Welcome to iGEM 2015

6/10/15 Competent Cell Preparation
- 10g of Stock LB made w/ Vennor powder (500 uL of ddH_2O)
- Inoculated 5 tubes w/ 5mL LB + 10uL of cells
- Shaking until we get an OD of 0.4
  - ⊗ We got an OD of .842, in 4C! -

6/12/15 Gel Electrophoresis
- Digest plasmid backbone (psBIC3) w/ (EcoRI & Pst I) and the front insert w/ (EcoRI & SpeI) and back insert w/ (XbaI & Pst I)
- Ran a gel to verify if we did things right
  - Unsuccessful :(

Comp. Cell Preparation 7.1x
- Streak (not glass beads) the growths on a plate of plain LB - Michelle will inoculate colonies on Sunday for us

Amp Plate Creation

6/30/15 Plate creation
- 500 mL of LB agar (10g LB, 7.5g agar)
- Also autoclaved water
- Thought the 3rd autoclave was broken. It is not. You have to exit out of all the menus before starting cycle.

CB

Promoter cloning

7/01/2015

Plate Creation (Amp)

Autoclaved 250°F 30min Sterile time

1) Made 500ml of LB Agar (10g LB, 7.5g agar)
   - Was slightly above 500ml due to the volume change from the powders
2) Labeling to be done inside the hood? (Not sure)
   ↳ (Labeling of the plates)

July 1, 2015

James Blondin

Heat Shock Time Test #1

DNA: well 2015-4-16G

Sketch Notes

	1	2	3	4	5
DNA	2uL	~2uL (totaled light when right)	2uL	2uL	2uL
comp	40µL	27uL	31.5uL	<10uL	19uL
planned shock time	50s	40s	45s	30s	35s
actual time	~49	~49	~49	~34	~42

← the well should now be empty

varying concentrations times might run in the test's viability, but oh well...

Note to self: each tube needs its own floatation device, or this will never work. I'm not fast enough.

- Also, our pre-growth time was far longer than 2 minutes.
- ~~Transfer~~ Transfer the comp cells to a 15mL tube when adding LB after heat shock. It will save you so much trouble!

Todd's ~~Advise~~ Advice

- Having good comp cells can give you 100-fold more efficiency
- Ideal heatshock time/other steps can give you 2-fold more
- He suggests we just measure our efficiencies and see if we need new comp. cells

Notice to self: practice experiments are extremely helpful for learning about pit-falls.

I'm canceling this experiment for now. It's not worth it...
- we should use pUC19
- we should have the same concentrations
- the times need to be redone (use the single hole floatation devices in the drawer near the 42°C bath)

Digestion to test SpeI

500 ng DNA (20 uL of backbone)
.2 uL SpeI
3 uL 10x buffer (cutsmart)
7 uL water

7/2/15 TE Equation: TE = Colonies / µg / Dilution

Colonies: The number colonies counted on plate
µg: The amount of DNA transformed expressed in µg
Dilution: Total dilution of the DNA before plating

We are transforming 100pg of BBa_J04450 into ~50uL of cells, outgrow w/ 950uL of LB

Plate 50uL

µg DNA: 0.0001
Dilution: 50/1000 = 0.05
# of colonies: x

TE = x / 0.0001 / 0.05 = Our TE in cfu/µg

(*) NOTE: ONLY ALLOWED TO GROW FOR ~20 MIN
NOTE: ~50mL of cells, 950 LB; cells varied from 40-50mL by our estimates

Put in 37°C Shaker → will count Colonies tomorrow & take to a grad student. takes ~18hrs for full expression

(7/2/2015 (cont.)

Digestion to test SpeI

~~500 ng RFP (10 uL · 50 ng/uL)~~
~~2 uL SpeI~~ 0.5 uL SpeI (iGEM restriction digest protocol, NOT Trello's) ☆
3 uL Cutsmart
7 uL water
250 ~~500~~ ng pSB1K3 (~~20~~ 10 uL · 25 ng/uL)
↳ We chose this one instead of the RFP part because it had a higher concentration (25 ng/uL vs 50 ng/uL) and that it was a plasmid backbone

iGEM Protocol

Tube 1: pSB1K3 and SpeI GQ (not sure what GQ is, it was one of the tubes)

Tube 2: pSB1K3 and SpeI

Tube 3: pSB1K3 and water (~~10~~ 5 uL pSB3, ~~15~~ 15 uL H_2O)

"Lane 4": Log ladder
↑ for the gel

~~We put 2013 batch 2 in the first tube, ran out, so used 2014 batch 1 in the 2nd and 3rd; and only 5 uL of each~~

Tube 1: 10 uL of 2013
Tube 2: 5 uL of 2013 + 5 extra H_2O
Tube 3: 5 uL of 2014 + 5 extra H_2O

To have correct total volume

7/12/2015

MD JC

Running a Gel for SpeI

First, we are going to prepare the gel. Measure 1g of agarose (note: this makes 1% agarose gel, we can make 2% agarose gel to resolve closer sized bands, or 0.7% to separate larger bands)

Pour agarose powder into a microwavable flash along with 100mL of 1xTAE (see TAE protocol if it's not there) (See Gel Electrophoresis Notes 0)

Microwave for 1-3 minutes in spurts of 30-45 seconds until agarose is completely dissolved and there is a nice rolling boil (1:30)

Let agarose solution cool down for 5 minutes

Add ~~EtBr~~ Midori Green to a final concentration of approximately ~~0.2-0.5 ug/mL (typically 2-3uL of lab stock solution per 100mL gel)~~ 10 uL/100mL

Place the newly poured gel in the 4C for 10-15 minutes or set at room temperature for 20-30 minutes @ 11:12 am

Pour agarose into a gel tray with the well comb in place ← switch order!

Second, we are going to load samples and run the gel. Add loading buffer in a 1:5 ratio to each of our digest samples, as well as EtBr (if you forget to do this, soak the gel in EtBr and rinse with water to even out the staining differential that will appear) (see Notes 1, 2, and 2.5)

Once the gel is solidified, place the agarose gel into the gel box

Fill the gel box with 1xTAE until the gel is covered

Carefully load a molecular weight ladder to the first lane of the gel (see Notes 2)

Carefully load samples into the additional wells of the gel (see Notes 3 and 4)

Run the gel at 80-150V until the dye line is ~75%-80% of the way down the gel (see Notes 5 and 6)

Turn off the power and remove the gel from the gel box

alize our gel using the UV machine

7/12/2015

① Transformation from 7/1/15 failed; we are going to prepare solutions for making Competent cells on Monday

- Michelle will grow up a culture of cells for Monday on Sunday
- 100mL of LB made in 2 250mL flasks has been autoclaved (50mL each) → Stored in 4°C
- ~~100mL of LB made in 2 250mL flasks has been autoclaved (50mL each) → Stored in 4°C~~

(*)(*)(*) E Coli strain for competency is HST08 from ClonTech (we grew it up from a tube Erin made Competent → so not E directly from the Company)

- Took 4mL of sterile filtered $CaCl_2$ (1M) + 36mL H_2O to make 0.1M $CaCl_2$
  ↳ Stored in 4°C

Order of gel: Ladder II III I III

II was poorly pipetted!

② Continuation of SpeI digest

Add loading buffer (6x) to the digested samples

- Run the gel
- Load 12μL of ladder
- Load 2μL of loading dye per 10μL of sample
- ~85 Volts for 80 minutes

(*) Tube contents on Pg 19!

③ Another Comp Cell test b/c I found puc19 and have hope (7 tubes of Comp Cells)

1) Spin down transformation efficiency kit tubes for 30 sec at 8,000 rpm
2) Thaw cells on ice
3) Pipet 1 uL of DNA from kit & 2uL of puc19 into Comp cell tube → MIX BY GENTLY SHAKING!!!
4) Heat Shock for 60 seconds at 42°C (Recommended by ClonTech)
5) Place tubes on ice for 2 minutes → (DO GO IMMEDIATELY)
6) Add 500 uL of LB
7) Shake for 45 minutes
8) Plate Cells on appropriate antibiotic plate → 37°C Shaker overnight
   ↳ 30uL plated, Sam (Chris's lab) will put it in the 4°C for us

(3.5) Sit on ice for 30 minutes

7/6/15

① Looked at comp cell test
- heavy growth on control
~60 very small colonies on each puc19 plate
About this big →.

Efficiency calculator: (sciencegateway.org/tools/transform.CB
.000050 ng/uL DNA
2 uL DNA added
550 uL total volume
30 uL plated
60 colonies
1.1 e7 transformants / ng DNA
"Garbage." Remake!

*OD when taken out .28 A

7/6/15 - Testing Competent Cells made w/ Steve

① puc19 spun down w/ baby centrifuge — Prewarmed
② Comp cells thawed for 30 min / LB plates (Amp / No Antibiotic)
③ 100 ng of puc19 added to a tube of Comp cells (2 tubes; one is the control)
④ Heatshocked for 30 seconds (as suggested by Life Technologies for Top10 cells)
⑤ 500 uL of LB immediately added (room-temperature LB)
⑥ Incubated on ice for 5 minutes (as per Life Technologies' protocol)
⑦ 37°C growth for an hour was skipped b/c we are plating on Amp
- Ampicillin affects synthesis of peptidoglycan → it won't have an impact until cell division starts. This is unlike CM or KM where they impact ribosome function
⑧ 50uL plated on each plate
⑨ 37°C o/n

- Jg et. al.

* Someone who was here fill this out pls ☺ (JamesBlondin)

7/6/15 Comp Cell Creation

- 1% inoculation of (I think) pre-cultured cells in LB (someone from the morning confirm the cell source please)
  - Made into 200mL LB (still a 1% dilution)
- Inoculated for ~~about~~ 1.5 hours in 37°C shaker (____ rpm)
  - O.D. at 1 hour: 0.135
  - O.D. at 1.5 hours: ~~0.28~~ 0.28
- ☆ Transfered 50mL each into four 50mL Falcon tubes
- ☆ Centrifuged ~~for~~ 10 minutes at 3,000 rpm at 4°C
- Removed supernatant (by pouring). Resuspended in 5mL 0.1M $CaCl_2$ each.
- Left on ice for 1 hour (60 minutes)
- ☆ Centrifuged for 10 minutes at 3,000 rpm at 4°C
- ☆ Removed supernatant (by pipetting). Resuspended in 2.5mL 0.1M $CaCl_2$, 15% glycerol (each).
- Aliquoted 50uL to microcentrifuge tubes (in ice buckets, in the cabinet).
  - ~~About~~ 94 50uL tubes were put into one box and stored in -80°C.
  - The other cells were aliquoted at 100 uL or 1mL and put in a second box in the -80°C (were out of autoclaved tubes)

*Note: we had help from Steve for certain parts of the procedure.

James Blondin 7/7/2015 Comp Cell Test Results

Sample	Observations (17 hours later)	Possible Reasons:
puc19, 100 ng/mL amp.	no visible growth, no moisture	• not all of the transformants were plated
puc19, LB	~~no visible growth~~ no visible colonies, moisture in plate	• our puc19 might not be ~~viable~~ working
no plasmid, LB	no visible colonies, moisture in plate	• the LB was too hot / added too soon
		• the comp cell protocol was ~~it done~~ quickly enough

James Blondin 7/7/2015

- We autoclaved an empty jar, small pipette tips, and a jar of microcentrifuge tubes.

7/8/15

Transformed the following parts:

CB	B0032	R0010	K592016
MD	B0034	K592008	K911003
James and Drew	B0015	R0040	K592006
	B0040	R0062	K914003
	J33201	I7404006	K561001
	K1404006	R0084	K554000
	K54001	K346002	K873002

Using the following protocol:

1. Pre-warm plates and LB in 37°C
2. Thaw comp cells (50 µL each) on ice for 5 min
3. Add 2 µL DNA
4. Incubate on ice for 5-30 min.
5. Heat shock at 42°C for 30 s
6. Incubate on ice 2 min.
7. Add 500 µL warm LB
8. Place in 37° shaker for 1-2 hrs
9. Plate ~~30~~ 50 µL in the 37° overnight.

- We kept the cells in the shaker for 1 hr.
- Plated on ~~ampicil~~ CM plates

All parts except for B0032, ka11003, K1404006 and B0034 were also spun down and plated a 2nd time after the 1st plating:

1. Centrifuge at 3000 rpm for 10 min.; drain
2. resuspend in ~~50 µL LB~~ 55 µL dH2O

~~Transformation~~ protocol (from Steve)

7/7/2015

0. Thaw
1. Add DNA (2 µL) and gently swirl to mix
2. Incubate on ice for 5-30 minutes
3. Heat Shock @ 42°C for 30 seconds
4. Add 1mL LB and incubate 37°C for 1-2 hrs
5. ~~Spin down~~ Plate cells with 30 µL
6. Remove liquid from tube
7. spin down cells at
8. Add LB and plate on another dish

rest in ice 2 mins

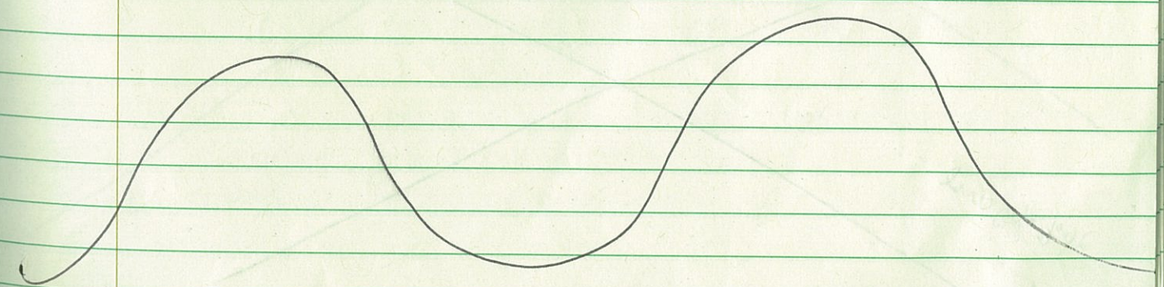
3. Plate 50 µL on CM plates

All were placed in the 37° incubator overnight

Note - spun cells say "spun"
- The first plating is labeled "30 µL" because we initially thought we'd need that much. We actually used 50 µL on all plates that say "30 µL" ← CB

As for the "spun" plates, we used 55µl to resuspend the cells and transfer 50µl to plates.

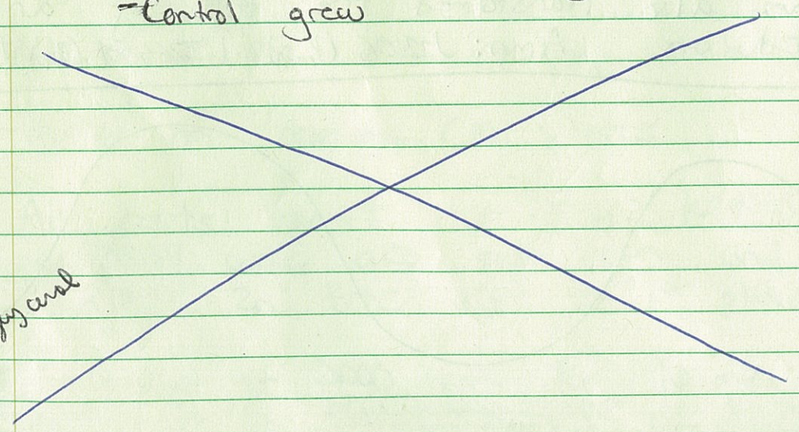
James also transformed the following and plated on Amp: J22106 (P_{tacM}) and I765001 (P_{LtetO}) (backbone: pSB1A2)



7/9/15 → REDO ON 7/10, NOT DONE

+ -Made CM plates (10 g LB, 7.5 g agar, 500 mL H_2O)
-Used new CM 34 stock solution we made
- 10 mL 100% ethanol
- .34 g CM
- filtered with .22 μM filter
- Keep stored in -20°C
+ -Added 250 μL of CM 34 to make 17 μg/mL plates
+ -Stored in 4°.

Checked growth of promoters after 18 hrs in 37°.
Observations:
- All displayed some growth except B0034 and E0040. Both of these came from the 4th kit plate in the 2015 distribution. Is something wrong with this plate?
- The spun down cells all grew better than the 50 μL ones, but in no case was a 50 μL plate unusable while the spun down plate was. To save plates, we might not need to inclut the spindown plating in the future.
- Control grew



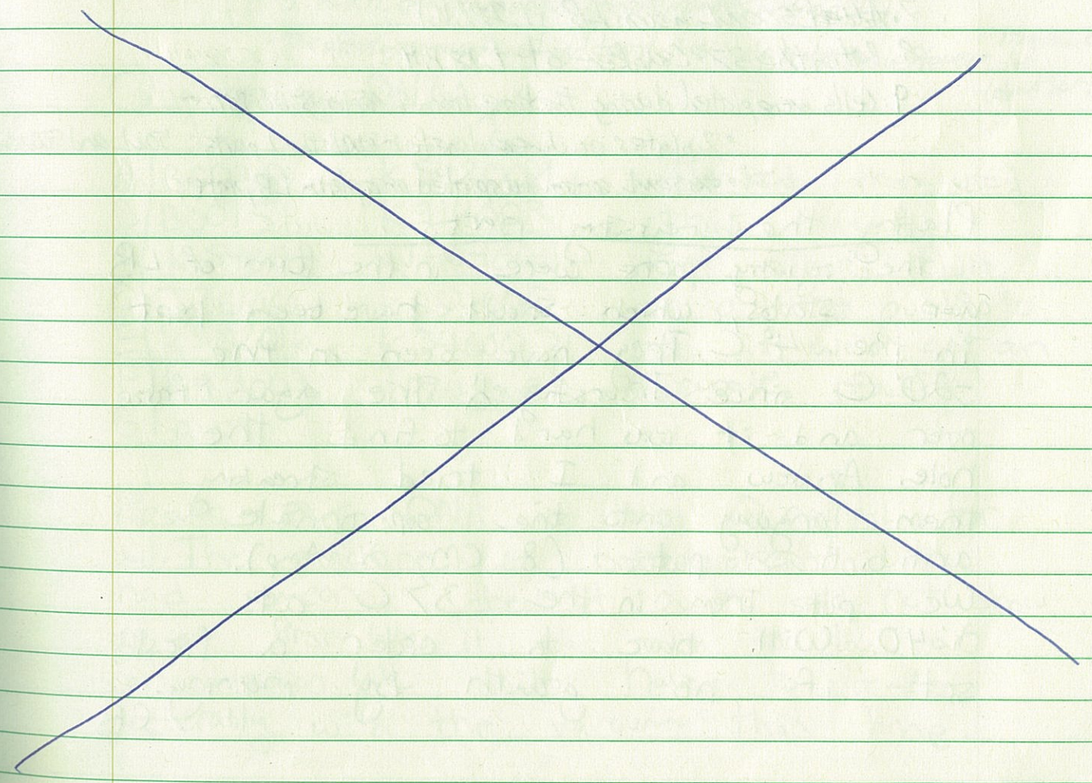
500 mL of grown up → 2% glycerol in LB 500 mL of culture

7/10/15 Promoter Inoculation
- Took 30 mL plates and picked top colony in each one (colony circled on plate)
- Added 5 mL LB (treated with CM 17 μg/mL), 3 AMP plates, as well 100 μg/mL
- Put into falcon tubes
- Shaken overnight in 37°C
- Colonies picked were circled on the plates
* Two Transformations failed (B0034, E0040)
- Placed in 37°C at 4:30 p.m.

Notes:
- there may be labeling issues with K592008, K911003, I and J33201

7/10/2015 Plasmid Purification

for cell storage
- Transfer 600 μl culture into tubes
- Add 400 μL 50% glycerol into each tube, then store in the -80°C



7/13/15

James Blondin

Transformed the following parts:

E0240 2015-2-24-B (RBS 32, GFP, Terminator 15)

I13504 2015-3-17C (RBS 34, GFP 40, Terminator 15)

1. Added 10uL dH_2O to each well, mixed by pipetting. Red color observed (good).
2. Added 2uL to a 50uL competent cell tube (1 DNA sample per tube)
3. Put on ice at 1:04 P.M.
4. Put LB in a 15mL tube in the 37°C to warm it.
5. Heat shocked for 30 seconds at ~1:24 P.M. (1:24 + 30 seconds?) (added 2uL dH_2O to a cell tube just before the heat shock)
6. Put on ice for 2 minutes (1:25 - 1:27)
7. Added 500uL warm LB (1:27 P.M.)
8. Put in the 37°C shaker at 1:28 P.M.
9. Cells were plated during the time from 2:45 to 3:06 P.M.
   - 2 plates each were done for each of the 2 parts: 50uL and 500uL
   - the 50uL control was plated on a plain LB plate.

Plating the registry parts

The registry parts were in the form of LB agar stabs, which should have been kept in the 4° C. They have been in the -20° C since Thursday :(The agar froze over and it was hard to find the hole. Andrew and I tried streaking them anyway onto the appropriate antibiotic plates (8 CM, & Amp). We put them in the 37°C at 8:40. Will have to order a fresh set if no growth by morning. CB

James Blondin 7/14/15

- The 5 plates from yesterday's transformations were removed from the 37°C around 12:36 P.M. (23 hours later)
- All 5 plates showed significant growth, as is expected by hour 23. Satellite colonies are easily spotted.
  - Only the two 50uL plates should be inoculated.
  - In the future, plates should be removed earlier (probably by hour 18).

10/12 in 37 by 2:32 P.M. (before) (CM plasmids)

- The 2 transformed plates and the 10 plated cultures from the Registry were inoculated in 5mL LB with 17 ug/mL CM or 100 ug/mL amp. The 10 CM tubes were put in by 2:32 P.M. The 2 amp tubes were put in by 3:22 P.M. (probably a dozen or more minutes before 3:22).
- The plates were parafilmed and stored at 4°C, along with the leftover 17ug/mL CM LB (about 16mL)

Quantifying DNA

	1	2	
A	B0032	K542008	Blanked then used this layout:
B	B0015	K914003	
C	K54001	K1404606	Got weird result, so added puc19 to see if we'd get an accurate reading.
D	J221061	K554000	
E	K592016	K346002	
F	K911003	I746361	
G	puc19		

Results - Our DNA is bad! 0 ng/mL for all. The machine is not broken, we had someone check it. puc19 did not show up at first b/c it was also too low a concentration. We will have to re-do, preferably with the vacuum this time.

* I labeled the top of each bad tube Caroline with a "1" and returned to its box in the Blazzick -20.

note: this protocol has been rewritten since. The original in blue ink is what was actually done that day.

STAT 7/14/15

~~Seed Cultures, Take II~~

	MiniKit I	MiniKit II
Solution I	250 uL	500 uL
Solution II	250 uL	500 uL
microcentrifuge tube	1.5 mL	2.0 mL
Solution III	350 uL	700 uL

James Blendin 7/15/15 Miniprep (12 samples)

1.) Took the 5mL tubes out of the 37°C shaker. Transfered 600 uL cells into 400 uL 50% glycerol to create glycerol stocks. This was done in the cabinet, and the stocks were stored in the -80°C.

2.) Centrifuged the 5mL tubes at max speed (3,500 rpm ~3,200 rcf) for 10 minutes.

3.) Removed the supernatant by pouring.

Tip: do this quickly (one big "dump" movement into the waste), before the cells "soften"

4.) Added 250 uL Solution I. Resuspended by pipetting. Aru's tip: vortex for 20 sec (really vortex it!)

Tip: do step 5 at the same time.

5.) Transfered the suspensions to new 2mL microcentrifuge tubes. (if using MiniKit II)

- Will need 2mL tubes, 1.5mL tubes are too small.

tip: these steps are time-sensitive! Transition quickly!

6.) Added 250 uL Solution II. Added to each tube in a rapid succession then inverted to mix all simultaneously. (Pipette tip, wait 2-3 minutes for better results but never exceed 5 minutes.)

7.) Added 350 uL Solution III, using the same technique as step 6.

8.) Centrifuged at max speed (15,000 rpm) for 10 minutes.

9.) Transfered 700 uL of the supernatants into mini columns inside 2mL collection tubes. Transfered by pipetting, making sure not to touch the precipitate.

