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CRISPR-CAS9 SYSTEM IN VITRO AND DNA MODIFICATIONS USING HOMOLOGY-DIRECTED REPAIR MECHANISM

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Narrative Questions

1. **Describe how you came to choose your topic, specifically noting any pre-research that you did. What sources did you use in this pre-research? To what extent did you consult with librarians, faculty, or others? How did this pre-research lead you to your topic?**

   I have always been a curious Biochemistry student who wants to work in the pharmaceutical and healthcare-related fields. Therefore, I spend time exploring discoveries in these fields to cultivate my knowledge and prepare myself as a Ph.D. candidate after graduation. In several introductory biology courses, mutation-based diseases appear to be one of the most complicated and almost-impossible-to treat illnesses. Therefore, I shifted my attention from global infections to mutation diseases due to genetic disorders. I was fortunate to be in the Intro to Biological Inquiry class of Professor Martha Grossel, which prepared me with knowledge of genetics. However, since I craved hands-on research in gene editing, this course could not satisfy my needs. 2 years ago, I came across an article from Nature called "Pioneers of revolutionary CRISPR gene editing win chemistry Nobel", which presents the ground-breaking discovery by Emmanuelle Charpentier and Jennifer Doudna on the CRISPR-Cas9 gene-editing system (Ledford and Callaway 2020, 346–47). The ability to replace damaged genes with healthy genes and correct mutation was so fascinating that I could not stop reading. I talked to Professor Deborah Eastman about my interest in researching the gene-editing mechanism, for which there are three nucleases TALENs, CRISPR/Cas9, and ZFNs (Li et al. 2020, 1). She advised me to read some review articles to understand their pros and cons before choosing the right one for me. Since the Nature article impressed me deeply, it inspired me to do research in the CRISPR-Cas9 gene-editing system. However, since this system has been used on a variety of animal models, including mouses, rats, and even humans (Wu et al. 2013, 659–62), I struggled with finding my research subject. I asked Professor Deborah Eastman for advice, and she recommended I research the effect of CRISPR-Cas9 on the alteration of the lacZ gene in Escherichia coli. I started by watching the "Gene Editing Mechanism of CRISPR-Cas9" from the Wyss Institute of Harvard University, which gave the visualization of how gene modification works. Then, I commenced my preliminary research by reading several articles from Science Direct, PubMed, Scopus, etc, which were recommended by Professor Sardha Suriyapperuma. Since this was pre-research, I searched for articles using very general terms, such as "gene-editing mechanism," "genome modification," "lacZ gene alteration," etc. She also advised me to read more review articles on gene drive technology and DNA repair mechanism to get the big picture of how my research can be. I also seek help from the two passionate librarians, Andrew Lopez and Lori Looney, to help with my pre-research. They provided me with tips on how to get the best out of One Search and also introduced me to several scientific databases, including Academic One File and PubMed Central. After doing the pre-research, I drew a brainstorming diagram

to connect all the pieces and finalized my research. By reading different experimental designs and confirming tests in several journal articles, I concluded that I would evaluate the success of lacZ gene alteration based on the color of colonies and perform PCR as well as gel electrophoresis to confirm my results.

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2. **Describe your process of finding information for your project. Note specifically the tools you used to undertake your research, as well as the specific search strategies you used within these tools. (Note: “Ebsco,” being an umbrella vendor, is not a specific enough response when identifying tools; listing the “library database” is also an unacceptably vague answer. Specific tools include JSTOR, America: History & Life, Web of Science, etc., along with OneSearch, the new library system.)**

I was fortunate to attend a session on citations, bibliographies, and research resources in Shain Library, where I was introduced to One Search, in my FYS-119K: Virtual Realities in Japan. Besides searching for general key terms, such as "gene drive technology," "genome-editing mechanisms" in One Search, Professor Deborah Eastman recommended that I should use more specific terms if I want results closer to my expectations. Therefore, I changed my strategy to searching using specific terms such as "RNA-guided CRISPR-Cas9," "lacZ gene alteration," "DNA breakage," to write a better background section. She also advised me to read review articles, which provide a general understanding and cite the original articles as evidence. This worked as a very effective resource for my research. After the preliminary research, I narrowed down my reading to the CRISPR-Cas9 system and the use of RNA and donor template DNA to design the experimental procedures. Since I was new to research, I wanted to look for examples in reliable scientific journal articles on PubMed and Science Direct to get an idea of the experimental setup. This time, I focused heavily on the Materials and Methods section to start designing the experiment. Since my subject was Escherichia coli, I tried to look for experiments that were done on either Escherichia coli or Streptococcus Pyogenes (a very similar species to E.coli) first. Once I obtained enough information on the experimental methods and achieved certain results, I needed evidence from credible

