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Fakultet for teknologi, kunst og design
Institutt for maskin, elektronikk og kjemi

TILGJENGELIGHET
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BACHELOROPPGAVE

BACHELOROPPGAVENS TITTEL	DATO
CRISPR/Cas9 gene-editing tools construction	25.05.2022
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Hybrid technology Hub, Domus Medica tilbygg, Universitetet i Oslo	Chencheng Wang Dr. Hanne Scholz

SAMMENDRAG
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The combined vector showed that it did perform cutting, and future research will reveal if the constructed donor DNA template vector results in a knock-in of GFP in the proglucagon gene.

3 STIKKORD
CRISPR/Cas9
Type 1-diabetes
Green fluorescent protein reporter gene

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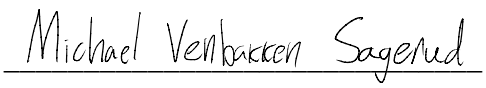

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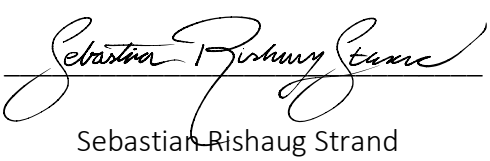

Sebastian Rishaug Strand

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Abstract

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Abbreviations and symbols

bp	Base pair
Cas	CRISPR-associated
Cas9	CRISPR-associated protein 9
Cas9-HF	High-fidelity Cas9
CFD	Cutting frequency determination
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
DMEM	Dulbecco's Modified Eagle's Medium
dNTP	deoxyribonucleotide triphosphate
DSB	Double strand break
E. coli	*Escherichia coli*
eCas9	Enhanced Cas9
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
ES cells	Embryonic stem cells
FP	Fluorescent proteins
GCG	Proglucagon gene
GFP	Green fluorescent protein
GLP-1	Glucagon-like peptide-1
GLP-2	Glucagon-like peptide-2
gRNA	Guide RNA
GRPP	Glicentin-related pancreatic polypeptide
HDR	Homology directed repair
HEK293 cells	Human embryonic kidney 293 cells
hiPSC	Human induced pluripotent stem cells
IP-1	Intervening peptide-1
IP-2	Intervening peptide-2
kb	Kilo base pair (1000 bp)
LB-medium	Lysogeny broth medium
MIT	Massachusetts Institute of Technology
MPGF	Major proglucagon fragment
MQ	Milli-Q™
mRNA	Messenger RNA
NHEJ	Non-homologous end joining
PAM	Protospacer adjacent motif
PC1/3	Prohormone convertase 1/3
PC2	Prohormone convertase 2
PCR	Polymerase chain reaction
PGDP	Proglucagon derived peptide
Pre-crRNA	Precursor CRISPR RNA
PSC	Pluripotent stem cells
RFP	Red fluorescent protein
RPM	Revolutions per minute
sgRNA	Single-guide RNA
Sp	Streptococcus pyogenes
SOC Medium	Super Optimal broth with Catabolite repression Medium
ssODN	Single-stranded oligonucleotides
T1D	Type 1-diabetes
T7E1	T7 Endonuclease I
TAE	Tris-acetate EDTA

tracrRNA	Trans-activating CRISPR RNA
UV	Ultraviolet
WT DNA	Wild type DNA
YFP	Yellow fluorescent protein

1. Introduction

1.1 Type 1-diabetes and the body

Type 1-diabetes (T1D) is a disease where the body's immune system destroys the pancreatic islets' insulin-producing beta cells (Figure 1). The production of insulin can either be gone entirely or not sufficient. The lack of insulin in the body will disrupt the cells' ability to take up nutrients from the bloodstream in the form of glucose. The higher blood sugar levels can lead to multiple physiological reactions that could harm or even be fatal for the patient without treatment (1-4).

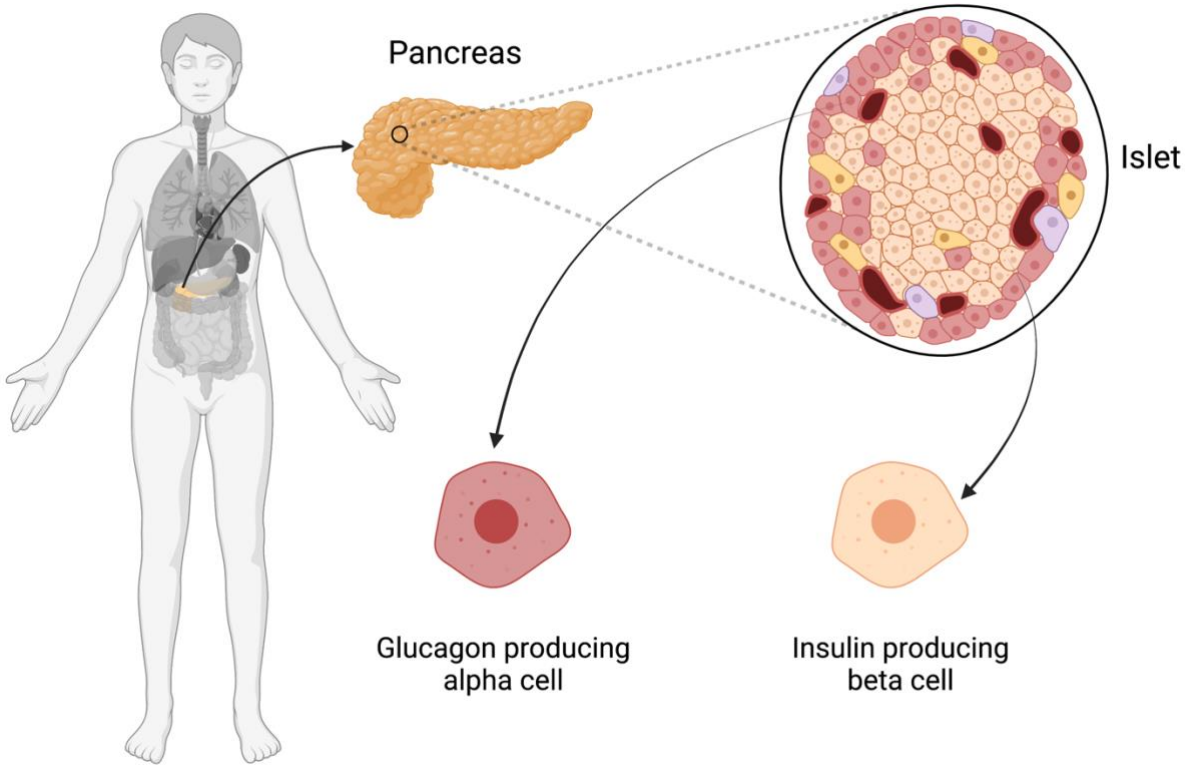

Figure 1. Illustration of the pancreatic islets and their most essential cells concerning blood sugar regulation. Created with BioRender.com.

1.1.1 Blood sugar-lowering hormone

Insulin is a hormone that attaches to a receptor on the cell surface of the receiving cell. With insulin connected to the receptor, a series of signals start resulting in the cell letting glucose through the cell membrane. Figure 2 shows the difference between a healthy person and how insulin works in their body and a person with T1D where the insulin production has stopped fully, resulting in no insulin present in the bloodstream.

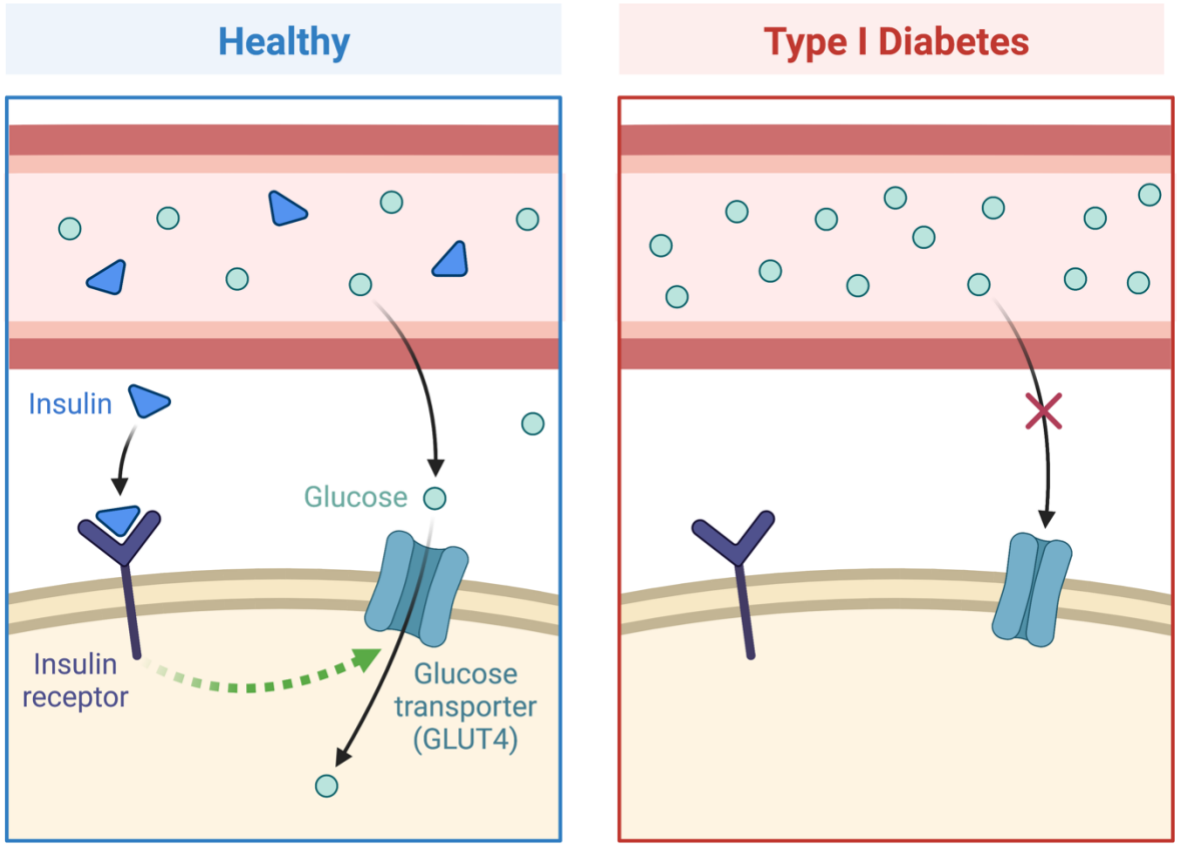

Figure 2. Left: A healthy person with average insulin production makes the glucose transporter open. Right: A person with type 1-diabetes does not have insulin that allows nutrients to enter the cells. Created with BioRender.com.

To this day, T1D is treated with insulin injections which help patients to live a life closer to people with normal insulin production. T1D patients are given diet recommendations, like higher consumption of unsaturated fat and whole-grain products over saturated fats and finer baked goods, to reduce the need for insulin injections as well as the risk for other bodily complications (4, 5).

1.1.2 Blood sugar-elevating hormone

The pancreas does not only produce insulin, but also other hormones, like glucagon. The alpha cells do the opposite job than the beta cells, elevate the blood sugar. Alpha cells produce proglucagon, a prohormone responsible for glucagon and many other glucagon-like peptides (6). Proglucagon is also expressed in other parts of the body, like the gut and the brain (7, 8), but the hormone profile is not the same in the different body parts because of tissue specific enzymes processing the prohormone differently. The schematic overview from Lafferty et al (9) review article illustrates perfectly how the genetic data, expressed in different body parts, end up with different proglucagon derived peptide (PGDP) profiles (Figure 3).

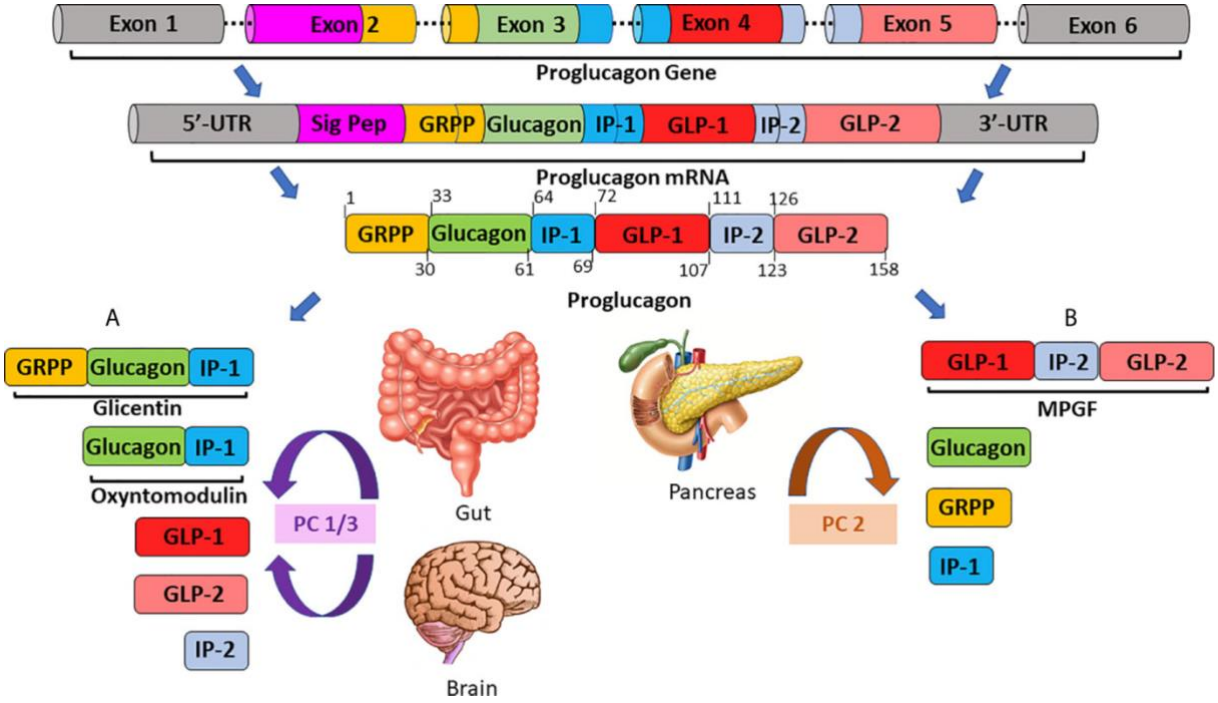

Figure 3. A schematic overview from Lafferty et al (9) of the tissue specific processing resulting in different PGDP profiles in the different body parts that express proglucagon. The proglucagon gene (GCG) is located in chromosome 2 and consists of six exons (10). After GCG is transcribed into the proglucagon messenger RNA (mRNA), the mRNA is translated into the prohormone proglucagon, consisting of 158 residues. (A) In the brain and gut, the prohormone convertase 1/3 (PC1/3) processes proglucagon into glicentin, oxyntomodulin, glucagon-like peptides-1 and -2 (GLP-1 and GLP-2) and intervening peptide-2 (IP-2). (B) The pancreas' prohormone convertase 2 (PC2) converts proglucagon into major proglucagon fragment (MPGF), glucagon, glicentin-related pancreatic polypeptide (GRPP) and intervening peptide-1 (IP-1).

1.2 CRISPR/Cas9 technology and double-strand breaks

Gene-editing was greatly advanced by the discovery of clustered regularly interspaced short palindromic repeats (CRISPR), a special type of repeated sequences of DNA in bacteria and archaea which can serve as the organisms' immune systems against foreign DNA when combined with a protein from the CRISPR-associated (Cas) nuclease family. Some bacteria and archaea respond to invading DNA by integrating short fragments of the foreign DNA into their chromosomal genome so that the sequence can be transcribed into a noncoding precursor CRISPR RNA (pre-crRNA), which will process into crRNA. The mature crRNA will, together with another noncoding RNA sequence called trans-activating CRISPR RNA (tracrRNA), sometimes referred to as the "scaffold", form a guide RNA (gRNA) (11). In the type II CRISPR/Cas system, the gRNA assembles with a CRISPR-associated protein Cas9 and together surveys the intracellular space for nucleic sequences matching those of its prior transgressors and destroys them by inducing a double-strand break (DSB) (12-15).

1.2.1 DNA repair mechanisms

In natural cycling cells, DNA replication occurs so the genomic data of the cell can be copied and divided equally between the two, new, identical daughter cells. If a DSB occurs during the

replication, the cell will seek to repair the damage in one of two ways: either by homology-directed repair (HDR) or non-homologous end joining (NHEJ) (Figure 4). HDR is possible if there is a DNA template for the repair system to recognise. If there is no template, NHEJ is the only possible outcome. Even if there is a DNA template present and both pathways can occur, NHEJ is the most frequent one to happen (16).

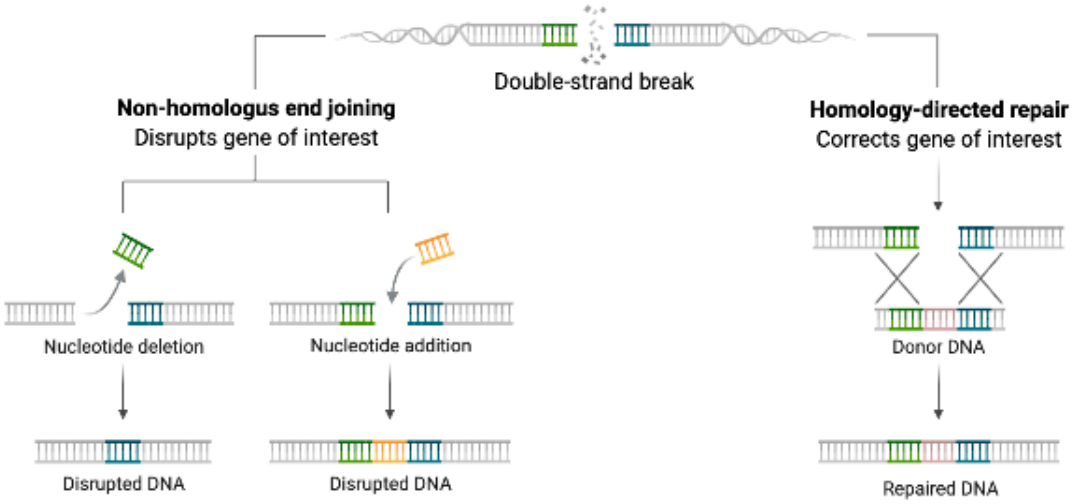

Figure 4. The two different repair pathways of a double-strand break: non-homologous end joining on the left and homology-directed repair on the right. Illustration created in BioRender.com

NHEJ is a useful DSB repair mechanism for identifying gene function or disrupting a gene's expression permanently from the genome of the cell. When a DSB occurs and NHEJ is the chosen pathway, the DNA often displays short, random sequence deletions and/or insertions, often referred to as indels. Indels can lead to frameshift mutations if the NHEJ repair happened in a coding area of the genome. When disrupted DNA leads to disruption of the reading frame and gives rise to a stop codon, the gene function is lost. The process of removing a gene and/or its function is known as knock-out. Knock-in is the process of introducing a new gene into the genome (16).

HDR is useful for genetic manipulations where a DNA template is present. By exploiting the natural repair pathway of HDR, it is possible to introduce new genetic information into the genome if the goal sequence has regions up- and downstream that are homologous to the cut region. Salsman and Dellaire report that even though NHEJ is a better option for larger knock-outs, and lacks some preciseness, NHEJ-mediated knock-ins do exist. HDR can also be used to introduce point mutations, as well as gene deletion, depending on what template is chosen

for the task (11). A DNA template donor for HDR can either be a donor vector for larger sequences or synthetic single-stranded DNA oligonucleotides (ssODN) (16).

1.2.2 Using CRISPR/Cas9

To perform gene-editing with CRISPR/Cas9 there are two requirements: the Cas9 nuclease and the gRNA. If HDR is the goal of inducing the DSB, a DNA template would be the additional third requirement. Since the crRNA is the part of the sgRNA that hones the nuclease to the correct location by RNA-DNA hybridisation, choosing a good sequence is crucial. The optimal crRNA to the target sequence is one with high specificity and low risk of off-target effects. The target sequence for a potential DSB is located upstream of a protospacer adjacent motif (PAM) sequence, which is -NGG for *Streptococcus pyogenes* (Sp)Cas9 (where N is any nucleotide) (11). The DSB only occurs after correct hybridisation between the gRNA and target sequence because it causes a conformational change in the Cas9 nuclease. The cut is generally located three bp (base pairs) upstream of the PAM site, meaning that the cut site could vary in each case, but three bp is the most common (16).

Using software, like SnapGene, allows finding suitable locations for a target sequence by searching for a PAM sequence in the genome. The 20 nucleotides on the 5' side of the PAM site will be a potential crRNA sequence (17). After finding a possible site, it is essential to analyse the area for off-target effects. Sites like CRISPOR (http://crispor.tefor.net/) will help select a sequence suitable for an experiment based on their on-target efficiency and off-target sites. The result is based on specificity scores, predicted efficiency, outcome, and off-targets for 0-1-2-3-4 mismatches (18). CRISPOR will also show the potential PAM sites in the sequence put into the programme, so finding a PAM site could also be done without SnapGene.

The gRNA consists of a target and a scaffold, the target being the mature crRNA and the scaffold being the tracrRNA. SnapGene's eBook on gRNA design mentions that a gRNA can either be a crRNA:tracrRNA duplex or a crRNA-tracrRNA chimeric RNA, often referred to as a single guide RNA (sgRNA) (17) (Figure 5).

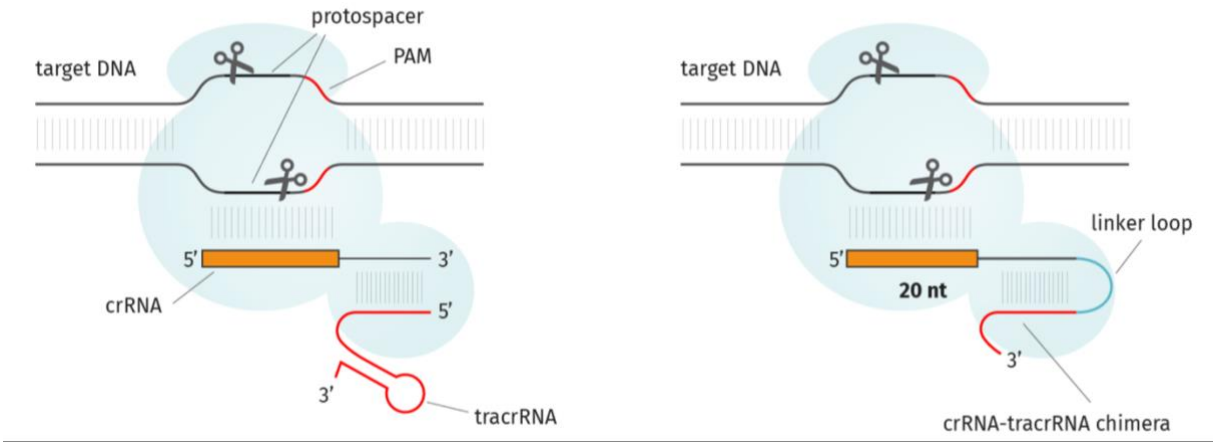

Figure 5. The two different gRNA options mentioned in SnapGene's Guide to gRNA design. To the left, a crRNA:tracrRNA duplex, and a crRNA-tracrRNA chimera to the right. The blue shadow represents the Cas9 nuclease.

Gene-editing with CRISPR/Cas9 has its limitations. For the SpCas9, the PAM site must be present 3' to the target sequence (19). The off-target effects are also an area of limitation and possible improvement when talking about the Cas9 nuclease. Off-target effects can be avoided by choosing a target site with the smallest homology possible in other places in the genome but engineering the nuclease to have less non-RNA-DNA interactions would make the nuclease more dependent on a perfect gRNA-DNA hybridisation. The dependency would in its turn improve the specificity of the nuclease and lower the risk of off-target binding and cleavage. Slaymaker et al. (20) and Kleinstiver et al. (21) were able to produce two of these engineered Cas9 nucleases, 'enhanced' Cas9 (eCas9) and 'high-fidelity' Cas9 (Cas9-HF) respectively (16).

The extensive knowledge gathered by using Cas9 variants that induce DSB were found to have high frequencies of mutagenesis in human cells (22). As CRISPR's gene-editing ability was discovered it introduces the possibility of removing certain genetic mutations in humans that lead to disease. The high frequency of mutagenesis in human cells using regular Cas9 caught the attention of researchers since this could be a dealbreaker for gene therapy use. The discovery that each of the two Cas9 nuclease subunits had domains that were homologous to the endonucleases HNH and RuvC, able to cut one DNA strand each, introduced the nickases – engineered Cas9 nucleases only able to break one strand. The nickases were produced by introducing inactivating point mutations to either the HNH or RuvC-like domains, making one of the nickases able to cut one strand, whereas the other nickase would cut the complementary strand (12) (Figure 6).