10.) Centrifuged at max speed (15,000 rpm) for 1 minute.

11.) Discarded the filtrate (in the collection tube), reused the collection tubes.

12.) Repeated steps 9-11.

(done by Aru Singh, recorded by James Blendin)

13.) Added 500 uL HBC Buffer.

14.) Centrifuged at max speed (15,000 rpm) for 1 minute.

15.) Discarded the filtrates, reused the collection tubes.

16.) Added 700 uL DNA Wash Buffer.

17.) Repeated steps 14 & 15.

(optional) 18.) Repeated steps 16 & 17.

19.) Centrifuged the mini columns for 2 minutes at max speed to dry.

20.) Transfered the mini columns to new 1.5mL tubes.

21.) Added 50 uL pH 7 50°C dH_2O (Tip: pH 8.5 dH_2O or Elution Buffer might work better)

22.) Left at room temperature for 1 minute.

23.) Centrifuged at max speed for 1 minute.

24.) Stored the DNA at -20°C. (note: they were left out for ~~a couple hours~~ before storing)

7/16/15

DNA Quantification of yesterday's minipreps

	1	2
A	L1166000	K387003
	121.2 ng/uL 1.86	47.1 ng/uL 1.85
B	K1071004	K1509001
	61.5 ng/uL 1.74	61 ng/uL 1.83
C	I138504	K896005
	49.9 ng/uL 1.8	65.2 ng/uL 1.87
D	I>65600	K62900 | motion distorted AVG
	46.8 ng/uL 1.83	57 ng/uL 1.78
[illegible]	K2?6004 [illegible]	K[illegible]3760[illegible]3 [illegible]
[illegible]	63?60?g/uL [illegible]	5[illegible].2 ng/uL [illegible]
[illegible]	K1?6600 [illegible] E[illegible]2440	[illegible]
[illegible]	14[illegible].1 ng/uL [illegible]	56.3 ng/uL 1.83
[illegible]	[illegible]	88.1 [illegible]
[illegible]	Put [illegible] 50 mL fresh LB into the autoclave	
[illegible]	[illegible]	[illegible]

Spectinomycin arrived. Was placed in the -20°C as indicated on package.

[illegible] [illegible] [illegible]

Note: 3 Amp ones were done too!

Seeded the plates from the 10th again into 5 mL of 17 ug/mL CM. PSLB. Put in the 37° Shaker [illegible] 12:50.

AJJ gene [illegible] plasmid was [illegible] see daily sheet with them.

They can't match [illegible] (Plate: I746361, [illegible]

Tubes [illegible] I7464006

7/17 7/17/15

- Create glycerol Stocks of Following Samples:

K346002	•I765001	I746006 } Both on same Tube	K561001
54001 *	•J22106	I746361 }	K730032
B0015	•KSQ2008	K554600	R0010
K911003	K1404006	R0040	R0084 (PFF745) addgene plasmid
KSQ2006	K914003	R0062	
B0032	J33201	KSQ2016	

Using 500ul of glycerol (50%) Stock and 500ul of Cells.

② Note: Use screwcap cryovial tubes for frozen glycerol stocks

* J22106 Did not have growth so we did not proceed w/ glycerol stock or purification

DNA Quantification

Parts	ng/μ	Ratio	Parts	ng/μ	Ratio
X K346003 (Mercury)	10.8	1.61	KSQ000 Cobalt	142.3	1.91
K911003 (Ribosuicin)	171.9	1.92	K592016	447.6	1.86
K873002 (HSP)	152.5	1.92	J33201 Lacrs Repressor	85.9	1.91
R0010 (Lac)	213.5	1.88	K561001 Vegetamicrographic	91.6	1.93
Addgene	174.2	1.87	K1404006	31	1.85
X K592008 (TS-Lac)	9.5	1.83	K554000 Soxs	96.1	1.84
K914003 (L-Rhamnose)	186.1	1.91			
R0084 Amp R	188.6	1.88			
I765001 UV	16.4	2.02			
B0015 Term	177.3	1.91			
B0032 RBS	129.8	1.89			
R0040 Tet	212	1.88			
I746361 PDPnm from P2 pmy	144.8	1.9			
add K592008 FixK2	63.4	1.71			
R0062 Lux	138.9	1.91			

7-20-2015 GFP Full construct Assembly

Digestions

1) in 1.5ml tube combine: 500 ng DNA, 0.8 μL each restriction enzyme, 3 μL of 10x buffer, and X μL of dH_2O to bring total volume up to 30 μL

2) Mix gently by pipetting

3) Incubate at appropriate temp (check manufacturer's instructions, typically 37°C) for 1 hour

4) Heat kill for 20 minutes (70°C)

Amounts of each:	DNA (μL)	H_2O (μL)	
R0040	~~2.36~~ 2.4	23	Also:
K540001	3.5	21.9	I765001
I13504	10.0	15.4	
K629001	8.8	16.6	
R0010	2.3	23.1	
K896005	7.7	17.7	
K873002	3.3	22.1	
K109004	8.1	17.3	
K911003	2.9	22.5	
I746361	3.5	21.9	
L1166000	4.1	21.3	
K554000	5.2	20.2	
K561001	3.5	19.9	
K914003	2.7	22.7	
E0240	8.9	16.5	
J33201	5.8	19.6	
R0084	2.7	22.7	
R0062	3.6	21.8	

Amounts of each:	DNA(uL)	H_2O(uL)
K1509001	8.2	17.2
K387003	10.6	14.8
K116601	3.5	21.9
K376003	9.8	15.6
Amp I765600	~~30.48~~ 30.5	none
Amp K216004	7.7	17.7

- [x] water
- [x] DNA
- [ ] ~~Restrict~~ CM restriction enzymes
- [ ] Amp restriction enzymes
- [x] Buffer

(X)(X) ONLY SOME OF THE DIGESTIONS WERE DONE; CHECK ~~37~~ 37° Waterbath to SEE WHICH ONES WERE DONE

- Digestions were done o/n at 37° (J22106) (I712074) (K117002)
- 3 parts were transformed & plated: Rad promoter, T7, and LsrA on appropriate plates, Incubation step was 30 minutes pre-heat shock, on ice, and allowed to grow for 30 minutes post-shock.
- 5 mL of ~~plated~~, GFP (x2), and K512008 (? Positive Ctrl) were grown up o/n

Digestions: Amp BB — Promoter: EcoRI / SpeI; GFP: EcoRI / XbaI
CMBB — Promoter: SpeI / PstI; GFP: XbaI / PstI

7/21/15

- O/n digestions were put in 80C to heat kill the enzymes for 20 min
- Plasmid purification began w/: K346002, E02540, J22106, another E02040, K592008
  ↳ OD seems a bit low :((all of them)
  - Buffer PE (Qiagen) was used instead of DUA wash buffer
- GFP that we had is getting digested w/ XbaI & PstI (The GFP that will be inserted CMBB's)
  - Began digestions at 1:10pm → End at 7:00pm

Note: Digestions yesterday were of: K561001, R0062, I746361, ~~I35504~~ I13504 (on 64?), K896005, E02040 (GFP)

- 1uL of rSAP (phosphatase) was added to the above digestions (excluding GFP) at 1:10pm
- At 2:~~00~~25pm the phosphatases were put in the 80C to be heat killed → removed at 2:31pm
  ↳ K561001, R0062, I746361, I13504, K896005 have been digested & phosphorylated, E02040 has been digested

- Four inoculations of colonies plated last night (No antibiotic added → to overcome poor o/n growth), K117002 (LsrA), 2 inoculations of J22106 (RecA), and I712074 (T7) → Incubated in 37°C

→ Results are recorded on the next page. RecA & T7+Lac were subpar. The rest were successful

- Beginning digestions of more promoters: J33201, K387003, K561001, K914003, I765000, R0040, I746361, R0062, R0010, K376003, K554000, K873002, K629001, R0084, K1071001, K1509001, K911003, K116600

Begin: 7pm

- 3uL Buffer, 1uL of ~~R&I~~ (each), ~~Some~~ 1ug of DUA (not strong), Water to 30 uL. Incubate for ~~8~~ 80 hours (probably o/n at this rate)
  → ~~80~~ 8uL of SpeI b/c low supply

NOTE: Plasmids K629001 & K1071004 are out! We need to purify more

7/21/2015 DNA Quantification

Part	Concentration (~~ng~~ng/μl)	Ratio
E0240	178.9	1.96
J22106	15.3	2.64
~~K592008~~	~~31.1~~ 17.1	~~1.96/2.04~~ 1.93
K346002	157.5	2.15
E0240	108.9	

Note - caps w/ "D" are dyetions. an additional "P" means ones with phosphatase.

7/22/15

- The remaining promoters (except Amp) have been dephosphorylated & digested & heat killed. WE CAN NOW LIGATE ONCE GFP HAS BEEN GEL PURIFIED

- Set up PCR w/ Lac-For, Lac-Rev, Pro-For, Kan-For, Kan-Rev w/ the following conclusions and notes:

Lac-For: 236 nmol
Lac-Rev: 24.6 nmol
Pro-For: 23.6 nmol
Pro-Rev = = Lac-Rev
Kan-For: 28.6 nmol
Kan-Rev: 25.6 nmol
Gfp-For: 22.4 nmol
Gfp-Rev: 23.5 nmol

cap names — Purified following parts w/ protcol from page 30.

		conc.	ratio
"LSRA"	LSRA pSB1C3	130.4	1.84
RecA1	J22106 RecA pSB1A2 (cracked)	100.9	1.82
RecA2	J22106 recA pSB1A2	101.2	1.79
"T7"	I712074-T7 pSB1KAB	140.2	1.82

~~Start at 20~~

Took the 4 parts list at bottom of page 36 and ran digestion.

Added this much of everything: (NL)

	DNA	H_2O	Enzyme 1		Enzyme 2		cutsmart
LSRA	7.8	17.6	0.8	Pst1	0.8	SpeI	3
RecA1	10	15.4	0.8	EcoRI	0.8	SpeI	3
RecA2	10	15.4	0.8	EcoRI	0.8	SpeI	3
T7	7.2	18.2	0.8	EcoRI	0.8	SpeI	3
	✓	✓	✓		✓		✓

- Mix gently by pipetting
- Incubate at 37° at 4:00
- heat kill for at

James Blondin

July 22, 2015 Digested DNA Purification (22 samples)

1.) Added 250 μL PB buffer (into ~50 μL DNA)
2.) • Transferred to a mini column (QIAquick column) in a 2mL collection tube.
• Centrifuged for 1 minute at max (15,000 rpm).
• Discarded the filtrate, reused the collection tube.
3.) • "Washed the sample."
• Added 200 μL DNA wash buffer.
• Centrifuged for 1 minute at max.
• Discarded the filtrate, reused the collection tube.
4.) "Washed the sample" again (repeated all of step 3).
5.) Centrifuged for 1 minute at max to dry.
6.) Placed the column in a 1.5mL microcentrifuge tube.
7.) "Eluted the sample"
• Added 15 μL H_2O
• Waited 1 minute* (*in reality, the wait was ~1 hour. Don't wait this long)
• Centrifuged for 1 minute at max (one of the caps came off. Place them sideways!)
8.) Stored the sample (1.5mL microcentrifuge tube) in the -20°C.

7/23 Making stock spectinomycin and plate

We want a 100 mg/mL stock solution.
~~We have 50 mg/mL~~ This appears to be what we ordered.

1000x dilution into plate = 1 µL per mL of gel.
So add 500 µL to the 500 mL of gel? Doesn't seem right - we'd use it all up. Ask Anu.

Running gel of GFP.
Add 10 µL of 6x loading dye to each 50 µL tube of GFP

Loaded 10 µL Loading buffer and ~100 µL GFP into 1% 2-well gel.
Ran for 30 min.

Put in tube labelled "GFP from gel"
It is in tube rack on bench awaiting purification.

Gel DNA Recovery

Add 600µl ADB to gel
10°C ~~Invert~~ Incubate 10 min, mix a couple times.
Load column. Spin 30s.
200µl DNA wash buffer Spin 30s.
200µl DNA wash buffer spin 1min
70µl dH2O. spin 1min.

(Some gel doesn't resuspend in ADB, so do it again)

680 470 605 637.5 515

Length of msr/msd regions
- w/ promoter: 284bp
- w/o promoter: 210bp

7/23 PCR (7 Rxns)

Each tube will have the following:
~~2.5µL~~ 25µL next time! :)
~~2.5µL~~ Q5 2x Master Mix
2.5µL Forward Primer
2.5µL Reverse Primer
2.87 µL pFF745 (500ng DNA)
39.63µL H2O

GG-Rev
GG-For & Rev will not have template
H2O to 42.5µL

The 7 Reactions will use the following primers:
① BB GG KanR Forward
BB GG KanR Rev → Anneal at 64°C
②③ BB Gap-msr For
BB lac promoter For
} BB msd (front) Rev → Anneal at 65°C
④ Lac-For
Lac-Rev
} Anneal at 62°C
⑤ Pro-For
Lac-Rev
} Anneal at 65°C
⑥ Kan-For
Kan-Rev
} Anneal at 63°C
⑦ GG-For
GG-Rev
} 95°C for 2min
Lower 0.1°C/sec → To 16°C for ∞

Each Primer was diluted to 20pmol/µL
- [primer]/20 = mL of H2O to add → Resuspend by pipetting

Using The Following Conditions for Thermocycling: (NOT GG-For, GG-Rev)

Initial Denaturation: 98°C for 2 min
Cycles: 25x
- 98°C for 7 seconds (7 b/c hairpins)
- Use above annealing times for 30 seconds (bk hairpins)
- 72°C for 10 seconds (~5-7 seconds by the standard 20-30/kb, but that seems so small :)

Do 35 next time

Final Extension: 72°C for 2 minutes

Note: Reaction ④ has an unknown amount of plasmid DNA bk we ran out
Reactions are in thermocycler 1+2

7/23 - 2 pFF745 Colonies were inoculated in 17 ug/mL LB at 6:30pm

7/24 Put 100 mL LB and 100 mL LB agar in autoclave B
Made a 1% gel with lots of wells and a 2% gel with 2 wells

Quantified GFP purified sample

GFP 1	10 ng/uL	5.56
GFP 2	2.6 ng/uL	.96

Added 100 uL spectinomycin to 100 mL LB agar and poured 9 plates. They are labelled and in the 4°

Took 7 PCR tubes out of thermocycler and put them on bench.

James Blondin July 24, 2015

- Miniprepped two samples of the Addgene plasmid (see my July 15, 2015 protocol), Note: Noah did steps 1-8, and I observed significant amounts of milky solid floating in the tubes after centrifugation (possibly a colloid), 15,000 rpm was insufficient at separating the solution from the milky solid particles.

DNA Quantification Results:

pFF745 (sample 1): 61.2 ng/uL, 1.85 ratio

pFF745 (sample 2): 37.8 ng/uL, 1.76 ratio

James Blondin July 24, 2015

- Quantified the 22 purified, digested DNA samples

#	Code	Value (ng/uL)	Value (ratio)
1	K376003	90.9	1.88
2	R0010	83.3	1.92
3	K914003	63.4	1.88
4	R0084	74.5	1.83
5	K873002	74.7	1.92
6	I13504 (GFP)	39.8	1.94
7	K56100	50.3	1.82
8	I746361	50	1.92
9	R0062	48.4	1.79
10	K896005	36	1.91
11	I746361	86.1	1.92
12	R0040	75.6	1.88
13	K397003	74.4	1.83
14	K107004	69	1.92
15	K56100	73.4	1.98
16	J33201	57.9	1.98
17	K150900	66.7	1.91
18	K629001	76.9	1.93
19	K554000	65.9	1.92
20	K911003	60.2	1.85
21	R0062	77	1.92
22	K1166000	74.8	1.95

7/24 PCR Take 2 (6 Runs) → No Gx (#7)

- Same as 7/23, except:
  - 25μL of master mix
  - 500pg of DNA
    - New DNA Conc is 61.2 ng/μL
      - Serially dilute, 10μL to 90μL, giving 6.12 ng/μL
      - Do this again to get 0.612 ng/μL
      - Take .81μL of this to get .5ng

→ 25μL + 2.5μL for + 2.5μL Rxn + 0.81μL dna + 19.19 μL H_2O

FINAL AMOUNTS

→ 20 pmol/μL, 20μM → $C_1V_1 = C_2V_2$ (What Qs using)

$(20\mu M)(X) = (0.5\mu M)(50\mu L)$

$X = 1.25\mu L$

① Add 1.25 μL of appropriate each primer to the 6 tubes
② Add .81 μL of the serially diluted DNA to each tube
③ Add 25 μL of 2x master mix
④ Add 19.19 μL of H_2O

bkan blac
b gap

"Bad microwaving" Gel messed up

Gel #1

1) Ladder 2) GFP 1 3) GFP 2 4) PCR 7

+ Diagnostic digestion (GFP digested to verify it)

10μL sample, 2μL loading Dye

10 μL Sample
2 μL loading Dye

~~Have not~~ Will be done tomorrow

Check on pc for results → There were a preliminary diagnostics

Gel #2

1) Log 2 Ladder; 2-7) PCRs 1-6

50 μL Sample, 10 μL Loading Dye → used 45 μL for each wells

Anything larger overflowed

James Blanden Very Quick Transformation

- Used J13002 (Plac+RBS34), pSB1A2, 2013-5-13B (will help w/ sugar application)
- Added 10μL H_2O to well, → 2μL DNA to 50μL comp cell (Thawed mostly in hand)
- After minutes (2-3) on ice, → 30 seconds in 42°C bath
- Plated 50μL directly onto 100 ng/mL carb plate. Did this in hood, put in 37°C.

7/25/2015

9:00 am Autoclaved glassware in autoclave B - MD

Cast .8% gels (x2) w/ B-well comb - MD + Andrew

We don't have EcoRV!!! - MD + Andrew

11 am Meeting

Discovered strains from sterile paper

Advice - skip the "control" w/o antibiotic

Strain may only be on part of the paper.

Put in the rest

Tips on gels/PCR

After PCR, run small (~2 μL) diagnostic gel

Then run column purification

Gel can be done - more work, lower yields, higher purity

Inoculation Notes - MD (of 4M17 strains)

↳ We did not record this last week! People involved were Aru, myself, + Andrew

Andrew and I made 8 tubes, 4 had SOB only + 4 had SOB w/ spectomycin. We used 5 μL of spectomycin / 5 mL of SOB. We ripped up the paper and put each of the 4 strains in 1 tube of SOB + 1 tube of SOB + spectomycin.

Aru Miranda Andrew

The next few days Ann was in charge of seeing if we had growth + found only 2 of the 8 tubes had growth. Here is a record of what we did

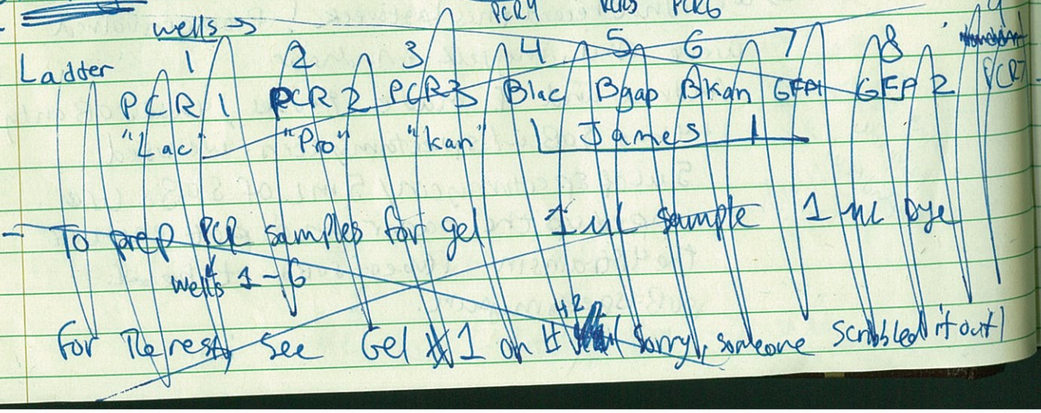
↙ Note → it was unnecessary to do these 4 tubes

	Strain		w/o specto	w/ specto	x = no growth
1	F144	KanR off	x	x	
2	F774	KanR off gal on	grew! (but unusable)	x	
3	F762	gal K	x	grew!	
4	F798	lacZ off	x	x	sw

Thoughts → Did they only put cells on part of the paper? Maybe the papers are putin didn't have cells. @ We should be using Spectomycin in all. There was no real advantage in using plain LB, just unnecessary due to our time/resource constraints

→ Primers arrived. Placed info in folder and primers in -20

Pierce carried → Ran gel (for Friday's PCR) 1% gel (120V)
& Miranda
12pm



To prep PCR samples for gel 1 uL sample 1 uL dye
wells 1-6

For the rest see Gel #1 on [illegible] (Sorry, someone scribbled it out)

* Run for 15min - take pic + check, then run it for 5min + check. (Todd said this is best for 1st couple times)

Lengths of PCR fragments to expect

Lac	210 bp
Pro	135 bp
Kan	170 bp
Blac	210 bp
Bgap	135 bp
Bkan	100 bp
GFP 1	720 bp
GFP 2	720 bp
PCR 7	

Should be same DNA just a different batch

Revised Info:

wells
1 Ladder
2 PCR1 (Lac)
3 * not enough added. Redo PCR2 on well 12
4 PCR3
5 PCR4 (Kan)
6 PCR5 (Blac)
7 * accidentally mixed 2 wells
8 * (Bgap)
9 ⊛
10 GFP1
11
12 GFP2
13 PCR7
14
15 PCR2 (Pro)

PCR 6 Ladder (Bkan)
⊛ 9th well was messed up. 7th overflowed above 9

Preparation of our dye/sample

PCR 1 ("Lac")
PCR 2 ("Pro")
PCR 3 ("Kan")
Blac (PCR4)
Bgap (PCR5)
Bkan (PCR6)
→ 1 uL sample for 1 uL dye

GFP1
GFP2
PCR7
→ 10 uL sample for 2 uL dye

10 Ladder

Added ~1.5 uL of our mixed sample/dye to each well

1/2pm Took an image of the gel after 15 min then ran 5 more min
↳ Image Lab 5.0

Final Images Saved as: (under Documents → iGEM → iGEM 2015 → 7-27-2015)
" 7-27-2015 Diagnostic Gel GFP, PCR7 and PCR1-6 "
.tiff and image lab images

Printed Miranda James

James Blanton 7/27/15

Resuspension Protocol (gBlock & primers)
1.) Centrifuge 3-5sec above 3,000g (I did 3,000 rpm above)
2.) Add TE buffer (I did water: 100uL for gBlock 1, diluted to 100 uM. for GFP for/rev & BB RTB for/rev)
3.) Vortex briefly
4.) Incubate at 50°C (waterbath) for 20 minutes.
5.) Vortex & centrifuge briefly

•Diluted the primers for the templates by 10 once (10uM) → 10uL of

Serial Dilutions
• Diluted 10uL EO240 and gBlock 1 (separately) in 90uL H_2O then repeated.
• Ending concentrations: 0.1 $\frac{ng}{uL}$ (gBlock 1), 0.563 $\frac{ng}{uL}$ (EO240)

PCR Prep

Volumes	BB RTB PCR	BB RTB PCR (sample #2)	GFP
Master Mix	25uL	25 uL	25uL
Forward Primer	1.25uL	1.25uL	1.25uL
Reverse Primer	1.25uL	1.25uL	1.25uL
DNA	5uL	5uL	1uL
Water	17.5uL	17.5uL	21.5uL
totals:	50uL	50uL	50uL

(I used 10uM primers, 0.563 $\frac{ng}{uL}$ EO240, and 0.1 $\frac{ng}{uL}$ gBlock 1)

PCR
• I setup the PCR for 2 samples of BB RTB and 1 sample of GFP.