journals to back them up and cross-check. In this stage, I expanded my search to other experimental designs as well to see if they still had the same results like mine and referred to them in my Results and Discussion section. I also took advantage of the helpful library service by visiting the Reference Desk. I asked librarian Andrew Lopez for some advice on the databases I should use since using a credible scientific database would ensure the reliability of the articles. He advised me to use PubMed, Science Direct, and Nature, all of which contributed largely to my reference sources. He also showed me how to select specific types of articles, such as peer-reviewed articles and review articles, on PubMed and Science Direct. Librarian Lori Looney showed me how to save all of the articles in Refworks and keep them in a separate folder. I am very grateful for their help in my citations and bibliographies as well since without them, I would not know about alphabetical rearrangements of references and proper citation formats. In addition to that, I went to Professor Sardha Suriyapperuma's office hours to seek more advice. She informed me that PubMed Central provides free access to scientific articles, which helped me substantially when finding the resources. I mainly used PubMed, PubMed Central, Nature, and Science Direct since they not only offer reliable scientific articles, but most of their journal articles are free and up-to-date.

3. **Describe your process of evaluating the resources you found. How did you make decisions about which resources you would use, and which you wouldn't? What kinds of questions did you ask yourself about resources in order to determine whether they were worthy of inclusion?**

   After having read several journal articles, I was overwhelmed by loads of information. Therefore, I calmed myself by writing an outline of what I planned to do and compared it to what I found. There were times when the research I found matched my interest, but it was done on another species (i.e humans) while I wanted to research on CRISPR-Cas9 effect on Escherichia coli, which are substantially different species. Therefore, the background and discussion sections of that article were the only parts I looked at. Since Professor Deborah Eastman strongly required journal articles that supported my results for cross-checking, I limited my findings from CRISPR-Cas9 use in general to CRISPR-Cas9 use in Escherichia coli or Streptococcus pyogenes (a similar species) and referred to their Materials and Method and the Results and Discussion sections only. The DNA modification does not only include the alteration of the lacZ gene but it also requires confirmation if the gene changing was a success. Therefore, I focused on reading the Results and Discussion section more to see the similarities and differences between my results and theirs. I always ask myself: What do I want to prove to the scientific community? What is my hypothesis? What evidence do I need to back it up? Since I hypothesized that the lacZ gene could be successfully edited by CRISPR-Cas9 using sgRNA and HDR, I only read the journal articles that not only used the CRISPR-Cas9 system but also utilized HDR instead of NHEJ to match with my research. Using the term "Gene Drive Technology" when searching for journal and

review articles might be too ambitious because of the four available nucleases used for this mechanism, so I limited my reading to RNA-guided CRISPR-Cas9 mechanism only to save time. Since I already used credible resources from the Shain Library, my professors, and well-known scientific databases, I did not have to judge the credibility of the journals. Instead, I would evaluate their usefulness to my research based on some criteria. How similar their experimental design is to my procedure? It would be even better if they had a different experimental procedure but still matched up with my results. Therefore, I would also look at papers that researched the same topic but in different set-ups and achieved the same results as I did as excellent evidence for my research paper. For example, according to Jiang et al. (2013, 233–39), whose research subjects were Escherichia coli and Streptococcus pyogenes, the CRISPR-Cas9 provided efficient gene editing using crRNA and the donor template DNA. Even though they used a complex of dual-RNA: Cas9, which is different from my complex of sgRNA: Cas9, my results were still comparable to them in terms of the general concept. I also talked to Professor Emily Tarsis and Professor Jacob Stewart on ways to find the most effective resource. Their advice for me was that I can evaluate the usefulness of the articles based on the abstract, which gives an overview of what the article was about, and the Results and Discussion section, which provides the outcomes of the research and the analysis. Thanks to these valuable resources, I am confident that I can work on my honors thesis in my senior year and design my experimental procedure.

