СОФИЙСКИ УНИВЕРСИТЕТ
„СВ. КЛИМЕНТ ОХРИДСКИ“
БИОЛОГИЧЕСКИ ФАКУЛТЕТ

SOFIA UNIVERSITY
St. KLIMENT OHRIDSKI
FACULTY OF BIOLOGY

Sofia University "St. Kliment Ohridski"

Faculty of Biology

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"Design and application of functional nucleic acids for synthetic control of gene expression"

ABSTRACT

dissertation thesis for the Doctoral degree in the professional field 4.3 Biological Sciences - "Genetics- Bioinformatics"

Scientific supervisor: **prof. Robert Dimitrov Penchovsky**

Sofia

2023

The dissertation was discussed at an extended meeting of the Department of Genetics, Faculty of Biology, Sofia University "St. Kliment Ohridski" held on 13.07.2023, on the basis of the Rector's order № 38-317/05.07.2023.

The dissertation is scheduled for approbation with a scientific jury, confirmed by Order No ..... of the Rector of Sofia University "St. Prof. Anastas Gerdzhikov.

Scientific Jury:
Internal scientific jury members:
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2. .....

External scientific jurors:
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5. .....

The dissertation approbation will take place on ..... at ..... hours in the building of the Faculty of Biology of Sofia University "St. Kliment Ohridski".

The thesis written by Georgi Yordanov Miloshev on "Design and application of functional nucleic acids for synthetic control of gene expression" contains:
Pages: 132
Figures: 61
Tables: 4
Citations: 184

The work is based on 2 scientific publications with the participation of the PhD student with a total impact factor of 0.45. Both papers were published in SCOPUS refereed journals and according to Scimago Journal and Country Rank (SJR) with a total of -32 points. Published chapter of a 15-point book.

1. Engineering a Plasmid as a Reporter System for Quantifying Gene Expression in Escherichia Coli - Georgi Y. Miloshev, Martina Traykovska, Dimitrios Kaloudas & Robert Penchovsky, *Proceedings of the Bulgarian Academy of Sciences* 75 (1), 2022, vol. 75, no. 1, pp. 56-61, ISSN: 13101331

IF: 0.326
Q3, 15 points
DOI: https://doi.org/10.7546/CRABS.2022.01.07,
https://www.scimagojr.com/journalsearch.php?q=31728&tip=sid
http://www.proceedings.bas.bg/index.php/cr/article/view/7/7

2. Small RNA-based systems for sensing and therapeutic applications - Robert Penchovsky, Georgi Y. Miloshev, Nikolet Pavlova, Katya B Popova, Aikaterini Valsamatzi-Panagiotou, Lozena A. Otcheva, Traykovska. Martina, *New Frontiers and Applications of Synthetic Biology*, 2022, Paperback ISBN: 9780128244692, eBook ISBN: 9780323859868, p.103-121, book chapter, 15 points

DOI: https://doi.org/10.1016/B978-0-12-824469-2.00004-X
https://www.sciencedirect.com/science/article/pii/B978012824469200004X?via%3Dihub

3. Various therapies against SARS-CoV-2 - Aikaterini Valsamatzi-Panagiotou, Martina Traykovska, Georgi Y. Miloshev, Robert Penchovsky, *Acta Microbiologica Bulgarica* Volume 39 / 1 (2023), 39(1), pp. 3-11

IF: 0.11
Q4, 12 points
https://actamicrobio.bg/archive/issue-2-2020/amb-2-2020-article-1.pdf
https://www.scimagojr.com/journalsearch.php?q=34601&tip=sid&clean=0

Total: IF: 0,45
Quartile (Q) according to SCOPUS: 42 points.

Words of gratitude

I would like to express my gratitude to Prof. Dr. Robert Penchovsky and the members of the Department of Genetics, Faculty of Biology, St. Kliment Ohridski", for the opportunity to work on the topic "**Design and application of functional nucleic acids for synthetic control of gene expression**" at the Department of Genetics.

The research activity was carried out in the laboratory of Prof. Dr. Robert Penczowski, and was supported by the following research projects:

1. "Design of functional nucleic acids for synthetic gene regulation in prokaryotes and eukaryotes", KP-06-N31/18/13.12.2019, funded by the Scientific Research Fund, Biological Sciences Committee, project leader Prof. Dr. Robert Dimitrov Penchovsky - **MAIN PROJECT.**

2. "Design and experimental testing of chimeric antisense oligonucleotides as antibacterial agents", DN13/14/20.12.2017, funded by the Scientific Research Fund, Medical Sciences Committee, project leader Prof. Dr. Robert Dimitrov Penchovsky.

3. "Creation of antisense oligonucleotides that specifically inhibit bacterial growth of *Helicobacter pylori* and *Porphyromonas gingivalis*", KP-06-M33/5/18.12.2019, funded by the Scientific Research Fund, Committee on Medical Sciences, project leader prof. asst. Dr. Martina Trajkovska

CONTENTS

1. INTRODUCTION

The use of antisense oligonucleotides (ASOs) in biology and medicine is key to solving a number of problems related to the treatment and prevention of various diseases, as well as the emergence of antibiotic resistance, leading to ineffectiveness of the prescribed therapy, prolonged hospital stay, high hospital costs and even death. Antisense oligonucleotides are short single-stranded molecules with an optimal length of 20-25 nucleotides that can directly bind to structures in the genome, for example bacterial riboswitches, and regulate their expression. Their study and research are important because they have a number of potential applications. It is possible to create antisense oligonucleotides whose targets are pathogenic bacteria, SARS-CoV-2 and many other pathogens. To ensure their penetration into a bacterium, they can bind to a carrier, either a cell-penetrating peptide (CPP) or a lipoprotein (Traykovska et al., 2018; Traykovska and Penchovsky, 2022). Based on their design, ASOs hybridize complementarily to the target sequence and as a result are able to regulate gene expression. The mechanism of action of antisense oligonucleotides has been well studied, and in the event of emerging antibiotic resistance or the emergence of mutations, their design can be readily redesigned and allow for optimized subsequent administration. There are already approved ASOs on the market for use in the treatment of neurodegenerative diseases and atherosclerosis (Bennett et al., 2021; Amanat et al., 2022).

COVID-19 contains a single-stranded RNA in which all essential genes are located at the 5'-end of the RNA, which is transcribed into mRNA. (Valsamatzi-Panagiotou, Traykovska, Georgi Y. Miloshev, Penchovsky, 2023). By finding the nucleotide sequence of these genes, which is done by specialized software programs, we can design an ASO that binds specifically to the complementary sequence of the target mRNA. The 5'-end of the mRNA is responsible for the synthesis of the replication and transcription genes. In this case, we could inhibit the translation and synthesis of key proteins involved in genomic RNA replication. These advantages are significant and make them a future therapeutic agent against various bacterial and viral infections (Penchovsky and Traykovska, 2015; Penchovsky, 2019; Valsamatzi-Panagiotou, 2020; Popova and Penchovsky, 2021).

Regulation of riboswitches by ASO enables informative RNA molecules to regulate their expression without the need for regulatory proteins. Thus, vital metabolites for bacteria and viruses will not be synthesized by the cells or transported inside them from the external environment. The result of this interference is the death of the respective viruses and bacteria. This, in turn, necessitates the use of a method for designing ribozymes (enzymes made of RNA). Our methods are very efficient in terms of time and laboratory labor because we can produce within a few minutes the desired sequences of high-speed allosteric ribozymes that can work in the cell. The

methods and techniques I rely on in my dissertation are from the fields of genetics, bioinformatics, synthetic biology, and microbiology. I also use cloning techniques. All of this has huge practical applications.

Another way to regulate gene expression is the practical use of new approaches for the analysis and design of complex networks in living cells with many applications in the pharmaceutical and biotechnology industries. The development of new versatile strategies for exogenous control of gene expression is based on designer allosteric ribozymes that can function within the cell. The synthetic riboswitches are produced by a proprietary computational system that provides fast operation and accurate modular designs with different Boolean logic functions. The riboswitches can be designed to function in the cell in the presence or absence of RNA(s) or small molecules, and also to activate or inhibit gene expression of any exogenous protein. In addition, riboswitches can be designed to induce RNA interference or microRNA pathways that can conditionally regulate the expression of key proteins in the cell. This can lead to the prevention of the development of various diseases. Therefore, synthetic riboswitches can be used as versatile cellular biosensors. With the help of modern technologies, the indicator RNA(s) can be accurately identified using next-generation sequencing technologies and with high accuracy. The methods can be used not only for exogenous control of gene expression, but also for cell death reprogramming, anticancer, and antiviral gene therapies. Such approaches could be used as powerful molecular medicines of the future.

Gene inhibition technology based on antisense oligonucleotides (ASOs) is widely adopted. As previously described, ASOs are chemically modified single-stranded short nucleic acids that bind specifically to target RNAs and inhibit their expression by two different mechanisms. They can cleave the target RNA through the action of RNase H or prevent the translation of the mRNA (Martinovich et al., 2018). The length of antisense oligonucleotides typically ranges from 13 to 50 nucleotides with an optimal range of 20 - 25 nucleotides (Chen et al., 2020; Katzmann et al., 2020). As synthetic oligonucleotides, ASOs are designed to be complementary to the target mRNA. There are three generations of ASOs based on their chemical modifications. First-generation ASOs have a phosphorothioate backbone modified by replacing the unpaired oxygen in the phosphodiester bond with a sulfur atom. This modification allows the cleavage of the mRNA by RNase H. Second-generation ASOs have increased medical protection but do not induce cleavage of the mRNA by RNase H. They have 2 prim alkyl modifications of ribose and 2 prim O-methyl or 2 prim O-methoxyethyl substitution. Protein nucleic acids and other locked nucleic acids are part of the third generation of ASOs. They have high chemical resistance to DNases and high affinity and specificity for RNA binding. However, they also cannot induce RNase H to cleave the target RNA. A combination of first- and second-generation modifications of an ASO affects the specific

degradation of mRNA by RNase H, providing prolonged stability due to methylation and delayed enzymatic hydrolysis of DNA/RNA due to the sulfur atom. ASOs that act via RNase H have a multi-turn function, opposite to pure second- and third-generation ASOs, which only work in a single-turn manner.

ASOs, with a specific unpaired CCA sequence at the 3 prim ends, can work by the action of ribonuclease P (RNase P), resulting in mRNA cleavage and subsequent inhibition of protein expression. Peptide nucleic acids or locked nucleic acids are used to prevent translation. These bind complementarily to the mRNA and cause spatial disruption of the target RNA (Penchovsky and Traykovska, 2015; Valsamatzi-Panagiotou et al., 2020). ASOs are chemically modified DNA oligomers. They form DNA and RNA hybrids with the target molecule (Bajan and Hutvagner, 2020; Rinaldi and Wood, 2018). ASOs have been widely used over the past 20 decades in the treatment of many different diseases, such as macular degeneration, cytomegalovirus retinitis, chylomicronemia, Duchenne-type muscular dystrophy and spinal muscular atrophy, and hypercholesterolemia. So far, there are six certified ASO drugs, including eteplirsen, fomivirsen, mila sen, nusinersen, pegaptanib, and volanesorsen (Martinovich et al., 2018; Khetarpal et al., 2019; Lim et al., 2017). ACOs are some of the most promising bacterial chemical agents that have many advantages, including rapid, extensive, and accurate procedures for rational drug design and specific targeting against global problems such as bacterial resistance. A cell-penetrating peptide, such as pVEC, can be attached to and penetrate any cell type (Penchovsky and Traykovska, 2015).

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is an RNA-guided DNA endonuclease system known as CRISPR-associated bacterial adaptive immune system. This system captures and stores viral sequences in the genome that are expressed as guide RNAs that directly bind CAS proteins to disrupt viral infection (Jakhanwal et al., 2021). The CRISPR/Cas9-mediated genome editing system is based on short RNA molecules. This system can be used as a specific DNA sequence cleavage and ligation for gene editing *in vivo (*Zhang et al., 2017a). Short RNA molecules are non-coding short RNAs that bind to target DNA sequences (Kaloudas et al., 2018).

First, they bind to the CAS9 enzyme, and the short RNA sequences direct the complex to the target site of the DNA, where CAS9 cuts the target DNA chain because it acts as an endonuclease. Short RNAs can come in two forms. The first is synthetic trans-activating CRISPR RNA and synthetic CRISPR RNA. It is designed to cleave the target site. The second form is a syhetic RNA that consists of both trans-activating CRISPR RNA and synthetic CRISPR RNA as a single construct. The complex formed by trans-activating CRISPR RNA and synthetic CRISPR RNA has a leading role for the CAS9 enzyme. Trans-activating CRISPR RNA is responsible for the endonuclease activity of CAS9 and synthetic CRISPR RNA binds to the specific DNA region.

Trans-activating CRISPR RNA binds to the enzyme, forming a stem loop. Synthetic CRISPR RNA identifies the specific complementary region and induces its cleavage by CAS9 after binding to the effector complex. The RNAs determine the activity of CAS9 (Jakhanwal et al., 2021).

CRISPR-Cas has an essential role in genetic engineering and genome editing (Jakhanwal et al., 2021; Schmidt and Platt, 2017; Stella et al., 2017; Kim and Lu, 2019; Marchisio and Huang, 2017). Guide RNAs can bind antisense RNA sequences and regulate RNA modification. Short RNAs can inhibit target RNA in eukaryotic cells and require the exogenous expression of CAS9, unlike RNase H and RNase P, which are present in eukaryotic cells. They are also used in RNA mutagenesis where they replace adenosine with inosine at the specific target site and modify the genetic code (Lee et al., 2016).

RNA synthetic biology is one of the most developing fields of modern synthetic biology. This is mainly due to the various RNA engineering and synthesis tools that have been developed and refined over many years. Such tools include SELEX of RNA aptamers and allosteric ribozymes, computer-aided design of allosteric ribozymes, and RNA-based sensors. In addition, synthetic RNAs can be readily synthesized by *in vitro* transcription. The future prospect of RNA synthetic biology is very promising because it will enable the use of RNA aptamers, allosteric ribozymes, and externally targeted RNA sequences for many different applications in nanobiotechnology and the pharmaceutical industry. RNA-based nanomachines can be used for molecular computers and molecular sensors that can work *in vitro* and *in vivo*. Apoptosis can be reprogrammed using synthetic control of gene expression based on allosteric ribozymes. RNA and DNA aptamers can be used to inhibit viral infections. ASO and externally targeted RNA sequences can inhibit the expression of any RNA in the cell and stop viral and bacterial infections. Therefore, there are countless opportunities for synthetic RNA biology to develop revolutionary applications based on already available tools.

RNA synthetic biology is a growing research field that is not becoming obsolete due to the development of many useful tools that have valuable applications. Such small RNA-based tools include molecular sensors, RNA computing devices, exogenous gene control elements, ASOs, and. These molecular tools based on RNA aptamers, allosteric ribozymes and modified oligonucleotides have been used in various pharmaceutical and biotechnological applications. They include various therapeutic agents based on aptamers, ribozymes, ASOs and short RNAs. In addition, aptamers and allosteric ribozymes have been used for exogenous control of gene expression *in vivo* and for biorecognition and computational applications *in vitro*. Advanced engineering methods of functional small RNAs are key in the development and application of these molecules in a variety of biotechnological applications.

Synthetic biology is a research field that has gained popularity in recent years. Applications of synthetic biology span the fields of pharmaceutical biotechnology and drug discovery through novel methods and strategies such as metabolic engineering, cell fate reprogramming, drug production in genetically modified organisms, molecular adhesives, functional nucleic acids, and genome editing (Pavlova et. al. 2022). Synthetic biology is an interdisciplinary research field with novel concepts for designing different biological systems *in vitro* and *in vivo* (Traykovska et.al. 2018; Penchovsky et. al. 2000; Penchovsky 2012). It uses engineering principles to create and assemble biological components, including tools of various research disciplines such as nanotechnology, functional nucleic acid engineering, molecular biology, reproductive medicine, gene therapy, genetic engineering, systems biology, biophysics, molecular computing, gene editing tools, drug design, and pharmaceutical engineering (Chappell et. al. 2015; Hollywood et. al. 2018; Liu & Pakrasi 2018; MacDonald & Deans 2016; Alnasser 2021; Kopka & Fernie 2018; Jagadevan et. al. 2018; Cantelli et. al. al. 2021). It also provides an opportunity to address climate change through biofuel production and pollutant removal through bioremediation (Jagadevan et. al. 2018; Kaloudas et. al. 2021). Two of the main goals of synthetic biology are to reprogram cells or organisms as well as to create new living cells. The former uses metabolic/genetic engineering and molecular biology techniques. Creating a fully living cell *de novo* by self-assembly of synthetic molecules is one of the most fundamental and complex goals for building artificial cells known as protocells or synthetic cells (Buddingh, van Hest 2017). The bacterial genome has been redesigned with added predefined characteristics such as genetically modified organisms (GMOs) that produce chemicals for drug production, environmental pollution cleanup, drug delivery tools, and biosensing functions (Liu, 2021; Deckers et.al. 2020).

After the creation of the first synthetic genome, the next step is to engineer gene expression circuits in prokaryotic and eukaryotic organisms with small molecules such as drugs, nutrients or ligands. It is extremely important and essential to produce metabolites, new methods for gene therapy, and do environmental monitoring (Penchovsky, Stoilova 2013). Production of beta-lactams, penicillin, terpenoids (milliradian, oleanolic acid, hydrocortisone), omega-3 eicosapentaenoic acid, flavonoids (naringenin), alkaloids (benzylisoquinoline alkaloids), stilbenoids (resveratrol) and vitamin C are made in yeast using standardized components known as BioBricks (BBs) (Deepika M, Sumathy 2020; Staal et.al. 2019). BBs consist of DNA sequences with different functions, easily combined into different gene expression circuits. Based on the clustered common intersystem short palindromic sequences (CRISPR)/CAS9, the genome editing tool opens up new possibilities to rewrite the genome of any living cell, which expands our reprogramming capabilities (van der Weyden et.al. 2021; Liang et.al.2020; Ghaemi et. al. 2021; Hartz et.al. 2021; Sharmae rt.al. 2021). CRISPR-CAS9 has been used as a powerful tool to

efficiently engineer *Saccharomyces cerevisiae*, cell lines and others (Rainha et.al. 2020). All the mentioned tools or technologies have been implemented or will be adopted soon. The prediction of new molecules with bioactivity is essential to optimize the drug discovery process which requires molecular representation (Kumar et.al. 2022).

2. AIMS AND TASKS

2.1. Aim

The aim of this Ph.D. thesis is to establish a new universal method for control of gene expression in *Escherichia coli* using synthetic antisense oligonucleotides that inhibit LacZ expression.

2.2. Tasks

In order to achieve the objective, the following tasks were set:

1. Design of antisense oligonucleotides to inhibit gene expression in *Escherichia coli.*
2. Cloning of a gene expression control construct in an *Escherichia coli* expression plasmid with a LacZ reporter gene.
3. Design of an OFF Switch control strategy for gene expression in *Escherichia coli.*
4. Experimental testing of antisense oligonucleotides for OFF Switch control of gene expression in *Escherichia coli.*
5. Design of an OFF Switch gene expression control strategy using the synthetic hammerhead ribozyme in *Escherichia coli.*
6. Experimental testing of antisense oligonucleotides for OFF Switch control of gene expression in ribozyme in *Escherichia coli.*

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Bioinformatics tools

I used the following open-access databases to perform bioinformatic analyses:

1. Foreign databases.

NCBI - National Center of Biotechnology Information - https://www.ncbi.nlm.nih.gov/. NCBI is one of the largest open-access databases and contains information that is separated into individual databases:

- Pubmed (scientific publications);
- Gene;
- Genome;
- Nucleotide;
- Protein;
- PubChem (chemical processes);
- SNP;
- BLAST (search for similar areas).

I used some of these databases of publications to write the Literature Review and to dig deeper into the issue.

Rfam 14.8 - https://rfam.xfam.org/

A database that contains a collection of RNA families, each represented by multiple sequence alignments, consensus secondary structures, and covariate patterns. I used it for the nucleotide sequences of bacterial riboswitches and ribozymes.

RNAfold web server - http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi

Freely available software that calculates the folding of single-stranded RNA and DNA secondary structures. I used it to calculate and visualize the folding of secondary structures of antisense oligonucleotides in the LacZ nucleotide sequence, as well as mutations used for gene expression.

KEGG: Kyoto Encyclopedia of Genes and Genomes, (http://www.genome.jp/kegg/) - KEGG - Kyoto Encyclopedia of Genes and Genomes.

From the KEGG PATHWAY section, I took information about all the enzymes and metabolites that are involved in the biochemical reactions of bacteria.

2. Databases and applications developed by Prof. Dr. Robert Penchovsky

Motif searcher - https://penchovsky.atwebpages.com/applications.php?page=43

The program allows searching for one or more motifs in DNA, RNA and protein sequences that we feed to the program ourselves.

RevComOligo - https://penchovsky.atwebpages.com/applications.php?page=41

The program is used to obtain a complementary sequence to a predefined one in the 5'-3' direction.

Restriction Mapping - https://penchovsky.atwebpages.com/applications.php?page=44

The program is used to detect restriction enzyme sequences by a predefined nucleotide sequence. The database recognizes 1502 enzymes. You can also choose to search the database only for enzymes that cut at one, two, or three positions. In addition, you can also select just the name(s) of the enzyme from a list, and so display its sequence.

PCR Annealing Temperature Calculator - https://penchovsky.atwebpages.com/applications.php?page=49

The program is used to calculate the binding temperature in a polymerase chain reaction (PCR) by inputting the sequence of the two primers (reverse and forward).

3.1.2. Materials used in the experiments

1. Bacterial media used:

LB medium - To make 1 liter of this, I followed the following protocol - 10g tryptone, 5g yeast extract and 10g NaCl.

2. Bacterial strains used:

For the purpose of the experiment, I used competent *E. coli* HB101 cells, 1 set (100 µl x10), which we ordered from TAKARA Bio INC, Japan.

3. Nucleic acids used:

We used antisense oligonucleotides - 9 different concentrations, which we designed and submitted for nucleotide synthesis to the American company GeneLink (http://www.genelink.com/). Different concentrations were used for different percentage of inhibition of bacterial growth. Experiments with each concentration were repeated 3 times and an average was taken.

Used primers (DNA sequences) - Oligonucleotides were ordered from Microsynth AG - Schützenstrasse 15 - P.O. Box - 9436 Balgach - Switzerland.

