

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*

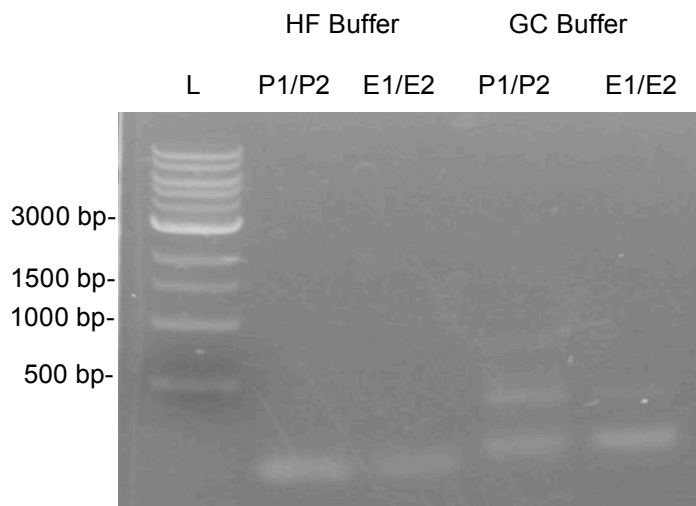


Figure 1: Amplification of *mmoG*. 20 μ l of PCR were checked on 1% (w/v) agarose gel. Primer combinations P1/P2 and E1/E2 were tested with Phusion HF Buffer and Phusion GC Buffer. Expected size: *mmoG*-1708 bp. As ladder (L) 1kb Ladder (NEB) was used.

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

- Inoculate one clone with 5 ml LB+Kan [50 μ g/ml] and incubate overnight at 37 $^{\circ}$ 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 OD₆₀₀ of each preculture
- Inoculate an 5 ml expression culture (LB+Kan [50 μ g/ml]) with an OD₆₀₀ of 0.3
- Grow the expression culture for 1 hour at 37 $^{\circ}$ 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

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 glass 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 % Separating Gel
 - load 10 µl
- Stain for 1 hour in Coomassie Staining Solution
- Destain overnight

