Plasmid Construction

Second-Generation Black Box Plasmid

Description

By constructing a second-generation black-box plasmid, we aim to create a controllable RNA polymerase-guided gene-editing system. This system functions as follows: In our plasmid design, CBE is linked to nMag and the RNAP N-terminal, while pMag is linked to the RNAP C-terminal. The binding of RNAP's N- and C-terminals depends on the light-sensitive proteins nMag and pMag, which only bind in the presence of blue light. Therefore, gene editing occurs when blue light is present, forming the CBE-nMag-pMag-RNAP complex, and stops when the light is absent. To assess mutation efficiency, an EGFP reporter system is included; high mutation rates weaken the green fluorescence of EGFP, while low mutation rates result in minimal fluorescence reduction. So, the low intensity in fluorescence indicates high mutation rates, and the high intensity in fluorescence indicated low mutation rates (as shown in Figure 1). This system allows us to precisely control and evaluate the effectiveness of gene editing.

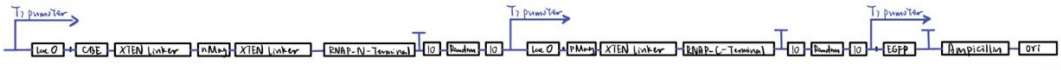

Figure 1 | The plasmid construction of second-generation black-box plasmid.

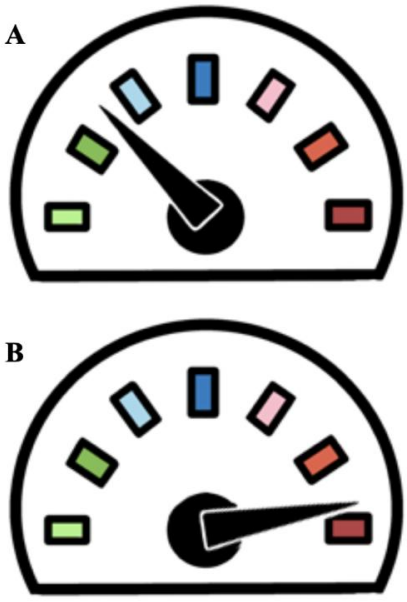

Figure 2 | The EGFP report system. A. The high intensity in fluorescence indicated low mutation rates. **B.** The low intensity in fluorescence indicates high mutation rates.

Elements

pBR322 ori

The pBR322 ori is the origin of replication in plasmids, responsible for initiating the autonomous replication of the plasmid within host cells (e.g., *E. coli*). It ensures that the plasmid DNA is replicated and transmitted during cell division by interacting with the host's replication proteins. The pBR322 ori typically maintains a moderate copy number (20-30 copies per cell), ensuring stable plasmid presence and gene expression within the host cell.

Rop

Rop (repressor of primer) is a regulatory protein in plasmids like pBR322. Its primary function is to facilitate the binding of RNA I and RNA II, reducing the initiation of plasmid replication and thereby lowering the plasmid's copy number within the cell. Excessive plasmid copies can impose a metabolic burden on the host cell, affecting growth, viability, and plasmid stability, potentially leading to plasmid loss. Rop protein plays a critical role in modulating plasmid replication stability, ensuring that the plasmid does not accumulate excessively in the host cell, thus maintaining stable transmission and normal function.

LacI

LacI is a repressor protein in the lactose operon (lac operon), which inhibits gene expression by binding to LacO. The LacI protein binds tightly to the LacO sequence, blocking RNA polymerase from binding to the promoter, thereby inhibiting the transcription and subsequent expression of downstream genes. In the presence of lactose or its analogs (e.g., IPTG), these molecules bind to LacI, causing a conformational change that releases LacI from LacO. This allows RNA polymerase to bind to the promoter and initiate transcription, leading to the expression of enzymes necessary for lactose metabolism. In the plasmid system, LacI expression ensures that the initiation of mutations is controllable.

