

# iGEM 2018: NUS Singapore-A

## WetLab Group 2\_August 2018 Logbook

### 2018.08.01

- Plasmid extraction & Sequencing
  - pA8C-ANS (Assembled A1, A2 & GelExtracted G1, G2)
- BL Characterisation
  - ON culture, OD 0.2; measured using Nanodrop
    - refreshed, (360ul in 9ml LB+K)

OD600 after 1hr refreshing	
Bind: 0.50	Brep: 0.42
ID: 0.46	RD: 0.36
IY: 0.38	RY: 0.34
IA: 0.40	

- Thus, diluted to OD0.2 & added to 12-well plate
  - 2hr ON, 6hr OFF (as suggested by Modelling; Bind is ON first)
- Cloning of Bind/rep into pSB1C3
  - Primer ordered

### 2018.08.02

- Biosynthesis of Luteolin 1.3 (07/31 culture)
  - 48hr extraction of 07/31 culture: 10,000 rpm, 3min
  - Decontamination protocol on LB+K, but not LB agar plates
    - Experimental Naringenin +ve
- Analysis of BL characterisation data 20180801
  - additional 21st hr reading was taken (Bind plate was left in 37 shaking & covered; Brep was left outside, 20 and covered)
    - Bind promoter cannot be off even when kept in dark overnight.
    - YbaQ is too strong, Bind promoter is being consistently suppressed (which is also observed in 07/17).
    - AAV could be a better deg tag for us. Planning of a 3~4hr ON and 4~5hr OFF pattern for next time round.
- Set up 1st Base account
- Re-Pick colonies for co-transformed Brep-F3'H (2) & pA8C-FNS (2) on K/C plate
  - inoculated into 50ml LB+50ul K+50ul C

### 2018.08.03

- Plasmid extraction of Re-Picked colonies & send for Sequencing
  - for co-transformed Brep-F3'H (2) & pA8C-FNS (2) in 50ml LB+50ul K+50ul
- Cloning of Bind/rep into pSB1C3

- 6 pairs of primers received
  - 1: Brep-F3'H-pTet-FNS Frag 1 [DNA: 7ng/ul]
  - 3: Brep-F3'H-pTet-FNS Frag 2 [5]
  - 4: pSB1C3 Frag 1 [34]
  - 6: pSB1C3 Frag 2 [32]
  - 5: E8K-Brepin-RFP [40]
  - 2: Truncated CPR (gBlock amplified, can be used for assembly directly)
- 25
- Look up Spinach
  - ask for advice; with/without scaffold
- Look up Stress promoter sequence

### 2018.08.06

- Decontamination protocol on LB agar plate
  - Experimental Naringenin +ve
- Luteolin 1.2, 1.3 products, filtered
- Plasmid Assembly
  - 1: Brep-F3'H-pTet-FNS Frag 1 + 2: Truncated CPR + 3: Brep-F3'H-pTet-FNS Frag 2
  - 4: pSB1C3 Frag 1 + 5: E8K-Brepin-RFP + 6: pSB1C3 Frag 2
  - Transformation, Recovery, Plate
    - Brep-F3'H-pTet-FNS-CPR, LB+K
    - pSB1C3-Brepin-RFP, LB+C
  - (Nuo'en) \*Mutagenesis, restriction site requirement check
- Check/ Buy co-factors/ supplements to help Luteolin synthesis
- Spinach, check origin in iGEM Registry

### 2018.08.07

- Luteolin 1.2, 1.3 filtered products passed for HPLC
- Pick colonies, inoculation (no colonies observed)
  - Brep-F3'H-pTet-FNS-CPR, LB+K
  - pSB1C3-Brepin-RFP, LB+C
  - \*Re-construct CPR and pSB1C3
- Co-transformation Sequencing result
  - Failed FNS again, F3'H is present
  - \*Re-do Co-transformation (1ul of each purified plasmid was added to competent cells)
- Modelling updates & Plans for Next characterisation
  - 1hr ON and OFF all the way

