QGEM Laboratory Notes

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Project: SAA Expression, ANP Expression, and the Binding Construct

June 16, 2009

- meeting between Bogdan, Bryant, James and I
- finished the sequence for the surface expression construct
- figured out the to-do list
- mini-prep the following:
  - linker K157013 *Plate 3, well 3G, plasmid Bba_K157000 (A)*
  - TEV protease
    - I712078 (C-terminus) *Plate 2, well 14M, plasmid J70003 (A)*
    - I712077 (N-terminus) *Plate 2, well 14K, plasmid J70003 (A)*
  - pLux
    - R0062 (not leaky) *plate 1, well 6O, pSB1A2 (A)*
    - R1062 (median strength in the absence of luxR/HSL) *plate 1, 8G, pSB1A2 (A)*
  - HO-pcyA
    - K098010 *plate 3, well 11N, plasmid pSB3K3 (K)*
  - terminator
    - B0015 *plate 1, well 23L, plasmid pSB1AK3 (AK)*
  - RFP
    - E1010 *plate 1, well 18F, plasmid pSB2K3 (K)*

2009-06-17

- transformed the following parts into Top10

1.	K157013	*Plate 3*	*well 3G*	*K157000*	*Resis:A*
2.	I712078	*Plate 2*	*well 14M*	*J70003*	*Resis:A*
3.	I712077	*Plate 2*	*well 14K*	*J70003*	*Resis:A*
4.	R0062	*Plate 1*	*well 6O*	*pSB1A2*	*Resis:A*
5.	R1062	*Plate 1*	*well 8G*	*pSB1A2*	*Resis:A*
6.	K098910	*Plate 3*	*well 11N*	*pSB3K3*	*Resis:K*
7.	B0015	*Plate 1*	*well 23L*	*pSB1AK3*	*Resis:AK*
8.	E1010	*Plate 2*	*well 18F*	*pSB2K3*	*Resis:K*

- left in 37C for four hours and left on the lab bench in room temperature overnight

2009-06-18

- no colonies were observed on the plates
- left the plates in 37C for four hours
- Picked one colony from each plate and made overnight broth culture for glycerol stock.
- K098010 plate did not grow, so it was left in the 37C overnight.

2009-06-19
- made the glycerol stocks from the overnight broth cultures
- started broth culture for K098010
- re-transformed the E1010 in Top10

2009-06-22
- re-started the broth culture for K098010 because it was overgrown (already in 67stationary phase)
- Observed no colonies on the E1010 plate. We found out that E101 should be on Kanamycin resistance plate.

Things to do
- get out the standard plasmid backbones
- High copy number assembly plasmid backbone
  - pSB1A3 *Plate 1, well 1K,*

2009-6-23
- submitted the VLA construct to Mr. GENE for synthesis
- placed orders for PCR primers

2009-6-24
- transformed the following parts into Top10

9.	RBS-luxR	*J37033*	*Plate 3*	*4O*	*pSB1A2*	*Resis: A*
10.	RBS-LuxI-ter	*F1610*	*Plate 2*	*24G*	*pSB1AK3*	*Resis: AK*
11.	RBS-LuxI	*K081008*	*Plate 2*	*10L*	*pSB1A2*	*Resis: A*
12.	RBS-LuxR-ter	*I0462*	*Plate 1*	*8O*	*pSB1A2*	*Resis: A*
13.	Pconst	*J23119*	*Plate1*	*18A*	*pSB1A2*	*Resis: A*
14.	LuxR	*C0062*	*Plate1*	*4O*	*pSB1A2*	*Resis: A*

2009-6-25
- purchased a QIAminiprep kit from BioBar
- picked colonies from plates and re-cultured in broth; left overnight
- ordered ITGA4 cDNA plasmid from OpenBioSystem.

2009-6-26
- made glycerol stocks for the parts transformed on 6-23
- designed primer for PCR out VLA cDNA plasmid and primer for pLux-RBS-HindIII
- Transformed the following parts into Top10 and left the plates in 37C overnight.

15.	RBS	*B0034*	*Plate1*	*2M*	*pSB1A2*	*Resis: A*
16.	HemeC	*I716154*	*Plate1*	*17B*	*pSB1A2*	*Resis: A*
17.	HemeD	*I716155*	*Plate1*	*17D*	*pSB1A2*	*Resis; A*

2009-6-27
- picked colonies from plates and made glycerol stocks

2009-6-28
- transformed glycerol stocks into broth culture

2009-6-29
- purified plasmids containing parts 1-17 from Top10 using Qiagen Spin Miniprep Kit
- performed Agarose Gel Electrophoresis to check the size of the parts. Bends did not migrate very far, possibly due to the fact that circular plasmids migrate very slowly. We decided to digest the plasmids with a BioBrick restriction enzyme and then run gel again.

2009-7-2
- We rerun the Agarose Gel Electrophoresis to check the size of the parts. It worked.

2009-07-06
- We transformed the following parts into Top10

18.	GFP	*E0040*	*Plate1*	*14K*	*pSB1A2*	*A*
19.	GFP constr.	*E0840*	*Plate1*	*12O*	*pSB1A2*	*A*
20.	pTet+GFP	*I13522*	*Plate2*	*8A*	*pSB1A2*	*A*
21.	LuxR construct	*K091204*	*Plate2*	*8J*	*pSB1A2*	*A*
22.	luxI + GFP	*J37034*	*Plate2*	*7I*	*pSB1A2*	*AK*
23.	pSB1AC3		*Plate1*	*11A*	*pSB1AC3*	*AC*
24.	pSB1AK3		*Plate1*	*13A*		*AK*
25.	pSB1AT3		*Plate1*	*15A*		*AT*

2009-07-07
- We picked colonies from the plates and made broth culture, which was left to grow overnight.

2009-07-08
- We purified the plasmids of part 18 to 25 using QIA spin miniprep kit.

