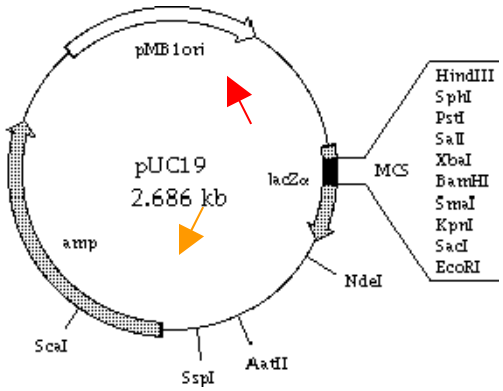
1/28/08

PCR to test primers and pUC19

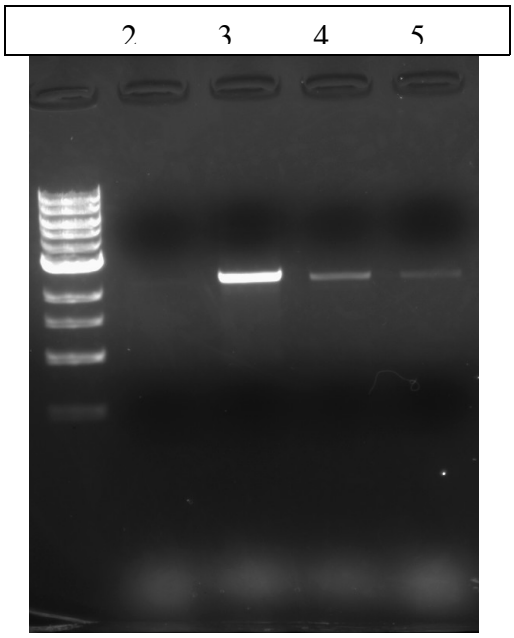


PCR Product should be 2.4 kB in length.

- Calculating primer concentration:
  - MW = 330 g/nucleotide * 30 mers = 10000 g
  - Concentration of primer: 10 uM
  - 10e-6 M *10000g = .1 g/L = .1 ug/uL = 100 ng?ul

PCR protocol:

Water	37
10x PfuTurbo Cx Buffer	5
10 mM dNTP	1
DNA Template	4
Primer 1	1
Primer 2	1
PfuTurbo Cx	1
	50



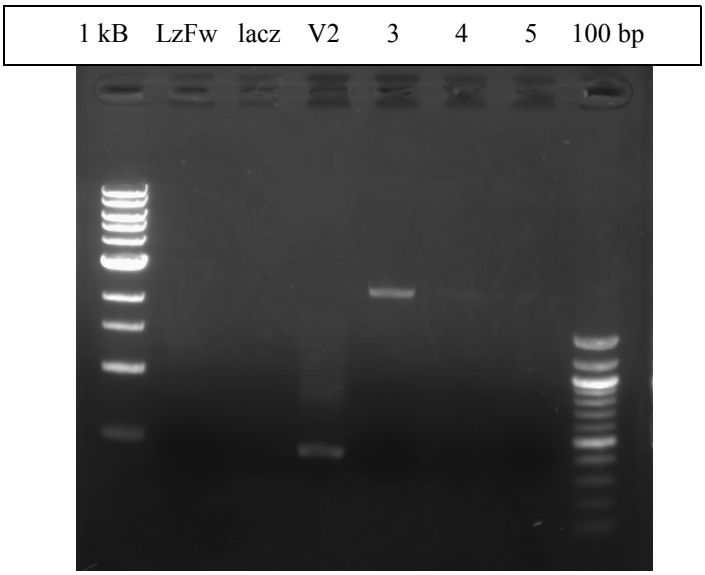
Cycle	Step	Temperature	Time
1	x1	95ºC	2 min
2	x30	95ºC	30 s
		Primer Tm-5ºC (55ºC)	30 s
		72ºC	2.5 min
3	x1	72ºC	10 min

List of primers for the gel:

1. pUC19Fw, pUC19Rv
2. pUC19ct1Fw, LzgfRv
3. pUC19Fw, pUC19ctlRv
4. pUC19Fwcat2, pUC19RvHindIII
5. pUC19FwphaB, pUC19RvHindIII

3, 4 , 5 were present and were gel purified

Gel Analysis after purification to see which DNA fragments present



*We are only interested in 5 which is not very apparent

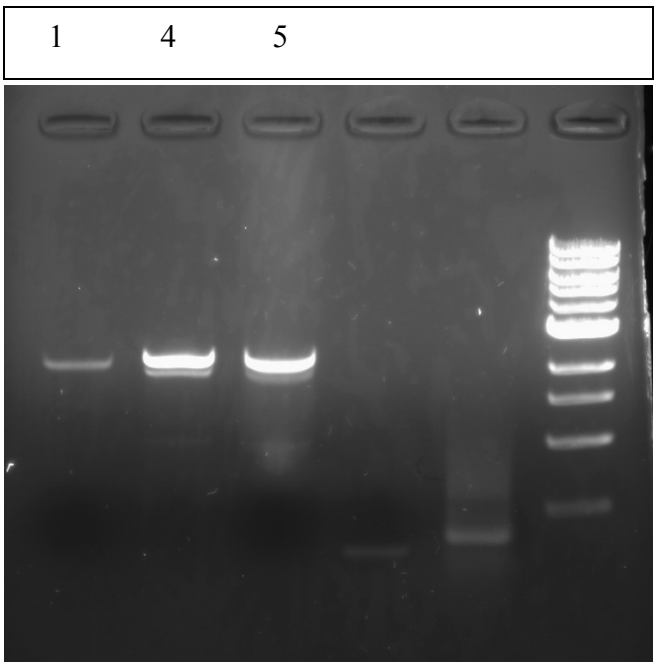
Redo PCR for 1,4,5

Water	35
10x PfuTurbo Cx Buffer	5
10 mM dNTP	1
DNA Template	6
Primer 1	1
Primer 2	1
PfuTurbo Cx	1
	50

Cycle	Step	Temperature	Time
1	x1	95ºC	2 min
2	x30	95ºC	30 s
		50ºC	30 s
		72ºC	2.5 min
3	x1	72ºC	10 min

1/29/08

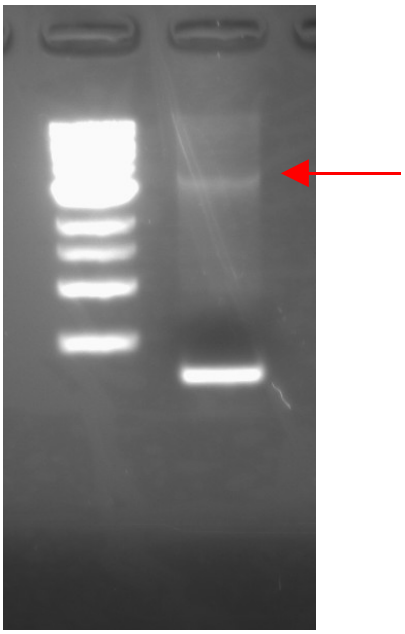
Gel of Redo PCR for 1,4,5



*Much greater increase in product for 4 & 5

PCR Genomic DNA

Water	40
10x PfuTurbo Cx Buffer	5
10 mM dNTP	1
DNA Template	1
Primer 1	1
Primer 2	1
PfuTurbo Cx	1
	50



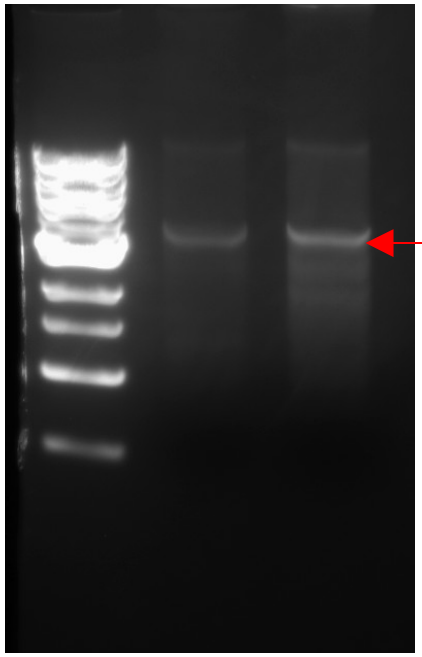
Cycle	Step	Temperature	Time
1	x1	95°C	2 min
2	x30	95°C	30 s
		55°C	30 s
		72°C	2.5 min
3	x1	72°C	10 min

*Didn't take longer extension times into account so major product too small. But the genomic DNA did seem to PCR out.

Redid PCR Genomic DNA

PCR Genomic DNA

Water	40	39
10x PfuTurbo Cx Buffer	5	5
10 mM dNTP	1	1
DNA Template	1	2
Primer 1	1	1
Primer 2	1	1
PfuTurbo Cx	1	1
	50	50



Cycle	Step	Temperature	Time
1	x1	95ºC	2 min
2	x30	95ºC	30 s
		55ºC	30 s
		72ºC	4 min
3	x1	72ºC	10 min

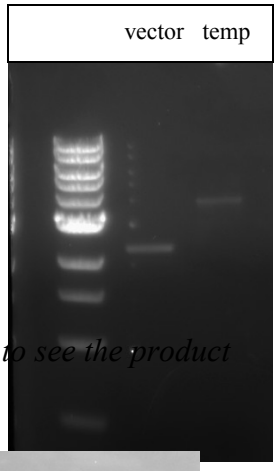
(Gel taken on 1/30/08)

The gel showed that the 2 ul template had a slightly more distinct band but not by much

1/30/08

Gel Purification and PCR product

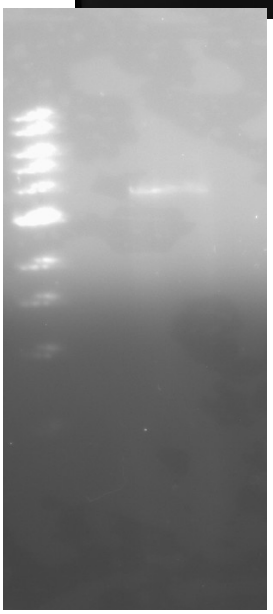
This is the image of the DNA after it had been gel purified. The template DNA consists of the 1 ul and 2 ul DNA from the previous gel combined into one tube and thus 1 lane.



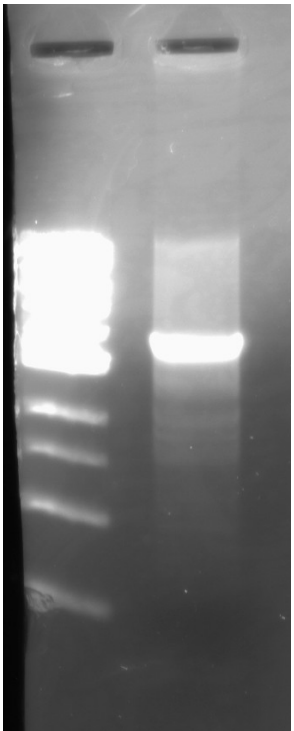
Vinny did another PCR and I looked at the gel of that PCR to see the product

Gel of 2nd PCR:

There was something wrong with the gel, perhaps the TAE buffer? The remaining PCR product was examined in a new gel.



This gel was very successful and showed a very clear band. This band was cut and put away for future extraction.



USER Ligation

With the first gel, that showed product (see first image in 1/30/08), a ligation was carried out.

	C	E
Water	12	0
Taq Buffer	1	1
phaCAB insert	0	12
#5 pUC19 vector	6	6
USER Enzyme	1	1
	20	20

Transformation of the plasmids into bacteria

Plated 5 plates:

1. pUC19 vector control
2. USER ligation control (100 ul)
3. USER ligation control (rest of bacteria)
4. Vector + insert (100 ul)
5. Vector + insert (rest of bacteria)

Nothing grew except for the pUC19 vector control which suggests that there was nothing wrong with the transformation itself.

2/4/08

Gel Purified previous PhaCAB insert

1. Used 3 volumes of Resuspension buffer & 10 ul of agarose beads
2. Didn't vortex DNA sample & resuspended by flicking instead
3. 20 ul of EB buffer used to elute.
4. Measured DNA came out to 5.9 uL

Ran PCR of insert and vector

Water	37	32
10x PfuTurbo Cx Buffer	5	5
10 mM dNTP	1	1
DNA Template	1	6
Primer 1	2.5	2.5
Primer 2	2.5	2.5
PfuTurbo Cx	1	1
	50	50

Forgot dNTP!