Table 1. Used primers and antisense oligonucleotides

	Oligonucleotides	Concentration
1	5′-GATCCTACAACAAGGTACCGACCTAGCAGGAGGTATTAATATGGTCCTCGAGGTC-3′	41,7 nmol/707,8 µg
2	5′-GATCGACCTCGAGGACCATATTAATACCTCCTGCTAGGTCGGTACCTTGTTGTAG-3′	42,4 nmol/715,5 µg
3	5′-GATCTGTGAGCTTTGACATAAATACCACTGGCGGTGATACTGGTAC-3′	41,3 nmol/586,8 µg
4	5′-CAGTATCACCGCCAGTGGTATTTATGTCAAAGCTCACA-3′	81,0 nmol/941,3 µg
5	5′-CATCCTTGGTACTTACAGCTTACGAGTCCCAAATAGGACGAAACGCGACACTAAACGTGCAGTGTTTCGGTCCTGTAATCCACAAGGAGGTATTAATATGC-3′	80,9 nmol/2732,9 µg
6	5′-TCGAGCATATTAATACCTCCTTGTGGATTACAGGACGCGAAACACTGCACGGTTTAGTGGTGTGTCGCGTTTCGTCCTATTTGGGACTCGTAAGCTGTAAGTACCAAGGATGGTAC-3′	81,6 nmol/2979,6 µg
7	5′-CATCCTTGGTACTTACAGCTTACGAGTCCCAAATAGGACCAAACGCGACACTAAACGTGCAGTGTTTCGGTCCTGTAATCCACAAGGAGGTATTAATATGC-3′	77,8 nmol/2627,8 µg
8	5′-TCGAGCATATTAATACCTCCTTGTGGATTACAGGACGCGAAAC	81,9 nmol/ 2992,4 µg

	ACTGCACGGTTTAGTGGTGTGTCGCGTTTGGTCCTATTTGGGA CTCGTAAGCTGTAAGTACCAAGGATGGTAC-3′	
9	5′- ACAGCGCGTCGTGATTAGC - 3′	33,9 nmol/197,6 µg
10	5′- ACAAGGTACCGACCTAGCAG - 3′	45,4 nmol/277,6 µg
11	5′- ATACCACTGGCGGTGATAC - 3′	249,8 nmol/429,8 µg
12	5′- CACCACTAAACCGTGCAGTG - 3′	36,2 nmol/ 219,8 µg
13	5′- CAGGCTCGCATGGCTCGCC - 3′	33,6 nmol/ 239,3 µg
14	5′- GTCCGAGCGTACCGAGCGG - 3′	43,0 nmol/ 249,8 µg
15	5′- CTATTTGGGACTCATCAG - 3′	36,2 nmol/ 219,8 µg

4. Apparatus used:

Refrigerator with freezer HOTPOINT - used for storage of various reagents.

ARCTICO -86°C freezer - used for storage of glycerol cultures.

Autoclave - BIOBASE Table Top Autoclave Class N Series - used for sterilization of bacterial media, instruments and consumables

Electronic balance - XS Instruments BALANCE mod. BL 224 BASIC - 220 gr. - used for precise measurement of the amount of different reagents used for the experiments.

Spectrophotometer - ONDA UV-21 is a UV/VIS spectrophotometer used to measure the optical density of bacterial culture growth.

pH meter - XS Instruments Cond 50 VioLab bench - used to optimize the pH of the various components and reagents used in Beta-Galactosidase analysis.

Vortex - Fisherbrand™ Analog Vortex Mixer - used to homogenize various reagents in Eppendorf tubes.

Shaker - Argolab Shaker Incubator SKI 4 - used to maintain and optimize conditions for inoculation of bacterial cultures.

Thermostat - Biosan Bio TDB-100, Dry block thermostat - used to incubate and maintain a specific temperature for the different reactions - restrictase, ligase, Beta-Galactosidase Assay.

Nanodrop - BioDrop - Micro-Volume Measurement Platforms - used to measure the concentration of plasmid DNA after its isolation, as well as to measure the concentration of various antisense oligonucleotides.

Centrifuge - NEYA centrifuge 16 R - used to homogenize under certain conditions (temperature, speed).

PCR - LifeECO Thermal Cycler - used for multiplication of cloning fragments.

Electrophoresis - multiSUB Midi, Midi Horizontal Electrophoresis System - is used to determine the size of different fragments as well as visualization of results after polymerase chain reaction.

Power Supplies consort EV3330 - used to power the electrophoresis.

Transilluminator - Analytik Jena UVP Benchtop Transilluminators - used to visualize agarose gel results after electrophoresis.

3.2. Methods

3.2.1. Software methods

Foreign software programs:

Clustal W/Clustal X - http://www.clustal.org/

This is a program that is used to compare and align several different sequences. The program makes it easy to find matches in sequences. To make the sequences easier to process, I pre-wrote the sequences in Notepad++. The file is saved in fasta format. Thus, by coloring the identical nucleotides in one color, the alignment between the analyzed sequences is seen and motifs are created.

Notepad++ - https://notepad-plus-plus.org/

Used to store nucleotide sequences as fasta format files. A fasta file can contain countless many sequences. To be read and understood correctly by the processing program, the sequences entered in the file need to follow the following rules - a file can contain an infinite number of lines. Up to 80 characters can be written on each line. Entering a new sequence starts with ">" followed by the sequence name, then, at the bottom, manually type or paste the copied sequence. The created fasta files are then used for sequence alignment (multiple alignment).

Basic Local Alignment Search Tool - BLAST search algorithm,

(http://blast.ncbi.nlm.nih.gov/Blast.cgi). The Basic Local Alignment Search Tool detects and compares regions of similarity between nucleotide sequences. I used it to compare different antisense oligonucleotide sequences, bacterial sequences,and human sequences.

SnapGene Viewer - I used this program to conveniently view plasmid sequences and to visualize the results after sequencing. The cloned fragments were sent to Macrogen Europe (Meibergdreef 57, 1105 BA Amsterdam, Netherlands) for sequencing.

OriginLab - I used this program to make the graphs for my dissertation, as well as the scientific publications. I also used it to easily analyze the results of the experiments.

3.2.2. Used kits and protocols

1) protocols

Chemical transformation of competent E. coli cells with plasmid pRS414 using the following protocol:

1. Competent cells are put on ice
2. 3 microliters of lprs414 plasmid
3. Incubate on ice for 30 min
4. Incubate at 42 degrees for 30 sec.
5. Incubate on ice for 5 min
6. Add 800 microliters of SOC (900 LB + 100 0.2 M Glu)
7. Incubate on a rocker for 1 hour at 37 degrees
8. Applied on petritis

Electroporation - an Eppendorf Eporator® was used with the following protocol:

1. Competent cells are put on ice
2. 3 microliters of plasmid are put into 40 microliters of CC.
3. The cuvette is placed in the 1700 V electroporator.
4. Add 1 milliliter SOC
5. Incubate for 50-60 minutes on a shaker at 37 degrees.
6. Apply in Petri dishes

Restrictase reaction (in a final volume of 50 microliters) - I have used three restrictases (BamH I, Xho I, Kpn I).

20 µl plasmid DNA

5 µl buffer

5 µl distilled water

10 µl first restrictase

10 µl second restrictase

Ligase reaction (in a final volume of 20 microliters) - several reactions are performed in parallel. Thermo Fisher Scientific T4 DNA Ligase enzyme was used

2 µl T4 DNA ligase enzyme

2 µl DNA ligase buffer

6 µl vector DNA,

4 µl insertional DNA

6 µl water

Restrictive reaction -

PCR reaction - a standard protocol in a final volume of 25 µl is used

10 x so buffer - 2.5 µl

10 mM mixture of dNTPs - 0.5 µl

10 mM straight primer - 0.5 µl

10 mM reverse primer - 0.5 µl

Matrix DNA - 1 µl

So polymerase - 0.5 µl

Water - 19.5 µl

The conditions for the PCR reaction were heated LTD at 112 °C, coupling at 94 °C for 1 min, denaturation at 94 °C for 30 s, gradient at 55 °C for 30 and coupling at 72 °C for 1 min, 30 cycles. After the reaction, the samples were run on a 2% agarose gel for 1 h and 30 min. Initially, the electrophoresis was run at 40V for 15 min to allow the samples to more easily enter the gel, then run at 65V. The first sample contains a ladder - 1000/3000 bp of DNA, which is used to determine the length of the amplified product.

Beta-galactosidase assay.

The beta-galactosidase assay was performed to quantify the level of promoter activity. *E. coli* were allowed to grow overnight in LB medium containing ampicillin (100 mg/L) and incubated at 37°C with shaking. A permeabilizing solution (80 µL) of (100 mM dibasic sodium phosphate ($Na2HPO4$), 20 mM KCl, 2 mM $MgSO4$, 0.8 mg/mL CTAB (hexadecyltrimethylammonium bromide), 0.4 mg/mL sodium deoxycholate, 5.4 µL/mL beta-mercaptoethanol) was added, and then the absorbance was measured at 600 nm (Abs 600). 20 µL of ON culture was added to tubes containing the permeabilization solution and 600 µL of substrate solution containing ONPG. Once a sufficiently saturated colour was obtained, 700 µl of stop solution (1 M sodium carbonate ($Na2CO3$)) was added and homogenized well. The tubes were then centrifuged at full speed for 5-10 minutes. The supernatant was poured into cuvettes and the absorbance was measured at 420 nm (Abs 420).

2) Kits:

Isolation of plasmid DNA with the following kits:

EURx - GeneMATRIX Plasmid Miniprep DNA Purification Kit

Sigma-Aldrich - GenElute™ Plasmid Miniprep Kit

Qiagen - QIAquick Gel Extraction Kit

Bioanalysis - Plasmid DNA purification kit

Other kits:

Thermo Fisher Scientific Inc - GeneJET Gel Extraction and DNA Cleanup Micro Kit

Sigma-Aldrich - GenElute Bacterial Genomic DNA | NA2110-1KT

4. RESULTS

4.1. Design of antisense oligonucleotides to inhibit gene expression in *Escherichia coli*.

Bacterial operons are polycistronic transcripts that can produce multiple proteins from a single iRNA transcript (S´aenz-Lahoya et al., 2019). The lac operon (lac operon) is required for lactose transport and metabolism in *Escherichia coli* (*E. coli*) (Clark et al., 2019). The lacZ gene is translated using beta-galactosidase, an intracellular enzyme that degrades the disaccharide lactose to glucose and galactose, which are used as energy sources (Juers et al., 2012). A color indicator of beta-galactosidase activity is ortho-nitrophenyl-b-D-galactopyranoside (ONPG), which dissolves and produces an intense yellow compound (orthonitrophenol). This compound absorbs at a wavelength of 420 nm, at which point measurement of beta-galactosidase activity becomes possible (Smale, 2010). B-galactosidase hydrolyzes the o-nitrophenyl-β-D-galactopyranoside substrate, which yields a stoichiometric yellow o-nitrophenol coloration and colorless galactose. LacZ can be used as a reporter gene in various gene expression systems, not only in *E. coli*, but also in other microbial species (Evans et al., 2013; Ju´arez-Rodr´ıguez et al., 2013; Son et al., 2008; Lewandoski and Smith, 1988). Using the lacZ gene together with the initiating ATG codon and the consensus sequence of *E. coli* - Schein-Dalgarno, was used for early studies to generate lacZ reporter systems for monitoring transcription at operon fusions (Lewandoski, Smith, 1988). In my work, I used the plasmid pRS414, which consists of about 10 kb, to generate a novel lacZ reporter system including a promoter, a Shine-Dalgarno, and the ATG codon in the lacZ gene frame. The inserted promoter element is a PL sequence of 38 base pairs of bacteriophage λ (Tomich et al., 1988). Analysis of the beta-galactosidase revealed high levels of activity in the samples compared to the negative control lacking bacteriophage λ, which showed no activity. Beta-galactosidase monitoring can also be used as a marker to study the effects of allosteric ribozyme and oligonucleotide activity. The newly developed system can be applied in synthetic biology for control of gene expression in synthetic designer ribozymes and antisense oligonucleotides (Brunner and Bujard, 1987; Penchovsky and Breaker, 2005; Penchovsky, 2014). Other gene control elements can act as switches to control gene expression.

A DNA fragment carrying the KpnI restriction site and the Schein-Dalgarno sequence, as well as an in-frame ATG transcription start codon with lacZ, was created by hybridization with two deoxyoligomers (*Figure 1)*. The first fragment, introducing the KpnI site in the pRS414 plasmid, uses BamHI and XhoI restriction sites located on the plasmid (*Figure 1*).

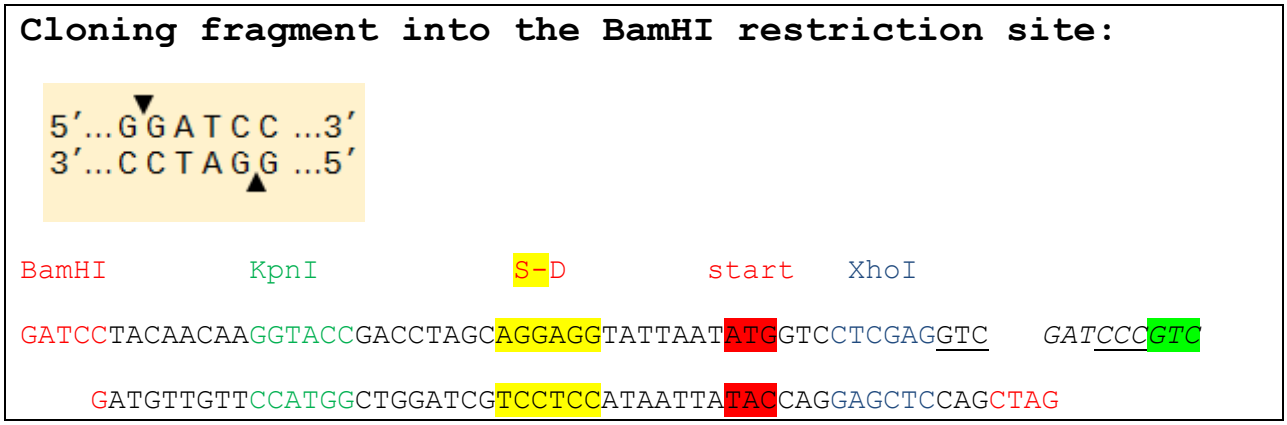


Figure 1. DNA fragment showing the location of the BamHI, KpnI, XhoI restrictionases, the Shine-Dalgarno sequence and the ATG codon on plasmid pRS414. Restrictase sequences are marked with different colors.

Source: Miloshev et.al. (2021) Engineering a plasmid as a reporter system for quantifying gene expression in Escherichia coli

The KpnI restriction site entry was verified by restriction mapping of KpnI endonuclease-generated clones *(Figure 2*). A 1% agarose gel stained with GelRed (Biotium, Fremont, CA) was used to visualize the restriction mapping.

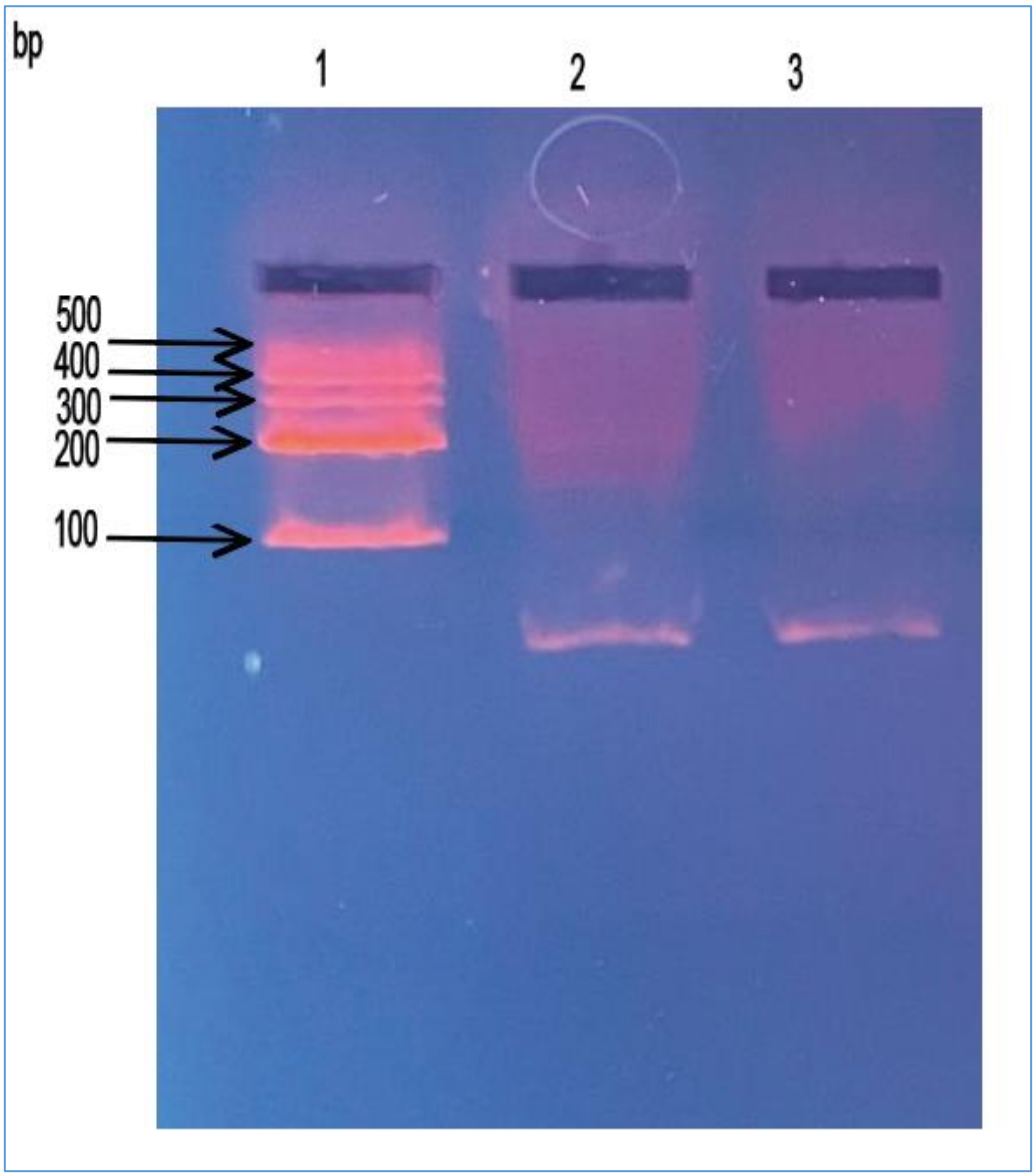


Figure 2. Restriction mapping of KpnI endonuclease clones generated. (1% agarose gel). Sample 1 contains the 500bd control ladder, which was used to size the remaining samples. Samples 2 and 3 contain clones amplified by PCR reaction involving KpnI endonuclease.
Source: Miloshev et.al. (2021) Engineering a plasmid as a reporter system for quantifying gene expression in Escherichia coli

The PL promoter sequence of 38 base pairs was cloned between BamHI and the novel KpnI restriction site (*Figure 3*). The promoter fragment was generated by heating the deoxyoligomers. The introduction of the KpnI and BamHI restriction sites was verified by restriction mapping of the generated clones from KpnI and BamHI endonucleases (*Figure 4*). A 1% agarose gel stained with GelRed (Biotium, Fremont, CA) was used to visualize the restriction mapping.

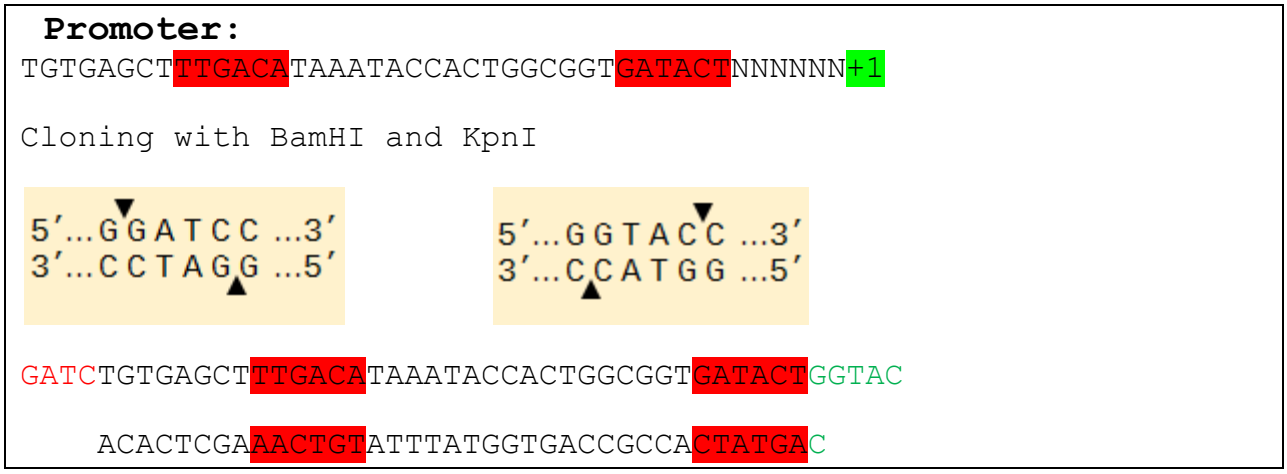


Figure 3. Promoter cloning with BamHI and KpnI restriction sites. BamHI and KpnI are used to insert the promoter fragment. *Sequences of the restrictases are marked with different colors.*
Source: Miloshev et al. (2021) Engineering a plasmid as a reporter system for quantifying gene expression in Escherichia coli

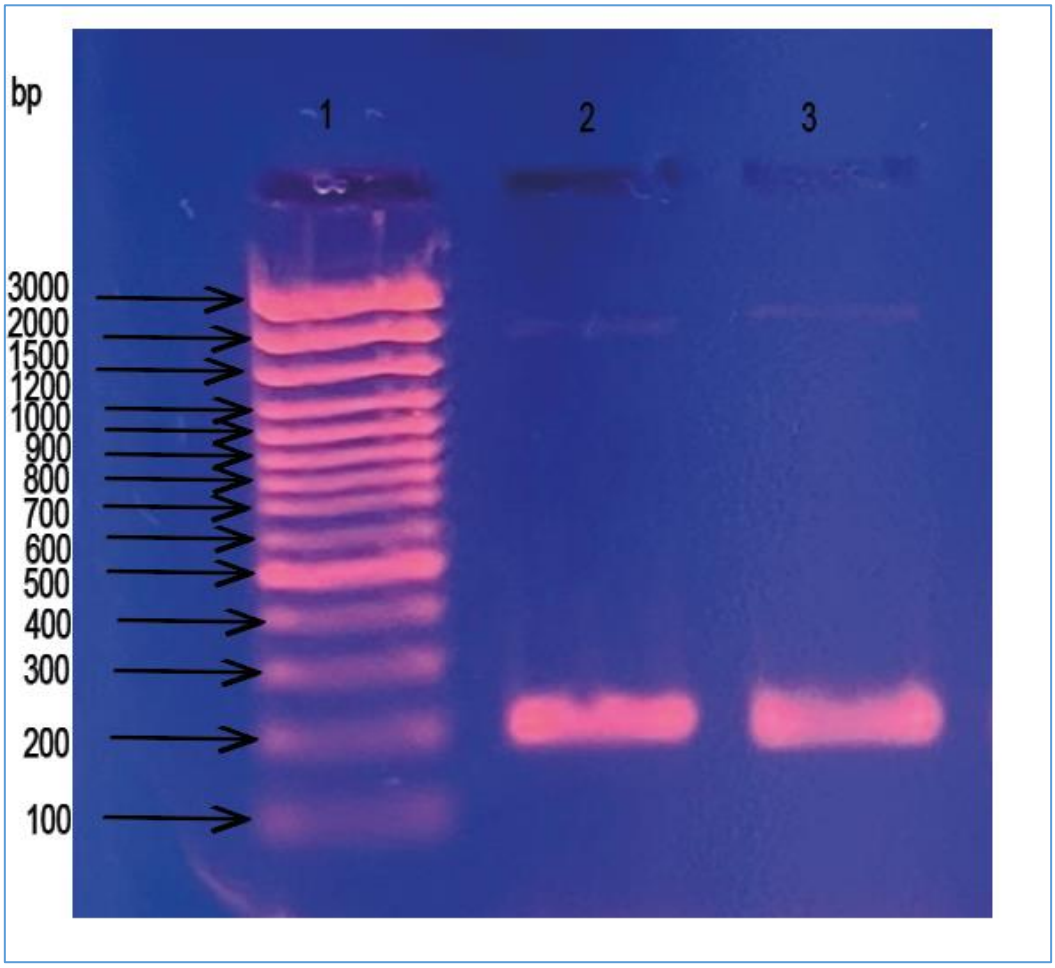


Figure 4. Restriction mapping of clones generated by KpnI and BamHI endonucleases. (1% agarose gel). Sample 1 contains the control ladder of 100bd-3000bd, which was used to size the remaining samples. Samples 2 and 3 contain PCR-amplified clones including KpnI and BamHI endonucleases.

