Lab Notebook - Week 10 (8/14/2017-8/20/2017)

Project: NU iGEM 2017 Shared Project
Authors: Lulu
Dates: 2017-08-14 to 2017-08-26

MONDAY, 8/14/17

PelB-saCas9-His6

Purpose: Repeat Jack's experiment with gradient temperature, add a PelB SS by PCR and religate the linearized plasmid
Primers: P59, P60
Plasmids: pC60, (all at conc. 5 ng/uL)

Primers - PelB Additi…

	A	B	C
1	**Primer Name**	**Sequence**	**Melting Temperature**
2	P59 (FW)	TGCTGCTCCTCGCTGCCCAGCCGGCGATGGCCaagagaaattacattctgggga	49.0, 74.2°C
3	P60 (REV)	GACCAGCAGCAGCGGTCGGCAGCAGGTATTTCATttgtcccctctttcgtg	52.9, 72.0°C

PCR reaction
Materials for each PCR:

- ddH20: adjust volume to 50 uL (18 uL)
- 2.5 uL 10 uM P59 (Phosphorylated)
- 2.5 uL 10 uM P60 (Phosphorylated)
- add 10 ng miniprepped plasmid DNA (2 uL each)
- 25 uL of Phusion HS Flex 2x MM
- PCR Tube

Procedure:

1. Add NF Water first
2. Add P59, P60, plasmid in any order * Vortex briefly after all added
3. Add MM
4. spin quickly to collect in bottom of tube

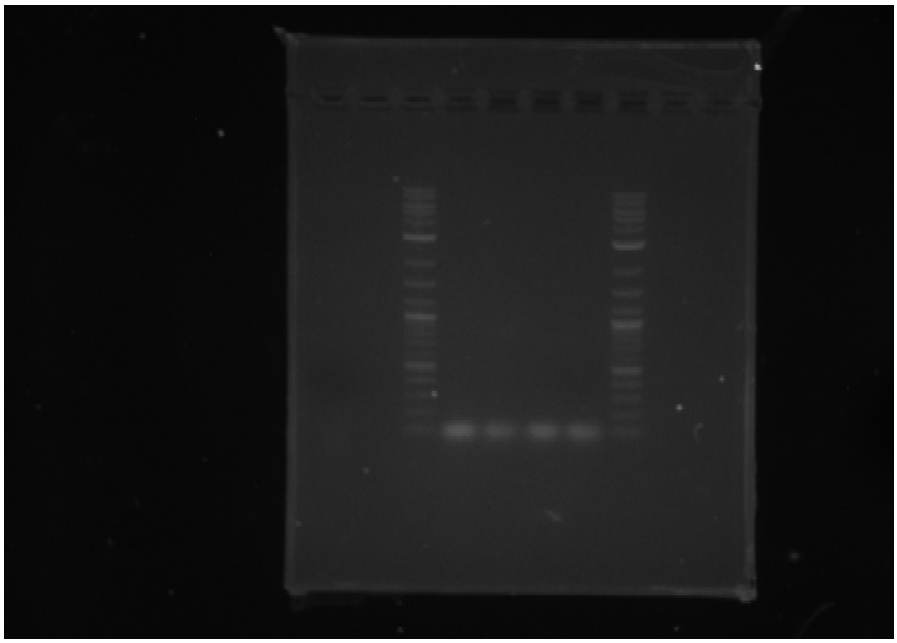
Gel electrophoresis (for identification of the correct linearized fragment)

- Load 4uL of Ladder into first well
- Load 3uL of PCR product and 1uL of dye onto each well (parafilm method)
- Expected band at **5.5kb**
- Run for ~60 mins

iPCR: pC60 (DsbA-saCas9-His6) [Gradient]
98C - 30 seconds (5447)
10 Cycles:
98C - 15s
50.8C / 53.2C / 56.5C / 58.0C- 30s
72C - 2.75 minutes
20 Cycles:
98C - 15s
69.6C - 30s
72C - 2.75 minutes
72C - 5 minutes

4C - inf.