Cycle	Step	Temperature	Time
1	x1	95°C	2 min
2	x30	95°C	30 s
		55°C	30 s
		72°C	4 min
3	x1	72°C	10 min

Repeated PCR

	vector	gel purified	insert 1/31	insert 2/4
Water	32	37	37	37
10x PfuTurbo Cx Buffer	5	5	5	5
10 mM dNTP	1	1	1	1
DNA Template	6	1	1	1
Primer 1	2.5	2.5	2.5	2.5
Primer 2	2.5	2.5	2.5	2.5
PfuTurbo Cx	1	1	1	1
	50	50	50	50

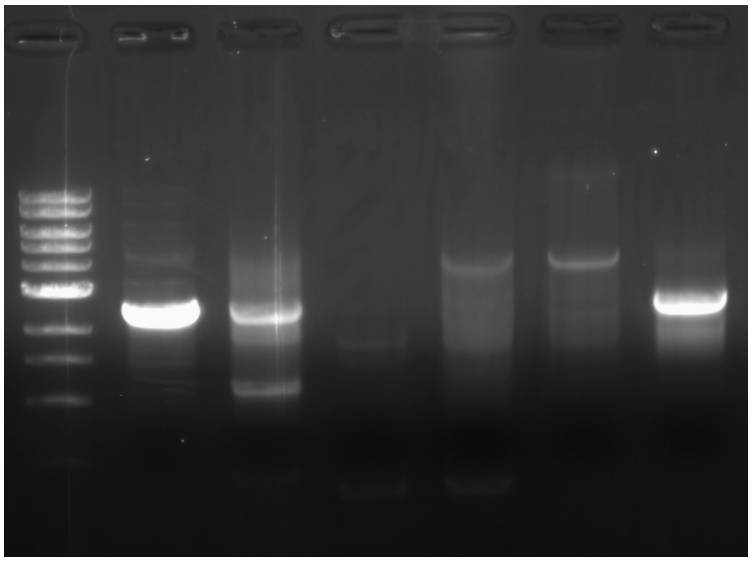
*The insert from 2/4 was made by either Vinny or Zhongying
and was verified by gel purification

Cycle	Step	Temperature	Time
1	x1	95°C	2 min
2	x30	95°C	30 s
		65	0s
		55°C	30 s w/ 1°C/s ramp
		72°C	4 min
3	x1	72°C	10 min

2/5/08

Gel from 2/4/08 PCR

vector	gel puri. vector	1/31 insert	2/4 insert	genomic insert	vector2



The genomic insert and vector 2 were done by Zhongying separately.
The 2/4/08 insert showed some product and the pUC19 had a very strong band.

<u>Gel purification and measurement of DNA concentrations</u>

DNA	ng/ul	260/280
phaCAB vector (lane 6)	17.65	1.81

phaCAB insert (lane 5)	3.34	2.39
vector phaCAB (lane 1)	27.01	1.96

USER Ligation

Used pUC19 vector from lane 1 & Zhongying's genomic DNA from lane 5

PCR Product:

Took it straight from PCR and not from gel purified product

	C	E
Water	13.5	0
10x Turbo Buffer	.5	0
phaCAB insert	0	15
pUC19 vector	4	4
USER Enzyme	1	1
	20	20

Gel Purified Product

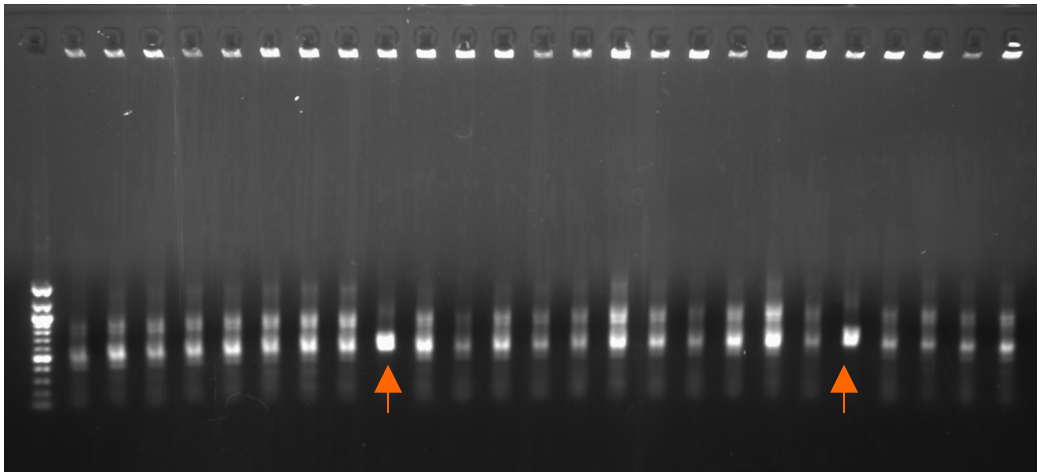
	C	E
Water	15	0
10x Turbo Buffer	2	2
phaCAB insert	0	15
pUC19 vector	2	2
USER Enzyme	1	1
	20	20

2/6/08

PCR of Colonies to check for insert

*Used FwC and RvB primers so should be 3.9 kB

1. 25 colonies total all from PCR USER ligation and none from gel purified.
2. PCR seemed to suggest 2 matches: 9 & 21



Inoculation of colonies 9 & 21

1. 3 mL LB broth and 60ug/mL ampicillin

2/7/08

Vinny did:
Plasmid preps

Digestion and gel to check plasmids
Gels leaked for some reason

2/8/08

Plasmid Purification of TOPO cloning

1. Zhongying tried new method using blunt end cloning and PCR TOPO vector and got many colonies
2. Grew up the colonies for a while.
3. We screened 4 colonies and purified the plasmids (1,13,15,18,9,21)

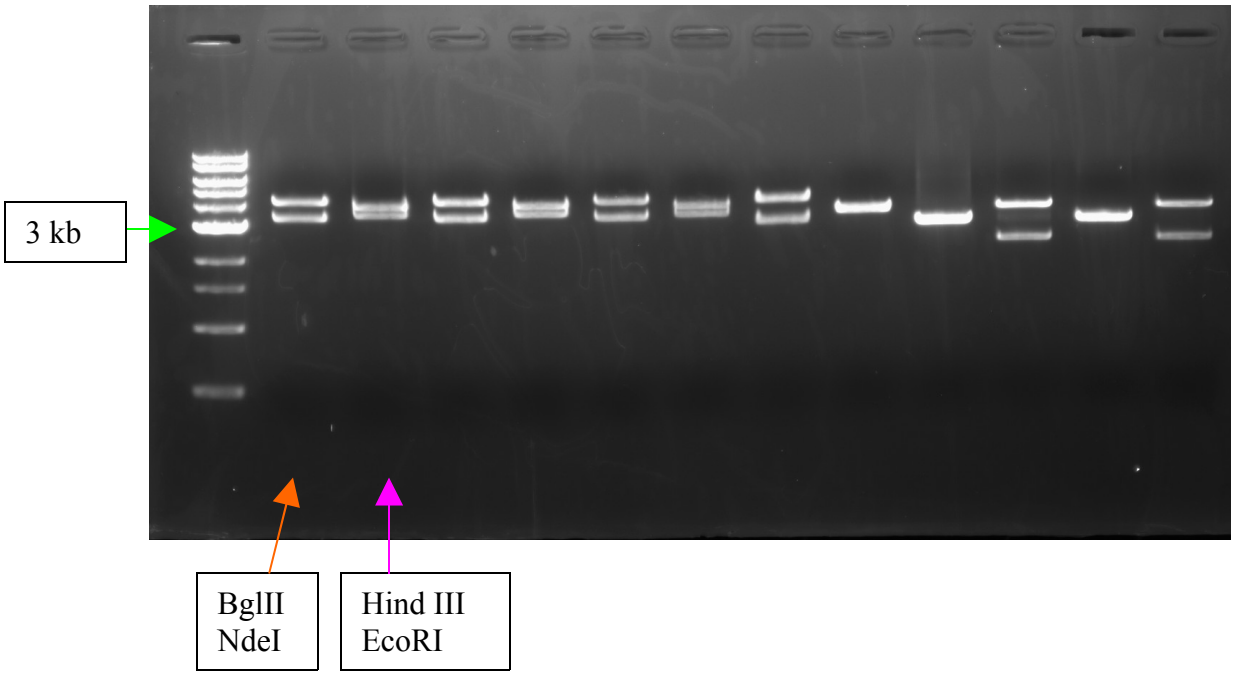
Digestion of the screened plasmids

2 checks were made

DNA	8
Buffer 2	1
Bg1 II	.5
Nde I	.5
	10

DNA	7
BSA	1
Buffer 2	1
Hind III	.5
EcoRI	.5
	10

1 13 15 18 9 21



9 & 21 (User Enzyme): BglII + NdeI → 3.0 kb & 3.3 kb
HindIII + EcoRI → 3.9 kb & 2.4 kb
1,13, 15, 18 (TOPO): BglII + NdeI → 3.3 kb & 4.1 kb
HindIII + EcoRI → 3.5 kb & 3.9 kb

2/12/08

Digestion to check direction of TOPO plasmids
(1, 13, 15, 18)

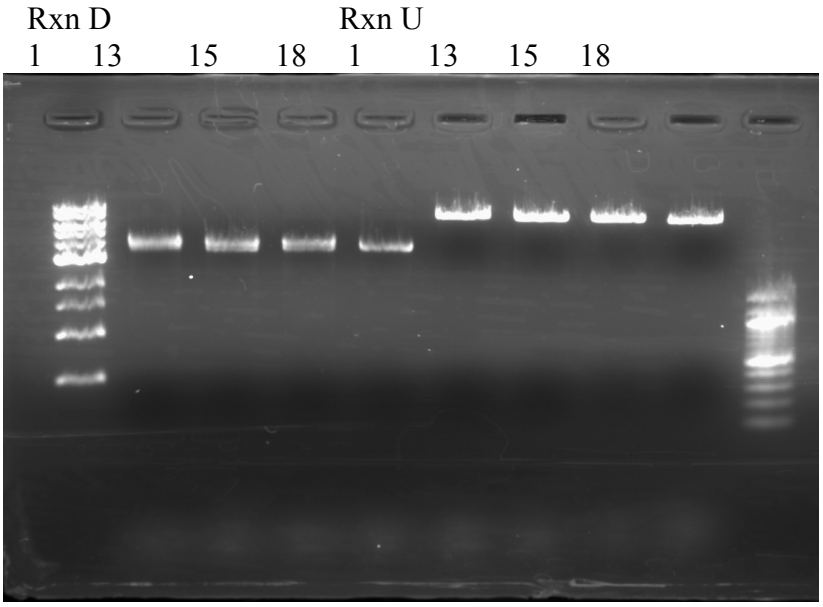
2 checks were made

Rxn D (check direction)

DNA	7
Buffer 2	1
BamHI	.5
Nde I	.5
BSA	1
	10

Rxn U (check if u from primers still there)

DNA	8
Buffer 2	1
Hind III	1
	10



If correct, should have 2 bands, 1 at: 4059 and another at 3328
Rxn D seems to be correct because the bands are rather close in size. Thus, they would be hard to discern. Rxn U also seems correct because there's a band.

5/19/08

PCRs of Rxns 1, 2, 5, 6, 7, 8 while adjusting for the annealing temperatures of each primer

H_2O	28.5
L Primer	2.5
R Primer	2.5
Template (diluted)	2
5x Buffer	10
DNTP	4
Phusion	.5
	50 ul

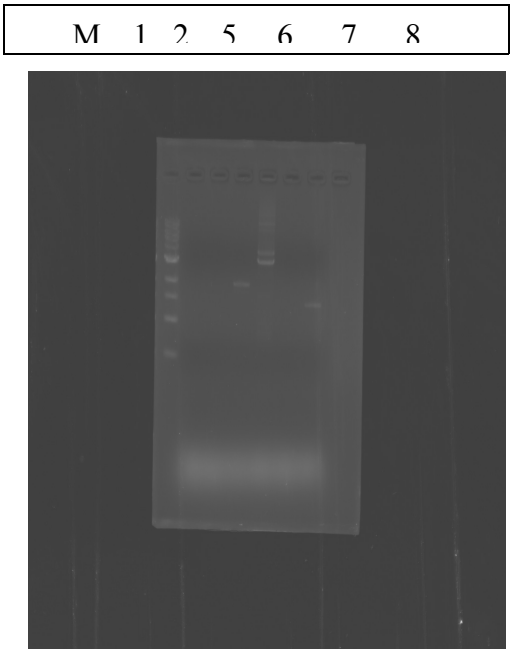
Rxns 1, 2, 6, 7

98ºC	30s	x1
98ºC 60ºC 72ºC	10s 30s 1 m	x5
98ºC 68ºC 72ºC	10s 30s 1 m	x25
72ºC 4ºC	10 m ∞	x1

Rxns 5, 8

98ºC	30s	x1
98ºC 58ºC 72ºC	10s 30s 30s	x5
98ºC 66ºC 72ºC	10s 30s 30s	x25
72ºC 4ºC	10 m ∞	x1

Gel Picture:



*Only 5 & 8 had bands and 6 had multiple bands. 6 could be because undiluted template was used.

