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„Expansion and characterization of pathway reporter plasmids“

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1.Introduction

1.1. Plasmid

One of the main reasons why bacteria are viable, is their ability to transfer parts of their genome to other bacteria. This process is called conjugation and is essential for their survival. In 1952 J. Lederberg and his team found out that the causer of the conjugation, the F-Factor, is located extrachromosomal and called it Plasmid **(1)**. Plasmids are small, circular double stranded DNA molecules, which can replicate independently from chromosomal DNA. Bacteria are using them to exchange important information for survival such as antibiotic resistance. After the discovery of restriction enzymes and gel electrophoresis, plasmids have been used for creation of recombinant DNA with the gene of interest. By using the recombinant DNA technology, high amounts of the gene of interest can be replicated easily and in a short time **(2).**

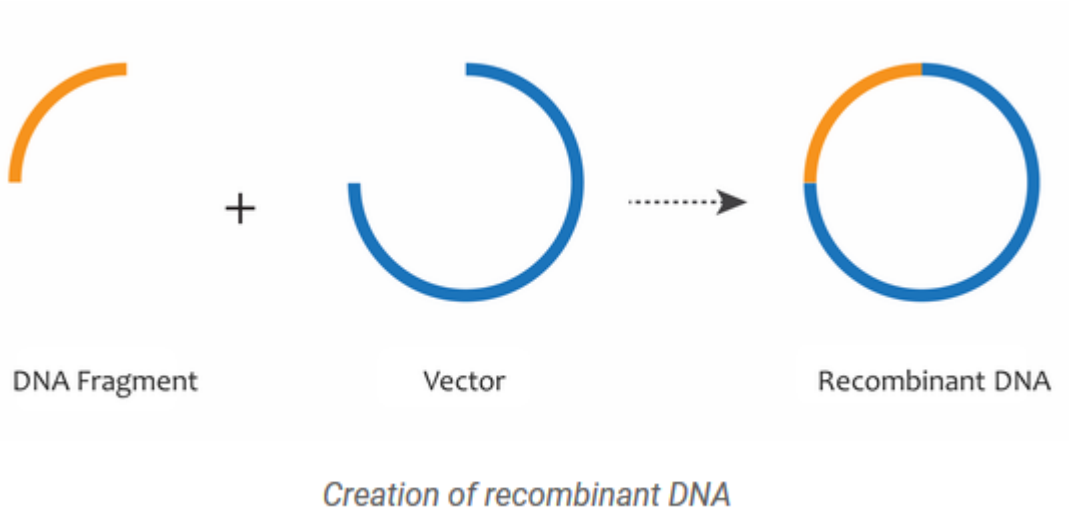

Figure 1: Creation of recombinant DNA.(Figure from Addgene.org)

Plasmids are available in different sizes and vary broadly in their functionality. All of them have an origin of replication (ori), an antibiotic resistance gene and multiple cloning site. The ori site is responsible for the initiation of the replication, the antibiotic resistance gene allows the host bacteria containing the plasmid to grow in or on media containing that antibiotic. The most commonly used antibiotics for selection are ampicillin, kanamycin, tetracycline and spectinomycin **(3).** Another important sequence inside the plasmid is the Multiple cloning site (MCS). Within this short segment of DNA several unique restriction sites for restrction enzyme are present. By forming blunt ends any blunt-ended fragment can be inserted into the MCS, whereas with sticky ends DNA fragments digested with the same restriction enzyme can be inserted **(4)**. The fragment which is inserted in to the MCS is called Insert. The Promoter Region initiate the transcription of the Insert and if the plasmid is designed for human/ mammalian cells the promoter should show activity in human/ mammalian cells. Some plasmids contain a selectable marker which is used to select for cells that successfully taken up the plasmid and showing the successfully expressing of the insert. The Primer binding site is another short single stranded DNA sequence used as an activating point for PCR amplification or DNA sequencing of the plasmid **(5)**.

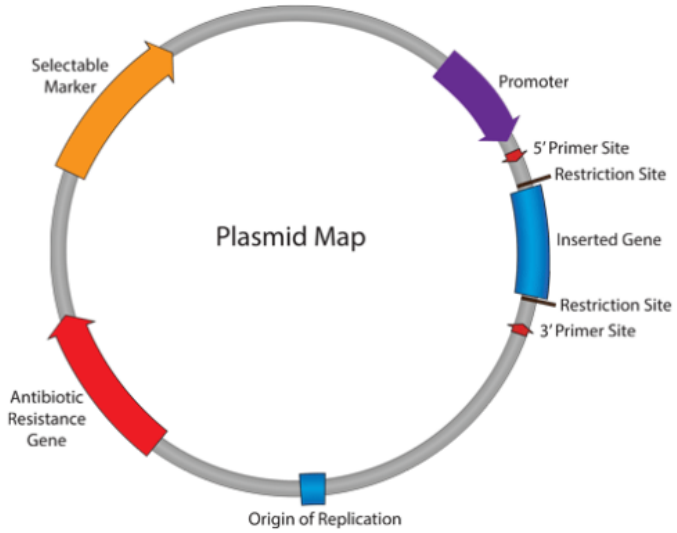

Figure 2: Plasmid map showing important components. (Figure from Addgene.org)

1.2. Reporter Plasmids

Another way of using plasmids is to study the function of genetic elements. Such type of plasmids are called reporter plasmids. These plasmids contain the gene of interest, which can be a reporter gene that makes it possible to read out the activity of the genetic element **(4,6).** The reporter gene is under the control of a promoter which is inducible by Transcription factors. Reporter genes are used to characterize and identify promoter elements, to identify interacting proteins and to monitor the transfection efficiency. Engineering reporter genes into viral vectors makes it possible to track the activity of viral genes in host cells and improved the understanding for the control of viral genes. Other processes in cells like, RNA processing, gene targeting, and signal transduction pathways have been monitored by using reporter genes. Reporter plasmids are introduced into cells by transfection methods and cells which have integrated the reporter gene can be selected during the propagation through selectable markers which are included in to the transfection vector. The transfer of the reporter plasmids in-to eukaryotic cells can be done by viral transduction, carrier-mediated transfection or direct injection of the plasmid DNA into cells. Detection of the reporter can be done by measuring the reporter mRNA or measuring the reporter proteins. Methods like Northern Blot analysis or reverse transcription PCR are used to detect reporter mRNA. But since the measuring of mRNA is more complex, a lot of assays are available for measuring the reporter protein. Assays for measuring the enzymatic activity, spectrophotometric characteristics and indirectly antibody-based assays are often used. The most sensitive assay is the enzymatic assay, but it is limited if the cells have endogenous enzymatic activity. Antibody based assays are less sensitive but will detect the reporter protein even if it is not active and it can be used to visualize protein expression via immunohistochemistry. Some of the most commonly used reporter gene assays are mentioned below **(4,26).**

β- Galactosidase Assay:

Beta galactosidase is an enzyme which catalyses the hydrolysis of β-galactoside. The reporter gene for this assay is the lacZ gene from E.coli which encodes for β-Galactosidase. Using various substrates like o-nitrophenyl-β-D-galactopyranoside (ONPG) or chlorophenol red β-D-galactopyranoside (CPRG) the enzymatic activity can be measured with a spectrophotometer, luminometer or fluorometer. β-methyl umbelliferyl galactoside (MUG) or fluorescein digalactoside (FDG) are substrates used for fluorescent-based assays, which allow the detection in single cells **(4,26).**

Green Fluorescent Protein (GFP) Assay:

The GFP was first isolated from Aequorea Victoria and contains a chromophore group in the center of the protein. Depending on the excitation peak it emits green (395nm) or blue (475nm) light. Because GFP does not need any substrates or cofactors for fluoresce it can be monitored in living organisms and cells. By changing amino acids, several mutants with different excitation peak can generated such as blue fluorescent protein (447nm). Fusion proteins of GFP can be used to detect protein-protein interaction or as biosensors. Stability to heat, chemical denaturants and to extreme pH change are some of the advantages of GFP **(4,26).**

Luciferase Assay:

The most frequently used reporter gene is the luc gene for luciferase cloned from the Photinus pyralis. Luciferase catalyses light emission by using D-Luciferin, ATP,O2 and Magnesium. The light emission is measured with a luminometer and the amount of signal correlates with the activity of the promoter. By including coenzyme A into the assay the light reaction is more sustained and allows reproducible results. The sensitivity of this assay is higher than the previous mentioned assays and the results can be received in minutes compared to other assays **(4,7,26).**

1.3. Plasmid validation

After propagating, isolating and purifying the plasmid, it is important to confirm the identity of the plasmid. Sanger Sequencing is a quick method to determine the precise order of nucleotides within the plasmid. For this sequencing method backbone-specific primers, nucleotides and small amount of dideoxy nucleotides are needed for inducing chain break. The resulting DNA sequences are separated by gel electrophoresis. The most common used technique for quickly verifying a plasmid is the diagnostic restriction digest (DRD). It is performed by digesting DNA with restriction enzymes, which are naturally occurring bacterial endonucleases that recognize a huge broad of DNA sequences. For a successful digest, so called restriction sites are necessary on which the restriction enzyme cleave the DNA. The diagnostic restriction digest (DRD) is divided in two parts, incubation and agarose gel electrophoresis. During the first part the plasmids will be incubated with restriction enzymes in a so-called thermocycler to allow enzymatic digestion followed by heat inactivation of the enzymes. After the inactivation, the digested plasmids are going to be separated by gel electrophoresis and the size of the resulting fragments can be determined easily by comparing them to the DNA standard containing fragments of length **(9).**

1.4. Cancer and Gene Therapy

Cancer is the leading cause of death in 21st century. The incidence and mortality rate are rapidly growing, and the leading reasons are population growth, changes in lifestyle and increased longevity. **(10).** Nearly 40000 new incidences have been registered in 2017 in Austria **(11)**. Since the discovery of oncogenes and the functional loss of tumor suppressor genes, it is known that tumorigenesis is a multistep process leading to malignant transformation of normal human cells. The so called six hallmarks of cancer are suggested to be the reason of malignant growth **(12)**.

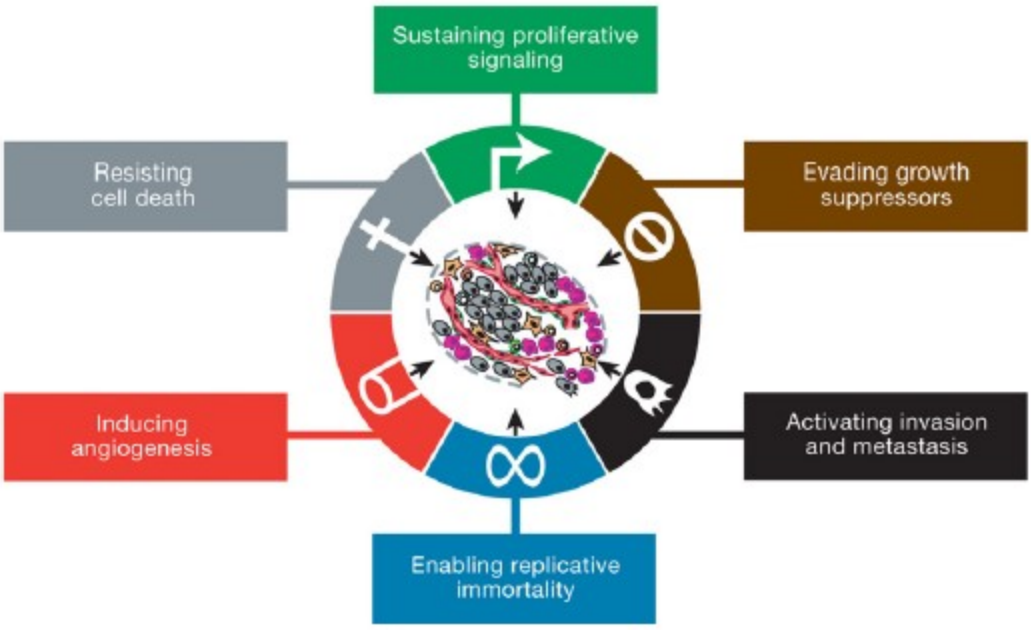

Figure 3: The six Hallmarks of cancer. (Figure from Hanahan and Weinberg 2011)

Current treatment methods such as surgical removal, radiotherapy and chemotherapy have limited therapeutic effects, not enough for treating tumour metastasis or tumour resistance. To overcome these problems, cancer gene therapy seems to be a promising method **(13)**. Gene therapy involves the exchange of defective gene with a functional copy of that gene to correct the underlying disorder. It was initially proposed in 1960 as a method for treating diseases caused by single-gene defects. In 1966, Edward Tantum described in his paper the use of viruses for genetic manipulation and the possible advantages **(14)**. The first successful therapeutic application of gene therapy was achieved in 1990 from Rosenberg and his team. Since then, gene therapy has been investigated for treating thalassaemia, Duchenne muscular dystrophy, hypercholesterinaemia, cystic fibrosis, haemophilia, neurodegenerative diseases like Morbus Parkinson, Morbus Alzheimer or multiple sclerosis and also infectious diseases such as HIV **(14,15,16,17,18,19)**.

Due to recent technological advances, the field of gene therapy has been expanded and leaded to the creation of cancer gene therapy **(14).** There are several possibilities to target and eliminate cancer cells with gene therapy such as: delivering apoptosis causing genes, inserting tumor suppressor genes or using anti-sense RNA/DNA to down regulate oncogenes and promoting immune response against cancer cells **(14).**

Gene delivery is possible by using physical targeting methods like electroporation, microinjection or using viral and non-viral vectors which are modified to bind selectively to cancer cells **(14,20)**.

Vectors for application in gene therapy

Viral vectors are very efficient delivery methods for nucleic acid therapeutics. They can be separated in two categories: the integrating vectors like Retroviral and Lentiviral vectors and the non- integrating vectors like Adenoviral vectors and Adenovirus associated virus vectors. Gene transfer with viral vectors is the most efficient method nowadays. However, the pathogenicity of viral proteins, pre-existing immunity due to vaccination and previous infection and adenovirus-induced liver toxicity are limiting factors for their use **(14,20)**.

To avoid the previous mentioned disadvantages of viral vectors, several methods for gene delivery have been developed. Electroporation is one physical method which uses high-voltage impulses to enable therapeutic DNA to enter the cells but can be only used for ex-vivo modification since it is not possible to target specific population of cells **(21)**. Another very successful way for delivering nucleic acids is to deliver the therapeutic gene in form of plasmid DNA inside of cationic lipids (lipoplexes) or polymers (polyplexes). Both carriers are positively charged and bind to the negatively charged DNA due to electrostatic interaction. For successful packaging and delivery, the surface of the carriers can be modified with different substances. Polyethyleneimine (PEI) based polymeric vectors are protecting the DNA from degradation and enables endosomal escape. They can be also coupled with targeting proteins, to bind on surface protein which are commonly overexpressed by tumor cells. Polyethylenglycol (PEG) based polymers prevents the aggregation of complexes and increases the stability **(14,20,22,23,24,25)**.

The delivered plasmid DNA contains all important characteristics for plasmids like origin of replication, multiple cloning sites, antibiotic resistance gene for selection and the therapeutic gene inserted in to the multiple cloning site. After covering the plasmid DNA into polyplexes or lipoplexes it can be delivered into the targeted tissue and leads after transcription and translation of the therapeutic gene, to the desired therapeutic effect. Compared to viral vectors, the non-viral vectors can be produced at lower costs, are less immunogenic and it is possible to repeat the administration several times **(20)**.

1.5. Wnt, Hedgehog &Notch

Wnt, Hedgehog and Notch are developmental pathways which are important for embryonic development, cell death, motility and migration. Due to scientific research it is known that the deregulation of all three pathways play an important role in the development of cancer and especially cancer stem cells. That's the reason why these three pathways are attractive targets for anticancer therapies **(27)**.

1.5.1. Wnt Pathway

The Wnt pathway belongs to the signalling pathways which are key mediators for embryonic development **(28,29)**. It was discovered during a mutagenesis screen of Drosophila melanogaster and was called the wingless gene because a mutation on this gene leads to wingless type of Drosophila **(30)**. The Int-1 gene promotes the development of breast cancer in mice **(28)**. After binding of Wnt to its receptor Frizzled (FZ)/ low density lipoprotein receptor related protein (LPR) complex on the cell surface, the pathway gets activated. The receptor complex inactivates a so-called destruction complex containing glycogen synthase kinase-3β (GSK-3), Axin, Adenomatous Polyposis Coli (APC) which are responsible for the degradation of β-catenin. Usually the cytoplasmatic β-catenin levels are kept low (Fig. 4) by the previously mentioned destruction complex, but because the protein complex is inactivated, β-catenin accumulates in the cytoplasm and nucleus. Inside the nucleus, β-catenin interacts with transcription factors (TCF)/ lymphoid enhancer-binding factor (LEF) and leads to growth and proliferation **(31,32)**.

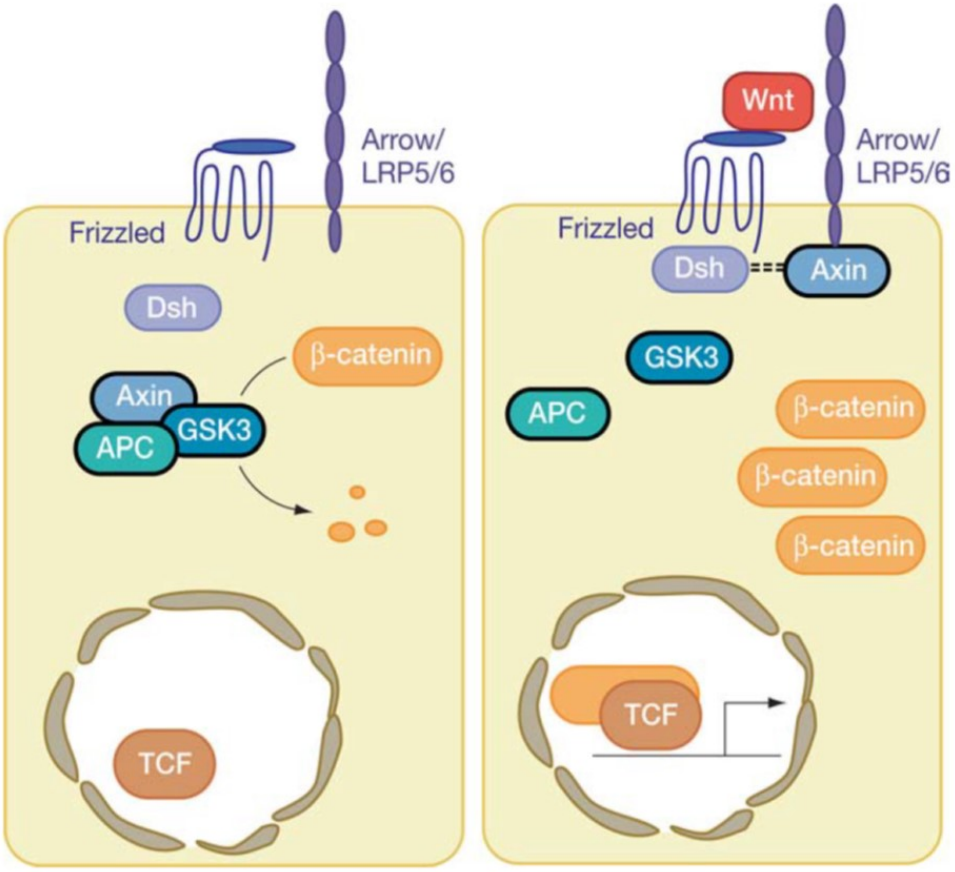

Figure 4: Wnt signaling pathway. (Left) In cells not exposed to β-catenin. (Right) Transduction of β-catenin in to the nucleus after binding of wnt to the FZ/LPR complex. (Figure from Catriona Y. Logan and Roel Nusse 2004)

Since the Wnt pathway is active in the development of cancer, several transgenic reporters containing multimerized TCF binding site have been developed over the past years to track the activity of this pathway **(32)**. The first reporter with an multimerized TCF binding site was a luciferase- expressing transfection construct called TOPFLASH **(33)**. Since then the most commonly used Wnt reporter is a synthetic promoter with TCF/LEF binding sites **(32)**.
The reporter plasmid pMuLE_ENTR_TOP-NL1.1_L5-L4 (TOP-NLuc) which has been developed in the MMCT laboratory has a TCF/LEF binding site and leads to transcription and translation of luciferase after activation of TCF/LEF.

1.5.2. Notch Pathway

The Notch signaling is very important for metazoan development, tissue regeneration and allows short range communication between cells. It can promote or suppress proliferation of cells, cell death and activation of differentiation in a wide range of tissues. Because of all these mentioned properties, defects in Notch signalling leads to multiple human disorders. Some of them are developmental syndromes like, Alagille syndrome, adult-onset diseases such as cerebral autosomal dominant arteriopathy with subcortical infarct and leukoencephalopathy (CADASIL) and of course cancer. Notch has an interesting mechanism of signal transduction, which sets it apart from other signaling pathways. For the activation of this pathway, a signal sending cell binds with its ligand to the Notch receptor and leads to an intramembrane proteolysis. After proteolysis, the Notch intracellular domain (NICD) translocate to the nucleus and forms a transcription complex with an DNA binding protein like CBF, leading to activation of downstream target genes **(34)**. Since CBF is important for the signal transduction, reporter with CBF binding site can be used for measuring the Notch activity **(35)**.

