

CRISPR-Cas9 knockout of AXL in bladder cancer cells: Establishing an AXL-deficient T24 cell line

Student: Madina Yernur

Supervisor: Eugene Tulchinsky, PhD

Bachelor of Medical Sciences
Nazarbayev University School of Medicine

Astana, 2025

Abstract

AXL is a member of the TAM receptor tyrosine kinase, normally activating many important pathways that enhance cell proliferation, survival, and inhibit apoptosis in cell signalling. In cancer, however, the dysfunction of AXL might lead to drug resistance in therapies, which will eventually lead to cancer deterioration, metastasis, and poor survival of patients. AXL inhibition or knockout experiments in mouse models significantly improve those adverse effects. Additionally, studies show that AXL inhibition impaired phagocytosis, efferocytosis, and induced male infertility in mouse models. To study the function of AXL in more detail in cell signaling, especially in bladder cancer, AXL gene knockout needs to be done with CRISPR-Cas9 technologies, to obtain a T24 cell line without AXL expression. Then, single cell cloning will be screened with Western Blotting, so that an AXL-deficient T24 cell line can be established.

Table of Content

Introduction

- 1. Overview of Bladder Cancer**
- 2. TAM receptors and AXL**
 - 2.1 Overview of TAM Receptor Kinase Family
 - 2.2 Structure and Function of TAM
- 3. AXL**
 - 3.1 AXL activation
 - 3.2 AXL in normal physiological condition
 - 3.3 AXL expression in Cancer
 - 3.4 AXL as a Therapeutic Target
- 4. Research Design**
 - 4.1 CRISPR-Cas9
 - 4.2 Hypothesis
 - 4.3 Aims

Materials and Methods

Results

Discussions

Conclusion

References

Introduction

1. Overview of Bladder Cancer

Bladder cancer is the 10th most common cancer worldwide, has a high prevalence rate. Around the world, there are more than 1.6 million people who are suffering from bladder cancer, making it a critical health risk, causing 550,000 new diagnosis and 200,000 deaths each year (Richters et al., 2020). Despite its global impact, the screening program is not available worldwide because of the low incidence of aggressive types of bladder cancer and the absence of a proper tool for screening (Dobruch & Oszczudłowski, 2021). Currently, the prognosis and treatment for bladder cancer is primarily based on the tumor and the nodal stage, and treatment for localized bladder cancer often involves cystectomy (Mao et al., 2020). However, due to the lack of more novel and efficient treatment, and because of the shortcomings of prognosis tools, the long-term survival rate for bladder cancer have shown only little improvement over past 30 years (Mao et al., 2020). Therefore, new therapeutic methods are needed to develop for more efficient treatment and more accurate forecasting of the disease. TAM receptor tyrosine kinase families, including AXL, TYRO3, and MERTK, are closely associated with cancer recently, and studying this family of receptors lead to the new potential therapeutic target in many other types of cancers (Dufour, 2019). Nevertheless, the role of TAM in bladder cancer is still under explored.

2. TAM Receptors and AXL

2.1 Overview of TAM Receptor Kinase Family

TAM receptors are a receptor tyrosine kinase (RTK) family contains 3 members, AXL, TYRO3, and Mer, that have various downstream effects in signaling pathways, such as promoting phagocytosis of apoptotic cells, activating PI3K-AKT, AKT/GSK3 β / β -catenin, MAPK-ERK pathways, and regulating immune responses (Yadav et al., 2025). In cancer, however, overexpression

of TAM receptors, especially AXL, hyperactivates those pathways, and induces anti-apoptotic conditions, promoting cell survival, proliferation, and invasion of cancer cells to other tissues, by developing resistance towards therapies (Yadav et al., 2025). Unlike other RTKs, the overexpression of TAM receptors is not due to the mutation in the receptor, but the tumor microenvironment can amplify the corresponding gene of the receptor (Yadav et al., 2025). Therefore, inhibition of those receptors shows improved cancer prognosis and decreased drug resistance in animal models (Yeo et al., 2023). That is why TAM family, especially AXL, are actively studied as a therapeutic target since they may not be prone to mutations and have lower risk of developing drug resistance. Therefore, in this research, the member of the TAM family, AXL, is aimed to knock out in the T24 bladder cancer cell lines by using CRISPR-Cas9 techniques; to obtain a cell line without AXL expression for further research.

2.2 Structure and Function of AXL

All three members of the TAM family have a similar structure and function. All three receptors are single-pass transmembrane proteins, having one extracellular domain, containing two immunoglobulin-like (Ig) domain and two fibronectin type III like (FNIII) repeats responsible for ligand recognition and binding; one intracellular kinase domain that can be autophosphorylated at the tyrosine residues, and one hydrophobic alpha helix domain serving as a linkage between extracellular and intracellular domains (Lemke & Rothlin, 2008). The ligands for TAM receptors are Growth Arrest-Specific protein 6 (GAS6), Protein S1 (PROS1), Tubbby, Tubbby-like protein 1 (Tulp-1), and galectin-3. GAS6 binds to all three TAM receptors, but with AXL shows the highest affinity; and PROS1 binds only with TYRO3 and MER, but not AXL (Wium et al., 2018). The role of Tubbby, Tubbby-like protein 1 (Tulp-1), and galectin-3 in TAM receptors in signaling is still underexplored (Yadav et al., 2025). Binding of ligands to extracellular domains triggers dimerization of two

receptors, like other RTKs, leads to autophosphorylation at tyrosine residues in the intracellular domain. The phosphorylation in intracellular domains triggers the recruitment of adaptor and effector proteins and activates many intracellular signaling pathways such as PI3K-AKT, AKT/GSK3 β / β -catenin, MAPK-ERK pathways (Yadav et al., 2025). However, binding of ligands with the receptors is not enough to activate the cell signaling pathways. The full activation of TAM receptors requires the binding of ligands and phosphatidylserine (PtdSer) (Auyez et al., 2021), only then the dimerization of receptors may occur.

The *AXL* gene encodes 20 exons of 894 amino acids, and it is located at the locus 19q13.2 (University of California Santa Cruz Genome Browser, n.d.). Exon 1 encodes a signaling peptide, Exon 2-5 encodes an Ig-like domain, Exon 6-9 encodes FNIII-like repeats, Exon 10 has 9 amino acids and encodes the cleavage site for ADAM 10 or ADAM 17 (Shen et al., 2020; Lu et al., 2017). This cleavage by ADAMs in exon 10 makes a soluble form of AXL to secrete to extracellular environment, binding of AXL ligands GAS6 and provide negative feedback by blocking receptor activation, which is also a potential biomarker for cancer prognosis (Lu et al., 2017). The two isoforms of AXL differ at the Exon 10 region, this region is missing in isoform 2, making them uncleavable for ADAM compared to isoform 1 (Lu et al., 2017). Exon 11 encodes the transmembrane domain, and γ -secretase cuts to form the nuclear form of AXL that contains a nuclear localization signal (NLS), to allow the shedded intracellular AXL to enter the nucleus to suppress or arouse the gene transcription (Lu et al., 2017). Exons 13-20 encode the intracellular tyrosine kinase domain. Understanding the *AXL* gene encoding the receptor is important in this research because CRISPR-Cas9 can be used to specifically target certain Exon regions to perform gene knockout with this knowledge.

3. AXL

3.1 AXL activation

Activation of AXL can happen under several circumstances, ligand-dependent and ligand-independent (homophilic interaction and heterophilic activation). Ligand-dependent activation includes the binding of GAS6 and AXL in a 1:1 ratio to activate the receptor. The optimum activation also requires the binding of the GLA domain of GAS6 with PtdSer. Full activation of receptors in this form triggers the activation of downstream canonical pathways, such as activation of PI3K/Akt, MAPK/ERK pathways (Yadav et al., 2025). Activation of AXL also occurs without the presence of GAS6 or other ligands. This type of activation is called ligand-independent activation and it triggers non-canonical pathways. Homophilic interaction is a type of ligand-independent activation, which indicates the activation of the pathway after the interaction of AXL monomers on neighboring cells. Another example of ligand-independent activation is heterophilic activation, which is the self-dimerization occurring between AXL and other receptors, such as c-MET, HER2, and EGFR (Yadav et al., 2025). This type of activation leads to further cancer deterioration according to Yadav et al. (2025). Therefore, the activation of AXL can happen in many ways.

3.2 AXL in normal physiological condition

There are several ways to study the normal function of AXL. One example can be shown in the research that aimed to study the phosphorylated residues of AXL by mutating tyrosine to phenylalanine and study phosphor-deficient mutants; then, the function of each tyrosine can be studied. Activation of receptors after ligand binding and autophosphorylation at Y779 (tyrosine 779 residue), Y821, and Y866 produces sites for the binding of proteins containing SH2 domains. Specifically, phosphorylation at Y821 recruits phospholipase C γ , regulatory subunits of PI3K such as

p85, SRC, LCK, and GRB2; and phosphorylation of Y779 makes the docking site for p85αβ, and Y866 makes the docking site for phospholipases C (Braunger et al., 1997). This result from this study indicates that AXL is associated with cell proliferation and survival, by activating pathways such as PI3K-AKT, AKT/GSK3β/ β-catenin, MAPK-ERK. Moreover, apart from those pathways that AXL activates, studies also show that the receptor can enhance cell survival by inhibiting apoptosis-inducing caspase 3 and BAD, and activating the inhibitors of apoptosis such as survivins and BCL-2 (Zhu et al., 2019).

Apart from studying AXL by mutating tyrosine residues to phenylalanine, gene knockout studies in mice were also conducted. In normal physiological conditions, the studies with mice show that AXL serves the function of correct functioning of phagocytosis, efferocytosis (the phagocytosis of apoptotic cells), and limiting innate immune response (Auyez et al., 2021). TAM receptors on the surface of professional and unprofessional phagocytes bind to their ligands GAS6 and PROS1 that are already linked to cells presenting PtdSer, a signal for being phagocytosed, and the receptors get activated to enhance phagocytosis. In studies of TAM triple knockout (all *AXL*, *TYRO*, and *MER* are knocked out) models, phagocytosis was severely impaired. This can be seen from male infertility in mice, where TAM's absence in Sertoli cells makes phagocytosis impossible, leading to the aggregation of dead cells. Blocking of the clearance of aggregated dead cells in seminiferous tubules halts the formation of spermatozoa, which eventually leads to male infertility (Deng et al., 2016). Efferocytosis is also impaired in TAM knockout mice causing the accumulation of apoptotic cells in tissues that triggers autoimmune responses (Zhu et al., 2019). These studies show the importance of TAM receptors in the proper functioning of phagocytosis in professional and unprofessional phagocytes. To conclude, the normal function of AXL in normal physiological conditions is guiding the activation of PI3K-AKT, AKT/GSK3β/ β-catenin, MAPK-ERK pathways for cells to proliferate

and survive, and also making phagocytosis and efferocytosis possible by giving signals to professional and unprofessional phagocytes. `

3.3 AXL expression in Cancer

AXL is overexpressed in cancer, giving the adverse effects on important physiological functions. The studies inducing monoclonal antibodies to AXL show that inhibition of this receptor induces apoptosis of cancer cells in renal cell carcinoma (Yu et al., 2015). Thus, confirming the hypothesis on the importance of this receptor in cancer cell proliferation. Moreover, AXL is also linked to the expression of Rho, Rac, and PI3K/Akt pathways, which are responsible for cell migration and invasion. Experiments to decrease the expression of AXL significantly reduced cancer cell invasion and migration (Yadav et al., 2025). Additionally, studies also show that AXL initiates the epithelial to mesenchymal transition (EMT). Overstimulation of AXL is correlated with the enhancement of mesenchymal markers and downregulation of pre-epithelial markers enhances cell invasiveness and metastasis (Thiery et al., 2009). Apart from these, AXL is also linked with producing growth hormones, stimulating the growth and tube formation. In cancer, reducing the expression of AXL also decreases the expression of DKK3 and Ang-2 proteins, which are the proteins responsible for angiogenesis (Yadav et al., 2025). This can confirm that AXL expression can be correlated with angiogenesis in cancer. Therefore, overexpression of AXL not only leads to the proliferation, survival of cancer cells, but also migration, invasion of cells to other tissues, and contributes to the EMT and angiogenesis; this all theoretically favor the deterioration of cancer in patients.

