

# May-June 2013

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## 5/31/2013

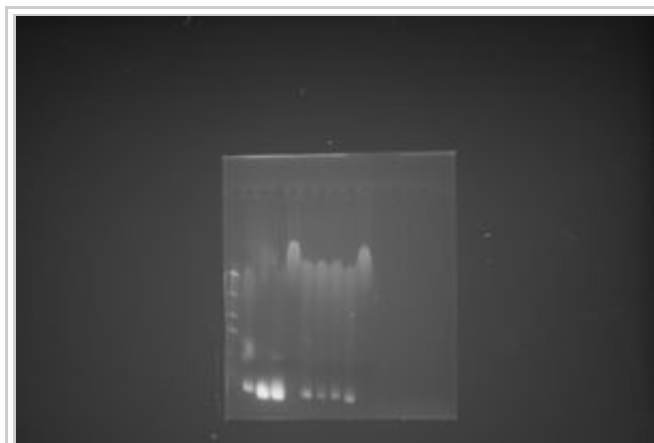
### A. PCRs of Glucosyltransferase from *Arabidopsis thaliana*

- We prepared PCRs on the Arabidopsis GTs (glucosyltransferase)(9:30 AM)
- Master Mix for the PCRs created by Hojae

I prepped the PCR (Phusion) for the At\_GT\_AT1G05530 (pIGEM13\_003)

### B. Set up Gels

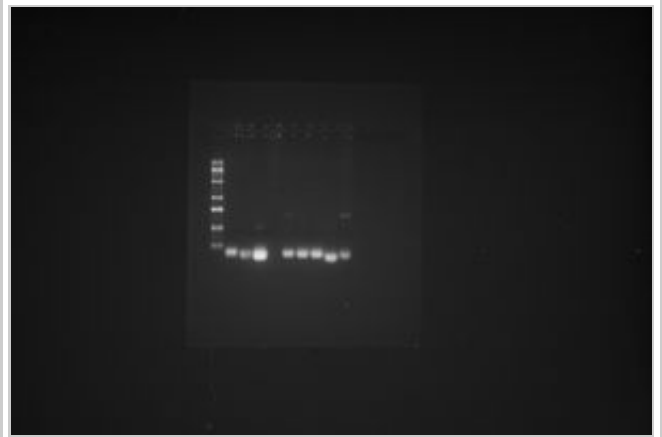
- Pour/Set up the gel for the PCRs (Done by Ramya at 12:25 PM)
- We took the PCR products out of the thermocycler and loaded them into the wells in the gel



**Picture 1** This Gel did not run properly because the speed setting was set too high. Also note the high presence of primer dimers

## C. Expand PCR

- After the gel purifications, we decided to change from a phusion PCR to a Expand PCR since many primer dimers were present (evident in photos of gel)
  - One problem source that we identified was using wrong concentrations of primers
  - Another problem source we thought could be possible was
  - We determined that the cDNA template for the arabinadopsis was a possible problem, so we managed to acquire the arabinopsis Genome form the Somerville Lab

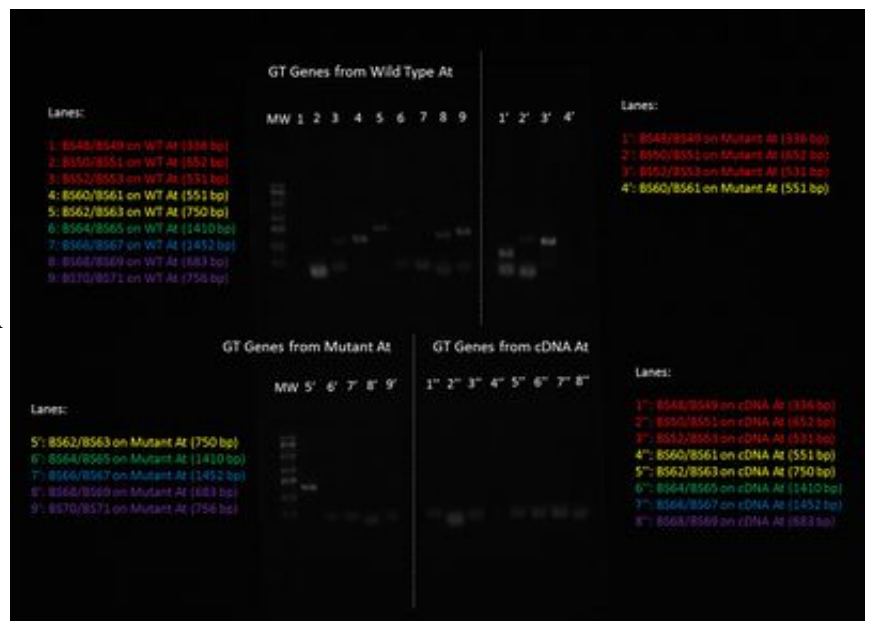


**Picture 2** Even after running the Gel at a lower speed, there were many primer dimers present as evident in the photo. From this observation, we will run a expand PCR instead of a phusion PCR

One of wild type (of lower quality) and another not a wild type (higher quality, but increased chance of mutations in genome)

- After acquiring the genome for the arabinadopsis, we did the following PCRS (6:00 PM)
  - PCR(expand) with wt arabinadopsis genome
  - PCR(expand) with non-wt arab genome
  - PCR(expand) with complementary DNA
- Master Mix created by Hojae

6/01/2013



## A. Gel purification of WT, M, and cDNA

## B. DNA extraction from Gels

- Helped Ramya and Hojae extract GT DNA from WT, M, cDNA of the purifications that worked

**6/02/2013**

## A. Golden Gate Assembly

- Ran 2 Golden Gate Assemblies for PR6/PR7 and PB1/PB2 on Lefty B (11:40 AM Start)

