Hamburg University of Applied Sciences

Faculty of Life Sciences

A triple-LentiCRISPR system targeting EGFR and BIRC5 in Glioblastoma multiforme

Master Thesis

Pharmaceutical Biotechnology

submitted by

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[redacted]

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This master thesis was conducted and written in the period from September 2023 to March 2024. The experiments were carried out in the laboratory group of Dr. Schreiber in the department of virology at the Bernhard Nocht Institute for Tropical Medicine in Hamburg.

1. Supervisor: Prof. Dr. Claus-Dieter Wacker (HAW Hamburg)

2. Supervisor: Dr. Michael Schreiber (BNITM Hamburg)

Preface

This thesis is the final submission for the attainment of the Master of Science in Pharmaceutical Biotechnology at the University of Applied Sciences (HAW) in Hamburg. Before I started my thesis, I worked for more than two years in the lab group of Dr. Schreiber at the Bernhard Nocht Institute for Tropical Medicine (BNITM). During this time, I completed my internship semester and my bachelor thesis as well as worked as a student trainee during my master studies. This allowed me to deepen my knowledge in microbiological and molecular biological methods. Since then, I have published three papers on my research with one as a first author. I was excited to apply my knowledge to this master's thesis.

The topic of my master's thesis was to develop sgRNAs that target specific mutations in EGFR and BIRC5 genes that are present in glioblastoma tumor cells. These sgRNA sequences were cloned into a triple sgRNA CRISPR-Cas9 plasmid. This plasmid was then used to generate a lentiviral vector that has been pseudotyped with a modified Zika virus envelope. The lentiviral vectors were tested in infection studies with tumor cells isolated from glioblastoma multiforme.

Together with my supervisor, Dr. Michael Schreiber, I developed the topic of my thesis and optimized the strategies to achieve the results presented in this thesis. The knowledge I have gained over the past two years has helped me to optimize protocols, optimize my strategies and o work largely independently. I have received important help and scientific input during my research from my work colleagues as well as from Dr. Dr. med. Birco Schwalbe (Asklepios Clinic Nord-Heidberg). My first supervisor Prof. Dr. Claus-Dieter Wacker has been following my work for the past three years and has been a supporter of my career and our project.

I hope you enjoy reading this master thesis.

Celine Pöhlking

Hamburg, 15th of March 2024

Table of content

List of abbreviations

Table 1 Abbreviations

AKH	*Asklepios Klinik Nord-Heidberg*
Amp	Ampicillin
AmpR	Ampicillin resistance
ATP	Adenosine triphosphate
BamHI	BamHI restriction enzyme
BIRC5	Baculoviral IAP repeat containing 5
BLAST	Basic Local Alignment Search Tool
BNITM	Bernhard Nocht Institute for Tropical Medicine
BsmBI-v2	BsmBI-v2 restriction enzyme
CA	Capsid
CAR	Chimeric antigen receptor
Cas	CRISPR associated
CMV	immediate/early promoter enhancer of cytomegalovirus
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CY	Cytoplasma
ddH_2O	Double distilled water
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dsDNA	Double strand DNA
dYT	Double yeast tryptone
EcoRI	EcoRI restriction enzyme
EDTA	ethylenediaminetetraacid
EF	Elongation factor
EF-1α	Elongation factor 1-alpha
EGFR	Epidermal growth factor receptor
ESCRT	Endosomal sorting complex required for transport
Gag	Group specific antigen
GBM	Glioblastoma multiforme
gp41	Glycoprotein 41
gRNA	Guide RNA
GSC	Glioblastoma stem cell
hCSF	Human cerebrospinal fluid

HEK	Human embryonic kidney cell
HF	High fidelity
HIV-1	Human Immunodeficient virus 1
IAP	Inhibitor of apoptosis protein
kb	Kilo base pairs
LTR	Long terminal repeat
LV	Lentiviral vector
LV-ZIKV-3BIRC5	LV with Zika virus envelope containing a CRISPR-Cas9 against BIRC5
LV-ZKV-3EGFR	LV with Zika virus envelope containing a CRISPR-Cas9 against EGFR
MA	Matrix
mAb	Monoclonal antibody
MLV	Murine leukemia virus
MOPS	3-(N-Morpholino)propanesulfonic Acid
mRNA	Messenger RNA
NC	Nucleo capsid
NEB	New England Biolabs
NES	Nuclear export signal
NIH	National Institute of Health
OV	Oncolytic virus
P2A	2A peptide
pAb	Polyclonal antibody
PAM	Protospacer adjacent motif
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with 0.1% Tween
pCMV-dR8.2 dvpr	2nd generation lentiviral packaging plasmid
PCR	Polymerase chain reaction
PEI	Polyethyleneimine
Pol	Polymerase gene HIV-1
ppt	Polypurine tract
psPAX2	2nd generation lentiviral packaging plasmid
Rev	Regulator of expression of virion proteins
RNA	Ribonucleic acid
rpm	Rounds per minute
RRE	Rev response element
SFA	ScreenFect A
sgRNA	Single-guide ribonucleic acid
ssRNA	Single strand RNA

SVPolyA	Simian virus 40 PolyA
TAE	Tris-acetate-EDTA
Tat	Trans-Activator of Transcription
TCGA	The cancer genome atlas
TFB	Transformation buffer
TM	Transmembrane
TME	Tumor microenvironment
TracrRNA	Trans activating crRNA
UV	Ultraviolet light
VSV-G	Vesicular stomatitis virus glycoprotein
WPRE	Woodchuck hepatitis virus posttranscriptional regulatory element
YT	Yeast tryptone
YT	Yeast tryptone
ZIKV	Zika virus

List of figures

List of tables

1. Introduction

Glioblastoma multiforme (GBM) is one of the most aggressive and widespread brain tumors. The prognosis for people diagnosed with GBM is very poor, as the average survival rate is only 15 months[1–3]. The current standard treatment, known as the Stupp protocol[4], consists of a combination of surgery followed by temozolomide treatment and radiotherapy. However, this approach often fails due to the resilience of GBM stem cells, which are remarkably resistant to conventional therapies, leading to relapses[5,6]. To make matters worse, the tumor infiltrates healthy brain tissue, making complete surgical removal practically impossible. To date, existing treatments focus primarily on symptom relief rather than cure, underscoring the urgent need for innovative therapeutic strategies.

In recent years, GBM research has developed considerably through new approaches. Among these, immunotherapy has emerged as a promising new approach, with strategies such as checkpoint inhibitors, CAR-T-cell therapy[7–10], vaccine therapy[11–13] and oncolytic viruses (OV)[14–18] being studied to combat GBM. A key element actively employed in these treatment approaches is the CRISPR-Cas system, which has revolutionized the field of genetic engineering. This adaptable system allows precise targeting of specific genes and mutations, offering the potential to disrupt key signaling pathways responsible for GBM progression[8,19,20]. By harnessing the gene editing capabilities of CRISPR, researchers can target genes that are overexpressed or associated with high mutation rates in GBM cells, effectively blocking the tumor's growth machinery[20].

With CRISPR technology, the gene editing machinery can be completely packaged in vectors, such as lentiviral vectors[21]. These can be equipped with viral envelopes that facilitate specific entry into GBM cells through high tropism for GBM cells [22,23].

The present study describes the development of a CRISPR-Cas9 system with three single-guiding RNAs (sgRNAs). The sgRNA target sequences are specifically designed against the epidermal growth factor receptor (EGFR) and the Baculoviral IAP repeat containing 5 (BIRC5) genes which play a crucial role in GBM development. The CRISPR-Cas9 system is located on a lentiviral plasmid which will be used in three plasmid pseudotype infection experiments using freshly isolated GBM tumor cells. In order to verify the mutations already published in the TCGA-GBM data base[24] and to search for other novel mutations, genomic DNA isolated from GBM cells will be analyzed at respective sgRNA target sites.

1.1. Glioblastoma multiforme

Glioblastoma multiforme is the most common and deadliest primary tumor of the central nervous system. GBM continues to pose a challenge to neuro-oncology despite the progress made over the last 15 years[1,25,26]. Long-term survival rates have seen only moderate improvements, and the treatment primarily aims at decreasing growth and alleviating symptoms rather than achieving a cure. The current standard treatment established by Stupp et al. is based on safe surgical resection followed by intensive chemotherapy with temozolomide and radiotherapy[4]. However, resection of the tumor mass is limited and leaves behind tumor cells, especially GBM stem cells (GSC), which infiltrate the healthy brain tissue. It is suspected that GSCs are the main reason for the recurrence of the tumor after resection, due to the high resistance to chemotherapy and radiotherapy[5,27–29]. Other challenges include immunosuppression caused by the tumor microenvironment, low mutational burden, rapid tumor growth, regenerative compartments and heterogeneity within the tumor itself as the name multiforme indicates[28,29]. GBM heterogeneity is observed not only inter patient, but also intra patient.

The heterogeneity or “multiforme” nature of GBM impedes the development of effective therapeutic strategies, making GBM a persistent and elusive target in the realm of neuro-oncology. Clinical research is constantly looking for alternative solutions, recognizing the limitations of the current approach. Various options are being explored, including novel anti-cancer approaches. These encompass CAR-T cell therapy[7,8,25], cancer vaccines[13,30], checkpoint inhibitors[30,31] and oncolytic viruses[32–35], reflecting a multifaceted strategy aimed at enhancing the body's natural defenses against malignant cells.

These therapies offer strategies at genomic level that enable selective elimination of GBM cells. Sequencing of GBM samples allows comparison of and insight into genomic mutations leading to tumor development[36]. Mutations can cause an increase or decrease in the expression of the respective gene and give GBM cells their dangerous characteristic. However, the silencing of genes by mutations can also enable permanently active signaling pathways and thus promote progression of the tumor. Since mutations in GBM grade IV also affect certain receptors, a similar dependency can be sought and targeted. CRISPR-Cas9 can inactivate the corresponding gene for the enzyme and specifically influence tumor growth, while the healthy cells can use the second metabolic pathway[20]. In addition, point mutations are also suitable targets, as these are selectively recognized and cleaved by the CRISPR-Cas9 system. In the case of so-called lethal genes, this can make the decisive difference to healthy brain cells.

1.2. CRISPR-Cas – Revolution in GBM research

In 2007, Barrangou et al. discovered the CRISPR-Cas bacterial system during experiments with *Streptococcus thermophilus*. They observed resistance to phages, which is the first adaptive immune system ever documented[37]. The CRISPR-Cas immune response is based on a three-step reaction consisting of acquisition, transcription, and interference. In the first step, the foreign deoxyribonucleic acid (DNA) is acquired during phage infection and integrated into the CRISPR locus of the host genome. The CRISPR locus harbors the genomic DNA for the Cas enzyme as well as the so-called spacers and repeats. (Figure 1, A). The CRISPR locus produces mature crRNA (CRISPR-derived RNA) and Trans-activating CRISPR (tracrRNA). An active Cas complex always consists of a Cas9 endonuclease and a guide RNA (gRNA). The gRNA consists of the crRNA: tracrRNA hybrid, which is bound by the Cas9 enzyme. As soon as the gRNA/Cas9 system detects a suitable sequence on the foreign DNA, it introduces a double strand break about 3 bp upstream of the protospacer adjacent motif (PAM) (Figure 1, A). The PAM sequence is recognized by Cas9 and is essential for cleavage. This mechanism protects the cell's genome from cutting all the crDNAs integrated into the genome, as the PAM sequence is not part of the target sequences stored in the genomic DNA[38].

Overall, the prerequisites for DNA cleavage firstly are site specific to the 20 bp nucleotide target sequence and secondly a PAM sequence, in the case of Cas9 the three-nucleotide sequence NGG. The knockout of genes is achieved by mistakes in cellular self-repair mechanisms. Non-homologous end joining, and microhomology-mediated end joining are typically known to disrupt protein coding. Furthermore, the cleavage activity of the Cas9 enzyme can also be used to integrate donor DNA into the genome[20,37,39,40]. The simplicity and limitless customization of the CRISPR-Cas9 system enables the establishment and development for mutation specific strategies against GBM.

As a genetic editing tool, the CRISPR-Cas9 system can be designed to target any 20-bp sequence in front of the PAM sequence. In this work a lentiviral CRISPR-Cas9 system is used that combines the crRNA and tracrRNA into one sgRNA (Figure 1, B). The function of the sgRNA corresponds to the gRNA of the original CRISPR-Cas9 system and enables a simple way of cloning Cas9 target sequences. This demonstrates the simplicity of the CRISPR-Cas9 system, as the sgRNA spacer-sequence can be replaced by any sequence/PAM combination required for therapeutic purpose.

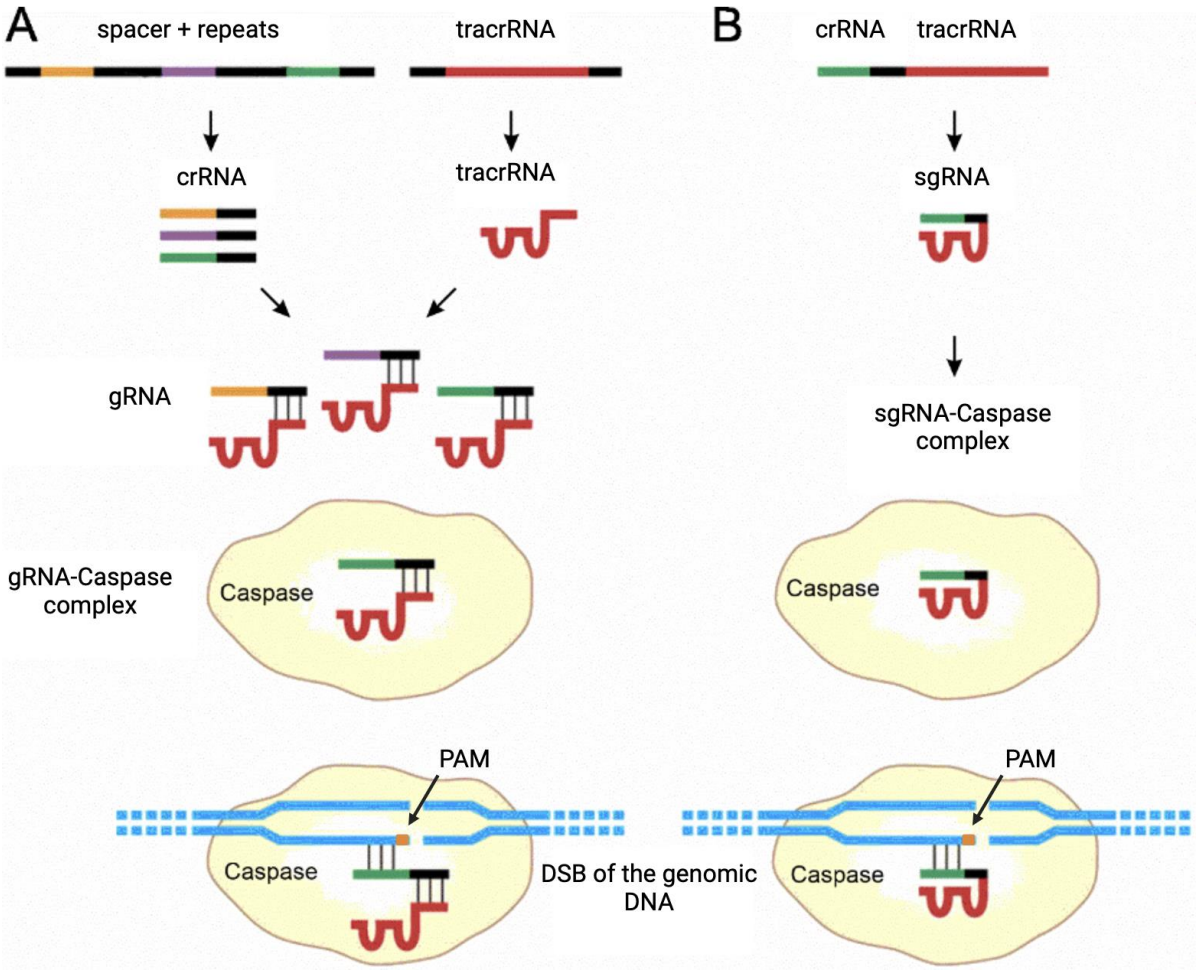

Figure 1 **Components and function of the CRISPR-Cas9 system.**

A In *Streptococcus thermophilus* the spacer and the repeats code for crRNA, which binds to tracrRNA. The hybrid of crRNA and tracrRNA is called gRNA. Different gRNAs can also be generated from different spacers. The gRNA binds to a caspase enzyme. The so-called gRNA-caspase complex then searches for matching sequences on the genomic DNA. If a suitable sequence is bound with a neighboring PAM sequence, the caspase enzyme induces a double strand break.

B As a genetic tool, one gene codes for the sgRNA, a combination of crRNA and tracrRNA as a single RNA molecule. The sgRNA binds to a caspase and the sgRNA-caspase complex then functions like the gRNA-caspase complex in the original organism.

1.3. CRISPR-Cas9 targets in GBM and sgRNA design

CRISPR-Cas9-based genome-wide screening is of utmost importance to identify the optimal and best functioning sgRNA sequences[8,36]. Especially in tumor research, mutations play an essential role in tumor growth, differentiation and immunology[41,42]. Mutations lead to overexpression, lack of gene expression or dysfunction. Although GBM is considered one of the most heterogeneous tumors, parallel mutations are found in up to 50% of patients[41,43]. They are often characterized by identical deletions or point mutations.

The signaling pathway of the EGFR plays a decisive role in the development of various types of cancer. The increased expression of both the wild type and the mutated EGFR has been the subject of studies for decades. This signaling pathway is also important for the pathogenesis of GBM, which makes it an attractive target for therapeutic intervention. In GBM, overexpression of EGFR is a prominent feature found in 40% of tumors, while 50% of GBM cases have mutations in EGFR, called EGFRvIII[44–46]. This involves the deletion of exons 2 to 7, resulting in a frame deletion of 267 amino acids in the extracellular domain (Figure 2, A).

EGFRvIII is unable to bind ligands but remains constitutively active and contributes to certain downstream signal transductions that increase tumorigenicity. Understanding the complexity of EGFR signaling, its mutations and its impact on GBM underscores the potential for targeted therapeutic strategies to combat this aggressive form of cancer. For CRISPR-Cas9, the tyrosine kinase portion of the protein is a highly effective target due to its role in downstream signaling. The realization that mutations occur in parallel in multiple patients has led to an approach in which a single mutation can be targeted. This approach can be used with CRISPR gene knockout by selecting a high-frequency parallel mutation. This mutation can either be integrated directly into the spacer-sequence of the sgRNA, or if a mutation leads to two consecutive guanines, a new PAM sequence is created, that can be used to select a 20 bp target sequence next to it.

In both cases, CRISPR-Cas9 cleaves the target site only if the mutation is present and therefore does not cleave the non-mutated DNA in healthy cells. As described in literature, mutations occur at the same site in multiple patients[24]. Therefore, a CRISPR-Cas9 system with multiple EGFR sgRNAs may be helpful for targeting more than one mutation in GBM cells[43]. Additionally, to EGFR, the BIRC5 gene is another important gene that can also be targeted by a multi sgRNA CRISPR-Cas9 system (Figure 2, B).

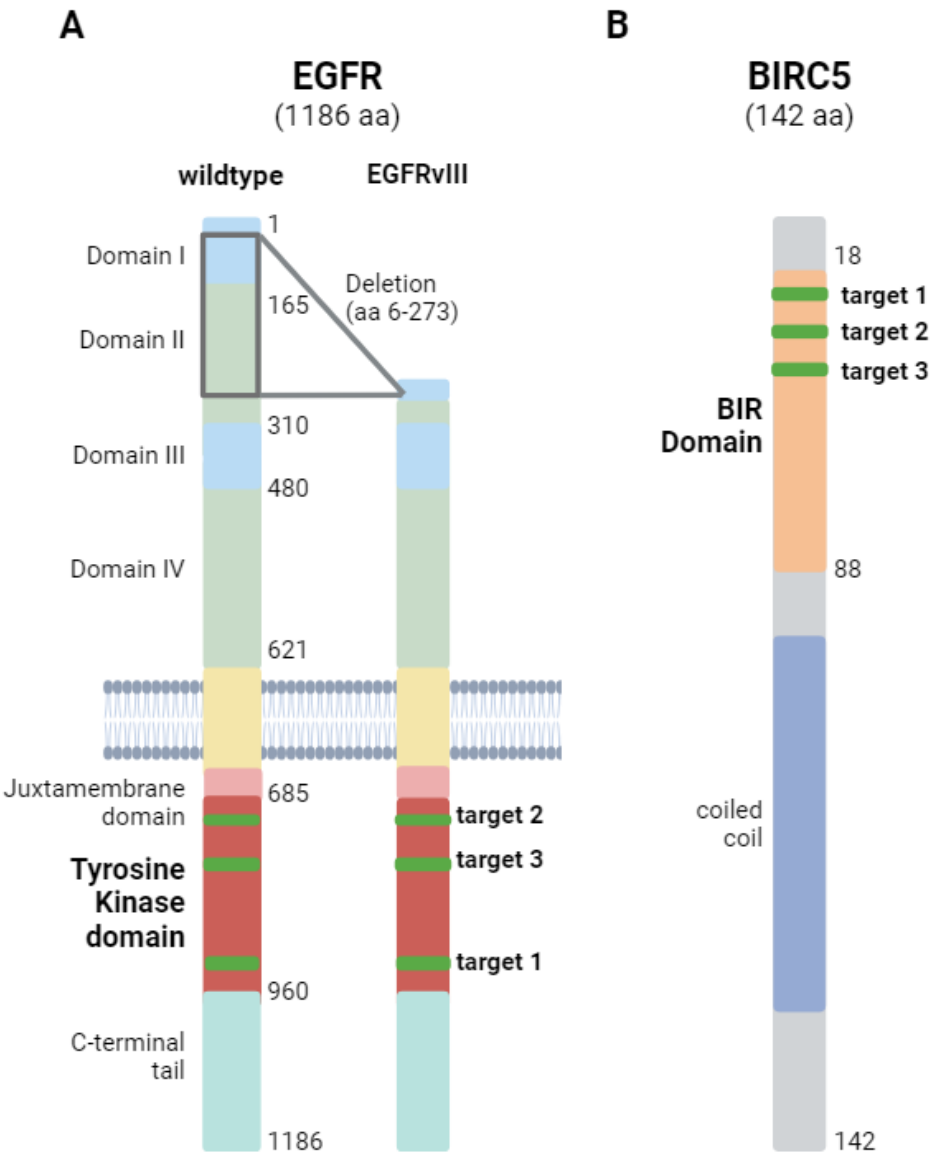

Figure 2 **EGFRvIII and BIRC5 as targets of a triple CRISPR-Cas9 system.**

A EGFR is highly mutated in GBM. The deletion of exons 2-7 found in EGFRvIII is one significant mutation (amino acids 6-273). This deletion leads to a permanently active EGFR, because ligand binding is no longer required for downstream signaling. A deletion cannot be targeted with a CRISPR sgRNA thus a different target needs to be found in EGFR/EGFRvIII. Here the tyrosine kinase domain is a potential target due to its crucial role in downstream signaling inside the cell. A design of three sgRNAs targeting parallel mutations in this domain may lead to inactivation of downstream signaling, inactivating the overexpressed and thus damaging role of the EGFR gene in GBM.

B BIRC5 belongs to the apoptosis inhibitors and plays a role in regulating cell division and inhibition of apoptosis. Its role in GBM progression is due to its anti-apoptotic effects and contribution to GSCs escaping cell death. Thus, its inactivation can be of relevance for patient survival. Three sgRNAs were designed to target the BIR domain. Leading to the inactivation of survivin and its anti-apoptotic effects.

Numbers indicate amino acid positions. (The image from Figure 2 A was designed based on a figure from An et al.[43], the image was created with BioRender.com)

BIRC5 is an immune-related gene that inhibits apoptosis and promotes cell proliferation. It is highly expressed in most tumors and leads to a poor prognosis in cancer patients. Therefore, the development of sgRNAs against the BIRC5 gene is another approach to combat GBM. The BIR domain in BIRC5 has no mentioned mutations in literature and GBM databases, making it an optimal region to cleave the DNA in all patients[24].

Through its inhibition, the progression of the tumor can be reduced, and the patient's survival time might therefore be extended. BIRC5 codes for the protein survivin, that belongs to a family of apoptosis inhibitors. In GBM, survivin has been shown to play a role in tumor progression and resistance to current treatment options. Its apoptotic effects and its overexpression in GBM contribute to GSCs escaping from programmed cell death, which contributes to tumor growth and relapses. Thus, the role of survivin in regulating cell division and the cell cycle leads to uncontrolled GBM tumor cell proliferation[47,48].

Elevated levels of survivin are directly related to the aggressiveness of the tumor and correlate with a poor prognosis in GBM patients. One of the most important roles in current therapy could be the correlation of survivin with resistance to chemotherapy and radiotherapy. A BIRC5 knock-out could therefore also be combined with standard therapy. Finally, angiogenesis, which describes the process of new blood vessel formation, could also be utilized by survivin[47,48]. In view of these crucial aspects, BIRC5 continues to be investigated as a target molecule in GBM and was also one of the selected targets in the master's thesis.

In this thesis, sgRNAs specifically targeting either EGFR or BIRC5 were developed. To achieve greater efficacy in gene silencing, a triple sgRNA vector was generated, one for each of the two described genes. Silencing the target gene at three sites may lead to a higher probability of loss of gene function due to the generation of deletions, as large intron sequences will be excised. The spacers for the sgRNAs for the triple-sgRNA CRISPR-Cas9 system were designed during my bachelor thesis, as they were already used in a single-sgRNA system[49]. For the sgRNA design, GBM databases from the cancer genome atlas program (TCGA-GBM) through the cBioPortal were checked for GBM parallel mutations. During this search, it became evident that EGFR carries a large number of parallel mutations[24]. Therefore, sgRNAs were designed with one of the parallel mutations being part of the target sequence (Figure 3). Thus, the CRISPR-Cas9 system only cleaves DNA if the parallel mutation is present in the GBM cells. For the BIRC5 gene, sgRNAs were selected from literature, as a knock-out was successfully achieved through their use (Figure 4). During the design process of the sgRNAs, care was taken to ensure that a PAM sequence was present at the 3' end of the spacer, and possible off-target sites were analyzed with the basic local alignment search tool from NIH (BLAST)[50].

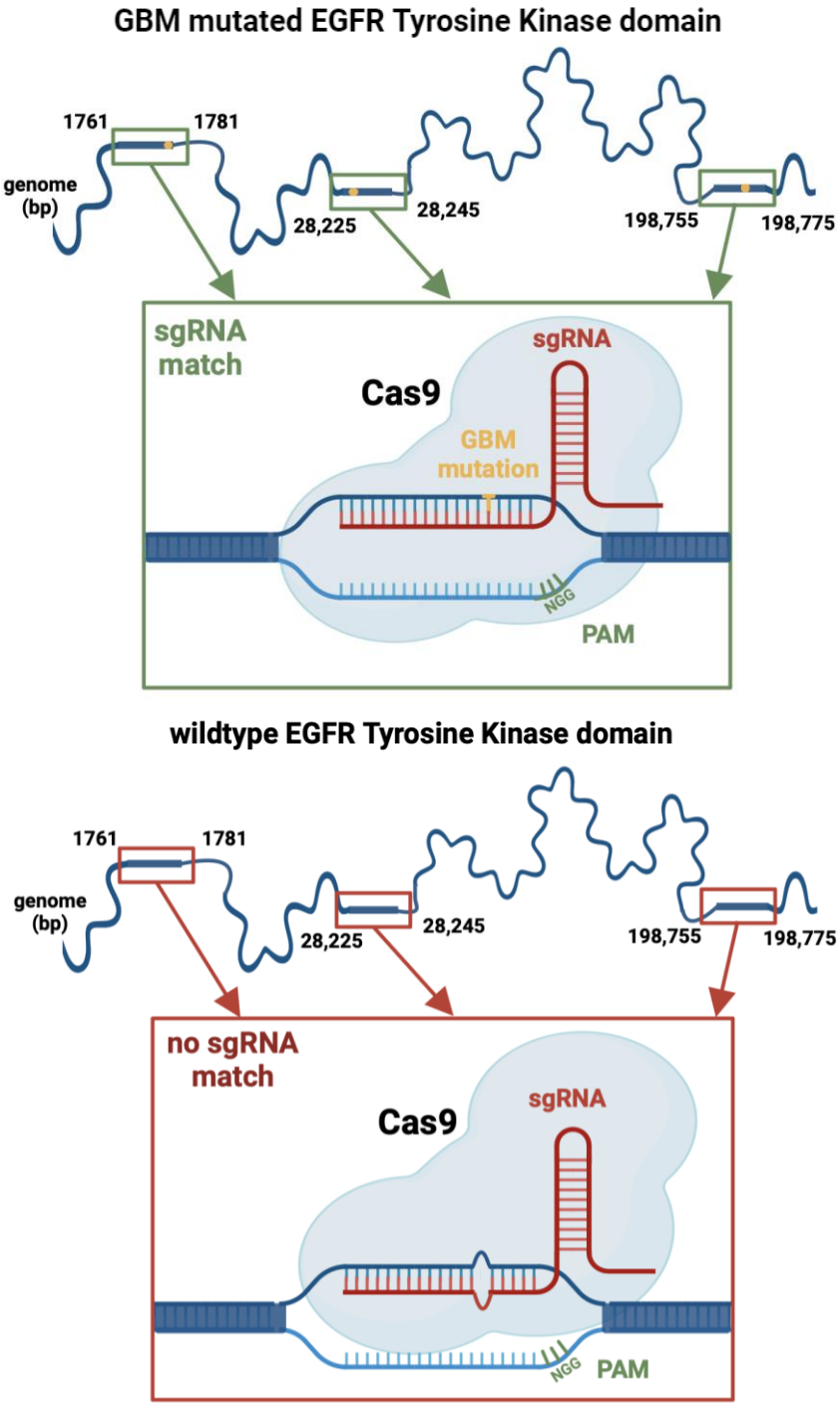

Figure 3 **CRISPR-Cas9 activity based on mutations found in EGFR.**
The EGFRvIII and other mutated variations of the EGFR gene found in GBM have shown parallel mutations found in patient wide DNA screenings and sequencing results. Three parallel mutations with the highest probability were chosen to be integrated into the sgRNA design, thus the mutated base is also found on the corresponding spacer sequence of the sgRNA. In the mutated EGFR tyrosine kinase domain, the CRISPR-Cas9 system can fully bind to the target sequence and initiate double strand break.
The wildtype EGFR found in healthy neuronal cells has less to no mutations compared to the GBM cells. Because of that, the mutations integrated into the spacer sequence are not found on the genome. The Cas9 could therefore find no perfect match and it will therefore not find any matching target sequence.

(The image was created with BioRender.com)

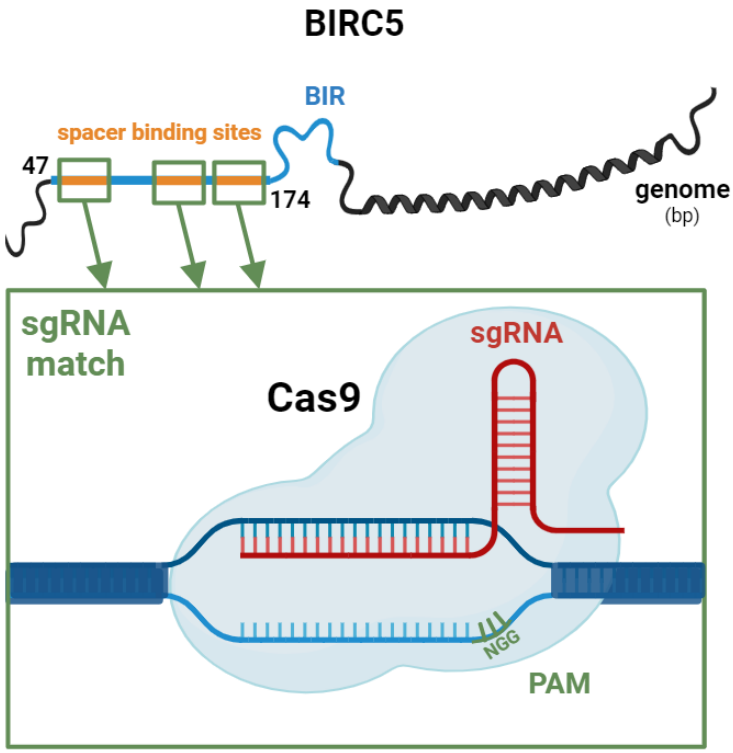

Figure 4 **CRISPR-Cas9 activity of the triple sgRNA plasmid at BIRC5 target sites.** The BIRC5 target sites are located in the BIR domain (blue) of the BIRC5 genome. The target sequences are close to each other, which allows cleavage of the BIR domain into two gene fragments and thus preventing successful repair of the genetic material after Cas9 activity. The target sequences are not specific to the GBM cells. Therefore, no specificity is achieved with this CRISPR-Cas9 system, so it relies on a GBM-specific vector.

1.4. Lentiviral vectors

The use of oncolytic viruses (OVs) has shown great potential since the virus envelope proteins can be manipulated to specifically infect target cells as for example GBM[51]. OVs exert a dual mechanism of antitumor response by inducing tumor-specific cell killing and systemic antitumor immunity. Preclinical studies have demonstrated the efficacy of OVs such as the adenovirus[52,53], herpes simplex virus[15], measles virus[54], parvovirus[55] and Zika virus (ZIKV)[22,56]. Clinical studies showed low side effects and a significant increase in survival time in a subset of GBM patients[14,22,56,57]. As OV therapies pose a potential risk due to further virus replication in the patient, the so-called lentiviral vector (LV) approach has emerged from this strategy. By using LVs, therapeutic genes such as the CRISPR-Cas9 system can be transferred into the target cell. Figure 5 shows the lentiviral genome with the genes required for HIV-1 virus production. The LV is based on the HIV-1 genome, while in a 2nd generation LV the relevant genes of the virus are split across three plasmids. The three-plasmid system requires a packaging plasmid, the transfer plasmid and a plasmid coding for the viral envelope.

The transfer plasmids carry the viral genome from the 5'-LTR to the 3'-LTR. Since the 5'-U3 promoter region is missing in some constructs, the lentiviral transfer plasmid needs a promoter in front of the 5'-R-U5 LTR. The viral genome is transcribed by the CMV promoter, and carries the rev response element (RRE), HIV-1 ψ, polypurine tract (ppt) and the transfer gene. The RRE is a sequence that occurs in retroviruses and plays a crucial role in the export of unspliced or partially spliced RNA from the nucleus into the cytoplasm[58]. The rev protein, which is encoded by the two exons rev.1 and rev.2, binds to the RRE sequence, and facilitates nuclear export of the unspliced viral genome and the partially spliced RNA. The RRE is therefore essential for the incorporation of the lentiviral genome into the virus particle. The HIV-1 ψ sequence is essential for the proper packaging of the two viral RNAs during the assembly of the virus particle. It is required for efficient encapsulation of the vector genome into the particle. HIV-1 ψ is located in the gag region. During viral replication ψ interacts with the gag nucleo capsid (NC) domain. The ppt sequence plays a role during reverse transcription[58]. The 18 bp long sequence serves as a RNAse H resistant RNA primer molecule, initiating the synthesis of the 2nd viral DNA strand.

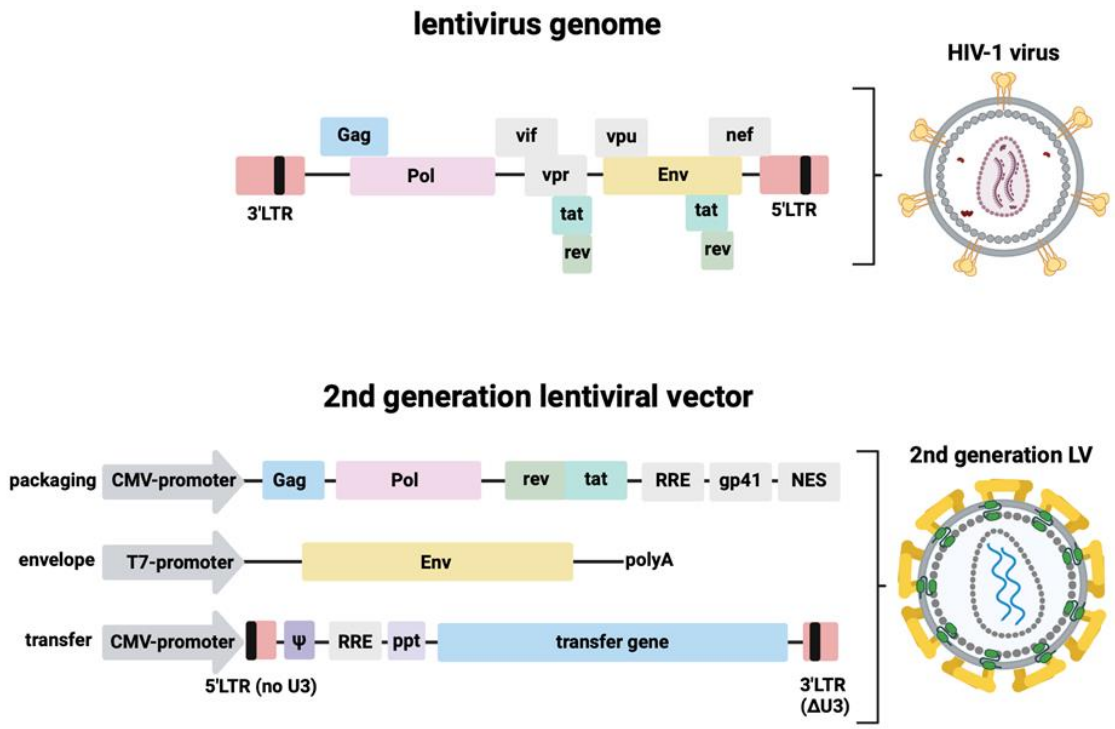

Figure 5 **Components of the three-plasmid 2nd generation lentiviral vector.**
The lentiviral three-plasmid vector system is used to generate pseudotype particles consisting of three plasmids: one containing the required genes for packaging, one for the viral envelope, and one containing the transfer DNA. The lentiviral packaging vector codes for the HIV gag/gagpol, rev and tat and carries the RRE, Δgp41 and NES coding sequences. The envelope plasmid contains the gene for the ZIKV-E protein which is chosen for a most-specific cell infection. The transfer plasmid contains the viral genome with the HIV ψ, RRE, ppt sequences, the transfer gene and the 3'-LTR including a non-functional U3 region (ΔU3). (The image was created with BioRender.com)

A 2nd generation packaging plasmid (psPAX2) contains the group-specific HIV-1 antigen (gag), the HIV-1 polymerase (pol), the RRE, the non-functional glycoprotein subunit 41 (Δgp41), rev, tat, protease and the nuclear export signal (NES). The gag protein is cleaved by the HIV-1 protease to form structural proteins after the budding of the viral peptide. These proteins include the p17 matrix protein (MA), the p24 capsid protein (CA), the p7 nucleocapsid (NC) and the p6 protein. Gag is essential for the assembly and release of viral particles by interacting with the viral RNA that contributes to the formation of the viral core. It also contributes to the budding process when the viral particle detaches from the cell, a process that is driven by the endosomal sorting complex required for transport (ESCRT) machinery. The HIV-1 pol gene encodes the precursor protein for the three viral enzymes that are necessary for the replication of the virus. The most important enzyme is reverse transcriptase (RT), which is essential for the reverse transcription of viral RNA into dsDNA. It also codes for the integrase, which is responsible for the integration of DNA into the host genome. The protease is relevant since it cuts the gag and gagpol precursor proteins to allow maturation of the viral particle from non-infectious to its infectious state. The gp41 plays a role in the infection of the viral particle as it contains the fusion domain that inserts into the host cell membrane and initiates the fusion of the viral and cellular membranes, allowing the viral core to enter. The tat gene is essential for the 2nd generation system as the RNA transcription from the 5'-LTR is tat dependent. The NES is a short peptide containing four 4 hydrophobic residues in a protein that targets it for export from the nucleus to the cytoplasm[58,59].

