iGEM 2018: NUS Singapore-A
WetLab Group 2_July 2018 Logbook

2nd July 2018, Monday

- **Pick colonies**
  - Brep-AAV 2

3rd July 2018, Monday

- **Plasmid extraction**
  - Brep-AAV 2
- **Arrival of gblocks from IDT**
  - Brep-34-F3'H
  - F3H-34-DRF
  - LCR-Laccase
  - Cph8

4th July 2018, Tuesday

- **Plasmid construction &**
  - pLasl-F3H-pLasl-DFR
- **Cell inoculation for Blue light characterisation (5th July)**
  - From Glycerol stock*
  - Bind control, ID2, IA1, IY1
  - Brep control, RD2, RY2

5th July 2018, Wednesday

- **Blue light characterisation**
  - 3-hr-ON-3hr-OFF for both Bind & Brep
  - 1hr Refreshing (200ul Overnight culture into 5ml LB+K)

OD600 after 1-hr Refreshing			
Bind		Brep	
Bind	0.38	Brep	0.32
ID2	0.38	RD1	0,33
IA1	0.35	RY2	0,35

IY1	0.47		

- **Pick colonies**
    - pLasl-F3H-pLasl-DFR-TOP10
- **Sequencing result**
    - pLasl-F3H-DFR, missing pLasl promoter
    - Plasmid backbone was verified okay

6th July 2018, Thursday

- **Plasmid extraction**
    - pLasl-F3H-DFR-TOP, [27.6ng/ul]

9th July 2018, Monday

- **Primers received**
    - EL222-Brep-FWD1
    - EL222-Brep-FWD2
    - EL222-Brep-REV2
    - pBbE2K REV
- **Gene fragment amplification**
    - Brep-F3’H-pTet-FNS

10th July 2018, Tuesday

- **Plasmid construction**
    - Brep-F3H-DFR
    - Brep-F3’H-pTet-FNS
    - pLasL-F3H-DFR backbone
- **Gblock amplification**
    - F3H+DFR
    - F3’H
    - FNS
- **Gel extraction**
    - F3’H-FNS backbone fragment
- **Preparation for Blue light characterisation**
    - Inoculation of overnight cultures

12th July 2018, Thursday

- **Arrival of primers**
    - F3H-DFR-FWD & REV
    - Brep-34-F3’H FWD & REV
    - FNS FWD & REV
- **Gel extraction for amplified glock**

    - F3H+DFR
    - F3'H
    - FNS
- **Plasmid extraction**
    - Brep-F3'H-FNS, TOP10

13th July 2018, Friday

- **Synchronised Blue light characterisation**
    - Brep-RFP
    - Brep-RFP-YbaQ
    - Brep-RFP-DAS
- **Plasmid construction**
    - Brep-F3'H-pTet-FNS
        - *Assembly was performed at 45 °C, 60 min
- **Arrival of gblocks**
    - 3GT
    - ANS
- **Extraction of T7 RNA polymerase from iGEM 2017 kit**
    - Plate 4, Well E1 & C1

16th July 2018, Monday

- **Preparation for Blue light characterisation**
    - Inoculation of overnight cultures from glycerol stocks
        - Bind-RFP
        - Bind-RFP-DAS
        - Bind-RFP-AAV
        - Bind-RFP-YbaQ

17th July 2018, Tuesday

- **Synchronised Blue light characterisation**
    - Bind-RFP
    - Bind-RFP-DAS
    - Bind-RFP-AAV
    - Bind-RFP-YbaQ
- **Make agar plates**
    - Chloramphenicol, 25 ng/ul
- **Arrival of primers & Plasmid construction**
    - F3'H-FNS (BB) V1 FWD & REV
    - F3'H-FNS (BB) V2 FWD & REV
    - F3'H-FNS (BB) V3 FWD & REV
    - QsR1-dRBS-RFP V2 FWD & REV

