# Foundations of Synthetic Biology Laboratory Report

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## Experiment 1 - CRISPR genome editing of BFP to GFP in the yeast genome

1) Our original designs:

| | |
|---|---|
| **gRNA** | TTTGGTCTCACGCACTAGTAACAACTTTATCTCAGTTTTAGAGCTAGAAATAGCAAGTTA |
| **Forward Primer** | GGGTAAATTACCTGTTCCTTGGCCAACCCTAGTAACAACCTTGACTTATGGTGTTCAATG |
| **Reverse Primer** | GCCTTTTCATATGGTCTGGGTATCTTGAGAAACATTGAACACCATAAGTCAAGGTTGTTA |

2) The provided designs:

| | |
|---|---|
| **gRNA for BFP FWD** | TTTGGTCTCACGCACTAGTAACAACTTTATCTCAGTTTTAGAGCTAGAAATAGCAAGTTA |
| **BFP-GFP swap FWD** | ACTACGGGTAAATTACCTGTTCCTTGGCCAACCCTAGTAACAACTTTGACATATGGTGTT |
| **BFP-GFP swap REV** | CATGCCTTTTCATATGGTCTGGGTATCTTGAGAAACATTGAACACCATATGTCAAAGTTG |

a. The designs for the gRNA are the same, as the 20 bases needed were inserted into the template provided, so would both guide the Cas9 protein to the BFP target site for DNA cleavage. However, for the other two, the designs are quite similar in bases and size of overlap but differ slightly like a 'frameshift' (an additional codon downstream). While making our original design, the steps demonstrated in Class 1 were followed but we decided to increase the editing region by changing an additional base in the adjacent codon upstream to ensure that the CRISPR would not recognise the region once it had been repaired. We also wanted to keep the 28 base overlap between the forward and reverse primer as we thought it was an optimal amount. Overall, our design had a difference of 12 bases (highlighted in purple) compared to the one provided to us.

b. The differences are unlikely to result in a significantly different outcome as the size of the base overlap between the two primers are the same and as most of the bases were the same, except the differences that ensure the same amino acid is used (e.g. Threonine) during translation but this would not cause much difference as they are silent changes. However, it might have either increased or decreased the chance of CRISPR working, depending on G/C or A/T content due to hydrogen bonding. Factors like binding efficiency and specificity would be altered, resulting in a potentially higher or lower chance of the yeast not surviving. Overall, I think that there would not be much of a difference in the results, assuming that the experiment went well.

3) Results obtained from Lab 2:

