Journal - Pheophorbrides

Project: Journals

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SUNDAY, 4/28/2019

Micha, Chris, and Mike were enjoying the day off.

WEDNESDAY, 5/1/2019

First day of work! Subunit still unassigned.
Project discussion.

WEEK 1 SUMMARY

Whole team worked on literature review and talked to Dr. LV about the feasibility of a biofilm matrix approach to extracting canola from oil.

MONDAY, 5/6/2019

Subunits still unassigned.
Looked at various chlorophyll binding proteins (CP43, CP47, CP43', CP26, CP29, CB2A_SPIOL), and chlorophyll binding domains, biofilm matrix binding. Looked at structures of CP43 and CP47 which were membrane proteins.
Read papers regarding chlorophyll isolation: mainly using ethanol, acetone or other organic solvents. Looked at potential repurposing applications for chlorophyll to be isolated.

TUESDAY, 5/7/2019

Subunits assigned.
Team had discussion about whether *E.coli or B. subtilis* or Yeast should be used for protein secretion. Team also looked at whether we should degrade all chlorophyll to chlorophyll a or to pheophorbide a, and read papers about which enzymes to use int he pathway. Found that pheophorbide a is more expensive and used in anti-cancer research so team decided to focus on pheophorbide a.

WEDNESDAY, 5/8/2019

Team is still debating on whether proteins should be secreted or isolated from chassis. Team looked for papers to figure out how to obtain compound profiling before and after the enzyme treatment, because chances are there are more compounds other than pheophorbide in the final solution. Thought spectrophotometry would be a good way of measure but Chlorophyll a and pheophytin absorbances overlap. Team realized that chlorophyllide a was overlooked from the pathway, so the chlorophyll degradation pathway was expanded and chlorophyllase added.
Team needs to find another way to determine relative concentrations/contents of the isolated solution.

THURSDAY, 5/9/2019

Team still deciding between *B. subtilis* or *E. coli*. Team had a mini crisis: reverse reactions were not accounted for. Team decided not to provide chlorophyll a synthase and chlorophyllide a oxygenase to prevent reverse reactions from ocurring. Team gathered the protein sequences of Chlorophyll b reductase, 7-hydroxymethyl chlorophyll a reductase, chlorophyllase, and pheophytinase.

FRIDAY, 5/10/2019

Team decided that a minimum of four enzymes would be used, but unsure with which ones. Team will be purifying the proteins instead of secreting.

WEEK 2 SUMMARY

This week we were finally divided into our subgroups. We were assigned to extracting the chlorophyll from the binding proteins. We looked at the chlorophyll degradation pathway and decided to concentrate our chlorophyll derivatives to pheophorbide a. The team looked at ways to obtain the compound profiles using spectrophotometry. We also decided to pursue protein purification instead of secretion.

MONDAY, 5/13/2019

Team identified the chlorophyll degradation pathway to pheophorbide A. Decided to work on a 2-step process, with four enzymes to facilitate pathway:

1. Chlorophyllase (Chlorophyll a -> pheophorbide a)
2. Pheophytinase (Pheophytin a -> pheophorbide a)
3. Chlorophyll b reductase
4. 7-Hydroxymethyl chlorophyll a reductase

We also decided we would need to supply Ferredoxin and NADPH to our batches. We found a chlorophyllase BioBrick in the registry, then did more research on the marketability of pheophorbide a.

Chris took the day off.

TUESDAY, 5/14/2019

Found that pheophorbide a market was more concentrated to research groups, particularly in photodynamic therapy (PDT) for cancer and wrinkle prevention. Team did some research on the activation mechanism of photosensitizers, looked at some patents involving use of pheophorbide a in therapeutics.

Chris took the day off.

WEDNESDAY, 5/15/2019

Team looked at periplasmic extraction and chose chloroform extraction over cold osmotic shock (cleaner and efficient). We also made a timeline for our experiment.

1. Sf-GFP registry transform + study secretion
    - BL21 *E.coli* + sf-GFP + pET
2. Order Parts
3. Digest & ligate parts
4. Transform separate enzyme parts (4)
5. Protein Extraction + Purification
    - Extraction : secretion
    - Purification: His Tag
6. Optimize sf-GFP secretion
    - DNA level?
7. Enzyme Assays (Individual)
8. Total enzyme trial
9. Pheophorbide A isolation
    - HPLC
10. Anti-cancer treatment potential
11. Future Directions and scalability.

THURSDAY, 5/16/2019

The team had a crisis because a wiki pathway page showed that chlorophyll B had an Mg-dechelatase. Team left with the question 'Does Chlorophyll B get degraded into pheophytin B naturally?'.

Mike took the day off.

FRIDAY, 5/17/2019

Team discovered that in higher plants, chlorophyll B had to be converted into chlorophyllide B first or 7-hydroxymethyl chlorophyll A or be exposed to low pH for it to degrade. Found that Arabidopsis SGR and SGRL sequences could not be expressed in *E. coli* Decided to use CrSGR sequence instead and use cell lysate instead if protein purification does not work out like in paper. We looked at alternative uses of other chlorophyll catabolites (Anti-fungal, insecticide, acne treatment).
Team had a crisis because pathway would change with the new CrSGR sequence since it does not recognize chlorophyllide a.

Team unsure about periplasmic secretion because of auto-secretion sfGFP method would secrete proteins automatically and be a method of protein quantification.

WEEK 3 SUMMARY

This week we decided on a pathway to stick to and looked at more papers supporting enzyme activities. Based on these we chose to use *E. coli* as our chassis since Chlorophyllase, Pheophytinase, Chlorophyll b reductase, and 7-Hydroxymethyl chlorophyll a reductase have been expressed in them. We started aggregating parts for our gene circuit using parts from the registry and sequences in the papers. We also found more uses for pheophorbide a in terms of photodynamic therapy.

MONDAY, 5/20/2019

Victoria Day (Everyone Away)

TUESDAY, 5/21/2019

Today, we decided to focus only on the outer pathway (chlB -> chlA -> Pheophitin A) and disregard chlorophyllase. We have spent the majority of the day looking for sequences of the needed enzymes (Particularly Mg-Dechelatase and Pheophytinase) that have been successfully expressed in *E. coli.* We have figured out the general structure of our G-block. We are considering using a periplasmic signal peptide (DsbAss), which we need to find the sequence for.

WEDNESDAY, 5/22/2019

We decided not to use sf-GFP for secretion in E. coli due to its limited characterization and a professor contact that did not observe the auto-secretion properties in her experiments. We found the DsBA signal sequence and pheophytinase sequence. We decided to use E.coli (BL21) as our chassis becase the sequences have been expressed in it. We will be using psB1C3 as our vector and T7 promoter. We also found data/papers for Sebastian to refer to in kinetic modelling.

THURSDAY, 5/23/2019

Looked at different uses of photosensitizers and other uses of pheophorbide a. We also looked at how HPLC worked, solvents and absorbances of the compounds of interest for it. We also looked at reaction buffers needed for the enzymes.

Parts were still being finalized but at proposal at the moment are the following:

BL21 *E. coli* cells
- Has the T7 RNA polymerase integrated into the genome under the control of IPTG induction

Part Design - gBlock:
- HO - DNA buffer - **Restriction sites** (x3) - T7 promoter - RBS - **DsbAss** signal peptide for periplasm localization - Enzyme cDNA (4 different things) - Spacer (Ala x **12 residues**) - His tag (6 residues) - bidirectional terminator - Restriction sites (x3) - DNA buffer - OH

Vector:
- pSB1C3

FRIDAY, 5/24/2019

Today we spent most of the day continuing to construct the parts needed to complete our objective. We have decided to model a few different options for spacers between our GOI and the His-tag in each of our constructs, to inform the direction we take with our parts. Experimental planning for individual protein assays was also conducted. We also searched for more uses for Pheophorbide. Protein sequences were also BLASTed.

WEEK 4 SUMMARY

We realigned our pathway and decided on using DsbA as our signal sequence. Team also figured the general G-block structure, along with our parts. Additionally, we gathered literature on reaction buffers, and solvents and absorbances required for HPLC. Protein sequences were blasted and constructs were modified.

MONDAY, 5/27/2019

Skype call with Dr. Randall Weselake. Chris and Mike had a meeting with the education group and worked on the Webber Presentation. Micha reached out to PDT experts. Mike did some spacer modelling on pymol.

TUESDAY, 5/28/2019

Team organized the journal entries. Codon optimization of all the enzymes was done and implemented into the final part design. Made protein models in pymol to be used by the modeling team in GROMACS.

WEDNESDAY, 5/29/2019

The team worked with Andrew in protein modelling. Team reorganized/updated the timeline and cleared the things to research page on onenote. Model worked after adding more spacers. Contacted Dr. Lisa Gieg to ask for HPLC help in the future.

THURSDAY, 5/30/2019

Whole subgroup went to Webber. Chris and Mike presented the constructs to Jake and got his approval. Chris tried to reduce the IDT score to 0. Mike worked on the assessment package for Innovates Calgary, and codon deoptimizing the ends (his tag & proteolitic site). Micha searched for papers relating to the enzyme assays. We will be using **Lichtenthaler and Wellburn**'s equations to quantify chlorophyll a and/or b.

1. Chlorophyll b reductase
    - Put in CBR proteins in chl a + b soup.
    - Calculate the drop in chl b.
        - Assume the drop in chl b is the activity of CBR.
        - https://www.researchgate.net/post/Chlorophyll_and_cartonoid_quantification
2. 7 Hydroxymethyl Chlorophyll A Reductase
    - Use **new** chl a + b solution from spinach.
    - Measure with spec.
    - Equation: quantify chl a.
    - Add 7-HMCR in the **new** soup.

FRIDAY, 5/31/2019

Mike trained Dry lab. Chris was a downer. Chris attempted to decrease the complexity score below 7 (again). Micha did more research on enzyme assays.

WEEK 5 SUMMARY

This week the team worked on more pymol modelling, reducing the IDT score, organizing OneNote, and working on enzyme assays. Jake also gave his tentative approval (waiting for IDT score to decrease).

MONDAY, 6/3/2019

-Chris and Micha finished the fridge stock inventory.

-Chris reached out to IDT regarding the sequence.
-Mike worked with JD and Andrew to build the TelusSpark project and chop some wood.
-Team also had an HP meeting which mostly involved planning the philosophical discussion event.
-Phone call with Alyssa Lam from the LSI to talk about the event.
-Micha sent a follow-up email to Dr. Moore from U of A.

TUESDAY, 6/4/2019

Team met with Dr. Gijs from Genome Alberta and had a phone call with Dr. Veronique who is involved with oil processing/manufacturing. Chris and Mike had a *box* meeting to discuss the Telus Spark project. Micha talked to Dr. L.V. about what she missed and what the team is up to presently. Team then had an outreach meeting.

WEDNESDAY, 6/5/2019

Team finalized the parts and ordered them. Chris was hurt so he lashed out at Micha. Mike fixed an ancient laboratory laptop. Chris and Mike had a box meeting and an education meeting. Team quantified how much Ferredoxin and NADPH are needed to be ordered. Team also asked Deirdre for Calcium Carbonate but she had none and got methanol. Team started working on a proper protocol document.

THURSDAY, 6/6/2019

-Chris made P1 and P2 buffers.
-Micha and Mike worked on plasmid miniprep and restriction digests.
-Team then looked at verifying 7-HMCAR activity.
- Dr. Mayi met Dr. Lisa Gieg, who told her that we are welcome to drop by and use HPLC machine but would be better to have our compounds ready in tubes.

FRIDAY, 6/7/2019

-Team met with farmer Craig.
-Cleaned up the computer area and got all the devices back together.
-Chris took a photoshoot of the wonderful Sarah Walker.
-Helped women in science achieve greatness.
-We had a wet lab meeting, everyone got on the same page.
- We got BL21 cells and the parts that came in went to the wrong campus. Came up with the pageant idea -> Sarah Walker is our top model. Her pdb is on fire. Mike looked at some computer vision stuff, Chris looked at some HPLC stuff.

WEEK 6 SUMMARY

Team had an HP-heavy week, reaching out/talking to Dr. Moore, LSI hub, Dr. Gijs from Genome Alberta, Box meeting for Telus Spark. Parts needed were finalized and ordered. Team started doing lab work (plasmid minipreprs and restriction digests). Team got the BL21 cells donated by Dr. Mayi's friend.

MONDAY, 6/10/2019

- Make overnight cultures of BL21 for chemically competent cells
- Clone in our parts (2, 4) into pSB1C3 using EcoRI and Pst1 (Digestion, Ligation, Transformation into DH5alpha)
    - Part 1 is being done by the Extraction subgroup since we are using the same part 1 (Promoter)

TUESDAY, 6/11/2019

- Make glycerol stock for BL21 cells
- Make the BL21 cells chemically competent
- The transformation plates did not yield any colonies, thus we are replating the ligations, redoing the digestion + ligation, and letting those initial plates continue trying to grow

- Clone in our parts (2, 4) into pSB1C3 using EcoRI and Pst1 (Digestion, Ligation, Transformation into DH5alpha)

WEDNESDAY, 6/12/2019

- Tested our chemically competent BL21 (DE3) cells and DH5-a cells using RFP construct pSB1C3.
-Redoing transformations from June 11, 2019 were redone because they did not work.
-New digestions and ligations were made, with positive controls added.
-Chris made linearized backbone

THURSDAY, 6/13/2019

- cPCR parts colonies -> gel -> found 3 colonies and 4 colonies that worked for End Sequence and DsbA, respectively
-Control plates and ES transformations grew.
-Based on the BL21 plates, the BL21 stock was not very competent.
-Overnight from June 12, 2019 colonies were made.
-New chemically competent BL21 cells were made.
-Digestions and ligations were made for the four new parts were made and transformed.
-Parts were also rehydrated.

FRIDAY, 6/14/2019

-Ligations from June 13 were transformed.
-BL21 cells were retested for chemical competency using RFP test kit from iGEM.
-Overnights for colonies from June 12 (done on June 16) were made.
-7-HCAR and PPH were retransformed as a control.
-ES showed bands, DsbA A showed no bands, and DsbA C showed smears.

WEEK 2 SUMMARY

Team made a stock of chemically competent DH5-alpha cells which were found to not be very competent. Another batch of chemically competent cells were created. Our subgroup digested and ligated our 4 enzyme parts.

MONDAY, 6/17/2019

- Miniprepped the (21) overnights containing the pSB1C3-ES and pSB1C3-DsbA parts from June 16, 2019.
-Sent ES, DsBA samples for sequencing.
-June 14, 2019 transformations were cPCRd.
-Glycerol stocks of miniprepped overnights were made.

TUESDAY, 6/18/2019

- Today Christian spent the whole day preparing for our faculty talk presentation
- Mike helped do some cPCR with Sara "Life of the Party Far"
    * redid some the parts from the previous cPCR with better gel
    * Looks like we got some dope bands yo
- Mike is also redigest/ligating HCAR and CBR since they did not bode well on the cPCR
- Mike also dropped some dankass memes on the "Important Documents" page

Micha dropped by to have the phone call with Dr. Moore.

WEDNESDAY, 6/19/2019

-Mike transformed CBR and HCAR
-Sent PPH and HCAR for sequencing
-SGR transformed too

Micha took the day off.

THURSDAY, 6/20/2019

-Sequencing results from ES, DsBA sent June 17, 2019 all came negative
-PSB1C3 + SGR had growth.
-PSBIC3 + HCAR had no growth.
-pSB1C3+ CBR had very minimal growth.
-RFP plate that Mike fucked up yesterday before plating grew quite a lot.
-Sequencing for PPH came back positive.
-DSBA and ES were redigested and ligated at a 2:1 and 3:1 ratio of insert: 50ng pSB1C3.
-CBR was retransformed, and replated from June 19, 2019.

Micha took the day off.

FRIDAY, 6/21/2019

-HMCAR might be okay in sequence. There are gaps though.
-ES DSBA
-ES and DSBA ligations from June 20, 2019 were transformed.
-CBR and HCAR transformations were redone.
-SGR and CBR were cPCRd, alone with the older ES and DsBA.

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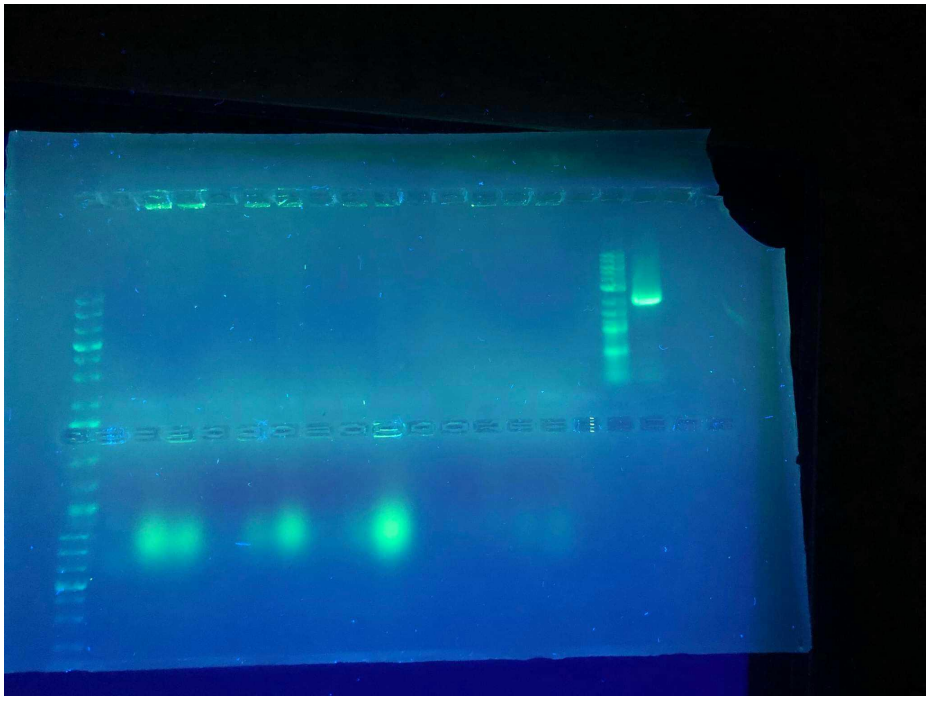

Figure 1. Mike bit the gel. Ladder (1 kb plus - NEB)

CBR_SGR.jpg

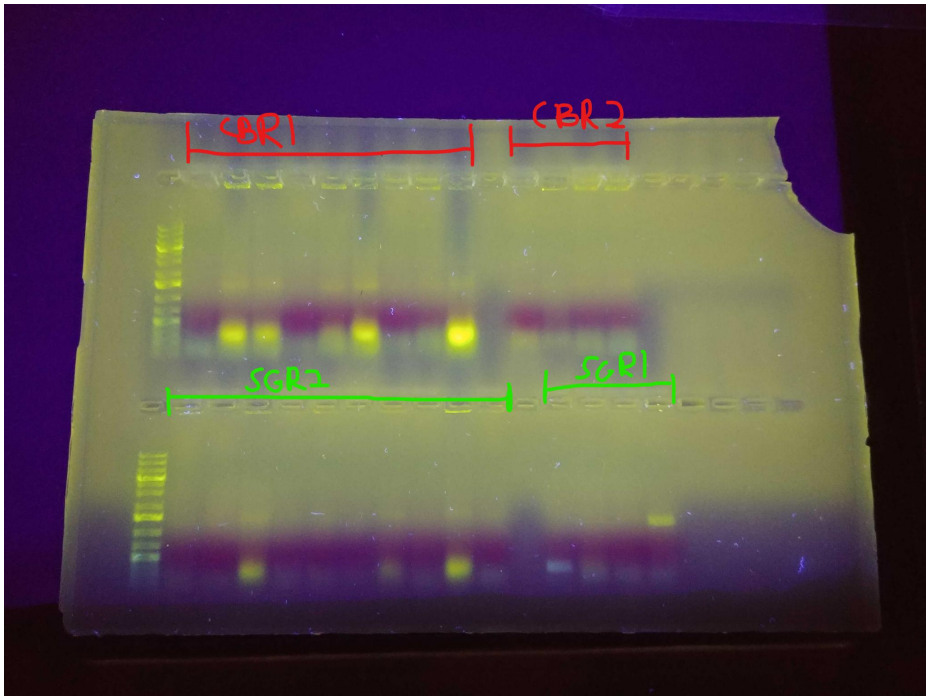

Figure 1. 1% Agarose gel of pSB1C3-CBR and pSB1C3-SGR cPCR reactions of CBR and SGR gBlocks ran at 100V for 30 min. cPCR reactions used VR and VF2 primers and proceeded as per the "colony PCR" protocol. pSB1C3-CBR and pSB1C3-SGR ligation products (#AMOUNTOFDNA) that were ligated in a 1:1 (insert:backbone) ratio were transformed into chemically competent DH5α cells as per the "Bacterial Transformation" protocol. Top Row: Ladder - NEB 1kb plus | Lanes 2-9: pSB1C3-CBR | Lanes 11-14: pSB1C3-CBR. Bottom Row: Ladder - NEB 1kb plus | Lanes 2-10: pSB1C3-SGR | Lanes 12-15: pSB1C3-SGR.

