1. Perform all steps on ice.

2. Resuspend cell pellet in 30m Binding Buffer (20mM Tris-HCl, 500mM NaCl, 1mM EDTA, pH 8.0).

3. Sonicate cell suspension for 10 mins (100 amplitude; 1 second on, 2 seconds off). Put tube in beaker with ice.
   a) Take sample of raw lysate for later SDS-PAGE - Store at 4°C.

4. Clarify lysate via centrifugation (16,000 rpm, 4°C, 20 mins in a Beckman Coulter Optima TM L-100 XP Ultrafuge).
   a) Take sample of cell pellet for later SDS-PAGE - Store at 4°C.
   b) Sample Supernatant.

5. Filter Supernatant via Syringe method using a 0.22µm filter/cup.

6. Equilibrate StrepTrap HP 5mL column (GE Healthcare, UK) with binding buffer.
   a) Keep waste flow through for later SDS-PAGE - Store at 4°C.

7. Load filtered supernatant onto equilibrated column.

8. Elute Sepharose-bound protein using a gradient of binding buffer and elution buffer (20mM Tris-HCl, 500mM NaCl, 1mM EDTA, 2.5mM desthiobiotin, pH 8.0).

9. Clean column using dd-H2O and regenerate with 0.5M NaOH then store in 20% ethanol at 4°C.

10. Perform an SDS-PAGE on clarified lysate, cell pellet, flow-through and purified fractions determined via UV peaks in elution profile.
Escherichia Coli Transformation Protocol

1) Defrost 50 µl of chemically competent E. Coli
2) Add plasmid DNA
   - 2 µl if sequence verified plasmid
   - 5 µl for ligations
3) Incubate on ice for 30 minutes
4) Heat shock at 42°C for 1 minute
5) Incubate on ice for 2 minutes
6) Add 200 µl of LB.
7) Incubate at 37°C for 1 hour
8) Plate entire contents onto LB plate with X
9) Incubate plates at 37°C overnight
Agarose Gel Electrophoresis Protocol

Karen

1) Measure 0.5g of agarose

2) Mix agarose powder with 50ml 0.5X TBE in a 250ml microwavable flask

3) Microwave for 1 minute in 30 Second intervals, stop and swirl, until the agarose is completely dissolved (do not overboil the solution as some buffer will evaporate and thus alter the final percentage of agarose in the gel)

4) Allow the agarose solution to cool to about 50°C (when you can comfortable keep your hand on the flask for 10 seconds)

5) Add 5µl of 10,000x Gel Red and gently mix to prevent formation of bubbles.

6) Pour the agarose into a gel tray with the well comb in place

7) Let sit for 20-30 minutes until completely solidified

8) Once solidified, place the agarose gel into the gel box

9) Fill gel box with 0.5X TBE until the gel is covered

10) Add 2µl of loading buffer to each of your DNA Samples

11) Carefully load a molecular weight ladder into the first lane of the gel

12) Carefully load your samples into the additional wells of the gel

13) Run the gel at 100V for 1-1.5 hours (until the dye line is approximately 75-80% of the way down the gel)

14) Turn off power, disconnect the electrodes from the power source and carefully remove the gel from the gel box

15) Visualise your DNA fragments using any device that has UV light
1) MgCl₂ - 0.1 M
   - Measure 9.5211 g
   - Add 80 mL of dH₂O
   - Top up to 100 mL
   - Take 10 mL and add 90 mL dH₂O = 0.1 M
   - Autoclave

2) CaCl₂ - 0.1 M
   - Measure 11.1 g CaCl₂
   - Add to 80 mL of dH₂O
   - Top up to 100 mL
   - Take 10 mL and add 90 mL dH₂O = 0.1 M
   - Autoclave

3) 100 mL LB broth in a 250 mL conical flask
   - To be autoclaved

**GENE CONSTRUCTS - IDT**

- Glycine activator 944 bp
  1000 ng => 1.715 fmoi

- PEPD
  1000 ng => 0.67 fmoi

- Eicosane 1547 bp
  1000 ng => 1.076 fmoi

- GCUR TF
  500 ng => 1.094 fmoi

- ChaC
  1000 ng => 1.773 fmoi

- GCVA TF
  1000 ng => 1.426 fmoi

- Eicosanol RFP
  1000 ng => 1.709 fmoi

- Nitroxoline
  1000 ng => 1.708 fmoi
1) Centrifuge the tube for 3-5 seconds at a minimum 3000×g to pellet the material to the bottom of the tube.

