Week 7: 20. July 2015- 24. July 2015

20. July 2015

1) Digest of *medh2, phi* and pSC1B3 with EcoRI/ PstI
   - Pipetting scheme according to the protocol Restriction Digest
   - Adding directly both enzymes and incubate the Reaction at 37 °C for 1 hour.
   - Verify 10 ul of the digestion on agarose gel

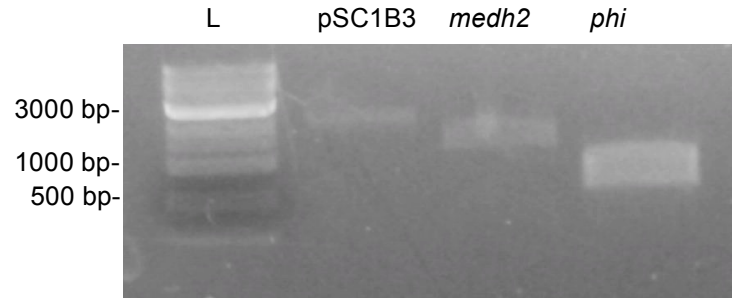

Figure 1: Digest of *medh2* and *phi* with EcoRI/ PstI. 10 µl of Digest were checked on 1% (w/v) agarose gel. Expected sizes: pSC1B3- 2033 bp, *medh2*-1197 bp *and phi*-665 bp. As lader (L) 1kB Ladder (NEB) was used.

2) Purification of digested *medh2, phi* and pSC1B3
   - Using Wizard® SV Gel and PCR Clean-Up System (Promega) and following the provided manual
3) Dephosphorylation of digested pSC1B3 using shrimp alkaline phosphatase
   - Pipetting scheme according to the protocol Shrimp Alkaline Phosphatase Treatment (Fermentas).

4) Ligation of *medh2* and *phi* into pSC1B3
   - Pipetting scheme according to the Protocol Ligation with T4 DNA Ligase (NEB)
   - Use dephosphorylated pSC1B3
   - Control Reaction: pSC1B3 Selfligation
   - Incubate the reaction at 16 °C overnight

21. July 2015

1) Transformation of *E. coli* TOP10 cells with pSC1B3+*medh2*, pSC1B3+*phi* and pSC1B3 selflligated
   - Add 5 µl of ligation reaction to chemically competent *E. coli* TOP10 cells.
   - Following the protocol for transformation of chemical competent *E. coli* cells

- Plate on LB+Clm [25 µg/ml] and incubate overnight at 37 °C

2) Inoculation of preculture culture for pilotexpression of MMOX, MMOY, MMOZ, MMOB, MMOC, MMOD, MMOG
   - Inoculate one clone with 5 ml LB+Kan [50 µg/ml] and incubate overnight at 37 °C shaking at 220 rpm.
   - As negative control inoculate a 5 ml preculture of *E. coli* BL21 cells containing the selfligated pET-30
3) Pilotexpression of MMOX, MMOY, MMOZ, MMOB, MMOC, MMOD, MMOG,
   - Measure OD_{600} of each preculture
   - Inoculate an 5 ml expression culture (LB+Kan [50 µg/ml]) with an OD_{600} of 0.3
   - Grow the expression culture for 1 hour at 37 °C shaking at 220 rpm.
   - Measure OD_{600} again
   - Take a 1 ml sample in an uninduced stage and harvest the cell by centrifugation at 8,000 x g for 5 minutes
   - Induce protein expression by adding IPTG (Final Concentration: 1mM).
   - Incubate the culture for 3 hours at 37 °C shaking at 220 rpm.
   - Take 1 ml sample 3 hours after induction and harvest the cells by centrifugation at 8,000 x g for 5 minutes
   - Keep the cell pellets at 4 °C
   - Treat the negative control in the same way!

