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From IGEM 2010

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Notebook 4/9/10

Insert: pRFPEC 2.6kb: resistance: Ampicillin
Vector: pET28b 5.4kb: resistance: Kanamycin
Inserting 718bp RFP gene into pET28b vector using ecoRI and BamHI

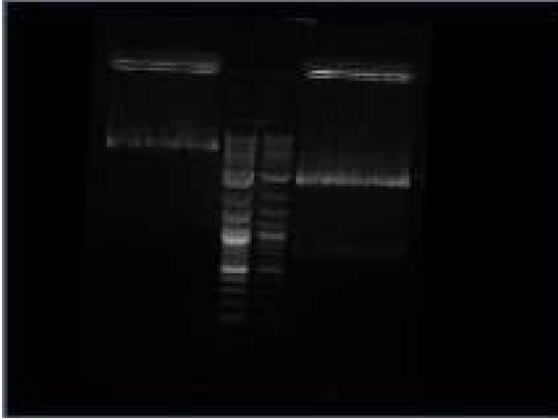
1)Miniprepped vector and insert with kit. Ran check gel to estimate concentrations.

2) Ran a double digest of insert and vector with ecoRI HF and BamHI HF with buffer 4.

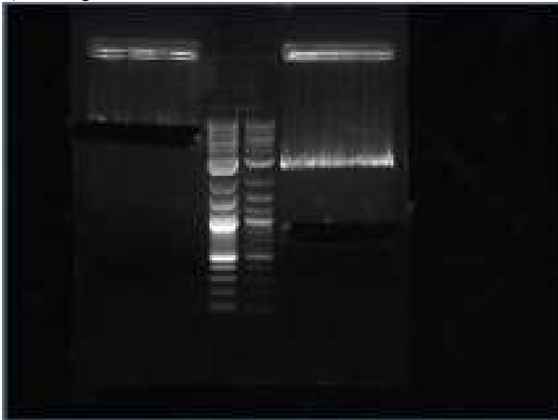
BamHI HF	2.5uL
EcoRI HF	2.5uL
Buffer 4	5uL
DNA	40uL

Ran overnight.

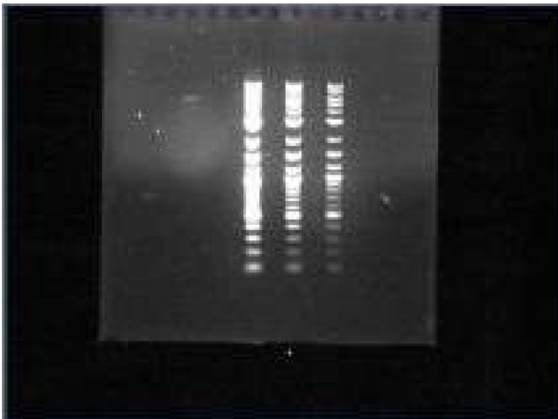
3) Ran test gel of digest.



It wasn't very much to work with, but it was visible. On the right is the pRFPEC gene, on the left is the pET28b.
4) Sliced gel.



5) Purified gel extract using kit. Washed columns two more times with ethanol, in addition to the once in the kit protocol. Eluted with 30uL 70C EB buffer to increase concentration.



On the left is pRFPEC on right is pET28b. The ladders are 1X, .5X, .25X respectively.
6) Performed ligation at 16C for 2.5 hours. Used in a total of 10 uL reaction:

```
Plate 1: 2 vector, no insert, 1 buffer, 1 ligase, 6 water
Plate 2: no vector, 6 insert, 1 buffer, 1 ligase, 2 water
Plate 3: 2 vector, 6 insert, 1 buffer, 1 water, no ligase
Plate 4: 2 vector, 5 insert, 1 buffer, 1 ligase
Plate 5: 1 vector, 7 insert, 1 buffer, 1 ligase
```

7) Transformed cells (saved some DNA from ligation just in case). Time constant was on average, about 3 +/- .3, no arcs. Streaked 6 plates to screen. The 2:5 and 1:7 showed bacteria growth the next day on the Kan Plates (1:7 had about 30 colonies, 2:5 had about 7 colonies). All other plates showed no growth.
8) Inoculated three cultures of 1:7 colonies and 1 culture of 2:5. Streaked a plate with these four cultures.

==Screening: April 20==
9) Inocculated 6 colonies from plates 5-1, 5-2, 3 (should have done more). Colonies were found on all plates, whereas there were no colonies on April 10, which means either the plates didn't have adequate antibiotics and some bacteria without the Kanamycin resistance were taking advantage of that or my transformations failed. 20 uL Kan in 5ml LB. Plate 3 served as the control: the bacteria should *not* grow in Kanamycin. Colonies from 5-1 and 5-2 should grow in Kanamycin if the transformation worked correctly. The following is a picture of the cultures:

The first three on the left are from plate 5-2, the next two are from 5-1, and the far right is from plate 3.
Plate 3 showed no growth in Kanamycin, as expected since the bacteria shouldn't have the plasmid for Kan resistance. This is evidence that the bacteria on it were simply taking some sort of advantage on the Kan plates
10) I then ran an overnight digest on bacteria from 5-1 and 5-2 by mini-prepping and then digesting all the DNA with BamHI HF and EcoRI HF in 10% NEB buffer 4.

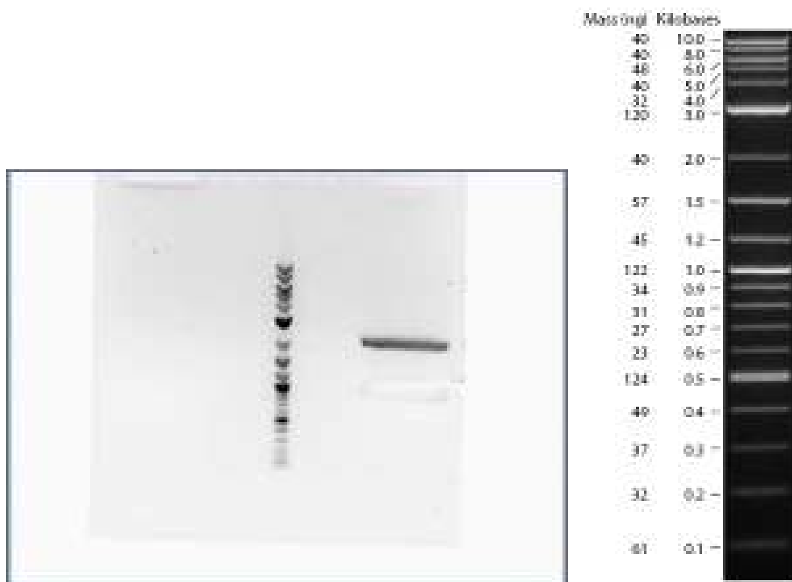
April 21
11) Ran a gel of the digest from previous day.
Which yielded a single bands at about ~5.5 kb, which means for that colony, the vector probably just re-ligated on itself, along with the Kan resistance. Still need to do a large screening of many colonies to try to find one that worked.

Notebook 6/9/10

BOB & POP Clones
-Minipreped BOB and POP integration sites (BBa_11022 & BBa_11023 in pSB2k3 plasmid)
-Concentrations were found to 28.2 ng/uL for 11022 and 35.6 ng/uL for 11023. Very low, however it was found that the pSB2k3 is an inducible copy number plasmid inducible with IPTG. -New liquid cultures inocculated (5mL) and induced with 0.5uL of 1M IPTG, to make a concentration of 100uM of IPTG. -Digests of BBa_11022, BBa_11023, BBa_E0240, BBa_K199021 for 2 hr

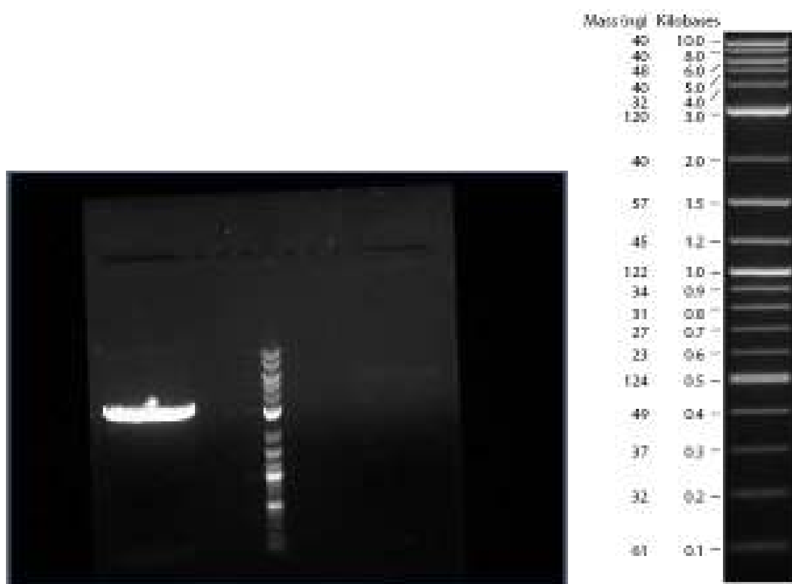
11022 (BOB)	11023 (POP)	E0240 (RBS-GFP-T-T)	K199021 (RBS-RFP inverted)
1 SpeI	1 EcoRI HF	1 XbaI	1 SpeI
1 PstI	1 XbaI	1 PstI	EcoRI HF
0.5 BSA	0.5 BSA	0.5 BSA	0.5 BSA
5 Buffer 4	5 Buffer 4	5 Buffer 4	5 Buffer 4
40 DNA	40 DNA	4.79 DNA	7.53 DNA
2.5 H20	2.5 H20	37.7 H20	35 H20
50 uL	50 uL	50 uL	50 uL

The resulting gel was this:



above: BB_I1022 (BOB backwards), 2log ladder, BB_E0240 (RBS-GFP-T-T)

The BOB dna is non-existent (the concentration may have been too low). The GFP band at 500bp is too small, the part is more than 800 bp.



above: BB_K199021 (RBS RFP backwards), 2log ladder, BB_I1023 (POP)

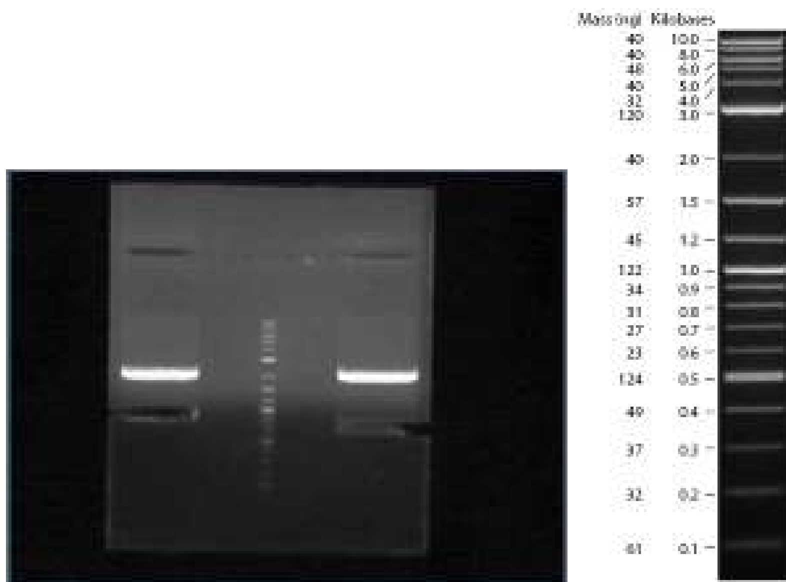
The RFP didn't seem to cut. But the POP has a very faint band at 4.6kb, which is where it should be, however it is too small to work with.

Notebook 6/10/10

-started second miniprep of 11022, 11023 (BOB & POP) and eluted with 40uL H20. Concentrations for 11022 and 11023 were 37.8 ng/uL and 30.4 ng/uL. Did another miniprep and received concentrations of 20.9 and 22.2 for 11022 and 11023. -combined minipreps and did DNA precipitation:

```
1. add 0.1 volume 3M NaAC
2. add 2 volumes cold EtOH and cool for 1 hr in freezer
3. centrifuge for 15 min.
4. remove as much supernatant as possible.
5. add 200uL cold 70% EtOH
6. centrifuge 5 minutes
7. removes supernatant and evaporate ethanol
8. re-suspend pellet in water
```

-inocculated liquid cultures of 11022 and 11023 to be used in a midiprep
-began digest of E0240 and K199021 (RBS GFP T T & RBS RFP backwards)



left to right: RBS-GFP-T-T, ladder, RBS-RFP backwards

Notebook 6/15/10

Sequencing of Cre and Hin Cassette and pBAD33 and pBAD35

Began sequencing Reaction of:
1. B1006 (http://partsregistry.org/Part:BBa_B1006) + 1718008 (http://partsregistry.org/wiki/index.php?title=Part:BBa_I718008) + B1006 (http://partsregistry.org /Part:BBa_B1006) (Cre Cassette T+araC RBS cre+T) clone. See part page here.
2. B1006 (http://partsregistry.org/Part:BBa_B1006) + 1718008 (http://partsregistry.org/wiki/index.php?title=Part:BBa_I718008) clone.
3. pLac (http://partsregistry.org/wiki/index.php/Part:BBa_K200021) + HinLVA (http://partsregistry.org/Part:BBa_J31000) clone.See part page here.
4. pBAD33 mutation (from lactase project).
5. pBAD35 mutation (from lactase project).

