Overview

1. Introduction

This lab book with its appendix describes all the necessary considerations, protocols and experimental steps we used for the creation of the described system. It focuses on the practical work, the scientific background won't be discussed extensively but you find a short introduction to the theme in this first chapter. A detailed description of the theoretical background as of the modeling and animation parts is available at our iGEM website; its consideration will contribute to the understanding of our project.

Sequences of all mentioned genes and primers are shown in the appendix. The concept of the primer design is also included.

The lab book provides a general description of the used protocols (chapter 2), followed by a part-wise documentation of the experimental procedure where we show how each Biobrick has been created (chapter 3). Then the detailed protocols for the assembly of these Biobricks to larger ones are explained (chapter 4). In the final part of this lab book, the experimental steps for the expression are represented (chapter 5).

For the summer project iGEM 2010 the plan was to create a light-controllable specific protein degradation system. The system contains several parts: the bacterial ClpXP protease from *E. Coli*, the photoreceptor protein Phytochrome B and the Phytochrome Interacting Factor (PIF 3 or 6) from *A. thaliana*, and the DAS+4/LAA/λO recognition sequences for ClpX. Those are the main parts of our system.

The ClpXP proteases consist of three main parts: the ClpX unit and two units of ClpP. The ClpX is a hexamere consisting of six identical subunits. The ClpX is responsible for recognizing proteins containing a specific degradation tag, unfolding and leading them into the catalytic core of the enzyme, the two ClpP subunits.
We decided to use in this project ClpX knockdown cells provided from Tanja Baker from the MIT, in which a plasmid with three expressed units of ClpX. These subunits, connected through a 20aa long linker, will assemble in the cell to the full hexameric ClpX. For the light-dependent part of the system the photoreceptor protein Phytochrome B (PhyB) is fused to the N-terminal side of each of these two trimers. PhyB is characterised by a red/far-red photochromicity. Through red-light absorption (650–670 nm)PhyB undergoes a rapid conformational change from its ground state P_r to its active state P_{fr}. The structural change allows the binding of different interacting factors (PIF).The process is completely reversible through absorption in the near infra-red spectrum (705-740nm).

Target proteins are fused to the PIF and tagged with the degradation sequence which, through light activation, brings the degradation sequence in proximity to ClpX and guides them to the catalytic core of the protease. Therefore a specific degradation of proteins containing the degradation sequence can be induced by a light signal.

To test protein degradation GFP was fused to PIF 3/6 and the specific degradation tag LAA, DAS+4 or λO. LAA is the native degradation sequence of *E.coli* for ClpX, the DAS+4 sequence is artificially altered so that it have weakened interactions with ClpXP and depends on an adaptor. The λO- tag is the N-terminal equivalent to the DAS+4 tag. In *E. coli*, the adaptor SspB tethers specifically tagged substrates to the ClpXP protease, causing a modest increase in their rate of degradation. In our system, the role of the adaptor-protein SspB has been assumed by Pif3/6. So only light-induced activation can lead to binding and efficient degradation of DAS+4 bearing constructs.

2. Overview of Methods and Material

Though background and application are different for each BioBrick, the experimental procedure for their creation contains a number of common steps in general. The principal stages are listed below:

1. **Plasmid amplification / Amplification from genome (for ClpX)**
2. **Integration in iGEM-Plasmid**
3. **Mutagenesis (for ClpX, PhyB900 and Pif3)**
4. **Plasmid amplification (stock)**

In the following we give you an overview of the protocols of which our experiments are based on. The listed protocols present the standard form we will refer to in next chapters. Nevertheless the variables have been adapted and modified in many cases; all changes will be noted in the individual protocols.

Before the work on the principal experiments can start the groundwork have to be lain. This includes the preparation of cell culture media and required chemical compounds as the production of competent cells if you have no possibility to use commercial cells. Therefore we will start this overview with the overview of acquired materials for cell media and the optimized protocol for the preparation of competent cells.

Materials for cell culture media

LB liquid medium (1L)
10g tryptone
5g yeast extract
10g NaCl
add 800ml water dissolve (check pH between 7-8 if not adjust with NaOH)
adjust to 1L
autoclave

LB agar medium (1L)
10g tryptone
5g yeast extract
10g Nacl
add 800ml water dissolve (check pH between 7-8 if not adjust with NaOH)
add 15g agar
melt agar into solution heating (or microwave)
adjust to 1L
autoclave

SOB medium (1L)
20g tryptone
5g yeast extract
0.5g NaCl
dissolve in 950mL dH2O
add 10mL 250mM KCl
bring to pH 7 with NaOH
adjust volume to 1L
autoclave
add 5ml sterile 2M MgCl2

SOC medium (100ml)
Add 2ml sterile 1M glucose solution to 100 ml SOB

Protocol for preparation of competent cells

Background:

Preparation of competent cells is an essential step to realize successful cloning. It is important to be able to have quickly at disposition cells that can be efficiently transformed. Therefore the following protocol describe how to create E. coli competent cells that can be frozen at – 80°C maintaining a transformation efficiency about 10^7-10^8 colony forming units (cfu)/µg DNA.

Required material:

- Room at 37°C
- Cold room at 4°C
- E. Coli liquid culture
- 0,1M CaCl2
- Transformation buffer (16% Glycerol, 0,08M $CaCl_2$: 2,5ml 80% Glycerol +10ml 0,1M $CaCl_2$)
- CASO-Bouillon-PEG stérile et froid (85 % de milieu de culture CASO-Bouillon, 10 % PEG (8000), 5 % DMSO, 50mM MgCl2).

Protocol Steps:

- Transfer 4ml of your 4ml into 500ml of fresh and sterile LB medium

- Incubation at 37°C and 150rpm shaking until reaching the optimal optical density of OD=0,5-0,6 (measurement at 600nm).

- Transfer the 500 ml into 10 falcon tubes of 50 ml.

- Centrifuge for 10min at 4°C and 4000rpm, throw the supernatant

- Resuspension of the pellet in 20ml cooled 0,1M $CaCl_2$

- Centrifuge for 10min at 4°C and 4000rpm, throw the supernatant

- Resuspension of the pellet in 50ml cooled 0,1M $CaCl_2$

- Suspension on ice for 30min

- Centrifugation at 4°C, 4000rpm for 10min

- Resuspension of the pellet in 12 ml cooled transformation buffer (16% Glycerol, 0,08M $CaCl_2$: 2,5ml 80% Glycerol +10ml 0,1M $CaCl_2$)
- Aliquotisation: 100µl per 1,5ml Tube, in 4°C-room as fast as possible, or plunged into dry ice.
- Storage under -80°C
- Thaw cells on ice.
- Use 50µl per transformation
- Add between 0,01µg and 1µg DNA depending on the DNA source (plasmid transformation, ligation…)
- Add 50µl of PEG-DMSO previously thaw on ice.
- Let the cells incubate 30 minutes in ice.
- Heat-shock 1 minute at 42°C
- Plunged cells in ice for two minutes
- Resuspend cells into 900µl of SOC for a better efficiency, SOB or LB medium work too.
- Incubate at 37°C for 1h00
- Plate on LB agar with the right antibiotic resistance
- Incubation overnight at 37°C

⇨ Using only 50µl of those frozen bacteria gives rise to a transformation efficiency of about 10^8 colony forming units /µg DNA used. The test were done with the plasmid pUC19 and the E. coli strain were either the ATCC 8739 or some E. coli ClpX – gotten from Tania Baker's laboratory (MIT)

Troubleshooting:

At the beginning, several protocols have been tried, arisen either from personal internship experiences of the team members, from the practical cours of Enzyme-free Cloning or from the openwetware website directed by the MIT and recommended by the iGEM supervisors.

The combination of several protocols worked the best and allowed us to optimize our protocol integrating the following crucial steps:

- Using PEG-DMSO for the first incubation. Indeed, skipping this step decrease the transformation efficiency by 10^2 cfu/µg DNA.
- Respecting the 30 minutes incubation on ice before the heat shock
- Using SOC medium instead of LB medium allows a better recovery and quicker growth of the cells

Plasmid Amplification

a) Transformation (Heat-choc)

Thaw cells slowly on ice

Ad 50µl of LB-PEG/DMSO to 50µl of your cells

Add 100µl of cells/LB-PEG to 1 ng of DNA

Leave on ice for 10-30 min

Heat shock 1min, 42°C, cool on ice for 2 to 5 min

Add 1 ml SOC or LB , incubate at 37°C for 30-60 min

b) Miniprep

Following the instructions of the manufacturer

Integration in an iGEM-Plasmid

a) PCR with primers containing the standard BioBrick prefix and suffix

Prepare the reaction mix, briefly vortex and centrifuge all reagents before starting

Calculate necessary volume of template DNA for an amount of 1to 10 ng (depending on the measured concentration!)

Dilute primer to 6pmol/µl
Prepare reaction mix (here for Roche Expand High Fidelity PCR system)

5 µl High Fidelity Buffer (10x conc. with 25mM MgCl2)
1 µl Desoxynucleotide mix (10mM of each dNTP)
2 µl upstream primer (6pmol/µl)
2 µl downstream primer (6pmol/µl)
_µl template DNA
39,25 – $V_{template\ DNA}$ of sterile water
0,5 µl DNA polymerase (High Fidelity Enzyme Mix)

Vortex gently and centrifuge briefly before adding the polymerase

Thermal cycling:

As the primers contained the BioBrick standard pre- and suffixes for the integration in the iGEM-plasmid the primer presented lengths between 40 and 55 bp and Tm-values up to 90°C. Consequently the annealing temperature had to be chosen as high as possible but different to the extension temperature that favors polymerase activity. As such high temperatures generally favor the specificity but not the yield of the PCR products we chose the touch-down principle for

the setting of the thermal cycler and decreased the annealing temperature about 0,2°C at each step.

	Temperature (°C)	Time (min)
1) Initial Denaturation	**94**	**8**
2) Denaturation	**94**	**0.5**
3) Annealing	**65**	**0.5**
4) Extension (for 1kb)	**72**	**1**
Go to Step 2, 25 times decreasing temperature by 0.2°C per cycle		
5) Final extension	**72**	**7**
Cooling	**4**	**Infinity**

b) Control (Gel electrophoresis)

In most of the cases 1%-agarose-gels were used allowing an optimal separation from 200 to 10000 basepairs.

c) Purification of PCR-Products

Following the instructions of the manufacturer

d) Digestion with EcoRI and PstI

Prepare reaction mix :

For double digestions the optimal buffer promoting maximal activity of each of the restriction enzyme has to be determined in a first place, e.g. using the NEBuffer Activity Chart for Restriction Enzymes.

5 µl template (≈500ng)
5 µl NEB Buffer 2
0,5 µl BSA
1 µl EcoRI HF 20 U/µl (NEB)
1 µl PstI 20 U/µl(NEB)
37,5 µl sterile H2O

Digestion in 50 µl reaction volume for 10 min at 37°C

Heat inactivation of the restriction enzymes at 80°C for 20 min

e) Ligation

Ligation in the *EcoRI/PstI*-precutted vector psB1C3

Different insert-vector ratios in 20 µl reaction volume

Amount of insert and vector according to size and concentration
2 µl T4 DNA Ligase Buffer
1 µl T4 DNA Ligase (NEB)
Filling up to 20 µl with sterile H2O

Ligation for at least 10 min at room temperature

Heat inactivation of the ligase for 10 min at 65°C

f) Transformation >> See 1.a) !

g) Isolation on Agar-Plates

Plate on selective medium

Incubate overnight at 37°C (inverse the plate!)

h) PCR Colony Screening

Prepare an hot water-bath to 99°C !

Prepare a fresh agar-plate, use a marker to divide it in 4 sections and number them

Work under sterile conditions under a flame

Prepare 4 eppendorf-tubes by adding 20µl of sterile water and number them

Choose a colony from your transformation-plate, use a 200 micropipette-cone to transfer the half of it to the first prepared eppendorf-tube (pipette up and down several times)

Take the rest and position it to the corresponding quarter of your prepared agar-plate

Repeat this experience for the remaining (2-4) colonies

Incubate the plate overnight at 37°C (inversed!), the “mother”-plate is conserved at 4°C

Continuation with the bacteria-containing eppendorf-tubes : Vortex intensely

Place the tube in the hot water-bath for 5 min

Centrifuge 1 min at 10 000 rpm

Transfer the supernatant with a 200 micropipette to a PCR-tube (mark the corresponding number)

Follow the standard PCR-protocole (see 2.a), to economize material the volume of the samples can be adjusted to 25µl.

Prepare a preculture of the colonies which you got positively tested

Incubate overnight at 37°C

Mutagenesis (for PhyB900, ClpX, Pif3)

a) Miniprep

Following the instructions of the manufacturer

b) Pfu-Mutagenesis

For the mutagenesis PCR reaction a Pfu-polymerase was used, at the beginning the PfuTurbo DNA polymerase from Stratagene with was provided by Dr. Maria Zeniou and later the Pfu-DNA polymerase from Fermentas.

Thus the variables and parameters of the standard protocol *(see 2.a)* were adapted following the instructions of the manufacturer.

c) Digestion with DpnI

Add 1 µl (10U) of the *Dpn* I restriction enzyme directly to each
Amplification tube, mix by pipetting the solution up and down several times

Centrifuge for 1 minute

Incubate at 37°C for 1 hour

d) Control (Gel-Electrophoresis)

e) Transformation >> See 1.a)!

f) Isolation on Agar-Plates

g) PCR Colony screening >> See 2.h) !

h) Sequencing

Plasmid amplification (stock) >> See point 1)!

3. Biobrick Creation

The term "BioBrick" describes a standard for interchangeable parts, developed with a view to building biological systems in living cells. Each part is flanked by a prefix and suffix that have to be compatible with the BioBrick standards. The standard suffix contains a EcoRI (g^aattc (5'->3')) and a XbaI (t^ctaga (5'->3')) restriction site, connected via a NotI (NotI gcggccgc (5'->3')) site. The standard prefix contains a SpeI (a^ctagt (5'->3')) and a PstI (ctgca^g (3'->5')) restriction site, also connected via a NotI site.

The BioBrick standard prefix and suffix with its easy cloning strategy offer an excellent and universal way to combine various parts, e.g. promoter region, gene of interest, terminator etc. However, also proteins consist of different parts and the combination of fusion proteins is an important task in Synthetic Biology. However, this modular assembly from BioBrick standard parts is not possible due to the generation of a stop codon at the SpeI/Xba scar (...act**actagag**ca...).

Therefore, some of the former iGEM-teams developed new fusion standards for the modular construction of protein fusion parts compatible with the first BioBrick version. We chose the Fusion Protein BioBrick Assembly Standard developed by the iGEM Team Freiburg 2007, which contains two additional compatible restriction sites NgoMIV (**g^ccggc (5'->3')**) and AgeI (a^ccggt (5'->3')). The resulting scar codes for amino acids, which are compatible with flexible linkers as well as with the N-end rule for protein stability.

BioBricks Standard

	PREFIX	SUFFIX
With ATG	gaattcgcggccgcttctag	tactagtagcggccgctgcag
Without ATG	gaattcgcggccgcttctagag	

Fusion Protein BioBrick Assembly Standard

	PREFIX	SUFFIX
Fusion	gaattcgcggccgcttctagatg**gccggc**	accggttaatactagtagcggccgctgcag

In the following we show how each Biobrick has been created, starting with a short presentation of its background and conception.

Below you find the complete list of the single biobricks parts we created.

- **ClpX**
- **Phytochrome B (1-650)**
- **Phytochrome B (1-900)**
- **PIF6**
- **PIF3**
- **Linker20**
- **DAS+4**
- **LAA+4**
- **Lambda**

ClpX(-N) biobrick

Background

ClpXP is a part of an E.coli protease which consists of three parts, the hexametric ClpX and two heptametrical ClpP subunits. ClpX consists of six identical subunits, each 1092bp long. ClpX recognizes and unfolds protein containing certain tags like LAA and leading them into the catalytic center of this protein complex, the two ClpP units. ClpX has two internal restriction sides for EcoRI and two restriction sides for AgeI.

Conception

ClpX has two internal restriction sides for EcoRI and two restriction sides for AgeI. The purpose of this first experimental part was to extract the ClpX gene out of the E.coli genome, to alter the internal EcoRI and AgeI sides in the ClpX gene and to fuse iGEM fusion pre- and suffixes to the ClpX sequence in order to get an iGEM Biobrick with standard prefix and suffix standard without internal EcoRI, Not, XbaI, AgeI, SpeI and PstI sides.

The sequence was obtained from the following database for *DH5α* E.coli cells.

- http://www.ncbi.nlm.nih.gov/gene/945083
- http://www.ncbi.nlm.nih.gov/nuccore/49175990?from=456650&to=457924&report=gbwithpars

gaattcgcggccgcttctagatggccggcCGCAGTGCGCTACCGACGCCGCATGAAATTCGCAACCACCTGGACGATTACGTT
ATCGGCCAGGAACAGGCGAAAAAAGTGCTGGCGGTCGCGGTATACAACCATTACAAACGTCTGCGCAACGGCGAT
ACCAGCAATGGCGTCGAGTTGGGCAAAAGTAACATTCTGCTGATCGGTCCGACCGGTTCCGGTAAAACGCTGCTG
GCTGAAACGCTGGCGCGCCTGCTGGATGTTCCGTTCACCATGGCCGACGCGACTACACTGACCGAAGCCGGTTATG
TGGGTGAAGACGTTGAAAACATCATTCAGAAGCTGTTGCAGAAATGCGACTACGATGTCCAGAAAGCACAGCGTG
GTATTGTCTACATCGATGAAATCGACAAGATTTCTCGTAAGTCAGACAACCCGTCCATTACCCGAGACGTTTCCGGT
GAAGGCGTACAGCAGGCACTGTTGAAACTGATCGAAGGTACGGTAGCTGCTGTTCCACCGCAAGGTGGGCGTAAA
CATCCGCAGCAGGAATTCTTGCAGGTTGATACCTCTAAGATCCTGTTTATTTGTGGCGGTGCGTTTGCCGGTCTGGA
TAAAGTGATTTCCCACCGTGTAGAAACCGGCTCCGGCATTGGTTTTGGCGCGACGGTAAAAGCGAAGTCCGACAA
AGCAAGCGAAGGCGAGCTGCTGGCGCAGGTTGAACCGGAAGATCTGATCAAGTTTGGTCTTATCCCTGAGTTTATT
GGTCGTCTGCCGGTTGTCGCAACGTTGAATGAACTGAGCGAAGAAGCTCTGATTCAGATCCTCAAAGAGCCGAAA
AACGCCCTGACCAAGCAGTATCAGGCGCTGTTTAATCTGGAAGGCGTGGATCTGGAATTCCGTGACGAGGCGCTG
GATGCTATCGCTAAGAAAGCGATGGCGCGTAAAACCGGTGCCCGTGGCCTGCGTTCCATCGTAGAAGCCGCACTG
CTCGATACCATGTACGATCTGCCGTCCATGGAAGACGTCGAAAAAGTGGTTATCGACGAGTCGGTAATTGATGGTC
AAAGCAAACCGTTGCTGATTTATGGCAAGCCGGAAGCGCAACAGGCATCTGGTGAAaccggttaatactagtagcggccgc
tgcag

Problem: 2 AgeI and 2 EcoRI sides

Primers for cloning ClpX out of the E.Coli genome

These primes were used to amplificate ClpX from the *E.coli* genome.

Forward primer (5'->3') : 31bp
CGCAGTGCGCTACCGACGCCGCATGAAATTC
Reverse primer (5'->3') : 32bp
TTCACCAGATGCCTGTTGCGCTTCCGGCTTGC

Primers for Pfu-mutagenesis

1. ACC=Thr AC(A,T,G,C), GGT=Gly GG(A,T,G,C) AgeI site
   Forward primer (5'->3') (31 bp)
   CTGATCGGTCCGACTGGTTCCGGTAAAACGC
   Reverse primer (5'->3') (31 bp)
   GCGTTTTACCGGAACCAGTCGGACCGATCAG

2. GAA=Glu GA(A,G), TTC=Phe TT(C,T) EcoRI site
   Forward primer (5'->3') (28 bp)
   CATCCGCAGCAGGAGTTCTTGCAGGTTG
   Reverse primer (5'->3') (28 bp)
   CAACCTGCAAGAACTCCTGCTGCGGATG

3. GAA=Glu GA(A,G), TTC=Phe TT(C,T) EcoRI site
   Forward primer (5'->3') (25 bp)
   CGTGGATCTGGAGTTCCGTGACGAG
   Reverse primer (5'->3') (25 bp)
   CTCGTCACGGAACTCCAGATCCACG

4. ACC=Thr AC(A,T,G,C), GGT=Gly GG(A,T,G,C) AgeI site
   Forward primer (5'->3') (24 bp)
   GGCGCGTAAAACTGGTGCCCGTGG
   Reverse primer (5'->3') (24 bp)
   CCACGGGCACCAGTTTTACGCGCC

Primers for amplification of ClpX with fusion pre- and suffix

After mutagenesis of internal restriction sides, the fusion pre- and suffixes were added to the ClpX gene.

Forward primer (5'->3'):
NNNNNNgaattcgcggccgcttctagatg**gccggc**CGCAGTGCGCTACCGACGCCGC
Reverse primer (5'->3'):
NNNNNNctgcagcggccgctactagtattaaccggtTTCACCAGATGCCTGTTGCGC

Followed procedure to obtain this biobrick:

The experimental procedure to obtain the ClpX-Biobrick can be divided in three principal parts:

1. **Isolation of the ClpX-gene out of E.coli genome**
2. **Cloning of ClpX into the pUC19 vector**
3. **Site directed mutagenesis of restriction sites**

Part 1: Isolation of ClpX out of E.coli genome

The DNA of E.coli was extracted by the following protocol from a previous TP of Molecular genetics. Then the ClpX gene extracted and amplified via PCR.