2009-07-09
- We ran a 1% Agarose gel electrophoresis to confirm the plasmid lengths of part 18 to 25.
- Gel loading and concentrations:

I.	E0040	120ug/uL	loaded 12ul
II.	pSB1AC3	120ug/uL	loaded 12ul
III.	R0062	120ug/uL	loaded 12ul
IV.	pSB1AT3	120ug/uL	loaded 12ul
V.	J23119	120ug/uL	loaded 12ul
VI.	J37034	120ug/uL	loaded 12ul

VII.	K091204	120ug/uL	loaded 12ul
VIII.	E0840	120ug/uL	loaded 10ul
IX.	B0034	50ug/uL	loaded 10ul
X.	F1610	50ug/uL	loaded 10ul
XI.	I13522	120ug/uL	loaded 10ul

- We figured out the relative concentrations of the parts by comparing the bands with the ladder (The 5,000bp ladder is about 120ng/ul)

- Parts digested

I.	R0062	P_{lux}	prefix	EcoR1+Spe1
II.	pSB1AT3		backbone	EcoR1+Pst1
III.	J23119	P_{const}	prefix	EcoR1+Spe1
IV.	B0034	RBS	suffix	Xba1+Pst1
V.	F1610	RBS-LuxI-STOP	suffix	Xba1+Pst1
VI.	B0015	Terminator	prefix	EcoR1+Spe1

- restriction digestion mix recipe:
  - 600ng of DNA
  - 4ul restriction buffer
  - 0.5ul EcoR1 and 0.5ul Spe1, or
  - 0.5ul Xba1 and 0.5ul Pst1
  - Up to 35ul of ddH_2O
- We digested the parts using corresponding BioBrick restriction enzymes, and then purified the parts using QIAquick PCR Purification Kit.
- Stored the purified DNA in -20C overnight.

2009-07-10

- We ran a 1% Agarose gel electrophoresis to confirm the products of restriction digestions.
- The expected bands of the parts did not show up. This is probably due to the fact that most of the parts have lengths within 100bp and the QIA quick PCR Purification Kit removes DNA below 100bp length.
- The miniprep plasmids of the parts are digested again with corresponding restriction enzymes for 2 hours.
- Enzymes are deactivated (denatured) by heating at 65C

2009-07-13

- ligated the parts into the following constructs using the *T4 ligase protocol*.
  - P_{const} – RBS – LuxI – 2xSTOP
  - P_{lux} – RBS
  - P_{const} – RBS (Heme Oxygenase)
  - Terminator – RBS
- Transformed the ligated constructs into Top10
- made broth culture from the ITGA4 cDNA glycerol stock

- Transformed ANP construct and kill switch (I716462) into Top10.
  I716462 *plate 2* *well 3C* *Amp*
-

2009-07-14

- purified the plasmid for ITGA4 using QIAminiprep spin kit.
- Recultured ITGA4 containing cells in broth.
- Picked colonies and made broth culture for the ligated constructs in triplicates.
- Picked colonies and made broth culture for ANP construct and the kill switch (I716462)
  - ANP construct plate was overgrown

2009-07-15

- PCR the colonies to check ligations
- Broth culture for ANP construct did not contain cells. Therefore transformed Top10 cells were cultured in broth overnight again. Broth culture was also made for I716462.
- Run gel to confirm ligations and ITGA4
- working constructs from ligation
  - C1 (P_{const} – RBS)
  - PL3 (P_{lux} – RBS)
  - T3 (Ter- RBS)

  Note: BioBrick primers worked for pSB1AT3 backbone.

2009-07-16

- Broth culture for ANP construct and I716562 did not grow. This was due to defective Amp plates.
- We retransformed ANP construct and I716562 in cells.
- We PCR the miniprep samples of ligation to confirm proper ligations.
- Working constructs from ligation:
  - T3 (Ter-RBS)
  - PL2 (P_{lux} – RBS)
  - I3 (LuxI construct)
  - C1 (P_{const} – RBS)

2009-07-17

- remade broth culture for ANP construct and I716562
- PCR TEV proteases C-terminal and N-terminal separately
- PCR ITGA4 fragment from cDNA plasmid.
- Gel showed correct bands for TEV C and N termini
- ITGA4 band did not show up; plasmids also did not show up.

2009-07-20

- Took out the following parts and gave them to Kate & Mike

26.	pBad/araC	I0500	Plate 1	14N	pSB2K3 (K)
27.	pBad	I13453	Plate 1	1F	pSB1A3 (A)
28.	pLac	R0010	Plate 1	1D	pSB1A2 (A)

- Run gel to confirm PCR ITGA4 fragment and full length
    - Correct bands were observed for ITGA4 fragment

2009-07-21

- miniprep ANP construct and the Kill switch I716462.
- Digestion scheme:
    - pSB1AT3 digested with XbaI and SpeI
    - ANP construct digested with XbaI and BamHI
    - ITGA4 digested with BamHI and SpeI
    - pTet-GFP construct digested with XbaI and SpeI
- Ligated the ANP construct and ITGA4 into backbone pSB1AT3 (VLA construct plasmid)
- Ligated the pTet-GFP construct into backbone pSB1AT3
    - Buffer 2

2009-07-22

- digested the VLA construct plasmid with SpeI and treated with CIP phosphatase for 10min.
- ligated it with pTet-GFP construct
- transformed the plasmid into cells; plated on TET resistance plate overnight.

2009-07-23

- Observed lots of red colonies on the VLA construct plate, which indicates defective ligations.
    - This might be because the backbone pSB1AT3 was not digested properly and VLA construct was not inserted into the backbone.
- repeated the 2nd step of TEV protease PCR stitch with phfusion DNA polymerase
    - gel showed a band at around 700bp, which was the length of the expected PCR product.

2009-07-24

- plated the glycerol stock for pSB1AT3 and pSB1AC3 on Amp resistance plates

2009-07-27

- digested TEV protease and pSB1AT3 with Xba1 and Spe1; ligated the insert into the backbone.

2009-07-28

- ligated TEV protease to Ter-RBS in pSB1AC3

- digestion scheme:
  - TEV: XbaI + PstI + Buffer 2
  - Ter-RBS: EcoR1 + Spe1 + Buffer 2
  - pSB1AC3: EcoR1 + Pst1 + Buffer 2
- PCR:
  - ITGA1 – use Partial VLA tube; VLA Partial FWD + new ITGA REV primers
  - ANP construct – use I716462; new VLA backbone FWD + VLA backbone REV

2009-07-29
- Transformed the following plasmids into DH5alpha:
  - TEV protease in pSB1AT3
  - Ter-RBS-TEV in pSB1AC3
- Run gel to check PCR for ANP construct and ITGA4 fragment
  - Gel confirmed that ANP and I716462 miniprep samples were switched.
  - Bands for ANP construct did not show up
  - One very faint band was observed for ITGA4 fragment
- Run PCR to check primers for VLA backbone and ITGA fragment
  - Old ANP: use original VLA backbone FWD and REV primers
  - New ANP: use new VLA backbone FWD and old REV primers
  - Old ITGA4: use original VLA partial FWD and REV primers
  - New ITGA4: use old VLA partial FWD and new REV primers

2009-07-30
- make broth cultures (in triplicate) for TEV-pSB1AT3 and Ter-RBS-TEV-pSB1AC3
- make broth cultures for ITGA4 cDNA plasmid and ANP construct
- Run gel to confirm PCR in 2009-07-39
- Ligations:
  - P_{lux} (R0062) – [RBS-GFP-Ter] (E0840)
  - P_{const} (J23119) – [RBS=LuxI-RBS-GFP-Ter] (J37034)

2009-07-31
- one of the broth culture for TEV-RBS-TEV-pSB1AC3 was red (defective ligation); the other two broth cultures had red pellets after spinning down.
- none of TEV-pSB1AT3 broth cultures grew
- PCR
  - C1 (P_{const} – RBS)
  - I3 (LuxI construct)
  - P2 (P_{Lux} – RBS)
  - T3 (Ter – RBS)
  - pSB1AT3
  - -CT
  - ANP construct (with VLA backbone primers)
  - ITGA4 Partial

  - ANP construct (with SAA primers)
- Gel results:
  - PCR products for C1, I3, P2 and T3 did not show up – suggested that primer might not have worked.
  - SAA primers gave correct product. This suggests that the ANP construct was actually the SAA construct.

