Signal transduction and amplification

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Cloning

Level 0

Estimated time: 3 days (obtaining plasmids) + 3 days (cloning)

As the first step in the Type IIS assembly process, the basic parts were cloned into the Level 0 (pSB1C00) backbone (See Parts). pSB1C00_J04452 was acquired from the 2019 Distribution Kit. To take up DNA from the Distribution kit, we followed the corresponding protocol (**Distribution Kit DNA resuspension**). After taking up the DNA, the pSB1C00_J04452 plasmid was transformed into *E. coli* DH5α competent cells using the **transformation protocol**. Then, the plasmid was isolated from **overnight cultures** using the **GeneJET Plasmid Miniprep protocol**.

The basic parts were either synthetized or taken from the 2019 Distribution Kit. Some of the basic parts, the T7 polymerase and the T7 lysozyme were isolated from the BL21 pLysS strain via PCR (see Section 1.1.1).

The cloning of the basic parts into the Level 0 backbone was done using the **Type IIS cloning protocol** with SapI restriction enzyme. Once the cloning has been completed, 5 ul of the cloning reaction was transformed into *E. coli* DH5α competent cells using the **transformation protocol**. The transformed cells were plated on chloramphenicol plates. The plates were left to grow overnight and the white colonies were picked and screened by colony PCR the following day. The **colony PCR** and **agarose gel protocols** were followed to confirm the size of the integrated fragment. Overnight cultures of the positive clones were grown and the plasmid DNA was harvested following the **GeneJET Plasmid Miniprep protocol.** The concentration of DNA was assessed by Nanodrop (ThermoFisher). The extracted plasmids were sent for sequencing with the **Eurofins Sequencing Kit.**

Isolation of T7 polymerase and T7 lysozyme

Estimated time: 3 days (DNA isolation) + 3 days (cloning into Level 0)

The BL21 pLysS strain was used to get the sequences of the T7 polymerase and the T7 lysozyme by PCR amplification. The strain was obtained through an internal university source. The genomic DNA and the plasmid DNA were extracted from overnight culture using the **Genomic extraction** and the **GeneJET Plasmid Miniprep protocol**s. For the genomic extraction 15.5 mg pellet was used.

Once the genomic and plasmid DNA were isolated, the T7 polymerase and T7 lysozyme sequences were amplified through PCR following the **Phusion PCR protocol**. T7 lysozyme had the correct Type IIS fusion sites and SapI restriction sites after this PCR. The primers for the PCR (T7pol_G_Fw and Rv, T7lysFS-Fw and Rv) were ordered from IDT. To confirm the length of the amplified sequences, the PCR reaction was tested by gel electrophoresis.

In a second PCR step, the correct fusion and restriction sites were added to the T7 polymerase sequence (T7polFS-Fw and Rv). Then, T7 polymerase and T7 lysozyme were cloned into the Level 0 backbone with the SapI enzyme using **the Type IIS cloning protocol**.

QB3′ and Terminator fusion

Estimated time: 1 day (fusion PCR) + 3 days (cloning into Level 0)

In the Type IIS assembly, 4 basic parts can compose a transcriptional unit. The QB3′ and the terminator were fused together to fit within the 4 basic part limit for certain constructs.

Primers were designed to add the correct terminator Type IIS fusion sites and SapI recognition sites to the 5′ end of the QB3′ (Qbeta3'_w_SapI_Fw) and to the 3′ end of the terminator sequences (Ter_w_SapI_Rv). Other primers were used to add SapI recognition sites on the 3′ end of the QB3′ (Qbeta3'_w_SapI_rv) and the 5′ end of the terminator (Ter_w_SapI_Fw). These recognition sites were positioned that when the enzyme cuts, the two sequences can ligate scarlessly. The primers were ordered from IDT and the sites were added by PCR, following the **Phusion PCR protocol**.

After the PCR, the QB3′ and terminator sequences with the added overhangs were assembled and cloned into the Level 0 backbone through the Type IIS assembly. As a result of one cloning reaction, the QB3′ and the terminator sequences fused together scarlessly and were inserted into the Level 0 backbone.

Level 1

Estimated time: 3 days

To assemble the transcriptional units (TU), the parts in the pSB1C00 were cloned into one of the Level 1 backbone (pSB1K0#_J04454). The Level 1 backbones were obtained from the 2019 Distribution Kit as described above for the Level 0 backbone. The cloning was done using the previously assembled basic parts in Level 0 backbone with the BsaI restriction enzyme following the **Type IIS cloning protocol,** and you can see all the composite parts on the Parts page.

Once the cloning has been completed, 5 ul of the cloning reaction was transformed into *E. coli* DH5α competent cells using the **transformation protocol**. The transformed cells were plated on kanamycin plates. The plates were left to grow overnight and the white colonies were picked and screened by colony PCR the following day. The **colony PCR** and **agarose gel protocols** were followed to confirm the size of the inserted fragment. Overnight cultures of the positive clones were grown and the plasmid DNA was harvested following the **GeneJET Plasmid Miniprep protocol**. The concentration of DNA was assessed by Nanodrop (ThermoFisher). The extracted plasmids were sent for sequencing with the **Eurofins Sequencing Kit**.

Level 2

Estimated time: 3 days

Up to 4 TUs in pSB1K0# (Level 1) plasmids can be assembled together to form multi-transcriptional units (MTUs) in pSB3C1# (Level 2) plasmids. pSB3C1# plasmids are obtained by modifying pSB3C0# plasmids (see Development of iGEM Type IIS standard). This is done by using the **Type IIS cloning protocol** and SapI restriction enzyme. The cloned MTUs can be found on the Parts page.

After cloning, 5uL of the cloning reaction were transformed to *E.coli* DH5α using the **Transformation protocol** and left growing overnight. The next day, white colonies were picked for screening following the **colony PCR** and **agarose gel protocols**. Overnight cultures of the positive clones were grown overnight, following the **Overnight culture protocol**. Afterwards, plasmid DNA was extracted from the cultures using the **GeneJET Plasmid Miniprep Kit** and protocol and the concentration of DNA was assessed by Nanodrop (ThermoFisher).. The resulting plasmids were sent for sequencing with the **Eurofins Sequencing Kit**.

Sequencing

Estimated time: 1 day (over night)

Sequence confirmation of cloned parts was performed with the use of Mix2Seq Kit OVERNIGHT (Eurofins) according to manufacturer's instructions (found in the **sequencing protocol**). Purified DNA of appropriate concentration was mixed with one of the sequencing primers (VF2, VR or custom primers) and nuclease free water. The results were analysed with the help of SnapGene 5.1.5. Version and Benchling online tool.