### Protocol for Golden Gate Assembly

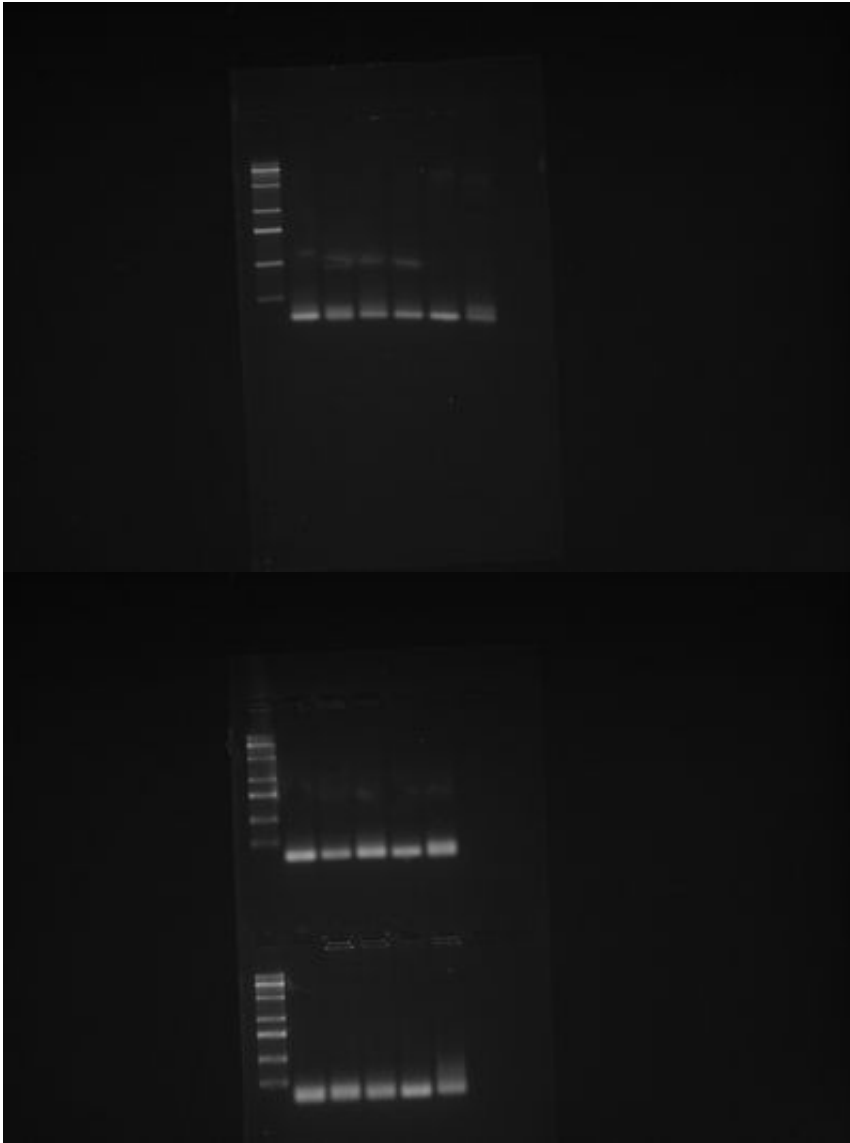
1. 6.5 uL of ddH<sub>2</sub>O
2. 1 uL of T4 Ligase Buffer
3. 0.5 uL of T4 Ligase
4. 0.5 uL of pGG01
5. 0.5 uL of BsmBI
5. 0.5 uL of each product (1uL total) From the Yellow Rack in -2

## B. Transformation by Heat Shock

- Began transformation of E. Coli cells for the Golden Gate Assemblies (done by Hojae on 6/1/2013) with Bernardo
- Left GT1 cells with LB + CAM resistance in culture on 3:00 PM

## C. Gel Purification of diluted cDNA, WT, Mutant

- Nothing with the cDNA template seemed to be working (as evident in the gels)
  - Picture 1 (on left): the bands did not line up correctly as expected
  - Picture 2 (on right): almost no bands present



#### **D. Considerations when Co-transforming FMO + GT genes**

- We don't want both the FMO and the GT genes to have the ColE1 origin, since their copy numbers then will be the same
  - In addition, the likelihood of problems occurring increases if we use both ColE1 origins
- After some consideration, we will apply the p15a origin of replication to FMO (lower copy number) while applying the ColE1 origin to the GT genes

**6/03/2013**

#### **A. Serine/Threonine Kinases**

- This paper has a table of Serine/Threonine kinases and substrates [1]  
(<http://www.microbiology.columbia.edu/faculty/pdf/pereiraetal2011.pdf>)

##### **a. PknG**

## ■ What is it?

- Present in: *Mycobacterium tuberculosis*
- Function: Modulate phagosome-lysosome function, virulence, regulation of glutamate metabolism
- Substrate: PknG acts on GarA (Glycogen accumulation regulator) [2] (<http://www.uniprot.org/uniprot/P64897>)
  - Possible Problem: GarA binds with gdh (NAD-specific glutamate dehydrogenase) and kgd (Multifunctional 2-oxoglutarate metabolism enzyme)

## ■ Some general notes about PknG

- autophosphorylation has no effect on kinase activity, but promotes binding to GarA
- The kinase domain of PknG is inactive by itself (means the flanking domains are important). It does not have a transmembrane domain and shown to be localized to bacterial cytosol and membrane.
- Made of N-terminal Trx domain, a kinase domain, and a C-terminal TPR domain with the typical two lobed organization characterized in eukaryotic serine/threonine kinases.
- Source: <http://www.jbc.org/content/284/40/27467.long>

Table 1. Identification of phosphopeptides within the N-terminal sequence of PknG.

