Constructing a CRISPR-Cas9 Vector for Mutagenesis of a Potential Phosphorylation Site of the Dsn1 Kinetochore Protein

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Introduction

Kinetochores are large protein complexes that assist in chromosomal segregation by attaching one end to the centromere of the chromosome and the other end to the spindle microtubules during cell division in either mitosis or meiosis. Kinetochores couple sister chromatids to dynamic microtubules during chromosomal alignment and anaphase, allowing their separation during cell division into daughter cells.

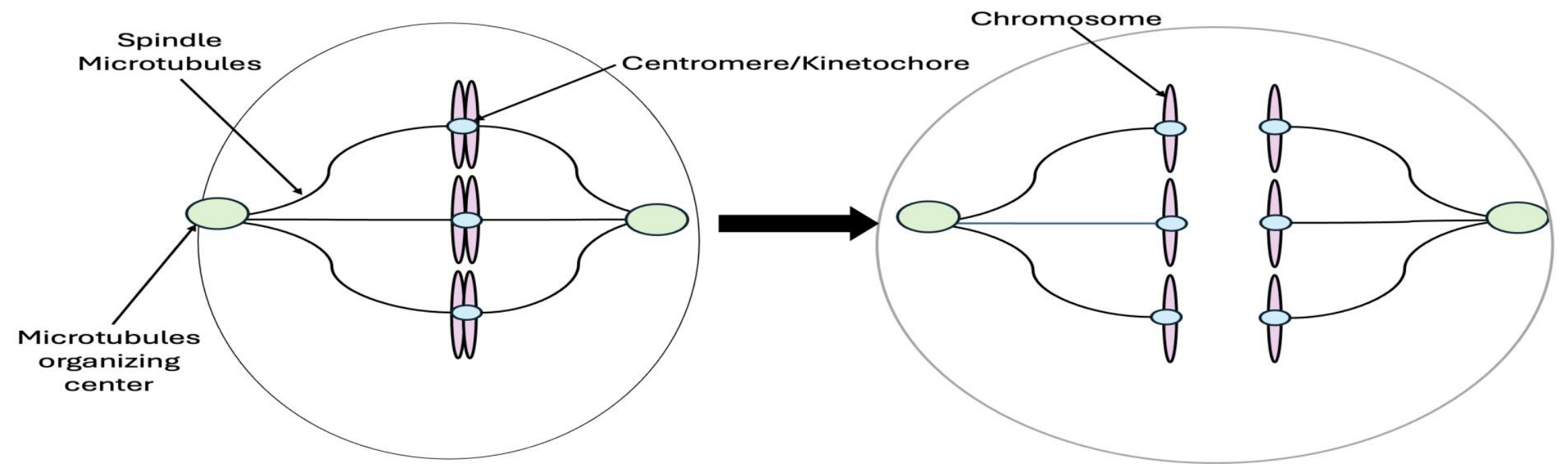


Figure 1. Normal chromosomal segregation when the spindle microtubules attach to the centromere/kinetochore and pull the sister chromatids.

If kinetochores are not properly attached to the spindle microtubules, it can result in mis-segregation during anaphase, an abnormal number of chromosomes, and can lead to genetic disease, cancer, or cell death. Kinetochore attachments to spindle microtubules are a crucial part in monitoring the Spindle Assembly Checkpoint (SAC), which ensures that anaphase only proceeds after all kinetochores are attached correctly to the spindle microtubules.