PelB gradient.png



TorA backbone PCR (take 1)

Purpose: Re-do TorA backbone PCR with lower primer concentrations
Primers: P61, P62 (100 uM)
Plasmids: pC60 (10 ng/uL)

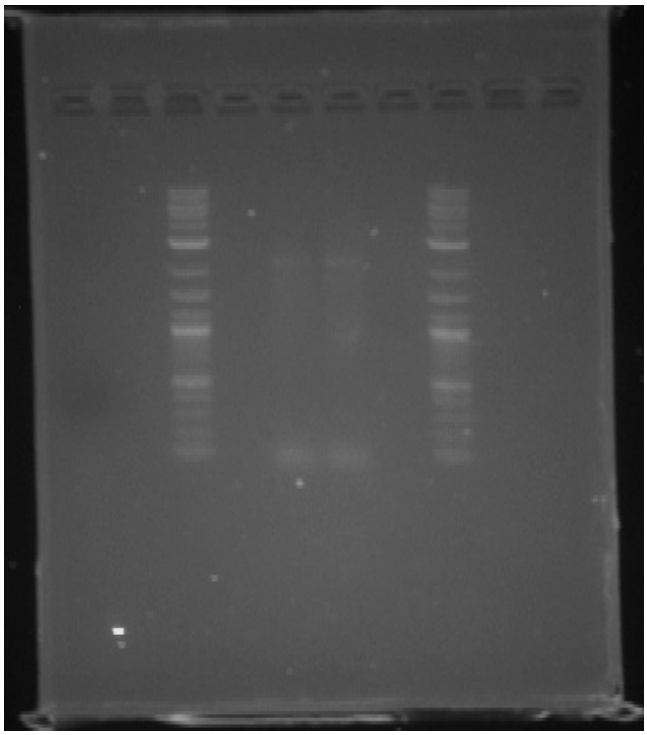
Materials:

Table3

	A	B
1	**nf water**	18 uL
2	**P61 (100 uM)**	2.5 uL
3	**P62 (100uM)**	2.5 uL
4	**pC60 (10 ng/uL)**	2 uL
5	**Phusion HS MM**	25 uL

Gel:
1 hour @ 96-97 V
4 uL per well (including dye; 3 uL of PCR, 1 uL of dye)

TorA backbone 1kb ladder



TUESDAY, 8/15/17

PCR TorA insert

PCR for TorA:

Table1

	A	B
1	**nf water**	18 uL
2	**P63 (100uM)**	2.5 uL
3	**P62 (100 uM)**	2.5 uL
4	**G-block (10 ng/uL)**	2 uL
5	**Phusion HS MM**	25 uL