22. July 2015

- Only the Ligation of medh2 into pSC1B3 seems to be successful no colonies were grown on the plate containing transformed *E. coli* Top10 cells with pSC1B3+*phi*

1) SDS-PAGE and Coomassie staining to verify successful Pilotexpression
   - Prepare Samples for SDS-Page:
     - Add 100 µl 1x SDS-Loading Buffer
     - Boil the sample at 95 °C for 20 minutes
   - Run SDS-Page
     - 12 % Seperating Gel
     - load 10 µl
   - Stain for 1 hour in Coomassie Staining Solution

- Destain overnight

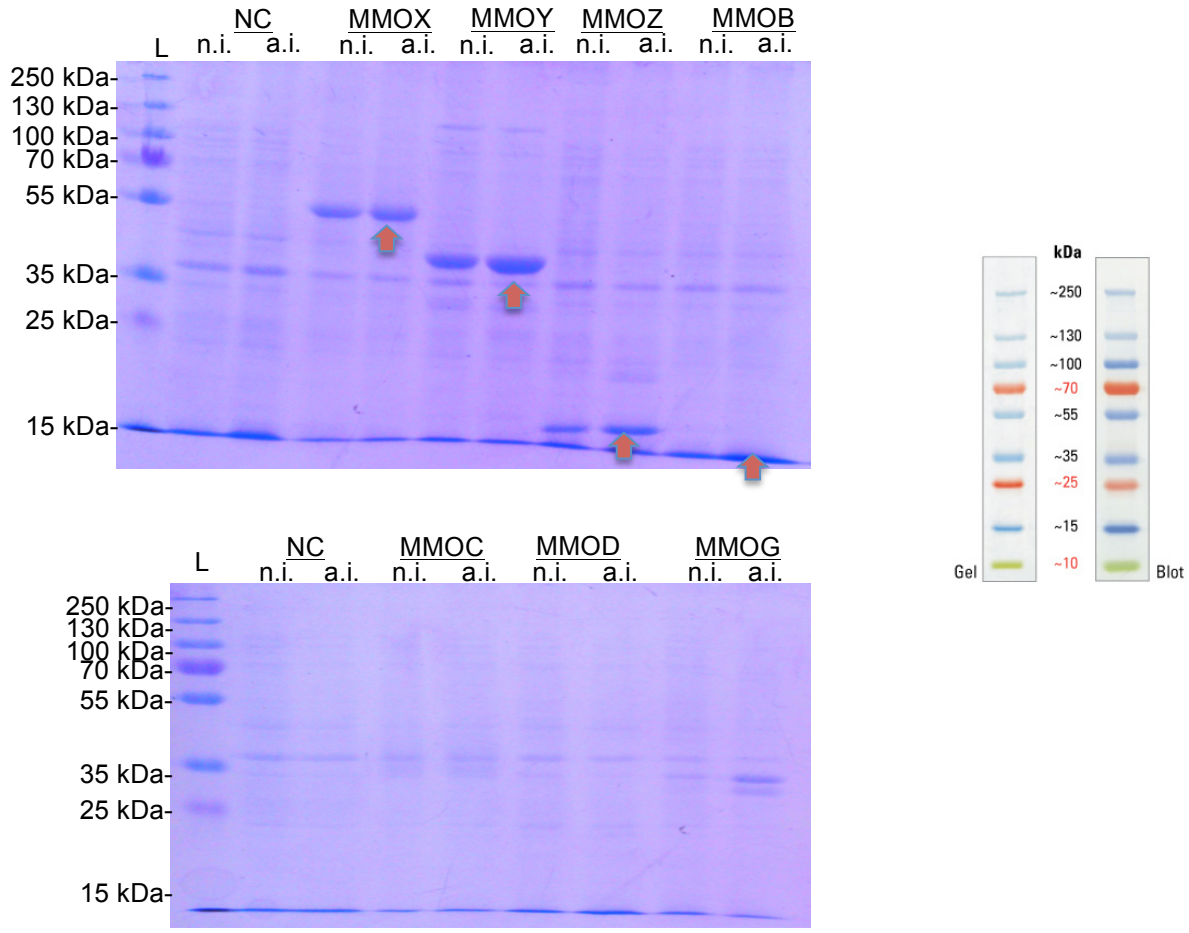

Figure 2: Pilotexpression of MMOX, MMOY, MMOZ, MMOB, MMOC, MMOD, MMOG in *E. coli* BL21. Coomassie staining of heterologously expressed MMOX, MMOY, MMOZ, MMOB, MMOC, MMOD, MMOG, in pET-30. 1 ml samples were taken in an uninduced stage (n.i.) and 3 hours after induction (a.i.) with 1 mM IPTG. Theoretical weights: MMOX-60.56 kDa, MMOY-45.06 kDa, MMOZ-19.81 kDa, MMOB-15.95 kDa, MMOC-38.48 kDa, MMOD-11.91 kDa, MMOG-59.38 kDa. Orange arrows determine the appropriate protein. As ladder PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (ThermoScientific) was used. As negative control (NC) selfligated pET-30 was used for expression in *E. coli* BL21. Proteins were separated by SDS-PAGE.