2009-08-01

- broth cultures for Ter-RBS-Ter ligation and Lux I construct were pink.
- LuxI construct culture had intense pink color.

2009-08-04

Digestion:

1.	P_{lux}	R0062 (4)	EcoR1 + Spe1 + Buffer (EcoR1)
2.	RBS(i)	B0034 (15)	EcoR1 + Spe1 + Buffer (EcoR1)
3.	RBS(ii)	B0034 (15)	Xba1 + Pst1 + Buffer 3
4.	P_{const}	J23119 (10)	EcoR1 + Spe1 + Buffer (EcoR1)
5.	Termin	B0015 (6)	Xba1 + Pst1 + Buffer 3
6.	RBS-LuxI	F1610 (12)	Xba1 + Pst1 + Buffer 3
7.	LuxI-GFP	J37034	Xba1 + Pst1 + Buffer 3
8.	pSB1AT3		EcoR1 + PstI + Buffer (EcoR1)
9.	GFP	E0840	Xba1 + Pst1 + Buffer 3
10.	Kill switch	I716462	EcoR1 + Spe1 + Buffer (EcoR1)
11.	Tev in pSB1AT3		Xba1 + Pst1 + Buffer 3

- Ran a 2% gel to check the bands; the gel did not work.
- Ran a 1% gel for 30min; imaged the gel, and then ran for another 30min.

Gel Results:

- pSB1AT3, GFP, and I716462 gave correct bands.
- P_{lux}, RBS, P_{const} and Ter were inconclusive because the correct bands
- RBS-LuxI, LuxI-GFP, and Tev did not give correct bands.

2009-08-05

Ligations:

1.	P_{lux} + RBS(ii) + pSB1AT3	insert length = 67
2.	RBS(i) + Tev + pSB1AT3	750
3.	I716462 + Ter + pSB1AT3	2050
4.	P_{const} + J37034 + pSB1AT3	1579
5.	P_{const} + RBS(ii) + pSB1AT3	47
6.	P_{lux} + GFP + pSB1AT3	933
7.	P_{const} + LuxI + pSB1AT3	833
8.	+CT: I716462 + GFP + pSB1AT3	2796
9.	–CT: pSB1AT3	

2009-08-06
- Digested ligation products 2, 3, 4,6,7 and 8 with EcoR1 + Pst1
- Run agarose gel to check ligations.
- Gel results were inconclusive because the bands were very faint.
- Transformed the ligation products into DH5alpha competent cells.

2009-08-07
- ran a SDS-PAGE for SAA samples: (loaded 20ul of sample)
  - transformed DH5alpha cells with the SAA construct
  - grew the cells in broth culture for 1, 2, and 3 days.
  - At each time point, spun the cells and collected the supernatant.
- The gel shows an increase in band intensity across days 1, 2, and 3.
- stained with coomassie for
- made broth cultures for the ligation plates; left in 37C overnight.

2009-08-08
- transferred the broth cultures to 4C bridge.

2009-08-10
- Miniprep the following broth cultures, which grew properly; made glycerol stocks.
  - P_{lux} – RBS in pSB1AT3 1A
  - RBS – TEV in pSB1AT3 2B
  - P_{const} – LuxI in pSB1AT3 7A
- Picked colonies and made broth cultures for the rest of ligation samples.

2009-08-11
- miniprep the following broth cultures, which grew properly; made glycerol stocks.
  - I716462 – Ter in pSB1AT3 3C
  - P_{const} – J37034 in pSB1AT3 4C
  - P_{const} – RBS – pSB1AT3 5C
  - P_{lux} – GFP in pSB1AT3 6C

Note: miniprep samples for #4 and #5 were both labelled identically. We need to use sequencing to determine which is which.

- Sent all seven ligation minipreps for sequencing.
- Digestion:
  - RBS – TEV EcoR1 + Spe1 + Buffer (EcoR1)
  - I716462 – Ter Xba1 + Pst1 + Buffer 3
- ligated the two parts; stored the ligation product in -20C overnight.

2009-08-12
- transformed the ligation product (RBS-TEV-I716462-Ter) into DH5alpha

- digested the ligation with EcoR1 and PstI and ran the sample on agarose gel to confirm ligation.

2009-08-17
- ran a SDS-PAGE on SAA samples and negative control samples (pSB1AT3); stained gel with Coomassie Blue.
- Both SAA samples and negative control samples showed bands.
- After checking the sequence, we realized that SAA construct does not have promoter or RBS. Therefore we need to ligate P_{lux}-RBS with SAA construct.
- Grew up broth cultures from ligation glycerol stock.

2009-08-18
- miniprep the ligation cultures.
  - Ligation #3 did not grow; ligation #2 had red pellet; ligation #6 had very low concentration.
- obtained SAA primary antibody from Tom
- ordered primers for CS2+MT Myc tag
- ran SDS-PAGE for SAA and block overnight in blocking buffer (TBST + 2% milk)
  - loading: day1(sup), day2(sup), day2(lys), day1(-ve), day2(-ve), day2(lys)
- measuring DNA concentrations of miniprep samples
  - Pconst-RBS 308 ng/uL
  - RBS-TEV 289
  - Pconst-J37034 73
  - Pconst-RBS 60
  - Plux-GFP 24 (too low)
  - Pconst-LuxI 158

2009-08-19
- incubated blot with primary antibody (1:500) in 2% milk (dissolved in TBST) at RT for 2 hours.
- Washed with 3X TBST
- Incubated the blot with anti-rabbit secondary antibody (1:10,000) for 1hour at RT.
- Chemiluminescence
- Film showed multiple non-specific bands in all lanes, including –CTLs.
  - Reason to this is that SAA construct does not contain promoter and RBS.

