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Gene Editing Experiment Using the CRISPR-Cas9 System

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ABSTRACT

An experimental report on using the CRISPR-Cas9 system to edit the gene and change the trait of E. coli

Keywords: CRISPR, CRISPR-Cas9, E. coli, gene editing, gene, DNA, RNA, gRNA, crRNA

1. INTRODUCTION

CRISPR origins in nature - (bacteria) The Clusters of Regularly Interspaced Short Palindromic Repeat (CRISPR) system was first discovered in archaea and later in bacteria. It is known to serve as a part of the bacterial immune system, which defends against invading viruses. When the presence of viral DNA is detected inside a cell, it is incorporated into the CRISPR region of the bacterial DNA. The next time, it is encountered, two short strands of RNA, called the spacer RNA and crRNA, together called gRNA, are produced. In the spacer RNA, a sequence is contained which matches that of the viral DNA. The RNA strands then create a complex with an enzyme called the CAS9. This causes a conformational change that allows the complex to bind to the matching sequence in the viral DNA. The CAS9 contains tiny molecular scissors that are capable of cutting strands of DNA. The complex attaches to the viral DNA at the target sequence, and disables its functions by causing a Double Stranded Break (DSB).

2. CRISPR in Scientific Research

Since eukaryotic cells have the natural ability to repair DNA, scientists take advantage of this nature to purposely create mutations and observe specific functions of DNA. Eukaryotic cells can repair their DNA through two general pathways: the Non Homologous End Joining (NHEJ) and Homology Directed Repair (HDR). The NHEJ frequently causes deletions or insertions at the DSB, but is more active in the cell and efficient, whereas the HDR is more desirable for controlled mutations, since errors are minimum and results are more accurate and specific. However, HDR has lower efficiency than NHEJ. Scientists often use the NHEJ when turning off a gene or to prevent a certain gene from functioning. The HDR is implemented when specific mutations are desired.

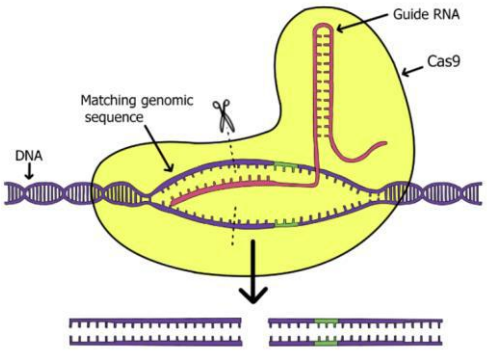

Figure 1. Mechanism of the CRISPR-Cas9 system

3. Steps of the CRISPR Reaction

1. Cas9 binds the gRNA molecule

2. The Cas9/gRNA complex finds the DNA sequence that matches the gRNA

3. Cas9 cuts the DNA

4. The cell's repair machinery fixes the break and using our supplied template

5. The cell has a new DNA sequence

Making Competent Cell with CaCl2 and PEG8000

Though an uncertain fact, PEG8000 is thought to play several functions in transformation. DNA and cell walls are both negatively charged, and naturally repel each other. In theory, PEG8000 shields the negative charge of the DNA in order to make it easier to permeate the cell wall. Moreover, PEG8000 is believed to help transport DNA into the cell and make the cell membrane more porous. Similarly, it is often perceived that CaCl2 shields and neutralizes the negative charge of DNA.

4.

5. SECTIONS

5.1 Bacteria Streaking Method

Before streaking, use a Bunsen burner to sterilize the inoculation loop. Then open the lid of the plate just enough that the loop can go in. Pick a colony and scrape off the bacteria with the loop in a zig-zag motion until one-third of the plate is covered. Then sterilize the loop again with the burner. Rotate the plate and streak bacteria again.

5.2 Agarose Gel: a Medium for E. Coli

Agar is a jelly-like substance used to feed and grow bacteria; agar is usually indigestible to microorganisms. In the experiment, this agarose gel will be put to use in order to create a substance called LB agar media. LB media is an imperative factor of the experiment since it is a nutritionally rich medium in which bacteria are able to grow.

5.3 Mechanism of Action of Streptomycin(antibiotic)

Streptomycin is an antibiotic that binds to ribosomes, causing the prevention of protein synthesis. When bacteria are grown in the presence of streptomycin, they're going to die. Using CRISPR, we can introduce a point mutation in the DNA of the bacteria which will cause a mutation in the rRNA so that the ribosomes cannot bind to streptomycin. The experiment will prove to be successful if the bacteria continues to live and function properly in the presence of streptomycin.

