# May 31st, 2013 (Friday)

From Dueber Lab Wiki

## Contents



## Extraction of Glycosyl Transferase (GT) from Abrabidopsis *thaliana* (At) via PCR

### Trial No. 1

The Berkeley iGEM 2013 team has 6 slightly different GT genes to test, 5 of which could be extracted from the genomic At. Some of these 5 GT genes will be assembled via Golden Gate Assembly of a total of 9 PCR products. I took charge of making **pIGEM13_001 (At_GT_AT4G15550, total size 3087 bp)**, which involves Golden Gating the following PCR products and backbone template:

- pcr01: Using oligos BS48/BS49 (336 bp)
- pcr02: Using oligos BS50/BS51 (652 bp)
- pcr03: Using oligos BS52/BS53 (531 bp)

**Steps taken:**

1. Diluted all primers to 100 uM.
2. Created the PCR mixture found here (https://dueberlab.com/wiki/General_Protcols#PCR) for all 9 PCR products.
3. Program was modified to:

98 degrees Celsius for 5 min

98 degrees Celsius for 10 sec
50 degrees Celsius for 30 sec
72 degrees Celsius for 1 min 15 sec
72 degrees Celsius for 10 min
12 degrees Celsius forever
Steps 2-4 repeated 29 times.

1. PCR products were gel purified. Here is the following image:

**Figure 1.** Trial 1 Results. All lanes seem to have excess primer dimers. Decided to switch Phusion pol to Expan pol to generate desired PCR products.

## Trial No. 2

1. Created the PCR mixture found here (https://dueberlab.com/wiki/Template:IGEM_Expand_PCR) for all 9 PCR products.

Note: Instead of 1.5 uL of 10 uM Oligos, used 0.5 uL of 100 uM Oligos instead.

1. Created the PCR mixture found here (https://dueberlab.com/wiki/Template:IGEM_Expand_PCR) for all 5 GT genes, by only using the first and last oligos of each gene.

Note: The reason why we have several PCR products for 1 GT gene is to avoid restriction sites and introns in genomic At. However, since the template we used was At cDNA (which doesn't include introns), we tried to extract the entire GT gene. If it works, we can skip the Golden Gate Assembly of PCR products.

*Please see June 1st, 2013 (Saturday) for PCR Program and Gel Purification results.*

## Trial No. 3

1. Obtained genomic Wild Type At gene and genomic Mutant At.
2. Created the PCR mixture found here (https://dueberlab.com/wiki/Template:IGEM_Expand_PCR) for all 9 PCR products with WT At gene.

Note: WT At was diluted 1:5 in order to conserve stock template DNA.

1. Created the PCR mixture found here (https://dueberlab.com/wiki/Template:IGEM_Expand_PCR) for all 9 PCR products with Mutant At gene.

Note: Mutant At was diluted 1:5 in order to conserve stock template DNA.

*Please see June 1st, 2013 (Saturday) for PCR Program and Gel Purification results.*

# Literature Research on Tyrosine Kinase in E. *coli*

Structure of Escherichia coli tyrosine kinase Etk reveals a novel activation mechanism (http://www.nature.com/emboj/journal/v27/n12/full/emboj200897a.html#B39)



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# June 1st, 2013 (Saturday)

From Dueber Lab Wiki

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# Results from GT Extraction from Abrabidopsis *thaliana* (At) via PCR

## Summary of Previous Work (May 31st, 2013)

Because of the lack of desired product using Phusion, we decided to improve by doing the following:

1. Switch to Expand polymerase from Phusion polyermase.
2. Use Expand polyermase PCR protocol for cDNA At to extract whole GT gene. (Trial 2 in May 31st, 2013 Notes)
3. Use Expand polyermase PCR protocol for cDNA, Wild Type and Mutant At. (Trial 3 in May 31st, 2013 Notes)

Hojae, Thomas and Roy prepared the above protocols. Hojae was in charge of Master Mix, while Thomas and Roy were in charge of preparing the oligos.

## Results

**Note:** 15 uL of PCR products was used for each lane.

# Gel No. 1

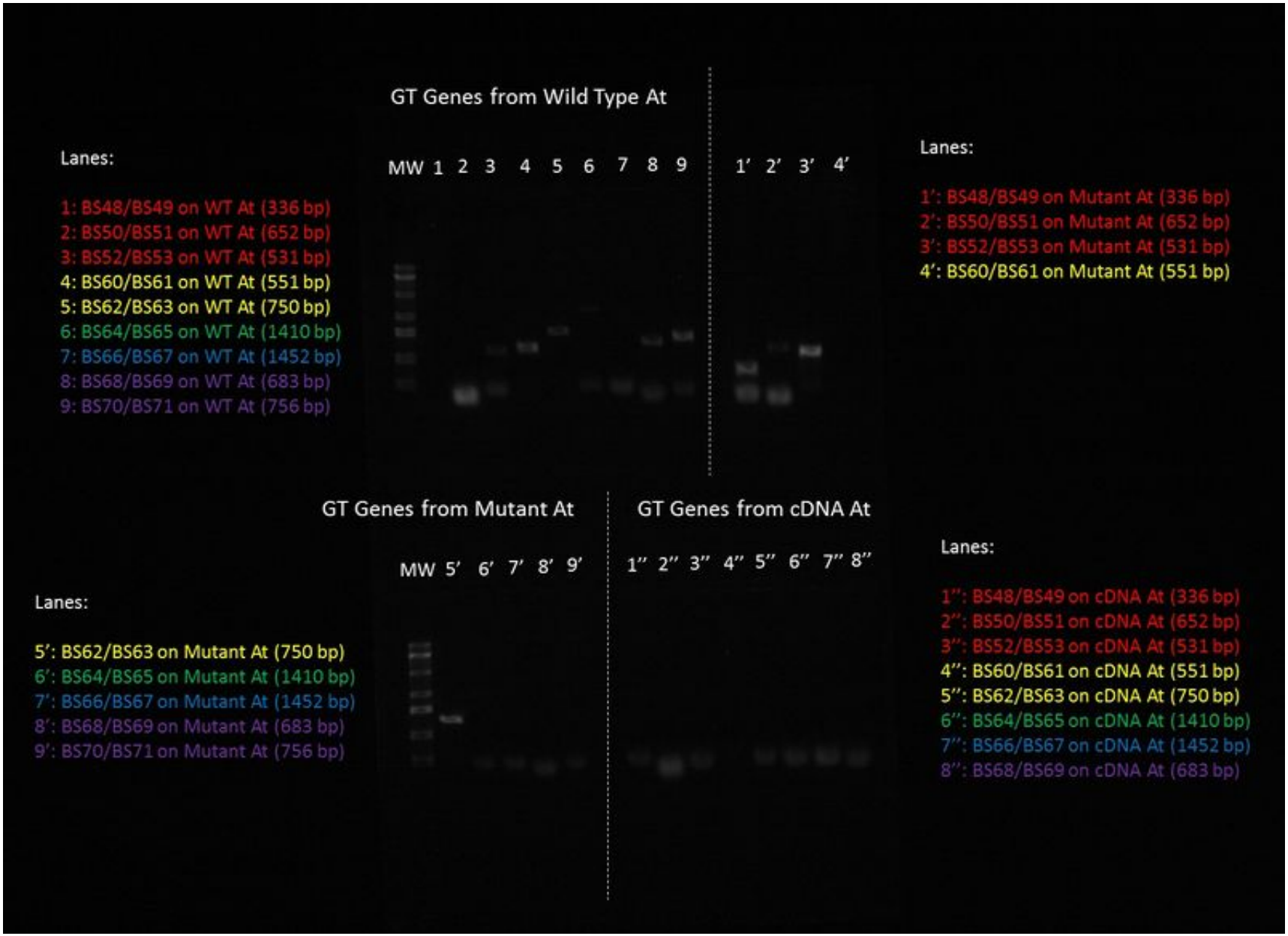


**Figure 1.** Gel Image of PCR products of GT Gene fragments extracted from Wild Type (WT), Mutant (M) and cDNA At. Color coded lanes indicate that the PCR products must be Golden Gated to form the full GT gene of interest.