Thermocycler Protocol:
1) 95C 3 mins
2) 95C 30s
3) 50C for 10s
4) 60C for 4min
5) Go to 2 (35 times)
6) Hold 4C

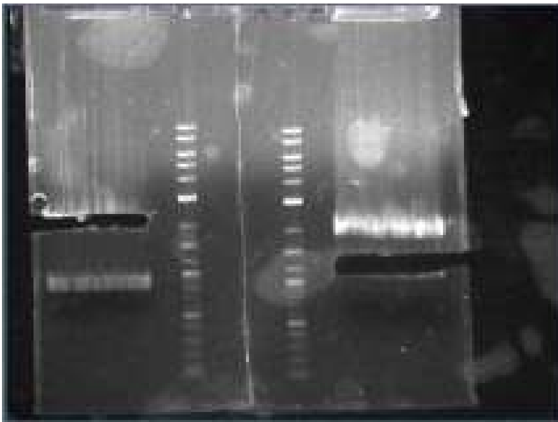
P22 integrase parts and new RBS-Lac parts
Did liquid cultures of I11033 (http://partsregistry.org/Part:BBa_I11033) (POP derived from p22), I11032 (http://partsregistry.org/Part:BBa_I11032) (BOB derived from p22), S03520 (http://partsregistry.org/Part:BBa_S03520) (promoter and lacI)

Notebook 6/16/10

Cloning of BBa_S03520 (http://partsregistry.org/Part:BBa_S03520) and BBa_J23100 (http://partsregistry.org/Part:BBa_J23100) (Promoter$_{const.}$ + RBS + lacIq)

- Nate digested BBa_S03520 (http://partsregistry.org/Part:BBa_S03520) and BBa_J23100 (http://partsregistry.org/Part:BBa_J23100) by cutting out the RFP contained in the BBa_J23100 (http://partsregistry.org/Part:BBa_J23100) with SpeI and PstI and cutting out the RBS lacIq part from the BBa_S03520 (http://partsregistry.org/Part:BBa_S03520) with XbaI and PstI.

- Gel extracted the two parts:



Its important to note that the J23 promoter has an odd digest patters, see BBa_J23100 (http://partsregistry.org/Part:BBa_J23100) .
On the left, the bottom band is the RFP contained in the vector backbone, the top band is the vetor and the promoter we want.
On the right, the bottom band is the RBS+lacIq and the top is the rest of the plasmid.

The gel extraction yielded the following concentrations:

```
J23: 18.4 ng/uL

S035: 6.4 ng/uL
```

- Preformed ligations:

Rxn 1	Rxn 2	Control
1 ligase buff	1 ligase buff	0 ligase buff
1 ligase	1 ligase	0 ligase
6 uL S035	7 uL S035	no insert
2 uL J23	1 uL J23	3 uL J23

- Proceeded to transform cells:

```
Transformation 1: 4.3ms

Transformation 1a: 4.00ms

Transformation 2: 5.9ms

Transformation Control: 4.8ms
```

- Plated transformations and stored in 37C incubator

Notebook 6/17/10

Promoter Lac Clone

Only plates from reaction 2 of ligation grew, so liquid cultures of three colonies were done. None on other plates.

- Redid ligation using 3 backbone (J23):5 insert (S035)
- Retransformed cells from yesterdays ligation:

```
transformation 1 (yesterday's ligation): 3.8 ms

transformation 2 (yesterday's ligation): 3.3 ms

transformation 3 (today's ligation): 3.8 ms

control: 4.4 ms
```

- Let outgrow for 1hr and plated cells.

Sequencing

Received sequencing information (see 6/18/10)

Notebook 6/18/10

The BBa_J23100 (http://partsregistry.org/Part:BBa_J23100) and BBa_S03520 (http://partsregistry.org/Part:BBa_S03520) clone (the Promoter$_{const.}$+RBS+lacIq)

Plates from the previous day's transformations yielded lots of growth, with quite a few colonies on the control.
At least 8-10 times as many colonies on the ligated transformations compared to the control. Liquid cultures
were inocculated for 6 colonies and one for the control (to test the control plate) in Ampicillin. The colonies on the
Amp plates suggest that they may be losing their resistance, or at least on that one plate.

Sequencing of Cre and Hin Cassette

Began Sequencing Alignment of Cre Cassettes and Hin Cassette (K20+J31)
Strip tube labels:

1- Af	B1 + I7 clone w/ forward primer
2 - Ar	B1 + I7 clone w/ reverse primer
3 - Bf	K20+J31 forward primer
4 - Br	K20+J31 reverse primer
5 - Cf	Cre Cassette forward primer
6 - Cr	Cre Cassette reverse primer
7 - pBAD33f	pBAD33 forward primer
8 - pBAD33r	pBAD33 reverse primer
9 - pBAD35f	pBAD35 forward primer
10 - pBAD35r	pBAD35 reverse primer

1 - Af B1 + I7 clone w/ forward primer
2 - Ar B1 + I7 clone w/ reverse primer
Bf,r - K20 + J31
Cf,r Cre Cassette B1 + I7 + B1

See Completed Parts for more sequencing information.

The reverse sequencing reactions for Cre Cassette had a very weak signal. We may consider doing the reverse seq. rxn. over again to determine correct sequence.

6/19/10

Screening of BBa_J23100 (http://partsregistry.org/Part:BBa_J23100) and BBa_S03520 (http://partsregistry.org/Part:BBa_S03520) clone (the Promoter$_{const.}$+RBS+lacIq)

Began screening of liquid cultures of the BBa_S03520 (http://partsregistry.org/Part:BBa_S03520) and BBa_J23100 (http://partsregistry.org/Part:BBa_J23100) clone using the alkaline lysis miniprep procedure, however, due to the nature of the J23 vector plasmid, J61002 (http://partsregistry.org/Part:BBa_J61002) , a rigorous enzymatic screening was not necessary. Since any uncut backbone, would simply result in a contituitive promoter and RFP, the colonies on the screening plate that resulted from uncut backbone would result in red colonies. So selection was based on the colony color and plasmid resistence, since the biobrick enzymes were non-compatible, there was no chance of vector religation or insert religation.
So if the digest shows the expected banding pattern, then we can be fairly certain that the cloning was successfull and enzymatic screening is not necessary.

Cloning of BBa_J23100 (http://partsregistry.org/Part:BBa_J23100) and BBa_S03520 (http://partsregistry.org/Part:BBa_S03520) + B1006 (http://partsregistry.org/Part:BBa_B1006)

Also, inocculated the B1006 (http://partsregistry.org/Part:BBa_B1006) "terminator" part for finishing the lacIq cassette, which would consist of a cont. Promoter + RBS + lacIq + Terminator.

6/20/10

6/21/10

Cloning of BBa_J23100 (http://partsregistry.org/Part:BBa_J23100) and BBa_S03520 (http://partsregistry.org/Part:BBa_S03520) + B1006 (http://partsregistry.org/Part:BBa_B1006) continued

Miniprep:

The miniprep concentrations:

```
B1006 (http://partsregistry.org/Part:BBa_B1006)  123.1 ng/uL

BBa_S03520 (http://partsregistry.org/Part:BBa_S03520)  and BBa_J23100 (http://partsregistry.org/Part:BBa_J23100) : 83.5 ng/uL.
```

Digest:
Biobrick Digests:

Part	BBa_S03520 (http://partsregistry.org/Part:BBa_S03520) + BBa_J23100 (http://partsregistry.org/Part:BBa_J23100)	B1006 (http://partsregistry.org/Part:BBa_B1006)
DNA	33uL	33uL
Enzyme 1	1.5uL EcoRI HF	1.5uL EcoRI HF
Enzyme 2	1.5uL SpeI	1.5uL XbaI
Buffer 4	4	4
BSA	.4 (100X)	.4 (100X)
Total	~40uL	~40uL

Gel Extraction:
Gel Extracted the parts. The BBa_S03520 (http://partsregistry.org/Part:BBa_S03520) + BBa_J23100 (http://partsregistry.org/Part:BBa_J23100) clone had an expected bands at 1.1kb and ~2kb (due to the 2.94kb J61002 (http://partsregistry.org/Part:BBa_J61002) plasmid minus the RFP cut out). The 1.1 kb is the promoter + J23 + S035 clone, and was a very faint band. The B1006 vector was extracted at the 3.2kb band.

Both slices were 0.14 grams, so kit purification was done. Resulting in the following concentrations:

```
J23 + S035: 2.2 ng/uL very low concentration!

B1006: 23 ng/uL also low concentration
```

<b>Ligation:

Even though concentrations were very low, a single ligase reaction was done, perhaps lady luck will smile upon my ligation reaction and bring the birth of new successful transformations.

Ligation	Control
1 uL ligase	0 ligase
1 uL ligase buffer	0 ligase buffer
7 J23+S035 insert	7 H20
1 B1006 backbone	3 B1006 backbone (to exaggerate control)

Note that the control has more backbone than the first ligation, this is because having only 1uL of backbone may not have adequate DNA for a tranformation and may result in a false negative, (having no colonies on the control plate when in fact there was some undigested vector present). So the backbone was exaggerated, so that, if there was undigested backbone, it would have a higher chance of being revealed on the control plate, so that the amount of undigested vector could be quantified.

Tranformation:

```
lac cassette clone: 3.5ms

control: 5.6 ms
```

Ligation was left to outgrow in 37C incubator for just over 60 minutes, before they were plated.

Hin Cassette Screening

Picked 12 colonies, A,B, & C for HIn Cassette Screening and innocculated liquid cultures. On the Hin Cassette control plate, there were no colonies, and a few hundred on the ligation plate, so only 3 colonies were screened.

6/22/10

Results of lac generator transformation (BBa_J23100 (http://partsregistry.org/Part:BBa_J23100) and BBa_S03520 (http://partsregistry.org/Part:BBa_S03520) + B1006 (http://partsregistry.org/Part:BBa_B1006))

The control, which consisted of 3uL backbone (5.6 ms during transformation) had about as many colonies as the ligation, which had 1uL backbone (3.5 ms). This indicates there was a significant amount of uncut vector B1006 (http://partsregistry.org/Part:BBa_B1006) . However, the control, having more backbone than the ligation will be "exageratted" in respect to the quantification of the uncut vector. The control plate had about 300 larger colonies, while the ligation rxn had about 300 smaller colonies, with a few large colonies similar in appearance to the ones on the control plate. **So screening of 12 colonies, A through L were chosen throughout the plate.**

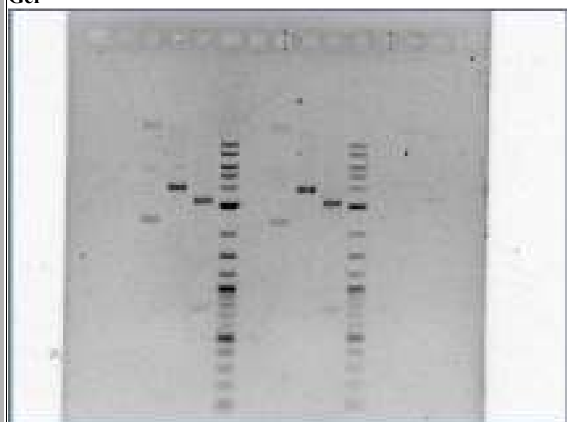
In case that the transformation was unsuccessful, more of the J23+S035 clone was inocculated for potential gel extraction later in the week depending on the results of the screening.