Beyond the role of favoring cancer progression as previously mentioned, AXL also favors cancer by modulating the immune system, causing not only the suppression of immunity to allow cancer cells to escape, but also by inducing drug resistance in cancer immunotherapy. Firstly, AXL overexpression blocks the MHC-1 complex, which is responsible for recognition of self. As a result, it triggers the adverse immune response (Auyez et al., 2021). Secondly, AXL overexpression causes

therapeutic resistance by upregulating Twist and BCL-2, reducing the production of chemokines and cytokines, and activating the PI3K/Akt pathway, all of this action can help cancer to evade therapeutical interventions (Zhu et al., 2019). This shows that AXL has a great but complex role in immunity in terms of cancer.

3.4 AXL as Therapeutic Target

AXL is a promising therapeutic target because of its involvement in cell proliferation, growth, and its role in metastasis and drug resistance in cancer. AXL knockdown experiments in animal models showed slower cancer progression by reducing cell proliferation and promoting apoptosis. It also improved the performance of immunotherapy and chemotherapy in experimental settings (Yadav et al., 2025). The current therapeutic interventions include small-molecule AXL tyrosine kinase inhibitors, anti-AXL monoclonal antibodies (mAb) and antibody–drug conjugates (ADC), anti-AXL chimeric antigen receptor (CAR) therapies, and other therapeutic agents are available or currently being studied (Yadav et al., 2025). However, this still cannot fully prevent the acquired drug resistance based on AXL receptors. This is why the combination of multiple therapies is recommended to increase the efficiency while decreasing the chances of getting resistance (Yadav, 2025). Therefore, further investigations of AXL should be done in different types of cancers to obtain novel therapeutic strategies.

4. Research Design

4.1 CRISPR-Cas9

CRISPR-Cas9 is an efficient gene editing tool commonly used by researchers. Originally, CRISPR-Cas is responsible for the “immune system” for microbes. This method consists of several elements, such as Cas9 – a cutter to induce double double-stranded break (DSB) at the place that is

guided by another element – sgRNA. Another essential element is a protospacer adjacent motif, or PAM sequence. The Cas9 protein has a PAM-interacting domain, which is serving as a marker for the enzyme to induce a DSB several nucleotides upstream from it. DNA repairing pathways such as non-homologous end joining (NHEJ) or homology directed repair (HDR) are activated after inducing DSB, and by using different properties of both pathways, whole gene knockout or precise genome editing can be achieved. To be more specific, NHEJ is an error-prone pathway, triggered when there is no repair template. NHEJ re-ligates the two DSB together and creates an insertion/deletion (indel). This property can be used to obtain a knockout of a gene, because an indel in the coding region can induce a frameshift mutation or a premature stop codon. Similarly, by introducing two DSBs, a certain gene fragment can be deleted. On the other hand, HDR is a pathway occurs less frequently than NHEJ, and generates the genes in the genome based on the template the subject introduces allowing to perform precise gene editing (Ran et al., 2013). In this research, the NHEJ pathway is aimed at creating an indel to further knockout the whole *AXL* gene.

Comparing with other gene engineering tools like ZFNs and TALENs, CRISPR-Cas9 is useful in many ways, since it has higher specificity and is easier to engineer compared to complicated ZFNs; and easier to customize compared to TALENs, because the latter one requires the presence of two new TALEN genes, while CRISPR-Cas9 requires a designed 20 bp sgRNA (Ran et al., 2013). Due to the fact that CRISPR-Cas9 is a system that is easier to design with higher specificity and more efficient compared to other gene editing tools, it is suitable for generating a gene knockout cell line for this research.

4.2 Hypothesis

AXL can be knocked out with CRISPR-Cas9 techniques in T24 cell lines by introducing sgRNA that is complementary to the Exon 2 region of *AXL*. By creating INDELs, the knockout form can be tested with PCR or western blotting.

4.3 Aims

To knock out *AXL* in bladder cancer cells using the CRISPR-Cas9 method by inducing sgRNAs targeting the Exon2 region. To establish an AXL-deficient T24 bladder cancer cell line for further research.

Specific aims:

- To design sgRNA for *AXL* gene knockout in the Exon2 region.
- To obtain a plasmid containing sgRNA using bacterial transformation, restriction digestion, and ligation. To amplify the vector containing sgRNA after ligation by bacterial transformation.
- To transfect bladder cancer cells with a plasmid containing sgRNA.
- To select the AXL knockout cell lines by screening with Western Blotting.

Materials and Methods

To design sgRNA for Axl gene knockout in the Exon 2 region

sgRNA pairs were designed by using the Santa Cruz Genome Browser by the University of California (UCSC genome browser, <https://genome.ucsc.edu/index.html>). First, the PAM sequence was searched. UCSC genome browser automatically searches for this sequence when the function “CRISPR target” is activated, and suggests potential sgRNA with a specificity score and hypothetical efficiency. For this research, 5'-NGG is the favored PAM sequence. Next, the individual suggested sgRNA were eliminated by several factors: if the sequence is not 20 base pairs long; if the GC content is not between 30-80%; if the sequence is not starting with a G in the 5' in sense strand, otherwise, RNA Polymerase III (PolIII) will not be activated; and sequence containing more than four consecutive thymine (T) were excluded, because it might interfere the PolIII function for transcribing with U6 promoter in the plasmid that was selected. Then, a complementary sequence of selected sgRNA was obtained, so that the two sgRNAs complementary to each other can anneal together and form gRNA, or the term “oligo” will be used further. The next step was to add overhangs, 5'-CCAC-3' for the sense strand, and 5'-AAAC-3' for the antisense strand. This will help oligo to ligate to the plasmid that was cut by the BsaI restriction enzyme, allowing us to obtain the plasmid containing the designed oligos. The oligos were ordered from the National Center for Biotechnology, Kazakhstan, and the Nazarbayev University Core Facility. One pair of sgRNA can be used for knocking out the gene; in this case, the outcome can be detected with Western Blotting to check if the corresponding protein is expressed. Another case is using two pairs of sgRNA with a certain number of nucleotides between them. This will allow the deletion of the fragment of gene in between those sgRNAs, and create a frameshift mutation or premature stop codon as previous case. The advantage of using two pairs of sgRNAs is that it is easier to detect using PCR, unlike a more complicated and time-consuming Western Blotting.

To design and check primers for deletion

If two pairs of sgRNA are going to be used for knocking out the gene, primers are needed to detect the deletion. For example, the length of a certain gene is 400 base pairs, and two pairs of sgRNAs were designed with 100 base pairs in between. Forward and reverse primers can be designed upstream and downstream of this gene. Therefore, if sgRNA and Cas9 worked well, the expected outcome of PCR is a fragment of 300 base pairs, while the wildtype shows a 400-base-pair fragment, this can be the indication of the knockout of the gene. For designing primers, the gene sequence of AXL was obtained from the UCSC genome browser, and the fragments upstream and downstream of Exon 2 was selected as forward and reverse primers. The forward primer followed the sense strand, while the reverse primer was taken from the antisense strand. Some inclusion criteria are: the length is 17-24 base pairs; the difference between the melting temperature of forward and reverse primers is not too much, ideally similar; the GC content is around 40-60%. Then, the amplification result, and the secondary structure were checked in the Snap Gene program with the PCR function before ordering primers from Nazarbayev University Core Facility.

After obtaining the primers, they were checked with doing PCR with DNA obtained from trypsinizing T24 cells. Since there are two forward primers and two reverse primers, four different combinations (F1+R1, F1+R2, F2+R1, F2+R2; F stands for forward, R stands for reverse) were tested.

Plasmid selection

pRG-2 plasmids were selected in this research (Addgene plasmid, Catalog # 104174). This plasmid contains the U6 promoter, and BsaI cutting sites immediately after the promoter, and another similar cutting site located around 670 nucleotides downstream from the previous BsaI cutting site. U6 promoter allows the transcription of the whole plasmid. This plasmid also contains the Ampicillin resistance gene, making sure to kill all bacteria that are not transformed with this plasmid in the

ampicillin-containing media. Also, the Cas9-containing plasmid is p3S-cas9-1 (Addgene Plasmid, Catalog # 104173), containing the gene for the Cas9 protein and the ampicillin resistance gene for similar purposes.

LB agar plate and LB Broth preparation

LB Agar (Lennox L Agar, Catalog # 22700041) powder from Invitrogen was used in a proportion of 37g per 1000 mL of water to prepare LB agar, and LB Broth powder was used in a proportion of 40g per 1000 mL of water to prepare LB Broth solution, according to the manufacturer's suggestions. Solutions were sent to the autoclave for 20 minutes before use. For preparing LB agar plates, first, the solution was heated to reach the liquid state, and after cooling down to around 40-50°C, ampicillin was added in the volume ratio of 1µL of 100X ampicillin per 1 mL of LB Agar solution. The same ratio was used to prepare the LB broth solution with antibiotics. After mixing antibiotics gently, the solution was poured into petri dishes and left for some time under the hood for solidifying and vaporizing excess water.

Amplifying pRG2 and Cas9 plasmids

pRG2 and Cas9 plasmids were obtained from the stock in the lab, and it is transformed into the GH5-α strain of *Escherichia coli* bacteria for amplification. Competent GH5-α cells for each transfection were put on ice for 30 minutes. Then, pRG2 and p3S cas9-1 plasmids were induced separately into each competent cells separately and again put on ice for 30 minutes. Next, cells went through a heat shock procedure, 42°C for 45 seconds, then put in ice for 5 minutes, according to Dr Guschin's protocol (2024). Furthermore, bacteria were seeded to agar plates contain ampicillin. The plates were incubated at 37°C overnight. The next day, 1-2 colonies grown in the plate were selected, and mixed in 2 mL of LB Broth containing ampicillin to grow overnight in the shaker at 37°C. The next day, Miniprep plasmid isolation was performed with the Plasmid Miniprep 2.0 Kit (Evrogen, Catalog # BC121S) according to the protocol provided in the kit. Purity and concentration were tested

with a Nanodrop (Nanodrop 2000C spectrophotometer, Thermo Scientific). Also, 1% TAE gel electrophoresis was performed with 1kb DNA ladder (NEB, #N3232L) and DNA loading dye (NEB, #B7025S; Thermo Scientific™, #R1161) to test if the molecular weight of the isolated plasmid aligned with the original plasmid from the stock.

Restriction Digestion and Gel Extraction

The isolated pRG2 plasmid was digested with the BsaI enzyme (NEB #104173) to form a fragment that can be further ligated with oligos we designed. SmartBuffer (NEB # B6004S) was selected for digestion according to the NEBcloner (<https://www.neb.com/en>). pRG2 plasmid isolate was added to the solution containing SmartBuffer, BsaI enzyme, and distilled water. The solution was incubated at 37°C for 1 hour to perform digestion, and enzyme inactivation was done by allowing the temperature to reach 65°C and incubating for 10 minutes. After treatment with BsaI, two fragments weighing approximately 700 and 2500 should be formed. Restriction digestion for Cas9 was performed similarly, but with the HindIII enzyme (NEB #R3104S). 1% TAE agarose gel electrophoresis with 100 ng DNA materials loaded under the condition of 60V for an hour was performed, and Imaging was done with the Chemidoc apparatus (The ChemiDoc MP Imaging System, BioRad) with UV lights on since the gel contained Ethidium Bromide.