CRISPR-CAS9 SYSTEM *IN VITRO* AND DNA MODIFICATIONS USING HOMOLOGY-DIRECTED REPAIR MECHANISM

By Binh Vo

Abstract

The CRISPR-Cas9 system is one of the brightest candidates in genome editing in several species (Wu et al. 2013, 659–62). The purpose of this study is to research the possibility of the *lacZ* gene alteration using CRISPR-Cas9 in *Escherichia coli* and test the precision of the DNA modification. It is possible to decide if the *lacZ* gene was altered based on the color of the colonies formed as a result. However, to ensure the success of the DNA modification, multiplex PCR was used. The yielded amplicons from the PCR test can be visualized using gel electrophoresis. The results showed that the overall study was a success with some complications. The *lacZ* gene was successfully edited in most samples with the donor template DNA; however, the study presented some limitations during the procedure.

I. Introduction

It has never been thought that humans can genetically engineer organisms and biological systems to correct unwanted mutations. It was not until the first genetic modifications in mice and yeast in the 1970s and 1980s that the scientific community started to believe in this incredible ability (Carroll 2017, 653–59). This fascinating breakthrough opens the door for the gene-editing mechanism and allows scientists to exceed the limitation of clinical interference on the human body. Scientific advancement has never stopped growing, giving hopes to invent such gene alteration technology that satisfies two criteria: simplicity and efficiency. The CRISPR-Cas9 system stands out as one of the best tools via the gene-editing mechanism (Xia et al. 2018, 699–

709) due to its high specificity and convenience. Recent studies show that the CRISPR-Cas9 can correct unwanted mutations leading to severe diseases, such as sickle cell anemia (Demirci et al. 2019, 37–52) and cystic fibrosis (Marangi and Pistritto 2018, 396). There is also evidence of how CRISPR-Cas9 contributes to the research of enhancing the efficacy of cancer therapeutic drugs (Behan et al. 2019, 511+).

This study was conducted to test the practicality of the CRISPR-Cas9 system *in vitro* and observe the DNA modifications. Generally, it can serve as one of the contributors to the knowledge of CRISPR-Cas9 and its clinical applications. In this study, *Escherichia coli* utilized the CRISPR-Cas9 system to alter the *lacZ* gene, which is responsible for the coding of the enzyme β-galactosidase (β-gal). The *lacZ* gene is a constituent of the lac operon, a group of genes that enable the bacteria to consume lactose. As a site for DNA insertion, the *lacZ* gene allows the bacterial colony to have color as a result of the reaction between β-gal and X-gal (Juers, Matthews, and Huber 2012, 1792–1807), signaling the completion of lacZ editing. This CRISPR-based method involves Cas9 endonuclease, which cuts the genomic DNA at the targeted sites. The sgRNA directed the CRISPR-associated Cas9 endonuclease to the desired DNA region recognized by the protospacer-adjacent motif (PAM) sequence (Costa et al. 2004; Jiang et al. 2013, 233–39). After the cut, the DNA suffers from a double-stranded break, which requires either homology-directed repair (HDR) or nonhomologous end-joining (NHEJ) to repair (Ran et al. 2013, 2281–2308). It is hypothesized that the *lacZ* gene can be successfully edited using the CRISPR-Cas9 system with donor template DNA, sgRNA via the HDR mechanism.

II. Materials and Methods

Bacterial Strain

In this study, the *lacZ* gene of *Escherichia coli* served as the target mutated by the CRISPR-Cas9 system. The bacterial strain utilized in the experiments was *E. coli* HB101-pBRKan, which possesses a functional *lacZ* gene in its natural formation. For the purpose of this study, this strain had undergone engineering modifications to express Cas9 and had had a plasmid containing antibiotic-resistance genes. The plasmid served as a means for introducing sgRNA and donor template DNA. For safety purposes, the bacteria were modified to disable the NHEJ performance.

Designing sgRNA and donor template DNA

The sgRNA includes a 20-nucleotide protospacer in the given *lacZ* gene followed by a PAM sequence (5'-NGG, where N can be any nucleotide of interest). The protospacer was then pasted to the tracer sequence to complete the sgRNA.

The donor template DNA had a length of 40 base pairs, including the deleted bases for a frameshift mutation to the *lacZ* gene. Two homology arms of 15 base pairs, 5' and 3' homology arms, were obtained to frame the *lacZ* sequence. The sequence for insertion was 10 base pairs long with deleted bases.