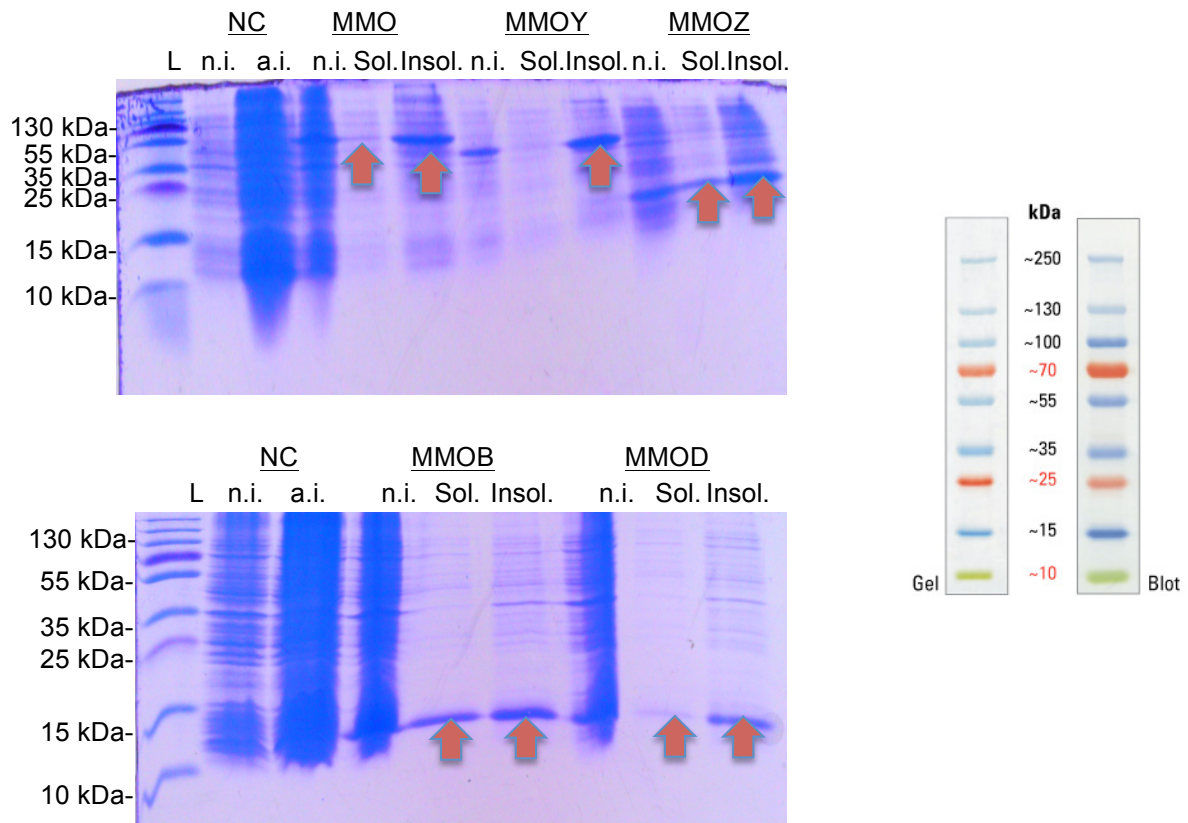


Figure 2: Solubility assay MMOX, MMOY, MMOZ, MMOB, MMOD. Coomassie staining of solubility assay of MMOX, MMOY, MMOZ, MMOB and MMOD. Protein expression was performed for 3 hours. Cell pellet was lysed and separated into soluble (sol) and insoluble (insol) Fraction. As control 1 ml sample was taken in an uninduced stage. Theoretical 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 appropriate protein. As ladder PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (ThermoScientific) was used. As negative control (NC) selfigated pET-30 was used for expression in *E. coli* BL21. Proteins were separated by SDS-PAGE.

6. August 2015

- 1) Amplification of *hps* 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: *hps_E1/E2*,
 - Template: pCR4+*hps*-mut XbaI/PstI

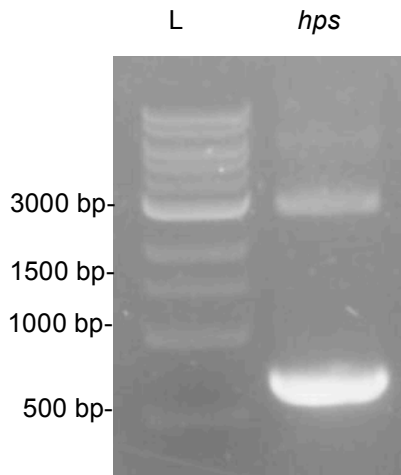


Figure 3: Amplification of *hps* adding restriction sites *NcoI/EcoRI* for cloning into pET-28. 20 μ l of PCR were checked on 1% (w/v) agarose gel. Expected size: *hps*. As ladder (L) 1kB Ladder (NEB) was used.

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 μ l of the digestion on agarose gel

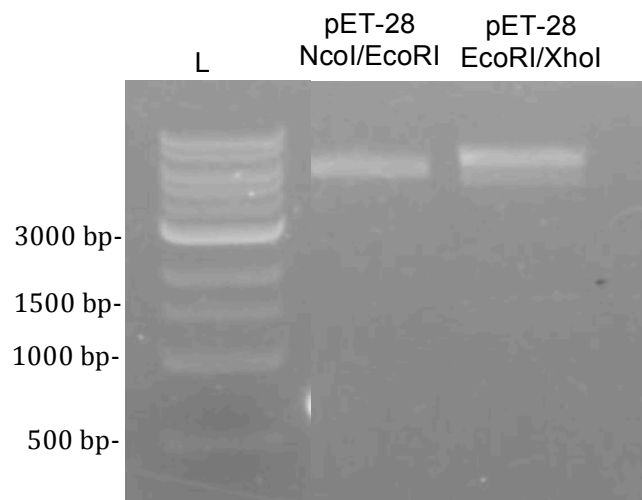


Figure 4: Digest of pET-28 with *NcoI/EcoRI* and *EcoRI/XhoI*. 10 μ l of Digest were checked on 1% (w/v) agarose gel. Expected sizes: pET-28. As ladder (L) 1kB Ladder (NEB) was used.

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 *hps*, *phi* and *medh2* for BioBrick System

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

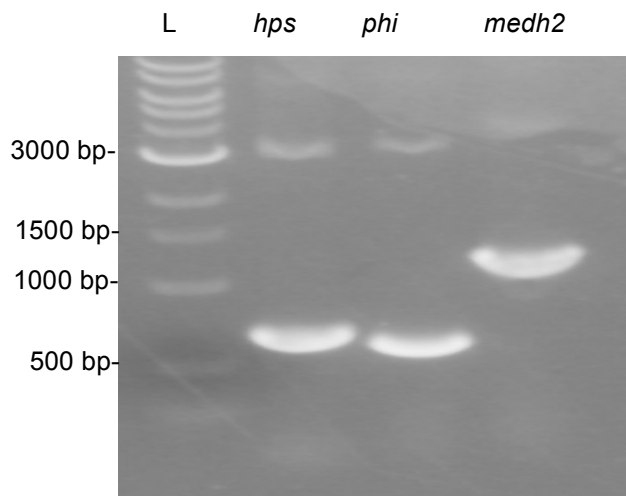


Figure 5: Amplification of *hps*, *phi* and *medh2* for BioBrick system. 20 ul of PCR were checked on 1% (w/v) agarose gel. Expected size: *hps*, *phi* and *medh2*. As ladder (L) 1kb Ladder (NEB) was used.

2) Purification of generated *hps*, *phi* and *medh2* PCR product

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