Week of July 1

SUNDAY, 7/1/2018

strains info

Table1

	A	B	C	D	E	F	G	H
1	Strain	Type	Phylum	Application	Kan susceptible	Neo susceptible	pBBR1	RSF1010
2	Escherichia coli (MG1655, DH10B, Nissle 1917)	Gram-negative	Proteobacteria	Model gram-negative	yes	yes	Yes	Yes
3	Corynebacterium glutamicum	Gram-negative	Proteobacteria	Industrial production of amino acids	yes	yes	probably	probably
4	Shewanella oneidensis	Gram-negative	Proteobacteria	Bioelectronics	yes	yes	probably	probably
5	Pseudomonas putida	Gram-negative	Proteobacteria	Bioremediation	yes	yes	Yes	probably
6	Sinorhizobium meliloti	Gram-negative	Proteobacteria	Agriculture	yes	yes	Yes	probably
7	Vibrio natriegens	Gram-negative	Proteobacteria	Industrial production of various compounds	low level resistance; look for faster growing colonies	?	probably	probably
8	Bacillus subtilis	Gram-positive	Firmicutes	Model gram-positive	yes	yes	maybe	?
9	Lactococcus lactis	Gram-positive	Firmicutes	Therapeutics, cheese production	yes	?	?	?

MONDAY, 7/2/2018

People present: Soohyun, Stefanie, Katherine
Time: 11:30AM-1:45PM and 2:15-5:30PM (5.5 hours)
Location: ABL 126
Goals: PCR for Shewanella, purify 2 genomes, make primers for our bacterial strains
Anna's to-do list of the day

1. Do PCRs for ribosomal operon of Shewanella: make two reactionsCheck which primers to use
    a. Standard protocol (don't forget to check the size of the product and Tm)

b. Touchdown (don't forget to adjust times and temperatures if needed)

Done

1. Purify the rest of the genomes. Albert has liquid cultures for them (pick up the kit in Keck)

Done

1. Go to "Orthogonal translation" folder and open the file with MG1655 rrnB - see what primers it has to amplify the operon (iG013 and iG014). Design primers to amplify operons for all other bacteria (primers should bind to annotated genes on either side of the operon)

Done

Nanodrop dNTP

Goal: Find the concentrations of the DNA we have.

Materials

- NF H2O
- Aliquots:
  - Part 449
  - Part 423
  - Part 447
  - Part 639
  - Part 616
  - Part 479
  - Ribosomal E. coli
    - TD
    - 1
    - 2
    - 3

Procedure

- Used 1uL water to blank
- Nanodropped 1uL of: (in ABL 128)
  - Part 449: 99.7 ng/uL
  - Part 423: 45.3 ng/uL
  - Part 447: 62.8 ng/uL
  - Part 639: 74.9 ng/uL
  - Part 616: 22.2 ng/uL
  - Part 479: 65.6 ng/uL
  - Ribosomal E. coli TD: 14.8 ng/uL
  - Ribosomal E. coli 3: 5.9 ng/uL
  - Ribosomal E. coli 1: 8.1 ng/uL
  - Ribosomal E. coli 2: 9.2 ng/uL

Make more dNTP mix

Goal: Make more dNTPs for PCR reactions

Materials

- Bases: (keep on ice)
  - dTTP
  - dGTP
  - dCTP
  - dATP
- nfH2O

Procedure

Desired 1mL of 10mM dNTP mix

Used https://www.elabprotocols.com/protocols/#!protocol=179

Calculations to check

$c_1v_1=c_2v_2$

100mM * (v1) = 10mM * 1000uL

v_1 = 100uL

- Added 100uL of each base and 600uL to microcentrifuge tube
- Shook and tapped to mix
- Made 200uL aliquots
- Stored at -20C in ABL 126

Summary: The process was simple and didn't take much time. We didn't encounter any issues.

PCR

Goal: Perform PCR on Shewanella like Soohyun and Katherine did last week on E. coli. We want to create many copies of the Shewanella DNA, using the 2 different methods in order to get 2 different concentrations and see which program works best.

Materials

- 2 PCR tubes (standard and touchdown)
- Primers: iG011 and iG012
- Shewanella DNA
    - Length: 8196 bp
    - Anneal temp: 64C
    - Elongation time: 4 minutes 6 seconds

In PCR tubes, combined
20 uL Q5 buffer
40 ng DNA (0.3 uL)
5 uL of each primer
1 uL Q5 polymerase --> add last and keep in the rack when in use
2 uL dNTPs
up to 100 uL (nf) H2O (64 uL)

Procedure

- Added to tube:
    - 64.3uL H2O
    - 2.74uL Shewanella DNA
    - 5uL of forward primer and backward primer
    - 2uL dNTPs
    - 20uL Q5 buffer
    - 1uL Q5 polymerase
- Split the contents of the large tube amongst 2 smaller PCR tubes
- Placed in Thermal Cycler
    - Standard: iGEMPCR3 (ABL 126)

iGEMPCR3

	A	B	C
1	Lid Temp: 105		
2	**Step**	**Temperature (C)**	**Time**
3	1	98	0:30
4	2	98	0:10
5	3	64	0:30
6	4	72	4:06
7	5	Go to Step 2	Repeat 34x
8	6	72	2:00
9	7	12	Hold

- Touchdown: (Keck 201)

iGEMTD

	A	B	C
1	Lid Temp: 105C		
2	**Step**	**Temperature (C)**	**Time**
3	1	98	30
4	2	98	10
5	3	74	30
6	4	72	4:06
7	5	Go to Step 2	Repeat 14x
8	6	98	10
9	7	59	30
10	8	72	4:06
11	9	Go to Step 6	Repeat 30x
12	10	72	5:00
13	11	12	Hold

Summary: We had some issues determining what programs to use for the PCR. But aside from that, the process was fairly simple. Again, touchdown proved to be the optimal programs from high concentration results from PCR.

Purify Vibrio Natriegens and Corynebacterium Glutamicum Genomes

Goal: Similar to DNA miniprep, we want to extract the genome from the cells for us to use in later experiments.

Materials

- Heat block

- Water bath
- 1.5mL microcentrifuge tube
- Vibrio and Coryne. genomes
- Wizard SV Genomic DNA Purification from Bacteria Kit

Procedure

Followed Wizard SV Genomic DNA Purification from Bacteria protocol

- Pelleted 0.5mLof culture by centrifuging it at 14,000 rpm for 2 minutes
- Placed nfH2O on heat block set at 65C
- Began to warm water bath to 80C (in preparation for later step of protocol)
- Resuspended material pellet in 500uL Nuclei Lysis/RNase Solution
- Incubate in 80C water bath for 10 minutes
- Added ~500uL Wizard SV Lysis Buffer to tube and mixed with a pipette
- Labeled a minicolumn/collection tube for the 2 samples
- Transferred bacterial cell lysate to the minicolumn
- Centrifuged at 13,000rpm for 3 minutes
- Discarded collected fluid
- Added 650uL Wash Solution to minicolumn*
- Centrifuged at 13,000rpm for 1 minute*
- Discarded liquid*
- Repeated starred (*) steps 3x
- After last wash, discarded fluid and reassembled the minicolumn
- Centrifuged at 13,000rpm for 2 minutes
- Removed minicolumn and placed in a new microcentrifuge tube
- Added 200uL warmed nfH2O
- Incubated the samples for 2 minutes at room temperature
    - Note: Coryne. sample sat for 4 min
- Centrifuged the samples at 13,000rpm for 1 minute
- Removed minicolumn and capped microcentrifuge tube
- Stored at -20C

Summary: The procedure was straightforward, we simply followed the procedure in the kit.

Make Primers for Bacterial Strains on Benchling

Goal: Use benching to make primers for the set of DNA in each bacterial strain that contains the 3 codes of DNA we want to copy in order to express orthogonal ribosomes.

Procedure

- Looked for Gene X and Gene Y (unique genes) in each strain
- Used benchling to make a primer
    - Primer must be <70% GC content, ~ 65C annealing temperature, and contain part of the unique genes
    - NOTE: Made sure to enclose 16S rRNA, 23S rRNA, and 5S rRNA
    - NOTE: Overlapped the primer with part of the unique genes
        - Did this in order to make sure that the primers will only bind in specific places that we want, not multiple areas in the DNA (DNA contains many copies of the operon we want to clone)

Summary: We had difficulty understanding what to do at first, but we eventually figured out what we needed to do. Any sequence can be made into a primer.

OD Curves:

1. Took colony from each strain (from plates made on 6/18) and cultured in 2 mL base medium + 0.5% glucose (+ascorbate for C. glutamicum).
    a. in appropriate shaking incubator at 5:35 pm

TUESDAY, 7/3/2018

People present: Stefanie, Soohyun, Anna
Time: 11:15AM-1PM and 2-5:30 (5.25 hours)
Location: Keck 201, ABL 126
Goals: Gel electrophoresis of the Shewanella DNA

Gel Electrophoresis

Goal: Perform an electrophoresis of the genomes to make sure our PCRs worked correctly.

Materials

- Pre-prepped 0.8% agarose gel

Make Gel

Procedure

- Microwaved 0.8% agarose
- Added 5uL of SYBER Safe dye to 50mL aliquot of the gel
- Poured gel into caster
- Waited ~30 minutes

Prep Samples

- Located samples
- Added loading buffer to samples
    - NOTE: It's a 6x concentration loading buffer, we want a total of 1x concentration loading buffer
    - Calculations:
      c1v1=c2v2
      6x * v1 = 1x * 50uL
      8.3uL
- Added 8.3uL loading buffer to each sample

Make More 1x TAE

- NOTE: When we want to cut the gel we need clean TAE, if we just want to run the PCR then we don't need clean TAE
- Calculations:
  c1v1=c2v2
  50x * v1 = 1x * 1L
  v1 = .02L = 20mL
- Added 20mL of 50x TAE to a bottle
- Added 1L of diH2O to the bottle
- Shook to mix

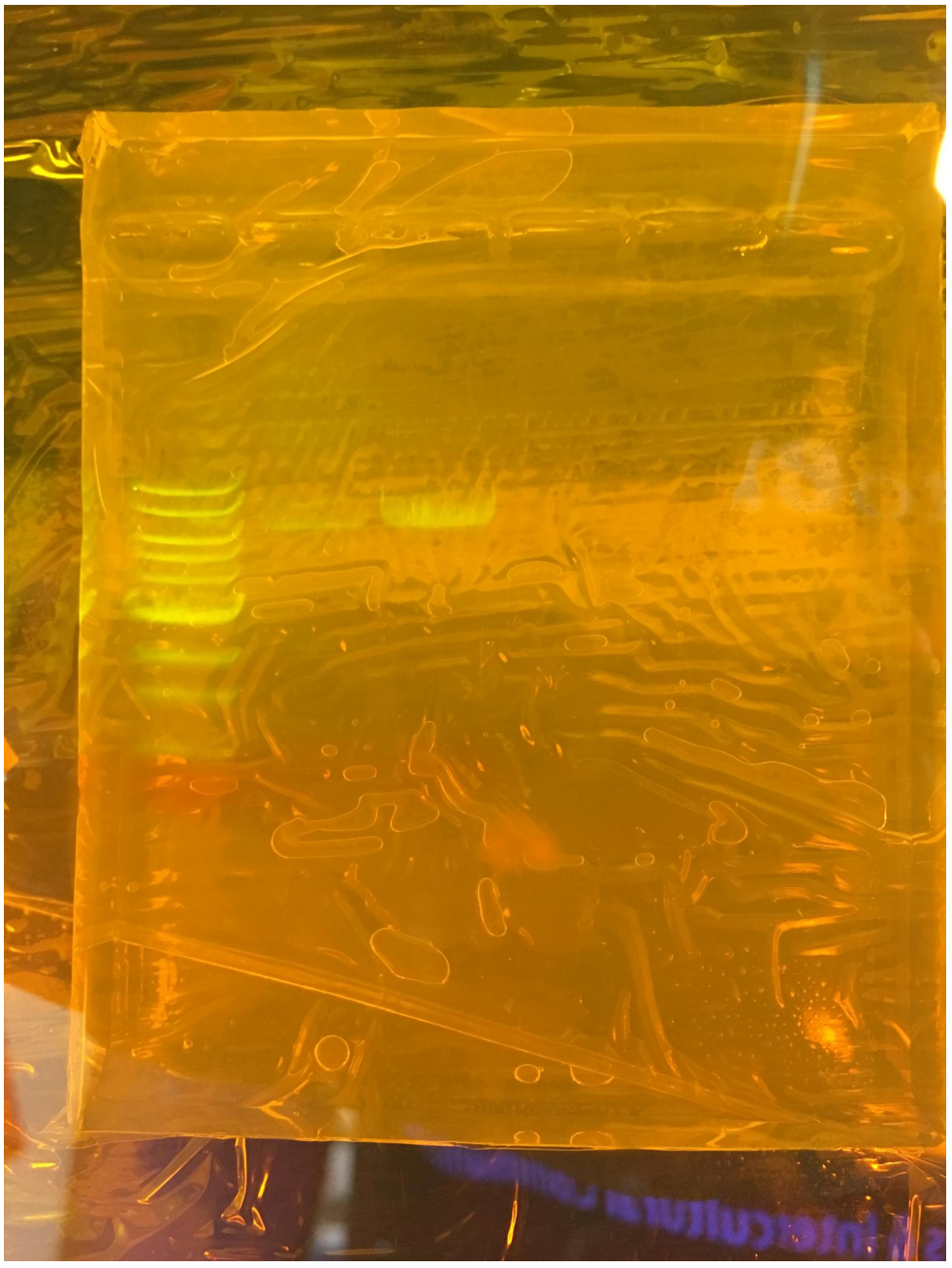
Running PCR

- Poured 1x TAE into the chamber, up to fill line
- Placed gel into the chamber
- Covered and left for lunch
- Mixed samples using pipette tip (pipetted up and down)
- Inserted samples into gel
- Covered the chamber
- Turned on machine (115 volts)
- Left to run

Gel Electrophoresis Well Arrangement

	A	B	C
1	Well 1	Well 2	Well 3
2	DNA Ladder	Touchdown	Standard

Genome Electrophoresis Gel



Summary: The Electrophoresis took a long time, we waited about an hour and the strands were still only about 1/2 way down the gel. But the gel was successful and so were our PCR.

Talk with Anna about our next Steps

Goal: Go over our next steps, what it means for our project, and make sure everyone is on the same page.

UBER with new origin; Cloning- Easy goal

- WAY1: We have old UBER that has Ampicilin Resistance and E. coli origins. We don't want either of those. So we will remove those and insert KanR, RSF1010, and mKate bio brick
    - In this approach, we can't change any part of the mKate brick if it happens to not be working
- WAY2: We could assemble the same as above, but instead of inserting an mKate bio brick we will make everything ourselves... It will be a 9 part assembly -- NO, FAILURE
    - Instead, we will use cassette vectors (what David explained a while back -- main backbone and a single part, single part falls off backbone and we insert what we want to).
    - Cassette vector is something we will make ourselves that will have GFP, UBER, RSF1010, KanR -- we want to clone this

H.W. Elements

- We will make these cassette vectors: GFP, SPACER (GRP and KanR cannot assemble together), KanR, + (origin)
    - RSF1010
    - pBBR1
    - RKZ
- Then we will incorporate one of the H.W. elements in place of GFP
- We will test these origins in other strains
- It will let us know what origins work (HOPEFULLY we will have a single origin that works across all of the strains)and then from there we will test that origin with different H.W. elements -- this is just to see the different levels of expression of the elements so we can characterize it

Changed UBER

- We want to make a part plasmid of tetracycline, H.W., and T7 RNAP
- We can't get the H.W. like we want it so we ordered 2 parts
    - Oligios that we need to phosphorylate and anneal
    - H.W. G block

Medium Goal

- The primers we made yesterday will allow use to cut there
- Then we will use Gibson to ligate 2 pieces together
- So the backbone has sticky ends that match the sticky ends on the operon so we can ligate them together (the operon is the 16S rRNA and the backbone is our cassette vector)

Gel Purify the DNA

Goal: Recover the DNA from the Gel Electrophoresis we did earlier.

Materials

- Zymoclean Gel DNA Recovery Kit
- Gel slice from electrophoresis

Procedure

Followed Zymoclean Gel DNA Recovery Kit Protocol

- Cut DNA out of the gel using a razor
- Placed in a microcentrifuge tube
- Added 300uL of ADB to the gel slice
- Incubated the tubes in a heat block at 42C for ~15-20 minutes
    - NOTE: added ~100uL more ADB to each tube
    - NOTE: there was still a clump of gel left at the bottom of both tubes
- Transferred the melted gel with a pipette to the Zymo-spin column
- Centrifuged for 60 seconds
- Discarded the flow through
- Added 200uL of Wash Buffer to the column and centrifuged for 30 seconds
- Discarded the flow through and repeat
- Added 15uL of water to the column

- Placed the column in a new microcentrifuge tube and centrifuged for 60 seconds

Summary: The process was simple and straight forward

Table3

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	639	4300	74.9	28.282	1.41 µL			0.354						5.74 µL		6.76
2	449	5393	99.7	30.017	1.33 µL			0.375						5.41 µL		7.09
3	616	55	22.2	655.382	0.06 µL			8.192						0.25 µL		12.25
4	479	2770	65.6	38.453	1.04 µL			0.481						4.22 µL		8.28
5	447	955	62.8	106.773	0.37 µL			1.335						1.52 µL		10.98
6	423	1555	45.3	47.301	0.85 µL			0.591						3.43 µL		9.07

OD Curves:

1. Took out all cultures at 10:45 am
2. Accidently left P. putida and S. oneidensis in 37C instead of 30C
    a. P. putida grew but not S. oneidensis
3. Set up OD curve plate:
    a. In table:
        I. 1 = B. subtilis
        II. 2 = Nissle 1917
        III. 3 = DH10B
        IV. 4 = MG1655
        V. 5 = V. natriegens
        VI. 6 = P. putida
        VII. 7 = L. lactis
        VIII. 8 = C. glutamicum
        IX. 9 = S. oneidensis
        X. red = base medium + glucose
        XI. blue = base medium + glucose + ascorbate
    b. Put breath EZ membrane on plate
    c. incubation temp = 33.5C

Table2

	A	B	C	D	E	F	G	H	I	J	K	L
1	1	1	1	2	2	2	3	3	3	4	4	4
2	5	5	5	6	6	6	7	7	7	8	8	8
3	9	9	9	blank	blank	blank						
4												
5	1	1	1	2	2	2	3	3	3	4	4	4
6	5	5	5	6	6	6	7	7	7	8	8	8
7	9	9	9	blank	blank	blank						
8												

UBER part plasmid

Annealing and phosphorylation of gBlock part

1)Added 1 uL of each iG014 and iG013 to 100 uL of water and 100 uL of cut smart buffer and incubated for 80 min with temperature decreasing from 98C to 25C

2)Prepared the annealing reaction:

a) 1 uL T4 ligase buffer, 0.5 uL T4 PNK, 1 uL of oligo anneal mix, 7.5 uL of water.

b) 1 uL T4 ligase buffer, 0.5 uL T4 PNK, 1 uL of oligo anneal mix (from the one with CutSmart), 7.5 uL of water.

c) 1 uL T4 ligase buffer, 0.5 uL T4 PNK, 8.5 uL of oligo anneal mix

Incubated at 37C for 30 min

Cassette Assemblies

Multigene and cassette assembly with RSF1010; multigene and cassette assembly for pBBR1

1)Cassette vector with UBER: 449, 639, 437, 447 (RSF1010)
2) Cassette vector with UBER 479, 639, 437, 447 (pBBR1)
3) Cassette vector for RSF1010: 449, 616, 437, 447
4) Cassette vector with pBBR1 479, 616, 437, 447
5) Multigene vector with RSF1010: 449, 616, 436, 447
6) Multigene vector with pBBR1: 479, 616, 436, 447

UBER part plasmid

430, T7 pcr product, UBER gblock, oligo part, TetR pcr product

Harris Wang part plasmids
16415 and

Golden Gate Assemblies

Table4

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
1	**Label**	***LengthDNA [bp]***	***ρDNA [ng/µL]***	***VDNA opt.***	**≈[DNA], ±0.01 nM**	4.0e-14	mol* =40 fmol		***Dil. Factor***								
2	639	4300	74.9		**28.282**	**1.41**	**µL**		**0.354**						**5.74**	**µL**	**6.76**
3	449	5393	99.7		**30.017**	**1.33**	**µL**		**0.375**						**5.41**	**µL**	**7.09**
4	616	55	22.2		**655.382**	**0.06**	**µL**		**8.192**						**0.25**	**µL**	**12.25**
5	479	2770	65.6		**38.453**	**1.04**	**µL**		**0.481**						**4.22**	**µL**	**8.28**
6	447	955	62.8		**106.773**	**0.37**	**µL**		**1.335**						**1.52**	**µL**	**10.98**
7	423	1555	45.3		**47.301**	**0.85**	**µL**		**0.591**						**3.43**	**µL**	**9.07**
8	430	2000	78		**63.324**	**0.63**	**µL**		**0.792**								
9	HW 16415	165	66.6		**655.382**	**0.06**	**µL**		**8.192**								
10	HW 31399	164	12.8		**126.727**	**0.32**	**µL**		**1.584**								