Redo PCR for rxns 1,2, 6, 7

H_2O	28.5
L Primer	2.5
R Primer	2.5
Template (original/undiluted)	2
5x Buffer	10
DNTP	4
Phusion	.5
	50 ul

Condition 1:

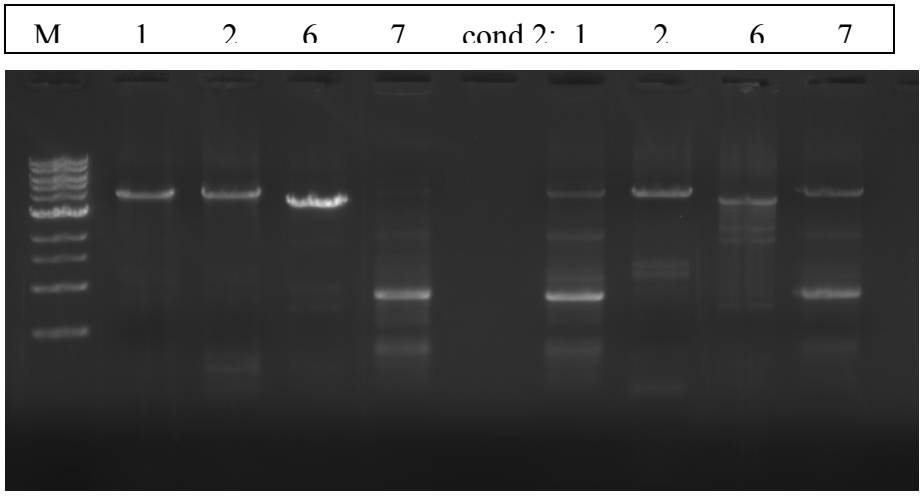
98ºC	30s	x1
98ºC 59ºC 72ºC	10s 30s 2 m	x30
72ºC 4ºC	10 m ∞	x1

Condition 2:

98ºC	30s	x1
98ºC 66ºC 72ºC	10s 30s 2 m	x30
72ºC 4ºC	10 m ∞	x1

*For reaction 6, the template phaC (colony 2) was diluted 1:10 and 2 ul was used as the template

Gel Picture:



5/20/08

PCR Purification of 1, 2, and 6 for condition 1. Read concentrations and performed digestion

Digestion:

Rxn 1:

	Insert (70)	PASK vector (60 ng)
H_2O	15	15
10x buffer	2	2
DNA	1	1
XbaI	1	1
BamHI	1	1
	20	20

Rxn 2:

	Insert (70)	PASK vector (60 ng)
H_2O	15	15
10x buffer	2	2
DNA	1	1
EcoRI	1	1
BamHI	1	1
	20	20

Rxn 5&6:

	Insert 5 (60)
H_2O	13
10x buffer	2
DNA	3
XbaI	1
EcoRI	1
	20

	Insert 6 (60)
H_2O	15
10x buffer	2
DNA	1
EcoRI	1
XhoI	1
	20

	PASK vector (60 ng)
H_2O	15
10x buffer	2
DNA	1
XbaI	1
XhoI	1
	20

37°C for 2 hrs and then heat shock at 80°C for 5 min
PCR purification following digestion

Ligation

Reaction 1:

	No ligase	No Insert	Exp
Insert	5.5	X	5.5
Vector	3	3	3
Ligase	X	.5	.5

Reaction 2:

	No ligase	No Insert	Exp
Insert	5.5	X	5.5
Vector	3	3	3
Ligase	X	.5	.5
Buffer	1	1	1
H_2O	.5	5.5	X
	10	10	10

Buffer	1	1	1
H_2O	.5	5.5	X
	10	10	10

Reaction 5&6:

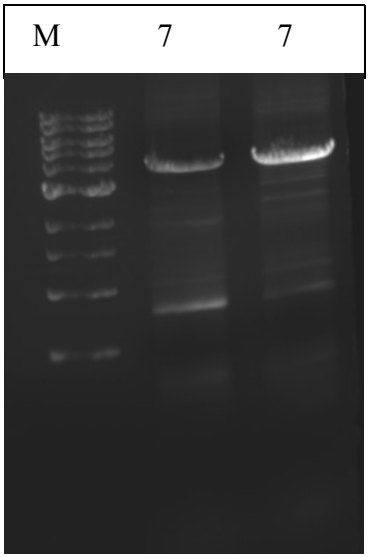
	No ligase	No Insert	Exp
Insert (5) Insert (6)	3 3.5	X	3 3.5
Vector	2	2	2
Ligase	X	.5	.5
Buffer	1	1	1
H_2O	.5	6.5	X
	10	10	10

Room temperature for 1 hour

Redo PCR for 7 using condition 2

H_2O	28.5
L Primer	2.5
R Primer	2.5
Template (original/undiluted)	2
5x Buffer	10
DNTP	4
Phusion	.5
	50 ul

98°C	30s	x1
98°C 66°C 72°C	10s 30s 2 m	x30
72°C 4°C	10 m ∞	x1



This PCR is to get more product. The product from this reaction will be combined with the PCR from yesterday (condition 2) and gel purified to get Rxn 7.

Transformation

- 50 ul cells
- 150 ul SOC medium
- 2 ul ligation product
- 9 plates total (3 per reaction)
- Plated on chloramphenicol Nile Red plates

5/21/08

Transformation from yesterday

-The transformation seemed successful. There were many colonies on the experimental plates→ more than on the control plates.
-1: more colonies on no insert plate than there should've been
-2: very good results: few colonies on no insert and a lot on experimental
-5&6: more colonies on experimental but also a good number on control

Digestion of 7, 8

	Insert 7 (60)
H_2O	13
10x buffer	2
DNA	3
XbaI	1
EcoRI	1
	20

	Insert 8 (65)
H_2O	11
10x buffer	2
DNA	5
EcoRI	1
XhoI	1
	20

	PASK vector (60 ng)
H_2O	15
10x buffer	2
DNA	1
XbaI	1
XhoI	1
	20

37°C for 2 hrs and then heat shock at 80°C for 5 min
PCR purification following digestion

Ligation

	No Insert	Exp
Insert (7) Insert (8)	X	3.5 2.5
Vector	2.5	2.5
Ligase	.5	.5
Buffer	1	1
H_2O	6	X
	10	10

Transformation

- 50 ul cells
- 150 ul SOC medium, 2 ul ligation product
- 2 plates total (1 control, 1 experimental)
- Plated on chloramphenicol Nile Red plates

Inoculation

- 20 ug/ml chloramphenicol for 3 mL total
- 1: 8 colonies
- 2: 4 colonies
- 5&6: 8 colonies

5/22/08

Plasmid Miniprep

- Miniprep of 1, 2, 5&6: 20 total
- Forgot to save bacteria! Remember to do that next time
- Spun for 4 min at max speed

Restriction Enzyme Digestion

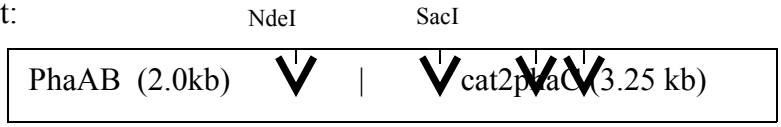
Rxn 1: BamHI, XbaI
- insert + vector: 3.87 kb + 2.92 kb

Rxn 2: EcoRI, BamHI
- insert + vector: 3.85 kb + 3.0 kb

Rxn 5&6: SacI, NdeI
- 1.3 kb + .86 kb+.57kb + 5.47 kb
- SacI cuts twice on phaC as well

	All clones
H_2O	15
10x buffer	2
DNA	1
Enzyme 1	1
Enzyme 2	1
	20

Insert:

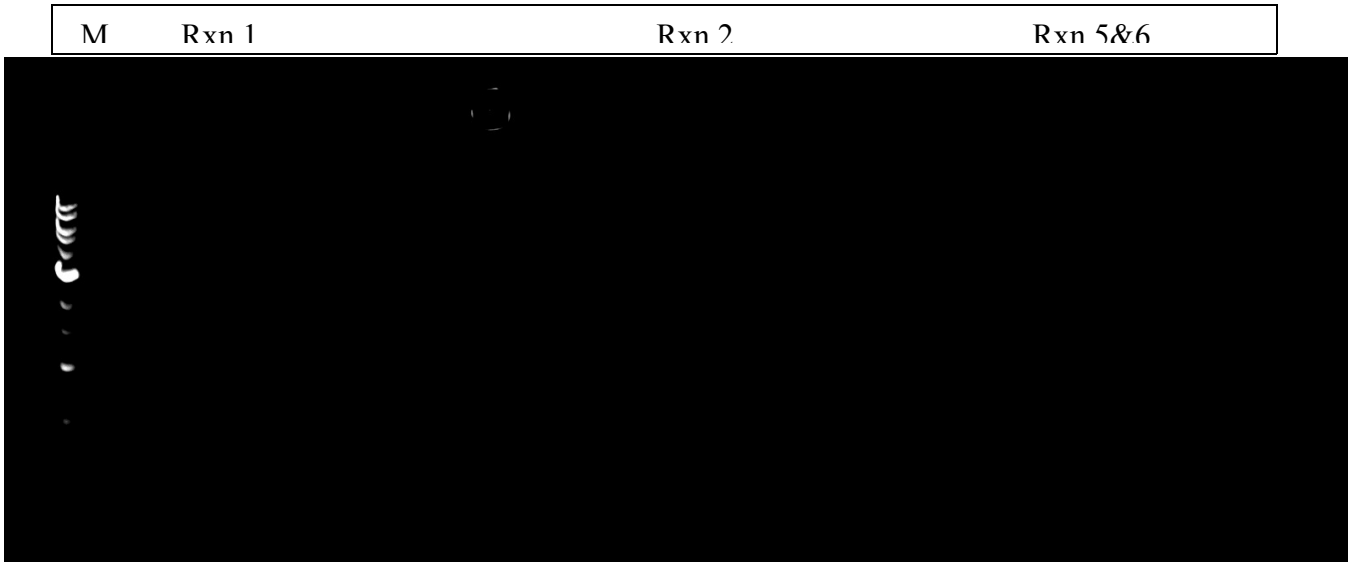


Vector: 2.9 kb

*The vector originally contains a site for SacI but it was digested when XhoI and XbaI were used

Digestion carried out for 1 hr at 37ºC, heat shock 5 min @ 80ºC

Gel Picture:



Transformation from yesterday for 7& 8

- No colonies→ retry later

Inoculation of Rxn #4 from 4/16/08

- Reexamination of plate revealed that one colony was particularly red→ grew in carbenicillin at 100 ug/ml in 3 ml overnight

5/23/08

Read Concentrations of plasmid DNA and found them to be really low

Redo Digestion with adjusted DNA concentrations

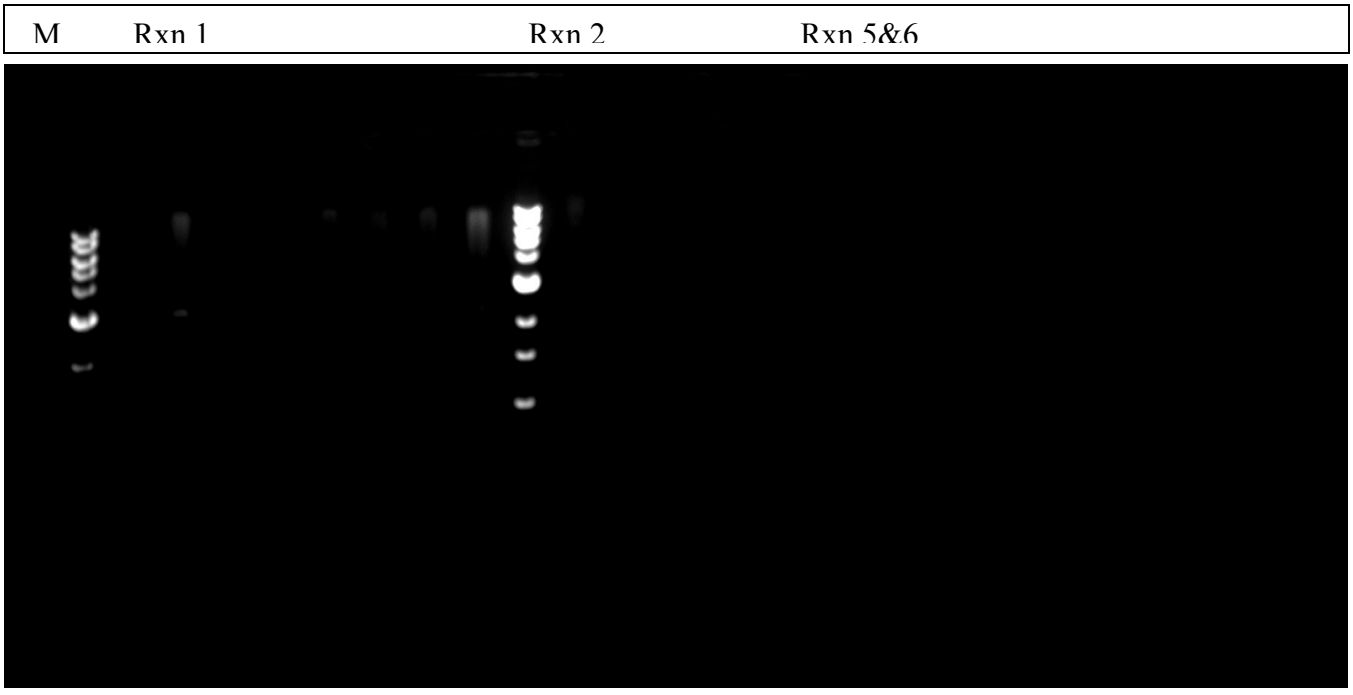
Rxn 1: BamHI, XbaI
- insert + vector: 3.87 kb + 2.92 kb

Rxn 2: EcoRI, BamHI
- insert + vector: 3.85 kb + 3.0 kb

Rxn 5&6: NotI, NdeI
- Each enzyme only cuts once
- 1.466+6.684

	All clones
H_2O	6
10x buffer	2
DNA	10
Enzyme 1	1
Enzyme 2	1
	20

Gel Picture:
*#1 first lane seemed to work. Everything else seemed to have failed. Each lane corresponds to colony number. (for Rxn 1, first lane is col #1)



Plasmid Miniprep of #4 col #11→ bacteria also stored in freezer: 1 mL +.3 mL glucose
- Much better yield of 95 ng/ul

