

Synthetic Biology Lab Report

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1 CRISPR Gene Editing of BFP to GFP in the Yeast Genome

1.1 Our Guide and Donor DNA Designs

Name	Sequence
gRNA for BFP FWD	TTTGGTCTCACGCA CATGAGATAAAGTTGTACT GTTTAGAGCTAGAAA TAGCAAGTTA
BFP-GFP swap FWD	TACGGGTAAATTACCTGTTCCCTGGCCAACCCTAGTAACAACGTT GACTT ATGGTGTCA
BFP-GFP swap REV	TTTCATATGGTCTGGGTATCTTGAGAACATTGAACACC ATAAGTC AAC GTTGTTACTA

Table 1: Designed gRNA sequences along with BFP-GFP donor DNA forward and reverse primers by me (Harsh) and my lab partner (Felix). In the gRNA, the textcolor **blue** indicates the variable detection sequence. In the donor DNAs, the textcolor **blue** indicates the original codons for BFP (S and H) whereas **green** indicates the BFP → GFP edit.

1.2 Comments on our CRISPR Construct Design

1.2.1 Differences in the design

- We assumed that we couldn't use the PAM site immediately next to the edit site and thus used a PAM site (5'-AGG-3') on the complementary strand 13 bases downstream of the edit site. Our assumption was wrong and in retrospect, we should have chosen the PAM site we identified originally that was close to the edit site.
- Our donor DNA forward primer was almost identical to the one provided in the lab but using different two different Threonine codons ACT instead of ACA (for the Serine to Threonine switch) and ACG rather than ACT (for another Threonine 3 bases upstream of the edit site). More over, our forward primer began two bases later than the one provided in the lab and thus spanned an extra two bases at the end.

1.2.2 Differences in Outcome if we used our gRNA and donor DNA sequences

- Due to our PAM site being further away from the edit site (as compared to the one provided in the lab), Cas9 cleavage efficiency would decrease, potentially lowering the number of fluorescent colonies or preventing editing entirely.

- While there is difference in relative abundances of the different codons in both designs, I believe the difference in outcome might not be as significant as efficiency decrease due to wrong choice of PAM site placement would dominate the edit.

1.3 Annotated Photo of the Agar Plates

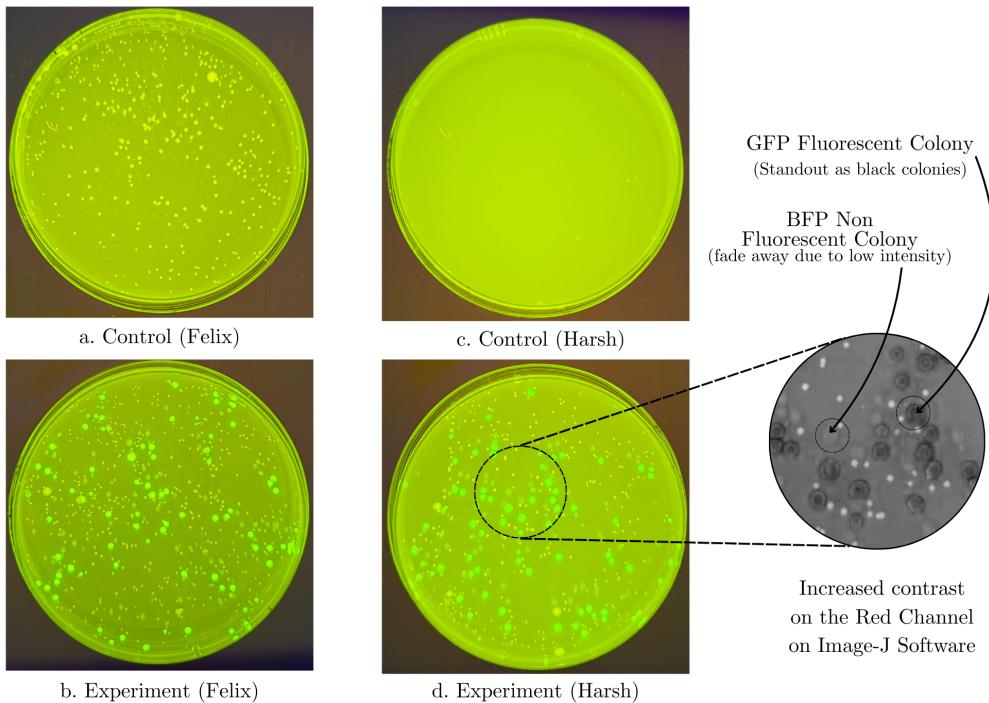


Figure 1: Annotated photos of the Agar Plates for me (Harsh) and my partner (Felix). The image was converted to the red-channel in ImageJ with high contrast to highlight the green fluorescent colonies from non-fluorescent colonies as labeled in the image above.

