**Week 6: 13. July 2015- 17. July 2015**

**13. July 2015**

1) Digest of *medh2* and pET-30 with EcoRI/XhoI
   - Pipetting scheme according to the protocol Restriction Digest
   - Adding directly both enzymes and incubate the Reaction at 37 °C for 1 hour.
   - Verify 10 µl of the digestion on agarose gel

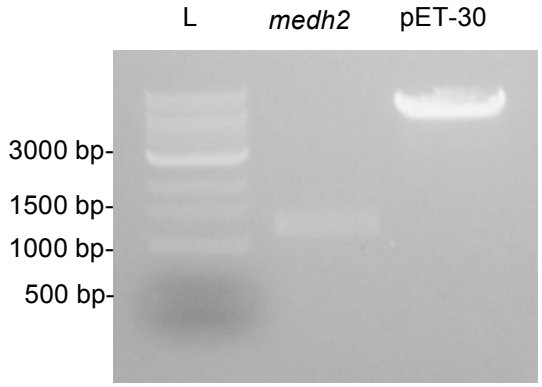


**Figure 1: Digest of *medh2* and pET-30 with EcoRI/XhoI.** 10 µl of Digest were checked on 1% (w/v) agarose gel. Expected sizes: *medh2*-1197 bp, pET-30-5269 bp. As ladder (L) 1kB Ladder (NEB) was used.

2) Purification of digested *medh2* and pET-30
   - Using Wizard® SV Gel and PCR Clean-Up System (Promega) and following the provided manual
3) Dephosphorylation of opened pET-30 using shrimp alkaline phosphatase
   - Pipetting scheme according to the protocol Shrimp Alkaline Phosphatase Treatment (Fermentas).
4) Ligation of *medh2* into pET-30
   - Pipetting scheme according to the Protocol Ligation with T4 DNA Ligase (NEB)
   - Use dephosphorylated pET-30
   - Control Reaction: pET-30 Selfligation
   - Incubate the reaction at 16 °C overnight
5) Inoculation of preculture culture for pilotexpression of MMOX, MMOY, MMOZ, MMOB, MMOC, MMOD, MMOG, HXLB in *E. coli* BL21
   - 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

6) Amplification of *medh2* and *hxlB* for BioBrick System

- Pipetting scheme and PCR program according to PCR with Phusion-HF DNA Polymerase Protocol
- Primer: medh2-BioBrick Fwd/Rev, hxlB-BioBrick Fwd/Rev
- Template: pCR4+*medh2*, pCR4+*hxlB*

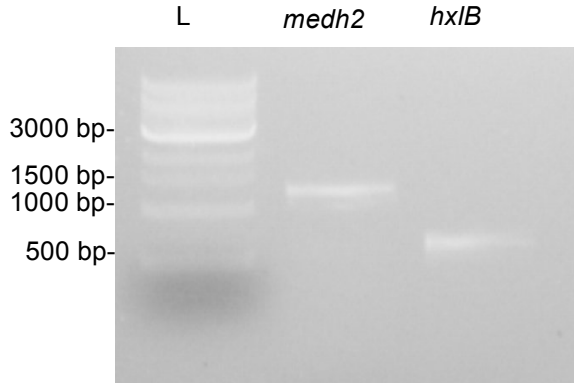


**Figure 2: Amplification of *medh2* and *hxlB* for BioBrick System.** 20 µl of PCR were checked on 1% (w/v) agarose gel. Expected size: *medh2*-1203 bp, *hxlB*-591 bp, As ladder (L) 1kB Ladder (NEB) was used.

7) Purification of generated *hxlB and medh2* PCR products

- Using Wizard® SV Gel and PCR Clean-Up System (Promega) and following the provided manual

**14. July 2015**

1) Transformation of *E. coli* TOP10 cells with pET-30+*medh2* and pET-30 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+Kan [50 µg/ml] and incubate overnight at 37 °C

2) Invitro mutagenesis of *hxlA* to remove PstI Restriction Site for BioBrick System

- Use QuickChange Site-Directed Mutagenesis Kit (Agilent) and follow the provided manual
- Primer: hxlA_mut3/mut4
- Template: pCR4+ hxlA-mut XbaI

a) DpnI Treatment

- Add 1 µl of DpnI to the PCR Reaction and incubate at 37 °C for 1 hour

b) Transformation of *E. coli* TOP10 cells with DpnI treated PCR reaction

- Add 1 µl of DpnI treated PCR reaction to chemically competent *E. coli* TOP10 cells.
- Following the Protocol for Transformation of chemical competent *E. coli* cells
- Plate on LB+Amp [100 µg/ml] and incubate overnight at 37 °C