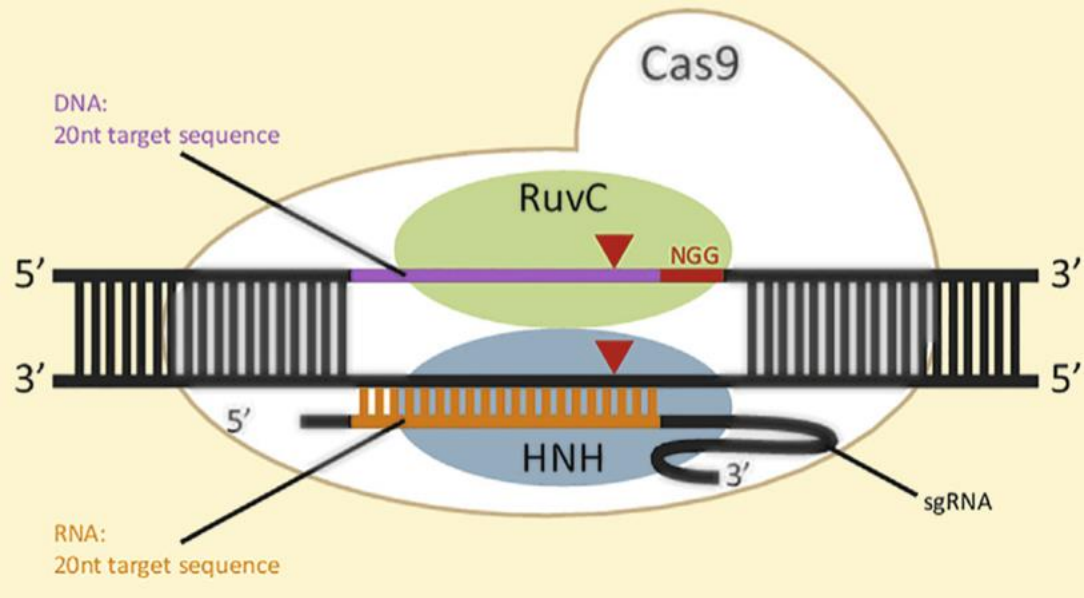

Figure 6. Cas9 nuclease bound to DNA by perfect sgRNA-DNA pairing The RuvC and HNH-like domains are visualised in green and blue respectively (16).

The nickases made possible a new way of inducing DSB. Using two different sgRNA for each of the nickases would lead them to different binding sites and make cuts resulting in long overhangs instead of the blunt ends the regular Cas9 makes. The long overhang provides greater predictability of correct insertion and integration because it decreases the risk of homology elsewhere in the genome (19, 23, 24).

As previously mentioned, the CRISPR/Cas9 system's efficiency and simplicity have made it into one of the most studied and widely used gene-editing variations in the field. The successful genome editing in *Drosophila* (25), *C. elegans* (26), zebrafish (27), mouse (28, 29), rat (30), primate (31) and human (32-34) showed that mutant allele repair was possible, and could mean reduction of disease symptoms or even fully rid an organism of a disease, if done early in the development of the organism (29). This would also mean that heritable diseases could be removed from a person's genome, making it impossible for a parent to pass it on to their offspring. Wu et al (35) recorded in 2013 that they corrected a genetic disease in a mouse with CRISPR/Cas9, making them one of the first to prove that Cas9 could help us solve the problem of genetic diseases in humans.

Liang et al. demonstrated in 2015 that CRISPR/Cas9 could be used in human embryos for genetic editing, even though their study had multiple errors, like mosaic genetic results and off-target effects (36). The study initiated ethical disagreement both in the public and among scientists on whether human germline modification should be supported or not (37). As

mentioned above, germline modification could mean ridding a human of heritable diseases, scientists fear abuse of the technology and that germline modification would rather benefit parents wanting nonmedical traits and result in “designer babies”, rather than being used in therapeutic, lifesaving situations (15). Most scientists critical of editing the human germline mention that the gene-editing tools we have today are too experimental because of their unpredictable effects on future generations. The possibility of exploiting the technology for unethical needs could hinder scientists from developing new, better techniques, like noninheritable genetic changes (38).

1.3 Fluorescence proteins as reporter genes

A DNA template donor intended for HDR in cells would benefit from being selective. One way of making cells selective is by reporter genes. The general use of reporter genes is to report the occurrence of a specific, parallel action. This means that a successful reporter gene needs to be dependent on the specific action to be expressed for the gene to be considered a reporter gene.

One type of reporter gene widely used in molecular and cellular processes is fluorescence proteins. Fluorescent proteins (FPs) originate from some marine creatures’ ability of bioluminescence and can come in a variety of different colours, like green (GFP), yellow (YFP), red (RFP) and so on. Making the FP dependent on the promoter of another gene, by, for example, making the gene of interest and the FP into one fusion protein, makes it possible to monitor gene expression by measuring the fluorescence intensity. Making the reporter into a fusion protein allows for visualisation of the expressed target protein, but also allows for localisation within the cell. Two different fusion proteins with different chromophores will allow for the visualisation of potential dynamics and interactions with each other (39).

1.4 Vector construction for Cas9 and In-Fusion cloning technology

As mentioned in the previous chapter (Using CRISPR/Cas9), CRISPR/Cas9 needs a DNA template to perform DNA repair using HDR. Larger sequence inserts require a vector plasmid (16). Depending on the goal of the gene editing, there are multiple ways of constructing your vector plasmid. If multiplex engineering is the end goal, making a guide vector with multiple sgRNA cassettes could be beneficial. Since the Cas9 nuclease can be utilised multiple times in a cell after being made, transfecting a vector with multiple sgRNA could give sufficient results. This could also require multiple DNA templates for the HDR process.

1.4.1 Combined Cas9 and sgRNA vector

In this case for single gene editing a combined vector is sufficient. Yumlu et al (16) method for gene editing with CRISPR/Cas9 in human induced pluripotent stem cells (hiPSC) shows a higher percentage of successful single gene mutagenesis using a combined vector coding for the sgRNA cassette, Cas9 nuclease alongside the fluorescent reporter. The fluorescent reporter in this case is to report on the Cas9 existence. After finding the sgRNA target sequence in the genome, a donor vector for Cas9 is required. The vector supplier Addgene (www.addgene.org) has multiple CRISPR/Cas9 expression vectors available for use. For this experiment the donor plasmid will be the plasmid pU6-(BbsI)sgRNA_CAG-Cas9-venus-bpA, which is optimised for mammalian expression (Figure 7). Using enzyme digestion, the plasmid can be cut, and the synthesised sgRNA can be designed with ends matching the sticky ends of the digested plasmid, allowing it to insert itself into the plasmid.

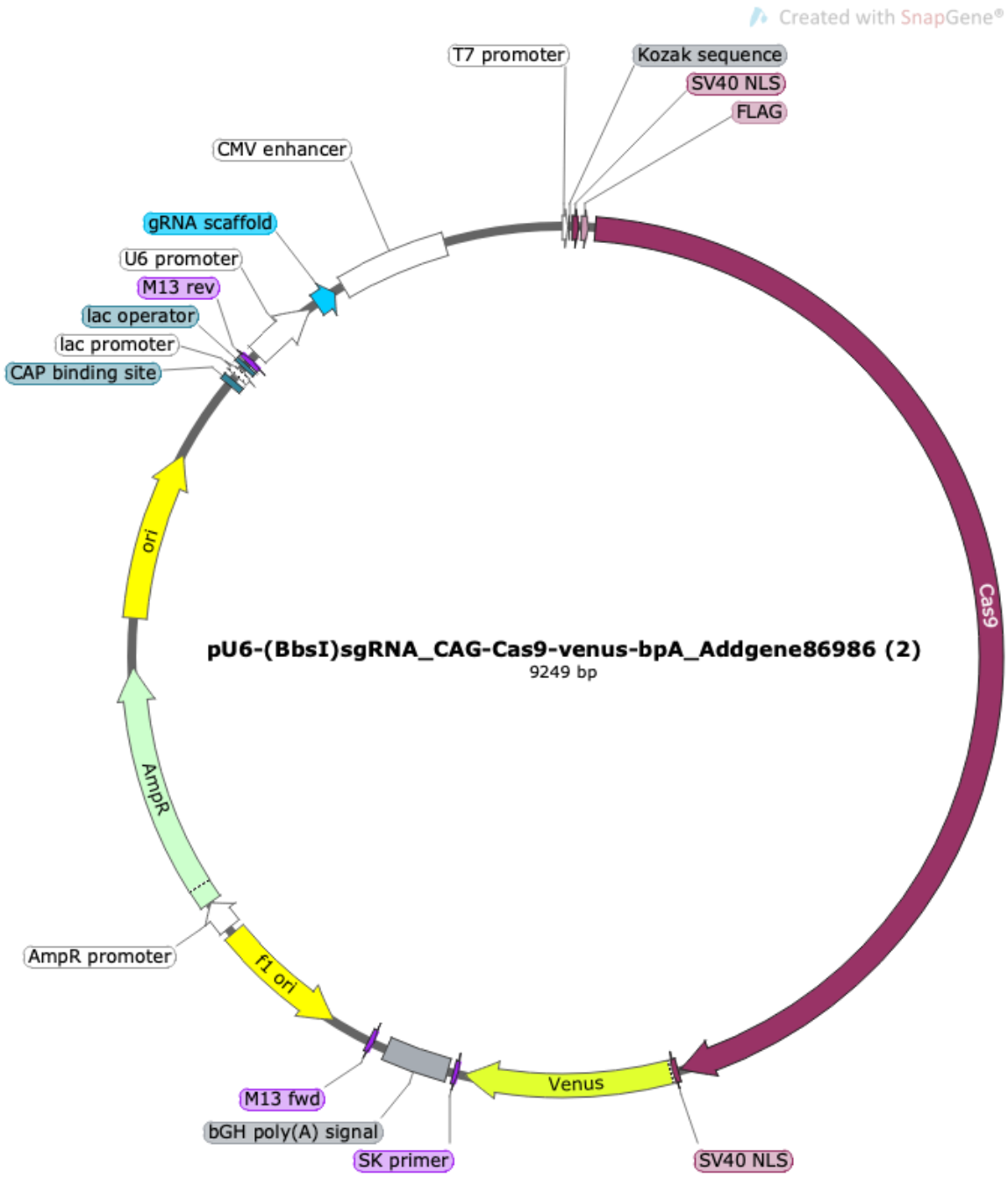

Figure 7. Map of donor plasmid that will express Cas9 as a fusion protein with the GFP variant "Venus". Plasmid map was created with SnapGene. BbsI restriction enzyme cut site is in between U6 promoter and gRNA scaffold are and will result in a sticky end.

1.4.2 Multi-fragment vector construction with In-Fusion

In-Fusion is a cloning technology developed by Takara Bio Inc. that allows for easy and effective construction of a vector with any insert desirable. As long as the insert has 15 base pairs homologous to the cut site on each side, the In-Fusion master mix will remove the 3'-prime ends, allowing the insert to anneal with the already linearised vector. In-Fusion does not require ligase or polymerase. The lack of ligase also makes it impossible for the vector to

close up on itself, minimising background. The easy to handle technology makes multi-fragment cloning a simple task, resulting in quick, customisable vectors (40) (Figure 8).

Figure 8. Takara's own illustration of streamlined multi-fragment cloning. The coloured parts represent the homologous, overlapping end that will anneal together (40).

The vector used in this experiment as a DNA template will need homologous arms with the cut site so the HDR pathway will have a chance to happen. The area we want to cut is located in the coding sequence for human proglucagon gene (GCG), therefor the homologous arms for HDR is to be found here. The donor plasmid to be used as a backbone is a PAX6 plasmid (Figure 9).

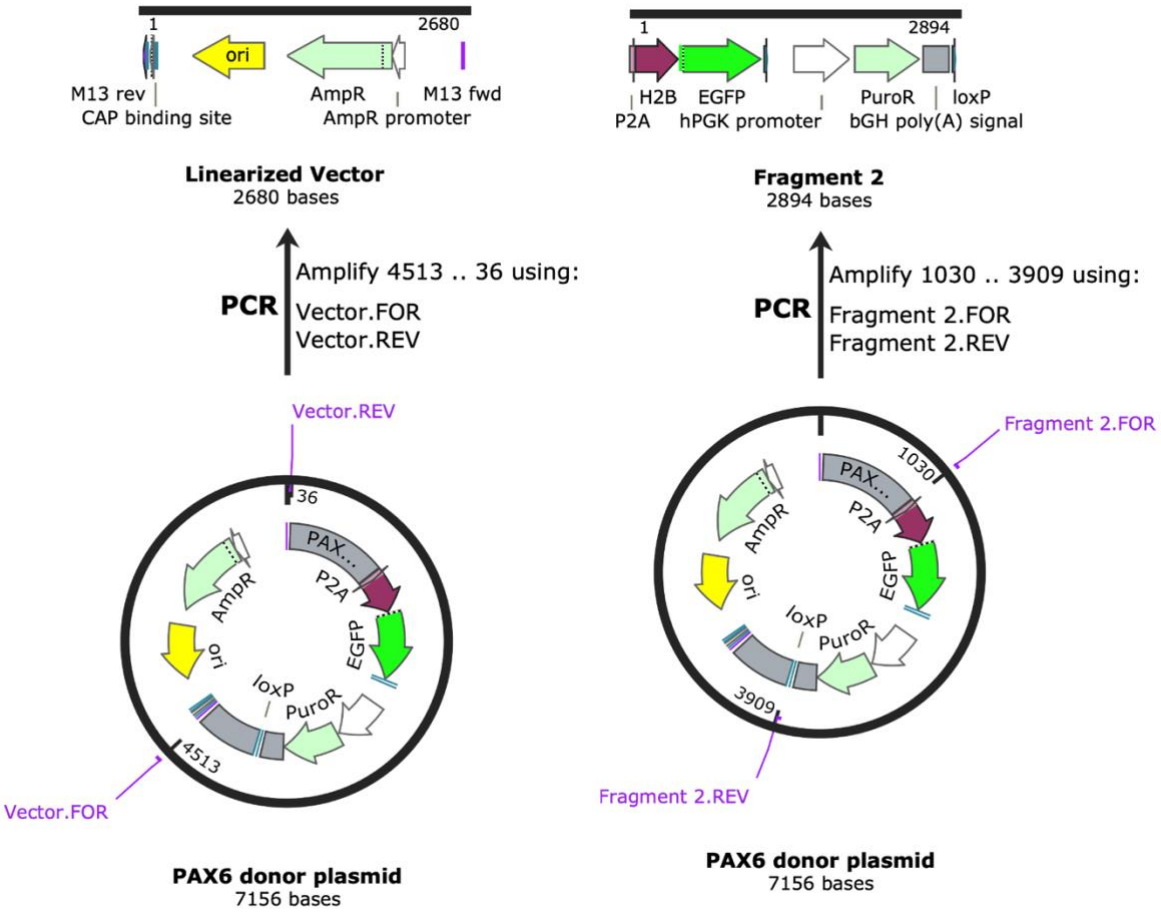

Figure 9. Linearised Vector: SnapGene map of PAX6 donor plasmid and the part that will be used as a backbone for the new plasmid. The linearised vector backbone includes ampicillin resistance and origin of replication. Fragment 2: Insertion fragment also from PAX6 plasmid. This fragment had the enhanced green fluorescent protein (EGFP) reporter gene and will need to be flipped so the translation direction is the same as the others. The purple representing the forward and reverse primers. Created in SnapGene.

The two remaining fragments are the homologous arms (Figure 10). The arms come from the human genome already isolated.

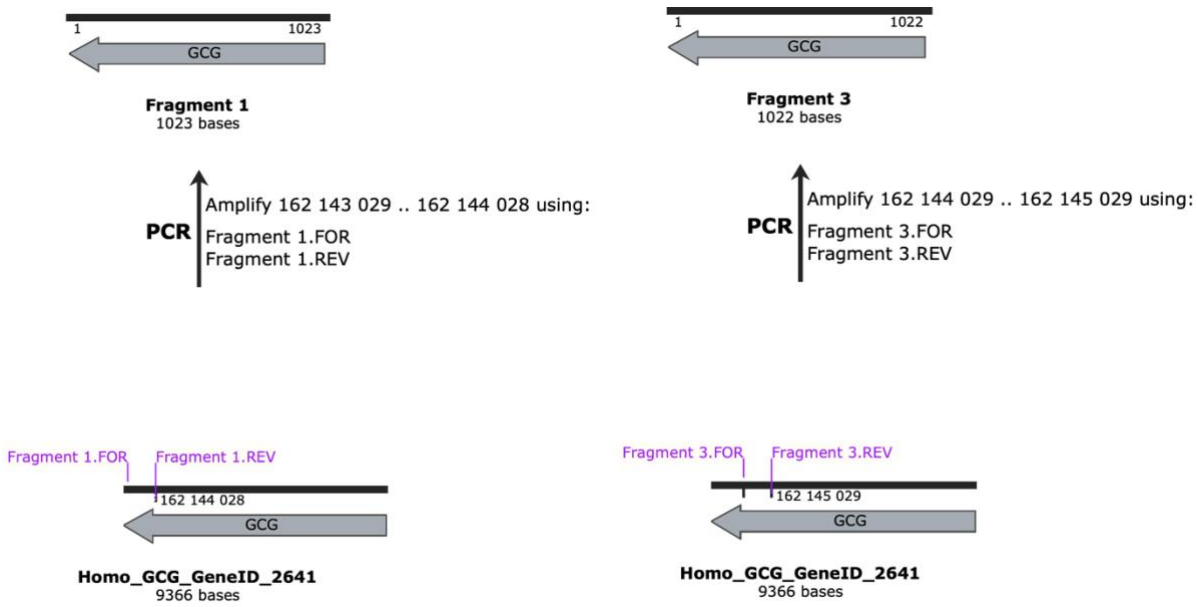

Figure 10. Fragment 1 and 3 consisting of GCG homologous arms for the multi-fragment cloning. Created in SnapGene.

Performing In-Fusion multi-fragment construction on these fragments will result in the finalised DNA donor template for our Cas9 directed DSB repair with HDR. As Figure 11 shows, all translations are made to go in the same direction after fusion. The reporter gene and puromycin resistance is in between the homologous arms, making it the insert in the DSB after repair.

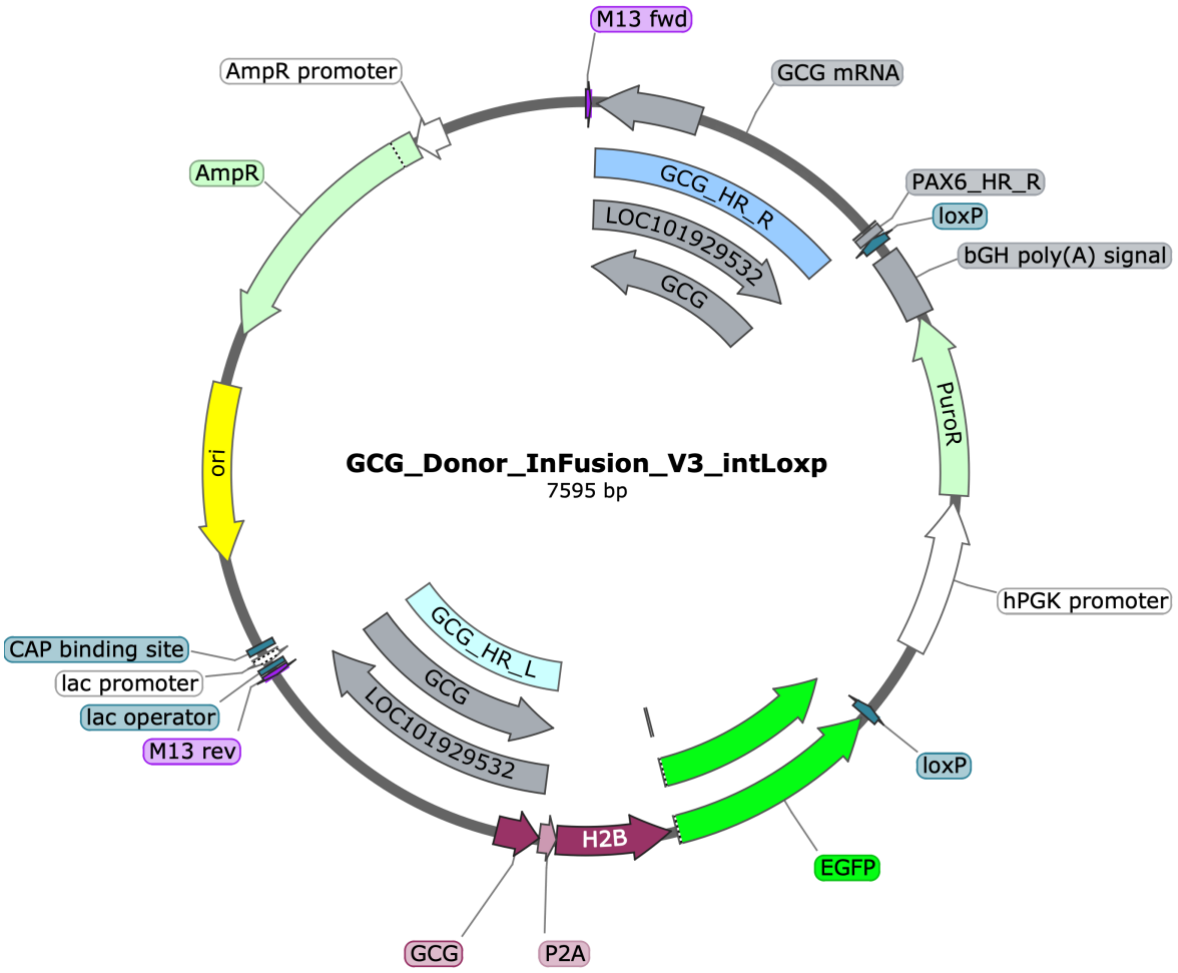

Figure 11. Finished product after In-Fusion multi-fragment cloning.

1.5 Stem cells and HEK293

The generic term "stem cell" refers to a type of cell with the ability to change into any cell of its organism. Subcategories divide stem cells into groups based on their potency. Totipotent stem cells can turn into any cell in an organism including the placenta and pluripotent stem cells (PSC) can turn into any cell in an organism excluding the placenta. Other categories like multipotent and unipotent stem cells have a lower degree of potency and can turn into different cell types in the same tissue or only one type of cell respectively (41).

1.5.1 From embryonic to induced pluripotent stem cells

Embryonic stem (ES) cells were the first type of pluripotent stem cell used in research because of their ability to divide indefinitely without losing their pluripotency (42). Therefore, ES cells derived from human blastocysts were viewed and proved useful when studying human developmental biology, drug discovery, use, and effects (43). However, the potential

ethical issues and rising controversy with using human embryos for research and testing posed a threat to further research and development. To get around the ethical issue, as well as getting cells that are more patient- and disease-specific, inducing somatic cells to express pluripotency by reprogramming them could be a viable option (44). Human induced pluripotent stem cells (hiPSC) are created by exposing somatic cells to growth factors which over time will reverse the cell's differentiated nature and reintroduce the pluripotent status (45). Inducing a patient's somatic cells to become pluripotent is helpful in researching possible drug effects on a patient and their organs without harming the patient, considering the cells can be grown in a dish.

1.5.2 Human embryonic kidney 293 cells

Mammalian cells are an important research playground for testing clinical drugs (46). The reason for using mammalian cells in research is that they produce host cells containing posttranslational modifications like those we find in humans, the most important ones being glycosylation, acetylation, sulfation, and phosphorylation (47). Mammalian cells have a high glycosylation modification compared to prokaryotes, yeast, and insect cells, making them a more ideal host cell (48).

Human cell lines would be the best cell lines to do research on for drugs meant for human use. Human embryonic kidney 293 (HEK293) is a type of human cell widely used in research. Because of their human origin, they possess the same posttranslational modifications and are able to produce proteins in a similar way that proteins are produced in humans naturally (49). HEK293 have a high growth rate, effective metabolism, are easy to transfect using common transfection reagents while upholding a high protein production (50, 51). HEK293 were made into immortalised cells by integrating 4,35 kbp of adenovirus type 5 DNA fragments into chromosome 19 by Graham around 1977, the number 293 being the experiment number the first successful HEK293 colony originated from – the 293rd experiment (52).

1.6 T7 Endonuclease I assay

When wanting to perform gene editing it is essential to confirm the activity of the cutting nuclease to measure the efficiency of the sgRNA. Documenting the activity allows for comparing the results with future attempts and improvements. The T7 Endonuclease I (T7E1) assay is one way to measure DNA cleavage and possible gene editing. As previously mentioned, after a DSB without a DNA template available the only possible DNA repair

pathway is NHEJ. With NHEJ there is a possibility of small insertions and deletions during the repair. By amplifying the wild-type (WT) DNA and the sequence of interest after sgRNA directed cutting, denaturing it and allowing it to re-anneal, there is a possibility of heteroduplex formation. The T7E1 will recognise the mismatch and cut at the site. Running the full-length WT DNA and the cut pieces along a ladder and controls, the results can be used to predict and measure the cutting efficiency of the sgRNA (53) (Figure 12).