PCR protocol: TorA insert

98C - 30 seconds

10 Cycles:

98C - 15s

63.6C - 30s

72C - 15s

20 Cycles:

98C - 15s

71.2C - 30s 71

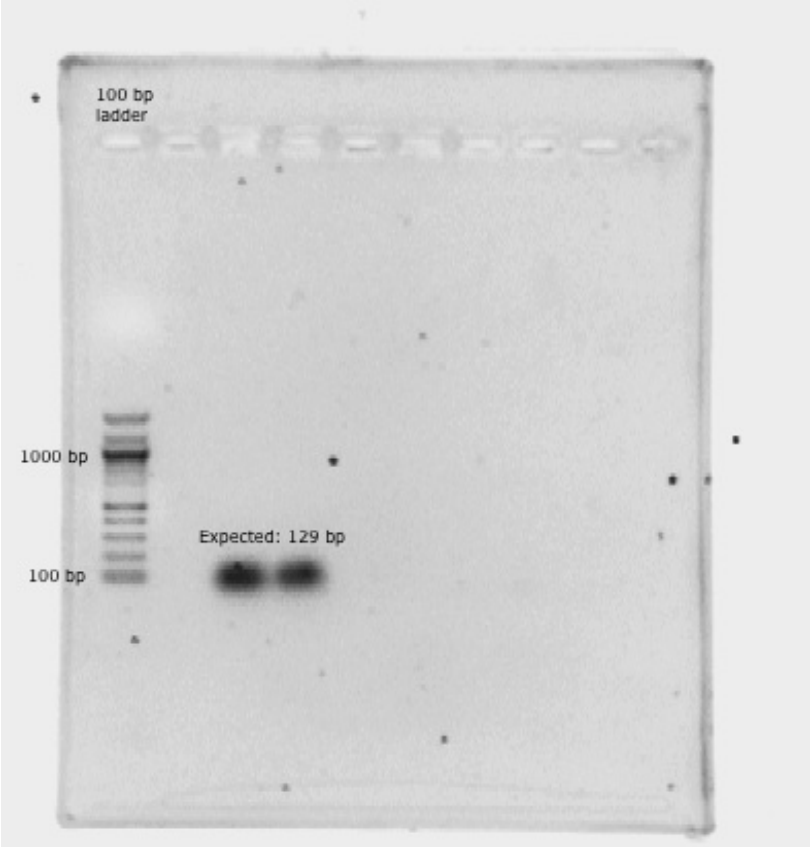
72C - 15s

72C - 5 minutes

4C - inf.

Gel:
1 hour @ 96-97 V
4 uL per well (including dye; 3 uL of PCR, 1 uL of dye)

TorA Insert PCR 100bp ladder.png



Jack's His6 protocol to re-run:

PCR to add His6

Purpose: Add a His tag by PCR and religate the linearized plasmid
Primers: P4 and P5 (non-phosphorylated)
Plasmids: pC53 (at conc. 1 ng/uL)

Primers - His6 additi…

	A	B	C
1	**Primer Name**	**Sequence**	**Melting Temperature**
2	P4 (FW)	TCATCATCATgtactagtagcggccgct	56.6, 62.71°C
3	P5 (REV)	TGGTGGTGCATtcagccctttttaataatctgcg	53.5, 64.31°C

PCR reaction

Materials for each PCR:

- ddH20: adjust volume to 50 uL (17.5 uL)
- 2.5 uL 10 uM P4 (Phosphorylated)

- 2.5 uL 10 uM P5 (Phosphorylated)
- 1.5 uL DMSO
- add 1 ng miniprepped DNA (1uL)
- 25 uL of Phusion HS Flex 2x MM
- PCR Tube

Procedure:

1. Add NF Water first
2. Add P4, P5, DMSO, pC34 in any order * Vortex briefly after all added
3. Add MM (Vortex briefly and spin down for a second or two to get material out of lid)
4. Vortex final reaction briefly and spin quickly to collect in bottom of tube
5. Place in Thermocycler at the following conditions:
    - 98 C for 30s
    - 98 C for 15s (Repeat Red 10x)
    - 57 C for 30s
    - 72 C for 2.5 minutes
    - 98 for 15s (Repeat Green 20x)
    - 65 for 30s
    - 72 for 30s
    - 72 C for 5 min
    - 4 C for inf. Time

TorA Backbone PCR

Purpose: Re-do TorA backbone PCR with lower primer concentrations
Primers: P61, P62
Plasmids: pC60 (10 ng/uL)