Hin Cassette Screening

Digests:

Colony	A_{uncut}	A_{PstIHF}	$A_{PstI\ HF\ \&\ EcoRI\ HF}$	B_{uncut}	B_{PstIHF}	$B_{PstI\ HF\ \&\ EcoRI\ HF}$	C_{uncut}	C_{PstIHF}	$C_{PstI\ HF\ \&\ EcoRI\ HF}$
DNA	5	5	5	5	5	5	5	5	5
PstI HF	0	1	1	0	1	1	0	1	1
EcoRI HF	0	0	1	0	0	1	0	0	1
H20	11	10	9	11	10	9	11	10	9
Total	20	20	20	20	20	20	20	20	20

Gel



6/23/10

Screening of lacI generator (BBa_J23100 (http://partsregistry.org/Part:BBa_J23100) and BBa_S03520 (http://partsregistry.org/Part:BBa_S03520) + B1006 (http://partsregistry.org/Part:BBa_B1006)) clone

Miniprep Miniprepped the twelve liquid culture from colonies A through L using the alkaline lysis protocol. The DNA pellet was very visible, so concentrations were not measured and digest was proceeded with immediately

---

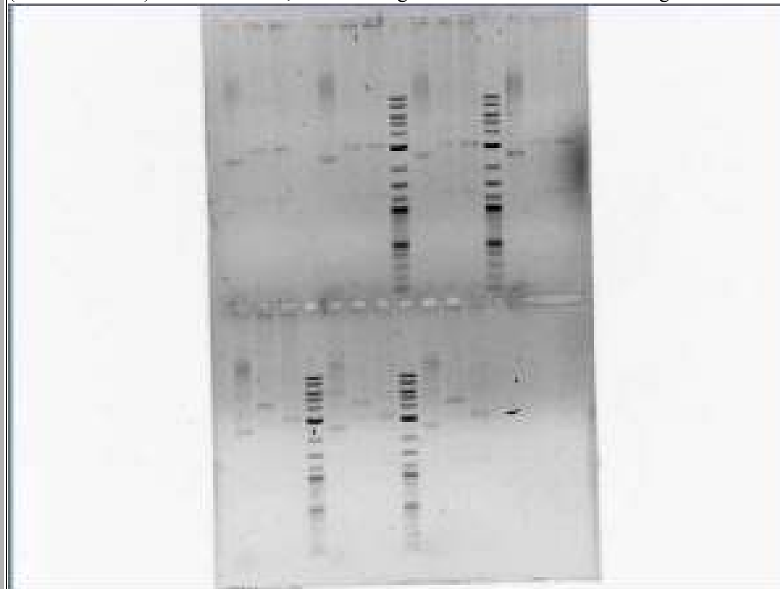
Digest:
Overnight digest of the following:

Colonies A through L	Single Cut with PstI	Double Cut with PstI and EcoRI
DNA	7uL	7uL
Buffer 4	2	2
BSA (10X)	2	2
EcoRI HF	0	1
PstI HF	1	1
H20	8	7
Total	20	20

6/24/10

Proof of Completed LacI generator: Screening of lacI generator (BBa_J23100 and BBa_S03520 + B1006) clone continued

The expected banding pattern of the LacI generator using biobrick enzymes EcoRI and PstI is 1.1 kb and 3.2 kb and about a 4.3kb band with single cut enzymes. The format of the gel is: colonies are in alphabetical order, separated by ladders and each colony "triplet" is in the following order: uncut, single cut (PstI), and double cut (PstI and EcoRI). ith this in mind, the following are the results of the screening:



Above: Starting at lane 5 (1st three lanes are colony A), Colonies A through F with 2 log ladder
Colony E above may be successful, but the 1.1kb band is kind of faint, however there is more success in gel 2 below.

Colonies G through L starting at lane 1 with 2 log ladder

Colonies H (2nd lane triplet), J (4th triplet), and K (5th triplet) were successful! As you can see, those colonies had a single band (middle lane in triplet) of about 4.3 kb and double cut bands of 3.2 kb and 1.1 kb, which means those colonies were successful and **the lac generator is completed.**

Colony H will be selected as the lacI generator bacteria line and will be innocculated and plated.

See LacI Cassette Completed Parts section.

This week (6/19 through 6/24) summary

1. Hin Cassette was successful and plated, the gel pictures and proof for that or on the fried computer. The fresh plate is in the 37C incubator as of 6/25 and needs to be glycerol stocked.
2. LacI generator was successful and plated, colony H was selected and need to be glycerol stocked. The liquid culture was inocculated with A+K in the 37 incubator as of 6/25.
We now have all of our major "key" clones prepared and ready for testing as soon as our "lock" construct is finished being synthesized. The last thing we have to do is combine the lacI generator with the hin cassette, to produce the final hin generator cassette. Then we will have to place the hin and cre completed parts into plasmids of our choosing.

Summary of 6/28 through 7/1

Attempt of Hin Cassette + LacI cassette clone

Three attempts to get LacI plasmid prepped resulted in no plasmid band whatsoever on a gel. Plasmid was kit prepped twice, and alkaline lysed, all of which resulted in no plasmid band on a gel after an attempted digest. Hin cassette, which was prepped in parallel in order to clone, did show expected banding. The following gel is after a kit prepping and digest (Hin: SpeI and PstI & and Lac: XbaI and PstI):

Further screening will be done on the following colonies from the original ligation: D,E,F,H,J,K,L. These were the colonies that looked promising during the first screening of the lacI cassette. See LacI Cassette for further information on the LacI Cassette Screening. These colonies will be cultured in LB with Ampicillin and Kanamycin. A control for Kanamycin was thrown in, the Pc+LacI clone which has only Ampicillin resistence (if it grows, then Kanamycin is bad). I don't believe the Ampicillin is bad, but it is possible.

Sequencing of Hin, Cre, and Lac Cassettes

- Lac cassette sequencing will be put on hold until the second screening.
- Hin Cassette will be put on hold until Cre cassette shows that a reverse sequencing reaction can be done through the last terminator (see below).
- The Cre Cassette sequencing failed on the reverse reaction. We have reason to believe that the reverse reaction cannot read through the terminator at the end of the cassette, and so we are going to sequence the Cre Cassette in reverse again. If it fails, then we will design a forward sequencing primer in the cre gene to read through the rest of the cassette; we will do the same for the Hin abd Lac cassettes since they also have the same terminator at the end of the gene.

Notebook 7/1/10

Cloning of Hin Generator (Hin Cassette + LacI Cassette)

Miniprepping:
Kit prepped Cre Cassette, Hin Cassete, and LacI Cassettes:

```
concentrations:
cre: 95 ng/uL
hin: 46.3 ng/uL
LacI: 58.3 ng/uL
```

Digests:

LacI Cassette (insert)	Hin Cassette (backbone/vector)
30uL DNA	30uL DNA
3.6 Buffer 4	3.6 Buffer 4
0.36 BSA (100X)	0.36 BSA (100X)
1uL XbaI	1uL SpeI
1uL PstIHF	PstIHF

Gel Extraction:
There was a band for the Hin Cassette, but no DNA whatsoever (again) in the LacI Cassette. Since, this has happened multiple times, where the Hin Cassette band is there, and there is no LacI Cassette band at all, re-screening of the LacI cassette will be done.

Re-Screening of LacI Cassette

Innocculations of D,E,F,H,J,K,L were done from the original screening plate. These were the colonies that looked promising in the initial screening. Please see LacI Cassette for information on the original screening.

Notebook 7/2/10

Re-Screening of LacI Cassette

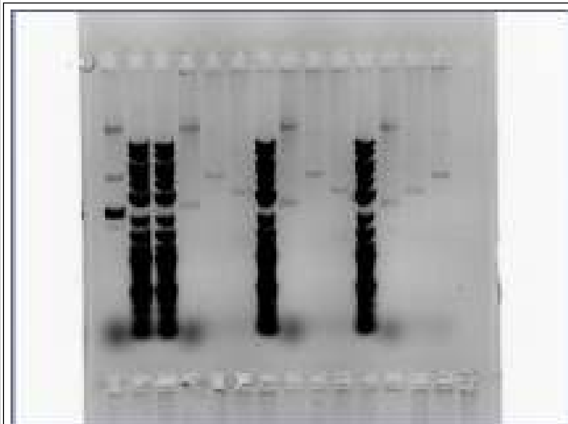
Digest of LacI Cassette Screen

```
Solution 1: 35 Buff 4, 3.5 BSA (10X), 140 H20
Solution 2: 77 Solution 1, 7 PstI HF
Solution 3: 77 Solution 2, 7 PstI HF, 7 EcoRI HF
```

Colonies D, E, F, H, J, K, L in 7 strip tubes, 8uL DNA + 10uL Solution 2 and in 7 other strip tubes, 8uL DNA + 10uL Solution 3.

Gel Of Re-Screeing

Top Gel



Gel Channels:

4	D_{uncut}
5	D_{PstIHF}
6	$D_{PstIHF+EcoRIHF}$
7	2log

Bottom Gel

15	H_{uncut}
16	H_{PstIHF}
17	$H_{PstIHF+EcoRIHF}$
18	2log
19	J_{uncut}
20	J_{PstIHF}

8	E_{uncut}
9	E_{PstIHF}
10	$E_{PstIHF+EcoRIHF}$
11	2log
12	F_{uncut}
13	F_{PstIHF}
14	$F_{PstIHF+EcoRIHF}$

21	$J_{PstIHF+EcoRIHF}$
22	2log
23	K_{uncut}
24	K_{PstIHF}
25	$K_{PstIHF+EcoRIHF}$
26	L_{uncut}
27	L_{PstIHF}
28	$L_{PstIHF+EcoRIHF}$

Picking Colonies to Plate for New Cell Line of LacI Cassette
According to the gel above, all the colonies that were re-screened showed the correct banding pattern. The expected banding pattern of the LacI generator using biobrick enzymes EcoRI and PstI is 1.1 kb and 3.2 kb and about a 4.3kb band with single cut enzymes. So, colonies E and J were chosen as the new cell line of the LacI Cassette.

Notebook 7/3/10

Cloning of Hin Generator (Hin Cassette + LacI Cassette)

Innocculated Colonies E and J from the re-screening of LacI Cassette and Hin Cassette for the Cloning of the Hin Generator (Hin Cassette + LacI Cassette)

Notebook 7/4/10

Cloning of Hin Generator (Hin Cassette + LacI Cassette)

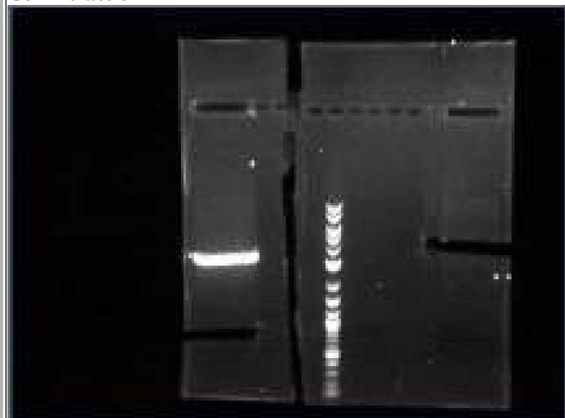
Miniprep
Miniprepped innocculations of LacI Cassette Colonies E & J and Hin Cassette using Quiagen kit protocol.

Digest
Overnight digest of LacIq Cassette Colony E, Hin Cassette, and Colony J

Notebook 7/510

Cloning of Hin Generator (Hin Cassette + LacI Cassette)

Gel Extraction



Above: Hin Cassette (left), 2 log ladder (middle), lacIq Cassette (right)

Gel purified the slices:

```
lacIq slice: 8.9ng/uL
Hin slice: 8.1ng/uL
```

Ligation
Set up benchtop ligation (0.5 hours)

Ligation 1	Ligation 2	Control
1uL LacIq DNA	2uL LacIq DNA	2uL lacIq DNA
7uL Hin DNA	6uL Hin DNA	8uL H20
1uL Buffer	1uL Buffer	---
1uL T4 ligase	1uL T4 ligase	---

Transformation of DH10B cells

```
1:7  3.80ms (2.4 uL DNA)
2:6A arced (2.4uL DNA)
2:6B 3.80ms (2uL DNA)
Control 5.60ms (5uL DNA)
```

Left cells to out grow in water bath at 37C with occasional stirring then plated cells. There were only 2 A/K plates left, so I decided to plate the 1:7 on the A/K as well as the control, a control on a Kan plate, then the 2:6 ligation on a Kan plate.

Notebook 7/6/10

Cloning of Hin Generator (Hin Cassette + LacI Cassette)

Results from transformations
The 1:7 transformation on the A/K plate had a single colony. The 2:6 plate on the Kan plate had over 30 colonies. Both of the controls had no colonies. So colonies 1,2,3,5 were chosen from the 2:6 transformation, and colony 5 was chosen from the 1:7 transformation to be innocculated in Amp and Kan liquid LB.

Notebook 7/7/10

Cloning of Hin Generator (Hin Cassette + LacI Cassette)

Inoculations from 7/6
Colonies 1,2,3,5 (from the 2:6 transformation on the Kan plate) yielded no growth while colony 4 (from the 1:7 transformation on the A/K plate) yielded growth. A lesson to be learned here is to always use the exact antibiotics as the plasmid has resistance for.
Colony 4 growth as mini-prepped for screening.

Digest
37C for 2hrs

8uL DNA
1uL Buff 4
1uL BSA (10X)
0.5uL PstI HF
0.5uL EcoRI HF
11uL total

Gel Extraction
Ran a gel of the digest. The gel only showed the ladder. I suspect that the DNA is not sinking to the bottom of the channel.

Ligation
I redid the ligation using 3uL insert:5uL backbone which is about 3X insert to 1X backbone.

Transformation

```
Transformation 1 of ligation: 2.4uL DNA 3.50ms
Transformation 2 of ligation: 2.2 uL DNA 3.50ms
Control: 3.0uL DNA 5.60ms
Control: 3.2uL DNA 4.7ms
```

After outgrowth, the transformations were all plated on A/K plates.

