

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

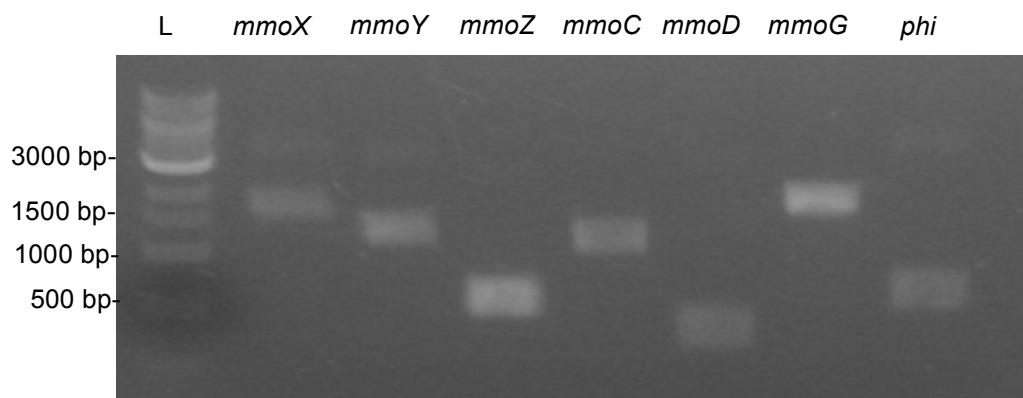


Figure 1: Digest of *mmoX*, *mmoY*, *mmoZ*, *mmoC*, *mmoD*, *mmoG* and *phi* with NdeI/ EcoRI. 10 µl of digest were checked on 1% (w/v) agarose gel. Expected sizes: *mmoX*- 1605 bp, *mmoY*- 1191 bp, *mmoZ*- 546 bp, *mmoC*-1082 bp, *mmoD*- 333 bp, *mmoG*- 1702 bp and *phi*- 585 bp. As ladder (L) 1kB Ladder (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 μ l of pSC1B3+*mmoX*, pSC1B3+*mmoY*, pSC1B3+*mmoZ*, pCR4+*mmoC*, pSC1B3+*mmoD*, PCR fragment *mmoG*, pSC1B3+*phi*
- Negative Control: add 1 μ l MilliQ Water