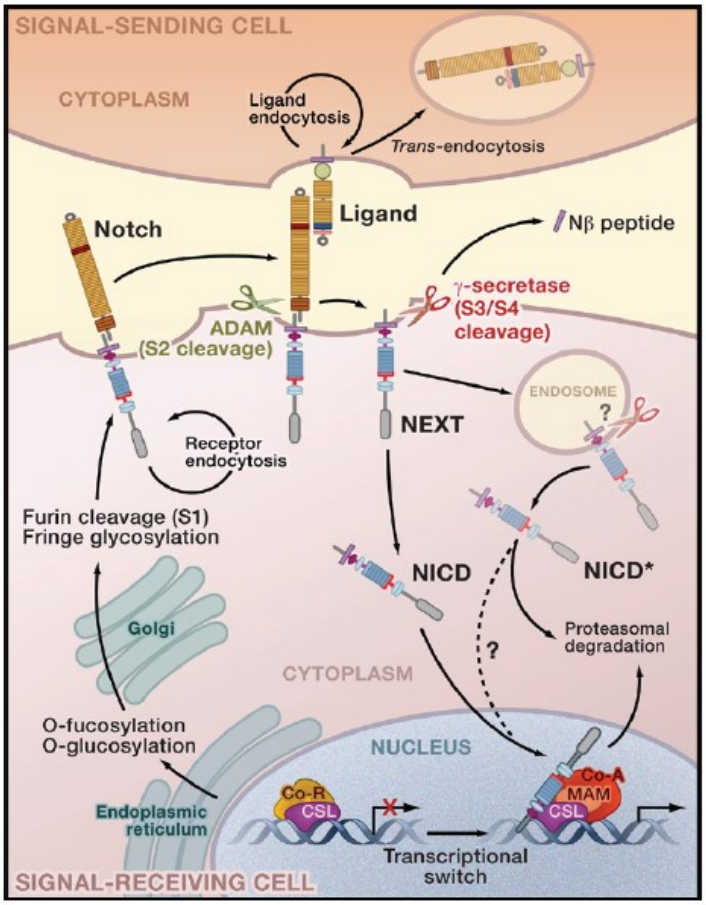

Figure 5: Notch signaling pathway.(Figure from Kopan R. and Ilagan MX. 2009)

1.5.3. Hedgehog Pathway

Another very important signaling pathway during the embryogenesis is the Hedgehog pathway. It regulates body patterning and organ development during embryogenesis and is involved in physiological and tumorigenic control of postnatal cellular events. An inappropriate reactivation of this pathway can lead several cancer types. There are tree Hedgehog ligands, Sonic (SHH), Indian (IHH) and Desert (DHH), which are secreted from different tissues at different stages of development. The Sonic (SHH) Hedgehog pathway gets activated after binding of Sonic protein to its transmembrane receptor Patched (PTCH). After binding, the Smoothened protein gets released and activates Gli transcription factors. Gli transduce in to the nucleus and stimulate transcription of the Hedgehog target genes. At the end PTCH and Hedgehog are degraded in lysosomes **(36)**. Because of the importance of Gli, the reporter of the Hh pathway contains a multiple Gli binding sites **(37)**.

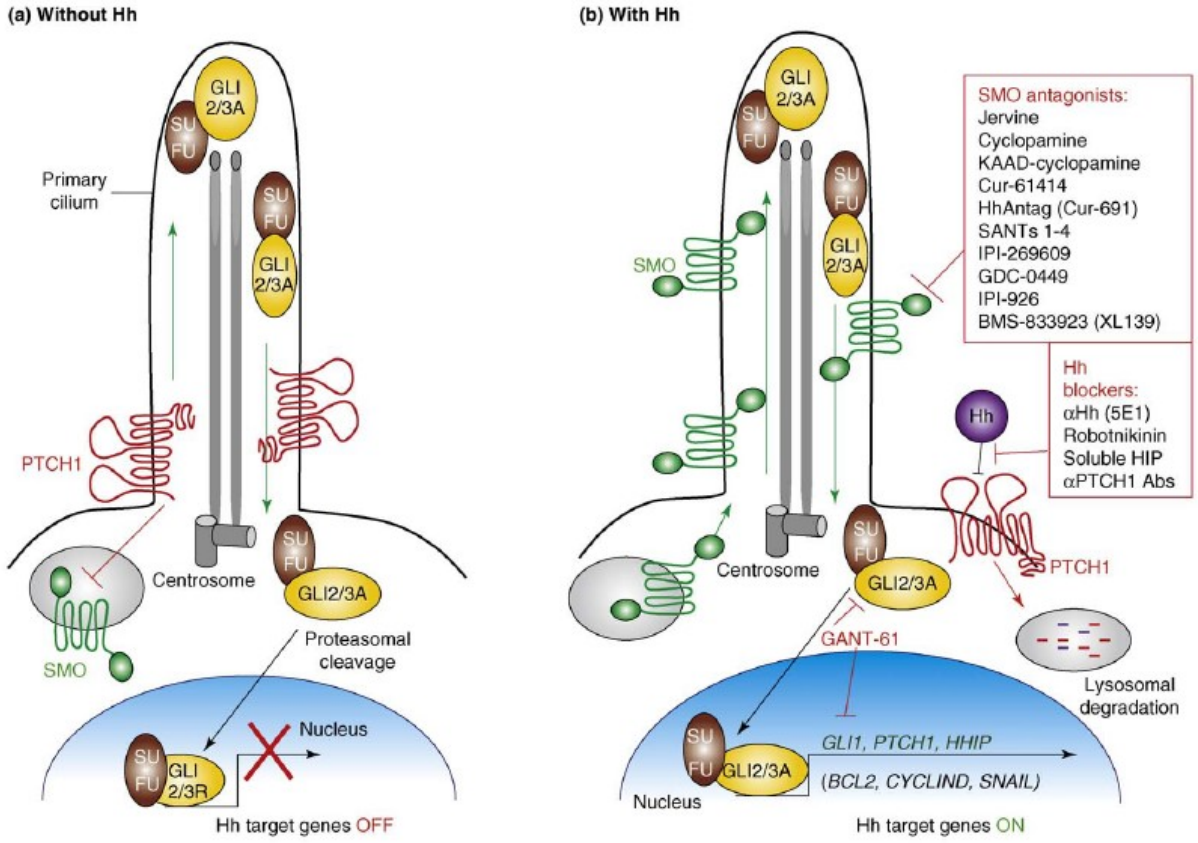

Figure 6: Hedgehog Pathway: (a) inactivated pathway. (b) activated pathway: after binding of Hh to its receptor PTCH, the SMO gets released and moves in to the cilium. SMO activates Gli2 and Gli3 and prevents their cleavage. Activated Gli2/3 translocate in to the nucleus and stimulate the transcription of target genes such as GLI1, PTCH1 and HHIP. (Figure from Scales SJ. and de Sauvage FJ. 2009)

1.5.4. Other available reporter plasmids

Pathway	Reporter Plasmid	Binding Sites	Addgene
Wnt-Pathway	Lentiviral-TOP-dGFP-reporter	LEF-1/TCF-responsive promoter	#14715
	M50 Super 8x TOPFlash	TCF/LEF binding site	#12456
Hedgehog-Pathway	pGL3b-8XGliBS:EGFP	8x Gli-binding site	#84602
	7Gli:GFP	Gli-binding site	#110494
Notch-Pathway	CBF:H2B-Venus	CBF1-Responsive-Element	#44211
	CBFRE (mt) EGFP	CBF1-reponsive element	#26870

Table 1: Table showing other available reporter plasmids for the 3 pathways.

The reporter plasmids showing in Table 1 are available on addgene. Lentiviral-TOP-dGFP-reporter is a reporter plasmid for the Wnt pathway containing enhanced GFP as reporter gene under the control of a LEF-1/TCF responsive promoter. This reporter plasmid was cloned into a lentiviral vector with self-inactivating characteristic and was used to investigate the role of Wnt signaling pathway in self-renewing hematopoietic cells **(38).** M50 Super 8x TOPFlash is another reporter plasmid for the Wnt pathway with a TCF/LEF binding site and luciferase as reporter gene to study the noncanonical Wnt/Fz signaling in zebrafish prickle **(39).** Both reporter plasmids for the Hedgehog pathway contain Gli binding site which control the expression of GFP genes. pGL3b-8XGliBS:EGFP was used during a screening for Hedgehog pathway inhibitors and 7Gli:GFP was applied to test whether Ubr3,a ubiquitin ligase has an impact on Sonic Hedgehog signaling in mammals **(40,41)**. The Notch pathway reporter plasmids have been used for investigating the Notch signaling in transgenic mice. The CBFH2B-Venus construct contains a CBF1 binding site linked to the simian virus 40 (SV40) promoter and to yellow fluorescent protein (YFP) as reporter. This construct was used to report the Notch pathway activity in transgenic mice and mouse stem cells. For investigating the Notch signaling during cerebral development the CBFRE(mt)EGFP reporter plasmid was constructed containing enhanced GFP as reporter and CBF1-responsive simian virus 40 promoter to generate transgenic mice for in vivo experiments **(42,43)**.

2. Aim of the thesis

Signaling pathways play crucial roles in cancer and are very important for the development and proliferation of cancer cell. Towards this, plasmid based reporter systems have been developed in the MMCT laboratory to track the activity of these signaling pathways in cancer cell lines.

The aim of the present work was to expand, characterize and validate different luciferase based plasmids which were originally developed for tracking the activity of Wnt, Hedgehog and Notch signalling pathways. Towards this, first plasmids were expanded by bacterial culture and isolation of plasmid DNA, which was quantified by UV-Vis spectrophotometry. Then the isolated plasmid was subjected to diagnostic restriction digestion (by a varitety of restriction enzymes) and the restriction map validated for identification of the plasmid and for confirmation of the presence of desired sequences/features responsible for tracking the respective pathway.

3. Materials

3.1. Enzymes

The following enzymes have been used during the diagnostic restriction digest.

Enzymes	Source	Lot	Catalog #
Fast Digest KpnI	Thermo Scientific	00360782	FD0524
Fast Digest XbaI	Thermo Scientific	00269798	FD0684
Fast Digest XhoI	Thermo Scientific	00047820	FD0694
Fast Digest Mph1103I	Thermo Scientific	00185839	ER0731
Fast Digest BamHI	Thermo Scientific	00031073	ER0051
Fast Digest MbiI	Thermo Scientific	00346615	FD1274
Fast Digest NotI	Thermo Scientific	00363432	FD0594
Fast Digest PvuI	Thermo Scientific	00286755	FD0624
Fast Digest PvuII	Thermo Scientific	00279627	FD0634
Fast Digest NheI	Thermo Scientific	00380372	FD0974
Fast Digest EcoRI	Thermo Scientific	00274229	FD0274
Fast Digest HindIII	Thermo Scientific	00250958	FD0504
Fast Digest SmaI	Thermo Scientific	00294700	FD0663
Fast Digest DraI	Thermo Scientific	00237891	FD0224
Fast Digest BglII	Thermo Scientific	00594040	FD0084

Table 2: Enzymes used during this work.

3.2. Buffer and Ladder

The following Buffer and Ladder have been used for the diagnostic restriction digest.

Buffer & Ladder	Source	Lot	Catalog #
10xFast Digest Green Buffer	Thermo Scientific	00263153	B72
GeneRuler 1kb Plus DNA Ladder	Thermo Scientific	00771333	SM1331

Table 3: Buffer and Ladder used during this work.

3.3. Chemicals and Kit

The following chemicals and kit have been used during this work.

Chemical or Kit	Source	Lot	Catalog #
GeneJet Plasmid Maxiprep Kit	Thermo Scientific	00781761	K0492
GeneJet Plasmid Maxiprep Kit	Thermo Scientific	00756277	K0492
20xSB Buffer pH: 8,09	MMCT	03072019	-
Agarose DNA Pure Grade	Electran®	0000397414	443666A
EtBr-Solution	Sigma	1510JM	-
LiChrosolv EtOH	Sigma Aldrich	K50447027832	64175
Iso-Propanol 99,9%	Sigma Aldrich	SHBK8324	67630
Glycerol 50%	Sigma Aldrich	-	-
Ampicillin	Sigma Aldrich	BCBM5574V	A0166
Kanamycin	Sigma Aldrich	SLBW6738	K1377
Ultra-pure water (MQ-H_2O)	Sartorius arium® pro	-	-

Table 4: Chemicals and kits used for this work.

3.4. Device

The following devices have been used during this work.

Device	Manufacturer
NanoVue Plus Spectrophotometer	GE Healthcare
GeneQuant 1300 Spectrophotometer	GE Healthcare
Centrifuge (Ser.-No.: 41607553)	Thermo Fisher Scientific
MaxQ4450 (Shaking incubator)	Thermo Fischer Scientific
Incubator	WTC binder
TC412 Thermal Cycler	Techne
Molecular Imager® Gel Doc™ XR System	Bio-Rad

Table 5: Devices used for this work.

3.5. Medium for bacterial culture

Medium	Composition
LB medium with ampicillin (LB+ amp.)	1% Tryptone, 0.5% Yeast extract, 0.5% NaCl, 50mg/ml Ampicillin
LB medium with kanamycin (Lb+ kan.)	1% Tryptone, 0.5% Yeast extract, 0.5% NaCl, 25mg/ml Kanamycin
LB+ amp. Plates	1% Tryptone, 0.5% Yeast Extrakt, 0.5% NaCl, 1.5% agarose, 100µg/ml Ampicillin
LB+ kan. Plates	1% Tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agarose, 50µg/ml Kanamycin

Table 6: Components of the LB Medium and LB Plates.

3.6. Plasmids

The following plasmids have been expanded during this work.

Plasmid Name	Short name	Characteristics	Source
pMuLE_ENTR_TOP-NL1.1_L5-L4	TOP-NLuc	Reporter gene for Wnt pathway; kanamycin resistance	MMCT, University of Vienna
pMuLE_ENTR_12xGLI-FLuc_R4-R3	GLI-FLuc	Reporter gene for Hedgehog (Hh) pathway; kanamycin resistance	MMCT, University of Vienna
pMuLE_ENTR_CBF-Gluc_L3-L2	CBF-GLuc	Reporter gene for Notch pathway; kanamycin resistance	MMCT, University of Vienna
pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc	3P-Luc	Reporter gene for all three pathways (Wnt, Hh, Notch); ampicillin resistance	MMCT, University of Vienna
PUC-19	PUC-19	Control plasmid; pUC origin; ampicillin resistance	MMCT, University of Vienna
pMuLE_ENTR_CMV-NL1.1_L1-R5	CMV-NLuc	Inducer plasmid; kanamycin resistance	MMCT, University of Vienna
pAd-wnt3a	pAd-wnt3a	Inducer plasmid for Wnt pathway; kanamycin resistance	Addgene #12518
phGli1	phGli1	Inducer plasmid for Hh pathway; ampicillin resistance	Addgene #84922
phlCN1	phlCN1	Inducer plasmid for Notch pathway; ampicillin resistance	Addgene #17623

Table 7: Plasmids expanded during this work.

4. Methods

4.1. Preparation of culture media

4.1.1. Preparation of LB Agar Plates

The LB Plates were prepared as follow: 600ml of ultra-pure H2O have been mixed with 21g of LB Agar (Lennox) and autoclaved and let cool down to 45°C on the water bath. After the cooling process the appropriate antibiotics have been added in to the solution according to Table 8.

Antibiotic	Stock solution (mg/ml)	Working concentration (µg/ml)
Ampicillin	50	100
Kanamycin	25	50

Table 8: Antibiotics have been used for LB plates and LB Medium.

The prepared solution has been mixed properly and nearly 20ml have been poured in to petri dishes. For cooling down faster the petri dishes were left half open and after 30 min they were closed, sealed with Parafilm® and stored at 4°C till further use.

4.1.2. Preparation of LB Medium

The LB medium was used for the liquid culture (Pre- culture and O/N culture). According to manufacturer's protocol 12g of LB medium powder have been mixed with 600ml ultra-pure H2O. The solution was autoclaved and stored at room temperature. Before use the needed amount of appropriate antibiotics was calculated according to Table 8.

4.2. Plasmid expansion

4.2.1. Streaking

To produce single colonies, bacterial cultures were streaked with an inoculation loop on the LB plate with the appropriate antibiotic and incubated for at least 16h at 37°C.

Plasmid name	Antibiotic	Temperature
TOP-NLuc	Kanamycin	37°C
GLI-FLuc	Kanamycin	37°C
CBF-GLuc	Kanamycin	37°C
3P-Luc	Ampicillin	30°C
PUC19	Ampicillin	30°C
CMV-NLuc	Kanamycin	37°C
pAd-wnt3a	Kanamycin	30°C/48h
phGli1	Ampicillin	37°C
phlCN1	Ampicillin	30°C

Table 9: Appropriate antibiotics for every plasmid.

4.2.2. Pre-culture

After 16h of incubation one CFU was picked and placed in 15ml of medium with appropriate antibiotic and have been incubated on the shaking incubator for 4h and 180rpm.

4.2.3. Overnight culture

To receive high amounts of plasmid an overnight culture was prepared by mixing 500µl of pre culture with 250ml of LB medium with the appropriate antibiotic and have been incubated overnight on the shaking incubator at 37°C and 180rpm.

4.2.4. Glycerol stock

For long term storage 500µl of overnight culture was mixed with 500µl of sterile filtered 50% glycerol in a cryotube. The resulting mixture have been stored at -80°C. By using this method, E.coli strains can be stored for years.

4.2.5. Plasmid isolation

After overnight incubation the optical density (OD600) was measured with GeneQuant 1300 Spectrophotometer to detect the optimal growth for the purification kit as recommended. The maximum culture volume to use was determined using formula:

Maximum culture volume (mL) = 750/OD600

The bacterial suspension was centrifuged for 10 min. at 5,000 x g to harvest the cells. After centrifugation the supernatant was discarded and the purification steps were performed with the cell pellet. The plasmids have been purified by using the GeneJet Plasmid maxiprep kit (Thermo scientific) according to the manufacturer's protocol.

4.2.6. DNA quantification

For the quantification 4µl of DNA solved in elution buffer was measured with NanoVue Plus Spectrophotometer. The elution buffer was used as blank.

4.2.7. Isopropanol precipitation

To purify the plasmid DNA obtained from Maxiprep's, isopropanol precipitation was performed.

500 µl of plasmid solution was transferred into a fresh sterile tube and was mixed with 1000µl isopropanol and incubated overnight at -20°C. The resulting mixture was centrifuged at 15000g and 4°C for 15 minutes. The supernatant was discarded, and the tube was dried on a paper towel. The pellet was washed with 750 µl cold 96% ethanol and mixed by vortexing for 20sec. until the pellet floats. The resulting mixture was centrifuged at 15,000 g for 5 minutes at 4°C. The previous 2 steps have been repeated and the supernatant was discarded. The pellet was air dried for 30 min. and an appropriate volume of nuclease free water was added to dissolve the pellet. The solution was kept at 4˚C for 1-2 days. After measuring the concentration with NanoVue Plus Spectrophotometer the tubes were stored at -80°C.

4.3. Plasmid validation

4.3.1. Diagnostic restriction digest (DRD)

Using the diagnostic restriction digest the identity of the plasmids and certain features of the plasmids were detected. For this, 255ng of Plasmid DNA were mixed with 2µl of 10xFast digest Green Buffer, 1µl of the appropriate restriction enzyme and was filled up to 20µl with ultra-pure water. The mixture was incubated at 37°C for 40 minutes to activate the enzyme, followed by an inactivation step at 80°C for 10 minutes. The finished product was kept at 4°C until analysis with gel electrophoresis.

Plasmid	Enzymes
TOP-NLuc	HindIII
	XbaI
	NheI
	XhoI
	EcoRI
	PvuII

Table 10: Restriction enzymes have been used for TOP-NLuc.

Plasmid	Enzymes
GLI-FLuc	BamHI
	PvuII
	XbaI
	DraI
	Mph1103I
	NheI
	EcoRI
	PvuI

Table 11: Restriction enzymes have been used for Gli-FLuc.

Plasmid	Enzymes
CBF-GLuc	EcoRI
	Mph1103I
	HindIII
	PvuII
	NheI
	PvuI

Table 12: Restriction enzymes have been used for CBF-GLuc.

Plasmid	Enzymes
3P-Luc	SmaI
	NheI
	EcoRI
	BamHI
	PvuII

Table 13: Restriction enzymes have been used for 3P-Luc.

Plasmid	Enzymes
PUC19	HindIII
	PvuI
	DraI
	MbiI
	PvuII
	XbaI

Table 14: Restriction enzymes have been used for PUC19.

Plasmid	Enzymes
CMV-NLuc	BglII
	DraI

Table 15: Restriction enzymes have been used for CMV-NLuc.

Plasmid	Enzymes
pAd-wnt3a	KpnI
	MbiI
	PvuII

Table 16: Restriction enzymes have been used for pAd-wnt3a.

Plasmid	Enzymes
phGli1	XbaI
	EcoRI
	PvuII
	BamHI
	XhoI
	MbiI

Table 17: Restriction enzymes have been used for phGLi1.

Plasmid	Enzymes
phlCN1	KpnI
	NotI
	PvuII
	HindIII

Table 18: Restriction enzymes have been used for phlCN1.

4.3.2. Gel electrophoresis

Agarose gel electrophoresis was used to separate and identify the DNA fragments. For this 0,96g agarose was solved in 120ml of SB Buffer by using a microwave oven. After cooling down 6µl of ethidium bromide stock solution (10mg/ml) was added, which intercalates between the bases and acts as a fluorescent dye to indicate the position of the fragments. The mixture was poured into gel trays and let stand until it was hardened. The samples were loaded into the pockets alongside a DNA standard (GeneRuler 1kb Plus DNA Ladder). The electrophoresis was performed at 80V for 60 minutes. The fragments were made visible by using the Molecular Imager® Gel Doc™XR System and their sizes were determined by comparing to the DNA standard.

5. Results

5.1. Propagation of the plasmids

5.1.1. Bacterial growth

The agar plates (Fig.7 - Fig.15), which were streaked with suspension of different bacterial stocks transfored with plasmids were examined visually and showed the expected growth after overnight incubation at the appropriate temperature.

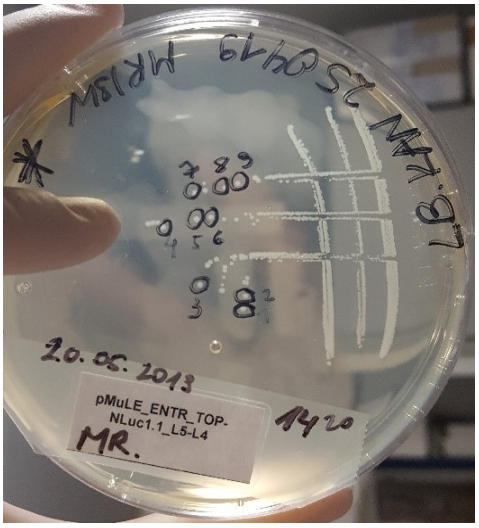

Figure 7: TOP-NLuc: Bacterial growth after incubation at 37°C on LB plate containing kanamycin.

