

Welcome to iGEM 2015

~~Michelle's # 813 999 5156~~

6/10/15 Competent Cell Preparation

- 10g of Sterile LB made w/ Yennox powder (500 mL of ddH₂O)
- Inoculated 5 tubes w/ 5 mL LB + 10 uL of cells
- Shaking until we get an OD of 0.4
↳ We got an OD of .842, in 4C! - ~~18~~

6/12/15 Gel Electrophoresis

- Digest plasmid backbone (psBIC3) w/ (EcoRI & Pst I) and the first insert w/ (EcoRI & Spe I) and have got insert w/ (Xba I & Pst I)
- Ran a gel to verify if we did things right
↳ Unsuccessful :c

Comp. Cell Preparation

- Streak (not glass beads) the genomes on a plate of plain LB - Michelle will inoculate colonies on Sunday for us

Amp Plus 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
30 min
sterile
time

- 1) Made 500 ml 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 Blandin

Heatshock Time Test #1 DNA: well 2015-4-166

Sketch Notes	1	2	3	4	5
DNA	2uL	2uL (not all get mixed in right)	2uL	2uL	2uL
comp	40uL	27uL	31.5uL	40uL	19uL
planned shock time	50s	40s	45s	30s	35s
actual time	~49	~49	~49	~34	~42

the well should now be empty
concentrations varying times might ruin 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 the comp cells to a 5ml tube when adding LB after heat shock. It will save you so much trouble!

Todd's 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 pitfalls.

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 (coty mart)
7 uL water~~

7/2/15 TE Equation: $TE = \text{Colonies} / \mu\text{g} / \text{Dilution}$
 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 in 950uL of LB

1uL / 100uL (100uL left) (1uL in LB to plate 50uL)
 Plate 50uL

$\mu\text{g DNA} = 0.0001$
 Dilution: $1000 / 1000 = 0.001$

of colonies: x
 $TE = x / 0.0001 / 0.001 = \text{Our TE in cfu} / \mu\text{g}$

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

Put in 57°C Shaker → will count colonies tomorrow & take to a grad student. takes ~18hrs for full expression

7/2/2015

MD + JC Running a Gel for Spe I

First, we are going to prepare the gel. Measure 1g of agarose (note: 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 to a final concentration of approximately 0.2-0.5 ug/mL (typically 2-3uL of lab stock solution per 100mL gel)
 Midori Green 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

Visualize our gel using the UV machine

7/2/2015

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 (50 mL each) -> stored in 4°C

② E. coli strain for competency is HST08 from Clontech (we grew it up from a tube Eric made competent -> so not E. directly from the company)
 - Took 4mL of sterile filtered CaCl2 (1M) + 36mL H2O to make 40mL CaCl2
 -> stored in 4°C
 It was poorly prepared!

③ Continuation of Spe I digest
 Order of gel: Ladder II III I III

④ Add loading buffer (6x) to the digested samples

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

⑤ Tube contents on Pg A!

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 8000 rpm

(3:5) Sit on ice for 30 minutes

2) Throw cells on ice

④ NOTE: WE WILL TEST COMPETENCY OF 2 CELLS, Box ONE & Box TWO

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. Streak overnight

-> 30uL plated, Sam (Zhu's lab) will put it in the 4°C for us.

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/μL DNA
 2 μL DNA added
 550 μL total volume
 30 μL plated
60 colonies
 1.1 e7 transformants / ng DNA
 "Garbage." Remake!

*OD when taken out .28 A

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

- ① puc19 spun down at 1000 rpm
 ② Comp cells treated for 30 min / LB plates (Amp / No Antibiotic) Revised
 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~~ 500 μL of LB immediately added (room-temperature LB)
 ⑥ Incubated on ice for 5 minutes (see 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 50 μL plated on each plate
 ⑨ 37°C o/n

- J.B. et. al.

② Someone who was here fill this out pls (James Blandin)

7/6/15 Comp Cell Creation

- 1% inoculation (dilution specific) of (I think) pre-cultured cells in LB (someone from the morning confirm the cell source please)
- Made into 200 mL 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
- * Transferred 50 mL each into four 50 mL falcon tubes
- * Centrifuged for 10 minutes at 3,000 rpm at 4°C
- Removed supernatant (by pouring). Resuspended in 5 mL 0.1 M CaCl₂ 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 2.5 mL 0.1 M CaCl₂, 15% glycerol (each).
- Aliquoted 50 μL to microcentrifuge tubes (in ice buckets, if the cabinet).
 - About 94 50 μL tubes were put into one box and stored in -80°C.
 - The other cells were aliquoted at 100 μL or 1 mL and put in a second box in the -80°C (we ran out of autoclaved tubes)
- * Note: we had help from Steve for certain parts of the procedure.

James Blandin

7/7/2015 Comp Cell Test Results

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

James Blandin

7/7/2015

- we auto claved an empty jar, small pipette tips, and a jar of microcentrifuge tubes.

7/8/15

CB	B0032	R0010	K592016
MD	B0034	K592008	K911003
James	B0015	R0040	K592006
Andrew	E 0040	R0062	K914003
	J33201	I7404006	K561001
	K1404006	R0084	K554000
	K54001	K 346002	K873002

Using the following protocol:

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

- We kept the cells in the shaker for 1 hr.

- Plated on ~~ampicillin~~ CM plates

All parts except for B0032, K911003, K1404006 and B0034 were also spun down and plated a 2nd time after the 1st plating:

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

7/7/2015

Thaw

- Add DNA (2 µL) and gently swirl to mix.
- Incubate on ice for 5-30 minutes
- Heat shock @ 42°C for 30 seconds
- Add 1 mL LB and incubate ^{37°C} for 1-2 hrs
- ~~Spin down~~ Plate cells with 30 µL
- Remove liquid from tube
- spin down cells at
- Add LB and plate on another dish

rest in ice 2 mins

Plate 50 µL on CM plates

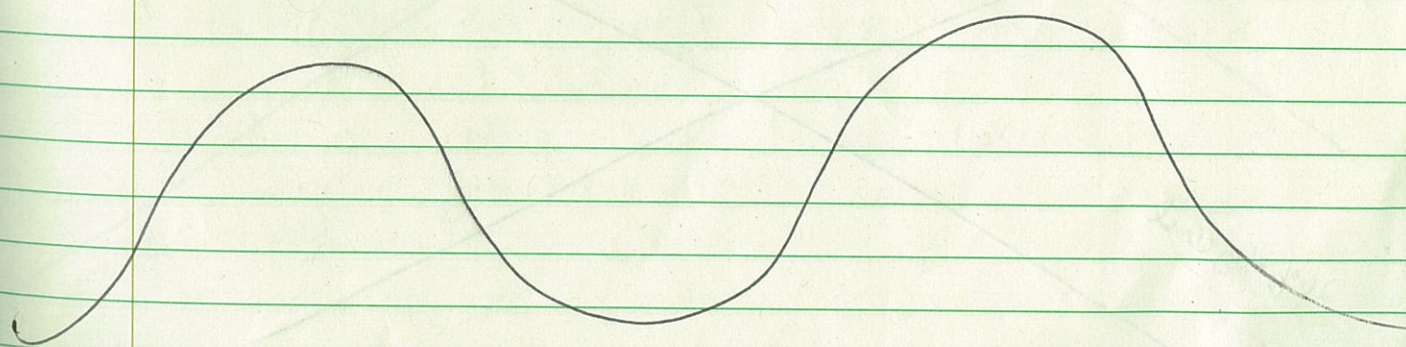
All were placed in the 37° incubator overnight

As for the Spin plates, we used 55 µL to resuspend the cells and transfer to 11 to plates.

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

James also transformed the following and plated on Amp: J22106 (P_{trcA}) and I765001 (P_{uv}) (backbone: pSB2A2)



7/9/15 → 2 EDO
on 7/10, 1001
same

- Made CM plates (10 g LB, 7.5 g agar, 500 mL H₂O)
- 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 spin down cells all grew better than the 50 μL ones, but in no case was a 50 μL plate unusable while the spin down plate was. To save plates, we might not need to include the spin down plating in the future.
- Control grew

20% glycerol

500 μL of growth up → 20% glycerol LB
500 μL of culture

7/12/15 Promoter Inoculation

- Took 30 mL plates and picked top colony in each one (colony ~~was~~ 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°
- Colonies picked were circled on the plates
- * Two Transformations failed (B0034, E0040)
- Placed in 37° 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 10 μ L dH₂O to ^{each} well, mixed by pipetting. Red color observed (good).
2. Added 2 μ L to a 50 μ L comp cell tube (DNA sample per tube)
3. Put on ice at 1:04 P.M.
4. Put LB in a 15 μ L tube in the 37°C to warm it. ^{added 2 μ L dH₂O to a control cell tube before the heat shock}
5. Heat shocked for 30 seconds at ~1:24 P.M. (1:24 \pm 30 seconds?)
6. Put on ice for 2 minutes (1:24 - 1:26)
7. Added 500 μ L 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~~ the 2 parts: 50 μ L and 500 μ L
- 50 μ L 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 0:40. 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 50 μ L plates should be inoculated.
- In the future, plates should be removed earlier & probably by hour 18).

^{before}
10/12 in 37°C by 2:32 P.M.
(CM plasmids)

- The 2 transformed plates and the 10 plated cultures from the Registry were inoculated in 5 mL LB with 17 μ g/mL CH or 100 μ g/mL amp. The 10 CH 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 parafilm and stored at 4°C, along with the leftover ^{17 μ g/mL CH} LB (about 16 mL)

Quantifying DNA

	1	2	
A	B0032	K542008	Blanked then used
B	B0015	K914003	this layout:
C	K54001	K1404606	Got weird result,
D	J221061	K554000	so added puc19 to
E	K592016	K346002	see if we'd get an
F	K911003	I746301	accurate reading.
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 Dad tube Caroline with a "1" and returned to its box in the Blastack

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

Still 7/14/15

Seed Cultures, Take II

James Blandin

7/15/15 Miniprep (12 samples)

no observed decent spagueness.

- 1.) Took the 5mL tubes out of the 37°C shaker. Transferred 600µL cells into 400µL 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 ref) for 10 minutes.
- 3.) Removed the supernatant by pouring.

Tip: do this quickly (one big dump into the waste), before the cells soften. vortex for 20 sec (really vortex it)

★ 4.) Added 500µL solution I. Resuspended by pipetting. Aru stip.

Tip: do step 5 at the same time.

★ 5.) Transferred the suspensions to new 2mL microcentrifuge tubes. using MiniKit II

• will need 2mL tubes. 1.5mL tubes are too small.

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

★ 6.) Added 500µL solution II. Added to each tube in a rapid succession then inverted to mix all simultaneously. in a row quickly, do so gently. (tip: wait 2-3 minutes for better results but never exceed 5 minutes.)

★ 7.) Added 200µL solution III, using the same technique as step 6.

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

9.) Transferred 700µL of the supernatants into minicolumns inside 2mL collection tubes. Transferred 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 tubes), reused the collection tubes.

12.) Repeated steps 9-11.

13.) Added 500µL HBC Buffer.

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

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

16.) Added 700µL 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.) Transferred the mini columns to new 1.5mL tubes.

21.) Added 50µL pH 7 dH₂O (tip: pH 8.5 dH₂O or Elution Buffer might work better)

22.) Left at room temperature for 1 minute.

(done by Aru Singh recorded by James Blandin)

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

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 storage)

7/16/15

DNA Quantification of yesterday's miniprep

	1	2
A	L1166000 121.2 ng/µL [1.86]	K387003 47.1 ng/µL [1.85]
B	K1071004 61.5 ng/µL [1.74]	K1509001 61 ng/µL [1.83]
C	I13504 49.9 ng/µL [1.8]	K896005 65.2 ng/µL [1.87]
D	I765600 46.8 ng/µL [1.83]	K629001 57 ng/µL [1.78]
E	K216004 19.1 6350 ng/µL [1.83]	K326003 51.2 ng/µL [1.84]
F	K1166000 14.1 120 ng/µL [1.87]	E0240 56.3 ng/µL [1.83]
G	J.18 88.1 2.815	
H	Put 50µL in 1.5mL fresh LB into the autoclave	
I	1.0 000000 88.1 2.815	

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

Seeded the plates from the 10th agar plate. 5mL of 17 µg/mL Cm.PSLB. Put in 37° Shaker. Add gene plasmid was seeded along with them. They can't match. Plate: I746361, Tubes: I7464006

7/17 7/17/15

Create glycerol stocks of following samples:

K346002	I765001	I746006	Both	K561001
S4001	J22106	I746361	on same tube	K73002
B0015	K592008	K554600		R0010
K911003	K1404006	R0040		R0084
K592006	K914003	R0062		(PFF745) addgene plasmid
B0032	J33201	K592016		

Using Serial of glycerol (50%) Stock and Serial of Cells.

- ⊗ 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 K346002 (Mycu)	10.8	1.61	K590001 ^{control}	142.3	1.91
K911003 (Riboswitch)	171.9	1.92	K592016	445.6	1.86
K873002 (HSP)	152.5	1.92	J33201 ^{trans reporter}	85.9	1.91
R0010 (Lac)	213.5	1.88	K561001 ^{very microbic}	91.6	1.93
Addgene	174.2	1.87	K1404006	31	1.85
X K592008 (T5-Lac)	9.5	1.83	K154000 ^{SoxS}	96.1	1.84
K914003 (L-Phenase)	186.1	1.91			
R0084 Amp ^R	188.6	1.88			
I765001 UV	16.4	2.02			
B0015 term	177.3	1.91			
B0032 RBSS	129.8	1.89			
R0040 Tet	212	1.88			
I746361 ^{DDP from P2}	144.8	1.9			
old K592008 FixK2	63.4	1.71			
R0062 Luf	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 dH2O 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 ₂ O (μL)	
R0040	2.36	2.4	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	
K1091004	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	5.5	19.9	
K914003	2.7	22.7	
K0240	8.9	16.5	
J33201	5.8	19.6	
R0084	2.7	22.7	
R0062	3.6	21.8	

Amounts of each:	DNA (μL)	H ₂ O (μL)
K1509001	8.2	17.2
K387003	10.6	14.8
K1166001	3.5	21.9
K376003	9.8	15.6
<hr/>		
Amp	I7656003	30.48 30.5
Amp	K216004	7.7
		none
		17.7

- water
- DNA
- ~~Restr~~ CM restriction enzymes
- Amp restriction enzymes
- Buffer

(X) ONLY SOME OF THE DIGESTIONS WERE DONE; CHECK 37° WATERBATH TO SEE WHICH ONES WERE DONE

- Digestions were done o/n at 37° (J22106) (I712074) (K117002)
- 3 parts were transformed & plated: Lac 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.
- 5ml of pRed, GFP (x2), and K592003 (? PstI check) were grown up o/n

Digestions: Amp BB
 Promoter: EcoRI / SpeI
 GFP: EcoRI / XbaI

CM BB
 Promoter: SpeI / PstI
 GFP: XbaI / PstI

7/21/15

- digestions*
- O/n ~~digestions~~ were put in 80°C to heat kill the enzymes for 20 min
 - Plasmid purification began w/ K346002, E62040, J22106, another E02040, K592003
 ↳ OD same a bit low i.e. (all of them)
 - Buffer PE (Qiagen) was used instead of DNA wash buffer
 - GFP that we had is getting digested w/ XbaI & PstI (The GFP that will be inserted CM BB's)
 - Began digests at 1:10pm → ended 7:00pm

Note: Digestions yesterday were w/ K561001, R0062, I746361, ~~J33201~~ I13504 (in 64?), K896005, E02040 (GFP)

- 1μL of rSAP (phosphatase) was added to the above digestions (excluding GFP) at 1:10pm
- At 2:30pm the phosphatases were put in the 80°C to be heat killed → removed at 2:31pm
 ↳ **K561001, R0062, I746361, I13504, K896005 have been digested & phosphorylated, E02040 has been digested**
- Four inoculations of cultures plated last night (No antibiotic added → to overcome poor o/n growth), K117002 (LsrA), 2 inoculations of J22106 (RCA), and I712074 (T7) → **Inoculated in 37°C**

- Results are recorded on the next page. Pect & T7+Lac were skipper. The rest were successful
- Beginning digestions of more promoters: J33201, K387003, K561001, K914003, I768003, Pect10
 ↓
 I746361, R0062, R0010, K376003, K554000, K873002, K629001
 Begin: Jan
 R0084, K1071004, K1509001, K911003, L1166000
- 3μL Buffer, 1μL of ~~PstI~~ ~~(GFP)~~ ~~1μg of DNA (not strong)~~, water to 30μL. Incubate for ~~2 hrs~~ (probably o/n at this rate)
 ↳ 40μL 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/ul)	Ratio
E0240	178.9	1.96
J22106	15.3	2.64
K392008	17.1 31.1	1.96 / 2.04
K346002	157.5	1.93
E0240	108.9	2.15

Note - caps w/ "D" are digests, 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-Fox, Lac-Rev, Pro-Fox, Kan-Fox, Kan-Rev w/ the following conditions and ratios:

- Lac-Fox: ~~236~~ 236 nmol
- Lac-Rev: 24.6 nmol
- Pro-Fox: 23.6 nmol
- Pro-Rev = Lac-Rev
- Kan-Fox = 23.6 nmol
- Kan-Rev = 25.6 nmol
- Gtr-Fox = 22.4 nmol
- Gtr-Rev = 23.5 nmol

cap names Purified following parts w/ protocol from page 30:

cap names		conc.	ratio
"LSRA"	LSRA pSBIC3	130.4	1.84
RecA1	J22106 RecA pSBIA2 (beaded)	100.9	1.82
RecA2	J22106 recA pSBIA2	101.2	1.79
"T7"	I712074-T7 pSBKAG	140.2	1.82

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

Added this much of everything: (NL)

	DNA	H ₂ O	Enzyme 1	Enzyme 2	cutsmart
LSRA	7.8	17.6	0.8 PstI	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 uL PB buffer (into ~50 uL DNA)
- 2.) Transferred to a minicolumn (Qiagen) in a 2 mL collection tube.
 - Centrifuged for 1 minute at max (15,000 rpm).
 - Discarded the filtrate, reused the collection tube.
- 3.) "Washed the sample."
 - Added 200 uL 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. ~~37A~~ wood bbp
- 6.) Placed the column in a 1.5 mL microcentrifuge tube.
- 7.) "Eluted the sample"
 - Added 15 uL H₂O
 - 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 (1.5 mL microcentrifuge tube) in the -20°C.

7/23 Making stock spectinomycin and plates
 We want a 100 mg/ml stock solution.
~~We have 50 mg/ml~~ This appears to be what we ordered.

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

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

Loaded 10 μ L loading buffer and ~100 μ L 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 600 μ L ADB to gel
 70°C Inocul Incubate 10 min, mix a couple times.
 Load column, spin 30s
 200 μ L DNA wash buffer spin 30s
 200 μ L DNA wash buffer spin 1 min
 100 μ L dt20 spin 1 min.