Another fragment containing an antisense oligonucleotide that inhibits bacterial growth was then created. The structure of the fragment can be seen in Figure 5. The antisense oligonucleotides have the following nucleotide sequence:

1 - 5′-CAGGCTCGCATGGCTCGCC-3′

2 - 5′-GTCCGAGCGTACCGAGCGG-3′

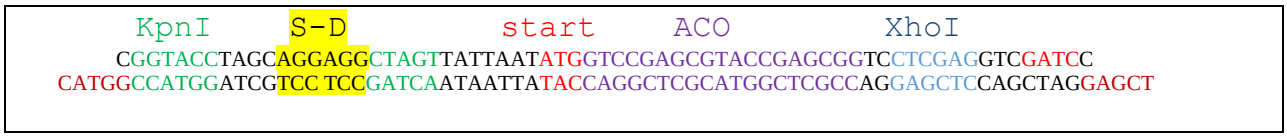


Figure 5. Structure of the fragment containing an antisense oligonucleotide that inhibits bacterial growth*. DNA fragment showing the location of the KpnI and XhoI restricases, the Shine-Dalgarno sequence, and the ATG codon on plasmid pRS414. Restrictase sequences are marked with different colors.*

Source: Miloshev et al. (2021) Engineering a plasmid as a reporter system for quantifying gene expression in Escherichia coli

The putative secondary structure of the sequence was determined with the freely available software RNA Fold http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi (*Figure 6*). The secondary structure of the ASO sequence in the whole plasmid structure can be seen in Figure 7.

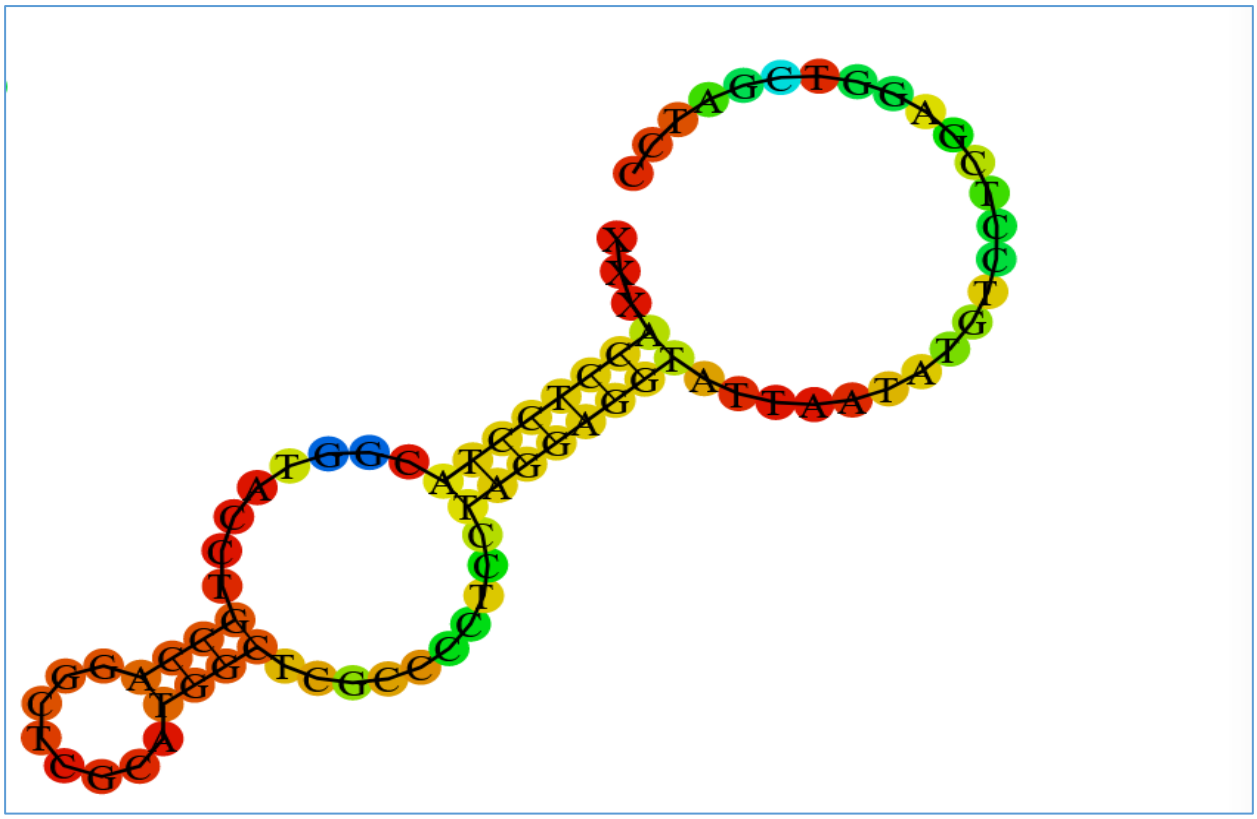


Figure 6. Putative secondary structure of the fragment obtained by RNA fold.

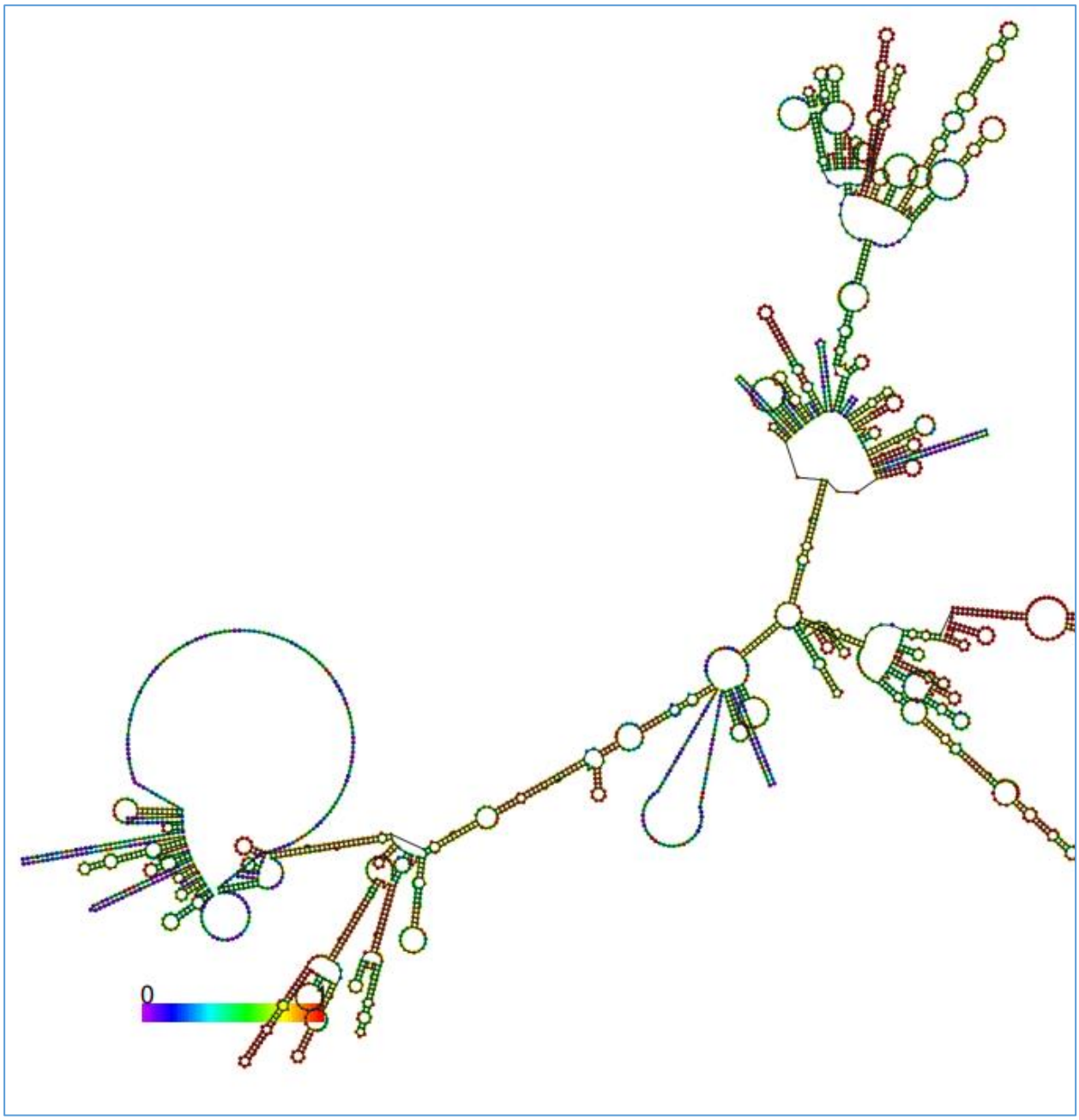
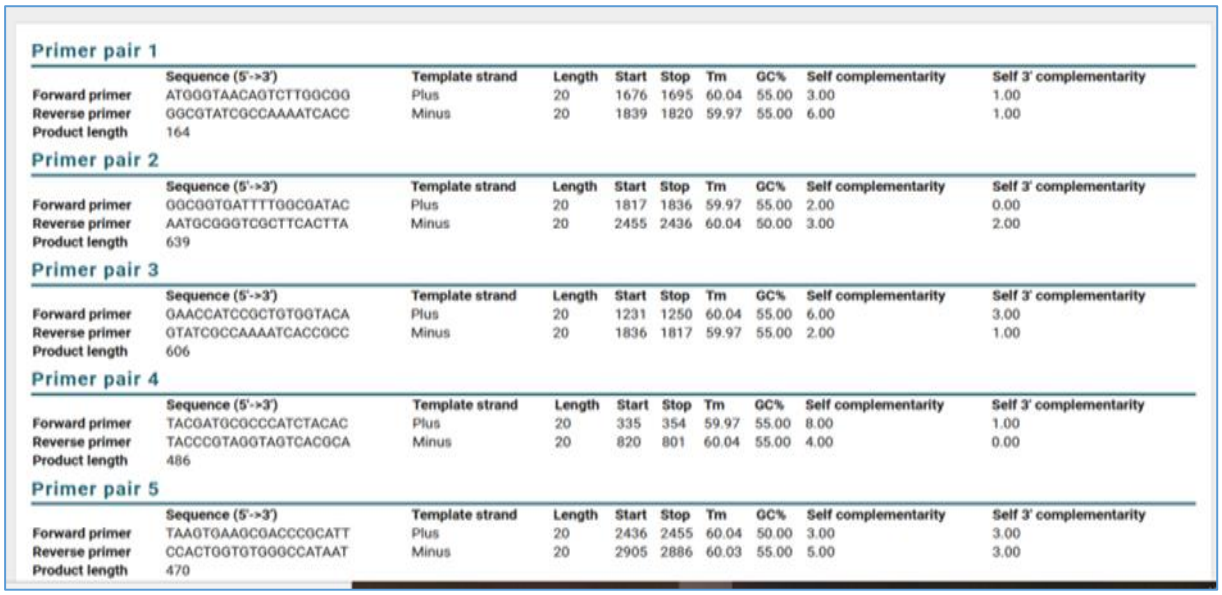


Figure 7: Putative secondary sequence structure in the whole plasmid structure obtained by RNA fold.

The goal of cloning is to obtain enzymes to test as allosteric ribozymes for synthetic control of gene expression.

Oligonucleotides and primers were ordered from Invitrogen by Thermo Fisher Scientific. Primers were selected using the BLAST algorithm (*Figure 8*).



Primer pair 1

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	ATGGGTAACAGTCTTGGCGG	Plus	20	1676	1695	60.04	55.00	3.00	1.00
Reverse primer	GGCGTATCGCCAAAATCACC	Minus	20	1839	1820	59.97	55.00	6.00	1.00
Product length	164								

Primer pair 2

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GGCGGTGATTTTGGCGATAC	Plus	20	1817	1836	59.97	55.00	2.00	0.00
Reverse primer	AATGCGGGTCGCTTCACTTA	Minus	20	2455	2436	60.04	50.00	3.00	2.00
Product length	639								

Primer pair 3

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GAACCATCCGCTGTGGTACA	Plus	20	1231	1250	60.04	55.00	6.00	3.00
Reverse primer	GTATCGCCAAAATCACCGCC	Minus	20	1836	1817	59.97	55.00	2.00	1.00
Product length	606								

Primer pair 4

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TACGATGCGCCCATCTACAC	Plus	20	335	354	59.97	55.00	8.00	1.00
Reverse primer	TACCCGTAGGTAGTCACGCA	Minus	20	820	801	60.04	55.00	4.00	0.00
Product length	486								

Primer pair 5

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TAAGTGAAGCGACCCGCATT	Plus	20	2436	2455	60.04	50.00	3.00	3.00
Reverse primer	CCACTGGTGTGGGCCATAAT	Minus	20	2905	2886	60.03	55.00	5.00	3.00
Product length	470								

Figure 8. The selected primers were obtained by the BLAST algorithm.

4.2. Cloning of a gene expression control construct in an Escherichia coli expression plasmid with a Lac Z reporter gene.

For the purpose of cloning, competent cells of *E. coli* strain HB101 (Takara Bio INC, Japan). The bacteria were grown in LB medium containing ampicillin (100 mg/L). The accuracy of the cloned sequences was verified by sequencing (Macrogen Europe, The Netherlands). The map of the pRS414 plasmid is depicted in Figure 9. The complete sequence of the pRS414ge plasmid is given at https://penchovsky.atwebpages.com/research.php?page=11.

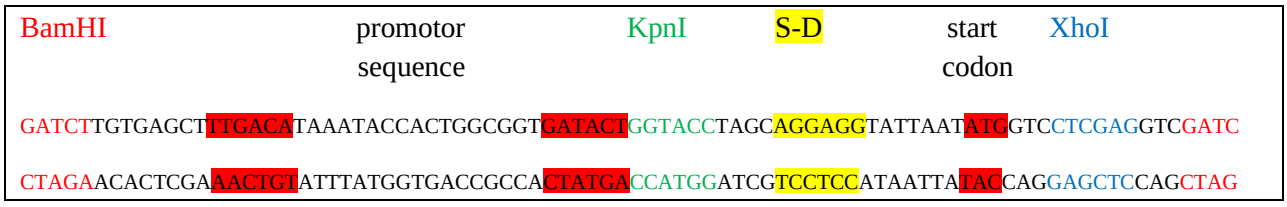


Figure 9. Map of pRS414 plasmid.
Source: Miloshev et al. (2021) Engineering a plasmid as a reporter system for quantifying gene expression in Escherichia coli

A beta-galactosidase assay was then performed to quantify the level of promoter activity. *E. coli* were allowed to grow overnight in LB medium containing ampicillin (100 mg/L) and incubated at 37°C with shaking. Permeabilization solution (80 µL) of (100 mM dibasic sodium phosphate ($Na2HPO4$), 20 mM KCl, 2 mM $MgSO4$, 0.8 mg/mL CTAB (hexadecyltrimethylammonium

bromide), 0.4 mg/mL sodium deoxycholate, 5.4 µL/mL beta-mercaptoethanol) was added, then the absorbance at 600 nm (Abs 600) was measured. 20 µL of ON culture was added to tubes containing the permeabilization solution and 600 µL of substrate solution containing ONPG. The time of addition is recorded. Three replicates were made of each sample for three-time points of 15, 20 and 30 minutes respectively. Once a sufficiently saturated color was obtained, 700 µl of stop solution (1 M sodium carbonate (Na_2CO_3)) was added and homogenized well. The tubes were then centrifuged at full speed for 5-10 minutes. The supernatant was poured into cuvettes and absorbance was measured at 420 nm (Abs 420.) The Miller formula (Jonoska, Seeman 2001) was used to calculate the Miller units (MU) of enzyme activity:

$$MU = 1000 \times \frac{\text{Abs } 420}{\text{Abs } 600 \times H \times T},$$

where T is the reaction time in minutes and V is the culture volume used in the analysis. Each experiment was triplicated, and the average of A420 was used to calculate the MU for each sample.

High levels of enzyme activity were recorded in all beta-galactosidase assays during the three-time points tested of 15, 20, and 30 minutes, respectively. For the 15th-minute measurement, 774.56 MU were obtained. Similar activity was obtained at the other time points with 773.99 MU and 763.48 MU at 20 and 30 minutes, respectively (Table 2).

Table 2. Results of all Beta-galactosidase assays during the three tested time points of 15, 20, and 30 minutes, respectively. Three replicates were performed and an average value was reported.

		λ = 600 nm (Abs 600)	λ = 420 nm (Abs 420)	ME
1.	15 min.	0,587	0,911	775,97
2.	15 min.	0,587	0,905	770,87
3.	15 min.	0,587	0,915	779,39
4.	20 min.	0,587	0,913	777,68
5.	20 min.	0,587	0,910	775,13
6.	20 min.	0,587	0,903	769,17
7.	30 min.	0,587	0,912	759,79
8.	30 min.	0,587	0,902	768,31
9.	30 min.	0,587	0,895	762,35

The strong promoter of the constitutive bacteriophage λ expressing the lacZ gene gave similar results for all spots in triplicate analysis. All three-time points of the PL promoter assay showed MU above 760, indicating a higher level of enzyme activity. The negative control sample lacking the λ phage promoter did not show any beta-galactosidase activity at the 30-min time point, as expected due to the lack of promoter from the plasmid *(Figure 10)*.

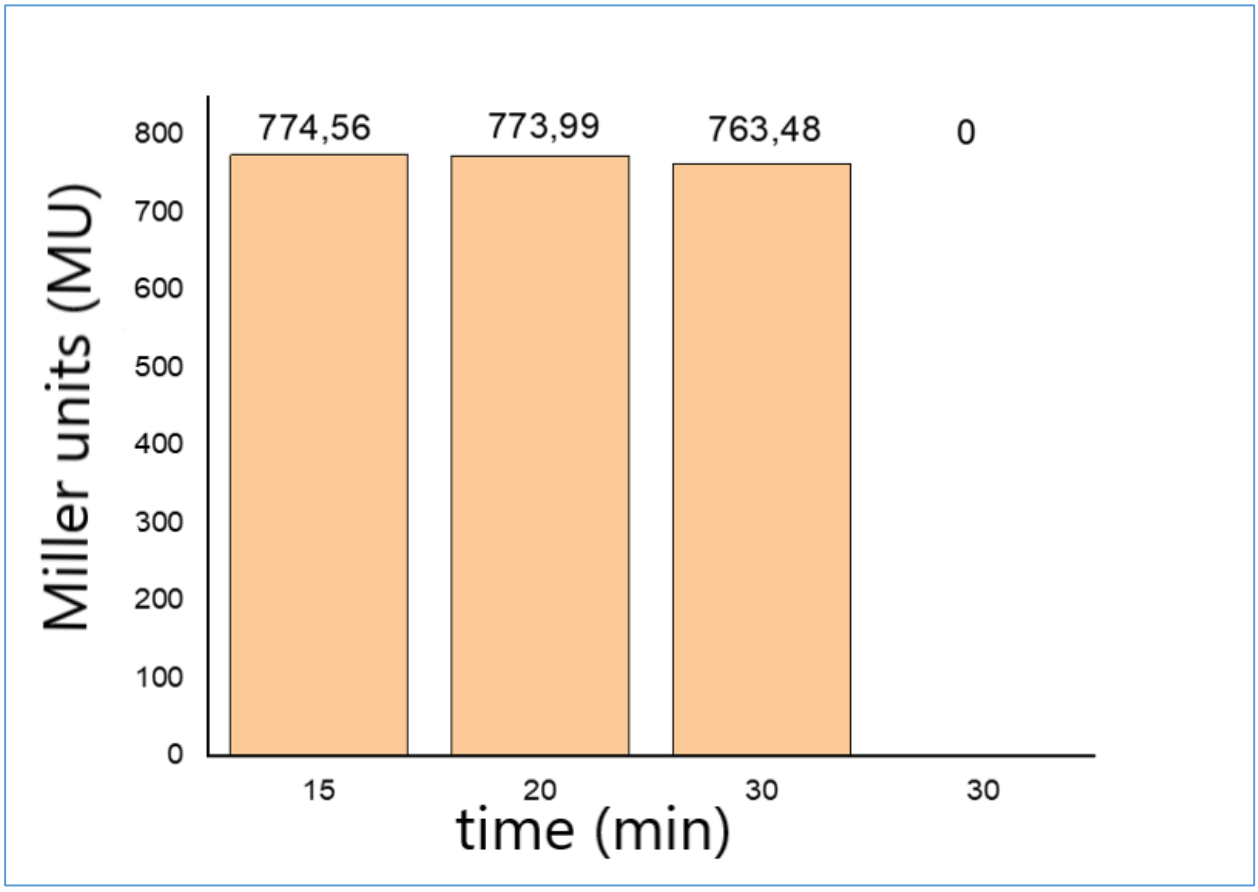


Figure 10. Graphical representation of beta-galactosidase enzyme activity over the three-time points tested of 15, 20, and 30 minutes.
The negative control sample lacking the λ phage promoter showed no beta-galactosidase activity at the 30-min time point.

In the absence of the promoter, beta-galactosidase assays showed 11.08 MU, but only as a background signal. These results indicate that the generated reporter gene construct using the pRS414 plasmid worked efficiently and gave the expected results regarding the enzymatic activity of beta-galactosidase among the tested sample and the negative control lacking the PL promoter at the times that were tested (*Figure 11*). The experiments were repeated 3 times.

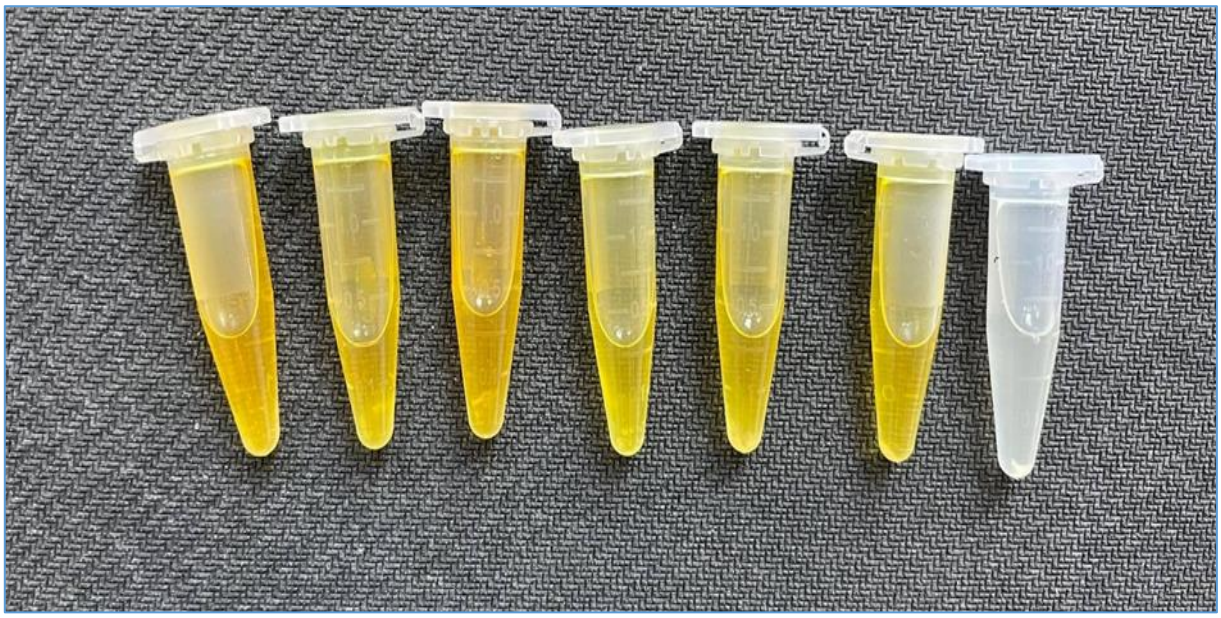


Figure 11. Results of experimental testing of beta-galactosidase activity*. Yellowed tubes showed high levels of beta-galactosidase activity. The colorless tube showed no staining in the absence of the promoter and only as a background signal (negative control).*

With this experiment, I proved that the new lacZ reporter system for RNA synthetic biology research had created a novel pRS414 gene reporter plasmid. A DNA fragment carrying a KpnI restriction site, the Shine-Dalgarno sequence, and a transcription-initiating ATG codon in the lacZ gene frame was used to create the reporter system. I also added the BamHI and XhoI restriction sites of the plasmid. The present study introduces the promoter sequence of λ phage PL of 38 base pairs into the plasmid as a BamHI - KpnI fragment using the new KpnI site introduced in the previous step. Analysis of beta-galactosidase activity confirmed the accuracy of the expression system, as enzyme activity was obtained in all samples tested, except the negative control. The strong promoter of λ phage PL expressed the lacZ reporter gene constitutively throughout, providing similar results during the activity assay. The newly generated system was successfully used for synthetic control of gene expression for designer ribozymes and an antisense oligonucleotide. The plasmid used for the experiment is not the novel 4788 base pair pRS414 plasmid and was designed to apply synthetic gene expression in *E. coli*. The complete sequence of our novel pRS414ge plasmid containing the PL promoter (*Figure 12*) of the λ phage and has a length of 10715 bp is given. The new pRS414ge is a unique tool for genetic control experiments in the field of RNA synthetic biology as it offers single-step cloning of various allosteric ribozymes, a Schein-Dalgarno sequence, and target sites for antisense oligonucleotides, which is a very convenient and accurate gene expression kit. The submitted plasmid is available for academic research upon request.