DsBA_ES.jpg

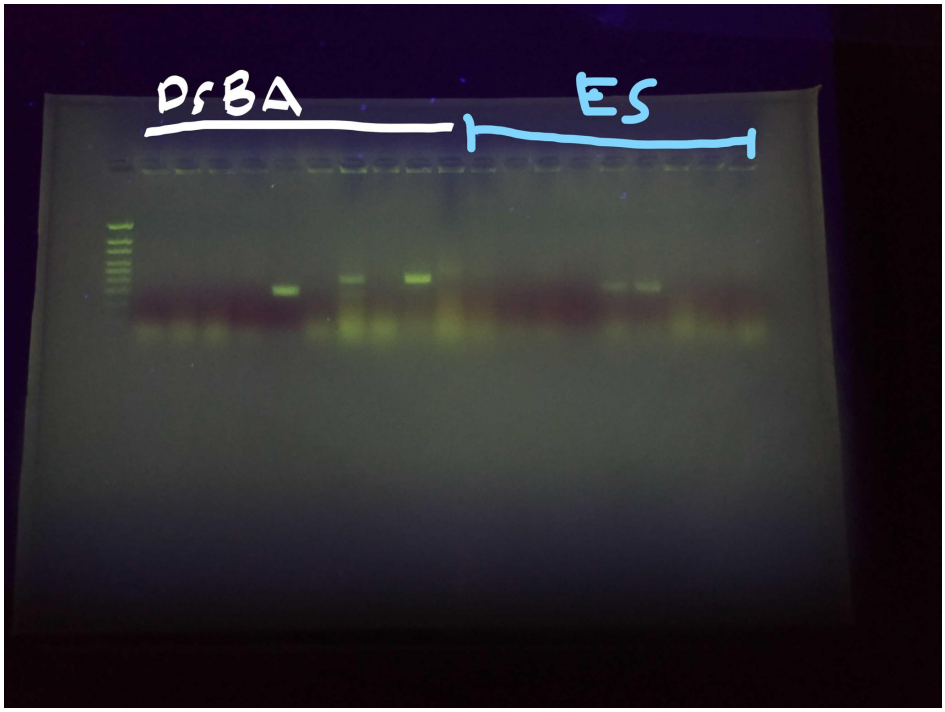

Figure 2. 2% Agarose gel of pSB1C3-DsBA and pSB1C3-ES cPCR reactions of DsBA and ES gBlocks ran at 100V for 30 min. cPCR reactions used VR and VF2 primers and proceeded as per the "colony PCR" protocol. pSB1C3-DsBA and pSB1C3-ES ligation products (#AMOUNTOFDNA) that were ligated in a 3:1 (insert:backbone) ratio were transformed into chemically competent DH5α cells as per the "Bacterial Transformation" protocol. Ladder - NEB 1kb plus | Lanes 2-10: pSB1C3-DsBA | Lanes 11-14: pSB1C3-ES.

WEEK 3 SUMMARY 17-21

This week our sequencing results for PPH and HCAR in pSB1C3 part came back positive. More digests, ligations, and transformations for DsBA and ES signal peptides in 2:1 and 3:1 vector: insert ratios were made. The team also had a phone call with Dr. Ronald Moore, a doctor and photobiology expert from the University of Alberta to discuss pheophorbide and photodynamic therapy.

MONDAY, 6/24/2019

-cPCRs did not work and transformations did not grow.
-SGR and CBR (1:1), ES, DsbA (3:1) were redigested and ligated using EcoRI-HF and PstI, pSB1C3.

TUESDAY, 6/25/2019

-Transformed ES, DSBA, SGR, CBR.
-Golden Gate was executed for the first time using gel purified PCR products (CBR, HCAR, PPH).
-SGR needed to PCRd with Kappa BioBasic HF instead of NEB Taq, and gel purified.
-Serial dilutions of chlorophyll stock (extraction) were done.

Digests and ligations, and transformations for SGR and CBR 1:1, ES 1:1, and DsbA 3:1 using EcoRI-HF and PstI and pSB1C3 plasmid were made. A Golden Gate reaction using gel-purified CBR, HCAR, and PPH PCR products was done. Chlorophyll extract stocks were also serially diluted. The team also started to incorporate DMSO in PCR reactions.

WEDNESDAY, 6/26/2019

-CanolaPalooza happened

THURSDAY, 6/27/2019

-Transformations from Tuesday showed colonies

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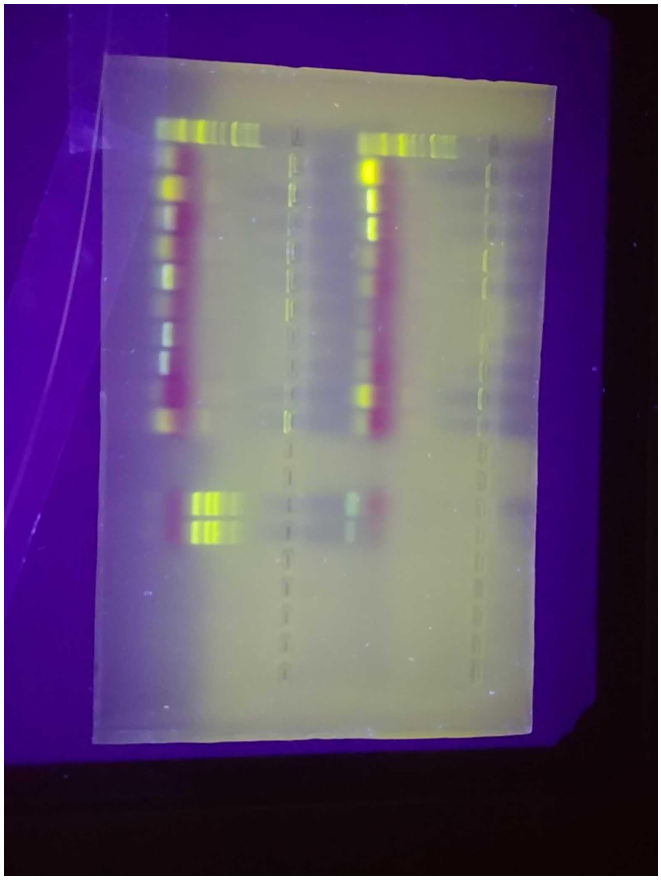

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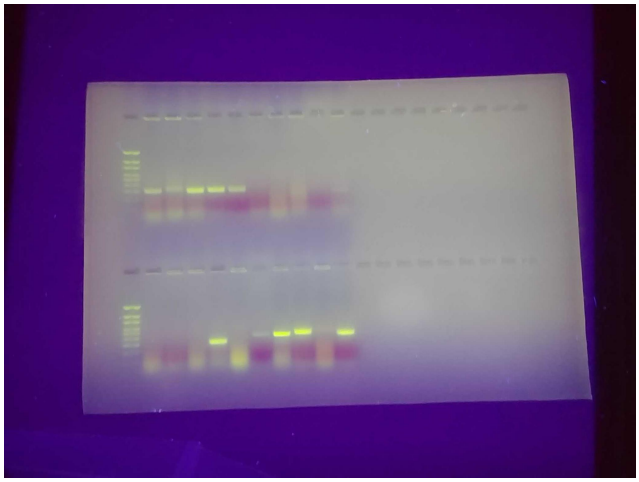

FRIDAY, 6/28/2019

-cPCR the SGR masterplate from June 27/19. Micha forgot to cPCR CBR
-Did PCR of the SGR with DMSO-- brand was very bright
-Chris did minipreps ES and DSBA
-Tested the different ladders: Invitrogen 1kb+ with less contents not as good as the one with more volume

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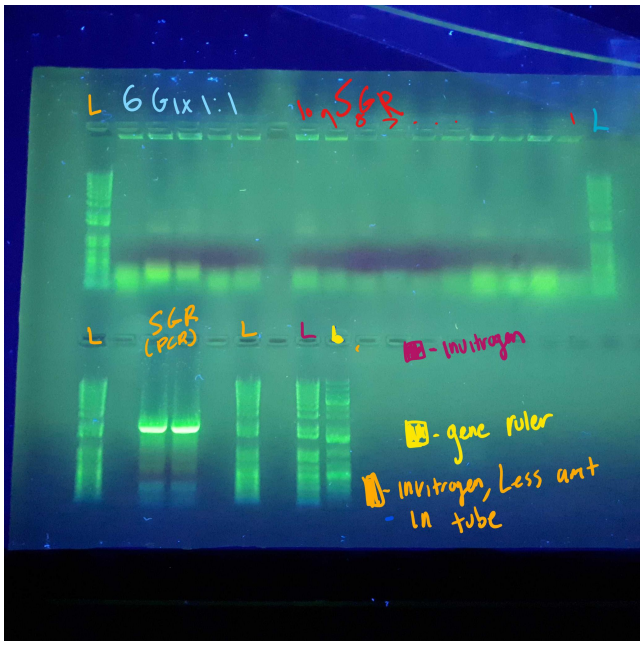


Figure 3. 1% Agarose gel of pSB1C3-SGR cPCR reactions and SGR gBlocks ran at 100V for 30 min. cPCR reactions used VR and VF2 primers and proceeded as per the "colony PCR" protocol. pSB1C3-SGR ligation products (#AMOUNTOFDNA) that were ligated in a 1:1 (insert:backbone) ratio were transformed into chemically competent DH5α cells as per the "Bacterial Transformation" protocol. Also shown, SGR gBlock which has been PCR amplified as per the "PCR" protocol. This gel also ran several ladders to test the efficacy of the ladder stocks in our lab. Top Row: Ladder - NEB 1kb plus | Lanes 7-16: pSB1C3-SGR | Lane 17: Ladder - NEB 1kb plus. Bottom Row: Ladder - NEB 1kb plus| Lanes 3-4: PCR SGR | Lane 6: Ladder - Invitrogen 1 kb plus | Lane 8: Invitrogen 1kb plus | Lane 9: Gene ruler 1 kb plus.

ES DsbA DC.jpeg

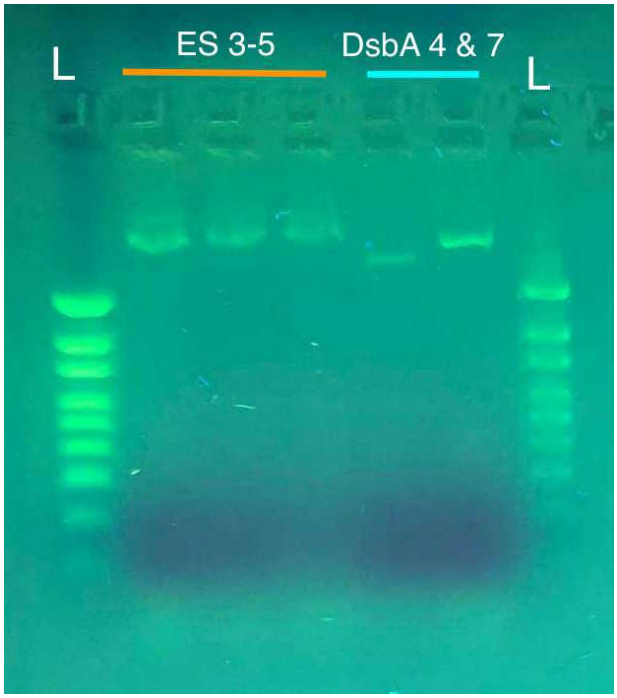


Figure 4. 2% agarose gel confirmation of digested ES and DsBA gBlocks ran at 100V for 30 min. Both constructs were cut with Eco-RI and SpeI restriction enzymes as per the "Restriction Digest Confirmation" protocol. Expected lengths of bands are 300 bp for digested ES and 119 for digested DsBA Ladder - NEB 100-1500 bp | Lanes 2-4: digested ES | Lanes 5-6: digested DsBA | Lane 7: Ladder - NEB 100-1500 bp.

WEEK 4 SUMMARY 24-28

This week we went to Canolapalooza where we got to do a wealth of HP work. We got to meet Ward Toma and Dr. Veronique Barthet IN THE FLESH. This week we continued to dig-lig-transform our parts. We also attempted to do Golden Gate cloning for the first time. We also aided the Gixie chicks in making serial chlorophyll dilutions to aid in their characterization of chlorophyll 'n shit.

MONDAY, 7/1/2019

-Canada Day!
-Team took the day off.

canada flag.jpg

TUESDAY, 7/2/2019

-Redid the digest for ES and DSBA
-GG assembly for all parts
-Ran cPCR of CBR on gel which was forgotten by Micha from June 28, 2019.
-Gel purified SGR (pcr product)
-Redid the digest confirmations because the gel was shaken up a bit.
-Made overnights for SGR 10 (10.1, 10.2, 10.3) and SGR 2 (2.1, 2.2) but Micha accidentally made 10.4 and only one of 2.

CBR CPCR.jpg

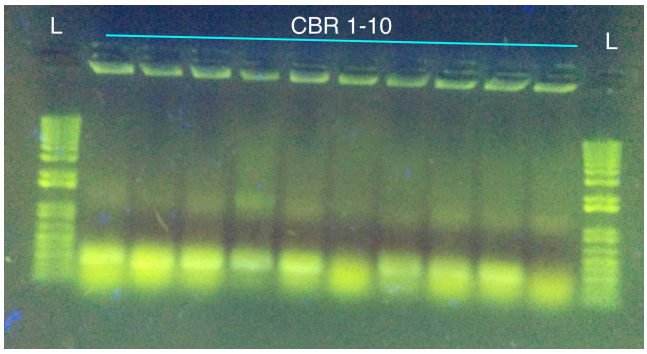


Figure 5. 1% Agarose gel of pSB1C3-CBR cPCR reactions of and SGR gBlocks ran at 100V for 30 min. cPCR reactions used VR and VF2 primers and proceeded as per the "colony PCR" protocol. pSB1C3-SGR ligation products that were ligated in a 1:1 (insert:backbone) ratio were transformed into chemically competent DH5α cells as per the "Bacterial Transformation" protocol. Also shown, SGR gBlock which has been PCR amplified as per the "PCR" protocol. This gel also ran several ladders to test the efficacy of the ladder stocks in our lab. Top Row: Ladder - NEB 1kb plus | Lanes 7-16: pSB1C3-SGR | Lane 17: Ladder - NEB 1kb plus. Bottom Row: Ladder - NEB 1kb plus| Lanes 3-4: PCR SGR | Lane 6: Ladder - Invitrogen 1 kb plus | Lane 8: Invitrogen 1kb plus | Lane 9: Gene ruler 1 kb plus.

ES DSBA DC.jpg

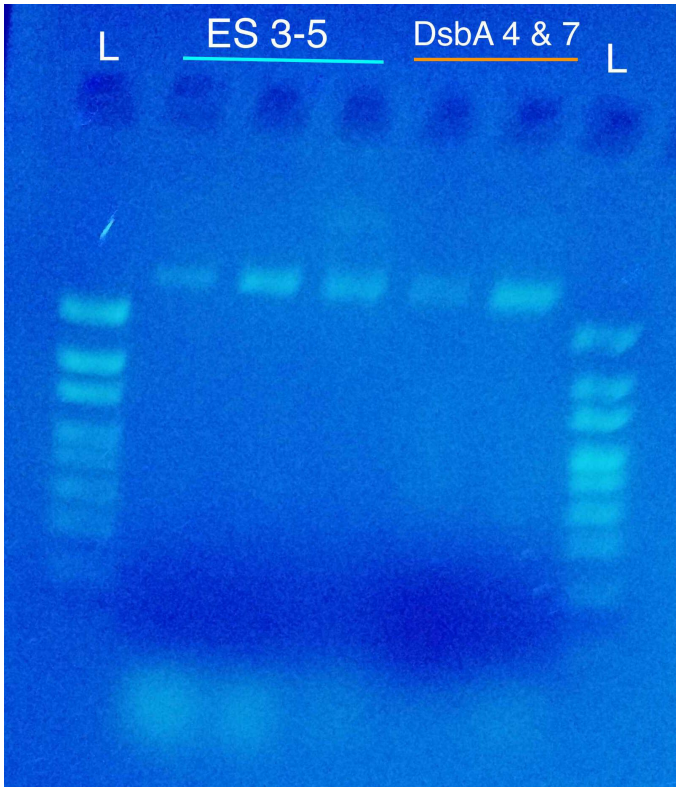


Figure 2. digest confirms of ES 3,4,5 and DsbA 4, 7 (100 bp biobasic)

FUTURE IMPROVEMENTS:

-Add undigested samples: backbone only, PCR fragment with similar size

WEDNESDAY, 7/3/2019

-Made Glycerol Stocks for CBR and SGR. The Glycerol stucks were made with 300:300 ratio instead of 500:500 because of insufficient glycerol amounts

-Transformed GG constructs (ligations) : pSB1A3 + parts for CBR, SGR, HCAR, SGR, and PPH.

-Made minipreps of CBR 4.1, 4.2, 4.3, SGR 10.1, 10.2, 10.3, 10.4, CBR 8 and 10. (Micha's first solo Miniprep! Was sad 👷)

THURSDAY, 7/4/2019

-Made digest confirmations for SGR 10.1, 10.4 and CBR 4.1, 8, 10 Minipreps from 07/03 Wednesday; Based on the bands they did not work and only have the vector (unligated products) (see Figure 3). The PCR and Digest confirmations disagree, potentially because primer used could be amplifying gene on non-specific amplification.
-Sent ES 5, DSBA7, CBR 4.1, and SGR 10.1 for sequencing
-Made new 50% Sterile glycerol

FUTURE IMPROVEMENTS:
-Do negative controls in PCR using untransformed DH5-a

SGR CBR DC.jpg

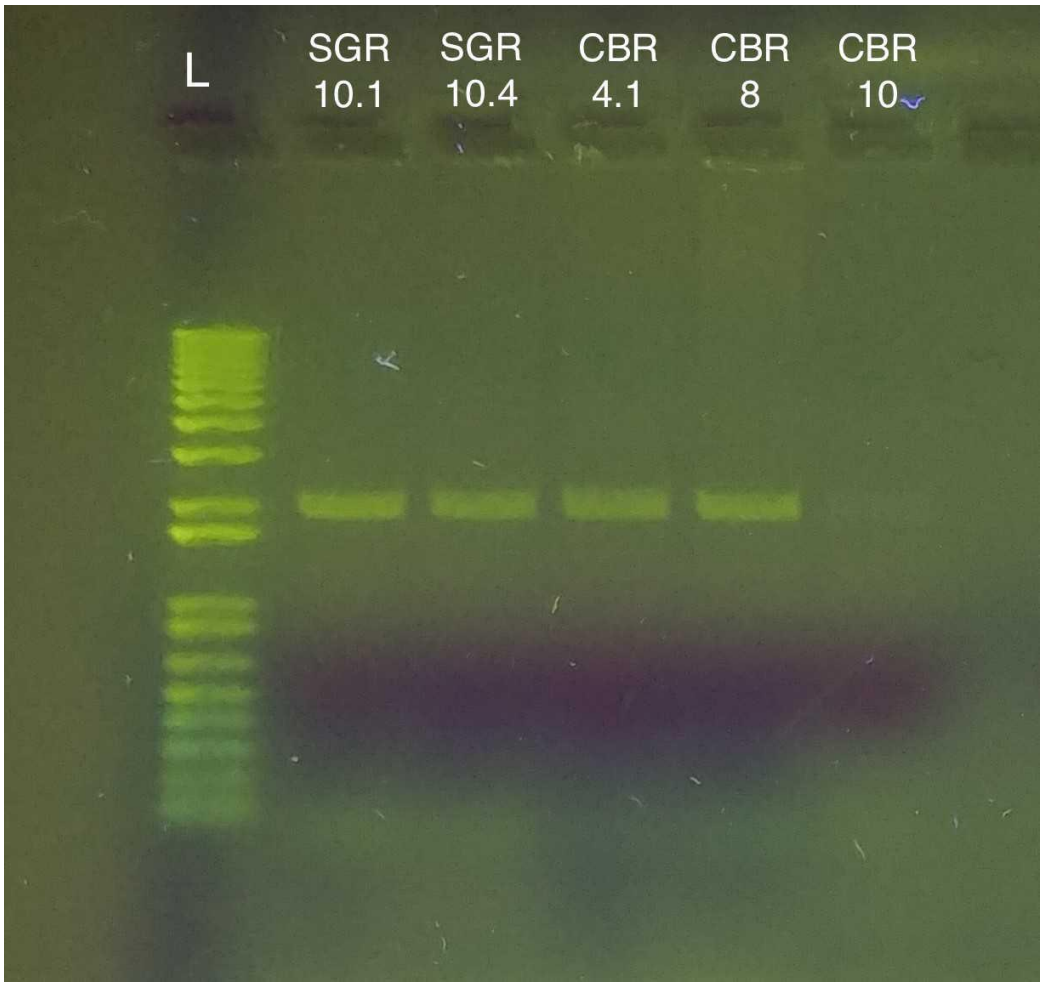


Figure 3. SGR 10.1, 10.4 and CBR 4.1, 8, 10 Digest Confirmation w/ EcoRI-HF and PstI

FRIDAY, 7/5/2019

-Sent ES 5 and DSBA 7 for sequencing.
-Masterplates made for GG satellite colonies (Marked with green marker on original plate) for HCAR, SGR, CBR, and PPH.
-cPCR for different colonies on original SGR and CBR plates that were ligated 24/06/19 and 25/06/19, marked with a red marker.

WEEK 1 SUMMARY

This week the team attempted to clone all the parts (pSB1A3 + SGR, PPH, CBR, or HCAR) using golden gate assembly into pSB1A3 instead of pSB1C3 due to insufficient plasmid stocks. More ES and DSBA digests, ligations and transformations were done. More cPCR reactions were done for SGR, CBR, ES and DSBA were made. SGR PCR-product from the previous week was also gel-purified. After consulting with our research assistant, we will be adding negative and positive controls as part of troubleshooting our colony PCR reactions in the future.

MONDAY, 7/8/2019

-Did cPCR on Friday 07/05 Colonies (GG satellite colonies and different original SGR and CBR)
-Sequencing results of ES 5 and DSBA 7 are negative.
-cPCR + masterplates from transformations from 07/03 Wednesday for HCAR, SGR, CBR, and PPH with different ampicillin concentrations (plated by Sara on Saturday 7/06). White colonies were present in each plate but did not grow until two days after
-The cPCR results of the transformations from 07/03 Wednesday were messy.
-Tested the ladder aliquots in the fridge.