2) Add the following volume of TE buffer to get a final concentration of DNA:
   - Glycine detector - 100 µL
   - PepD gene - 100 µL
   - Eicosane → I-Eicosanoı Forward - 100 µL
   - Nitroxoline RFP biosensor - 100 µL
   - GCUR TF - 50 µL
   - ChaC gene - 100 µL
   - GCUA TF - 100 µL
   - Eicosanoı RFP - 100 µL

3) Briefly vortex and centrifuge.

**Notes**
- Plated out E. Colı K12 (MC11655) grown o/N in LB. (Plated on LB agar at 37°C)
- Need to inoculate 10mL of LB.
Chemically Competent cells Protocol

Amount of Substance

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgCl₂</td>
<td>0.1 M</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.1 M</td>
</tr>
<tr>
<td>Glycerol</td>
<td>100%</td>
</tr>
</tbody>
</table>

Procedure

1. Inoculate a single colony of appropriate cells into 10ml LB in a sterile tin. Add antibiotic if needed, and culture O/N at 37°C, 200 rpm.
2. Inoculate 100ml LB with 1ml O/N culture.
3. Incubate at 37°C, 200rpm until OD₆₀₀ = 0.3 - 0.6 (~2 hours).
4. Transfer to 2x 50ml Falcon tubes and leave on ice for 30 mins.
5. Centrifuge at 4000 xg, 5 mins, 4°C.
6. Re-Suspend pellet gently in 2.5ml ice cold 0.1M MgCl₂.
7. Incubate on ice for 30 mins.
8. Centrifuge at 4000 xg, 5 mins, 4°C.
9. Re-Suspend pellet gently in 2.5ml ice cold 0.1M MgCl₂.
10. Incubate on ice for 30 mins.
11. Centrifuge at 4000 xg, 5 mins, 4°C.
12. Re-Suspend pellet gently in 2ml ice cold 0.1M CaCl₂ / Glycerol solution (1.7ml 0.1M CaCl₂, 0.3ml 100% Glycerol).
13. Aliquot (50μl) and flash freeze in liquid nitrogen.
Calculations of amounts of DNA in fmol for the reaction (McCl)

Concentration of DNA is in ng/µl, each one = 10 ng/µl DNA
Must convert to fmol/µl. Need to use DNA amount of 20 fmo

- Glycine activator = 10 ng/µl = 17.15 fmol/µl
  \[ \frac{10 \text{ fmol}}{17.15} = 0.58 \]

- PepD = 10 ng/µl = 9.67 fmol/µl
  \[ \frac{10 \text{ fmol}}{9.67} = 1.03 \mu l \]

- Eicosane = 10 ng/µl = 10.46 fmol/µl
  \[ \frac{10 \text{ fmol}}{10.46} = 0.96 \mu l \]

- GCUR TF = 10 ng/µl = 21.88 fmol/µl
  \[ \frac{10 \text{ fmol}}{21.88} = 0.46 \mu l \]

- ChaC = 10 ng/µl = 17.53 fmol/µl
  \[ \frac{10 \text{ fmol}}{17.53} = 0.57 \mu l \]

- CCGA TF = 10 ng/µl = 14.26 fmol/µl
  \[ \frac{10 \text{ fmol}}{14.26} = 0.7 \mu l \]

- Eicosan RFP = 10 ng/µl = 17.08 fmol/µl
  \[ \frac{10 \text{ fmol}}{17.08} = 0.59 \mu l \]

- Nitroxiline = 10 ng/µl = 17.08 fmol/µl
  \[ \frac{10 \text{ fmol}}{17.08} = 0.59 \mu l \]

DUK - AF = 2203 bp
\[ = 111.21 \text{ fmol} \]

\[ C_1, U_1 = C_2, U_2 = 10 \mu l \]

\[ 111.21 \times 1 = 10.21 \times 1 \]

\[ 0.09 \mu l + 6.91 \text{ EB} \]
Level 1

DNA parts - add µl (calculate - 1 µl plasmid+ DNA (µl))

10x ligase buffer - 1 µl
Ligase at 20 U/µl - 1 µl
BsaI at 10 µ/µl - 1 µl
ddH₂O - Make to 20 µl

glycine activator = ddH₂O (µl) 15.42
PepD = 14.97
Eicosane = 15.04
GCVR TF = 15.54
ChAc = 15.43
CCVA TF = 15.3
Eicosanol RFP = 15.41
Nitroxoline = 15.41
DNA ligase = 3.77 ng/µl = 260/280 = 0.66
Testing composite cells

Incubate on ice at
1) 14:53 → take out at 15:23
2) 14:57 → take out at 15:27

Transform with DUK AE SpT RFP Bo015

Take 1 out at 4:28 pm
Take 2 out at 4:32 pm
Transformations

1) Glycine Activator - bad
2) Pep D - bad
3) Eicosane - bad
4) GCUR TF - bad
5) Cha C - good
6) GCVA TF - good
7) Eicosanol RFP - good
8) Nitroxoline - good