(65°C) x RTB RTB
(72°) x RBS GFP x

Goals for tomorrow: gel test on the 3 PCR products (use 1uL) , column purify the reaction products, 4 BB SCRIBE subparts (use 10uL each), quantify (2uL), digest 3 of them (not Tan) (use 30uL each), ligate, transform

7/28 PCR Purification and Digestion (SCRIBE assembly)

Purification
4 samples: lac, gap, RTB ①, RTB ②
① Put 40 µL of sample in QIAquick column, inside 2 mL collection tube
② Add 200 µL HBC buffer and centrifuge at max speed (13000 rpm) for min (discard filtrate and reuse collection tube)
③ Add 250 µL DNA wash buffer, centrifuge (discard/reuse as ②)
④ Repeat ③
⑤ Centrifuge at max speed for a minute to dry
⑥ Move columns to 1.5 mL ~~eppi~~ microcentrifuge tubes
⑦ Add 20 µL H_2O, wait for 1-5 minutes
⑧ Centrifuge at max speed for a minute
⑨ Quantify DNA sample

Data:

Sample	260	280	Conc. ng/µL	Ratio
lac	.0859	.0468	85.9	1.84
gap	.021	.0112	21	1.87
RTB ①	.047	.0257	47	1.83
RTB ②	.0462	.0261	46.2	1.77

Digestion
6 samples: lac, gap, RTB ①, RTB ②, pSB1C3 ①, pSB1C3 ② (25 ng/µL)

Sample	lac	gap	RTB ①	RTB ②	pSB1C3 ①	pSB1C3 ②
500ng Quantity to add (µL)	5.82	23.8	10.6	10.8	20	20
(µL) H_2O to add	36.18	18.2	31.4	31.2	22	22

① Add 500 ng of DNA ↑ to 1.5 mL microcentrifuge tubes
② Dilute with H_2O to 42 µL
③ Add 5 µL CutSmart buffer (added 1.2 µL into GAP)
④ For lac and gap: add 1 µL of EcoRI and 1 µL of SpeI
For RTB: add 1 µL of XbaI and 1 µL of PstI
For pSB1C3: add 1 µL of EcoRI and 1 µL PstI
⑤ Add 1 µL of DpnI to each digestion
⑥ Mix by vortex / pipet
Time: ⑦ ~~Control~~ Incubate at 37°C (water bath works too)

[redacted] PCR II

Notes

gBlocks we have: msd/ssrA (4)
- Lac
- Pro*] → EcoRV BB
* RT/B

Primers we have: gBlock w/ overhangs & homology
EcoRV pCR2.1 ones

7/28 PCR II : Amplification of gBlocks; SCRIBE Construction & Assembly (Pg 1/3)

[redacted]

Notes: - gBlocks, as well as the Addgene (pFF745) plasmid are viable templates
- Digestions need to occur before Gibson on pSB1C3!
- Remember to dephosphorylate the digestion
- Use DpnI (eats methylated DNA) on pSB1C3 for the Gibson to get rid of plasmid
- Transform 5 μL of assembly

Abbreviations → - "Lac" = 1/2 msd w/ Lac Promoter ; "Pro*" = 1/2 msd w/ No promoter; "Kan" = 2/2 msd w/ KanR-on
"GG" = 2/2 msd w/ NO KanR-on, just BsaI sites ; "RTB" = Rev. Transcriptase & β recombinase

PCR5

[redacted] To Be Done: ① Lac + Kan + RTB] → Gibson'd straight into pSB1C3
② Lac + GG + RTB]

Depends on which primers we have.
③ Pro* + GG] → Golden Gate'd into PI pACYC (plasmid for GG → given by Todd)
④ Pro* + Kan
⑤ Pro*, Kan + RTB] → Gibson'd into pSB1C3
⑥ Pro*, GG + RTB]

General Overview: ① Resuspend gBlocks
② Amplify gBlocks (5 Amplifications, 6^2 primers sets)
③ Do Gibson assembly on the gBlocks
④ Touchdown PCR on [redacted]

PCR II: Amplification of gBlocks; SCRIBE Construction & Assembly (Pg 2/2)

(5) [redacted]

① Spin down primers + gBlocks briefly (until it hits max speed, then stopped)

② Diluted primers that have not been resuspended yet to 20 μM

Part 1 - For: 31.3 nmol → 31.3 nmol / [redacted] = 1.565/2 = 0.78 [redacted], add [redacted] twice this in mL H_2O

Rev: 43.1 nmol → 31.3 nmol / 1.565 mL

↓ Add 1.075 mL twice = 20 nmol/mL = 20 μM ✓

Part 2: For - 25.1 nmol, add 0.6275 mL twice

Rev - 29.2 nmol, add 0.73 mL twice

Master mix

→ X ③ Resuspended gBlocks to 10 ng/μL (500 ng of dried DNA: Add 25 μL H_2O) ④ 2d is 5 ng/μL

Part 2a-2c are [redacted] - Add 50 μL H_2O → copy (2d) is now 5 ng/μL

Part 2d is 250 ng

④ [redacted] Set up the amplification PCR as follows: (Can be scaled to any number)

- 25 μL OS 2x Master Mix
- 0.5 μM [redacted] For - Primer (1.25 μL w/ my dilutions)
- 0.5 μM Rev - Primer
- 500 ng of [redacted] Template → 500 ng ÷ 10 ng/μL = 0.05 μL } Dilute the gBlock so we don't need to pipet this
- 12.5 μL of H_2O (a-c) (& will use 2.5 μL water) 500 ng ÷ 5 ng/μL = 0.1 μL
- 50 μL ✓

↳ $C_1V_1 = C_2V_2$

$(10\ ng/\mu L)(5\ \mu L) = C_2(995\ \mu L)$

→ Dilute 5 μL of [redacted] in 995 μL H_2O to get

35x [redacted]

98°C 30 sec

98°C 7 sec

[redacted]°C for P2, 72°C for P1 for 30 sec

72°C for 10 sec

72°C for 2 min

a gBlock w/ Conc. 0.05 ng/μL → use 10 μL of this (20 μL for Gib)

Continued on Page 52!

7-28-15

Miranda **Made Gels** (1 mg - 8% from yesterday not put in fridge, threw out)

2:04pm 2x 1% gels & wells → in fridge ✓

3:00pm 4x 1% gels & wells → in fridge ✓

James Blondin **Ran Resuspensions of pSB1C3 Linearized Backbones**

Quantification Results:

2012: 1.96 ng/μL, 2.34 ratio

2012: 2.76 ng/μL, 2.46 ratio

2013: 15.66 ng/μL, 2.23 ratio

2014: 10.66 ng/μL, 2.31 ratio

2015: 11.26 ng/μL, 2.12 ratio

Steps:

1.) Add 50 μL H_2O, mix by pipetting.

2.) Vortex for 1 min, rest 10 min, vortex 1 min, "short" for ~5 seconds (missing).

3.) Run a quantification test. (Nanodrop)

4.) Store in -20°C.

MD **Ran a GFP Gel** - 1% gel @ 120V for 20 min. in & comb

#1	#2	#3 - 8 empty
adder	GFP	X
2 μL	12 μL	

↑ 10 mL sample 2 mL dye

Result → NOTHING ☹ ☹, we failed to PCR the GFP

MD **Ran a "RTB" gel** (Reverse Transcription/ Bioengineered)

1% gel @ 120V for 20 min in & comb

10 μL	10 μL	10 μL	
Ladder	BRTB	BRTB	X

same thing according to same

→ 1 μL of sample & 1 μL dye

→ used 10 μL in each well except used 4 μL in last well

Results: ☹ Not right band, primer dimers

PCR II Continued (Pg 3/5)

⑤ 10μL product was put in a gel (1%) running at 50/cm for 20 minutes

⑥ 1μL phosphatase was added to pSB1C3 vector → Incubated for 1 hr

⑦ ~~Redone GFP PCR + Xbal Xho Xba NEBuilder X for X full X construction~~

Notes on NE Builder:

2-3 Fragment Assembly: 1:2 Vector: Insert ratio

Total Fragment amount: 0.03-0.2 pmols w/ optimization w/ 50-100ng vectors & 2x insert; total vol. of unpurified PCR fragments < 20%

10μL Master Mix

Water to 20μL

→ Incubate samples in thermocycler for 15 Min at 50°C, transform immediately

⑧ PCR Purified the 5 Reactions. Eluted w/ 15μL H_2O. → I may have forgotten to elute C; added extra 15μL.

⑨ Amplification of Part 1 failed; the rest were successful

⑩ Phosphatase reaction was not heat killed!

→ Part 1: 129.3 ng/μL, 1.8

2a: 111.4 ng/μL, 1.88

2b: 133.3 ng/μL, 1.89

2c: 84.3 ng/μL, 1.85

2d: 102.1 ng/μL, 1.86

7/28/2015

James Blaidin

Summary of BB Method Progress (SCRIBE Assembly)

- The pSB1C3 backbone has been located, resuspended, quantified, and digested. Dpn1 was added, but not phosphatase. Enzymes have been denatured.
- B lac and B gap have been successfully PCRed, gel tested, column purified, quantified, and digested (Dpn1 added), and ~~the~~ enzyme denatured.
- B RTB 1 and 2 failed the gel test, and so are useless. Thus, ligation and transformation can't be done until B RTB is successfully PCRed.
- I setup another PCR of B RTB (3):
  - (500 pg total) • 5μL of 0.1 ng/μL gBlock 1 (I vortexed for 1 min, waited 10 min, vortexed for 1 min, and centrifuged gBlock 1 and the primers for BB RTB)
  - (25 pmol) • 2.5μL forward primer
  - (25 pmol) • 2.5μL reverse primer
  - • 25μL 2x Q5 MasterMix
  - • 15μL dH_2O
  - 50μL total
- Settings: melt at 98°C, anneal at 65°C, rest at 72°C, 35 cycles (7sec, 30sec, 10sec), hold at 4°C until ~~[illegible]~~ removed from Thermocycler
- I've ~~done~~ made sure that gBlock 1 and its primers are resuspended.
- I've confirmed their ordered sequences to see if they would assemble a ~2000bp fragment as desired.
- Aru tried amplifying the 5 gBlocks (1 and 2a-2d) ~~[illegible]~~ using old amplification primers. Everything but Part 1 succeeded.
- Thus, there might be a problem with part 1 itself.

Possible Solutions:

- Redo the PCR of B RTB (3) and run a gel test.
- ~~Order new primers to assemble B RTB in 2 halves, then assemble them into~~ ~~[illegible]~~
- If gBlock 1 really is a failure, I have no way of PCRing out B RTB for the biobrick assembly method. If this is the case, then we'll have to use (as the only viable option) Aru's golden gate SCRIBE assembly method. To think that gBlock cost $700 in Origo Card money...

7/29 GFP PCR

$500\,pg \cdot \frac{1\,ng}{1000\,pg} \cdot \frac{1\,\mu L}{.563\,ng} \approx 1\,\mu L$

Added to PCR tube

2.5 µL	for. primer	1 µL	GFP
2.5 µL	rev. primer	25 µL	Q5 mix
	19 µL	H_2O	

Put in thermocycler w/ "scribe" program

Inoculation of MIT strains (2nd time)

MD & JB

3 strains we inoculated in [3 tubes of 5mL of SOB + 5µL of spect8]

Tube #1 → F144
Tube #2 → F774
Tube #3 → F798 (our strain of interest)

Placed in 37° shaker at 11:55

1:45 measured OD_{600} against SOB blank

Blank	.000
F798	.005
F144	.005
F774	.014

4:00 Eyeball check. F774 is visibly cloudier.
F798 and F144 look like plain SOB.

7/29

Redo PCR II → Part 1

① Setting up the following reactions:
- a) Redo of yesterday
- b) DMSO Buffer G
- c) Touchdown
- d) Control

} Each of which has a modified extension time!

② Run the redo first; if it fails, run the Buffer G & Touchdown
③ Run Heatkilled pSB1C3 dephosphorylation
④ Assembly Notes: Size of SCRIBE Components:

Part 1: 1972 bp 19.4 ng/µL BB: 2070 bp 2.3 ng/µL
2a: 280bp, 111.4 ng/µL
2b: 354bp 133.3 ng/µL
2c: 280bp 84.3 ng/µL
2d: 206bp 102.1 ng/µL

pSB1C3 Digestion:

⑤ PCR Purified part 1 & pSB1C3 Digestion, eluted w/ 15µL H_2O
→ 3 ng/µL & 0 ng/µL, what the fuck? Digesting more pSB1C3 + PCRing more part 1
⑥ Ran a gel on pSB1C3, GFP, and Part 1 pSB1C3 & Part 1 were successful!
⑦ Redo the PCR b/c low yield/shitty purification. Three variants:
1) Same as last time
2) Buffer G variant
- 25µL Buffer G
- 12 µL H_2O
- 1.25µL Primer (Each)
- 10µL template
- 0.5µL Q5 Polymerase

3) Some add touchdown attempt
⑧ Requantified initial PCR
⑨ I give up.

Digestions of pSB1C3

1) Combine 100ng (instead of 500 b/c we don't have enough), .8 uL each restriction enzyme, 3uL of 10x buffer, X H2O to go to 30uL total volume
   .8 uL EcoRI
   .8 uL Pst I
2) Mix gently by Pipetting
3) Incubate for 2hrs (~37°C)
4) Heat ~~Kill~~ Kill 20m (70°C)

(using a Previous Protocol)

☆ Due to time constrictions, the product was put in -20°C after step 3.

☆ STILL need to heat kill

↳ In Primer box labeled "Heat Kill ASAP" on the top

7/30 Measured OD_{600} (:)

	Diluted w/ 250 uL H2O, 500 uL cells	actual	(x1.5)
Blank	0.000		
F798	1.585		
F144	1.474		
F774	1.558		

7/30 ① Quantified PCR Prt 2 attempts of PSB1C3 →
- 13.3 ng/uL 1.96 (Messed up purification yesterday) → 2.24 ng/uL 3.43
- 48.3 ng/uL 1.89 (T.D attempt)
- 19.4 ng/uL 1.62 (First one Restore) → Best one on the gel!

Digestion of I13504 (59.8 ng/µl)

2 hr digest
20 minutes Heat kill

DNA 500/59.8 = 12.6 µl
Smartcut 3 µl
XbaI. PstI 0.8 µl
Water 30 - 0.8x2 - 3 - 12.6 = 12.8

2:28-4:28.

- Made Glycerol Stocks of F144, F774, F798
  - 500uL 50% glycerol, 500uL of strain
  - placed in -80°C

- Diluted Strains of F144, F774, F798
  - Spectinomycin LB (6uL antibiotic, 6mL LB)
  - 2uL Strain with 1998uL of LB to give 1/1000 concentration
  - plated using 5uL and spread with pipette tip
  - placed in 37°C

② pSBIC3 Come up at a low [illegible] Concentration - plated in Spectros to get a higher concentration
③ Digestion move pSBIC3 w/ PstI & SpeI
④ [illegible] PCR Products were run on a gel again for my own sanity, the redo & transformation
- The redo of the first one is the best looking one - 19.4 ng/μL
→ ⑤ Since we barely have any Vector left:
a) Digest a part & run a gel purification (use PstI & SpeI)
b) Transform some pSBIC3 & purify on Monday
⑥ Rerun a PCR on the gBlock again bc I am a freak
⑦ Combined remaining pSBIC3 in a tube: 10.1 ng/μL, 1.93

⑧ pSBIC3 Transformation
- Using [illegible] 25 μL of pSBIC3 (~[illegible] 30 ng)
- Thaw cells on ice for 5 min
- Pipet [illegible] 25 μL of cells ([illegible]) (30 ng) (B/c not to exceed 5% of Volume)
- Incubate on ice for 5 min
- Heat shock at 42° C for 30 sec
- Ice 2 min
- 500 μL LB
- 1 hr Recovery + Plate

⑨ PCR Done Again
- Same as before. Thermocycler 4
⑩ Added 4 μL to Vacuum spun pSBIC3 & quantified 2 μL: Stock concentration
⑪ Digested Lac promoter w/ EcoRI - PstI o/n (1000 ng) (1 μL of enzyme each) to be gel purified!

TO DO: ① Heat kill the digestion
② Gel purify out pSBIC3
③ Gel confirm PCR digestion in thermocycler 4
④ Order pSBIC3 primers

James Blanden 7/30/2015 Part 1 → BB RTB PCR: temperature test

(seconds, °C) cycletimes:	Sample	Temperature, °C
30 at 98	6	58
35x [7 at 98, 30 at (varies), 60 at 72]	7	59.3
60 at 72	8	61.2
hold at 4°C	9	63.3
	10	65
	11	65.8

Gel Run: 20150730_JB_BBRTB3_6to11)

1	2	3	4	5	6	7	8
12μL ladder	6μL 7	6μL 6, 6μL 7	12μL 8	12μL 9	12μL 10	12μL 11	12μL 12

PCR Prep:
30 μL 2xQ5 M.M.
18 μL H_2O
6 μL 0.1 ng/μL gBlock 1
3 μL forward primer
3 μL reverse primer

Results:
- virtually no 2,000 base pair DNA detected in any sample

Next steps:
- try it again (maybe I used the same primer twice?)
- try new annealing PCR times, buffers, etc.
- switch to a new method (try PCR-ing the extraction and lysis from the pfu's separately)

3:47 - stuck the F798, F144, and F774 1/1000 dilutions in the 37°

Poured CM plates in the hood. 250 μL CM Stock (34) into 500 mL LB agar. Labeled and stored in 4°C.

CB

F13504 gel

1% agarose gel, 15 well

Lane	1	2	3
	2 log ladder	F13504	2 log ladder

1 μL F13504 1 μL dye

Lane	1	2	3	4	5	6	7
	2log	2log	F13504	2log		2log	

So many 2logs b/c Practice

gel → 20 minutes

7/31 Autoclaved 2 flasks and 1 box medium tips in autoclave C

Ran 1%, 12-comb/well long gel of last night's PCR products. 2 uL of each sample.

Lane	1	2	3	4	5
	ladder 1		II	III	ladder

Ran for 20 min. at ~~40V~~ 120 V. Then another 10 min.

Also quantified PCR

	ng/uL	ratio
I	555.5	1.78
II	567.3	1.84
III	535.4	1.81

Good. there is DNA there. should purify well.

Did I13504 digest from ~~yesterday~~. Put in 37° (waterbath) at 10:27.

—

Imaged gel. Bands were ~100 bp. Some smearing but not too bad. Saved on computer under today's date.

Put PCR products in GG box. They have "I" "II" and "III" in addition to today's date.

7/31/2015

MD (12pm ish)
- → Made 2x 1% gels with 15 wells
- → Heat Killed "Lac digestion" for 20 min.
- → 1uL of rSAP (shrimp phosphatase), then put in 37°C waterbath for 1 hour, then another 5 min heat kill

"rSAP" = phosphotase (prevents self-ligation) stored in Golden Gate Box

---

Protocol: I13504 Gel

* not much was left for this well

Lane	1	2	3	4	5	6	7	8	9
	2 log Ladder		~~I~~ I13504		I13504		I13504		2 log Ladder

- 2 uL of each sample in wells (15 well gel)
- 2 uL Ladder
- for part, 1 uL Part 1 uL dye
  - total → 3 uL part, 3 uL dye

Settings: 110 V, 20 minutes

1000fmol = 1pmol

0.03pmol = 30fmol

0.2pmol = 200fmol

To Do (Anu)

① PCR pSB1C3

② a) Digest digest!

③ b) Gel to confirm size

c) PCR Purify

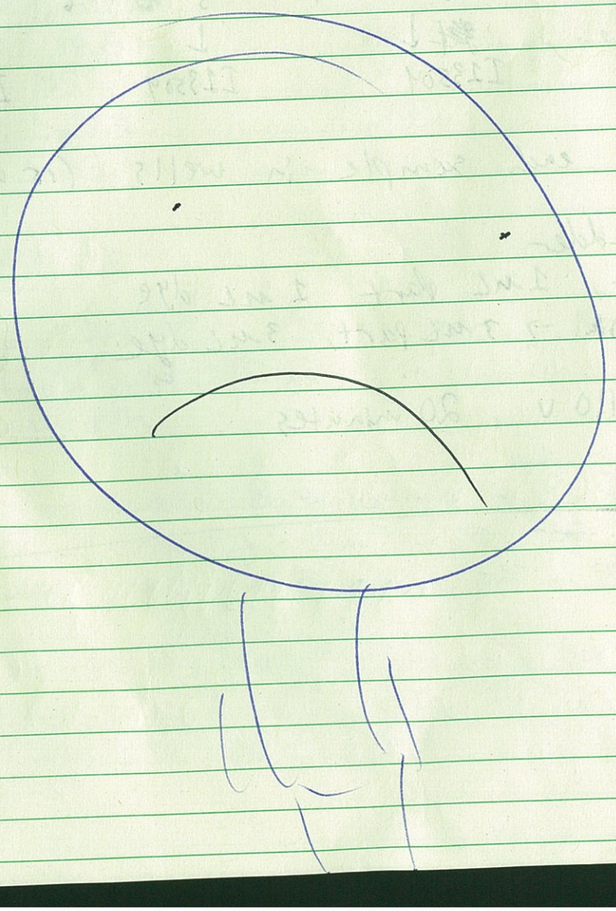
d) Quantify

② If PCR fails: Gel purify the lac promoter digestion

③ Final all PCRs or just run new ones → make sure you keep these!

- Found!

④



James Blondin

7/31/2015 BB RTB Temperature Test, Attempt 2

Sample	2X MasterMix	Primer	my (Kaul) (tubes)	Template	H2O	Temp, °C
12	5nL	0.5nL of each	"	0.5ng/nL (or template) gBlock 1	3nL	58.0
13	5nL	"	"	"	"	59.3
14	5nL	"	"	"	"	61.2
15	5nL	"	"	"	"	63.3
16	5nL	"	"	"	"	65.0
17	5nL	"	"	"	"	65.8
18	5nL	"	"	2nL (Aric's template) gBlock 1	1nL	58.0
19	5nL	"	"	"	"	59.3
20	5nL	"	"	"	"	61.2
21	5nL	"	"	"	"	63.3
22	5nL	"	"	"	"	65.0
23	5nL	"	"	"	"	65.8

Call PCR samples are 10nL reactions)

cycle:

Results:

- I tested 12-17 on a pre-made gel from the 4°C, and I saw only primer dimers (no 3000 base DNA).
- I tested 18-23 on a gel I made myself (52mL 1X TAE buffer, 0.52g agarose, 5.2nL midori green), and still only got primer dimers.

Going forward:

- Clearly, something is wrong with my primers, or gBlock 1.
- I've decided to design new primers using the Addgene plasmid only.
  - I'll make a "GG retron part" using 3A-based assembly.
  - I'll make a beta recombinase intermediate (RBS-ORS-Terminator) using golden gate assembly.

7/31/15

① Setting up pSB1C3 ⊕ PCR (×2)
- 25 µL mastermix
- ~~424~~ 424 pg of DNA (1 µL) (pSB1C3 from Lac)
$\left(212 \frac{ng}{\mu L}\right)\left(\frac{1}{2}\mu L\right) = X \cdot 1000 \mu L$
$= .212 \frac{ng}{\mu L} = 212 \frac{pg}{\mu L}$ → ~2 µL of dilution
- 1.25 µL PSB1C3-for
- 1.25 µL BBB-rev
- 11.5 µL H_2O

Tm = 74°C & 79°C → T_A = 72°C b/c temp has to be < 72°C for QS!

Both are in Thermocycler 1, one is at 72°C, one is at 77°C

② B/c of the temperature of the primers, a Buffer G Rxn was made!
- 25 µL Buffer G
- 424 pg (1 µL) DNA Template (Lac, R0040) 0.5 µL GXS Polymerase
- 1.25 µL For/Rev primers
- 20.5 µL H_2O

Done at 68°C & 72°C in Thermocycler 2

③ Digest all PCR samples out of the Thermocycler w/ PstI & SpeI for 30 minutes
- All of DNA
- 1 µL enzyme (PstI for longer overhangs)
- 5 µL NEB Cutsmart
- x µL to 50 µL

Heat Killed for 20 min! In GG Box

Q: But the gBlocks don't have that! → Go get the current gBlock sequence

④ Diagnostic gel on the 4 pSB1C3 PCRs + the 3 part 1 pcrs (G) (G)

Ladder	Ladder	P1	P1	P1	PSB	PSB	PSB	PSB	Ladder

- Volume is now ~45 µL → PSB(1) & Both PSB(G)'s worked!

