# End of the Note Book

**16 September 2016**

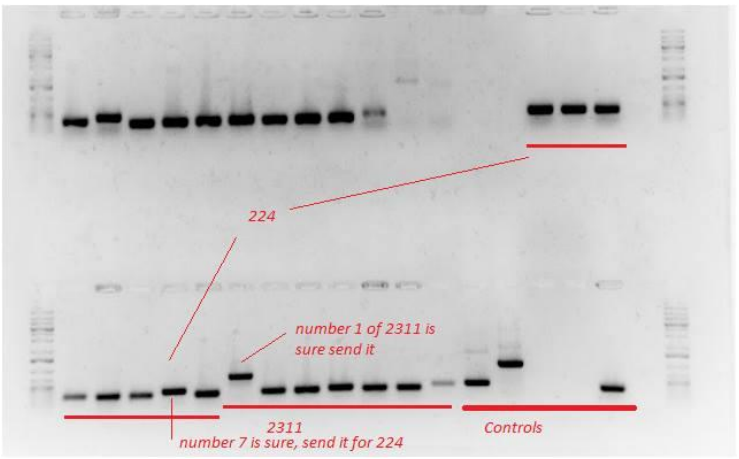
Colonies PCR

Mix (25 µL total volume reaction):

- 12.5 µL DreamTaq PCR Mastermix 2X (ThermoScientific)
- 2.5 µL 10X DreamTaq Green Buffer (ThermoScientific)
- 0.25 µL Forward Primer (0.5 µM) iG001
- 0.25 µL Reverse Primer (0.5 µM) iG002
- 9.5 µL H2O filtrated (qs 25 µL)

+ clones

Agarose gel 1 %




Precultures

Positive clones selected from the agarose gel results were picked and used to inoculate 3 mL of LBC (25 µg/mL chloramphenicol) medium.

Observation

➔ All the precultures have grown

- PCR colony

Mix:

- Dream Taq MM 2X
- Fw
- Rv
- DNA
- H2O

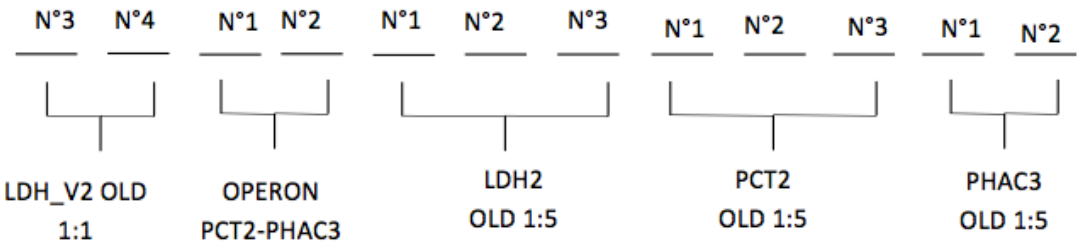
| | |
|---|---|
| 10 µL | 280 µL |
| 1.5 µL | 42 µL |
| 1.5 µL | 42 µL |
| 1 µL | - |
| 6 µL | 168 |

X28

Just to remind:

Position E5: Mix without clone
Position E6: Mix with pLAC into pSB1C3
Position E7: Mix with mRF-HisTag into Psb1C3

Migration map:



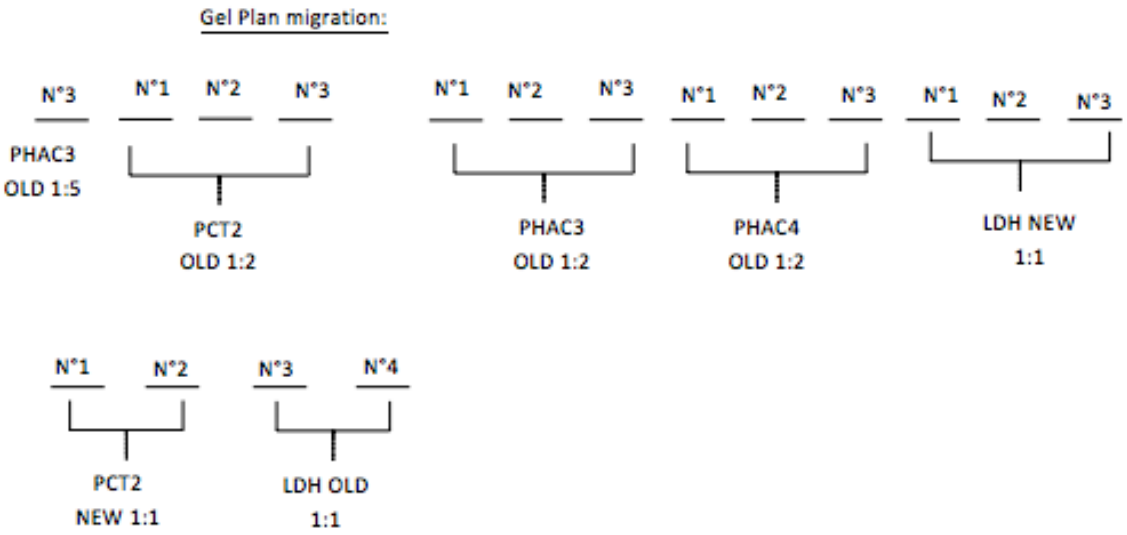

➢ Expected Bands

LDH_V2= ~1550
PCT_V2=~1950
PHAC_V3=~2050
PHAC_V4=~2000

➢ Cycle

| | |
|---|---|
| 95 °C | 3 min |
| 95 °C | 30 sec |
| 50 °C | 30 sec |
| 72 °C | 1.15 min |
| 72 °C | 5 min |
| 10 °C | ∞ |

X30




**20 September 2016**

Precultures

3 mL LB medium in 15 mL Falcon tube+ 1 µL *P. putida* KT2440 from -80 °C stock

Incubation 30 °C overnight with shaking.

- PCR Gibson of pct_V2, phac_V3, phac_V4, pSB1B3 HQ, pSB1C36 mRFP-HisTag

Mix:

Vf= 50 µL

- 5X Q5 Reaction Buffer
- 10 mM dNTP
- 10 µM Fw
- 10 µM Rv
- DNA
- Q5 (0.2 U/µL)
- H2O

| | |
|---|---|
| 10 µL | 70 µL |
| 1 µL | 7 µL |
| 2.5 µL | - µL |
| 2.5 µL | - µL |
| 1 µL | - |
| 0.5 µL | 3.5 µL |
| 32.5 µL | 227 µL |

X7

- Solubilization of primers

For PR_IG075: Add 209 µL H2O for [100 µM]

For PR_IG076: Add 198 µL H2O for [100 µM]

For PR_IG077: Add 242 µL H2O for [100 µM]

For PR_IG078: Add 236 µL H2O for [100 µM]

For PR_IG079: Add 257 µL H2O for [100 µM]

For PR_IG080: Add 300 µL H2O for [100 µM]

For PR_IG081: Add 235 µL H2O for [100 µM]

For PR_IG082: Add 229 µL H2O for [100 µM]

For PR_IG083: Add 238 µL H2O for [100 µM]

For PR_IG084: Add 266 µL H2O for [100 µM]

Aliquot 1/10 of each primer

- Gel purification of PCT2, PhaCv3, PhaCv4
- Purification

## 21 September 2016

- Gel Extraction pSb1C3 + mRFP-Histag

Gel weight= 300 mg

- PCR clean-up pct2, phac_v3, phac_v4

Nanodrop:

| Genes | Concentration (ng/µL) | Ratio 260/280 |
|---|---|---|
| PCT_V2 | 152 | 1.82 |
| PHAC_V3 | 130.7 | 1.84 |
| PHAC_V4 | 132.9 | 1.83 |
| pSB1C3 IHQ | 108.3 | 1.80 |
| pSB1C3 Biobrick | 14.7 | 1.74 |

- Gibson
    - Ratio Calculation

➔ Pct2-phac3 into pSB1C3

| | |
|---|---|
| pSB1C3 IGHQ | 0.92 µL |
| H2O | 6.75 µL |

Mix:

| | |
|---|---|
| PCT2 | 1.04 µL |
| PHAC3 | 1.29 µL |

➔ Pct2-phac4 into pSB1C3 IGHQ

Mix:

| | |
|---|---|
| pSB1C3 IGHQ | 0.92 µL |
| H2O | 6.76 µL |
| PCT2 | 1.04 µL |
| PHAC4 | 1.27 µL |

➔ Pct2-phac3 into pSB1C3 Biobrick

Mix:

| | |
|---|---|
| pSB1C3 Biobrick | 6.80 µL |
| H2O | 0.87 µL |
| PCT2 | 1.04 µL |
| PHAC3 | 1.29 µL |

➔ Pct2-phac4 into pSB1C3 Biobrick

Mix:

| | |
|---|---|
| pSB1C3 Biobrick | 6.80 µL |
| H2O | 0.89 µL |
| PCT2 | 1.04 µL |
| PHAC4 | 1.27 µL |

Add 10 µL of HIFI DNA Assembly MM in each.
And 10 µL of hifi with 10 µL of positive control
Keep on ice!

