

CONTENTS

A. Buffers:

1. **1X TAE Buffer (Tris acetate EDTA), 1:50 dilution from 50X stock**

50X stock recipe:

242 g Tris-free base dissolved in MilliQ water

57.1 mL glacial acetic acid

18.61 g Disodium EDTA

Make volume up to 1 L with MilliQ water

To make 1 L 1X TAE, 20 mL 50X stock added to 980 mL MilliQ water

2. **5X Laemmli Buffer**

Stock recipe:

5 mL 10% w/v SDS solution

2.5 mL 100% v/v glycerol

1 mL 1 M Tris-Cl (pH 6.8)

500 μ L 1% w/v bromophenol blue

Make volume up to 10 mL with MilliQ water

To make 1 mL 5X Laemmli buffer, 900 μ L stock added to 100 μ L fresh β -mercaptoethanol

1. **1X SDS Protein Running Buffer, 1:10 dilution from 10X stock**

10X stock recipe:

288 g glycine dissolved in MilliQ water

60 g Tris

20 g SDS

Make volume up to 2 L with MilliQ water

To make 1 L 1X SDS Protein Running Buffer, 100 mL 10X stock added to 900 mL MilliQ water

2. **50 mM HEPES Buffer (pH 8.0), 1:20 dilution from 1 M stock**

11.91 g HEPES powder dissolved in MilliQ water

Adjust pH to 8.0 with 10N NaOH

Make volume up to 50 mL with MilliQ water

To make 50 mL 50 mM HEPES Buffer (pH 8.0), 2.5 mL 1 M stock added to 47.5 mL MilliQ water

3. **NaPi Sonication Buffer (pH 7.6)**

50 mM Na₃PO₄

300 mM NaCl

10 mM imidazole

0.1 mM MgCl₂

4. **NaPi Wash Buffer (pH 7.6)**

50 mM Na₃PO₄

500 mM NaCl

30 mM imidazole

5. NaPi Elution Buffer (pH 7.6)

50 mM Na₃PO₄
500 mM NaCl
500 mM imidazole

6. HEPES Sonication Buffer (pH 8.0)

50 mM HEPES
300 mM NaCl
10 mM imidazole
0.1 mM MgCl₂

7. HEPES Wash Buffer (pH 8.0)

50 mM HEPES
500 mM NaCl
30 mM imidazole

8. 1X PBST Buffer

100 mL 10X PBS
1 mL Tween 20 (0.1% v/v final concentration)
Make volume up to 1 L with MilliQ water

9. 1X Western Blot Transfer Buffer, 1:10 dilution from 10X stock

10X stock recipe (without methanol):

288 g glycine dissolved in MilliQ water
60 g Tris
5 g SDS

Make volume up to 2 L with MilliQ water

To make 1 L 1X Western Blot Transfer Buffer, 100 mL 10X stock added to 200 mL methanol
+ 700 mL MilliQ water

10. Western Blot Blocking Buffer

3 g skim milk powder dissolved in 100 mL 1X PBST (3% w/v final concentration)

11. Western Blot Stripping Buffer

15 g glycine dissolved in MilliQ water
1 g SDS
10 mL Tween 20 (1% v/v final concentration)
Adjust pH to 2.2 with 10N HCl
Make volume up to 1 L with MilliQ water

B. Others:

1. **LB broth**

20 g Luria broth powder dissolved in 1 L MilliQ water
Autoclaved at 121°C for 15 mins

2. **LB agar**

30 g Luria agar powder suspended in 1 L MilliQ water
Heated to a boil with stirring
Autoclaved at 121°C for 15 mins

3. **Terrific broth**

47.6 g Terrific broth powder dissolved in MilliQ water
0.4 mL 100% v/v glycerol
Make volume up to 1 L with MilliQ water
Autoclaved at 121°C for 15 mins

4. **50 mg/mL Kanamycin**

1 g kanamycin dissolved in 20 mL MilliQ water
Filter sterilized with 0.22 µm filter

5. **100 mg/mL Ampicillin**

2 g ampicillin dissolved in 20 mL MilliQ water
Filter sterilized with 0.22 µm filter

6. **0.5 M IPTG**

2.383 g IPTG dissolved in 20 mL MilliQ water
Filter sterilized with 0.22 µm filter

7. **1 M PMSF**

0.0174 g PMSF dissolved in 1 mL DMSO

8. **CBB Destaining Solution**

100 mL glacial acetic acid
300 mL methanol
600 mL MilliQ water

Constructs:

4 µg lyophilized , dissolved in 20 µL sterilised water.

Vial numbers: (Original GenScript vials)

C1: dCBD + SpyCatcher (858 bp)

C2: OpdA + SpyTag (2058 bp)

The above was already cloned in pUC57-Kan vector and was our stock.

Primers:

100 µM stock was diluted to 10 µM in Eppendorf Tubes.

(10 µL of 100 µM stock in 90 µL sterile water)

Vial numbers:

P144: C1_FOR

P145: C1_REV

P146: C2_FOR

P147: C2_REV

A. Resuspending Constructs:

- The vials were centrifuged at 6000 rpm and 4°C for 1 min.
- 20 µL sterile water was added to each vial.
- The vials were vortexed for 1 min.
- The vials were heated at 50°C for 10 mins to ensure that pellets were not present.

B. PCR Amplification of constructs:

- **PCR Mix:**

Materials	1 Reaction	Master Mix (for 5 reactions)
10X KOD Buffer	2.5 µL	12.5 µL
25 mM MgSO ₄	1.5 µL	7.5 µL
2 mM dNTPs	2.5 µL	12.5 µL
10 µM FOR Primer (FP)	1 µL	-
10 µM REV Primer (RP)	1 µL	-
Template DNA	0.5 or 1 µL	-
KOD Polymerase	0.5 µL	2.5 µL
Nuclease-Free H ₂ O	15.5 or 15 µL	75 µL

Total reaction volume is 25 μ L per PCR tube.

After all the components for the master mix were added, it was spun down.

Note: Template DNA here were our stock plasmids pUC57-Kan containing the constructs C1 or C2.

Reaction Setup: 22 μ L of master mix was added to 4 tubes, which were labelled and filled with the remaining components as follows:

Tube No.	Template DNA (Construct)	Primers
1	C1 (0.5 μ L)	C1_FOR + C1_REV (1 μ L each)
2	C1 (1 μ L)	C1_FOR + C1_REV (1 μ L each)
3	C2 (0.5 μ L)	C2_FOR + C2_REV (1 μ L each)
4	C2 (1 μ L)	C2_FOR + C2_REV (1 μ L each)

So that all tubes contained 25 μ L of reaction mixture, to Tube 1 and 3, 0.5 μ L more water (NF H₂O) was added.

After adding all the components, they were mixed by flicking and pipetting, then the tubes were spun down.

Reaction Times and Temperatures:

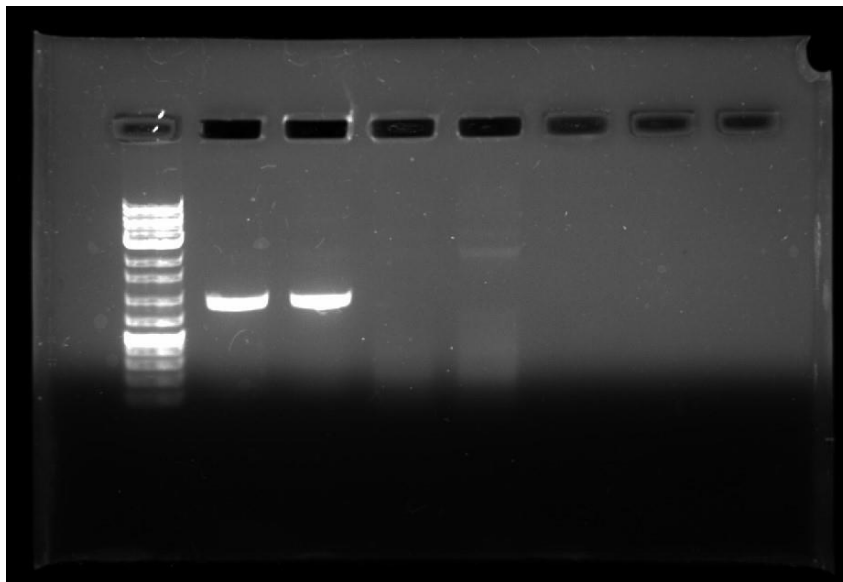
Tube No.	Stage	Temp.	Time
1 and 2	Denaturation (initial) + Polymerase Activation	95°C	2 mins
	Denaturation	95°C	30 s
	Annealing	58°C	30 s
	Extension	70°C	30 s
3 and 4	Denaturation (initial) + Polymerase Activation	95°C	2 min
	Denaturation	95°C	30 s
	Annealing	58°C	30 s
	Extension	70°C	1 min

For all the 4 tubes, 35 cycles of denaturation, annealing and extension were carried out. More extension time was given for tubes 3 and 4 as C2 is much longer.

C. Agarose Gel Electrophoresis for PCR verification:

1. 1% Agarose Gel was prepared by adding 0.35 g Agarose to 35 mL 1X TAE buffer. The solution was boiled till it was clear.
2. The solution was cooled down until the flask could be touched.
3. 2 μ L of 1 mg/mL EtBr was added to the solution and mixed by swirling, and then the solution was poured into the cast and allowed to solidify.
4. Lanes 2-5 were loaded with 4 μ L NF H₂O, 1 μ L 6X loading dye, and 1 μ L PCR product. Samples were as follows:
 - Lane 1: 1.2 μ L 1 kb ladder
 - Lane 2: Tube 1
 - Lane 3: Tube 2
 - Lane 4: Tube 3
 - Lane 5: Tube 4
5. The gel was run for around 10 mins at 150 V in 1X TAE running buffer.

Gel Results:



Lanes 2 and 3 showed a band slightly smaller than 1 kb. However, Lanes 4 and 5 did not show significant bands. A very faint band around 2 kb was visible in lane 5.

So, the PCR for C1 had seemed to work, but not for C2.

The C1 PCR products, i.e., the contents of tubes 1 and 2 were pooled together (total volume 50 μ L) and stored at -20°C (**labelled C1 PCR**).

Suggestions: Annealing temperature/extension time needed to be changed for C2.

D. Transforming DH5α with pUC57-Kan vectors carrying C1 & C2:

Strain to be transformed: *E. coli* DH5α, made chemically competent using CaCl₂.

1. 3 tubes were taken, and each was filled with 120 μL of competent cell suspension. The following components were then added:
 - Tube C1: 1 μL of C1 stock
 - Tube C2: 1 μL of C2 stock
 - Tube NC: 1 μL sterile water
2. The tubes were kept on ice for 30 min after addition of DNA.
3. Heat shock at 42°C was given for 1.5 min.
4. The tubes were returned to ice, and kept there for 5 min.
5. The cells were allowed to recover by adding 1 mL LB broth to each tube and incubating the tubes at 37°C, with shaking at 190 rpm for 1 hr.
6. The tubes were taken out and centrifuged at 4000 rpm for 3 min to pellet the cells.
7. 0.9 mL supernatant media was taken out from each tube. The cell pellet was resuspended in the remaining media.
8. The cells were then plated on LB + Kan agar (spread using 8-10 glass beads per plate), and the plates were incubated at 37°C for 14 hrs.

A. PCR Amplification of C2:

PCR Mix: Same as on 21 June. No master mix was prepared. Instead, the mix composition for 1 reaction was used. For template, 1 μ L of C2 was added, and for primers, 1 μ L each of C2_FOR and C2_REV primers were added.

Reaction Times and Temperatures: 35 cycles of PCR as before.

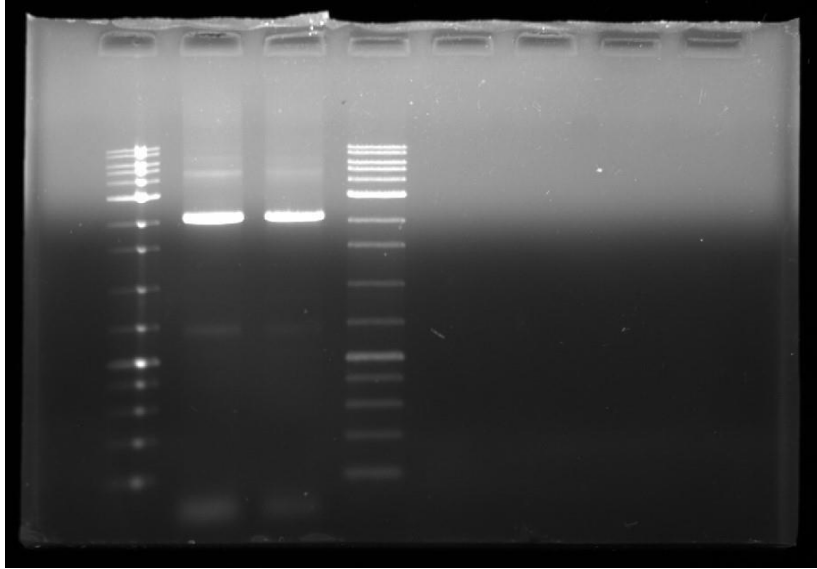
Tube No.	Stage	Temp.	Time
3	Denaturation (initial) + Polymerase Activation	95°C	2 mins
	Denaturation	95°C	30 s
	Annealing	58°C	30 s
	Extension	70°C	2.5 mins
4	Denaturation (initial) + Polymerase Activation	95°C	2 mins
	Denaturation	95°C	30 s
	Annealing	55°C	30 s
	Extension	70°C	1.5 mins

B. Agarose Gel Electrophoresis for PCR verification:

Composition - 1% agarose gel, 0.35 g agarose dissolved in 35 mL 1X TAE. 2 μ L of 1 mg/mL EtBr was added.

- Samples were loaded as follows:
 - Lane 1: 1.2 μ L 1 kb ladder
 - Lane 2: Tube 3
 - Lane 3: Tube 4
 - Lane 4: 1.2 μ L 1 kb ladder
- The gel was run for around 10 mins at 150 V in 1X TAE running buffer.

Gel Results:

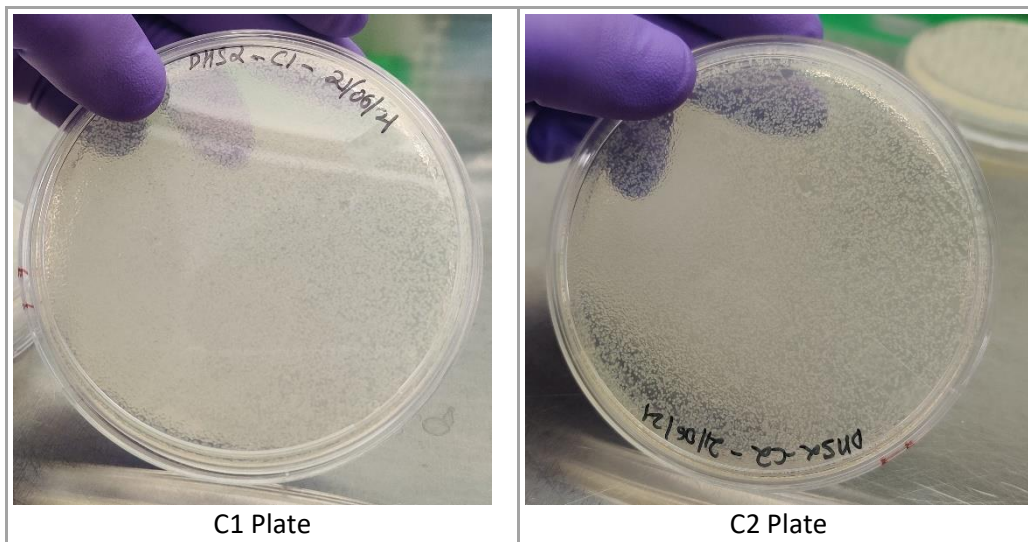


Bands around 2 kb were visible in Lanes 2 and 3. So, the PCR for C2 had also seemed to work.

The C2 PCR products, i.e., the contents of tubes 3 and 4 were pooled together (total volume 50 μ L) and stored at -20°C (**labelled C2 PCR**).

A. Inoculation of transformed DH5 α (continued from 21 June, D):

1. The plates were taken out from 37°C and transferred to the hood at room temperature for inoculation. Pictures of the two plates (corresponding to C1 and C2) are given below:



2. A colony from each plate was inoculated into separate test tubes containing 6 mL LB broth + 6 μ L of 50 mg/mL Kan (working concentration 50 μ g/mL). The plates were returned to 4°C.
3. The tubes were incubated at 37°C with shaking at 180 rpm for 15 hrs.

D. pETMCN Digestion with NdeI:

Master Mix:

Materials	Volume
10X CutSmart Buffer	3 μ L
NdeI	3 μ L
Sterile water	9 μ L
pETMCN vector	15 μ L

Total reaction volume was 30 μ L (**labelled cut pETMCN**).

The above components were mixed then incubated at 37°C for 4 hrs.

A. Miniprep of pUC57-Kan vectors carrying C1 & C2 (continuing after 22 June, C):

Miniprep was done using **Thermo's GeneJET Plasmid Miniprep Kit (K0503)**.

1. 6 mL culture from each test tube was transferred to 3 Eppendorf tubes each of volume 2 mL.
2. The tubes were centrifuged at 8000 rpm for 2 mins to pellet the cells. The supernatant was discarded.
3. To the first tube for each culture, 250 μ L Resuspension Buffer was added, and cells were resuspended by pipetting in and out.
4. The above was transferred to the next tube, and resuspension was carried out again. This was repeated until all of the resuspended cells resided in the last tube for each culture.
5. 250 μ L Lysis Buffer was added to the tubes containing the resuspended cells. The tube contents were mixed by inverting a few times, and the suspension became clearer.
6. 350 μ L Neutralization Buffer was added to the tubes. The tube contents were mixed by inverting a few times, and some white precipitate was observed.
7. The tubes were centrifuged at 14000 rpm for 5 mins. The debris was pelleted, with the plasmid DNA staying in the supernatant.
8. The supernatant from each tube was transferred to separate spin columns.
9. The spin columns were centrifuged at 14000 rpm for 1 min. The flowthrough collected in the collecting tubes was discarded.
10. 500 μ L Wash Buffer was added to the spin columns. Step 9 was repeated.
11. Step 10 was repeated.
12. The spin columns were centrifuged at 14000 rpm for 1 min (empty spin).
13. The collecting tubes were then removed, and the spin columns were placed into Eppendorf tubes.
14. 40 μ L Elution Buffer was added to the spin columns, and allowed to stand for 10 mins.
15. The tubes were centrifuged at 14000 rpm for 1 min. The flowthrough was collected inside the Eppendorf tubes (**labelled C1 MP and C2 MP**).
16. Concentration of plasmid DNA was measured using a Nanodrop machine (elution buffer was taken as blank). Loading volume was 2 μ L for all samples.
17. The Eppendorf tubes were stored at -20°C.

Nanodrop results:

Sample	Concentration (ng/ μ L)	A260/A280	A260/A230	A260
C1 MP	306.45	1.932	2.253	6.151
C2 MP	340.70	1.923	2.284	6.860

Since $A260/A280 > 1.8$, the miniprep samples can be considered pure. $A260/A230$ is around 2.2-2.3, which is very slightly high.

B. Gel Extraction of PCR amplified constructs & cut pETMCN vector:

Composition - 1% agarose gel, 1 g agarose dissolved in 100 mL 1X TAE. 1.5 µL of 1 mg/mL EtBr was added.

Samples were loaded as follows:

- Lane 1: 2 µL 1 kb ladder
- Lane 2: 50 µL C1 PCR + 11 µL 6X loading dye
- Lane 3: 50 µL C2 PCR + 11 µL 6X loading dye
- Lane 4: 30 µL cut pETMCN + 7 µL 6X loading dye
- Lane 5: 1 µL uncut pETMCN + 1 µL 6X loading dye + 4 µL NF H₂O

Gel Results:

This gel is not to be imaged under UV, to minimise exposure (whole volume of the PCR products and the cut vector were loaded).

Some non-specific bands were visible in Lanes 2 and 3 in addition to the proper bands. Lane 4 had one band corresponding to the cut pETMCN vector. Lane 5 had two very faint bands just below and above the band in lane 4, which correspond to the circular and supercoiled forms of plasmid DNA. Linear form was not observed as the vector was uncut here.

- The correct bands (corresponding to the size of C1 and C2) were excised from lanes 2, 3 and 4 using a scalpel, and placed in 3 separate tubes.

The following steps were carried out using **Qiagen's QIAquick Gel Extraction Kit (28704)**.