Gel 1 Mike.jpg

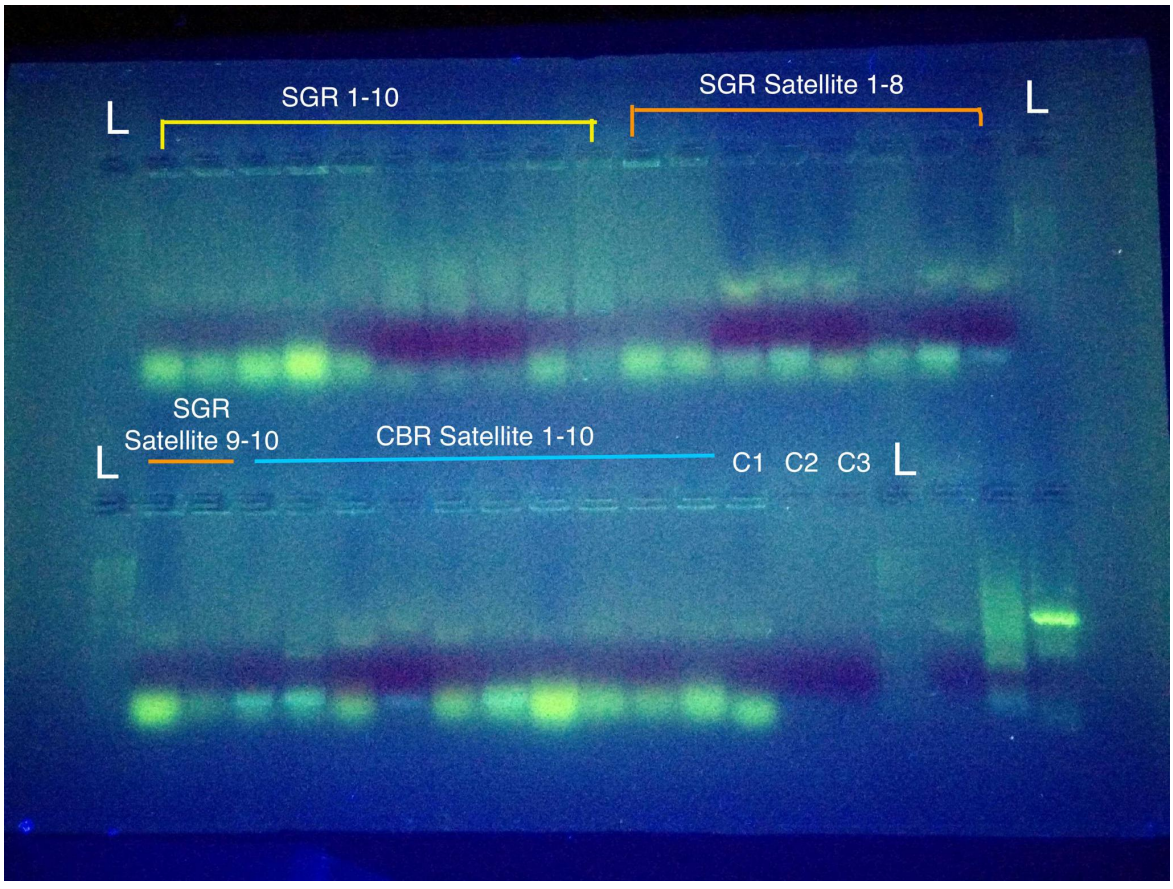


Figure 1. Mike's Gel TOP(L | SGR 1-10 | GG Satellite SGR 1-8| L). BOTTOM: (L| GG Satellite SGR 9-10 | GG Satellite CBR 1-10 | Chemically Competent DH5-a | Empty Vector (CBR 4.1 miniprep) | cPCR Mastermix | L)

Gel 2 micah.jpg

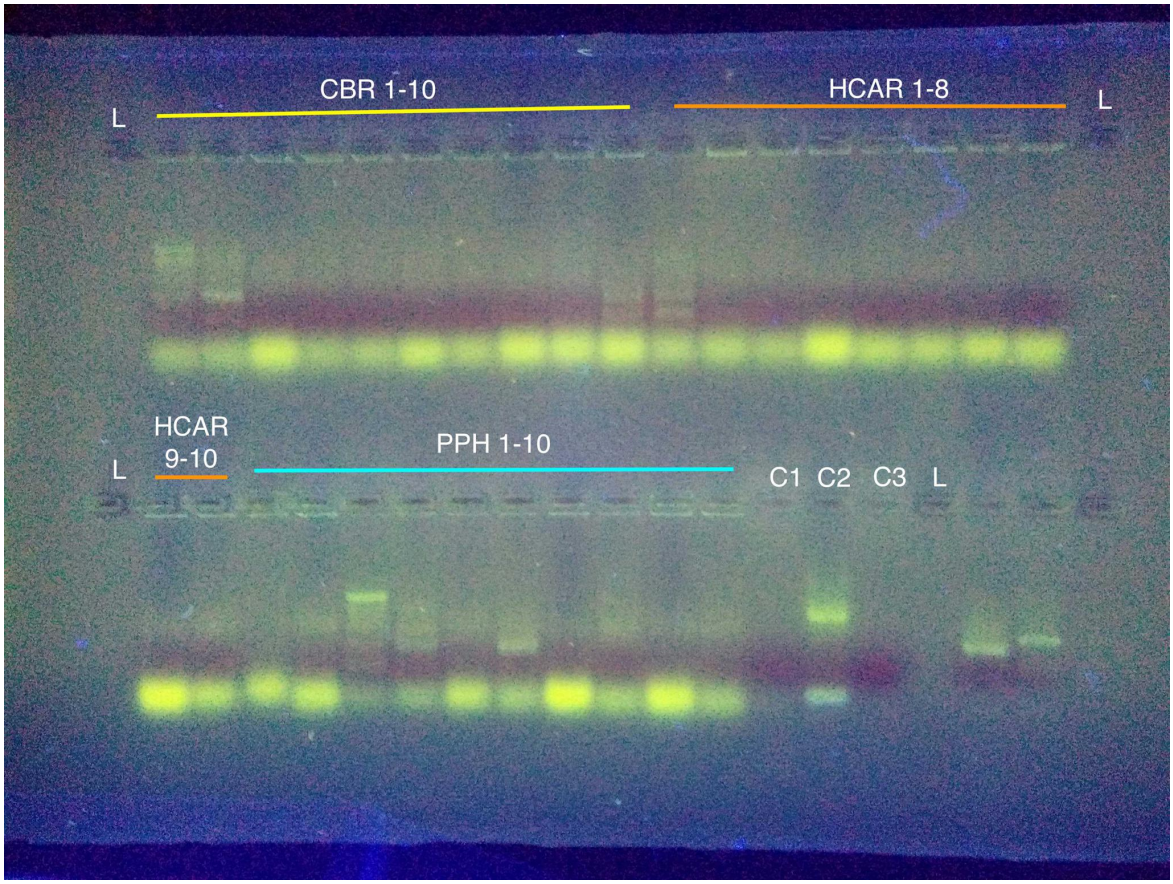
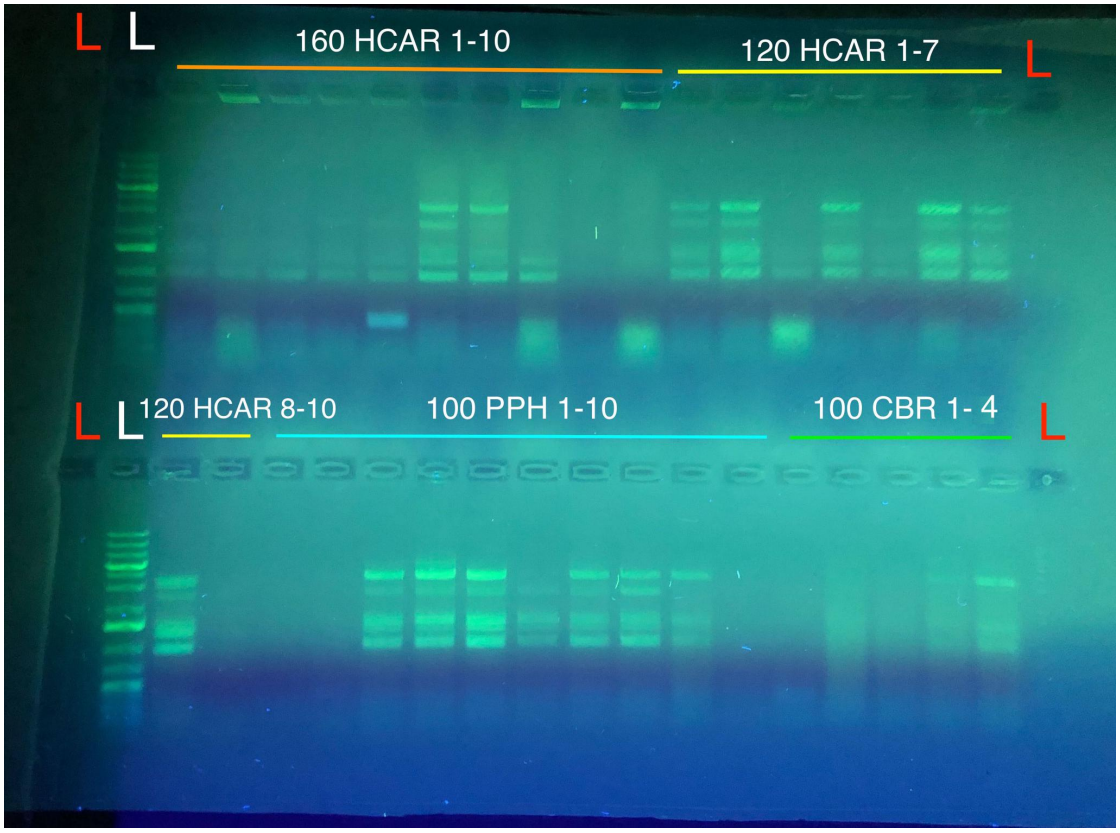
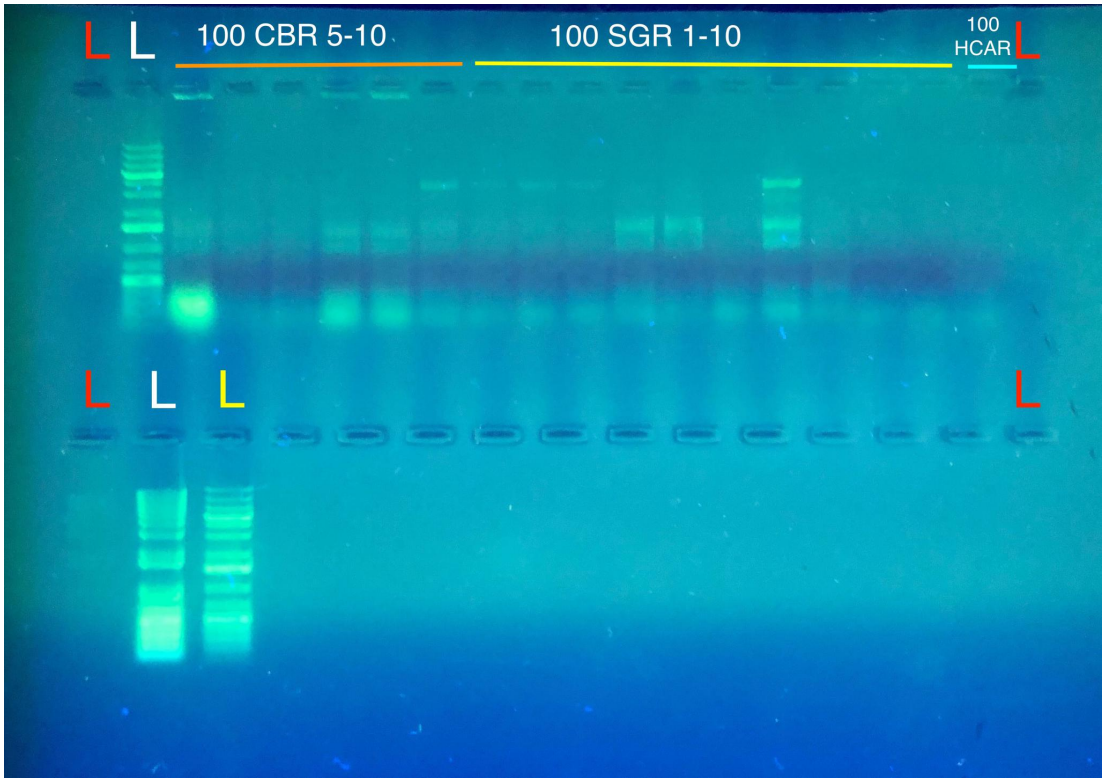


Figure 2. Micha's Gel (L | CBR 1-10 | HCAR 1-8| L) Bottom: (L| HCAR 9-10 | PPH 1-10 | Chemically Competent DH5-a | Empty Vector (CBR 4.1 miniprep) | cPCR Mastermix | L)

Gel 1 Amp GG.jpg



GEL 2 AMP GG.jpg



To do:
Overnights
Glycerol Stocks
Streak on a plate

TUESDAY, 7/9/2019

-DsbA sequence was positive. ES (weak), CBR, SGR were negative.
-Golden Gate cPCRs from July 8, 2019 came out with many bands. CBR had little but incorrect ones. HCAR, PPH had a few defined ones but SGR had one defined band.
-ES5 sequencing showed that it was not in the plasmid and did not have the prefix as a part, and has changes all over the suffix. Marija thinks there was a problem at the digestion level.
-DsBA7 was confirmed to be in.
-Still need pSB1C3-CBR,SGR, ES still need to get in.

Micha took the day off.

WEEK 11 SUMMARY

Did cPCR of golden gate satellite colonies and normal SGR and CBR colonies. Sequencing for ES signal sequence, CBR, and SGR came back negative but DSBA came back positive. Our Golden Gate colony PCRs had multiple incorrect bands. We did more digestions, ligations, and transformations for CBR, SGR, and ES.

MONDAY, 7/15/2019

-Made a mega cPCR for ES, SGR and CBR.
-RFP contamination was visible for and CBR

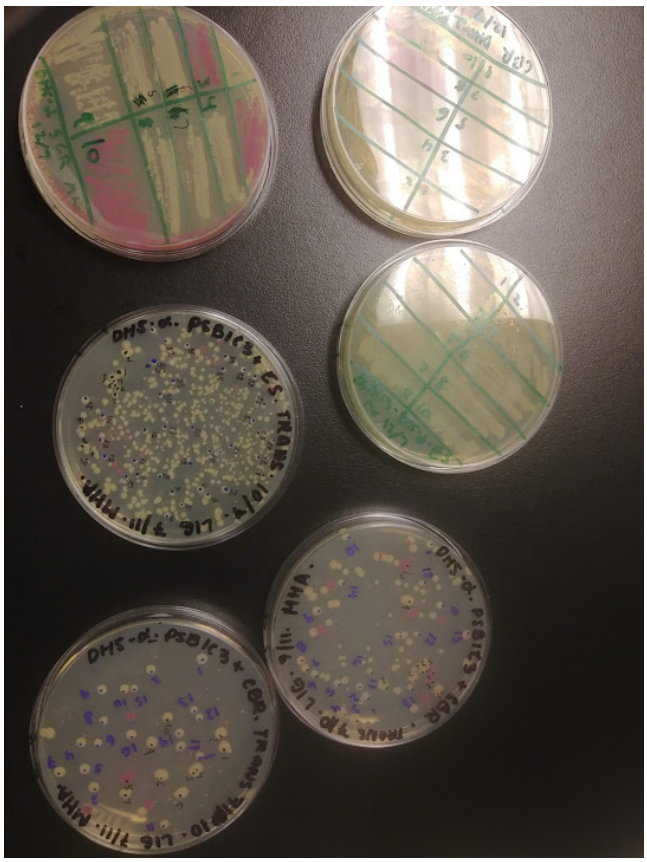
-Master plates grew but have RFP contamination

-Minipreps of overnights of GG made on thursday were PCRd.

-Gels from cPCR of pSB1C3-CBR,SGR,ES showed that ES1#3 and SGR#10 had bands of interest but nothing for CBR worked.

-Sequencing results from July 11,2019 showed ES-3, CBR-8. and SGR-10.4 were not in.

image.png



TUESDAY, 7/16/2019

-Loaded the gels for the mega cPCR

-Mike PCR'd HCAR, CBR, SGR, PPH Minipreps and ran digests on sequence-confirmed HCAR to get pSB1C3.

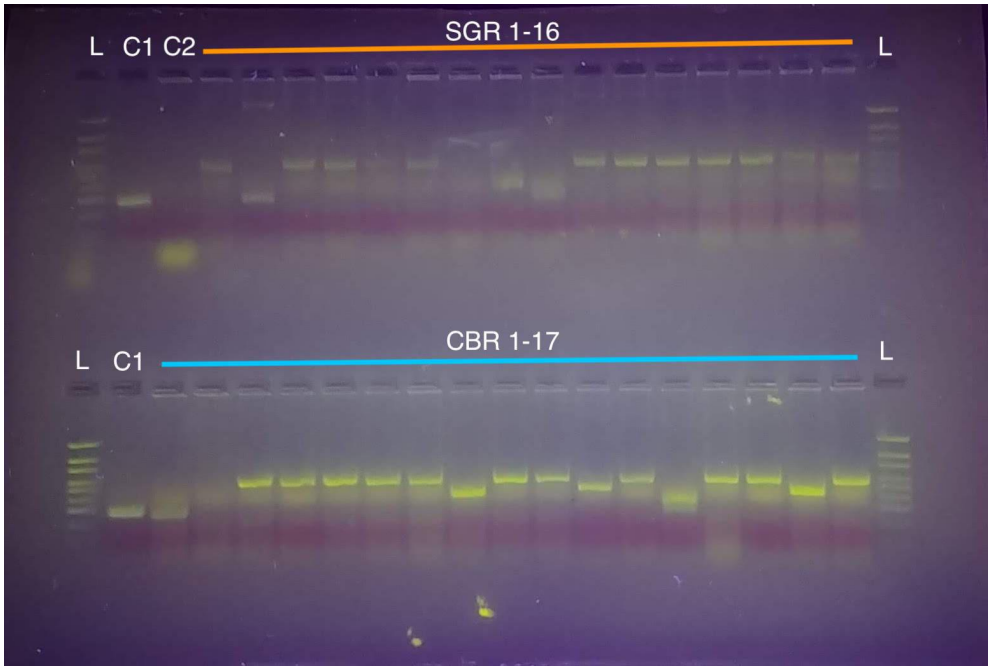
-Micha swapped the ladders, so the SGR and CBR 1% gel contains the 100-1500bp Bio Basic ladder and the ES 2% Gel contains the 1kbplus Gene Ruler ladder.

-Overnights for SGR (1, 2, 10, 11), CBR (3, 4, 15, 17), and ES (15, 19, 31) were done.

-Bands for both SGR and CBR look generally shorter so it is a possibility that the extension time during cPCR was insufficient. Marija suggested increasing it to 2.5 minutes.

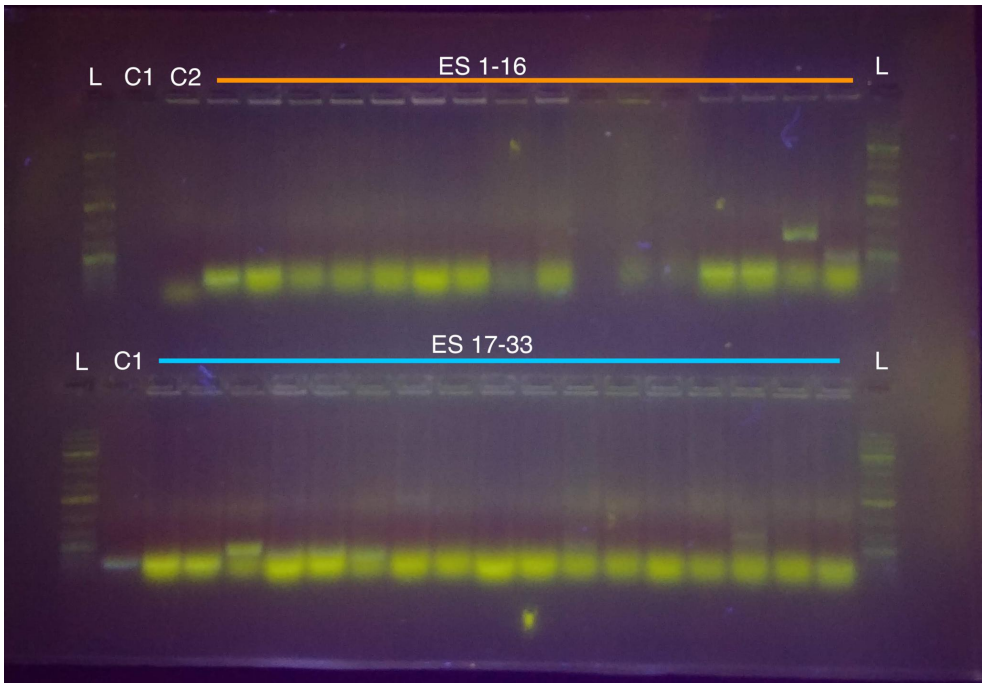
-Got the new constructs from IDT.

SGR CBR 7:15.jpg



SGR and CBR cPCR. C1- Glycerol Stock for CBR 8. C2- DH5-a

ES 7:15.jpg



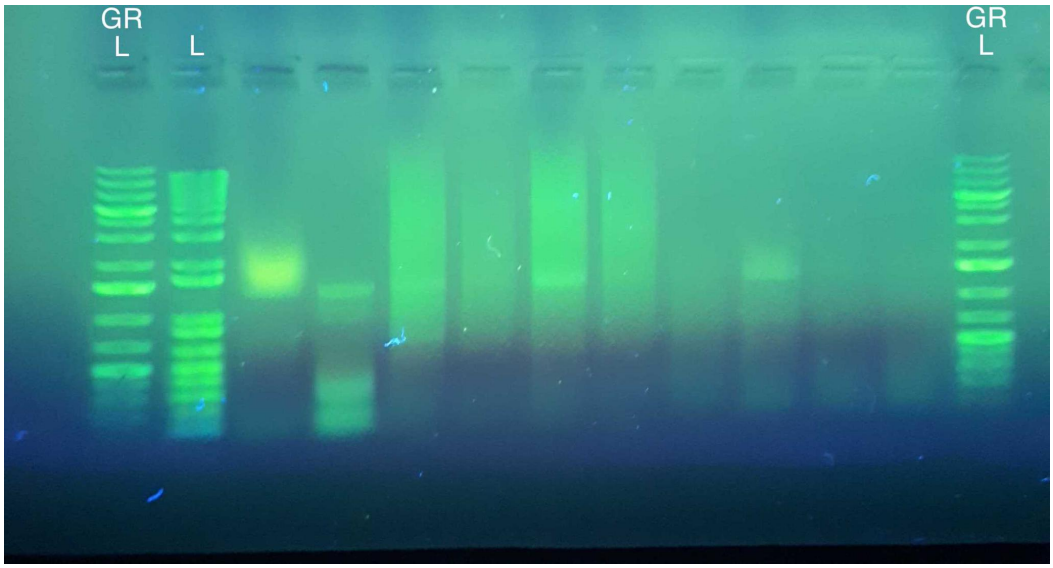
ES cPCR. C1- Glycerol Stock for CBR 8. C2- DH5-a.