26/7/19
1) Pep D - bad
2) Eicosane - bad
3) GCUR TF - bad
1/8/19

Karen

** Kanamycin LB plates poured **

** Gel electrophoresis - 1% agarose gel **

\[
\text{1g agarose + 100ml of 1xTBE} \quad 0.5g + 50ml (1xTBE) \\
\text{1g + 50ml (0.5xTBE)} \\
\]

** Gel 1 (10 wells) **

<table>
<thead>
<tr>
<th>Well</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Ladder</td>
<td>-ve Control</td>
<td>+ve Control</td>
<td>Cysine amino</td>
<td>Pep D</td>
<td>Ecosone</td>
<td>CCUR</td>
<td>Redo Pep D</td>
<td>Ladder</td>
<td>-</td>
</tr>
</tbody>
</table>

- 2 µl of sample + 2 µl of loading buffer (Gel loading dye purple (6x))
- 10 µl of ladder (1 Kb)
- 100V, 1 hour => only band on +ve control.

** Gel 2 (10 wells) **

<table>
<thead>
<tr>
<th>Well</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>Ladder</td>
<td>+ve Control</td>
<td>-ve Control</td>
<td>ChaC</td>
<td>GCUR</td>
<td>Ecosone</td>
<td>Redo RFP</td>
<td>Nonlinear Ladder</td>
</tr>
</tbody>
</table>

- 2 µl sample + 2 µl loading buffer
- 10 µl of ladder (1 Kb)
- 100V, 50 mins (out of time)
Calculations for MoClo

To do calculations
- Glycine activator
- Pep D
- Eicosane
- GCUR TF
- Cha C

MoClo transformations

- Make 10 fmol/µl of DUK-AF stock
  1) 2203 bp / 111.21 fmol
     0.09 µl of plasmid and resuspend in 0.91 EB
  1) 10 fmol/µl
  1) x5: 0.45 µl of plasmid + 4.55 µl EB Enough for 5 reactions.

- Glycine activator
  1) 1 µl of DUK-AF 10 fmol/µl stock (10 fmol)
  1) 0.63 µl of glycine activator stock (11 fmol/µl)
  1) 1 µl 10 x ligase buffer
  1) 1 µl ligase at 20 U/µl
  1) 1 µl BsaI at 10 U/µl (add last)
  1) 15.97 µl ddH₂O

- Pep D
  1) 1 µl DUK-AF
  1) 1.14 µl Pep D
  1) 14.86 µl ddH₂O

- Eicosane
  1) 1 µl DUK-AF
  1) 1.06 µl Eicosane
  1) 14.94 µl ddH₂O

- GCUR TF
  1) 1 µl DUK-AF plasmid
  1) 0.51 µl GCUR
  1) 15.49 µl ddH₂O

- Cha C
  1) 1 µl DUK-AF plasmid
  1) 0.63 µl Cha C
  1) 15.37 µl ddH₂O
QIAprep Spin Miniprep Kit

1. Pellet 1-5 ml bacterial overnight culture > 8000 rpm, 3 min, RTP

2. Resuspend pelleted bacterial cells in 250 µl Buffer P1, transfer to microcentrifuge tube

3. Add 250 µl Buffer P2 and mix thoroughly by inverting tube 4-6 times until clear

4. Add 350 µl Buffer N3, mix immediately and thoroughly by inverting tube 4-6 times

5. Centrifuge 10 min, 13000 rpm table top microcentrifuge

6. Apply 800 µl supernatant (steps) in the QIAprep 2.0 spin column by pipetting. Centrifuge 30-60 s, discard flow through.

7. Wash QIAprep spin column by adding 0.5 ml Buffer PB, centrifuge 30-60 s, discard flow through

8. Wash QIAprep spin column by adding 0.75 ml Buffer PE, centrifuge 30-60 s, discard flow through

9. Centrifuge 1 min to remove residual wash buffer.

10. Place QIAprep 2.0 column in clean 1.5 ml microcentrifuge tube. To elute DNA, add 50 µl Buffer EB or water to column, let stand for 1 min, centrifuge for 1 min

Overnight culture of E.coli DH5α DUK-AF

Glycerol stocks made.
Seqencing GCUA, eicosanol, nitroxoline

TS00533602 - GCUA (F)
TS00533603 - GCUA (R)
TS00533604 - Eicosanol (F)
TS00533605 - Eicosanol (R)
TS00533606 - Nitroxoline (F)
TS00533607 - Nitroxoline (R)

MoClo repeat (level 1) (doubled amount of insert)

