

## CRISPR in Yeast

**Introduction**

In this part of the CRISPR project, the main goal was to transform yeast cells using gRNA + HDR combinations to allow CRISPR editing. The way it works is using a gRNA to guide Cas9 to make a double stranded cut at the gRNA binding site. Then, the damaged DNA can repair itself either through non homologous end joining (NHEJ), in which damaged strands ligate to each other directly, or homologous repair (HR), in which damaged strands use a homologous strand as a template to repair (Addgene). Both ways can destroy certain unwanted genes or replaced them by a premature stop codon. In this experiment, we used pCas plasmids containing gRNA1 so that the Cas9 produced can make the cut. We want the damaged plasmid to repair itself via HR, so homology directed repair (HDR) templates were designed to be the homologous strands. The HDR1 template contained mutations at different sites, including one that would introduce a TAA stop codon and destroy the PAM sequence (LM 70). The HDR4 template contained a single nucleotide deletion at the PAM sequence so that PAM would be destroyed and a frame shift would create a stop codon.

Different plasmid-HDR combinations were used to transform yeast, as shown in Table 1. pCas-gRNA1 was mixed with HDR1, 4, or nothing. pML104 empty was mixed with either HDR1 or 4. There are also negative controls, including pCas empty with nothing and water with nothing. After transformation, red colonies were grown in liquid cultures and had the genomic DNA extracted and amplified using PCR. The sequences would be analyzed by comparing them with expected sequences to locate mutations and observe any NHEJ and unexpected edits. For the whole experiment, I hypothesized that HDR frequency would be higher than NHEJ frequency and that mutations closer to the double stranded break would be easier to be incorporated via HR.

**Methods**

- **Transforming yeast (pg. 95-96, Protocol 14)**

Transformation was planned using different plasmids and HDR templates, as shown in Table 1. The combinations were added to competent yeast cells, and warm EZ 3 buffer was added to decrease cell integrity to allow plasmids to go through. After a first incubation, YPD was added as nutrients. After a second incubation, the reactions were plated and incubated.

- **Setting up liquid cultures (pg. 96, Protocol 14)**

After transformation, colony phenotypes were analyzed. Three red colonies were picked up and transferred into liquid YPD cultures. G418 was added to cultures containing pCas-transformed yeast, and URA<sup>-</sup> was added to those containing pML-transformed yeast.

- **Yeast gRNA extraction and setting up PCR (pg. 98-99, Protocols 15-16)**

After yeast growth, three cultures were centrifuged, and NaOH was added to complete lysis. After incubation in heat block, PCR was set up using GoTaq, forward and reverse primers, water, and the three extracted DNA. A negative control with only GoTaq, forward and reverse primers and water was also set up. All the reactions were put into the thermocycler.

- **Column clean-up of PCR product (pg. 75-76, Protocol 8)**

The PCR products were cleaned using columns, binding buffer, wash buffer, and warm elution buffer. Cleaned products was collected for Nanodrop and future use.

- **Sending for sequencing (pg. 89, Protocol 12)**

For each cleaned product, a 10 µl aliquot of 20 ng/µl was prepared and sent for sequencing.

- **Analyzing sequencing results (pg. 100, Protocol 17)**

Sequences were compared with expected sequences regarding mutations at edited regions, regions upstream and downstream from the edited regions, and any unexpected edits.

## Results

Table 1. Yeast Transformation Plan and Results

|                         | #1            | #2            | #3            | #4         | #5                              | #6                              | #7                           |
|-------------------------|---------------|---------------|---------------|------------|---------------------------------|---------------------------------|------------------------------|
| Plasmid                 | pCas<br>gRNA1 | pCas<br>gRNA1 | pCas<br>gRNA1 | pCas empty | pML104<br>empty                 | pML104<br>empty                 | water                        |
| HDR                     | HDR1          | HDR4          | None          | None       | HDR1                            | HDR4                            | None                         |
| # of YPD<br>plates      | 3xG418        | 3xG418        | 3xG418        | 3xG418     | 3xURA <sup>-</sup>              | 3xURA <sup>-</sup>              | 1xG418<br>1xURA <sup>-</sup> |
| What this<br>is testing | Cut+HDR1      | Cut+HDR4      | Cut+NHEJ      | No cut     | No cut but<br>endogenous<br>HDR | No cut but<br>endogenous<br>HDR | Nothing<br>should<br>happen  |
| Red<br>colonies         | 16            | 68            | 0             | 0          | 0                               | 0                               | 0                            |
| White<br>colonies       | 350           | 776           | 816           | 1248       | 1168                            | 1992                            | 0                            |
| Total<br>colonies       | 366           | 844           | 816           | 1248       | 1168                            | 1992                            | 0                            |