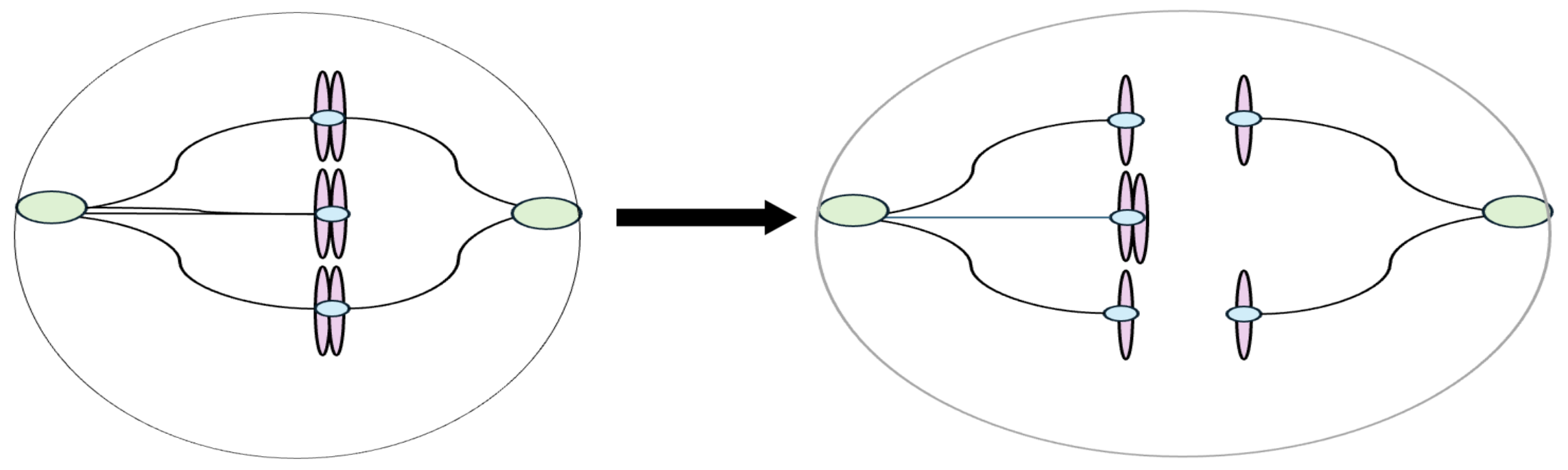


Figure 2. Incorrect attachment of spindle microtubules can allow for the incorrect amount of DNA, leading to aneuploidy.

Dsn1 protein is a crucial component of the kinetochore of the Mis12 complex and helps mediate chromosomal segregation by linking spindle microtubules to the chromosome. Mps1 kinases are involved in SAC and are known to phosphorylate the kinetochore at areas with acidic R-groups. Threonine 491 (T491) is a potential Mps1 target due to its two neighboring acidic amino acids. To investigate the potential role of the phosphorylation site, we plan to introduce a phosphomimetic mutation through the CRISPR-Cas9 system by changing Dsn1-T491 to Dsn1-T491DD.

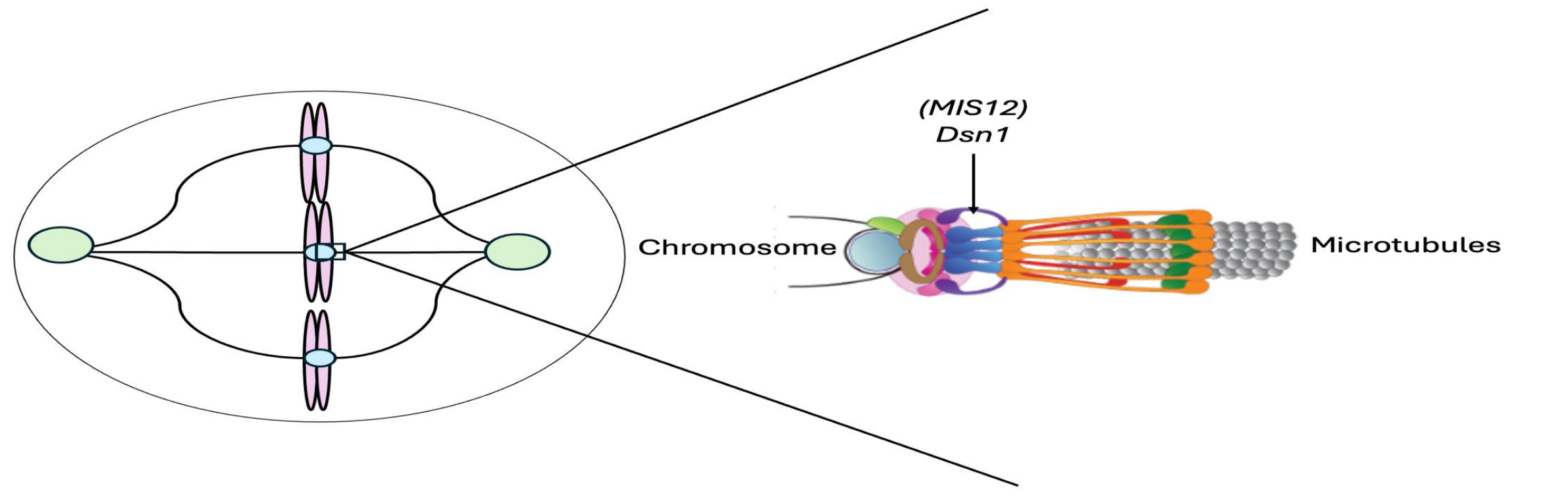
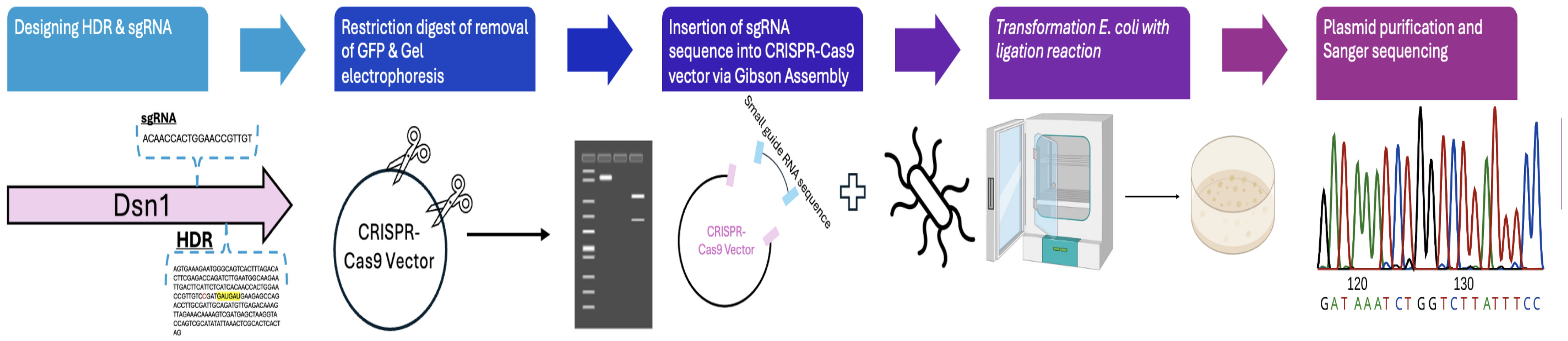