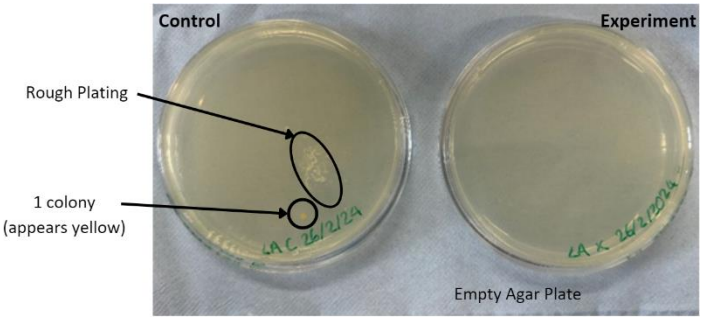


FIGURE 1: CONTROL AND EXPERIMENT PLATES 1

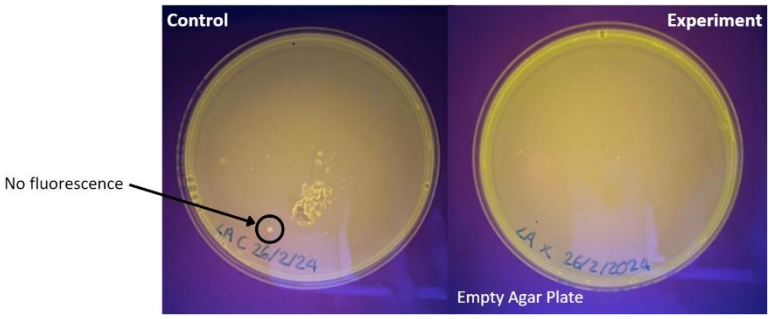


FIGURE 2: PLATES 1 UNDER BLUE LIGHT

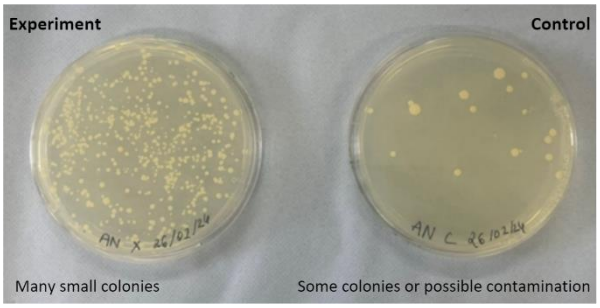


FIGURE 3: EXPERIMENT AND CONTROL PLATES 2

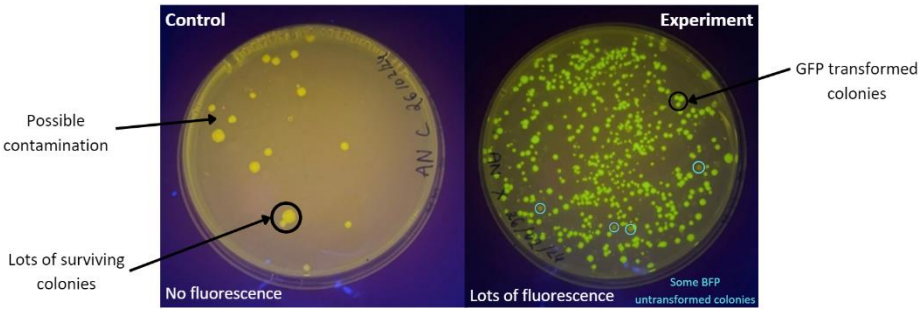


FIGURE 4: PLATES 2 UNDER BLUE LIGHT

4)

TABLE 1: SUMMARY OF RESULTS

| Case | Control | | Experiment | |
|---|---|---|---|---|
| | Colonies | Green | Colonies | Green |
| **1** | 1 | No | 17 | No |
| **2** | No | No | Yes, a large amount | Yes |

5) Looking at the results we obtained, we can see that they are very different. In Case 1, as seen in Figures 1 and 2, there were little to no colonies grown on both plates, not even contamination. This means that the CRISPR/Cas9 did not efficiently edit the GFP gene into the yeast genome. It could be due to an insufficient amount of donor DNA being present in the sample or low translation efficiency. On the other hand, it is most likely due to human error: potentially, the competent yeast cells were not added, the solution was not mixed well before plating or inaccurate pipetting.

Looking at Case 2 (right side of Figure 3), there are a small amount of yeast colonies on the control plate, which suggests there's incomplete editing. Ideally, there should be no colonies because the CRISPR/Cas9 should be cutting the DNA sequence and distorting its function and as a result, the yeast should not survive. Or this could be due to contamination via human error and plating methods. On the right side of Figure 4, we can clearly see that there is a lot of green fluorescence present, but some yeast colonies with the BFP gene remain (as they do not fluoresce, circled in blue), which suggests that there was also incomplete or inefficient editing of the BFP gene to GFP. A potential reason is that the donor DNA was not fully integrated into the sequence. The large amount of yeast colonies with GFP suggests that the experiment worked well as when the exposed to blue light, it fluoresced green and when the control was exposed, no fluorescence was present.

## Experiment 2 - Golden Gate Assembly of defined and combinatorial metabolic pathways

For this experiment, two types of designs for DNA assembly reactions were designed, where two are defined and two are combinatorial. The promoter strengths we used are found in the table below:

| | | Defined | | Combinatorial | |
|---|---|---|---|---|---|
| **Promoter** | Gene | D Tube 1 | D Tube 2 | C Tube 1 | C Tube 2 |
| **W, M, S or X** | CrtE | M | M | M | X |
| **W, M, S or X** | CrtI | M | S | M | X |
| **W, M, S or X** | CrtYB | M | M | X | X |

Where W is weak, M is medium and S is strong.

