AUGUST

Date: Wednesday, 8/1

Objective(s):

Check the protein expression for F3H+DFR & F3'H+FNS.

Day Summary:

The protein gel was left for overnight shaking for the bands to clearly appear.

Procedure:

1. 15% separating gel was prepared in the following manner:

Content	*Volume*
40% Acrylamade	3.75ml
H_2O	3.75 ml
4x Separating Buffer	2.5 ml
10% APS	50 µl
TEMED	9 µl

2. The mixture was quickly poured into the casting glass till the first brim and the bubbles were burst using isopropanol.

3. Separating gel was made to set for thirty minutes.

4. The stacking gel was prepared in the following manner:

Content	*Volume*
40% Acrylamade	0.6 ml
H_2O	3.1 ml
4x Stacking gel Buffer	1.25 ml
10% APS	50 µl
TEMED	12 µl

5. Once the separating gel was set, the isopropanol was poured out.

6. The stacking gel was poured into the casting glass till the second brim. The comb was inserted into the casting gel carefully and was used to burst the bubbles.

7. The gel was made to set for thirty minutes.

8. Eight samples were used for this experiment:

   F3H-DFR control at 30℃ for two days
   F3H-DFR I : Induced with 1mM AHL inducer at 30℃ for two days
   F3'H-FNS control at 30℃ for two days
   F3'H-FNS I: Induced with 200 nM tetracycline and kept in the dark at 30℃ for two days
   F3H-DFR control at 37℃ for two days
   F3H-DFR I : Induced with 1µM AHL inducer at 37℃ for two days
   F3'H-FNS control at 37℃ for two days
   F3'H-FNS I: Induced with 200 nM tetracycline and kept in the dark at 37℃ for two days

9. The samples were placed in a centrifuge and spun down for six minutes.

10. 1X Binding buffer was produced in the following way:

Ingredient	*Concentration*
Imidazole	5 mM
NaCl	500 M
Tris-HCl, pH 7.9	20 mM
$MgCl_2$	5 mM

11. The cells were resuspended using 300µl of 1x binding buffer and then all the contents were then transferred into their respective Eppendorf tubes.

12. The first Eppendorf tube (F3H-DFR control) was placed inside a beaker filled with ice and the cells were then lysed by sonication.

13. Sonicator settings:

Amplitude	10
Process Time	1 minute
Pulse ON Time	5 seconds
Pulse OFF Time	15 seconds

14. The sonication was repeated for the other seven Eppendorf tubes.

15. Once all cell samples were lysed, they were spun down for 10 minutes in a refrigerated centrifuge.

16. 16 Eppendorf tubes were prepared, two for each sample, one for the supernatant and one for pallete. All the sixteen Eppendorf tubes were filled with 10µl of 2x loading dye.

17. Once the refrigerated centrifuge was done, the supernatant was transferred to separate Eppendorf tubes. The pallets were then resuspended using 300µl 1x binding buffer.

18. 100µl of suspended pallets and supernatants were transferred to their respective Eppendorf tubes (the ones with loading dyes).

19. All the sixteen tubes were placed in a heat block at 95℃ for five minutes. After five minutes the tubes underwent a short spin. The gel ran for sixty minutes at 150V.

20. Once the electrophoresis was done, the gel was carefully extract the gel from the glass casting, the stacking layer was removed and then the remaining gel was submerged in water.

21. The submerged gel was then swirled using an orbital shaker at 50 rpm for 5 minutes. After 5 minutes, the water was changed. This process was repeated two more times and the final waste water was rinsed out.

22. BLS was poured into the container until the gel was submerged. It was the heated in a microwave at high for one minute.

23. Once heated, the gel was then shaken in an orbital shaker at fifty rpm for one hour.

24. Bls was then poured out and the gel was rinsed thoroughly.

25. The container was once again filled with water till the gel was submerged and was left for overnight shaken in an orbital shaker at 50 rpm.

Date: Friday, 8/3

Day Summary:

dsDNA concentrations for Plasmid 2 (ANS 1 & ANS 2) were low after plasmid purification (<60 ng/µL) but were still sent for sequencing. Plasmid 1 (Denovo A & Denovo B) were sent for sequencing.

Procedure:

DNA Purification for Plasmids 1 and 2

1. DNA purification was carried out for the 4 plasmids (Denovo A, Denovo B, ANS 1 & ANS 2) as described in 'QIAprep Plasmid DNA Purification Using QIAprep Spin Miniprep Kit and a Microcentrifuge' protocol.

