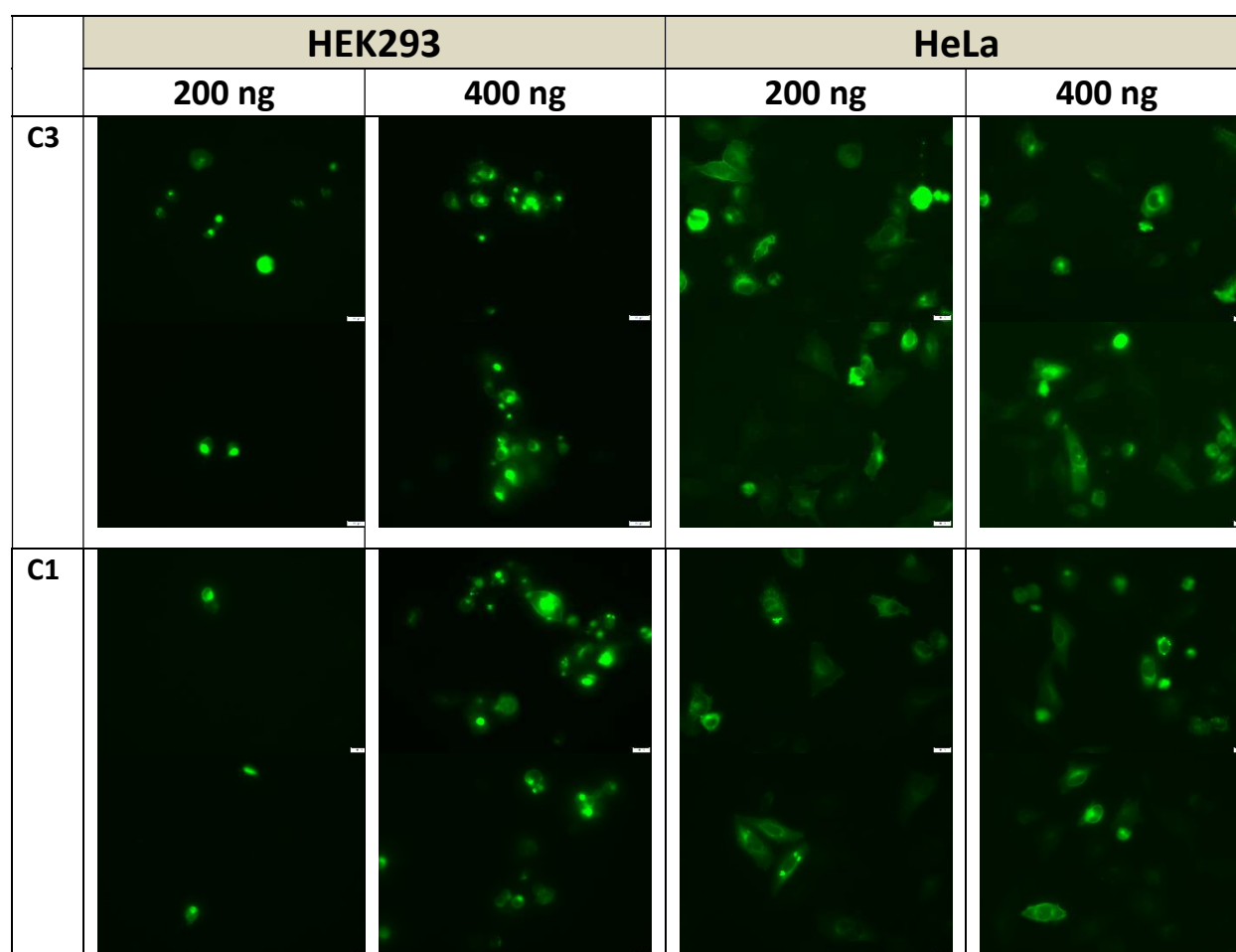


Figure 11. Qualitative comparison of transfection efficiency between HEK293 cells and HeLa cells. This figure shows FLM images of transfected HEK293 cells and HeLa cells with EGFR-EGFP plasmid (200 ng or 400 ng dose of plasmid DNA [clone 3 – C3 or clone 1 – C1 EGFR-EGFP plasmid] in LPEI polyplexes prepared at 20 µg/mL polyplexing concentration). The cells were fixed and imaged approx. 24 hours post transfection. Both cell lines were seeded seeding density of 10 000 cells per well. The transfection was carried out at 5% FCS concentration, with FCS being added 4 hours post transfection. The figure presents two representative field views at 40X magnification out of total 9 field views taken (3 field views for each of 3 replicate wells in 96-well plate).

The second optimisation round (**Figure 12**) compared HEK293 cells and HEK293T cells. As in the previous round, the cells were seeded at 10 000 cells per well and the cells were transfected with 200 ng or 400 ng dose of plasmid DNA. In this round the EGFR-EGFP clones used for transfection were clones expanded in this work, H3 P1 and N3 P2. Furthermore, the polyplexing concentration was increased to 40 µg/mL.

As in the previous round, the difference between 200 ng and 400 ng dose was observable (**Figure 12**). This was especially true for HEK293T cells, which transfected very well with 400 ng dose of plasmid. Moreover, the transfection efficiency of the HEK293T cells was higher compared to HEK293 cells at both doses. Therefore, the decision was taken to move forward with HEK293T cells rather than HEK293 cells. When comparing the EGFR-EGFP clones, the difference didn't seem to be very high, but it seemed that transfection with H3 P1 plasmid worked a little better. However, this difference could well be within variability between experiments. Nevertheless, the H3 P1 plasmid was used in further transfections. It was also possible to compare the differences in transfection efficiency between HEK293 cells transfected with 20 µg/mL polyplexes (**Figure 11**) and with 40 µg/mL polyplexes (**Figure 12**). From the results it seemed that the polyplexing concentration didn't have effect on transfection efficiency that would be outside of variability between experiments.

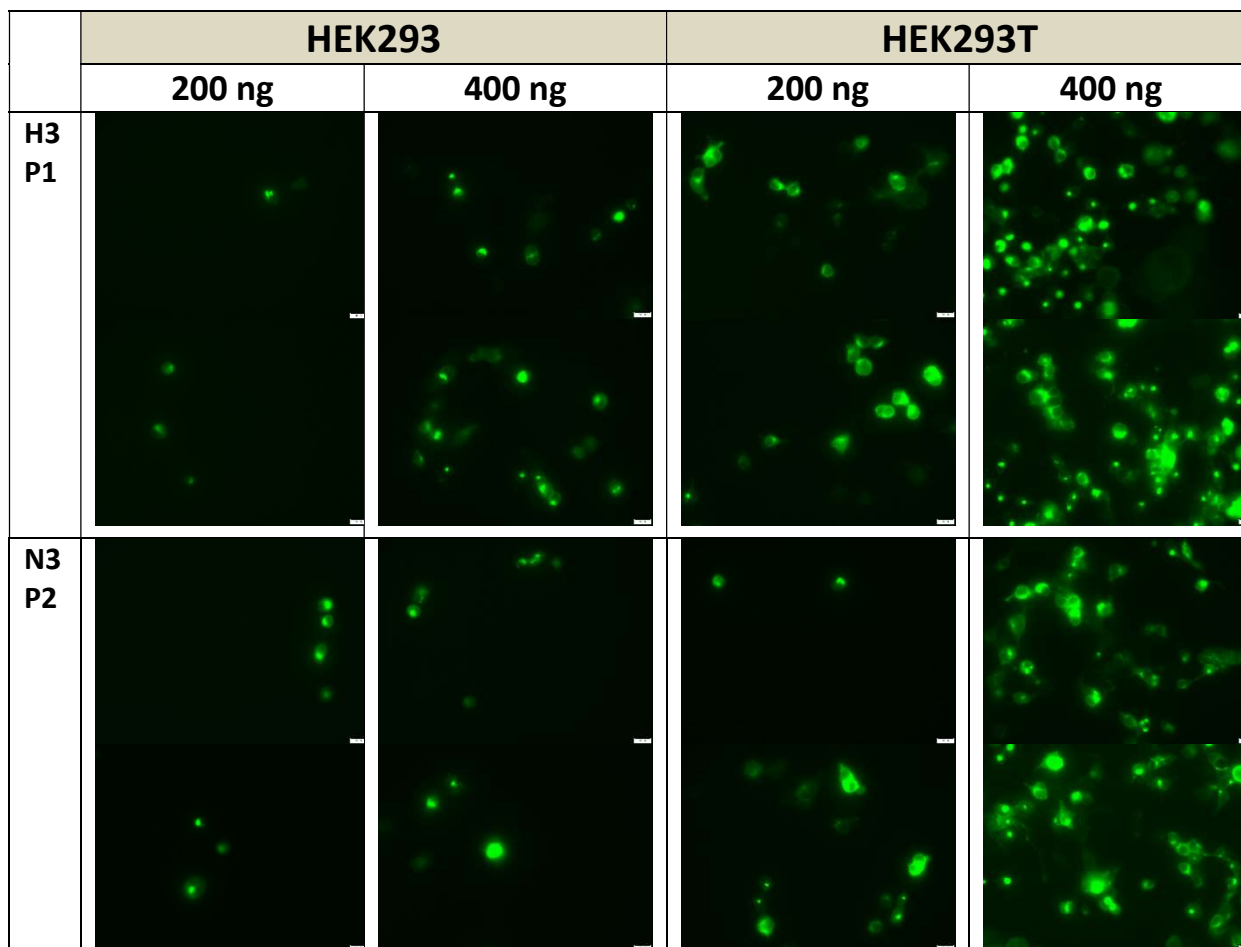


Figure 12. Qualitative comparison of transfection efficiency between HEK293 cells and HEK293T cells. This figure presents FLM images of transfected HEK293 and HEK293T cells with EGFR-EGFP plasmid (200 ng or 400 ng of plasmid DNA [H3 P1 and N3 P2 clones of EGFR-EGFP] complexed in LPEI polyplexes, which were prepared at 40 µg/mL polyplexing concentration). The cells were fixed and imaged ~ 24 hours post transfection. Both cell lines were seeded at density of 10 000 cells per well. The transfection occurred at 5% FCS concentration, which was established 4 hours post transfection. The figure shows two representative field views at 40X magnification per each condition. In total, 9 field views were taken (3 field view per each of 3 replicate wells in 96-well plate).

On the side note, the confluency of the cells seeded at 10 000 cells per well seeding density appeared to be quite low. Therefore, before the third optimisation round, the seeding density was increased to 15 000 cells per well. This decision was based on the confluency that HEK293T and A549 cells reached approx. 24 hours after seeding (**Figure 13**). In this experiment, the cells were seeded in three different seeding densities. On day 2, the confluency was observed and the best results were reached with 15 000 cells per well seeding density, with 10 000 cells per well being too little and 20 000 cells per well too much, especially for A549 cells that reached nearly 100% confluency.

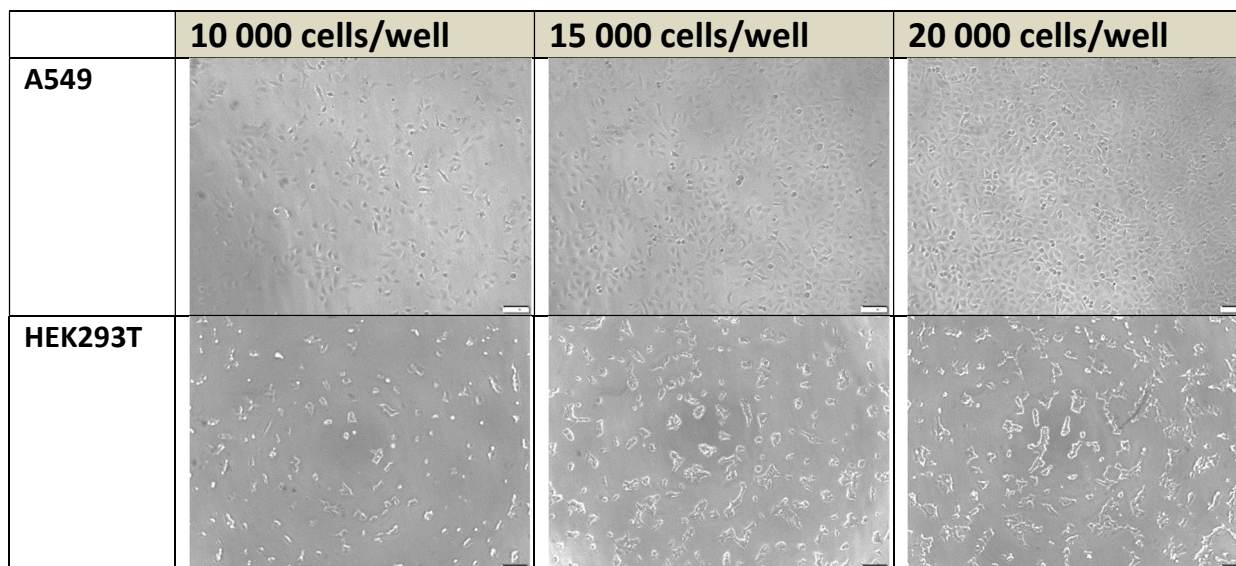


Figure 13. Day 2 confluency of A549 and HEK293T cells at different seeding densities. Confluency of A549 and HEK293T cells is shown approx. 24 hours post seeding at different seeding densities. The figure shows phase contrast images taken by light microscopy at 10X magnification. Representative images are shown.

In the third optimisation round (**Figure 14**), HEK293T and A549 cells were seeded at the revised seeding density. As in the previous step, the cells were transfected with 200 ng or 400 ng of H3 P1 EGFR-EGFP clone complexed in LPEI polyplexes prepared at 40 $\mu\text{g}/\text{mL}$ polyplexing concentration. As in the second optimisation round (**Figure 12**), the HEK293T cells transfected really well with strong signal. The 400 ng dose resulted in higher transfection efficiency compared to 200 ng dose. On the other hand, there wasn't a clear difference between 200 ng and 400 ng dose with the A549 cells. Furthermore, the signal was much weaker and higher exposure times had to be used for imaging. For comparison the HEK293T cells were imaged at exposure times $\sim 20 - 30$ ms, while the A549 cells were imaged at exposure times ~ 200 ms. This resulted also in higher background signal in A549 images. Similarly as with HeLa cells, the difference could have been caused by more spread out localisation of EGFR-EGFP molecules, compared to compactly clustered HEK293T cells. All in all, the strength of the observable signal in HEK293T cells points strongly to their use in demonstrative transfection within the realms of laboratory practical course.

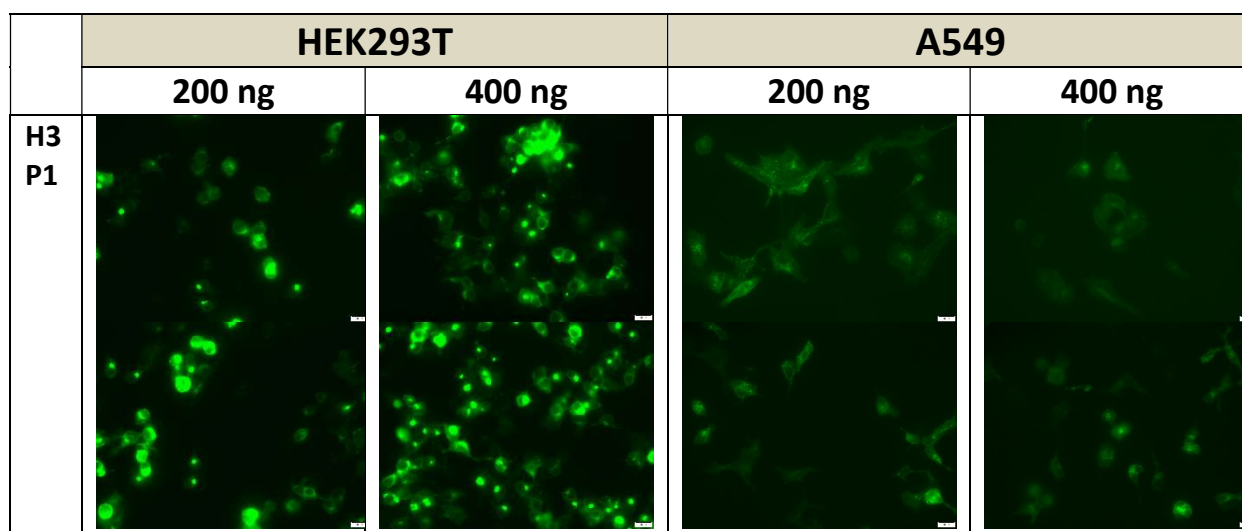


Figure 14. Qualitative comparison of transfection efficiency between HEK293T cells and A549 cells. This figure shows the FLM images of HEK293T and A549 cells transfected with EGFR-EGFP plasmid (200 ng or 400 ng dose of plasmid DNA [H3 P1 clone] complexed with LPEI in polyplexes at 40 μ g/mL polyplexing concentration). The cells were fixed and imaged ~ 24 hours post transfection. Both cell lines were seeded at seeding density of 15 000 cells per well. The transfection occurred in presence of 5% FCS, which was supplied 4 hours post treatment with polyplexes. The figure shows two representative field views per each condition at 40X magnification. In total, 9 field views were taken per each condition (3 field view per each of 3 replicate wells in 96-well plate). In the case of A549 cells, the two field views shown come from two separate replicate experiments.

As mentioned in the beginning of this section, the toxicity of polyplex transfection was assessed via resazurin assay, which determined the cell viability ~ 24 hours post transfection. The main objective of resazurin assay measurement was to compare 200 ng and 400 ng plasmid DNA dose to untransfected control group. This was done for HEK293 cells (**Figure 15a**), HEK293T cells (**Figure 15b**) and A549 cells (**Figure 15c**). The cell viability decreased more or less in concentration dependant manner. For HEK293 cells, viability at 200 ng dose was ~ 87%, while at 400 ng dose it was ~ 91%. For HEK293T cells this was ~ 97% viability at 200 ng dose and ~ 90% viability at 400 ng dose. Viability of A549 cells at 200 ng dose was ~ 93% and at 400 ng it was ~ 81%. The cell viability was surprisingly good especially with HEK293T cells. This raised the possibility to increase the dose even higher in further experiments to reach higher transfection efficiency. However, it needs to be added that the variability between experiments could be quite high, therefore one has to be careful to overly interpret these results as only polyplex transfection dependent, but other variables, such as variability in seeding accuracy, have to be taken into account.