~~⑤ Running another PCR on Part 1 again; 3 are at 72°C, one uses Buffer G at 67.2°C~~

⑤ Running another PCR on Part 1 again; 3 are at 72°C, one uses Buffer G at 67.2°C

To Do Monday

① PCR Purify the 3 pSB1C3 things that worked
② Quantify the above
③ Quick diagnostic gel on PCR-1s which are "Gibson Ready" (in GG-Box) for my own mental sanity (use 3 µL of DNA, 0.5 µL loading dye))
④ On the same gel, run a diagnostic gel on the stuff in PCR 1 ⇒
- if it shows a ~2kb fragment, purify that size! Quantify also. Use the small columns & elute w/ 15 µL; if you aren't 100% sure about how to do this, don't do it.

8/2/2015

Michelle - Inoculate 1 ml of LB + 100 µg/ml Spectinomycin with the following:
- F762
- F144
- F774
- F798

Grow @ 37°C overnight in shaker

Caroline

Comp cells from MIT strains

- Make 1 M $CaCl_2$

Online protocol says 147.02 g of $CaCl_2 \cdot 2H_2O$ in 1 L H_2O
We'll do 1.47 g in 10 mL H_2O
↳ Sterilized with .22 µM filter

Materials needed -
200 mL LB
20 mL .1 M $CaCl_2$

10 mL .1 M $CaCl_2$ - 15% glycerol
↳ - 1 mL 1 M $CaCl_2$
3 mL Sol. glycerol
6 mL dH_2O

Put .5 mL of overnight culture of all 4 strains in 49.5 mL LB in 37° shaker @ 3:57 PM

	OD_{600}:	F762	F144	F774	F798
time	4:40	.032	.72 * .016 = .046	.034	.036

* P967

8/3/15

① Running a gel on the components of the assembly for final verification

Ladder Redo 1' 2' 3' Buffer G Old Redo 1 pSB1C3 Ladder

② While the gel is running, PCR purify the other tubes

- Old Redo 1 was the best at 19.4 ng/μL (1972 bp)
- pSB1C3 Normal: 103.7 ng/μL (Best one, the 2 Buffer G ones were 69.2 & 69.7 ng/μL) (2070bp)
- Part 2.1: 111.4 ng/μL (280bp)
- 2.2: 133.3 ng/μL (354bp)
- 2.3: 84.3 ng/μL (280bp)
- 2.4: 102.1 ng/μL (206bp)

~~1μL pSB1C3 · 81.07 fmol / 0.5μL · 39.08 fmol~~

[redacted]

③ Gibson Reno Setups

- Using 100ng of pSB1C3: 78.18 fmol ; 75ng = 58.63 fmol

We need 2:1 ratio of insert:BB for part 1 → 190.5ng for 156.36 fmol ; 117.26 fmol = 142.9ng

Going w/ a 3:1 ratio of part 2:BB → 51.31ng for 234.54 fmol ; 175.89 fmol = 38.48ng (2.1)

Final Setups

75ng of pSB1C3 (.723μL) (58.63 fmol) } 8.089μL
142.9 ng of Part 1 (7.366 μL) (117.26 fmol)

Part 2

We need 175.89 fmol

		TOTAL VOLUME SO FAR	Water To Add
2.1:	30.44 ng → 0.273μL	=> 8.362μL	1.638μL
2.2:	38.48 ng → 0.289 μL	=> 8.378 μL	1.622μL
2.3:	30.44 ng → 0.361μL	=> 8.450 μL	1.55μL
2.4:	22.40 ng → 0.219μL	=> 8.308 μL	1.692μL

• 10μL of HiFi DNA Assembly Master Mix to each tube

8/3/15

④ PCR Purifying the Buffer G PCR of Part 1 just in case we run out of redo pII

- 35.3 ng/μL conc.

OD measurements from page 65

☆ for F144, we put the cuvette in the wrong orientation. Measurement is 0.046 (grew a little bit between measurements (~40 minutes growth total) -PH

	F762	F144	F774	F798
5:00	.04	.622	.05	.048
5:30	0.056	0.028	0.070	.068
6:00	~~0.095~~ 0.090	0.044	.116	0.122

James Blondin

08/03/2015

(Aris's suggestion: purify → quantify → run a gel on some of it

PCR Experiments

- I'm running one more onset of PCR experiments to test my primers before I order an entire new set. I have ordered new primers to PCR out the same "Part 1" gBlock for 3A assembly in two stages (in case my primers were too short in homology or too long overall).

		template	forward primer	reverse primer	water	Q5 MM	bands (strongest → weakest)
Order:	1 mine (primers)	5 nL	2.5 nL	2.5 nL	15 nL	25 nL	~0.1 kb, 2 kb, 1 kb
(control)	2 Aris's primers	5 nL	1.25 nL	1.25 nL	17.5 nL	25 nL	2 kb
	3 forward	0	2.5 nL	0	22.5 nL	25 nL	none
	4 reverse	0	0	2.5 nL	22.5 nL	25 nL	none
	5 both	0	2.5 nL	2.5 nL	20 nL	25 nL	~0.1 kb

Cycle: 2 min 98°C, 35x [0s/15sec 98°C, 30 sec 64.9°C (71.9 for Aris's), 60sec 72°C], 2 min 72°C

For next time: run a gel on the rest of my sample one from today, gel purify it (WEAR A LONG SLEEVE SHIRT), quantify it, and use some of the purified template as a new template (try to use 500 pg DNA for PCR, if you can).

8/3/15

⑤ Gibson was done for 50 min at 50°C, held at 4°C

⑥ 2a & 2c were attempted to be transformed by electroshock; failed b/c we didn't clean up → ARCed ::

⑦ 2b → 2d were heat shocked into NEB 5α Comp Cells (2 μL of assembled product added)

- Note: They incubated w/ DUH on ice for ~45 min
  This was not my best transformation protocol! May fail Either way, 2a needs to be done.

⑧ Plated 100 μL of the cells. Stored rest in 4°C. Plates in 37°C

⑨ Ran a 90V 30 min 1% gel on assembled products

- Only BB & 2a/b/c/d parts seemed to show. Why did no part 1 show?
  ↳ Part 1 was the sketchest one!

8/4/15

① OD of FF144/774 measured at 12:39pm

114: 0.097

744: 0.130

Placed back in 37°C, will check at 1:10pm

② Colony PCR on the 3 transformed constructs (2b-2d + RTB in pSB1C3)

- Using 8 colonies per plate (So 24 total)

a) Pick a colony & dilute it in 50 μL of LB

b) Combine 12.5 μL of the Q5 Master Mix w/ the appropriate amount of Part-2-For & Part-1-Rev primer.

0.5 μM = final conc. of primers

20 μM = Current conc. of primers

$C_1V_1 = C_2V_2$

$(20\,\mu M)V_1 = (0.5\,\mu M)(10\,\mu L)$

$V_1 = 0.25\,\mu L$ → (0.25)(24) = 6 μL each primer | 96 μL H_2O

Each tube will have:
5 μL 2x Q5 MM
0.5 μL primers
H2O
0.5 μL template (cells)

c) Aliquot this into each (24) PCR tube

d) Add 0.5 μL of (a) dilution in each tube

e) Run the following thermocycler program

98°C for 5 minutes to lyse cells

40x { 98°C for 10 sec; 72°C for 30 sec; 72°C for 90 sec (~3 kb) }

72°C for 2 min

4°C hold

f) Verify PCRs by gel → Inoculate correct tubes from (a) in 5 mL LB

g) Store plates w/ parafilm

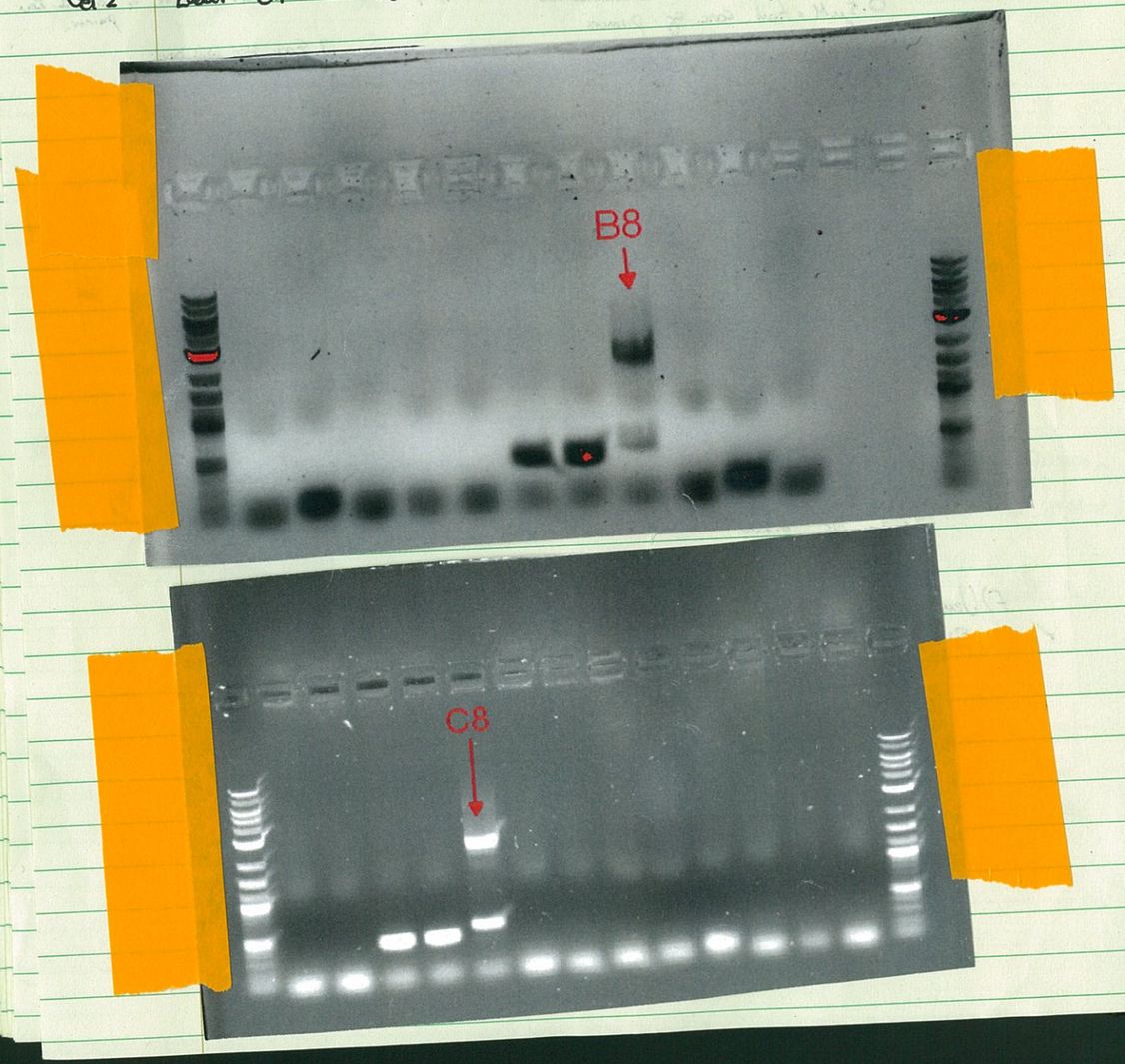
8/14/15

③ 2 μL of Assembly 2a + RTB + PSB1C3 was transformed, but it was a super stolen transformation...

④ Gel Electrophoresis

Gel 1 Ladder B1 B2 .... B8 C1 C2 C3 Ladder

Gel 2 Ladder C4 ... C8 D1 D2 D3 D4 D5 D6 D7 D8 Ladder



Competent Cells (8/14/15)

① OD Measurements

Time	FF144	FF774
12:30pm	0.097	0.130
1:20pm	0.212	0.278
~2:00pm	~0.27	In 4°C

James Blondin

08/04/2015

Mass

tube1 1.03g (1.03)

tube1 + gel 1.27g (∴ 0.24g gel)

tube2 1.01g

tube2 + gel 1.27g (∴ 0.26g gel)

Gel Purification

- I used a 2% agarose gel to separate out my ~2kb DNA from the 45uL of sample 1 from yesterday. 45uL PCR mixture, 9uL dye, 3uL ladder.
- I used a razor blade to cut out the desired band in two halves for purification (each in a 1.5mL tube).

Protocol (Zymo Clean)

1.) Add 3uL ADB Buffer per mg gel to the sample (ex: for a 0.25g gel, add 750uL ADB Buffer). Incubate for 10 minutes at 50°C, ensuring the gel is completely dissolved (mix as needed).

2.) Transfer to a "Zymo-Spin I Column" (mini column) in a 2mL collection tube.

3.) Centrifuge at max for 1 minute. ("spin"). Discard the filtrate, recycle the tube.

4.) "Wash" (add 200uL DNA Wash Buffer), spin, discard the filtrate, and reuse the collection tube.

5.) Repeat step 4.

6.) Transfer the column to a 1.5mL microcentrifuge tube. Add ___ uL H_2O (I used ___ uL H_2O) and spin (wait 1 minute before spinning).

7.) Store the tube now containing purified, eluted DNA.

Results:

- 15.4 ng/uL DNA (quite high - undissolved gel might be this...)
- 51.33 ratio

- I did two serial dilutions on this DNA (1.54 ng/uL & 0.154 ng/uL)

PCR Setup:	template	primers (my OG RTB primers)	Master Mix	H_2O	Annealing Temp
1 Direct RTB (0G template)	5uL 0.1 ng/uL 0G template	2.5uL each	25uL	15uL	64.9
2 G1 RTB (gel purified)	3uL 0.154 ng/uL purified PCR	2.5uL each	25uL	17uL	71.9
3 G2 RTB (gel PCR2)	3uL 0.154 ng/uL purified PCR	2.5uL each	25uL	17uL	71.9

cycle: same as yesterday

- I used 72°C for the gel purified PCR DNA because my forward primer should now have 60 bases of homology (much more homology).
- I may have had undissolved gel contaminating my purified DNA. If this is the case, I can restart with PCR sample 1 from today.

todo tomorrow: test 5uL of each reaction. Do gel purification on any successful 2kb bands, quantify, and possibly digest overnight.

James Blondin

8/05/2015 & 8/06/2015

	mass w/o gel	mass w/ gel	uL ADB Buffer Added	(quantification) Final Data:
1	1.05g	1.33g (0.28g)	840uL	5.4 ng/uL
2	1.05g	1.32g (0.27g)	810uL	2.84 ratio
3	1.01g	1.30g (0.29g)	870uL	

James Blondin

- I did a PCR on my original template (0.1 ng/uL, 5uL), 3uL of the 0.154 ng/uL gel purified DNA from 8/04/2015 (diluted 100x), and 1uL of the 5.4 ng/uL gel purified DNA from 8/05/2015 (not diluted (my mistake)).
- All reactions except the direct one failed to show any significant amounts of DNA in the 2kb range, and the direct one barely showed any.

Conclusion: these primers I'm using don't work. Try the new primers I ordered on Monday and got on 8/05/2015.

- I resuspended the new primers: "shorted".
- Added 10uL H_2O per manufacturer's instructions.
- Vortexed.
- shorted.
- Left in the 50°C bath for ~1 hour.
- Vortexed.
- shorted.
- Stored. I have not yet quantified them.

Going forward: I only have one day left in lab before leaving for 2 weeks. At this point, I see little reason to keep working on this BBA assembly in wet lab directly.

- At this point, I'd rather just start transitioning to dry lab / prepare for those 2 weeks.
- If I can get someone else up to speed on what I'm doing, that'd be great.

8/5/15

To Do

① ~~Redo~~ colony PCR on ~~previous~~ colonies (30 min prep ~~and~~ 2.5 hour run)
- Positive hits
- Plate D remnants } Run a gel (40 min incl. prep)
- 8 from plate A

② Assemble D ~~again~~ and A again (60 min)

X - Find/make/digest/~~dephosphorylate~~ pSB1C3 (Miniprep, 1.5 hr PCR, 2.5 hrs enzyme treatment, 20 min purification) ← Found some!

③ Purify successful transformations (1 hr or less) (Run a diagnostic to see if unspecific → Also digest!)

④ Retransform A/B/C/D except do it properly you fuck. (~~start~~ 1 hr wait + ~30 min actual work)

Plan of Action

- Set up colony PCRs and run them ✓
- Have someone pour 2 gels while this happens ✓
- Find pSB1C3 / Set up what needs to happen for assembly ASAP (PCR + Digest + Dephosphorylate + Purification) (+Gel Confirmation) ✓
- Retransform ✓
- Purify + quantify ✓
- Colony PCRs should be done by then → Run the gel ✓
- Assemble while gel is running ✓
- Inoculate successful colonies
- Transform assembly ✓ (before 4pm is the goal; before 6pm is the reality)

---

① pFF745 + pac19 transformed into FF744 + FF144 ~~Ran~~
- Thawed for ~5 min
- Incubated for ~10 min
- Shocked for 30 sec
- Rest is standard.

~~Extraction~~

8/5/15

Start: 2:15pm

(*) Idea: Lab Assistant python script
↳ Incl. protocol times too
↳ Weekly <3 hrs to make
→ Live app + telegraf interface

Ex: "Colony PCR"
↳ Tells you to remember to make a scaffold

① Set up colony PCRs — 26 PCR tubes for PCR itself; 23 tubes for 50 µL inoculations

- ~~G~~ A Colonies (A1-A6) (6)
- B8 - B9-B15 (8)
- ~~D5~~ - D9 - D10 D11 (4)
- C8 - C9 - C15 (8)

✓ - Aliquot 50 µL LB in A1-A6, B9-B15, C9-C15, D9-D11
✓ - Pick the colonies & Inoculate
- Prepare "Master Mix" & aliquot 9.5 µL to each tube
- Put 0.5 µL each template in → BE EXTRA CAREFUL w/ B8 & C8

② Do Gibson on A & D again (A = 280bp D = 206bp)

75 ng of pSB1K3 = 58.63 fmol

29.56 ng of D

(*) Attempting a 4:1 ratio for the smaller parts: $\frac{58.63}{\frac{4}{234.52}} = 234.52$ fmol) = 40.59 ng of A

↳ Part 2a & 2d (i.e. parts w/o KanR, so the smallest ones) were the ones that failed, so higher molar ratio is good 8)

↳ Jk, D A = C in size, my transformation was just retarded.

29.56 ng of D = 0.289 µL ; 40.59 ng of A = 0.364 µL

142.9 ng of Part 1 = 4.04 µL } 4.763 µL
75 ng of pSB1K3 = .723 µL }

✓ H_2O to add to A: 4.873 µL, D: 4.948 µL } → 10 µL total

+10 µL Master Mix

(*) 50°C for 30 minutes

③ Purified & Quantified ~~Gibson~~ C8 & B8

B8: 90.4 ng/µL → 1.81
C8: 85.1 ng/µL → 1.89

GFP PCR (GFP Prime 1)

Dilutions: (to 20 nm)

GFP forward: 28 nmol / 20 = 1.4 ml (0.7 x2)
GFP Reverse: 29.8 nmol / 20 = 1.49 ml (0.745 x2)

GFP Forward (w/ Prefix): 26.7 nmol / 20 = 1.34 ml (0.668 x2)
GFP Reverse (w/ Suffix): 33.1 nmol / 20 = 1.66 ml (.828 x2)

~~50 µl~~

for 50 µL rxn

Add: 1) 25 µL Q5 Master Mix
2) 1.25 µL Forward
3) 1.25 µL Reverse
4) 500 Pg DNA → ~~9.17 µL~~ 9.17 µL
5) 13.33 µL H_2O

math

| 27.5 |
| 9.17 |
| 36.67 |

50 − 36.67 = 13.331

500 Pg · 1 ng / 1000 Pg · 1 µL / 54.5 ng = 0.00917 µL

Math

→ ~~10 micro to 90 micro water~~
↳ ~~10 to 90 ⇒ 9~~
→ 10 micro sample to 90 H_2O
↳ ~~9.17 µL~~ 9.17 µL

Anneal at 70°C
Extension → 20 seconds

Todd's GFP → 54.5 ng/µL

8/5/15

④ Gel on Colony PCRs, 110V 20min 0.8% gel

(Spillover)
Ladder A1 A2 A3 A4 A5 A6 B8 B9 B10 B11 B12 B13 B14 B15 Ladder
Ladder C8 C9 C10 C11 C12 C13 C14 C15 D5 D9 D10 D11 Ladder

REFER TO ⑧ FOR AN ACCURATE LIST OF THINGS TRANSFORMED

⑤ At 8:35, NEB 5α for retransformations of old A/D + new A/D assemblies & FF144/FF774 for purified B&C plasmids were put on ice to thaw for transformations

NOTE: FF144 & FF744 will be heat shocked for 45 seconds b/c they are DH5α derivatives.

NEB Cells will be heat shocked for 30 seconds per NEB's protocol

⑥ pFF745 will be retransformed w/ 45 seconds of heat shock b/c why not, puc19 too. → 8:50pm was the time they were put on ice.

9:06: Mixing w/ DNA.

9:55 In 37C Incubator. → Take out after ~45 min

Things Transformed

A (new) A (new) D (new) D (old) in NEB 5α

pFF745, C8, B8 in FF144/FF774

puc19 in FF144

8/6/15

Run digestion on parts C8+ B8*

↳ Scaled down to 10 ul

1.5 ul CutSmart buffer

.25 ul of EcoRI

.25 ul of PstI

125 total ng of DNA → for C8 = 1.5 ul for B8 = 1.4 ul

fill to 10 ul → H_2O = 6.5 ul 6.6 ul

- after Combining, mix gently + centrifuge briefly

Samer

MP Labelled C8*, 138*

Ran Digestion to add PstI:

1.5 uL CutSmart
.25 uL EcoRI
.25 uL PstI
.25 uL PstI
1.5 uL C8 / 1.4 uL B8
to 5 uL H_2O / 6.6 uL H_2O in C8 in B8
mix, centrifuge +
thermocycler #4

8/6/15 To Do (D) ⑩, ③, ⑨, ①, ④, ⑥, ⑤, ②, ⑦ in that order (or concurrently, just ⑤ ⑧ & ④ are most important to do today)

① In ~4 hours (3pm - 4pm) tease out all the parts & see which successful (Some FF144/FF774 ones may be good; Andrea should be)

② For all successful transformations in FF144/FF774, inoculate 3 colonies from each

- Note: There are doubles of the plates, you don't need to inoculate the repeat plates, but do clearly mark what plate you used!

Wait to do this b/c we may not begin experiments tomorrow!