### 2018.08.08 (Tue)

- Pick colonies, inoculation (no colonies observed at 11am)
  - Brep-F3'H-pTet-FNS-CPR, LB+K
  - pSB1C3-Brepin-RFP, LB+C
  - Co-transformed Brep-F3'H & pA8C-FNS, LB+K+C
    - No colonies observed

- flavone synthase II (FNS II), a membrane bound cytochrome P450 dependent monooxygenase
- Troubleshoot\*\*\*\*\*
  - missing Terminators at the end of F3'H, which is the primer binding site for CPR insertion
- Luteolin synthesis
  - Order chemicals, enhanced production
  - HPLC result check check

### 2018.08.10 (Fri)

- Blue Light Characterisation
  - Bind covered, kept in 30 degree, over 2 nights
  - 200ul into 5ml refreshed LB+K
  - Bind: 45min ON, OFF all the way (takes reading every hour)
  - Brep: 45min covered, Light on=Repressed all the way
- Repeat & Optimised PCR: Brep-F3'H-pTet-FNS-CPR
  - 2ul Template
  - 2ul dNTP
  - 1ul each Primer
  - 46ul Mastermix
  - \*Failed; smears and multiple bands observed (although the faint right sized are there)
  - re-send for Sequencing, as there may be mutations at the terminator/primer binding site
- Plasmid extraction & Sent for Sequencing (Primer: pBAD, EL222 Reverse)
  - Co-transformed Brep-F3'H and pA8C-FNS (37 degree 6hr, kept in Fridge)
  - put in incubator grow a while first
- ANS & 3GT Construct troubleshoot
  - ANS has failed twice
  - possible mis-binding, no matter what
- Getting Quotations for Co-factors\*

### 2018.08.13 - 08.17 Weekly plan

- Sequencing
  - Re-confirmation: Brep-F3'H-pTet-FNS B V3
    - possible mutations at terminator, which is also primer binding site for inserting CPR
    - Seq primers: ColE1 FWD & Kanamycin FWD (universal primer, gRNA)
  - Co-transformed Brep-F3'H & pA8C-FNS 2
    - Seq primers: ELR, pBAD FP (1st base universal)
- New deg tag, strength: AAV<new tag<YbaQ
  - LAA~LVA, > YbaQ
  - iGEM registry
  - design primer\*
  - Try again: Bind-LVA
    - using previous plasmid & cell
  - Increase Blue-light intensity to 80%

- Change EL222 Promoter: over expression of EL222 may affect RFP expression
  - 106 or rmbp1 (can be done concurrently)
  - Modify the existing backbones with deg tags
  - PCR cut into 2 fragments, assembly
  - Bind AAV, YbaQ (as part improvement)
  - design primer\*
  - \*new experiment plan: Brep 3hr light ON to repress, then OFF to see the increase
- Dye synthesis
  - Re-run Negative control: Wild type +/- Naringenin and positive control
  - ccaS & ccaSR... has arrived-> Characterisation can be carried out with GFP
    - where to put it in the gene circuit
  - Construct ANS-3GT (waiting for primer)
  - Waiting for co-factors to arrive to do more synthesis
  - \*HPLC: all samples contain traces of Luteolin & Fungal contamination
- Spinach aptamer check check\*
  - iGEM registry