Gel extraction was done with the E.Z.N.A® Omega Gel Extraction Kit. 1% TAE gel electrophoresis with previously digested pRG2 vectors was performed for an hour, and a higher-weighted band was extracted with a scalpel under a UV light source. Then, the gel cut was further processed to purify DNA content by using the protocol suggested in this kit. Results were again checked with the Nanodrop apparatus for purity and concentration, and 1% agarose gel electrophoresis with a 1kb DNA ladder to test the approximate weight. After this step, the sgRNA can be inserted into the fragment that was extracted from the gel.

Transformation of Plasmid with Oligos to Competent Cells

To obtain the plasmid containing our sgRNAs designed before, annealing of sgRNAs, ligation, and transformation should be done. For annealing, sense and antisense sgRNA were added to SmartBuffer according to Prof. Guschin's protocol (2024). After mixing with the shaker, solutions were put in the T-100 PCR machine with the following program: +95°C for 3 min, +95°C to +85°C in - 2°C /sec. increment, +85°C to +25°C in - 0.1°C/sec., Hold at +4°C. After annealing and obtaining gRNA, ligation was performed with the Invitrogen kit (Anza™ #IVGN2108). Annealed oligos and previously isolated vector fragment was added together with ligase mixture. To test whether insertion of sgRNA was successful, a negative control was also obtained, with water added instead of oligos. The solution was kept at room temperature for 30 minutes. Then, transformation was done with the TOP10 chemically competent cells protocol. Bacterial mixture was added to LB Broth with the correct volume of ampicillin and put in an incubator at 37°C for 30 minutes. Then, bacteria were seeded onto an agar plate and incubated overnight in a CO₂-rich environment. The colonies grown in experimental plates should be 5-10 times more than the negative control, according to Prof. Guschin (2024).

Colony PCR

To make sure that bacteria grown in a plate contain the oligo inserted, colony PCR was performed, and the results were checked with gel electrophoresis. For colony PCR, 1 colony from agar plate was suspended in water, and was added to the solution containing U6 as forward primer and antisense of oligo as reverse primer with Red Taq Master Mix (Life Science, #5200300-1250). This will allow the generation of 100 base pair fragment (the length between U6 primer and antisense sequence in the plasmid) if the oligo were successfully ligated into plasmid. This can be confirmed by performing 2% TBE gel electrophoresis with 100 base pair DNA ladder (NEB, #N3231L).

Isolating Plasmid Containing Oligo Inserted

Miniprep isolation (Plasmid Miniprep 2.0 Kit, Evrogen, Catalog # BC121S) of plasmids was performed first to check the quality of the plasmid with insertion, following the Nanodrop to test purity and concentration, and imaging to see the weight. Then, Midiprep (Plasmid Midiprep 3.0 Kit, Evrogen, Catalog # BC224) or Maxiprep (E.Z.N.A® Plasmid DNA Maxi Kit, Omega, Catalog # D6922-00S) was done following the company's recommended protocol for isolating a higher amount. Nanodrop was done to test purity and concentration, and imaging was done to see the weight, following precipitation to reach a higher volume.

Gene Sequencing

Isolated plasmids containing oligos were sent for sequencing at the National Center for Biotechnology, Kazakhstan. Before sending the plasmids, the concentrations were obtained with Nanodrop. Plasmid was added to U6 primer and BigDye (BigDye™, Thermo Fisher Scientific, Catalog # 4336917) with water, then samples were put in a PCR machine, set up as 1 min of denaturation at 95°C, then 25 cycles consisting of 10 s denaturation, 5 s annealing, and 4 min elongation, mixture was cooled to 4°C lastly. The solution was then sent for Sanger Sequencing in the facilities at the National Center for Biotechnology (Astana, Kazakhstan).

Preparing Cell Culture for Transfection

T24 bladder cancer cell line was used in this experiment. This cell line was obtained in 1973 from a female patient who had transitional cell carcinoma in her bladder (Harding & Theodorescu, 2007). T24 bladder cancer cell line was obtained from the stock, thawed from the cryovial, DMSO were removed by centrifugation, seeded in T25 flask, and grown in the medium consisting Dulbecco's Modified Eagle Medium (DMEM) with high glucose (Gibco™, Catalog # 41965120), in addition with 10% Fetal Bovine Serum (FBS), 1% Penicillin-Streptomycin (CORNING, Catalog #

30002367), 1% MEM Non-essential amino acids (MEM NEAA, Gibco™, Catalog # 11140050), and placed in an incubator with 37°C degree and 5% CO₂ level. After reaching 90% or more confluency, cells were passaged to a T75 flask by trypsinizing with Trypsin EDTA for 3-5 minutes in an incubator, and neutralized by adding twice the volume of complete medium. Cell counting was performed before seeding cells into 24-well plates with a hemocytometer. For freezing the cells, cells were mixed in 9 mL complete medium in addition to 1 mL DMSO, and frozen at -80°C, to obtain more T24 cells for the experiment. For washing the cells, PBS solution was used. All cell works were done under the biosafety cabinet, and all lab consumables were disinfected with ethanol and sterilized with UV light under the hood before usage.

Transfection

To test if all plasmids with oligos are suitable for transfection, all plasmids were transfected with the pEGFP plasmid when cells reached 90% or more confluence in 24-well plates with Lipofectamine 3000 Reagent (Invitrogen, Catalog # 2966989) Protocol. 2 rows of tubes were prepared and labeled for each oligo. In the first row, tubes contained Opti-MEM (Gibco™, 31985-070) and Lipofectamine 3000 reagent; in the second row, tubes contained OptiMEM medium, plasmids (oligo+pEGFP), and enhancer P3000™ (Invitrogen, Catalog # 2293284) reagent. Solutions from row 2 were added to the corresponding tubes in row 1, after vortexing at maximum for 5 seconds, and solutions were incubated at room temperature for 10 minutes. Then, solutions were added on top of complete media containing cells. Transfection efficiency was obtained by observing under the fluorescence microscope.

For CRISPR-Cas9 transfection of the plasmid containing oligo, approximately 8000 T24 cells were seeded into 24-well plates with 500 µL complete media. Transfection was performed when cells reached different confluence, or double transfection at both 30% and 50%, was performed. This time, plasmids containing gRNA with plasmid containing Cas9 were transfected together, with a control

pEGFP plasmid to check if the transfection happened. Having two oligos with some nucleotides (not a factor of three) in between will allow the deletion of the genes and make a frameshift mutation leading to a similar Indel outcome. Whether transfection occurred was observed under the microscope, the pEGFP sent green signals, and was checked after 24 hours and 48 hours. Then, whether gene knockout was performed was tested by using Western Blotting after growing single-cell clones.

Single Cell Cloning

Cells 48 hours after transfection were passaged to 96-well plates as 1 cell per well, 2 cells per well, and 0.5 cells per well. Cells grown in 24-well plates were counted with a disposable hemocytometer, and calculations were done to make sure that the number of cells in each plate was correctly achieved. Each well of 96-well plates contains 100-150 μ L of complete media, and they are changed every 3-4 days. After the 5th day of seeding into 96-well plates, wells that did not contain any colonies or contained more than one colony were excluded from the experiment. The cells were grown for 15 days before transferring to 24 well plates, and the total number of colonies left was 25 out of 288 wells. 18 clones were successfully transferred to 24-well plates. After cells reach more than 80% confluence, cells were further split into two 6-well plates.

Combining the sgRNA to test the knockout with PCR

Since cloning and Western Blot screening take time, this step of the experiment was done in collaboration with a Master's in Molecular Medicine student at Nazarbayev University, Sandugash Myrzagali, who is also doing her thesis work aiming to perform AXL whole gene knockout, using sgRNA targeting Exon 1 of the gene (sgRNA: 5'-CACCGGGGCGTGCATGGCCCCCAG-3'). For transfection, around 100 ng of sgRNA targeting Exon 1 designed by her, around 100 ng of sgRNA targeting Exon 2 designed by me (oligo 1), and around 100 ng of Cas9 plasmid were transfected into T24 cells in 24-well plates. With two pairs of sgRNA, if both worked well with Cas9 and induced two DSBs, the site for cut will anneal back together, but deleting the gene between breaks. 48 hours after

transfection, some samples from wells were taken for PCR with the forward primer my colleague designed (TGGGAGCCCAACAACCTTCTG) and the reverse primer I designed (CTTTCTGCCCTCTGTGTGACG). The expected outcome after PCR is a band in gel electrophoresis around 380 base pairs, meaning the sgRNAs work well, and there is a chance that the gene might be knocked out. By this experiment, we can test if the sgRNA is working.

Western Blotting Screening

Western blotting is needed to perform to screen the single clones. If any clone did not show AXL protein expression, this clone is obtained for further experiments, and meaning the goal of this project is to be reached. Before western blotting, cells were transferred from 96 well plates to 24 well plates. And after reaching more than 80% confluence at 24-well plates, cells were further split into two 6-well plates. After reaching more than 80% confluence, cells can be collected, and samples can be prepared for western blotting.

Results

To design sgRNA for Axl gene knockout in the Exon 2 region

Table 1. sgRNAs targeting the Exon 2 region of AXL, complementary to sense and antisense, were designed with the USCS Genome Browser, and were ordered from the National Center for Biotechnology (Astana, Kazakhstan) and the Nazarbayev University Core Facilities.

sgRNA	Sense/Antisense	Sequence (5'-3')	Concentration
AXL Exon 2 S1	Sense	CACCGCTGAAGAAAGTCCCTTCGT	199.9 pmol/ul
AXL Exon 2 A1	Antisense	AAACACGAAGGGACTTTCTTCAGC	244.1 pmol/ul
AXL Exon 2 S2	Sense	CCACGAGGTACATTGGCTTCGGGA	133.8 pmol/ul
AXL Exon 2 A2	Antisense	AAACTCCCGAAGCCAATGTACCTC	155.6 pmol/ul

To design and check primers for deletion

Table 2. Primers were designed to be located in the Exon 2 region of AXL after selection based on length, melting temperature, GC content, and potential secondary structure.

Primer	Sequence (5'-3')	Concentration
Forward 1	CAAGGACAGGGTGGAACTGAG	97.1 pmol/ul
Reverse 1	CTTTCTGCCCTCTGTGTGACG	248.8 pmol/ul
Forward 2	GGGGTTCCTAAGCTAACTCTTCC	135.2 pmol/ul
Reverse 2	CGGCTGACAAGTGGTCAAACCTG	229.0 pmol/ul
U6 Primer	TGGACTATCATATGCTTACC	314.5 pmol/ul

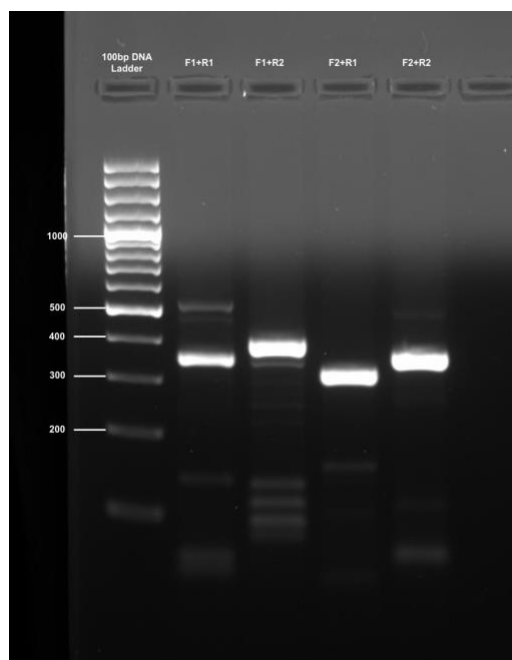


Figure 1. Gel electrophoresis of PCR using combinations of primers to test the primers. Columns from left to right: 100 base pair DNA Ladder, Forward 1 + Reverse 1, Forward 1 + Reverse 2, Forward 2 + Reverse 1, Forward 2 + Reverse 2. Solid bands corresponding to the actual distance between forward and reverse primers, meaning all primers working correctly.