1.4 Table of the Results

Colony Type	Felix C	Felix X	Harsh C	Harsh X
Non-Fluorescent	51	23	0	71
Fluorescent	0	69	0	71
Total	51	92	0	142

Table 2: Counts for fluorescent and non-fluorescent colonies on each plate with assistance from IMAGEJ software.

1.5 Discussion of the Results

**Note: I have Deutanopia (red-green colorblindness), due to which I couldn't visually identifying any fluorescent colonies. For the same reason, I chose to convert the image to the red-channel in ImageJ with high contrast to highlight the green fluorescent colonies from non-fluorescent colonies (it seems counterintuitive as I should have used a green channel but the red channel had better contrast). The colonies mentioned on the table are tallied from my lab*

partner for correctness. This has also affected my ability to properly identify colors in the later experiments and thus there my errors in my judgement.

The results from this transformation experiment, where CRISPR/Cas9 was used to transform yeast BFP to GFP, show distinct outcomes for my plates and my lab partner's. My Control (C) plate had 0 colonies, indicating a failure in uptake of the CRISPR construct or poor growth competency of my control cells. Since an identical CRISPR construct was used for both, this error should primarily be due to procedural / pipetting error.

Felix's control (C) plate had 51 non-fluorescent colonies, indicating successful CRISPR construct uptake and repair via non-homologous end joining by the yeast cells due to the absence of donor DNA. This clearly shows that CRISPR construct uptake allowed growth on uracil-deficient media, but the BFP-to-GFP editing did not occur as expected.

My experimental (X) plate had 142 colonies (71 fluorescent, 71 non-fluorescent), indicating $\approx 50\%$ success rate in the editing of BFP to GFP. This indicates that half of the cell colonies underwent non-homology end joining to repair the cut DNA whereas the other half included the CRISPR edit via homology directed repair. Felix's experimental (X) plate had 92 colonies (69 fluorescent, 23 non-fluorescent), reflecting a 75% editing success rate due to efficient homologous recombination. While the number of colonies on Felix's plate is less than mine, the success rate is higher.

Moreover, the colonies on Felix's Experimental (X) plate was almost double the amount in the Control (C) plate indicating that the control cells might have continually underwent DNA cleavage, and died therefore, due the presence of the CRISPR construct but not the donor DNA.

Since all the experimental setup was identical along with the reagents used, the difference in colony and efficiency between mine and Felix's cells can most likely be attributed to procedural differences.

2 Golden Gate Assembly of the Defined and Combinatorial Metabolic Pathways

2.1 Reasoning behind the Defined (D) Design choices

Gene	Harsh Strength Choice (Promoter)	Felix Strength Choice (Promoter)
Crt-E	Strong (pHTB2)	Weak (pRAD27)
Crt-I	Strong (pTEF2)	Medium (pALD6)
Crt-YB	Strong (pHHF2)	Strong (pHHF2)

Table 3: Genes and their promoters used (indicating strength) in the defined (D) design.

Here are the reasons for the choice of promoters by me (Harsh):

- The primary reason was to maximize metabolic flux through the defined pathway. Selecting all strong promoters ensures a high initial flux of geranylgeranyl diphosphate (via CrtE), rapid conversion of phytoene to lycopene (via CrtI), and efficient cyclization to beta-carotene (via CrtYB). This would test the hypothesis that transcription is the rate-limiting step in beta-carotene accumulation. If successful, this would lead to a high accumulation of orange-yellow colonies.
- Another reason was to enquire which gene was the rate-limiting step in the pathway. For example, if CrtYB is overexpressed but beta-carotene levels remain low (e.g., red colonies indicating lycopene buildup), it could indicate insufficient activity of CrtI or

CrtE, despite their strong promoters. This mirrors strategies in metabolic engineering where over-expression highlighted bottlenecks for optimization.

- The final reason was to test the metabolic stress on the yeast cells. Small or sparse colonies might be indicative of this.

Felix's choice of the promoters in increasing order of strength was aimed to limit early pathway expression, preventing competition with native pathways like ergosterol biosynthesis for precursors like FPP, reducing metabolic burden. Compared to a direct design with all strong promoters, this weak-medium-strong gradient tests whether escalating expression toward the end improves balance and yield by mitigating bottlenecks, offering a more nuanced approach to optimization.