DNA Parts
1. Glycine activator
2. PepD
3. Eicosane
4. CcUA
5. ChaC
6. CcUA
7. Eicosanol
8. Nitroxoline

Plasmid DVK-AF nanodrop (2203bp)
1. 23.6 ng/µl
2. 23.4 ng/µl  ⇒ 23.6 ng/µl → 16.51 fmol
3. 23.8 ng/µl

⇒ 0.61 µl of plasmid for 10 fmol
1) Glycine Activator
- 0.61 µL DVA-F
- 1.16 µL insert
- 1 µL 10x Ligase buffer
- 1 µL Ligase at 20U/µL
- 1 µL BsaI at 10U/µL
- 15.2 µL ddH₂O

2) PepD
- 0.61 µL DVA-F
- 2.06 µL insert
- 1 µL 10x Ligase buffer
- 1 µL Ligase at 20U/µL
- 1 µL BsaI at 10U/µL
- 14.33 µL ddH₂O

3) Eicosane
- 0.61 µL DVA-F
- 1.92 µL insert
- 1 µL 10x Ligase buffer
- 1 µL Ligase at 20U/µL
- 1 µL BsaI at 10U/µL
- 14.47 µL ddH₂O

4) GCVR-TF
- 0.61 µL DVA-F
- 0.92 µL insert
- 1 µL 10x Ligase buffer
- 1 µL Ligase at 20U/µL
- 1 µL BsaI at 10U/µL
- 15.47 µL ddH₂O

5) ChaC
- 0.61 µL DVA-F
- 1.14 µL insert
- 1 µL 10x Ligase buffer
- 1 µL Ligase at 20U/µL
- 1 µL BsaI at 10U/µL
- 15.25 µL ddH₂O

6) GCVA
- 0.61 µL DVA-F
- 1.14 µL insert
- 1 µL 10x Ligase buffer
- 1 µL Ligase at 20U/µL
- 1 µL BsaI at 10U/µL
- 14.99 µL ddH₂O

7) Eicosanol
- 0.61 µL DVA-F
- 1.18 µL insert
- 1 µL 10x Ligase buffer
- 1 µL Ligase at 20U/µL
- 1 µL BsaI at 10U/µL
- 15.21 µL ddH₂O

8) Nitramine
- 0.61 µL DVA-F
- 1.18 µL insert
- 1 µL 10x Ligase buffer
- 1 µL Ligase at 20U/µL
- 1 µL BsaI at 10U/µL
- 15.21 µL ddH₂O
Gel electrophoresis of all new MoClo assemblies

<table>
<thead>
<tr>
<th>Well</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Ladder</td>
<td>Uve</td>
<td>+ Ve</td>
<td>Glycine activator</td>
<td>Pep D</td>
<td>Eicosane</td>
<td>GCUR</td>
<td>ChaC</td>
<td>GCUA</td>
<td>Eic-RFP</td>
<td>Nitro</td>
<td>Ladder</td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>+ Ve</td>
<td>+ Ve</td>
<td>Duk-KAF</td>
<td>Duk-KAF</td>
<td>Duk-KAF</td>
<td>Duk-KAF</td>
<td>Duk-KAF</td>
<td>Duk-KAF</td>
<td>Duk-KAF</td>
<td>Duk-KAF</td>
<td>Duk-KAF</td>
<td></td>
</tr>
</tbody>
</table>

Transformations -
Transformed E. coli DH5α with the following assembled plasmids:
- Glycine activator
- Pep D
- Eicosane
- GCUR
- ChaC
+ 1 control

Re-plated K12
**MoClo assembly of Glycine activator and Eicosane**

- 2 µL loading buffer (6x) + 2 µL sample
- 8 µL ladder

<table>
<thead>
<tr>
<th>Well</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>ladder</td>
<td>-ve</td>
<td>+ve</td>
<td>Gly</td>
<td>Eic</td>
<td>ladder</td>
</tr>
</tbody>
</table>
14/8/19

Aims - Take out + check transformations
- Miniprep ChaC + glyA (gly A didn’t work)
- Send miniprep for sequencing

Transformations
None worked

Miniprep
Overnight of ChaC worked. GlyA didn’t

Miniprep ChaC
ChaC(F) - TS00533669
ChaC(R) - TS00533670

Transformation
5 PepD, Eicosane, GCUR - used Sul instead of 2µ
Aims - Maclo of PepD, Eicosane and glycine activator
- Transform bacteria
- Plate Kanamycin plates.