T7 promoter

The T7 promoter is a DNA sequence specifically recognized by T7 RNA polymerase to initiate transcription. In this experiment, its role is to allow RNA polymerase to

bind and begin the transcription and translation of downstream genes, resulting in the production of the desired protein.

LacO

LacO is the operator sequence within the lactose operon, serving as the binding site for the LacI repressor protein, thereby regulating the expression of downstream genes. LacO is simply a specific DNA region and does not encode any protein or RNA itself, so it is not expressed.

RBS

RBS (Ribosome Binding Site) is a critical element in plasmids that directs the ribosome to bind to the mRNA, initiating protein translation. The RBS is typically located between the promoter and the start codon (AUG), acting as a specific nucleotide sequence recognized by the ribosome. This recognition ensures that translation begins at the correct location, leading to the synthesis of the target protein. In this experiment, the RBS is essential for the expression of downstream CBE and RNAP proteins from the plasmid, making it a key element in the translation process.

rAPOBEC_43.1 (CBE)

rAPOBEC_43.1 is a cytidine deaminase used in plasmid-based base editing. It converts cytosine (C) in DNA sequences into uracil (U), which is recognized as thymine (T) during subsequent DNA replication, resulting in a C-to-T mutation. This enzyme is used as a tool for gene editing in this experiment. Additionally, it will be linked to nMag and the N-terminal of RNAP to facilitate RNA polymerase-guided gene editing in the experiment.

XTEN Linker

The XTEN Linker is a polypeptide sequence typically used in plasmids to connect two proteins or functional domains. Its main role is to provide sufficient space and flexibility, allowing the connected proteins to fold correctly and function properly while minimizing interference between them. In this experiment, the XTEN Linker connects CBE-nMag-RNAP’s N-terminal and pMag-RNAP’s C-terminal, enabling RNA polymerase-guided gene editing.

nMag

nMag is a light-sensitive protein that can bind to pMag, leading to the combination of the N-terminal and C-terminal of RNAP. Because nMag is light-sensitive, blue light is required to promote its binding with pMag. When blue light is present, the RNAP-nMag-pMag-CBE complex forms and induces mutations in downstream genes; without blue light, the mutations cannot be initiated. This allows for the controlled assembly and disassembly of RNAP by blue light, enabling controllable RNA polymerase-guided gene editing.

RNAP N-terminal

The RNAP N-terminal is the initial portion of the RNAP protein. In this experiment, it combines with the RNAP C-terminal to form a complete, functional RNAP, which performs transcription (DNA to RNA), laying the foundation for subsequent protein synthesis.

TAA

TAA is a stop codon, also known as a termination signal or stop codon. During translation, the TAA signal appears at the end of the mRNA sequence. When the ribosome encounters TAA, it halts protein synthesis and releases the newly synthesized polypeptide chain. This ensures the precise termination of protein synthesis, preventing the disorderly extension of the polypeptide chain.

T7 terminator

The T7 terminator is a DNA sequence that halts transcription driven by T7 RNA polymerase. When RNA polymerase encounters the T7 terminator sequence during mRNA synthesis, transcription stops, resulting in the production of a complete mRNA molecule. The T7 terminator ensures accurate transcription termination, preventing the synthesis of unnecessary RNA fragments. This is particularly important in gene expression systems to produce accurate and complete mRNA.

10

T7 RNA polymerase gene, also known as T7 gene 10, encodes T7 RNA polymerase. This polymerase is a strong transcription enzyme from T7 bacteriophage that

specifically recognizes the T7 promoter and drives efficient transcription of downstream genes.

Random

A "Random insertion sequence" is an unspecified nucleotide sequence that can interrupt the function of specific genes, study gene function, or insert specific elements at non-specific locations.

pMag

pMag is a light-sensitive protein that can bind to nMag, leading to the combination of the N-terminal and C-terminal of RNAP. Because pMag is light-sensitive, blue light is required to promote its binding with nMag. When blue light is present, the RNAP-nMag-pMag-CBE complex forms and induces mutations in downstream genes; without blue light, the mutations cannot be initiated. This allows for the controlled assembly and disassembly of RNAP by blue light, enabling controllable RNA polymerase-guided gene editing.

