Lab Notebook - Week 14 (9/11/2017-9/28/2017)

Project: NU iGEM 2017 Shared Project
Authors: Katerina Cheronis
Dates: 2017-09-11 to 2017-09-17

MONDAY, 9/11/17

things are going pBAD today

Sequencing Sent:

Table1

	A	B	C
1	Name	Plasmid	Primer
2	iPY1	pC86	P2
3	iPY2	pC87	P2
4	iPY3	pC88	P2

Nanodrop results
PK90: pet28a
37.8 ng/uL
260/280: 1.91
260/230: 1.99

PC91: pBAD
9.9 ng/uL or 40 ng/uL
260/280: 1.90
260/230: 1.48

TUESDAY, 9/12/17

NEW DAY GOOD DAY PRODUCTIVE DAY

cjCas9 gBlock into a random vector

Selected random vector: YcdB_saCas9_His6 (pSB1C3)

Phosphorylation:

- Treat the DNA with T4 polynucleotide kinase (PNK) to phosphorylate the 5’-ends
    - 10x T4 DNA ligase buffer (1 µL)
    - linearized DNA (2 µL)
    - T4 PNK (1 µL)
    - ddH2O (6 µL)
- Incubate at 37 °C for 30 min, then cool to room temperature.

Single digest:

1. Add components to a clean tube in the order shown:
   1 µL DNA (concentration 1 µg/µL)

2 µL 10x buffer
1 µL restriction enzyme
16 µL sterile water

2. Incubate the reaction at digestion temperature (usually 37°C) for 1 hour.
3. Stop the digestion by heat inactivation (65°C for 15 minutes) or addition of 10mM final concentration EDTA.
4. The digested DNA is ready for use in research applications.

Ligation:

- Add the following to the reaction mix:
    - (Total Volume: 20 uL)
        - ddH2O: 8 µL
        - 10x T4 DNA ligase buffer: 1 µL
        - T4 DNA ligase: 1 µL
- Incubate at room temperature for 60 min.

Gel electrophoresis:

- Run gel for linearized plasmid and ligated product with a 2-log ladder
-

Double Restriction Digest - cjCas9 into pET28a Expression Vector

Plasmid goal:

pET28a_cjCas9

Restriction sites: BamHI and NotI

Digestion Setup

	A	B
1	Insert (cjCas9)	Vector (pET28a)
2	100 ng DNA (10 uL)	600 ng DNA (15 uL)
3	10x Buffer (3uL)	10x Buffer (3 uL)
4	NF Water (15 uL)	NF Water (15 uL)
5	BamHI (1 uL)	BamHI (1 uL)
6	NotI (1 uL)	NotI (1 uL)

- Mix above components into separate 1.5 mL microcentrifuge tubes
- Incubate at 37 C for 1 hour
- Heat inactivate at 65 C for 15 mins

WEDNESDAY, 9/13/17

ClyA-saCas9-His6 PCR

INSERT PCR:

Plasmid: pBAD (plasmid pC37 and pC38)

Primers

PCR Primers - Insert

	A	B	C
1	**Primer Name**	**Sequence**	**Melting Temperature**
2	P77 (FW)	gagcacgaaagaggggacaaTTAAAGAGGAGAAAGGTCATGA	51.7, 64.5
3	P78 (REV)	agaatgtaatttctcttcatGACTTCAGGTACCTCAAAGA	56.4, 65.9

PCR Materials:

- nf water 22 uL
- 1 uL 10uM P77
- 1 uL 10uM P78
- 1 uL plasmid (~1 ng/uL)
- 25 uL Phusion HS Flex 2xmm
- PCR tube

Total volume = 50 uL

PCR protocol (3 tubes used)
98 deg C for 30s
Repeat 10x:
98 deg C for 15s
54.7 deg C for 30s
72 deg C for 30s
Repeat 25x:
98 deg C for 15s
67.5 deg C for 30s
72 deg C for 30s
72 deg C for 5 minutes
4 deg C for inf

Gel of pC37 and pC38:
pC38 worked the best

BACKBONE PCR:
Plasmid: try pC59 and pC74 --> dilute to 1ng/uL
Primers

Table2

	A	B	C
1	**Primer Name**	**Sequence**	**Melting Temperature**
2	P79 (FW)	TCTTTGAGGTACCTGAAGTCatgaagagaaattacattctggga	50.6, 66.6
3	P80 (REV)	ATGACCTTTCTCCTCTTTAAttgtcccctctttcgtgctc	50.4, 61.7

PCR Materials:

- nf water 22 uL
- 1 uL 10uM P79
- 1 uL 10uM P80
- 1 uL plasmid (~1 ng/uL)

- 25 uL Phusion HS Flex 2xmm
- PCR tube

Total volume = 50 uL

PCR protocol (3 tubes used)
98 deg C for 30s
Repeat 10x:
98 deg C for 15s
53.4deg C for 30s
72 deg C for 2:00 mins
Repeat 25x:
98 deg C for 15s
64.7 deg C for 30s
72 deg C for 2:00 mins
72 deg C for 5 minutes
4 deg C for inf

Gibson Assembly:
DPNI Digest (1uL added) of backbone and insert at 37deg C for 1 hour
PCR wash: Backbone, Insert

- 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

Gibson concentrations

	A	B	C	D	E
1		**ng/uL**	**bp length**	**nmol/uL**	**nM**
2	Backbone	63.8	6103	6.37123e-5	63.71231483
3	Insert	36.0	3159	1.9743e-5	19.7429951

Gibson Volumes

	A	B	C
1		**Assembly**	**Negative control**
2	BB	1 uL	1 uL
3	Insert	1 uL	1 uL
4	nf water	8 uL	18 uL
5	Gibson MM	10 uL	---

Place Gibson mix into PCR machine, 15 minutes @ 50°C
Transformation using gibson protocol attached. Transformation successful.