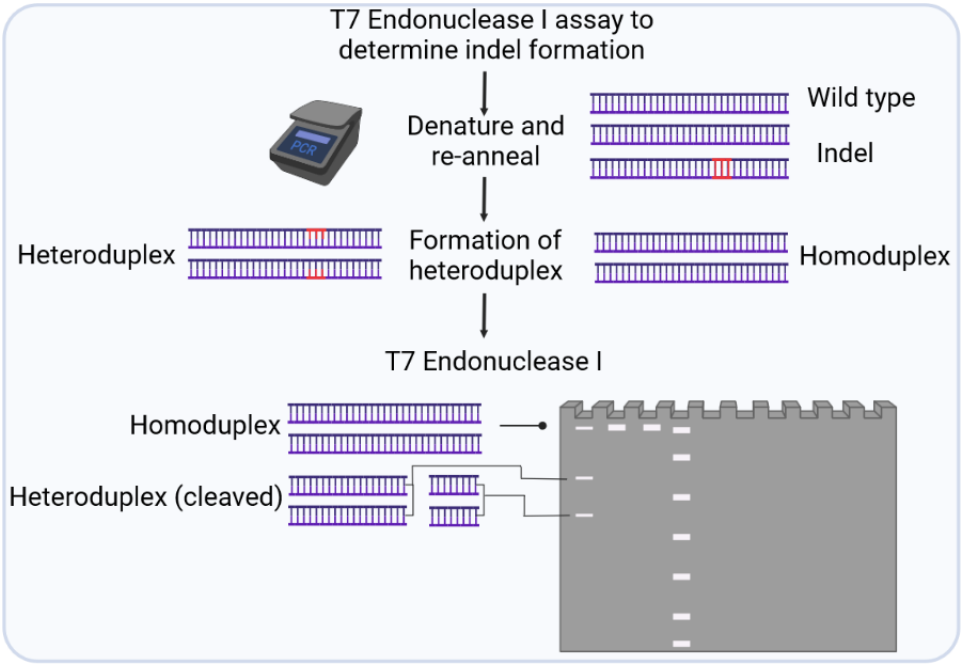

Figure 12. The wild type DNA and mutated DNA are amplified and re-anneal making heteroduplexes. The T7E1 cut at the mismatch site, resulting in a homoduplex and the cut pieces left of the heteroduplex.

Using gel quantification software, the intensity of each cut band can be used to estimate cleavage intensity using Equation 1 (19).

$$Cleaved\ PCR\ product\ (f_{cut}) = \frac{b+c}{a+b+c} \times 100\%$$

*Equation 1. Formula for fraction of PCR product cleaved. Where **a** is the intensity of the band of the uncut product, **b** and **c** are the intensity of the bands for the cut fragments.*

Based on the binominal probability distribution of duplex formation the indel occurrence can be calculated with Equation 2 (19).

$$Indel\ occurrence\ = 1 - \sqrt{1 - f_{cut}} \times 100\%$$

Equation 2. Indel occurrence formula, where f_{cut} is the product from Equation 1.

2. Materials and methods

2.1 Equipment and instruments

New Brunswick Scientific Innova 4000 Incubator Shaker

Thermo Scientific Fresco 21 Microcentrifuge

VWR Ministar Silverline

Techne Touchgene Gradient

Thermo Scientific Drybath Stdrd 2blck 200-240V

Thermo Scientific NanoDrop 2000C Spectrophotometer

Termaks Incubator B8054

Hoefer™ HE33 Mini Horizontal Agarose Electrophoresis Unit

Amersham Biosciences Electrophoresis Power Supply EPS 301

Nuair LabGard ES Class II Biosafety Cabinet

ZEISS Axiocam 202 mono

ZEISS Axio Vert.A1

ZEISS Colibri 7

Grant Instruments GD100 Thermostatic Water Bath

Labinco L46 Power Mixer

Sartorius ED822 Extend Laboratory Scale

VWR MegaStar 600

Nunc™ EasYFlask™ Cell Culture Flasks 175 cm^2, Thermo Fisher Scientific, cat.no. 159920

2.2 Kits

PrimeSTAR HS DNA Polymerase kit (54), Takara, cat.no R010A

- PrimeSTAR HS DNA Polymerase [2.5 U/µL]
- 5X PrimeSTAR Buffer (Mg^{2+} plus)
- dNTP Mixture [2.5 mM]

QIAprep® Spin Miniprep Kit, QIAGEN, cat.no. 27106

- QIAprep 2.0 Spin Columns
- Collection tubes (2 ml)
- Buffer P1
- Buffer P2
- Buffer N3*
- Buffer PB*
- Buffer PE (concentrate)
- Buffer EB
- LyseBlue®
- RNase A †

NucleoSpin® Gel and PCR Clean-Up kit, Takara, cat.no. 740609.50

- NucleoSpin® Gel and PCR Clean-Up Columns
- Collection Tubes (2 mL)
- Binding Buffer NTI
- Wash Buffer NT3 (Concentrate)*
- Elution Buffer NE**

In-Fusion® HD Cloning Kit (55), Takara, cat.no. 102518

- 5X In-Fusion HD Enzyme Premix
- Linearised pUC19 Control Vector (50 ng/µL)
- 2 kb Control Insert (40 ng/µL)

GenElute™ Mammalian Genomic DNA Miniprep Kit, Sigma-Aldrich, cat.no. G1N70-KT

- Resuspension Solution
- Lysis Solution T
- Lysis Solution C
- Column Preparation Solution
- Wash Solution Concentrate
- Elution Solution

- Proteinase K
- RNase A Solution
- GenElute™ Miniprep Binding Columns in tubes
- Collection Tubes, 2.0 mL capacity

Lipofectamine™ 3000 Transfection Reagent kit, Thermo Fisher Scientific, cat.no L3000-001

- Lipofectamine™ 3000 Reagent
- P3000TM Enhancer Reagent

DH10B Competent Cells kit, Thermo Fisher Scientific, EC0113

- DH10B Competent Cells
- pUC19 DNA (10 pg/µL)
- S.O.C. Medium

2.3 Chemicals and vectors

PAX6 donor plasmid was a gift from Lorenz Studer (56) (Addgene plasmid # 105239 ; http://n2t.net/addgene:105239 ; RRID:Addgene_105239), received in NEB Stable Competent E. coli as agar stab.

pU6-(BbsI)sgRNA_CAG-Cas9-venus-bpA was a gift from Ralf Kuehn (57) (Addgene plasmid # 86986 ; http://n2t.net/addgene:86986 ; RRID:Addgene_86986) received in DH5α Competent E. coli as agar stab.

pEGFP-C1 Vector; GenBank Accession #6084-1

pUC19 DNA, Thermo Fisher Scientific, cat.no. SD0061

Human Genome DNA Template, previously isolated from HMGUi001-A-1 hiPSC stem cell line

SeaKem ® LE Agarose, Lonza, cat.no. 50004

Human Embryonic Kidney (HEK)-293 cells, CRL-1573™, from American Type Culture Collection (ATCC)

Tris-Acetate EDTA electrophoresis buffer, produced and supplied by Oslo Universitetssykehus

LB-medium, produced and supplied by Oslo Universitetssykehus

Carbenicillin, Invitrogen, cat.no. 10177-012

99,1% ethanol, Antibac Absolutt alkohol prima, cat.no. 600068

BbsI Restriction Enzyme, New England Biolabs, cat.no. R3539S

Ethidium Bromide, Sigma-Aldrich, cat.no. E1510

6X DNA Loading Dye, Thermo Scientific, cat.no. R0611

Cut Smart® Buffer 10X, New England Biolabs, cat.no B7204S

Mass Ruler DNA Ladder High Range, Fermentos, cat.no. SM0398

O'GeneRuler Express DNA Ladder, Thermo Scientific, cat.no SM1563

Milli-Q® Purified water

RNase-Free water, QIAgen, cat.no. 1017979

5X Phusion HF Buffer, New England Biolabs, cat.no B0518S

10X T4 Ligase Buffer, New England Biolabs, cat.no B0202S

T4 Ligase, New England Biolabs, cat.no M0202S

Dulbecco's Modified Eagle's Medium-high glucose, Sigma-Aldrich, cat.no D6429

Opti-MEM™ I Reduced Serum Medium, Gibco™, cat.no 31985062

Fetal Bovine Serum, Gibco™, cat.no 10270-106

Penicillin-Streptomycin, Gibco™, cat.no 15070-063

Trypsin EDTA, Thermo Fisher Scientific, cat.no 25300054

10X NEBuffer 2, New England Biolabs, cat.no B7002S

T7 Endonuclease I enzyme, New England Biolabs, cat.no. M0302

2.4 Custom synthesised oligonucleotides ordered from Eurofins Genomics

Table 1. Primer names, 5'->3' DNA sequences and functions of custom synthesised oligonucleotides ordered from Eurofins Genomics to be used for construction of sgRNA and In-Fusion plasmids.

Custom synthesised oligonucleotides ordered from Eurofins Genomics		
Primer name	Sequence (5'->3')	Function
Fragment 1.FOR	TCGAGCTCGGTACCCTGTCAGATCGGAATAATTTACATTTACAAATTATATCATAGAAAA	Right homology arm of the glucagon gene.
Fragment 1.REV	GAAGTTATAGGTGACTGCTTTTTAGTTAATTCTGAAAACC	
Fragment 2.FOR	TCACTGACGCCACCAACTTCAGCC	Fragment containing EGFP gene.
F2-3.Rev	GTCACCTATAACTTCCTCTTAAATTCGTGGCAAAGCTTGTTGA	
Fragment 3.FOR	TGGTGGCGTCAGTGATTTTGGTCTGAATCAACC	Left homology arm of the glucagon gene.
Fragment 3.REV	GGTCGACTCTAGAGGATGTTGATAAAATGATATTCAAAGTACAAATAGGTAAAAACATCC	
Vector.FOR	CCTCTAGAGTCGACCTGCAG	Vector backbone containing ampicillin resistance.
Vector.REV	GGGTACCGAGCTCGAATTCACT	
gN20F	CACCGTTCAGACCAAAATCACTGAC	Oligo for cloning sgRNA to direct Cas9 enzyme where to cut. Contains 20 nucleotides.
gN20R	AAACGTCAGTGATTTTGGTCTGAAC	
gN21_F	CACCGCAGTCACCTGTCAGTGATTT	Oligo for cloning sgRNA to direct Cas9 enzyme where to cut. Contains 21 nucleotides.
gN21_R	AAACAAATCACTGACAGGTGACTGC	
OnT_F	TCTGTGTTTCTAGCTTCTTGACTCT	Primers for On-Target analysis of indel formation.
OnT_R	TGCAGGCTTGGTATTAGATCCCA	

Table 2. Sequencing primers used in Sanger sequencing of an In-Fusion cloned plasmid, sorted by order of appearance of the primers from the point of origin in the plasmid. The primer number is based on the name of the primers as they appear in the plasmid map.

Sequencing Primers			
Primer name	Sequence (5'->3')	Sequencing area/function	Number
Puro-F_Seq3	GCAACCTCCCCTTCTACGAGC	Puromycin resistance gene	3
hPGK_F_Seq4	GGTGTTCCGCATTCTGCAAG	Human PGK promoter	4
EGFP-C_Seq5	CATGGTCCTGCTGGAGTTCGTG	EGFP gene end	5
EGFP_N_Seq6	GAGCTGGACGGCGACGTAA	EGFP gene start	6
GCGL_Seq7	GGCATGAAATTATGCAGGCTTGGTAT	Glucagon left homology arm	7
M13rev-49	GAGCGGATAACAATTTCACACAGG	Standard Sanger primer	8
M13uni-43	AGGGTTTTCCCAGTCACGACGTT	Standard Sanger primer	1

2.5 Workflow

2.5.1 Constructing the sgRNA expression vector

Figure 13 depicts the workflow for constructing the sgRNA expression vector. Frozen bacterial stock containing the Cas9-Venus plasmid was cultured, plasmid DNA was isolated, and an agarose gel electrophoresis was performed to verify that the plasmid had been successfully isolated. The Cas9-Venus plasmid was linearised by BbsI restriction enzyme to later be able to ligate the guide sequence to the vector. The enzyme reaction containing the linearised vector was loaded on an agarose gel to confirm the correct base pair length and that linearisation was complete, and the gel band was cut out, isolated, and purified. Custom guide sequences were ordered and annealed to form a double-stranded DNA sequence, ligated into the linearised Cas9-Venus vector to create the sgRNA expression vector. This vector was transformed into competent DH10B cells, and the cells were spread onto carbenicillin containing agar plates to select for transformed cells. Clones were picked from the agar plates and cultured, and then the plasmid was isolated and purified to obtain more of the sgRNA expression vector. The sgRNA was transfected into HEK293 cells, and the genomic DNA was isolated and purified to determine the indel formation by T7 Endonuclease I assay.

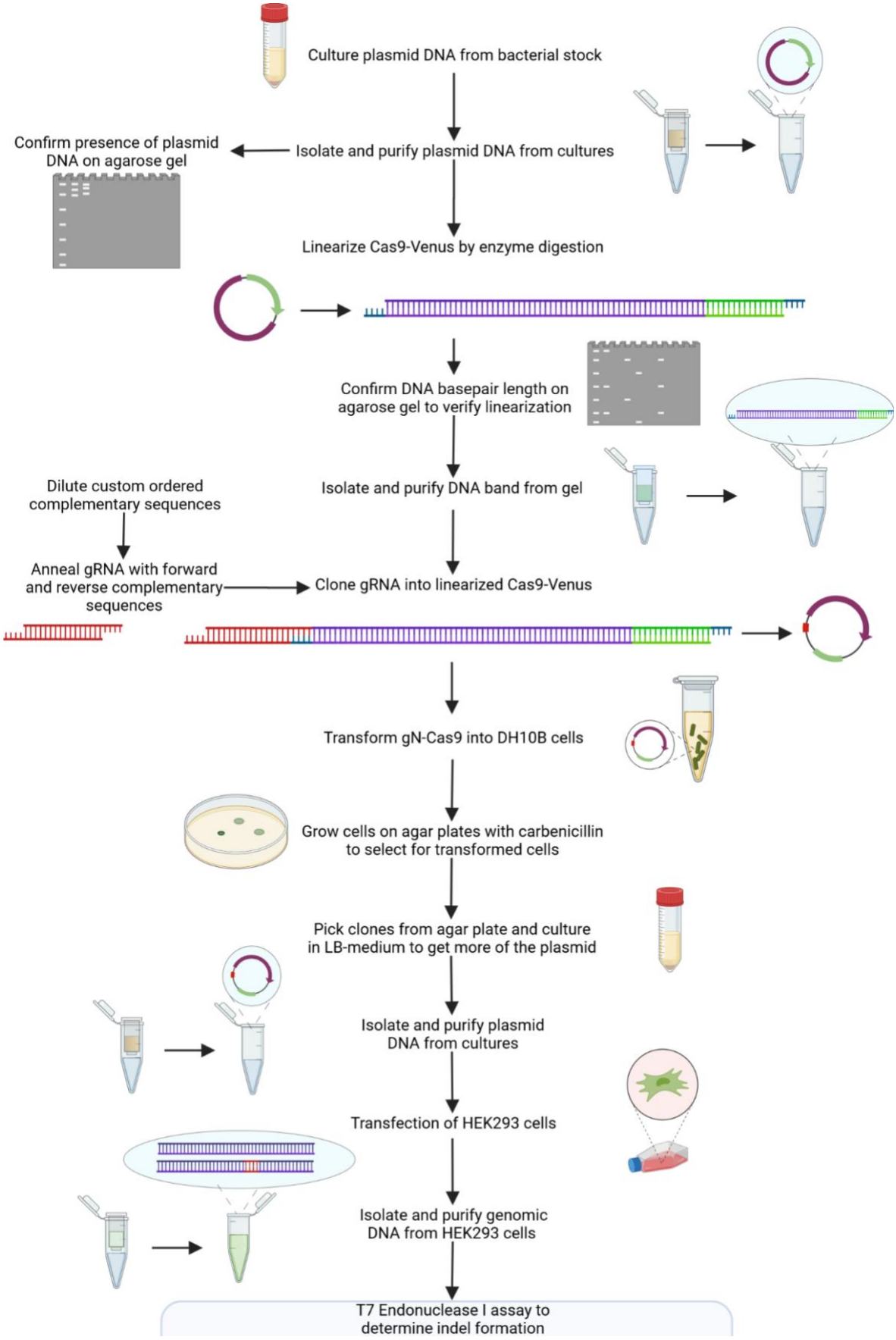

Figure 13. Workflow depicting the construction of a sgRNA-Cas9 expression vector. Created with BioRender.com

2.5.2 Constructing a template plasmid from four DNA fragments using In-Fusion cloning

Figure 14 depicts the workflow for constructing the In-Fusion plasmid containing the four fragments consisting of the right and left glucagon homology arms, insert section and linearised PAX6 donor vector backbone. Frozen bacterial stock containing the PAX6 donor vector plasmid was cultured, isolated, and purified to obtain more of the desired plasmid. An agarose gel electrophoresis was performed to verify that the plasmid had been successfully isolated. The four DNA fragments were amplified in a PCR machine together with custom ordered forward and reverse primers. The ampicillin resistance gene and the vector backbone were amplified from the PAX6 donor vector, and the left and right glucagon homology arms were amplified from the Human Genome DNA template. After PCR, the reaction mixtures were loaded in separate wells on an agarose gel to confirm the length of the fragments, and the bands were cut out from the gel and purified. The four fragments were cloned together to create via an In-Fusion cloning reaction. DH10B competent cells were transformed with the plasmid and spread onto carbenicillin containing agar plates to select for transformed cells. Transformed clones were picked from the agar plates and cultured, purified and verified by Sanger sequencing.

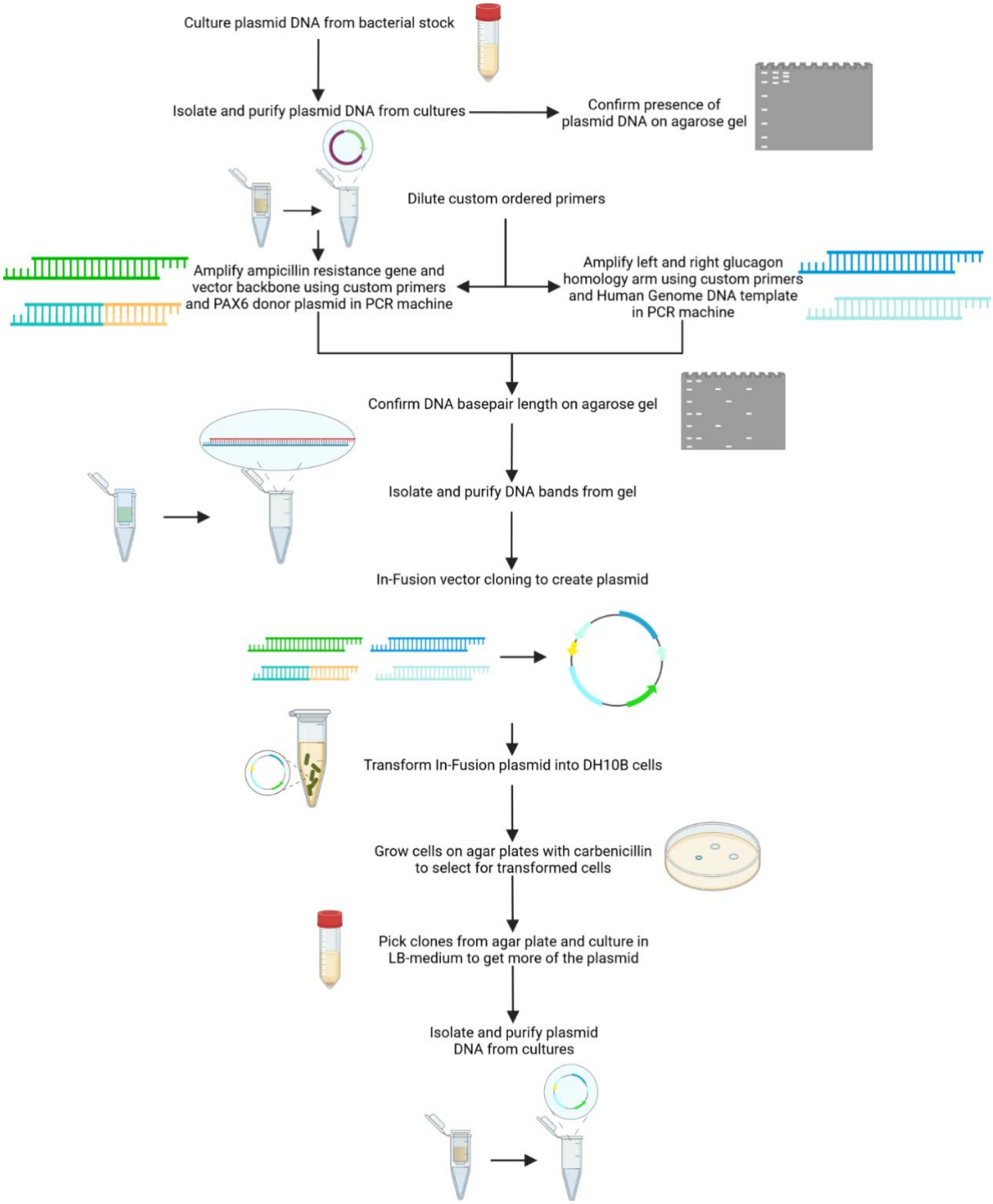

Figure 14. Workflow depicting the construction of an In-Fusion vector with four DNA fragments. Created with BioRender.com

2.6 Experimental

2.6.1 Culturing plasmid samples

To amplify plasmid DNA for further DNA isolation, four 15 mL Falcon tubes were filled with 5,0 mL of LB-medium containing 100 µg/mL of carbenicillin. These were inoculated with frozen DH5α competent *E. coli* containing Cas9-Venus (2X), and frozen NEB stable competent *E. coli* containing PAX6 donor plasmid (2X). The lids of the tubes were screwed halfway loose to allow air exchange, placed in a rotating incubator at 37 °C overnight to allow bacterial growth, and stored at 4° C.

2.6.2 Isolating plasmid DNA from bacteria

2 mL of the two Cas9-Venus samples was pipetted into two separate 2 mL microcentrifuge tubes, as were the two PAX6 donor plasmid samples. The samples were pelleted in a centrifuge at 3000 x g for 2 minutes. The supernatant was aspirated off. An additional 2 mL of each of the four samples was pipetted onto the pellets in the Eppendorf tubes, and the process was repeated. The remainder of the liquid in the Falcon tubes was pipetted onto the pellets and then centrifuged, and then the process was repeated until all bacteria were harvested.

Using the QIAprep® spin miniprep kit (58), 250 µL of Buffer P1 containing LyseBlue reagent was used to resuspend the four pelleted samples. 250 µL of Buffer P2 was then added to each of the samples, and the tubes were inverted five times to mix them thoroughly. 350 µL of Buffer N3 was then added to each of the tubes, and tubes were again inverted five times to mix them thoroughly. The samples were then centrifuged at 17900 x g for 10 minutes. The supernatant from each sample was then pipetted into their respective QIAprep spin columns, placed in centrifuge tubes, and centrifuged at 17900 x g for 30 seconds, and the flowthrough was discarded. 7,5 mL of Buffer PE was added to the spin columns, and the tubes were centrifuged at 17900 x g for 30 seconds to wash the columns, and the flowthrough was discarded. The samples were centrifuged at 17900 x g for 1 minute to remove residual wash buffer, and the flowthrough was discarded. The spin columns were placed in clean 1,5 mL centrifuge tubes, and 50 µL of MQ-water was added to the centre of the spin columns, and after 1 minute, the samples were centrifuged at 17900 x g for 1 minute to elute the DNA. The samples were analysed with a NanoDrop spectrophotometer to determine the DNA concentration.

To confirm the presence of plasmid DNA in the samples, an 0,8% agarose gel was prepared by mixing 0,42 g of agarose with 50 mL 1x TAE buffer in an Erlenmeyer flask. The mixture was warmed in a microwave oven for approximately 1 minute until all the agarose was dissolved. 4 µL of ethidium bromide was added for staining the DNA for visualisation under UV light. The flask was held under running cold water to cool it down. The mixture was poured into a mould to create the agarose gel. After the gel had solidified, 3 µL of each DNA sample was mixed with 0,5 µL of 6X DNA loading dye and added to their respective wells in the agarose gel. Electrophoresis was performed at 90 V for approximately 1 hour. The remaining DNA samples were then stored at -20 °C.

2.6.3 Linearising of Cas9-Venus plasmid

A Cas9-Venus plasmid sample was linearised by BbsI restriction enzyme digestion at restriction sites 8329 bp and 8351 bp that produced sticky ends with four base overhangs, shown in Figure 15, so it could later be used guide sequence ligation.