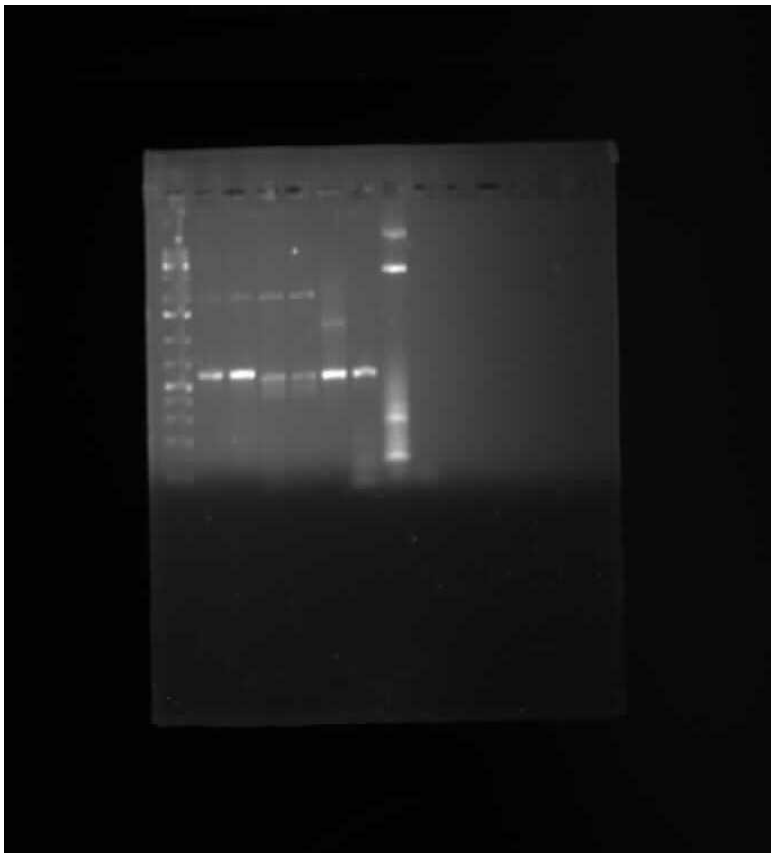
2009-08-21
- ran PCR with the new SAA primers

2009-08-22
- ran Agarose gel to check PCR – got strong bands
- Digestion using Fermentas restriction enzymes:

  - 16ul water
  - 2ul Buffer Tango (10X)
  - 1ul enzyme 1
  - 1ul enzyme 2
- Digestion scheme:
  - Pconst – RBS Digest with SpeI for 1 hour in tango, then EcoR1 for 1 hour
  - SAA XbaI + PstI in Tango buffer for 2 hours
  - pSB1AC3 EcoR1 + PstI in Buffer O for 2 hours
- Ligation (T4 Quick ligase)
  - 4ul Ouick ligase buffer
  - 1ul T4 ligase
  - 6ul X2 inserts
  - 2 ul pSB1AC3

2009-08-24
- miniprep J36846 (LppOmpA) part
- grew up pSB1AC3
- plate Pconst-RBS-SAA ligation

2009-08-25
- plates for SAA ligation did not contain any white colonies – need to redo ligation procedure.
- Search literature on LppOmpA; found that a weak promoter is required to express it in bacteria.
- **PCR#1:** SAA, CS1-MT (myc tag), and LppOmpA
- **Agarose Gel #1**



Loading: Ladder, LppOmpA1, LppOmpA2, SAA1, SAA2, SAA3, -CTL, Myc1, -CTL2

- Digestion:
  1. Pconst-RBS (4) EcoR1 + Spe1 + EcoR1 buffer
  2. Pconst-RBS (5) EcoR1 + Spe1 + EcoR1 buffer
  3. Plux-RBS EcoR1 + Spe1 + EcoR1 buffer
  4. pSB1AC3 EcoR1 + Pst1 + EcoR1 buffer
  5. Myc-tag Xba1 + Pst1 + Buffer 3
  6. SAA Xba1 + Pst1 + Buffer 3

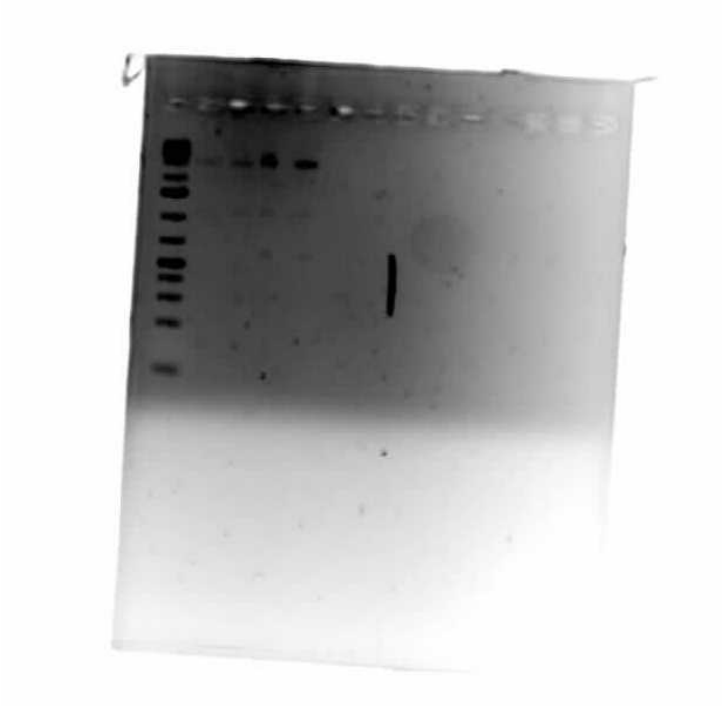
- Ligaton
  7. 1+6+4 Pconst-RBS-SAA
  8. 2+6+4 Pconst-RBS-SAA
  9. 3+5+4 Plux-RBS-Myc
  10. 3+6+4 Plux-RBS-SAA

- **PCR#2 –** LppOmpA with V2F FWD primer and RV2 primer
- **Agarose #2**
  11. Bands are faint – 2 possible errors:
      - PCR#1 did not work properly
      - PCR#2 did not work
  12. Need to run PCR#1 and #4 again

- Repeated PCR#2 (samples labelled LppOmpA1.1 and LppOmpA1.2)

2009-08-26

- Run gel to check PCR#2 repeat
  13. Correct bands showed up
- Digested the 4 ligations with EcoR1+Pst1 to check length
- Run gel to check ligations



- Loading: 1+6+4, 2+6+4, 3+5+4, 3+6+4, LOC2.1, LOC2.2,
- Bands seemed to indicate defective digestions of the ligation plasmids.

