Week 5: 6. July 2015 – 10. July 2015

6. July 2015:

1) Digest of *mmoX, mmoY, mmoZ, mmoC, mmoD, mmoG, phi* with NdeI/EcoRI
    - Pipetting scheme according to the protocol Restriction Digest
    - First adding NdeI and incubate the Reaction at 37 °C for 1 hour, afterwards heat inactivation of NdeI at 65 °C for 2 minutes. Adding 1 ul of EcoRI and incubate the digest again for 1 hour at 37 °C
    - Verify 10 µl of the digestion on agarose gel

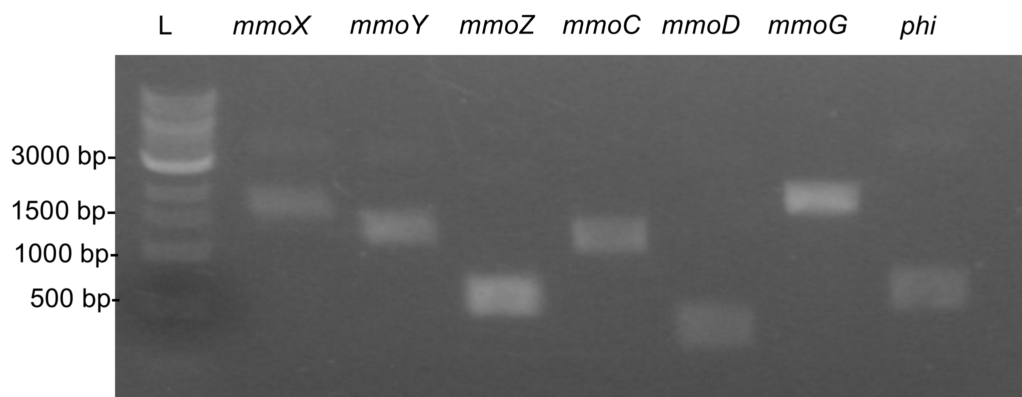

Figure 1: Digest of *mmoX, mmoY, mmoZ, mmoC, mmoD, mmoG* and *phi* with NdeI/ EcoRI. 10 µl of digest were checked on 1% (w/v) agarose gel. Expected sizes: *mmoX*- 1605 bp, *mmoY*- 1191 bp, *mmoZ*- 546 bp, *mmoC*-1082 bp, *mmoD*- 333 bp, *mmoG*- 1702 bp and *phi*- 585 bp. As ladder (L) 1kB Ladder (NEB) was used.

2) Purification of digested *mmoX, mmoY, mmoZ, mmoC, mmoD, mmoG, phi*
    - 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 *mmoX, mmoY, mmoZ, mmoC, mmoD, mmoG, phi* 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

7. July 2015

1) Transformation of *E. coli* TOP10 cells with pET-30+*mmoX*, pET-30+*mmoY*, pET-30+*mmoZ*, pET-30+*mmoC*, pET-30+*mmoD*, pET-30+*mmoG*, pET-30+*phi*
   - 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 *hps* to remove XbaI Restriction Site
   - Use QuickChange Site-Directed Mutagenesis Kit (Agilent) and follow the provided manual
   - Primer: hps_mut1/mut2
   - Template: pCR4+*hps*

   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. 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+*mmoX*, pET-30+*mmoY*, pET-30+*mmoZ*, pET-30+*mmoC*, pET-30+*mmoD*, pET-30+*mmoG*, pET-30+*phi*
   - Constructs: pET-30+*mmoX*, pET-30+*mmoY*, pET-30+*mmoZ*, pET-30+*mmoC*, pET-30+*mmoD*, pET-30+*mmoG*, pET-30+*phi*
   - Pipetting scheme and PCR program according to PCR with Taq-DNA Polymerase Protocol
   - Primer: mmoX_E1/E2, mmoY_E1/E2, mmoZ_E1/E2, mmoC_E1/E2, mmoD_E1/E2, mmoG_E1/E2, phi_E1/E2
   - Check 6 Clones per Construct