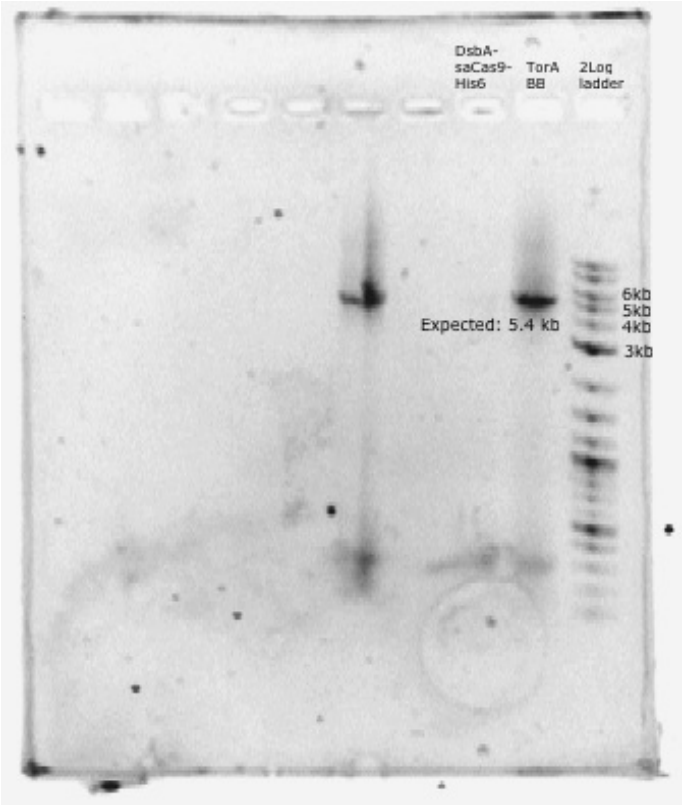
Materials:

Table2

	A	B
1	**nf water**	16.5 uL
2	**P61 (10 uM)**	2.5 uL
3	**P62 (10uM)**	2.5 uL
4	**pC60 (10 ng/uL)**	2 uL
5	**Phusion HS MM**	25 uL
6	**DMSO**	1.5 uL

Gel:
1 hour @ 96-97 V
4 uL per well (including dye; 3 uL of PCR, 1 uL of dye)

TorA backbone PCR with Jack take2.png



2-log ladder
Lane with band: TorA backbone
3rd lane from left: His6

WEDNESDAY, 8/16/17

PCR: TorA Insert (take 2)

Purpose: Repeat TorA insert PCR with lower concentration of primers
Primers: P63, P64 (10 uM)
DNA: SS G-block (10 ng/uL)

Table4

	A	B
1	**nf water**	20.5 uL
2	**P63 (10uM)**	1 uL
3	**P62 (10 uM)**	1 uL
4	**G-block (10 ng/uL)**	1 uL
5	**Phusion HS MM**	25 uL
6	**DMSO**	1.5 uL

PCR protocol:
98C - 30 seconds
10 Cycles:

98C - 15s
63.6C - 30s
72C - 15s
20 Cycles:
98C - 15s
71.2C - 30s 71
72C - 15s
72C - 5 minutes
4C - inf.

Gel:
1 hour @ 96-97 V
4 uL per well (including dye; 3 uL of PCR, 1 uL of dye)

PCR: YcbK Insert

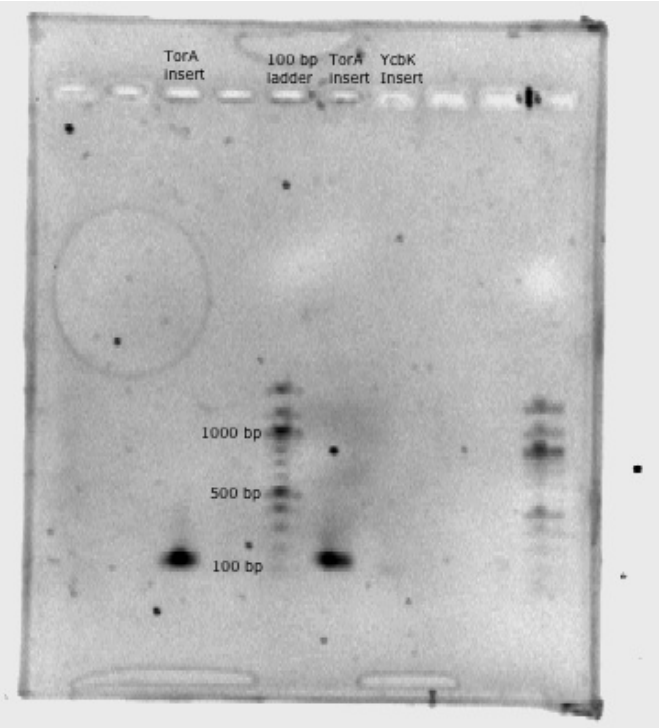
Purpose: Replace DSBA with YcbK
Primers: P75, P76 (10 uM)
DNA: pC60 (10 ng/uL)