RNAP C-terminal

The RNAP C-terminal is the terminal portion of the RNAP protein. In this experiment, it combines with the RNAP N-terminal to form a complete, functional RNAP, which performs transcription (DNA to RNA), laying the foundation for subsequent protein synthesis.

EGFP

EGFP (Enhanced Green Fluorescent Protein) is an optimized green fluorescent protein derived from the original GFP of the jellyfish (*Aequorea victoria*). After significant mutagenesis, the EGFP signal weakens and can be observed under a microscope, indicating that our black-box system has achieved significant mutations and is effective. Therefore, EGFP is an important component of the reporter system in this experiment.

8xHis

8xHis is a protein tag consisting of eight consecutive histidine residues, primarily used for protein purification and detection. By adding the 8xHis tag to either the N- or

C-terminus of the target protein, the protein can be easily isolated from cell lysates using affinity chromatography techniques such as nickel or cobalt affinity columns.

Ampicillin

In this experiment, ampicillin resistance is used as the selectable marker to accurately select high-copy *E. coli* during plasmid amplification. The plasmid is transformed into *E. coli* and then cultured in an environment containing ampicillin. *E. coli* that fail to acquire the plasmid will lack ampicillin resistance and die. Conversely, *E. coli* that successfully acquire the plasmid will express ampicillin resistance and survive in the presence of the antibiotic. This allows for the selection of *E. coli* containing the plasmid, which can then be further propagated in monoclonal cultures.

First-generation black box plasmid

Description

This plasmid was constructed to serve as a positive control for our black box second generation plasmid, Its original sources are Cravens, A., Jamil, O.K., Kong, D. et al. Polymerase-guided base editing enables in vivo mutagenesis and rapid protein engineering. *Nat Commun*12, 1579 (2021). https://doi.org/10.1038/s41467-021-21876-z

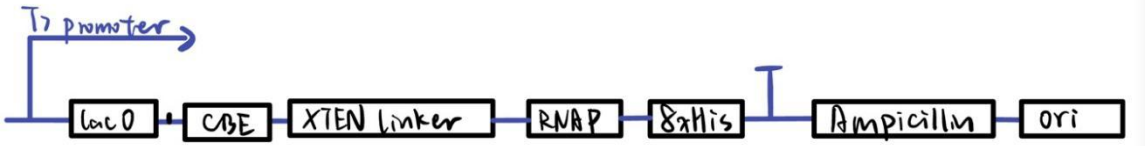

Figure 3 | The plasmid construction of first-generation black box plasmid.

Nicotine metabolic plasmid

Description

The plasmid carries genes for four enzymes necessary to degrade nicotine, a toxic substance. Because these enzymes are not present in E. coli, we wanted to use mutations in these enzymes to identify the most efficient combinations of genes for degrading nicotine. We then constructed this foreign plasmid and transferred it into E. coli. Through the mutation of the black box system, these four enzymes can be mutated to facilitate our subsequent screening.

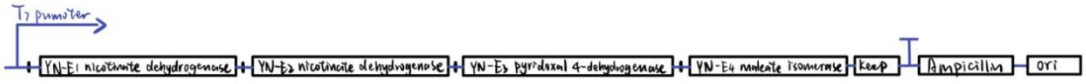

Figure 4 | The plasmid construction of nicotine metabolic plasmid.

Elements

YN-E1 Nicotinate Dehydrogenase

YN-E1 Nicotinate Dehydrogenase is the initial enzyme in nicotine metabolism, responsible for converting nicotine into 3-pyridinecarboxylic acid (niacin). This reaction marks the first step in nicotine degradation through an oxidative dehydrogenation process. The 3-pyridinecarboxylic acid produced serves as the substrate for subsequent enzymatic reactions.