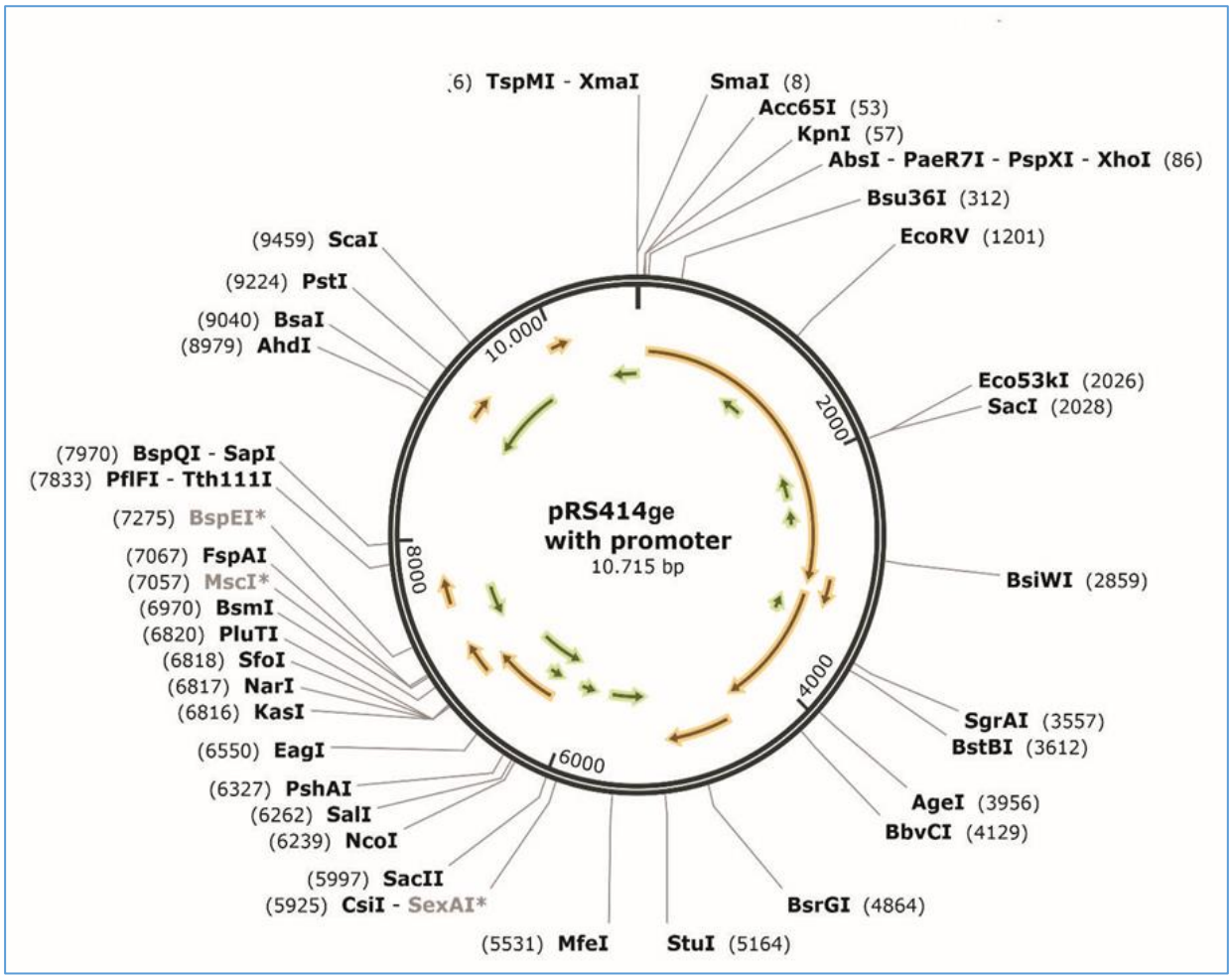


Figure 12. Restriction site map of the generated plasmid pRS414 (10715 kb), including the restriction site for KpnI and PL promoter.

Source: Miloshev et al. (2021) Engineering a plasmid as a reporter system for quantifying gene expression in Escherichia coli

A restriction reaction was performed, followed by ligation, then chemical transformation and electroporation. The results of chemical transformation and electroporation can be seen in Figure 13, Figure 14, Figure 15, and Figure 16. The experiments were repeated several times in order to isolate a good and visible single colony. The electroporation results show visibly better results after transformation compared to the chemical transformation results.

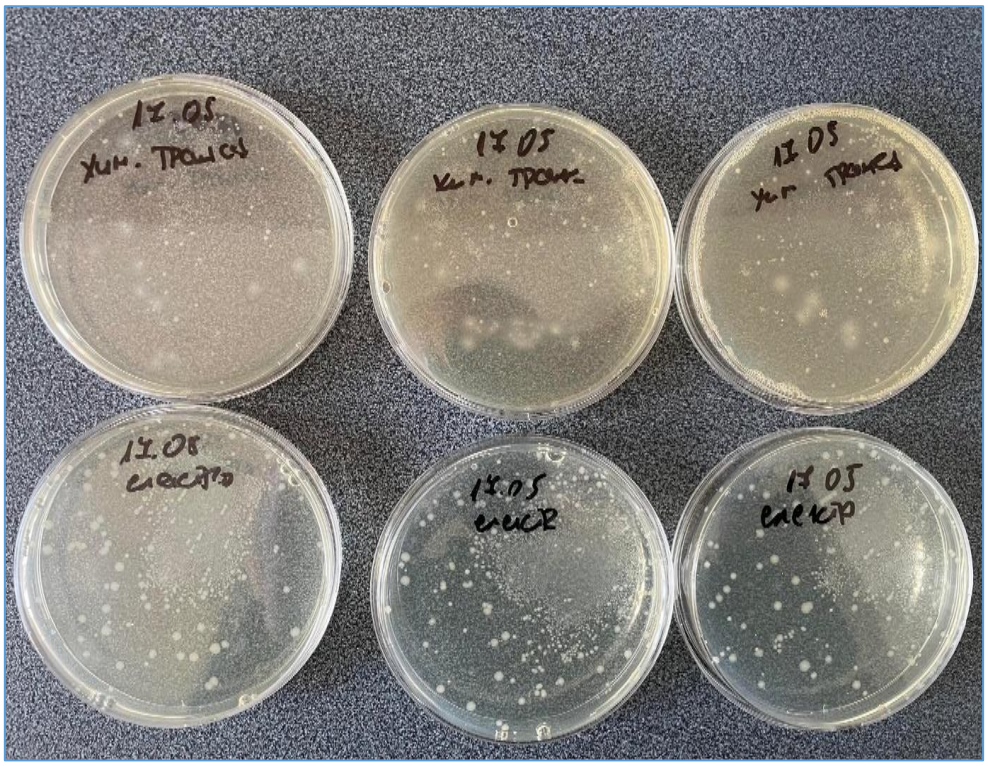


Figure 13. Comparison of results from petris treated with chemical transformation and electroporation. Better results are visualized with electroporation. *The first row is petri dishes after chemical transformation, the second row is petri dishes after electroporation.*

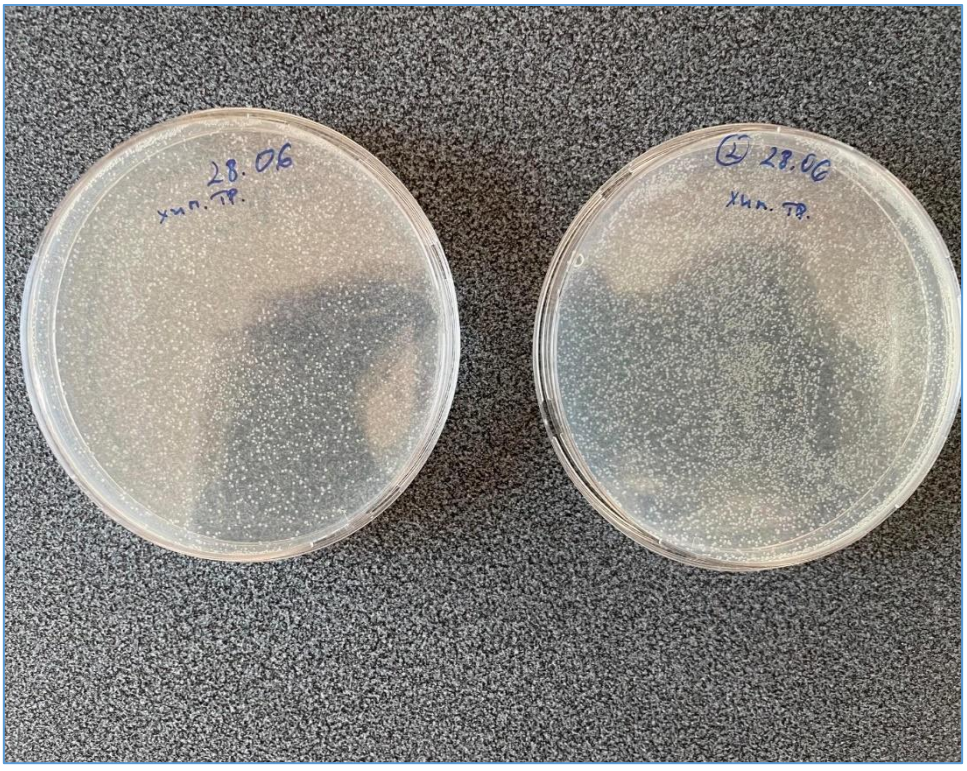


Figure 14. Petri dishes results after chemical transformation.
Multiple colonies are observed.

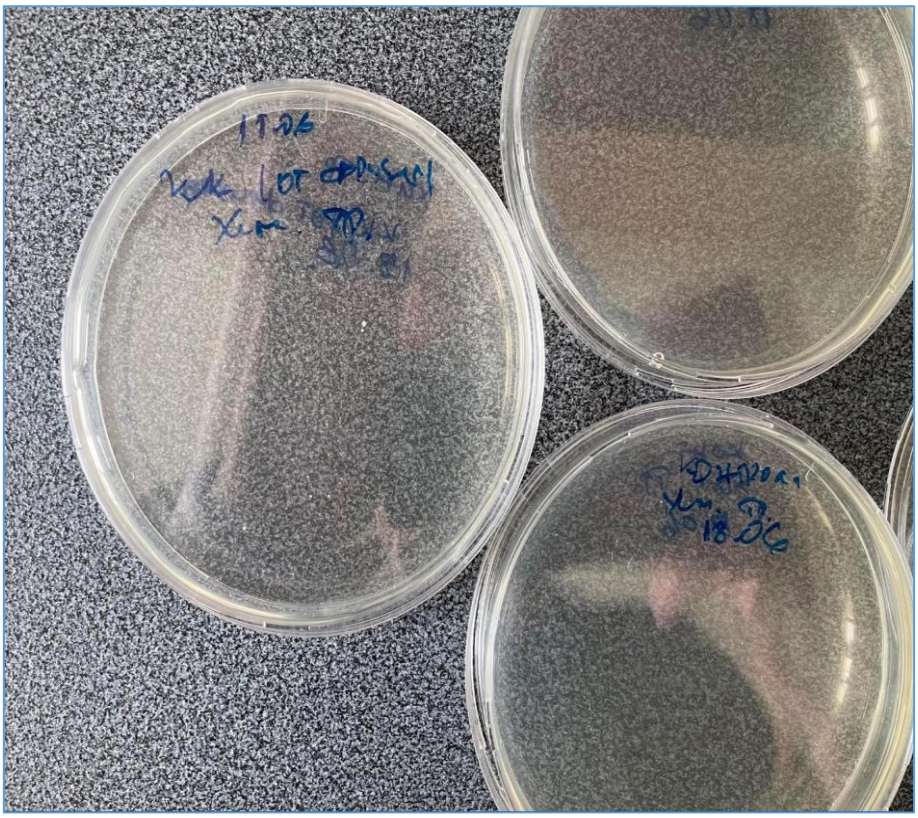


Figure 15. Single colonies.
The experiment was repeated several times to isolate a single colony.

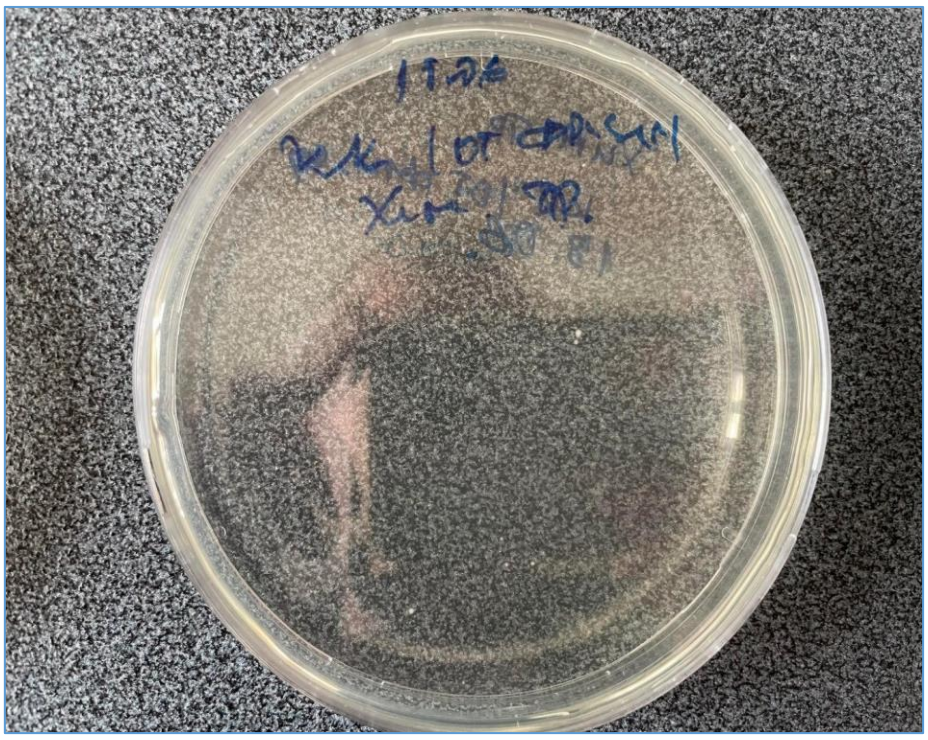


Figure 16. Single colonies.
The experiment was repeated several times to isolate a single colony.

A few clones were selected that were shortened by the KpnI enzyme and sent to Macrogene Europe for sequencing. The sequencing results are visible in Figures 17, 18, and 19. The sequencing results proved the presence of the nucleotide restriction sequences used, the Schein-Dalgarno sequence, the start codon, and the presence of the ASO used. Plasmid DNA was isolated using the *Sigma-Aldrich - GenElute™ Plasmid Miniprep Kit* to perform the sequencing. Twenty different samples were prepared in a final volume of 10 microliters and a concentration of 150 nanograms per microliter. The concentration of isolated plasmid DNA was detected with a nanodrop from *BioDrop*. The final sequencing result was obtained 2 months after shipment.

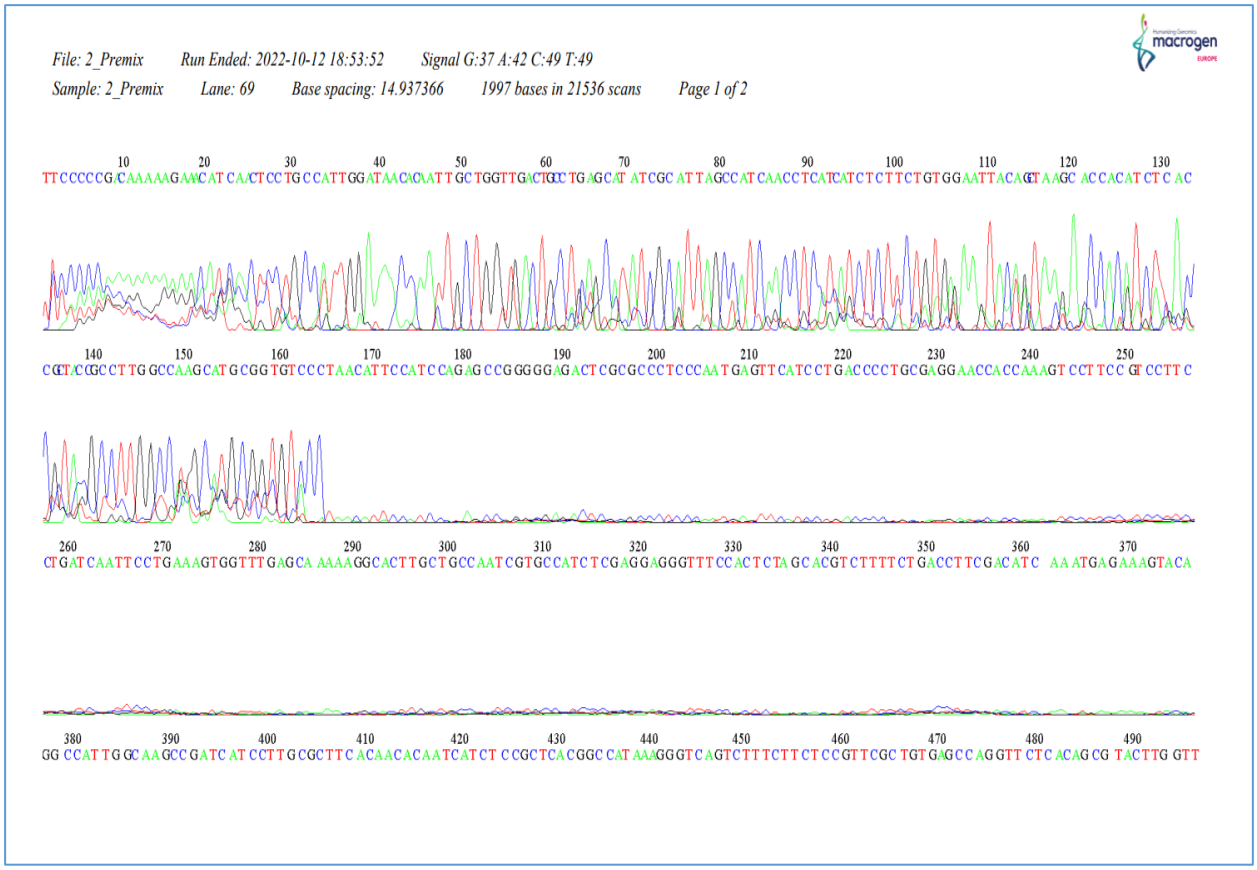


Figure 17. Sequencing performed by Macrogene Europe.
Pronounced colour peaks indicate the presence of a good result after PCR. The different peaks correspond to individual nucleotides.

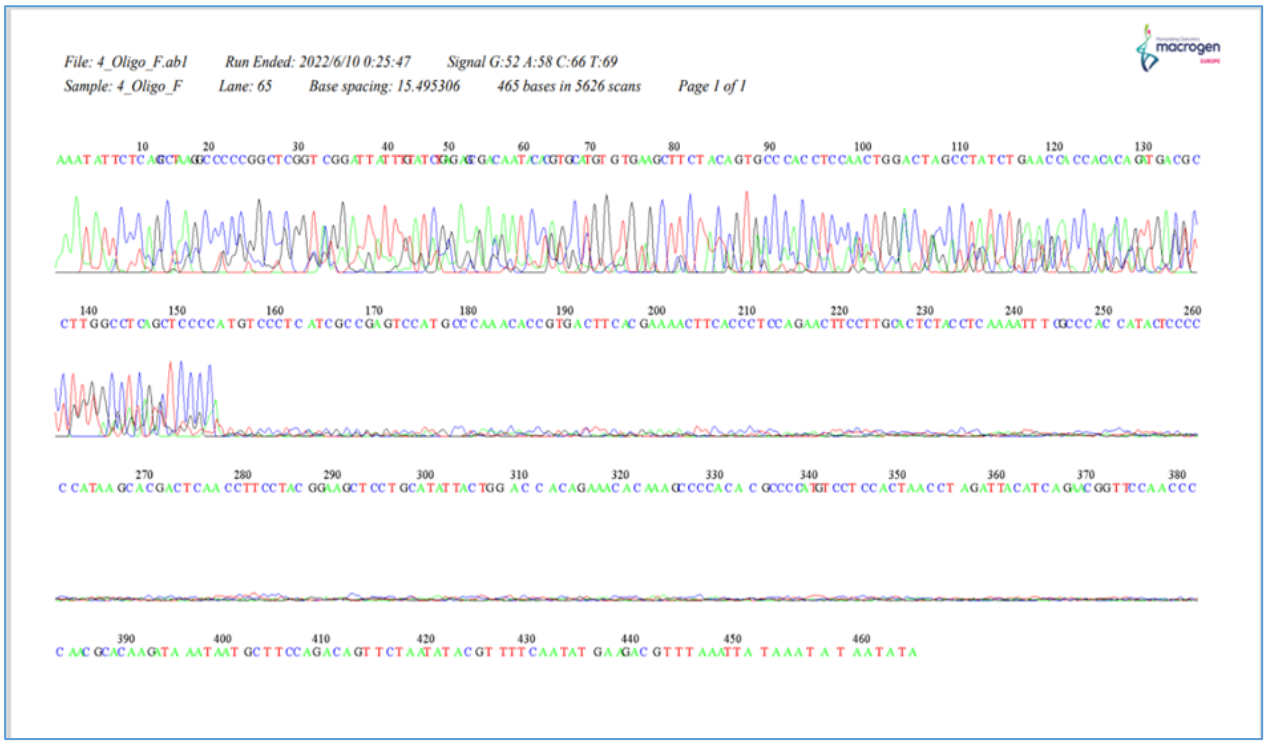


Figure 18. Sequencing performed by Macrogene Europe
Pronounced colour peaks indicate the presence of a good result after PCR. The different peaks correspond to individual nucleotides.