Editing the *lacZ* gene with the sgRNA and the donor template DNA

The samples of this experiment obtained the colonies from the two starter plates, IPTG/X-gal and IPTG/X-gal/ARA. Arabinose was added to activate the HDR mechanism and edit the *lacZ* gene. Half of the samples had pLZDonorGuide plasmids, which provide the sgRNA and donor template DNA for HDR mechanism activation and *lacZ* gene transformation. The other half received

pLZDonor plasmids, which only supplied the donor template DNA. The blue-white screening technique was used to confirm the success of *lacZ* editing.

PCR test for confirming DNA modifications

The samples were tested using multiplex PCR to detect the presence of the target DNA sequence. This test involves simultaneous amplification of several amplicons in one reaction utilizing a unique primer set of each. Most of the PCR tubes obtained one blue colony each from either the IX/ARA plate or the product samples of the previous stage. The remaining also received a white colony each from the latter source. These PCR tubes became the materials for the PCR samples.

Besides the PCR samples receiving the supernatant from their compatible PCR tubes, the two PCR controls were included in this test. The positive PCR control shows the standard of the PCR test and yields amplicons. On the other hand, the negative PCR control can detect reagent contamination or foreign DNA introduction to the samples. It will not yield any amplicons and template or only have one or the other primer (forward/reverse primer).

Gel electrophoresis for visualizing the PCR products

Since each primer set yields a specific PCR amplicon, these amplicons of the PCR samples can be separated using gel electrophoresis. The produced DNA bands on each PCR sample were compared to the base pair ruler for the size and the PCR controls for the standard and contamination detection.

III. Results

The results from the *lacZ* gene transformation show that there was a mix of edited and unedited lacZ genes. The modified *lacZ* gene produced white bacterial colonies, while its unmodified counterpart yielded blue colonies. The amplicon amplification gave more insights in confirming if the DNA was altered. In the multiplex PCR, the disruption of the primer binding sites or the absence of the donor template DNA will inhibit the target PCR sequence from amplification. Each PCR sample yielded specific amplicons by the primer sets. The produced 1,100 bp amplicon signaled the failure of modifying the Cas9 cut site. The yielded 650 bp amplicon showed that the target cut site was repaired using the donor DNA. PCR samples producing 350 bp amplicon were determined to have the chromosomal DNA extracted and a successful PCR test. However, there was no information on whether the *lacZ* gene was edited deduced from this amplicon. The gel electrophoresis helped visualize the PCR products as DNA bands. The band of fluorescent dye indicated that the PCR test was successful. The copied sequence of interest showed up as a single DNA band. If there is more than the desired sequence, the gel will have more than a band.

1. Guide RNA and Donor template DNA design:

a) Single Guide RNA

A sgRNA directs the Cas9 endonuclease enzyme to the DNA of interest. Its guiding region is designed to be complementary to the target DNA sequence, which signals to the Cas9 enzyme the location of cutting sites.

DNA target:

5’tacaccaacg tgacctatcc cattacggtc aatccgccgt ttgttcccac ggagaatccg 3’

3’atgtggttgc actggatagg gtaatgccag ttaggcggca aacaagggtg cctcttaggc 5’

20-nucleotide protospacer:

3’gttgc actggatagg gtaat 5’

5’caacg tgacctatcc catta 3’

3’ guugc acuggauagg guaau 5’

5’caacg ugaccuaucc cauua 3’

The designed sgRNA:

5’caacg ugaccuaucc cauua3’5’GUUUUAGAGCUAGAAAUAGC AAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGU GCUUUUUU3’

b) Donor template DNA

Inserted sequence: tgcgcccatc

3’ |homology arm |

5’tacaccaacg tgacctatcc cattacggtc aatccgccgt ttgttcccac ggagaatccg 3’

5’ |homology arm|

3’atgtggttgc actggatagg gtaatgccag ttaggcggca aacaagggtg cctcttaggc 5’

5’ homology arm: ctatcc cattacggt

3’ homology arm: tacggtc aatccgcc

(The PAM sequence is colored in yellow, and the target sequence is colored in green.)