# Gel No. 2

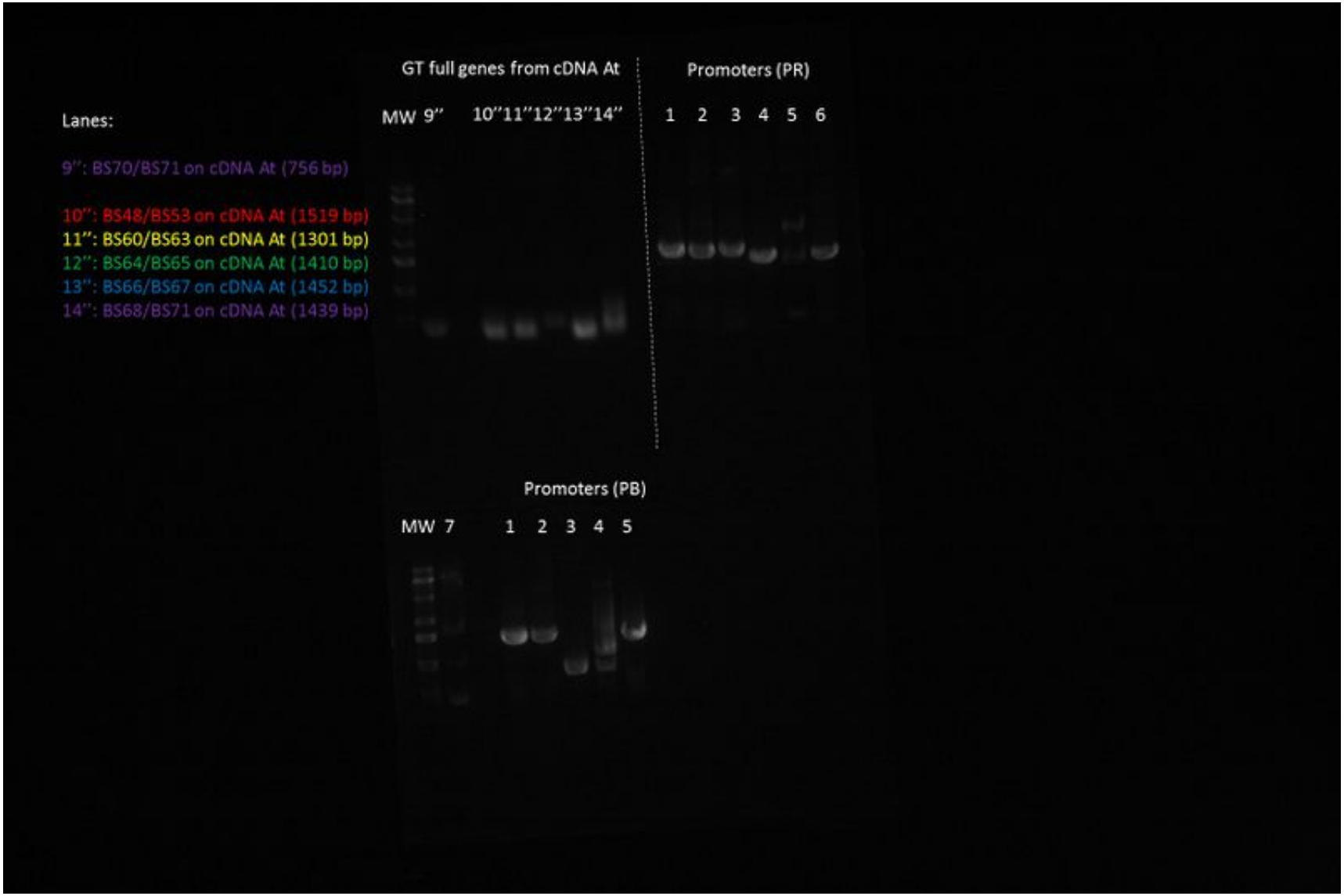


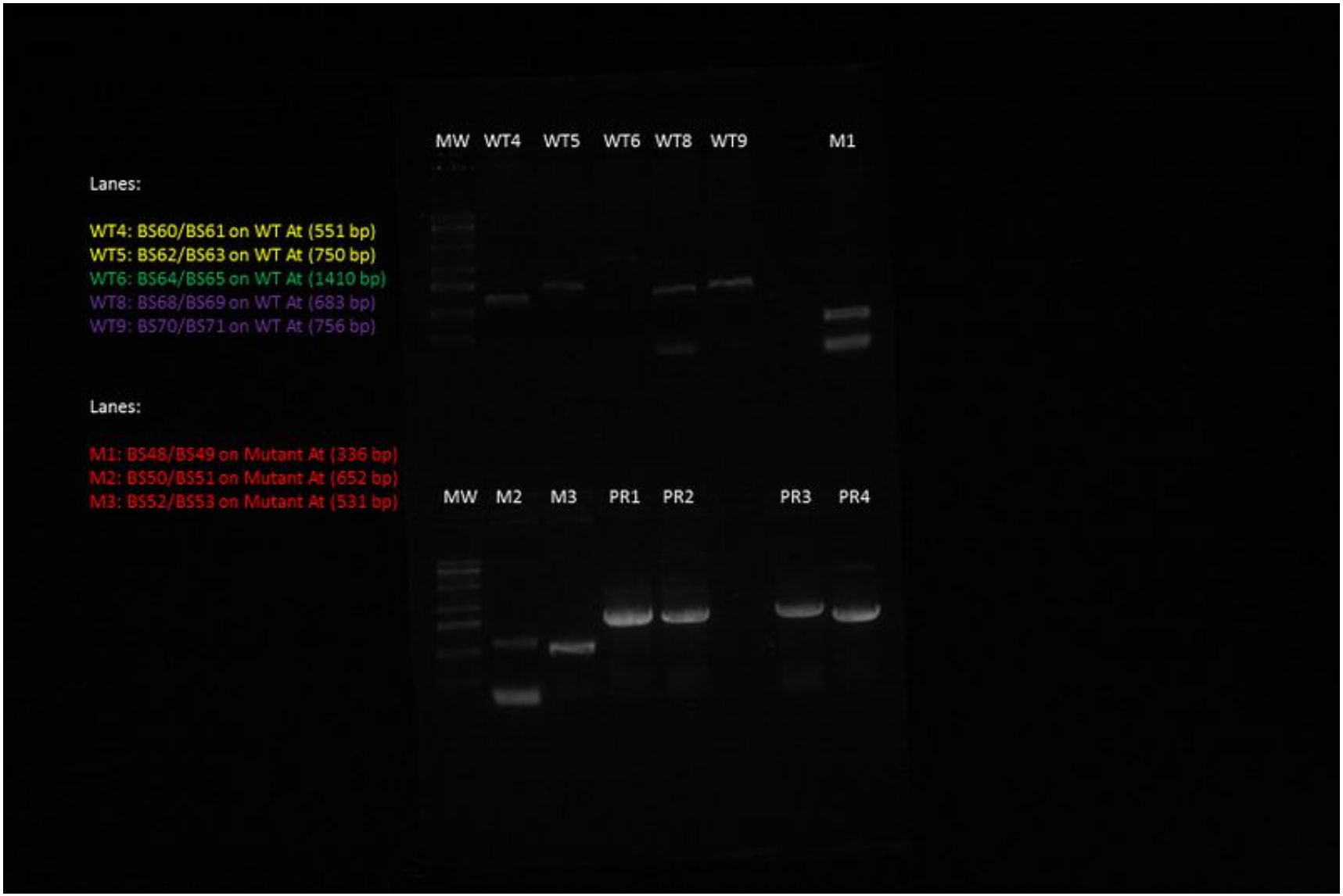

**Figure 2.** Gel Image of PCR products of full GT Gene extracted from cDNA At and Promoters. PR indicates that the PCR mixture was prepared by Ramya, PB indicates that the PCR mixture was prepared by Bernardo.

Based on the results above, we decided to extract the following:

| Gel No. | WT/M/cDNA and Lane No. | Description |
|---|---|---|
| 1 | WT 4 | BS60/BS61 on WT At (551 bp) |
| 1 | WT 5 | BS62/BS63 on WT At (750 bp) |
| 1 | WT 6 | BS64/BS65 on WT At (1410 bp) |
| 1 | WT 8 | BS68/BS69 on WT At (683 bp) |
| 1 | WT 9 | BS70/BS71 on WT At (756 bp) |
| 1 | M 1 | BS48/BS49 on Mutant At (336 bp) |
| 1 | M 2 | BS50/BS51 on Mutant At (652 bp) |
| 1 | M 3 | BS52/BS53 on Mutant At (531 bp) |
| 2 | PR 1 | BT01/BT02 on pWCD0502 (1309bp) |
| 2 | PR 2 | BT01/BT04 on pHL1232 (1268bp) |
| 2 | PR 3 | BT01/BT05 on pHL1232 (1288bp) |
| 2 | PR 4 | ? |
| 2 | PR 5 | ? |
| 2 | PB 1 | BT06/BT07 on pWCD0327 (1091bp) |
| 2 | PR 3 | ? |
| 2 | PR 5 | BT12/BT13 on pZNR0448 (983bp) |

# Additional PCR for Gel Purification

**Note:** Purification of Gel No. 1 and Gel No. 2 did not go very well (Ethanol was not properly evaporated), verified by Nanodrop. Ran another Gel with the remaining PCR products (20 uL for GT Genes, 35 uL for Promoters). Gel purification was done by Ramya around 5:00 PM. Note that we ONLY re-ran the 16 PCR products that worked (i.e. listed on the Table above).




# Golden Gate Assembly of GT genes and Promoters

**Note:** Set up 12 Golden Gates Total (4 for GTs and 8 for Promoters).

## Set-Up for GT Gene Golden Gate

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 3.5 uL | ddH2O | 5.5 uL | ddH2O | 3.5 uL | ddH2O | 1.5 uL | ddH2O |
| 1 uL | T4 Ligase Buffer | 1 uL | T4 Ligase Buffer | 1 uL | T4 Ligase Buffer | 1 uL | T4 Ligase Buffer |
| 0.5 uL | T4 Ligase | 0.5 uL | T4 Ligase | 0.5 uL | T4 Ligase | 0.5 uL | T4 Ligase |
| 0.5 uL | BsmBI | 0.5 uL | BsmBI | 0.5 uL | BsmBI | 0.5 uL | BsmBI |
| 2 uL | PCR Product: WT 4 | 2 uL | PCR Product: WT 6 | 2 uL | PCR Product: WT 8 | 2 uL | PCR Product: M 1 |
| 2 uL | PCR Product: WT 5 | | | 2 uL | PCR Product: WT 9 | 2 uL | PCR Product: M 2 |
| | | | | | | 2 uL | PCR Product: M 3 |
| 0.5 uL | pGG01 | 0.5 uL | pGG01 | 0.5 uL | pGG01 | 0.5 uL | pGG01 |

**Update:** There is a mistake in setting up Golden Gate for Column 1. The mixture is missing 2 uL of Oligo Anneal made by Ramya on May 31st, 2013. This has been re-done by Bernardo on June 3rd, 2013 (Monday).

### Set-Up for Promoter Golden Gate

| | |
|---|---|
| 7 uL | ddH2O |
| 1 uL | T4 Ligase Buffer |
| 0.5 uL | T4 Ligase |
| 0.5 uL | BsmBI |
| 0.5 uL | PCR Product: PR 1/PR 2/PR 3/PR 4/PR 5/PB 1/PB 3/PB 5 |
| 0.5 uL | pGG01 |

**Update:** There are several mistakes in setting up the Golden Gate.

1. PR 4 and PR 5 should have been combined together in one tube. This has been re-done by Roy on June 2nd, 2013 (Sunday).
2. PB 1 and PB 3 should have been combined together in one tube. This has been re-done by Roy on June 2nd, 2013 (Sunday).

# Future Work

1. E. *coli* transformation (Tomorrow, Bernie and Roy)
2. Re-try forming GT Gene 1 and GT Gene 4 using diluted cDNA (In Progress, Bernie)

3. Picking colonies (Tomorrow, Ramya)

# June 3rd, 2013 (Monday)

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## Summary of Morning Meeting

### Previous Work over Weekend

- Saturday:

1. Extracted GT Genes 1 (from Mutant At), 2 (from WT At), 3 (from WT At), and 5 (from WT At) and Promoters.
2. Golden Gated by Hojae at night.

- Sunday:

1. Re-Golden Gate two Promoters by Roy.
2. Transformation of 8 correct Golden Gates.
3. Designed 6 oligos to extract p15A Origin of Replication.

## In-Progress (Today To-Do)

1. Picking Colonies - Done by Ramya on 9:00 AM.
2. Re-Golden Gated GT Gene 1 and create Golden Gate for pCon - Done by Bernardo, started Thermocycler on 10:00 AM.
3. Transformation of two above Golden Gates around 2:00 PM.
4. Miniprep the colonies picked by Ramya around 5:00 PM.
5. Test digest.
6. If possible, Golden Gate the GT and Promoters together. (For Tuesday)

## Future Work (Tuesday, June 4th ~ Thursday, June 6th)

- Tuesday:

1. Extraction of p15A Origin of Replication via PCR.
2. Golden Gate the PCR products.

- Wednesday:

1. Transformation of p15A Origin of Replication.
2. Miniprep the colonies.
3. Golden Gate the p15A Origin of Replication and FMO.

- Thursday:

1. Transformation with just FMO.
2. Co-Transformation with FMO and GT.

# Literature Research: Tyrosine Kinases in E. coli

**Background:**

1. **Protein tyrosine kinase (PTK)** is an enzyme that that transfer phosphate of ATP to tyrosine residues.
2. **BY Kinase** stands for **B**acterial t**Y**rosine kinase.

## "Cells of Escherichia coli Contain a Protein-Tyrosine Kinase, Wzc, and a Phosphotyrosine-Protein Phosphatase, Wzb" (1999)

Link: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC93815/

**Summary:**

1. Wzc and Wzb are known to participate in the export of the extracellular polysaccharide colanic acid from the cell to medium.
2. Wzc is an inner membrane protein that possesses an ATP-binding domain and three predicted transmembrane segments.
3. Wzb has an amino acid sequence homologous to that of acid phosphatases.
4. Wzc-Wzb pair of E. coli is homolog of the Ptk-Ptp pair of A. *johnsonii*.
5. For wzc gene cloning, the sequences of the two primers were 5′-GCGGGATCCACAGAAAAAGTAAAACAACATGCCGCTCCGG-3′ at the N terminus (the BamHI site is italicized; the second codon of wzc is underlined) and 5′-CCGGAATTCTTATTTCGCATCCGACTTATATTCG-3′ at the C-terminus (the EcoRI site is italicized; the stop codon of wzc is underlined).
6. For wzb gene amplification, the sequences of the primers used were 5′-TATGGATCCTTTAACAACATCTTAGTTGTCTGTGTCGGC-3′ at the N terminus (the BamHI site is italicized; the second codon of wzb is underlined) and 5′-CGGGGTACCTTATACCTGCTCTGCGTTCAATGC-3′ at the C terminus (the KpnI site is italicized; the stop codon of wzb is underlined).
7. The main result of this study is the demonstration that two proteins of E. coli, Wzc and Wzb, carry an autophosphorylating protein-tyrosine kinase activity and a phosphotyrosine-protein phosphatase activity, respectively.