What makes the LV approach so powerful is that the envelope plasmid used in 2nd generation systems can be easily replaced with other envelopes. This means, that the tropism of the pseudotype particle achieved by this system can be changed by choosing a different specific viral envelope. In the 1990s, Page et al. developed an HIV vector for the first time by replacing the original HIV envelope glycoprotein with the MLV envelope glycoprotein and successfully produced infectious vectors[60]. Only six years later, VSV-G was discovered as an efficient glycoprotein for coating LVs. Its ability to support entry into almost all cell types, combined with high LV stability, makes it an important and standard tool in gene therapy strategies[61,62]. However, its broad cell tropism also leads to non-specific infection, which makes LV-selectivity for a specific target cell difficult. Especially in GBM, selectivity for tumor cells is required to avoid infection of healthy brain cells. In this case the ZIKV envelope proved to be a candidate with GBM specificity[33,63].

1.5. A ZIKV E pseudotyped LV containing CRISPR for GBM targeting

The aim of this work was to develop a ZIKV E pseudotyped LV including the Cas9 endonuclease and three sgRNAs for the transfer of the CRISPR-Cas system into GBM tumor cells. ZIKV has a high specificity for GBM cells, making it a prime candidate for targeted therapy[63,64]. Studies have shown that ZIKV infections pose a significant risk during pregnancy, particularly in the first month when fetal brain cells are rapidly multiplying. This can lead to microcephaly, which is characterized by an underdeveloped brain and an abnormally small head size[65]. Interestingly, ZIKV has a specific tropism for rapidly dividing brain cells which expresses receptors that are weakly present in healthy, normally dividing brain cells[66]. Ongoing receptor studies focusing on candidates such as Axl/Gas6 and integrin *αvβ5*, which are found on GBM cells, are critical for understanding ZIKV infections[23,67–69]. The development of ZIKV-E pseudotyped LVs is essential for the study of infectivity in cell culture systems, considering the heterogeneity of tissues involved in this complex process.

The envelope plasmid pE41.2 has shown promising results in previous infection studies and is therefore used in GBM tumor cell transfection experiments[70,71]. The pE41.2 envelope plasmid was cloned to include the E protein of ZIKV, which is linked to the transmembrane (TM) and cytoplasmic (CY) region of HIV-1 gp41 (Figure 6). Using this envelope, pseudotype budding and particle formation all take place together at the internal cell membrane. The three plasmids are combined in one transfection protocol to form ZIKV-E pseudotyped LVs containing CRISPR (LV-ZIKV-3EGFR or LV-ZIKV-3BIRC5). In this process, the triple sgRNA CRISPR-Cas9 system is packed into the ZIKV-E pseudotyped LV. The tropism of the ZIKV envelope and the specificity of the triple sgRNA CRISPR-Cas9 system allows for low off-target effects which are necessary for therapeutic approaches concerning the brain.

Figure 6 shows the infection with the ZIKV-E pseudotyped LV. The LV can enter the cell due to its ZIKV E envelope, which leads to fusion of the particle with the cell membrane (Figure 6.1) and release of the two ssRNA genomes (Figure 6.2). The ssRNA is reverse transcribed into dsDNA (Figure 6.3) and transported into the cell nucleus. There, the dsDNA is integrated into the genome (Figure 6.4), from which it is transcribed by the cell's own machinery (Figure 6.5). Cas9 is expressed under the control of the EF-1*α* core promotor and the sgRNAs are transcribed by their U6 promoter. The Cas9 mRNA is translated outside the nucleus (Figure 6.6) and the Cas9 enzyme then returns to the nucleus to bind to one of the sgRNAs. The now active Cas9-sgRNA complex searches for target sequences in the cell's genome and cleaves the DNA if the target and PAM sequences are present (Figure 6.7).

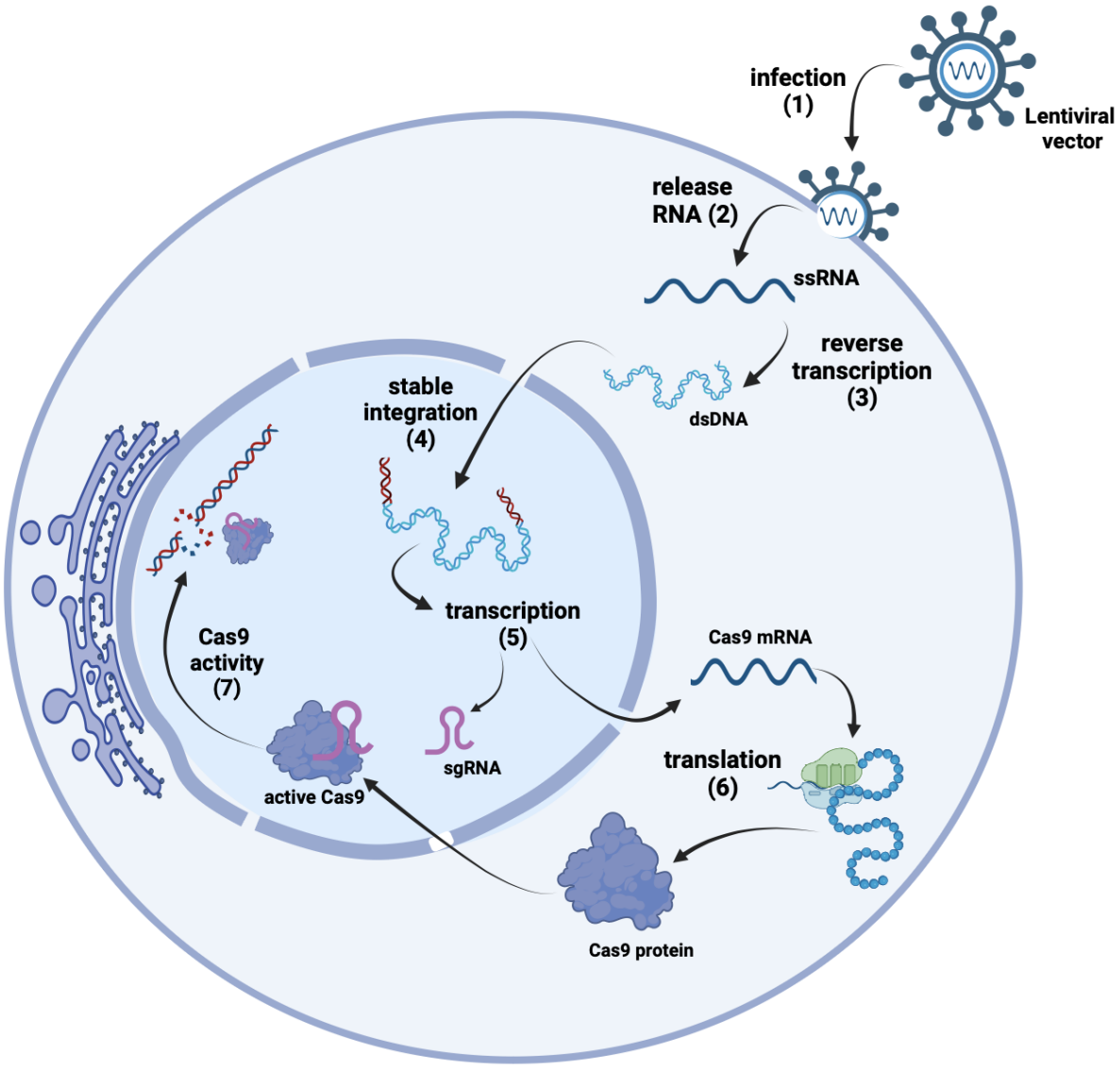

Figure 6 **Lentiviral vector infection with a CRISPR-Cas9 transfer gene.**

1 Cell infection is initiated through the viral particle containing the ZIKV envelope.
2 The virus particle fuses with the cell membrane and the ssRNA is released from the capsid.
3 Reverse transcription of the mRNA into dsDNA.
4 The dsDNA is transported into the cell nucleus and integrated into the cell's genome.
5 Transcription from the genome leads to mRNA for Cas9 and the respective sgRNA.
6 The mRNA for Cas9 is transported from the cell nucleus and translated into the protein.
7 The Cas9 enzyme is transported back into the cell nucleus and binds to an sgRNA. The now active CRISPR-Cas9 complex searches for matches on the genome and, if found, introduces a double strand break. In the case of an LV, no infectious virus particles can be produced by the cell, which is referred to as a single round infection.

(The image was created with BioRender.com) (Plasmid map exported from Snapgene)

1.6. Aim of thesis

This thesis aims to develop two LentiCRISPRv2 vectors containing a triple sgRNA system targeting either mutations in the EGFR or sequences in the BIRC5 gene. The sgRNAs targeting EGFR have been designed to specifically target parallel mutations that occur in GBM. The strategy used to target cancer cells is shown in figure 7. The sgRNA (green), gRNA scaffold (light blue) and U6 promoter (white) region is highlighted on the LentiCRISPRv2-mCherry-3EGFR plasmid (Figure 7.1). Additionally, the mCherry reporter (red) will be replaced by the E2-Crimson reporter to verify if its fluorescence is better suited for the transfection and pseudotype infection experiments. The plasmid is transfected into HEK293T cells, and the viral RNA will be transcribed (Figure 7.2). The viral RNAs are packaged into the ZIKV-E pseudotyped LV. The LV containing the CRISPR system will be used for infection studies (Figure 7.3). Validation of transfection and infection will be performed by fluorescence microscopy due to the mCherry or E2-Crimson reporter gene expression. In addition, freshly isolated GBM tumor cells and HEK293T cells as a control will be used for PCR analysis to study the sequences present at the sgRNA target sites. The amplified DNA fragments will be cloned and sequenced to analyze the presence and frequency of mutations and to detect new mutations. To study the EGFR expression, immunostaining with a commercial EGFR-specific antibody will be performed.

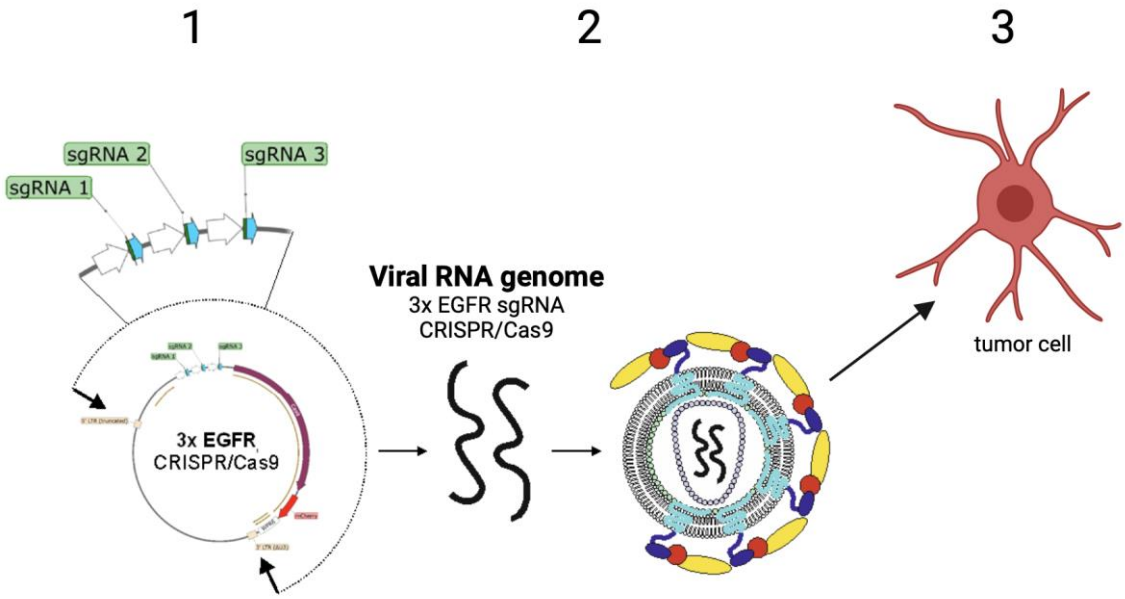

Figure 7 **LentiCRISPR-ZIKV E pseudotype for tumor cell infection.**

1 LentiCRISPRv2 plasmids are cloned carrying E2-Crimson three U6 promotor-sgRNA regions.

2 The viral RNA genome is transcribed from the 5'-LTR to the 3'-LTR including the transgene. The transgene of the LentiCRISPRv2-mCherry-3EGFR contains the genes for the triple sgRNA CRISPR-Cas9 system and the reporter. Transfected cells produce the pseudotype by packaging the transfer gene into the ZIKV-HIV particle.

3 The pseudotyped particle is used for infection. Infections are detected by fluorescence microscopy through the mCherry or E2-Crimson reporter.

2. Material

2.1. Devices

Table 2 Devices

Name	Description	Manufacturer
Centrifuge	Biofuge pico	Heraceus Holding GmbH
	Centrifuge 5810 R, Rotor FA-45-6-30	Eppendorf AG
	Avanti Centrifuge J-26XP	Beckman Coulter GmbH
	Centrifuge 5415C	Eppendorf AG
Electrophoresis chamber	Horizon 58	Life Technologies
Fluorescence microscope	EVOS FL Auto/ EVOS M5000/ EVOS M7000	Life Technologies
Light Microscope	Diavert	Leitz
Incubator	CB 150	Binder
	INE 500	Memmert
	Shaking Incubator 3031	GFL
Pipettes	Research plus (different sizes)	Eppendorf
	Pipetman (different sizes)	Gilson
Scalpel	Metal blade	B. Braun
Sterile workbench	Sterilgard III Advance	Baker Company
Thermocycler	Professional Thermocylcer	Biometra
	Mastercycler Gradient	Eppendorf AG
Thermomixer	Thermomixer comfort, 1,5 mL Tubes	Eppendorf AG
Vortex mixer	VF2	IKA Labortechnik
Spectral photometer	IKAMAG RET-GS	IKA-Werke-GmbH
Analysis scale	Extend ED3202S-CW	Sartorius AG
Magnet stirrer	IKAMAG RET-GS	IKA-Werke GmbH & Co. KG

2.2. Consumables

Table 3 Consumables

Name	Description	Manufacturer
Pipette tips	Biosphere filter tips (different sizes)	Sarstedt AG & Co. KG
Serological pipettes	Costar stripette (different sizes)	Corning
Lab bottles	different sizes	Schott Duran
Reaction tubes	Screwable micro reaction vessels and Safe seal reaction tubes (different sizes)	Sarstedt AG & Co. KG
	Screwable micro reaction vessels 1.5 mL	Sarstedt AG & Co. KG
	Centrifuge tubes 50 mL, 120x7 mm, PP 15 mL, 114x28 mm, PP	Sarstedt AG & Co. KG
Petri dishes	92x16 mm 82.1473	Sarstedt AG & Co. KG
Cuvette	1 mL, OS, QS	Helma
Cell culture bottles	TC-bottle T75, Stand, Bel. Kap. (red)	Sarstedt AG & Co. KG
	TC-bottle T75, Cell+, Bel. Kap. (yellow)	Sarstedt AG & Co. KG
Cell culture plates	TC-plate 24-well, standard (red)	Sarstedt AG & Co. KG
	TC-plate 96-well, standard (red)	Sarstedt AG & Co. KG
	TC-plate 96-well, Cell+ (yellow)	Sarstedt AG & Co. KG

2.3. Chemicals

Table 4 Chemicals

Name	Manufacturer
Ampicillin sodium salt	Carl Roth GmbH & Co. KG
Ethanol 99.8%	Carl Roth GmbH & Co. KG
Ethidium bromide 10 mg/mL	Carl Roth GmbH & Co. KG
Di-sodium hydrogen phosphate dihydrate	Carl Roth GmbH & Co. KG
Potassium dihydrogen phosphate	Carl Roth GmbH & Co. KG
LB-agar	Carl Roth GmbH & Co. KG
LE-agarose	Biozym
TRIS	Carl Roth GmbH & Co. KG
Acetic acid	Carl Roth GmbH & Co. KG

Ethylenediaminetetraacetic acid	Carl Roth GmbH & Co. KG
Tryptone	Carl Roth GmbH & Co. KG
Yeast extract	AppliChem
Potassium acetate	Carl Roth GmbH & Co. KG
Rubidium chloride	Sigma Aldrich
Magnesium chloride	Carl Roth GmbH & Co. KG
Calcium chloride	Carl Roth GmbH & Co. KG
Glycerol	Carl Roth GmbH & Co. KG
Na-MOPS	Carl Roth GmbH & Co. KG
Sodium chloride	Carl Roth GmbH & Co. KG
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co. KG
Isopropanol	Carl Roth GmbH & Co. KG

2.4. Reagent kits

Table 5 Reagent kits

Name	Manufacturer
NucleoBond Xtra Plasmid DNA purification	Machery-Nagel
NucleoSpin Gel and PCR Extraction kit	Machery-Nagel
QIAquick Gel Extraction kit	Qiagen
NEB® PCR Cloning kit	NEB

2.5. Cells

Table 6 Cells

Name	Organism	Origin
DH5α	*Escherichia coli (E. coli)*	BNITM
AKH-XX	Primary cell culture from GBM patients, XX stands for the samples 05, 09, 10, 11, 13, 14, 15, 19, 20, 23.	Asklepios clinic Nord-Heidberg
HEK293T	*Homo sapiens*, human embryonic kidney cell	Friedrich-Löffler-Institut, Insel Riems-Greifswald
U138, U87, U343	*Homo sapiens*, human glioblastoma cell lines	Cell Lines Services (CLS)

2.6. Software

Table 7 Software

Name	Manufacturer
Snapgene	GSL Biotech LLC
CorelDRAW Essentials 2021	Corel Corporation
BioRender	BioRender.com

2.7. Plasmids

Table 8 Plasmids

Name	Description	Manufacturer
LentiCRISPRv2-mCherry	LV, Cas9, mCherry reporter, (Addgene#99154)	Addgene
LentiCRISPRv2-E2-Crimson	LV, Cas9, E2-Crimson reporter	Constructed in this thesis
LentiCRISPRv2-E2-Crimson-3EGFR	LV, Cas9, E2-Crimson reporter, triple sgRNA against EGFR	Constructed in this thesis
LentiCRISPRv2-E2-Crimson-3BIRC5	LV, Cas9, E2-Crimson reporter, triple sgRNA against BIRC5	Constructed in this thesis
LentiCRISPRv2-mCherry-3EGFR	LV, Cas9, mCherry reporter, triple sgRNA against EGFR	Constructed in this thesis
LentiCRISPRv2-mCherry-3BIRC5	LV, Cas9, mCherry reporter, triple sgRNA against BIRC5	Constructed in this thesis
psPAX2	2nd generation lentiviral packaging plasmid (Addgene#12259)	Addgene
pCMV-dR8.2 dvpr	2nd generation lentiviral packaging plasmid (Addgene#8455)	Addgene
pE41.2	ZIKV envelope plasmid with ZIKV E protein and HIV-1 gp41 TM and CP polypeptide	Constructed by Vivien Grunwald

2.8. Reagents

Table 9 Reagents

Name	Description	Manufacturer
Antibiotic	Ampicillin sodium salt	Roth
Buffer	10x T4 ligase buffer	NEB
	5x Phusion HF reaction buffer	NEB
	CutSmart buffer	NEB
	NEBuffer r3.1	NEB
DNA marker	Gene Ruler 1 kb DNA ladder	Thermo Scientific
	Gene Ruler 50 kb DNA ladder	Thermo Scientific
	Gene Ruler 100 bp DNA ladder	Thermo Scientific
Loading dye	Loading dye purple 6X	NEB
Nucleotides	dNTP	NEB
Enzymes	Trypsin/EDTA	Pan Biotech
	Phusion HF DNA-Polymerase	NEB
	BsmBI-v2	NEB
	BamHI-HF	NEB
	BsrGI-HF	NEB
	T4 DNA Ligase	Fermentas
Transfection reagent	PEI MAX-Polyethylenimine, linear (MW 40,000)	Polysciences Inc
Transfection buffer	ScreenFect®A dilution buffer	Incella GmbH
DAPI stain	ROTI Mount FlourCare DAPI	Carl Roth GmbH & Co. KG
Cell culture media	Dulbecco's Modified Eagle Medium (DMEM)	Pan-Biotech GmbH
Media supplement	Fetal bovine serum (FBS)	Pan Biotech GmbH
	Human Cerebrospinal Fluid (hCSF)	Asclepios clinic Nord-Heidberg,
Primary antibody	EGFR Mouse mab, [1 mg/mL]	Proteintech
Secondary antibody	Polyclonal goat ab to mouse IgG, [2 mg/mL], Alexa Fluor 488 conjugate	Abcam

3. Methods

3.1. Production of competent *E. coli* DH5*α*

In a test tube, 3 mL of yeast tryptone (YT) medium was added and inoculated with a single colony of *E. coli* DH5α. The test tube was incubated overnight under rotation (37 °C, Memmert incubator INE 500). In a 1000-mL Erlenmeyer flask, 100 mL of YT medium and 2 mL of the overnight culture were combined. The culture was incubated in a laboratory shaker (37 °C, 180 rpm, incubator GFL, 3031) until the optical density (OD_{600}) was between 0.3 and 0.4. Cell growth was stopped by rotating the Erlenmeyer flask on ice. For each Erlenmeyer flask, 100 mL of TFB I buffer and 5 mL of TFB II buffer were required and placed on ice in advance. 50 mL of the chilled culture was added to each 50-mL centrifuge tube and centrifuged for 15 min at 10,000 x g and 4 °C (Eppendorf, Centrifuge 5810 R, rotor FA-45-6-30). In order to avoid reheating of the bacterial culture, the centrifuge was cooled to 4 °C prior to utilization. After centrifugation, the supernatants were discarded, and the bacterial pellets were each suspended in 50 mL of TFB I buffer. The centrifuge tubes were then incubated on ice for 15 min and centrifuged again at 10,000 x g at 4 °C for 15 min (Eppendorf, Centrifuge 5810 R, rotor FA-45-6-30). The supernatant was discarded, and the pellets were dissolved in 2.5 mL each of TFB II buffer. The resulting competent bacteria were frozen at -70 °C in PCR reaction tubes of 100 µL each until further usage.

1 L YT medium	8	g	Tryptone
	5	g	Sodium chloride
	5	g	Yeast extract

TFB I buffer	30	mM	Potassium acetate
	100	mM	Rubidium chloride
	50	mM	Manganese chloride
	10	mM	Calcium chloride
	15	%	Glycerol

The buffer solution was adjusted to a pH of 5.8 with acetic acid and sterile filtered through a 0.2 µm filter.

TFB II buffer	10	mM	Na-MOPS
	10	mM	Rubidium chloride
	75	mM	Calcium chloride
	15	%	Glycerol

The buffer solution was adjusted to a pH of 6.8 with acetic acid and sterile filtered through a 0.2 µm filter.

3.2. Transformation

The competent *E. coli* DH5*α* generated in Method 3.1 were used for transformation to introduce plasmid DNA into the cells. For this purpose, heat shock was performed. Beforehand, a YT-Amp agar plate was retrieved from the refrigerator and heated to 37 °C in the incubator (Memmert, GFL 3031). 50 µL of competent cells (-70 °C) were thawed on ice and pipetted into a PCR tube together with the appropriate amount of DNA (1 µL midi-prep, 5 µL mini-prep, complete ligation set) followed by incubation on ice for 10 minutes. The Thermocycler (Eppendorf, Master cycler Gradient) was then brought to 42 °C and the PCR reaction tube was incubated for 3 min. The PCR tube was then placed back on ice and incubated for an additional 20 min. In the meantime, 1 mL of YT medium was added to a 1.5-mL reaction tube and warmed in a shaker at 37 °C (Eppendorf, Thermomixer comfort). After the incubation period, the competent cells were pipetted into the 1.5-mL tube, and the culture was incubated at 37 °C and 700 rpm for 30 min (Eppendorf, Thermomixer comfort). The YT-Amp agar plate was removed from the incubator. The cell suspension was centrifuged at 8,000 x g for 1 min (Haraeus, Biofuge pico), and the medium was discarded to 100 µL. The cell pellet was dissolved in the remaining medium and the solution was pipetted and spread onto the agar plate. The agar plate was incubated overnight at 37 °C (Memmert, GFL 3031). The next day, the plate was examined for colonies and stored in the refrigerator at 7 °C for up to 3 weeks.

1 L YT medium	8	g	Tryptone
	5	g	Sodium chloride
	5	g	Yeast extract
YT-Amp agar	8	g	Tryptone
	5	g	Sodium chloride
	5	g	Yeast extract
	16	g	Agar
	1000	mL	ddH_2O
	6	mL	Ampicillin (60 mg/mL)

The YT agar was autoclaved without ampicillin. After autoclaving, ampicillin was added at a temperature of approximately 45 °C. The YT agar was poured into petri dishes and incubated overnight at 37 °C to check for possible contamination (Memmert, GFL 3031).

3.3. DNA sequencing

For DNA sequencing, 10 µL of plasmid DNA, from the Mini plasmid purification (3.6), and 5 µL of primer solution (1:10 diluted oligonucleotide from Metabion, Table 10) were pipetted into a 1.5-mL reaction tube. Sequence analysis was performed by LGC-Genomics

(Berlin, Germany). The tube was labeled with a label provided by the company and shipped by courier. The next day, samples were sequenced and viewed online.

Table 10 **Primers for sequencing.**
The sequencing primer were ordered as oligonucleotides from Metabion at a concentration of 100 µM (desalted). Upon arrival, the oligonucleotides were stored at -20 °C.

Name	Description	Sequence 5' – 3'
psPAX_Primer1	CMV enhancer for	ACTTGGCATATGATACACTTGA
psPAX_Primer2	HIV-1 gag rev	CACTGTGTTTAGCATGGTGTTT
psPAX_Primer3	HIV-1 pol for	AATTGGAGAGCAATGGCTAGTG
dR8.2_Primer1	CMV enhancer for	ACTTGGCATATGATACACTTGA
dR8.2_Primer2	CMV-F for	CGCAAATGGGCGGTAGGCGTG
dR8.2_Primer3	HIV-1 pol for	AATTGGAGAGCAATGGCTAGTG
Seq_Primer	Verification of all 3 sgRNAs in LentiCRISPRv2 plasmid	GGGTTTATTACAGGGACAGC
WPRE-R	Verification E2-Crimson in LentiCRISPRv2	CATAGCGTAAAAGGAGCAACA
pMiniT2Seq	Verification PCR scan cloning results in the pMiniT2 plasmid	ACCTGCCAACCAAAGCGAGAAC

3.4. Agarose gel electrophoresis for DNA analysis

For the separation of the different DNA fragments according to their size, a 0.9% agarose gel was used. For this purpose, 0.9 g of agarose was weighed into a 200 mL laboratory bottle. To the agarose, 100 mL of TAE buffer was added. The laboratory bottle was heated in a microwave until the agarose was dissolved. In a 50-mL centrifuge tube, 1 µL ethidium bromide and 20 mL 0.9% agarose were added. The mixture was poured into the gel tray of the electrophoresis chamber (Horizon 58, Life Technologies) (7 cm x 7 cm). Finally, one or two combs (8 pockets, 16 pockets) were inserted into the spaces provided. After 15 min, the agarose gel was completely solidified, and placed into the electrophoresis chamber. TAE buffer was added to the electrophoresis chamber until it reached just above the gel. A DNA marker (1 kb, 100 bp, Thermo Scientific) was pipetted into the first pocket to classify the DNA fragments by size. Loading dye (Purple (6X), NEB) was added to the sample and filled up to 10 µL with ddH_2O. DNA was separated at 140 V for 20 min (small DNA fragments expected) or 40 min (large DNA fragments expected). The DNA bands were analyzed under UV light.

TAE buffer	40	mM	TRIS
	20	mM	Acetic acid 99%
	2	mM	EDTA

3.5. PCR purification

PCR DNA clean-up and gel extraction were performed using the NucleoSpin® gel and PCR clean-up kit from Macherey-Nagel. For gel extraction, the gel was weighed and 200 µL of NTI buffer was added per 100 mg gel weight. The gel was then completely dissolved by incubating the tube at 50 °C for 1 h (Eppendorf, Thermomixer comfort). For PCR purification, two volumes of binding buffer (NTI) were added to 1 volume of sample. The next steps included PCR purification and gel extraction. Up to 700 µL of sample were loaded onto a clean NucleoSpin® column at a time. The column was centrifuged at 11,000 x g for 30 s (Haraeus, Biofuge pico). The flow through was then discarded and 700 µL of wash buffer (NT3) was applied. The column was centrifuged again at 11,000 x g for 30 s (Haraeus, Biofuge pico). The wash step was repeated. The column was then dried at 11,000 x g for 1 min (Haraeus, Biofuge pico), the tube discarded and replaced with a new 1.5-mL reaction tube. For elution, 30 µL of elution buffer (NE) was added to the column. The column was incubated at room temperature for 1 min, and the solution was eluted at 11,000 x g for 1 min (Heraeus, Biofuge pico). The DNA was stored at -20 °C until further use.

3.6. Mini plasmid extraction

4 mL of dYT-Amp medium was added to a test tube. A colony was taken from an agar plate with a glass rod and transferred to the test tube by stirring it in the medium for a few seconds. The test tube was closed with an aluminum cap and incubated overnight at 37 °C. The bacterial suspension was gradually transferred to a 1.5-mL reaction tube and centrifuged at 11,000 x g for 30 s to pellet the cells (Haraeus, Biofuge pico). The medium was discarded. This procedure was repeated until all 4 mL of the cell suspension was centrifuged off. Cells were then resuspended in 250 µL of A1 resuspension buffer (stored at 7 °C after addition of RNAse). After resuspension, no cell clumps should remain in the buffer or at the bottom of the tube. To the suspension, 250 µL of A2 lysis buffer was added and the tube was inverted three times. Cell lysis was achieved by incubating the tube for 5 min. Then 300 µL of A3 neutralization buffer was added and the tubes were rolled on a test tube roller until the suspension was completely colorless (no vortexing or shaking). The lysate was separated from the cells by centrifugation at 11,000 x g for 10 min (Haraeus, Biofuge pico). The lysate was then transferred to a NucleoSpin® column, and DNA was bound to the column during centrifugation at 11,000 x g for 1 min (Haraeus, Biofuge pico). The flow-through was discarded and 500 µL of AW wash buffer was added to the column. The column was centrifuged again at 11,000 x g for 1 min (Haraeus, Biofuge pico) and afterwards the wash buffer was discarded. Then 600 µL of A4 wash buffer was added to the column and centrifuged at 11,000 x g for 1 min. The silica membrane of the column was dried by centrifuging again at 11,000 x g for 2

min. The collection tube was removed and replaced with a clean 1.5-mL reaction tube. For the DNA solution, 50 µL of the AE elution buffer was added and the column was incubated at room temperature for 1 min. Elution was then performed by centrifuging the column at 11,000 x g for 1 min. The DNA was stored at -20 °C until further use.

dYT-Amp medium	16	g	Tryptone
	5	g	Sodium chloride
	10	g	Yeast extract
	1000	mL	ddH_2O
	5	mL	Ampicillin (60 mg/mL)

3.7. Maxi plasmid extraction

Maxi plasmid extraction was performed using the NucleoBond® Xtra plasmid DNA purification kit from Macherey-Nagel. To a 1 L Erlenmeyer flask, 250 mL of dYT-Amp medium was added and a colony of transformed *E. coli* DH5α containing the desired plasmid. The bacterial culture was incubated overnight at 37 °C and 180 rpm (incubator GFL, 3031, Memmert). Gradually, 50 mL of the overnight culture was added to a 50 mL tube and centrifuged at 10,000 x g for 2 min (5810 R, rotor FA-45-6-30, Eppendorf). This was repeated five times until all cells were collected. To the cell pellet, 12 mL of resuspension buffer (stored at 7 °C with RNAse) was added, and the cell pellet was completely dissolved until no lumps remain. Then 12 mL of lysis buffer was added, and the tube was inverted three times. After 5 min of incubation at room temperature, 12 mL of neutralization buffer was added, and the suspension was rolled on a test tube roller until it was neutralized indicated by changing its color from blue to white. The lysate was then separated by centrifugation at 10,000 x g for 15 min (5810 R, Rotor FA-45-6-30, Eppendorf). During the centrifugation time, the filters were loaded with 15 mL of equilibration buffer. The lysate was then added to the filter wool. The filter wool was then reloaded with 15 mL of equilibration buffer and then discarded. The column was washed with 25 mL of wash buffer while 12 mL of isopropanol was added to a 50-mL centrifugation tube. After the washing step, the centrifugation tube containing isopropanol was placed under the column, and the DNA was eluted by adding 15 mL of elution buffer to the column. The centrifugation tube was vortexed to disperse eluted DNA and isopropanol. The centrifugation tube was then placed in the centrifuge and the DNA was precipitated at 15,000 x g for 30 min (5810 R, rotor FA-45-6-30, Eppendorf). The supernatant was carefully discarded. The DNA pellet was then dissolved in 500 µL ddH_2O and transferred to a 1.5-mL reaction tube.

3.7.1. Precipitation

The DNA solution was placed on ice and 50 µL sodium acetate was added. Up to 1.5 mL of ethanol (-20 °C) was added to the 1.5-mL reaction tube. The solution was shaken to achieve optimal distribution of sodium acetate and ethanol. The reaction tube was incubated on ice for 15 min. The tube was then centrifuged at 13,000 x g for 10 min (Haraeus, Biofuge pico) and again placed on ice. After another 10 min of incubation, the tube was centrifuged again at 13,000 x g for 10 min (Heraeus, Biofuge pico). The supernatant was discarded, and the plasmid pellet was incubated at 90 °C with the lid open for 1 h. To evaporate remaining isopropanol 500 µL of ddH_2O was added and the tube was incubated for 1 h at 80 °C and 700 rpm (Eppendorf, Thermomixer comfort). After incubation, the DNA was checked to see if it was completely dissolved; if not, shaking was continued, if the solution was too viscous, more water was added. After the DNA was dissolved, the concentration was measured and adjusted.

3.8. DNA measurement and concentration adjustment

The DNA measurement was performed with a photometer (Spectral photometer, UV 160 A, Shimadzu GmbH). Here, black quartz cuvettes with a volume of 100 µL were used. For zero adjustment, 100 µL of ddH_2O was added to a cuvette. To the other cuvette, 95 µL of ddH_2O and 5 µL of the DNA sample were added and mixed by pipetting up and down. The DNA concentration was determined by measuring the OD_{260}. If the OD_{260} was 1, the DNA concentration had the desired concentration of 1 µg/µL. If the OD_{260} was above 1, ddH_2O was added to the DNA sample, which was then measured again and adjusted until the desired concentration was achieved.

3.9. Mammalian cell cultivation

The eukaryotic cells were grown at 37 °C and 5% CO_2 in red or yellow capped T75-bottles (Sarstedt). The cell culture protocol for the freshly isolated GBM cells from AKH, was adapted from Pöhlking et al.[70]. For HEK cells the red bottles were used while for the AKH-XX cells yellow (Cell+) bottles were used. The HEK cells were grown with DMEM media + 10% FBS. For the AKH-XX cells DMEM media + 10% FBS was supplemented with 50% human cerebrospinal fluid (hCSF). After sufficient confluence the cell cultures were split. First the cells were washed by discarding the media and by the addition of 2 mL PBS. Afterwards 2 mL of Trypsin/EDTA were added and incubated for 5 min to detach the cells. 8 mL medium (DMEM + 10% FBS) were added to the detached cells and used for cell separation by carefully pipetting up and down using a serological pipette. For the next passage, a part of the detached cells was transferred to a new bottle together with 35 mL of fresh medium. Cells were also transferred to a centrifuge tube, diluted, and transferred into 24-well or 96-well plates.

3.10. Immunofluorescence staining of EGFR

3.10.1. Antibody staining with primary and secondary antibodies

Cell fixation

The cells were grown on 96-well plates overnight. When the cells had a density of 70-90% the medium was discarded by turning the plates upside down. The wells were carefully washed with 200 µL PBST which was again discarded by turning the plate over. The wells were then covered with 50 µL 3.7% formaldehyde and incubated for 20 min to fixate the cells. The formaldehyde was carefully pipetted from the wells, and it was again washed twice with 200 µL PBST.

Primary antibody

The fixed cells were incubated with 30 µL Triton-X-100 per well for five minutes. The wells were washed twice with 200 µL PBST afterwards. Blocking was performed with 30 µL 5% BSA/PBST for 1 h. For the primary antibody, the EGFR antibody was diluted 1:250 with 1% BSA/PBST. For each cell line, at least three wells were used for each antibody. One well was left blank as a negative control. The antibody was incubated overnight at 7 °C.