2.2 Reasoning behind the Combinatorial (C) Design choices

Gene	Harsh Strength (Promoter)	Felix Strength (Promoter)
Crt-E	Strong (pHTB2)	Mixed (pPAB1, pHTB2, pRAD27)
Crt-I	Mixed (pTEF2, pALD6, pHHF1)	Strong (pTEF2)
Crt-YB	Weak (pRET2)	Weak (pRET2)

Table 4: Genes and their promoters used (indicating strength) in the combinatorial (C) design.

My choice of a strong promoter for Crt-E (pHTB2), mixed promoters for Crt-I (pTEF2, pALD6, pHHF1), and a weak promoter for Crt-YB (pRET2) hypothesized that making flux dominant in the early part of the beta-carotene pathway would optimize overall production. This involved driving robust geranylgeranyl diphosphate (GGPP) synthesis with a strong Crt-E promoter to ensure a strong initial flux, varying Crt-I expression to fine-tune the conversion of phytoene to lycopene and prevent bottlenecks, and limiting Crt-YB activity with a weak promoter to test whether reduced downstream expression could efficiently utilize intermediates without causing accumulation (e.g., avoiding red lycopene colonies). This approach contrasts with a direct design using uniform strong promoters, aiming to explore if early-pathway dominance yields better efficiency or healthier colonies by balancing metabolic load.

Felix's selection of a medium promoter for Crt-E, a strong promoter for Crt-I, and a weak promoter for Crt-YB aimed to balance precursor availability and intermediate conversion while minimizing downstream stress. The medium Crt-E promoter was likely chosen to moderate GGPP production, reducing competition with native pathways like ergosterol synthesis and alleviating metabolic burden. A strong Crt-I promoter ensured high phytoene desaturase activity, maximizing lycopene production to prevent bottlenecks at this intermediate step. The weak Crt-YB promoter limited the final cyclization to beta-carotene, matching the moderate upstream flux and avoiding overproduction stress, potentially enhancing overall pathway efficiency.