Donor template:

Deleted base

ctatcc cattacggt tgcgcccatc tacggtc aatccgcc

5’homology arm inserted gene 3’homology arm

2. Plate Results

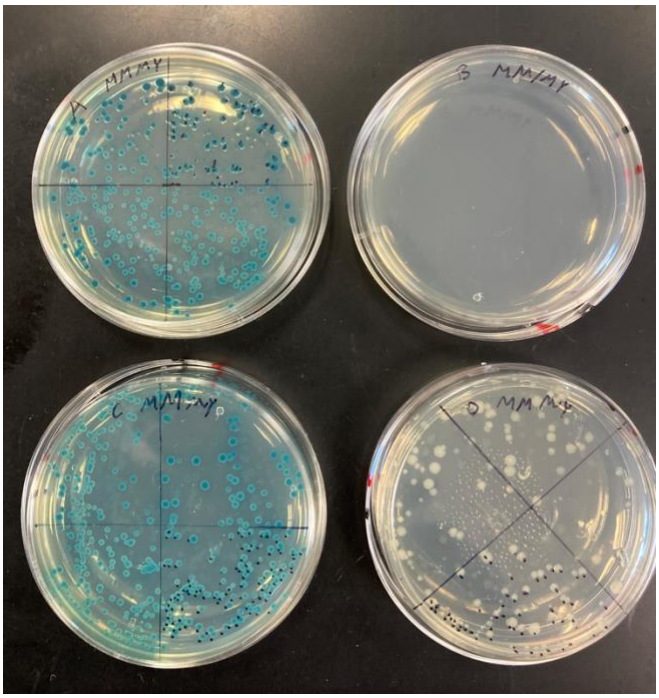


Figure 1

The above figure presents the visualization for the CRISPR samples A, B, C, and D. The small dots on each plate are the bacterial colonies formed as a result of editing lacZ by using either plZDonor or pLZDonorGuide plasmids for the HDR activation and DNA insertion.

Table 1. The summary of the results of the CRISPR plates in Figure 1

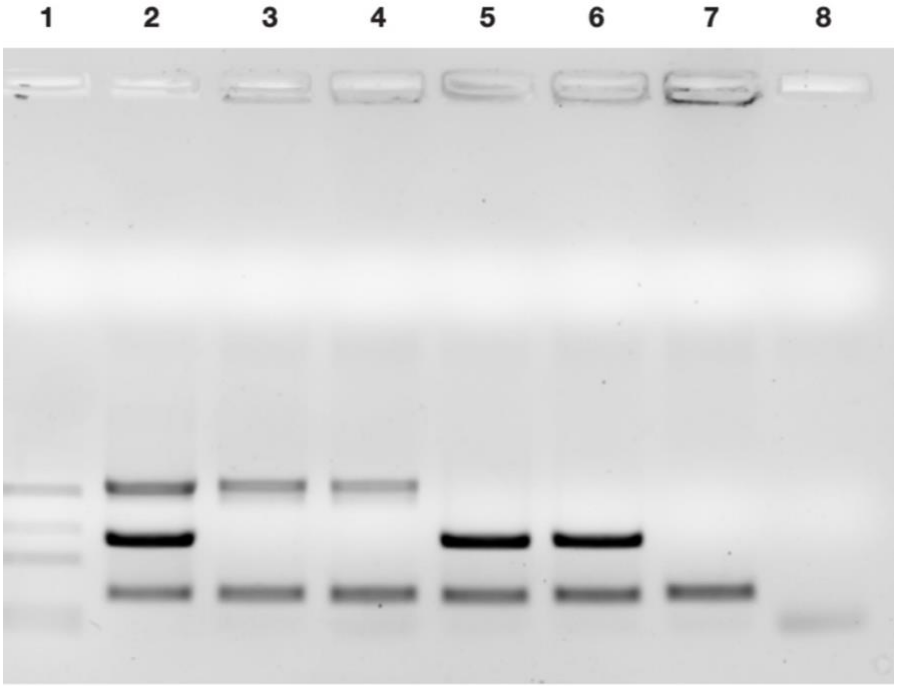
Plate	Bacteria source	Plasmids	Cas9	HDR system	sgRNA	Donor Template DNA	Predicted *lacZ* change	Color of colonies	Number of colonies
A	IX	pLZDonor	+	OFF	-	+	Fail to be cut by Cas9	Blue	160
B	IX	pLZDonorGuide	+	OFF	+	+	Can be cut by Cas9	N/A	N/A
C	IX/ARA	pLZDonor	+	ON	-	+	Fail to be cut by Cas9	Blue	200
D	IX/ARA	pLZDonorGuide	+	ON	+	+	Can be cut by Cas9	White	220

Table 1

The above table gives detailed data yielded from the *lacZ* gene transformation. By using different plasmids, sgRNA were supplied, and the HDR system was either activated or deactivated, resulting in a *lacZ* gene change. The color and the number of bacterial colonies were reported to support the visualization shown in Figure 1. If the *lacZ* gene was confirmed to be modified, the bacterial colonies yielded would have a blue color; otherwise, it gave a white color.

