Task 2

Obtain Gene of Interest

Gene synthesis

- We use this method when the Gene sequence is unknown, as we know the sequence to use PCR.

PCR

- eutS
- eutR
- Amplified eutS eutR using PCR are known as primary PCR products.
- eutS + eutR(Primary PCR product was used again in PCR to make it into a single insert)
    - Troubleshoot when combined PCR is done it was not amplified because the region we chose was not in particular to the DH5 alpha in nature while we designed primer we used the snapgene file to generate premier but when we checked for the DH5 alpha strain of E coil it is not the actual sequence from the snapgene sequence besacially it was not matching with the primer designed with DH5 Alpha.

Cell Lysate PCR

Independent amplification of eutR and peutS genes using the designed primers:

- eutR and eutS genes are naturally present in the E. coli genome. So, to insert these genes it should first be isolated and amplified and then inserted into the vector.
- For that, Resuspend one colony from the DH5 Alpha E.coli cells agar plate in 20 µL of distilled water in a PCR tube
- Heat shock the suspension at 98°C to break the cell membrane and release the cellular components (used the thermal cycler to lyse the cell and remove the DNA)
- Centrifuge/spin down
- Discard the pellet and use the supernatant as a DNA template for PCR.

PCR protocol:

- Thaw the PCR master mix at room temperature
- Vortex the master mix then spin it down briefly in a microcentrifuge to collect the material at the bottom of the tube.
- For a 25 µL of PCR reaction:

Components	Amount to be added	Final concentration
PCR master mix 2X	12.5 µL	1X

Upstream primer 10 µM	0.25 - 2.5 µL	0.1 - 1.0 µM
Downstream primer 10 µM	0.25 - 2.5 µL	0.1 - 1.0 µM
Template DNA	1 - 5 µL	< 250 ng
Molecular biology PCR grade water	Add to make the total volume up to 25 µL	-

Making the stock solution of primers

Primers	Volume of water to be added to get 100pm/µL or 100 µL concentration
eutR_CFW1	238.9 µL
eutR_CFW2	311.82 µL
eutR_CRE	274.9 µL
eutS_CFW	338.7 µL
eutS_CRE	336.36 µL

These primer solutions were then diluted to make for 25 µL of PCR reaction.

PCR Mixture prep:

- Add 12.5 µL of master in a PCR tube
- Add upstream primer (2 µL for eutS and 4 µL for eutR)
- Add downstream primers (2 µL)
- Add template DNA of 1 µL
- Add PCR-grade water to make the total volume up to 25 µL

Two different PCR reactions were conducted for eutS and eutR separately, with two replicates. This was because the annealing and extension temperatures for the two gene amplifications were different.

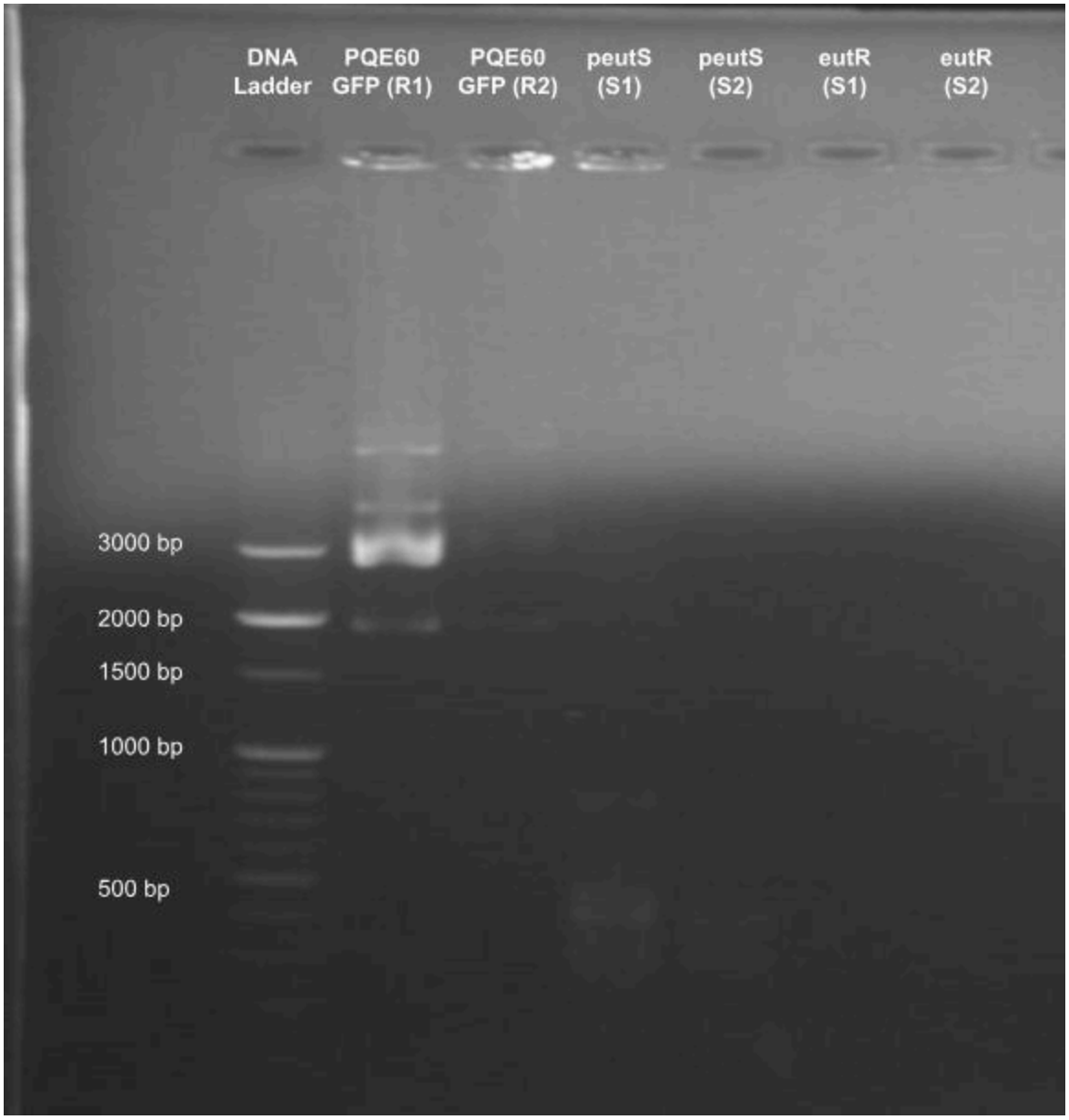
Setting the PCR:

