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UG Research Fall 2020

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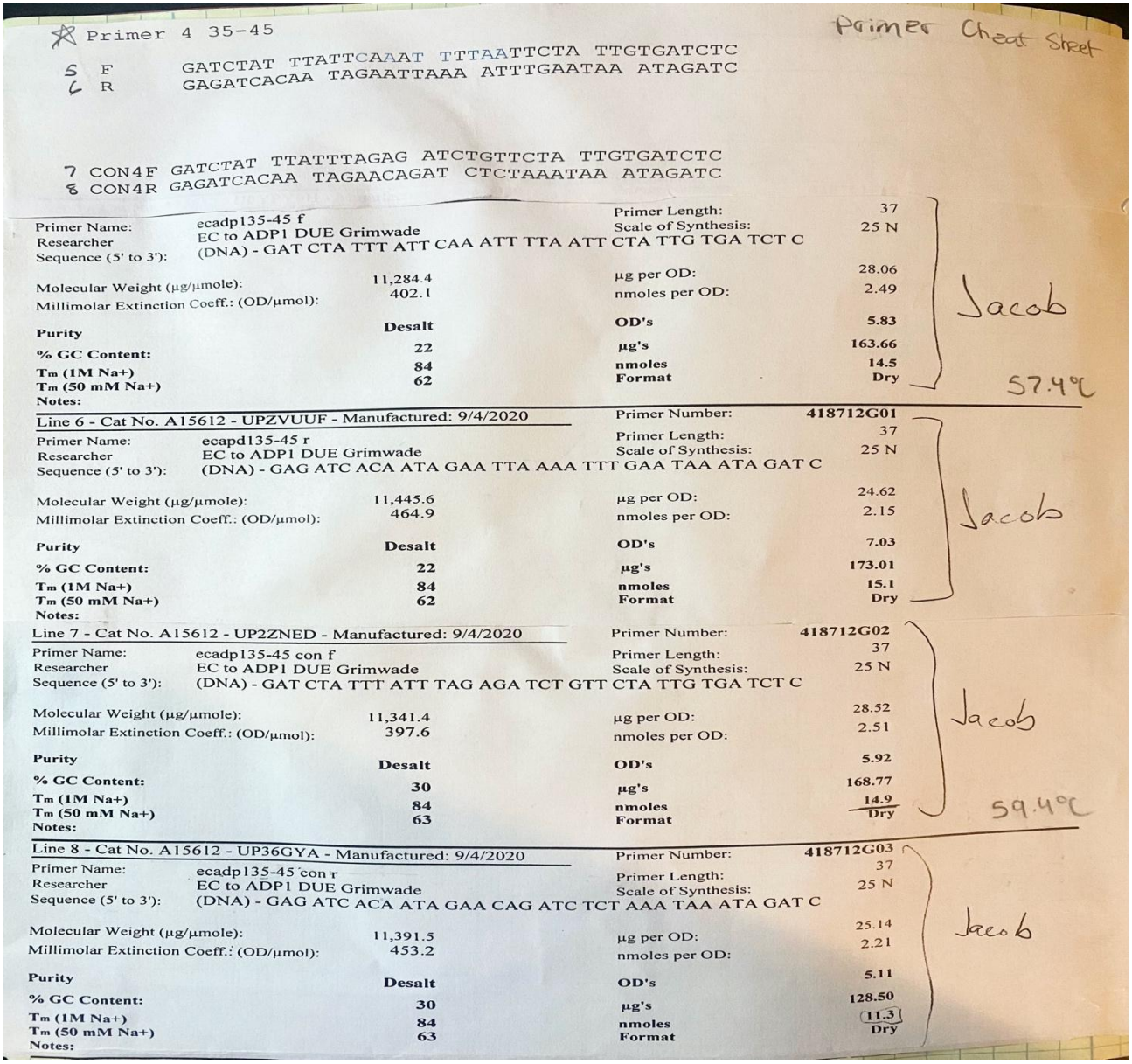
UG Research Lab Paper. Fall of 2020

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).