- Incubation: 50 °C, 15 minutes then on ice

- Chemically Competent Cells Transformation

➔ Add 2 µL of the chilled assembled to 25 µL competent cells
➔ Waiting 30 min in ice
➔ Heat shock at 42 °C for 30 sec
➔ Transfer the tube on ice for 2 min
➔ Add 950 µL LB for each
➔ Incubation: 60 min, 37 °C, 250 rpm
➔ Spread 100 µL on the plate

➔ Incubation 37 °C overnight

**23 September 2016**

- Gibson plate observation

➔ DH5-alpha pct2-phac3-pSB1C3 iGHQ 14 clones
➔ DH5-alpha pct2-phac4-pSB1C3 iGHQ 13 clones
➔ DH5-alpha pct2-phac3-pSB1C3 Biobrick 1 clones
➔ DH5-alpha pct2-phac4-pSB1C3 Biobrick 5 clones

- PCR Colony

Mix:

- DreamTaq Master Mix 2X
- PR_IG001
- PR_IG002
- DNA
- H2O

| | |
|---|---|
| 10 µL | 350 µL |
| 1.5 µL | 52.5 µL |
| 1.5 µL | 52.5 µL |
| 1 µL | - |
| 6 µL | 210 µL |

X35

Cycle:

| | |
|---|---|
| 95 °C | 3 min |
| 95 °C | 30 sec |
| 50 °C | 30 sec |
| 72 °C | 1.30 min |
| 72 °C | 2 min |
| 10 °C | ∞ |
| | |

X25

- Result Gibson

N°2 pct2 phac4 pSB1C3 iGEM HQ

N°1 pct2 phac4

N°5 pct2 phac4 pSB1C3 Biobrick

- Preculture in LB+ Cam (10 µg/mL)
- Miniprep of the cultures
- Gel Verification of Gibson PCR

➔ Don't work

Growth experiments

Stock in 50 % glycerol stored at -80 °C

Experiments performed in triplicates on 96 wells plate with LB medium as blank.

The plate was incubated at 30 °C with shaking during 20 h after inoculating with 1/1000 dilution of preculture.

The OD at 600 nm was read every 20 s.

The experiments were repeated 3 consecutive days from fresh precultures started the day before.

The results were sent to Imperial College IGEM team for collaboration along with the results of carbon source tests performed in July.

**27 September 2016**

- Sequencing

➔ WatchBox 1887819 (27.09.16)

**28th September 2016**

- Gibson

➔ Calculation for Ratio 1:1

Mix DNA 1:

| | |
|---|---|
| pct2 | 0.62 µL |
| Phac3 | 0.76 µL |
| H2O | 8.62 µL |

Mix DNA 2:

| | |
|---|---|
| pct2 | 0.66 µL |
| Phac3 | 0.81 µL |
| H2O | 8.63 µL |

Mix DNA3:

| | |
|---|---|
| pct2 | 0.62 µL |
| Phac4 | 0.75 µL |
| H2O | 8.63 µL |

Mix DNA 4:

| | |
|---|---|
| pct2 | 0.66 µL |
| Phac4 | 0.80 µL |
| H2O | 8.54 µL |

➔ Calculation for Ratio 1:2
Mix 5:

| | |
|---|---|
| Diluted pct2 | 1.04 µL |
| Diluted Phac3 | 1.29 µL |
| H2O | 6.75 µL |
| IGHQ Vector | 0.92 µL |

➔ Mix 6:

| | |
|---|---|
| Diluted pct2 | 1.04 µL |
| Diluted Phac4 | 1.27 µL |
| H2O | 6.76 µL |
| IGHQ Vector | 0.92 µL |

➔ Mix 7:

| | |
|---|---|
| Diluted pct2 | 1.04 µL |
| Diluted Phac3 | 1.29 µL |
| H2O | 4.27 µL |
| Biobrick Vector | 3.40 µL |

➔ Mix 8:

| | |
|---|---|
| Diluted pct2 | 1.04 µL |
| Diluted Phac4 | 1.27 µL |
| H2O | 4.29 µL |
| Biobrick Vector | 3.40 µL |

➔ Put 15 min to 50 °C and after at -20 °C

**29th September 2016**

- Transformation

➔ 2 µL product + 25 µL of Dh5-alpha cells+ 950 µL SOC Medium
➔ 1 h Recovery

- Culture plate LB Cam (10 µg/mL)
- Preparing plate

Ci= 50 mg/mL $vi = \frac{10\ \mu g * 25\ mL}{50000} = 5\ \mu l$

Cf= 10 µg/mL

Vf= 25 mL

- Purification PCR of gBlock fragment
- Nanodrop

  Nothing ☹

**30th September 2016**

- Concentration / SpeedVac
- Verification Gel of purifications
- PCR amplification gBlock

**4 October 2016**

<u>Gibson assembly 2nd step</u>

pSB1C3 IGEM Headquarters 110.3 ng/µL

pSB1C3 mRFP 14.1 ng/µL

*Mix Gibson from the 1st step giving the operon:*

1) PCT2+PhaC3 65.5 ng/µL
2) PCT2+PhaC4 60.8 ng/µL
3) PCT2+PhaC3 63.4 ng/µL
4) PCT2+PhaC4 65.7 ng/µL

