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CRISPR/Cas9 Gene Editing in Bacteria: Leading to Hematopoietic Stem Cell Editing in Pakistan

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Abstract

Background: Deletion of LacZ gene in E. coli HB101-pBRKan using Clustered Regularly Inter-Spaced Short Palindromic Repeats-Cas9 (CRISPR/Cas9) gene-editing tool.

Methodology: Using BioRadTM USA "Out of the blue" gene editing kit, we demonstrated in vitro knock-out model for deletion of LacZ gene in E. coli HB101-pBRKan by employing CRISPR/Cas9 gene-editing tool. We followed an already designed modular plasmid assembly strategy and tested a single nuclease. Editing genomic locus was achieved within three days of minimal lab work.

Results: We were successful in gene editing of the E. coli LacZ gene using CRISPR/Cas9.

Conclusion: With the practical experience gained, we are confident enough to experiment in animal models to tackle the hematological disease.

Keywords: CRISPR-Cas9, genome engineering, DNA

Background

CRISPR/Cas9 gene editing is relatively a new entrant in the research arena of Pakistan. Few review articles have been written by people who have minimal hands-on experience of using this editing tool. Experiments have been performed and published using plant models. Still, nothing significant has been done to use this technique on animal models, ultimately leading to experimentation with human cells. The ultimate target is successful gene editing of a hematopoietic stem cell to delete/insert the β-globin gene to find a cure for hemoglobinopathies including β-Thalassemias and Sickle cell disease. Furthermore, gene editing will be used to experiment with solid tumors like B - Cell lymphomas.

Methodology

The study was conducted after taking permission from the Institutional Ethical Committee. Bio-RadTM (6000 James Watson Drive, Hercules, CA 94547) "Out of the blue" CRISPER kit with Catalog number 12012608EDU was used to alter the *E.coli LacZ gene*. The non-pathogenic bacterial strain *E. coli* HB101-pBRKan included in this kit has undergone genetic modification to prevent it from growing unless cultivated on an enriched medium. *LacZ*, a specific bacterial gene present only in bacteria, was chosen as the target gene. This gene was cut using Cas9, which was included in the package. The goal of the experiment was to remove the chromosomal *lacZ* gene in *E. coli* by transforming them using plasmid-based expression systems for donor template DNA and guide RNA; The second step was to calculate gene editing efficiency using blue-white screening and then to confirm the results using PCR.[1]

Preparation of Lysogeny Broth (LB Agar) plates

Three days before the start of the experiment, the first step was to prepare LB agar plates. The steps were carried out as per manufacturer's directions. A 500 mL KIX flask and a 1 Liter KIX/SPT flask were labeled. The arabinose vial was filled with three ml of deionized water. This procedure was repeated until the arabinose was completely dissolved, which took around 10 minutes. After that, 500 ul of deionized water was added to the spectinomycin vial. The mixture was vortexed until it was thoroughly dissolved. The flask labeled KIX/SPT was then filled with 700 mL of deionized water. Then 3 mL of deionized water was added to the KIX Mix vial, then recapped and shaken for 5 seconds to mix. In the KIX/SPT flask, this KIX Mix slurry was added and rinsed twice. The vial was examined to ensure that all of the contents had been transferred successfully to the flask. This spinning process lasted for 20 seconds until the powder was evenly distributed throughout the solution. Next 200 ml of this solution were instantly put into the

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500-ml KIX flask. After that, 7 g LB agar powder was added to the KIX flask.

In KIX/SPT flask, the remaining LB agar powder was added. Both flasks were autoclaved for 30 minutes on the liquid cycle till boiling, being careful not to overcook. Eight plates were labeled IX, while the remaining eight plates were labeled IX/ARA. IX/SPT was assigned to forty plates. The antibiotic kanamycin (K) was present in KIX Mix and was used to select E. coli HB101-pBRKan. Kanamycin was found in all of the LB agar plates used in this experiment. With molten KIX LB agar, eight IX plates were quickly filled to one-third to one-half (10 ml).

One milliliter of rehydrated arabinose was mixed into the remaining molten KIX LB agar, and eight IX/ARA plates were filled about one-third to one-half full (10 ml) with this molten agar. Five hundred ul rehydrated spectinomycin was added and stirred to mix. Fill minimum of 40 IX/SPT plates with the molten agar. Because spectinomycin would be destroyed by the extreme heat of more than 60°C, it was not added until the agar had cooled enough to handle. Also, because the agar firms were at 27°C, the plates were poured before they cooled too much. After 30 minutes, the agar solidified, and the plates were left to dry at room temperature for two days, unwrapped, and in the dark. This step increases the liquid transformation's uptake. The stacks of plates were coated in aluminum foil to keep them away from light after drying and then wrapped in plastic. The plates were kept at 4°C in an upside-down manner in the refrigerator.

