1. Overview DNA-Switch Syntheses

Toehold switches were constructed by two PCR-based steps, in which the recognition and hairpin region was attached to a LacZ reporter element. The construction was amplified from a circular DNA Template. For this we used the ZIKV_Sensor_28B_LacZ Plasmid. [1]

Primer P1 and P2 are specifically designed for different Toehold switches (Abb. 1). These include the Recognition-Structure, the characteristic Hairpin, Ribosome binding site and Linker. The Switches were constructed by ligating them to the *lacZ* reporter gene.

A specific third step was used to purify the preceding amplifications and also attach a T7 Promotor region.

1.1. Step 1 Extention-Primer_P1 PCR

1.1.1. PCR-Protocol

For Switch-Syntheses three PCR-Steps are needed. The first two are used to ligate two extension primer to the LacZ reporter gene, while the third step attaches a T7-Promotor region.
All PCR reactions should be set up on ice. The Master mix must be prepared for the appropriate number of samples as shown in the table given below. (*Table 1*) The assembly should start with the biggest volume, in this case water. Phusion DNA Polymerase should be pipetted last to prevent primer degradation. The finished mix needs to be put into the Mastercycler at run at program iGEM60C.cyc.

Components	**general information for 25 µl PCR-solution (given in µl)**
ddH2O	16.75
AccuPrime Buffer A (GC-rich) 5X LOT: 1895683	5
Phusion from AG Schuelke LOT: 380550	0.25
DNA Template (2.5 pg/µl)	1
Primer Reverse uR.2 LacZ (10pmol/µl)	1
total	24
add idividually	
forward primer WS (10 pmol/µl)	1

Table 1: Pipetting instructions for 25 µl P1 PCR-assembly

1.1.2. Gel-Electrophoresis-Protocol:

The PCR-Product then shall be evaluated by Agarose gel-electrophoreses. Electrophoresis uses an electrical field to move the negatively charged DNA through the matrix. The DNA therefore will be separated by length. As our product has an expected length of about 3.000bB we use a 1kB ladder for comparison.

A big Gel-Cast shell be prepare with 120ml 0.5x TBE-Buffer and 1.2 g of Agarose (1%). After boiling 6.5 µl RotiSafe are added and the mixture is spread in prepared cast.
Add a mixture of 2 µl loading dye and 3 µl PCR product into wells.

If bands are shown one can proceed with the PCR Clean up and step P2.

[1] available through Addgene (plasmid number: 75006)

1.1.3. Clean up

For the PCR-Clean up we use the Wizard SV Gel and PCR Clean-up System by Promega. PCR purification is an important step to remove excess nucleotides, salts and additives.
Add an equal volume of Membrane Binding Solution to the PCR amplification. Transfer the mix onto the spin column and place column on 2ml collecting tube. Incubate for 1min.
Spin 1 min at 14.000 rpm and discard the flow through. Add 700 µl wash buffer (B) for the first washing step and spin 1 min at 14.000 rpm. The flow-through shell be discarded again. For second washing step add 500 wash Buffer (B) and spin 5 min at 14.000 rpm. After discarding the flow through, dry by spinning 1 min at 14.000 rpm. Place spin column into new 1.5 ml micro centrifuge tube and add 50 µl HPLC water. Incubate 1 min at room temperature and centrifuge 1 min at 14.000 rpm
No filter tips or nuclease-free water is needed for P1.
Centrifuge needs to be balanced for every use.

Determine concentrations in NanoDrop. Therefore blank the spectrophotometer with 1 µl HPLC water. Afterwards 1µl clean PCR Product can be evaluated. The ratio at 260nm and 280nm absorption is used to validate the purity of DNA. A A260/A280 of about 1.8 is considered pure for DNA.[2]

1.2. Step 2 Extention-Primer_P2 PCR

All P1 products need to be diluted to 2.5ug/ml for further use.
For step P2 PCR reactions as shown in Table 3 should be set in the same manner as P1. The diluted P1-product (template) and P2-forward primer are added individually or every switch. Again Mastercycler and Programm IGEM60C.cyc need to be used.

Components	general information for 50 µl PCR-solution
ddH2O	36.5
AccuPrime Buffer A 5X	10
Phusion	0.5
Primer Reverse uR.2 LacZ (10pmol/µl)	1
total	48
add individually	
forward primer2 WS (10 pmol/µl)	1
diluted Produkt Primer1 (2.5 pg/ µl)	1

Table 2: Pipetting instructions for 50 µl P2 PCR-assembly

Agarsose gel electrophoresis and clean up are conducted as step P1 (1.1.2 - 1.1.3.) Concentration is determined in a NanoDrop spectrophotometer.

1.3. Step 3 T7+6-Primer_P3 PCR

Dilute all P2-products to 2.5ug/ml and proceed to step P3-PCR in the same manner as P1 and P2 (see 1.1.1). Master mix components are given in Table 4 below.

Components	general information for 25 µl PCR-solution
ddH2O	16.75
NEW AccuPrime Buffer A (GC-rich) 5X LOT: 1895683	5
Phusion from AG Schuelke LOT: 380550	0.25

[2] *https://www.thermofisher.com/de/de/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/ultraviolet-visible-visible-spectrophotometry-uv-vis-vis/uv-vis-vis-instruments/nanodrop-microvolume-spectrophotometers.html*

T7+6 HPLC forward primer WS (10 pmol/µl)	1
Primer Reverse uR.2 LacZ (10pmol/µl)	1
total	24
add individually	
DNA Template (2.5 pg/µl)	1

Table 3: Pipetting instructions for 25 µl P3 PCR-assembly

Put the mix into the Mastercycler and run program IGEM40C.cyc.

Run agarose gel electrophoresis as before to interpret PCR results. In a 1.5 ml microcentrifuge tube, add 125 µl of DNA Binding Buffer to PCR product.. Mix briefly by vortexing. Transfer the assembly to a Spin Column in a Collection tube. This shell be centrifuged for 30 seconds. After discarding the flow through ass 200 µl DNA Wash Buffer and centrifuge again for 30 seconds. Repeat the wash step. Add 12 µl HPCL water and incubate at room temperature for 1 minute. Transfer the column into a 1.5ml tube and centrifuge for 30 minutes. Concentration of the eluted DNA must be determent in NanoDrop.

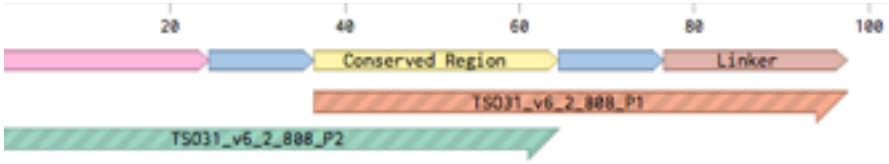

Abb. 1: Primer P1 and P2