Construction of a “leakless” promoter prevents skewing of Cas9 gRNA libraries

Background/Objective

Propagation of guide RNA (gRNA) libraries in the presence of Cas9 due to a leaky inducible promoter can skew library member abundance during outgrowth prior to selection, affecting the ability to accurately identify causative genes and/or mutations linked to phenotypes in high throughput (HT) screens. We showed that a multilayered inducible promoter prevents this skew by reducing leaky Cas9 expression.

Approach

- A heat-inducible promoter, PL, was combined with a heat-responsive ribosomal binding site from cyanobacterial heat shock protein *hsp17* (termed an “RNA thermometer”[1], or RNAT) and was used to control Cas9 expression in *E. coli* in the presence of either functional or nonfunctional gRNAs.
- A library containing 1000 empirically validated gRNAs (500 functional, 500 nonfunctional) was designed and evaluated for changes in member abundance in either repressive (30°C) or permissive (42°C) conditions for Cas9 expression.

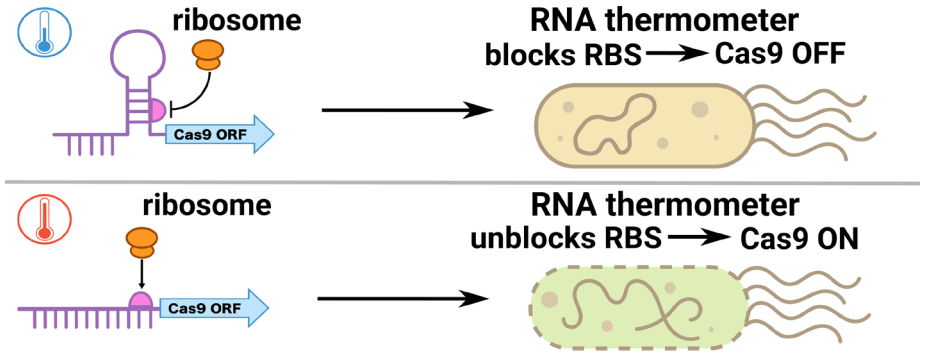
Results

- When tested with a single functional gRNA, the PL promoter with RNAT prevented leaky Cas9 expression at 30°C, reducing cell death due to unrepairable double strand breaks that are thought to be the source of library skew.
- When tested using the 1000-member library, the PL promoter and RNAT also prevented skew of the functional gRNA members of the library at 30°C, while the PL promoter alone exhibited high amounts of cell death and resulting skew even after 24 hours of culture.
- The PL promoter with RNAT, when induced at 42°C, was still able to mediate a high degree of killing, demonstrating the ability to more tightly control Cas9 expression necessary for HT library studies.

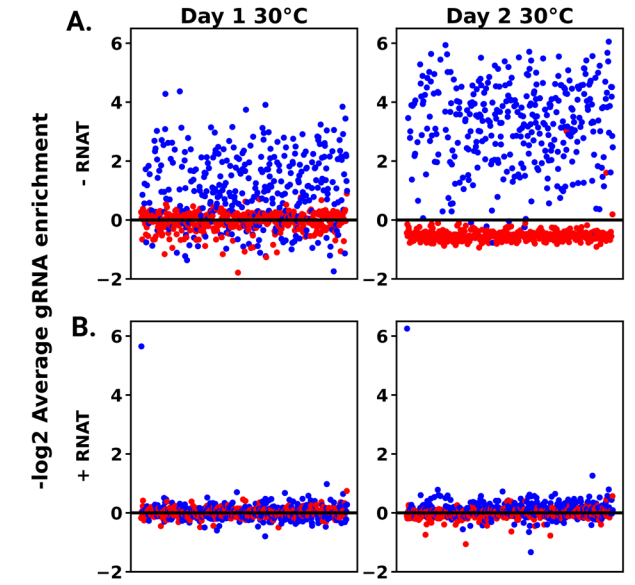
Significance/Impacts

Use of this inducible promoter-RNAT fusion allows for the safe propagation of gRNA libraries for HT Cas9-based studies in bacteria, greatly streamlining the production of these HT libraries and improving reproducibility of HT screens for genotype-phenotype discovery.

1. Kortmann et al., (2010). *Nucleic Acids Res.* https://doi.org/10.1038/nrmicro2730.
2. Kammerdiener et al., (2026). *Nucleic Acids Res.* https://doi.org/https://doi.org/10.1093/nar/gkaf1495.



Promoter-RNA Thermometer Construct



Preventing Cas9 Leak Eliminates Library Skew