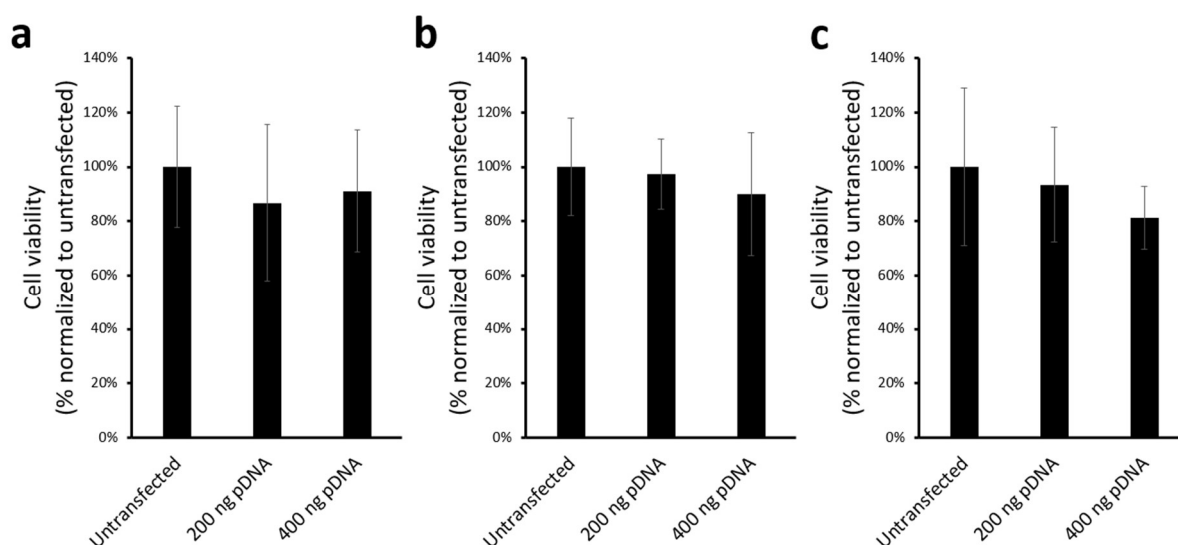


Figure 15. Cell viability 24 hours post transfection. The graphs presented in this figure show cell viability of **a)** HEK293, **b)** HEK293T and **c)** A549 cells 24 hours post transfection determined by resazurin assay. The graphs show mean % cell viability $\pm$ standard deviation normalized to untransfected control. The mean and standard deviation is calculated from $n=2$ independent experiments. Each experiment had at least 3 technical replicates.

7.1.3. Upscaling EGFR-EGFP *in vitro* transfection to 6-well plate format

The *in vitro* transfection optimisation rounds in 96-well plate format helped to establish the cell line to be used (HEK293T), as well as the seeding density. Moreover, information on toxicity was gained, which helped further decisions on dosage. The next step was to utilise the information gained in 96-well plate format and to upscale the transfection parameters to 6-well plate format.

In order to reach similar cell confluency upon transfection, the seeding density needed to be increased in 6-well plate, as there is more surface area available for the cells to grow. The seeding density was upscaled by simple linear relationship between seeding density and total surface area available and was calculated as follows: $Seeding\ density_{6-well\ plate} = (Seeding\ density_{96-well\ plate} / Surface\ area_{96-well\ plate}) \times Surface\ area_{6-well\ plate}$. This calculation gave $\sim 415\ 000$ cells/well as a 6-well plate seeding density corresponding to the 15 000 cells/well in 96-well plate format. Based on the observation of confluency ~ 24 hours after seeding, it could be concluded that the respective seeding densities for 96-well plate and 6-well plate are matching (**Figure 16**).

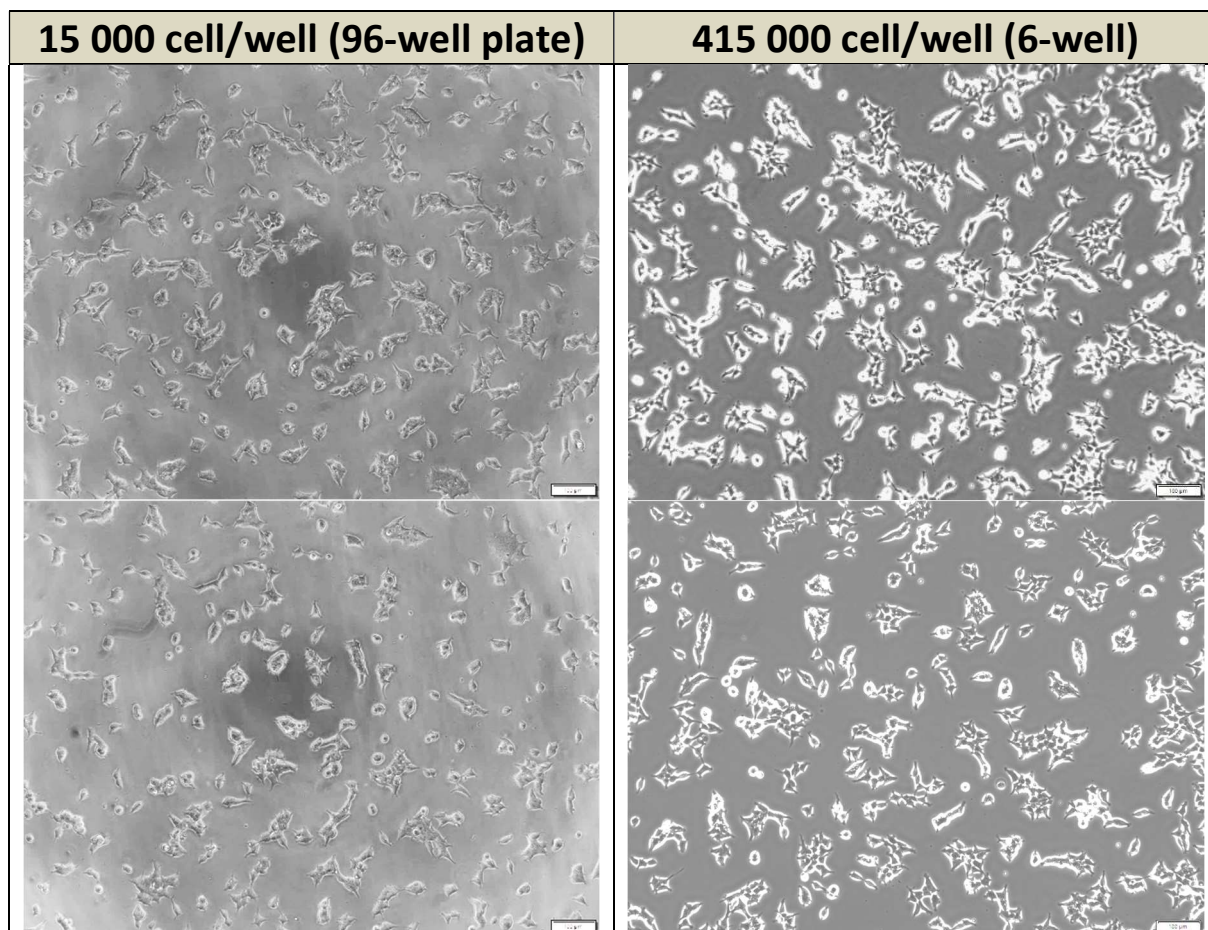


Figure 16. Upscaling seeding density from 96-well plate format to 6-well plate format. The seeding density of 15 000 cells/well in 96-well plate was upscaled linearly by increase in total surface area available to 415 000 cells/well in 6-well plate. This figure compares day 2 confluency of HEK293T cells seeded at respective densities in 96-well plate and 6-well plate. Two respective phase contrast images take approx. 24 hours post seeding at 10X magnification are shown.

After establishing the seeding density for 6-well plate, another key parameter for transfection needed to be upscaled. This was the dosage of plasmid DNA. Similarly to seeding density, the dosage was adjusted linearly. In 96-well plate the working volume was 200 μ L, whereas in 6-well plate this volume was 2 mL, a 10-fold increase. Therefore, the dose was also increased by 10-fold, meaning that 200 ng dose in 96-well plate would be 2 μ g in 6-well plate. However, since the transfection efficiency was much higher with the 400 ng dose and the cell viability was still very good (**Figure 12, 14 and 15**), it was decided to increase the dose to 8 μ g, which would correspond to 800 ng in 96-well plate experiment.

As can be seen in **Figure 17**, the FLM readout of the transfection didn't reveal a big difference between the 4 μ g and 8 μ g doses of the EGFR-EGFP plasmid. Nonetheless, the transfection efficiency appeared to be very good as there was plenty of green EGFP signal in areas of cell

growth indicated by the blue DAPI signal. The transfection with 4 μg EGFR-EGFP was in line with transfections with 400 ng EGFR-EGFP in 96-well plate format, however the increase to 8 μg didn't offer an obvious benefit that would be visible by naked eye. In terms of cell viability, the resazurin assay detected difference between 4 μg and 8 μg doses, as the cell viability decreased in dose dependant manner (**Figure 18**). The cell viability after transfection with 8 μg EGFR-EGFP was $\sim 10\%$ lower when compared to 4 μg treatment. Nevertheless, the cell viability was still reasonably high. After 4 μg transfection the cell viability was $\sim 92\%$, while after 8 μg transfection it was $\sim 82\%$. The approx. 92% cell viability after 4 μg transfection also corresponds to the $\sim 90\%$ cell viability of HEK293T cells in 96-well experiments after transfection with 400 ng EGFR-EGFP (**Figure 15**). This further indicated that the upscaling to 6-well plate didn't affect the behaviour of the transfections in HEK293T cells, as the results are quite reproducible between 96-well and 6-well plate transfections. However, one thing that must be taken into consideration is that, while for both 400 ng dosage in 96-well plate and 4 μg dosage in 6-well plate the polyplexes were prepared at 40 $\mu\text{g/mL}$ polyplexing concentration, for the 8 μg dose the polyplexes were prepared at 80 $\mu\text{g/mL}$ polyplexing concentration. Therefore, care should be taken when extrapolating results of 8 μg transfection in 6-well plate back to 96-well plate.

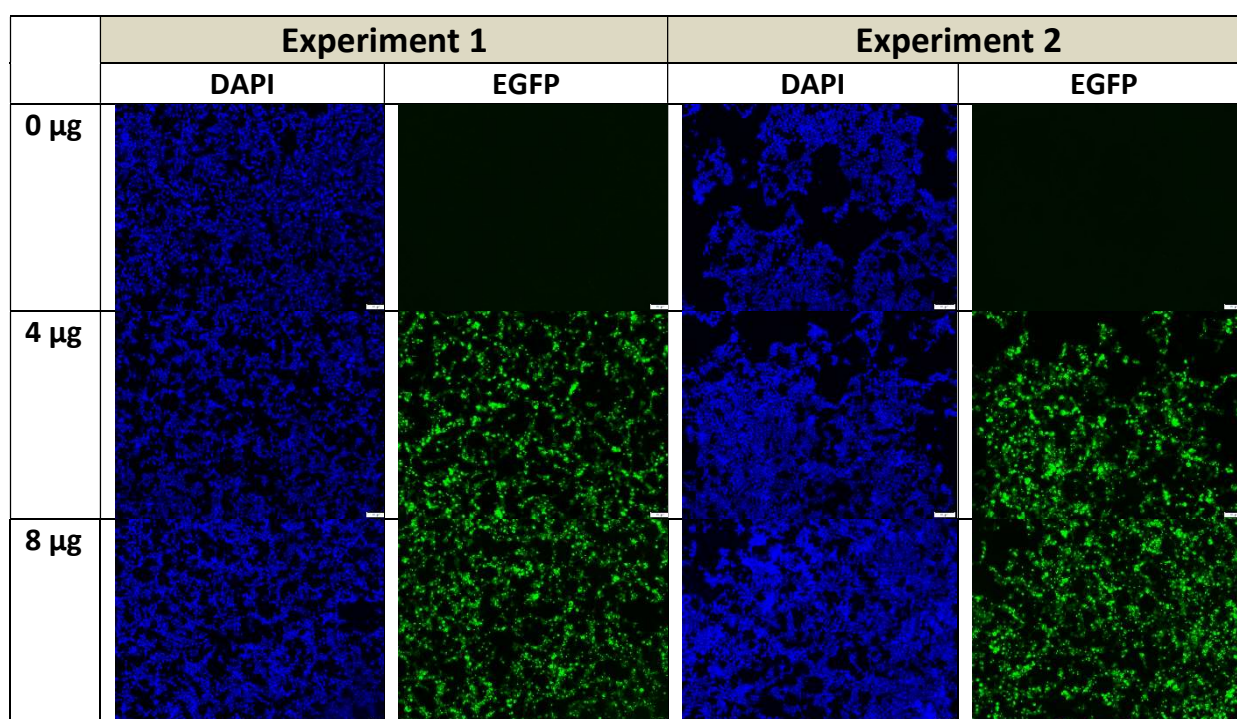


Figure 17. FLM images of HEK293T cells transfected with EGFR-EGFP plasmid. FLM images of two independent transfections with EGFR-EGFP plasmid. The figure shows representative images in DAPI and EGFP channels of untransfected HEK293T cells (0 μg dose) and HEK293T cells transfected with 4 μg (40 $\mu\text{g}/\text{mL}$ polyplexing concentration) and 8 μg (80 $\mu\text{g}/\text{mL}$ polyplexing concentration) dose of EGFR-EGFP plasmid DNA. The cells were fixed, stained with DAPI and imaged ~ 24 hours post transfection. The transfection was done at 5% FCS medium, supplied 4 hours after polyplex addition. The images were taken at 10X magnification.

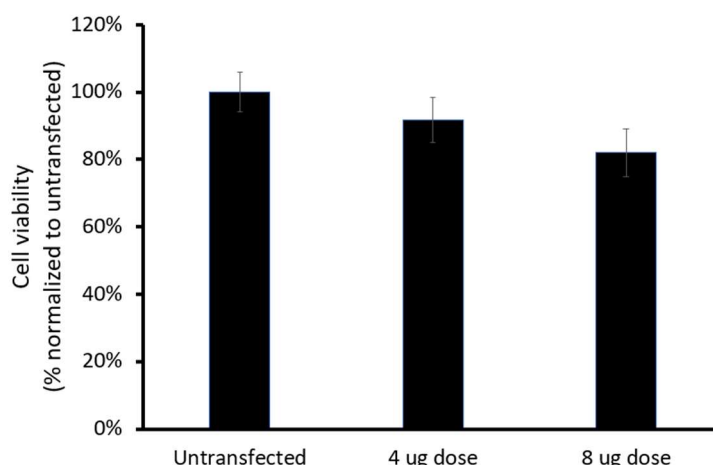


Figure 18. Cell viability of HEK293T cell 24 hours post transfection. The graph presented in this figure shows % cell viability normalized to untransfected cell 24 hours post transfection with 4 μg or 8 μg pDNA dose in 5% FCS medium. The cell viability was determined by resazurin assay. The % cell viability is shown as mean $\pm$ standard deviation of $n = 2$ independent experiments. Each experiment had 3 technical replicates.

As the results of the resazurin assay indicated differences in cell viability between 4 and 8 μg doses, it seemed likely that there are also differences in transfection efficiency, that are not easily observed by FLM. Hence, the transfection efficiency was also assessed quantitatively via flow cytometry, which allowed precise information on proportion of transfected cells. Additionally, the cells were stained with DAPI, which allowed assessment of toxicity that is related only to transfection itself. Together with resazurin cell viability readout, this offered a fuller picture on toxicity introduced by polyplex transfection. Moreover, in the flow cytometry experiments, effects of FCS concentration in medium on transfection efficiency and toxicity were assessed.

In **Figure 19a**, it can be seen that there is indeed a difference in transfection efficiency between 4 μg and 8 μg dose. In 5% FCS the transfection efficiency reached $\sim 42\%$ transfected cells in 4 μg transfection group and $\sim 53\%$ transfected cells in 8 μg transfection group. Interestingly, the transfection efficiency with 1% FCS was a little lower with $\sim 35\%$ transfected cells in 4 μg transfection group and $\sim 45\%$ transfected cells in 8 μg transfection group. This

showed the difference between 4 and 8 μg dose to be around 10% in transfection efficiency. Although clear reproducible increase in transfection efficiency could be seen after increase of the dose to 8 μg , this increase failed to show statistical significance. The only statistically significant difference was between the transfected groups and untransfected control group. The same applies for the decrease in transfection efficiency after reducing the FCS concentration to 1%, as no statistical significance was observed between transfections with 5% or 1% FCS.

When it comes to transfection related toxicity, the increase in dose of plasmid DNA also meant an increase in toxicity (**Figure 19b**), as expected. Following transfection with 4 μg EGFR-EGFP in 5% FCS there were $\sim 87\%$ live cells in the sample, while after transfection with 8 μg EGFR-EGFP in 5% FCS there were $\sim 84\%$ live cells. This is quite in line with the previous resazurin readout and indicates that even with 8 μg dose the toxicity is not really high. As with the transfection efficiency, reduction of FCS concentration to 1% caused changes to toxicity as well. In this case, the toxicity was lowered in comparison to cells transfected in presence of 5% FCS. The mean % of live cells in the 4 μg group in 1% FCS was $\sim 92\%$, while in the 8 μg group in 1% FCS it was $\sim 89\%$. This data suggest that relationship between transfection efficiency and toxicity exists, where the more cells are transfected the more die as a result. Again, the differences between 4 μg and 8 μg doses, as well as the 5% and 1% FCS groups didn't reach statistical significance. However, a clear difference exists between this groups, as the decrease of toxicity in 1% FCS group caused loss or reduction of statistical significance between transfected groups and untransfected control group.