③ Find a digestion for determining if B8/C8 are in B8/C8, then run a gel on it (currently inoculating in B8/C8 w/ pstI & EcoRI; plates have been sterilized! they were kept in fridge, first bleached, then autoclaved)

- Find an enzyme that cuts B8/C8 in the insert but not the vector itself

④ For successful colonies in NEB 5α cells (New A / Old D / New D), pick 8 colonies from each & do a Colony PCR (Erasmany)

- Use Part-1-Rev & Part-2-For, on bench/in Golden Gate Box

w/ Michelle

- Protocol is in depot in lab notebook & on Illinois iGEM email from Todd

- Mark each colony you pick from the plate and be very organized when doing this → it will end up being ~32 PCR reactions involving ~64 PCR tubes total

⑤ Make a bunch of plates of CM/KM/LB, ~20/30 of each (use 10 uL per plate, swirl around to cover the bottom of the plate, you'll use less LB (i.e., have to autoclave less LB))

⑥ Run a GFP PCR using GFP-For-Wan-Prefix & GFP-Rev-With-Suffix → this will give us a GFP we can use for assembly

Patricia

- Run a quick diagnostic on the GFP template PCR to verify ~750 bp band

- ~~Do a PCR cleanup first, then quantify to know how much template to add~~

- Do a PCR cleanup first, then quantify to know how much template to add

GFP Amplification Gel Test (GFP Prime 1)

~~Lane 1 2 3 4~~

Lane	1	2	3	4	5	6	7	8	9	10	11	12	13
	Ladder		GFP		GFP		GFP		GFP		GFP		Ladder

1% Gel, 20m.
5 mL GFP and 5 mL Dye
Add 2 mL into each well

↓

Gel! Very succesful

Put here

GFP Prime 1 Quantification

First try GFP 14.4 ng/mL
ratio 1.76

Second try → 20.7 ng/mL
1.75 ratio

↓

Try this measurement

GFP PCR (GFP Prime 2)

* Adds prefix, suffix, rbs

Run 8 x 50 mL Rxn

Add: 1) 25 mL Q5 Master Mix
2) 1.25 mL Forward
3) 1.25 mL Reverse
4) 500 pg DNA → 2.4 mL
5) 20.1 mL H_2O

Anneal at 72°C

22 sec extension time

Math
$500\ pg \cdot \frac{1\ ng}{1000\ pg} \cdot \frac{1\ mL}{20.7\ ng} = 0.02415\ mL$

10 micro sample to 90 micro water

8/7/15

To Do (+Whatever y'all have planned)

① Run a gel on the digestions from yesterday on 88 wells of the 96 well plate if we see the band pattern we expect, then proceed to ②

② Run a gel on the Colony PCRs
- This will be a lot of well loading, so do be careful. I typically add 2 μL of buffer then actually put ~7 μL into the well. Add tape to the bottom of the tray (one of them has that done) to see the wells easier.
- Showing a band at ~2,000 bp = success!

③ For all successful colonies, take the contents of the 50 μL inoculation done in the PCR tube (i.e., where you got your template from) & inoculate that in 5 mL LB + CM (2.5 μL of 34 mg/mL CM stock), incubate in the 37°C, ~~④~~ ASK MICHELLE OR SOMEONE ELSE TO TAKE IT OUT ON SATURDAY ~~FRI~~

④ Count the # of plates we have on reserve of each type & ~~so~~ send that count over chat or something
- We may need to make more plates

⑤ Compile all the promoters we want to use into one box/rack that we can all easily access (Not 100% sure what lysate ones were placed, listing them all)
- Compile the concentrations/amounts of each one too. Just estimate the amounts left.
- L1166000, 121.2 ng/μL, ~50 μL left, drives gene expression under acute & chronic hypoxia, psB1C3
- K117002, ____ ng/μL, ~50 μL left, AI-2 inducible quorum sensing promoter, psB1A2
- I765000, 46.8 ng/μL, ~~some~~ ~70 μL left, Fe inducible promoter, ILLEGAL PstI SITE !, psB1A2
- K540001, ____ ng/μL, ~50 μL left, Cobalt sensitive promoter, psB1A2
- R0040, ____ ng/μL, ~50 μL left, Tet promoter, psB1C3
- K376003, 51.2 ng/μL, <10 μL left, $JG O_2$ sensitive promoter, ~~[redacted]~~ psB1C3
- K387003, 74.4 ng/μL, <10 μL left, fdhF hypoxia promoter, psB1C3
- J33201, ~~[redacted]~~ 85.9 ng/μL, ~50 μL left, arsenic promoter/repressor (sodium arsenate), psB1C3
- R0010, 213.5 ng/μL, ~50 μL left, Lac promoter, psB1C3
- ⑥ Transform SoxR from above into ↓
- ⑥ K55400, 96.1 ng/μL, ~50 μL left, SoxS promoter (BUT IT IS REGULATED BY SoxR (Well 21I, 2015 plate I)
  - SoxR in the presence of nitrous oxide will allow SoxS to promote transcription
- R0062, ~~[redacted]~~ 138.9 ng/μL, ~50 μL, Lux promoter
- K1071004, 61.5 ng/μL, empty ????, nitrate sensor (nitrate has to be the only N_2 source → ammonia will do this if no other N_2 is present)
- K561001, 91.6 ng/μL, ~30 ~~[redacted]~~ μL, vgb microaerobic promoter

Ignore this one for now, I'll handle it. →

⑥

⑦ Run a PCR on BBa using addgene no ~~[redacted]~~ promoter primers to generate 2 cut PSB construct.

8/10/2015 Do a Gel for Colony PCR

2μl Loading Dye (6X) + 10μl Sample.

S D8 D7 D6 D5 D4 D3 S | S D2 D1 A1 A2 A3 NewD S

X
(I broke this well)

~~The~~ failed protocol of Colony PCR

① Pick colonies from plates by tips and put the tip into 25μl LB.
② Mix { 13×5 = 65μl QS buffer
13×0.25 = 3.25μl FW Primer (Part2)
13×0.25 = 3.25μl RV Primer (Part1)
13×3.5 = 45.5μl dH2O
③ Add 9μl of the mixture to each PCR tube.
④ Add 1μl templete LB into each tube
(D1-D8, A1-A3. NewD)
⑤ PCR { 98°C 5min
98°C 10sec
72°C 30sec
72°C 90sec } 35X
72°C 2min
4°C

8/10/15

① Set up PCR on B8 to generate a C construct using:
- Part_Pro primer for top strand
- Part_1_Rev for bottom strand
} Note: This will give a very high Tm for Part 1. Consider using Buffer G • too.

Note2: This part will have BbsI on the 5' end! We will need to cut off

B8: 90.8 ng/μL
Ld 2μL in 998 μL H2O = 1:500 dilution = 0.18 ng/μL → 2μL = ? 360pg

Setting up 3 PCR Reactions:

① 25μL QS 2x	② 25μL Buffer G
2μL B8	2μL B8
1.25μL Primer 1+2	1.25μL Primers
20.5μL H2O	0.5μL QS Polymerase
	20μL H2O

} Will run at 72° & 68°C

→ Put in thermocycler 2 at 2:15

② Digestion of B8 & C8 showed nothing → redoing the digestion. (MD♡)

mixture

B8
1.25μL Buffer 2.1
0.25μL EcoRI
0.25μL PstI
0.25μL BsiI
125ng DNA (1.4μL B8)
6.6μL H2O
10μL Total Volume

Also running a positive & negative control using pFF745 & no plasmid.
(61.2 ng/μL · x μL = 125 ng, x = 2.2047 μL) we added more than 1μL
Use 7.032μL water for pFF745
Use no DNA & 7.75μL H2O instead

Ran "Dig" in Thermocycler #1 @ 3:55pm for B8, positive, negative control.

8/10/15

③ Redoing the Colony PCR w/ Buffer G on NewA_1, NewA_2, New_D & ~~New~~ Old_D

- Just kidding. Using whatever colonies I can to do it

④ Picked 3 biological replicates from ~~FF745/144~~ plates w/ pFF745/B8 added to them. Added to 5mL of LB+ 30μg/mL CM. → 37°C Shaker, 8:00pm

- Total of 12 tubes shaking

③ Positive Control: ~~B8 from X first round~~
Picked another B8 to be safe (B18)
Repicked C8 just in case (C18)

New Colonies Picked: C16, C17, C18, C19
New_A 1,2,3
New_D 1
~~Old~~ Old D 9-13
New_A2 1-5

19 PCR tubes for inoculation
19 tubes for PCR itself → 38 tubes

26.5: 100μL of QS 2x
4μL of primers 1/2
80μL H_2O
} 9.5μL in each tube

LB Tubes: C16, C17, C18, ~~C19~~ Old D9 ~~D10~~ D13 D12 D11 NewD1, NewA 1-3, NewA2 1-5, B18, B8

⑤ Extension time = 1:00 not 90sec! Also add 20sec at 98°C at start of cycle not 10sec!

⑤ Running a gel on B→C 1,2,3 (QS 2x, Buffer G, Buffer G) & Digestion 1,2,3 (B8, Control, Control) 72°C 66°C

Setup for Experiments/Assays on 8/11/15

- We will have ~~5~~ 3 biological replicates
- ~~5~~ 5 different concentrations → ~~25~~ 15 plates
- Need to delete 7x → 85 plates (KM plates)
- Then 4 deletions on Spec/LB plates (4 → 60 plates (Spec. or LB, TBD)

=> 145 plates needed. Use 96 well plate for growing up.

Dilutions (Todd's suggestion)

10^0_x 10^1_x 10^2_x 10^3_c 10^4_x 10^5_c 10^6_x 10^7_x

Kan

LB

$LB = 5_{\text{plates}} \times \frac{2_{\text{replicates}}}{8\ 1} \times \frac{4_{\text{concentration}}}{2} \times 2_{\text{strains}}$

$Kan = \frac{5}{2}_{\text{plates}} \times \_\__{\text{replicates}} \times \_\_\_\__{\text{concentrations}} \times \_\__{\text{strains}}$

Digestion

0.75μl buffer
0.75μl enzyme 1
0.75μl enzyme 2
5 μl DNA

run 1 hr, heat kill 10-20 minutes
Load 5μl → 1-2μl
(load less ladder) ~~1-2μl~~, thin comb

	Tues	Wed	Thurs	Fri
	~~Prelming~~	Prelming		
Prelming	Start	Plate	(Count)	
Full	inoculate	Start	Plate	Count

8/11/15 Setting Up Lac Testing

2.383g 238.3 g/mol

2.383 = (238.3)·10^-2

IM 1000x → mol/liter

10^-2 mol / L → 1000x

10^-3 mol · 1L → 100mL

100mM = 24 mg/ml

Summary

① Prepare 54 tubes Using:
- 1 replicate (i.e., one of the colonies from the plate)
- 2 Concentrations of IPTG → Use 1mM IPTG & 0.1mM IPTG
- Both plasmids

4 = 1×2×2

Powder is in the -20°C, look online for how to make stock (1000x) solution → you will have to delete this further in separate tubes. Do the math to figure out how much IPTG to add to 1mL of prewarmed LB

① Put 1mL of LB into 4 tubes → put in 30°C shaker

② Make 1000x stock IPTG

10uL (1:5)

③ Do math/delete appropriately

8/13/15 We forgot to add CM !!

④ Take a (1:1000) dilution of feed culture & inoculate that in LB → 37°C shaker, take 1mL of seed culture in 1mL of LB first.

⑤ Add inducer (WRITE TIME DOWN) 5:28pm

⑥ → 30°C shaker (4 tubes in back)

TUBES (4 total)
B8(1) with .1mM
" " 1mM
PP745 with .1mM
" " 1mM

1mM 238.30 grams / 1 mole × 1 mole / 1000 moles = .23830 grams

.1mM × 238.39 / 1 mole × 1 mole / 1000 moles = .023830 grams

Maths for 1000x stock of IPTG

1 Molar = 1 mole / 1L × 2383g / 1 mole × 1L / 1000mL × 10mL

2.383g of IPTG / 10mL water

* PCR Purification of GFP Prime 2
- will put into KM backbone later (will be decided in later pg)

* Use Jane's protocol for PCR Purification on page 37

Changes: 7) Elute to 30 uL H_2O
- Warm the water in waterbath first
- wait 5 minutes

8) Quantify Each Tube
9) Store in -20°C

GFP prime 2 (Part w/ Prefix, suffix, RBS)
Quantification Results:

GFP	1:	107.2 ng/uL	Ratio 1.82
	2:	113.3 ng/uL	1.89
	3:	119.6 ng/uL	1.92
	4:	106.7 ng/uL	1.87
	5:	96.2 ng/uL	1.89
	6:	115.6 ng/uL	1.88
	7:	116.3 ng/uL	1.90
	8:	104.5 ng/uL	1.83

Eluted w/ 30 uL,
took 2 for quant,

Unused tubes have ~ approx 28 uL

(side notes) 238mg = .238g
2380 mg = 2.38 → 1000mM (1000x, b/c 1mM is the standard) ERGO
↓ ↓
ten 1uL 999uL = 1mM Dilute a 1000x solution y 1000 to get 1x

8/11/15

① Digestions / Ligations to set up: (Purify first!)
a) Digest the C's from yesterday w/ EcoRI & PstI } Ligate! (ratio
b) Digest some BB too (pSB1C3) then dephosphorylate

② Run a gel on Colony PCRs

~~③ Digestions GFP~~ ③ Digestions
3 a) GFP (for CM purposes): [XbaI & BtsI]
~~Digestions~~
3 b) GFP for 3A (EcoRI & BtsI)] Ligate together!
car
3 c) Kanamycin BB (EcoRI & PstI) }
4) Part C ("EcoRI & PstI ") } → use
5) PSB1C3 (EcoRI & PstI)

GFP Protocol for Plate reader → use TEB Buffer 2!

Look at next page
Plate Reader Machine:
Black Plate w/ Clear bottom

wash [Spin down cells X2?
~~Digestions~~ PB card enforcement
Resuspend in water or PB
↳ ~~250mL~~ ~500mL

Dilute to OD=1 (Just not 3!)
Dilutions to not "overload"
Plate reader → GFP program Set OD600 GFP
Select part?
Make sure EGFP otherwise Change excitation range

1st well → 200uL (
2nd well → take x amt from #1 then dilute to 200
↓
Could dilute all tubes 3x and run machine add buffer to #1 to put back at 200uL)

OD/GFP Protocol

Pierce Hadley

8-11-2015

Note 1: Dilutions only matter in the sense that we are putting the OD/Fluorescence in the machine's range. Fluorescence is always normalized to OD (Flu/OD) so that we do not need to mark down the dilution in our measurements. (range for OD is .1 → 3)

Note 2: You should have already grown your cultures in a deep well plate or in a tube (hypoxia testing). Your LB growth volume should be 1 ml.

1. Wash your cells to create stock solution
   a. Spin down your cells
   b. Discard supernatant
   c. Re-suspend in 500 microliter of water or phosphate buffer (doesn't matter, be consistent though) For future steps, we will refer to your choice simply as "buffer"
   d. Repeat steps a-c
   e. These washed cells in buffer will now be referred to as stock solution.
2. Load on black well plate with clear bottom
   a. Each well will have 3 dilutions (purpose of this is to put the OD and fluorescence in range of the machine. Ex. Machine's OD range is .1 to 3.)
      i. Well 1 – Add 200 microliter of your stock solution (from the deep well or tube)
      ii. Well 2 – 2x dilution of stock: Take 100 microliters of stock and add 100 microliters of buffer
      iii. Well 3 – 4x dilution of stock: Take 50 microliters of stock and add 150 microliters of buffer
   b. Even if all of your dilutions are in range, they should give approximately the same normalized measurement when you take fluorescence/OD.
3. Load and run JN_GFP_OD
   a. Make sure that our GFP is EGFP
      i. If not, you will have to change your excitation range. Find what kind of GFP you have and change the range accordingly
   b. When you run the program, you will get two tables, one for OD and the other for fluorescence
4. Make your graphs!
   a. X axis will be what you are testing (inducer concentration, time, ect.)
   b. Y axis will be fluorescence normalized to OD (fluorescence/OD)

5 Digestion Rounds

See 3a, 3b, 3c, 4, and 5 from page 91

note: for 3a, use all of ~~the~~ GFP Full Part tube #1 (pg 89)
digest

for 3b, use all of GFP full part tube #2
multiple digestion

(1) Digestion Protocol for 3a

1. Add ~~500~~ 500ng of DNA
2. Add 5 uL of NEB Buffer 2.1
3. Add 1 uL of BSA (b/c Buffer 2.1 doesn't have BSA like cutsmart does)
4. Add 1 uL of Xba 1
5. Add 1 uL of Pst 1
6. Add dH_2O so final volume is 40 uL

(2) Digestion Protocol for 3b, 3c, 4, and 5

1. " "
2. " "
3. " "
4. Add 1 uL of EcoR1
5. Add 1 uL of Pst 1
6. " "

Make a master mix for (2) so you don't have to do by hand

8/11/15

③ Purified B→C Constructions:
(1): 70.7 ng/μL
(2): 28.6 ng/μL
(3): 28.1 ng/μL
} Eluted w/ 50μL H_2O!

④ Digestion both B→C (1) & pSB1C3 w/ EcoRI + PstI in NEBuffer 2.1 + BSA
Using the protocol on pg 93! (Rewriting here for simplicity)

~~⑤ Running a gel on Colony PCR tubes in the screw down oven~~

Using iGEM's Digestion / Todd's Digestion (Pg 87)

For GFP:

⑥ Add 500 ng DNA:
- 4.66μL of GFP(1), 4.4μL of GFP(2)
- 4 μL of NEBuffer 2.1
- 1μL BSA (for 1 tube) (for 5 tubes)
- 1μL XbaI/PstI or EcoRI/PstI
- 27.34μL H_2O for GFP(1), 27.59μL H_2O for GFP(2)

Make a 5x master mix for each GFP(1) & (2) → 23.3μL GFP(1), 22.05 GFP(2), 136.7 H_2O(1), 137.95 H_2O(2), 5μ enzymes
→ Aliquot into 10 tubes for digestion 20μL of 2:1

For pSB1C3 & B→C (1) (70.7 ng/μL)
① Add 5μL DNA
② 1 μL NEBuffer 2.1
③ 0.25 μL EcoRI / 0.5μL PstI
④ 0.25 μL BSA
⑤ 4 μL H_2O

Make a 1x only

→ In 37°C o/n (Not enough PCR tubes!)

8.12.15 Ligations + Controls

Summer

1) PSB1C3 to itself for Control
2) GFP(1) + PSB1C3 for GFP Control
3) ~~all other ligations~~ one PSB1C3 backbone promoter ligation

10 uL rxns Following NEB T4 ligase protocol:

10x T4 DNA ligase buffer - 1 uL
Vector DNA (2kb) - 4 uL 50ng (from the digestion yesterday of PSB1C3 @ 94.500ng/40uL)
* insert DNA - 53.5 ng
Nuclease free water (into box)
DNA ligase (Thick) - .5 uL

for 3) use J33201 → 85.9 ng/uL
* need 52.5 ng in total for ligation per in Silico Calculation
52.5/85.9 = 0.611 uL

1) *1 = PSB1C3 = Backbone, fill to water (2.5 uL)

2) *2 = GFP @ 100ng/uL, use .5 uL of GFP

3) *3 - aforementioned

8-12-15 Gel on 8/11 Colony PCR

Miranda

11:50 am => Prepared 2 1% gels with 15 combos

Gel #1 (3 uL in each well) (5 uL of Ladder)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Ladder	1	2	3	4	5	6	7	8	9	Ladder				

Gel #2

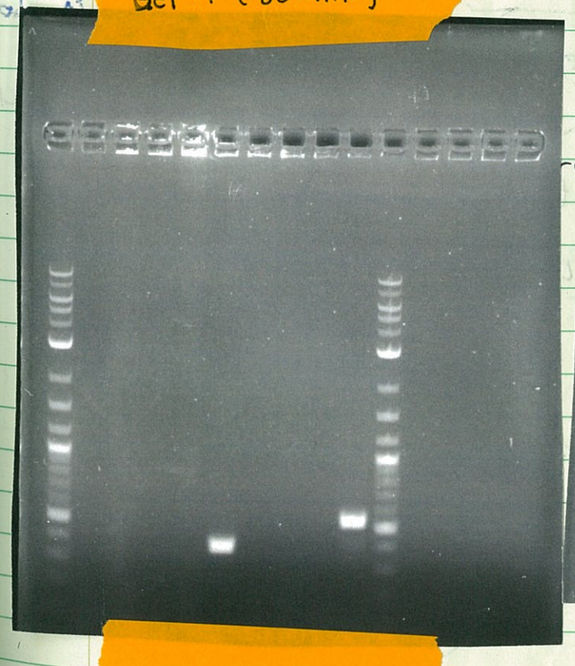
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Ladder	10	11	12	13	14	15	16	17	18	Ladder	Ladder			

12:30 pm => Ran gel for ~30 minutes. (20 min. checked)

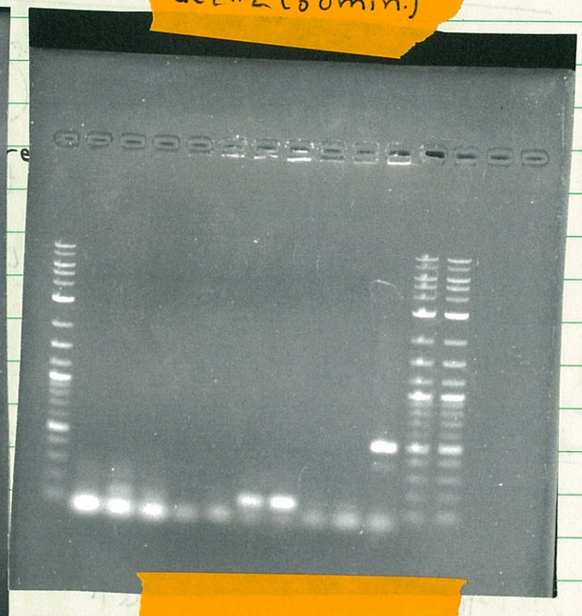
NOTE!!! → someone messed with configuration and the gel was run funny. Ask me (Mo) for details

Results:

Gel #1 (30min.)



Gel #2 (30min.)



8/12/15 Heat Kill Digestion / Plate Prep

Joshua
10:45 AM

① Heat kill digestions (4) from 8/11 (P.94) in 80°C for 20 minutes.
② Put in large ice box

→ Autoclaved 2 flask of 250 mL LB/agar in autoclave ②
with ~~water~~ a glass bottle of water.

1:30pm
Miranda
Andrew

~25 LB + 25 Kanamycin Plates

(1.) Took 2 flasks of 250mL LB agar from autoclave
Stored in 80C.
(2.) Prepared 22 plain LB plates.
(3.) Added 100 uL of Kanamycin from
antibiotics box to 250mL of LB agar.
(4.) Prepared 23 KM plates.
(5.) Stored in 4°C

⑥ ~~Re~~-Stored those tubes in ice box
to -20°C

MD

TOOK induced seed culture (pg 88)
out at 5:29pm

Dilutions

10^0 — 10^{-4} on KM + 10^{-3} – 10^{-7} on LB

10^0 50 uL of each of tubes in a KM plate

10^1 10uL of stock + 90uL LB → KM plate

10^2 10uL of 10^1 + 90uL LB → KM plate (2x)

10^3 10uL of 10^2 + 90uL LB → LB plate, KM Plate

10^4 " " 10^3 " " → LB plate, KM Plate

10^5 " " 10^4 " " → LB plate

10^6 " " 10^5 " " → LB Plate

10^7 " " 10^6 " " → LB Plate

PLATES Key

① = B8 (1) FF144 @ .1mMole IPTG
② = PP745 (1) FF144 @ .1 mMole IPTG
③ = PP745 (1) FF144 @ 1mMole IPTG
④ = B8 (1) FF144 @ 1mMole IPTG

* (second number) is a reference to ← this

* first number = dilution number
so 10^0=0, 10^1=1, 10^2=2

* Instructions

0.1
0.2
0.3
0.4

* Read this as follows
0.1 = nondilute .1mMole IPTG with B8
0.2 = nondilute (10^0) of .1mMole with PP745
etc.

IPTG: 1mM = 238ug/ml

KM Plates go ...
0.1 - 0.4
1.1 - 1.4
2.1 - 2.4
3.1 - 3.4
4.1 - 4.4
(20 plates of KM)

LB Plates go ...
3.1 - 3.4
4.1 - 4.4
5.1 - 5.4
6.1 - 6.4
7.1 - 7.4 ⑤
(20 plates of LB) +1 potential contaminated 7.3

⑤ Note 7.3 was done twice. The one w/ 4 X might be contaminated

Put plates in 37°C @ 7:33.

8-13-2015

Note: For today, GFP full part tubes 3-8, as well as the original GFP template, are in the misc. box of parts.

8-13-2015 (MD) RESULTS from 8-12-15 plating

Plates are funky, we didn't let them chill enough? Also, I would try them in the hood next time.

OBSERVATIONS:
Growth more robust on LB, barely any growth on KM, as expected.