Table5

	A	B
1	**nf water**	22
2	**P63 (10uM)**	1 uL
3	**P62 (10 uM)**	1 uL
4	**G-block (10 ng/uL)**	1 uL
5	**Phusion HS MM**	25 uL

PCR protocol:
98C - 30 seconds
10 Cycles:
98C - 15s
54.9C - 30s
72C - 15s
20 Cycles:
98C - 15s
68.4C - 30s
72C - 15s
72C - 5 minutes
4C - inf.

TorA insert and YcbK insert PCR.png



100 bp ladder
Expected: TorA 129, YcbK 93

Continue with Gibson for TorA

Digestion with DpnI: Backbone and TorA

- Add 1uL of Dpn1 to each vial, incubate for 1 hour at 37C

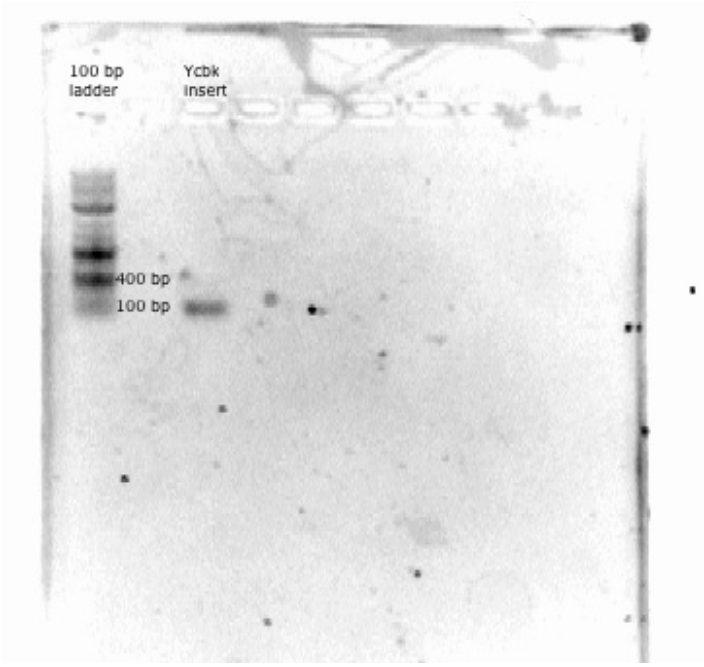
PCR wash: Backbone, TorA

- Add 250uL of PB and mix well with each PCR reaction
- Pipette everything into a column from the miniprep kit and spin for 1 minute
    - Dump out PB into miniprep waste
- Wash with CWC (500 uL) and spin for 1 minute
    - Dump out CWC into miniprep waste
- Spin dry column for 1 minute
    - Dump out any elution
- Add 30uL of nf water into sterile 2mL tube, spin for 1 minute to elute

Gel again for YcbK:

30 minutes @ ~99 V
4 uL of 100 bp ladder
Add 1 uL of dye to 3 uL of DNA
Mix on parafilm

YcbK PCR take 2.png



100 bp ladder with YcbK band at around ~100 (expecting ~93, close enough)

iPCR: PelB

Purpose: iPCR for PelB
Primers: P59, P60 (10 uM)
Plasmid: pC60

Table6

	A	B
1	**nf water**	21 uL
2	**P59 (10uM)**	1 uL
3	**P60 (10 uM)**	1 uL
4	**pC60 (5 ng/uL)**	2 uL
5	**Phusion HS MM**	25 uL

PCR protocol:
98C - 30 seconds
10 Cycles:
98C - 15s
59C/57.1C/53C/49.9C - 30s
72C - 2:45
20 Cycles:
98C - 15s
72C - 30s
72C - 2:45
--
72C - 5 minutes

4C - inf.