- 600 µL Buffer QG was added to all 3 tubes.
- The tubes were incubated at 50°C for 10 mins. The contents were mixed by inverting regularly in order to completely dissolve the gel pieces.
- 300 µL isopropanol was added to each tube and mixed by inverting.
- The tube contents were transferred to separate spin columns.
- The spin columns were centrifuged at 14000 rpm for 1 min. The flowthrough collected in the collecting tubes was discarded.
- 750 µL Buffer PE was added to the columns for washing. Step 6 was repeated.
- The spin columns were centrifuged at 14000 rpm for 1 min (empty spin).
- The collecting tubes were then removed, and the spin columns were placed into Eppendorf tubes.
- 50 µL Buffer EB was added to the tubes corresponding to C1 and C2; 35 µL Buffer EB was added to the tube corresponding to cut pETMCN. The tubes were allowed to stand for 10 mins.
- The tubes were centrifuged at 14000 rpm for 1 min. The flowthrough was collected inside the Eppendorf tubes (**labelled C1 PCR GE, C2 PCR GE and cut pETMCN GE**).
- After taking samples for verification gel, the tubes were stored at -20°C.

C. Agarose Gel Electrophoresis for Gel Extraction verification:

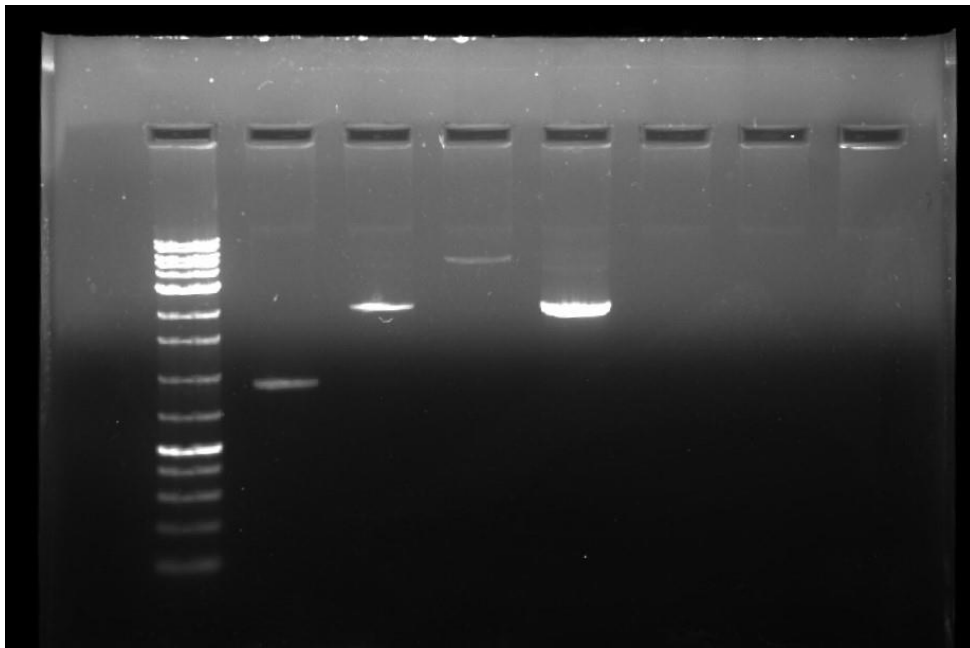
Composition - 1% agarose gel, 0.35 g agarose dissolved in 35 mL 1X TAE. 2 μ L of 1 mg/mL EtBr was added.

Lanes 2-5 were loaded with 4 μ L NF H₂O, 1 μ L 6X loading dye, and 1 μ L gel extracted product.

Samples were loaded as follows:

- Lane 1: 1.2 μ L 1 kb ladder
- Lane 2: C1 PCR GE
- Lane 3: C2 PCR GE
- Lane 4: cut pETMCN GE
- Lane 5: C2 PCR GE

Gel Results:



The band in lane 2 was faint, possibly due to a loading mistake.

The band in lane 3 was irregular, again possibly due to a loading mistake.

The band in lane 4 was faint, thus, the cut vector seemed to be quite dilute, which is desirable.

The band in lane 5 is bright.

Since the results are not very consistent, this gel was repeated on 24 June 2021.

A. Agarose Gel Electrophoresis for Gel Extraction verification:

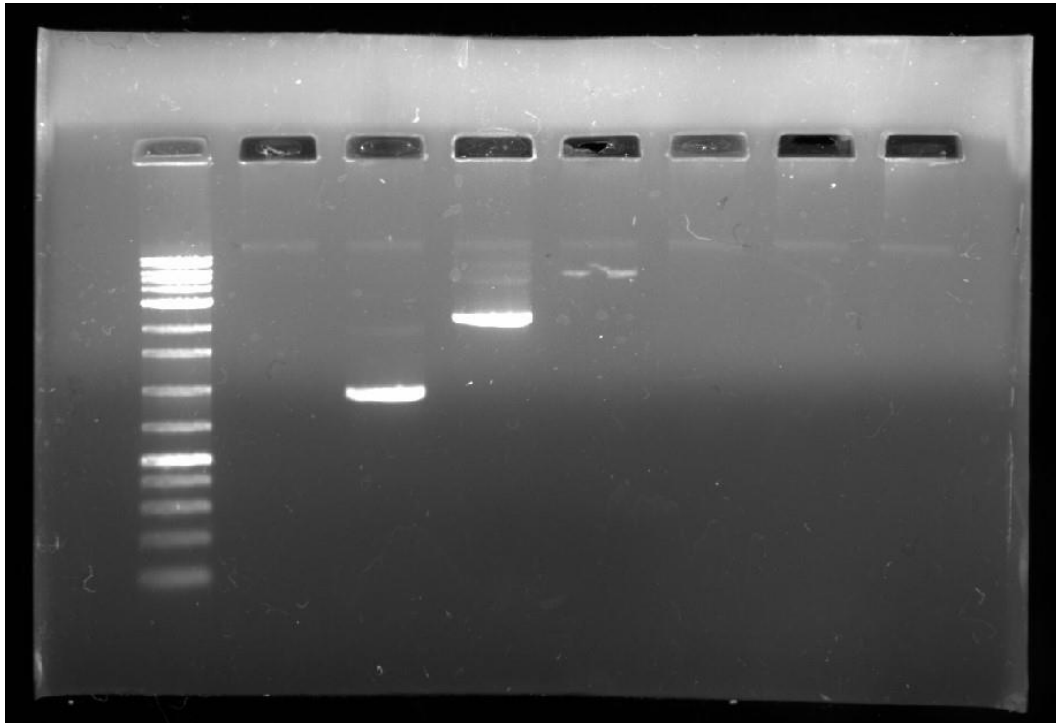
Composition - 1% agarose gel, 0.35 g agarose dissolved in 35 mL 1X TAE. 2 μ L of 1 mg/mL EtBr was added.

Lanes 3-5 were loaded with 4 μ L NF H₂O, 1 μ L 6X loading dye, and 1 μ L gel extracted product.

Samples were loaded as follows:

- B. Lane 1: 1.2 μ L 1 kb ladder
- C. Lane 3: C1 PCR GE
- D. Lane 4: C2 PCR GE
- E. Lane 5: cut pETMCN GE

Gel Results:



The bands in lane 3 and 4 were similarly bright, suggesting the concentration of both PCR products was high. 1 or 2 very faint contaminant bands could also be observed.

The band in lane 5 was comparatively faint, thus, the cut vector seemed to be quite dilute, which is desirable. The irregular shape might have been due to defects in the gel.

Since a roughly 1:3 to 1:4 ratio of construct to vector is most appropriate for Gibson Assembly, the above gel seemed to suggest Gibson Assembly could now be carried out using our constructs and cut vector.

B. Gibson assembly of constructs:

Gibson Assembly was done using **NEB's NEBuilder HiFi DNA Assembly Master Mix (E2621)**.

1. The following components were added sequentially to separate PCR tubes in the same order as below: (EVC = empty vector control)
 - Tube C1: 1 μ L 2X Master Mix + 0.5 μ L C1 PCR GE + 0.5 μ L cut pETMCN GE
 - Tube C2: 1 μ L 2X Master Mix + 0.5 μ L C2 PCR GE + 0.5 μ L cut pETMCN GE
 - Tube EVC: 1 μ L 2X Master Mix + 0.5 μ L NF H₂O + 0.5 μ L cut pETMCN GE

Total reaction volume in each tube was 2 μ L. After adding all the components, they were mixed by flicking, then spun down.

2. The tubes were incubated at 50°C for 1 hr.

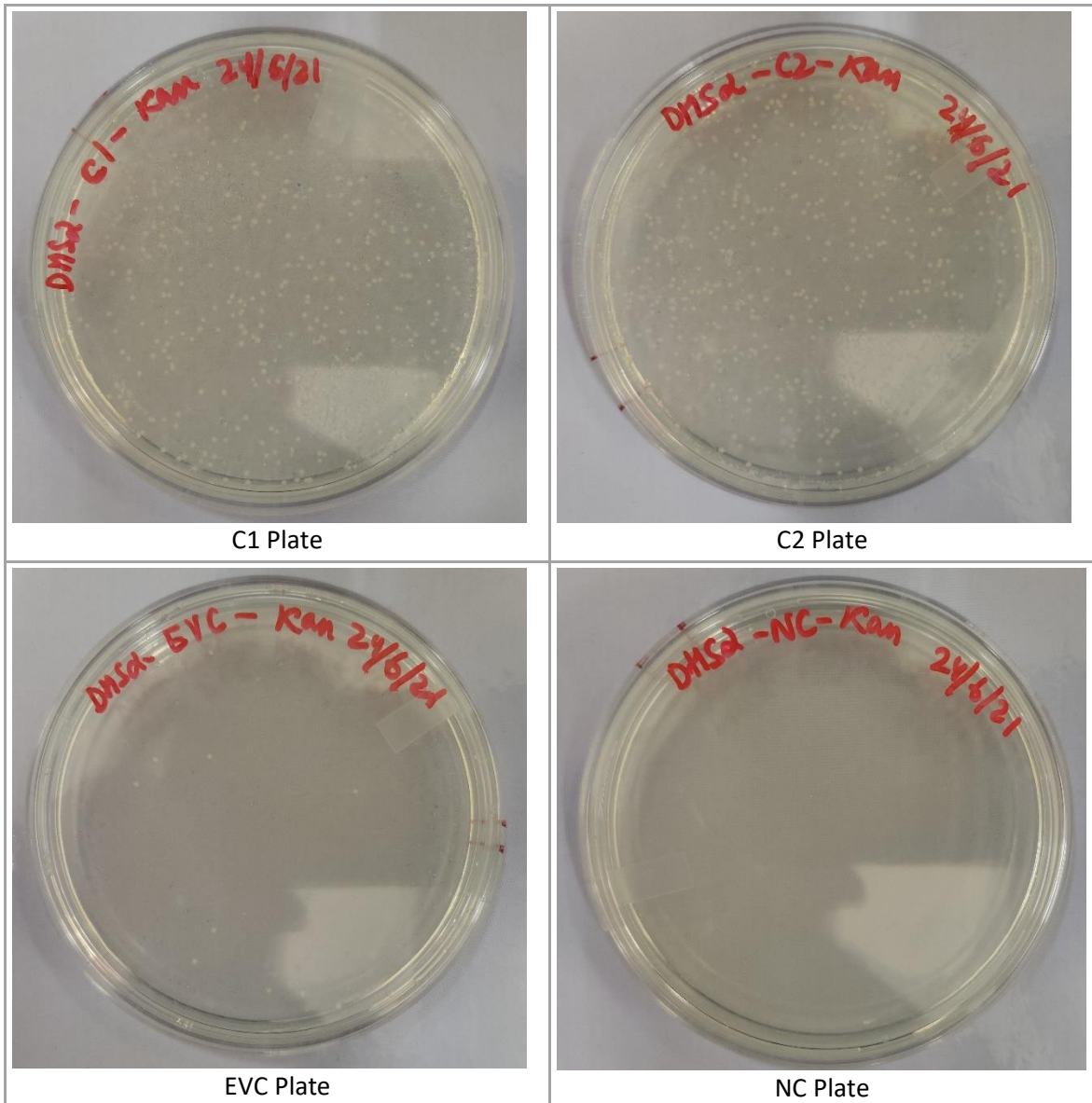
C. Transforming DH5 α with Gibson assembled constructs:

Strain to be transformed: *E. coli* DH5 α , made chemically competent using CaCl₂.

1. 4 tubes were taken, and each was filled with 100 μ L of competent cell suspension. The following components were then added:
 - Tube C1: 2 μ L of C1 Gibson product
 - Tube C2: 2 μ L of C2 Gibson product
 - Tube EVC: 2 μ L of EVC Gibson product
 - Tube NC: 2 μ L NF H₂O
2. The tubes were kept on ice for 30 mins after addition of DNA.
3. Heat shock at 42°C was given for 1.5 mins.
4. The tubes were returned to ice and kept there for 5 mins.
5. The cells were allowed to recover by adding 1 mL LB broth to each tube and incubating the tubes at 37°C, with shaking at 190 rpm for 1 hr.
6. The tubes were taken out and centrifuged at 4000 rpm for 3 mins to pellet the cells.
7. 0.9 mL supernatant media was taken out from each tube. The cell pellet was resuspended in the remaining media.
8. The cells were then plated on LB + Kan agar (spread using 8-10 glass beads per plate), and the plates were incubated at 37°C for 14 hrs.

A. Inoculation of transformed DH5α (continued from 24 June, C):

The plates were taken out from 37°C and transferred to the hood at room temperature for inoculation. Pictures of the four plates are given below:



- B. 8 colonies each from plates C1 and C2 were inoculated into separate test tubes containing 4 mL LB broth + 4 μ L of 50 mg/mL Kan (working concentration 50 μ g/mL). The plates were returned to 4°C.
- C. The tubes were incubated at 37°C with shaking at 180 rpm for 17 hrs.

A. Miniprep of Gibson assembled plasmids (continuing after 25 June, A):

Miniprep was done using **Thermo's GeneJET Plasmid Miniprep Kit (K0503)**.

1. 4 mL culture from each test tube was transferred to 2 Eppendorf tubes each of volume 2 mL.
2. The tubes were centrifuged at 8000 rpm for 2 min to pellet the cells. The supernatant was discarded.
3. To the first tube for each culture, 250 μ L Resuspension Buffer was added, and cells were resuspended by pipetting in and out.
4. The above was transferred to the next tube, and resuspension was carried out again. This was repeated until all of the resuspended cells resided in the last tube for each culture.
5. 250 μ L Lysis Buffer was added to the tubes containing the resuspended cells. The tube contents were mixed by inverting a few times, and the suspension became clearer.
6. 350 μ L Neutralization Buffer was added to the tubes. The tube contents were mixed by inverting a few times, and some white precipitate was observed.
7. The tubes were centrifuged at 14000 rpm for 5 mins. The debris was pelleted, with the plasmid DNA staying in the supernatant.
8. The supernatant from each tube was transferred to separate spin columns.
9. The spin columns were centrifuged at 14000 rpm for 1 min. The flowthrough collected in the collecting tubes was discarded.
10. 500 μ L Wash Buffer was added to the spin columns. Step 9 was repeated.
11. Step 10 was repeated.
12. The spin columns were centrifuged at 14000 rpm for 1 min (empty spin).
13. The collecting tubes were then removed, and the spin columns were placed into Eppendorf tubes.
14. 35 μ L Elution Buffer was added to the spin columns and allowed to stand for 10 mins.
15. The tubes were centrifuged at 14000 rpm for 1 min. The flowthrough was collected inside the Eppendorf tubes (**labelled 1 through 8 for C1 and 1* through 8* for C2**).
16. Concentration of plasmid DNA was measured using a Nanodrop machine (elution buffer was taken as blank). Loading volume was 1 μ L for all samples.
17. The Eppendorf tubes were stored at -20°C.

Nanodrop results:

C1 samples:

Sample	Concentration (ng/μL)	A260/A280	A260/A230	A260
1	58.5	1.940	1.918	1.178
2	64.2	1.922	1.891	1.283
3	10.35	1.865	0.489	0.287
4	55.25	1.939	1.703	1.126
5	63.4	1.921	1.730	1.285
6	57.75	1.909	1.729	1.176
7	51.25	1.912	1.718	1.043
8	65.65	1.951	1.728	1.333

- Sample 3 was probably improperly inoculated.
- Since A260/A280 > 1.8, the miniprep samples can be considered pure.
- A260/A230 < 2, so there was a lot of salt contamination or similar.

C2 samples:

Sample	Concentration (ng/μL)	A260/A280	A260/A230	A260
1*	63.8	1.939	1.708	1.304
2*	65.95	1.931	1.714	1.352
3*	227.3	1.926	2.099	4.589
4*	67.05	1.938	1.827	1.367
5*	261.8	1.924	2.147	5.278
6*	72.2	1.928	1.875	1.470
7*	75.9	1.918	1.721	1.547
8*	67.3	1.926	1.753	1.383

- Samples 3 and 5 are inconsistent.
- Some A260/A280 < 1.8, so some protein contamination may be present.
- In most cases A260/A230 < 2, so there was heavy salt contamination or similar.

B. Agarose Gel Electrophoresis of Gibson assembled plasmids:

Composition - 0.8% agarose gel, 0.56 g agarose dissolved in 70 mL 1X TAE. 2 μ L of 1 mg/mL EtBr was added.

Samples were loaded as follows:

Lane 1 on both top and bottom rows was loaded with 2 μ L of 1 kb ladder.

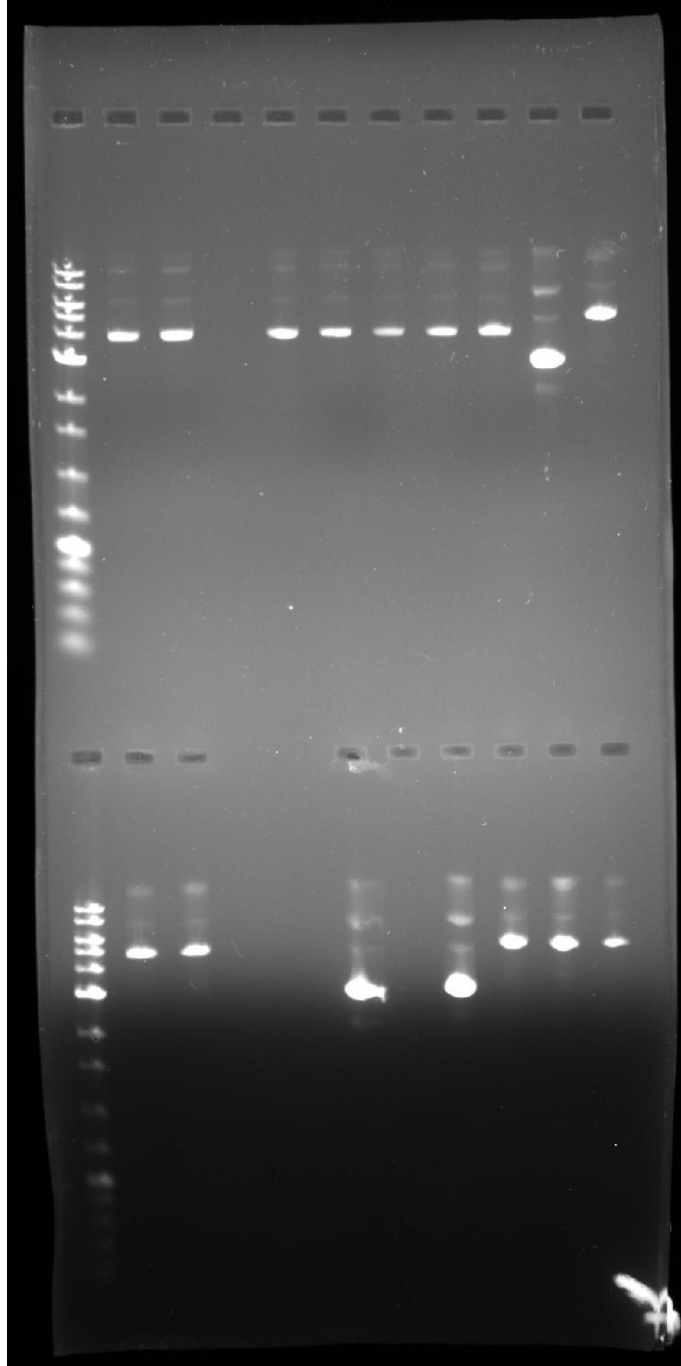
Top row:

- Lanes 2 through 9: C1 tubes 1 through 8 respectively
- Lanes 10 & 11: C2 tubes 3 & 4 respectively

Bottom row:

- Lanes 2 & 3: C2 tubes 1 & 2 respectively
- Lanes 4 & 5: Empty
- Lane 6: Misloaded C2 tube 3
- Lane 7: Empty
- Lanes 8 through 11: C2 tubes 5 through 8 respectively

Gel Results:



No band was observed for C1 tube 3, hence, this was rejected for further experiments. All the other C1 tubes had the same, single prominent band (supercoiled form). Linear and circular forms were also faintly visible.

C2 tubes 3 and 5 showed bands which ran farther than the rest. Other samples were consistent, and slightly larger than those for C1, which is expected.