Cassette vector with UBER

	A	B	C	D	E
1					
2	**RSF1010**			**pBBR1**	
3	**Part**	**µL**		**Part**	**µL**
4	449	1.33		479	1.04
5	639	1.41		639	1.41
6	437	0.5		437	0.5
7	447	0.37		447	0.37
8					
9					
10	BsaI	0.5		BsaI	0.5
11	T4 DNA Ligase	0.5		T4 DNA Ligase	0.5
12	T4 Buffer	1.5		T4 Buffer	1.5
13	10X BSA	1.5		10X BSA	1.5
14	H20	7.18		H20	7.47
15					

Cassette vector without UBER

	A	B	C	D	E
1					
2	**RSF1010**			**pBBR1**	
3	**Part**	**µL**		**Part**	**µL**
4	449	1.33		479	1.04
5	616	0.1		616	0.1
6	437	0.5		437	0.5
7	447	0.37		447	0.37
8					
9					
10	BsaI	0.5		BsaI	0.5
11	T4 DNA Ligase	0.5		T4 DNA Ligase	0.5
12	T4 Buffer	1.5		T4 Buffer	1.5
13	10X BSA	1.5		10X BSA	1.5
14	H20	8.49		H20	8.78
15					

Thermocyling protocol for cassette vectors: end-on ligation to preserve BsaI cut sites

End ligation.png

End-On-Ligation Golden Gate Assembly

	A	B	C	D
1		Step	Temp	Time
2		Initial Digestion (opt.)	37°C	10 min
3	Repeat25×	Digestion	37°C	1.5 min
4		Annealing & Ligation	16°C	3 min
5		Storage	16°C	∞

Multigene Vector

	A	B	C	D	E
1					
2	**RSF1010**			**pBBR1**	
3	**Part**	**µL**		**Part**	**µL**
4	449	1.33		479	1.04
5	616	0.1		616	0.1
6	436	0.5		436	0.5
7	447	0.37		447	0.37
8					
9					
10	BsaI	0.5		BsaI	0.5
11	T4 DNA Ligase	0.5		T4 DNA Ligase	0.5
12	T4 Buffer	1.5		T4 Buffer	1.5
13	10X BSA	1.5		10X BSA	1.5
14	H20	8.49		H20	8.78
15					

UBER part plasmid

	A	B
1		
2	**Part**	**µL**
3	430	0.63
4	T7 PCR product	0.3
5	UBER gblock	0.5
6	oligo part	1
7	TetR PCR product	0.3
8		
9		
10	BsaI	0.5
11	T4 DNA Ligase	0.5
12	T4 Buffer	1.5
13	10X BSA	1.5
14	H20	8.06
15		

Harris Wang

	A	B	C	D	E
1					
2	**HW 16415**			**HW 31399**	
3	**Part**	**µL**		**Part**	**µL**
4	430	0.63		430	0.63
5	HW 16415	0.1		HW 16415	0.32
6					
7					
8	BsaI	0.5		BsaI	0.5
9	T4 DNA Ligase	0.5		T4 DNA Ligase	0.5
10	T4 Buffer	1.5		T4 Buffer	1.5
11	10X BSA	1.5		10X BSA	1.5
12	H20	10.06		H20	9.84
13					

Thermocycling protocol for other assemblies: Standard BsaI protoocol.

THURSDAY, 7/5/2018

People present: Katherine, Soohyun, Stefanie, Anna
Meeting at 1:00, wetlab at 2:00
Agenda:
- Golden gate assembly for Harris Wang and UBER part plasmid
- Preparing liquid cultures

These parts to assemble the UBER part plasmid

Table5

	A	B	C
1			
2	**Part**	**µL**	
3	430	0.63	-
4	T7 PCR product	0.3	-
5	UBER gblock	0.5	-
6	oligo part	1	-
7	TetR PCR product	0.3	-
8			
9			
10	Esp3I	0.5	-
11	T4 DNA Ligase	0.5	
12	T4 Buffer	1.5	-
13	10X BSA	1.5	-
14	h20	8.06	-
15			

Two assemblies, two UBER part plasmids one with each of the oligo parts.
U1- W1
U2- WLA6

Table6

	A	B	C
1	**HW 16415**		
2	**Part**	**µL**	
3	430	0.63	-
4	HW 16415	0.3	-
5			
6			
7	Esp3I	0.5	-
8	T4 DNA Ligase	0.5	-
9	T4 Buffer	1.5	-
10	10X BSA (+PEG)	1.5	-
11	NF H20	9.86	-

Prepared liquid cultures of the stuff we did yesterday

FRIDAY, 7/6/2018

People present: Katherine, Stefanie, Soohyun, Anna
Started 10:30 ended at 2:06 pm

1. Miniprep 436, 437, and RSF1010 multigene

- done, accidentally added 1000 uL buffer 1, centrifuged for one minute to correct mistake, discarded top bit and then continued process- made a mistake with miniprepping 436. I added 1000 uL P1, but caught the mistake. We repelleted and discarded the liquid. But then I accidentally added P2 first, and then quickly added p1 and mixed it all together at once

1. Calculate the amounts of 436 and 437 needed for 40 fmol

2. Repeat the assemblies for: - cassettes with UBER, pBBR1, RSF1010, pBBR1 multigene with 2x the amount of DNA compared to previously

Wait for the assemblies to be done

1. Use clean and concentrate kit to purify DNA from the assemblies. I want to do this to transform more DNA than 0.3 uL. For this we need to clean the assemblies or it will ark.
2. Transform 5.0 uL of purified assemblies. Try with one first - if it arks, decrease to 2 uL.
3. Pick colonies (if any) for transformations of HW and UBER part plasmids (I did them yesterday) and make liquid cultures

Repeat the assemblies for: - cassettes with UBER, pBBR1, RSF1010, pBBR1 multigene with 2x the amount of DNA compared to previously

Cassette vector with UBER (2)

	A	B	C	D	E
1					
2	**RSF1010**			**pBBR1**	
3	**Part**	**µL**		**Part**	**µL**
4	449	2.66	-	479	2.08
5	639	2.82		639	2.82
6	437	0.28		437	0.28
7	447	0.74		447	0.74
8					
9					
10	BsaI	0.5		BsaI	0.5
11	T4 DNA Ligase	0.5		T4 DNA Ligase	0.5
12	T4 Buffer	1.5		T4 Buffer	1.5
13	10X BSA	1.5		10X BSA	1.5
14	H20	4.29	-	H20	4.87
15					

Cassettes vectors w/o UBER (2)

	A	B	C	D	E
1					
2	**RSF1010**			**pBBR1**	
3	**Part**	**µL**		**Part**	**µL**
4	449	2.66	-	479	2.08
5	616	0.2	-	616	0.2
6	437	0.28	-	437	0.28
7	447	0.74	-	447	0.74
8					
9					
10	BsaI	0.5		BsaI	0.5
11	T4 DNA Ligase	0.5		T4 DNA Ligase	0.5
12	T4 Buffer	1.5	-	T4 Buffer	1.5
13	10X BSA	1.5	-	10X BSA	1.5
14	H20	6.91	-	H20	7.49
15					

Ran end ligation protocol

pBBR1 Multigene (2)

	A	B
1	**pBBR1**	
2	**Part**	**µL**
3	479	2.08
4	616	0.2
5	436	0.2
6	447	0.74
7		
8		
9	BsaI	0.5
10	T4 DNA Ligase	0.5
11	T4 Buffer	1.5
12	10X BSA	1.5
13	H20	7.57
14		

Ran standard BsaI Golden Gate cycle

Determining Antibiotic Resistance of Strains

1. Streaked out all strains on plain LB plate (V. natriegens, B. subtilis, C. glutamicum, nissle1917, mg1655, DH10B, L. lactis, P. putida, S. oneidensis, S. meliloti)
    a. In appropriate incubators at 5:20 pm

SATURDAY, 7/7/2018

People present: Soohyun, Anna, Stefanie

Transformed the assemblies made on Friday into *E. coli* MG1655 electrocompetent cells using electroporation
Plated on Kan50

SUNDAY, 7/8/2018

People present: Anna

Picked colonies for all assemblies; streaked out on a new plate the construct with pBBR1 multigene and pBBR1+Uber since there was a lawn

Week of July 9

MONDAY, 7/9/2018

People present: Stefanie, Soohyun, Katherine
Time: 10:30AM-12:30PM, 1-5:45PM (6.75 hours)
Location: ABL 126
Goals: Count cells (interlab), miniprep last week's assemblies and H.W. cultures,

Recap from last week: Katherine and Soohyun finished the interlab, we will count cells today. They made assemblies that were successful! Today we will miniprep them.

Miniprep Assemblies and H.W. Cultures

Goal: Extract the DNA from the cells for later use.

Materials

- Qiagen Miniprep Kit
- Liquid cultures from Keck 201
    - UBER part plasmid #1, #2, #3, #4
    - pBBR1 + UBER
    - RSF1010 cassette
    - pBBR1 cassette
    - H.W. 16514

Procedure

pBBR1 + UBER, RSF1010 cassette, pBBR1 cassette

- Transferred part of cultures to microcentrifuge tubes and centrifuged for 30 seconds at 13,200rpm. Repeated twice.
- Labeled spin columns as above^ with iGEM Miniprepped 7/9
- Resuspended bacteria in 250uL of buffer P1 by pipetting up and down
    - Just to resuspend
- Added 250uL of buffer P2 to tube and invert tube 6 times
    - Actually lyses the cells
    - NOTE: May have added a little more/less than 250 to 2 of the cultures
- Added 350uL of buffer N3 to tube and insert 6 times
- Centrifuged for 10 minutes at 13,000rpm

Repeat above for UBER pp #1, #2, #3, #4

- NOTE: When pelleting DNA, UBER #4 was centrifuged 4 times
- NOTE: When inverting tubes to mix, the tubes were inverted 5 times

Repeat above for H.W. 16514

- NOTE: When pelleting DNA, tube was centrifuged 4 times
- NOTE: Katherine used PB Buffer when minipreping this sample

LEFT for presentation in GRBW (12:00-1:00)

Resumed and completed all remaining steps for the 3 sets of minipreps

- Applied supernatant (liquid result from previous step
- Centrifuged for 60 seconds at 13,200rpm; discard flow-through
- Centrifuged for 60 seconds at 13,200rpm; discard flow-through
- Added 750uL of Buffer PE to the spin column
- Centrifuged for 60 seconds at 13,200rpm; discard flow-through
- Transferred the spin column to a new micro centrifuge tube
- Centrifuged for 2 minutes at 13,200rpm; discard flow-through

- Placed the spin column in a new micro centrifuge tube
- Added 50uL of buffer EB to the spin column
- Let stand for 4 minutes
- Centrifuged for 30 seconds

Create Cassettes

Materials

- Benchling files:
    - pBBR1 cassette vector
    - cassette vector RSF1010
    - pSPB501 ConLS (1)
    - H.W. 29572
    - pSPB460 mKate2 (4)
    - pSPB463 tVoigtS6 L3S3P00 (4)
    - pSPB512 ConR2 (6)

Procedure

RSF1010 cassette, ConLS connector, 29572 Harris Wang element, mKate, mKate terminator, ConR2 connector
pBBR1 cassette, ConLS connector, 29572 Harris Wang element, mKate, mKate terminator, ConR2 connector

- Opened up origin
- Near bottom right of the page, clicked Assembly Wizard, Create New Assembly
- Clicked Golden Gate in pop up
- Selected backbone at bottom center (slightly to the left) of the page
- Selected Set Fragement
- Selected insert and opened up ConLS connector*
- Selected Set Fragment*
- Repeated starred (*) bullets for the remaining components as listed above
- Saved to "Cassettes" in iGEM 2018 - Orthogonal Transcription and Broad Host Parts
- Saved as:
    - pBBR1 + 29572 H.W. + mKate
    - RSF1010 + 29572 H.W. + mKate

Nanodrop Assemblies, H.W., and UBER Minipreps

Goal: Determine the concetration of DNA we extracted from the cells from our digest

Materials

- Miniprep results
- nfH2O

Procedure

- Took 1uL nfH2O and nanodropped as a blank
- Wiped nano drop with kimwipe
- Took 1uL of _____ and measured:
    - UBER pp #1 = 148.6 ng/uL
    - UBER pp #2 = 154.3 ng/uL
    - UBER pp #3 = 124.0 ng/uL
    - UBER pp #4 = 327.3 ng/uL
    - pBBR1 cassette = 112.1 ng/uL
    - pBBR1 + UBER = 96.5 ng/uL
    - RSF1010 cassette = 63.4 ng/uL
    - H.W. 16514 = 93.4 ng/uL

Digest Test of UBER pp's with BsaI

Goal: Perform a digest so we can run an electrophoresis to make sure that the parts we wanted to assemble are correct.

Materials

- BSAI
- UBER pp DNA
- Cut Smart Buffer
- NF Water

Procedure

- Labeled PCR tubes: 1, 2, 3, 4, C
- Added volumes listed in table below to PCR tubes
    - Water and DNA added while waiting for Cut Smart Buffer to defrost in the 4C fridge (can't defrost at room temperature)

Table2

	A	B	C	D	E	F
1	*Volumes = uL*	**BsaI (5 units)**	**DNA (0.5 µg)**	**Cut Smart Buffer**	**NF Water**	**Total**
2	UBER1	0.5	4.0	2.5	18	25
3	UBER2	0.5	4.0	2.5	18	25
4	UBER3	0.5	4.0	2.5	18	25
5	UBER4	0.5	2.0	2.5	20	25
6	Control (440)	0.5	5.6	2.5	16.4	25

- Incubated PCR tubes for 1hr at 37C in PCR machine (ABL 126)

Sending DNA for Sequencing

Materials

- DNA
    - pBBR1
    - pBBR1 + UBER
    - RSF1010
    - H.W. 16514
- nfWater
- Primers
    - C80
    - B18

Procedure

- Labeled tubes: 1, 2, 3, 4
- Added respective amounts of water, primer, DNA to tubes

Sample Calculations: pBBR1 Cassette
500ng DNA / 112.1 ng/uL = 4.46uL DNA

Table3

	A	B	C	D	E	F	G
1	**Sample #**	**Sample**	**Sample Length**	**Primer**	**DNA Amount (uL)**	**Primer Amount (uL)**	**Water Amount (uL)**
2	1	pBBR1 cassette	4001-6000	C80	4.46	2.5	8.04
3	2	RSF1010 cassette	6001-8000	C80	7.89	2.5	4.61
4	3	pBBR1 + UBER	8001-10000	C80	5.18	2.5	7.32
5	4	H.W. 16514	2001-4000	B18	5.35	2.5	7.15

Count Cells- Interlab

Goal: FINISH INTERLAB; count the colonies on the plates that Soohyun and Katherine made the other day

Materials

- Plates from 2nd part of Interlab (ABL 126 4C fridge bottom shelf)

Procedure

- Obtained plates
- Wiped off condensation
- Counted colonies using sharpie to mark colonies already counted
- Recorded number of colonies

Number of Colonies- Interlab Pt. 2

	A	B	C	D	E	F	G	H	I	J	K	L	M
1		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
2	**D3**	>300	>300	>300	>300	180	>300	161	>300	>300	>300	>300	>300
3	**D4**	18	43	11	64	54	81	84	105	62	103	18	66
4	**D5**	1	8	2	11	24	5	10	10	10	5	14	3

Summary: It was very difficult to count all of the colonies, especially when they were clumped together. It was time consuming, but not difficult in an effort sense. Finally done with interlab! (Yay!)

Gel Electrophoresis of Digest

Goal: Run the digests that we did earlier, based on the lengths we see we will know whether our parts assembled correctly (if we see the right length).

Materials

- Agarose gel mix
- DNA ladder
- Samples
- Loading dye
- Gel dye

Procedure

Katherine made agarose gel

- Removed PCR tubes from the PCR machine (1 hours later)
- Added 5 uL of SYBR-safe gel stain to liquid agarose gel
- Added 10uL loading dye to each sample (1,2,3,4 and C) and mixed with a pipette

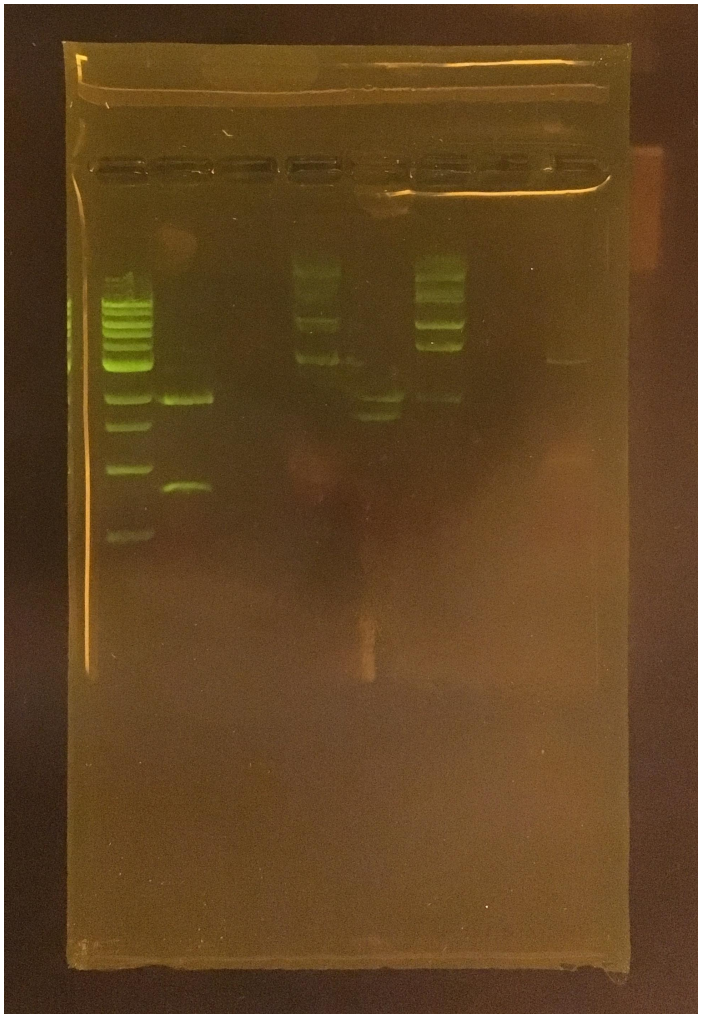
- Removed gel from caster and placed in chamber with 1x TBE
- Inserted 15uL of 1kb DNA ladder in the far right well
- Inserted samples in the following arrangement
- NOTE: Some the the samples splashed out of their respective wells but otherwise the process worked well

Gel Electrophoresis Well Arrangement

	A	B	C	D	E	F	G	H
1	**Well 1**	**Well 2**	**Well 3**	**Well 4**	**Well 5**	**Well 6**	**Well 7**	**Well 8**
2	DNA Ladder	UBER pp 1	(Space)	UBER pp 2	UBER pp 3	UBER pp 4	(Space)	Control

- Ran gel on 100 volts (3:52PM)
    - NOTE: We want the ladder to run about 2/3 of the way down the gel
    - NOTE: Stopped running gel at 4:06PM -- there was a lot of bubbling near the negative node -- Changed liquid in chamber entirely -- Restarted a few minutes later at 125 volts
- Stopped gel and placed on UV light

IMG_8369.jpg



Summary: We kept running into issues when running the gel with the TBE bubbling near the negative electrode. It also took a long time, but eventually we got decent results. We sent in sample 3 for sequencies.