Transformations
⇒ GlyA, PepD, Eicosane, GCUR ⇒ none worked
⇒ Jasmines M6 + 0.974
⇒ Freeze shock 30 mins. Take out at 15:43

- How Long Shock lasts - glutathione 1.5mmol/l

16/8/19

Gel Electrophoresis

<table>
<thead>
<tr>
<th>Well</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample ladder</td>
<td>-ve</td>
<td>+ve</td>
<td>Glycine</td>
<td>Activator</td>
<td>PepD</td>
<td>Eicosane</td>
<td>GCUR</td>
<td>ChaC</td>
<td>Cas1</td>
<td>Cas2</td>
<td>Cas3</td>
<td>ladder</td>
</tr>
</tbody>
</table>
Aims - MoClo of Glycine activator, PepD, Eicosane, GCUR + ChAC
- Ratio of plasmid:G block

**Glycine Activator**

<table>
<thead>
<tr>
<th>Ratio</th>
<th>DUK-AF (µL)</th>
<th>Insert (µL)</th>
<th>ddH₂O (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:4</td>
<td>0.61</td>
<td>12.27</td>
<td>9.12</td>
</tr>
<tr>
<td>1:5</td>
<td>0.61</td>
<td>11.24</td>
<td>5.15</td>
</tr>
<tr>
<td>2:1</td>
<td>1.22</td>
<td>14.75</td>
<td>0.61</td>
</tr>
</tbody>
</table>

**GCUR**

<table>
<thead>
<tr>
<th>Ratio</th>
<th>DUK-AF (µL)</th>
<th>Insert (µL)</th>
<th>ddH₂O (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>0.46</td>
<td>15.93</td>
<td>0.61</td>
</tr>
<tr>
<td>1:2</td>
<td>0.92</td>
<td>15.47</td>
<td>0.61</td>
</tr>
<tr>
<td>1:3</td>
<td>1.38</td>
<td>15.01</td>
<td>0.61</td>
</tr>
</tbody>
</table>

**PepD**

<table>
<thead>
<tr>
<th>Ratio</th>
<th>DUK-AF (µL)</th>
<th>Insert (µL)</th>
<th>ddH₂O (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>0.61</td>
<td>15.36</td>
<td>0.03</td>
</tr>
<tr>
<td>1:2</td>
<td>0.61</td>
<td>14.33</td>
<td>1.06</td>
</tr>
<tr>
<td>1:3</td>
<td>0.61</td>
<td>13.30</td>
<td>3.09</td>
</tr>
</tbody>
</table>


## Eicosane (LadA)

<table>
<thead>
<tr>
<th>Ratio</th>
<th>DUK-AF</th>
<th>Insert</th>
<th>ddH₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>0.61 µL</td>
<td>0.57 µL</td>
<td>15.82 µL</td>
</tr>
<tr>
<td>1:2</td>
<td>0.61 µL</td>
<td>1.14 µL</td>
<td>15.25 µL</td>
</tr>
<tr>
<td>1:3</td>
<td>0.61 µL</td>
<td>1.71 µL</td>
<td>14.68 µL</td>
</tr>
<tr>
<td>1:4</td>
<td>0.61 µL</td>
<td>2.28 µL</td>
<td>14.11 µL</td>
</tr>
<tr>
<td>1:5</td>
<td>0.61 µL</td>
<td>2.85 µL</td>
<td>13.54 µL</td>
</tr>
<tr>
<td>2:1</td>
<td>1.22 µL</td>
<td>0.57 µL</td>
<td>15.21 µL</td>
</tr>
</tbody>
</table>

## PCR

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |
|------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Sample | Ladder | -ve | +ve | GA | GA | CA | CA | GA | GA | CA | CA | GA | GA | CA | CA | GA | GA | GA | GA | GA | GA | GA | GA | GA | GA |
|        |      |     |     | 1  | 2  | 3  | 4  | 5  | 1  | 2  | 3  | 4  | 5  | 1  | 2  | 3  | 4  | 5  | 1  | 2  | 3  | 4  | 5  | 1  | 2  | 3  | 4  |

---

Note: The table above shows the PCR results for various samples with labels 1 to 24. The lanes are divided into groups with corresponding labels for easy reference.
20/8/19

**Miniprep Cas repeat (suspended in T6)**

- 1% gel ran - 100V
  - 80min + extra 30mins 2µL loading dye
  - (0.71 agarose next time)

**Nanodrop result of miniprep**

- 35.18ng/µL
- 55.77ng/µL

**Transforming BL21DE3 E.coli**

- 50µL of chemically competent E.coli was defrosted
- 2µL plasmid DNA was added
- This was incubated on ice for 30 mins
- This was then heat shocked at 42°C for 1min
- The mixture was incubated on ice for another 2mins
- 200µL of LB was added
- This was incubated at 37°C for 1 hour
- The contents was plated onto an LB plate with Chloramphenicol
- Plates were incubated overnight at 37°C
21/8/19

→ Ran gels from yesterday - bands present

→ Transformations of 'Gly-active', ChaC, PepD, GCUR - none worked

→ Transformed E. coli with DUK-AF → worked.