C. Digestion of Gibson assembled plasmids with XbaI:

Master Mix:

Materials	1 Reaction	Master Mix (for 15 reactions)
10X Fast Digest Green Buffer	1 μ L	15 μ L
XbaI (Fast Digest - Fermentas)	1 μ L	15 μ L
Nuclease-Free H ₂ O	5 μ L	75 μ L

7 μ L Master Mix was added to 15 PCR tubes each (7 samples of C1, 8 samples of C2). 3 μ L of each of the minipreped plasmid samples were added to corresponding tubes. Total reaction volume in each tube was 10 μ L.

The above components were mixed then incubated at 37°C for 1 hr.

D. Agarose Gel Electrophoresis of digested Gibson assembled plasmids:

Composition - 0.8% agarose gel, 0.8 g agarose dissolved in 100 mL 1X TAE. 2 μ L of 1 mg/mL EtBr was added.

Control: cut pETMCN vector, gel extracted (**cut pETMCN GE**).

8 μ L cut pETMCN GE + 4 μ L F.D. Green Buffer + 28 μ L NF H₂O (total volume 40 μ L, for loading 4 wells)

Samples were loaded as follows:

Lanes 1 & 12 on both top and bottom rows were loaded with 2 μ L of 1 kb ladder.

Lanes 2 & 14 on both top and bottom rows were loaded with 10 μ L of cut pETMCN control.

Top row:

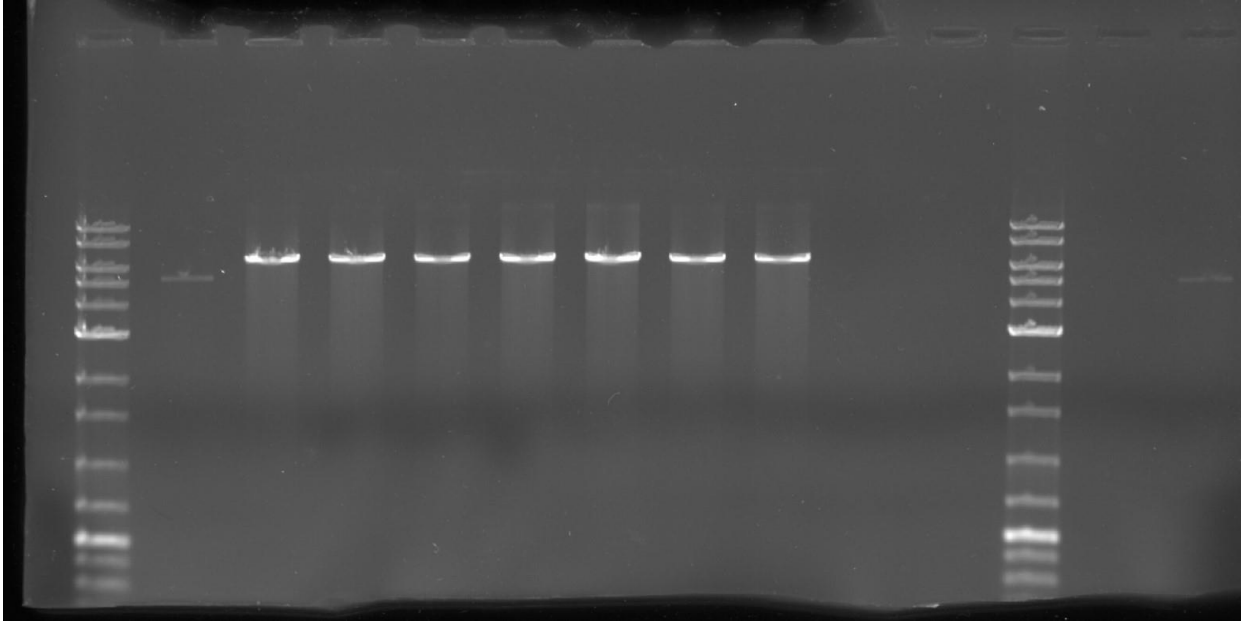
- Lanes 3 through 9: C1 tubes 1 through 8 resp. (except tube 3)

Bottom row:

- Lanes 3 through 10: C2 tubes 1 through 8 resp.

Gel Results:

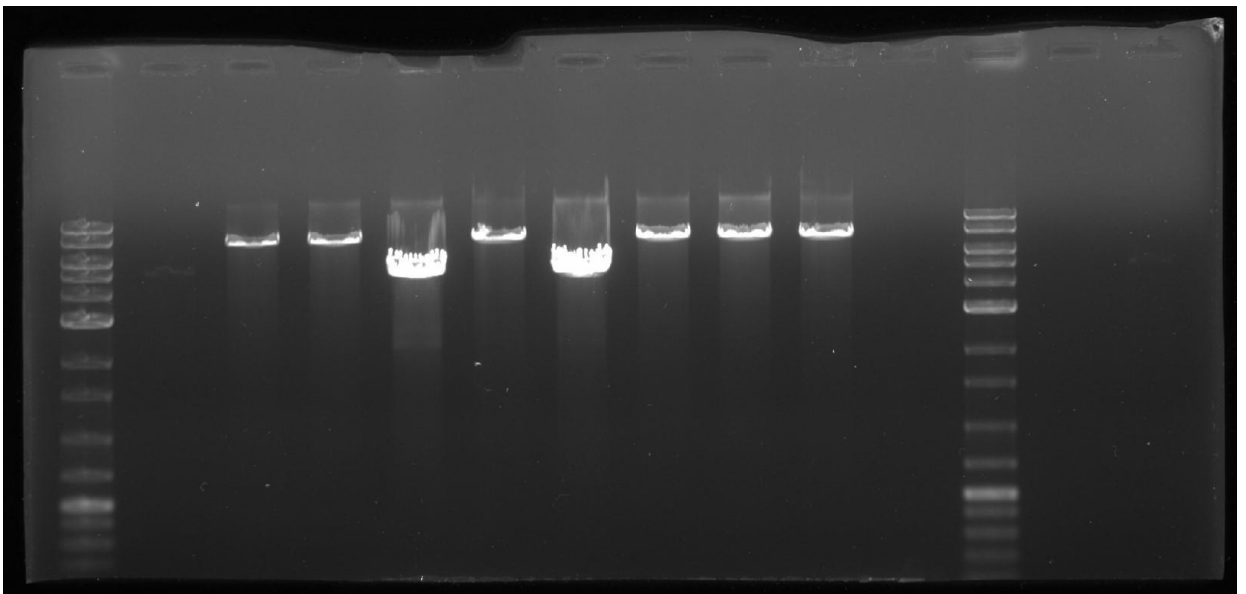
Top gel:



Faint bands were observed for the cut pETMCN vector, which was expected since it was dilute.

All C1 bands are consistently larger than the cut vector band.

Bottom gel:



Faint bands were observed for the cut pETMCN vector, which was expected since it was dilute.

All C2 bands except for tubes 3 & 5 were consistently larger than the cut vector band. For these tubes, the size was approximately the same as the cut vector.

This result seemed to suggest that C1 samples 1, 2, 4, 5, 6, 7 and 8, and C2 samples 1, 2, 4, 6, 7 and 8 were positive clones, while the rest were not.

A. Double Digestion of Gibson assembled plasmids with BglII & NheI:

Master Mix:

Materials	1 Reaction	Master Mix (for 16 reactions)
10X CutSmart Buffer	1 μ L	16 μ L
BglII (NEB)	0.25 μ L	4 μ L
NheI-HF (NEB)	0.25 μ L	4 μ L
Nuclease-Free H ₂ O	5.625 μ L	90 μ L

7 μ L Master Mix was added to 15 PCR tubes each (7 samples of C1, 8 samples of C2). 2 μ L of each of the miniprep plasmid samples were added to corresponding tubes. Total reaction volume in each tube was 9 μ L.

The above components were mixed then incubated at 37°C for 3 hrs.

B. Agarose Gel Electrophoresis of double digested Gibson assembled plasmids:

Composition - 1% agarose gel, 1.5 g agarose dissolved in 150 mL 1X TAE. 2 μ L of 1 mg/mL EtBr was added.

1 μ L 6X loading dye was added to each 9 μ L reaction mixture before loading.

Control: cut pETMCN vector, gel extracted (**cut pETMCN GE**).

4 μ L cut pETMCN GE + 2 μ L 6X loading dye + 6 μ L NF H₂O (total volume 12 μ L, for loading 2 wells)

Samples were loaded as follows:

Lanes 1 & 14 on both top and bottom rows were loaded with 1.5 μ L of 1 kb ladder.

Lane 12 on both top and bottom rows was loaded with 6 μ L of cut pETMCN control.

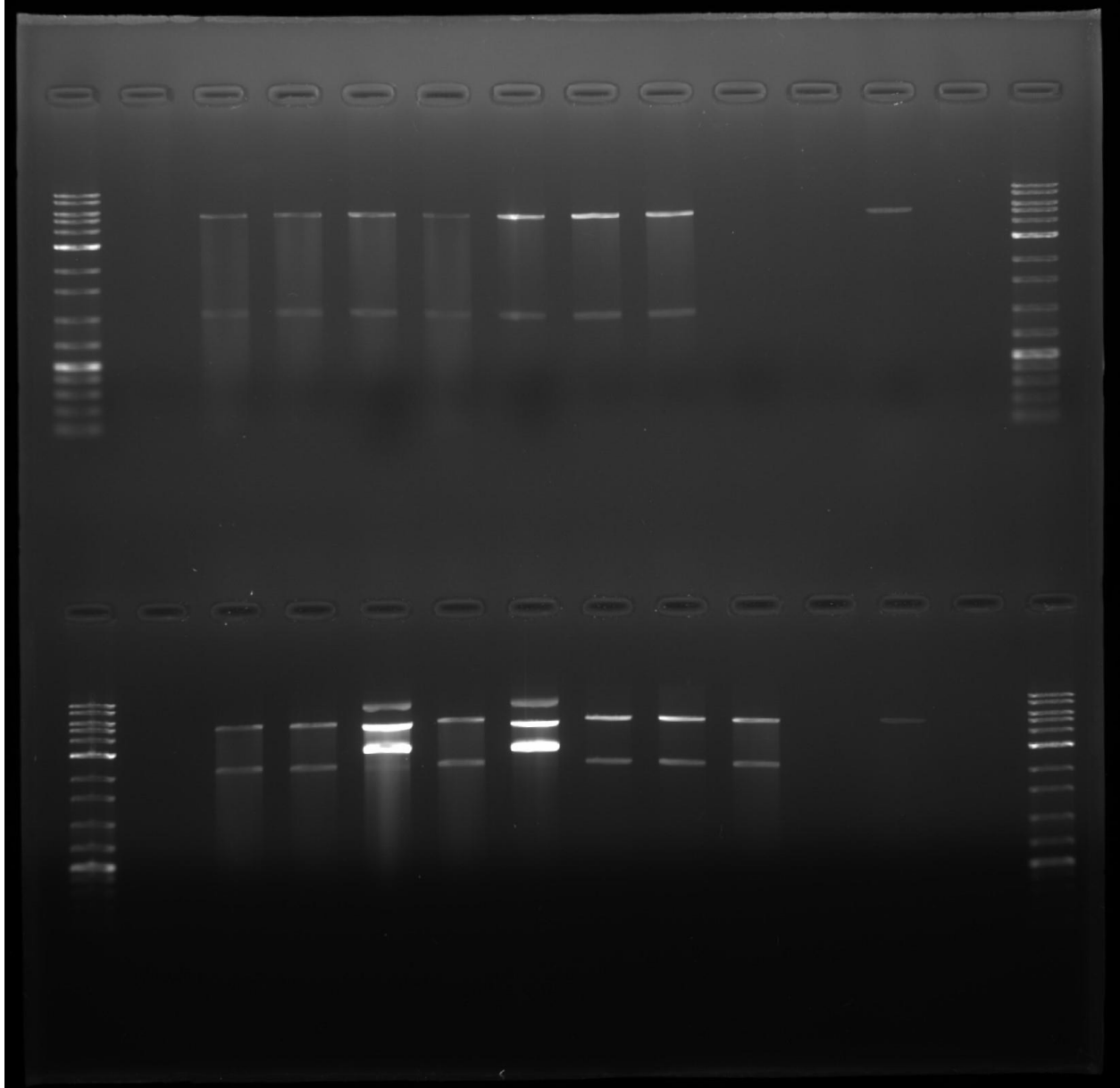
Top row:

- Lanes 3 through 9: C1 tubes 1 through 8 resp. (except tube 3)

Bottom row:

- Lanes 3 through 10: C2 tubes 1 through 8 resp.

Gel Results:



Faint bands were observed for the cut pETMCN vector, which was expected since it was dilute.

All C1 bands showed consistent bands (2 each, corresponding to the vector backbone and the insert). Tubes 7 & 8 gave the brightest bands.

C2 tubes 3 & 5 showed 3 bands each. The top bands were larger than the cut vector, thus, some of the plasmids had probably not been doubly digested. The lowest bands were also larger than the proper insert. Except for tubes 3 & 5, all C2 tubes showed consistent bands (2 each, corresponding to the vector backbone and the insert). Tubes 6 & 7 gave the brightest bands.

A slight smear could also be observed in many lanes, which seemed to suggest some contamination.

This result again seemed to suggest that C1 samples 1, 2, 4, 5, 6, 7 and 8, and C2 samples 1, 2, 4, 6, 7 and 8 were positive clones, while the rest were not.

Two clones each for C1 and C2 were chosen for further experiments:

C1: Tubes 7 & 8

C2: Tubes 6 & 7

A. Double Digestion of selected positive Gibson clones with BglII & NheI:

Master Mix:

Materials	1 Reaction	Master Mix (for 5 reactions)
10X CutSmart Buffer	1 μ L	5 μ L
BglII (NEB)	0.25 μ L	1.25 μ L
NheI-HF (NEB)	0.25 μ L	1.25 μ L
Nuclease-Free H ₂ O	5.5 μ L	27.5 μ L

7 μ L Master Mix was added to 4 PCR tubes each (2 samples of C1, 2 samples of C2). 2 μ L of each of the miniprep plasmid samples (C1 tubes 7 & 8, C2 tubes 6 & 7) were added to corresponding tubes. Total reaction volume in each tube was 9 μ L.

The above components were mixed then incubated at 37°C for 3 hr.

B. Agarose Gel Electrophoresis of double digested positive clones:

Composition - 1% agarose gel, 0.7 g agarose dissolved in 70 mL 1X TAE. 2 μ L of 1 mg/mL EtBr was added.

2 μ L 6X loading dye was added to each 9 μ L reaction mixture before loading.

Controls:

cut pETMCN vector, gel extracted (**cut pETMCN GE**).

4 μ L cut pETMCN GE + 2 μ L 6X loading dye + 6 μ L NF H₂O (total volume 12 μ L, for loading 2 wells)

C1 PCR product, gel extracted (**C1 PCR GE**).

2 μ L C1 PCR GE + 1 μ L 6X loading dye + 3 μ L NF H₂O

C2 PCR product, gel extracted (**C2 PCR GE**).

2 μ L C2 PCR GE + 1 μ L 6X loading dye + 3 μ L NF H₂O

Samples were loaded as follows:

Lane 1 on both top and bottom rows were loaded with 1.5 μ L of 1 kb ladder.

Lane 2 on both top and bottom rows was loaded with 6 μ L of cut pETMCN control.

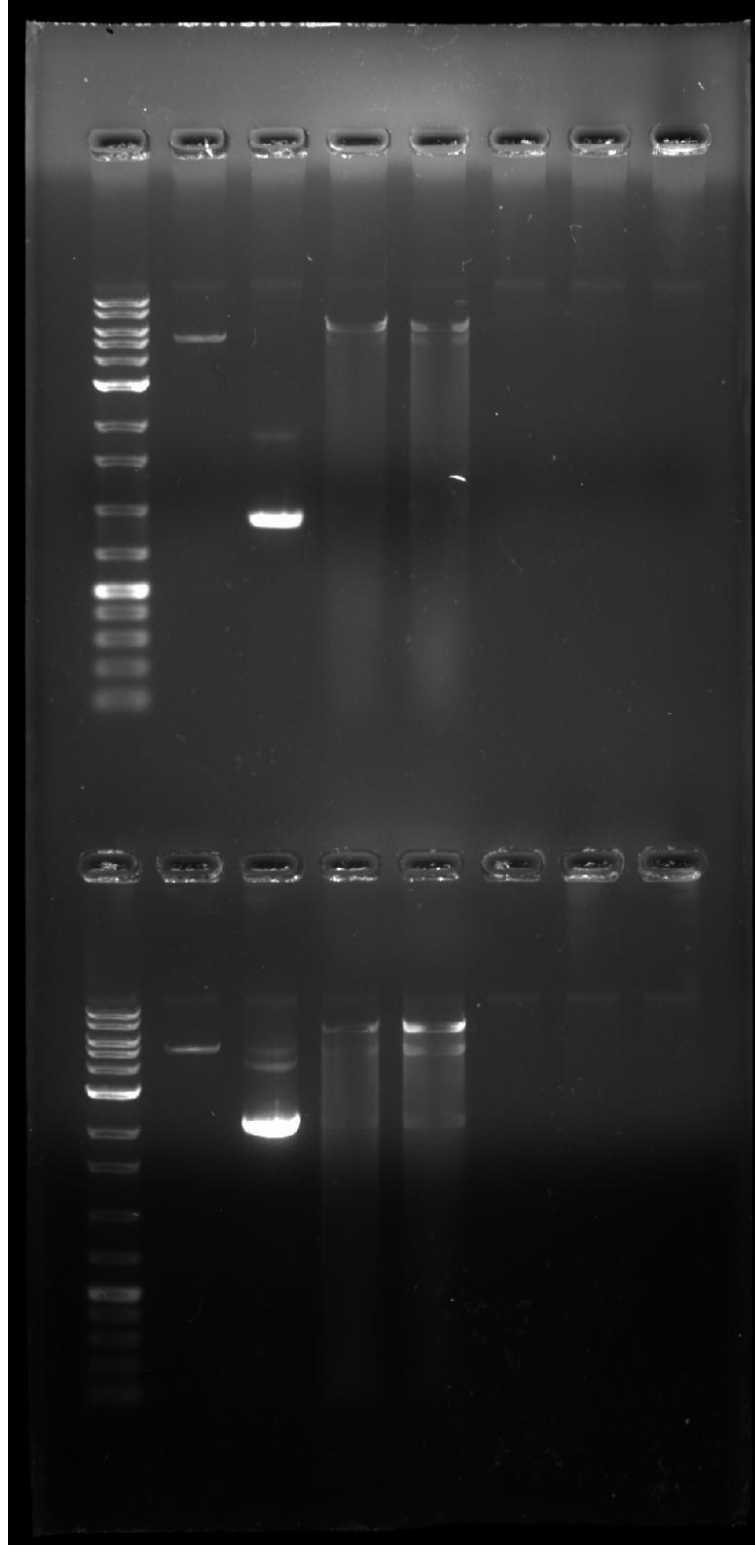
Top row:

- Lane 3: C1 PCR GE
- Lane 4: C1 tube 7
- Lane 5: C1 tube 8

Bottom row:

- Lane 3: C2 PCR GE
- Lane 4: C1 tube 6
- Lane 5: C1 tube 7

Gel Results:



For C1, a slight contaminating band could be observed in lane 3. In lanes 4 & 5, no vector backbone and insert bands were apparent. Double digestion was probably unsuccessful, as the prominent bands were slightly larger than the cut vector backbone.

For C2 again, slight contaminating bands could be observed in lane 3. In lanes 4 & 5, the vector backbone and insert bands were could be observed but were quite faint. The larger bands on top seemed to suggest that most of the plasmids were not doubly digested, since they are larger than the cut vector backbone.

A considerable smear could also be observed in many lanes, which seemed to suggest some contamination.

This gel has to be run again.

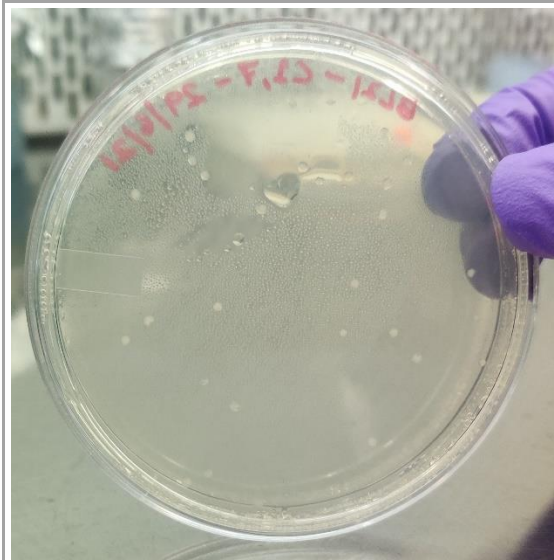
C. Transforming BL21(DE3) with positive Gibson clones:

Strain to be transformed: *E. coli* BL21(DE3), made chemically competent using CaCl₂.

