PHOCUS

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Troubleshooting for phage engineering, a guide based on experience

TU Delft

iGEM

Introduction

To show that we can successfully engineer a phage we made a proof of concept using the bacteriophage T7 and *E. coli* BL21 (DE3), replacing a non-essential gene with a GFP reporter gene. In order to construct our engineered phage, we went through several iterations of the engineering design cycle *(see Engineering success page)*. We encountered several problems during this part of the project that were solved by troubleshooting. Since the multiple difficulties we faced during our phage engineering path, we thought it could be of great help to the iGEM community to provide a short and simple guide about troubleshooting phage engineering

General principle of Bacteriophage Recombineering of Electroporated DNA (BRED)

To engineer our bacteriophage, we used Bacteriophage Recombineering of Electroporated DNA (BRED) [1]. The first step of BRED involves electroporating double stranded DNA (dsDNA) into cells containing bacteriophages. This dsDNA contains homologous regions to the bacteriophages' DNA. DNA recombination occurs inside the cell causing the dsDNA fragment to be inserted into the bacteriophages in the target position.

The efficiency of this method is 10%, therefore screening is needed to verify that the insertion is successful . A mixture of engineered [2] and non-engineered phages, is added to agar plates containing bacteria. The phages infect the bacteria, replicate inside them, resulting in plaque formation. These plaques are regions on the plate where cells have been destroyed due to phage infection and only phages are present. These plaques could be then screened via PCR to check if the phage engineering has succeeded.

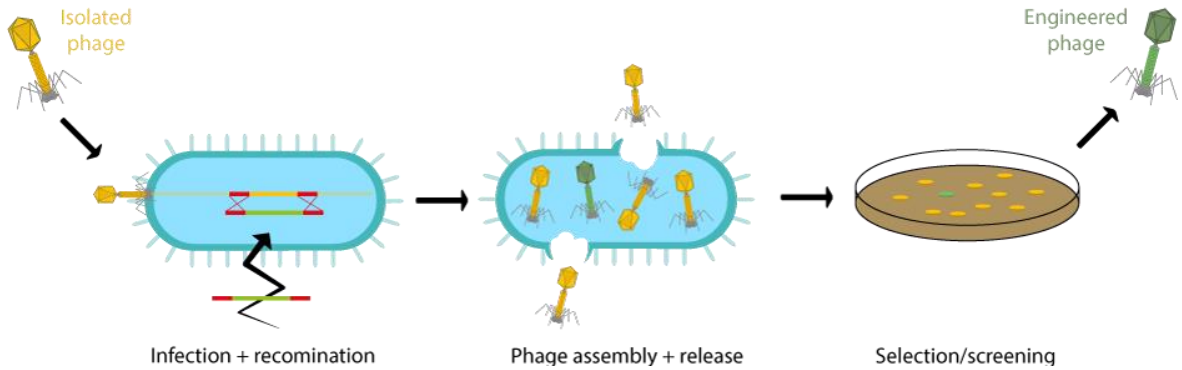

Figure 1: dsDNA (green) containing homologous regions to the bacteriophage (red) are electroporated into cells previously infected with bacteriophages. Recombination results in recombinant bacteriophages, containing the dsDNA fragment. Adapted figure from Kilcher and Loessner (2019) [3].

Advices for phage engineering with BRED

1. Before starting your experiment, determine the optimal Plaque Forming Unit (PFU). This is the amount of phages needed to obtain individual plaques. If the PFU is too high, no plaques form (Figure 2, left), making PCR screening impossible. If the PFU is within the good range, this will result in plaque formation (Figure 2, right). For our phages (T7) this was 10^8 PFU. Each plaque can be screened by performing a PCR experiment to identify if the phages are engineered.

