Week 9: 3. August 2015- 7. August 2015

3. August 2015

1) Amplification of *mmoG* for cloning
   - Pipetting scheme and PCR program according to PCR with Phusion-HF DNA Polymerase Protocol
   - Testing Phusion HF Buffer and Phusion GC Buffer
   - Primer: mmoG_P1/P2, mmoG_E1/E2
   - Template: gDNA *Methylococcus capsulatus*

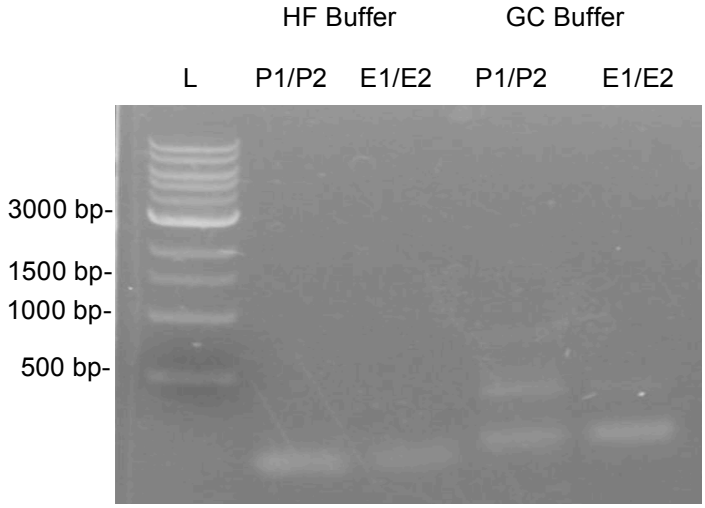

Figure 1: Amplification of *mmoG.* 20 µl of PCR were checked on 1% (w/v) agarose gel. Primer combinations P1/P2 and E1/E2 were tested with Phusion HF Buffer and Phusion GC Buffer. Expected size: *mmoG*-1708 bp. As lader (L) 1kB Ladder (NEB) was used.

2) Inoculation of preculture culture for solubility assay of MMOX, MMOY, MMOZ, MMOB and MMOD
   - 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

4. August 2015

1) Expression of MMOX, MMOY, MMOZ, MMOB and MMOD for solubility assay
   - 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

2) Solubility Assay, SDS-PAGE and Coomassie staining to verify soluble expression of MMOX, MMOY, MMOZ, MMOB and MMOD
   - Solubility Assay
     - Resuspend the cell pellet in 600 µl PBS buffer
     - Add glas beads
     - Use machine to disrupt the cells
     - Centrifuge at 16,000 x g for 5 minutes
     - Transfer 600 µl supernatant to a new tube—> soluble fraction
     - Resuspend the pellet in 300 µl PBS buffer → Insoluble fraction
   - Prepare Samples for SDS-Page:
     - Add 200 µl 4x SDS-Loading Buffer
     - Boil the sample at 95 °C for 20 minutes
   - Run SDS-Page
     - 18 % Seperating Gel
     - load 10 µl
   - Stain for 1 hour in Coomassie Staining Solution
   - Destain overnight