Figure 9: CBF-GLuc: Bacterial growth after incubation at 37°C on LB plate containing kanamycin.

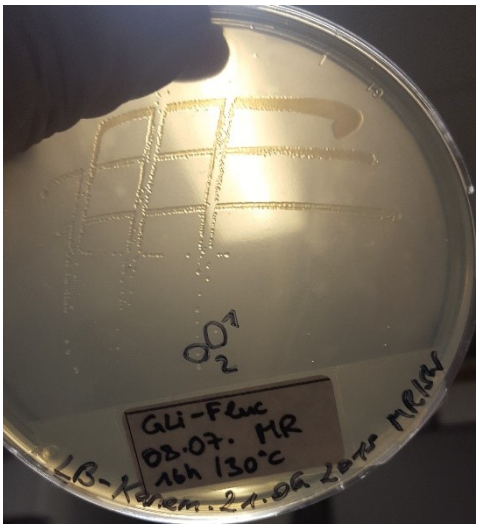

Figure 8: GLI-FLuc: Bacterial growth after incubation at 37°C on LB plate containing kanamycin.

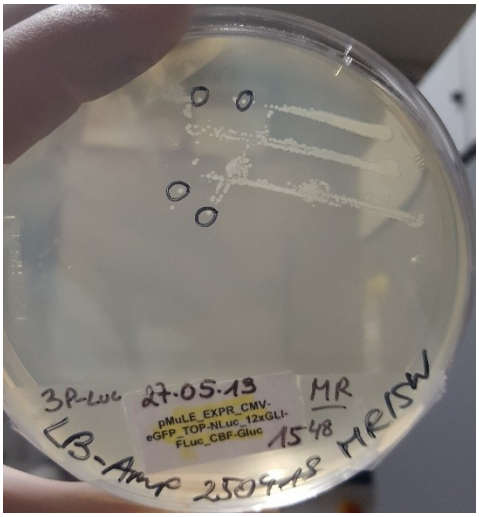

Figure 10: 3P-Luc: Bacterial growth after incubation at 30°C on LB plate containing ampicillin.

Figure 11: CMV-NLuc: Bacterial growth after incubation at 37°C on LB plate containing kanamycin.

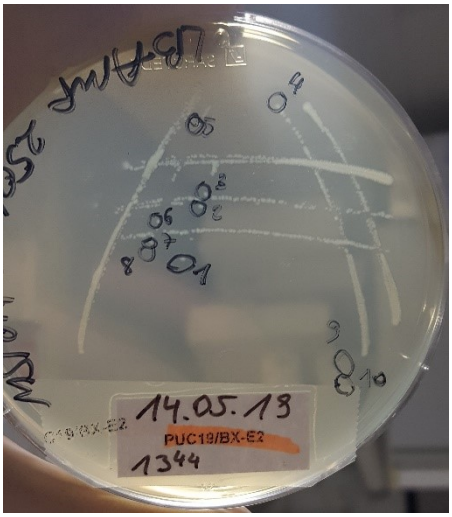

Figure 12: PUC19: Bacterial growth after incubation at 30°C on LB plate containing ampicillin.

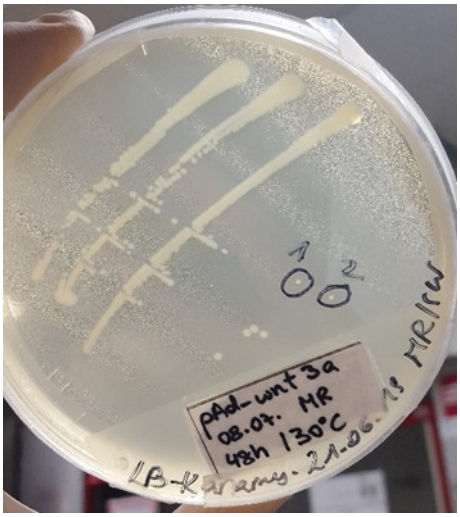

Figure 13: pAd-wnt3a: Bacterial growth after 24h of incubation at 30°C, even though the prescribed incubation time was 48h. LB plate containing kanamycin

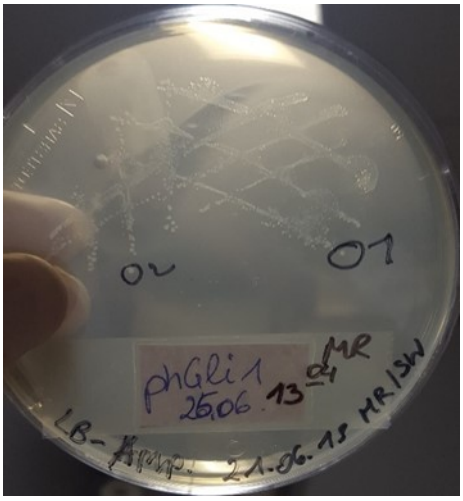

Figure 15: phGli1: Bacterial growth after incubation at 37°C on LB plate containing ampicillin.

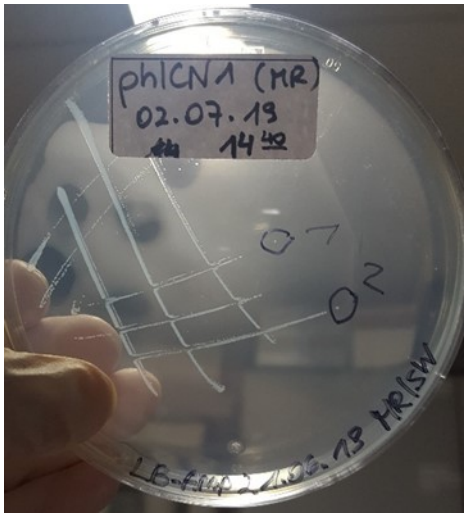

Figure 14: phlCN1: Bacterial growth after incubation at 30°C on LB plate containing ampicillin.

5.1.2. Plasmid isolation

After the bacteria has grown sufficiently, the plasmids were purified by using the GeneJet Plasmid maxiprep kit (Thermo scientific) according to the manufacturer's protocol. Later, the concentration of the purified plasmid was measured with NanoVue Plus Spectrophotometer. The results are visible on Table 19.

Plasmid	Number of Maxiprep	Date	Concentration
TOP-NLuc	8	08.05.2019	272,5µg/ml
	3	22.05.2019	411,0µg/ml
	3	22.05.2019	448,5µg/ml
GLI-FLuc	5	10.07.2019	623,0µg/ml
	5	10.07.2019	691,5µg/ml
CBF-GLuc	5	08.07.2019	191,0µg/ml
	5	08.07.2019	190,0µg/ml
3P-Luc	4	09.05.2019	259,0µg/ml
	3	29.05.2019	363,5µg/ml
	3	29.05.2019	318,0µg/ml
	4	26.06.2019	418,5µg/ml
	4	26.06.2019	469,5µg/ml
PUC19	1	16.05.2019	62,5µg/ml
	1	16.05.2019	66,5µg/ml
	1	20.05.2019	78,0µg/ml
	1	20.05.2019	93,5µg/ml
CMV-NLuc	5	02.07.2019	470,0µg/ml
	5	02.07.2019	540,5µg/ml
pAd-wnt3a	6	12.07.2019	162,0µg/ml
	6	12.07.2019	155,5µg/ml
phGli1	5	27.06.2019	706,5µg/ml
	4	27.06.2019	558,5µg/ml
phlCN1	4	04.07.2019	416,5µg/ml
	4	04.07.2019	381,0µg/ml

Table 19: DNA quantification after Maxiprep.

5.1.3. Isopropanol precipitation

The isopropanol precipitation was performed to obtain ultra-pure plasmids.

Plasmid	Total plasmid amount before	Total plasmid amount after	DNA yield in %
TOP-NLuc	681,25µg	464µg	68
	1027,5µg	799,5µg	77,8
	1121,25µg	485,25µg	43
3P-Luc	388,5µg	338µg	99
	259µg	87,9µg	33,9
	872,4µg	122,4µg	14
	763,2µg	159µg	20
PUC19	48,75µg	35,3µg	72
	51,87µg	40,8µg	78,6
	60,84µg	51,5µg	84,6
	72,93µg	52,5µg	72

Table 20: Results after isopropanol precipitation.

5.2. Plasmid validation via DRD

5.2.1. Diagnostic restriction digest of TOP-Nluc

TOP-NLuc is the reporter plasmid for the wnt pathway. To determine this plasmid, the DRD was performed by using the restriction enzymes which were selected following the restriction map shown in Figure 16, that was prepared using Snap Gene®.

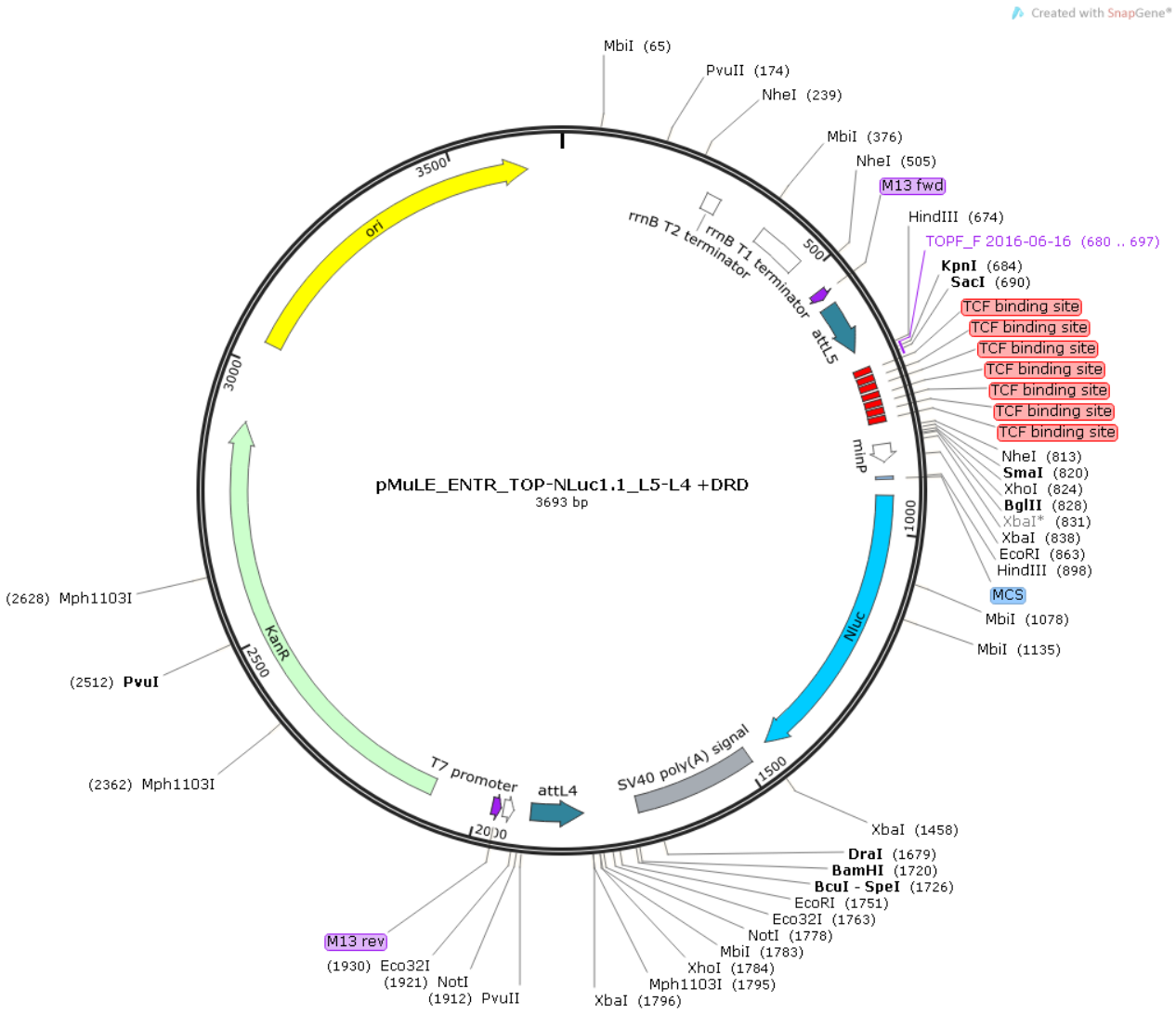

Figure 16: Restriction map for TOP-Nluc which shows the restriction enzymes able to cleave the plasmid, as well as the number and location of the recognition sequences. (Created with Snap Gene®).

For TOP-NLuc a combination of single cutters and multiple cutters were used, shown in Table 21.

Column	Enzyme	Number of Bands	Number of Base Pair
1	uncut	1	3693 bp
2	HindIII	2	3469 bp 224 bp
3	XbaI	3	2735 bp 620 bp 338 bp
4	NheI	3	3119 bp 308 bp 266 bp
5	XhoI+NheI	5	2148 bp 960 bp 308 bp 266 bp 11 bp
6	NheI+EcoRI	5	2181 bp 888 bp 308 bp 266 bp 50 bp
7	PvuII+XhoI	4	1955 bp 960 bp 650 bp 128 bp
8	PvuII+PvuI	3	1738 bp 1355 bp 600 bp

Table 21: Restriction enzymes applied to determine TOP-NLuc.

The mentioned enzymes were mixed with 255ng of plasmid DNA, 10xFast Digest Green Buffer and ultra-pure water. Afterwards the prepared mixture was incubated at 37°C for 40 minutes to activate the enzyme, followed by an inactivation step at 80°C for 10 minutes. The finished product was kept at 4°C until analysis with gel electrophoresis. The electrophoresis was performed at 80V for 60 minutes. The fragments were made visible by using the Molecular Imager® Gel Doc™XR System and their sizes were determined by comparing to the DNA standard.

5.2.1.1. Agarose gel electrophoresis of TOP-NLuc (A) 1160µg/ml.

All the bands in Figure 17A were matching with the simulation on Snap Gene® in Figure 17B. In lane 1 with the uncut plasmid, are 2 more bands visible which might be the nicked and linear form of the plasmid. They appear due to replication or harsh alkaline lysis and migrate slower than the supercoiled form.

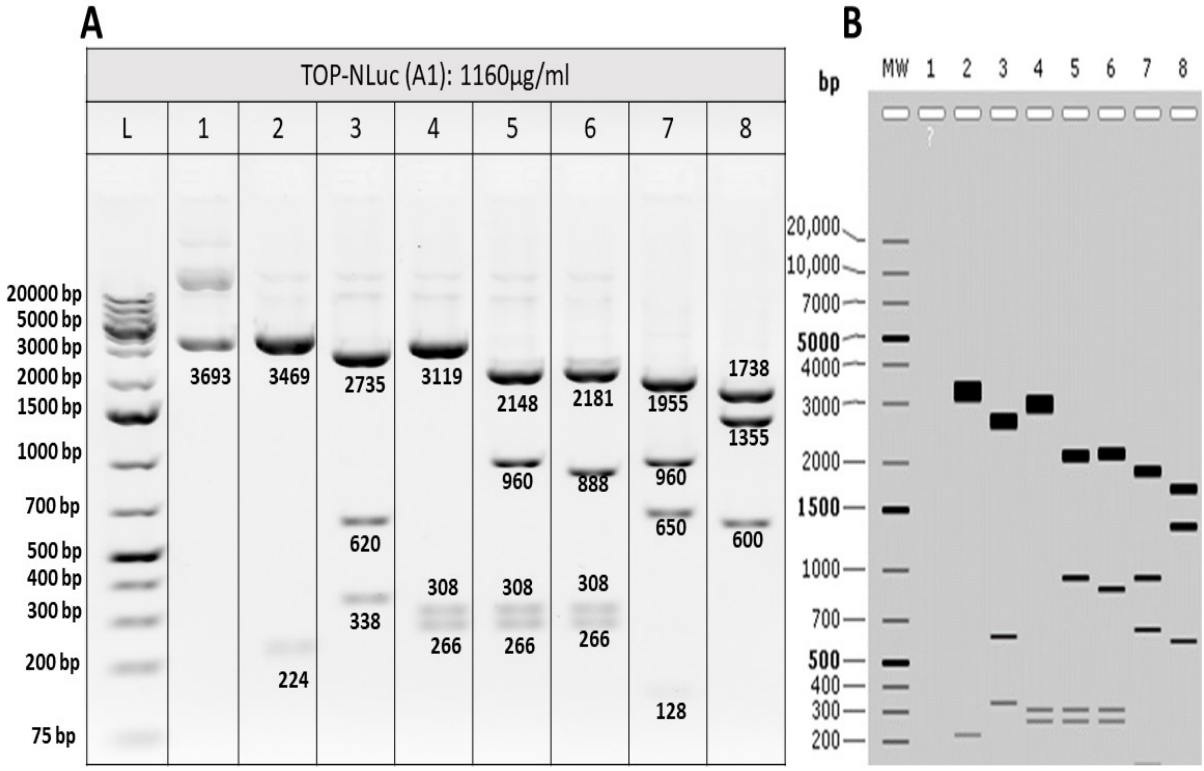

Figure 17: Agarose gel electrophorese of TOP-NLuc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Uncut
Lane 2: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with HindIII.
Lane 3: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with XbaI
Lane 4: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with NheI
Lane 5: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digetsed with XhoI+NheI
Lane 6: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with NheI+EcoRI
Lane 7: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with PvuII+XhoI
Lane 8: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with PvuII+PvuI

As shown in Figure 18A the gel electrophoresis was matching with the simulation on Snap Gene® Figure 18B. In lane 1 with the uncut plasmid, are 2 more bands visible which might be the nicked and linear form of the plasmid. They appear due to replication or harsh alkaline lysis and migrate slower than the supercoiled form.

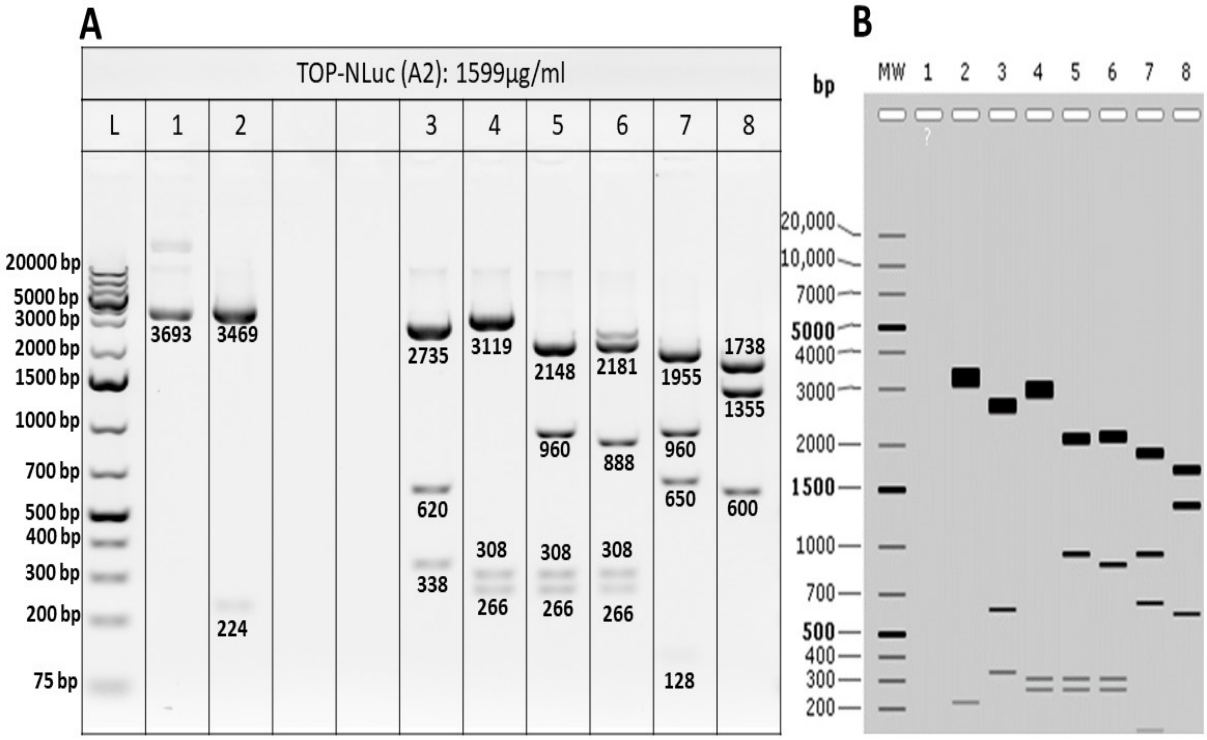

Figure 18: Agarose gel electrophoresis of TOP-NLuc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Uncut
Lane 2: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with HindIII.
Lane 3: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with XbaI
Lane 4: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with NheI
Lane 5: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digetsed with XhoI+NheI
Lane 6: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with NheI+EcoRI
Lane 7: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with PvuII+XhoI
Lane 8: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with PvuII+PvuI

Because the bands in lane 4, 5 and 6 (Figure 19A) didn't match simulation (Figure 19B) the electrophoresis was repeated and showed the same results. That's why the stocks were removed from the storage.