Amplifying pRG2 and Cas9 plasmids

Bacterial plates containing pRG2 and Cas9 were obtained (Figure 2), and 1-2 colonies were grown overnight in LB Broth, and Miniprep plasmid isolation was successfully performed. The concentration and purity of plasmids were calculated with Nanodrop (Table 3), and gel electrophoresis was performed to test that the plasmid isolated has the same weight as the original plasmids obtained from the stock (Figure 3).

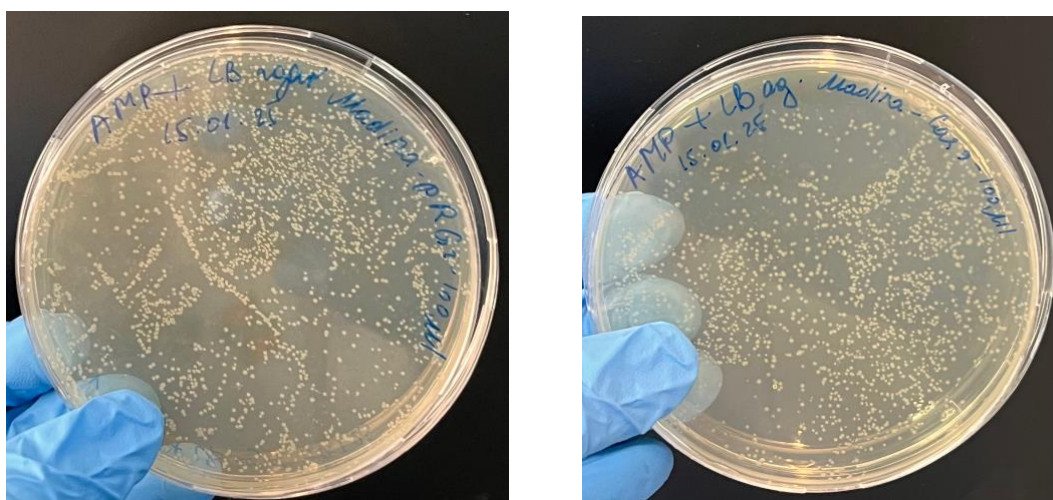


Figure 2. Bacterial plates containing pRG2 and Cas9.

Table 3. Nanodrop results of pRG2 and Cas9 isolation

Plasmid	Concentration (ng/ μ L)	260/280
pRG2	18.7	2.14
Cas9	56.2	1.99

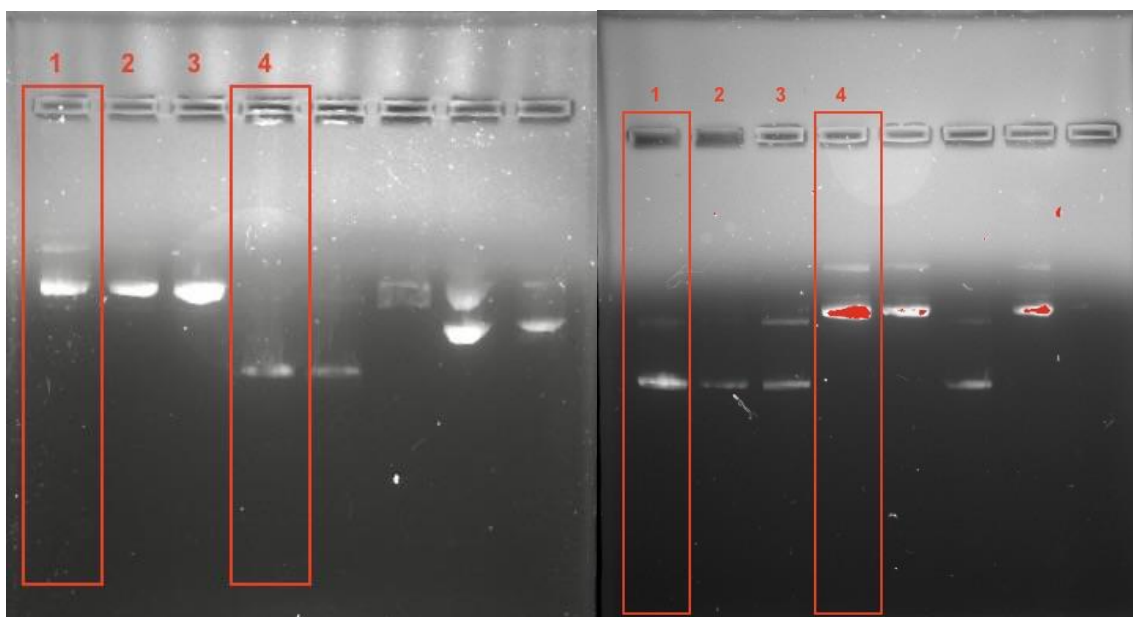


Figure 3. The gel electrophoresis result of the plasmid purification. The column numbered 1 is the control from stock, and column 4 is the results I obtained. On the left, the control plasmid is Cas9; on the right, the control plasmid is pRG2.

pRG2 Restriction Digestion and Gel Extraction

Since the concentration was low and the band showing in gel electrophoresis was not correct, plasmid isolation was done another time before starting restriction digestion, and the concentration and purity are stated in Table 4. Digestion was done for both plasmids selected before with low concentration with low purity, and a newly selected one with higher concentration, and the result of digestion was shown with gel electrophoresis (Figure 4).

Table 4. Nanodrop results of the second time pRG2 isolation

Plasmid	Concentration (ng/ μ L)	260/280
pRG2-1	158.2	1.88
pRG2-2	155	1.89

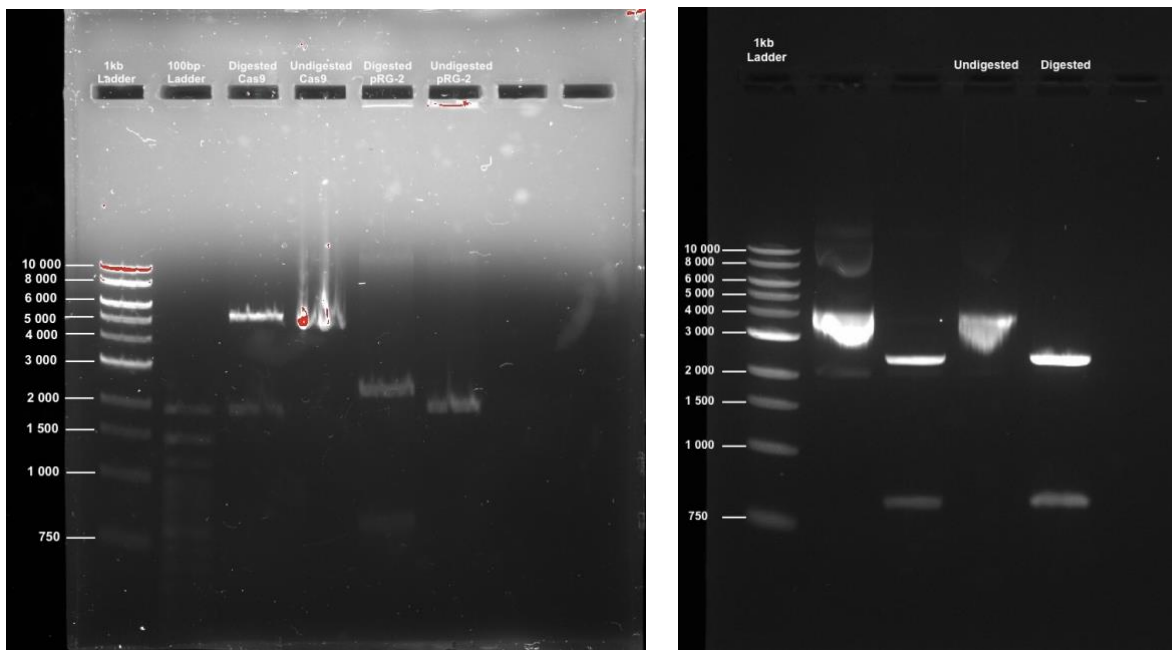


Figure 4. Gel electrophoresis results from restriction digestion. On the left, the pRG2 isolated had a low concentration, and there were non-specific bands on the top. On the right, there are newly selected plasmids with higher concentration and purity, showing clearer bands at 2000 and 750 base pairs, corresponding to the weight of digested fragments.

Gel extraction was performed in the digested plasmid in the electrophoresis. The nanodrop results (Table 5) and gel electrophoresis (Figure 5) were used to check the results.

Table 5. Nanodrop analysis of the concentration of the extracted vector from the gel.

Concentration (ng/ μ L)	Volume (μ L)	Mass (ng)	260/280
37.4	15	0.56	1.96

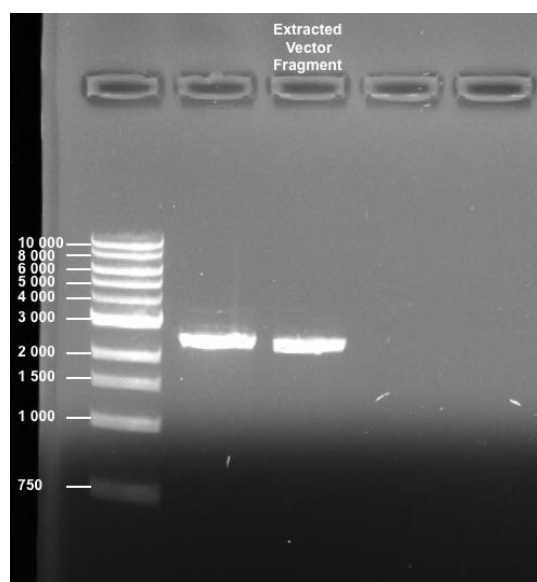


Figure 5. Gel electrophoresis of an extracted vector with a 1kb DNA ladder.

Transformation of Oligos to Competent Cells

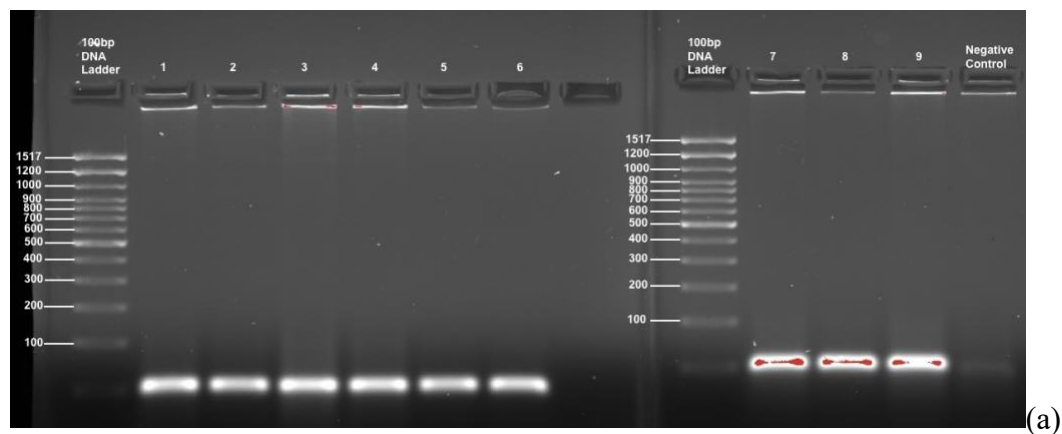
Annealing of 2 sgRNA pairs and ligation of the oligo to the extracted vectors was performed. Two transformations were performed for oligo 1, because bacteria did not grow in the first transfection of oligo 1, and the transformation without any plasmids was used as a negative control. The second transformation with oligo 1 was performed with a higher volume of plasmid with oligo and a higher volume of competent cells. The ratio of experimental plate and negative control is visible. Transformation of oligo 2 was done, and the results are shown in Table 6 as the ratio of number of colonies in comparison with negative control.