1) For the defined designs, these promoter strengths were chosen as we wanted D Tube 1 (MMM) to be considered as a control tube, while D Tube 2 (MSM) was used to observe if there was an increase in gene expression. Our main aim was to increase the expression of Lycopene, as we expected that the yeast would appear more red. As a result, this was decided so that the two results could be compared for our hypothesis to be determined.

2) For the combinatorial designs, in C Tube 1 (MMX), we wanted to observe the effect of a change in the CrtYB promoter strength as it can be found at two points in the carotene production pathway and to see if the pathway that was taken could be predicted. To elaborate, if it is weak, we would expect no colour or red colonies, as it will be converted into ergosterol or lycopene only. If expression is medium, then we would expect orange colonies. If expression is strong, we would expect the majority to be (dark) orange colonies. In C Tube 2 (XXX), we wanted to see all the possible combinations ($3^3 = 27$) or see if one is favoured more than another, based on the majority of colours expressed in the colonies and to see the variety of different levels of colour strength.

3) Results obtained from Lab 3:

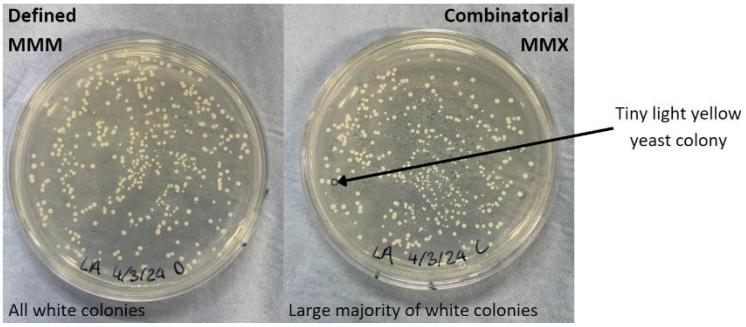


FIGURE 5: DEFINED AND COMBINATORIAL PLATES 1

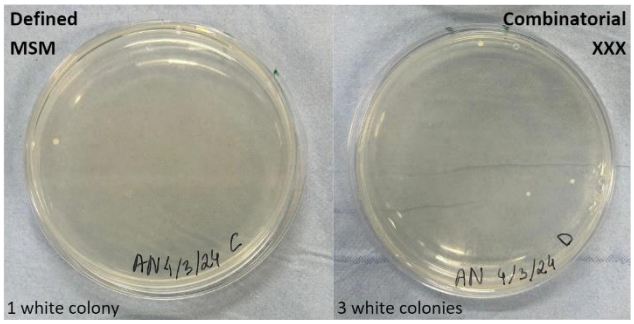


FIGURE 6: DEFINED AND COMBINATORIAL PLATES 2

Results obtained by peers from Lab 3:

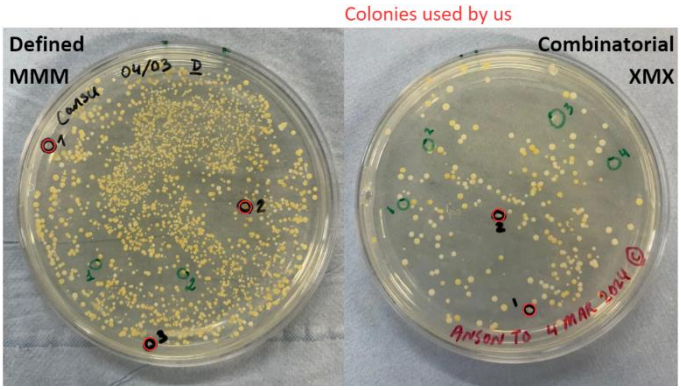


FIGURE 7: PEERS PLATE RESULTS

4) For Case 2 (Figure 6), the fact that there is a very little amount of colonies present would suggest that after the DNA parts were included into the mixture and incubated in the thermocycler, the DNA parts did not form at all into the desired multigene plasmid. Resulting in inefficient Golden Gate Assembly and the yeast not surviving. This could be due to human error, technique issues or the use of the incorrect promoters. Regarding Case 1 (Figure 5), there were many colonies present but a large lack of colour, they were all white. This would also suggest that there was inefficient Golden Gate Assembly, where the cassettes did form into a plasmid but did not express what we expected. It is also possible that the promoters did not bind well to the plasmid as no additional colour was expressed, or the assembled constructs were unstable.