YN-E2 Nicotinate Dehydrogenase

YN-E2 Nicotinate Dehydrogenase follows YN-E1 in the pathway, further oxidizing 3-pyridinecarboxylic acid into pyridine acid or other related compounds. This step advances the degradation of nicotine into smaller, simpler molecules that can be further processed in other metabolic pathways.

YN-E3 Pyridoxal 4-Dehydrogenase

YN-E3 Pyridoxal 4-Dehydrogenase is involved in vitamin B6 metabolism but plays a role in nicotine metabolism by likely providing essential cofactors required for the dehydrogenase activities of YN-E1 and YN-E2. These cofactors enable efficient oxidative reactions, facilitating the initial stages of nicotine degradation.

YN-E4 Maleate Isomerase

YN-E4 Maleate Isomerase converts maleate into fumarate, a crucial step in integrating the degradation products of nicotine into the tricarboxylic acid (TCA) cycle. This integration allows the cell to harness energy from the degradation products. YN-E4's role is to process the intermediates produced by YN-E1 and YN-E2, converting them into forms that can be utilized within the cell's energy metabolism.

Keep

The function of "Keep" in a plasmid typically refers to maintaining the plasmid's replication and stability. For a plasmid to remain stably within a host cell, specific genes or sequences are required to ensure proper replication and transmission during cell division. Specifically, "Keep" may involve mechanisms for low-copy number maintenance, partitioning systems, or encoding related proteins that help retain the plasmid within the host, ensuring its continued presence and functionality.

Ampicillin

In this experiment, kanamycin resistance is used as a selectable marker to accurately select high-copy *E. coli* during plasmid amplification. The plasmid, along with the black-box system plasmid, is introduced into *E. coli*. To more precisely select *E. coli* that retain and amplify both plasmids, kanamycin, rather than ampicillin, is added to the nicotine plasmid. The use of dual antibiotics allows for more accurate selection of *E. coli* containing both desired plasmids, which are then further amplified in monoclonal cultures.

Spermidine generating plasmid

Description

This plasmid carries genes for three enzymes essential for converting other substances into spermidine, a compound known for its potential to extend lifespan. Since these enzymes are not naturally present in *E. coli*, we aim to utilize mutations of these enzymes to determine the most effective gene combination for producing higher levels of spermidine. We constructed this exogenous plasmid and transferred it into *E. coli*. The black-box system allows for mutations of the three enzymes, facilitating subsequent screening efforts.

Figure 5 | The plasmid construction of spermidine generating plasmid.

Elements

Spermidine synthase

Spermidine synthase is an enzyme that catalyzes the conversion of putrescine into spermidine by transferring an aminopropyl group from decarboxylated S-adenosylmethionine (dcSAM) to putrescine, making this a crucial step in spermidine synthesis.

S-adenosylmethionine decarboxylase

S-adenosylmethionine decarboxylase is the key enzyme responsible for producing dcSAM by decarboxylating S-adenosylmethionine (SAM), providing the aminopropyl donor essential for the function of spermidine synthase.

Agmatinase

Agmatinase hydrolyzes agmatine to produce putrescine, which serves as a precursor for spermidine synthesis, thereby supporting the activity of spermidine synthase. These enzymes work closely together in the biosynthesis of spermidine.

Ampicillin

In this experiment, kanamycin resistance is used as a selectable marker to accurately

select high-copy *E. coli* during plasmid amplification. The plasmid, along with the black-box system plasmid, is introduced into *E. coli*. To more precisely select *E. coli* that retain and amplify both plasmids, kanamycin, rather than ampicillin, is added to the nicotine plasmid. The use of dual antibiotics allows for more accurate selection of *E. coli* containing both desired plasmids, which are then further amplified in monoclonal cultures.