Table 6. Number of colonies in bacterial plates after transformation.

Plate + oligo	Number of colonies in experimental plate	Number of colonies in negative control
Oligo 1	0	0
Oligo 1 second transformation	~ 200	5-10
Oligo 2	~ 300	~ 300

Colony PCR

The results of colony PCR for oligo 1 are shown in Figure 6a. The result of colony PCR for oligo 2 is shown in Figure 6b, and since the gel electrophoresis result contained many non-specific bands, solid negative results were excluded, and parameters for PCR were adjusted, the result is shown in Figure 6c.



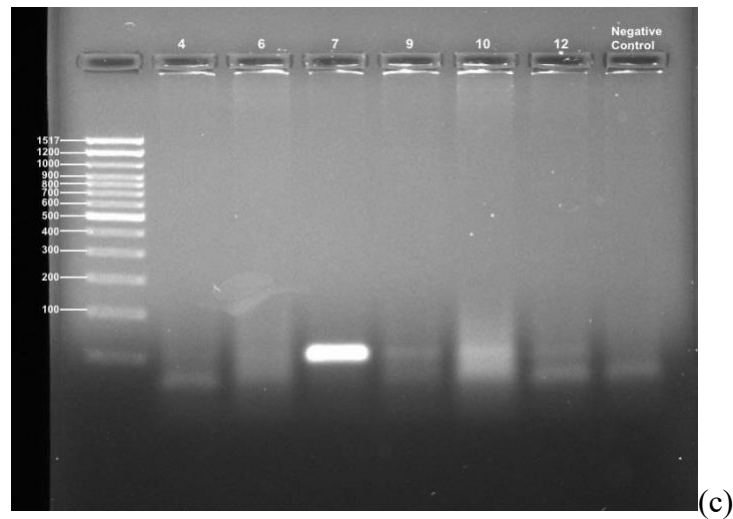
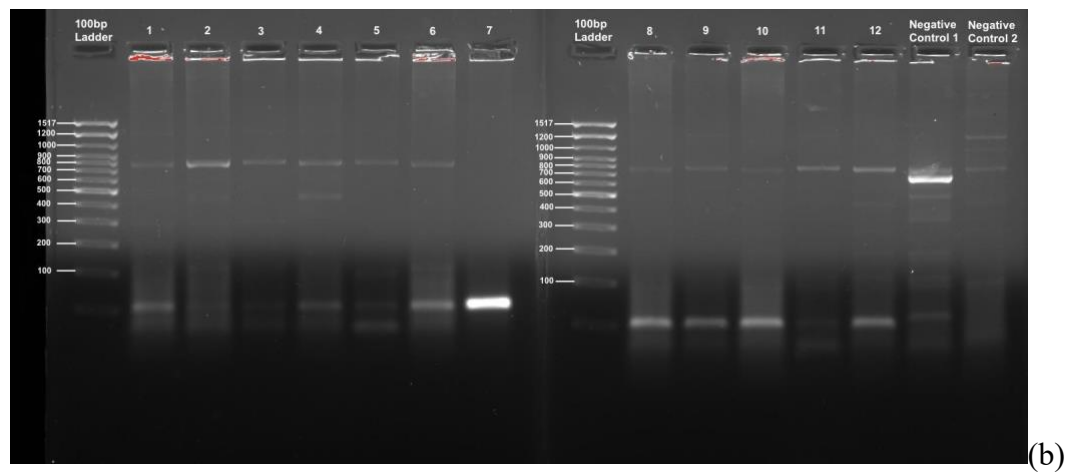


Figure 6. Results from colony PCR. (a) unsuccessful PCR for colonies containing oligo 1. (b) successful PCR for colonies containing oligo 1. (c) PCR for colonies containing oligo 2, first try. (d) PCR for colonies containing oligo 2, second try.

Isolating Plasmid Containing Oligo Inserted

After making sure the plasmid contains the oligo we designed, plasmid isolation was done with Miniprep first. Gel electrophoresis for plasmid with oligo 1 was also done to test the weight of the isolated plasmid (Figure 7).

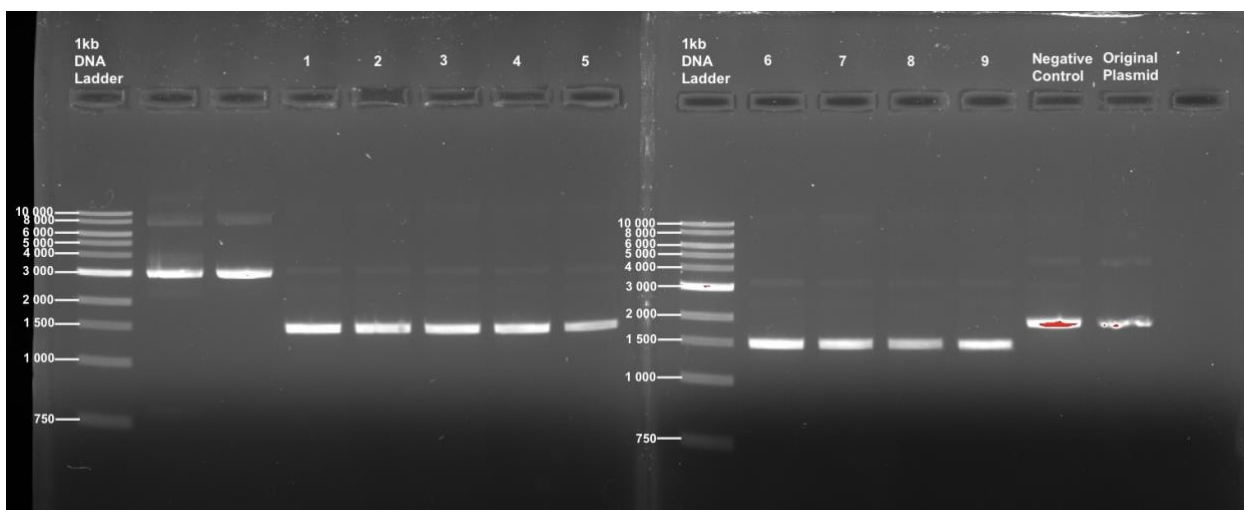


Figure 7. Gel electrophoresis results of a plasmid containing oligo 1 isolated with Miniprep.

Miniprep isolation of plasmid was also performed for plasmids in several colonies containing oligo 2. Gel electrophoresis was performed to test the weight of the isolated plasmid (Figure 8).

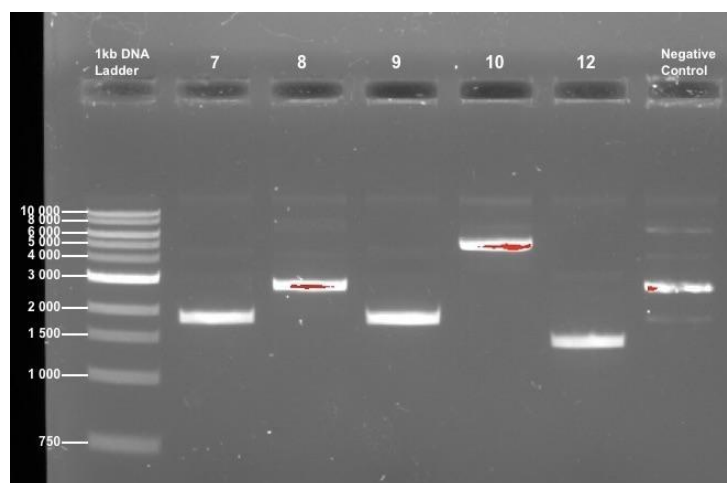


Figure 8. Gel electrophoresis results of Miniprep isolation of plasmids isolated from several colonies of bacteria contain oligo 2 transformation.

Furthermore, Midiprep isolation was done for isolating plasmids containing oligo 1 from 1 colony, concentration and purity were tested with Nanodrop (Table 7), and gel electrophoresis was done to test the weight (Figure 9a). Maxiprep isolation of plasmids with oligo 2 transformation was done with colonies 7 and 12, tested with Nanodrop (Table 7), and gel electrophoresis (Figure 9b).

Precipitation was performed to reach higher concentration for further experiment, final results are also shown in Table 7.

Table 7. Nanodrop results for Midiprep and Maxiprep isolation of plasmids containing oligos 1 & 2.

Sample	Initial Concentration (ng/ μ L)	260/280	Final Concentration (ng/ μ L)
Oligo 1 (Midiprep)	87.8	1.90	782.2
Oligo 2.7 (Maxiprep)	187.6	1.86	1222.4
Oligo 2.12 (Maxiprep)	126.4	1.85	1253.7

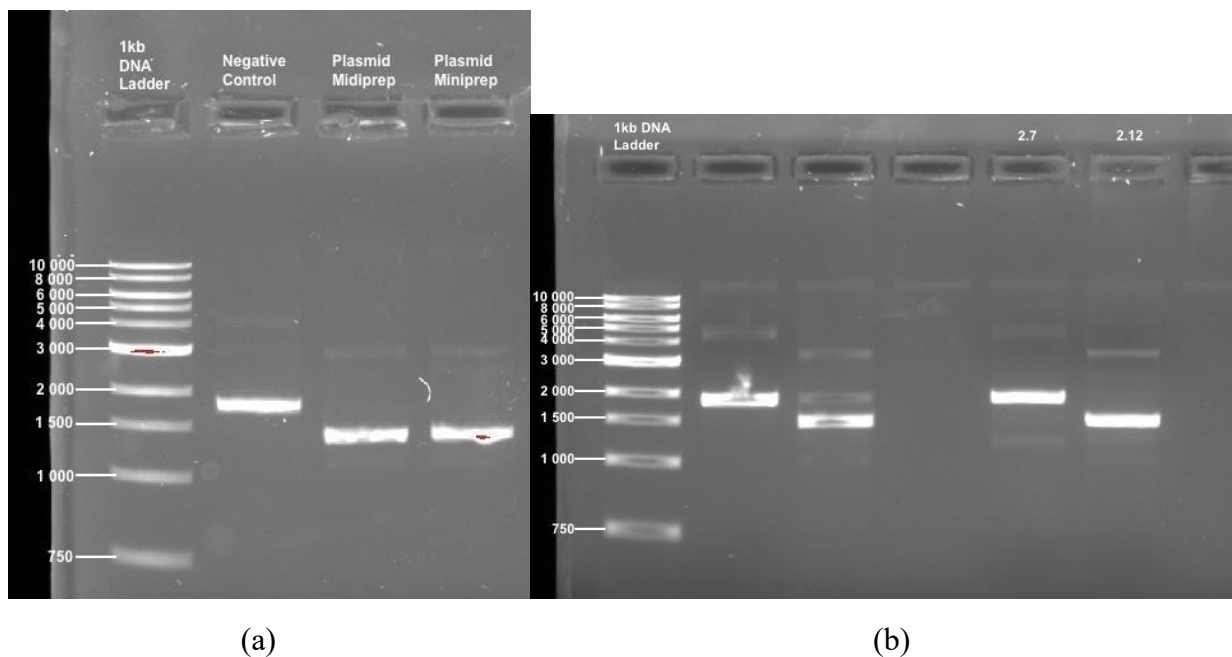


Figure 9. (a) Gel electrophoresis results of plasmid containing oligo 1 with Midiprep, in comparison to negative control and plasmid isolated from miniprep, with 1 kb DNA ladder as reference. (b) Gel electrophoresis results of two plasmids from bacterial transformation with oligo 2, colonies #7 and #12, with a 1kb DNA ladder.