2. Plasmid concentrations of samples Denovo A, Denovo B, ANS 1 and ANS 2 were measured and recorded in the table below.

Sample	*Concentration (ng/µL)*
Denovo A	143
Denovo B	156
ANS 1	52.08
ANS 2	47.8

Date: Tuesday, 8/7

Objective(s):
Running HPLC of luteolin standard and lab produced luteolin

Day Summary:
Overnight running of HPLC of ten different luteolin samples

Procedure:

1. 50mM of luteolin was prepared using DMSO.

2. Eight HPLC vials were prepared and 50µL of the following was filled into each of the vials:

    a. Negative control with naringenin, sample 1.3
    b. Negative control without naringenin, sample 1.3
    c. Experimental with naringenin, sample 1.3
    d. Experimental without naringenin, sample 1.3
    e. pABC - FNS, sample 1.2
    f. Brep - F3'H, sample 1.2
    g. 20mM of standard luteolin
    h. 5mM of standard luteolin

3. The buffer system used was 100 mM Ammonium acetate and 100% acetone. The pumps were then purged with their respective buffer.

4. The column was connected to the system and the samples were keyed in.

5. The column was attached to the HPLC machine using the arrow on the column as reference. It was ensured that there was no leakage and that the pressure was maintained below 100 bar.

6. HPLC cycle was conducted in the following manner:

Action	*Time (mins)*	*Volume (µL)*	*Remarks*
Flush	15	1	Methanol
Equilibrate	15	1	Methanol

Blank - DMSO	46	10	-
Equilibrate	15	1	Methanol
Negative control with naringenin, sample 1.3	46	10	-
Equilibrate	15	1	Methanol
Negative control without naringenin, sample 1.3	46	10	-
Equilibrate	15	1	Methanol
Experimental with naringenin, sample 1.3	46	10	-
Equilibrate	15	1	Methanol
Experimental without naringenin, sample 1.3	46	10	-
Equilibrate	15	1	Methanol
pABC - FNS, sample 1.2	46	10	-
Equilibrate	15	1	Methanol
Brep - F3'H, sample 1.2	46	10	-
Equilibrate	15	1	Methanol
20mM of standard luteolin	46	10	-
Equilibrate	15	1	Methanol
5mM of standard luteolin	46	10	-
Equilibrate	15	1	Methanol
Flush	15	1	Methanol

Date: Friday, 8/10

Objective(s):

Check the protein expression for F3H+DFR & F3'H+FNS.

Day Summary:

The protein gel was left for overnight shaking for the bands to clearly appear.

Procedure:

1. 15% separating gel was prepared in the following manner:

Content	*Volume*

40% Acrylamade	3.75ml
H_2O	3.75ml
4x Separating Buffer	2.5ml
10% APS	50µl
TEMED	9µl

2. The mixture was quickly poured into the casting glass till the first brim and the bubbles were burst using isopropanol.

3. Separating gel was made to set for thirty minutes.

4. The stacking gel was prepared in the following manner:

Content	*Volume*
40% Acrylamade	0.6ml
H_2O	3.1ml
4x Stacking gel Buffer	1.25ml
10% APS	50µl
TEMED	12µl

5. Once the separating gel was set, the isopropanol was poured out.

6. The stacking gel was poured into the casting glass till the second brim. The comb was inserted into the casting gel carefully and was used to burst the bubbles.

7. The gel was made to set for thirty minutes.

8. Eight samples were used for this experiment:

   F3H-DFR control at 37℃ for two days
   F3H-DFR I: Induced with 2mM AHL inducer at 30℃ for two days
   F3'H-FNS control at 30℃ for two days
   F3'H-FNS I: Induced with 50 nM tetracycline and kept in the dark at 30℃ for two days
   F3'H-FNS I: Induced with 100 nM tetracycline and kept in the dark at 30℃ for two days
   F3'H-FNS I: Induced with 150 nM tetracycline and kept in the dark at 30℃ for two days

9. The samples were placed in a centrifuge and spun down for six minutes.

10. 1X Binding buffer was produced in the following way:

Contents	*Concentration*
Imidazole	5 mM
NaCl	500 M
Tris-HCl, pH 7.9	20 mM
$MgCl_2$	5 mM

11. The cells were resuspended using 300µl of 1x binding buffer and then all the contents were then transferred into their respective Eppendorf tubes.

12. The first Eppendorf tube (F3H-DFR control) was placed inside a beaker filled with ice and the cells were then lysed by sonication.