Residue	Sequence	MR <sup>+</sup> Average theoretical	MR <sup>+</sup> Average measured	Number of phosphate groups	Phosphoresidue(s)
10-60	SGPGTQPADAQATATSATVRPLSTQAVFRPDFG DEDNFFPHPTLGPDTPEQDR	5395.7	5395.2 5475.3 5555.4	0 1 2	Location not identified
29-60	PLSTQAVFRPDFGDEDNFFPHPTLGPDTPEQDR	3597.8	3597.5 3677.6	0 1	T32
10-30	SGPGTQPADAQATATSATVRPL	2027.2	2027.3 2107.3	0 1	T23
10-28	SGPGTQPADAQATATSATVR	1816.9	1885.8	1	T23
61-73	MATTSRVRPPVRR	1527.6	1507.5 1687.6	1 2	T63 and/or T64 and/or S65

Phosphopeptides within the N-terminal of PknG

of "Key Residues in *Mycobacterium tuberculosis* Protein Kinase G Play a Role in Regulating Kinase Activity and Survival in the Host"

<http://www.ncbi.nlm.nih.gov/pubmed/19019160> of "Regulation of glutamate metabolism by protein kinases in mycobacteria."

## B. Mini Prep

- Ran a mini-prep with Thomas for the GT genes and the promoters

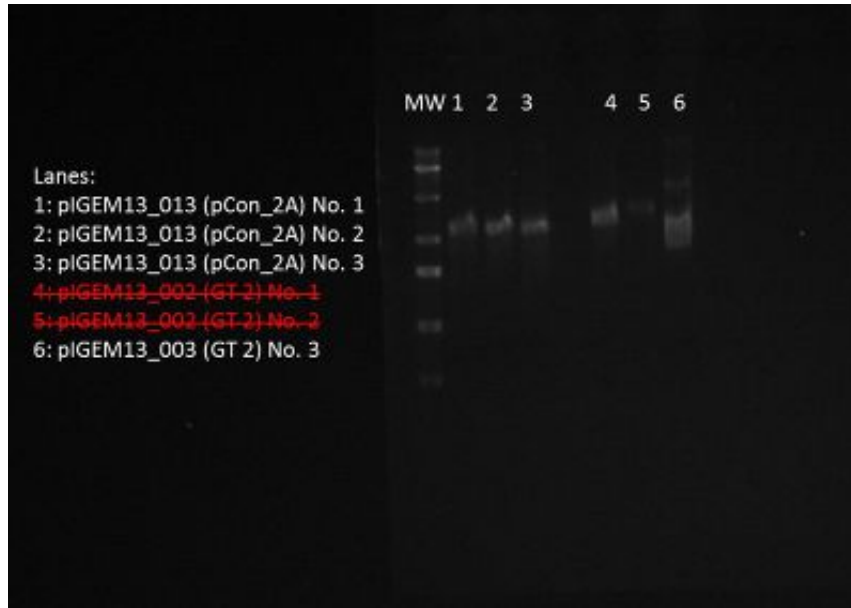
## 6/04/2013

## A. Learning how to use Foldit

- Chris taught us how to use Foldit to help us understand protein kinases and come up with possible designs for the Glu protein

## B. Mini Prep + Digest Test

- Note: Switched over to a newer mini-prep kit
- Did a mini prep for the PCon 2a and the GT 2 and ran a digest test with BsaI



## 6/05/2013

### A. Transformation (Part 1) B. Poured Plates

- Poured plates for Kan, Cam, and Amp with Ramya and Bernardo (stored in iGEM fridge)

### C. Transformation (Part 2)

- Did a transformation of piGEM13\_024 and piGEM13\_025

## 6/06/2013

### A. Sequencing

- Set up sequencing for piGEM22 (S23 primer) and piGEM23 (S23 primer)
- Will update soon...

## 6/07/2013

### A. Results of piG19, piG22 with p450 and FMO

- Checked the experiment with differing tryptophan levels, arabinose (inducer), Glucose (C -- repressor) on the pCon promoter
  - Everything seemed to be working as expected, but to make sure, we centrifuged them and froze them

in the -80C freezer

- Pelleting the cells allowed us to compare the color of the cells with respect to the color of the media (Note that some of the solution is actually more pink/red than others due to the production of the side product indirubin)

## B. Indican Extraction Research

- The main methods to indican extraction appear to be HPLC/MS and HPLC/ELSD
- ELSD (Evaporative light sensing detectors) --> identifies/analyzes any compound that has lower volatility than the mobile phase
- Some reference literature:
  - **Beta-glucosidase-catalyzed hydrolysis of indican from leaves of *Polygonum tinctorium*. [3]**



**Test Digest of piG15/16** Lane 1: ladder; Lane 2,3: piG15a & piG15b; Lane 4,5: piG16a & piG16b

([https://dueberlab.com/w/images/b/b8/Maugard\\_2002.pdf](https://dueberlab.com/w/images/b/b8/Maugard_2002.pdf))

- A new HPLC-ELSD method to quantify indican in *Polygonum tinctorium* L. and to evaluate beta-glucosidase hydrolysis of indican for indigo production. File:indican extraction 1.pdf
- Determination of indican, isatin, indirubin and indigotin in *Isatis indigotica* by liquid chromatography/electrospray ionization tandem mass spectrometry File:indican extraction 2.pdf
- Tissue and Intracellular Localization of Indican and the Purification and Characterization of Indican Synthase from Indigo Plants File:Plant Cell Physiol-2000-Minami-218-25.pdf
- This paper also extracted indican with the HPLC-ELSD method File:indican extraction 3.pdf