Expected band sizes:
SGR - 2202 bp
CBR - 1380 bp
ES - 573 bp

WEDNESDAY, 7/17/2019

-Cassie made us realize we do not have to clone into pSB1C3, and can use pSB1A3
-Chris made glycerol stocks of SGR (1, 2, 10, 11), CBR (3, 4, 15, 17), and ES (15, 19, 31).
-Mike PCRd the golden gate constructs. Gel image showed no clear, distinct bands.
-Mike did digestions + ligations for HCAR and PPH full constructs.
-Chris + Mike + Nimaya + Sravya made P1 buffer
-Micha made minipreps for SGR (1, 2, 10, 11), CBR (3, 4, 15, 17), and ES (15, 19, 31).
-Sent SGR __, CBR __, and ES ___ for sequencing.
-Full constructs for CrSGR and CBR arrived.

MIKE PCR.jpg



Gel image of PCR products for Golden Gate constructs. GR L represents Thermofischer Gene Ruler 1kb plus, L represents Invitrogen 1 kb plus.

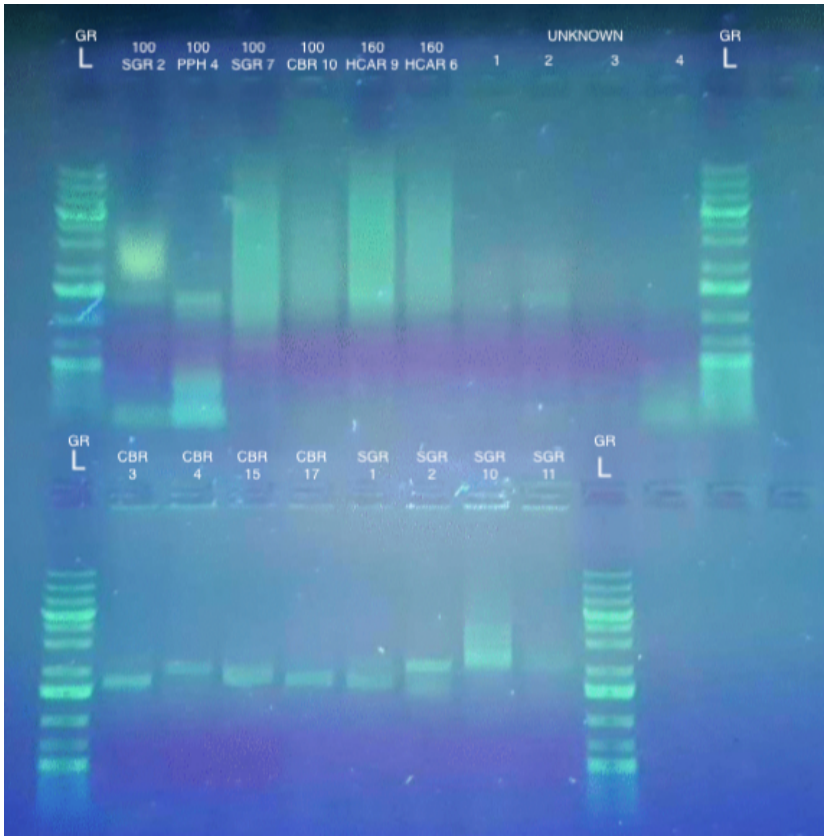
THURSDAY, 7/18/2019

-Team did EcoRI-HF/PstI digestions, salt/ethanol DNA precipitations, and ligations for CBR and SGR in both 1:1 and 2:1 ratios of insert to vector.
-CBR 2:1 was lost because the centrifuge tube exploded in the cold room centrifuge.
-Human Practices Meeting
-Salvaged some remaining T4 DNa ligase and diluted it to ±2U/uL

FRIDAY, 7/19/2019

-Transformed the ligations from July 18 and July 17 (PPH, HCAR, SGR 1:1 and 2:1, CBR 1:1)
-PCR products from July 17 were rerun on the gel.
-Digest Confirmations for SGR, CBR, and ES Minipreps from July 17 (all) were made.
-Made aliquots for Plain LB.
-Sequencing results from July 17, 2019 came back negative for ES, SGR, and CBR.
-Wasted four mini gels for ES.
-ES digest confirmations showed no bands.
-Based on the gel we have concluded that Mike's PCR was not good and has to be redone (top row).

PCR and DC 7:19.png



Gene Ruler. 1% Gel. Top row: PCR products. Bottom row: Digest Confirmations for CBR, SGR.

Chris took the day off.

WEEK 12 SUMMARY WEEK 3 JULY 15-19

We did a huge masterplate for ES, SGR and CBR and they ended up having quite a bit of RFP contamination. This week, on Dr. Marija's suggestion, we increased the elongation time from 2 min to 2.5 min. The full constructs that we ordered (HCAR, SGR, CBR, PPH) arrived, so me and Micha began the process of digesting and ligating.

MONDAY, 7/22/2019

-We resuspended the failed transformations from friday and replated them to verify that we did something wrong.
-We realized that the linearized pSBIA3 from friday was not digested so the failed transformations from friday must have been due to blunt ends.
-As part of the troubleshooting process, we planned a 1:1, 3:1, and 5:1 ratio for our digestion and ligations. Dr. Marija said that her previous lab technician mentioned that ethanol precipitation causes almost half of the DNA contents to be lost, so she suggested to double the concentrations. Despite the DNA spike, the concentrations were mostly still less than 10 ng/uL.
-More troubleshooting: CBR is so big (larger than pSB1A3) so next time in ligation calculators, it will be treated as the vector, and pSB1A3 the fragment.
-HP write-ups

Ethanol Precipitation (NanoDrop)

	A	Concentration (ng/uL)	A 280/260	D
1	CBR 5:1	15.6	1.625	✔
2	CBR 3:1	10.7	1.574	
3	CBR 1:1	5.0	1.333	
4	SGR 5:1	6.85	1.671	
5	SGR 3:1	8.2	1.577	✔
6	SGR 1:1	6.55	1.456	

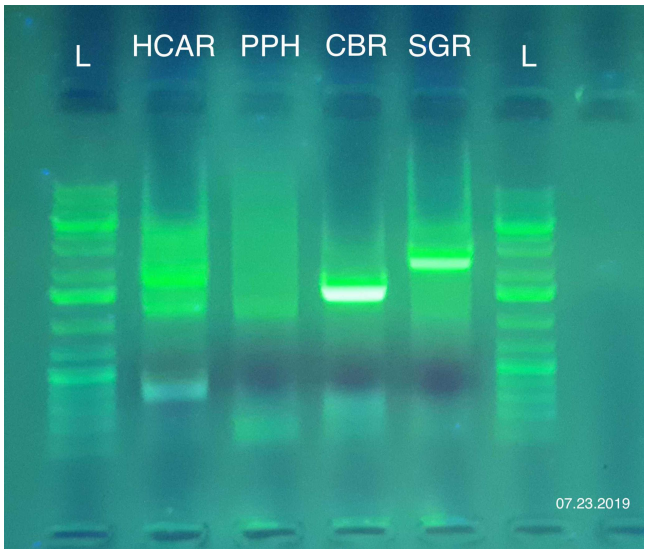
Chris took the day off.

TUESDAY, 7/23/2019

- [x] PCR : HCAR, PPH, SGR, and CBR g-blocks
- [x] Check Plates for Growth
- [x] 1:1 Ligations using CBR 5:1 and SGR 3:1.
- [x] Digest pSB1A3 backbone
- [x] Dig-cip-lig PPH and HCAR (use large batch for digestion, NanoDrop after digestion)
- [ ] Talk to Marija to design optimization of workflow
- [x] The Seb talk

-PCR'd HCAR, PPH, SGR, and CBR g-blocks. PCR for CBR and SGR worked; extension time was set to 2.5 minutes. PPH and HCAR did not show up well.
-Plates with friday (7/19) transformations showed no growth, as expected.
-pSB1A3 backbone was digested, for ligations.
-1:1 Ligations using CBR 5:1 and SGR 3:1 were made.
-Digested, precipitated, and ligated PPH and HCAR. Concentrations were:
-Discussed alternative pheophorbide proposal with Sebastian.

PCR products 7:23.jpg



Gel of PCR products. Ladder used was Gene Ruler 1 Kb plus. CBR and SGR were excised.

WEDNESDAY, 7/24/2019

- [x] Make SOB, glucose solution and Magnesium solution to make SOC
- [ ] Re-do digligs with new ethanol precipitation protocol for _____ _____ ____?
- [x] PCR HCAR and PPH
- [ ] Retransform yesterday transformations with SOC
- [x] Run Gel for today and yesterday PCR
- [x] Make AMP plates
- [x] Gel for PCR things (Micha)
    - 1% gel
    - also load in the CBR and SGR, PPH and HCAR from yesterday so we can gel purify that
    - Load in the new ladder as well to compare

-The plates from yesterday did not grow at all
    - We did make sure to digest the back bone this time, so that was not the problem
    - We are going to leave the plates in the incubator through the day

-We will re-transform and re-plate the digligs/transformations from yesterday but we need to figure out what to do differently
-Troubleshooting: for ethanol precipitation, the A230/260 values were incredibly low which meant the presence of a lot of organic solvents. Dr. Mayi and Dr. Marija said that it is because we have not added a wash step for our ethanol precipitation. From Now on, we will be removing the supernatant (100% cold ethanol) then **washing it with 200-500µL 70% ethanol**, then drying it. Dr. Mayi mentioned that we should **vacufuge for 10-15 minutes only**, otherwise it would be hard to resuspend the DNA.
-New protocol: **Making SOB** and **SOC** and using that in place of LB for transformation.
-More troubleshooting: Marija changed the PCR protocol based on the specific TAQ Polymerase we used. This method was tested for HCAR and PPH today. The gels show smears for HCAR and PPH from 7/24/19 and 7/23/19. Dr. Mayi said that it may be due to the primer binding elsewhere. She and Dr. Marija said that we should design specific primers. Clearly there are multiple bands for HCAR and PPH.
-Micha missed some lab work
-Chris accidentally added glucose twice, so the SOC was very concentrated. It will need to be diluted.
-Mike accidentally used the stock (undiluted) ligase for the ligations.
-Chris transformed and plated PPH and HCAR in 3:1 and 1:1 ratios, standard and pelleted.

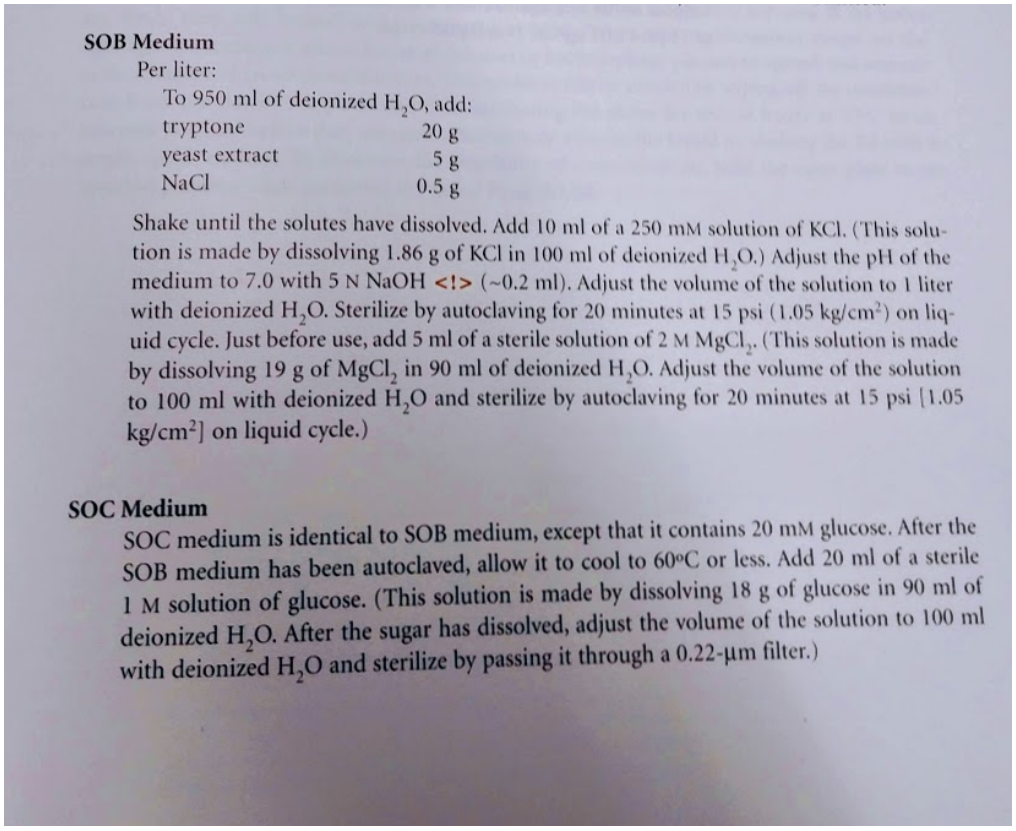
SOC

1. Autoclave SOB medium. Cool to 60˚C.
2. Add 20 mL Sterile 1 M Glucose
    a. Dissolve 18g glucose in 90 mL of deionized H2O
    b. Adjust volume of solution to 100mL with deionized H2O
    c. Sterilize by passing it through a 0.22µm filter

SOB

1. Per litter, add:
    a. 950 mL deionized H2O, 20g tryptone, 5g yeast extract, 0.5g NaCl.
2. Shake until dissolved. Add 10 mL of 250 mM KCl solution:
    a. 250 mM KCl solution: dissolve 1.86g KCl in 100mL of deionized H2O.
3. Adjust pH of medium to 7.0 with 5N NaOH (±0.2 mL).
4. Adjust volume of solution to 1.0L with deionized H2O.
5. Autoclave.
6. Add 5mL of sterile 2M MgCl2 solution
    a. 2M MgCl2 solution: dissolve 19g MgCl2 in 90 mL deionized H2O.
7. Adjust volume of solution to 100mL with deionized H2O. Sterilize by autoclaving.

image.png



SOB Medium

Per liter:

To 950 ml of deionized H_2O, add:

tryptone	20 g
yeast extract	5 g
NaCl	0.5 g

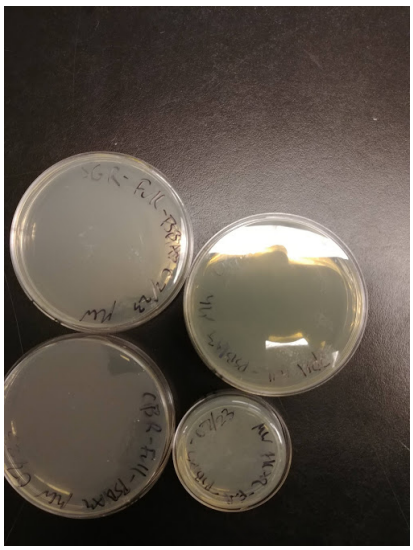
Shake until the solutes have dissolved. Add 10 ml of a 250 mM solution of KCl. (This solution is made by dissolving 1.86 g of KCl in 100 ml of deionized H_2O.) Adjust the pH of the medium to 7.0 with 5 N NaOH <!> (~0.2 ml). Adjust the volume of the solution to 1 liter with deionized H_2O. Sterilize by autoclaving for 20 minutes at 15 psi (1.05 kg/cm^2) on liquid cycle. Just before use, add 5 ml of a sterile solution of 2 M $MgCl_2$. (This solution is made by dissolving 19 g of $MgCl_2$ in 90 ml of deionized H_2O. Adjust the volume of the solution to 100 ml with deionized H_2O and sterilize by autoclaving for 20 minutes at 15 psi [1.05 kg/cm^2] on liquid cycle.)

SOC Medium

SOC medium is identical to SOB medium, except that it contains 20 mM glucose. After the SOB medium has been autoclaved, allow it to cool to 60°C or less. Add 20 ml of a sterile 1 M solution of glucose. (This solution is made by dissolving 18 g of glucose in 90 ml of deionized H_2O. After the sugar has dissolved, adjust the volume of the solution to 100 ml with deionized H_2O and sterilize by passing it through a 0.22-µm filter.)

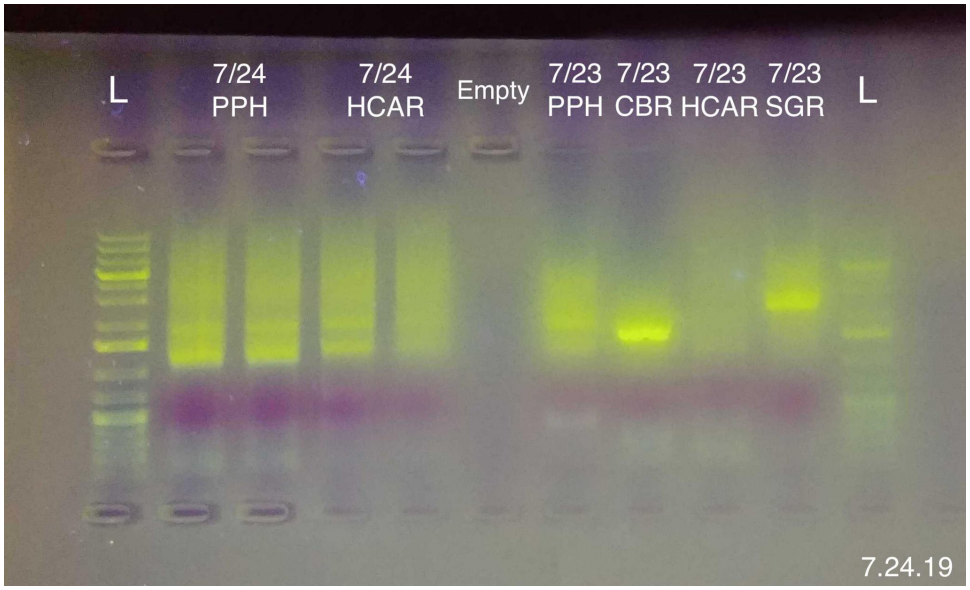
SOC and SOB media. Reference: Green, Michael R., and Joseph Sambrook. Molecular Cloning a Laboratory Manual. Cold Spring Harbor Laboratory Press, 2012.

image.png



No growth for the plates that were retransformed (7/22/19)

PCR 7:24.jpg



L: Gene Ruler 1 kb plus ladder. Gel is for PCR products.

Need to start wiki writeups for how HP meetings helped subgroup decide and design our project

THURSDAY, 7/25/2019

-Mega cPCR was done for PPH and HCAR 1:1 and 3:1 for both standard and pelleted transformation treatments. The other was pelleted because to increase concentrations.

-Excised CBR and SGR were gel purified and will need to be NanoDropped.

- Micha washed the spin column with wash buffer twice instead of once-- first without waiting and the second left for five minutes.

-Chris made digests for CBR and SGR and ethanol precipitated them. These will need to be NanoDropped.

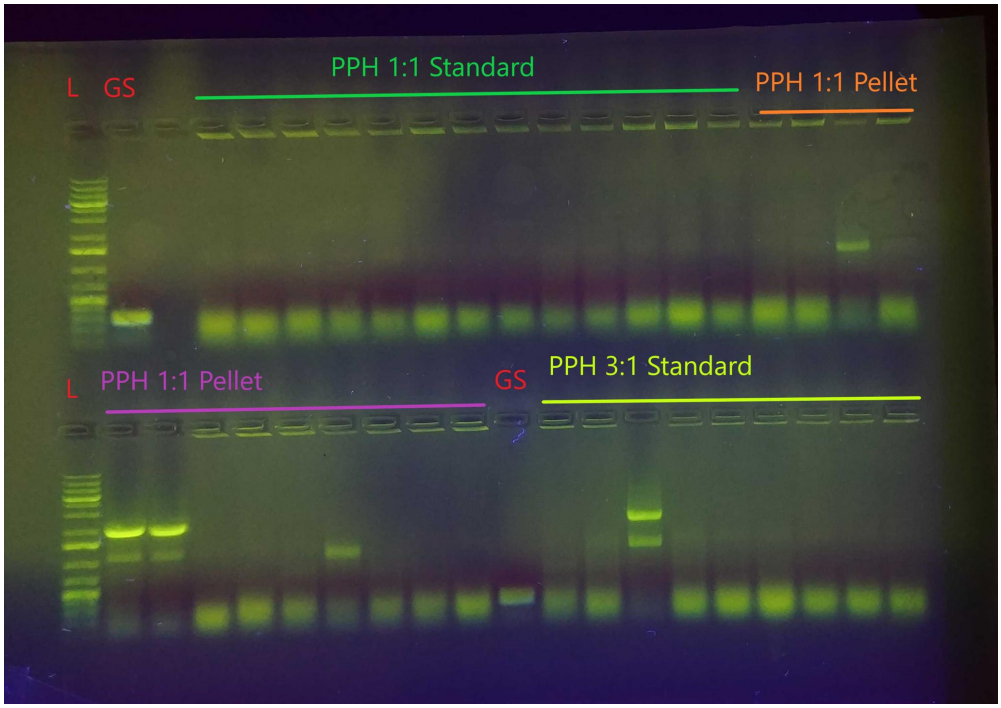
-Mike made LB + Amp plates.

-Gel #2 was not made due to time constraints and PCR well limitations. cPCR will be done for PPH 3:1 Pelleted. In the same gel, glycerol stock and mastermix control will be run, alongside PCR products.

-For PPH, 1:1 pelleted colonies #3, 5, 6 and 3:1 standard #3 will need overnights.