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

Length of msr/msd regions

- w/ promoter: 254bp
- w/o promoter: 210bp

7/23 PCR (7 Rxns)

Each tube will have the following:

- 2.5 μ L ~~Q5~~ ^{2.5 μ L not time!} 2x Master Mix
- 2.5 μ L Forward Primer
- 2.5 μ L Reverse Primer
- 2.87 μ L pFF745 (200ng DNA)
- 39.63 μ L H₂O

The 7 reactions will use the following primers:

- BB GG KanR Forward
 BB GG KanR Rev \rightarrow Anneal at 64°C
- BB Gap-msr For } BB msd (front) Rev
 BB lac promoter For \rightarrow Anneal at 65°C
- Lac-For } Anneal at 62°C
 Lac-Rev }
- Pro-For } Anneal at 65°C
 Lac-Rev }
- GG-For & Rev will not have template
 H₂O to 42.5 μ L
- Kan-For } Anneal at 63°C
 Kan-Rev }
- GG-For } 95°C for 2 min
 GG-Rev } Lower 0.1C/sec \rightarrow To 16°C for 10

Each Primer was diluted to 20 pmol/ μ L
 - [primer] / 20 = mL of H₂O to add \rightarrow Resuspend by pipetting

Using The Following Conditions for Thermocycling: (NOT ~~95°C~~ 65°C For, GG-Rev)

Initial Denaturation: 98°C for 2 min
 Cycles: { 98°C for 7 seconds (7 bic hairpins)
 25x { Use above annealing times for 30 seconds (bic hairpins)
 72°C for 10 seconds (~5-7 seconds by the standard 20-30/kb, but that seems too small :))
 Final Extension: 72°C for 2 minutes

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

7/23 - 2 pFF745 colonies were inoculated in 17 μ g/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/ μ L 5.56
GFP 2 2.6 ng/ μ L .96

Added 100 μ L spectinomycin to 100 mL LB agar and
poured 9 plates. They are labelled and in the 4^o

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

James
Blondin

July 24, 2015

• Mini-prepped 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 $\frac{\text{ng}}{\mu\text{L}}$, 1.85 ratio

pFF745 (sample 2): 37.8 $\frac{\text{ng}}{\mu\text{L}}$, 1.76 ratio

July 24, 2015

James
Blondin

• Quantified the 22 purified, digested DNA samples

#	Code	Value (ng/ μ L)	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 \checkmark	1.94
7	K561001	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	K1071004	69	1.92
15	K561001	73.4	1.98
16	J33201	57.9	1.98
17	K1509001	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

7124 PCR Tube 2 (6 Runs) → No GG (#7)

- Same as 7123, except:

- 25 μL of master mix

- 500pg of DNA

- New DNA Conc is 61.2 μg/L

- Serially dilute, 10 μL to 990 μL, giving 6.12 μg/L

- Do this again to get 0.612 μg/L

- Take .81 μL of this to get .5ng

→ 25 μL + 2.5 μL for + 2.5 μL Rev + 0.81 μL dm = 19.19 μL H₂O

FINAL AMOUNTS

① Add 25 μL of each primer to the 6 tubes

② Add .81 μL of the serially diluted DNA to each tube

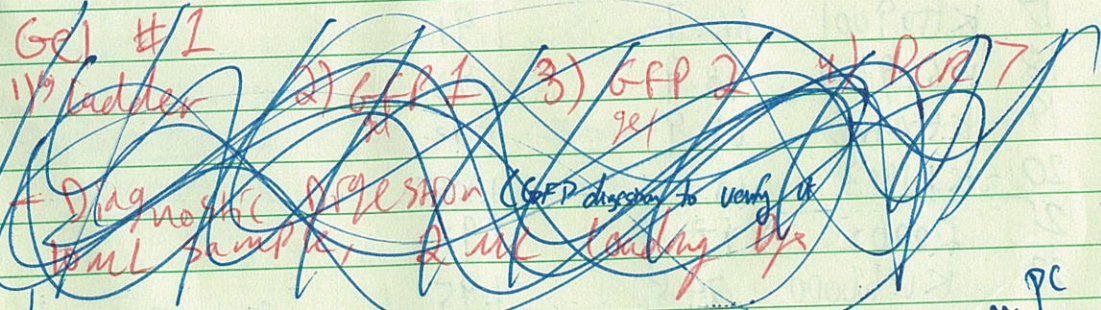
③ Add 25 μL of 2x master mix

④ Add 19.19 μL of H₂O

20 $\frac{pmol}{\mu L}$ 20 μM → C₁V₁ = C₂V₂
(20 μM X) = (0.5 μM X 50 μL)
X = 1.25 μL

bkan b lac
b gap

"Bad microwave-ing"
Gel messed up



10 μL sample
2 μL loading dye

Gel #2

1) log2 ladder; 2-7) PCRs 1-6

50 μL sample, 10 μL loading dye → used 45 μL for gel wells

Check on PC for results → These are primary diagnostics

Anything larger over flow

AVU
Miranda
Andrew

James Blunden Very crude transformation

• Used J13002 (P_{lac}+RBS3A), pSB1A2, 2013-5-13B (will help w/ sugar application)

• Added 10 μL H₂O to well, ⇒ 2 μL DNA to 50 μL comp cell (thawed mostly in hand)

• After minutes (2-3) on ice, ⇒ 30 seconds in 42°C bath

• Plated 50 μL directly onto 100 μg/ml amp plate, did this in hood, put in 37°C.

7/27/2015

9:00am Autoclaved glassware in autoclave B - run

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

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

11am 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 4 M17 strains)

↳ we did not record this last week! People involved were AVU, 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.

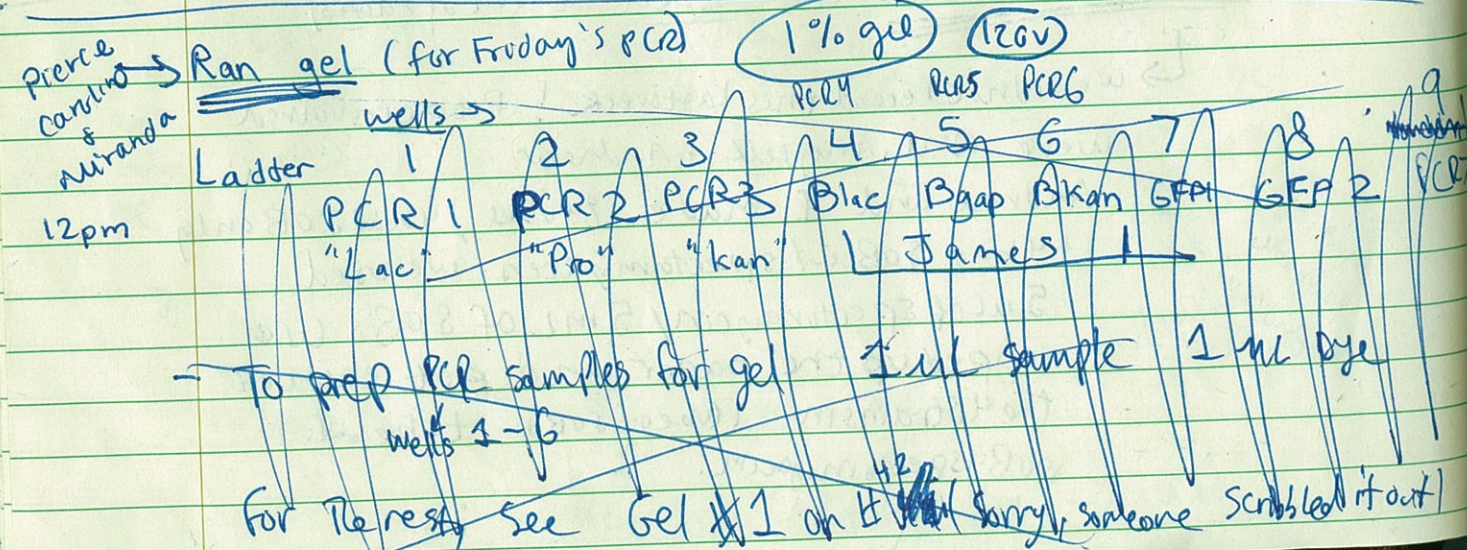
The next few days Anu was in charge of seeing if we had growth + found only 2 of the 8 tubes had growth. Here is a record of what we did

Note → it was unnecessary to do these 4 tubes

Strain	w/o spec	w/ spec	x = no growth
1 F144 <small>Kan^R off</small>	x	x	
2 F774 <small>Kan^R off galK</small>	grew! <small>(bot unusable)</small>	x	
3 F762 <small>galK</small>	x	grew!	
4 F798 <small>lac^R off</small>	x	x	slw

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

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

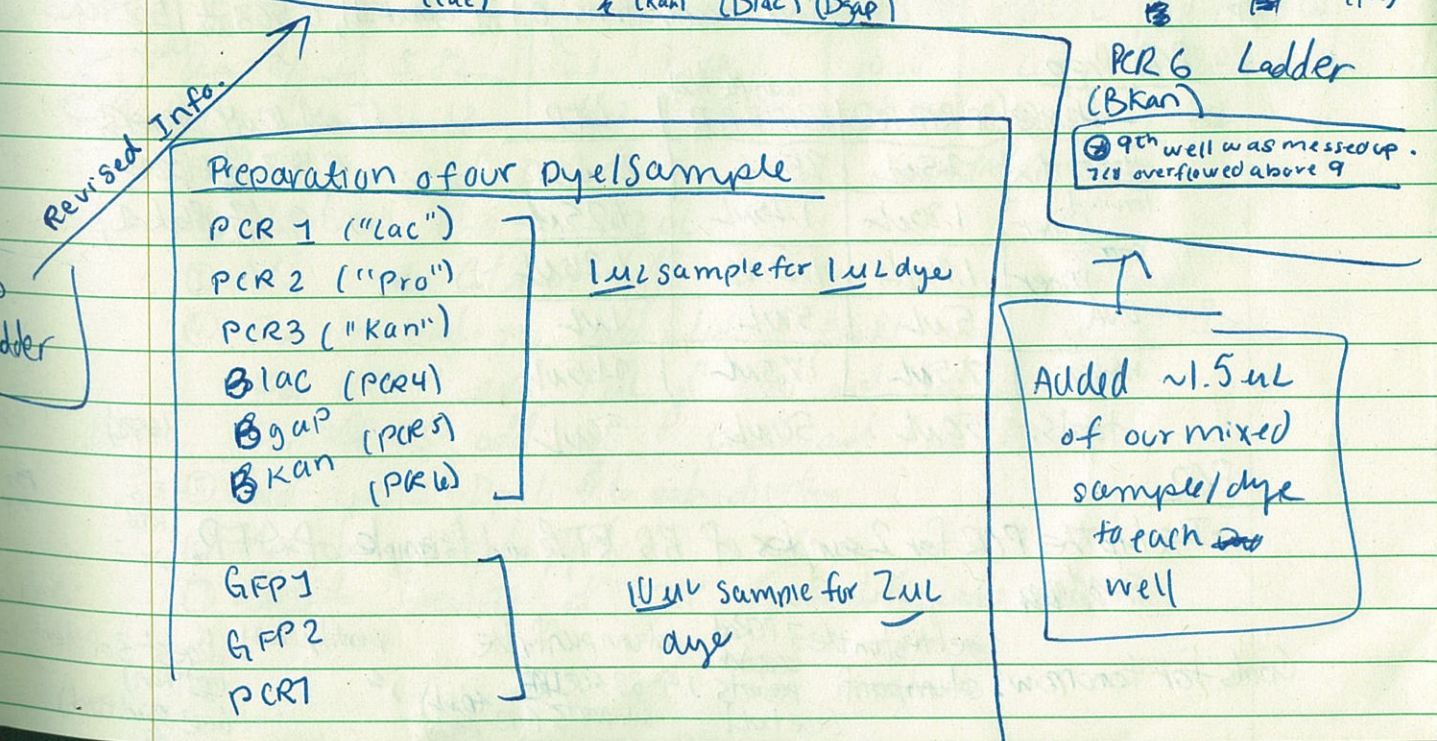
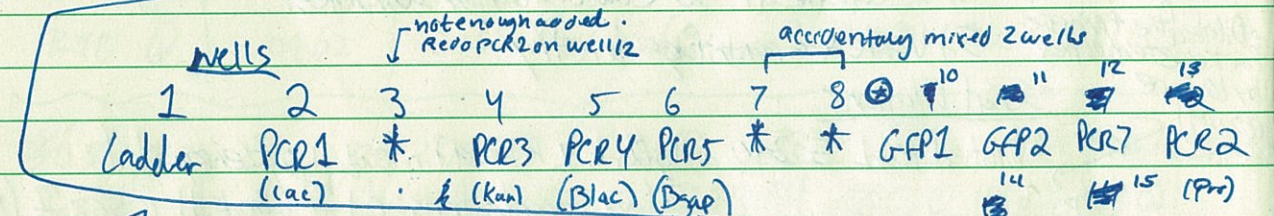


⊗ Run for 15min - take pic + check, then run it for 5min + check. (Todd said this is best for 1st couple times)

Lengths of PCR fragments to expect

Lac	210 bp
Pro	135 bp
Kan	170 bp
B lac	210 bp
B gap	135 bp
B Kan	100 bp
GFP 1	720 bp
GFP 2	720 bp
PCR 7	

Should be same DNA, just a different batch



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

Pirvax
 Mramdar
 James

Final Images Saved as: (under Documents → 16 EM → 16 EM 2015 → 7-27-2015)

" 7-27-2015 Diagnostic Gel GFP, PCR1 and PCR 1-6

tiff and image lab images

James
 Blenkin

7/27/15

Resuspension Protocol (gBlock & primers)

- 1.) Centrifuge 3-5 sec above 3,000g (Idid 3,000 rpm)
- 2.) Add TE buffer (Idid water: 100 µL for gBlock 1, diluted to 100 µL for GFP for rev & BB RTB for rev)
- 3.) Vortex briefly
- 4.) Incubate at 50°C (water bath) for 20 minutes.
- 5.) Vortex & centrifuge briefly

10 µL hot
 Diluted the templates for these templates by 10 once (10 µL)

Serial Dilutions

- Diluted 10 µL Eo240 and gBlock 1 (separately) in 90 µL H₂O then repeated.
- Ending concentrations: 0.1 µg/µL (gBlock 1), 0.563 µg/µL (Eo240)

PCR Prep

	BB RTB PCR (sample #1)	BB RTB PCR (sample #2)	GFP
Master Mix	25 µL	25 µL	25 µL
Forward Primer	1.25 µL	1.25 µL	1.25 µL
Reverse Primer	1.25 µL	1.25 µL	1.25 µL
DNA	5 µL	5 µL	1 µL
Water	17.5 µL	17.5 µL	21.5 µL
Totals	50 µL	50 µL	50 µL

(Used 10 µM primers, 0.563 µg/µL Eo240, and 0.1 µg/µL gBlock 1)

PCR

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

Goals for tomorrow: column purify the products (use 1 µL), 4 BB Scribe templates (use each), digest 3 of them (not Kan) (use 20 µL each)

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 HBE buffer and centrifuge at max speed (15000 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 ~~micro~~ microcentrifuge tubes
 - ⑦ Add 20 µL H₂O, 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, pSBIC3 ①, pSBIC3 ② (25 µg/µL)

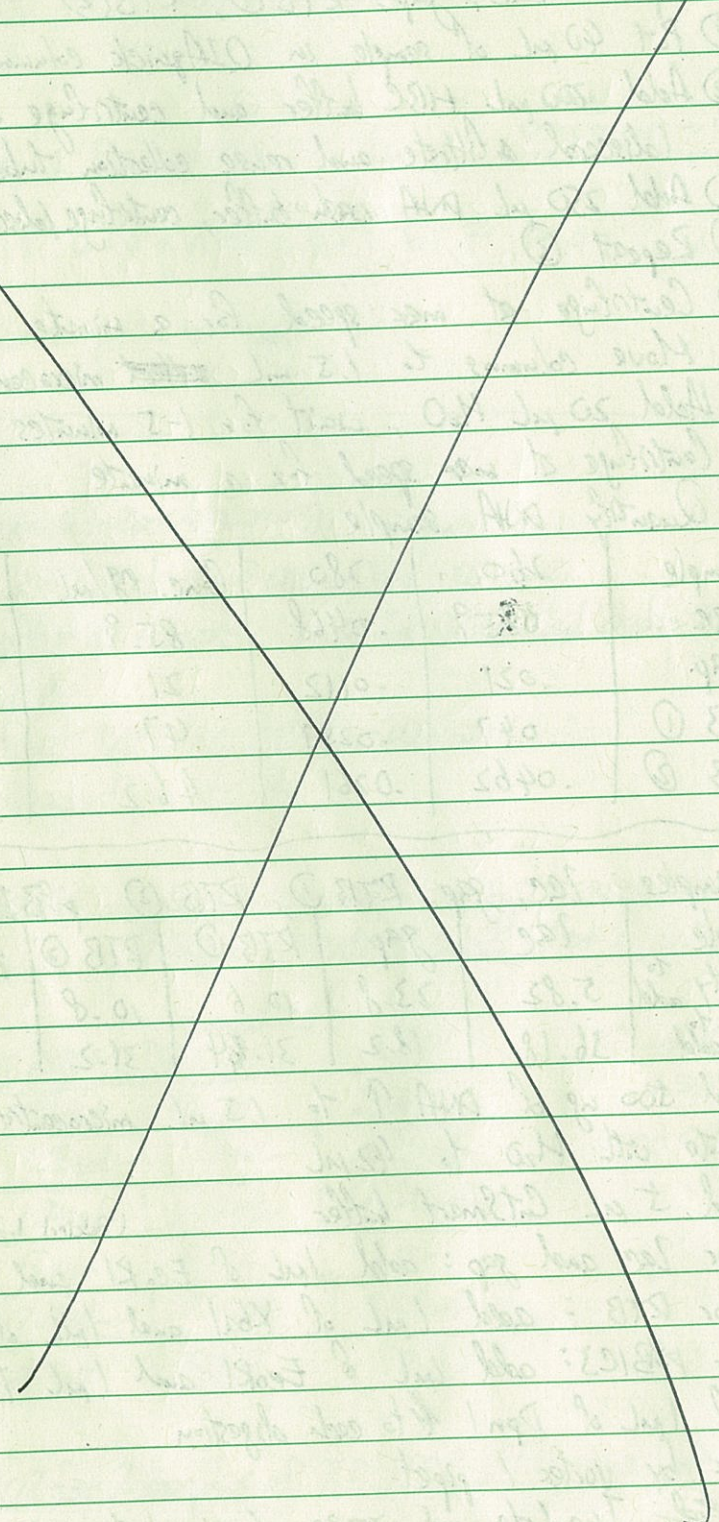
Sample	lac	gap	RTB ①	RTB ②	pSBIC3 ①	pSBIC3 ②
Quantity to add (µL)	5.82	23.8	10.6	10.8	20	20
Quantity to add (µL H ₂ O)	36.18	18.2	31.44	31.2	22	22

- ① Add 500 µg of DNA ↑ to 1.5 mL microcentrifuge tubes
- ② Dilute with H₂O 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 pSBIC3: add 1 µL of EcoRI and 1 µL PstI
- ⑤ Add 1 µL of DpnI to each digestion
- ⑥ Mix by vortex / pipet
- ⑦ Incubate at 37°C (water bath works too)

Time:

~~PCR II~~

~~Notes~~



gBlocks we have: msd/errsr (4)

- Lac
- Pro] → EcoRV
BB

Primers we have: gBlock w/ overhangs & homology
gBlock primers over

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

~~Notes~~

- gBlocks, as well as the Addgene (pFF745) plasmid are viable templates
- Digestions need to occur before Gibson on pSB1C3!
- Remember to dephosphorylate the digests
- Use DpnI (cuts methylated DNA) on pSB1C3 for the Gibson to get rid of plasmid
- Transform 5 μ L 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 recoding

PCR

Assembly To Be Done: ① Lac + Kan + RTB] → Gibson'd straight into pSB1C3

② Lac + GG + RTB

③ Pro* + GG] → Golden Gate'd into PI pACYC (plasmid for GG → given by Todd)

Depends on which primers we have.

