iGEM TEAM

VILNIUS-LITHUANIA

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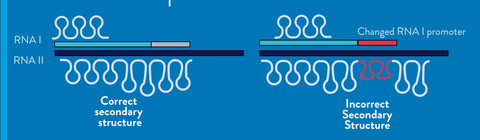
PLASMID COPY NUMBER CONTROL

RNA I can be used to regulate plasmid copy number. Yet RNA II secondary structures, important for replication, make modifying RNA I promoter difficult.

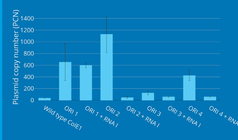


Modifying the RNA I promoter in the origin of replication can often be detrimental to replication, because changes in the promoter's sequence (point mutations or switching to another type) also alters the secondary structures of RNA II which are crucial to the formation of replication primer.

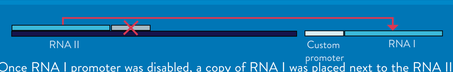
Disabling the native RNA I promoter and placing a copy of RNA I next to RNA II is an elegant solution to this problem.

Five RNA I promoter mutants were picked using *in silico* analysis[2]. The alghorithm searched for and prioritized mutations which: **A.** Reduce the binding rate of RNA polymerase the most **B.** Keep the complementry RNA II secondary structure unchanged for successful replication initiation.

	-10 consensus	-35 consensus
WT	AGTGTA	CTTCAA
ORI1	AGTAAG	AAATGG
ORI2	AGTAAG	TCCGGG
ORI3	AGTAAG	CTTCAA
ORI4	AGTGTA	AAATGG
ORI5	AGTGTA	TCCGGG



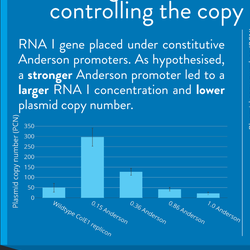
The selected promoters were validated experimentally. We looked for a knock-out mutant promoter which yields the highest plasmid copy number (lowest RNA I concentration). Also, it was important for copy number to go back to wild type levels after a **WT** RNA I gene was placed next to the RNA II gene, which would show that RNA II secondary structures were unchanged. Having in mind the mentioned requirements ORI 2 was perfect - it had both a very high copy number with mutated RNA I promoter and an almost equal copy number to wildtype when **WT** RNA I was cloned in.

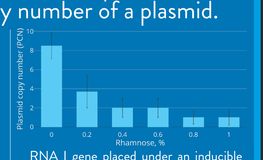


Once RNA I promoter was disabled, a copy of RNA I was placed next to the RNA II gene. Consequently, this allowed to place RNA I under any custom promoter.

Placing RNA I under custom promoters - controlling the copy number of a plasmid.

RNA I gene placed under constitutive Anderson promoters. As hypothesised, a **stronger** Anderson promoter led to a **larger** RNA I concentration and **lower** plasmid copy number.



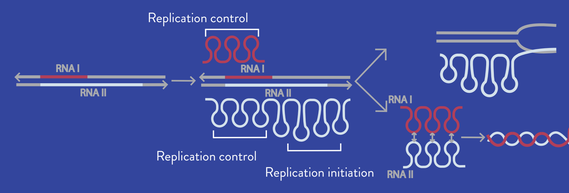


RNA I gene placed under an inducible Rhamnose promoter. As inductor concentration increased, RNA I concentration increased as well. As a result, larger inductor concentrations lead to lower plasmid copy numbers.

INTRODUCTION

Plasmids are a key tool in synthetic biology, however there is still a lack of means to create precise and flexible plasmid systems. Proper control of plasmid copy number, compatible multiple plasmid groups, and the ability to keep the plasmids stable are at minimum eagerly anticipated in current synthetic biology. Here we present a novel framework for easy, flexible and standardized work with plasmid systems.

BACKGROUND. ColE1 plasmid copy number control

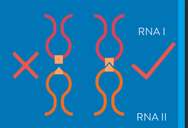


3' end of RNA II forms a primer for DNA polymerase to bind - **Replication initiation**

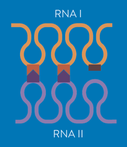
RNA I binds to RNA II and forms a complementary duplex, making primer formation impossible - **Replication inhibition**

PLASMID GROUPS

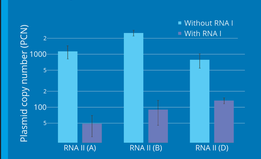
Different recognition sequences were added to the end of RNA I and RNA II loops to engineer compatible, independently controlled plasmids.