## 2018.08.13 - 08.14

- Biosynthesis of Luteolin
  - HPLC Result
    - Control samples was observed with Luteolin-like component
- Biosynthesis of Luteolin 1.4 (repetition of 1.3, prof's order)
  - cells inoculated:
    - Top10 glycerol stock for Negative control; LB
    - Brep-F3'H-pTet-FNS stock as Positive control; LB+K, covered
  - Refreshed in LB +/- K, start at 9:50; 37, 250rpm, covered; 2 control 2 experimental
  - New Protocol, Luteolin\*
    - paper: Strain Improvement of Recombinant E coli for Production of Plant Flavonoids
    - Overnight culture in LB, 37, 250rpm
    - Refresh in 50ml LB with Antibiotics: starting OD 0.1, ending OD 0.6
    - Induction (50ul 200uM ATC, Max Induction) at 30 degree, 3hr; START 14:00
    - Centrifuge to collect cells, culture fresh M9 with Antibiotics at OD 1.8-2.0
    - Addition of Substrate (50ul 0.2mM Naringenin) & Inducer
    - Continue Incubation at 30 degree, 36hr; START 08/14 18:30, END 08/16 9:00
- Sequencing result
  - Co-transformed Brep-F3'H & pA8C-FNS failed (missing FNS again)
  - Brep-F3'H-pTet-FNS backbone, confirmed F3'H & FNS seq OKAY,
    - mutations are at the Terminator-> primers re-designed
- PCR fragment amplification
  - 1,2,3: Brep-F3H-DFR-F3'H
  - 4,5,6,7: pA8C-ANS-3GT

- Spinach gblock & primers are delivered
- pSB1C3-Brepin-RFP, on LB+C plate
  - no colonies observed

### **2018.08.15 (Weds)**

- Gel electrophoresis of 08/14 PCR samples (gel photo taken)
  - Brep-F3H-DFR-F3'H failed
    - Wrong backbone was used when designing primers
    - Nonetheless, Fragment 3 can still be used (Brep-F3'H)
  - pA8C-ANS-3GT success
    - pA8C-1 Fragment was observed with smear, absence of distinct band, [18ng/ul]; pA8C-2 is okay [51]
    - 3GT gBlock: [7]; ANS gBlock: [58]
    - Assembly ratio: 4:2:2:2
    - Transform, Heat shock, recovery, Plate on K plate
- Spinach Assembly
  - Backbone: pSB1C3 Frag 1 & 2 from previous experiment [~30ng/ul]
  - gBlock Spinach [7ng/ul]
  - Assembly ratio: 1:2:6 (gBlock)
- Biosynthesis of Luteolin 1.4
  - equally yellowish

### **2018.08.16 (Thurs)**

- Biosynthesis of Luteolin 1.4 Harvest\*
  - filtered supernatant and pellet, ready for HPLC
- Pick colonies
  - pA8C-ANS-3GT
  - pSB1C3-Spinach (no colonies)
  - Re-transformed Brep-F3'H-pTet-FNS\*
- Spinach gBlock ~3ul left
  - gBlock amplification
  - Test pSB1C3 cells
    - pSB1C3 backbone only is transformed into TOP10, to verify the viability of backbone\*
  - transformation & plate on C plate

### **2018.08.16 (Fri)**

- Plasmid extraction & sent for Sequencing
  - Re-transformed Brep-F3'H-pTet-FNS\* (F1, F2)
  - pA8C-ANS-3GT (A1, A2)
- Spinach gblock amplified
  - Gel ran & extracted, sent for Sequencing (S)
- pSB1C3 plasmid backbone amplified from T7 RNA Pol plasmid

### **2018.08.20 (Mon)**

- pSB1C3 plasmid backbone amplified from T7 RNA Pol
  - run gel check & send for sequencing
- BL21\* obtained from Syncti Dr LingHua
  - inoculated into LB, overnight
- Assembled Spinach + pSB1C3
- Sequencing result:
  - pA8C-ANS-3GT (A1, A2) FAILED
  - Re-transformed Brep-F3'H-pTet-FNS\* (F1, F2) OKAY
    - \*missing Terminator again\*

#### **2018.08.21 (Tues)**

- Prepared BL21 star\* Competent cells
- Transformed assembled Spinach + pSB1C3 into Top10
- Biotin (4 degree) & Thiamine (rtp) obtained from YanPing
- Prepared new M9 medium
  - 100ml 10mM Biotin stock: 0.2443g in 100ml H2O (10ul added into 1L M9)
  - 100ml 6mM Thiamine stock: (not fully dissolved): 0.20238g in 100ml H2O (1ul added into 1L M9)
  - Excluded casimino acid