3. PCR results:

Figure 2. The visulization of gel electrophoresis for the PCR products. The supporting figure legends were arranged on the right of Figure 2.



Lane	Sample
1	Molecular weight ruler (MWR)
2	Positive PCR control (+)
3	PCR sample S
4	PCR sample C
5	PCR sample D1
6	PCR sample D2
7	PCR sample D3
8	Negative PCR control (-)

Figure 2

All the PCR samples were put on an agarose gel to be tested by gel electrophoresis. Each PCR sample yielded specific amplicons by the primer sets, which could provide the information about the DNA insertion and the success of the PCR test. The bands on each lane represent the amplicons yielded by each PCR sample. The far-left lane served as standard of size for the bands on the remaining lanes. The specific data can be found in Table 2.

Table 2. The detailed data for Figure 2.

No.	PCR sample	Amplicons			*lacZ* gene status
		1,100 bp	650 bp	350 bp	
1	MWR	✓	✓		Active
2	Positive PCR control (+)	✓	✓	✓	Active
3	PCR sample S	✓		✓	Active
4	PCR sample C	✓		✓	Active
5	PCR sample D1		✓	✓	Inactive
6	PCR sample D2		✓	✓	Inactive
7	PCR sample D3			✓	N/A
8	Negative PCR control (-)				Inactive

Table 2

The above table reports the specific amplicons that each PCR sample produced. From these data, the *lacZ* gene status can be confirmed as either "active" or "inactive." If there were no data or the amplicons expected were not produced, the *lacZ* gene status would be reported as "N/A."

IV. Discussion and Conclusion

Generally, the findings of the study indicate that the CRISPR-Cas9 system allowed the editing in the *lacZ* gene of *Escherichia coli* with the help of the donor template DNA and sgRNA via the HDR mechanism (Deligianni and Kiamos 2021, 111415). Thus, the hypothesis of the study was proved as correct since the use of sgRNA and donor template DNA via HDR mechanism showed successful transformation in the *lacZ* gene and correct DNA insertion, which were confirmed by

the multiplex PCR and visualized by gel electrophoresis. The sgRNA and donor template DNA presented as effective contributors to the gene-editing since the overall results showed that the *lacZ* gene was edited successfully. In the plate results, most of the samples during the usage of sgRNA and donor template DNA showed the expected color of colonies with one exception. This outlier factor affected the data report of the number of colonies since it had no colonies observed. The PCR results pointed out the limitation of the study as one of the PCR samples failed to yield the expected amplicon and present the activity of the *lacZ* gene. All other PCR samples successfully matched the predictions. The hypothesis could also be confirmed by the published result of Jiang et al. (2013, 233–39). This study indicated that the CRISPR-Cas9 system requires the engineering of crRNA (or sgRNA) and the donor template DNA to edit genes and, therefore, serves as a powerful gene-editing tool.

There might have been contamination during the sample preparation or the overall procedure that caused one of the plates to have no colonies shown. The unexpected result in the PCR visualization may be due to human error - there is a possibility that the gel electrophoresis was not handled and processed accurately. While these limitations did not heavily affect the overall outcome of this study, it is crucial to acknowledge these errors and consider better laboratory techniques. The samples could have been prepared better with sterile tools and appropriate reagents to prevent contamination. Also, the handling of gel electrophoresis could have been done with cautious and high precision for recording results.

The scientific community is constantly surprised with new technologies using the gene-engineering mechanism. Recent works showed that these technologies are currently utilized in

high-throughput screening applications and present several potentials (Costa et al. 2004). Among them, the CRISPR-Cas9 system has proved to be one of the most efficient gene-editing tools with such simplicity and versatility. Researchers believe that it may have several advancements, which expose its potential to the fullest.

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