6. Materials

6.1 Tools

250 mL glass bottle, 10-100 µL variable volume adjustable pipette, 1-200 µL pipette tips, petri plates, microcentrifuge tube rack, inoculation loops, plate spreader, nitrile gloves, 1.5 mL microcentrifuge tubes, 1.5 mL microcentrifuge tubes containing LB Broth, 50mL centrifuge tube

6.2 Chemicals

LB Agar, LB Strep/Kan/Arab Agar (Strep (50 µg/ml), Kan (25 µg/ml) and Arabinose (1mM)) 1 mL Bacterial Transformation Buffer 25mM CaCl2, 10% PEG 8000, 55µL of 100ng/µL Cas9 Plasmid Kanr, 55µL of 100ng/µL gRNA Plasmid Ampr, 55µL of 1mM Template DNA, non-pathogenic E. coli bacteria Freeze-Dried Tube (DH5α).

7. Method

7.1 Day 1: Making LB Agar Gel Media

1. Mix LB Agar media powder and 150 mL water in a 250mL glass bottle. Shake it vigorously.

2. Heat the agar to dissolve it. Put the bottle in the microwave for a few seconds at a time, being careful not to let the bottle boil over. Keep heating until the agar is translucent and there are no visible suspended particles.

3. Let the bottle cool for 20-30 minutes.

4. While the bottle remains somewhat warm, pour agar solution into the plates. (Figure. 2)

5. Let it cool and solidify overnight at room temperature.

7.2 Day 2: Making LB Strep Media, Streaking E. Coli

1. Repeat steps for making LB agar gel media with LB Strep/Kan/Arab Agar powder.

2. Take a freeze-dried DH5α tube and add 100µL of water to the bacteria tube.

3. Using an inoculation loop, spread bacteria onto a new LB Agar plate in a zig-zag formation.

4. Wait until the plate is dry before putting on the lid.

5. Let the plate grow overnight at 30°C.

7.3 Day 3: Making Competent Cell

1. Pipette 100µL of bacterial transformation mix(25mM CaCl2, 10% PEG 8000) into a new microcentrifuge tube.

2. Using an inoculation loop, gently scrape a little bacteria off of your fresh plate until the loop is filled, and mix it into the transformation mix until any big clumps have disappeared.

3. Store the tube at 4°C in the fridge.

Figure 2. Pouring agar solution into plates

7.4 Day 4: Making Gene-Edited E. Coli

1. Add 10µL of competent cell mixture to Cas9, gRNA, template DNA tube. Remember to change out the pipette tip for a new one between steps.

2. Incubate the tube in the fridge for 30 minutes. Then incubate it for 30 seconds in 42°C water. (Figure. 3)

3. Add 1.5mL of room temperature water to one of the LB media microcentrifuge tubes and shake to dissolve the LB.

4. Add 250µL of LB media to a competent cell mixture containing DNA. (Figure. 4)

5. Incubate the tube at 30°C for 12 hours.

Figure 3. 42°C water

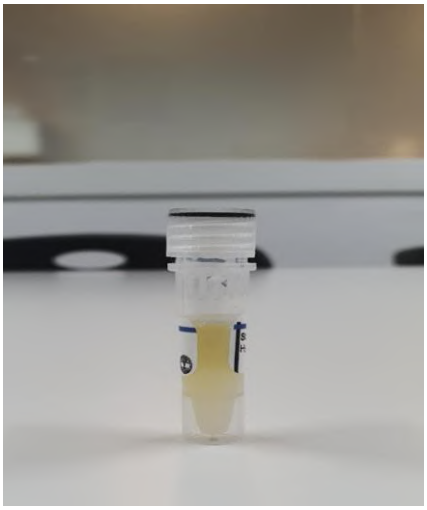

Figure 4. Competent cell mixture with LB media

7.5 Day 5: Streaking Gene-edited E. Coli

1. Take an LB/Strep/Kan/Arab plate and add all of the CRISPR transformation mixture on it.

2. Using an inoculation loop, gently spread the bacteria around the plate and let dry for 10 minutes before putting the lid back on.

3. Incubate the plate at room temperature for 24-48 hours.

8. Results

With streptomycin present, the growth of bacteria should abate since the antibiotic binds to ribosomes, preventing protein synthesis. In the experiment, I implemented the CRIPSR-Cas9 system in order to introduce a point mutation in the DNA of the bacteria which would eventually cause a mutation in the rRNA, prohibiting the streptomycin from binding to the ribosomes. In a successful experiment, the bacteria would continue to grow and function despite the presence of streptomycin. As a result, I can conclude that the experiment was a success as evinced by the clear growth of bacteria on the petri dish with small, white spots.

9. Discussion

There are also several possible errors to be mindful of during the experiment. Some errors which can be avoided with meticulous attention include too much/little cooling, not consistent heating, death of bacteria from aggressive mixing, reuse of pipettes, and careless streaking. There are also some errors that may be difficult to avoid with the extents of human prowess. These include difference in temperature, and approximative timing. In all, the experiment demonstrates that genetic engineering can be exploited for countless purposes which can possibly benefit humans. Genetic engineering offers the possibility of alleviating diseases such as cystic fibrosis, sickle cell disease, hemophilia and many other likewise. Subsequently, thorough discussion is required among those who have expertise in the field of genetic engineering in order to clearly differentiate between where genetic engineering can be applied and not.

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