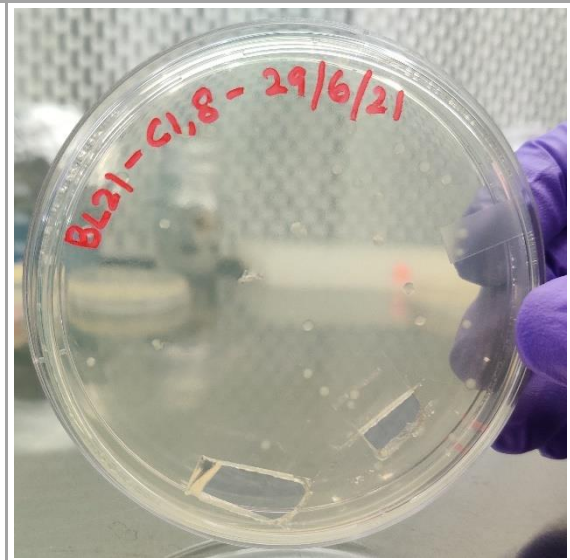
1. 5 tubes were taken, and each was filled with 60 µL of competent cell suspension. The following components were then added:
 - Tube C1,7: 1 µL of C1 clone, tube 7
 - Tube C1,8: 1 µL of C1 clone, tube 8
 - Tube C2,6: 1 µL of C2 clone, tube 6
 - Tube C2,7: 1 µL of C2 clone, tube 7
 - Tube NC: 1 µL sterile water
2. The tubes were kept on ice for 30 mins after addition of DNA.
3. Heat shock at 42°C was given for 1.5 mins.
4. The tubes were returned to ice and kept there for 5 mins.
5. The cells were allowed to recover by adding 1 mL LB broth to each tube and incubating the tubes at 37°C, with shaking at 190 rpm for 1 hr.
6. The tubes were taken out and centrifuged at 4000 rpm for 3 mins to pellet the cells.
7. 0.9 mL supernatant media was taken out from each tube. The cell pellet was resuspended in the remaining media.
8. The cells were then plated on LB + Kan agar (spread using 8-10 glass beads per plate), and the plates were incubated at 37°C for 15 hrs.

A. Inoculation of transformed BL21(DE3) (continued from 29 June, C):

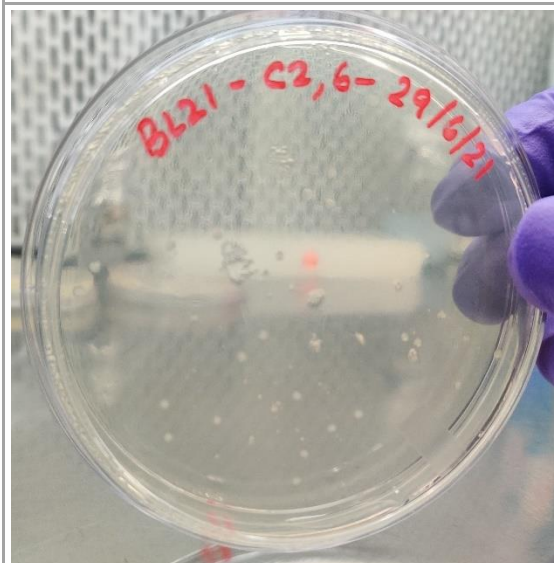
- The plates were taken out from 37°C and transferred to the hood at room temperature for inoculation. Pictures of the four plates are given below:



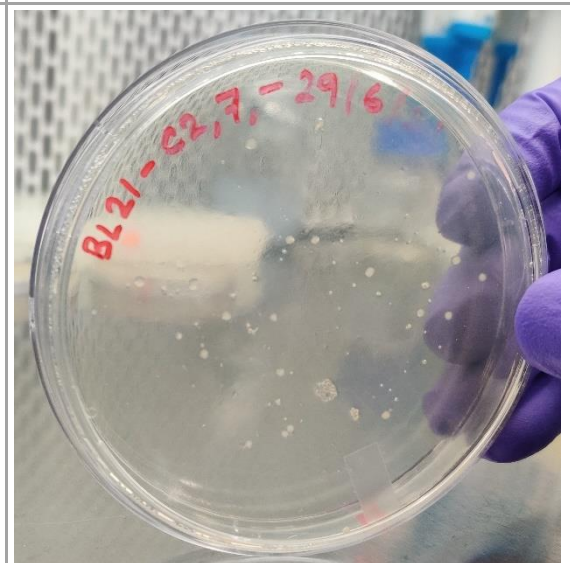
C1,7 Plate



C1,8 Plate



C2,6 Plate



C2,7 Plate

- 2 colonies from each plate were inoculated into separate test tubes containing 3 mL LB broth + 3 μ L of 50 mg/mL Kan (working concentration 50 μ g/mL). The plates were returned to 4°C.
- The tubes were incubated at 37°C with shaking at 180 rpm for 15 hrs.

B. Transforming DH5α with positive Gibson clones:

Strain to be transformed: *E. coli* DH5α, made chemically competent using CaCl₂.

1. 5 tubes were taken, and each was filled with 60 µL of competent cell suspension. The following components were then added:
 - Tube C1,7: 1 µL of C1 clone, tube 7
 - Tube C1,8: 1 µL of C1 clone, tube 8
 - Tube C2,6: 1 µL of C2 clone, tube 6
 - Tube C2,7: 1 µL of C2 clone, tube 7
 - Tube NC: 1 µL sterile water
2. The tubes were kept on ice for 30 mins after addition of DNA.
3. Heat shock at 42°C was given for 1.5 mins.
4. The tubes were returned to ice and kept there for 5 mins.
5. The cells were allowed to recover by adding 1 mL LB broth to each tube and incubating the tubes at 37°C, with shaking at 190 rpm for 1 hr.
6. The tubes were taken out and centrifuged at 4000 rpm for 3 mins to pellet the cells.
7. 0.9 mL supernatant media was taken out from each tube. The cell pellet was resuspended in the remaining media.
8. The cell suspension was then added to a corresponding test tube containing 4 mL LB broth + 4 µL of 50 mg/mL Kan (working concentration 50 µg/mL).
9. The tubes were incubated at 37°C with shaking at 180 rpm for 15 hrs.

A. Expression tests with transformed BL21(DE3):

Subculturing: From the 4 tubes of liquid culture, separate secondary cultures (1:100) were started, according to the following scheme:

60 μ L of each of the primary cultures was added to similarly labelled tubes, each containing 6 mL LB broth + 6 μ L of 50 mg/mL Kan (working concentration 50 μ g/mL).

- Tube C1,7: For 0.25 mM IPTG induction
 - Tube C1,8: For 0.5 mM IPTG induction
 - Tube C2,6: For 0.25 mM IPTG induction
 - Tube C2,7: For 0.5 mM IPTG induction
1. The above tubes were incubated at 37°C, with shaking at 180 rpm for around 1.5 hrs, until OD₆₀₀ reached about 0.5.
 2. From each of the 4 above tubes, the following 4 setups were made: (16 tubes in total)
 - Uninduced (23°C): 1 mL
 - Uninduced (30°C): 1 mL
 - Induced (23°C): 1 mL
 - Induced (30°C): 1 mL
 3. For 0.25 mM IPTG induction, 0.5 μ L of 0.5 M IPTG was added. For 0.5 mM IPTG induction, 1 μ L of 0.5 M IPTG was added.
 4. The 23°C tubes were incubated for 6 hrs with shaking at 180 rpm. The 30°C tubes were incubated for 4 hrs with shaking at 180 rpm.
 5. The tubes were centrifuged at 5000 rpm for 15 mins at 4°C to pellet the cells. The pellets and supernatants were stored separately at -20°C.

B. Miniprep of positive Gibson assembled clones (continuing after 30 June, B):

Miniprep was done using **Thermo's GeneJET Plasmid Miniprep Kit (K0503)**.

1. 4 mL culture from each test tube was transferred to 2 Eppendorf tubes each of volume 2 mL.
2. The tubes were centrifuged at 8000 rpm for 2 min to pellet the cells. The supernatant was discarded.
3. To the first tube for each culture, 250 μ L Resuspension Buffer was added, and cells were resuspended by pipetting in and out.
4. The above was transferred to the next tube, and resuspension was carried out again. This was repeated until all of the resuspended cells resided in the last tube for each culture.
5. 250 μ L Lysis Buffer was added to the tubes containing the resuspended cells. The tube contents were mixed by inverting a few times, and the suspension became clearer.

6. 350 μ L Neutralization Buffer was added to the tubes. The tube contents were mixed by inverting a few times, and some white precipitate was observed.
7. The tubes were centrifuged at 14000 rpm for 5 mins. The debris was pelleted, with the plasmid DNA staying in the supernatant.
8. The supernatant from each tube was transferred to separate spin columns.
9. The spin columns were centrifuged at 14000 rpm for 1 min. The flowthrough collected in the collecting tubes was discarded.
10. 500 μ L Wash Buffer was added to the spin columns. Step 9 was repeated.
11. Step 10 was repeated.
12. The spin columns were centrifuged at 14000 rpm for 1 min (empty spin).
13. The collecting tubes were then removed, and the spin columns were placed into Eppendorf tubes.
14. 40 μ L Elution Buffer was added to the spin columns, and allowed to stand for 10 mins.
15. The tubes were centrifuged at 14000 rpm for 1 min. The flowthrough was collected inside the Eppendorf tubes (**labelled C1,7 MP and C1,8 MP for C1, and C2,6 MP and C2,7 MP for C2**).
16. Concentration of plasmid DNA was measured using a Nanodrop machine (elution buffer was taken as blank). Loading volume was 1 μ L for all samples.
17. The Eppendorf tubes were stored at -20°C.

Nanodrop results:

C1 samples:

Sample	Concentration (ng/ μ L)	A260/A280	A260/A230	A260
7	5.35	1.877	0.374	0.113
8	5.95	1.676	0.388	0.128

The plasmid concentration was very low, probably due to an unsuccessful transformation, or directly inoculating into liquid culture instead of plating first.

C2 samples:

Sample	Concentration (ng/ μ L)	A260/A280	A260/A230	A260
6	3.5	1.892	0.361	0.076
7	4.95	1.707	0.452	0.108

Again, the plasmid concentration was very low, probably due to an unsuccessful transformation, or directly inoculating into liquid culture instead of plating first.

A. SDS PAGE of cell pellets (continuing after 1 July, A):

Mix for 4 gels:

Materials	Resolving gel (15%)	Stacking gel (4%)
Acrylamide-bisacrylamide (30:1)	10 mL	1.3 mL
Distilled H ₂ O	4.7 mL	6.2 mL
1.5 M Tris (pH = 8.8)	5 mL	-
0.5 M Tris (pH = 6.8)	-	2.5 mL
10% SDS	200 µL	100 µL
10% APS*	100 µL	50 µL
TEMED*	10 µL	10 µL

*added at last, immediately before adding to cast

Total volume is 20.01 mL for resolving gel, 10.16 mL for stacking gel.

Gels were 1 mm in thickness (1 mm Bio-Rad glass plates). After adding the resolving gel mix to the cast, a 2-3 mm thick layer of isopropanol was added on top to flatten the gel as well as for removing air bubbles. After the resolving gel had solidified, the stacking gel mix was added. The width of the stacking gel from the bottom of the wells to the top of the resolving gel layer was around 1 cm.

Sample preparation:

1. The cell pellets were resuspended in 100 µL NF H₂O in each tube.
2. 30 µL of 5X Laemmli buffer was added to each cell suspension.
3. The above mixtures were boiled at 95°C for 10 mins.
4. The tube contents were centrifuged at 14000 rpm for 7 mins.
5. 15 µL of each of the supernatants was loaded according to the following scheme (8 µL of marker was loaded):

C1 gel:

Sample	-	Stained marker	Uninduced	Induced	Uninduced	Induced	Uninduced	Induced	Uninduced	Induced
IPTG conc. used (in mM)	-	-	-	0.25	-	0.5	-	0.25	-	0.5
Temp. of culture (in °C)	-	-	23	23	23	23	30	30	30	30
Lane no.	1	2	3	4	5	6	7	8	9	10

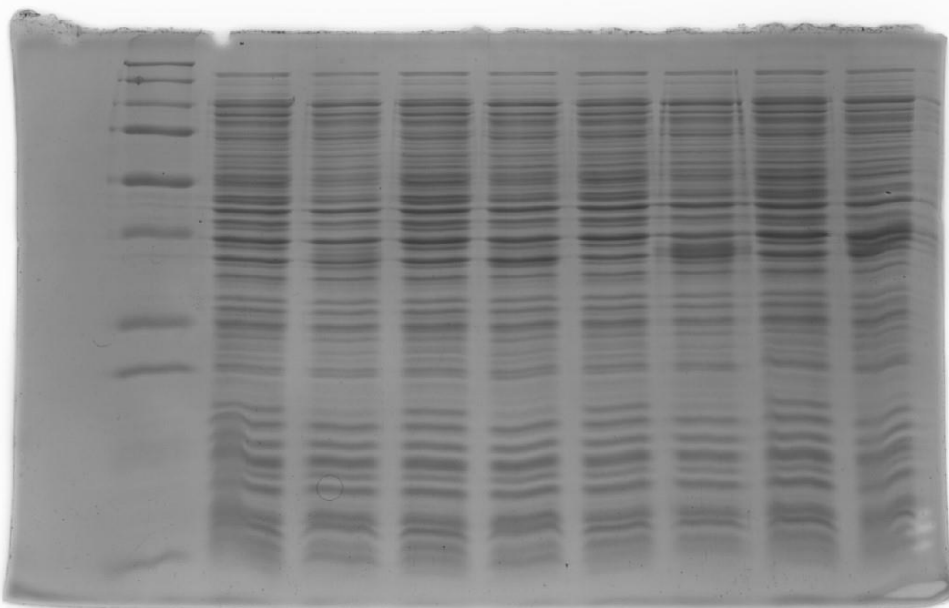
C2 gel:

Sample	Unstained marker	Uninduced	Induced	Uninduced	Induced	Uninduced	Induced	Uninduced	Induced	Induced
IPTG conc. used (in mM)	-	-	0.25	-	0.5	-	0.25	-	0.5	0.25
Temp. of culture (in °C)	-	23	23	23	23	30	30	30	30	30
Lane no.	1	2	3	4	5	6	7	8	9	10

- The gels were run at 100 V while the dye front was in the stacking gel, and at 150 V while it was in the resolving gel.
- After the gels had run (dye front reaching about 0.5-1 cm from bottom), they were taken out from the apparatus and the stacking gel was cut off. The gels were transferred to boxes containing MilliQ water.
- The water was then drained off, and Coomassie Blue staining solution was added to cover the gels. Staining was done for 10 mins on a shaker.
- The Coomassie Blue stain was drained off, and destaining was done for around 30 mins. The gels were then imaged.

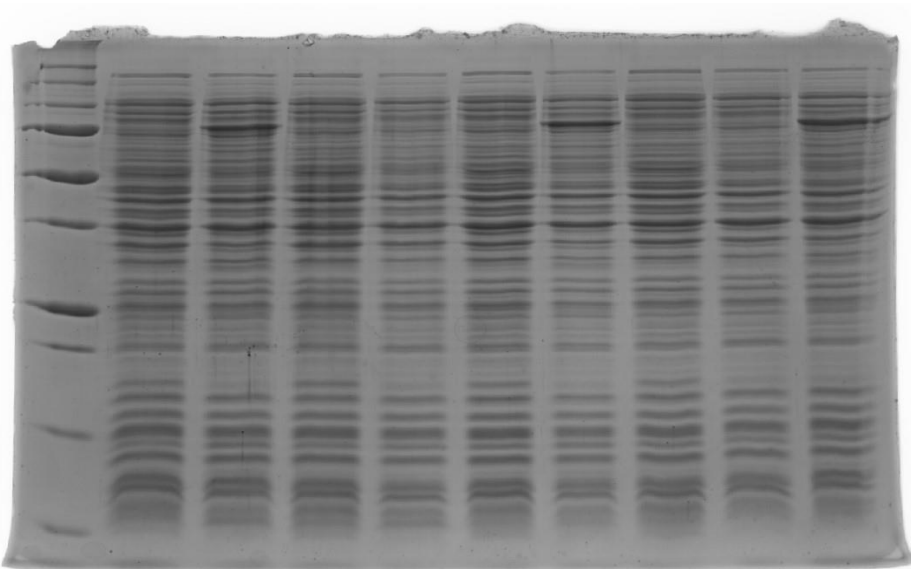
Gel results:

C1 gel:



There was no brighter band in the induced sample lanes compared to the uninduced sample lanes around the 25 kDa band (C1 mol wt. Around 29 kDa). Expression could not be verified.

C2 gel:



Brighter bands could be observed around the 75 kDa band, in lane 3 (compared to 2), lane 7 (compared to 6) and also lane 10 which has the same sample as lane 7. Thus, expression was verified at 0.25 mM IPTG induction for both temperatures 23°C and 30°C.

A. SDS PAGE of supernatants (continuing after 1 July, A):

Sample preparation:

Concentrating proteins in supernatant (extracellular proteins):

Merck's Amicon Ultra Centrifugal Filters (10,000 NMWL) and Sartorius' VIVASPIN 20 (10,000 NMWL) kit were used for concentrating proteins in the supernatant. The following samples (1 mL each) were taken forward for SDS PAGE:

Tube 11: C1, induced at 0.25 mM IPTG at 23°C
Tube 12: C1, induced at 0.5 mM IPTG at 23°C
Tube 13: C1, induced at 0.25 mM IPTG at 30°C
Tube 14: C1, induced at 0.5 mM IPTG at 30°C
Tube 15: C1, uninduced at 30°C
Tube 16: C1, uninduced at 23°C

Tube 21: C2, induced at 0.25 mM IPTG at 23°C
Tube 22: C2, induced at 0.5 mM IPTG at 23°C
Tube 23: C2, induced at 0.25 mM IPTG at 30°C
Tube 24: C2, induced at 0.5 mM IPTG at 30°C
Tube 25: C2, uninduced at 30°C
Tube 26: C2, uninduced at 23°C

1. The above tube contents were divided into two and added to corresponding spin columns (500 µL capacity).
2. The spin columns were centrifuged at 14000 rpm for around 8.5 mins (C1 tubes) or 6 mins (C2 tubes) at 4°C, until about 40 µL of supernatant was left in the column.
3. The flowthrough collected in the collecting tubes was discarded. The empty collecting tubes were again spun down and the remaining liquid was also discarded.
4. The spin columns were inverted and placed into the collecting tubes, and were centrifuged at 5000 rpm for 1.5 mins at 4°C.
5. The corresponding concentrated supernatants were pooled together, such that each 1 mL sample was concentrated to about 80 µL.
6. 22 µL of 5X Laemmli buffer was added to each of the tubes. The mixtures were boiled at 95°C for 5 mins.
7. 20 µL of each of the samples was loaded according to the following scheme (8 µL of marker was loaded; gel: 4% stacking, 15% resolving, 1 mm gel):

C1 gel:

Sample	Unstained marker	Uninduced	Induced	Induced	Induced	Uninduced	Induced	Uninduced	Unstained marker
IPTG conc. used (in mM)	-	-	0.25	0.25	0.5	-	0.25	0.5	-
Temp. of culture (in °C)	-	23	23	23	23	30	30	30	-
Lane no.	1	2	3	4	5	6	7	8	9

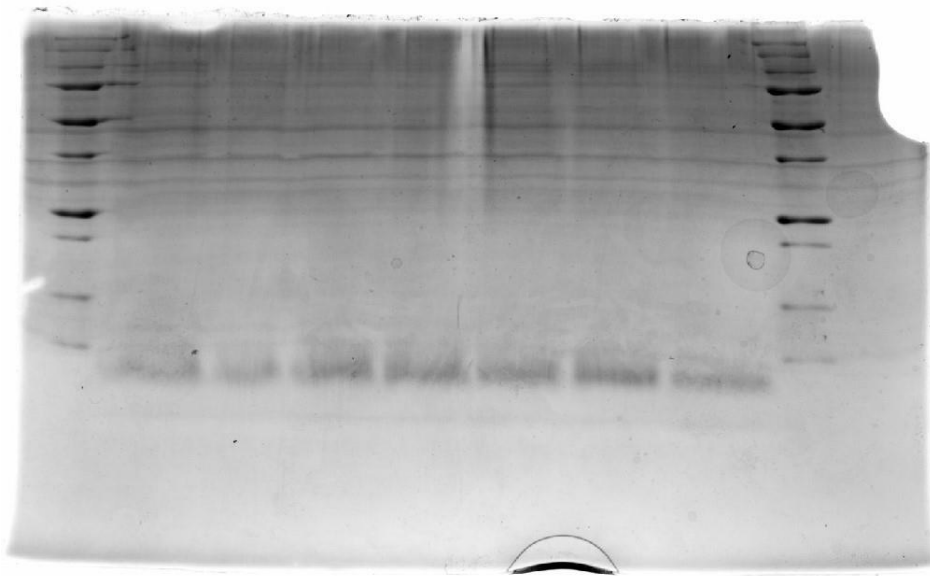
C2 gel:

Sample	Unstained marker	Uninduced	Induced	Induced	Uninduced	Induced	Uninduced	Unstained marker
IPTG conc. used (in mM)	-	-	0.25	0.5	-	0.25	0.5	-
Temp. of culture (in °C)	-	23	23	23	30	30	30	-
Lane no.	1	2	3	4	5	6	7	8

8. The gels were run at 100 V while the dye front was in the stacking gel, and at 150 V while it was in the resolving gel.
9. After the gels had run (dye front reaching about 0.5-1 cm from bottom), they were taken out from the apparatus and the stacking gel was cut off. The gels were transferred to boxes containing MilliQ water.
10. The water was then drained off, and Coomassie Blue staining solution was added to cover the gels. Staining was done for 10 mins on a shaker.
11. The Coomassie Blue stain was drained off, and destaining was done for around 30 mins. The gels were then imaged.