Figure 3. The *Saccharomyces cerevisiae* kinetochore structure indicating the location of Mis12 in Dsn1 (Biggins Lab)

Methodology/Experiment Plan



Designing HDR & sgRNA → Restriction digest of removal of GFP & Gel electrophoresis → Insertion of sgRNA sequence into CRISPR-Cas9 vector via Gibson Assembly → *Transformation E. coli with ligation reaction* → Plasmid purification and Sanger sequencing

Results

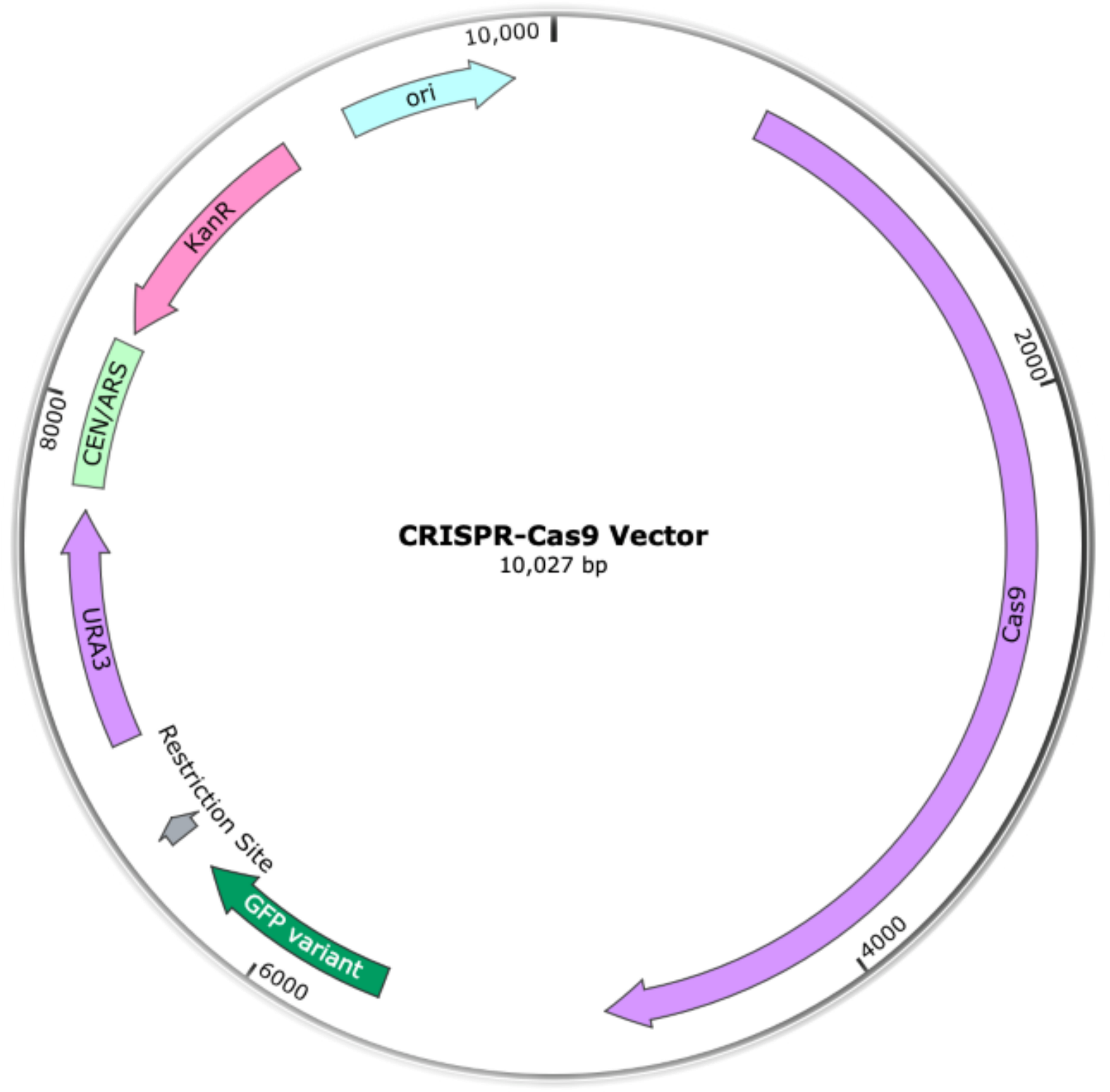


Figure 5. The map of the CRISPR-Cas9 vector with GFP

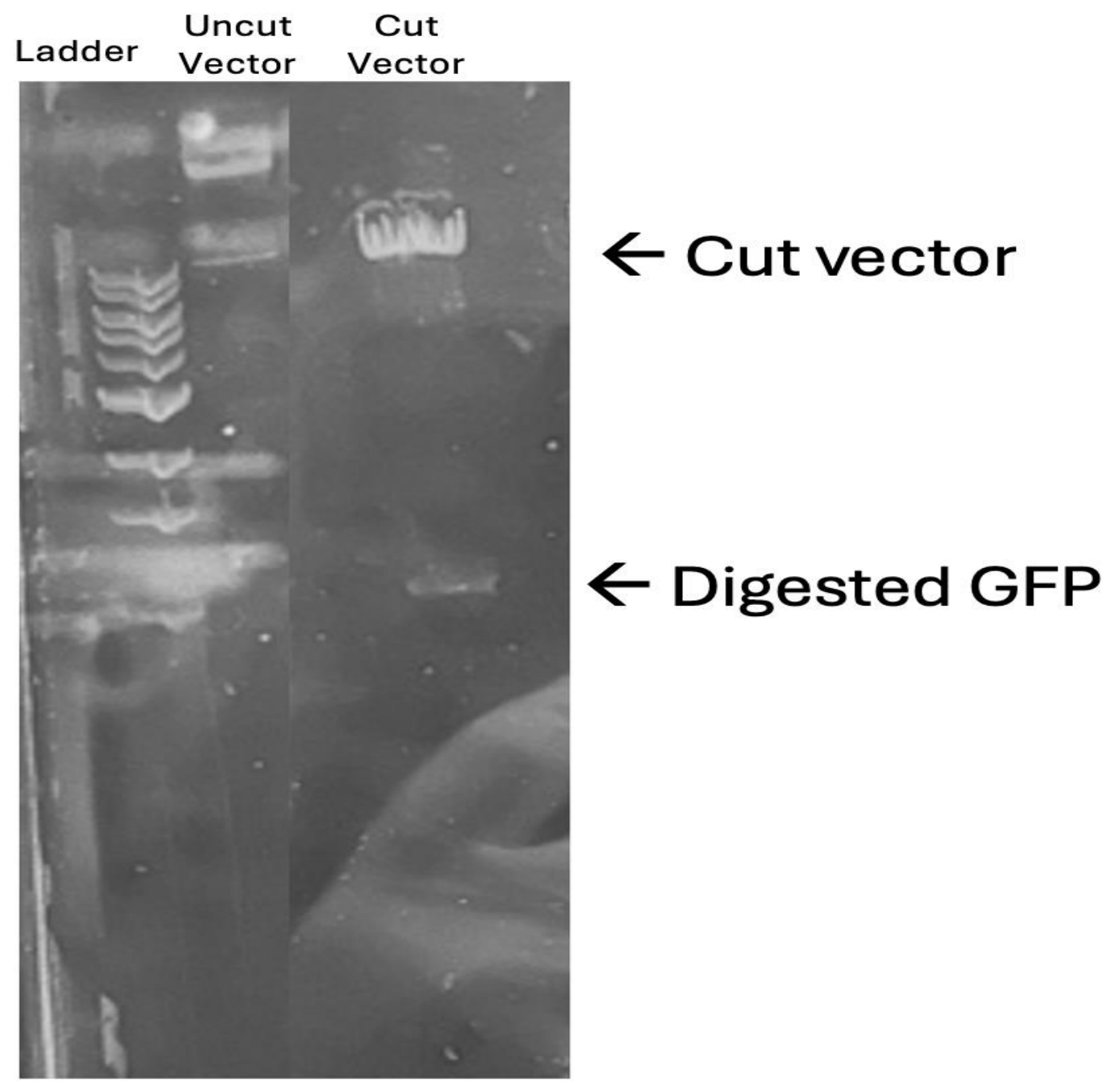
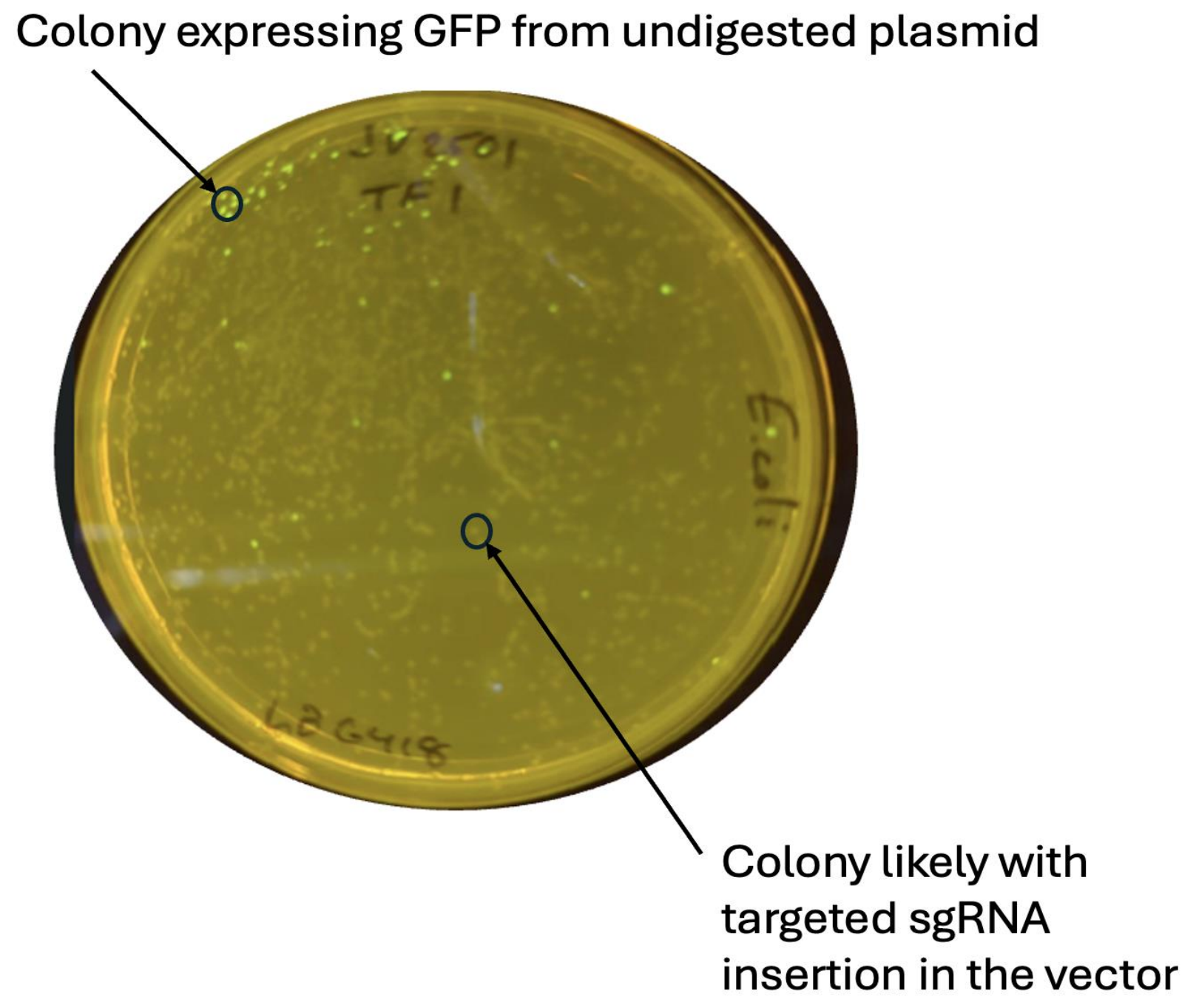