THURSDAY, 8/17/17

Gel again for YcbK and PelB gradient PCR:

1 hour @ ~105V

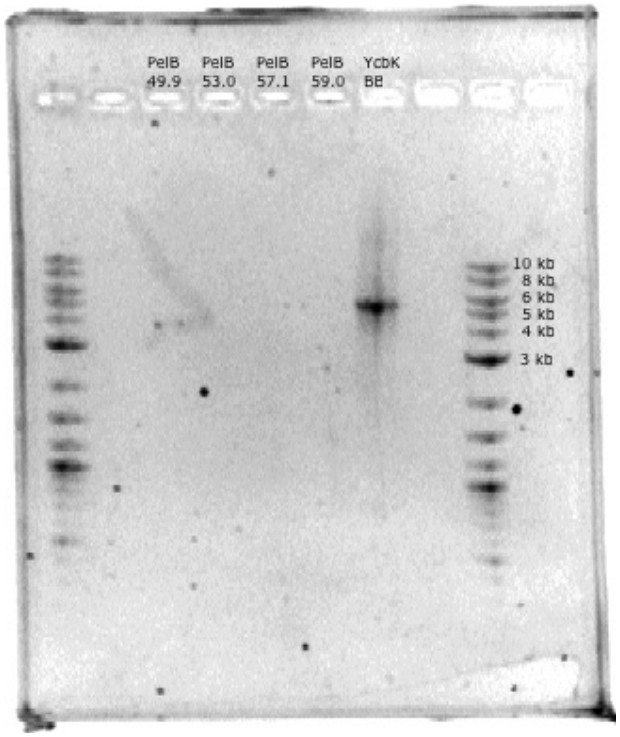
4 uL of 100 bp ladder

Add 1 uL of dye to 3 uL of DNA

Mix on parafilm

Each PelB is marked by gradient temp (°C)

PelB grad and YcbK bb PCR.png



Gel successful for YcbK, another failure with PelB

PCR for NapA insert

Purpose: PCR NapA insert

Primers: P67, P68 (10 uM)

Plasmid: G-block (10 ng/uL)

