

Overview

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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/ λ O 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 hexamer 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 λ O. 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 λ O- 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 10⁷-10⁸ 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 CaCl₂: 2,5ml 80% Glycerol +10ml 0,1M CaCl₂)
- 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 CaCl₂
- Centrifuge for 10min at 4°C and 4000rpm, throw the supernatant
- Resuspension of the pellet in 50ml cooled 0,1M CaCl₂
- 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 CaCl₂: 2,5ml 80% Glycerol +10ml 0,1M CaCl₂)
- 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 10⁸ 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 10² 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

1. 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

2. 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 T_m-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

3. *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 μ l (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

4. 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 ($g^a a a t t c$ (5’->3’)) and a XbaI ($t^a c t a g a$ (5’->3’)) restriction site, connected via a NotI ($N o t I$ $g c g g c c g c$ (5’->3’)) site. The standard prefix contains a SpeI ($a^a c t a g t$ (5’->3’)) and a PstI ($c t g c a^g$ (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/XbaI scar ($\dots a c t a c t a g a g c a \dots$).

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 ($g^a c c g g c$ (5’->3’)) and AgeI ($a^a c c g g t$ (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	$g a a t t c g c g g c c g c t t c t a g$	$t a c t a g t a g c g g c c g c t g c a g$
Without ATG	$g a a t t c g c g g c c g c t t c t a g a g$	

Fusion Protein BioBrick Assembly Standard

	PREFIX	SUFFIX
Fusion	$g a a t t c g c g g c c g c t t c t a g a t g c c g g c$	$a c c g g t t a a t a c t a g t a g c g g c c g c t g c a g$

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 hexameric 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 sites for EcoRI and two restriction sites for AgeI.

Conception

ClpX has two internal restriction sites for EcoRI and two restriction sites 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 sites 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 sites.

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/nucore/49175990?from=456650&to=457924&report=gbwithpars>

```
gaattcgggcccgttctagatggccggcCGCAGTGCCTACCGACGCCGCATGAAATTCGCAACCACCTGGACGATTACGTT
ATCGGCCAGGAACAGGCGAAAAAAGTGCTGGCGGTTCGCGGTATACAACCATTACAAACGTCTGCGCAACGGCGAT
ACCAGCAATGGCGTCGAGTTGGGCAAAAGTAACATTCTGCTGATCGGTCCGACCGGTTCCGGTAAAACGCTGCTG
GCTGAAACGCTGGCGCGCTGCTGGATGTTCCGTTACCATGGCCGACGCGACTACACTGACCGAAGCCGGTTATG
TGGGTGAAGACGTTGAAAACATCATTGAAAGCTGTTGCAGAAATGCGACTACGATGTCCAGAAAGCACAGCGTG
GTATTGTCTACATCGATGAAATCGACAAGATTTCTCGTAAGTCAGACAACCCGTCCATTACCGGAGACGTTTCCGGT
GAAGGCGTACAGCAGGCACTGTTGAAACTGATCGAAGGTACGGTAGCTGCTGTTCCACCGCAAGGTGGGCGTAAA
CATCCGAGCAGGAATTCGTCAGGTTGATACCTCTAAGATCCTGTTTATTTGTGGCGGTGCGTTTGCCGGTCTGGA
TAAAGTGATTTCCACCGTGTAGAAACCGGCTCCGGCATTGTTTTGGCGCGACGGTAAAAGCGAAGTCCGACAA
AGCAAGCGAAGGCGAGCTGCTGGCGCAGGTTGAACCGGAAGATCTGATCAAGTTTGGTCTTATCCCTGAGTTTATT
GGTCGTCTGCCGTTGTCGCAACGTTGAATGAACTGAGCGAAGAAGCTCTGATTCAGATCCTCAAAGAGCCGAAA
AACGCCCTGACCAAGCAGTATCAGGCGCTGTTAATCTGGAAGGCGTGGATCTGGAATTCGTCGACGAGGCGCTG
GATGCTATCGCTAAGAAAGCGATGGCGCGTAAAACCGGTGCCGTGGCCTGCGTTCCATCGTAGAAGCCGCACTG
CTCGATACCATGTACGATCTGCCGTCCATGGAAGACGTCGAAAAAGTGGTTATCGACGAGTCGGTAATTGATGGTC
AAAGCAAACCGTTGCTGATTTATGGCAAGCCGGAAGCGCAACAGGCATCTGGTGAAaccggttaatactagtagcggccgc
tgcag
```

Problem: 2 AgeI and 2 EcoRI sites

Primers for cloning ClpX out of the E.Coli genome

These primers were used to amplify 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) Agel site

Forward primer (5'->3') (31 bp)

CTGATCGGTCCGACTGGTTCCGGTAAAACGC

Reverse primer (5'->3') (31 bp)

GCGTTTTACCGGAACCACTCGGACCGATCAG

2. GAA=Glu GA(A,G), TTC=Phe TT(C,T) EcoRI site

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

CATCCGCAGCAGGAGTCTTGCAGGTTG

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)

CGTGGATCTGGAGTTCCTGACGAG

Reverse primer (5'->3') (25 bp)

CTCGTCACGGAATCCAGATCCACG

4. ACC=Thr AC(A,T,G,C), GGT=Gly GG(A,T,G,C) Agel site

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

GGCGCGTAAAACGGTGCCCGTGG

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

CCACGGGCACCACTTTACGCGCC

Primers for amplification of ClpX with fusion pre- and suffix

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

Forward primer (5'->3'):

NNNNNNgaattcgcgccgcttctagatgcccggcCGCAGTGCGCTACCGACGCCGC

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 rpm for 5min.
- water phase were put in a new tube and 400 µl chloroform and 400 µl phenol was added and centrifuged by 12000 rpm 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.