④ Pro* + Kan

⑤ Pro*, Kan + RTB] → Gibson'd into pSB1C3

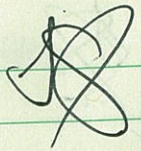
⑥ Pro*, GG + RTB]

General Overview: ① Resuspend gBlocks

② Amplify gBlocks (5 Amplifications, 2 primers sets)

③ Do Gibson assembly on the gBlocks

④ Turn down PCR on



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

③ PCR

- ① Spin down primers + gBlocks briefly (until it hits max speed, then stopped)
- ② Diluted primers that have not been resuspended yet to $20 \mu\text{M}$
 Part 1 - For: $31.3 \text{ nmol} \rightarrow 31.3 \text{ nmol} / 1.565 \text{ mL} = 1.998 \text{ nmol/mL} = 0.78 \text{ }^{25} \mu\text{M}$, add twice this in mL H_2O
 Rev: $43.1 \text{ nmol} \rightarrow 31.3 \text{ nmol} / 1.565 \text{ mL}$
 Add $1.075 \text{ mL} = 20 \text{ nmol/mL} = 20 \mu\text{M}$

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

Rev: 29.2 nmol , add 0.73 mL twice

Misc note

- \rightarrow X ③ Resuspended gBlocks to 10 ng/mL 2d is 500 ng/mL
 Part 2a-2c are 10 ng/mL of direct DNA. Add $25 \mu\text{L H}_2\text{O}$
 Part 2d is 250 ng - Add $50 \mu\text{L H}_2\text{O} \rightarrow$ conc. (2d) is now 5 ng/mL

- ④ Set up the amplification PCR as follows:
 - $25 \mu\text{L}$ OS 2x Master Mix
 - $0.5 \mu\text{M}$ For-Primer ($1.25 \mu\text{L}$ w/ my dilutions)
 - $0.5 \mu\text{M}$ Rev-Primer
 - 500 ng of Template $\rightarrow 500 \text{ ng} = 10 \text{ ng/mL} = 0.05 \mu\text{L}$ } Dilute the gBlock so we don't need to pipet this
 - $12.5 \mu\text{L}$ of H_2O (a-c) $500 \text{ ng} = 5 \text{ ng/mL} = 0.1 \mu\text{L}$
 - $50 \mu\text{L}$ (I will use $2.5 \mu\text{L}$ water)

98°C 30 sec

98°C 7 sec

35x 68°C for P2, 72°C for P1 for 30 sec

72°C for 10 sec

72°C for 2 min

Continued on Page 52!

7-28-15

Miranda

Made Gels

- $2:04 \text{ pm}$ 2x 1% gels @ wells \rightarrow in fridge
- $3:00 \text{ pm}$ 4x 1% gels @ wells \rightarrow in fridge

James Blending

Resuspensions of pSB1C3 Linearized Backbones

Quantification Results:

- 2012: $1.96 \frac{\text{ng}}{\mu\text{L}}$, 2.31 ratio
- 2012: $2.76 \frac{\text{ng}}{\mu\text{L}}$, 2.46 ratio
- 2013: $15.66 \frac{\text{ng}}{\mu\text{L}}$, 2.23 ratio
- 2014: $10.66 \frac{\text{ng}}{\mu\text{L}}$, 2.31 ratio
- 2015: $11.26 \frac{\text{ng}}{\mu\text{L}}$, 2.12 ratio

Steps:

- 1.) Add $50 \mu\text{L H}_2\text{O}$, mix by pipetting.
- 2.) Vortex for 1 min, rest 10 min, vortex 1 min, "short" for ~5 seconds (mixing).
- 3.) Run a quantification test. (Nanodrop)
- 4.) Store in -20°C .

MD Rana^{GFP} Gel - 1% gel @ 120V for 20 min. in 8 comb

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

\uparrow 10 μL sample
2 μL dye

Result \rightarrow NOTHING :-(, we failed to PCR the GFP

MD Ran a "RTB" gel (Reverse Transcriptase/Bromomerase)

1% gel @ 120V for 20 min in 8 comb

10 μL	10 μL	10 μL	
Ladder	BRTB	BRTB	X

\uparrow same thing according to James

\rightarrow full of sample & dye
 \rightarrow used 10 μL in each well
 used 4 μL in last well

Results: :(Not right band, primer dimers

PCR II Continued (Pg 3/3)

- ⑤ 10 μ L product was put in a gel (1%) running at 50/cm for 20 minutes
- ⑥ 1 μ L phosphatase was added to pSB1C3 Vector \rightarrow incubated for 1 hr
- ⑦ Reading GFP PCR + Setting up NEBuilder X for full 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%

10 μ L Master Mix

Water to 20 μ L

\rightarrow Inoculate samples in thermocycler for 15 Min at 50°C, transform immediately

- ⑧ PCR Purified the 5 reactions. Eluted w/ 15 μ L H₂O. \rightarrow I may have forgotten to elute C; added extra 15 μ L.
- ⑨ Amplification of Part 1 failed; the rest were successful
- ⑩ Phosphatase reaction was not heat killed!

\rightarrow Part 1: 129.3 ng/ μ L, 1.8
2a: 111.4 ng/ μ L, 1.88
2b: 133.3 ng/ μ L, 1.89
2c: 84.3 ng/ μ L, 1.85
2d: 102.1 ng/ μ L, 1.86

James
Blondin

7/28/2015

Summary of BB Method Progress (SCRIBE Assembly)

- The pSB1C3 backbone has been located, resuspended, quantified, and digested. DpnI was added, but not phosphatase. Enzymes have been denatured.
- B lac and B gap have been successfully PCR'd, gel tested, column purified, quantified, and digested (DpnI added), and ~~denatured~~ 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 PCR'd.

I setup another PCR of B RTB (3):

(500 μ g total) • 5 μ L of 0.1 $\frac{ng}{\mu$ L} 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) • 2.5 μ L forward primer

(25 pmol) • 2.5 μ L reverse primer

• 25 μ L 2x Q5 Master Mix

• 15 μ L dH₂O

50 μ L total

• Settings: melt at 99°C, anneal at 65°C, rest at 72°C, 35 cycles (7 sec, 30 sec, 10 sec), hold at 4°C until removed from Thermocycler

- I've ~~been~~ 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.
- Arn tried amplifying the 5 gBlocks (1 and 2a-2d) ~~using~~ using old amplification primers. Everything but Part 1 succeeded.
- Thus, there might be a problem with part 1 itself.

Possible Solutions:

- Re-run 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 B RTB.
- 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) Arn's golden gate SCRIBE assembly method. To think that gBlock cost \$700 in ~~disgusting~~ money...

7/29 GFP PCR

$$500 \text{ pg} \cdot \frac{1 \text{ ng}}{1000 \text{ pg}} \cdot \frac{1 \text{ NL}}{.563 \text{ ng}} \approx 1 \text{ NL}$$

Added to PCR tube

2.5 NL for. primer 1 μ L GFP
 2.5 NL rev. primer 25 NL QS mix
 19 NL H₂O

Pot in thermocycler w/ "scribe" program

Inoculation of MIT strains (2nd time)MD
OB3 strains we inoculated in ^{3 tubes of} [5 mL of SOB + 5 μ L of spectra]Tube #1 \rightarrow F144Tube #2 \rightarrow F774Tube #3 \rightarrow F798 (our strain of interest)

Placed in 37 shaker at 11:55

1:45 measured OD₆₀₀ against SOB blank

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

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

7/29

Redo PCR II \rightarrow Part 1

① Setting up the following reactions:

a) Redo of yesterday

b) AMBA 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 Vials: Size of SCRIBE Components:

Part 1: 1972 bp ^{19.4} ~~19.4~~ ng/ μ L BB: 2070 bp 2.4 ng/ μ L2a: 280bp, 111.4 ng/ μ L2b: 354bp 133.3 ng/ μ L2c: 280bp 84.3 ng/ μ L2d: 206bp 102.1 ng/ μ L

pSB1C3 Digestion:

⑤ PCR Purified part 1 & pSB1C3 Digestion, eluted w/ 15 μ L H₂O \rightarrow 3 ng/ μ L & 0 ng/ μ L, what the fuck? Digesting more pSB1C3 + PCRing more part 1

⑥ Run a gel on pSB1C3, GFP, and Part 1 pSB1C3 & Part 1 were successful!

⑦ Redo the PCR b/c low yield/stuffy purification. Three variants:

1) Same as last time

2) Buffer G variant

25 μ L Buffer G

12

20 μ L H₂O1.25 μ L Primer (EcoR)10 μ L template0.5 μ L QS Polymerase

3) Same old touchdown attempt!

⑧ Requantified initial PCR

⑨ I give up.

Digestions of pSB1C3

instead of 500 b/c we don't have enough

1) Combine 100ng, 0.8 mL each restriction enzyme, 3 mL of 10x buffer, X H₂O to go to 30 mL total volume

2) Mix gently by pipetting
0.8 mL EcoRI
0.8 mL PstI

3) Incubate for 2 hrs (~37°C)

4) Heat ~~kill~~ 20 min (70°C)

(using a previous protocol)


★ Due to time constraints, 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 OD₆₀₀ ☺

	Diluted w/ 250 µL H ₂ O, 500 µL cells	actual (x1.5)
Blank	0.000	
F798	1.585	
F144	1.474	
F774	1.558	

7/30 ① Quantified PCR Prod 2 attempts of pSB1C3 → 

- 13.3 ng/µL 1.96 (Mesocel ~~from~~ up purification yesterday) → 2.4 ng/µL 3.43
- 48.3 ng/µL 1.89 (T.D. attempt)
- 19.4 ng/µL 1.62 (First one Redone) → Best one on the gel!

Digestion of 113504 (39.8 ng/µL)

DNA 500/39.8 = 12.6 µL 2 hr digest
Smartcut 3 µL 20 minutes Heat kill
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

- 500 µL 50% glycerol, 500 µL of strain
- placed in -80°C

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

② pSBIC3 Come up at a low concentration - plated in spreads to get a higher concentration

③ Digesting more pSBIC3 w/ PstI & SpeI

④ PCR Products were run on a gel again for my own sanity, the redo & transcription

- The ratio of the first one is the best looking one - 19.4 ng/ul

⑤ Since we barely have any vector left:

a) Digest a pair & run a gel purification (use PstI & SpeI)

b) Transform some pSBIC3 & purify on Monday

⑥ Run a PCR on the gBlock again b/c I am a twerk

⑦ Combined remaining pSBIC3 in a tube: 10.1 ng/ul, 1.93

⑧ pSBIC3 Transformation me 30

- Using 2.5 ul of pSBIC3 (~100ng)

- Thaw cells on ice for 5 min (1.5g BM 8.9g) + 0.25 I to moisten

- Pipet 2.5 ul of cells (30ng) (b/c not to exceed 5% of volume)

- Inoculate on ice for 5 min

- Heat shock at 42°C for 30 sec

- Ice 2 min

- 500ul LB 8.51 = 2.5 - 8 - 5 x 8.5 - 0.5

- 1hr Recovery + Plate

⑨ PCR Done Again

- Same as before. Thermocycler 4

⑩ Added 4ul to vacuum spin pSBIC3 & quantified 2ul: that concentration

⑪ Digested lac promoter w/ EcoRI: PstI ofn (100ng) (1ul of enzyme each) to be gel purified!

TO DO: ① Heat kill the digester

② Gel purify out pSBIC3

③ Gel confirm PCR digester in thermocycler 4

④ Order pSBIC3 primers

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

(seconds, °C) cycles times	Sample	Temperature, °C	Gel Run: (20150730 JB, BB RTB, 6 to 11)
30 at 48	6	58	1ul ladder 6ul 7 6ul 6 12ul 8 12ul 9 12ul 10 12ul 11 12ul 12
30 at 48 30 at 72	7	54.3	
60 at 72	8	61.2	
60 at 72 hold at 40C	9	63.3	
	10	65	
	11	65.8	

Prep:
30mL 2x0.5M H₂O
1.5mL H₂O
6uL 0.1M gBlock I
3uL forward primer
3uL reverse primer

Results:
• virtually no 2,000 base NTA detected in any sample

Next steps:
• try it again (maybe I used the same method twice?)
• try new dds PCR times, buffers, etc.
• switch to a new method (try PCR-ing the retron and beta from the pIF745 separately)

3:47 - stuck the F790, F144, and F774 1/1000 dilutions in the 37°

Poured CM plates in the hood. 250 uL CM Stock (34) into 500 mL LB agar. Labeled on d stored in 40°C.

CB

F13504 gel

1% agarose gel, 15 well

Lane	1	2	3
	2 log ladder	F13504	2 log ladder

1uL F13504 1uL dye

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

So many for 2 logs b/c practice

gel → 20 minutes

7/31 Autoclaved 2 flasks and 1 box medium tins 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 I II III ladder

Ran for 20 min. at ~~10V~~ 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° at 10:27.
waterbath

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

→ Made 2x 1% gels with 15 wells

(12pmish) → Heat killed "Lac digestion" for 20 min.

→ 1 uL of rSAP (shrimp phosphatase), then put in 37°C waterbath for 1 hour, then another 5 min heat kill

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

Preced. I13504 Gel

Lane 1 2 3 4 5 6 7 8 9
2 log Ladder ↓ I13504 ↓ I13504 ↓ I13504
* not much was left for this well

- 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

109190

1000 fmo1 = 1 pmol
 0.03 pmol = 30 pfmo1
 0.2 pmol = 200 fmo1



To Do (Am)

① PCR pSB1C3

② a) Don't forget! ~~at 94°C~~

b) Gel to confirm size

c) PCR Purify

d) Quantity

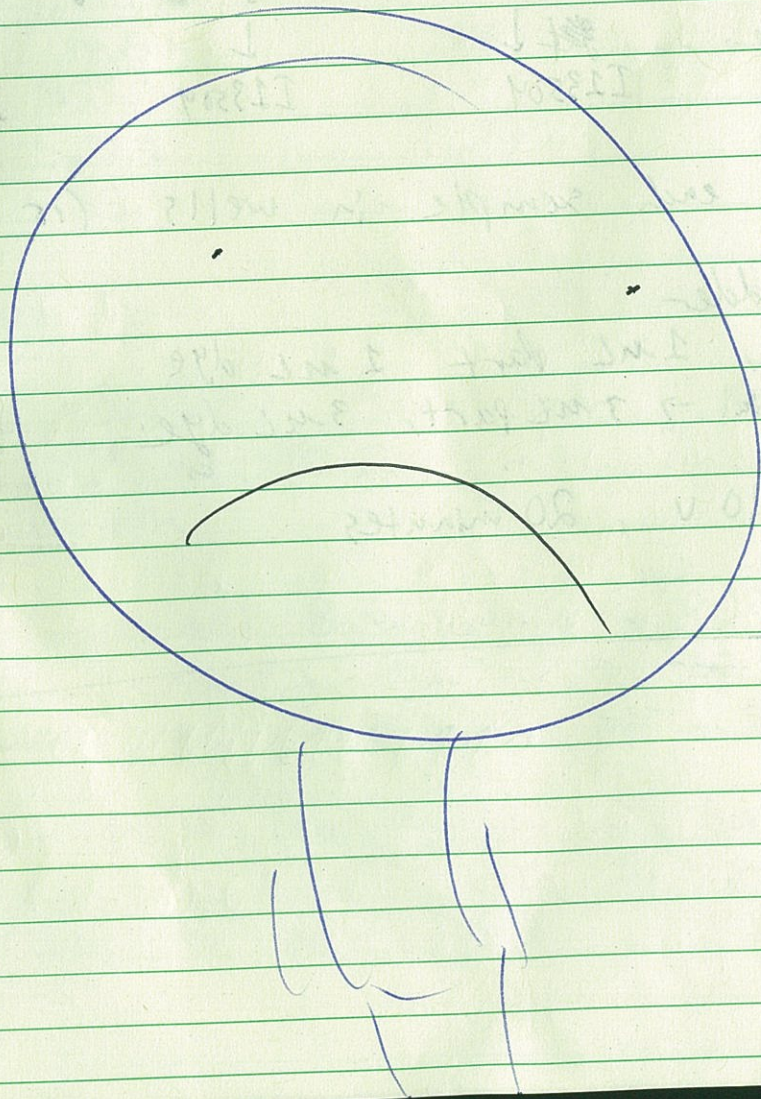
② If PCR fails: Gel purify the low primer digestion

③ Find old PCRs or just run new ones → make sure you keep track!

- Done!

④ Amplify/Clone

→ Don't forget! Change BB x 5 μL



James
Blondin

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

Sample	2x MasterMix	Primer	my back (tubes)	Template	H ₂ O	Temp, °C
12	5 μL	0.5 μL of each (tubes)	0.1 μL	0.1 μL (mtemplate) 98 block 1	3 μL	58.0
13	5 μL	"	"	"	"	59.3
14	5 μL	"	"	"	"	61.2
15	5 μL	"	"	"	"	63.3
16	5 μL	"	"	"	"	65.0
17	5 μL	"	"	"	"	65.8
18	5 μL	"	"	2 μL (Arus) template 98 block 1	3 μL	58.0
19	5 μL	"	"	"	"	59.3
20	5 μL	"	"	"	"	61.2
21	5 μL	"	"	"	"	63.3
22	5 μL	"	"	"	"	65.0
23	5 μL	"	"	"	"	65.8

(all PCR samples are 10 μL reactions)

cycle:

Results:

- I tested 12-17 on a pre-made gel from the 40C, and I saw only primer dimers (no 3000 base DNA).
- I tested 18-23 on a gel I made myself (52 mL TAE buffer, 0.52g agarose, 5.2 mL midori green), and still only got primer dimers.