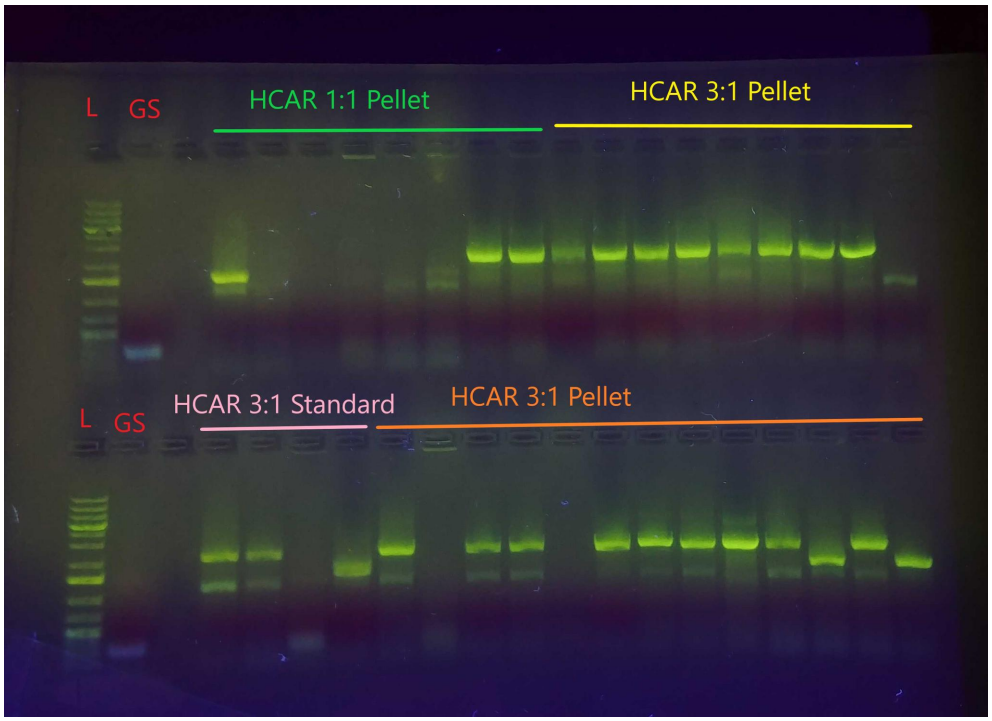
-For HCAR, most colonies look identical so HCAR 1:1 Pelleted #13, 3:1 Standard #4, 8, and 3:1 Pelleted #9 and 7 will need overnights.

Gel 1 7:25.jpg



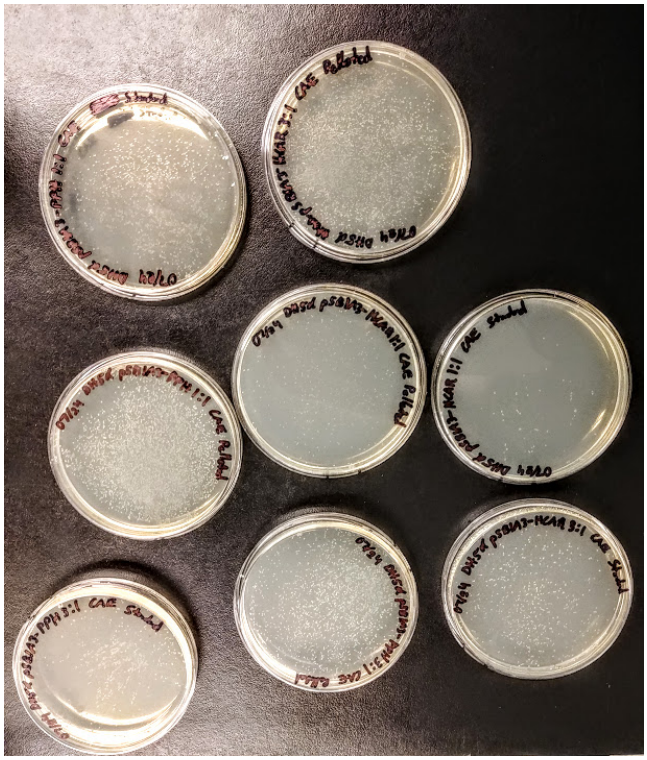
Gel 1 cPCR. 1% Gel. Third lane is empty. L is ladder. GS used was CBR 4.1 Glycerol Stock which was sequence-confirmed to have recircularized pSB1A3. Top third lane is empty.

Gel 3 7:25.jpg



Gel 3. Top and bottom third lane are empty. L is ladder. GS used was CBR 4.1 Glycerol Stock which was sequence-confirmed to have recircularized pSB1A3.

image.png



Colonies are small because they were plated late on 7/24.

FRIDAY, 7/26/2019

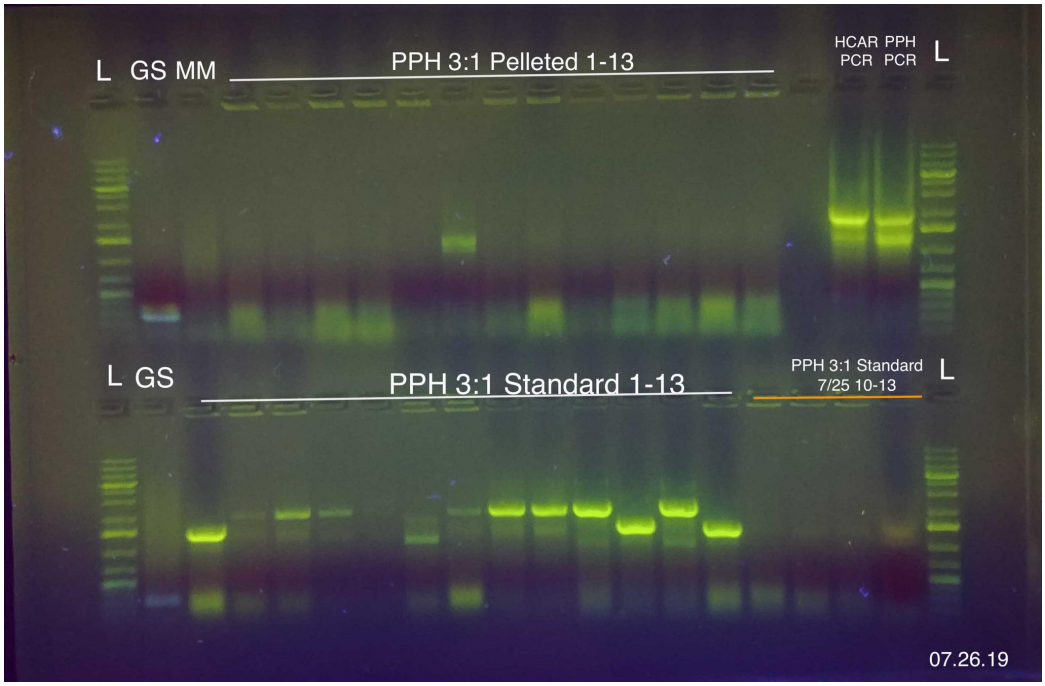
-cPCR will be done for PPH 3:1 Pelleted. In the same gel, glycerol stock and mastermix control will be run alongside PCR products.

-Overnights will be set up. Dr. Mayi and Marija can put them away on Saturday.

- [x] PPH 1:1 Pelleted #3, 5, 6
- [x] PPH 3:1 Standard #3
- [x] HCAR 1:1 Pelleted #13
- [x] HCAR 3:1 Standard #4, 8
- [x] HCAR 3:1 Pelleted #9, 7

-Continue ligations for 7/25 digestions and then transform, plate them.

cPCR and PCR 7:26.jpg



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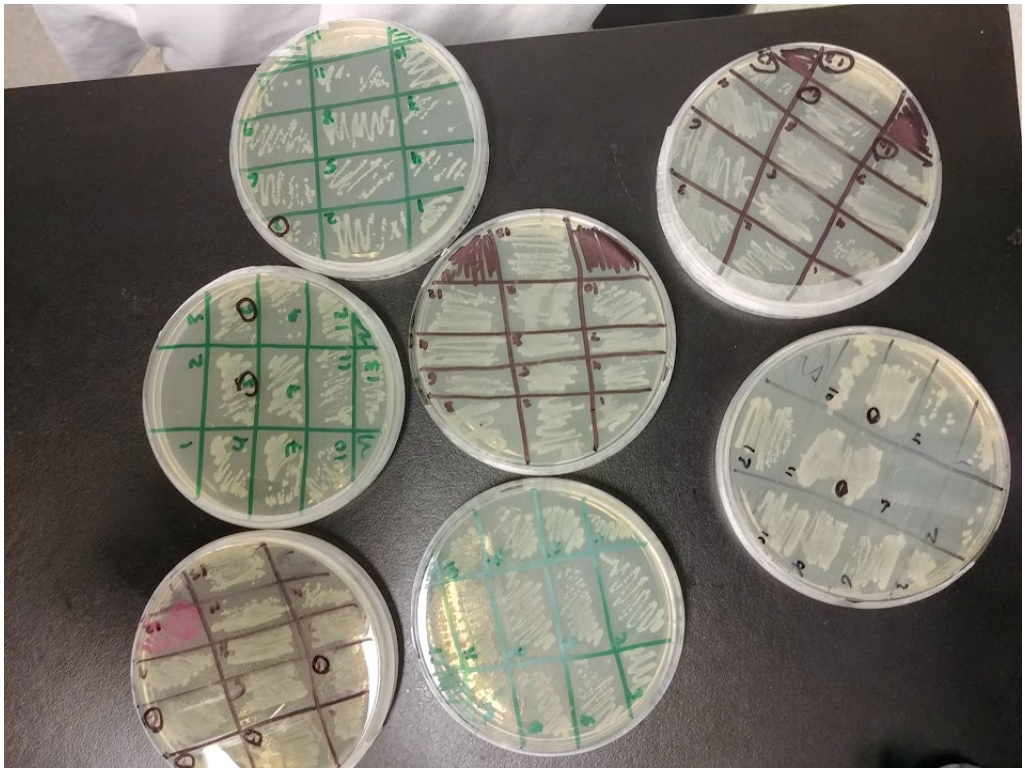


WEEK SUMMARY

This week was dedicated to troubleshooting results and protocols. The team started ethanol and salt precipitating DNA after the digests with double the amount of DNA. A wash step was also added to the protocols after precipitation, with the vacufuge step reduced to 15 minutes maximum. Additionally, SOC protocols were added for use in place of standard LB for transformations. Each PCR step was adjusted to increase activity and specificity based on our constructs and ingredients. Since DNA amounts were doubled for digests, the team did a PCR spree on the full constructs. More pSBIA3 backbone was digested since those last week were not, causing the failed transformations.

MONDAY, 7/29/2019

-Gel purified PPH and HCAR from 7/26.
-Miniprepped overnights from Friday, 7/26.
-Sent H1P #8, H3S #3, P3S 3 and P ___ for sequencing
-Transformations for CBR and SGR were made.
-Made glycerol stocks for the overnights from 7/26.
-Troubleshooting: CBR and SGR gel-purified parts had weird absorbances because Micha used elution buffer instead of water to resuspend the DNA.
-Mike went to the Hunter Hub meeting with Prabhu to discuss the Synbio Discussion.

No lab photo today!!! Am sad.

TUESDAY, 7/30/2019

-Sara Far did cPCRs for us
-Team did no lab work.
-Team did meetings the whole day

WEDNESDAY, 7/31/2019

-cPCR was redone for the ones yesterday because temperatures were different, and bands were not long enough.
-PCR of SGR and CBR full constructs were done
-Digestion, precipitation and ligations for SGR and CBR full constructs.

THURSDAY, 8/1/2019

-Ligations in the freezer because no CC cells for pSBIA3 CBR and SGR
-Analyze CBR and SGR old pSBIA3 gels
-Transformed PPH and HCAR + pSBIA3 into BL21.
-Nanodropped gel-purified SGR and CBR full constructs.
-Sequencing results came in: PPH and HCAR full constructs were in.
-Chemically competency test

Micha took the day off.

FRIDAY, 8/2/2019

-Did Minipreps of 8/01 overnights.
-Made glycerol stocks of the minipreps.
-Made LB + Amp Agar Plates
-Retransformed PPH and HCAR into BL21 (P3S.3 and H3S.3)
-Retested DH5-a for chemical competency.
-Sent CBR 1:1 Standard 4, SGR 1:1 Standard 8, SGR 3:1 Pelleted 3, SGR 1:1 Pelleted 12 for sequencing
-Transformations from 8/01 lawned because they were plated on LB + No anti-biotic plates. Sad.

WEEK 14 SUMMARY Aug 1 -2 WEEK 1

This week we amplified DNA and successfully cloned in PPH and HCAR into DH5-α. We then cloned PPH and HCAR into BL21 cells and redid digests, ethanol precipitations, and ligations for SGR and PCR. Team had a lot of meetings about pheophorbide application. We received an e-mail from Dr. Addy about the fungal cultures they had available and are willing to give us.

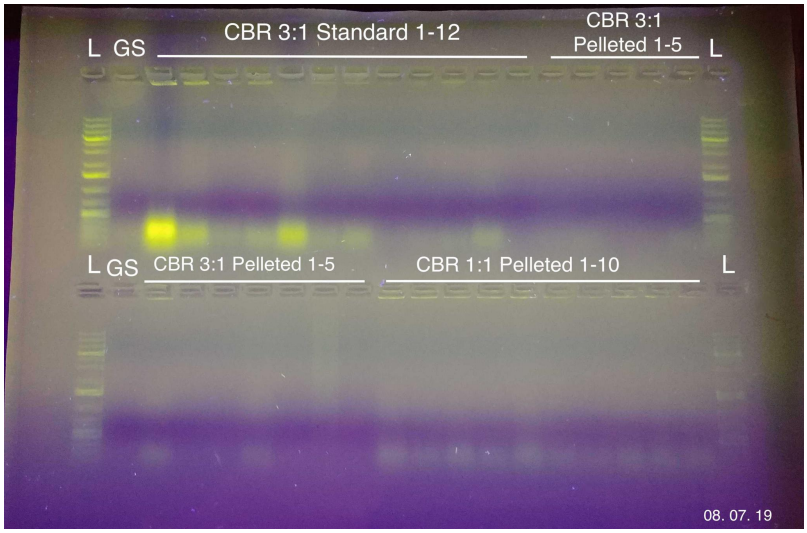
MONDAY, 8/5/2019

-Chris made transformations of CBR and SGR in DH5-α
-Chris made overnights for protein start-ups (BL21, BL21 pSB1A3, BL21 pSB1A3-HCAR, BL21 pSB1A3-PPH)
-Cleaned Mr. Clean out of the lab

TUESDAY, 8/6/2019

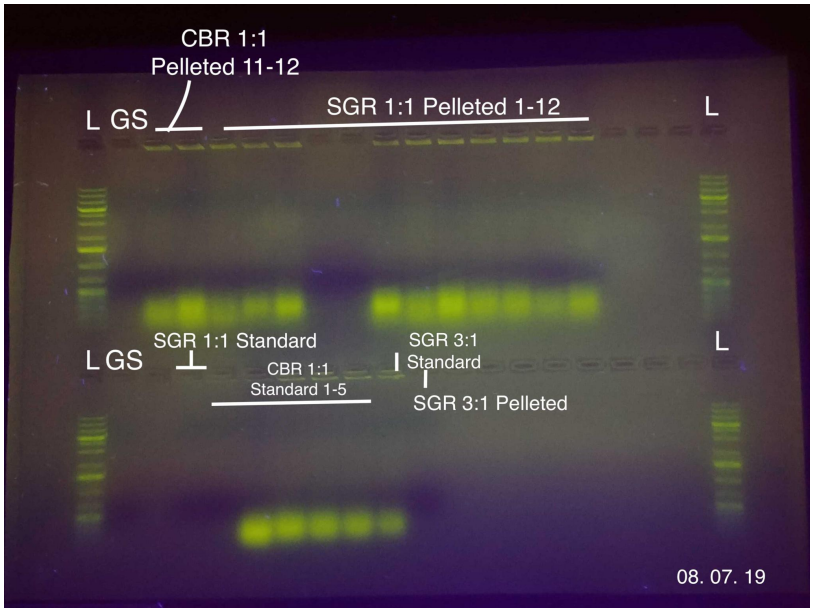
-cPCR of CBR and SGR transformations from August 5, 2019
-Started protein production and induced with IPTG from overnights on August 5, 2019
-There were almost no bands in Gel 1. This is because Micha used the tray that was not working and had to transfer the whole gel, with most of the contents being washed out.
-Troubleshooting: the band sizes were all the wrong size (not amplified). We suspect that this is due to increasing the annealing temperature to 68°C when we have been doing 57°C.

GEL 1 8:7.jpg



Gel 1.

GEL 2 8:7.jpg



WEDNESDAY, 8/7/2019

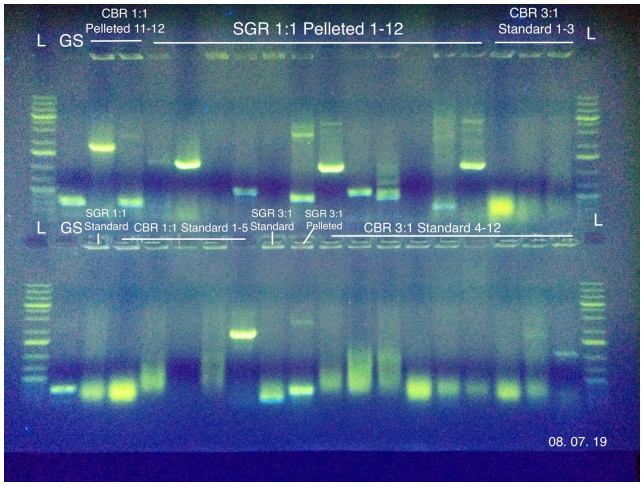
-Continued protein production from August 6, 2019

-Resuspend pellet, freeze/thaw, sonicate, run on gel

-Redid the cPCRs that failed from August 6, 2019 in the same format. However, this time the annealing temperature is set to 57*C.

-cPCR gel showed varied band sizes, rendering it unsuccessful.

cPCR 8:7.jpg



FRIDAY, 8/9/2019

-Chris went with Prabhu to talk to Dr. Baker about the SynBio discussion

WEEK 15 SUMMARY AUG 5-9 WEEK 2

This week the team did transformations and colony PCR on CBR and SGR (into DH5α). Colony PCR showed no bands initially but when redone, had the wrong band sizes which we suspect was due to increasing the annealing temperature to 68°C from the usual 57°C. After another colony PCR set to 57°C annealing temperature, we saw bands but still with varying sizes. We prepared more proteins (PPH, HCAR, and native BL21 pSBIA3 proteins).

MONDAY, 8/12/2019

-Made glycerol stocks from Sravya overnights (No date mentioned)
-Made transformations for pSBIA3-CBR and pSBIA3-SGR.
-Did protein inductions from the overnights.

Micha took the day off.

TUESDAY, 8/13/2019

Our pheophorbide came in today.
-Checked the OD of induction Overnights: BL21 pSBIA3 = 1.673, BL21 HCAR = 1.685, BL21 PPH 1.684.
-Ran cPCR for colonies from pSBIA3-CBR and pSBIA3=SGR DH5-a colonies.
-Ran 10% SDS Gel on samples from Augusts 12, 2019.
-Ran inductions for August 12, 2019 overnights overnights.

- Mike went throuh the protein purification protocol for BL21-psb1a3, HCAR and PPH
  - The gels ran for these samples did not have a positive match for the protein of interest.
  - We did not complete the sonication steps this day, but rather completed it the following day

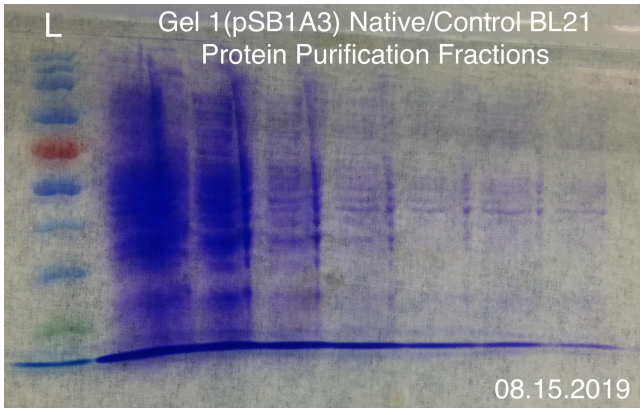
WEDNESDAY, 8/14/2019

-Overnights from August 13, 2019 were induced, with the following ODs: pSB1A3 = 0.744, pSB1A3-HCAR = 0.617, pSB1A3-PPH = 0.366 and induced with 1M IPTG (50uL for 1mM IPTG)
-Protein purifications from August 13, 2019 were continued, starting from sonication (refer to Mike or Nimaya's Notebook)
-Made more 10% SDS-PAGE gels for protein purification.
-Did not see our protein on the SDS-PAGE gel

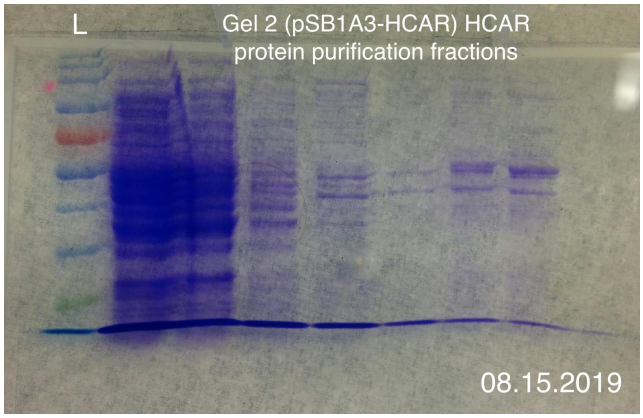
Today, Mike and Chris met with Fran Cusack in regards to obtaining Fungus samples to test the anti-fungal properties of pheophorbide. She provided us with samples of *P. Neglecta (?)* and *Sclerotinia* sclerotiorum as well as PDA (Potato Dextrose Agar) plates and CMA (Corn Meal) broths for culturing.