2009-08-27

- PCR #3 did not work for the 3rd time – need to redesign primers
- Miniprep ligation plasmids, digested with EcoR1 + PstI
- Made broth cultures for Pconst-RBS from glycerol stock

2009-08-28

- ran gel to check ligations
- miniprep Pconst-RBS 1, 2
- Digestion:
  1. Pconst – RBS EcoR1 + Spe1
  2. Plux – RBS EcoR1 + Spe1
  3. SAA Xba1 + Pst1
  4. Myc Xba1 + Pst1
  5. K091101 EcoR1 + Spe1
  6. R0040 EcoR1 + Spe1

7. RBS Xba1 + Pst1
8. pSB1AC3 EcoR1 + Pst1
9. pSB1AT3 EcoR1 + Pst1

- Ligation:
  I. Pconst-RBS-SAA 1+3+8
  II. Ptet-lac – RBS 5+7+9
  III. Ptet-RBS 6+7+9

Note: each ligation was done in duplicate (one was done using normal T4 ligation protocol and the other was done using Fermentas T4 Quick ligation protocol)

- Transformation:
  - Normal ligation (N) of (i) was plated on chloramphenicol plates.
  - Normal ligation (N) of (ii) and (iii) were plated on AMP plates due to plate shortage.
  - Quick ligations (Q) for (ii) and (iii) were plated on Tet plates.

2009-08-29

- made broth cultures from the plates

2009-08-30

- miniprep samples
  1. Pconst-RBS-SAA (N)
  2. Pconst-RBS-SAA (N)
  3. Ptet-RBS (Q)
  4. Ptet-lac – RBS (Q)
  5. Ptet-RBS (Q)
  6. Ptet-lac – RBS (Q)
  7. Pconst-RBS-SAA (Q)
  8. Pconst-RBS-SAA (Q)
- digest miniprep 1, 7, 3, 4 with fast Xba1/Pst1
- Run gel to confirm lengths
  1. 1 & 7 – 556bp (as well as miniprep 164+264)
  2. 3 – 66bp (confirmed)
  3. 4 – 95bp (confirmed)
- Gel loading:
  S1, S3, S4, S7, 1+6+4, 2+6+4
- observations:
  - bands were very faint – seemed like digestion did not proceed very well.
  - Need to try the gel again

- Digestions:
  - Pconst-RBS-SAA (1&7) E/S
  - Ter (B0015) X/P

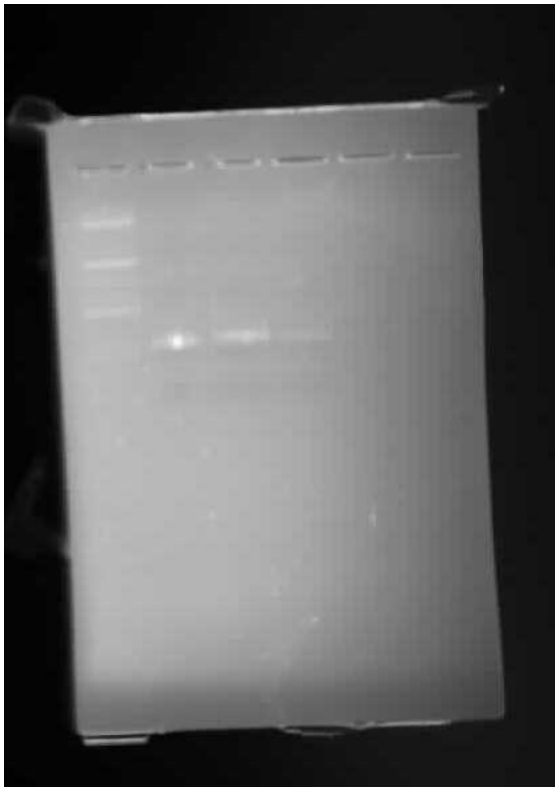
  - LppOmpA (J36846) X/P
  - Ptet-RBS E/S
  - Ptet-lac – RBS E/S
- ligations:
- Pconst-RBS-SAA (1) + B0015
- Pconst-RBS-SAA (7) + B0015
- Ptet-RBS (S3) + J36846
- Ptet-lac – RBS (S4) + J36846
- Agarose gel showed that ligations for Pconst-RBS-SAA-Ter did not work; but ligations for Ptet-RBS-Lpp-OmpA and Ptet-lac – RBS – Lpp-OmpA worked.
- We decided to ligate Ptet-RBS (S3) to SAA instead, because Ptet can be used as a constitutive promoter.

2009-09-01
- Digested miniprep tube S2 and S8 (see 2009-08-30) to check for Pconst-RBS-SAA
- Gel showed that S2 and S8 worked.
- Digestion:
  - Ptet-RBS (S3) with EcoR1 and Spe1
  - SAA with Xba1 and Pst1
  - pSB1AC3 with EcoR1 and Pst1
- Ligation:
  - Ptet-RBS (3) ligated to SAA (1) in pSB1AC3 in tube 9
  - Ptet-RBS (3) ligated to SAA (2) in pSB1AC3 in tube 10

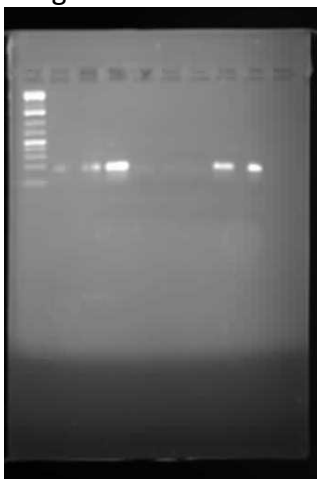
Summary
- *Pconst-RBS-SAA tubes 1 and 7 did not work.*
- *Pconst-RBS-SAA tube 2 and 8 worked!*

2009-09-02
Summary:
- All Ptet-RBS-LppOmpA broth cultures were red upon harvest spin
- All Ptet-lac – RBS-LppOmpA were also red
- Ptet-RBS-SAA plates yielded some white colonies

Actions taken:
- regrew broth cultures for Ptet-RBS-LppOmpA and Ptet-lac – LppOmpA plates
- started serial PCR using VF2 and R2 and the products from PCR round A
  - tubes labelled LOB1, LOB2
- PCR purify LOB1 and LOB2
- Started PCR round 3 for LOC1 and LOC2 using VF2 and R3
- Ran gel on LOB samples



- PCR purify LOC1 and LOC2
- Started PCR round 4 for LOD1 and LOD2 using VF2 and R4
- Ran gel on LOC and LOD
  - Gel showed bands around 200bp for LOC samples – need to repeat PCR
- PCR Round 4
  New samples called LOD1, 2, -ive
  Imaged round 3 on gel
  Loading: ladder, 1, 2, -ive
  Observations:
  - 1st set bands from previous gel ~600 vanished
  - 2nd set ~100-200
- Ran gel for PCR round 3 and 4