Gene Sequencing

Results of Sanger sequencing were obtained from NCBI. The summary of sequencing is provided below in Table 8.

The result shows that the plasmid with oligo 1 ligated is the plasmid containing the correct sgRNA. However, the plasmid with oligo 2.7 did not contain any oligos, but the original sequence, and the plasmid with oligo 2.12 does not contain oligo 2 but oligo 1.

Table 8. Summary of the results from Sequencing

Plasmid	Expected sequence after 5'-CCAC	Sequence after 5'-CCAC	Final result
Oligo 1	GCTGAAGAAAGTCCCTTCGT	GCTGAAGAAAGTCCCTTCGT	Oligo 1
Oligo 2.7	GAGGTACATTGGCTTCGGGA	Original plasmid	None
Oligo 2.12	GAGGTACATTGGCTTCGGGA	GCTGAAGAAAGTCCCTTCGT	Oligo 1

PCR to check the unexpected results

Unexpected results were obtained with the samples 2.7 and 2.12; those samples without oligo 2 still showed bands after PCR with U6 primers and the antisense strand of oligo 2. Therefore, PCR needs to be done with both antisense from oligo 1 and 2, which were obtained from the stock, to eliminate the possibility of contamination. Plasmids containing “oligo 1” and “oligo 2” were individually amplified with U6 + antisense from oligo 1, and with U6 + antisense from oligo 2 to test which oligo this plasmid contains. The results from gel electrophoresis show that both plasmids containing “oligo 1” and “oligo 2” showed amplification when treated with U6 + antisense from oligo 1 as primers (Figure 10).

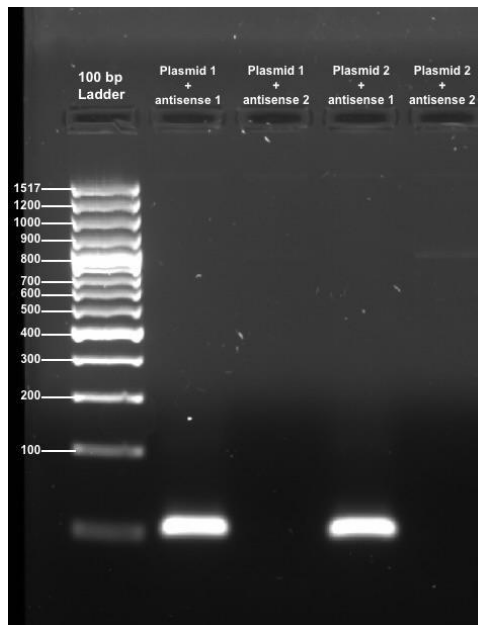


Figure 10. Additional PCR experiment to check how the unexpected results happened.

Transfection

Before starting actual CRISPR-Cas9 transfection, all 3 plasmids (Oligo 1, Oligo 2.7, Oligo 2.12) were transfected into T24 cells with the pEGFP plasmid to test the efficiency of each vector. The results are shown in Figure 11. Transfection with Oligo 1 (Figure 11b) is less efficient than transfection with only pEGFP (Figure 11a), which can be seen from the ratio of fluorescent cells and the plate. The transfection of Cas9 is low, only showing several cells in the fluorescence field.

Transfection of plasmid with oligo and Cas9 is performed at lower (Figure 12b) or higher (Figure 12f) confluence for single transfection, and at 30% and 50% confluence for double transfection (Figure 12d).

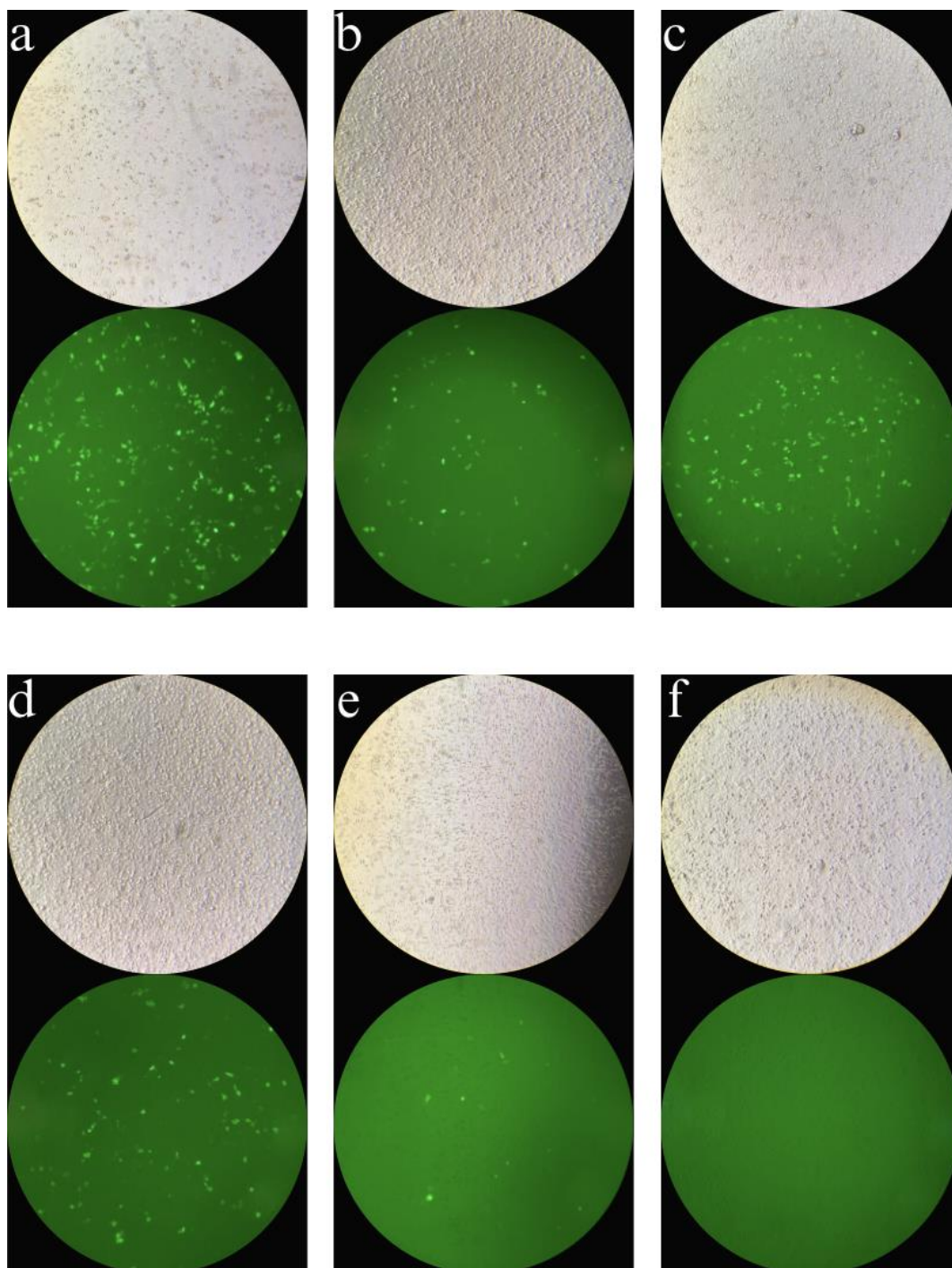


Figure 11. Results from transfection after 24 hours: (a) only pEGFP plasmid; (b) pEGFP+oligo 1; (c) pEGFP+oligo 2.7; (d) pEGFP+oligo 2.12; (e) pEGFP+Cas9; (f) no plasmid. The top observations are with light fields, and the bottom ones with fluorescence filters.

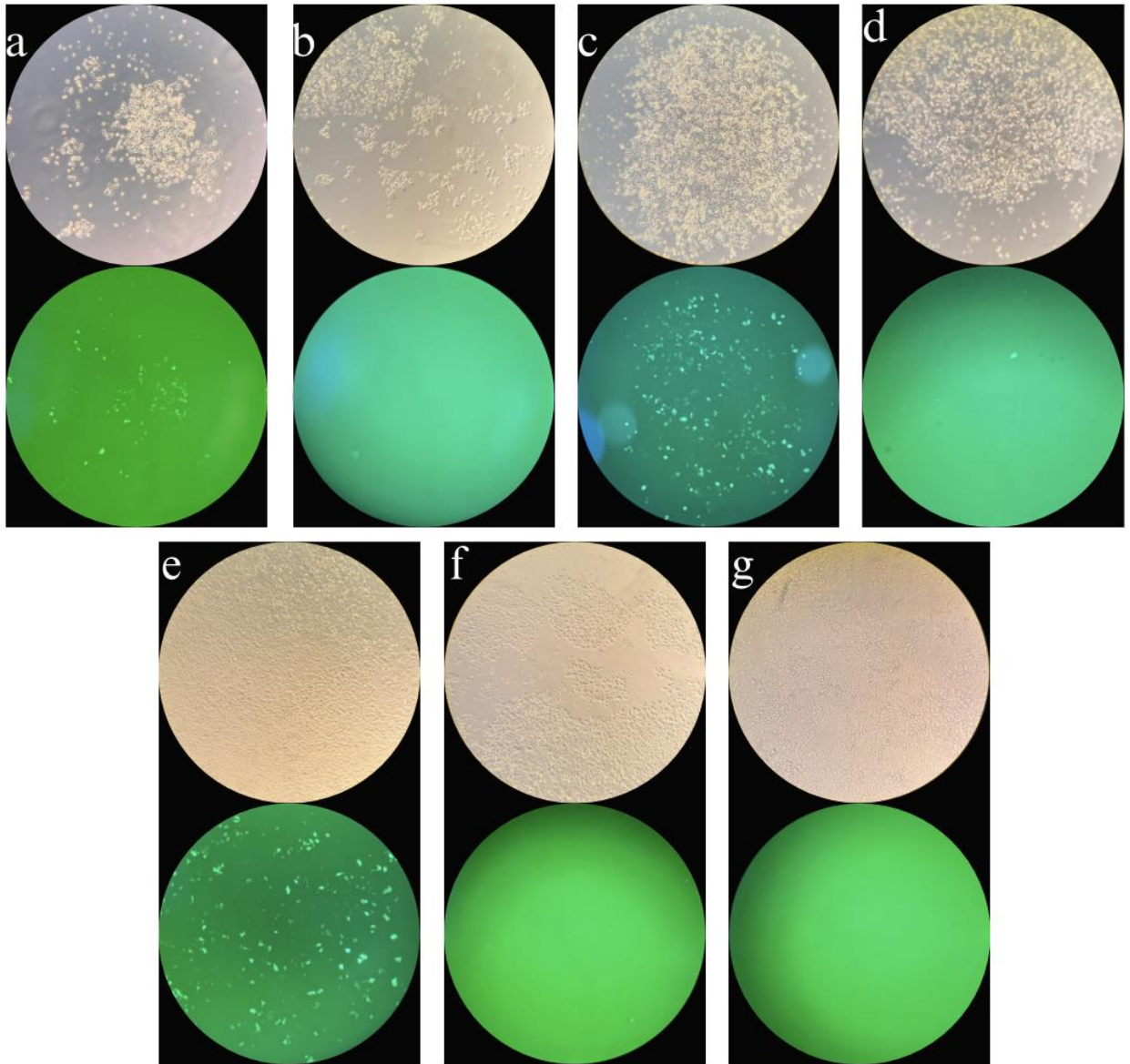


Figure 12. Results from transfection after 24 hours: (a) transfection of pGFP at lower confluence; (b) transfection of Oligo1+Cas9 at lower confluence; (c) double transfection of pEGFP at 30% and 50% confluence; (d) double transfection of Oligo1+Cas9 at 30% and 50% confluence; (e) transfection of pGFP at higher confluence; (f) transfection of Oligo1+Cas9 at higher confluence; (g) cells without any transfection. The top observations are with light fields, and the bottom ones with fluorescence filters.