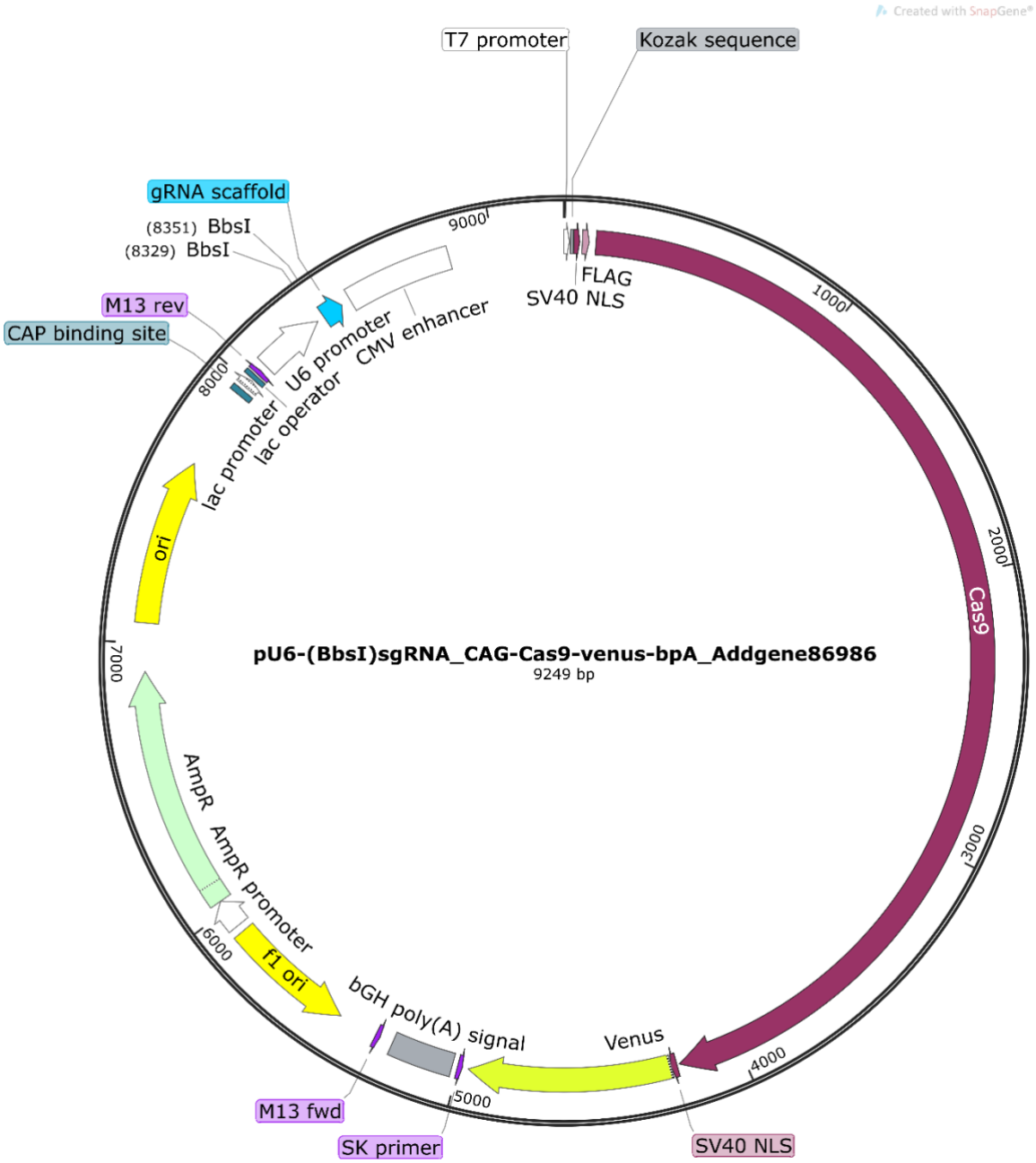

Figure 15. Plasmid map of pU6-(BbsI)sgRNA_CAG-Cas9-venus-bpA, acquired from Addgene (#86986). The plasmid was cut with restriction enzyme BbsI to create a linearised vector. Cut sites for BbsI can be seen at 8329 and 8351 bp.

3,76 µL of the Cas9-Venus sample containing 532,1 ng/µL of DNA was pipetted into a PCR tube to get approximately 2000 ng of DNA. The added volume of each reagent is shown in Table 3.

Table 3. The volume of each reagent added to a PCR tube for linearising a Cas9-Venus vector by restriction enzyme digestion with BbsI.

Cas9-Venus (2) Linearising	
Reagent	Volume [µL]
Cas9-Venus (2) sample	3,76
BbsI Restriction Enzyme	0,50
CutSmart ® Buffer 10X	2,50
Milli-Q water	18,24
Total volume:	**25**

After the addition of all the reagents, the PCR tube was centrifuged for a few seconds to collect all reagents at the bottom of the tube, and it was then incubated at 37 °C for 30 minutes to linearise the Cas9-Venus plasmid.

2.6.4 Isolating DNA from agarose gel

To ensure the plasmid was cut correctly and had the correct length, it was run on an agarose gel. Afterwards, the DNA was isolated and purified from the gel as it was suspended in the gel matrix and contained DNA loading dye and ethidium bromide, so that it could be used later in cloning the sgRNA plasmid.

5 µL of DNA loading dye was added to the PCR tube containing the linearised Cas9-Venus plasmid, and the sample was added to a well in a prepared 0,8% agarose gel. A DNA Ladder with a 1500 to 10000 bp range was added to a well in the agarose gel, and electrophoresis was performed at 90 V for approximately 1 hour. A band in the gel was visible right above 10000 bp in the well loaded with plasmid. The gel band was cut out from the agarose gel, weighed (100 mg) and placed in a 1,5 mL Eppendorf tube.

A NucleoSpin® Gel and PCR Clean-Up kit was used to purify the Cas9-Venus sample by following the “DNA extraction from agarose gels”-protocol (59).

200 µL of NTI buffer was added to the tube, and then heated to 50 °C in a heating block for 9 minutes until the gel was dissolved. The mixture was added to a NucleoSpin® spin column and centrifuged at 11000 x g for 30 seconds, and the flowthrough was discarded. 700 µL of buffer NT3 was added to the spin column, then centrifuged at 11000 x g for 30 seconds, and the flowthrough was discarded. The tube was centrifuged again at 11000 x g for 60 seconds and then placed in a heating block at 70 °C for 2,5 minutes to ensure there was no buffer left in the column and to dry the silica membrane. The spin column was placed in a clean microcentrifuge tube, and 20 µL of Milli-Q water was added to the spin column. After 1

minute, the spin column was centrifuged at 11000 x g for 60 seconds to elute the DNA into a collection tube. A NanoDrop spectrophotometer was used to determine the DNA concentration of the linearised Cas9-Venus sample.

2.6.5 Dilution of primers and complementary sequences of gRNA

Custom synthesised oligonucleotides for PCR and sequencing primers, as well as forward and reverse complementary sequences for gRNA were ordered and received as powders. RNase-Free water was added to all tubes according to the synthesis report to produce a concentration of 100 pmol/µL for all samples. 20 µL aliquots of the PCR and sequencing primers were further diluted by adding 20 µL of the stock solution to 180 µL of RNase free water to produce a 10 µM working primer solution. The gRNA forward and reverse complementary sequences were not further diluted. All primer solutions were stored at -20 °C.

2.6.6 Preparing donor fragments

Four DNA fragments consisting of the right and left glucagon homology arms, insert section containing green fluorescent protein, and linearised PAX6 donor vector backbone were prepared by amplifying the relevant sections in a PCR machine with the custom synthesised primers, to be used later for In-Fusion vector cloning.

The amplified vector backbone and GFP insert regions of the PAX6 donor plasmid can be seen in Figure 16.

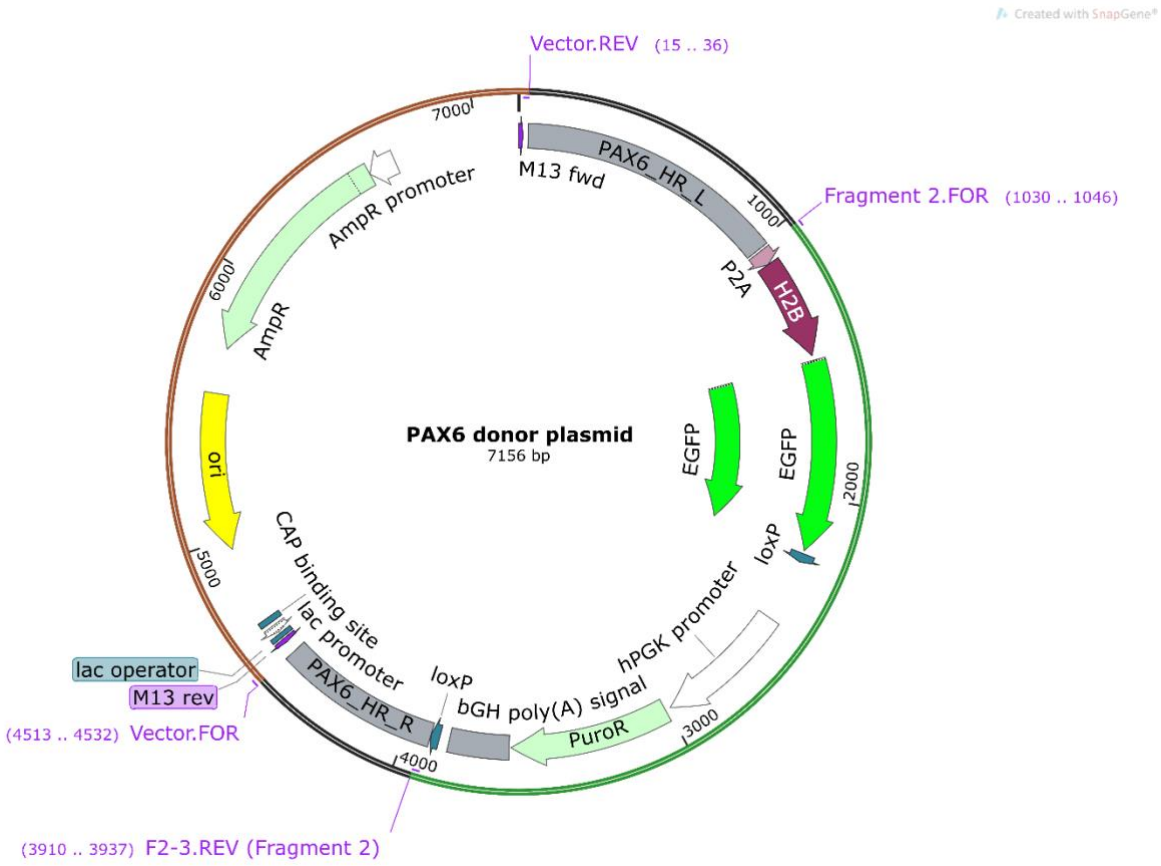

Figure 16. A PAX6 donor plasmid used to amplify two sections to produce a vector backbone and an insert section containing a green fluorescent protein reporter gene, with forward and reverse primers to indicate the segments to be amplified. The brown line between the forward and reverse primers named "Vector" was used as a vector backbone containing ampicillin resistance and the ori DNA replication genes. The green line between the forward and reverse primers for "Fragment 2" was used as a green fluorescent protein marker. Created with SnapGene

A human genome DNA template previously isolated from HMGUi001-A-1 hiPSC stem cell line containing the glucagon gene was used to amplify the homology arms, as seen in Figure 17.

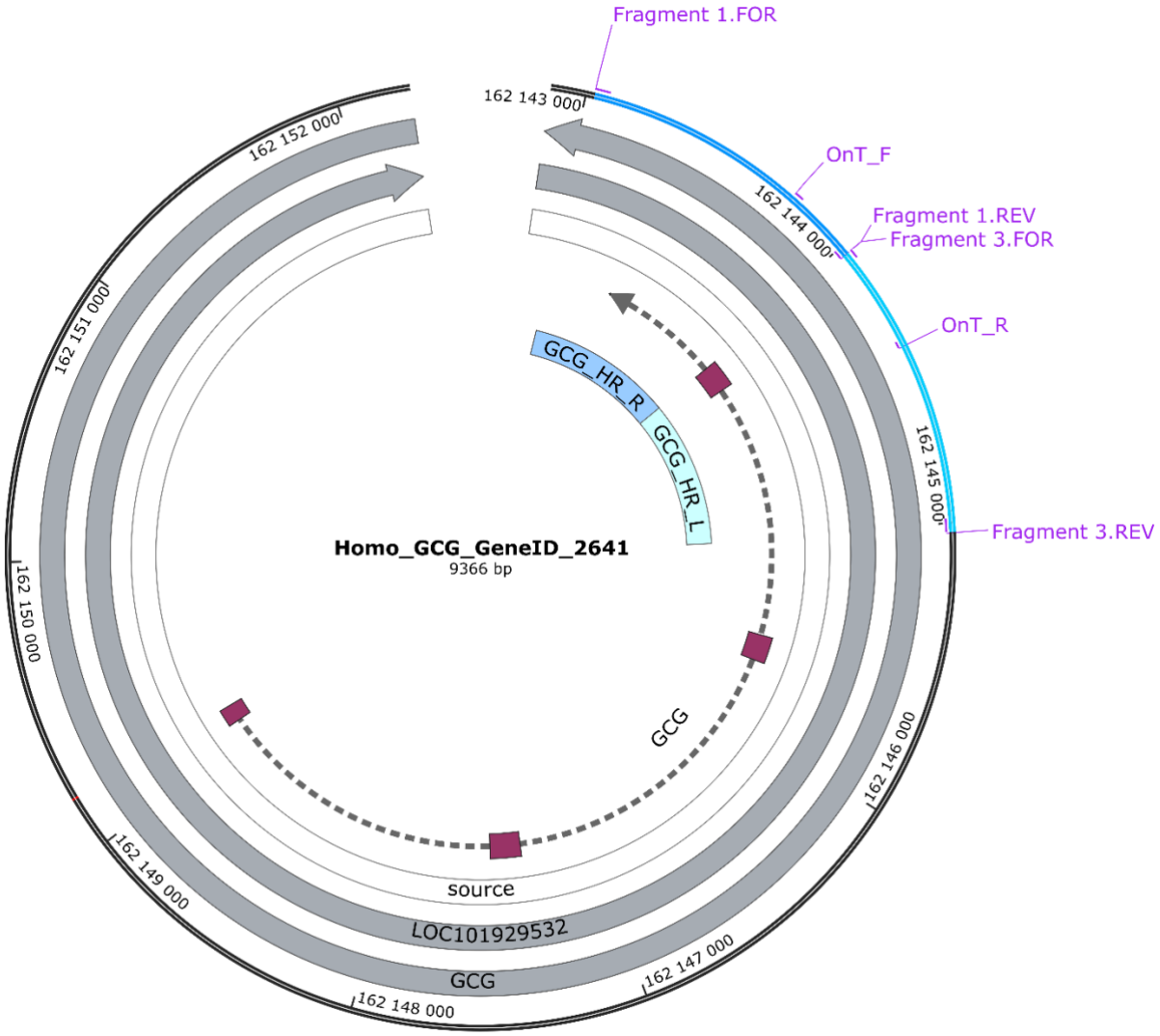

Figure 17. A 9366 bp fragment of a human genome DNA template previously isolated from HMGUi001-A-1 hiPSC stem cell line containing a left and right homology arm of the glucagon gene. The right and left homology arms were amplified in a PCR machine with the fragment 1 primers for the right homology arm, and the fragment 3 primers for the left homology arm. Also shown are On-Target (OnT) primers that were used to estimate the indel occurrence of an sgRNA. Created with SnapGene.

2.6.6.1 Fragment 1 & 3: Right and left glucagon homology arms

PrimeSTAR buffer, dNTP mixture, Human genome DNA template previously isolated from HMGUi001-A-1 hiPSC stem cell line, and PrimeSTAR HS DNA polymerase were added to two PCR tubes along with forward and reverse primers for the right and left glucagon homology arms in the amounts shown in Table 4 and Table 5 to produce two 50 µL PCR reaction mixtures.

Table 4. Volume of reagents added to a PCR tube for creating a right homology arm of the glucagon gene in a PCR reaction.

Fragment 1: GCG_HR_R		
Component	Amount (µl)	Final concentration
5X PrimeSTAR Buffer (Mg^{2+} plus)	10	1 X
dNTP Mixture (2,5 mM each)	4	200 µM each
Fragment 1.FOR (10 µM)	1	0,25 µM
Fragment 1.REV (10 µM)	1	0,25 µM
Human genome DNA template (77,6 ng/µL)	0,6	To 50 ng
PrimeSTAR HS DNA Polymerase (2,5 U/µL)	0,5	1,25 U/50 µL
ddH_2O	32,9	
Total	50	

Table 5. Volume of reagents added to a PCR a tube for creating a left homology arm of the glucagon gene in a PCR reaction.

Fragment 3: GCG_HR_L		
Component	Amount (µl)	Final concentration
5X PrimeSTAR Buffer (Mg^{2+} plus)	10	1 X
dNTP Mixture (2,5 mM each)	4	200 µM each
Fragment 3.FOR (10 µM)	1	0,25 µM
Fragment 3.REV (10 µM)	1	0,25 µM
Human genome DNA template (77,6 ng/µL)	0,6	To 50 ng
PrimeSTAR HS DNA Polymerase (2,5 U/µL)	0,5	1,25 U/50 µL
ddH_2O	32,9	
Total	50	

The reaction mixtures were placed in a PCR machine and PCR were performed using the program shown in Table 6 to amplify the DNA fragments.

Table 6. PCR program for amplifying of the right and left homology arms in a PCR reaction.

PCR Reaction Fragment 1 & 3		
Temperature	Duration	Comments
75°C	Hot start	
98°C	5 min	
98°C 60°C 72°C	10 sec 5 sec 1 min/kb (70 sec)	30 cycles
72°C	10 min	
4°C	Hold	

The finished PCR products were stored at -20 °C temporarily.

2.6.6.2 Fragment 2 & 4: Insert section and linearised PAX6 donor vector

PrimeSTAR buffer, dNTP mixture, PAX6 donor vector and PrimeSTAR HS DNA polymerase were added to two PCR tubes along with forward and reverse primers for the insert section containing the green fluorescent protein gene, and the donor vector containing the ampicillin resistance gene in the amounts shown in Table 7 and Table 8 to produce two 50 µL PCR reaction mixtures.

Table 7. Volume of reagents added to a PCR a tube for creating an insert section from the PAX6 donor vector in a PCR reaction.

Fragment 2: Insert section		
Component	Amount (µl)	Final concentration
5X PrimeSTAR Buffer (Mg^{2+} plus)	10	1 X
dNTP Mixture (2,5 mM each)	4	200 µM each
Fragment 2.FOR (10 µM)	1	0,25 µM
Fragment 2-3.REV (10 µM)	1	0,25 µM
Pax6_Donor vector (210,2 pg/µL)	0,5	To 100 pg
PrimeSTAR HS DNA Polymerase (2,5 U/µL)	0,5	1,25 U/50 µL
ddH_2O	33	
Total	50	

Table 8. Volume of reagents added to a PCR a tube for creating a linearised vector of the PAX6 donor vector in a PCR reaction.

Fragment 4: Linearised Vector		
Component	Amount (µl)	Final concentration
5X PrimeSTAR Buffer (Mg^{2+} plus)	10	1 X
dNTP Mixture (2,5 mM each)	4	200 µM each
Vector.FOR (10 µM)	1	0,25 µM
Vector.REV (10 µM)	1	0,25 µM
Pax6_Donor vector (210,2 pg/µL)	0,5	To 100 pg
PrimeSTAR HS DNA Polymerase (2,5 U/µL)	0,5	1,25 U/50 µL
ddH_2O	33	
Total	50	

The reaction mixtures were placed in a PCR machine, and PCR were performed using the program shown in Table 9 to amplify the DNA fragments.

Table 9. PCR program for amplifying of the linearised PAX6 donor vector and insert section in a PCR reaction.

PCR Reaction Fragment 2 & 4		
Temperature	Duration	Comments
75°C	Hot start	
98°C	5 min	
98°C 60°C 72°C	10 sec 5 sec 1 min/kb (3 min)	30 cycles
72°C	10 min	
4°C	Hold	

The finished PCR products were stored at -20 °C.

2.6.7 Gel electrophoresis and DNA extraction of donor fragments

Donor fragments were thawed and loaded in wells in a 0,8% agarose gel, and electrophoresis was performed for 1 hour at 90 V to confirm that the fragments produced had the expected length, and to isolate and purify the fragments of the expected length.

When DNA is loaded on an agarose gel, and a current is applied across the gel, the negatively charged DNA will travel through the gel matrix toward the cathode (positive electrode). Smaller DNA fragments will travel through the gel with lesser resistance than bigger fragments, and the fragments can be seen visually by staining the DNA with a dye prior to starting the electrophoresis. The smaller fragments will have travelled further down the gel than the larger fragments because of the lower resistance. By having a DNA ladder with known base pair lengths in another well in the gel, the fragment lengths can be accurately estimated by comparing the sample to the DNA ladder. The PCR reaction mix may contain DNA fragments that are not amplified as well as unused PCR reagents. Excising the gel band at the expected fragment length and purifying the sample can be done to isolate the desired DNA fragment.

After electrophoresis, each band containing the fragments were cut out of the agarose gel for purification following the NucleoSpin® Gel and PCR Clean-Up protocol chapter 5.2.

Each gel band corresponding to each fragment was placed in its own 1,5 mL microcentrifuge tube, and Buffer NTI was added to each tube in amounts equal to twice the gel weight. The microcentrifuge tubes were placed in a heat block at 50 °C for 10 minutes to completely dissolve the agarose gel.

Four NucleoSpin ® Gel and PCR Clean-Up Columns were placed in their own clean 2 mL collection tube, and each of the four fragment samples were loaded in their own separate column. The columns were centrifuged at 11000 x g for 30 seconds, and the flowthrough was discarded.

To wash the silica membrane in the spin columns containing the DNA fragments, 700 µL of Buffer NT3 were added to each spin column and centrifuged at 11000 x g for 30 seconds. The flowthrough was discarded, and the columns were centrifuged for an additional 1 minute at 11000 x g to ensure no residual buffer was remaining. The spin columns were then placed in a heat block for 2 minutes at 70 °C for total removal of the ethanol contained in the buffer.

The spin columns were placed in four new 1,5 mL microcentrifuge tube, and 10 µL of Milli-Q water were added to each column. After 1 minute of incubation at room temperature, the spin columns were centrifuged at 11000 x g for 1 minute to elute the DNA in each sample. 20 µL of Milli-Q water was added to each spin column, and the elution was performed once more to ensure elution of most of the DNA in the columns.

The concentration of DNA for each of the four samples was determined with NanoDrop spectrophotometry.

2.6.8 Donor vector cloning

Following the In-Fusion® HD Multiple-Insert Cloning protocol, the four fragments consisting of left and right homology arm of glucagon coding gene, linearised PAX6 donor vector and insert section were cloned into a plasmid vector.

To two PCR tubes, Fragment 1 (GCG_HR_R), Fragment 2 (Insert section), Fragment 3 (GCG_HR_L) and Fragment 4 (Linearised vector) were added in a 2:1:2:1 molecular ratio along with 5X In-Fusion HD Enzyme Premix and Milli-Q water to produce two parallel 20 µL reaction mixtures. The volume added of each reagent is shown in Table 10.

Table 10. Volume of reagents and DNA fragments added to a PCR tube for In-Fusion cloning.

Donor vector cloning		
Component	Amount (µl)	Final concentration
5X In-Fusion HD Enzyme Premix	4	1 X
Fragment 1 GCG_HR_R (6,4 ng/µL)	8,260	52,86 ng
Fragment 2 Insert section (30,7 ng/µL)	2,322	71,29 ng
Fragment 3 GCG_HR_L (16,3 ng/µL)	3,088	50,33 ng
Fragment 4 Linearised vector (41,8 ng/µL)	1,579	66 ng
ddH_2O	0,751	
Total	20,00	

A positive control consisting of enzyme premix, pUC19 control vector and a pUC19 DNA control insert was also produced using the volumes of each reagent shown in Table 11. The PCR tubes were labelled In-Fusion 1 and In-Fusion 2.

Table 11. Volume of reagents and DNA fragments added to a PCR tube to act as a positive control for In-Fusion cloning.

Donor vector cloning Positive Control		
Component	Amount (µl)	Final concentration
5X In-Fusion HD Enzyme Premix	2	1 X
Control inserts (2 kb) (40 ng/µL)	2	80 ng
pUC19 control vector (50 ng/µL)	1	50 ng
ddH_2O	5	
Total	10,00	

All three reaction mixtures were incubated for 15 minutes at 50 °C to complete the In-Fusion reaction, and then placed on ice.

The In-Fusion plasmid parallels and the positive control were transformed into competent *E. coli* cells following the DH10B Competent Cells protocol (60).