2.3 Annotated Photos of the Results

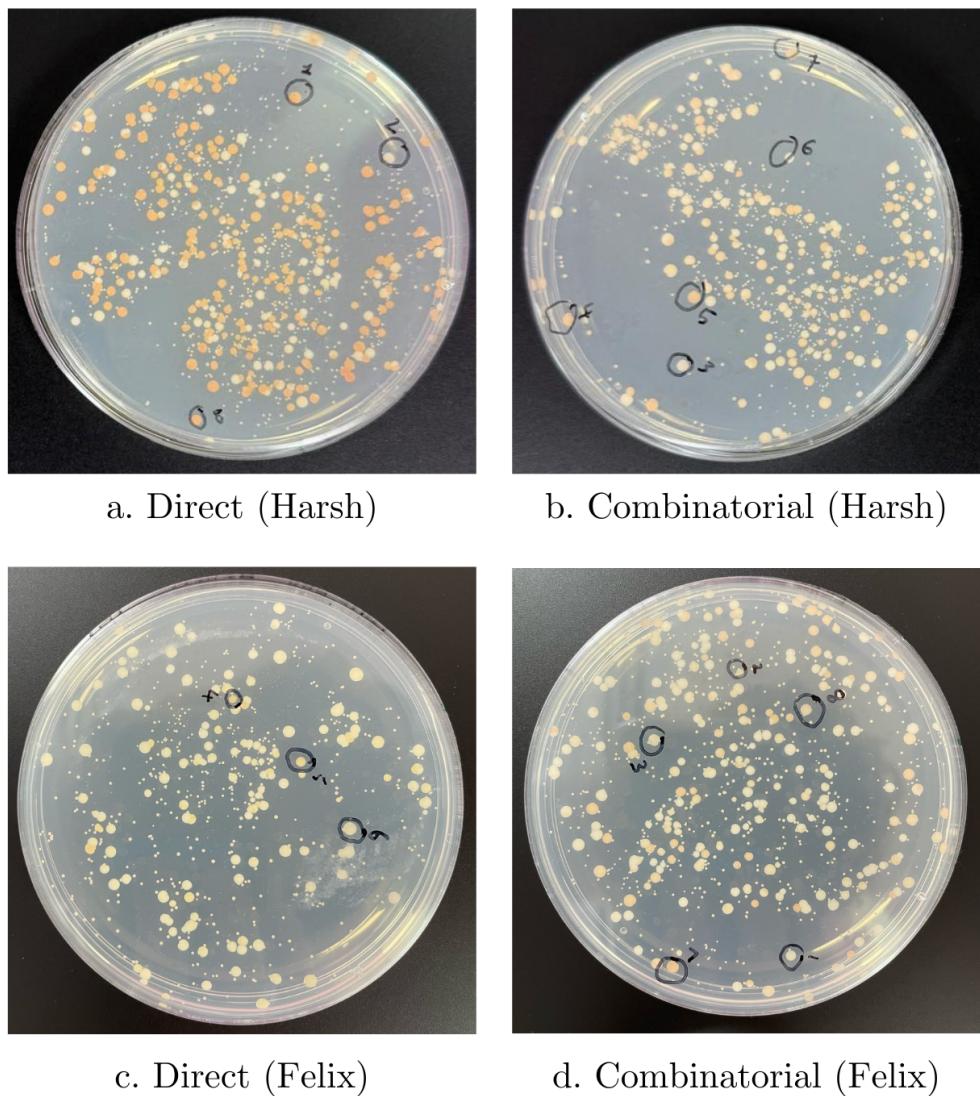


Figure 2: Annotated photos showing the results of both the direct (D) and combinatorial (C) designs. The colonies chosen for streaking are marked on each plate with an associated number.

2.4 Discussion of Results in Context of Assembly and Transformation

The number of colonies on all four plates seem be quite high. This suggests that the CRISPR transformation efficiency was quite high. This also suggests that the PAM site must have modified as expected to prevent excessive cleavage DNA (leading to death / low number of colonies as seen in agar plates of other experiments). All four plates seem to show a mix range of colors from light yellow to orange suggesting that the knockout rate was also quite high. In the Direct (D) design, most colonies are saturated Orange apart from a few colonies that are completely white in color. This suggests either that these colonies underwent non-homologous end joining or that the CRISPR construct was not able to make the edit. There also seems to be a lot of small white colonies (contaminants) that seem to have accumulated equally on all plates.

2.5 Choice of Colonies for Streaking

To validate the hypothesis outline above, we carefully chose colonies for streaking in order to maximize for color diversity from each of the colonies. Colonies 1 and 8 (chosen from Harsh's Direct (D) design) were chosen as they were the most orange in color and thus would be the best to validate whether all strong promoters would lead to the highest accumulation of beta-carotene as compared to other strategies. Colony 2 was taken as an outlier from the Direct (D) design as it was one of the few colonies that wasn't either completely saturated with orange or was white in color.

Colonies 3–7 were chosen from the Combinatorial (C) design to display the different range of colors that can be seen in the Combinatorial (C) design with mixed promoters. More colonies were chosen from the Combinatorial (C) design from both me and Felix's plate.