DNA extraction

- 1,5ml of bacteria culture were centrifuged by 10000rpm for 2min.
- Pellet was resuspended in 567 µl TE buffer (10mM Tris at pH 8 and 1mM EDTA) with 30 µl SDS 10% and 3 µl protein K (20mg/ml) and incubated at 37°C for 1h.
- 100 µl NaCl 5M were added.
- 80 µl CTAB/NaCl were added and incubated at 65°C for 10min.
- 780 µl chloroform was added and centrifuged by 12000 rmp for 5min.
- water phase were put in a new tube and 400 µl chloroform and 400 µl phenol was added and centrifuged by 12000 rmp for 5min.
- water phase was put in a new tube, 480 µl of isopropanol was added carefully. Then the tube was inverted to mix the content and incubated at room temperature for 2min.
- DNA was collected with a pipette and put into a new tube containing 1,5 ml of a mixture of 75% EtOH/ 25% TE.
- This DNA pellet was then transferred to a new tube containing 100 µl TE

PCR to extract the ClpX gene

- PCR mixes according to Roche High fidelity PCR.(https://www.roche-applied-science.com/pack-insert/1732641a.pdf)
- Genomic E.coli DNA as template
- PCR program :

	Temperature (°C)	Time (min)
Step 1	**94**	**8**
Step 2	**94**	**0.5**
Step 3	**65**	**0.5**
Step 4	**72**	**1**
Go to Step 2, 25 times temperature decreased by 0.2 each new cycle		
Step 5	**72**	**7**
Step 6	**4**	**Infinity**

After PCR, reaction tubes were put on a 1% agarose gel to verify the bands.

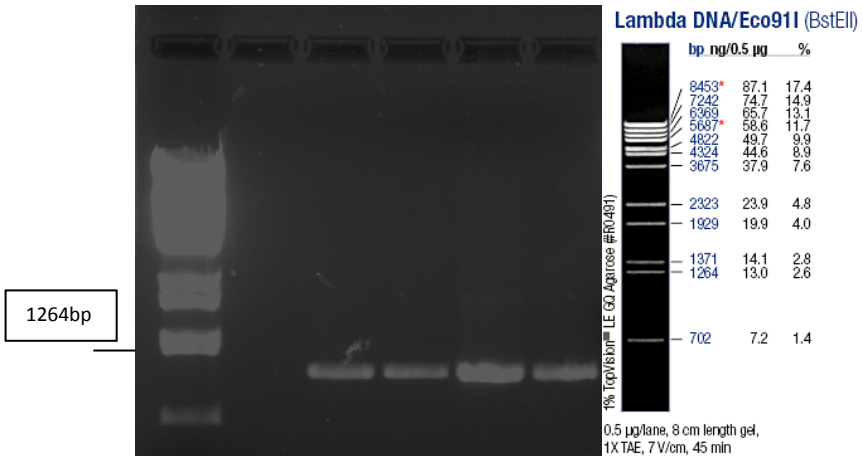


Figure: Left site: Marker and then the three PCR reaction. Right site. Description of the used marker.

- PCR products (~1000 bp) have the size of ClpX (1092 bp)
- Qiagen purification of PCR sample 1A and 1B (lane 3 & 4)

Verification of PCR products (ClpX gen) by AgeI and EcoRI digestion

- **Total reaction volume: 20 µl**
- **6 µl H_2O**
- **10 µl PCR**
- **2 µl 10x NEB buffer**
- **1 µl EcoRI**
- **Incubation at 37°C for 2h**
- **Heat inactivation for 20 min at 65°C**
- **Verification by gel electrophoresis**

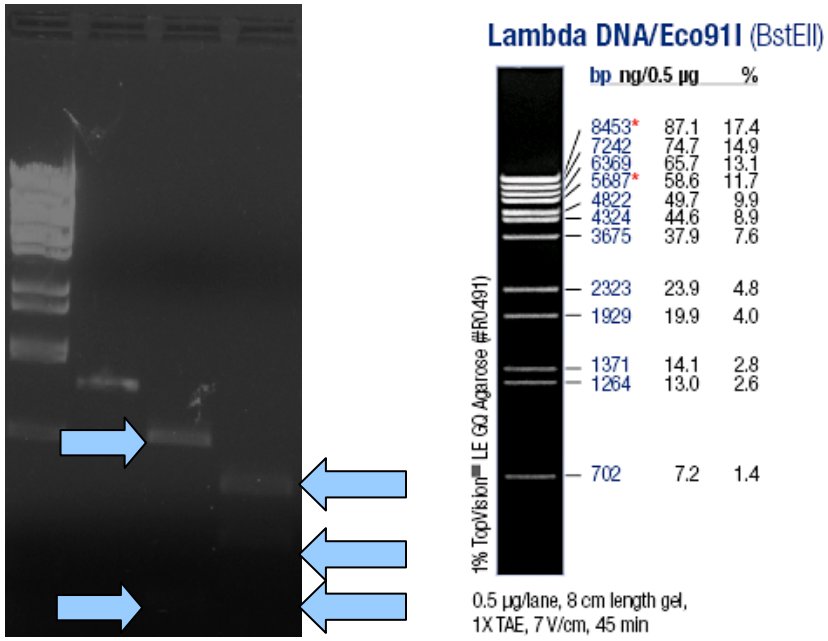


Figure: First line marker then ClpX, ClpX digested with AgeI, ClpXdigested with EcoRI

- Band size of the digested ClpX PCR products matches with the expectations
  - AgeI: 738 bp, 181 bp, 173 bp
  - EcoRI: 520 bp, 345 bp, 227 bp
- ClpX has the right length and the restriction digestion revealed the right restriction fragments.

ClpX was successfully extracted from the E.coli genome and purified.

Part II: Cloning ClpX into the pUC19 vector

The next step was to clone the ClpX gene into a standard pUC19 vector in which the Pfu mutagenesis to alter the restriction should concur. First problems and obstacles were observed as the first dephosphorylation protocol of the plasmid and plasmid transformation into the cells were not effective. After changing the parameters of the protocols the cloning step was successfully achieved. In this part the successful steps are described. The transformations of the plasmids were done into competent ClpX knockdown cells (*WM3100*)**.**

pUC19 EcoRI digestion

Reason: Cut the vector so it is prepared for blunt end generation.

- **4 µl pUC19 (1µg/µl)**
- **2 µl 10x NEB 4 buffer**
- **13 µl water**
- **1 µl EcoRI (20 U/µl) → 5x overdigestion**
- **Incubation for 1h at 37°C**
- **Heat inactivation for 20 min at 65°C**

Blunt-end creation

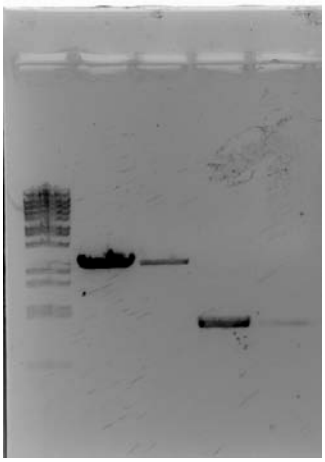
Reason: To generate a blunt end vector for cloning the ClpX insert into the vector. This is done because ClpX does not have yet restriction sides in the beginning/end of its sequence for sticky end ligation

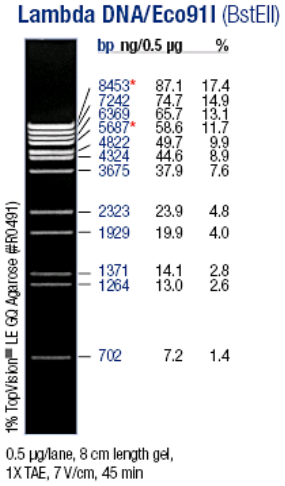
- **30µl reaction**
- **Take totality of EcoRI digestion and add:**
- **1µl NEB 4 buffer**
- **0.5 2mM dNTPs → 33 µM dNTPs**
- **1µl Klenow (5U/µl)**
- **7.5 µl water**
- **Incubate 15 min at 25°C**
- **Add 6µl 50mM EDTA**
- **Heat inactivation for 20 min at 75°C**
- **Purification with Qiagene PCR Product purification kit**
- **Elute with 35 µl**

Quantification of insert and vector

Reason: To know the amount/concentration of plasmid/insert for correct vector:insert ratio calculation

- Gel electrophoresis





(from left to right) 1 µg marker, 5 µl pUC19 EcoRI digested & blunt end, 5µl 1:10 dilution of pUC19 EcoRI digested & blunt end, 5µl ClpX PCR product, 5 µl 1:10 diluted ClpX PCR product

- Vector 1:10 band equals highest marker band = 17.4 % of 0.5 µg → 87 ng diluted, 870 ng non diluted → 870ng/5µl=174 ng/µl
- Insert 1:10 band equals lowest marker band = 1.4 % of 0.5 µg → 7 ng diluted, 70 ng non diluted → 70ng/5µl=14 ng/µl

Insert phosphorylation

Reason: To be sure that insert is phosphorylated.

- Total volume 90µl à 14 ng/µl → 1260 ng insert
- Reaction volume 100µl
  - 90µl insert
  - 9 µl 10x ligation buffer
  - 1 µl T4 Polynucleotide Kinase
- Incubation 30 min at 37°C
- Heat inactivation 20 min at 70°C

Vector dephosphorylation

Reason: Dephosphorylation prevents the vector from self-ligation. So vector can only ligates into a circular form when integrating the insert.

- Total volume 26 µl à 174 ng/µl → 4524 ng
- 2 different phosphatase assays
  - Shrimp alkaline phosphatase
    - 13 µl vector
    - 2 µl 10x SAP buffer
    - 1 µl water
    - 4 µl SAP (1U/µl)
  - Thermosensitive alkaline phosphatase
    - 13 µl vector
    - 2 µl 10x TSAP buffer
    - 1 µl water
    - 4 µl TSAP (1U/µl)
- Incubation at 37°C for 30 min (2x more reaction time) for both reaction mixes
- Heat inactivation at 70°C for 20 min for both reactions

Ligation

Reason: To ligate the vector with the insert.

- Insert: 12.6 ng/µl → 0.017 pmol/µl
- Vector: 113.1 ng/µl → 0.063 pmol/µl
- Ligation mixes x2 (one time SAP and once TSAP)

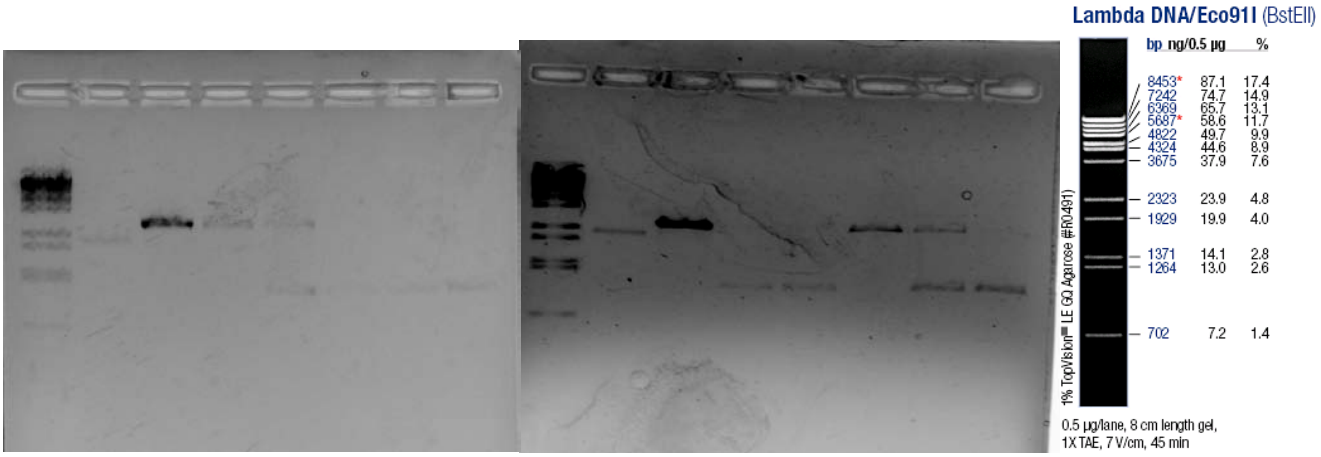
Insert	vector	insert	vector
1	1	3.7µl	1µl
3	1	1.11µl	1µl (1:10 dilution)
5	1	1.85µl	1µl (1:10 dilution)
10	1	3.7µl	1µl (1:10 dilution)

- Add 2µl 10x ligation buffer and 2µl T4 ligase and fill up to 20µl
- For a control the dephosphorylated vector without insert was ligated. (1µl vector from one time SAP and once TSAP, 1µl ligase, 1µl buffer, 7µl water)
- Incubation overnight at 16°C

Verification of ligated plasmids

Reason: Test if plasmids for transformation carry the insert.

- Plasmids directly taken from the ligation mix. Each time a volume of 5 µl with 1 µl loading buffer 6x.



Left: plasmids dephosphorylated with shrimp. First lane 0.5 µg marker (1 µl), second lane pUC19 plasmid circular, third lane pUC19 plasmid digested with EcoRI, fourth lane ligation mix vector:insert 1:3, fifth lane v:i 1:5, sixth lane vector without insert, seventh lane v:i 1:3, eighth lane v:i 1:10.

Right: plasmids dephosphorylated with TSAP. First lane 0.5 µg marker (1 µl), second lane pUC19 plasmid circular, third lane pUC19 plasmid digested with EcoRI, fourth lane vector without insert, fifth lane v:i 1:1, sixth lane v:i 1:3, seventh lane v:i 1:5, eighth lane v:i 1:10.

--> Ligation with low efficiency. By shrimp just lane 5 could have in ligated vector with insert. By the TSAP a ligated is possible in mixture resembled by the line seven.

Transformation

Reason: To transform the plasmid with the ClpX plasmid into the E.coli cells.

- Thaw competent cells on ice
- Add 50 µl cells to your DNA
- Leave on ice for 10 min
- Heat shock 1min at 42 °C
- Leave on ice for 10 min
- Add 1 ml SOC medium
- Incubation at 37°C for 30 min
- Plating 500 µl of control (pUC19), ligation mix v:i 1:3 shrimp and TSAP on ampicillin selection plates. The shrimp and TSAP dephosphorylated vector with a insert ratio v:i 1:1, 1:5, 1:10 and the vector without insert were plated each on half an ampicillin selection plate with 200 µl of the mixture. So for example shrimp and TSAP with the same v:i ratio are on the same plate. Growth overnight.

Control of the transformants

All plates showed colonies.

- Efficiency of the transformation was $6{,}1*10^5$ (control plate).
- Calculation: 6100 colonies on plate. Added vector was 10ng. So 610 colonies per ng of vector, so $6.1*10^5$ of colonies per µg.

--> Transformation was successful

- Total colonies on plates
  - Only vector 8-10 only vector 1
  - 1:1 shrimp 1 1:1 TSAP >22 (40% plated)
  - 3:1 shrimp 14 3:1 TSAP 8
  - 5:1 shrimp 7 5:1 TSAP 17 (40% plated)
  - 10:1 shrimp 7 10:1 TSAP 13 (40% plated)
- 19 colonies after the following scheme were selected.
- Number of the colony= insert: vector ratio then shrimp or TSAP
- 1= 1:1 shrimp
- 2-4= 1:1 TSAP
- 5-8= 3:1 shrimp
- 9-12= 3:1 TSAP
- 13-15= 5:1 shrimp
- 16-17= 5:1 TSAP
- 18= 10:1 shrimp
- 19= 10:1 TSAP

All colonies were grown in LB-ampicillin liquid cultures overnight.

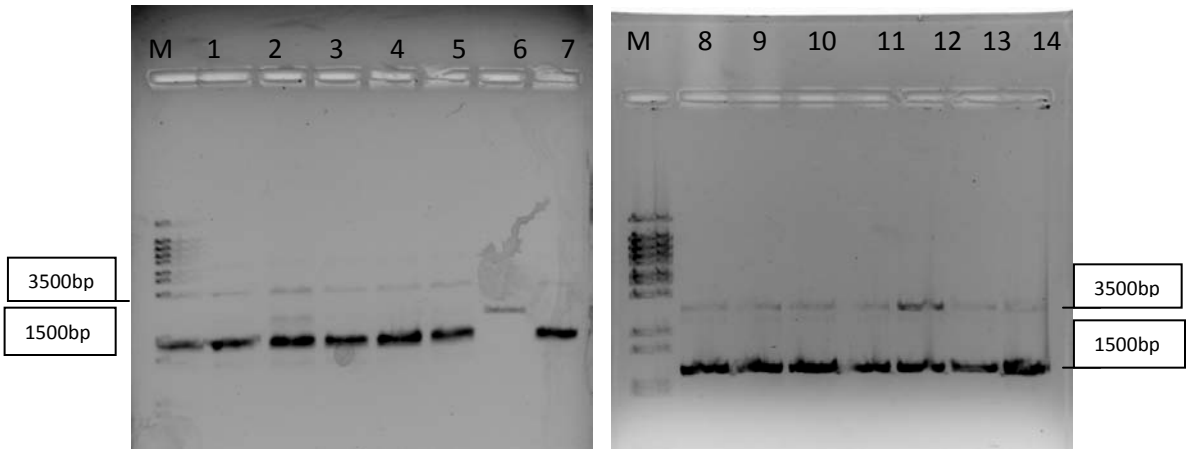
Plasmid isolation

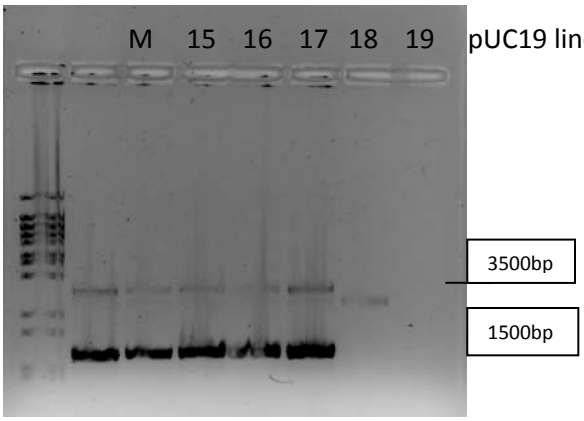
- Minipreps Nucleospin® Plasmid QuickPure (Macherey nagel)

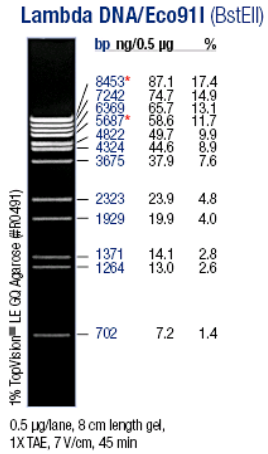
Screen of colonies for inserts

Reason: To verify if the plasmid in the colonies carries the insert. Plasmid was purified and later digested with EcoRI. Because the ClpX gene begins with a C, a blunt end created vector with the insert will again contain an EcoRI restriction side. The ClpX gene also contains two EcoRI restrictions sides, so several bands should be visible.