2.6.9 Transformation of In-Fusion plasmids and sgRNA into DH10B competent cells

Six Eppendorf tubes containing 50 µL of competent cells were thawed on ice. 1 µL of the two parallels of In-Fusion plasmid and the positive control were added to their own tube of competent cells. 5 µL of gN20-Cas9, gN21-Cas9, and a negative control were added to their respective tubes of DH10B cells as well. All tubes were mixed by gently flicking the tubes.

The cells were incubated for 30 minutes on ice, and then heat shocked for 30 seconds in a 42 °C water bath, and again incubated on ice for 2 minutes for recovery. 250 µL of room temperature S.O.C. medium was added to each tube, and the tubes were placed in a shaking incubator at 225 RPM for 1 hour at 37 °C. After incubation, each of the six tubes were diluted

with 200 µL of S.O.C. medium. 50 µL of each sample, as well as 250 µL of the In-Fusion plasmid samples was spread onto labelled carbenicillin containing agar plates. The plates were then inverted and placed in a 37 °C incubator overnight.

The following day, 5 colonies from each of the three plates containing the In-Fusion plasmids were picked and placed in separate Falcon tubes containing 5 mL of LB-medium with 100 µg/mL carbenicillin. The Falcon tubes were placed in a shaking incubator at 225 RPM and 37 °C overnight. After incubation, the QIAprep® Spin Miniprep kit was used to isolate the In-Fusion cloned donor plasmid DNA from all the samples. NanoDrop spectrophotometry was used to determine the DNA concentration of each sample, and 50 ng of DNA of two parallels of both the In-Fusion 1 and In-Fusion 2 were sent to Eurofins for Sanger sequencing to confirm if the cloning was successful.

2.6.10 Designing guide RNAs

To design the guide RNA, a PAM sequence was chosen near the desired cut site of an isolated section of a human DNA template containing the left and right homology arms of the glucagon gene. Isolated section of the human DNA template is previously shown in Figure 17.

The PAM sequence for the SpCas9 system is NGG, so the search function of the SnapGene software was utilised to find any NGG sequences in the human DNA template, as shown in Figure 18. A section of 20 nucleotides upstream for the two NGG sequences closest to the site between the left and right homology arms of the glucagon gene was then copied and inputted into the CRISPOR web tool.

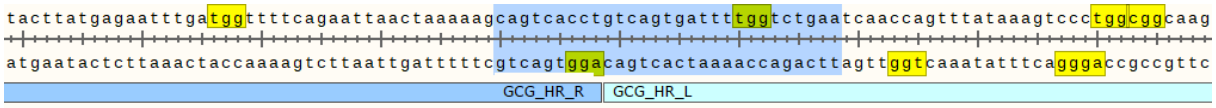

Figure 18. Section of a human genome DNA template isolated from a HMGUi001-A-1 hiPSC stem cell line containing the left and right homology arms of the glucagon gene. NGG DNA sequences are marked in yellow (N signifies any nucleotide), to identify PAM sequences for a SpCas9 system. Sequence marked in blue is a section of 20 base pairs up stream of the two closest NGG sequences to the desired cut site of the SpCas9 system. Created with SnapGene.

The CRISPOR web tool identified the two PAM sequences and provided forward and reverse complementary sequences that could be used to produce two different guide sequences when annealed. These sequences were then custom ordered as oligonucleotides from Eurofins Genomics.

Forward and reverse complementary sequences are listed in Table 1 in the 5’ to 3’ direction for all sequences.

Listed in Table 12 are the forward and reverse complementary sequences aligned to illustrate the sticky ends that were later used as ligation points with a linearised Cas9-Venus plasmid.

Table 12. Forward and reverse complementary sequences for two guide RNAs aligned to illustrate sticky ends that will form upon annealing.

				gN20																							
C	A	C	C	G	T	T	C	A	G	A	C	C	A	A	A	A	T	C	A	C	T	G	A	C			
				C	A	A	G	T	C	T	G	G	T	T	T	T	A	G	T	G	A	C	T	G	C	A	A	A

				gN21																							
C	A	C	C	G	C	A	G	T	C	A	C	C	T	G	T	C	A	G	T	G	A	T	T	T			
				C	G	T	C	A	G	T	G	G	A	C	A	G	T	C	A	C	T	A	A	A	C	A	A	A

Figure 19 shows where the BbsI restriction enzyme cuts the Cas9-Venus plasmid to create sticky ends where the annealed gRNA was later ligated.

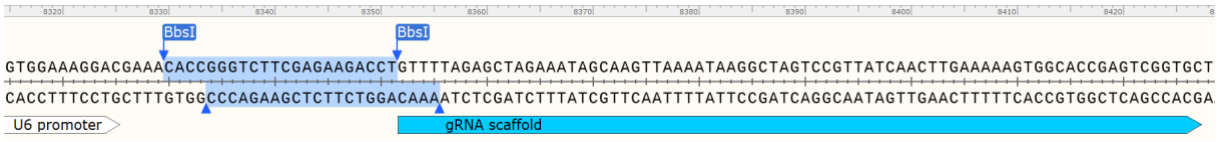

Figure 19. Zoomed in section of plasmid pU6-(BbsI)sgRNA_CAG-Cas9-venus-bpA displaying cut sites of the restriction enzyme BbsI, creating sticky ends with 4 nucleotide overhangs at 8329 and 8351 bp. Created with SnapGene

2.6.11 Preparation of sgRNA expression construct

The gRNA nucleotides gN20 and gN21 were annealed by mixing the forward and reverse complementary sequences with a 5X Phusion HF buffer and water in PCR tubes in the amounts shown in Table 13 and Table 14.

Table 13. Volume of reagents and primers added to a PCR tube for annealing of the gN20 guide RNA.

gN20 Annealing		
Component	Amount (µl)	Final concentration
gN20_F	2	10 µM
gN20_R	2	10 µM
5X NEB Phusion HF Buffer	4	1x
ddH_2O	12	
Total	20	

Table 14. Volume of reagents and primers added to a PCR tube for annealing of the gN21 guide RNA.

gN21 Annealing		
Component	Amount (µl)	Final concentration
gN21_F	2	10 µM
gN21_R	2	10 µM
5X NEB Phusion HF Buffer	4	1x
ddH_2O	12	
Total	20	

The tubes were loaded in a PCR machine, and a ramp down program as shown in Table 15 was performed to anneal the guide RNA. The products were diluted 1:200 by adding 1 µL of PCR product to 199 µL of Milli-Q water and named gN20_D and gN21_D.

Table 15. Ramp down program used to anneal gN20 and gN21 guide RNAs in a PCR machine.

Ramp down program for gRNA			
Step	Temperature	Ramp rate	Duration
Initial denaturation	95°C		10 min
Annealing	95-85°C	-2°C/second	
	85°C		1 min
	85-75°C	-0,3°C/second	
	75°C		1 min
	75-25°C	-0,3°C/second	
	25°C		1 min
	25-4°C	-0,3°C/second	
Hold	4°C		Hold

To clone the gRNAs into the linearised Cas9-Venus vector, annealed guide sequence was added together with linearised Cas9-Venus vector, T4 ligase buffer, T4 ligase, and Milli-Q water in PCR tubes in the amounts shown in Table 16 and Table 17, and briefly centrifuged to collect all liquid to the bottom of the tubes.

Table 16. Volume of reagents and DNA added to a PCR tube for cloning the gN20 guide RNA into a linearised Cas9-Venus vector.

Cloning diluted gN20 into linearised Cas9-Venus vector		
Component	Amount (µl)	Final concentration
gN20_D	2	
Cas9_Venus_Vector (Linearised) 28,5 ng/µL	3,5	to 100 ng
10X T4 Ligase buffer	2	1 x
T4 Ligase	0,3	
ddH_2O	12,2	
Total	20	

Table 17. Volume of reagents and DNA added to a PCR tube for cloning the gN21 guide RNA into a linearised Cas9-Venus vector.

Cloning diluted gN21 into linearised Cas9-Venus vector		
Component	Amount (µl)	Final concentration
gN21_D	2	
Cas9_Venus_Vector (Linearised) 28,5 ng/µL	3,5	to 100 ng
10X T4 Ligase buffer	2	1 x
T4 Ligase	0,3	
ddH_2O	12,2	
Total	20	

A sample without any gRNA or T4 ligase was also prepared to act as a negative control in the amounts shown in Table 18.

Table 18. Volume of reagents and DNA added to a PCR tube to act as a negative control when cloning the gN20 and gN21 guide RNA into a linearised Cas9-Venus vector.

Negative control		
Component	Amount (µl)	Final concentration
gRNA	0	
Cas9_Venus_Vector (Linearised) 28,5 ng/µL	3,5	to 100 ng
10X T4 Ligase buffer	2	1 x
T4 Ligase	0	
ddH_2O	14,5	
Total	20	

The ligation reactions were incubated for one hour at 22 °C, followed by inhibition at 65 °C for 10 minutes, and were then set to hold at four °C. The products were then named gN20-Cas9 and gN21-Cas9.

2.6.12 Inspecting for correct insertion of sgRNA

Five colonies from each agar plate containing sgRNA transformed cells were picked with a sterile pipette tip and placed in 5 mL of LB-medium containing 100 µg/mL of carbenicillin and placed in a shaking incubator at 37 °C overnight. After incubation, four of the tubes containing the gN20-Cas9 plasmid and two of the tubes containing gN21-Cas9 plasmid had produced viable cultures. The tubes that produced bacterial cultures were named gN20-Cas9_1 through gN20-Cas9_4 and gN21-Cas9_1 to gN21-Cas9_2.

The QIAprep® Spin Miniprep Kit was again used to isolate plasmid DNA from each of the samples.

The concentration of purified DNA was determined with a NanoDrop spectrophotometer. 50 ng of purified DNA from gN20-Cas9 _1, gN20-Cas9_2, gN21-Cas9_1 and gN21-Cas9_2 was sent to Eurofins for Sanger sequencing to confirm if the cloning was successful.

2.6.13 Cell culture

In a laminar flow cell culture hood, cell culture medium was prepared by adding 500 mL of DMEM High Glucose medium, 50 mL of Fetal Bovine Serum (FBS) along with 5 mL of Penicillin Streptomycin antibiotic to a flask. 12 mL of the medium was transferred to a T175 cm^2 EasyFlask (Nunc™).

Trypsin EDTA was used to dissociate the HEK293 to be used, split at 90% confluence upon daily observation. The cells were resuspended with 10 mL of the cell culture media to inactivate enzyme activity, followed by centrifugation at 200 x g for 3 minutes. The supernatant was aspirated off, and the pelleted cells were resuspended in 3 mL of the DMEM-FBS medium. The suspension was passaged to a T175 flask at 1:3 ratio, along with an additional 10 mL of the DMEM FBS medium in each cell culture flask. The suspension and medium were gently mixed with a cross-pattern movement on the internal surface of the flask.

The flask was placed in an incubator at 37 °C overnight to allow cell growth.

2.6.14 Transfection with Lipofectamine 3000

HEK293 cells were seeded at 1.0 * 10^5 cells/cm^2 on 24 well plates. One day after seeding the HEK293 cells, the Lipofectamine 3000 protocol (61) was followed to transfect the cells with gN20-Cas9 _1, gN21-Cas9_1 and a pEGFP-C1 Vector to act as a positive control.

To create dilutions of the Lipofectamine™ 3000 reagent, 4,5 µL Lipofectamine™ 3000 reagent and 75 µL Opti-MEM™ Medium was added two 1,5 mL centrifuge tubes, and to a third tube there was added 1,5 µL Lipofectamine™ 3000 reagent and 25 µL Opti-MEM™ Medium.

To prepare a master mix of diluted DNA, 150 µL of Opti-MEM™ Medium and 6 µL of P3000™ reagent was added to two 1,5 mL microcentrifuge tubes, marked tube 1, 2 and 3. To the third tube, there was added 50 µL of Opti-MEM™ Medium and 2 µL of P3000™ reagent. 1 µg of the gN20-Cas9 _1, gN21-Cas9_1 and positive control plasmids was added to each tube.

The diluted Lipofectamine™ 3000 reagent and diluted DNA master mixes were then mixed in their separate tubes in a 1:1 ratio, and then incubated at room temperature for 10 minutes.

50 µL of DNA-lipid complexes were added to three separate wells containing seeded HEK293 cells for the gN-Cas9 plasmids. To a seventh well, 50 µL of DNA-lipid complex containing the positive control were added. One additional well had no DNA added to act as a negative control. The well plate was placed in an incubator at 37 °C, cell media was exchanged after 24 hours, and the cells were analysed at 48 hours after transfection.

2.6.15 Isolating genomic DNA from transfected HEK293 cells

Following the Sigma-Aldrich GenElute™ Mammalian Genomic DNA Miniprep Kit protocol (62) and reagents, the sgRNA was isolated from the transfected HEK293 cells.

The HEK293 cells transfected with gN20-Cas9_1, gN21-Cas9_1 and positive control from the 24-well plate were transferred to their own 1,5 mL microcentrifuge tubes and pelleted by centrifuging at 300 x g for 5 minutes. The culture medium was removed and discarded. Each of the pellets were then resuspended in 200 µL of Resuspension Solution, and 20 µL of Proteinase K was added to each tube along with 200 µL of Lysis Solution C. The tubes were thoroughly vortexed and placed in a heat block to incubate at 70 °C for 10 minutes to lyse the cells.

500 µL of Column Preparation Solution was added to seven pre-assembled GenElute™ Miniprep Binding Columns, and the columns were centrifuged at 12000 x g for 1 minute. The flowthrough was then discarded.

After incubation of the lysate, 200 µL of 95% ethanol was added to each of the tubes and then mixed thoroughly by vortexing. The contents of the tubes were then transferred to their own treated binding column and centrifuged at 6500 x g for 1 minute. The collection tubes with the flowthrough were discarded, and the columns were placed in new 2 mL collection tubes.

To wash the columns containing DNA, 500 µL of diluted Wash Solution was added to each column and then centrifuged at 6500 x g for 1 minute. The collection tube containing the flowthrough was discarded and the columns were placed in new 2 mL collection tubes. 500 µL of diluted Wash Solution were added to each column, and then centrifuged at 16000 x g for 3 minutes to further wash the binding columns. The collection tubes with flowthrough were discarded, and the binding columns were then placed in new 2 mL collection tubes.

To elute the genomic DNA, 200 µL of Elution Solution was added to the centre of each binding column, and after incubating for 5 minutes at room temperature, they were centrifuged at 6500 x g for 1 minute. A second elution was performed by adding 200 µL of Elution Solution to each column, and again letting the tubes incubate at room temperature for 5 minutes before centrifuging at 6500 x g for 1 minute. NanoDrop spectrophotometry was performed on each of the seven samples to determine the DNA concentration.

2.6.16 Determining Genome Target Efficiency by T7 Endonuclease I assay

A T7 Endonuclease I assay was performed in order to evaluate the gene editing efficiency of the sgRNA at the cut site. The genomic DNA of transfected cells were isolated, and an area of 691 base pairs spanning approximately one third of the left and right homology arms of the glucagon with the target cut area in the centre was amplified with PCR. The On-Target amplified area are shown in Figure 17. The PCR products were denatured and annealed in a PCR machine to form heteroduplexes between wild type and mutated DNA. T7 Endonuclease I was added to annealed product to cleave any formed heteroduplexes at the point of mismatch, and agarose gel electrophoresis was performed to separate cleaved products. Imaging software was used to estimate the cleavage intensity, which in turn was used to estimate the indel occurrence.

PrimeSTAR buffer, dNTP mixture, PrimeSTAR HS DNA polymerase, forward and reverse On-Target PCR primers were added in the amounts shown in Table 19 to seven PCR tubes, along with 10 ng of genomic DNA extracted from transfected HEK293 cells with enough Milli-Q water to bring the total reaction volume to 50 µL. Table 20 shows the volume of isolated genomic DNA and Milli-Q water added to achieve 10 ng of genomic DNA added and the volume of water required to reach 50 µL of reaction volume.

Table 19. Volume of reagents, primers and DNA added to a PCR tube for amplifying the on-target area to determine genome target efficiency.

Determining genome target efficiency		
Component	Amount (µl)	Final concentration
5X PrimeSTAR Buffer (Mg^{2+} plus)	10	1 X
dNTP Mixture (2,5 mM each)	4	200 µM each
OnT_L (10 µM)	1	0,25 µM
OnT_R (10 µM)	1	0,25 µM
Genomic DNA (gN20, gN21, Negative control)		10 ng
PrimeSTAR HS DNA Polymerase (2,5 U/µL)	0,5	1,25 U/50 µL
ddH_2O		
Total	50	

Table 20. Amount of genomic DNA isolated from HEK293 cells, with volumes used in the PCR reactions for each sample in table 19.

Genomic DNA isolated from HEK293 cells		
Sample (Concentration)	µL for 10 ng DNA	µL ddH_2O for 50 µL
gN20-Cas9_1 (25,4 ng/µL)	0,394	33,106
gN20-Cas9_2 (29,6 ng/µL)	0,338	33,162
gN20-Cas9_3 (28,3 ng/µL)	0,353	33,147
gN21-Cas9_1 (27,3 ng/µL)	0,366	33,134
gN21-Cas9_2 (28,8 ng/µL)	0,347	33,153
gN21-Cas9_3 (19,5 ng/µL)	0,513	32,987
Negative control	0,219	33,281

The seven PCR tubes containing the reaction mixtures were placed in a PCR machine, and PCR were performed according to the program displayed in Table 21.

Table 21. PCR program for amplifying of the target area of genomic DNA in a PCR reaction.

Determining genome target efficiency		
Temperature	Duration	Comments
75°C	Hot start	
98°C	5 min	
98°C 60°C 72°C	10 sec 5 sec 1 min/kb (45 sec)	30 cycles
72°C	10 min	
4°C	Hold	

5 µL of each PCR products was placed in its own well in a 1,6 % agarose gel alongside a DNA ladder, and electrophoresis was performed for 1 hour at 90 V to confirm that DNA fragments of the expected length had been amplified. The bands in the gel were cut out and purified

following the NucleoSpin® Gel and PCR Clean-Up protocol chapter 5.2, eluting the DNA in the final step using 20 µL of Milli-Q water. NanoDrop spectrophotometry were performed on each of the seven samples to determine the concentration of DNA.

Seven annealing reaction mixtures were prepared by adding 2 µL of 10X NEBuffer 2 to each of seven PCR tubes, along with the amount of DNA purified from gel as shown in Table 22 and enough Milli-Q water to bring the total reaction volume to 20 µL.

Table 22. Amount and volume of DNA added to an annealing reaction of seven samples.

Annealing reaction			
Sample (Concentration)	Volume added [µL]	ddH_2O added [µL]	DNA amount [ng]
gN20-Cas9_1 (19,8 ng/µL)	10,1	6,9	200
gN20-Cas9_2 (9,5 ng/µL)	14	3	133
gN20-Cas9_3 (6,9 ng/µL)	14	3	96,6
gN21-Cas9_1 (10,1 ng/µL)	12,5	4,5	126
gN21-Cas9_2 (10,8 ng/µL)	12,5	4,5	135
gN21-Cas9_3 (25,9 ng/µL)	7,7	9,3	199
Negative control (12,9 ng/µL)	14,04	2,96	181

The PCR products were placed in a PCR machine with an annealing program as shown in Table 23 to denature the DNA strands and allowing them to hybridise again.

Table 23. PCR ramp down program for annealing and hybridisation of samples to determine off-target effects.

Hybridisation			
Step	Temperature	Ramp rate	Duration
Initial denaturation	95°C		5 min
Annealing	95-85°C	-2°C/second	
	85-25°C	-0,1°C/second	
Hold	4°C		Hold

1 µL of T7 Endonuclease I was added to each of the seven PCR tubes containing the hybridised products to cleave any formed heteroduplexes. The reactions were incubated at 37 °C for 15 minutes, and then stopped by adding 1,5 µL of 0.25 M EDTA to each tube.

10 µL of each reaction were added to their own well in a 2% agarose gel, and electrophoresis were performed for 45 minutes at 90 V. Photos were taken of the gel under UV light, and imaging software were used to estimate the cleavage intensity of the T7 Endonuclease I. The cleavage intensity was then used to estimate the indel occurrence of our guideRNA-Cas9 complex.

3. Results

3.1 Isolating plasmid DNA from bacteria

After isolation of DNA from two replicates each of the Cas9-Venus plasmid and PAX6 donor plasmid from bacteria cells cultured from bacterial stocks, the samples were analysed with NanoDrop spectrophotometry to determine the DNA concentration of each sample as well as their purity. Table 24 shows the measured concentration and the ratio of absorbance at 260 nm and 280 nm. The absorbance ratio indicates that the DNA samples were pure, since values obtained were close to 1,8, which is generally accepted as pure DNA.

Table 24. Concentration and 260/280 ratio of isolated and purified samples of a Cas9-Venus plasmid and a PAX6 donor vector plasmid measured by NanoDrop spectrophotometry.

NanoDrop measurements		
Sample	Concentration [ng/µL]	260/280
Cas9-Venus (1)	429,8	1,85
Cas9-Venus (2)	532,1	1,87
PAX6 Donor plasmid (1)	141,8	1,87
PAX6 Donor plasmid (2)	210,2	1,86

200 ng DNA of each DNA sample was loaded in a 0,8% agarose gel. Figure 20 shows the four samples along with a 1,5 – 10 kb DNA ladder. However, the plasmids are not cut, therefore still holding on to their secondary structures and that in turn makes their resistance through the gel greater. This means that they have not travelled as far as their linearised versions would have done, and the distance travelled does not reflect the plasmids size. Uncut plasmid has several conformations including relaxed, supercoiled and nicked, which were all visible on the gel (3 bands).

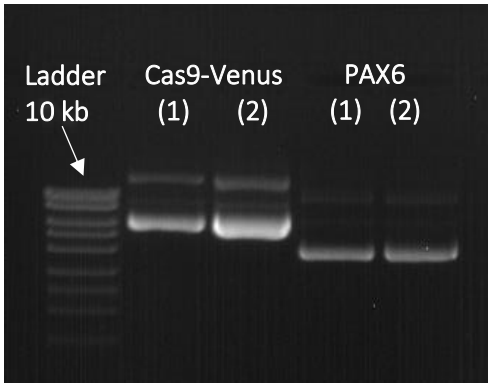

Figure 20. Agarose gel depicting a 1,5 – 10 kb DNA ladder and two parallels each of a Cas9-Venus plasmid and a PAX6 Donor vector plasmid. The plasmids are not cut, and the ladder are not representative for length of the DNA.

3.2 Linearised Cas9-Venus

Linearisation of the Cas9-Venus plasmid replicate labelled (2) was performed with a BbsI restriction enzyme reaction, and the entire reaction volume was added to a 0,8% agarose gel along with a 1,5 – 10 kb DNA ladder. The agarose gel electrophoresis confirmed that the Cas9-Venus plasmid had been correctly cut and that the fragment had the expected base pair length of just over 10 kb as shown in Figure 21.

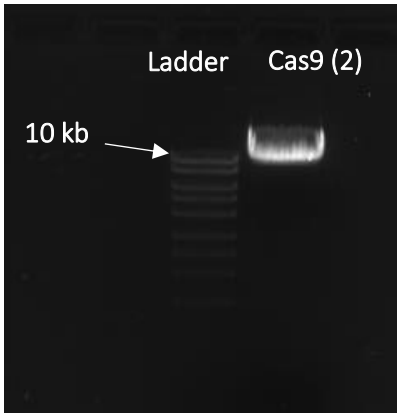

Figure 21. Agarose gel depicting a 1,5 – 10 kb DNA ladder and a linearised Cas9-Venus plasmid. The topmost band in the DNA ladder represents a bp length of 10 kb.

The gel band was cut out and purified, and NanoDrop spectrophotometry determined that the DNA concentration was 28,5 ng/µL, and the sample had a 260/280 ratio of 1,83, indicating a pure DNA sample.

Two gRNA, named gN20 and gN21, was annealed and ligated into the linearised vector to produce two sgRNA construct vectors: gN20-Cas9 and gN21-Cas9.

3.3 CRISPOR scores for gRNA

The CRISPOR web tool that provided the primers for designing the gRNA also provided specificity scores, predicted efficiency, outcomes and off-target counts for the gRNAs, as shown in Table 25.

Table 25. Scores predicted by the CRISPOR web tool for two guide sequences used in producing an sgRNA. All scores range from 0-100 except for the off-target count. The table displays specificity scores ranging from 0 – 100, where a higher value means lower likelihood of off-target effects. Predicted efficiency scores range from 0 – 100, where a higher score indicates a higher likelihood of cleavage at the specified position. Out-of-frame deletions percentage and probability of a frameshift caused by indels ranges from 0 – 100. The total off-target mismatches are a numerical value and not a range.