THURSDAY, 9/14/17

Table3

	A	B	C	
1	**SS**	**Primers**	**Sequence**	
2	NapA (1 a,b)	REV	TGCCACACCGGGGACCGACAGA	With pC
3		FWD	aagagaaattacattctgggattagatataggcattactagcgttgggtacgga	
4	YcbK (2 a,b)	REV	AGCGAAGGCAGGAGTAGGCAGGAT	With pC (pSB1C
5		FWD	aagagaaattacattctgggattagatataggcattactagcgt	
6	YcdB (3 a,b)	REV	GGCGTGTGCCACGGGGCATGA	With pC
7		FWD	aagagaaattacattctgggattagatataggcattactagcgttgggtacggaata	

- Materials (Per RXN)
  - 1 uL 10 uM FW
  - 1 uL 10 uM REV
  - 1.5 uL DMSO
  - 1 uL plasmid DNA (1ng/uL)
  - 25 uL of Phusion HS Flex 2x MM
  - PCR Tube

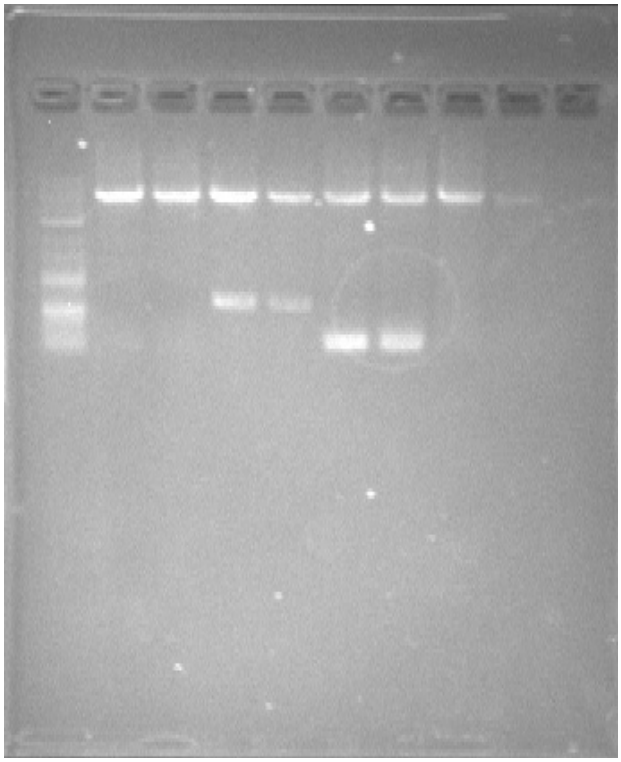
Thermocycler conditions:
98 C for 30s
98 C for 15s (Repeat Red 35x)
64 C for 30s
72 C for 2.2 minutes
72 C for 10 min
4 C for inf. Time

Gel results

Table4

	Lane number	Description
1	1	NapA a
2	2	NapA b
3	3	YcbK a
4	4	YcbK b
5	5	YcdB a
6	6	YcdB b
7	7	pBAD A
8	8	pBAD B

Gel results



Digestion with DpnI

- Add DpnI (1uL) to the purified PCR product and incubate for 1-4 hours (37 degC)
    - Incubated for 60 minutes

PCR wash

- Add 250uL of PB and mix well with each PCR reaction
    - Used the one in the Qiagen kit
- Pipette everything into a column and spin for 1 minute
- Wash with CWC (500 uL) and spin for 1 minute
- Elude using 30uL of ddH2O

Nanodrop results:

Nanodrop

	A	B	C	D
1		**Concentration**	**260/280**	**260/230**
2	**NapA**	88 ng/uL		
3	**YcbK - pSB1C3**	27 ng/uL		
4	**YcdB**	55 ng/uL		
5	**YcbK - pBAD18cm**	84ng/ul		

FRIDAY, 9/15/17

Ligation

Procedure:

1. Calculate the volume of the DpnI-treated PCR product containing 50-150 ng and fill in the table below.
2. Calculate the volume of water to make up to 10 µL.
3. Mix the components below:
4. Incubate for 120 min at room temperature (~22°C).

Reaction Volumes for Ligat…

	A	B	C	D	E
1	**Reagent**	**Nap A**	**YcbK - pSB1C3**	**YcdB**	**Ycbk - pBad18cm**
2	ddH2O	7 uL	6 uL	7 uL	7 uL
3	Purified PCR product (50 ng)	1 uL	2 uL	1 uL	1 uL
4	10x reaction buffer	1 uL	1 uL	1 uL	1 uL
5	T4 DNA ligase, 5U/uL	1 uL	1 uL	1 uL	1 uL
6	**Total:**	10 uL	10 uL	10 uL	10 uL

Perform Transformations:

Volumes for iPCR Transformations

	A	B	C
1	30 mL C-cells	5 uL iPCR ligated product	35 uL rescue media

Transformations performed and cells incubated at 37 deg C.

SUNDAY, 9/17/17

Overnight cultures for: ClyA-saCas9-His6, NapA-saCas9-His6, YcdB-saCas9-His6, YcBK-saCas9-His6 (pBad and pSB1C3) started (2 of each). Cm antibiotic used.

For TEM experiment:
3 cultures (5 mL) of Top10 cells and 3 cultures of JC8031 were started

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