Rehydrating the bacteria

This procedure was completed two days before the experiment. A 150–250 ml bottle was filled with 50 ml of distilled/deionized water and LB broth capsule. This bottle was loosely capped and autoclaved three times to get it to boil. The soup was then allowed to cool to room temperature. The bottle cap was tightened, and the bottle was kept at 4°C. Before using, it was brought to room temperature. Using a sterile pipette tip, 250 ml of this broth was added to the vial of lyophilized *E. coli* HB101-pBRKan. To resuspend the bacteria, the vial was gently shaken and incubated at 37°C for 24 hours.

Streaking and incubation of starter plates

A sterile plastic inoculation loop inoculated eight IX and eight IX/ARA plates with rehydrated *E. coli* HB101-pBRKan. The initial streak pattern was from side to side near the quadrant's boundary. The plate was then rotated for about a quarter turn, and the loop was passed from one side to another through the previously made streaks a few times before extending them into next quadrant. This treatment was carried out twice more. The starter plates were stored at 4°C after being incubated in upside down position in a 37°C incubator oven for 24 hours.

Preparation of Plasmids

The next step was to introduce 25 ul of pLZDonor plasmid to all of the eight pD tubes and 25 ul of pLZDonorb Guide plasmid to each pDG tube. Until ready to use, all solutions were kept refrigerated at 4°C. All reagents were brought to room temperature prior to their use, except for the transformation solution (TS).

Results

Experiment 1: The *lacZ* gene and blue-white screening

The *lacZ gene*, which is naturally located in the chromosome of *E. coli* HB101-pBRKan, was damaged in first experiment employing CRISPR-Cas9 technology. *LacZ* is the gene encoding for b-galactosidase (b-gal), an enzyme that permits *E. coli* to hydrolyze lactose. X-gal is a colorless lactose analog that can be degraded by b-gal and turned into a blue pigment. When bacteria with functional *lacZ* were cultivated on media with X-gal, *LacZ* gene editing was perceived as successful when blue or white colonies appeared: those with functional lacZ turned blue. In contrast, those with inoperable *lacZ* genes remained white. (Figure 1)

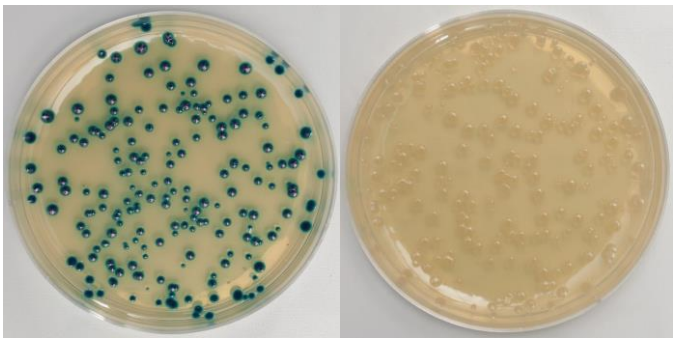

Figure 1. E. coli plated on media containing X-gal. Bacteria with a functional lacZ gene (left) are blue. Bacteria with a disrupted, nonfunctional lacZ gene (right) are white. (BioRad)™

Experiment 2: Transformation of *E. coli* HB101-pBRKan by using plasmids

Bacterial culture was initiated on two separate starter plates (IX and IX/Ara). Bacteria grown on the IX plate did not express the Cas9 enzyme essential for HDR (repair system turned off); however, bacteria grown on the IX/ARA plate did (repair system ON). **(Table I)**

Table I: Media additives for starter and experimental LB agar plates. (BioRad)™

Plate	Kanamycin	IPTG	x-gal	Arabinose	Spectinomycin
IX	✚	✚	✚		
IX/ARA	✚	✚	✚	✚	
IX/SPT	✚	✚	✚		✚

Bacteria present on both starter plates were transformed using either pLZDonor or pLZDonorGuide. The sgRNA required to guide Cas9 to cleave lacZ was not present in bacteria transformed with pLZDonor. As a result, no gene editing occurred, and the transformants remained blue regardless of whether the repair system was turned off or on.

The sgRNA along with donor template DNA were found in bacteria transformed using pLZDonorGuide. Cas9-mediated cutting occurred on the plates missing HDR system expression (arabinose was absent), but DNA repair did not occur, resulting in bacterial mortality and no growth.[2]Cas9-mediated cutting happened in plates with Homology Dependent Recombination (HDR) system expression (arabinose was present). HDR machinery employed donor template DNA from pLZDonorGuide to patch the cut and add a stop codon into the *lacZ* gene's codon. The transformants that resulted were white. Cutting and repair mechanisms mediated by Cas9 commenced almost quickly after transformation. Spectinomycin, which was present in the IX/SPT agar media, was used to select the successfully converted bacteria. (Figure 2)

Validation of the findings

The *lacZ* gene was targeted by plating the cells on IPTG and X-gal plates. The number of white colonies to total colonies was used to calculate apparent editing efficiency. Moreover, the results were confirmed by colony PCR and DNA sequencing to identify white colonies. True editing effectiveness was determined, equal to the ratio of appropriately edited colonies to total colonies.