Figure 19. Sequencing performed by Macrogene Europe.
Pronounced colour peaks indicate the presence of a good result after PCR. The different peaks correspond to individual nucleotides.

LacZ expression was also tested by the X gal screening method. 0.2 g (200 mg) along with 10 ml. DMF (N,N-dimethylformamide). 0.238 g of 100 mM isopropyl-beta-D-thiogalactopyranoside was also added. Add 100 mL of distilled water and pour into a petri dish containing culture medium (Lysogen broth) and agar. The petri dishes were allowed to stand overnight at 37 degrees. After 12 hours, I report the presence of the specific blue color characteristic of the X gal method. This was done to confirm the results. The results are visible in figures 20 and 21.

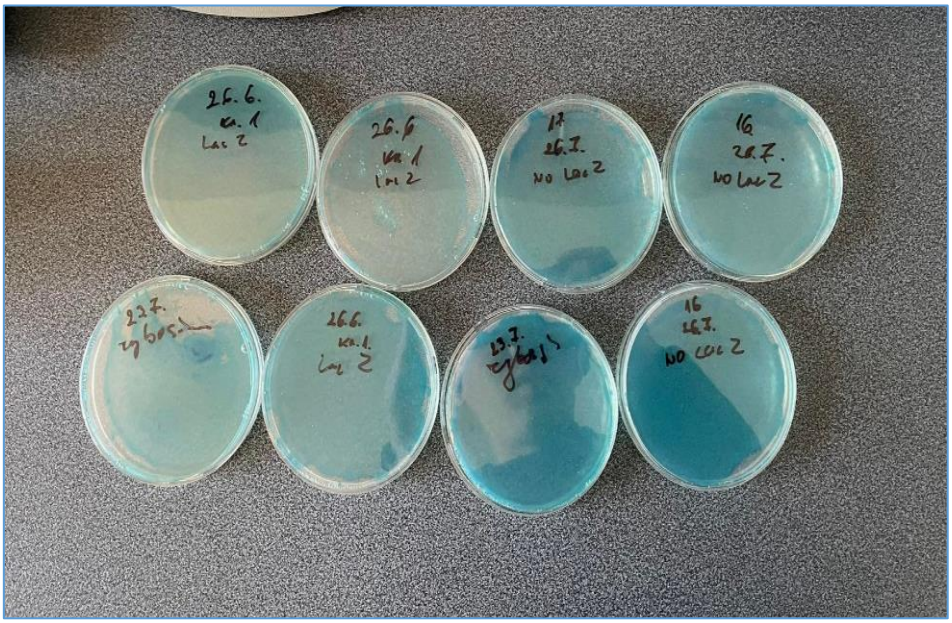


Figure 20. LacZ expression results tested by X gal screening method.
A characteristic blue coloration is observed.

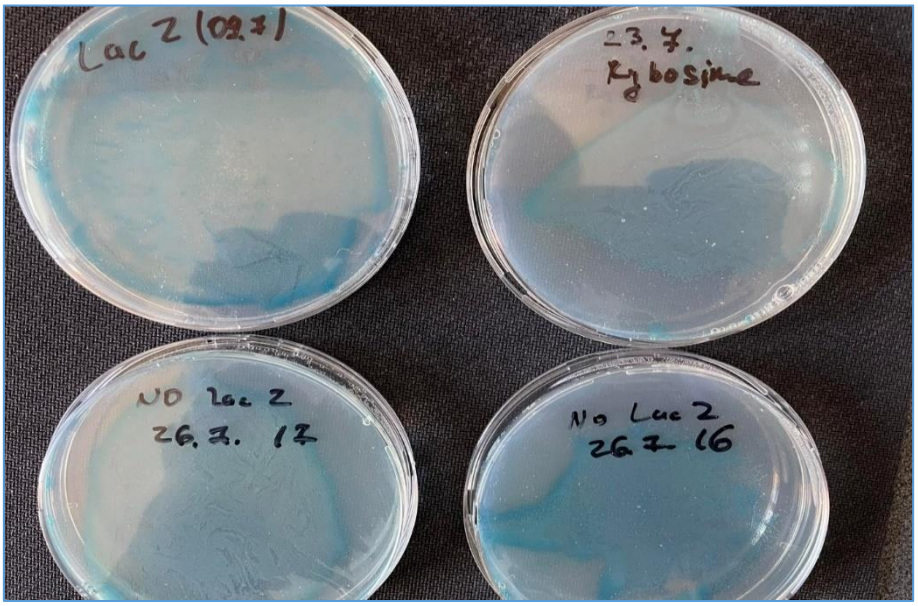


Figure 21. LacZ expression results tested by X gal screening method.
A characteristic blue coloration is observed.

The sequences were bioinformatically analyzed with the ClustalX32 and SnapGene programs to confirm the cloning results. The results are visible in Figures 22. and 23. The results of multiple alignments of the plasmid sequence with the plasmid sequence containing the cloned fragments are visible.

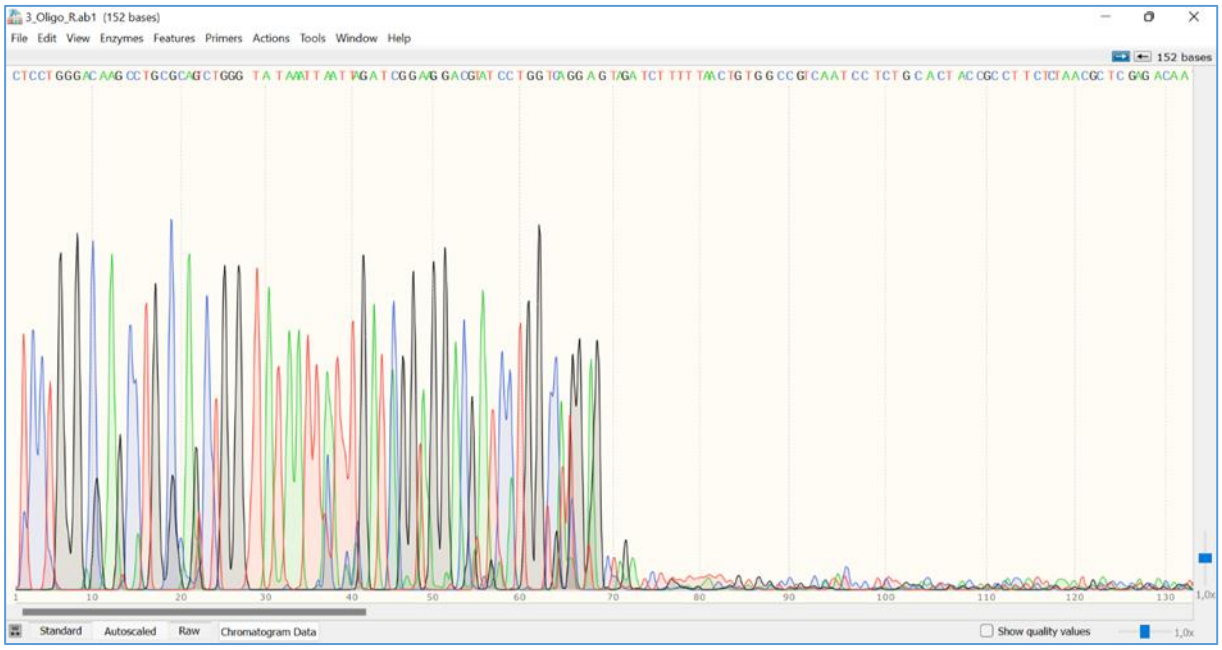


Figure 22 - Bioinformatic analysis with SnapGene.
The distinct color peaks show the matching of the bases between the different sequences.

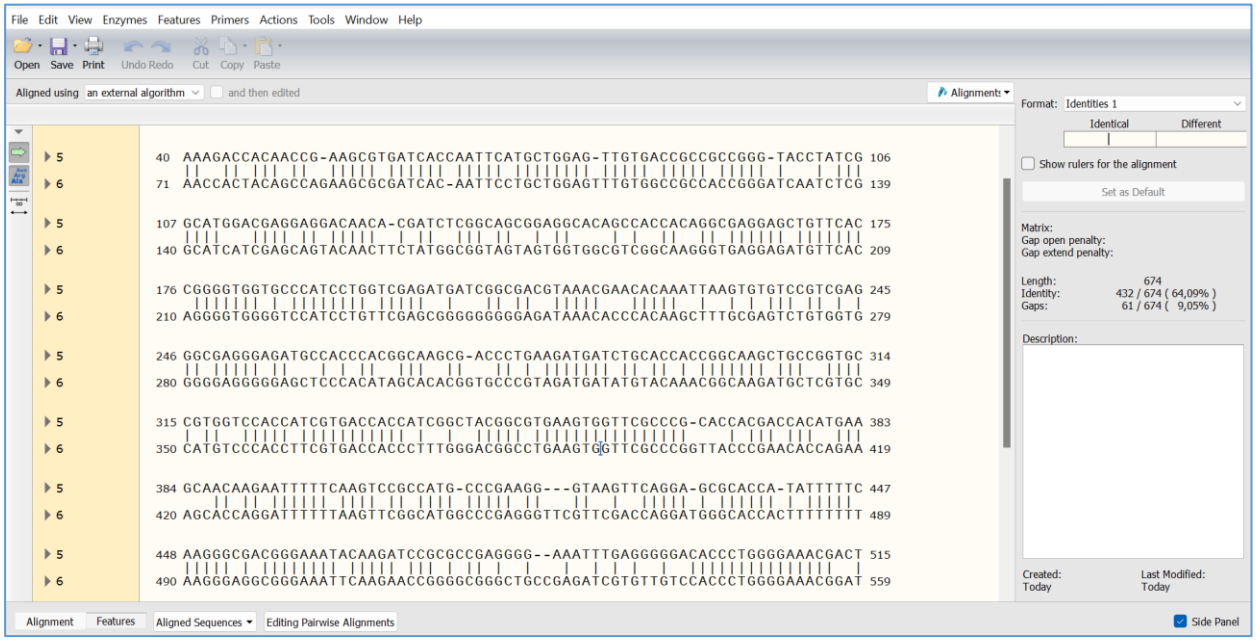


Figure 23. Bioinformatic analysis with ClustalX 32.
The linking between them shows the matching of bases between the different sequences.

4.3. Design of strategy for OFF Switch control of gene expression in *Escherichia coli.*

In designing the OFF Switch gene expression control strategy in *Escherichia coli*, I have used a combination of first and second-generation. The strategy aims to inhibit bacterial growth by hybridizing the ASO to the target sequence. The designed ASO is 19 nucleotides long and has the following sequence: 5′- CAGGCTCGCATGGCTCGCC- 3′. The structure of the fragment can be seen in Figure 24.

Figure 24. Structure of the fragment containing an antisense oligonucleotide that inhibits bacterial growth. *Restrictase sequences are marked with different colors.*
Source: Miloshev et al. (2021) Engineering a plasmid as a reporter system for quantifying gene expression in Escherichia coli

To be recognized by RNase H endonuclease and to hybridize with it to trigger enzymatic degradation of mRNA and thus inhibit bacterial growth, the ASO was modified in the middle 11 nucleotides. In such a modification, one of the oxygen atoms of the phosphodiester bond is replaced by a sulfur atom. This modification is characteristic of first-generation ASOs. This enables RNase H to function, unlike second and third-generation ASOs. Second-generation modifications increase the stability of ASO against mononucleases and exonucleases. Thus, terminal nucleotides are modified from the 5′ and 3′ ends. After the modifications, the sequence of ASO is 5′- C2A2G2G2C1T1C1G1C1A1T1G1G1C1T1C2G2C2C2- 3′.

The OFF Switch gene expression control strategy in *Escherichia coli* is shown in Figure 25. When the ASO binds to the target sequence, the Schein-Dalgarno sequence is disrupted, and accordingly, no gene expression is observed.

ACO is covalently bound to a cell-penetrating peptide, pVEC (CPP). A chimeric molecule is formed. The pVEC CPP can pass through the cell wall of prokaryotic cells, but also through the cell wall of eukaryotic cells. The concentration of pVEC CPP used is not toxic to prokaryotic cells, in this case, *Escherichia coli* cells.

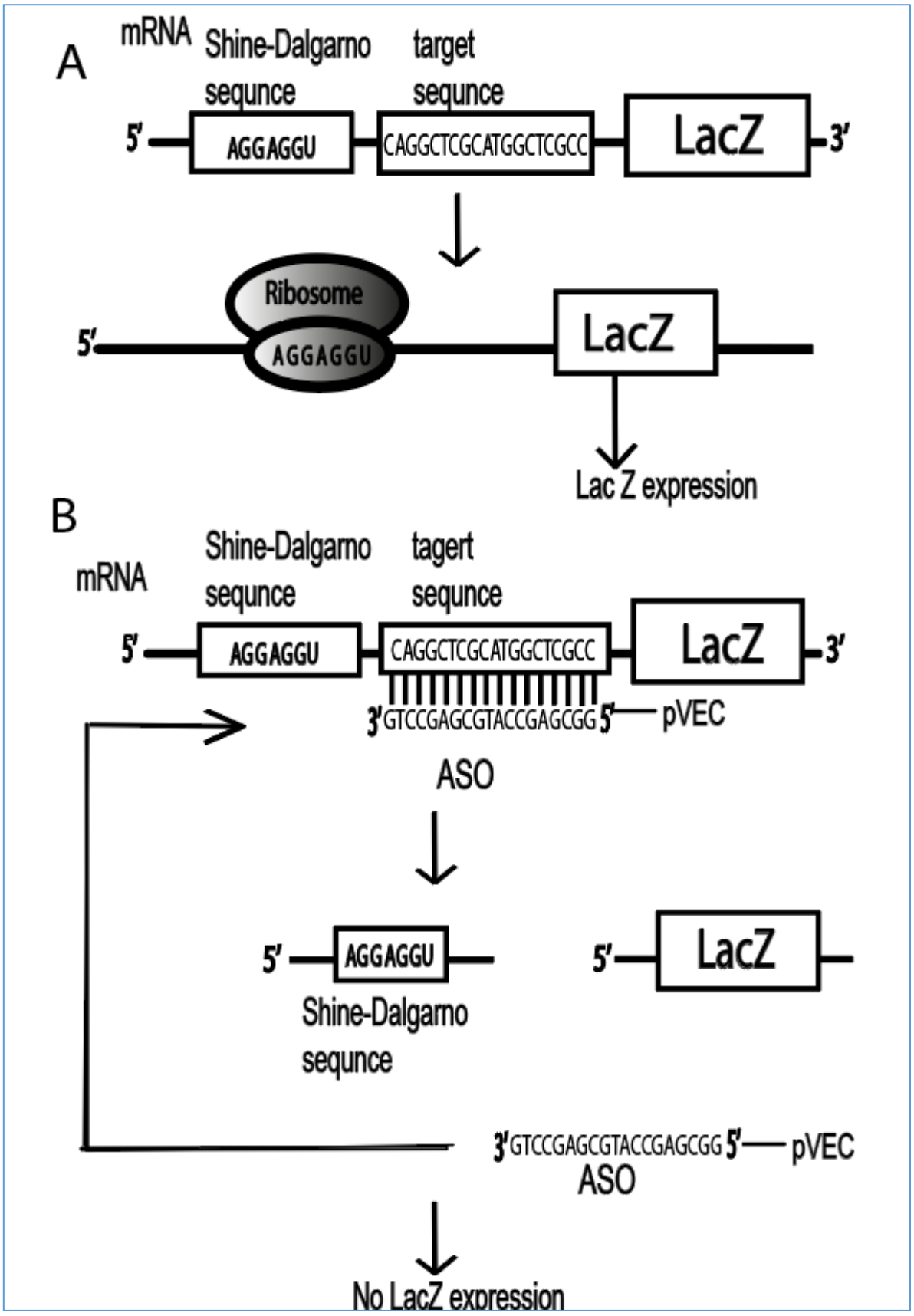


Figure 25. Schematic representation of an OFF Switch control strategy for gene expression in Escherichia coli

A - Gene expression of LacZ - in the absence of ASO. B-When ASO binds to the target sequence, the Shine-Dalgarno sequence is disrupted, and accordingly, no gene expression is observed.

pVEC CPP does not require a specific temperature to enter through a cell that wall. The CPP has the following sequence LLIILRRRIRKQAHAHSK, which is encoded by the following amino acids H2N-Leu-Leu-Ile-Ile-Leu-Arg-Arg-Arg-Ile-Arg-Lys-Gln-Ala-His-Ala-his-Ser-Lys-OH. pVEC is composed of 18 amino acids. The nitrogen end of the CPP is hydrophobic, and the carbon end is hydrophilic. Its middle part is positively charged because there are many arginine residues. Once it enters the bacterium, only the ASO hybridizes to the target sequence.

For the experiment, cells of E. coli strain HB101 (Takara Bio INC, Japan). The bacteria were grown in LB medium containing ampicillin (100 mg/L).

4.4. Experimental testing of antisense oligonucleotides for OFF Switch control of gene expression in *Escherichia coli.*

To accomplish this task, I tested antisense oligonucleotides with 9 different concentrations (table) to find the percentage of inhibition of bacterial growth of *Escherichia coli* at different concentrations. The experiments were repeated 3 times and an average value was taken.

Table 3. 9 different concentrations of ACO were used to establish the percentage of inhibition of bacterial growth of Escherichia coli

1.	100 micrograms/millilitre
2.	200 micrograms/millilitre
3.	279 micrograms/millilitre
4.	559 micrograms/millilitre
5.	840 micrograms/millilitre
6.	1119 micrograms/millilitre
7.	1440 micrograms/millilitre
8.	1678 micrograms/millilitre
9.	2238 micrograms/millilitre

The experiment was started with an antisense oligonucleotide concentration of 100 micrograms/milliliter. For the experiment, a bacterial culture (within 8-9 hours) was plated in a vial containing 20 milliliters of Lysogeny broth and 20 microliters of ampicillin (100 mg/L). Culturing was performed on a shaker at a constant temperature of 37°C and 200 rpm. After incubation, a dilution of the bacterial culture is made at a ratio of 1:1000, and testing is done at an interval of 6-8 hours. Plate 3 tubes containing bacterial culture without antisense oligonucleotide and 2 tubes containing bacterial culture and 100 micrograms/milliliter of antisense oligonucleotide. Every 30 minutes, the growth of the bacterial culture is counted. When cessation of bacterial growth is detected, a beta-galactosidase assay is performed according to the protocol described previously. The mean value of beta-galactosidase activity of bacterial cultures containing ASO was 502.84 MU, and that of bacterial cultures without ASO was 510.84 MU. At this concentration of antisense oligonucleotide, there was 98.44% LacZ expression and 1.56% inhibition of bacterial growth, respectively (*Figure 26*).

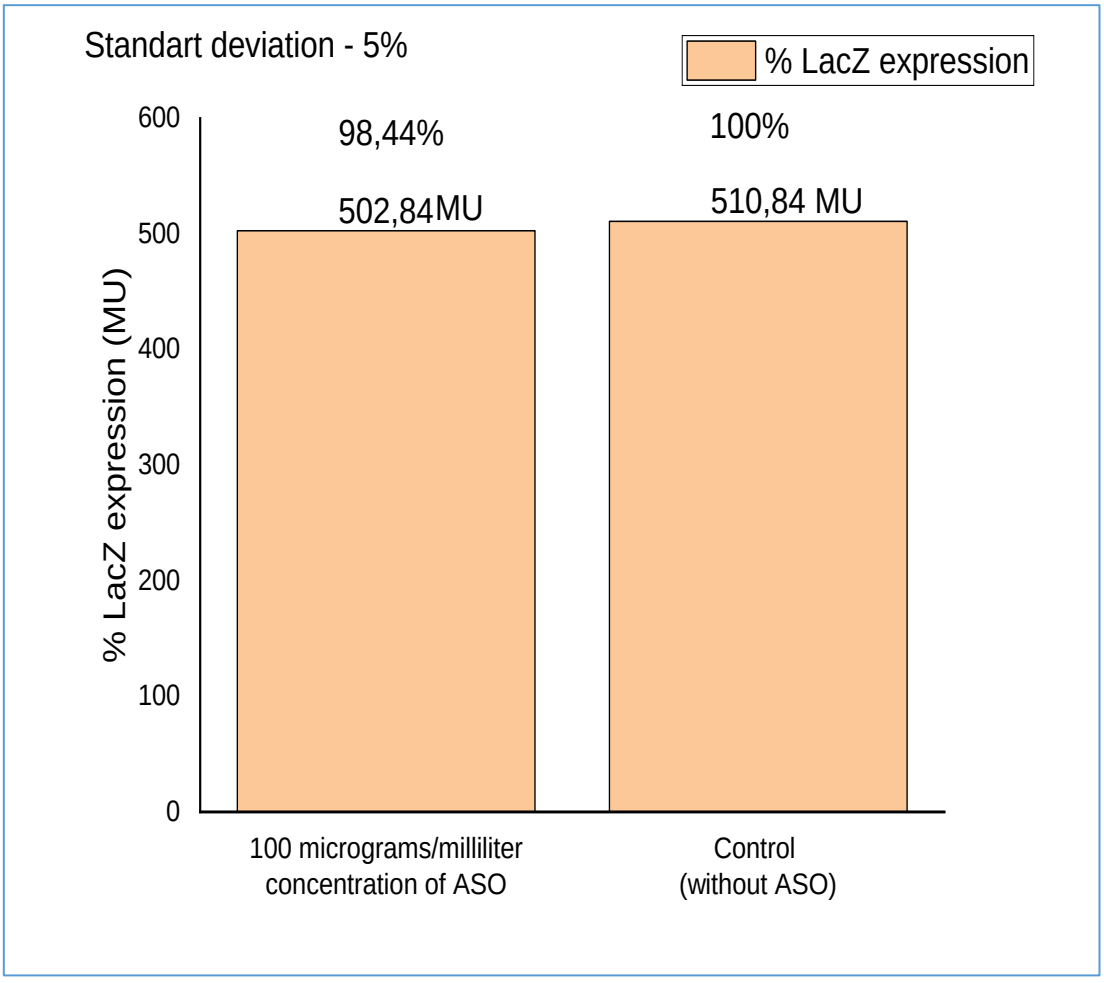


Figure 26. At an ASO concentration of 100 micrograms/milliliter, 98.44% LacZ expression (502,84 MU) and 1.56% bacterial growth inhibition were observed, respectively.