Notebook 7/8/10

Cloning of Hin Generator (Hin Cassette + LacI Cassette)

Transformation Results:

```
Plate 1 (transformation 1): 26 colonies
Plate 2 (transformation 2): 6 colonies
Control 1: 0 colonies
Control 2: 1 colony
```

Innocculated 4 colonies from plate 1 and 1 colony from yesterday's transformation screen of the single colony ("colony 4") as to redo screening.

Sequencing of "Hin Cassette"
Started sequencing reaction for "Hin Cassette"

Notebook 7/9/10

Cloning of Hin Generator (Hin Cassette + LacI Cassette)

Miniprep
Miniprepped colonies from 7/8 inoculations:

```
Colony 1: 244.8ng/uL
Colony 2: 42.2ng/uL
Colony 3: 223.4ng/uL
Colony 4: 771.ng/uL
Colony 5 (Original Transformation colony): 79.3ng/uL
```

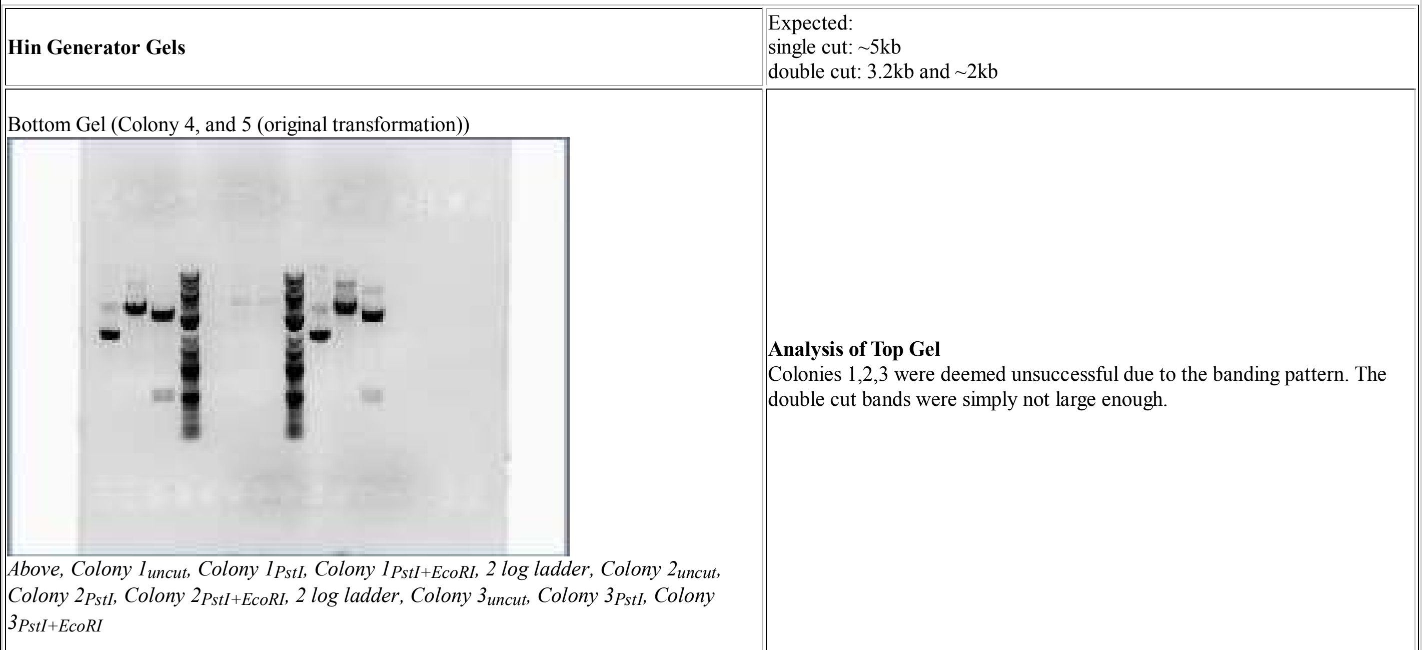
Digest

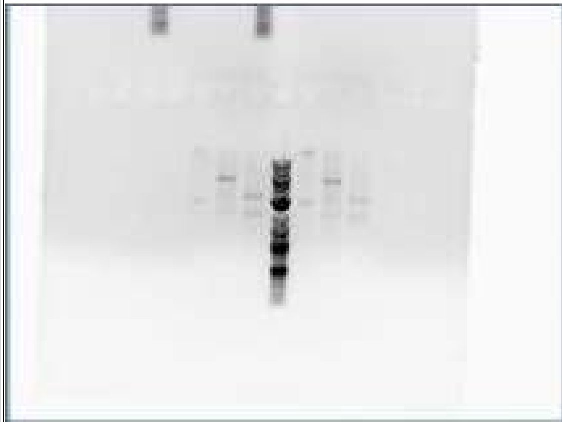
Digests for all colonies 1-5	
Single Cut	Double Cut
1.4 DNA	1.4 DNA
8.6uL Solution 1	8.6uL Solution 2

Enzyme Solution 1
36.6H20
6 Buff 4
6 BSA (10X)
3 PstI HF

Enzyme Solution 2
36.6H20
6 Buff 4
6 BSA (10X)
3 PstI HF
3 EcoRI HF

Results of Screening
Two colonies were deemed successful due to a restriction digest banding pattern:

Hin Generator Gels	Expected: single cut: ~5kb double cut: 3.2kb and ~2kb
Bottom Gel (Colony 4, and 5 (original transformation))  *Above, Colony 1_{uncut}, Colony 1_{PstI}, Colony $1_{PstI+EcoRI}$, 2 log ladder, Colony 2_{uncut}, Colony 2_{PstI}, Colony $2_{PstI+EcoRI}$, 2 log ladder, Colony 3_{uncut}, Colony 3_{PstI}, Colony $3_{PstI+EcoRI}$*	**Analysis of Top Gel** Colonies 1,2,3 were deemed unsuccessful due to the banding pattern. The double cut bands were simply not large enough.



Above, Colony 4_{uncut}, Colony 4_{PstI}, Colony $4_{PstI+EcoRI}$, 2 log ladder, Colony 5_{uncut}, Colony 5_{PstI}, Colony $5_{PstI+EcoRI}$

Analysis of Bottom Gel
The diffusion of the log ladder makes it difficult to discern the ladder bands, but one can see the Hin Generator Composite is significantly large at around 2kb (double cuts). One can also see that the single cut produces a band at around 5kb. From this evidence, it was determined that the Hin Generator was successful for colonies 4 & 5.

Notebook 7/10/10

Cloning of Key Construct

Digests Using the minipreps from the previous day:

Hin Generator colony #4	Original Hin Generator	Cre Cassette
21uL DNA (77ng/uL)	21uL DNA (74ng/uL)	25uL DNA (94ng/uL)
1.5uL XbaI	1.5uL XbaI	1.5uL EcoRI HF
1.5uL EcoRI HF	1.5uL EcoRI HF	1.5uL SpeI
5uL Buffer 4	5uL Buffer 4	5uL Buffer 4
5uL BSA(10X)	5uL BSA(10X)	5uL BSA(10X)
16 H20	16 H20	13 H20
50uL total	50uL total	50uL total

Gel Extraction
Concentrations ended up being much too low for a ligation.

Notebook 7/11/10

Notebook 7/12/10

Cloning of Key Construct

Miniprep
Miniprepped cultures of Cre Cassette and Hin Generator (Hin Cassette + LacI Cassette) using kit:

```
"Cre Cassette": 191 ng/uL
"Hin Cassette": 115 ng/uL
```

Double Digest 37C for 2 hours

Hin Generator	Cre Cassette
1.5uL PstI HF	1.5uL PstI HF
1.5uL XbaI	1.5uL SpeI
22.6 H20	29.5 H20
5 Buff 4	5 Buff 4
5 BSA(10X)	5 BSA(10X)
18 DNA	10.5 DNA

Sequencing of Completed Parts

Sequencing of Hin and Cre Cassettes using new piGEM primers (these primers are farther away from the terminators then our other pSB1AK3 primers we have been using).
Ran a 10uL Big Dye Reaction for sequencing overnight.

Notebook 7/13/10

Sequencing Results for Hin and Cre Cassette

The sequence covered by the sequencing reaction confirmed the correctness of the Cre Cassette, however, the Hin Cassette revealed that the RBS was missing from the K20 (http://partsregistry.org/wiki/index.php/Part:BBa_K200021) part. *So the cloning of the Key Construct was ceased.*

Notebook 7/14/10

Sequencing of LacI Cassette, P22 test cassette, Int cassette

Ran sequencing reaction for LacI Cassette, P22 test cassette, Int cassette with forward and reverse primers:

```
1 primer, 1 big dye, 1.5 buffer, 1 DNA, 5.5 H20
```

Notebook 7/16/10

Ordered Primers to add RBS to the Hin Cassette:
Hin Fix Reverse 5' TTTCTCCTCTTTCTCTAGTATGTGCTCAGTATCTTG 3'
Hin Fix Forward 5' TACTAGATGGCTACTATTGGGTATATTCG 3'
Both Tms of 51.6 and 51.9°C

Notebook 7/20/10

Screening of Cre Test Cassette

The Cre Test Cassette needed to be rescreened since the bands on the gel ran off. The DNA for the screens were from Alkaline lysed cells picked off of the transformation plate.

Digests: @37C for 2hrs

Single Cut

	Screen #	1	2	3
Rxn Solution	14.5uL Solution 1	14.5uL Solution 1	14.5uL Solution 1	14.5uL Solution 1
DNA	3uL	3uL	3uL	3uL

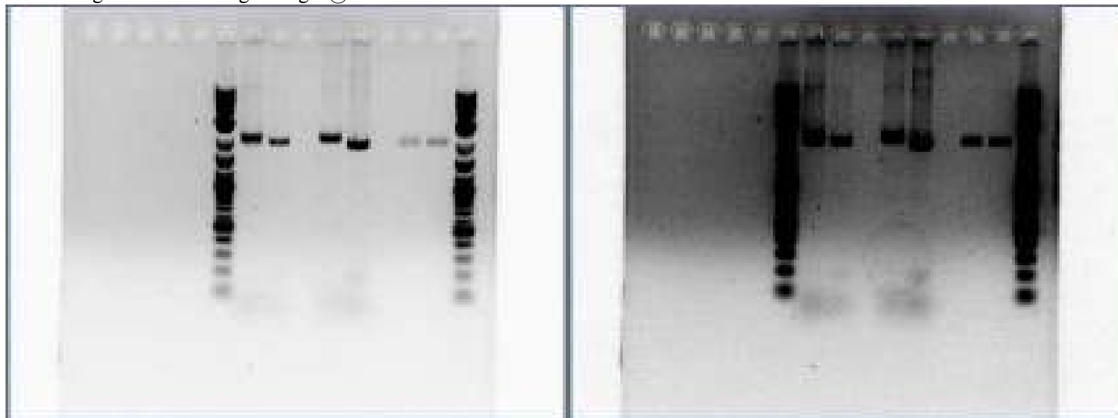
Double Cut

Screen #	1	2	3
Rxn Solution	14.5uL Solution 1 + 0.5uL EcoRI HF	14.5uL Solution 1 + 0.5uL EcoRI HF	14.5uL Solution 1 + 0.5uL EcoRI HF
DNA	3uL	3uL	3uL

Solution 1

100uL H20
14.4 NEB Buffer 4
4PstIHF

Ran the digests on a 1.2% agarose gel @60V for 75 minutes.



above: Left: 2 log ladder, Screen 1 PstI cut, Screen 1 EcoRI and PstI cut, 2P, 2E+P, 3P, 3E+P, ladder; Right: same gel but darker.

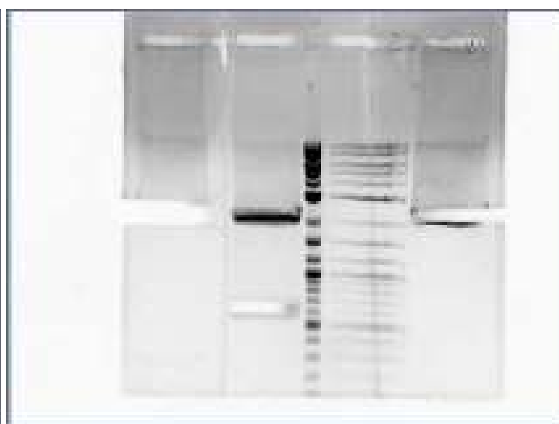
With a single cut, the band should be 2.2kb. With a double E+P cut, the bands should be 200bp and 2kb. We observe that in screens 1 and 2, but not 3. So we conclude that screens 1 and 2 are correct. Screen 1 was chosen as the cell line.

Cloning of Cre Test, Hin Test, and Lock Cassette + (RBS+RFP+T+T)

The plan was to cut out the RBS-RFP from the J23100 (http://partsregistry.org/Part:BBa_J23100) part and insert it behind the Cassettes.