5) In Lab 3, we decided to streak out the two colours we obtained (white and yellow) and we were able to find peers who used the same or a very similar combination of cassettes as us (refer to Figure 7). This would allow us to observe the colours that we should have obtained and compare them with what we have. When selecting which colonies to streak out, we selected ones that were quite isolated with no other colonies nearby as this could affect what colour would appear.

6) Results obtained from Lab 4:

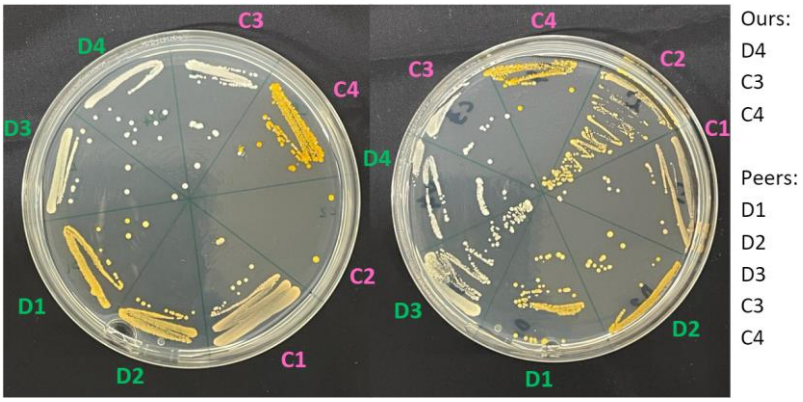


**FIGURE 8: STREAKED-OUT COLONY AGAR PLATES**

7) Colonies of orange and yellow were expected from both the designed and combinatorial plates but were not obtained from this experiment. The lack of colour and colonies implies that there were experimental errors when carrying this out. On the right side of Figure 5, we did obtain a very small yellow colony, this could be because the plasmid was able to form for this singular yeast cell and therefore it was able to survive then it grew. Errors could also arise due to our original use of the incorrect type of tubing (microfuge tubing instead of PCR tubing) so we had to extract and transfer the contents and as a result, we may have lost some material in the final product. As a result, the experiment did not turn out well or as predicted.

Regarding the defined side of the colour wheel (in green), D4 is the colour we obtained from our plate which is not what is expected, considering all promoters were medium. Referring to D1, D2 and D3 (obtained from peers), we would expect these colours as the pathway would go directly to β-carotene with no change in the colour strength. We would expect a medium orange colour, but some lighter shades of orange (D3) appeared meaning that there could have been impurities, or the cassettes did not bind well to form the plasmid, but it also didn't result in killing the yeast. For these peers, it was successful as they obtained the colours expected.

Regarding the combinatorial side of the colour wheel (in pink), C3 and C4 refer to the white and yellow colonies we obtained, respectively. If we refer to C4, (the colour turned out to be darker compared to at first glance) this is a colour we would have expected with our combinatorial design choice of MMX. By looking at how dark the orange is, this outcome means we can predict that the CrtYB promoter was of a strong strength by also looking at the metabolic pathway as β-carotene (orange colour) is the final product. Looking at the right side of Figure 7, we can see that there was a large variety of different colours and shades: white, orange and yellow. Now referring to C1 and C2, we would have expected similar results for the XXX combination as the strength of the promoter for CrtYB would affect the darkness of the colour yellow/orange and would cause many different colours expressed.