Secondary antibody

The first antibody was discarded and the wells were washed three times with 200 µL PBST. The goat pAb to Ms IgG secondary antibody was diluted with a 1:500 dilution of 1% BSA/PBST. For staining each well was covered with 30 µL of the antibody solution. The secondary antibody was incubated for 1 h at room temperature in the dark. It was then discarded and the wells were washed three times with 200 µl PBST. Afterwards cells could be further processed with DAPI staining.

3.10.2. DAPI-staining eukaryotic cells

With DAPI-staining, cell nuclei can be made visible through fluorescence microscopy. Before coloring the buffer was discarded and one drop of ROTI® Mount FlourCare DAPI was added to each well. Afterwards the cells were used in fluorescence microscopy.

3.11. Transfection of HEK293T cells

3.11.1. Poly-L-Lysine coating of 24-well plates

The poly-L-lysine was diluted 1:10 with ddH_2O and 300 µL was added to each well of a 24-well plate. After 5 minutes, the solution was pipetted off and each well was washed two times with 1 mL of PBS. After the final washing step, the remaining PBS was removed. The plate was then incubated at 37 °C for two hours. The plate can be used directly or stored at 7 °C for up to one year.

3.11.2. Transfection

Transfection experiments were performed to verify the function of the E2-Crimson reporter compared to the mCherry reporter first. Afterwards the LentiCRISPRv2 triple-sgRNA plasmids were used for infection following transfection. For the three-plasmid pseudotype system, the pE41.2 envelope plasmid and the packaging plasmids psPAX and pCMV-dR8.2 dvpr were used. The LentiCRISPRv2 plasmids were used as the transfer gene (Figure 8).

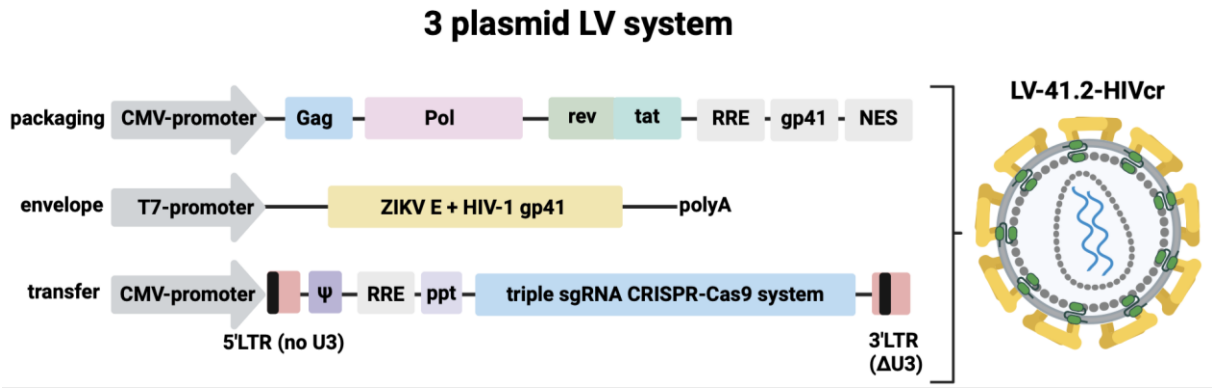

Figure 8 **Components of the three-plasmid 2nd generation lentiviral vector.**
The lentiviral three-plasmid vector system is used to generate pseudotype particles consisting of three plasmids: one containing the genes for the envelope proteins (gp41.2), one containing the transfer DNA to be packaged (LentiCRISPRv2) and a third containing the genes required for packaging the transfer DNA into the envelope (psPAX2/ pCMV-dR8.2 dvpr). The lentiviral packaging vector contains the DNA for the HIV-1 gag, gagpol, RRE which are crucial for packaging the RNA into the pseudotype. The envelope plasmid contains the gene for the envelope protein which is chosen for specific cell infection. The transfer plasmid contains the genes for the 5'LTR, HIV-1 psi, the transfer gene, and the 3'LTR. The image was created with BioRender.com.

Day 1

The detached HEK cells from Method 3.9 were transferred to a centrifugation tube and diluted 1:20 with ddH_2O. To each well of a poly-L-Lysine coated 24-well plate (Method 3.9) 1 mL of cells were added and the cells were incubated overnight at 37 °C and 5% CO_2.

Day 2

The medium was removed and 100 µL of fresh medium (DMEM + 10% FBS) was added. A confluence of around 80% was needed for the subsequent steps. For transfection a PEI mix and DNA mix was prepared (Table 11). Afterwards the PEI mix was added in 10 µL steps into the DNA mix and mixed by slowly pipetting up and down after each transfer. The PEI/DNA mix was incubated for 20 min. The 20 µL were added to a well of the 24-well plate. The plate was gently moved in circular motions to distribute the DNA/PEI mix in the media. The plate was incubated at 37 °C and 5% CO_2 for 3 h. Afterwards the medium was gently pipetted out of the well and discarded. Care was taken to not detach cells during these steps.

Table 11 DNA and PEI reactions for transfection.

	Single plasmid			
Plasmid name	**DNA [µL]**	**SFA buffer [µL]**	**PEI [µL]**	**SFA buffer [µL]**
LentiCRISPRv2-XXX	1	39	3	37

	Three-plasmid			
Plasmid name	**DNA [µL]**	**SFA buffer [µL]**	**PEI [µL]**	**SFA buffer [µL]**
LentiCRISPRv2-3EGFR psPAX/ pCMV-dR8.2 dvpr pE41.2	0.5 3 1.5	35	10	30
LentiCRISPRv2-3EGFR psPAX/ pCMV-dR8.2 dvpr pE41.2	1 6 3	30	15	25
LentiCRISPRv2-3EGFR psPAX/ pCMV-dR8.2 dvpr pE41.2	1 10 10	19	25	15
LentiCRISPRv2-3EGFR PsPAX/ pCMV-dR8.2 dvpr pE41.2	1 10 6	23	30	10
LentiCRISPRv2-3EGFR psPAX/ pCMV-dR8.2 dvpr pE41.2	1 6 10	23	30	10
LentiCRISPRv2-3EGFR psPAX/ pCMV-dR8.2 dvpr pE41.2	1 1 1	37	6	34

Day 3

The cells were now used in fluorescence microscopy (Method 3.11). Transfection rate was documented by overlapping the transparent pictures with the TxRed filter (585/624 nm).

3.12. Infection of tumor cells

Day 1

AKH-10, AKH-13 and U87 cells were diluted according to their cell growth. AKH-10 was diluted 1:10, AKH 13 1:15 and U87 1:5. The diluted cells were used to prepare 96-well plates, by adding 200 µL of cell dilution to each well. The cells were incubated overnight at 37 °C and 5% CO_2.

Day 2

The cells were now 60% confluent. 150 µL of medium was removed from each well, leaving only a small amount of medium on the cells. The suspension from the 24-well transfections was carefully transferred to a 1.5 mL microcentrifugation tube. The tubes were then centrifuged at 14,000 x g for 4 min. The suspension was again transferred to another tube and centrifuged again at 16,000 x g for 4 minutes. 150 µL of the transfection medium was added to each well of the prepared 96-well plates. The 96-well plates were incubated at 37 °C and 5% CO_2.

Day 5 - 8

Three days after infection, the cells were examined using a fluorescence microscope according to Method 6.5.3. The infection rate was documented by overlaying the transparent images with the TxRed filter (560-50 nm). The images were taken at 4x, 10x and 20x magnification.

3.13. Fluorescence microscopy

With the EVOS M7000 microscope, the 24-well and 96-well plates were analyzed under sterile conditions. Microscopy was performed at room temperature using 4x and 20x magnification. Immunostaining fluorescence was analyzed using the DAPI- and GFP filters. Transfection and infection experiments were analyzed using the TxRed (585/624 nm) filter.

3.14. Sequence Analysis of EGFR and BIRC5 target sites

To verify EGFR mutations in the isolated GBM cells, PCR analysis and sequencing of the cloned PCR fragments was performed.

3.14.1. Cell lysis for template preparation

Cell lysis was performed using a commercial direct PCR cell lysis reagent (Viagen Biotech Inc.). The cells from one cell culture (Method 3.11.2) were washed with 2 mL PBS and detached by using Trypsin/EDTA. Afterwards, the cells were transferred into a 50 mL tube, 20 mL of PBS were added and the cells were centrifuged for 10 min at 8,000 x g. The cells were then suspended in 300 µL direct PCR Lysis reagent (Cell) containing 0.3 mg/mL freshly prepared Proteinase K. The mixture was transferred to a 1.5-mL reaction tube and incubated at 37 °C for 6 h until no clumps were visible. Afterwards the cell lysate was incubated at 85 °C for 50 min to inactivate Proteinase K (Thermomixer comfort, Eppendorf).

3.14.2. PCR amplification of the EGFR and BIRC5 sgRNA target sites

For the PCR analysis of the EGFR and BIRC5 genes, primers were designed that amplify an approximately 1,000 bp long sequence containing the target sites used in the LentiCRISPRv2 plasmids. Because of low amplification with raw cell lysate, two primer sets are designed for each fragment amplification. In Figure 9-11 the design for the amplification of the EGFR gene containing the sgRNA target sites of EGFR are shown. In Figure 12, the amplification site for the three BIRC5 sgRNA target sites are given. The outside primers are used for amplification from the genomic DNA of the cell lysate first. The amplification mix was then again used in a second PCR using the inside primer pair. This ensures a higher fragment yield and visible DNA bands in agarose gel electrophoresis.

The annealing temperatures of the primer pairs were matched for optimal annealing during PCR (Table 12). The primer design was based on the DNA sequence from NIH sequences for BIRC5 and EGFR[72,73]. Therefore, HEK293T cells were used as a positive control. After the second amplification, the fragments were used in gel electrophoresis for detection and subsequent gel purification.

Figure 9 **PCR analysis of the EGFR sgNRA1 target site.**
Both primer pairs are shown in purple and the EGFR sgRNA1 target site is highlighted in red. Amplification leads to a 950 bp fragment being amplified for further PCR cleanup and cloning. (The sequence map was exported from Snapgene)

Figure 10 **PCR analysis of the EGFR sgNRA2 target site.**
Both primer pairs are shown in purple and the EGFR sgRNA2 target site is highlighted in red. Amplification leads to a 770 bp fragment being used for further PCR cleanup and cloning. (The sequence map was exported from Snapgene)

Figure 11 **PCR analysis of the EGFR sgNRA3 target site.**
Both primer pairs are shown in purple and the EGFR sgRNA3 is highlighted in red. Amplification leads to an 820 bp fragment being amplified for further PCR cleanup and cloning. (Sequence was exported from Snapgene)

Figure 12 **PCR analysis of the BIRC5 sgRNA target sites.**
Both primer pairs are shown in purple and the BIRC5 sgRNAs targe sites are highlighted in red. Amplification leads to a 1024 bp fragment being amplified for further PCR cleanup and cloning. (The sequence map was exported from Snapgene)

Table 12 Primer for PCR analysis of target sites.

Name	Sequence 5' – 3'	Annealing temp.
Amplification of the sgRNAEGFR1_fragment (998 bp)		
For1_EGFR1	GGTCCCCGCCACCCCCCACC	72 °C
Rev1_EGFR1	GAGGGGCCTGGCTGTGGGAGGGCAT	72 °C
For2_EGFR1	CCACATGCAGGAAGGTCAGCCTGGC	67 °C
Rev2_EGFR1	GCCTGACAGGGGACGCGCAC	67 °C
Amplification of the sgRNAEGFR2_fragment (820 bp)		
For1_EGFR2	CACTGCACAGAGACTTGCCACC	62 °C
Rev1_EGFR2	GCTCTTTTGTCCCCCTACCCTCC	63 °C
For2_EGFR2	CACTGGAGAAACACTCATGTGGG	61 °C
Rev2_EGFR2	GCAGCATTGCACAACACAGGC	63 °C
Amplification of the sgRNAEGFR3_fragment (867 bp)		
For1_EGFR3	CCCATGCTGGCACTGTGGCGAAGGCG	72 °C
Rev1_EGFR3	GGGCTGTGACGCCCCCGCAGCC	73 °C
For2_EGFR3	CGCCACAGCCCAGTCCTGTGC	68 °C
Rev2_EGFR3	TGAGCCCATTTCAGCTGGGCTGC	66 °C
Amplification of the sgRNABIRC5_fragment (1024 bp)		
For1_BIRC5	CCCAGAAGGCCGCGGGGGGTGG	73 °C
Rev1_BIRC5	CGGGCGTGCGCTACCATGCCCGG	72 °C
For2_BIRC5	GCCTAAGAGGGCGTGCGCTCCC	67 °C
Rev2_BIRC5	GGGTTTCGCCAAGTTGGCCAGGC	67 °C

Below, the two master mix reactions for the first and second PCR are given. For each reaction the corresponding primers from Table 12 were used.

	1	**2**		
Reaction master mix	32.5	28.5	µL	ddH_2O
	10.0	10.0	µL	5x Phusion HF reaction buffer
	2.5	-	µL	For1 (10 µM)
	2.5	-	µL	Rev1 (10 µM)
	-	2.5	µL	For2 (10 µM)
	-	2.5	µL	Rev2 (10 µM)
	1.0	-	µL	Cell lysate
	-	5.0	µL	Reaction master mix 1
	0.5	0.5	µL	Phusion HF DNA polymerase (2U/µL)
	1.0	1.0	µL	dNTP
	50.0	50.0	µL	

Thermocycler protocol	Step 1	98 °C, 30 s	
	Step 2	XX °C, 30 s	35 cycles
	Step 3	72 °C, 1 min	
	Step 4	15 °C, hold	

Annealing temperatures were based on the annealing temperature calculated using Snapgene (Table 12). DNA amplification was checked using gel electrophoresis (Method 3.4).

3.14.3. Cloning of PCR fragments into pMiniT2

Fragments obtained from the nested PCR analysis were ligated using the NEB PCR cloning kit. For ligation of a PCR fragment into pMiniT2 a 1:3 ratio of plasmid and PCR fragment is recommended and was calculated using the NEBioCalculator. The fragment DNA concentration was measured at a OD_{260} (Method 3.8) which led to the following cloning mix shown below.

	sample	**control**		
Reaction master mix	1	1	µL	Linearized pMiniT2 Plasmid
	5	1	µL	Sample/control
	1	1	µL	Cloning mix 1
	3	3	µL	Cloning mix 2
	-	4	µL	ddH_2O
	10	10	µL	

Incubation was carried out at 25 °C for 15 min then transferred on ice and incubated for 2 min. Afterwards, the reaction mixtures were transformed into *E. coli* DH5α (Method 3.2). From the transformed bacteria, five colonies were picked and plasmid DNA was purified as described before (Method 3.6). For sequencing, the DNA was sent to LGC Genomics using the pMiniT2seq primer (Method 3.3).

4. Results

4.1. Cloning of the LentiCRISPRv2-mCherry-3EGFR plasmid

Two plasmids containing triple sgRNA CRISPR-Cas system were generated. Additionally, the mCherry reporter was replaced with the E2-Crimson reporter to study differences in fluorescence in transfected and infected cells.

As a template, the LentiCRISPRv2-mCherry plasmid (Addgene #99154) encodes for a full CRISPR-Cas9 system. This includes the U6 promoter for the transcription of the sgRNA, the single-molecule combination of the spacer sequence and gRNA scaffold as well as the EF-core-promoters for the transcription of Cas9 and mCherry. The gRNA scaffold on the LentiCRISPRv2 plasmid combines the crRNA and tracrRNA. Normally a Golden Gate cloning method is applied which uses BsmBI restriction sites to clone one spacer RNA between the U6 promoter and the gRNA scaffold.

In this work, three sgRNAs were cloned into the BsmBI restriction site. For a functioning CRISPR-Cas9 system, each spacer needs its own U6 promoter and gRNA scaffold. Therefore, two fragments were designed to encode for the three sgRNAs combined with two more U6 promoters. To achieve this, primers were designed to amplify the U6 promoter and gRNA scaffold separately with BsmBI restriction sites at the start of the U6 promoter and at the end of the gRNA scaffold (Figure 13.1). BsmBI-v2 was selected as an enzyme because it recognizes asymmetric DNA sequences and cleaves outside this recognition sequence. Thus, during BsmBI restriction, the recognition sites for BsmBI were excised from the fragments and the plasmid, which prevents any BsmBI restriction activity in the subsequent cloning steps. Furthermore, the overhang can be designed to ligate fragments in the correct 5’-to-3’ orientation. In this case the end of the gRNA scaffold was ligated to the start of the U6 promoter producing one fragment containing both genes (Figure 13.2 and 13.3). The fragment was then used for two separate PCR amplifications. In the first amplification, a forward primer was used which includes the DNA sequence for the sgRNA1 (Figure 13.4, green) as well as a BsmBI recognition site (red) at the start. The reverse primer includes the first half of the sgRNA2 (Figure 13.4, blue) and another BsmBI recognition site. In the second amplification the forward primer included the second half of the sgRNA2 (Figure 13.4, blue) as well as a BsmBI recognition site. The reverse primer includes the sgRNA3 (Figure 13.4, orange) and a BsmBI recognition site. After amplification (Figure 13.5), the fragments were digested with BsmBI (Fig. 13.6). Due to the unique overhangs generated, the two fragments could immediately be ligated into the BsmBI digested LentiCRISPRv2-mCherry plasmid (Figure 13.6).

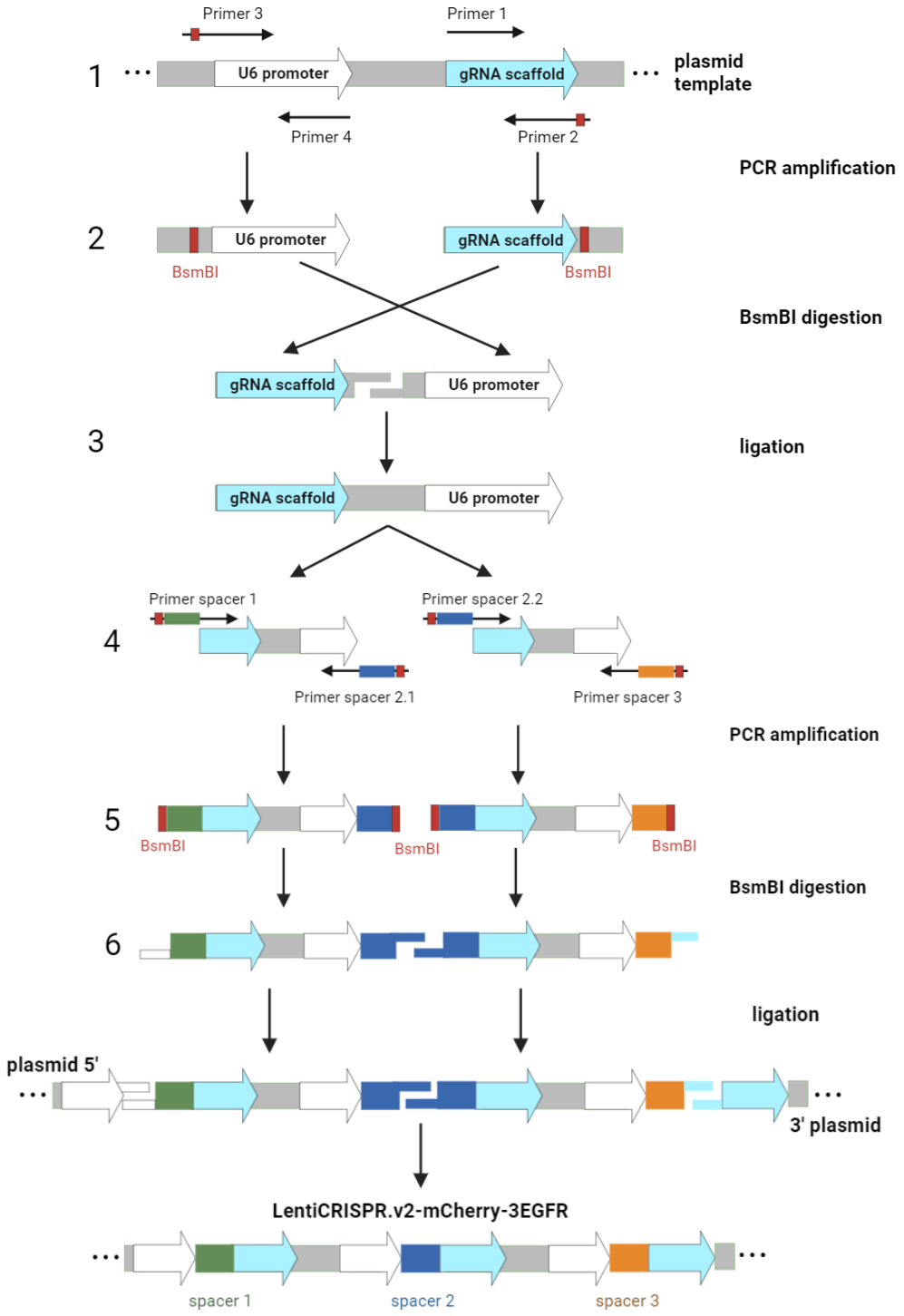

Figure 13 **LentiCRISPRv2-mCherry-3EGFR cloning.**

1 Primers were designed to amplify the gRNA scaffold and U6 promoter and to introduce BsmBI restriction sites at the start of the U6 promoter and end of the gRNA scaffold.
2 The two fragments were BsmBI digested.
3 BsmBI sticky ends match and are ligated to generate one U6 fragment.
4 From this template, two separate PCRs were run. One including the spacer 1 and spacer 2.1 primers. The second PCR used spacer 2.2 and spacer 3 primers. Each primer included a BsmBI restriction site and the spacer sequence for sgRNA 1,2 and 3.
5 Both fragments were digested with BsmBI leading to unique overhangs.
6 The two fragments were ligated into the BsmBI digested LentiCRISPRv2-mCherry plasmid. (Ligation can only occur in one direction as the BsmBI sticky ends are different)

4.1.1. Primer design

Scaffold and U6 promoter amplification

For the cloning protocol, primers were needed to amplify the gRNA scaffold and the U6 promotor separately. Here it was necessary to design BsmBI recognition sites at the end of the gRNA scaffold fragment and at the beginning of the U6 promoter fragment to digest and ligate the two fragments together to generate an scU6 fragment needed to integrate more than one sgRNA. For that, the needed base pair amount was calculated between the scaffold and U6 promoter. Then BsmBI restriction sites were designed to generate sticky ends. Additionally, annealing temperatures for the primer pairs were kept as similar as possible during design. The only restriction for primer design was that they could not be longer than 60 bp. In Table 13, forward and reverse primers for the gRNA scaffold and U6 promoter amplification are displayed. The recognition sites for BsmBI are highlighted in green. Since the recognition sites of BsmBI are outside of the restriction site, successful digestion and ligation lead to the excision of the recognition sits, resulting in a finished fragment lacking them.

sgRNA-fragment amplifications

For the triple sgRNA plasmid, three sgRNAs were needed. For that, the EGFR sgRNAs were used which were designed during my bachelor thesis (Table 13)[49]. The BsmBI recognition sites were not part of the cleaved, amplified fragments as they were removed during digestion. This means that several such fragments could be produced and assembled without being cut by BsmBI in the final DNA construct. Additionally, the sgRNAs needed to be integrated as well as enough base pairs to overlap with the scU6 fragment. Here the primer design from Cao et. al. was adapted considering their similar approach on designing a multi sgRNA system with a similar CRISPR vector[74]. The primers used are shown in Table 14. In Table 14, the sgRNA target sequences are highlighted in green, blue and orange. The BsmBI recognition sites are shown in red.

Table 13 EGFR spacer sequences with integrated GBM specific mutations.

Name	Sequence (5'-3')	PAM	Mutation	Amino acid change
Spacer 1	TCAAGATCACAGATTTTGGG	(C**G**G)	T2572G	Leu858Arg
Spacer 2	ACCTGCCCGGCAG**T**AGTCAT	(GGG)	G1793T	Gly598Val
Spacer 3	GCCGAACGCACCGGAG**T**CC	(CGG)	G2156A	Gly719Asp

Table 14 **Primers for PCR amplification.**
The primers were ordered as oligonucleotides from Metabion at a concentration of 100 µM (desalted). Upon arrival, the oligonucleotides were stored at -20 °C.

Name	Description	Sequence
gRNA scaffold amplification		
Primer 1	Amplification gRNA scaffold fragment forward primer	**ACGACGACGACGCTCTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCG**
Primer 2	Amplification gRNA scaffold fragment reverse primer, with BsmBI restriction site (red)	**ATATATATACGTCTCGCCTCACTGACGGGCACCGGAGCC**
U6 Promoter amplification		
Primer 3	Amplification U6 promoter fragment forward primer, with BsmBI restriction site (red)	**ACGACGACGCGTCTCGGAGGGCCTATTTCCCATGATTCCTTCATATTTGCAT**
Primer 4	Amplification U6 promoter fragment reverse primer	**ACGACGACGACGACGACGATTTCTCCGGTGTTTCGTCCTTTCCACAAGATA**
sgRNA fragment amplification		
Primer spacer 1	Amplification scU6 fragment forward primer with spacer 1 (green), with BsmBI restriction site (red)	**ACGCGTCTCACACCGTCAAGATCACAGATTTTGGGGTTTTAGAGCTAGAAATAGCAAGTT**
Primer spacer 2.1	Amplification scU6 fragment reverse primer with spacer 2.1 (blue), with BsmBI restriction site (red)	**ACGCGTCTCCTGCCGGGCAGGTCGGTGTTTCGTCCTTTCCAC**
Primer spacer 2.2	Amplification scU6 fragment forward primer with spacer 2.2 (blue), with BsmBI restriction site (red)	**ACGCGTCTCCGGCAGTAGTCATGTTTTAGAGCTAGAAATAGC**
Primer spacer 3	Amplification scU6 fragment reverse primer with spacer 3 (orange), with BsmBI restriction site (red)	**ACGCGTCTCAAAACTGCCGAACGCACCGGAGTCCCGGTGTTTCGTCCTTTCCAC**

4.1.2. PCR amplification of the gRNA scaffold and U6 promoter regions

The LentiCRISPRv2-mCherry plasmid was used as a template to amplify the gRNA scaffold and U6 promoter separately (Figure 14). For the gRNA scaffold amplification, Primer 1 and Primer 2 were used in the reaction master mix. For the U6 promoter amplification Primer 3 and Primer 4 were used. The fragment size of the gRNA scaffold (fragment 2) is 155 bp and the size of the U6 promoter fragment (fragment 1) is 273 bp long. For the ligation of both fragments, two BsmBI-v2 recognition sites were included in the Primer 2 and 3 (Figure 9, red).

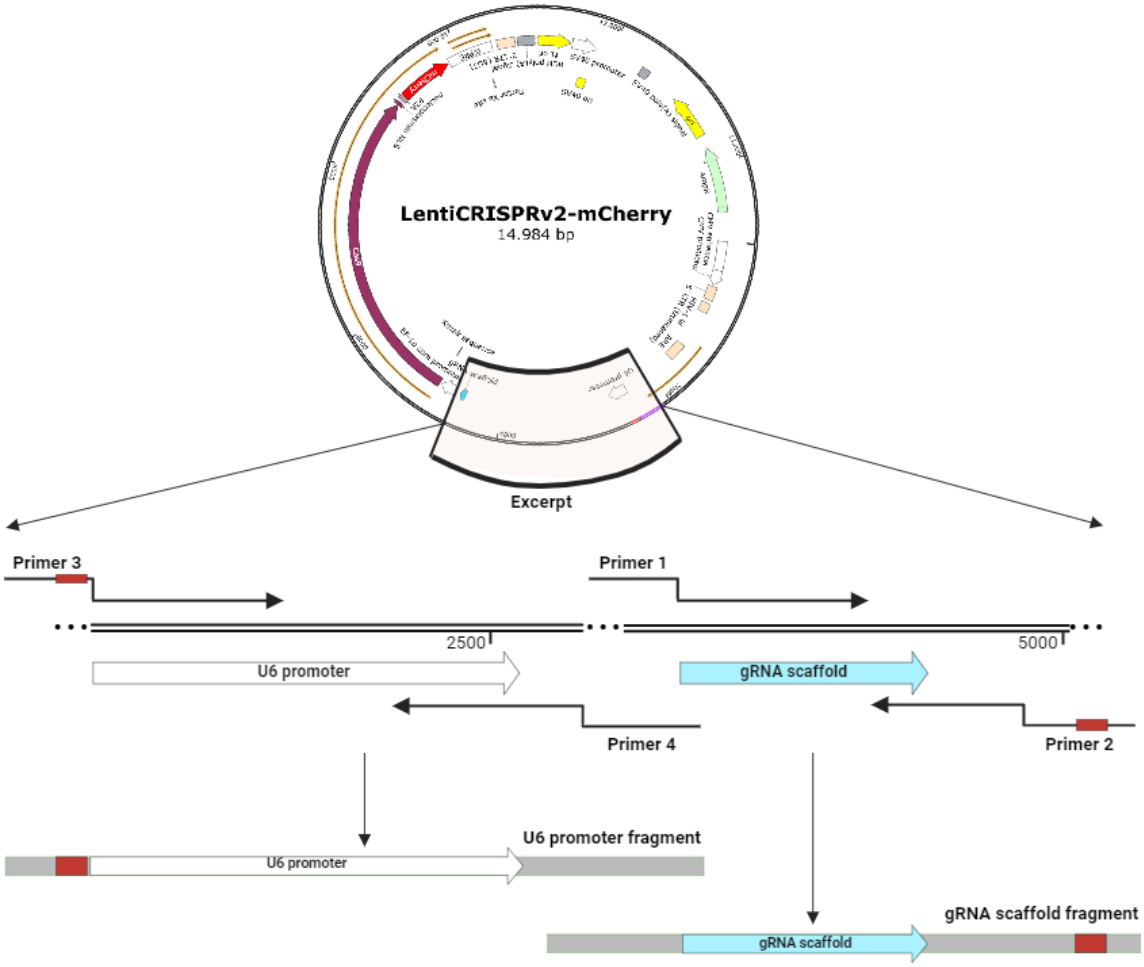

Figure 14 **Strategy for U6 and gRNA isolation from LentiCRISPRv2-mCherry.**
To generate the fragments between the three sgRNAs, a fragment containing the U6 promoter and gRNA scaffold needed to be generated. For that the LentiCRISPRv2-mCherry plasmid was used as a template. Two primers for the U6 promoter were designed which amplified only the promoter (U6 promoter fragment) containing a BsmBI restriction site in the forward primer. The gRNA scaffold was amplified with a BsmBI restriction site in the reverse primer (gRNA scaffold fragment).

(The figure was designed using BioRender.com)

Reaction master mix	32.5	µL	ddH_2O
	10.0	µL	5x Phusion HF reaction buffer
	2.5	µL	Primer 1 or 3 (10 µM)
	2.5	µL	Primer 2 or 4 (10 µM)
	1.0	µL	LentiCRISPRv2-mCherry (1 µg/µL)
	0.5	µL	Phusion HF DNA polymerase (2 U/µL)
	1.0	µL	dNTP
	50.0	µL	

Thermocycler protocol	Step 1	98 °C, 30 s	35 cycles
	Step 2	55 °C, 30 s	
	Step 3	72 °C, 1 min	
	Step 4	15 °C, hold	

The two fragments were applied to gel electrophoresis (Figure 15, Method 22). The gRNA scaffold fragment and U6 promoter fragment were detected at the expected positions for their size, cut out of the gel and purified using Method 3.5.

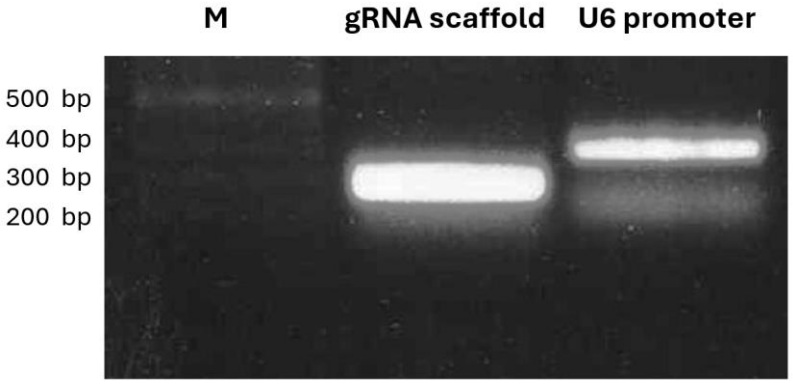

Figure 15 **Gel electrophoresis of gRNA scaffold and U6 promotor PCR fragments.**
The gel showed the amplified gRNA scaffold fragment and the amplified U6 promoter fragment compared to a 100 bp marker from Thermo Scientific (#SM0321) (M). The theoretical size of the gRNA scaffold fragment was 155 bp and the theoretical size of the U6 promoter fragment is 273 bp. A 0.9% agarose gel was used and run at 140 V for 20 min.

4.1.3. Ligation of the gRNA scaffold and U6 promoter fragments

One fragment which contains the gRNA scaffold and one fragment which contained the U6 promoter were purified using the PCR purification kit (see Method 3.5). The fragments were then digested with BsmBI (Figure 16, Step 1).

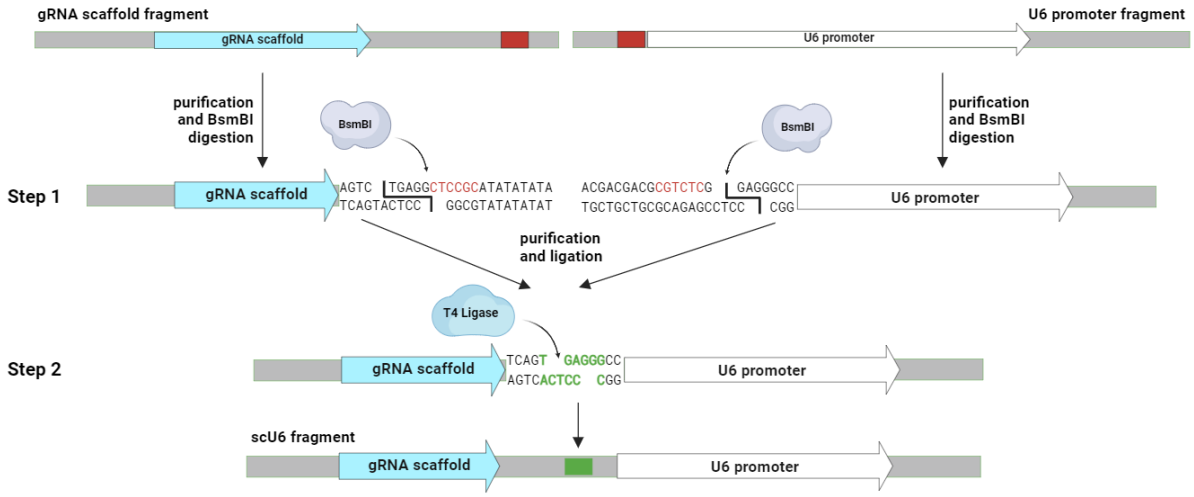

Figure 16 **BsmBI digestion and ligation of gRNA scaffold and U6 promoter.**
The two fragments were purified using a PCR purification kit and digested with BsmBI at 55 °C. The enzyme was inactivated, and the two fragments were purified. The fragments were ligated overnight using the T4 DNA ligase. Ligation led to the scU6 fragment which will further be used in sgRNA assembly via PCR.

(The figure was designed in BioRender.com)

Digestion mix

1	µL	BsmBI (10 U/µL)
20	µL	Purified fragment 1
20	µL	Purified fragment 2
5	µL	10 x NEB r3.1 reaction buffer
4	µL	ddH_2O
50	µL	

Digestion was performed at 55 °C for 20 min. The BsmBI restriction enzyme was then deactivated at 80 °C for 20 min (Thermocycler, Biometra).

The digested fragments were loaded on an agarose gel and run at 140 V for 20 min (Method 3.4). The two DNA bands (273 bp for fragment 1 and 155 bp for fragment 2) were cut from the gel and transferred into a 1.5-mL reaction tube. The two gel fragments were purified according to the method described in section 3.5. Ligation of the two plasmids was performed using T4 DNA ligase overnight at 16 °C (Thermocycler, Biometra) (Figure 16, step 2). The T4 DNA ligase was deactivated afterwards at 80 °C for 20 min (Thermocycler, Biometra).

Ligation mix

1	µL	T4 DNA ligase (1 U/µL)
20	µL	Purified fragment 1
20	µL	Purified fragment 2
5	µL	10 x T4 DNA ligase buffer (NEB, 10 mM ATP)
4	µL	ddH_2O
50	µL	

After ligation, the DNA fragments in the ligation mix were separated using a 0.9% agarose gel (140 V for 20 min) (Method 3.4, Figure 17). Since not all gRNA scaffold fragments and U6 promoter fragments were completely ligated, the DNA band appearing at a gel position between 400 and 500 bp, as shown in Figure 17 (red frame) was cut from the gel and further purified as described (Method 3.5). The expected size of the scU6 fragment was 428 bp.

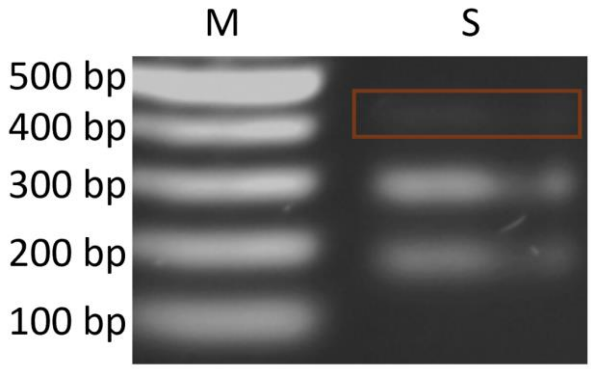

Figure 17 **Ligation of gRNA scaffold and U6 promoter fragments.**
The gel showed the ligation reaction of the gRNA scaffold and U6 promoter fragments after BsmBI digestion. The theoretical size of the ligated U6sc fragment was 428 bp. A 0.9% agarose gel was used and run at 140 V for 20 min.