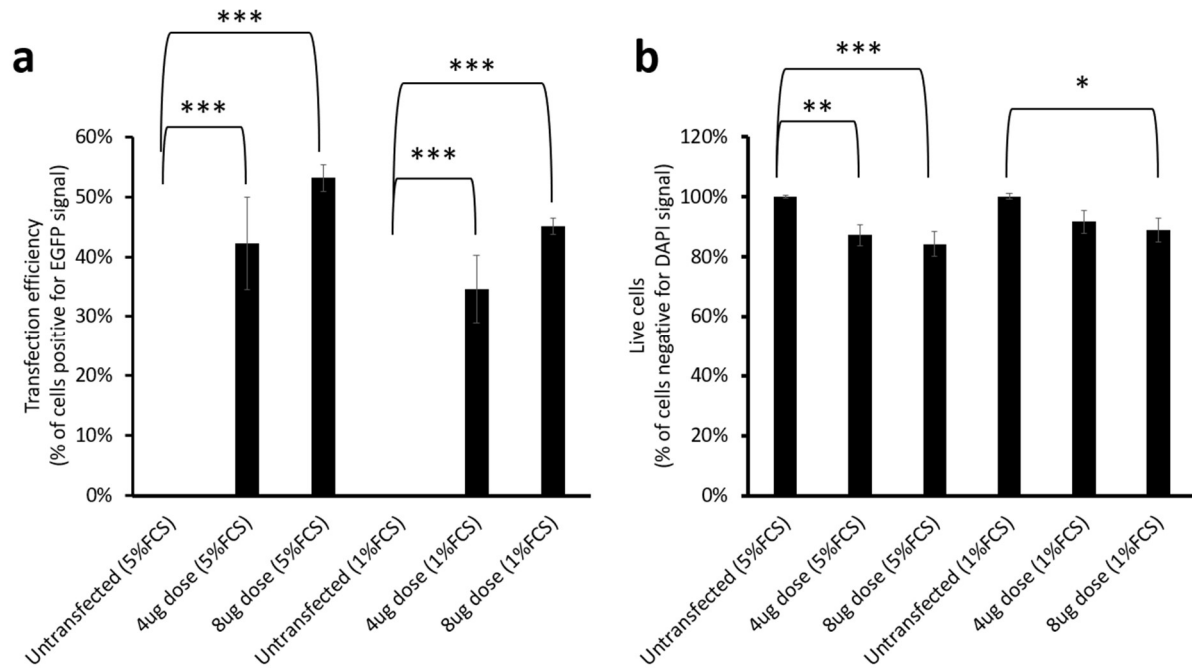


Figure 19. Transfection efficiency and transfection related toxicity of HEK293T cells 24 hours post transfection. a) Transfection efficiency of HEK293T cells transfected by LPEI polyplexes with EGFR-EGFP plasmid (4 or 8 µg dose in 5% or 1% FCS). Transfection efficiency is shown as a mean ± standard deviation of $n = 3$ independent experiments. The transfection efficiency is represented by the percentage of cells positive for EGFP signal. **b)** Visualisation of transfection related toxicity in HEK293T. Percentage of live cells, normalized to untransfected control in 5% FCS, following transfection with 4 µg or 8 µg dose of EGFR-EGFP plasmid in LPEI polyplexes in 5% or 1% FCS medium is shown. Live cells are shown as mean ± standard deviation of $n = 3$ independent experiments. Each experiment contained 2 technical replicates. * = $p < 0.5$, ** = $p < 0.1$, *** = $p < 0.001$. Data analysed by two-way ANOVA followed by Tukey multiple comparisons test.

When examining the distribution of cells according to their EGFP signal intensity, there were two populations of cells visible on the histogram after transfection for both doses (**Figure 20a and b**). The smaller population exhibited strong EGFP signal, indicating strong expression of the EGFR-EGFP construct. On the other hand the larger population only shifted in comparison to untransfected population, indicating very weak expression. Diagonal gating didn't indicate that this shift would be caused by unspecific fluorescence. With 8 µg dose the strongly fluorescent population was proportionally slightly bigger than with 4 µg dose. It appears that the ~ 10% difference in transfection efficiency came from this increase in the more strongly fluorescent population rather than in the shift of the weakly fluorescent population, as this shift was minimal. On the other hand, the difference caused by 1% FCS concentration, seemed to originate both in smaller shift of weakly fluorescent population, as well as smaller proportion of strongly fluorescent population (**Figure 20b**). It is worth mentioning, that the toxicity appeared only in strongly fluorescent population, as a clear cut-off could be seen after gating for live cells in diagonal graph of DAPI (VioBlue) and EGFP (FITC) signal (**Figure 21a**).

Also, the diagonal graphs of VioGreen or PercP-Vio700 channels against FITC channel reveal a clear break between the two populations occurring after transfection (**Figure 21b and c**). This might suggest that only the population showing strong EGFP emission is truly transfected, while the shift in the main population could be caused by other factors, such as intake of extracellular vesicles containing expressed EGFR-EGFP fusion protein.

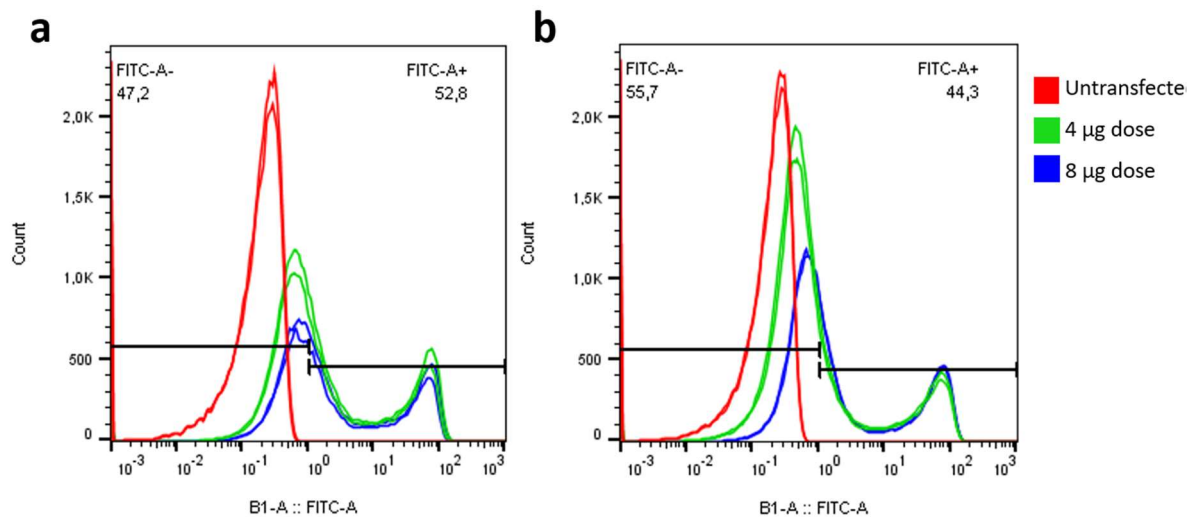


Figure 20. Distribution of HEK293T population by intensity of EGFP fluorescence. These graphs show the distribution HEK293T cells in respect to the intensity of EGFP fluorescence (recorded in FITC channel). Red lines represent cells that were not transfected, green lines represent cells transfected with 4 µg plasmid DNA and blue lines represent cells transfected with 8 µg plasmid DNA in **a**) 5% FCS medium or **b**) 1% FCS medium. The graphs also show gate cut-off for positive/negative cells for EGFP signal. Each histogram shows two technical replicates.

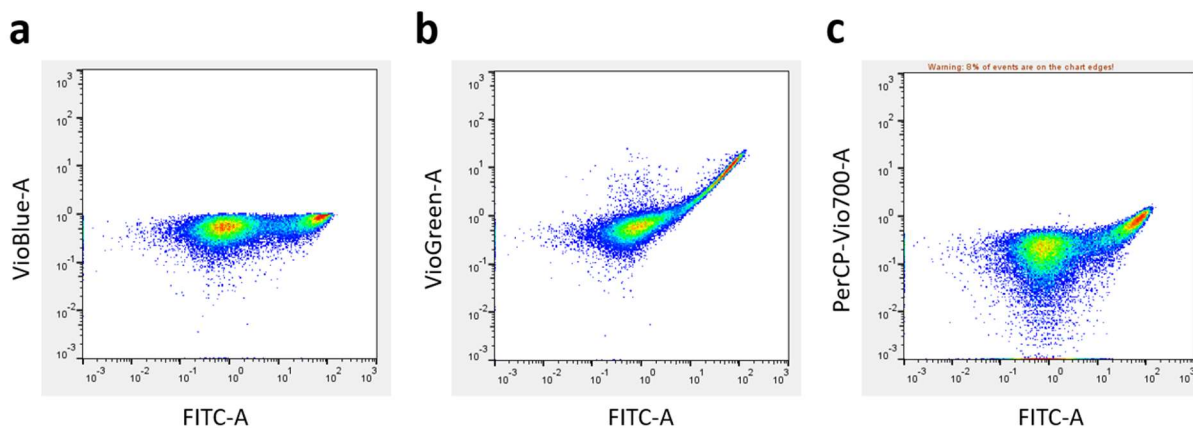


Figure 21. Diagonal gating of single live HEK293T cells in different fluorescence channels. **a**) diagonal gating by VioBlue vs. FITC channel. **b**) diagonal gating by VioGreen vs. FITC channel. **c**) diagonal gating by PercP-Vio700 vs. FITCS channel. The examples shown in this figure are based on transfection with 8 µg of plasmid DNA.

To have a clearer picture on how cell viability is affected by the transfection also in 1% FCS media, additional resazurin assays were conducted (**Figure 22**). The crucial difference between

results from resazurin assay and DAPI live/dead staining in flow cytometry is that resazurin assay is a readout of metabolic activity and therefore includes other factors, such as cell growth and therefore cell viability can be observed, whereas DAPI live/dead staining only differentiates live from dead cells. In 5% FCS media, the same dose dependent decrease in cell viability could be observed with ~ 97% cell viability in 4 μ g group and ~ 81% cell viability in 8 μ g group. Most importantly, the use of 1% FCS medium resulted in lower cell viability even in untransfected group, compared to the same group in 5% FCS. This can be explained by the fact that with lower serum concentrations the cells are not as stimulated to grow and therefore the overall cell count is lower, resulting in lower readout. Similarly, the metabolic activity isn't as stimulated with lower concentrations of growth factors and stimulants present in 1% FCS medium. Interestingly, the transfection didn't introduce an additional decrease in cell viability in 1% FCS medium, as there wasn't any clear trend present (untransfected ~ 85% cell viability, 4 μ g dose ~ 90% cell viability, 8 μ g dose ~ 82% cell viability). It appears that the decrease in cell viability in 1% FCS media masks the low toxicity introduced by polyplex transfection observed in the flow cytometry DAPI staining. It also suggests that the resazurin assay is more sensitive to changes in growth rate of cells which can mask effects of treatments. This reiterates the importance of having both resazurin readout and live/dead readout to see a full picture of toxicity and to differentiate the toxicity that is introduced only by the treatment.

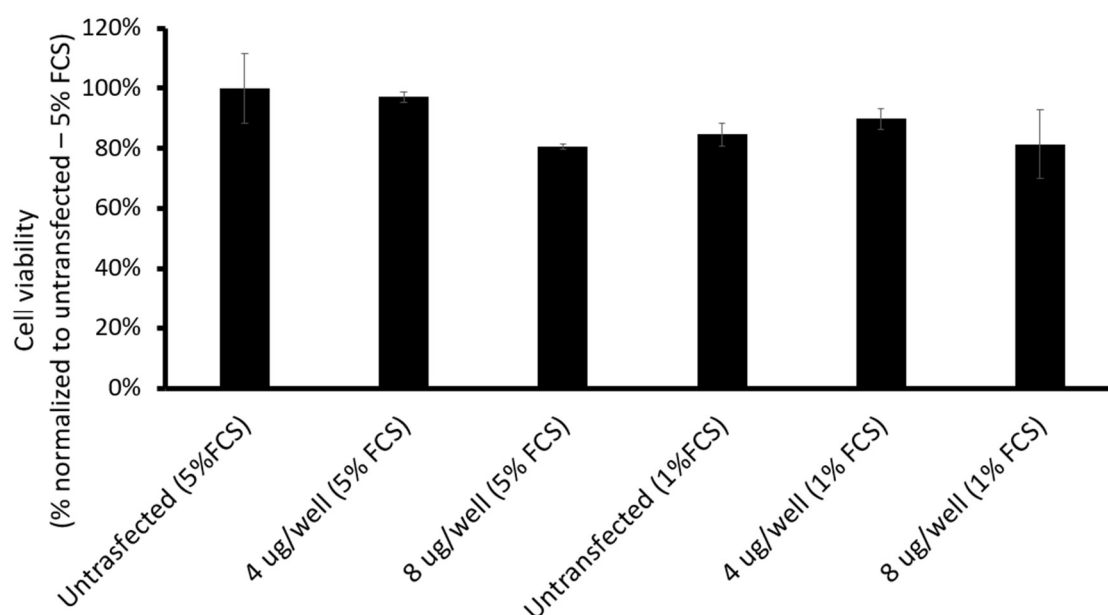


Figure 22. Cell viability of HEK293T cells 24 hours post transfection – 5% vs 1% FCS. The following graph shows % mean cell viability $\pm$ standard deviation of HEK293T cells, 24 hours post transfection as determined by resazurin assay. The cell viability was normalized to untransfected (5% FCS control). The graph compared cell viability after transfection with 4 or 8 μ g of plasmid DNA in 5% or 1% FCS medium. The mean and standard deviation is based of $n = 2$ independent experiments, with each experiment comprising of 3 technical replicates.

7.1.4. Investigating the effect of polyplexing concentration on transfection efficiency

As discussed previously, the polyplexing concentration differed between the 4 μ g and 8 μ g transfections. This means that in comparing these two conditions the polyplexing concentration must be taken into account. It is widely accepted that the protocol of polyplex formation can greatly influence the resultant transfection efficiency. Different factors, such as polymer to DNA ratio, polymer complexity, polymer charge or molecular weight influence the complexation and condensation process, which in turn influence nanoparticle size and number resulting in effected transfection efficiency ⁴⁹. Moreover, the medium in which polyplexes are prepared also has effects, which can be seen in the example of HBS vs. HBG buffer, where HBG buffer resulted in superior transfection efficiency ⁵². Therefore, it seems likely that polyplexing concentration will have an impact on transfection efficiency. Here we hypothesised that increase in polyplexing concentration will results in more efficient complexation and nanoparticle formation, which in turn will lead to superior transfection efficiency without changes in plasmid DNA dosage.

To test this hypothesis, two sets of LPEI polyplexes with EGFR-EGFP plasmid were prepared. The one set was prepared at polyplexing concentration of 20 $\mu\text{g/mL}$, while the other at 80 $\mu\text{g/mL}$. The HEK293T cells were transfected with these polyplexes so that the plasmid DNA dose was 4 μg . FCS was supplied four hours post transfection making the final concentration 5%. The transfection efficiency was measured by flow cytometry.

The results showed no significant difference in transfection efficiency caused by different polyplexing concentrations (**Figure 23a**). While the transfection efficiency reached with 20 $\mu\text{g/mL}$ polyplexes was $\sim 38.5\%$, the transfection efficiency reached with 80 $\mu\text{g/mL}$ polyplexes was little lower at $\sim 36.5\%$. However, this small difference wasn't statistically significant. The difference in measured toxicity by DAPI staining was also minimal (**Figure 23b**). The % of live cells following transfection with 20 $\mu\text{g/mL}$ polyplexes was $\sim 90\%$ and following transfection with 80 $\mu\text{g/mL}$ polyplexes it was $\sim 91\%$. Even though this difference was really small, statistical analysis revealed significance with p-value < 0.05 . Considering the earlier pattern, where lower transfection efficiency followed lower toxicity, it might be that the higher polyplexing concentration results in lower transfection efficiency. Nevertheless, since the differences were really small it seems unlikely that any major benefit can be achieved by decreasing of polyplexing concentration.

Also the distribution of the HEK293T cells according to their EGFP signal didn't reveal a major benefit (**Figure 24**). As in previous experiments, the transfection gave two populations, one with strong EGFP signal and one with weak EGFR signal. Following transfection with 20 $\mu\text{g/mL}$ or 80 $\mu\text{g/mL}$ polyplexes no apparent shift in either of these population, or in proportions of these populations was visible.

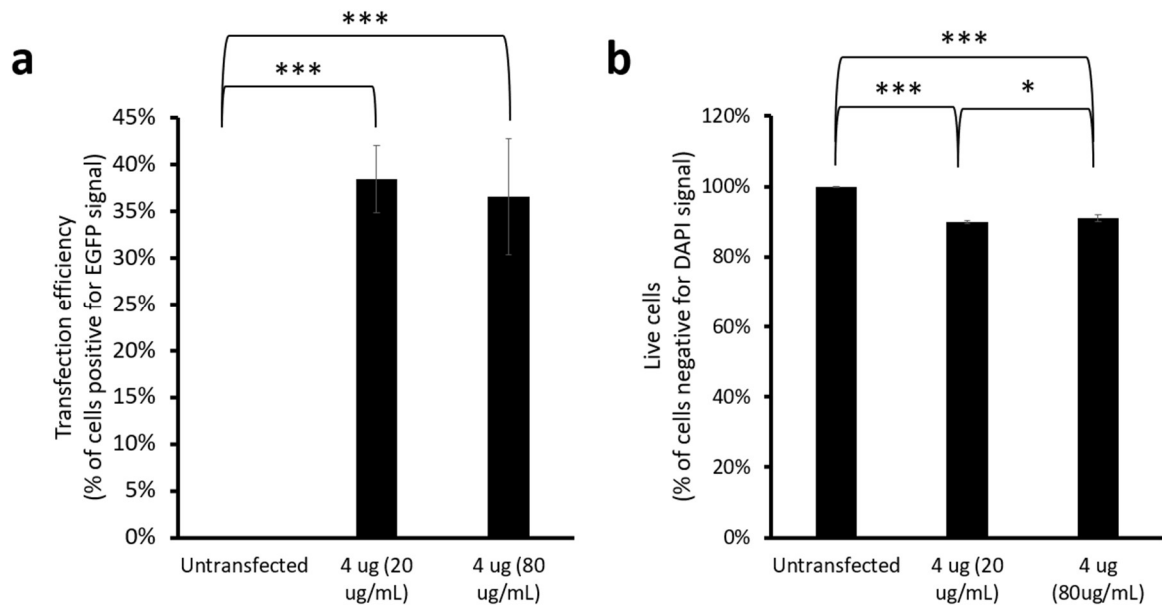


Figure 23. Comparison of transfection efficiency and transfection related toxicity of 20 µg/mL vs. 80 µg/mL polyplexes at the same dosage. This figure compares **a)** transfection efficiency and **b)** transfection related toxicity of 20 µg/mL and 80 µg/mL LPEI polyplexes with EGFR-EGFP plasmid DNA. The cells (HEK293T) were treated with the same dose of plasmid DNA – 4 µg. The measurements were done 24 hours post transfection. Transfection efficiency is shown as a mean % of cells positive for EGFP signal $\pm$ standard deviation. Transfection related toxicity is shown as mean % of live cells (cells negative for DAPI signal) $\pm$ standard deviation. The data were analysed by one-way ANOVA. * = $p < 0.5$; *** = $p < 0.001$. Untransfected $n = 2$ replicates (2 independent experiments); 4 µg (20 µg/mL) $n = 5$ replicates (2 independent experiments); 4 µg (80 µg/mL) $n = 4$ replicates (2 independent experiments). Each replicate had two sample measurements.