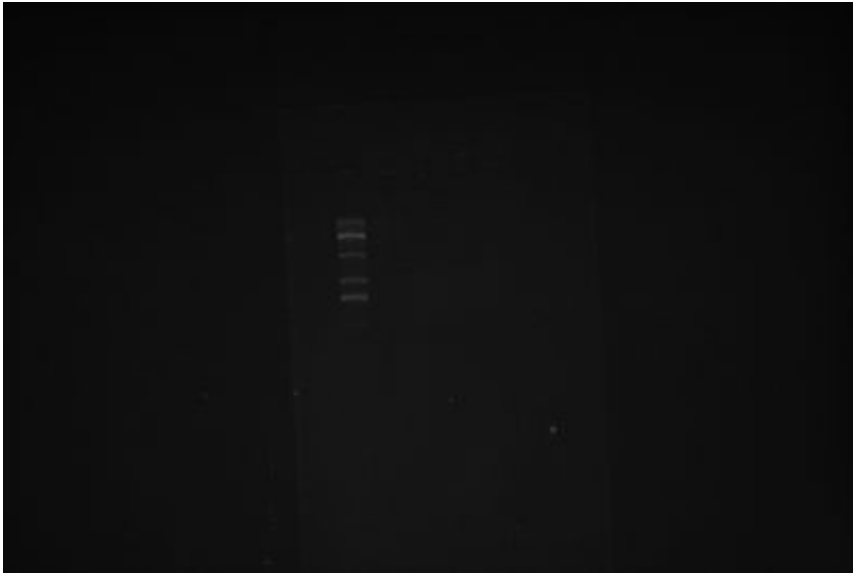
## C. Test Digest of piG15 and piG16

- Also, ran a test digest on piG15 and piG16

# 6/09/2013

## A. Concentrating piGEM15 and piGEM16

- Nanodropped the piGEM15/16 and reconcentrated them with the Zippy concentration kit with Bernardo
  - The best concentrations achieved for both piGEM15 and piGEM16 were around 30 ug/ml
  - We digested them again now that they were more concentrated, but didn't see any bands



## B. Design of RFP Tagged FMO/p450 experiment

- Confirmed plasmid GTs --> piG 18,19,20 on pBad promoter
- Indigo Genes with RFP tag: FMO(ZNR379) && p450 (ZNR380) on pCon promoter
- Variables: Tryptophan, Glucose, Arabinose



piGEM	FMO/p450	Tryptophan (H/L)	Glucose/Arabinose
19	FMO	H	G
19	FMO	H	A
19	FMO	L	G
19	FMO	L	A
19	p450	H	G
19	p450	H	A
19	p450	L	G
19	p450	L	A
19	N/A	H	G
19	N/A	H	A
19	N/A	L	G
19	N/A	L	A
N/A	p450	H	G
N/A	p450	H	A
N/A	p450	L	G
N/A	p450	L	A
N/A	FMO	H	G
N/A	FMO	H	A
N/A	FMO	L	G
N/A	FMO	L	A

### C. Transformation of piG19, 19/FMO, 19/p450, FMO, and p450

- piG19 on KanR, FMO and p450 on Amp
- Finished plating the transformations at 5:50 PM
  - 15 ul used on controls && 25 ul used on transformations

## 6/10/2013

### A. Picking colonies for Experiment

- Picked colonies in order to culture them with Hojae
  - Note: Separate colonies were used for each tube, so discrepancies might occur with the results

### B. Mini prep

- Helped Thomas mini-prep the pGG01-pGG96 to create our own stock of pGG templates
  - Eluted them in 30ul of water, and nano-drop concentration was around ~65 ng/ug