13. Sonicator settings:

Amplitude	10
Process Time	1 minute
Pulse ON Time	5 seconds
Pulse OFF Time	15 seconds

14. The sonication was repeated for the other five Eppendorf tubes.

15. Once all cell samples were lysed, they were spun down for 10 minutes in a refrigerated centrifuge.

16. Twelve Eppendorf tubes were prepared, two for each sample, one for the supernatant and one for pallete. All the sixteen Eppendorf tubes were filled with 10 µl of 2X loading dye.

17. Once the refrigerated centrifuge was done, the supernatant was transferred to separate Eppendorf tubes. The pallets were then resuspended using 300 µl 1x binding buffer.

18. 100 µl of suspended pallets and supernatants were transferred to their respective Eppendorf tubes (the ones with loading dyes).

19. All the sixteen tubes were placed in a heat block at 95℃ for five minutes. After five minutes the tubes underwent a short spin.

20. The samples and the ladder were injected into the wells of the gel in the following way: The gel ran for sixty minutes at 150 V.

21. Once the electrophoresis was done, the gel was carefully extract the gel from the glass casting, the stacking layer was removed and then the remaining gel was submerged in water.

22. The submerged gel was then swirls using an orbital shaker at 50 rpm for 5 minutes. After 5 minutes, the water was changed. This process was repeated two more times and the final waste water was rinsed out.

23. BLS was poured into the container until the gel was submerged. It was the heated in a microwave at high for one minute.

24. Once heated, the gel was then shaken in an orbital shaker at 50 rpm for one hour.

25. Bls was then poured out and the gel was rinsed thoroughly.

26. The container was once again filled with water till the gel was submerged and was left for overnight shaken in an orbital shaker at 50 rpm.

Date: Tuesday, 8/13

Objective(s):

Organic extraction of luteolin from media samples provided by Group 2.

Day Summary:

Organic extraction of samples completed.

Procedure:

1. 10 ml of each media was mixed with 1 ml of 6M HCl to acidify the media.

2. 2 ml of ethyl acetate was added to mixture and it was inverted 4-6 times to mix.

3. The mixture was spun down in a centrifuge for 3 minutes at 600 rpm to form two immiscible layers; top layer was the organic layer and the bottom layer was the media.

4. The top layer pipetted out.

5. The organic layer was then centrifuged under vacuum for 40 minutes.

6. The steps were repeated four times to obtain all the organic material.

7. Collect the dry powder.

Date: Tuesday, 8/14

Objective(s):

Running HPLC of luteolin standard and lab produced luteolin

Day Summary:

Overnight running of HPLC of 12 different luteolin samples

Procedure:

1. 50mM of luteolin was prepared using DMSO.

2. Twelve HPLC vials were prepared and 50µL of the following was filled into each of the vials:

    a. Negative control with naringenin, sample 1.3
    b. Negative control without naringenin, sample 1.3
    c. Experimental with naringenin, sample 1.3
    d. Experimental without naringenin, sample 1.3
    e. pABC – FNS, sample 1.2
    f. Brep – F3'H, sample 1.2
    g. 2.5mM of standard luteolin
    h. 1mM of standard luteolin
    i. 0.5mM of standard luteolin
    j. 0.1mM of standard luteolin
    k. 40µM of standard luteolin
    l. Blank (DMSO + Methanol)

3. The buffer system used was 100 mM Ammonium acetate and 100% acetone. The pumps were then purged with their respective buffer.

4. The column was connected to the system and the samples were keyed in.

5. The column was attached to the HPLC machine using the arrow on the column as reference. It was ensured that there was no leakage and that the pressure was maintained below 100 bar.

6. HPLC cycle was conducted in the following manner:

Action	*Time (mins)*	*Volume (µL)*	*Remarks*
Flush	15	1	Methanol
Equilibrate	15	1	Methanol
Blank - DMSO	46	10	-
Equilibrate	15	1	Methanol
Negative control with naringenin, sample 1.3	46	10	-
Equilibrate	15	1	Methanol
Negative control without naringenin, sample 1.3	46	10	-
Equilibrate	15	1	Methanol
Experimental with naringenin, sample 1.3	46	10	-

Equilibrate	15	1	Methanol
Experimental without naringenin, sample 1.3	46	10	-
Equilibrate	15	1	Methanol
pABC - FNS, sample 1.2	46	10	-
Equilibrate	15	1	Methanol
Brep - F3'H, sample 1.2	46	10	-
Equilibrate	15	1	Methanol
2.5mM of standard luteolin	46	10	-
Equilibrate	15	1	Methanol
1mM of standard luteolin	46	10	-
Equilibrate	15	1	Methanol
0.5mM of standard luteolin	46	10	-
Equilibrate	15	1	Methanol
0.1mM of standard luteolin	46	10	-
Equilibrate	15	1	Methanol
40µM of standard luteolin	46	10	-
Equilibrate	15	1	Methanol
Flush	15	1	Methanol

Date: Tuesday, 8/14

Objective(s):

Transformation of Plasmid 1 into DH10β via heat shock

Plating of transformed DH10β into [1] LB and [2] LB + Strep50.

