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Engineering Chromosomal Ribosome Binding Site Libraries

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Introduction

To connect genetic circuits or control flux through a metabolic pathway, microbial engineering frequently necessitates careful control of protein expression. We've created a predictive design strategy for synthetic ribosome binding sites that allows for proportionate control of a protein's synthesis rate. Over 100 predictions in *Escherichia coli* were tested, and the method was found to be accurate to within a factor of 2.3 over a 100,000 fold range. Reusing a ribosome binding site sequence in multiple genetic circumstances might result in varied protein expression levels, as the design technique properly predicts. By rationally adjusting a protein's expression level to connect a genetic sensor to a synthetic circuit, we illustrate the method's utility.

Multiplexed gene expression optimization by Ribosome Binding Site (RBS) engineering is a useful method for improving artificial features in bacteria, such as genetic circuits and production routes. Established approaches construct smart RBS libraries from a single partially degenerate sequence that efficiently samples the whole space of translation initiation rates. However, DNA Mismatch Repair (MMR) systems significantly limit the sequence space available when integrating the library through CRISPR/Cas 9 based genome editing. MMR efficiency is determined by the type and length of the mismatch, and so effectively eliminates potential library members. Rather than working in MMR deficient strains, which accumulate off-target mutations, or relying on temporary MMR inactivation, which necessitates additional steps, we overcome this limitation by developing a pre-selection rule of Genome Library Optimized Sequences (GLOS), which allows large functional diversity to be introduced into MMR proficient strains with sequences that are no longer subject to MMR-processing. We employ *Escherichia coli* to create numerous GLOS libraries and show that they do retain diversity during genome editing and that they may be used in sophisticated genome editing procedures like simultaneous deletions. We claim that by using this method, chromosomal functions can be fine-tuned in a stable and efficient manner with minimal effort [1,2].

Description

In prokaryotic systems, modifying the strength of the Ribosome Binding Site (RBS) is a popular and effective way to adjust gene expression levels. Translation is up or down regulated by small alterations in as little as 6 to 8 bp in a spatially well-defined region. RBS engineering is a useful tool for rationally optimising a wide range of artificial functions, such as the signalling characteristics of genetic circuits or pathway fluxes for the production of valuable products, because the Translation Initiation Rate (TIR of any RBS can be roughly predicted solely from its nucleotide sequence. In many circumstances, the required level of translation is unknown, and the RBS strength forecast is inaccurate. Confounding factors such as metabolic burden, essentiality of related genes, side product generation, and toxicity may also be present, and projected expression levels may not be met due to a lack of free ribosomes. As a result, optimising the TIR for each of the relevant genes frequently necessitates the use of a library method. Due to the fact that full randomization of even a small portion of the RBS produces a set of sequences that is too large to evaluate for a single gene, let alone for gene combinations, and is heavily biassed towards non-functional or weak RBSs, a number of tools exist that use the previously mentioned approximate prediction of RBS strengths to design smaller libraries with a high level of functional sequences whose predicted TIRs still span the entire RBS. "RBS library calculator," "MAGE Oligo Design Tool" (MODEST, "Empiric Model and Oligos for Protein Expression Changes" (EMOPEC, and "Reduced Libraries" are a few of them (RedLibs [3].

The crucial functions that need tuning are often located on the chromosome, posing a significant practical impediment to efficient engineering, particularly in circumstances where genetic stability of created strains is important, such as in industrial biotechnology. However, the development of Multiplex Automated Genome Engineering (MAGE, which allows for the efficient introduction of small changes in the prokaryotic genome using lambda red-supported exploitation of single stranded DNA oligonucleotides as fake Okazaki fragments, has allowed RBS engineering to be extended to chromosomal genes, including large-scale multiplexing. The mechanism of action of this targeted mutagenesis method, on the other hand, inherently results in effects that are dependent on the sequence of the mutagenic oligonucleotide: the target cell counters mutagenesis by removing mismatches during replication using its MMR enzyme MutS, and the efficiency of this process is dependent on the length and nature of the mismatch. As a result, when the target cell is transformed, the members of mutagenic oligonucleotide

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libraries will have very varied outcomes. In MMR deficient (MMR) strains, the impact can be avoided, although this leads in a higher overall mutation rate (about four undesired point mutations in the genome per MAGE cycle, which can impair selection). The MMR system can be temporarily disabled, reducing the risk of unwanted mutations, but this comes at a cost [4].

We present an alternative genome engineering methodology that allows us to completely bypass the MMR system, which

- Is optimally suited for manipulations in practically relevant MMR+ strains and
- Eliminates the risk of sequence-based bias from library tactics. MutS, in particular, does not recognise insertions or mismatches of more over 5 bp.

Using oligonucleotides that introduce the intended mutation (s) on a (minimum) 6 bp-long mismatch should thereby reduce sequence bias. In practise, this criteria, which we name the GLOS rule (for genome library optimised sequences), can be simply implemented as an additional requirement for existing oligonucleotide selection algorithms, such as the RedLibs algorithm for RBS engineering [5].

Conclusion

The GLOS rule presented here enables fast and efficient construction of smart RBS libraries in MMR+ strains while reducing the amount of off-target effects and retaining a higher tendency to repair oligonucleotide-induced indels. Moreover it was previously shown that an active MMR system leads to higher killing efficiency for the CRISPR/Cas 9 counter selection 33. We show that complex modifications such as RBS library construction with simultaneous sequence deletion can be performed in a single step with high AR efficiencies, demonstrating the potential of this method

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