Jacob Kaplan

Dr. Grimwade

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1 Introduction

The target for research done in this lab this semester was aimed towards finding the mechanisms and targets behind all initiation of DNA replication. In order to do this, a specific targeting site for the initiation of DNA replication in bacterial cells was selected as a base for research and a possibility for the development of a novel antibiotic. In any bacterial cell, orisomes are assembled by the initiator protein DnaA to build an origin of replication (oriC). (Grimwade & Leonard, 2017) The problem here is that only a few bacterial orisomes have been fully discovered, so the short-term aim for our research was to synthetically make an oriC on a different bacterium by combining mutations in the duplex unwinding element (DUE).

Poc170 was the main plasmid used, which Poc170 contains E. coli oriC and pBR 322 origin were given in primers sequences in different regions of oriC. The exact primer design information is given below in (Figure 1).

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Figure 1: Primer Sheet

2 Methods

Plasma Purification (poc170): To harvest the cultured bacteria first transfer a total of 4 mL of cultured cells into a microcentrifuge tube. Centrifuge down and discard the supernatant. Next, resuspend the pellet in 200 μl of PD1 buffer and suspend the cell via vortex. To lyse the cells, add 200 μl PD2 buffer and invert 10 times. Neutralizing the plasmid entailed adding 300 μl of PD3 buffer which is mixed by inversion 10 times. Adding the supernatant from before, centrifuge the tube and discard flow through. Placing a PD column into the collection tube add 600 μl of wash buffer, centrifuge, and discard flow through. Dry column by 3 min centrifuge and place PD column into a new microcentrifuge tube. To elute the DNA, add 50 μl of elution buffer into the center of the PD matrix. Let the column stand for 2 minutes then centrifuge for another 2 minutes. Transfer flow through back into the center of the column and centrifuge again for eluded purified plasmid DNA.

To read the results from Plasmid Purification, then blank sensor for Nanodropanalysis. Transfer 1 μl to sensor and record and label results.

Site-Directed Mutagenesis: Store suspended Primers in EB 10mm Tris pH of 8.5 at a concentration of 250 pmol/μl. Dilute an aliquot of primer to 15 pmol/μl.

To calculate the annealing temp, use the Thermo Fisher website and enter the sequence to get the annealing temperature.

PCR: dilute poc170 concentration to 50 ng/μl. Follow (Figure 2) to set PCR tube solutions.

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Figure 2: Solution Guide for PCR

After adding all contents to the PCR tube place 4 PCR tubes in the thermocycler Biorad PCR machine. Step 1 95°C to heat lid. Step 2 14 cycles at 95°C for 30 seconds then the annealing temperature 55°C for 1 minute and then to 72°C for 4 minutes. Step 3 extension at 72°C for 8 minutes. After the PCR product will be kept in storage in fridge.

Gel Electrophoresis: Make a standard 1% agarose gel mold. Each channel gets 5 μl of sample and 3 μl of loading dye. 1st channel gets 2 μl of 100kb DNA ladder. Let gel run at 100v for 45-50 minutes. After the gel is stained with ethidium bromide for 20 minutes. Analyze gel with trasn UV rays.

Competent Cells, and Transformation: Take one colony of DH5a colonies from a plate and resuspend in 10 mL of LB in a shaking water bath overnight at 37°C. Inoculate 40 mL of LB with 0.4 mL of recipient cells, and grow the cells in 37°C shaking water bath till cells are in exponential growth but under 0.4 OD600 using the spectrophotometer. Rest the flask on ice for 5 minutes keep everything cold. Next, put cells in a sterile centrifuge tube, and pellet cells into Stovall centrifuge 5000 rpm at 4°C. Pour off media, and resuspend cells into 20 mL of ice-cold 100mM CaCl2. Pellet cells again in centrifuge same settings and resuspend cells into 1 mL of CaCl2. Lastly, leave cells on ice in the refrigerator overnight. For transformation and plating follow the steps in (Figure 3)

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Figure 3: Transformation Set-up and Procedures

3 Results

In this semester the product was often used as a component for the next part of the lab procedure. In the plasmid purification protocol, a result of 207.4 ng/μl was given which is more than acceptable. The poc170 at 207.4 ng/μl concentration was then diluted and used as a template for following PCR reaction.

PCR was a step that was needed to be repeated several times in order to get results. In 6 attempts at PCR only once results were visible in gel under UV. If PCR was successful, the gel mold would have shown a clear band at 3800 bp on the experimental lane of the gel along with the bands shown for the control primers. Pictures of the gel were stored on a computer kept in lab.

Preparing the competent cells and transformation have gotten results once from the successful run of PCR. Very few colonies were present after plating, which gave a yielding result far below an acceptable level. Diverging out, in an experimental run trying to improve the yield of control poc170 colonies results were drastically better. A result of around 4000 colonies per μg was a better yield, but still not at an acceptable yield. All of the researchers in the lab was having similar yielding issues after transformation.

4 Conclusion

Ultimately, if everything had gone correctly the region of mutation in the center of oriC along with the right half of E. coli DUE and with the right half of ADP1 DUE would have made a fully synthetic oriC. The newly mutated oriC would have been placed onto a different bacterium and undergo further experimental research.

The part that continuously failed for primer 4 was the site-directed mutagenesis. Making a few intentional mutations in the center of the primer (in this case 8) and undergoing PCR and gel electrophoresis allows these mutations to be inserted into cloned DNA with the modified sequence. In the first few attempts, the annealing temperature was found to be too high, a lower annealing temperature was used to allow time for the mutated primer to bind to the template. Further fails of PCR was not able to be debunked successfully but will be further researching in the upcoming semester.

In the experimental run that was designed to increase the colonial yield of the product after competent cell transformation fared better results. Using verified ultrapure H2O to make LB and CaCl2 for competent cell and transformation of poc170 control. The yield was still not high enough to be considered successful. More techniques to increase yield will be further researched in the upcoming semester.

5 Works Cited

Grimwade, J. E., & Leonard, A. C. (2017). Targeting the Bacterial Orisome in the Search for New Antibiotics. *Frontiers in Microbiology,* *8*. doi:10.3389/fmicb.2017.02352