5/27/08

Digestion of #4 col #11 to see if insert present

DNA: 60 ng (1 ul DNA + 2 ul H_2O)→ 30 ng/ul→ use 2 ul

	#4
H_2O	14
10x buffer	2
Diluted DNA	2
BamHI	1
EcoRI	1
	20

Single Colony PCR –**preemptive screening to see if insert present**

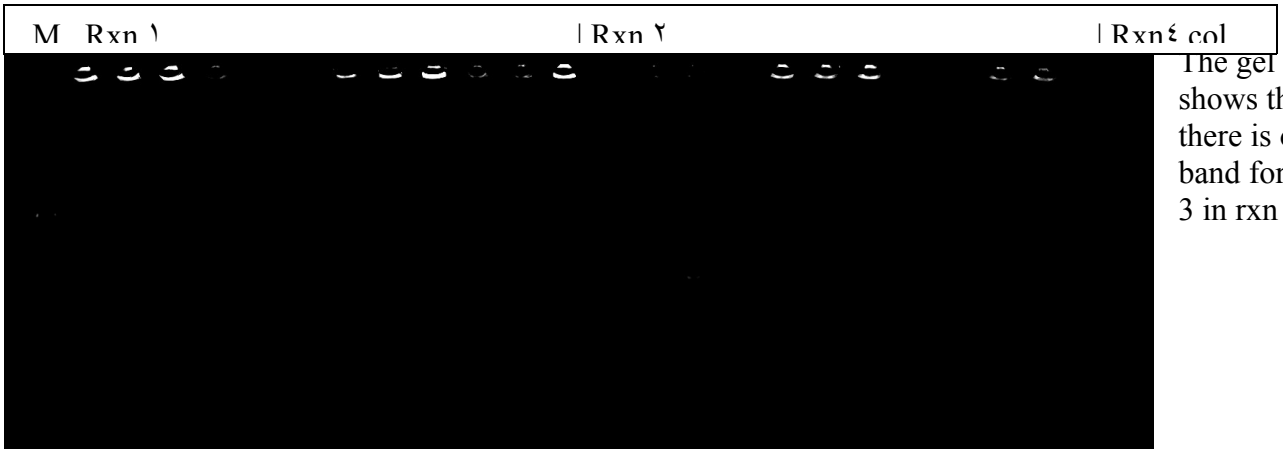
*Used primer phaAFw and phaARv → the insert should be about 1.2 kb

H_2O	6.7
phaAFw	.5
phaARv	.5
Standard PCR Buffer	1
dNTP	.8
Homemade Taq	.5
	10 ul

94ºC	5 m	x1
94ºC 60ºC 72ºC	30s 30s 1 m 50s	X40
72ºC 4ºC	10 m ∞	x1

***Picked up 1 colony per tube**
*12 colonies from Rxn 1
*12 colonies from Rxn 2

Gel Picture:



The gel shows that there is one band for col 3 in rxn 2.

This band is slightly larger than expected: 1.3 instead of 1.2. However, the colony was picked up and inoculated for further analysis.

Inoculation

- 8 colonies from number 1 including original 1 in lane 1 were picked up
- 8 colonies from number 2 also picked up including col 3 (lane 3 in Rxn 2)

5/28/08

Miniprep of 16 colonies inoculated the day before. Concentrations read and recorded.

Digestion of miniprep to see if insert present

	#1		#2
H_2O	14	H_2O	14
10x buffer	2	10x buffer	2
Diluted DNA	2	Diluted DNA	2
BamHI	1	BamHI	1
XbaI	1	EcoRI	1
	20		20

1 hr 37°**C,** 5 min at 80°**C**

PCR of Reaction 6

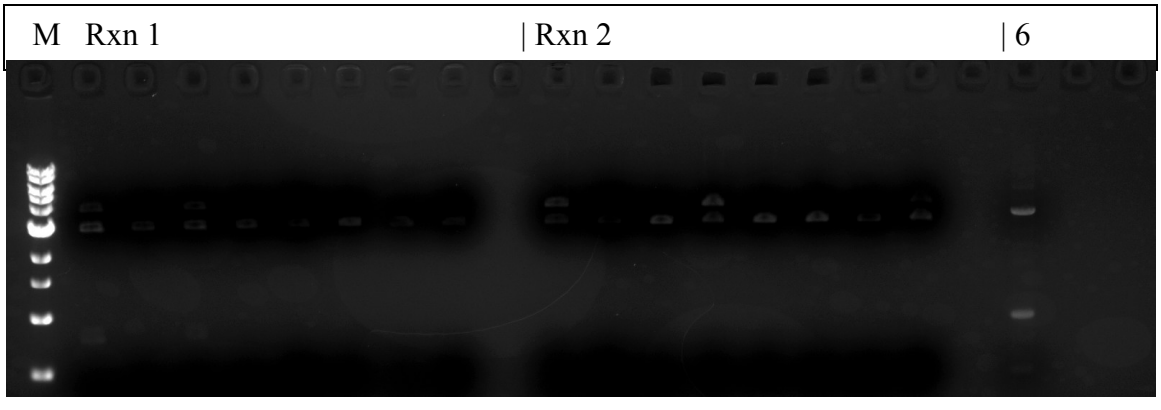
*Used col 11 from Rxn 4 → the colony that formed the most plastic→ glowed very red on Nile Red plates

For the template DNA, diluted original DNA (95 ng/ul): 2 ul DNA + 1 ul H_2O to yield ~30 ng/ul. The final concentration of template is then around 60 ng/ul

H_2O	28.5
L Primer	2.5
R Primer	2.5
Template (diluted)	2
5x Buffer	10
dNTP	4
Phusion	.5
	50 ul

98°C	30s	x1
98°C 59°C 72°C	10s 30s 2 m	x30
72°C 4°C	10 m ∞	x1

Gel Picture



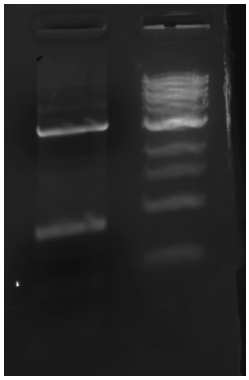
*Lane 1 and 3 for Rxn 1 seem to be successful: insert size should be 3.8 kb and vector size should be 3.0 kb
*Lane 1 and 4 for Rxn 2 also seem to be successful; insert size should be 3.8 kb and vector size should be 3.0 kb
*Rxn 6 was also successful: 3.25 kb but there are 2 bands→ gel purification

5/29/08

Ran gel for Rxn 6 for gel purification

*Previous to loading onto gel, Rxn 6 was PCR purified

Rxn 6 M



Digestion

Rxn 5&6:

	Insert 5 (70)
H_2O	12.5
10x buffer	2
DNA	3.5
XbaI	1
EcoRI	1
	20

	Insert 6 (80)
H_2O	8
10x buffer	2
DNA	8
EcoRI	1
XhoI	1
	20

	PASK vector (60 ng)
H_2O	15
10x buffer	2
DNA	1
XbaI	1
XhoI	1
	20

37°C for 2 hrs and then heat shock at 80°C for 5 min
PCR purification following digestion

Ligation

	No Insert	Exp
Insert (5) Insert (6)	X	3 3.5
Vector	2	2
Ligase	.5	.5
Buffer	1	1
H_2O	6.5	X
	10	10

Overnight, 16°C

5/30/08

Transformation

- 2 ul DNA
- 50 ul cells, 200 ul SOC medium
- 2 plates: control (no insert) & experimental

*The plates were pretty good. There were more colonies on the experimental than in the control. However, the control also had a number of colonies.

6/2/08

Inoculation

- 10 ug/ul → use 70 ul total for 35 mL of LB broth → makes 10 tubes

6/2/08

Miniprep of colonies grown over the weekend

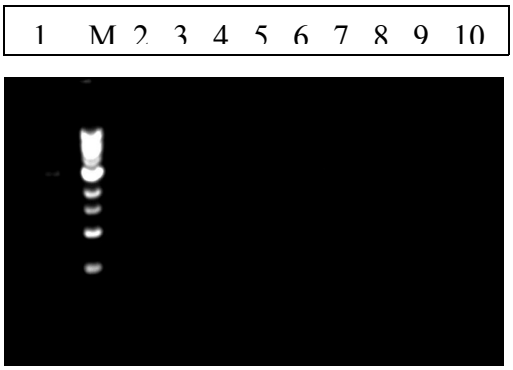
Read Concentrations

Digestion to check if insert present in the colonies

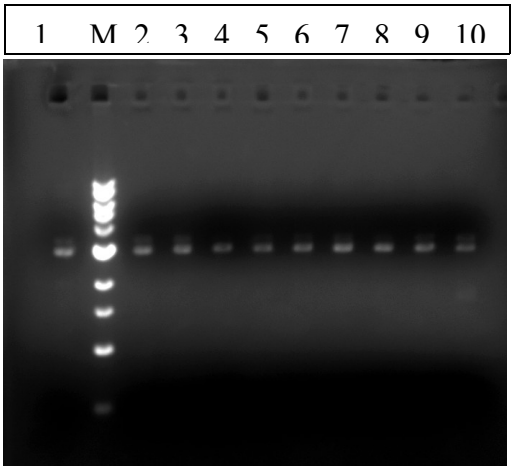
	Clone 5 (~150 ng)
H_2O	10
10x buffer	2
DNA	6
NdeI	1
NotI	1
	20

Band sizes should be:
3.0 kb, 1.7 kb, and 3.6 kb

Gel Pic 1: (30 min)



Gel Pic 2: (50 min)



*Gel Pic 2 shows that lane 10 has 3 bands and all the other colonies have 2 bands. Lane 10 seems to be correct!

6/3/08

Redo ligation by mixing PCR purified digested product in equimolar concentrations:

DNA fragment	Molecular weight (based on kb size)	ng used in digestion	Molar concentration (in 30 ul b/c of PCR purification)	Mixture volume to get .0236 pmol
5	1235.9 kDa	70 ng	.00189 pmol/ul	12.5 ul
6	2008.4 kDa	80 ng	.00133 pmol/ul	17.7 ul
V	1792.1 kDa	60 ng	.00118 pmol/ul	20 ul

- Vacuum dry equimolar product for 1 hr to further concentrate it
- Dissolve dry product in 8.5 ul H_2O

Ligation Rxn:

	Clone 5
DNA (redissolved)	8.5
T4 ligase	.5
Buffer	1
	10

Room temperature for 1 hr

Transformation
- 2 ul DNA product, 200 ul SOC medium
- The SOC medium was a little cloudy but I didn't notice till after I'd put it into the bacteria

Pour plates containing 20 ug/ml chloramphenicol, .5 ug/ml Nile Red, and 45 ng/ul tetracycline

*250 ml volume→ each plate holds ~20 ml → I poured 11 plates

Addition	Initial Conc	Desired Conc	Volume
Chloramphenicol	10 ug/ul	15 ug/ml	375 ul
Nile Red	.25 mg/ml	.5 ug/ml	500 ul
Tetrocycline	2 ug/ul	45 ng/ml	6 ul

Stamped Plate 1 and Plate 2 onto new Tet + plates
- Used a weigh boat to transfer colonies
- Did it wrong b/c pressed too hard! Should lightly touch colonies and then lightly touch onto new plate!!

6/4/08

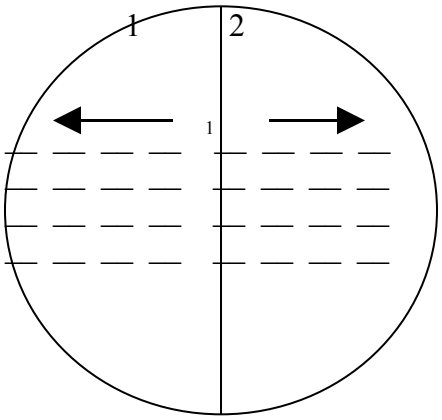
Serial increase in Tetrocycline concentration to see maximum induction of bioplastic production

Diluted Tet from 2mg/ml→ 2 ng/ul: 1 ul undiluted Tet + 999 ul EtOH

Concentrations ng/ml	ul diluted Tet	ul EtOH
0	0	200
50	5	195
100	10	190
150	15	184.5
200	20	180

- 200 ul total volume
- Mix EtOH with Tet → then put on pre-warmed plates (pre-warmed for 20 min)
- Spread with cell spreader
- Picked colonies from original 1E and 2E plates and put on new plates

*150 ng plate→ accidentally put on EtOH and then Tet w/out first mixing



Colonies on original plate were marked by underlining and labeling with a number

<u>Inoculation</u>

- 10 colonies picked up
- 60 ul of 10 ug/ul → 20 ug/ul final concentration

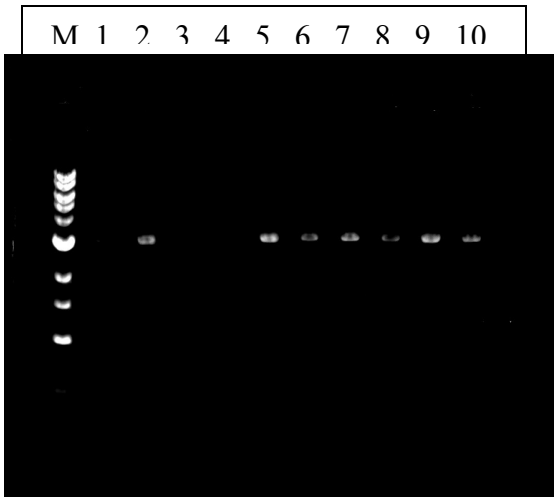
6/5/08

Miniprep of previously transformed 10 colonies

Restriction Enzyme Digestion to check if insert present

	Clone 5
BSA	1
Buffer 3	1
H_2O	1
NdeI	.5
NotI	.5
DNA	6
	10

*37°C for 1 hr, heat shock 80°C



Seems as if all the tested colonies did not contain the insert.