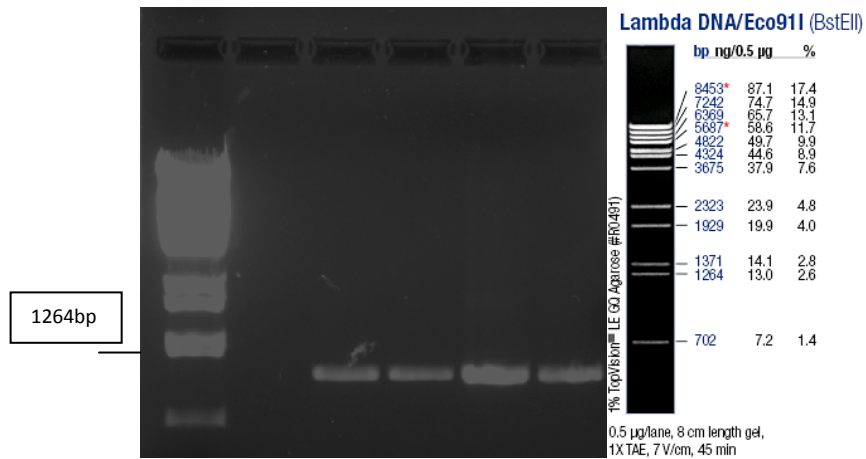


Figure: Left site: Marker and then the three PCR reaction. Right site. Description of the used marker.

- 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 H₂O**
- **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**

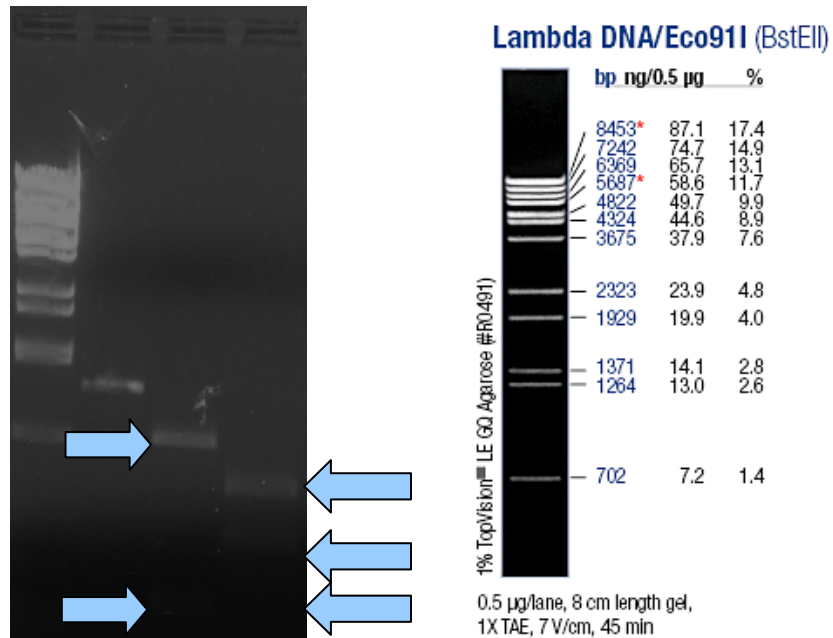


Figure: First line marker then ClpX, ClpX digested with AgeI, ClpXdigested with EcoRI

- 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

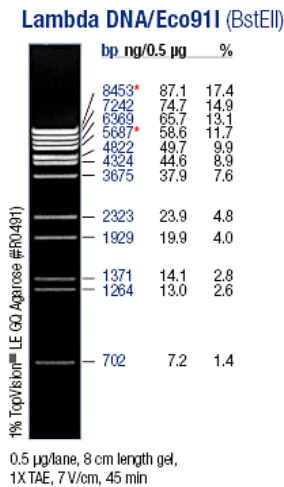
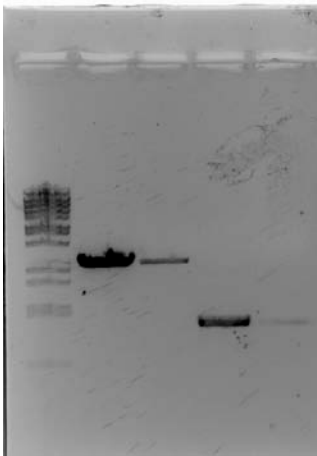
Reason: To generate a blunt end vector for cloning the ClpX insert into the vector. This is done because ClpX does not have yet restriction sites in the beginning/end of its sequence for sticky end ligation

- 30µl reaction
- Take totality of EcoRI digestion and add:
- 1µl NEB 4 buffer
- 0.5 mM 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, 5 µl 1:10 dilution of pUC19 EcoRI digested & blunt end, 5 µl 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

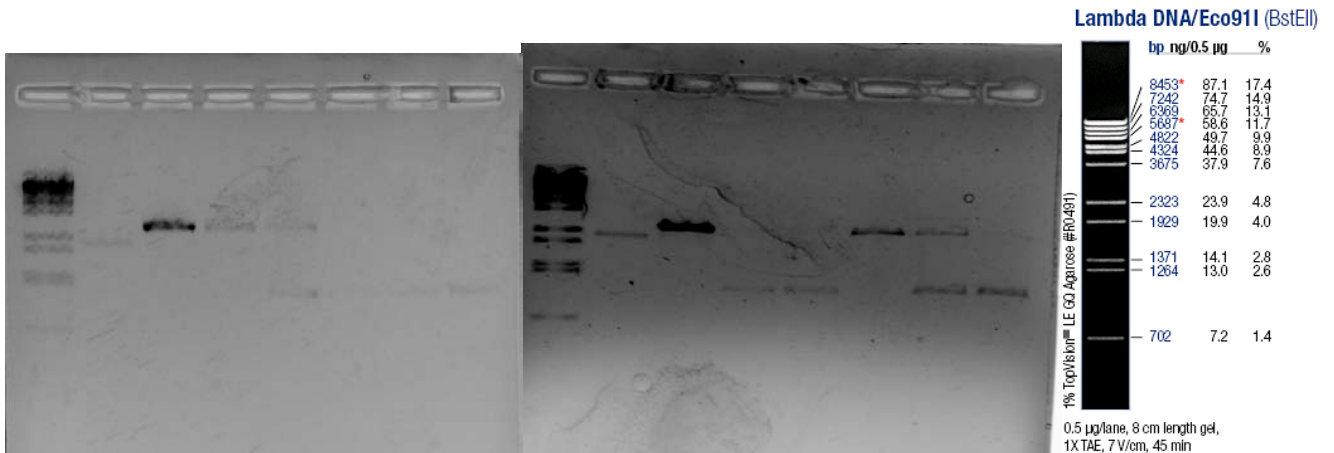
Reason: To ligate the vector with the insert.

- 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 μ l with 1 μ l loading buffer 6x.