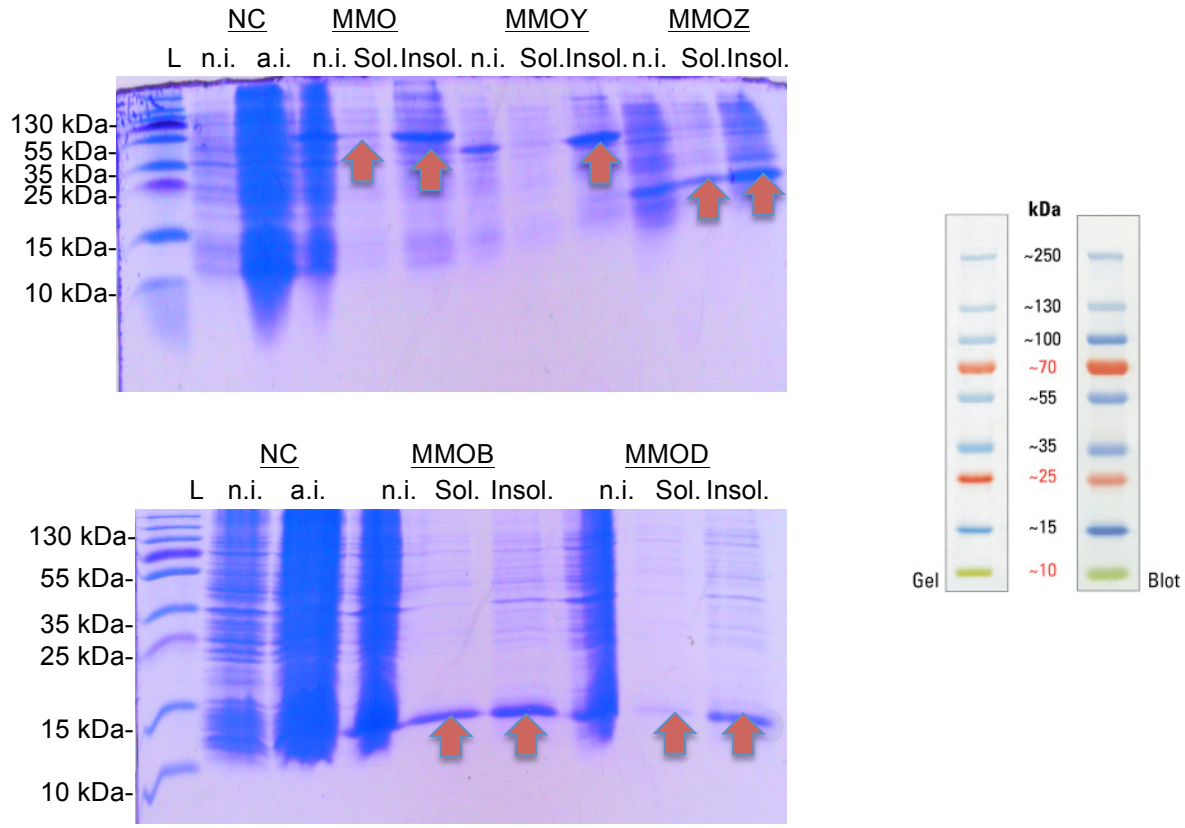

Figure 2: Solubility assay MMOX, MMOY, MMOZ, MMOB, MMOD. Coomassie staining of solubility assay of MMOX, MMOY, MMOZ, MMOB and MMOD. Proteinexpression was performed for 3 hours. Cell pellet was lysed and separated into soluble (sol) and insoluble (insol) Fraction. As control 1 ml sample was taken in an uninduced stage. Theorteical molecular weight: MMOX-60.56 kDa, MMOY-45.06 kDa, MMOZ-19.81 kDa, MMOB-15.95 kDa, MMOD-11.91 kDa. Proteins were separated by SDS-PAGE. Orange arrows determine the appropiate 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.

6. August 2015

1) Amplification of *hxlA* adding restriction sites (NcoI/ EcoRI) for cloning into pET-28

- Pipetting scheme and PCR program according to PCR with Phusion-HF DNA Polymerase Protocol
- Primer: hxlA_E1/E2,
- Template: pCR4+*hxlA*-mut XbaI/PstI

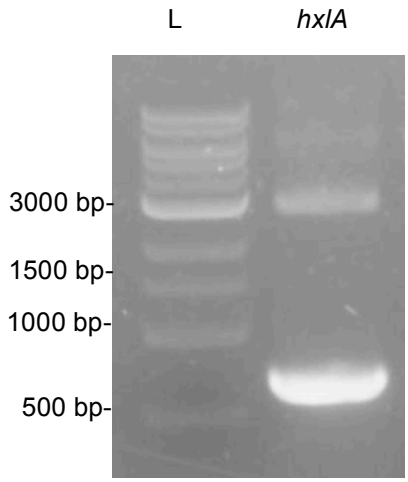

Figure 3: Amplification of *hxlA* adding restriction sites NcoI/EcoRI for cloning into pET-28. 20 µl of PCR were checked on 1% (w/v) agarose gel. Expected size: *hxlA*. As lader (L) 1kB Ladder (NEB) was used.