- **gblock amplification**
    - F3H-34-DRF FWD & REV
    - FNS FWD & REV

18th July 2018, Wednesday

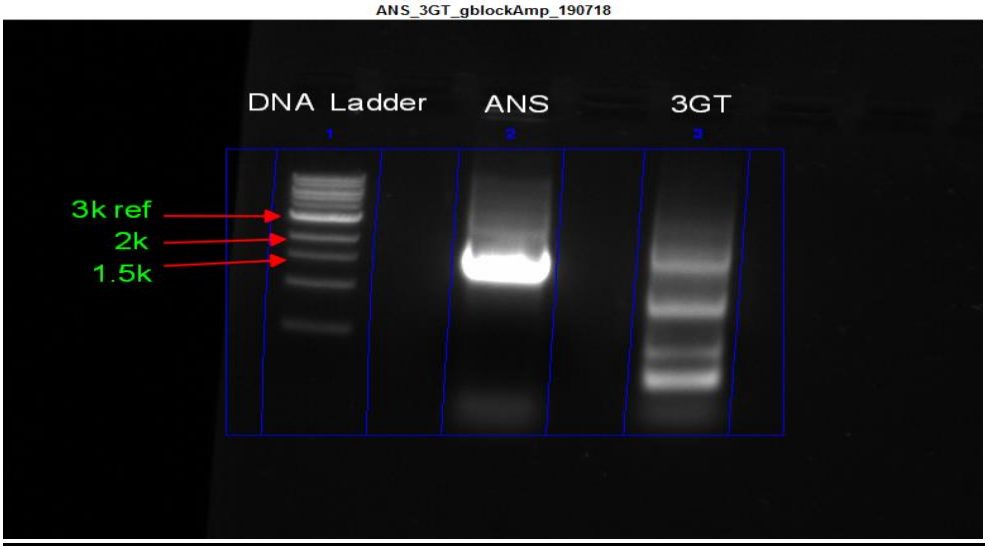
- **Plasmid extraction for T7 RNA polymerase**
    - 1C1 & 2
    - 1E2 & 2
- **Pick colonies for**
    - Brep-F3'H-pTet-FNS V1
    - Brep-F3'H-pTet-FNS V2
    - Brep-F3'H-pTet-FNS V3
    - pLasl-F3H-DFR

19th July 2018, Thursday

Objective(s):

- Amplification of gblocks ANS and 3GT
- Plasmid Extraction and sequencing for:
    - F3'H-FNS v2.0 plasmid
    - F3'H-FNS v3.0 plasmid
    - F3H-DFR v2.0 plasmid

Results



Analysis
Smear of bands observed in lane 3 (3GT), cut the 1.7k band. Low concentration of DNA is expected.

Nanodrop measurement

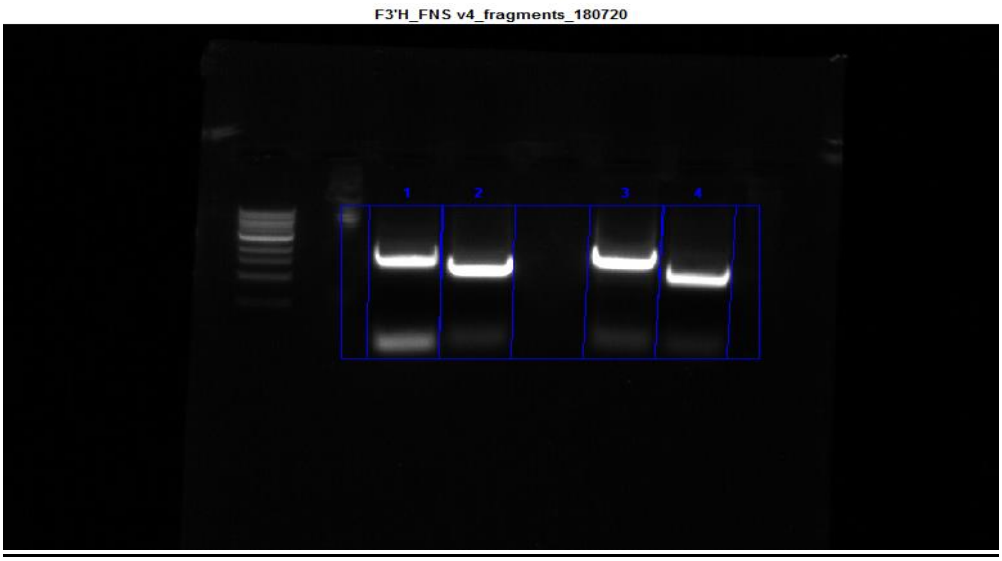
Fragment	ng/μL	A260/A280
ANS	51.6	1.86
3GT	7.6	1.94