Guide RNA, sequence and protospacer adjacent motif		Specificity score		Predicted Efficiency		Outcome		Off-target
gRNA	Guide Sequence + PAM	MIT	CFD	Doench '16	Moreno-Mateos	Out-of-Frame	Lindel	Count
gN20	TTCAGACCAAAATCACTGAC +AGG	74	86	59	39	79	91	171
gN21	CAGTCACCTGTCAGTGATTT +TGG	72	88	28	50	61	69	180

According to the CRISPOR user manual, the specificity scores range from 0 – 100, where a higher number means lower likelihood of an off-target effect. The MIT score measures the uniqueness of the guide sequence in the genome (Human in this case). The CFD score behaves like the MIT score, but correlates better with the total off-target cleavage fraction of a guide.

The predicted efficiency also ranges from 0 – 100, where a higher number indicates higher likelihood of cleavage at the specified position. The CRISPOR user manual recommends the Doench ’16 for guides expressed in cells with U6 promoter, and the Moreno-Mateos is recommended for guides transcribed in-vitro with T7 promoter.

Percentage of clones carrying Out-of-Frame deletions, and probability of a frameshift caused by indels are predicted in the outcome columns. The CRISPOR tool also predicted a total of 171 and 180 off-target mismatches for gN20 and gN21 respectively.

3.4 sgRNA plasmid analysis

The sgRNA construct vectors were transformed into competent DH10B cells and cultured, then spread onto carbenicillin containing agar plates.

Four colonies of competent DH10B cells transformed with the gN20-Cas9 construct vector, and two colonies of competent DH10B cells transformed with the gN21-Cas9 construct vector were picked and cultured, and plasmid DNA was isolated from all the samples.

NanoDrop spectrophotometry was performed on each sample to determine the DNA concentration and 260/280 ratio as shown in Table 26.

Table 26. Concentration and 260/280 ratio of isolated plasmid DNA from transformed DH10B cells and purified samples of six guideRNA-Cas9 complexes measured by NanoDrop spectrophotometry.

NanoDrop measurements		
Sample	Concentration [ng/µL]	260/280
gN20-Cas9_1	589,5	1,87
gN20-Cas9_2	585,1	1,86
gN20-Cas9_3	595,8	1,87
gN20-Cas9_4	567,3	1,87
gN21-Cas9_1	660,8	1,85
gN21-Cas9_2	567,4	1,87

gN20-Cas9_1, gN20-Cas9_2, gN21-Cas9_1 and gN21-Cas9_2 plasmid samples were sent to Eurofins for Sanger sequencing.

Sequencing results were compared to the sgRNA-Cas9-Venus complex, shown in Figure 22 and Figure 23, in the SnapGene software.

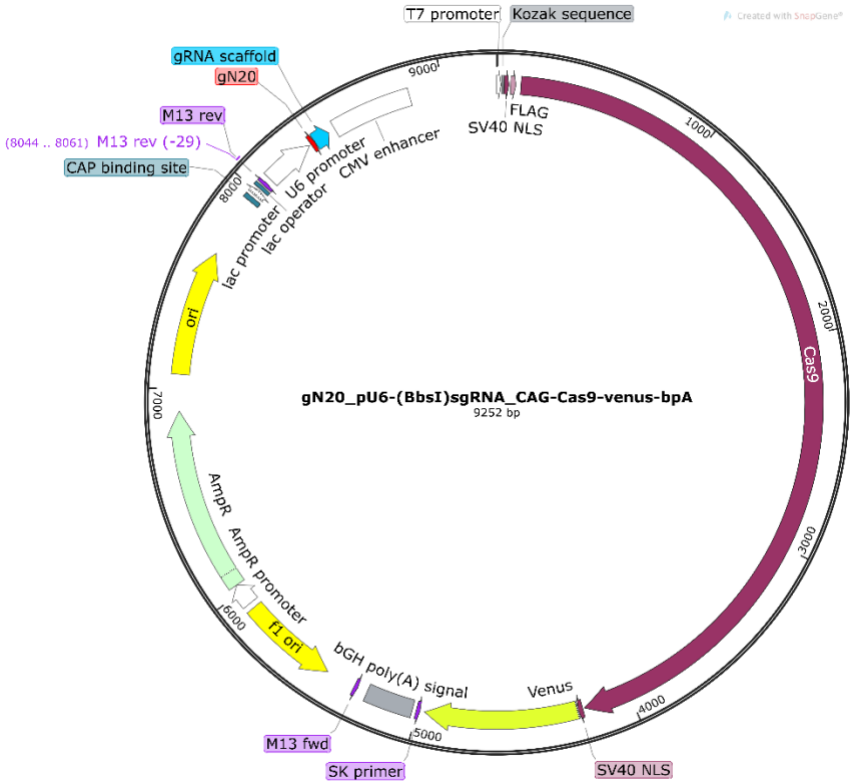

Figure 22. Plasmid map of an sgRNA constructed with a custom gN20 guide RNA and a pU6-(BbsI)sgRNA_CAG-Cas9-venus-bpA Cas9-Venus plasmid. Created with SnapGene.

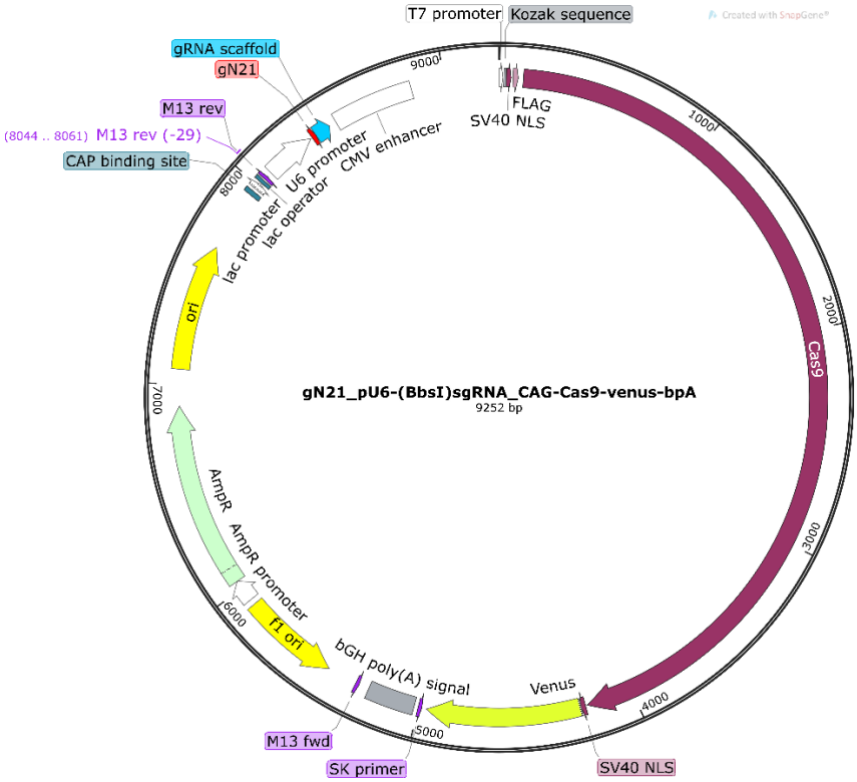

Figure 23. Plasmid map of an sgRNA constructed with a custom gN21 guide RNA and a pU6-(BbsI)sgRNA_CAG-Cas9-venus-bpA Cas9-Venus plasmid. Created with SnapGene.

The SnapGene software outlined zero mismatches and two gaps/insertions in the gN20-Cas9_1 sample. The gN20-Cas9-2 had one mismatch and one gap/insertion.

One of the gaps in the gN20-Cas9_1 sample was one bp wide and occurred at 8099 bp of the original sequence, but the gN20-Cas9_2 sample did not have a gap at this location as shown in Figure 24.

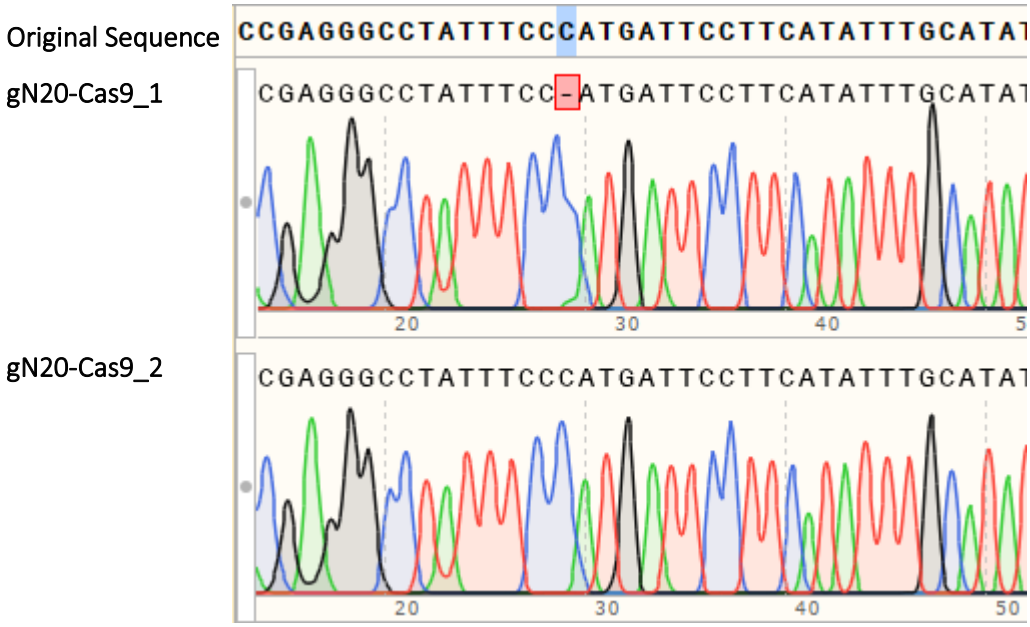

Figure 24. Chromatogram of two sections of Sanger sequenced plasmids compared to the original sequence of the plasmid. gN20-Cas9_1 shows one base pair gap in the sequencing. gN20-Cas9_2 is sequenced correctly. Created with SnapGene.

Both gN20-Cas9 samples had an insertion between base pair 8845 and 8846 in the plasmid. gN20-Cas9_1 had a 178 bp length insertion, while gN20-Cas9_2 had a 217 bp length insertion as shown in Figure 25.

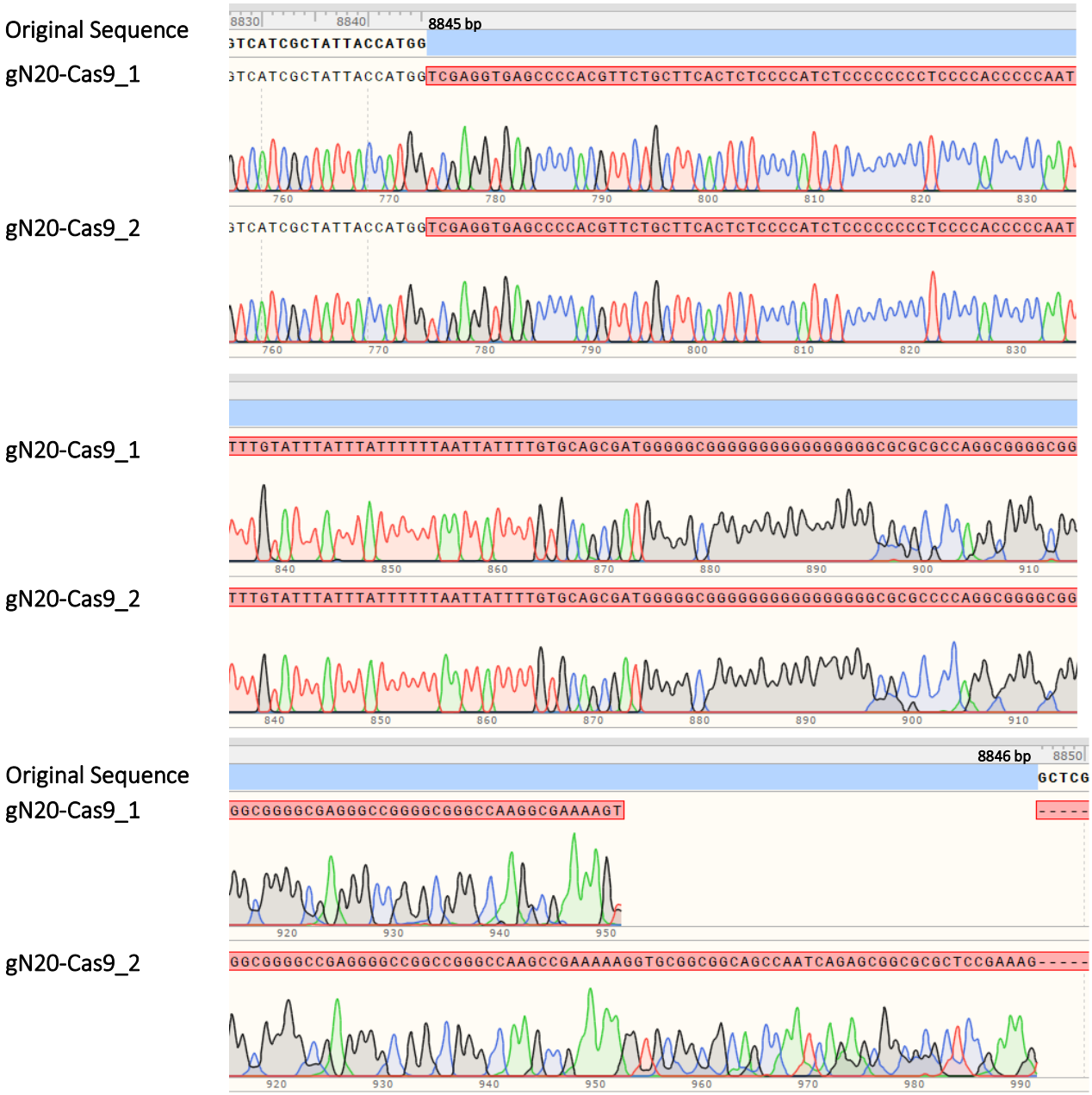

Figure 25. Chromatogram of two sections of Sanger sequenced plasmids compared to the original sequence of the plasmid. gN20-Cas9_1 and gN20-Cas9_2 both had an insertion between bases 8845 and 8846 compared to the original plasmid sequence. Insertion length was 178 bp for the former and 217 for the latter. Created with SnapGene.

The gN20-Cas9_1 sample had a gap of 1784 base pairs spanning from base pair 8846 to base pair 1377 around the origin point of the plasmid, followed by 11 aligned base pair spanning from base pair 1378 to 1388 as shown in Figure 26.

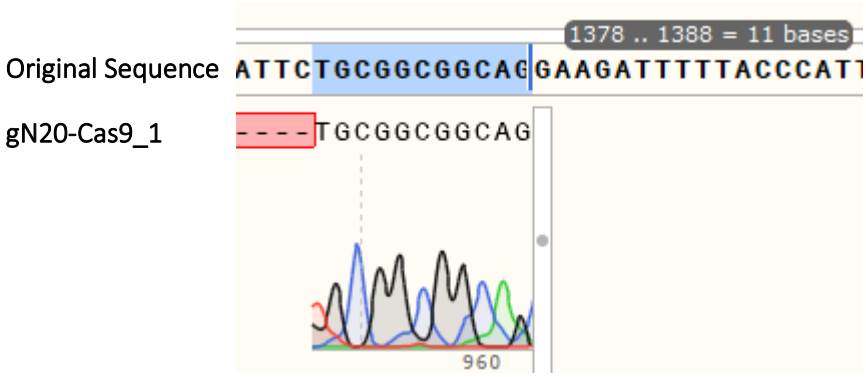

Figure 26. Chromatogram of a section of a Sanger sequenced plasmid compared to the original sequence of the plasmid. gN20-Cas9_1 shows an alignment of 11 base pairs in the sequencing at positions 1378 to 1388 of the plasmid. Created with SnapGene.

The gN20-Cas9_2 sample had a gap of 67 base pairs spanning from base pair 8846 to base pair 8912, followed by 13 aligned base pairs containing one mismatch as shown in Figure 27, where a guanine base in the original sequenced was replaced by a thymine base in the sequenced sample.

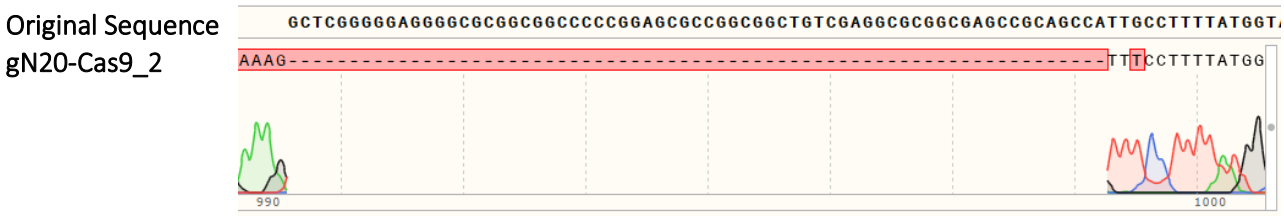

Figure 27. Chromatogram of a section of a Sanger sequenced plasmid compared to the original sequence of the plasmid. gN20-Cas9_2 shows a gap 67 base pairs spanning base pair 8846 to base pair 8912, followed by an alignment of 13 base pairs containing one mismatch where an expected guanine base has been sequenced as a thymine base. Created with SnapGene.

The inserted gRNA is shown in Figure 28, and the sequencing results shows that the guide RNA is inserted and successfully sequenced.

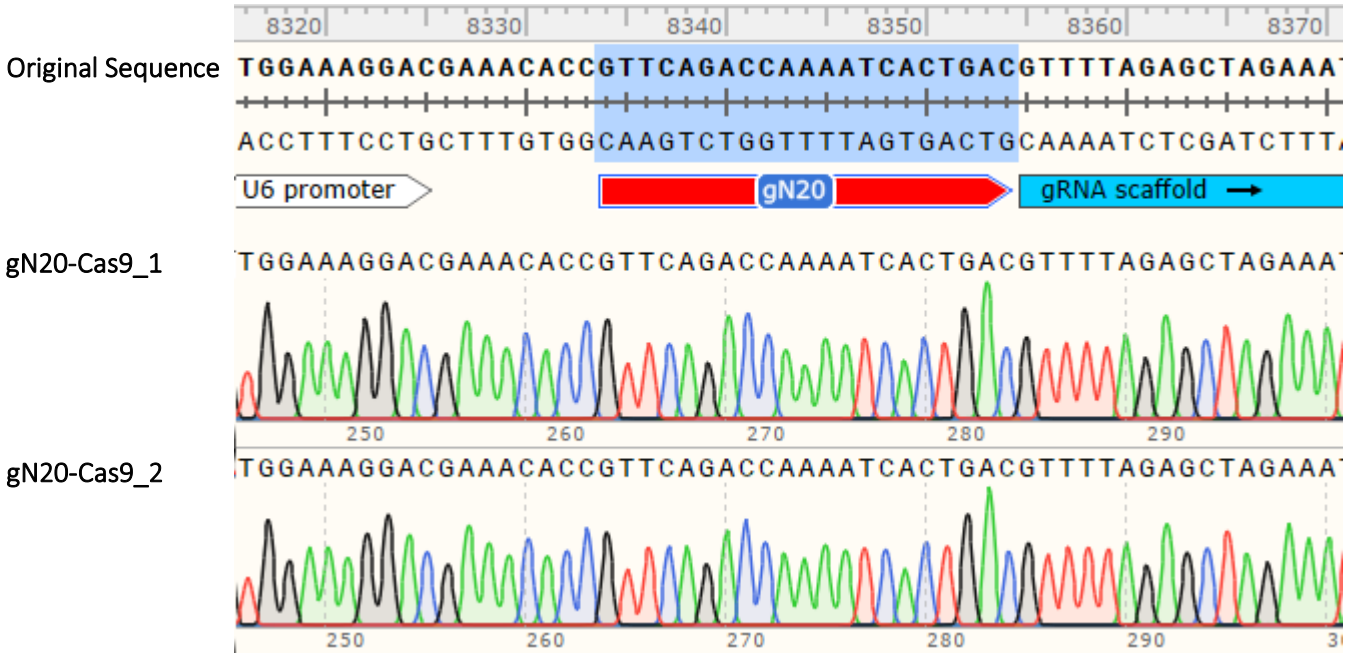

Figure 28. Chromatogram of two sections of Sanger sequenced plasmids compared to the original sequence of the plasmid. A guide RNA named gN20 is seen to be successfully inserted and sequenced. Created with SnapGene.

The gN21-Cas9-Venus complex was compared to the sequenced gN21-Cas9 samples. The gN21-Cas9_1 sample three gaps/insertions compared to the original sequence. The gN21-Cas9-2 sample had one gap/insertion. None of the two samples had any mismatches.

The gN21-Cas9_1 sample had a gap of one bp at the 8099 bp position of the original sequence, and the gN21-Cas9_2 sample had a gap of two bp spanning bp position 8081 to 8082 in the original sequence, as shown in Figure 29.

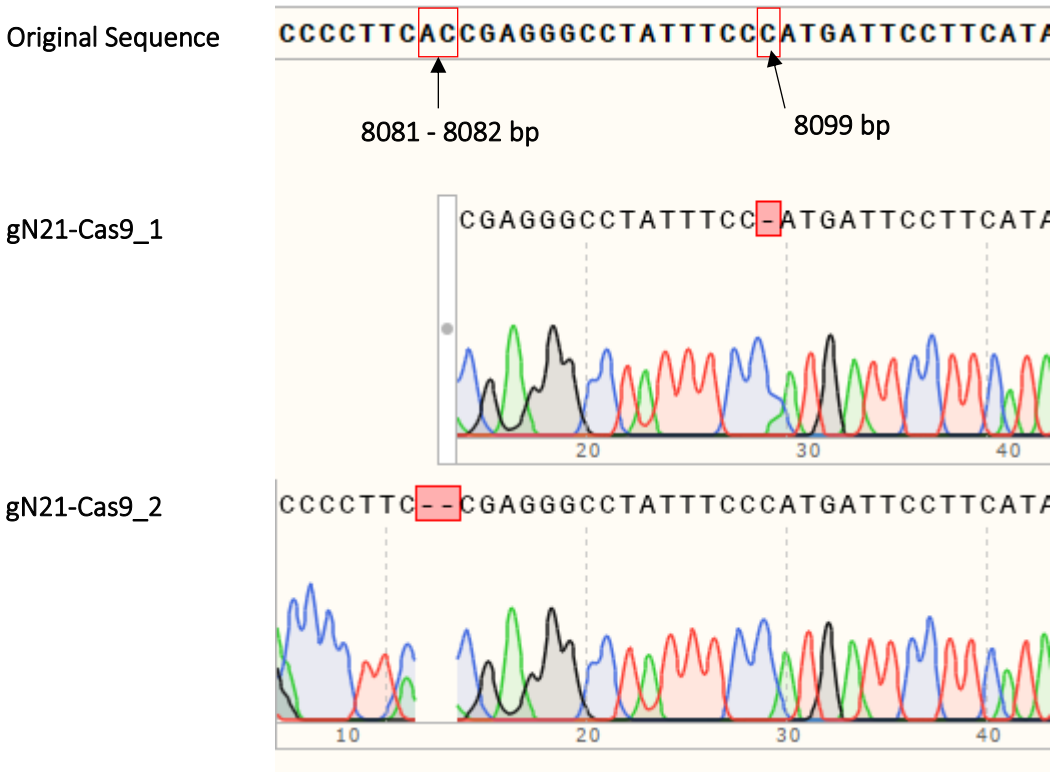

Figure 29. Chromatogram of two sections of Sanger sequenced plasmids compared to the original sequence of the plasmid. gN21-Cas9_1 shows one base pair gap in the sequencing at base pair 8099 of the original plasmid sequence. gN21-Cas9_2 shows a two base pair gap in the sequencing at base pairs 8081 to 8082 of the original plasmid sequence. Created with SnapGene.

The gN21-Cas9_1 sample had an insertion of 257 base pairs between bp 8845 and 8846 of the original sequence as shown in Figure 30, followed by a gap of 175 base pairs spanning from base pair 8846 to 9020 in the original sequence.

Figure 30. Chromatogram of a section of a Sanger sequenced plasmid compared to the original sequence of the plasmid. gN21-Cas9_1 shows an insertion of 257 base pairs in the sequencing between base pair 8845 and 8846 of the original plasmid. Created with SnapGene.

This gap was followed by 10 aligned base pair spanning from base pair 9021 to 9030, and a new insertion of 168 base pairs between base pair 9030 and 9031. This insertion was followed by a gap of 1331 base pairs spanning from base pair 9031 to 1109 around the origin point of the plasmid, ending with 11 aligned bases spanning base pair 1110 to 1120 as shown in Figure 31.