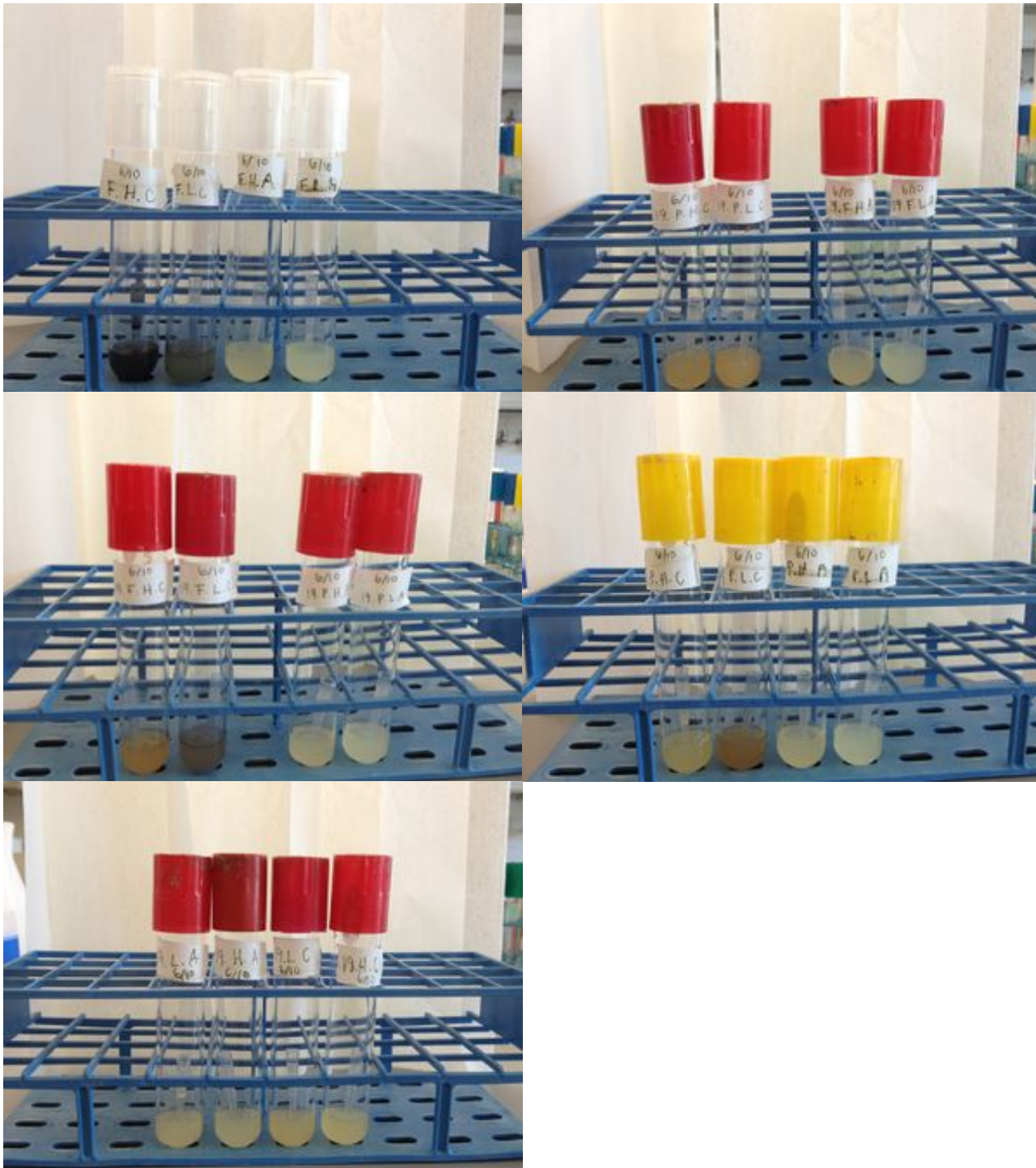
### C. Sequencing

- Sequenced piG15,21,23,pGG53,30,78

## 6/11/2013

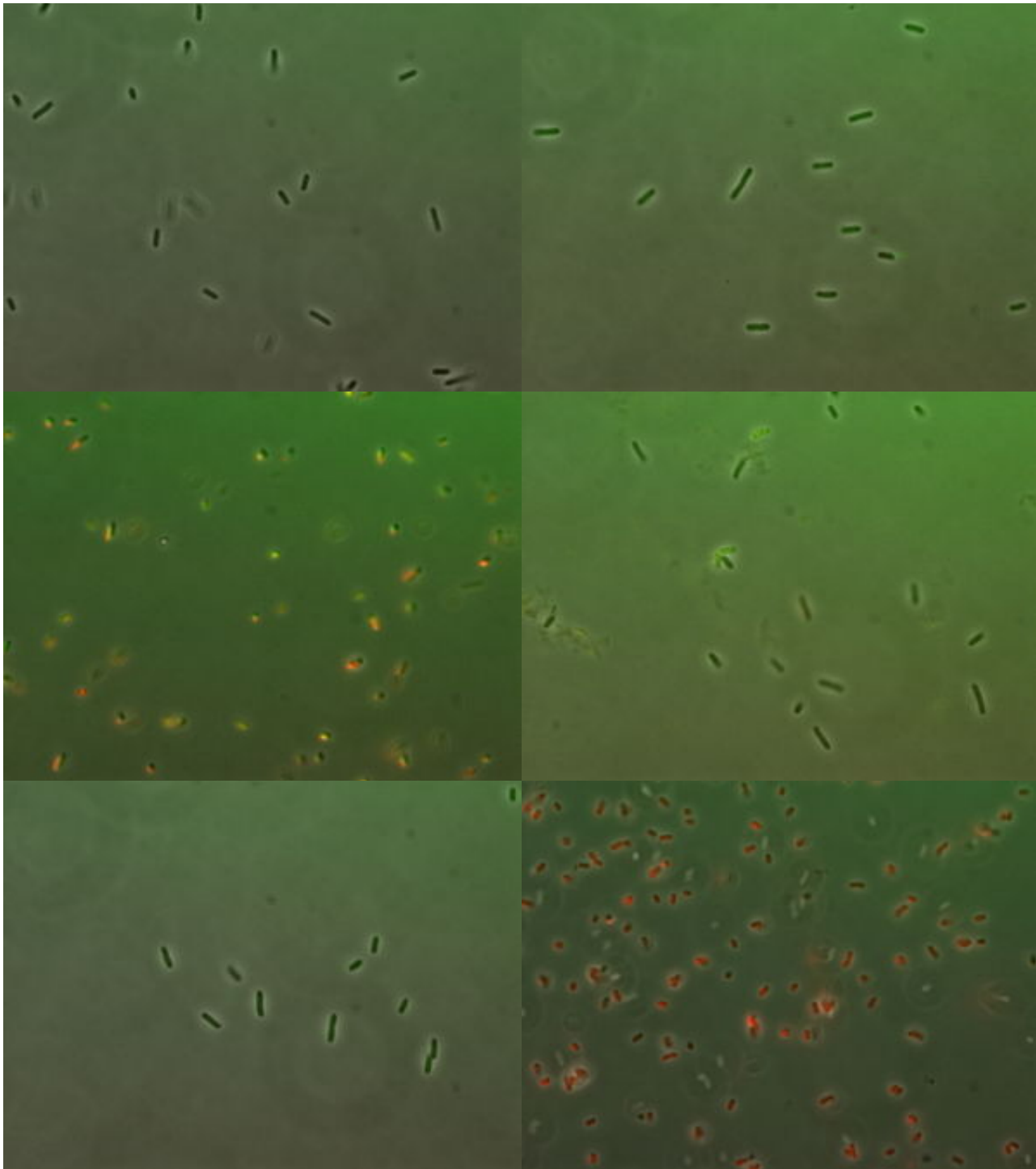
### A. Results of Experiment

- Looked at the results of the experiemnt from piG19+FMO/p450 with RFP tag experiment (pictures below)



## B. Microscopy of experiments

- Bernardo and I took photos of the cell cultures from the experiment (some photos below)



## C. Sequencing

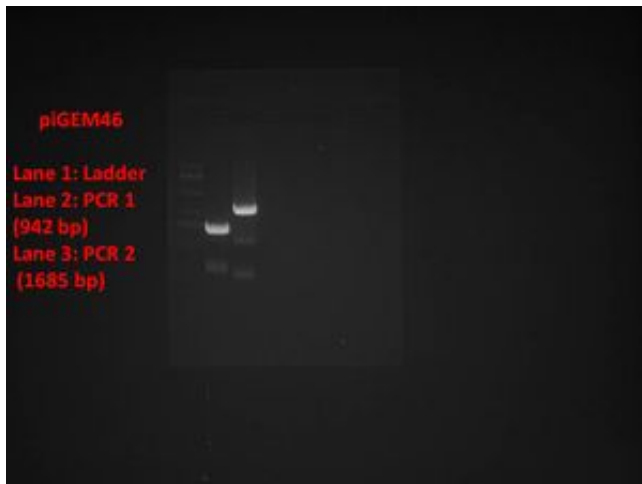
- Sequenced piG38-45

Name	Primer
piG38	BQ53
piG39	BM78
piG40	V38
piG41	V38
piG42	V38
piG43	V38
piG44	V38
piG45	V38