Left: plasmids dephosphorylated with shrimp. First lane 0.5 μ g marker (1 μ l), 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 μ g marker (1 μ l), 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 μ l cells to your DNA
- Leave on ice for 10 min
- Heat shock 1min at 42 $^{\circ}$ C
- Leave on ice for 10 min
- Add 1 ml SOC medium
- Incubation at 37 $^{\circ}$ C for 30 min
- Plating 500 μ l 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 μ l 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 $6,1 \cdot 10^5$ (control plate).
- Calculation: 6100 colonies on plate. Added vector was 10ng. So 610 colonies per ng of vector, so $6,1 \cdot 10^5$ 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.

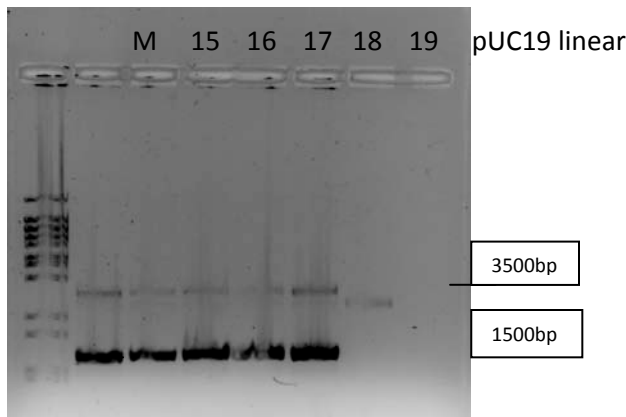
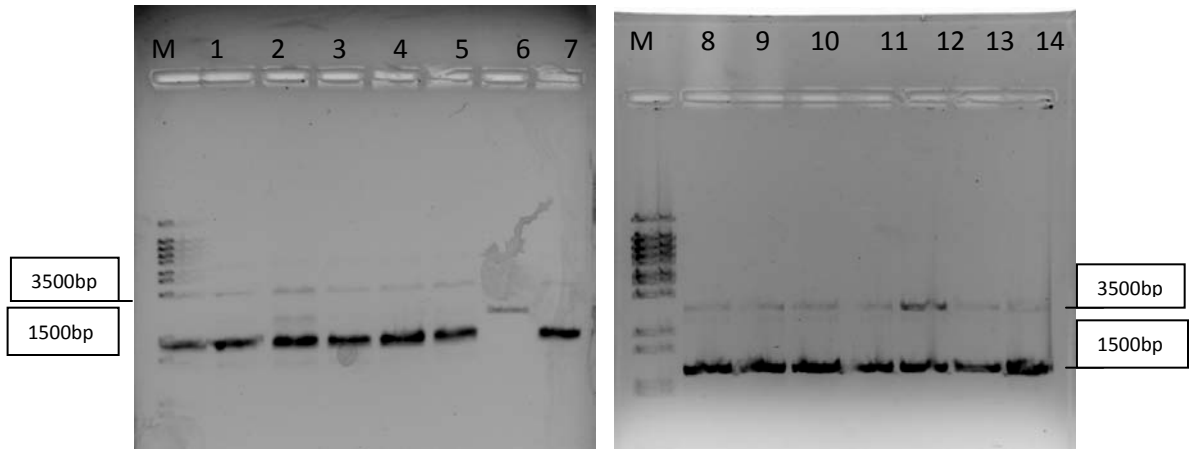
Plasmid isolation

- Minipreps Nucleospin® Plasmid QuickPure (Macherey nagel)

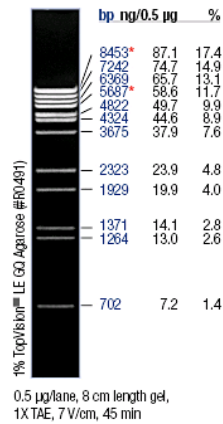
Screen of colonies for inserts

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.

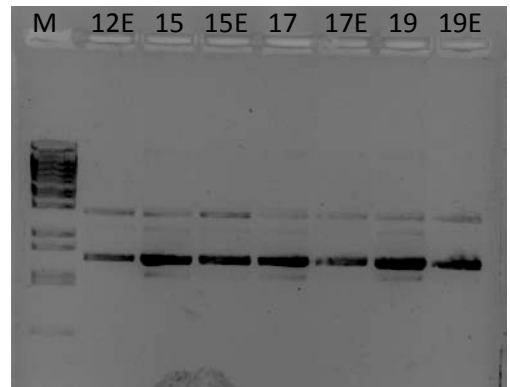
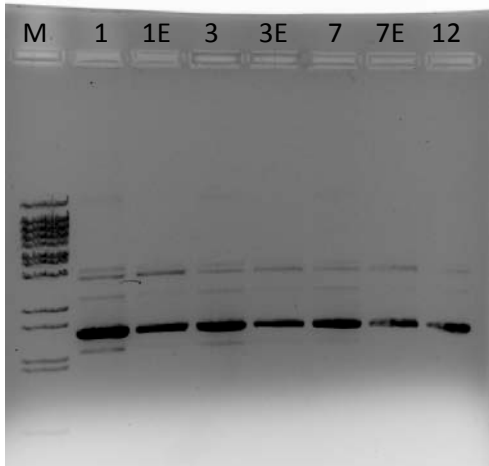
- Plasmid miniprep for all 19 colonies by Nucleospin purification (Maschery-Nagel)
- EcoRI digestion for all 19 colonies and pUC19 plasmid and ClpX insert for control:
 - 10 μl of purified plasmid
 - 2 μl of NEB 4 buffer
 - 7 μl water
 - vortex briefly, then centrifuge to get the liquid down.
 - 1 μl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



Lambda DNA/Eco91I (BstEII)