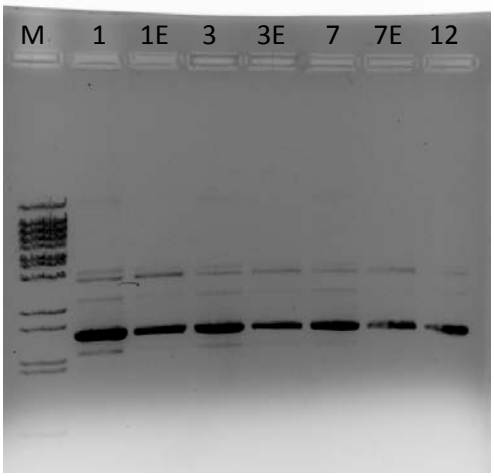
- Plasmid miniprep for all 19 colonies by Nucleospin purification (Maschery-Nagel)
- EcoRI digestion for all 19 colonies and pUC19 plasmid and ClpX insert for control:
  - 10 µl of purified plasmid
  - 2 µl of NEB 4 buffer
  - 7 µl water
  - vortex briefly, then centrifuge to get the liquid down.
  - 1 µl EcoRI was added and incubated at 37°C for 2h.
- After digestion all 21 samples were put on a gel

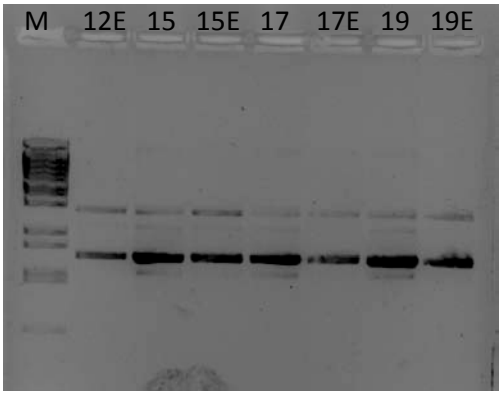






- All colonies show linear plasmid bands of approximately 3500 bp, which is greater than the linearized pUC19 vector (2686 bp). However all lanes contain a very strong band at approximately 1500-1600 bp.
- To compare the undigested with the digested plasmids, a new gel electrophoresis had been performed, but only with best samples (1, 3, 7, 12, 15, 17, 19)





- Only number = non-digested sample, number+E = EcoRI digested sample

- Digested samples contain all only 2 bands, the desired band at 3500 bp and the unknown band at 1500-1600 bp. The circular plasmid is cleaved by EcoRI (visible especially for samples 1 and 3 between non-digested and digested samples)
  ➔ Transformants contain ClpX

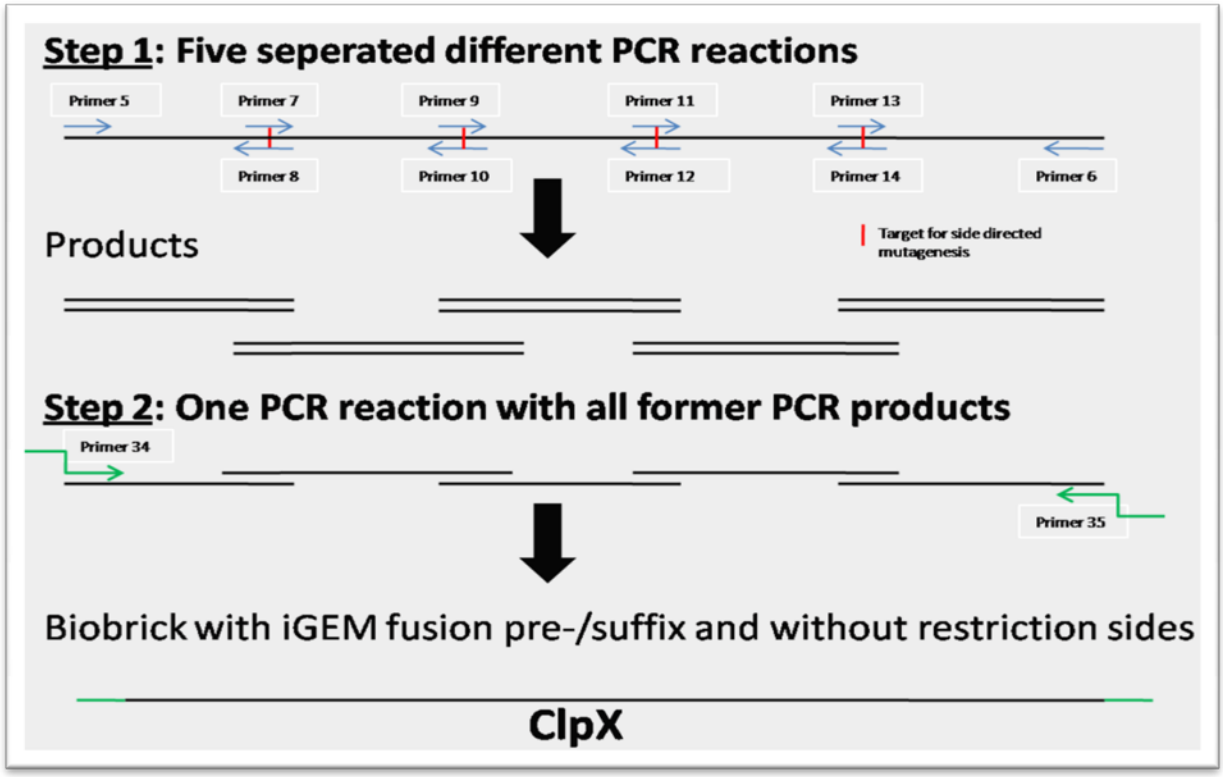
Part III: Site-directed mutagenesis

ClpX contains two EcoRI and two AgeI restrictions sides. As the fusion pre- and suffix for iGEM Biobricks has these sides as well, this internal restrictions sides had to be altered to ensure these standards. The first approach was a standard Pfu mutagenesis for the altering of two restriction sides per PCR reaction. Unfortunately in this part of Biobrick construction major problems were encountered. The first mutagenesis did not alter the restrictions sides and not even changing of protocol parameters did successfully alter the restriction sides. Other protocols were tried. A single strand single restriction side approach was also unsuccessful and a promising protocol from the open wet page could not be done due to the lack of a thermo stabile ligase (taq ligase). The detailed protocol of these failed efforts is presented in *chapter 5 (Troubleshooting)*.

After several weeks it was decided to order the ClpX Biobrick without restriction sides by GeneArt. However, the successful alteration of the restriction sides in ClpX was achieved by using a complete new approach, weeks the synthesized gene arrived.

The new approach

The principle of this technique is two PCR reaction steps. In the first step five separated PCR reactions were conducted which lead to five different PCR products. So primer 5 and 8 give one product, 7 and 10 the next. The primers were already designed for the mutagenesis step. In the second step the PCR products were put together. Because the primers are overlapping the fragments will anneal during the PCR reaction. The primers used in this reaction are primers which have the same sequence as the primers in the beginning and the end of the ClpX gene but additionally they have also the iGEM pre and suffix. So just when all fragments are completely annealed they can be amplified. This amplified fragment already contains now the fusion pre- and suffix.



Results

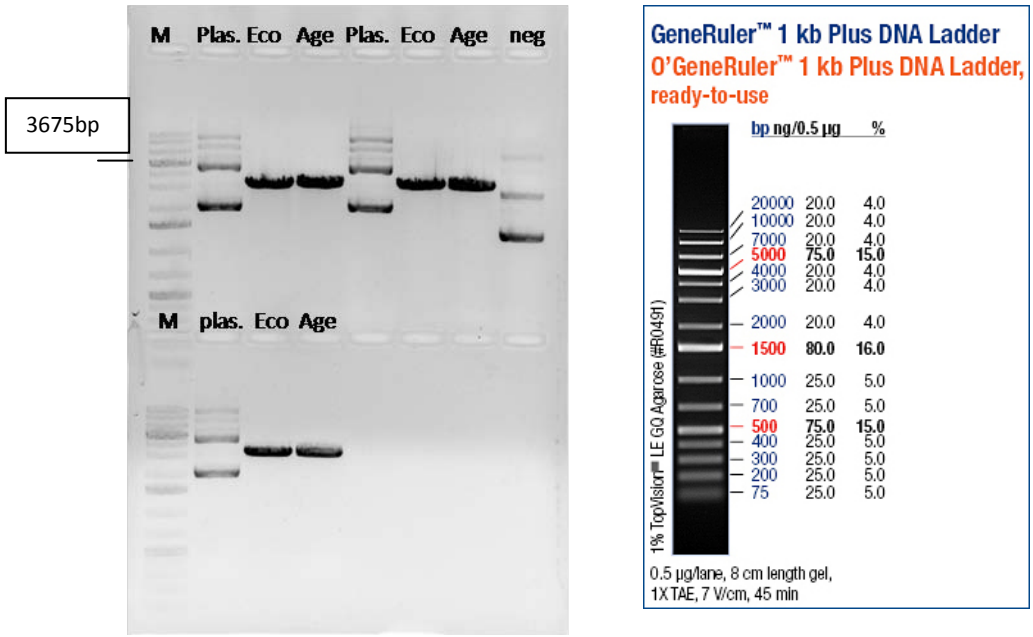


Figure Gelelectrophosresis of the result. First column marker followed by undigested PSB1C3 plasmid with ClpX, plasmid digested in EcoRI, plasmid digested in AgeI. Eights columm on the top is the negative control consistibng of the PSB1C3 plasmid without insert.

Comparing the new plasmid with the negative control, it is obvious that the new plasmid consists the ClpX fragment. Digestion of this fragment with either EcoRI or AgeI showed no digestion. ClpX was therefore successfully mutated an so without restriction sides.

Phytochrome B (900) Biobrick

Background:

Phytochromes characterized by a red/far-red photochromicity. Through red-light (650–670 nm) absorption the phytochrome undergoes a rapid conformational change from its ground state P_r to its active state P_{fr}. The structural change allows the binding of the PIF. This light-sensitive interaction has been mapped to the 650-residue amino-terminal photosensory core of PhyB (*Khanna et al., 2004*). The process is completely reversible through absorption in the near infra-red spectrum (705–740 nm).

The photoreceptor protein PhyB serves for the light-dependent activation of the system, therefore it will be fused to the N-teminal of the ClpX-trimer.

Conception:

In in-vivo applications it has been shown that the PIF-interaction with the PhyB photosensory core (residues 1–650) is irreversible in infrared light. Lim & Voigt (2009) demonstrated by assaying PIF6 (which has the strongest interactions of all previously reported PIF domains) against different variants of PhyB that the tandem C-terminal PAS domains (residues 1-908)of plant phytochromes are necessary to confer rapid photoreversibility under infrared light (*Lim & Voigt, 2009*).
The original sequence contains a SpeI restriction within the first 908 residues,

NNNNNNgaattcgcggccgcttctagATGGTTTCCGGAGTCGGGGGTAGTGGCGGTGGCCGTGGCGGTGGCCGTGGCG
GAGAAGAAGAACCGTCGTCAAGTCACACTCCTAATAACCGAAGAGGAGGAGAACAAGCTCAATCGTCGGGAACG
AAATCTCTCAGACCAAGAAGCAACACTGAATCAATGAGCAAAGCAATTCAACAGTACACCGTCGACGCAAGACTCC
ACGCCGTTTTCGAACAATCCGGCGAATCAGGGAAATCATTCGACTACTCACAATCACTCAAAACGACGACGTACGG
TTCCTCTGTACCTGAGCAACAGATCACAGCTTATCTCTCTCGAATCCAGCGAGGTGGTTACATTCAGCCTTTCGGAT
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TCAGGCTCTATTGACTGCAAACAAGCGGGTTAGCCTCGAGGGAAAGGTTATTGGGGCTTTCTGTTTCTTGCAAATC
CCGAGCaccggttaatactagtagcggccgctgcagNNNNNN

The plasmid containing the PhyB-sequence was provided by the laboratory of Wilfried Weber from the University of Freiburg. To create the BioBrick part the sequence was amplified with primers containing the standard prefix with ATG and the fusion suffix of the Fusion Protein Assembly Standard.

Forward primer (5'->3'): (41bp)

GGATCCgaattcgcggccgcttctagATGGTTTCCGGAGTC

Reverse primer (5'->3'): (52 bp)

CAGCTGctgcagcggccgct**t**actagt**atta**accggtGCTCGGGATTTGCAAG

In order to get a sequence without an internal restriction sites of one of the BioBrick standards the SpeI restriction site was altered without changing the encoded amino acid (ACT=Threonine (AC(T,A,G,C)).

Primers for Pfu-mutagenese:

Forward primer (5'->3'): (28 bp)
GGACAAGACGTTACGAGTCAGAAAATCG

Reverse primer (5'->3'): (28 bp)
CGATTTTCTGACTCGTAACGTCTTGTCC

Followed procedure to obtain this biobrick:

1. Amplification and addition of the pre- and suffixes
   PCR with Roche Expand High Fidelity PCR system following the standard protocol (30 sec for extension step 4)
2. Digestion with EcoRI and PstI
   - To prevent damage of the pre- and suffixes during PCR-amplification the primers have been orders with a 5'overhang of six additional nucleic acids so the sequence has to be digested to create the NgoMIV and AgeI-overhangs
   - Digestion of 500ng in 50 µl reaction volume for 10 min at 37°C
   - Heat inactivation of the restriction enzymes at 80°C for 20 min
3. Ligation in the *EcoRI/PstI*-precutted vector psB1C3
   - Different insert-vector ratios: 1:1 / 2:1 in 20 µl reaction volume
     - 1/2 µl insert (≈10/20 ng)
     - 3,5 µl vector (≈70ng)
     - 2 µl T4 DNA Ligase Buffer
     - 1 µl T4 DNA Ligase (NEB))
     - 12,5/11,5 µl H2O
4. Transformation
   - 5 µl of the ligation assay were transformed with 50 µl of competent cells and plated on chloramphenicol-containing LB-agar plates
   - Incubation at 37°C overnight
5. Screening by PCR on colony
6. Preculture
   - Positively-tested colonies have been set in 5ml chloramphenicol-containing LB-medium
7. Miniprep with QIAquick PCR Purification Kit from Quiagen
8. Verification
   - Restriction enzyme digestion with EcoRI and SpeI separately and double digestion.
9. Pfu-Mutagenesis
   - PCR with Fermentas Pfu-DNA polymerase (5 min for extension step 4)
10. Digestion with DpnI
11. Transformation
    - 1µl of the PCR products were transformed with 50 µl of competent cells and plated on chloramphenicol-containing LB-agar plates
    - Incubation at 37°C overnight
12. Screening by PCR on colony

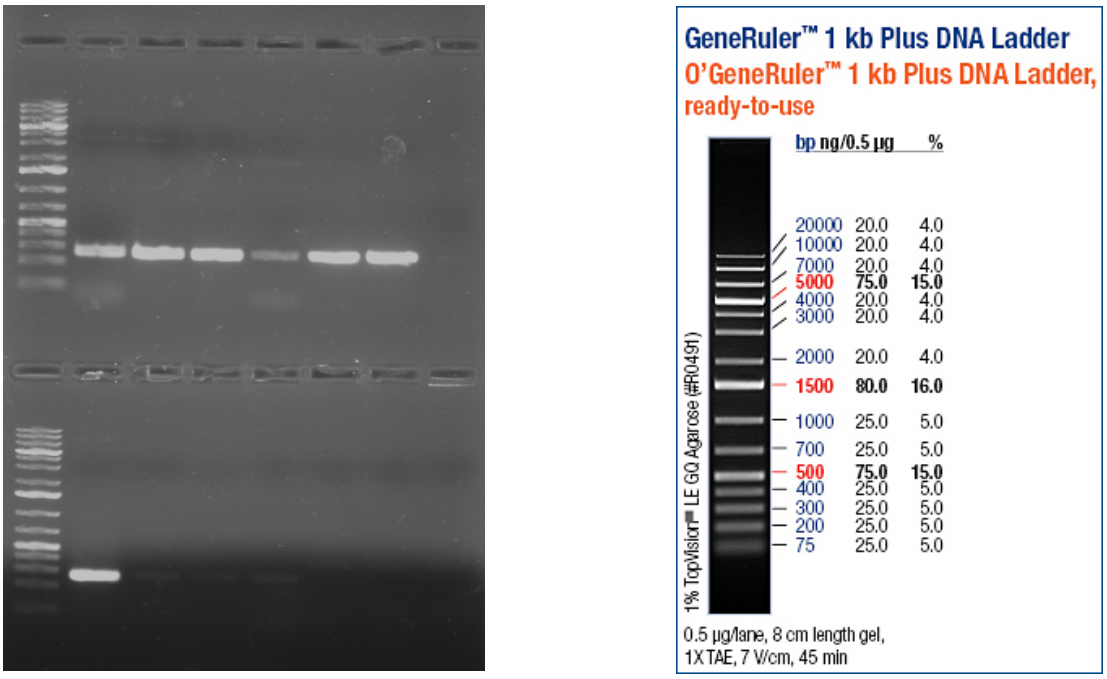

Figure 1: The band at 300bp is characteristic for the PhyB insert after amplification

13. Preculture
    - Positively-tested colonies have been set in 5ml chloramphenicol-containing LB-medium
14. Miniprep with QIAquick PCR Purification Kit from Quiagen
15. Verification
    - Restriction enzyme digestion with EcoRI and SpeI separately and double digestion.

    Amplification and addition of the pre- and suffixes.
16. Sequencing

atggtttccggagtcgggggtagtggcggtggccgtggcggtggccgtggcggagaagaagaaccgtcgtcaagtcac
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Phytochrome B (1-650) Biobrick

Background:

Phytochromes characterized by a red/far-red photochromicity. Through red-light (650–670 nm) absorption the phytochrome undergoes a rapid conformational change from its ground state P_r to its active state P_{fr}. The structural change allows the binding of the PIF. This light-sensitive interaction has been mapped to the 650-residue amino-terminal photosensory core of PhyB (*Lim & Voigt, 2009*). The process is completely reversible through absorption in the near infra-red spectrum (705–740 nm).

The photoreceptor protein PhyB serves for the light-dependent activation of the system, therefore it was fused to the N-terminal of the ClpX-trimer.

Conception:

As mentioned before it has been shown that the PIF-interaction with the PhyB photosensory core (residues 1–650) is irreversible in infrared light in in vivo-application (*Lim & Voigt, 2009*). Nevertheless, the binding strength and kinetic parameters depend on the composition and nature of the individual system, so we decided to include also this shorter variant of PhyB in our tests.

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GTTTACAGTTAAACATGGAATTGCAGTTAGCTTTGCAAATGTCAGAGAAACGCGTTTTGAGAACGCAGACACTGTT
ATGTGATATGCTTCTGCGTGACTCGCCTGCTGGAATTGTTACACAGAGTCCCAGTATCATGGACTTAGTGAAATGTG
ACGGTGCAGCATTTCTTTACCACGGGAAGTATTACCCGTTGGGTGTTGCTCCTAGTGAAGTTCAGATAAAAGATGT
TGTGGAGTGGTTGCTTGCGAATCATGCGGATTCAACCGGATTAAGCACTGATAGTTTAGGCGATGCGGGGTATCCC
GGTGCAGCTGCGTTAGGGGATGCTGTGTGCGGTATGGCAGTTGCATATATCACAAAAAGAGACTTTCTTTTTTGGT
TTCGATCTCACACTGCGAAAGAAATCAAATGGGGAGGCGCTAAGCATCATCCGGAGGATAAAGATGATGGGCAAC
GAATGCATCCTCGTTCGTCCTTTCAGGCTTTTCTTGAAGTTGTTAAGAGCCGGAGTCAGCCATGGGAAACTGCGGA
AATGGATGCGATTCACTCGCTCCAGCTTATTCTGAGAGACTCTTTTAAAGAATCTGAGGCGGCTATGAACTCTAAAG

TTGTGGATGGTGTGGTTCAGCCATGTAGGGATATGGCGGGGGAACAGGGGATTGATGAGTTAGGTaccggttaatact agtagcggccgctgcagNNNNNN

The plasmid containing the PhyB-sequence was provided by the laboratory of Wilfried Weber from the University of Freiburg. To create the BioBrick part the sequence was amplified with primers containing the standard prefix with ATG and the fusion suffix of the Fusion Protein Assembly Standard.

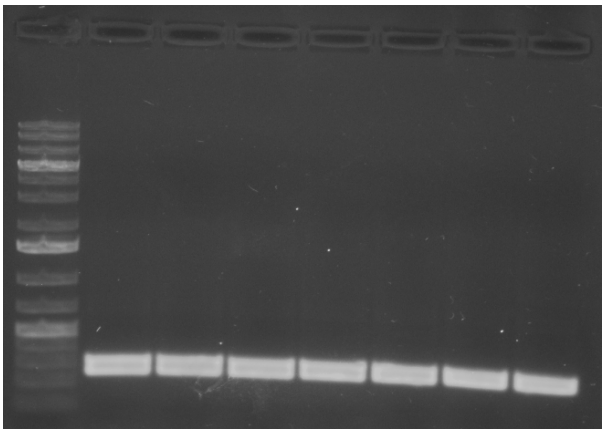
Forward primer (5'->3'): (42 bp)
GGATCCgaattcgcggccgcttctagATGGTTTCCGGAGTC

Reverse primer (5'->3') : (53 bp)
CAGCTGctgcagcggccgc**t**actagt**atta**accggtCCCCGCCATATCCCTAC

Followed procedure to obtain this biobrick:

1. Amplification and addition of the pre- and suffixes
   - PCR with Roche Expand High Fidelity PCR system following the standard protocol (30 sec for extension step 4)
2. Digestion with EcoRI and PstI
   - To prevent damage of the pre- and suffixes during PCR-amplification the primers have been orders with a 5'overhang of six additional nucleic acids so the sequence has to be digested to create the NgoMIV and AgeI-overhangs
   - Digestion of 500ng in 50 µl reaction volume for 10 min at 37°C
   - Heat inactivation of the restriction enzymes at 80°C for 20 min
3. Ligation in the *EcoRI/PstI*-precutted vector psB1C3
   - Different insert-vector ratios: 1:1 / 2:1 in 20 µl reaction volume
     - 1/2 µl insert (≈10/20 ng)
     - 3,5 µl vector (≈70ng)
     - 2 µl T4 DNA Ligase Buffer
     - 1 µl T4 DNA Ligase (NEB))
     - 12,5/11,5 µl H2O
4. Transformation
   - 5 µl of the ligation assay were transformed with 50 µl of competent cells and plated on chloramphenicol-containing LB-agar plates
   - Incubation at 37°C overnight

5. Screening by PCR on colony



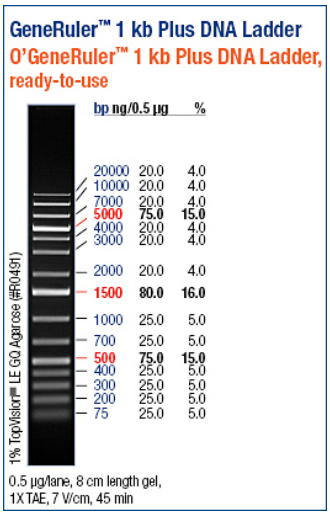


Figure 2:The band at 300bp is characteristic for the PhyB insert after amplification

6. Preculture
   - Positively-tested colonies have been set in 5ml chloramphenicol-containing LB-medium
7. Miniprep with QIAquick PCR Purification Kit from Quiagen
   - Verification
   - Restriction enzyme digestion with EcoRI and SpeI separately and double digestion.
8. Sequencing

atggtttccggagtcgggggtagtggcggtggccgtggcggtggccgtggcggagaagaagaaccgtcgtcaagtcac
actcctaataaccgaagaggaggagaacaagctcaatcgtcgggaacgaaatctctcagaccaagaagcaacactgaa
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tgggaaactgcggaaatggatgcgattcactcgctccagcttattctgagagactcttttaaagaatctgaggcggct
atgaactctaaagttgtggatggtgtggttcagccatgtagggatatggcgggg

Pif3

Background:

PIF3 is a downstream transcription factor in a well studied signaling pathway of A. thaliana, upon stimulation with red (650 nm) light, it binds directly to PhyB and translocates to the nucleus as a heterodimer where it modulates the transcription of response genes. PIF3 binds only the red-light-exposed form of phytochrome, Pfr, and shows no measurable binding affinity for the dark- or infraredexposed Pr state12.
In our system target proteins are fused to PIF3 and tagged with the DAS+4 degradation sequence which, through light activation, brings the degradation tag in proximity to ClpX.