*Gibson ratio 1:1*

| | | | |
|---|---|---|---|
| Vector amplified from pSB1C3-mFRP | 1) | Vector | 3.40 µL (50 ng) |
| | | Insert (mix 1) | 1.21 µL |
| | | H2O (qs 10 µL) | 5.40 µL |
| | 2) | Vector | 3.40 µL (50 ng) |
| | | Insert (mix 2) | 1.30 µL |
| | | H2O (qs 10 µL) | 5.30 µL |

| | | | |
|---|---|---|---|
| | 3) | Vector | 3.40 µL (50 ng) |
| | | Insert (mix 3) | 1.25 µL |
| | | H2O (qs 10 µL) | 5.35 µL |
| | 4) | Vector | 3.40 µL (50 ng) |
| | | Insert (mix 4) | 1.20 µL |
| | | H2O (qs 10 µL) | 5.40 µL |
| Linear vector pSB1C3 provided by IGEM Headquarters | 5) | Vector | 0.91 µL (100 ng) |
| | | Insert (mix 1) | 2.42 µL |
| | | H2O (qs 10 µL) | 6.68 µL |
| | 6) | Vector | 0.91 µL (100 ng) |
| | | Insert (mix 2) | 2.60 µL |
| | | H2O (qs 10 µL) | 6.49 µL |
| | 7) | Vector | 0.91 µL (100 ng) |
| | | Insert (mix 3) | 2.50 µL |
| | | H2O (qs 10 µL) | 6.60 µL |
| | 8) | Vector | 0.91 µL (100 ng) |
| | | Insert (mix 4) | 2.41 µL |
| | | H2O (qs 10 µL) | 6.68 µL |

Add 10 µL NEB HiFi DNA Assembly Mastermix 2X
Incubation 50 °C, 15 min
Cool on ice

Transformation

- Thaw 50 µL chemo-competent DH5-alpha *E. coli*
- Add 2 µL DNA (mix Gibson)
- Incubation 30 min on ice
- Heat shock: 30 s at 42 °C
- Incubation 2 min on ice
- Add 950 µL NEB medium (#B9020S)
- Incubation 1 h at 37 °C with shaking
- Centrifugation 7000 rpm 1 min
- Resuspend cells with rest of supernatant after throwing away the most of it
- Spread all the content on plates LBC
- Incubation overnight 37 °C

**7 October 2016**

Solubilisation of IDT gBlocks in 100 µL of nuclease-free water and short centrifugation

Incubation 50 °C during 20 min

Vortexing and then, short centrifugation again

-PCR of gBlock genes

Mix:

- 10 µL Q5 reaction buffer 5X
- 1 µL dNTP mix
- 2.5 µL 10 µM Forward Primer (iG063)
- 2.5 µL 10 µM Reverse Primer (iG064)
- 1 µL DNA template
- 0.5 µL Q5 DNA polymerase
- 32.5 µL Nuclease-free water (qs 50 µL)

Mix for 5 reactions

*PCR program:*

1) 98 °C 30 s
2) 98 °C 30 s
3) 55 °C 30 s
4) 72 °C 1 min
   35 repeats of the steps bloc from 2) to 4)
5) 72 °C 2 min
6) 10 °C infinite

Colony PCR on Gibson transformation

- 12.5 µL DreamTaq Green PCR Master Mix 2X (Thermo Scientific, #K1081)
- 1.25 µL 10 µM Forward Primer (iG001)
- 1.25 µL 10 µM Reverse Primer (iG002)
- 10 µL Nuclease-free water (qs 25 µL)

+ clone

Mix for 23 reactions

*PCR program:*

1) 95 °C 5 min
2) 95 °C 30 s
3) 50 °C 30 s
4) 72 °C 3 min
   30 repeats of the steps bloc from 2) to 4)
5) 72 °C 10 min
6) 10 °C infinite

-Nanodrop of PCR genes purification of 3.09.16

| Genes | Concentration (ng/µL) | Ratio 260/280 |
|---|---|---|
| LDH_V2 | 138.2 | 1.88 |
| PCT_V2 | 115.6 | 1.92 |
| PHAC_V3 | 125.4 | 1.91 |
| PHAC_V4 | 134.7 | 1.91 |

**8 October 2016**

➔ Problem with several PCR reaction tubes → we do them again

Colony PCR on Gibson transformation

- 12.5 µL DreamTaq Green PCR Master Mix 2X (Thermo Scientific, #K1081)
- 1.25 µL 10 µM Forward Primer (iG001)
- 1.25 µL 10 µM Reverse Primer (iG002)
- 10 µL Nuclease-free water (qs 25 µL)