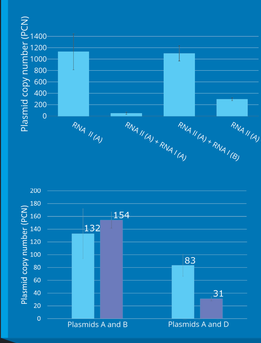
Only 2 out of 3 interacting stem loop sequences were modified for each new plasmid group, as mutations in the third loop of RNA II have a high chance of impairing replication[3]. Yet, that does not mean that the third loop of RNA I shouldn't be changed. When the RNA I gene is placed next to RNA II, the two genes are not constricted to being complements of each other. If RNA I sequence at the third loop is not modified, every plasmid group cross-interacts and causes background inhibition of all plasmid replication. This background inhibition can be minimized by adding mutations to the RNA I third loop, which reduce RNA II binding efficiency.



Experimental validation of plasmid groups



Three plasmid groups were investigated with and without their appropriate RNA I genes. As expected in every unique group the RNA I gene dramatically decreased plasmid copy number. This shows that added specific sequences keep the RNA I molecule functional.



Then, cross interactions between RNA II from group A and every group's RNA I were investigated. As initially thought, RNA I (A) inhibits replication to wildtype levels. RNA I (B) does not show any significant interaction, which is the best possible outcome. Yet, RNA I from group D significantly inhibits the replication.

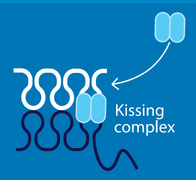
Plasmids were co-transformed in pairs. A and B groups worked perfectly as they achieved the same copy number as when transformed alone. Although A and D were both reduced in copy number, this was expected as the cross-interaction experiment showed strong interactions between these two groups.

GLOBAL COPY NUMBER CONTROL

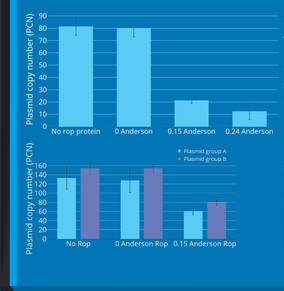
Modulating the copy number of all plasmid groups at once can act as an additional global control parameter, and ColE1 replicon gives a perfect hint on achieving this goal as it codes a protein called Rop[4].

The Rop protein dimer is a bundle of four tightly packed alpha-helices that are held together by hydrophobic interactions[5]. It recognises the stem-loop kissing complex of RNA I-II and binds to it.

Rop binding to the stem-loop complex increases the RNA I-II interaction affinity, consequently lowering the plasmid replication initiation rate.



Rop recognizes the secondary structure of the complex, rather than the specific sequences. Therefore, we have theorized that this protein should affect all plasmid groups simultaneously.

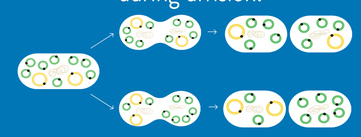


Placing the *rop* gene under different anderson promoters, using only one plasmid group. Results show that using a stronger Anderson promoter successfully produced more Rop protein and thus reduced the plasmid copy number.

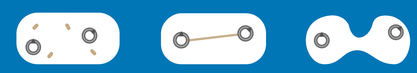
Using Rop protein in a two plasmid group system. Plasmid from group A is carrying the *rop* gene under different Anderson promoters. Results demonstrate that the Rop protein does not discriminate between groups, yet it lowers the copy number of both, while keeping the initial copy number ratio.

ACTIVE PARTITIONING SYSTEM

Low copy number plasmids can be easily lost during division.

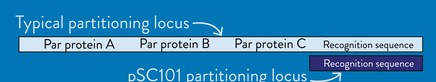


Plasmid coded proteins ensure plasmid stability in low copy number plasmids[6].



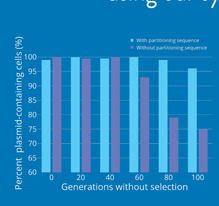
In natural systems, special plasmid-encoded proteins attach to each of the plasmid recognition sites, and separate the pair to opposite cell poles.