Analysis
Result for 3GT is expected as smear is observed in gel electrophoresis. Won't be sending for sequencing because we still have the stock solution, and we will be using the stock solution for plasmid construction.

20th July 2018, Friday

Objective(s):

- Construction of F3'H-FNS v4.0 plasmid (1 gene 1 plasmid)



Analysis
All bands are correct.

Nanodrop measurement

Fragment	ng/μL	A260/A280
pA8C-FNS Fragment 1	41.5	1.88
pA8C-FNS Fragment 2	77.9	1.89
Brep-F3'H Fragment 1	71.9	1.89
Brep-F3'H Fragment 2	66.5	1.50

Analysis
Concentration of all plasmid is satisfactory, however, the purity of Brep-F3'H Fragment 2 is too low. However, Gibson assembly was carried out anyway.

24th July 2018, Tuesday

Objective(s):

- Construction of F3'H-FNS v4.0 plasmid (1 gene 1 plasmid)

Day Summary:

1. Biosynthesis of Luteolin 1.0
2. Plasmid purification for pA8C-FNS and Brep-F3'H plasmids
3. Construction of pBAD ANS plasmids
4. Blue light characterisation: always on/off
5. Inoculation of Brep-F3'H-pTet-FNS cells for Biosynthesis of Luteolin 1.1

1. Biosynthesis of Luteolin 1.0

Procedure
See "Biosynthesis of Luteolin" in our protocol book!

Note: We accidentally added ATC into both flasks so we do not have a control in this experiment. Hence, we incubated 1 flask at 37°C and another at 30°C instead.

To retrieve the dyes, we spun the tube at 10000 rpm for 3 minutes. We then decontaminated the supernatant by following our "Decontamination Protocol" (Under safety) to verify if our dye is safe to be brought out of the lab.

Observations

- Intensity of yellow is higher in flask incubated at 37°C than at 30°C
- Incubating flask for 48h results a more intense yellow than 24h
- However, the overall colour is still undesirable, we expect a much more intense yellow.
- Plate that is plated with decontaminated dyes do not show any colony, this proves the efficiency of our decontamination protocol.

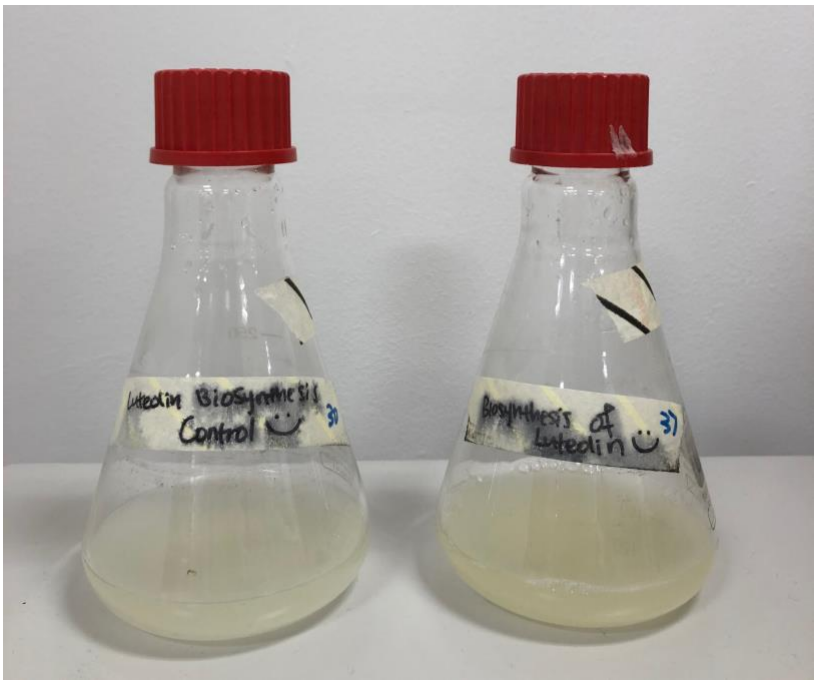


Figure 1: Culture of F3'H-FNS E. coli at 30°C (left) vs 37°C (right)