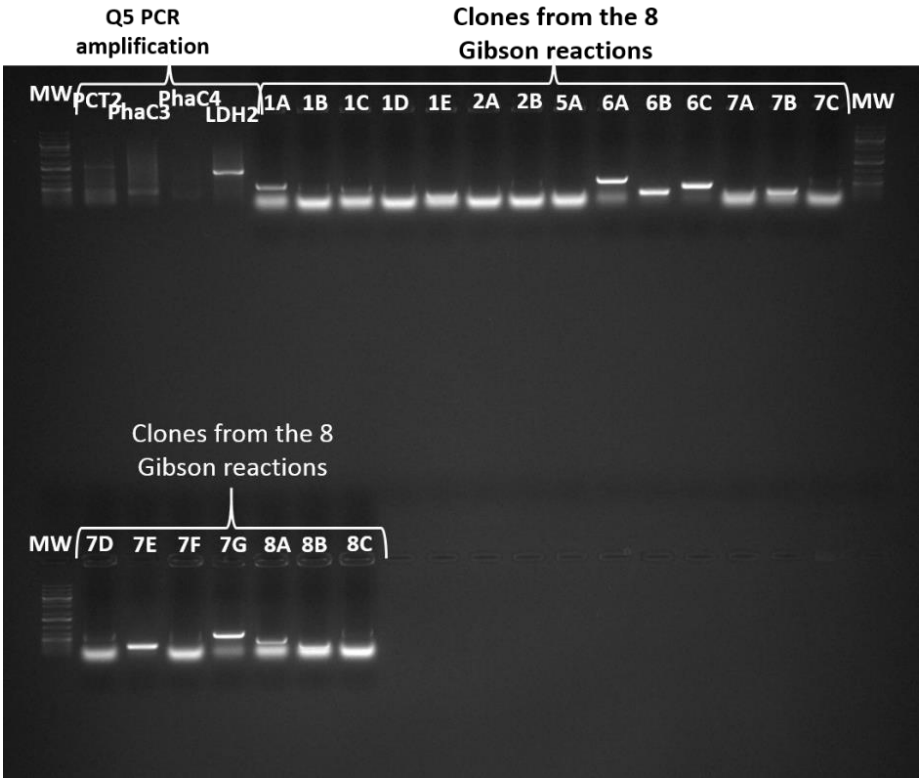
+ clone

Mix for 9 reactions

*PCR program:*

1) 95 °C 5 min
2) 95 °C 30 s
3) 50 °C 30 s
4) 72 °C 3 min
   30 repeats of the steps bloc from 2) to 4)
5) 72 °C 10 min
6) 10 °C infinite

Agarose gel 1 %




Digestion E+P

Mix: Vf= 100 µL

| | |
|---|---|
| DNA | 50 µL |
| Buffer 2.1 | 10 µL |
| EcoR1 | 1.5 µL |
| Pst1 | 1.5 µL |
| H2O | 37 µL |

PCR clean-up

- Nanodrop

| 7 October | | |
|---|---|---|
| Genes | Concentration (ng/µL) | Ratio 260/280 |
| LDH_V2 | 37.7 | 2.04 |
| PCT_V2 | 29.8 | 2.07 |
| Phac_V3 | 35.7 | 2.04 |
| Phac_V4 | 40.3 | 2.05 |

| 30 September | | |
|---|---|---|
| Genes | Concentration (ng/µL) | Ratio 260/280 |
| LDH_V2 | 8.8 | 2.49 |
| PCT_V2 | 7.7 | 2.32 |
| Phac_V3 | 11.0 | 2.68 |
| Phac_V4 | 8.1 | 2.46 |
| 21 September | | |
| Genes | Concentration (ng/µL) | Ratio 260/280 |
| LDH_V2 | 7.9 | 2.54 |
| PCT_V2 | 7.8 | 2.59 |
| Phac_V3 | 7.0 | 2.35 |
| Phac_V4 | 7.3 | 2.31 |

| 3 September | | |
|---|---|---|
| Genes | Concentration (ng/µL) | Ratio 260/280 |
| LDH_V2 | 16.3 | 2.20 |
| PCT_V2 | 7.1 | 2.79 |
| Phac_V3 | 8.2 | 2.30 |
| Phac_V4 | 9.0 | 2.52 |

**9 October 2016**

Pouring of 46 LBC (10 µg/mL chloramphenicol) plates + 5 LBK (50 µg/mL kanamycin) plates + 2 LBS (50 µg/mL spectinomycin) plates

**10 October 2016**

Concentration measurements:

LDH 7.10: 37.7 ng/µL
PCT 7.10: 29.8 ng/µL
PhaC3 7.10: 35.7 ng/µL
PhaC4 7.10: 40.3 ng/µL