THURSDAY, 8/15/2019

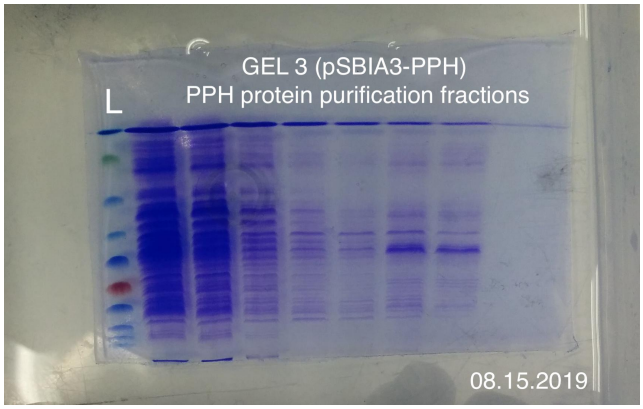
GEL 1 CONTROL.jpg



Gel 2 HCAR.jpg

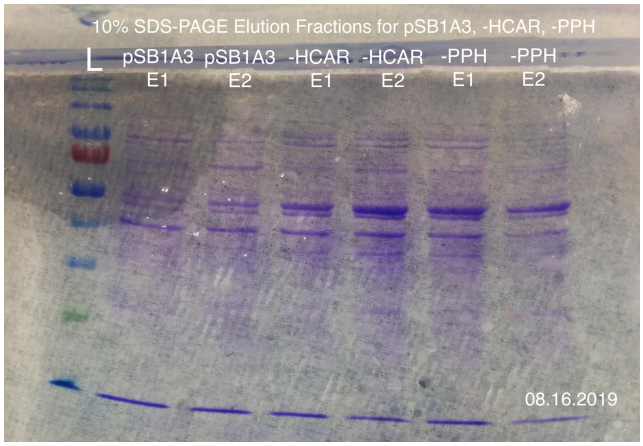


GEL3PPH.jpg



FRIDAY, 8/16/2019

Gel 1 Aug 16.jpg



Confirmation #1 for PPH and HCAR purification.

Micha off to Seattle.

WEEK 16 WEEK 3

Subcultures for pSB1A3, HCAR, and PPH were induced with IPTG and protein purifed, then ran on 10% SDS-PAGE Gel. The band sizes correspond to PPH and HCAR protein sizes which confirm our first replicate for PPH and HCAR purification. Team also obtained pheophorbide a from Sta. Cruz Biotech and potato dextrose plates, fungal cultures (*Sclerotinia sclerotorium* and *Pestialotopsis microspora*), and corn meal broths for culturing from Ms. Fran Cusack, a laboratory technician at the University of Calgary.

MONDAY, 8/19/2019

-Transformations of ligations done from August 16, 2019 into

TUESDAY, 8/20/2019

-Ran cPCR for RFP, SGR, and CBR but where is the picture was not great.
-Did PCR on CBR and SGR.

WEDNESDAY, 8/21/2019

-Redid the colony PCR from August 20, 2019 due to poor screening.
-

cPCR 6GIX CBR SGR.jpg

Gel ____. Ruler:: Gene Ruler. Top = RFP. Bottom: RFP and CBR, SGR.

THURSDAY, 8/22/2019

-Chris and Micha talked to Dr. Addy about fungi in general. She referred us to some plant pathologists and her summer students.
-Micha had the PURE lunch event.

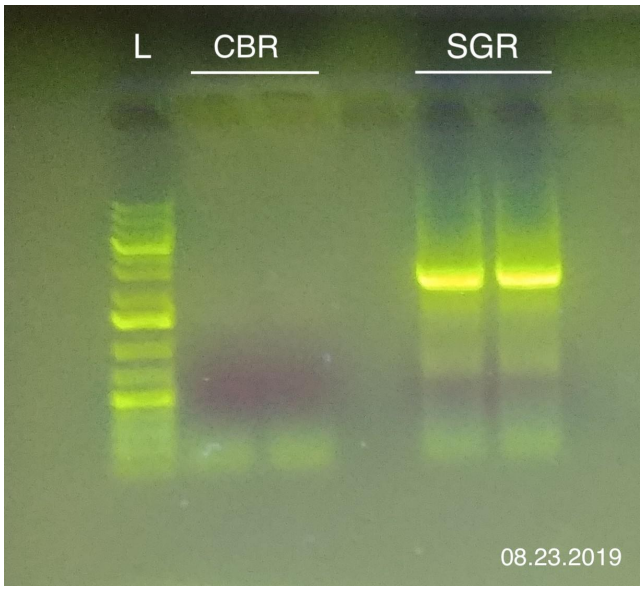
-Redid the cPCR for CBR and SGR, alongside 6GIX.
-Team did some write-ups, Micha worked on Falling Walls writing.
-Continue protein purification starting with sonication step from yesterday (Stock from August 14, 2019 and from August 21, 2019).
-Make overnights for MW CBR 1:1 #2.

Mike took the day off.

FRIDAY, 8/23/2019

-Did minipreps of the overnight for MW CBR 1:1 #2.
-PCR'd SGR and CBR. There were no bands for CBR. SGR was gel-purified.
-Finished washing the Ni-NTA.
-Ran protein purification fractions (PPH and HCAR) on 10% SDS-page gel
-

pcr cbr sgr.jpg



Gene Ruler | CBR | CBR | Empty lane | SGR | SGR

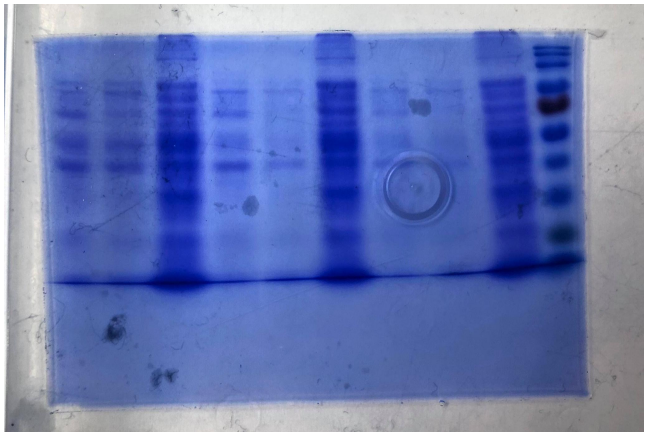
Mike took the day off.

WEEK 17 SUMMARY WEEK 4

MONDAY, 8/26/2019

-Gel destained, shows the 2/3 confirmationof PPH and HCAR expression.
-Sent CBR MW 1:1 Ptd #1.1 and #2.1 minipreps for sequencing
-Made mastermix for stacking gels and 10% Resolving gels.
-Made 3 10% resolving gels.
-Made new digests, precipitation and ligation for CBR and SGR, but this time with double the vector for SGR (?) since the fragment is larger than the vector.

received_912841059050114_2.jpeg



Gel #2. Protein purification confirmation #2 for PPH and HCAR. No n

Gel ___. 2/3 confirmation for PPH and HCAR.

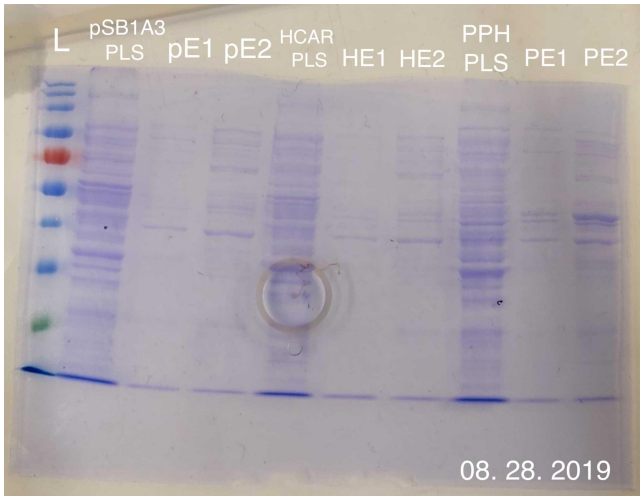
TUESDAY, 8/27/2019

-Ran PCR products from August 26, 2019 on the gel. Bands were very faint so gel was discarded.
-Ligations from August 26, 2019 were transformed and plated.
-Proteins from August 26, 2019 were sonicated, then eluted.

WEDNESDAY, 8/28/2019

-Ran cPCR for colonies from August 26, 2019. However, no bands were visible even for the eight ladders that were put up. The team suspects that it might be the new RedSafe called "FireRed" which was blue. We looked at the instructions after to troubleshoot but there was nothing wrong with it.
-A 10% SDS Page gel containing PPH and HCAR elution fractions was prepared. Bands were visible for PPH but band sizes for HCAR were the same as plasmid proteins. Samples for the second elutions had more bands than the first elution.

GEL AUGUST 28.jpg



Gel # 3 : Protein purification confirmation #3 for PPH, not for HCAR.

Gel __.

THURSDAY, 8/29/2019

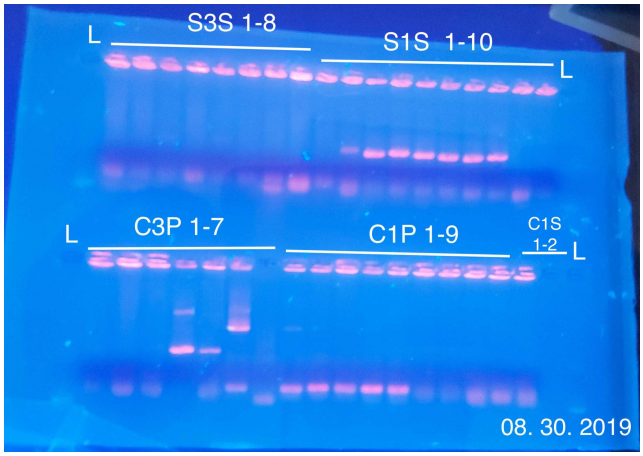
-cPCR from August 28, 2019 masterplates were redone.
-Made reaction buffers for PPH and HCAR
-Sonicated proteins (PPH and HCAR) for purification.
-Ammonium Sulfate Precipitation for PPH and HCAR from August 22, 2019.

FRIDAY, 8/30/2019

-Make overnights for the colonies with bands.

-Ammonium Sulfate Precipitated HCAR and PPH proteins

Gel 1 redid.jpg



Gel 2. I don't know/have the annotations. not in wiki

SATURDAY, 8/31/2019

-Figured out how to make 7-Hydroxymethyl chlorophyll a by reacting chlorophyll b with NaBH4.

-Miniprepped overnights and made glycerol stocks for cPCR wells with bands.

-Redid the cPCR with bands that were roughly at length of interest (unknown due to invisible ladder).

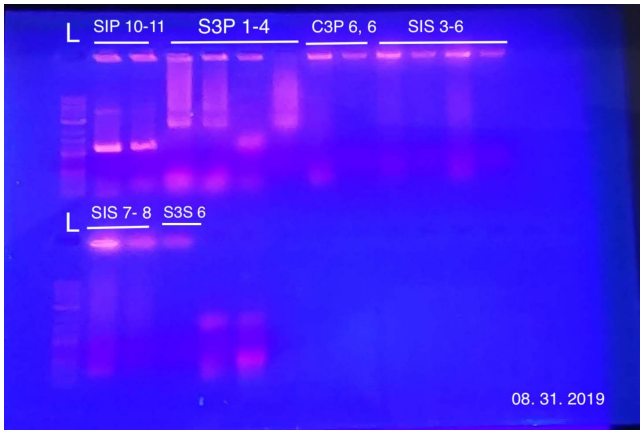
-Protein purified HCAR and PPH.w

-Extracted chlorophyll using 80% Acetone

-Protocol used:

1. Add 80% acetone to spinach
2. Grind using mortar and pestle
3. Place in 50 mL Falcon Tube (A) with the crushed leaves. Centrifuge at 3400g for 5 minutes.
4. Remove supernatant using serological pipette. Keep in a new Falcon Tube (B). Do NOT throw the pellet.
5. Spin Falcon Tube with Pellet (A) at 3400g for 5 minutes.
6. Place supernatant into Falcon Tube B.
7. Spin pellet, repeat steps 4-6 until pellet is white.

LATES GEL .jpg



cPCR redone. Ladder does not look good; seems degraded. Marija thinks it is because of the buffer. At the bottom, wells 5&6 were not loaded anything so that is weird.

WEEK 18 SUMMARY WEEK 5

This week the team column purified, and ammonium sulphate precipitated more PPH and HCAR proteins. More digests, ethanol/salt DNA precipitation and ligations were done for CBR and SGR. We decided to double the vector ratio in SGR digests since the fragment is larger than the vector. Team faced difficulties in visualizing the gel because of the new nucleic acid stain SafeRed. Minipreps were made for colonies with bright bands. HCAR and PPH were also ammonium sulphate precipitated. Chlorophyll was prepared using 80% acetone and spinach.

MONDAY, 9/2/2019

Labour Day. Team took day off.

TUESDAY, 9/3/2019

-Sent S3P 1, 2, and 4 for sequencing.
-Made new 3:1 digestions, precipitations and ligations for two different aliquots of gel-purified CBR.
-Concentrations after precipitations were very low (3.5 and 6.00) and thus discarded.
-Could not find pheophytin; will do that tomorrow.
-Did mINTERLAB collaboration swabbing micha and prabhu's eyeglasses + doorknob on LB Agar plate.

WEDNESDAY, 9/4/2019

-Made new digests and ethanol/salt precipitations for CBR
-Sequencing results for S3P 1, 2, and 4 results came back negative. They were 29% match.
-Column purified PPH and HCAR.
-Ethanol/Salt precipitations for CBR will need to be vacufuged tomorrow.
-Ordered $NaBH_4$ and $CaCO_3$.

THURSDAY, 9/5/2019

None

FRIDAY, 9/6/2019

-Continued vacufuge step for ethanol precipitation.
-Ligated CBR
-Team looked at TLC methods for enzyme assays
-Team made pheophytin a using diluted HCl.

-Team will perform TLC tomorrow

SATURDAY, 9/7/2019

PHEOPHORBIDE WRITE-UPS DUE:

- [ ] HP writeup
- [ ] Profit proposal (due Wednesday)
- [ ] Wiki write-ups (Weekly Summaries)
- [ ] MDSC 507 Comments

WEEK 19 SUMMARY

Sequencing results for SGR 3:1 Pelleted were negative. More digests and ligations for CBR and SGR 3:1 insert: vector were made. PPH and HCAR were column purified again. Chlorophyll a and b was extracted from spinach using 80% acetone, then acidified with 30 mM to make HCl. Team did a collaboration with SoundBio iGEM for mINTERLAB.

MONDAY, 9/9/2019

-Prepared paper discs for disc test by using hole puncher, placing them on beaker, covering it with foil and sending them to dry autoclave.
-Transformations for SGR 1:1 and SGR 2:1 standard and pelleted were re-streaked on different plates.

DISC TEST PROTOCOL:

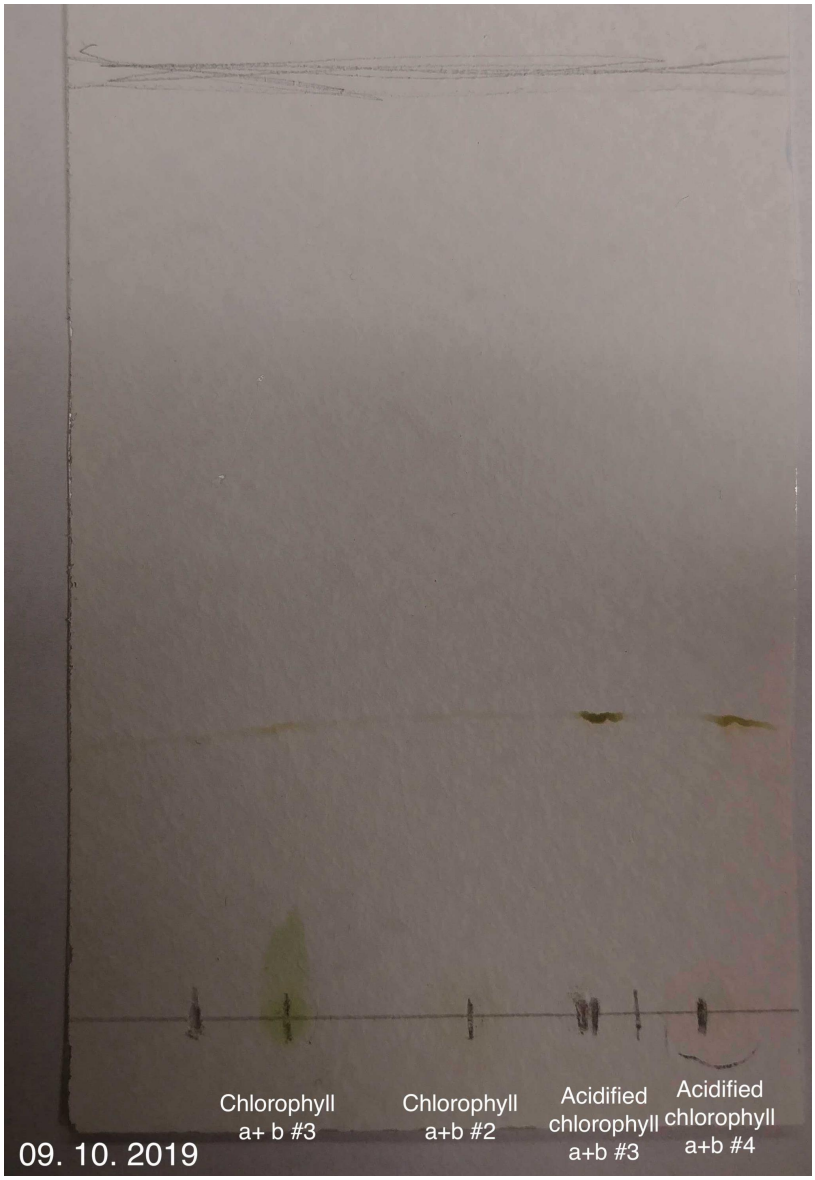
1. Use puncher to punch holes on Whatman filter paper
2. Place in beaker and cover with foil.
3. Dry autoclave. Use asceptically.

TUESDAY, 9/10/2019

-Ran 4th set of column-purified proteins and stained the gel.
-Ran cPCR of SGR 1:1 and SGR 2:1 standard and pelleted.
-Ran first TLC plate trial using flexible cellulose TLC plate and 100% hexane as solvent. Team faced problems with TLC because hexane was not fully moving to top of the plate, and only chlorophyll a + b #3 showed visible movement. This might be due to unconcentrated samples and/or not using a silica plate. Team is also unsure of what to make of the orange colour seen on solvent front on top of chlorophyll a+b #3, and acidified chlorophyl a+b #3 and #4. This might be due to pheophytin or chlorophyll running all the way to the solvent front. Pigment-wise, we suspect that it is pheophytin due to its warm tones.
-Chris acidified Micha chlorophyll sample to create pheophytin a. However, absorbance values before and after acidification all decreased, suggesting chlorophyll a and b, and pheophytin a and b all decreased.

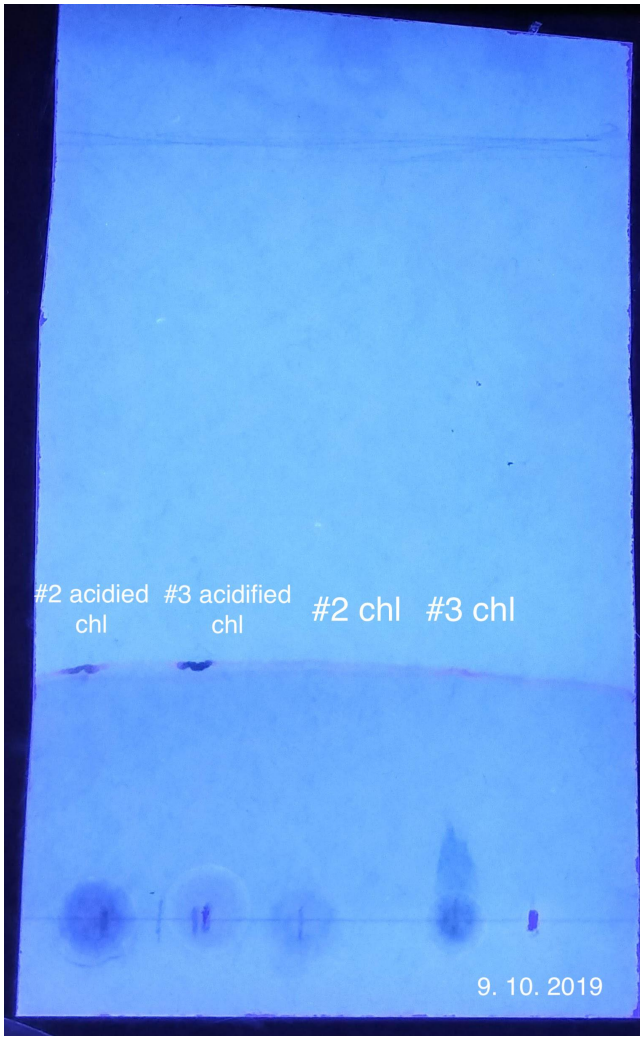
Paper discs, autoclave.

TLC 1.jpg



First TLC trial. 100% Hexane on flexible cellulose TLC plate. Chlorophyll a and b compared to acidified chlorophyll a and b. Acidified #3 and #4 are pheophytin because they are grey and higher up. The topmost streak on chlorophyl a+b #3 is chlorophyll a, and chlorophyll b would be at the bottom.

20190911_170653_HDR (1).jpg

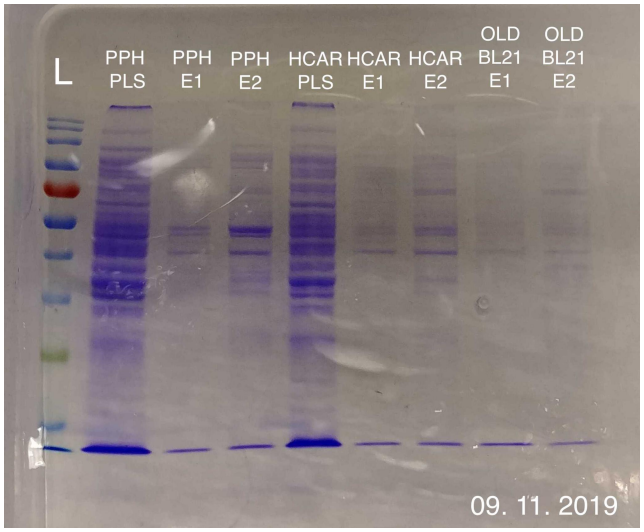


WEDNESDAY, 9/11/2019

-Ran cPCR samples from September 10, 2019 on the gel. Micha dropped the 96-well plate so contamination occured.
-Destained SDS-PAGE gel from September 10, 2019 show fourth confirmation of PPH and third confirmation of HCAR production and purification.
-Overnights were made for _____ cultures with bands of interest.
-Team solubilized pheophorbide a in 25% acetone. Bottle containing pheophorbide now has 35ng/uL with 1.42 mL of 25% acetone.
-Team started paper disc tests fungal plates that were one day old. Discs with varying pheophorbide concentrations were place 2.5 cm away from center of fungal culture.