Going Forward:

- Clearly, something is wrong with my primers, or g Block 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 recombinaase intermediate (RBS-ORF-Terminator) using golden gate assembly.

7/31/15

① Setting up pSBIC3 & PCR (x2)

- 25 μ L mastermix
- $\frac{424}{2}$ of DNA (1 μ L) (pSBIC3 from Lac)
- $(212 \frac{ng}{\mu L})(1 \mu L) = X \cdot 100 \mu L$

$\frac{424}{2} = 212 \frac{ng}{\mu L} \rightarrow \sim 1 \mu L$ of dilution

- 1.25 μ L pSBIC3-for
- 1.25 μ L PSB-100
- 11.5 μ L H₂O

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

② B/c of the temperatures of the primers, a Buffer G was made:

- 25 μ L Buffer G
- 424 μ g (1 μ L) DNA Template (Lac, P0040) 0.5 μ L GS Polymerase
- 1.25 μ L For/Rev primers
- 20.5 μ L H₂O

Done at 68°C & 72°C in Thermocycler 2.

③ Digest both samples out of the Thermocycler w/ PstI for 30 minutes

- All of DNA
- 1 μ L enzyme (PstI for longer overhangs)
- 3 μ L 1000 Cotanet
- $x \mu$ L to 50 μ L

Heat killed for 2 min! in GG Box

④ Diagnostic gel on the 4 pSBIC3 PCRs + the 3 part 1 pers (G) (G) Ladder

- Volume is now ~ 45 μ L \rightarrow PSB(1) & Both PSB(G)'s worked!

⑤ Purify ~~the~~ ~~PCR~~ ~~products~~ ~~and~~ ~~quantify~~

⑥ Running another PCR on Part 1 again; 3 are at 72°C, one uses Buffer G at 67.2°C.

Q: But the gBlocks don't have that!
 -> Go get the correct gBlock sequence

Put samples in the

To Do Monday

- ① PCR Purify the 3 pSBIC3 things that worked
- ② Quantify the clones
- ③ Quick diagnostic gel on PCR-1s ~~from~~ which are "Gibson Ready" (in GG-Box) for my current mental sanity (use 3 μ L of DNA, 0.5 μ L loading dye)
- ④ On the same gel, run a diagnostic gel on the stuff in PCR1 \rightarrow
 - if it shows a ~2kb fragment, purify that size! Quantify also Use the small columns & elute w/ 15 μ L; if you ~~are~~ ^{arent} 100% sure about how to do this, do it.

8/2/2015

Michelle - Inoculate 1ml of LB + 100 μ g/ml Spectinomycin with the following:

- F762
 - F144
 - F774
 - F798
- Grow @ 37°C overnight in shaker

Caroline - Comp cells from MIT strains

- make 1 M CaCl₂
 Online protocol says 147.02 g of CaCl₂ · 2H₂O in 1L H₂O
 We'll do 1.47 g in 10 mL H₂O
 \rightarrow Sterilized with .22 μ m filter

- Materials needed -
- 200 mL LB
 - 20 mL .1 M CaCl₂
 - 10 mL .1 M CaCl₂ - 15% glycerol
 - \rightarrow - 1 mL 1M CaCl₂
 - 3 mL 50% glycerol
 - 6 mL dH₂O

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
4:40	.032	.72 * .016	.034	.036
		.046		
		PS67 \downarrow		

James
Blondin

08/03/2015

(Ari's suggestion: purify, quantify, run gel on some of it)

PCR Experiments

I'm running one more ~~more~~ set 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).

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

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

For next time: run gel on the ~~most~~ rest of my sample one from today, gel purify it (WEAR ALONG 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 ~~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 electroporation; failed bc we didn't clean up → ARCCB

⑦ 2b → 2d were heat shocked into ~~the~~ UEB 50x Comp Cells (2 μL of assembled product added)

- Note: They incubated w/ DNA 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 50 min gel on assembled products

- Only BB & 2a/b/c/d parts seemed to show. Why did no part 1 show?

↳ Part 1 was the sketchiest one!

8/8
8/4/15

① OD of FF144/774 measured at 12:30 pm

114: 0.097

744: 0.130

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

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

- ~~using~~ Using 8 colonies per plate (So 24 total)

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

✓ b) Combine $\frac{125}{250}$ 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 → $C_1V_1 = C_2V_2$

$C_1V_1 = C_2V_2$

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

$V_1 = 0.25 \mu L \rightarrow (0.25)(24) = 6 \mu L$ each primer $96 \mu L$ H₂O

Each tube will have:

5 μL 2x 0.5 μM

0.5 μL primers

4 μL H₂O

0.5 μL template (cell)

✓ c) Aliquot this into each (24) PCR tube

✓ d) Add 0.5 μL of (c) dilution in each tube

✓ e) Run the following thermocycler program

98°C for 5 minutes to lyse cells

98°C for 10 sec

40x { 72°C for 30 sec

72°C for ~~30~~ 90 sec (~3 kb)

72°C for 2 min

4°C hold

f) Verify PCR by gel → Inoculate correct tubes from (a) in 5 μL LB

✓ g) Store plates w/ parafilm

70 8/14/15

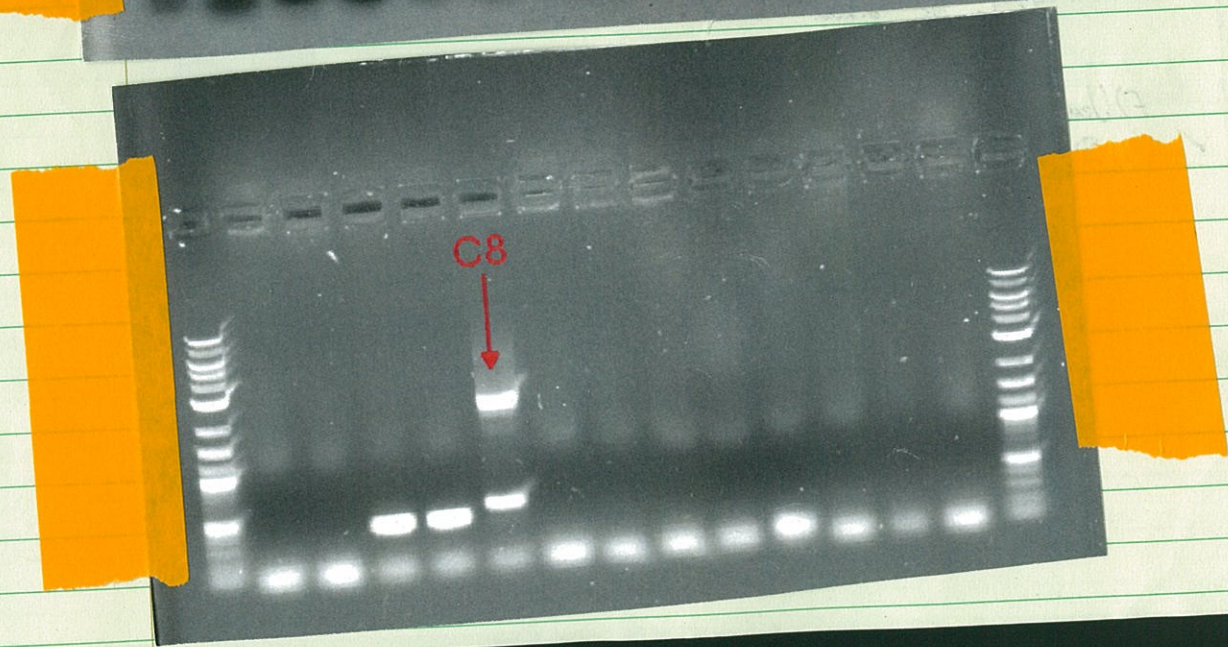
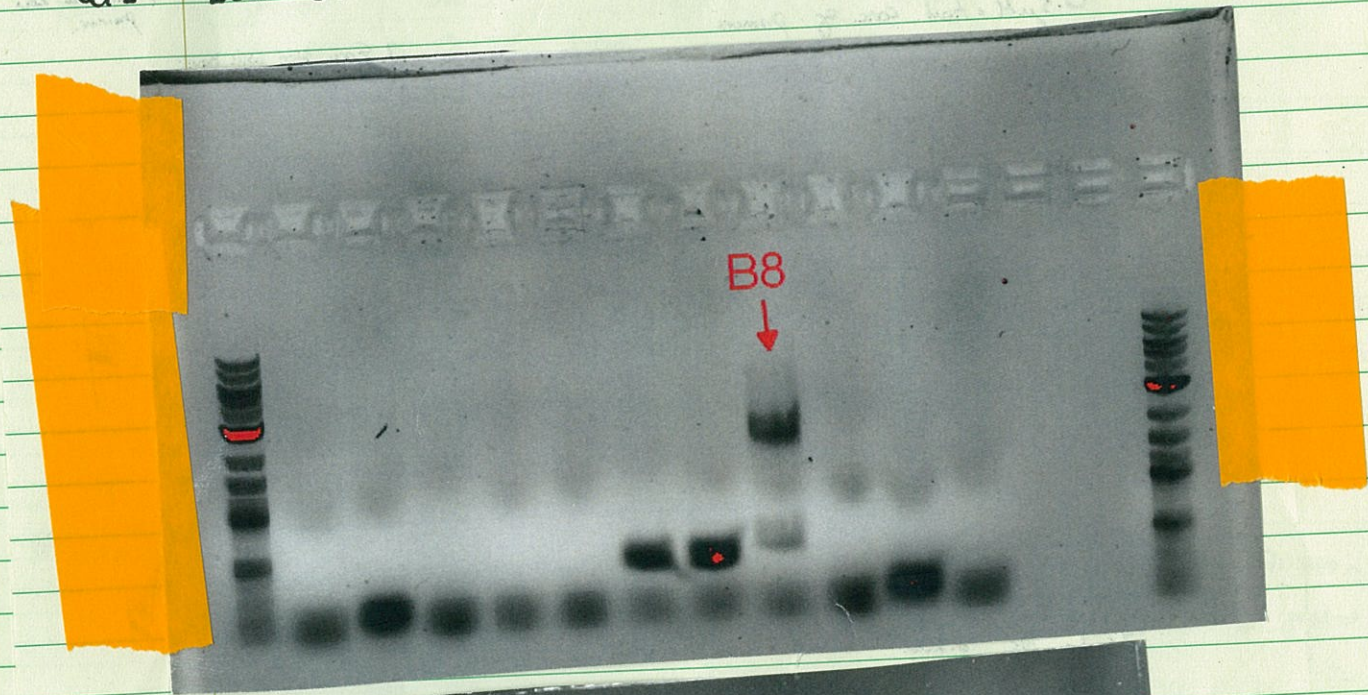
~~8~~

③ 2 μ L of Assembly 2a + RTB + PSB1c3 was transformed, but it was a super status 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	0.248
~2:00pm	~0.27	in 4°C

James
Blondin

08/04/2015

Mass

Gel Purification

tube 1 1.03g (1.03)
tube 2 + gel 1.27g (∴ 0.24g gel)
tube 2 1.01g
tube 2 + gel 1.27g (∴ 0.26g gel)

• I used a 2% agarose gel to separate out my 2kb DNA from the 45µL of sample from yesterday, 45µL PCR mixture, 9µL dye, 3µL 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 3µL per mg gel to the sample (ex: for 0.25g gel, add 750µL 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, reuse the tube.
- 4.) "wash" (add 200µL 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 1µL H₂O and spin (wait 1 minute before spinning).
- 7.) Store the tube now containing purified, eluted DNA.

(I used 1µL H₂O)

Results:

• 15.4 ng/µL ^{DNA}
• 51.33 ratio ^(quite high - undissolved)
_(this might be gel)

• I did two serial dilutions on this DNA (1.54 ng/µL & 0.154 ng/µL)

PCR Setup:	template	primers	Master Mix	H ₂ O	Annealing Temp	cycle:
1 Direct RTB (no template)	5µL 0.154 ng/µL template	2.5µL each	25µL	15µL	64.9	same as yesterday
2 G1 RTB (gel PCR)	3µL 0.154 ng/µL purified PCR	2.5µL each	25µL	15µL	71.9	
3 G2 RTB (gel PCR)	3µL 0.154 ng/µL purified PCR	2.5µL each	25µL	15µL	71.9	

• I used 72°C for the gel PCR, DNA because my forward primer should have 60 bases of homology ^(much longer)

• I may have had undissolved gel contaminating my purified DNA. If this is the case, I can restart with sample 1 from today.

to do tomorrow: test 5µL of each reaction. Do gel purification on any successful 2kb bands, quantify, and possibly over digest.

James
Blondin

8/05/2015 & 8/06/2015

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

James Blondin ~~did a gel test on~~ ^{did a gel test on}

• I did a PCR on both original template (0.154 ng/µL, 5µL) and 3µL of the 15.4 ng/µL gel purified DNA from 8/04/2015 (diluted 100x) and 1µL of the 5.4 ng/µL gel purified DNA from 8/05/2015 (not diluted).
• All reactions except the direct one failed to show any significant amounts of DNA in the 2kb range, and only 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: ~~didn't~~ "shorted"
- Added 10µL H₂O per. n mole digo in the tube.
- 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 ~~two weeks~~ one day left in lab before leaving for 2 weeks. At this point, I see little reason to keep working on this BTA assembly in wet lab directly.

- At this point, I'd rather just start transitioning to dry lab ~~that~~ 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

S

To Do

① ~~Run~~ colony PCR on ~~plates~~ (30 min prep @ 2.5 hour run)

- Positive hits
- Plate D remains } Run a gel (40 min ^{incl.} prep)
- 8 from plate A

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

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

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

④ Re-transform A/B/C/D except do it properly you fuck. (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 + Dephosphorylation + Purification) (+ Gel Confirmation)
- Re-transform ✓
- Purify + quantify ✓
- Colony PCRs should be done by then → Run the gel ✓
- Assemble while gel is running ✓
- Inoculate successful colonies ✓
- Transform assemblies (before 4pm is the goal; before 6pm is the reality)

⑤ pFF745 + puc19 transformed into FF744 + FF194

- Thawed for ~5 min
- Incubated for ~10 min
- Shocked for 30 sec
- Rest is standard.

FF744
 FF194

8/5/15

* Idea: Lab Assistant python script
↳ incl. protocol times too
↳ library < 3 hrs to make
→ Java app + tablet interface

Ex: "Colony PCR"
↳ Tells you to remember to wear a suit

① Set up colony PCRs

- 6 A colonies (A1-A6) (6) } 26 PCR tubes for PCR itself
- B8 - B9 - B15 (8) } 23 tubes for 50 μL reactions
- D5 - D9 - D10 - D11 (4)
- C8 - C9 - C15 (8)

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

② Do Gibson on A & D again (A = 290bp D = 206bp)

75 ng of pSB1K3 = 58.63 fmo 29.56 ng of D

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

↳ Part 2 = 2A (i.e., parts w/o KanR, so the smallest ones) were the ones that failed,

so higher molar ratio is good B)

↳ Jk, A = C in size, any 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

H₂O to add to A: 4.873 μL } 10 μL total
D: 4.948 μL

③ Purified & Quantified ~~CS & B8~~

B8: 90.4 ng/μL
1.81 OD

C8: 85.1 ng/μL
1.89

+ 10 μL Master Mix
* 50°C for 30 minutes

GFP PCR (GFP Prime 1)

Dilutions: (to 20 nm)

GFP Forward: 28 nmol / 20 = 1.4 μL (0.7 x 2)

GFP Reverse: 29.8 nmol / 20 = 1.49 μL (0.745 x 2)

GFP Forward (w/ prefix): 26.7 nmol / 20 = 1.34 μL (0.668 x 2)

GFP Reverse (w/ suffix): 33.1 nmol / 20 = 1.66 μL (0.828 x 2)

for 50 μL rxn

- math
- | | | |
|------|------------------------------|---------|
| Add: | 1) 25 μL Q5 Master mix | 27.5 |
| | 2) 1.25 μL Forward | 9.17 |
| | 3) 1.25 μL Reverse | 36.67 |
| | 4) 500 pg DNA → 9.17 μL | 9.17 μL |
| | 5) 13.33 μL H ₂ O | |

$$500 \text{ pg} \cdot \frac{1 \text{ ng}}{1000 \text{ pg}} \cdot \frac{1 \text{ μL}}{54.5 \text{ ng}} = 0.00917 \text{ μL}$$

$$50 - 36.67 = 13.33$$

Math

→ 10 micro to 90 micro water
→ 10 to 90 → 9
→ 10 micro sample to 90 H₂O
→ 9.17 μL 9.17 μL

Anneal at 70°C
Extension → 20 seconds

Todd's GFP → 54.5 ng/μL

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 μ L GFP and 5 μ L Dye
 Add 2 μ L into each well

Gel Very successful

Put here

GFP Prime 1 Quantification

First try GFP 14.4 ng/ μ L
 ratio 1.76

Second try \rightarrow 20.7 ng/ μ L
 1.75 ratio

\downarrow
 Try this measurement

GFP PCR (GFP Prime 2)

* Adds prefix, suffix, rbs

Run 8 x 50 μ L Rxn

- Add:
- 1) 25 μ L @ 5 Master Mix
 - 2) 1.25 μ L Forward
 - 3) 1.25 μ L Reverse
 - 4) 500 pg DNA \rightarrow 2.4 μ L
 - 5) 20.1 μ L H₂O

Anneal at 72°C

22 sec extension time

$$\text{Math } 500 \text{ pg} \cdot \frac{1 \text{ ng}}{1000 \text{ pg}} \cdot \frac{1 \mu\text{L}}{20.7 \text{ ng}} = 0.02415 \mu\text{L}$$

10 micro sample to
 90 micro water

8/2/15 To Do (+Whatever y'all have planned)

1) Run a gel on the digestions from yesterday on B8 & B8 ³⁹ if we see the band pattern we expect, we're good

2) Run a gel on the colony PCRs

- This will be a bit of well loading, so do be careful. I typically add 2µL of buffer than actually put ~7µL 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 50µL incubation done in the PCR tube (i.e., where you got your template from & inoculate that in 5mL LB+CM (2.5µL of 39 mg/mL CM stock), incubate in the 37°C, ~~ASK MICHELLE OR SOMEONE ELSE TO TAKE IT OUT ON SATURDAY~~

4) Count the # of plates we have on reserve of each type & 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 (100% Don't save what happens ones were placed, listing them all)

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

- L1166000, 121.2 mg/mL, ~50µL left, drives gene expression under acute & chronic hypoxia, pSB1C3

- K117002, mg/mL, ~50µL left, AI-2 inducible quorum sensing promoter, pSB1A2

- I765000, 46.8 mg/mL, ~50µL left, Fe inducible promoter, ILLEGAL PSI SITE!, pSB1A2

- K540001, mg/mL, ~50µL left, Cobalt sensitive promoter, pSB1A2

- R0040, mg/mL, ~50µL left, Tet promoter, pSB1C3

- K376003, 51.2 mg/mL, <10µL left, JG O₂ sensitive promoter, pSB1C3

- K587003, 74.4 mg/mL, <10µL left, FdhF hypoxia promoter, pSB1C3

- J33201, 85.9 mg/mL, ~50µL left, arsenic promoter/repressor (Sodium arsenite), pSB1C3

6) Transform SoxR from distnb.