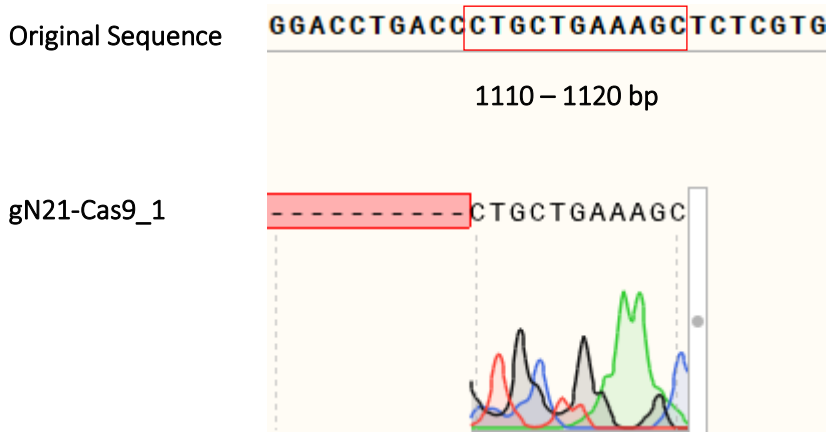

Figure 31. Chromatogram of a section of a Sanger sequenced plasmid compared to the original sequence of the plasmid. gN21-Cas9_1 shows an alignment of 11 base pairs in the sequencing at positions 1110 to 1120 of the plasmid. Created with SnapGene.

Shown in Figure 32 are the successful sequencing of the inserted gN21 in the Cas9-Venus plasmid.

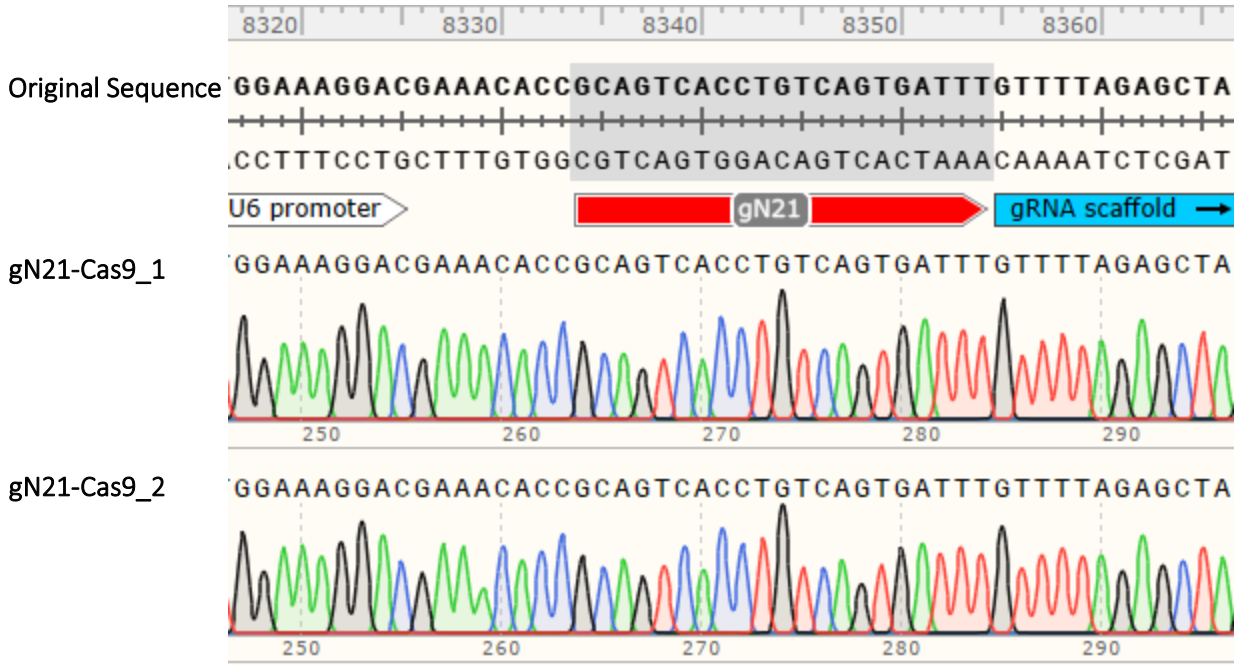

Figure 32. Chromatogram of two sections of Sanger sequenced plasmids compared to the original sequence of the plasmid. A guide RNA named gN21 is seen to be successfully inserted and sequenced. Created with SnapGene.

3.5 Agarose gel electrophoresis of amplified fragments used for In-Fusion cloning

Figure 33, Figure 34 and Figure 35 shows the agarose gels after electrophoresis of the PCR reaction mixes containing the four fragments used in cloning the In-Fusion plasmid, a 100 – 5000 bp DNA ladder was used in all of the agarose gels. The reaction volumes were split along wells due to the limit of the volume in the wells. Fragments 1 and 3 had twice the reaction volumes as fragments 2 and 4 because of the ratio used of each fragment in the In-Fusion cloning reaction.

Figure 33 shows Fragment 1: Right homology arm of the glucagon gene. The expected fragment length was 1023 bp and can be seen slightly above the 1000 bp marker of the DNA ladder.

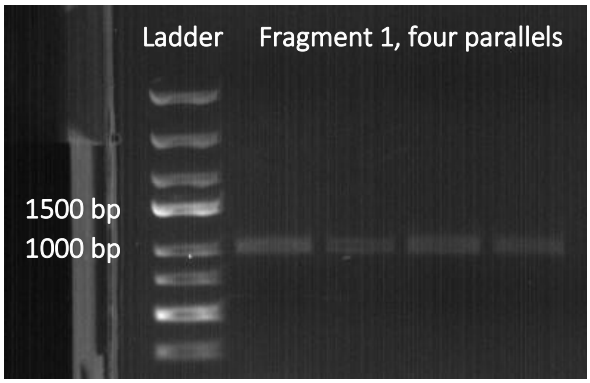

Figure 33. Agarose gel depicting a 100 – 5000 bp DNA ladder and four parallels of a 1023 bp fragment of a right homology arm of the glucagon gene amplified with PCR from a human genome template. The brightest band in the DNA ladder above the fragment parallels represent a 1500 bp length, and the band below represents a 1000 bp length.

Figure 34 shows Fragment 2: Insert section from the PAX6 donor plasmid containing the EGFP reporter gene. The expected fragment length was 2894 bp but is seen slightly in line with the 3000 bp marker of the DNA ladder.

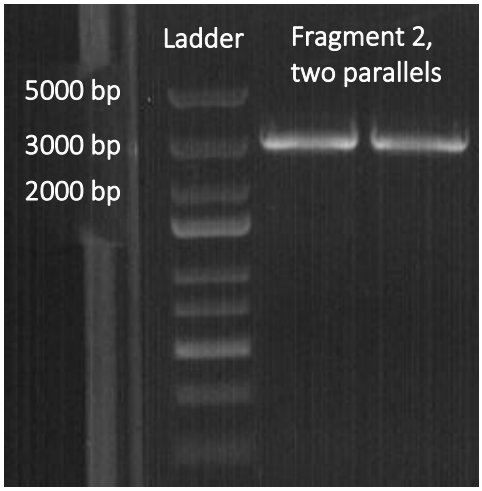

Figure 34. Agarose gel depicting a 100 – 5000 bp DNA ladder and two parallels of a DNA fragment containing a green fluorescent protein gene amplified with PCR from a PAX6 donor plasmid. The topmost band of the DNA ladder represents a base pair length of 5000 bp.

Figure 35 shows Fragment 3: Left homology arm of the glucagon gene and Fragment 4: Linearised vector backbone of the PAX6 donor plasmid.

The expected length of fragment 3 was 1022 bp and can be seen faintly along the 1000 bp marker of the DNA ladder.

The expected length of fragment 4 was 2680 bp and can be seen clearly below the 3000 bp marker of the DNA ladder.

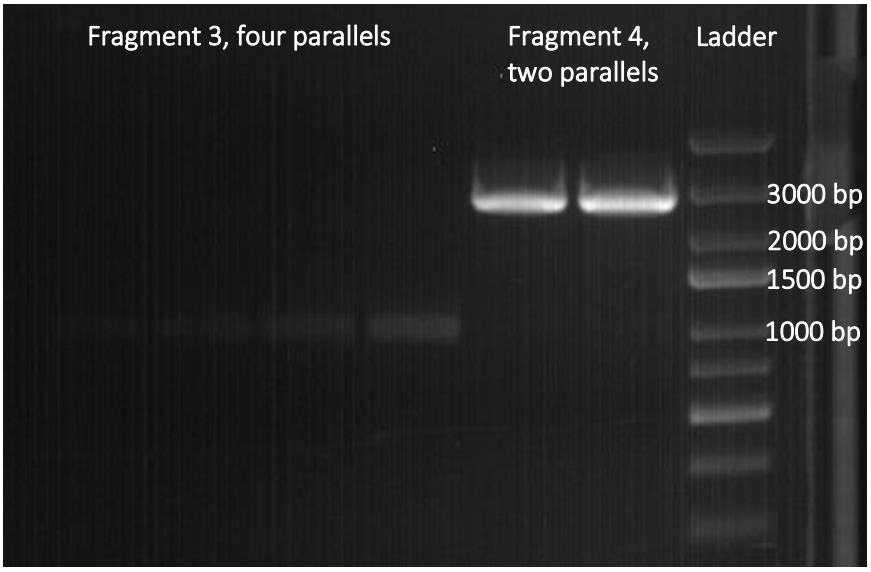

Figure 35. Agarose gel depicting a 100 – 5000 bp DNA ladder and four parallels of a 1022 bp fragment of a left homology arm of the glucagon gene (fragment 3) amplified with PCR from a human genome template, and two parallels of a 2680 bp fragment of a donor vector backbone (fragment 4) amplified with PCR from a PAX6 donor plasmid. Numbers marked next to the DNA ladder represents the bp length of the band next to the numbers.

All the gel bands containing the fragments were cut out, isolated, and purified, and NanoDrop spectrophotometry was performed to determine the DNA concentration and 260/280 ratio as shown in Table 27.

Table 27. Concentration and 260/280 ratio of isolated and purified samples of four PCR amplified fragments containing right and left glucagon homology arms, insert section and linearised PAX6 donor vector backbone measured by NanoDrop spectrophotometry.

NanoDrop measurements		
Sample	Concentration [ng/µL]	260/280
Fragment 1	6,4	2,07
Fragment 2	30,7	1,81
Fragment 3	16,3	1,99
Fragment 4	41,8	2,00

3.6 Designing the In-Fusion reaction

According to the In-Fusion user guide the insert to vector molecular ratio should be 2:1. To calculate how much of each fragment should be added to the In-Fusion reaction, we had to

consider the concentration of DNA for each fragment we had in solution, the maximum reaction volume available in a PCR tube, and making sure that the threshold of DNA amount was reached for each fragment to produce the desired reaction of combining all fragments together.

The molecular weight for each fragment was calculated assuming 499,5 average g/mol of a base pair, as shown in Table 28. Picomole per µL was then calculated using the known concentration and the calculated molecular weight. As the fragment 3 had the highest molar concentration of DNA, approximately 50 ng of DNA from this fragment was taken as the starting point to calculate the amount of DNA needed for the rest of the fragments, as this was the lowest DNA amount that could be used in the reaction. The volume required of each component was then calculated to reach the desired ratio of 2:1:2:1.

Table 28. Four DNA fragments used in an In-Fusion cloning reaction, with desired ratio, measured concentration and known base pairs. Also shown are calculated molecular weight (assuming 499,5 g/mol of a nucleotide), molar concentration, required DNA amount with volume, and moles for each component.

Donor vector cloning calculations								
Component	Ratio	Concentration [ng/µL]	Base pairs	MW [g/mol]	[pmol / µL]	[ng]	[µL] for ratio	[pmol]
Fragment 1 GCG_HR_R	2	6,4	1023	510988,5	0,01252	50,39	7,873	0,10
Fragment 2 Insert section	1	30,7	2894	1445553	0,02124	71,27	2,322	0,05
Fragment 3 GCG_HR_L	2	16,3	1022	510489	0,03193	50,34	3,088	0,10
Fragment 4 Linearised vector	1	41,8	2680	1338660	0,03123	66,00	1,579	0,05

The actual volume and, in turn, added DNA amount deviated slightly from the calculated amounts, as shown in Table 29.

Table 29. Volume and DNA amount used in an In-Fusion cloning reaction of four DNA fragments, with calculated molar ratio of each component.

Donor vector cloning [Actually used]				
Component	Calculated ratio	[ng]	[µL] used	[pmol]
Fragment 1 GCG_HR_R	2,1	52,86	8,260	0,103
Fragment 2 Insert section	1,0	71,29	2,322	0,049
Fragment 3 GCG_HR_L	2,0	50,33	3,088	0,099
Fragment 4 Linearised vector	1,0	66,00	1,579	0,049

3.7 In-Fusion plasmid analysis

After culturing transformed competent DH10B cells with two replicates of the In-Fusion cloned plasmid along with a positive control, plasmid DNA was isolated from all the samples.

NanoDrop spectrophotometry was performed on each sample to determine the DNA concentration and 260/280 ratio as shown in Table 30.

Table 30. Concentration and 260/280 ratio of isolated and purified samples of ten parallels of an In-Fusion cloned plasmid and four parallels of a positive control measured by NanoDrop spectrophotometry.

NanoDrop measurements		
Sample	Concentration [ng/µL]	260/280
In-Fusion 1_1	470,8	1,89
In-Fusion 1_2	415,9	1,91
In-Fusion 1_3	387,7	1,92
In-Fusion 1_4	610,0	1,89
In-Fusion 1_5	353,1	1,90
In-Fusion 2_1	485,2	1,91
In-Fusion 2_2	190,0	1,85
In-Fusion 2_3	439,8	1,90
In-Fusion 2_4	545,3	1,90
In-Fusion 2_5	544,3	1,90
Positive control 1	441,8	1,90
Positive control 2	53,2	1,89
Positive control 3	277,1	1,89
Positive control 4	495,7	1,89

In-Fusion 1_1, In-Fusion 1_2, In-Fusion 2_1 and In-Fusion 2_2 plasmid samples were sent to Eurofins for Sanger sequencing with the primers shown in Table 2 (Primer number is based on primer name, sorted by order of appearance throughout the plasmid map from point of origin).

Sequencing results were compared to the In-Fusion plasmid constructed in the SnapGene software, as shown in Figure 36.

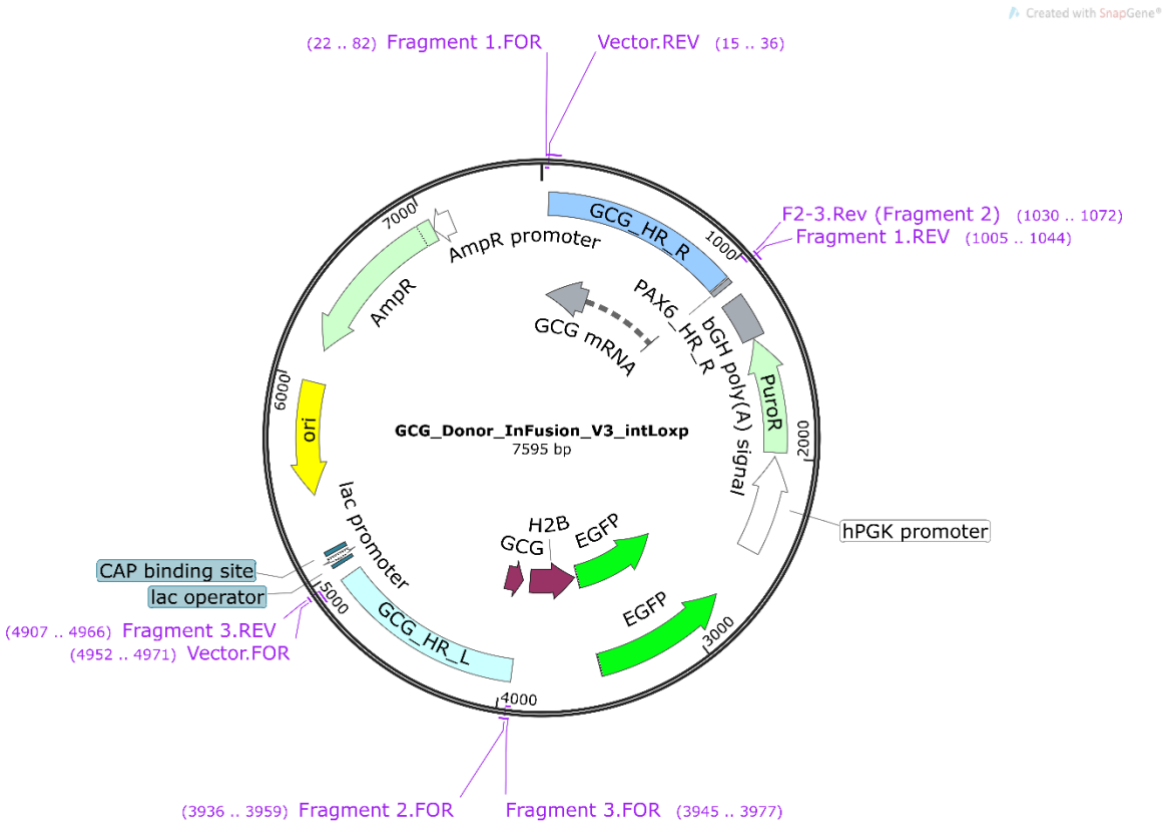

Figure 36. A plasmid constructed with four different DNA fragments in an In-Fusion reaction. The forward and reverse primers display where the added fragment starts and ends. Created with SnapGene.

For the primers used in sequencing of the In-Fusion_1 sample, the average number of base pairs produced during the sequencing were 1380 base pairs, where the average section of the aligned base pairs of the sequence were bases 9 to 1300. The average number of mismatches were 14, and the average number of gaps/insertions were 10. Detailed numbers of bases, alignments, mismatches, and gaps/insertions for each primer are shown in Table 31.

Table 31. Primer names and numbers, bases, alignments, mismatches, and gaps/insertions of an In-Fusion cloned plasmid sample named In-Fusion 1 that have been sequenced by Sanger sequencing.

In-Fusion 1					
Primer name	Primer	Bases	Aligned	Mismatches	Gaps/Insertions
Puro-F_Seq3	3	1364	14 - 1309	14	14
hPGK_F_Seq4	4	1276	3 - 1276	11	10
EGFP-C_Seq5	5	1422	11 - 1289	22	7
EGFP_N_Seq6	6	1394	9 - 1246	10	4
GCGL_Seq7	7	1392	6 - 1342	16	6
M13rev-49	8	1374	9 - 1339	15	19
M13uni-43	1	1440	10 - 1297	11	8

Figure 37 shows the sequenced area above the sequence of the plasmid, with primer numbers corresponding to primer names in Table 2 next to the start of the sequenced area. The filled section of an arrow indicates alignments in the sequenced area, while an empty section indicates a gap or gap/insertion in the sequenced area.

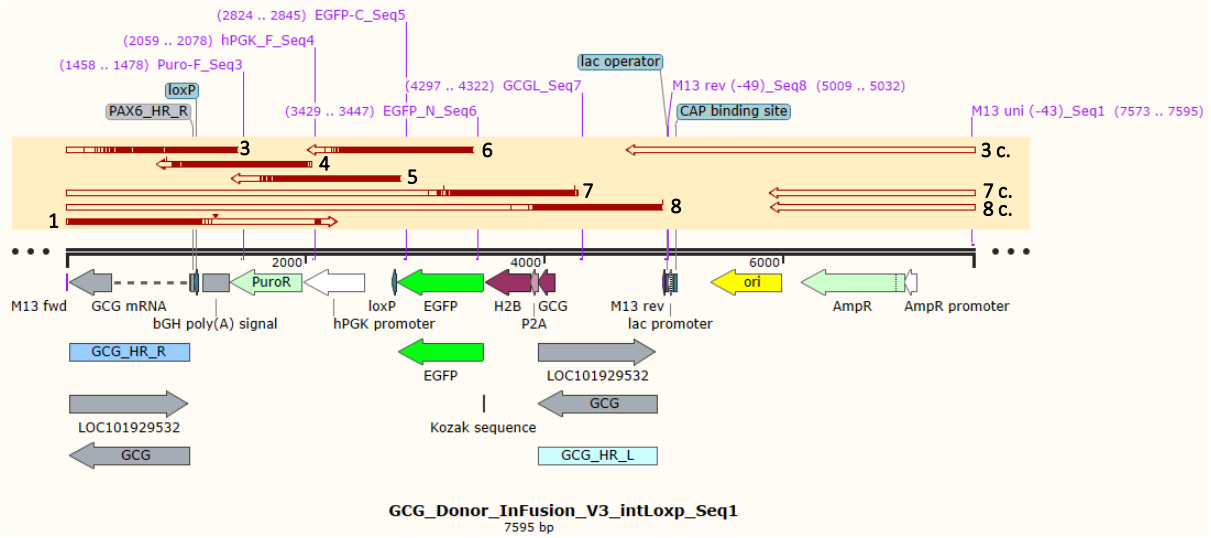

*Figure 37. Horizontal plasmid map of an In-Fusion cloned plasmid named In-Fusion 1, with sequenced areas shown above the plasmid map as arrows. A filled section of an arrow indicates alignments in the sequenced area, while an empty section indicates a gap or gap/insertion in the sequenced area. Each sequenced area is marked with a number at the start position of the sequence, corresponding to primer numbers as seen in the end of primer name in purple text. Primer numbers with c. attached means that the sequence spanned the origin point of the plasmid and are **c**ontinuing from the other end of the horizontal map. Created with SnapGene.*

For the primers used in sequencing of the In-Fusion_2 samples, the average number of base pairs produced during the sequencing were 1382 base pairs, where the average section of the aligned base pairs of the sequence were bases 10 to 1252. The average number of mismatches were 14, and the average number of gaps/insertions were 9. Detailed numbers of bases, alignments, mismatches, and gaps/insertions for each primer are shown in Table 32.

Table 32. Primer names and numbers, bases, alignments, mismatches, and gaps/insertions of an In-Fusion cloned plasmid sample named In-Fusion 2 that have been sequenced by Sanger sequencing.

In-Fusion 2					
Primer name	Primer	Bases	Aligned	Mismatches	Gaps/Insertions
Puro-F_Seq3	3	1427	17 - 1322	13	10
hPGK_F_Seq4	4	1501	5 - 1235	5	5
EGFP-C_Seq5	5	1500	11 - 1338	19	16
EGFP_N_Seq6	6	1189	13 - 1135	10	14
GCGL_Seq7	7	1399	8 - 1259	9	4
M13rev-49	8	1244	9 - 1243	13	5
M13uni-43	1	1413	10 - 1232	28	7

Figure 38 shows the sequenced area above the sequence of the plasmid, with primer numbers corresponding to primer names in Table 2 next to the start of the sequenced area. The filled section of an arrow indicates alignments in the sequenced area, while an empty section indicates a gap or gap/insertion in the sequenced area.

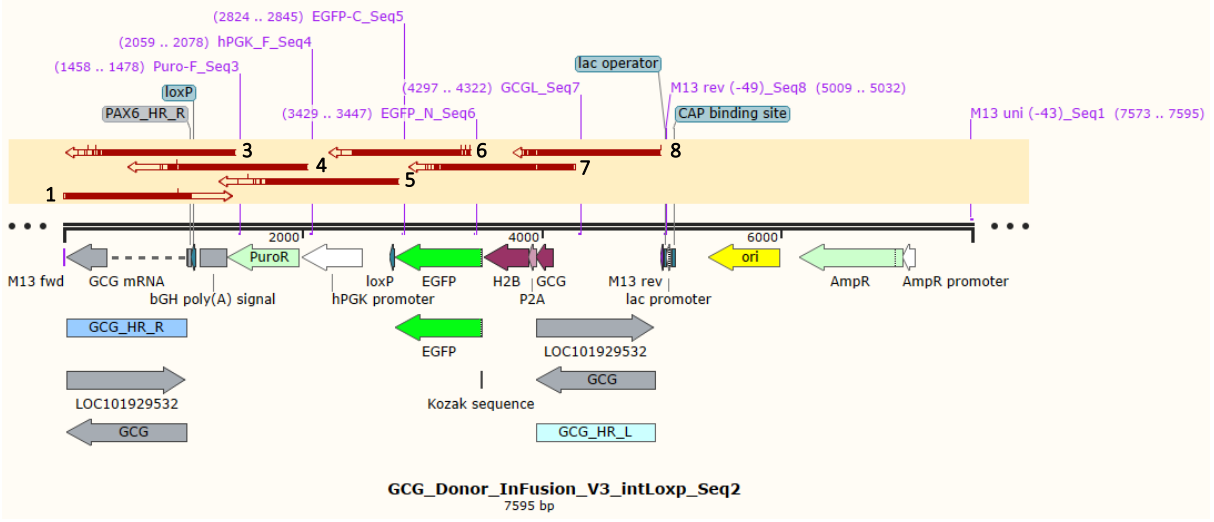

Figure 38. Horizontal plasmid map of an In-Fusion cloned plasmid named In-Fusion 2, with sequenced areas shown above the plasmid map as arrows. A filled section of an arrow indicates alignments in the sequenced area, while an empty section indicates a gap or gap/insertion in the sequenced area. Each sequenced area is marked with a number at the start position of the sequence, corresponding to primer numbers as seen in the end of primer name in purple text. Created with SnapGene.