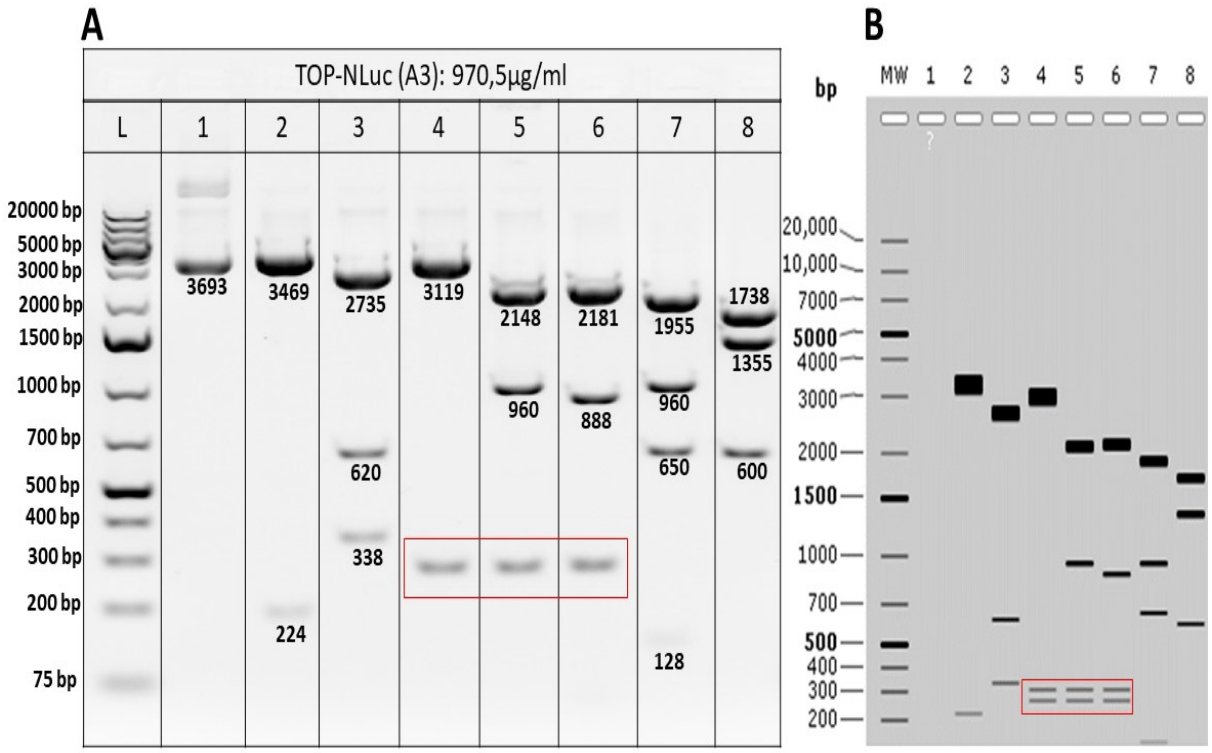

Figure 19: Agarose gel electrophoresis of TOP-NLuc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Uncut
Lane 2: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with HindIII.
Lane 3: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with XbaI
Lane 4: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with NheI
Lane 5: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digetsed with XhoI+NheI
Lane 6: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with NheI+EcoRI
Lane 7: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with PvuII+XhoI
Lane 8: pMuLE_ENTR_TOP-NL1.1_L5-L4 -Digested with PvuII+PvuI

5.2.2. Diagnostic restriction digest of GLI-FLuc.

GLI-FLuc is the reporter plasmid for the Hedgehog (Hh) pathway. To determine the identity of this plasmid, DRD was performed by using the restriction enzymes which were selected following the restriction map shown in Figure 20, that was prepared using Snap Gene®.

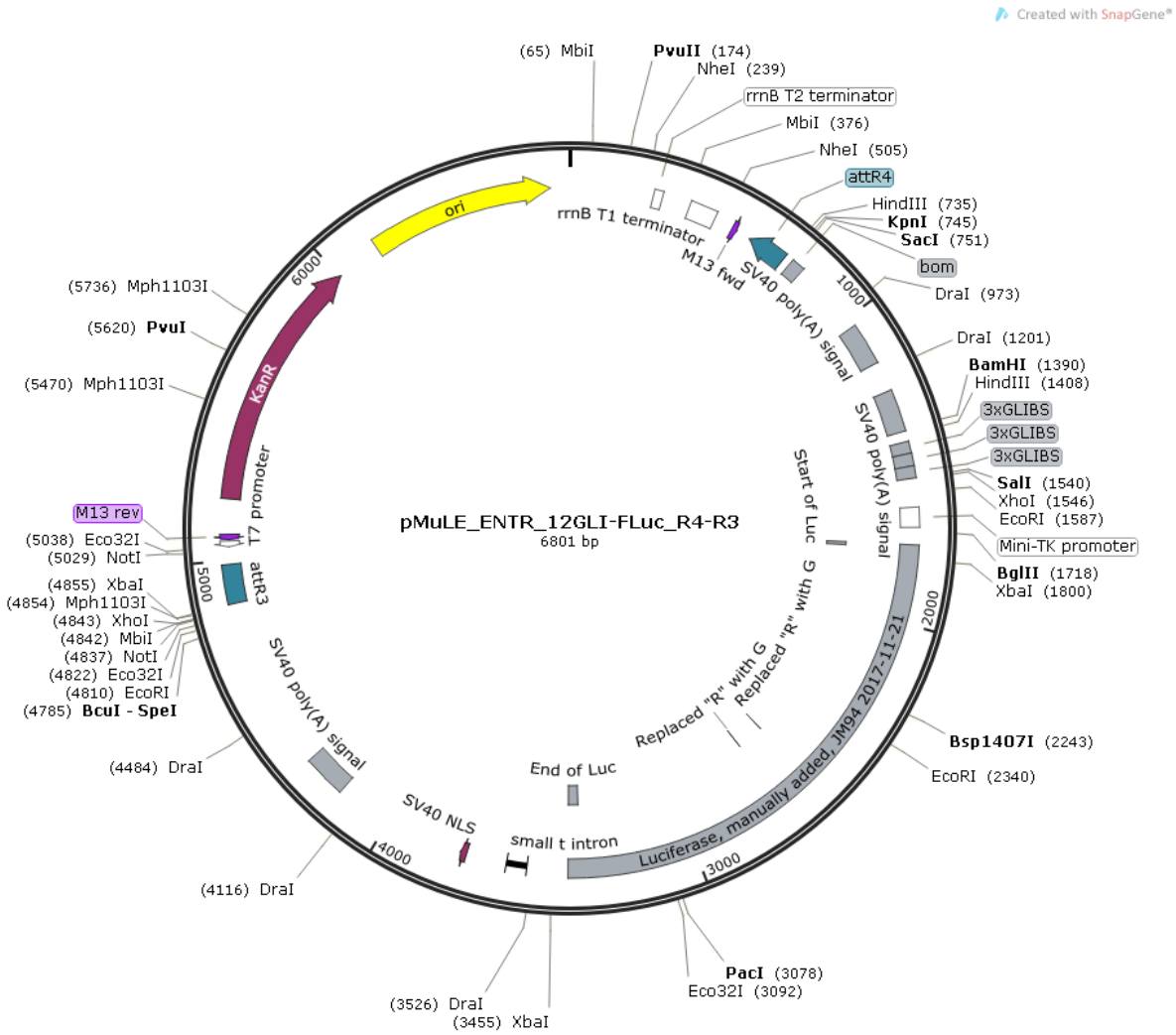

Figure 20: Restriction map for Gli-FLuc which shows the restriction enzymes able to cleave the plasmid, as well as the number and location of the recognition sequences. (Created with Snap Gene®).

For GLI-FLuc a combination of single cutters and multiple cutters were used, shown in Tab. 22.

Column	Enzyme	Number of Bands	Number of Base Pair
1	uncut	1	6801
2	BamHI	1	6801
3	PvuII+BamHI	2	5585 1216
4	XbaI+DraI	8	2919 1655 599 590 371 368 228 71
5	Mph1103I+NheI	5	4349 1304 616 266 266
6	EcoRI+PvuI	4	2768 2470 810 753

Table 22: Restriction enzymes applied to determine GLI-FLuc.

The mentioned enzymes were mixed with 255ng of plasmid DNA, 10xFast Digest Green Buffer and ultra-pure water. Afterwards the prepared mixture was incubated at 37°C for 40 minutes to activate the enzyme, followed by an inactivation step at 80°C for 10 minutes. The finished product was kept at 4°C until analysis with gel electrophoresis. The electrophoresis was performed at 80V for 60 minutes. The fragments were made visible by using the Molecular Imager® Gel Doc™XR System and their sizes were determined by comparing to the DNA standard.

5.2.2.1. Agarose gel electrophoresis of GLI-FLuc.

Although the ladder was not completely divided in both Figures (Fig. 21A& Fig. 22A), the number and position of the bands match the simulation (Fig.21B& Fig.22B). In lane 1 (Fig. 21A and Fig. 22A) with the uncut plasmid, are 2 more bands visible which might be the nicked and linear form of the plasmid. They appear due to replication or harsh alkaline lysis and migrate slower than the supercoiled form.

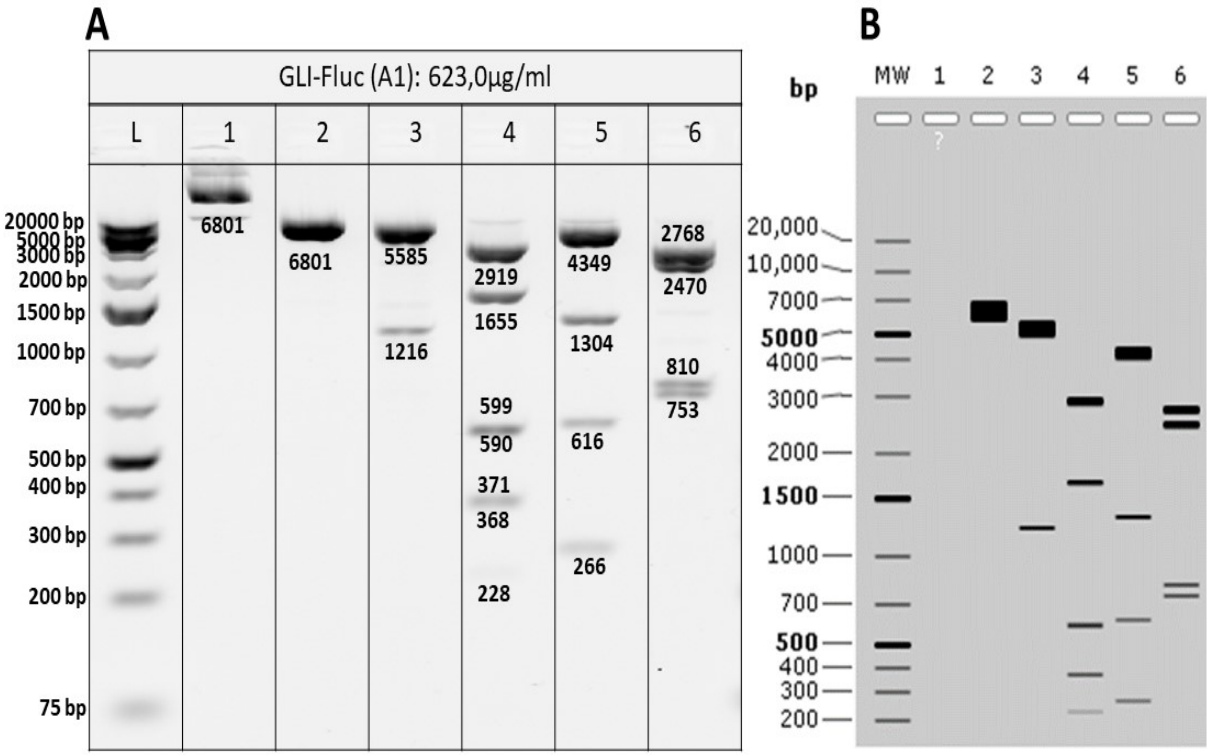

Figure 21: Agarose gel electrophoresis of Gli-FLuc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_ENTR_12xGLI-FLuc_R4-R3 -Uncut
Lane 2: pMuLE_ENTR_12xGLI-FLuc_R4-R3 -Digested with BamHI
Lane 3: pMuLE_ENTR_12xGLI-FLuc_R4-R3 -Digested with PvuII+BamHI
Lane 4: pMuLE_ENTR_12xGLI-FLuc_R4-R3 -Digested with XbaI+DraI
Lane 5: pMuLE_ENTR_12xGLI-FLuc_R4-R3 -Digested with Mph1103I+NheI
Lane 6: pMuLE_ENTR_12xGLI-FLuc_R4-R3 -Digested with EcoRI+PvuI

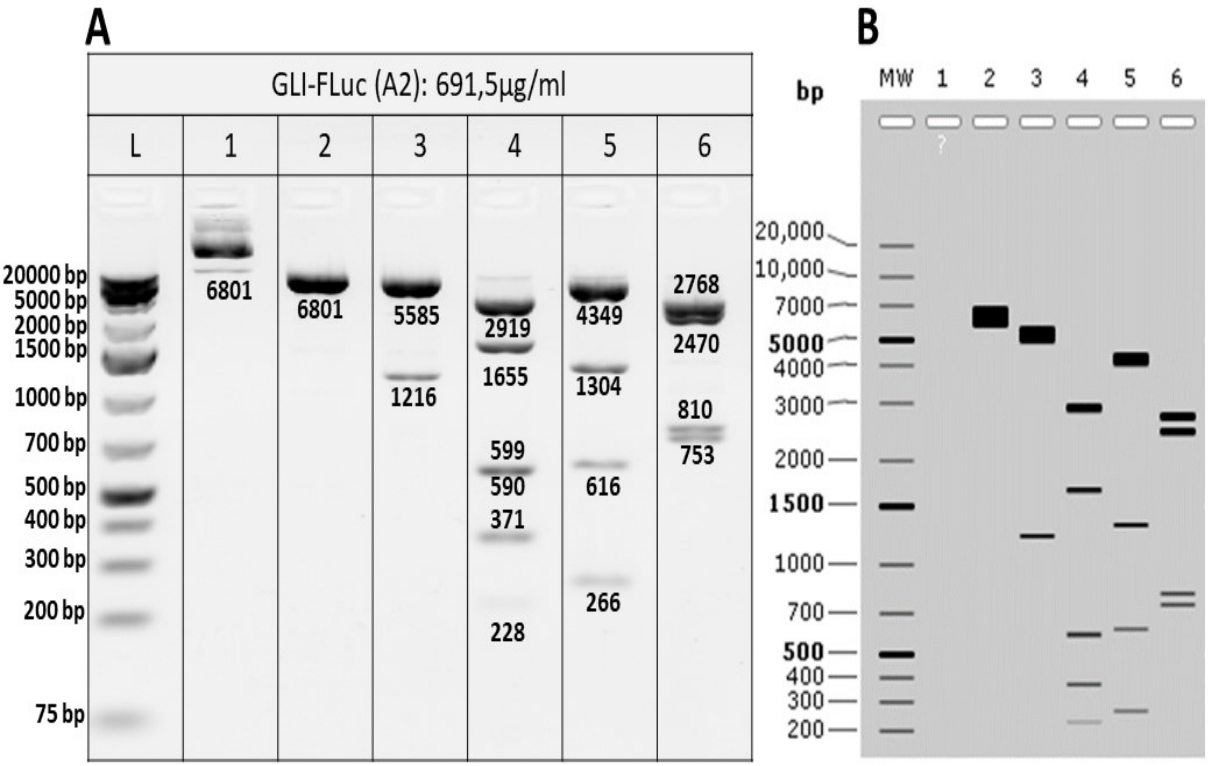

Figure 22: Agarose gel electrophoresis of Gli-FLuc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_ENTR_12xGLI-FLuc_R4-R3 -Uncut
Lane 2: pMuLE_ENTR_12xGLI-FLuc_R4-R3 -Digested with BamHI
Lane 3: pMuLE_ENTR_12xGLI-FLuc_R4-R3 -Digested with PvuII+BamHI
Lane 4: pMuLE_ENTR_12xGLI-FLuc_R4-R3 -Digested with XbaI+DraI
Lane 5: pMuLE_ENTR_12xGLI-FLuc_R4-R3 -Digested with Mph1103I+NheI
Lane 6: pMuLE_ENTR_12xGLI-FLuc_R4-R3 -Digested with EcoRI+PvuI

5.2.3. Diagnostic restriction digest of CBF-GLuc.

CBF-Gluc is the reporter plasmid for the Notch pathway. To determine this plasmid the DRD was performed by using the restriction enzymes which were selected following the restriction map shown in Figure 23, that was prepared using Snap Gene®.

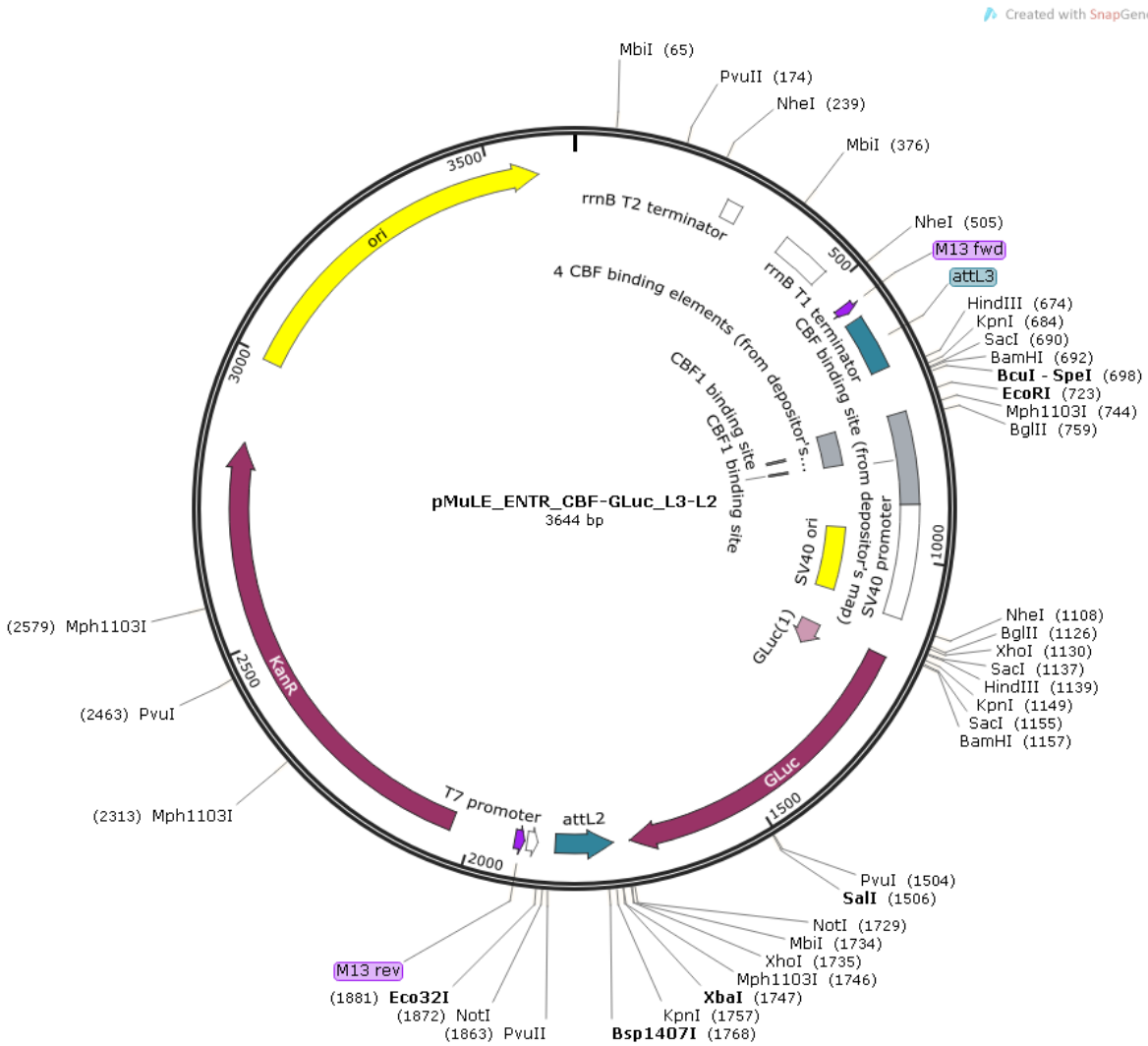

Figure 23: Restriction map of CBF-GLuc which shows the restriction enzymes able to cleave the plasmid, as well as the number and location of the recognition sequences. (Created with Snap Gene®).

For CBF-GLuc a combination of single cutters and multiple cutters were used, shown in Table 23.

Column	Enzyme	Number of Bands	Number of Base Pair
1	uncut	1	3644
2	EcoRI	1	3644
3	Mph1103I+HindIII	6	1739 607 567 395 266 70
4	PvuII+HindIII	4	1955 724 500 465
5	NheI+PvuI	5	1420 959 603 396 266

Table 23: Restriction enzymes applied to determine CBF-GLuc.

The mentioned enzymes were mixed with 255ng of plasmid DNA, 10xFast Digest Green Buffer and ultra-pure water. Afterwards the prepared mixture was incubated at 37°C for 40 minutes to activate the enzyme, followed by an inactivation step at 80°C for 10 minutes. The finished product was kept at 4°C until analysis with gel electrophoresis. The electrophoresis was performed at 80V for 60 minutes. The fragments were made visible by using the Molecular Imager® Gel Doc™XR System and their sizes were determined by comparing to the DNA standard.

5.2.3.1. Agarose gel electrophoresis of CBF-GLuc.

In Figure 24 (A1) all the bands were matching the simulation in Figure 24B. But in the second gel in Figure 24 (A2) there were two extra bands in lane 5 at nearly 2500bp-3000bp which were not visible in the simulation in Figure 24B. In lane 1 (Fig. 24A1 and A2) with the uncut plasmid, are 2 more bands visible which might be the nicked and linear form of the plasmid. They appear due to replication or harsh alkaline lysis and migrate slower than the supercoiled form.