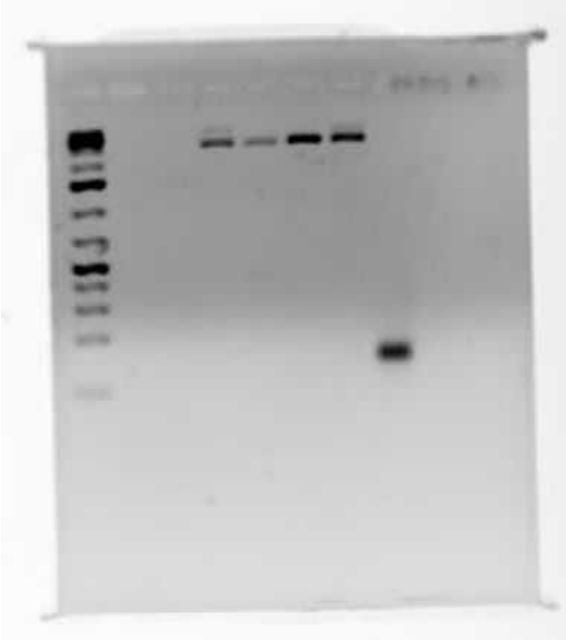


Loading:
Ladder, LOD1, LOD2, -ive, LOC1, LOC2, -ive, LOD1, LOD2

Observations:
- showed bands at 200bp – repeat PCR from step 2
- Tubes labelled LOB1 rep 2, LOB2, rep2, and –ive rep2
- PCR running w/ DEPC H_2O

2009-09-03

- PCR purified LOB1+2, rep2 (and gel)
- Miniprep
  - Ptet-RBS-SAA
  - Ptet-RBS-Lpp-OmpA
- Digestion:
  - Pconst-RBS-SAA (S2) E/S
  - S8 E/s
  - B0015 X/P
- Run PCR #3 on LOD1 + LOD2, RBS
- Run gel for LOC1, LOC2 and –CTL



Loading:
Ladder, LOC1 (rep2), LOC2, (rep2), S2-B0015, S8-B0015, 9-B0015(1), 9-B0015(2), -CTL

- Troubleshooting (talking to Ian)
  - Lower annealing temp to 3.0°C below Tm of R3
  - Use 2:10 use 2ul/rxn of purified R2 for R3
  - Try to re-amplify R2 and if ok use R3 template
  - Remove aliquots at 25 cycles

Protocol named IGEM-°C

PCR R2 and R3

- A – dilute R2 – for R3
- B – regular R2 – for R3
- C – dilute R2 – reamplify
- D – regular R2 – reamplify

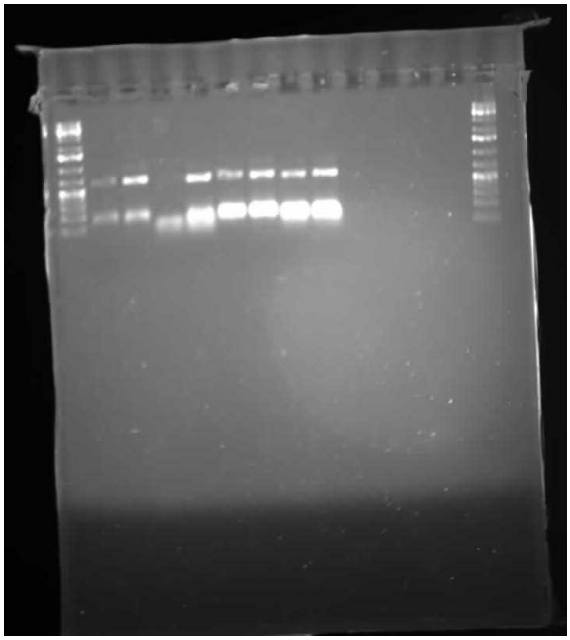
} Purified band

- E – dilute R2 – for R3
- F – regular R2 – for R3
- G – dilute R2 – reamplify
- H – regular R2 – reamplify

} PCR purification kit

- Fast digest may not have worked – try again with regular digestion
- Repeated PCR 3 with updates
- Checked plates: left ligations to grow longer
- Removed 7ul of PS0 PCR sample at end of cycle 28 and deactivated at 72°C for 10min.

Gel run
- cycle 28 aliquot loaded
-



Loading:
Ladder, A – H, space, ladder

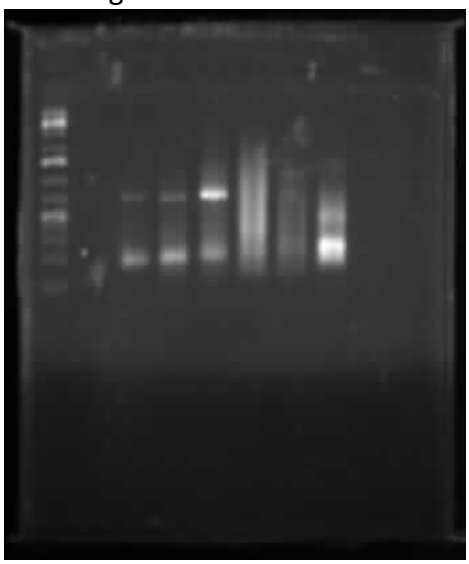
Observations
- all bands for PCR1 showed
- excised R3 bands
- Ian suggests hot start and separate mm for +ctl (ie reamplification)

2009-09-05
- Ran PCR 4 on A, B, E, and F
- Picked colonies from P_{tet}-RBS-SAA-TER (1+2) and P_{const} – RBS – SAA – Ter (S8 – B0015)
- Plates for S8-B0015 and S2-B0015 were put back into 37°C due to the lack of colonies.
- Minipreped pSB1AC3 and pSB1AT3
- PCR purification
  - LODA, LODB, LODE, LODF

2009-09-06
- pick colonies from S8-B0015 plate (tube 2+3)
- pick colonies from S2-B0015 (tube 1)
- made glycerol stocks for tube 1
- minipreped P_{tet}-RBS-SAA-Ter

2009-09-07

- Ran gel for R4 PCR



Loading

Ladder, A, B, E, F, B

Observations

- No bands >500bp. R4 did not amplify
- Check temp and rerun on Tue

2009-09-08

- miniprep SAA construct – called "SAA Full"
- SAA +ive ctl growing for set up tomorrow
- Digest SAA to confirm

2009-09-09

SAA experiment started at 11:30am

- T2 is at 1:30pm
- T4 is at 3:30pm
- Overnight = 11:30am

OD Instruction (OD600)

- cuvette with 1ml media (blank)
- place cuvette sideways
- press ref to blank
- OD>1 means dilute sample

OD 600	T0	T2	T4	T24
pSB1AT3 sup	0.058	0.560	1.601	2.716
pSB1AT3 wcl	0.063	0.600	1.582	2.822
SAA sup	0.040	0.330	1.306	1.358
SAA wcl	0.036	0.324	1.321	2.841

- must control the vol of sample removed when resuspending whole cell lysate to lyse. You need to keep the same concentration of cells.
    - if 3mL removed, resuspend relative to sample if 1.5ml removed.