Single Cell Cloning

Cells were seeded in three 96-well plates with a concentration of two cells per well, one cell per well, and 0.5 cells per well. Five days after seeding, the wells did not contain a single colony were excluded from the research. In the end, only 25 clones out of 288 wells were obtained. These clones were seeded into 24-well plates, in total 18 clones were successfully growing in 24 well plates. All results are shown in Figure 13. After cells reached 80% confluence in 24 well plates, cells were split into two 6-well plates, one for storing clones, and another for western blotting.

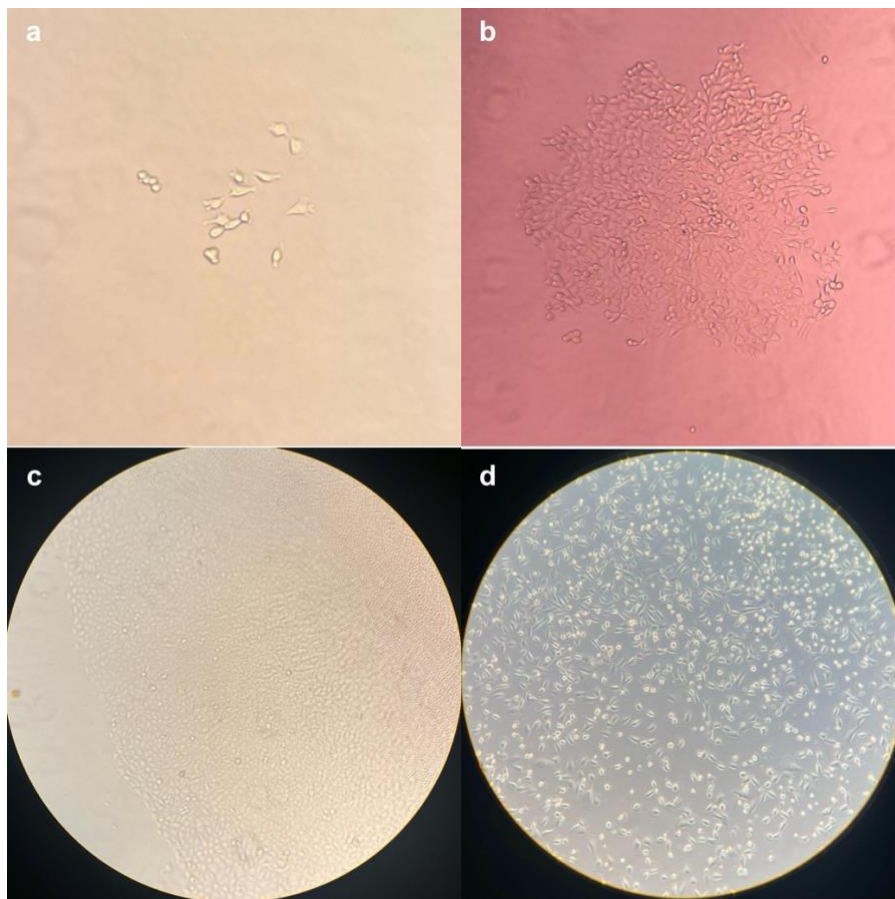


Figure 13. Results of single cell cloning. (a) single cell clone 4 days after seeded to 96-well plates; (b) clone 8 days after seeded to 96-well plates; (c) clone 15 days after seeded to 96-well plates; (d) clone after transferred to 24-well plates

Combining sgRNAs

The results of PCR indicated in the gel electrophoresis showed that there was a deletion combining two sgRNA pairs (Figure 14). After amplifying with forward primer designed by my colleague (sequence: TGGGAGCCCAACAACCTTCTG) and reverse primer 1 designed by me (sequence: CTTTCTGCCCTCTGTGTGACG), the wildtype only showed single band around 1500 bp, while cells transfected with two sgRNA shows one band at 1500 bp, another band at 400bp.

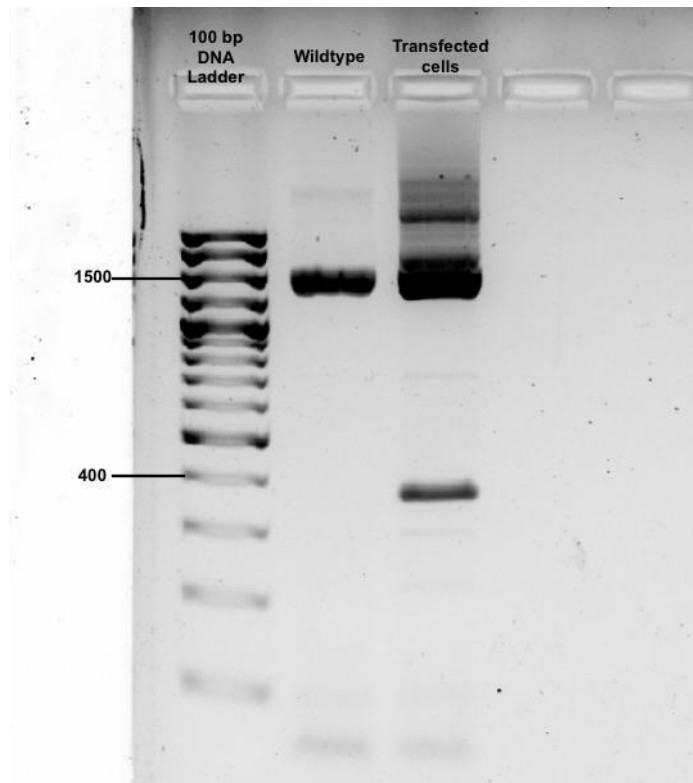


Figure 14. Results from PCR in gel electrophoresis, from left to right: 100 bp DNA ladder, wildtype, and transfected cells.

Western Blotting Screening

The results of Western blotting have not been obtained yet because of the time limitation.

Discussion

The T24 clone without AXL expression has not been obtained yet, because the experiment is not finished. The clones should be screened further with Western Blotting to find the one without AXL expression. However, based on the gel electrophoresis results of combining two pairs of sgRNA located in Exon 1 and Exon 2 of AXL, we can confirm that the deletion happened between those two pairs of sgRNAs' target sites, and this can suggest that the sgRNA designed were working. According to Figure 14, the wildtype only shows around 1500 bp, while cells transfected with two sgRNA containing plasmids, one band at 1500 bp, another band at around 400 bp. After confirming that the primers designed works properly (Figure 1), they can be used in PCR. The length of the fragment between 2 primers according to UCSC Genome Browser is 1519 base pairs, and if the sequence between two pairs of sgRNA are deleted, the length between those two primers is 380 base pairs, which corresponds to the Figure 14. This is the evidence that in some cells, sgRNA worked well to induce DSB. AXL might be knocked out successfully. Since both pairs of sgRNA are working in knocking out the AXL, there is a possibility that the clone without AXL expression can be obtained after Western Blotting.

The experiment was going smoothly on previous steps most of the time, but some aspects also failed, and the results needed to be discussed in detail. Firstly, during the step of Miniprep plasmid isolation of Cas9 and pRG2 for the first time, those two plasmids were accidentally mixed, and therefore showed different bands in comparison to the original plasmid in Figure 3. Also, the Cas9 vector (here I assumed this is Cas9 because it shows a higher band compared to pRG2) has non-specific bands, which can be a clue of contamination with other DNA materials. Moreover, the concentration and purity were low, less than 50 ng/ μ L, and more than 2.00 260/280 absorbance ratio (Table 3). It is recommended to have purity around 1.80 in the 260/280 absorbance ratio for "pure" DNA (Lucena-Aguilar et al., 2016). Therefore, another plasmid isolation was done, and the

concentration with purity is good enough, that is more than 150 ng/ μ L (Table 4). Also, in gel electrophoresis, those two plasmids showed the correct weight compared with the 1kb DNA Ladder (Figure 3). Meaning that those two plasmids are ready to use for restriction digestion, which is the next step.

For restriction digestion, the expected weight after digestion is around 670 and 2500 base pairs for pRG2, and around 2000 and 5000 base pairs for Cas9. This result is confirmed with gel electrophoresis after digestion, as shown in Figure 4. There were two bands for digested plasmid, and only one band is shown in the undigested plasmid. This confirms that digestion with BsaI was successful. Another gel with only digested and undigested pRG2 was run, as shown in Figure 4b. However, the undigested band is located lower than the higher band of the digested one, this can be explained by the supercoiling nature of plasmids when they are not digested. Here, the conclusion is correct restriction digestion was made. Further, plasmid extraction from gel is performed with the Omega plasmid extraction kit, and an analytical gel with 1kb DNA Ladder was run, showing that the extracted plasmid has only one clear band at around 2500 bp (Figure 5). The concentration was near 40 ng/ μ L. And the purity is also near perfect, showing 1.96 on the 260/280 absorbance ratio (Table 5). To conclude, extracted pRG2 plasmid fragment that is treated with BsaI enzyme is ready to be ligated with sgRNAs we designed, and then the results can be tested by bacterial transformation, because the pRG2 plasmid contains ampicillin resistance gene, which can help bacteria to survive the environment contains this antibiotic. This is also a good selection for bacterial, only those who has successfully obtained the plasmid will survive.

Bacterial transformation was done after annealing of sense and antisense sgRNA pairs, and ligation of oligos to BsaI treated vector. The results of the first bacterial transformation with the first oligos did not show any results; bacteria did not grow in the plate, where all the conditions favored the growth of bacteria. There might be any reasons at any stage: annealing, ligation, or transformation

is inefficient. To test which step was failed, transformation was done again with more ligated plasmid and more competent cells, and used the competent cell from a new source, because the efficiency of cells might be low. The results shows that the transfection was successful with visible ratio between plasmid contains oligo and negative control without oligo, because according to Professor Guschin's protocol, 5-10 times more colonies in experimental group is expected in comparison with negative control (Results were summarized in Table 6). If the transformation still fails for a higher volume of cells and plasmid, it means that the ligation and annealing need to be done again. For oligo 2, however, the transformation seemed to be successful, but the problem is negative control also contained with almost equal number of bacteria. This can be explained by contamination in the negative control.

Then, to test if the ligation is truly successful, colony PCR was done. Colony PCR requires U6 primer and antisense sequence of corresponding oligo as primers, and if the ligation occurred, there should be a band in gel electrophoresis with the length between U6 and antisense sgRNA in the plasmid, which is near 100 base pairs (Snap Gene). The result of the PCR shows that all colonies, except for the negative control, show a band less than 100 base pairs (Figure 6a). This means that oligo 1 was successfully inserted into the plasmid, and the plasmids are ready for amplification. The first PCR for the second oligo also showed non-specific bands (Figure 6b) by increasing the melting temperature in the PCR machine's settings. The results were still unclear (Figure 6c), because except colony 7, all other colonies did not show a strong band. The reason is unknown, but several colonies, after excluding the ones without a clear band, were selected to perform miniprep isolation of plasmids.