22/8/19

→ Overnight culture of DUK-AF
21/8/19

Protein expression testing - overnight culture

Day 1

1) Aliquot 10mL liquid LB into 50mL falcon tube
2) Add antibiotic (Chloramphenicol 10 µL)
3) Take a single colony of transferred E-coli (BL21 DE3) Cas13a - add to the falcon tube.
4) Loosely cap
5) Incubate at 37°C with shaking.

Day 2 - 22/8/19

6) Subculture cells 1/100 dilution in 10mL liquid LB in a sterilin (100 µL overnight culture in 10mL LB)
7) Incubate at 37°C with shaking until OD_{600} is between 0.4 - 0.6 (monitored hourly)

Start - 9:10am

10:00 OD_{600} = 0.035
11:00 OD_{600} = 0.071
12:00 OD_{600} = 0.179
13:00 OD_{600} = 0.476 - Final measure.

8) Remove 500 µL into an eppendorf and centrifuge at 13,000 rpm for 2 mins. Discard Supernatant and label cells as 'time = 0' control.
9) Split the culture into 2 sterilins - 2.5mL each with 3 µL IPTG added.
10) Incubate at 37°C with shaking for 3-4 hours

Start - 13:30
End - 16:45

11) Remove 500 µL of each sample into a 1.5mL eppendorf respectively.
   - Centrifuge samples at 13,000 rpm for 2 mins.
   - Discard the supernatant
   - Label and refrigerate.
Day 3 - 23/8/19

SDS-PAGE prepared by Connor (acrylamide at 12%)

12) Add 50µL of SDS-PAGE loading buffer to each pellet and thoroughly re-suspend them - include the ‘time = 0’ pellet

13) Seal the eppendorf tubes. Incubate samples at 100°C on a heatblock for 10 mins. (This solubilises the pellet)

14) Vortex every 2 mins to mix
   - careful of pressure build up
   - open and close gently before vortexing.

Centrifuge samples at 13,000rpm for 2 mins

15) Prepare SDS-PAGE with appropriate acrylamide % (15% recommended)

16) Assemble the gel in the gel-running tank with a buffer dam on the other side of the gel holder

17) Remove the comb from the gel

18) Add 1 x SDS-PAGE running buffer to the internal reservoir
   - make sure there are no air bubbles in the lanes
   - check that the reservoir doesn't leak

19) Fill external reservoir to the line with 1 x SDS-PAGE running buffer.

20) Load 5-10 µL of protein ladder into the first well.

21) Load 15 µL of each sample into separate wells.

   Well #1 = ladder, 
   2 = ‘time = 0’ 
   3 = control 
   4 = With IPTG

22) Run the gel at 200 volts for 40 mins or until blue dye starts to leak from the bottom of the gel

23) Stain the gel with Coomassie blue to see the protein bands

First SDS-PAGE was unsuccessful due to glass breakage and the gel being ripped.

A second attempt was made with Annie's guidance.
24) Add 0.5 g coomassie brilliant blue r-250 to a clean 500 mL duran bottle, this is for the coomassie stain.

25) Add 200 mL ethanol to the duran and mix.

26) Carefully add 250 mL of acetic acid to the duran and mix.

27) Add 50 mL distilled water to the duran and mix.

28) Make destain by adding 1 L ethanol and 500 mL acetic acid to a 10 L carboy, filling to 5 L with distilled water and mixing.

Both stains were available were pre-available in the lab for general use.
Gel Staining

1) Carefully remove the SDS-PAGE from the glass and place it in a plastic tray (dd-water makes this easier).
2) Rinse the gel with dd-water to remove excess buffer.
3) Cover the gel in buffer - it should be fully submerged.
4) Place a cracked lid on the plastic tray and microwave for 40 seconds. Immediately cover and take to the fume hood.
5) Open in the fume hood for 5 mins, then re-cover and leave to stain for ~1 hour.
6) Remove the stain (pour into original bottle), and rinse the gel with dd-water.
7) Repeat with destain, but decant the used destain into a carboy with an activated charcoal filter.
8) Image gel using x
   If bands are faint, repeat stain/destain as necessary.
23/8/19

→ Miniprep DUK-AT bacteria
   2×Jasmine, 2×iCEM

→ Make agar plates - minimal media different concentrations of glycine, cysteine and tryptophan.