- P0010, 215.5 mg/mL, ~50µL left, Lac promoter, pSB1C3

7) K55400, 96.1 mg/mL, ~50µL 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, 138.9 mg/mL, ~50µL, Lux promoter

- K1071004, 61.5 mg/mL, empty ???, nitrate sensor (Nitrate has to be the only N₂ source → ammonia will do this if no other N₂ is present)

- K561001, 91.6 mg/mL, ~30µL, vgb microaerobic promoter

6)

7) Run a PCR on B8 using ~~additive~~ no promoter primers to generate 2c+RBS 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

- Pick colonies from plates by tips and put the tip into 25µl LB.
- Mix
 - 13 x 5 = 65µl QJ buffer
 - 13 x 0.25µl FW Primer (Part 2)
 - 13 x 0.25µl RW Primer (Part 1)
 - 13 x 3.5 = 45.5µl dH2O
- Add 9µl of the mixture to each PCR tube.
- Add 1µl template LB into each tube (D1-D8, A1-A3, NewD)
- PCR

98°C	1min
98°C	10sec
72°C	30sec
72°C	90sec
72°C	2min
4°C	

8/10/15

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

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

Setting up PCR Reactions:

- | | | |
|--------------------|---------------------|-------------------------|
| ① 25µl Q5 X | ② 25µl Buffer G | } Wd run at 72°C & 68°C |
| 2µl B8 | 2µl B8 | |
| 1.25µl Primers 1+2 | 1.25µl Primers | |
| 20.5µl H2O | 0.5µl Q5 Polymerase | |
| | 20µl H2O | |

→ Put in thermocycler at 2:15

Digestion of B8 & C8 showed nothing → redoing the digestion (MDV)

- minutes
- 1.8µl Buffer 2.1
 - 0.25µl EcoRI
 - 0.25µl PstI
 - 0.25µl PciI
 - 125ng DNA (1.4µl B8)
 - 6.6µl H2O
 - 10µl Total Volume
- Also running a positive & negative control using PFF745 & no plasmid. we added more than 10µl
- Use 7.032µl water for PFF745
 Use no DNA & 7.75µl H2O instead

Ran "Orig" 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 & (Old-D)
 - Just kidding. Using whatever colonies I can to do it

④

④ Picked 3 biological replicates from FF795/144 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 first vial
 Picked another B8 to be safe (B18)
 Required C8 just in case (C18)

New Colonies Picked: C16, C17, C18, C19
 New-A 1, 2, 3
 New-D 1
 Old D 9-13
 New-A2 1-5

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

20.5 = 100µL of GS2x } 9.5µL in each tube
 4µL of primer 1/2 }
 80µL H₂O }

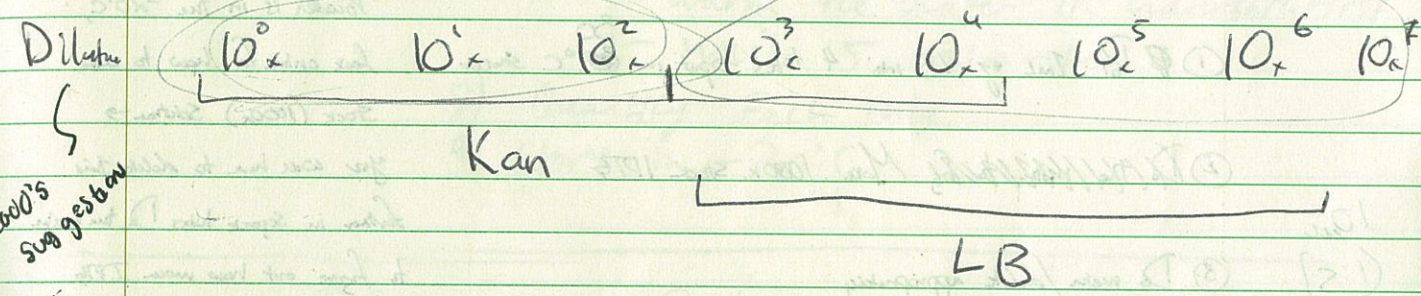
LB Tubes: C16, C17, C18, Old D 9-13, New D1, New A 1-3, New A2 1-5, B18, B8

④ Extension time = 1:00 not 90sec! Also 20sec at 98°C at start of cycle not 10sec!

⑤ Running a gel on B→C 1, 2, 3 (GS2x, 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 3 biological replicates
- 5 different concentrations → 15 plates
- Need to dilute 7x → 85 plates (KM plates)
- Then 4 dilutions on Spec/LB plates → 60 plates (Spec. or LB, TBD)
- ⇒ 145 plates needed. Use 96 well plate for growing up.



LB = $\frac{2}{5}$ plates × $\frac{2}{1}$ replicates × $\frac{4}{2}$ concentrations + 2 stars

Kan = $\frac{2}{5}$ plates × $\frac{2}{1}$ replicates × $\frac{4}{2}$ concentrations × 2 stars

Digestion

0.75µL buffer
 0.75µL enzyme 1
 0.75µL enzyme 2
 5µL DNA

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

	Tues	Wed	Thurs	Fri
Prelmy	Prelmy	Prelmy		
Start		plate	Count	
Full	inoculate	Start	Plate	Count

8/11/15

Setting Up Lac Testing

Summary

① Prepare 4 tubes using:

- 1 replicate (i.e., one of the colonies from the plate)
- 2 concentrations of IPTG
- Both plasmids

4 = 1x2x2

2.583g 258.3 g/mol

$$2.583 = (258.3) \cdot 10^{-2}$$

1M 1000x
↓
mol/liter
→ 1000x
10⁻² mol/L
→ 10⁻² mol/L
→ 1L → 100mL

100 mM = 24 mg/ml

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

② Add IPTG to make 1000x stock IPTG

10x

(1:5)

③ Do math/delete appropriately

④ Take a (1:1000) dilution of seed culture & inoculate that in LB

8/13/15 We forgot to add CH₂

⑤ Add inducer (WRITE TIME DOWN)

5.28 pm

⑥ → 30°C shaker (4 tubes in back)

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 prepared LB

TUBES (4 total)	
B8711	with 1mM
1111	" 1mM
PP745	with 1mM
1111	" 1mM

$$1mM \frac{238.30 \text{ grams}}{1 \text{ mole}} \times \frac{1000 \text{ moles}}{1m \text{ Mole}} = 238.30 \text{ grams}$$

$$1mM \times \frac{238.3 \text{ g}}{1 \text{ mole}} \times \frac{1000 \text{ moles}}{1m \text{ mole}} = 238.30 \text{ grams}$$

Maths for 1000x stock of IPTG

$$1000 \text{ Molar} = \frac{238.3 \text{ g}}{1 \text{ mole}} \times \frac{1 \text{ mole}}{1000 \text{ Molar}} \times 1000 = 2.383 \text{ g of IPTG} / 10 \text{ mL water}$$

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 mL H₂O
- 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/mL	Ratio 1.82
2:	113.3 ng/mL	1.89
3:	119.6 ng/mL	1.92
4:	106.7 ng/mL	1.87
5:	96.2 ng/mL	1.89
6:	115.6 ng/mL	1.88
7:	116.3 ng/mL	1.90
8:	104.5 ng/mL	1.83

Eluted w/ 30 mL, took 2 for quant,

Unused tubes have approx 28 mL

[Faint handwritten notes on page 90, including "PCR protocol for PCR..." and "Change: 1) Blank of 30 ml H₂O..."]

(side notes) 238mg = .238g
 2380 mg = 2.38g → 1000mM (1000x, b/c 1mM is the standard)
 ↓
 for 1mL 999.44L = 1mM
 ERG0
 ↓ Dilute a 1000x solution by 1000 to get 1x

- 8/11/15
- Digestions/Ligations to set up:
 - a) Digest the C's from yesterday w/ EcoRI & PstI } Ligate! later
 - b) Digest some BB too (pSBK3) then dephosphorylate

② Run a gel on Colony PCR

- ③ Digestions
- a) GFP (for cu purposes): [XbaI & PstI]
 - b) GFP for 3A (EcoRI & PstI)
 - c) Kanamycin BB (EcoRI & PstI)
 - 4) Part c ("EcoRI & PstI")
 - 5) pSBK3 (EcoRI & PstI)
- Ligate together! → use

GFP Protocol for plate reader ~~use~~ use IEB Refer 21

[Circled note:] look at next page

Plate Reader Machine:
 Black plate w/ clear bottom

wash | Spin down cells X2?
 PB card supernatant
 Resuspend in water or PB
 ~ 200µl ~ 500µl

Dilute to 0.001 (Just not 3!)
 Dilutions to not "overload"
 put reader → GFP program J6 = OD600 GFP
 Select part 3
 Make sure EGFP alternative
 Change excitation range

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

OD/GFP Protocol

Pierce Hadley

3-11-2015

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 ^{tube} GFP Full Part #1 _{digest} ^{pg 91}

for 3b, use all of GFP Full Part tube #2

multiple digestion

① Digestion Protocol for 3a

1. Add ~~500~~ 500 μ g of DNA
2. Add 5 μ l of NEB Buffer 2.0
3. Add 1 μ l of BSA (b/c buffer 2.1 doesn't have BSA ^{the cutsmart does})
4. Add 1 μ l of Xba I
5. Add 1 μ l of Pst I
6. Add H₂O so final volume is 40 μ l

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

1. " "
2. " "
3. " "
4. Add 1 μ l of EcoRI
5. Add 1 μ l of Pst I
6. " "

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

8/11/15

③ Pooled B→C constructions:

- (1): 70.7 ng/μL
 - (2): 28.6 ng/μL
 - (3): 28.1 ng/μL
- } Eluted w/ 50 μL H₂O!

④ Digesting both B→C (1) & pSBIC3 w/ EcoRI + PstI in NEBuffer 2.1 + BSA
Using the protocol on pg 93! (Round here for simplicity)

~~⑤ Running a gel on Colony PCR → tubes w/ the same volume as ④~~

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

For GFP:

① Add 50 μL DNA:

- 4.6 μL of GFP(1), 4.4 μL of GFP(2)
- 4 μL of NEBuffer 2.1

Make a 5x master mix

- 1 μL BSA (for 1 tube) (for 5 tubes)
- 1 μL XbaI/PstI or EcoRI/PstI

For each GFP(1) & (2) → 27.34 μL H₂O for GFP(1), 27.59 μL H₂O for GFP(2)

→ Aliquot into 10 tubes for digestion (20 μL of 2-1)

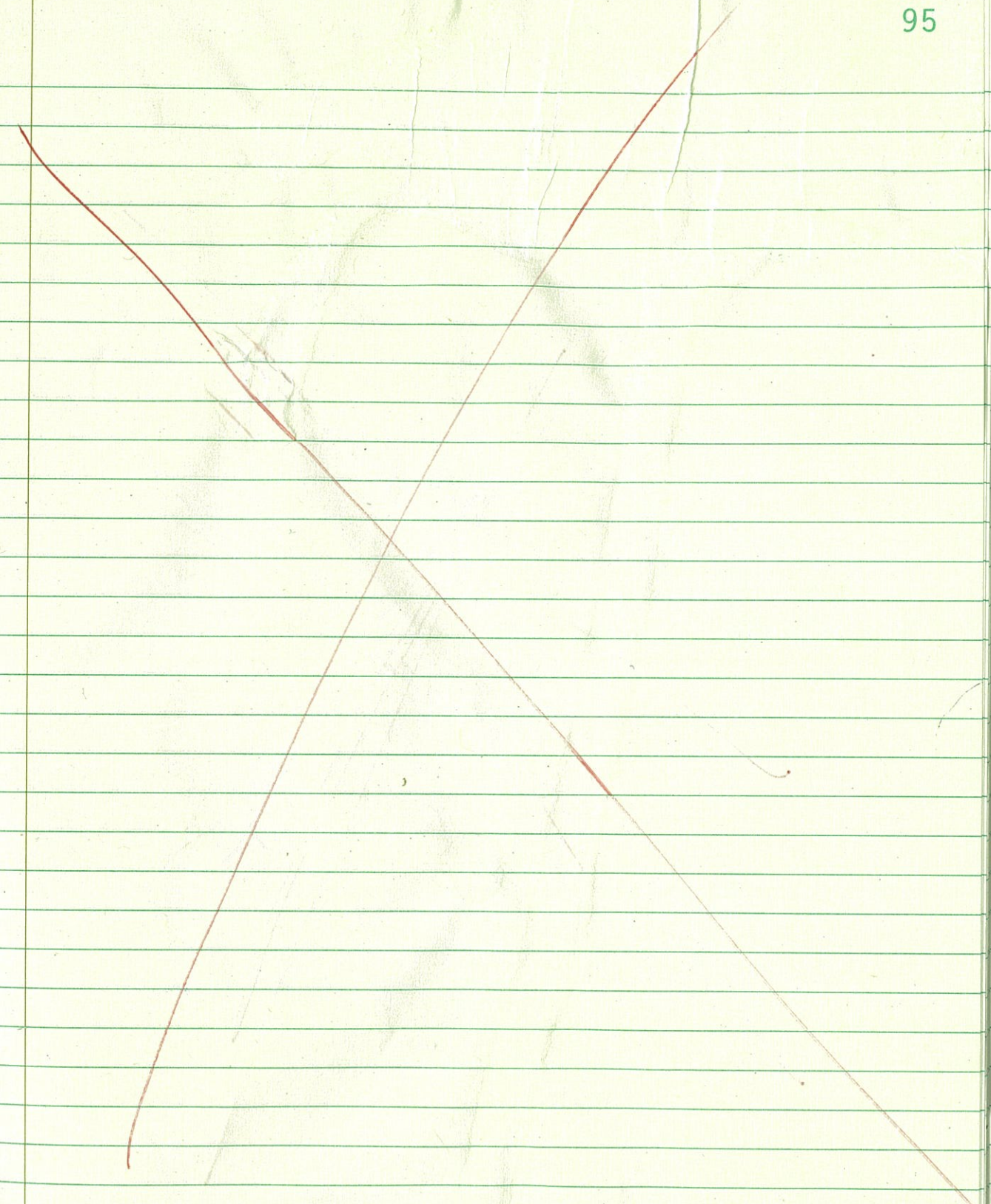
pSBIC3
For B→C (1) (70.7 ng/μL)

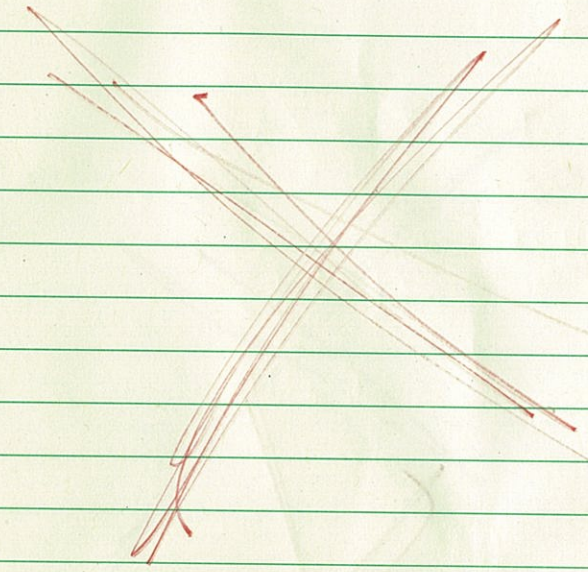
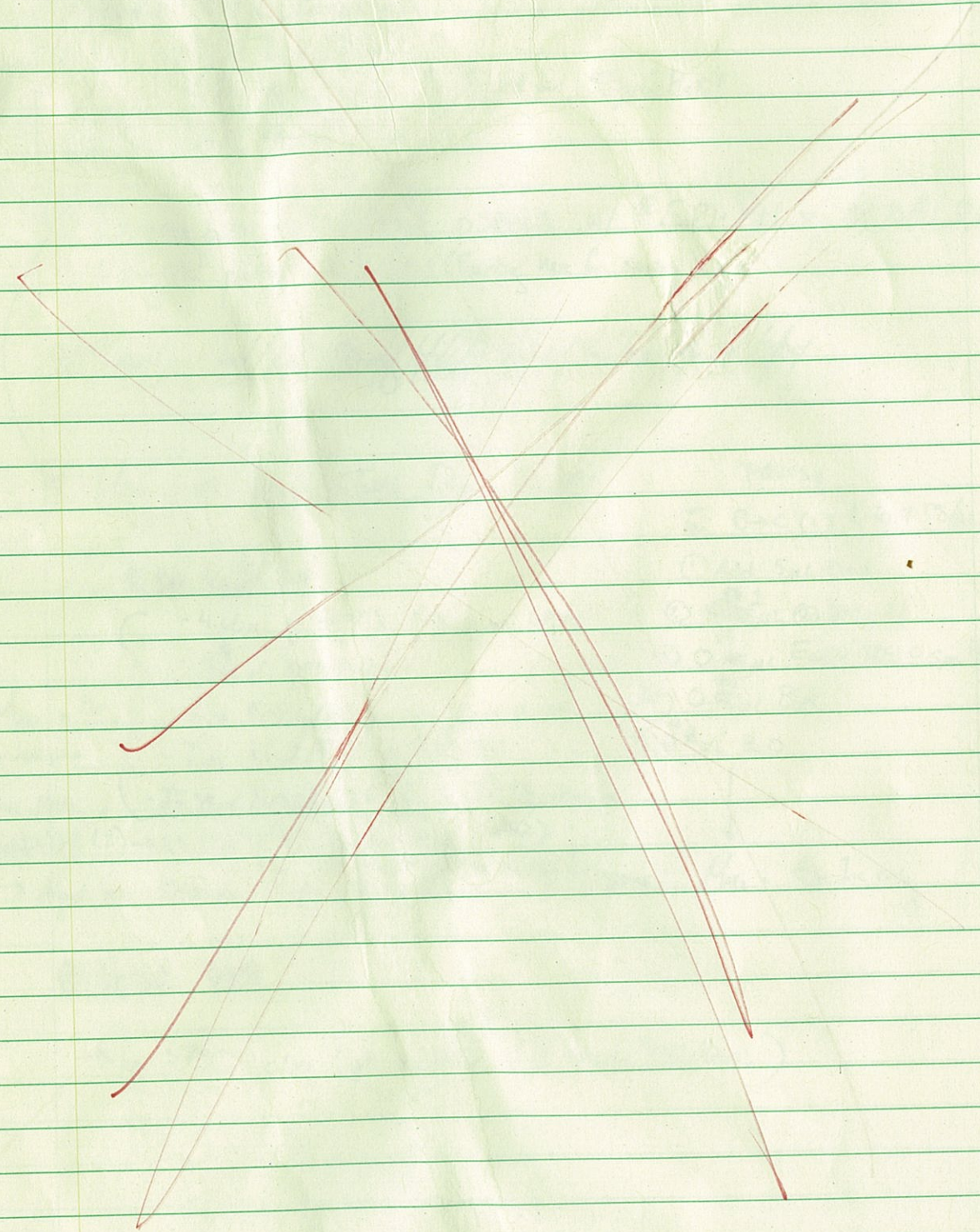
- ① Add 5 μL DNA
- ② 1 μL Buffer 2.1
- ③ 0.25 μL EcoRI / 0.5 μL PstI
- ④ 0.25 μL BSA
- ⑤ 4 μL H₂O

↓
Make a 1x only

~~⑤~~

→ In 37°C o/n (use release Not enough PCR tubes!)