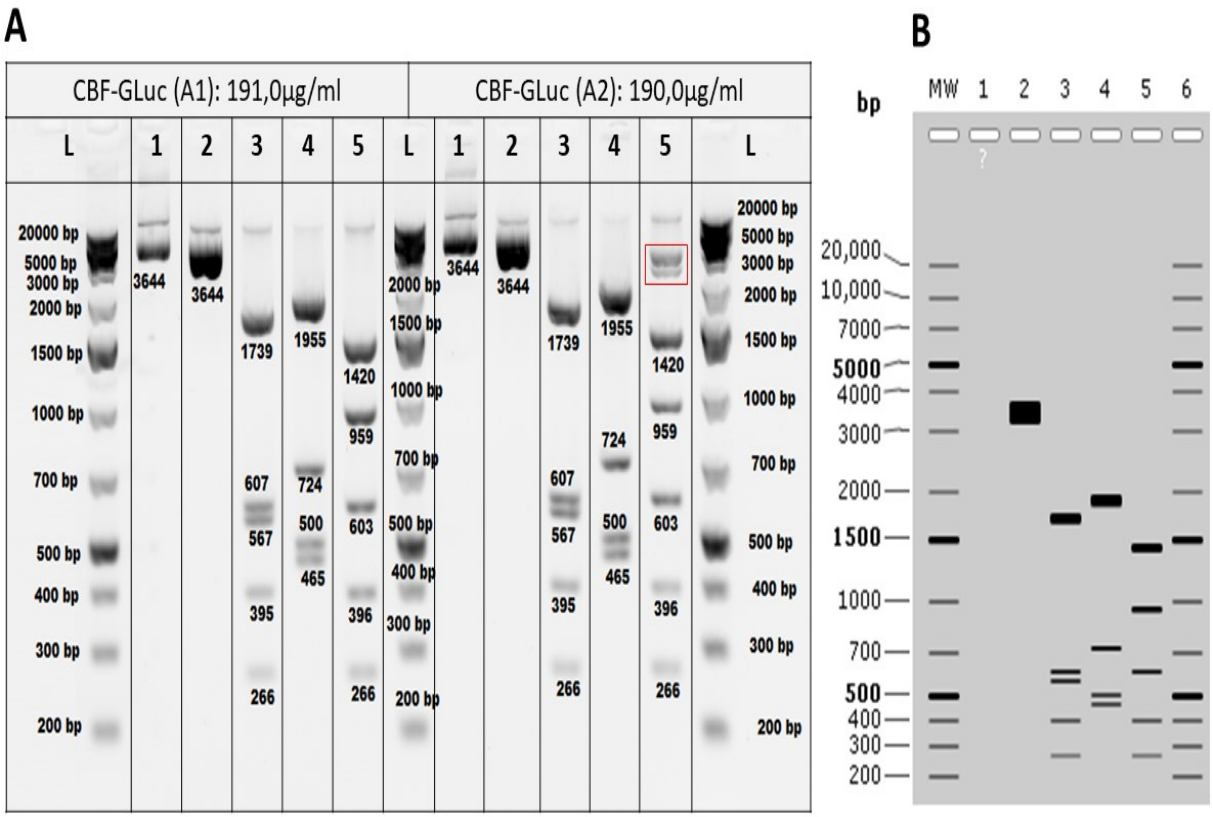

Figure 24: Agarose gel electrophoresis of CBF-GLuc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_ENTR_CBF-Gluc_L3-L2 -Uncut
Lane 2: pMuLE_ENTR_CBF-Gluc_L3-L2 -Digested with EcoRI
Lane 3: pMuLE_ENTR_CBF-Gluc_L3-L2 -Digested with Mph1103I+HindIII
Lane 4: pMuLE_ENTR_CBF-Gluc_L3-L2 -Digested with PvuII+HindIII
Lane 5: pMuLE_ENTR_CBF-Gluc_L3-L2 -Digested with NheI+PvuI

5.2.4. Diagnostic restriction digest of 3P-Luc

3P-Luc is the reporter plasmid for all 3 pathways (Wnt, Hh, and Notch). To determine this plasmid, the DRD was performed by using the restriction enzymes which were selected following the restriction map shown in Figure 25, that was prepared using Snap Gene®.

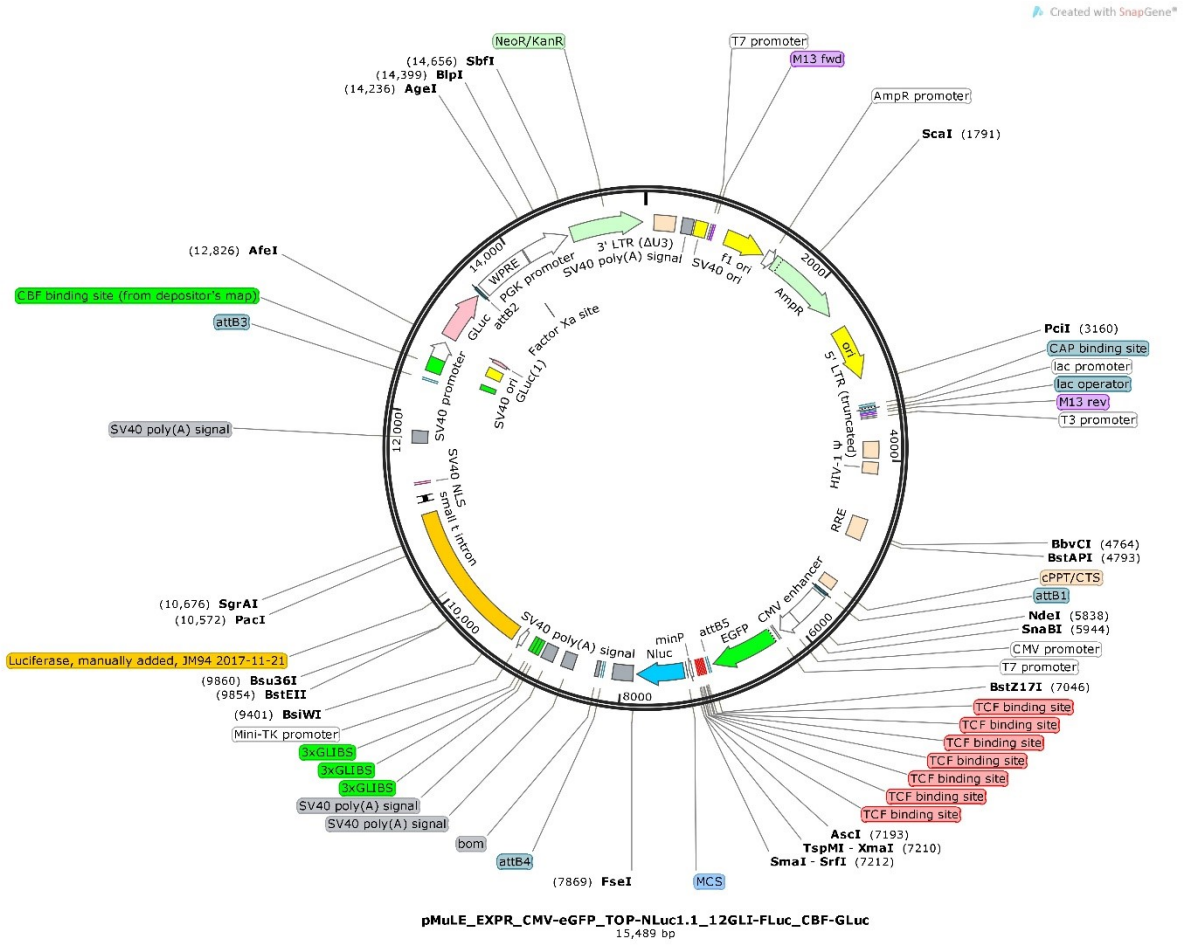

Figure 25: Restriction map of 3P-Luc which shows the restriction enzymes able to cleave the plasmid, as well as the number and location of the recognition sequences. (Created with Snap Gene®).

To determine 3P-Luc a combination of single cutters and multiple cutters were used, shown in Table 24.

Column	Enzyme	Number of Bands	Number of Base Pair
1	uncut	1	15489
2	SmaI	1	15489
3	NheI	3	7104 5616 2769
4	EcoRI	8	6893 2470 1735 1680 938 888 753 132
5	BamHI	5	8880 3521 1851 772 465
6	PvuII	4	11561 2519 767 642

Table 24: Restriction enzymes applied to determine 3P-Luc.

The mentioned enzymes were mixed with 255ng of plasmid DNA, 10xFast Digest Green Buffer and ultra-pure water. Afterwards the prepared mixture was incubated at 37°C for 40 minutes to activate the enzyme, followed by an inactivation step at 80°C for 10 minutes. The finished product was kept at 4°C until analysis with gel electrophoresis. The electrophoresis was performed at 80V for 60 minutes. The fragments were made visible by using the Molecular Imager® Gel Doc™XR System and their sizes were determined by comparing to the DNA standard.

5.2.4.1. Agarose gel electrophoresis of 3P-Luc

This gel electrophoresis has been running at 80V for 60 minutes. The results (Fig.26A- Fig.34A) are matching the simulation on Snap Gene® (Fig.26B- Fig.34B). In lane 1 (Fig.26A- Fig. 34A) with the uncut plasmid, are 2 more bands visible which might be the nicked and linear form of the plasmid. They appear due to replication or harsh alkaline lysis and migrate slower than the supercoiled form.

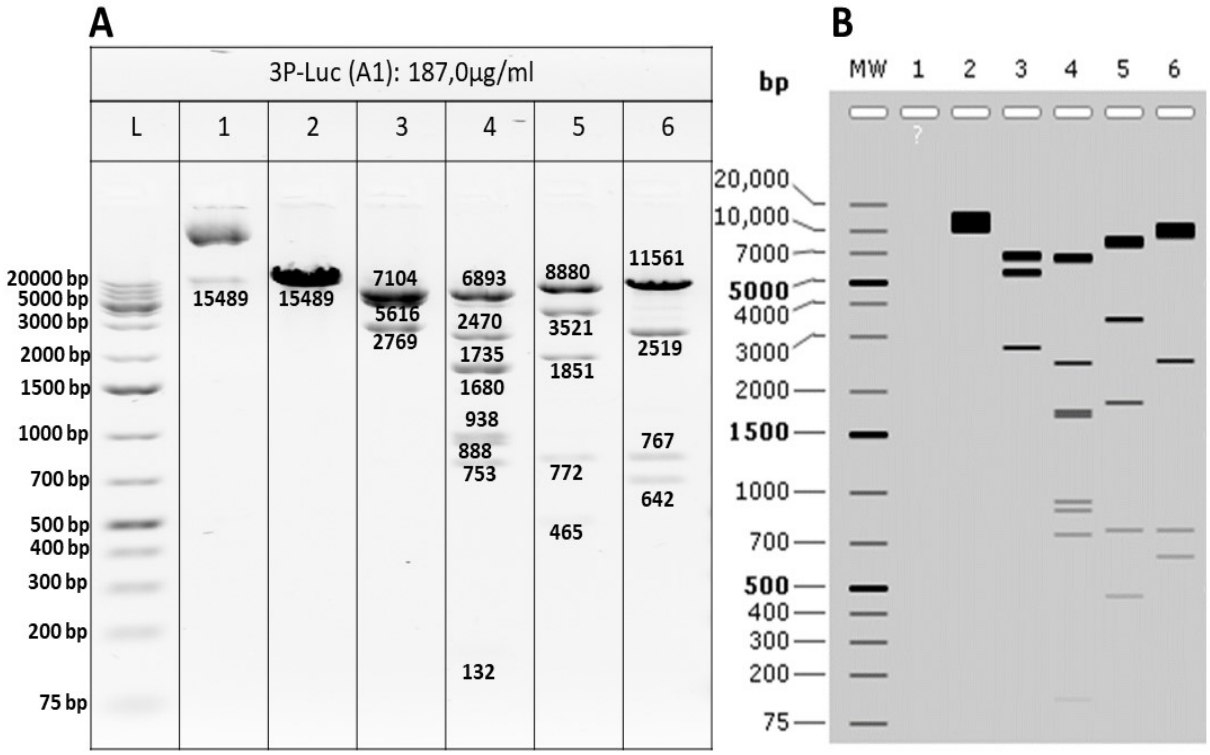

Figure 26: Agarose gel electrophoresis of 3P-Luc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Uncut
Lane 2: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with SmaI
Lane 3: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with NheI
Lane 4: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with EcoRI
Lane 5: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with BamHI
Lane 6: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with PvuII

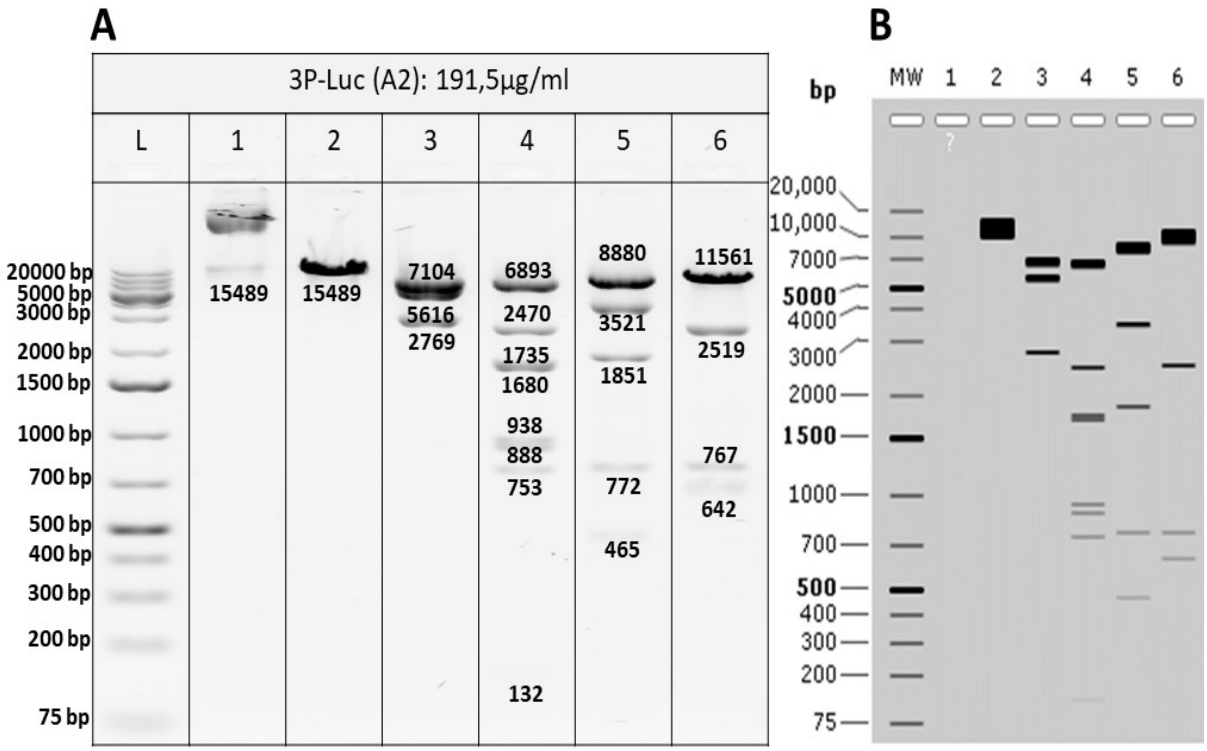

Figure 27: Agarose gel electrophoresis of 3P-Luc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Uncut
Lane 2: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with SmaI
Lane 3: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with NheI
Lane 4: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with EcoRI
Lane 5:pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with BamHI
Lane 6: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with PvuII

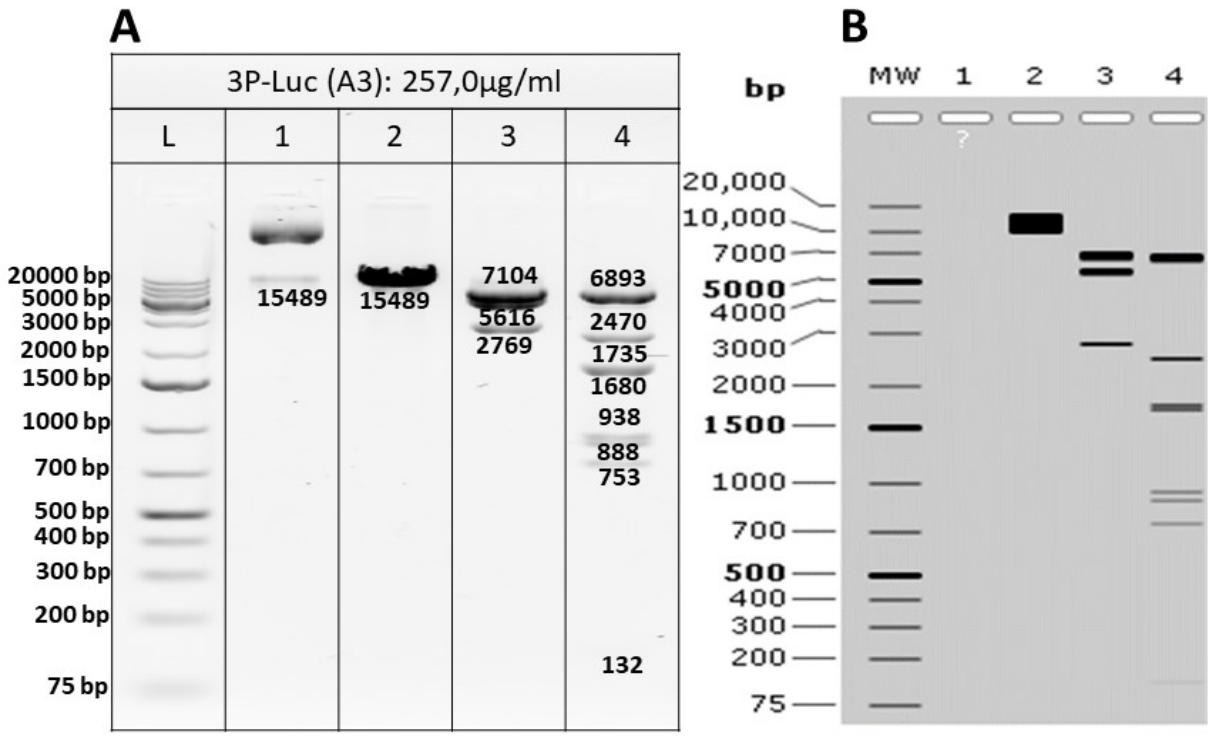

Figure 28: Agarose gel electrophoresis of 3P-Luc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Uncut
Lane 2: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with SmaI
Lane 3: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with NheI
Lane 4: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with EcoRI
Lane 5: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with BamHI
Lane 6: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with PvuII

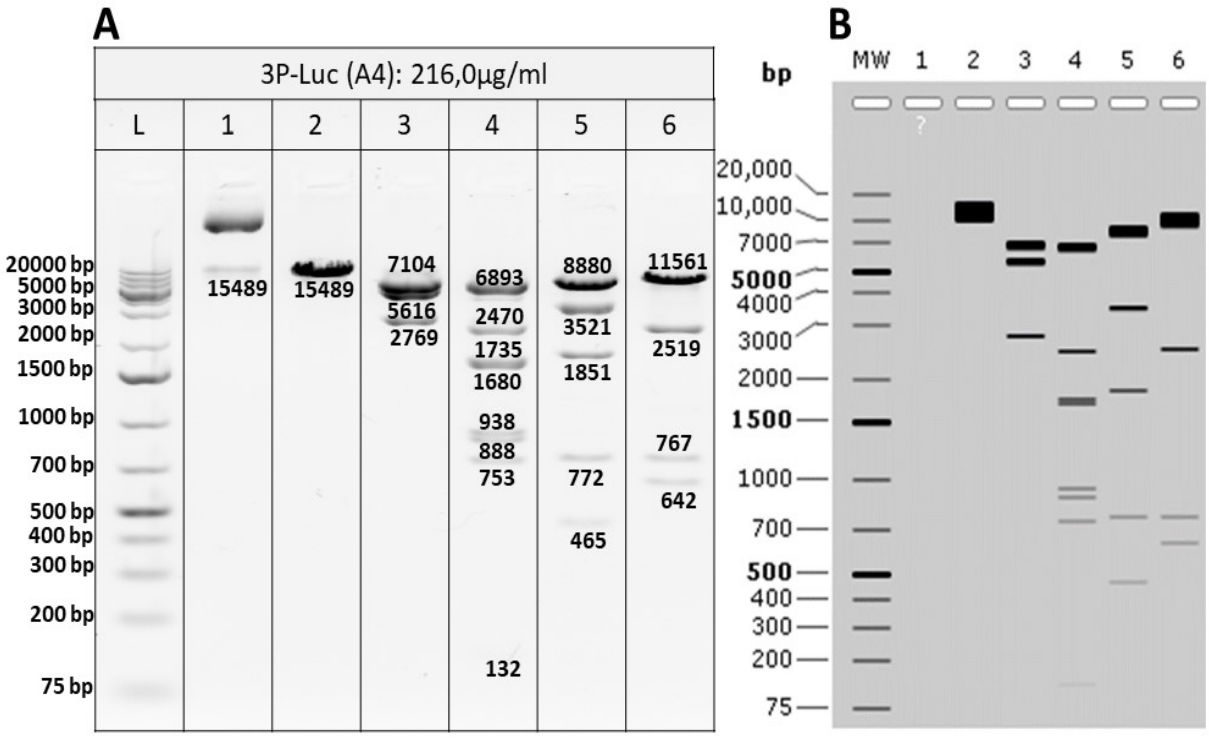

Figure 29: Agarose gel electrophoresis of 3P-Luc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Uncut
Lane 2: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with SmaI
Lane 3: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with NheI
Lane 4: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with EcoRI
Lane 5: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with BamHI
Lane 6: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with PvuII

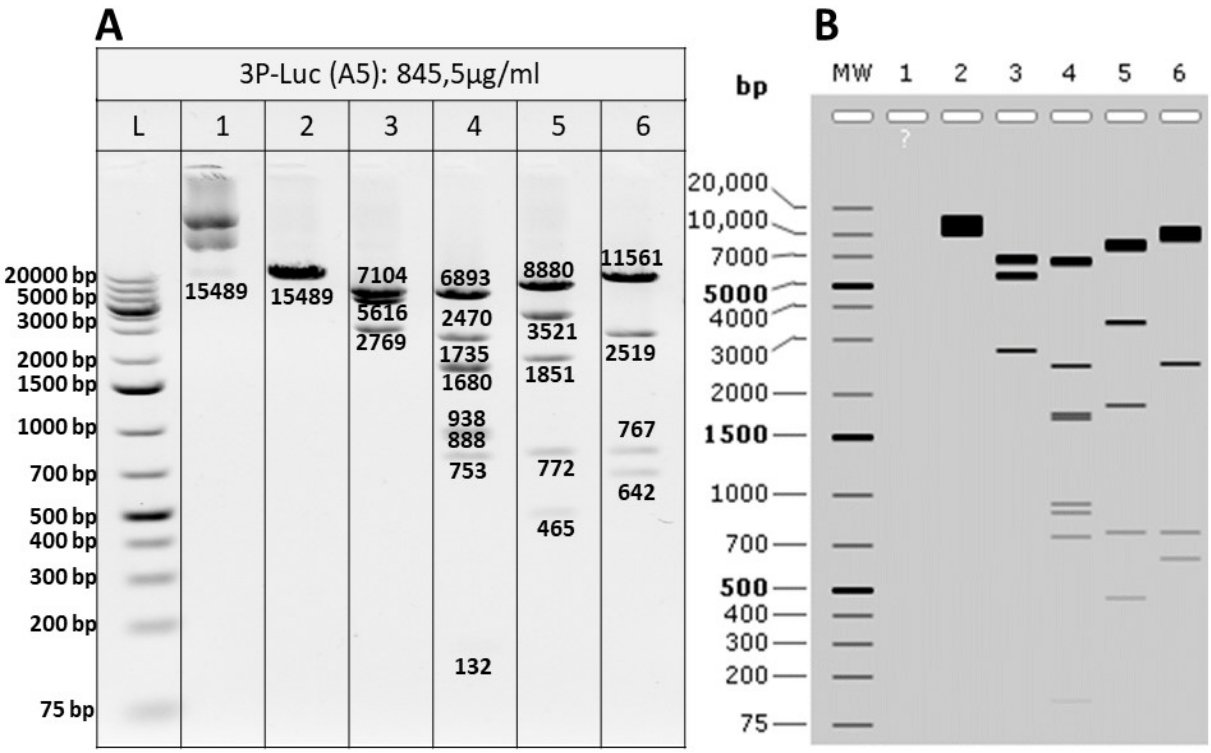

Figure 30: Agarose gel electrophoresis of 3P-Luc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Uncut
Lane 2: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with SmaI
Lane 3: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with NheI
Lane 4: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with EcoRI
Lane 5: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with BamHI
Lane 6: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with PvuII