The experiment was continued with an antisense oligonucleotide concentration of 200 micrograms/milliliter. For the experiment, a new bacterial culture was plated (within 8-9 hours) in a vial containing 20 milliliters of Lysogeny broth and 20 microliters of ampicillin (100 mg/L). Culturing was performed on a shaker at a constant temperature of 37°C and 200 rpm. After incubation, a dilution of the bacterial culture is made at a ratio of 1:1000 and testing is done at an interval of 6-8 hours. Plate 3 tubes containing bacterial culture without antisense oligonucleotide and 2 tubes containing bacterial culture and 200 micrograms/millilitere of antisense oligonucleotide. Every 30 minutes, the bacterial culture growth is counted. When cessation of bacterial growth is detected, a beta-galactosidase assay is performed according to the protocol described previously. The mean value of beta-galactosidase activity of bacterial cultures containing ASO was 449.19 MU and that of bacterial cultures without ASO was 498.44 MU At this concentration of antisense oligonucleotide, 90.10% LacZ expression and 9.90% inhibition of bacterial growth were observed, respectively *(Figure 27*)

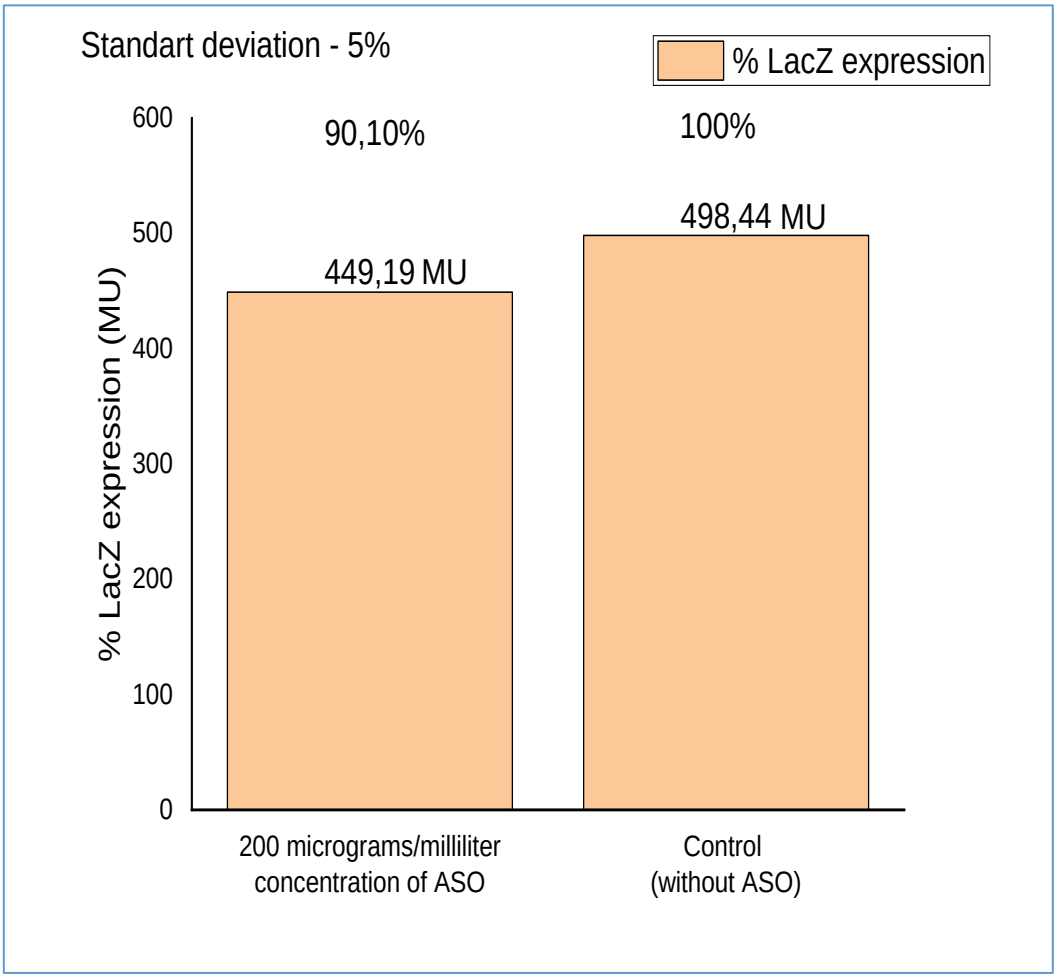


Figure 27. At an ASO concentration of 200 micrograms/milliliter, 90.10% LacZ expression (449,19 MU) and 9.90% bacterial growth inhibition were observed, respectively.

I then tested bacterial survival with an antisense oligonucleotide concentration of 279 micrograms/milliliter. For the experiment, a new bacterial culture was plated (within 8-9 hours) in a vial containing 20 milliliters of Lysogeny broth and 20 microliters of ampicillin (100 mg/L). Culturing was performed on a shaker at a constant temperature of 37°C and 200 rpm. After incubation, the bacterial culture was diluted 1:1000 and tested at 6-8 hours intervals. Plate 3 tubes containing bacterial culture without antisense oligonucleotide and 2 tubes containing bacterial culture and 279 micrograms/milliliter of antisense oligonucleotide. The bacterial culture growth was counted every 30 minutes. When cessation of bacterial growth is detected, a beta-galactosidase assay is performed according to the protocol described previously. The mean value of beta-galactosidase activity of bacterial cultures containing ASO was 436.74 MU and that of bacterial cultures without ASO was 510.84 MU. At this concentration of antisense oligonucleotide, 85.50% LacZ expression and 14.50% inhibition of bacterial growth were observed, respectively (*Figure 28*).

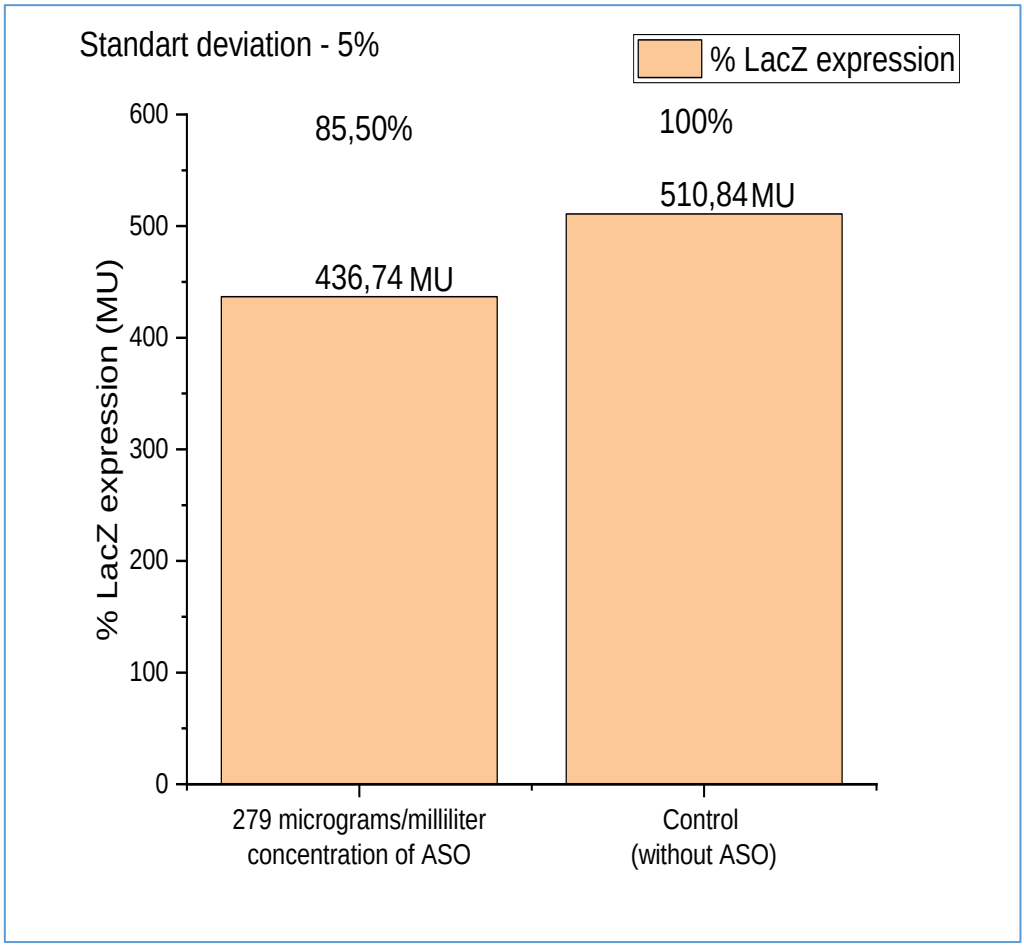


Figure 28. At an ASO concentration of 279 micrograms/milliliter, 85.50% LacZ expression (436,74 MU) and 14.5% bacterial growth inhibition were observed, respectively.

The experiment was continued with an antisense oligonucleotide concentration of 559 micrograms/milliliter. For the experiment, a new bacterial culture was plated (within 8-9 hours) in a vial containing 20 milliliters of Lysogeny broth and 20 microliters of ampicillin (100 mg/L). Culturing was performed on a shaker at a constant temperature of 37°C and 200 rpm. After incubation, the bacterial culture was diluted 1:1000 and tested at 6-8 hours intervals. Plate 3 tubes containing bacterial culture without antisense oligonucleotide and 2 tubes containing bacterial culture and 559 micrograms/milliliter antisense oligonucleotide. The bacterial culture growth was counted every 30 minutes. When cessation of bacterial growth is detected, a beta-galactosidase assay is performed according to the protocol described previously. The mean value of beta-galactosidase activity of bacterial cultures containing ASO was 407.66 MU and that of bacterial cultures without ASO was 510.84 MU. At this concentration of antisense oligonucleotide, 79.80% LacZ expression and 20.20% inhibition of bacterial growth were observed, respectively (*Figure 29*).

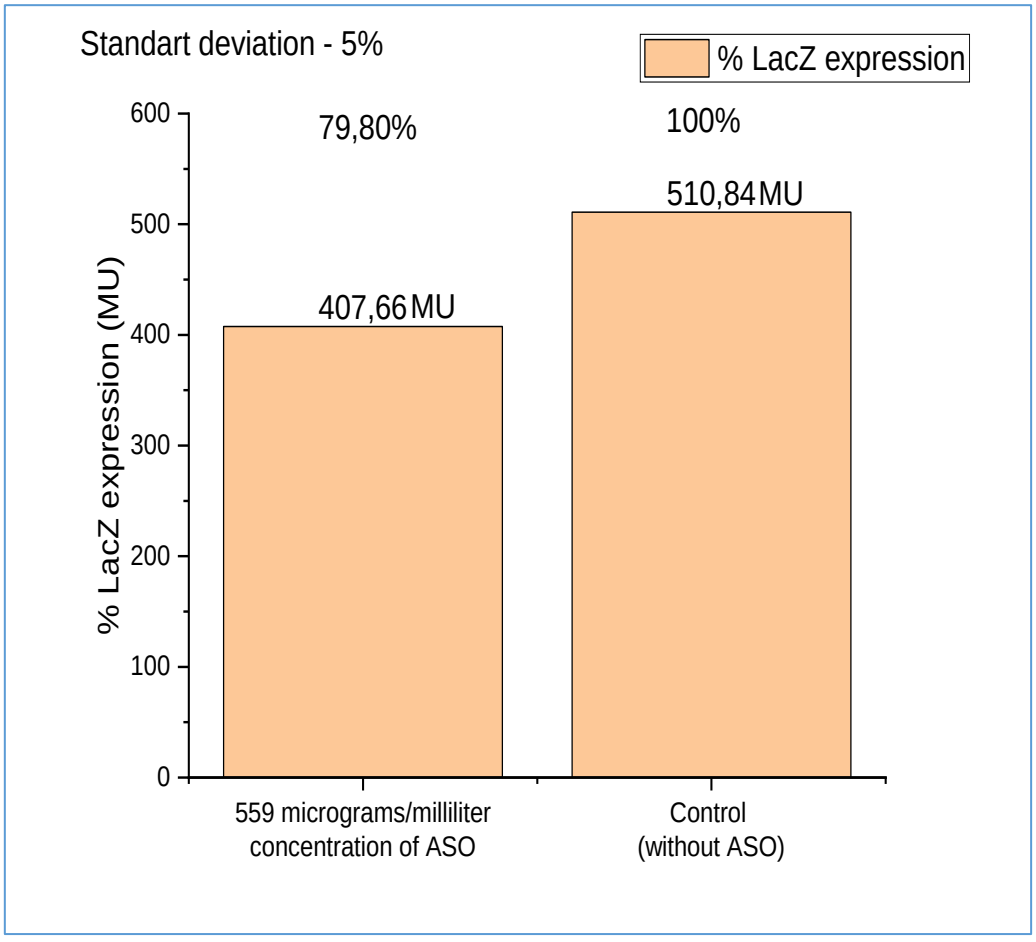


Figure 29. At an ASO concentration of 559 micrograms/milliliter, 79.80% LacZ expression (407,66 MU) and 20.20% bacterial growth inhibition were observed.

The percentage of bacterial growth inhibition was tested at an antisense oligonucleotide concentration of 840 micrograms/milliliter (*Figure 30*). For the experiment, a new bacterial culture was plated (within 8-9 hours) in a vial containing 20 milliliters of Lysogeny broth and 20 microliters of ampicillin (100 mg/L). Culturing was performed on a shaker at a constant temperature of 37°C and 200 rpm. After incubation, the bacterial culture was diluted at a ratio of 1:1000 and tested at an interval of 6-8 hours. Plate 3 tubes containing bacterial culture without antisense oligonucleotide and 2 tubes containing bacterial culture and 840 micrograms/millilitre of antisense oligonucleotide. Every 30 minutes, the growth of the bacterial culture is counted. When cessation of bacterial growth is detected, a beta-galactosidase assay is performed according to the protocol described previously. The mean value of beta-galactosidase activity of bacterial cultures containing ASO was 340.40 MU, and that of bacterial cultures without ASO was 498.44 MU At this concentration of antisense oligonucleotide, 68.29% LacZ expression and 31.71% inhibition of bacterial growth were observed, respectively (*Figure 30*).

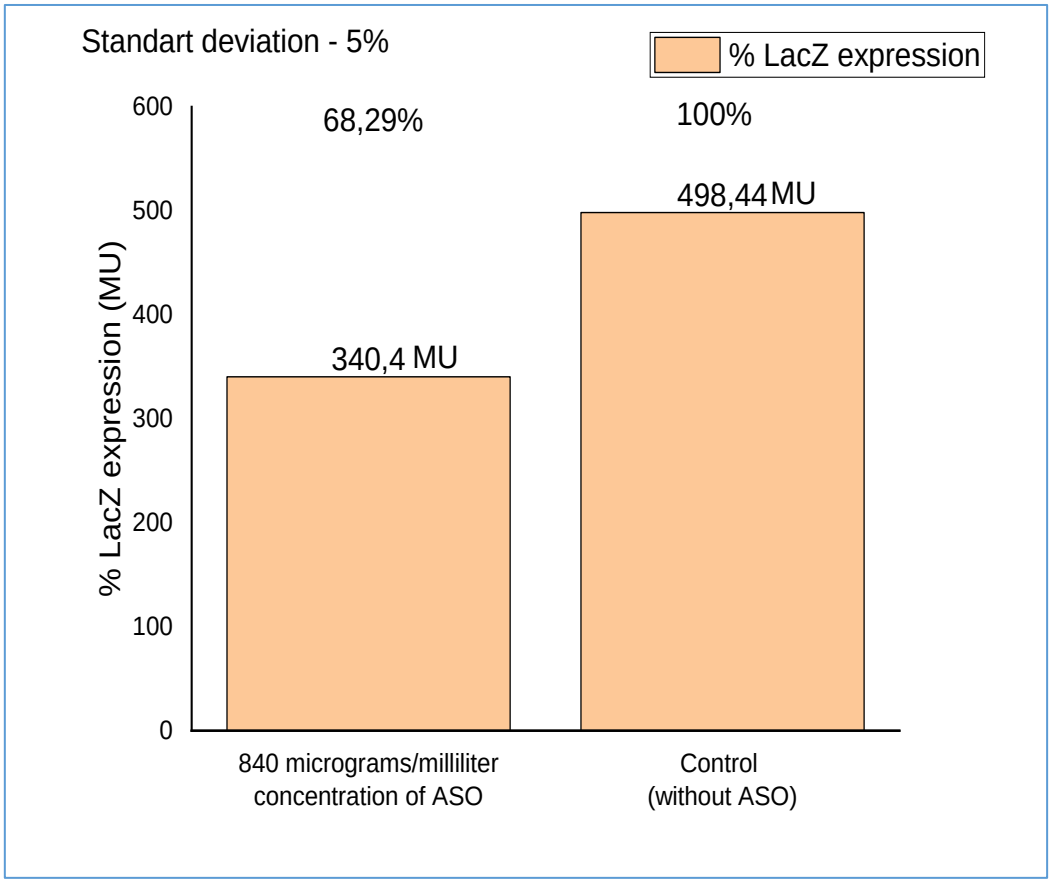


Figure 30. At an ASO concentration of 840 micrograms/milliliter, 68.29% LacZ expression (340,40 MU) and 31.71% bacterial growth inhibition were observed, respectively.

I continued the experiment to test bacterial growth's inhibition percentage at an antisense oligonucleotide concentration of 1119 micrograms/milliliter (*Figure 31*). For this purpose, a new bacterial culture (within 8-9 hours) was plated in a vial containing 20 milliliters of Lysogeny broth and 20 microliters of ampicillin (100 mg/L). Culturing was performed on a shaker at a constant temperature of 37°C and 200 rpm. After incubation, the bacterial culture was diluted at a ratio of 1:1000 and tested at an interval of 6-8 hours. Plate 3 tubes containing the bacterial culture without antisense oligonucleotide and 2 tubes containing the bacterial culture and 1119 micrograms/milliliter of antisense oligonucleotide. The bacterial culture growth was counted every 30 minutes. When cessation of bacterial growth is detected, a beta-galactosidase assay is performed according to the protocol described previously. The mean value of beta-galactosidase activity of bacterial cultures containing ASO was 304.38 MU and that of bacterial cultures without ASO was 520.76 MU. At this concentration of antisense oligonucleotide, 58.45% LacZ expression and 41.55% inhibition of bacterial growth were observed, respectively (*Figure 31*).

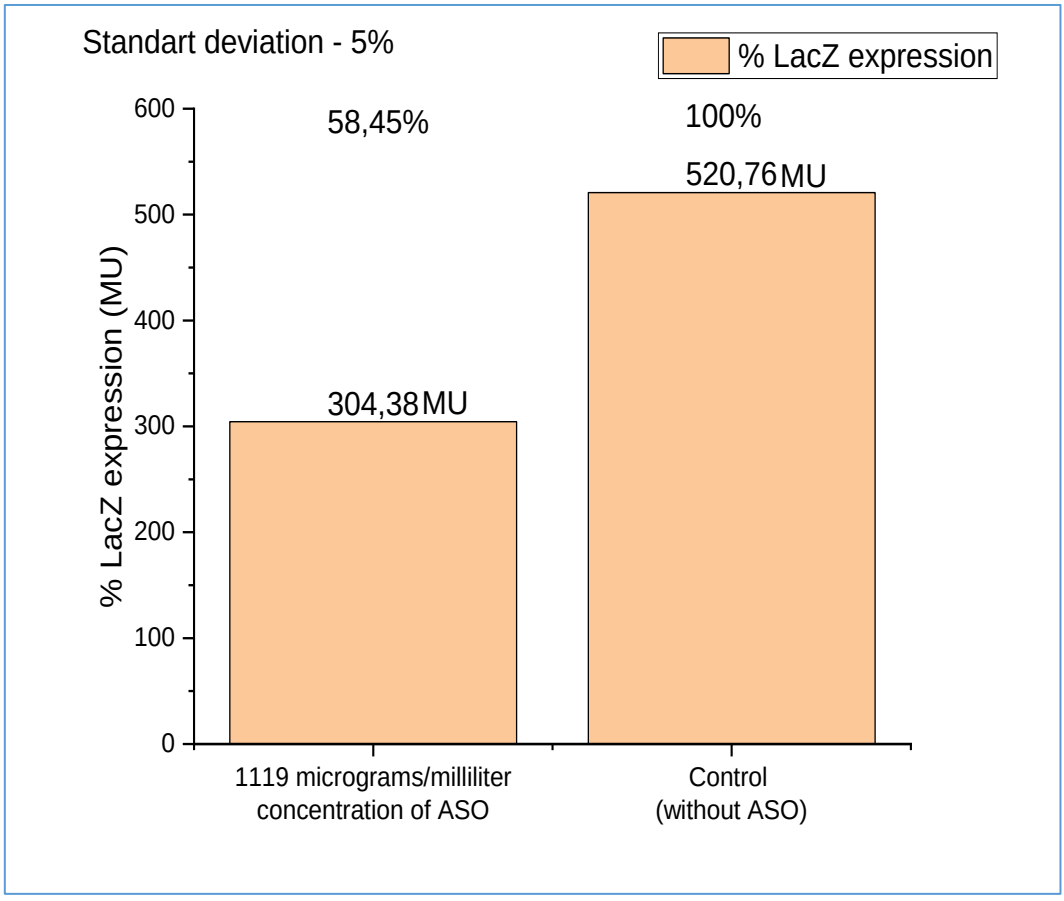


Figure 31. At an ASO concentration of 1119 micrograms/milliliter, 58.45% LacZ expression (304,38 MU) and 41.55% bacterial growth inhibition were observed, respectively.

The percentage of bacterial growth inhibition was tested at an antisense oligonucleotide concentration of 1440 micrograms/milliliter (*Figure 32*). For the experiment, a new bacterial culture was plated (within 8-9 hours) in a vial containing 20 milliliters of Lysogeny broth and 20 microliters of ampicillin (100 mg/L). Culturing was performed on a shaker at a constant temperature of 37°C and 200 rpm. After incubation, the bacterial culture was diluted at a ratio of 1:1000 and tested at an interval of 6-8 hours. Plate 3 tubes containing bacterial culture without antisense oligonucleotide and 2 tubes containing bacterial culture and 1440 micrograms/millilitre of antisense oligonucleotide. Every 30 minutes, the growth of the bacterial culture is counted. When cessation of bacterial growth is detected, a beta-galactosidase assay is performed according to the protocol described previously. The mean value of beta-galactosidase activity of bacterial cultures containing ASO was 269.40 MU, and that of bacterial cultures without ASO was 498.44 MU. At this concentration of antisense oligonucleotide, 54.05% LacZ expression and 45.95% inhibition of bacterial growth were observed, respectively (*Figure 32*).

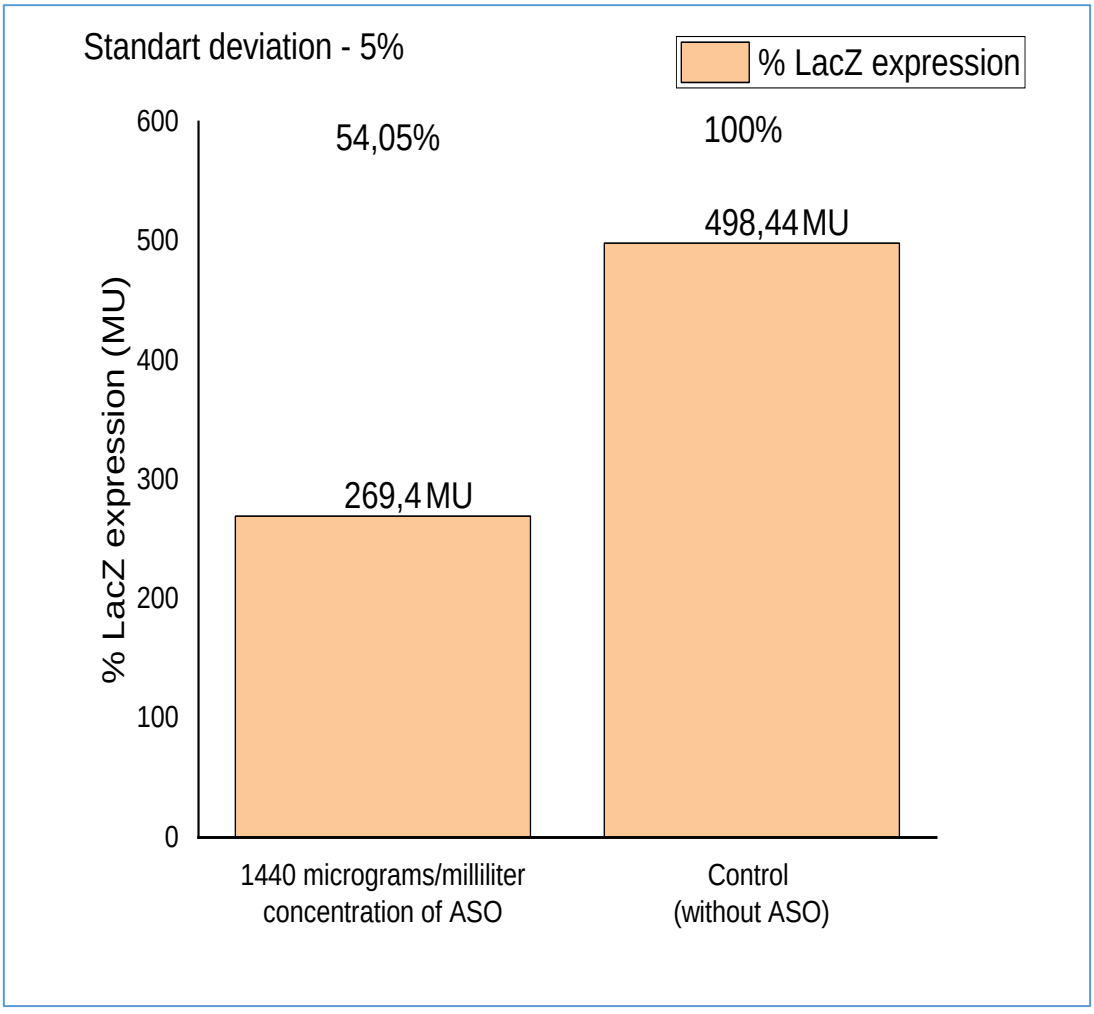


Fig hurrah 32. At an ASO concentration of 1440 micrograms/milliliter, 54.05% LacZ expression (269,40 MU) and 45.95% bacterial growth inhibition were observed, respectively.