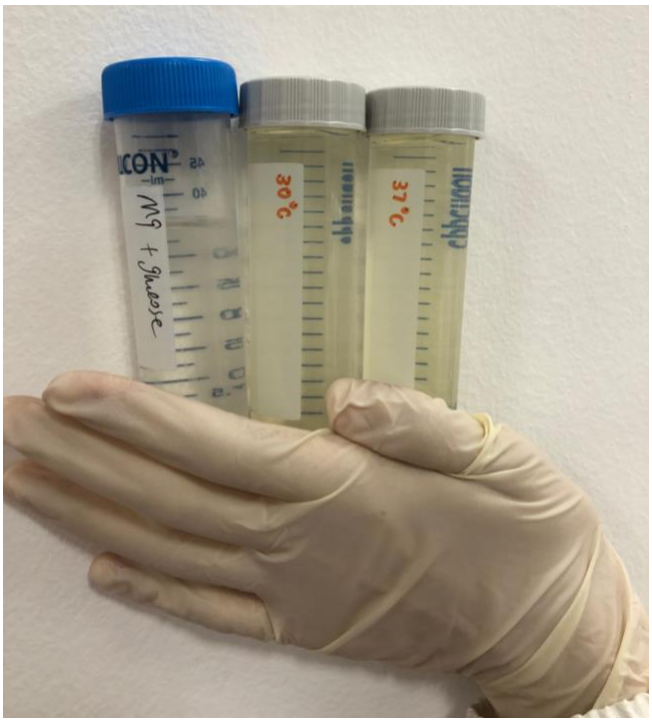


Figure 2: Comparing supernatant with M9 shows that the yellow we have gotten from our culture is significant

Conclusions

- Experiment has to be repeated to include a control
- Decontamination protocol needs to be repeated because the plate is not aesthetically appropriate to be featured on our wiki

2. Plasmid purification for pA8C-FNS and Brep-F3'H plasmids

Procedure

See "Plasmid Extraction (Miniprep)" in our protocol book!

Primers used for sequencing

-insert primers used for sequencing here-

Results

-insert plasmid concentration here-

- Construction of both plasmid is successful after sequencing.

Conclusion

Co-transformation of plasmids need to be carried out into *E. coli* Top-10.

3. Construction of pBAD-ANS plasmids

Procedure
See "Construction of Plasmid" in our protocol book!

Results
-insert DNA concentration table here-

Analysis and Conclusion
Construction failed. Repeated procedure till assembly step on 28 July 2018.

4. **Blue light characterisation 07/24**

Procedure
Results
- Inconsistent starting OD, hard for trend prediction

Analysis and Conclusion
- 2 fold difference…..
- but high non-induced/ repressed RFP expression-> Leakiness
- Required OD for stabilised RFP production

25th July 2018, Wednesday

Day Summary:
1. Pick & Inoculate colonies transformed with ANS in pA8C plasmid (*3GT to be inserted if success) and pA8C-EL222 backbone
2. Biosynthesis of Luteolin 1.0 & 1.1

1. **Pick & Inoculate colonies transformed with ANS in pA8C plasmid (*3GT to be inserted if success) and pA8C-EL222 backbone**

Procedure
Refer to protocol

2. **Biosynthesis of Luteolin 1.0 & 1.1**

Procedure:
- Luteolin 1.0
    - 30℃ incubator was found opened (not-shaking) in the morning…..
    - Yellowish cell culture was checked using new Microplate reader, results to be analysed later
- Making Luteolin 1.1
    - 1ml Overnight culture is inoculated into 50ml M9 + 50ul Kanamycin
    - Starting OD600: 0.06, measured using Nanodrop

- 11am: starts, 37℃ shaking 250rpm
- 3:30pm: OD 1.50-> pipetted out 25ml culture and replenished with new M9+K-> OD0.80
- 3:45pm: fed with 50ul 0.2mM Naringenin for Flask 1 & 2; only 1 is induced with 50ul 200uM ATC; 37℃ shaking 250rpm (should have changed to 30, to favour cell production…..)