## "Tyrosine phosphorylation: an emerging regulatory device of bacterial physiology" (2007)

**Table 1. Biochemically characterized tyrosine kinases, phosphotyrosine phosphatases and phosphotyrosine protein substrates and their cellular roles in different bacterial species**

| Organism | Tyrosine kinase | LMW-PTP | PHP | Phosphotyrosine protein | Function |
|---|---|---|---|---|---|
| *Escherichia coli* | Wzc (BY-kinase) | Wzb | None | Ugd | Capsular polysaccharide and colanic acid production |
| | Etk (BY-kinase) | Etp | | RpoH, RseA | Heat shock response |
| | BipA (potential BY-kinase) | | | | Antibiotic resistance |
| | | | | | O-antigen capsule |
| | | | | | Virulence regulation |

[a]Abbreviations: BY-kinase, bacterial tyrosine kinase; HPK, histidine protein kinase; LMW-PTP, low-molecular-weight phosphatase; PHP, histidinol phosphate phosphoesterase.

**Summary**:

1. Endogenous substrates of the BY-kinases include:

- UDP-sugar dehydrogenases
- Glycosyltransferase (GT)

1. Phosphorylation of the above increases their activity and thereby stimulating the formation of precursors for polysaccharide production. Note that the pohsphorylation has other implications such as Polymyxin resistance.

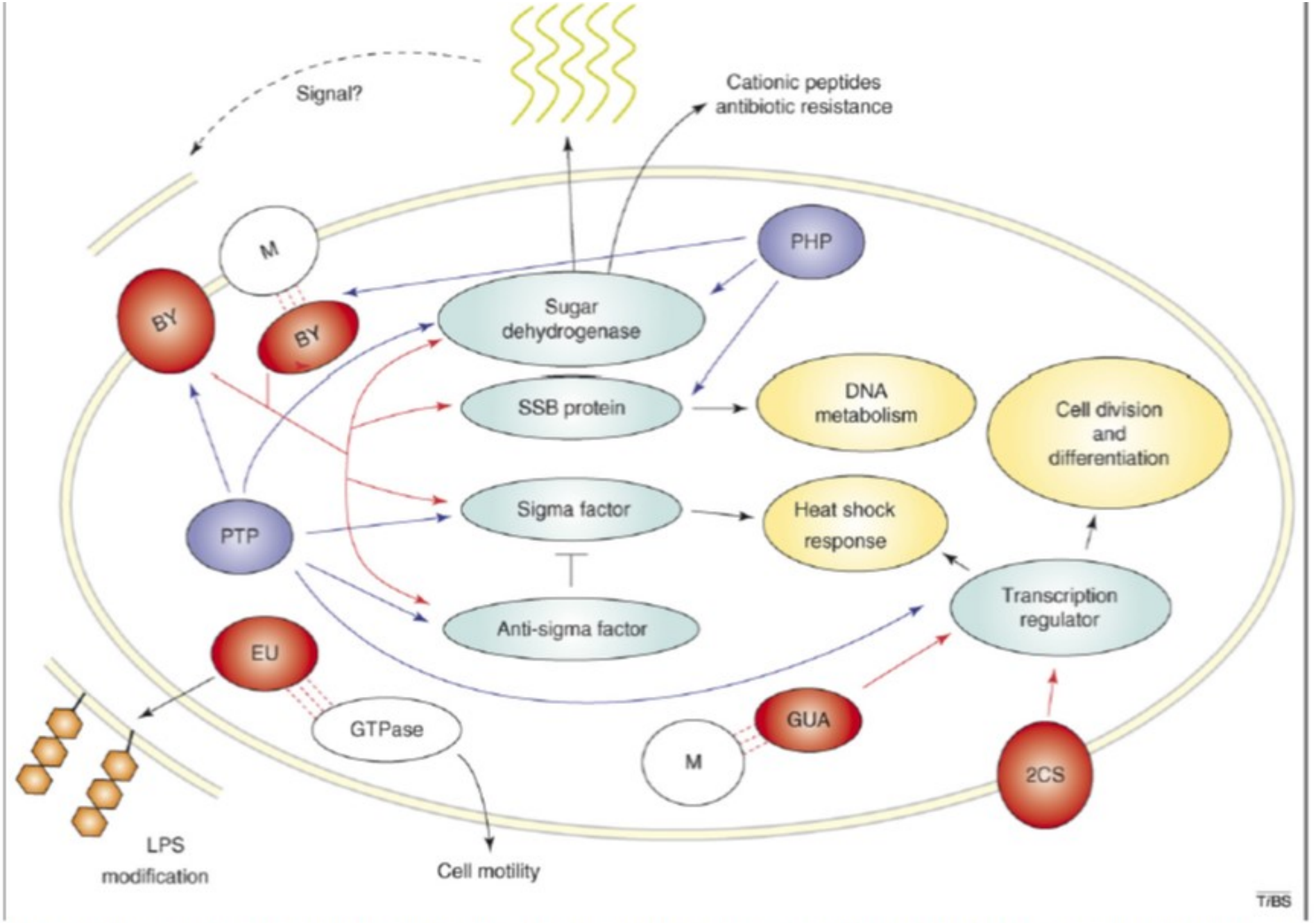


**Figure 3.** Schematic model of the main tyrosine-phosphorylation events and effects in a virtual bacterial cell. Abbreviations: BY, BY-kinases; EU, eukaryotic-like PTK; GUA, guanidino-like PTK; 2CS, histidine Kinase-like PTK; PHP, PHPs; PTP, LMW-PTPs. Broken red lines indicate interactions between tyrosine kinases and their modulators (M) or cellular partners. BY-kinases phosphorylate sugar dehydrogenases, SSB proteins, and sigma and anti-sigma factors. Bacterial tyrosine kinases of the GUA and 2CS type phosphorylate transcription regulators, and the eukaryotic-type tyrosine kinases affect the cell motility (via protein-protein interactions) and possibly modify lipopolysaccharides. The phospho-substrates of bacterial tyrosine kinases are dephosphorylated by the phosphatases of the PHP and LMW-PTP class. Tyrosine

phosphorylation events regulate important cellular processes ranging from DNA metabolism and the cell cycle, to stress response and synthesis of various cell-surface compounds.

### "Autophosphorylation of the Escherichia coli Protein Kinase Wzc Regulates Tyrosine Phosphorylation of Ugd, a UDP-glucose Dehydrogenase" (2003)

**Summary**:

1. UDP-glucose dehydrogenase (Ugd) participates in the synthesis of the exopolysaccharide colanic acid.
2. The process of phosphorylation of Ugd by Wzc was shown to be stimulated by previous autophosphorylation of Wzc on **tyrosine 569** (Y569).
3. When Wzc is phosphorylated, no colanic acid is synthesized by bacteria, whereas when Wzc is dephosphorylated by Wzb, colanic acid is produced.
4. Has protocols on Phosphorylation Assay, Protein Dephosphorylation Assay and Phosphotransferase Assay which we may potentially use?

### "Kinetic and Catalytic Mechanisms of Protein Kinases" (2001)

**Table 1. Several Protein Kinases and Their Substrate Specificities[a]**

| | name | consensus sequence | ref |
|---|---|---|---|
| | serine protein kinases | | |
| PKA | cAMP-dependent protein kinase | -R−R-X-**S/T**-hyd- | 173 |
| PhK | phosphorylase kinase | -R−X-X-**S/T**-F−F- | 38 |
| cdk2 | cyclin-dependent kinase-2 | -**S/T**-P−X-R/K | 32 |
| ERK2 | extracellular-regulated kinase-2 | -P−X-**S/T**-P- | 36−38 |
| | tyrosine protein kinases | | |
| c-Src | cellular form of the transforming agent of the Rous sarcoma virus | -E−E-I-**Y**-E/G−X-F- | 30 |
| v-Fps | transforming agent of the Fujinami sarcoma virus | -E−I-**Y**-E−X-I/V- | 30, 174 |
| Csk | C-terminal Src kinase | -I-**Y**-M−F−F−F- | 175 |
| InRK | Insulin receptor kinase | -**Y**-M−M-M- | 30 |
| EGFR | Epidermal growth factor receptor | -E−E-E-**Y**-F- | 30 |

[a] Phosphorylation sites (P-sites) are shown in bold type.

# Literature Research: Indoxyl-UDPG-Glucosidase

### "Characterization of an indican-hydrolyzing enzyme from Sinorhizobium *meliloti*" (2010)

**Summary:**

1. Beta-glucosidase was determined to be a **monomer** with MW of 52 kDa.

**Experimental Procedure to clone Beta-glucosidase:**
The strain S. meliloti was obtained from MicroBank (www.microbank.re.kr).
Escherichia coli XL1-Blue (Stratagene) was used as the host for the gene cloning and protein expression.
The pMAL-c2X (New England Biolabs) plasmid was used to induce the soluble expression of the enzyme.
The recombinant cells were routinely cultured in LB medium supplemented with ampicillin (50 g/ml) at 37◦C unless otherwise stated.

This article sites http://www.sciencedirect.com/science/article/pii/S1381117708002117 ***(EXTREMELY useful)***

**Table 1**
List of strains, genes and primers used in this study.

| Strain | Acession no. | Primer sequence |
|---|---|---|
| *T. caldophilus* | AAO15361 | 5′-TAGAATTCAACGCCGAAAAGTTT-3′<br>5′-TAAAGCTTTCACTCTGGCTG GGG-3′ |
| *T. thermophilus* | YP145326 | 5′-TAGAATTCAACGCCGAAAAATTC-3′<br>5′-TAAAGCTTTTAGGTCTGGGCCCG-3′ |
| *F. johnsoniae* | ZP01246844 | 5′-GCCCATGGGTAAAATTGAAAACTCATTT-3′<br>5′-GCAAGCTTTTAAGATAAAAAATCTTTAAA-3′ |
| *S. meliloti* | NP386997 | 5′-ATCCATGGTGATCGAAGCCAAGA-3′<br>5′-ATAAGCTTTCATCCCGGCTTGT-3′ |

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# June 4th, 2013 (Tuesday)

From Dueber Lab Wiki

## Contents



## Transformation Results/Colony Picking of GT 2, pCON_2A, pTRC_2A and pTRC_strongRBS


**Figure 1.** Transformation of plasmids GT 2 (pIGEM13_002), pCON_2A (pIGEM13_013), pTRC_2A (pIGEM13_010) and pTRC_strongRBS (pIGEM13_009). Since the colonies from pTRC_2A and pTRC_strongRBS are red (indicating presence of RFP), they were abandoned.

At 9:30 AM, Roy and Hojae **picked 3 colonies each from GT 2 and pCON_2A,** labeled GT 2 No. 1, GT 2 No. 2, GT 2 No. 3 && pCON_2A No.1, pCON_2A No. 2, pCON_2A No. 3.

# RBS Library

### PNK Treatment of Small Oligos"

1. Phosphorylate the 5' end of each oligo separately by treating with PNK - let following mixture sit for 1 hr at 37oC:

| | |
|---|---|
| 1 uL | oligo (100 uM) |
| 1 uL | 10x ligase buffer |
| 7 uL | H2O |
| 1 uL | T4 PNK |

1. Mix the 10 ul of each phosphorylated oligo together and bring the total volume to 200 ul with water (so, 10 + 10 + 180)
2. Take 50 ul of the mixture and run it on the thermocycler using the "Anneal" program (described below).
3. Use 2 ul of the mixture to ligate into the desired vector.

Anneal Program: 96˚C for 6:00 .1˚C per second ramp down to 23˚C Hold at 23˚C forever.

***Storage:*** Annealed RBS Library is stored at -20 degrees Celsius in the iGEM Fridge (in PCR tube). Non-annealed RBS (150 uL) is stored at -20 degrees Celsius in the iGEM Fridge (in 1.5 mL Eppendorf Tube).