A light system is preferred, and the stabilization system from pSC101 ensures it.



Most of known partitioning systems can be as large as 2000 base pairs. Yet we have stumbled upon and characterized a pSC101 stabilization system, which is only 400 bp long and uses cell's protein machinery[7].

pSC101 partitioning system was investigated using our synthetic vector.



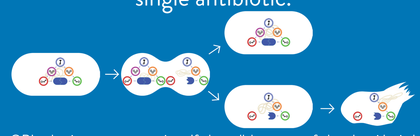
Stability of SynORI vector was investigated with and without an active partitioning system. The vector was modulated to 20 plasmids per cell with an Anderson promoter. After 60 generations there was a significant stability drop when partitioning system was not used. In contrast, with pSC101 system stability has not diminished even after 100 generations.

SYNORI SELECTION SYSTEM

In multiple plasmid systems different antibiotics for each plasmid group are commonly used.

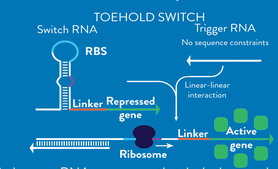
Simultaneous use of multiple antibiotics can place excessive stress on the host, increase the costs of growth media and also raise biosafety risks.

We propose an alternative approach which allows to maintain up to 5 plasmid groups with a single antibiotic.

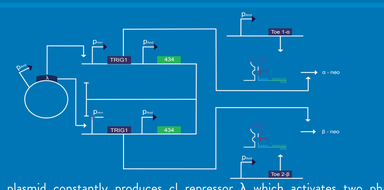


SynORI selection system overview. If the cell loses any of the plasmids, it loses antibiotic resistance and dies.

System consists of single transcription factor, two toehold switches, their triggers and a split resistance protein.



Toehold switches are RNA sequences that lock the translation of downstream genes by sequestering the ribosome binding sites and the AUG start codon into its secondary structure[8] (translation OFF). When a trigger RNA, complementary to the Toehold's 5' end, is presented, it forms a duplex with the switch sequence, disrupting the lock state (translation ON).



The first plasmid constantly produces cI repressor λ which activates two phage promoters regulating the Toehold triggers present in the second and third plasmids. The fourth and the fifth plasmids generate split antibiotic resistance gene transcripts regulated by toehold switches. Trigger RNA activates the translation and an active antibiotic resistance protein is produced.

SYN ORI

A FRAMEWORK FOR MULTI-PLASMID SYSTEMS

EVERYDAY LAB WORK

Limiting your research to a particular plasmid copy number due to small selection of replicons is no more. SynORI enables scientists to pick any required number of copies.

SMART ASSEMBLY OF PROTEIN COMPLEXES

Easy manipulation of plasmid copy number allows the induction of each plasmid group at appropriate times, making complex protein formation and modification resource efficient.

BIOLOGICAL COMPUTING

Plasmid copy number regulation is equivalent to adding a global parameter into a computer system. It enables the coordination of multiple gene expression.

METABOLIC ENGINEERING

In addition to currently widely used control types - transcriptional and translational - SynORI offers simultaneous multiple gene control, making the search for ideal parameters of large metabolic circuits more efficient and accessible.

METHODS

PLASMID COPY NUMBER DETERMINATION

Copy number was estimated using Real-Time PCR. Two qPCR standard curves were generated – one for a nucleoidal gene and one for a plasmid-specific gene. By employing different standard curves we were able to evaluate total bacteria and plasmid number in the qPCR reaction. The plasmid copy number per cell is thus found by dividing the total plasmid number by the cell number.

STABILITY ASSAY

The plasmid loss rate was investigated by patching single colonies from different generations on LB agar plates with antibiotic. Only cells containing plasmids grew on the plated regions. Plated areas with no growth indicated plasmid loss. The percentage of cells that have lost their plasmids was estimated by using the ratio of total patches to patches that did not grow any cells.

ACHIEVEMENTS

1. Engineered a synthetic origin of replication which allows:
   a. Plasmid copy number control (in a constitutive, inducible manner)
   b. Creation of specifically controllable synthetic plasmid groups.
2. Constructed a global (group-unspecific) plasmid copy number device.
3. Characterized and applied an active partitioning system for low copy number plasmid stabilization.
4. Designed and constructed a selection system that can hold up to five different plasmid groups in the cell using only one antibiotic.

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