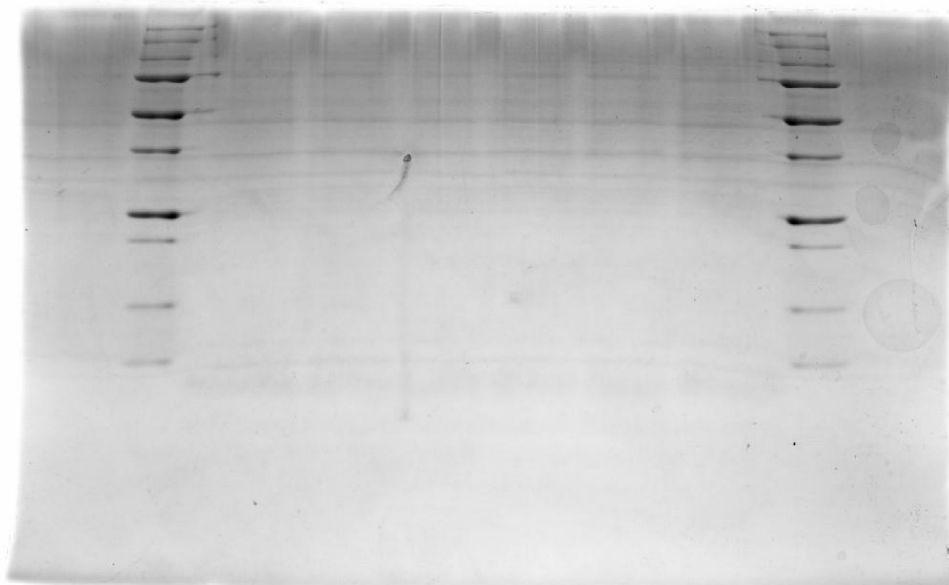
Gel results:

C1 gel:



There was no brighter band in the induced sample lanes compared to the uninduced sample lanes around the 25 kDa band (C1 mol wt. Around 29 kDa). Expression could not be verified.

C2 gel:



There was no brighter band in the induced sample lanes compared to the uninduced sample lanes around the 75 kDa band (C1 mol wt. around 74 kDa). Expression could not be verified. Neither C1 nor C2 seemed to be exported out of the cells.

Date: 05.07.2021

Dr. Palani's Lab

A. Western Blot of cell pellets (continuing after 1 July, A):

Sample preparation:

1. The same samples from 2 July, A, were used for loading. 20 μ L of each of the supernatants was loaded according to the following scheme (10 μ L of marker was loaded; gel: 4% stacking, 15% resolving, 1 mm gel):

Gel setup:

Sample	C1, Uninduced	C1, Induced	C1, Induced	C1, Induced	C1, Induced	C1, Induced	Stained marker	C2, Uninduced	C2, Induced	
IPTG conc. used (in mM)	-	0.25	0.5	-	0.25	0.5	-	-	0.25	-
Temp. of culture (in °C)	23	23	23	30	30	30	-	30	30	-
Lane no.	1	2	3	4	5	6	7	8	9	10

2. The gel was run at 100 V while the dye front was in the stacking gel, and at 150 V while it was in the resolving gel.
3. After the gel had run (dye front reaching the bottom), it was taken out from the apparatus and the stacking gel was cut off. The gel was transferred to a box containing 1X transfer buffer.
4. The following layered setup was assembled in a Bio-Rad Trans Blot machine (from bottom to top): 3 Whatman papers (wetted in 1X transfer buffer) -> nitrocellulose membrane -> SDS PAGE gel -> 3 Whatman papers (wetted in 1X transfer buffer).
5. The transfer was done at 0.11 A limited to 13 V, for 1 hr 15 mins.
6. The nitrocellulose membrane was taken out and kept in distilled H₂O.
7. The membrane was then stained in Ponceau S stain for a few seconds.
8. Destaining was done by placing the membrane in distilled H₂O, followed by 1X PBST buffer.
9. The membrane was then transferred to blocking buffer (3% w/v skim milk solution in 1X PBST), and kept on a rocker for 1 hr at 37°C.
10. The blocking buffer was then drained. Primary antibody solution of 1 μ L mouse anti-His antibody (sc-8036, 200 μ g/mL stock) in 4 mL blocking buffer (1:4000 antibody dilution, working concentration 50 ng/mL) was then added to the membrane. This was kept on a rocker overnight at 4°C.

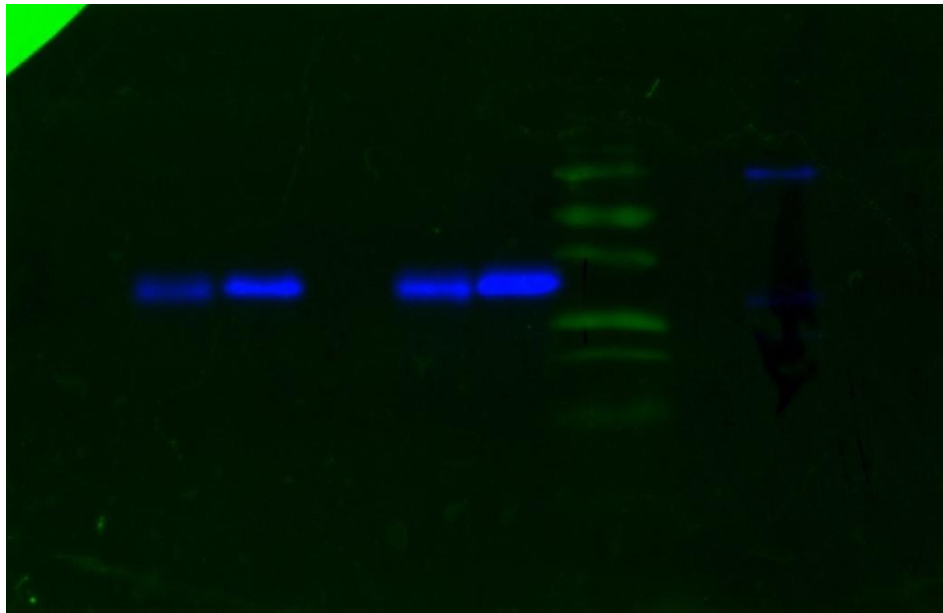
Date: 06.07.2021

Dr. Palani's Lab

A. Western Blot of cell pellets (continued from 5 July, A):

1. The primary antibody solution was drained. The membrane was covered with 1X PBST and kept on a rocker for 7 mins at 37°C, after which the PBST was drained.
2. The above wash step was repeated two more times.
3. Secondary antibody solution of 1.5 μ L goat anti-mouse antibody conjugated to HRP (Genei #HPO6) in 5 mL blocking buffer (1:3333 antibody dilution) was then added to the membrane. This was kept on a rocker for 1 hr at 37°C.
4. The above wash step was performed three times.
5. The membrane was next covered in 500 μ L peroxide + 500 μ L ECL substrate solution, and imaging was done immediately.

Western blot results:



Bands just above 25 kDa were observed in lanes 2, 3, 5 and 6, which showed successful induction of C1 at these conditions. Induction was strongest at 30°C for 0.5 mM IPTG induction.

A faint band was also visible around 75 kDa in lane 9 which showed induction of C2.

A. Inoculation of BL21(DE3):

1. The BL21(DE3) plates (C1,7 and C1,8; C2,6 and C2,7) were taken out from 4°C and transferred to the hood at room temperature for inoculation.
2. 2 colonies from each plate were inoculated into separate test tubes containing 10 mL LB broth + 10 µL of 50 mg/mL Kan (working concentration 50 µg/mL). The plates were returned to 4°C.
3. The tubes were incubated overnight at 37°C with shaking at 180 rpm.

A. Expression tests with BL21(DE3):

Subculturing: From the 4 tubes of liquid culture, separate secondary cultures (1:50) were started, according to the following scheme:

- 120 μL of each of the primary cultures was added to similarly labelled tubes, each containing 6 mL LB broth + 6 μL of 50 mg/mL Kan (working concentration 50 $\mu\text{g}/\text{mL}$).
 - Tube C1,7
 - Tube C1,8
 - Tube C2,6
 - Tube C2,7
- The above tubes were incubated at 37°C, with shaking at 180 rpm for around 1.5 hrs, until OD_{600} reached about 0.5.
- From each of the 4 above tubes, the following 4 setups were made: (16 tubes in total)
 - Uninduced: 1 mL
 - Induced with 0.25 mM IPTG: 1 mL
 - Induced with 0.5 mM IPTG: 1 mL
 - Induced with 1 mM IPTG: 1 mL
- For 0.25 mM IPTG induction, 0.5 μL of 0.5 M IPTG was added. For 0.5 mM IPTG induction, 1 μL of 0.5 M IPTG was added. For 1 mM IPTG induction, 2 μL of 0.5 M IPTG was added.
- The tubes were then incubated at 30°C for 4 hrs with shaking at 180 rpm.
- The tubes were centrifuged at 5000 rpm for 15 mins at 4°C to pellet the cells. The supernatants were discarded, and the pellets were stored for SDS PAGE.

A. Solubility tests with BL21(DE3):

Subculturing: From the primary cultures C1,7 and C2,6, separate secondary cultures (1:50) were started, according to the following scheme:

- 1 mL of each of the primary cultures was added to similarly labelled flasks, each containing 50 mL LB broth + 50 μL of 50 mg/mL Kan (working concentration 50 $\mu\text{g}/\text{mL}$).
 - Tube C1,7
 - Tube C2,6
- The above flasks were incubated at 37°C, with shaking at 180 rpm for around 1.5 hrs, until OD_{600} reached about 0.5.
- 50 μL of 0.5 M IPTG was added to each of the flasks, for induction at 0.5 mM IPTG.
- The flasks were then incubated at 30°C for 4 hrs with shaking at 180 rpm.
- The cultures were collected in Falcon tubes, and were centrifuged at 5000 rpm for 15 mins at 4°C to pellet the cells. The supernatants were discarded, and the pellets were stored at -20°C.

A. SDS PAGE of cell pellets (continuing after 7 July, A):

Sample preparation:

1. The cell pellets were resuspended in 100 μ L NF H₂O in each tube.
2. 30 μ L of 5X Laemmli buffer was added to each cell suspension.
3. The above mixtures were boiled at 95°C for 10 mins.
4. The tube contents were centrifuged at 14000 rpm for 7 mins.
5. 7.5 μ L of each of the supernatants was loaded according to the following scheme (5 μ L of marker was loaded; gel: 4% stacking, 15% resolving, 1 mm gel):

Gel 1: C1,7 and C2,6

Sample	C1, Uninduced	C1, Induced	C1, Induced	C1, Induced	Stained marker	C2, Uninduced	C2, Induced	C2, Induced	C2, Induced	
IPTG conc. used (in mM)	-	0.25	0.5	1	0.25	-	0.25	0.5	1	-
Lane no.	1	2	3	4	5	6	7	8	9	10

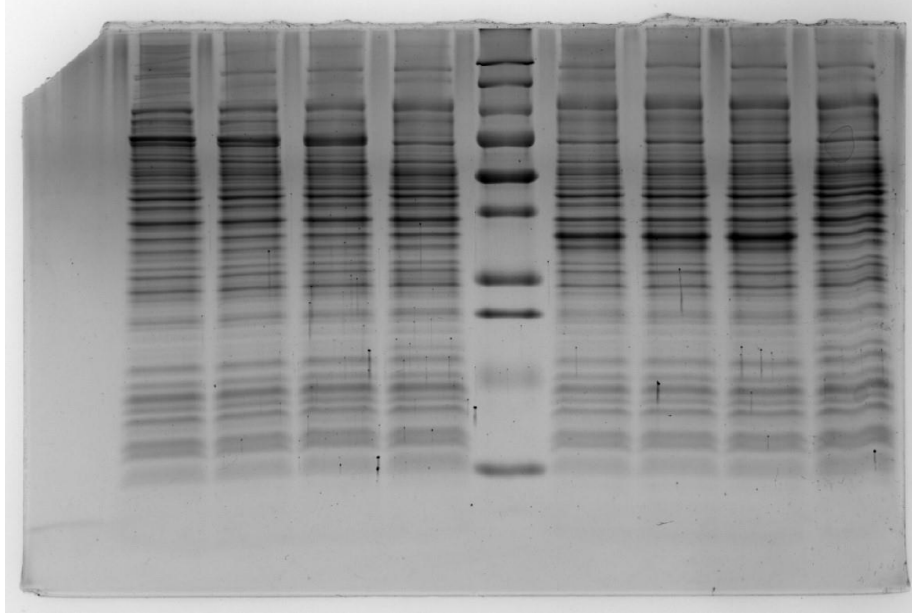
Gel 2: C1,8 and C2,7

Sample	C1, Uninduced	C1, Induced	C1, Induced	C1, Induced	Stained marker	C2, Uninduced	C2, Induced	C2, Induced	C2, Induced	
IPTG conc. used (in mM)	-	0.25	0.5	1	0.25	-	0.25	0.5	1	-
Lane no.	1	2	3	4	5	6	7	8	9	10

6. The gels were run at 100 V while the dye front was in the stacking gel, and at 150 V while it was in the resolving gel.
7. After the gels had run (dye front reaching about 0.5-1 cm from bottom), they were taken out from the apparatus and the stacking gel was cut off. The gels were transferred to boxes containing MilliQ water.
8. The water was then drained off, and Coomassie Blue staining solution was added to cover the gels. Staining was done for 5 mins on a shaker.
9. The Coomassie Blue stain was drained off, and destaining was done overnight. The gels were then imaged.

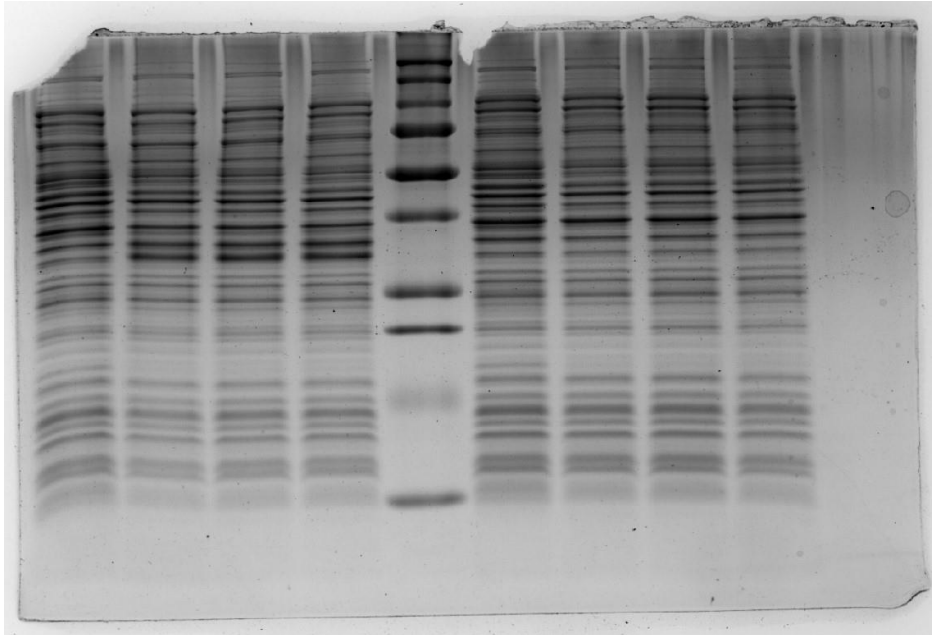
Gel results: (on 8 July 2021)

Gel 1:



There was no brighter band in the induced sample lanes compared to the uninduced sample lanes around the 25 kDa band. Expression could not be verified.

Gel 2:



There was no brighter band in the induced sample lanes compared to the uninduced sample lanes around the 75 kDa band. Expression could not be verified.

Since expression could not be verified using SDS PAGE, a Western blot was performed on 8 July.

A. Western Blot of cell pellets (continuing after 7 July, A):Sample preparation:

1. The same samples from 7 July, C, were used for loading. 7.5 μ L of each of the supernatants was loaded according to the following scheme (5 μ L of marker was loaded; gel: 4% stacking, 15% resolving, 1 mm gels):

Gel 1: C1,7 and C2,6

Sample	C1 Uninduced	C1, Induced 0.25 mM IPTG	C1, Induced 0.5 mM IPTG	C1, Induced 1 mM IPTG	Stained marker	C2 Uninduced	C2, Induced 0.25 mM IPTG	C2, Induced 0.5 mM IPTG	C2, Induced 1 mM IPTG	-
Lane no.	1	2	3	4	5	6	7	8	9	10

Gel 2: C1,8 and C2,7

Sample	C1 Uninduced	C1, Induced 0.25 mM IPTG	C1, Induced 0.5 mM IPTG	C1, Induced 1 mM IPTG	Stained marker	C2 Uninduced	C2, Induced 0.25 mM IPTG	C2, Induced 0.5 mM IPTG	C2, Induced 1 mM IPTG	-
Lane no.	1	2	3	4	5	6	7	8	9	10

2. The gels were run at 100 V while the dye front was in the stacking gel, and at 150 V while it was in the resolving gel.
3. After the gels had run (dye front reaching the bottom), they were taken out from the apparatus and the stacking gel was cut off. The gels were transferred to boxes containing 1X transfer buffer.
4. The following layered setup was assembled in a Bio-Rad Trans Blot machine (from bottom to top): 3 Whatman papers (wetted in 1X transfer buffer) -> nitrocellulose membrane -> SDS PAGE gel -> 3 Whatman papers (wetted in 1X transfer buffer).
5. The transfer was done at 0.22 A limited to 13 V, for 45 mins.
6. The nitrocellulose membranes were taken out and kept in distilled H₂O.
7. The membranes were then stained in Ponceau S stain for a few seconds.
8. Destaining was done by placing the membranes in distilled H₂O, followed by 1X PBST buffer.
9. The membranes were then transferred to blocking buffer (3% w/v skim milk solution in 1X PBST), and kept on a rocker for 1 hr at 37°C.

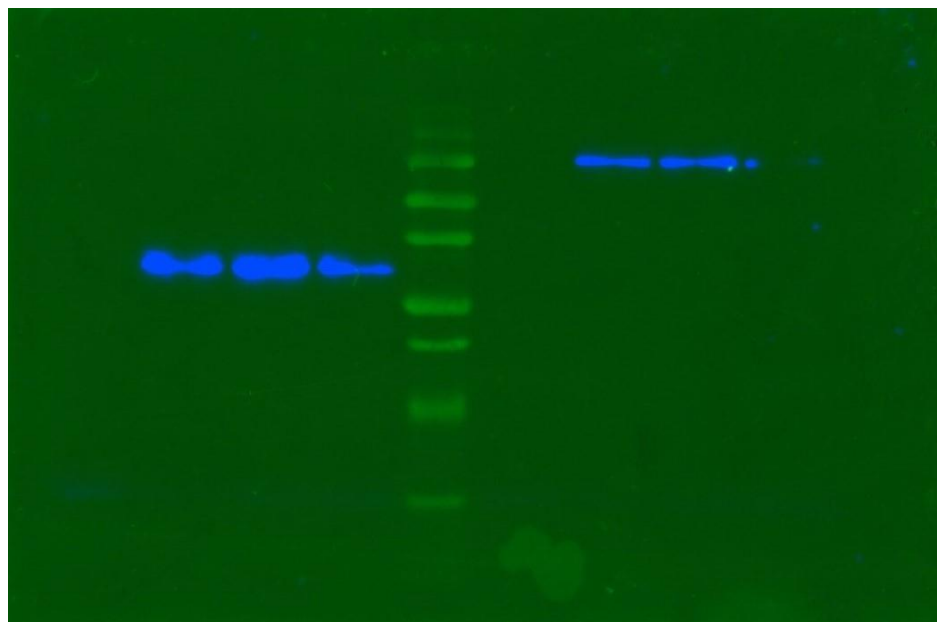
10. The blocking buffer was then drained. Primary antibody solution of 1 μL mouse anti-His antibody (sc-8036, 200 $\mu\text{g}/\text{mL}$ stock) in 4 mL blocking buffer (1:4000 antibody dilution, working concentration 50 ng/mL) was then added to each of the membranes. They were then kept on a rocker overnight at 4°C.