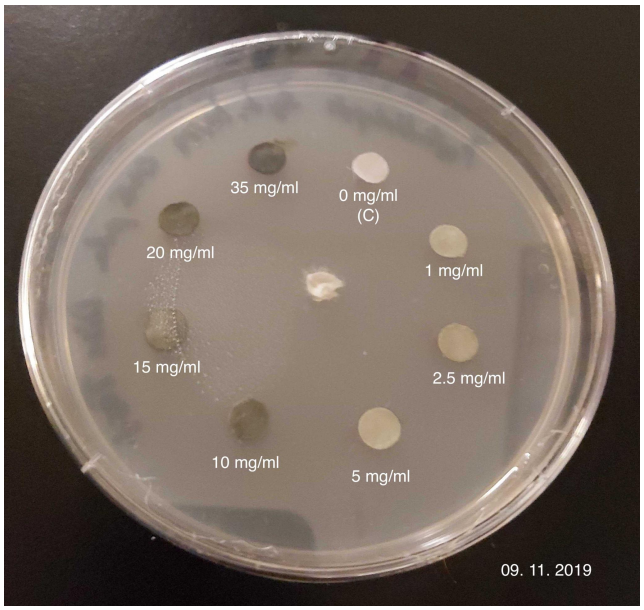
1. Work in an asceptic environment. Dip meetal tweezers in 70% ethanol and flame it to sterilize.
2. Using sterilized tweezers, grab a single autoclaved paper disc and dip into tube containing the solution of interest.
3. Allow solution to fully coat the disc. Tap disc on sides of the test tube to remove excess.
4. Place on desired location on agar plate. Gently tap disc until firm.

GEL 4.jpg

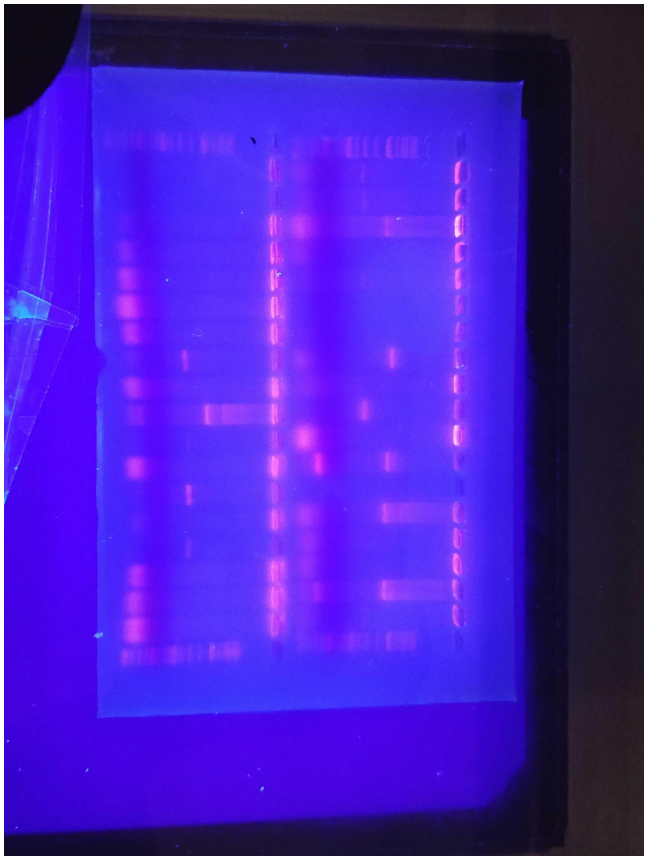


FOURTH PROTEIN GEL. 4/4 Confirmation of PPH and 3/4 Confirmation of HCAR. Samples run were from September 4, 2019 Column purifications. OLD BL21 was from September 14, 2019 (?).

Pheophorbide disc 1 .jpg



20190911_191030.jpg

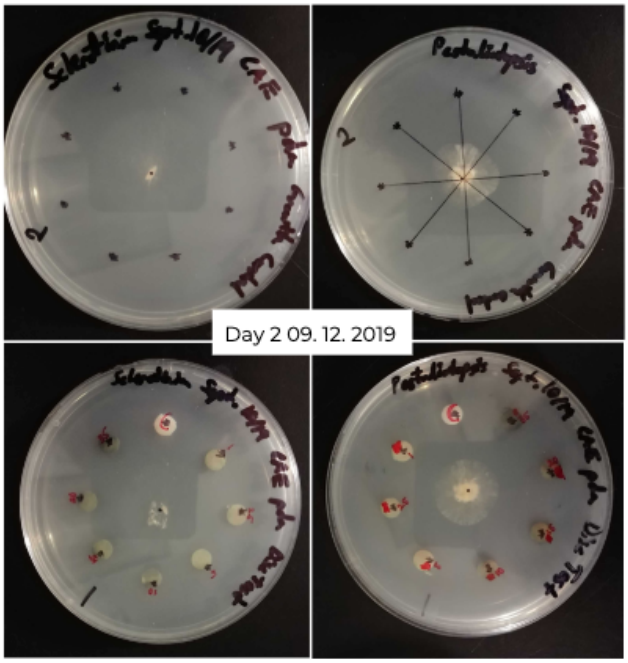


no label!!?? not in wiki

THURSDAY, 9/12/2019

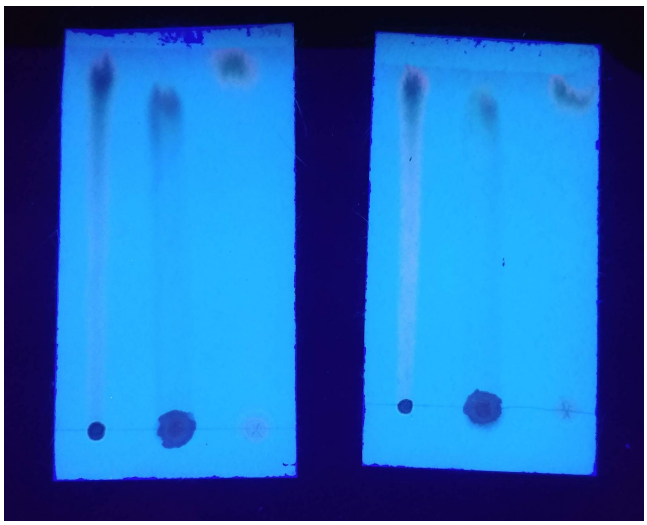
-Day 2 of pheophorbide dark test trial

Screen Shot 2019-09-12 at 7.39.24 PM.png



Day 2!

20190917_215255.jpg



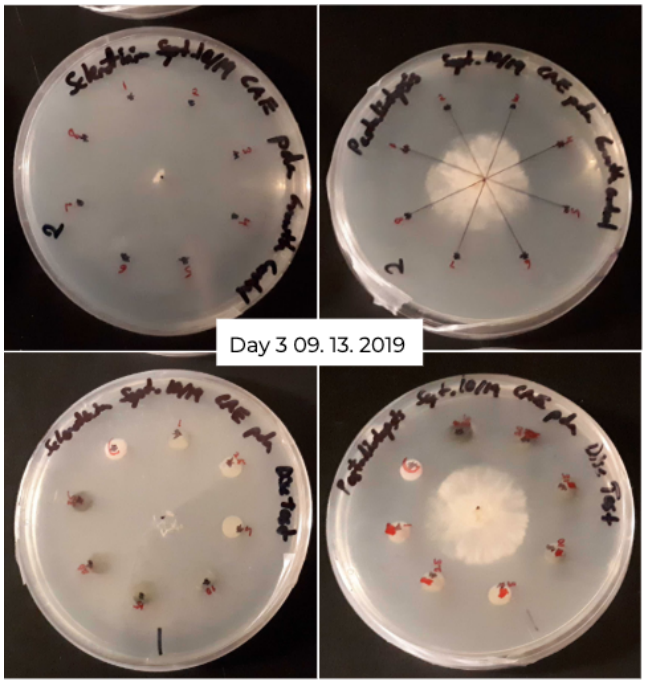
Solvent: 100% Hexane. Pb, Pn, chlorophyll. FIGURE A1

Pb,
Solvent:100% Hexane

FRIDAY, 9/13/2019

-Day 3 of pheophorbide test trial

Screen Shot 2019-09-17 at 3.36.04 PM.png

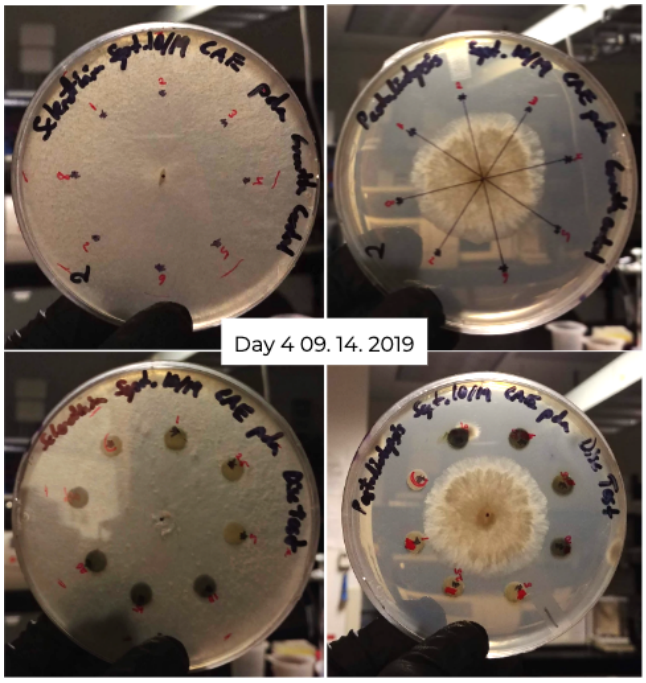


Day 3

SATURDAY, 9/14/2019

-Day 4 of dark pheophorbide testing. Our tests show no apparent effect of pheophorbide a on sclerotinia but some degree of inhibition on pestaliopsis based on the difference in distance the mycelium grew. These results show that pheophorbide show no cytotoxic effect on sclerotinia in the dark. The pestaliopsis will be observed for a couple more days to confirm the dark cytotoxicity of pheophorbide a.
-Micha extracted chlorophyll using 80% acetone, and some using DMSO. 12.5mL of 80% acetone was added to 50g of spinach, and ground thoroughly for 10 minutes. DMSO amounts were not measured. DMSO showed promising chlorophyll a and b extraction, but when ran on the TLC faced problems. DMSO is polar and thus interfered with elution. Micha will never use DMSO in chlorophyll extraction again. Additionally, DMSO has a high boiling point, and so did not evaporate from the cellulose TLC plate.
-Micha tested 25 mg/mL pheophorbide on TLC, it did not run.
-Micha tried the big TLC chamber with the big cellulose TLC plates. Chamber was not saturated so the solvent kept evaporating.

Screen Shot 2019-09-17 at 3.35.56 PM.png



Day 4

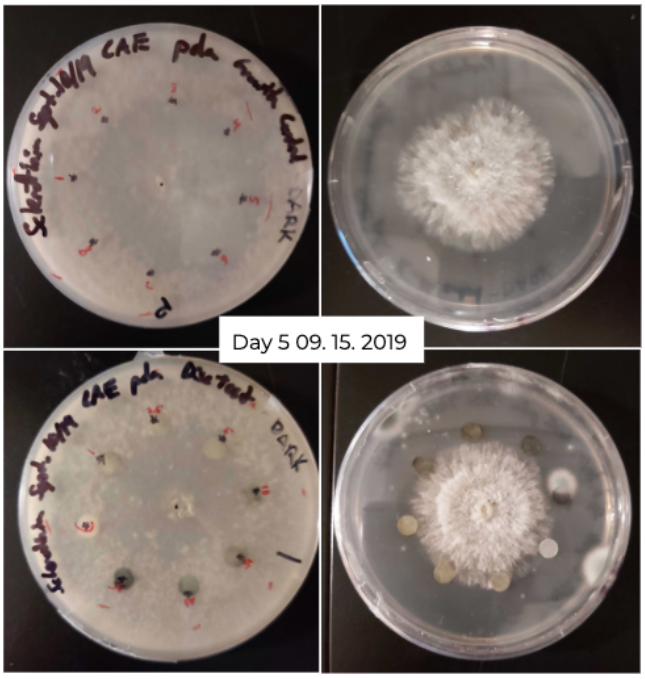
SUNDAY, 9/15/2019

-Chris cultured new fungi plates with double disks at 1.5 cm away from the center of the fungus to the center of the disk, with less pheophorbide concentrations (0, 5, 15, 25, 35 mg/ml). The double disks are to account for the possibility of pheophorbide a diffusing into the agar. Another plate was made, with less concentrations (insert concentrations here) at blank cm away from the center of the fungi to the center of the disc. These two plates are contained in a well-lit box (1400 lumens, white LED) at room temperature for our light trials.

WEEK 20 SUMMARY WEEK 2 SEPT 9-15

More transformations and cPCR were done for SGR. The first thin layer chromatography experiment was run using flexible cellulose TLC plates with 100% hexane as solvent. However, the first plate did not run well because the chamber was not saturated enough. We were unsure of what the orange color seen on the solvent fron on top of chlorophyll #3 and acidified chlorophyll #3 and #2 were. This might be due to pheophytin or chlorophyll all the way to the solvent front. Chris acidified chlorophyll sample to create pheophytin, and absorbance values corresponding to chlorophyll wavelengths decrease and pheophytin wavelengths increased. Pheophorbide did not run on the cellulose plates with 100% hexane. Team also attempted to use the big TLC chamber and run cellulose sheets on 100% hexane but the chamber was not saturated enough so the solvent kept evaporating from the plate. Another SDS-PAGE gel was run, and showed fourth confirmation of PPH and third confirmation of HCAR purification. Pheophorbide a was solubilized in 25% acetone. Dark treatments with pheophorbide paper disc tests across different concentrations on fungal cultures were started. More fungal cultures for light treatments (1400 lumens, white LED) were made with double disks at 1.5 cm away from the center of the fungi to the disk center, with concentrations 0mg/ml, 5 mg/ml, 25mg/ml, and 35mg/ml. The double disks were done to account for the possibility of pheophorbide diffusion into the agar plates.

Screen Shot 2019-09-17 at 3.35.10 PM.png

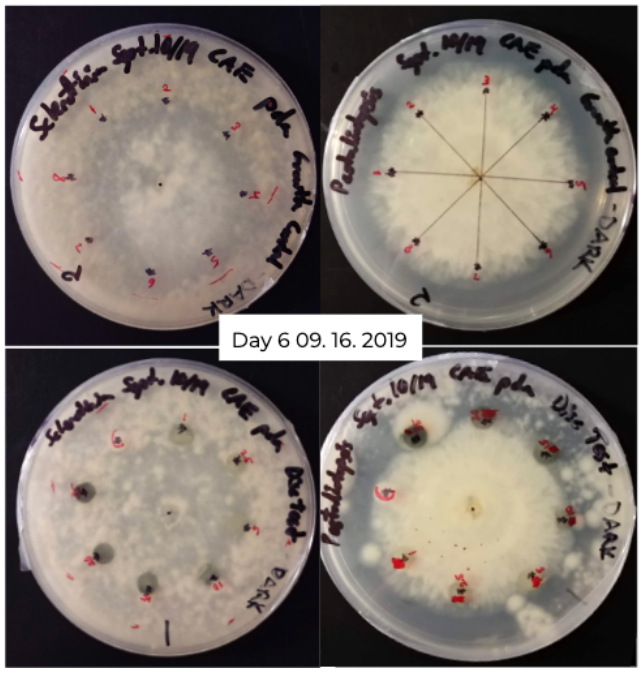


Day 5

MONDAY, 9/16/2019

-Team saw promising results from pheophorbide a light trial

Screen Shot 2019-09-17 at 3.34.21 PM.png



Day 6

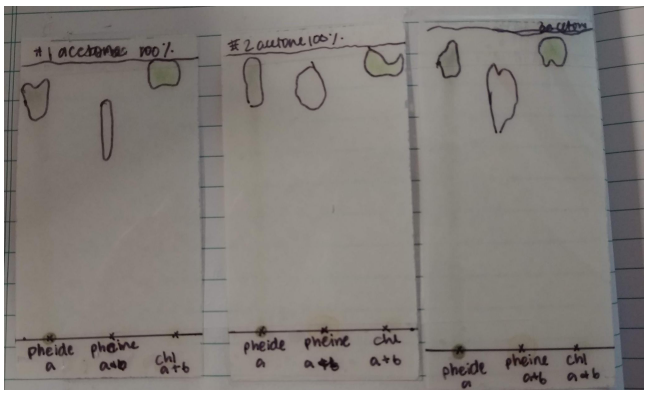
TUESDAY, 9/17/2019

-Ran TLC on 100% acetone solven, using cellulose sheets. Our TLC sheets show that pheophorbide a runs higher than pheophytin a and b.

-Chris made new fungal cultures and added pheophorbide discs to fungal cultures

-Ran cPCR gel but ladder did not appear.

20190917_222037.jpg



Solvent: 100% Acetone FIGURE A2

WEDNESDAY, 9/18/2019

-TLC: Comparison for 100% acetone and 80-20 acetone was done on cellulose plates
-Fungal data collection for Sept. 14 day 4
-Fungal data collection for Sept. 16 Day 2
-CBR cPCR band sizes were promising, made overnights.

72346911_407263233286519_248609367270621184_n.jpg

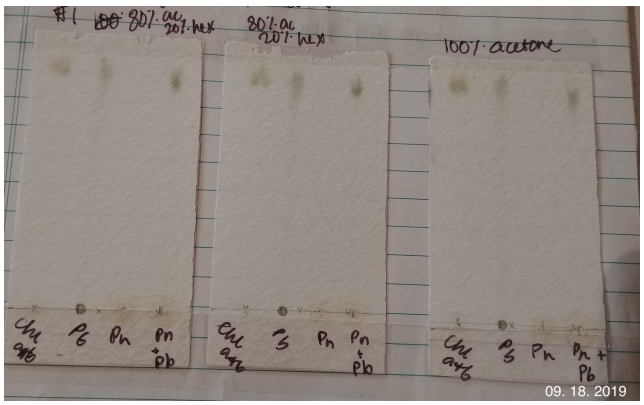


FIGURE A3

SATURDAY, 9/21/2019

AGEM.

Week Summary

This week we collected fungal data and troubleshooted TLC experiments. We tried using 100% acetone and 80:20 acetone:hexane as our solvent which eluted our compounds better than 100% hexane did, but were not separated distinctly. We also did colony PCR for CBR and SGR, and prepared some overnights of CBR colonies.

MONDAY, 9/30/2019

TUESDAY, 10/1/2019

-Digests and ligations for CBR and SGR 2:1 without alcohol precipitations were done.
-Attempted to make new chlorophyll by adding drops of 1M HCl but corroded the silica plates among other things; discarded.
-PPH from elution fraction 2 that was long stored in the -20C fridge without being resuspended in glycerol , or ammonium sulfate precipitated was tested for pheophytin reactions.
The kinetics were observed on Nanodrop under wavelength of 409 but did not show any differences.

- Either protein is dead, or the pH of pheophytin is really bad.
    - Pheophytin used was the TLC pheophytin 1.

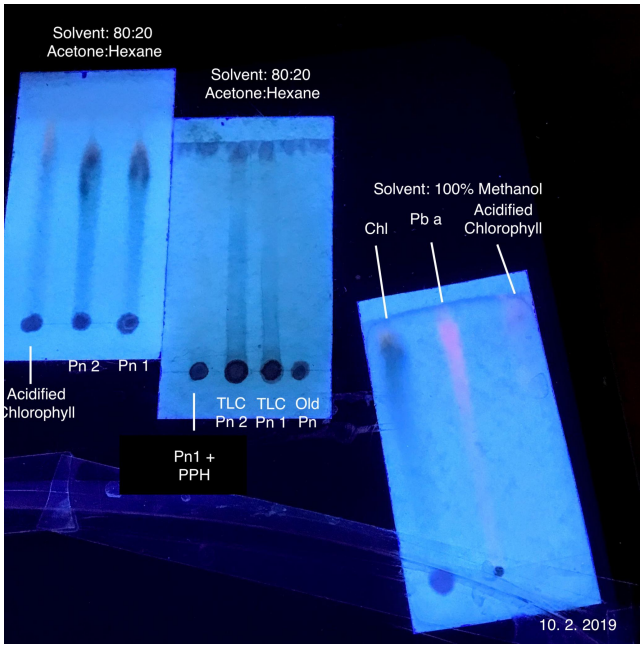
WEDNESDAY, 10/2/2019

-Transformations of CBR and SGR ligations done from (October 1) into DH5-a.
-Sent CBR for sequencing
-New fungal cultures made :

1. 2nd + 3rd replicate of trial that worked (1.5cm, double disc x5)
2. Light growth control (now with closer light source)
3. Half Plates reattempted with 1.5cm + single disc + 2 disc + 35mg/ml vs 0 mg/ml
4. Light treatment: 5 discs double, 1.5cm.