2) Colony-PCR to screen for clones containing the pSC1B3+*medh2* construct

- Constructs that needed to be identified: pSC1B3+*medh2*
- Pipetting scheme and PCR program according to PCR with Taq-DNA Polymerase Protocol
- Primer: medh2_E1/E2, BioBrick_Sequencing Primer Fwd/Rev
- Check 6 Clones
- Positive Control: add 1 µl of pCR4+medh2, pSC1B3+mmoB
- Negative Control: add 1 µl MilliQ Water

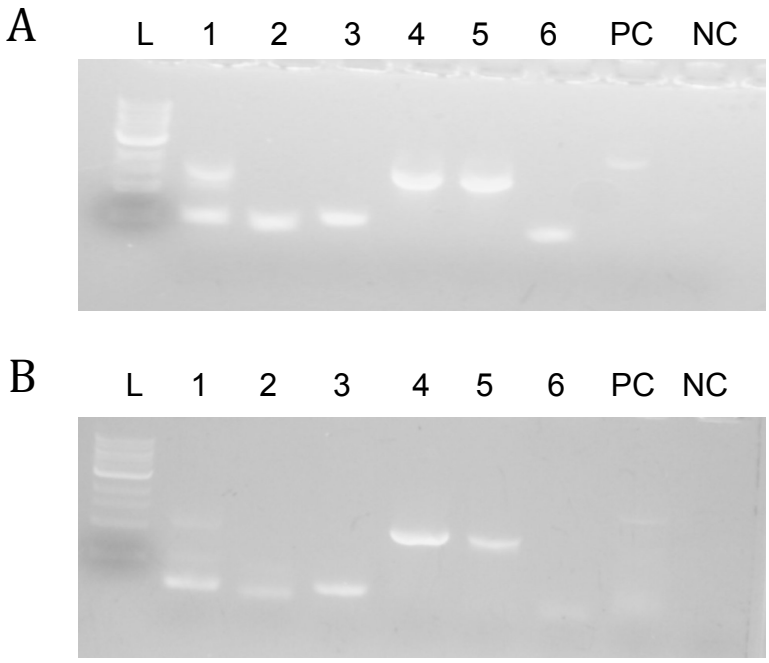

Figure 3: Colony-PCR to identify clones containing pSC1B3+*medh2* Biobrick construct.
PCR with Taq-DNA Polymerase to identify pSC1B3+*medh2*. Numbers 1-6 determine the checked clone. (A) Primer medh2_E1/E2 were used. (B) BioBrick sequencing primer were used. As positive control (PC) 1 µl pCR4+*medh2* (A) and pSC1B3+*mmoB (B) was used*. As negative control (NC) 1 µl MilliQ Water was added. 10 µl of PCR were analzyed on 1 % (w/v) agarose gel. Expected sizes: *medh2*-1203 bp (A) (B). As ladder (L) 1 kB Ladder (NEB) was used.

3) Inoculation of liquid culture for plasmid isolation of pSC1B3+*medh2*

- Clone 4 was positive in the Colony-PCR with both different primer combinations, therefore this one was chosen for Plasmid isolation
- Inoculate clone with 5 ml LB+Kan [50 µg/ml] and incubate overnight at 37 °C shaking at 220 rpm.

23. July 2015

1) Plasmid Isolation of pSC1B3+*medh2*

- Use the QIAprep Spin Miniprep Kit (Qiagen) and follow the provided manual.
- Send the plasmids for sequencing with T7-FWD primer
    - Sequencing evealed a misdesigned forward primer, so we ordered new primer for cloning into BioBrick Vector.