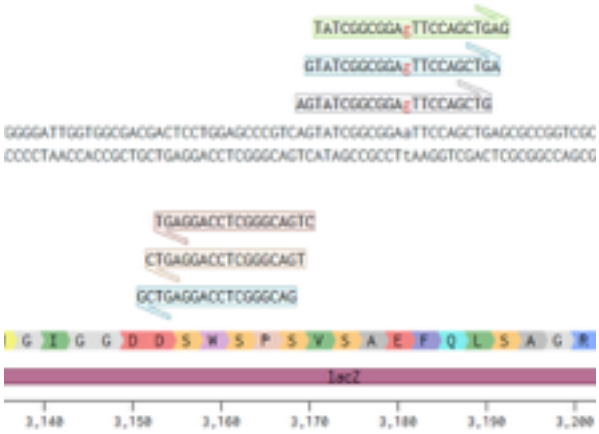
Preparation of Sensors for iGEM-Submission:

1) Mutagenesis

Goal:
Introduce a silent mutation at position (3181;G>A) in lacZ to remove EcoRI restriction site.

Methods:
We used Q5 mutagenesis kit (NEB) and designed primers using NEBaseChanger software. The software recommended a forward-primer introducing the mutation and a reverse primer with suitable tm to complete the plasmid. Since the software neither provided a ranking of other primer pairs nor detailed information on how the primers are chosen, we designed two more primer pairs introducing the mutation. Mutagenesis was carried out according to the manufacturers protocol with 1ng, 8ng and 20ng of plasmid being used for each primer pair. Successful mutagenesis was confirmed by sequencing.

Results: (Möglichst Sequencing ergebnisse noch hochladen; esther hatte aber heute frei :-/ Alternativ einfach verweis, dass mittels Sequencing confirmed wurde aber Upload aufgrund einer Up-Geschwindigkeit von 2KB/h auf den iGEM-Server nicht möglich war..)



2) Cloning into pSB1C:

Goal:
All of our experiments were PCR-Based. For this reason, we had to clone our sensors into pSB1C for iGEM-Submission only and were selecting two strategies to reach this goal:

(i) Cloning of our PCR-Product into pGEM®-T Easy vector and subsequent insertion of the insert to pSB1C via Not1-Restriciton sites.

(ii) Amplifying our PCR-Product using tailed primers introducing the restriction sites SpeI and XbaI. Direct insertion into pSB1C

Methods:
For TA-Cloning 5,5 uL of PCR-Product, generated by Phusion PCR according to our SOPs, were incubated with 0,5 uL Units GoTaq® Flexi DNA Polymerase (5U/uL) from Promega, 2ul dATP (2mM), and 2uL GoTaq® Flexi Buffer (5X) for 30 minutes at 70°C. Ligation was performed over night at 4°C using T4-Ligase (M180A Promega) at a molar vector:insert ratio

of 1:1 and transformed to ultracompetent DH5a-Cells (NEB). Positive clones were identified by colony PCR using a primer annealing to the T7-promoter as forward-primer and two reverse primers annealing to lacZ. These primers worked well for sequencing the switches already and thus we decided to use them again.
FW_T7: GCGAATTAATACGACTCACTATAGGG
Rev_Seq1 (310BP-Product): TGAATGGCGAATGGCGCTTTG
Rev_Seq2 (445 BP-Product): CATCTACACCAACGTGACCTATC
5mL bacterial cultures were grown from 4 positive clones per sensor. DNA was isolated the next day using NucleoSpin MiniPrep System (Merchery-Nagel) according to the manufacturer's instructions. DNA was digested using NotI HF (1U/ug of DNA in CutSmart Buffer) for one hour and heat-inactivated for 20 minutes at 65°C. 2,5 ug of digested DNA was loaded on a gel, bands were excised and cleaned up using ZymoClean™ Gel DNA recovery kit according to the manufacturer's instructions. Recovery of DNA was low (12-30ng/uL). Never the less we tried to clone the Gel-Purified, dephosphorylated pSB1C-Backbone, which was a kind gift from iGEM Potsdam. Ligation was performed using Quick Ligase (NEB) with a Backbone:Insert Ration of 1:1 and 1:3. Subsequent transformation into ultracompetent DH5a-Cells and growth on Agar plates containing Chloramphenicol did not result in any colonies.

For direct cloning into pSB1C using tailed primers, we designed primers introducing XbaI to the 5' end and SpeI to the 3' end of the PCR Product. For forward primers two variations were designed to speed experiments up:
FW1: XbaI.T7 GCCGCTTCTAGAtaatacgactcactatagg,
FW2: XbaI.T7.6NT GCCGCTTCTAGAGCGAATTAATACGACTCAC
Rev1: SpeI.LacZ.stop GCCGCTACTAGTTTATTTTTGACACCAGACC

Inserts were amplified using Phusion DNA Polymerase (Thermo Fisher) and cleaned up using ZymoClean™ PCR CleanUp-Columns (Zymo Research). Purified PCR products were digested using SpeI and XbaI for 1 hour (all restriction enzymes and Cutsmart Buffer were purchased by NEB).Following heat inactivation at 80°C for 20 minutes the PCR product was purified as described above. The pSB1C-Backbone was cut using the restriction enzymes SpeI and XbaI. To decrease the chance for re-ligation of the vector, we dephosphorylated the vector backbone using calf intestinal alkaline phosphatase (CIP) according to the manufacturer's instructions (NEB). The digested and phosphorylated backbone plasmid was run on a 1% agarose gel and the aprox. 2.2kb band was cut and purified using the Gel Extraction ZymoClean Kit (Zymo Research). Subsequently, we ligated the purified digested PCR products with the phosphorylated pSB1C backbone using Quick Ligase for 15 minutes at room temperature and transformed the ligated product into ultracompetent DH5a cells. Positive colonies were identified using Colony PCR as mentioned above but with a different forward primer, that binds to the iGEM backbone (VF2 primer ATTACCGCCTTTGAGTGAGC). Bacterial cultures were grown over night and DNA was purified using Quick DNA universal Kit (ZymoResearch) according to the manufacturer's instructions.