But to reapply pheophorbide** not sure which one
-TLC of old and new pheophytin and new pheophytin + PPH

IMG_3531.jpg



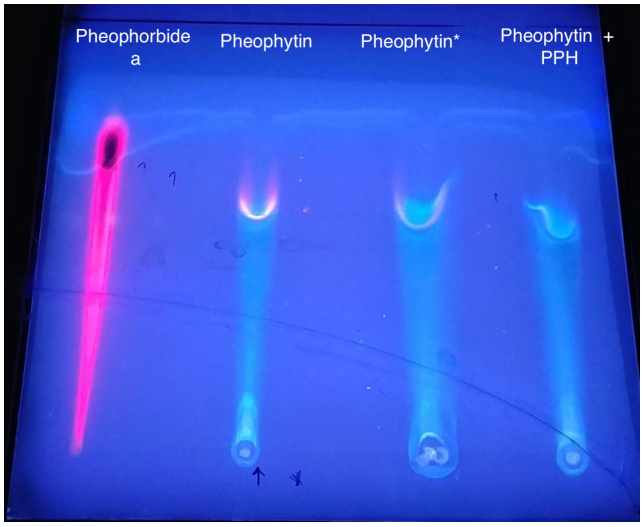
Trying methanol for the first time!?@!>!@#

THURSDAY, 10/3/2019

FRIDAY, 10/4/2019

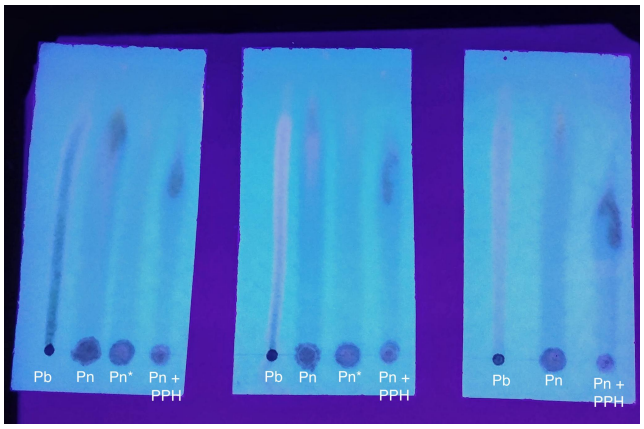
SATURDAY, 10/5/2019

20191005_192234.jpg



Solvent: methanol

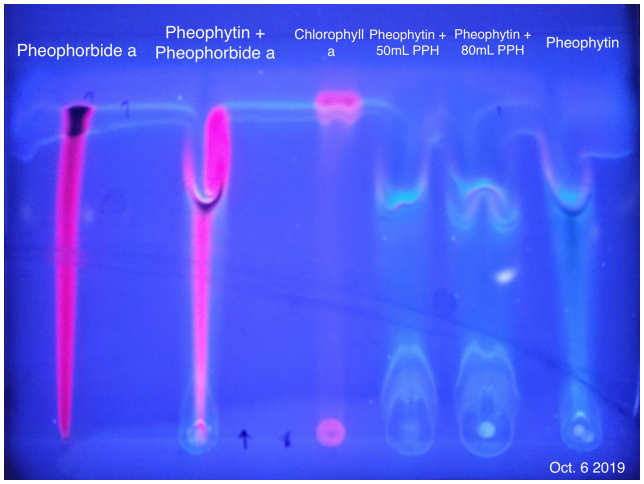
20191005_172809.jpg



Solvent: Methanol

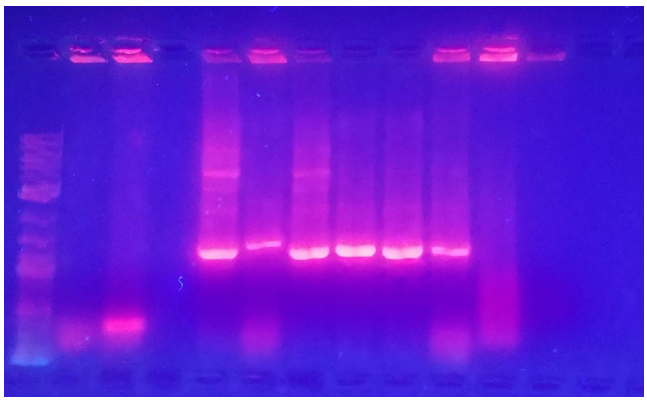
SUNDAY, 10/6/2019

20191006_192513.jpg



Solvent: Methanol. Old pheophytin elution fraction, not ammonium sulfate precipitated. (E2) from september 22

20191006_191019.jpg



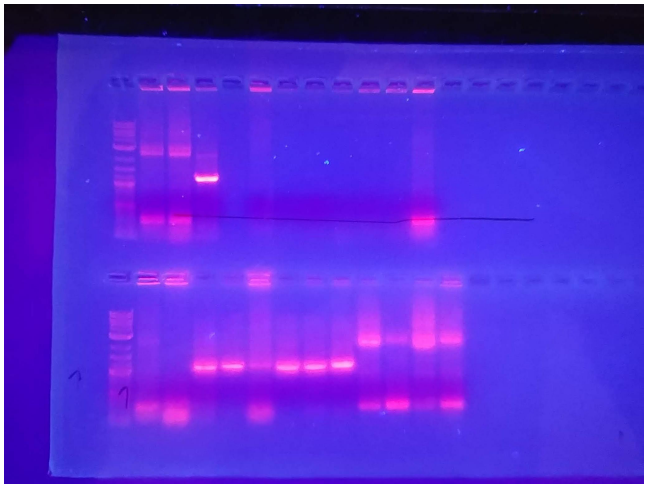
WEEK 23 SUMMARY WEEK 1 OCTOBER 1-6

More CBR and SGR digests, ligations, and transformations (without salt/ethanol precipitation to conserve DNA) in 2:1 ratios into DH-5α were made. The plasmid minipreps were then sent for sequencing, which returned negative. PPH from elution fraction 2 which was long stored in the -20C fridge without being resuspended in glycerol , or ammonium sulfate precipitated was tested for pheophytin reactions using the kinetics function in NanoDrop machine under wavelength of 409nm. However, there was no change observed across 40 minutes, potentially indicating non-functional proteins. Team tried using 100% methanol and 80:20 Acetone:Hexane as a solvent in cellulose plates for Thin Layer Chromatography. Pheophytin was still not eluting fully from the spotting line, so we tried using silica plates for the first time to eliminate the possibility of high adsorption of the compounds to the cellulose plate. Using the silica plate, we were able to visualize the fluroescence of chlorophyll, pheophytin, and pheophorbide for the first time. We ran our first pheophytinase reactions using the aforementioned non-functional proteins with pheophorbide a without reaction buffer with 100% methanol as the solvent but there was little to no red fluorescence.

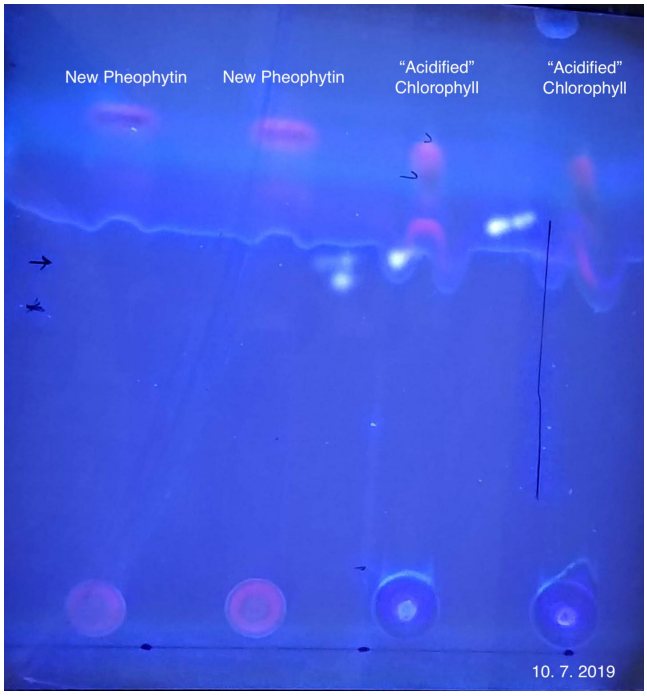
MONDAY, 10/7/2019

-cPCR for SGR and CBR were made
-Oct 2 fungal data day 5 was collected
-Minipreps were made
-Overnights for CBRS 4, CBRP 7, SGRS 2 were made

20191007_222200.jpg

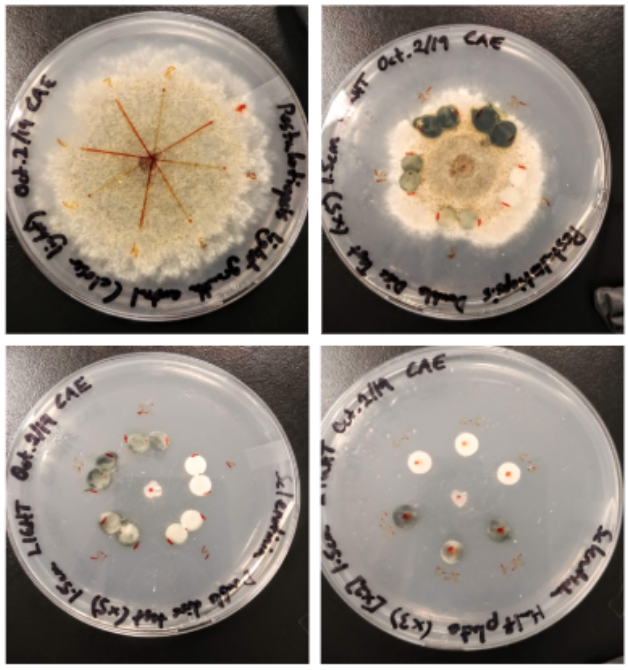


20191007_220643.jpg



Solvent: 100% Methanol

Screen Shot 2019-10-10 at 7.41.10 PM.png



TUESDAY, 10/8/2019

-Fungat data for oct 2 day 6 cultures were gathered
-Overnights for two of each: pSB1A3 BL21, HCAR, and PPH were made in 2mL LB-AMP.

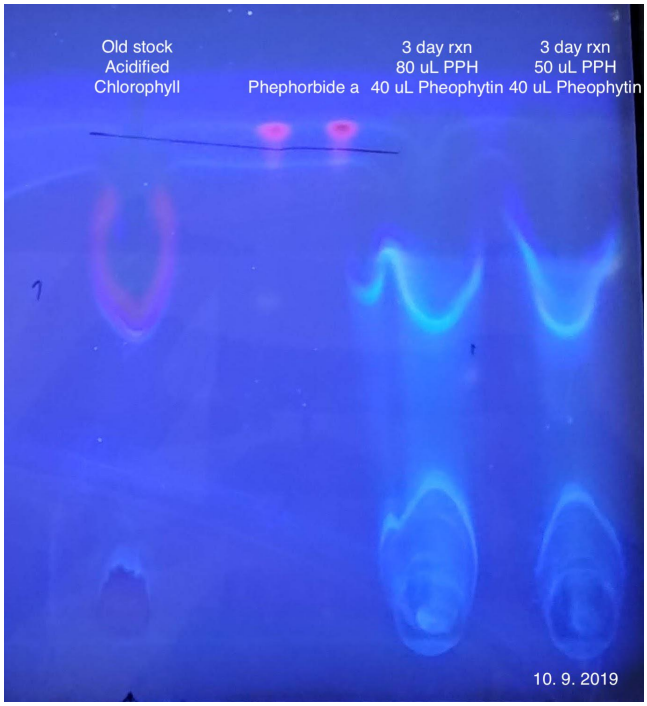
WEDNESDAY, 10/9/2019

-Ammonium Sulfate-containing proteins were spun down because the ammonium sulfate was not fully dissolved. The supernatants from elutions 1, 2, and 3 were aggregated in a tube
-Two of the following: pSB1A3 BL21, HCAR, and PPH were subcultured

THURSDAY, 10/10/2019

-Attempted to make Pheophytin by extracting fresh chlorophyll from spinach in the freezer using 80% acetone but did not work; only brown and green-orange pigments were extracted.
-Subcultures from October 9 were pelleted and put in the freezer.

20191009_225808 (1).jpg

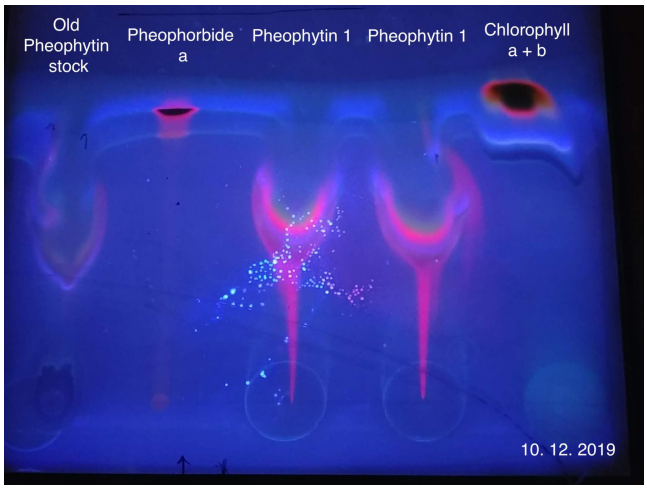


Solvent: Methanol

z

FRIDAY, 10/11/2019

20191011_221557.jpg



Pheophytin, pheophorbide, and chlorophyll a + b visualization. Samples were eluted by Thin Layer Chromatography using methanol:hexane (70:30) solvent on a silica plate.

-Mike sonicated and added lysozyme to the subcultures which will be column purified-- HCAR, PPH, and BL21 pSB1A3 which were prepared.
-Proteins were also sonicated.
-Micha made a fresh batch of chlorophyll using the following protocols:

CHLOROPHYLL EXTRACTION:

- Add 1g fresh spinach leaves without the stem to 20mL 80% acetone.
- Grind using mortal and pestle until spinach leaves is ground up.
- Centrifuge spinach + acetone mixture at -10 degrees, 3750 rpm, for 9 minutes.
- Transfer supernatant to new falcon tube covered with foil.

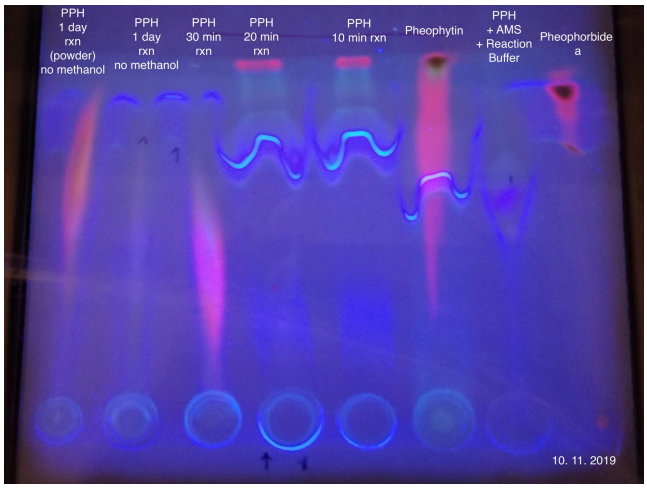
PHEOPHYTIN PRODUCTION

- Add HCl to centrifuged chlorophyll solution (80% acetone + spinach) until the final concentration of HCl in solution is 20mM. Leave for 2 minutes.
- Add NaOH to neutralize the solution until its final concentration in solution is 20mM.
- Check pH with pH probe and add more NaOH or HCl as necessary to achieve desired pH. For pheophytinase reactions, do this until pH 8.0 is obtained.
- Add foil to the container to protect against light.

SATURDAY, 10/12/2019

- Proteins from pSB1A3 BL21, HCAR, PPH subcultures were column purified
- Ran TLC with and without weird protein mix on 70:30 methanol: hexane solvent, silica plate.

20191012_165353.jpg



Solvent used: 70-30 Methanol: Hexane but some hexane might have evaporated because pheophorbide ran lower than pheophytin did, unlike the previous plate. We will need more replicates of this because the first plate (prev) might have been a false positive. Use the weird aggregate

SUNDAY, 10/13/2019

-Massive wiki-writeup session
-Ammonium-sulfate precipitated the proteins for pSB1A3 BL21, HCAR, and PPH elutions 1, 2, and 3.

WEEKLY SUMMARY WEEK 3 OCT 7-13

Team did more digests, ligations, transformations, cPCR, and minipreps for SGR and CBR. pSBIA3, PPH, and HCAR proteins were ammonium sulfate precipitated, but the salt was not fully dissolved so the supernatants from the three elutions were aggregated in a falcon tube. We were unsure wether the proteins would still be functional, but ran the solutions on silica plates using methanol as a solvent. Since pheophorbide and pheophytin were still not easily distinguishable from each other, a solvent mixture of 70:30 methanol:hexane was used. More subcultures were made, induced, protein-purified, and ammonium-sulfate precipitated. New pheophytin stock was made using chlorophyll from fresh spinach, and neutralized to pH 8.0 to ensure pheophytinase will not denature.

MONDAY, 10/14/2019

-Sent CBRS-4, CBRP-7, SGRS-2 for sequencing.

-Ran sets 1 and 2 of Elutions 1, 2, and 3 of pSB1A3, HCAR, and PPH on 10% SDS-PAGE gels. Loaded 15 uL sample + 5uL 4X SDS-loading + 1uL B-mercaptoethanol, which was boiled for 10 minutes.

TUESDAY, 10/15/2019

-TLC: ran PPH reactions from October 14, 2019 using 70:30 methanol:hexane solvent.

-Ran Ammonium-sulfate precipitated pSB1A3 proteins, PPH, and HCAR on 10% SDS-PAGE gel.

-Made new protein overnights to be purified: 2 sets of PPH, HCAR, and pSB1A3 BL21.

-Made new PPH reactions with 1mM EDTA to remove magnesium in solution and account for possibility of pheophorbide a being converted to chlorophyllide a.

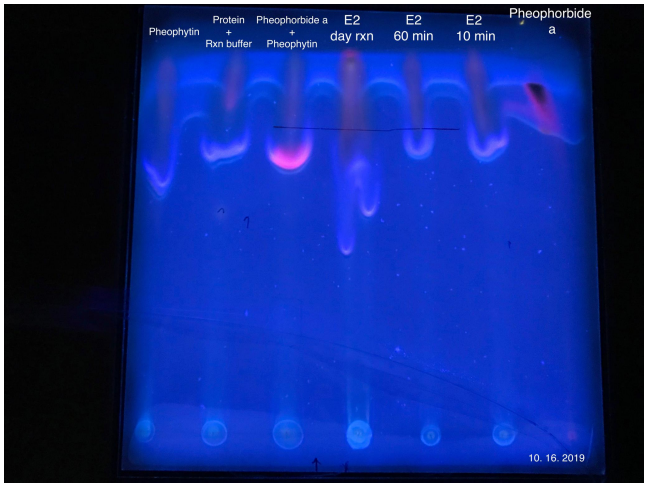
WEDNESDAY, 10/16/2019

-Got plates from Fran, made new fungal cultures.

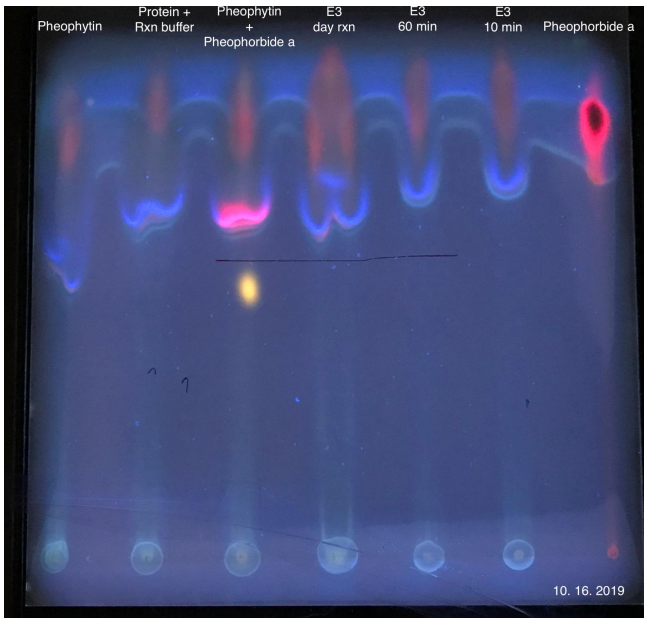
-Subcultured the overnights and induced with IPTG.

-Ran TLC of Oct. 15 2019 reactions with PPH in silica plate using 70:30 methanol:hexane solvent.

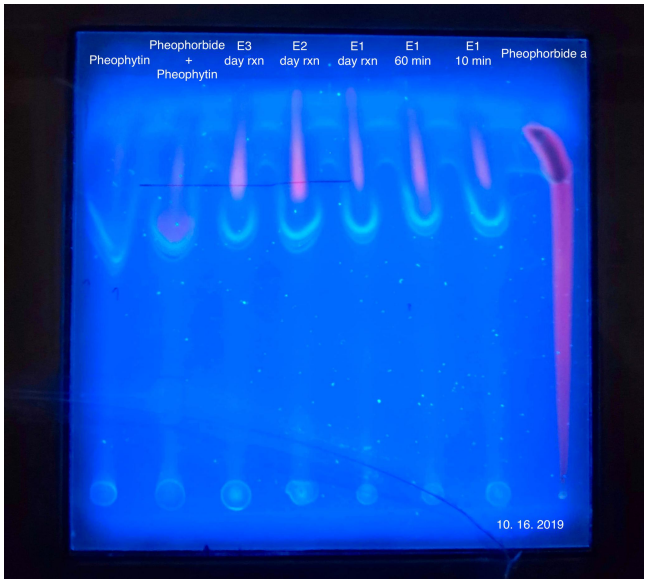
TLC 2.jpg



tlc 3.jpg



TLC1.jpg



THURSDAY, 10/17/2019

-Made new TLC, re-run E2 and E3 because E3 day rxn on tlc #3 from Oct. 16 2019 looks "promising". THis is day 1 rxn. Also I put in only 10 uL for all of the spots.

FRIDAY, 10/18/2019

- Prepared PPH cell lysate and column purifications without ammonium sulfate precipitations.
- Made new pheophytinase reactions using cell lysate and non-ammonium sulfate precipitated pheophytinase elution fractions.
  - 100uL cell lysate or elution fraction
  - 100uL pheophytin

    - 50uL reaction buffer
- Ran day 2 reactions from October 17, 2019 samples on the TLC however it showed no pheophorbide production.

SATURDAY, 10/19/2019

- Ran day 1 pheophytinase reactions from October 18, 2019 on silica plate with 70:30 methanol:hexane as the solvent. Saw pheophorbide production for the first time predominantly in

WEEKLY SUMMARY

This week we sent CBR and SGR minipreprs for sequencing which came back negative. We also ran our two sets of PPH, HCAR, and pSB1A3 column-purified elution fractions in 10% SDS-PAGE gel which showed our bands of interest. A western blot was also made which showed antibody fluorescence for HCAR, and bands without fluorescence for PPH. We started adding pheophorbide a + pheophytin mixtures to our TLC plates as a control. Our reactions still do not show any signs of pheophorbide production. We suspect that the ammonium-sulfate precipitations either precipitated or denatured our proteins, so we prepared cell lysate and non-ammonium sulfate precipitated elution fractions for our pheophytinase reactions. The reactions were left at 37°C for a day then run on silica plates with 70:30 methanol:hexane. We saw pheophorbide a production by pheophytinase for the first time, which was more prominent in the cell lysates than the elution fractions.