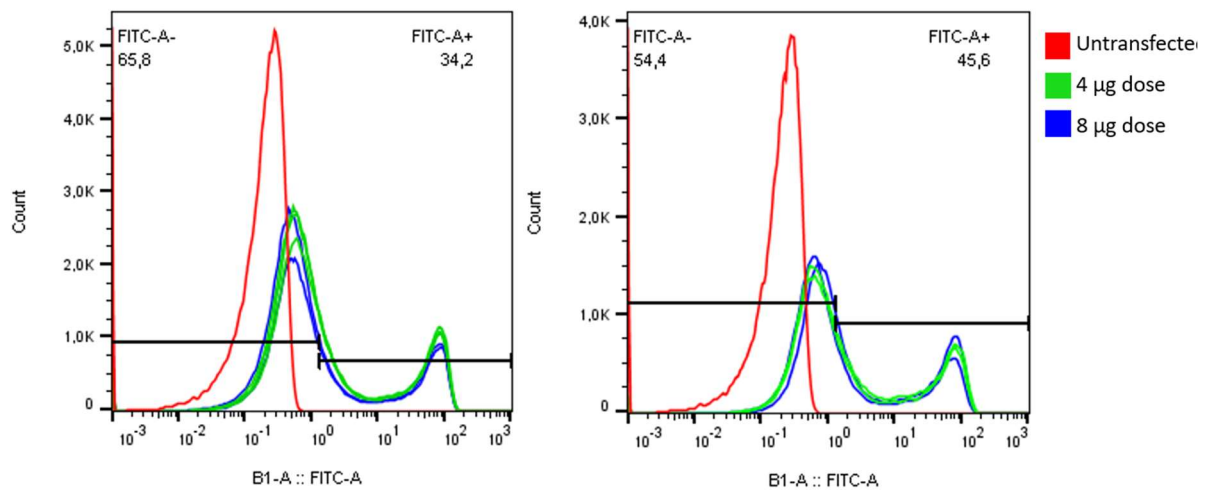


Figure 24. Comparison of distribution of HEK293T population in terms of EGFP fluorescence following transfection with 20 µg/mL or 80 µg/mL polyplexes. These graphs show distribution of HEK293T cells according to their emitting EGFP signal intensity in FITC emission spectrum 24 hours post transfection. Red line shows distribution of untransfected cells. Green line shows cells transfected with polyplexes prepared at 20 µg/mL polyplexing concentration, while blue line shows distribution of cells transfected with 80 µg/mL polyplexes. All transfected cells were transfected with 4 µg of plasmid DNA in 5% FCS concentration. The two graphs shown represent two independent experiments and two measured samples are shown of 4 µg and 8 µg transfections in each graph.

The most interesting result came from the assessment of cell viability through resazurin assay (**Figure 25**). The cell viability decreased with increasing polyplexing concentration. At 20 µg/mL polyplex treatment the cell viability was ~ 86% and at 80 µg/mL polyplex treatment it was ~ 75%. Moreover, this difference exhibited statistical significance. This suggests that the polyplexes prepared at 20 µg/mL and 80 µg/mL are not the same, as a clear biological response can be observed. However, before drawing conclusions on effects of polyplexing concentration, one has to also consider the amount of HBG buffer added to the cells. Naturally, to reach the same dose of plasmid DNA, larger volume of polyplexes in HBG buffer needed to be added. Hence, the differences in cell viability might not be a direct consequence of different polyplex formation caused by different polyplexing concentration, but also the amount of HBG added to the cell can play a part. Nevertheless, this is a very intriguing result, even more so considering the fact that no clear effect on transfection efficiency could be observed and that the toxicity observed in flow cytometry appeared to show a reverse trend.

At this stage it is difficult to formulate hypothesis that could explain this behaviour, as more information is needed, particularly on the properties of polyplexes formed at 20 and 80 µg/mL. One possibility could be that the mitochondrial function, which is also required for resazurin conversion is more impaired after treatment with polyplexes prepared at higher concentrations. It has been found that positively charged polyplexes interact with negatively charged membrane of mitochondria, which impairs their function ⁵⁵. Moreover, the oxygen consumption rate was found to decrease with increasing concentration of PEI ⁵⁶. Therefore, it can be speculated that higher polyplexing concentration leads to formation of polyplexes with higher LPEI content, which subsequently impairs mitochondrial function. Additionally, higher interaction with mitochondria might impair the expression of the EGFR-EGFP construct, resulting in no increase in perceived transfection efficiency. However, as mentioned earlier, more data are necessary in order to draw any conclusions.

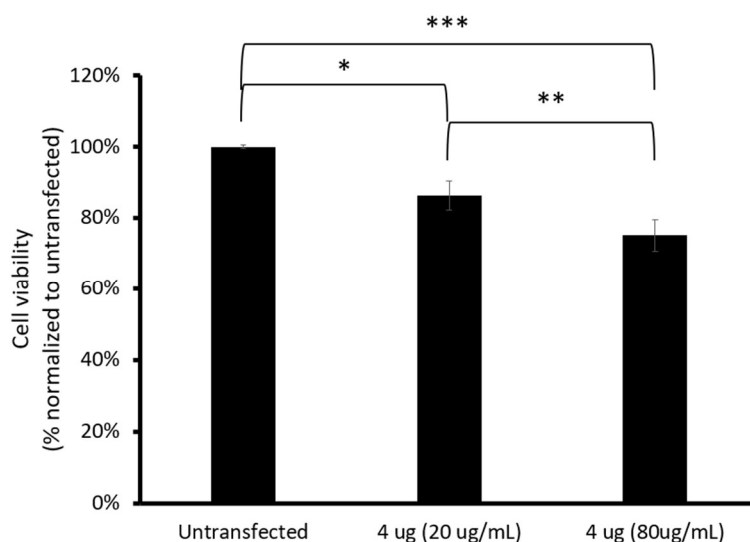


Figure 25. Cell viability of HEK293T cells 24 hours post transfection with 20 $\mu\text{g}/\text{mL}$ or 80 $\mu\text{g}/\text{mL}$ polyplexes. This figure shows HEK293T viability determined by resazurin assay 24 hours after transfection with polyplexes prepared either at 20 $\mu\text{g}/\text{mL}$ or 80 $\mu\text{g}/\text{mL}$. All cells were transfected with the same dose of 4 μg plasmid DNA in 5% FCS concentration. The cell viability is shown as a mean % normalized to untransfected control $\pm$ standard deviation. The data were analysed for statistical significance by one-way ANOVA. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. Untransfected $n = 2$ replicates (2 independent experiments); 4 μg (20 $\mu\text{g}/\text{mL}$) $n = 5$ replicates (2 independent experiments); 4 μg (80 $\mu\text{g}/\text{mL}$) $n = 4$ replicates (2 independent experiments). Each replicate had two sample measurements.

7.2. In vitro transfections with T790M EGFR-EGFP for studying tyrosine kinase inhibitors

One of the main aims was to observe effects of pharmacological inhibition of EGFR receptor. To do this western blot readout with detection of both EGFR and phospho-EGFR is used. Therefore, another key aspect of this thesis was to optimise the EGFR-EGFP transfection for western blot detection, as well as the western blot itself. Moreover, in order to understand the complexity of targeting and inhibition of EGFR, a pharmacologically relevant mutant T790M EGFR is used in transfections, so that the differences in TKI treatment between WT and the T790M version can be observed. To be able to carry out these transfections, expansion of T790M EGFR-EGFP plasmid was another key objective of this thesis.

This chapter first addresses the expansion and validation of the T790M EGFR-EGFP plasmid clone. Subsequently, focus is taken on functional validation in transfection, which was observed by flow cytometry. Finally, optimisation of the western blot transfection parameters is discussed.

7.2.1. Expansion and validation of T790M EGFR-EGFP plasmid

In order to obtain EGFR-EGFP plasmid which carried the T790M mutation, the WT H3 P1 EGFR-EGFP clone expanded in this work was subjected to SDM, which introduced this mutation. The SDM was carried out by Dominik Peischl and more information on this will be available in his upcoming thesis. But briefly, SDM was carried out with two different sets of primers, one commercial set (NE) and one custom designed set by Dominik Peischl (DP), resulting in two different plasmid clones. Moreover, after the SDM, two different KLD reaction times were tried (5 min or 15 min). This gave, in total, four different SDM products.

The SDM products were then expanded in this work. To expand these SDM products, competent bacteria were transformed with these plasmids via heat-shock transformation. Subsequently, successfully transformed bacteria were selected on kanamycin selection plates (**Figure 26**). In total, three runs of SDM and subsequent transformation were done. In the first run, all four transformation reactions with different SDM products yielded colonies, with most colonies appearing on NE15 (commercial primers, 15 min KLD) plate. The second SDM/transformation run was less successful, as only two colonies appeared only on DP15 (custom primers, 15 min KLD) plate. In the third run, the 15 min KLD reaction times were abandoned and therefore only two transformation reactions were carried out, the NE5 (commercial primers, 5 min KLD) and DP5 (custom primers, 5 min KLD). Both of these reactions were successful as multiple colonies appeared on the respective selection plates.

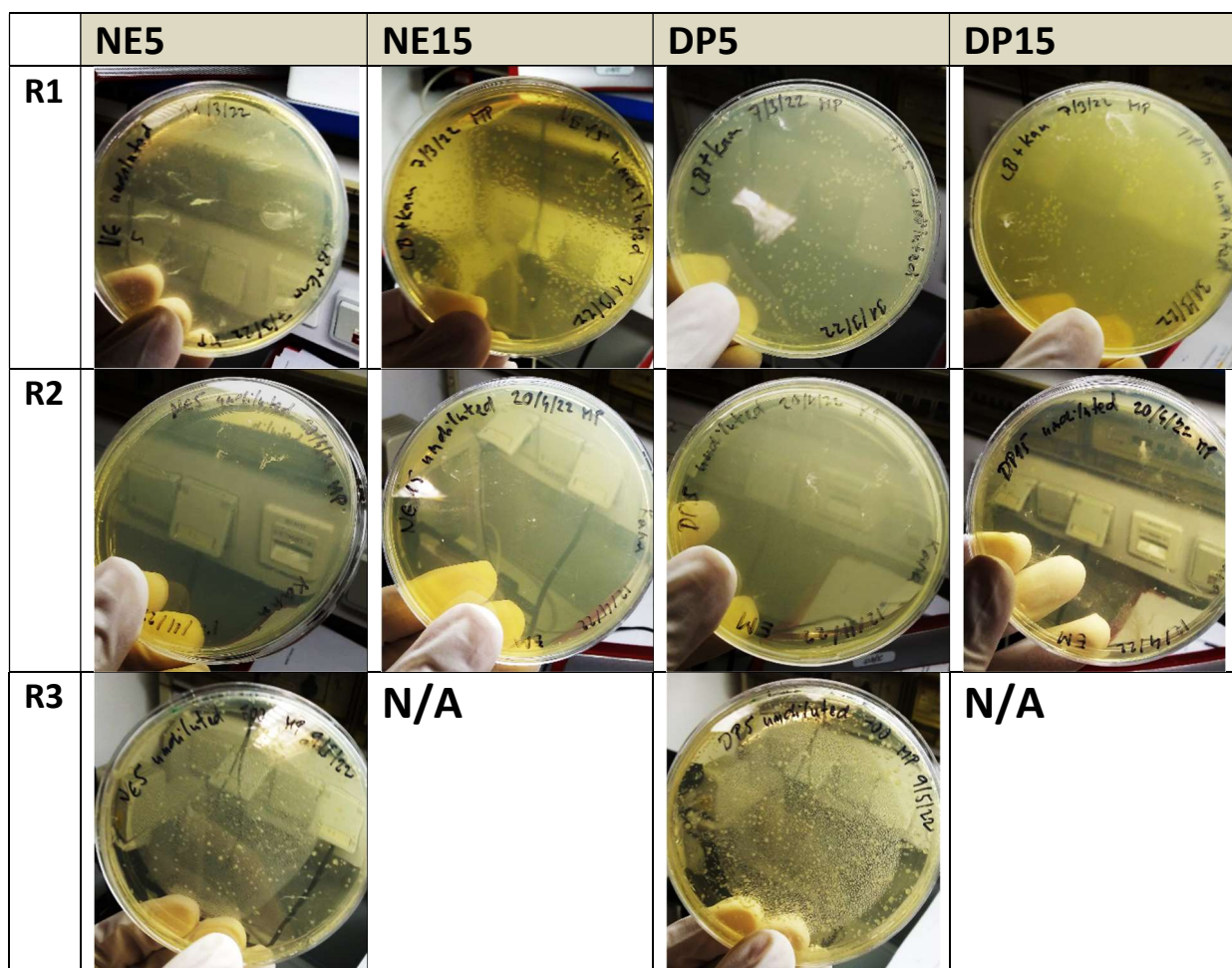


Figure 26. Transformation of competent *E.coli* with T790M EGFR-EGFP plasmid produced by site-directed mutagenesis. This figure shows kanamycin selection agar plates on which competent bacteria were cultured after heat-shock transformation with T790M EGFR-EGFP plasmid that was produced by site-directed mutagenesis. In total, four transformation reactions were carried out (NE5, NE15, DP5 and DP15) in three runs (R1, R2, R3). The plasmids in NE5, NE15, DP5 and DP15 transformation reactions were produced by using different primers for site-directed mutagenesis (NE or DP) and underwent KLD reaction for 5 or 15 minutes. Please note, in run 3 only two transformation reactions (NE5 and DP5) were carried out. N/A = not applicable.

The transformed bacteria from the selection plates were then cultured in liquid pre-culture and O/N culture to obtain suitable growth for subsequent maxiprep isolation of the plasmid. The summary of the cultures can be seen in **Table 14**. In the first run, four cultures were processed, one for each of the SDM/transformation reactions. The total culture time (pre-culture and O/N culture combined) was 21 hours. The OD600 values were high enough to get two pellets for maxiprep extraction from NE5, NE15 and DP5 cultures. The OD600 of the DP15 culture allowed only one pellet for maxiprep extraction. In the second run, the two colonies that appeared on the DP15 selection plate were cultured, resulting in cultures DP15C1 and DP15C2. After total culture time of 22.5 hours, the OD600 values were rather low (1.478 and

3.604 respectively). This allowed one pellet to be obtained for maxiprep extraction from DP15C2 culture. However, it wasn't possible to get one full pellet from the DP15C1 culture due to the low OD600. The two cultures derived from the NE5 and DP5 reactions of the third run (NE5C3 and DP5C3), again reached high OD600s (8.045 and 9.875 respectively). These OD600 values were measured after total culture time of 23 hours. This also allowed two pellets for maxiprep extraction to be prepared from NE5C3 culture and three pellets from the DP5C3 culture.

Table 14. Summary of T790M EGFR-EGFP cultures. This table summarises the cultures of T790M EGFR-EGFP plasmid after transformation. Culture ID refers to respective transformation reaction. From which run of transformation the culture comes from is specified. Measured OD600 after specified pre-culture and overnight (O/N) culture time is shown.

Culture ID	Run	OD600	Pre-culture time (hrs)	O/N culture time (hrs)
NE5	R1	7.065	5	16
NE15	R1	6.875	5	16
DP5	R1	9.375	5	16
DP15	R1	3.945	5	16
DP15C1	R2	1.478	5.5	17
DP15C2	R2	3.604	5.5	17
NE5C3	R3	8.045	6	17
DP5C3	R3	9.875	6	17

The overall yield of the plasmids from maxiprep were very solid (**Table 15**). The average concentration of plasmid DNA obtained from one maxiprep pellet was 698.4 µg/mL, with average yield of 838.1 µg of plasmid DNA. The purities of the plasmid DNA determined by A260/A280 ratio was also good, as it ranged from 1.843 to 1.863. As was the case in WT EGFR-EGFP expansion, the A260/A230 ratio was little higher than generally accepted 2.2 value. Here the ratios ranged from 2.215 to 2.3095. By individual cultures, in the first run the NE5, NE15, DP5 and DP15 cultures yielded 1.804 mg, 1.837 mg, 1.457 mg and 1.488 mg of plasmid DNA respectively (the yield from the two pellets combined). In run 2, the DP15C2 culture yielded 720 µg of plasmid DNA. The NE5C3 and DP5C3 cultures of the run 3 yielded in total 1.926 mg and 2.384 mg of plasmid DNA per culture respectively. Also, an interesting trend emerged in the data, where the NE cultures always yielded better per pellet than the DP cultures, suggesting that some differences in the plasmids produced by the respective primer pairs might exist.

Table 15. Overview of T790M EGFR-EGFP plasmid yields. This table summarises plasmid yields from cultures summarised in table 14. From some of the cultures, multiple pellets for Maxiprep processing were obtained. These are denoted as P followed by number shown in plasmid ID, which refers to culture ID from which the plasmid was purified. For each plasmid, concentration in $\mu\text{g/mL}$, as well as total yield in μg is shown. The purity of obtained plasmids is indicated by A260/A280 and A260/A230 ratios.