- Positive Control: add 1 µl of pSC1B3+*mmoX*, pSC1B3+*mmoY*, pSC1B3+*mmoZ*, pCR4+*mmoC*, pSC1B3+*mmoD*, PCR fragment *mmoG*, pSC1B3+*phi*
- Negative Control: add 1 µl MilliQ Water

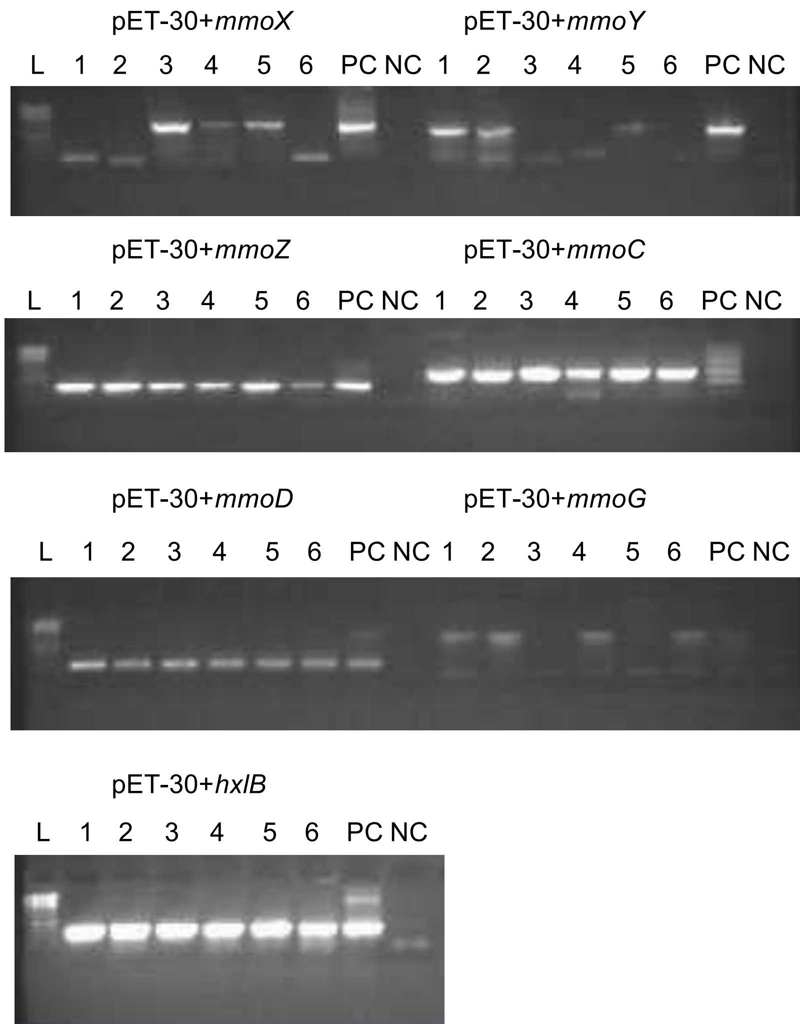

Figure 2: Colony-PCR to identify clones containing pET-30+*mmoX*, pET-30+*mmoY*, pET-30+*mmoZ*, pET-30+*mmoC*, pET-30+*mmoD*, pET-30+*mmoG*, pET-30+*phi* expression constructs. Numbers 1-6 determine the checked clone. As positive control (PC) 1 µl pSC1B3+*mmoX*, pSC1B3+*mmoY*, pSC1B3+*mmoZ*, pCR4+*mmoC*, pSC1B3+*mmoD*, PCR fragment mmoG, pSC1B3+*phi 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: *mmoX*-453 bp, *mmoY*-1197 bp, *mmoZ*-552 bp, *mmoC*-1088 bp, *mmoD*-339 bp, *mmoG*-1708 bp, *phi*-591 bp. As ladder (L) 1 kB Ladder (NEB) was used.