Part	Cre Test Cassette Screen 1	Cre Test Cassette Screen 2	Hin Test Cassette	Lock Cassette	J31000
DNA	12uL	12uL	18uL	22uL	10uL
Buffer 4	3uL	3uL	3uL	3uL	3uL
BSA (10X)	3uL	3uL	3uL	3uL	3uL
H20	12uL	12uL	6uL	2uL	14uL
SpeI	1	1	1	1	0
EcoRI HF	0	0	0	0	1
PstI HF	1	1	1	1	1

Above: J23100 and Cre Test Cassette



Above: Hin Test Cassette, J23100, and Lock Test Cassette

OPPS! The banding looked correct, but it was at this point that I realized that cutting J23100 (http://partsregistry.org/Part:BBa_J23100) with X and P would bring along the promoter with it. I should have cut everything with S+P to clone it.

New Cloning Strategy

1. Cloning of I13507 (RBS+RFP+T+T) (http://partsregistry.org/Part:BBa_I13507) into plasmid vectors pSB3T5 (10-15 copies) (http://partsregistry.org/Part:pSB3T5) and pSB4C5 (~5 copies) (http://partsregistry.org/Part:pSB4C5) . This is to determine if the copy number will affect the recombination.
2. Cloning of junk DNA lengths 300-500bp, 900-1.1kb, 1.5-2.0kb into the SapI sites of the Test Cassettes and Lock Construct. This is to determine the affects of the lengths on the inversion of the Cre/lox and Hin/hix sites and the deletion of the Cre/lox. This will get our test cassettes and lock constructs in the long, medium, and small versions.
3. Then the small, medium, and long versions of the test cassettes will be inserted into the I13507 (RBS+RFP+T+T) (http://partsregistry.org/Part:BBa_I13507) in pSB3T5 (10-15 copies) (http://partsregistry.org/Part:pSB3T5) and I13507 (RBS+RFP+T+T) (http://partsregistry.org/Part:BBa_I13507) in pSB4C5 (~5 copies) (http://partsregistry.org/Part:pSB4C5) backbones.

Some of this cloning can be done in parallel while we are testing one of the test cassettes. For example, we can do the cloning for the hin test cassette while we are running our tecan experiments on the cre test cassette.

CLONES	I13507 (RBS+RFP+T+T) (http://partsregistry.org/Part:BBa_I13507) in pSB3T5 (10-15 copies) (http://partsregistry.org/Part:pSB3T5)	I13507 (RBS+RFP+T+T) (http://partsregistry.org/Part:BBa_I13507) in pSB4C5 (~5 copies) (http://partsregistry.org/Part:pSB4C5)
Cre Test Cassette Small Cre Test Cassette Medium Cre Test Cassette Long	Small length, medium copy Medium length, medium copy Long length, medium copy	Small length, low copy Medium length, low copy Long length, low copy
Hin Test Cassette Small Hin Test Cassette Medium Hin Test Cassette Long	Small length, medium copy Medium length, medium copy Long length, medium copy	Small length, low copy Medium length, low copy Long length, low copy
Lock Construct Small Lock Construct Medium Lock Construct Long	Small length, medium copy Medium length, medium copy Long length, medium copy	Small length, low copy Medium length, low copy Long length, low copy

Notebook 7/21/10

Cloning of I3507 (RBS+RFP+T+T) (http://partsregistry.org/Part:BBa_I13507) into PSB4C5 (low copy number) (http://partsregistry.org/Part:pSB4C5) and PSB3T5 (med. copy number) (http://partsregistry.org/Part:pSB3T5) plasmids

Innoculated pSB3T5, pSB4C5, I13507, Cre Test Cassette, Hin Test Cassette, and Lock Construct.

DIGEST
Used old minipreps of I13507 (259ng/uL), pSB3T5 (158ng/uL), pSB4C5 (over 1000ng/uL????) to begin digests @37 for 2hrs.<i>I find that the pSB4C5 concentration being over 1000 hard to believe since it is supposed to be a low copy number plasmid.

Part	I13507	pSB3T5	pSB4C5
DNA	8.3uL	19uL	10uL
H20	13.7uL	13.7uL	12uL
Buff 4	3	4	3
BSA (10X)	3	3	3
PstIHF	1	1	1
EcoRIHF	1	1	1
Total	30	40	30

GEL EXTRACTION Gel pictures were saved over by someone else before I could email them to myself. For the vectors, there were two bands, the backbone at 3.2kb and a RFP coding device (J04450 (http://partsregistry.org/Part:BBa_J04450)) at 1kb which is what was inside of the backbone in the biobricked vector. The top 3.2kb bands were cut out of the gel. The I13507 bands were ~900bp (insert) and 2kb (pSB1A2 vector). The bottom 900bp band was cut out.

```
Gel Extraction Concentrations:
  I13507: 20ng/uL
  pSB4C5: 152ng/uL (woah alot! the band was very very dark! I though it was supposed to be a low copy number plasmid???)
  pSB3T5: 20ng/uL
```

LIGATION (@25C for 1hr)

```
Ligation 1:
  4.4uL pSB3T5 (backbone)
  3.5uL I13 (insert)
  1uL ligase buffer
  1ul ligase
Ligation 2:
  1uL pSB4C5 (backbone)
  5uL I13 (insert)
  2uL
  1uL ligase buffer
  1uL ligase
Control 1:
  4.4uL psB3T5
  6.6uL H2O
Control 2:
  1uL pSB4C5
  9uL H20
```

TRANSFORMATIONS

```
Control 1: w/ 0.63uL; 5.4 ms
Control 2: w/ 1 uL; 5.2 ms
Ligation 1:
            w/ 0.7uL; 4.7ms
Ligation 2:
            w/ 1.1uL; arced
            w/ 0.8uL; arced (bad curvet?)
            w/ 0.5uL 5.4ms
```

Let cells outgrow for 1hr in 37C incubator with occational tube inversion.

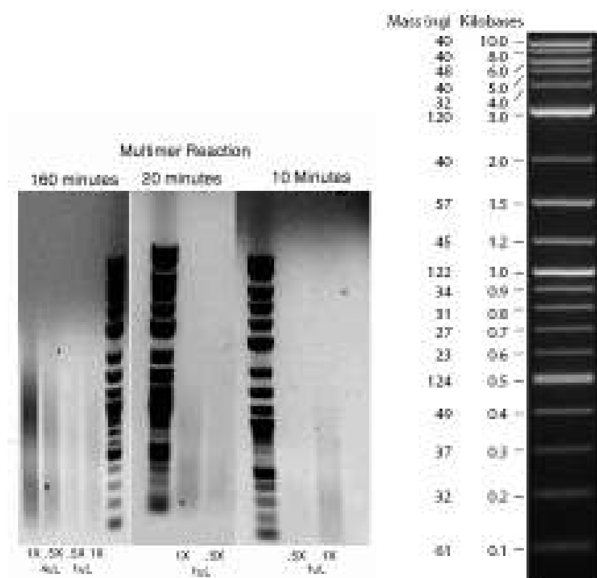
PLATING
Plated ligation 1 (5.4ms and one of them that arced) and control 1 on a regular LB plate and spread 25uL of tetracycline to simulate a normal tetracycline plate (which we don't have). Plated ligation 2 and control 2 on a canymycin plate.

Mock Multimer Reaction

A mock multimer reaction was performed to test the multimer reaction conditions.

```
Annealing (95C for 20sec, then ramped down to 25C at 0.1C/SEC):
  Full:
    5uL Multimer FWD
    5uL Multimer BWD
  Half:
    2.5uL Multimer (forward)
    2.5uL Multimer (reverse)
    5uL H20
Kinase (incubated at 37C for 0.5 hour):
  Added:
    1.2uL T4 Ligase Buffer
    1uL Kinase
```

```
After Kinase Reaction, 1uL ligase was added and incubated at 35C for varied amounts of time.
```



There was not much difference between the 10 and 20 minute reaction, as the smear was centered at about 500bp. But after 2.5 hours, the smear was centered at 1kb, so the reaction appeared to be a success.

Sequencing of LacI Cassette

The sequencing information got back for the LacI Cassette. Everything aligned correctly to the expected sequence. More information can be found in the LacI Cassette page.

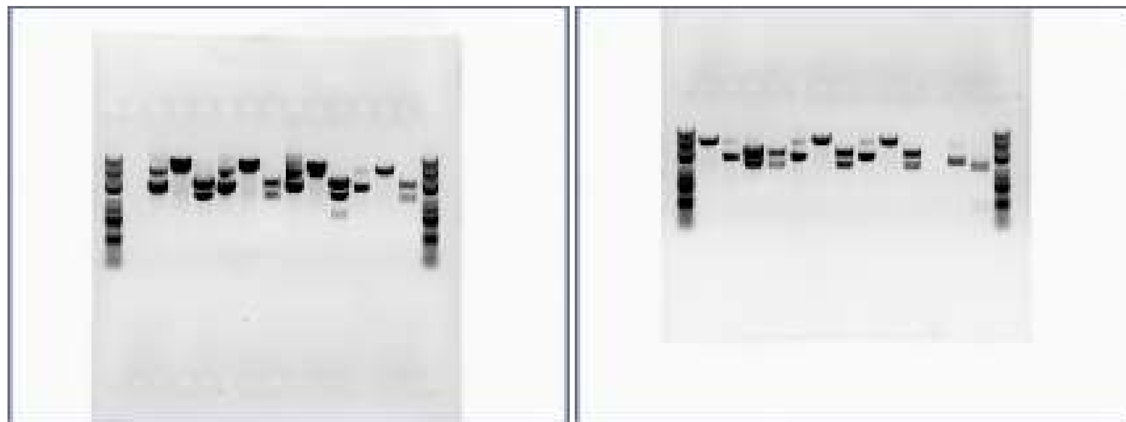
Notebook 7/22/10

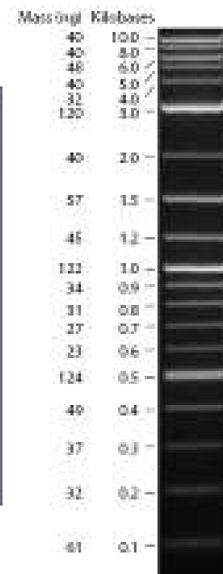
Cloning of I3507 (RBS+RFP+T+T) (http://partsregistry.org/Part:BBa_I13507) into PSB4C5 (low copy number) (http://partsregistry.org/Part:pSB4C5) and PSB3T5 (med. copy number) (http://partsregistry.org/Part:pSB3T5) plasmids

TRANSFORMATION RESULTS

The controls had no colonies. Ligation 1 had ~50 colonies. Ligation 2 had about ~150 colonies. On control 1 and ligation 1, there was a "lawn" of bacteria on the edges of plates (from the competent cells), while the center had distinct colonies. This is attributed to the fact that tetracycline was squirted onto the plate in the center to simulate a TC resistent plate. (This is the regular LB plate that had 25uL TC squirted on it).

SCREENING





Lane #	Screen and Enzyme
1	2 log ladder
2	none
3	Screen 4A$_{\text{uncut}}$
4	Screen 4A$_{\text{PstI}}$
5	Screen 4A$_{\text{EcoRI + PstI}}$
6	Screen 4B$_{\text{uncut}}$
7	Screen 4B$_{\text{PstI}}$
8	Screen 4B$_{\text{EcoRI + PstI}}$
9	Screen 4C$_{\text{uncut}}$
10	Screen 4C$_{\text{PstI}}$
11	Screen 4C$_{\text{EcoRI + PstI}}$
12	Screen 3A$_{\text{uncut}}$
13	Screen 3A$_{\text{PstI}}$
14	Screen 3A$_{\text{EcoRI + PstI}}$
15	2 log ladder

Lane #	Screen and Enzyme
1	2 log ladder
2	3B$_{\text{PstI}}$
3	Screen 3B$_{\text{uncut}}$
4	Screen 4A$_{\text{PstI + EcoRI}}$
5	Screen 3B$_{\text{EcoRI + PstI}}$
6	Screen 3C$_{\text{PstI}}$
7	Screen 3C$_{\text{EcoRI + PstI}}$
8	Screen 3C$_{\text{uncut}}$
9	Screen 3D$_{\text{PstI}}$
10	Screen 3D$_{\text{EcoRI + PstI}}$
11	Screen 3D$_{\text{uncut}}$
12	None
13	Lock Cassette in pSB1A2$_{\text{PstI}}$
14	Lock Cassette in pSB1A2$_{\text{PstI + EcoRI}}$
15	2 log ladder

The screening results were confusing. What was expected with a double cut was a 840bp (I13) band and a 3200bp band (backbone). Apparently something large inserted inserted in all the backbones. Perhaps in the original gel extraction (the ones where my pictures got saved over) I was careless and didn't realize that digestion of I13 resulted in a 2kb band, but since I13 is in pSB1A2 (a 2kb backbone), digestion of I13 resulted in a single 2kb band with both the insert and the backbone, but I believe I remember seeing two bands in the gel extraction. Unfortunately there is no gel picture.

I also tried cloning I13 behind the Cre Test Cassette and Hin Test Cassette. In this cloning, I cut I13 with XbaI and PstI and the Test Cassettes with SpeI and PstI. This is the picture of the gel extraction:

Above: left: I13; middle: Cre Test Cassette; right: Hin Test Cassette

The Test Cassettes have the expended band of 2.2kb when cut with S and P. But I13 has only one band at around 2kb. This make me think, from the screening and this gel, that I13 is not the correct part. I think it would be most beneficial to cease using that part and use something we know definitely works.