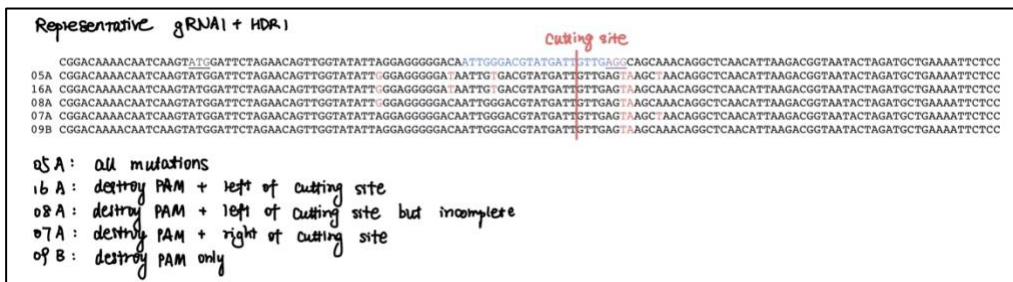


Figure 1. Aligned representative sequences with gRNA1 + HDR1 combination.



Figure 2. Aligned representative sequences with gRNA1 + HDR4 combination.

According to Table 1, Plate #1 and #2 yielded red colonies, Plates #3-6 had only white colonies, and Plate #7 had no colonies.

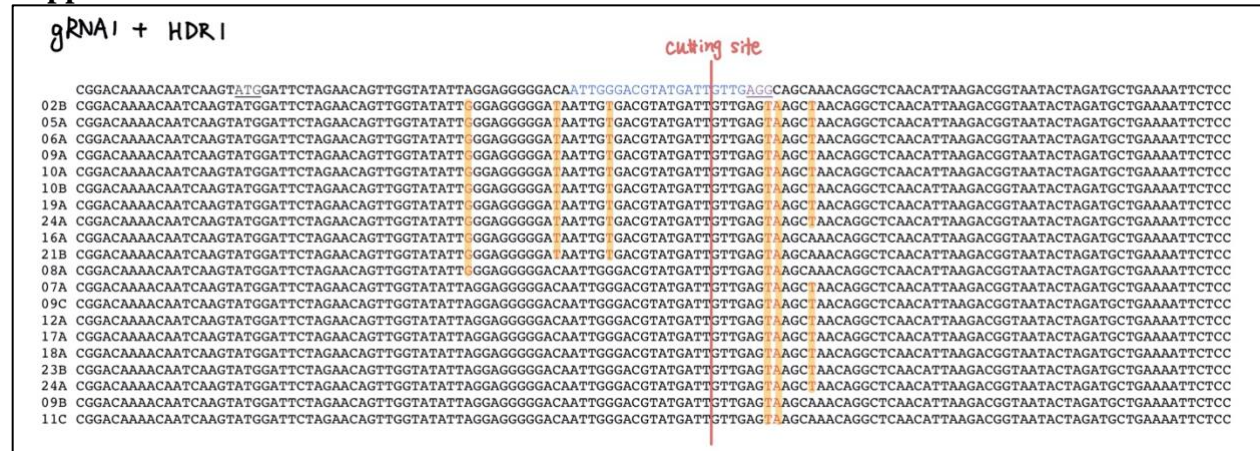
According to Figure 1&2 and Supplemental Figure 1&2, there were multiple ways the HDR template got incorporated. For HDR1, about half of the sequences had all the mutations, and half of them had only mutations on right of the cutting site. For HDR4, some sequences had mutations only on the left of the cutting site, some had mutations only on the right of the cutting site, and some had mutations on both sides. All of them had a destroyed PAM sequence.

## Discussion

According to Table 1, the frequency of HDR is  $(16+68)/(366+844)=6.9\%$ , and the frequency of NHEJ is 0%. As a result, HDR frequency was higher than NHEJ frequency. To me, this is reasonable because although the HDR pathway is inefficient, it is more targeted. As long as HR happens, the designed template can be incorporated with high fidelity. In comparison, NHEJ is more common and efficient, but it cannot guarantee desired repairs.

Combining Figure 1 and Supplemental Figure 1, it is confirmed that mutations introducing the TAA stop codon were all incorporated, and it can somehow match the expectation that mutations closer to the cutting site would be easier to be incorporated. However, Figure 2 and Supplemental Figure 2 showed that the mutations could actually be incorporated in very different ways, such as showing up on different sides of the cutting site, and this was not exactly what I expected. For those that had incomplete mutations, one possible explanation is some mutations got fixed by polymerases with the exonuclease activity during proof reading. In order to get a more confident conclusion, I believe more data should be collected in future experiments.

## Supplemental Results



Supplemental Figure 1. Aligned class sequences with gRNA1 + HDR1 combination.



Supplemental Figure 2. Aligned class sequences with gRNA1 + HDR4 combination.

**References**

M. Butler et al. "Plasmids used in the lab" in: Recombinant DNA Laboratory Manual., UC San Diego, pp27-32 (2020)

Addgene. CRISPR Guide. <https://www.addgene.org/guides/crispr/>