# Mini-Prep of pIGEM13_002 (GT 2) and pIGEM_013 (pCON_2A)

**Note:** Roy and Hojae have used the new Miniprep protocol below for GT 2 No. 1, GT 2 No. 2, GT 2 No. 3 && pCON_2A No. 1, pCON_2A No.2, pCON_2A No. 3.

## Zyppy Plasmid Miniprep Kit Protocol

1. Add 100 uL of 7X Lysis Buffer (Blue) to 600 uL of *E. coli* culture in a 1.5 mL microcentrifuge tube. Mix by inverting the tube 4-6 times.
2. Add 350 uL of cold Neutralization Buffer (Yellow), mix thoroughly.
3. Centrifuge at 11,000 - 16,000 x g for two minutes.

4. Transfer the supernatant into the Zymo-Spin IIN column.
5. Place the column into a Collection tube and centrifuge for 15 seconds. Discard the flow-through and place the column back into the same Collection tube.
6. Add 200 uL of Endo-Wash Buffer to the column. Centrifuge for 15 seconds.
7. Add 400 uL of Zyppy Wash Buffer to the column. Centrifuge for 30 seconds.
8. Transfer the column into a clean 1.5 mL microcentrifuge tube then add **50 uL of ddH2O** directly into the column matrix and let it stand for one minute at room temperature. Centrifuge for 15 seconds to elute the DNA.

## Test Digest

To confirm that the Miniprepped plasmids of GT 2 and pCON_2A has been performed correctly, 2 uL out of 50 uL of Miniprepped plasmids have been used for a test digest using BsaI.

For each Miniprepped plasmids (GT 2 No. 1, GT 2 No. 2, GT 2 No. 3, pCON_2A No. 1, pCON_2A No.2 and pCON_2A No. 3), the following tubes were made and incubated at 37˚C for 20 minutes.

| | |
|---|---|
| 6.5 uL | ddH2O |
| 2 uL | Miniprepped Plasmid |
| 1 uL | 10x NEB Buffer #4 |
| 0.5 uL | BsaI |

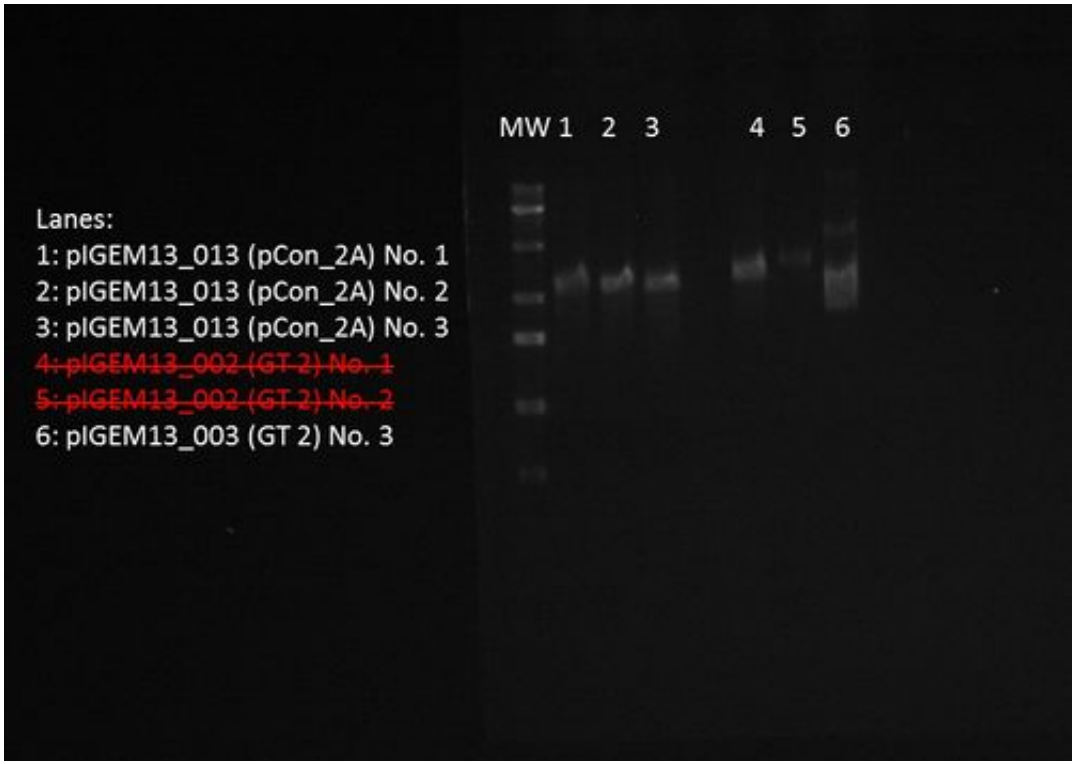
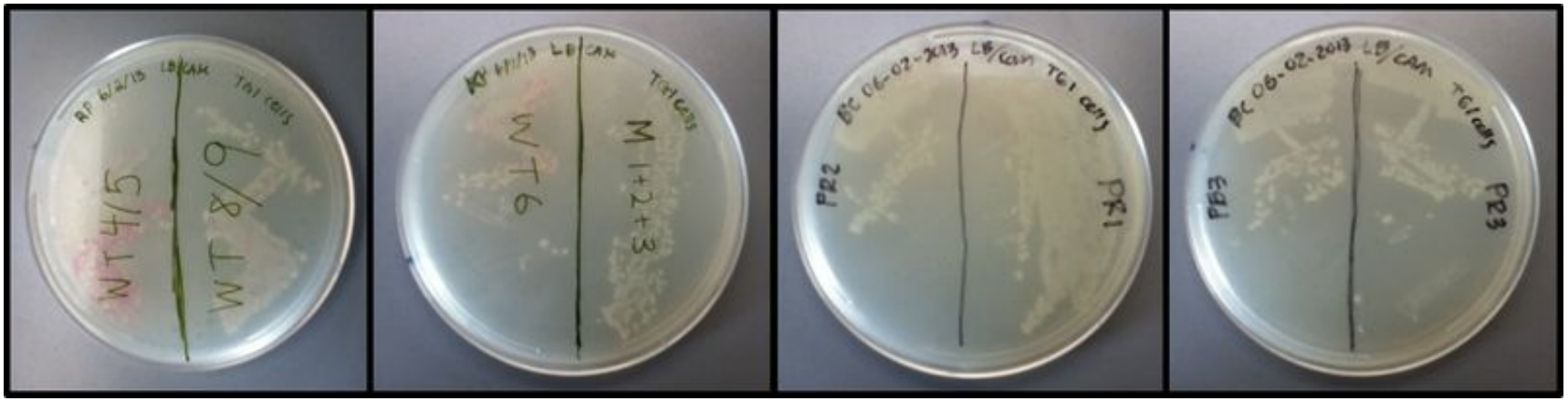



**Figure 2.** The expected sizes after digesting pCON_2A with BsaI was ~1500 bp and ~150 bp, for GT 2 with BsaI was ~1600 bp and ~1300 bp. Based on the test digest, it was determined that GT 2 No.1 and GT 2 No. 2 should not be used, while all pCON_2A were in good conditions.

# Transformation Results, Mini-prep and Cassette Assembly (pIGEM13_018 ~ pIGEM13_023)




Based on the above results, Ramya made 6 Cassette Assemblies (pIGEM13_018 ~ pIGEM13_023).

- Promoters: pBAD_strongRBS, pCON_RBS_BACTERIAL
- Genes: GT 1, GT 3, GT 5

Please see Notes on Wednesday, June 5th, 2013 for results from:

1. Transforming TG1 cells with FMO with ColE1 Origin of Replication.
2. Transforming TG1 cells with pIGEM13_018 ~ pIGEM13_023 ONLY.
3. Co-Transforming TG1 cells with FMO and pIGEM13_018 ~ pIGEM13_23, both ColE1 Origin of Replication.

# Cassette Assemblies with RBS Library (pIGEM13_024 ~ pIGEM13_037)

These are separate from Ramya's Cassette Assemblies since we could now use Promoter 2A parts with Annealed RBS Library.

Hojae and Zack prepared these Cassette Assemblies.

| Tube No. | Plasmid Name | Promoter | Glycosyl Transferase (GT) Gene |
|---|---|---|---|
| 1 | pIGEM13_024 | pBad_strongRBS | pIGEM13_002 (GT 2) |
| 2 | pIGEM13_025 | pCon.rbs bacterial (pZNR0462) | pIGEM_002 (GT 2) |
| 3 | pIGEM13_026 | pBad_2A & RBS Library | pIGEM_001 (GT 1) |
| 4 | pIGEM13_027 | pBad_2A & RBS Library | pIGEM_002 (GT 2) |
| 5 | pIGEM13_028 | pBad_2A & RBS Library | pIGEM_003 (GT 3) |
| 6 | pIGEM13_029 | pBad_2A & RBS Library | pIGEM_005 (GT 5) |
| 7 | pIGEM13_030 | pCon_2A & RBS Library | pIGEM_001 (GT 1) |
| 8 | pIGEM13_031 | pCon_2A & RBS Library | pIGEM_002 (GT 2) |
| 9 | pIGEM13_032 | pCon_2A & RBS Library | pIGEM_003 (GT 3) |
| 10 | pIGEM13_033 | pCon_2A & RBS Library | pIGEM_005 (GT 5) |
| 11 | pIGEM13_034 | pTet_2A & RBS Library | pIGEM_001 (GT 1) |
| 12 | pIGEM13_035 | pTet_2A & RBS Library | pIGEM_002 (GT 2) |
| 13 | pIGEM13_036 | pTet_2A & RBS Library | pIGEM_003 (GT 3) |
| 14 | pIGEM13_037 | pTet_2A & RBS Library | pIGEM_005 (GT 5) |

# June 5th, 2013 (Wednesday)

From Dueber Lab Wiki

## Contents



## Exp 1, Trial 1: Co-Transforming FMO with GTs: pIGEM13_018 to pIGEM13_023


Please see Ramya's or Bernardo's Notebooks for further experiments done with pIGEM13_018 to pIGEM13_023.

## Transformation of pIGEM13_024 to pIGEM13_037 via Electroporation

**Note:** Transformation of pIGEM13_024 and pIGEM13_025 has been done by Roy Park via Heat Shock. Transformation of pIGEM13_027 to pIGEm

## Transformation by Electroporation Protocol

**Note:** Please ask Zack Russ for where Electrically Competent cells are.

### Materials and Location:

- 100uL aliquots of commercially-bought competent cells (in -80 degrees Celsius).

    1. Thaw and keep on ice like chemically competent cells.
    2. Dilute the cells 10x in ice-cold 10% glycerol (1 mL final volumne). Glycerol can be found in Deli fridge.

- White-capped Cuvettes. Can be found in the Deli fridge.

    1. Keep on ice and label the caps.

- DNA to transform the cells. Aliquot 1 uL into a new PCR tube and keep on ice.
- Sterile LB broth for rescue. Keep at room temperature.
- Electroporation Machine. Can be found next to the Microwave in the Gel Station.

### Steps:

1. Add 30 uL of thawed, 10x diluted, electrically competent cells into 1 uL of DNA. Avoid forming air bubbles. Try centrifuging tubes if you introduce air bubbles.
2. Immediately transfer cell-DNA mixture to cuvette to the middle groove on the side of the cuvette with a semi-circular protrusion ("Pointy-Edge"). **Do NOT go beyond the first stop or else you WILL introduce air bubbles!**
3. Tap the cuveete 5-6 times to even out the cells along the bottom of the cuvette. The dark liquid should travel from top of the groove to middle of the groove.
4. Set up Electroporation Machine to 1800 V.
5. Load the cuvette with "Pointy Edge" facing toward the machine. Keep the cap on.
6. Press "Pulse."
7. Machine will beep within 5 seconds and beep to indicate the end. Read the time constant. Time constant greater than 4.0 indicates successful transformation.