Andrew

Run a Gel of the ligation yesterday
① Ladder Sample 2 Sample 3 Ladder
Sample 3 → Promoter+GFP (J33201)

Store the Sample 2, 3 in Ligation Box

Transformation of GFP+PSB1C3 GFP+J33201+PSB1C3 & a control

Add 2.5 µL GFP / GFP+J33201 to competent Cells.
5 min ~~incubation~~ incubation on ice.
30s. heat shock.
2 min incubation on ice.
add 700 µL LB to each tube.
4:03 37°C incubation
4:03 Pre-warm the plate in 37°C
Do GFP/GFP+J33201 on both LB and CM, do a Control on LB

8/13

① Digesting BB/(B→C) overnight w/ XbaI:

~2 µL BB, ~3 µL (B→C)	37°C o/n
1 µL XbaI	
1 µL Cutsmart	
6 µL / 5 µL H2O	

~~Prepare~~ Colony PCR for ① J33201+GFP Plate (8/13/15) and ② GFP (no promoter, 8/13/15)

Tube 1 → J33201 A→H (~~1A~~ 1A, 1B, ...)
Tube 2 → GFP only A→H (" ")

8/14/15

① Setting up the following PCR reactions using GoTaq 2x Green MM

a) R0040 (to get pSB1C3) (50 µL Reaction) (1.25 µL primers)
b) B8 (w/ colony PCR pII & pI primers) (10 µL Rxn) (.625 µL primers)
c) [redacted] C8 (w/ pII & pI primers) (15 µL)
d) All of the assembly (A→D) using PII & PI primers (10 µL)
e) B→C
f) Part 1 & Part 2b

PSB1C3 Tubes have a P on top

1	2	3	4	5	6	7	8	9	10
~~[redacted]~~	~~[redacted]~~	II	III	IV	B8	C8	B→C	P.1	P.2B

Colony PCR Mix
5 mL GoTaq MasterMix
.25 Colony For
.25 Colony Rev
4 mL H_2O

And .5 mL Template (our ~~6~~ inoculated colonies)

START OF THE REDOS

8/13/15

① pSB1C3 was amplified (2 tubes, 50µL reaction) using P0040 as a template.
- 30 cycles
- ≈ 500ng DNA
- 10sec denaturation per cycle
- 0.5µM each primer
- 20 sec annealing ↪ @ 30sec initial denaturation
- 40 sec extension → Thermocycler 1

10sec denaturation!

② Part 1 & 2B will also be reamplified (50µL Rxn)
- Using Part_Two_Amp_Fr & Rev & Part_1 For/Rev
- Same as above except PCR will be done w/ 20 sec @ annealing, 68°C for pII B, 72°C for pI_E
- ≈ 500ng DNA

Note: A lot of PCR Ext protocols heat 2min @ 98°C.....
Could that be the reason for all of our failures???

③ Run an gel of PCR-BB Product.

Ladder	BB1 (5µl+1µl Loading Dye)	BB2	Ladder
	band @ ~2 kb as it should be		

④ Purify PCR-BB Product
- Used page 37 protocol.
- eluted w/ 30 µL H_2O, not IS

⑤ Quantify PCR-BB product

	conc.	ratio
BB1	9.9 ng/µL	2.3
BB2	17 ng/µL	1.98

↳ PIIB was successful!

⑥ ~~PI~~ PI PCR failed again: Redoing w/ Buffer G

a) Making new working stock: 1µL of gBlock (10ng/µL) in 9µL H_2O

Therm 1 → - 50µL Rxn w/ Buffer G at 72°C w/ 500pg template

Therm 2 [- 50µL Rxn w/ Buffer G at 68°C "

- 50µL Rxn w/ ~~Buffer~~ B8 as template at 70°C (1µL template)

Therm 3 [- 50µL rxn w/ previously okay PI

- 50µL Rxn w/ "Gibson Ready PI"

↑ All w/ Buffer G!

70°C!

⑦ Digestion of pSB1C3 for Gibson Assembly

Mix BB1 and BB2

B: 40 · 56µl DNA.
2.5µl XBA] 2.5µl PstI.
7µl ~~DW~~ Buffer. 2.1

→ 37°C incubation.

3:10 Added 1µL of Dpn1 and returned to 37°

3:40 Added rSAP (1µL) and returned to 37°

4:00 placed in 80° to heat kill enzymes

4:20 Removed from 80° and PCR purified w/ small column to increase concentration.

350 µL HBC, spin 1 min

1 min spin to dry → Wash sample twice - 200 µL wash BUFFER 30s spin

elute w/ 20 µL H_2O

⑧ Running a gel on the (6) PCR's

Ladder - 72°C Buffer G - 68°C G - 70°C B8 - 70C Gibson - 70C PI 35.3 ng/µL tube

⑨ Quantify BB digest

concentration: 15.1 ng/µL ratio: 1.82

Store the tube in box afor the week of 8/17/17.

⑩ From (6), 1, 2, 3 were successful

↳ Running a gel on 1 & 2 for gel purfection purposes

⑪ Gel Purfection on 1:

- 450µL ADB added to gel
- Incubated for 10 min at 50°C (mixed sporadically)
- Spun 30 sec
- 200µL wash buffer → Stand for 2min prior to centrifugation
- Spun 30sec
- 200µL wash buffer → Stand for 2 min prior to centrifugation
- Spun 1 min → Spun 1 min again w/ the cap open to dry the column!)
- Eluted w/ 10µL H_2O (prewarmed to 70°C) ((Using "water heater" in thermocycler)

=> 10µL of Part 1 at 6.2 ng/µL

⑫ PCR Purfection of Part 2B: 33.1 ng/µL

(13) Setting up Gibson reaction:

Part 1: 6.2 ng/μL

Part 2: 33.1 ng/μL

Vector: 15.1 ng/μL → Optimized cloning efficiency: 50 ng of DNA = 3.31 μL = 39.09 fmol

We want 78.18 fmol of Part 1 & 117.27 fmol of Part 2
(1972BP)

↳ 78.18 fmol part 1 = 95.27 ng = 15.37 μL ← Very problematic. W/a 1:1 ratio we need 47.64 ng = 7.68 μL
(3548BP)

↳ 117.27 fmol part 2B = 25.66 ng = 0.775 μL

We exceed the total rxn volume by 1.765 μL. We will proceed regardless, and scale the rxn for 10μL:
(Using a 1:1 ratio for Part 1)

19.54

=> 1.655 μL pSB1C3 (fmol)

3.84 μL Part 1 (19.54 fmol)

0.3875 Part 2B (58.64 fmol)

} 0.097 fmol total of fragments

5 μL Assembly Master Mix

10.88 μL (~9% more than is allowed)

Incubating at 50°C for 60 minutes.

(14) Transformed 2μL to 100μL of NEB 5α Cells

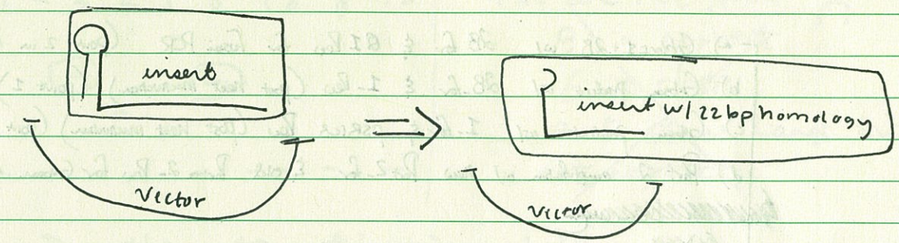
(15) Plated 30μL, spun rest down (10 min 3900 rpm), resuspended in 100 μL H2O, streaked on plate.

100 μL was waaaay too much...

8/18 PCR of Hairpin Structure Revision 8-18-15

MD

(1) Overview of Purpose of PCR



→ Added:

25 mL of G Buffer

1.25 mL forward primer (22 bp 5' homology for part 2B)

1.25 mL reverse primer (part 1)

21 mL H_2O

.5 mL Q5 polymerase

1 mL DNA (2B + part 1 combination)

8

(2) Ran PCR on assembled part, gel only amplified w/ PII & PII primers, not PI rev & PII for.

(3) Picked 5 Colonies from the 2 plates yesterday & ran a PCR using GoTaq protocol.

(4) Notes from calling NEB:

① Ligase eats 3'-5', so our primers may be getting eaten up before they anneal
- We don't see primers on the gel, lends support to this idea (other DNA is somehow covalently bonded to vector using ligase)
- Fix w/ a 98°C 2 min heat inactivation

② Gblock 1+2 should give product all by itself w/ the primers we are using

③ Use P.1 for & pSB1C3 Rev to verify what's inside

(4.5) Ran a gel on (2) → Smearing!

(4.75) 98°C for 2 min, heat inactivated the exonuclease.

⑤ Setting up the following PCRs:

a) Gblock 1+2B w/ 2B for & Q1 Rev for fusion PCR (point 2 in NEB Notes)
b) Gibson product w/ 2B for & 1 Rev (post heat inactivation) (point 1)
c) Gibson product w/ 1 for & pSB1C3 Rev (post heat inactivation) (point 3) (~4kb, worm. Do ~2 min ext? 10sec/kb)
d) Part 2 amplification w/ new Part 2 for & old Partex 2 Rev for Gibson again

[redacted]

→ Add PI & PIIB in an equimolar ratio: 250ng of PIIB = 1.143 fmol (250ng : 5μL pIIB)
1.143 fmol of PI = 1.393ng : 1.393μL of PI

Therm 2 ↔ So, for fusion PCR:

✓ 2.5μL ~~5μL~~ of pIIB (1.143 fmol) 0.5715 fmol
✓ 0.697 ~~1.393~~μL of PI (1.143 fmol) 0.5715 fmol
25μL Buffer G
0.5μL Q5 Polymerase
[redacted]
✓ 1.25μL 2B-for-new
✓ 1.25μL 1-Rev
✓ 18.08 ~~15.61~~μL H_2O
50μL Reaction

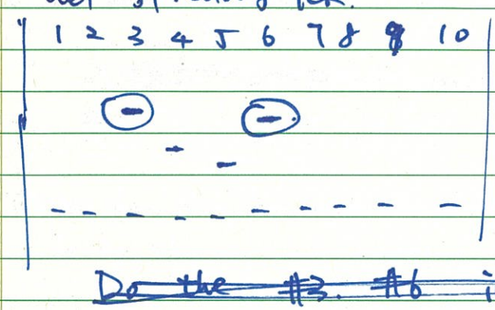
Therm 4 ↑

For (b)-(c), we will use ~0.2μL of Gibson product w/ normal reaction conditions
For (d) we will use 10μL (800ng) of PIIB w/ normal reaction conditions
(b)(c) ✓✓ ✓✓
0.2μL Gibson, 1.25μL per primer, 0.5μL Q5 Polymerase, 5μL Buffer G, 21.8μL H_2O
(d)
10μL PIIB stock, 1.25μL per primer, 0.5μL Polymerase, 25μL Buffer G, 12μL H_2O

↓ Therm 3

AT

⑥ Gel of Colony PCR.



All are not good ~~^~~
3 is correct.
6 is close.
4. 5 show bands but not correct.

~~Do the #3, #6 inoculation.~~

CB ⑦ PCR purified the "d" product (Part 2) and eluted w/ 20 μL dH_2O

Quantify
- 1⑤ Concentration: 12.6 ng/μl
OD: 1.8

⑧ Ran a gel on the 4 PCR reactions, (d) & (c) showed w/3 → Not sure what (c) gives us. (d) was purified to the above (⑦) ratio. (a) showed only primers, (b) showed smearing. Perhaps too much time w/ success? I dunno.

⑨ Setting up ~~Gblo~~ Gibson reaction:

1.655μL pSB1C3 (19.54 fmol) ✓
3.84μL Part 1 (19.54 fmol) ✓
1.02μL Part 2B (58.54 fmol) (Neg. w/ no 5' hairpin) ✓
5μL Assembly Master Mix
11.52μL total Volume (~12% over suggested volume)

Run at 50°C for 1 hour

(10) Setting up the following ~~reaction~~ PCRs:

50µL pIIB (~~1ng~~ 1ng; 4.571 fmol)
5.57µL DI (4.571 fmol)
30µL Q5 2x Hot Start MM
~~10~~ 4.43µL H_2O
60µL total → Split into 4 tubes, each will have a stage 1 annealing temp of 60°C, 62°C, 64°C 66°C (Tm of 40 BP overlap is 73°C)

Then, second phase, ~~add~~ make a mastermix of 1.50µL each primer + 1µL Q5 polymerase and add it to the mix → Then run a normal PCR at 72°C for annealing temp w/ ~2x ext. time.

Here is the thermocycler program used for the overlap extension PCR:

98°C	30 seconds		98°C 30 seconds
98°C	10 seconds		98°C 10 seconds
60-66°C	30 seconds	x10	72°C 20 seconds x25
72°C	60 seconds		72°C 45 seconds
72°C	120 seconds		72°C 120 seconds
PAUSE → ADD PRIMERS & MORE Q5			4°C Hold

(11) Also setting up these PCRs:
-) More Part 1 pcr w/ Q5 2x hot start (2 50µL reactions)
~~b) Normal 2B+1 pcr w/ Q5 2x hot start~~

(12) Once Gibson Rxn is done, added 2µL to 50µL comp cells, heat-killed the exonuclease for the rest.

(13) Performed a transformation; Incubated w/ DNA on ice for 30 minutes, 950µL LB post heat shock (30 sec heat shock) outgrowth step for ~~60~~ 30 minutes ~~b/c I went to go home~~. Plated 100µL on CM → 37°C, then plated the rest

(14) Using the ~~Gibson~~ heat inactivated Gibson reaction, set up the following PCR:
0.5µL Gibson reaction
2B for- primer
1- Rev primer

(15) Ran a gel on all the PCR products. Part 1 was strong, Gibson failed to produce anything (98°C 2 min before doing full PCR protocol), 64°C Overlap Extension seems promising!!!

(¥) FIGURE OUT WHAT THE ~~PCR~~ IS UP WITH THE NONSPECIFIC AMPLIFICATION! ~~THIS~~ THIS IS PROBABLY BAD.

(16) Colony PCR gel

ladder 1 2 3 gap 4 5 6 7 8 9 10 ladder
↑ accidently punctured the well

8/19/15

① Setting up the following OE-PCRs: (20 µL Reactions)

a) 64°C w/ Q5 2x Hot Start } Note: Using the 2 gblocks themselves

b) 64°C w/ Buffer G & Q5 Polymerase }

c) d) Repeats of the above, but w/ Todd's recommended scheme.

Round One: 4 tubes

A: 10 µL Q5 2x Hot Start ✓

0.2 µL GBlock 2B (2ng, 9.141 fmol) ✓

1.114 µL GBlock 1 (11.14ng, 9.141 fmol) ✓

4.8 µL H_2O ✓

B: Same thing

C: 10 µL Q5 2x Buffer G ✓

0.2 µL GBlock 2B ✓

1.114 µL GBlock 1 ✓

0.5 µL Q5 Polymerase

4.3 µL H_2O ✓

D: Same thing

② Ran Part 1 PCR from yesterday in a .7% gel, cut out and gel purified w/ Standard protocol

450 µL ADB, incubate 10 min, Mix @ 50°C

Load column, spin 30 s

Wash twice w/ 200 µL wash

elute w/ 50 µL H_2O

Prepped 2 tubes

③ Round 2 of OE-PCR

- Take 4µL from Run B & D, set up a Q5 2x MM & Buffer G Run:

  4µL B/D
  10µL Buffer G/Q5 ✓
  0.75µL 2-for ✓
  0.75µL 1-Rev ✓
  0.5µL Q5 (if Buffer G) ✓
  3-3.5µL H_2O ✓
  } => E & F

- Add 0.75µL Primers & 0.75µL Q5 Polymerase to A & C
- Add 0.5µL Primers & 0.5µL Q5 Polymerase to B & D

④ Setting up another NEBuilder Run because b/c WHY THE FUCK IS IT NOT WORKING D:<

Using 75ng of backbone: 58.63 fmol
117.26 fmol part 1: 142.9ng = 14.29ng of gBlock...
232.92 fmol part 2 (4x molar) = 50.96ng
↓ Way too high across the board.

Current Conc: gBlock 1: 10ng/µL
Part 2: 12.6 ng/µL
Vector: 15.1 ng/µL

OPTIMIZED! .1995 pmol total! ~9.67µL total!

50ng Vector: 39.09 fmol = 3.31µL
78.18 fmol Part 1: 95.27ng = 9.527µL → Need to speedvac this so 5.33µL = 95.27ng (17.87ng/µL)
78.18 fmol Part 2B: 17.10ng = 1.36µL

↓ Speedvac'd it to ~5µL

20.47µL → 2.048
5.48 → 0.548
1.48µL H2O

Three of these were done:

20µL → ① Vector, Part 2 (PCR Prod), Part 1 (Speedvac + Vbar)
10µL IBeam → ② Part 2 (PCR Prod) + PCR Prod/Gel Purified Part 1
10µL → ③ Part 2 (GBlock) + Part 1 (gBlock)
IBeam

⑤ Gel on OE-PCR showed nothing. Setting up exact rxn from yesterday (#10 from 8/15)
exact
- 557µL of P1 Strepsi from gBlock source & more H2O to compensate → all rxn at 64°C w/ Q5 2x MM

⑥ PCR of Result of Fusion PCR C from 8/18/15

1uL Template
.25uL forward (Part 2a 22bp Vector Ho)
.25uL reverse (Part 1 Rev)
5uL QSMasterMix
3.5uL water

(Labeled tube as "3-2")

Notes and what we can do

① Run a gel on OE-PCR reaction before & after the first stage
- If we see a band intensity inc of things near 300bp, the old primers are messing things up → ∴ gel purify 2B.

② Run OE-PCR just on the normal gBlocks, so no primer issues

③ Amplify 2B for gel purification. Use new forward primer, and we only have one new primer

④ Use middle primers from the Addgene (or Seg. ones) construction in conjunction w/ what we have been using to see if maybe the primers are the problem for confirmation

⑤ Go ahead w/ the 2D GG screens, b/c we know that totally works

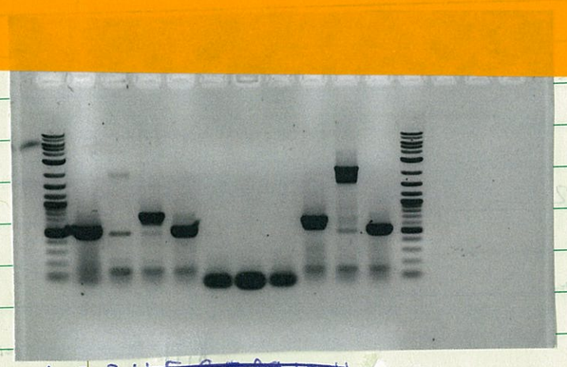
⑥ Run a TD PCR on OE-PCR, do (1) (2) as also!

(7) Gel for OE (#5 from pg 119) & Fusion PCR C (#6 " ") 3-2

3 uL sample, 0.6 uL dye

Wells	1	2	3	4	5	6	7	8	9	10	11	12	13
	2-Lg ladder	A		B		C		D		3-2		2-Lg ladder
		↓								↓		↓	
		1	—	—	—	—	—	—	—	—	—	PCR C	
		OE											

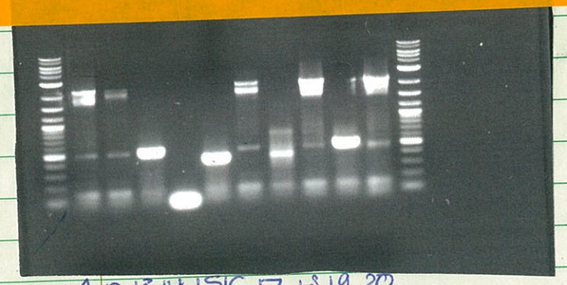
(8)



Colony PCR gel

1 2 3 4 5 6 7 8 9 10

2, 9, 11, 12, 16, 18, 20 all showed ~ ~ 2.2Kb bands, indicating successful assembly.



11 12 13 14 15 16 17 18 19 20

(8.5) Running 2B+2TB amplification attempt from the 2 test Gibson assemblies b/c they started before we saw colony PCR success

(9) Given success of colony PCRs, we will proceed w/ assembly of 2a/c/d.

Note: OE PCR A was mildly successful, PCR C & OE 2-4 were not. We are abandoning OE-PCR for now.

Amplify w/ the following conditions/setup. (NOTE: SPIN DOWN & VORTEX ~~EVERYTH~~ ALL DNA PRIOR TO USE)

500pg template DNA (10 µL diluted sample)
1.25 µL 2-for
1.25 µL 2-Rev
25 µL ~~QS~~ QS 2x Hot Start MM
12.5 µL H_2O

98°C	30 sec	(Should be the "Sanbe" protocol & on the thermocycler)
98°C	10 sec	} 30x
72°C	20 sec	
72°C	10 sec	
72°C	2 minutes	

(10) (8) Making 25% Glycerol stocks of the successes → Done in the hood, put in cryovials, and stored in -80C (500 µL @ 50% Glycerol & 500 µL Cells) at 10:00pm per Steve's suggestion. ☿

(11) PCR Purifying 2B/A/C/D while running a gel on 2A(D + the Gibson amplifications (the Gibsons were heat killed at 98°C for 20 min b/c of exonucleases)

- Eluting w/ 10 µL H_2O pre-warmed to 50°C

(*) 2A: 188.2 ng/µL
2C: 176.8 ng/µL
2D: 146.4 ng/µL

(*) 2A(D showed strong bands on the gel, as did the Gibson ~~amplific~~ reaction w/ just the gBlocks. The one w/o gBlocks (using PCR derived DNA) did not work, instead we saw amplification of Part 2B!!! ☿☿

Assembles + Transformation will have to be tomorrow ☹

8/20/15

① Spin purifying the 7 hits from yesterday

Colony 2: 42.5 ng/μL 12: 45.1 ng/μL 20: 27.2 ng/μL

9: 43.2 ng/μL 16: 45.5 ng/μL

11: 99.1 ng/μL 18: 33.1 ng/μL

② Running 10μL Gibson reactions w/ the purified 2 parts from yesterday.

2A: 188.2 ng/μL 2806bp 1: 48.2 ng/μL 1972bp

2c: 176.8 ng/μL 2886bp

2d: 146.4 ng/μL 2066bp

Too many total moles!
75ng pSB1C3: 58.63 fmol
117.26 fmol Part 1: 142.9ng =
175.89 fmol Part 2A: 30.44ng =
2c: 30.44ng
2d: 22.40ng

Too many total moles, but lowest possible vector amount... The assembly did work w/ a 1:1 P1: Vector...
50ng pSB1C3: 39.09 fmol = 3.31μL
78.18 fmol Part 1: 95.27ng = 1.98μL
117.27 fmol Part 2A: 20.29ng = 0.108μL
2c: 20.29ng = 0.115μL
2d: 14.93ng = 0.102μL

Total: 0.234 pmol ~4.5μL DNA ~5.5μL H_2O

Since we have minimal Gibson, we were set up a 10μL total reaction, so we are cutting everything by 1/2

First, diluting 2μL of Part 2 in 18μL H_2O to be able to pipet more reasonable amounts

↳ CONTINUED ON NEXT PAGE

③ Digesting 4 of the plasmid w/ two different schemes: 1) EcoRI/PstI to see the 2.2kB insert

	Approx Conc of Plasmid			2) XhoI to generate 3 bands to see if the insert is there.
	~50ng/μL	~100ng/μL	<50ng/μL	
Amount of DNA	5μL	5μL	10μL	(XhoI cuts the RT & vector
RE1	0.75μL	0.75μL	0.75μL (prepared twice)	
RE2 (if applicable)				
H_2O (if app)	0.5μL	0.5μL	0.5μL	
Buffer	0.75μL	0.75μL	1.1 - 1.25μL (dep. on H_2O added)	

③ Continued

Note: XhoI did not exist in sufficient quantities for the amounts needed. ~3µL of enzyme was used (total) when 5.25µL total should have been.

Metermine also ran out (kind of) for tubes 7, so they may be skewed (both digestions)

NEBuffer 2.1 was used w/ EcoRI + PstI

Cutsmart w/ XhoI

Both in thermocycler 1 for 60min at 37°C, heatkilled 80°C for 20min

Tubes w/ blue hinge: double digest.