Golden Gate Assemblies of Cassette Vectors

Goal: Perform golden gate assemblies that we made earlier on Benchling

Materials

- DNA
  - pBBR1 cassette origin
  - RSF1010 cassette origin
  - ConLS
  - H.W. 29572
  - pSPB460 mKate2
  - Terminator: pSPB463 tVoigtS6 L3S3P00 (4)
  - ConR2 (6)
- BsaI
- 10x BSA
- T4 DNA ligase
- T4 Ligase Buffer
- diWater

Procedure

- Used Shyam's wet lab calculator to determine what volume of DNA we need to add to get 40fmol
  - **NOTE** Anna said when DNA volumes are <0.2uL, just add 0.2uL
  - Other NOTE: all three 0.2 uL micropipettes ABL don't work consistently so we used one from Keck

Wetlab Calculator Inputs/Results

	A	B	C	D	E	F	G
1	**Label**	***LengthDNA[bp]***	***ρDNA [ng/μL]***		**≈[DNA], ±0.01 nM**	4.0e-14	mol*=40 fmol
2	RSF1010 origin	6429	63.4		**16.012**	**2.5**	**μL**
3	pBBR1 origin	3763	112.1		**48.370**	**0.83**	**μL**
4	pSPB501 ConLS (1)	154	175.7		**1852.486**	**0.02**	**μL**
5	H.W. 29572	168	112.1		**1083.428**	**0.04**	**μL**
6	pSPB460 mKate2 (4)	697	112.6		**262.307**	**0.15**	**μL**
7	pSPB463 tVoigtS6 L3S3P00 (4)	59	234.9		**6464.499**	**0.01**	**μL**
8	pSPB512 ConR2 (6)	165	237.8		**2340.086**	**0.02**	**μL**

- Added respective amounts of components listed in the table below to tubes
  - diWater
  - 10x BSA
  - T4 Ligase Buffer
  - DNAs
  - BsaI
  - T4 DNA Ligase

GGA Component Volumes

	A	B	C	D	E
1		**RSF1010 Cassette**		**pBBR1 Cassette**	
2	**Component**	**Volume (uL)**		**Volume (uL)**	
3	diWater	7.5		9.17	
4	BsaI	0.5		0.5	
5	T4 DNA Ligase	0.5		0.5	
6	10x T4 Ligase Buffer	1.5		1.5	
7	10x BSA	1.5		1.5	
8	DNA's	3.5		1.83	*Values adjusted to Anna's directions
9		*2.5*		*---*	*RSF1010 origin*
10		*---*		*0.83*	*pBBR1 origin*
11		*0.2*		*0.2*	*pSPB501 ConLS (1)*
12		*0.2*		*0.2*	*H.W. 29572*
13		*0.2*		*0.2*	*pSPB460 mKate2 (4)*
14		*0.2*		*0.2*	*pSPB463 tVoigtS6 L3S3P00 (4)*
15		*0.2*		*0.2*	*pSPB512 ConR2 (6)*

- Placed PCR tubes in the thermocycler (ABL 126) under the program: iGEMGGB

iGEMPCR3

	A	B	C
1	Lid Temp: 105		
2	**Step**	**Temperature (C)**	**Time**
3	1	98	0:30
4	2	98	0:10
5	3	64	0:30
6	4	72	4:06
7	5	Go to Step 2	Repeat 34x
8	6	72	2:00
9	7	12	Hold

TUESDAY, 7/10/2018

People present: Soohyun, Stefanie, Katheirne
Time: 11AM-, 11:30AM- 3:00
Location: ABL 126
Goals: Miniprep RSF1010+UBER/ pBBR1 Multigene, send ____ for sequencing, resuspend primers 15/16 and PCR with pRSFDuet1-mKate, resuspend 16/18 and PCR with UBER gBlock, (repeat assembly??), prepare liquid cultures of yesterday's assembly, wiki progress

Miniprep RSF1010 + UBER and pBBR1 Multigene

Goal:

Materials

- Liquid cultures from Keck 201
    - RSF1010 + UBER
    - pBBR1 multigene
- Qiagen Miniprep Kit

Procedure

- Transferred part of cultures to microcentrifuge tubes and centrifuged for 30 seconds at 13,000rpm. Repeated twice.
- Labeled spin columns as above^ with iGEM Miniprepped 7/10
- Resuspended bacteria in 250uL of buffer P1 by pipetting up and down
- Added 250uL of buffer P2 to tube and invert tube 6 times
- Added 350uL of buffer N3 to tube and insert 6 times
- Centrifuged for 10 minutes at 13,000rpm
- Applied supernatant (liquid result from previous step
- Centrifuged for 30 seconds at 13,000rpm; discard flow-through
- Centrifuged for 30 seconds at 13,000rpm; discard flow-through
- Added 750uL of Buffer PE to the spin column
- Centrifuged for 30 seconds at 13,000rpm; discard flow-through
- Transferred the spin column to a new micro centrifuge tube
- Centrifuged for 1 minute at 13,000rpm; discard flow-through
- Placed the spin column in a new micro centrifuge tube
- Added 50uL of nfH2O to the spin column
- Let stand for 1 minute
- Centrifuged for 1 minute

Summary:

Sending DNA for Sequencing

Materials

- DNA
    - pBBR1 multigene
    - UBER pp #3 (from yesterday 7/9)
    - RSF1010 + UBER
- nfWater
- Primers
    - C80
    - P1 T7 seq

Procedure

- Labeled tubes: 1, 2, 3
- Added respective amounts of water, primer, DNA to tubes

Sample Calculations: pBBR1 multigene
500ng DNA / 105.0 ng/uL = 4.76 uL DNA

Table1

	A	B	C	D	E	F	G	H
1	**Sample #**	**Sample**	**Sample Length**	**Primer**	**DNA Amount (uL)**	**Primer Amount (uL)**	**Water Amount (uL)**	**Total (uL)**
2	1	UBER pp #3	6001-8000	T7 P1 seq	4.03	2.5	8.47	15
3	2	RSF1010 + UBER	>10000	C80	3.49	2.5	9.01	15
4	3	pBBR1 multigene	4001-6000	C80	4.76	2.5	7.74	15

Antibiotic titration for all strains

Table4

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	0 ng/mL Kan	1	1	1	2	2	2	3	3	3	4	4	4
2	12.5 ng/mL Kan	1	1	1	2	2	2	3	3	3	4	4	4
3	25 ng/mL Kan	1	1	1	2	2	2	3	3	3	4	4	4
4	50 ng/mL Kan	1	1	1	2	2	2	3	3	3	4	4	4
5	0 ng/mL Kan	5	5	5	6	6	6						
6	12.5 ng/mL Kan	5	5	5	6	6	6						
7	25 ng/mL Kan	5	5	5	6	6	6						
8	50 ng/mL Kan	5	5	5	6	6	6						

Table5

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	0 ng/mL Neo	7	7	7	0 ng/mL Kan	8	8	8	9	9	9			
2	25 ng/mL Neo	7	7	7	12.5 ng/mL Kan	8	8	8	9	9	9			
3	50 ng/mL Neo	7	7	7	25 ng/mL Kan	8	8	8	9	9	9			
4	100 ng/mL Neo	7	7	7	50 ng/mL Kan	8	8	8	9	9	9			
5														
6														
7														
8														

1. Prepared 96-well plate as shown above
    a. Each well was filled with 1 mL base medium + 0.5% glucose (+ ascorbate for C. glutamicum)
    b. Each row contains medium w/ diff. concentration of antibiotic
    c. picked a colony of each strain into wells in pattern shown above
        I. 1 = DH10B
        II. 2 = MG1655
        III. 3 = NIssle1917
        IV. 4 = C. glutamicum

V. 5 = L. lactis
VI. 6 = B. subtilis
VII. 7 = S. meliloti
VIII. 8 = S. oneidensis
IX. 9 = P. putida

d. S. meliloti is resistant to kanamycin; it gets tested in different concentrations of neomycin
e. No Vibrio b/c it doesn't want to grow on plates anymore.

2. Streaked Vibrio on plate again

WEDNESDAY, 7/11/2018

Antibiotic titration for all strains

1. Vibrio did not grow on plate
2. Transferred 100 uL of each well of antibiotic resistance titration to shallow 96-well plate
   a. MG1655, C. glutamicum, L. lactis susceptible to 50 ug/mL of kanamycin
   b. Nissle1917 and S. oneidensis maybe susceptible; need to check higher concentrations
   c. DH10B, B. subtilis, P. putida don't seem affected by 50 ug/mL kanamycin
   d. S. meliloti didn't grow at all, even in plain medium
3. Made new kanamycin aliquots (50 mg/mL)

Repeat assemblies for RSF1010, pBBR1 cassettes and multigenes

from miniprepped samples:

Cassette vector without UBER

	A	B	C	D	E
1					
2	**RSF1010**			**pBBR1**	
3	**Part**	**µL**		**Part**	**µL**
4	449	1.33	-	479	1.04
5	616	0.1	-	616	0.1
6	437	0.5		437	0.5
7	447	0.37		447	0.37
8					
9					
10	BsaI	0.5		BsaI	0.5
11	T4 DNA Ligase	0.5		T4 DNA Ligase	0.5
12	T4 Buffer	1.5		T4 Buffer	1.5
13	10X BSA	1.5		10X BSA	1.5
14	H20	8.49	-	H20	8.78
15					

End ligation.png

End-On-Ligation Golden Gate Assembly

	A	B	C	D
1		Step	Temp	Time
2		Initial Digestion (opt.)	37°C	10 min
3	Repeat25×	Digestion	37°C	1.5 min
4		Annealing & Ligation	16°C	3 min
5		Storage	16°C	∞

Multigene Vector

	A	B	C	D	E
1					
2	**RSF1010**			**pBBR1**	
3	**Part**	**µL**		**Part**	**µL**
4	449	1.33		479	1.04
5	616	0.1		616	0.1
6	436	0.5		436	0.5
7	447	0.37		447	0.37
8					
9					
10	BsaI	0.5		BsaI	0.5
11	T4 DNA Ligase	0.5		T4 DNA Ligase	0.5
12	T4 Buffer	1.5		T4 Buffer	1.5
13	10X BSA	1.5		10X BSA	1.5
14	H20	8.49		H20	8.78
15					

from original parts (Anna):
(try with multigenes)

Table6

	A	B	C	D	E
1					
2	**RSF1010**			**pBBR1**	
3	**Part**	**µL**		**Part**	**µL**
4	449	0.5		479	0.5
5	616	0.5		616	0.5
6	436	0.5		436	0.5
7	447	0.5		447	0.5
8					
9					
10	BsaI	0.5		BsaI	0.5
11	T4 DNA Ligase	0.5		T4 DNA Ligase	0.5
12	T4 Buffer	1.5		T4 Buffer	1.5
13	10X BSA	1.5		10X BSA	1.5
14	H20	8.79		H20	8.79
15					

send for sequencing: UBER #4 part plasmid
RSF1010 multigene

Table7

	A	B	C	D
1		water	DNA	primer
2	RSF1010	5.8	6.7	2.5
3	UBER #4	10.9	1.6	2.5

Primer resuspension

Table8

	A	B	C
1	**Organism**	**Primer**	**H2O (uL)**
2	bacillus	iG025	294
3	bacillus	iG026	234
4	c. glut	iG027	283
5	c. glut	iG028	282
6	lactis	iG029	227
7	lactis	iG030	239
8	putida	iG031	307
9	putida	iG032	264
10	meliloti	iG033	256
11	meliloti	iG037	267
12	vibrio	iG034	298
13	vibrio	iG035	261
14		AG102	216

THURSDAY, 7/12/2018

People present: Soohyun, Katheirne
Time: 11:11-20 AM, 12:00- 3:45 pm
Location: ABL 126
Goals: Transform and plate DH5-a cells with yesterday's assemblies; PCR of bacillus, lactis, and putida

Assemblies were take from ABL thermocyclers and stored in 4 degree fridge for later use. After the day, put in the 20- freezer.

PCR of non-model organism for ribosomal operons

Materials

- 0.2 uL PCR tubes
- Primers: see table from yesterday
- Genomic DNA

Table9

	A	B	C	D	E	F	G
1		**Bacillus**	**C. glut.**	**Lactis**	**Putida**	**Meliloti**	**Vibrio**
2	**Length**	5447	6175	5451	6250	7134	5963
3	**Annealing Temp (C)**	68.0	69.0	67.0	69.0	71.0	69.0
4	**Elongation time**	181.6	205.8	181.7	208.3	237.8	198.8

Procedure

- Added to tube:
  - up to 50.0 uL H2O
  - ____ uL of DNA
  - 5 uL of forward primer and backward primer
  - 2 uL dNTPs
  - 20 uL Q5 buffer
  - 1 uL Q5 polymerase
- NOTE: we could not find the DNA for the CG, Melitloti, and the Vibrio. We will PCR these when we can find this

Table10

	A	B	C	D	E	F	G
1		**Bacillus (stock 74.5 ng/uL)**	**C. glut.**	**Lactis (stock 107.1 ng/uL)**	**Putida (stock 90.8 ng/uL)**	**Meliloti**	**Vibrio**
2	H20	16.46		16.63	16.54		
3	DNA	0.54		0.37	0.46		
4	Primers	5.0 each of 025, 026	5.0 each of 027, 028	5.0 each of 030, 029	5.0 each of 031, 032	5.0 each of 037, 033	5.0 each of 034, 035
5	dNTPS	2.0	2.0	2.0	2.0	2.0	2.0
6	Q5 buffer	20.0	20.0	20.0	20.0	20.0	20.0
7	Q5 polymerase	1.0	1.0	1.0	1.0	1.0	1.0

- Placed in Thermal Cycler
  - Standard: iGEMPCR3 (ABL 126) --> Adjust Extension Time!
    - We will only be running the standard protocol today because both standard and touchdown were successful with Shewanella
    - Ran these PCRs in ABL

FRIDAY, 7/13/2018

People Present: Sooyhun, Anna

Transformation Procedure: Turns regular cells into genetically engineered cells

Procedure:
- Use DNA Clean and Concentrate kit (follow those instructions, per the last step elute with 8 uL of water)
- Take 2 uL of that and transform it by
- getting DH5 from competent cells in Keck (-80 freezer, ask Jordan/Albert) DO NOT LET THEM SIT AT ROOM TEMPERATURE, KEEP IN ICE
- Get SOB to rescue the cells
- Put cuvettes on ice before using them to have them be chilled
- Grab the stuff to be transformed from ABL (pBBR1 mutligene, UBER + pBBR1, RSF1010, pBBR1 mutligene with orig parts, RSF1010 multigene, RSF1010 multigene with orig parts)
- When materials gathered, turn on shock machine and hit button to set to milliseconds (probably just do this in Keck)
- Take 2 uL of the DNA and pipette into one alloquote of 50 uL each of the competent cells (tube of the competent cells)

- Close it and flick it to mix
- Pipette out everything in the tube, depress until just the liquid (no air bubbles)
- Tilt cuvette towards you, pipette everything between the plates
- Tap cuvette on the table to knock liquid between the plates
- Wipe the sides with a kimwipe
- Pull out slider, stick the cuvette inside
- Shock the cells, then quickly pull out and add 1 mL of SOB
- Pipette up and down four times
- Make sure the machine reading is above 5.4 ms
- Transfer the liquid from pipetting into a labelled Falcon tube
- Leave the tubes in the shaker/incubator at 37* for an hour to recover
- After that hour, plate the cells (Kan plates)

Also, run those PCRs on a gel.

Collecting Strains

1. Obtained BL21 from Dr. Beason

SATURDAY, 7/14/2018

Golden Gate assembly transformations

Transformed the assemblies made on Wednesday into E. coli DH5a cells. Plated on Kan50 plates and put into 37C incubator in ABL.

PCR T7 promoter + mKate + terminator out of PRSFDuet-mKate

Table11

	A	B	C	D	E	F	G	H
1		Phusion HF buffer	dNTPs	Fwd Primer	Rev Primer	Phusion DNA polymerase	mKate template	H2O
2	mKate	10	1	2.5	2.5	0.5	0.2	33.3

band of expected size (~ 900 bp) was observed. Purified using Zymo kit

SUNDAY, 7/15/2018

People Present: Anna

Part plasmids digest

Digest part plasmids and gel purify to elmiminate E. coli origin

Table12

	A	B	C	D	E	F
1	*Volumes = uL*	**Bsal (5 units)**	**DNA (0.5 µg)**	**Cut Smart Buffer**	**NF Water**	**Total**
2	447	0.5	3.0	2.5	19	25
3	639	0.5	6.5	2.5	15.5	25
4	616	0.5	22	2.5	0	25
5	437	0.5	4.0	2.5	18	25
6	436	0.5	3.0	2.5	19	25
7	449	0.5	6.0	2.5	16	25
8	479	0.5	7.7	2.5	14.3	25

Incubate at 37C for 1 hour

RSF1010-mKate cassette assembly

Colony PCR on old assemblies

Do colony PCR on UBER + RSF1010, UBER part plasmid, and RSF1010 multigene
Miniprep colonies formed for RSF1010 cassette vector and do PCRs on them (since we only got 3 colonies)

Primers:

1. UBER + RSF1010: D51, D53 // Tm = 64 C // length = 2200
2. UBER part plasmid: D62, P1 T7 seq //Tm = 59 C // length = 2050
3. RSF1010 multigene: D51, D53 // Tm = 64 C // length = 2200

do separate PCRs to check RSF1010 multigene and UBER part plasmid

Get D62 from Shyam, D51, D53, P1 T7 seq from ABL

Labelled 3 sets of PCR strips
Put 5 uL of LB into 5 tubes for each strip
Picked a colony for each tube (lightly touch it with toothpick, avoid grabbing too much, avoid picking agar)
Placed into the incubator for 20 min
Used 1 uL of culture from each well as PCR template
Put tubes with cultures into the incubator

Table13

	A	B	C	D	E	F	G	H
1		Phusion HF buffer	dNTPs	Fwd Primer	Rev Primer	Phusion DNA polymerase	template	H2O
2	PCR components	10	1	2.5	2.5	0.5	1	32.5
3								
4	MM for 5 rxns	54	5.4	13.5	13.5	2.7		175.5
5								

Note that initial denaturation time should be 3 min

Table 2: Thermocycling Conditions for colony PCR

	A	B	C	D	E
1	**STEP**	**TEMP**	**TIME for UBER + RSF1010**	**TIME for UBER part plasmid**	**TIME for RSF1010 multigene**
2	Initial Denaturation	98°C	**3:00 min**	3:00 min	3:00 min
3	35 Cycles	98°C	10 seconds	10 seconds	10 seconds
4		65°C	30 seconds	30 seconds	30 seconds
5		72°C			
6	Final Extension	72°C	2 minutes	2 minutes	2 minutes
7	Hold	4–10°C	infinite	infinite	infinite

If colony PCR fails -> repeat the assembly with digested fragments

Part plasmids digest

Bands for 436, 437, and 447 look faint, so will repeat the digests with larger amount of DNA. pBBR1 part plasmid digest looks strange:

Table14

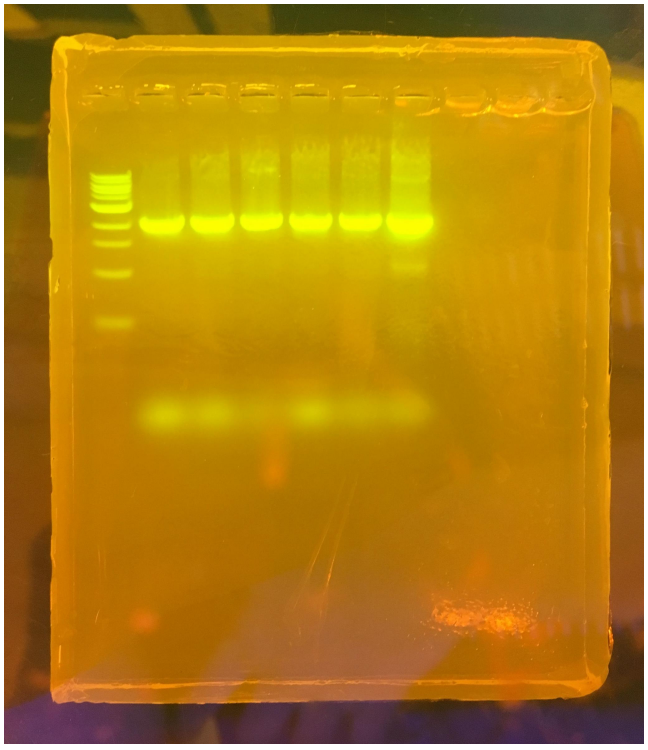
	A	B	C	D	E	F
1	*Volumes = uL*	**Bsal (5 units)**	**DNA (0.5 µg)**	**Cut Smart Buffer**	**NF Water**	**Total**
2	447	0.5	8.0	2.5	14	25
3	437	0.5	8.0	2.5	14	25
4	436	0.5	8.0	2.5	14	25

gel purified the fragments

Colony PCR results

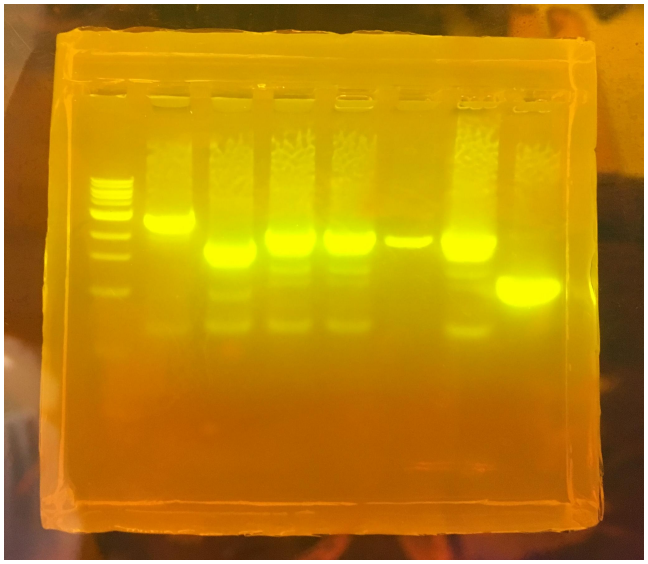
RSF1010 multigene

IMG_8392.jpg



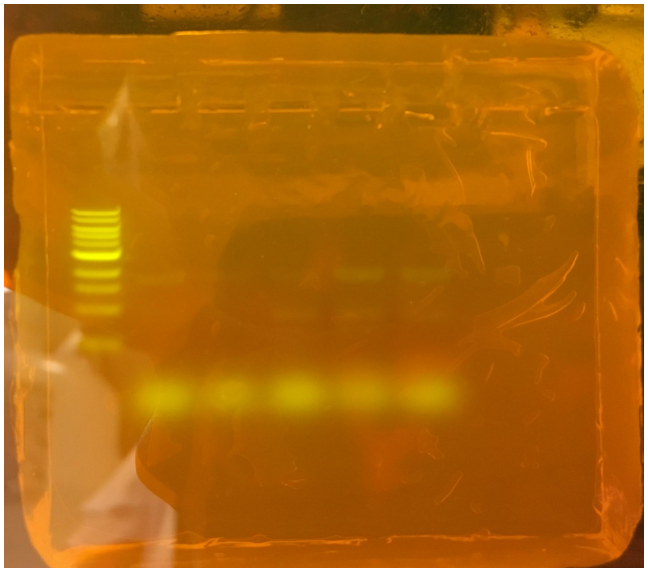
UBER part plasmid

IMG_8394.jpg



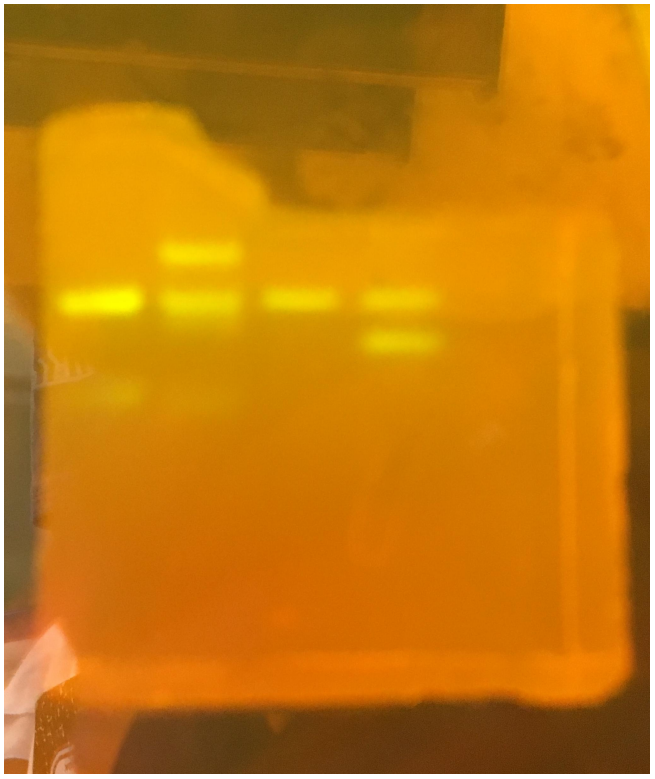
RSF1010 + UBER

IMG_8398.jpg



pBBR1 part plasmid

IMG_8399.jpg



Week of July 15

SUNDAY, 7/15/2018

Repeat of antibiotic titration for all strains

Table4

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	0 ug/mL Kan	1	1	1	2	2	2	3	3	3	4	4	4
2	12.5 ug/mL Kan	1	1	1	2	2	2	3	3	3	4	4	4
3	25 ug/mL Kan	1	1	1	2	2	2	3	3	3	4	4	4
4	50 ug/mL Kan	1	1	1	2	2	2	3	3	3	4	4	4
5	0 ug/mL Kan	5	5	5	6	6	6	10	10	10			
6	12.5 ug/mL Kan	5	5	5	6	6	6	10	10	10			
7	25 ug/mL Kan	5	5	5	6	6	6	10	10	10			
8	50 ug/mL Kan	5	5	5	6	6	6	10	10	10			

Table5

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	0 ug/mL Neo	7	7	7	0 ug/mL Kan	8	8	8	9	9	9			
2	25 ug/mL Neo	7	7	7	12.5 ug/mL Kan	8	8	8	9	9	9			
3	50 ug/mL Neo	7	7	7	25 ug/mL Kan	8	8	8	9	9	9			
4	100 ug/mL Neo	7	7	7	50 ug/mL Kan	8	8	8	9	9	9			
5														
6														
7														
8														

1. Prepared 96-well plate as shown above
    a. Each well was filled with 1 mL base medium + 0.5% glucose (+ ascorbate for C. glutamicum)
    b. Each row contains medium w/ diff. concentration of antibiotic
    c. picked a colony of each strain into wells in pattern shown above
        I. 1 = DH10B
        II. 2 = MG1655
        III. 3 = NIssle1917
        IV. 4 = C. glutamicum
        V. 5 = L. lactis
        VI. 6 = B. subtilis
        VII. 7 = S. meliloti
        VIII. 8 = S. oneidensis
        IX. 9 = P. putida
        X. 10 = BL21
    d. S. meliloti is resistant to kanamycin; it gets tested in different concentrations of neomycin

e. In appropriate incubator at 2:30 pm

MONDAY, 7/16/2018

People present: Stefanie, Soohyun, Anna
Time: 10:45AM-3:15PM and 3:45-6:45PM (7.5 hours)
Location: ABL 126, Keck 201
Goals: Organize ABL Boxes, Run minipreps in preparation of other things, Run gel of operons to see if they worked, Run digest to prep for analyzation later, Gel purify parts for Anna

Recap: Katherine and Soohyun reperformed the failed assemblies (RSF1010 and pBBR1 and multilines from the week of July 1st (reference iGEM notebook)), performed a PCR of Bacilus lactis and Sudimonus putida.

Organize Primer Boxes in ABL

Goal: Hopefully find the missing genomes (?), organize for easier location of needed materials

- Made a box with:
  - Primers
  - Antibiotics
  - H.W., UBER, parts
  - Supplies: dNTPS, operons, ribosomal *things*, buffers

Summary: We did not find the missing genomes, but organizing things will hopefully help

Miniprep UBERpp, RSF1010 & pBBR1 cassettes

Materials

- Liquid cultures from Keck 201
  - UBER pp 2.1
  - RSF1010 C 2.1
  - RSF1010 C 2.3
  - RSF1010 C 2.2
  - RSF1010 + UBER 2.4
  - RSF1010 + UBER 2.5
  - pBBR1 C 1
  - pBBR1 C 2
- Qiagen Miniprep Kit

Procedure

- Transferred part of cultures to microcentrifuge tubes and centrifuged for 30 seconds at 13,200rpm. Repeated twice.
- Labeled spin columns as above^
- Resuspended bacteria in 250uL of buffer P1 by pipetting up and down
- Added 250uL of buffer P2 to tube and invert tube 6 times
  - NOTE: We noticed some white filaments floating in the P2 buffer, so we switched to a new P2 buffer
    - RSF1010 C 2.2, UBER pp 2.1, pBBR1 C 1 received the old P2 buffer
- Added 350uL of buffer N3 to tube and insert 6 times
- Centrifuged for 10 minutes at 13,200rpm
- Applied supernatant (liquid result from previous step)
- Centrifuged for 30 seconds at 13,200rpm; discard flow-through
- Centrifuged for 30 seconds at 13,200rpm; discard flow-through
- Added 750uL of Buffer PE to the spin column
- Centrifuged for 30 seconds at 13,200rpm; discard flow-through
- Transferred the spin column to a new micro centrifuge tube
- Centrifuged for 1 minute at 13,200rpm; discard flow-through

- Placed the spin column in a new micro centrifuge tube
    - Labeled as above^^ with miniprep iGEM 7/16
- Added 50uL of nfH2O to the spin column
- Let stand for 1 minute
- Centrifuged for 1 minute

Summary: We had an issue with possibly contaminated P2. Anna brought us another bottle to use; hopefully the samples that received the old P2 will still turn out well. Otherwise, the process was normal.

Gel Purify Parts

Materials

- Gel slices from Keck 201
    - 639
    - 447
    - 437
    - 436
    - 449
- Zymoclean Gel Kit

Procedure

Followed ZymoClean Gel Kit protocol

- Added 300uL of ABD to each gel containing tube
- Incubated on 55C heat block for 10 minutes
    - NOTE: All of the gel was melted (highest temperature settings may work better)
- Labeled Collection Tubes
- Transferred melted gel to Zymo-Spin Columns in Collection Tubes
- Centrifuged for 1 minuts at 13,200rpm
- Discarded the flow through
- Added 200uL of DNA Wash Buffer to the Spin Columns*
- Centrifuged for 30 seconds at 13,200rpm*
- Discarded flow through*
- Repeated starred (*) bullets
- Labeled microcentrifuge tubes
- Placed the spin columns in the new microcentrifuge tubes
- Added 15uL of nfH2O to the column
- Centrifuged for 1 minute
- Labeled tubes: ### gel purified DNA 7/16
- Placed in green "iGEM Parts" box in tall -20C fridge in ABL 126 --- Removed and took to Anna

Summary: Using a higher temperature for the incubation helped to remove the issue with unmelted gel. The process was normal.

Nanodrop Parts, UBER, & RSF1010 cassette

Materials

- DNA (listed below)
- nfH2O

Procedure

- Took 1uL nfH2O and nanodropped as a blank
- Wiped nano drop with kimwipe
- Took 1uL of _____ and measured:
    - UBER pp 2.1 = 257.6 ng/uL
    - 436 = 0.4 ng/uL
    - 437 = 0.3 ng/uL
    - 447 = 2.0 ng/uL

- 639 = 7.2 ng/uL
- 449 = 9.2 ng/uL
- RSF1010 C 2.2 = 23.8 ng/uL

Summary: The Uber had a good reading, as did RSF1010. But, all of the parts had really low readings.

Run Gel of Ribosomal Operons

Goal: See if we see any bands. It will let us know whether or not our standard protocol PCRs worked -- if not we will need to redo them

Materials

- Ribosomal Operons (PCR products of Genomes -- standard protocol)
  - Lactis
  - Putida
  - Bacillus
- DNA Ladder
- TAE
- 0.8% Agarose mix

Procedure

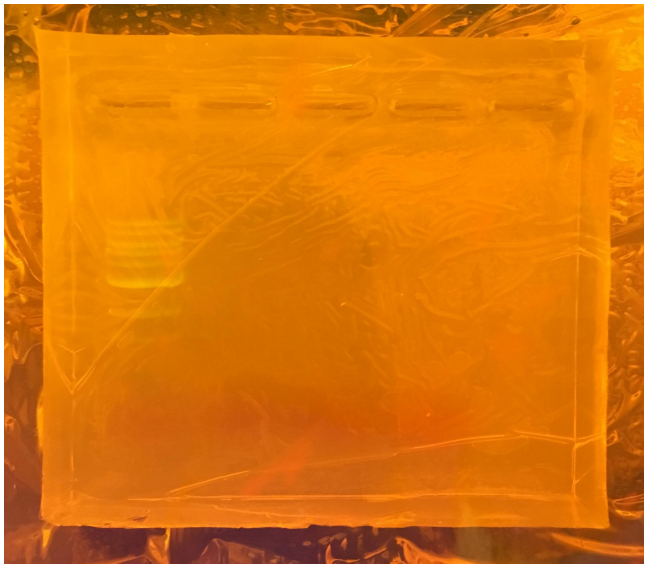
- Microwaved 0.8% agarose
- Poured ~50mL aliquot of gel
- Added 5uL of SYBER Safe dye to aliquot
- Poured gel into caster and add well mold
- Waited ~40 minutes (3:10-3:50)
- Poured 1x TAE into the chamber, up to fill line
- Placed gel into the chamber
- Added 8.3uL loading buffer to each sample
- Mixed samples using pipette tip (pipetted up and down)
- Inserted samples into gel
- Covered the chamber
- Turned on machine (122 volts)
- Left to run -- 4:12PM

Gel Electrophoresis Well Arrangement

	A	B	C	D
1	Well 1	Well 2	Well 3	Well 4
2	DNA Ladder	Lactis	Putida	Bacillus

- Stopped power source
- Removed gel and placed on UV light

IMG_8400.jpg



Summary: No bands were visible. We will need to redo the PCR, but with touchdown.

Nanodrop Miniprep Results

Materials

- Miniprep results (listed below)
- nfH2O

Procedure

- Took 1uL nfH2O and nanodropped as a blank
- Wiped nano drop with kimwipe
- Took 1uL of _____ and measured:
    - RSF1010 + UBER 2.5 = 263.3 ng/uL
    - RSF1010 C 2.3 = 52.3 ng/uL
    - pBBR1 C 1 = 121.0 ng/uL
    - RSF1010 + UBER 2.4 = 252.0 ng/uL
    - RSF1010 C 2.1 = 51.0 ng/uL
    - pBBR1 C 2 = 224.9 ng/uL

Digest of Miniprepped Cassettes

Goal:

Materials

- BsaI
- DNA (listed in table)
- Cut Smart Buffer
- nfH2O

Procedure

- Labeled PCR tubes: (corresponds to the table below)
    - RU2.4
    - RU 2.5
    - RC 2.1
    - RC 2.3
    - pBC1

- pBC2
- Added volumes listed in table below to PCR tubes: water, DNA, cut smart buffer, BsaI
  - Water and DNA added while waiting for Cut Smart Buffer to defrost in ice (can't defrost at room temperature)

Restriction Digest Volumes

	A	B	C	D	E	F
1	*Volumes = uL*	**BsaI (5 units)**	**DNA (0.5 µg)**	**Cut Smart Buffer**	**NF Water**	**Total**
2	RSF1010 + UBER 2.4	0.5	2	2.5	20	25
3	RSF1010 + UBER 2.5	0.5	1.90	2.5	20.1	25
4	RSF1010 C 2.1	0.5	9.80	2.5	12.2	25
5	RSF1010 C 2.3	0.5	9.56	2.5	12.44	25
6	pBBR1 C 1	0.5	4.13	2.5	17.87	25
7	pBBR1 C 2	0.5	2.22	2.5	19.78	25

- Incubated PCR tubes for 1hr at 37C in PCR machine (Keck 201)

Summary: Simple process, didn't run into any issues.

Assembling RSF1010 + UBER

Ligation protocol:

Table1

	A	B	C	D	E
1	Reagents	Volume (uL)		Thermocycling Temp	Time
2	447	7.7 (25 fmol)		16C	16:00
3	437	5.13 (25 fmol)		4C	hold
4	449	9.2 (25 fmol)			
5	639	9.62 (25 fmol)			
6	T4 Buffer	4			
7	T4 Ligase	2			

Repeat of antibiotic titration with higher concentrations of kan

Table3

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	0 ng/mL Kan	1	1	1	2	2	2	3	3	3			
2	50 ng/mL Kan	1	1	1	2	2	2	3	3	3			
3	100 ng/mL Kan	1	1	1	2	2	2	3	3	3			
4	200 ng/mL Kan	1	1	1	2	2	2	3	3	3			
5													
6													
7													
8													

1. Prepared 96-well plate as shown above
    a. Each well was filled with 0.5 mL base medium + 0.5% glucose
    b. Each row contains medium w/ diff. concentration of antibiotic
    c. picked a colony of each strain into wells in pattern shown above
        I. 1 = Nissle1917
        II. 2 = B. subtilis
        III. 3 = P. putida

TUESDAY, 7/17/2018

People present: Stefanie, Anna, Katherine
Time: 11:00- (lunch at 1:20-1:50) 3:00
Location: ABL 126, Keck 201
Goals: Run touchdown PCRs for bacillus, lactis, and putida, send purified strains of RSF1010 cassette 2.1 and 2.3. (with C80 primer) in for sequencing

PCR of non-model organism for ribosomal operons (Attempt 2)

Materials

- 0.2 uL PCR tubes labeled Bac TD (top B), Lact TD (top L), and Put TD (top P)
- Primers (this table shows the primers for each bacteria)

Table8

	A	B	C
1	**Organism**	**Primer**	**H2O (uL)**
2	bacillus	iG025	294
3	bacillus	iG026	234
4	c. glut	iG027	283
5	c. glut	iG028	282
6	lactis	iG029	227
7	lactis	iG030	239
8	putida	iG031	307
9	putida	iG032	264
10	meliloti	iG033	256
11	meliloti	iG037	267
12	vibrio	iG034	298
13	vibrio	iG035	261
14		AG102	216

- Genomic DNA

Procedure

- Added to tube:
  - up to 50.0 uL H2O
  - ____ uL of DNA (specified below)
  - 5 uL of forward primer and backward primer
  - 2 uL dNTPs
  - 20 uL Q5 buffer
  - 1 uL Q5 polymerase
- NOTE: we could not find the DNA for the CG, Melitloti, and the Vibrio. We will PCR these after we purfiy those genomes today
- Caculated elongation time by using 30 sec/kb, used the linked primers on benchling to find the length to do math on

Table9

	A	B	C	D	E	F	G
1		**Bacillus**	**C. glut.**	**Lactis**	**Putida**	**Meliloti**	**Vibrio**
2	**Bases**	5443	6158	5426	6254	7109	5944
3	**Annealing Temp (C)**	68.0	69.0	67.0	69.0	71.0	69.0
4	**Elongation time**	2 min 43 sec	3 min 5 sec	2 min 43 sec	3 min 8 sec	3 min 33 sec	2 min 58 sec

Table10

	A	B	C	D	E	F	G
1		**Bacillus (stock 74.5 ng/uL)**	**C. glut.**	**Lactis (stock 107.1 ng/uL)**	**Putida (stock 90.8 ng/uL)**	**Meliloti**	**Vibrio**
2	H20	16.46		16.63	16.54		
3	DNA	0.54		0.37	0.46		
4	Primers	5.0 each of 025, 026	5.0 each of 027, 028	5.0 each of 030, 029	5.0 each of 031, 032	5.0 each of 037, 033	5.0 each of 034, 035
5	dNTPS	2.0	2.0	2.0	2.0	2.0	2.0
6	Q5 buffer	20.0	20.0	20.0	20.0	20.0	20.0
7	Q5 polymerase	1.0	1.0	1.0	1.0	1.0	1.0

- Placed in Thermal Cycler in Keck
  - Touchdown:
    - We will run the touchdown protocol today because there were no visible strands when we ran the standard protocol PCRs
    - Using TD with elongation and anealing times as specified above
    - Note, the things in green are the parts of the cycle specific to this PCR

iGEMTD

	A	B	C
1	Lid Temp: 105C		
2	**Step**	**Temperature (C)**	**Time**
3	1	98	30
4	2	98	10
5	3	(start at 77, decrease to 67)	30
6	4	72	3:08
7	5	Go to Step 2	Repeat 14x
8	6	98	10
9	7	59	30
10	8	72	3:08
11	9	Go to Step 6	Repeat 30x
12	10	72	5:00
13	11	12	Hold

Sending for Sequencing

Goals: Send in RSF1010 cassette 2.1 and 2.3. (with C80 primer) for sequencing

Materials

- DNA
  - RSF1010 cassette 2.1
  - RSF1010 cassette 2.3
- nfWater
- Primers
  - C80

Procedure

- Labeled tubes: 1 and 2 (2.1 and 2.3 respectively)
- Added respective amounts of water, primer, DNA to tubes

Sample Calculations: RSF1010 Cassettes
500ng DNA / 51 and 52.3 respectively ng/uL

Table2

	A	B	C	D	E	F	G	H
1	**Sample #**	**Sample**	**Sample Length**	**Primer**	**DNA Amount (uL)**	**Primer Amount (uL)**	**Water Amount (uL)**	**Total (uL)**
2	1	RSF1010 2.1	7445 (51.0)	C80	9.8	2.5	2.7	15
3	2	RSF 10101 2.3	7445 (52.3)	C80	9.56	2.5	2.94	15

Assembling RSF1010 + UBER

1. Transformed 1 uL ligation product into DH5a cells
   a. electroporated, let rest in 1 mL SOB
   b. in 37C shaking at 3:30 pm, out at 4:50 pm
   c. Plated 100 uL of 1/100 dilution of culture on Kan50 plate
   d. in 37C incutator at 5:00 pm

Repeat of antibiotic titration using neomycin for all strains

Table6

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	0 ug/mL Neo	1	1	1	2	2	2	3	3	3	4	4	4
2	50 ug/mL Neo	1	1	1	2	2	2	3	3	3	4	4	4
3	100 ug/mL Neo	1	1	1	2	2	2	3	3	3	4	4	4
4	200 ug/mL Neo	1	1	1	2	2	2	3	3	3	4	4	4
5	0 ug/mL Neo	5	5	5	6	6	6	7	7	7			
6	50 ug/mL Neo	5	5	5	6	6	6	7	7	7			
7	100 ug/mL Neo	5	5	5	6	6	6	7	7	7			
8	200 ug/mL Neo	5	5	5	6	6	6	7	7	7			