The best and most sure way to solve this problem will be to do the following: we have a promoter part J23100 (http://partsregistry.org/Part:BBa_J23100) inside of a test plasmid J61002 (http://partsregistry.org/Part:BBa_J61002) . The promoter is flanked on the left by EcoRI and XbaI and on the right by SpeI. To the right of the SpeI site, is a RBS+RFP+T, and on the right of that the PstI site (E--X--Promoter--S--RBS+RFP+T--P). If we cut the Test Cassettes and the Lock Consturct with S+P and the J61002 plasmid with S+P, we can insert the RBS+RFP+T directly behind the Test Cassettes and Lock, while restoring the SpeI site between the Cassettes and the RFP device (--E-X--Test Cassette--S--RBS+RFP+T--P--). We know for sure that the RBS+RFP+T device works since the J23100 part had red colonies. After addition of the RFP device behind the cassettes, we can cut them with SapI and add the junk DNA to adjust the cassettes to desired lengths, and then finally add them to the desired plasmid.

Cloning of junk DNA into Cre, Hin Test Cassettes and Lock Construct

DIGESTION

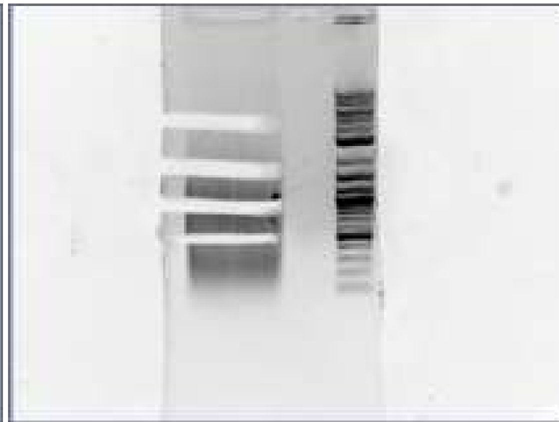
```
Lock:
  1.5uL SapI
  10uL DNA (219ng/uL)
  3uL Buff 4
  3uL BSA (10X)
  12.5uL H20
Cre Test Cassette:
  1.5uL SapI
  10uL DNA (219ng/uL)
  3uL Buff 4
  3uL BSA (10X)
  12.5uL H20
Hin Test Cassette:
  1.5uL SapI
  10uL DNA (219ng/uL)
  3uL Buff 4
  3uL BSA (10X)
  12.5uL H20
```

MULTIMER REACTION

Before I realized the problem above, I had tried to clone the junk DNA into the Test Cassettes and Lock Constructs.

```
Multimer Reaction Protocol:
 1. Anneal at 95C then ramp down to 25C at 0.1C/sec
     25uL MULTI FWD
     25uL MULTI BWD
 2. Kinase Reaction at 35C for 1/2 hours
      5uL T4 Kinase (PNK) (~10% T4 Kinase)
      6uL T4 Ligase Buffer (~10% 10X Ligase buffer)
 3. Ligation at 25C for 2.5 hours
      4uL T4 Ligase (~10% T4 Ligase)
```

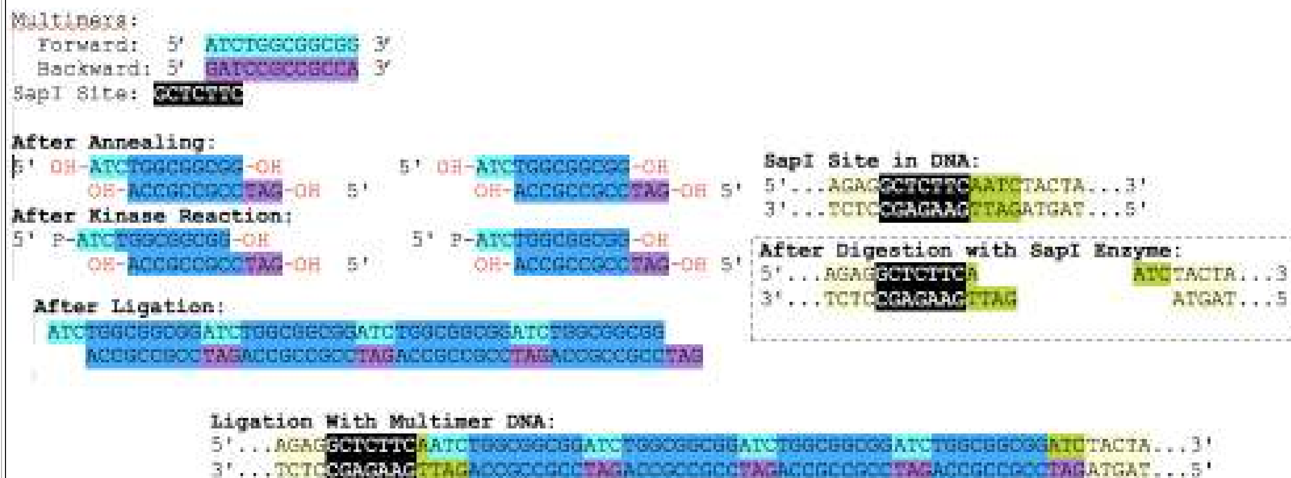
Above: Lock Cassette (SapI), 2 log ladder, Cre Test Cassette (SapI)



Above: Multimer Reaction Cut Picks

Multimers:

```
Forward:  5' ATCTGGCGGCGG 3'
Backward: 5' GATCCGCCGCCA 3'
```



GEL EXTRACTION Gel Extracted 500bp Junk, 1000bp Junk, 1500bp Junk, and 4000bp Junk:

```
500bp: 14ng/uL
1000bp: 10.6ng/uL
1500bp: 7.5ng/uL
4000bp: 11.0ng/uL
```

Left gel extract in -20.

Notebook 7/23/10

Notebook 7/25/10

Cloning of Junk DNA into Cre Test Cassette and Lock

Preformed the following ligation at room temperature for 1hr:

LIGATION	small insert (500bp) 14ng/uL	medium insert (1000bp) 10.6ng/uL	large insert (1500bp) 7.5ng/uL	extra large insert (4000bp) 11.9ng/uL
Cre Test Cassette (21.5ng/uL)	#1 2.4uL Insert 2.3uL CTC 3.3 H20	#2 5.7uL Insert 2.3uL CTC	#3 6.64uL Insert 1.35uL CTC	#7 5uL Insert 3uL CTC
Lock (13.9ng/uL)	#4 2.4uL Insert 3.5uL Lock 2.3uL H20	#5 5.7uL Insert 3.5uL Lock	#6 5uL Insert 3uL Lock	#8 5uL Insert 3uL Lock

Innocculated J23100 in J61002 and Hin Test Cassette. The J23 will be used for its RBS+RFP+T and the hin test cassette for insertion of Junk DNA.

Notebook 7/26/10

Ran sequencing of Cre Test Cassette and Hin Test Cassette.

Cloning of Junk DNA into Cre Test Cassette and Lock

From overnight ligation DH10B cells were transformed:

```
Ligation 1: [illegible]
Ligation 2: 1.4uL--3.5ms
Ligation 3: 1.9uL--3.5ms
Ligation 4: 1.8uL--3.7ms
Ligation 5: 1.4uL--4.1ms
Ligation 6: [illegible]
Ligations 7&8: did not transform
```

Notebook 7/27/10

Cloning of Junk DNA into Cre Test Cassette and Lock

TRANSFORMATION RESULTS FAILED: ALL LAWNS ON ALL PLATES.

Even the control had lawns, which seems odd since it was just cut vector.

New Cloning:

Nate had already done another multimer reaction and gel extraction, so a digest of Cre Test Cassette with SapI was done:

DIGEST

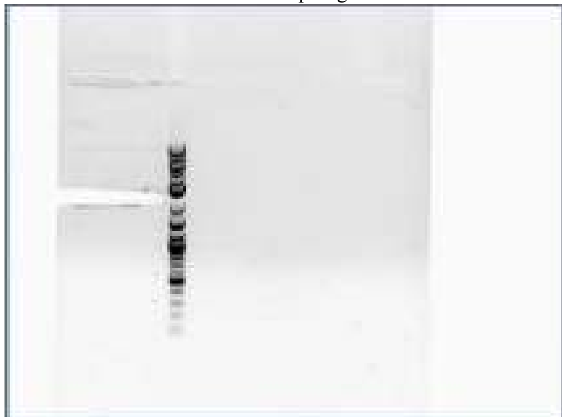
@37C for 2hrs.

```
2ug (9.13uL) Cre Test Cassette DNA
3uL Buffer 4
3uL BSA (10X)
1.5uL SapI
15.37uL H20
```

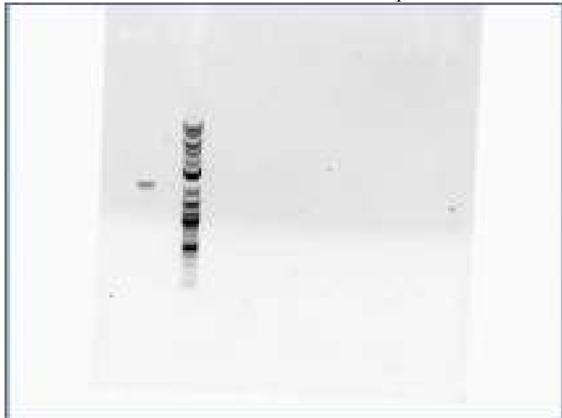
PHOSPHATASE
added the following to the digest:

```
3.4uL Antarctic Buffer
1.3uL Antactic Phosphatase
   incubated 37C 1hr
```

GEL EXTRACTION
Gel Extracted Cre Test Cassette SapI digested:



Accidentally eluted into ethanol contaminated tube. Added elution to buffer and
bound DNA to column and re-eluted. Nanodrop concentration said 12ng/uL. Ran a gel to confirm DNA:



LIGATION

LIGATION	500bp	1000bp	1500bp	Control
Cre Test Cassette	5uL	3.56uL	2.5uL	4uL
Insert	10uL	6.44uL	8.5uL	0uL
Ligase Buffer	2uL	2uL	2uL	2ul
Ligase	2uL	2uL	2uL	2uL
H20	8uL	8ul	8uL	8uL

TRANSFORMATIONS

```
CTC 500: 2uL=13.4ng--4.2ms
CTC 1000: 2.8uL=15ng--4.2ms
CTC 1500: 4.7uL=15ng--2.9ms
Control: 6.25uL=15ng--3.9ms
```

Phusion Reaction for Adding RBS to Hin Cassette and Hin Generator

Miniprepped Hin Generator and Hin Cassette for Phusion reaction:

```
Hin Generator: 169.5ng/uL
Hin Cassette: 232.5ng/uL
```

Phusion Protocol:
Did the following protocol for Hin Generator and Hin Cassette:

```
MIX:
 5X Phusion HF Buffer: 4uL
 DNTP's (10mM) (10%): 4uL
 Phusion (10%): 2.5uL
 Primer FWD (10%): 1.7uL
 Primer BWD (10%): 1.7uL
 Template (10%): 2uL
 H20: 4.1uL
 Total: 20uL
```

```
THERMOCYCLER:
 (1) 98C 45 secs
 (2) 98C 120 secs
 (3) 54.4C 25 secs
 (4) 72C 1:15
 (5) Go To 2 29 times
 (6) 72C 5:00
 (7) 4C forever
```

Ran a gel of 1uL of each reaction:

Above: 2 log ladder, Hin generator (1st reaction), Hin Cassette (1st reaction),
Hin Generator (2nd reaction), Hin Cassette(2nd reaction, the one with blue marker on it).

Tomorrow I will gel extract, then Kinase treat followed by blunt end ligation and transformation.
Then screening and finally sequencing...

7/28/10

Screening of E0240 in pSB3T5 and pSB4C5

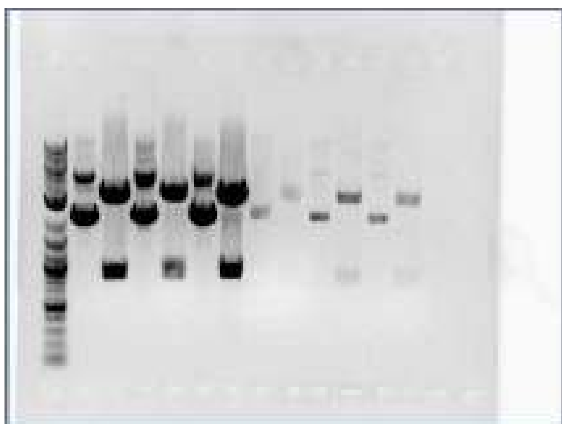
MINIPREP
Miniprepped screens of E0240 in pSB4C5 (A,B,C) and pSB3T5 (D,E,F).
DIGESTION
For screens A through F:

Screens A through F
3uL DNA
17uL Solution 1

Solution 1
12.9 Buff 4
90 H20
3uL EcoRI HF
3uL PstI HF

I forgot to do a single cut screen, but I think a double cut is sufficient to show that the E0240 part is in the vector.

```
Expected Bands:
  Double Cut: 3.2kb (backbone); 876bp (E0240)
```



<i>From left to right:2 log ladder, uncut(screens A through f), E+P(screens A through F)
I chose screens A and F for the new cell line. They were innocculated and plated 7/29/10.