A. Western Blot of cell pellets (continued from 8 July, A):

1. The primary antibody solutions were drained. The membranes were covered with 1X PBST and kept on a rocker for 7 mins at 37°C, after which the PBST was drained.
2. The above wash step was repeated two more times.
3. Secondary antibody solution of 1.5 μ L goat anti-mouse antibody conjugated to HRP (Genei #HPO6) in 5 mL blocking buffer (1:3333 antibody dilution) was then added to each of the membranes. They were then kept on a rocker for 1 hr at 37°C.
4. The above wash step was performed three times.
5. Each of the membranes were then covered in 500 μ L peroxide + 500 μ L ECL substrate solution, and imaging was done immediately.

Western blot results:

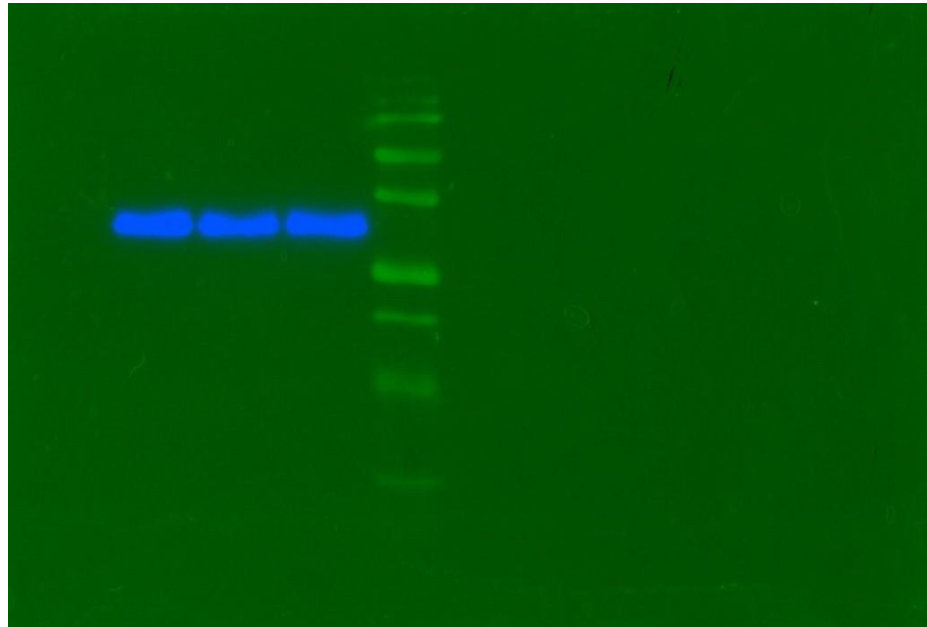
Blot 1:



Bands just above 25 kDa were observed in lanes 2, 3 and 4, which showed successful induction of C1 at these conditions. Induction was strongest for 0.5 mM IPTG induction.

Bands around 75 kDa were observed in lanes 7 and 8 which showed successful induction of C2 at these conditions. A faint band was also visible in lane 9, which was possibly due to poor substrate application. Induction was strongest for 0.5 mM IPTG induction.

Blot 2:



The results for C1 were very similar to those observed in blot 1 above. However, no bands were visible for C2.

Overall, it could be concluded that there was good induction at 30°C for 0.5 mM IPTG induction for both C1 & C2, and this condition was used for further experiments.

A. Solubility tests with BL21(DE3) (continuing after 7 July, B):

Preparing Ni-NTA beads:

1. 100 μ L of Ni-NTA bead slurry was taken in two Eppendorf tubes and was mixed with 400 μ L NaPi wash buffer each.
2. The above tubes were centrifuged at 2000 rpm for 2 mins at 4°C. The supernatant was discarded.
3. The above wash step was performed two more times.
4. The washed beads were kept resuspended in around 100 μ L of NaPi wash buffer, on ice.

Sample preparation:

5. The cell pellets from the 50 mL cultures were each resuspended in 2 mL NaPi sonication buffer and kept on ice.
6. 40 μ L of 50X Protease Inhibitor Cocktail (PIC) (working dilution 1X) and 150 μ L of 20 mg/mL lysozyme (working concentration 1.5 mg/mL) were added, and the tubes were kept on ice for 30 mins.

7. The resuspended cell suspensions were each sonicated for 5 cycles of 1 min total pulse on time, with 5 sec on, 10 sec off cycles.
8. The whole cell lysates were centrifuged at 14000 rpm for 30 mins at 4°C. The supernatants were taken out carefully and transferred to tubes labelled TCE and kept on ice, while the pellets were each resuspended in 200 µL NF H₂O in tubes labelled **S2**.
9. 40 µL of TCE samples were aliquoted into tubes labelled **S1**.
10. The rest of the TCE samples were mixed with the Ni-NTA beads and incubated at 4°C for 2 hrs on a rotating stand.
11. The above tubes were centrifuged at 2000 rpm for 2 mins at 4°C. The supernatants were transferred to Eppendorf tubes. 40 µL from these tubes was aliquoted into tubes labelled **S3**.
12. The beads were washed thrice with NaPi wash buffer as before.
13. The beads used for C2 were observed to be quite green (due to presence of an sfGFP), and hence were not subjected to elution. 70 µL of NaPi wash buffer and 30 µL of 5X Laemmli buffer were added to these tubes (labelled **E1**) and boiled at 95°C for 5 mins.
14. The beads for C1 in one of the two tubes were incubated with 70 µL NaPi elution buffer for 5 mins, and then centrifuged at 2000 rpm for 2 mins at 4°C. The supernatant was subjected to a Bradford assay, and a positive result was obtained.
15. 70 µL of NaPi wash buffer was added to the other tube. 30 µL of 5X Laemmli buffer was added to each of the two C1 tubes containing beads (labelled **E1 - E for eluted, and B for directly boiled**), and boiled at 95°C for 5 mins.
16. 12 µL of 5X Laemmli buffer was added to tubes S1 and S3, and 60 µL of 5X Laemmli buffer was added to tubes S2. They were boiled at 95°C for 5 mins.
17. All of the above samples were stored at -20°C.

A. SDS PAGE for solubility tests (continuing after 9 July, B):

Sample preparation:

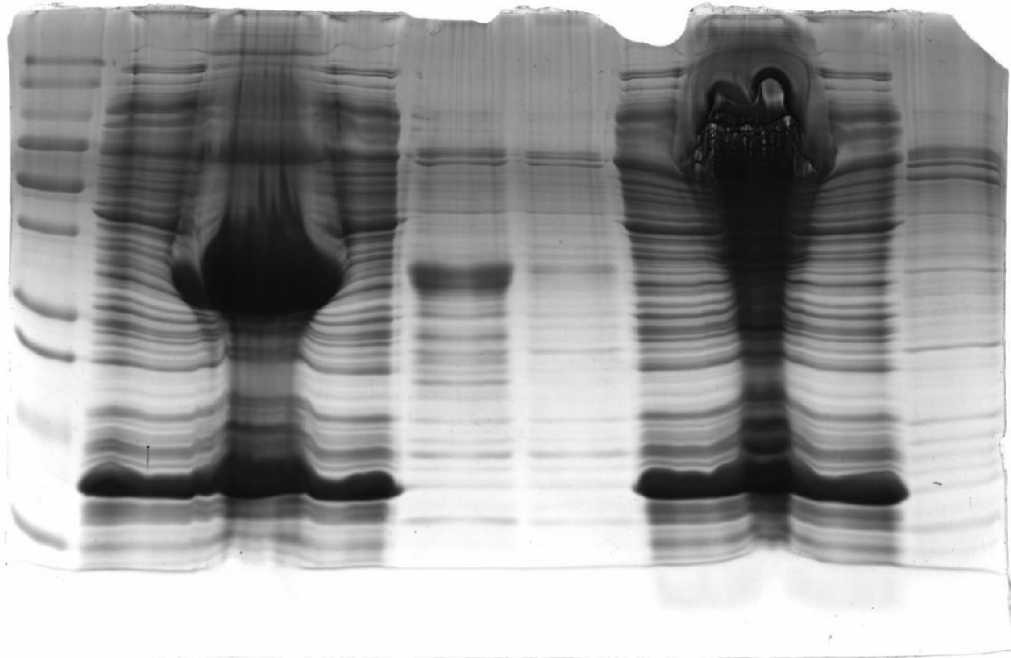
1. The boiled samples, which were stored at -20°C, were thawed and centrifuged at 14000 rpm for 7 mins.
2. 10 µL of each of the supernatants was loaded according to the following scheme (4 µL of marker was loaded; gel: 4% stacking, 15% resolving, 0.75 mm gel):

Gel setup:

Sample	Stained marker	C1 S1	C1 S2	C1 S3	C1 E1, boiled	C1 E1, eluted	C2 S1	C2 S2	C2 S3	C2 E1, boiled
Lane no.	1	2	3	4	5	6	7	8	9	10

3. The gels were run at 100 V while the dye front was in the stacking gel, and at 150 V while it was in the resolving gel.
4. After the gels had run (dye front reaching about 0.5-1 cm from bottom), they were taken out from the apparatus and the stacking gel was cut off. The gels were transferred to boxes containing MilliQ water.
5. The water was then drained off, and Coomassie Blue staining solution was added to cover the gels. Staining was done for 5 mins on a shaker.
6. The Coomassie Blue stain was drained off, and destaining was done for 3 hrs. The gels were then imaged.

Gel results:



Lanes 3 and 8 showed large blobs, indicative of a large amount of protein in these samples. Amount loaded was thus reduced in subsequent experiments. Sonication was also probably not complete, as there are large bands near 25 kDa in lane 3 and 75 kDa in lane 8, indicative of unlysed cells.

Lanes 4 and 9 showed a lot of contaminating bands, which suggested that the beads needed to be washed better.

Lane 5 showed a large band near 25 kDa, which was much more prominent than the other bands. This suggested that the C1 protein was appreciably soluble.

Lane 6 showed a faint band near 25 kDa, which also suggested that elution needed to be better.

Lane 10 seemed to show a band near 75 kDa, which could confirm the presence of the C2 protein, or it could have also been contaminating proteins.

Suggestions: Amount of protein loaded in the S1, S2 and S3 lanes should be lower. A Western blot would more clearly show the presence or absence of our proteins in the E1 lanes, as opposed to contaminants.

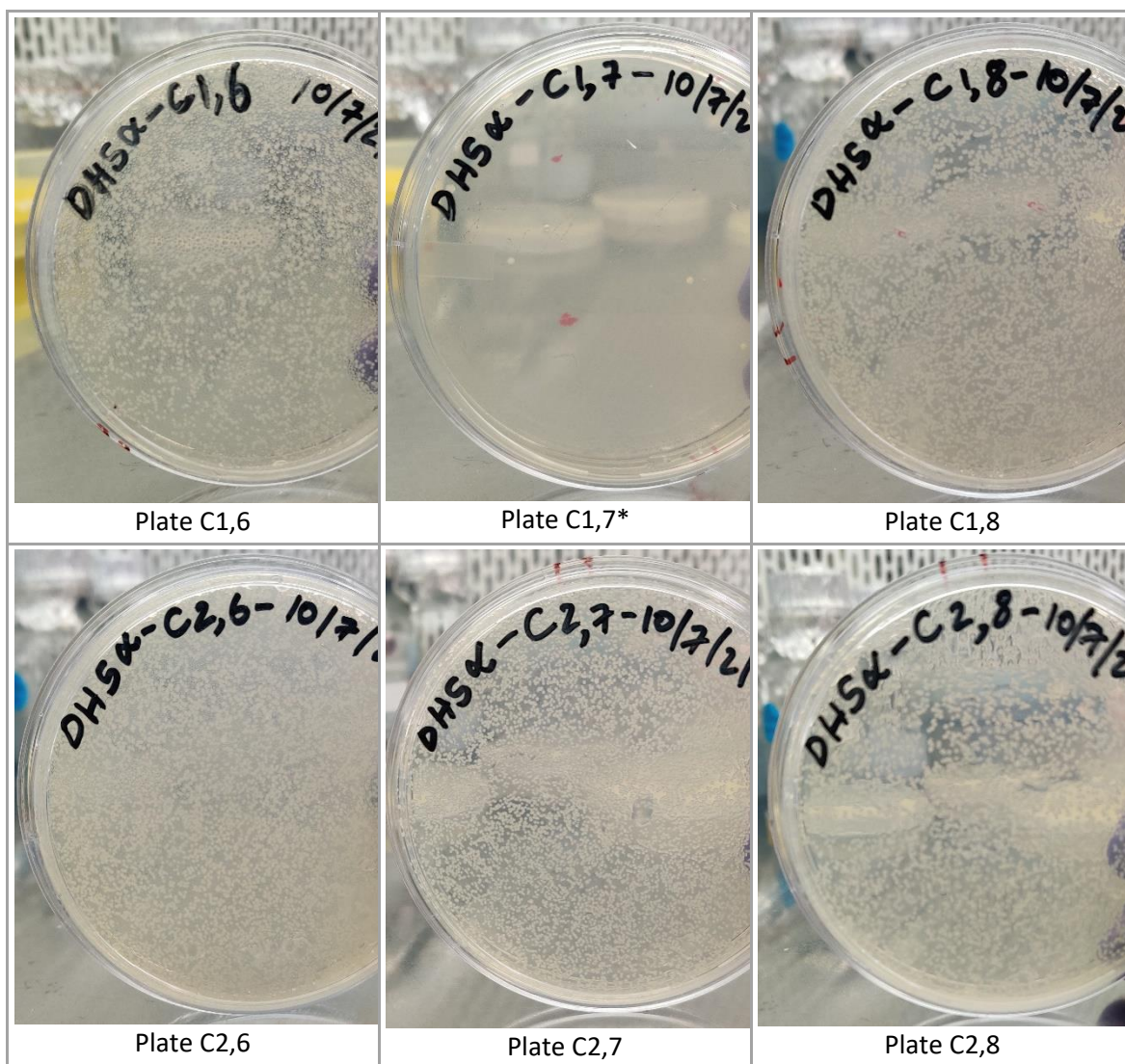
A. Transforming DH5α with positive Gibson clones:

Strain to be transformed: *E. coli* DH5α, made chemically competent using CaCl₂.

1. 7 tubes were taken, and each was filled with 80 µL of competent cell suspension. The following components were then added:
 - Tube C1,6: 2 µL of C1 clone, tube 6
 - Tube C1,7: 2 µL of C1 clone, tube 7
 - Tube C1,8: 2 µL of C1 clone, tube 8
 - Tube C2,6: 2 µL of C2 clone, tube 6
 - Tube C2,7: 2 µL of C2 clone, tube 7
 - Tube C2,8: 2 µL of C2 clone, tube 8
 - Tube NC: 2 µL sterile water
2. The tubes were kept on ice for 30 min after addition of DNA.
3. Heat shock at 42°C was given for 1.5 min.
4. The tubes were returned to ice, and kept there for 5 min.
5. The cells were allowed to recover by adding 1 mL LB broth to each tube and incubating the tubes at 37°C, with shaking at 190 rpm for 1 hr.
6. The tubes were taken out and centrifuged at 4000 rpm for 3 min to pellet the cells.
7. 0.9 mL supernatant media was taken out from each tube. The cell pellet was resuspended in the remaining media.
8. The cells were then plated on LB + Kan agar (spread using 8-10 glass beads per plate), and the plates were incubated at 37°C for 14 hrs.

A. Inoculation of transformed DH5 α (continued from 10 July, B):

1. The plates were taken out from 37°C and transferred to the hood at room temperature for inoculation. Pictures of the six plates (corresponding to the three clones each of C1 and C2) are given below:



* = no apparent colonies, possibly contamination

2. A colony from each plate was inoculated into separate test tubes containing 4 mL LB broth + 4 μ L of 50 mg/mL Kan (working concentration 50 μ g/mL). The plates were returned to 4°C.
3. The tubes were incubated at 37°C with shaking at 180 rpm for 15 hrs.

A. Western Blot after solubility tests (continuing after 9 July, B):

Sample preparation:

1. The same samples from 10 July, A, were used for loading. The supernatants were loaded according to the following scheme (4 μ L of marker was loaded; gel: 4% stacking, 15% resolving, 0.75 mm gel):

Gel setup:

Sample	Stained marker	C1 S1 (5 μ L)	C1 E1, boiled (3 μ L)	C1 E1, boiled (5 μ L)	C1 E1, boiled (10 μ L)	C1 E1, eluted (3 μ L)	C1 E1, eluted (5 μ L)	C1 E1, eluted (10 μ L)	C2 S1 (5 μ L)	C2 E1 (3 μ L)	C2 E1 (5 μ L)	C2 E1 (10 μ L)
Lane no.	1	2	3	4	5	6	7	8	9	10	11	12

1. The gel was run at 100 V while the dye front was in the stacking gel, and at 150 V while it was in the resolving gel.
2. After the gel had run (dye front reaching the bottom), it was taken out from the apparatus and the stacking gel was cut off. The gel was transferred to a box containing 1X transfer buffer.
3. The following layered setup was assembled in a Bio-Rad Trans Blot machine (from bottom to top): 3 Whatman papers (wetted in 1X transfer buffer) -> nitrocellulose membrane -> SDS PAGE gel -> 3 Whatman papers (wetted in 1X transfer buffer).
4. The transfer was done at 0.2 A limited to 10 V, for 30 mins.
5. The nitrocellulose membrane was taken out and kept in distilled H₂O.
6. The membrane was then stained in Ponceau S stain for a few seconds.
7. Destaining was done by placing the membrane in distilled H₂O, followed by 1X PBST buffer.
8. The membrane was then transferred to blocking buffer (3% w/v skim milk solution in 1X PBST), and kept on a rocker for 1 hr at 37°C.
9. The blocking buffer was then drained. Primary antibody solution of 1 μ L mouse anti-His antibody (sc-8036, 200 μ g/mL stock) in 4 mL blocking buffer (1:4000 antibody dilution, working concentration 50 ng/mL) was then added to the membrane. It was then kept on a rocker overnight at 4°C.

A. Transforming BL21(DE3) with clones C1,8 and C2,7:

Strain to be transformed: *E. coli* BL21(DE3), made chemically competent using CaCl₂.

1. 3 tubes were taken, and each was filled with 50 µL of competent cell suspension. The following components were then added:
 - Tube C1,8: 1 µL of C1 clone, tube 8
 - Tube C2,7: 1 µL of C2 clone, tube 7
 - Tube NC: 1 µL sterile water
2. The tubes were kept on ice for 30 mins after addition of DNA.
3. Heat shock at 42°C was given for 1.5 mins.
4. The tubes were returned to ice and kept there for 5 mins.
5. The cells were allowed to recover by adding 1 mL LB broth to each tube and incubating the tubes at 37°C, with shaking at 190 rpm for 1 hr.
6. The tubes were taken out and centrifuged at 4000 rpm for 3 mins to pellet the cells.
7. 0.9 mL supernatant media was taken out from each tube. The cell pellet was resuspended in the remaining media.
8. The cells were then plated on LB + Kan agar (spread using 8-10 glass beads per plate), and the plates were incubated at 37°C for 15 hrs.

B. Miniprep of C1 and C2 clones 6, 7 and 8 (continuing after 11 July, A):

Miniprep was done using **Thermo's GeneJET Plasmid Miniprep Kit (K0503)**.

1. 4 mL culture from each test tube was transferred to 2 Eppendorf tubes each of volume 2 mL.
2. The tubes were centrifuged at 8000 rpm for 2 min to pellet the cells. The supernatant was discarded.
3. To the first tube for each culture, 250 µL Resuspension Buffer was added, and cells were resuspended by pipetting in and out.
4. The above was transferred to the next tube, and resuspension was carried out again. This was repeated until all of the resuspended cells resided in the last tube for each culture.
5. 250 µL Lysis Buffer was added to the tubes containing the resuspended cells. The tube contents were mixed by inverting a few times, and the suspension became clearer.
6. 350 µL Neutralization Buffer was added to the tubes. The tube contents were mixed by inverting a few times, and some white precipitate was observed.
7. The tubes were centrifuged at 14000 rpm for 5 mins. The debris was pelleted, with the plasmid DNA staying in the supernatant.
8. The supernatant from each tube was transferred to separate spin columns.
9. The spin columns were centrifuged at 14000 rpm for 1 min. The flowthrough collected in the collecting tubes was discarded.
10. 500 µL Wash Buffer was added to the spin columns. Step 9 was repeated.
11. Step 10 was repeated.
12. The spin columns were centrifuged at 14000 rpm for 1 min (empty spin).