Table7

	A	B	C	D	E	F	G	H	I	J	K	L	M
1	0 ug/mL Neo	8	8	8	9	9	9	10	10	10			
2	50 ug/mL Neo	8	8	8	9	9	9	10	10	10			
3	100 ug/mL Neo	8	8	8	9	9	9	10	10	10			
4	200 ug/mL Neo	8	8	8	9	9	9	10	10	10			
5													
6													
7													
8													

1. Prepared 96-well plate as shown above
    a. Each well was filled with 1 mL base medium + 0.5% glucose (+ ascorbate for C. glutamicum)
    b. Each row contains medium w/ diff. concentration of antibiotic
    c. picked a colony of each strain into wells in pattern shown above
        I. 1 = DH10B
        II. 2 = MG1655
        III. 3 = NIssle1917
        IV. 4 = C. glutamicum
        V. 5 = L. lactis
        VI. 6 = BL21
        VII. 7 = B. subtilis
        VIII. 8 = S. meliloti
        IX. 9 = S. oneidensis
        X. 10 = P. putida
    d. in appropriate incubator at 5:00 pm

WEDNESDAY, 7/18/2018

People present: Stefanie, Anna, Katherine, Soohyun
Time: 11:15, Meeting at 1:00, back to wetlab at 2:15 pm-
Location: Keck 201
Goals: Run the PCRs from yesterday on a gel, run another PCRs for bacillus, lactis, and putida but with 100 ng of DNA and Touch-Up cycle

Repeat of antibiotic titration using neomycin for all strains

1. Took out 96 well plates at 11:00 am
2. Transferred 100 uL of culture to shallow-well 96-well plate
3. Read OD

Assembling RSF1010 + UBER

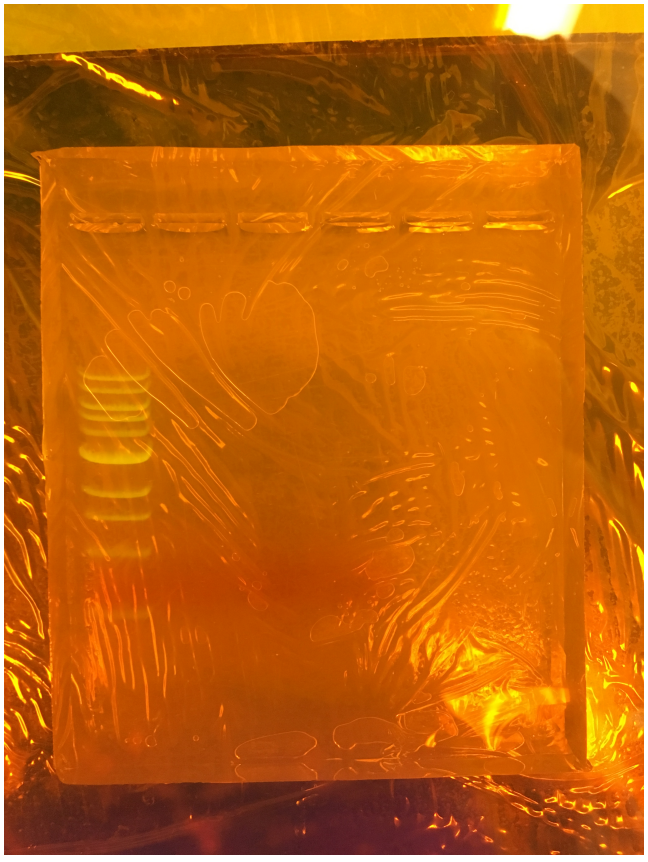
1. Took out plate at 11:00 am
2. No colonies grew on plate
    a. replated with 500 uL of undiluted transformation

b. in 37C incubator at 4:50 pm

Running PCRs on Gel attempt 2

Aand it did not work. Cool.

image.png



PCR Attempt 3

Table11

	A	B	C	D	E	F	G
1		**Bacillus (74.5 ng/uL)**	**Bacillus with DSMO**	**Lactis (107.1 ng/uL)**	**Lactis with DSMO**	**Putida (90.8 ng/uL)**	**Putida with DSMO**
2	H20	20.66	18.16	21.07	18.57	20.87	18.4
3	DNA	1.34	1.34	0.93	0.93	1.13	1.10
4	Primers	2.5 ea.	2.5 ea.	2.5 ea.	2.5 ea.	2.5 ea.	2.5 ea.
5	dNTPS	2.0	2.0	2.0	2.0	2.0	2.0
6	Q5 buffer	20.0	20.0	20.0	20.0	20.0	20.0
7	Q5 polymerase	1.0	1.0	1.0	1.0	1.0	1.0
8	DSMO	0	2.5	0	2.5	0	2.5

*Accidentally added 0.93 uL DNA initially to Putida (no DMSO). Could only add 0.2 uL additional DNA for total seen in table

(Math from Monday 7/9 calculator)

Table12

	A	B
1	**Parts**	**Volume for 40 fmol**
2	cassette vector RSF1010 (concentration 51 ng/uL)	1.29
3	pSPB501 ConLS (1)	0.02
4	HW 29572	0.04
5	pSPB460 mKate2 (4)	0.15
6	pSPB463 tVoigtS6 L3S3P00 (4)	0.01
7	pSPB512 ConR2 (6)	0.02
8	BsaI	0.5
9	T4 DNA ligase	0.5
10	T4 Buffer	1.5
11	10x BSA	1.5
12	H2O	9.47

Started in ABL thermocycler under IGEMBSAI

Purifying genomic DNA

1. Picked one colony each of C. glutamicum and S. meliloti into 4 mL of base medium + 0.5% glucose (+ ascorbate for C. glut)
2. in 37C shaking incubator at 5:00 pm

THURSDAY, 7/19/2018

People present: Stefanie, Soohyun
Time: 11:15AM-3PM (3.75 hours)
Location: ABL 126, Keck 201
Goals: Run a gel of PCR attempt 3, clean assembly, purify genome, transform assembly

Cleaned Assembly

Goal: Clean the assembly so we have the DNA to transform into cells

Materials

- Zymo DNA Clean and Concentrator Kit
- DNA: RSF1010 + H.W. 29572 + mKate assembly

Procedure

- Added assembly to a microcentrifuge tube (~ 16.7uL)
- Added 5 volumes of DNA Binding Buffer to each volume of DNA
    - Calculations
      Sample : Buffer
      1 : 5
      16.7uL : 83.5uL
- Transferred the mix to a spin column (pipetted up and down 3 times to mix)
- Centrifuged for 30 seconds at 13,000rpm
- Discarded flow through
- Added 200uL of DNA Wash Buffer to the column*
- Centrifuged for 30 seconds at 13,000rpm*
- Discarded flow through*
- Repeated starred (*) bullets
- Transferred the column to a new microcentrifuge tube
- Added 8uL of nfH2O to the column
- Incubated it for 1 minute at room temperature
- Centrifuged the tubes for 30 seconds at 13,000rpm
- Discarded column
- Labeled tube: RSF1010 assembly -- RSF1010 + H.W. 29572 + mKate iGEM cleaned 7/19

Summary: The process was simple. I didn't run into any issues.

Made More 0.8% Agarose Stock

Procedure

Desired 500mL gel at 0.8% agarose

- Measured 4.0012g agarose powder
      Calculations: 500mL * 0.008 = 4g
- Measured 500mL 1x TAE in a graduated cylinder
- Mixed agarose and TBE in a bottle
- Microwaved and observed for 30 seconds + 5 seconds + 5 seconds, swirled in between

Run Gel of Redone Ribosomal Operons- Attempt 3

Goal: Hopefully we will see some bands this time. We performed the PCRs with a touchup protocol and we also tried both with and without DMSO.

Materials

- PCR results from yesterday (Ribosomal operons)
    - Bacillus: with and without DMSO
    - Lactis: with and without DMSO
    - Putida: with and without DMSO
- DNA Ladder
- TAE
- 0.8% Agarose mix
- Loading dye (6x Gel Loading Dye)
- SyberSAFE Gel Stain

Procedure

- Microwaved 0.8% agarose stock
- Poured 2 ~50mL aliquot of gel
- Added 5uL of SYBER Safe dye to the aliquots, shook to mix
- Poured gels into casters and add well molds
- Waited

Transform Cleaned Assemblies

Goal: We want to insert our correct sequences into cells and hopefully see them expressed.

Materials

- DH5a competent cell aliquots
- Cleaned RSF1010 assembly DNA
- Cuvette
- SOB

Procedure

- Labelled cuvette and tube: RSF1010 assembly and RSF1010 + H.W. 29572 + mKate iGEM transformed 7/19
- Obtained DH5a cells from iGEM box in Keck 201 -40C fridge (middle section, 2nd from left, 2nd down)
- Waited for cells to thaw, kept on ice. Kept cuvettes on ice
- Added 2uL of DNA to cells
- Flicked to mix
- Pipetted 50uL out of cells and transferred into cuvette
- Flicked to spread cells
- Transformed cells
    - NOTE: I wasn't sure what settings to put on the machine so I didn't get a reading (I need to change the setting to milliseconds), but it began to smell like something was burned. I may have fried the cells.
- Added 1000uL LB to cuvette
    - LB not kept on ice
- Pipetted up and down to mix
- Transferred cells to a falcon tube
- Placed tubes in shaker at 37C to incubate for 1 hour (2:31)

Summary: I performed the transformation twice, the first time after transforming the cells I realized the SOB I was going to use was contaminated with bacteria. At that point the cells had waited too long and most likely would not have grown, so I started over. The second time I performed the transformation and placed the cells in a tube for incubation. The cells may have sat out to long, and as stated above^ the area smelled slightly burned after, so I don't think the transformation will be successful. I used Jordan's technique which Anna does not approve of (adding the cells to the cuvette at the bench and then moving to transform them -- Anna doesn't trust letting the cells sit in the cuvette), NEXT TIME add the cells to the cuvette right before transforming them.

Running Gel Cont.

Procedure

- Poured 1x TAE into the chambers, up to fill line
    - Made more 1x TAE stock
- Placed 1 gel into the chamber
    - 1 turned out good, the other ripped (had to redo)
- Added 8.3uL loading buffer to each sample
- Mixed samples using pipette tip (pipetted up and down)
- Inserted ladder and samples into gel
    - 1st gel had non-DMSO samples
    - 2nd gel had DMSO samples
    - Made more dyed 1 kb ladder
        - 8ul nf-water
        - 2uL DNA ladder
        - 2uL dye
        - Scaled 10x: 80uL water, 20uL ladder, 20uL dye
- Covered the chamber
- Turned on machine (122 volts)
- Left to run

Well Arrangement-3.1

	A	B	C	D
1	Well 1	Well 2	Well 3	Well 4
2	DNA Ladder	Lactis	Putida	Bacillus

Well Arrangement-3.2

	A	B	C	D
1	Well 1	Well 2	Well 3	Well 4
2	DNA Ladder	Lactis with DMSO	Putida with DMSO	Bacillus with DMSO

Summary: No bands observed in either DMSO or non-DMSO gels

Purifying genomic DNA

1. Took out culture of C. glutamicum at 11:00 am
2. Culture of S. meliloti is still clear
    a. left in 30C incubator
3. Purified C. glutamicum using Promega Wizard SV genomic purification kit
    a. Stored in ABL -20 freezer in blue iGEM box
    b. Made master mix by combining 500 uL Nuclei Lysis Solution and 7 uL RNase in 1.5 mL tube
    c. Incubated at 80 C in heatblock with water added to one of the wells

DNA Gel of Bacillus, lactis, and putida

No bands in either with DMSO or without DMSO samples
Note: Soohyun might have let the DMSO gel run too long. It's probably fine.

Assembling RSF1010 + UBER

1. Took out plate at 11:00 am
2. Colonies grew on plate
    a. Under blue light, about 10 green colonies could be seen
3. Picked 3 of the green colonies into 4 mL LB
4. Put cultures in 37C shaking incubator at 4:00 pm

Antibiotic titration with plates

1. PIcked one colony of all strains into 2 mL base medium + 0.5% glucose (+ ascorbate for C. glut)
    a. in appropriate shaking incubator at 5:00 pm
2. Prepared 250 mL of base medium agar (LB + 220 mg/L MgSO4 + 0.5% glucose + 1% vitamin and mineral supplements)
    a. per Shyam's instructions:
        I. added Kan to 200 ug/mL in 80 mL of agar (A)
        II. used 40 mL of that to make two Kan200 plates
        III. added 40 mL of plain base medium agar (B) + mixed

IV. used 40 mL of new mixture A to make two Kan100 plates
V. added 40 mL B to A again
VI. used 40 mL of A to make two Kan50 plates
VII. added 40 mL B to A again
VIII. used 40 mL of A to make two Kan12.5 plates
IX. let plates cool

Making competent cells

1. Made 5 mL cultures of DH10B, BL21, MG1655, Nissle1917
    a. in shaking 37C incubator at 5:00 pm
2. Autoclaved LB and 10% glycerol

Transforming piG016 (RSF1010 + HW 29572 + mKate)

1. Stefanie transformed via electroporation DH5a cells with RSF1010 + HW 29572 + mKate assemblies made yesterday
    a. Prediction: We're not sure if the cells will grow due to complications with transforming the cells.
2. Incubated for 1 hr in shaking incubator at 37 C
3. Plated on KanR plate and left in ABL 37 C incubator at 4:30 pm. Will check this at 10:00 am tomorrow.

Part transformations

Transformed 479 (a,b) and 450 parts (to miniprep later) into E. coli MG1655 cells. Plated on Chl34 after 1h of incubation

Making Competent Cells

Anna: making corynebacterium and Lactococcus competent cells

Preparing media:

- 500 mL of LB with 0.5 % glucose
- 15 % glycerol (150 mL + 850 mL)
- LBG + 2.5% glycine + 4 mg/ml isonicotinic acid hydrazid
- 10% glycerol + 0.5 M sucrose
- LB + 1% glycine
- LB + 0.5M sucrose + 10 mM MgCl2 + 2 mM CaCl2

Made 5 mL liquid cultures of Corynebacterium and Lactococcus

Also streaked out Sinorhizobium and P. putida on LB plate (took P. putida directly from Eric's stock and made a negative control plate with Kan50)

FRIDAY, 7/20/2018

RK2 and pBBR1 cassette vector assemblies

Cassette vector without UBER

	A	B	C	D	E
1					
2	**RK2**			**pBBR1 (a,b)**	
3	**Part**	**µL**		**Part**	**µL**
4	480	0.5		479	0.5
5	616	0.3		616	0.3
6	437	0.2		437	0.2
7	447	0.37		447	0.37
8					
9					
10	BsaI	0.5		BsaI	0.5
11	T4 DNA Ligase	0.5		T4 DNA Ligase	0.5
12	T4 Buffer	1.5		T4 Buffer	1.5
13	10X BSA	1.5		10X BSA	1.5
14	H20	9.42		H20	9.42
15					

Used end-on ligation thermocycling protocol

thermocycling protocol:

End ligation.png

End-On-Ligation Golden Gate Assembly

	A	B	C	D
1		Step	Temp	Time
2		Initial Digestion (opt.)	37°C	10 min
3	Repeat25×	Digestion	37°C	1.5 min
4		Annealing & Ligation	16°C	3 min
5		Storage	16°C	∞

Samples labeled RK2 (R), 479 a (a), and 479 b (b) and running in ABL 126 thermocycler

C. glut. PCR

Prepared standard and Touchdown samples in single tube and split into two tubes

Table13

	A	B
1		**C. glut.**
2	H20	19
3	DNA	3
4	Primers	5.0 each of 027, 028
5	dNTPS	2.0
6	Q5 buffer	20.0
7	Q5 polymerase	1.0

Standard protocol

iGEM3

	A	B	C
1		Temperature (C)	Time
2	Initial Denaturation	98	30 sec
3	Denaturation	98	10 sec
4	Annealing	69	30 sec
5	Extension	72	3 min, 5 sec
6	Repeat 34 times		
7	Final Extension	72	2 min
8	Hold	12	

Touchdown

Table15

	A	B	C
1	Lid Temp: 105C		
2	**Step**	**Temperature (C)**	**Time**
3	1	98	30
4	2	98	10
5	3	(start at 72, decrease to 64)	30
6	4	72	3:05
7	5	Go to Step 2	Repeat 14x
8	6	98	10
9	7	59	30
10	8	72	3:05
11	9	Go to Step 6	Repeat 30x
12	10	72	5:00
13	11	12	Hold

Antibiotic titration with plates

1. Spotted 25 uL of cultures of all strains onto plates prepared on 7/20
    a. P. putida, S. oneidensis, and S. meliloti were put on the same Kan0, Kan12.5, Kan25, Kan50, Kan100, and Kan200 plates
    b. DH10B, MG1655, BL21, Nissle1917, L. lactis, C. glutamicum, B. subtilis on the same Kan0, Kan12.5, Kan25, Kan50, Kan100, and Kan200 plates
    c. In appropriate incubator at 1:55 pm

Assembling RSF1010 + UBER

1. Took out cultures at 11:15 am
2. Miniprepped all three, eluted with 50 uL dH2O
    a. [RSF1010+UBER 1] = 80.7 ng/uL
    b. [RSF1010+UBER 2] = 101.5 ng/uL
    c. [RSF1010+UBER 3] = 91.1 ng/uL
3. Digest w/ BsaI:

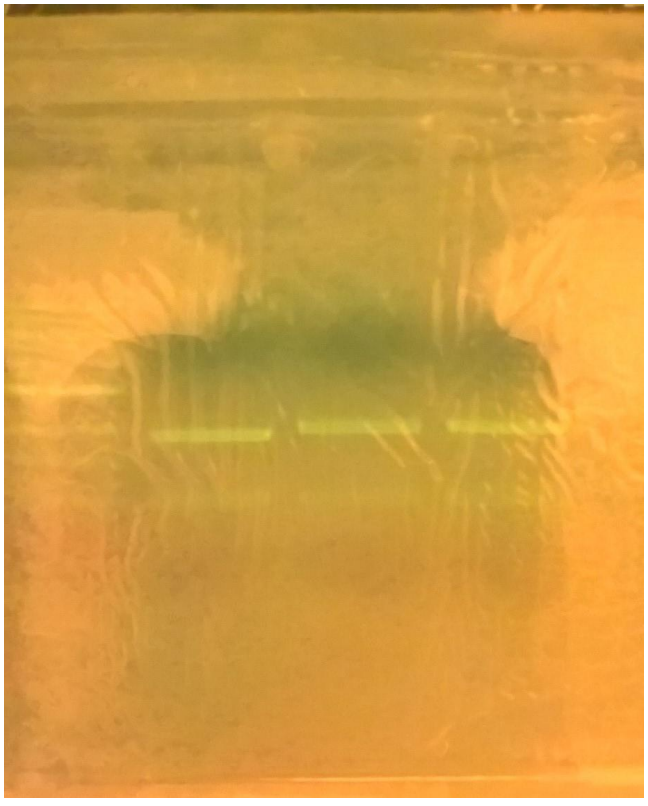
Table14

	A	B	C	D
1	Reagent	RSF1010UBER1	RSF1010UBER2	RSF1010UBER3
2	DNA	6.2	4.9	5.5
3	10X Cutsmart Buffer	2	2	2
4	BsaI	0.5	0.5	0.5
5	dH2O	16.3	17.6	17
6	Total	20 uL	20 uL	20 uL

4. Ran on 0.8% agarose gel, 100V
    a. wells from left to right: 1 kb ladder, RSF1010+UBER1, RSF1010+UBER2, RSF1010+UBER3
    b. start 6:10, end 6:50 pm

Expected band sizes: 1 band at 10 kB, 1 band at 1 kB
Results: 1 band at 1 kB, 1 band at 2 kB

RSF1010 UBER BsaI digest.jpg



Parts Digest

1. piG012 - modified UBER part plasmid

2. 639 (original UBER part plasmid)
3. 436
4. 437
5. 447 (KanR)
6. 449 (RSF1010)

Table16

	A	B	C	D	E	F
1	*Volumes = uL*	**BsaI (5 units)**	**DNA (0.5 µg)**	**Cut Smart Buffer**	**NF Water**	**Total**
2	piG012	0.5	3.0	2.5	19	25
3	639	0.5	6.5	2.5	15.5	25
4	437	1	10	2.5	11.5	25
5	436	1	10	2.5	11.5	25
6	449	1	12.0	2.5	9.5	25

Transformations

Transformed assemblies for cassettes and 639 part plasmid into E. coli DH5a. Plated assemblies on Kan50 plates and part plasmid on Chl34.