LDH 3.09: 16.3 ng/µL
PCT 3.09: 7.1 ng/µL
PhaC3 3.09: 8.2 ng/µL
PhaC4 3.09: 9.0 ng/µL

LDH 21.09: 7.9 ng/µL
PCT 21.09: 7.8 ng/µL
PhaC3 21.09: 7.0 ng/µL
PhaC4 21.09: 7.3 ng/µL

LDH 30.08: 8.8 ng/µL

PCT 30.08: 7.7 ng/µL
PhaC3 30.08: 11.0 ng/µL
PhaC4 30.08: 8.1 ng/µL

**11 October 2016**

- Preparation 1L water+ 0.1 % HCOOH
- Test of solubility -> 10 µL of standard in 90 µL Solvent

  - Anthocyanin

➔ Isobutanol = not soluble
➔ DMSO = not soluble
➔ Ethyl Acetate = not soluble
➔ $CH_3CN$ 60 % = not soluble
➔ $CH_3CN$ 100 % = not soluble
➔ ETOH = not soluble
➔ HCL = ~soluble
➔ Methanol + Water + HCL = ~soluble
➔ Methanol+ HCL= ~soluble

  - Quercetin

➔ EtOH = soluble

  - Maldivin

➔ Soluble in Methanol HCL

Bettencourt Collaboration = we decided to do spectrometry masse instead of HPLC because it is more fast in time and the quercetin will be passed in negative mode but the maldivin and anthocyanin will be passed in negative mode.

**12 October 2016**

- Solution Stock

➔ At 2 mg/mL in EtOH 100 %

2 mg -> 1 mL EtOH

12.3 mg -> 6.15 mL EtOH

- Standard range in H2O mQ

- Quercetin

Isotopic mass = 302.042664 Da

Formula = $C_{15}H_{10}O_7$

Solution Stock: [2000 mg -> 1000 mL]

- [20 mg/1000 mL]

➔ 6 µL in 594 µL $H_2O$ mQ -> Solution 1

- [10 mg/L]

➔ 300 µL of 1 + 300 µL $H_2O$ -> Solution 2

- [5 mg/L]

➔ 300 µL of 2 + 300 µL $H_2O$ -> Solution 3

- [1 mg/L]

➔ 100 µL of 3 + 400 µL $H_2O$ -> Solution 4

- [0.5 mg/L]

➔ 250 µL of 4 + 250 µL $H_2O$ -> Solution 5

- [0.1 mg/L]

➔ 50 µL of 5 + 200 µL $H_2O$ -> Solution 6

- Maldivin

Isotopic mass = 330.073955 Da

Formula = $C_{17}H_{15}O_7$

Solution Stock: 50 µL + 450 µL MeOH-HCl 2 M -> [100 mg/L]

- [20 mg/1000 mL]

➔ 100 µL in 400 µL $H_2O$ mQ or $CH_3CN$ 60 % -> Solution 1

- [10 mg/L]

➔ 250 µL of 1 + 250 µL $H_2O$ mQ or $CH_3CN$ 60 %-> Solution 2

- [5 mg/L]

➔ 250 µL of 2 + 250 µL $H_2O$ mQ or $CH_3CN$ 60 %-> Solution 3

- [1 mg/L]

➔ 100 µL of 3 + 400 µL $H_2O$ mQ or $CH_3CN$ 60 %-> -> Solution 4

- [0.5 mg/L]

➔ 250 µL of 4 + 250 µL $H_2O$ mQ or $CH_3CN$ 60 %-> -> Solution 5

- [0.1 mg/L]

➔ 50 µL of 5 + 200 µL $H_2O$ mQ or $CH_3CN$ 60 %-> -> Solution 6

- Plaque MS

➔ 75 µL of each dilution + 75 µL Solution C

Solution C = 95 % acetate + 5 % carbonate ammonium 10 mM

- Vial

➔ 200 µL of each dilution in insert into vial for test HPLC

**13th October 2016**

- Ligation 1:10 with different Vf= 25 µL or Vf= 20 µL

Length

- GB3 = 2038 bp
- IGHQ = 2037 bp

DNA Mix:

7 October 2016

| LDH + IGFQ (100 ng) | |
|---|---|
| Vector | 0.92 µL |
| H2O | 2.92 µL |
| LDH | 16.2 µL |

| LDH + GB3 (50 ng) | |
|---|---|
| Vector | 2.16 µL |
| H2O | 9.77 µL |
| LDH | 8.08 µL |

| PCT + IGHQ (75 ng) | |
|---|---|
| Vector | 0.69 µL |

| | |
|---|---|
| H2O | 0.78 µL |
| PCT | 20.09 µL |

| PCT + GB3(50 ng) | |
|---|---|
| Vector | 2.16 µL |
| H2O | 4.46 µL |
| PCT | 13.39 µL |

| PHAC3 + GB3 (75 ng) 1:5 | |
|---|---|
| Vector | 2.16 µL |
| H2O | 2.44 µL |
| PHAC3 | 25.9 µL |

| PHAC3 + IGHQ (75 ng) | |
|---|---|
| Vector | 0.69 µL |
| H2O | 1.45 µL |
| PHAC3 | 17.85 µL |

| PHAC3 + GB3(50 ng) | |
|---|---|
| Vector | 2.16 µL |
| H2O | 5.95 µL |
| PHAC3 | 11.90 µL |

| PHAC4 + IGHQ (75 ng) | |
|---|---|
| Vector | 0.92 µL |
| H2O | 2.05 µL |
| PHAC4 | 21.12 µL |

3rd September 2016

| LDH + GB3 (50 ng) 1:10 | |
|---|---|
| Vector | 2.16 µL |
| H2O | 0.89 µL |
| LDH | 18.68 µL |

21th September 2016

| PHAC4 + GB3(50 ng) | |
|---|---|
| Vector | 2.16 µL |
| H2O | 7.29 µL |
| PHAC4 | 10.56 µL |

30th September 2016

| PHAC3 + GB3 (75 ng) 1:5 | |
|---|---|
| Vector | 2.16 µL |
| H2O | 2.51 µL |
| PHAC3 | 30.33 µL |

- Preparation of standard range for MS

20 mg/L; 10 mg/L; 5 mg/L; 1 mg/L

- Preparation of samples
  75 µL of supernatant of each + 75 µL of solution C.

**14 October 2016**

- Transformation of the ligation product by heat shock
  3 µL of ligation product into DH5-alpha strain.
  Recovery 1 hour.
  spread on LB + chloramphenicol plates (25 µg/µL).

**15 October 2016**

<u>Digestion PhaC3_only and PCT2_only</u>

- 30 µL IDT gBlock gene
- 5 µL buffer NEB CutSmart 10X
- 1 µL EcoRI
- 1 µL SpeI
- 13 µL nuclease-free water (qs 50 µL)

Incubation 1 h 37 °C

DNA clean & concentrator-5 kit (Zymo Research, D4003S)
DNA binding buffer added with 5:1 ratio
1 min incubation after adding 20 µL of nuclease-free water before centrifugation for elution

<u>Ligation</u>

| | **Quick ligase** | **T4 DNA ligase** |
|---|---|---|
| **pSB1C3 digested by E+S (from clone B → 13.3 ng/µL)** | 6 µL | 6 µL |
| **gene digested by E+S** | 3 µL | 3 µL |

| | | |
|---|---|---|
| **Buffer** | 10 µL Quick ligase buffer 2X (NEB) | 2 µL T4 DNA ligase buffer 10X (NEB) |
| **ligase** | 1 µL Quick ligase (NEB) | 1 µL T4 DNA ligase |
| **H2O (nuclease-free water)** | - | 8 µL |

Transformation ligation mixes in pSB1C3

- Thaw 50 µL chemo-competent DH5-alpha *E. coli*
- Add 2 µL DNA (mix ligation)
- Incubation 20 min on ice
- Heat shock: 45 s at 42 °C
- Incubation 3 min on ice
- Add 450 µL LB medium
- Incubation 1 h at 37 °C with shaking
- Centrifugation 7000 rpm 3 min
- Throw away supernatant
- Resuspend cells with rest of supernatant (around 50 µL)
- Spread all the content on plates LBC (10 µg/mL chloramphenicol)
- Incubation overnight 37 °C

Transformation ligation mixes in pSEVA 224, 424 or 2311

- Thaw 50 µL chemo-competent DH5-alpha *E. coli*
- Add 2 µL DNA (mix ligation)
- Incubation 20 min on ice
- Heat shock: 45 s at 42 °C
- Incubation 3 min on ice
- Add 450 µL LB medium
- Incubation 1 h at 37 °C with shaking
- Centrifugation 7000 rpm 3 min
- Throw away supernatant
- Resuspend cells with rest of supernatant (around 50 µL)
- Spread all the content on plates LBK (50 µg/mL kanamycin) for pSEVA 224 or 2311 or LBS (50 µg/mL spectinomycin) for pSEVA 424
- Incubation overnight 37 °C

DNA PCR clean-up kit (NEB) for gBlock genes amplified by PCR

**16 October 2016**

➔ Our strain of DH5-alpha bacteria seems already resistant to spectinomycin → test by preculture in 3 mL LBS (50 µg/mL spectinomycin) liquid medium

