CRISPR Bacterial Gene Knockout Service (Cat. No. C424)

Case Study: CRISPR-assisted knockout of chloramphenicol resistance cassette (CAT) in *E. coli*.

Summary

- A genomically encoded chloramphenicol resistance cassette (referred to as CAT - chloramphenicol acetyl transferase) was knocked out using CRISPR-assisted genome editing.
- *E. coli* transformants were screened for sensitivity to chloramphenicol and correct chromosomal insertion of repair template.
- CAT knockout was confirmed by sequencing.

Phase 1: Cas9 and sgRNA Design and Cloning

- To improve recombination rates in bacteria, phage-derived (λ red) recombinases were employed alongside Cas9 in pCas to carry out enhanced homologus recombination **(Figure 1)**.
- sgRNAs were designed against the CAT gene which was previously introduced into the *E. coli* genome at the *yeeR* locus (accession number: NP_416505). Each sgRNAs was individually cloned into pTarget **(Figure 1)**.
- Repair templates were designed as single-stranded oligonucleotides containing homology to the CAT gene. The repair template also contains three stop codons for the early termination of CAT and a unique restriction site for screening purposes **(Figure 3)**. Importantly, the repair template eliminates the PAM site, preventing Cas9 re-targeting and cleavage of edited cells.

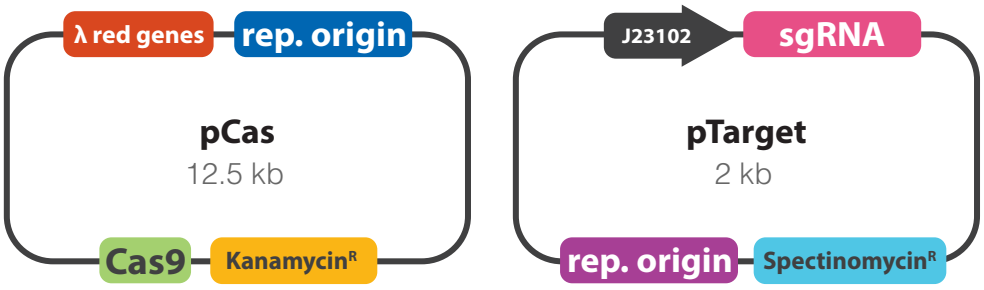

Figure 1 *Vector maps of pCas and pTarget. pCas9 constitutively expresses Cas9, whereas the λ red genes are inducible. pTarget constitutively expresses the sgRNA to guide Cas9 to the target locus.*

Phase 2: Preparation of λ red-induced electrocompetent cells and transformation

- pCas, carrying the λ red genes and Cas9, was transformed into *E. coli* cells. These cells were then made electrocompetent and the λ red genes were induced prior to co-transformation of pTarget and the repair template.

Phase 3: Screening and Sequencing - Knockout of genomically-encoded chloramphenicol resistance cassette (CAT)

A) Screening for sensitivity to chloramphenicol

- Transformants were replica picked onto kanamycin and chloramphenicol agar plates to assess sensitivity to chloramphenicol.
- Successful knockout and inhibition of the CAT gene is indicated by growth on kanamycin plates, but no growth on chloramphenicol plates **(Figure 2)**.

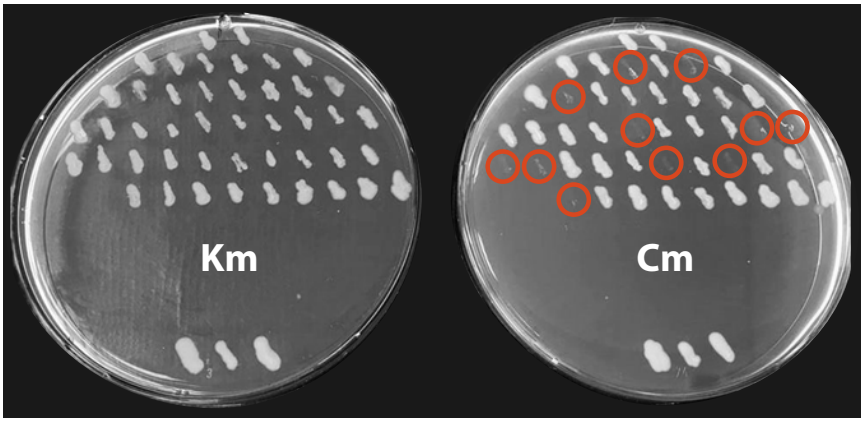

Figure 2 *Replica plates of potential CAT knockouts. Replica plates demonstrate 11/45 transformants were successfully edited (circled in red). The kanamycin plate is shown on the left, and the chloramphenicol plate on the right. Wild type controls (carrying the kanamycin plasmid and CAT gene integrated into the chromosome) are shown at the bottom of each plate.*

B) Screening for correct chromosomal insertion of repair template by restriction digest

- Successfully edited transformants can be verified by restriction enzyme digest using the unique SpeI site **(Figure 3)**.