SATURDAY, 7/21/2018

RK2 and pBBR1 cassette assemblies with UBER

Cassette vector with UBER

	A	B	C	D	E
1					
2	**RK2**			**pBBR1**	
3	**Part**	**µL**		**Part**	**µL**
4	480	0.5		479	0.5
5	639	0.5		639	0.5
6	437	0.5		437	0.5
7	447	0.37		447	0.37
8					
9					
10	BsaI	0.5		BsaI	0.5
11	T4 DNA Ligase	0.5		T4 DNA Ligase	0.5
12	T4 Buffer	1.5		T4 Buffer	1.5
13	10X BSA	1.5		10X BSA	1.5
14	H20	8.92		H20	8.92
15					

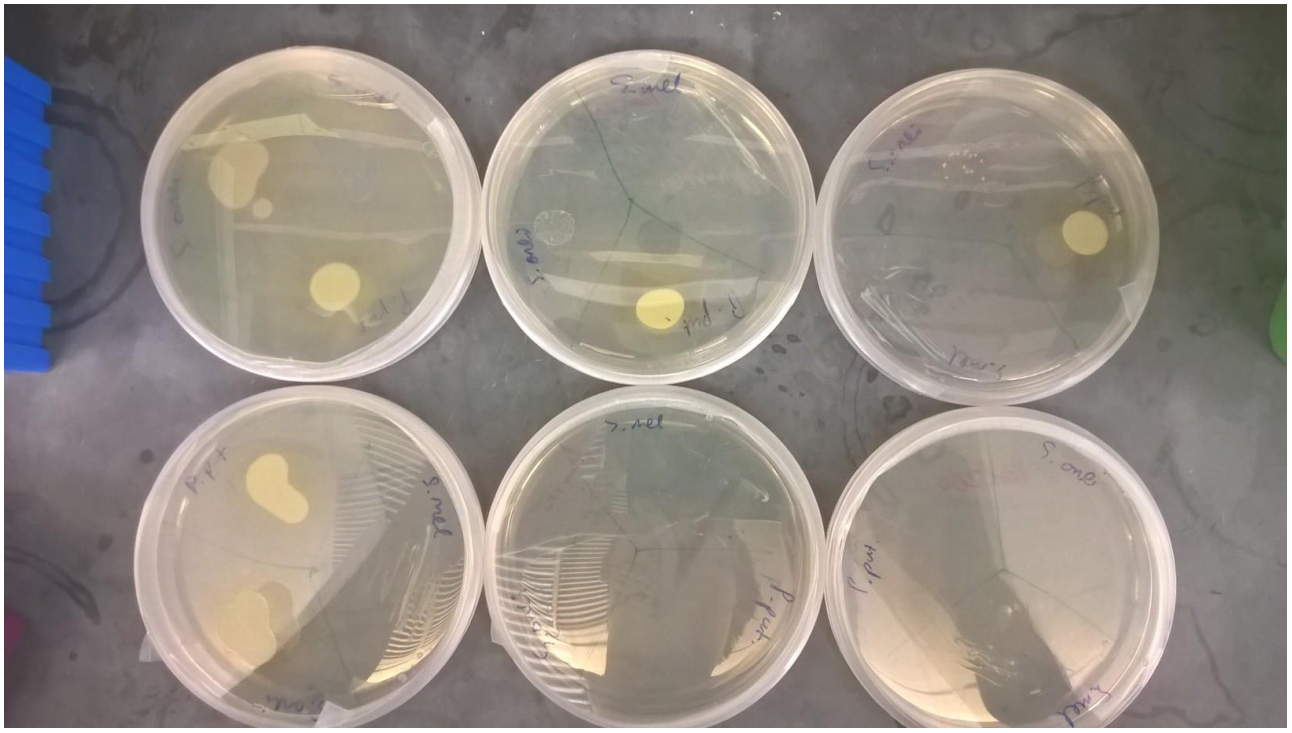
RK2 and pBBR1 cassette vector assemblies

picked 3 green colonies for each plate and cultured in 4 mL LB + Kan50. Also made a liquid culture for pSBP430 as a control (cassette vectors should be less green)

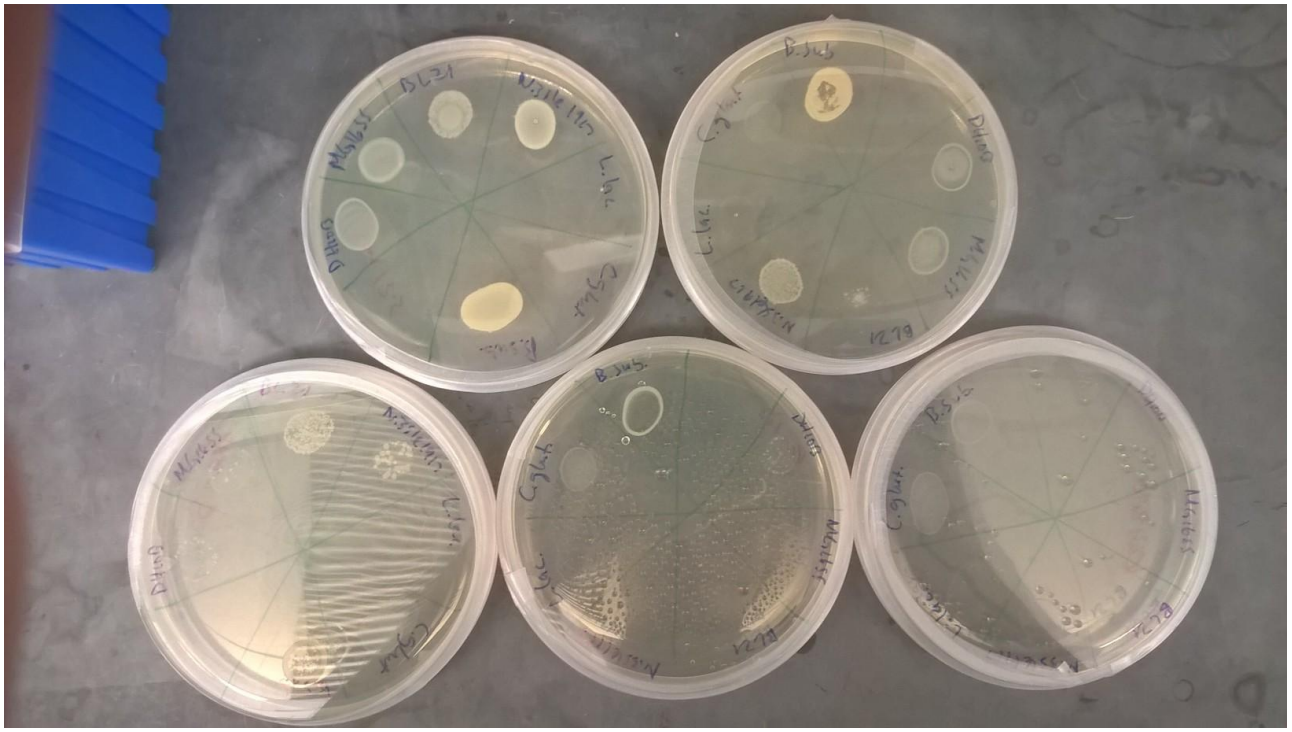
Antibiotic titration with plates

1. Took out plates at 10:30 am
2. Results

kan plate titration 30C.jpg



kan plate titration.jpg



SUNDAY, 7/22/2018

Week of July 23

MONDAY, 7/23/2018

People present: Stefanie, Anna, Stefanie
Time: 11:30AM-2:30PM and 3-6:15 (6.25 hours)
Location: Keck 201, ABL 126
Goals: Make liquid cultures of Anna's transformed assemblies and cassettes, Miniprep RK2 and 639 and nano drop, run digest of parts and run gel to analyze

Results: We think the transformations from Thursday worked! We got red colonies, meaning we at least were able to transform mKate into the cells successfully.

Make Liquid Cultures

Goal: Make liquid cultures of transformed cells Anna made yesterday so we can see more clearly whether the transformations worked or not.

Materials

- Plates:
  - pBBR1 + UBER
  - RK2 + UBER
  - pBBR1 Cassette a & b
- Kan50
- LB

Procedure

- Anna labeled microcentrifuge tubes:
  - pBBR1 (1, 2, 3) iGEM 7/23
  - RK2 (1, 2, 3) iGEM 7/23
  - pBBR1 + UBER (1, 2, 3, 4, 5) iGEM 7/23
- (Worked in hood) Added 4mL LB
- Added 4uL Kan50 and shook slightly
- Picked green colonies from the plates (Anna had them circled)
  - 3 from pBBR1 + UBER
  - 3 from RK2 + UBER
  - 5 from pBBR1 cassette A and B
  - NOTE: tried to make sure I didn't gauge into the plate, worked for some, not others
- Placed toothpick in tube
- Placed all tubes in 37C shaker in Keck 201 at about 12:15PM

Summary: I'm curious to see how everything turns out. I didn't run into any issues; just the normal process.

Miniprep RK2 and 639

Goal: Miniprep RK2 for sequencing, and 639 to run a digest on with other parts.

Materials

- Qiagen Miniprep Kit
- Liquid cultures from Keck 201
  - RK2 cassette vector
  - 639

Procedure

- Transferred part of cultures to microcentrifuge tubes and centrifuged for 30 seconds at 13,200rpm. Repeated twice.
- Labeled spin columns as above^
- Resuspended bacteria in 250uL of buffer P1 by pipetting up and down

- Added 250uL of buffer P2 to tube and inverted tube 6 times
- Added 350uL of buffer N3 to tube and inverted 6 times
    - NOTE: 639 may have gotten less than 350uL, when pipetting out the liquid seemed to stop (like if there was a bubble) and then continue. When I compared the 2 microcentrifuge tubes for approx. volumes they looked the same
- Centrifuged for 10 minutes at 13,000rpm
- Applied supernatant (liquid result from previous step) to spin column
    - NOTE: white particles floating in supernatant, so I centrifuged again for about 45 seconds. Particles were still floating so I moved on to the next step
- Centrifuged for 30 seconds at 13,000rpm; discarded flow-through
- Centrifuged for 30 seconds at 13,000rpm; discarded flow-through
- Added 750uL of Buffer PE to the spin column
- Centrifuged for 30 seconds at 13,000rpm; discarded flow-through
- Transferred the spin column to a new micro centrifuge tube
- Centrifuged for 1 minute at 13,000rpm; discarded flow-through
- Placed the spin column in a new micro centrifuge tube
    - Labeled as above^^ with miniprep iGEM 7/23
- Added 50uL of nfH2O to the spin column
- Let stand for 1 minute
- Centrifuged for 1 minute at 13,000rpm

Nanodrop

Procedure

- Took miniprepped DNA to Keck 301
- Took 1uL nfH2O and nanodropped as a blank
- Wiped nano drop with kimwipe
- Took 1uL of _____ and measured:
    - RK2 cassette vector = 196.6ng/uL
    - 639 = 465.1ng/uL

Summary: Ran into some issues: 1- suspicious of the amount of N3 added to 639 due to weird pipetting 2- white particles were floating in both supernatant liquids, I'm not sure if it affected the minipreps. Based on nano drop results, the minipreps turned out well.

Restriction Digest

Goal: Cut DNA so that it can be analyzed in a gel.

Materials

- BsaI
- Cut Smart Buffer
- nfH2O
- DNA
    - piG012
    - 639
    - 437
    - 436
    - 449

Procedure

- Labeled PCR tubes as shown in the table below
- Added volumes listed in table below to PCR tubes: water, DNA, cut smart buffer, BsaI
    - Water and DNA added while waiting for Cut Smart Buffer to defrost in ice (can't defrost at room temperature)

Table14

	A	B	C	D	E	F
1	*Volumes = uL*	**Bsal (10units)**	**DNA (1000 ng)**	**Cut Smart Buffer**	**NF Water**	**Total**
2	piG012	1	3.06	5	40.94	50
3	639	1	2.15	5	41.85	50
4	437	2	8	5	35	50
5	436	2	8	5	35	50
6	449	2	7.39	5	35.61	50

piG012 = (refactored UBER part plasmid)
NOTE: nfH2O was rounded to tenths place

- Incubated PCR tubes for 1hr at 37C in PCR machine (ABL 126)

Summary: We didn't run into any issues. Normal, simple process.

Soohyun prepared RK2 DNA for sequencing.

Run Gel of Digest

Goal: Run the samples to see if our sequences are correct.

Materials

- 1% agarose mix
- Gel stain
- DNA ladder
- Loading dye
- DNA:
  - piG012
  - 639
  - 437
  - 436
  - 449

Procedure

- Soohyun made 1 gel (stock bottles were empty)

Made More 1.0% Agarose Stock

Desired 500mL gel at 1.0% agarose

- Measured g agarose powder
  Calculations: 500mL * 0.01 = 5g
- Measured 500mL 1x TAE in a graduated cylinder

Make More 1x TAE

NOTE: Used all of stock bottle (~300mL)

- Calculations:
  c1v1=c2v2
  50x * v1 = 1x * 1L
  v1 = .02L = 20mL
- Added 20mL of 50x TAE to a bottle
- Added 1L of diH2O to the bottle
- Shook to mix

Made More 1.0% Agarose Stock Cont.

- Mixed agarose and TBE in a bottle
- Microwaved and observed for 30 seconds + 5 seconds + 5 seconds, swirled in between

NOTE: Anna said we made too much, so make less stock next time.

Run Gel Cont.

- We made a second gel
- Waited for gels to solidify
- Added new 1x TAE to two electrophoresis chambers
- Added gels to the chambers
- Added 8.3uL of loading dye to the samples
- Pipetted up and down to mix
- Added 15uL of 1kb DNA ladder and DNA samples to the gels

Well Arrangement- Gel 1

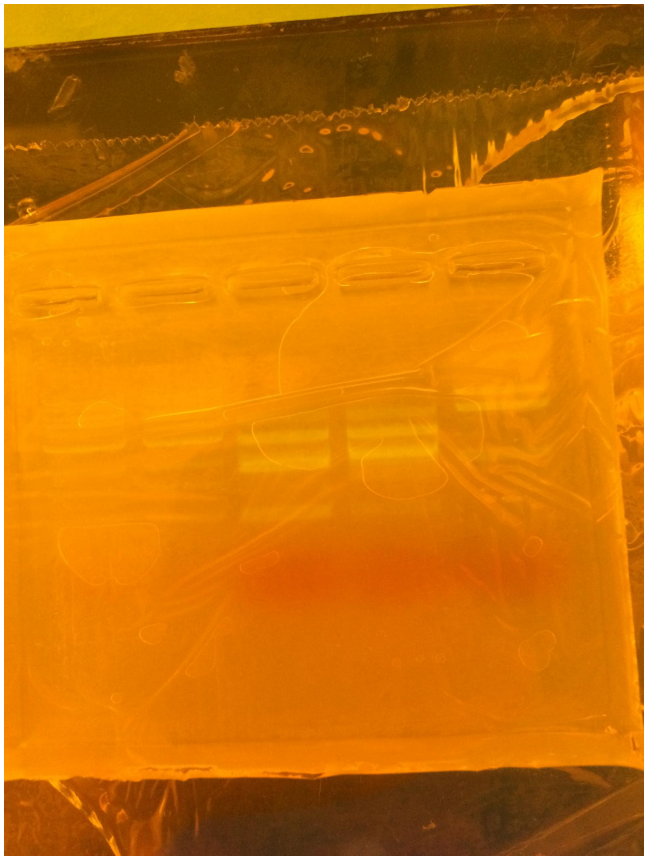
	A	B	C	D	E
1	Well 1	Well 2	Well 3	Well 4	Well 5
2	DNA Ladder	DNA Ladder	437	436	639

Well Arrangement- Gel 2

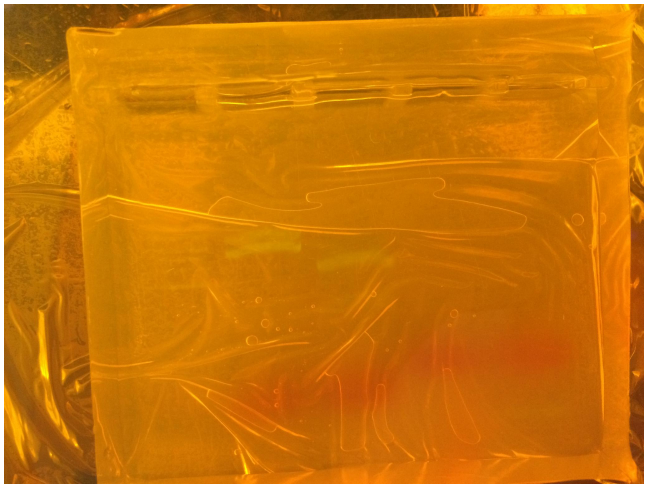
	A	B	C	D	E
1	Well 1	Well 2	Well 3	Well 4	Well 5
2	DNA Ladder	449	piGo12	Corynebacterium PCR	Corynebacterium PCR- TD

- Turned on machine and let the gel run
- Turned off machine and placed gels on UV reader

Gel 1.jpg



Gel 2.jpg



Band Locations

	A	B	C	D
1	**Gel**	**Sample**	**Band**	**Location**
2	Gel 1	437	1	4 kb
3			2	3 kb
4			3	1.8 kb
5			4	1 kb
6		436	1	4 kb
7			2	3 kb
8			3	2 kb
9			4	1 kb
10		639	1	above 1st line on ladder
11			2	5 kb
12			3	4 kb
13			4	1.6 kb
14	Gel 2	449	1	8 kb
15			2	5.5 kb
16		piG012	1	4.5 kb
17			2	past 3.0 kb (~2kb)
18	Cornynebactrium	PCR & PCR-TD	None	N/A

- Covered gels in plastic wrap and left in 4C fridge in Keck 201:
  - Gel #1 iGEM 7/23
  - Gel #2 iGEM 7/23

Summary: We kept running out of stock and had to keep remaking it; the process took much longer than it normally would (and should have).

Parts Digest

Table16

	A	B	C	D	E	F
1	*Volumes = uL*	**Bsal (10units)**	**DNA (1000 ng)**	**Cut Smart Buffer**	**NF Water**	**Total**
2	piG012	1	3.06	5	40.94	50
3	639	1	2.15	5	41.85	50
4	437	2	8	5	35	50
5	436	2	8	5	35	50
6	449	2	7.39	5	35.61	50

Incubated at 37 C for 1 hr (2:30 - 3:30 PM)

- Stopped cycle at 2:35 to add 2 uL more DNA to 437 and 436; replaced them immediately back in thermocycler

RK2 Sequencing

Mixed:

- 2.54 uL DNA
- 2.5 uL C80 primer
- 9.96 uL NF H2O

TUESDAY, 7/24/2018

People present: Stefanie, Soohyun, Anna
Time: 11:45AM-1:45PM and 2:15-4:15 (4 hours)
Location: Keck 201, ABL 126
Goals: Miniprep pBBR1 + UBER and send it for sequencing, Resuspend new primers we received, Assemble the multigenerational vector with UBER

Miniprep pBBR1 + UBER

Materials

- pBBR1 + UBER liquid culture from Keck 201
- Qiagen Miniprep kit

Procedure

- Transferred part of cultures to microcentrifuge tubes and centrifuged for 30 seconds at 13,000rpm. Repeated twice.
- Labeled spin column as above^
- Resuspended bacteria in 250uL of buffer P1 by pipetting up and down
- Added 250uL of buffer P2 to tube and inverted tube 6 times
- Added 350uL of buffer N3 to tube and inverted 7 times
- Centrifuged for 10 minutes at 13,000rpm
- Applied supernatant (liquid result from previous step) to spin column
- Centrifuged for 30 seconds at 13,000rpm; discarded flow-through
- Centrifuged for 30 seconds at 13,000rpm; discarded flow-through
- Added 750uL of Buffer PE to the spin column
- Centrifuged for 30 seconds at 13,000rpm; discarded flow-through
- Transferred the spin column to a new micro centrifuge tube
- Centrifuged for 1 minute at 13,000rpm; discarded flow-through
- Placed the spin column in a new micro centrifuge tube
    - Labeled as above^^ with miniprep iGEM 7/24
- Added 50uL of nfH2O to the spin column
- Let stand for 1 minute
- Centrifuged for 1 minute at 13,000rpm

Nanodrop

Procedure

- Took miniprepped DNA to ABL 128
- Took 1uL nfH2O and nanodropped as a blank
- Wiped nano drop with kimwipe
- Took 1uL of _____ and measured:
    - pBBR1 + UBER = 198.6 ng/uL

Summary: I didn't run into any issues. Simple process; went relatively quickly with only 1 sample.

Send Miniprep results for Sequencing

Goal: Sequence our pBBR1 + UBER assembly to see if the parts are assembled correctly.