2.6 Annotated Photos of the Streaked Plates

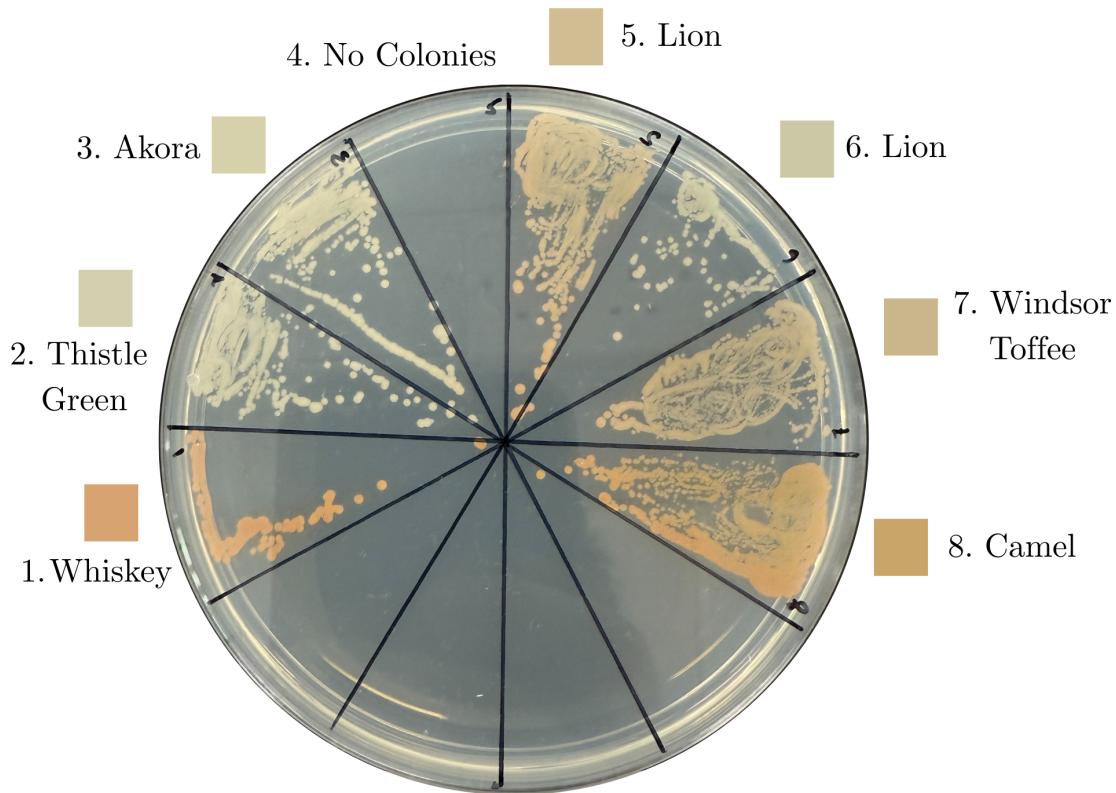


Figure 3: Annotated photos showing the results of the streaked plates.

2.7 Discussion of the Results in Context of the Design Choices

**Note: Felix's streaking plate was lost (unsure how) and so I'll primarily be biasing the analysis on my results.*

The results from the Direct (D) design (Plate a) strongly support Harsh's hypothesis that transcription might be the rate-limiting step in beta-carotene accumulation. Colonies 1 and 8, which were streaked out, suggest that upregulating all promoters (pHTB2 for Crt-E, pTEF2 for Crt-I, pHHF2 for Crt-YB) leads to a higher accumulation of beta-carotene compared to the other strategies (Combinatorial - Harsh, Direct - Felix, and Combinatorial - Felix). The colonies from the Direct (D) design were significantly more orange in color compared to all other

plates, indicating that the strong promoters drove high expression of all three genes, resulting in efficient flux through the pathway and minimal accumulation of intermediates like lycopene (which would appear red). This can be visually seen in the streaked colonies 1 and 8.

In contrast, the Combinatorial (C) design (Plate b) showed more variability in colony color, with some orange colonies but also beige and reddish ones. The reddish colonies likely indicate lycopene accumulation, which could result from the weak promoter for Crt-YB (pRET2) limiting the conversion of lycopene to beta-carotene (can be seen in the colonies 2 and 3). The mixed promoters for Crt-I (pTEF2, pALD6, pHHF1) introduced variability in phytoene desaturase activity, which may have led to inconsistent flux through the pathway. While this design successfully generated a range of expression profiles, it did not achieve the same level of beta-carotene production as the Direct design, suggesting that a balanced but lower expression profile (especially for Crt-YB) was less effective in this context.

The Combinatorial (Felix) plate (Plate d) also shows a mix of colors, but with less orange intensity than Harsh's plates, reflecting Felix's choice of a medium promoter for Crt-E (mixed: pPAB1, pHTB2, pRAD27), a strong promoter for Crt-I (pTEF2), and a weak promoter for Crt-YB (pRET2). This design similarly struggled with bottlenecks, likely due to insufficient Crt-E activity and limited Crt-YB expression.

The observation that I believe has the strongest validity is that **having all strong promoters doesn't cause a severe metabolic stress on the yeast cells**. This is evidenced by the fact that all the colonies are of a similar color and are of a similar size. This suggests that the metabolic stress is not too high and the cells are able to handle the strong promoters.

Apart from these observations, no other significant difference was observed between the four plates.

3 Promoter Modification of the Carotenoid Biosynthetic Pathway

3.1 Our Guide RNA and Donor DNA Sequences

We chose gRNA 18 with the RAP1 binding site on KL-TEF2p to edit. The Donor DNA design is shown below:

Name	Sequence
Forward Primer	GGTCTTTCTCCGCTCTCTCGAATAACAATGAA CACTCGTACACTCA TAGCCTACAC
Reverse Primer	CCTGTATAAACGCTACTCTGTTCACCTGTGTAGGCT ATGAGTGTACGAGT GTTCATGGTT

Table 5: Donor DNA sequences for the RAP1 binding site modification. The site of edit is highlighted in red.