8.12.15

Ligations + Controls

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

Summer

10 ul runs following NEB T4 ligase protocol:

- 10x T4 DNA ligase - 1 ul
- Vector DNA (2ul) - 4 ul 50ng (from the digestion yesterday of PSB1C3 @ 94, 500ng/40ul)
- * insert DNA - 53.5 ng
- Nuclease free water (veto base)
- DNA ligase (heat) 0.5 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 = 0.611 ul + water (2.5 ul)
- 2) #2 = GFP @ 100ng/ul, use 0.5 ul of GFP
- 3) #3 - as per mentored

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

Miranda

11:50 am ⇒ Prepared 2 1% gels with 15 combs

Gel #1 (3 ul in each well) (15 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				

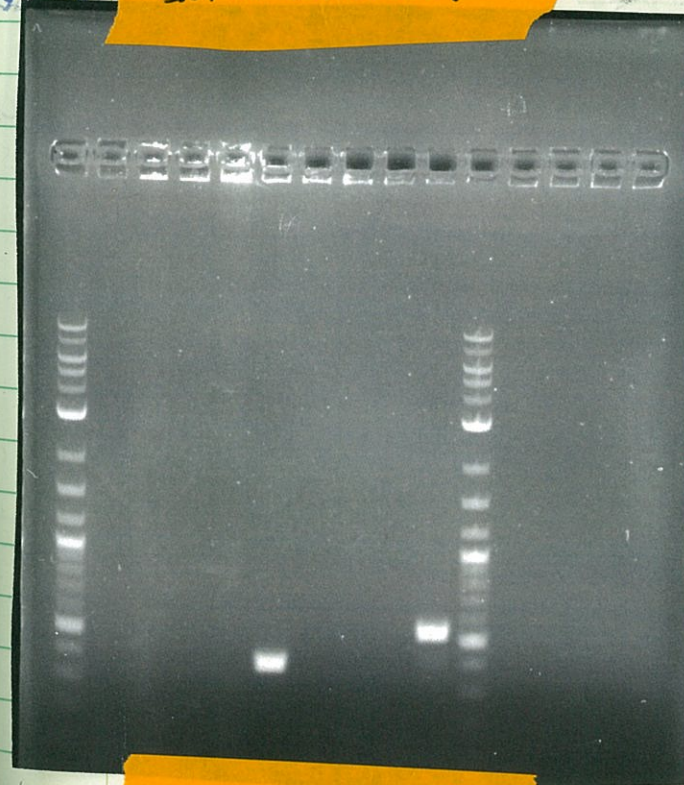
Gel #2

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 un-funny. Ask metmol for details

Results:

Gel #1 (30 min.)



Gel #2 (30 min.)



8/12/15 Heat Kill Digestion / Plate Prep

Joshua ① Heat kill digestions (4) from 8/11 (P.94) in 80°C for 20 minutes.
10:45 AM 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 ~25 LB + 25 Kanamycin Plates
Miranda ① Took 2 flasks of 250 mL LB agar from autoclave stored in 80°C.
Andrew ② Prepared 222 plain LB plates.
③ Added 100 µL of Kanamycin from antibiotics box to 250 mL of LB agar.
④ Prepared 233 KM plates.
⑤ Stored in 4°C ⑥ Stored those tubes in ice box in -20°C

NO TOOK induced seed culture (pg 88) out at 5:29pm (10⁰ - 10⁴ on KM + 10³ - 10⁷ on LB)

Dilutions	Procedure	Plate Type
10 ⁰	50 µL of each of tubes in a KM plate	KM Plate
10 ¹	10 µL of stock + 90 µL LB →	KM plate
10 ²	10 µL of 10 ¹ + 90 µL LB →	KM plate
10 ³	10 µL of 10 ² + 90 µL 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 @ .1 mM IPTG
 - ② = PP745 (1) FF144 @ .1 mM IPTG
 - ③ = PP745 (1) FF144 @ 1 mM IPTG
 - ④ = B8 (1) FF144 @ 1 mM IPTG
- * (second number) is reference to ← this

~~NON DILUTIONS~~

0.1
0.2
0.3
0.4

* Read this as follows
0.1 = non dilute .1 mM IPTG with B8
0.2 = non dilute (10⁰) of .1 mM with PP745 etc.

* first number = dilution number
so 10⁰ = 0, 10¹ = 1, 10² = 2

* Instructions

IPTG: 1 mM = 238 µg/ml

KM Plates go ...	LB Plates go ...
0.1 - 0.4	3.1 - 3.4
1.1 - 1.4	4.1 - 4.4
2.1 - 2.4	5.1 - 5.4
3.1 - 3.4	6.1 - 6.4
4.1 - 4.4	7.1 - 7.4

(20 plates of KM) (20 plates of LB) + 1 potential contaminated 7:3

Note 7:3 was done + will. The one with X might be contaminated

Put plates in 37°C @ 7:33. Note that if no induction in any of the tubes at 8:15 look for induction at 8:30. Do not look for induction at 8:45. Do not look for induction at 9:00. Do not look for induction at 9:15. Do not look for induction at 9:30.

PIIB was successful!

⑥ PI PCR failed again: Redoing w/ Buffer G
 a) Making new working stock: 1 μL of gBlock (10ng/μL) in 9 μL H₂O

- Therm 1 → - 50 μL Run w/ Buffer G at 72°C w/ 500 μg template
 Therm 2 [- 50 μL Run w/ Buffer G at 68°C
 - 50 μL Run w/ ~~reduced~~ BB as template at 70°C (1 μL template)
 Therm 3 [- 50 μL run w/ previously okay PI
 - 50 μL Run w/ "Gibson Ready PI" 70°C!
- ↑ All w/ Buffer G!

⑦ Digestion of PSB103 for Gibson Assembly
 Mix BBI and BB2

- 3:40
 - 56 μL DNA
 - 2 μL XbaI 2.5 μL PstI
 - 7 μL Buffer 2.1

37°C incubation.

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

3:40 Added rSAP (1 μL) 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 H₂O

⑧ Running a gel on the (6) PCR's

Ladder - 72°C Buffer G - 68°C G - 70°C PSB - 70°C Gibson - 70°C PI SS3 ns/μL tube

⑨ Quantify BB digest

concentration: 15.1 ng/μL ratio: 1:82
 Store the tube in box after the week of 8/17/15.

⑩ From (6), 1, 2, 3 were successful

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

⑪ Gel Purification on 1:

- 450 μL ADB added to gel
- Incubated for 10 min at 50°C (mixed sporadically)
- Spin 30 sec
- 200 μL wash buffer → Stand for 2 min prior to centrifugation
- Spin 30 sec
- 200 μL wash buffer → Stand for 2 min prior to centrifugation
- Spin 1 min ~~(@ 5000)~~ → Spin 1 min again w/ the cap open to dry the column!
- Eluted w/ 10 μL H₂O (prewarmed to 70°C) (Using "water heater" in thermocycler)

⇒ 10 μL of Part 1 at 6.2 ng/μL

⑫ PCR Purification 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 DNA₀ ⇒ 3.31 μL = 39.09 fmoles

We want 78.18 fmoles of Part 1 & 117.27 fmoles of Part 2

→ 78.18 fmoles part 1 = 95.27 ng = 15.37 μL
 → 117.27 fmoles part 2B = 25.66 ng = 0.775 μL

Very problematic w/ a 1:1 ratio we need 47.64 ng = 7.68 μL

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

⇒ 1.655 μL PSB1C3 (19.54 fmoles)
 3.84 μL Part 1 (19.54 fmoles)
 0.3875 μL Part 2B (58.64 fmoles)

5 μL Assembly Master Mix
 10.88 μL (~9% more than is allowed)

0.097 fmoles total of fragments

Incubating at 50°C for 60 minutes.

⑭ Transformed 2 μL to 100 μL of NEB Sex Cells

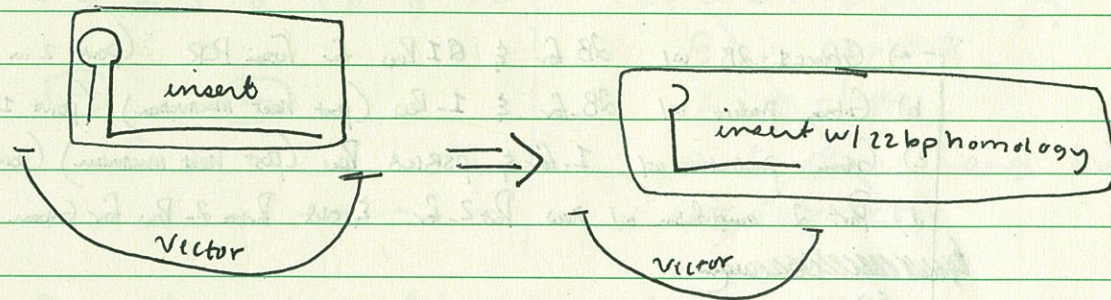
⑮ Plated 30 μL, spun rest down (10 min 3000 rpm), resuspended in 100 μL H₂O, streaked on plate.
 ↓
 100 μL was way too much...

8/18 PCR of Hairpin Structure Revision

8-18-15

MD

① Overview of Purpose of PCR



→ Added:

- 25 μL of G Buffer
- 1.25 μL forward primer (22 bp 5' homology for part 2B)
- 1.25 μL reverse primer (part 1 reverse)
- 21 μL H₂O
- .5 μL Q5 polymerase
- 1 μL DNA (2B + part 1 combination)

8

② Re-run PCR on assembled part, get only amplified w/ P1I & P1R primers, not P1rev & P1I 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 safe bc covalently bonded to vector)
 - Fix w/ a 98°C 2 min heat inactivation
- ② GBlock 1+2 should give product all by itself as the primers we are using
- ③ Use P.1 for & PSB1C3 Rev to verify what's inside

④.5 Ran a gel on (2) → Smeary!

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

⑤ Setting up the following PCRs:

- a) Gblock 1 + 2B w/ 2B for & 01 Rev for fusion PCR (point 2 in NEB Udes)
- b) Gibson product w/ 2B-for & 1-Rev (post heat inactivation) (point 1)
- c) Gibson product w/ 1-for & psBIC3 Rev (post heat inactivation) (point 3) (~4kb, w/ 2B)
- d) Part 2 amplification w/ new Part 2-for & old Part 2-Rev for Gibson again

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

So, for fusion PCR:

- 2.5 μL 5 μL of PIB (1.143 fmol) 0.5715 fmol
- 0.697 1.393 μL of PI (1.143 fmol) 0.5715 fmol
- 25 μL Buffer G
- 0.5 μL 05 Polymerase
- 1.25 μL 2B-for-new
- 1.25 μL 1-Rev
- 18.08 μL 15.61 μL H₂O
- 50 μL Reaction

For (b)-(c), we will use ~0.2 μL of Gibson product w/ normal reaction conditions

For (d) we will use 10 μL (500pg) of PIB w/ normal reaction conditions

(b)(c) ✓ ✓
 → 0.2 μL Gibson, 1.25 μL per primer, 0.5 μL Polymerase, 25 μL Buffer G, 21.8 μL H₂O

(d) ✓
 → 10 μL PIB stock, 1.25 μL per primer, 0.5 μL Polymerase, 25 μL Buffer G, 12 μL H₂O

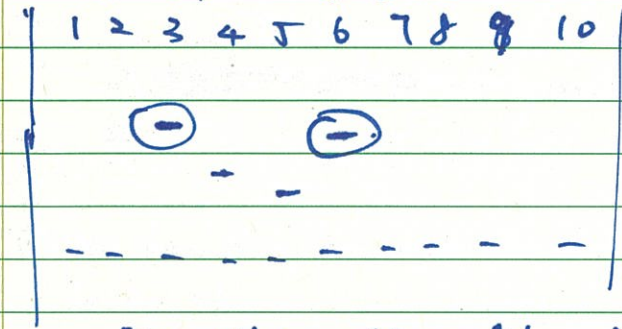
Then 3

Then 2

Then 4

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 dH₂O

Quantify

Concentration: 12.6 ng/μL
 OD: 1.8

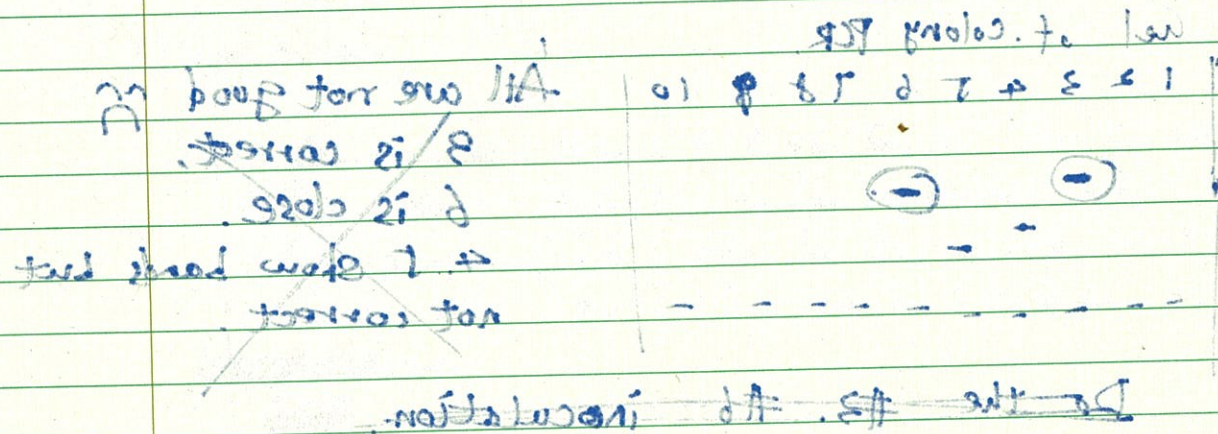
⑧ Ran a gel on the 4 PCR reactions, (d) & (c) showed bands → Not sure what (c) gives us. (d) was purified to the above (7) ratio. (a) showed only primers, (b) showed smearing. Perhaps too much time w/ nuclease? I doubt.

① Setting up Gibson reaction:

- 1.655 μL psBIC3 (19.54 fmol) 4
- 3.84 μL Part 1 (19.54 fmol) 8
- 1.02 μL Part 2B (58.84 fmol) (Nba, w/ no 5' overhang) 8
- 5 μL Assembly Master Mix
- 11.52 μL total volume (~12% over suggested volume)

Run at 50°C for 1 hour

TA



⑩ Setting up the following reaction PCR:

- 40 μ L pIB (4.571 pmol)
- 5.57 μ L PI (4.571 pmol)
- 30 μ L Q5 & Hot Start MM
- 4.43 μ L H₂O

60 μ L total \rightarrow Split into 4 tubes, each will have a stage 1 anneal of 60°C, 62°C, 64°C, 66°C (T_m of 40BP)

Then, second phase, all were a mixture of 1.50 μ L each primer + 1 μ L ^{overlap is 73°C} add it to the mix \rightarrow Then run a normal PCR at 72°C for annealing temp w/ ~2.20 ext. time.

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

98°C 30 seconds	} x 10	x 25	98°C 30 seconds
98°C 10 seconds			98°C 10 seconds
60-66°C 20 seconds			72°C 20 seconds
72°C 60 seconds			72°C 45 seconds
72°C 120 seconds			72°C 120 seconds
PAUSE \rightarrow ADD PRIMERS & MORE Q5			4°C Hold

⑪ Also setting up these PCR:

\rightarrow Move Part 1 PCR w/ Q5 hot start (2 50 μ L reactions)

~~b) \rightarrow ~~2B + 1 part of Q5 hot start~~~~

⑫ Once Gibson Rxn is done, added 3 μ L to 50 μ L comp cells, heat-killed the exonuclease for the rest.

⑬ Performed a transformation; Incubated w/ DNA on ice for 30 minutes, ^{30 sec heat shock} outgrowth step for ^{30 sec heat shock} 30 minutes ~~but I want to go home~~. Plated on CM \rightarrow 37°C, ^{spread plate & resuspended} then ^{add 50 μ L} ^{then pour the rest} ~~spread plate & resuspended~~

⑭ Using the Gibson heat inactivated Gibson reaction, set up the following PCR:

- 0.5 μ L Gibson reaction
- 2B for - primer
- 1. Rev primer

⑮ Run 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 ~~IS~~ 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 a 10 ladder
 \rightarrow accidentally punctured the well

8/19/15

① Setting up the following Q5-PCRs: (20 μ L Reactions)

- a) 64°C w/ Q5 2x Hot Start
 - b) 64°C w/ Buffer G & Q5 Polymerase
 - c) ~~64°C w/ Buffer G & Q5 Polymerase~~
 - d) Repeats of the above, but w/ Todd's recommended scheme.
- Note: Using the gblocks themselves

~~Excess~~
~~Q5~~ ~~Q5~~

Panel One: 4 tubes

A: 10 μ L Q5 2x Hot Start ✓
~~0.2 μ L GBlock 2B~~ 0.2 μ L GBlock 2B (2ng, 141 fmol)
~~1.114 μ L GBlock 1~~ 1.114 μ L GBlock 1 (11.14ng, 9.141 fmol)
 4.8 μ L H₂O ✓

B: ~~10 μ L~~ Some thing

C: 10 μ L Q5 Buffer G ✓
 0.2 μ L GBlock 2B ✓
 1.114 μ L GBlock 1 ✓
 0.5 μ L Q5 Polymerase
 4.3 μ L H₂O ✓

D: Some thing

② Ran Part 1 PCR from yesterday in a .7% gel, cut out and gel purified w/ standard protocol
 450 μ L ADB, incubate 10 min, Mix
 Load column, spin 30s @ 50°C
 Wash twice w/ 200 μ L wash
 elute w/ 50 μ L H₂O
 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 ✓ ~~se-OR~~
 - 0.75μL 1-Rev ✓
 - 0.5μL Q5 (if Buffer G) ✓
 - 3-3.5μL H₂O ✓✓
- } ⇒ 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

Using 75ng of backbone: 58.63 fmoles
 117.26 fmoles part 1 = 142.9ng = 14.29ng of gBlock...
 232.92 fmoles part 2 (4x molar) = 50.96ng

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

50ng Vector: 39.09 fmoles = 3.31μL
 78.18 fmoles Part 1: 95.27ng = 9.527μL → Need to speed up this so 5.33μL = 95.27ng (17.87ng/μL)
 78.18 fmoles Part 2B: 17.10ng = 1.36μL
 OPTIMIZED! 9.67μL total!
 ↓ Speed up it to ~5μL

Three of these were done:

- 20μL → ① Vector, Part 2 (PCR Prod), Part 1 (Spacer + Ubs)
- 10μL → ② Part 2 (PCR Prod) + PCR Prod/Gel Purified Part 1
- 10μL → ③ Part 2 (gBlock) + Part 1 (gBlock)

20.475 → 2.0475
 5.4075 → 0.54075
 1.40175 → 0.140175

⑤ ~~PCR on OE-PCR~~ Gel on OE-PCR showed nothing. Setting up exact run from yesterday (#10 from 8/18) except .557μL of PI straight from gBlock source & more H₂O to compensate → all run at 64°C w/ Q5 2x MM

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

1μL Template

- 25μL forward (Part 2a 22bp Vector Ho)
- 25μL reverse (Part 1 Rev)
- 5μL Q5 Master Mix
- 3.5μL water

(Labelled tube as "3-2")

⑦ 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 priming issues
- ③ Amplify 2B for gel purification. Use new forward primer, and we only have one rev primer (or seq. ones)
- ④ Use middle primers to from the Addgene 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 GS screens, b/c we know that totally works
- ⑥ Run a TD PCR on OE-PCR, do (1) (2) as also!