Conception:

The light-sensitive interaction with PhyB has been mapped to the last 100-residue N-terminal activated phytochrome binding (APB) domain of PIF3 (*Lim & Voigt, 2009*).
We chose this sequence, as it has already been successfully used in different synthetic in vitro applications (10, 14, 15) that benefitted from its light-sensitive interactions with PhyB. The original sequence contains a XbaI restriction site.

GaattcgcggccgcttctagatggccggcATGCCTCTGTTTGAGCTTTTCAGGCTCACCAAAGCTAAGCTTGAATCTGCTCAA
GACAGGAACCCTTCTCCACCTGTAGATGAAGTTGTGGAGCTGGTGTGGGAAAATGGTCAGATATCAACTCAAAGTC
AGTCAAGTAGATCGAGGAACATTCCTCCACCACAAGCAAACTCTTCTAGAGCTAGAGAGATTGGAAATGGCTCAAA
GACGACTATGGTGGACGAGATCCCTATGTCAGTGCCATCACTAATGACGGGTTTGAGTCAAGACGATGACTTTGTT
CCATGGTTGAATCATCATaccggttaatactagtagcggccgctgcag

The plasmid containing the PIF3-sequence was provided by the laboratory of Stephan Kircher from the University of Freiburg. For the synthesis of the BioBrick part primers containing the sites of the Fusion Protein BioBrick Assembly Standard were used.

Forward primer (5'->3'): 51 bp
GGATCCgaattcgcggccgcttctagatggccggcATGCCTCTGTTTGAGC

Reverse primer (5'->3'): 51 bp
ctgcagcggccgctactagtattaaccggtATGATGATTCAACCATGGAAC

In order to get a sequence without an internal restriction sites of one of the BioBrick standards the XbaI-restriction site was altered without changing the encoded amino acid(TCT=Serin (TC(T,A,G,C)).

Primers for Pfu-mutagenese:

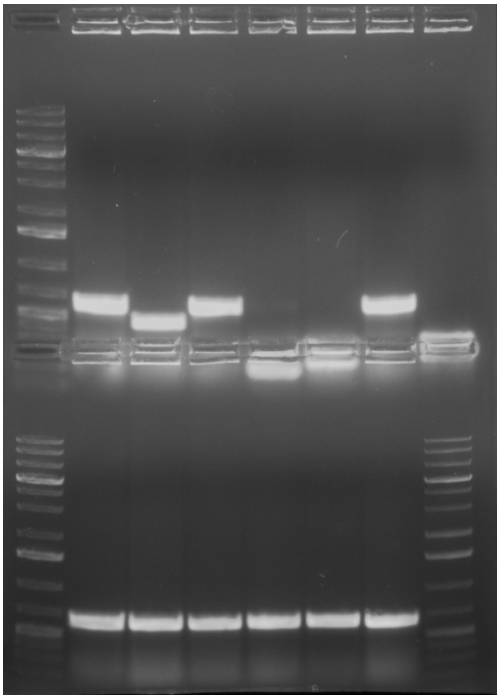
Forward primer (5'->3') (24 bp)
GCAAACTCTTCAAGAGCTAGAGAG

Reverse primer (5'->3') (24 bp)
CTCTCTAGCTCTTGAAGAGTTTGC

Followed procedure to obtain this biobrick:

1. Amplification and addition of the pre- and suffixes
    - PCR with Roche Expand High Fidelity PCR system following the standard protocol (30 sec for extension step 4)
2. Digestion with EcoRI and PstI
    - To prevent damage of the pre- and suffixes during PCR-amplification the primers have been orders with a 5'overhang of six additional nucleic acids so the sequence has to be digested to create the NgoMIV and AgeI-overhangs
    - Digestion of 500ng in 50 µl reaction volume for 10 min at 37°C
    - Heat inactivation of the restriction enzymes at 80°C for 20 min
3. Ligation in the *EcoRI/PstI*-precutted vector psB1C3
    - Different insert-vector ratios: 1:1 / 2:1 / 4:1 in 20 µl reaction volume
        - 1/2/4 µl insert (≈10/20/40 ng)
        - 3,5 µl vector (≈70ng)
        - 2 µl T4 DNA Ligase Buffer
        - 1 µl T4 DNA Ligase (NEB))
        - 12,5/11,5/9,5 µl H2O
4. Transformation
    - 5 µl of the ligation assay were transformed with 50 µl of competent cells and plated on chloramphenicol-containing LB-agar plates
    - Incubation at 37°C overnight
5. Screening by PCR on colony
6. Preculture
    - Positively-tested colonies have been set in 5ml chloramphenicol-containing LB-medium
7. Miniprep with QIAquick PCR Purification Kit from Quiagen
8. Verification
    - Restriction enzyme digestion with XbaI and Pst1 separately and double digestion.
9. Pfu-Mutagenesis
    - PCR with Fermentas Pfu-DNA polymerase (5 min for extension step 4)
10. Digestion with DpnI
11. Transformation
    - 1µl of the PCR products were transformed with 50 µl of competent cells and plated on chloramphenicol-containing LB-agar plates
    - Incubation at 37°C overnight

12. Screening by PCR on colony



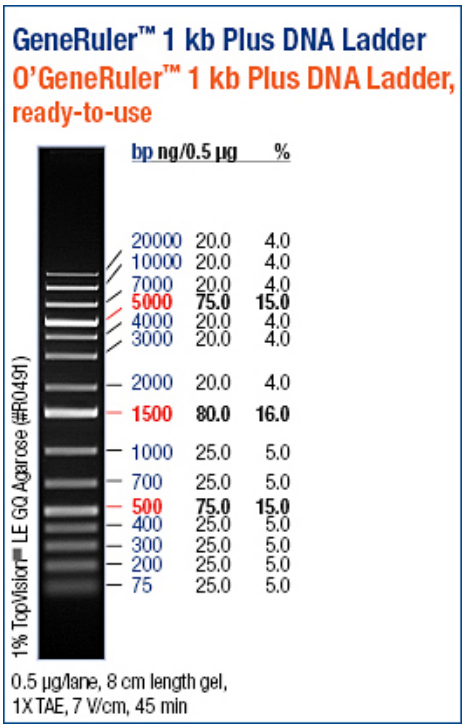


Figure 3: The bands at 600bp are characteristic for PIF3/6 amplified with VF and VR, the band at 300bp are the plaamids without insert.

13. Preculture
    - Positively-tested colonies have been set in 5ml chloramphenicol-containing LB-medium
14. Miniprep with QIAquick PCR Purification Kit from Quiagen
15. Verification
    - Restriction enzyme digestion with XbaI and Pst1 separately and double digestion.
16. Amplification and addition of the pre- and suffixes

17. Sequencing

```
atgcctctgtttgaacttttcaggctcaccaaagctaagcttgaatctgctcaagacaggaacccttctccacctgta
gatgaagttgtggagctggtgtgggaaaatggtcagatatcaactcaaagtcagtcaagtagatcgaggaacattcct
ccaccacaagcaaactcttcaagagctagagagattggaaatggctcaaagacgactatggtggacgagatccctatg
tcagtgccatcactaatgacgggtttgagtcaagacgatgactttgttccatggttgaatcatcat
```

Pif6

Background:

For the design of the first engineered system that achieved to enable the spatiotemporal control of PhyB-PIF interactions in in-vivo experiments, Lim and Voigt (2009) screened multiple potential phytochrome–PIF pairs by a fluorescence translocation assay in NIH3T3 cells. They measured the red-light-induced translocation of yellow fluorescent protein (YFP) fused to PIF domains to co-expressed phytochrome domains fused through a flexible linker to mCherry and localized to the plasma membrane by a carboxyterminalpolybasic, prenylation sequence from Kras. Of all previously reported PIF domains, only the N terminus of PIF6 is strong enough to cause significant translocation of YFP to the membrane.

Fig.1: Implied system of Lim and Voigt (2009) to screen potential phytochrome-PIF pairs in a fluorescence translocation assay.

Conception:

We chose used the same sequence of the last 100-residue N-terminal activated phytochrome binding (APB) domain of PIF6, which was already successfully used by Lim and Voigt (2009).

gaattcgcggccgcttctagatggccggcATGATGTTCTTACCAACCGATTATTGTTGCAGGTTAAGCGATCAAGAGTATATG
GAGCTTGTGTTTGAGAATGGCCAGATTCTTGCAAAGGGCCAAAGATCCAACGTTTCTCTGCATAATCAACGTACCA
AATCGATCATGGATTTGTATGAGGCAGAGTATAACGAGGATTTCATGAAGAGTATCATCCATGGTGGTGGTGGTG
CCATCACAAATCTCGGGGACACGCAGGTTGTTCCACAAAGTCATGTTGCTGCTGCCCATGAAACAAACATGTTGGA
AAGCAATAAACATGTTGACaccggttaatactagtagcggccgctgcag

The plasmid containing the PIF6-sequence was provided by the laboratory of Wilfried Weber from the University of Freiburg. For the synthesis of the Pif6 BioBrick primers containing the sites of the Fusion Protein BioBrick Assembly Standard were used.

Forward primer (5'->3'): 54 bp

GGATCCgaattcgcggccgcttctagatggccggcATGATGTTCTTACCAACCG

Reverse primer (5'->3'): 58 bp

CAGCTGctgcagcggccgctactagtattaaccggtGTCAACATGTTTATTGCTTTCC

Followed procedure to obtain this biobrick:

1. Amplification and addition of the pre- and suffixes
    - PCR with Roche Expand High Fidelity PCR system following the standard protocol (30 sec for extension step 4)
2. Digestion with EcoRI and PstI
    - To prevent damage of the pre- and suffixes during PCR-amplification the primers have been orders with a 5'overhang of six additional nucleic acids so the sequence has to be digested to create the NgoMIV and AgeI-overhangs
    - Digestion of 500ng in 50 µl reaction volume for 10 min at 37°C
    - Heat inactivation of the restriction enzymes at 80°C for 20 min
3. Ligation in the *EcoRI/PstI*-precutted vector psB1C3
    - Different insert-vector ratios: 1:1 / 5:1 in 20 µl reaction volume
        - 1/5 µl insert (≈10/20 ng)
        - 3,5 µl vector (≈70ng)
        - 2 µl T4 DNA Ligase Buffer
        - 1 µl T4 DNA Ligase (NEB))
        - 12,5/7,5 µl H2O
4. Transformation
    - 5 µl of the ligation assay were transformed with 50 µl of competent cells and plated on chloramphenicol-containing LB-agar plates
    - Incubation at 37°C overnight
5. Screening by PCR on colony
6. Preculture
    - Positively-tested colonies have been set in 5ml chloramphenicol-containing LB-medium
7. Miniprep with QIAquick PCR Purification Kit from Quiagen

8. Verification
    - Restriction enzyme digestion with EcoRI and Pst1 separately and double digestion.
9. Sequencing

```
atgatgttcttaccaaccgattattgttgcaggttaagcgatcaagagtatatggagcttgtgtttgagaatggccag
attcttgcaaagggccaaagatccaacgtttctctgcataatcaacgtaccaaatcgatcatggatttgtatgaggca
gagtataacgaggatttcatgaagagtatcatccatggtggtggtggtgccatcacaaatctcggggacacgcaggtt
gttccacaaagtcatgttgctgctgcccatgaaacaaacatgttggaaagcaataaacatgttgac
```

Linker biobrick

Background:

The linker biobrick is used to join the three ClpX subunits covalently in order to build a ClpX trimer and to link the degradation tags and PIF3/6 with the protein destined for degradation.

Conception:

We chose to use the same linker, which was already successfully used by Baker and Sauer (2009) to construct the ClpX trimer. It is a twenty amino acid linker (ASGAGGSEGGGSEGGTSGAT). The codon usage of *E. coli* (http://www.geneinfinity.org/sp_codonusage.html) has been used to decide the DNA sequence; in addition RFC 25 fusion prefix and suffix have been added to the sequence.

gaattcgcggccgcttctagatg**gccggc**GCGAGCGGCGCGGGCGGCAGCGAAGGCGGCGGCAGCGAAGGCGGCACCAGC
GGCGCGACCaccggttaatactagtagcggccgctgcag

The linker has been order as six separate, *Eco*RI+*Age*I precut primers, which were hybridized in order to obtain the complete linker sequence.

Forward strand (5'->3'): (89 bp)

aattcgcggccgcttctagatg**gc|cggc**GCGAGCGGCGCGGGCGGCAGCGAAGGCGGCGGCAG|CGAAGGCGGCACCAG
CGGCGCGACCa

Reverse strand (5'-3'): (89 bp)

ccggtGGTCGCGCCG|CTGGTGCCGCCTTCGCTGCCGCCGCCTTCGCTGCC|GCCCGCGCCGCTCGC**gccggc**catctaga
agcggccgcg

Ordered primer:

Forward: 5'aattcgcggccgcttctagatg**gc** 3'

Forward: 5'**cggc**GCGAGCGGCGCGGGCGGCAGCGAAGGCGGCGGCAG 3'

Forward: 5'CGAAGGCGGCACCAGCGGCGCGACCa 3'

Reverse: 5'ccggtGGTCGCGCCG 3'

Reverse: 5'CTGGTGCCGCCTTCGCTGCCGCCGCCTTCGCTGCC 3'

Reverse: 5'GCCCGCGCCGCTCGC**gccggc**catctagaagcggccgcg 3'

Followed procedure to obtain this biobrick:

1. Primer phosphorylation
    - Each primer is phosphorylated in a single reaction to ensure equal phosphorylation efficiency
    - Reaction volume 20 µl
        - 1 nmol (10µl of a 100µM stock solution) of each primer
        - 2 µl NEB ligation buffer 10x
        - 1 µl T4 polynucleotide kinase (NEB) 10U/µl
        - 7 µl dH_20
    - Incubation for 30 min at 37°C
2. Hybridization
    - Mixture of the single phosphorylated primers in an equimolar ratio
    - Heating to 65°C for 10 min
    - cooling slowly down to room-temperature
3. Restriction enzyme digestion
    - In order to prevent linker polymers due to restriction site overlapping, the hybridized sample was digested by *Eco*RI (20U) and *Age*I (20U)
    - Heat inactivation of the restriction enzymes at 65°C for 20 min
4. Verification by gel electrophoresis

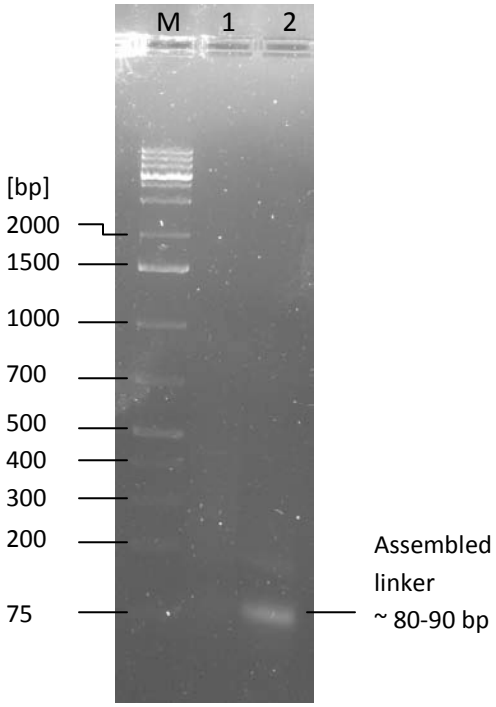


Figure 4: Verification of linker hybridization by 2% agarose gel electrophoresis after primer hybridization and EcoRI+AgeI digestion. M: 0.25 µg GeneRuler 1kb Plus DNA Ladder (Fermentas), 1: 2µl sample, 2: 5µl sample. The assembled linker construct is visible in lane 2 at 80-90 bp, which correlates to the expected size.

5. Ligation
    - Two different insert vector ratios (1:1 and 3:1) in a 10 µl reaction volume
    - A RFC 25 compatible pSB1C3 vector cut by *Eco*RI and *Age*I was used

6. Transformation
    - The complete ligation assay was used in transformation with 50 µl of competent cells
    - Incubation at 37°C overnight
7. Screening by PCR on colony
    - Standard protocol using iGEM VF and VR primers in a touch-down PCR with annealing temperatures from 60°C to 55°C over 25 cycles

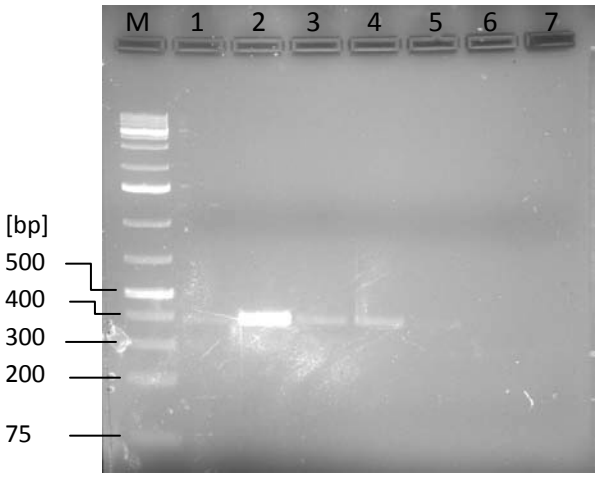


Figure 5: Analysis of PCR products of seven colonies by 1% gel electrophoresis. M: 0.25 µg GeneRuler 1kb Plus DNA Ladder (Fermentas), 1-7: colony number. Colonies 1 to 4 show PCR products at approximately 400 bp (~300 bp vector + 89 bp linker). Precultures of colony 2 and 4 were made.

8. Restriction enzyme digestion of the minipreped plasmid as verification
    - Restriction enzyme digestion with *Eco*RI and *Age*I separately and double digestion.
9. Sequencing

```
gcgctggcggcgtaataa
```

DAS-Tag BioBrick

Background:

The DAS+4 tag presents a C-terminal recognition sequence that has been artificially altered so that it has weakened interactions with ClpXP and depends on an adaptor (Baker & Sauer, 2006). In *E. coli*, the adaptor SspB tethers specifically tagged substrates to the ClpXP protease, causing a modest increase in their rate of degradation. In our system, the role of the adaptor-protein SspB has been assumed by Pif3/6. So only light-induced activation can lead to binding and efficient degradation of DAS+4 bearing constructs.

Conception:

We decided to use the same tag, which was already successfully used by Baker and Sauer (2006). It is a eleven amino acid tag (AANDENYADAS). The codon usage of *E. coli* (http://www.geneinfinity.org/sp_codonusage.html) has been used to decide the DNA sequence, in addition NgoMIV and PST1 restriction sites have been added to the sequence.
The tag has been order as two separate NgoMIV + Pst1 precut primers, which were hybridized in order to obtain the tag sequence.

Ordered primer:

Forward: 5'**CCGGC**GCGGATGCGAGCTAATAATACTAGTAGCGGCCGC 3'

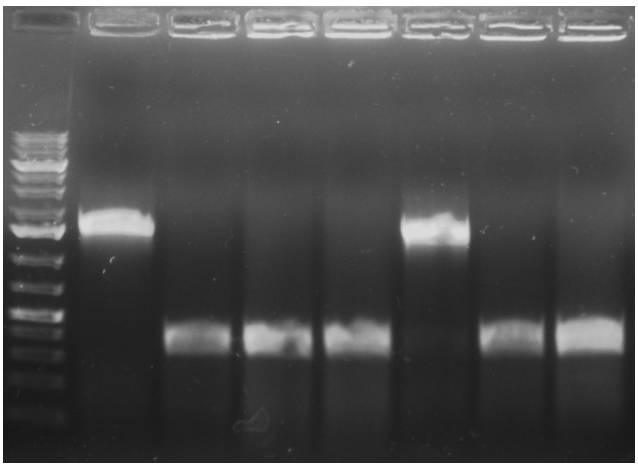
Reverse: 5'TGCAGCGGCCGCTACTAGTATTATTAGCTCGCATCCGCG 3'

For the synthesis of the PstI-site a mistake occurred in the command of the primers, as we did not consider that PstI cuts in the (3' -> 5') sens, contrary to the other restriction enzymes of the BioBrick standard. A supplementary step of ligation digestion in the experimental procedure can fix this mistake.