#### **2018.08.23 (Thurs)**

- Order primers
- Pick colonies: pSB1C3-Spinach
- View spinach backbone seq results - success
- Transformed confirmed plasmid
  - F3'H-FNS into BL21\*
  - F3H-DFR into BL21\*
  - Brep-F3'H into BL21\*
  - pA8C-FNS into BL21\*
- Adapted wild type BL21\*
- Amplified psb1c3
- Transformed psb1c3-spinach into beta10
- Transformed pT7-RBS34-RFP into BL21\* and BL21
- Inoculated F3'H-FNS to get more plasmid

#### **2018.08.24 (Fri)**

- Pick colonies for:
  - BL21\*:
    - F3'H-FNS
    - F3H-DFR
    - Brep-F3'H
    - pA8C-FNS
    - pT7-RFP
  - BL21: pT7-RBS34-RFP
  - Beta10: spinach-pSB1c3
- Run gel for pSB1c3 amplification and extract

- failed
- Construct EL222, Bind-RFP individual plasmids
  - Aiyang has 2 plasmids mixed in one tube: EL222 & Brep-RFP
  - PCR to individual amplified out EL222 & replace Brep-RFP into Bind-RFP
    - Aim to Test baseline expression level of Bind-RFP, without the assistance of EL222
  - Fragments are amplified (DAS, AAV, YbaQ, LVA & Bind)
    - to be ran gel & assembled, transformed on 25/08
- Sub-culture M9-adapted BL21\* cells
  - round 2 adaptation
- Investigate effect of Naringenin
  - 200ul M9-adapted BL21\* into 5ml LB
- Brep-F3'H-FNS plasmid extracted from Glycerol stock
- Confirm NTU collab
- Confirm pT7 construction

### 2018.08.25 (Sat)

- Biosynthesis of Luteolin 1.5 (1st detection of Luteolin)
  - cells inoculated (not covered in Dark)
    - BL21\* 20/08
    - BL21\* Brep-F3'H-pTet-FNS 1 24/08
    - BL21\* Brep-F3'H 1 24/08
    - BL21\* pA8C-FNS 1 24/08
  - Refreshed in LB +/- K or C, start at 10:35; 37, 300rpm, covered; 2 control 2 experimental
  - New Protocol, Luteolin\*
    - paper: Strain Improvement of Recombinant E coli for Production of Plant Flavonoids
    - Overnight culture in LB, 37, 300rpm
    - Refresh in 50ml LB with Antibiotics: starting OD 0.1, ending OD 0.6
    - Induction (50ul 200uM ATC, Max Induction) at 30 degree, 3hr; START 14:00
    - Centrifuge to collect cells, culture fresh M9 with Antibiotics at OD 1.8-2.0
    - Addition of Substrate (50ul 0.2mM Naringenin) & Inducer
    - Continue Incubation at 30 degree, 36hr; START 08/25 18:00, END 08/27 17:00
  - Plasmid extraction:
    - BL21\* Brep-F3'H-pTet-FNS 1&2
    - BL21\* Brep-F3'H 1&2 24/08
    - BL21\* pA8C-FNS 1&2 24/08
    - TOP10-pSB1C3-Spinach 1&2&3
  - Gel extraction (of PCR'd plasmid fragment 1 & 2) -> Assembly -> Transformation into Beta-10
    - \*Extracted bands are of right size; Photo taken
    - Bind-Das-RFP
    - Aav
    - YbaQ

- LVA
- Bind
- pAC-EL222
- Co-transformation of Prem's plasmid
  - Bind-RFP & EL222

#### **2018.08.27 (Mon)**

- Samples sent for Sequencing
  - BL21\* Brep-F3'H-pTet-FNS 1&2
  - BL21\* Brep-F3'H 1&2 24/08
  - BL21\* pA8C-FNS 1&2 24/08
  - TOP10-pSB1C3-Spinach 1&2&3
- Pick colonies & Inoculation into LB+K/C
  - Beta-10 series:
    - Bind-Das-RFP
    - Aav
    - YbaQ
    - LVA
    - Bind
    - Chez-YbaQ
    - pAC-EL222
  - Co-transformed Bind-RFP & EL222: NO colonies observed
- Harvest of Luteolin 1.5 (BL21\* 1st try) & sent for HPLC