Phusion Reaction for Adding RBS to Hin Cassette and Hin Generator

PHUSION REACTION

Master Mix
0.85uL Hin Fix FWD
0.85uL Hin Fix BWD
2uL DNTP's
20uL HF
74uL H20

Hin Generator
48.85uL Master Mix
0.62uL Phusion
0.5uL Hin Generator

Hin Cassette
48.85uL Master Mix
0.62uL Phusion
0.5uL Hin Cassette

```
THERMOCYLCER:
 (1) 98C 45secs
 (2) 98C 120secs
 (3) 54.4C 25secs
 (4) 72C 1:15
 (5) Go To 2 29 times
 (6)72C 5:00
 (7) 4C forever
```

GEL EXTRACTION
Ran all of the reaction in a gel; 0.7%; 85V; 30mins:

Above: Hin Generator after Phusion; Hin Cassette after Phusion
It is now apparent that the thermocycler or master mix could be further optimized to prevent smearing.

```
Gel Extraction Concentrations:
  Hin Generator: 14.4ng/uL
  Hin Cassette: 21.1ng/uL
```

KINASE PHOSPHORYLATION
@37C for $^{1}/_{2}$ hours

Hin Generator	Hin Cassette
6.44uL DNA (100ng)	4.74uL DNA (100ng)
1uL T4 Kinase	1uL T4 Kinase

1.1uL T4 Ligase Buffer	1.1uL T4 Ligase Buffer
1.56uL H20	3.26uL H20
10ul total	10uL total

LIGATION
added 1uL Ligase to each reaction and incubated at 16C for 3 hours, followed by 4C overnight.

7/29/10

Plated Screens A and F for E0240 in pSB3T5 and pSB4C5. Mike S. made freezer stocks of these.

Cloning of Junk DNA into Cre Test and Hin Test Cassettes

TRANSFORMATION

```
HIN TEST CASSETTE:
  control: 1uL>>3.9ms
  HTC 500: 1uL>>3.8ms
  HTC 1000: 1uL>>3.7ms
  HTC 1500: 1uL>>3.8ms
```

```
CRE TEST CASSETTE:
  control: 6.5uL>>arced
  CTC 500: 2uL>>4.00ms
  CTC 1000: 2.8uL>>3.50ms
  CTC 1500: 4.7uL>>3.50ms
```

Let outgrow for 1hr at 37C, then plated cells on Amp 100 plates.

Phusion Reaction for Adding RBS to Hin Cassette and Hin Generator

TRANSFORMATION

```
Hin Generator 1.5uL>>3.40ms
Hin Cassette 1.5uL>>3.60ms
```

Let outgrow for 1hr at 37C, then plated cells on Amp/Kan plates.

7/30/10

Phusion Reaction for Adding RBS to Hin Cassette and Hin Generator

The plates showed no colonies.
Ideas to optimize reaction:

1. lower primer concentration
2. increase aneealing temperature, use gradient
3. Reduce cycles
4. denature for shorter periods of time
5. optimize magnesium concentration

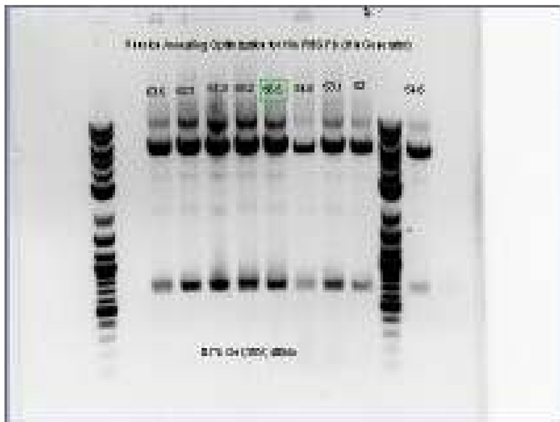
Ran a new Phusion Reaction:

```
MASTER MIX
  0.86 Primer FWD
  0.86 Primer BWD
  20 HF buffer
  74 H20
  2 Dntp

REACTION MIX
  48.9 Master mix
  0.6 Phusion
  0.5 Hin Generator DNA

THERMOCYCLER CONDITIONS:
  1) 98C 30s
  2) 98C 15s
  3) Tm 10s
  4) 72C 1:18
  5) Go To (2) 29 Times
  6) 72C 10:00
  7) 4C forever
```

Used one reaction mix for annealing optimization using the following annealing temperatures: 63.5C, 62.7C, 61.3C, 59.2C, 56.5C, 54.6C, 53.1C, 52C.



It appears around 54.6C is the best temperature for annealing.
optimize annealing temp...

Cloning of Junk DNA into Cre Test Cassette and Hin Test Cassette

TRANSFORMATION RESULTS

Transformation	Number of Colonies
CTC 500	23
CTC 1000	30
CTC 1500	0
CTC Control	over 200
HTC 500	1
HTC 1000	5
HTC 1500	1
HTC Control	25

It appears that our cloning was a failure. However, I innocculated some colonies for screening to determine the problem. The vector is probably self-ligating despite the phosphatase treatment. Also, the multimer reaction is probably still occurring, using up the ligase.
Innocculated 5 colonies from CTC 500, 6 from CTC 1000, 3 from HTC 1000, 1 from HTC 500, 1 from HTC 1500.

7/31/10

Phusion Reaction for Adding RBS to Hin Cassette and Hin Generator

PHUSION REACTION
There were still no colonies on the Hin Generator from the plate (7/29/10), but there was a single colony on the Hin Cassette. That colony was picked and a liquid culture was made with that colony for screening tomorrow.
Another Phusion reaction was run with the Hin Generator. Two Master Mixes were made:

```
MASTER MIX HF:
  74 h20
  0.7 FWD Primer
  0.7 REV Primer
  2 DNTP's
  20 HF Buffer
MASTER MIX GC:
  74 h20
  0.7 FWD Primer
  0.7 REV Primer
  2 DNTP's
  20 GC Buffer
REACTION MIX:
  49 Master Mix
  0.6 Phusion
  0.5 Hin Generator DNA
```

Two reaction mixes were made, one with the GC master mix and one with the HF master mix. The thermocycler condition are the same from yesterday.
GEL EXTRACTION

Above: GC buffer reaction mix, 2 log ladder, HF buffer reaction mix

```
Concentrations:
  GC Master mix: 41.7 ng/ul
  HF Master mix: 38.5 ng/ul
```

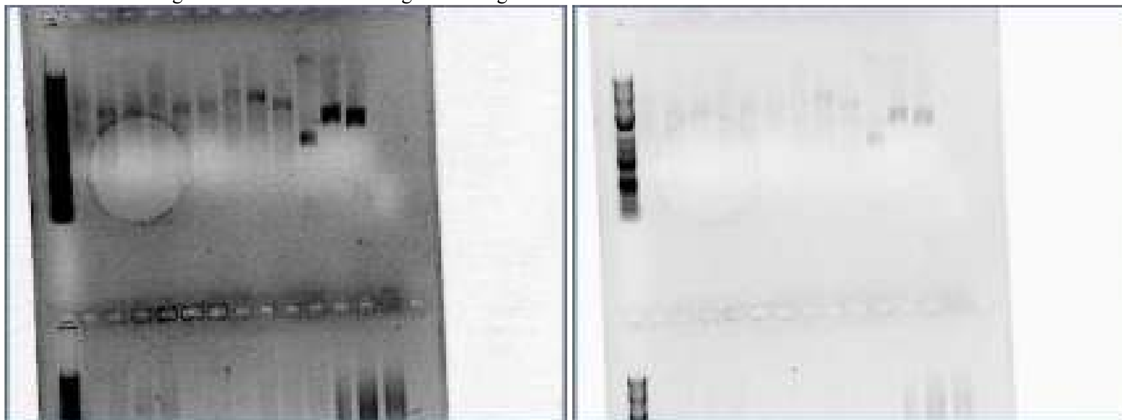
KINASE PHOSPHORYLATION

GC Reaction	HF Reaction
1uL Kinase	1uL Kinase
1uL T4 Ligase Buffer	1uL T4 Ligase Buffer
3uL GC Master Mix	3uL HF Master Mix
5uL H20	5uL H20

incubated at 37C for 45 minutes **LIGATION OF BLUNT ENDS**
Added 3uL Ligase and 0.3uL Ligase buffer to each reaction.
Ran ligation at room temperature for 2-3 hours, then at 16C for 1 hr, then 4C overnight.

Screening of Junk DNA and Test Cassettes

Only screens A,B,C,E,F,J,N,O grew in the liquid culture. D,G,H,I,K,L,M,P did not grow in the Amp liquid culture. Miniprepped A,B,C,E,F,J,N,O. Digest each with just PstI and a double digest of EcoRI and PstI using a 20uL digestion reaction and 3uL DNA.



It appears there was not very much DNA in the minipreps. So the digestion was redone, this time with 16uL DNA for each reaction and just a double digest.

It appears that none of the screens worked. I could detect no second band on any of the screens. We will have to rethink our cloning strategy.

8/1/10

Screening of Hin Cassette PCR fix

Miniprepped Hin Cassette from the Phusion PCR plate (the single colony from two days ago). Ran a digest of the Hin Cassette DNA to determine whether to continue to sequencing.

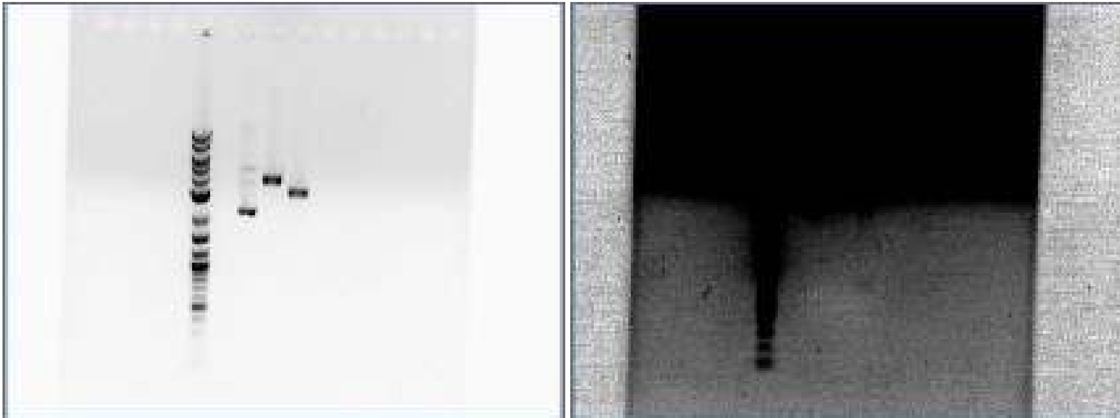
Single Digest	Double Digest
2uL Hin Cassette	2uL Hin Cassette
2uL Buffer 4	2uL Buffer 4
0.5 PstI HF	0.5 PstI HF
15.5 H20	15ul H20

Cloning of Hin Generator PCR fix

TRANSFORMATION

```
Ligation #1: 1uL 3.50ms
Ligation #2: 1uL 3.60ms
Ligation #3: 1uL 3.60ms
Ligation #4: 1uL 3.70ms
```

GEL



```
EXPECTED BANDING PATTERN:
  For a single cut PstI is: 3.189kb (pSB1AK3) + .575kb (J31) + 75bp (K20) + small terminators (78bp) = ~3.9kb
```

```
  For the PstI and EcoRI is about 700bp and 3.2kb
```

Its difficult to see the second band, but the picture on the right shows a band at 700bp. This confirms that this DNA contains the Hin Cassette, but does not confirm that the RBS is cloned in. Sequencing will be run on this.

SEQUENCING
Ran Big Dye sequencing reaction on the Hin Cassette Fix.

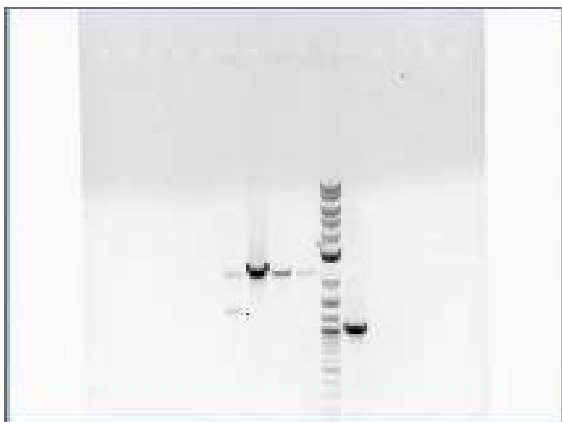
8/2/10

Sequencing Confirmation of RBS fix on Hin Cassette

The sequencing results confirm that the RBS fix using phusion followed by a blunt end ligation has successfully added an RBS in the location desired.