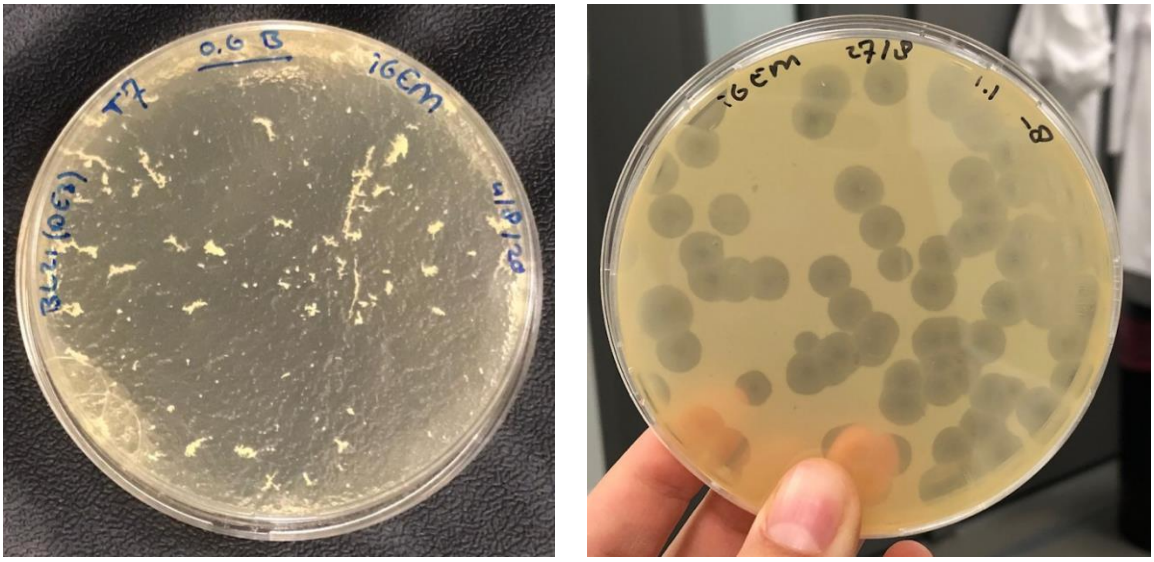

Figure 2: Left: Result of adding a too high PFU, no plaques form, all cells die. Right: Result of adding a correct PFU, separate plaques are visible

2. When performing a PCR experiment to screen for the phages, it is important that the correct primers are used. We advise to create two different sets of primers. Of the pair, For instance, one primer hybridizes the regions flanking the insert and the other hybridizes with part of the inserted gene (Figure 3). After the PCR is performed, a gel electrophoresis of the amplified products could show whether the gene was successfully inserted, as well as whether the sample contains wild-type phage or not.

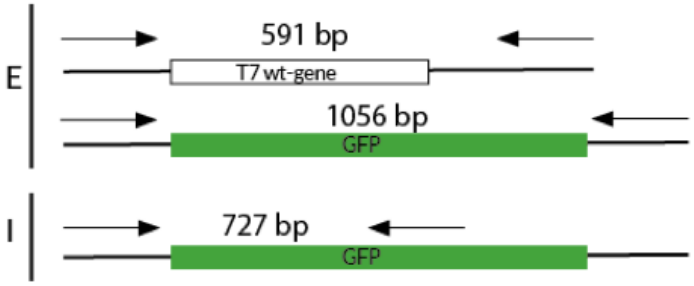

Figure 3: Screening of engineered phages by PCR amplification. A) The two different sets of primers needed to verify correct insertion. The first set (E) hybridizes externally. The second set of primers (I) binds internernally. B) Possible results that can be obtained after performing the screening PCR with these primers.

3. If the PCR results are negative, the phage engineering efficiency was probably too low. In this case, we recommend to screen the entire sample obtained after BRED. This alternative method is referred to as phage pool PCR (Figure 4).