13. The collecting tubes were then removed, and the spin columns were placed into Eppendorf tubes.
14. 35 μL Elution Buffer was added to the spin columns and allowed to stand for 10 mins.
15. The tubes were centrifuged at 14000 rpm for 1 min. The flowthrough was collected inside the Eppendorf tubes (**labelled accordingly**).
16. Concentration of plasmid DNA was measured using a Nanodrop machine (elution buffer was taken as blank). Loading volume was 1 μL for all samples.
17. The Eppendorf tubes were stored at -20°C .

Nanodrop results:

C1 samples:

Sample	Concentration (ng/ μL)	A260/A280	A260/A230	A260
6	89.15	1.895	1.938	1.790
7	99.0	1.915	1.932	1.987
8	61.05	1.881	1.963	1.237

C2 samples:

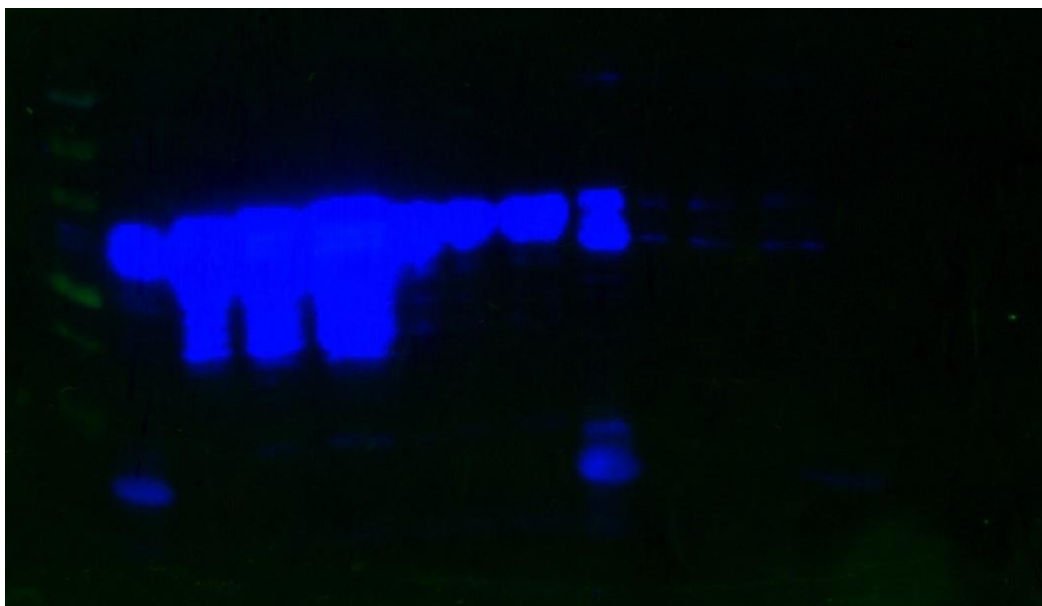
Sample	Concentration (ng/ μL)	A260/A280	A260/A230	A260
6	16.8	1.349	0.672	6.361
7	87.65	1.889	1.689	1.766
8	97.85	1.928	1.838	1.988

Except C2 clone 6, the plasmid concentrations as well as A260/A280 ratio and A260/A230 were acceptable.

A. Western Blot after solubility tests (continued from 12 July, A):

1. The primary antibody solution was drained. The membrane was covered with 1X PBST and kept on a rocker for 7 mins at 37°C, after which the PBST was drained.
2. The above wash step was repeated two more times.
3. Secondary antibody solution of 1.5 µL goat anti-mouse antibody conjugated to HRP (Genei #HPO6) in 5 mL blocking buffer (1:3333 antibody dilution) was then added to the membrane. It was then kept on a rocker for 1 hr at 37°C.
4. The above wash step was performed three times.
5. The membrane was then covered in 500 µL peroxide + 500 µL ECL substrate solution, and imaging was done immediately.

Western blot results:



Bands just above 25 kDa were observed in lanes 2 through 8, which confirmed presence of C1 protein. These bands in lanes 3 through 5 were considerably brighter, which showed successful binding of C1 with the Ni-NTA beads, and hence C1 was soluble.

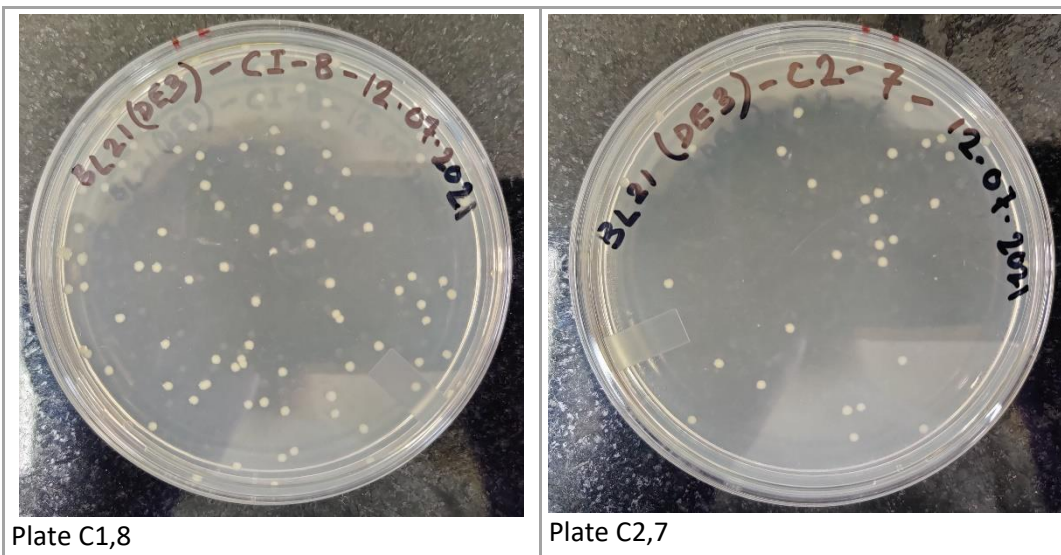
Lanes 6 through 8 showed comparatively fainter bands, which seemed to suggest that elution had not been performed properly.

Lanes 2 through 5 also had a smear of bands for lower molecular weights, since these samples had not been eluted, and contaminating proteins might still have been present on the beads. Lanes 6 through 8 were free of this contamination.

Lanes 9 through 12 did not show bands near 75 kDa (even though the beads were observed to be green in colour during purification). Some faint bands around 25 kDa could be observed, which was not expected. This could have been due to protein contamination, since elution had not been performed, or even due to degradation of the C2 protein during purification.

A. Inoculation of transformed BL21(DE3) (continued from 12 July, B):

1. The plates were taken out from 37°C and transferred to the hood at room temperature for inoculation. Pictures of the two plates (corresponding to C1,8 and C2,7) are given below:



2. A colony from each plate was inoculated into separate test tubes containing 10 mL LB broth + 10 μ L of 50 mg/mL Kan (working concentration 50 μ g/mL). The plates were returned to 4°C.
3. The tubes were incubated at 37°C with shaking at 180 rpm for 15 hrs.

A. Large scale C1 culture + C1 and C2 expression tests with transformed BL21(DE3) (continued from 13 July, B):

Subculturing:

1. From the C1 primary culture, 2 mL each was added to 4 flasks, each containing 500 mL LB broth + 500 μ L of 50 mg/mL Kan (working concentration 50 μ g/mL) (1:250 secondary cultures).
From the C2 primary culture, 2 mL was added to 98 mL LB broth + 100 μ L of 50 mg/mL Kan (working concentration 50 μ g/mL) (1:50 secondary culture).
2. The above flasks were incubated at 37°C, with shaking at 180 rpm for around 1.5-2 hrs, until OD₆₀₀ reached about 0.5.
3. From each of the above flasks, a 1 mL uninduced sample was taken, after which they were induced at 0.5 mM IPTG.
4. The flasks were incubated at 30°C for 4 hrs with shaking at 180 rpm.
5. The flask contents were centrifuged at 5000 rpm for 15 mins at 4°C to pellet the cells. The supernatants were discarded, while the pellets were stored at -20°C.

A. Solubility tests for C2 (continuing after 14 July, A):

Preparing Ni-NTA beads:

1. 100 μ L of Ni-NTA bead slurry was taken in an Eppendorf tube and was mixed with 400 μ L NaPi wash buffer.
2. The above tube was centrifuged at 2000 rpm for 2 mins at 4°C. The supernatant was discarded.
3. The above wash step was performed two more times.
4. The washed beads were kept resuspended in around 100 μ L of NaPi wash buffer, on ice.

Sample preparation:

5. One of the cell pellets (corresponding to 50 mL culture) was resuspended in 2 mL NaPi sonication buffer and kept on ice.
6. 40 μ L of 50X Protease Inhibitor Cocktail (PIC) (working dilution 1X) and 150 μ L of 20 mg/mL lysozyme (working concentration 1.5 mg/mL) were added, and the tube was kept on ice for 30 mins.
7. The resuspended cell suspension was sonicated for 10-15 cycles of 15 secs total pulse on time, with 1 sec on, 2 sec off cycles.
8. The whole cell lysate was divided into two Eppendorf tubes and centrifuged at 14000 rpm for 30 mins at 4°C. The supernatants were taken out carefully and transferred to tubes labelled TCE and kept on ice, while the pellets were resuspended in 500 μ L NF H₂O. 40 μ L was aliquoted from the resuspended pellets in tubes labelled **S2**.
9. 40 μ L of TCE samples were aliquoted into tubes labelled **S1**.
10. The rest of the TCE samples were mixed with the Ni-NTA beads and incubated at 4°C for 2 hrs on a rotating stand.
11. The above tubes were centrifuged at 2000 rpm for 2 mins at 4°C. The supernatants were transferred to Eppendorf tubes. 40 μ L from these tubes was aliquoted into tubes labelled **S3**.
12. The beads were washed thrice with NaPi wash buffer as before.
13. 70 μ L of NaPi wash buffer and 30 μ L of 5X Laemmli buffer were added to these tubes (labelled **E1**) and boiled at 95°C for 5 mins.
14. 12 μ L of 5X Laemmli buffer was added to tubes S1, S2 and S3. They were boiled at 95°C for 5 mins.
15. A Western blot was performed with the above samples, after which they were stored at -20°C.

A. Western Blot after C2 solubility test:

Sample preparation:

1. The above samples were used for loading. The supernatants were loaded according to the following scheme (4 μ L of marker was loaded; gel: 4% stacking, 15% resolving, 0.75 mm gel; C1 was used as a control):

Gel setup: The following setup was repeated in the next half of the gel. One of the halves was used for detection via anti-His primary antibody, while the other was used for detection via anti-GFP primary antibody.

Sample	Stained marker	C2 S1 (5 μ L)	C2 S2 (5 μ L)	C2 S3 (5 μ L)	C2 E1, boiled (5 μ L)	C2 E1, boiled (10 μ L)	C1 E1, boiled (5 μ L)
Lane no.	1	2	3	4	5	6	7

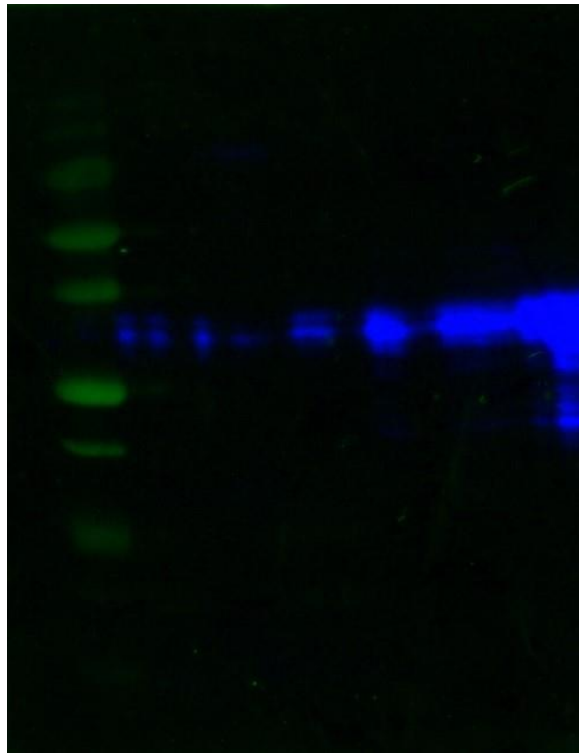
2. The gel was run at 100 V while the dye front was in the stacking gel, and at 150 V while it was in the resolving gel.
3. After the gel had run (dye front reaching the bottom), it was taken out from the apparatus and the stacking gel was cut off. The gel was transferred to a box containing 1X transfer buffer.
4. The following layered setup was assembled in a Bio-Rad Trans Blot machine (from bottom to top): 3 Whatman papers (wetted in 1X transfer buffer) -> nitrocellulose membrane -> SDS PAGE gel -> 3 Whatman papers (wetted in 1X transfer buffer).
5. The transfer was done at 0.2 A limited to 10 V, for 30 mins.
6. The nitrocellulose membrane was taken out and kept in distilled H₂O.
7. The membrane was then stained in Ponceau S stain for a few seconds.
8. Destaining was done by placing the membrane in distilled H₂O, followed by 1X PBST buffer.
9. The membrane was then transferred to blocking buffer (3% w/v skimmed milk solution in 1X PBST), and kept on a rocker for 1 hr at 37°C.
10. The blocking buffer was then drained. The membrane was cut from the middle, and the two halves were incubated with two different primary antibody solutions - 1) 1 μ L mouse anti-His antibody (sc-8036, 200 μ g/mL stock) in 4 mL blocking buffer (1:4000 antibody dilution, working concentration 50 ng/mL), and 2) 1 μ L mouse anti-GFP antibody (sc-9996, 200 μ g/mL stock) in 4 mL blocking buffer (1:4000 antibody dilution, working concentration 50 ng/mL). It was then kept on a rocker overnight at 4°C.

A. Western Blot after C2 solubility test (continued from 15 July, B):

1. The primary antibody solutions were drained. The membranes were covered with 1X PBST and kept on a rocker for 7 mins at 37°C, after which the PBST was drained.
2. The above wash step was repeated two more times.
3. Secondary antibody solution of 1.5 μ L goat anti-mouse antibody conjugated to HRP (Genei #HPO6) in 5 mL blocking buffer (1:3333 antibody dilution) was then added to each of the membranes. They were then kept on a rocker for 1 hr at 37°C.
4. The above wash step was performed three times.
5. Each of the membranes were then covered in 500 μ L peroxide + 500 μ L ECL substrate solution, and imaging was done immediately.

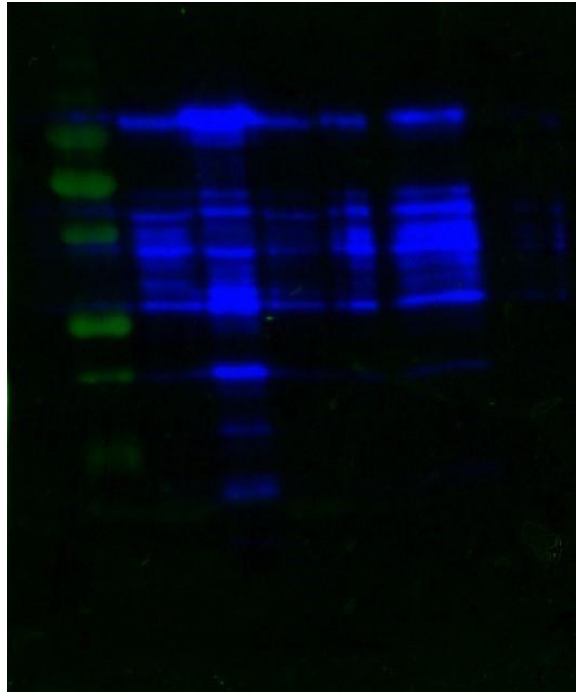
Western blot results:

Blot 1: anti-His



No bands around 75 kDa were visible. A consistent, somewhat smeared band was observed just below 37 kDa. This might have been caused due to protein degradation, or contamination.

Blot 2: anti-GFP



A prominent band was visible around 75 kDa in the boiled sample, which seemed to confirm the presence. There were a lot of other bands as well for lower molecular weights, which might have been caused due to protein degradation, or contamination.

A lot of non-specific bands were observed, and hence the above was repeated for a lower antibody concentration. For this, the membranes were stripped by incubation with stripping solution for 15 mins, followed by 2 washes of 5 mins each with 1X PBST.

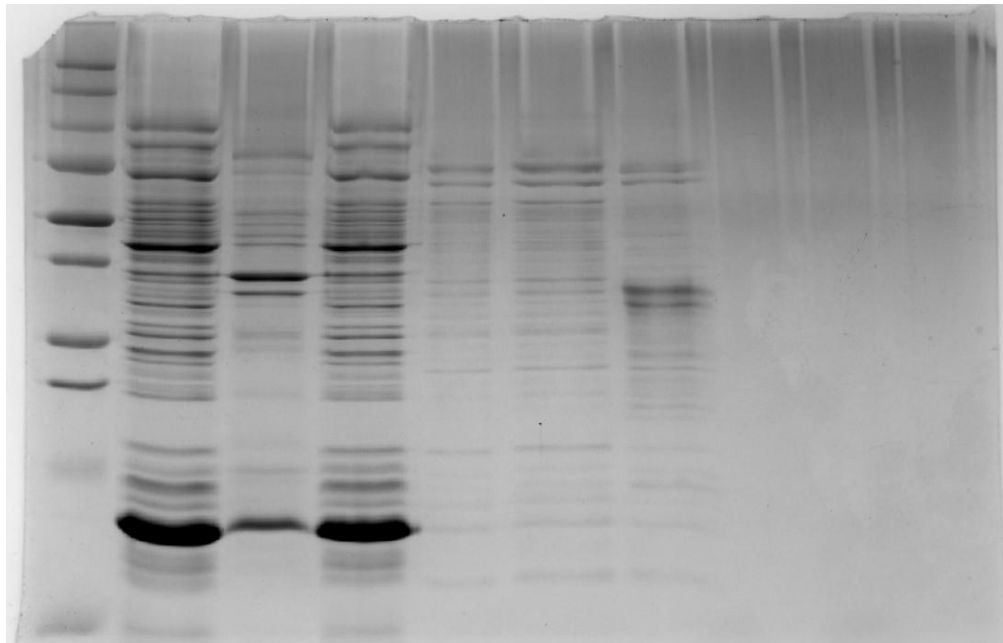
After this, blocking was done similarly for 45 mins, followed by overnight incubation with 5 mL each of 1:10000 dilutions of the corresponding antibodies.

A. SDS PAGE for C2 solubility test (continuing after 15 July, A):

Sample preparation:

1. The boiled samples, which were stored at -20°C , were thawed and centrifuged at 14000 rpm for 7 mins.
2. The same gel setup was used as for one half of the Western on 15 July, B.
3. The gels were run at 100 V while the dye front was in the stacking gel, and at 150 V while it was in the resolving gel.
4. After the gels had run (dye front reaching about 0.5-1 cm from bottom), they were taken out from the apparatus and the stacking gel was cut off. The gels were transferred to boxes containing MilliQ water.
5. The water was then drained off, and Coomassie Blue staining solution was added to cover the gels. Staining was done for 5 mins on a shaker.
6. The Coomassie Blue stain was drained off, and destaining was done overnight. The gels were then imaged.

Gel results: (on 17 July 2021)



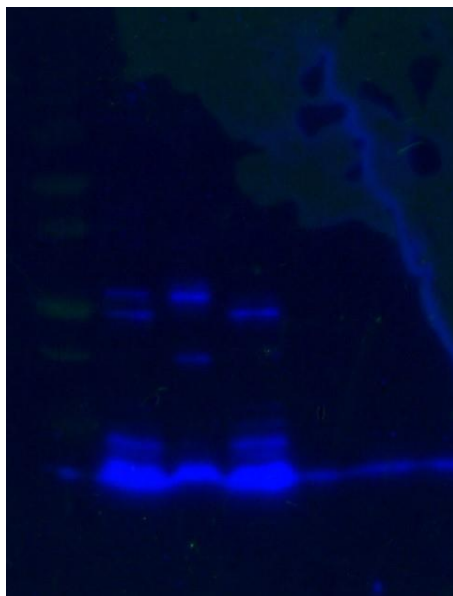
Samples S1 and S3 seemed to show prominent bands around 75 kDa, but the purified samples did not. Expression could not be verified.

A. Western Blot after C2 solubility test (continued from 16 July, A):

1. The primary antibody solutions were drained. The membranes were covered with 1X PBST and kept on a rocker for 7 mins at 37°C, after which the PBST was drained.
2. The above wash step was repeated two more times.
3. Secondary antibody solution of 1.5 μ L goat anti-mouse antibody conjugated to HRP (Genei #HPO6) in 5 mL blocking buffer (1:3333 antibody dilution) was then added to each of the membranes. They were then kept on a rocker for 45 mins at 37°C.
4. The above wash step was performed three times.
5. Each of the membranes were then covered in 500 μ L peroxide + 500 μ L ECL substrate solution, and imaging was done immediately.