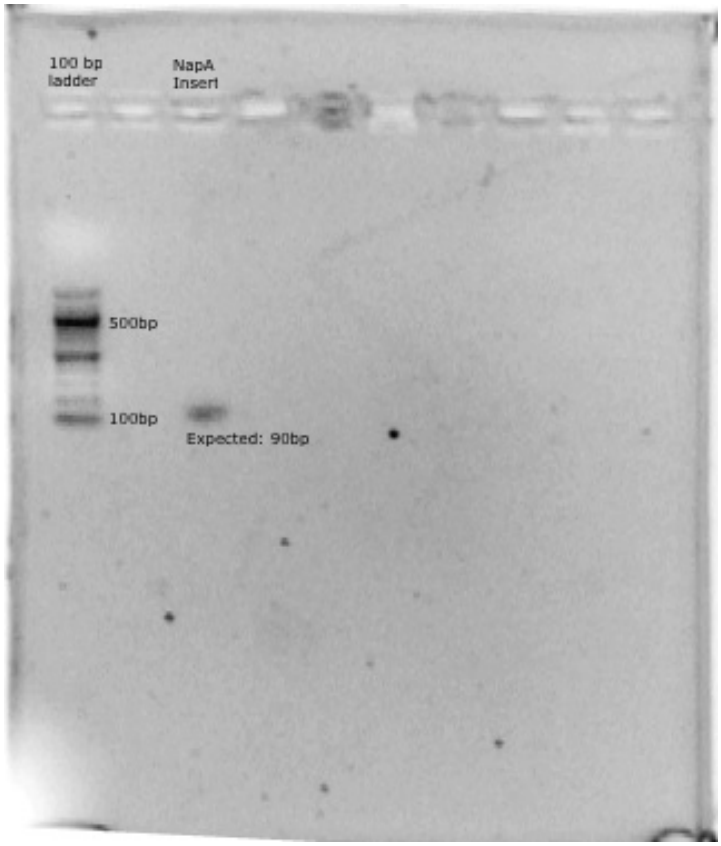
Table7

	A	B
1	**nf water**	22 uL
2	**P67 (10uM)**	1 uL
3	**P68 (10uM)**	1 uL
4	**G-block (10 ng/uL)**	1 uL
5	**Phusion HS MM**	25 uL

PCR protocol (NapA insert):
98C - 30 seconds
10 Cycles:
98C - 15s
60C - 30s
72C - 15s
20 Cycles:
98C - 15s
69.6C - 30s
72C - 15s
--
72C - 5 minutes
4C - inf.

Gel for NapA insert PCR:
1 hour @ ~105V
4 uL of 100 bp ladder
Add 1 uL of dye to 3 uL of DNA
Mix on parafilm

NapA insert PCR.png



Gibson for TorA

Gibson Concentration TorA

	A	B	C	D	E
1		ng/uL	bp length	nmol/uL	nM
2	TorA BB	138	5391	4.21e-5	42.14
3	TorA Insert	54.1	129	6.89e-4	689.1

Gibson reaction mix TorA

	A	B	C
1		**TorA Assembly**	**Negative control**
2	pC60	1.19 uL	1.19 uL
3	TorA	0.22 uL	0.22 uL
4	nf water	8.60 uL	18.60 uL
5	Gibson MM	10 uL	----

Transformation?

Gibson for YcbK

Gibson Concentration YcbK

	A	B	C	D	E
1		**ng/uL**	**bp length**	**nmol/uL**	**nM**
2	YcbK BB	60.1	5391	1.84e-5	18.35
3	YcbK insert	14.6	93	2.57e-4	257.7

Dilute YcbK insert into 20nM:
(29 uL)(257.7 nM) = (x uL)(20 nM)
x = 373.72 uL
373.72 - 29 = **344.72 uL** water added to YcbK insert

Gibson reaction mix YcbK

	A	B	C
1		**YcbK Assembly**	**Negative control**
2	YcbK BB (18 nM)	1 uL	1 uL
3	YcbK insert (20 nM)	3 uL	3 uL
4	nf water	6 uL	16 uL
5	Gibson MM	10 uL	---

Transformation YcbK Gibson
Cells: Top10
Protocol: Gibson product transformation (see attached protocol)
Plated onto Chr plates

PCR for NapA backbone

Purpose: PCR NapA backbone
Primers: P65, P66 10 uM)
Plasmid: pC60 (5 ng/uL)

Table8

	A	B
1	**nf water**	21 uL
2	**P65 (10uM)**	1 uL
3	**P66 (10uM)**	1 uL
4	**pC60 (5 ng/uL)**	2 uL
5	**Phusion HS MM**	25 uL

PCR protocol (NapA insert):
98C - 30 seconds

10 Cycles:
98C - 15s
55C - 30s
72C - 2:45
20 Cycles:
98C - 15s
69.6C - 30s
72C - 15s
--
72C - 5 minutes
4C - inf.

FRIDAY, 8/18/17

Transformation with Gibson Product

Materials:
50uL comp cells for each experiment /Gibson mix
15uL of Gibson assembly DNA
65uL of SOC media

1. Place 50uL comp cells in a microcentrifuge tube (or use original vial) -- keep on ice.
2. Pipette 25uL of Gobson DNA into each tube.
3. Incubate on ice for 20 min.
4. Heat shock tubes in water bath at 42°C for 60s
5. Add 65uL of SOC media, rescue for 1 hr & 15 min at 37°C in shaker.
6. Plate 80uL on Cm plate

PCR YcdB backbone:

Primers: P69, P70
DNA: pC60 (5ng/uL)

-resuspend primers to 100uM
-dilute from 100uM to 10uM
    -2.5uL of P69 and P70
    -22.5uL of nf water (each)

components:
-nf water: 21 uL
-P70: 1uL (10uM)
-P71: 1uL (10uM)
-pC60: 2uL (5ng/uL)
-Phusion HS MM: 25uL
TOTAL: 50uL

YcdB backbone PCR settings:

98°C - 30s
10 cycles:
    98°C - 15s
    55°C - 30s
    72°C - 2:45
25 cycles:
    98°C - 15s
    68.2°C - 30s
    72°C - 2:45
72°C - 5min

4°C - inf

PCR YcdB insert:

Primers: P71, P72

DNA: G-block (10 ng/uL)

resuspend primers to 100uM

dilute from 100uM to 10uM

- 2.5uL of P71, P72
- 22.5uL of nf water each

components:

- nf water: 22uL
- P71: 1uL (10uM)
- P72: 1uL (10uM)
- G-block: 1uL (10 ng/uL)
- Phusion HS MM: 25uL

TOTAL: 50uL

PCR settings:

98°C - 30s

10 cycles:

- 98°C - 15s
- 62.6°C - 30s
- 72°C - 15s

25 cycles

- 98°C - 15s
- 70.8°C - 30s
- 72°C - 15s

72°C - 5 min

4°C - inf.

Overnights:

Put in @ 4:35pm

- 3 TorA DH5alpha
- 3 YcbK Top 10

SATURDAY, 8/26/17

Miniprep of 3 TorA (pC70-72) and 3 YcbK (pC73-75)

- using updated Promega protocol and kit.

Nanodrop Results

Table9

	Plasmid	Concentration	260/280	260/230
1	pC70	323.2 ng/uL	1.91	2.47
2	pC71	324.7 ng/uL	1.89	2.25
3	pC72	356.7 ng/uL	1.90	2.45
4	pC73	383.0 ng/uL	1.88	2.32
5	pC74	282.3 ng/uL	1.81	1.60
6	pC75	238.4 ng/uL	1.88	2.20

Transformation Protocol (Chelsea's)

Introduction

Transformation protocol as recommended by Chelsea Hu.

Materials

- Competent cells
- DNA
- 2 mL microcentrifuge tube
- Agar plates (with right antibiotic resistance if needed)
- Ice (in bucket)

Procedure

1. Thaw comp cells (more than 50 uL) on ice
2. 10 uL of competent cells in each tube (20 uL for JC8031)
3. Remove agar plates (containing the appropriate antibiotic) from storage at 4°C and let warm up to room temperature
4. .5 uL of 1 pg-10 ng of each plasmid DNA in tubes, do not disturb in anyway (not even flick)
    a. Dilute stocks of 50 ng to 10 ng
5. Sit on ice for 20 mins
6. Heat shock at 42 C for 60 secs
7. Chill on ice for 5 mins
8. Add 10 uL (20 uL if using JC8031) of SOC (no antibiotics) and rescue for an hour at 37 C in shaker
9. Plate on appropriate antibiotic -agar plates

Transformation with Gibson Product

Introduction

Transformation into competent cells after Gibson assembly
Protocol given by Chelsea

Materials

- 50 uL competent cells per Gibson assembly DNA
- 15 uL of GIbson assembly DNA
- 65 uL SOC media
- Agar plates

Procedure

Transformation protocol

1. Place 50 uL of competent cells into a microcentrifuge tube

   (Or use original vial, if it already has 50 uL)
   Keep on ice
2. Pipette 15 uL of Gibson assembly DNA into each tube
3. Incubate on ice for 20 minutes
4. Heat shock tubes in water bath at 42°C for 60s
5. Incubate on ice for 5 minutes
6. Add 65 uL of SOC media, rescue for 1 hour & 15 minutes at 37°C in shaker
7. Plate 80 uL onto plate with correct antibiotics