Followed procedure to obtain this biobrick:

1. Primer phosphorylation
   - Each primer is phosphorylated in a single reaction to ensure equal phosphorylation efficiency
   - Reaction volume 50 µl
     - 7µl of each primer from a 10µM stock solution => 500ng
     - 5 µl NEB ligation buffer 10x
     - 1 µl T4 D kinase (NEB) 10U/µl
     - 30 µl dH_20
   - Incubation for 30 min at 37°C•
2. Hybridization
   - Heating of the phosphorylation mixture to 98°C for 2 min
   - cooling slowly down to room-temperature

3. Ligation
    - In order to debug the mistake of the wrong PstI-extension that was made with the creation of the primers ; the following digestion step will create the right overhang
    - Addition of 1µl T4 DNA Ligase (Fermentas) to the hybridization mixture
    - Heat inactivation of the Ligase enzyme at 70°C for 5 min
4. Restriction enzyme digestion
    - By NgoMIV and Pst1, this will create the right PstI-overhang
    - Prevents polymers formation due to restriction site overlapping
    - Heat inactivation of the restriction enzymes at 80°C for 20 min
5. Ligation in the *NgoMIV/PstI*-precutted vector psB1C3
    - Insert vector ratio 10:1 in a 20 µl reaction volume
        - 5 µl insert (≈50ng)
        - 1 µl vector (≈10ng)
        - 2 µl T4 DNA Ligase Buffer
        - 1 µl T4 DNA Ligase (Fermentas)
        - 11 µl H2O
6. Transformation
    - 5µl of the ligation assay were transformed with 50 µl of competent cells and plated on chloramphenicol-containing LB-agar plates
    - Incubation at 37°C overnight
7. Screening by PCR on colony



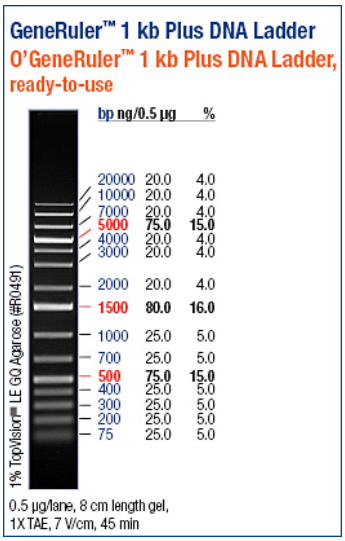


Figure 6: Bands at 400bp are characteristic for the plasmid with inserted tag.

8. Preculture
    - Positively-tested colonies have been set in 5ml chloramphenicol-containing LB-medium
9. Verification
    - Restriction enzyme digestion with EcoRI and Pst1 separately and double digestion.
10. Sequencing

```
gcggatgcgagctaataa
```

LAA-Tag BioBrick

Background:

LAA tag is a C-terminal region of the natural ssrA-recognition sequence of E. Coli that interacts with the ClpXP protease. A protein fused with this tag will be preferentially degraded by the ClpX protease without need of an adaptor protein (Baker & Sauer, 2006). This tag serves as positive control for the functionality of the composed ClpXP and the PhyB-ClpXP fusion protein.

Conception:

We chose to use the same tag, which was already successfully used by used by Baker and Sauer (2006). It is a eleven amino acid tag (AANDENYALAA). The codon usage of *E. coli* (http://www.geneinfinity.org/sp_codonusage.html) has been used to decide the DNA sequence and NgoMIV and Pst1 restriction sites have been added to the sequence.

The tag has been order as two separate NgoMIV + Pst1 precut primers, which were hybridized in order to obtain the tag sequence.

Ordered primer:

Forward: 5' **CCGGC**GCGCTGGCGGCGTAATAATACTAGTAGCGGCCG**C** 3'

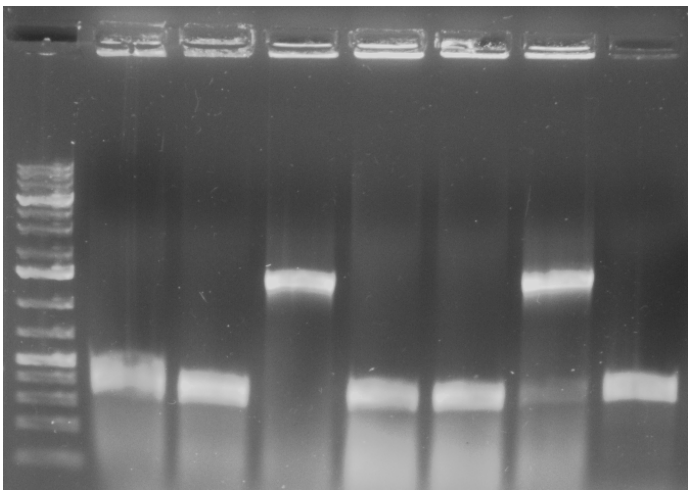
Reverse: 5' **TGCAG**CGGCCGCTACTAGTATTATTACGCCGCCAGCGC**G** 3'

For the synthesis of the PstI-site a mistake occurred in the command of the primers, as we did not consider that PstI cuts in the (3' -> 5') sens, contrary to the other restriction enzymes of the BioBrick standard. A supplementary step of ligation digestion in the experimental procedure can fix this mistake.

Followed procedure to obtain this biobrick::

1. Primer phosphorylation
   - Each primer is phosphorylated in a single reaction to ensure equal phosphorylation efficiency
   - Reaction volume 50 µl
     - 7µl of each primer from a 10µM stock solution => 500ng
     - 5 µl NEB ligation buffer 10x
     - 1 µl T4 D Kinase (NEB) 10U/µl
     - 30 µl dH_20
   - Incubation for 30 min at 37°C
2. Hybridization
   - Heating of phosphorylation mixture to 98°C for 2 min
   - cooling slowly down to room-temperature

3. Ligation
    - In order to debug the mistake of the wrong PstI-extension that was made with the creation of the primers ; the following digestion step will create the right overhang
    - Addition of 1µl T4 DNA Ligase (Fermentas) to the hybridisation mixture
    - Heat inactivation of the Ligase enzyme at 70°C for 5 min
4. Restriction enzyme digestion
    - By NgoMIV and Pst1, this will create the right PstI-overhang
    - Prevents polymers formation due to restriction site overlapping
    - Heat inactivation of the restriction enzymes at 80°C for 20 min
5. Ligation in the *NgoMIV/PstI*-precutted vector psB1C3
    - Insert vector ratio 10:1 in a 20 µl reaction volume
        - 5 µl insert (≈50ng)
        - 1 µl vector (≈10ng)
        - 2 µl T4 DNA Ligase Buffer
        - 1 µl T4 DNA Ligase (Fermentas)
        - 11 µl H2O
6. Transformation
    - 5µl of the ligation assay were transformed with 50 µl of competent cells and plated on chloramphenicol-containing LB-agar plates
    - Incubation at 37°C overnight
7. Screening by PCR on colony



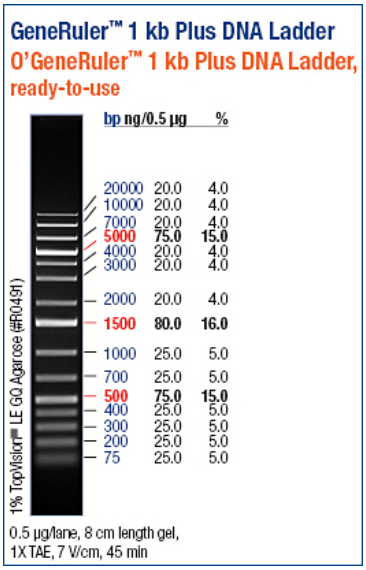


Figure 7:Bands at 400bp are characteristic for the plasmid with inserted tag.

8. Preculture
    - Positively-tested colonies have been set in 5ml chloramphenicol-containing LB-medium
9. Verification
    - Restriction enzyme digestion with EcoRI and Pst1 separately and double digestion.
10. Sequencing

gcggatgcgagctaataa

λO-Tag Biobrick

Background:

The λO tag is the N-terminal equivalent to the DAS+4 tag. Degradation of proteins bearing the N-terminal λO tag normally requires the N-domain of ClpX, which is missing in the PhyB-linker-$[ClpX]_3$ variant.

Baker and Sauer (2009) used this tag to test an artificial tethering system and demonstrated that it can serve as degradation signal for substrates that are tethered to ClpX.

Conception:

We chose to use the same sequence, which was already successfully used by used by Baker and Sauer (2009): NH_2-TNTAKILNFGR. The codon usage of *E. coli* (http://www.geneinfinity.org/sp_codonusage.html) has been used to decide the DNA sequence, in addition NgoMIV and AgeI restriction sites have been added to the sequence.

The tag has been order as two separate NgoMIV + AgeI precut primers, which were hybridized in order to obtain the tag sequence.

Ordered primer:

Forward: 5' **AATTC**GCGGCCGCTTCTAGATGACCAACACCGCGAAAATTCTGAACTTTGGCCGCA 3'
Reverse: 5' **CCGGT**GCGGCCAAAGTTCAGAATTTTCGCGGTGTTGGTCATCTAGAAGCGGCCGCG3'

Followed procedure to obtain this biobrick:

1. Hybridization
   - Mixture of the primers in an equimolar ratio in 50µl
     - 7µl of each primer from a 10µM stock solution => 500ng (10ng/µl)
     - 36 µl dH_20
   - Heating to 98°C for 2 min
   - cooling slowly down to room-temperature
2. Ligation in the *NgoMIV/AgeI*-precutted vector psB1C3
   - Insert vector ratio ≈15:1 in 20 µl reaction volume
     - 4 µl insert (≈40ng)
     - 2 µl vector (≈20ng)
     - 2 µl T4 DNA Ligase Buffer
     - 1 µl T4 DNA Ligase (Fermentas)
     - 11 µl H2O
3. Transformation
   - 5µl of the ligation assay were transformed with 50 µl of competent cells and plated on chloramphenicol-containing LB-agar plates
   - Incubation at 37°C overnight

4. Screening by PCR on colony

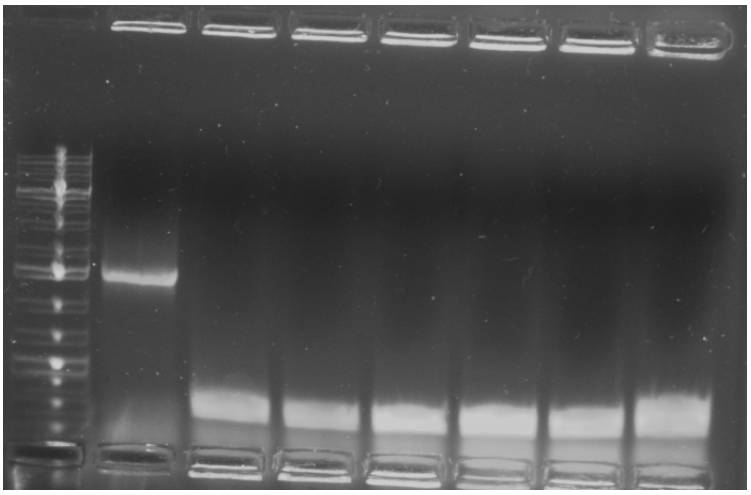
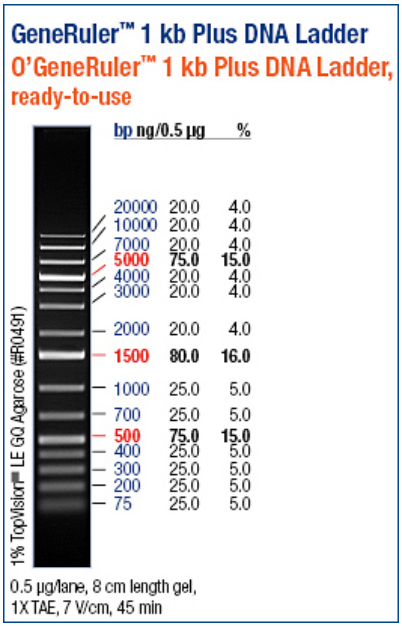


Figure 8: Bands at 400bp are characteristic for the plasmid with inserted tag.



5. Preculture
   - Positively-tested colonies have been set in 5ml chloramphenicol-containing LB-medium
6. Verification
   - Restriction enzyme digestion with EcoRI and Pst1 separately and double digestion.
7. Sequencing

```
atgaccaacaccgcgaaaattctgaactttggccgc
```

4. Assembly

In the following we show how the different BioBricks have been assembled. As for the single parts, we will start with a short presentation of background and conception of each assembled BioBrick.

Below you find the complete liste of the assembled constructs we created.

- **PIF3/6-His Tag and PhyB 650/900-His Tag**
- **ClpX-Trimer ($[ClpX(-N)]_3$)**
- **PhyB 650 - $[ClpX(-N)]_3$**
- **PhyB 900 - $[ClpX(-N)]_3$**
- **GFP-Pif3-DAA**
- **GFP-Pif3-LAA**
- **GFP-Pif6-DAA**
- **GFP-Pif6-LAA**

PIF3/6-His Tag and PhyB 650/900-His Tag

Background:

The association of the PIFs and PhyBs proteins with the histidine tag allows the purification of those proteins quickly and efficiently through a nickel column. The purification of those factors is important for their characterization.

Conception:

The conception of the biobrick was done through the assembly of the initial PIF3/PIF6/phyB650/PhyB900 biobricks and the His-tag biobrick. For a better yield, it is better to use two different plasmid resistances from the two initial biobricks. For instance, in this case, the His-tag biobrick was taken directly from the biobrick sent by the MIT; it is the biobrick BBa_K157011 with the Freiburg fusion restriction sites.

In orofr to realize this biobrick, it was ofciofd to insert the PIFs or PhyBs part into the hit-tag biobrick and this in a way to have the his-tag in the C-terminal end of the protein. All the protocols used were the standard protocol with a variation in the molar concentration from both parts. The PIF biobrick with a chloramphenicol resistance was cut with EcoRI and AgeI. On the other siof, the his-tag biobrick with an ampicillin resistance was cut with the EcoRI and NgoMIV restriction enzymes. The ligation was done without any purification with several molar concentration mixes from both biobricks:

- Equal molar concentration from both biobricks
- Twice as much initial biobrick than his-tag biobrick
- Four times as much initial biobrick than his-tag biobrick

The ligations were transformed and platted on LB plates with an ampicillin resistance. Therefore, only the colony transformed with the initial-histag plasmid or the histag plasmid alone will be viable. After an overnight incubation, the colonies are screened with the VR and VF primers and the clones which have integrated the PIF part are chosen and after purification, sent to sequencing. For the PhyB, the PCR control was done using two primers ofsigned for the sequencing.

Followed procedure to obtain this biobrick:

1. Restriction enzyme digestion
    a. Mix (50µl reaction volume):
        i. 5µl of biobrick (80ng/µl)
        ii. 5µl of NEB2 buffer
        iii. 1µl of each restriction enzyme (See the conception part)
        iv. 0,5µl BSA 100X
        v. 37,5µl of H_2O
    b. 30 minutes incubation at 37°C
    c. 20 minutes inactivation at 80°C
2. Ligation
    a. Mix (20µl reaction volume):
        i. 1µl of his-tag biobrick
        ii. Either 1µl, 2µl or 4µl of initial biobrick
        iii. 2µl ligation buffer 10X
        iv. 0,5µl ligase
        v. Either 15,5µl, 13,5µl or 11,5µl H_2O
    b. Incubation at room temperature (20°C-25°C) for 20 minutes
    c. Inactivation 15 minutes at 65°C
3. Transformation following the standard protocol.
4. Colony screening
    a. Each colony is resuspenofd into 20µl H_2O
    b. PCR mix (50µl reaction volume):
        i. 2µl adapted primer 10µM
        ii. 2µl adapted primer 10µM
        iii. 1µl dNTPs 10mM each
        iv. 5µl PCR Buffer 10X
        v. 5µl MgCl2 25mM
        vi. 1µl Taq polymerase (2.5U/µl)
        vii. 15µl resuspenofd cells
        viii. 19µl H2O
    c. PCR conditions
        i. 94°C for 5 minutes
        ii. 94°C for 30 seconds
        iii. 55°C for 30 seconds
        iv. 68°C for 1 minute
        } 25 times
        v. 68°C for 5 minutes
        vi. 20°C forever
    d. Gel electrophoresis
        i. 10µl PCR + 2µl Loading Dye 6X

ii. Run 120V for 40 minutes

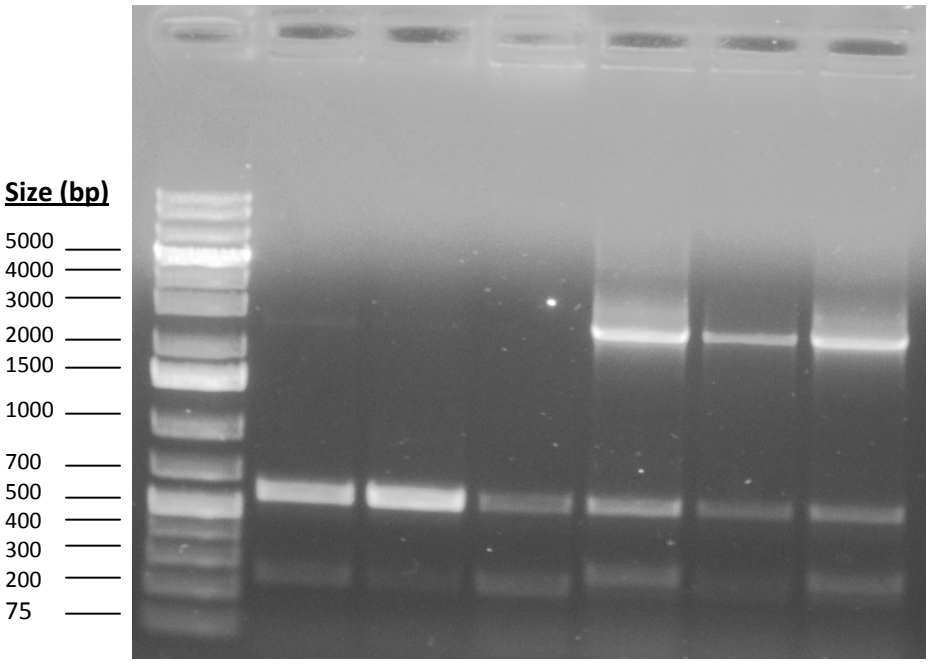


Figure 9: exemple of an agarose gel after PCR verification of the PIF factor, the band at about 600 base pair is the band ofsired. line 2 and 3 are typical from a good insertion.

iii. Characteristic bands at 600 (PIF) or 900 (PhyB) base pair.

5. Precultures of the selected colonies
    a. 5ml of LB medium
    b. 5µl of Ampicillin 1000X
    c. Incubation overnight at 37°C
6. Purification of the plasmid using a miniprep kit
7. Restriction enzyme digestion test
    a. Mix (20µl reaction volume):
        i. 5µl of purified plasmid
        ii. 2µl of NEB Buffer 2
        iii. Either 1µl of EcoRI + 1µl PstI or 1µl EcoRI only
        iv. 0,5µl BSA 100X
        v. 10,5µl H2O or 11,5µl H2O
    b. Incubation 15 minutes at 37°C
    c. Gel electrophoresis (see above)

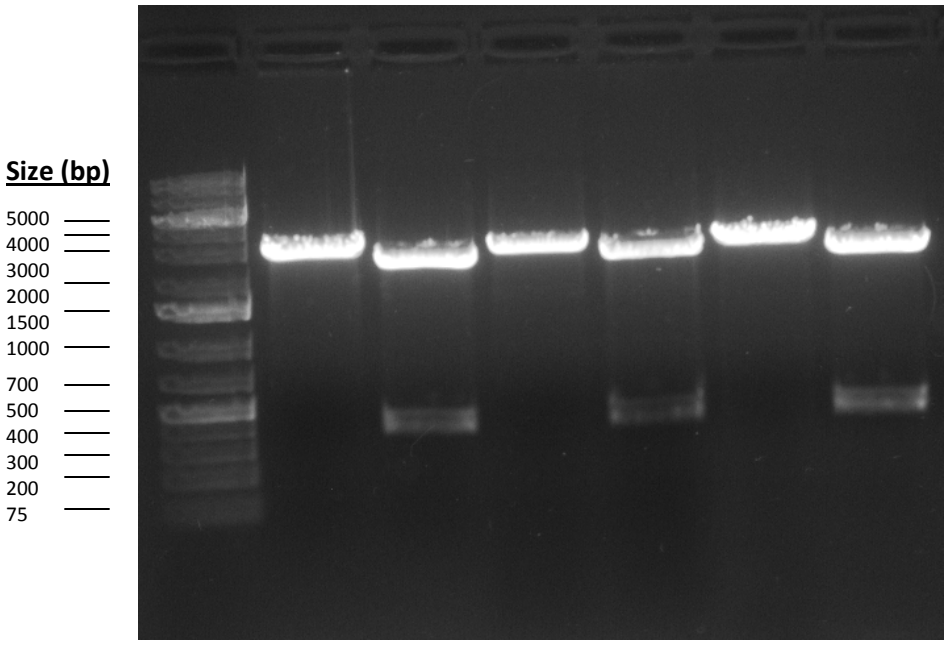


Figure 10: exemple of an agarose gel after digestion of the PIF-Histag biobrick. Line 2,4,6 is the biobrick digested with EcoRI. The line 3,5,7 are the corresponding biobrick digested by EcoRI and PstI. The characteristic band for PIF is at about 350 base pair, about 2000 bp for PhyB650 and about 2800 bp for PhyB900.

i. Characteristic bands can be seen at 300-400 bp

8. Sequencing

Troubleshooting:

There were some problems due to the assembly of those biobricks. However it was not very evident to find the causes of the mistakes. It was therefore decided to do the experiment again and it finally worked. Out of the colonies tested, about half had the insert and one of each was sent to sequencing.