④ Prepare a 1.5% gel, planning on running the gel for 30min at 110V GEL RESULTS ON NEXT PAGE

Colony: 2 9 11 12 16 18 20

Best ones

② Continued

- Since the Part 2's are so concentrated, we will have to dilute them so we can use pipet reasonable amounts
  - A 1:10 dilution puts us at pipetting ~1µL of the dilution into the Gibson, so 1µL comes Part 2 MED into 9µL H_2O. Labeled these tubes w/ "2A dilute", "2C Dilute", "2D Dilute"

Actually doing a half reaction. for each tube

PSB1C3 - 1.66 µL ✓

Part 1 - 0.99 µL ✓

~~Part 2 - 0.5 µL~~ (diluted 10x)

Master Mix - 5 µL ✓

	2A	2C	2D
2A	.54 µL ✓		
2C		.575 µL ✓	
2D			.51 µL ✓
H_2O	1.81 µL ✓	1.775 µL ✓	1.84 µL ✓

3A Assembly for Eindhoven - Caroline + Miranda 8/20/15

Overview:

Combining Kanamycin backbone (PSB3K3.M1), LacPromoter (R0010), and a yellow chromatoprotein + RBS (I13103 and #23LinKit4)

Protocol:

⑤ Transformed Gibson products (2A, 2C, and 2D) as well as 113C03 (for 3A assembly)
- Each Gibson product went in 50 µL of NEB comp cells
- 113C03 went in homemade comp cells

Both heat shocked for 30 seconds, grown up for 1 hour, plated on their respective prewarmed plates

⑥ PSB1C3 BB is getting PCR'd in 2 50µL Reactions w/ OS 2x Hot Start MM using R0040 as a template in thermocycler 3

⑦ Gel for the digestions of the colonies whose plasmids were purified on the right;

!!! ✱ 11, 12, 16, 18 all produced banel patterns we expected for both EcoRI+PstI digestions & XhoI digestions (w/ Colony 18 potentially being a victim of partial digestion. ✱ !!!

⑧ 2 tubes w/ 5mL LB 17µg/mL CM of cols 11, 12, 16 were inoculated o/n for further purifcn & Sequencing tomorrow

⑨ The 2A/C/D & 113C03 transformations were plated w/ 100µL for the CM ones & 20µL for amp ones. The rest of the tube for both were also plated to recover all the transformants

2B + RTB Colonies 2, 9, 11, 12, 16, 18, 20 digested with (EcoRI, PstI) (wells 2-8) and (XhoI) (wells 9-15) - 11, 12, 16, 18 succ

well 14 of 15

AJ

Colony PCR of SCRIBE 2.0

Reaction Buffer

- 25µl ~~Taq~~ Master Mix
- 2.5µl primer Part_2A_22bp_Hu 67µl
- 2.5µl Primer Part 1 Rev ~~Part 2 Rev~~ 67µl ?
- 20µl Autoclaved Water ~~20~~ 15µl

650 µl X (number of colonies +1)
25 +1 = 26

Use tips to ~~touch~~ pick the colonies and touch the bottom of PCR tubes

Transfer ~~the~~ 15µl Reaction buffer to each PCR tubes
(Use pipette to mix them)

PCR Cycle
- ~~94°C 3min~~ 95°C 1min
- 95°C 15s
- 59°C 30s
- 72°C 2min 30s
- 72°C 2min

} x30

CB Purified plasmids from colonies 11, 12, and 16.
Eluted w/ 50 µl dH_2O.

	~~conc.~~ conc.	ratio
11-A	116.1	1.79
11-B	112.6	1.86
12-A	66.3	1.9
12-B	33.3	1.83
16-A	39.8	1.86
16-B	33	1.91

Constructs are in "box of the week" and labelled with SCRIBE + their number (11-A, 12-B, etc)

Set up tube 11-A for sequencing since it showed strongest results.

AJ [illegible]

Plate	Colonies Picked From It
2A (Everything Else)	1-6
2A (100µL)	7
2BC (100µL)	8-9
2C (Everything Else)	10-18
2D (100µL)	19
2D (Everything Else)	20-25

Note: I messed up & accidentally tried to use the wrong primers; 1-10 tips were used to scrape the bottom of the tube again, 11-25 were not affected. Sry :(

② Gel on colony PCRs gave no hits for 2A/C/D. Checking to see what was done differently

A) [illegible]

B) Successful assembly was done w/ a 1:1:3 Vector: Part 1: Part 2, these other ones were done w/ a 1:2:3 ratio

B) Colony PCR was done w/ 2x the conc. of primers we normally use

③ Retransformations of 2A/C/D+RBS in Top 10 are occurring.

④ Using Pro ~~Pro~~ For & P1 Rev on 11A to generate a 2CRBS equivalent & construct assembly
- 50µL GXS 2x H.S. (Hot Start) Master Mix

⑤ Plan of action: - Try and generate the 2C analog
- Digest it w/ XhoI & EcoR1/PstI ~~or~~ or some ~~mid~~
- Pick some more colonies & do colony PCR ~~gross~~ (Maybe 0.1μL of the stuff growing in the 37?

⑤ Attempted B→C PCR w/ QS, purified

⑥ Digesting ~~PCR~~ 2μL 11-A & (B→C) to compare SCRIBE types

⑦ Picked 10 more colonies for colony PCR in thermocycler 4
(20 tubes are incubating in 37°C)

⑧ Transformed 2008 (2A/2C/2D) in top 10 & plated

8/21/2015

James Blanchard

- Good to be back at work :)

- I transformed 11-A (SCRIBE 1, $P_{lac} \rightarrow kanR_{ON}$, etc.) onto CM plates.
  - Put them in at 3:00P.M. (plates in 37°C)
  - Four plates
    - no DNA (control), 50nL plated into plain LB
    - 0.5nL 116.1ng/nL DNA, 50nL plated into CM (identically after all other steps)
    - 1.0nL 116.1ng/nL DNA, 50nL plated into CM
    - 1.0nL 116.1ng/nL DNA, 250nL plated into CM
  - 25min on ice ⇒ add DNA to 50nL compcells (FF144), put on ice ~25min, heat shock in 42°C bath for 30 sec, 2min on ice, add 250nL pre-warmed LB, grow in 37°C shaker for 45min, plate, grow overnight
  - We should be able to inoculate these for testing our new iGEM part!

- I set up a PCR (on 11-A as a template) to get SCRIBE$_{kanR_{ON}}$ without a promoter:
  - 5nL 0.1161 ng/nL 11-A (I did serial dilutions)
  - 25nL 2x HotStart MasterMix
  - 1.25nL of Aru's primer "Prof for"
  - 1.25nL of Aru's primer "Part 1 Rev"
  - 17.5nL H_2O (50nL total volume now)
  - 'Big' protocol (Aru set it up in advance)
- I did PCR purification on the 11-A PCR (see Page 37)
- Aru digested it (see Aru's test on the previous page, 130)

8/24/15

To Do

① Induce MIT ~~Dd~~ strain w/ Addgene & 28 construct
② Gibson some ~~stuff~~
③ Desperately hope colony PCR worked out
④ Prepare cloning things where are lacking

8/24/15 AJ

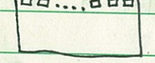
① Poured 4 1% 15 comb gels for use throughout the day.
② Dephosphorylating psB1C3 w/ rSAP for 1 hr, heat killing 5min @ 37°C
③ Picked 24 colonies from retransformation of 24/C/D
④ Running the 2nd Colony PCR done today on a gel along w/ the (B→C) test.
⑤ - Everything failed; ~~[redacted]~~ surprise
⑤ Set up a new colony PCR w/ the freshly picked things (1-26)
- New PCR conditions → will be discussed tomorrow

AJ ~~Do~~ make ~~[redacted]~~ 10mL 100mM IPTG for induction
NF (mix 1ml 1000x (1M) IPTG and 9ml ~~dH2O~~ sterile water)
∴ If we want to do a 1mM induction, we can add 10µl 100mM IPTG to 2ml LB.

8/24/15
NF ① Materials: Addgene Plasmid → pFF745 } CM plate
MIT Strain → FF144
② Inoculated 1 colony (FF144; pFF745) into 5mL LB (25µg/mL CM)
③ Incubate in 37°C shaker overnight

8/24/15 SCRIBE 1.0 Induction
NF ① Prepared 10mL 100mM IPTG
② Added 2mL LB to one tube (①) and 2mL LB + 20µL 100mM IPTG to a second tube (labeled ②)
③ "Strain F144 SCRIBE 1.0 8/23/15" removed from 37°C shaker. 2µL of strain solution added to each tube (1000x dilution).
④ Both tubes placed in 30°C shaker at 4:24 P.M. to incubate for exactly 24 hours
⑤ Strain solution placed in 4°C

NF Ran gel digest on dephosphorylated psB1C3 — 110V, 20minutes



A is good
B is sketch

8/24/15 JA Assembly
JC

Master mix for backbone	Master Mix for part A	Master Mix for part B
5µL Nebuffer 2.1	5µL Nebuffer 2.1	5µL Nebuffer 2.1
0.5µL EcoRI-HF	0.5µL EcoRI-HF	0.5µL XhoI
0.5µL PstI, DPNI	0.5µL SpeI	0.5µL PstI
18.5µL dH_2O	19µL dH_2O	19µL dH_2O

Part B : 26.9 ng/µL, Ratio: 2.01

~~Digest~~ Assembly : - BB- 4 µL of enzyme Master Mix + 4.44 µL of BB (Miranda)
A- 2µL of 37ng/µL LMC, 2µL of H_2O
B- 4µL of chromoprotein

22.5 ng/µL of plasmid PSB1K3.ms (Miranda)

Oxgen

3A Digest

PartA → waterbath 37°C @ 5:58pm

PartB → 37°C @ 5:58pm

BB → 37°C @ 6:23pm

~~Take out @ 6:28 & put in 80C for 30min~~

~~Take out @ 6:53 and put in 80C for 20min~~

Take out tomorrow, Remember to heatkill 20min @ 80C before ligation!!!

8/25/15 Thermocycler To Dos:

① Set up Gibson rxn (10μL total) in a 1:1:5 ratio w/ the Gibson MM Steve gave

② Set up amplifications of gBlocks using Jenna's primers

Innoculations of Colony PCRs ☆ marked w/ # & date

Colonies 1-9 2A + RTB - CM 30mL

10-17 2A + RTB (~~40~~ mL) CM

18-19 2C + RTB 30mL

20-23 2C + RTB ~~14~~ mL

24-26 2D + RTB

Use: 2, 3, 4, 6, 7, 13

15, 16, 18, 19, 20, 26

Use 2.5 mL CM LB

AJ Transformation of 3A Assembly Test

7:32 PM Add 2.5μl "3A" Assembly to 50μl Top 10 Competent Cells

Incubation on ice for 20min

7:52 30s heat shock, incubation on ice for 2min

7:54 add 700μl LB to each tube

incubation in 37°C for 1 hour

8:05 Pre-warm the LB & CM Plates

8:54 Transformation. CM Plate labeled "3A"

9:00 Return Purple DNA Ladder to PCR Box

SA purified inoculated Colonies, Labeled & put in -20°C

quantified	using	tecan ng/μL	260/280		ng/mL	260/280
2	77.7		1.83	15	82.7	1.92
3	74.6		1.89	16	80.1	1.88
4	80.7		1.84	18	69.5	1.86
6	77.4		1.84	19	63.4	1.94
7	59.6		1.9	20	66.7	1.87
13	72.2		1.85	26	58.8	1.87

8/26 Plan

① Gibson w/ pSB1C3, ~~[illegible]~~ 2B (PCR Product), Part 1 (gBlock) ② Necessary to resample 2A0003? Probably, then

↳ Run the rxn for 4 hours, end it when you want to go home

② Digest 2A/c/D constructs Screen purified

0 Verify the BBs or see if we have enough of ours left.

0.5 PCR purify the BBs

AJ Transformation of ?
The same protocol in B5.
4:30. Incubation in 37°C for 1 hour

8/26 ① Diluted gBlocks 2A/B/C/D to 0.1 ng/μL using same nuclease free H_2O I found in the -20C

② Amplifying 2A/B/C/D, 50μL Rxn, 12 cycles!

③ Ran 2A/B/C/D on a gel w/ pSB1C3 that Noah amplified + digested + dephosphorylated

④ PCR purified all of them & eluted w/ 10μL H_2O at 50°C to get:
2A:
2B:
2C
2D:
pSB1C3 (A):
pSB1C3 (B):

⑤ Ran a 10μL Gibson reaction, set up like this:
5μL Gibson

⑥ 12 Cycle PCR gel looks good. pSB1C3 is kind of sketchy, making more.

AJ
7:30 Run a Gel of 2A. 2B. 2C. 2D. Psb1c3-A. Psb1c3-B.

Digestion of purified DNA.
Mix 9.7μl PstI 9.7μl EcoRI-HF. 3μl 2.1 Buffer. 32.5 H_2O.
add 7μl to each tube.
add 5μl DNA to each tube.

August 27, 2015

James Blondin

- I analyzed the plates I prepared two days ago to test $P_{LlacO-1}$ ↱ sCRIBE(kanR)$_{ON}$ (2B+RTB).
- Based on the Sanger sequencing results, we know now that our construct has several mutations that should render it incapable of functioning.

(50

	45 nL on KH (no dilution)	45 nL on KH (no dilution)	Diluted by 10, 45 nL plated on KH
no IPTG induction	0 colonies	0 colonies	0 colonies
	1 colony	1 colony	0 colonies
1mM IPTG induction	11 colonies	0 colonies	1 colony
	10 colonies	1 colony	0 colonies

	10^5 Dilution, 45 nL on LB	10^6 Dilution, 45 nL on LB	10^7 Dilution, 45 nL on LB
no IPTG	(hundreds)	~140	15
1mM IPTG	(hundreds)	~84	12

- Note that these values were observed & recorded two days after plating (and the day after they were removed from the 37°C).

Uninduced cells

No significant colony count means a recombinant frequency of zero.

Induced 1mM IPTG cells

$$\frac{11 \text{ colonies} + 10 \text{ colonies}}{2} = 10.5 \text{ colonies}$$

$$\frac{10.5 \text{ mutant colonies}}{45 \text{ nL} * 10^{-1}} = 0.0233 \frac{\text{mutant colonies formed}}{\text{nL cells grown}}$$

$$\frac{12 \text{ colonies}}{45 \text{ nL} * 10^{-7}} = 2.67 * 10^{6} \frac{\text{colonies formed}}{\text{nL cells grown}}$$

$$\frac{84 \text{ colonies}}{45 \text{ nL} * 10^{-6}} = 1.87 * 10^{6} \frac{\text{colonies formed}}{\text{nL cells grown}}$$

average: $2.27 * 10^{6}$ colonies formed / nL cells grown

Recombinant Frequency:

$$\frac{0.0233 \frac{\text{mutant colonies formed}}{\text{nL cells grown}}}{2.27 * 10^{6} \frac{\text{colonies formed}}{\text{nL cells grown}}} = 1.03 * 10^{-8}$$

Results:

- 1mM IPTG led to a recombinant frequency of ~10^{-8}, while no IPTG led to zero recombinants.
- Considering If the background rate is 10^{-9} (it's either 10^{-9} or 10^{-10}, if my memory's correct), then this is above the limit of quantification.
- This is surprising, since our sequencing analysis failed to demonstrated drastic mutations in our construct. Either something else is going on, or our construct still somehow works... (to a very small degree)

- I also ran a DNA quantification on some purified DNA I was working with:

	Concentration, ng/mL	Ratio		Concentration, ng/mL	Ratio
2A	14.8	1.85	2B	26.4	1.87
2C	14.5	1.71	2D	8	1.95
P(1)	55.2	1.82	P(2)	109	1.87
P(3)	85	1.88	P(4)	80.7	1.83
P(5)	85.6	1.83			

8/17/15

① Gibson Assembly
- Using the two cycle amplification part 2's
- Using direct gBlock 1
- Using P(2) as the vector

Ron suggested a 1:1:2 ratio, NEB recommends 1:2/3 or up to 5x when <200bp

⊛ Maintaining a 1:1 ratio b/t BB & Part 1, showed our only "success" so far.
⊛ Our only "success" has used 3x Part 2B. We will maintain that ratio for this Gibson.

50 ng Vector = 39.09 fmol = 0.458 µL
39.09 fmol Part 1: 47.64 ng = 4.764 µL
117.27 fmol Part 2A: ~~14.93 ng~~ 20.29 ng = 1.37 µL
2B: 25.66 ng = 0.971 µL
2C: 20.29 ng = 1.399 µL
2D: 14.93 ng = 1.866 µL

For a 20 µL Rxn → We want a 10 µL one, so drop everything by ½

~~Reaction Assembly~~

~70 ng DNA in 10 µL of stock = 7 ng/µL → Okay to transfer 4 µL of Gibson into 50 µL calls.

Make a 4x Master Mix w/ 20 µL Gibson MM

30 ng Vector = 23.45 fmol (Being cheeky here :)): 0.275 µL
23.45 fmol P1 = 28.58 ng = 2.858 µL (1
70.35 fmol • 2A: 12.17 ng = 0.859 µL (1 µL H_2O) MM (No P.2)
2B: 15.34 ng = 0.581 µL (1.29 µL H_2O) - 1.1 µL Vector
2C: 12.17 ng = 0.839 µL (1.03 µL H_2O) - 11.43 µL P1
2D: 8.958 ng = 1.12 µL (0.747 µL H_2O) - 20 µL Gibson MM

⊛ Running for 1 hr at 50°C (Amplify product w/ Taq?)

② Transformed 4μL of the Scaled down Gibson to 50μL Comp cells
- Thawed on ice for 20 min
- Incubated for 30 min
- Shocked for 30 sec
- Grew up for 60 min
- 50μL plated & 450μL plated
- Labelled "2__ + RTB in Top10 (X μL plated) 8/27/15 "JB"

(2.5) Emailed Jess that we left at ~10:10pm James B.; Plate sputin 37°C at ~10:15 P.M. (I suggest removal around 2:00 P.M. tomorrow)

③ Going home early (9pm)

Update, I took them out around 1:00 P.M. (on 15 LabB in the fridge)

AJ 3A DNA Purification
Follow the "E.Z.N.A. Plasmid DNA Mini Kit Protocol P10-P12.
Store Sample 2-10 in the Blue rack in -20°C
(Sample #1 is dropped because wrong reagent was added)

8.28. AJ. Quantification of 3A

	Conc	Ratio
2	32.3	1.9
~~3~~ 3	89.6	1.9
4	39.5	1.91
5	91	1.82
6	73.9	1.89
7	53.9	1.92
8	73.2	1.92
9	78.4	1.73
10	85.4	1.88

Digestion of 3A.

Mix 7.7μl EcoRI-HF 7.7μl PstI, 1.7μl 2.1 Buffer and 2.1μl dH2O add 7μl the mixture and 7μl DNA to each tube

5:43 Set up the digestion in cycler.
Return 3A-2~10" tube and digestion Enzymes to the blue rack in -20°C.

8/28/15 ① Colony PCR on the 2A→D Constructs.
- 8 colonies from ACD } 34 colony PCRs → Set up three 9x & 8x 1Cox MM
- 15 from B

PCR Conditions
1.25μL 2-for
1.25μL 1-Rev
Template
2μL H_2O
5μL Taq Gran MM
9.5μL for 1x MM → Then add 0.5μL template.

9x MM			1Cox MM		
11.25μL	2-for	9.5μL each tube	20μL	2-for	9.5μL each tube
11.25μL	1-Rev		20μL	1-Rev	
18μL	H_2O		32μL	H_2O	
45μL	Gran Taq		80μL	Gran Taq	

② Inoculated ALL of the colonies blc [redacted] waiting 3 hours for colony PCR. Asking Michelle to put them in 4C tomorrow

* USED 34μg/mL to INOCULATE

AJ. 8/31/15

~~Run a Gel of the Colony PCR. and 3A Digestion and the~~

Gel A	B1	~~B2~~	~~B3~~	~~B4~~	~~B5~~	~~B6~~	B7	B8	~~B9~~	B10	B11	B12	B13
~~Gel B~~	~~B14~~	~~B15~~	~~C1~~	~~C2~~	~~C3~~	~~C4~~	~~C5~~	~~C6~~	~~C7~~	~~C8~~	~~D1~~	~~D2~~	~~D3~~
~~Gel C~~	~~D4~~	~~D5~~	~~D6~~	~~D7~~	~~D8~~	~~A1~~	~~A2~~	~~A3~~	~~A4~~	~~A5~~	~~A6~~	~~A7~~	~~A8~~

Run a Gel of the Colony PCR

2x 5well in one Gel

B1	–	B13										
~~B14~~	C1	–	C5	D1	–	D4	A1	–	A4			

Other Samples that are not tested are stored in cycler 3.

9/1/15

AT Do a Plasmid Purification of #13 Sample of Colony PCR

Concentration: 8189.9 ng/μl Ratio: 1.87

Do a digestion of #13 plasmid

DNA ~~Strong~~ 189.9 = 13μl

Enzyme PstI 0.8μl EcoRI-HF 0.8μl

2x1 Buffer 5μl

Water: 0.1μl

INTERESTING NOTE:
SMALL COLONIES CAME UP GOOD...

9/2/15 Quantification of Sample

17	107.66	1.87
22	81.26	1.94
23	44.26	1.87
27	117.56	1.88
X 37	25.66	1.93
X 38	88.16	1.83

Not good digest

① Rerun colony PCR. Success at:
17 23 36/37 (maybe)
22 27

9/2/15 ① Purifying colony PCR successes (6)

② Digesting all 5 ins in the following ways

a) EcoRI + PstI

0.75 μL EcoRI
0.75 μL PstI
0.75 μL Buffer 2.1
0.5 μL H2O
5 μL Template (If template conc << 50 ng/μL, use 2x template (so 10 μL), use same amount of enzyme, adjust H2O & Buffer 2.1 amounts.)

Symbol for much less than. So like 20 ng/μL

b) XhoI (Ask Steve for it, his phone # is in the latest email he sent to the iGEM account)

0.75 μL XhoI
0.75 μL Buffer 2.1
1 μL H2O
5 μL Template (Same instr. as above for template)

→ 7 Lts (#samples + 1)

Note: Make a mastermix of 7x everything you want to add (except template) & it'll be faster to do this.

- Incubate for 1 hr at 37°C, heat kill for 20 min at 80°C.
- Run a 1.5% gel at 110V for ~35 minutes

9-2-15
SA

Colony PCR on C+D
primers: 22bp 2A handoff (F)
part 1 rev (R)
to actually add template, pick part of colony + mix inside of tube!

.25 ul F primer		4.5 ul
.25ul R primer	x 16 rxns =	4.5 ul
5ul green tag		90ul
4.5ul dH_2O	18 rxns	81ul
10 ul		

THE PCR TUBES FOR C + D are REVERSED!

in thermocycler 5

0.25 P1
0.25 P2
0.5 T
5 mL Gm

9/2
③ Gels showed:
- 4 expected digestions of purified 2B (w/ EcoRI + PstI & XhoI)
- 1 successful 2C colony
- No 2D successes.

9/3
① Sent #27 for sequencing

② Running a 14 colony Colony PCR on Plates A+D (Set up for 30 → 7.5 mL Primer 1, 120 μL H2O, 7.5 mL Primer 2, 150 mL Green Tag)

TO DO → ③ Transformed plasmids (17, 22, 23, 27) B into MIT Strain FF14C1 (James Blondin)

Comments:
- 0.5 μL of each DNA sample used ~~except~~ ~~for [illegible]~~ (see page 144).
- I'm not sure how long it was between adding DNA & heat shock (I was distracted) (30 min or less...)
- Plates put in 37°C at 6:15 P.M.
- Plated 500 μL instead of 450 μL for 1 of the plates, those (9/3/15)

- 20 min thaw on ice
- 30 min incubation w/ DNA
- 30 sec heat shock
- 2 min recovery on ice
- 500 μL LB
- 1 hour outgrow (put 8 CM plates in 37°C too)
- Plate 50 μL & 450 μL (i.e., the rest of it)
- 37°C o/n

④ Purified plasmid C7 (double check gel! I'm going off memory)
→ 203.9 ng/μL

Do at the same time! 5 & 6

⑤ Digest C7 w/ EcoRI & PstI (10 μL Rxn)
- 1.5 hr digestion 20 min heat kill
- Use Buffer 2.1

⑥ Run a gel on digestion → 35 min @ 90 V w/ 1.5% gel!
*USE 10 μL digest for

⑥ Digest another C7 w/ XbaI & PstI. Check WEB Double Digest for Buffer! Let this run for 90 min → DO NOT GEL THIS.
- Make a 20 μL Rxn

(7) If you're closing [illegible] to go on from here, message me.