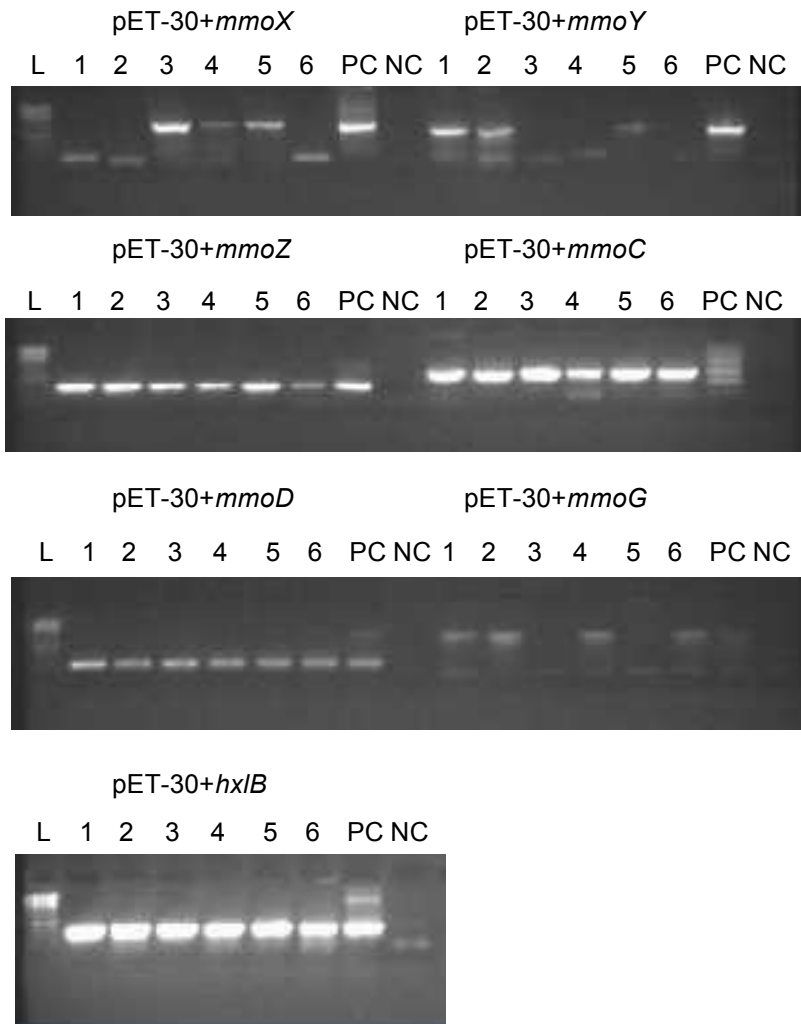


Figure 2: Colony-PCR to identify clones containing pET-30+*mmoX*, pET-30+*mmoY*, pET-30+*mmoZ*, pET-30+*mmoC*, pET-30+*mmoD*, pET-30+*mmoG*, pET-30+*phi* expression constructs. Numbers 1-6 determine the checked clone. As positive control (PC) 1 μ l pSC1B3+*mmoX*, pSC1B3+*mmoY*, pSC1B3+*mmoZ*, pCR4+*mmoC*, pSC1B3+*mmoD*, PCR fragment *mmoG*, pSC1B3+*phi* 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: *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 μ g/ml] and incubate overnight at 37 $^{\circ}$ C shaking at 220 rpm.

3) Inoculation of liquid culture for plasmid isolation of pCR4+hps-mut XbaI

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

4) Amplification of *mmoG*, *medh2* and *phi* for BioBrick System

- 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*

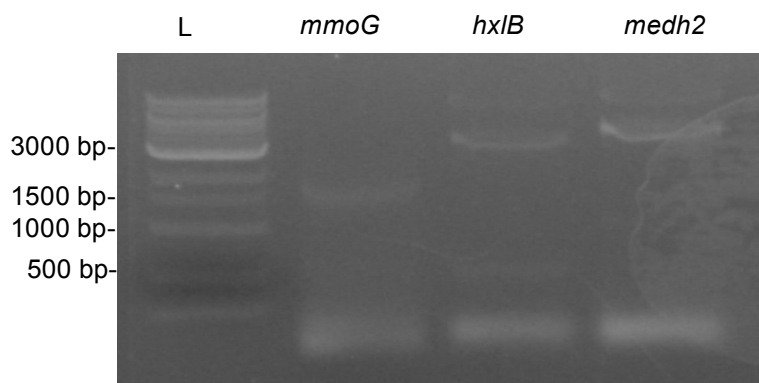


Figure 3: Amplification of *mmoG*, *phi* and *medh2* for BioBrick System. 20 µl of PCR were checked on 1% (w/v) agarose gel. Expected sizes: *mmoG*-1708 bp, *phi*-591 bp, *medh2*-1203 bp. As ladder (L) 1kb Ladder (NEB) was used.

9. July 2015

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

- 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) Plasmid Isolation of pCR4+hps-mut XbaI

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

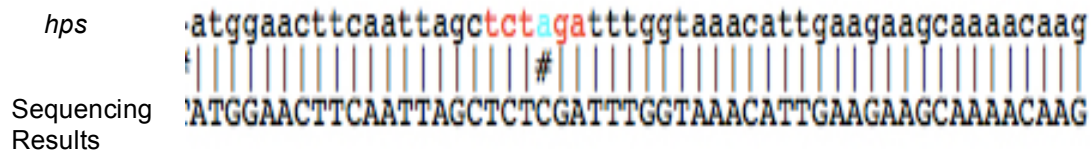


Figure 4: Alignment of *hps* original sequence with sequencing results of *hps* invitro mutagenesis to remove *Xba*I restriction site. Red colour indicates the *Xba*I restriction site in the *hps* sequence. Cyan labeled A in the *hps* sequence indicates the base we wanted to change into a C.

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+Amp [100 μg/ml] and incubate overnight at 37 °C