ClpX- Trimer Biobrick

Background:

Phytochrome B needs to be fixed to the Clpx hexameric part of the protease. It is possible to link one phytochrome B per ClpX monomer but this could lead to sterical problems. So the decision was made to follow the idea of the publication of Tanja Baker 2009. This permits to have only one phytochrome B for three ClpX units. Moreover the publication proved that the speed of the assembly of two ClpX trimers is quite the same as with ClpX monomers. So the Clpx hexameric part is composed of two ClpX trimers each one coupled with a phytochrome B.

Conception:

ClpX primer was constructed due to cloning methods. The new approach in this method is that the primer was built from a PCR product. The construction of the ClpX dimer was conducted with standard methods. Then in a PCR reaction the ClpX primers located inside of the dimer were used to construct this new primer. The product of this PCR was used as primer for constructing the ClpX trimer.

Followed procedure to obtain this construct:

1. Amplification by PCR of ClpX linker with primer VF&VR
   - 1µl de ClpX (linker)
   - 1µl de dNTP
   - 1,5µl de primer 19 (VF)
   - 1,5µl de primer 20 (VR)
   - 5µl de buffer pfu
   - 1µl de pfu
   - 39µl d'H_2O

2. Digestion of VF-ClpX-VR by NgOMIV
   - 5µl VF-ClpX-VR
   - 3µl buffer NEB 4 10x
   - 21µl of H_2O
   - 1µl NgOMIV

   -> 1h at 37°C then inactivation and purification with column (30µl final volume)

   Digestion du VF-linker-VR par Age1
   - 5µl VF-linker-VR
   - 3µl buffer fast digest
   - 21µl d'H_2O
   - 1µl Age1

   -> 30 min at37°C then inactivation and purification with column (30µl at the end)

3. Ligation VF-ClpX-VR NgOMIV + VF-Linker-VR Age1
   - 10µl VF-ClpX-VR NgOMIV dig
   - 10µl VF-linker-VR Age1 dig
   - 3µl ligase buffer
   - 6µl H_2O
   - 1µl ligase

   -> 10 min at room temperature then inactivation 20 min at 80 °C and purification with column (30µl at the end)

4. Amplification: by PCR of VF-linker-ClpX-VR with primer VF&VR
   - 2µl de VF-linker-ClpX-VR
   - 1µl de dNTP
   - 1,5µl de primer 19 (VF)
   - 1,5µl de primer 20 (VR)
   - 5µl de buffer pfu
   - 1µl de pfu
   - 38µl d'H_2O

5. Purification with miniprep

6. Test on Gel : VF-ClpX-VR vs VF-linker-ClpX- VR

7. Digestion of VF-linker-ClpX-VR by NgOMIV
   a. 5µl VF-linker-ClpX-VR
   b. 3µl buffer NEB 4 10x
   c. 21µl d'H_2O
   d. 1µl NgOMIV

   -> 1h at 37°C then inactivation and purification with miniprep (30µl at the end)

   Digestion of VF-linker-ClpX-VR by Age1
   - 5µl VF-linker-ClpX-VR
   - 3µl buffer fast digest
   - 21µl d'H_2O
   - 1µl Age1

   -> 30 min at 37°C then inactivation and purification with column (30µl at the end)

8. Ligation VF-linker-ClpX-VR NgOMIV + VF-Linker-ClpX-VR Age1
   - 10µl VF-linker-ClpX-VR NgOMIV dig
   - 10µl VF-linker-ClpX-VR Age1 dig
   - 3µl ligase buffer
   - 6µl d'H_2O
   - 1µl ligase

   -> 10 min at room temperature then inactivation 20 min at 80 °C and purification with column (30µl at the end)

-> Failure of the experiment -> lot of non specific strips

->extraction of the good trip on a LMP gel but a lot of strips are present where we expect us strip.

9. Solution: Creation of a new primer by PCR on a fragment of the first PCR thanks a interne primer of ClpX.
   - 1μl de ClpX -linker-ClpX
   - 1μl de dNTP
   - 1,5μl de intern ClpX primer
   - 1,5μl de intern ClpX primer
   - 5μl de buffer pfu
   - 1μl de pfu
   - 39μl d'H_2O

10. Amplification: Creation of VF-linker-ClpX-linker-ClpX-VR

    A
    - 1μl de VF-linker-ClpX-VR dig Age1
    - 1μl de VF-linker-ClpX-VR dig NgoMIV
    - 1μl de dNTP
    - 1,5μl de primer Clpx-linker-ClpX
    - 1,5μl de primer 19 (VF)
    - 5μl de buffer pfu
    - 1μl de pfu
    - 38μl d'H_2O

    B
    - 1μl de VF-linker-ClpX-VR dig Age1
    - 1μl de VF-linker-ClpX-VR dig NgoMIV
    - 1μl de dNTP
    - 1,5μl de primer Clpx-linker-ClpX
    - 1,5μl de primer 20 (VR)
    - 5μl de buffer pfu
    - 1μl de pfu
    - 38μl d'H_2O

    C
    - 1μl de A
    - 1μl de B
    - 1μl de dNTP
    - 1,5μl de primer 19 (VF)
    - 1,5μl de primer 20 (VR)
    - 5μl de buffer pfu
    - 1μl de pfu
    - 38μl d'H_2O

11. Insertion in PSB1C3 of linker-ClpX-linker-ClpX with restriction enzyme

12. Insertion of ClpX the beginning of the construct with restriction enzyme

PhyB - [ClpX(-N)]$_3$ Biobricks

As the applied strategy for the assembly of the phytochromes with the ClpX-Trimer was the same for PhyB650 and PhyB900, we summarized the protocol for these two constructs.

Background:

The linker-ClpX-Linker-ClpX-Linker-ClpX biobrick is used to build a ClpX trimer and to build the PhyB650-linker-ClpX-Linker-ClpX-Linker-ClpX and PhyB900- linker-ClpX-Linker-ClpX-Linker-ClpX. The assembly of two of these iofntical subunits leads to the ClpX hexamer of the final ClpXP protease.

Conception:

We chose to use the same method, which was already successfully used by Tanja Baker (Davis JH, Baker TA, Sauer RT, J Biol Chem. 2009 Aug 14;284(33):21848-55) to construct the ClpX trimer.

Followed procedure to obtain this construct:

13. Amplification by PCR of ClpX linker with primer VF&VR
    - 1µl of ClpX (linker)
    - 1µl of dNTP
    - 1,5µl of primer 19 (VF)
    - 1,5µl of primer 20 (VR)
    - 5µl of buffer pfu
    - 1µl of pfu
    - 39µl d'H_2O

14. Digestion of VF-ClpX-VR by NgOMIV
    - 5µl VF-ClpX-VR
    - 3µl buffer NEB 4 10x
    - 21µl of H_2O
    - 1µl NgOMIV

    -> 1h at 37°C then inactivation and purification with miniprep kit (30µl final volume)

15. Digestion du VF-linker-VR par Age1
    - 5µl VF-linker-VR
    - 3µl buffer fast digest
    - 21µl d'H_2O
    - 1µl Age1

    -> 30 min at37°C then inactivation and purification with column (30µl at the end)

16. Ligation VF-ClpX-VR NgOMIV + VF-Linker-VR Age1
    - 10µl VF-ClpX-VR NgOMIV dig
    - 10µl VF-linker-VR Age1 dig
    - 3µl ligase buffer
    - 6µl H_2O
    - 1µl ligase

-> 10 min at room temperature then inactivation 20 min at 80 °C and purification with column (30µl at the end)

17. Amplification: by PCR of VF-linker-ClpX-VR with primer VF&VR
    - 2µl of VF-linker-ClpX-VR
    - 1µl of dNTP
    - 1,5µl of primer 19 (VF)
    - 1,5µl of primer 20 (VR)
    - 5µl of buffer pfu
    - 1µl of pfu
    - 38µl d'H_2O

18. Miniprep purification

19. Test on Gel : VF-ClpX-VR vs VF-linker-ClpX- VR

    ->We can see many different stripes and one of them can match with what we expect.

20. Digestion of VF-linker-ClpX-VR by NgOMIV
    - 5µl VF-linker-ClpX-VR
    - 3µl buffer NEB 4 10x
    - 21µl d'H_2O
    - 1µl NgOMIV

    -> 1h at 37°C then inactivation and purification with column (30µl at the end)

21. Digestion of VF-linker-ClpX-VR by Age1
    - 5µl VF-linker-ClpX-VR
    - 3µl buffer fast digest
    - 21µl d'H_2O
    - 1µl Age1

    -> 30 min at 37°C then inactivation and purifictaion with column (30µl at the end)

22. Ligation VF-linker-ClpX-VR NgOMIV + VF-Linker-ClpX-VR Age1
    - 10µl VF-linker-ClpX-VR Age1 dig
    - 3µl ligase buffer
    - 6µl d'H_2O
    - 1µl ligase
    - 10µl VF-linker-ClpX-VR NgOMIV dig

    -> 10 min at room temperature then inactivation 20 min at 80 °C and purification with column (30µl at the end)

    -> Failure of the experiment -> lot of non specific strips

    -> Extraction of the good strip on a LMP gel but a lot of strips are present where we expect our strip.

23. Solution: Creation of a new primer by PCR on a fragment of the first PCR thanks to an intern primer of ClpX.
    - 1µl of ClpX -linker-ClpX
    - 1µl of dNTP
    - 1,5µl of intern ClpX primer
    - 1,5µl of intern ClpX primer
    - 5µl of buffer pfu
    - 1µl of pfu
    - 39µl d'H_2O

24. Amplification: Creation of VF-linker-ClpX-linker-ClpX-VR

    A
    - 1µl of VF-linker-ClpX-VR dig Age1
    - 1µl of VF-linker-ClpX-VR dig NgoMIV
    - 1µl of dNTPs
    - 1,5µl of primer Clpx-linker-ClpX
    - 1,5µl of primer 19 (VF)
    - 5µl of buffer pfu DNA polymerase
    - 1µl of pfu DNA polymerase
    - 38µl H_2O

    B
    - 1µl of VF-linker-ClpX-VR dig Age1
    - 1µl of VF-linker-ClpX-VR dig NgoMIV
    - 1µl of dNTP
    - 1,5µl of primer Clpx-linker-ClpX
    - 1,5µl of primer 20 (VR)
    - 5µl of buffer pfu DNA polymerase
    - 1µl of pfu DNA polymerase
    - 38µl d'H_2O

    C
    - 1µl of A
    - 1µl of B
    - 1µl of dNTP
    - 1,5µl of primer 19 (VF)
    - 1,5µl of primer 20 (VR)
    - 5µl of buffer pfu DNA polymerase
    - 1µl of pfu DNA polymerase
    - 38µl d'H_2O

25. Insertion in PSB1C3 of linker-ClpX-linker-ClpX with restriction enzyme

26. Insertion of ClpX a the beginning of the construct with restriction enzyme

27. Insertion of PhyB 650-linker and PhyB900- linker in the plasmid with restriction enzyme

GFP(CFP)-linker-Pif3/6- linker-DAS/LAA BioBricks

Background:

These final assemblies will enable to test the feasibility of the degradation according to different factors (the tag, the protein to be degraded, the PIF)

Conception:

We chose to proceed by successive steps, thus building intermediary assemblies.

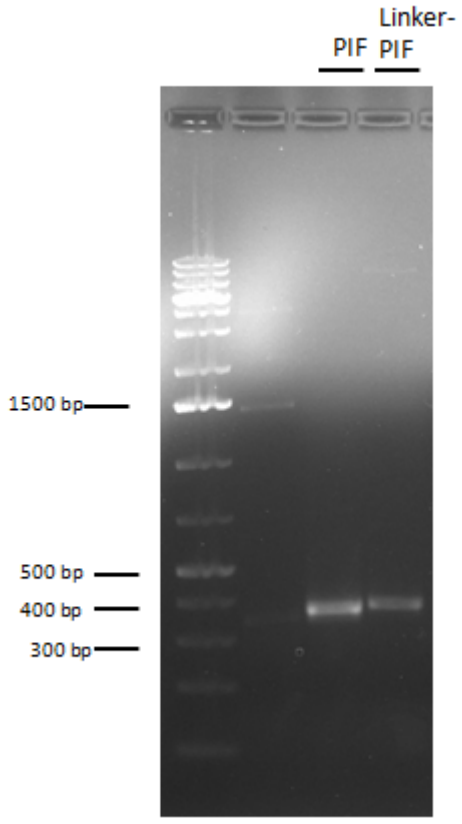
Followed procedure to obtain the linker-PIF3/6 biobrick (in PSB1C3) :

Remark : The procedure was done according to the available stock (linear linker, linear PIF3/6) insofar as the strategies to use were limited and not necessary the best ones for obtaining this biobrick.

1. Digestion of the Linker by AgeI
   Digestion of PIF3/6 by NgoMIV

Remark : Each digestion is always followed by a purification on column before any other use

2. Ligation : 20min at 24 °C
3. Electrophoresis on agarose gel



The linear PIF has been compared with the ligation product. A small difference is notable (the linker measure 60bp). The linear PIF does not contain the VR part. That is why the band is only at about 400bp.

4. PCR-Amplification

i. PCR- Amplification of Linker-PIF with VF/PIF suffix

ii. PCR-Amplification of the PSB1C3 vector (about 10kb) from the ClpX biobrick with the primers 13 and 8 (from the ClpX mutagenesis)

Reaction mix	
Template	1µL
dNTP 10mM	1µL
Primer 8 (1:10)	1,5µL
Primer 13 (1:10)	1,5µL
Pfu Buffer (MgSO4)	5µL
Pfu	1µL
H20	39µL
Whole volume	50 µL

94 °C	5min	1 X
94 °C	45sec	18 X
65°C	45sec	
72°C	5min	
72°C	10min	1 X

5. Digestion

i. Digestion of the PSB1C3 amplified by EcoRI,PstI

Reaction mix	
Template	20 µL
NEB 4 buffer	3µL
BSA 10X	3µL
EcoRI	1µL
PstI	1µL
H20	2µL
Whole volume	30 µL

→ 2hours at 37°C then inactivation during 20 min at 65°C

ii. Digestion of Linker-PIF amplified by EcoRI,PstI

Purification on column of the digestion products

6. Ligation

Reaction mix		
Insert	1µL	2 µL
Vector	1µL	
Ligation Buffer (MgCl2)	1µL	
T4 DNA ligase	1µL	
H20	6µL	5 µL

→5min at room temperature

+ 2 µL vector

→2,5min at room temperature

+ 2 µL vector

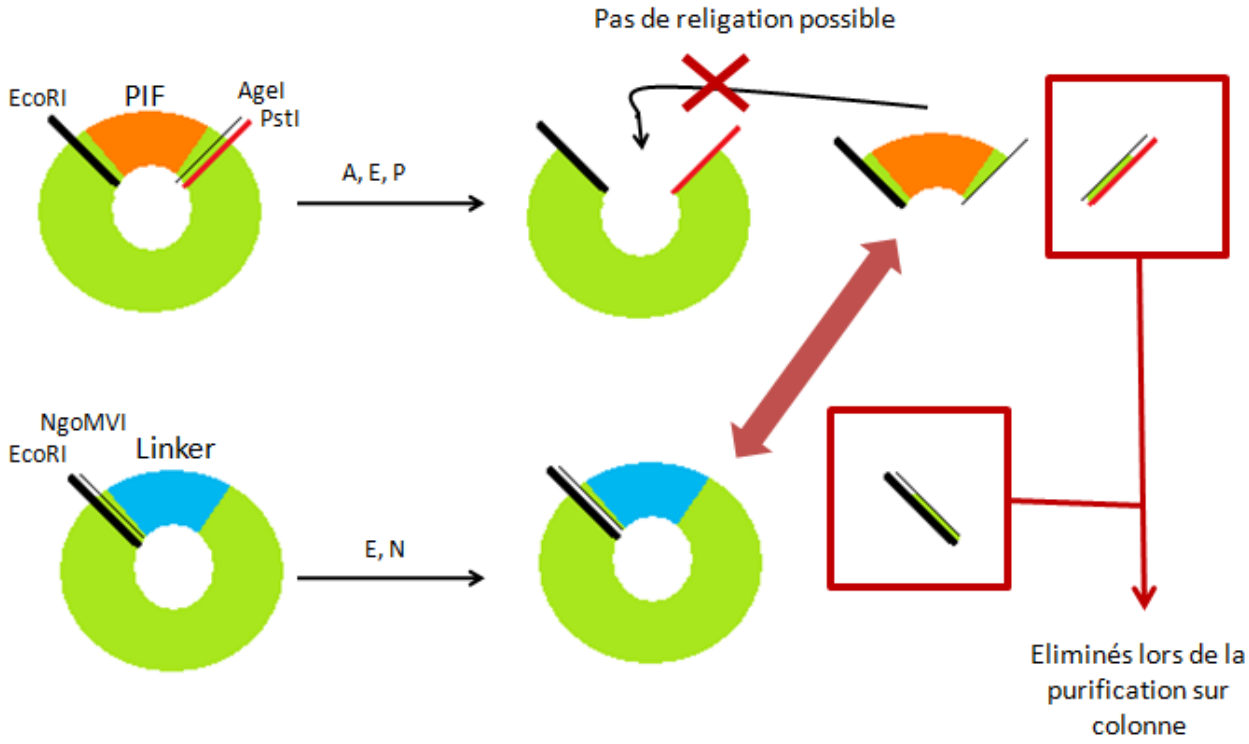
→2,5min at room temperature

7. Transformation
8. PCR colony (precultures performed in parallel)

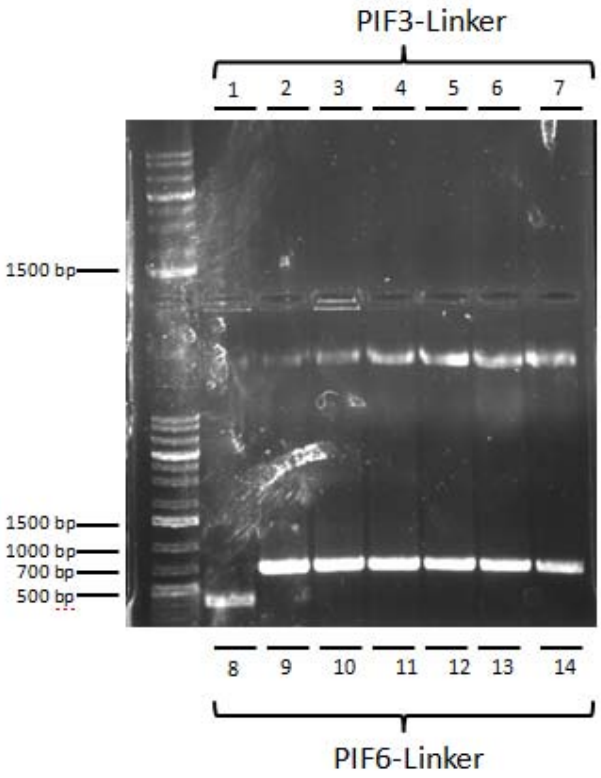
9. Electrophoresis
10. Miniprep
11. Sequencing: No sequencing has been performed at this time for management reason.

Followed procedure to obtain the PIF 3/6 –linker biobrick (dans PSB1C3) :

Strategy:



1. Digestion of the Linker biobrick with NgoI and EcoRI
   Digestion of the PIF3/6 biobrick with AgeI, EcoRI and PstI
2. Ligation
3. Transformation
4. PCR colony (Minicultures are done in parallel)
5. Gel electrophoresis



Except for the number 8, the size of the bands (700bp) for PIF6-Linker corresponds to the expected size (300-400bp for PIF + 70-80bp for the Linker + 2*150bp for VF/VR). Nevertheless further verification need to be done. Concerning PIF6, the gel has too much run. However, as the bands have all the same size and as the majority of the bands of PIF3-Linker seems okay, the chances are that it is similar for PIF6-Linker (everything has been done in parallel for the both construction

6. TO DO : Minipreparation for two samples of each construction (with PIF 3 and with PIF6), PCR with primer forward in VF and a reverse primer corresponding to the linker
   → Sequencing

Followed procedure to obtain the GFP(CFP)-PIF 3/6 –linker biobrick (in PSB1C3) :

1st Method applied

1. PCR-Amplification of the DNA needed

i) PCR Amplification of the CFP gene (750bp) contained in PSB1C3 by VF/VR

Reaction mix	
Template (10ng/µL)	1µL
dNTP 10mM	1µL
Primer 19 (1:10)	1,5µL
Primer 20 (1:10)	1,5µL
Pfu Buffer (MgSO4)	5µL
Pfu	1µL
H20	39µL
Whole volume	50 µL

94 °C	1min30	1 X
94 °C	30sec	25 X
65°C	30sec	
	→ -0,4°C/cycle : Touchdown 65°C -> 55°C	
72°C	2min	
72°C	7min	1 X

Remark: Elongation time : 2min/1kb with the Pfu DNA polymerase

ii) PCR-Amplification of the Linker-PIF (from the first method) by VF/47(PIF3) or 16 (PIF6)

Reaction mix	
Template	2µL
dNTP 10mM	1µL
Primer 19 (1:10)	1,5µL
Primer 47/16 (1:10)	1,5µL
Pfu Buffer (MgSO4)	5µL
Pfu	1µL
H20	38µL
Whole volume	50 µL

Thermal cycling		
94 °C	1min	1 X
94 °C	30sec	18 X
65°C	30sec	
	→ -0,3°C/cycle : Touchdown 60°C -> 55°C	
72°C	1min	
72°C	6min	1 X

Remark: Several PCR have been performed without leading to results because of a problem with the dNTP.