Plasmid ID	Run	Concentration ($\mu\text{g/mL}$)	Yield (μg)	A260/A280	A260/A230
NE5 P1	R1	698.5	838.2	1.860	2.3095
NE5 P2	R1	804.5	965.4	1.8575	2.2565
NE15 P1	R1	723.5	868.2	1.860	2.2715
NE15 P2	R1	807	968.4	1.853	2.2665
DP5 P1	R1	595	714	1.8625	2.2625
DP5 P2	R1	619	742.8	1.8505	2.231
DP15 P1	R1	640	768	1.860	2.262
DP15C2	R2	600	720	1.843	2.269
NE5C3 P1	R3	788.5	946.2	1.8465	2.215
NE5C3 P2	R3	816.5	979.8	1.860	2.284
DP5C3 P1	R3	608.5	730.2	1.847	2.2415
DP5C3 P2	R3	700	840	1.859	2.219
DP5C3 P3	R3	678	813.6	1.863	2.302

After the plasmids were isolated, the presence of the T790M mutation was validated by exon 20 PCR and subsequent DRD of the amplified exon 20. This was again done by Dominik Peischl and more detail will be included in his upcoming thesis. The plasmid clones that were positive for the desired mutation were then validated by DRD of the overall plasmid (**Figure 27**). The plasmid clones positive for the mutation were NE5, NE15, DP5 and DP15 from run 1, as well as NE5C3 and DP5C3 from run 2. The DRD revealed that only the NE5, NE15, DP15 and NE5C3 matched the predicted fragment pattern (**Figure 27a and b**). The DP5 and DP5C3 clones both lacked several bands in EcoRI and PvuII restriction reactions. Additionally, the uncut fragments appeared to be shorter than uncut fragments of other plasmid clones. As the missing bands corresponded to restriction sites of EcoRI and PvuII that appear in the region of EGFR part of the plasmid, it seems that the whole EGFR part of the plasmid was lost. Moreover, this DRD result also could partly explain why the yields of the DP clones were lower, as the missing EGFR part would lower the amount of DNA. All in all, the DP5 clones were not to be used in further applications.

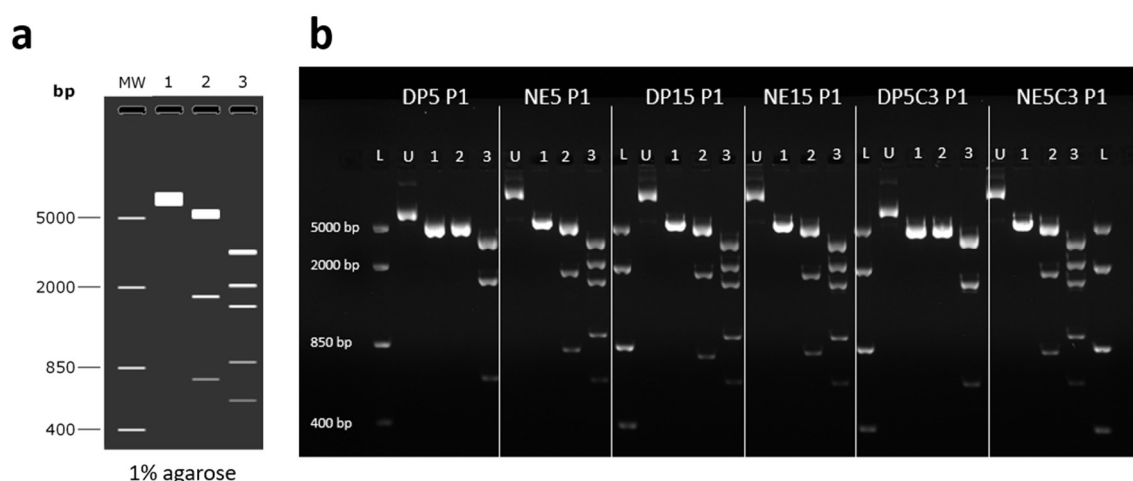


Figure 27. Validation of EGFR-EGFP plasmids positive for T790M mutation by diagnostic restriction digest. **a)** Predicted fragment pattern by simulation of diagnostic restrictive digest in Snap Gene. **b)** Diagnostic restriction digest of EGFR-EGFP plasmids positive for the T790M mutation (DP5 P1, NE5 P1, DP15 P1, NE15 P1, DP5C3 P1 and NE5C3 P1). Labels: MW/L = molecular weight/ladder; U = uncut plasmid; 1 = restriction reaction with BglII; 2 = restriction reaction with EcoRI; 3 = restriction reaction with PvuII.

7.2.2. Comparison of transfection efficiency between parent wild-type EGFR-EGFP clone and multiple T790M EGFR-EGFP clones

The expansion of the T790M EGFR-EGFP yielded four clones that passed validation by DRD and which possessed the T790M mutation. These were the NE5, NE15, DP15 and NE5C3 clones. The next step was to validate these plasmid clones in transfection to assess their transfection efficiency. In theory, since the plasmids should differ only in one nucleotide, which causes the T790M mutation, from the parent WT H3 EGFR-EGFP clone, the transfection efficiency should remain the same. To confirm this, HEK293T cells were transfected with 4 μ g of each of the clones complexed in LPEI polyplexes prepared at 40 μ g/mL polyplexing concentration. The cells were supplied with 1% FCS medium four hours post transfection and the transfection efficiency was measured by flow cytometry ~ 24 hours post transfection.

Surprisingly, the results of the flow cytometry showed that only DP15 clone reached the same level of transfection efficiency as the parent WT H3 clone (**Figure 28a**). Both of these plasmids reached transfection efficiency of ~ 28%. The other plasmids showed transfection efficiency of only 5.3% to 7.5%. The T790M plasmid clones were also compared to T790M EGFR-EGFP plasmid that was already used in B17 course (this plasmid is referred to as B17). This clone reached transfection efficiency of ~ 15%, which was lower than the WT H3 and the DP15 clones, but more importantly, this transfection, as the transfections with H3 and DP15 clones,

resulted in appearance of the cell population emitting strong EGFP signal (**Figure 29b**). This wasn't the case with the other clones, which didn't even cause the shift of the main population (**Figure 29a and b**). With these plasmids, only small bump in the main population at the edge of gate separating EGFP positive and negative cells was visible, which account for the ~ 5 – 7% transfection efficiency. Furthermore, only the WT H3 plasmid and the T790M DP15 plasmid exhibited some level of toxicity (**Figure 28b**). Transfection with these plasmids resulted in ~ 92 – 93% live cells compared to untransfected control, whereas transfection with the other clones resulted in ~ 99 – 100% live cells. Thus, it seems that the transfection didn't really occur. Curiously, transfection with the B17 plasmid also didn't cause toxicity at the level of the WT H3 and T790M DP15 plasmid. This could be explained by the fact, that the population of cells with strong EGFP signal isn't as shifted as the other populations produced by the H3 and DP15 transfections (**Figure 29b**). As mentioned earlier in this thesis, the toxicity occurred only in cells with strong EGFP signal. Hence, it appears that the transfection by B17 plasmid wasn't strong enough to cause this toxicity.

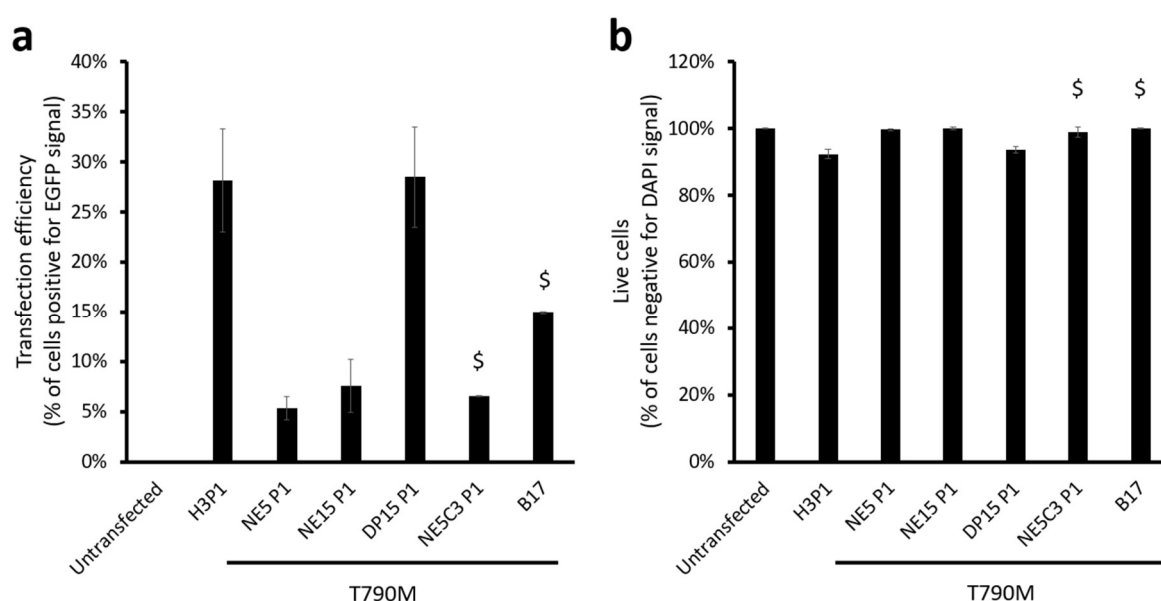


Figure 28. Transfection efficiency and transfection related toxicity of HEK293T cells transfected with different T790M EGFR-EGFP plasmid clones. This figure shows **a)** transfection efficiency reached with different clones of T790M EGFR-EGFP plasmid produced by site-directed mutagenesis, as well as **b)** their transfection related toxicity. Transfection efficiency is represented by mean of % of cells positive for EGFP signal $\pm$ standard deviation. Toxicity is represented by the mean of % of live cells (cells negative for DAPI) $\pm$ standard deviation. The data presented are based on $n = 2$ independent experiments, unless stated otherwise (\$). Each experiment had 2 measurements. The transfection was carried out with 4 μ g pDNA dose (40 μ g/mL polyplexing concentration) and 1% FCS.

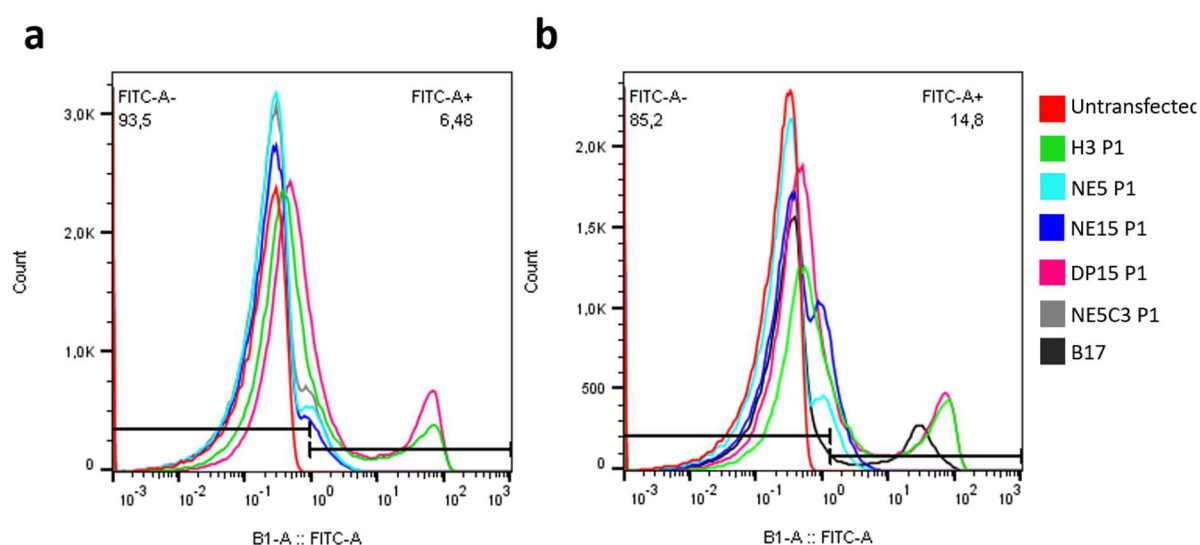


Figure 29. Distribution of HEK293T cell population by intensity of EGFP signal 24 hours post transfection with different T790M EGFR-EGFP clones. This figure shows the distribution of HEK293T cells with respect to emitting EGFP signal recorded in FITC channel 24 hours post transfection with 4 μ g dose of plasmid DNA complexed in LPEI polyplexes (40 μ g/mL). The figure shows two graphs: **a)** experiment A and **b)** experiment B – replicate of experiment A. Colouring scheme: red = untransfected; green = H3 P1; light blue = NE5 P1; dark blue = NE15 P1; purple = DP15 P1; grey = NE5C3 P1; black = B17. Please note: in experiment B, NE5C3 P1 was replaced by B17. Cut-off gate separating EGFP positive from EGFP negative cells is also shown on the graph.

In addition to toxicity measured by flow cytometry, cell viability was assessed by resazurin assay. Following the transfections, there wasn't any apparent decrease in cell viability (**Figure 30**). This is the case even for the strongly transfecting plasmid clones H3 and DP15. As these transfections were carried out in the presence of 1% FCS in the medium, this result is in line with previous observations, where no apparent loss of cell viability could be seen on top of the cell viability already lost due to decrease in FCS concentration (**Figure 22**).

In summary, from all the SDM and subsequent transformation reactions and runs, only one plasmid clone positive for the T790M mutation passed the validation in transfection, the DP15 clone. Therefore, only this plasmid clone was used in further experiments, and glycerol stock of only this clone should be used in further expansions of the T790M EGFR-EGFP plasmid. The fact that the other clones passed the DRD but failed in *in vitro* transfections suggests that, while the overall architecture of the plasmid remained intact, some mutations, most likely frame shifts, were introduced which prevent expression of functional EGFR-EGFP construct. However, in order to fully understand the underlying cause, these plasmid clones would have to be sequenced.

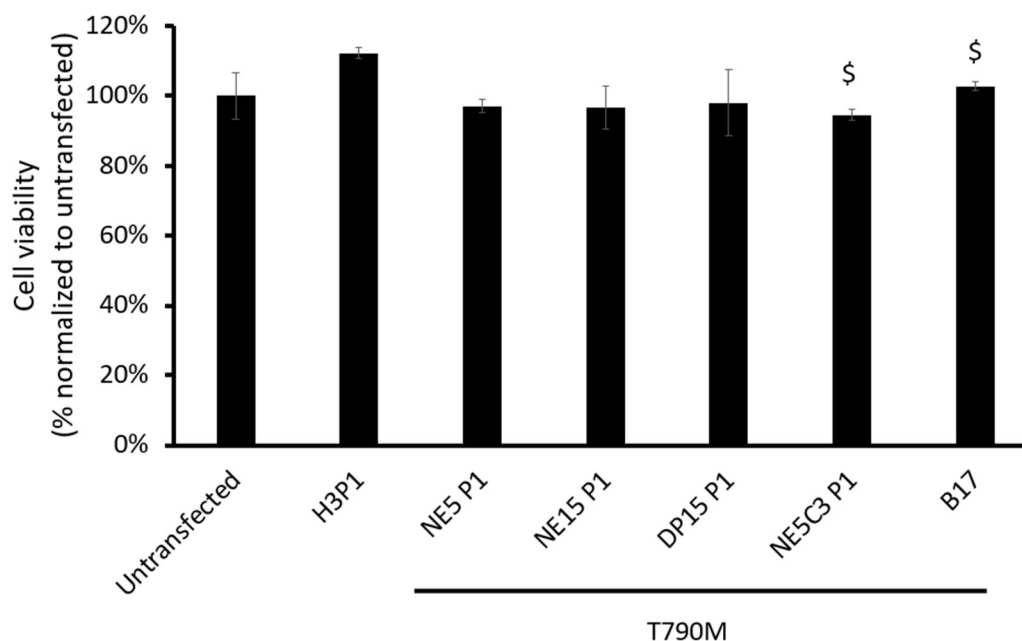


Figure 30. HEK293T cell viability 24 hours post transfection with different clones of T790M EGFR-EGFP plasmid. This figure show the average viability of HEK293T cells 24 hours post transfection with T790M EGFR-EGFP clones specified in the graph. The cells were transfected with 4 μ g of plasmid DNA complexed in LPEI polyplexes (40 μ g/mL). The total FCS concentration in transfection was 1%. The cell viability is shown as mean % normalized to untransfected control $\pm$ standard deviation. The data is based on $n = 2$ independent experiments, unless stated otherwise (\$). Each experiment had 3 measurements.

7.2.3. Optimisation of transfection with WT and T790M EGFR-EGFP for western blot readout

After expansion and validation of the T790M EGFR-EGFP plasmid, the next objective was to optimise the western blot protocol, so that clear effects of TKIs on both WT and T790M EGFR could be observed. The optimisation processes focused on several aspects, namely transfection conditions, hEGF stimulation, TKI treatment, protein sample preparation and antibody dilutions.

For the first western blot experiment, HEK293T cells were transfected with 4 μ g of the WT EGFR-EGFP. Four hours post transfection, FCS was supplied to reach the final FCS concentration of either 5% or 1%. As FCS contains growth factors, this was done to assess background EGFR phosphorylation caused by FCS. Approximately 24 hours post transfection, the phosphorylation of EGFR was stimulated by 30 min incubation in the presence of 1ng/mL hEGF, after which the cells were lysed and protein samples were prepared for the western blot, where phospho-EGFR was detected.

As can be seen in **Figure 31**, the phospho-EGFR protein appeared as smear on the membrane, rather than defined bands. This can be quite common occurrence with membrane proteins such as EGFR, as they tend to aggregate upon boiling ⁵⁷, which is one of the steps in preparation of the sample for SDS-PAGE separation. In this experiment, the protein sample was boiled at 95°C for 10 min. Therefore in further iterations it was decided to lower the boiling time to 5 min. In terms of signal intensity, there was no phospho-EGFR signal detected in untransfected samples. Some background signal was visible in both 5% and 1% FCS samples, with no hEGF stimulation, where the 5% FCS phospho-EGFR signal appeared to be stronger. The hEGF stimulation caused a marked increase in intensity of the phospho-EGFR signal, and again, the sample where cells were transfected in 5% FCS was stronger in intensity. Hence, in order to minimise background activation of EGFR by growth factors present in FCS, further iterations of transfections for wester blot were done in presence of 1% FCS medium.