6/6/08

Continued monitoring the colonies although none of them seems to glow red on the Nile Red Plates

6/9/08

Transformation of col #11 and col #5 ON CARBENICILLIN plates→ check to see if #11 is actually bright

6/10/08

The PCR was performed with 5 tubes:

1. 5
2. 6 (using col 5 as template)
3. 6 (using col 11 as template)
4. 7
5. 8

H_2O	28.5
L Primer	2.5
R Primer	2.5
Template (diluted)	2
5x Buffer	10
DNTP	4
Phusion	.5
	50 ul

Rxns 5, 8

98ºC	30s	x1
98ºC 58ºC 72ºC	10s 30s 30s	x5
98ºC 66ºC 72ºC	10s 30s 30s	x25
72ºC 4ºC	10 m ∞	x1

Rxn 6

98ºC	30s	x1
98ºC 59ºC 72ºC	10s 30s 2 m	x30
72ºC 4ºC	10 m ∞	x1

Rxn 7

98ºC	30s	x1
98ºC 66ºC 72ºC	10s 30s 2 m	x30
72ºC 4ºC	10 m ∞	x1

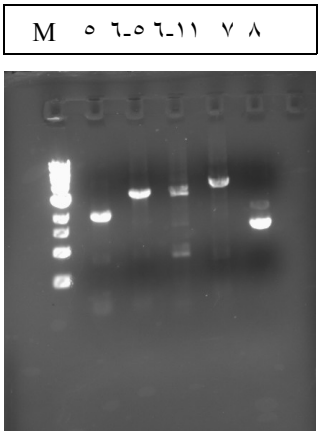
*Rxn 6 was run using diluted DNA template

- col 5: 1 ul DNA + 9 ul H_2O (60 ng→ 120 ng used total)
- col 11: 2 ul DNA + 1 ul H_2O (30 ng→ 60 ng used total)

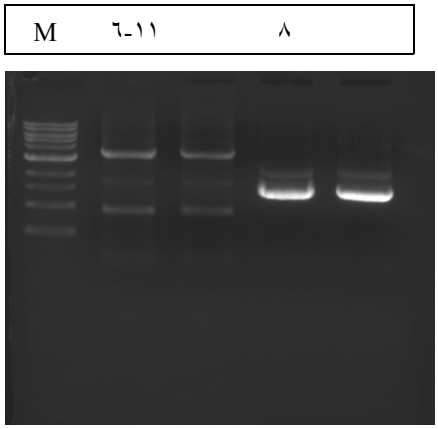
*phaCAB (asterisked) used as template

*PSOScat2#9 undiluted used as template

Gel Picture:



This gel showed PCR products that were all of the correct size. The only problem was that 6-11 and 8 required gel purification.



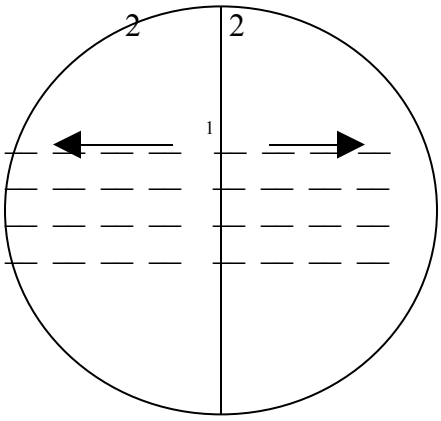
All of the PCR product was run in this gel so that the correct size band could be cut out

Redid Serial Tetrocycline Induction

- Poured plates so put Tet directly into plate
- Pouring plates (300 ml total for ~18 plates):
    - 450 ul Chloramph. (15 ug/ml)
    - 600 ul Nile Red (.5 ug/ml)

Addition of Tet to 5 plates assuming each plate has 20 mL:
*dilution of Tet made: 2 ul Tet + 2 ul H_2O → 1 ug/ul Tet

Concentrations ng/ ml	ul Tet
0	0
50	1 dil
100	1
150	1.5 dil
200	2



6/11/08

Read DNA concentrations of gel purified and DNA purified→ all pretty high

- 6-11 and 8 had trouble reading concentrations→ gave error about bubble

Redid Digestion

Rxn 5&6:

	Insert 5 (80)
H_2O	15
10x buffer	2
DNA (dil)	1
XbaI	1
EcoRI	1
	20

	Insert 6-5 (80)
H_2O	15
10x buffer	2
DNA (dil)	8
EcoRI	1
XhoI	1
	20

	Insert 6-11 (80)
H_2O	15
10x buffer	2
DNA	1
EcoRI	1
XhoI	1
	20

	PASK vector (60 ng)
H_2O	15
10x buffer	2
DNA	1
XbaI	1
XhoI	1
	20

37°C for 2 hrs and then heat shock at 80°C for 5 min
PCR purification following digestion

Rxn 7&8

	Insert 7 (65)
H_2O	15
10x buffer	2
DNA	1
XbaI	1
EcoRI	1
	20

	Insert 8 (66)
H_2O	14
10x buffer	2
DNA	2
EcoRI	1
XhoI	1
	20

	PASK vector (60 ng)
H_2O	15
10x buffer	2
DNA	1
XbaI	1
XhoI	1
	20

37°C for 2 hrs and then heat shock at 80°C for 5 min
PCR purification following digestion

Ligation

	No Insert	Exp
Insert (5) Insert (6)	X	3 3.5
Vector	2	2
Ligase	.5	.5
Buffer	1	1
H_2O	6.5	X
	10	10

	No Insert	Exp
Insert (7) Insert (8)	X	4 2
Vector	2	2
Ligase	.5	.5
Buffer	1	1
H_2O	6.5	X
	10	9.5

Transformation

- 6 clone was good→ few colonies on control but many on experimental
- 5 clone not so good→ too many colonies on control

<u>6/12/08</u>

Redo digestion for clone 5:

<u>Rxn 5&6:</u>

	Insert 5 (80)
H_2O	14
Buffer 2	2
DNA (dil)	1
XbaI	1
BSA	2
	20

	Insert 5 (80)
H_2O	14
EcoRI buffer	5
DNA (PCR pur)	30
EcoRI	1
	50

*XbaI and EcoRI shouldn't be double digested→ do XbaI first for 1 hr, heat shock at 80 for 5 min and then PCR purify then proceed with EcoRI with the same protocol.

	Insert 6-5 (80)
H_2O	13
EcoRI Buffer	2
DNA (dil)	1
EcoRI	1
XhoI	1
BSA	2
	20

	Insert 6-11 (80)
H_2O	13
EcoRI Buffer	2
DNA	1
EcoRI	1
XhoI	1
BSA	2
	20

	PASK vector (60 ng)
H_2O	13
Buffer 2	2
DNA	1
XbaI	1
XhoI	1
BSA	2
	20

37ºC for 2 hrs and then heat shock at 80ºC for 5 min
PCR purification following digestion

Ligation

	No Insert	Exp
Insert (5) Insert (6)	X	3 3.5
Vector	2	2
Ligase	.5	.5
Buffer	1	1
H_2O	6.5	X
	10	10

16ºC Overnight

<u>6/13/08</u>

Transformation

- .5 ul DNA
- 200 ul cells

6/16/08

Miniprep

Digestion to check if insert present

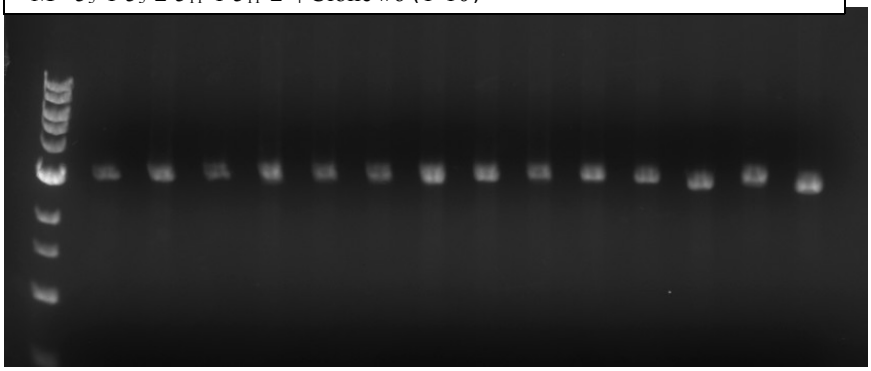
	Clone 5	Clone 6
Buffer 3	1	1
DNA	7	7
NdeI	.5	.5
NotI	.5	.5
BSA	1	1
	10	10

For Clone 5: Band sizes should be:
3.0 kb, 1.7 kb, and 3.6 kb

For Clone 6: Band sizes should be:
3.6, 3.4, 1.1 kb

Gel Picture:

M 5_5-1 5_5-2 5_{11}-1 5_{11}-2 | Clone #6 (1-10)



None of these had desired insert

Plating again using increased concentration of Tet

Made Tet that was 200 ug/ul → 1 g Tet into 5 ml → .2 g/ml → 200 ug/ul
Made 50, 100, 200 ug/ml plates

6/17/08

2-part method of digestion:
*The same protocol was used for both insert (70 ng) and vector (80 ng)

	Part 1
H_2O	14
Buffer 2	2
DNA (dil)	1
XbaI	1
BSA	2
	20

	Part 2
H_2O	14
EcoRI buffer	5
DNA (PCR pur)	30
EcoRI	1
	50

*XbaI and EcoRI shouldn't be double digested→ do XbaI first for 1 hr, heat shock at 80 for 5 min and then PCR purify then proceed with EcoRI with the same protocol.

Ligation

	No Insert	Exp
Insert (5)	X	5.5
Vector	3	3
Ligase	.5	.5
Buffer	1	1
H_2O	5.5	X
	10	10

Transformation
- .5 ul DNA
- 50 ul cells

6/18/08

2-part method of digestion:
*The same protocol was used for both insert (70 ng) and vector (80 ng)

	Part 1
H_2O	14
Buffer 2	2
DNA (dil)	1
XbaI	1
BSA	2
	20

	Part 2
H_2O	14
EcoRI buffer	5
DNA (PCR pur)	30
EcoRI	1
	50

*XbaI and EcoRI shouldn't be double digested→ do XbaI first for 1 hr 40 min, heat shock at 80 for 5 min and then PCR purify then proceed with EcoRI with the same protocol except heat shocked at 65 for 20 min

Ligation

	No Insert	Exp
Insert (5)	X	5.5
Vector	3	3
Ligase	.5	.5
Buffer	1	1
H_2O	5.5	X
	10	10

Transformation
- .5 ul DNA
- 50 ul cells

Inoculation
- one colony for clone 1 was red on 100 ug/ml Tet plate → picked up and grew

Tet
- Made 1 plate at 10 ug/ml to see if this is effective concentration

6/19/08

Miniprep of clone #1 col #13 (mislabeled as col. #9!)

Digestion to check if clone #1 had insert

	Part 1
H_2O	5
10x Buffer	2
Template	11
XbaI	1
BamHI	1
	20

37ºC for 1 hr, 80ºC for 5 min

Single Colony PCR to check for phaA insert
Used primer phaAFw and phaARv → the insert should be about 1.2 kb

H_2O	6.7
phaAFw	.5
phaARv	.5
Standard PCR Buffer	1
dNTP	.8
Homemade Taq	.5
	10 ul

94ºC	5 m	x1
94ºC 60ºC 72ºC	30s 30s 1 m 50s	X40
72ºC 4ºC	10 m ∞	x1

***Picked up 1 colony per tube, 23 colonies picked up total, 1 colony from control**

Gel Picture**:**

M Clone1 | Clone 5 (Colonies 1-23)

6/23/08

- Designed Primers
- Tried 1 ug & 500 ng concentrations of Tet to grow colonies
    - 1 ug – didn't grow much
    - 500 ng – all grew

6/24/08

- Planned growing the bacteria and purifying plastic.

Protocols:

Growing Bacteria (from Valentin et. al.)

1. Grow 3 ml overnight
2. Use 1 ml of overnight culture in 50 ml of LB broth at 30°C. Grow until the cells reach an optical density of .7 (600 nm)
3. Add glucose to a final concentration of 1% and transfer to orbital incubator at 37°C. Cells harvested after 48 h of incubation

Harvest Cells (from Valentin et. al.)

1. Centrifuge at 3500 rpm and suspend in .9% saline solution. Recentrifuge to obtain a washed pellet
2. Freeze the pellet and dry by lyophilization
3. Shake the pellet overnight at 37°C in chloroform
4. Remove bacterial cell through filtration using Whatman paper
5. Precipitate polyester from the filtered solution by adding 10 volumes of ethanol
6. Redissolve polymer in chloroform
7. Reprecipitate with 10 volumes methanol
8. Filter using a Whatman paper filter.
9. Redissolve polymer in deuterochloroform (NMR) or chloroform (gel permeation chromatography)

Harvest Cells (from Hein et. al.)

