Directed evolution of targeted Cas9 cleavage to the LCA10 splice donor mutation

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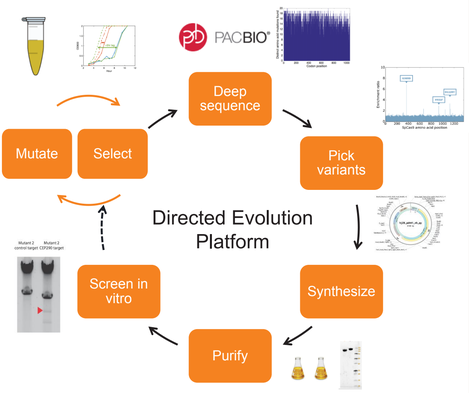
Abstract

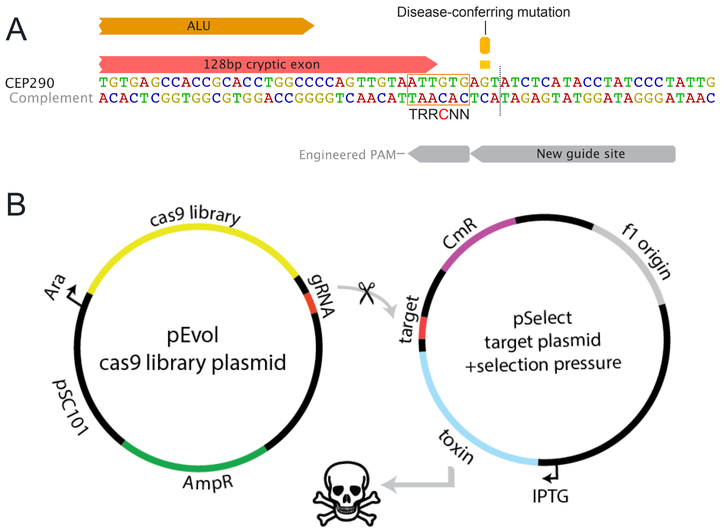
CRISPR/Cas9 has wide reaching scientific and therapeutic applications, but exactly targeting cutting sites efficiently remains difficult. Several cas9 variants have been engineered to target alternate PAMs; however, generation of new variants is often laborious. Additionally, the cutting ability of nucleases is often highly guide-dependent and shows a large amount of variation between sites. In order to quickly engineer new nuclease variants targeted to cleave a given site, we developed a directed evolution platform to select for DNA cleavage in bacteria. Our system allows rapid mutation of all amino acids throughout the protein, expanding potential diversity of functional mutants. These mutant libraries are challenged with phage containing target sites in a competitive pool. Using this method, we are developing *S. aureus* cas9 variants which have altered PAM preferences to target cleavage to the LCA10 IVS26 splice donor mutation. This engineered mutant can be efficiently packaged into AAV, allowing potential therapeutic use.

FIGURE 1. Directed Evolution Overview



Our directed evolution platform first begins with generating SaCas9 libraries and subjecting them to several rounds of selection. After deep sequencing of our libraries, we synthesize and purify mutants of interest for further downstream characterization.

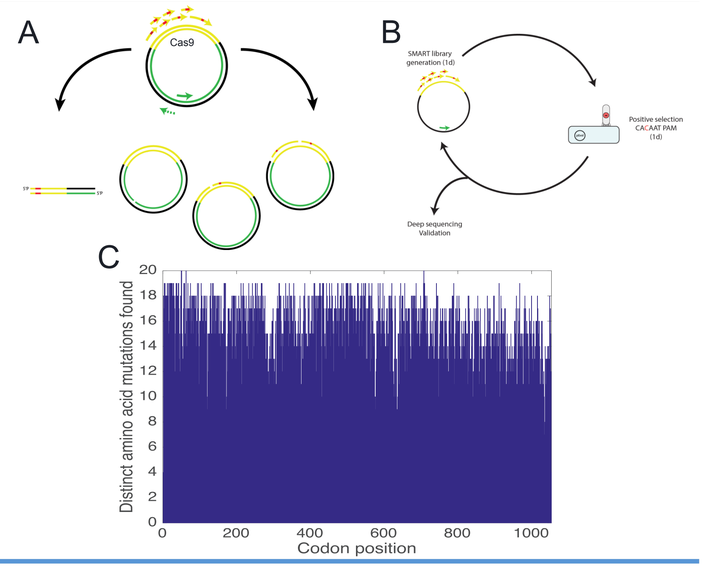
FIGURE 2. Adapting Directed Evolution Method for CEP290



A single point mutation at the CEP290 locus introduces a disease causing splice donor site in patients with Leber's congenital amaurosis (LCA10) (Figure 2A). Using SaCas9 to generate a nearby indel would disrupt the cryptic exon. However, there are few canonical PAMs close to the mutation, making access to the region difficult. Currently, we are engineering an SaCas9 to recognize an NNCRRT PAM near the mutation site. Our directed evolution setup includes an SaCas9 library and gRNA on one plasmid and a toxin and target site on a second plasmid (Figure 2B). Successful cleavage of the target site by a mutant SaCas9 will ensure that it outcompetes other variants in each round of directed evolution.