3.2 Choice of Promoter Modification

RAP1, or Repressor Activator Protein 1, is an essential transcription factor in *Saccharomyces cerevisiae*, known for binding to upstream activating sequences (UAS) in promoters. Literature, such as González et al. [2020], highlights RAP1's role in depleting nucleosomes from its binding sites, increasing accessibility for other transcription factors and enhancing gene expression. This is particularly relevant for highly expressed genes, including those involved in metabolic pathways. For instance, Miura et al. [1998] discuss engineering yeast for carotenoid production,

emphasizing the importance of promoter strength, which aligns with modifying RAP1 binding sites to boost transcription. Recent work by Tang et al. [2020] has further elucidated the architecture of yeast promoters and the role of transcription factors like RAP1 in regulating gene expression, providing valuable insights for synthetic biology applications.

gRNA-18 was chosen because it targets a potential RAP1 binding site, offering a direct approach to enhance transcriptional activation. The edited sequence provided shows changes to make the target region more similar to the RAP1 consensus sequence, suggesting an intent to increase RAP1 binding affinity. This is supported by the experimental design, where primers were designed 40 base pairs upstream and downstream of the edited region, ensuring precise homology-directed repair (HDR) to implement the modification.

In contrast, gRNA-16 and gRNA-14 target TA-rich regions, which are likely involved in nucleosome positioning or other regulatory mechanisms but not directly in transcription factor-mediated activation. TA-rich regions, as discussed in promoter architecture studies (e.g., González et al. [2020]; Tang et al. [2020]), can affect chromatin accessibility, but their impact on transcription factor binding, especially RAP1, is less direct. Therefore, gRNA-18 was preferred for its specificity to a functionally critical region for upregulating CrtYB expression.

3.3 Concentration and Quality of Donor DNA

Measurement	Value (Harsh)	Value (Felix)
DNA Concentration (ng/ul)	303.6	402
Quality (A260/A280)	1.832	1.805

Table 6: Concentration and quality of the donor DNA for me and my lab partner.

3.4 Calculation of Annealing Temperature

The annealing temperature was calculated to be 51.8°C for the DNA sequence where both primers are complementary to each other. This was the predicted annealing temperature between both the donor DNA primers.

3.5 Comment on the the calculated annealing temperature

The calculated annealing temperature of 51.8°C for our donor DNA primers was very well-suited for the PCA reaction, which used an annealing step of 50°C. Since the calculated temperature is only slightly higher than the protocol temperature, the primers likely annealed efficiently without significant non-specific binding. This closeness helped ensure robust amplification of high-quality, double-stranded donor DNA.

Moreover, the success of my PCA reaction was supported by the Nanodrop results, which showed a high DNA concentration (303.6 ng/μl) and a good purity ratio (A260/A280 = 1.832)—indicating that the synthesized DNA was ample and clean (free from protein or phenol contamination).