M 100 bp marker from Thermo Scientific (#SM0321) (2 µL)
S gRNA scaffold and U6 promoter fragment ligation mixture (5 µL)

4.1.4. Amplification of the scU6 fragment with sgRNA primers

The purified scU6 fragment was used as a template for two separate PCR amplifications (Figure 18). PCR A was performed with the two primers spacer1 and spacer2.1. Primer spacer2.1 is contained the first half of the spacer sequence 2. PCR B was performed with the spacer2.2 primer containing the second half of the spacer sequence 2 and the spacer3 primer containing the complete spacer sequence 3.

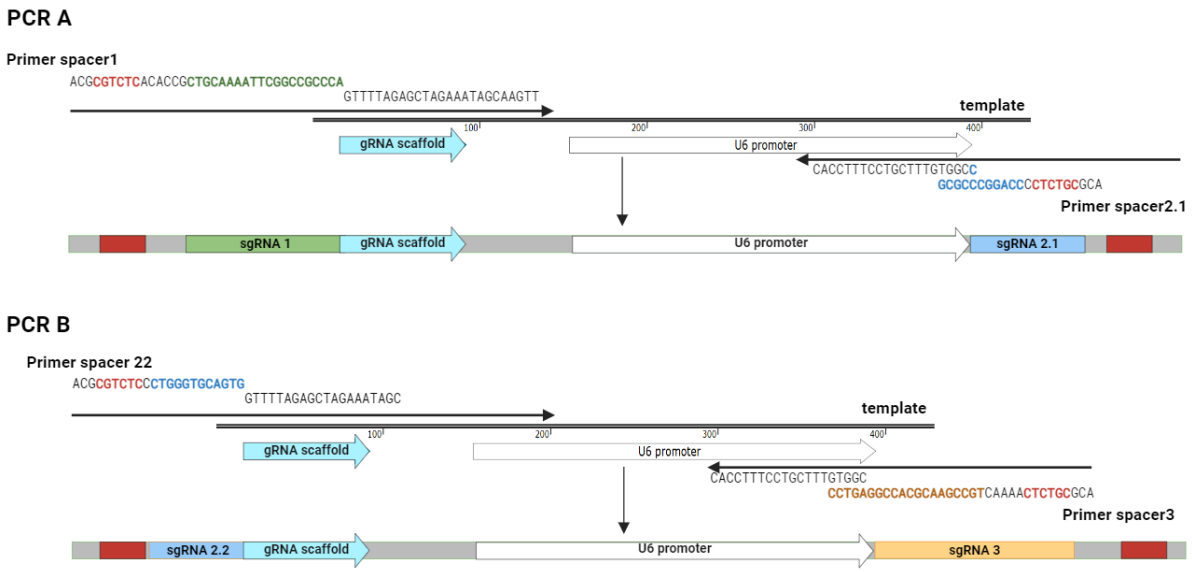

Figure 18 **PCR assembly of the spacer sequences 1-3.**

A This amplification contained the scU6 fragment as a template. Amplification was performed with the Primer spacer1 and Primer spacer2.1. Both primers have a BsmBI restriction site (red) as well as spacer 1 (green) or the first half of spacer 2 (blue). Each amplification was done with an optimal amplification temperature for both primers. The amplified sgRNA1/2.1 fragment was purified and used for ligation.

B In the second amplification the scU6 fragment was used as a template. Amplification was performed with the Primer spacer1 and Primer spacer2.1. Both primers have a BsmBI restriction site (red) as well as spacer 2.2 (blue) or the first half of spacer 3 (orange). Each amplification was done with an optimal amplification temperature for both primers. The amplified sgRNA2.2/3 fragment was purified and used for ligation.

BsmBI-v2 restriction sites were designed to enable cloning into the LentiCRISPRv2 plasmid in the right order from 5' to 3'. (The figure was designed in BioRender)

Reaction master mix

28.5	µL	ddH_2O
10.0	µL	5x Phusion HF reaction buffer
2.5	µL	Primer spacer 1 or Primer spacer 2.2 (10 µM)
2.5	µL	Primer spacer 2.1 or Primer spacer 3 (10 µM)
5.0	µL	Purified scU6 fragment
0.5	µL	Phusion HF DNA polymerase (2U/µL)
1.0	µL	dNTP
50.0	µL	

Thermocycler protocol	Step 1	98 °C, 30 s	35 cycles
	Step 2	55 °C, 30 s	
	Step 3	72 °C, 1 min	
	Step 4	15 °C, hold	

The sgRNA1/2.1 and sgRNA2.2/3 fragments were applied to gel electrophoresis (Figure 19, Method 22). The amplified fragments were detected at the expected positions in the gel, cut out of the gel and purified using Method 3.5.

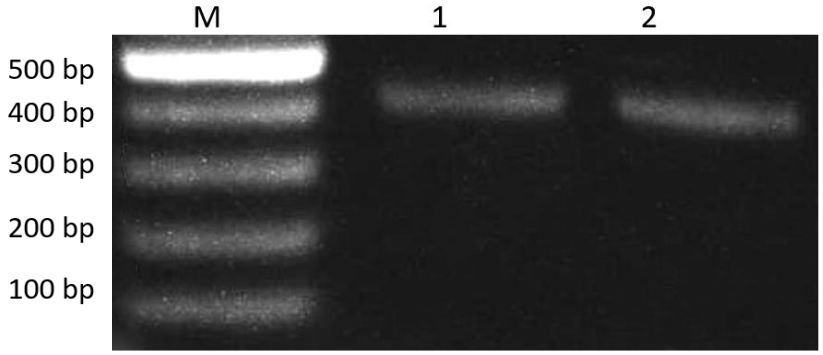

Figure 19 **Gel electrophoresis of amplified sgRNA1/2.1 and sgRNA2.2/3 fragments.**
The gel showed the sgRNA1/2.1 amplified fragment (1) and sgRNA2.2/3 fragment (2) compared to the 100 bp marker (M). The theoretical size of the fragments was 418 bp. A 0.9% agarose gel was used and run at 140 V for 20 min.

M	100 bp marker from Thermo Scientific (#SM0321) (4 µL)
1	sgRNA1/2.1 fragment (2 µL)
2	sgRNA2.2/3 fragment (2 µL)

4.1.5. Assembly of the LentiCRISPRv2-mCherry-3EGFR plasmid

The LentiCRISPRv2-mCherry plasmid and the two fragments (sgRNA1/2.1 and sgRNA2.2/3) were separately digested using BsmBI (Figure 20, plasmid (1), sgRNA1/2.1 fragment (2), sgRNA2.2/3 (3)). Digestion was performed at 55 °C for 20 min and enzyme inactivation was performed at 80 °C for 20 min (Thermocycler, Biometra). Digestions were afterwards purified using the PCR purification kit (Method 3.5).

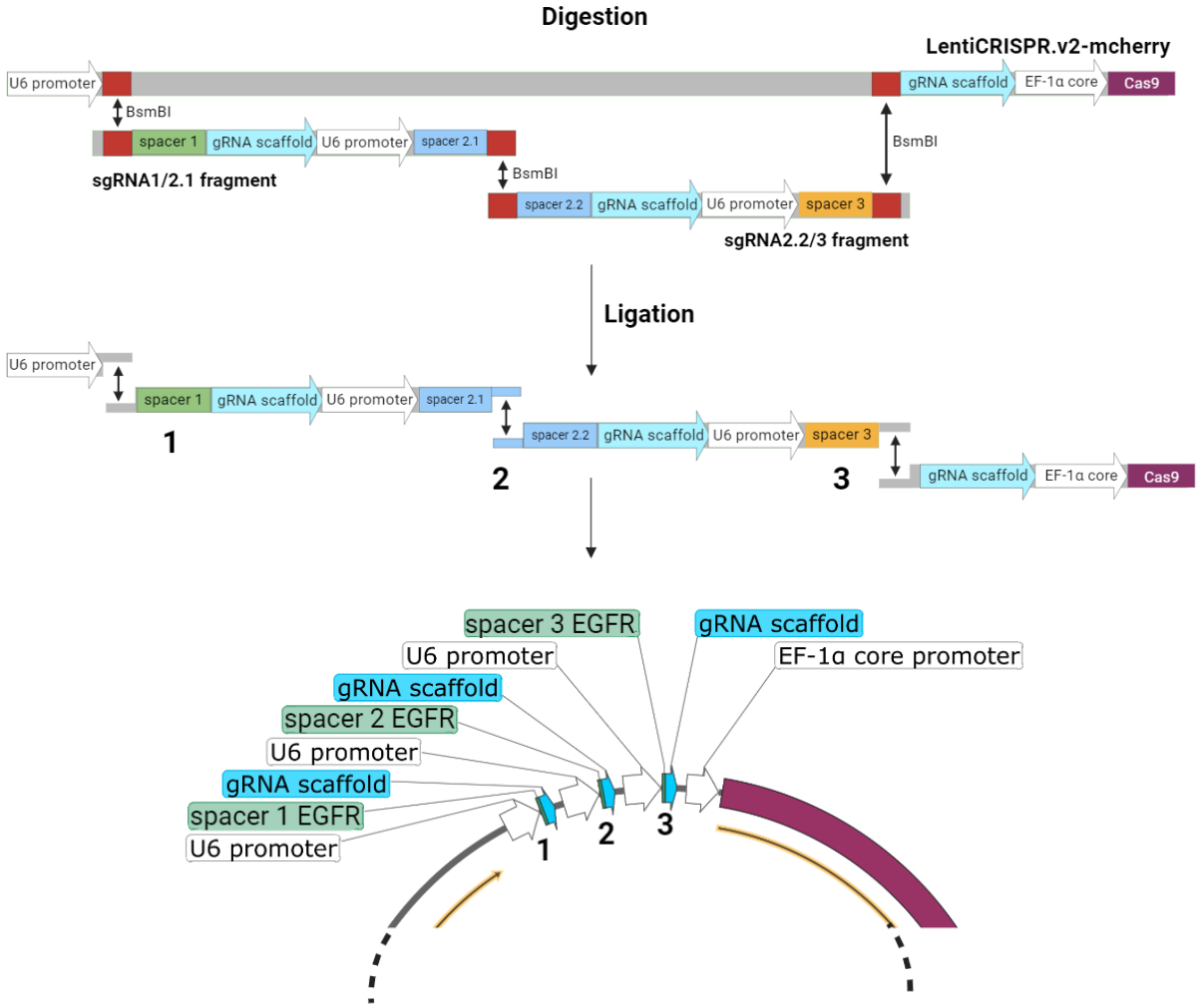

Figure 20 **Assembly of LentiCRISPRv2-mCherry-3EGFR.**
Digestion of the LentiCRISPRv2-mCherry plasmid, sgRNA1/2.1 fragment and sgRNA2.2/3 fragment with BsmBI. Ligation of the plasmid with the two fragments to the LentiCRISPRv2-mCherry-3EGFR. The sticky ends of the fragments and the plasmid only match in the order shown above.

(Plasmid map was made using Snapgene. The figure was made using BioRender.com)

Digestion mix F1R1/F2R2

1	µL	BsmBI (10 U/µL)
20	µL	Fragment (sgRNA1/2.1 or sgRNA2.2/3)
5	µL	10 x NEB r3.1 reaction buffer
14	µL	ddH_2O
40	µL	

Digestion mix plasmid

1	µL	BsmBI (10 U/µL)
2	µL	LentiCRISPRv2-mCherry (1 µg/µL)
5	µL	10 x NEB r3.1 reaction buffer
32	µL	ddH_2O
40	µL	

The purified BsmBI fragments and the BsmBI cleaved plasmid were then used in a single ligation using T4 DNA ligase. Ligation was performed at 16 °C overnight (Thermocycler, Biometra). The enzyme was inactivated at 80 °C for 20 min (Thermocycler, Biometra).

Ligation mix

1	µL	T4 DNA ligase (1 U/µL)
10	µL	sgRNA1/2.1 fragment
10	µL	sgRNA2.2/3 fragment
5	µL	Purified and digested LentiCRISPRv2-mCherry
5	µL	10 x T4 DNA ligase buffer (NEB, 10 mM ATP)
19	µL	ddH_2O
50	µL	

The ligation mix was fully transformed into *E. coli* DH5α (Method 3.2). From the 11 cultivated colonies, all were picked after transformation and used for mini plasmid extraction (Method 3.6). The plasmid DNA was used for sequencing (Method 3.3). Two colonies showed the right sequencing results (Figure 21). The DNA of one colony was then used for another transformation (Method 3.2) and maxi plasmid extraction (Method 3.7). The plasmid DNA was then stored at -20 °C. In Figure 21, the sequencing result is compared to the theoretical DNA sequence of the triple sgRNA LentiCRISPRv2-mCherry vector. The sequencing results showed full alignment to the theoretical plasmid, with no mutations. The full sequencing results are given in appendix 10.2.

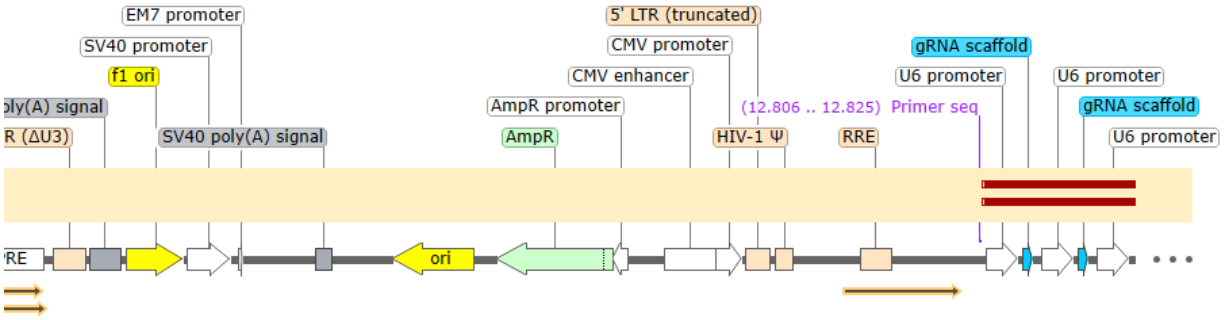

Figure 21 **Alignment sequencing results of LentiCRISPRv2-mCherry-3EGFR.**
The sequencing results were aligned with the theoretical sequence of the LentiCRISPRv2-mCherry-3EGFR plasmid. From 11 colonies, two showed the correct sequence. Aligned sequences are shown in red and include all three sgRNAs, their U6 promotors and gRNA scaffolds. Alignment was performed using Snapgene. The full sequence is shown in appendix 10.2.

4.2. Cloning of the triple BIRC5-sgRNA CRISPR vector

Since the efficacy and overall procedure of the cloning method for the LentiCRISPRv2-mCherry-3EGFR plasmid gave low yields, another method for sgRNA cloning was applied. For this method two synthetic DNA fragments (Figure 22.1) were ordered from Eurofins who synthesized and delivered the BIRC5 fragments as BsmBI inserts in the pEX-A (#187600) plasmid. This method simplified the cloning process by shortening the number of steps.

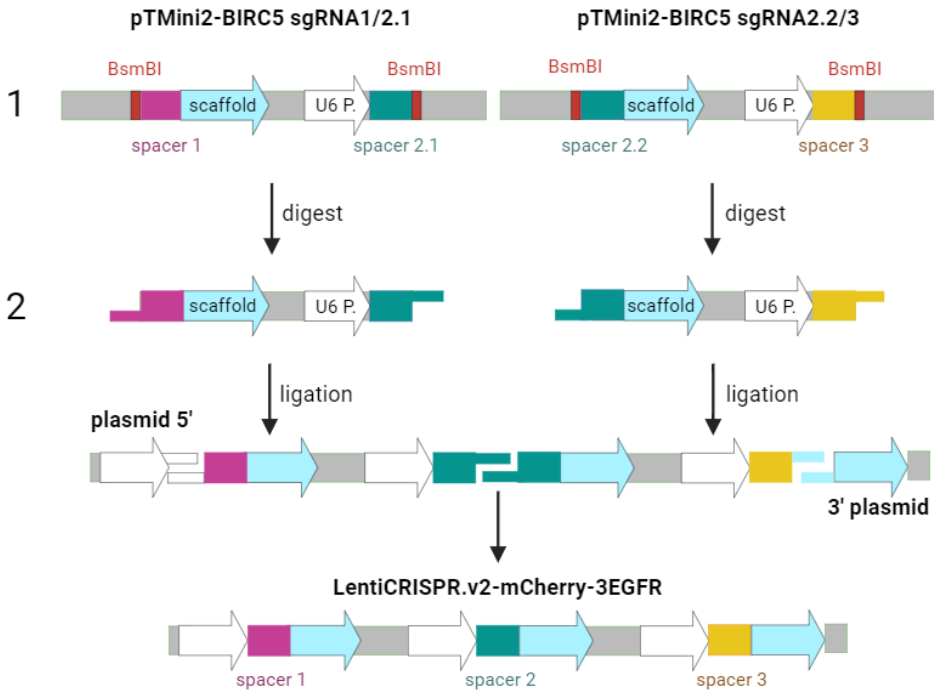

Figure 22 LentiCRISPRv2-mCherry-3EGFR cloning.

1 Two fragments were designed. The first fragment had the genes for the spacer 1 (pink), gRNA scaffold (light blue), U6 promoter (white) and spacer 2.1 (blue). The second fragment had the genes for the spacer 2.2 (blue), gRNA scaffold (light blue), U6 promoter (white) and spacer 3 (yellow). The fragments were ordered in a pTMini2 plasmid with BsmBI-v2 recognition sites at each end (red). The restriction overhangs were designed to match the overhangs between spacer 2.1 and spacer 2.2 and the overhangs from the LentiCRISPRv2 plasmid after BsmBI-v2 digestion.

2 The digested fragments could then be ligated in one specific direction into the LentiCRISPRv2-mCherry plasmid.

4.2.1. BIRC5 triple sgRNA fragment design

The fragments were designed with BsmBI restriction sites at the 5'- and 3'-end. After BsmBI restriction, the different sticky ends will match to ligate both fragments 1 and 2 at the same time into the LentiCRISPRv2-mCherry vector. The design for fragment 1 is shown in Figure 23 and the design for fragment 2 is shown in Figure 24. Both fragments were designed and ordered from Eurofins. Fragment 1 contained the sequence for the first spacer (red) and one gRNA scaffold and U6 promoter. Fragment 2 contained the sequence for the second and

third spacer (red) as well as one gRNA scaffold and U6 promoter. Ligation was carried out with the LentiCRISPRv2-mCherry and the two fragments.

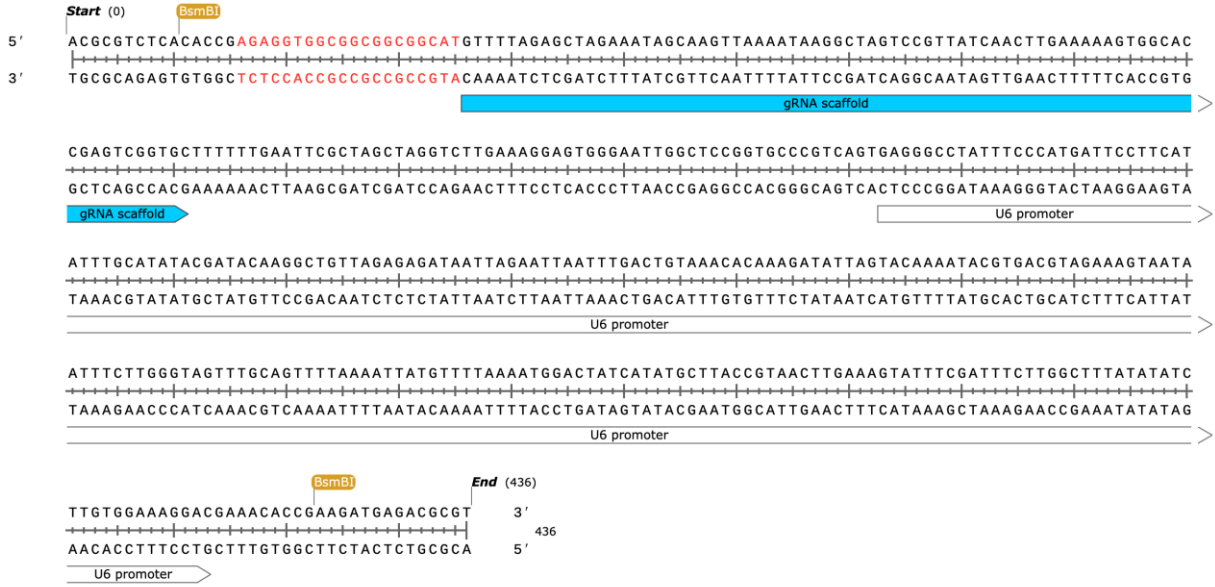

Figure 23 **Fragment 1 design for LentiCRISPRv2-mCherry-BIRC5 cloning.**
The fragment had two BsmBI restriction sites to be cloned together with fragment 2 into the Golden Gate BsmBI sites of the LentiCRISPRv2-mCherry plasmid. The first spacer is marked in red. (The sequence maps were exported using Snapgene)

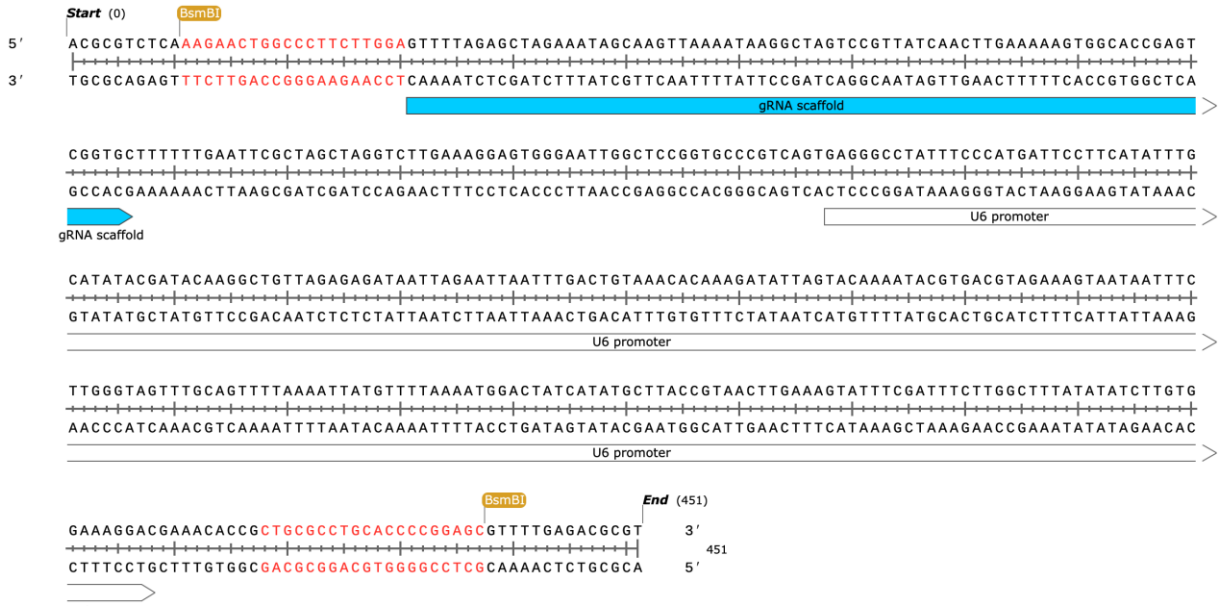

Figure 24 **Fragment 2 design for LentiCRISPRv2-mCherry-BIRC5 cloning.**
The fragment had two BsmBI restriction sites to be cloned together with fragment 1 into the Golden Gate BsmBI sites of the LentiCRISPRv2-mCherry plasmid. The second and third spacers are marked in red. (The sequence maps were exported using Snapgene)

4.2.2. BsmBI restriction of sgRNA inserts and plasmid

The LentiCRISPRv2-mCherry plasmid and the two sgRNA inserts, called fragments 1 and 2 were each digested with BsmBI. Digestion was performed at 55 °C for 20 min and enzyme inactivation was performed at 80 °C for 20 min (Thermocycler, Biometra). The three digestions were afterwards applied to agarose gel electrophoresis (Figure 25), and the sgRNA fragments at the 450 bp position were cut from the gel (Fig. 25, red frame) and purified using the PCR purification kit (Method 3.5).

Digestion mix fragments	1	µL	BsmBI-v2 (10 U/µL)
	5	µL	Fragment (1 or 2)
	5	µL	10 x NEB r3.1 reaction buffer
	29	µL	ddH_2O
	40	µL	

Digestion mix plasmid	1	µL	BsmBI-v2 (10 U/µL)
	2	µL	LentiCRISPRv2-mCherry (1 µg/µL)
	5	µL	10 x NEB r3.1 reaction buffer
	32	µL	ddH_2O
	40	µL	

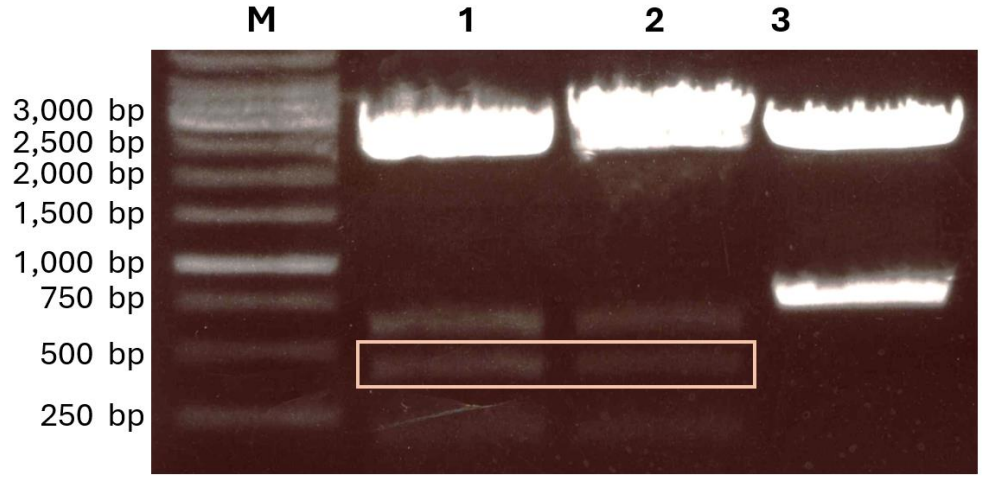

Figure 25 **Isolation of BsmBI fragments for BIRC5 sgRNA.**
The gel showed the BsmBI restriction of the pEX-A plasmids with the BIRC5 fragment 1 (1) and BIRC5 fragment 2 (2). The theoretical size of fragment 1 was 436 bp and the theoretical size of fragment 2 is 451 bp (red frame). Both fragments were cut from the agarose gel for purification (0.9% agarose, 140 V, 20 min).

M 1 kb DNA ladder from Fermentas (#SM0311) (4 µL)
1 BsmBI restriction pEX-A-BIRC5-fragment 1 (50 µL)
2 BsmBI restriction pEX-A-BIRC5-fragment 2 (50 µL)

The purified fragments and plasmids were then used in a single ligation using the T4 DNA ligase. Ligation was performed at 16 °C overnight (Thermocycler, Biometra). The enzyme was inactivated at 80 °C for 20 min (Thermocycler, Biometra).

Ligation mix		
1	µL	T4 DNA ligase
10	µL	Purified and BsmBI-v2 digested fragment 1
10	µL	Purified and BsmBI-v2 digested fragment 2
5	µL	Purified and BsmBI-v2 digested LentiCRISPRv2-mCherry
5	µL	10 x T4 DNA ligase buffer
19	µL	ddH_2O
50	µL	

The ligation mix was fully transformed into *E. coli* DH5*α* (Method 3.2). Five of the 53 colonies were picked after transformation and used for mini plasmid purification (Method 3.6). The plasmid DNA was used for sequencing (Method 3.3). Three colonies showed the correct sequence (Figure 26). The DNA of one colony was then used for another transformation (Method 3.2) and followed by maxi plasmid extraction (Method 3.7). The plasmid DNA was then stored at -20 °C. In Figure 26, the sequencing results are compared to the theoretical DNA sequence of the LentiCRISPRv2-mCherry-BIRC5 vector. The full sequencing result is given in appendix 10.3.

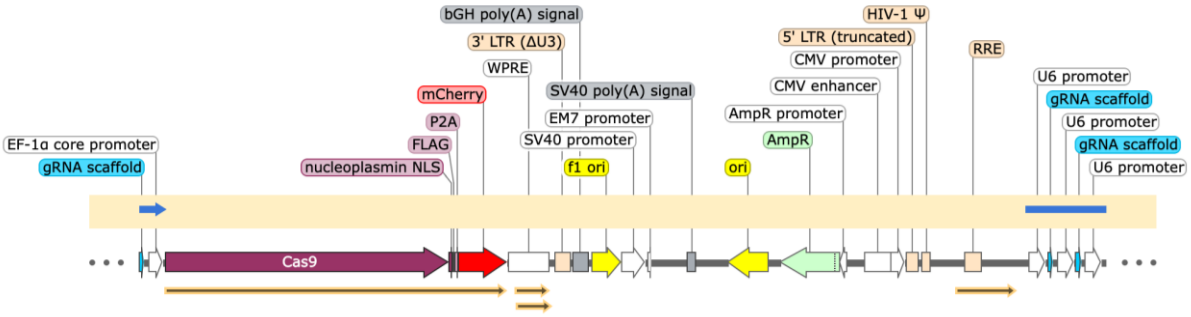

Figure 26 **Alignment sequencing results LentiCRISPRv2-mCherry-3BIRC5.**
The sequencing results were aligned with the theoretical sequence of the LentiCRISPRv2-mCherry-3EGFR plasmid. From 11 colonies picked, two showed the right sequence. Aligned sequences are shown in blue and include all three sgRNAs, their U6 promotors and gRNA scaffolds. Alignment was performed in Snapgene. The full sequence can be found in appendix 10.3.

4.3. Exchange of the mCherry reporter with E2-Crimson

The mCherry reporter is known to be very sensitive and previous infection experiments have not shown any mCherry fluorescence. It was therefore planned to replace the mCherry reporter with the more stable E2-Crimson reporter.

4.3.1. E2-Crimson fragment design

To exchange the mCherry reporter with the E2-Crimson reporter, two restriction sites were found at each site of the mCherry gene. For restriction the BamHI and BsrGI enzymes were chosen. Due to the location of the BamHI enzyme, the P2A sequence is also cut out of the LentiCRISPRv2 vector and must be included with the E2-Crimson fragment. The E2-Crimson was then designed to match the restriction sites of the LentiCRISPRv2 vector (Figure 27).

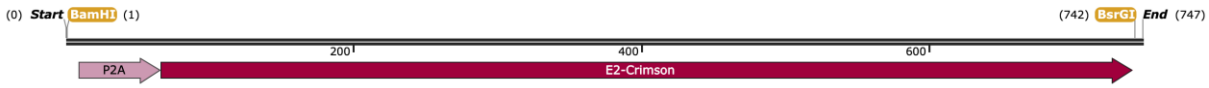

Figure 27 **E2-Crimson fragment design for ligation into LentiCRISPRv2-mCherry.** The E2Crimson fragment was designed to have the P2A sequence which is needed to translate the reporter together with the CRISPR sequence. The restriction sites for cloning were matched to the restriction sites on the LentiCRISPRv2 vector. The fragment is cut out of the pEX-E2-Crimson vector using BsrGI and BamHI. The sequence figure was designed in Snapgene.

4.3.2. BsrGI and BamHI digestion and ligation of fragment and vector

The pEX-E2-Crimson, LentiCRISPRv2-mCherry and both triple LentiCRISR.v2-mCherry plasmids were used for restriction with BsrGI and BamHI. In Figure 28 the main cloning protocol is shown. The E2-Crimson and P2A gene were cut out from the pEX-E2-Crimson plasmid and the mCherry and P2A gene was taken from the LentiCRISPRv2 plasmid. Both digestions were performed with BsrGI and BamHI leading to matching sticky ends. The E2-Crimson fragment was then ligated into the LentiCRISPRv2 plasmids.

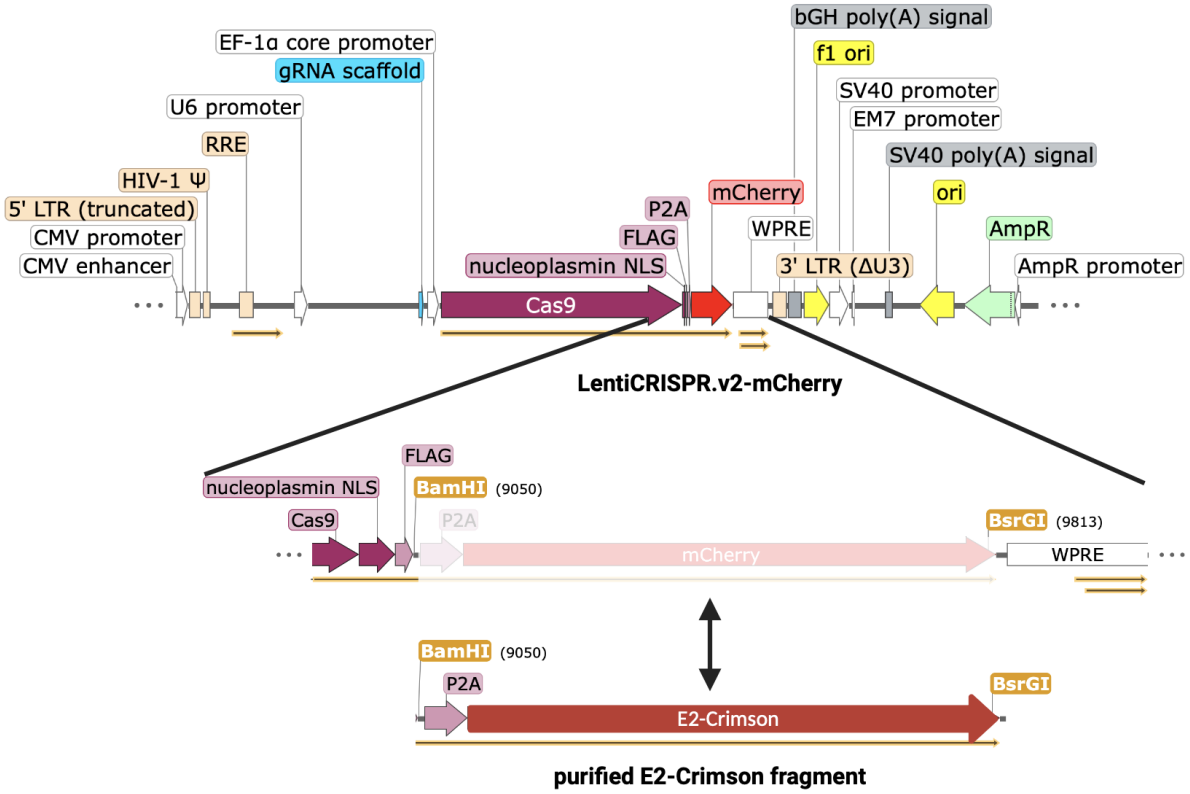

Figure 28 **Cloning procedure to exchange the mCherry reporter with E2-Crimson.** BamHI and BsrGI were used to digest the LentiCRISPRv2-mCherry plasmids and the pEX-E2-Crimson plasmid. The digested LentiCRISPRv2-mCherry plasmid and digested E2-Crimson fragment are purified and ligated.

(The sequence maps were made using Snapgene and the figure was designed using BioRender.com)

Enzymatic restriction was performed in four reactions (1, 2, 3 and 4) at 37 °C for 1 h (Thermocycler, Biometra). The restriction enzymes were inactivated at 80 °C for 20 min (Thermocycler, Biometra). The following four reactions were performed.

Digestion	**1**	**2**	**3**	**4**		
	1	1	1	1	µL	BsrGI-HF (20 U/µL)
	1	1	1	1	µL	BamHI-HF (20 U/µL)
	8	8	-	-	µL	pEX-E2-Crimson (from Mini plasmid extraction, method 3.6)
	-	-	3	-	µL	LentiCRISPRv2-mCherry (1 µg/µL)
	-	-	-	3	µL	LentiCRISPRv2-mCherry-EGFR3 (1 µg/µL)
	4	4	4	4	µL	10 x cut smart buffer
	26	26	31	31	µL	ddH_2O
	40	40	40	40	µL	

After digestion, the complete digestion mix was separated on a gel electrophoresis at 140 V for 20 min in a 0.9% agarose gel. In Figure 29 the restriction results are shown.

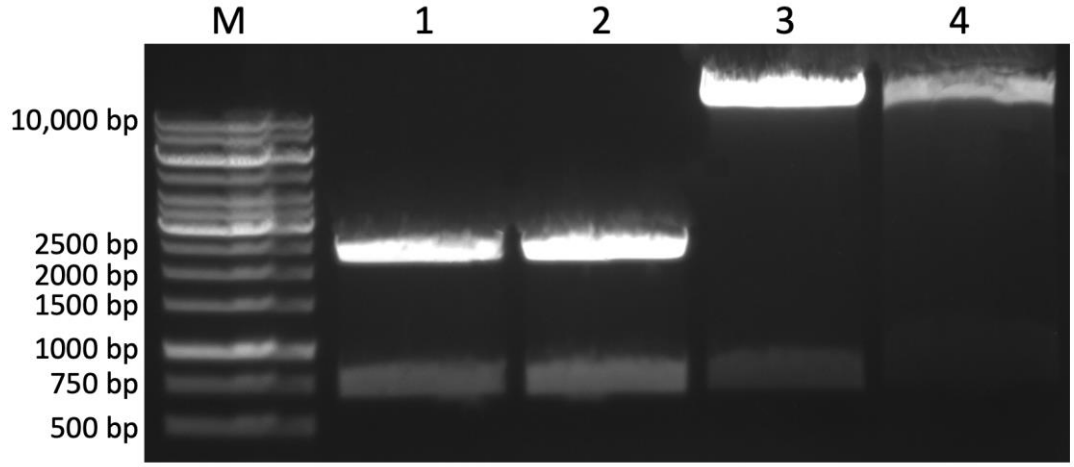

Figure 29 **BsrGI and BamHI restriction of pEX-E2-Crimson and LentiCRISPRv2.**
The gel showed digestion reactions of the pEX-E2-Crimson (1,2), the LentiCRISPRv2-mCherry (3) and LentiCRISPRv2-mCherry-EGFR3 (4) with BamHI and BsrGI. A 1 kb marker was used as comparison. A 0.9% agarose gel was used and run at 140 V for 20 min. The pEX-E2-Crimson plasmid has a size of 2450 bp. After digestion a 759 bp fragment with the E2-Crimson gene was cut out of the plasmid. The LentiCRISPRv2-mCherry plasmid had a size of 14,984 bp. After digestion the mCherry gene is cut out and the vector had a remaining size of 13,201 bp. The LentiCRISPRv2-mCherry-EGFR3 plasmid had a size of 13,938 bp. After digestion the remaining size of the vector was 13,155 bp. The E2-Crimson fragments and both cut LentiCRISPRv2 plasmids were used for further ligation.