## Experiment 3 - Promoter modification of the carotenoid biosynthesis pathway

1) The design choices:

| | |
|---|---|
| **gRNA (G7)** | TTTGGTCTCACGCAAGAATTAATAAAAAGATCTAGTTTTAGAGCTAGAAATAGCAAGTTA |
| **Forward Donor DNA** | AGAGTTACTTCAGAAATAACAAAAAAATCGATCAAGAATTGATCAAAAGGAGTATGGGAA |
| **Reverse Donor DNA** | GATAGCTGTGGGTTTATCCTGATCTTGTTCTTTTCCCATACTCCTTTTGATCAATTCTTG |

2) These designs were chosen as we wanted to increase gene expression which would result in darker/more colour expression. Based on papers that we had read, some studies investigated how expression is affected depending on its similarities compared to the Kozak consensus sequence in yeast. For example, "the efficiency of protein synthesis is highly influenced by a purine at position -3 and/or an adenine at position -1". For the sequence given, we made appropriate changes (highlighted in purple) to the Kozak sequence (underlined in blue), hoping it would increase expression. We also wanted to increase and use a more optimal amount of guanine and cytosine bases (30%) to increase binding as this can lead to enhanced transcription, without adding too many that it will affect the annealing and melting temperature and make it too high due to the triple hydrogen bonding property that it has.

3) For this experiment, we used the first case of Donor DNA

| Case | DNA concentration ($ng/\mu l$) | 260/280 ratio |
|---|---|---|
| **1** | 121.45 | 1.874 |
| **2** | 123.10 | 1.879 |

4) Using the calculator provided and inputting the 28 bases overlap between our two donor DNAs in the 3' to 5' direction, as well as the primer concentrations provided on the specification sheet, the predicted annealing temperature is 53.2℃.

5) The calculated temperature for the PCA reaction was found to be 3.2℃ higher than the cooling temperature used in the thermocycler. Usually in PCA reactions, the annealing temperature used is set slightly below the actual melting temperature of the primers. This is to promote the complementary binding of the forward and reverse primers which form stable base pairs within the overlapping region. Consequently, this enhances the specificity of the primer binding to the target sequences and therefore minimise non-specific binding sites. As a result, these optimised conditions will maximise the amplification efficiency, specificity, and the yield of the desired products.

   Regarding the success of good quality DNA production, the annealing temperature of 50℃ allowed for a successful PCA reaction. At this temperature, the two primers were able to bind well and effectively at the overlapping region, resulting in a successful insertion of the designed promoter sequence into the DNA construct.

6)