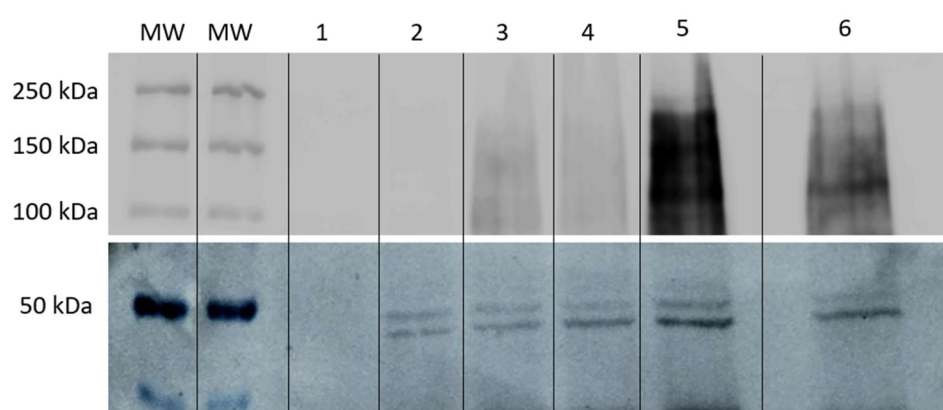


Figure 31. Phospho-EGFR visualised by western blot following unoptimized sample preparation. HEK293T cells were transfected with 4 μ g of EGFR-EGFP plasmid. 24 hours post transfection, the cells were stimulated with hEGF. This figure shows phospho-EGFR signal (top) on western blot, following unoptimized protocol. The protein sample was cooked for 10 min at 95°C prior the western blot. As a loading control, α/β -tubulin was detected (bottom). Labels: **MW** = molecular weight; **1** = untransfected cells, 5% FCS; **2** = untransfected cells, 1% FCS; **3** = 4 μ g EGFR-EGFP, 5% FCS, no hEGF; **4** = 4 μ g EGFR-EGFP, 1% FCS, no hEGF; **5** = 4 μ g EGFR-EGFP, 5% FCS, 1 ng/mL hEGF (30 min); **6** = 4 μ g EGFR-EGFP, 1% FCS, 1 ng/mL hEGF (30 min). For all samples, 20 μ g of protein was loaded on SDS-PAGE gel.

In the next experiments, WT EGFR was compared to T790M EGFR. For the T790M transfection, cells were transfected with either NE5C3 clone or DP15 clone of the T790M EGFR-EGFP plasmid. Also, as low toxicity was shown in flow cytometry experiments, the dose was increased to 8 μ g. To decrease the background EGFR phosphorylation, the FCS concentration was lowered to 1% as mentioned previously. Furthermore, lowering hEGF stimulation time to 10 min and concentration to 0.1 ng/mL was tried. If sufficient stimulation was achieved with

these conditions, this could save considerable time and resources. Increasing antibody dilution ratios was also tried. Finally, the protein boiling time was reduced as discussed previously.

First of all, the reduction of boiling time helped significantly to reduce the smears, as both phospho-EGFR and EGFR appeared as more defined bands (**Figure 32 and 33**). Therefore, boiling time of 5 min at 95°C is recommended. Secondly, no EGFR (or phospho-EGFR) signal could be detected in cells transfected with NE5C3 T790M clone (**Figure 32**). This further supports the results of the flow cytometry, where the transfection efficiency with this plasmid clone was very low. Moreover, since no EGFR signal could be detected, this means that expression of the whole EGFR-EGFP construct is faulty and the lack of green fluorescent signal in flow cytometry cannot be only attributed to fault in EGFP part of the protein. On the other hand transfection with the DP15 T790M clone resulted in nice expression of the EGFR (**Figure 33**). There was still some background activation of the EGFR visible also in 1% FCS (**Figures 32 ad 33, lanes 2 and 8**). Therefore, 5% FCS concentrations are not recommended for transfections for this kind of western blot experiment, even though the cell viability and transfection efficiency were higher in flow cytometry readouts. Nonetheless, the opportunity to increase the dose of the plasmid DNA to 8 µg counteracts the loss of transfection efficiency to lower FCS concentration. In terms of hEGF stimulation, there wasn't any visible difference between incubation with 1 ng/mL hEGF for 30 min and incubation with 0.1 ng/mL hEGF for 10 min (**Figure 32 and 33**). Hence, the lower concentration and incubation time seemed to be sufficient for EGFR activation and phosphorylation and therefore could be used for purposes of visualising this effect.

Another aspect of the western blot experiment were the antibodies and their dilution factors. All antibodies were diluted in 5% BSA in TBST. Increasing the antibody dilution ratio was tried to achieve nicer signal as well as to save resources. In **Figure 32**, anti-EGFR and anti-pEGFR antibodies were diluted in 1:2000 ratio and the secondary anti-mouse (for EGFR) and anti-rabbit (for phospho-EGFR) antibodies were diluted in 1:5000 and 1:10 000 ratio respectively. In **Figure 33**, the dilution factor for primary antibodies stayed the same, but the secondary antibodies dilution factor was increased to 1:10 000 and 1:20 000 for each respective antibody. As can be seen this increase in ratio still resulted in nice signal and also the bands appeared to be more refined. Thus these dilution factors can be used in further experiments. On a side note, if possible, it is recommended to use freshly prepared dilutions of antibodies,

as reusing antibody dilutions can result in unspecific binding, as demonstrated by tubulin signal in **Figure 32**. When the tubulin was detected with freshly prepared antibody dilution, the unspecific binding disappeared (**Figure 33 and 34**).

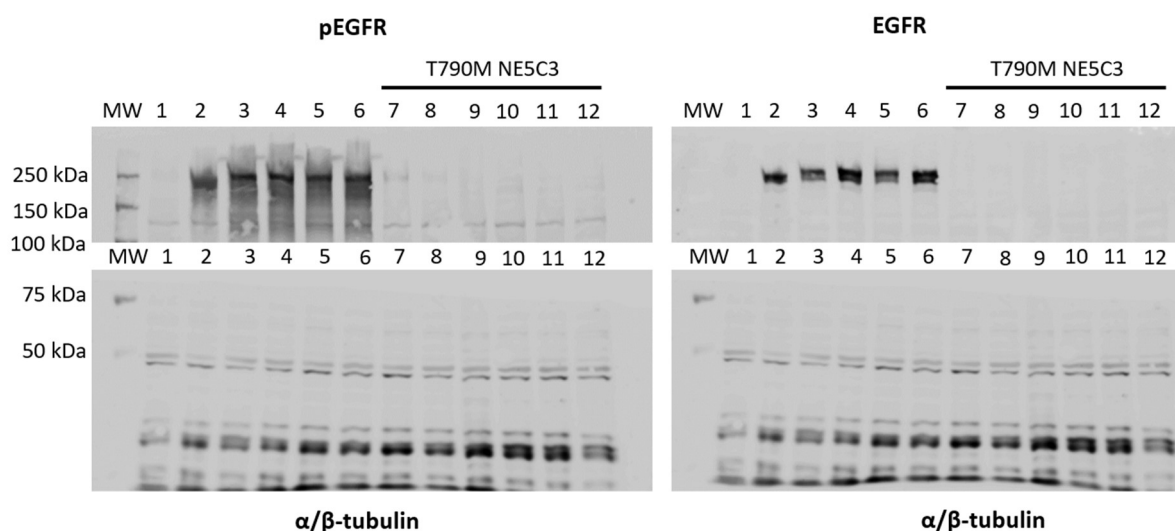


Figure 32. Western blot readout comparing transfection with WT EGFR-EGFP and NE5C3 clone of T790M EGFR-EGFP. In this western blot experiment, HEK293T cells were transfected with 8 μ g WT or NE15C3 T790M EGFR-EGFP in 1% FCS. 24 hours post transfection the cells were stimulated with 0.1 ng/mL or 1 ng/mL hEGF for 10 or 30 min. The cooking time of the protein sample was reduced to 5 min. On the top left, phospho-EGFR signal is shown, while on the top right, EGFR signal is shown. 20 μ g of protein sample was loaded to the SDS-PAGE gel. As a loading control, α/β -tubulin was detected (bottom images - ~ 50 kDa mark). The figure also shows unspecific binding detected by the tubulin antibodies. Labels: **MW** = molecular weight; **1** = untransfected; **2** = WT EGFR, no hEGF; **3** = WT EGFR, 1 ng/mL hEGF (30 min); **4** = WT EGFR, 1 ng/mL hEGF (10 min); **5** = WT EGFR, 0.1 ng/mL hEGF (30 min); **6** = WT EGFR, 0.1 ng/mL hEGF (10 min); **7** = untransfected; **8** = T790M EGFR, no hEGF; **9** = T790M EGFR, 1 ng/mL hEGF (30 min); **10** = T790M EGFR, 1 ng/mL (10 min); **11** = T790M EGFR, 0.1 ng/mL hEGF (30 min); **12** = T790M EGFR, 0.1 ng/mL hEGF (10 min).

After the above optimisation of western blot parameters, the effect of TKI treatments was observed. HEK293T cells were transfected with WT and T790M EGFR-EGFP as per above mentioned conditions. Four hours after the transfection, together with FCS addition, the cells were treated with either gefitinib or Osimertinib at 0.1 μ M or 1 μ M concentration. The following day EGFR phosphorylation was stimulated by 10 min incubation with 0.1 ng/mL hEGF.

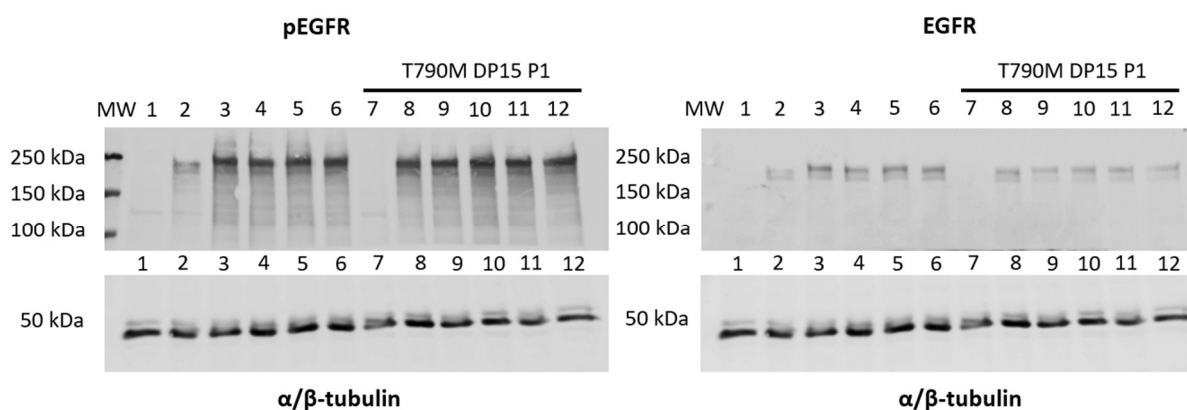


Figure 33. Western blot readout of transfection with WT EGFR-EGFP and DP15 clone of T790M EGFR-EGFP. In this experiment, HEK293T cells were transfected with 8 μ g of WT EGFR-EGFP or DP15 clone of T790M EGFR-EGFP in 1% FCS. The cells were stimulated with hEGF 24 hours post transfection. The concentration of hEGF used was either 0.1 ng/mL or 1 ng/mL. The duration of hEGF stimulation was 10 min or 30 min. Top left image shows phospho-EGFR signal. Top right image shows EGFR signal. Bottom images show α/β -tubulin signal, which was used as a loading control (20 μ g of protein was loaded onto the SDS-PAGE gel). Fresh anti- α/β -tubulin antibody dilution was used resulting in loss of unspecific binding. Labels: **MW** = molecular weight; **1** = untransfected; **2** = WT EGFR, no hEGF; **3** = WT EGFR, 1 ng/mL hEGF (30 min); **4** = WT EGFR, 1 ng/mL hEGF (10 min); **5** = WT EGFR, 0.1 ng/mL hEGF (30 min); **6** = WT EGFR, 0.1 ng/mL hEGF (10 min); **7** = untransfected; **8** = T790M EGFR, no hEGF; **9** = T790M EGFR, 1 ng/mL hEGF (30 min); **10** = T790M EGFR, 1 ng/mL (10 min); **11** = T790M EGFR, 0.1 ng/mL hEGF (30 min); **12** = T790M EGFR, 0.1 ng/mL hEGF (10 min).

The results of the western blot clearly demonstrated the inhibitory effects of the TKIs (**Figure 34**). The intensity of the WT phospho-EGFR signal visibly decreased following the treatments with gefitinib and Osimertinib, while only Osimertinib decreased the intensity of T790M phospho-EGFR signal. This behaviour is expected, as the T790M mutation renders the EGFR protein resistant to inhibition caused by first generation TKIs, such as gefitinib, but not to third generation TKIs, such as Osimertinib. This result is also significant from the aspect, that it demonstrated functionally the presence of the T790M mutation introduced by the SDM. Therefore, the DP15 clone can be used for future expansions of the T790M EGFR-EGFP plasmid. Coming back to the inhibition by the TKIs, a dose dependent relationship could also be visually seen, as the phospho-EGFP signal appeared to be gradually weaker with increasing the TKIs concentration from 0.1 μ M to 1 μ M. For the purposes of demonstration of TKIs effects, it would certainly be beneficial to use both concentrations to show this dose dependent effect.

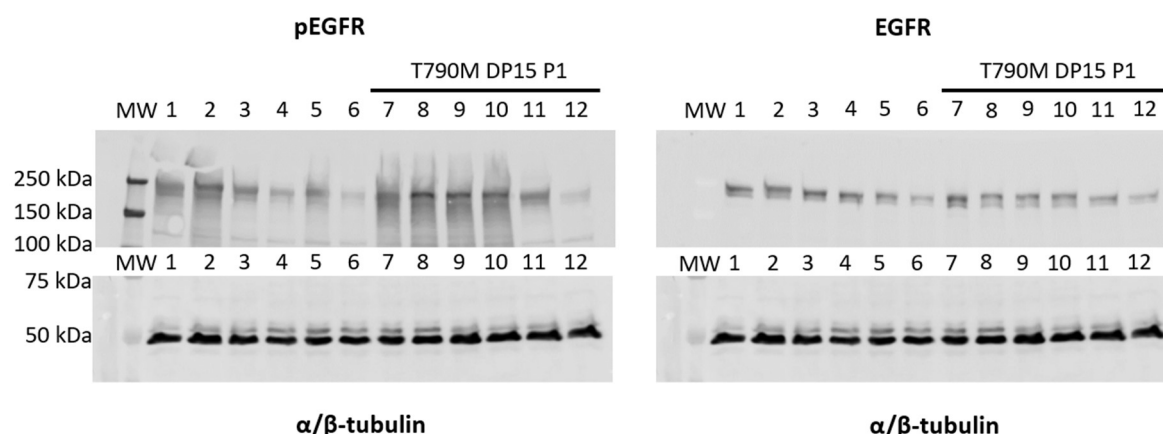


Figure 34. Inhibition of EGFR phosphorylation by gefitinib and Osimertinib. This figure shows the effects of two TKIs, gefitinib and Osimertinib, on phosphorylation of EGFR via western blot. HEK293T cells were transfected with 8 µg of WT or T790M EGFR-EGFP plasmid. After 4 hours, the cells were treated with 0.1 µM or 1 µM gefitinib or Osimertinib. DMSO was used as a control treatment. 24 hours after transfection, the cells were stimulated with 0.1 ng/mL hEGF for 10 min. The cells were lysed and the lysates were analysed by western blot. Top left image shows phospho-EGFR signal, while top right image shows EGFR signal. Bottom images show α/β-tubulin signal, which was used as a loading control (20 µg of protein for all samples was loaded onto the SDS-PAGE gel). Labels: **MW** = molecular weight; **1** = WT EGFR-EGFP, no hEGF stimulation, DMSO control; **2** = WT EGFR-EGFP, 0.1 ng/mL hEGF (10 min), DMSO control; **3** = WT EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 0.1 µM gefitinib; **4** = WT EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 1 µM gefitinib; **5** = WT EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 0.1 µM Osimertinib; **6** = WT EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 1 µM Osimertinib; **7** = T790M EGFR-EGFP, no hEGF stimulation, DMSO control; **8** = T790M EGFR-EGFP, 0.1 ng/mL hEGF (10 min), DMSO control; **9** = T790M EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 0.1 µM gefitinib; **10** = T790M EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 1 µM gefitinib; **11** = T790M EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 0.1 µM Osimertinib; **12** = T790M EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 1 µM Osimertinib.

7.3. Expansion and functional validation of best performing WT and T790M EGFR-EGFP clones

The final objective of this work was to demonstrate reproducibility of the optimised parameters in the previous sections. In order to do this, the WT and T790M EGFR-EGFP clones that were used in previous steps were expanded from their respective glycerol stocks to obtain new clones for the final validation. This expansion was done also to identify these glycerol stocks as suitable for further expansions. Moreover, in parallel to maxiprep expansion, gigaprep expansion was set up, so that large amounts of plasmid DNA could be obtained.

The overview of the expansion can be found in **Table 16**. The H3G2 culture is derived from the H3 culture from which the main WT EGFR-EGFP plasmid clone was obtained. On the other hand the DP15G2 culture is derived from the DP15 culture, from which the working DP15 T790M EGFR-EGFP plasmid was obtained. As mentioned, in parallel to maxiprep culture, a

gigaprep culture was set up. One gigaprep culture consisted of 4x 350 mL cultures and therefore the OD600 value shown in **Table 16** is shown as a range. The maxiprep and gigaprep cultures were all inoculated from the same respective preculture, therefore the plasmids obtained from maxiprep and gigaprep should be identical. The OD600 value of the O/N H3G2 maxiprep culture reached 9.11. This was after total culture time of 23.5 hours (pre-culture and O/N culture combined). The parallel gigaprep culture had OD600 range of 5.77 – 9.08. As a result two pellets were obtained for maxiprep isolation (each pellet prepared from 82 mL of culture) and one pellet for gigaprep isolation (1.4 L of culture) was prepared. The DP15G2 maxiprep culture reached OD600 of 7.1 after total culture time of 23 hours and thus also two pellets for maxiprep isolation were obtained (one pellet made of 105 mL of culture). The parallel gigaprep cultures had an OD600 range of 4.67 – 6.68 and one pellet from the total volume of 1.4 L was prepared for the gigaprep isolation.

Table 16. Overview of cultures in expansion of WT and T790M EGFR-EGFP clones. This table summarises the expansion of best clones of WT and T790M EGFR-EGFP plasmid. The WT H3G2 culture was derived from H3 clone, while the T790M DP15G2 culture was derived from DP15 clone. OD600 is shown of the O/N cultures. For H3G2 (Giga) and DP15G2 (Giga) a range of OD600 s shown, as these were cultured in multiple flasks. The Maxi and Giga O/N cultures for H3G2 and DP15G2 were derived from the same pre-culture. The total pre-culture and O/N culture times are shown as well.