2) Inoculation of liquid culture for plasmid isolation of pET-30+*mmoX*, pET-30+*mmoY*, pET-30+*mmoZ*, pET-30+*mmoC*, pET-30+*mmoD*, pET-30+*mmoG*, pET-30+*phi*

- Inoculate one clone with 5 ml LB+Kan [50 µg/ml] and incubate overnight at 37 °C shaking at 220 rpm.

3) <u>Inoculation of liquid culture for plasmid isolation of pCR4+*hps*-mut XbaI</u>

- Inoculate one clone with 5 ml LB+Amp [100 µg/ml] and incubate overnight at 37 °C shaking at 220 rpm.

4) <u>Amplification of *mmoG, medh2* and *phi* for BioBrick System</u>

- Pipetting scheme and PCR program according to PCR with Phusion-HF DNA Polymerase Protocol
- Primer: mmoG-BioBrick Fwd/Rev, medh2-BioBrick Fwd/Rev, phi-BioBrick Fwd/Rev
- Template: PCR Fragment *mmoG* for TOPO cloning, pCR4+*medh2*, pCR4+*phi*

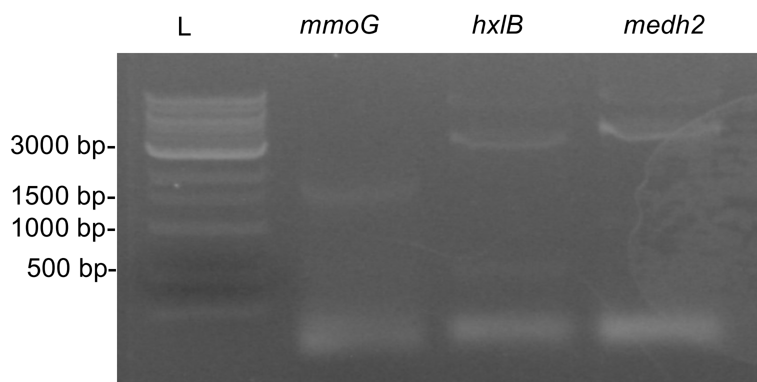

Figure 3: Amplification of *mmoG, phi* and *medh2* for BioBrick System. 20 µl of PCR were checked on 1% (w/v) agarose gel. Expected sizes: *mmoG*-1708 bp, *phi*-591 bp, *medh2*-1203 bp. As ladder (L) 1kB Ladder (NEB) was used.

<u>9. July 2015</u>

1) <u>Plasmid Isolation of pET-30+*mmoX*, pET-30+*mmoY*, pET-30+*mmoZ*, pET-30+*mmoC*, pET-30+*mmoD*, pET-30+*mmoG*, pET-30+*phi*</u>

- Use the QIAprep Spin Miniprep Kit (Qiagen) and follow the provided manual.
- Send the plasmids for sequencing with T7-FWD primer
    - Expression constructs seem to be correct

2) <u>Plasmid Isolation of pCR4+*hps*-mut XbaI</u>

- Use the QIAprep Spin Miniprep Kit (Qiagen) and follow the provided manual.
- Send for Sequencing
    - Mutagenesis was successful, XbaI restriction site was successfully mutat

hps atggaacttcaattagctctagatttggtaaacattgaagaagcaaaacaag

Sequencing Results ATGGAACTTCAATTAGCTCTCGATTTGGTAAACATTGAAGAAGCAAAACAAG

Figure 4: Alignment of *hps* original sequence with sequencing results of *hps* invitro mutagensis to remove XbaI restriction site. Red colour indicates the XbaI restriction site in the hps sequence. Cyan labeled A in the *hps* sequence indicates the base we wanted to change into a C.

3) Transformation of *E. coli* BL21 cells with expression plasmids

- Expression Plasmids: pET-30+*mmoX*, pET-30+*mmoY*, pET-30+*mmoZ*, pET-30+*mmoB*, pET-30+*mmoC*, pET-30+*mmoD*, pET-30+*mmoG*, pET-30+*phi*
- Add 1 µl of Expression Plasmid to chemically competent *E. coli* BL21 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