M	1 kb marker from Fermentas (#SM0311) (2 µL)
1	pEX-E2-Crimson BamHI-HF and BsrGI-HF digest with 8 µL DNA from Mini DNA extraction (40 µL)
2	pEX-E2-Crimson BamHI-HF and BsrGI-HF digest with 10 µL DNA from Mini DNA extraction (40 µL)
3	LentiCRISPRv2-mCherry BamHI-HF and BsrGI-HF digest (40 µL)
4	LentiCRISPRv2-mCherry-3EGFR (40 µL)

The two fragments in lanes 1 and 2 at around 750 bp and the two fragments in lanes 3 and 4 were cut from the gel and used in PCR purification (Method 3.5). The purified fragments and plasmids were then used in two ligations (A and B) using the T4 DNA ligase. Ligation was performed at 16 °C overnight (Thermocycler, Biometra). The enzyme was inactivated at 80 °C for 20 min (Thermocycler, Biometra).

Ligation	**A**		**B**		
	1	µL	1	µL	T4 DNA ligase (1 U/µL)
	10	µL	10	µL	Purified and BamHI-HF and BsrGI-HF digested E2-Crimson fragment
	-	µL	10	µL	Purified and BamHI-HF and BsrGI-HF digested LentiCRISPRv2-mCherry
	10	µL	-	µL	Purified and BamHI-HF and BsrGI-HF digested LentiCRISPRv2-mCherry-EGFR3
	5	µL	5	µL	10 x T4 DNA ligase buffer (NEB, 10 mM ATP)
	24	µL	24	µL	ddH_2O
	50	µL	50	µL	

The complete ligation mixes were transformed into *E. coli* DH5α (Method 3.2). From each transformation five colonies were picked and used for plasmid extraction (Method 3.6). The DNA was then sent to sequencing using the WPRE-R primer (Method 3.3). Two clones showed the expected sequence (Figure 30). The DNA of one clone was then used for retransformation (Method 3.2) and maxi plasmid purification (Method 3.7). The plasmid DNA was then stored at -20 °C. In Figure 30 the sequencing result is compared to the theoretical DNA sequence of the triple sgRNA LentiCRISPRv2-E2-Crimson vector. The full sequencing result is given in appendix 10.4.

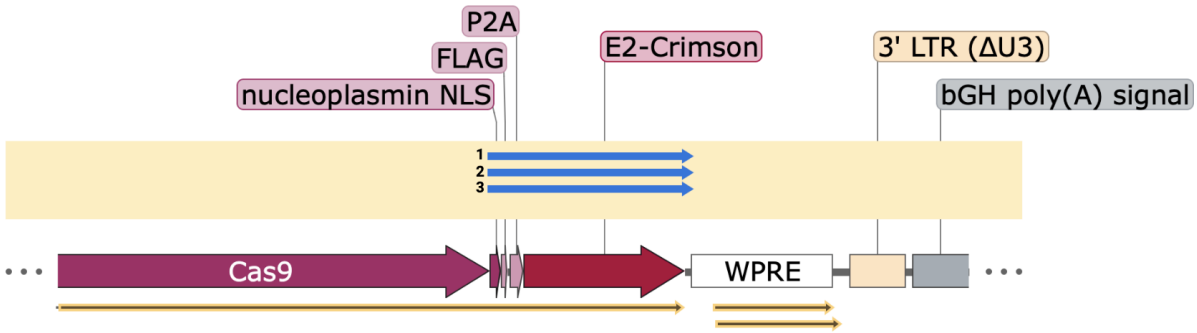

Figure 30 **Sequencing results aligned to theoretical LentiCRISPRv2-E2-Crimson.** The sequencing results of the empty LentiCRISPRv2 (1) and both triple sgRNA LentiCRISPRv2-E2-Crimson plasmids (2,3) were compared to the theoretical plasmid map in Snapgene. All three sequencing results showed the right sequence compared to the theoretical DNA sequence. The full sequence can be found in appendix 10.4.

As a control, the three candidates, vector (no sgRNA), vector including 3EGFR and 3BIRC5 were fully sequenced. The top alignment was the LentiCRISPRv2-E2-Crimson (1), the second alignment was the LentiCRISPRv2-E2-Crimson-3EGFR (2) and the third alignment is the LentiCRISPRv2-E2-Crimson-3BIRC5 (3). All sequencing results showed no mutations and were 100% identical to the original DNA template. Alignment was carried out using Snapgene.

4.4. Detection of EGFR expression

Immunofluorescence staining was performed with the EGFR primary mouse and anti goat Alexa Fluor 488 secondary antibody to detect the expression of EGFR on the cell membrane of the isolated tumor cells and HEK293T cells (Method 3.10). The cells were further stained with DAPI and fluorescence was detected using an M7000 microscope (Method 3.13).

Expression of the EGFR antibody was detected for the HEK293T and AKH-5,10,13,19,23 cells (Figure 31). Fluorescence was shown under the GFP filter while the nuclei were analyzed with the DAPI filter. The two images generated from both filters were overlapped.

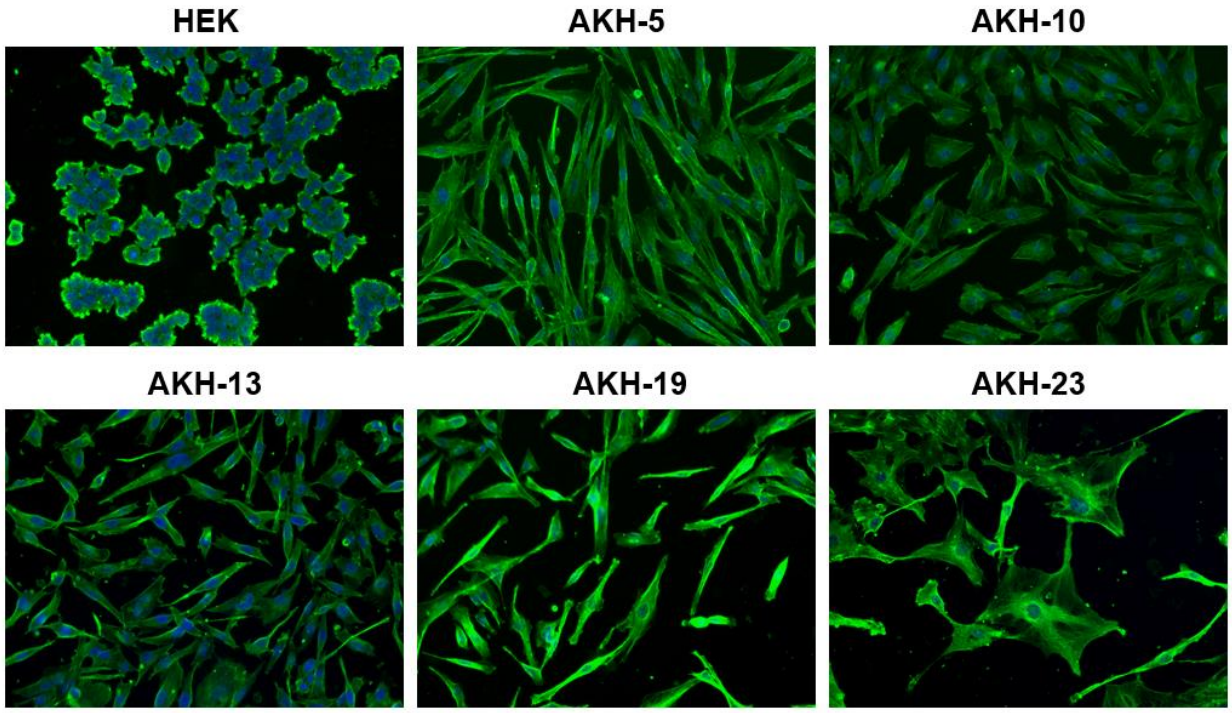

Figure 31 **Immunofluorescence images of EGFR antibody staining.**
The cells were stained with a primary and secondary antibody using the monoclonal anti EGFR goat antibody and anti goat Alexa Fluor secondary antibody. Afterwards, the cell nuclei was stained with DAPI. Images were taken at 20x magnification with the GFP (470/525 nm) and DAPI (357/447 nm) filters. The images generated with both filters were overlapped.

4.5. Transfection and infection of cells

4.5.1. Sequence verification of the packaging plasmid

Due to no results the three-plasmid infection in the beginning, the packaging vectors pCMV-dR8.2 dvpr and psPAX2 were verified using sequencing with three primers. The sequencing results are given in Figure 32 for pCMV-dR8.2 dvpr and Figure 33 for psPAX2. The theoretical sequences compared to the sequencing results matched 100%. All six sequencing results can be seen in appendix 10.5 and 10.6.

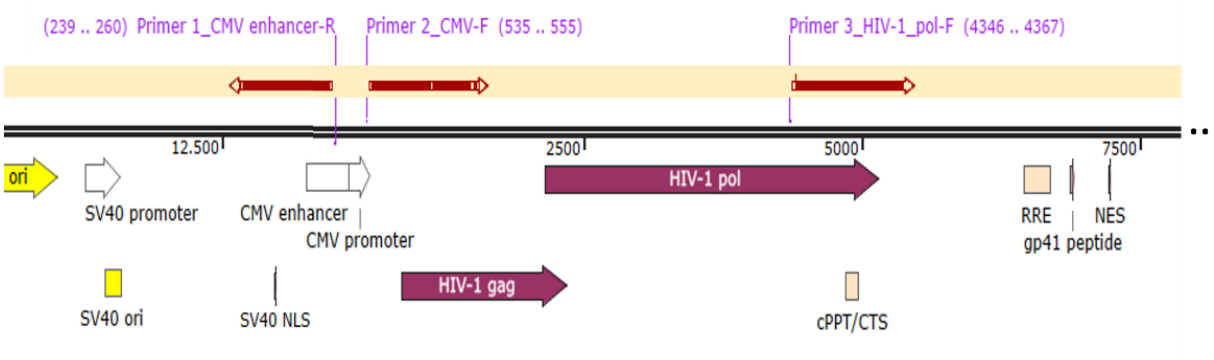

Figure 32 **Sequencing of pCMV-dR8.2 dvpr with three primers for plasmid verification.** The pCMV-dR8.2 dvpr plasmid was verified using primers to amplify the CMV enhancer (primer 1), the CMV-promoter and HIV-1 gag (primer 2) and HIV-1 pol (primer 3). Separate sequencing with each of the primers and comparison to the theoretical gene of pCMV-dR8.2 dvpr was performed using Snapgene. The full sequences can be found in appendix 10.5.

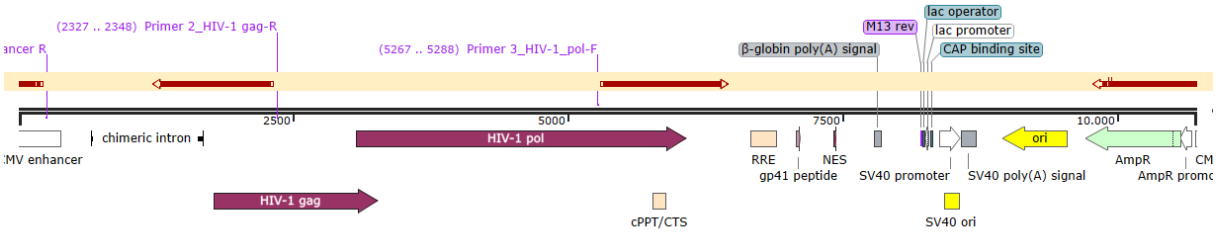

Figure 33 **Sequencing of psPAX2 with three primers for plasmid verification.** The psPAX2 plasmid was verified using primers to amplify the CMV enhancer (primer 1), the CMV-promoter and HIV-1 gag (primer 2) and HIV-1 pol (primer 3). Separate sequencing with each of the primers and comparison to the theoretical gene of psPAX2 was performed in Snapgene. The full sequences can be found in appendix 10.6.

4.5.2. mCherry and E2-Crimson reporter comparison

Transfection experiments were performed with the LentiCRISPRv2 plasmids containing either the mCherry or E2-Crimson reporter (Method 3.11). For the one plasmid transfection 1 µg of DNA was used for a well on a 24-well plate. Fluorescence of the plasmids was detected by the TxRed filter used in fluorescence microscopy (Method 3.13). Images from the transparent filter were used to analyze cell viability and to determine overall transfection rate. The results are shown in Figure 34. The light intensity for all images was kept constant to show differences in fluorescence strength by both reporters. Both plasmids were successfully transfected into HEK293T cells. Cell viability was high and cell growth continued after transfection. Fluorescence was checked for a week each day after transfection. Cell viability decreased as cell growth continued and cell density reached a critical point. Fluorescence intensity was stable over time and transfected cells remained viable.

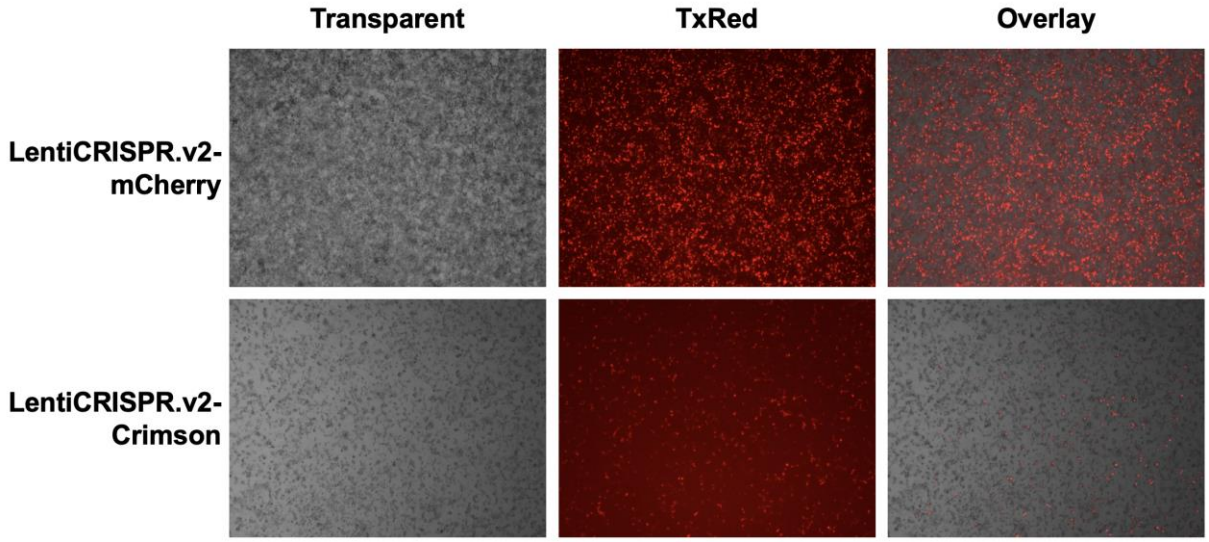

Figure 34 **Transfection results of LentiCRISPRv2 with mCherry and E2-Crimson.** Transfection was performed with HEK293T cells. PEI was used as a transfection agent in combination of 1 µg DNA together with 3 µL PEI 40,000. After transfection the cells were incubated overnight at 37 °C and 5% CO_2. Afterwards the cells were used in fluorescence microscopy under the TxRed filter and 4x zoom. Cell viability was verified with translucent light by assessing cell morphology. Overall transfection rate was compared between translucent light and the TxRed filter (585/624 nm).

4.5.3. Three plasmid transfection and infection of AKH-10 cells

For the infection experiments, different conditions were tested with varying amounts and mixing of the three plasmids and PEI. The ratios were determined based on the results of Grunwald *et al*. using a two plasmid ZIKV-E vector[71]. The concentration of the transfer gene was kept constant as transfection could be detected for the plasmid through the TxRed filter and sufficient transfection was reached with the amount of 1 µg. For all conditions either the psPAX or pCMV-dR8.2 dvpr plasmids were used separately (Method 3.11).

As for the results, the transfection always showed a 40-60% transfection rate. But only for the experiment with 0.5 µg LentiCRISPRv2-mCherry-EGFR3, 3 µg psPAX2 and 1.5 µg pE41.2 with 10 µL PEI one infected AKH-10 cell was detected (Figure 35). A repeated experiment with identical conditions led to no further infections. Additionally, variations in the time of transfection and cell incubation after transfection showed no infected cells.

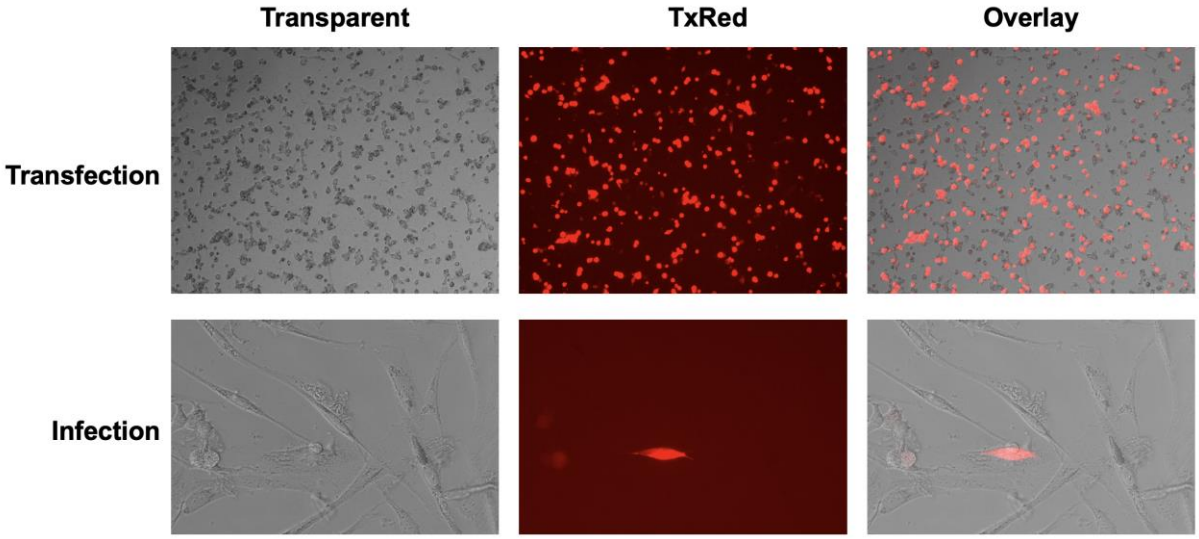

Figure 35 **Transfection and infection results of the three plasmid pseudotype.** Transfection was performed with the LentiCRISPRv2-mCherry-EGFR3 (transgene), psPAX2 (packaging plasmid) and pE41.2 (envelope plasmid). The three-plasmid transfection was performed with HEK cells and transfected cells were grown for three days. The supernatant from the HEK cells was used to infect the AKH cells. Here, a single infected AKH-10 cell is shown. Images were generated with translucent light and the TxRed filter (585/624 nm).

4.6. EGFR and BIRC5 mutations isolated GBM tumor cells

4.6.1. PCR amplification of EGFR sgRNA target sites

For the verification of the EGFR and BIRC5 target sites as well as to detect mutations described in literature as well as novel mutations, PCR amplifications were performed, resulting in 800-950 bp DNA fragments. For the EGFR spacer binding sites, three separate PCRs were performed to verify the mutations included in the sgRNA design. For the BIRC5 spacer fragments, one PCR amplification was performed, as the spacers were located in close perimetry to each other. In Figure 36 the gel electrophoresis results for the EGFR fragments amplified in PCR 2 are shown (Method 3.4). The fragments were cut out of the gel and used for PCR DNA purification (Method 3.5). Based on the results from Figure 36, eleven AKH cells, three U cell lines and HEK293T cells were analyzed for the EGFR target sequences to compare mutations. The PCR results are shown in Figure 37. For all gel separations, a 50 bp marker was used. Positive scans are marked in green, and the DNA bands were cut out and purified (Method 3.5). Scans marked in blue were positive but had to be repeated to achieve a higher DNA concentration for purification. This was due to low DNA yield. Scans which were negative were repeated (Figure 38). The repeated runs are shown in Figure 38. Positive runs which were also purified were marked in green. The other runs were repeated a third time, resulting in no visible bands. All DNA band purifications were done using Method 3.5. The fragments were suitable for PCR cloning. Overall, 13 PCR fragments were purified for the EGFR target 1, 14 samples for the EGFR target 2 and 17 samples for EGFR target 3. All purified fragments were used for cloning into the pMiniT2 plasmid for sequencing analysis.

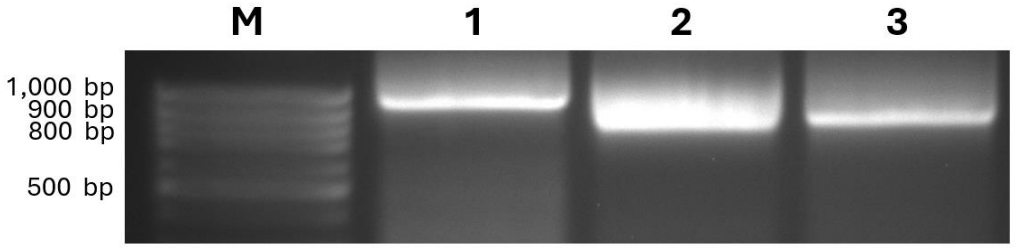

Figure 36 **Gel electrophoresis PCR analysis of the EGFR target sites.**
The gel showed the PCR analysis results of EGFR target sites in AKH-10 cell lysate. A 0.9% agarose gel was used and run at 140 V for 20 min. The EGFR target site for spacer 1 had an expected fragment size of 950 bp (1), The EGFR target site for spacer 2 had an expected fragment size of 770 bp (2), The EGFR target site for spacer 3 had a fragment size of 820 bp (1).

M 50 bp marker from Fermentas (#SM0378) (4 µL)
1 PCR scan EGFR target site of sgRNA1 (50µL)
2 PCR scan EGFR target site of sgRNA2 (50µL)
3 PCR scan EGFR target site of sgRNA3 (50µL)

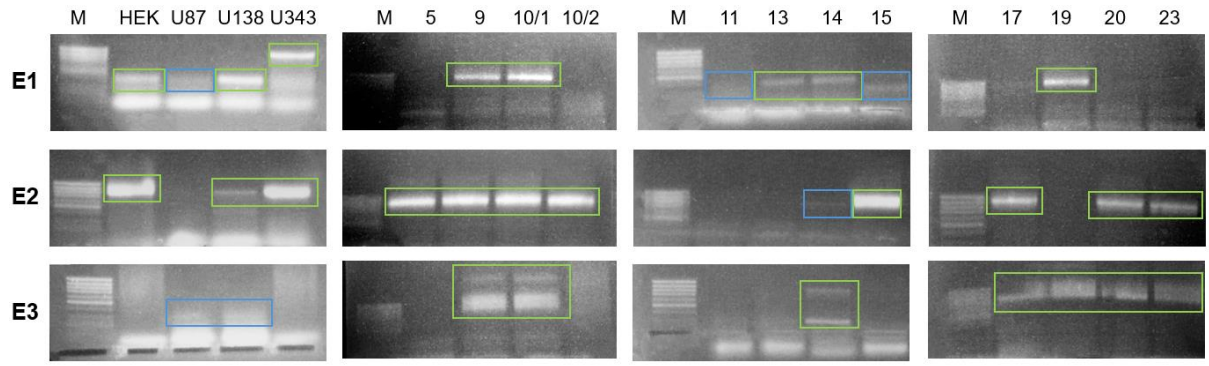

Figure 37 **PCR analysis of EGFR target sites AKH, HEK293T and U-cells.**
The gel showed the PCR analysis results of the EGFR target sites in HEK, U87, U138, U343 and AKH cell lysates. A 0.9% agarose gel was used and run at 140 V for 20 min. As a marker (M) 4 µL 50 bp marker from Fermentas (#SM0378) was used.

E1 PCR fragments amplified with the primers for the EGFR target site 1. The theoretical fragment had a size of 950 bp.

E2 PCR fragments amplified with the primers for the EGFR target site 2. The theoretical fragment had a size of 770 bp.

E3 PCR fragments amplified with the primers for the EGFR target site 3. The theoretical fragment had a size of 820 bp.

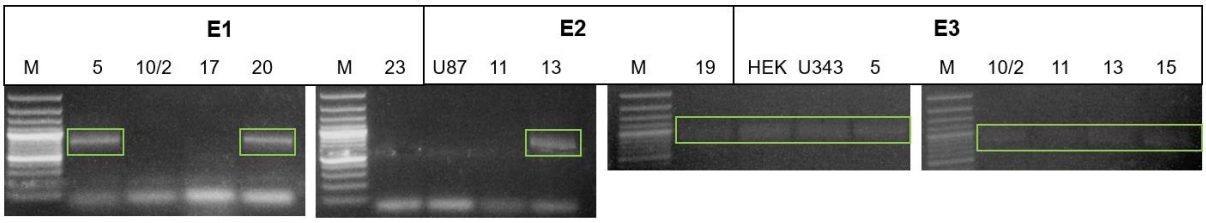

Figure 38 **Repeated PCR analysis for EGFR target sites.**
The gel showed the repeated PCR analysis results of the EGFR target sites in HEK, U87, U343 and AKH cell lysates. A 0.9% agarose gel was used and run at 140 V for 20 min. As a marker (M) 4 µL of the 100 bp marker from Fermentas (#SM0321) was used.

E1 PCR fragments amplified with the primers for the EGFR target site 1. The theoretical fragment had a size of 950 bp.

E2 PCR fragments amplified with the primers for the EGFR target site 2. The theoretical fragment had a size of 770 bp.

E3 PCR fragments amplified with the primers for the EGFR target site 3. The theoretical fragment had a size of 820 bp.

4.6.2. PCR amplification of BIRC5 sgRNA target sites

In Figure 39, the three gels are shown of the fragments amplified with the BIRC5 target site PCR analysis. From 16 samples overall 10 samples were amplified. Further repeats of the non-amplified reactions led to no results. The ten fragments which were shown at approximately 1,000 bp in the gel were cut out and used in PCR purification (Method 3.5).

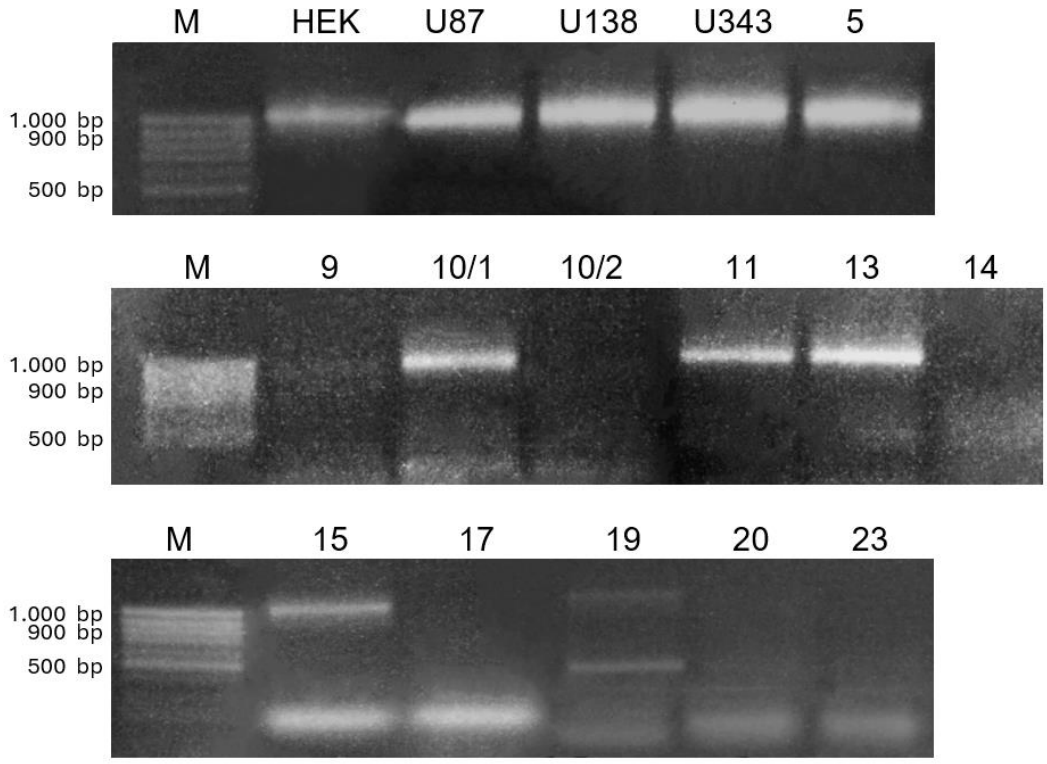

Figure 39 **PCR analysis of BIRC5 sgRNA target sites.**
The gel showed the PCR analysis results of the BIRC5 target sites. A PCR amplification was successful in HEK, U138, AKH-10,13 and 14. A 0.9% agarose gel was used and run at 140 V for 20 min. All three target sites are in the same PCR analysis leading to one 1042 bp fragment. As a marker (M) 4 µL of the 100 bp marker from Fermentas (#SM0321) was used.

4.7. Sequence analysis for EGFR and BIRC5 target sites

The PCR analysis fragments were cloned into the pMiniT2 plasmid using the NEB® PCR Cloning kit and further sequenced with the pMiniT2Seq primer (Method 3.3). The sequencing results were aligned to the theoretical DNA sequences from NCBI and the HEK293T genome[75,76]. The alignments can be found in appendix 10.7. In Figures 40-42 the sequencing results are shown with the mutations and target sites they inhabited. The target sites are each shown in blue, and deletions are highlighted in orange and mutations in red. For simplifications only the missing or exchanged base pairs were marked.

4.7.1. EGFR mutations

Eight cell lines were successfully sequenced for target site 1 (Figure 40), numbers at the end of the sequence indicate the number of sequences obtained. The cloning of the PCR fragments of cells U343, AKH-5, -11, -15, -20 and -23 showed faulty or incorrect DNA sequences. A total of two deletions and six mutations were found, with one mutation in AKH-14 and AKH-19 at the same position. The mutation inserted in spacer sequence 1 at position 414 could not be detected in any cell.

A total of 13 cell lines were sequenced at the target sequence for spacer 2 (Figure 41). Five deletions and twenty mutations were found. One of these mutations, which was found in AKH-10, is the mutation known from the literature to be built into spacer 2. Furthermore, two different AKH-23 sequencing results were obtained. The sequencing of AKH-20 cells did not lead to any analyzable results.

Eleven cell lines were sequenced for target site 3 (

Figure 42), numbers at the end of the sequence indicate the number of sequences obtained. The cloning of the PCR fragments of cell lines AKH-5, -11, -15, -17 and -19 showed faulty or incorrect sequencing results. A total of three deletions and nine mutations were found. The mutation inserted in spacer sequence 3 at position 496 could not be detected in any cell. For the AKH-10 cell line, two different sequencing results were obtained. All EGFR sequencing results can be viewed in appendix 8.7.

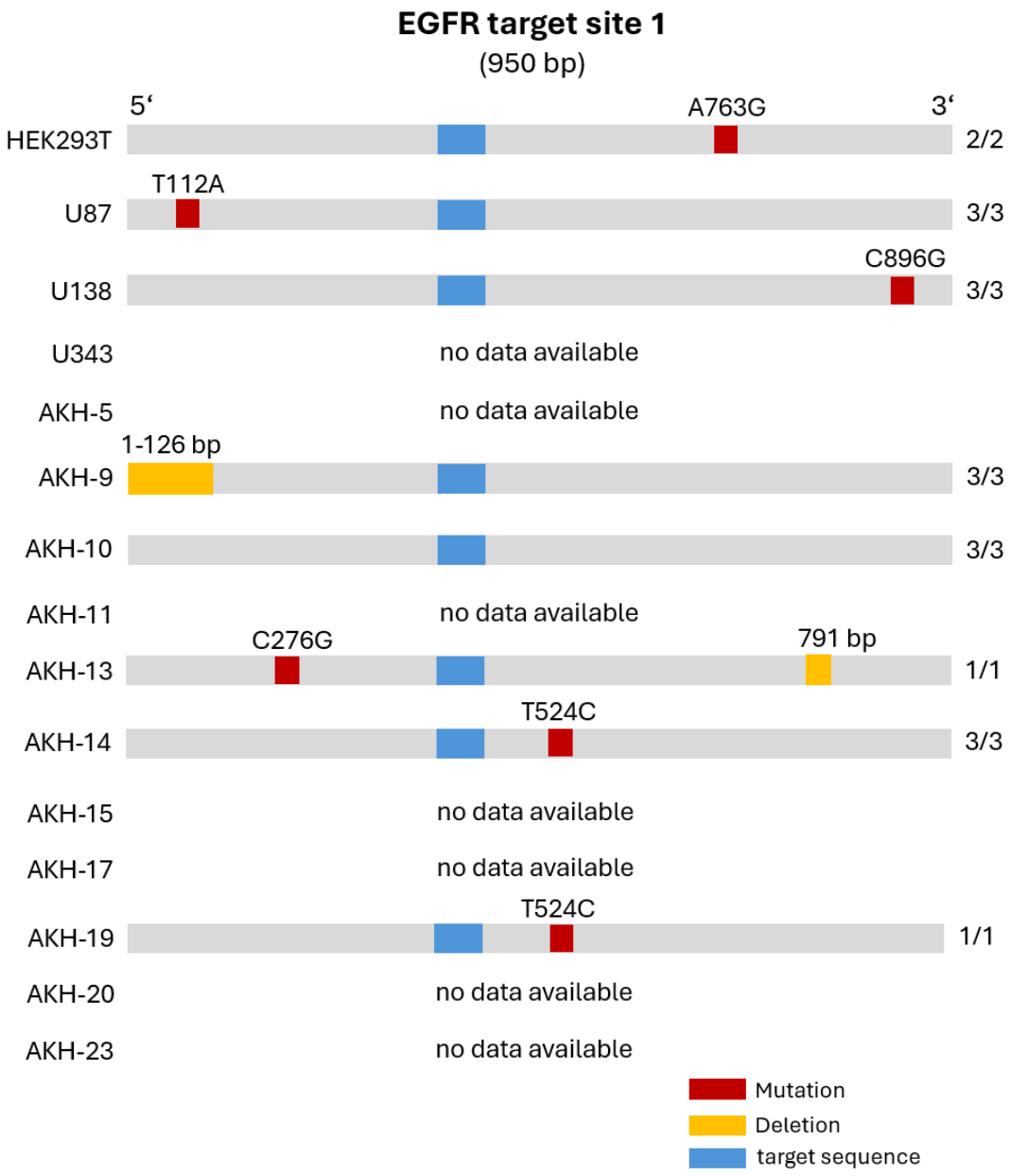

Figure 40 **Sequencing results of PCR analysis EGFR1 spacer binding site.**
The spacer target site is located from 391-411 bp (blue). Mutations are highlighted in red and deletions in orange. At the end of each sequence, it is indicated how many sequencing results were achieved. Data was not available if either the PCR amplification or the sequencing results were faulty or negative.

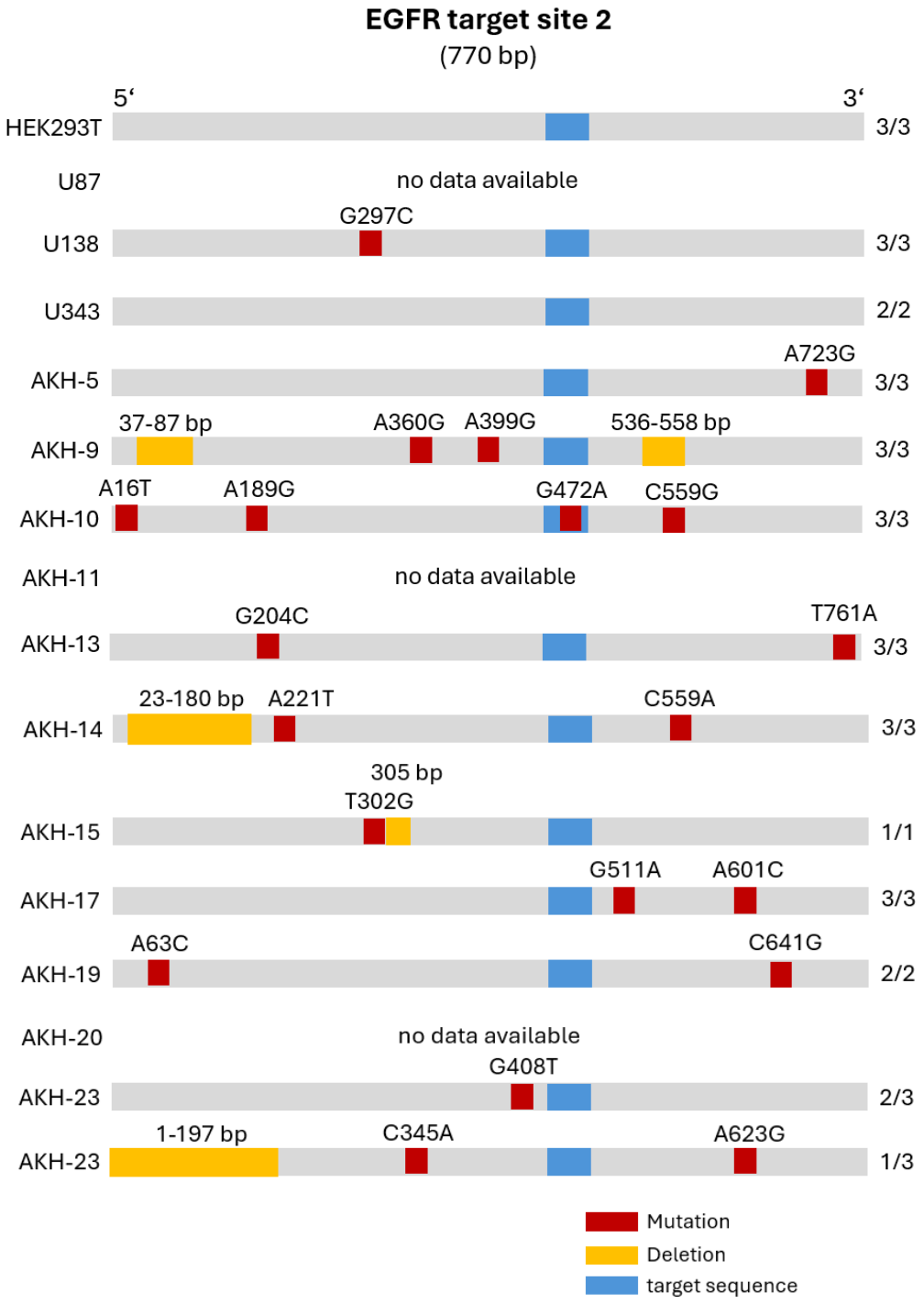

Figure 41 **Sequencing results of PCR analysis of the EGFR2 spacer binding site.** The spacer target site is located from 458-478 bp (blue). Mutations are highlighted in red and deletions in orange. At the end of each sequence, it is indicated how many sequencing results were achieved. Data was not available if either the PCR amplification or the sequencing results were faulty or negative.