Culture ID	WT/T790M	OD600	Pre-culture time (hrs)	O/N culture time (hrs)
H3G2 (Maxi)	WT	9.11	6	17.5
H3G2 (Giga)	WT	5.77 – 9.08	6	17.5
DP15G2 (Maxi)	T790M	7.1	6	17
DP15G2 (Giga)	T790M	4.67 – 6.68	6	17

In this work, only the maxiprep isolation was carried out to immediately test the expanded plasmids. The pellets prepared for the gigaprep isolation were stored at -20°C, until they are needed for future work. The obtained yields of the plasmids from maxiprep can be seen summarised in **Table 17**. The measured concentrations of the plasmid eluates were reasonably high and in the range of previous expansions of the same plasmid. The total yield of the WT H3G2 EGFR-EGFP plasmid was 1.438 mg (both pellets combined). The total yield of the T790M DP15G2 EGFR-EGFP plasmid was somewhat higher at 1.795 mg. The A260/A280 ratio showed good purity achieved in all maxiprep isolations with values of 1.8505 and 1.8515 for WT and 1.8475 and 1.8495 for T790M plasmid clones. Again, as in previous expansions the A260/A230 ratio was higher as expected with values ranging from 2.2575 to 2.322. However,

as no issue was detected in previous experiments carried out with plasmids with similar A260/A230 ratios, this doesn't seem to pose a big problem.

Table 17. Summary of plasmid yields from the expansion of best working clones. In this table, yield of plasmids from the cultures shown in Table 16 obtained by Maxiprep extraction are shown. The plasmid ID refers to respective culture ID (H3G2 P1/P2 derived from H3G2 (Maxi) and DP15G2 P1/P2 derived from DP15G2 (Maxi)). Whether the plasmid is WT or contains T790M mutation is specified. The concentration and total yield is shown, as well as the A260/A280 and A260/A230 markers of purity.

Plasmid ID	WT/T790M	Concentration (µg/mL)	Yield (µg)	A260/A280	A260/A230
H3G2 P1	WT	587	704.4	1.8515	2.284
H3G2 P2	WT	611.5	733.8	1.8505	2.316
DP15G2 P1	T790M	728	873.6	1.8475	2.2575
DP15G2 P2	T790M	767.5	921	1.8495	2.322

After isolation of the plasmids, the overall plasmid identity was checked by DRD. The DRD was carried out in the same way as in previous EGFR-EGFP expansions. The resulting fragment pattern matched the predicted fragment pattern for both the WT and T790M plasmids (**Figure 35**). There were two additional bands appearing in the DP15G2 restriction reactions with EcoRI. However, these were most likely a result of star activity of the enzyme described earlier as the bands matched the previously described bands and were comparatively weaker in intensity. Thus the identity of the plasmid seemed to be confirmed.

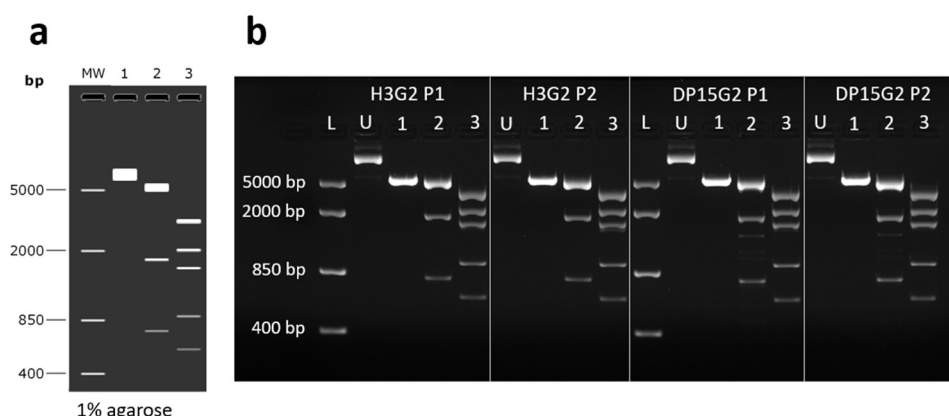


Figure 35. Validation of expanded WT and T790M EGFR-EGFP plasmid by diagnostic restriction digest. a) Predicted fragment pattern by simulation of restriction digest in SnapGene software. **b)** Diagnostic restriction digest of WT (H3G2 P1/P2) and T790M (DP15G2 P1/P2) EGFR-EGFP plasmids that were expanded from the clones used throughout this work. Labels: L = ladder; U = uncut; 1 = restriction reaction with BglII; 2 = restriction reaction with EcoRI; 3 = restriction reaction with PvuII.

Once the plasmids were validated by DRD, final *in vitro* transfections experiments were carried out to confirm functionality of these plasmids. The transfection efficiency was determined by

flow cytometry. HEK293T cells were transfected with 4 μ g or 8 μ g of WT or T790M EGFR-EGFP plasmid as previously. The FCS concentration was 5%. The results showed transfection efficiency of $\sim$ 26% at 4 μ g dose for both plasmids and $\sim$ 37% and $\sim$ 35% at 8 μ g dose for WT and T790M plasmid respectively (**Figure 36a**). While the transfection efficiency for the T790M plasmid at 4 μ g was similar to that of the parent plasmid clone (**Figure 28a**), the transfection efficiency of the WT was lower than the mean transfection efficiency reached with its parent clone (**Figure 19a**). Nevertheless, it was still within the range of measured transfection efficiencies with its parent H3 plasmid clone (**Figure 28a**). Moreover, the trend of $\sim$ 10% increase in transfection efficiency upon raising the dose to 8 μ g was maintained. Also, the distribution of HEK293T cells according to the intensity of the emitted EGFP signal closely resembled the previous experiments (**Figure 37**). One possible explanation for the decrease in transfection efficiency could be the age of the cells. While in the original transfections with the WT H3 P1 plasmid the passages of HEK293T cells were between p43 to p49, the passage of HEK293T cells used in this experiment was p65.

In terms of toxicity, there were between 91% and 92% live cells after transfection with 4 μ g of the plasmid DNA for both WT and T790M plasmid and between 87% and 88% live cells after transfection with 8 μ g of the plasmid DNA (**Figure 36b**). This level of toxicity is again on par with the previous experiments as it fall within the range of previously measured values. Therefore, these results showed the reproducibility also with new clones of both WT and T790M EGFR-EGFP plasmid DNA. These results also support the low toxicity of the polyplex transfection, as well as the connection between transfection efficiency and toxicity observed, where higher transfection efficiency means also higher toxicity. Since the toxicity appears predominantly in the population of cells emitting strong EGFP signal, one can hypothesise that high level of EGFR-EGFP expression leads to disruption of the plasma membrane which leads to death of the cell.

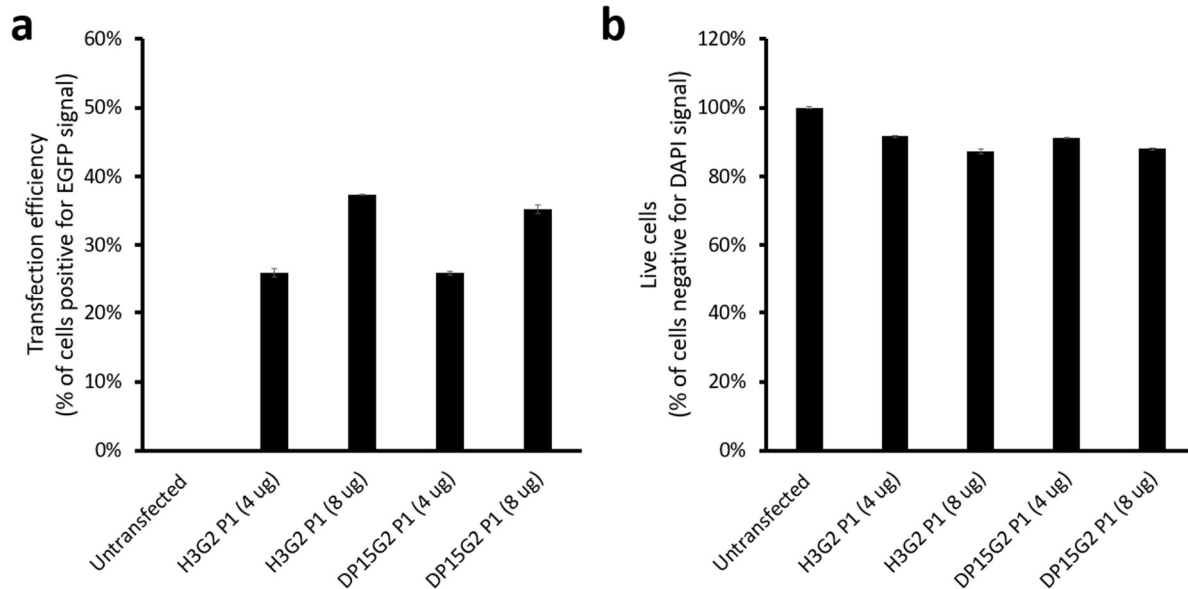


Figure 36. Transfection efficiency and transfection related toxicity of newly expanded plasmid clones. This figure presents the **a)** transfection efficiencies and **b)** transfection related toxicity of WT (H3G2 P1) and T790M (DP15G2 P1) EGFR-EGFP plasmid clones. The transfected cells were HEK293T cells and the parameters were measured ~ 24 hours post transfection. The cells were transfected with either 4 or 8 μg of respective plasmid DNA complexed in LPEI polyplexes. The polyplexing concentrations were 40 and 80 $\mu\text{g}/\text{mL}$ respectively for each dose. Four hours post transfection, complete medium was added to the cells, so the final FCS concentration was 5%. The transfection efficiency is shown as mean % of live cells positive for EGFP signal measured in FITC spectrum $\pm$ standard deviation. The transfection related toxicity is shown as mean % of live cells (cells negative for DAPI signal) $\pm$ standard deviation. The data shown in this figure is based on $n = 1$ biological replicate, with $n = 2$ technical replicates.

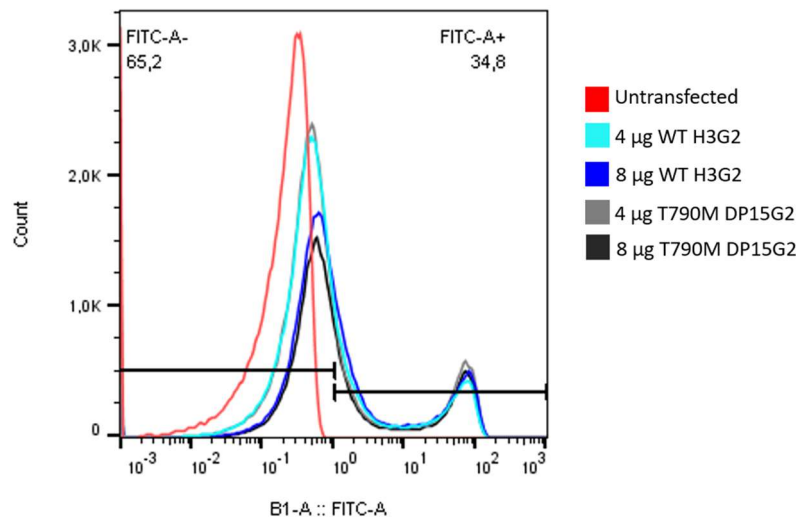


Figure 37. Distribution of HEK293T cells in respect to the intensity of EGFP signal measured in FITC spectrum 24 hours post transfection with newly expanded EGFR-EGFP plasmid clones. The distribution of HEK293T cells emitting EGFP signal recorded in FITC spectra is shown in this figure. The cells were transfected with either 4 or 8 μg of WT or T790M EGFR-EGFP plasmid DNA complexed in LPEI polyplexes. The polyplexing concentration was 40 or 80 $\mu\text{g}/\text{mL}$ for each respective dose. Four hours after transfection FCS was supplied to reach final 5% FCS concentration. The measurement was done ~ 24 hours post transfection. Colour code: Red = untransfected; Light blue = 4 μg of WT H3G2 P1; Dark blue = 8 μg of WT H3G2 P1; Grey = 4 μg of T790M DP15G2 P1; Black = 8 μg of T790M DP15G2 P1.

When it comes to cell viability, the results of resazurin assay showed a dose dependent relationship (**Figure 38**). At 4 μg dose, the cell viability stayed practically at the same level with untransfected control, as the cell viability was between 99% and 100%. After transfection with 8 μg of the plasmid DNA the cell viability dropped to 82% and 84% for WT and T790M EGFR-EGFP respectively. This dose dependent decrease in cell viability is in agreement with previous transfections, which were carried out in 5% FCS medium. Although, cell viability was observed to decrease more after 4 μg treatment previously, this still appears to be within range of experimental variation, as in previous experiments the cell viability in 5% FCS after 4 μg dose moved between 91% and 98%, with standard deviations of up to 6.5%. And since the values in **Figure 38** are based on one biological replicate, one has to rely more on observed trends rather than on absolute values in such case. Thus, the cell viability shown in **Figure 38** is a good representation of results that can be expected of single one experiment.

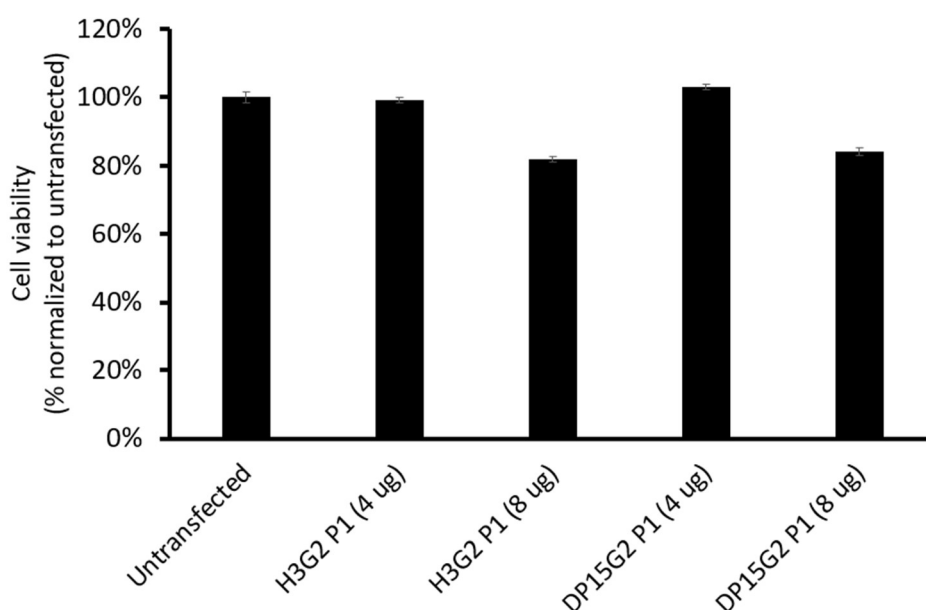


Figure 38. HEK293T viability 24 hours post transfection with newly expanded WT and T790M EGFR-EGFP clones. This graph shows the cell viability as determined by resazurin assay 24 hours post transfection with 4 or 8 μg of WT (H3G2 P1) or T790M (DP15G2 P1) plasmids complexed in LPEI polyplexes (40 $\mu\text{g}/\text{mL}$ or 80 $\mu\text{g}/\text{mL}$ respectively). 4 hours post transfection, complete medium was supplied to the cells so the final FCS concentration would be 5%. The cell viability is shown as mean % viable cells normalized to untransfected control $\pm$ standard deviation. The values are based on one biological replicate with three technical replicates.

The final step was to try the newly expanded plasmids in western blot experiment. The experiment was conducted according to the optimised parameters introduced in previous section of this thesis. The HEK293T cells were transfected with 8 μg of both WT and T790M

plasmid and four hours post transfection FCS was added to reach 1%. The cells were treated with 0.1 μ M and 1 μ M gefitinib or Osimertinib. EGFR activation was stimulated with 0.1 ng/mL hEGF (10 min incubation). The results are presented in **Figure 39**. Examination of the phospho-EGFR signal revealed the expected pattern of EGFR activation and inhibition by TKIs. The fact that gefitinib failed to inhibit activation of T790M EGFR further confirmed the identity of the expanded plasmids. Therefore, the stocks newly expanded plasmids as well as the stocks of their parent plasmids can be used in further expansions and further experiments, as results presented in this section validated the identity and functionality of these plasmids. Unfortunately, either the protein transfer to nitrocellulose membrane or the incubation with anti-EGFR and anti-tubulin antibodies was ineffective in this last experiment, as the signals of both EGFR and α/β -tubulin was quite low. Nevertheless, the phospho-EGFR signal still showed quite clear trend, indicating the reproducibility of the results.

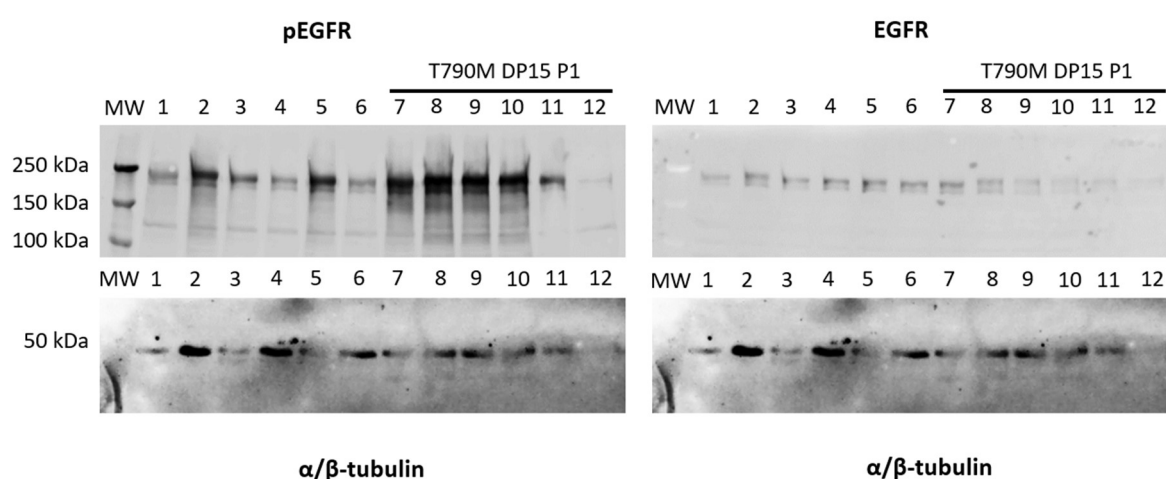


Figure 39. Validation of transfection with new plasmid clones via western blot. This figure shows western blot readout of transfection of HEK293T with newly expanded WT (H3G2) and T790M (DP15G2) EGFR-EGFP clones. As usual, the HEK293T cells were transfected with 8 μ g of plasmid DNA complexed in LPEI polyplexes at 80 μ g/mL polyplexing concentration. Four hours after transfection complete medium was added to reach final 1% concentration. Moreover, the cells were treated with 0.1 μ M or 1 μ M gefitinib and Osimertinib to visualise functionality of the T790M mutation. The phosphorylation of EGFR was achieved by stimulation with 0.1 ng/mL hEGF for 10 min. Top left image shows phospho-EGFR signal. Top right image shows EGFR signal. Bottom images show α/β -tubulin signal, which was detected for purposes of loading control. Total of 20 μ g of protein per each sample was loaded onto the gel. Labels: **MW** = molecular weight; **1** = WT EGFR-EGFP, no hEGF stimulation, DMSO control; **2** = WT EGFR-EGFP, 0.1 ng/mL hEGF (10 min), DMSO control; **3** = WT EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 0.1 μ M gefitinib; **4** = WT EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 1 μ M gefitinib; **5** = WT EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 0.1 μ M Osimertinib; **6** = WT EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 1 μ M Osimertinib; **7** = T790M EGFR-EGFP, no hEGF stimulation, DMSO control; **8** = T790M EGFR-EGFP, 0.1 ng/mL hEGF (10 min), DMSO control; **9** = T790M EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 0.1 μ M gefitinib; **10** = T790M EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 1 μ M gefitinib; **11** = T790M EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 0.1 μ M Osimertinib; **12** = T790M EGFR-EGFP, 0.1 ng/mL hEGF (10 min), 1 μ M Osimertinib.