2009-09-14
- western blotting samples normalized against OD=0.324
- samples were boiled w/ SDS sample buffer for 3mins
- loading (T0 and T2 were ran on gel 1, T4 and T24 on gel 2)
    - Media ctl
    - pSB1AT3 sup
    - SAA sup
    - pSB1AT3 wcl
    - SAA wcl
    - Ladder
    - pSB1AT3 sup
    - SAA sup
    - pSB1AT3 wcl
    - SAA wcl

2009-09-15
- Prepared overnight broth culture for cells containing Lpp-OmpA-TEVx2-linker in pSB1AT3
- Continued western blot
    - Washed blots 3X15min in TBST
    - Incubated blots with 1° Ab (1:500 dilution in 2% skim milk TBST) for 2hrs
    - Washed blots 3X15min in TBST
    - Incubated with 2°Ab (1:10,000 in 2% skim milk dissolved in TBST) overnight at 4°C.

2009-09-16
- broth culture made on 09-15 did not grow – need to remake broth cultures
- Digested RBS and Myc tag with DraI restriction enzyme
- Purified digestion products using PCR purification kit.
- Ligated RBS and Myc tag and inserted into pSB1AT3

2009-09-17
- The Tet plate for RBS-Myc ligation overgrew – defective antibiotic plate
- Digestion:
    - Ptet-RBS-SAA-Ter (A, B, F) digested with E/P
    - pSB1AT3 with E/P
    - RBS with Dra1
    - Myc with Dra1

2009-09-28
- received SAA positive control sample (1ug/ml) from Tom Tam in Dr. Roger Deeley's lab at Queen's Cancer Research Institute

  - HDL-SAA derived from inflamed mice
- Received SAA polyclonal antibody (1:1000 dilution)

2009-09-29
- Ran SDS-PAGE on SAA samples
- Sample preparation:
  - +CTL (1ug/ml)
    - Concentration 10: mixed 55ul of +CTL with 11ul of 6XSDS buffer
    - Concentration 1: mixed 5.5ul of +CTL with 49.5ul of ddH_2O and 11ul of 6XSDS buffer

    This method was used to prepare all samples; boiled samples at 100°C for 10min
  - made 120ul of SAA T0 and T4 whole cell lysate (wcl)
- Run SDS-PAGE at constant Amp (40mA) for 90min
- Blocked blots in 5% milk for 1hr
- Incubated blots with 1°Ab overnight at 4°C.

2009-10-2
- Ordered new primers for making the binding construct
- *PCR round 1:*
  - PCR out Ptet-RBS from Part () using BioBrick primers (V2F and VR)
  - PCR out Lpp-OmpA-Linker-TEVx2-Linker fragment from Mr. Gene construct using primer "Ptet Stitch FWD" and "VLA stitch REV"
  - PCR amplify the whole Mr. GENE construct using primer: "PCR Whole FWD" and "PCR Whole REV"
- *PCR round 2:*
  - PCR stitch Ptet-RBS fragment to Lpp-OmpA-TEVx2-Linker fragment using primer: V2F and "VLA stitch REV"
  - PCR out ITGA4 fragment from ITGA cDNA using primer: "VLA Partial FWD + REV"
- Run 1% agarose gel to confirm *PCR round 1*:

Loading scheme (left to right): ladder, Ptet-RBS fragment #1 (lane 2 & 3), Construct fragment for stitching, Amplified construct

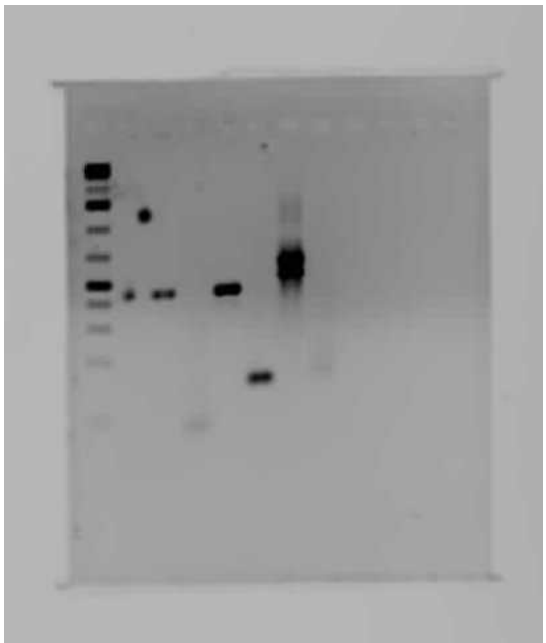
Observations
- samples floated out of the wells during loading
- might suggest that samples contained ethanol from PCR purification step.

2009-10-05

- *PCR round 3:*
  - PCR stitch ITGA4 fragment to Ptet-RBS-construct
    - Template: VLA stitch fragment Oct. 2 + Ptet-construct Oct. 2
    - Primer: V2F + VLA Partial REV
  - PCR ANP fragment out of the Mr. Gene construct
    - Template: Amplified construct Oct. 2
    - Primer: ANP FWD + PCR Whole REV
- Ran 1% Agarose gel to confirm *PCR round 2* from 10-02

Loading scheme:
Ladder, Ptet-RBS-Construct-VLA, ANP, Blank, ANP, -CTL, Ptet-RBS-Construct, VLA4

Observations:
- samples floated out of the wells when loading
- bands were very faint
- This suggested that PCR purification was defective.



2009-10-06

- Re-amplify the products from PCR round 1,2 and 3.
  - Construct fragment for stitching
  - Ptet-RBS-construct-VLA
  - ANP
  - VLA4 fragment
  -
- Agarose gel:

Loading scheme:

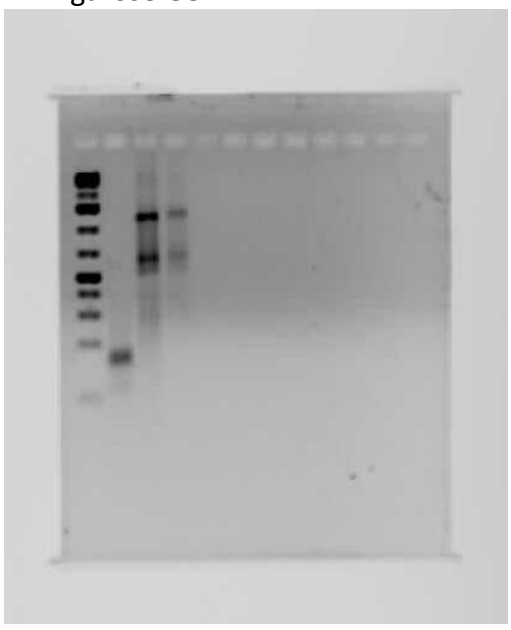
- ladder, VLA 1, VLA2, Ptet-construct, VLA 1, ANP, construct fragment, Ptet-construct-VLA

Observations:

- samples floated out of the wells again!
  - Need to use a different PCR purification kit/protocol
- Ptet-construct stitch and Ptet-construct-VLA stitch did not work!