The percentage of bacterial growth inhibition was also tested at an antisense oligonucleotide concentration of 1678 micrograms/milliliter (*Figure 33*). For the experiment, a new bacterial culture was plated (within 8-9 hours) in a vial containing 20 milliliters of Lysogeny broth and 20 microliters of ampicillin (100 mg/L). Culturing was performed on a shaker at a constant temperature of 37°C and 200 rpm. After incubation, a dilution of the non-bacterial culture was made at a ratio of 1:1000 and testing was done at an interval of 6-8 hours. Plate 3 tubes containing the bacterial culture without antisense oligonucleotide and 2 tubes containing the bacterial culture and 1678 micrograms/milliliter of antisense oligonucleotide. The bacterial culture growth was counted every 30 minutes. When cessation of bacterial growth is detected, a beta-galactosidase assay is performed according to the protocol described previously. The mean value of beta-galactosidase activity of bacterial cultures containing ASO was 131.15 MU and that of bacterial cultures without ASO was 520.86 MU. At this concentration of antisense oligonucleotide, 25.24% LacZ expression and 74.76% bacterial growth inhibition were observed, respectively (*Figure 33*).

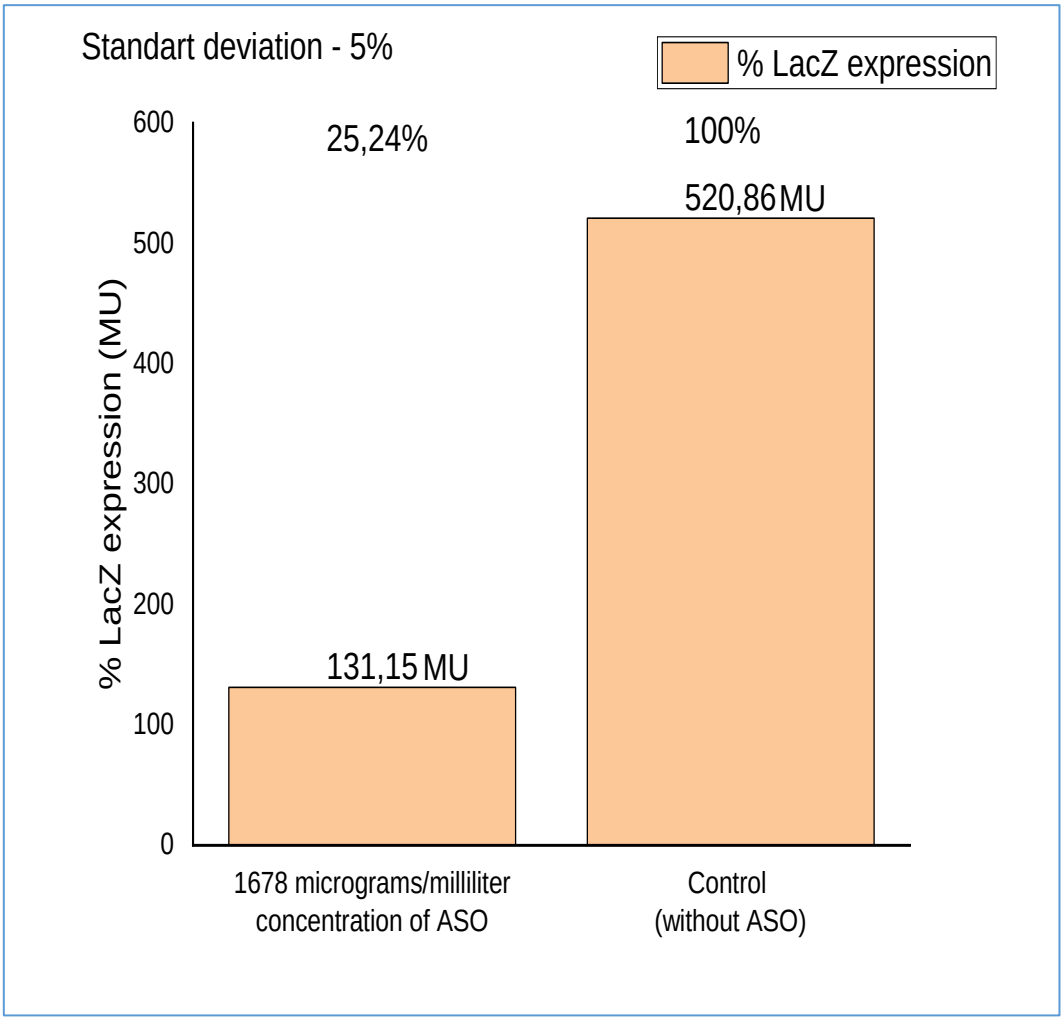


Figure 33. At an ASO concentration of 1678 micrograms/milliliter, 25.24% LacZ expression (131,15 MU) and 74.76% bacterial growth inhibition were observed, respectively.

The last antisense oligonucleotide I used for the experiment was at a concentration of 2238 micrograms/milliliter (*Figure 34*). For the experiment, a new bacterial culture was plated (within 8-9 hours) in a vial containing 20 milliliters of Lysogeny broth and 20 microliters of ampicillin (100 mg/L). Culturing was performed on a shaker at a constant temperature of 37°C and 200 rpm. After incubation, the bacterial culture was diluted at a ratio of 1:1000, and testing was done at an interval of 6-8 hours. Plate 3 tubes containing bacterial culture without antisense oligonucleotide and 2 tubes containing bacterial culture and 2238 micrograms/milliliter of antisense oligonucleotide. The bacterial culture growth was counted every 30 minutes. When cessation of bacterial growth is detected, a beta-galactosidase assay is performed according to the protocol described previously. The mean value of beta-galactosidase activity of bacterial cultures containing ASO was 27.33 MU and that of bacterial cultures without ASO was 657.003 MU. At this concentration of antisense oligonucleotide, 4.15% LacZ expression and 95.85% inhibition of bacterial growth were observed, respectively (*Figure 34*).

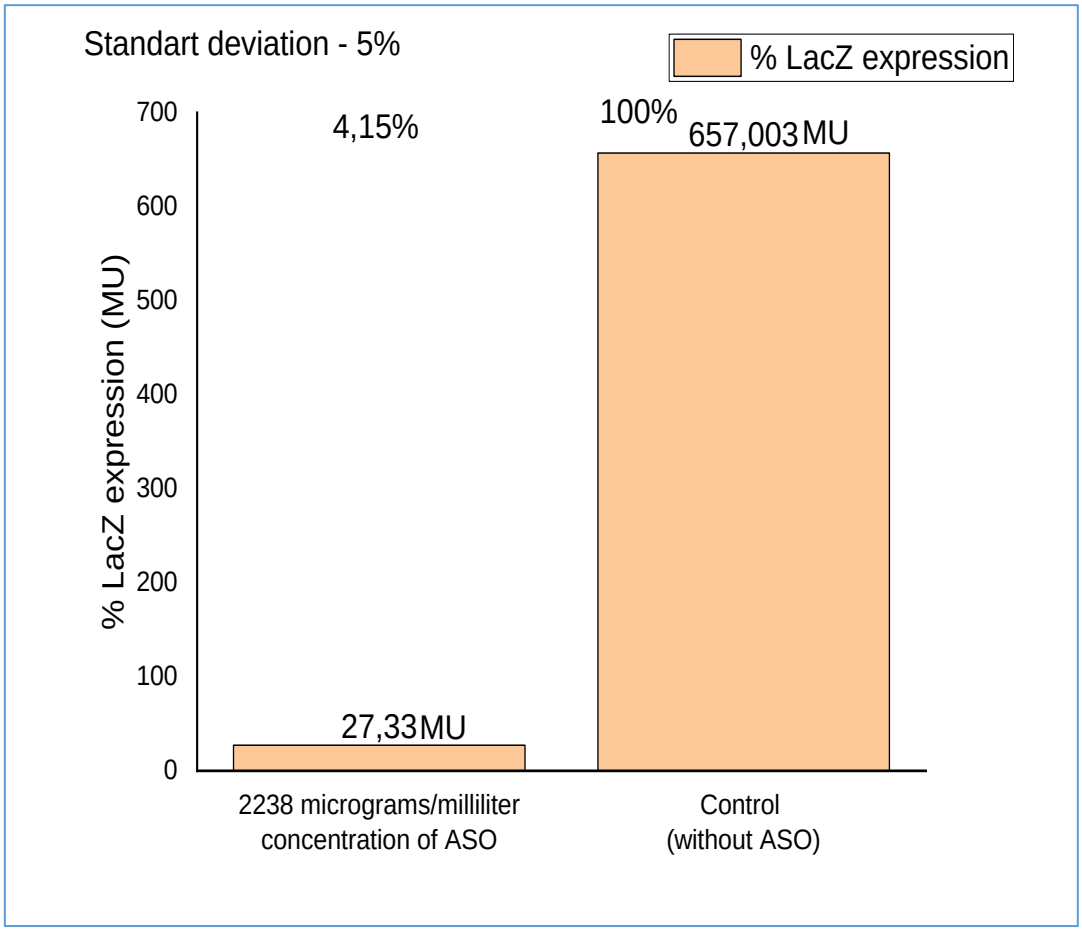


Figure 34. At an ASO concentration of 2238 micrograms/milliliter, 4.15% LacZ expression (27,33 MU) and 95.85% bacterial growth inhibition were observed, respectively.

I found that as the concentration of ASO increased, the LacZ expression decreased, and the bacterial growth inhibition rate increased accordingly. Experiments with each concentration were repeated 3 times and an average value was taken. For each experiment, I also used a control containing a bacterial culture without an adherent oligonucleotide. A summary graph can be seen in Figure 35. The ASO was ordered from Invitrogen by Thermo Fisher Scientific.

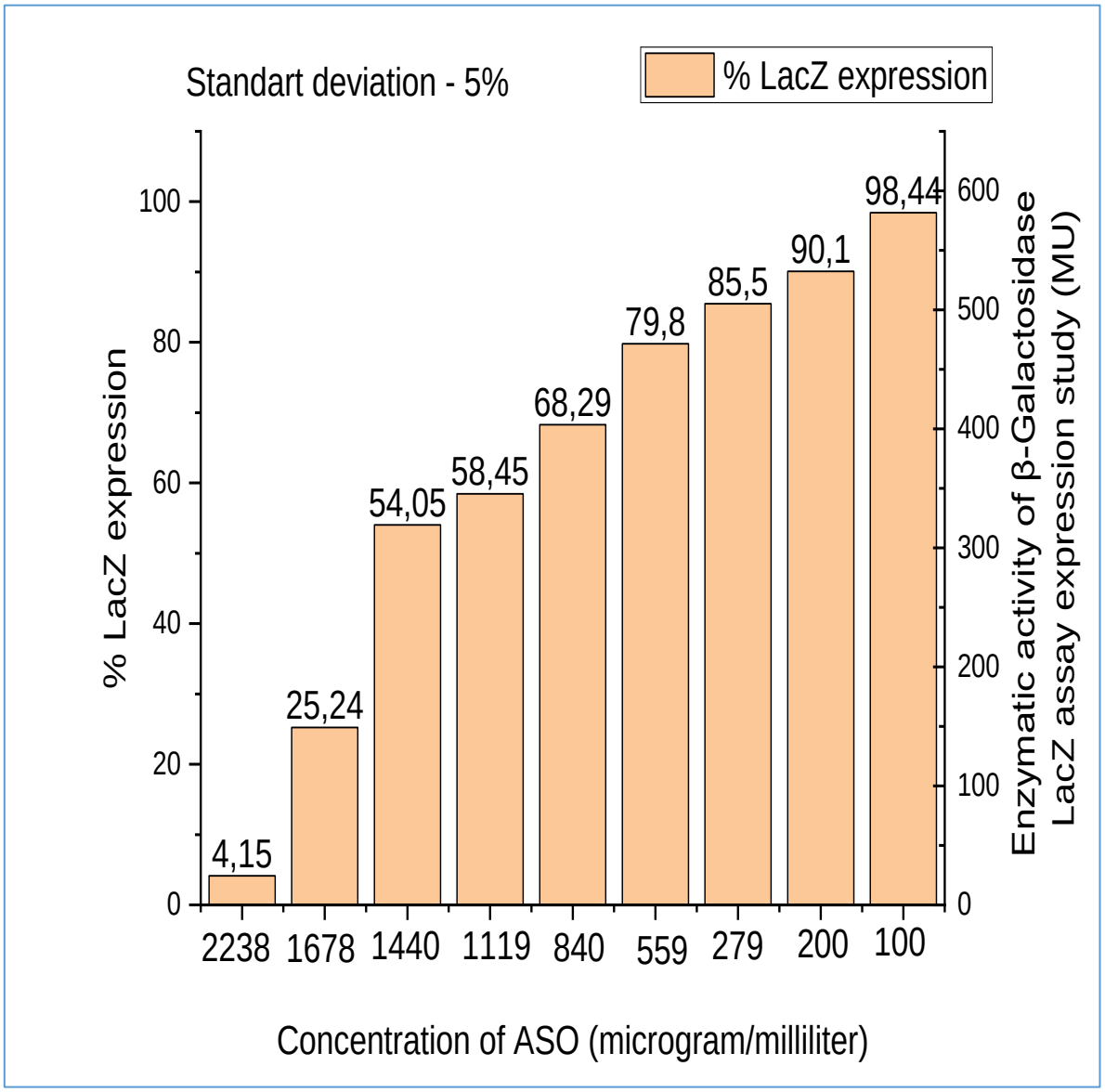


Figure 35. Summary graph showing the percentage of LacZ expression using different concentrations of the antisense oligonucleotide.

4.5. Design an OFF Switch gene expression control strategy using the synthetic hammerhead ribozyme in *Escherichia coli.*

In designing the OFF Switch gene expression control strategy using the synthetic hammerhead ribozyme in *Escherichia coli,* I have used a combination of first and secosecond-generations. The strategy aims to inhibit bacterial growth by hybridizing ASO with allosterian c ribozyme. The designed ASO is 18 nucleotides long and has the following sequence: 5′-CTATTTGGGACTCATCAG - 3′.

An allosteric hammerhead ribozyme is used. A pVEC CPP is used for the introduction of ASO. A chimeric molecule is formed. The pVEC CPP can pass through the cell wall of prokaryotic cells, but also through the cell wall of eukaryotic cells. The concentration of pVEC CPP used is not toxic to prokaryotic cells, in this case *Escherichia coli* cells.

The pVEC CPP does not require a specific temperature to enter through the cell wall. CPP has the following sequence LLIILRRRIRKQAHAHSK, which is encoded by the following amino acids H2N-Leu-Leu-Ile-Ile-Leu-Arg-Arg-Arg-Ile-Arg-Lys-Gln-Ala-His-Ala-his-Ser-Lys-OH. pVEC is composed of 18 amino acids. The nitrogen end of the CPP is hydrophobic, and the carbon end is hydrophilic. Its middle part is positively charged because there are many arginine residues. Once it enters the bacterium only the ASO hybridizes to the target sequence.

For the experiment, cells of E. coli strain HB101 (Takara Bio INC, Japan). The bacteria were grown in LB medium containing ampicillin (100 mg/L).

The OFF Switch gene expression control strategy in *Escherichia coli* was created using Adobe Illustrator, as shown in Figure 36. When the allosteric hammerhead ribozyme is not bound to the ASO, the allosteric ribozyme is not torn, and the Shine-Dalgarno sequence is not blocked. Therefore it is torn, and gene expression is observed. When the allosteric hammerhead ribozyme interacts with ASO between strain I and strain II rupture of the allosteric ribozyme is blocked, the Schein-Dalgarno sequence is blocked, and consequently, no gene expression is observed.

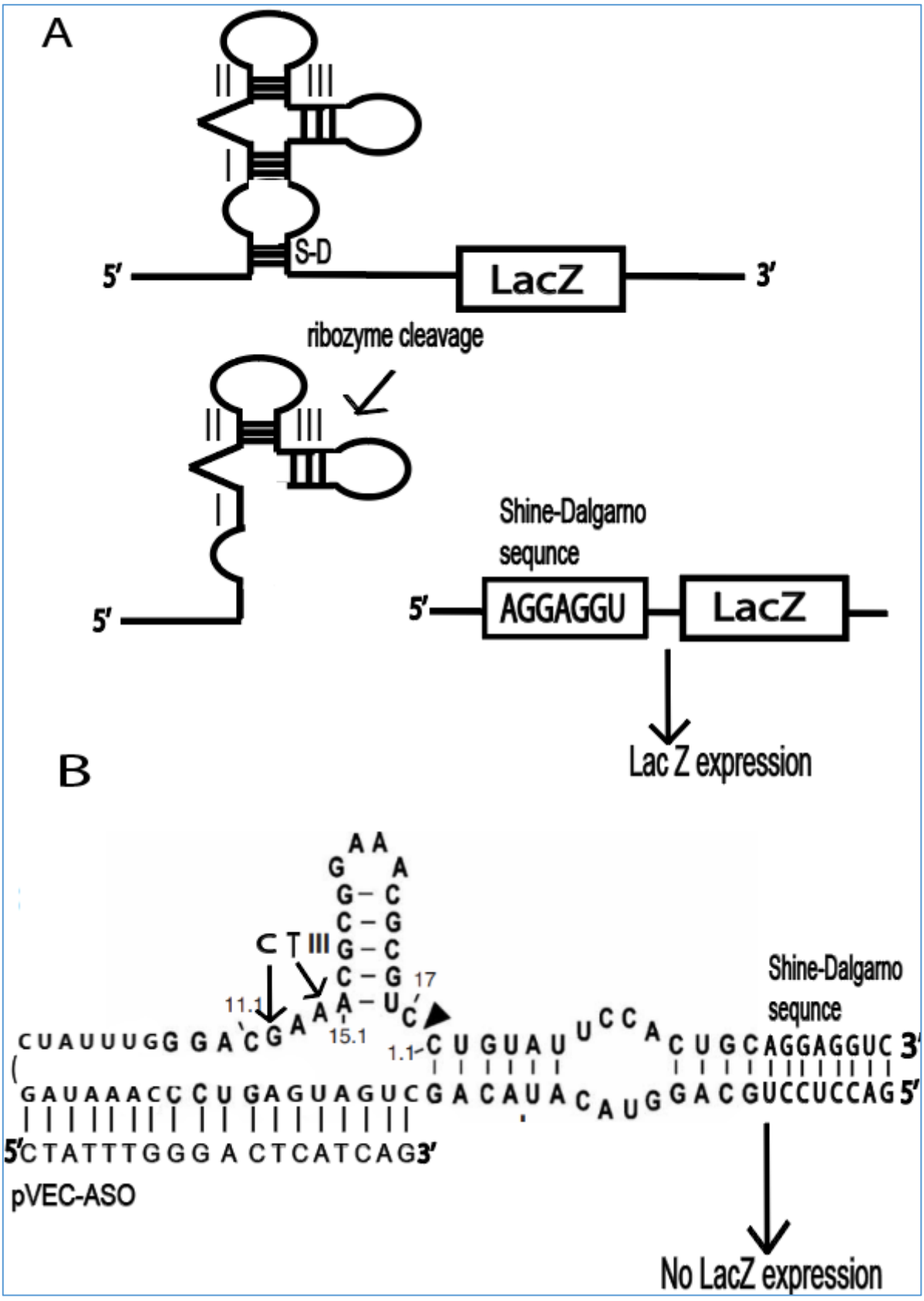


Figure 36. Schematic representation of an OFF Switch control strategy for gene expression in ribozyme in Escherichia coli.

(A) In the presence of an allosteric hammerhead ribozyme that is not associated with ASO, disruption of the allosteric ribozyme is not blocked, and gene expression is observed. (B). In the presence of an allosteric ribozyme that interacts with ASO, cleavage of the allosteric ribozyme is blocked, the Shine-Dalgarno sequence is blocked, and no gene expression is observed.

A DNA fragment carrying the KpnI and XhoI restriction sites and the Shine-Dalgarno sequence, as well as an in-frame ATG transcription start codon with lacZ, was created by hybridization with two deoxyoligomers (*Figure 37*).

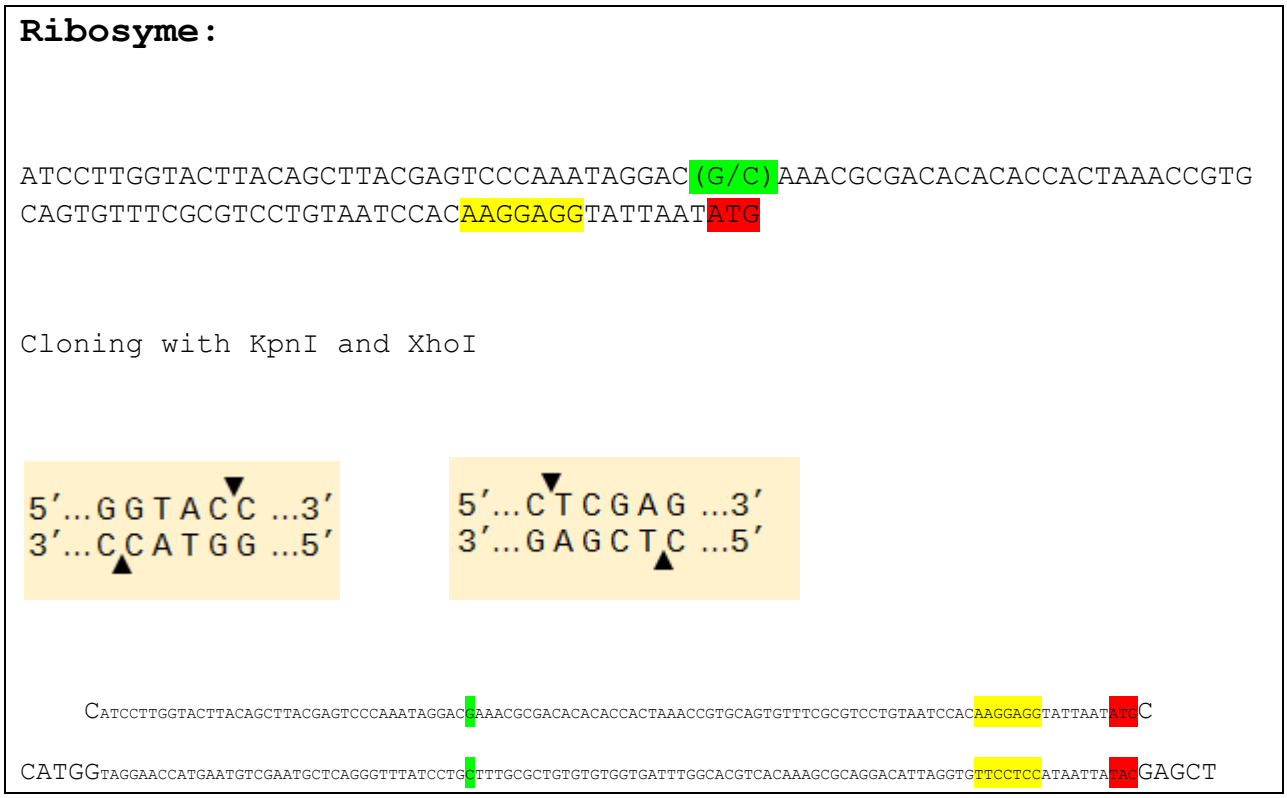


Figure 37. Cloning of an alstoreic ribozyme with KpnI and XhoI restriction site. *KpnI and XhoI are used to insert the ribozyme fragment. The sequences of the restrictases used for cloning are marked with different colors.*

The OFF Switch control strategy of gene expression in ribozyme in *Escherichia coli* was tested by 5 different ASO (mismatch) mutations. Testing was done using freely available RNAcofold Webserver software - http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAcofold.cgi. I found that when mutations exist in the ASO sequence, the desired effect is not observed. The ASO sequence is as follows:

5′- CTATTTGGGACTCATCAG -3′. By RNAcofold Webserver, the result of the thermodynamic stability value of the ASO and the ribozyme sequence is **-43.70** kcal/mol.