Multiplex PCR for Gene Editing Detection

At the DNA level, multiplex PCR was employed to corroborate these findings. Each colony was first subjected to rapid DNA extraction, followed by multiplex PCR to determine the presence of the donor template DNA insertion. Three primer sets were used in the multiplex PCR experiment. The "TAA" and "CACCACCACCACCAC" DNA sequences were directly integrated into primers for amplification of both upstream as well as downstream homologous arms required for mutation of the *lacZ* initiation codon and labeling with an N-terminal His-tag, respectively.[3,4]

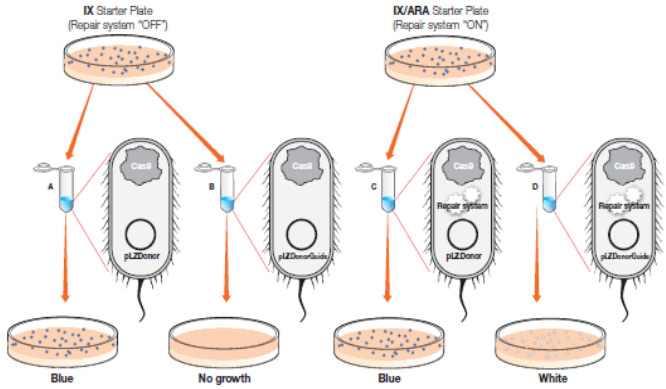

Figure 2. Transformations performed with expected results (BioRad)™

The first primer was created to detect *lacZ* that had not been changed. One of the primers in the set is coupled to the Cas9 target cut site directly. The primer will not bind if the desired cut site has been altered. This primer combination was expected to yield a 1,100 bp amplicon if the target Cas9 cut site was not modified.[5]

The second primer set was created to detect *lacZ* that had been changed. One of the primers in the set would bind to the donor template DNA insert. A successful repair of the intended cut site would result in a 650 bp amplicon with this primer set if donor template DNA were used. As a control, the third pair of primers amplified an unrelated region well downstream of the lacZ gene to ensure that the sample contained chromosomal DNA. This primer set would give a 350 base pair amplicon if chromosomal DNA extraction and

PCR were successful, regardless of whether *lacZ* was changed or not.

LacZ was presumably knocked off in about 91 percent of the altered strains. Only 12% of the samples were found to have shorter *lacZ* gel bands by colony PCR, indicating that the editing was done precisely as intended. Others had no band or smear on a gel, indicating a change in the chromosomal state around the *lacZ* locus. We were unable to sequence these strains to explore for genetic changes due to funding restrictions.[6,7,8]

Discussion

As always, a journey of a thousand miles starts with a single step, and we started successfully experimenting with bacterial models to establish and practice already established procedures in CRISPR/Cas9 Gene editing in bacterial models. We were venturing into unchartered waters and wanted that our initial experiment yielded positive results, which it did. In the process, we were able to develop protocols for our next venture, that is, to experiment with animal cell lines in either mouse or Zebrafish models. Hopefully, we want to proceed to do projects relating to knock out -knock-in models to edit human β-globin gene and explore treatment options of curing β- Thalassaemia and Sickle Cell disease.

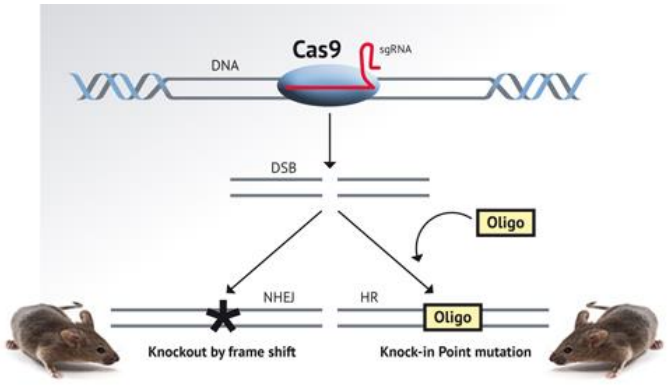

Figure 3. Mouse Knock out -knock-in models

Gene editing for knock-out models of solid tumors like B - Cell Lymphomas is also on the cards. Ultimately, we may manipulate Human Hematopoietic Stem Cells to find a cure for different diseases we worked upon in the animal models. [9,10]

Conclusion

The road to success is arduous and full of challenges. Still, with sound technical knowledge and human resource at hand, our team is fully capable of facing challenges ahead and tackling hematological diseases.

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