#### **2018.08.28 (Tues)**

- Sequencing result
  - OKAY
    - BL21\* Brep-F3'H-pTet-FNS 1&2
    - BL21\* Brep-F3'H 1&2 24/08
    - BL21\* pA8C-FNS 1&2 24/08
  - Failed
    - TOP10-pSB1C3-Spinach 1&2&3
- PCR for pT7-Brep-F3'H-FNS, and FNS-CPR

#### **2018.08.29 (Wed)**

- Plasmid extraction
  - Beta-10 series:
    - Bind-Das-RFP
    - Aav
    - YbaQ
    - LVA
    - Bind
    - pAC-EL222
  - DH5a
    - ANS-3GT-B
- Gel Electrophoresis of PCR pT7-Brep-F3'H-FNS & FNS-CPR



- Failed
- Biosynthesis of Luteolin 1.6
  - cells inoculated (not covered in Dark)
    - BL21\*
    - BL21\* Brep-F3'H-pTet-FNS (1)
    - BL21\* Brep-F3'H which one? (1)
    - BL21\* pA8C-FNS which one? (1)

### 2018.08.30 (Thurs)

- Biosynthesis of Luteolin 1.6 (BL21\* + 3 co-factors)
  - Refreshed in LB +/- K or C, start at TIME; 37, 300rpm, covered; 2 control 2 experimental
  - New Protocol, Luteolin\*
    - paper: Strain Improvement of Recombinant E coli for Production of Plant Flavonoids
    - Overnight culture in LB, 37, 300rpm
    - Refresh in 50ml LB with Antibiotics: starting OD 0.1, ending OD 0.6
    - Induction (50ul 200uM ATC, Max Induction) at 30 degree, 3hr; start at TIME
    - Centrifuge to collect cells, culture fresh M9 with Antibiotics at OD 1.8-2.0
    - Addition of Substrate (50ul 0.2mM Naringenin) & Inducer & Co-factors to induce Biotransformation:
      - 0.5 mM 2-oxoglutaric acid, 0.5 mM FeSO<sub>4</sub> and 0.5 mM sodium ascorbate (50uL each into the culture)
    - Continue Incubation at 30 degree, 36hr; START 08/29 TIME, END 08/31 TIME
- Co-transformation
  - F3H-DFR and ANS-3GT into BL21\* & cell culture inoculated and in shaking
- Prepare Co-factors Stock
  - 0.5M Sodium ascorbate (RTP)
  - 0.5M Iron II sulphate (FeSO<sub>4</sub>) (RTP): crystal seems to be formed —> put in water bath to dissolve next time
  - 0.5M alpha-ketoglutaric acid (in -20degC)
  - using \*Sigma Molarity calculator-> Graphpad Molarity calculator
- Send for Sequencing
  - Bind-Das-RFP
  - Aav
  - YbaQ
  - LVA
  - Bind
  - pAC-EL222
  - ANS-3GT-B
- SDS-PAGE procedure
- Pick colonies from Brep-F3H-DFR-BL21\*
  - keep in 4 degree & take up to make Glycerol stock\* seq confirmed

**2018.08.31 (Fri)**

- SDS-PAGE 2nd try on Luteolon 1.5 pellets
  - plenty protein present near 25kDa
- Picked colonies for Co-transformed F3H-DFR and ANS-3GT into BL21\*
- Sequencing result
  - Bind-DAS-RFP Failed; previous Bind-DAS was okay
  - AAV Failed 1&2 (several mutations at Deg tag); previous Bind-AAV was okay
  - YbaQ 1&2 OKAY
  - LVA 2 OKAY
  - Bind 2 OKAY