Primer Cheat Sheet

Primer 4 35-45

5 F GATCTAT TTATTCAAAT TTTAATTCTA TTGTGATCTC
6 R GAGATCACAA TAGAATTAAA ATTTGAATAA ATAGATC

7 CON4F GATCTAT TTATTTAGAG ATCTGTTCTA TTGTGATCTC
8 CON4R GAGATCACAA TAGAACAGAT CTCTAAATAA ATAGATC

Primer Name: ecadp135-45 f
Researcher EC to ADP1 DUE Grimwade
Sequence (5' to 3'): (DNA) - GAT CTA TTT ATT CAA ATT TTA ATT CTA TTG TGA TCT C

Primer Length: 37
Scale of Synthesis: 25 N

Molecular Weight (µg/µmole): 11,284.4
Millimolar Extinction Coeff.: (OD/µmol): 402.1

µg per OD: 28.06
nmoles per OD: 2.49

Purity: Desalt
% GC Content: 22
Tm (1M Na+): 84
Tm (50 mM Na+): 62
Notes:

OD's: 5.83
µg's: 163.66
nmoles: 14.5
Format: Dry

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57.4°C

Line 6 - Cat No. A15612 - UPZVUUF - Manufactured: 9/4/2020

Primer Number: 418712G01

Primer Name: ecapd135-45 r
Researcher EC to ADP1 DUE Grimwade
Sequence (5' to 3'): (DNA) - GAG ATC ACA ATA GAA TTA AAA TTT GAA TAA ATA GAT C

Primer Length: 37
Scale of Synthesis: 25 N

Molecular Weight (µg/µmole): 11,445.6
Millimolar Extinction Coeff.: (OD/µmol): 464.9

µg per OD: 24.62
nmoles per OD: 2.15

Purity: Desalt
% GC Content: 22
Tm (1M Na+): 84
Tm (50 mM Na+): 62
Notes:

OD's: 7.03
µg's: 173.01
nmoles: 15.1
Format: Dry

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Line 7 - Cat No. A15612 - UP2ZNED - Manufactured: 9/4/2020

Primer Number: 418712G02

Primer Name: ecadp135-45 con f
Researcher EC to ADP1 DUE Grimwade
Sequence (5' to 3'): (DNA) - GAT CTA TTT ATT TAG AGA TCT GTT CTA TTG TGA TCT C

Primer Length: 37
Scale of Synthesis: 25 N

Molecular Weight (µg/µmole): 11,341.4
Millimolar Extinction Coeff.: (OD/µmol): 397.6

µg per OD: 28.52
nmoles per OD: 2.51

Purity: Desalt
% GC Content: 30
Tm (1M Na+): 84
Tm (50 mM Na+): 63
Notes:

OD's: 5.92
µg's: 168.77
nmoles: 14.9
Format: Dry

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59.4°C

Line 8 - Cat No. A15612 - UP36GYA - Manufactured: 9/4/2020

Primer Number: 418712G03

Primer Name: ecadp135-45 con r
Researcher EC to ADP1 DUE Grimwade
Sequence (5' to 3'): (DNA) - GAG ATC ACA ATA GAA CAG ATC TCT AAA TAA ATA GAT C

Primer Length: 37
Scale of Synthesis: 25 N

Molecular Weight (µg/µmole): 11,391.5
Millimolar Extinction Coeff.: (OD/µmol): 453.2

µg per OD: 25.14
nmoles per OD: 2.21

Purity: Desalt
% GC Content: 30
Tm (1M Na+): 84
Tm (50 mM Na+): 63
Notes:

OD's: 5.11
µg's: 128.50
nmoles: 11.3
Format: Dry

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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.