1) Digest of pET-28 with NcoI/ EcoRI and 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

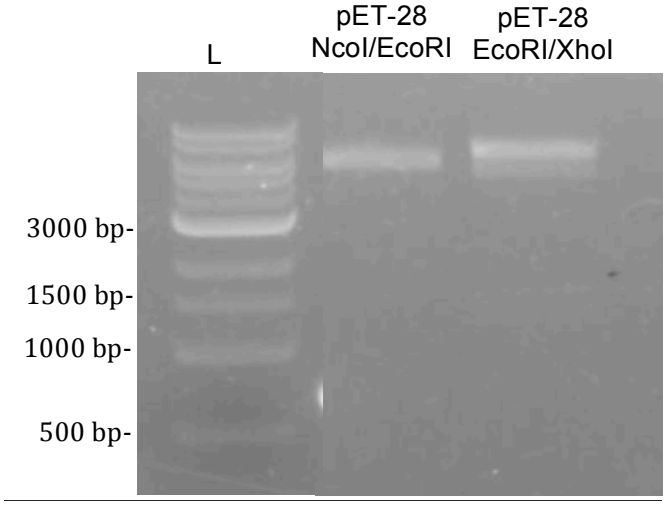

Figure 4: Digest of pET-28 with NcoI/EcoRI and EcoRI/XhoI. 10 µl of Digest were checked on 1% (w/v) agarose gel. Expected sizes: pET-28. As ladder (L) 1kB Ladder (NEB) was used.

3) Purification of digested pET-28
   - Using Wizard® SV Gel and PCR Clean-Up System (Promega) and following the provided manual

4) Dephosphorylation of opened pET-28 using shrimp alkaline phosphatase
   - Pipetting scheme according to the protocol Shrimp Alkaline Phosphatase Treatment (Fermentas).

7. August 2015

1) Amplification of *hxlA, hxlB* and *medh2* 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, hxlA-BioBrick Fwd/Rev
   - Template: pCR4+medh2, pCR4+hxlB, pCR4+hxlA-mut XbaI/PstI

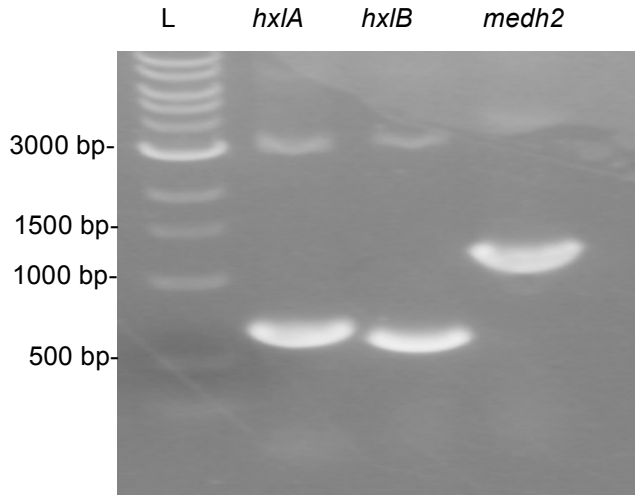

Figure 5: Amplification of *hxlA, hxlB* and *medh2 for BioBrick system.* 20 ul of PCR were checked on 1% (w/v) agarose gel. Expected size: *hxlA, hxlB* and *medh2*. As lader (L) 1kB Ladder (NEB) was used.

2) Purification of generated *hxlA, hxlB* and *medh2* PCR product
   - Using Wizard® SV Gel and PCR Clean-Up System (Promega) and following the provided manual