(6) Gel on colony PCR showed 5 hits for A, 3 for D

(roughly all in 5mL LB + 5μL CM. (34 mg/mL)

→ 37°C

9/03/2015 JamesBlanden

- Two days ago, I restarted amplifying Part 1 and Part 2B for assembly because our first colony PCR failed. Despite our later success, I've decided to continue working on this "pipeline" until I see one of our DNA sequences passes sequencing.

(done on 9/01/2015)

DNA Quantification of Primers & Templates

- To ensure this back-up went smoothly, I did all protocols in the cabinet except for the DNA quantification itself.
- After quantifying the DNA, I observed something I don't understand: the concentrations change over time following particular trends:

- Note: the only difference between trials is the time I ran the nanodrop (same 2uL drops each time)

		Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
primers for Part 2	Primer 2a 22bp VectorHo	66.4, 1.62	67.7, 1.63	69.6, 1.63	72.2, 1.64	75.8, 1.63
	Primer Part 2 amp Rev	110, 1.72	115.4, 1.72	118.7, 1.72	122.5, 1.73	129.6, 1.74
primers for Part 1	Primer Part 1 For	59.2, 1.78	60.3, 1.78	60.7, 1.79	63.9, 1.8	67.7, 1.79
	Primer Part 1 Rev	57.4, 1.49	58.5, 1.53	59.7, 1.52	61.9, 1.52	65.9, 1.53
	Template gBlock 1 (diluted)	18.7, 1.5	18.5, 1.54	16.4, 1.55	7.7, 1.79	3.3, 1.74
	2B diluted gBlock (done by Aru)	0, 0.87	0, 0	0, 0.62	0, 0	0.7, 7

	Trial 6	Trial 7	Trial 8
2a 22bp VectorHo	82.3, 1.64	84.3, 1.63	117.8, 1.61
Part 2 amp Rev	139, 1.74	146, 1.74	169.5, 1.78
Part 1 For	71.4, 1.82	77, 1.81	93.2, 1.79
Part 1 Rev	70.4, 1.52	75.6, 1.51	84.5, 1.51
gBlock 1 (diluted)	3.4, 2.27	4.5, 1.87	6.3, 2.17
2B diluted gBlock (done by Aru)	.0, 1	0.3, NaN	0.1, NaN

- I would say, the upward trend in concentration over time possibly was due to a gradual rise in droplet temperature across trials, but that wouldn't explain why gBlock 1's concentration dropped so dramatically...

more ↓

James Blandin 9/03/2015

PCR Setup (done on 9/1/2015)

- Because of the unclear primer and template concentrations, I set up two PCR reactions per DNA fragment, each at different concentrations.
  - One would set the primer concentrations to 0.5 nM if the primers were at the highest concentrations observed.
  - The other assumed the lowest primer concentrations observed.
- Unfortunately, I've since lost track of which reaction was which. So I still don't know/reliably what concentrations were really in these reactions...
- I set the template ~~concentrations~~ masses to be 500 pg or less if the templates were at their highest concentrations observed.
  - Looking at it now, I should have gone for 100 pg instead. (10 uL reactions, not 50 uL reactions)
- I also set up controls with no template in them.

(all volumes in uL)	Part 2B α	Part 2B β	Part 2B control (no template)	Part 1 α	Part 1 β	Part 1 control (no template)
forward primer	0.909	0.510	0.409	0.725	0.463	0.725
reverse primer	0.417	0.272	0.417	0.746	0.510	0.746
@5 Hot Start 2x MM	5.000	5.000	5.000	5.000	5.000	5.000
template (note: this template was used & later by 10 again before being added)	0.714	0.714	0	0.400	0.400	0
add'd dH2O	2.960	3.504	3.674	3.129	3.627	3.529

- If pipettes didn't have enough decimal digits to select, I rounded.

PCR settings: (same)

Part 2B α, β, & control:

- 98°C for 30 sec
- 12x [98°C for 10 sec; 66°C for 20 sec; 72°C for 10 sec]
- 72°C for 2 min

Part 1 (2kb gen 2 design) α, β, & control:

- 98°C for 30 sec
- 15x [98°C for 10 sec; 69°C for 20 sec; 72°C for 45 sec]
- 72°C for 2 min

James Blandin 9/03/2015

Gel Set-Up (done on 9/3/2013)

- Poured 60 mL of 1x TAE buffer and 0.6 g agarose into a 250 mL flask (for a 1% gel). Covered top with aluminum foil, mixed, microwaved until boiling, waited until cool enough to touch through nitrile gloves on, added 6 uL Midori Green, poured into container, and waited to solidify (put paper towels on top to use paper towels to reduce the dye's light exposure).

Gel Test

- I added 1 uL of each PCR reaction and 0.2 uL 6x green loading dye in this order: (left to right) Part 1, Part 1, Part 1 control, Part 2B, Part 2B, Part 2B control
- I added our purple ladder (2 log DNA) into both side wells.
- I ran the gel at 110 V (7 cm long gel), 400 mA, 20 minutes.

Results:

- Both Part 2B reaction wells showed a considerable amount of DNA around the 400 base % range.
- No DNA was observed anywhere else in the gel (except in the ladder).
- In the future, I'll just run all remaining 9 uL of each reaction on a gel and use gel purification if I observe sufficient DNA in the 2,000 base range.
- Note to self! Am put the PCR tubes on the yellow rack in the 40°C around

James B. 9/04/2015

- I ran a gel on the other 9 uL of each reaction.
- Both Part 1 & Part 2B were successfully amplified (decent band observed).
- I'll need to repeat the reactions, but with 50 uL instead, to get enough DNA to work with.
- Since the sequencing was almost perfect, this may be moot...

AJ * Gel of a ~~dig~~ digestion sample
* Inoculation of A. D. (8 samples total(ly).
~~Tube~~ (5 ml LB + 1 µl 34 µg/µl CM)
~~LABEL~~ Labeled A2, A5, A9, A11, A14, D2, D8, D9
in 37°C shaker

for (i=0; i<4x; i++)
i=0
while (i<x)
i++

TO DO

① ~~Digest~~ Purify A & D Colonies
② Digest A & C & D, use B27 as a control; EcoRI/PstI for all, do XhoI for A & B as well.
③ If correct → Send to sequencing. ↳ 1.5% gel for ~30min
④ If incorrect, do more Colony PCR & ~~grow~~ inoculate the successes
⑤ ~~Make gel, digest, run gel, cut out, purify~~ Email Michelle after to see
⑥ Move plates to 4C → Email Michelle to see if she can inoculate on Labor Day
⑦ Move a fraction of KM & CM plates; count how many there are first!
- Read old experiments to see how many plates we need in total.

9/3/15 ① Purifying the A+D Colonies. (8 in total). ~~Make sure to label the tubes.~~
A3, A5, A9, A11, A14
D2, D8, D9

A3	189.5	1.87
A5	521.8	1.91 ④
A9	470.9	1.92
A11	586.9	1.87 ④
A14	447.8	1.86
D2	438.7	1.91
D8	456.3	1.92
D9	310.2	1.87

② Setting up the following digestions: Call 10μL

0.75μL EcoRI
0.75μL PstI
0.75μL Buffer 2.1
2.5μL DNA
3μL H_2O
7.75μL total

10x MM = (PCR tubes w/ a ● are the ones w/ this digest!)
7.5μL EcoRI
7.5μL PstI
7.5 Buffer 2.1
50μL H_2O
5.25μL into each tube, 9 total.

(III)
6x MM for A
0.75μL XhoI
0.75μL Cutsmart
3.5μL H_2O
2.5μL Template
5μL each template
4.5μL XhoI
4.5μL Cutsmart
21μL H_2O

4x MM (ξ)
0.75μL BsaI
0.75μL Buffer
3.5μL H_2O
2.5μL Template
3μL BsaI
3μL Buffer (Cutsmart)
15μL H_2O
5μL each tube

Gel 1: AAAAA DDD } → In numerical order!
2: C A(5) D(3)

③ First ran a gel on A w/ XhoI → ALL GOOD & D w/ BsaI → Not sure
Then on C, A, D w/ EcoRI+PstI, gel was run at 0.7% so it wasn't clear, but C looks good, and maybe the others?

9/7/15 AJ 15:20 Inoculation of MIT Strains and Our Part B.

Pick one ~~from~~ colony from FF144 2B #17, 22, 23, 27, & Addgene and put the tips into 5ml ~~4B~~ + 5µl CM (34mg/µl) in 8 tubes

9/8/15 AJ		#017	#22	#23	#27	Addgene
	OD_{600}	0.911/2 =	0.861/2	0.889/2	0.867/2	1.027/2

IPTG Induction

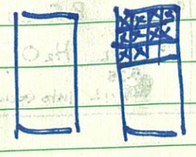
~~Add 1mM IPTG (20µl 100mM)~~
~~Make too~~
Make 1mM IPTG. LB. CM Medium (20µl 100mM IPTG +2mL LB + 2µl 34mg/µl

	#17	#22	#23	#27	Addgene
+IPTG	✓	✓	✓	✓	✓
-IPTG	✓	✓	✓	✓	✓

Totally 10 tubes.

30°C, 24hours inoculation.

The location is



9-9 Make 20 Kanamycin plates

20 mg/mL each plate × 20 plates = 400 mg of Km in total

Stock is 50mg/mL

$\frac{400\,mg}{1} \times \frac{1g}{1000mg} \times \frac{1mL}{50mg} \times \frac{1000\,\mu L}{1mL} = 8000\,\mu L$ of

$\frac{20\,mg}{1mL}$ 20 plates, 10mL = 200 mL of LB + agar

$\frac{2\,mg}{4000\,mg}$ of Km in total

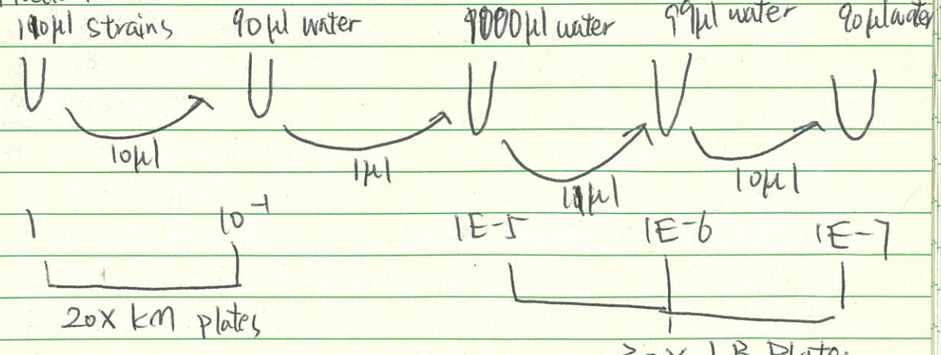
$4000mg \times \frac{1g}{1000mg} \times \frac{1mL}{50mg} \times \frac{1000\,\mu L}{1mL} = 80\,\mu L$ of Km in total

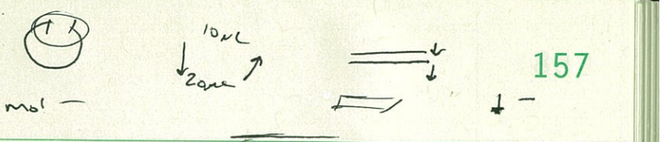
~~Add~~ AJ 11:23

~~Add~~ Make KM plate.
Add 200µl 10 mg/ml KM to 100 ml LB to make 20µg/ml

Finally, we lack 2 LB Plates, so we use two old LB plates for #17 10^7X IPTG+ & #22 10^7X IPTG+

Dilution





9/12/15 To Do

1. Make LB agar ✓
2. Retransform old Gibson assemblies (In case plates are [redacted] now)
3. Try and find green tag ∴ → FOUND!!!
4. Redo Gibson assembly on B + Transform (MIT Strain!) (In case assembly was [redacted] → gives us more to screen!)
5. Digest C+ Ligate into BB → Transform to MIT strain (To see if it works)
6. OE-PCR trials & errors, see if we can get it to work
7. Set up mastermixes for [redacted] stuff
8. Make sure we have sufficient BB / DpnI treat it

Make more backbone

Ordered To Do

1. Set up Gibson → Inv → Leave one in (MM, Part 1, 2B (PCR))
2. Digest / Part C → Inv → Ligate DpnI C (XbaI, PstI)
3. + Ligase → Digest — Gel purify C, (4) Ligate C
4. ~~Mass transformation~~
5. Mass trans

1. Digesting part C w/ XbaI / PstI

20 µL Digestion >10%

2 µL 2.1
1 [redacted] µL XbaI
1 µL PstI
~~[redacted]~~ 2.5 µL Part C ~500ng
13.5 µL H_2O

~~[redacted]~~

2. Resetting up the 40+ Colony PCRs

- 10.75 µL Part 2a Primer_for
- 10.75 µL Part 1 Rev
- 215 µL goTaq
- 150.5 µL H_2O

} 9 µL into each tube + 1 µL LB Colony

③ Made CM34 plates (x25)
④ Digestion of Part C was a bust
⑤ Transformed old B & new B ligaion to FF144
⑥ 10 positive colony PCR hits → 1, 16, 18, 21, 22, 25, 35, 39, 40, 42 → Inoculated in CM34LB
⑦ PCR'd more BBs → gel confirmed!

9/13/15

To Do

~~[redacted]~~

① Set up Colony PCRs on new transformations (as many as you can)
② Purify plasmids (10 of them)
③ Digest purifications (1 hr digestion is good enough)
④ Run a gel on Colony PCRs
⑤ Inoculate the hits from colony PCR ⅔ the successful digests.
⑥ Set up the good plasmids to be Sequenced. ● If not high enough conc, leave a strong note to remind tmw.
⑦ Pour holle KM plates!

→ Should be done to leave by 10pm. Remember to induce tomorrow!

9/13

⑥ Since no plates showed maximal / No growth, retransform old & new B into FF144 AND Top10!
- Just in case it is the CM34 plates, I will grow them up in CM17 LB or make more LB agar... Maybe it was too high for a transformation... → MAKE NEW CM [redacted]

① Purifying the 10 plasmids first, so I can work to remedy ● (○)
- Eluted w/ 50µL 50°C H_2O
- 20sec vortex w/ RNAse A followed by a 20sec bath & Vortex (Keeps low yields get impacted)
- ~~Added Sol III directly to the middle of solution, immediately inverted until~~ (<5! Very Important!)
- Let Sol II sit w/ Cells+RNAse A for 3-4min, inverting & rotating very gently the entire time. (If you invert too much, the genomic DNA will shear → Compromising your purity (but is wait to obtain on Nanodrop; it was read ALL DNA, not just plasmid.
- Added Sol III directly to the middle of solution, immediately inverted until I was ready to pipet in more (4-8 times)
- Took out supernatant 100µL at a time, stopping once I ~~came to~~ disturb the pellet. If it got disturbed, ditch what you pipetted if it got in the pipet. This way, being the one to get ALL of the DNA is not that risky. If I feel I will disturb the pellet, I put what I have in the column, ~~and~~ leave the µL I'm pulling in, and try again.
- DNA Wash Buffer twice
- Let 50°C water sit on middle of column for 5 min before final spin.

→ I think one (or multiple) of these things lead to very high plasmid concentrations. Will record the concentrations below
↳ Ir. Precipitate was in the middle. Maybe Sol III to the middle is bad. I thought I did it last time... Maybe on the bottom!

1: 172.5 ng/µL
16: 104.9 ng/µL
18: 342.8 ng/µL
21: 235 ng/µL
22: 195.9 ng/µL
25: 241.6 ng/µL
35: 104.5 ng/µL
39: 89.1 ng/µL
40: 136.8 ng/µL
42: 232.8 ng/µL

- Still worked out kind of okay. #18 had the strongest band too!!! Looks promising

② Retransforming old & new B to FF144, along w/ a ~~control~~, new B in Top 10, and B18 - But only 5 min incubation for that one b/c time, and B25 along w/ it

a) I'm a scrub.... There is a chance ~~there~~ there is a mixup. But, considering my normal pipetting habits, I think the order of the tubes are,
1, 2, B18, B25, B_2 (Top10)
If not, & they are in the opposite order. If B_2 works for one cell & not the other, we'll know.

Due to culture tubes being in a different order than the heat shock.

③ Digest all 10 plasmids w/ XhoI using appx 250ng DUA & then BB w/ EcoRI, PstI
↳ Diagnostic ↳ for pcr work
- PCR purified pSB1C3 (b/c digestion w/ EcoRI is really bad in CS buffer) & the following concentrations:
1: 16.7ng/μL (HBC was not mixed, just put on top) 2: HBC was mixed before spun, 43.5ng/μL

XhoI Digestion:
0.75μL XhoI
0.75μL CutSmart
→ All of them
11x MM
8.25μL XhoI } x 1.5μL each tube
8.25μL CutSmart }

(250ng) DNA(μL) used: 1: 1.5 16: 2.5 18: 0.70 21: 1.1 22: 1.5 25: 1
35: 2.5 39: 3 40: 2 42: 1.1

Water to use = (7.5)-(1.5 + μL DNA used)
= (In order): 4.5, 3.5, 5.3, 4.9, 4.5, 5, 3.5, 3, 4, 4.9

EcoRI + PstI Digestion: 1x pSB1C3(2) b/c higher conc.
Digesting in 5 ~250ng Rxns:

0.75μL	EcoRI	x5 → 3.75μL	→ 7.75μL in each
0.75μL	PstI	3.75μL	
0.75μL	Buffer 2.1	3.75μL	
5μL	DNA	25μL	
0.5μL	H_2O	2.5μL	

③ Contd → In Thermocycler 1, all of them are a 3 hour digestion.

JL 09/14/2015 4pm
Run gel on sample from digestions

Well	1	2	3	4	5	6	7	8	9	10	11	12
Sample	Ladder	1	16	18	21	22	25	35	39	40	42	Ladder

7/14 ① 7/10 colonies showed expected digestion patterns
② Those colonies were transformed to FF144
③ THESE WERE NOT PLATED

DONT FORGET
To Do
① Make CM17 LB/agar plates (7 transformations = 14 plates; Plate 30-100μL on one & the rest on the other
② AND PLATE!!!
~~Plate colonies using GreenTaq when they are used~~
② Pick 8 colonies from B_2 (new) & 8 colonies from B_1 (old) & Run colony PCR

0.25μL	2a-22bp-homology (For. Primer)	17x MM: 4.25μL Primer For
0.25μL	Part 1 Rev (Rev Primer)	4.25μL Rev
3.5μL	H_2O	85μL GreenTaq
5μL	GreenTaq	59.5μL H_2O
1μL	Template (Touch colony & inoculate in 100μL LB)	↓ 9μL Each Tube

③ Prep B18 and B25 (at a minimum, but B1 & B16 had good digestions) for Sequencing
- The sequencing center wants 5μL of volume w/ a conc. of 100-200 ng/μL → So you'll have to dilute a bit. Put those in Eppendorf tubes w/ SCRIBE-B18 & SCRIBE-B25 clearly labeled on the top
- 5μL of Sequencing Primers 1-4 at a conc of 5-10pmol. These are in the blue rack. They are diluted to 100pmol currently. Put in 8 (4 for each) separate labelled Eppendorf tubes

↳ Located in the ice box w/ the comp cells
↳ Put them all in the -20 when done

④ Inoculate colonies from the B18 & B25 transformation plates (the are w/ a Δ on them; i.e., not the ones used for colony PCR) in 5mL LB + 17 μg/μL CM

⑤ Run a gel on the colony PCRs then inoculate the successes in 5μL LB+17μg/mL CM
OR if you can't stay here until the colony PCR is done &
run a gel, inoculate everything in 5mL LB+CM.

9/15

① Reprepped B18 & B25 for sequencing & sent it in
② PCR'd Ken w/ GG sites
③ Retransforming B1, 16, 21, 22, 25, 42
④ Site Directed Mutagenesis + transform

9/15/15 James Blaylin

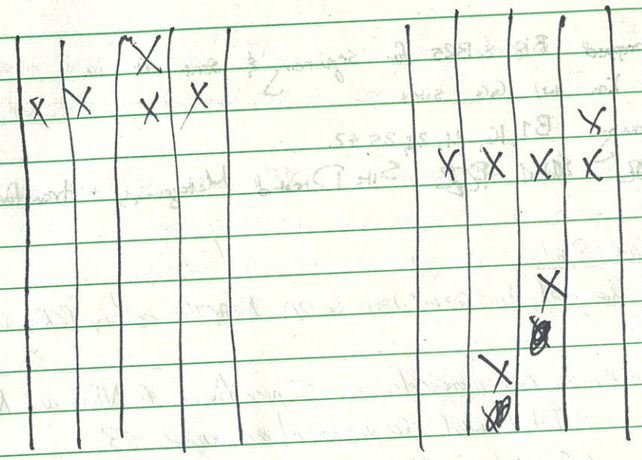
- I ~~not~~ analyzed the gel Andrew ran on the colony PCRs, New and Old 'B.'
- Based on Aru's recommendation, I purified ~~4~~ NB6 and NB8. (N for Ken guessing)
  - I followed the protocol on page 33.
  - I made one error: I did not do a 2-minute spin to dry the mini column before the final elution.

⑤ GG assembly + transform ✓ (Done. at 11:00)
⑥ IPTG inductions on B18/25 (~8:00pm)
⑦ Made XM20 plates

9/16 ① Plated 30μL of B18/B25 (IPTG induced) at ~8am after 12 hrs of induction
② 8:00am: B1, B16, B22, B21, B22, B42 were inoculated w/ CM17+IPTG
Using LB that was open all night (THIS IS ME TRYING SOMETHING; IGNORE)
③ 11:00am: Using fresh LB, the following were put in the 37C in CM17/LB

B1	B21	B42	SDM-3 ①	SDM-3 ③	GG-11
B16	B22	NG	SDM-3 ②	GG-5	SDM-3 ④

④ At night: · B1/16/21/22/42 were plated after (2)
· ③ was induced w/ IPTG
· ① was plated after ~24 hrs of induction
⑤ Next day morning: ④ Inductions were plated ~2pm
B1/22 & B25 (from ①) showed growth. → Seq 1/22
→ Growing 1/22/25 to mid log growth (0.5-0.7 OD)
Put ④ Inductions back in the 30°C at ~3pm (to be plated at noon)



Summary

B18/25 were initially transformed, grown up, induced, plated, seq'd
- Failed (X in Red) → B18 is fucked, B25 has R→H in RT
B1/16/21/22/[redacted]/42 were transformed into FF144
- Grown up for 12 hours WITH IPTG (1mM) (~ 9:00am)
- Grown up for ~ 9/10 hours WITHOUT IPTG (Whats supposed to happen)
- Induced w/ IPTG for ~16 hrs
- Plated at 2:00 Thursday

[redacted]

After growing w/ IPTG for ~12hrs, these were plated
- B1/22/[redacted] Showed growth on [redacted] Kan 20 plates
- We will compare this against the "proper" 12 induction/plating done today
- [redacted]

B25/18 were plated at the same time, but after 24 hour induction
- B25 showed growth

9/16
① B1/16/21/22/42 Were ~~also~~ plated after 12hrs growth & 12 hrs IPTG
② " " Put back in 30C for more induction
③ B1/B22 showed KM growth → Sending in for sequencing
④ B1/B22/B25/Addgene inoculated in 5ml LB + CM17
- Will grow until midlog, then will induce
⑤ Preparing B1/B22/B25 for iGEM part submission