- 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

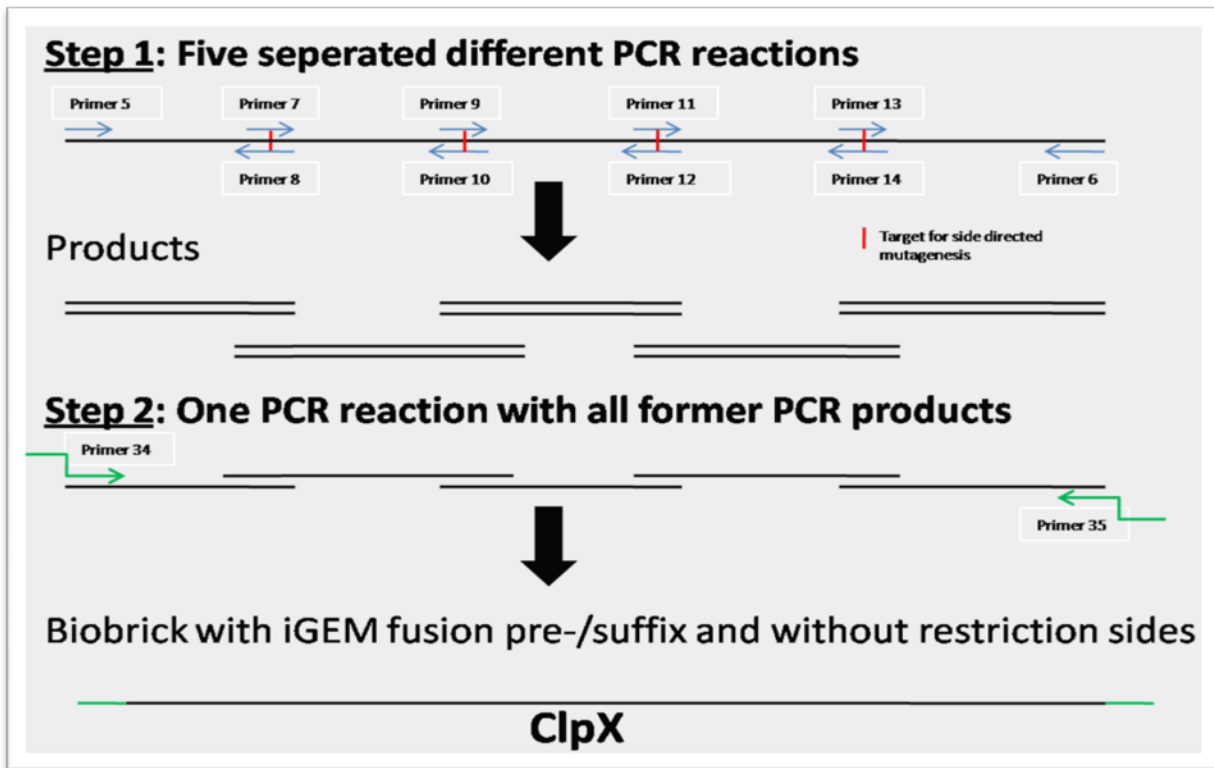
Part III: Site-directed mutagenesis

ClpX contains two EcoRI and two AgeI restriction sites. As the fusion pre- and suffix for iGEM Biobricks has these sites as well, these internal restriction sites had to be altered to ensure these standards. The first approach was a standard Pfu mutagenesis for the altering of two restriction sites per PCR reaction. Unfortunately in this part of Biobrick construction major problems were encountered. The first mutagenesis did not alter the restriction sites and not even changing of protocol parameters did successfully alter the restriction sites. Other protocols were tried. A single strand single restriction site approach was also unsuccessful and a promising protocol from the open wet page could not be done due to the lack of a thermo stable 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 sites by GeneArt. However, the successful alteration of the restriction sites 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

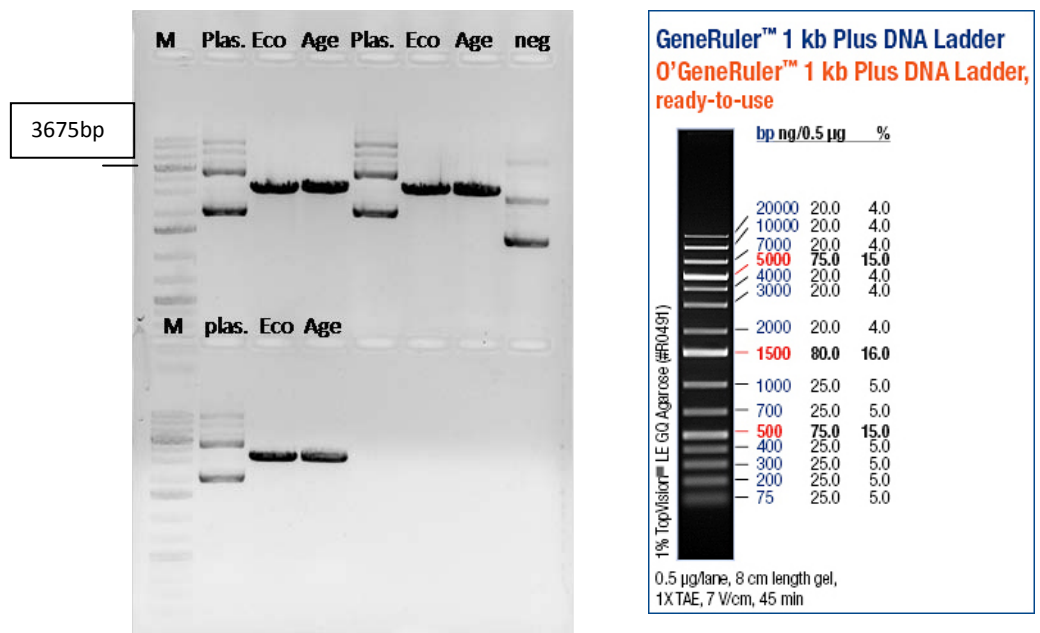


Figure Gelelectrophoresis of the result. First column marker followed by undigested PSB1C3 plasmid with ClpX, plasmid digested in EcoRI, plasmid digested in AgeI. Eighth column on the top is the negative control consisting of the PSB1C3 plasmid without insert.