3.6 Annotated Photos of the Agar Plates

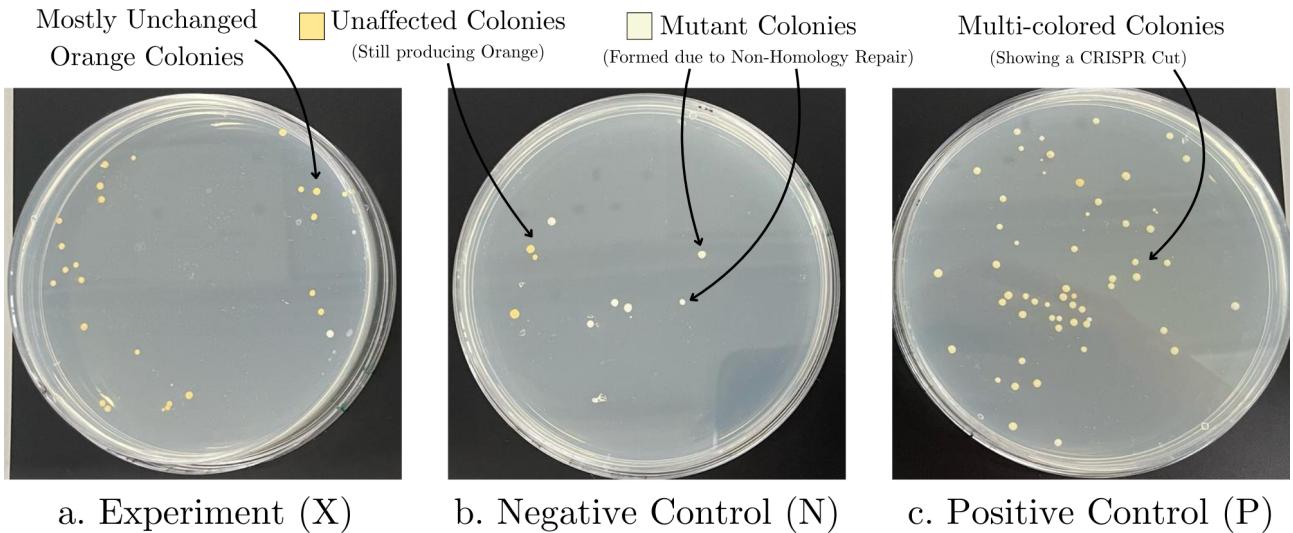


Figure 4: The figures above show three plates: Experiment (X), Control (C), and Negative (N).

3.7 Discussion of the Results

The Experimental plate (X) had our donor DNA and gRNA choice; negative control (N) had no donor DNA and our CRISPR gRNA-18 construct; positive control (C) had gRNA-9 construct with donor DNA prepared by the GTAs. The Negative Control (N) plate shows a few orange colonies (2 to 3) where as the rest of the colonies seem off white to light yellow in color. Difference from the original Orange hue suggests that the CRISPR construct was successful in making the DNA edit but due to the absence of a donor DNA, non-homologous end joining was used to repair the cut DNA which led to random mutations (INDELS) leading to off-color colonies. As the PAM site was not modified (again due to the absence of donor DNA), the DNA seemed to have been repeatedly cut again and again leading to death of many colonies. This explains the low number of colonies on the Negative Control (N) plate.

The Positive Control (C) plate has significantly more amount of colonies as compared to the other two plates. This plate has the gRNA-9 construct with donor DNA prepared by the GTAs. The colonies on this plate also display a small diversity of colors (primarily light yellow to orange). This suggests that the CRISPR transformation (with G-9) was successful in making transforming the CRT-I gene. The results, however, do not show a significant difference in the B-carotene produced as compared to the saturated orange colonies from the Negative Control (N) plate that were supposedly unaffected by the CRISPR construct.

The Experimental (X) plate also shows very few colonies with most being saturated orange in color (with the exception of a few light yellow and off-white colonies). This suggests that the CRISPR transformation was successful in making the most of DNA edits but the colonies were not able to grow as compared to the Positive Control (C) plate. This suggests that the PAM site was not modified as expected and the DNA went multiple rounds of cleavage and repair leading to death of many colonies. There also doesn't seem to be any difference in the amount of B-carotene produced as compared to the Positive Control (C) plate.

3.8 What would you have done with more time in the lab?

With more time in the lab, I would have liked to do a redundant copy of the entire experiment. In retrospect, the strategy I used to identify the colonies to be streaked was still not properly calibrated and was a bit random. With more time, I would probably carefully identify the colonies in the increasing order of their color gradient to better judge the outcomes of the earlier experiments.

4 Yeast BioArt Picture

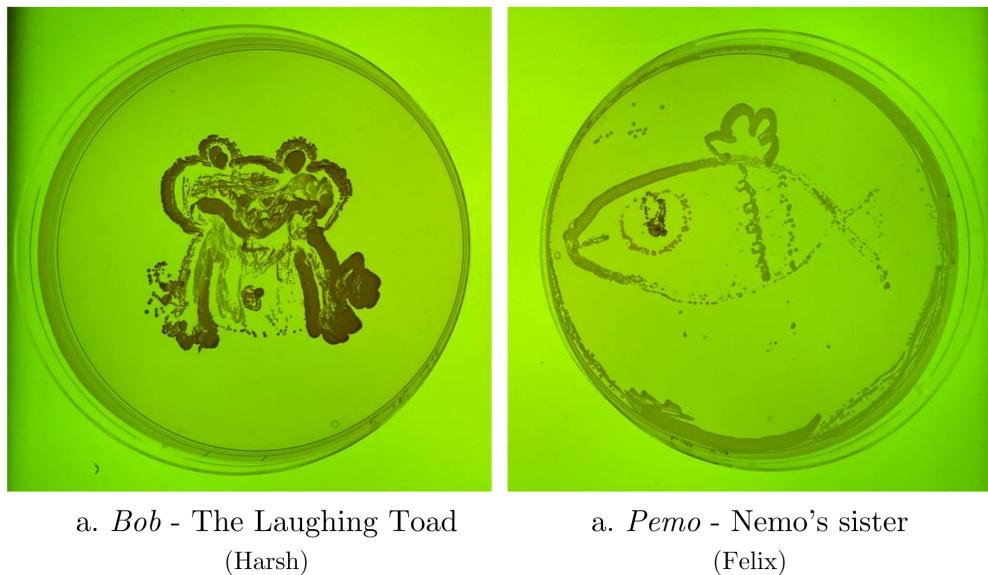


Figure 5: The figures above show our attempt in Yeast Bioart.