Figure 6. Gel electrophoresis after restriction digestion was performed to remove GFP from the CRISPR vector.



Colony expressing GFP from undigested plasmid

Colony likely with targeted sgRNA insertion in the vector

Figure 7. *E. coli* transformation with our ligated plasmid. The fluorescent colonies have GFP present, and the non-fluorescent colonies likely have sgRNA present that was inserted via Gibson assembly.

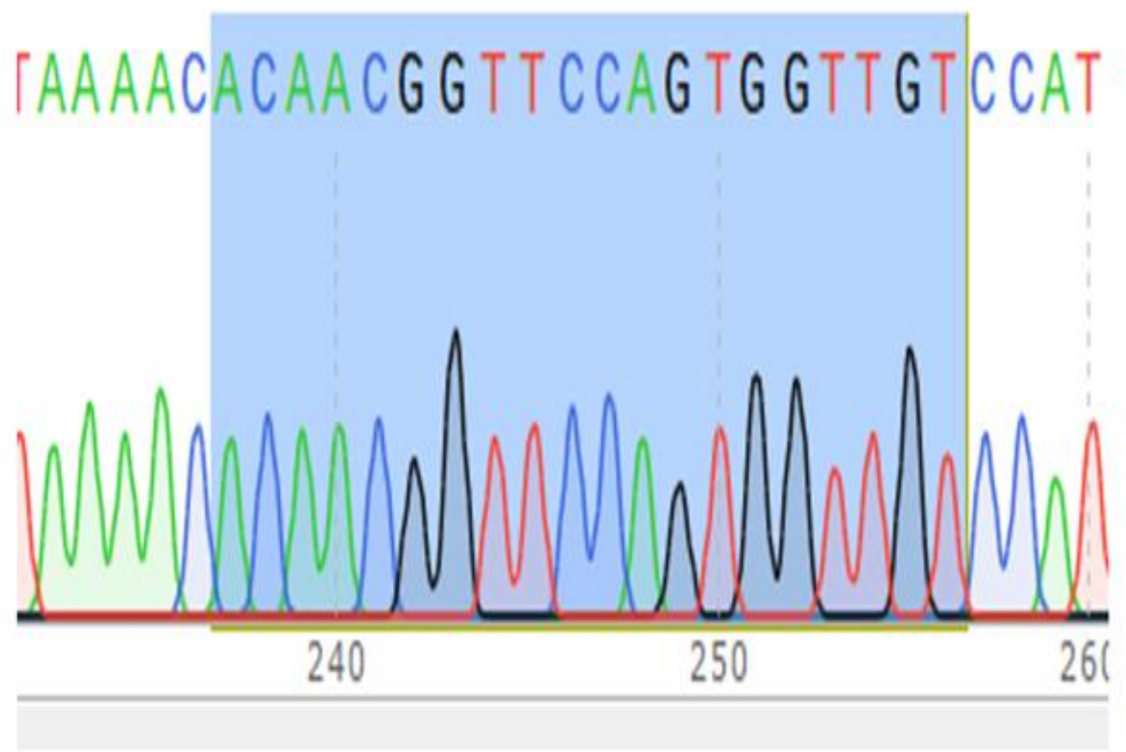


Figure 8. Sanger sequencing results of the digested vector, showing that our sgRNA was correctly inserted into the vector.