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 and 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-terminal 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,

```
NNNNNNgaattcgcggccgcttagATGGTTCCGGAGTCGGGGTAGTGGCGGTGGCCGTGGCGGTGGCCGTGGCG  
GAGAAGAAGAACCCTCGTCAAGTCACACTCCTAATAACCGAAGAGGAGGAGAACAAGCTCAATCGTCGGGAACG  
AAATCTCTCAGACCAAGAAGCAACTGAATCAATGAGCAAAGCAATTCAACAGTACACCGTCGACGCAAGACTCC  
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ATACAAAGAGAATGAAGCAACTGTCAATAAGCTTCTTCTCGTGCTTTGAGAGGGGACGAGGAAAAGAATGTGGA
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TCAGGCTCTATTGACTGCAAACAAGCGGGTTAGCCTCGAGGGAAAGTTATTGGGGCTTTCTGTTTCTTGCAAATC
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)

CAGCTGctgcagcggccgctactagtagtattaccggtGCTCGGGATTGCAAG

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)

CGATTTTCTGACTCGTAACGTCTTGTC

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 μ l 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 μ l reaction volume
 - 1/2 μ l insert (\approx 10/20 ng)
 - 3,5 μ l vector (\approx 70ng)
 - 2 μ l T4 DNA Ligase Buffer
 - 1 μ l T4 DNA Ligase (NEB))
 - 12,5/11,5 μ l H₂O
4. Transformation
 - 5 μ l of the ligation assay were transformed with 50 μ l 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
 - 1 μ l of the PCR products were transformed with 50 μ l of competent cells and plated on chloramphenicol-containing LB-agar plates
 - Incubation at 37°C overnight
12. Screening by PCR on colony

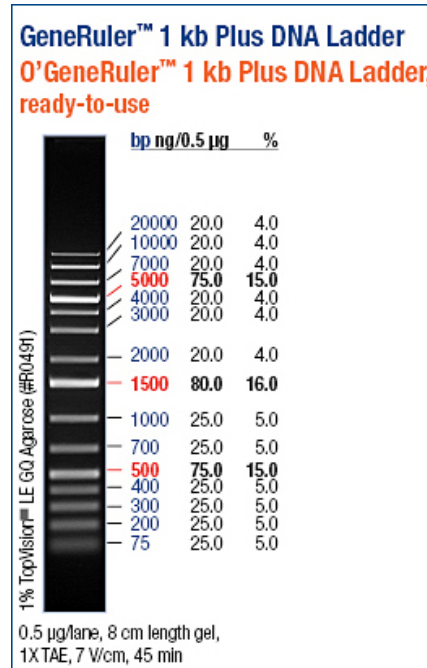
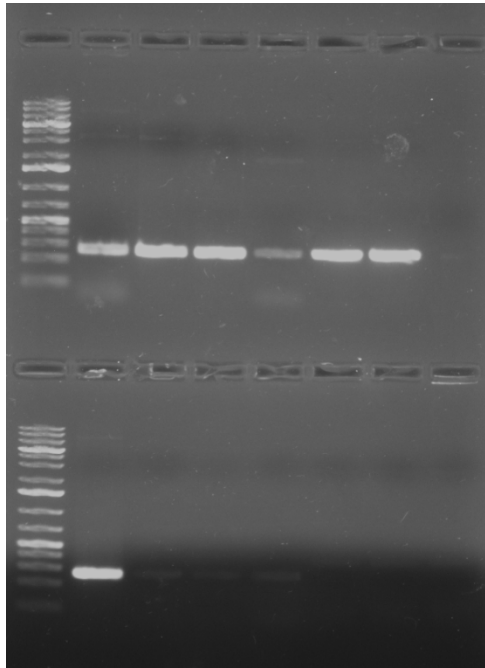


Figure 1: The band at 300bp is characteristic for the PhyB insert after amplification

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 Spel separately and double digestion.

Amplification and addition of the pre- and suffixes.

16. Sequencing

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atggtttccggagtcgggggtagtggcgggtggccgctggcgggtggccgctggcgggagaagaagaaccgctcgtcaagtcac
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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.

```
NNNNNNgaattcgcggccgcttctagATGGTTCCGGAGTCGGGGTAGTGGCGGTGGCCGTGGCGGTGGCCGTGGCG  
GAGAAGAAGAACCCTCGTCAAGTCACACTCCTAATAACCGAAGAGGAGGAGAACAAGCTCAATCGTCGGGAACG  
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```

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)

GGATCCgaattcgcggccgcttctagATGGTTCCGGAGTC

Reverse primer (5'→3') : (53 bp)

CAGCTGctgcagcggccgctactagtattaaccggtCCCCGCCATATCCCTAC

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

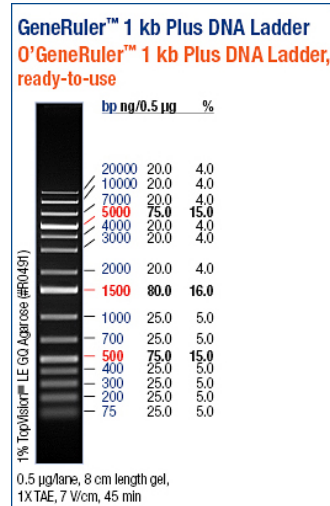
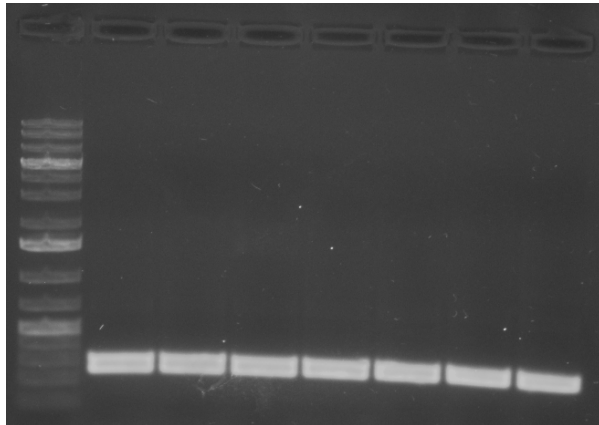


Figure 2: The band at 300bp is characteristic for the *PhyB* insert after amplification

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

```
atggtttccggagtcgggggtagtggcgggtggccgtggcgggtggccgtggcgggagaagaaccgctcgtcaagtccac  
actcctaataaccgaagaggaggagaacaagctcaatcgtcgggaacgaaatctctcagaccaagaagcaacactgaa  
tcaatgagcaaagcaattcaacagtaacccgtcgacgcaagactccacgccgttttcgaacaatccggcgaatcaggg  
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atcgggttacagtgaaaacgccagagaaatgttagggattatgcctcaatctgttccactcttgagaaacctgagatt  
ctagctatgggaactgatgtgagatctttggtcacttcttcgagctcgattctactcgagcgtgctttcgttgctcga  
gagattaccttgtaaatccggtttggtatccattccaagaatactggtaaacggttttacgccattcttcataggatt  
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tgggaaactgcggaaatggatgcgattcactcgcctccagcttattctgagagactcttttaagaatctgaggcggct  
atgaactctaaagttgtggatggtgtggttcagccatgtagggatatggcggg
```