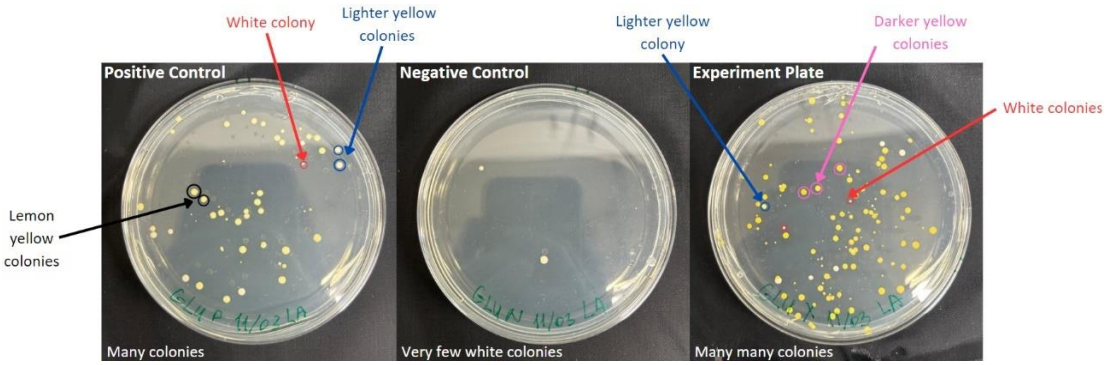


FIGURE 9: YEAST COLONIES OBTAINED

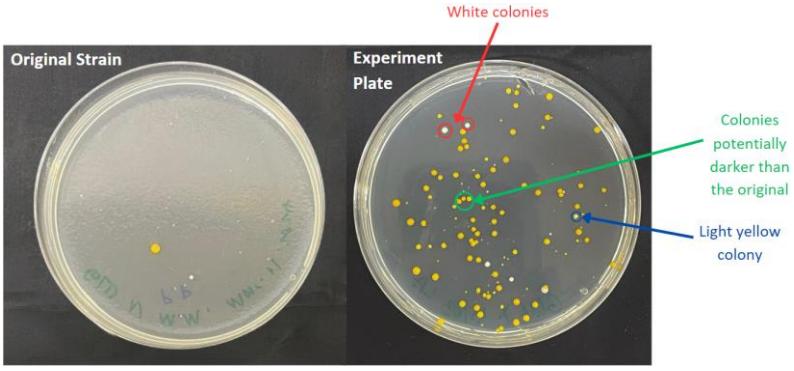


FIGURE 10: ORIGINAL VS EXPERIMENT YEAST COLONIES

7) Our design aimed to increase gene expression by modifying the Kozak sequence of KL-PGK1p in CrtI. By optimising the translation efficiency of the gene via increasing the binding strength between the two primers, resulting in a boost in transcription. This would result in an elevated amount of mRNA produced and therefore higher protein/pigment expression. The objective was to achieve a difference in colour expression, ideally, darker or stronger.

In this experiment, the observed results closely match the expected results for both the positive and negative control plates. The positive control plate (refer to left side of Figure 9) showed an expected majority of lemon-yellow coloured yeast colonies, which represents the success of the CRISPR/Cas9 process with the provided gRNA and donor DNAs (a promoter edit). Additionally, the presence of some white colonies reflects the result of non-specific Crt gene deletions. For the negative control plate (refer to middle of Figure 9), we expected very few colonies due to the cleavage of the donor DNA so the yeast wouldn't survive. Regarding our experimental plate (refer to right side of Figure 9), we did obtain lots more colonies varying in shades of yellow/orange, which implies that the CRISPR/Cas9 process was successful. The reasons for the white colonies present are the same as explained above.

To gain a more detailed view for colour comparison, the lids of the agar plates were taken off and the pictures of the plates of the original strain and our experimental plates were placed side by side (Figure 10). Looking at some of the colours of the colonies, it is quite difficult to see but some colonies are potentially darker than the original (circled in green). This suggests that the promoter edit was also quite successful as more Lycopene was expressed. However, we can also see that some colonies are lighter yellow or white (circled in blue and red), which means that the edit was not effective. Overall, this experiment went well as we obtained the expected results.

8) If more time in the lab was provided, other procedures could have been carried out or the steps of the experiment could be reordered. For example, regarding the purification process, the solution could be centrifuged more times to isolate the DNA from the water more effectively to then obtain a higher concentrated DNA solution. This in turn would increase the efficiency of the CRISPR/Cas9 reaction and therefore more effective editing. It could also improve the specificity so that the DNA would be able to be integrated into the target area better. As a result, we would possibly end up with a large majority of yellow and orange colonies with little to no white colonies. Additionally, regarding the Nanodrop spectrophotometer, this device could have been used initially for us to determine the concentrations, 260/280 ratio and the position of the ethanol peak for the two DNA samples before they were used so that we could use the "better", more effective one for the experiment.

## Experiment 4 - Yeast BioArt Picture

1)