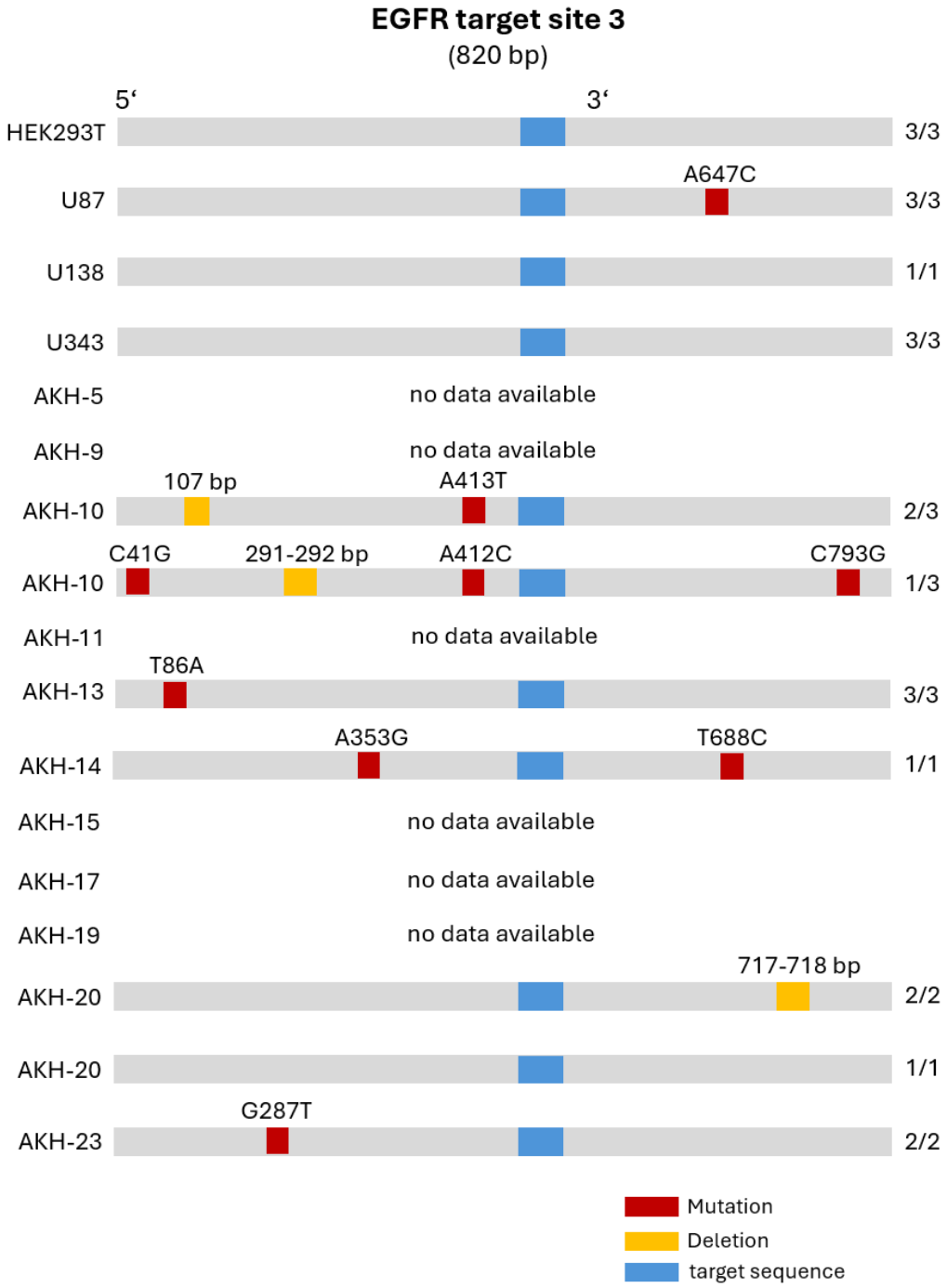

Figure 42 **Sequencing results of PCR analysis of the EGFR3 spacer binding site.**
The spacer target site is located from 485-505 bp (blue). Mutations are highlighted in red and deletions in orange. At the end of each sequence, it is indicated how many sequencing results were achieved. Data was not available if either the PCR amplification or the sequencing results were faulty or negative.

4.7.2. BIRC5 mutations

The BIRC5 PCR fragments from PCR analysis were sequenced, which led to the results shown in Figure 43. Displayed are the 1024 bp long fragment results which were compared to the reference sequence of BIRC5 from NIH[72]. Overall, three clones were sequenced per cell line and as seen in Figure 43. All three clones showed the same DNA sequence in one cell line. One mutation was found in HEK293T at position G256C of the fragment. No mutations were found in the fragment for the GBM cells. The sequencing results can be viewed in appendix 8.8.

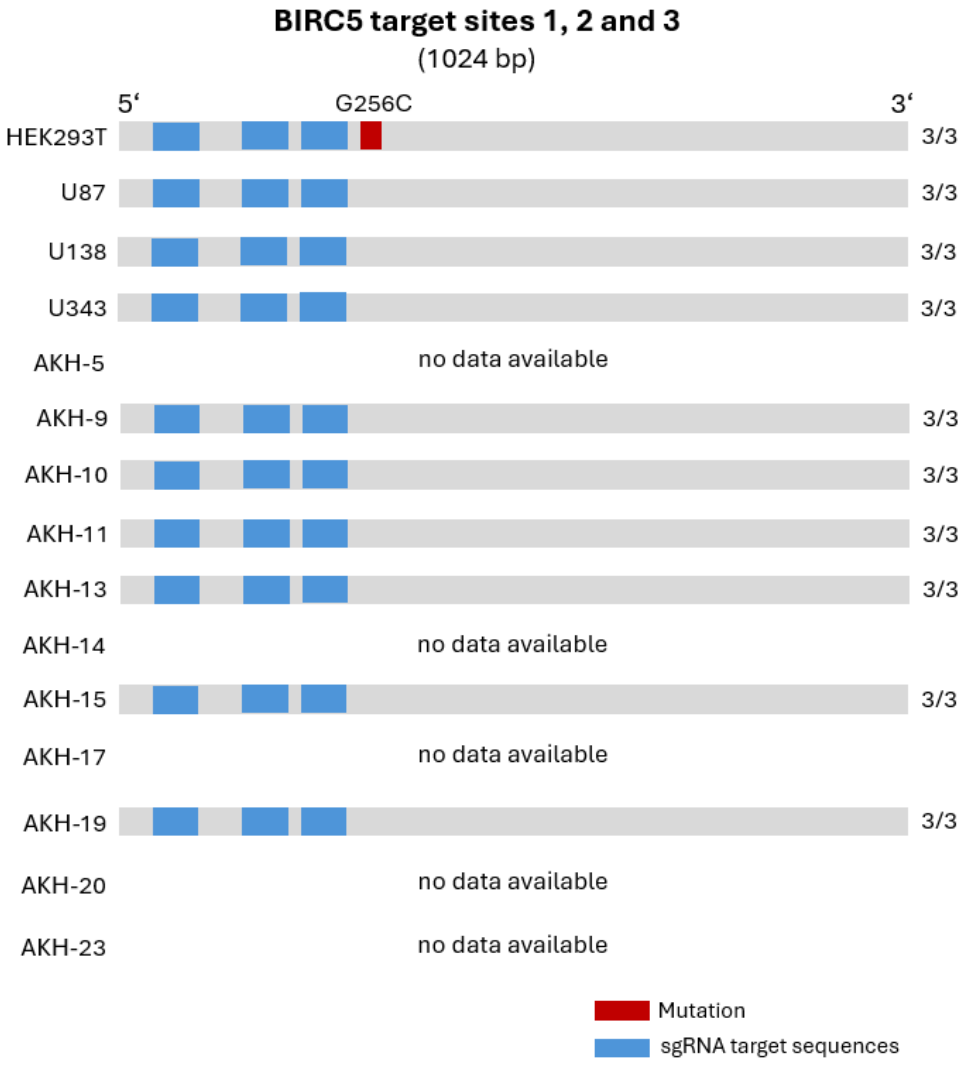

Figure 43 **Mutation of the BIRC5 sgRNA sites.**
The sgRNA target sites are highlighted in blue and mutations found in red. A total of three sequences were analyzed for each cell line. One mutation was detected at G256C of the fragment from the HEK293T analysis.

5. Discussion

5.1. Cloning of the triple sgRNA region

The discovery of the bacterial CRISPR defense system has permanently changed the possibilities of molecular biology and oncology with regard to the modification of the eukaryotic genome. The ease of use and adaptability of the CRISPR-Cas9 system allows targeted, sequence-specific interventions[39]. CRISPR-Cas9 works by designing a 20 bp long search sequence that corresponds to an identical sequence in the genome. The search sequence as part of the Cas9-binding sgRNA can be adapted and optimized as required[39,40]. Due to these properties, CRISPR is already being used in CAR-T cell therapy approaches as well as genome-wide gene scanning[8,36]. These approaches allow to target, modify, or study genes by direct in-cell genome modifications. Moreover, the complete CRISPR-Cas9 system can also be transferred into cells using a viral or lentiviral vector. To produce LVs, sgRNA-CRISPR-Cas9 containing LV genomes have been developed that carry also different sgRNA designs and these genomes can then be packaged into an LV[77]. Through the design of the LV envelope, an LV enables to transfer the CRISPR-Cas9 system especially into those cells that contain the respective envelope receptor. However, cutting a defined gene at only one location, by using one specific sgRNA, inactivation of the gene is not guaranteed due to the cell's DNA repair mechanisms[78]. Some studies already focused on the construction of plasmids with multiple sgRNAs to achieve an enhanced destruction of the gene of interest[20].

Cloning of multiple sgRNAs into the lentiviral CRISPR plasmid LentiCRISPRv2 was presented by Cao et al. (2016)[74]. In the scope of this study a U6 promoter sgRNA-DNA element can be placed in series to generate single, double or triple sgRNA-expressing plasmids. Since the U6 promoter is different to SV40 or other eukaryotic promoters like CMV, the U6 promoter can be present on a single DNA molecule in multiple copies being still active. On this basis, two triple sgRNA plasmids were cloned, using also the U6 promoter for sgRNA expression. Another important part of the cloning strategy was the use of the restriction enzyme BsmBI. Type IIS restriction enzymes like BsmBI bind to their respective 6-bp long recognition site but digest the DNA downstream of this site at a non-sequence specific location. Thus, a single BsmBI cut can be used to generate DNA fragments with individual, and different 4-bp long 5'-sticky ends on each site of a DNA fragment. The BsmBI digested fragments are assembled by annealing the constructed complementary four-base 5'-overhangs to the neighboring fragments. The digested fragments and the final construct no longer contain any BsmBI sites, restricting further cutting of the final DNA construct. The final product therefore also assembles and accumulates over time during ligation. This method can be used through generation of sgRNA-U6 promotor elements manually by using an appropriate template and primers to introduce the target sequence or by using a synthetic DNA element that fits into the

BsmBI strategy[79]. It should be noted that the assembly of a synthesized gene, is achieved by the ligation of smaller DNA fragments. Since the fragment contains repetitive sequences due to the U6 promoter and gRNA scaffold, mismatches could occur during assembly. Therefore, it was not possible to synthesize a triple U6 promotor-sgRNA BsmBI-insert containing all three spacer sequences in one step as one single molecule. Thus, the idea of the first cloning technique was adopted by design of two fragments already containing the spacer sequences and BsmBI restriction sites. Both cloning methods for the U6 promotor-sgRNA BsmBI-inserts were successfully used in this study and to target EGFR or BIRC5 by three targets simultaneously. However, the use of synthetic sub-fragments is easier and faster and would allow the preparation of a large library of U6 promotor-sgRNA elements for a variety of targets in GBM tumor cells. During cloning, it was important to purify the individual BsmBI fragments to achieve a higher yield at each ligation step. It was important to perform PCR and gel purification steps to eliminate the resulting BsmBI-cleaved DNA fragments, especially those resulting from the PCR products after BsmBI digestion. Otherwise, ligation of U6 promoter-sgRNA elements would have been blocked by the cleaved ends of the PCR fragments.

5.2. Exchange of the mCherry reporter with E2-Crimson

Fluorescent reporters are often used to monitor transfection rates and infections rates of LVs[80]. Ning et al. showed that neuronal cells are sensitive to mCherry, the reporter used in the LentiCRISPR.v2-mCherry plasmid. Red fluorescent proteins are considered toxic to neurons and are not very suitable for detection in neuronal cells due to their aggregation and low fluorescence intensity. To counteract this, Ning et al. developed a fluorescent protein that remains stable in transfected cells in the long term and does not exhibit aggregation or toxicity. In addition, the E2-Crimson reporter reported to have a 28% higher fluorescence intensity[81,82]. In this study, the mCherry reporter in the LentiCRISPRv2-mCherry plasmids was replaced by the E2-Crimson reporter. For this purpose, an E2-Crimson fragment with Bam-HI and EcoRI restriction sites was designed and synthesized by Eurofins. For the design it had to be considered that the P2A motif, a 22 bp sequence located between the Cas9 enzyme and the reporter, must be intact as indicates for the ribosomal skipping. This allows both proteins to be expressed under the same promoter but leads to the separation of them during translation. Moreover, a positive fluorescence signal indicates that Cas9 was successfully expressed. Due to the location of the digestion sites, the P2A sequence had to be included in the E2-Crimson fragment. The fragment and the LentiCRISPRv2 plasmids were digested and ligated in a two-step process. This approach demonstrates that synthetic DNA can be used for rapid and simple exchange of reporter genes in lentiviral plasmids, especially when unique six-cleavage sites such as BamHI and EcoRI are present. Fluorescence analysis of the transfection images showed that the mCherry reporter led to significantly higher fluorescence

intensities than the E2-Crimson when transfected with the same amount of plasmid. No significant changes in cell viability were observed for either plasmid under the transmitted light microscope. The growth of transfected HEK293T cells was also largely identical to that of the non-transfected cells. The two E2-Crimson plasmids showed the expected red fluorescence signals and can be utilized for further studies with neuronal cells and GBM tumor cells.

5.3. Analysis of EGFR and BIRC5 mutations

GBM databases such as TCGA-GBM provide information on the frequency and location of mutations that determine the lethal characteristics of this brain tumor[24]. In the case of mutations, it is also possible to see on which genes they are located and how frequently they occur in a series of sequences. Identical mutations which occur in different patients, parallel mutations, are often responsible for the negative characteristics of GBM. CRISPR-Cas9 screenings have shown that such genes can be used as targets for the development of immuno- or gene therapeutical approaches[36].

The triple CRISPR-Cas9 plasmids developed in this study were designed to inactivate exactly two of these genes, called EGFR and BIRC5. The first plasmid is based on sgRNAs, into each of which a point mutation that frequently occurs in GBM has been integrated. This means that in the mutated brain cells, cutting of the genomic DNA strand occurs only at this predetermined point. The EGFR mutations that were included in the target sequences were selected via the cBioPortal and the TCGA database as very frequently occurring mutations (Tab. 13, page 39). According to these data bases, the selected mutations for the three EGFR sgRNAs occur in up to 50% of patients[24,83]. Due to its properties, EGFR is also a frequent target for other GBM therapeutic approaches. One mutated EGFR, EGFRvIII is the most common EGFR mutant and an important target in cancer cells in general. EGFRvIII-positive cancer cells are challenging to treat due to their aggressive and resistant nature to conventional therapies[10,42,46]. However, targeting EGFRvIII specifically has been a focus of research for developing novel treatment strategies for GBM and other EGFRvIII-positive cancers. Deletions in the outer membrane region enable this receptor, which occurs to be constantly active in GM, even without ligands and to exhibit constitutive tyrosine kinase activity. The exact effects of EGFRvIII mutations are not yet fully understood but are associated with increased tumor growth and stem cell renewal[43]. As deletion via CRISPR-Cas9 is difficult to achieve, the tyrosine kinase domain of the receptor was selected as the target in this study. According to the literature, parallel mutations of the receptor occur in up to 50% of patients who undergo sequencing[24].

In this study, the construction of the EGFR sgRNAs alongside the corresponding LentiCRISPRv2 plasmid was based on the mutation analysis through the TCGA database. In this regard, during an internship in the Schreiber-Lab in the summer of 2021, I developed a

method for the culturing of GBM cells from tissue samples, the so-called primary GBM cell cultures[70]. Since that time, 24 primary cell cultures have been established, 12 of which were used in this work for genomic DNA isolation and mutation analysis. The concept was based on the amplification of the corresponding regions by PCR followed by cloning into a sequencing plasmid such as pMiniT2. Cloning was carried out using the NEB cloning kit and proved to be a good and reproducible method, resulting in high yields of clones in most cases. The method proved to be reproducible, but it was not possible to amplify the genome of all cell lines with the primers created. In the figures 40-42 (pages 65-67) this was indicated as “no data available”. One possibility for this may be that mutations on the genome might have prevented some of the primers used for the amplification by the nested PCR from binding. The sequence analysis of several clones would then be used to determine the type and frequency of the respective mutations. As a control, HEK293T cells as well as three other GBM derived cell lines U87, U138 and U343 cells were used for this kind of analysis. The sequencing was intended to show whether the mutations listed in the literature were present and whether new mutations had emerged. Although only three sequences were shown in the figures (Figure 40-42, pages 65-67), a high degree of variability within the patient collective was apparent.

The sequencing of the EGFR gene carried out in this study showed that only one of the examined cell cultures (AKH-10, target site 2) showed the mutation described in the literature that was also chosen for the cloning of the triple EGFR plasmid (spacer 2). The EGFR mutation G193T in EGFR which leads to the amino acid change Gly598Val was found in all three AKH-10 PCR clones (spacer 2). The mutations integrated into the target sequences could not be detected in any other AKH cell culture U cell line. Nevertheless, it could be shown how heterogenous mutated the tyrosine kinase region is and that there are differences in the mutations of analyzed cell cultures. In addition to individual point mutations, deletions were detected outside the sgRNA target sequences. Only in one case a parallel mutation was found in AKH-14 and AKH-19 cells at the target site 1. Another similar mutation was found at the target site 2, at an identical location, being a C559G mutation in AKH-10 and aC559A mutation in AKH-14. As the EGFR target sites lay in a genomic area of around 293,000 bp, many introns and exons are at hand for the tyrosine kinase domain. Comparing this to the final EGFR protein, parts of the amplified fragments are in introns, thus these mutations will not show any effect on the final EGFR protein. This is additionally demonstrated by the EGFR expression. The immunofluorescence results prove, that EGFR is highly expressed in the cell lines AKH-05, AKH-10, AKH-13, AKH-19 and AKH-23 thus the mutations had no negative effect on EGFR expression. Even though mutations located in the introns have no direct effect on the phenotype of the cells, mutations may be used for GBM specific targeting to cleave larger genomic DNA fragments out of the cellular genome, thus making extra-large deletions. While a single sgRNA-CRISPR-Cas9 system is only able to cleave at one location on the genome, it

is often limited to the exons, in order to achieve gene inactivation. The multi-sgRNA-CRISPR-Cas9 system, on the other hand, can be used to recognize several target sequences, in exons but also mutations present in introns. In a strategy in which several sites are cut at wide intervals, large gene segments are cut out, which makes functional EGFR repair almost impossible.

For the inactivation of the BIRC5 gene, three sgRNAs against the BIR domain of BIRC5 were developed in this study. BIRC5 encodes the protein survivin, which is overexpressed in GBM cells and thereby stimulates tumor growth and inhibits apoptosis. Survivin is also associated with chemoresistance[48,72]. The inactivation of BIRC5 can be achieved by destruction of the BIR domain. Therefore, sequences for sgRNAs were chosen from the sequence database and literature. BIRC5 is not a mutated gene that is different in GBM and healthy cells. However, it is overexpressed in GBM and must therefore be destroyed to block GBM development. The cBioPortal shows that there are no mutations in the BIR domain making this domain a constant CRISPR target[24]. The sequence analysis made in this study confirms the data retrieved from cBioPortal. Analysis of the BIR target sequences showed that only one mutation was present in HEK293T cells, and no mutations were found in cells of GBM origin. This mutation from a guanine base to a cytosine base detected in the HEK293T cells, G256C, can be confirmed from the literature as a typical point mutation identified in HEK293T[84]. Although, BIRC5 is constant in GBM, this assumption is only made for the open reading frame, the exons for BIRC5. BIRC5 (survivin) plays a crucial role in carcinogenesis as it is highly overexpressed in most cancers, including GBM and therefore it is an important achievement to lower survivin activity in tumors[47,48]. Survivin is associated with therapy resistance and tumor growth due to its apoptosis-inhibiting properties. This makes BIRC5 an ideal target for a CRISPR approach. However, as the gene itself has few mutations and therefore differs only slightly from healthy cells, a decisive specificity for GBM compared to healthy cells is not present. For example, the triple sgRNA CRISPR-Cas9 against BIR has no GBM specificity. That, when such a tool will be applied the lentiviral vector must have a very high specific for GBM tumor cells. Actually, the GBM to non-GBM (healthy brain cells) transduction ratio for a LV coated with a ZIKV E is about 200:1, which is at the current state of knowledge the best off-target ratio available. However, it is not clear if this is good enough for in vivo applications. In order to avoid the need for vector specificity, DNA sequencing of the entire BIRC5 genomic region could be performed, as potential mutations with GBM specificity might be located in BIRC5 introns or other non-coding areas outside of the BIRC5 exons. Most of the sequences derived from databases only describe the transcriptome of cancer cells and therefore leave non-coding areas of the genome unexplored[24]. In general, a multiple CRISPR-Cas9 system with specific targets at the DNA level that also consider exon and intron mutations is likely to be a more advanced tool for CRISPR-dependent applications in the future.

6. Outlook

The results of this study show that, on an exemplary basis, that the EGFR plasmid in a form of a designated LentiCRISPRv2-mCherry-3EGFR, could be used for LV generation and thus be transferred into tumor cells. However, an important question that remains, is the off-target efficiency of an anti-cancer tool like an LV in general. As shown, different genes can be targeted that are mutated and therefore cancer specific or genes that are constant in tumor cells but unfortunately also in healthy cells. It was thought that tumor specific mutated genes can be used as overall, universal targets but as shown in this study, a high rate of very different mutations were identified. That the assumption that can be made because of the data presented in the literature is not confirmed in the present analyses using cells from grade 4 GBM tumors. A CRISPR approach targeting EGFR should be possible, nevertheless it must be adapted precisely to the patient-specific mutations. An alternative strategy would offer to target important but constant genes, like BIRC5, but here the LV used for transferring the therapeutic genes into tumor cells must be highly cell specific. Therefore, further studies are needed to demonstrate a low and preferably non-existent off-target activity of such an LV CRISPR tool. To study off-target effects non-tumor cells are needed, which presents difficult due to the advanced cell structure of brain cells. Thus, any intervention in vivo, in the human brain must be without any severe side-effects. Off-target studies are therefore the next important step leading a way to a successful LV-therapy for GBM.

The analysis of mutations in EGFR is made by using primary cell cultures made from GBM samples that are composed of a large variety of tumor cells. Since the isolated genomic DNA is from different tumor cells it is not clear whether an identified mutation or combination of mutations in the three CRISPR target sites belong to a specific tumor cell. As the method used on this study is based on the analysis of cells that are growing as adherent cells it would be interesting to use other selective methods. One method is the use of 3D cultures, for example the culturing of organoid structures. It is thought that these structures are more relevant since they are mainly composed of GBM stem cells. Thus, such a cell culture system should be used to isolate genomic DNA for analysis of GBM specific mutations and the analysis of their frequency based on a more relevant number of sequences. Another strategy would be to sort cells from fresh tumor samples based on specific markers on the cellular surface. However, the present study shows that EGFR mutations are frequently present in GBM tumors but are highly patient i.e. tumor specific. A high tumor specificity is a preferred property, whereas the high patient specificity is an unwanted property that contradicts the development of a simple broad-spectrum application. In contrast, the targeted knockdown of a highly sensitive gene such as BIRC5 leads to the destruction of tumor cells but is unfortunately not tumor specific. Such an approach will show a high off-target activity, unless the LV has no off-target activity, transferring the anti-tumor tool only in the tumor cells.

7. Summary

The brain tumor called Glioblastoma multiforme grade 4 is very heterogeneous on a genomic level. Although many genes in GBM tumors cells are mutated there are also promising targets that when inactivated are candidates for cancer treatment. The EGFR genes belong to a group of cancer targets that is mutated and is therefore a perfect target for a highly specific CRISPR-Cas9 strategy. In contrast, wild type genes are also proposed as candidates for cancer treatment like the apoptosis inhibition protein survivin, also known as BIRC5.

In a first part, plasmids were constructed with a CRISPR-Cas9 system targeting EGFR as well as BIRC5. The strategy used consisted of creating three different cleavage sites for the CRISPR "gene scissors". To achieve this goal, three different sgRNAs were inserted in series, each controlled by a U6 promoter. The three sgRNA target sites for EGFR were taken from databases where they are described as so-called parallel mutations, that are frequently identified in GBM tumors. The three sgRNA target sites for BIRC5 taken from literature data demonstrate a complete inactivation of the gene. The U6 promotor–sgRNA elements were cloned into the BsmBI site of the LentiCRISPRv2 plasmid. In addition, the mCherry reporter in LentiCRISPRv2 was replaced with the fluorescence protein called E2-Crimson. Thus, for each target, EGFR and BIRC5, two triple sgRNA-CRISPR-Cas9 plasmids were generated.
Two different approaches were used for cloning, whereby it has proven to be advantageous that sgRNA-U6 promoter fragments, as shown in Figure 13.5, can also easily be produced synthetically. This makes it easier to assemble multiple sgRNAs-U6 promoter elements in one plasmid by using the special BsmBI cleavage properties.

In a second part, tumor cells isolated from 12 GBM patients were analyzed for EGFR and BIRC5 mutations. In addition, three laboratory-adapted GBM derived cell lines, U-87, U138 and U343 were studied. HEK293T cells were used as a control. The mutations described in literature and used for construction of the triple sgRNA-LentiCRISPRv2 plasmids were identified only in AKH-10 cells at the EGFR target site 2. All other tumor cell lines are characterized by different mutations and deletions. In total, 32 different point mutations and 10 deletions were found in the 16 cell samples. Only in two tumors, AKH-14 and AKH-19, an identical point mutation T524C was identified. The analysis therefore showed a very heterogeneous picture of mutations and deletions, without a uniform pattern in the population of tumor samples examined. In contrast, except for a G256C mutation, all BIRC5 genes were identified as wild-type and therefore not mutated in the GBM tumor cells.

8. References

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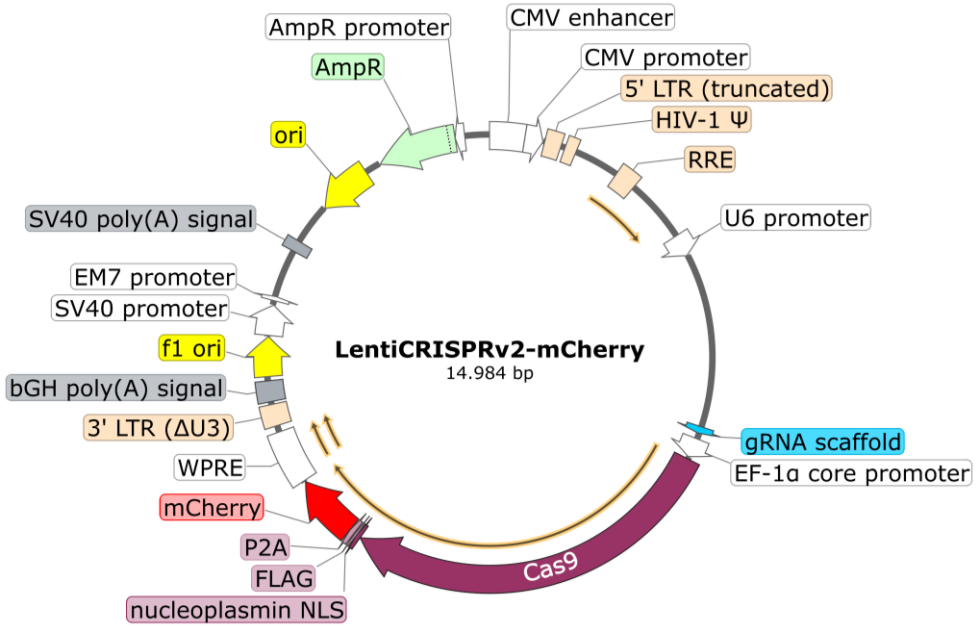
9. Appendix

9.1. Plasmid maps

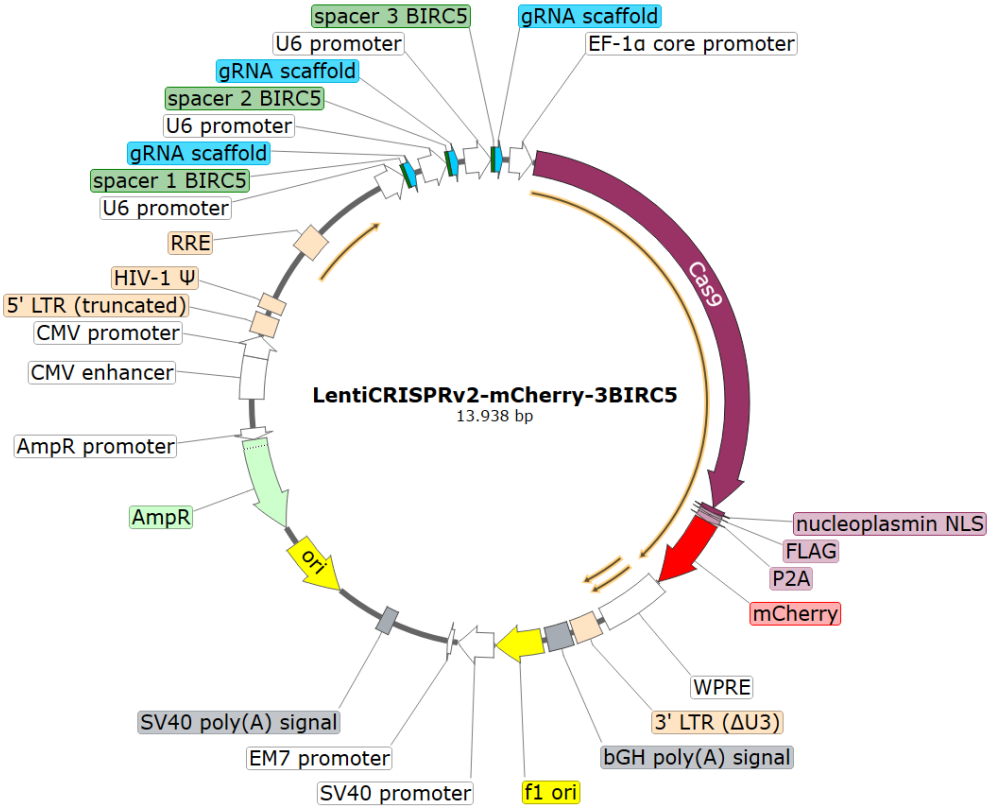
LentiCRISPRv2-mCherry

(lentiviral transfer plasmid)



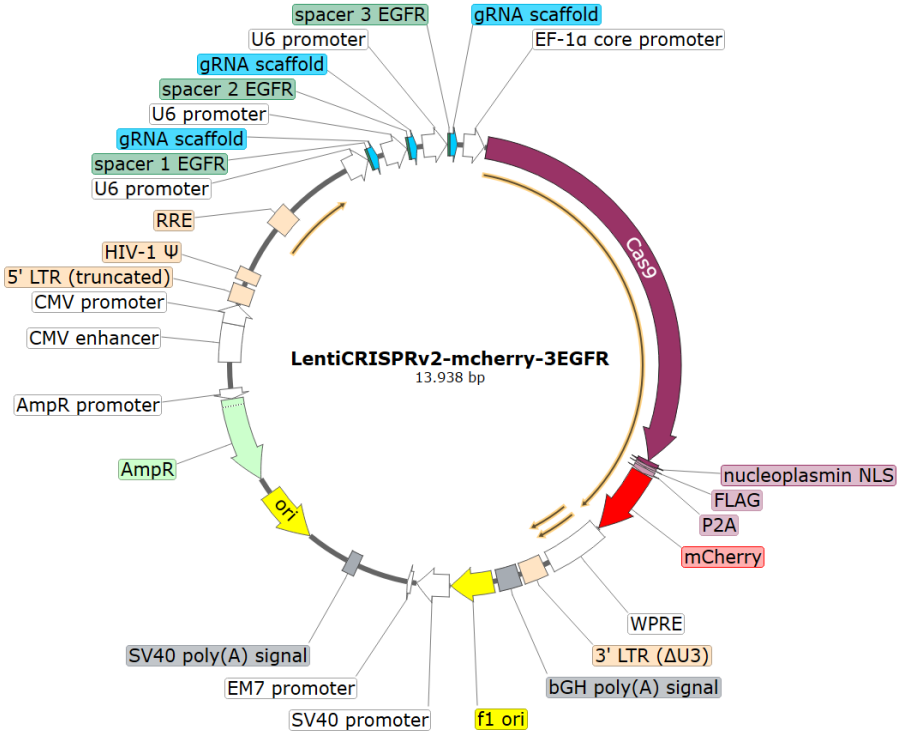
LentiCRISPRv2-mCherry-BIRC53

(lentiviral transfer plasmid)



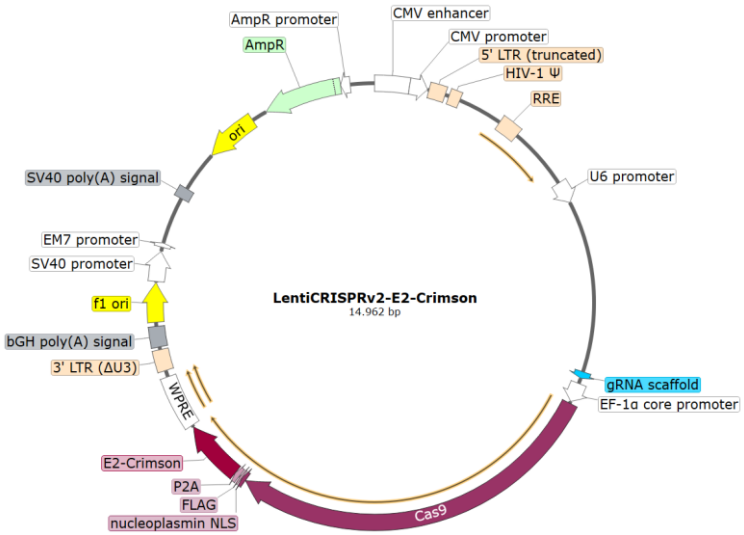
LentiCRISPRv2-mCherry-3EGFR

(lentiviral transfer plasmid)



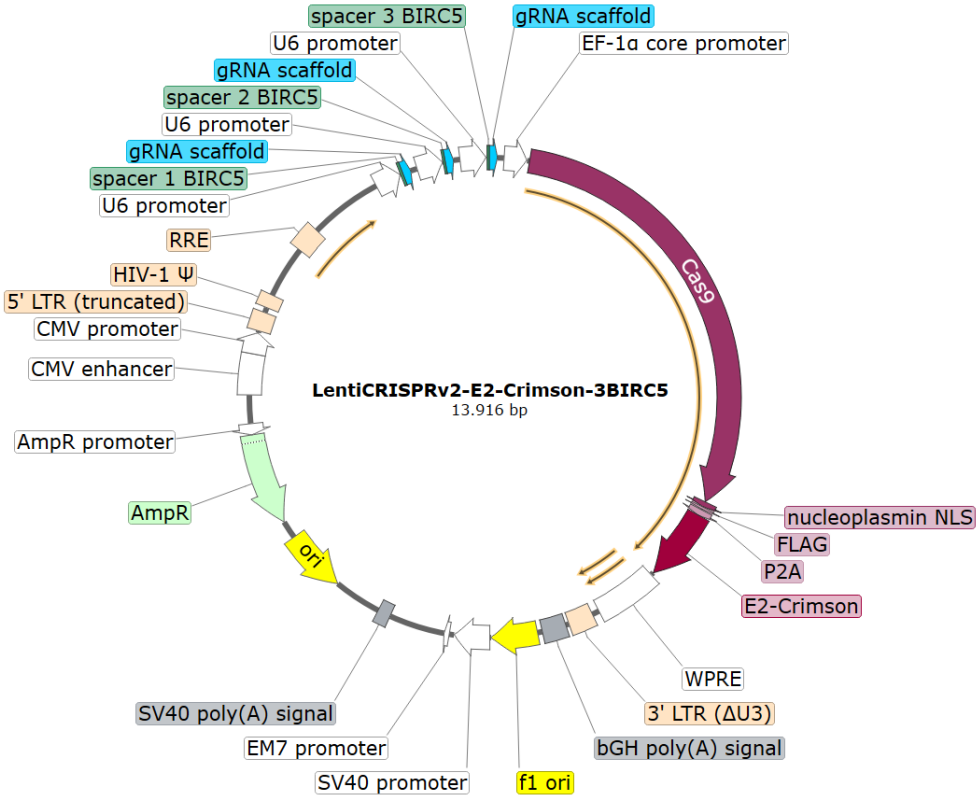
LentiCRISPRv2-E2-Crimson

(lentiviral transfer plasmid)