Overnight inoculation of transformed DH10β for storage at -80°C.

Procedure:

1. Plasmid 1 was reassembled via Gibson's Assembly with missing PAL fragment.

2. Plasmid 1 was transformed into DH10β using the following heat shock protocol:

Procedure	*Duration*
Ice	30 mins

Heat at 42 °C	35s
Ice	2 mins

3. DH10β cells were allowed to recover with 200µL of LB at 37°C for 30 mins.

4. 2 tubes were prepared for overnight inoculation under the 2 conditions [1] LB and [2] LB + Strep50. The following were added to each tube:

	Tube 1	*Tube 2*
LB	4 ml	4ml
Strep50	-	4µL
DH10β	4µL	4µL
Plasmid 1	3µL	3µL

5. DH10β cells were plated under the 2 conditions [1] LB and [2] LB + Strep50.

Date: Wednesday, 8/15

Objective(s):
Grow up Group 2's sample and analyse HPLC results for luteolin

Day Summary:
Inoculation done and sample left to grow for two days; retention time for luteolin was identified.

Procedure:

1. Add the sample to 50 ml of LB.

2. Add 25µg/ml of streptomycin into the sample.

3. HPLC. Data derived from graphs.

Concentration	*Area*
40 µm	246567
100 µm	757328
500 µm	2975132
1 mM	6772853

The retention for luteolin was identified as 30.3 min.

Date: Wednesday, 8/28

Objective(s):
To do organic extraction for (a) BL21* + Naringenin, (b) F3'H + Naringenin, (c) FNS + Naringenin and (d) Experimental + Naringenin, and perform PCR for the deNoVo-PAL plasmid.

Day Summary:
Organic extraction was completed successfully and ready for HPLC.

Procedure for HPLC:

1. Add 1ml of 6N HCl to 10 ml of the sample in an 15 ml eppendorf tube.
2. Add 2ml of ethyl acetate to the mixture.
3. Centrifuge the mixture for 3 minutes
4. Pipette the top layer out carefully into 2 ml Eppendorf tubes. Do not pipette the bottom layer!
5. Vacuum Centrifuge it for 35 mins with the caps open.
6. Add 1.5ml of ethyl acetate to the original mixture to the remaining sample.
7. Centrifuge the mixture for 3 minutes.
8. Pipette the top layer to the same 2 ml Eppendorf tube.
9. Vacuum centrifuge for 40 mins with the caps open.
10. Repeat steps 6-9.
11. Dissolve the organic solute obtained in DMSO and transfer them to HPLC vials.

Procedure for PCR:

1. PCR for deNovo. Temp - 72°C, Time - 82 secs

PrimeSTAR Max Premix	25µL
Con-MCS Rev	0.75µL
New PAL-4CL For	0.75µL
DeNoVo Lace	0.8µL
ddH_2O	25µL

2. PCR for new PAL. Temp - 72°C, Time - 82 secs

PrimeSTAR Max Premix	25µL

Con-MCS Rev	0.75µL
New PAL-4CL For	0.75µL
PEaat 5 mini prep	1.5µL
ddH_2O	22µL

3. PCR for PhtpG1-RBS. Temp - 72°C, Time - 5 secs

PrimeSTAR Max Premix	25µL
PCDF-htp61 F	0.75µL
RFP-htp61 R	0.75µL
PhtpG1-RBS gblock	0.5µL
ddH_2O	23µL

4. PCR for PhtpG1-GFP. Temp - 72°C, Time - 5 secs

PrimeSTAR Max Premix	25µL
PhtpG1-GFP F	0.75µL
tepCDF-GFP R	0.75µL
GFP backbone	0.5µL
ddH_2O	23µL

5. PCR for PhtpG1-RFP. Temp - 72°C, Time - 37 secs, Pressure - 3617 bp

PrimeSTAR Max Premix	25µL
PhtpG1-RFP F	0.75µL
PCDF-RFP R	0.75µL
AHL COLE backbone	1.8µL
ddH_2O	21.7µL

6. PCR for htp61-RFP. Temp - 72°C, Time - 37 secs, Pressure - 3617 bp

PrimeSTAR Max Premix	25µL
RFP-PCDF F	0.75µL

PhtpG1-RFP R	0.75µL
PCDF	1.5µL
ddH_2O	22µL

Date: Wednesday, 8/29

Objective(s):

Gibson assembly of Plasmids (1) Denovo + PAL (2) PCDF-htpG1-RFP (3) PSBIC3-plaC-GFP-p15a. Transformation of Plasmid (1) Denovo + PAL (2) PCDF-htpG1-RFP (3) PSBIC3-plaC-GFP-p15a into DH5α competent cells before plating them..