I was trying to make a laughing toad (which I named Bob). Brown color was chosen for the outline with green infill. The outcome resembles my initial design inspiration but didn't turn out as magnificent as I hoped. My lab partner tried to draw a fish with a 'Sharingan (A visual ability from Naruto Anime)' in its eye. While the fish's scaffold is prudent, I warned him that drawing the eye was too ambitious.

5 Optimizing the Beta-Carotene Pathway in E. Coli using RBS Calculator

5.1 RBS Sequences for the Genes

Gene	Sequence
Crt-E	ATACTAGAG GAGGTACTAG ATGACGGCTCGCGAAAAAAACACGTTCATCTCACTCGCGATGCTGCGGA
Crt-B	CAGGCCTGGTTGACAAAAACTCGCTGCCGTCAAGTTAATAACTAGAG CTCAAGGAGGTACT AGAT GAATAATCCGTCGTTACTCAATCATGCCGTGAAACGATGGCAGTTGG
Crt-I	CCTCCCCGCCCTGCGCATCTCTGGCAGCGCCCGCTCTAATAACTAGAG CTCAAGGAGGTACT AGAT GAAACCAACTACGGTAATTGGTGAGGCTTCGGTGGCCTGGCACTGGC
Crt-Y	AAAGCGACAGCAGGTTGATGCTGGAGGATCTGATATAATAACTAGAG GAGGTACTAG ATGC AACCGCATTATGATCTGATTCTCGTGGGGCTGGACTCGCGAATGG

Table 7: Table shows the RBS sequences for the genes (highlighted in blue) and their flanking sequences (≈ 50 bp each) upstream and downstream (highlighted in gray). The start codon of the CDR region is highlighted in black.

5.2 RBS Strength Calculation Results

Gene	Translation Rate	ΔG_{total}
Crt-E	2672.46	-1.72
Crt-B	21787.90	-6.38
Crt-I	676.81	1.33
Crt-Y	6069.20	-3.54

Table 8: Table shows the predicted Translation Rate and ΔG_{total} for the genes.

5.3 Rationale behind the gene to optimize

The analysis of the RBS binding strengths for the beta-carotene biosynthesis genes in table 8 shows that CRT-I, with a low translation rate of 676.81 and a positive Delta G of 1.33 kcal/mol, is a potential bottleneck in the pathway, limiting phytoene desaturation compared to the higher rates of CRT-E (2672.46), CRT-B (21787.99), and CRT-Y (6069.20). To address this, the most apt target is CRT-I, targeting a translation rate of 8000 to 10000 to enhance binding stability and align CRT-I expression with upstream (CRT-E) and downstream (CRT-Y) flux, while avoiding the excessive overexpression suggested by CRT-B.

5.4 Optimized RBS Sequence for CRT-I

Gene	Optimized Sequence	Translation Rate	ΔG_{total}
CRT-I	CAAGAGAAATCACATAGGGATCATTAA	8578.85	-4.31

Table 9: Table shows the original and optimized RBS sequences for CRT-I.

5.5 Discussion of the Results

Optimizing the CRT-I RBS to increase its translation rate from 676.81 to 8578.85 would likely alleviate the bottleneck in the beta-carotene biosynthesis pathway by enhancing phytoene desaturase production, ensuring better conversion of phytoene to lycopene and supporting downstream beta-carotene synthesis. The potential downside, however, might be that the predicted translation rate of the new RBS sequence might be too high, potentially causing lycopene accumulation if downstream CRT-YB cannot keep up, risking intermediate toxicity or metabolic burden, as seen with smaller colonies. However, this is difficult to comment out without experimental validation.

References

- A. González, M. N. Hall, S. C. Lin, and J. S. Hardwick. Rap1p and other transcriptional regulators can function in defining distinct domains of gene expression. *Nucleic Acids Research*, 48(22):12534–12555, 2020. doi: 10.1093/nar/gkaa1129.
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