## 6/12/2013

### PCR/Purification/GG assembly

- Ran a PCR reaction for piG46 and ran a gel purification
- Expect to see gel lengths of 942 bp and 1685 bp



- After the gel purification was completed, the GG assembly for piG46 was run

## 6/13/2013

### A. Mini Prep/Test Digest of pTET promoter

- Completed miniprep of pTET promoter (piG14)
  - Nano-dropped concentration of pTET is around 40 ng/ul
- Ran a test digest with *bsa*I since this is a part
  - Expected band sizes to be around ~990 bp and ~1660 bp
  - the digestion seemed not to be sufficient with around 30 minutes, so left in the incubator for around ~2

hours for a complete digest



## B. Cassette Assembly

- Did cassette assemblies for GTs with the pTET promoter

## 6/14/2013

### A. Multi-Gene Cassette Assemblies/Transformations

- Ran multi-gene cassette assemblies with FMO + GT on the pglpT.rbs promoter
- After the multi-gene assemblies finished, transformed them into TG1 cells

## B. Project Re considerations

## 6/16/2013

### A. Colony picking

- Picked colonies for the multi-gene assemblies in a 24 well block

#### Layout of the well block

105b	107b	109b	
105a	107a	109a	
104b	106b	108b	110b
104a	106a	108a	110a

## B. Mini-prep of well block

- Mini-prep failed in blue lysis buffer step (too much shaking when there should have been only a couple

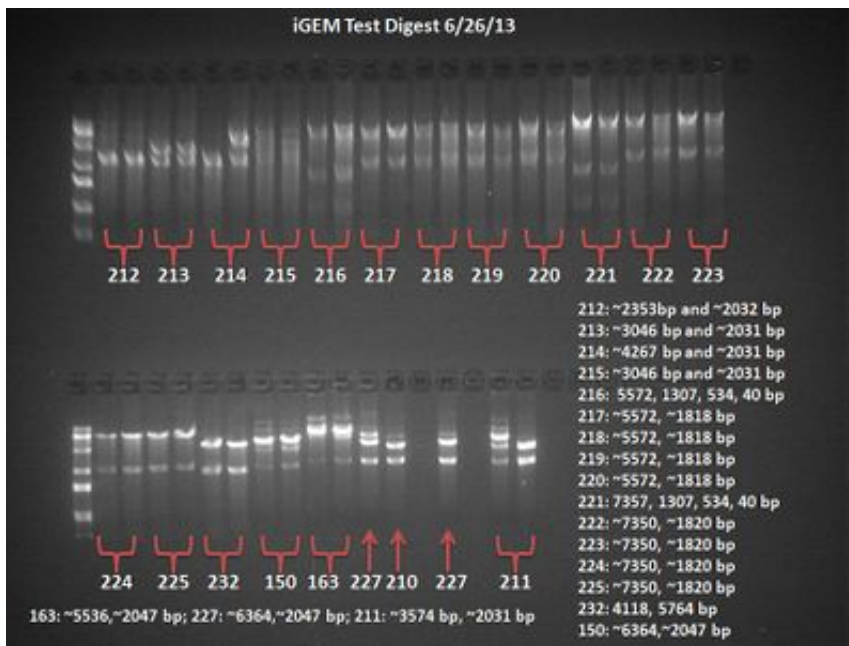
inverts)

- The test-digest for the mini-prep looked like there might have been genomic DNA present, so Thomas re-picked colonies for the multi-gene assemblies

### Layout of the well block

104a	104b	105a	105b	106a	106b
107a	107b	108a	108b	109a	110a
109b	110b	GFP	GFP	RFP	RFP

## 6/26/2013



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# July 2013

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The gaps in the notebook will be filled eventually with more detail (most likely it was just cloning days though)

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## 7/8/2013

### A. Design of Cell Lysate Experiment

- We realized the cassettes designed were on yeast promoters, so we designed some more cassettes so that this experiment can be carried out in E. Coli as well



Templates
pGG006
pGG078
pGG081
piGEMXIII240
pYGEMXIII001
pYGEMXIII002
pYGEMXIII003
pYGEMXIII005
pYGEMXIII006
pYGEMXIII007
pYGEMXIII010
pYGEMXIII015
pYGEMXIII016
pYGEMXIII017
pYGEMXIII034
pYGEMXIII043
pYGEMXIII044

pYGEMXIII061 (AmpR): pGG006, piGEMXIII240, pYGEMXIII044, pYGEMXIII005, pYGEMXIII006, pGG078, pGG081 using Bsal
pYGEMXIII062 (AmpR): pGG006, piGEMXIII240, pYGEMXIII010, pYGEMXIII005, pYGEMXIII006, pGG078, pGG081 using Bsal
pYGEMXIII063 (AmpR): pGG006, piGEMXIII240, pYGEMXIII007, pYGEMXIII005, pYGEMXIII002, pGG078, pGG081 using Bsal
pYGEMXIII064 (AmpR): pGG006, piGEMXIII240, pYGEMXIII016, pYGEMXIII005, pYGEMXIII006, pGG078, pGG081 using Bsal
pYGEMXIII065 (AmpR): pGG006, piGEMXIII240, pYGEMXIII043, pYGEMXIII005, pYGEMXIII006, pGG078, pGG081 using Bsal
pYGEMXIII066 (AmpR): pGG006, piGEMXIII240, pYGEMXIII017, pYGEMXIII005, pYGEMXIII006, pGG078, pGG081 using Bsal