The two PCR have been controlled by electrophoresis on agarose gel.

2. Digestion

i) Digestion of CFP by AgeI

Reaction mix	
Template	5 µL
Fast Digest buffer	3µL
AgeI	1µL
H20	21µL
Whole volume	30 µL

→ 30min at 37°C then inactivation at 80°C during 20min

ii) Digestion of L-PIF by Ngo

Reaction mix	
Template	15 µL
NEB 4 buffer	3µL
NgoI	1,5µL
H20	10,5µL
Whole volume	30 µL

→ 30min at 37°C then inactivation at 80°C during 20min

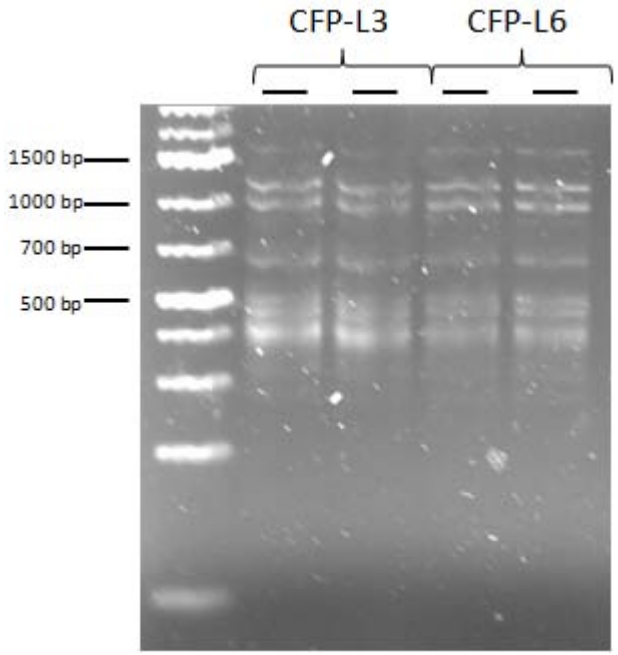
Purification of all the digestion with the "Quiakit PCR purification"

3. Ligation

Reaction mix	
L-PIF /A	15µL
CFP/N	5µL
Ligation Buffer (MgCl2)	3µL
T4 DNA ligase	1,5µL
H20	5,5 µL

→30min at about 20°C

4. Extraction on gel LMP 1,5% (after a first agarose gel)



→ Lots of bands -> Selection of 1 band at 1200 and one at 1700, extraction by the kit PCR Fermentas

5. PCR amplification VF/VR
6. Electrophoresis : No band at 1200 or 1700 anymore, only at 400bp

Remark : Many other members of the group had also difficulties with the purification on LMP gel

2nd Method applied

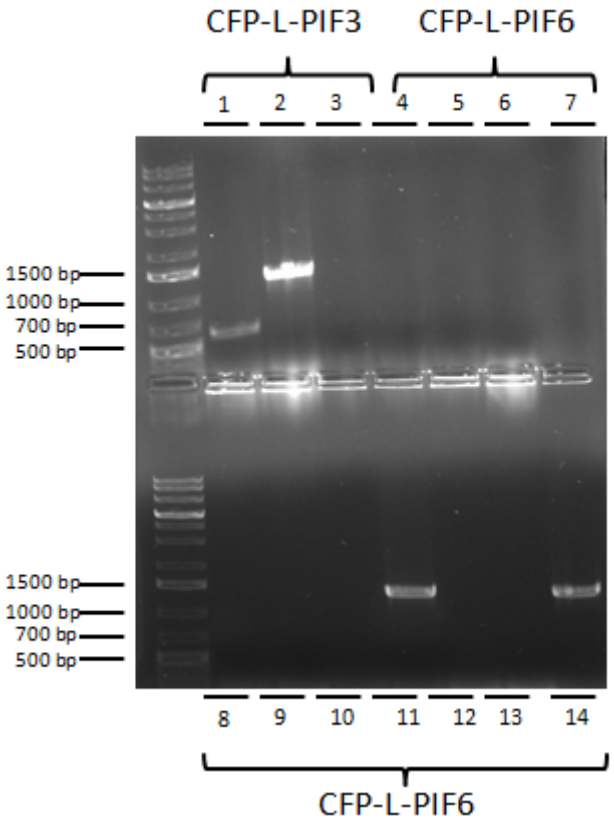
1. Digestion of the biobrick Linker-PIF3/6 (C3) by Ngo and EcoRI
   Digestion of the CFP(GFP) amplified by PCR by AgeI and EcoRI
2. Ligation
3. Transformation
4. PCR colony (Minicultures were done in parallel)

Reaction mix	
Template	10µL
dNTP 10mM	1µL
Primer 19 (1:10)	1,5µL
Primer 20 (1:10)	1,5µL
Taq Buffer	2µL
MgCl2	2 µL
Taq	1µL
H20	1µL
Whole volume	20 µL

94 °C	5min	1 X
94 °C	30sec	25 X
65°C	30sec	
	→ -0,4°C/cycle : Touchdown 65°C -> 55°C	
72°C	45sec	
72°C	5min	1 X

Achievement of the template: One colony in 20µL of water → boiled → taking of 10µL

5. Electrophoresis



We obtained only 1 positive result for CFP-L-PIF3 (other colonies were tested for this construction but laid on another gel) and two positive results for CFP-L-PIF6. But we needed only one positive results for each, so it was okay.

6. Minipreparation
7. Sequencing

→ Unfortunately, the Linker was not in the construct. Nevertheless we will test this construct as the linker may not be necessary.

Followed procedure to obtain the PromRbs-GFP(CFP)-PIF 3/6 –linker biobrick (in PSB1A3) :

Remark: Two kinds of PromRbs have been used : the promoter inducible by tetracycline and the LAC promoter

1. Digestion of the biobrick GFP(CFP)-Linker-PIF3/6 (C3) by XbaI and PstI
   Digestion of the PromRbs Biobrick by EcoRI and SpeI
   Digestion of the PSB1A3 vector by EcoRI and PstI
2. Ligation

Reaction mix	
Digested PB1A3 vector (very few concentrated 25ng/µL)	3µL
Digested PromRbs biobrick	1µL
Digested GFP(CFP)-Linker-PIF3/6 (C3) biobrick	1µL
Ligation Buffer	1µL
T4 DNA ligase	0,5µL
H20	3,5 µL
Whole volume	10 µL

→ 15 min at room temperature

3. Transformation

Few colonies were obtained. Some satellite-colonies were noticed.

Plate	Number of colonies obtained	Number of colonies screened
PromLACRbs-GFP-PIF6 (A3)	2	2
PromLACRbs-GFP-PIF3(A3)	About 20	4
PromTETRbs-GFP-PIF6 (A3)	About 15	5
PromTETRbs-GFP-PIF3 (A3)	3	3

4. PCR colony
5. Gel electrophoresis

→ No positive results obtained for the time being

Followed procedure to obtain the final assembly :

Not yet performed but we will combine the biobrick Linker-LAA/DAS with our last assembly

5. Troubleshooting

This part will give you an overview of the problems we met during the work at the bench. The specific oftails to individual parts are enclosed to the documentation how it has been created; here we will summarize the most time-consuming and weightiest difficulties we have been faced with, as

- **Obtaining competent cells – the arduous path**
- **The ClpX-story (or Why digestion tests do not substitute regular sequencing)**
- **PCR colony screening (or DO look a gift horse in the mouth)**
- **PstI (or Never forget the devil is in the detail)**
- **Characterization**

Obtaining competent cells

A good efficiency of competent cells is the basis for a successful transformation. At the beginning of our work this condition was not given, consequently we did not achieve to perform successful transformation. We tried several protocols of different origins to overcome this obstacle, arisen either from personal internship experiences of the team members, from the practical course of Enzyme-free Cloning or from the openwetware website directed by the MIT and recommended by the iGEM supervisors.

Protocol 1(Source: University of Basel)

HTB (250ml)
0.6g HEPES
0.55g CaCl2
4.66g KCl
dissolve in 200mL dH2O
adjust pH to 6.7 with KOH
add 2.72g MnCl2.4H2O
adjust volume to 250 mL

50X TAE (1L)
242 g Tris Base
57.1 mL glacial acetic acid
100 mL of 0.5 M EDTA, pH 8.0
adjust to 1L with water

1X TE (1L)
10ml 1M tris Hcl
2ml 0.5M EDTA
adjust to

To get good results it's important to start with fresh cells. Strike a plate the day before the day before inoculation, and use directly from the 37°C stove. After cells have been spun down they should stay cold.

Day 1

Use about 10 large colonies from a freshly grown plate to start a 200ml culture in SOB medium (LB is ok too). Grow the cells overnight at room temperature or a bit below (18- 22°C) with vigorous shacking. Start at least 2 or 3 bottle with varying inoculation amounts, to be sure to have at least 1 OK during next day.

Day 2

1. The cells should be grown to an OD600 as close to 0.45 as possible, and therefore must be monitored carefully. When the correct ofnsity as been reached the culture should be put in 4 sterile 50mL falcon tube and placed on ice for 10min.
2. Spin for 15min at 3500-4000 rpm and carefully discard all the supernatant.
3. Resuspend each pellet in 16mL cold HTB and keep on ice for 10min.
4. Spin again 15 min at 3500 rpm and carefully discard all supernatant.
5. Resuspend each pellet in 4mL cold HTB
6. Slowly add 300ul (filter sterilized) DMSO while gently swirling the cell suspension in each falcon
7. Aliquot immediately into eppendorf tubes (100ul) and quick freeze in a -80°C fridge.

>> **gives around 120 Aliquots**

- ➔ This transformation technique gave a transformation efficiency of about **10^5-10^6 cfu/µg DNA** (cfu=colony forming unit)

Protocol 2(CaCl2-Method, Source Humboldt-University of Berlin)

Required Material:

30 ml + 500ml	LB Medium
50 µl	E. Coli Culture
110ml (+ 10ml for TB)	0,1M CaCl2
12 ml	Transformation buffer (16% Glycerol, 0,08M $CaCl_2$: 2,5ml 80% Glycerol +10ml 0,1M $CaCl_2$)

Day 1: Preculture

- Inoculate ≈ 50µl of your E. Coli culture (after unfreezing from -80 °C under mild conditions) or one colony from a cultivation plate into 30ml of sterile LB medium
- Incubation overnight at 37°C and 150rpm shaking

Day2:

- Transfer 4ml of your 4ml into 500ml of fresh and sterile LB medium
- Incubation overnight at 37°C and 150rpm shaking until reaching the optimal optical density of OD=0,6 (measurement at 600nm), this takes about 4 – 5 hours
- if OD=0,6: store cells on ice for 10min
- Centrifuge for 10min at 4°C and 4000rpm, throw the supernatant
- Resuspension of the pellet in 60ml cooled 0,1M $CaCl_2$
- Z Centrifuge for 10min at 4°C and 4000rpm, throw the supernatant
- Resuspension of the pellet in 50ml cooled 0,1M $CaCl_2$
- Suspension on ice for 30min
- Centrifugation at 4°C, 4000rpm for 10min
- Resuspension of the pellet in 12 ml cooled transformation buffer (16% Glycerol, 0,08M $CaCl_2$: 2,5ml 80% Glycerol +10ml 0,1M $CaCl_2$)
- Aliquotisation: 100µl per 1,5ml Tube, in 4°C-room as fast as possible
- (freeze in liquid N2), storage unofr -80°C

>> gives around 120 Aliquots

➔ This transformation technique gave a transformation efficiency of about **10^6 cfu/µg DNA** (cfu=colony forming unit)

Protocol 3 (variant of the Hanahan protocol using CCMB80 buffer, Source: openwetware)

Materials

- Detergent-free, sterile glassware and plasticware (see procedure)
- Table-top OD600nm spectrophotometer
- SOB

CCMB80 buffer

- 10 mM KOAc pH 7.0 (10 ml of a 1M stock/L)
- 80 mM $CaCl_2.2H_2O$ (11.8 g/L)
- 20 mM $MnCl_2.4H_2O$ (4.0 g/L)
- 10 mM $MgCl_2.6H_2O$ (2.0 g/L)

- 10% glycerol (100 ml/L)
- adjust pH DOWN to 6.4 with 0.1N HCl if necessary
- adjust pH up will precipitate manganese dioxied from Mn containing solutions.
- sterile filter and store at 4°C
- slight dark precipitate appears not to affect its function

Procedure

- **Preparing glassware and media :**

Eliminating detergent

Detergent is a major inhibitor of competent cell growth and transformation. Glass and plastic must be detergent free for these protocols. The easiest way to do this is to avoid washing glassware, and simply rinse it out. Autoclaving glassware filled 3/4 with DI water is an effective way to remove most detergent residue. Media and buffers should be prepared in detergent free glassware and cultures grown up in detergent free glassware.

Prechill plasticware and glassware

Prechill 250mL centrifuge tubes and screw cap tubes before use.

- **Preparing seed stocks :**

- Streak TOP10 cells on an SOB plate and grow for single colonies at 23°C
    - room temperature works well
- Pick single colonies into 2 ml of SOB medium and shake overnight at 23°C
    - room temperature works well
- Add glycerol to 15%
- Aliquot 1 ml samples to Nunc cryotubes
- Place tubes into a zip lock bag, immerse bag into a dry ice/ethanol bath for 5 minutes
    - This step may not be necessary
- Place in -80°C freezer indefinitely.

- **Preparing competent cells :**

- Inoculate 250 ml of SOB medium with 1 ml vial of seed stock and grow at 20°C to an OD600nm of 0.3
    - This takes approximately 16 hours.
    - Controlling the temperature makes this a more reproducible process, but is not essential.
    - Room temperature will work. You can adjust this temperature somewhat to fit your schedule
    - Aim for lower, not higher OD if you can't hit this mark
- Centrifuge at 3000g at 4°C for 10 minutes in a flat bottom centrifuge bottle.
    - Flat bottom centrifuge tubes make the fragile cells much easier to resuspend
    - It is often easier to resuspend pellets by mixing before adding large amounts of buffer
- Gently resuspend in 80 ml of ice cold CCMB80 buffer
    - sometimes this is less than completely gentle. It still works.
- Incubate on ice 20 minutes
- Centrifuge again at 4°C and resuspend in 10 ml of ice cold CCMB80 buffer.
- Test OD of a mixture of 200 µl SOC and 50 µl of the resuspended cells.
- Add chilled CCMB80 to yield a final OD of 1.0-1.5 in this test.

- Incubate on ice for 20 minutes
- Aliquot to chilled screw top 2 ml vials or 50 µl into chilled microtiter plates
- Store at -80°C indefinitely.
    - Flash freezing does not appear to be necessary
- Test competence (see below)
- Thawing and refreezing partially used cell aliquots dramatically reduces transformation efficiency by about 3x the first time, and about 6x total after several freeze/thaw cycles.

➔ This transformation technique gave a transformation efficiency of about **10^5 cfu/µg DNA**

(cfu=colony forming unit)

Measurement of competence:

- Transform 50 µl of cells with 1 µl of standard pUC19 plasmid
    - For high efficiencies the concentration should be about 10 pg/µl or 10^{-5} µg/µl
- Hold on ice 0.5 hours
- Heat shock 60 sec at 42C
- Add 250 µl SOC
- Incubate at 37 C for 1 hour in 2 ml centrifuge tubes rotated
    - using 2ml centrifuge tubes for transformation and regrowth works well because the small volumes flow well when rotated, increasing aeration.
- Plate 20 µl on LB-agar plates
    - Good cells should yield around 100 - 400 colonies
    - Transformation efficiency is (dilution factor=15) x colony count x 10^5/µg DNA
    - Th e transformation efficiency is acceptable starting from $5x10^7$ cfu/µg DNA.

➔ A transformation efficiency of 10^5-10^6 cfu/µg DNA is not enough sometimes to transform the sent biobricks from the MIT. Therefore it is important to have a transformation efficiency of about 10^8 cfu/µg DNA. You can see the protocol we used at the beginning of the lab-book. It gave very good results with a transformation efficiency of 10^8 cfu/µg DNA.

The combination of several protocols worked the best and allowed us to optimize our protocol integrating the following crucial steps:

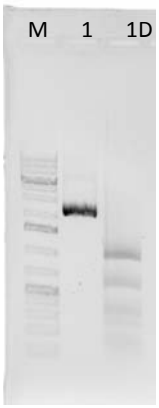
- Using PEG-DMSO for the first incubation. Indeed, skipping this step decrease the transformation efficiency by 10^2 cfu/µg DNA.
- Respecting the 30 minutes incubation on ice before the heat shock
- Using SOC medium instead of LB medium allows a better recovery and quicker growth of the cells.
-

The ClpX-story

This part will describe one mutagenesis protocol which was unsuccessful in order to give a general idea about what was the problem.

Test for DpnI digestion of the used plasmids

- DpnI digestion of 5 µl sample 1
  - 5µl sample 1
  - 1 µl 10x NEB4 buffer
  - 1 µl DpnI
  - 3 µl water
- Gel electrophoresis



- M: 0.25 µg marker, 1: undigested sample 1, 1D: DpnI digested sample

→ Plasmid DNA out of the DH5α cells is susceptible to DpnI digestion and can be used in pfu

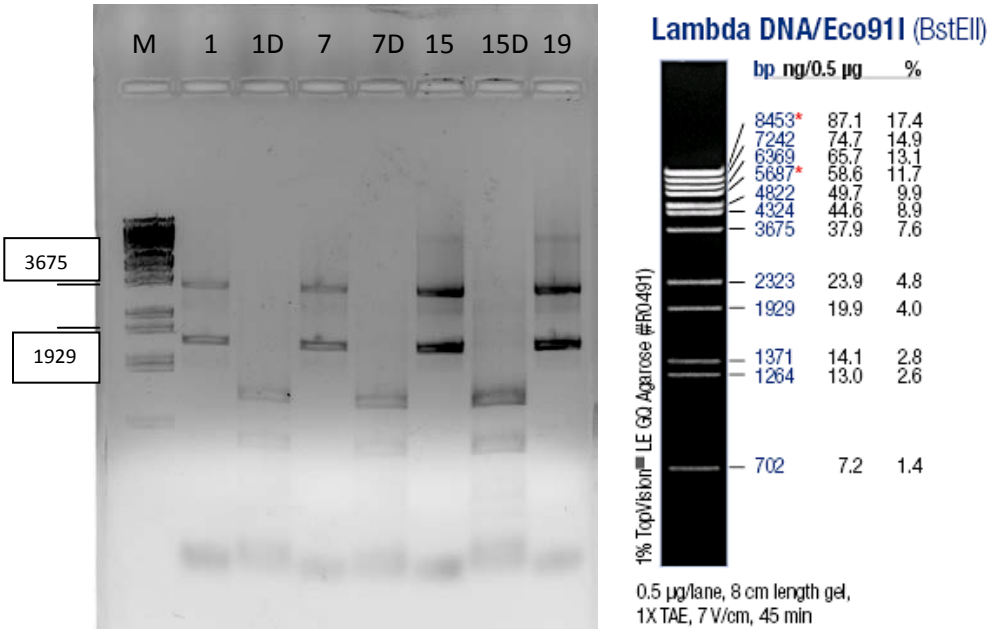
Mutagenesis of the two EcoRI sites

- Determination of the plasmid concentration within the samples
  - For sample 1: band intensity = 2.6% of 0.5 µg ladder → 13 ng/5µl=2.6 ng/µl
- Master mix x5
  - 5µl 10x Stratagene Pfu Turbo buffer 25 µl
  - 1µl dNTPs 5 µl
  - 1µl iGEM primer 9 (15 µM) → 300 nM final 5 µl
  - 1µl iGEM primer 10 (15 µM) 5 µl
  - 1µl iGEM primer 11 (15 µM) 5 µl
  - 1µl iGEM primer 12 (15 µM) 5 µl
  - 19 µl water 95 µl
- 29 µl master mix in 0.2 ml PCR tubes
- 4 PCR reactions for samples 1, 7, 15, 19
  - Sample 1: 5 µl sample + 15 µl water (~13 ng)
  - Sample 7: 10 µl sample + 10 µl water (~26 ng)
  - Sample 15: 15 µl sample + 5 µl water (~39 ng)
  - Sample 19: 20 µl sample (~52 ng)
- Addition of 1 µl Stratagene PfuTurbo polymerase to each reaction
- PCR Program
  - 94°C 2 min
    - 94°C 2 min
    - 65°C 30 sec
    - 72°C 4 min
    - 18 cycles
  - 72°C 7 min
  - 4°C ∞
- Take 40µl of each PCR reaction in a new 1.5 ml tube and add,
  - 5 µl 10x NEB buffer 4
  - 3 µl water
  - 1 µl DpnI
  - 1 µl EcoRI
- Incubation at 37°C for 1h

Analysis for successful PCR and DpnI, EcoRI digestion

Reason: DpnI will digest the old plasmid, EcoRI will digest all plasmids with two or one restriction sides. So just plasmids will be transformed which are new synthesized and by which the two EcoRI restriction sides were successfully altered.