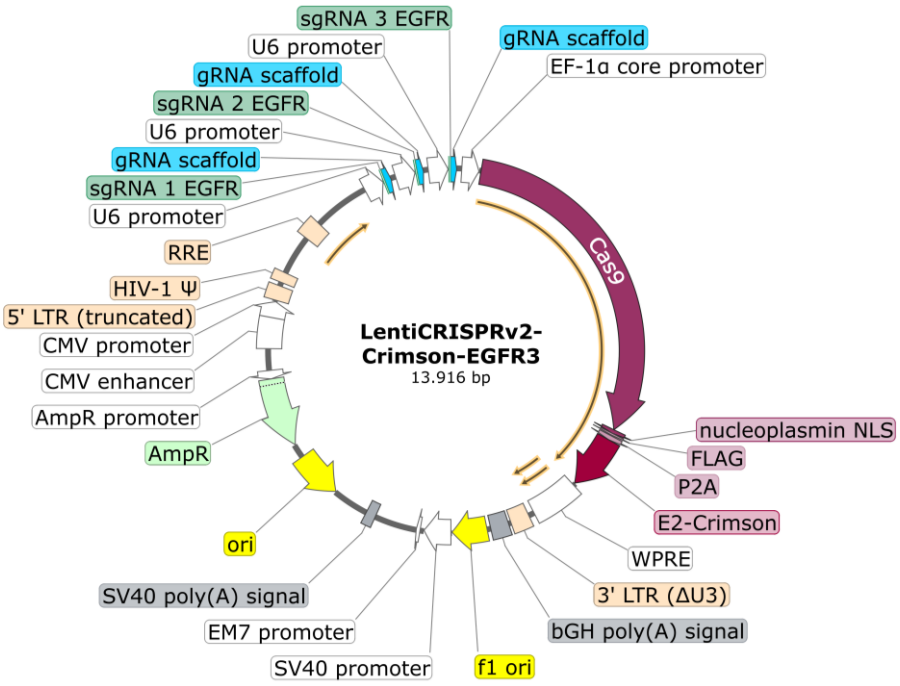
LentiCRISPRv2-E2-Crimson-3BIRC5

(lentiviral transfer plasmid)



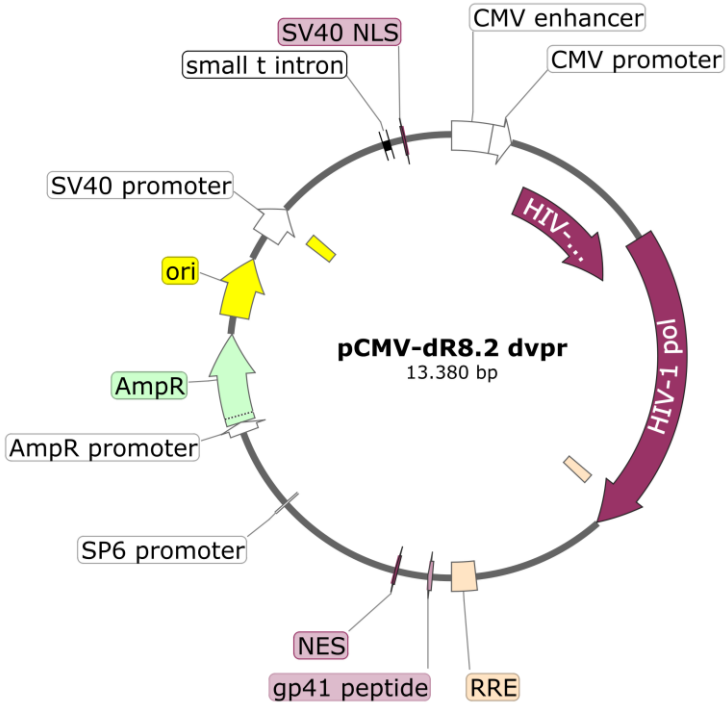
LentiCRISPRv2-E2-Crimson-3EGFR

(lentiviral transfer plasmid)



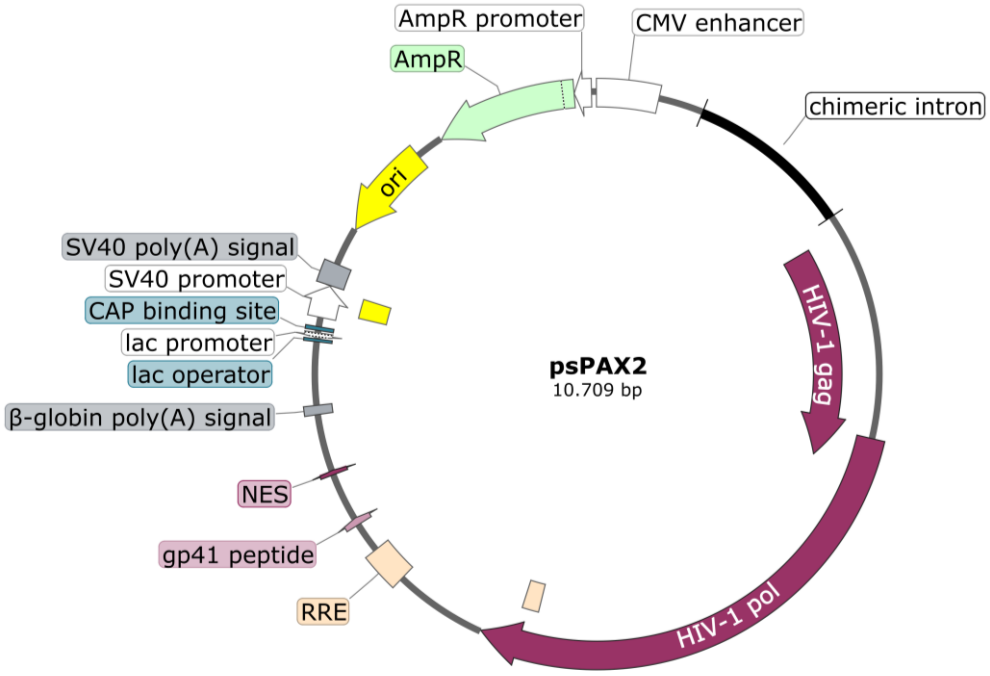
pCMV-dR8.2 dvpr

(lentiviral packaging plasmid, 2nd generation)



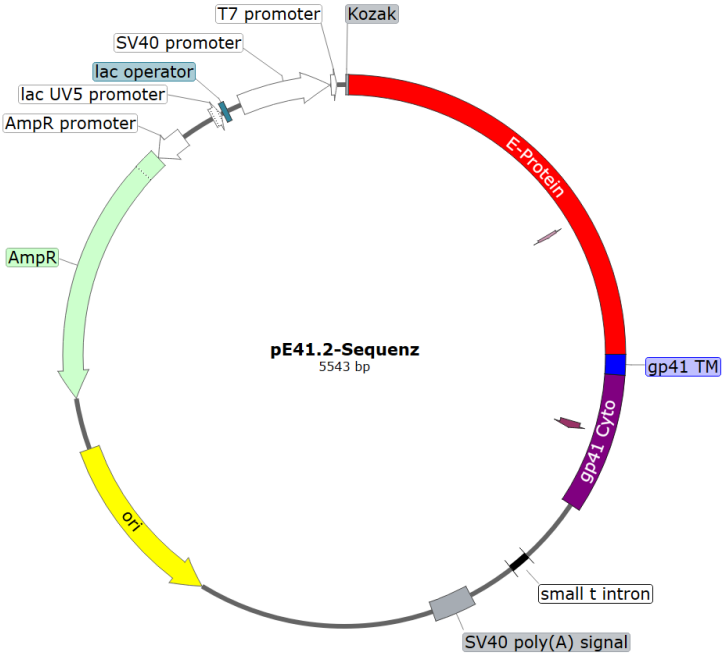
psPAX2

(lentiviral packaging plasmid, 2nd generation)



pE41.2

(ZIKV envelope plasmid)



9.2. LentiCRISPRv2-mCherry-3EGFR sequencing results

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9.3. LentiCRISPRv2-mCherry-3BIRC5 sequencing results

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9.4. LentiCRISPRv2-E2-Crimson sequencing results

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9.5. Sequencing results pCMV-dR8.2 dvpr

dR8.2_Primer1

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dR8.2_Primer2

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dR8.2_Primer3

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9.6. Sequencing results psPAX2

psPAX_Primer1

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TCAATGTCGACCCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTT
TTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATA
ATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTG
CGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAA
GATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGA
GAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCG
CGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAG
AATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAG
AGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGAC

psPAX_Primer2

TGCGGTATCACTTCTGGGCTGAAAGCCTTCTCTTCTACTACTTTTACCCATGCATTTAAAGT
TCTAGGTGATATGGCCTGATGTACCATTTGCCCCTGGATGTTCTGCACTATAGGGTAATTTT
GGCTGACCTGATTGCTGTGTCCTGTGTCAGCTGCTGCTTGCTGTGCTTTTTTCTTACTTTTG
TTTTGCTCTTCCTCTATCTTGTCTAAAGCTTCCTTGGTGTCTTTTATCTCTATCCTTTGATG
CACACAATAGAGGGTTGCTACTGTATTATATAATGATCTAAGTTCTTCTGATCCTGTCTGAA
GGGATGGTTGTAGCTGTCCCAGTATTTGTCTACAGCCTTCTGATGTTTCTAACAGGCCAGGA
TTAACTGCGAATCGTTCTAGCTCCCTGCTTGCCCATACTATATGTTTTAATTTATATTTTTT
CTTTCCCCCTGGCCTTAACCGAATTTTTTCCCATCGATCTAATTCTCCCCCGCTTAATACTG
ACGCTCTCGCACCCATCTCTCACCAGTCGCCTCCCCTCGCCTCTTGCCGTGCGCGTCAACGC
GGCCCGAATTCTTTGCCAAAATGATGAGACAGCACAATAACCAGCACGTTGCCCAGGAGCTG
TAGGAAAAAGAAGAAGGCATGAACATGGTTAGCA

psPAX_Primer3

TTGGTATTAAGGTACCGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGATACA
AGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAAT
ACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAAT
GGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGT
GGAAAGGACGAAACACCGTCAAGATCACAGATTTTGGGGTTTTAGAGCTAGAAATAGCAAGT
TAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGAAT
TCGCTAGCTAGGTCTTGAAAGGAGTGGGAATTGGCTCCGGTGCCCGTCAGTGAGGGCCTATT
TCCCATGATTCCTTCATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTAGAATTA
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GGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTG
AAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACCGACCTGCCCG
GCAGTAGTCATGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACT
TGAAAAAGTGGCACCGAGTCGGTGCTTTTTTGAATTCGCTAGCTAGGTCTTGAAAGGAGTGG
GAATTGGCTCCGGTGCCCGTCAGTGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATAT
ACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAG
TACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGT
TTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATA
TATCTTGTGGAAAGGACGAAACACCGGGACTCCGGTGCGTTCGGCAGTTTTAGAGCTGGAAT
AGCAAGTTAAAATAAGGCTAG

9.7. PCR analysis EGFR sequences

9.7.1. HEK293T

target site 1

AAGTCCAGTAAGTTCAAGCCCAGGTCTCAACTGGGCAGCAGAGCTCCTGCTCTTCTTTGTCC
TCATATACGAGCACCTCTGGACTTAAAACTTGAGGAACTGGATGGAGAAAAGTTAATGGTCA
GCAGCGGGTTACATCTTCTTTCATGCGCCTTTCCATTCTTTGGATCAGTAGTCACTAACGTT
CGCCAGCCATAAGTCCTCGACGTGGAGAGGCTCAGAGCCTGGCATGAACATGACCCTGAATT
CGGATGCAGAGCTTCTTCCCATGATGATCTGTCCCTCACAGCAGGGTCTTCTCTGTTTCAGG
GCATGAACTACTTGGAGGACCGTCGCTTGGTGCACCGCGACCTGGCAGCCAGGAACGTACTG
GTGAAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCTGGCCAAACTGCTGGGTGCGGA
AGAGAAAGAATACCATGCAGAAGGAGGCAAAGTAAGGAGGTGGCTTTAGGTCAGCCAGCATT
TTCCTGACACCAGGGACCAGGCTGCCTTCCCACTAGCTGTATTGTTTAACACATGCAGGGGA
GGATGCTCTCCAGACATTCTGGGTGAGCTCGCAGCAGCTGCTGCTGGCAGCTGGGTCCAGCC
AGGGTCTCCTGGTAGTGTGAGCCAGAGCTGCTTTGGGAACAGTACTTGCTGGGACAGTGAAT
GAGGATGTTATCCCCAGGTGATCATTAGCAAATGTTAGGTTTCAGTCTCTCCCTGCAGGATA
TATAAGTCCCCTTCAATGGCGCAATTGGGAAAGGTCACAGCTGCCTTGGTGGTCCACTGCTG
TCAAGGACACCTAAGGAACAGGAAAGGCCCCATGCGGACCCGAGCTCCCAGGGCTGTCTGTG
GCTCGTGGCTGGGACAGGCAGCAATGGAGTCCTTCTCTCCCTTCACTGGCTCGGTTTCTCTT
AGGGACCCTCACAGCACTAAGGG

target site 2

CACTGGAGAAACACTCATGTGGGTTTTCTTAAATTTGCCTCCCTCTGAGCTTCCCTTTAACT
TCAACTATAATATGCAAGAAAGACTATCTGACCATAAATACACATTTGGGCCAATCAAGATG
GTTTTGCCAAGGAAAGATGCCCACAATGGTTAAGCAGAATGCAATAATGTAGAGAATATCAT
TTCTTTCATGCTGGTGTATATCATATGCATTCAAAAACAGGGAGAACTTCTAAGCAACTAAC
AGTGACCATATCAAGCAGGTGCAATCACAGAATAACTGGTTTTCTCCTTTAAGAATTTTTCT
ATCATTTGGCTTTCCCCACTCACACACACTAAATATTTTAAGTAAAAAGTTACTTCCATTTT
GAAAGAGAAAAGAAAGAGACATGCATGAACATTTTTCTCCACCTTGGTGCAGGGACCAGACA
ACTGTATCCAGTGTGCCCACTACATTGACGGCCCCCACTGCGTCAAGACCTGCCCGGCAGGA
GTCATGGGAGAAAACAACACCCTGGTCTGGAAGTACGCAGACGCCGGCCATGTGTGCCACCT
GTGCCATCCAAACTGCACCTACGGGTGAGTGGAAAGTGAAGGAGAACAGAACATTTCCTCTC
TTGCAAATTCAGAGATCAAAAATGTCTCCCAAGTTTTCCGGCAACAAATTGCCGAGGTTTGT
ATTTGAGTCAGTTACTTAAGGTGTTTTGGTCCCCACAGCCATGCCAGTAGCAACTTGCTTGT
GAGCAGGCCTCAGTGCAGTGGGAATGACTCTGCCATGCACCGTGTCCCCGGCCGGGCCTGTG
TTGTGCAATGCTGC

target site 3

CGCCACAGCCCAGTCCTGTGCTGGAAGCCATGTTCTCTGGAAAGAGCAACCCAGCGGCTCAT
AAGCATAAGCGCGTGTGATGTGCCCCAACCAAACGACCGCCATGCACAACTTCCCTACCGGA
GTTTTCAATCCAGTTAATAGGCGTGGAAACAGACATAGAAATTGTGTTTGTTGAAAGGTAGC
TGTTCAGTTAAAGAACACCTGTATCAGAGCCTGTGTTTCTACCAACTTCTGTCAAGCTCTGT
AGAGAAGGCGTACATTTGTCCTTCCAAATGAGCTGGCAAGTGCCGTGTCCTGGCACCCAAGC
CCATGCCGTGGCTGCTGGTCCCCCTGCTGGGCCATGTCTGGCACTGCTTTCCAGCATGGTGA
GGGCTGAGGTGACCCTTGTCTCTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACC
CAGTGGAGAAGCTCCCAACCAAGCTCTCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGA
TCAAAGTGCTGGGCTCCGGTGCGTTCGGCACGGTGTATAAGGTAAGGTCCCTGGCACAGGCC

TCTGGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTGGGGAGCCCAGAGTCCTTGCAAGCTGT
ATATTTCCATCATCTACTTTACTCTTTGTTTCACTGAGTGTTTGGGAAACTCCAGTGTTTTT
CCCAAGTTATTGAGAGGAAATCTTTTATAACCACAGTAATCAGTGGTCCTGTGAGACCAATT
CACAGACCAAAGGCATTTTTATGAAAGGGGCCATTGACCTTGCCATGGGGTGCAGCACAGGG
CGGGAGGAGGGCCGCCTCTCACCGCACGGCATCAGAATGCAGCCCAGCTGAAATGGGCTCA

9.7.2. U87

Target site 1

AAGTCCAGTAAGTTCAAGCCCAGGTCTCAACTGGGCAGCAGAGCTCCTGCTCTTCTTTGTCC
TCATATACGAGCACCTCTGGACTTAAAACTTGAGGAACTGGATGGAGAAAAGATAATGGTCA
GCAGCGGGTTACATCTTCTTTCATGCGCCTTTCCATTCTTTGGATCAGTAGTCACTAACGTT
CGCCAGCCATAAGTCCTCGACGTGGAGAGGCTCAGAGCCTGGCATGAACATGACCCTGAATT
CGGATGCAGAGCTTCTTCCCATGATGATCTGTCCCTCACAGCAGGGTCTTCTCTGTTTCAGG
GCATGAACTACTTGGAGGACCGTCGCTTGGTGCACCGCGACCTGGCAGCCAGGAACGTACTG
GTGAAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCTGGCCAAACTGCTGGGTGCGGA
AGAGAAAGAATACCATGCAGAAGGAGGCAAAGTAAGGAGGTGGCTTTAGGTCAGCCAGCATT
TTCCTGACACCAGGGACCAGGCTGCCTTCCCACTAGCTGTATTGTTTAACACATGCAGGGGA
GGATGCTCTCCAGACATTCTGGGTGAGCTCGCAGCAGCTGCTGCTGGCAGCTGGGTCCAGCC
AGGGTCTCCTGGTAGTGTGAGCCAGAGCTGCTTTGGGAACAGTACTTGCTGGGACAGTGAAT
GAGGATGTTATCCCCAGGTGATCATTAGCAAATGTTAGGTTTCAGTCTCTCCCTGCAGGATA
TATAAGTCCCCTTCAATAGCGCAATTGGGAAAGGTCACAGCTGCCTTGGTGGTCCACTGCTG
TCAAGGACACCTAAGGAACAGGAAAGGCCCCATGCGGACCCGAGCTCCCAGGGCTGTCTGTG
GCTCGTGGCTGGGACAGGCAGCAATGGAGTCCTTCTCTCCCTTCACTGGCTCGGTTTCTCTT
AGGGACCCTCACAGCACTAAGGG

Target site 3

CGCCACAGCCCAGTCCTGTGCTGGAAGCCATGTTCTCTGGAAAGAGCAACCCAGCGGCTCAT
AAGCATAAGCGCGTGTGATGTGCCCCAACCAAACGACCGCCATGCACAACTTCCCTACCGGA
GTTTTCAATCCAGTTAATAGGCGTGGAAACAGACATAGAAATTGTGTTTGTTGAAAGGTAGC
TGTTCAGTTAAAGAACACCTGTATCAGAGCCTGTGTTTCTACCAACTTCTGTCAAGCTCTGT
AGAGAAGGCGTACATTTGTCCTTCCAAATGAGCTGGCAAGTGCCGTGTCCTGGCACCCAAGC
CCATGCCGTGGCTGCTGGTCCCCCTGCTGGGCCATGTCTGGCACTGCTTTCCAGCATGGTGA
GGGCTGAGGTGACCCTTGTCTCTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACC
CAGTGGAGAAGCTCCCAACCAAGCTCTCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGA
TCAAAGTGCTGGGCTCCGGTGCGTTCGGCACGGTGTATAAGGTAAGGTCCCTGGCACAGGCC
TCTGGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTGGGGAGCCCAGAGTCCTTGCAAGCTGT
ATATTTCCATCATCTACTTTACTCTTTGTTTCACTGAGTGTTTGGGAAACTCCAGTGTTTTT
CCCCAGTTATTGAGAGGAAATCTTTTATAACCACAGTAATCAGTGGTCCTGTGAGACCAATT
CACAGACCAAAGGCATTTTTATGAAAGGGGCCATTGACCTTGCCATGGGGTGCAGCACAGGG
CGGGAGGAGGGCCGCCTCTCACCGCACGGCATCAGAATGCAGCCCAGCTGAAATGGGCTCA

9.7.3. U138

Target site 1

AAGTCCAGTAAGTTCAAGCCCAGGTCTCAACTGGGCAGCAGAGCTCCTGCTCTTCTTTGTCC
TCATATACGAGCACCTCTGGACTTAAAACTTGAGGAACTGGATGGAGAAAAGTTAATGGTCA
GCAGCGGGTTACATCTTCTTTCATGCGCCTTTCCATTCTTTGGATCAGTAGTCACTAACGTT
CGCCAGCCATAAGTCCTCGACGTGGAGAGGCTCAGAGCCTGGCATGAACATGACCCTGAATT
CGGATGCAGAGCTTCTTCCCATGATGATCTGTCCCTCACAGCAGGGTCTTCTCTGTTTCAGG
GCATGAACTACTTGGAGGACCGTCGCTTGGTGCACCGCGACCTGGCAGCCAGGAACGTACTG
GTGAAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCTGGCCAAACTGCTGGGTGCGGA
AGAGAAAGAATACCATGCAGAAGGAGGCAAAGTAAGGAGGTGGCTTTAGGTCAGCCAGCATT
TTCCTGACACCAGGGACCAGGCTGCCTTCCCACTAGCTGTATTGTTTAACACATGCAGGGGA
GGATGCTCTCCAGACATTCTGGGTGAGCTCGCAGCAGCTGCTGCTGGCAGCTGGGTCCAGCC
AGGGTCTCCTGGTAGTGTGAGCCAGAGCTGCTTTGGGAACAGTACTTGCTGGGACAGTGAAT
GAGGATGTTATCCCCAGGTGATCATTAGCAAATGTTAGGTTTCAGTCTCTCCCTGCAGGATA
TATAAGTCCCCTTCAATAGCGCAATTGGGAAAGGTCACAGCTGCCTTGGTGGTCCACTGCTG
TCAAGGACACCTAAGGAACAGGAAAGGCCCCATGCGGACCCGAGCTCCCAGGGCTGTCTGTG
GCTCGTGGCTGGGACAGGCAGCAATGGAGTGCTTCTCTCCCTTCACTGGCTCGGTTTCTCTT
AGGGACCCTCACAGCACTAAGGG

Target site 2

TTTTCTTAAATTTGCCTCCCTCTGAGCTTCCCTTTAACTTCAACTATAATATGCAAGAAAGA
CTATCTGACCATAAATACACATTTGGGCCAATCAAGATGGTTTTGCCAAGGAAAGATGCCCA
CAATGGTTAAGCAGAATGCAATAATGTAGAGAATATCATTTCTTTCATGCTGGTGTATATCA
TATGCATTCAAAAACAGGGAGAACTTCTAAGCAACTAACAGTGACCATATCAAGCAGGTGCA
ATCACAGAATAACTGGTTTTCTCCTTTAAGAATTTTTCTATCATTTGCCTTTCCCCACTCAC
ACACACTAAATATTTTAAGTAAAAAGTTACTTCCATTTTGAAAGAGAAAAGAAAGAGACATG
CATGAACATTTTTCTCCACCTTGGTGCAGGGACCAGACAACTGTATCCAGTGTGCCCACTAC
ATTGACGGCCCCCACTGCGTCAAGACCTGCCCGGCAGGAGTCATGGGAGAAAACAACACCCT
GGTCTGGAAGTACGCAGACGCCGGCCATGTGTGCCACCTGTGCCATCCAAACTGCACCTACG
GGTGAGTGGAAAGTGAAGGAGAACAGAACATTTCCTCTCTTGCAAATTCAGAGATCAAAAAT
GTCTCCCAAGTTTTCCGGCAACAAATTGCCGAGGTTTGTATTTGAGTCAGTTACTTAAGGTG
TTTTGGTCCCCACAGCCATGCCAGTAGCAACTTGCTTGTGAGCAGGCCTCAGTGCAGTGGGA
ATGACTCTGCCATGCACCGTGTCCCCGGCCGG

Target site 3

CGCCACAGCCCAGTCCTGTGCTGGAAGCCATGTTCTCTGGAAAGAGCAACCCAGCGGCTCAT
AAGCATAAGCGCGTGTGATGTGCCCCAACCAAACGACCGCCATGCACAACTTCCCTACCGGA
GTTTTCAATCCAGTTAATAGGCGTGGAAACAGACATAGAAATTGTGTTTGTTGAAAGGTAGC
TGTTCAGTTAAAGAACACCTGTATCAGAGCCTGTGTTTCTACCAACTTCTGTCAAGCTCTGT
AGAGAAGGCGTACATTTGTCCTTCCAAATGAGCTGGCAAGTGCCGTGTCCTGGCACCCAAGC
CCATGCCGTGGCTGCTGGTCCCCCTGCTGGGCCATGTCTGGCACTGCTTTCCAGCATGGTGA
GGGCTGAGGTGACCCTTGTCTCTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACC
CAGTGGAGAAGCTCCCAACCAAGCTCTCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGA
TCAAAGTGCTGGGCTCCGGTGCGTTCGGCACGGTGTATAAGGTAAGGTCCCTGGCACAGGCC
TCTGGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTGGGGAGCCCAGAGTCCTTGCAAGCTGT
ATATTTCCATCATCTACTTTACTCTTTGTTTCACTGAGTGTTTGGGAAACTCCAGTGTTTTT
CCCAAGTTATTGAGAGGAAATCTTTTATAACCACAGTAATCAGTGGTCCTGTGAGACCAATT
CACAGACCAAAGGCATTTTTATGAAAGGGGCCATTGACCTTGCCATGGGGTGCAGCACAGGG
CGGGAGGAGGGCCGCCTCTCACCGCACGGCATCAGAATGCAGCCCAGCTGAAATGGGCTCA

9.7.4. U343

Target site 2

TTTTCTTAAATTTGCCTCCCTCTGAGCTTCCCTTTAACTTCAACTATAATATGCAAGAAAGA
CTATCTGACCATAAATACACATTTGGGCCAATCAAGATGGTTTTGCCAAGGAAAGATGCCCA
CAATGGTTAAGCAGAATGCAATAATGTAGAGAATATCATTTCTTTCATGCTGGTGTATATCA
TATGCATTCAAAAACAGGGAGAACTTCTAAGCAACTAACAGTGACCATATCAAGCAGGTGCA
ATCACAGAATAACTGGTTTTCTCCTTTAAGAATTTTTCTATCATTTGGCTTTCCCCACTCAC
ACACACTAAATATTTTAAGTAAAAAGTTACTTCCATTTTGAAAGAGAAAAGAAAGAGACATG
CATGAACATTTTTCTCCACCTTGGTGCAGGGACCAGACAACTGTATCCAGTGTGCCCACTAC
ATTGACGGCCCCCACTGCGTCAAGACCTGCCCGGCAGGAGTCATGGGAGAAAACAACACCCT
GGTCTGGAAGTACGCAGACGCCGGCCATGTGTGCCACCTGTGCCATCCAAACTGCACCTACG
GGTGAGTGGAAAGTGAAGGAGAACAGAACATTTCCTCTCTTGCAAATTCAGAGATCAAAAAT
GTCTCCCAAGTTTTCCGGCAACAAATTGCCGAGGTTTGTATTTGAGTCAGTTACTTAAGGTG
TTTTGGTCCCCACAGCCATGCCAGTAGCAACTTGCTTGTGAGCAGGCCTCAGTGCAGTGGGA
ATGACTCTGCCATGCACCGTGTCCCCGGCCGG

Target site 3

CGCCACAGCCCAGTCCTGTGCTGGAAGCCATGTTCTCTGGAAAGAGCAACCCAGCGGCTCAT
AAGCATAAGCGCGTGTGATGTGCCCCAACCAAACGACCGCCATGCACAACTTCCCTACCGGA
GTTTTCAATCCAGTTAATAGGCGTGGAAACAGACATAGAAATTGTGTTTGTTGAAAGGTAGC
TGTTCAGTTAAAGAACACCTGTATCAGAGCCTGTGTTTCTACCAACTTCTGTCAAGCTCTGT
AGAGAAGGCGTACATTTGTCCTTCCAAATGAGCTGGCAAGTGCCGTGTCCTGGCACCCAAGC
CCATGCCGTGGCTGCTGGTCCCCCTGCTGGGCCATGTCTGGCACTGCTTTCCAGCATGGTGA
GGGCTGAGGTGACCCTTGTCTCTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACC
CAGTGGAGAAGCTCCCAACCAAGCTCTCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGA
TCAAAGTGCTGGGCTCCGGTGCGTTCGGCACGGTGTATAAGGTAAGGTCCCTGGCACAGGCC
TCTGGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTGGGGAGCCCAGAGTCCTTGCAAGCTGT
ATATTTCCATCATCTACTTTACTCTTTGTTTCACTGAGTGTTTGGGAAACTCCAGTGTTTTT
CCCAAGTTATTGAGAGGAAATCTTTTATAACCACAGTAATCAGTGGTCCTGTGAGACCAATT
CACAGACCAAAGGCATTTTTATGAAAGGGGCCATTGACCTTGCCATGGGGTGCAGCACAGGG
CGGGAGGAGGGCCGCCTCTCACCGCACGGCATCAGAATGCAGCCCAGCTGAAATGGGCTCA

9.7.5. AKH-5

Target site 2

TTTTCTTAAATTTGCCTCCCTCTGAGCTTCCCTTTAACTTCAACTATAATATGCAAGAAAGA
CTATCTGACCATAAATACACATTTGGGCCAATCAAGATGGTTTTGCCAAGGAAAGATGCCCA
CAATGGTTAAGCAGAATGCAATAATGTAGAGAATATCATTTCTTTCATGCTGGTGTATATCA
TATGCATTCAAAAACAGGGAGAACTTCTAAGCAACTAACAGTGACCATATCAAGCAGGTGCA
ATCACAGAATAACTGGTTTTCTCCTTTAAGAATTTTTCTATCATTTGGCTTTCCCCACTCAC
ACACACTAAATATTTTAAGTAAAAAGTTACTTCCATTTTGAAAGAGAAAAGAAAGAGACATG
CATGAACATTTTTCTCCACCTTGGTGCAGGGACCAGACAACTGTATCCAGTGTGCCCACTAC
ATTGACGGCCCCCACTGCGTCAAGACCTGCCCGGCAGGAGTCATGGGAGAAAACAACACCCT
GGTCTGGAAGTACGCAGACGCCGGCCATGTGTGCCACCTGTGCCATCCAAACTGCACCTACG
GGTGAGTGGAAAGTGAAGGAGAACAGAACATTTCCTCTCTTGCAAATTCAGAGATCAAAAAT
GTCTCCCAAGTTTTCCGGCAACAAATTGCCGAGGTTTGTATTTGAGTCAGTTACTTAAGGTG
TTTTGGTCCCCACAGCCATGCCAGTAGCAACTTGCTTGTGGGCAGGCCTCAGTGCAGTGGGA
ATGACTCTGCCATGCACCGTGTCCCCGGCCGG

9.7.6. AKH-9

Target site 1

AGCGGGTTACATCTTCTTTCATGCGCCTTTCCATTCTTTGGATCAGTAGTCACTAACGTTCG
CCAGCCATAAGTCCTCGACGTGGAGAGGCTCAGAGCCTGGCATGAACATGACCCTGAATTCG
GATGCAGAGCTTCTTCCCATGATGATCTGTCCCTCACAGCAGGGTCTTCTCTGTTTCAGGGC
ATGAACTACTTGGAGGACCGTCGCTTGGTGCACCGCGACCTGGCAGCCAGGAACGTACTGGT
GAAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCTGGCCAAACTGCTGGGTGCGGAAG
AGAAAGAATACCATGCAGAAGGAGGCAAAGTAAGGAGGTGGCTTTAGGTCAGCCAGCATTTT
CCTGACACCAGGGACCAGGCTGCCTTCCCACTAGCTGTATTGTTTAACACATGCAGGGGAGG
ATGCTCTCCAGACATTCTGGGTGAGCTCGCAGCAGCTGCTGCTGGCAGCTGGGTCCAGCCAG
GGTCTCCTGGTAGTGTGAGCCAGAGCTGCTTTGGGAACAGTACTTGCTGGGACAGTGAATGA
GGATGTTATCCCCAGGTGATCATTAGCAAATGTTAGGTTTCAGTCTCTCCCTGCAGGATATA
TAAGTCCCCTTCAATAGCGCAATTGGGAAAGGTCACAGCTGCCTTGGTGGTCCACTGCTGTC
AAGGACACCTAAGGAACAGGAAAGGCCCCATGCGGACCCGAGCTCCCAGGGCTGTCTGTGGC
TCGTGGCTGGGACAGGCAGCAATGGAGTCCTTCTCTCCCTTCACTGGCTCGGTTTCTCTTAG
GGACCCTCACAGCACTAAGGG

Target site 2

TTTTCTTAAATTTGCCTCCCTCTGAGCTTCCCTTTA(ACTTCAACTATAATATGCAAGAAAG
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)GCCAATCAAGATGGTTTTGCCAAGGAAAGATGCCCACAATGGTTAAGCAGAATGCAATAAT
GTAGAGAATATCATTTCTTTCATGCTGGTGTATATCATATGCATTCAAAAACAGGGAGAACT
TCTAAGCAACTAACAGTGACCATATCAAGCAGGTGCAATCACAGAATAACTGGTTTTCTCCT
TTAAGAATTTTTCTATCATTTGGCTTTCCCCACTCACACACACTAAATATTTTAAGTAAAAA
GTTACTTCCATTTTGAAAGAGAAAGTCTCCACCTTGGTGCGGGGACCAGACAACTGTATCCA
GTGTGCCCACTACATTGACGGCCCCCACTGCGTCAAGACCTGCCCGGCAGGAGTCATGGGAG
AAAACAACACCCTGGTCTGGAAGTACGCAGACGCCGGCCATGTGTGCCACCT(GTGCCATCC
AAACTGCACCTAC)GGGTGAGTGGAAAGTGAAGGAGAACAGAACATTTCCTCTCTTGCAAAT
TCAGAGATCAAAAATGTCTCCCAAGTTTTCCGGCAACAAATTGCCGAGGTTTGTATTTGAGT
CAGTTACTTAAGGTGTTTTGGTCCCCACAGCCATGCCAGTAGCAACTTGCTTGTGAGCAGGC
CTCAGTGCAGTGGGAATGACTCTGCCATGCACCGTGTCCCCGGCCGG

9.7.7. AKH-10

target site 1

CCACATGCAGGAAGGTCAGCCTGGCAAGTCCAGTAAGTTCAAGCCCAGGTCTCAACTGGGCA
GCAGAGCTCCTGCTCTTCTTTGTCCTCATATACGAGCACCTCTGGACTTAAAACTTGAGGAA
CTGGATGGAGAAAAGTTAATGGTCAGCAGCGGGTTACATCTTCTTTCATGCGCCTTTCCATT
CTTTGGATCAGTAGTCACTAACGTTCGCCAGCCATAAGTCCTCGACGTGGAGAGGCTCAGAG
CCTGGCATGAACATGACCCTGAATTCGGATGCAGAGCTTCTTCCCATGATGATCTGTCCCTC
ACAGCAGGGTCTTCTCTGTTTCAGGGCATGAACTACTTGGAGGACCGTCGCTTGGTGCACCG
CGACCTGGCAGCCAGGAACGTACTGGTGAAAACACCGCAGCATGTCAAGATCACAGATTTTG
GGCTGGCCAAACTGCTGGGTGCGGAAGAGAAAGAATACCATGCAGAAGGAGGCAAAGTAAGG
AGGTGGCTTTAGGTCAGCCAGCATTTTCCTGACACCAGGGACCAGGCTGCCTTCCCACTAGC
TGTATTGTTTAACACATGCAGGGGAGGATGCTCTCCAGACATTCTGGGTGAGCTCGCAGCAG
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AACAGTACTTGCTGGGACAGTGAATGAGGATGTTATCCCCAGGTGATCATTAGCAAATGTTA
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CAGCTGCCTTGGTGGTCCACTGCTGTCAAGGACACCTAAGGAACAGGAAAGGCCCCATGCGG
ACCCGAGCTCCCAGGGCTGTCTGTGGCTCGTGGCTGGGACAGGCAGCAATGGAGTCCTTCTC
TCCCTTCACTGGCTCGGTTTCTCTTAGGGACCCTCACAGCACTAAGGGGTGCGCGTCCCCTG
TCAGGC

target site 2

CACTGGAGAAACACTCATGTGGGTTTTCTTAAATTTGCCTCCCTCTGAGCTTCCCTTTAACT
TCAACTATAATATGCAAGAAAGACTATCTGACCATAAATACACATTTGGGCCAATCAAGATG
GTTTTGCCAAGGAAAGATGCCCACAATAGAATGCAATAATGTAGAGAATATCATTTCTTTCA
TGCTGGTGTATATCATATGCATTCAAAAACAGGGAGAACTTCTAAGCAACTAACAGTGACCA
TATCAAGCAGGTGCAATCACAGAATAACTGGTTTTCTCCTTTAAGAATTTTTCTATCATTTG
GCTTTCCCCACTCACACACACTAAATATTTTAAGTAAAAAGTTAATCGTTAAGAACTTCCAT
TTTGAAAGAGAAAAGAAAGAGACATGCATGAACATTTTTCTCCACCTTGGTGCAGGGACCAG
ACAACTGTATCCAGTGTGCCCACTACATTGACGGCCCCCACTGCGTCAAGACCTGCCCGGCA
GTAGTCATGGGAGAAAACAACACCCTGGTCTGGAAGTACGCAGACGCCGGCCATGTGTGCCA
CCTGTGCCATCCAAACTGCACCTACTGGTGAGTGGAAAGTGAAGGAGAACAGAACATTTCCT
CTCTTGCAAATTCAGAGATCAAAAATGTCTCCCAAGTTTTCCGGCAACAAATTGCCGAGGTT
TGTATTTGAGTCAGTTACTTAAGGTCTTTTGGTCCCCACAGCCATGCCAGTAGCAACTTGCT
TGTGAGCAGGCCTCAGTGCAGTGGGAATGACTCTGCCATGCACCGTGTCCCCGGCCGGGCCT
GTGTTGTGCAATGCTGC

target site 3 sequence 1

CGCCACAGCCCAGTCCTGTGCTGGAAGCCATGTTCTCTGGAAAGAGCAACCCAGCGGCTCAT
AAGCATAAGCGCGTGTGATGTGCCCCAA(C)CAAACGACCGCCATCTACAACTTCCCTACCG
GAGTTTTCAATCCAGTTAATAGGCGTGGAAACAGACATAGAAATTGTGTTTGTTGAAAGGTA
GCTGTTCAGTTAAAGAACACCTGTATCAGAGCCTGTGTTTCTACCAACTTCTGTCAAGCTCT
GTAGAGAAGGCGTACATTTGTCCTTCCAAATGAGCTGGCAAGTGGCGTGTCCTGGCACCCAA
GCCCATGCCGTGGCTGCTGGTCCCCCTGCTGGGCCATGTCTGGCACTGCTTTCCAGCATGGT
GAGGGCTGAGGTGACCATTGTCTCTGTGTTCTTGTCCGCCCCAGCTTGTGGAGCCTCTTACA
CCCAGTGGAGAAGCTCCCACCAAGCTCTCTTGAGGATCTTGAAGGAATCTGAATTCAAAAAG
ATCAAAGTGCTGGGCTCCGGTGCGTTCGGCACGGTGTATAAGGTAAGGTCCCTGGCACAGGC
CTCTGGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTGGGGAGCCCAGAGTCCTTGCAAGCTG
TATATTTCCATCATCTACTTTACTCTTTGTTTCACTGAGTGTTTGGGAAACTCCAGTGTTTT
TCCCAAGTTATTGAGAGGAAATCTTTTTATAACCACAGTAATCAGTGGTCCTGTGAGACCAA