Golden Gate assemblies
pYGEMXIII051 (AmpR): pGG006, piGEMXIII240, pYGEMXIII034, pYGEMXIII005, pYGEMXIII001, pGG078, pGG081 using Bsal
pYGEMXIII052 (AmpR): pGG006, piGEMXIII240, pYGEMXIII015, pYGEMXIII005, pYGEMXIII003, pGG078, pGG081 using Bsal
pYGEMXIII053 (AmpR): pGG006, piGEMXIII240, pYGEMXIII044, pYGEMXIII005, pYGEMXIII003, pGG078, pGG081 using Bsal
pYGEMXIII054 (AmpR): pGG006, piGEMXIII240, pYGEMXIII010, pYGEMXIII005, pYGEMXIII003, pGG078, pGG081 using Bsal
pYGEMXIII055 (AmpR): pGG006, piGEMXIII240, pYGEMXIII034, pYGEMXIII005, pYGEMXIII002, pGG078, pGG081 using Bsal
pYGEMXIII056 (AmpR): pGG006, piGEMXIII240, pYGEMXIII016, pYGEMXIII005, pYGEMXIII003, pGG078, pGG081 using Bsal
pYGEMXIII057 (AmpR): pGG006, piGEMXIII240, pYGEMXIII043, pYGEMXIII005, pYGEMXIII003, pGG078, pGG081 using Bsal
pYGEMXIII058 (AmpR): pGG006, piGEMXIII240, pYGEMXIII017, pYGEMXIII005, pYGEMXIII003, pGG078, pGG081 using Bsal
pYGEMXIII059 (AmpR): pGG006, piGEMXIII240, pYGEMXIII007, pYGEMXIII005, pYGEMXIII001, pGG078, pGG081 using Bsal
pYGEMXIII060 (AmpR): pGG006, piGEMXIII240, pYGEMXIII015, pYGEMXIII005, pYGEMXIII006, pGG078, pGG081 using Bsal

- Since these constructs are intended for E.Coli and E.Coli lacks Protein Kinase A (PKA), we will have our ligands always turned on by changing the serine (S) to E

### Intended Procedures with Cell Lysate Experiment

- Take 50 ul of one culture (sonicated) and 50ul of another culture (sonicated) and add X-Gal to observe color change
- Alternatively, we will be using the cassettes with yeast promoters to build multi-genes in order to carry out the experiment in yeast
- These constructs are pYGEMXIII045-50

Templates
pGG090
pYGEMXIII011
pYGEMXIII012
pYGEMXIII020
pYGEMXIII021
pYGEMXIII022
pYGEMXIII023
pYGEMXIII032
pYGEMXIII033

Golden Gate assemblies
pYGEMXIII045 (KanR): pYGEMXIII020, pYGEMXIII032, pGG090 using BsmBI
pYGEMXIII046 (KanR): pYGEMXIII011, pYGEMXIII032, pGG090 using BsmBI
pYGEMXIII047 (KanR): pYGEMXIII021, pYGEMXIII032, pGG090 using BsmBI
pYGEMXIII048 (KanR): pYGEMXIII022, pYGEMXIII033, pGG090 using BsmBI
pYGEMXIII049 (KanR): pYGEMXIII012, pYGEMXIII033, pGG090 using BsmBI
pYGEMXIII050 (KanR): pYGEMXIII023, pYGEMXIII033, pGG090 using BsmBI

## B. Cloning and preparation of parts/cassettes necessary for yeast integration

- Looked like some of the cassettes failed to transform or GG correctly, so re-assembled them (pYG11-19)

7/9/2013

- Picked Colonies for

7/10/2013

7/11/2013

7/12/2013

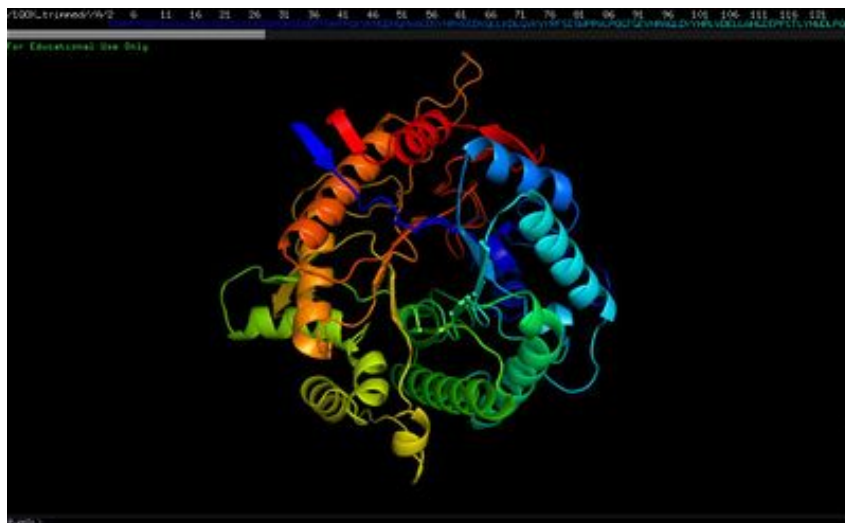
7/14/2013

7/15/2013

Beta-Glucosidase (BACILLUS CIRCULANS) PDB Accession/Download: 1QOX [1]  
(<http://www.ebi.ac.uk/pdbe-srv/view/entry/1qox/summary>)

### B-Glu (Circulans)

- TIM-barrel structure



- quaternary structure: octamer [2] (<http://www.sciencedirect.com/science/article/pii/S1047847799942065>)
- Active Site residues: ARG(77), HIS(121), ASN(165), GLU(166), CYS(169), ASN(294), TYR(296), GLU(355), TRP(402)
  - Basically, these residues are inside of the beta barrel