**Note:** If you hear a zap before a beep, it means the cells have died. The time constant will also be around 1.0 to 2.0, indicating a bad transformation. Repeat above steps and re-do the transformations.

1. After each transformation, immediately add 500 uL of LB into the groove of the cuvette. You may want a second person to do this while you transform. Gently pipette up and down, and transfer 500 uL of cells into a new, labeled 1.5 mL Eppendorf Tube.
2. Shake at 37 degrees Celsius for 30 to 60 minutes to rescue.
3. Plate 200 uL out of 500 uL of total rescue broth using glass beads.

### Future Steps

1. Colony pick/Culture pIGEM13_024 to pIGEM13_037 on June 6th, 2013.
2. Mini-prep pIGEM13_024 to pIGEM13_037 on June 6th, 2013.
3. Set up Multi-Cassette Assembly of FMO, p15A and pIGEM13_026 to pIGEM_037 together.

**Note:** Use p15A Origin of Replication for FMO.

## Golden Gate Assembly of pIGEM13_017 (Gblocks)

**Note:** Please ask Zach Russ for which Backbone to use.

Made a single tube containing the following:

| | |
|---|---|
| 2 uL (8 uL total) | GBlock 1, GBlock 2, GBlock 3, GBlock 4 |
| 1 uL | T4 Ligase Buffer |
| 0.5 uL | T4 Ligase |
| 0.5 uL | BsmBI |
| 0.5 uL | pZNR_419 |

**Note:** Total volume is 10.5 uL!
**Note 2:** With pZNR_419, the correct colonies express GFP! Check under UV to pick colonies.

## Transformation of pIGEM13_014 to pIGEM13_017 via Heat Shock

## Future Steps

1. Colony pick/Culture pIGEM13_014 to pIGEM13_017 on June 6th, 2013.
2. Mini-prep pIGEM13_014 to pIGEM_017 on June 6th, 2013.
3. pIGEM13_014 (pTET_strongRBS) may be Cassette Assemblied with GT 1 (pIG 1), GT 2 (pIG 2), GT 3 (pIG 3), GT 5 (pIG 5) and GBlocked (pIG 17).
4. pIGEM13_015 (p15A_Amp_sfGFP) and pIGEM13_016 (p15A_Kan_sfGFP) may be Cassette Assemblied with FMO.
5. pIGEM13_017 (Gblocked) may be Cassette Assembled with a bunch of Promoters.

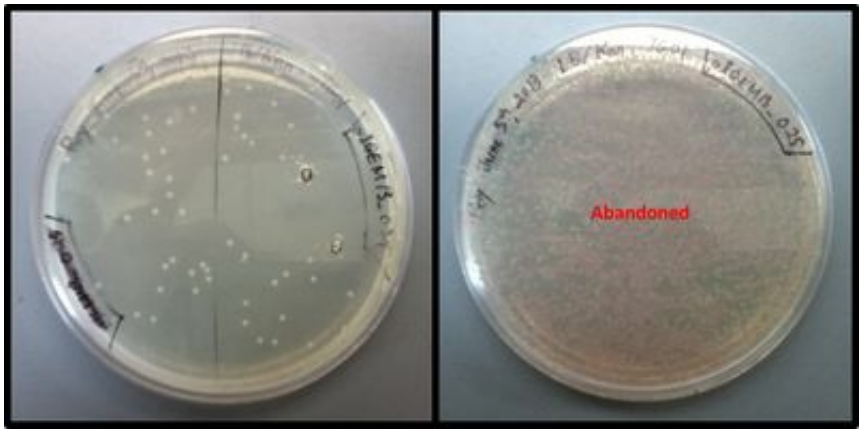
# June 6th, 2013 (Thursday)

From Dueber Lab Wiki

## Contents



## Colony Picking/Culturing pIGEM13_024 and pIGEM13_025

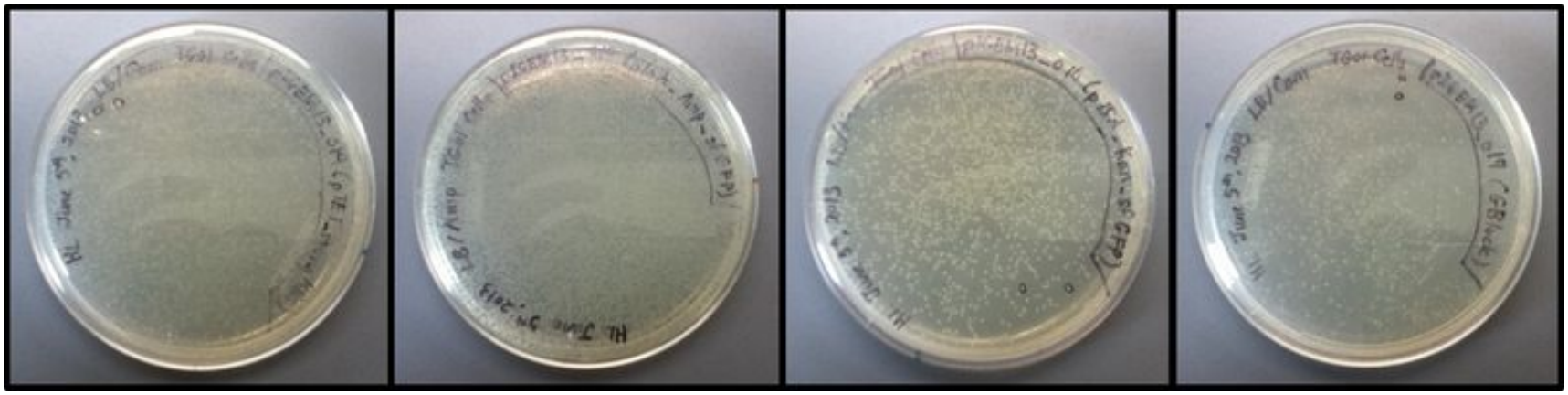



**Note:** Only pIGEM13_024 were picked.

### Future Steps

1. Mini-prep at 6:30 PM.
2. Co-tranformation with p15A/FMO.

# Colony Picking/Culturing pIGEM13_014 to pIGEM13_017



## Future Steps

1. Mini-prep at 6:30 PM.
2. Test-digest with BsaI.
    - pIGEM13_014: ~1000 bp and 1600 bp
    - pIGEM13_015: ~2000 bp and 900 bp
    - pIGEM13_016: ~1900 bp and 900 bp
    - pIGEM13_017: ~1450 bp and 1650 bp

# Failure of pIGEM13_026 to pIGEM13_037 (Cassette Assembly with RBS Library)

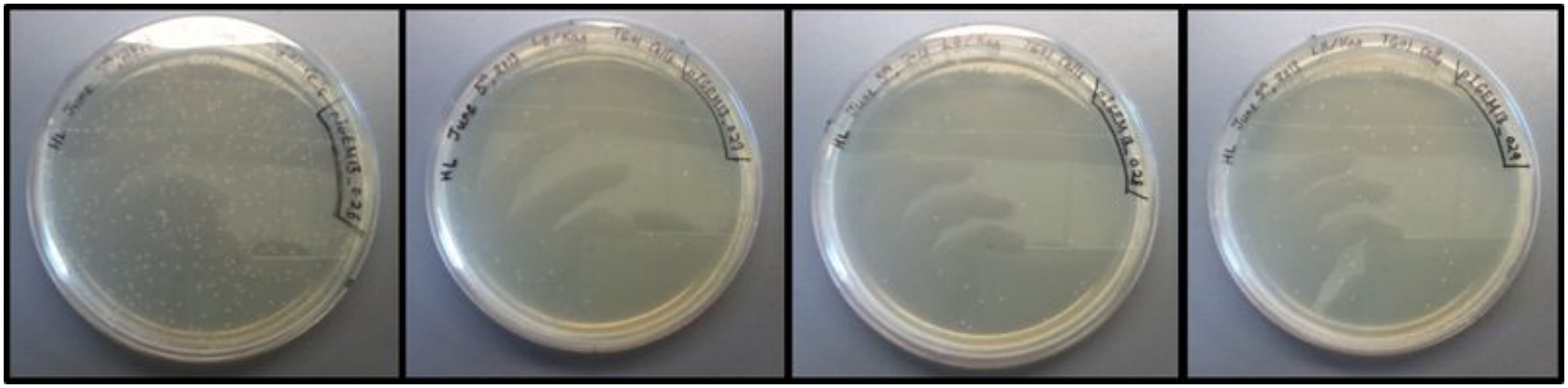


**Figure 1.** (Left to Right) pIGEM13_026, pIGEM13_027, pIGEM13_028, pIGEM13_029. These correspond to pBAD_2A with GT 1, 2, 3 and 5, respectively.

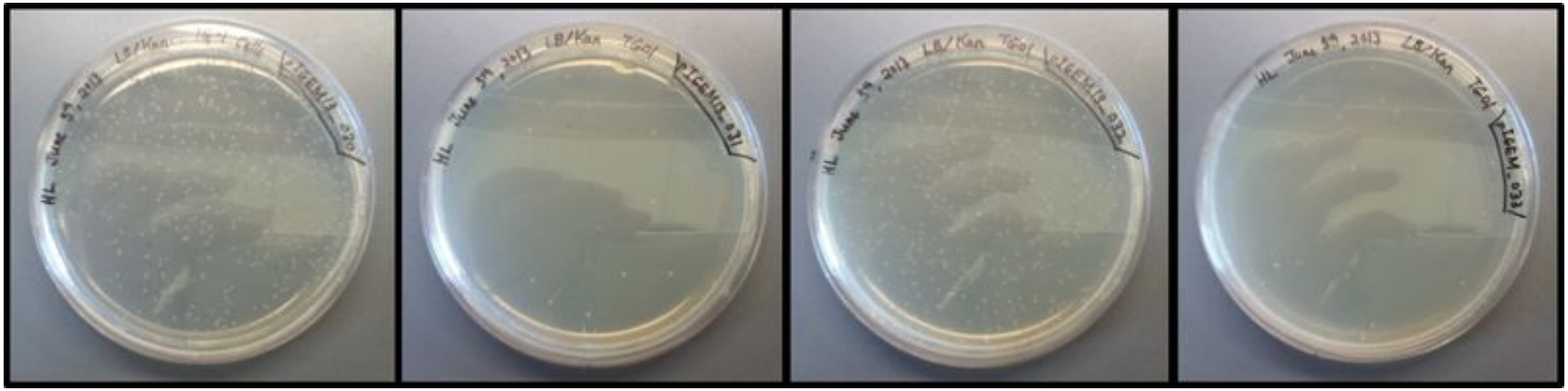


**Figure 2.** (Left to Right) pIGEM13_030, pIGEM13_031, pIGEM13_032, pIGEM13_033. These correspond to pCON_2A with GT 1, 2, 3 and 5, respectively.