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 infrared-exposed 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.

```
GaattcgcggccgcttctagatggcggcATGCCTCTGTTGAGCTTTTCAGGCTCACCAAAGCTAAGCTTGAATCTGCTCAA  
GACAGGAACCCTTCTCCACCTGTAGATGAAAGTTGTGGAGCTGGTGTGGGAAAATGGTCAGATATCAACTCAAAGTC  
AGTCAAGTAGATCGAGGAACATTCCTCCACCACAAGCAAAGCTCTCTAGAGCTAGAGAGATTGAAAATGGCTCAA  
GACGACTATGGTGGACGAGATCCCTATGTCAGTGCCATCACTAATGACGGGTTTGAGTCAAGACGATGACTTTGTT  
CCATGGTTGAATCATCATaccggttaataactagtagcggccgctgcag
```

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

```
GGATCCgaattcgcggccgcttctagatggcggcATGCCTCTGTTGAGC
```

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)

```
GCAAAGCTCTCAAGAGCTAGAGAG
```

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 μ l 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 μ l reaction volume
 - 1/2/4 μ l insert (\approx 10/20/40 ng)
 - 3,5 μ l vector (\approx 70ng)
 - 2 μ l T4 DNA Ligase Buffer
 - 1 μ l T4 DNA Ligase (NEB))
 - 12,5/11,5/9,5 μ l H₂O
4. Transformation
 - 5 μ l of the ligation assay were transformed with 50 μ l 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 *PstI* 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
 - 1 μ l of the PCR products were transformed with 50 μ l of competent cells and plated on chloramphenicol-containing LB-agar plates
 - Incubation at 37°C overnight

12. Screening by PCR on colony

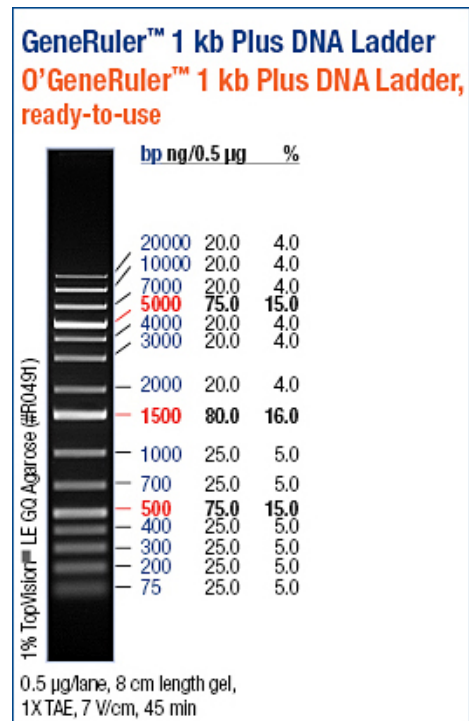
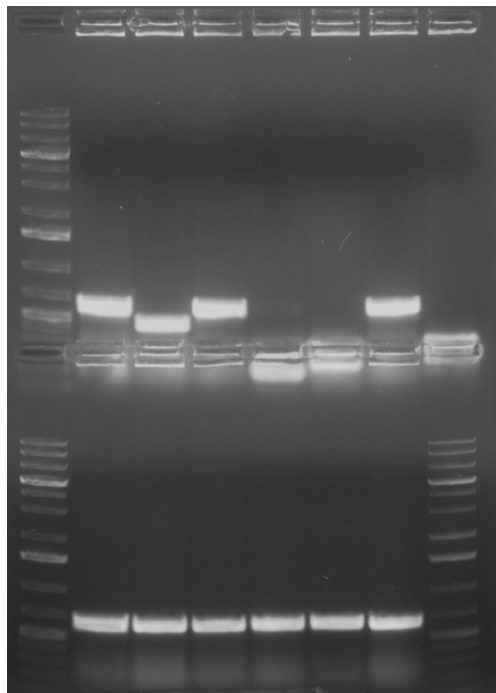


Figure 3: The bands at 600bp are characteristic for PIF3/6 amplified with VF and VR, the band at 300bp are the plaamids without insert.

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 PstI separately and double digestion.

16. Amplification and addition of the pre- and suffixes

17. Sequencing

```
atgcctctgtttgaacttttcaggctcaccaaagctaagcttgaatctgctcaagacaggaacccttctccacctgta
gatgaagttgtggagctgggtgtgggaaaatggtcagatatcaactcaaagtcagtcagtagatcgaggaacattcct
ccaccacaagcaaactcttcaagagctagagagattggaaatggctcaaagacgactatgggtggacgagatccctatg
tcagtgccatcactaatgacgggtttgagtcaagacgatgactttgttccatggttgaaatcatcat
```

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 carboxyterminal polybasic, 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.

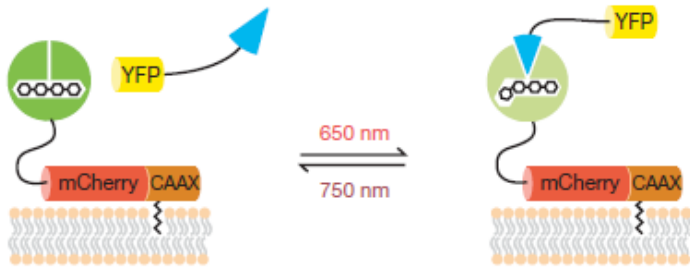


Fig.1: Implied system of Lim and Voigt (2009) to screen potential phytochrome-PIF pairs in a fluorescence translocation assay.

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  
AATCGATCATGGATTTGTATGAGGCAGAGTATAACGAGGATTTTCATGAAGAGTATCATCCATGGTGGTGGTGGTG  
CCATCACAATCTCGGGGACACGCAGGTTGTTCCACAAAGTCATGTTGCTGCTGCCCATGAAACAAACATGTTGGA  
AAGCAATAAACATGTTGACaccggttaataactagtagcggccgctgcag
```

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

```
CAGCTGctgcaggcggccgctactagtaaccggtGTC AACATGTTTATTGCTTTCC
```

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 *PstI* separately and double digestion.
9. Sequencing

```
atgatgttcttaccacccgattattgttgcaggttaagcgatcaagagtatatggagcttgtggttgagaatggccag  
attcttgcaaagggccaaagatccaacgtttctctgcataatcaacgtaccaaactgatcatggatttgtatgaggca  
gagtataacgaggatttcatgaagagtatcatccatggtggtggtggtgcatcaciaatctcggggacacgcagggt  
gtccaciaaagtcattgttgcctgctgcccataaacaacatggtggaaagcaataaacatggtgac
```

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.