③ Continued

Note: XhoI did not exist in sufficient quantities for the amounts needed. ~3 μ L of enzyme was used when 5.25 μ L total should have been. Mastermix also ran out for tubes 7, so they may be sketchy (both digestions)

NEBuffer 2.1 was used w/ EcoRI + PstI

Cutsmart w/ XhoI

Both in thermocycler 1 for 60min at 37°C, heatshock 80°C for 20min
Tubes w/ blue hinges: double digest.

④ Preparing 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

⑤ 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 ~1 μ L of the dilution into the Gibson, so 1 μ L conc. Part 2MED into 9 μ L H₂O. Labeled these tubes w/ "2A dilute", "2C Dilute", "2D Dilute"

Actually doing a half reaction, for each tube

PSB1C3 - 1.66 μ L ✓

Part 1 - 0.99 μ L ✓

Part 2 - 0.5 μ L ✓

Master Mix - 5 μ L ✓

	2A	2C	2D
	2A - .54 μ L ✓	2C - .575 μ L ✓	2D - .51 μ L ✓
H ₂ O	1.81 μ L ✓	1.775 μ L ✓	1.84 μ L ✓

3A Assembly for Eidhoven - Caroline + Miranda

8/20/15

Overview:

Combining Kanamycin backbone (PSB1K3.M1), LacPromoter (R0010), and a yellow chromato protein + RBS (113C03 and #23Linkit4)

Protocol:

⑤ Transformed Gibson products (2A, 2C, and 2D) as well as 113C03 (for 3A assembly)
- Each Gibson product went in 50 μ L 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 50 μ L reactions w/ Q5 & 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;

!!! ⑧ 11, 12, 16, 18 all produced band patterns we expected for both EcoRI+PstI digestions & XhoI digestions (w/ Colony 18 potentially being a victim of partial digestion. ⑨ !!!

⑧ 2 tubes w/ 5mL LB 17 µg/mL CM of each 11, 12, 16 were inoculated o/pn for further purification & sequencing tomorrow

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



Well 14 of 15

... 2A-C/D + 113C03 ...

AJ

Colony PCR of SCRIBE 2.0

Reaction Buffer
 25 μ l ~~Tag~~ Master Mix } 650 μ l
 2.5 μ l primer Part 2a-24p.Hu 67 μ l }
 2.5 μ l primer Part 1 Rev ~~Rev~~ 67 μ l }
 20 μ l Autoclaved water 20 μ l }
 X (number of colonies + 1)
 25 + 1 = 26

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

Transfer ~~the~~ 10 μ l Reaction buffer to each PCR tubes (Use pipette to mix them)

PCR Cycle	95°C	1 min
	95°C	15s
	59°C	30s
	72°C	2 minutes
	72°C	2 min

} x 30

CB Purified plasmids from colonies 11, 12, and 16.
 Eluted w/ 50 μ l dH₂O.

	116.1 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.

~~2A (Empty Etc) 1-6~~
~~2A (100 μ l) 7~~
~~2BC (100 μ l) 8-9~~
~~2C (Empty Etc) 10-18~~
~~2D (100 μ l) 19~~
~~2D (Empty Etc) 20-25~~

Plate	Colonies Picked From It
2A (Empty Etc)	1-6
2A (100 μ l)	7
2BC (100 μ l)	8-9
2C (Empty Etc)	10-18
2D (100 μ l)	19
2D (Empty Etc)	20-25

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

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

- A) 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/ ~~at~~ the conc of primers we normally use

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

④ Using ~~Pro~~ Pro For & P1 Rev on 11A to generate a 2CRIS equivalent construct using 50 μ l Q5 & H.S. (Hot Start) Master Mix

- Plan of action:
 - Try and generate the 2C analog
 - Digest it w/ XhoI & EcoRI/PstI or some other one
 - Pick some more colonies & do colony PCR (Maybe 0.1µl of the stuff growing in the 37°)

⑤ Attempted B→C PCR w/ GS, partial

⑥ Digesting ~~2A/2C/2D~~ 2A/2C/2D & (B→C) to compare SCRIBE sizes

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

⑧ Transformed ~~2A/2C/2D~~ 2A/2C/2D in top 10 & picked

8/21/2015

James Blandin

• Good to be back at work ;

• I transformed 11-A (SCRIBE 1, P_{lac}→kan^R etc.) onto CM plates.

• Input them in at 3:00 P.M. (plates in 37°C)

• Four plates ² ^{plain}

• no DNA control, 50µL plated into LB

• 0.5µL 116.1ng/µL DNA, 50µL plated into CM (identically ^{other steps})

• 1.0µL 116.1ng/µL DNA, 50µL plated into CM

• 1.0µL 116.1ng/µL DNA, 250µL plated into CM

• 25 min on ice → add DNA to 50µL comp cells (FF144), put on ice ~25 min, heat shock in 42°C bath for 30 sec, 2 min on ice, add 250µL prewarmed LB, grow in 37°C shaker for 45 min, plate, grow overnight

• We should be able to inoculate these for testing our new iGEM part!

• I setup a PCR (on 11-A as a template) to get SCRIBEkan^R without a promoter:

• 5µL 0.1161ng/µL 11-A (10⁵ serial dilutions)

• 25µL 2x Hot Start Master Mix

• 1.25µL of Aru's primer "Pro_{for}"

• 1.25µL of Aru's primer "Part 2 Rev"

• 17.5µL H₂O (50µL total vol)

• 2ig 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 text on the previous page, 130)

8/24/15

To Do

- ① Induce MIT ~~colony~~ strain w/ Addgene & 2B construct
- ② Grab some ~~stuff~~
- ③ Dependably huge colony PCR worked out
- ④ Prepare cloning things which are lacking

8/24/15

- ① Poured 4 15 comb ^{1%} gels for use throughout the day.
- ② Dephosphorylating psBIC3 w/ rSAP for 1 hr, heat killing ^{637C} swim 8X
- ③ Picked 24 colonies from retransformation of 2A/C/D
- ④ Running the 2nd Colony PCR done today on a gel along w/ the (B→C) test.
- ⑤ - Everything failed; ~~surprise~~ surprise
- ⑥ Set up a new colony PCR w/ the freshly picked things (1-26)
 - New PCR conditions → will be described tomorrow

AJ ~~Do~~ make 10 mL 100 mM IPTG for induction
 (mix 1 ml 100x (1M) IPTG and 9 ml ~~also~~ sterile water)
 - If we want to do a 1mM induction, we can add 10 μL 100 mM IPTG to 2 ml LB.

8/24/15

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

8/24/15 SCRIBE 1.0 Induction

- NF
- ① Prepared 10 mL 100 mM IPTG
 - ② Added 2 mL LB to one tube (①) and 2 mL LB + 20 μL 100 mM IPTG to a second tube (labeled ②)
 - ③ "Strain F144 SCRIBE 1.0 8/23/15" removed from 37°C shaker. 2 μL 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 psBIC3
 110V, 20 minutes

□ □ ... □ □ □	B A ladder
	A is good B is sketch

8/24/15 3A Assembly

Master mix for backbone	Master Mix for part A	Master Mix for part B
5 μL Nebuffer 2.1	5 μL Nebuffer 2.1	5 μL Nebuffer 2.1
0.5 μL EcoRI-HF	0.5 μL EcoRI-HF	0.5 μL XbaI
0.5 μL PstI, DPN1	0.5 μL SpeI	0.5 μL PstI
18.5 μL dH ₂ O	19 μL dH ₂ O	19 μL dH ₂ O

Part B: 26.9 ng/μL, Ratio: 2.01
 Digest Assembly: B- 4 μL of Master Mix + 444 μL of BB (Miranda)
 Digestion: A- 2 μL of 57 ng/μL LAC, 2 μL of H₂O
 B- 4 μL of chromoprotein

22.5 ng/μL of plasmid psBIC3.m3 (Miranda)
 Digest

3A Digest

- Part A → 37°C @ 5:58pm
- Part B → 37°C @ 5:58pm
- BB → 37°C @ 6:23pm

Take out @ 6:28 put in 80°C for 20min
 Take out @ 6:53 and put in 80°C for 20min

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

8/25/15

Thermocycler To Do's

- Set up Gibson rxn (10ul total) in a 1:1:5 ratio w/ the Gibson MM. Skew gene
- Set up amplifications of gBlocks using James' primers

Inoculations of Colony PCRs * marked w/ # & date

- Colonies 1-9 2A + RTB - CM 30uL
- 10-17 2A + RTB (400 uL) CM
- 18-19 2C + RTB 30uL
- 20-23 2C + RTB 140 uL
- 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 2.5ul "3A" Assembly to 50ul Top 10 Competent Cells
- Incubation on ice for 20min
- 7:52 30s heat shock, incubation on ice for 2min
- 7:54 add 100ul LB to each tube
- incubation in 37°C for 1 hour
- 8:05 Pre-warm the LB & CM Plates
- 8:14 Transformation CM Plate labeled "3A"

9:30

Return Purple DNA ladder to PCR Box

SA

purified inoculated Glones, labeled it per in -20°C

quantified	using Tecan	no/180	ng/ml	200/280
2	77.7	1.83	15	82.7
3	74.6	1.89	16	80.1
4	80.7	1.84	18	69.5
6	77.4	1.84	19	63.4
7	59.6	1.9	20	66.7
13	72.2	1.85	26	58.8

8/26 Plan

- Gibson w/ pSB1C3, ~~add~~ 2B (PCR Product), Part 1 (gBlock) → Run the rxn for 4 hrs, end it when you want to go home

Verify the BB or see if we have enough old ones left.

- Digest 2A/C/D constructs. Some are purified

PCR purify the BB's

AJ

Transformation of ?
The same protocol in B5.

4:30

Inoculation in 37°C for 1 hour

8/26

① Diluted gBlocks 2A/B/C/D to 0.1 ng/μL using same amount increase free H₂O
I found in the -20C

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

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

④ PCR purified all of them to 1/2 eluted w/ 10μL H₂O at 50°C to get:

⑤ Run a 10μL Gibson Reaction, set up like this:

⑥ 12 Gyr PCR gel looks good. pSB1C3 is kind of sketchy, mixing more.

AJ
1:50

Run a gel of 2A, 2B, 2C, 2D, pSB1C3-A, pSB1C3-B.

Digestion of purified DNA.

Mix 9.75μL PstI, 9.75μL EcoRI-HF, 13μL 2.1 Buffer, 32.5 H₂O

add 1μL to each tube.

add 1μL DNA to each tube.

August 27, 2015

James Blodin

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

	450 μ L on KH (no dilution)	45 μ L on KH (no dilution)	Diluted by 10, 45 μ L plated on KH
no IPTG induction	0 colonies	0 colonies	0 colonies
1M IPTG induction	1 colony	1 colony	0 colonies
no IPTG induction	11 colonies	0 colonies	1 colony
1M IPTG induction	10 colonies	1 colony	0 colonies

	10^5 Dilution, 45 μ L on LB	10^6 Dilution, 45 μ L on LB	10^7 Dilution, 45 μ L on LB
no IPTG	hundreds	~140	15
1M IPTG	hundreds	~84	12

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

Uninduced Cells	Induced 1M IPTG Cells
No significant colony count means a recombinant frequency of zero.	$\frac{11 \text{ colonies} + 10 \text{ colonies}}{2} = 10.5 \text{ colonies}$
	$\frac{10.5 \text{ mutant colonies}}{45 \mu\text{L} \times 10^7} = 0.0233 \frac{\text{mutant colonies formed}}{\mu\text{L cells grown}}$
	$\frac{12 \text{ colonies}}{45 \mu\text{L} \times 10^7} = 2.67 \times 10^{-6} \frac{\text{colonies formed}}{\mu\text{L cells grown}}$
	$\frac{84 \text{ colonies}}{45 \mu\text{L} \times 10^6} = 1.87 \times 10^{-6} \frac{\text{colonies formed}}{\mu\text{L cells grown}}$
	average: $2.27 \times 10^{-6} \frac{\text{colonies formed}}{\mu\text{L cells grown}}$

Recombinant Frequency: $\frac{0.0233 \text{ mutant colonies formed}}{\mu\text{L cells grown}} \div \frac{2.27 \times 10^{-6} \text{ colonies formed}}{\mu\text{L cells grown}} = 1.03 \times 10^{-8}$

Results:

- 1M IPTG led to a recombinant frequency of $\sim 10^{-8}$, while no IPTG led to ~~zero~~ zero recombination.
- Considering if the background rate is 10^{-9} (it's either 10^{-9} or 10^{-10} , if my memory is correct), then this is above the limit of quantification.
- This is ~~surprising~~ surprising, since our sequencing ~~failed~~ analysis ~~stand~~ 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 from working with:

	Concentration, ng/ μ L	Ratio	Concort	Concentration, ng/ μ L	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			

8/27/15

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, @ WEB recommends 1:2:3 or up to 5x when <200bp

* Maintaining a 1:1 ratio bit 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 fmoles = 0.458 μ L

39.09 fmoles Part 1: 47.64 ng = 4.764 μ L

117.27 fmoles Part 2A: ~~4.93 ng~~ 20.29 ng = 1.37 μ L

2B: 25.66 ng = 0.971 μ L

2C: 20.29 ng = 1.399 μ L

2D: 14.93 ng = 1.866 μ L

For a 20 μ L Rxn
 → We want a 10 μ L one,
 so drop everything by 1/2

Bacterial Culture

More a 4x [30 μ g Vector = 23.45 fmoles (Being cheeky here = 1): 0.275 μ L ^{7ng/ μ L → Okay to transfer 4 μ L of Gibson into 50 μ L cells.}
 Master Mix [23.45 fmoles P1 = 28.58 ng = 2.858 μ L (1)
 w/ 20 μ L Gibson Mix 70.35 fmoles • 2A: 12.17 ng = 0.859 μ L (1 μ L H₂O) MM (No P.2)
 2B: 15.34 ng = 0.581 μ L (1.29 μ L H₂O) - 1.1 μ L Vector
 2C: 12.17 ng = 0.839 μ L (1.03 μ L H₂O) - 11.43 μ L P1
 2D: 8.958 ng = 1.12 μ L (0.747 μ L H₂O) - 20 μ L Gibson MM

② Permitting for 1 hr at 50°C

(Amplify product w/ Tag?)

- ② Transformed 4 μ l of the Serial down Gibson to 50 μ l Comp cells
 - Thawed on ice for 20 min
 - Incubated for 30 min
 - Shocked for 30 sec
 - Grew up for 60 min
 - 50 μ l plctd & 450 μ l plctd
 - Labelled "2- + BTB in Top 10 (X μ l plctd) 8/27/15 JB"

②.5 Emerited Jess that we left at ~10:10pm James B.; Plates put in 37°C at ~10:15 AM.

③ Going home early (9pm)

(I suggest removal around 2:00 PM tomorrow)
 Update, I took them out around 1:00 PM. (15 hours in the 37°C)

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.

	Conc	Ratio
AJ.		
Quantification of 3A		
2	32.3	1.9
3A-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 7.7 μ l EcoRIHF 7.7 μ l PstI, ^{17 μ l} ~~17 μ l~~ and 27 μ l dH₂O
 add 17 μ l the mixture and 17 μ l BSA 2.1 Buffer to each tube

5:43

Set up the digestion in cycles.

Return 3A-2-10 tube and digestion Enzymes to the blue rack in -20°C.

8/28/15

① Colony PCR on the 2A-3D GUSINES.

- 8 Colonies from ACD } 39 Colony PCR's → Set up ^{three 9} 8x & 8 I 16x MM
- 15 from B

PCR Conditions

1.25 μ l 2-for

1.25 μ l 1-Rev

0.8 μ l Template

2 μ l H₂O

5 μ l Tag Green MM

9.5 μ l for 1x MM → Then add 0.5 μ l template

9x MM

11.25 μ l 2-for

11.25 μ l 1-Rev

18 μ l H₂O

45 μ l Gran Tag

16x MM

20 μ l 2-for

20 μ l 1-Rev

32 μ l H₂O

80 μ l Gran Tag

} 9.5 μ l each tube

Ord. b. l. t. c. has ^{left} ~~been~~ ~~set~~ ~~up~~ ~~for~~ ~~PCR~~ ~~and~~ ~~the~~ ~~digestion~~
1.5 ~~buffer~~ ~~and~~ ~~left~~ ~~has~~ ~~substituted~~ ~~the~~ ~~left~~ ~~hand~~ ~~of~~ ~~the~~ ~~gel~~ ~~and~~ ~~run~~ ~~it~~ ~~at~~ ~~each~~ ~~lane~~

set up at 20°C for 3 hours for colony PCR. Asking Maxwell
to put them in 4°C tomorrow

② Inoculated ALL of the colonies b/c waiting 3 hours for colony PCR. Asking Maxwell

to put them in 4°C tomorrow

③ USED 34 μg/ml to INOCULATE

AJ. 8/31/17

Run a Gel of the Colony PCR ~~and~~ ~~the~~ ~~digestion~~

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

2 x 1 well in one Gel

B1	-	B14
<hr/>		
B15 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: 189.9 μg/μl Ratio: 1.87

Do a digestion of #13 plasmid

DNA 200ng / 189.9 = 1.31 μl
 Enzyme PstI 0.8 μl EcoRI/HF 0.8 μl
 2x Buffer 1 μl
 Water: 0.1 μl

⑦ If you're planning to go on leave, message me.