2009-10-07

- Rerun *PCR round 2*
  - Template: Ptet-RBS stitch Oct 2 + Construct stitich Oct. 2
  - Primer: V2F + VLA stitch REV
  - sample labelled "Ptet-construct Oct. 7"
- PCR purification was done using () kit.
- Agarose Gel



Loading scheme:
Ladder, -CTL, unpurified sample, purified sample

Observations:

- one band around 1300bp
- one band around 600bp
- one faint band around 500bp

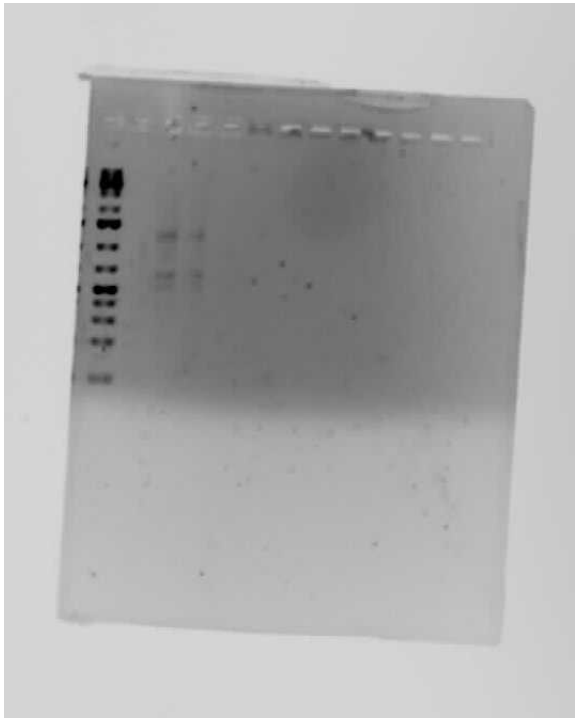
2009-10-08

- Repeated *PCR round 3*
  - Template:
    - Ptet-RBS-construct Oct. 7 (4ul) – used both purified and unpurified samples
    - VLA stitch frag 2 Oct. 6
  - Primer: V2F + VLA Partial REV

  - Sample labelled: Binding construct Oct. 7
- Ran Agarose gel to check PCR

2009-10-09

- repeated PCR stitch of Ptet-RBS to Construct
  - Prepared PCR reaction containing Ptet-RBS-GFP-Ter template and construct fragment template and ran PCR without primers for 15 cycles
  - Took out samples and added V2F primer only. Ran PCR for 20 cycles
  - Sample labelled: Ptet-Construct Oct. 9



Loading scheme
Ladder, -CTL, Ptet-construct-VLA1 and 2

Observation:
- one dark band at around 1000bp
- one dark band at around 600bp
- one weak band at around 500bp
- 600bp band might be the band for correct stitching.

2009-10-09

- Digestions:
  - ANP frag 2 Oct. 6 E/P
  - Amplified construct Oct. 2 E/P
  - VLA stitch frag 2 Oct. 6 E/P
  - pSB1AC3 E/P

- ligations using Quick ligation protocol:
  - ANP + pSB1AC3 (labelled ANP L Oct. 9)
  - Whole + pSB1AC3 (whole L)
  - VLA + pSB1AC3 (VLA L)
- Transformed into Top 10; plated on CM overlay plates

2009-10-10

- checked plates
  - only VLA plate yielded white colonies
  - overnight broth culture was made

2009-10-12

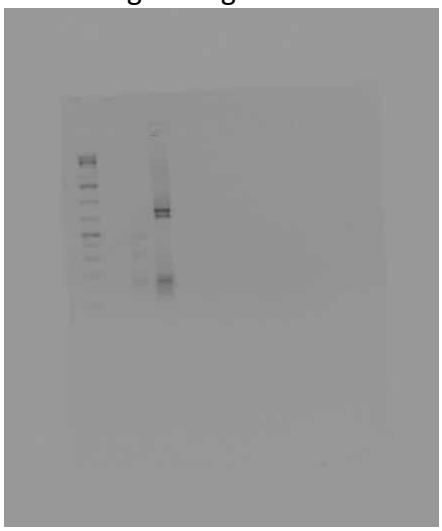
- re-plated the transformed cells from 10-09 on CM overlay plates
- made glycerol stock for VLA in pSB1AC3 (labelled VLA Oct. 10, stored in -80°C box)

2009-10-13

- repeated digestion:
  Fermentas digestion procedure:
  - 1ul EcoR1
  - 1ul Pst1
  - 5ul Buffer O (10X)
  - 10ul DNA
  - 33ul ddH_2O

  Total vol = 50ul
- Digested ANP and pSB1AC3 – treated with CIAP for 1hr
- PCR purified pSB1AC3 digestion
- Ligation using Quick ligation procedure
  - Ligated ANP to pSB1AC3 for 3hrs
- transformed cells into Top 10 and plated on CM overlay plates
- Ran gel for Ptet-Construct PCR stitch
  - Loaded 20ul of "Ptet-Construct Oct. 9" and 20ul of "Ptet-construct Oct. 7"
- Excised the bands 600bp; purified using Gel purification kit
- PCR
  - Reamplify Ptet-Construct using primers V2F for 30 cycles

  - PCR stitch VLA to Ptet-RBS-construct
  - PCR amplify Mr. Gene whole construct (running low)
- Ran Agarose gel



loading:
ladder, reamplification, stitch Oct. 13, whole construct, -CTL

Observation:
- Ptet-Construct-VLA stitch (lane 3) did not work
  - Expected band should be ~900bp

2009-10-14
- checked plates for ANP ligation
  - very few white colonies – likely due to phosphatase treatment of pSB1AC3
  - made overnight broth culture
- transformed ANP and Whole construct ligations into TOP10; plated on CM plates

2009-10-15
- made glycerol stock for ANP ligation from broth culture
- minipreped ANP
- made broth culture for Whole construct ligation.