## Current Split Sites

- Amino acids: 42,91,132,180,272,326,366,425

**7/16/2013**

## Continued Split-Proteins Research

-an 8-fold barrel enzyme: yeast phosphoribosyltranferase isomerase

**7/17/2013**

**7/18/2013**

**7/19/2013**

**7/20/2013**

-Finalized Cut sites for First round of Split Glu Testing

## First Round of Cut Sites From Top View

- 1) 258-259 (EY) deletion
- 2) 322-329 (KTDIGWEI) deletion
- 3) 228-231 (YRRT) deletion
- 4) 361-366 (DGLSLD) deletion
- 5) 272-276 (YKPPIV) deletion
- 6) 178-185 (VHAPGNK) deletion
- 7) 424-426 (YDT) deletion
- 8) Split after 431 (P)
- 9) Split after 133 (W)
- 10) 44-61 (VKNGDNGNVACDSYHRV) deletion

## From Bottom View

- 11) Split after 393 (I)
- 12) Split after 348 (N)
- 13) Split after 286 (P)
- 14) Split after 211 (G)
- 15) Split after 156 (GGK)
- 16) Split after 111 (ANG)
- 17) Split after 71 (DLG)

## 7/21/2013

- Set up some assemblies for Split Lac

## 7/22/2013

- Ran split lactamase assay with failed results

## 7/23/2013

- Attempted to troubleshoot the split-lactamase assay

[Place picture of test digest from pYGEM117-129]

## 7/24/2013

- Put info about the research about the split GFP done
- Received Oligos for the Split B-Glus (set up half of the PCR reactions [about 20])
- Discovered some problems in the part design for the split lactamase
  - One of the split lactamase parts had MWP protein, while another had some ~20 residues missing. This, however, still does not explain why the split-lactamase assay gave off false positives for each individual fragment of the lactamase

## 7/25/2013

[place pictures of designs of cassettes for split GFP]

- Received oligos for the Split GFP fragments today
  - Subsequently, PCRed, Gel purified, and set up the assemblies for these parts

## 7/26/2013

- During the meeting, we decided to put Split B-Glu put on hold indefinitely
- In addition, using split-lactamase as an assay is also put on hold indefinitely

## 7/30/2013

### A. Chimeric GTs

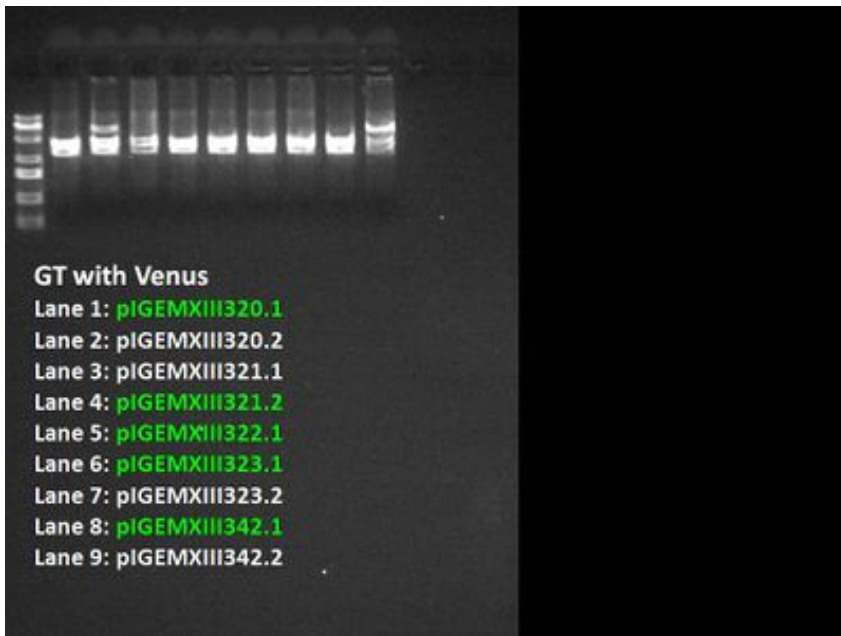
- A possible method to change substrate specificity for GTs is to swap/combine domains from different UDP-

## GTs

- Create around 10~20 different chimeric GTs, and use a color-metric screen assay (with less blue colonies having GTs that take in indoxyl as its substrate)

### B. Venus Tagged GTs

- Picked colonies for GTs tagged with Venus
- Thomas checked colonies for expression under the microscope, and did find that the GTs had low levels of expression (this lead to Ramya discovering that the mutant GTs were in fact designed incorrectly). Our previous conclusion that GTs were not expressing are not true



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

Ran Split GFP Assay, and cloning parts from scratch again for split lactamase

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

- Decided to abandon the split GFP assay (due to problems with parts + takes too long to see results→ what we are aiming for are results within minutes, not hours)
- Started Indigo Titer Conditions (different tryptophan levels, salts, PHs)
- Will run split lactamase assay again in yeast. Seems that E. coli in general tend to exhibit a false positive with nitrocefin

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

- After running tests with split lactamase in yeast, we got false positives again (seems to be a kinetic issue within the cells)
- Continued Indigo Titer experiments

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

- Ran more indigo titer experiments. Tried varying levels of tryptophan. Seems to be the case that adding more tryptophan titers the concentration of indigo
- Purified the FMO enzyme this week, to begin experiments on enzyme kinetic assays (Glucosidase already purified)

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

- Ran some initial kinetic assays with Glucoside and pure indican dissolved in ddH2O
- Finalizing indigo titer experiments to see the maximum amount of indigo production I can achieve

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

- Finalized data for indigo titer experiments, ran kinetic tests for indole, ONPG, PNPG

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

- Re-ran some kinetic assays for FMO and Glu with ONPG, since it seemed like the enzyme never saturated or the substrate concentration was too low
- Preparing and creating slides for powerpoint

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

- Had first public presentation and coded stuff for wiki