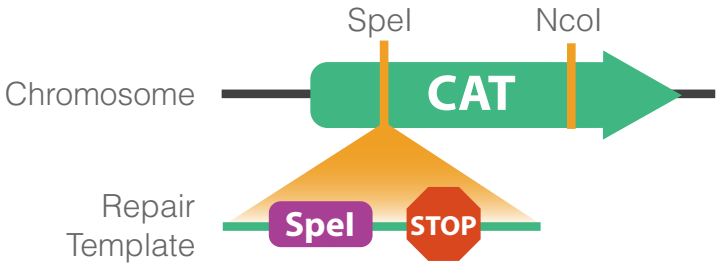

Figure 3 *Schematic of CAT gene knockout using a repair template containing stop codons and SpeI site.*

- The target locus was PCR amplified from the chloramphenicol-sensitive colonies and then digested using SpeI and NcoI to reveal a unique digest profile **(Figure 4)**.

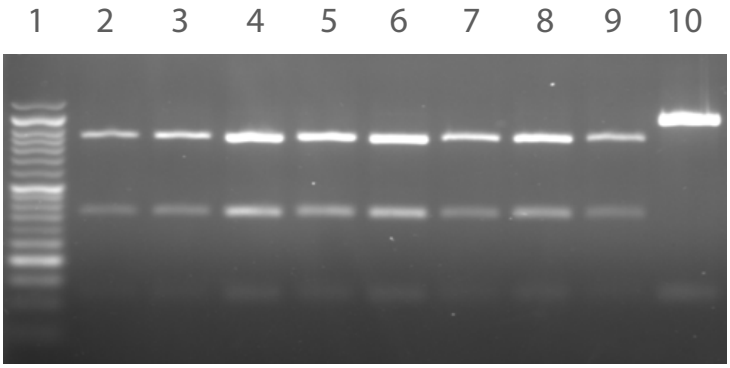

Figure 4 *Agarose gel depicting restriction digest profiles of the chloramphenicol- sensitive colonies. PCR products subjected to SpeI/NcoI restriction digest produces three bands for a positive clone and two bands for a negative clone. Lane 1: 100 bp Opti-DNA Marker. Lane 2-9: Colonies #1-8. Lane 10: Negative control.*

C) Sequencing of chloramphenicol-sensitive and restriction digest positive colonies

- PCR products were subjected to Sanger sequencing to confirm correct insertion and knock out of the CAT gene **(Figure 5)**.

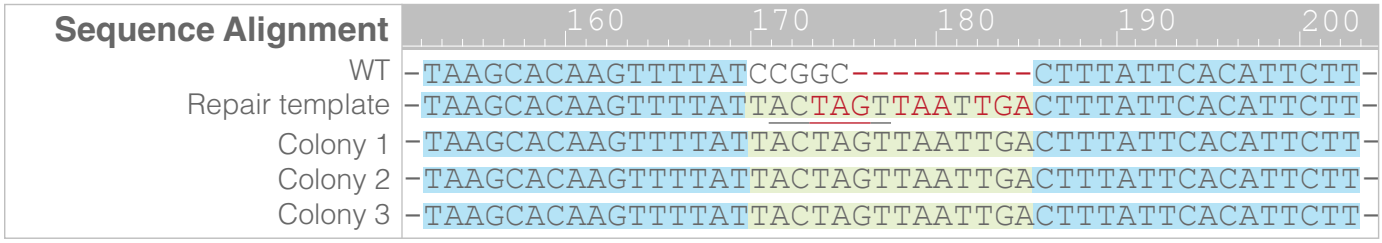

Figure 5 *Sequence alignment of CAT gene knockout colonies compared to wild type and repair template. The knockout insertion sequence (green) depicts the three stop codons (red) and the SpeI restriction site (underlined).*