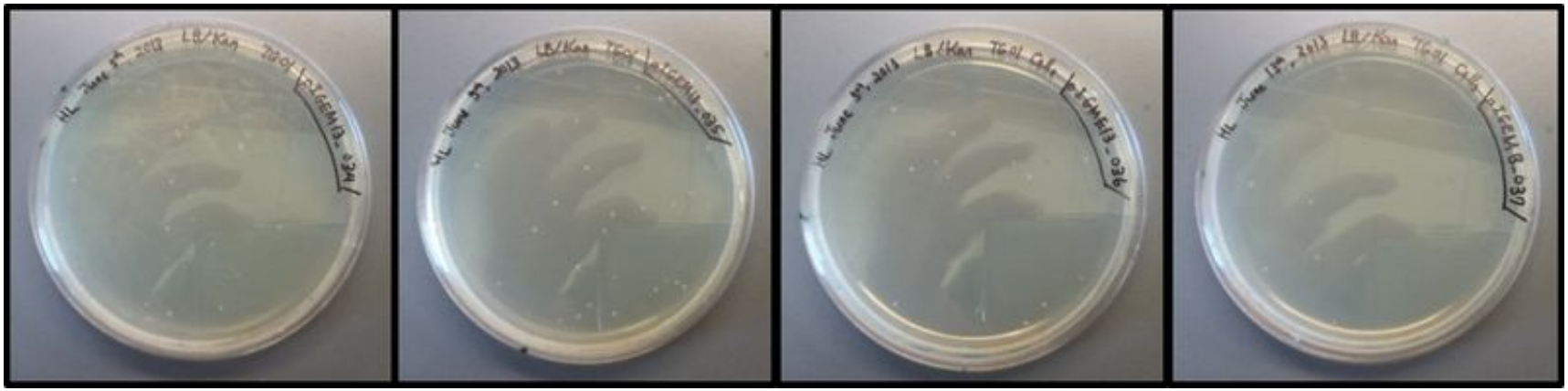


**Figure 3.** (Left to Right) pIGEM13_034, pIGEM13_035, pIGEM13_036, pIGEM13_037. These correspond to pTET_2A with GT 1, 2, 3 and 5, respectively.

There are several reasons why they failed.

1. Zach says that the last 6 base pairs of RBS library is most important, which is equivalent to 4^6 = 4096 different RBS sequences. Thus, we would like to see at least 4096 colonies, which is not the case based on the pictures above.
2. Apparently colonies that glow red under UV are not transformed correctly.

### Future Steps

1. Re-do the Cassette Assembly, making sure to even out the concentration of the parts.

# (Redo) Cassette Assembly of pIGEM13_026 to pIGEM13_037

| | Master Mix (x14) |
|---|---|
| 3.5 uL | ddH2O |
| 1 uL | T4 Ligase Buffer |
| 0.5 uL | T4 Ligase |
| 0.5 uL | BsaI |
| 0.5 uL | Annealed RBS Library |
| 0.5 uL | pGG006 (Robert's) |
| 1 uL | pGG062 (Zach's) |
| 1 uL | pGG078 (Zach's) |
| 1 uL | pGG083 (Zach's) |
| | **Individually** |
| 0.5 uL | Promoter 2A: pIGEM13_007, pIGEM13_012, pIGEM13_013 (1.5 uL) |
| 0.5 uL | GTs: pIGEM13_001, pIGEM13_002 (1.5 uL), pIGEM13_003, pIGEM13_005 |

# Transformation of pIGEM13_026 to pIGEM13_037 via Electroporation

Please follow protocol from Hojae Lee's Lab Notebook titled "June 5th, 2013 (Wednesday)."



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# June 7th, 2013 (Friday)

From Dueber Lab Wiki

## Contents



## Pigment Extraction from E. coli

Source: http://mic.sgmjournals.org/content/135/6/1507.long

This paper tried to express some plasmids found in *Rhodococcus* in E. coli, which produced blue and pink pigments (pretty similar to what we are doing).

Here is how they extracted these pigments. Not sure if it is applicable to Indican, but good reference nonetheless:

**Extraction, purification and characterization of pigments.** Pigments were extracted from pigmented E. coli strains grown in 100 ml LB broth containing 50 pg ampicillin ml-1. Cells were harvested by centrifugation, resuspended in 10 ml water and disrupted by sonication. Suspensions were shaken with an equal volume of chloroform for 2 h and centrifuged at 16000g for 10 min. Chloroform extraction was repeated and the extracts were pooled, evaporated to dryness and resuspended in chloroform. Pigment extracts were fractionated by preparative **partition chromomatography** on a 60 x 3.4 cm glass column packed with silica gel 60 (Merck) using chloroform as the eluant. Extracts were prepared and fractionated from cells of Rhodococcus sp. JLlO and Rhodococcus sp. ATCC 21 145 by the same procedure. Pigment was analysed by visible and UV light spectroscopy on a Beckman DU-40 spectrophotometer.

## Indican Analysis by HPLC

**Note:** We will need to think of a way for extracting Indican from E. *coli* (Sonication then some method to separate indican from everything else), but here are the conditions of how other people analyzed extracted Indican using HPLC.

Source: https://dueberlab.com/w/images/b/b8/Maugard_2002.pdf

## Identification via HPLC

Reactions were conducted in screwcap glass vials. In the standard procedure, fresh leaves were ground in a mortar and suspended in 1% (v/v) acetic acid in acetone (10 mL/g of fresh leaves) and stirred at 24 °C for 4 h. After filtration, acetone was evaporated under reduced pressure. The residue was dissolved (1)in a small volume of **methanol** and analyzed by TLC and HPLC or HPLC-MS, (2) in an aqueous solution **50 mM MES buffer pH 5.0**. Dye concentrations, yields of indigo precursors produced from of P. tinctorium and yields of indican hydrolysis were quantified by HPLC with reference to a **standard curve of products dissolved in methanol**.

Separation and identification of indigo precursors and dyes was conducted using an HPLC system from HewlettPackard (processor, pump, UV detector and injector model 1100). Analyses were carried out with an Ultrasep C18 (250 **?** 4 mm, 6 ím) reverse phase column from ICS (France), at 50 °C with a flow rate of 0.85 mL/min, using a gradient made of solvent A (CH3OH/TFA 0.2%) and solvent B (H2O/TFA 0.2%).

Program:
0-10 min, 85% B/15% A f 75% B/25% A;
10-30 min, 75% f 30% B;
30-40 min, hold at 30% B;
40-45 min, 30% f 85% B.

Products were detected and quantified using a diode array detector at **280 (precursors)**, 550 (indirubin, red dye), and **603 nm(indigo, blue dye)**. During the chromatographic analysis, UV/vis spectra of each individual peak were acquired online using a diode array detector. Further characterization of purified peaks was achieved by HPLC-MS analyses, with comparisons to the A280 traces (online) obtained with rapid-scanning UV analysis.

## Results from Identification of compounds in P. *tinctorium*

The major compounds produced in P. tinctorium were identified by their UV and mass spectra:

1. Indican:
    - HPLC t_R 7.6 min;
    - UV λ_max 218, 280;
    - MS m/z 296 (MH+).

HPLC t_R, mass spectrum, and UV spectrum were identical to those of commercially available indican (Sigma).

1. Isatin:

    - HPLC t_R 12.07 min;
    - UV Λ_max 210, 244, 305;
    - MS m/z 148 (MH+).

HPLC t_R, mass spectrum. and UV spectrum were identical to those of commercially available isatin (Sigma).

1. Flavonoid:

    - HPLC t_R 24-32 min.
    - UV spectra of these three compounds correspond to flavonoid spectra.

1. Indigo:

    - HPLC t_R 35.16 min;
    - UV Λ_max 603 nm.

1. Indirubin:

    - HPLC t_R 37.5 min,
    - UV Λ_max 552 nm.

1. In both dyes, mass spectrum yielded an apparent MH+ ion at m/z 263, indicating a molecular mass of 262. Fragmentation of the m/z 263 ion yielded m/z 235 (-CO).

# Golden Gate Assembly of pIGEM13_015, pIGEM13_016, pIGEM13_21, pIGEM13_23 and pIGEM13_25

**Note:** Did not end up doing Cassette Assembly of pIGEM13_025 due to lack of pGG006, pGG062, pGG078 and pGG083.

- This page was last modified on 9 June 2013, at 18:55.
- This page has been accessed 36 times.

# June 8th, 2013 (Saturday)

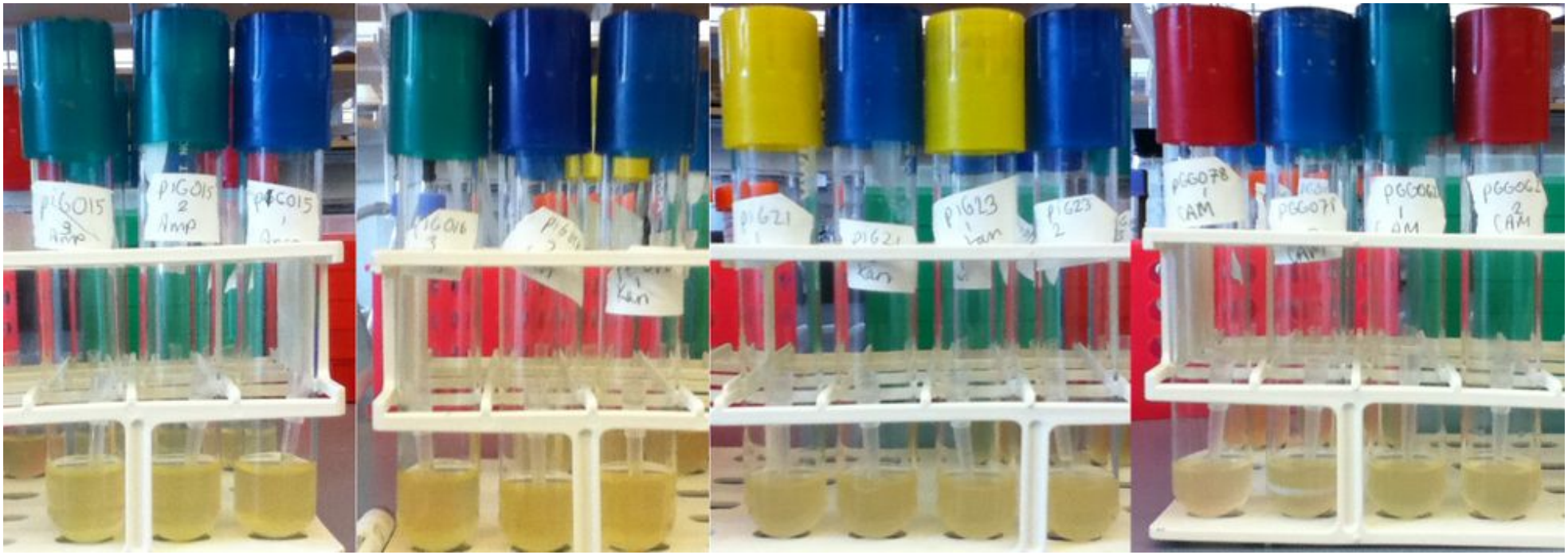
From Dueber Lab Wiki

## Mini-prep of pIGEM13_015, pIGEM13_016, pIGEM13_021, pIGEM13_023, pGG062 and pGG078

**Note:** Three colonies have been picked for pIGEM13_015 and pIGEM13_016 by Ramya with total volume of 7 mL. Two colonies have been picked for the rest with total volume of 5 mL.

**Note 2:** For each of the three colonies for pIGEM13_015 and pIGEM13_016, I collected FOUR 1.5 mL tubes (a.k.a. used 6 mL) to miniprep, labeled pIG 15_1.1, pIG 15_1.2, pIG 15_1.3, pIG 15_1.4, etc. At the elution step, I combined pIG 15_1.1 and pIG 15_1.2 together using 25 uL of water in order to get a higher concentration of p15A.

# Total of 32 Minipreps!!! (From 6:50 PM to 9:30 PM)



Retrieved from "https://dueberlab.com/w/index.php?title=June_8th,_2013_(Saturday)&oldid=18441"

- This page was last modified on 9 June 2013, at 20:14.
- This page has been accessed 12 times.