FIGURE 3. SMART Library Generation

Mutant SaCas9 libraries were engineered using the scanning mutagenesis at random targets (SMART) protocol (Figure 3A). The process begins with a modified PCR step, in which forward oligos, containing codon mismatches, introduce degeneracy at every amino acid position across the protein (Figure 3C). Undesired reaction products were removed from the library with a cocktail of nucleases. Libraries were then transformed into *E. coli* and infected with phage containing a toxic gene and a target site with a noncanonical NNCRRT PAM (Figure 3B).



Acknowledgement

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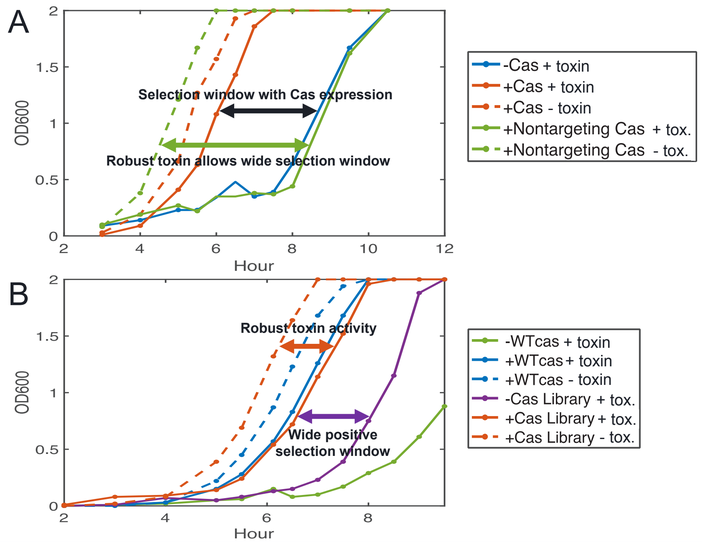
FIGURE 4. Inducible Toxin Allows for Tunable Selection Stringency



Growth curves were measured under varying conditions to assess the selection stringency of our system. The inducible toxin greatly reduces the growth rate of cells in liquid media, as indicated by the green arrow (Figure 4A). Successful Cas9 cleavage of the target provides a competitive advantage, as shown by the black arrow.

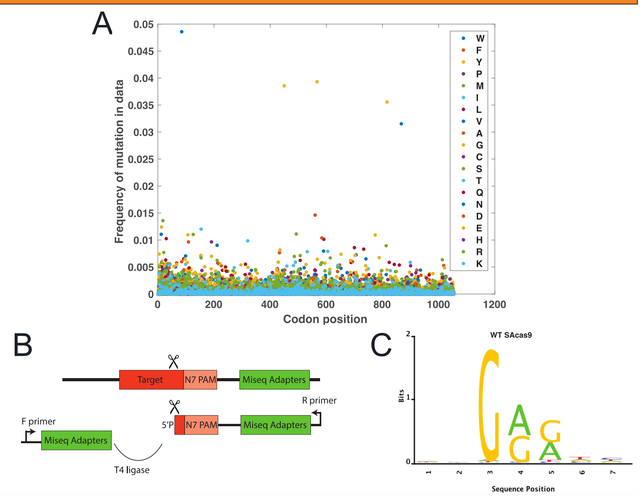
Subjecting our evolved mutants to the toxin yields similar results to that of WTCas9. Inducing SMART libraries also provides selective advantage to cells as demonstrated by the purple arrow, indicating library functionality (Figure 4B).

FIGURE 5. Identifying and Characterizing Novel Enzymes

After three rounds of directed evolution, libraries were subjected to NGS (Figure 5A). Multiple residues were enriched in distinct regions of the protein, most interestingly in the PI domain.

We are currently synthesizing and testing the efficiency of these mutants by cleaving at the CEP290 site with an NNCRRT PAM.

PAM for evolved enzymes will be verified by our PAM validation assay (Figure 5B). Here mutants will be exposed to a target with a completely degenerate PAM sequence. After ligating on a Miseq adapter, the sequencing results can be used to generate PAM logos, which can validate PAM preferences of our evolved variants (Figure 5C).



Conclusion

Our directed evolution platform allows for high yielding rapid generation of improved Cas9 variants. The SMART method provides considerable diversity and unbiased access across the entire SaCas9 gene, as opposed to single point mutation mutagenesis methods, such as error prone PCR. Rigorous selection parameters ensure high quality mutants that can be characterized quickly and efficiently in our downstream processing. The method can be easily adapted to other directed evolution projects, such as increasing cleavage efficiency, improving specificity, and introducing new enzyme functions.

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