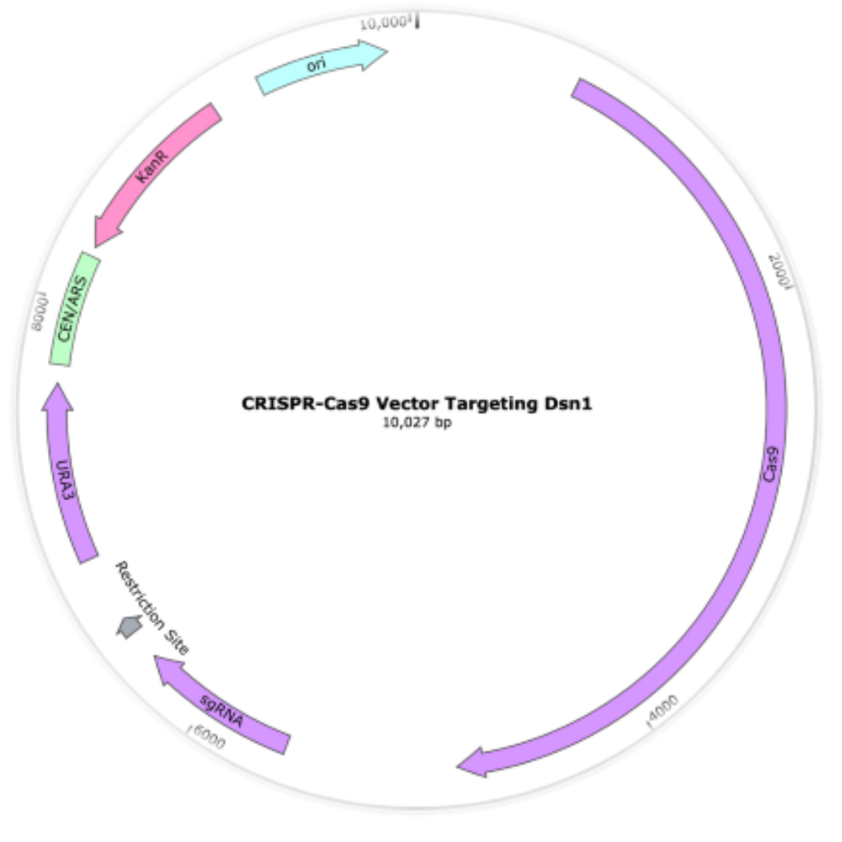
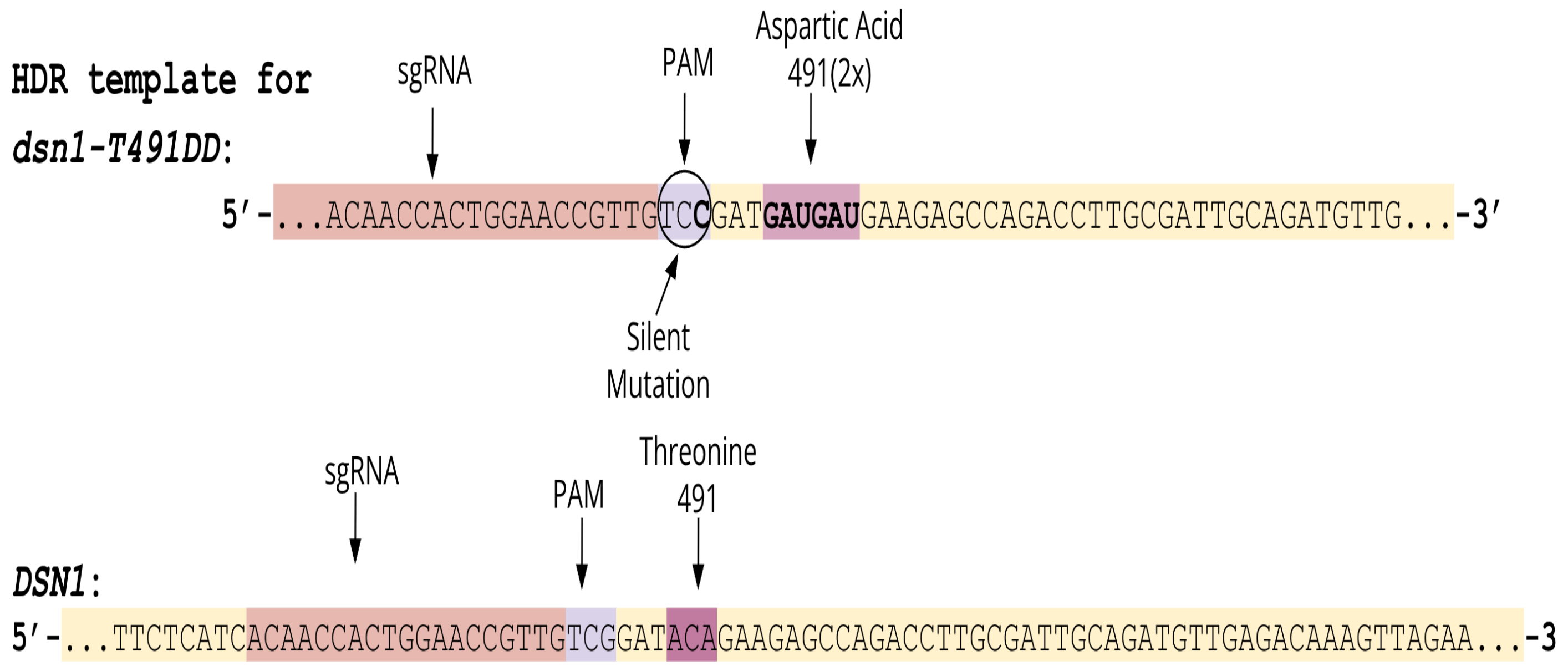