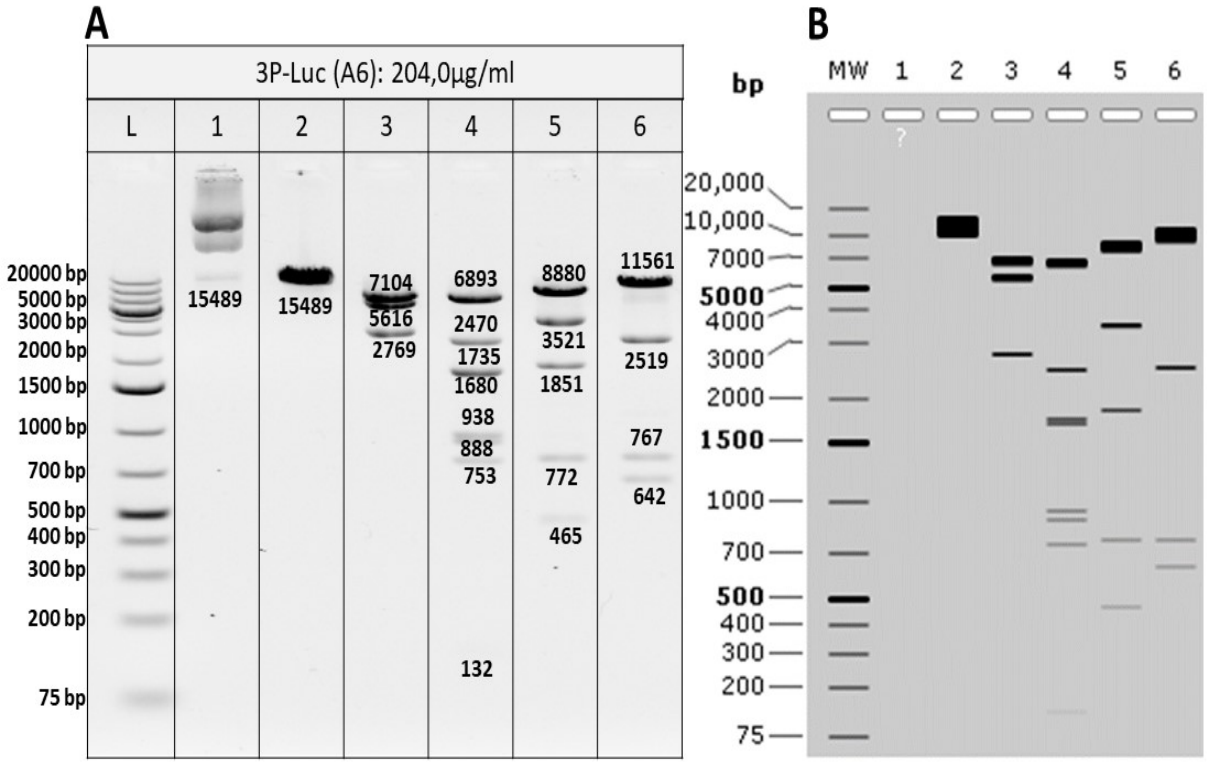

Figure 31: Agarose gel electrophoresis of 3P-Luc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Uncut
Lane 2: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with SmaI
Lane 3: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with NheI
Lane 4: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with EcoRI
Lane 5: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with BamHI
Lane 6: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with PvuII

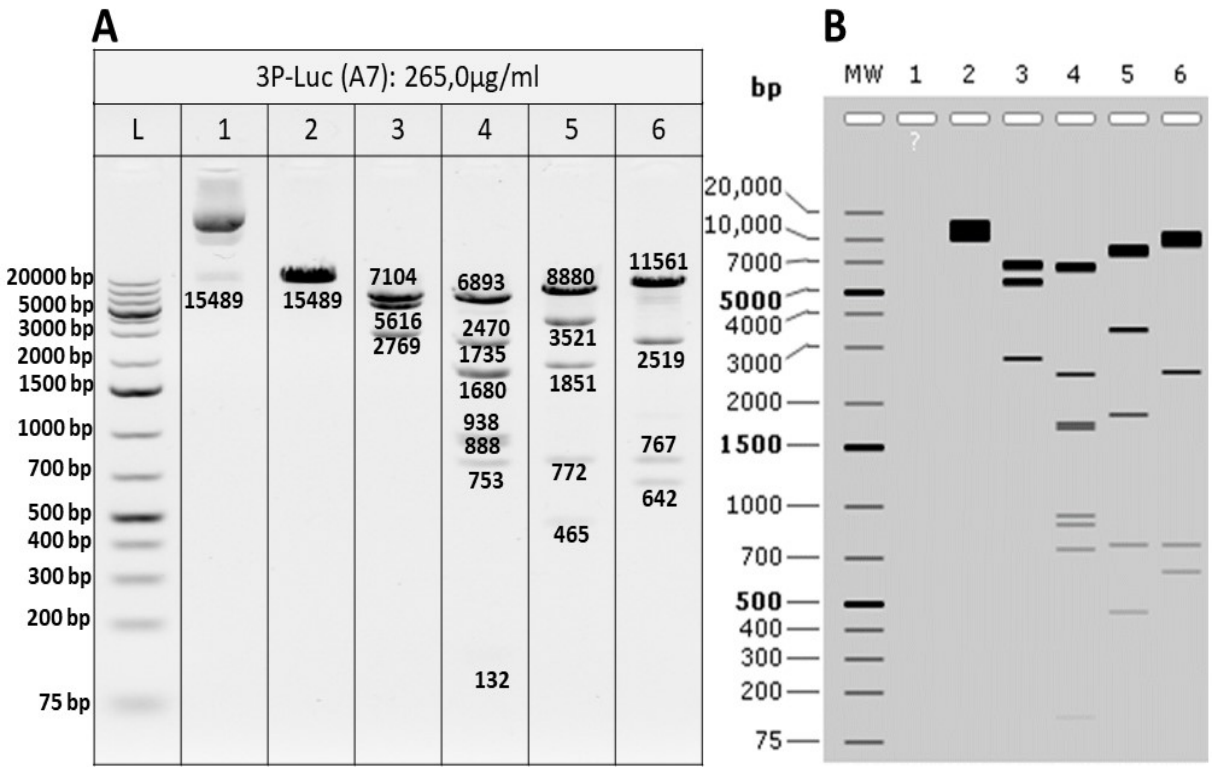

Figure 32: Agarose gel electrophoresis of 3P-Luc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Uncut
Lane 2: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with SmaI
Lane 3: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with NheI
Lane 4: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with EcoRI
Lane 5: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with BamHI
Lane 6: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with PvuII

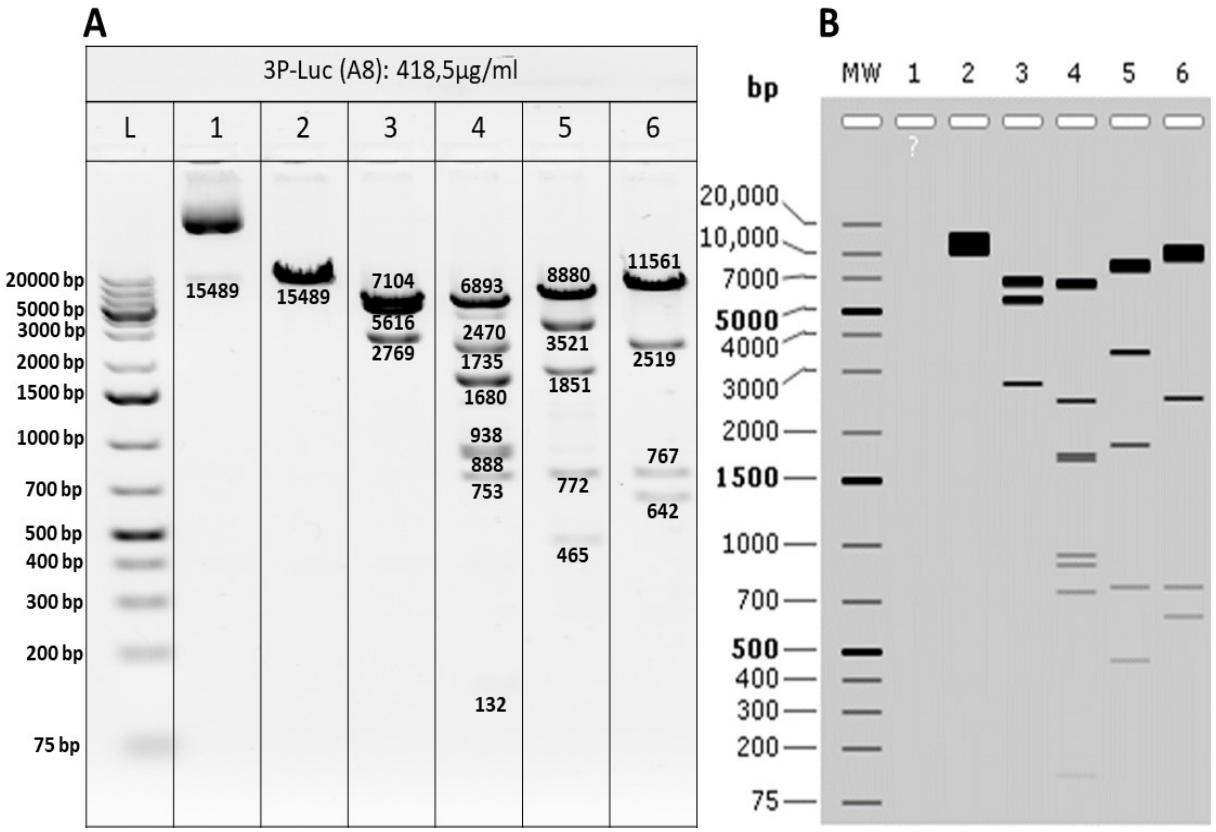

Figure 33: Agarose gel electrophoresis of 3P-Luc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Uncut
Lane 2: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with SmaI
Lane 3: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with NheI
Lane 4: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with EcoRI
Lane 5: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with BamHI
Lane 6: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with PvuII

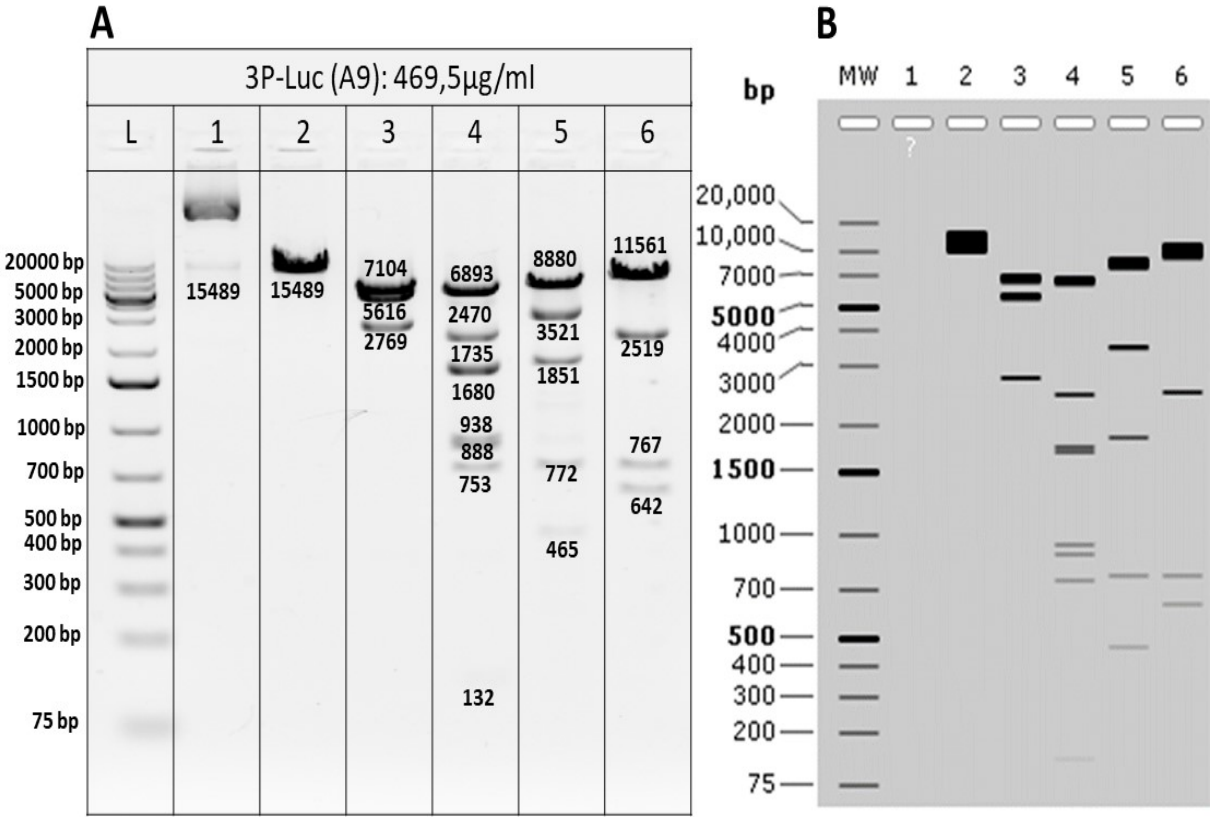

Figure 34: Agarose gel electrophoresis of 3P-Luc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Uncut
Lane 2: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with SmaI
Lane 3: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with NheI
Lane 4: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with EcoRI
Lane 5: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with BamHI
Lane 6: pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc -Digested with PvuII

5.2.5. Diagnostic restriction digest of PUC19

PUC19 is a cloning vector which was used as a negative control. Because there is no gene for luciferase on this plasmid, using it should not induce light emission, that's why it is used as a negative control. To determine this plasmid the DRD was performed by using the restriction enzymes which were selected following the restriction map shown in Figure 35 , that was prepared using Snap Gene®.

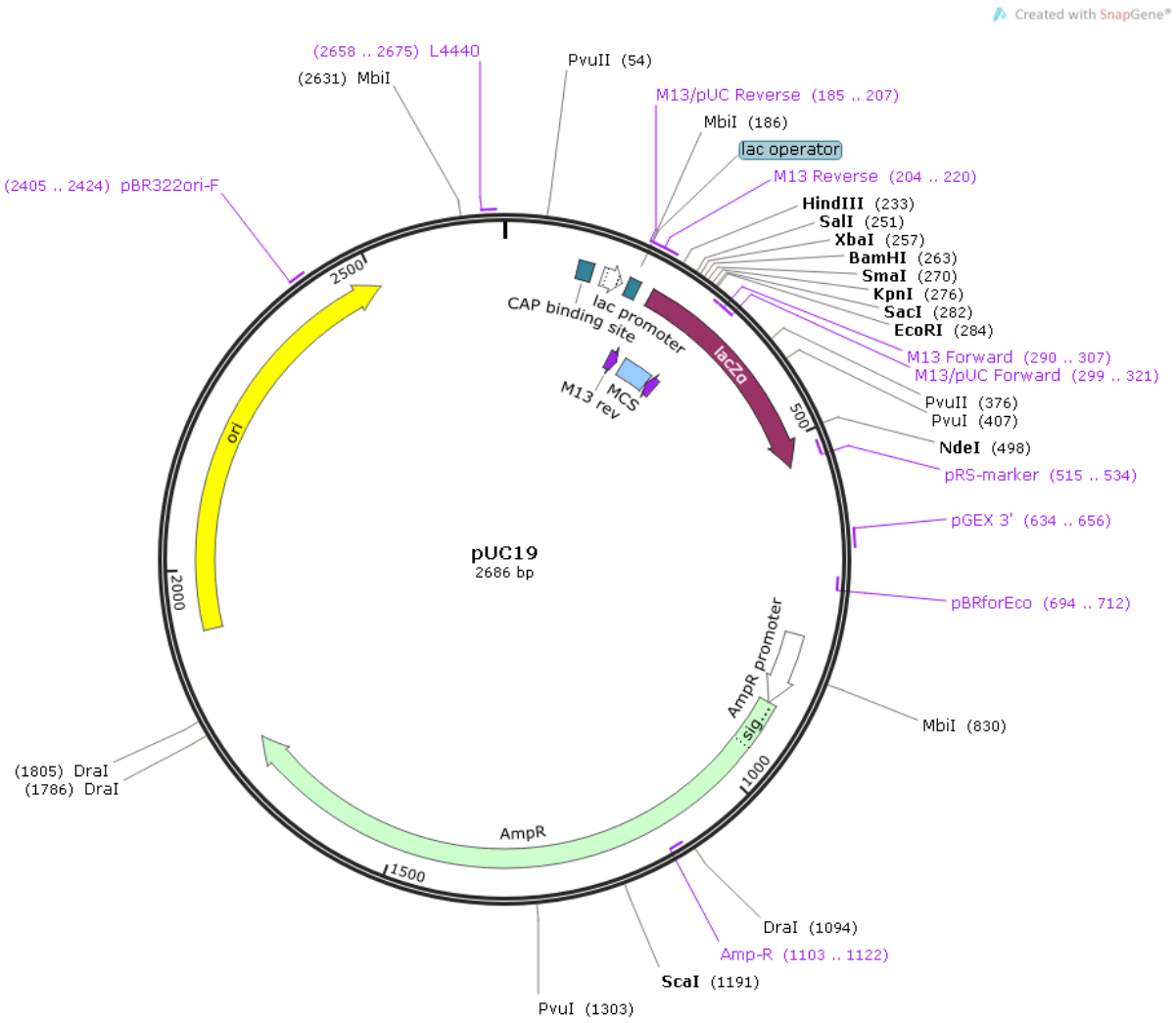

Figure 35: Restriction map of PUC19 which shows the restriction enzymes able to cleave the plasmid, as well as the number and location of the recognition sequences. (Created with Snap Gene®).

To determine PUC19, a combination of single cutters and multiple cutters were used, shown in Table 25.

Column	Enzyme	Number of Bands	Number of Base Pair
1	uncut	1	2686
2	HindIII	1	2686
3	HindIII+PvuI	3	1616 896 174
4	DraI+MbiI	6	826 692 644 264 241 19
5	XbaI+PvuII	3	2364 203 119

Table 25: Restriction enzymes applied to determine PUC19

The mentioned enzymes were mixed with 255ng of plasmid DNA, 10xFast Digest Green Buffer and ultra-pure water. Afterwards the prepared mixture was incubated at 37°C for 40 minutes to activate the enzyme, followed by an inactivation step at 80°C for 10 minutes. The finished product was kept at 4°C until analysis with gel electrophoresis. The electrophoresis was performed at 80V for 60 minutes. The fragments were made visible by using the Molecular Imager® Gel Doc™XR System and their sizes were determined by comparing to the DNA standard.

5.2.5.1. Agarose gel electrophoresis of PUC19

The gel electrophoresis has been running at 80V for 60minutes. All the bands in Fig.36A and Fig.37A were matching the simulation on Snap Gene ® in Fig.36B and Fig.37B. But there was an extra band in lane 5 in every sample. Therefore, the gel electrophoresis was repeated and showed the same results.