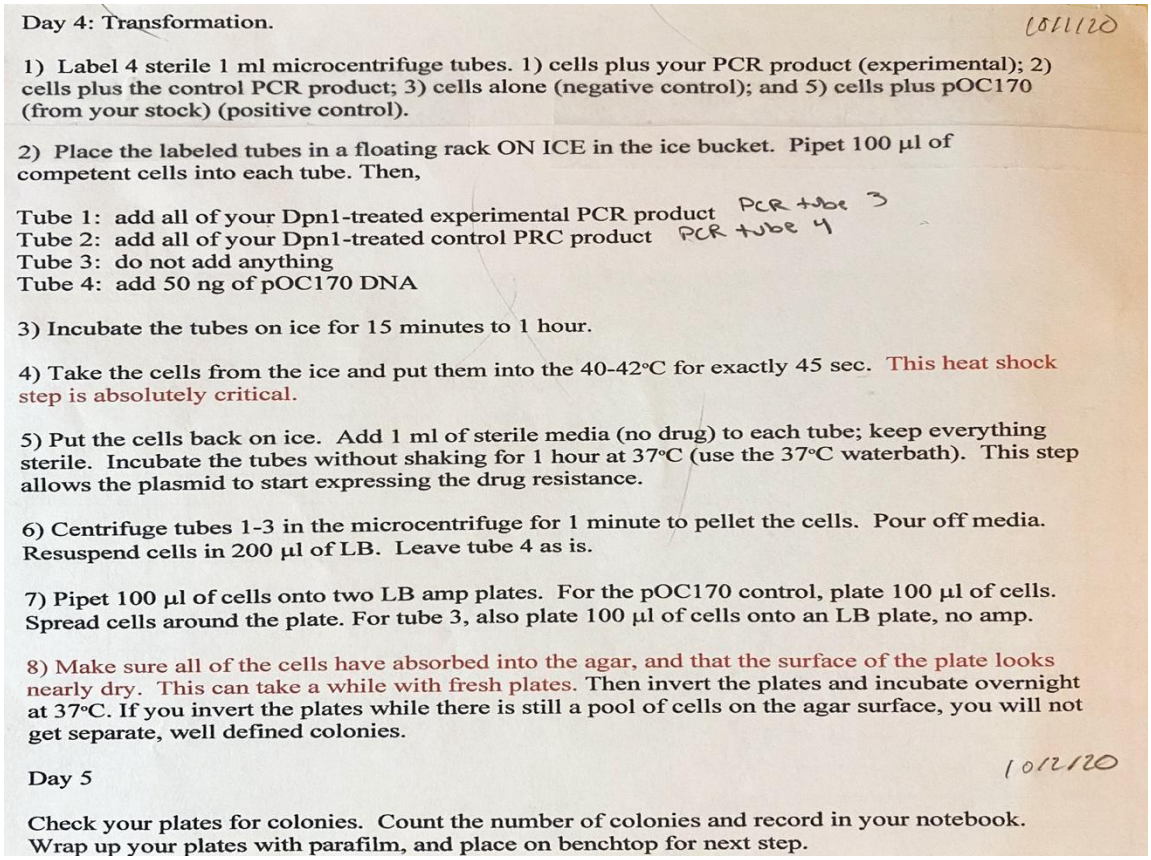
PCR Rxn. 50uL	PDNA Template	DNTPs	FP	RP	10x Poly Buffer	Vent poly	$MgSO_4$	DiH_2O
1) Template only	1uL	2uL	X	X	5uL	0.5uL	2.5uL	34uL
2) primer only	X	2uL	1.25uL	1.25uL	5uL	0.5uL	2.5uL	37.5uL
3) Exp primer	1uL	2uL	1.25uL	1.25uL	5uL	0.5uL	2.5uL	36.5uL
4) cont primer	1uL	2uL	cont 1.25uL	cont 1.25uL	5uL	0.5uL	2.5uL	36.5uL

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 $CaCl_2$. Pellet cells again in centrifuge same settings and resuspend cells into 1 mL of $CaCl_2$. Lastly, leave cells on ice in the refrigerator overnight. For transformation and plating follow the steps in (Figure 3)



Day 4: Transformation. 10/11/20

1) Label 4 sterile 1 ml microcentrifuge tubes. 1) cells plus your PCR product (experimental); 2) cells plus the control PCR product; 3) cells alone (negative control); and 5) cells plus pOC170 (from your stock) (positive control).

2) Place the labeled tubes in a floating rack ON ICE in the ice bucket. Pipet 100 µl of competent cells into each tube. Then,

Tube 1: add all of your Dpn1-treated experimental PCR product PCR tube 3
Tube 2: add all of your Dpn1-treated control PRC product PCR tube 4
Tube 3: do not add anything
Tube 4: add 50 ng of pOC170 DNA

3) Incubate the tubes on ice for 15 minutes to 1 hour.

4) Take the cells from the ice and put them into the 40-42°C for exactly 45 sec. This heat shock step is absolutely critical.

5) Put the cells back on ice. Add 1 ml of sterile media (no drug) to each tube; keep everything sterile. Incubate the tubes without shaking for 1 hour at 37°C (use the 37°C waterbath). This step allows the plasmid to start expressing the drug resistance.

6) Centrifuge tubes 1-3 in the microcentrifuge for 1 minute to pellet the cells. Pour off media. Resuspend cells in 200 µl of LB. Leave tube 4 as is.

7) Pipet 100 µl of cells onto two LB amp plates. For the pOC170 control, plate 100 µl of cells. Spread cells around the plate. For tube 3, also plate 100 µl of cells onto an LB plate, no amp.

8) Make sure all of the cells have absorbed into the agar, and that the surface of the plate looks nearly dry. This can take a while with fresh plates. Then invert the plates and incubate overnight at 37°C. If you invert the plates while there is still a pool of cells on the agar surface, you will not get separate, well defined colonies.

Day 5 10/12/20

Check your plates for colonies. Count the number of colonies and record in your notebook. Wrap up your plates with parafilm, and place on benchtop for next step.

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