eutS

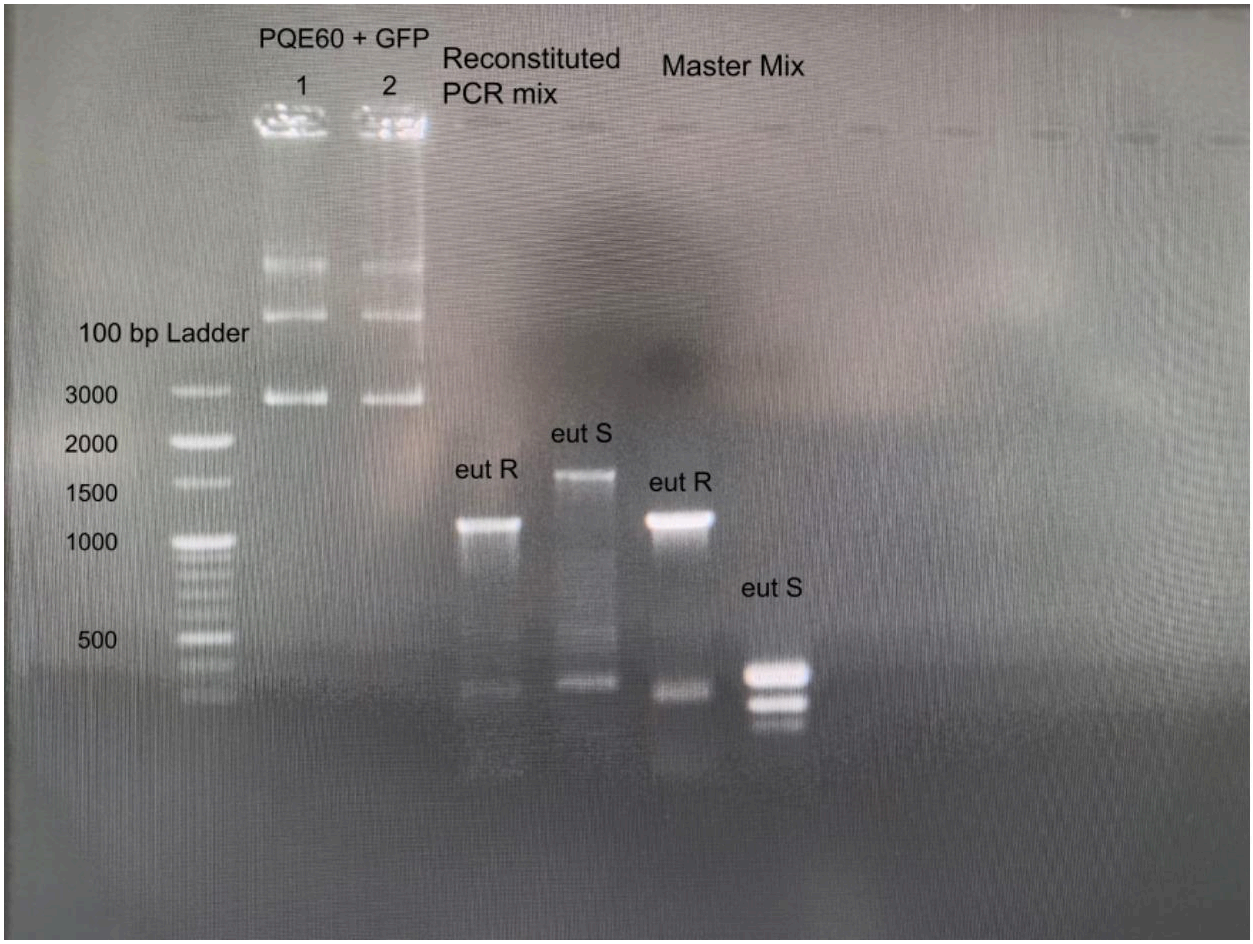
Step	Temperature (°C)	Time (min)
Initial denaturation	95	3:00
Denaturation	95	0:30
Annealing	60	0:30
Extension	72	1:30
30 cycles		
Final extension	72	5:00
Hold	12	Infinity
Total time for PCR =		1:48:27