TTCACAGACCAAAGGCATTTTTATGAAAGGGGCCATGTGCAGCACAGGGCGGGAGGAGGGCC
GCCTCTCACCGCACGGCATCAGAATGCAGCCCAGCTGAAATGGGCTCA

target site 3 sequence 2

TGGAAGCCATGTTCTCTGGAAAGAGCAACCCAGCGGCTCATAAGCATAAGCGCGTGTGATGT
GCCCCAACCAAACGACCGCCATGCACAACTTCCCTACCGGAGTTTTCAATCCAGTTAATAGG
CGTGGAAACAGACATAGAAATTGTGTTTGTTGAAAGGTAGCTGTTCAGTTAAAGAACACCTG
TATCAGAGCCTGTGTTTCTACCAACTTCTGTCAAGCTCTGTAGAGAAGGCGTACATTTGTCC
TTCCAAATGAGCTGGCAAGTGCCGTGTCCTGGCACCCAAGCCCATGCCGTGGCTGCTGGTCC
CCCTGCTGGGCCATGTCTGGCACTGCTTTCCAGCATGGTGAGGGCTGAGGTGACCCTTGTCT
CTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACCCAGTGGAGAAGCTCCCAACCA
AGCTCTCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGATCAAAGTGCTGGGCTCCGGTG
CGTTCGGCACGGTGTATAAGGTAAGGTCCCTGGCACAGGCCTCTGGGCTGGGCCGCAGGGCC
TCTCATGGTCTGGTGGGGAGCCCAGAGTCCTTGCAAGCTGTATATTTCCATCATCTACTTTA
CTCTTTGTTTCACTGAGTGTTTGGGAAACTCCAGTGTTTTTCCCAAGTTATTGAGAGGAAAT
CTTTTATAACCACAGTAATCAGTGGTCCTGTGAGACCAATTCACAGACCAAAGGCATTTTTA
TGAAAGGGGCCATTGACCTTGCCATGGGGTGCAGCACAGGGCGGGAGGAGGGCCGCCTCTCA
CCGCACGG-CATCAGAAT

9.7.8. AKH-13

Target site 1

AAGTCCAGTAAGTTCAAGCCCAGGTCTCAACTGGGCAGCAGAGCTCCTGCTCTTCTTTGTCC
TCATATACGAGCACCTCTGGACTTAAAACTTGAGGAACTGGATGGAGAAAAGTTAATGGTCA
GCAGCGGGTTACATCTTCTTTCATGCGCCTTTCCATTCTTTGGATCAGTAGTCACTAACGTT
CGCCAGCCATAAGTCCTCGACGTGGAGAGGCTCAGAGCCTGGCATGAACATGACCCTGAATT
CGGATGCAGAGCTTCTTCCCATGATGATGTGTCCCTCACAGCAGGGTCTTCTCTGTTTCAGG
GCATGAACTACTTGGAGGACCGTCGCTTGGTGCACCGCGACCTGGCAGCCAGGAACGTACTG
GTGAAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCTGGCCAAACTGCTGGGTGCGGA
AGAGAAAGAATACCATGCAGAAGGAGGCAAAGTAAGGAGGTGGCTTTAGGTCAGCCAGCATT
TTCCTGACACCAGGGACCAGGCTGCCTTCCCACTAGCTGTATTGTTTAACACATGCAGGGGA
GGATGCTCTCCAGACATTCTGGGTGAGCTCGCAGCAGCTGCTGCTGGCAGCTGGGTCCAGCC
AGGGTCTCCTGGTAGTGTGAGCCAGAGCTGCTTTGGGAACAGTACTTGCTGGGACAGTGAAT
GAGGATGTTATCCCCAGGTGATCATTAGCAAATGTTAGGTTTCAGTCTCTCCCTGCAGGATA
TATAAGTCCCCTTCAATAGCGCAATTGGGAAAGGTCACAGCTGCCT(T)GGTGGTCCACTGC
TGTCAAGGACACCTAAGGAACAGGAAAGGCCCCATGCGGACCCGAGCTCCCAGGGCTGTCTG
TGGCTCGTGGCTGGGACAGGCAGCAATGGAGTCCTTCTCTCCCTTCACTGGCTCGGTTTCTC
TTAGGGACCCTCACAGCACTAAGGG

Target site 2

TTTTCTTAAATTTGCCTCCCTCTGAGCTTCCCTTTAACTTCAACTATAATATGCAAGAAAGA
CTATCTGACCATAAATACACATTTGGGCCAATCAAGATGGTTTTGCCAAGGAAAGATGCCCA
CAATGGTTAAGCAGAATGCAATAATGTAGAGAATATCATTTCTTTCATGCTGGTGTATATCA
TATGCATTCAAAAACAGCGAGAACTTCTAAGCAACTAACAGTGACCATATCAAGCAGGTGCA
ATCACAGAATAACTGGTTTTCTCCTTTAAGAATTTTTCTATCATTTGGCTTTCCCCACTCAC
ACACACTAAATATTTTAAGTAAAAAGTTACTTCCATTTTGAAAGAGAAAAGAAAGAGACATG
CATGAACATTTTTCTCCACCTTGGTGCAGGGACCAGACAACTGTATCCAGTGTGCCCACTAC
ATTGACGGCCCCCACTGCGTCAAGACCTGCCCGGCAGGAGTCATGGGAGAAAACAACACCCT
GGTCTGGAAGTACGCAGACGCCGGCCATGTGTGCCACCTGTGCCATCCAAACTGCACCTACG
GGTGAGTGGAAAGTGAAGGAGAACAGAACATTTCCTCTCTTGCAAATTCAGAGATCAAAAAT
GTCTCCCAAGTTTTCCGGCAACAAATTGCCGAGGTTTGTATTTGAGTCAGTTACTTAAGGTG
TTTTGGTCCCCACAGCCATGCCAGTAGCAACTTGCTTGTGAGCAGGCCTCAGTGCAGTGGGA
ATGACTCTGCCATGCACCGTGACCCCGGCCGG

Target site 3

CGCCACAGCCCAGTCCTGTGCTGGAAGCCATGTTCTCTGGAAAGAGCAACCCAGCGGCTCAT
AAGCATAAGCGCGGGTGATGTGCCCCAACCAAACGACCGCCATGCACAACTTCCCTACCGGA
GTTTTCAATCCAGTTAATAGGCGTGGAAACAGACATAGAAATTGTGTTTGTTGAAAGGTAGC
TGTTCAGTTAAAGAACACCTGTATCAGAGCCTGTGTTTCTACCAACTTCTGTCAAGCTCTGT
AGAGAAGGCGTACATTTGTCCTTCCAAATGAGCTGGCAAGTGCCGTGTCCTGGCACCCAAGC
CCATGCCGTGGCTGCTGGTCCCCCTGCTGGGCCATGTCTGGCACTGCTTTCCAGCATGGTGA
GGGCTGAGGTGACCCTTGTCTCTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACC
CAGTGGAGAAGCTCCCAACCAAGCTCTCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGA
TCAAAGTGCTGGGCTCCGGTGCGTTCGGCACGGTGTATAAGGTAAGGTCCCTGGCACAGGCC
TCTGGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTGGGGAGCCCAGAGTCCTTGCAAGCTGT
ATATTTCCATCATCTACTTTACTCTTTGTTTCACTGAGTGTTTGGGAAACTCCAGTGTTTTT
CCCAAGTTATTGAGAGGAAATCTTTTATAACCACAGTAATCAGTGGTCCTGTGAGACCAATT
CACAGACCAAAGGCATTTTTATGAAAGGGGCCATTGACCTTGCCATGGGGTGCAGCACAGGG
CGGGAGGAGGGCCGCCTCTCACCGCACGGCATCAGAATGCAGCCCAGCTGAAATGGGCTCA

9.7.9. AKH-14

Target site 1

AAGTCCAGTAAGTTCAAGCCCAGGTCTCAACTGGGCAGCAGAGCTCCTGCTCTTCTTTGTCC
TCATATACGAGCACCTCTGGACTTAAAACTTGAGGAACTGGATGGAGAAAAGTTAATGGTCA
GCAGCGGGTTACATCTTCTTTCATGCGCCTTTCCATTCTTTGGATCAGTAGTCACTAACGTT
CGCCAGCCATAAGTCCTCGACGTGGAGAGGCTCAGAGCCTGGCATGAACATGACCCTGAATT
CGGATGCAGAGCTTCTTCCCATGATGATCTGTCCCTCACAGCAGGGTCTTCTCTGTTTCAGG
GCATGAACTACTTGGAGGACCGTCGCTTGGTGCACCGCGACCTGGCAGCCAGGAACGTACTG
GTGAAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCTGGCCAAACTGCTGGGTGCGGA
AGAGAAAGAATACCATGCAGAAGGAGGCAAAGTAAGGAGGTGGCTTTAGGTCAGCCAGCATT
TTCCTGACACCAGGGACCAGGCTGCCTCCCCACTAGCTGTATTGTTTAACACATGCAGGGGA
GGATGCTCTCCAGACATTCTGGGTGAGCTCGCAGCAGCTGCTGCTGGCAGCTGGGTCCAGCC
AGGGTCTCCTGGTAGTGTGAGCCAGAGCTGCTTTGGGAACAGTACTTGCTGGGACAGTGAAT
GAGGATGTTATCCCCAGGTGATCATTAGCAAATGTTAGGTTTCAGTCTCTCCCTGCAGGATA
TATAAGTCCCCTTCAATAGCGCAATTGGGAAAGGTCACAGCTGCCTTGGTGGTCCACTGCTG
TCAAGGACACCTAAGGAACAGGAAAGGCCCCATGCGGACCCGAGCTCCCAGGGCTGTCTGTG
GCTCGTGGCTGGGACAGGCAGCAATGGAGTCCTTCTCTCCCTTCACTGGCTCGGTTTCTCTT
AGGGACCCTCACAGCACTAAGGG

Target site 2

TTTTCTTAAATTTGCCTCCCTCT(GAGCTTCCCTTTAACTTCAACTATAATATGCAAGAAAG
ACTATCTGACCATAAATACACATTTGGGCCAATCAAGATGGTTTTGCCAAGGAAAGATGCCC
ACAATGGTTAAGCAGAATGCAATAATGTAGAGAATATCATTTCTTTCATGCTGGTGT)ATAT
CATATGCATTCAAAAACAGGGAGAACTTCTAAGCATACTAACAGTGACCATATCAAGCAGGT
GCAATCACAGAATAACTGGTTTTCTCCTTTAAGAATTTTTCTATCATTTGGCTTTCCCCACT
CACACACACTAAATATTTTAAGTAAAAAGTTACTTCCATTTTGAAAGAGAAAAGAAAGAGAC
ATGCATGAACATTTTTCTCCACCTTGGTGCAGGGACCAGACAACTGTATCCAGTGTGCCCAC
TACATTGACGGCCCCCACTGCGTCAAGACCTGCCCGGCAGGAGTCATGGGAGAAAACAACAC
CCTGGTCTGGAAGTACGCAGACGCCGGCCATGTGTGCCACCTGTGCCATCCAAACTGCACCT
AAGGGTGAGTGGAAAGTGAAGGAGAACAGAACATTTCCTCTCTTGCAAATTCAGAGATCAAA
AATGTCTCCCAAGTTTTCCGGCAACAAATTGCCGAGGTTTGTATTTGAGTCAGTTACTTAAG
GTGTTTTGGTCCCCACAGCCATGCCAGTAGCAACTTGCTTGTGAGCAGGCCTCAGTGCAGTG
GGAATGACTCTGCCATGCACCGTGTCCCCGGCCGG

Target site 3

CGCCACAGCCCAGTCCTGTGCTGGAAGCCATGTTCTCTGGAAAGAGCAACCCAGCGGCTCAT
AAGCATAAGCGCGTGTGATGTGCCCCAACCAAACGACCGCCATGCACAACTTCCCTACCGGA
GTTTTCAATCCAGTTAATAGGCGTGGAAACAGACATAGAAATTGTGTTTGTTGAAAGGTAGC
TGTTCAGTTAAAGAACACCTGTATCAGAGCCTGTGTTTCTACCAACTTCTGTCAAGCTCTGT
AGAGAAGGCGTACATTTGTCCTTCCAAATGAGCTGGCAAGTGCCGTGTCCTGGCACCCAAGC
CCATGCCGTGGCTGCTGGTCCCCCTGCTGGGCCATGTCTGGCACTGCTTTCCAGCATGGTGG
GGGCTGAGGTGACCCTTGTCTCTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACC
CAGTGGAGAAGCTCCCAACCAAGCTCTCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGA
TCAAAGTGCTGGGCTCCGGTGCGTTCGGCACGGTGTATAAGGTAAGGTCCCTGGCACAGGCC
TCTGGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTGGGGAGCCCAGAGTCCTTGCAAGCTGT
ATATTTCCATCATCTACTTTACTCTTTGTTTCACTGAGTGTTTGGGAAACTCCAGTGTTTTT
CCCAAGTTATTGAGAGGAAACCTTTTATAACCACAGTAATCAGTGGTCCTGTGAGACCAATT
CACAGACCAAAGGCATTTTTATGAAAGGGGCCATTGACCTTGCCATGGGGTGCAGCACAGGG
CGGGAGGAGGGCCGCCTCTCACCGCACGGCATCAGAATGCAGCCCAGCTGAAATGGGCTCA

9.7.10. AKH-15

Target site 2

TTTTCTTAAATTTGCCTCCCTCTGAGCTTCCCTTTAACTTCAACTATAATATGCAAGAAAGA
CTATCTGACCATAAATACACATTTGGGCCAATCAAGATGGTTTTGCCAAGGAAAGATGCCCA
CAATGGTTAAGCAGAATGCAATAATGTAGAGAATATCATTTCTTTCATGCTGGTGTATATCA
TATGCATTCAAAAACAGGGAGAACTTCTAAGCAACTAACAGTGACCATATCAAGCAGGTGCA
ATCACAGAATAACTGGTTTTCTCCTTTAAGAATTTTTCTATCATTTGGCTTGTCC(C)CACT
CACACACACTAAATATTTTAAGTAAAAAGTTACTTCCATTTTGAAAGAGAAAAGAAAGAGAC
ATGCATGAACATTTTTCTCCACCTTGGTGCAGGGACCAGACAACTGTATCCAGTGTGCCCAC
TACATTGACGGCCCCCACTGCGTCAAGACCTGCCCGGCAGGAGTCATGGGAGAAAACAACAC
CCTGGTCTGGAAGTACGCAGACGCCGGCCATGTGTGCCACCTGTGCCATCCAAACTGCACCT
ACGGGTGAGTGGAAAGTGAAGGAGAACAGAACATTTCCTCTCTTGCAAATTCAGAGATCAAA
AATGTCTCCCAAGTTTTCCGGCAACAAATTGCCGAGGTTTGTATTTGAGTCAGTTACTTAAG
GTGTTTTGGTCCCCACAGCCATGCCAGTAGCAACTTGCTTGTGAGCAGGCCTCAGTGCAGTG
GGAATGACTCTGCCATGCACCGTGTCCCCGGCCGG

9.7.11. AKH-17

Target site 2

AAGTGTGTATCGCTCGAGGGATCCGAATTCAGGAGGTAAAAACCATGATGCAGCATTGCACA
ACACAGGCCCGGCCGGGGACACGGTGCATGGCAGAGTCATTCCCACTGCACTGAGGCCTGCT
CACAAGCAAGTTGCTACTGGCATGGCTGTGGGGACCAAAACACCTTAAGTAACTGACTCAAA
TACAAACCTCGGCAATTTGTTGCCGGAAAACTTGGGAGACATTTTTGATCTCTGAATTTGCA
AGAGAGGAAATGTTCTGTTCTCCTTCACTTTCCACTCACCCGTAGGTGCAGTTTGGATGGCA
CAGGTGGCACACATGGCCGGCGTCTGCGTACTTCCAGACCAGGGTGTTGTTTTCTCCCATGA
CTCCTGCCGGGCAGGTCTTGACGCAGTGGGGGCCGTCAATGTAGTGGACACACTGGATACAG
TTGTCTGGTCCCTGCACCAAGGTGGAGAAAAATGTTCATGCATGTCTCTTTCTTTTCTCTTT
CAAAATGGAAGTAACTTTTTACTTAAAATATTTAGTGTGTGTGAGTGGGGAAAGCCAAATGA
TAGAAAAATTCTTAAAGGAGAAAACCAGTTATTCTGTGATTGCACCTGCTTGATATGGTCAC
TGTTAGTTGCTTAGAAGTTCTCCCTGTTTTTGAATGCATATGATATACACCAGCATGAAAGA
AATGATATTATCTACATTATTGCATTCTGCTTAACCATTGTGGGCATCTTTCCTTGGCAAAA
CCATCTTGATTGGCCCAAATGTGTATTTATGGTCAGATAGTCTTTCTTGCATATTATAGTTG
AAGTTAAAGGGAAGCTCAGAGGGAGGCAAATTTAAGAAAACCCACATGAGTGTTTCTCCAGT
GACAGGAAGGTGGCAAGTCTCTGTGCAGTGGGAATGACTCTGCCATGCACCGTGTCCCCGGC
CGGGCCTGTGTTGTGCAATGCTGCCTGATAATAATTAATTAAGACGTCAGAATTCTCGAGGC
GGCCGCATGTGCGTCTCCCTATAGTGAG

9.7.12. AKH-19

Target site 1

AAGTCCAGTAAGTTCAAGCCCAGGTCTCAACTGGGCAGCAGAGCTCCTGCTCTTCTTTGTCC
TCATATACGAGCACCTCTGGACTTAAAACTTGAGGAACTGGATGGAGAAAAGTTAATGGTCA
GCAGCGGGTTACATCTTCTTTCATGCGCCTTTCCATTCTTTGGATCAGTAGTCACTAACGTT
CGCCAGCCATAAGTCCTCGACGTGGAGAGGCTCAGAGCCTGGCATGAACATGACCCTGAATT
CGGATGCAGAGCTTCTTCCCATGATGATCTGTCCCTCACAGCAGGGTCTTCTCTGTTTCAGG
GCATGAACTACTTGGAGGACCGTCGCTTGGTGCACCGCGACCTGGCAGCCAGGAACGTACTG
GTGAAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCTGGCCAAACTGCTGGGTGCGGA
AGAGAAAGAATACCATGCAGAAGGAGGCAAAGTAAGGAGGTGGCTTTAGGTCAGCCAGCATT
TTCCTGACACCAGGGACCAGGCTGCCTCCCCACTAGCTGTATTGTTTAACACATGCAGGGGA
GGATGCTCTCCAGACATTCTGGGTGAGCTCGCAGCAGCTGCTGCTGGCAGCTGGGTCCAGCC
AGGGTCTCCTGGTAGTGTGAGCCAGAGCTGCTTTGGGAACAGTACTTGCTGGGACAGTGAAT
GAGGATGTTATCCCCAGGTGATCATTAGCAAATGTTAGGTTTCAGTCTCTCCCTGCAGGATA
TATAAGTCCCCTTCAATAGCGCAATTGGGAAAGGTCACAGCTGCCTTGGTGGTCCACTGCTG
TCAAGGACACCTAAGGAACAGGAAAGGCCCCATGCGGACCCGAGCTCCCAGGGCTGTCTGTG
GCTCGTGGCTGGGACAGGCAGCAATGGAGTCCTTCTCTCCCTTCACTGGCTCGGTTTCTCTT
AGGGACCCTCACAGCACTAAGGG

Target site 2

AAGTGTGTATCGCTCGAGGGATCCGAATTCAGGAGGTAAAAACCATGATGCAGCATTGCACA
ACACAGGCCCGGCCGGGGACCCGGTGCATGGCAGAGTCATTCCCACTGCACTGAGGCCTGCT
CACAAGCAAGTTGCTACTGGCATGGCTGTGGGGACCAAAACACCTTAAGTAACTGACTCAAA
TACAAACCTCGGCAATTTGTTGCCGGAAAACTTGGGAGACATTTTTGATCTCTGAATTTGCA
AGAGAGGAAATGTTCTGTTCTCCTTCACTTTCCACTCACCCGTAGGTGCAGTTTGGATGGCA
CAGGTGGCACACATGGCCGGCGTCTGCGTACTTCCAGACCAGGGTGTTGTTTTCTCCCATGA
CTCCTGCCGGGCAGGTCTTGACGCAGTGGGGGCCGTCAATGTAGTGGACACACTGGATACAG
TTGTCTGGTCCCTGCACCAAGGTGGAGAAAAATGTTCATGCATGTCTCTTTCTTTTCTCTTT

CAAAATGGAAGTAACTTTTTACTTAAAATATTTAGTGTGTGTGAGTGGGGAAAGCCAAATGA
TAGAAAAATTCTTAAAGGAGAAAACCAGTTATTCTGTGATTGCACCTGCTTGATATGGTCAC
TGTTAGTTGCTTAGAAGTTCTCCCTGTTTTTGAATGCATATGATATACACCAGCATGAAAGA
AATGATATTATCTACATTATTGCATTCTGCTTAACCATTGTGGGCATCTTTCCTTGGCAAAA
CCATCTTGATTGGCCCAAATGTGTATTTATGGTCAGATAGTCTTTCTTGCATATTATAGTTG
AAGTTAAAGGGAAGCTCAGAGGGAGGCAAATTTAAGAAAACCCACATGAGTGTTTCTCCAGT
GAGAGGAAGGTGGCAAGTCTCTGTGCAGTGGGAATGACTCTGCCATGCACCGTGTCCCCGGC
CGGGCCTGTGTTGTGCAATGCTGCCTGATAATAATTAATTAAGACGTCAGAATTCTCGAGGC
GGCCGCATGTGCGTCTCCCTATAGTGAG

9.7.13. AKH-20

Target site 3 sequence 1

CGCCACAGCCCAGTCCTGTGCTGGAAGCCATGTTCTCTGGAAAGAGCAACCCAGCGGCTCAT
AAGCATAAGCGCGTGTGATGTGCCCCAACCAAACGACCGCCATGCACAACTTCCCTACCGGA
GTTTTCAATCCAGTTAATAGGCGTGGAAACAGACATAGAAATTGTGTTTGTTGAAAGGTAGC
TGTTCAGTTAAAGAACACCTGTATCAGAGCCTGTGTTTCTACCAACTTCTGTCAAGCTCTGT
AGAGAAGGCGTACATTTGTCCTTCCAAATGAGCTGGCAAGTGCCGTGTCCTGGCACCCAAGC
CCATGCCGTGGCTGCTGGTCCCCCTGCTGGGCCATGTCTGGCACTGCTTTCCAGCATGGTGA
GGGCTGAGGTGACCCTTGTCTCTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACC
CAGTGGAGAAGCTCCCAACCAAGCTCTCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGA
TCAAAGTGCTGGGCTCCGGTGCGTTCGGCACGGTGTATAAGGTAAGGTCCCTGGCACAGGCC
TCTGGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTGGGGAGCCCAGAGTCCTTGCAAGCTGT
ATATTTCCATCATCTACTTTACTCTTTGTTTCACTGAGTGTTTGGGAAACTCCAGTGTTTTT
CCCAAGTTATTGAGAGGAAATCTTTTATAACCACAGTAATCAGTGGTCCTGTGAGACCAATT
CACAGACCAAAGGCATTTTTATGAAAGGGGCCATTGACCTTGCCATGGGGTGCAGCACAGGG
CGGGAGGAGGGCCGCCTCTCACCGCACGGCATCAGAATGCAGCCCAGCTGAAATGGGCTCA

Target site 3 sequence 2

CACAGCCCAGTCCTGTGCTGGAAGCCATGTTCTCTGGAAAGAGCAACCCAGCGGCTCATAAG
CATAAGCGCGTGTGATGTGCCCCAACCAAACGACCGCCATGCACAACTTCCCTACCGGAGTT
TTCAATCCAGTTAATAGGCGTGGAAACAGACATAGAAATTGTGTTTGTTGAAAGGTAGCTGT
TCAGTTAAAGAACACCTGTATCAGAGCCTGTGTTTCTACCAACTTCTGTCAAGCTCTGTAGA
GAAGGCGTACATTTGTCCTTCCAAATGAGCTGGCAAGTGCCGTGTCCTGGCACCCAAGCCCA
TGCCGTGGCTGCTGGTCCCCCTGCTGGGCCATGTCTGGCACTGCTTTCCAGCATGGTGAGGG
CTGAGGTGACCCTTGTCTCTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACCCAG
TGGAGAAGCTCCCAACCAAGCTCTCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGATCA
AAGTGCTGGGCTCCGGTGCGTTCGGCACGGTGTATAAGGTAAGGTCCCTGGCACAGGCCTCT
GGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTGGGGAGCCCAGAGTCCTTGCAAGCTGTATA
TTTCCATCATCTACTTTACTCTTTGTTTCACTGAGTGTTTGGGAAACTCCAGTGTTTTTCCC
AAGTTATTGAGAGGAAATCTTTTATAACCACAGTAATCAGTGGTCCTGTGAGACCAATTCAC
AGACCAAAGGCATTTTTAT(G)AAAGGGGCCATTGACCTTGCCATGGGGTGCAGCACAGGGC
GGGAGGAGGGCCGCCTCTCACCGCACGGCATCAGAATGCAGCCCAGCTGAAATGGGCTCA

9.7.14. AKH-23

Target site 2 sequence 1

AAGTGTGTATCGCTCGAGGGATCCGAATTCAGGAGGTAAAAACCATGATGCAGCATTGCACA
ACACAGGCCCGGCCGGGGACACGGTGCATGGCAGAGTCATTCCCACTGCACTGAGGCCTGCT
CACAAGCAAGTTGCTACTGGCATGGCTGTGGGGACCAAAACACCTTAAGTAACTGACTCAAA
TACAAACCTCGGCAATTTGTTGCCGGAAAACTTGGGAGACATTTTTGATCTCTGAATTTGCA
AGAGAGGAAATGTTCTTTTCTCCTTCACTTTCCACTCACCCGTAGGTGCAGTTTGGATGGCA
CAGGTGGCACACATGGCCGGCGTCTGCGTACTTCCAGACCAGGGTGTTGTTTTCTCCCATGA
CTCCTGCCGGGCAGGTCTTGACGCAGTGGGGGCCGTCAATGTAGTGGACACACTGGATACAG
TTGTCTGGTCCCTGCACCAAGGTGGAGAAAAATGTTCATGCATGTCTCTTTCTTTTCTCTTT
CAAAATGGAAGTAACTTTTTACTTAAAATATTTAGTGTGTGTGAGTGGGGAAAGCCAAATGA
TAGAAAAATTCTTAAAGGAGAAAACCAGTTATTCTGTGATTGCACCTGCTTGATATGGTCAC
TGTTAGTTGCTTAGAAGTTCTCCCTGTTTTTGAATGCATATGATATACACCAGCATGAAAGA
AATGATATTATCTACATTATTGCATTCTGCTTAACCATTGTGGGCATCTTTCCTTGGCAAAA
CCATCTTGATTGGCCCAAATGTGTATTTATGGTCAGATAGTCTTTCTTGCATATTATAGTTG
AAGTTAAAGGGAAGCTCAGAGGGAGGCAAATTTAAGAAAACCCACATGAGTGTTTCTCCAGT
GACAGGAAGGTGGCAAGTCTCTGTGCAGTGGGAATGACTCTGCCATGCACCGTGTCCCCGGC
CGGGCCTGTGTTGTGCAATGCTGCCTGATAATAATTAATTAAGACGTCAGAATTCTCGAGGC
GGCCGCATGTGCGTCTCCCTATAGTGAG

Target site 2 sequence 2

(TTTTCTTAAATTTGCCTCCCTCTGAGCTTCCCTTTAACTTCAACTATAATATGCAAGAAAG
ACTATCTGACCATAAATACACATTTGGGCCAATCAAGATGGTTTTGCCAAGGAAAGATGCCC
ACAATGGTTAAGCAGAATGCAATAATGTAGAGAATATCATTTCTTTCATGCTGGTGTATATC
ATATGCATTCAA)CAAACCTCGGCAATTTGTTGCCGGAAAACTTGGGAGACATTTTTGATCT
CTGAATTTGCAAGAGAGGAAATGTTCTGTTCTCCTTAACTTTCCACTCACCCGTAGGTGCAG
TTTGGATGGCACAGGTGGCACACATGGCCGGCGTCTGCGTACTTCCAGACCAGGGTGTTGTT
TTCTCCCATGACTCCTGCCGGGCAGGTCTTGACGCAGTGGGGGCCGTCAATGTAGTGGACAC
ACTGGATACAGTTGTCTGGTCCCTGCACCAAGGTGGAGAAAAATGTTCATGCATGTCTCTTT
CTTTTCTCTTTCAAAATGGAAGTAACTTTTTACTTAAAATATTTAGTGTGTGTGAGTGGGGA
AAGCCAAATGATAGAAAAATTCTTAAAGGAGAAAACCAGTTATTCTGTGATTGCACCTGCTT
GATATGGTCACTGTTAGTTGCTTAGAAGTTCTCCCTGTTTTTGAATGCATATGATATACACC
AGCATGAAAGAAATGATATTATCTACATTATTGCATTCTGCTTAACCATTGTGGGCATCTTT
CCTTGGCAAAACCATCTTGATTGGCCCAAATGTGTATTTATGGTCAGATAGTCTTTCTTGCA
TATTATAGTTGAAGTTAAAGGGAAGCTCAGAGGGAGGCAAATTTAAGGAAACCCACATGAGT
GTTTCTCCAGTGACAGGAAGGTGGCAAGTCTCTGTGCAGTGGGAATGACTCTGCCATGCACC
GTGTCCCCGGCCGGGCCTGTGTTGTGCAATGCTGCCTGATAATAATTAATTAAGACGTCAGA
ATTCTCGAGGCGGCCGCATGTGCGTCTCCCTATAGTGAG

Target site 3

CGCCACAGCCCAGTCCTGTGCTGGAAGCCATGTTCTCTGGAAAGAGCAACCCAGCGGCTCAT
AAGCATAAGCGCGTGTGATGTGCCCCAACCAAACGACCGCCATGCACAACTTCCCTACCGGA
GTTTTCAATCCAGTTAATAGGCGTGGAAACAGACATAGAAATTGTGTTTGTTGAAAGGTAGC
TGTTCAGTTAAAGAACACCTGTATCATAGCCTGTGTTTCTACCAACTTCTGTCAAGCTCTGT
AGAGAAGGCGTACATTTGTCCTTCCAAATGAGCTGGCAAGTGCCGTGTCCTGGCACCCAAGC
CCATGCCGTGGCTGCTGGTCCCCCTGCTGGGCCATGTCTGGCACTGCTTTCCAGCATGGTGA
GGGCTGAGGTGACCCTTGTCTCTGTGTTCTTGTCCCCCCCAGCTTGTGGAGCCTCTTACACC
CAGTGGAGAAGCTCCCAACCAAGCTCTCTTGAGGATCTTGAAGGAAACTGAATTCAAAAAGA
TCAAAGTGCTGGGCTCCGGTGCGTTCGGCACGGTGTATAAGGTAAGGTCCCTGGCACAGGCC
TCTGGGCTGGGCCGCAGGGCCTCTCATGGTCTGGTGGGGAGCCCAGAGTCCTTGCAAGCTGT

ATATTTCCATCATCTACTTTACTCTTTGTTTCACTGAGTGTTTGGGAAACTCCAGTGTTTTT
CCCAAGTTATTGAGAGGAAATCTTTTATAACCACAGTAATCAGTGGTCCTGTGAGACCAATT
CACAGACCAAAGGCATTTTTATGAAAGGGGCCATTGACCTTGCCATGGGGTGCAGCACAGGG
CGGGAGGAGGGCCGCCTCTCACCGCACGGCATCAGAATGCAGCCCAGCTGAAATGGGCTCA

9.8. PCR analysis BIRC5 sequences

9.8.1. HEK293T

ATGCCCCGCGGCGCGCCATTAACCGCCAGATTTGAATCGCGGGACCCGTTGGCAGAGGTGGC
GGCGGCGGCATGGGTGCCCCGACGTTGCCCCCTGCCTGGCAGCCCTTTCTCAAGGACCACCG
CATCTCTACATTCAAGAACTGGCCCTTCTTGGAGGGCTGCGCCTGCACCCCGGAGCGGGTGA
GACTGCCCGGCCTCCTGGCGTCCCCCACGCCCGCCTTGCCCTGTCCCTAGCGAGGCCACTGT
GACTGGGCCTCGGGGGTACAAGCCGCCCTCCCCTCCCCGTCCTGTCCCCAGCGAGGCCACTG
TGGCTGGGCCCCTTGGGTCCAGGCCGGCCTCCCCTCCCTGCTTTGTCCCCATCGAGGCCTTT
GTGGCTGGGCCTCGGGGTTCCGGGCTGCCACGTCCACTCACGAGCTGTGCTGTCCCTTGCAG
ATGGCCGAGGCTGGCTTCATCCACTGCCCCACTGAGAACGAGCCAGACTTGGCCCAGTGTTT
CTTCTGCTTCAAGGAGCTGAAGGCTGGGAGCCAGATGAGACCCCATGTAAGTCTTCTCTGGC
CAGCCTCGATGGGCTTTGTTTTGAACTGAGTTGTCAAAAGATTTGAGTTGCAAAGACACTTA
GTATGGGAGGGTTGCTTTCCACCCTCATTGCTTCTTAAACAGCTGTTGTGAACGGATACCTC
TCTATATGCTGGTGCCTTGGTGATGCTTACAACCTAATTAAATCTCATTTGACCAAAATGCC
TTGGGGTGGACGTAAGATGCCTGATGCCTTTCATGTTCAACAGAATACATCAGCAGACCCTG
TTGTTGTGAACTCCCAGGAACGTCCAAGTGCTTTTTTTGAGATTTTTTAAAAAACAGTTTAA
TTGAAATATAACCTACACAGCACAAAAATTACCCTTTGAAAGTGTGCACTTCACACTTTCGG
AGGCTGAGGCGGGCGGATCACCTGAGGTCAGGAGTTCAAGACCTGCCTGG

9.8.2. U-cells and AKH cells

GGCGCGCCATTAACCGCCAGATTTGAATCGCGGGACCCGTTGGCAGAGGTGGCGGCGGCGGC
ATGGGTGCCCCGACGTTGCCCCCTGCCTGGCAGCCCTTTCTCAAGGACCACCGCATCTCTAC
ATTCAAGAACTGGCCCTTCTTGGAGGGCTGCGCCTGCACCCCGGAGCGGGTGAGACTGCCCG
GCCTCCTGGGGTCCCCCACGCCCGCCTTGCCCTGTCCCTAGCGAGGCCACTGTGACTGGGCC
TCGGGGGTACAAGCCGCCCTCCCCTCCCCGTCCTGTCCCCAGCGAGGCCACTGTGGCTGGGC
CCCTTGGGTCCAGGCCGGCCTCCCCTCCCTGCTTTGTCCCCATCGAGGCCTTTGTGGCTGGG
CCTCGGGGTTCCGGGCTGCCACGTCCACTCACGAGCTGTGCTGTCCCTTGCAGATGGCCGAG
GCTGGCTTCATCCACTGCCCCACTGAGAACGAGCCAGACTTGGCCCAGTGTTTCTTCTGCTT
CAAGGAGCTGGAAGGCTGGGAGCCAGATGACGACCCCATGTAAGTCTTCTCTGGCCAGCCTC
GATGGGCTTTGTTTTGAACTGAGTTGTCAAAAGATTTGAGTTGCAAAGACACTTAGTATGGG
AGGGTTGCTTTCCACCCTCATTGCTTCTTAAACAGCTGTTGTGAACGGATACCTCTCTATAT
GCTGGTGCCTTGGTGATGCTTACAACCTAATTAAATCTCATTTGACCAAAATGCCTTGGGGT
GGACGTAAGATGCCTGATGCCTTTCATGTTCAACAGAATACATCAGCAGACCCTGTTGTTGT
GAACTCCCAGGAACGTCCAAGTGCTTTTTTTGAGATTTTTTAAAAAACAGTTTAATTGAAAT
ATAACCTACACAGCACAAAAATTACCCTTTGAAAGTGTGCACTTCACACTTTCGGAGGCTGA
GGCGGGCGGATCACCTGAGGTCAGGAGT

9.9. Statutory declaration

I hereby declare that I have written this thesis independently and without outside help. Text passages that are based verbatim or in spirit on publications or lectures by other authors are marked as such.

Hamburg, 15th of March 2024

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Celine Pöhlking

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