FIGURE 11: YEAST BIOART

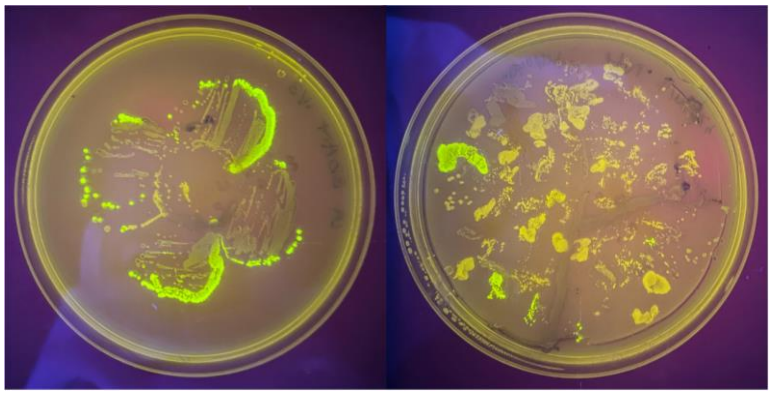

FIGURE 12: YEAST BIOART UNDER BLUE LIGHT

2) In the figures above, on the left side is an attempt at a drawing of a flower. On the right side is an attempt of a tree. Although we can see the overall idea of what they are showing, they both lack detail and do not look very clear. Nevertheless, they were relatively successful as when they were put under blue light, the yeast with the GPF gene fluoresced. Also, certain areas where colouring in between lines were attempted appeared to work well on some parts but could be improved by streaking more of the yeast. To improve, more colours could be used to showcase the yeast's ability to possess a large range of colours by editing a gene.

## Final Question - Optimising the same pathway in *E. coli* using the RBS calculator

1) The sequences identified are:

| Enzyme | RBS and CDS |
|---|---|
| **CrtE** | AGCGGATAACAAGATACTGAGCACATACTAGAGGAGGTACTAGATGACGGTCTGCGCAAAAA AACACGTTCATCTCACTCGCGATGCTGCGGAGCAGTTA |
| **CrtB** | GGTTTGACAAAAAACTCGCTGCCGTCAGTTAATAATACTAGAGCTCAAGGAGGTACTAGATGA ATAATCCGTCGTTACTCAATCATGCGGTCGAAACGAT |
| **CrtI** | CCTCCCCGCCCTGCGCATCTCTGGCAGCGCCCGCTCTAATAATACTAGAGCTCAAGGAGGTACT AGATGAAACCAACTACGGTAATTGGTGCAGGCTTCG |
| **CrtY** | TCGGCAAAAGCGACAGCAGGTTTGATGCTGGAGGATCTGATATAATAATACTAGAGGAGGTA CTAGATGCAACCGCATTATGATCTGATTCTCGTGGGGG |

2) The predicted translation rates and the change in Gibbs free energy identified:

| Enzyme | Translation rate | $\Delta G_{total}$ |
|---|---|---|
| **CrtE** | 31230.37 | -7.18 |
| **CrtB** | 21787.90 | -6.38 |
| **CrtI** | 1545.10 | -0.50 |
| **CrtY** | 4084.45 | -2.66 |

3) The aim is to redesign the CrtI RBS sequence in E. coli to improve the beta carotene biosynthesis pathway. Firstly, this is because it has the lowest change in Gibbs Free energy and translation rate (relative to the others), and assuming that a translation rate of 1000 is medium strength, obtaining a value of 1545.10 suggests that it is not as strong as the others. Additionally, since we're assuming that "the pathway and cell metabolism behave similarly in E. coli as it does in yeast" because it is a critical step, it catalyses the conversion of phytoene to lycopene. Improving this expression could increase the flux through this pathway, and therefore lead to a higher production of lycopene and therefore a redder expression. To redesign the RBS sequence for CrtI, we would have to look at the sequence and try to optimize it by matching it to the Shine-Dalgarno consensus sequence for E. coli.

4) The entire CrtI sequence was used in the RBS calculator (control translation rates). After following the steps and inputting a desired translation rate of 100,000, the results obtained are:

| New RBS sequence | Predicted Translation Rate |
|---|---|
| TCTATAAGGAATATACAAAAACACCATAAGGAGGTATTTTT | 100730.94 |

5) Based on the assumptions that we made, there may be similarities amongst yeast and E. coli but yeast is still classed as a eukaryote whereas E. coli is a prokaryote. This means that there would be a difference in metabolisms and actual/realistic translation rates, as well as their consensus sequences. Additionally, in E. coli, it consists of four Crt enzymes but yeast consists of only three, which overall could affect the pathway, if we assume that it is the same in both species. Furthermore, realistically there could be other factors involved that would affect the pathways, for example, enzyme kinetics, pathway regulation and metabolism. As a result, the impact of modifying the CrtI expression in E. coli may not completely reflect the effects observed in yeast.

## References

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