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DEVELOPMENT AND VALIDATION OF A REPORTER SYSTEM FOR MPRA OF CRISPR-NUCLEASE ACTIVITY IN ESCHERICHIA COLI

Background. CRISPR nucleases such as Cas9 and Cas12a are key genome-editing tools, yet their performance is constrained by restricted PAM (protospacer-adjacent motif) compatibility, off-target cleavage, and inefficient nuclear delivery. Bacterial selection systems coupling site-specific DNA cleavage to survival via a toxin under an inducible promoter provide a direct genotype-phenotype link, required for at-scale screening of large variant libraries to engineer optimized nucleases. **Aim.** To develop and validate an inducible ccdB toxin-based system for the massively parallel reporter assay (MPRA) of CRISPR-nuclease activity in *E. coli*, converting cell survival into a quantitative, sequenceable readout. **Methods.** ccdB was PCR-amplified from pTypeIIIS and inserted into a custom pRSFYF plasmid by Golden Gate assembly; an attenuated Shine-Dalgarno (SD) sequence was then introduced by site-directed mutagenesis. Mammalian codon-optimised ErCas12a and its catalytically dead variant (dErCas12a) were cloned into pGEX carrying a ccdB-targeting gRNA cassette under the J23119 promoter. For screening, 1 ng plasmid encoding functional or inactive ErCas12a under the tac promoter was transformed into DH10B cells harboring pRSFYF-ccdB and plated on LB agar with 0.2% arabinose and 50 µg/mL chloramphenicol (37 °C, overnight). Colonies were enumerated with Cellpose-SAM algorithm. **Results.** The first-generation pRSFYF-ccdB construct with native SD showed no transformant outgrowth in sensitive *E. coli* even without toxin induction. Introducing the attenuated SD and titrating arabinose inductor allowed to reduce ccdB leakage, achieving 7.25×10^7 CFU/µg recovery without induction. Comparative evaluation of WT ErCas12a (targeted and non-targeted crRNAs) versus dErCas12a showed no outgrowth on Ara+IPTG+ plates for WT with targeted crRNA, and comparable counts for WT with non-targeted crRNA and dErCas12a. However, WT constructs retained activity without IPTG induction, possibly from tac promoter leakage. Scaling the protocol yielded ~50 k colonies/14 cm plate, sufficient to quantitatively cover libraries of ~10³ functional variants on a single plate. **Conclusions.** We established an MPRA reporter system based on an arabinose-inducible ccdB toxin module and, using functional and inactive ErCas12a, demonstrated the feasibility of massively parallel screening of Cas12a variants. Future work should minimize basal ErCas12a expression through optimal IPTG levels and tighter promoter control.

Keywords: CRISPR-Cas, Cas12a, ErCas12a, ccdB, MPRA, *E. coli*.

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