8. Conclusion

To summarise, this thesis presents set of results that should improve the outcomes of transfection and western blot experiments for studying EGFR related biological questions. Firstly, the optimisation of *in vitro* transfection revealed HEK293T cells as the most easily transfectable model for EGFR-EGFP experiments. Moreover, the seeding density was established as 15 000 cells per well (in 96-well plate format). The assessment of cell viability revealed that the polyplex transfection at the initial doses of 200 ng and 400 ng of plasmid DNA didn't cause significant toxicity.

In upscaled conditions of 6-well plate, the transfection with HEK293T cells worked also very well. In order to reach similar confluency as in initial 96-well plate experiments the seeding density was upscaled to 415 000 cells per well. Thanks to low toxicity, it was possible to increase the plasmid DNA dose to 4 and 8 µg, which would be representative of 400 and 800 ng in 96-well plate format. The flow cytometry revealed that ~ 40 – 50% of cells emit EGFP signal after treatment with 4 and 8 µg plasmid DNA respectively. Decreasing the FCS concentration from 5% to 1% reduced the transfection efficiency, but not to great extent. Additionally, the toxicities even at 8 µg dosage were still low, with ~ 80% live cells following the treatment, as measured by DAPI staining in flow cytometry. This was confirmed also by cell viability measured by resazurin assay. Furthermore, this thesis revealed no difference in term of transfection efficiency by increasing the polyplexing concentration, although there was decrease in cell viability detected.

Another key aspect of this thesis was expansion of mutated version of EGFR-EGFP plasmid carrying the infamous T790M mutation. The expansion and subsequent validation yielded one T790M EGFR-EGFP clone, which passed the structural, as well as functional validation by DRD and transfection respectively. Moreover, western blot experiment with TKIs demonstrated the functionality of the mutation. Therefore, further expansion of T790M EGFR-EGFP plasmid should be done from this clone only.

In the process, western blot experiment parameters were optimised as well. It has been shown that cooking time of the protein sample should be reduced to 5 min at 95°C in order to produce clearer bands on the blot. Furthermore in order to reduce background activation of EGFR, the transfection should occur in 1% FCS rather than 5% FCS. To achieve visible

phosphorylation of EGFR, incubation with 0.1 ng/mL hEGF for 10 min is sufficient. Also using dilution factors of 1:2000 for primary anti-EGFR and anti-pEGFR antibodies, 1:10 000 for secondary anti-mouse (EGFR) and 1:20 000 for secondary anti-rabbit (pEGFR) antibodies is sufficient to obtain good signal on western blots. It is also recommended to use fresh antibody dilutions to avoid unspecific binding. Turning the focus to TKI treatment, both 0.1 μ M and 1 μ M dose of gefitinib and Osimertinib caused visible inhibition of WT EGFR activation, however, the inhibition at 0.1 μ M was much weaker. Nevertheless, it would still be desirable to proceed with both concentrations in order to show the relationship between dose and inhibition.

Finally, the work identified working WT and T790M EGFR-EGFP clones, which were validated by final expansion and subsequent structural and functional validation. The results showed reproducibility with certain degree of variability. Nonetheless, the results matched the trends observed by the earlier experiments. It is therefore recommended to use the clones described in this thesis in further transfections.

9. Appendix

9.1. Expansion and validation of Ext-Gluc plasmid

During the main EGFR-EGFP project a smaller side project was carried out. The aim of the side project was to expand Ext-Gluc plasmid, which was delivered on filter paper. Therefore, the first step in expansion was to elute the plasmid from the paper and then transform competent bacteria for further culturing.

9.3.1. Elution of plasmid from filter paper

The Ext-Gluc plasmid was eluted from filter paper using TE buffer (pH 8). The area of the filter paper containing the plasmid was cut into small pieces using autoclaved scissors. Around 90% of the filter paper pieces were put into single microcentrifuge tube for elution with 100 μ L TE buffer (elution 1). The remaining $\sim$ 10% of the filter paper pieces were eluted with 30 μ L TE buffer (elution 2). The filter paper was incubated with the TE buffer for 10 min at room temperature. After this incubation time, the tubes were spun down to separate the eluate from the filter paper. Moreover, the filter paper from elution 1 was transferred to another microcentrifuge tube for elution with 60 μ L elution buffer (elution 3). The same procedure for elution 3 was followed as with elution 1 and 2. The eluates were then immediately used in transformation reaction. Additionally, negative control was also included in the transformation, where eluate from blank (no plasmid) section of the filter paper was used. This blank was eluted with 30 μ L of TE buffer with the same incubation time as specified above.

9.3.2. Heat-shock transformation

The eluates described in the previous section were used in subsequent heat-shock transformation. To maximise chances of successful transformation, two different strains of competent bacteria were used. One strain were the DH5 α derived competent cells from the Q5[®] Site-Directed Mutagenesis kit by NEB (50 μ L per transformation reaction), while the other were in-house chemically competent stb13 cells (100 μ L of 1:1 stb13 and 50% glycerol mixture per transformation reaction). The transformations were done using 2 μ L of the eluates per transformation reaction. Otherwise, the protocol for the heat-shock transformation was the same as with T790M EGFR-EGFP transformation. For more information refer to section 6.2.1.

Following the outgrowth step, the bacteria were cultured on ampicillin selection plates to pick out transformants, as Ext-Gluc contains ampicillin resistance gene. For this the ampicillin plates were inoculated with 800 μL of the bacteria in SOC medium. Additionally, 100 μL was spread on LB only plates and zeocin plates to act as a positive and negative controls respectively. The plates inoculated with DH5 α derived cells were incubated at 37°C, while the stbI3 cells were incubated at 30°C. Both DH5 α plates and stbI3 plates were incubated for approximately 24 hours.

9.3.3. Liquid culture

The transformed bacteria were cultured in liquid pre-culture and overnight culture containing LB medium with 100 $\mu\text{g/mL}$ ampicillin. As was the case with plate incubation following the transformation, DH5 α derived cells were culture at 37°C, while stbI3 cells were cultured at 30°C. The pre-culture time was 6 hours and the overnight culture time was 16.5 – 17 hours. For more information on liquid culture see section 6.1.2.

9.3.4. Glycerol stock preparation

See section 6.1.3.

9.3.5. Plasmid extraction

See section 6.1.4.

9.3.6. Diagnostic restriction digest and plasmid validation

The DRD and plasmid validation was carried out as described in section 6.1.4. However, for the Ext-Gluc different restriction enzymes were chosen. The restriction reactions for the Ext-Gluc plasmid were:

- uncut control (no restriction enzyme added)
- reaction with BamHI
- reaction with Sall
- reaction with HindIII
- reaction with combination of BamHI and EcoRI

Successfully validated plasmids were approved for further applications.

9.1.2. Results

The plasmid DNA was eluted from filter paper using TE buffer (pH 8.0). Three eluates were made, which were used for the subsequent heat-shock transformation. Two different bacteria strains were tried. One was DH5 α derived strain from Q5 SDM kit by New England Biolabs (NEB) and the other was chemically competent stbl3 strain. These strains were transformed with the three eluates resulting in total of 6 transformation reactions. The successful transformations are shown in **Figure 40**. With the NEB cells, colonies were obtained from transformation with all three eluates of the plasmid, whereas only P1 eluate's transformation yielded colonies with stbl3 cells. In order to obtain suitable number of colonies for further expansion , the colonies on the stbl3 P1 plate were re-streaked on new plates (**Figure 40b**).

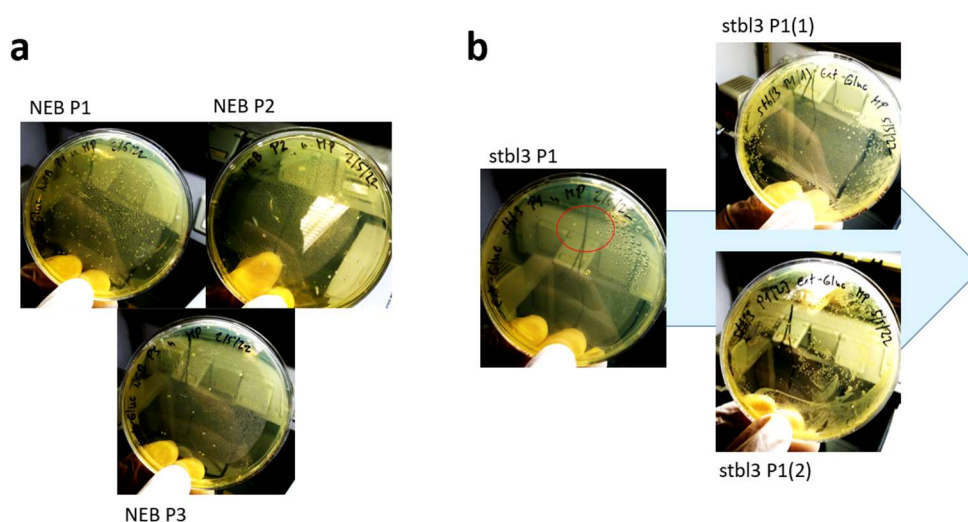


Figure 40. Transformation with eluted Ext-Gluc plasmid. This figure shows pictures of colonies transformed with Ext-Gluc plasmid on ampicillin selection plates. **a)** Transformed DH5 α derived competent cells from New England Biolabs (NEB). **b)** Transformed competent stbl3 cells. P1 = plasmid eluate 1, P2 = plasmid eluate 2, P3 = plasmid eluate 3. The two colonies on stbl3 P1 plate were re-streaked resulting in plates stbl3 P1(1) and stbl3 P1(2).

The colonies were picked from the selection plate for liquid culture (**Table 18**). The total culture time for the NEB cultures was 22.5 hours (pre-culture and O/N culture times combined), whereas for the stbl3 cultures it was 23 hours. Please note, that the incubation temperature was 37°C for NEB cultures and 30°C for stbl3 cultures (these temperatures apply also to solid culture on plates after transformation). After the O/N culture the OD600 values were measured to determine volume of the culture to be processed by the maxiprep. The

OD600 values were high enough to obtain 2 pellets for maxiprep from each culture, except for the NEBP3 culture.

Table 18. Overview of liquid bacterial culture in Ext-Gluc expansion. This table summarises the expansion of Ext-Gluc plasmid. Culture ID refers to the transformation plate from which the colony used for liquid culture was picked. The OD600 value represents growth of the O/N culture after the time specified in the table. Total pre-culture and O/N culture times are also shown.

Culture ID	OD600	Pre-culture time (hrs)	O/N culture time (hrs)
NEBP1	6.98	6	16.5
NEBP2	7.12	6	16.5
NEBP3	5.835	6	16.5
stbl3P1(1)	7.675	6	17
stbl3P1(2)	8.62	6	17

The maxiprep extractions yielded high amounts of plasmid DNA (**Table 19**). The average yield per pellet from the NEB cultures was 962.7 µg of plasmid DNA and the average yield per culture was 1.605 mg of plasmid DNA. The A260/A280 ratio also showed a good purity of the plasmid, however the A260/A230 ratio a little higher. The yields from the stbl3 cultures were considerably lower, with average yield per pellet of only 107.4 µg and average yield per culture of 214.8 µg. While the A260/A280 ratio was good, the A260/A230 ratio appeared to be rather low.

Table 19. Summary of yields of Ext-Gluc plasmid. This table presents the yields of maxiprep extractions of the Ext-Gluc plasmid. The plasmid ID refers to each respective culture from which the plasmid was extracted. As multiple pellets were obtained from each culture, the pellet is indicated by lowercase p followed by number. Measured concentration of plasmid DNA eluted from each pellet is shown, as well as the total yield. The measures of purity A260/A280 and A260/A230 are also shown.

Plasmid ID	Concentration (µg/mL)	Yield (µg)	A260/A280	A260/A230
NEBP1 p1	796	955.2	1.845	2.265
NEBP1 p2	756	907.2	1.844	2.312
NEBP2 p1	806	967.5	1.840	2.2545
NEBP2 p2	885.5	1062.6	1.8505	2.285
NEBP3 p1	767.5	921	1.854	2.315
stbl3P1(1) p1	70	84	1.867	2.059
stbl3P1(1) p2	99.5	119.4	1.8775	2.0725
stbl3P1(2) p1	87	104.4	1.871	1.958
stbl3P1(2) p2	101.5	121.8	1.8715	2.065

In the final step the extracted plasmid DNA was validated by DRD (**Figure 41**). The plasmid DNA extracted from the NEB cultures matched the predicted fragment pattern after digestion

with respective enzymes, confirming the structural identity of the plasmid as Ext-Gluc. The plasmid derived from the stbl3P1(1) culture also matched the predicted pattern. However, plasmid derived from the stbl3P1(2) culture lacked band after Sall digestion. Moreover, the digestion was incomplete as can be seen in **Figure 41c**. Considering this DRD result and the low yields of the stbl3 cultures, it is recommended to use only the plasmid clones derived from the NEB cultures. Similarly, further expansions should be done using the respective glycerol stocks prepared from these cultures.

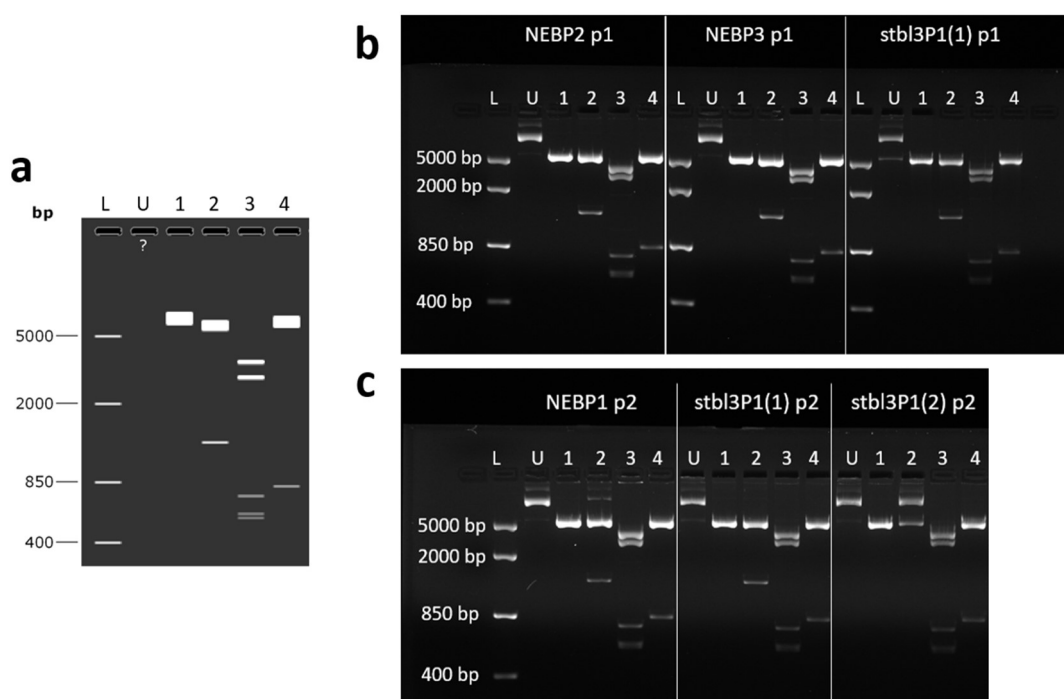


Figure 41. Structural validation of expanded Ext-Gluc plasmid by diagnostic restriction digest. a) Predicted fragment pattern by simulation of diagnostic restriction digest in Snap Gene software on 1% agarose gel. **b)** Diagnostic restriction digest of NEBP2, NEBP3 and stbl3P1(1) Ext-Gluc clones. **c)** Diagnostic restriction digest of NEBP1, stbl3P1(1) and stbl3P1(2) Ext-Gluc clones. Labels: L = ladder; U = uncut; 1 = restriction reaction with BamHI; 2 = restriction reaction with Sall; 3 = restriction reaction with HindIII; 4 = restriction reaction with BamHI + EcoRI.

10. List of abbreviations

B17 – 321031 PR Biochemical Methods and Advanced Pharmaceutical - Chemical Techniques

CAR – chimeric antigen receptor

DALYs – disability adjusted life years

DRD – diagnostic restriction digest

EGF – epidermal growth factor

EGFP – enhanced green fluorescent protein

EGFR – epidermal growth factor receptor

FLM – fluorescent light microscopy

GRB2 – growth factor receptor binding protein 2

hEGF – human epidermal growth factor

mAbs – monoclonal antibodies

O/N culture – overnight culture

pEGFR – phosphorylated EGFR

PEI – polyethyleneimine

PKC – protein kinase C

PLC- γ – phospholipase C- γ

SDM – site directed mutagenesis

STAT – signal transducers and activators of transcription

T790M – substitution of threonine for methionine at position 790 in EGFR

TKIs – tyrosine kinase inhibitors

WT – wild type

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