3.8 Transfection of HEK293 cells

gN20-Cas9_1 and gN21-Cas9_1 were transfected into three replicate dishes of HEK293 cells along with a positive and negative control. Fluorescence microscopy was performed to visualise the transfected cells and confirm successful transfection by verifying that the cells had produced the green fluorescent protein.

In the following image series are cells pictured first with no filters, followed by a filter to visualise the green fluorescent protein, and finally the two pictures overlayed upon each

other. The negative control produced, as expected, no fluorescent protein and is therefore not pictured.

Figure 39, Figure 40 and Figure 41 shows, respectively, HEK293 cells transfected with gN20-Cas9_1, gN21-Cas9_1 and the positive control under 10x magnification with a 150 µm scale bar.

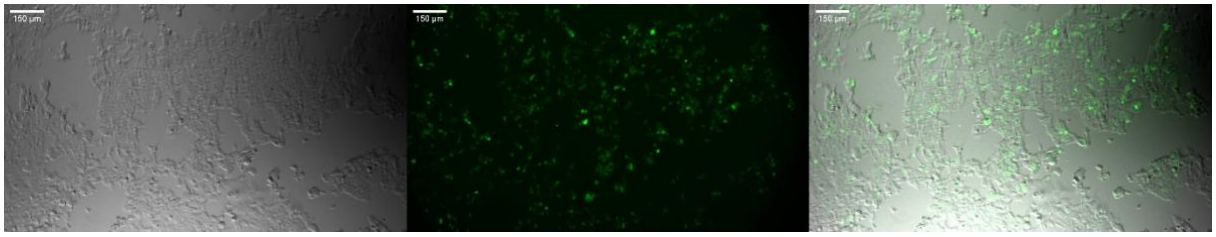

Figure 39. Fluorescence microscopy photos of HEK293 cells transfected with a gN20-Cas9 construct containing a green fluorescent protein gene under 10x magnification. First photo shows the cells without any filters, the second photo shows the green fluorescent protein produced by the cells, and the third photo is both previous photos imposed on each other. A white scale bar of 150 µm is shown in the top left of each photo.

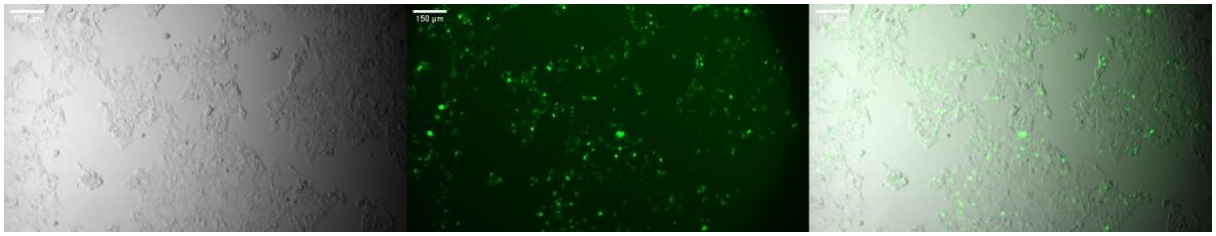

Figure 40. Fluorescence microscopy photos of HEK293 cells transfected with a gN21-Cas9 construct containing a green fluorescent protein gene under 10x magnification. First photo shows the cells without any filters, the second photo shows the green fluorescent protein produced by the cells, and the third photo is both previous photos imposed on each other. A white scale bar of 150 µm is shown in the top left of each photo.

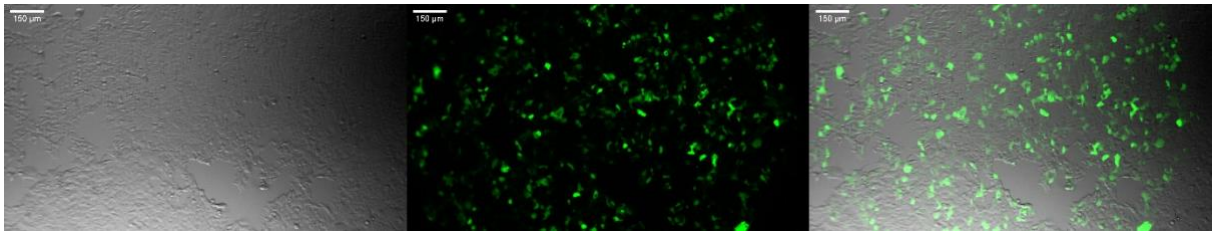

Figure 41. Fluorescence microscopy photos of HEK293 cells transfected with a positive control containing a green fluorescent protein gene under 10x magnification. First photo shows the cells without any filters, the second photo shows the green fluorescent protein produced by the cells, and the third photo is both previous photos imposed on each other. A white scale bar of 150 µm is shown in the top left of each photo.

Figure 42, Figure 43 and Figure 44 shows, respectively, HEK293 cells transfected with gN20-Cas9_1, gN21-Cas9_1 and the positive control under 32x magnification and with a 50 µm scale bar.

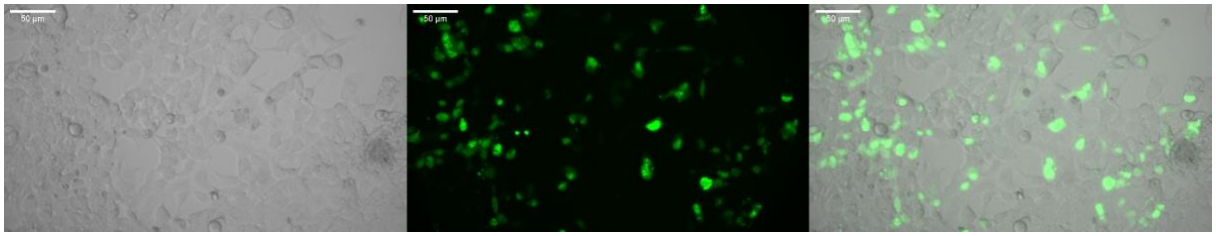

Figure 42. Fluorescence microscopy photos of HEK293 cells transfected with a gN20-Cas9 construct containing a green fluorescent protein gene under 32x magnification. First photo shows the cells without any filters, the second photo shows the green fluorescent protein produced by the cells, and the third photo is both previous photos imposed on each other. A white scale bar of 50 µm is shown in the top left of each photo.

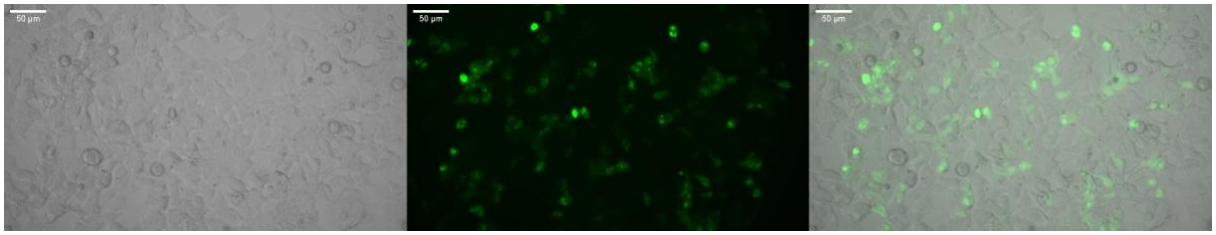

Figure 43. Fluorescence microscopy photos of HEK293 cells transfected with a gN21-Cas9 construct containing a green fluorescent protein gene under 32x magnification. First photo shows the cells without any filters, the second photo shows the green fluorescent protein produced by the cells, and the third photo is both previous photos imposed on each other. A white scale bar of 50 µm is shown in the top left of each photo.

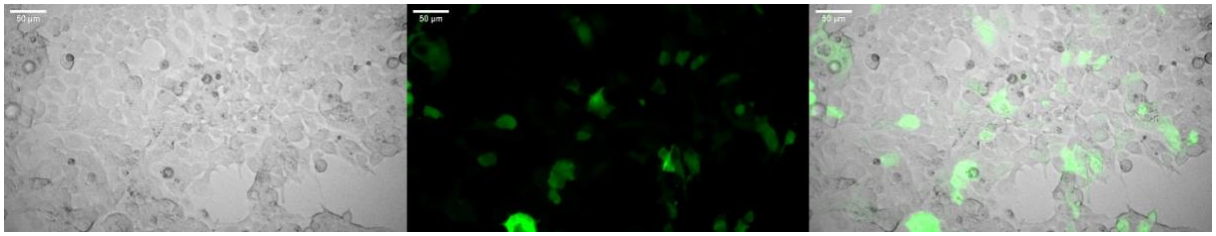

Figure 44. Fluorescence microscopy photos of HEK293 cells transfected with a positive control containing a green fluorescent protein gene under 32x magnification. First photo shows the cells without any filters, the second photo shows the green fluorescent protein produced by the cells, and the third photo is both previous photos imposed on each other. A white scale bar of 50 µm is shown in the top left of each photo.

3.9 Genomic DNA isolated from HEK293 cells

The genomic DNA was extracted from three replicates of the gN20-Cas9_1 and gN21-Cas9_1 transfected HEK293 cells, and from the negative control. NanoDrop spectrophotometry was performed on each sample to determine the DNA concentration and 260/280 ratio as shown in Table 33.

Table 33. Concentration and 260/280 ratio of isolated and purified samples of genomic DNA from HEK293 cells transfected with sgRNA-Cas9 complex and a negative control measured by NanoDrop spectrophotometry.

NanoDrop measurements		
Sample	Concentration [ng/µL]	260/280
gN20-Cas9_1_1 gDNA from HEK	25,4	1,56
gN20-Cas9_1_2 gDNA from HEK	29,6	1,91
gN20-Cas9_1_3 gDNA from HEK	28,3	1,98
gN21-Cas9_1_1 gDNA from HEK	27,3	1,76
gN21-Cas9_1_2 gDNA from HEK	28,8	1,94
gN21-Cas9_1_3 gDNA from HEK	19,5	1,90
Negative control gDNA from HEK	45,7	1,97

3.10 On-Target effect analysis

After amplification of the On-Target site of the genomic DNA in HEK293 cells for three replicates of gN20-Cas9_1_1 and three replicates of gN21-Cas9_1_1 isolated from the HEK293 cells, 5 µL of the reaction volume were loaded onto an agarose gel along with a 100 – 5000 bp DNA ladder. The expected fragment length was 691 bp and are very faintly visible between the 500 bp and 750 bp marker of the DNA ladder as shown in Figure 45.

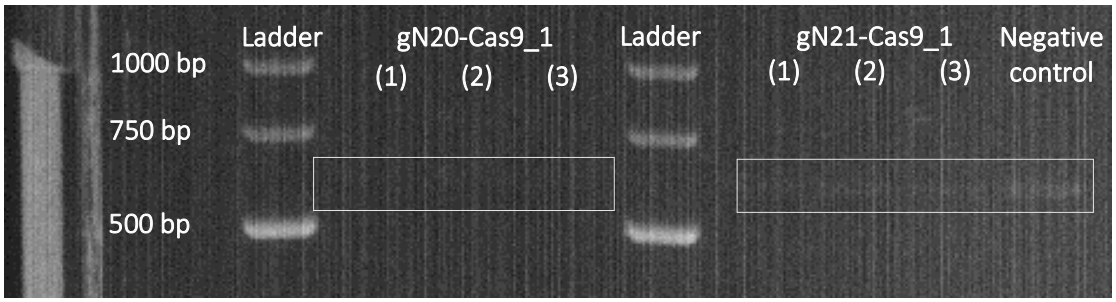

Figure 45. Agarose gel depicting three bands of two DNA ladders, and an amplified section of genomic DNA of HEK293 cells transfected with three parallels each of two different sgRNA-Cas9 complexes and one negative control. Bands are very faintly visible between 750 bp and 500 bp for the samples, highlighted with a grey box for better visualisation.

The gel bands were cut out, isolated, and purified, and NanoDrop spectrophotometry was performed to determine the DNA concentration and 260/280 ratio as shown in Table 34.

Table 34. Concentration and 260/280 ratio of isolated and purified samples of an amplified section of genomic DNA from HEK293 cells transfected with sgRNA-Cas9 complex and a negative control measured by NanoDrop spectrophotometry.

NanoDrop measurements		
Sample	Concentration [ng/µL]	260/280
gN20-Cas9_1 Purified	19,8	0,396
gN20-Cas9_2 Purified	9,5	0,191
gN20-Cas9_3 Purified	6,9	0,139
gN21-Cas9_1 Purified	10,1	0,203
gN21-Cas9_2 Purified	10,8	0,216
gN21-Cas9_3 Purified	25,9	0,518
Negative control Purified	12,9	0,258

3.11 Indel formation estimation by T7 Endonuclease I assay

The genomic DNA from transfected HEK293 cells with amplified on-target loci was denatured in a PCR machine, followed by a re-annealing program to form heteroduplexes. T7 Endonuclease I was added to each sample to detect and cleave the DNA where and if mismatches had formed.

The enzyme reaction was inactivated, and the reaction mixes were loaded onto an agarose gel as shown in Figure 46. The amplified on-target region had a base pair length of 691 bp. Fragments were expected to appear at approximately 240 and 360 bp and were faintly visible above and below the 300 bp marker of the DNA ladder for the amplified on-target region from the genomic DNA of HEK293 cells that had been transfected with gN20-Cas9. The amplified on-target region of gN21-Cas9 transfected cells produced no visible bands.

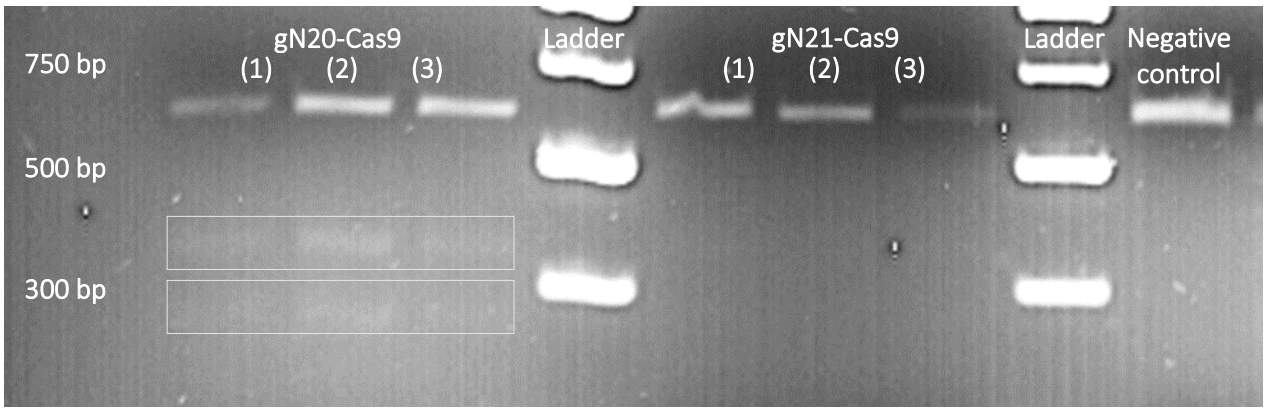

Figure 46. Agarose gel depicting two DNA ladders (brightest bands), and an amplified section of genomic DNA of HEK293 cells transfected with three parallels each of two different sgRNA-Cas9 complexes and one negative control that has been cleaved by T7 Endonuclease I after re-hybridisation in a PCR machine. Bands are faintly visible above and below 300 bp for the samples isolated from HEK293 cells transfected with the sN20-Cas9 vector construct, highlighted with a grey box for better visualisation. The gN21-Cas9 and negative control was not cleaved by T7 Endonuclease I and have not produced any bands except for the un-cleaved product visible between the 750 bp and 500 bp band of the DNA ladder.

To estimate cleavage by the T7 Endonuclease I enzyme, an image of the gel was analysed in the image software Fiji. The intensities of the gel bands were measured digitally as a grayscale value by the software, and Equation 1 was used to calculate an estimated average of 26,2% cleavage by the T7 Endonuclease I. The average indel occurrence was calculated with Equation 2 to be 14,4%.

4. Discussion and conclusion

By utilising powerful software like SnapGene and web tools like CRISPOR, two plasmids were designed and constructed. Successful plasmid cloning was confirmed by Sanger sequencing.

One of these plasmids was a sgRNA Cas9-Venus plasmid containing a guide sequence that can define a cut site in the human genome, specifically the glucagon gene, when translated, as well as the coding sequence for the SpCas9 nuclease to induce a double stranded break at the target site.

The other plasmid, the In-Fusion plasmid, was constructed to serve as an HDR DNA template for the DSB. The key feature of the constructed template plasmid is the insert section, which in this report is often referred to as "Fragment 2" in the In-Fusion plasmid. This insert section, planned located in exon 5 in the proglucagon gene, contains a green fluorescent protein. When proglucagon is produced by the cell, green fluorescent protein will also be produced.

By inserting this gene in the genome of human induced pluripotent stem cells with the CRISPR tools that we have constructed, a glucagon reporter human iPSC line could be generated. Making it easy to visualise proglucagon during pancreatic development. Additionally, by inserting the gene into an insulin reporter cell line like the hiPSC-INS-T2A-H2B-Cherry (63), a dual glucagon and insulin reporter cell line might be generated.

Reporter cell lines would be very useful for diabetic research. Using stem cells to understand the pancreatic development pathway in detail could aid the future development of type 1- and type 2-diabetes therapies. A dual reporter cell line in stem cells would be crucial to follow the differentiation process from stem cell into alpha or beta cell. A reporter line could also be useful in research cases like Thorel et al (64) where they studied the conversion of healthy alpha cells into beta cells by transdifferentiation in the case of extreme beta cell loss in mice. A human reporter cell line could help explore this opportunity in humans.

A dual reporter stem cell line in hiPSC could, in the long run, prove helpful in developing patient-specific therapies for T1D patients. By using the patient's own somatic cells to induce pluripotency, tracking the differentiation process into beta cells and why they do not function optimally, would give patient-specific insight into their biological processes. This insight is vital for treatment that could remove the disease from a patient. Reintroducing the blood sugar-regulating processes could also aid in lowering the risk of the other complications associated

with diabetes (65). However, these are all just personal hypothesises, and need to be researched further.

4.1 Agarose gel electrophoresis of Cas9-Venus and PAX donor plasmids

The relaxed, supercoiled, and nicked conformations of plasmid DNA was visible after loading isolated DNA from cultured bacterial stock on an agarose gel. Even though the distance travelled by the plasmids did not reflect the plasmids base pair size in respect to the DNA ladder loaded alongside them, it did indicate that the plasmid DNA structure was well maintained after isolation.

4.2 HEK293 cells transfected with single guide RNA

One thing to note one the photographs of HEK293 cells transfected with the sgRNA plasmid is that the green fluorescent proteins (GFP) are more concentrated than compared to the cells transfected with the pEGFP-C1 positive control vector. This is due to the SV40 Nuclear Localisation Signal (NLS) peptide gene located in the Cas9-Venus plasmid backbone, guiding the GFP to the cell nuclei. This is another indication that the backbone is working as expected.

4.3 Sanger sequenced single guide RNA plasmid

Even though all the sequenced sgRNAs had gaps and insertions, and one had a mismatch, all these errors occurred either near the start of the sequence where the primer binds, or at the end of the sequence where signal quality usually degrades. The errors may for that reason necessarily be an accurate representation of the actual sequence of the constructed sgRNA plasmid. (66)

Most importantly is that in both the gN20-Cas9 samples and gN21-Cas9 samples, all the guide sequence was successfully inserted and sequenced without errors.

4.4 T7 Endonuclease I assay of single guide RNA

By having the cut-site for the sgRNA off-set to the centre of the On-Target amplified area in the human genome DNA template previously shown in Figure 17, two bands could be expected in the agarose gel following a T7 Endonuclease I digestion. If the amplified area positioned the sgRNA in the exact centre, it would not be possible to distinguish if two fragments had formed as they would travel the same distance through the gel, making cleavage and indel estimation either impossible or highly inaccurate.

The gN20-Cas9 displayed two fragments in the agarose gel after electrophoresis of the enzyme digestion reaction, although the bands were very weak. The Fiji software was nevertheless able to detect and measure the fragments digitally, allowing for an estimation of cleavage and indel occurrence.

As the average estimated cleavage occurrence was 26,2% of the original plasmid for the gN20-Cas9 vector, weak bands are to be expected. A higher cleavage occurrence would result in the top band (original fragment) being weaker while the cleaved fragments bands would be stronger.

The fact that the gN21-Cas9 vector did not produce any perceivable bands in the agarose gel, either visually or digitally, may indicate that the single guide RNA was not able to cut at the desired location efficiently. This may be inferred as no indels occurred due to nonhomologous end-joining that would have followed a double stranded break by the sgRNA. As a green fluorescent protein was observed with fluorescence microscopy of HEK293 cells transfected with the vector, it is unlikely that unsuccessful transfection explains the lack of cleavage.

4.5 The In-Fusion reaction

According to the In-Fusion website, cloning three inserts into a vector with the In-Fusion® HD Multiple-Insert Cloning averages 42% efficiency (67). They compare this to ligation-based cloning of three inserts and determines a 0% efficiency.

Additionally, they were able to clone a plasmid with three inserts in three hours and fifteen minutes with the In-Fusion reaction. In comparison, ligation-based cloning took ten hours according to their experiment. None of these times take PCR amplification of fragments and vector purification into account.

There may be a bias to take into consideration as the documentation of this experiment seems to be hosted at Takara's website only.

Nevertheless, through reasonable molecular ratio design, the In-Fusion reaction worked as intended in the case of our experiment of cloning four fragments together, and ensured successful complex plasmid construction, while possibly saving both time and costly reagents.

4.6 Sanger sequenced template plasmid (In-Fusion plasmid)

Due to the sequence length limitation and inaccuracies in sequencing of DNA segments longer than 900 base pair (66), the template plasmid was sequenced with seven different

primers. These seven different primers spanned the most important fragments used in the plasmid, like the left and right homology arms of the glucagon gene, the green fluorescent protein gene and the puromycin resistance gene, with most primers overlapping in areas where the inserted fragment would be longer than 900 base pairs to ensure accurate sequencing results.

The vector backbone containing the ampicillin/carbenicillin resistance gene and the origin of replication are also of great importance, and the fact that we were able to culture cells transformed with the plasmid and select for them on carbenicillin containing agar plates indicates that the genes work as expected.

4.7 In summary

4.7.1 Culturing plasmid from bacterial stock

Plasmid DNA of Cas9-Venus and PAX6 donor vector was cultured from bacterial stock, isolated, and purified. Agarose gel electrophoresis verified that plasmid was present and DNA structure was well maintained after isolation.

4.7.2 Constructing a template plasmid from four DNA fragments using In-Fusion cloning

Left and right homology arms of the glucagon gene were amplified from a human genome DNA template with PCR. Ampicillin resistance gene, green fluorescent protein and vector backbone were amplified from the PAX6 donor plasmid with PCR. Agarose gel electrophoresis confirmed expected fragment length, and the bands were isolated and purified. All fragments were combined in an In-Fusion vector cloning reaction with a 2:2:1:1 ratio of left and right homology arms to insert section and vector backbone.

4.7.3 Constructing the sgRNA expression vector

Guide sequences were designed, and custom ordered as forward and reverse complementary sequenced and annealed together.

The Cas9-Venus plasmid was linearised by BbsI enzyme restriction, isolated and purified by agarose gel purification. Two sgRNA Cas9-Venus plasmids were constructed by ligating the guide sequence to the linearised Cas9-Venus plasmid.

4.7.4 Transformation and Sanger sequencing

The plasmids were transformed into DH10B cells and spread on carbenicillin containing agar plates to select for transformed cells. Transformed clones were picked and cultured in LB-medium to replicate the plasmids. Plasmid DNA were isolated and purified from cultured

cells. Sanger sequencing confirmed correct insertion of guide sequence into the Cas9-Venus plasmid, and successful annealing of the four fragments in the In-Fusion cloned template plasmid.

4.7.5 T7 Endonuclease I assay

The sgRNA Cas9-Venus plasmids were transfected into HEK293 cells to verify functional plasmid by observation of green fluorescent protein in a fluorescence microscope. The genomic DNA of the HEK293 was isolated and purified, and an area spanning the insertion site of the guide sequence was amplified with PCR. The amplified DNA was denatured and re-annealed to form heteroduplexes that were detected and cleaved by T7 Endonuclease I enzyme digestion. Digest reactions were loaded onto an agarose gel and band intensity of cleaved fragments was used to calculate estimated cleavage and indel formation of the sgRNA Cas9-Venus plasmid. The T7 Endonuclease I assay indicated that the guide sequence gN20 might be more efficient in cutting at the desired location, as the gN21 guide sequence did not produce any visible bands in the agarose gel.

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