Concentration of amplified gBlocks after PCR clean-up

PhaC4: 86.6 ng/µL
PhaC3: 120.6 ng/µL
LDH2: 202.5 ng/µL
PCT2: 154.6 ng/µL

Digestion by E+P of amplified genes

| | PhaC3 | PhaC4 | PCT2 | LDH2 |
|---|---|---|---|---|
| DNA genes | 7 µL | 7 µL | 5 µL | 4 µL |
| Buffer NEB CutSmart | 5 µL | 5 µL | 5 µL | 5 µL |
| EcoRI | 1 µL | 1 µL | 1 µL | 1 µL |
| PstI | 1 µL | 1 µL | 1 µL | 1 µL |
| H2O nuclease-free | 36 µL | 36 µL | 38 µL | 39 µL |

DNA clean & concentrator-5 kit (#D4003S Zymo Research)
Ratio 5:1 of DNA binding buffer
Incubation 1 min with 20 µL of nuclease-free water before centrifugation for elution

Precultures

2 mL LBC (25 µg/mL chloramphenicol) + clones from transformation of DH5-alpha *E. coli* with ligation mixes of pSB1C3 with PhaC3_only or PCT2_only

PCT_only: 9 clones from ligation with T4 DNA ligase and 5 clones from ligation with Quick ligase

PhaC3_only: 1 clone from Quick ligase and 2 clones from T4 DNA ligase

Incubation of the 17 precultures overnight at 37 °C with shaking.

**17 October 2016**

Miniprep

GenElute Plasmid Miniprep Kit (Sigma, PLN350-1KT)

3 clones for PhaC3 and 14 clones for PCT2 (see above)

Testing methods to detect the interest compound in HPLC

Samples run

- Preparation of samples
  - Vortex and samples centrifugation, 2 min, max speed.
  -Take 120 µL of supernatant of each and put them in each 1.5 tube.
  -Add 120 µL acetonitrile 100 %.
  -Centrifugation, 1 min, max speed.
  -Load 200 µL in the vials for HPLC.

**18 October 2016**

Colony PCR on the last trials of insertion of PhaC3, PhaC4, PCT2, LDH2, PhaC3_only and PCT2_only inside pSB1C3 and of insertion of PhaC4, PCT2 and LDH2 inside pSEVA224 and pSEVA2311 in process.

PCR Colonies:

20 µL mix per sample with:
- 10 µL Green Taq Master Mix 2X (#K1081 Thermo Scientific)
- 0.25 µL Forward primer iG001 (10 µM)
- 0.25 µL Forward primer iG002 (10 µM)
- 9.5 µL H2O (qs 20 µL)

+clone

*PCR program:*

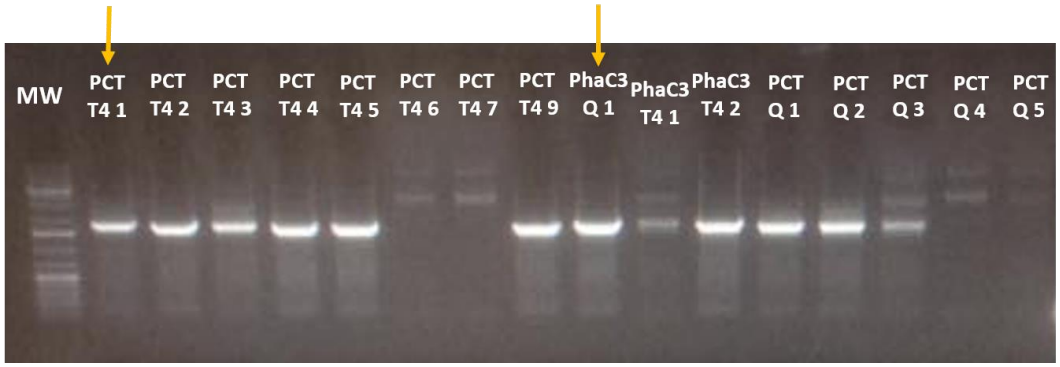
1) 95 °C 5 min
2) 95 °C 30 s
3) 50 °C 30 s
4) 72 °C 2 min
   30 repeats of the steps bloc from 2) to 4)
5) 72 °C 10 min
6) 10 °C infinite

HPLC Analysis (see Bettancout collaboration)

Sequencings of promoters isolated from 224 and 2311 plasmids and born by pSB1C3 came back positive.

**20 October 2016**

Gel electrophoresis with mixture from PCR on colonies obtained from PhaC3_only and PCT2_only ligations in pSB1C3.




*the two samples chosen are indicated by an arrow

Results for the sequencing of the promoters from pSEVA224 and pSEVA2311 came positives.

**21 October 2016**

Samples (promoters from pSEVA224 and pSEVA2311 and genes PhaC3_only and PCT2_only) sent to iGEM registry and to sequencing for PhaC3_only and PCT2_only.

**24 October 2016**

Sequencing results for PhaC3_oly and PCT2_only came positives.