Fixing Hin Generator

The PCR phusion cloning of the Hin Generator seemed to have worked with more ligase, followed by a longer ligation, and heat inactivation of the ligase! About 20-40 colonies. Ran a colony PCR on the Hin Generator. Picked four colonies A,B,C,D. Also made liquid cultures of the colonies and plated them.



Above: A,B,C,D, 2 log ladder, positive control (Hin Cassette), negative control (water).
All PCR amplification confirm presence of a 2kb insert, which is exactly what was expected. A had a second band, which was kind of odd. These will be sequenced tomorrow after the liquid culture miniprep

New Cloning Strategy for Junk DNA

So far, any attempt to clone the primer polymer (multimer) into our test cassettes with SapI has failed. This, I think, can be attributed to three main reasons:
Problems
(1) The vector is religating to itself, despite desphosphorylation attempts. SapI digestion makes the vector compatible with itself. This explains why there are so many colonies on our controls (vector + ligase).
(2) Though the multimerizing reaction worked on a gel, there is no guarantee that during the annealing step, that the primer are annealing in a 1:1 ratio. There will be some unannealed forwards or reverse primers floating around, and when the ligation step occurs, these will ligate to the ends of the multimer polymers, making their ends non-compatible with the SapI digested vector.

```
We want:
ATCprimerATCprimerATCprimerATCprimer
   primerTAGprimerTAGprimerTAGprimerTAG


But we are also getting (non-compatible ends in bold):

      ATCprimerATCprimerATCprimerATCprimer
primerTAGprimerTAGprimerTAGprimerTAGprimerTAG


ATCprimerATCprimerATCprimerATCprimerATCprimer
   primerTAGprimerTAGprimerTAGprimerTAG


      ATCprimerATCprimerATCprimerATCprimerATCprimer
primerTAGprimerTAGprimerTAGprimerTAGprimerTAG
```

(3) Even when the multimers do have the correct compatible ends, when we try to ligate them to the vector, the multimerizing reaction is still occurring between multimers, using the ligase. This explains when we have significantly less colonies on the ligations compared to the controls.

SOLUTION
With this in mind, the following cloning strategy has been developed to solve these problems:
(1) The multimerizing reaction will proceed as normal, except with a little bit more Reverse primer than the forwards. During the annealing step, most of the reverse primers will anneal to another forwards primer. However, there will not be enough FWD primer for ever REV primer, so some REV primer will remain unannealed. When the ligation step occurs, the annealed primers will multimerize as normal, however, the lone unannealed REV primers will ligate on one end of the multimers (they are compatible to the FWD primers), terminating the reaction on that end. Near the end of the ligation, the reaction will be quenched with REV primer even more, eventually terminating the reaction as all the annealed primers are used up. This will make for multimers that have non-compatible ends.

```
THIS IS WHAT WE ARE TRYING TO MAXIMIZE HERE IN THE MULTIMER REACTION:

      ATCprimerATCprimerATCprimerATCprimer
primerTAGprimerTAGprimerTAGprimerTAGprimerTAG

The ends of the multimers are no longer compatible, and the multimerizing can no longer proceed.
```

(2) The vector will be cut with SapI, however, before ligation with the multimers, it needs to be ligated with the FWD primer. We cannot completely remove vector re-ligation, but we can try to ligate the small FWD primer to the vector by flooding it with primer, making a primer+vector ligation encouraged.

```
VECTOR
 ---vector(sense)-----------ATC----------sense-------------
 ---vector(antisense)-------TAG----------antisense---------

VECTOR SAPI DIGESTED
 ---vector(sense)-----------      ATC----------sense-------------
 ---vector(antisense)-------TAG      ----------antisense---------

VECTOR SAPI DIGEST THEN FWD PRIMER LIGATION
 ---vector(sense)-----------ATCprimer      ATC----------sense-------------
 ---vector(antisense)-------TAG               ----------antisense---------
```

After the vector has the primer ligated to it, the vector cannot religate, it can only ligate with the multimer, which now has compatible ends.

```
FINAL LIGATION:
 MULTIMER
      ATCprimerATCprimerATCprimerATCprimer
primerTAGprimerTAGprimerTAGprimerTAGprimerTAG

 VECTOR
---vector(sense)-----------ATCprimer      ATC----------sense-------------
---vector(antisense)-------TAG               ----------antisense---------

---vector(sense)-----------ATCprimer  ATCprimerATCprimerATCprimerATCprimer    ATC----------sense-------------
---vector(antisense)-------TAG  primerTAGprimerTAGprimerTAGprimerTAGprimerTAG    ----------antisense---------

---vector(sense)-----------ATCprimerATCprimerATCprimerATCprimerATCprimerATC----------sense-------------
---vector(antisense)-------TAGprimerTAGprimerTAGprimerTAGprimerTAGprimerTAG----------antisense---------
```

PHOSPHORYLATION
Phosphorylated primers for multimers:

```
Reaction 1:
 40uL MULTI FWD
 5uL ligase buffer
 4uL Kinase
Reaction 2:
 40uL MULTI REV
 5uL ligase buffer
 4uL Kinase
@37C for 2 hours, then heat inactivation at 65C for 20 minutes, then freeze
```

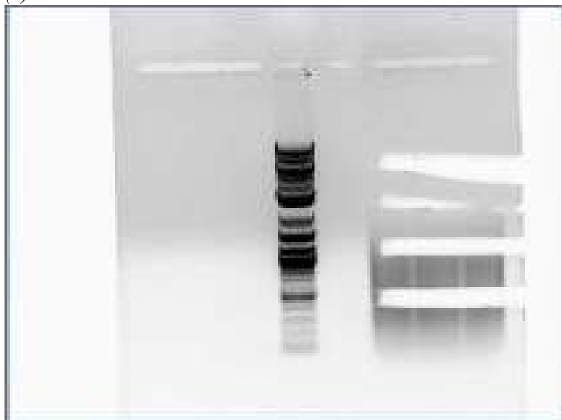
8/3/10

Sequenced the Hin Fix Generator colonies. Some of the sequencing was bad, but the sequencing results showed that at least one of the colonies were successful.

8/4/10

Cloning of Junk DNA into CTC

Yesterday: Primers were phosphorylated, heat inactivated, then frozen for use.
MULTIMER REACTION
(1) Annealling of primers:
25uL FWD primer and 30uL BWD primer (both phosphorylated); 95C to 25C at 0.1C/sec.
(2) Ligation of Primers:
Added 5.5uL ligase, 6.6uL ligase buffer; 25C for 2.5 hours.
(3) Ligation Quenching
Quenched ligation with 15uL phosphorylated REV primer
(4) Gel Extraction


Above: Multimer Rection with 2 log ladder.

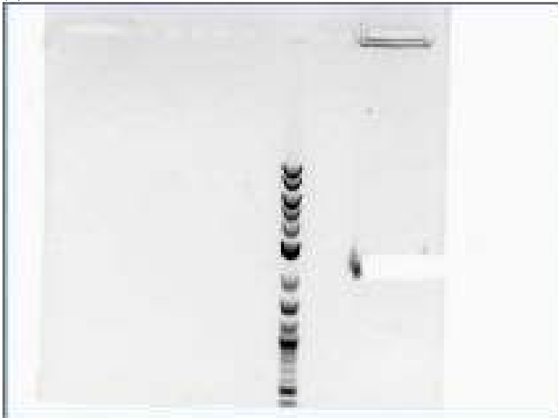
The reaction quench appeared to have worked, however, it may have been quenched too soon as there is no band smear much above 1kb.

```
Gel Concentrations:
  CTC 500: 30ng/uL
  CTC 1000: 7ng/uL
```

VECTOR PREPARATION
(1) Digestion

CTC DIGEST
18uL CTC (219ng/uL)
3 Buffer 4
3 BSA 10X
2 SapI
4 H20

Heat inactivated 20 minutes, 65C
(2) Phosphatase Treatment
Added 3.7uL Antarctic Phosphatase Buffer and 3.7uL Antarctic Phosphatase for 1hr at 37C, followed by heat inactivation at 65C for 30 minutes.
(3) Gel Extraction



```
Gel Concentration:
  CTC: 26.5ng/uL
```

(4) Primer Ligation

```
27uL CTC gel Extraction (26.5ng/uL)
6uL phosphorylated MULTIMER primer FWD
4uL T4 ligase
3.6 T4 ligase buffer
```

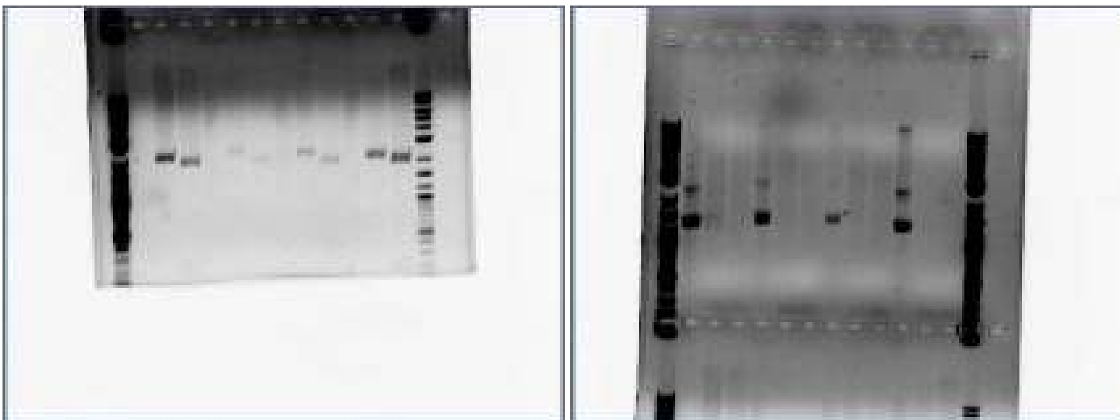
(5) PCR purification (primer removal) Used kit to do a PCR purification to remove excess primer.

```
Concentration: 19ng/uL
```

FINAL LIGATION
16C for 3hrs; 4C 9hrs; 50C 20 mins; 4C forever

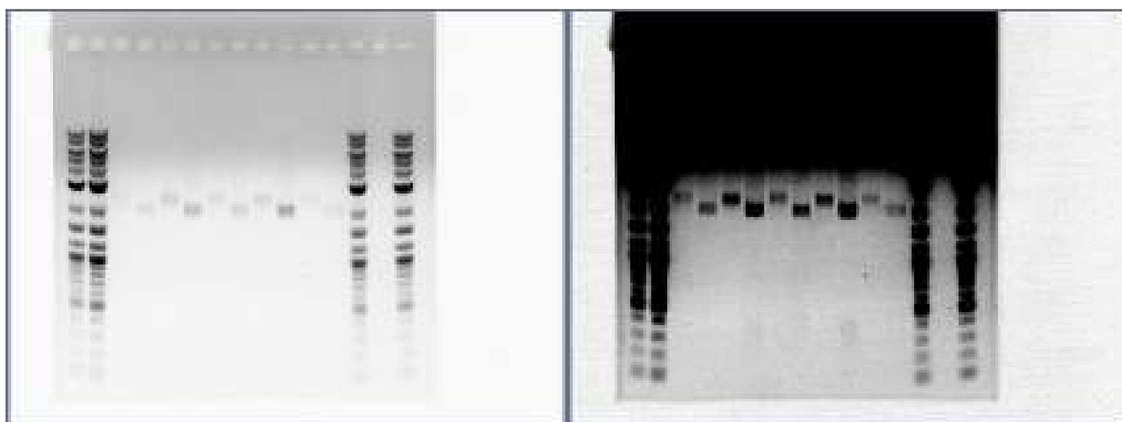
----------	Ligation 1	Ligation 2	Control 1	Control 2
Vector (CTC + primer ligation;19ng/uL)	5.26	1.5	5.26	5.26
Insert	2.74 (500bp;30ng/uL)	(1000bp;7ng/uL)	none	none
Ligase Buffer	1	1	1	0
Ligase	1	1	1	0
H20	0	0	0	4.7

8/10/10

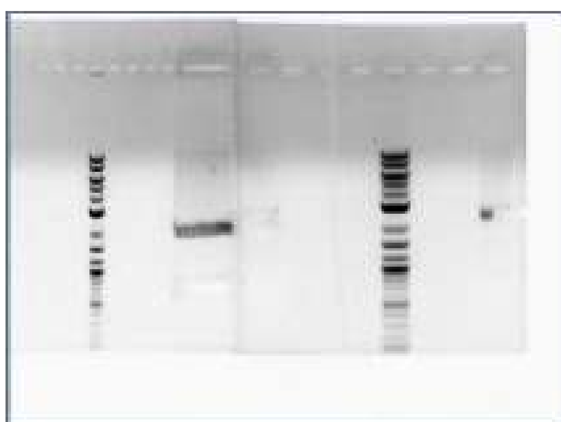


8/12/10

8/15/10



8/17/10



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