# June 9th, 2013 (Sunday)

From Dueber Lab Wiki

## Test Digest of pIGEM13_015, pIGEM13_016, pIGEM13_021, pIGEM13_023 and RBS Libraries

**Note:** pIGEM13_015 and pIGEM13_016 was digested with BsaI and NEB Buffer #4. The rest were digested with BsmBI and NEB Buffer #3.

### Results

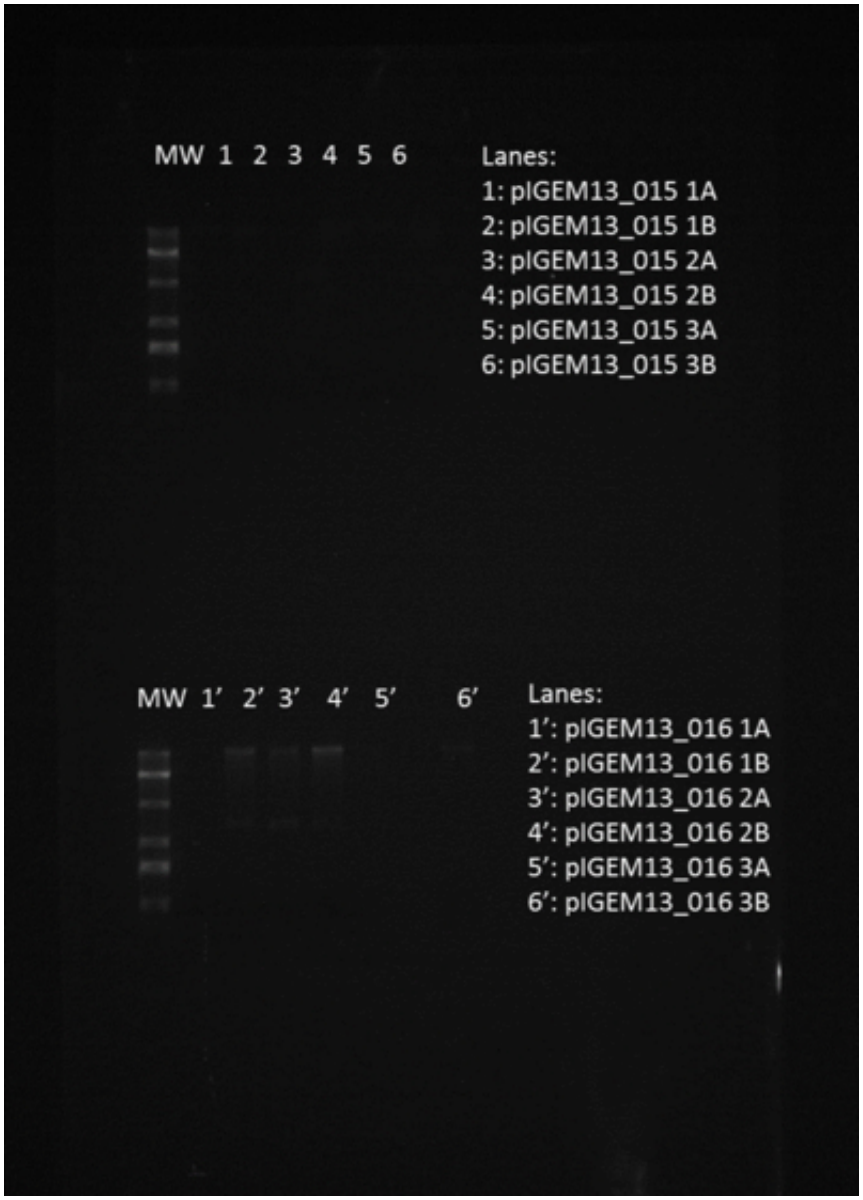



**Figure 1.** pIGEM13_015 did not work, pIGEM13_016 some are faint. These were also nanodropped - pIGEM13_015 had less than 10 ng/uL for each tube and pIGEM_016 had around 15 ~ 22 ng/uL. Bernardo and Roy will perform a procedure to concentrate the product.

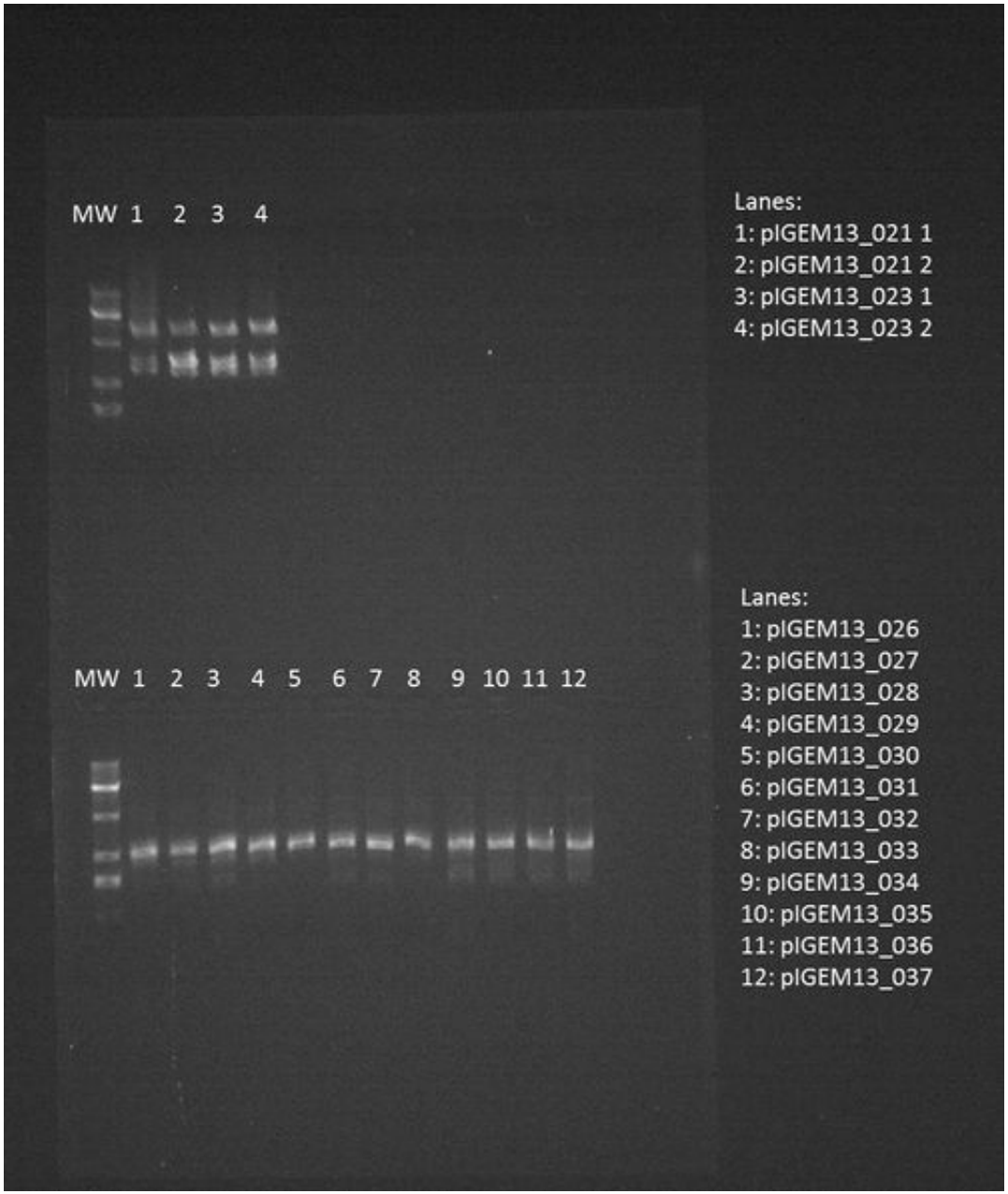



**Figure 1.** pIGEM13_021 and pIGEM13_023 seem to work. These will be sent for sequencing. RBS Library is suppose to have digestion products at around 3000 bp and 1800 bp for all of them, but only 1800 bp seem to show. pIGEM13_037 will be sent for sequencing to see what is going on.

# Other things to do

1. Concentrate pIGEM13_015 and pIGEM13_016 by Bernardo and Roy.
2. Own culture of pGG001 to pGG096 by Bernardo and Thomas at night.
3. Sequencing pIGEM13_015, pIGEM13_016, pIGEM13_021, pIGEM13_023 and pIGEM13_037 by Roy (tomorrow?)
4. Re-stocking ddH2O and cleaning tubes.

# June 13th, 2013 (Thursday)

From Dueber Lab Wiki

## Contents



## Mini-prep of pIGEM13_014 (pTET_strongRBS)

## Experiment: pIGEM13_018, pIGEM13_019, pIGEM13_020 and pIGEM13_024

## Cassette Assemblies and Electroporation of RBS Library

## Cassette Assemblies and Heat Shock of pIGEM13_014 with GTs



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# June 24th, 2013 (Monday)

From Dueber Lab Wiki

## Contents

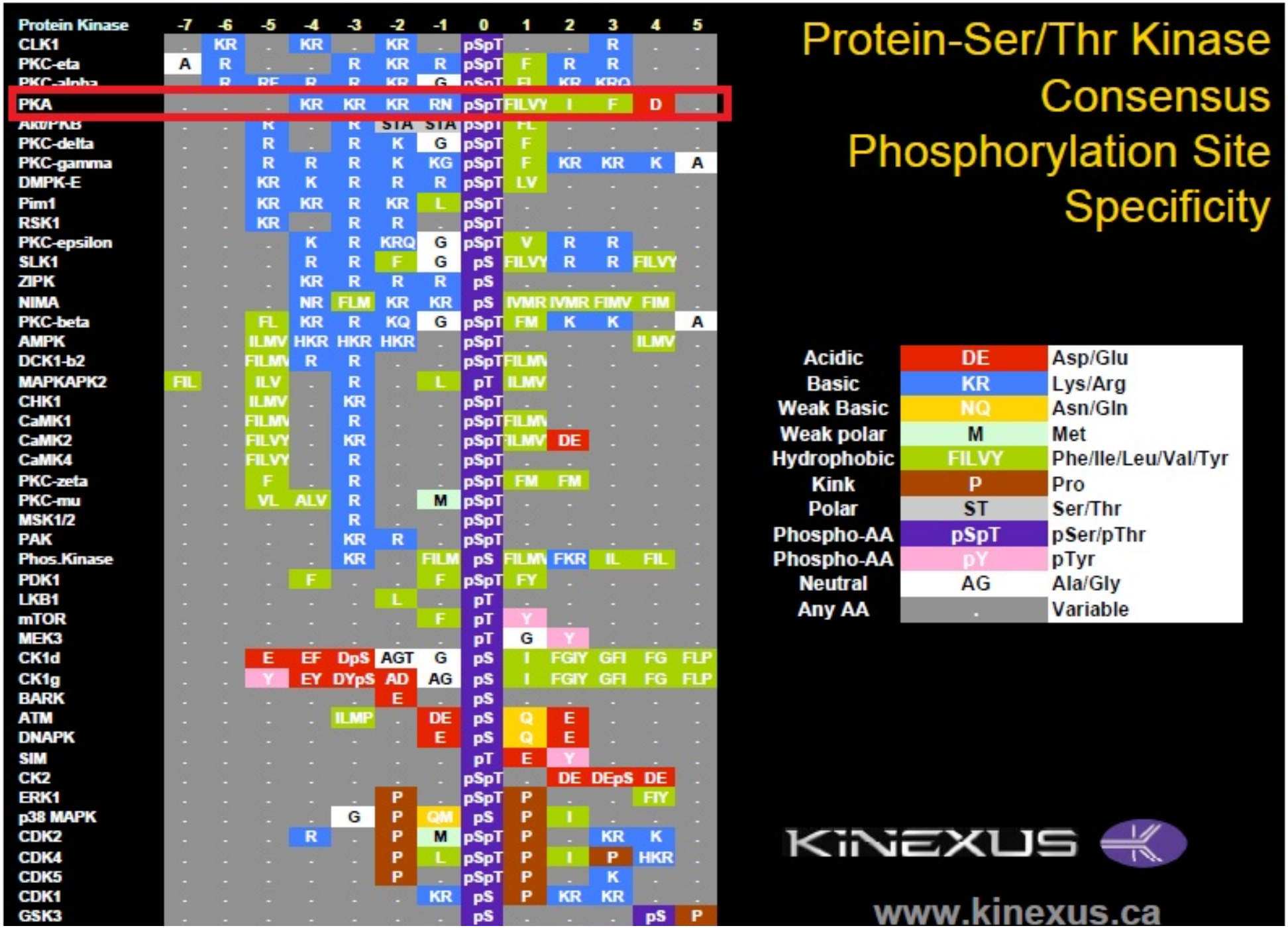


## Yeast Sensor Kinase System: PKA

Sources: Rewiring Cellular Morphology Pathways with Synthetic GEFs and Wikipedia Article on PKA.