- **Gel electrophoresis (10 µl undigested PCR products, 10 µl DpnI/EcoRI digested PCR)**



Number: undigested PCR sample, Number+D: DpnI and EcoRI digested PCR sample

- Pfu mutagenesis amplified DNA, as there are bands visible after DpnI digestion. The two bands for the undigested sample are linear and super coiled plasmid configurations. The bands of the digested samples are due to incomplete mutagenesis of EcoRI site (plasmid with only one of the two sites, which are mutated)

Transformation

- 2 µl of the EcoRI and DpnI digested pfu-PCR mix
- Add 50 µl competent DH5α cells
- 10 min on ice
- 1 min at 42°C
- 10 min on ice
- Add 500 µl SOC medium
- Incubate 45 min at 37°C
- Plate totality of the reaction on ampicillin selection plates

Liquid culture

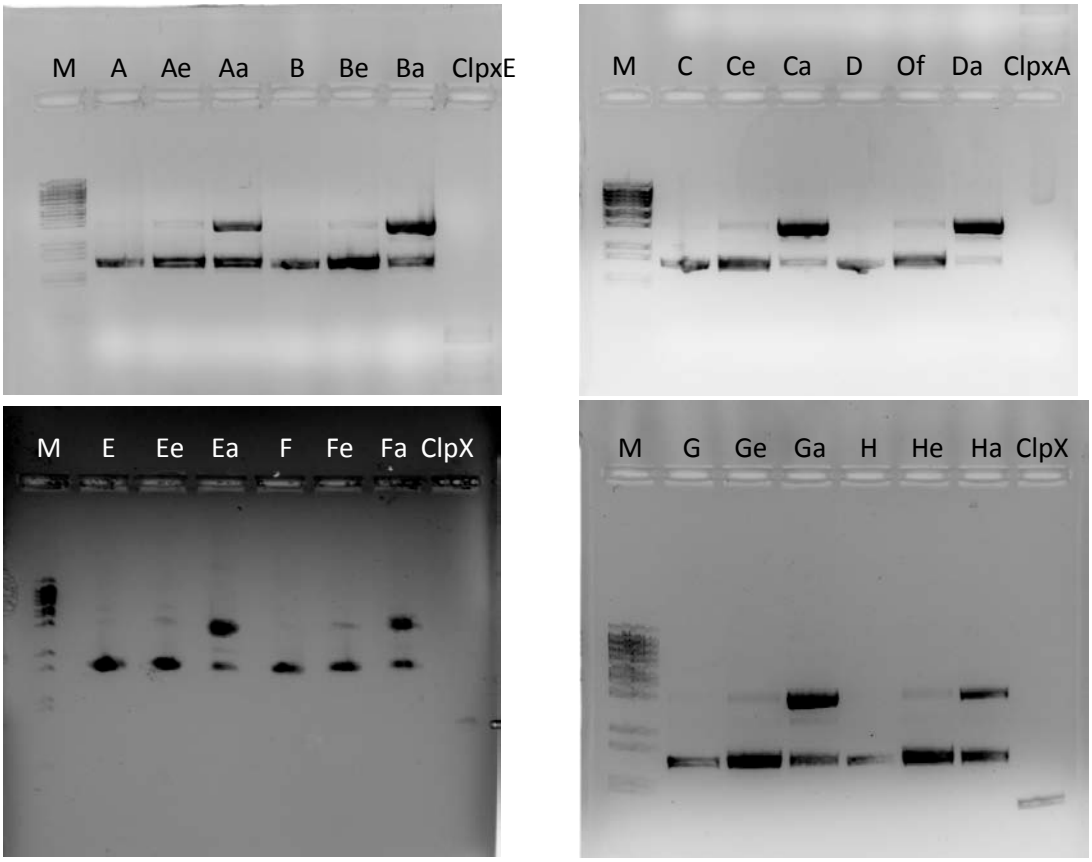
- Colonies for all 4 Pfu mutagenesis reactions
  - Plate of sample 1: 80 colonies samples A and B picked
  - Plate of sample 7: 100 colonies samples E and F picked
  - Plate of sample 15: 236 colonies samples C and D picked
  - Plate of sample 19: 1108 colonies samples G and H picked
- Ampicillin liquid culture of sample A-H

Plasmid purification

- Minipreps Nucleospin® Plasmid QuickPure (Macherey nagel)

Verification if EcoRI site eliminated

- EcoRI digestion
  - 10 µl reaction volume (5 µl of purified plasmid, 3 µl water,1 µl 10x buffer 4 NEB 1 µl EcoRI)
  - Incubation for 1h at 37°C
- Gel electrophoresis



M: Marker, A-H: samples undigested, Ae-He: EcoRI digestion, Aa-Ha: AgeI digestion

- The band pattern between undigested samples and EcoRI digested samples is equal, the uncut supercoiled plasmid migrates further (~1600-1700 bp) than the AgeI cut plasmid (~3600 bp),

small amounts of plasmids are also linear in undigested and EcoRI digested samples, but this can be occurred during the purification step

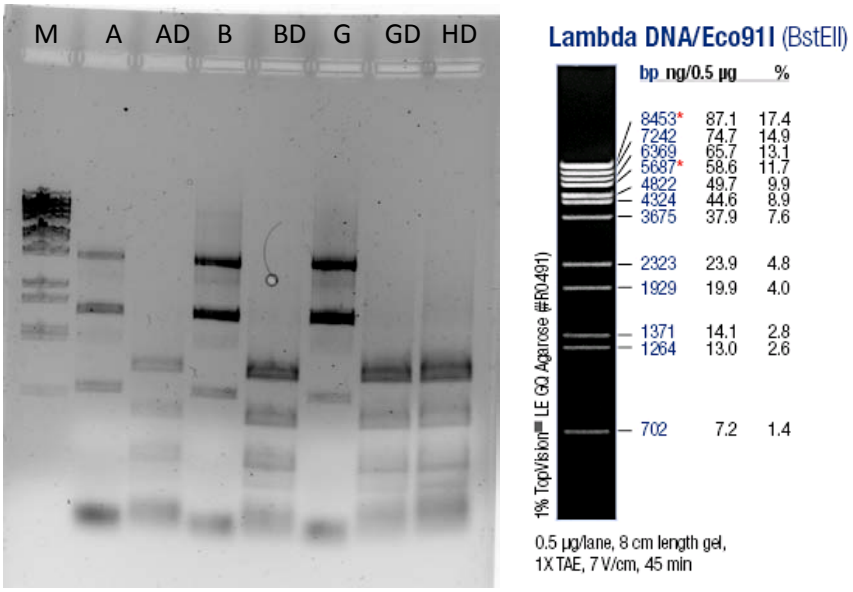
➔ **Pfu mutagenesis for EcoRI restriction sites showed positive results**

Mutagenesis of the two AgeI sites

- Master mix x5
  - 5µl 10x Stratagene Pfu Turbo buffer 25 µl
  - 1µl dNTPs 5 µl
  - 1µl iGEM primer 9 (15 µM) → 300 nM final 5 µl
  - 1µl iGEM primer 10 (15 µM) 5 µl
  - 1µl iGEM primer 11 (15 µM) 5 µl
  - 1µl iGEM primer 12 (15 µM) 5 µl
  - 19 µl water 95 µl
- 29 µl master mix in 0.2 ml PCR tubes
- 4 PCR reactions for samples 1, 7, 15, 19
  - Sample A: 5 µl sample + 15 µl water
  - Sample B: 10 µl sample + 10 µl water
  - Sample G: 15 µl sample + 5 µl water
  - Sample H: 20 µl sample
- Addition of 1 µl Stratagene PfuTurbo polymerase to each reaction
- PCR Program
  - 94°C 2 min
    - 94°C 2 min
    - 65°C 30 sec
    - 72°C 4 min
    - 18 cycles
  - 72°C 7 min
  - 4°C ∞
- Take 40µl of each PCR reaction in a new 1.5 ml tube and add,
  - 5 µl 10x NEB buffer 4
  - 2 µl water
  - 1 µl DpnI
  - 1 µl AgeI
  - 1 µl EcoRI (to be sure that there are no more EcoRI sites)
- Incubation at 37°C for 2h

Analysis for successful PCR and DpnI, AgeI, EcoRI digestion

- Gel electrophoresis (10 µl undigested PCR products, 10 µl DpnI/EcoRI digested PCR)



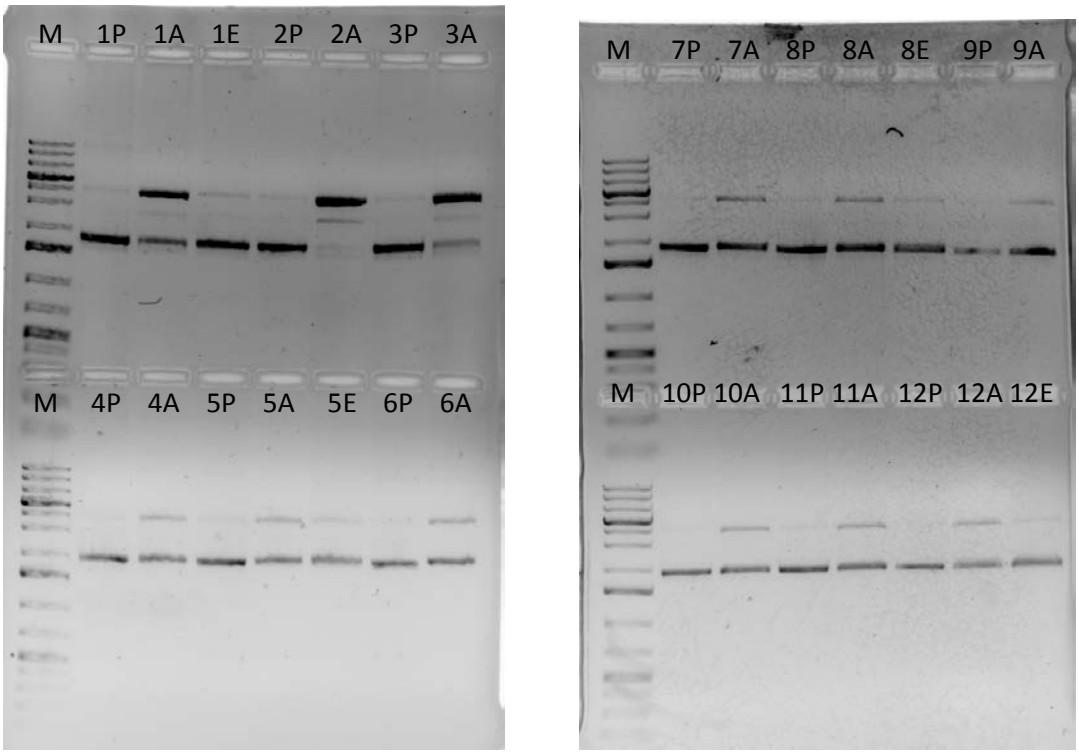
A-G: undigested PCR sample, A-H+D: DpnI, AgeI and EcoRI digested PCR sample

- Pfu mutagenesis amplified DNA, as there are bands visible after DpnI digestion. The two upper bands for the undigested sample are linear and super coiled plasmid configurations. The bands of the digested samples are due to incomplete mutagenesis of AgeI site (plasmid with only one of the two sites, which are mutated)
  --> Pfu mutagenesis for AgeI restriction sites showed positive results

Screen for ClpX insert in the vector

Reason: To verify if the plasmid in the colonies carries the insert. Plasmid is purified and later digested with AgeI. If the plasmid has no AgeI sides the band of the digested plasmid should be the same as the native pUC19 plasmid

- Plasmid miniprep for all 12 colonies by Nucleospin purification (Maschery-Nagel)
- AgeI digestion for this colonies:
  - 5 µl of purified plasmid
  - 1 µl of NEB 4 buffer
  - 3,5 µl water
  - vortex briefly, then centrifuge to get the liquid down.
  - 1 µl AgeI was added and incubated at 37°C for 2h.
- After digestion samples were put on a gel with pUC19 as a control



M: 0.25 µg marker, 1-12P: undigested sample, 1-12A: AgeI digested sample, 1E/5E/8E/12E: EcoRI digested samples

- AgeI digestion leads mainly to linear plasmid bands for samples 1-3
- AgeI digestion of samples 4-12 result in a strong circular band and a minor linear band

--> Pfu mutagenesis should have worked out for samples 4-12

PCR amplification of EcoRI/AgeI mutated Clpx by adding iGEM pre- and suffix

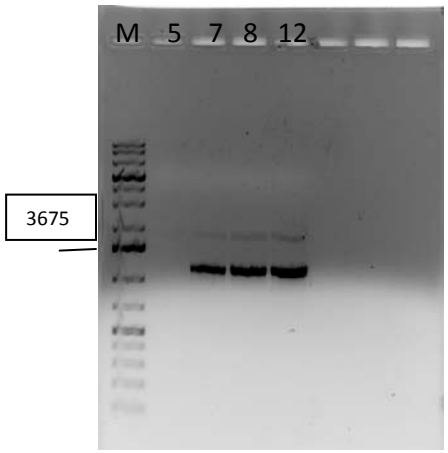
- 5x Master mix:
  - 25 µl 10x Pfu buffer
  - 5 µl 10 mM dNTPs
  - 5 µl iGEM primer 34
  - 5 µl iGEM primer 35
- Sample DNA
  - Sample 5: 1.25 µl (10 ng)
  - Sample 7: 3.2 µl (25 ng)
  - Sample 8: 6.25 µl (50 ng)
  - Sample 12: 6-8 ng (48-64 ng)
- Fill up with water to 50 µl
- Add 0.5 µl Pfu polymerase (native) (Fermentas)

- PCR program

	Temperature (°C)	Time (min)
Step 1	94	2
Step 2	94	0.5
Step 3	65	0.5
Step 4	72	2
Go to Step 2, 25 times temperature decreased by 0.2 each new cycle		
Step 5	72	7
Step 6	4	Infinity

Verification of PCR reaction

- **Gel electrophoresis with 5 µl of each sample**

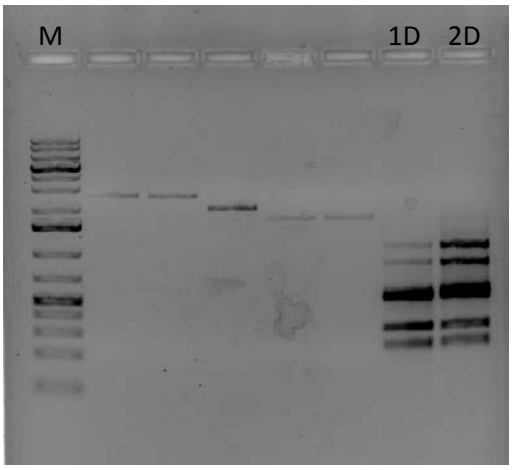


→ PCR reaction was successful, specific amplification bands of ClpX are visible and weak plasmid bands

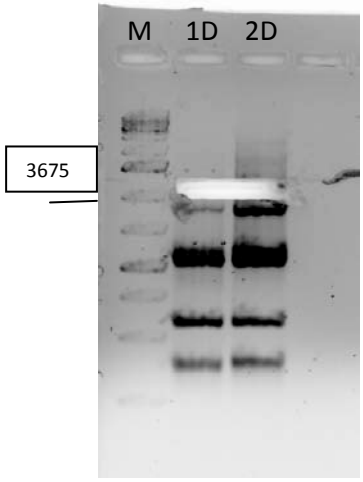
Gel electrophoresis and purification

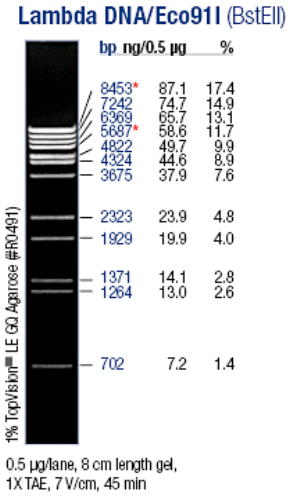
Reason: Before cloning the ClpX into the standard iGEM plasmids the ClpX insert was tested via overnight digestion. The expectation was a weak digested band and a strong undigested band. The undigested band should be extracted with gel purification which should avoid interference of undesired bands during ligation.

- Gel picture of the overnight digestion



- The overnight digestion reveals, that the desired ClpX band is stable to 10h EcoRI/PstI digestion
  → **This band is purified via gel electrophoresis**
- 2% low melting point electrophoresis





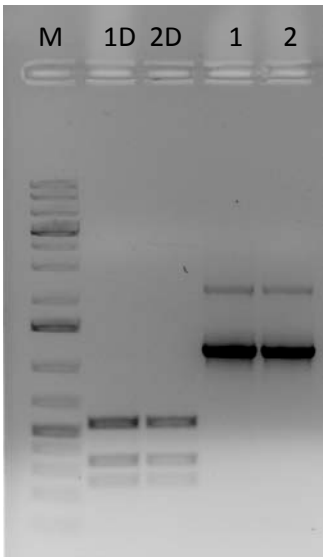
PCR amplification of the excised band

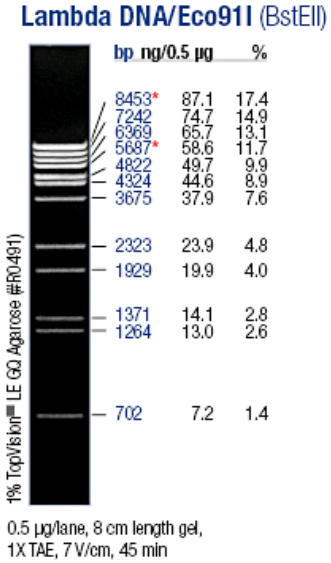
- Add 0.5-1 ml of 100mM Tris 1mM EDTA pH8 buffer to the excised gel fragment in a 2 ml tube
- Incubate for 15 min at 65°C to melt the gel fragment
- Add 1 volume of phenol/chloroform
- Vortex briefly
- Centrifugation for 10 min at 10000 rpm at room temperature
- Take the upper phase (aqueous phase) and repeat the phenol/chloroform extraction twice
- Add 1/10 volume of 5M NaCl and 3 volumes of EtOH absolute
- Incubate for 1h at 80°C
- Centrifugation for 15 min at 15000 rpm at 4°C
- Discard the supernatant and wash the pellet in 1 ml 70% EtOH
- Centrifugation for 10 min at 10000 rpm at 4°C
- Dry the pellet and dissolve it in an adequate volume of TE buffer

EcoRI/PstI restriction digestion of PCR products

Reason: In order to clone the ClpX fragment into the iGEM plasmid, ClpX has to be digested with PstI/EcoRI. In this step it was realized that the Pfu mutagenesis did not work as expected

- 40 µl reaction
  - 30 µl PCR products
  - 4 µl 10x NEB 2 buffer
  - 1.5 µl EcoRI
  - 1.5 µl PstI
  - 3 µl water
- Incubation 1h at 37°C, Heat inactivation for 20 min 65°C
- Purification with Qiagen PCR purification kit (Elution in 30 µl elution buffer)
- Gel electrophoresis





M: 0.25 µg marker; 1,2: PCR amplification of ClpX, 1D,2D: EcoRI and PstI digested samples 1,2

- Amplified ClpX band is completely digested by EcoRI.

→ Pfu mutagenesis did not work out for ClpX, remaining bands after each restriction enzyme digestion came probably from incomplete digestion

Some samples also other than described here were sequenced, but none of them had the alteration of nucleotide in their restriction sides. The problem why this mutagenesis did not work is not completely clear. The DpnI enzyme was working, as the EcoRI and AgeI restriction enzymes. Even long digestions ranging from 1h to 16h were not sufficient. Samples digested for 16h were put on AgeI, the non digested often strong band was extracted. This band should not contain any more the restriction sides, but after another PCR for amplification it showed the same digestion patterns with EcoRI and AgeI.

Maybe secondary structures of the plasmid prevented the restriction sides from digestion. A next reason could be the enzyme conditions were not sufficient for complete digestion, buffers could have changed by multiple use an therefore possible contaminations. Also during the mutagenesis step the Pfu polymerase could have not altered the side accordantly to the mispairing of primer and insert. Maybe a mutation was inserted and had created a new restriction side. Pfu polymerase has a very good proof reading but there is the possibility that a mutation is inserted. But as time was running out, the gene was ordered from GeneArt. But before the company was able to deliver, the ClpX without restriction sides was achieved by a new technique.

PCR colony screening

PCR colony screening seems to be an easy task. However it can become a vicious one if you do not have all the necessary data. We experienced this with the DNA polymerase. We gladly received some DNA polymerase from a lab in our school which was self produced and purified. They also gave us the corresponding buffer. During two to three weeks we couldn't succeed in cloning the PhyB part even though the PIF parts were already cloned and verified. Once, Mr Chatton came in our lab and we told him about our difficulties and our hypothesis that the taq was deficient. He asked how big our sequence was and when we told him that it was over 2000 base pairs, he explained us that it was hard to synthesize a fragment with more than 1000 base pair… That is the reason why we use two internal primers for colony sequencing with a DNA fragment of 300bp. This demonstrates that it is primordial to always work with products well known and well characterized.

PstI (or Never forget the devil is in the detail)

For the synthesis of the specific degradation Tags DAS and LAA we ordered two separate, NgoMIV + Pst1 precut primers, which were hybridized in order to obtain the degradation sequence.

For the synthesis of the PstI-site a mistake occurred in the command of the primers, as we did not consider that PstI cuts in the (3' -> 5') sens, contrary to the other restriction enzymes of the BioBrick standard.

Due to this error it took us several weeks to create these two C-terminal degradation tags, in spite of the basically simple synthesis steps. PCR screening of the transformed cells resulted in positively tested colonies, but in the following digestion tests we did not find a single product of the tag, only multiply enchained constructs. After several repetitions of these observations we proved the design of the ordered primers and finally found the problem.

A supplementary step of ligation digestion in the experimental procedure can fix this mistake.