

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

20. July 2015

1) Digest of *medh2*, *hxlB* 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

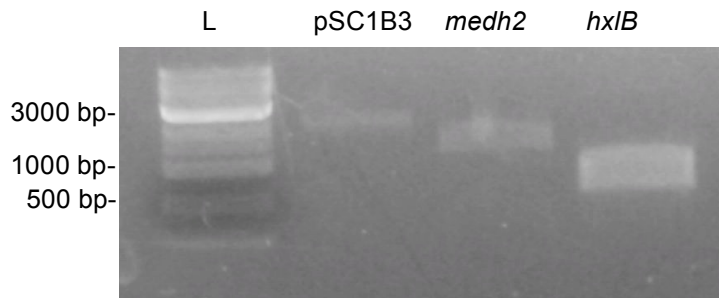


Figure 1: Digest of *medh2* and *hxlB* with EcoRI/ PstI. 10 µl of Digest were checked on 1% (w/v) agarose gel. Expected sizes: pSC1B3- 2033 bp, *medh2*-1197 bp and *hxlB*-665 bp. As lader (L) 1kB Ladder (NEB) was used.

2) Purification of digested *medh2*, *hxlB* 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 *hxlB* 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+*hxlB* and pSC1B3 selfligated

- 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 OD₆₀₀ of each preculture
 - Inoculate an 5 ml expression culture (LB+Kan [50 µg/ml]) with an OD₆₀₀ of 0.3
 - Grow the expression culture for 1 hour at 37 °C shaking at 220 rpm.
 - Measure OD₆₀₀ 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+hxIB
- 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 % Separating Gel
 - load 10 µl
 - Stain for 1 hour in Coomassie Staining Solution