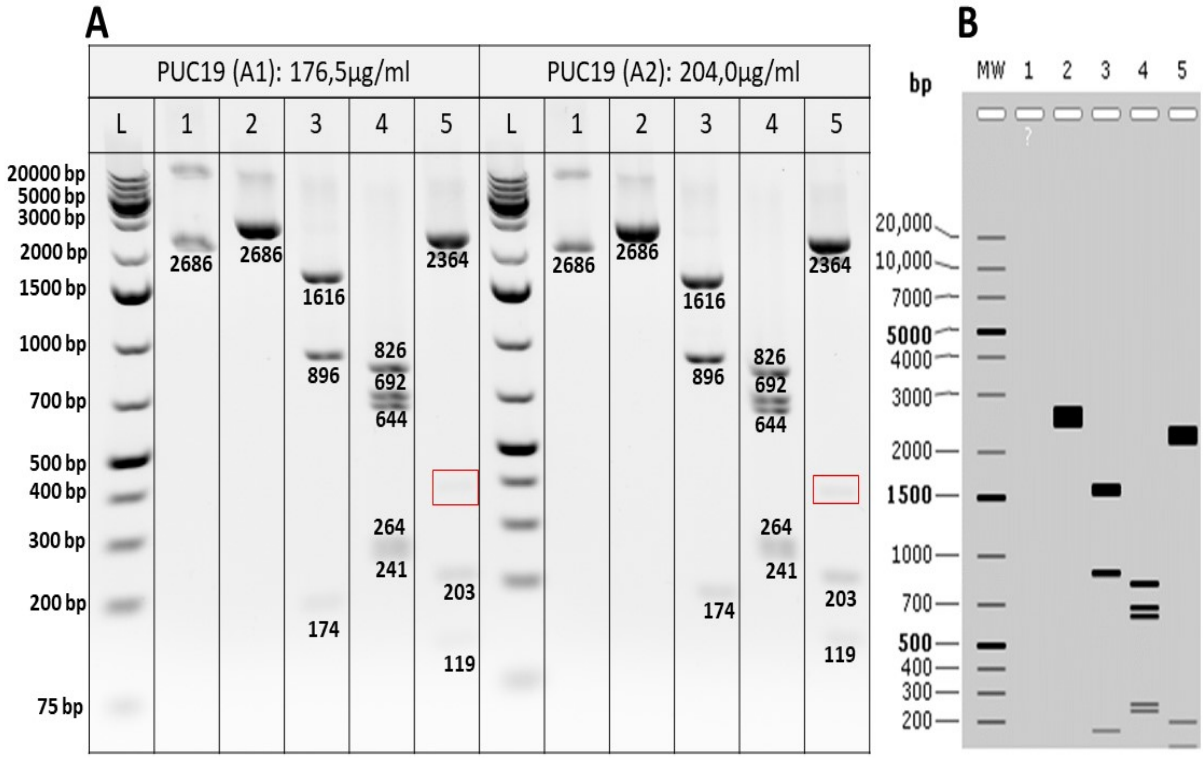

Figure 36 Agarose gel electrophoresis of PUC19 (A) and the simulation on Snap Gene® (B).
Lane 1: PUC19 -Uncut
Lane 2: PUC19 -HindIII
Lane 3: PUC19 -HindIII+PvuI
Lane 4: PUC19 -DraI+MbiI
Lane 5: PUC19 -XbaI+PvuII:

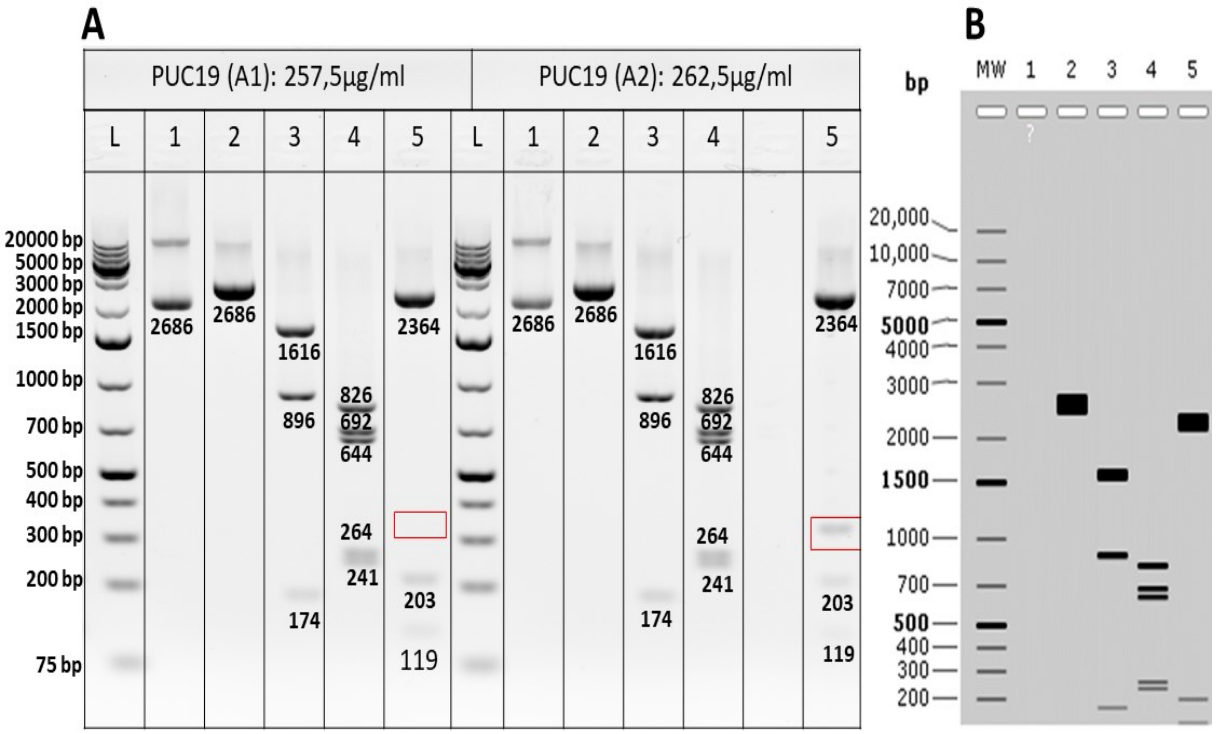

Figure 37: Agarose gel electrophoresis of PUC19 (A) and the simulation on Snap Gene® (B).
Lane 1: PUC19 -Uncut
Lane 2: PUC19 -HindIII
Lane 3: PUC19 -HindIII+PvuI
Lane 4: PUC19 -DraI+MbiI
Lane 5: PUC19 -XbaI+PvuII:

5.2.6. Diagnostic restriction digest of phlCN1

phlCN1 is an inducer plasmid for the Notch pathway. To determine this plasmid the DRD was performed by using the restriction enzymes which were selected following the restriction map shown in Figure 38 , that was prepared using Snap Gene®.

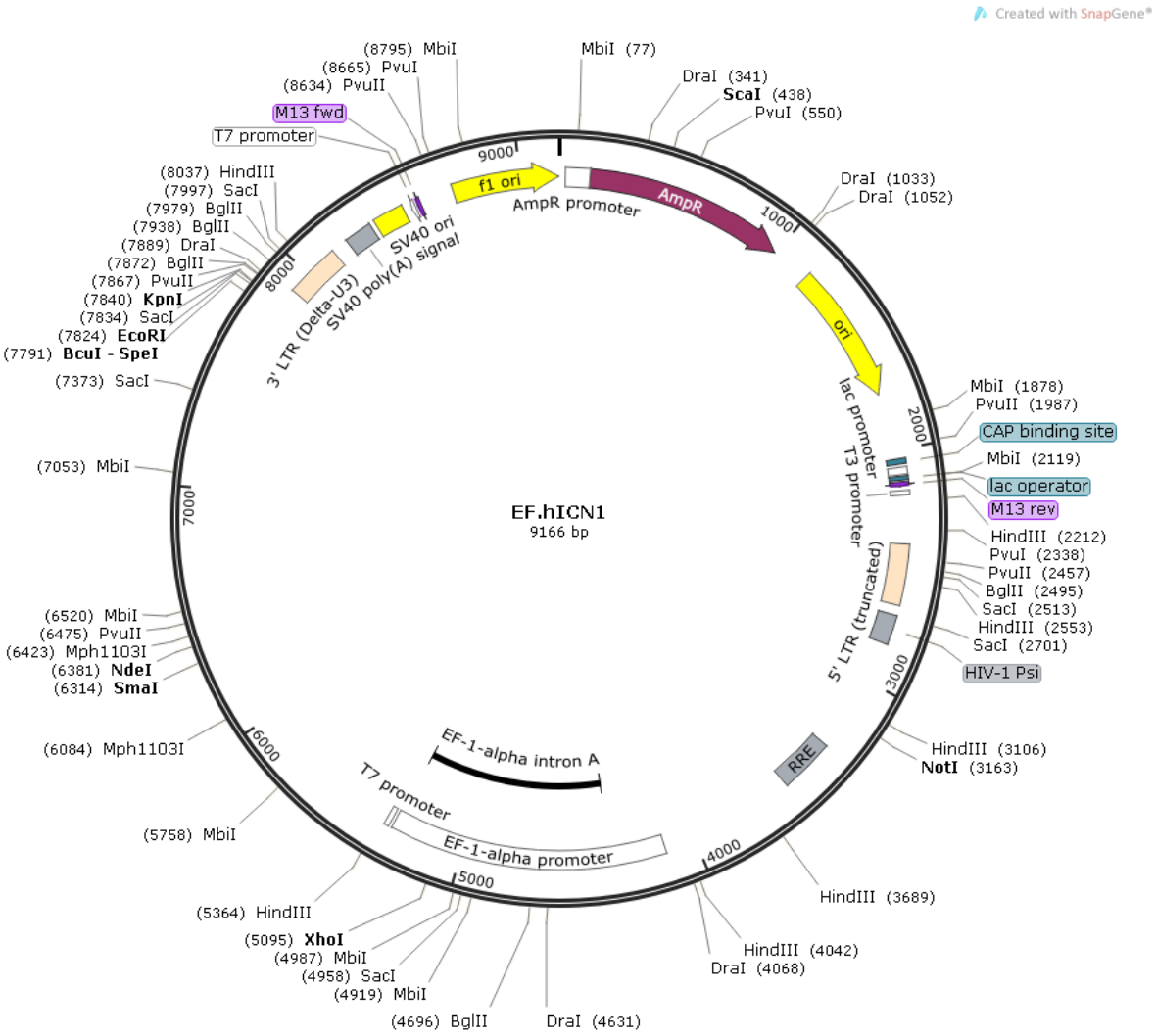

Figure 38: Restriction map of phlCN1 which shows the restriction enzymes able to cleave the plasmid, as well as the number and location of the recognition sequences. (Created with Snap Gene®).

To determine phlCN1, a combination of single cutters and multiple cutters were used, shown in Table 26.

Column	Enzyme	Number of Bands	Number of Base Pair
1	uncut	1	9166
2	KpnI	1	9166
3	KpnI+NotI	2	4677 4489
4	PvuII	5	4018 2519 1392 767 470
5	HindIII	7	3341 2673 1322 583 553 353 341

Table 26: Restriction enzymes applied to determine phlCN1

The mentioned enzymes were mixed with 255ng of plasmid DNA, 10xFast Digest Green Buffer and ultra-pure water. Afterwards the prepared mixture was incubated at 37°C for 40 minutes to activate the enzyme, followed by an inactivation step at 80°C for 10 minutes. The finished product was kept at 4°C until analysis with gel electrophoresis. The electrophoresis was performed at 80V for 60 minutes. The fragments were made visible by using the Molecular Imager® Gel Doc™XR System and their sizes were determined by comparing to the DNA standard.

5.2.6.1. Agarose gel electrophoresis of phlCN1

In both samples Fig.39 A1&A2 one band was (470bp) missing in lane 4, in lane 5 the bands 585 bp and 553 bp were not separated which should be separated according to the simulation (Fig.39B) and the bands 353bp and 341 were also not separated but it was matching the simulation. Under the band 341bp in lane 5 of both samples was an extra band which should not be there according to the simulation (Fig.39B).

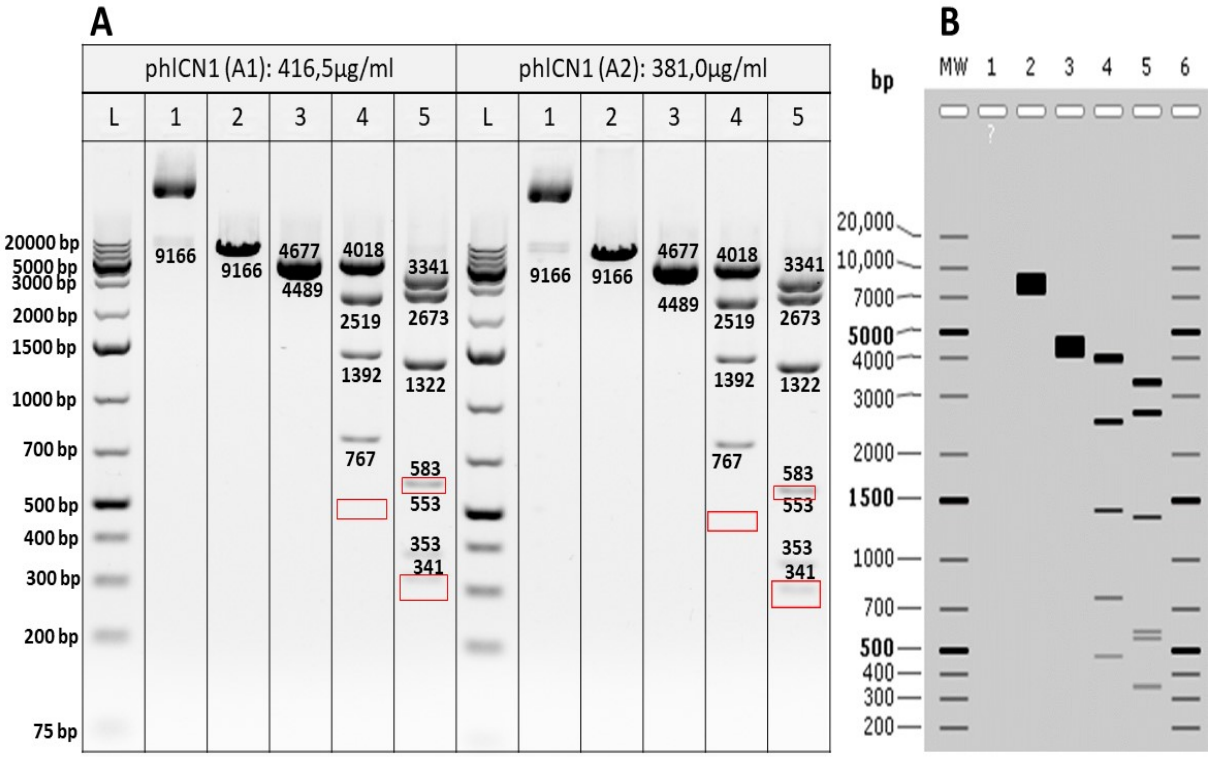

Figure 39: Agarose gel electrophoresis of phlCN1 (A) and the simulation on Snap Gene® (B).
Lane 1: phlCN1 -Uncut
Lane 2: phlCN1 -Digested with KpnI
Lane 3: phlCN1 -Digested with KpnI+NotI
Lane 4: phlCN1 -Digested with PvuII
Lane 5: phlCN1 -Digested with HindIII

5.2.7. Diagnostic restriction digest of pAd-wnt3a

pAd-wnt3a is an inducer plasmid for the Wnt pathway. To determine this plasmid the DRD was performed by using the restriction enzymes which were selected following the restriction map shown in Figure 40, that was prepared using Snap Gene®.

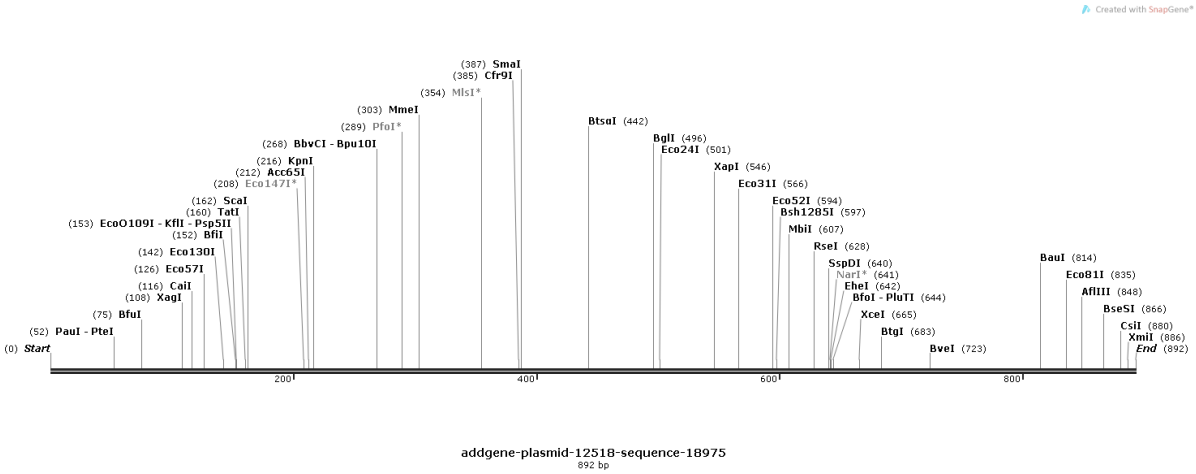

Figure 40: Restriction map of pAd-wnt3a which shows the restriction enzymes able to cleave the plasmid, as well as the number and location of the recognition sequences. (Created with Snap Gene®).

To determine pAd-wnt3a, a combination of single cutters and multiple cutters were used, shown in Table 27.

Column	Enzyme	Expected Number of Bands	Expected Number of Base Pair
1	uncut	1	892
2	KpnI	2	676 216
3	MbiI	2	607 285
4	PvuII	4	241 235 224 192

Table 27: Restriction enzymes applied to determine pAd-wnt3a.

The mentioned enzymes were mixed with 255ng of plasmid DNA, 10xFast Digest Green Buffer and ultra-pure water. Afterwards the prepared mixture was incubated at 37°C for 40 minutes to activate the enzyme, followed by an inactivation step at 80°C for 10 minutes. The finished product was kept at 4°C until analysis with gel electrophoresis. The electrophoresis was performed at 80V for 60 minutes. The fragments were made visible by using the Molecular Imager® Gel Doc™XR System and their sizes were determined by comparing to the DNA standard.

5.2.7.1. Agarose gel electrophoresis of pAd-wnt3a

In this gel the bands in Figure 41A were not matching the bands in the simulation (Fig.41B). The reason was that the whole plasmid map was not provided on addgene. (Addgene #12518). That's why more bands are visible. Both samples (A1) and (A2) were from different colony forming units but because their restriction pattern matches, it was assumed that both samples contained the same plasmid.

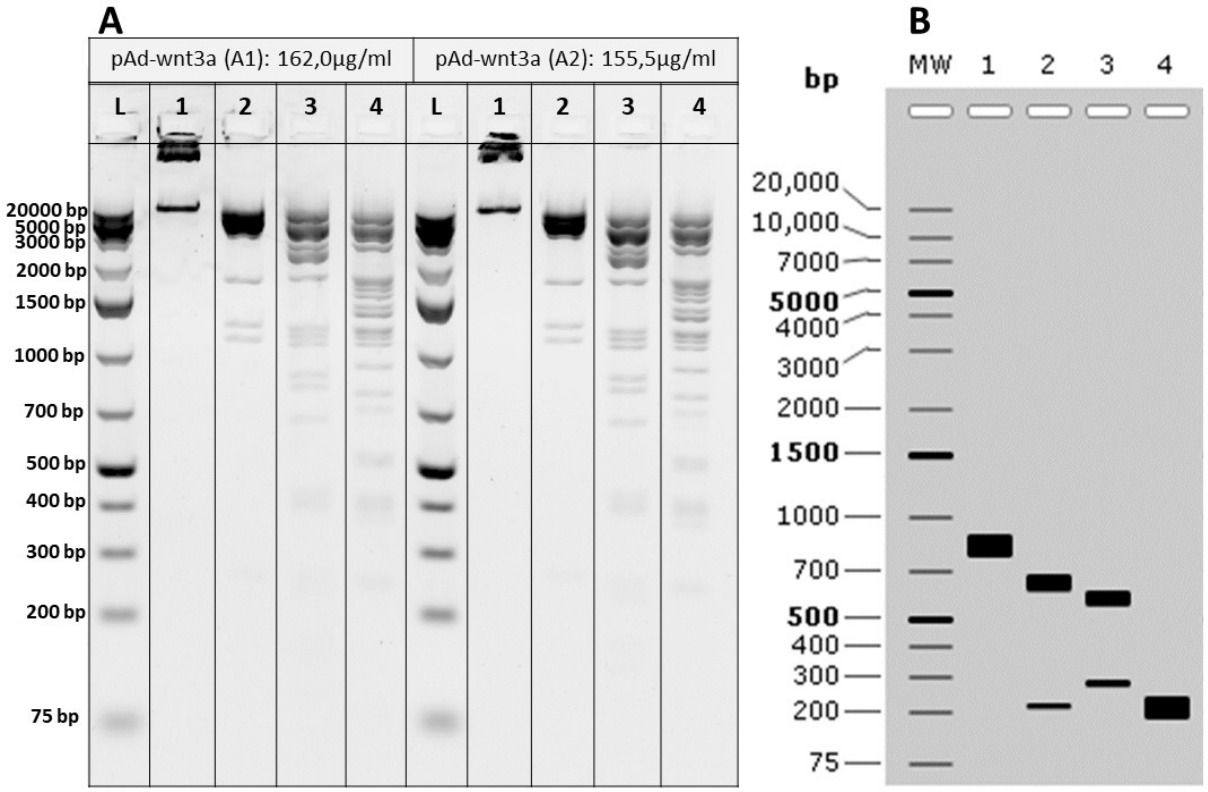

Figure 41: Agarose gel electrophoresis of pAd-wnt3a (A) and the simulation on Snap Gene® (B).
Lane 1: pAd-wnt3a -Uncut
Lane 2: pAd-wnt3a -Digested with KpnI
Lane 3: pAd-wnt3a -Digested with MbiI
Lane 4: pAd-wnt3a -Digested with PvuII

5.2.8. Diagnostic restriction digest of phGLi1

phGli1 is an inducer plasmid for Hedgehog pathway. To determine this plasmid the DRD was performed by using the restriction enzymes which were selected following the restriction map shown in Figure 42, that was prepared using Snap Gene®.

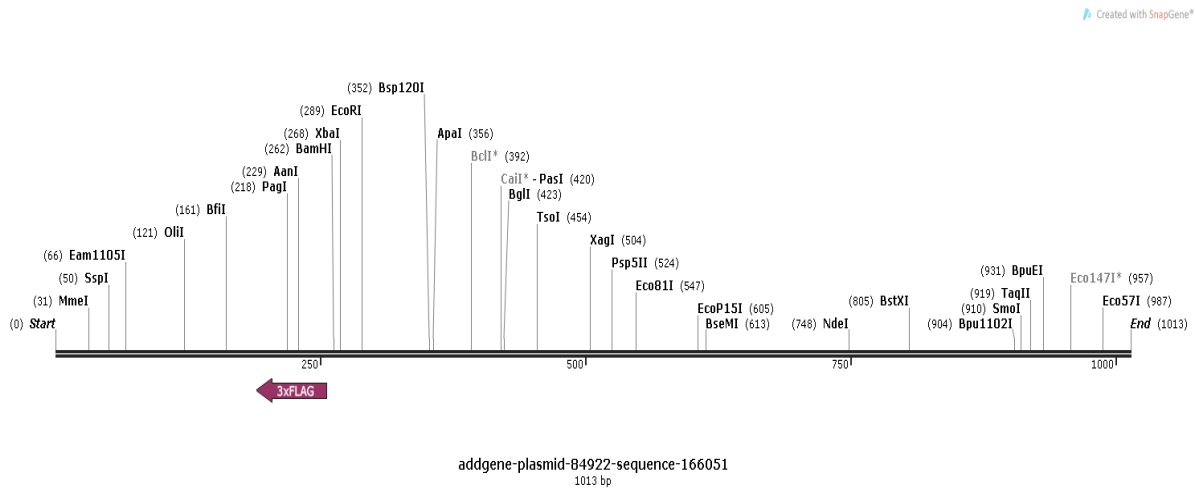

Figure 42: Restriction map of phGli1 which shows the restriction enzymes able to cleave the plasmid, as well as the number and location of the recognition sequences. (Created with Snap Gene®).