26th July 2018, Thursday

<u>Day Summary:</u>

- Biosynthesis of Luteolin 1.0 harvest & 1.1
- Sequencing result for extracted plasmid samples: Brep-F3'H and pA8C-FNS
- Plasmid extraction of pA8C-ANS 1&2 and pA8C-EL222 backbone
- Inoculation of Brep-F3'H-pTet-FNS cells for Biosynthesis of Luteolin 1.2

1. **Biosynthesis of Luteolin**

Procedure

- Luteolin 1.0 reaches 48hr
  - 30 & 37℃ cultures were centrifuged at 10,000rpm, 3 mins
  - Yellowish supernatant was checked using new Microplate reader, results to be analysed later
  - supernatant & cell pellet is kept in separate falcon tubes (4℃)
- Luteolin 1.1 reaches 24hr
  - Both flask 1 & 2 appeared as equally yellow with naked eyes, continue culturing

2. **Sequencing result**

Results

Brep-F3'H (2) and pA8C-FNS (2) were successful.

Procedure

- Inoculated into fresh culture medium
- co-transformed into one cell, plated on K/C-agar

3. **Plasmid extraction of pA8C-ANS 1&2 and pA8C-EL222 backbone & send for sequencing**

27th July 2018, Friday

Day Summary:

1. Biosynthesis of Luteolin 1.1 harvest & 1.2
2. Decontamination protocol test run 1
3. pH variation of filtered supernatant
4. Inoculation of Brep-F3'H-pTet-FNS cells for Biosynthesis of Luteolin 1.3
5. Pick colonies for co-transformed Brep-F3'H (2) & pA8C-FNS (2)
6. Glycerol stock made for pA8C-FNS and Brep-F3'H cells
7. PCR for synthesising pA8C-ANS (F&R fragments)
8. Sequencing result check

1. Biosynthesis of Luteolin

Procedure & Result

- Luteolin 1.1 reaches 48hr old
    - Centrifugation at 10,000 rpm, 3min
    - N-only supernatant appeared more yellowish than ATC+N
- Luteolin 1.2
    - one gene one plasmid culture: Brep-F3'H, pA8C-FNS
    - 37℃ shaking 250rpm
    - OD reached 1.00-> Diluted with M9+K to OD 0.47, measured using Nanodrop
    - Induced with 50ul ATC &/ fed with 50ul Naringenin-*OD
        - Wrong addition of inducers: Brep-F3'H should not added with any inducer, while pABC-FNS requires Arabinose to be induced.

2. Decontamination protocol test run 1

Procedure

Refer to safety protocol

3. pH variation of filtered supernatant

Procedure

- 6M HCl was added drop by drop into filtered supernatant, which is slightly yellowish originally

Result

- No visible colour change
- Change in absorbance spectra was observed, checked using old microplate reader

4. **Inoculation of Brep-F3'H-pTet-FNS cells for Biosynthesis of Luteolin 1.3**

Procedure
Refer to protocol

5. **Pick colonies for co-transformed Brep-F3'H (2) & pA8C-FNS (2)**

Procedure
- Co-transformation provide antibiotic resistance to both Kanamycin and Chloramphenicol
- Picked colonies was thus inoculated into LB broth added with 50ul K and 50ul C.