In the presence of one mutation in the sequence, 5′-CTATTTCGGACTCATCAG-3′, the thermodynamic stability value of the ASO and the ribozyme sequence was **-37.22** kcal/mol. In the presence of two mutations in the sequence 5′-CTATTTCGCACTCATCAG-3′ , the thermodynamic stability value of the ASO and the ribozyme sequence is **-33.03** kcal/mol. In the

presence of three mutations in the 5′-CTATTTCCCACTCATCAG-3′ sequence, the thermodynamic stability value of the ASO and the ribozyme sequence is **-17.43** kcal/mol. In the presence of four mutations in the 5′-CTATTTCCCACTGATCAG-3′ sequence, the thermodynamic stability value of the ASO and the ribozyme sequence was -11**.09** kcal/mol. In the presence of five mutations in the 5′-CTATTTCCCACTGATGAG-3′ sequence, the thermodynamic stability value of the ASO and the ribozyme sequence was **-6.43** kcal/mol.
This indicates that the presence of mutations in the ASO sequence does not show the desired effect.

4.6 Experimental testing of antisense oligonucleotides for OFF Switch control of gene expression in the ribozyme of *Escherichia coli.*

In experimental testing of the ASO for the OFF switch gene expression control strategy using the hammerhead ribozyme in *Escherichia coli,* I found that when there are mutations of the ribozyme, it does not cleave the target sequence and the Shayne-Dalgarno sequence is blocked, resulting in the absence of gene expression. Correspondingly, when there is a wild-type ribozyme (no hybridization with ASO), it is separated from the target sequence, the Schein-Dalgarno sequence is unblocked, and gene expression is observed. I repeatedly performed an OFF switch synthetic control analysis. After the design, a beta-galactosidase assay was performed to quantify the level of promoter activity. The cells of *E. coli* were allowed to grow overnight in LB medium containing ampicillin (100 mg/L) and incubated at 37°C with shaking. A permeabilizing solution (80 µL) of (100 mM dibasic sodium phosphate (Na_2HPO_4), 20 mM KCl, 2 mM $MgSO_4$, 0.8 mg/mL CTAB (hexadecyltrimethylammonium bromide), 0.4 mg/mL sodium deoxycholate, 5.4 µL/mL beta-mercaptoethanol) was added. Then the absorbance was measured at 600 nm (Abs 600). 20 µL of ON culture was added to tubes containing the permeabilization solution and 600 µL of substrate solution containing ONPG. The time of addition is recorded. Three replicates were made of each sample. Once a sufficiently saturated color was obtained, 700 µl of stop solution (1 M sodium carbonate (Na_2CO_3)) was added and homogenized well. The tubes were then centrifuged at full speed for 5-10 minutes. The supernatant was poured into cuvettes, and absorbance was measured at 420 nm (Abs 420.) The Miller formula (Jonoska, Seeman 2001) was used to calculate the Miller units (MU) of enzyme activity:

$$MU = 1000 \times \frac{\text{Abs } 420}{\text{Abs } 600 \times H \times T},$$

T is the reaction time in minutes and V is the culture volume used in the analysis. Each experiment was triplicated and the average of A420 was used to calculate the MU for each sample.

Twenty samples were tested, 18 containing only wild-type ribozyme and 2 with wild-type ribozyme hybridized with ASO. For the purpose of the experiment serving as controls containing only bacterial culture were also set. High levels of enzyme activity were recorded in all beta-galactosidase assays except for the two samples (*Figure 38*) containing the ribozyme and ASO (Table 4). They are colored in red in Table 4. In the three control samples, only background signal was reported. The values of all measurements are averaged and presented in Figure 39.

Table 4. Results of all Beta-galactosidase assays Three replicates were performed and an average value was reported.

	λ = 600 nm (Abs 600)	λ = 420 nm (Abs 420)	ME
1.	0,315	0,520	768,09
2.	0,287	0,660	1069,69
3.	0,293	0,686	1090,62
4.	0,325	0,738	1057,30
5.	0,295	0,716	1129,33
6.	0,338	0,724	997,24
7.	0,313	0,749	1114,58
8.	0,309	0,752	1132,53
9.	0,324	0,715	1027,29
10.	0,334	0,729	1025,31
11.	0,335	0,730	1013,88
12.	0,259	0,676	1215,82
13.	0,290	0,700	1123,59
14.	0,307	0,710	1075,75
15.	0,287	0,716	1160,45

16.	0,285	0,075	131,58
17.	0,273	0,068	119,30
18.	0,298	0,800	1250
19.	0,254	0,780	1428,57
20.	0,342	0,760	1034,01
21.	0,342	0,040	58,48
22.	0,395	0,045	56,81
23.	0,393	0,041	52,16

Figure 38. Results of experimental testing of beta-galactosidase activity.
Yellowed tubes (18) showed high levels of beta-galactosidase activity. Colorless tubes (2) showed no staining in the wild-type ribozyme and ASO presence.

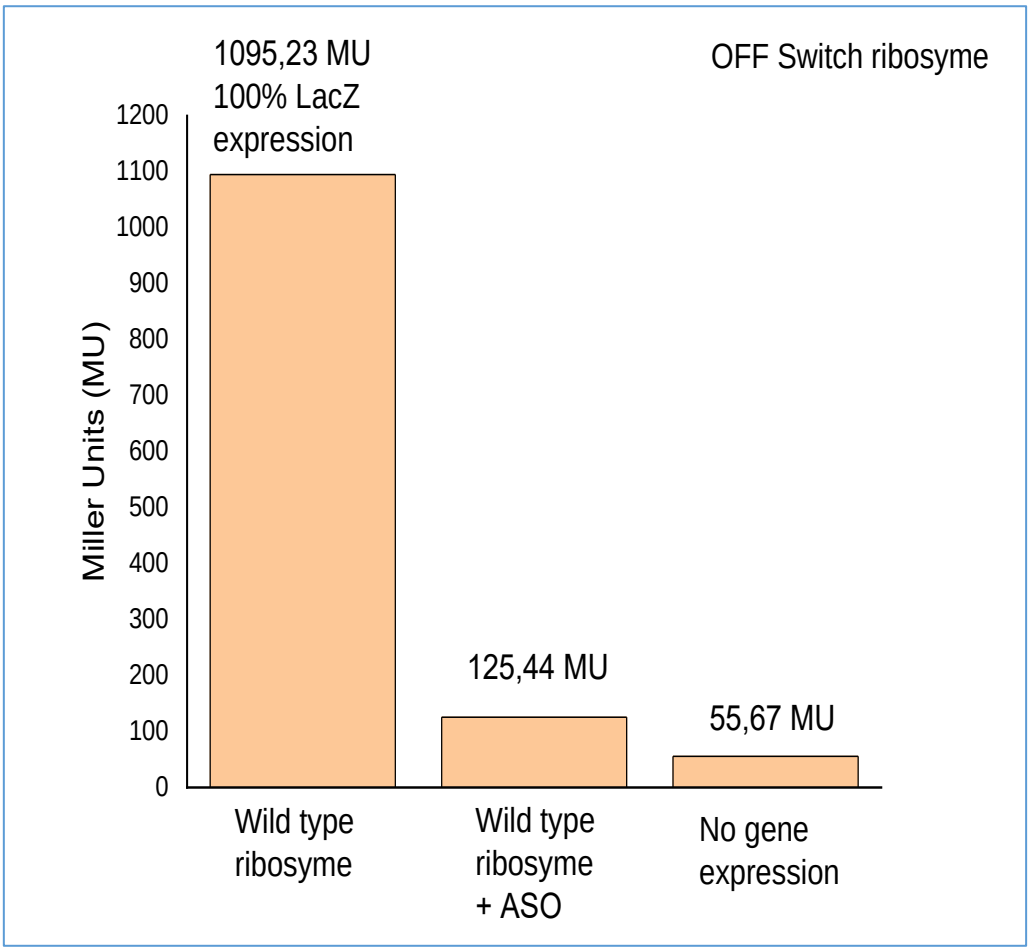


Figure 39. Results of experimental testing of beta-galactosidase activity of antisense oligonucleotides for OFF Switch gene expression control in Escherichia coli ribozyme.
The averaged values of beta-galactosidase activity in Miller units of wild type ribozyme (1095.23 MU), wild-type ribozyme with ASO (125.44 MU) and negative control (55.67) - background signal only were observed.

5. DISCUSSION

RNA synthetic biology is one of the most seriously developing branches of synthetic biology, thanks to the widely applicable variety of RNA engineering and synthesis tools. Small RNA-based tools include several allosteric ribozymes, molecular sensors, antisense oligonucleotides, RNA computing devices, exogenous gene control elements, and others, which have wide applications in medicine and pharmaceutics. They can be used as biosensors for disease screening, for developing therapeutic substances with marked antibiotic effects or others with drug effects in the supportive therapies of various groups of socially important diseases. Functional nucleic acids induce predetermined biochemical changes in different organisms and including humans. The first drug based on antisense oligonucleotide technology was approved in 1998 and named Fomivirsen. Six years later, the aptamer-based drug Pegaptanib was created and approved for use. ASOIs can regulate gene expression through their complementary binding to a sequence within the target mRNA. This occurs through 4 main mechanisms - translation prevention, transcription termination, trans-regulation, and bacterial ribozyme self-cleavage. Due to the deep knowledge of nucleic acid chemistry, various techniques are being applied for refinement and specific design to control gene expression synthetically. Endogenous and exogenous nucleic acids underlie most infectious diseases, congenital disabilities, age-related disorders, oncology, and autoimmune diseases. This expands their application possibilities.

In this Ph.D. thesis, a novel lacZ system for RNA synthetic biology research is demonstrated, which is a DNA fragment carrying the KpnI site, the Shine-Dalgarno sequence, and transcription initiation with an ATG start codon in-frame to the lacZ gene. The first fragment introducing the KpnI site into the pRS414 plasmid used BamHI and XhoI restriction sites located on the plasmid, and the PL promoter sequence of 38 base pairs was cloned between BamHI and the new KpnI restriction site. The new system was successfully applied and exerted synthetic control of gene expression on designer ribozymes and antisense oligonucleotides. The complete sequence of our novel plasmid, pRS414ge, contains the PL promoter of λ phage and has a length of 10715 base pairs. It represents a unique tool for experiments by offering one-step cloning of different allosteric ribozymes, the Schein-Dalgarno sequence, and target domains for antisense oligonucleotides, together with a very convenient and accurate gene expression array. In the OFF Switch synthetic gene expression control strategy design in *Escherichia coli, the* designed ASO represents a chimeric antisense oligonucleotide with characteristics inherent to first and second second-generation recognized by RNase H endonuclease and subsequently hybridized to it, and on the other hand, is modified to have greater stability towards exo- and endonucleases. Upon

complementary hybridization with the target sequence, ASO (5′-C2A2G2G2C1T1G1C1A1T1G1C1T1C2G2C2C2- 3′) and covalently attached cell-penetrating peptide pVEC (LLIILRRRIRKQAHAHSK, encoded by H2N-Leu-Leu-Ile-Ile-Leu-Arg-Arg-Arg-Ile-Arg-Lys-Gln-Ala-His-Ala-his-Ser-Lys-OH), provides regulation of gene expression and inhibits bacterial growth. For the first time, specific inhibition of a particular gene was observed. To establish the specific values and percentage of inhibition of bacterial growth of *Escherichia coli, the* antisense oligonucleotide was tested three times with 9 different concentrations. The results showed that with increasing concentration of ASO, bacterial survival decreased. At an ASO concentration of 2238 micrograms/milliliter, the inhibition of bacterial growth reached almost 96%. The high efficiency achieved suggests the subsequent widespread use of these versatile methods to control gene expression on any single gene selected to be targeted. After bioinformatics and genomic studies, a precisely designed chimeric ASO can be targeted for translation prevention, transcription termination, trans-regulation, and self-cleavage of the bacterial ribozyme.

In the design of the OFF Switch gene expression control strategy using the hammerhead ribozyme in *Escherichia coli, a* second ASO was applied with a sequence of 5′-CTATTTGGGACTCATCAG - 3′ to which a cell-penetrating peptide was again attached. A chimeric molecule is formed that penetrates the cell wall of prokaryotic cells, but it is also possible to penetrate eukaryotic cells. Analysis of 20 samples, 18 of which contained wild-type ribozyme alone and 2 samples containing wild-type ribozyme hybridized with ASO, revealed high enzyme activity levels in all beta-galactosidase assays except for the two samples containing ribozyme and ASO. This confirms the versatility of the method and the possibility of applying it in different bacterial lineages to regulate gene expression.

When the allosteric hammerhead ribozyme is not associated with the ASO, the allosteric ribozyme is not torn, and the Shayne-Dalgarno sequence is not blocked. Therefore it is torn and gene expression is observed. When the allosteric hammerhead ribozyme interacts with ASO between strain I and strain II the rupture of the allosteric ribozyme is blocked, the Schein-Dalgarno sequence is blocked, and consequently, no gene expression is observed. When Switch systems are applied, the control of gene expression is very rapid. When an effector is inserted, Switch action is observed immediately.

Once all tasks were accomplished and the set goals achieved in terms of design and application of functional nucleic acids for synthetic control of gene expression, the uniqueness and broad applicability of the methods in prokaryotes were confirmed. And, because of their versatility and high efficiency, they successfully extend the benefits that RNA synthetic-biology methods provide. What has been achieved should be seen as multifaceted in the process of discovering new

targets, and developing drugs and other therapeutic medications that, through regulation of gene expression, can have beneficial effects on society.

6. CONCLUSIONS

1. The newly designed system may have applications in synthetic biology for synthetic control of gene expression in designer ribozymes and antisense oligonucleotides.

2. We demonstrated that there were high levels of enzyme activity in all beta-galactosidase assays during the three-time points tested of 15, 20, and 30 minutes, respectively. For the 15^{th}-minute measurement, 774.56 MU were obtained. Similar activity was obtained at the other time points with 773.99 MU and 763.48 MU at 20 and 30 minutes, respectively.

3. A strategy was designed for OFF Switch synthetic control of gene expression in *Escherichia coli* using a combination of first and second-generation ASOs. A 19-nucleotide-long ASO was designed, which has the following sequence: 5′-CAGGCTCGCATGGCTCGCC- 3′. The ASO was modified in the middle 11 nucleotides to trigger the enzymatic degradation of mRNA and thus inhibit bacterial growth.

4. Through 9 different concentrations of ASO, its inhibitory effect on bacterial growth was demonstrated. As the concentration of ASO increased, the bacterial survival rate decreased, and the bacterial growth inhibition rate increased accordingly. At an ASO concentration of 2238 micrograms/milliliter, the inhibition of bacterial growth was 96%.

5. A strategy was designed for OFF Switch synthetic control of gene expression using the hammerhead ribozyme in *Escherichia coli.* The designed ASO is 18 nucleotides long and has the following sequence: 5′- CTATTTGGACTCATCAG - 3′.

6. By visualizing the putative secondary structure of the molecule, it was found that in the presence of mutations in the ASO sequence by hybridization with the hammerhead ribozyme does not have the desired effect. In the presence of five mutations in the sequence, the thermodynamic stability value of the ASO and the ribozyme sequence was -6.43 kcal/mol.

7. CONTRIBUTIONS

1. **Specific inhibition of gene expression has been demonstrated for the first time by a novel universal method for control of gene expression in *Escherichia coli* using ASO.**
2. **For the first time, the inhibitory action of specific ASOs hybridized with ribozyme in *Escherichia coli* was experimentally demonstrated. The constructs created represent universal methods and can be used to control the expression of any gene.**

ABBREVIATIONS USED

ASO - antisense oligonucleotides

ASOs - antisense oligonucleotides

GMO - genetically modified organism

DNA - deoxyribonucleic acid

iRNA - informational RNA

inRNA - interfering RNAs

CPP - cell-penetrating peptide

RNA - ribonucleic acid

XHR - Hammerhead ribozyme

BBs - BioBricks

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

CTAB - Cetyltrimethylammonium bromide

EGS - External guide sequence

glmS - glucosamine-6-phosphate activating ribozyme

HPC – High-performance computing

NGS - Next-generation sequencing

PAGE - Polyacrylamide gel electrophoresis

PCR - Polymerase chain reaction

SD - Shine-Dalgarno sequence

SELEX - Systematic evolution of ligands by exponential enrichment

SNP - single-nucleotide polymorphism

UTR - Untranslated region

3D - 3-dimensional space, 3-sided space

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Figure 27. At an ASO concentration of 200 micrograms/milliliter, 90.10% LacZ expression (449,19 MU) and 9.90% bacterial growth inhibition were observed, respectively.

Figure 28. At an ASO concentration of 279 micrograms/milliliter, 85.50% LacZ expression (436,74 MU) and 14.5% bacterial growth inhibition were observed, respectively.

Figure 29. At an ASO concentration of 559 micrograms/milliliter, 79.80% LacZ expression (407,66 MU) and 20.20% bacterial growth inhibition were observed.

Figure 30. At an ASO concentration of 840 micrograms/milliliter, 68.29% LacZ expression (340,40 MU) and 31.71% bacterial growth inhibition were observed, respectively.

Figure 31. At an ASO concentration of 1119 micrograms/milliliter, 58.45% LacZ expression (304,38 MU) and 41.55% bacterial growth inhibition were observed, respectively.

Figure 32. At an ASO concentration of 1440 micrograms/milliliter, 54.05% LacZ expression (269,40 MU) and 45.95% bacterial growth inhibition were observed, respectively.

Figure 33. At an ASO concentration of 1678 micrograms/milliliter, 25.24% LacZ expression (131,15 MU) and 74.76% bacterial growth inhibition were observed, respectively.

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Engineering a Plasmid as a Reporter System for Quantifying Gene Expression in *Escherichia Coli* - Georgi Y. Miloshev, Martina Traykovska, Dimitrios Kaloudas & Robert

Penchovsky, *Proceedings of the Bulgarian Academy of Sciences* 75 (1), 2022, Q3, IF: 0,326, vol. 75, no. 1, pp. 56-61, ISSN: 13101331, I am first author

DOI: https://doi.org/10.7546/CRABS.2022.01.07,

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Versatile tools of synthetic biology applied to drug discovery and production - Nikolet Pavlova, Georgi Y Miloshev, Antoniya V Georgieva, Martina Traykovska & Robert Penchovsky, *Future Medicinal Chemistry* Vol. 14, No. 18 Review 2022, Q2, IF: 4,7 https://doi.org/ 10.4155/fmc-2022-0063, second author am

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Various therapies against SARS-CoV-2 - Aikaterini Valsamatzi-Panagiotou, Martina Traykovska, Georgi Y. Miloshev, Robert Penchovsky, *Acta Microbiologica Bulgarica* Volume 39 / 1 (2023), Q4, IF: 0,11

https://actamicrobio.bg/archive/issue-2-2020/amb-2-2020-article-1.pdf

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2. "Design and experimental testing of chimeric antisense oligonucleotides as antibacterial agents", DN13/14/20.12.2017, funded by the Scientific Research Fund, Medical Sciences Committee, project leader Prof. Dr. Robert Dimitrov Penchovsky.

3. "Creation of antisense oligonucleotides that specifically inhibit bacterial growth of Helicobacter pylori and Porphyromonas gingivalis", KP-06-M33/5/18.12.2019, funded by the Scientific Research Fund, Committee on Medical Sciences, project leader prof. asst. Dr. Martina Trajkovska

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26.05.2022 - **Doctoral Student of the Year** for the academic year 2021/2022.

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Teaching:

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Teacher of biology and health education in junior high and high school in 125 Boyan Penev School since 2019.

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Duke of Edinburgh's International Award Leadership Training, 09.11-10.11.2019 in Sofia.

Training on "Training of Biology and Health Education Teachers who will teach the curricula for specialized training in classes XI and XII from the school year 2020/2021" under the National Programme "Qualification" 2020, held at the NCPCPS - town of. In the period 20.07-21.07.2020.

Training on "Innovative teaching methods, game, web tools", held by Shkoloto in the period 08.09-09.09.2020 at 125 Boyan Penev Secondary School.

Training on "Building skills to promote the personal development of children and students. Effective techniques for group and class management, assessment of results and measurement of

achievements" - 30.10-01.11.2020 in Sofia. Dupnitsa, Academy for Innovative Teachers "There's a Why!" - Startegia Ltd.

Training on "Google for Education", conducted by ORAC Engineering Ltd, online in the period 27.11-30.11.2020.

Training on "Google chromebook" - 28.08-29.08.2021, held at 125 Boyan Penev Secondary School by Google Center for Creative Learning.

Training on "Training of Biology and Health Education Teachers who will teach the curricula for specialized training in XII grade from the school year 2021/2022" under the National Qualification Programme 2020, held at the NCPCPS - Sofia. Bankya in the period 20.09-21.09.2021 and online on 13.11 and 20.11.2021.

Course on "Methods of teaching BDP in VIII - XII grade" - Ruse University "Angel Kanchev", completed on 27.10.2021 with a duration of 32 hours.

Training on "Individual Approach to Outstanding Students" - 18.12-19.12.2021 - IMI at BAS.

Training on "Teamwork for pedagogical specialists - development of skills for effective teamwork in a school team", held by Shkolos in the period 16.04-17.04.2022 in Borovets.

Training in CPR and AVD under the project "Shared Library for Life-Saving Skills" by the "First Three Minutes" Foundation - 21.03 (online) and 04.06.2022 (in person) in the city of. Sofia.

Participation in the competition "My Workplace" - BG Science.

Participation in the National Conference on Innovative Learning and Best Practices in Education "School for the Future" 5.0 - 21.,22.,23.10.2022 in Pamporovo

Training on "Stress Resilience and Burnout," conducted by Shkollo in the period 22.04-23.04.2023 in. Plovdiv.

Languages:

Bulgarian - mother tongue

English Language - B2 Certificate

Russian - basic level (written and spoken)

French - basic level (written and spoken)