⑥ Gel in colony PCR showed 5 hrs for A, 3 for D

(load all in 5µL LB + 5µL CM. (34 µg/ml))

→ 37°C

9/03/2015 James Blandin

• 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

• Note: the only difference between trials is the time I ran the nanodrop (same vol. drops each time)

• 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 changed over time, following ^{particular} trends:

	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
Primers for Part 2 Primer Part 2 amp Rev	66.4, 1.62	67.7, 1.63	69.6, 1.63	72.2, 1.64	75.8, 1.63
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, 1.78
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
2x 22bp Vector Ho	82.3, 1.64	89.3, 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 was due to ^{possibly} gradual rise in droplet temperature across trials, but that wouldn't explain why gBlock 1's concentration dropped so dramatically...

9/03/2015

James Blandin

PCR Setup (done on 9/1/2016)

- Because of the unclear primer and template concentrations, I setup two PCR reactions per DNA fragment, each at different concentrations.
 - One would set the primer concentrations to 0.5 μ M if the primers were at the highest concentrations observed.
 - The other assumed the lowest ^{primer} concentrations observed.
- Unfortunately, I've ~~been~~ ^{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 μ g or less if the templates were at their highest concentrations observed.
 - Looking at it now, I should have gone for 100 μ g instead. (10 μ L reactions, not 50 μ L reactions)
- I also setup controls with no template in them.

all (volumes μ L)	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	0.909	0.725	0.463	0.725
reverse primer	0.417	0.272	0.417	0.746	0.510	0.746
0.5 Hot start 2x HH	5.000	5.000	5.000	5.000	5.000	5.000
template	0.714 8	0.714	0	0.400	0.400	0
auto-primed Δ 120	2.960	3.504	3.674	3.129	3.627	3.529

note: all templates were added by 10am before being added

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

PCR settings:

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

(266 ^{igen 2} ~~changes~~)
Part 1 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 9/3/2015)

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

Gel Test

- I added 1 μ L of each PCR reaction and 0.2 μ L 6x green loading dye in this order: Part 1, Part 1, Part 1 control, Part 2^B, Part 2^B, Part 2^B control (left to right)
- I added ^{purple} ladder (2 log DNA) ^{into} onto both side wells.
- I ran the gel at 110V (7cm 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} put all remaining 9 μ L of each reaction on a gel and use gel purification if I observe sufficient DNA in the 2,000 base range.
- Not to self! Aru put the PCR tubes on the yellow rack in the -40°C.

James B.

9/04/2015

- I ran a gel on the other 9 μ L of each reaction.
- Both Part 1 & Part 2B were successfully amplified (no band observed).
- I'll need to repeat the reactions, but with 50 μ L instead, to get enough DNA to work with.
- Since the sequencing was almost perfect, this may be moot...

AJ * Gel of a ~~the~~ digestion sample

* Inoculation of A. D. (8 samples total)

(5ml LB + 1 μ L 34 μ g/ μ L CM)

LABELED Labeled A3, A7, A9, A11, A14, D2, D8, D9 in 37°C shaker


```

for (i=0; i<4x; i++)
{
  while (i<4x)
  {
    i++
  }
}
    
```

TO DO

- ① Digest Purify B 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 & ~~more~~ inoculate the successes
- ⑤ ~~inoculate B/B~~ / ~~inoculate B/B~~ / ~~inoculate B/B~~ / ~~inoculate B/B~~ / ~~inoculate B/B~~ → Email Michelle after to see old
- ⑥ Move plates to 4C → Email Michelle to see if she can inoculate at 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.

9/13/15 ① Purifying the A+D Colonies (8 in total). ~~Useful/Useful/Useful/Useful~~
 A3, A5, A9, A11, A14
 D2, D8, D9

A3	189.5	1.87	0.75 μL XhoI 0.75 μL BstI Control 3.5 μL H2O 2.5 μL Template 0.75 μL BstI 0.75 μL Buffer 3.5 μL H2O 2.5 μL Template 5 μL each template 4 μM MM (Σ) 3 μL BstI 3 μL Buffer (Control) 15 μL H2O 5 μL each tube
A5	521.8	1.91	
A9	470.9	1.92	
A11	586.9	1.87	
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 digests: (all 10 μL)

0.75 μL EcoRI	10x MM = (PCR tubes w/ a ● are the ones w/ this digest!) 7.5 μL EcoRI 7.5 μL PstI 7.5 μL Buffer 2.1 5.25 μL H2O 5.25 μL into each tube, 9 total.
0.75 μL PstI	
0.75 μL Buffer 2.1	
2.5 μL DWA	
3 μL H2O	
7.75 μL total	

9680 10 11A PA JA SA 10/15/15
 10/15/15 10/15/15

IA

Col 1: AAAAA DDD } → In numerical order!
 2: C A(5) D(3)

③ First run a gel on A w/ XhoI → All Good & D w/ BsaI → Not sure

Then on C, A, D w/ EcoRI+BstI, gel was run at 0.7% so it wasn't clear, but C looks good, and maybe the others?

9/7/15 Inoculation of MIT strains and our Part B.

AJ

Pick one ~~from~~ colony from FF144 2B #17, 22, 23, 27 & Addgene and put the tips into 5ml LB + 1µl CM (34µg/µl) in 5ml

15:20

9/8/15

AJ

	#017	#22	#23	#27	Addgene
OD ₆₀₀	0.911/2	0.865/2	0.889/2	0.867/2	1.027/2

IPTG Induction

~~Add 1ml IPTG (20µl 100mM)~~

~~Make too~~

Make 1mM IPTG LB, CM Medium (20µl 100mM IPTG

+2ml LB + 2µl 34µg/µl

	#17	#22	#23	#27	Addgene
+IPTG	✓	✓	✓	✓	✓
-IPTG	✓	✓	✓	✓	✓

Totally 10 tubes.

30°C, 24 hours inoculation.

The location is



9-9 Make 20 Km plates

~~20 µg/ml each plate
 + 20 plates
 400 µg of Km in total~~

Stock is 50 µg/ml

~~$$400 \mu\text{g} \times \frac{1 \mu\text{g}}{1000 \mu\text{g}} \times \frac{1 \text{ ml}}{50 \mu\text{g}} \times 1000 \times \frac{1000 \mu\text{l}}{1 \text{ ml}} = 8 \mu\text{l of}$$~~

$$\frac{20 \mu\text{g}}{1 \text{ ml}} \times 20 \text{ plates} \times 10 \text{ ml} = 200 \text{ ml of LB + aged}$$

$$\frac{20 \mu\text{g}}{4000 \mu\text{g of Km in total}}$$

$$4000 \mu\text{g} \times \frac{1 \mu\text{g}}{1000 \mu\text{g}} \times \frac{1 \text{ ml}}{50 \mu\text{g}} \times \frac{1000 \mu\text{l}}{1 \text{ ml}} = 80 \mu\text{l of Km in total}$$

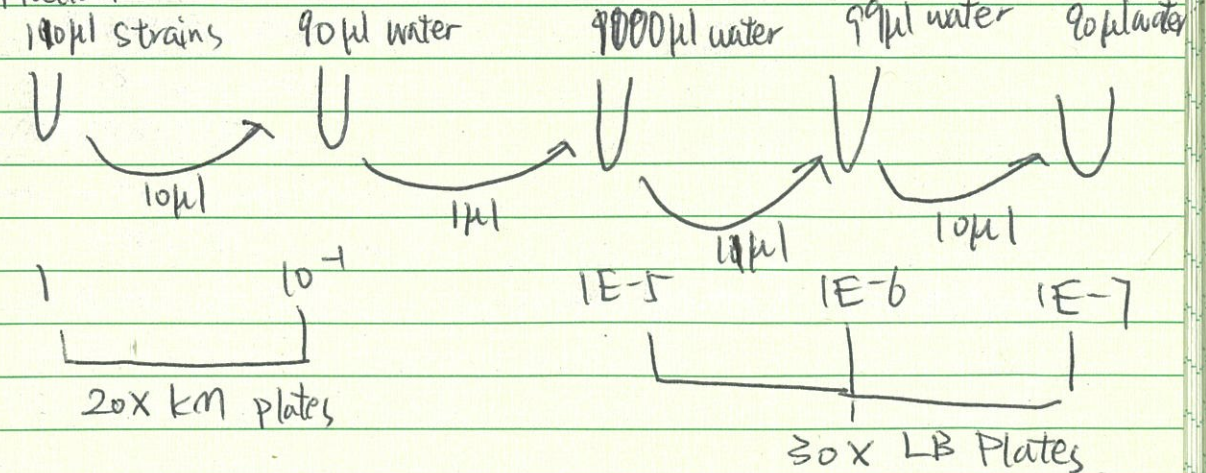
~~AJ~~
 AJ

Make KM plate.

Add 200 µl 10 µg/ml KM to 100 ml LB to make 20 µg/ml

Finally, we lack 2 LB plates, so we use two old LB plates for #17 10⁷X IPTG & #22 10⁷X IPTG

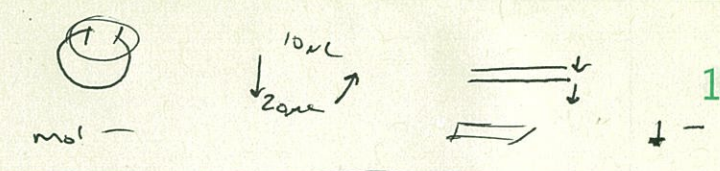
Dilution



11:23

9/12/15 To Do

- ① Make LB agar ✓
- ② Return from old Gibson assembly (In case plates are ~~not~~ new)
- ③ Try and find green tag \rightarrow FOUND!
- ④ Redo Gibson assembly on B + Transform (MT strain) (In case assembly was ~~not~~ \rightarrow gives us more to screen)
- ⑤ Digest C + Ligase into BB \rightarrow Transform to MT strain (To see if it works)
- ⑥ OE-PCR trials & errors, see if we can get it to work
- ⑦ Set up mastermix for ~~stuff~~
- ⑧ More sure we have sufficient BB/DpnI treat it



Ordered To Do

- ① Set up Gibson \rightarrow 2hr \rightarrow Leave me in (MM, Part 1, 2B(PCR))
- ② Digest Part C \rightarrow 2hr \rightarrow ~~to get~~ ^{D5 C} (XbaI, PstI)
- ③ * 2hr Gibson \rightarrow ~~stuff~~ Gel purify C, Lyse C
- ④ ~~Mass transformation~~
- ⑤ Mass trans

① Digesting part C w/ XbaI/PstI

- 20µL Digestion >10%
- 2µL 2.1
- 1µL XbaI
- 1µL PstI
- ~~2.5µL Part C~~ 2.5µL Part C ~500ng
- 13.5µL H₂O

② Reseting up the 40x Colony PCR

- 10.75µL Part 2a Primer for
 - 10.75µL Part 1 Rev
 - 215µL goTaq
 - 150.5µL H₂O
- } 9µL into each tube + 1µL LB Colony

- ③ ~~Prep~~ Made CM34 plates (x25)
- ④ Digestion of Part C was a bust
- ⑤ Transformed old B & new B Gibson to FF144
- ⑥ 10 positive colony PCR hits → 1, 16, 18, 21, 22, 25, 35, 39, 40, 42 → inoculate in CM34 LB
- ⑦ PCR'd more BB → gel confirmed!

9/15/15 To Do

- ① ~~Set up colony PCRs on new transformants (as many as you can)~~
- ② Purify plasmids (10 of them)
- ③ Digest preparations (1 hr digestion is good enough)
- ④ Run a gel on colony PCRs
- ⑤ Inoculate the hits from colony PCR & the successful digests
- ⑥ Set up the good plasmids to be sequenced. If not high enough conc, leave a strong note to re-prepare.
- ⑦ Box holds KM plates!

→ Should be able to leave by 10pm. Remember to induce tomorrow!

9/13/15

- ① Since no plasmid showed minimal/No growth, retransforming old & new B into FF144 AND Top10!
 - Just in case it is the CM34 plates, I will grow them up in CM17 LB or more max LB agr... Maybe it was too high for a transformation... → MAKE NEW CM

- ① Purifying the 10 plasmids first, so I can work to remedy ② (0)
 - Eluted w/ 50µL ~~50~~ 50°C H₂O
 - 20sec vortex w/ RNase A followed by a 20sec later vortex (Very low yields get expected)
 - ~~Added Solu III to the middle of column~~ (<5! Very important!)
 - Let Solu 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 prep (but it won't be obvious on Nanodrop; it will read ALL DNA, not just plasmid).
 - Added Solu III directly to the middle of column, immediately inverted vials. I was ready to pipet in more (4-8 times)
 - Took out supernatant 100µL at a time, stopping once I ~~was~~ disturb the pellet. If it got disturbed, ditch what you pipetted if it got in the pipet. This way, being the nice to get ALL of the DNA is a bit risky. If I feel I will disturb the pellet, I put what I have in the column, ~~and~~ lower 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 concentration below

↳ Jk. Precipitate was in the middle. Maybe Solu III to the middle is bad. I thought I did it last time... Maybe on the bottom!

1: 172.5 ng/µL	21: 235 ng/µL	35: 104.5 ng/µL	42: 232.8 ng/µL
16: 104.9 ng/µL	22: 195.9 ng/µL	39: 89.1 ng/µL	
18: 342.8 ng/µL	25: 241.6 ng/µL	40: 136.8 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 ~~that~~ there is a mixup. But, considering my normal pipetting habits, I think the order of the tubes are: 1, 2, B18, B25, B2 (Top 10). If not, & they are in the opposite order. If B2 works for one cell & not the other, we'll know.

③ Digest all 10 plasmids w/ XhoI using approx 250ng DVA & the BB w/ EcoRI. PCR purified PSB1C3 (b/c digestion w/ EcoRI is really bad in Q5 buffer) to the following concentrations:

1: 16.7ng/μL (HRC was not mixed, just put on top) 2: HRC was mixed before split, 43.5ng/μL

XhoI Digestion:

0.75μL XhoI
0.75μL CutSmart

11x MM

8.25μL XhoI } 1.5μL each total
8.25μL CutSmart }

(250ng) DVA(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 41: 1.1

Water to use = (7.5) * (1.5 + μL DVA Used)
= (in order): 4.5, 3.5, 5.3, 4.9, 4.5, 5, 3.5, 3, 4, 8.9

EcoRI + BstI Digestion: Use PSB1C3 (2) b/c higher conc.

Digesting in 5 ~ 250ng BstI:

0.75μL EcoRI x5 → 3.75μL → 7.75μL in each
0.75μL BstI 3.75μL
0.75μL Buffer 2.1 3.75μL
5μL DVA 25μL
0.5μL H2O 2.5μL

③ Cont'd → In Thermocycler 1, all of them are a 3 hour digestion.

JE 09/14/2015 4pm

Run gel on sample from digestions

Well	1	2	3	4	5	6	7	8	9	10	11	12
Sample Loader	1	16	18	21	22	25	35	39	40	42	Loader	

- 7/14
- ① 7/10 colonies showed expected digestion patterns
 - ② These colonies were transferred 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 Pick 8 colonies from B2 (new) & 8 colonies from B1 (old) & Run colony PCR

0.25μL 2a-22bp-homology (For-Primer)
0.25μL Part 1 Rev (Rev-Primer) } 17x MM = 4.25μL Primer For
3.5μL H2O } 4.25μL Rev
5μL GreenTag } 85μL GreenTag
1μL Template (Touch colony & inoculate in 100μL LB) } 57.5μL H2O
↓
9μL Each Tube

③ Prep B18 and B25 (at a minimum, but B1 & B16 had good digestions) for sequencing

↓

Located in the ice box w/ the comp cells

↓

Put them all in the blue rack. They are diluted to 100pmol currently. Put in 8 (4 for each) separate labelled Eppendorf tubes

• The sequencing center wants 5μL of volume w/ a conc. of 100-200 ng/μL → So you'll have to dilute a bit. Put these 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

④ Inoculate colonies from the B18 & B25 transformation plates (the one w/ a Δ on them; i.e., not the ones used for colony PCR) in 5ml LB + 17 μ g/ml CM

⑤ Run a gel on the colony PCRs then inoculate the successes in 5ml LB + 17 μ g/ml CM
OR if you can't stay here until the colony PCR is done $\frac{z}{z}$ run a gel, inoculate everything in 5ml LB + CM.

9/15

- ① ~~Gen~~ Repped B18 & B25 for sequencing & sent it in
- ② PCR'd Kan w/ GG sites
- ③ Retransforming B1, 16, 21, 22, 25, 42
- ④ ~~PCR'd~~ ~~SDM~~ PCR Site Directed Mutagenesis + transform

9/15/15 James Bladin

- I ~~did~~ the gel Andrew ran in on the colony PCRs, Neward Old 'B' analyzed
- Based on Aru's recommendation, I purified the NB6 and NB8. (N⁶ for ^{new} ^{guessing} in)
- 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 \checkmark (Done, at 11:00)
- ⑥ IPTG induction on B18/25 (~8:00pm)
- ⑦ Made X420 plates

9/16

- ① Plated 30 μ L of B18/B25 (IPTG included) ~~at~~ at ~8am after 12 hrs of induction
- ② 8:00am: B1, B16, ~~B25~~, B21, B22, B42 were inoculated w/ CH17/IPTG using LB that was open all night (THIS IS ME TRYING SOMETHING; IGMF)
- ③ 11:00am: Using fresh LB, the following were put in the 37C in CH17/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/ ~~recombined~~ 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. \rightarrow Seq 1/22
 - \rightarrow Growing 1/22/25 to mid log growth (0.5-0.7 OD)
 - Put ④ Inductions back in the 30C at ~3pm (to be plated at night)

			X				
	X	X	X	X			
							X
				X	X	X	X
							X
							X

211P

Summary

B18/25 were initially transformed, grown up, induced, plated, seq'd
 - Failed (X in Bod) → B18 is fucked, B25 has R→H in RT

B1/16/21/22/25/42 were transformed into FF144

- Grown up for 12 hours WITH IPTG (1mM) (~ 9:00am)
- Grown up for ~ 9/10 hours WITHOUT IPTG (What's supposed to happen)
- Induced w/ IPTG for ~ 16 hrs
- Plated at 2:00 Thursday

After growing w/ IPTG for ~ 12 hrs, these were plated

- B1/22/25 showed growth on Ken20 plates
- We will compare this against the "proper" induction/plating done today
- ~~B1/22/25~~

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/Adkgene inoculated in 5ml LB + CM17
 - Will grow until midlog then will induce
 - ⑤ Preparing B1/B22/B25 for iGEM part submission