6. **Glycerol stock made for pA8C-FNS and Brep-F3'H cells**

Procedure
Refer to protocol

7. **Sequencing result check**

Result
- pA8C-ANS 1 & 2 failed with the absence of inserted ANS gBLOCK)
- pA8C-EL222 backbone was successful

8. **PCR for synthesising pA8C-ANS (F&R fragments)**

Procedure
- Primers: ANS FWD with Chromo REV (1.3k); Chromo FWD & AND REV (1.6k);
- Template: pA8C-EL222
    - GEL ran, kept in 4℃ fridge

28th July 2018, Saturday

<u>Day Summary:</u>
1. LifeHacks Outreach, sample preparation

30th July 2018, Monday

<u>Day Summary:</u>

1. Biosynthesis of Luteolin 1.2 harvest
2. Inoculation of wild type *E.coli*, Brep-F3'H-pTet-FNS cells for Biosynthesis of Luteolin 1.3
3. Plasmid extraction of co-transformed Brep-F3'H and pA8C-FNS
4. Gel extraction of PCR fragments of pA8C-ANS backbones (F&R)
5. Transformation of assembled pA8C-ANS into Beta-10 competent cells
6. Decontamination protocol plate check

1. **Biosynthesis of Luteolin 1.2 harvest**

Procedure and Result

- 48+24hr old-> centrifuge to extract yellow out: 10,000 rpm, 3min
- pA8C-FNS is more yellowish than Brep-F3'H

2. **Inoculation of wild type *E.coli*, Brep-F3'H-pTet-FNS cells for Biosynthesis of Luteolin 1.3**

Procedure

Refer to protocol

- wild type *E.coli* (competent cell strain)
- Brep-F3'H-pTet-FNS cells inoculated from -80℃ glycerol stocks
- Overnight culture in LB broth

3. Plasmid extraction of co-transformed Brep-F3'H and pA8C-FNS

Procedure

Refer to protocol

- Samples sent for sequencing

4. **PCR fragments of pA8C-ANS backbones (F&R)**

Procedure and Result

- Gel extraction, size varification
- ~1.8ng/ul; 1.74
- ~4,200bp, gel photo in computer, have not export

5. **Transformation of pA8C-ANS into Beta-10 competent cells**

Procedure

Refer to protocol

6. **Decontamination protocol plate check**

Result
No colonies were observed on LB+K plate
Success!

31st July 2018, Tuesday

Day Summary:

1. Biosynthesis of Luteolin 1.3
2. Pick colonies for plated pA8C-ANS
3. Sequencing result of co-transformed Brep-F3'H and pABC-FNS
4. Modelling feedback and advices for next Blue Light characterisation
5. HPLC protocol write up

1. Biosynthesis of Luteolin 1.3

Procedure
See "Biosynthesis of Luteolin" in our protocol book!

- We have followed the protocol strictly
    - 37℃, LB+K, ON culture, 250rpm, **covered in dark**
    - 500ul ON culture + 50ml M9 + 50ul K into 250ml flask, to be refreshed
    - **37℃ to reach OD 0.40 -> Induction:** 50ul 200uM ATc, 50ul 0.02M Naringenin;
        - **Negative Control: 30**℃ after ATc Induction
            - a: wild type Naringenin +ve
            - b: wild type Naringenin -ve
        - **Experimental group: 30**℃ after ATc Induction, **covered in dark**
            - **1:** Brep-F3'H-pTet-FNS Naringenin +ve
            - **2:** Brep-F3'H-pTet-FNS Naringenin -ve

    - 11:00 Starting OD: 0.04, measured using Nanodrop
    - 13:40: Ctrl induced & fed at OD 0.37, 0.38;
    - Experimental continued to be incubated (OD 0.25, 0.28) & induced at 14:15, OD 0.47, 0.48
        - **Possible reason of yellowish**
            - Cell's native metabolites (wild type)
            - Naringenin colour interference
            - undesirable side products (from engineered plasmids)
        - Purpose of experiment

    - prove the origin of yellow…..

2. **Pick colonies for plated pA8C-ANS**

Procedure

Refer to Inoculation protocol

3. **Sequencing result of co-transformed Brep-F3’H and pABC-FNS**

Result

Failed.

F3’H was present, but not FNS.

4. **Modelling feedback and advices for next Blue Light characterisation**

Result

- similar findings after discussion?
- should try one round of 2hr ON, 6hr OFF