1. Cells were lyophilized and 3-5 mg used
2. Methanolysis was conducted in the presence of 15% sulfuric acid
3. The resulting compound was then analyzed by gas chromatography

6/25/08

Inoculation

- PCRBluntII-Topo #15 in 3 ml
- 15 ul Kanamycin in 3 ml for 50 ug/ml
- took from frozen stock→ supposed to just scoop a bit of the frozen bacteria into the 3 mL solution. We took 10 ul instead.

6/26/08

Followed Protocol for growing bacteria

- Grew 1 mL from yesterday's inoculation in 30°C incubator → 250 ul Kanamycin
- Attempted to read plates in microplate reader but failed

Assembly PCR

	Term	pASK, AB, Cat2C
H_2O	26.5	28.5
L Primer	2.5	2.5
R Primer	2.5	2.5
Template	4	2
5x Buffer	10	10
dNTP	4	4
Phusion	.5	.5
		50 ul

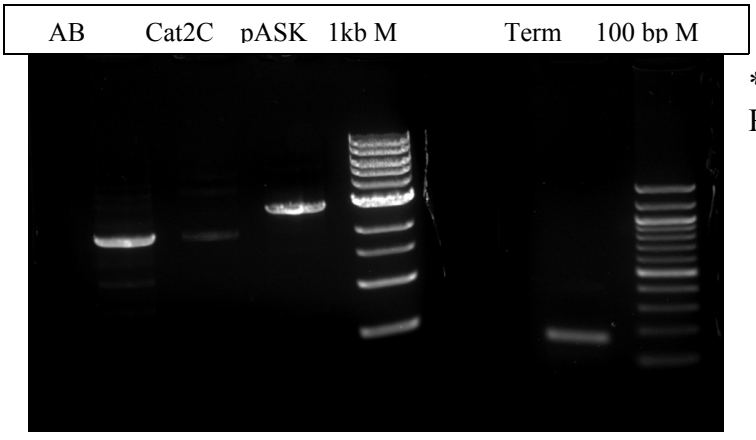
Term

98°C	30s	x1
98°C 63°C 72°C	10s 30s 6 s	x30
72°C 4°C	10 m ∞	x1

All else

98°C	30s	x1
98°C 66°C 72°C	10s 30s 1m 45 s	x30
72°C 4°C	10 m ∞	x1

Gel Picture:



*All bands are the correct size except for Cat2C. Realized we used the wrong template!

Repeated PCR for Cat2C

H_2O	28.5
L Primer	2.5
R Primer	2.5
Template	2
5x Buffer	10
dNTP	4
Phusion	.5
	50 ul

98ºC	30s	x1
98ºC 64.5ºC 72ºC	10s 30s 2 m	x30
72ºC 4ºC	10 m ∞	x1

6/27/08

Ran gel of Cat2 PCR

- The gel picture showed nothing

Added Glucose to Bacteria after 1 night of inoculation in 50 mL

- Added .5 g to 50 mL to make 1% glucose solution

Repeated PCR for Cat2phaC

- Diluted DNA:
  - pSOSCat2phaC #11: 2 ul DNA + 1 ul H_2O
  - pSOSCat2phaC #5: 1 ul DNA + 9 ul H_2O

For: Template # 5

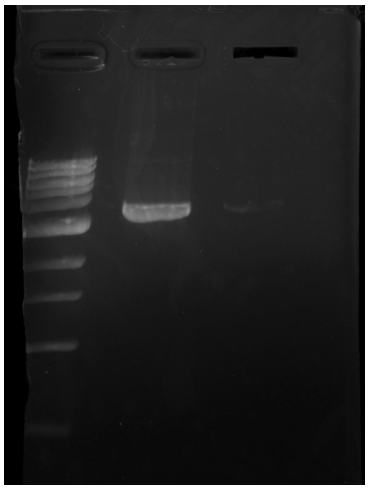
H_2O	28.5
L Primer	2.5
R Primer	2.5
Template (diluted)	2
5x Buffer	10
dNTP	4
Phusion	.5
	50 ul

For: Template #11

98ºC	30s	x1
98ºC 60ºC 72ºC	10s 30s 30s	x5
98ºC 66ºC 72ºC	10s 30s 30s	x25
72ºC 4ºC	10 m ∞	x1

98ºC	30s	x1
98ºC 65ºC 72ºC	10s 30s 2 m	x30
72ºC 4ºC	10 m ∞	x1

M Cat2C (5) Cat2C(11)



6/30/08

Gel Purification

- Purified the bands & read concentrations

Calculated Equimolar Concentrations

- Used 175 ng of pASK (recommended 200 ng)

PCR Reaction

	C	E
H_2O	12.75	.3
AB	X	2.24
Cat2phaC	X	9.5
pASK	X	5
Terminator	X	.72
5x Buffer	5	5
dNTP	2	2
Phusion	.25	.25
	25 ul	25 ul

98°C	30s	x1
98°C 60°C 72°C	10s 30s 55s	X40
72°C 4°C	10 m ∞	x1

*Used lowest Assembly Temperature

7/1/08

Transformation

- 1 ul DNA, 450 ul SOC medium
- Plated a total of 500 ul

7/2/08

Extraction of Polymer Protocol

1. Grew 3 ml (10 ul from frozen stock) overnight
2. Took 1 ml and grew in 50 ml @ 30°C overnight
3. Added 1% glucose and grew at 37°C for 48 h
4. Took out and incubated at 4°C overnight.
5. Pelleted cells @ 3500 rpm for 10 min; washed with 15 ml PBS
6. Froze in –80°C for 2 hr.
7. Lyophilization overnight
8. 5 ml chloroform used to extract polymer overnight at 37°C
    a. Pellet remained so we crushed the pellet using a spatula and then shook again for 4 hrs

9. Filtered out cell debris. Had to add more chloroform because not much left after filtration
    a. Added 30 ml of ethanol (> 10 volumes)
10. Nothing came out.

Reorder Primer for PCR

- Realized one primer was missing 2 bp

7/7/08

Inoculation of bacteria into 3 ml (50 ug/ml kanamycin)

7/8/08

Autoclaved glassware

- Gravity Cycle, place foil, no water
- If liquid cycle, place water in bin

Inoculated into 50 ml (50 ug/ml kanamycin)

- Around 4:00→ tried to reach OD of .7

7/9/08

Checked to see when bacteria grew to .7 Absorbance

- From previous night, bacteria at 1.7 A
- Diluted this culture: 15 mL + 45 mL LB = 60 mL total

Time	Absorbance
0	.486
85	.52
160	.765

- At .765 A, added 1% glucose and changed temperature to 37ºC

Repeat PCR of AB and Term

- Try to make 1 segment for Assembly

	Term	AB
H_2O	26.5	28.5
L Primer	2.5	2.5
R Primer	2.5	2.5
Template	4	2
5x Buffer	10	10
dNTP	4	4
Phusion	.5	.5
		50 ul

Term

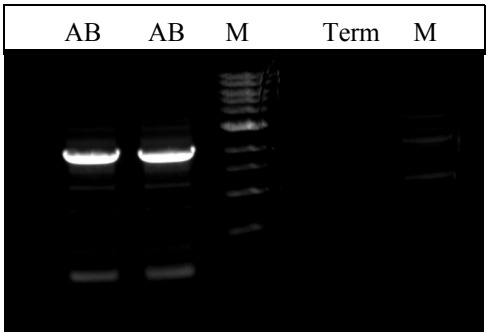
98ºC	30s	x1
98ºC 63ºC 72ºC	10s 30s 6 s	x30
72ºC 4ºC	10 m ∞	x1

AB

98ºC	30s	x1
98ºC 66ºC 72ºC	10s 30s 1m 45 s	x30
72ºC 4ºC	10 m ∞	x1

*Gel was thrown away!!! So AB product lost

Gel Picture:



*Using gel purified AB product from last week:

Assembly PCR

1st Cycle

H_2O	.3
AB	2.24
Terminator	.72
5x Buffer	5
dNTP	2
Phusion	.25
	25 ul

98°C	30s	x1
98°C 60°C 72°C	10s 30s 55s	X40
72°C 4°C	10 m ∞	x1

2nd Cycle

H_2O	28.5
L Primer	2.5
R Primer	2.5
PCR from 1st cycle	2
5x Buffer	10
dNTP	4
Phusion	.5
	50 ul

98°C	30s	x1
98°C 66°C 72°C	10s 30s 2 m	x30
72°C 4°C	10 m ∞	x1

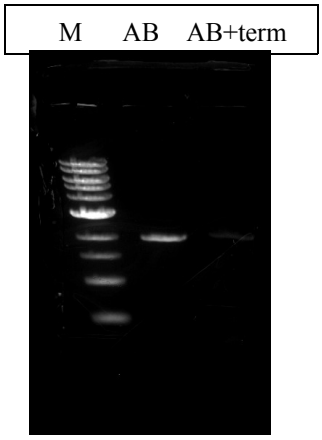
Inoculation of control (Zhongying's plasmid) and PCRTOPOII#15 into 3 ml (previous inoculation didn't have a control)

7/10/08

Ran gel of AB+Term

- Gel Purify

Gel Picture:

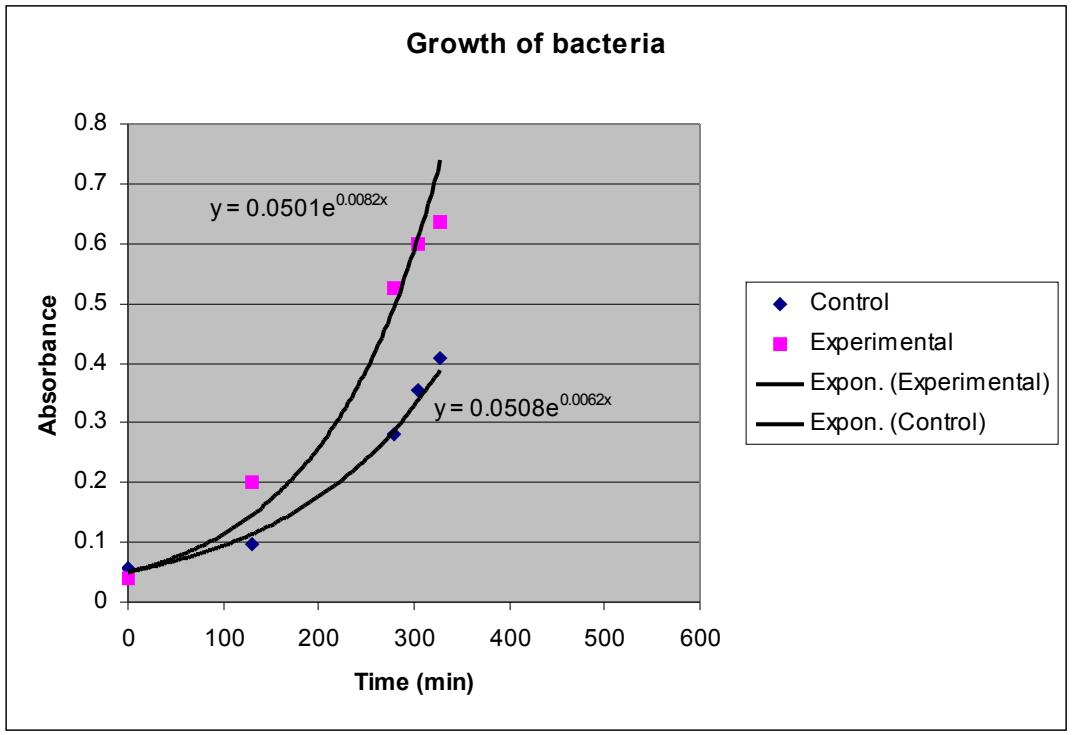


48 hr incubation continued→ will complete by 1:45 tmr

Put 1 ml into 50 for control and experimental

- Monitored for OD .7 at 600 nm

Time(min)	Control	Experimental
0	0.056	0.041
130	0.097	0.201
280	0.28	0.524
305	0.354	0.598
328	0.408	0.637



*Glucose added to experimental at 6:05 pm, which is approximately 338 min
*Glucose added to control at 7:40 pm which is approximately 438 min→ OD was around 1.4 by then

7/14/08

Extracting Plastic- Summary of Harvesting Cells

1. For first batch, 3 mL PCR-Blunt II Topo #15 grown overnight
2. 1 mL put into 50 mL in the afternoon and shaken overnight at 30°C.
3. Read OD and found that it was ~1.6 Abs. Diluted the cells by taking 15 mL and putting it into 60.
4. This made the OD ~.4. After 2 hrs growth, OD reached .765 and then glucose added and grown for 48 h at 37°C.
5. This batch was pelleted, washed with PBS, and cells were freeze-thawed 3X using dry ice and ethanol before being put in the freezer for 3 days.
6. Concurrently, grew another batch that included control bacteria from Zhongying following same procedure.
7. This time, 1 mL into 50 mL was monitored throughout day. For the experimental (PCR-Blunt II Topo #15), the glucose was added at ~OD .68. For the control, the glucose was added at ~OD . 1.4.
8. This batch was also grown for 48 h. Then, the cells were put in 4°C overnight.
9. Cells were pelleted at 3500 rpm for 15 min, washed with 15 mL PBS, and then freeze-thawed 3X using ethanol and dry ice. Pellets were frozen overnight.
10. Cells were lyophilized together in the machine overnight.
11. Cells were then suspended in 10 mL chloroform. Pellet was disturbed and shaken at 37°C overnight
12. Bacterial cell debris was removed using vacuum filtration and whatman paper. Experimental tubes combined. The filtrate for experimental split into 4 tubes. The filtrate for control split into 2 tubes.
13. 40 mL ethanol was added to the filtrate to precipitate out the plastic. White flaky substance did indeed precipitate out for both the control and the experimental but much more precipitated out for the experimental.
14. The solid was pelleted at 3800 rpm for 20 min and then redissolved in 5 mL chloroform.
15. Then 10 volumes of methanol used to reprecipitate the polymer.
16. Sample again pelleted and then frozen until further analysis.