Figure 9. The map of our constructed CRISPR-Cas9 vector



HDR template for *dsn1-T491DD*:

`5′-...ACAACCACTGGAACCGTTGTCCGATGAUGAUGAAGAGCCAGACCTTGCGATTGCAGATGTTG...-3′`

***DSN1*:**

`5′-...TTCTCATCACAACCACTGGAACCGTTGTCGGATACAGAAGAGCCAGACCTTGCGATTGCAGATGTTGAGACAAAGTTAGAA...-3`

Figure 10. We designed a homology-directed repair (HDR) template to be used for repairing DNA double-strand breaks made by our CRISPR-Cas9 system. Shown in this diagram is the position where the sgRNA will guide the double-strand break by Cas9. The protospacer adjacent motif (PAM) sequence and our mutation: insertion of two Aspartic Acid residues.

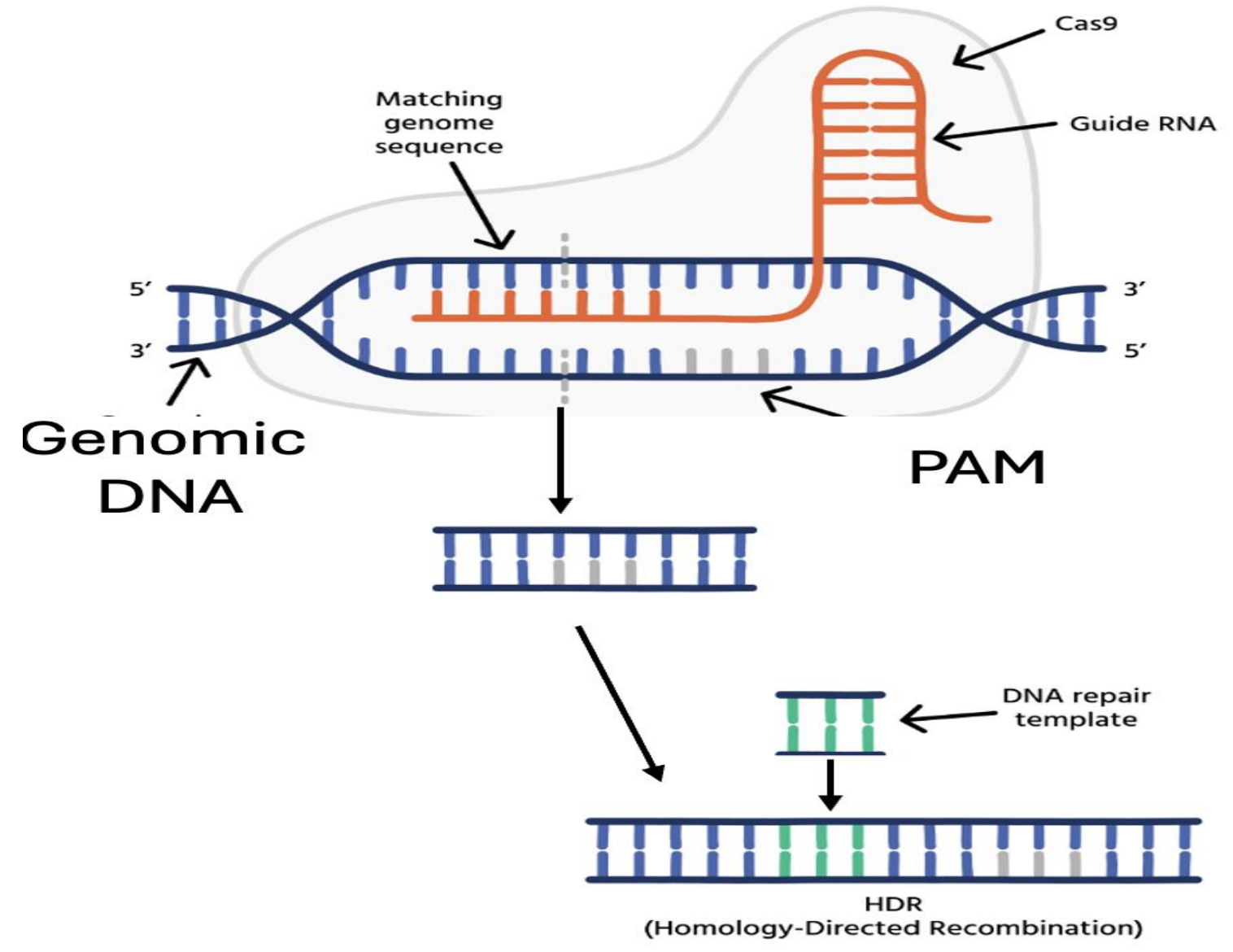


Figure 4. CRISPR is a gene editing technique that requires the Cas9 enzyme, which couples with a small guide RNA (sgRNA) that is complementary to a DNA sequence to be edited. Once sgRNA binds to the target DNA, Cas9 cuts the double-stranded DNA. A DNA repair template provided to the organism can facilitate repair of the break via homology-directed repair (HDR) (Image from iotaScience).

Conclusion