Materials

- DNA: pBBR1 + UBER
- nfWater
- Primer: C80

Procedure

- Labeled tubes: 1
- Added water, primer, DNA to tube

Calculations: pBBR1 + UBER

500ng DNA / 198.6 ng/uL = 2.517 uL DNA

Table15

	A	B	C	D	E	F	G	H	I
1	**Sample #**	**Sample**	**Sample Length**	**Primer**	**[DNA] (ng/uL)**	**DNA Amount (uL)**	**Primer Amount (uL)**	**Water Amount (uL)**	**Total (uL)**
2	1	pBBR1 + UBER	6001-8000	C80	198.6	2.52	2.5	9.98	15

- Printed Order Form
- Placed in bag with DNA

Summary: Simple process, no issues. My computer is now connected to the printer in Keck 201, so I can print order forms rather than having Anna do it every time.

Make Primer Aliquots

Goal: Make new primer aliquots for future use.

Materials

- Primer stock tubes
- nfH2O

Procedure

- Centrifuged stock primer tubes for 30 seconds at 13,000rpm

NOTE: performed starred (*) bullets individually for each tube and repeated

- Added water specified on papers accompanying the primers*
- Vortexed the stock tubes*
- Pipetted up and down several times*
- Transferred 10uL of primer to a microcentrifuge tube*
- Labeled the tubes E## or iG0## according to table below*
- Added 90 uL of water to each new tube
    - NOTE: added to all tubes consecutively)

Primer Resuspension

	A	B
1	Primer	Water Added (uL)
2	iGEM_E15	200
3	iGEM_E16	235
4	iGEM_E17	241
5	iGEM_E18	244
6	iGEM_E19	191
7	iGEM_E20	246
8	iGEM_E21	250
9	iGEM_E22	229
10	iGEM_E23	298
11	iGEM_E24	233
12	iGEM_E25	254
13	iGEM_E26	201
14	iGEM_E27	283
15	iGEM_E28	304
16	iGEM_E35*	212
17	iGEM_E36*	362
18	iGEM_iG049	271
19	iGEM_iG050	264
20	iGEM_iG052	268
21	iGEM_iG053	217

* (in table) = Anna took before I resuspended them

Soohyun performed Golden Gate Assemblies (Reference Week of July 23)

- RSF1010 + UBER
- RSF1010 + UBER modified
- RK2 multigene
- pBBR1 multigene

Multigene vectors with UBER

Multigene Vector

	A	B	C	D	E
1					
2	**RSF1010 + UBER (1)**			**RSF1010 + UBER (modified) (2)**	
3	**Part**	**µL**		**Part**	**µL**
4	449	**0.61**		449	0.61
5	639	**0.2**		piG012	**0.2**
6	436	**0.2**		436	0.2
7	447	**0.25**		447	0.25
8					
9					
10	BsaI	0.5		BsaI	0.5
11	T4 DNA Ligase	0.5		T4 DNA Ligase	0.5
12	T4 Buffer	1.5		T4 Buffer	1.5
13	10X BSA	1.5		10X BSA	1.5
14	H20	9.53		H20	9.53
15					

Multigene vectors RK2 and pBBR1:

Table1

	A	B	C	D	E
1					
2	**RK2 (3)**			**pBBR1 (4)**	
3	**Part**	**µL**		**Part**	**µL**
4	480	0.2		479	**0.2**
5	616	0.2		616	0.2
6	436	**0.2**		436	**0.2**
7	447	**0.25**		447	**0.25**
8					
9					
10	BsaI	0.5		BsaI	0.5
11	T4 DNA Ligase	0.5		T4 DNA Ligase	0.5
12	T4 Buffer	1.5		T4 Buffer	1.5
13	10X BSA	1.5		10X BSA	1.5
14	H20	9.94		H20	9.94
15					

Ran on BsaI protocol

BsaI digest of 480, 479, and pBBR1 + UBER cassette vector

Table2

	A	B	C	D
1		pBBR1 + UBER (uL)	479 (uL)	480 (uL)
2	DHA	2.5	2	2
3	Cutsmart	2	2	2
4	BsaI	0.5	0.5	0.5
5	H2O	15	15.5	15.5
6	Total	20	20	20

1. Put reagents in PCR tubes; amounts in table
2. Put in 37C incubator; start 4:45 pm, end 5:45 pm
3. Ran on gel

PCR of RSF1010 + 29572 HW + mKate

Table3

	A	B	C	D	E
1	Reagent	Amount (uL)		Thermocycler Temp (C)	Time
2	Template (RSF1010 + 29572 + mKate)	0.2		98	0:30
3	F primer (E35)	2	25X	98	0:10
4	R primer (E36)	2		60	0:30
5	dNTPs	2		72	1:30
6	5X HF buffer	10		72	10:00
7	Phusion	0.5		10	hold
8	dH2O	33.3			
9	Total	50			

1. Put all reagents into PCR tube as specified in table
2. Put tube in thermocycler; program specified in above table
3. Ran on gel w/ BsaI digests of 480, 479, and pBBR1 + UBER cassette vector

a very faint band observed, but below 2 kb. Repeat PCR with both phusion and Q5 + control

RSF1010 multigene: D51, D53 // Tm = 65 C // length = 2200
mKate cassette: E35, E35; Tm = 60 C; length = 2200

Table13

	A	B	C	D	E	F	G	H
1		Phusion HF buffer	dNTPs	Fwd Primer	Rev Primer	Phusion DNA polymerase	template	H2O
2	PCR components	10	1	2.5	2.5	0.5	0.2	33.3

Oligo Anneal for T7 promoter and terminator

anneal the two by combining the oligos at 1 µM in water, 1 µL of each in 100 µL
Run the rxn on the annealing program: 98° and cool to 25° at the minimum ramp rate, 0.1°/s.

make a 10–15 µL rxn with 1× T4 ligase buffer, 0.5 µL T4 PNK (polynucleotide kinase), and a tenth volume of the oligo anneal (final 100 nM). Incubate 30 min 37°. Use 0.5 µL in the Golden Gate (50 fmol).

16S rRNA PCR

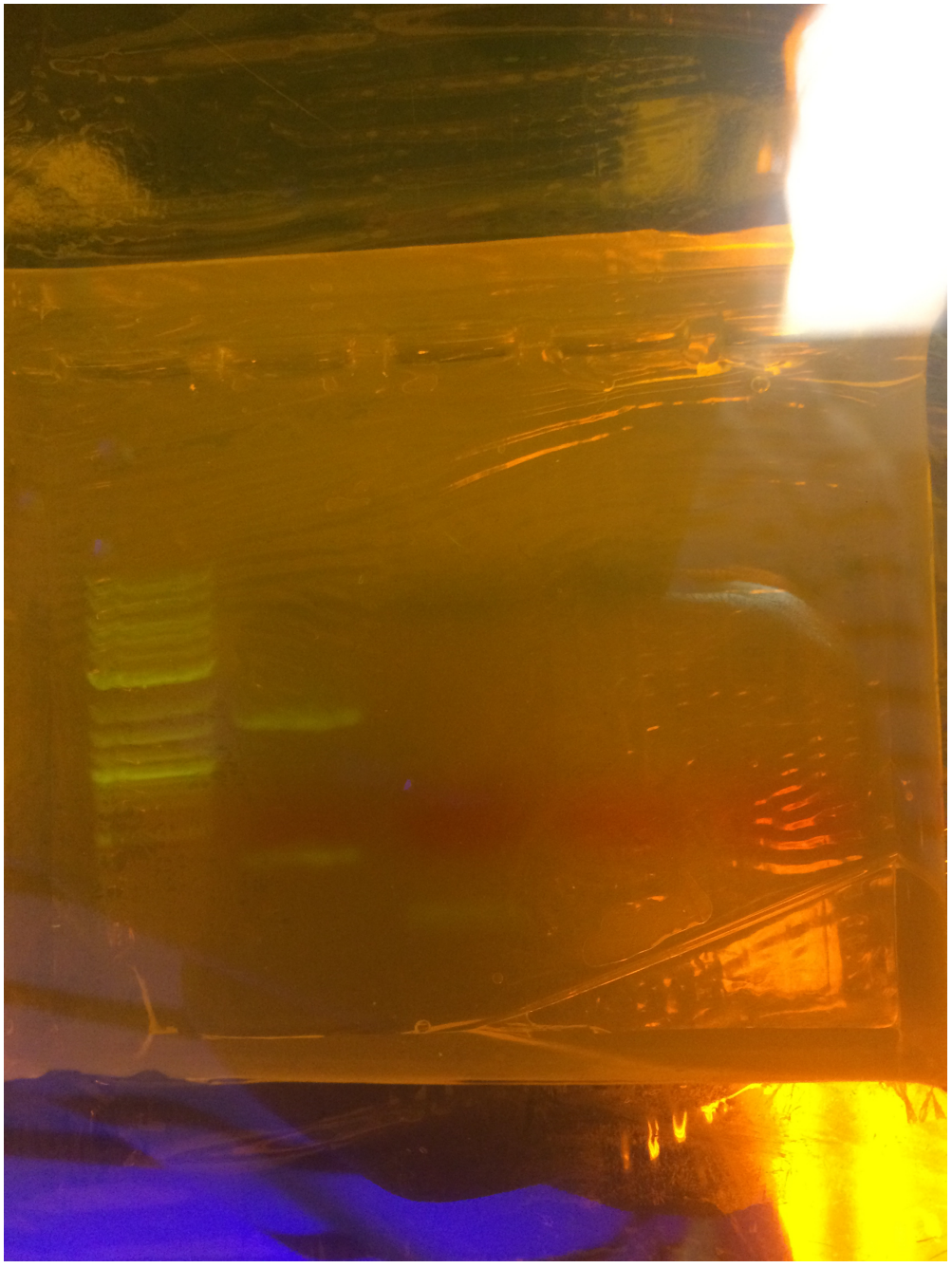
Table4

	A	B	C	D	E	F	G	H
1		Phusion HF buffer	dNTPs	Fwd Primer	Rev Primer	Phusion DNA polymerase	template	H2O
2	PCR components	10	1	2.5	2.5	0.5	0.2	33.3

Primers: 49,50 for the large fragment and 52, 53 for the small one.

WEDNESDAY, 7/25/2018

image.png



Assembling TZ terminator and LacO promoter

Instructions from Shyam:

"So for tZ, run 20 µL PCRs on P1/P2, P3/P4, and P5/P6, Tanl=65°, maybe 15 cycles (there's no exponential amplification here). You can overdo the primers a few-fold if hard to pipette; there's no mispriming probability without template.

These three fragments then must be spliced together by overlap extension (SOEing PCR), with terminal primers only exponentially amplifying the full, spliced product:

Combine like 0.5 µL of the three PCRs in a fourth PCR with P1/P6, TanI=65° for 10 cycles and no priming step for the remaining 20 cycles, since the spliced product should've formed enough by 10 cycles to be a template for the P1/P6.
15 s extension everywhere.

Then gel-purify the 250(?) bp band and GG into 430."

First PCR

Table6

	A	B	C	D	E	F	G	H
1		Phusion HF buffer	dNTPs	Fwd Primer	Rev Primer	Q5 DNA polymerase	template	H2O
2	PCR components	10	1	2.5	2.5	0.5	0	33.5
3	**for 20 uL**	4	0.4	1	1	0.2	0	13.4
4	MM	13.2	1.32	3.3	3.3	0.66	0	44.22

Table7

	A	B	C	D	E	F	G	H
1	**Step #**	1	2	3	4	5	6	7
2	**Temp**	98C	98C	65	72C	GO TO Step 2	72C	4C
3	**Time**	0:30	0:10	0:30	0:15	15X	5:00	hold

Second PCR

Thermocycling conditions

	A	B	C	D	E	F	G	H	I	J	K
1	**Step #**	1	2	3	4	5	6	7	8	9	10
2	**Temp**	98C	98C	65	72C	GO TO Step 2	65	72C	GO TO step 6	72C	4C
3	**Time**	0:30	0:10	0:30	0:15	10X	0:30	0:15	20X	5:00	hold

Golden Gate Assemblies

(1) Assembling part plasmids 445, 448, 632, and 642
(2) 16S rRNA part plasmid
(3) promoter and terminator part plasmids

Table5

	A	B	C	D	E	F
1						
2	**445**			**448**		
3	**Part**	**µL**		**Part**	**µL**	
4	445a	**0.5**		449a	0.27	
5	445b	**0.25**		448b	**2.2**	
6	430	**0.5**		430	0.5	
7						
8	H20	9.75		H20	8.03	
9						
10	**478**			**632**		
11	**Part**	**µL**		**Part**	**µL**	
12	478	**1**		632	**0.25**	
13	430	**0.5**		430	**0.5**	
14						
15						
16	H20	9.5		H20	10.25	
17						
18	**promoter**			**terminator**		
19	**Part**	**µL**		**Part**	**µL**	
20	promoter	**0.5**		terminator	**0.5**	
21						
22	430	**0.5**		430	**0.5**	
23						
24	H20	10		H20	10	
25						
26	**ribosomal operon**					
27	**Part**	**µL**				
28	short fragment	**0.2**				
29	long fragment	**1.22**				
30	430					
31						
32	H20	9.58				

amounts of Esp3I, T4 ligase buffer, T4 ligase, BSA as ususal.

purified using Zymo kit and transformed into *E. coli* MG1655

RSF1010 + HW + mKate digest

Digest looks correct.

Multigene vectors with UBER colony PCR

Primers:

(a) RK2 multigene: B05, D75 // Tm = 76 C // Product size = 2300 (Do this later, no green colonies for this assembly)
(b) RSF1010 + UBER: A38, D53 // Tm = 67C // Product size = 1400
(c) control: B17, B18// Tm = 66C // Product size = 1100

Table10

	A	B	C	D	E	F	G	H
1		Phusion HF buffer	dNTPs	Fwd Primer	Rev Primer	Phusion DNA polymerase	template	H2O
2	PCR components	10	1	2.5	2.5	0.5	1	32.5
3	PCR components	5	0.5	1.25	1.25	0.25	0.5	16.25
4	MM	76.5	7.75	19.375	19.375	3.875	7.75	251.875

THURSDAY, 7/26/2018

(0) Preparing solutions for competent cells

Prepare 500 mL of 1 M D-sorbitol (for Shewanella competent cells preparation)

(1) Making plates

1 sleeve of Chl34 plates
2 sleeves of Kan50 plates

(2) Send RSF1010 + HW 29572 + mKate for sequencing

Primers:

E36
B17
D53

(they are in Keck)

(3) Golden Gate Assemblies for RF1010 + other HW elements + mKate

Making plasmids the same as this: https://benchling.com/s/seq-RvaDUr5gk76731ER6X9U
but with different Harris Wang elements, specifically: 18567, 24117, 31422, 34701, 35539, 36709, 36850, 37769 (part plasmids that you miniprepped a while ago, they are in ABL)

Use the same exact procedure as previously:

Table12

	A	B
1	**Parts**	**Volume for 40 fmol**
2	cassette vector RSF1010 (concentration 51 ng/uL)	**2.61**
3	**pSPB501 ConLS (1)**	0.2
4	HW _	0.2
5	pSPB460 mKate2 (4)	0.15
6	**pSPB463 tVoigtS6 L3S3P00 (4)**	0.2
7	**pSPB512 ConR2 (6)**	0.2
8	BsaI	0.5
9	T4 DNA ligase	0.5
10	T4 Buffer	1.5
11	10x BSA	1.5
12	H2O	7.44

Table9

	A	B
1	HW 18567	**0.04**
2	HW 21177	**0.03**
3	HW 31422	**0.03**
4	HW 34701	**0.01**
5	HW 35539	**0.01**
6	HW 36709	**0.02**
7	HW 36850	**0.02**
8	HW 37769	**0.02**

transform the assemblies into E. coli EW11, recover for exactly 1 hrs, plate 75 uL on Kan50 plates
25 fmols each

Table8

	A	B
1		
2	**RK2 (3)**	
3	**Part**	**µL**
4	**480**	1.8
5	**616**	0.6
6	**436**	1
7	447	0.43
8		
9		
10	BsaI	0.5
11	T4 DNA Ligase	0.5
12	T4 Buffer	1.5
13	10X BSA	1.5
14	H20	6.96
15		

bolded were diluted by adding 0.2 uL DNA to 1.8 uL nf H2O; volume amounts are for 10-fold dilution

Bsal Standard Protocol

	A	B	C	D
1		Step	Temp	Time
2		Initial Digestion (opt.)	37°C	10 min
3	Repeat25× / 15×	Digestion	37°C	1.5 min
4		Annealing & Ligation	16°C	3 min
5		Digestion & Ligase Inact.	50°C	10 min
6		Inactivation	80°C	10 min
7		Storage	12°C	∞

(4) Gel Purification of TZ terminator PCR part

Get the part from Silberg lab 4C fridge (wrapped in foil, labelled Tz)

Oligo Anneal for T7-LacO promoter and T7-TetO promoter

anneal the two by combining the oligos at 1 µM in water, 1 µL of each in 100 µL
Run the rxn on the annealing program: 98° and cool to 25° at the minimum ramp rate, 0.1°/s.

Anneal the following:

(1) E21 and E22
(2) E23 and E24
(3) E25 and E26
(4) E27 and E28

made a 10 µL rxn with 1× T4 ligase buffer, 0.5 µL T4 PNK (polynucleotide kinase), and a tenth volume of the oligo anneal (final 100 nM). Incubated 30 min 37°.

Prepared 50% glycerol solution

- 5 mL glycerol
- 5 mL milli-Q H2O
- Mixed with stirplate for 5 min
- Filter purified

Prepared D-sorbitol (1 M)

- 100 mL milli-Q H2O
- 18.2 g D-sorbitol
- Mixed with stirplate until solution was clear
- Filter purified into two 50 mL Falcon tubes

FRIDAY, 7/27/2018

People present: Stefanie, Anna, Soohyun
Time: 11:45AM-2:30PM and 3:15-5 and 5:30-7:15 (6.5 hours)
Location: Keck 201
Goals: Miniprep Anna's cultures, perform restriction digests, and transform DNA into cells

Miniprep Parts from Anna

Materials

- Qiagen Miniprep Kit
- Pelleted liquid cultures from Anna

Procedure

- Anna had already transferred cultures to microcentrifuge tubes and pelleted them
- Resuspended bacteria in 250uL of buffer P1 by pipetting up and down
- Added 250uL of buffer P2 to tube and inverted tube 6 times
- Added 350uL of buffer N3 to tube and inverted 6 times
- Centrifuged for 10 minutes at 13,000rpm
- Applied supernatant (liquid result from previous step) to spin column
- Centrifuged for 30 seconds at 13,000rpm; discarded flow-through
    - NOTE: centrifuged for 13 seconds & 20 seconds
        - Stopped because I forgot to place the lid on the centrifuge
- Centrifuged for 30 seconds at 13,000rpm; discarded flow-through
- Added 750uL of DNA Wash Buffer (PE Buffer substitute) to the spin column
- Centrifuged for 30 seconds at 13,000rpm; discarded flow-through
    - NOTE: centrifuged for 26 seconds & 5 seconds
        - Centrifuge stopped on its own
- Centrifuged for 1 minute at 13,000rpm; discarded flow-through
- Placed the spin column in a new micro centrifuge tube
    - Labeled as above^^ with miniprep iGEM 7/27
- Added 40uL of nfH2O to the spin column
- Let stand for 1 minute
- Centrifuged for 1 minute at 13,000rpm
    - NOTE: Caps broke off while being centrifuged. I changed some of the liquid to new tubes.

Nanodrop

Procedure

- Took miniprepped DNA to Keck 301
- Took 1uL nfH2O and nanodropped as a blank
- Wiped nano drop with kimwipe
- Took 1uL of _____ and measured:
    - RK2 = 288.3 ng/uL
    - Promoter P.P. = 218.2 ng/uL
    - Terminator P.P. = 142.2 ng/uL
    - Ribosomal Operon = 425.9 ng/uL
    - pBBR1 (8) = 162.2 ng/uL
    - pBBR1 (9) = 273.9 ng/uL
    - pBBR1 (10) = 343.2 ng/uL
    - pBBR1 (11) = 64.9 ng/uL
    - pBBR1 (12) = 356.0 ng/uL
    - 445 = 87.8 ng/uL

- 448 = 350.8 ng/uL
- 478 = 286.9 ng/uL
- 632 = 467.7 ng/uL

Summary: The only issue I encountered was broken caps; if I can find a way to prevent them from breaking it would be ideal. The process took a long time due to the shear number of samples I was prepping, but aside from time taken the process was normal.

Restriction Digest

Goal: Cut DNA so that it can be analyzed in a gel.