8) Pilotexpression of MMOX, MMOY, MMOZ, MMOB, MMOC, MMOD, MMOG, HXLB into *E. coli* BL21
   - 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 (Optimal for induction of protein expression is an $OD_{600}$ between 0.4- 1.0)
   - Take a 1 ml sample in an uninduced stage and harvest the cell by centrifugation at 8000 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 8000 x g for 5 minutes
   - Keep the cell pellets at 4 °C
   - Treat the negative control the same way!
9) Amplification of *mmoG* for BioBrick System
   - Pipetting scheme and PCR program according to PCR with Phusion-HF DNA Polymerase Protocol
   - Primer: mmoG-BioBrick Fwd/Rev,
   - Template: PCR Fragment *mmoG* for TOPO cloning, pET-30+*mmoG*

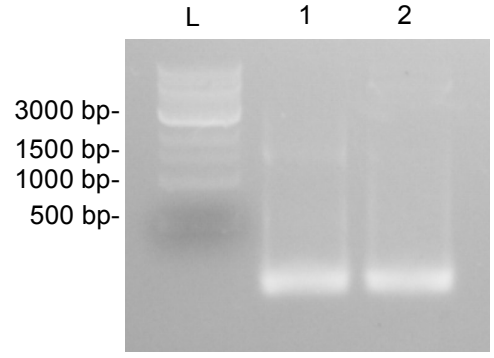

**Figure 3: Amplification of *mmoG* for BioBrick System.** 20 µl of PCR were checked on 1% (w/v) agarose gel. 1) PCR Fragment *mmoG* for TOPO cloning was used as template. 2) pET-30+*mmoG* was used as template. Expected size: *mmoG*-1708 bp, As ladder (L) 1kB Ladder (NEB) was used.

## 15. July 2015

- The ligation was successful, control reaction with selfligated pET-30 showed less clones compared to the ligation reactions

1) Colony-PCR to screen for clones containing the pET-30+*medh2* construct
   - Constructs that needed to be identified: pET-30+*medh2*
   - Pipetting scheme and PCR program according to PCR with Taq-DNA Polymerase Protocol
   - Primer: medh2_E1/E2
   - Check 6 Clones
   - Positive Control: add 1 µl of pCR4+*medh2*
   - Negative Control: add 1 µl MilliQ Water

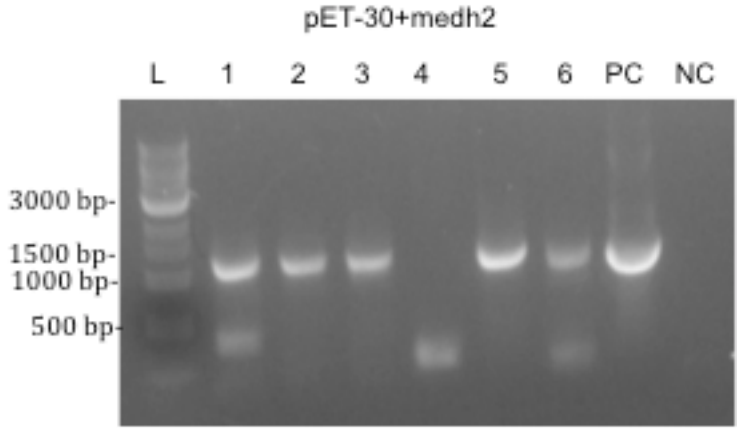


**Figure 4: Colony-PCR to identify clones containing pET-30+*medh2*.** Numbers 1-6 determine the checked clone. As positive control (PC) 1 µl pCR4+*medh2* was used. As negative control (NC) 1 µl MilliQ Water was added. 10 µl of PCR were analzyed on 1 % (w/v) agarose gel. Expected sizes: *medh2*- 1203 bp. As ladder (L) 1 kB Ladder (NEB) was used.

2) Inoculation of liquid culture for plasmid isolation of pET-30+*medh2*
   - Inoculate clone with 5 ml LB+Kan [50 µg/ml] and incubate overnight at 37 °C shaking at 220 rpm.
3) Inoculation of liquid culture for plasmid isolation of pCR4+*hxlA*-mut XbaI/PstI
   - Inoculate one clone with 5 ml LB+Amp [100 µg/ml] and incubate overnight at 37 °C shaking at 220 rpm.
4) 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

