Testing our NarX/NarL design in *E.coli*

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Purifying pSB1C3

The pSB1C3 plasmid was purified using GeneJET Plasmid Miniprep K0502 on an overnight culture of *E. coli* DH5α that had the pSB1C3 plasmid. 2 mL of the overnight culture was put in 2 mL Eppendorf tubes. In the harvest step, cells were centrifuged at 4000 rpm instead of 8000 rpm. Resuspension was used instead of vortexing in all steps. The plasmids were lastly eluted with a volume of 15 µL.

The plasmid was then linearized using PCR - Phusion™ High–Fidelity DNA Polymerase with a reaction volume of 20 µL. The primers used were FWD 1C3 Lin and RVS 1C3 Lin. The Phusion® High-Fidelity PCR Master Mix with HF buffer was used instead with 10 µL for each sample. In the PCR, 35 cycles were used with an annealing temperature at 56.8°C. This was performed on three samples and concentrations after the PCR were measured to 556.4 ng/µL, 530.2 ng/µL and 521.5 ng/µL respectively.

The samples were mixed into one tube and purified according to the GeneJET PCR Purification protocol (K0701). Isopropanol was not added in the optional step.

The concentration was measured to 100.7 ng/µL with the 260/280 value of 1.86 and the 260/230 value of 1.81.

Gibson Assembly

The sequence consisting of three fragments with the PyeaR-GFP and two VHHac-NarX was assembled with the purified pSB1C3 plasmid using Gibson Assembly. Two replicates, one negative control with only water and plasmid and one positive control with only Mastermix and NEB Positive Control were used. The NEBuilder HiFi DNA Assembly Reaction protocol was followed with the following changes.

- A ratio of 1:5 between plasmid and fragments were used.
- Half the amounts in the protocol were used with 10% extra to ensure a volume of 5 μL for each transformation.
- The incubation time was set to 60 min instead of 15 min.

Transformation

Chemically competent *E.coli* DH5α cells were transformed with the Gibson Assembly product following the "Transformation of chemically competent *E.coli* DH5α cells" protocol.

From the two replicates of the Gibson Assembly, two transformations were performed on one replicate and one transformation was performed on the other replicate. From the three transformations, cells were plated both in diluted and concentrated form using three plates for each form. The positive and negative control were also transformed.

Colony PCR

After overnight incubation, colony PCR on the transformed cells was performed following the "Colony PCR" protocol. Two and four colonies were taken from the diluted and concentrated plated cells, respectively.

Gel Electrophoresis

Gel Electrophoresis was performed according to the Gel Electrophoresis protocol.

Sequencing

The Mix2Seq kit was used for the preparation of the sequencing of colonies "O" and "Q". Five reactions per colony were prepared by making a mix which was then divided into five separate PCR tubes. Then 4 μL of the chosen primers were placed in its corresponding tube.

The primers used were:

- VF2, VR
- Bacillus Babes primers: Primer_seq_PyeaR_GFP_2x_acVHH_NarX_GB2_GB1_REV and Primer_seq_PyeaR_GFP_2x_acVHH_NarX_GB2_FOR .

All primers had an initial concentration of 5 µM.

Caffeine Assay

Caffeine Assay was performed on cultures from colonies “O” and “Q” according to the Caffeine Assay protocol. The cultures were incubated for 1.5h to let the cells reach an OD_{600} of 0.3. Note that this OD is not the same as in the protocol.

After incubation, 100 µL of the caffeine dilutions was added into 1.5 mL eppendorf tubes (with a total of 36 tubes). After the incubation time, 230 µL cells and 270 ddH20 were added to the tubes to reach an OD_{600} of 0.3. However, the cells reached an OD_{600} of 0.6 and it was diluted to 50% using LB-media before adding the prepared caffeine dilutions.

Absorbance and intensity was measured on the samples using a plate reader.

For the M9-medium assays, 15 mL Falcon tubes were used instead in order to increase oxygen levels for cell growth. Although the incubation time was increased, an OD_{600} of 0.3 was not reached before adding caffeine.