Materials

- Bsa1
- Cut Smart Buffer
- nfH2O
- Miniprepped DNA
  - RK2
  - Promoter P.P.
  - Terminator P.P.
  - Ribosomal Operon
  - pBBR1 (8)
  - pBBR1 (9)
  - pBBR1 (10)
  - pBBR1 (11)
  - pBBR1 (12)
  - 445
  - 448
  - 478
  - 632

Procedure

- Labeled PCR tubes as shown in the table below (corresponding to the list above)
- Added volume of each water for Master Mix and for individual p (11) and 445
- Added respective amounts od DNA to p (11) and 445
- Added 2uL of each respective DNA for remaining PCR tubes
- Added respective amounts of buffer to p (11), 445, and Master Mix
- Added respective amounts of Bsa1 to p (11), 445, and Master Mix
- Pipetted Master Mix up and down slowly 6 times to mix
- Made 11 aliquots 23uL of the Master Mix

Restriction Digest Volumes

	A	B	C	D	E	F
1	*Volumes = uL*	**Bsa1 (5 units)**	**DNA (500ng)**	**Cut Smart Buffer**	**NF Water**	**Total**
2	RK2	0.5	2	2.5	20	25
3	PPP	0.5	2	2.5	20	25
4	TPP	0.5	2	2.5	20	25
5	RO	0.5	2	2.5	20	25
6	p(8)	0.5	2	2.5	20	25
7	p(9)	0.5	2	2.5	20	25
8	p(10)	0.5	2	2.5	20	25
9	p(11)	0.5	**7.70**	2.5	14.3	25
10	p(12)	0.5	2	2.5	20	25
11	445	0.5	**5.69**	2.5	16.31	25
12	448	0.5	2	2.5	20	25
13	478	0.5	2	2.5	20	25
14	632	0.5	2	2.5	20	25
15	**Master Mix**	**5.65**	**--**	**28.25**	**226**	**282.5**

- Incubated PCR tubes for 1hr at 37C in PCR machine (ABL 126)

Summary: We originally were going to use NOT1 as the enzyme, but Anna couldn't find any. (We wanted to use an enzyme other than Bsa1 because we need that for assemblies, but its not necessary for digests.) We didn't run into any issues otherwise. Simple and easy process. Using a master mix saved a lot of time with needless pipetting.

Miniprep UBER + RSF1010 (correct) and N4

Materials

- Liquid cultures from Anna
  - UBER + RSF1010 (correct)
  - N4
- Qiagen Miniprep Kit

Procedure

- Transferred part of cultures to microcentrifuge tubes and centrifuged for 30 seconds at 13,000rpm. Repeated twice.
- Labeled spin column as above^
- Resuspended bacteria in 250uL of buffer P1 by pipetting up and down
- Added 250uL of buffer P2 to tube and inverted tube 6 times
- Added 350uL of buffer N3 to tube and inverted 7 times
- Centrifuged for 10 minutes at 13,000rpm
- Applied supernatant (liquid result from previous step) to spin column
- Centrifuged for 30 seconds at 13,000rpm; discarded flow-through
- Centrifuged for 30 seconds at 13,000rpm; discarded flow-through
- Added 750uL of Buffer PE to the spin column
- Centrifuged for 30 seconds at 13,000rpm; discarded flow-through
- Centrifuged for 1 minute at 13,000rpm; discarded flow-through
- Placed the spin column in a new micro centrifuge tube
  - Labeled as above^^ with miniprep iGEM 7/27

- Added 50uL of nfH2O to the spin column
- Let stand for 1 minute
- Centrifuged for 1 minute at 13,000rpm

Summary: The process went much more quickly than this morning. No issues.

Transform Assemblies into MG1655 Cells

Goal: Make more RSF1010 cassette and piG 202, we are running low.

Materials

- 3 MG1655 competent cell aliquots
- Assemblies:
  - RSF1010 cassette 51.0
  - piG 202
  - 430
- Cuvettes
- SOB

Procedure

- Obtained MG1655 cells from iGEM box in Keck 201 -40C fridge
- Waited for cells to thaw, kept on ice. Kept cuvettes on ice
- Labelled cuvettes and tubes as above
- Added 2uL of DNA to cells*
- Flicked to mix*
- Pipetted 50uL out of cells and transferred into cuvette*
- Flicked to spread cells*
- Transformed cells*
  - NOTE: 5.6 for RSF1010, 5.3 for piG, 5.6 for 430
- Added 1000uL SOB to cuvette
  - SOB not kept on ice
- Pipetted up and down to mix
- Transferred cells to a falcon tube
- Placed tubes in shaker at 37C to incubate for 1 hour (7:01PM)

Summary: The readings came out good for the most part. I followed Anna's protocol which will hopefully help the transformations go more successfully. The process was simple and easy.

Transformations of the assemblies from 7/26

Transformed the assemblies into E. coli MG1655 electrocompetent cells. Left in 37C shaker at 7:01. Plated 100 uL on Kan50 plates

Running digests on a gel

Prepared 1 % agarose gels and waited for 20 min for it to solidify.
Added 5 uL of loading dye to each sample

Order of samples on the gel:

Gel 1:

1. 445
2. 478
3. 448
4. 632
5. Ribosomal operon
6. Terminator part plasmid

7. Promoter part plasmid

Gel 2:

1. P (8)
2. P (9)
3. P (10)
4. P (11)
5. P (12)
6. RK2

SUNDAY, 7/29/2018

Golden Gate assembly mKate cassette vector, new pBBR1 multigene, new RSF1010 multigene

mKate cassette

	A	B
1	**Parts**	**Volume for 40 fmol**
2	438 cassette vector	0.34
3	pSPB501 ConLS (1)	0.02
4	mKate PCR product	0.25
5	pSPB512 ConRE (6)	0.02
6	BsaI	0.5
7	T4 DNA ligase	0.5
8	T4 Buffer	1.5
9	10x BSA	1.5
10	H2O	10.37

Table11

	A	B
1	**Parts**	**Volume for 40 fmol**
2	438 cassette vector	0.34
3	pSPB501 ConLS (1)	0.2
4	mKate PCR product	0.25
5	pSPB512 ConRE (6)	0.2
6	BsaI	0.5
7	T4 DNA ligase	0.5
8	T4 Buffer	1.5
9	10x BSA	1.5
10	H2O	10.01

Multigene vectors

	A	B	C	D	E
1					
2	**pBBR1 mob-**			**RSF1010 mob+**	
3	**Part**	**µL**		**Part**	**µL**
4	478	0.1		448	0.38
5	616	0.07		616	0.07
6	436	0.1		436	0.1
7	447	0.37		447	0.37
8					
9					
10	BsaI	0.5		BsaI	0.5
11	T4 DNA Ligase	0.5		T4 DNA Ligase	0.5
12	T4 Buffer	1.5		T4 Buffer	1.5
13	10X BSA	1.5		10X BSA	1.5
14	H20	10.15		H20	9.87
15					

Miniprep samples of RSF1010 HW + mKate

Miniprepped four samples using Quiagen kit according to the protocol .

Digest RSF1010 HW + mKate samples with NotI

Sinorhizobium competent cells preparation

Week of July 30

MONDAY, 7/30/2018

People present: Stefanie, Soohyun, Anna
Time: 10:30AM-1:45PM and 2:15-4:45PM (5.75 hours)
Location: ABL 126, Keck 201
Goals: Get everyone on the same page, send parts for sequencing, clean assemblies for transformation into cells, PCR piG202, plate transformations

Debrief with Anna

Testing Origins

- Anna has made all competent cells except for Coryne, Shewanella, Lacto (we're giving up on it because we don't have it and getting it is difficult)
- We are going to test the origins we know are working (pBBR1)

Orthogonal transcription -- we want to finish this this week so we can start testing in different strains

A)

- We tried to make cassette vectors which would allow us to put make directly into it, but it requires a harder protocol, so we switched to multigene vectors (simple GGA)
    - RSF1010+UBER Multigene vector
- Anna has prepared a T7 promoter + mKate +T7 terminator plasmid and today we will transform it into 2 e coli strains: normal e coli, and e coli that has been chromosomal modified to produced T7 RNAP
    - (on drawing colEJ is e coli's origin
    - If mkate is expressed in the modified e coli then we know that the plasmid is correct
    - We are transforming the plasmid into normal e coli in preparation for inserting the multigene vector

B)

- RSF1010 + highest HOMEWORK + mKate
    - we have one assembled, and we saw red colonies with the highest HOMEWORK ,
    - When lower Homework were expressed we didn't see red colonies
        - This means they are either wrong or don't express mkate at a detectable level
        - We are sending one for sequencing to make sure if its right or wrong
    - We are doing this because we don't want to want for the results from (A)
- When we get the results from (A) -- which origins work in which strains -- we will test all HOMEWORK elements in each strain

Orthogonal translation

- Full operon - we want to use the whole one because we aren't sure where we can cut the operon and it not interfere with the 16S production (we only know the truncation site in e coli)
- Purify genomes: Meloti, putida (next week -try to PCR out ribosomal -- once we get it then try it in the others)
    - We already have other genomes purified
- Need to design constructs before assembling
- A longside making the full operon we are going to try to express the 16S only -- we will try it with e coli and then test in other strains -- this would make a true universal translation system rather than having to make new constructs for each strain
    - 1) make a 16S part plasmid that contains an ASD mutation
        - Mutated it by doing a PCR that removed the whole operon, then using that as a template we amplified the region before the 16S and a small region after it, which resulted in 2 fragments. at the edge of the fragments we inserted a sequence.
    - 2) then we will take it and insert it into a plasmid that has a lac o promoter
        - Josh has a plasmid we can use to make the process faster
            - We will amplify the backbone of his plasmid and replace the gene with 16S

        - Then we do a 3 part assembly: 16S, backbone, terminator
    - 3) We need to test this, so we will take the RSF1010+KanR+HOMEWORK+RBS+mKate plasmid (we just made it) and put the mutation in the RBS
        - We do this by putting primers around the RBS and put the mutation in the primer overhangs
        - This will result in HOMEWORK+O-RBS+mKate+RSF1010

Send Samples for Sequencing

Materials

- DNA (listed in table below)
- nfWater
- Primer:
    - B17
    - B18

Procedure

- Labeled tubes: 1 ... 8
- Added water, primer, DNA to tubes

Sample Calculations: 445

500ng DNA / 87.8 ng/uL = 5.694 uL DNA

Table2

	A	B	C	D	E	F	G	H	I
1	**Sample #**	**Sample**	**Sample Length**	**Primer**	**[DNA] (ng/uL)**	**DNA Amount (uL)**	**Primer Amount (uL)**	**Water Amount (uL)**	**Total (uL)**
2	1	445	2001-4000	B18	87.8	5.69	2.5	6.81	15
3	2	448	6001-8000	B18	350.8	1.43	2.5	11.07	15
4	3	478	2001-4000	B18	286.9	1.74	2.5	10.76	15
5	4	632	2001-4000	B18	467.7	1.07	2.5	11.43	15
6	5	piG013	1001-2000	B18	142.2	3.52	2.5	8.98	15
7	6	piG014	1001-2000	B18	218.2	2.29	2.5	10.21	15
8	7	piG015	2001-4000	B18	425.9	1.17	2.5	11.33	15
9	8	RSF1010 + H.W.	6001-8000	B17	107.1	4.67	2.5	7.83	15

- Printed out order
- Placed order and tubes in the bag, and put it in the GeneWiz box outside Keck 201

Summary: Had issues with getting the liquid to congregate at the bottom. Normally I tap the tubes and the liquid falls, but this time the liquid remained on the side of the tubes. I kept accidnetally opening tubes that I didn't mean to. I may have had liquid come out of the tubes, but there were no droplets on the bench or liquid on my gloves, so I don't think this happened.

Clean Golden Gate Assemblies

Goal: Clean Anna's assemblies so we can transform them into E. coli cells later

Materials

- DNA:
    - Tz : Tz terminator part plasmid
    - Tet: TetO promoter
    - Lac: LacO promoter
    - 642: part plasmid for another broad host origin
    - mK1 and mK2: two assemblies of mKate cassette
    - P: multigene vector with a different version of pBBR1 origin
    - R: multigene vector with a different version of RSF1010 origin

- DNA Clean & Concentrate Kit

Procedure

- Determined volume in tubes: 15uL
- Added 30 uL of DNA Binding Buffer (2x volume)
- Vortexed for ~5 seconds
- Transferred volume to spin column
- Centrifuged for 30 seconds at 13,000rpm, and discarded flow through
    - NOTE: When placing tubes in centrifuge, I dropped Tz and it lost some liquid
- Added 200uL DNA Wash Buffer to ea. column
- Centrifuged column for 30 seconds at 13,000rpm. Repeated wash step
- Added 15 uL of nfH2O to column
- Incubated at room temperature for 1 minute
- Transferred spin column to microcentrifuge tube
- Centrifuged for 30 seconds at 13,000rpm

Summary: We didn't run into any issues, aside from me dropping Tz. The process was easy and simple.

Transform into MG1655 and Rosetta

Goal: Transform the following DNA (together and individually) to: 1- increase concentration of piG204 and piG205 2- to see if we get expression showing our sequences are correct

To do:

1. Transform (individually) all cleaned assemblies into MG1655
2. Transform (individually) mK1 and mK2 into Rosetta
3. Transform pRSFDuet-mKate into MG1655
4. Transform pBHR1 plasmid into MG1655
5. Transform (individually) piG204 and piG205 into MG1655
6. **Cotransform** pRSFDuet-mKate and piG012 into MG1655
7. **Cotransform** pRSFDuet-mKate and 639 into Mg1655

Materials

- DNA:
    - Golden Gate Assemblies (8)
    - pRSFDuet-mKate
    - pBHR1 plasmid
    - piG204
    - piG205
    - piG012
    - 639
- Electrocompetent cells:
    - 14 aliquots of MG1655
    - 2 aliquots of Rosetta
- Cuvettes
- Falcon Tubes

Procedure

- Obtained MG1655 and Rosetta cells from boxes in Keck 201 -80C fridge
- Waited for cells to thaw, kept on ice. Kept cuvettes on ice
- Labeled tubes
- Added 2uL of DNA to cells*
- Flicked to mix*
- Pipetted 50uL out of cells and transferred into cuvette*
- Flicked to spread cells*
- Transformed cells*

- Added 1000uL LB to cuvette*
    - LB not kept on ice
- Pipetted up and down to mix*
- Transferred cells to a falcon tube*
- Repeated starred (*) steps for ea. transformation
- Placed tubes in shaker at 37C to incubate for 1 hour (PM)

Summary: We didn't run into any issues. Simple process when there is 2 people.

Transformations

- mK1 (MG1655 and Rosetta)
- mK2 (MG1655 and Rosetta)
- pRSF Duet-mKate ONLY
- pRSF Duet-mKate w/ 639 (co-transformed)
- pRSF Duet-mKate w/ piG012 (co-transformed)
- 642
- pBHR1
- R
- P
- Tz
- Tet
- Lac
- piG204
- piG205

Plated all but piG204 and 205 at 6:00 pm (couldn't figure out what antibiotic plates to use)

Plated piG204 and 205 at 10:15 pm

PCR of piG202

Table1

	A	B
1		**uL**
2	H20	31
3	DMSO	1.5
4	piG202*	1.0
5	E35 Primer	2.5
6	E36 Primer	2.5
7	dNTPS	1.0
8	Q5 buffer	10.0
9	Q5 polymerase	0.5

*Dilute piG202 by 200 fold (199 uL H2O + 1 uL DNA) and use 1 uL
DMSO lowers annealing temp, go 2 deg. lower than predicted Tm

iGEMPCR3

	A	B	C
1	Lid Temp: 105		
2	**Step**	**Temperature (C)**	**Time**
3	1	98	0:30
4	2	98	0:10
5	3	58	0:30
6	4	72	1:17
7	5	Go to Step 2	Repeat 34x
8	6	72	2:00
9	7	12	Hold

iGEMTD

	A	B	C
1	Lid Temp: 105C		
2	**Step**	**Temperature (C)**	**Time**
3	1	98	0:30
4	2	98	0:10
5	3	68	0:30
6	4	72	1:17
7	5	Go to Step 2	Repeat 10x
8	6	98	0:10
9	8	72	1:17
10	9	Go to Step 6	Repeat 24x
11	10	72	5:00
12	11	12	Hold

TUESDAY, 7/31/2018

RSF1010 + UBER digest

Table16

	A	B	C	D	E	F	G
1	*Volumes = uL*	**XhoI**	**XbaI**	**DNA**	**Cut Smart Buffer**	**NF Water**	**Total**
2	piG204	0.5	0.5	15	2.5	6.5	25
3	piG205	0.5	0.5	15	2.5	6.5	25

Golden Gate Assemblies

a) mKate + Harris Wang elements on RSF1010 origin

Multigene vectors

	A	B	C	D	E
1					
2	**mKate with HW elements**		**Mastermix**		
3	**Part**	**µL**			
4	cassette vector RSF1010	0.65	5.85		
5	pSPB501	0.3	2.7		
6	HW elements	0.4			
7	pSPB463	0.3	2.7		
8	pSPB460	0.3	2.7		
9	pSPB512	0.3	2.7		
10					116.8
11	**BsaI**	0.5	4.5		
12	**T4 DNA Ligase**	0.5	4.5		
13	T4 Buffer	1.5	13.5		
14	10X BSA	1.5	13.5		
15	H20	8.54	76.86		
16			129.51	14.39	

amounts are indicated for 10x dilution

Table9

	A	B
1	HW 18567	
2	HW 21177	
3	HW 31422	
4	HW 34701	
5	HW 35539	
6	HW 36709	
7	HW 36850	
8	HW 37769	
9	HW29572	

WEDNESDAY, 8/1/2018

People present: Stefanie, Anna
Time: 1:30-4PM and 4:45-5:30PM (3.25 hours)
Location: ABL 126, Keck 201
Goals: Miniprep Anna's cultures and send some for sequencing

Miniprep

Materials

- Anna's liquid cultures- labeled iGEM
  - RSF1010 cassette vectors 1 & 2
  - RSF1010 mob + 1 & 2
  - pBBR1 mob - 1 & 2
  - piG204 & 205
  - 642
  - pBRH1
  - LacO
  - Tz
  - TetO
- Qiagen Miniprep Kit

Procedure

- Transferred part of cultures to microcentrifuge tubes and centrifuged for 30 seconds at 14,000rpm. Repeated 2/3 times.
- Labeled spin columns as above^
- Resuspended bacteria in 250uL of buffer P1 by pipetting up and down
- Added 250uL of buffer P2 to tube and invert tube 6 times
- Added 350uL of buffer N3 to tube and insert 6 times
- Centrifuged for 10 minutes at 13,000rpm
  - NOTE: Sat for a few minutes afterwards
- Applied supernatant (liquid result from previous step) to spin column
- Centrifuged for 30 seconds at 13,000rpm; discard flow-through
- Centrifuged for 30 seconds at 13,000rpm; discard flow-through
- Added 750uL of DNA Wash Buffer to the spin column
- Centrifuged for 30 seconds at 13,000rpm; discard flow-through

- Centrifuged for 1 minute at 13,000rpm; discard flow-through
- Placed the spin column in a new micro centrifuge tube
    - Labeled as above^^ with miniprep iGEM 7/23
- Added 50uL of nfH2O to the spin column
- Let stand for 1 minute
- Centrifuged for 1 minute at 13,000rpm

Summary: Didn't run into any issues. I made sure to have all of the tube tops set on the bottom of the centrifuge, and only 2 broke. I will keep experimenting to see what works best.

Send for Sequencing

Materials

- DNA
    - Tz
    - TetO
    - LacO
    - piG204
    - piG205
- Primers
    - B18
    - E11- P1 T7

Procedure

- **Soohyun added water and DNA**
- Added 2.5uL primer to each tube
- Printed order
- Added tubes and paper to bag, and left outside Keck 201

Golden gate assemblies transformations

Transformed the assemblies for HW elements and assemblies of mKate casssette with T7 promoter and terminator with 438 cassette vector into E. coli MG1655

Digest of multigene vectors with Esp3I and Digest with XmnI:

Expected bands observed forXmnI but not for Esp3I

THURSDAY, 8/2/2018

People present: Stefanie, Anna, Soohyun
Time: 7:30-8PM
Location: ABL 126
Goal: Clean assemblies for Anna

Clean Assemblies

Materials

- DNA:
    - 1
    - 2

    - 3
    - F
- DNA Clean & Concentrate Kit

Procedure

- Determined volume in tubes:
    - 11 uL
    - 12 uL
    - 13 uL
    - 11 uL
- Added 2x volume of DNA Binding Buffer
    - 22 uL
    - 24 uL
    - 26 uL
    - 22 uL
- Vortexed for ~5 seconds
- Transferred volume to spin column
- Centrifuged for 30 seconds at 13,000rpm, and discarded flow through
- Added 200uL DNA Wash Buffer to ea. column
- Centrifuged column for 30 seconds at 13,000rpm. Repeated wash step
- Added 8 uL of nfH2O to column
- Incubated at room temperature for 1 minute
- Transferred spin column to microcentrifuge tube
- Centrifuged for 30 seconds at 13,000rpm

Summary: No issues, simple and easy process.

PCR to amplify fragment from piG202 without HW element

Primers: iG054 and iG055 ; Tm = 68; length = 7568