After inserting oligos, miniprep isolation of plasmids were performed. The weight showing in gel electrophoresis for oligo 1 shows perfect results, meaning, the plasmids from any colonies are ready to be amplified again and isolated with Midiprep or Maxiprep (Figure 7). However, the second oligo after miniprep isolation showed completely different patterns. There might be some contamination during the experiment with oligo 2, which might be the reason for why colony PCR

also did not show a consistent result. Despite there might be some contamination, colony 7 and colony 12 were still chosen for next experiments, since they showed approximately 2000 base pairs in gel electrophoresis, which corresponds to the molecular weight of plasmid after insertion of oligos (Figure 8). After Miniprep isolation, those colonies showing potential good results were seeded into another new agar plate containing ampicillin, so that it can be used further for plasmid isolation.

After miniprep isolation for checking the molecular weight of oligo-inserted plasmids, Midiprep or Maxiprep was performed. The results are shown in Table 7 and Figure 9. For oligo 1, Midiprep (Evrogen) was performed and the concentration was reached 87.8 ng/ μ L. Results from gel electrophoresis to test the molecular weight also showed that it is the same with plasmid selected with miniprep. For oligo 2, colonies 7 and 12 were selected with Maxiprep (Omega), and the concentration was more than 100 ng/ μ L. The gel electrophoresis shows that oligo 2.7 has a higher band than oligo 2.12. The reason could be that the BsaI digestion of oligo 2.7 was not fully successfully performed. The results can be known only from sequencing. The isolated plasmids from Midiprep or Maxiprep are concentrated with performing precipitation according to the corresponding protocols, because for transfection, higher concentration is needed. The final concentrations were reached as shown in Table 7.

The results from gene sequencing shows that oligo 1 is successfully inserted into the pRG2 plasmid (Table 8). However, the plasmid containing oligo 2.7 has the same sequence as the original plasmid. Additionally, the plasmid containing oligo 2.12 did not show an expected sequence of oligo 2, but instead showed the sequence of oligo 1. Which is the indicator of contamination with the original vector without digestion, and contamination with oligo 1 during ligation of oligo 2. How did these colonies show amplified bands in colony PCR with U6 and antisense or oligo 2 is still unclear, another experiment with checking with different antisense primers is needed.

For the additional PCR experiment to check how the unexpected results happened, the results from gel electrophoresis shows that both plasmids containing “oligo 1” and “oligo 2” showed amplification when treated with U6 + antisense from oligo 1 as primers (Figure 10). Which confirms the results from sequencing, for both ligation, the vectors were ligated with oligo 1. Also, during ligation “oligo 2” contamination not only occurred with original pRG2 plasmids and plasmids containing oligo 1, but the primers were also contaminated with antisense of oligo 1. That is why, throughout the experiment, the wrong oligo inserted tested with wrong primers showed false positive results. Since the time was limited, I needed to give up with sgRNA pair 2, and only work with sgRNA 1.

Despite the plasmids isolated showing expected molecular weight and good concentration and purity, how efficient is the transfection of this plasmid is unknown. Therefore, the transfection of each isolated plasmids with pEGFP plasmid should be done. The results were obtained with comparison with transfection with control which is the transfection with only pEGFP plasmid (Figure 11a). Plasmid 1 showed significantly less fluorescence protein compared to control, this plasmid was isolated from Midiprep (Evrogen), which is less efficient to extract a plasmid for transfection (Figure 11b). Plasmid 2 selected with Maxiprep (Omega) shows better signal compared to plasmid 1 (Figure 11d). Transfection of Cas9 plasmid shows only little signal (Figure 11e), which means this plasmid is not usable, and another isolation is needed to be done. Figure 11f is the cell without any transfection, showing that it does not show false positive results and the cell does not contain fluorescence protein. From this experiment, plasmid 2 is selected for further experiments, and the need to re-isolate Cas9 was concluded.

After selecting the plasmid for further transfection, Plasmid 2 is transfected with Cas9 for different confluence of T24 cells in 24 well plates, and for another case, double transfection was done. Cells did not really grow and survive after transfection at a lower confluence, these cells were

discarded, and did not seed into 96 well plates. Both double transfection at 30% and 50% and single transfection at a higher confluence showed a good signal in pEGFP transfection (Figure 12), and these cells were selected to seed into 96 well plates. Clones are obtained after seeding to 96 well plates, as shown in Figure 13 (a), (b), (c), and successfully transferred to 24 well plates (Figure 13d). After successfully splitting cells from 24-well plates to 6-well plates, all clones need to be test with Western Blotting to see if *AXL* gene is knocked out.

Conclusion

The aim of this study was to generate a T24 bladder cancer cell line without AXL expression using CRISPR-Cas9. Establishing this cell line can provide a valuable tool for future research investigating the role of receptor in cancer progression, drug resistance, and cancer immunity. In this experiment, sgRNA is successfully targeting *AXL*, according to the PCR result performed with combined sgRNAs. However, due to time limitations, the cell line has not been established yet. For future recommendation, additional pairs of sgRNA can be designed and used as the combination to obtain deletions, which is easier to be validated with PCR.

References

- Auyez, A., Sayan, A.M., Kriaievska, M., & Tulchinsky, E. (2021). AXL receptor in cancer metastasis and drug resistance: When normal functions go askew. *Cancer*, 13, 4864. <https://doi.org/10.3390/cancers13194864>
- Braunger, J., Lothar Schleithoff, Schulz, A. S., Kessler, H., Lammers, R., Ullrich, A., Bartram, C. R., & Janssen, J. W. (1997). Intracellular signaling of the Ufo/Axl receptor tyrosine kinase is mediated mainly by a multi-substrate docking-site. *Oncogene*, 14(22), 2619–2631. <https://doi.org/10.1038/sj.onc.1201123>
- Deng, T., Chen, Q., & Han, D. (2016). The roles of TAM receptor tyrosine kinases in the mammalian testis and immunoprivileged sites. *Front Biosci Landmark*, 21, 316-327. doi: 10.2741/4390.
- Dobruch, J., & Oszczudłowski, M. (2021). Bladder Cancer: current challenges and future directions. *Medicina*, 57(8), 749. <https://doi.org/10.3390/medicina57080749>
- Dufour, F., Silina, L., Neyret-Kahn, H., Moreno-Vega, A., Krucker, C., Karboul, N., Dorland-Galliot, M., Maillé, P., Chapeaublanc, E., Allory, Y., Stransky, N., Haegel, H., Menguy, T., Duong, V., Radvanyi, F., & Bernard-Pierrot, I. (2019). TYRO3 as a molecular target for growth inhibition and apoptosis induction in bladder cancer. *British Journal of Cancer*, 120(5), 555–564. <https://doi.org/10.1038/s41416-019-0397-6>
- Gushchin, D., (2024). CRISPR/Cas9-mediated gene knockout in cell lines using double cut deletions. Protocol for MMM students.
- Harding, M. A., & Theodorescu, D. (2007). RhoGDI2: A new metastasis suppressor gene: Discovery and clinical translation. *Urologic Oncology Seminars and Original Investigations*, 25(5), 401–406. <https://doi.org/10.1016/j.urolonc.2007.05.006>

- Lemke, G., & Rothlin, C.V. (2008). Immunobiology of the TAM receptors. *Nat Rev Immunol*, 8.
<https://doi.org/10.1038/nri2303>
- Lu, Y., Wan, J., Yang, Z., Lei, X., Niu, Q., Jiang, L., Passtoors, W. M., Zang, A., Fraering, P. C., & Wu, F. (2016). Regulated intramembrane proteolysis of the AXL receptor kinase generates an intracellular domain that localizes in the nucleus of cancer cells. *the FASEB Journal*, 31(4), 1382–1397. <https://doi.org/10.1096/fj.201600702r>
- Lucena-Aguilar, G., Sánchez-López, A. M., Barberán-Aceituno, C., Carrillo-Ávila, J. A., López-Guerrero, J. A., & Aguilar-Quesada, R. (2016). DNA source selection for downstream applications based on DNA Quality Indicators analysis. *Biopreservation and Biobanking*, 14(4), 264–270. <https://doi.org/10.1089/bio.2015.0064>
- Mao, S., Wu, Y., Wang, R., Guo, Y., Bi, D., Ma, W., Zhang, W., Zhang, J., Yan, Y., & Yao, X. (2020). Overexpression of GAS6 promotes cell proliferation and invasion in bladder cancer by activation of the PI3K/AKT Pathway. *OncoTargets and Therapy*, 13, 4813–4824.
<https://doi.org/10.2147/ott.s237174>
- New England Biolabs. (n.d.). Double Digest Finder. <https://nebcloner.neb.com/#!/redigest>
- Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*, 8(11), 2281–2308.
<https://doi.org/10.1038/nprot.2013.143>
- Richters, A., Aben, K. K. H., & Kiemeny, L. a. L. M. (2019). The global burden of urinary bladder cancer: an update. *World Journal of Urology*, 38(8), 1895–1904.
<https://doi.org/10.1007/s00345-019-02984-4>
- Shen, L., Lei, S., Zhang, B., Li, S., Huang, L., Czachor, A., Breitig, M., Gao, Y., Huang, M., Mo, X., Zheng, Q., Sun, H., Wang, F. (2020). Skipping of exon 10 in Axl pre-mRNA regulated

by PTBP1 mediates invasion and metastasis process of liver cancer cells. *Theranostics*, 10(13), 5719-5735. <https://doi.org/10.7150/thno.42010>

Thiery, J. P., Acloque, H., Huang, R. Y. J., & Nieto, M. A. (2009). Epithelial-mesenchymal transitions in development and disease. *Cell*, 139(5), 871–890. DOI 10.1016/j.cell.2009.11.007

University of California, Santa Cruz. (n.d.). *UCSC Genome Browser*. Retrieved February 24, 2025, from https://genome.ucsc.edu/cgi-bin/hgTracks?db=hg38&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr19%3A41212021%2D41246220&hgssid=2466888589_aQ01YkSaEKFVIMFyn0yBbxZO6mfK

Wium, M., Pაცეც, J., & Zerbini, L. (2018). The dual role of TAM receptors in autoimmune diseases and cancer: An overview. *Cells*, 7(10), 166. <https://doi.org/10.3390/cells7100166>

Yadav, M., Sharma, A., Patne, K., Tabasum, S., Suryavanshi, J., Rawat, L., Machaalani, M., Eid, M., Singh, R., Choueiri, T.K., Pal, S., & Sabarwal, A. (2025). AXL signaling in cancer: From molecular insights to targeted therapies. *Sig Transduct Target Ther* 10 (37). <https://doi.org/10.1038/s41392-024-02121-7>

Yeo, X. H., Sundararajan, V., Wu, Z., Phua, Z. J. C., Ho, Y. Y., Peh, K. L. E., Chiu, Y.-C., Tan, T. Z., Kappei, D., Ho, Y. S., Tan, D. S. P., Tam, W. L., & Huang, R. Y.-J. (2023). The effect of inhibition of receptor tyrosine kinase AXL on DNA damage response in ovarian cancer. *Commun Biol* 6(660). <https://doi.org/10.1038/s42003-023-05045-0>

Yu, H., Liu, R., Ma, B., Li, X., Yen, H-y., Zhou, Y., Krasnoperov, V., Xia, Z., Zhang, X., Bove, A. M., Buscarini, M., Parekh, D., Gill, I. S., Liao, Q., Tretiakova, M., Quinn, D., Zhao, J., &

Gill, P. S. (2015). Axl receptor tyrosine kinase is a potential therapeutic target in renal cell carcinoma. *British Journal of Cancer*, 113(4), 616–625. <https://doi.org/10.1038/bjc.2015.237>

Zhu, C., Wei, Y., & Wei, X. (2019). AXL receptor tyrosine kinase as a promising anti-cancer approach: functions, molecular mechanisms and clinical applications. *Molecular Cancer*, 18(1). <https://doi.org/10.1186/s12943-019-1090-3>