**Minimal media agar plates**

1. 400ml of dH₂O, 22.56g minimal media (M9 salts) and 4g agar

2. Make 10mM stock solutions of glycine, cysteine + tryptophan
   - Glycine (75.07g/mol) = 0.0751g in 100ml dH₂O
   - Cysteine (121.16g/mol) = 0.12116g in 100ml dH₂O
   - Tryptophan (204.23g/mol) = 0.20423 in 100ml dH₂O
Eicosanol Stock Solution

10 cm³ Stock Solution at 50 mM

\[ C = 5 \times 10^{-5} \text{mol dm}^{-3} \]

\[ V = 0.01 \text{ dm}^3 \]

\[ n = 5 \times 10^{-7} \text{ moles} \]

\[ M_r = 298.62 \]

\[ m = 0.14931 \text{ g} \]

\[ \text{mass used} = 0.14931 \text{ g eicosanol} \]

To make stock 0.14931 g eicosanol was dissolved in a minimal amount of acetone and diluted appropriately with ddH₂O.

28/08/19

Minimal acetone was too much, ethanol and DMSO were tried and Ethyl Ether was found to be the most effective.
<table>
<thead>
<tr>
<th>GlyA</th>
<th>1:2 ratio: 1.92 µ insert</th>
<th>14.08 µ ddH₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:3 ratio: 2.88 µ insert</td>
<td>13.12 µ ddH₂O</td>
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</tr>
<tr>
<td></td>
<td>constant: 0.58 µ insert</td>
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<tr>
<td></td>
<td>1.00 µ DUK-AF</td>
<td></td>
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<tr>
<td></td>
<td>1.00 µ ligase buffer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00 µ ligase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00 µ BsaI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.42 µ ddH₂O</td>
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<table>
<thead>
<tr>
<th>Chac</th>
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<th>14.08 µ ddH₂O</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1:2 ratio: 1.38 µ insert</td>
<td>14.62 µ ddH₂O</td>
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<tr>
<td></td>
<td>1:3 ratio: 1.38 µ insert</td>
<td>14.62 µ ddH₂O</td>
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<tr>
<td></td>
<td>constant: 0.46 µ insert</td>
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<tr>
<td></td>
<td>1.00 µ DUK-AF</td>
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<td></td>
<td>1.00 µ ligase buffer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00 µ ligase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00 µ BsaI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.54 µ ddH₂O</td>
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</table>

<table>
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<tr>
<th>Eicosane</th>
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<th>14.86 µ ddH₂O</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>1:2 ratio: 1.4 µ insert</td>
<td>14.86 µ ddH₂O</td>
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<tr>
<td></td>
<td>1:3 ratio: 1.71 µ insert</td>
<td>14.29 µ ddH₂O</td>
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<tr>
<td></td>
<td>constant: 0.96 µ insert</td>
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<tr>
<td></td>
<td>1.00 µ DUK-AF</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00 µ ligase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00 µ l-Buffer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00 µ BsaI</td>
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</tr>
<tr>
<td></td>
<td>15.04 µ ddH₂O</td>
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</table>
Gel electrophoresis of Moclo

150 μl of 0.5 x TBE + 2.25 g agarose

| Lane | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  | 19  |
|------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Sample Ladder | +ve | G1 | G1 | +ve | GA | GA | Rep | Rep | Rep | Rep | E1 | E1 | E1 | G1 | G1 | G1 | G1 | G1 | G1 | G1 |
| Ladder | | | | | | | | | | | | | | | | | | | |
28/08/19

- Test the effects of Diethyl ether on the competence of E. coli DHSα
- Plate Eic-RFP DHSα bacteria
- Transformations

Stock solutions - 50%, 10mL
- 20% (0.2mL diethyl ether + 0.8mL water)
- 1% (10µL in 190µL water)
- 0.1% (20µL in 180µL water).

<table>
<thead>
<tr>
<th>Final Conc (%)</th>
<th>Diethyleneether (µL)</th>
<th>LB (µL)</th>
<th>E. coli (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.000</td>
<td>10 (20%)</td>
<td>188</td>
<td>2</td>
</tr>
<tr>
<td>0.500</td>
<td>5 (20%)</td>
<td>193</td>
<td>2</td>
</tr>
<tr>
<td>0.100</td>
<td>1 (20%)</td>
<td>197</td>
<td>2</td>
</tr>
<tr>
<td>0.050</td>
<td>10 (1%)</td>
<td>188</td>
<td>2</td>
</tr>
<tr>
<td>0.010</td>
<td>2 (1%)</td>
<td>196</td>
<td>2</td>
</tr>
<tr>
<td>0.005</td>
<td>10 (0.1%)</td>
<td>188</td>
<td>2</td>
</tr>
<tr>
<td>0.001</td>
<td>2 (0.1%)</td>
<td>196</td>
<td>2</td>
</tr>
<tr>
<td>0.000</td>
<td>20 (100%)</td>
<td>178</td>
<td>2</td>
</tr>
<tr>
<td>0.000</td>
<td>10 (100%)</td>
<td>188</td>
<td>2</td>
</tr>
</tbody>
</table>