Day Summary:

Gibson assembly was completed for Plasmids (1) Denovo + PAL (2) PCDF-htpG1-RFP (3) PSBIC3-plaC-GFP-p15a and plated respectively to form colonies overnight. 2 colonies of cells with Plasmid Denovo + PAL, and the only colony of cells with Plasmid PCDF-htpG1-RFP were chosen and cultured overnight. The samples were labelled N1, N2 and RFP respectively.

Procedure:

1. 3 Eppendorf tubes was labelled for Plasmid (1) Denovo + PAL (2) PCDF-htpG1-RFP (3) PSBIC3-plaC-GFP-p15a.

2. Reagents for the Gibson assembly for Plasmid (1) Denovo + PAL (2) PCDF-htpG1-RFP (3) PSBIC3-plaC-GFP-p15a were added to the Eppendorf tubes 1, 2 & 3 as per the tables below respectively.

   Eppendorf tube 1 for Gibson assembly for Plasmid Denovo + PAL:

Reagent	*Amount* (µL)
2X Gibson Assembly Master Mix	5.0
New Denovo PCR	2.1
New PAL PCR (28/8)	0.4
ddH2O	2.5

   Eppendorf tube 2 for Gibson assembly for Plasmid PCDF-htpG1-RFP:

Reagent	*Amount* (µL)
2X Gibson Assembly Master Mix	5.0
htpG1-RBS PCR	0.2
htpG1-RFP PCR	0.2

PCDF PCR	0.7
ddH2O	3.9

Eppendorf tube 3 for Gibson assembly for Plasmid PSBIC3-plaC-GFP-p15a:

Reagent	*Amount* (µL)
2X Gibson Assembly Master Mix	5.0
PSBIC3 PCR	0.3
plaC - GFP PCR	0.2
GFP-PSBIC3 PCR	0.2
P15a PCR	0.2
ddH2O	4.1

3. Spin down Eppendorf tubes 1, 2 & 3 for 10 secs to mix reagents thoroughly. Short spin only.

4. Carry out Gibson assembly for Eppendorf tube 1, 2 & 3. Settings: 50C for 1 hour and 12°C for infinite Hold

5. 3 electroporation cuvettes and 3 Eppendorf tubes 1a, 2a & 3a were labelled for Plasmid (1) Denovo + PAL (2) PCDF-htpG1-RFP (3) PSBIC3-plaC-GFP-p15a respectively.

6. 2 µL of Plasmid (1) Denovo + PAL (2) PCDF-htpG1-RFP (3) PSBIC3-plaC-GFP-p15a each were pipetted into 3 Eppendorf tubes containing 50µL of DH5α competent cells respectively. Keep on ice.

7. The mixture in all three Eppendorf tubes were resuspended. The entire contents of the 3 tubes were then pipetted into their respective electroporation cuvettes 1, 2 & 3. Wipe down the sides of the electroporation cuvettes. Electroporate.

8. The Bunsen burner was turned on to keep the surrounding environment sterile prior to opening the LB bottle. 500µL of LB was added to each of electroporation cuvettes 1, 2 & 3. Resuspend before transferring all contents to fresh Eppendorf tubes 1a, 2a & 3a respectively.

9. DH5α competent cells in Eppendorf tube 1a, 2a & 3a were allowed to recover for 45 min at 37°C and 220 rpm.

10. 100 µL of cells each transformed with Plasmid (1) Denovo + PAL (2) PCDF-htpG1-RFP (3) PSBIC3-plaC-GFP-p15a were plated respectively and incubated overnight at 37°C.

Date: Thursday, 8/30

Day Summary:

dsDNA concentrations for Plasmid (1) Denovo + PAL (2) PCDF-htpG1-RFP were sent for sequencing.

Procedure:

DNA Purification for Plasmids (1) Denovo + PAL (2) PCDF-htpG1-RFP

1. DNA purification was carried out for the 3 samples (N1, N2, RFP) as described in 'QIAprep Plasmid DNA Purification Using QIAprep Spin Miniprep Kit and a Microcentrifuge' protocol.

2. Plasmid concentrations of samples N1, N2, RFP were measured and recorded in the table below.

Sample	*Concentration (ng/μL)*
N1	352
N2	361
RFP	130