We were able to successfully clone the sgRNA-encoding sequence that will allow us to target the mutation in the *DSN1* gene into our CRISPR-Cas9 vector. We successfully removed the GFP to use Gibson assembly to insert our sgRNA into the vector through *E. coli* transformation. Then sent it off for sequencing to confirm that we successfully inserted our sgRNA into the vector.

Future Directions

The next step is to introduce our constructed CRISPR-Cas9 vector and our designed HDR template into *Saccharomyces cerevisiae,* confirm the presence of mutations through PCR and sequencing, and then take our mutant through a series of phenotypic tests that can reveal kinetochore dysfunction.

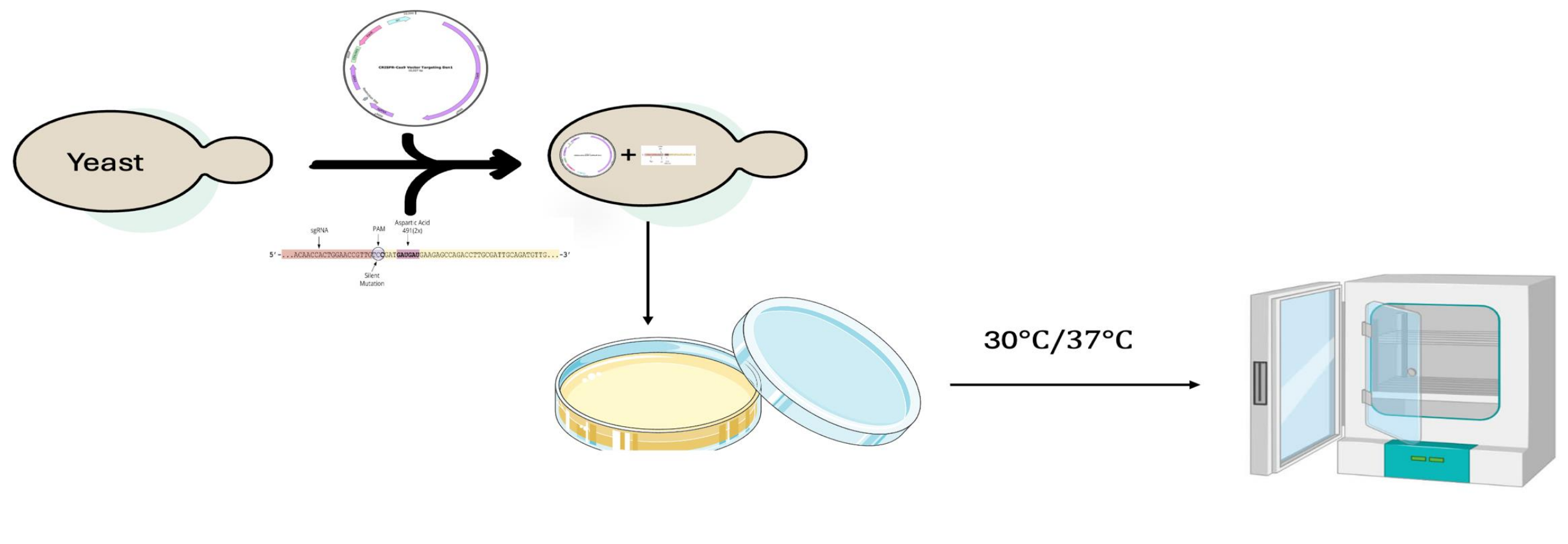


Figure 11. Introduce the CRISPR Vector with inserted sgRNA and HDR into yeast.

Acknowledgment

I would like to give a huge shout-out to my 495 classmates for making every week in lab an amazing experience. Thank you to the Sue Biggins lab for the CRISPR plasmid and hosting us at a lab meeting. Thank you to Ariele Barton for helping me codesigning the template for the HDR for us to use and Hannah Neir for providing the visual of the HDR mapping.

Resources