- Destain overnight

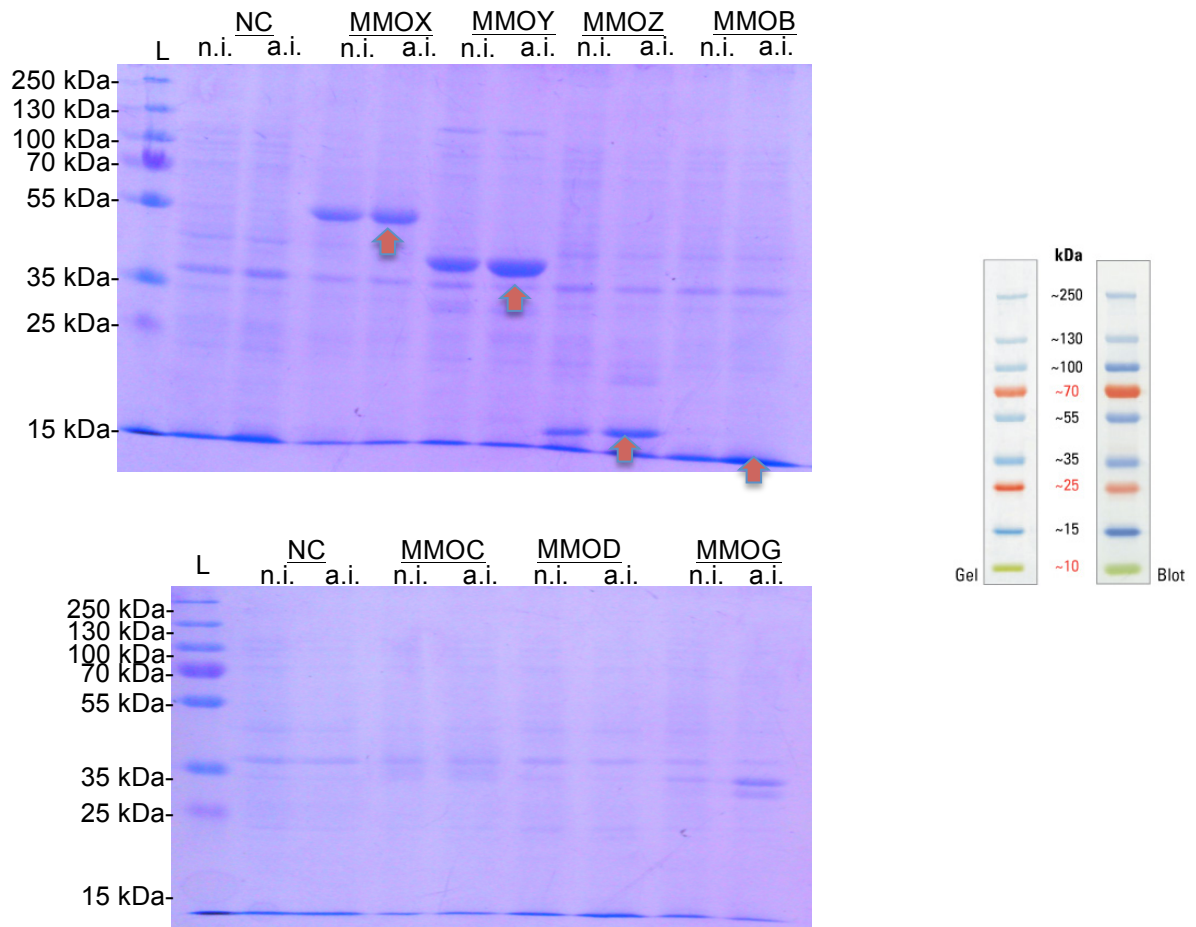


Figure 2: Pilotexpression of MMOX, MMOY, MMOZ, MMOB, MMOC, MMOD, MMOG in *E. coli* BL21. Coomassie staining of heterologously expressed MMOX, MMOY, MMOZ, MMOB, MMOC, MMOD, MMOG, in pET-30. 1 ml samples were taken in an uninduced stage (n.i.) and 3 hours after induction (a.i.) with 1 mM IPTG. Theoretical 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 μ l of pCR4+medh2, pSC1B3+mmoB
- Negative Control: add 1 μ l MilliQ Water

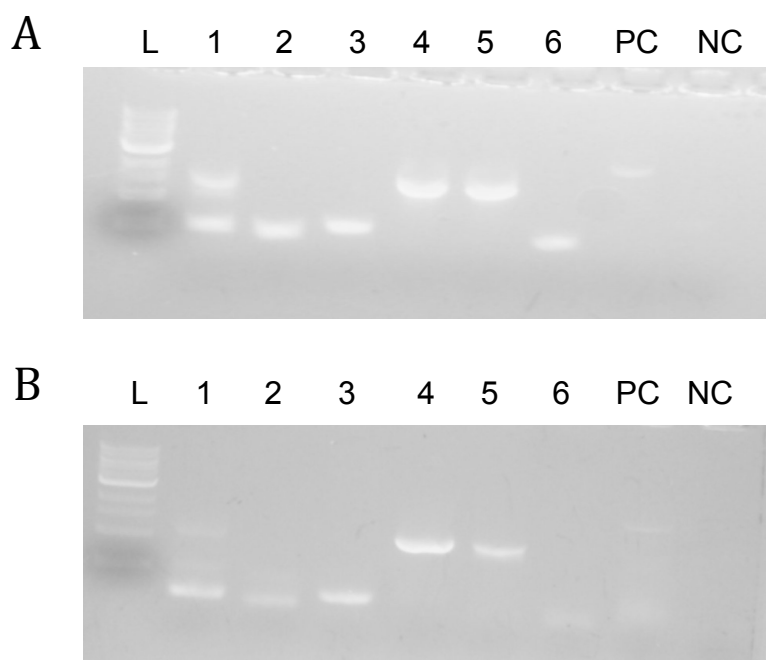


Figure 3: Colony-PCR to identify clones containing pSC1B3+*medh2* Biobrick construct.

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 μ l pCR4+*medh2* (A) and pSC1B3+*mmoB* (B) was used. As negative control (NC) 1 μ l MilliQ Water was added. 10 μ l of PCR were analyzed 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 μ g/ml] and incubate overnight at 37 $^{\circ}$ 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 revealed a misdesigned forward primer, so we ordered new primer for cloning into BioBrick Vector.