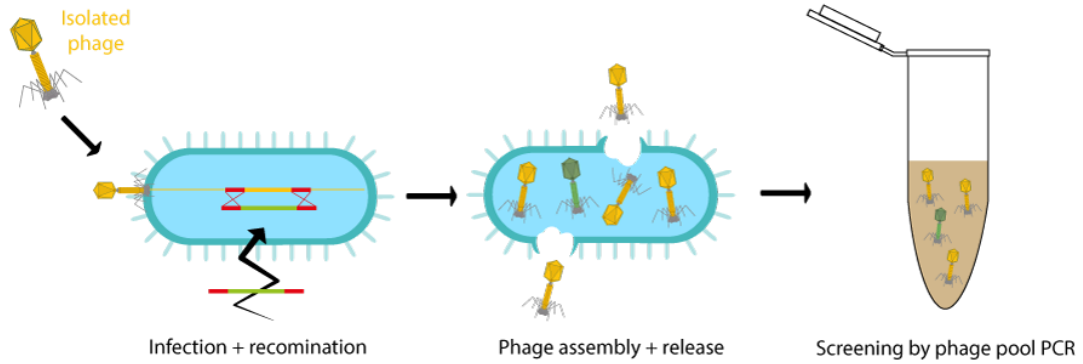

Figure 4: Scheme representing a phage pool PCR.

4. If you get a positive PCR result after the pool PCR, you have successfully engineered bacteriophages. You can now use the CRISPR-Cas9 system to isolate them (Figure 5). For this, you should design a single-guide RNA (sgRNA) sequence to target the gene you intend to replace. With CRISPR-Cas9, the genetic material of the non-engineered phages will be cleaved, and only engineered phages will be left. In order to confirm that, PCR screening as described in Step 2 should be performed.

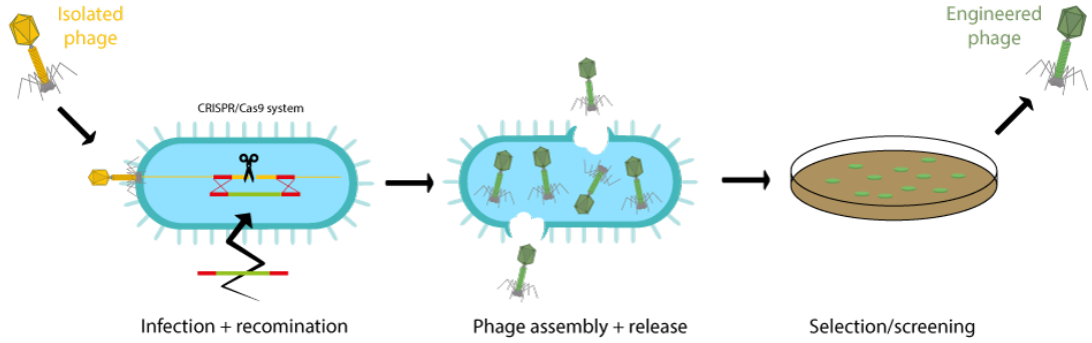

Figure 5: dsDNA (green) containing homologous regions to the bacteriophage (red) are electroporated into cells previously infected with bacteriophages. Recombination results in recombinant bacteriophages, containing the dsDNA fragment. Activated CRISPR/Cas9 system will cleave the wild-type phage DNA, therefore selecting the engineered phages. Adapted figure from Kilcher and Loessner (2019) [3].

References

[1] Marinelli, L. J., Piuri, M., Swigoňová, Z., Balachandran, A., Oldfield, L. M., van Kessel, J. C., & Hatfull, G. F. (2008). BRED: A simple and powerful tool for constructing mutant and recombinant bacteriophage genomes. *PLoS ONE*. https://doi.org/10.1371/journal.pone.0003957

[2] Chen, Y., Batra, H., Dong, J., Chen, C., Rao, V. B., & Tao, P. (2019). Genetic engineering of bacteriophages against infectious diseases. *Frontiers in Microbiology*. https://doi.org/10.3389/fmicb.2019.00954

[3] Kilcher, S., & Loessner, M. J. (2019). Engineering Bacteriophages as Versatile Biologics. *Trends in Microbiology*. https://doi.org/10.1016/j.tim.2018.09.006

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