```
gaattcgcggccgcttctagatggcggcGCGAGCGGCGGGCGGCAGCGAAGGCGGCGGCAGCGAAGGCGGCACCAGG  
GGCGCGACCaaccggttaataactagtagcggccgctgcag
```

The linker has been order as six separate, *EcoRI*+*AgeI* precut primers, which were hybridized in order to obtain the complete linker sequence.

Forward strand (5'→3'): (89 bp)

```
aattcgcggccgcttctagatggc|cggcGCGAGCGGCGGGCGGCAGCGAAGGCGGCGGCAG|CGAAGGCGGCACCAG  
CGGCGCGACCa
```

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

```
ccggtGGTCGCGCCG|CTGGTGCCGCCTTCGCTGCCGCCGCCTTCGCTGCC|GCCGCGCCGCTCGCgccggccatctaga  
agcggccgcg
```

Ordered primer:

Forward: 5'aattcgcggccgcttctagatggc 3'

Forward: 5'cggcGCGAGCGGCGGGCGGCAGCGAAGGCGGCGGCAG 3'

Forward: 5'CGAAGGCGGCACCAGCGGCGCGACCa 3'

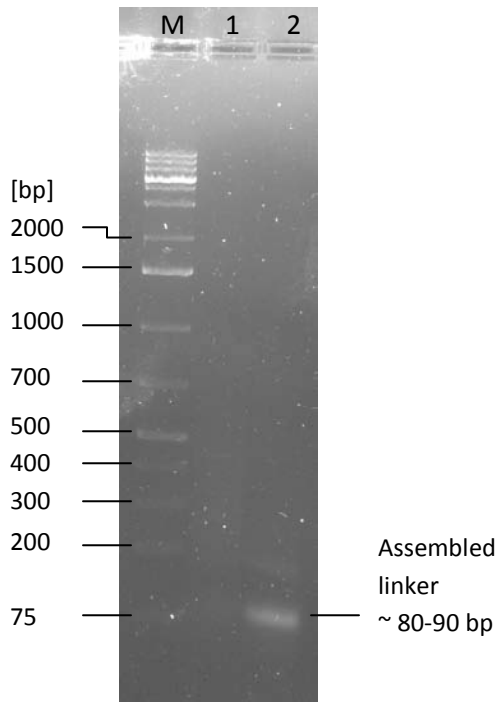
Reverse: 5'ccggtGGTCGCGCCG 3'

Reverse: 5'CTGGTGCCGCCTTCGCTGCCGCCGCCTTCGCTGCC 3'

Reverse: 5'GCCGCGCCGCTCGCgccggccatctagaagcggccgcg 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 μ l
 - 1 nmol (10 μ l of a 100 μ M stock solution) of each primer
 - 2 μ l NEB ligation buffer 10x
 - 1 μ l T4 polynucleotide kinase (NEB) 10U/ μ l
 - 7 μ l dH₂O
 - 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 *Eco*RI+*Age*I digestion. M: 0.25 μ g GeneRuler 1kb Plus DNA Ladder (Fermentas), 1: 2 μ l sample, 2: 5 μ l 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 μ l 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 μ l 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

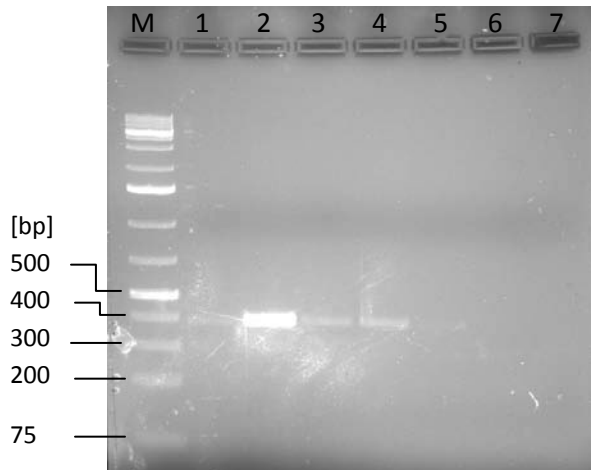


Figure 5: Analysis of PCR products of seven colonies by 1% gel electrophoresis. M: 0.25 μ g GeneRuler 1kb Plus DNA Ladder (Fermentas), 1-7: colony number. Colonies 1 to 4 show PCR products at approximately 400 bp (~300 bp vector + 89 bp linker). Precultures of colony 2 and 4 were made.

8. Restriction enzyme digestion of the miniprep plasmid as verification

- Restriction enzyme digestion with *Eco*RI and *Age*I separately and double digestion.

9. Sequencing

gcgctggcgcgtaataa

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

Ordered primer:

Forward: 5'**CCGGC**GCGGATGCGAGCTAATAATACTAGTAGCGGCC**GC** 3'

Reverse: 5'**TGCAG**CGGCCGCTACTAGTATTATTAGCTCGCATCC**GC** 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 dH₂O