Protein Kinase A, or PKA, is a Serine/Threonine sensor kinase found in yeast (*Saccharomyces cerevisiae*). It is inactive until cAMP level increases in the cell. In the paper by Yeh et al., cAMP level was increased by treatment with Forskolin. I briefly crossed an article somewhere that G-coupled protein (GCP) activity may increase cAMP level besides treatment with Forskolin, which is great since GCP activity can be stimulated by sensing various molecules, e.g. growth hormones. In short, anything that can increase cAMP level will be our target substrate (i.e. thing that we will sense).

According to the same Yeh et al. paper, the ideal PKA substrate is RRRRSIIFI (where S is phosphorylated), the below chart shows more sequences that PKA can recognize:




# Binding Domain: BRCT

We found this awesome website ([Pawson Lab (http://pawsonlab.mshri.on.ca/index.php?option=com_content&task=category&sectionid=3&id=40&Itemid=64%7CThe) ]) which lists Phosphorylated Serine binding domains (it has many, many other binding domains and their consensus sequences too). Of the 7 Phosphorylated-Ser binding domains listed, I found that the BRCT binding domain, found in the human BRCA1 (breast cancer type 1 susceptibility protein) gene can bind to a peptide sequence that contains pSXXF.

Using this information and another article [[1] (http://www.jbc.org/content/early/2003/10/24/jbc.C300407200.full.pdf%7CHere) ], we decided that the BRCT-PKA Consensus sequence will be **RRRRSYIFDK**. This sequence can phosphorylated by PKA, and then be bound to BRCT.

# Reporting System: B-GAL and B-LAC

Zach has posted two articles on split Beta-Galactosidase (B-GAL) and split Beta-Lactamase (B-LAC) in the Indican Discussion Pathway page.

1. Beta-Galactosidase cleaves X-Gal, which makes galactose and indoxyl-like molecule which dimerizes into a compound like indigo to make a blue color.
2. Beta-Lactamase acts on Nitrocefin, which turns a yellow solution into a red solution.

## Split B-GAL/B-LAC

Split protein parts are inactive until they come together. We are trying to split B-GAL and B-LAC into N-terminus B-GAL/C-terminus B-GAL and N-terminus B-LAC/C-terminus B-LAC (these have been done in the papers that Zach posted). We will bring these separate parts together by fusing N-terminus B-GAL to BRCT-PKA Consensus sequence and C-terminus B-GAL to BRCT domain. Thus, when the BRCT-PKA Consensus sequence is phosphorylated by PKA, the BRCT domain will try to bind to the consensus sequence, therefore bringing the split B-GAL parts together (same will be done for split B-LAC parts).

So far, we have made oligos/GBlocks to create split B-GAL and split B-LAC, which are named pYGEM13_001 to pYGEM13_008.

I think it may be a good idea to look into Split Glucosidases.



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# June 28th, 2013 (Friday)

From Dueber Lab Wiki

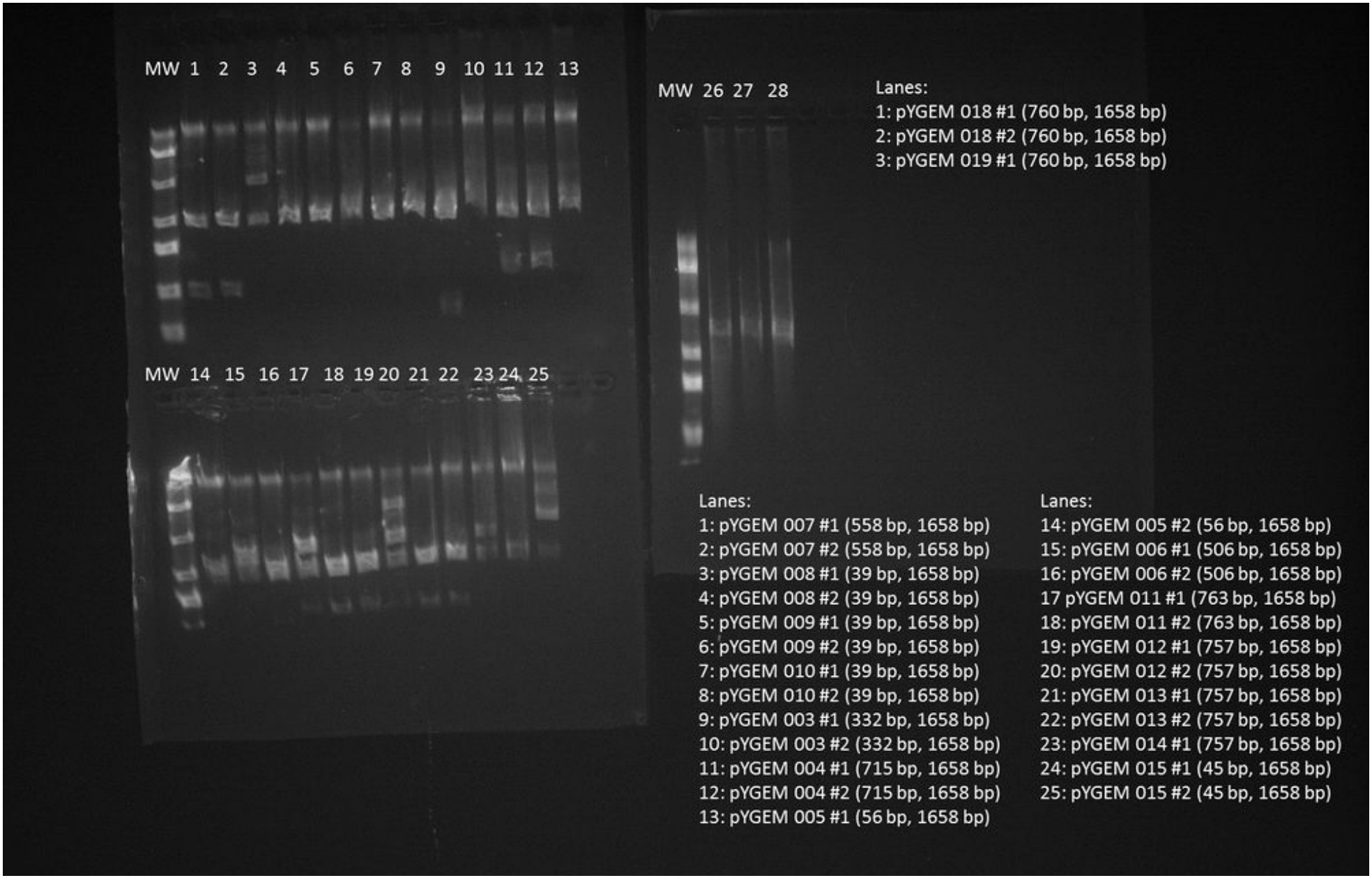

**Figure 1.** Test digests seem to look decent, but good idea to send everything for sequences - making sure that the sequence will include our gene of interest!!!



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1. Week of June 23rd to June 29th:

Started working on the Indigo Biosensor part of the project.

Overall Goal: To engineer a split-glucosidase enzyme that will act on indican upon sensing an external stimulus that can act through a G-Protein Couple Receptor (GPCR).

2. Week of June 30th to July 6th:

Designed and cloned two protein domains, BRCT and 14-3-3, which can bind to a consensus sequence to PKA (Protein Kinase A).

Designed and cloned consensus sequence, positive consensus sequence (in which serine was mutated to glutamate) and a negative consensus sequence (in which serine was mutated to histidine). Designed leucine zippers from UC Berkeley iGEM 2012 for positive and negative control.

Designed and cloned split beta-galactosidase (LacZ) and split lactamase based on from previous research who had success in cloning split enzymes.

3. Week of July 7th to July 13th:

Ran cell lysate experiment – unfortunately split lactamase had to be re-golden gated because the cassettes had AMP resistance, naturally giving rise to lactamase activity. Realized that we cloned the incorrect portion for split beta-galactosidase.

Confirmed activity of full beta-galactosidase.

4. Week of July 14th to July 20th:

Abandoned the split-lactamase system after another cell lysate experiment in which all samples turned from yellow to red, indicating false positives for all samples.

Encountered problems with cloning split beta-galactosidase due to size (>5,000 bp).
Started designing and cloning split-GFP based on paper that had success in cloning split-GFP with anti-parallel leucine zippers.

5. Week of July 21th to July 27th:

Cultured multi-gene assemblies of split-GFP (one split was constitutively expressed, the other was inducible with IPTG), and induced after 3 hours and cultured for 18 hours. Did not see expression of GFP under UV light.

6. Week of July 28th to August 3rd:

Revived the split-lactamase system.

Started cloning split-lactamase and split-galactosidase system compatible with yeast.

7. Week of August 4th to August 10th:

Continued cloning split-lactamase system in yeast.

Ran cell-lysate experiment with E. coli, found out that E. coli naturally has some lactamase activity (high background).

Friday: Field trip to Advanced Biofuels Process Demonstration Unit (ABPDU) and JBEI with Zach Russ, Chris Eiben, Terry Johnson and John Dueber. Met with Dr. Julio Baez for a tour of the facility.

8. Week of August 11th to August 17th:

Presented at the BioBuilder Workshop to high school teachers at 8$^{th}$ floor Stanley Hall.

Went to UC Davis for Nor-Cal iGEM team meet-up.

9. Week of August 18th to August 24th:

Ran the cell lysate experiment using split-lactamase in yeast. Induced PKA activity with Forskolin. Did not see noticeable pattern between Forskolin induced vs. Forskolin uninduced.

Stopped cloning for the Indigo-based Biosensor part of the project.

10. Week of August 25th to August 31st:

Selected Manufacturing track, updated UC Berkeley iGEM wiki with Project Description and Team Roster.

11. Week of September 1st to September 7th:

Uploaded the header for wiki, major update on the homepage and team page.
Designing official sweatshirt for UC Berkeley iGEM team
Friday: Conclusion of Indigo Biosensors Work.

12. Week of September 8th to September 14th:

Monday: First presentation meeting with Terry Johnson
Continued literature research and further developed a schematic for large-scale indigo dyeing process.

13. Week of September 15th to September 21st:

Monday: Second presentation meeting with Terry Johnson
Friday: First practice presentation for regional jamboree in front of Zach Russ, Chris Eiben, Terry Johnson and John Dueber

14. Week of September 22nd to September 28th:

Sunday: Major updates on the presentation slides
Monday – Tuesday: Practice presentations for regional jamboree
Wednesday: Presented at the SynBio Supergroup meeting in front of Berkeley graduate and postdoc students at 115 Energy Bioscience Building.
Thursday: Currently pulling an all-nighter to update the Berkeley iGEM Wiki before the wiki freeze.