Repeat Anhydrotetracycline Experiment

Concentration	ul Anhydro.
0 ng	200 EtOH
45 ng	.45 +199.55 EtOH
100 ng	1 +199 EtOH
200 ng	2 + 198 EtOH

*Picked 16 new colonies from the original plates. Marked these with an X

7/15/08

The plates from yesterday showed 3 promising colonies: two #2 red, 1 on 200 ng plate and 1 on 100 ng plate. One colony red for #1 on 100 ng plate. The 45 ng and 0 ng had no red colonies.

Continued Extracting polymers

Grew up the 3 colonies for further analysis

7/16/08

Miniprep of the 3 colonies

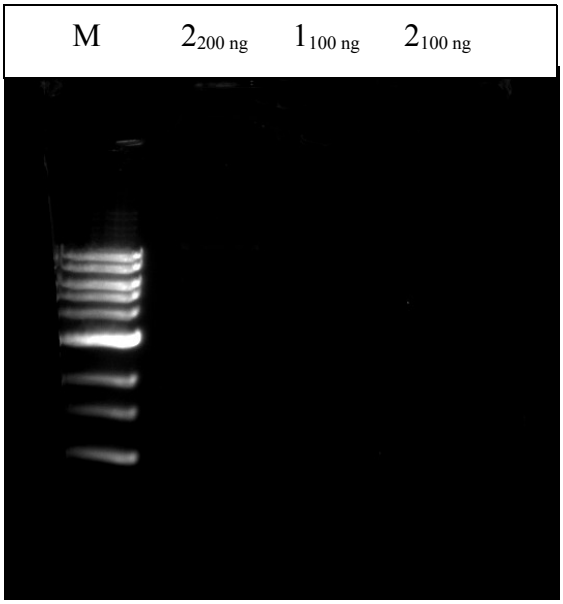
Digestion to check for insert

	Clone #1
BSA	1
Buffer 3	1
DNA	7
XbaI	.5
BamHI	.5
	10

	Clone #2
BSA	1
EcoRI Buffer	1
DNA	7
BamHI	.5
EcoRI	.5
	10

Ran for 1 hr 30 min. Heat shock for 5 min at 80°C.

Gel Picture:



*The gel seemed unsuccessful; one of the enzymes didn't seem to cut.

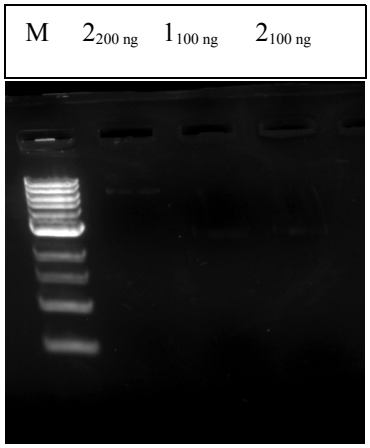
7/17/08

Digestion again. This time split up digestion and ran gel after digestion

	Clone #1 & #2
BSA	2
Buffer 3	2
DNA	15
BamHI	1
	20

*1 hr. 30 min followed by heat shock.

Gel Picture:



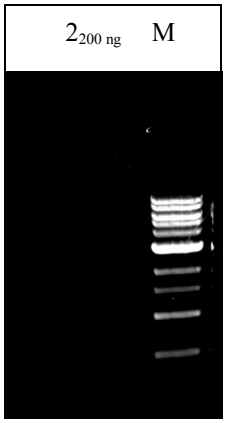
- BamHI seemed to have cut. Only the first lane seems promising
- PCR Purify and do another digestion for lane 1 only.

2nd Digestion

	Clone 2
H_2O	14
EcoRI buffer	5
DNA (PCR pur)	30
EcoRI	1
	50

1 hr 30 min. Ran gel.

Gel Picture:



*Product seems to have disappeared. Perhaps lost during PCR purification.

7/18/08

Finished extracting polymer

Transformed working colonies of 1, 2, 5

Grew up colonies (on Sunday night)

7/21/08

Digestion to check for insert in 1,2,5 → inserts should be present b/c they were verified previously

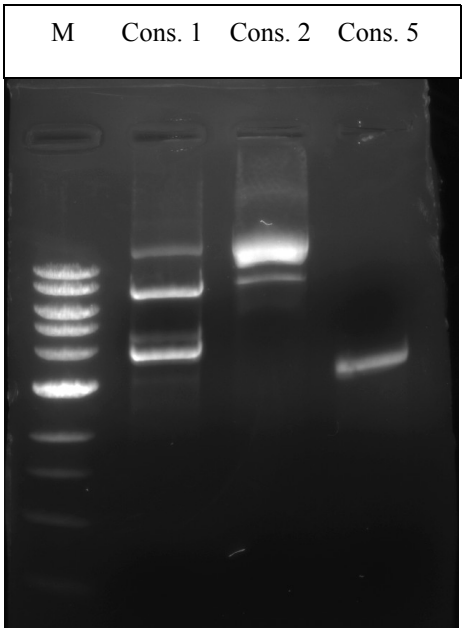
	Clone #1
BSA	2
Buffer 3	2
DNA	14
XbaI	1
BamHI	1
	20

Gel

	Clone #2
BSA	2
Eco Buffer	2
DNA	14
EcoRI	1
BamHI	1
	20

	Clone #5
BSA	2
Buffer 3	2
DNA	14
NdeI	1
NotI	1
	20

Picture:



*Gel didn't work. All the bands are the wrong size.

1 clone from each construct sent for sequencing:
*Protocol: 500 ng DNA, 12 ul total, 10 uM of primer

Clone	DNA	Primer	H_2O
1_3	6.5 ul	.5	5
2_1	6.6 ul	.5	4.9
3_1	2.5 ul	.5	9
4_{10}	1.45 ul	.5	10.05
5_{10}	8.26 ul	.5	3.24

7/22/08

Prepared samples for NMR

- Tried to measure out .020 g of each sample
- Put 600 ul into sample and then transferred to NMR tube

The control only had ~.008 g sample and 600 ul deuterochloroform put into sample anyway.

The experimental had > .040 g material

The experimental was greater than 2x the control which is a good sign.

*NMR will be run on Wednesday

7/23/08

NMR Analysis w/ Tony
H1 NMR spectroscopy at 300 MHz showed clean sample of polymer. However, peaks for control and experimental were very similar and did not match those found in literature for 3HB (no peaks at 2.5 ppm and 5.5 ppm).

Possibly plastic from pipet tips contaminated the sample? Or the pellet is another polymer or protein.

Re-plated on carb+NR plate to see if control turns pink:

(1/4 plate each:)
pUC19: control, one of Zhongying's bacteria
pSOScat2 #9, sample: from transformation plate
pSOScat2phaCAB #9: from –80C freezer
pSOScatphaC (construct 4, colony 11): from –80 freezer

ON at 37C

7/24/08

Took plate from 7/23/08 out of incubator. Placed on lab bench.

Sequencing results:
Constructs 1 and 2 show correct phaAFw sequence
Constructs 3 and 4 show correct phaCFw sequence
Construct 5 shows incorrect phaAFw sequence

Next -
Design primers for construct 1:
phaCmid1, phaCmid1, phaAmid

Design primers for construct 4:
phaCat2mid

Prepare sequencing solutions for Construct 1:
phaCFw, phaCRv, phaARv, phaBFw, and phaBRv

Prepare sequencing solutions for Construct 4:
PUC19seqFw, cat2Rv

Construct	**Colony**	**DNA**	Primer	**H2O**
1	3	6.5 ul	0.5 ul phaCXba1Fw	5 ul
1	3	6.5	0.5 ul phaCEcoR1Rv	5
1	3	6.5	0.5 ul phaARv	5
1	3	6.5	0.5 ul phaBFw	5
1	3	6.5	0.5 ul phaBBamH1Rv	5
4	10	1.45	0.5 ul pUC19seqFw	10.05 ul
4	10	1.45	0.5 ul cat2midFw	10.05
4	10	1.45 ul	0.5 ul cat2Rv2	10.05
4	10	1.45	0.5 ul phaCEcoR1Rv	10.05

7/25/08

Sent the 9 samples prepared yesterday out for sequencing.

Made 30 chlorenphenicol + Nile Red plates; placed in cold room
(1 mL of 10 mg/ml chlor + 1mL of 0.25 mg/ml NR + 500 mL of LB agar)

7/28/08

Drew 25-square grid on each of two plates. Picked 50 colonies from Maggie's assembly PCR attempt and streaked 4 times each, in preparation for colony PCR tomorrow. (Four streaks per sample – 1 streak per each of the 4 primer pairs to check for the different inserts.)

Grew ON.

7/29/08

Maggie complete assembly PCR of phaAB, cat2phaC, pASK, and terminator using the protocol:

Make phaAB from vector Topo 15 (1997bp).

PCR

5x HF Buffer	5ul
phaCAB Topo 15	0.25 ul
P1 (phaAB Fw)	2 ul
P2 (phaAB Rv)	2 ul
dNTPs (10mM)	2 ul
Phusion	**0.2 ul**
H2O	to 25 ul

98C	30sec	
30X		
	98C	7s
	63C	30s
	72C	30s
72C	5min	
4C	hold	

Conc. is 97.8 ng/ul

Make pASK from vector (3021 bp).

PCR

5x HF Buffer	5ul
phaCAB Topo 15	0.25 ul
P1 (pASK Fw)	2 ul
P2 (pASK Rv)	2 ul
dNTPs (10mM)	2 ul
Phusion	**0.2 ul**
H2O	to 25 ul

98C	30sec	
30X		
	98C	7s
	61C	30s
	72C	46s
72C	5min	
4C	hold	

Conc. is 167.3 ng/ul

7/29/08 (cont'd)

Make terminator (171 bp).

PCR	
5x HF Buffer	5ul
100xmRFP	0.25 ul
P1 (Term Fw)	2 ul
P2 (Term Rv)	2 ul
dNTPs (10mM)	2 ul
Phusion	**0.2 ul**
H2O	to 25 ul

98C	30sec	
30X		
	98C	7s
	62C	30s
	72C	3s
72C	5min	
4C	hold	

Make Cat2phaC (3279 bp).

PCR	
5x HF Buffer	10ul
phaCAB Topo 15	0.5 ul
P1 (pASK Fw)	3 ul
P2 (pASK Rv)	3 ul
dNTPs (10mM)	4 ul
Phusion	**0.4 ul**
H2O	to 50 ul

98C	30sec	
30X		
	98C	7s
	72C	1min
72C	5min	
4C	hold	

Conc. is 86.27 ng/ul

Maggie then transformed PCR2 product several days ago.

7/29/08 (cont'd)

Ran colony PCR

Make phaAB from vector Topo 15 (1997bp).
PCR

5x HF Buffer	5ul
Bacteria sample 1, 2, 3, 14, 15, 26, 27, 36, 37, 46	
P1 (phaAB Fw)	2 ul
P2 (phaAB Rv)	2 ul
dNTPs (10mM)	2 ul
Phusion	**0.2 ul**
H2O	to 25 ul

98C	30sec	
30X		
	98C	7s
	63C	30s
	72C	30s
72C	5min	
4C	hold	

Conc. is 97.8 ng/ul

Make pASK from vector (3021 bp).
PCR

5x HF Buffer	5ul
Bacteria sample 1, 2, 3, 14, 15, 26, 27, 36, 37, 46	
P1 (pASK Fw)	2 ul
P2 (pASK Rv)	2 ul
dNTPs (10mM)	2 ul
Phusion	**0.2 ul**
H2O	to 25 ul

98C	30sec	
30X		
	98C	7s
	61C	30s
	72C	46s
72C	5min	
4C	hold	

Conc. is 167.3 ng/ul

7/29/08 (cont'd)

Make terminator (171 bp).
PCR

5x HF Buffer	5ul
Bacteria sample 1, 2, 3, 14, 15, 26, 27, 36, 37, 46	
P1 (Term Fw)	2 ul
P2 (Term Rv)	2 ul
dNTPs (10mM)	2 ul
Phusion	**0.2 ul**
H2O	to 25 ul

98C	30sec	
30X		
	98C	7s
	62C	30s
	72C	3s
72C	5min	
4C	hold	

Make Cat2phaC (3279 bp).
PCR

5x HF Buffer	10ul
Bacteria sample 1, 2, 3, 14, 15, 26, 27, 36, 37, 46	
P1 (pASK Fw)	3 ul
P2 (pASK Rv)	3 ul
dNTPs (10mM)	4 ul
Phusion	**0.4 ul**
H2O	to 48 ul

98C	30sec	
30X		
	98C	7s
	72C	1min
72C	5min	
4C	hold	

Conc. is 86.27 ng/ul

7/29/08 (cont'd)

Ran 5ul of each sample on 1% agarose gel:

Gel 1
pASK and Cat2phaC PCR reactions
1kb marker, 10 lanes for pASK (colonies 1, 2, 3, 14, 15, 26, 27, 36, 37, 46), 10 lanes for Cat2phaC (colonies 1, 2, 3, 14, 15, 26, 27, 36, 37, 46)

Gel 2
phaAB and terminator PCR reactions
1kb marker, 10 lanes for phaAB (colonies 1, 2, 3, 14, 15, 26, 27, 36, 37, 46), 100 bp marker, 10 lanes for terminator (colonies 1, 2, 3, 14, 15, 26, 27, 36, 37, 46)

Results:
No positive bands for pASK fragment. Two high-specific bands for Cat2phaC fragmenet but not the correct size.
No positive bands for phaAB fragment. Two possible correct bands for terminator fragement.