- 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

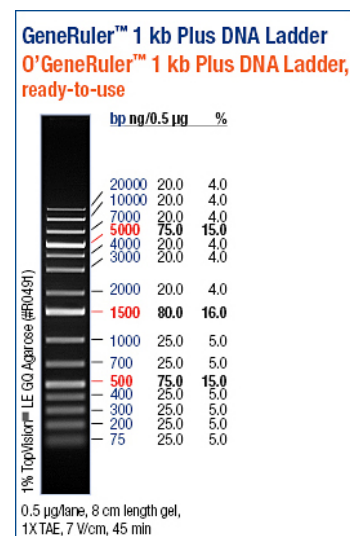
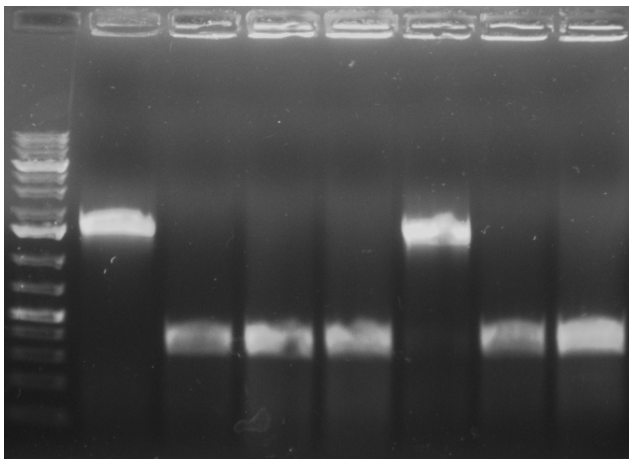


Figure 6: Bands at 400bp are characteristic for the plasmid with inserted tag.

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 Baker and Sauer (2006). It is an 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 linker has been ordered as two separate, NgoMIV + Pst1 pre-cut primers, which were hybridized in order to obtain the complete tag sequence.

Ordered primer:

Forward: 5' **CCGGC**GCGGATGCGAGCTAATAACTAGTAGCGGCCGC 3'

Reverse: 5' **TGCAG**CGGCCGCTACTAGTATTATTAGCTCGCATCCGCG 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') sense, 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
 - 7 µl of each primer from a 10 µM stock solution => 500ng
 - 5 µl NEB ligation buffer 10x
 - 1 µl T4 D Kinase (NEB) 10U/µl
 - 30 µl dH₂O
 - 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 PstI, 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

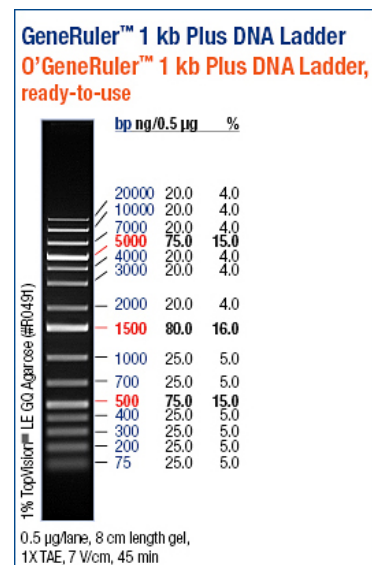
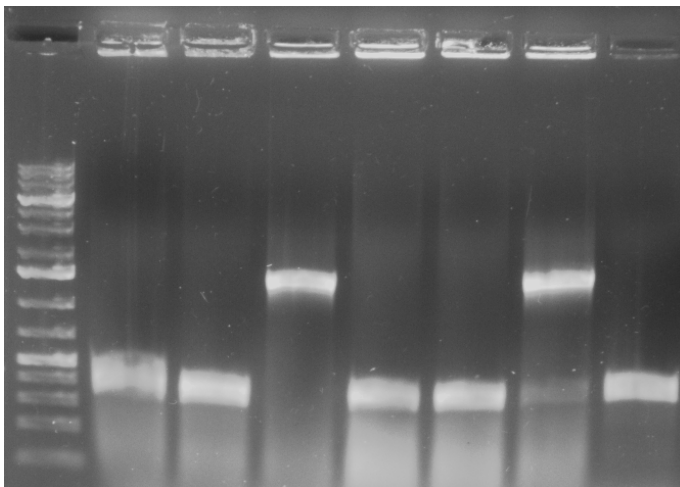


Figure 7: Bands at 400bp are characteristic for the plasmid with inserted tag.

8. Preculture

- Positively-tested colonies have been set in 5ml chloramphenicol-containing LB-medium

9. Verification

- Restriction enzyme digestion with EcoRI and PstI 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 Baker and Sauer (2009): NH₂-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 linker has been order as two separate, NgoMIV + AgeI precut primers, which were hybridized in order to obtain the complete linker sequence.

Ordered primer:

Forward: 5' **AATTC**GCGGCCGCTTCTAGATGACCAACACCGCGAAAATTCTGAACTTTGGCCGCA 3'
Reverse: 5' **CCGGT**GCGGCCAAAGTTCAGAAATTTTCGCGGTGTTGGTCATCTAGAAGCGGCCGCG3'

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 dH₂O
 - 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

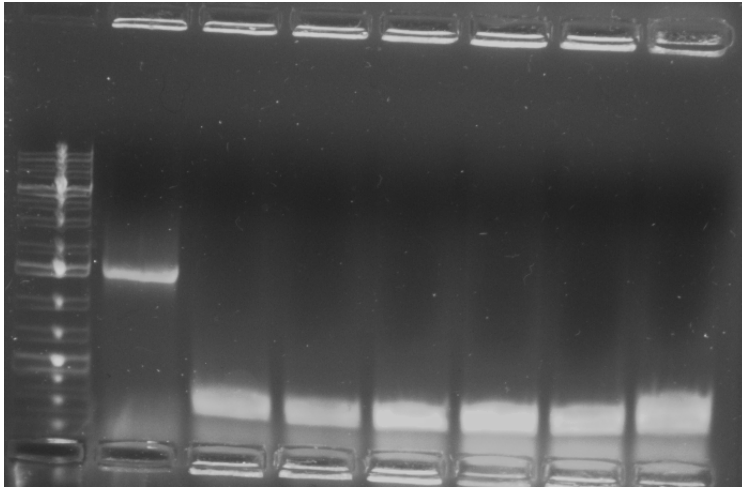
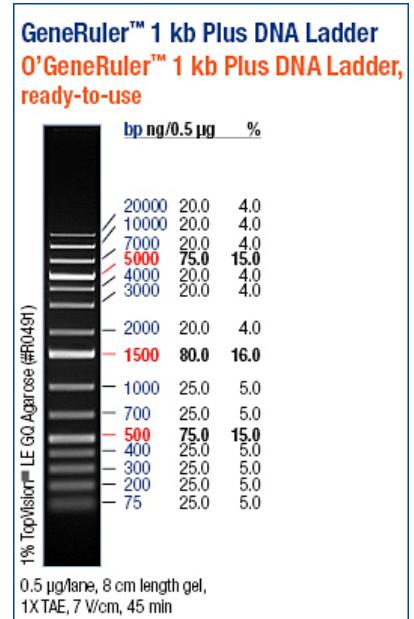


Figure 8: Bands at 400bp are characteristic for the plasmid with inserted tag.



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

atgaccaacaccgcaaaaattctgaactttggccgc