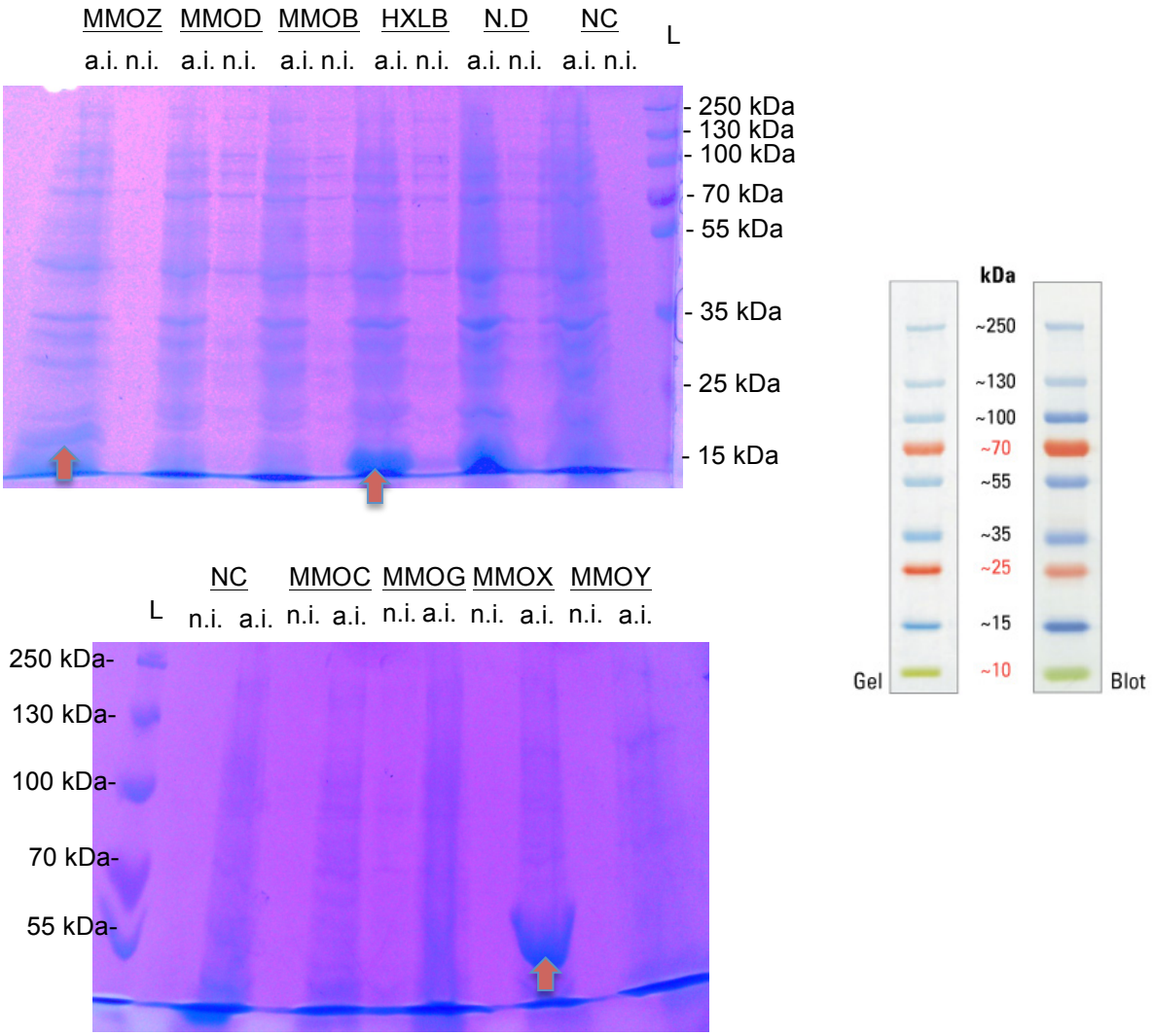


**Figure 5: Pilotexpression of MMOC, MMOG, MMOX, MMOY, MMOB, MMOD, MMOZ, HXLB in E. coli BL21.** Coomassie staining of heterologously expressed MMOC, MMOG, MMOX, MMOY, MMOB, MMOD, MMOZ, HXLB in pET-30. 1 ml samples were taken in an uninduced stage (n.i.) and 3 hours after induction (a.i.) with 1 mM IPTG. Theoretical weights: MMOC-38.48 kDa, MMOG-59.38 kDa, MMOX-60.56 kDa, MMOY-45.06 kDa, MMOB-15.95 kDa, MMOD-11.91 kDa, MMOZ-19.81 kDa, HXLB-20.23 kDa. Orange arrows determine the appropiate protein. As ladder PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (ThermoScientific) was used. N.D. determines a messed up sample. As negative control (NC) selfligated pET-30 was used for expression in *E. coli* BL21. Proteins were separated by SDS-PAGE.

**16. July 2015**

1) Plasmid Isolation of pCR4+*hxlA*-mut XbaI/PstI
   - Use the QIAprep Spin Miniprep Kit (Qiagen) and follow the provided manual.
   - Send for Sequencing
     - Sequencing Revealed a Fail in the Mutagenesis
2) Plasmid Isolation of pET-30+*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 Point Mutation in the pET-30 vector backbone