Also, grew up bacteria in preparation for harvesting reattempt:

1) Experimental: Constr. 1, colony #4 bacteria (from –80C)
2) Control: pASK vector (from –80C) ON

Grew both at 37C in 3 mL LB broth+chloranphenicol

7/30/08

For both experimental and control harvest bacteria, inoculated 1 mL into 50mL of LB broth + chloranphenicol. Shook at 30C until they reached 0.7 OD.

	1 hr. 15 min. OD	**3 hrs. 45 min. OD**	**5 hr. 15 min. OD**
Control	0.180 A	0.371 A	1.274 A
Experimental	0.168 A	0.345 A	1.172 A

At 1.2 A (ideally should have been 0.7 A), added 0.500 g of glucose and 5 ul of anhydrotetracycline (10 ug total) to the 50 mL broth. Shook at 37C for 48 hours.

Previous construct 4 colony 10 sequencing showed 4 possible mutations in pSOScat2.

Prepared sequencing samples for pSOScat2 #8 and pSOScat2 #9 to check if pSOScat2 has mutations:

Sample	**DNA**	Primer	**H2O**
pSOScat2 #8	2.52 ul	0.5 ul pUC19SeqFw	8.98 ul
pSOScat2 #8	2.52 ul	0.5 ul pUC19SeqRv	8.98 ul
pSOScat2 #9	1.38 ul	0.5 ul pUC19SeqFw	10.12 ul
pSOScat2 #9	1.38 ul	0.5 ul pUC19SeqRv	10.12 ul

However, Zhongying realized the template sequence used for sequencing comparison was an old version of the cat2 gene. A 2008 version that corrected the mutations in the old sequencing data matched up well with the sequencing results of cat2 from construct 4 colony 10.

7/31/08

Continued growing the experimental and control E. coli bacteria for harvesting at 37C.

--
Grew #1-39 of Maggie's colonies on chlor+NR plate with 100ug/L of anhydrotetracycline (5 ug total on 25 mL plate). Also, one colony from construct 1 colony #3 (from –80C) was plated as a positive control.

Appendix

Using restriction enzyme digestion reactions to cloning the plastic biosynthesis genes

#1: pASKphaCAB-noTag

Insert size: 3.87bp
PCR template: phaCAB in pCR Blunt II Topo #15

phaCXbaIFw: 5'-gc***tctaga***CGGCAGA GAG ACA ATC AAA TCA TG-3'
phaBBamHIRv: 5'-cg***ggatcc***TCA GCC CAT ATG CAG GCC G-3'

phaC gene start: tagtga**cggcagagagacaatcaaatcatg**gcgaccggcaaaggcgcggca
phaB gene end: tcgaccggcgccgacttctcgctcaacgg**cggcctgcatatgggctga**cctgccgg

Vector size: 2.92kb
:pASK-IBA7C digested with XbaI and BamHI

pASK-IBA7C (3021bp)
aagtgaaatgaatagttcgacaaaaa***tctaga***taacgagggcaaaaaatggctagctggagccacccgcagttcgaaaaaatcgaagggcgccg
agaccgcggtcccgaattcgagctcggtacccgg***ggatcc***ctcgaggtcgacctgcaggggacca

#2: pASKphaCAB-tag

Insert size: 3.85kb
PCR template: phaCAB in pCR Blunt II Topo #15

phaCEcoRIFw: 5'-cg***gaattc*** ATG GCG ACC GGC AAA GGC G-3'
phaBBamHIRv: 5'-cg***ggatcc***TCA GCC CAT ATG CAG GCC G-3'

phaC gene start: tagtgacggcagagagacaatcaaatc**atggcgaccggcaaaggcg**cggcagcttcca
phaB gene end: ctcgaccggcgccgacttctcgctcaacgg**cggcctgcatatgggctga**cctgccgg

Vector size: 3.0kb
pASK-IBA7C digested with EcoRI and BamHI

pASK-IBA7C (3021bp)
ccatcgaatggccagatgattaattcctaatttttgttgacactctatcattgatagagttattttaccactccctatcagtgatagagaaaagtgaaatgaata
gttcgacaaaaatctagataacgagggcaaaaaatggctagctggagccacccgcagttcgaaaaaatcgaagggcgccgagaccgcggtccc***g***
aattcgagctcggtacccgg***ggatcc***ctcgaggtcgacctgcaggggacc

Primers for Assembly PCR

#7: pASKphaAB-Ter-pLZCat2phaC

pASK-IBA7C (3021bp)
aagtgaaatgaatagtt**cgacaaaaa*tctaga*taacgagggcaa**aaaatggctagctggagccacccgcagttcgaaaaaatcgaagggcgccg
agaccgcggtcccgaattcgagctcggta**cccgggggatcc*ctcgag*gtcgacctgcag**ggggacca

pASKFw:

CAAAGCAGGCATGACCCTCGAGGTCGACCTGCAG

Phusion PCR 62
Assemble: 60

pASKRv:

CTTTCAATGGTTGCCCTCGTTATCTAGATTTTTGTCG

Phusion 61
Assemble: 60

PCR product: ~3kb

phaA Start:
Cccgttt**ccattgaaaggactacacaatgactgacg**ttgtcatcgtatccgccgcccgcaccgcggtcggcaagtttggcgg

phaB Stop:
gggcctgccggaagagatcgcctcgatctgcgcctggttgtcgtcggaggagtccggtttctcgaccggcgccgacttctcgctcaacgg**cggcctg
catatggctga**

PhaABFw:

AGATAACGAGGGCAACCATTGAAAGGACTACACAATGACTGACG
Phusion 63
Assemble: 60

PhaABRv:

GCTCTAGTATCAGCCCATATGCAGGCCGCC

Phusion 64
Assemble: 61

PCR product: ~2.0 kb

Terminator

tactagagccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacg
ctctctactagagtcacactggctcaccttt**cgggtgggcctttctgcgtttata**

TermFw:

CTGCATATGGGCTGATACTAGAGCCAGGCA TCA AAT AAA ACG

Phusion 62
Assemble: 61

TermRv:

GCTCACTGCCCGCTTTCCATATAAACGCAGAAAGGCC CAC CCG
Phusion 63
Assemble: 60

PCR product: 173bp

Cgactggaaagcgggcagtgagcgcaacgcaattaatgtgagttagctcactcattaggcaccccaggctttacacttt

Cat2phaCFw:

TGGAAAGCGGGCAGTGAGCGC

Phusion 65
Assemble: 60

atgcgcgctatcgcgcaatcgaacccgcgcctgg**gcgatacgtcaaagccaaggcatga**

Cat2phaCRv:

CGAGGGTCATGCCTGCTTTGACGTATCGC

Phusion 64
Assemble: 60

PCR product: ~3.3kb

#3: pASKphaC-tag

Insert size: 1.77kb
PCR template: phaCAB in pCR Blunt II Topo #15

phaCEcoRIFw: 5'-cg***gaattc*** ATG GCG ACC GGC AAA GGC G-3'
phaCBamHIRv: 5'-cg***ggatcc***TCA TGC CTT GGC TTT GAC GTA TC-3'

phaC gene start: tagtgacggcagagagacaatcaaatc**atggcgaccggcaaaggcg**cggcagcttcca
PhaC gene end: gcgctatcgcgcaatcgaacccgcgcctgggc**gatacgtcaaagccaaggcatga**

Vector size: 3.0kb
pASK-IBA7C digested with EcoRI and BamHI

pASK-IBA7C (3021bp)
ccatcgaatggccagatgattaattcctaatttttgttgacactctatcattgatagagttattttaccactccctatcagtgatagagaaaagtgaaatgaata
gttcgacaaaaatctagataacgagggcaaaaaatggctagctggagccacccgcagttcgaaaaaatcgaagggcggccgagaccgcggtccc***g***
aattcgagctcggtacccgg***ggatcc***ctcgaggtcgacctgcaggggacc

#4: pSOSCat2-phaC

phaC insert size: 1.77kb
PCR template: phaCAB in pCR Blunt II Topo #15

phaCBamHIFw: 5'-cg***ggatcc***gtgaataaCGGCAGA GAG ACA ATC AAA TCA TG-3'*
phaCEcoRIRv: 5'- cg***gaattc*** TCA TGC CTT GGC TTT GAC GTA TC-3'
*: tga and taa are the stop codons for the Cat2 protein

phaC gene start: tagtga**cggcagagagacaatcaaatcatg**gcgaccggcaaaggcgcggca
PhaC gene end: gcgctatcgcgcaatcgaacccgcgcctgggc**gatacgtcaaagccaaggcatga**

Vector size: 3.99kb
pSOSCat2 #9 digested with BamHI and EcoRI

pSOSCat2 #9 (complementary strand)
ggcttaactatgcggcatcagagcagattgtactgagagtgcaccatatgcggtgtgaaataccgcacagatgcgtaaggagaaaataccgcatcagg
cgccattcgccattcaggctgcgcaactgttgggaagggcgatcggtgcgggcctcttcgctattacgccagctcgcgaaaggggatgtgctgcaa
ggcgattaagttgggtaacgccagggttttcccagtcacgacgttgtaaaacgacggccagcgtaatcatggtcatt***gaattc***gagctcggtacccg
g***ggatcc***tcaatctctttttaaattcattcattaatgat

#5: pASKphaAB-pLZCat2phaC

phaAB insert size: 2.0kb
PCR template: phaCAB in pCR Blunt II Topo #15

phaAXbaIFw: 5'-gc***tctaga*** CCA TTG AAA GGA CTA CAC AAT GAC TG-3'
phaBEcoRIRv: 5'- cg***gaattc*** TCA GCC *CAT ATG* CAG GCC G -3'

PhaA gene start: t**ccattgaaaggactacacaatgactg**acgttgtcatcgtatccgccgccc
phaB gene end: tcgaccggcgccgacttctcgctcaacgg**cggcctg*catatg*ggctga**cctgccgg

pLZCat2phaC insert size: 3.25kb
PCR template: pSOSCat2-phaC

pLZEcoRIFw: 5'-cg***gaattC***GAC TGG AAA GCG GGC AGT G-3'
phaCXhoIRv: 5'-ccg***ctcgag***TCA TGC CTT GGC TTT GAC GTA TC-3'

pUC19 (part, complementary strand)
GTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCG
GAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTT
GCGCT**CACTGCCCGCTTTCCAGTCG**G

Vector size: 2.9kb
pASK-IBA7C digested with XbaI and XhoI

pASK-IBA7C (3021bp)
aagtgaaatgaatagttcgacaaaaa***tctaga***taacgagggcaaaaaatggctagctggagccacccgcagttcgaaaaaatcgaagggcgccg
agaccgcggtcccgaattcgagctcggtacccggggatcc***ctcgag***gtcgacctgcaggggacca

#6: pASKphaCAB-pLZCat2

phaCAB insert size: 3.86kb
PCR template: phaCAB in pCR Blunt II Topo #15

phaCXbaIFw: 5'-gc***tctaga***CGGCAGA GAG ACA ATC AAA TCA TG-3'
phaBEcoRIRv: 5'- cg***gaattc*** TCA GCC *CAT ATG* CAG GCC G -3'

phaC gene start: tagtga**cggcagagagacaatcaaatcatg**gcgaccggcaaaggcgcggca
phaB gene end: tcgaccggcgccgacttctcgctcaacgg**cggcctg*catatg*ggctga**cctgccgg

pLZCat2 insert size: 1.55kb
PCR template: pSOSCat2 #9

pLZEcoRIFw: 5'-cg***gaattc*** GAC TGG AAA GCG GGC AGT G-3'
Cat2BamHIRv: 5'-cg***ggatcc***ta aAAT CTC TTT TTA AAT TCA TTC ATT AAT GAT TCT C -3'

pSOSCat2 #9 (part, complementary strand)
tggtcattgaattcgagctcggtacccggggatcctc**aatctcttttaaattcattcattaatgattctc**

pSOSCat2 #9 (part, complementary strand)
GTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCG
GAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTT
GCGCT**CACTGCCCGCTTTCCAGTCG**G

Vector size: 2.9kb
pASK-IBA7C digested with XbaI and BamHI

pASK-IBA7C (3021bp)
aagtgaaatgaatagttcgacaaaaa***tctaga***taacgagggcaaaaaatggctagctggagccacccgcagttcgaaaaaatcgaagggcgccg
agaccgcggtcccgaattcgagctcggtacccgg***ggatcc***ctcgaggtcgacctgcaggggggacca