Detection Module Transformation in *B. subtilis*

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Description of the vectors used

For these experiments, 2 integration vectors were used: plasmids pBS1C and pBS2E (although the protocol optimization experiments were done only with pBS1C). All plasmids were linearized with a restriction enzyme prior to transformation, as it facilitates DNA intake by *B. subtilis*.

Plasmid pBS1C (https://www.addgene.org/55168/):

- Insertion in the AmyE locus
- Ampicillin resistance for *E.coli* transformation
- Contains mRFP for red/white screening in *E.coli*
- Chloramphenicol resistance for *B. subtilis* transformation (5µg/ml)
- Linearization by digestion with ScaI restriction enzyme
- Contains the PydfJ115_GFP insert

Plasmid pBS2E (https://www.addgene.org/55169/):

- Insertion in the LacA locus
- Ampicillin resistance for *E.coli* transformation
- Contains mRFP for red/white screening in *E.coli*
- MLS selection (1 µg/ml Erythromycin + 25 µg/ml Lincomycin) for *B. subtilis* transformation
- Linearization by digestion with PvuI restriction enzyme
- Contains the Rewired NarL + VHH-NarX insert

For double transformations, plates with 5µg/ml Chloramphenicol + 1 µg/ml Erythromycin + 25 µg/ml Lincomycin were used.

Assembling the constructs in their respective plasmids

The construct for the vector pBS1C was composed of just 1 fragment: PydfJ115_GFP, ordered from IDT as a gBlock. This plasmid needed to be linearized beforehand in order to remove the mRFP, thus making it suitable for Gibson Assembly.

The construct for the vector pBS2E was composed of 3 fragments: constitutive promoter + Rewired NarL; VHH-NarX; double terminator. The first and last fragment were ordered from IDT as gBlocks. VHH-NarX was instead extracted through PCR from one of the gBlocks ordered for the *E. coli* experiments. This plasmid also needed to be linearized beforehand.

Plasmid Linearization through PCR (removal of mRFP)

These vectors were isolated from an *E. coli* strain by using the GeneJET Plasmid Miniprep Kit, K0502. The PCR was done according to the "PCR - Phusion™ High–Fidelity DNA Polymerase" protocol, from ThermoFischer. This was then followed by DpN1 plasmid digestion (ThermoFischer protocol), which is recommended before a Gibson Assembly.

Primer Pair	*T_A*	*Amplicon Length*	*Elongation Time*
pBS1C_Linear_Fw *pBS1C_Linear_Rv*	*61 °C*	*6000bp*	*2min30s*
pBS2E_Linear_Fw *pBS2E_Linear_Rv*	*61 °C*	*6200bp*	*2min35s*

PCR Extraction of VHH-NarX

Done according to the "PCR - Phusion™ High–Fidelity DNA Polymerase" protocol, from ThermoFischer.

- Primers: *GAF22_Ext_Fw*; *GAF22_Ext_Rv*
  - T_A = 63 °C
- Fragment Length: 1100bp
  - 25s elongation time

Gibson Assembly

The NEBuilder HiFi DNA Assembly Reaction protocol was followed, but the incubation time was set to 60 min instead of 15 min. Additionally, before the assembly, all vectors and fragments that underwent PCR were purified using the Thermo Scientific GeneJET PCR Purification Kit K0701.

Vector:fragment molar ratios:

- PydfJ115_GFP + pBS1C construct 1:1 ratio
- Rewired NarL_VHH-NarX + pBS2E construct 1:3 ratio

Expressing the constructs in *B. subtilis*

E. coli transformation

The product of the 2 Gibson Assemblies was then separately cloned into *E. coli* according to the standard protocol. 13 pBS1C and 2 pBS2E clones were obtained, and all of them were submitted to colony PCR. Of these, 7 pBS1C and 2 pBS2E colonies were screened as positive. Afterwards, the positive clones were miniprepped (GeneJET Plasmid Miniprep Kit) and linearized through restriction enzyme digestion: pBS1C was digested with ScaI and pBS2E with PvuI, both provided by Promega.

B. subtilis double transformation #1

From the plasmids obtained in the last step, 1 pBS1C and 1 pBS2E were picked and transformed simultaneously into the same *B. subtilis* culture, following the ISN 2.0 protocol. This transformation yielded no colonies.

B. subtilis double transformation #2

In the second trial, the plasmids were transformed sequentially instead of simultaneously. In the first step, the pBS1C plasmids from all the 7 positive clones were transformed into *B. subtilis*. After incubation, all these plates had successful transformants (confirmed through colony PCR). A colony from each plate was cultured in liquid media overnight in order to conduct the second stage of the double transformation. 1 out of the 2 positive pBS2E clones had its plasmid miniprep transformed into these 7 overnights.

Since these colonies had already been transformed once and were growing in chloramphenicol, we decided to follow their growth –the timepoint at which natural competence is triggered could be delayed, which would imply changes in the transformation protocol. For these OD measurements, an Erlenmeyer flask was used with 10 ml of SP-media and 200uL of overnight culture. The values obtained were similar to the ones usually obtained for a wildtype culture. So, we did not change the incubation period mentioned in the ISN2.0 transformation protocol.

OD measurements:

OD 600nm	*Time (h) after incubation start*
0.184	1h15
0.626	2h15
1.28	3h15
1.628	4h10

After plating in double resistance plates, each plate yielded 1-3 colonies, including a negative control where a strain transformed only with pBS1C was inoculated in double resistance plates, pointing towards a failed transformation. The results of the colony PCR performed afterwards was also inconclusive.

Colony PCRs

Samples preparation and the thermocycler program are both described in the colony PCR protocol (Taq polymerase PCR), and the specific settings are as follows:

Primer Pair	*Role*	*T_A*	*Amplicon Length (if positive)*	*Elongation Time*
RNAse_HZ1_Fw *RNAse_HZ1_Rv*	*Colony PCR: Positive Control*	*48 °C*	*350bp*	20s
AmyE1_Fw *AmyE1_Rv*	*Colony PCR of pBS1C insert (PydfJ115_GFP)*	*54 °C*	*1800bp*	1min50s
AmyE2_Fw *AmyE2_Rv*	*Colony PCR of pBS1C insert (PydfJ115_GFP)*	*51 °C*	*1400bp*	1min25s
LacA1_Fw *LacA1_Rv*	*Colony PCR of pBS2E insert (Rewired NarL + VHH-NarX)*	*52 °C*	*700bp*	40s
LacA2_Fw *LacA2_Rv*	*Colony PCR of pBS2E insert (Rewired NarL + VHH-NarX)*	*54 °C*	*3600bp*	3min35s