Grow for 16hrs - 180rpm, 37°C, OD600
**Transformation**

Bacteria = DH5α E.coli

\[ \text{Gly A 1:1, Gly A 1:2, Pep D 1:1, Pep D 1:2, Eic 1:1, Eic 1:2, GCUR 1:1, GCUR 1:2, ChaC 1:1, ChaC 1:2.} \]

1. Add 5 μL of plasmid to chemically competent bacteria + put on ice for 30 mins
2. Heat shock for 1 min at 42°C
3. Place on ice for 1 min, then add 200 μL LB
4. Incubate at 37°C for 1 hour
5. Plate on Kanamycin plate overnight.

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**29/8/19**

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**Results**

- Transformations that worked:
  - Glycine activator 1:1
  - Pep D 1:2
  - Eic 1:1

- Transformations that didn't work:
  - ChaC
  - GCUR

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**To do**

- Make overnight cultures of Gly A, Pep D + Eic
- Minimal media
- Transform ChaC + GCUR
  - Use 1:1, 1:2 again.
30/8/19

- Transformations
  - Cha C and CCUR maybe worked
- Overnight
  - Eic and Pep D worked
  - Glycine Activator not grown. Have to transform again.

Today
- Miniprep Pep D + Eic

**Eic 1**

80.16 ng/µl 1.88 260/280
80.67 ng/µl 1.90 260/280
82.18 ng/µl 1.92 260/280.

**Eic 2**

70.10 ng/µl 1.89 260/280
71.10 ng/µl 1.91 260/280
70.72 ng/µl 1.92 260/280.

**Pep 1**

105 ng/µl 1.89 260/280
107 ng/µl 1.89 260/280
107.11 ng/µl 1.89 260/280.

**Pep 2**

100.66 1.89 260/280
100.99 1.91 260/280
101.03 1.92 260/280.
2/9/19

To do list

• Make/autoclave 100ml LB + 400ml LB Agar
• Plate reader assay (16h in plate reader)
• Overnight incubation of diethyl ether
• Inoculate 10µl LB overnight of DH5α
• Send DNA XX XX Sequence (PepD+Eic)

16h Plate reader assay

<table>
<thead>
<tr>
<th>Final Conc(%)</th>
<th>Diethyl Ether(µ)</th>
<th>LB(µ)</th>
<th>E. Coli(µ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.000</td>
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<td>178</td>
<td>2</td>
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<tr>
<td>5.000</td>
<td>10 (100%)</td>
<td>196</td>
<td>2</td>
</tr>
</tbody>
</table>

3x Control (LB+ Ecoli - OC) - Control A

Stocks

• 20% - 0.2ml of 100%, 0.8ml H2O
• 1% - 10µl of 20%, 190µl H2O
• 0.1% - 20µl of 1%, 180µl H2O.
Sent for sequencing
TS00942498 - Eic F0
TS00942499 - Eic F0
TS00942500 - Pep F0
TS00942501 - Pep F0
TS00942502 - Eic R0
TS00942503 - Eic R0
TS00942504 - Pep R0
TS00942505 - Pep R0

16hr incubation

Final volume = 600µL

<table>
<thead>
<tr>
<th>Final Conc (%)</th>
<th>Diethylether (µL)</th>
<th>LB (µL)</th>
<th>E. coli (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.000</td>
<td>30 (20%)</td>
<td>534</td>
<td>6</td>
</tr>
<tr>
<td>0.500</td>
<td>15 (20%)</td>
<td>564</td>
<td>6</td>
</tr>
<tr>
<td>0.100</td>
<td>3 (20%)</td>
<td>564</td>
<td>6</td>
</tr>
<tr>
<td>0.050</td>
<td>30 (1%)</td>
<td>579</td>
<td>6</td>
</tr>
<tr>
<td>0.010</td>
<td>6 (1%)</td>
<td>591</td>
<td>6</td>
</tr>
<tr>
<td>0.005</td>
<td>30 (0.1%)</td>
<td>564</td>
<td>6</td>
</tr>
<tr>
<td>0.001</td>
<td>6 (0.1%)</td>
<td>588</td>
<td>6</td>
</tr>
<tr>
<td>0.000</td>
<td>60 (100%)</td>
<td>564</td>
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</tr>
<tr>
<td>5.000</td>
<td>30 (100%)</td>
<td>588</td>
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