To determine phGli1, a combination of single cutters and multiple cutters were used, shown in Table 28.

Column	Enzyme	Expected Number of Bands	Expected Number of Base Pair
1	uncut	1	1013
2	XbaI	2	745 268
3	EcoRI	2	724 289
4	PvuII+BamHI	4	451 262 191 109

Table 28: Restriction enzymes applied to determine phGli1.

The mentioned enzymes were mixed with 255ng of plasmid DNA, 10xFast Digest Green Buffer and ultra-pure water. Afterwards the prepared mixture was incubated at 37°C for 40 minutes to activate the enzyme, followed by an inactivation step at 80°C for 10 minutes. The finished product was kept at 4°C until analysis with gel electrophoresis. The electrophoresis was performed at 80V for 60 minutes. The fragments were made visible by using the Molecular Imager® Gel Doc™XR System and their sizes were determined by comparing to the DNA standard.

5.2.8.1. Agarose gel electrophoresis of phGli1

Because the whole map of this plasmid was not available on addgene, the bands in Figure 43A were not matching the bands in the simulation in Figure 43B. Both samples (A1) and (A2) were from different colony forming units but because their restriction pattern matches, it was assumed that both samples contained the same plasmid.

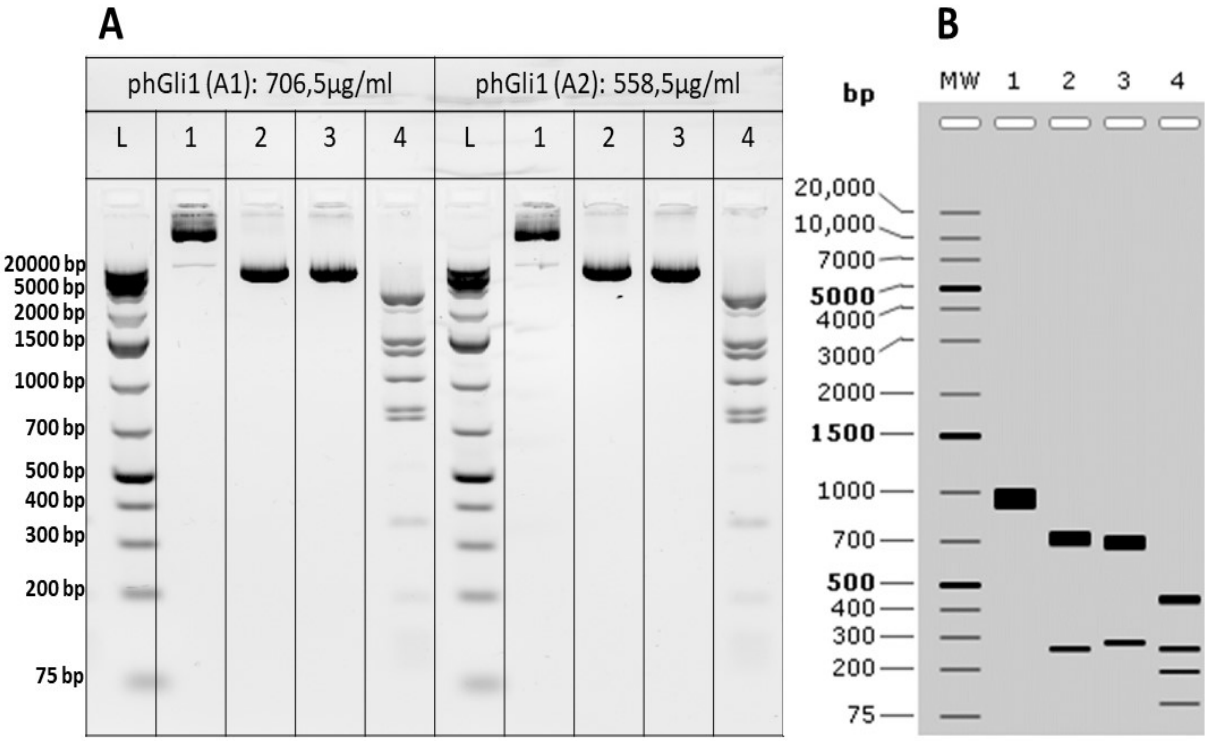

Figure 43: Agarose gel electrophoresis of phGli1(A) and the simulation on Snap Gene® (B).
Lane 1: phGli1 -Uncut
Lane 2: phGli1 -Digested with XbaI
Lane 3: phGli1 -Digested with EcoRI
Lane 4: phGli1 -Digested with PvuII+BamHI

5.2.9. Diagnostic restriction digest of CMV-NLuc

CMV-NLuc is a reporter plasmid for the Wnt pathway. To determine this plasmid the DRD was performed by using the restriction enzymes shown in Table 29.

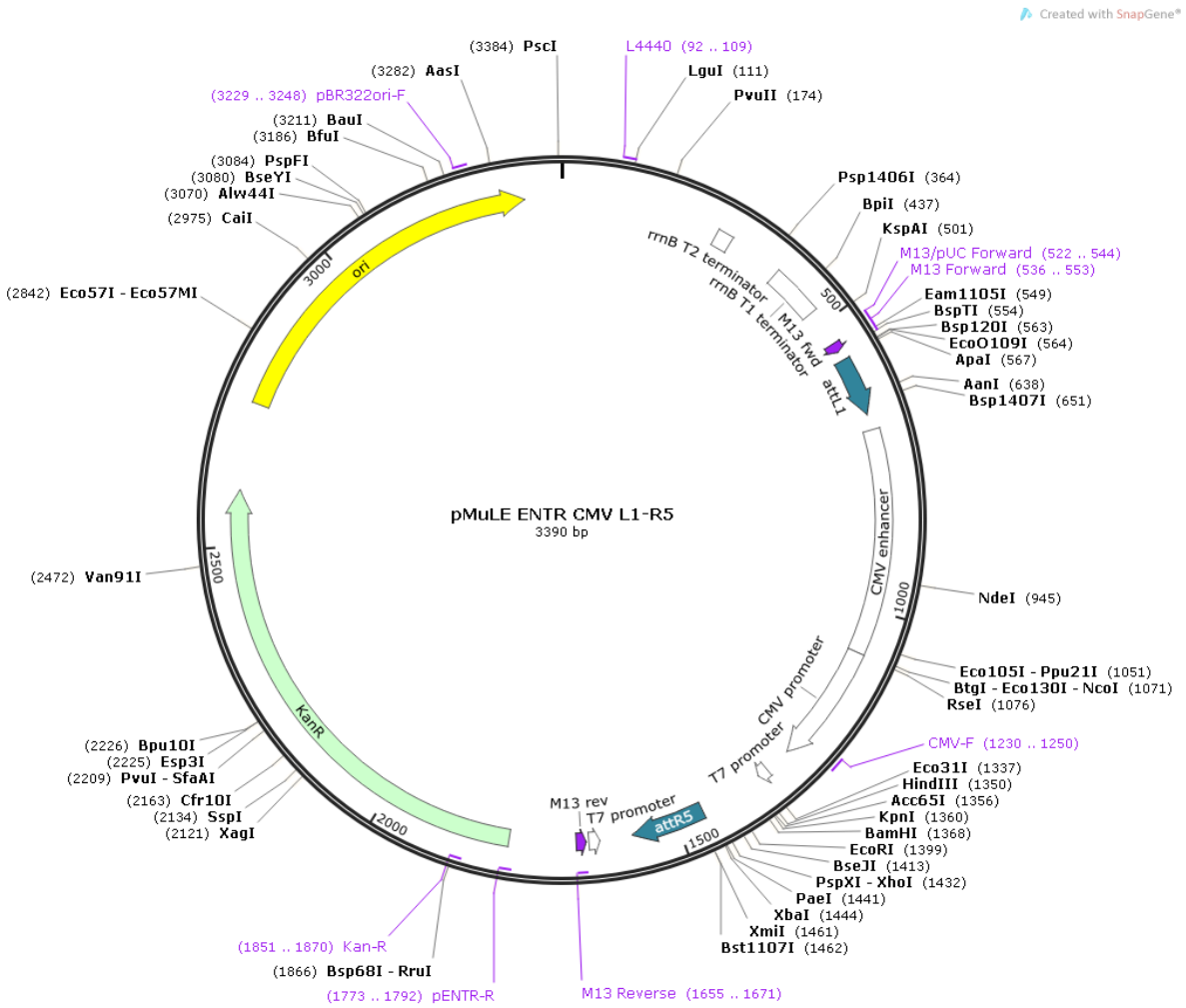

Figure 44: Incomplete restriction map of CMV-NLuc which shows the restriction enzymes able to cleave the plasmid, as well as the number and location of the recognition sequences. (Created with Snap Gene®).

Because the plasmid map of CMV-NLuc was not complete, the restriction enzymes which cut TOP-NLuc were used since both plasmids have the same plasmid backbone. Only single cutters which should not cut the plasmid due to the incomplete map, were chosen.

Column	Enzyme	Expected Number of Bands	Expected Number of Base Pair
1	uncut	1	3693
2	BglIII	1	3693
3	DraI	1	3693
4	BglII	2	2842 851

Table 29: Restriction enzymes which have been used to determine CMV-NLuc.

The mentioned enzymes were mixed with 255ng of plasmid DNA, 10xFast Digest Green Buffer and ultra-pure water. Afterwards the prepared mixture was incubated at 37°C for 40 minutes to activate the enzyme, followed by an inactivation step at 80°C for 10 minutes. The finished product was kept at 4°C until analysis with gel electrophoresis. The electrophoresis was performed at 80V for 60 minutes. The fragments were made visible by using the Molecular Imager® Gel Doc™XR System and their sizes were determined by comparing to the DNA standard.

5.2.9.1. Agarose gel electrophoresis of CMV-NLuc

Because the plasmid map of CMV-NLuc was incomplete, the gel Simulation on Snap Gene® in Figure 45B did not match with the electrophoresis results in Figure 45A. However, the two plasmids from the two different preps did match in their diagnostic restriction digest pattern.

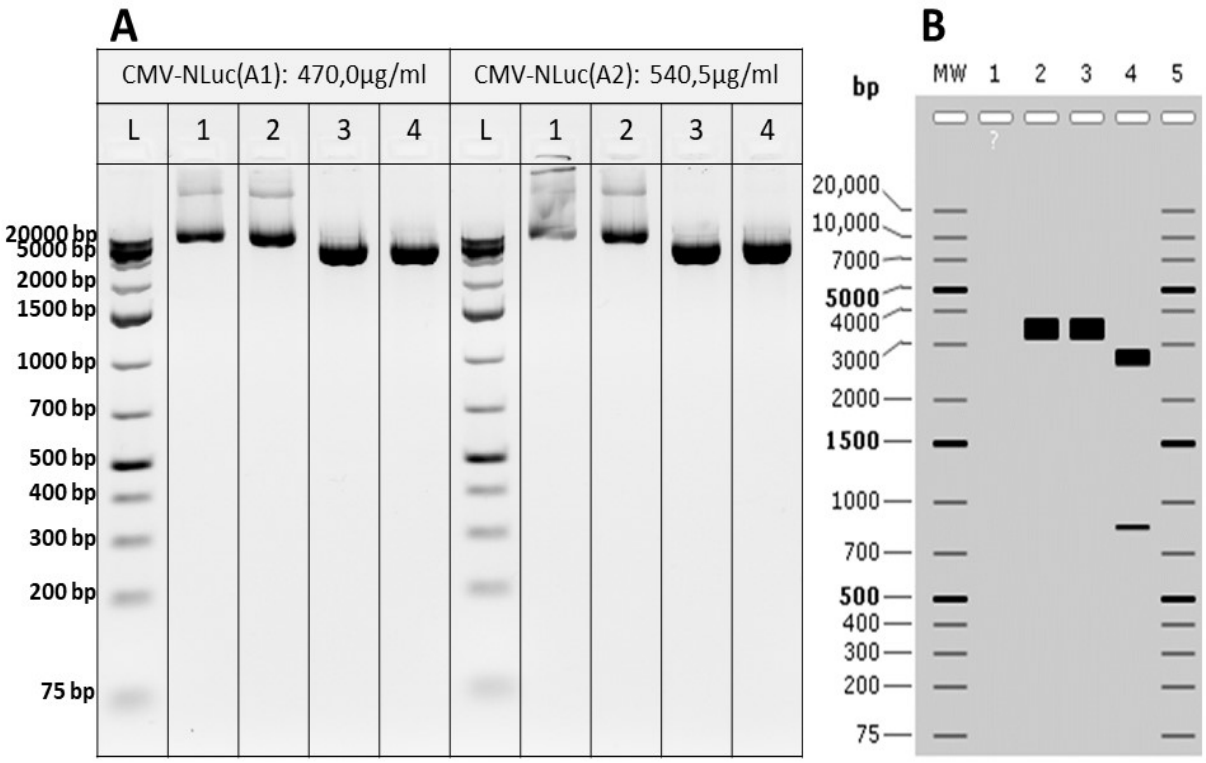

Figure 45: Agarose gel electrophoresis of CMV-NLuc (A) and the simulation on Snap Gene® (B).
Lane 1: pMuLE_ENTR_CMV-NL1.1_L1-R5 -Uncut
Lane 2: pMuLE_ENTR_CMV-NL1.1_L1-R5 -Digested with BglIII
Lane 3: pMuLE_ENTR_CMV-NL1.1_L1-R5 -Digested with DraI
Lane 4: pMuLE_ENTR_CMV-NL1.1_L1-R5 -Digested with BglII

5.3. Discussion

The purpose of this work was to create a stock of all the plasmids which were mentioned in Table 7 and to determine their identity by diagnostic restriction digest. To reach this, the bacteria were streaked on LB plates containing the appropriate antibiotic and were incubated at 37°C for 16h. After that, two liquid cultures have been produced to increase the total yield. Subsequently the bacteria have been lysed and purified using the GeneJet Plasmid Maxiprep Kit from Thermo Scientific. To determine the identity of the plasmid, 255ng of DNA have been mixed with 2µl 10xFast Digest Green Buffer, 1µl restriction enzyme and were filled up to 20µl with ultra-pure water. The prepared mixture was incubated at 37°C for 40 minutes to activate the enzyme, followed by an inactivation step at 80°C for 10 minutes. The finished product was kept at 4°C until analysis with gel electrophoresis. The electrophoresis was performed at 80V for 60 minutes. The fragments were made visible by using the Molecular Imager® Gel Doc™XR System and their sizes were determined by comparing to the DNA standard.

pMuLE_ENTR_TOP-NL1.1_L5-L4 (TOP-NLuc)

TOP-NLuc is a reporter plasmid for the Wnt pathway which was created by Julia Maier during her PhD Thesis. To produce this plasmid, pMuLE_ENTR_MCS_L5-L4, M50 Super 8xTOPFlash and pNL1.1 plasmid have been digested with several enzymes and were ligated together to yield the final plasmid. To determine the identity, the restriction enzymes mentioned in Table 21 have been used to digest the plasmid. Afterwards the gel electrophoresis results were compared to the gel simulation on Snap Gene®. In two of three gels (Fig. 17A and Fig. 18A) the results were matching the simulation (Fig. 17B and Fig.18B). Because the bands were not matching in the third gel (Fig. 19A) the digest was repeated and showed the same results. Reasons for a missing band could be incomplete digest, unfunctional restriction enzyme or incomplete plasmid. All the samples have been prepared and digested under the same condition which means that the digest was complete and because all the samples have been digested with the same enzymes (Table 21) and the results of the first two samples were matching the simulation (Fig. 17A and Fig. 18A), it is unlikely that the enzyme was not working. It seems like that this colony didn't contain the right plasmid. That was the reason why this stock was removed from the storage.

pMuLE_ENTR_12xGLI-FLuc_R4-R3 (GLI-FLuc)

GLI-FLuc is a reporter plasmid for the Hedgehog pathway which was also created by Julia Maier. For this plasmid a PCR was performed, and the resulting fragment was cloned into pMuLE_ENTR_MCS_R4-R3. The plasmid was digested with the restriction enzymes mentioned in Table 22. Afterwards the gel electrophoresis results were compared to the gel simulation on Snap Gene®. In both samples (Fig.21A and Fig.22A) an additional band was visible in lane 1 with the uncut plasmid, which was the nicked form of the plasmid that migrates slower than the supercoiled form. Because all the bands were matching the simulation (Fig.21B and Fig.22B) in Figure 21A and Figure 22A the stocks seems to contain the plasmid of interest.

pMuLE_ENTR_CBF-Gluc_L3-L2 (CBF-GLuc)

CBF-GLuc is a reporter plasmid for the Notch pathway. It was made by combining three plasmids. To determine the identity the restriction enzymes mentioned in Table 23 have been used to digest the plasmid. As can be seen in Fig. 24A all the bands were matching the simulation. There was an additional band in every column with the uncut plasmid, which was the nicked form of the plasmid that migrates slower than the supercoiled form.

In lane 5 (Fig. 24 A2) of the second sample, were 2 ghost bands at nearly 3000bp which should not be there due to the simulation. Ghost bands are denatured supercoiled DNA which appears because of alkaline lysis-based plasmid purification procedures **(44,45)**. Because the plasmid stock CBF-GLuc 190,0µg/ml contains denatured DNA it would be better not to use this stock since it will affect the results of future experiments.

pMuLE_EXPR_CMV-eGFP-TOP-NLuc_1.1_12xGLI-FLuc_CBF-Gluc (3P-Luc)

3P-Luc is the reporter plasmid for all three pathways (Wnt, Hedgehog und Notch). For the production, the three-luciferase reporter plasmid were ligated to one big plasmid, which should show the activity of all three pathways at the same time. To determine the identity, the restriction enzymes mentioned in Table 24 have been used to digest the plasmid. As can be seen in Fig.26A- Fig. 34A all the gel electrophoresis results are matching the simulation (Fig.26B- Fig.34B) on Snap Gene®. Some of them were already tested, for more information see the Diploma Thesis of Silvia Weiss.

PUC19

PUC19 is a cloning vector and was used as a negative control which should not shine during the measurement. PUC19 was determined by using the restriction enzymes mentioned in Table 25. In all the samples (Fig. 36A and Fig. 37A) was an extra band in lane 5 which did not match the simulation on Snap Gene® in Figure 36B and Figure 37B. The samples were already tested and showed no signal (see Diploma Thesis of Silvia Weiss) which means that the extra band could be a contamination with another enzyme during the preparation for the DRD.

phICN1

phICN1 is an inducer plasmid for the Notch pathway. To determine the identity, the restriction enzymes mentioned in Table 26 have been used to digest the plasmid. The results of gel electrophoresis from both samples were not matching the simulation (Fig.39B). In lane 4 one band was missing at 470bp, in lane 5 two bands (Fig. 39A, lane 5, 585bp and 553bp) were not separated and there was one extra band at nearly 300bp. Reason for the missing band (lane 4) and the extra band (lane 5) could be that the plasmid map is not complete. To be sure if the plasmid is working or not it should be tested.

pAd-wnt3a, phGli1 and CMV-NLuc

Two inducer plasmids pAd-wnt3a (Wnt pathway), phGli1 (Hedgehog pathway) and one reporter plasmid CMV-NLuc (Wnt pathway) were determined by using the restriction enzymes mentioned in Table 27- Table 29. Because the whole map of all three plasmids were not provided by the producer on addgene (Addgene #12518, Addgene #84922), the restriction digest was not complete, and the electrophoresis results did not match the simulation on Snap Gene®.

6. Conclusion

To sum up, all the stocks of TOP-NLuc, GLI-FLuc, 3P-Lu, PUC19 (were also tested in vitro by Silvia Weiss) and the CBF-GLuc stock with 191,0µg/ml contain the right plasmid as per the diagnostic restriction digest. Since the gel electrophoresis results of phICN1 did not match the simulation, both stocks should be tested in vitro to have further confirmation of the results. To have final statement about pAd-wnt3a, phGli1 and CMV-NLuc, these stocks should be tested as well since the complete plasmid map is not available.

7. Abstract

Cancer is one of the major causes of death in the 21st century. The mortality rate increases annually, hence it is important to understand this disease to develop targeted diagnosis and therapy. The reasons for the development of cancer are manifold. Deregulation of Wnt, Hedgehog and Notch pathways play an important role in the development of cancer and are active topics of interest to study the process and development of cancer. These three signaling pathways are usually responsible for embryonic development.

For this reason, reporter systems containing promoters for the expression of luciferases have been developed in the MMCT laboratory to monitor the activity of the above-mentioned signaling pathways.

In this work, those plasmids were amplified, purified and their identity was determined. Since it was necessary to validate stock solutions for further experiments, plasmids were subjected to diagnostic restriction digest and validated for their composition

Zusammenfassung

Krebs ist eine der häufigsten Todesursachen im 21. Jahrhundert. Die Sterblichkeitsrate steigt jährlich, daher ist es wichtig, diese Krankheit zu verstehen, um eine gezielte Diagnose und Therapie zu entwickeln. Die Gründe für die Entstehung von Krebs sind vielfältig. Die Deregulierung von Wnt-, Hedgehog- und Notch Signaltransduktionswegen spielt eine wichtige Rolle bei der Entstehung von Krebs und ist ein aktives Thema von Interesse, um den Prozess und die Entstehung von Krebs zu untersuchen. Diese drei Signalwege sind in der Regel für die embryonale Entwicklung verantwortlich.

Aus diesem Grund wurden im MMCT Labor, Reportersysteme entwickelt, die Promotoren für die Expression von Luciferasen enthalten um die Aktivität der oben genannten Signalwege zu beobachten.

In dieser Arbeit wurden jene Plasmide vervielfältigt, gereinigt und deren Identität geprüft. Da es notwendig war, eine saubere Stammlösung zu generieren, um sie für zukünftige weitere Experimente zu verwenden, wurden Plasmide einem diagnostischen Restriktionsverdau unterzogen und für ihre Zusammensetzung validiert.

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