eutR

Step	Temperature (°C)	Time (min)
Initial denaturation	95	3:00
Denaturation	95	0:30
Annealing	60	0:30
Extension	72	1:02
30 cycles		
Final extension	72	5:00
Hold	12	Infinity



The First PCR was a failed.



Observation

Sample	Main Band Observed (bp)	Expected Length (bp)
Plasmid (PQE60)	3000	3000
Reconstituted PCR Mix - eut R	≈1053	1053
Reconstituted PCR Mix - peut S	≈1500	1500
Master Mix - eut R	≈1053	1053
Master Mix - peut S	≈ 300	1500

The plasmid (PQE60) exhibits an observed main band matching its expected length of 3000 base pairs (bp), indicating intact DNA. Likewise, PCR mixes targeting the "eut R" and "peut S" genes show main

bands close to their expected lengths (≈1053 bp and ≈1500 bp, respectively), suggesting successful amplification. However, a discrepancy arises in the "peut S" gene PCR using the "Master Mix," where the observed main band (~300 bp) differs significantly from the expected size (1500 bp), indicating potential issues like incomplete or nonspecific amplification. There is nonspecific amplification at ~300 bp in every sample.

Prepare Vector

PQE60 Plasmid Isolation

- Grow E. coli DH5 Alpha strain and TG1 transformed with the plasmid of interest in 3 mL LB medium containing appropriate antibiotics in a tube After overnight (15–16 h) growth at 37°C, harvest the bacterial cells by centrifugation at top speed for 5 minutes at 5000 RPM at room temperature in a microcentrifuge.
- Resuspend the bacterial pellets completely in 1.5 mL ice-cold GTE (50mM glucose, 25mM Tris-Hcl, and 10mM EDTA of pH 8) by putting a toothpick in the tube and then vortex mixing for several seconds.
- Remove the toothpick and add 2.5 mL of freshly prepared lysis buffer (0.2 M NaOH, 1% sodium dodecyl sulfate [SDS]); mix quickly by inverting and swirling the tube several times until the contents become clear. Do not vortex.
- Precipitate cellular debris by adding 3 mL ice-cold 4 M potassium acetate solution (prepared by mixing 80 mL 5 M potassium acetate, 11.5 µL glacial acetic acid, and 8.5 mL H2O). Mix the contents thoroughly by immediately swirling the tube. Do not vortex mix.
- Centrifuge at top speed for 10 min and 10,000 RPM at room temperature and transfer the supernatant into a clean tube with a disposable pipet. Avoid collecting a floating film of denatured material by submerging the pipet tip well into the solution before suction.
- Add 4.8 mL isopropanol and invert tubes to mix well. Centrifuge at top speed for 10 min, 10,000 RPM and 4°C. Pour out the supernatant and place each tube back in the rotor in the original orientation. Quickly centrifuge the tubes at maximum speed for several seconds. Aspirate all residual supernatant from the tubes carefully.
- Rinse the pellet with 1 mL ice-cold 70% ethanol, resuspending and centrifuging for 1 min to remove PEG. Discard all the residual supernatant.
- Air-dry and dissolve the plasmid DNA in 100 mL TE.

Step	Material	Purpose
1. Alkaline Denaturation	-	It breaks hydrogen bonds in DNA, denaturing the double helix.

2. Cell Growth and Harvesting	-	Grows colonies overnight in liquid broth; cells harvested by centrifugation.
3. Resuspension	Glucose, Tris, EDTA, Lysozyme	Maintains osmolarity, and pH, prevents nuclease activity, and may digest bacterial cell walls.
4. Lysis using Lysis Buffer	NaOH, SDS	Breaks cell membrane and denatures DNA.
5. NeutRalization	Potassium/Sodium Acetate	NeutRalizes the solution, allowing plasmids to renature while larger DNA remains denatured.
6. Ethanol Precipitation	Chilled Ethanol	Precipitates plasmid DNA, which can then be collected through centrifugation.
7. Resuspension	Water/TE Buffer	Plasmid DNA is resuspended in a suitable buffer for storage or further use.