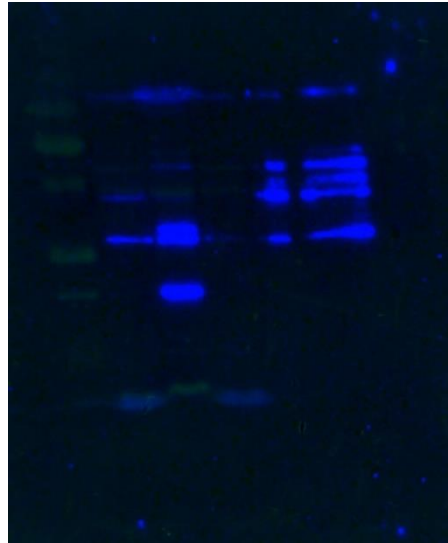
Western blot results:

Blot 1: anti-His



No bands around 75 kDa were visible in the lanes corresponding to the purified sample. A consistent band was observed instead, at a lower molecular weight. No conclusive results could be obtained.

Blot 2: anti-GFP



A prominent band around 75 kDa could be observed in the lane corresponding to the purified sample, but there were a lot of other, quite well-defined bands as well. Since the primary antibody concentration was very low, this seemed to suggest that there were quite a few, well-defined degradation products, and contamination was not the major cause of the smear in the original purified sample.

A. Inoculation of BL21(DE3) C2 clone (continued from 12 July, B):

1. The BL21(DE3) plate (C2,7 from 12 July) was taken out from 4°C and transferred to the hood at room temperature for inoculation.
2. 2 colonies from the plate were inoculated into a test tube containing 6 mL LB broth + 6 µL of 50 mg/mL Kan (working concentration 50 µg/mL). The plate was returned to 4°C.
3. The tube was incubated overnight at 37°C with shaking at 180 rpm.

A. C2 secondary culture + expression tests:

Subculturing:

1. 2 conical flasks were taken, to each of which 98 mL LB broth + 98 μ L of 50 mg/mL Kan (working concentration 50 μ g/mL) was added.
2. From the C2 primary culture, 2 mL was added to each conical flask above (1:50 secondary culture).
3. The above flasks were incubated at 37°C, with shaking at 180 rpm for around 1 hour, until OD₆₀₀ reached about 0.5.

Induction:

4. The flasks were taken out.
5. 2.38 g of CoCl₂.6H₂O was dissolved in 10 mL of water and filter sterilized with 0.22 μ m filter to prepare a 1 M solution. 10 μ L of this was added to each of the 2 flasks.
6. 50 μ L of 0.5 M IPTG was added to each of the 2 flasks, to obtain a working concentration of 0.25 mM.
7. One of the flasks was incubated for 4-5 hours at 30°C and 180 rpm.
8. The other flask was left to incubate overnight at 16°C and 180 rpm.
9. The 30°C was taken out and transferred to 2 centrifuge tubes and centrifuged for 15 minutes at 4°C and 3000 rpm.
10. The supernatant was discarded and the pellets for the 30°C were stored at -20°C

Preparing uninduced cultures for control:

11. 2 test tubes were taken, and each was filled with 6 mL LB and 6 μ L of 50 mg/mL Kan (working concentration 50 μ g/mL).
12. 120 μ L of C2 primary culture, 50 μ L of water and 0.6 μ L of 1 M CoCl₂.6H₂O solution was added to each tube.
13. Tubes were incubated at 37°C, with shaking at 180 rpm for around 2 hours, until OD₆₀₀ reached about 0.4-0.5.
14. Then, both the flasks were left to incubate at 180 rpm overnight at different temperatures (16°C and 30°C).

A. Transforming DH5 α with GST-GFP Nanobody plasmid piSP40:

Strain to be transformed: *E. coli* DH5 α , made chemically competent using CaCl₂.

- 2 tubes were taken, and each was filled with 100 μ L of competent cell suspension. The following components were then added:
 - Tube GST-GFP Nb: 1 μ L of plasmid piSP40
 - Tube NC: 1 μ L sterile water
- The tubes were kept on ice for 30 min after addition of DNA.
- Heat shock at 42°C was given for 1.5 min.
- The tubes were returned to ice, and kept there for 5 min.
- The cells were allowed to recover by adding 1 mL LB broth to each tube and incubating the tubes at 37°C, with shaking at 190 rpm for 1 hr.
- The tubes were taken out and centrifuged at 4000 rpm for 3 min to pellet the cells.
- 0.9 mL supernatant media was taken out from each tube. The cell pellet was resuspended in the remaining media.
- The cells were then plated on LB + Amp agar (spread using 8-10 glass beads per plate), and the plates were incubated at 37°C for 14 hrs.

A. Inoculation of BL21(DE3) C2 clone (continued from 12 July, B):

- The BL21(DE3) plate (C2,7 from 12 July) was taken out from 4°C and transferred to the hood at room temperature for inoculation.
- A colony from the plate was inoculated into a test tube containing 6 mL Terrific broth + 6 μ L of 50 mg/mL Kan (working concentration 50 μ g/mL) + 0.6 μ L of 1 M CoCl₂·6H₂O (working concentration 0.1 mM). The plate was returned to 4°C.
- The tube was incubated overnight at 37°C with shaking at 180 rpm.

C. Restriction Digestion of C2-pETMCN with KpnI:

Master Mix:

Materials	Volume
10X CutSmart Buffer	2.5 μ L
KpnI-HF	1 μ L
Sterile water	11.5 μ L
C2-pETMCN plasmid	10 μ L

Total reaction volume was 25 μ L. The above reaction was carried out for both C2 clones 6 and 7. The above components were mixed then incubated at 37°C for 3 hrs.

A. Gel Extraction of digested C2-pETMCN:

Composition - 0.8% agarose gel, 0.4 g agarose dissolved in 50 mL 1X TAE. 1.5 µL of 1 mg/mL EtBr was added.

Gel Results:

This gel is not to be imaged under UV, to minimise exposure.

Prominent bands could be observed near 6 kb and just above 1 kb, which seemed to confirm that the digestion was successful.

1. The correct bands were excised from the two appropriate lanes using a scalpel and placed in 2 separate tubes.

The following steps were carried out using **Qiagen's QIAquick Gel Extraction Kit (28704)**.

2. 600 µL Buffer QG was added to each of the tubes.
3. The tubes were incubated at 50°C for 10 mins. The contents were mixed by inverting regularly in order to completely dissolve the gel pieces.
4. 300 µL isopropanol was added to each tube and mixed by inverting.
5. The tube contents were transferred to separate spin columns.
6. The spin columns were centrifuged at 14000 rpm for 1 min. The flowthrough collected in the collecting tubes was discarded.
7. 750 µL Buffer PE was added to the columns for washing. Step 6 was repeated.
8. The spin columns were centrifuged at 14000 rpm for 1 min (empty spin).
9. The collecting tubes were then removed, and the spin columns were placed into Eppendorf tubes.
10. 30 µL Buffer EB was added to both tubes. The tubes were allowed to stand for 10 mins.
11. The tubes were centrifuged at 14000 rpm for 1 min. The flowthrough was collected inside the Eppendorf tubes.

E. Ligation of digested C2-pETMCN:

Master Mix:

Materials	Volume
10X T4 DNA Ligase Buffer	2 µL
T4 DNA Ligase	1 µL
Sterile water	7 µL
Digested C2-pETMCN plasmid	10 µL

Total reaction volume was 20 µL. The above reaction was carried out only for C2 clone 7. The above components were mixed then incubated at 16°C overnight.

On 24 July 2021:

The reaction mix was taken out from 16°C and kept at room temperature for 5 mins. The ligase was then heat inactivated by heating the reaction mix to 65°C for 10 mins. The tube (**labelled C2(2.0)-pETMCN**) was then stored at -20°C till further use.

A. Inoculation of DH5 α transformed with GST-GFP Nanobody plasmid piSP40 (continued from 23 July, A):

1. The GST-GFP Nb plate was taken out from 37°C and transferred to the hood at room temperature for inoculation.
2. A colony from the plate was inoculated into a test tube containing 6 mL LB broth + 6 μ L of 100 mg/mL Amp (working concentration 100 μ g/mL). The plate was returned to 4°C.
3. The tube was incubated overnight at 37°C with shaking at 180 rpm.

On 25 July 2021:

4. The tube contents were divided into 4 Eppendorf tubes, each containing 1.5 mL of the culture. They were then centrifuged at 5000 rpm for 15 mins at 4°C to pellet the cells. The supernatants were discarded, while the pellets were stored at -20°C.

A. Transforming DH5 α with C2(2.0)-pETMCN:

Strain to be transformed: *E. coli* DH5 α , made chemically competent using CaCl₂.

1. 2 tubes were taken, and each was filled with 100 μ L of competent cell suspension. The following components were then added:
 - Tube C2(2.0): 5 μ L of C2(2.0)-pETMCN
 - Tube NC: 5 μ L sterile water
2. The tubes were kept on ice for 30 min after addition of DNA.
3. Heat shock at 42°C was given for 1.5 min.
4. The tubes were returned to ice, and kept there for 5 min.
5. The cells were allowed to recover by adding 1 mL LB broth to each tube and incubating the tubes at 37°C, with shaking at 190 rpm for 1 hr.
6. The tubes were taken out and centrifuged at 4000 rpm for 3 min to pellet the cells.
7. 0.9 mL supernatant media was taken out from each tube. The cell pellet was resuspended in the remaining media.
8. The cells were then plated on LB + Kan agar (spread using 8-10 glass beads per plate), and the plates were incubated at 37°C for 14 hrs.

A. Resuspending *celG* primers:

1. The vials were centrifuged at 5000 rpm for 3 min.
2. Sterile water was added to each vial according to the following table, to make 100 μ M stocks

Primer name	Volume added (μ L)
celG KO FP	171
celG KO RP	84
celG CHECK FP	440
celG CHECK RP	276
celG EXP FP	540
celG EXP RP	390

3. The vials were stored at -20°C.

A. Miniprep of GST-GFP Nb plasmid (continuing after 24 July, A):

Miniprep was done using **Thermo's GeneJET Plasmid Miniprep Kit (K0503)**.

1. To the first tube, 250 μ L Resuspension Buffer was added, and cells were resuspended by pipetting in and out.
2. The above was transferred to the next tube, and resuspension was carried out again. This was repeated until all of the resuspended cells resided in the last tube.
3. 250 μ L Lysis Buffer was added to the tube containing the resuspended cells. The tube contents were mixed by inverting a few times, and the suspension became clearer.
4. 350 μ L Neutralization Buffer was added to the tube. The tube contents were mixed by inverting a few times, and some white precipitate was observed.
5. The tubes were centrifuged at 14000 rpm for 5 mins. The debris was pelleted, with the plasmid DNA staying in the supernatant.
6. The supernatant from the tube was transferred to a spin column.
7. The spin column was centrifuged at 14000 rpm for 1 min. The flowthrough collected in the collecting tube was discarded.
8. 500 μ L Wash Buffer was added to the spin column. Step 7 was repeated.
9. Step 8 was repeated.
10. The spin column was centrifuged at 14000 rpm for 1 min (empty spin).
11. The collecting tube was then removed, and the spin column was placed into an Eppendorf tube.
12. 30 μ L Elution Buffer was added to the spin column and allowed to stand for 10 mins.
13. The tube was centrifuged at 14000 rpm for 1 min. The flowthrough was collected inside the Eppendorf tube (**labelled GST-GFP Nb**).
14. Concentration of plasmid DNA was measured using a Nanodrop machine (elution buffer was taken as blank). Loading volume was 1 μ L.
15. The Eppendorf tube was stored at -20°C after further use.

Nanodrop results:

Sample	Concentration (ng/ μ L)	A260/A280	A260/A230	A260
GST-GFP Nb	285.65	1.933	2.245	5.744

16. Since $A260/A280 > 1.8$, the miniprep sample can be considered pure.
17. $A260/A230$ is just above 2.2, which is very slightly high.

A. Transforming BL21(DE3) with miniprep GST-GFP Nb plasmid:

Strain to be transformed: *E. coli* BL21(DE3), made chemically competent using CaCl_2 .

1. 2 tubes were taken, and each was filled with 100 μL of competent cell suspension. The following components were then added:
 - Tube GST-GFP Nb: 1 μL of miniprep GST-GFP Nb plasmid
 - Tube NC: 1 μL sterile water
2. The tubes were kept on ice for 30 min after addition of DNA.
3. Heat shock at 42°C was given for 1.5 min.
4. The tubes were returned to ice, and kept there for 5 min.
5. The cells were allowed to recover by adding 1 mL LB broth to each tube and incubating the tubes at 37°C , with shaking at 190 rpm for 1 hr.
6. The tubes were taken out and centrifuged at 4000 rpm for 3 min to pellet the cells.
7. 0.9 mL supernatant media was taken out from each tube. The cell pellet was resuspended in the remaining media.
8. The cells were then plated on LB + Amp agar (spread using 8-10 glass beads per plate), and the plates were incubated at 37°C for 14 hrs.

A. Inoculation of DH5 α transformed with C2(2.0)-pETMCN (continued from 25 July, A):

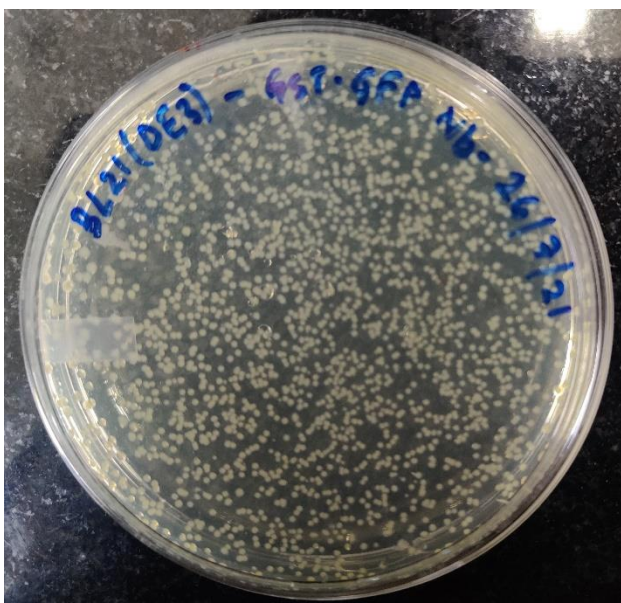
1. The C2(2.0) plate was taken out from 37°C and transferred to the hood at room temperature for inoculation. The picture of the plate is given below:



2. A colony from the plate was inoculated into a test tube containing 5 mL LB broth + 5 μL of 50 mg/mL Kan (working concentration 50 $\mu\text{g}/\text{mL}$). The plate was returned to 4°C .
3. The tube was incubated overnight at 37°C with shaking at 180 rpm.

A. Inoculation of BL21(DE3) transformed with GST-GFP Nb plasmid (continued from 26 July, B):

1. The GST-GFP Nb plate was taken out from 37°C and transferred to the hood at room temperature for inoculation. The picture of the plate is given below:



2. A colony from the plate was inoculated into a test tube containing 5 mL LB broth + 5 μ L of 100 mg/mL Amp (working concentration 100 μ g/mL). The plate was returned to 4°C.
3. The tube was incubated overnight at 37°C with shaking at 180 rpm.

A. Miniprep of C2(2.0)-pETMCN (continuing after 26 July, C):

Miniprep was done using **Thermo's GeneJET Plasmid Miniprep Kit (K0503)**.

1. The tube contents were divided into 4 Eppendorf tubes, each containing 1.25 mL of the culture. They were then centrifuged at 8000 rpm for 2 mins to pellet the cells. The supernatant was discarded.
2. To the first tube, 250 μ L Resuspension Buffer was added, and cells were resuspended by pipetting in and out.
3. The above was transferred to the next tube, and resuspension was carried out again. This was repeated until all of the resuspended cells resided in the last tube.
4. 250 μ L Lysis Buffer was added to the tube containing the resuspended cells. The tube contents were mixed by inverting a few times, and the suspension became clearer.

5. 350 µL Neutralization Buffer was added to the tube. The tube contents were mixed by inverting a few times, and some white precipitate was observed.
6. The tubes were centrifuged at 14000 rpm for 5 mins. The debris was pelleted, with the plasmid DNA staying in the supernatant.
7. The supernatant from the tube was transferred to a spin column.
8. The spin column was centrifuged at 14000 rpm for 1 min. The flowthrough collected in the collecting tube was discarded.
9. 500 µL Wash Buffer was added to the spin column. Step 8 was repeated.
10. Step 9 was repeated.
11. The spin column was centrifuged at 14000 rpm for 1 min (empty spin).
12. The collecting tube was then removed, and the spin column was placed into an Eppendorf tube.
13. 25 µL Elution Buffer was added to the spin column and allowed to stand for 10 mins.
14. The tube was centrifuged at 14000 rpm for 1 min. The flowthrough was collected inside the Eppendorf tube (**labelled C2(2.0) MP**).
15. Concentration of plasmid DNA was measured using a Nanodrop machine (elution buffer was taken as blank). Loading volume was 1 µL.
16. The Eppendorf tube was stored at -20°C after further use.

Nanodrop results:

Sample	Concentration (ng/µL)	A260/A280	A260/A230	A260
C2(2.0)	163.1	1.976	2.355	3.278

17. Since A260/A280 > 1.8, the miniprep sample can be considered pure.
18. A260/A230 is just above 2.2, which is very slightly high.

C. Double Digestion of miniprep C2(2.0)-pETMCN with BglII & BamHI:

Master Mix:

Materials	Volume
10X CutSmart Buffer	1 µL
BglII (NEB)	0.5 µL
BamHI-HF (NEB)	0.5 µL
Nuclease-Free H ₂ O	5 µL
C2(2.0)-pETMCN	3 µL

Total reaction volume in each tube was 10 µL.

The above components were mixed then incubated at 37°C for 2.5 hrs.

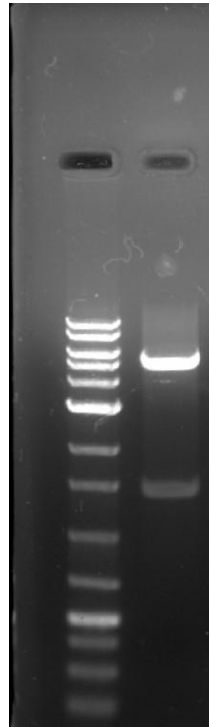
A. Agarose Gel Electrophoresis of double digested C2(2.0)-pETMCN:

Composition - 0.8% agarose gel, 0.4 g agarose dissolved in 50 mL 1X TAE. 1.5 μ L of 1 mg/mL EtBr was added.

2 μ L 6X loading dye was added to the reaction mixture before loading.

Samples were loaded as follows:

Gel Results:



Two prominent bands were observed, one just above 5 kb, which corresponds to the empty pETMCN vector fragment (5130 bp), and the other around 1.5 kb, which corresponds to the C2(2.0) fragment (1449 bp). A considerable smear could also be observed, which seemed to suggest some contamination. Overall, the digestion-ligation and the miniprep could be considered to be proper.

A. GST-GFP Nb secondary culture (continued from 27 July, A):

Subculturing:

From the GST-GFP Nb primary culture, 2 mL was added to 98 mL LB broth + 100 μ L of 100 mg/mL Amp (working concentration 100 μ g/mL) (1:50 secondary culture).

1. The above flask was incubated at 37°C, with shaking at 180 rpm for around 1.5-2 hrs, until OD₆₀₀ reached about 0.5.
2. From the above flask, a 1 mL uninduced sample was taken, after which it was induced at 0.5 mM IPTG.
3. The flask was incubated at 30°C for 4 hrs with shaking at 180 rpm.
4. The flask contents were centrifuged at 4500 rpm for 20 mins at 4°C to pellet the cells. The supernatants were discarded, while the pellets were stored at -20°C.

A. Transforming BL21(DE3) with miniprepped C2(2.0)-pETMCN:

Strain to be transformed: *E. coli* BL21(DE3), made chemically competent using CaCl₂.

1. 2 tubes were taken, and each was filled with 80 μ L of competent cell suspension. The following components were then added:
 - Tube C2(2.0): 2 μ L of miniprepped C2(2.0)-pETMCN
 - Tube NC: 2 μ L miniprep elution buffer
2. The tubes were kept on ice for 30 min after addition of DNA.
3. Heat shock at 42°C was given for 1.5 min.
4. The tubes were returned to ice, and kept there for 5 min.
5. The cells were allowed to recover by adding 1 mL LB broth to each tube and incubating the tubes at 37°C, with shaking at 190 rpm for 1 hr.
6. The tubes were taken out and centrifuged at 4000 rpm for 3 min to pellet the cells.
7. 0.9 mL supernatant media was taken out from each tube. The cell pellet was resuspended in the remaining media.
8. The cells were then plated on LB + Kan agar (spread using 8-10 glass beads per plate), and the plates were incubated at 37°C for 14 hrs.

A. Purification of GST-GFP Nb linked to Glutathione-Sepharose beads (continuing after 28 July, A):

Preparing Glutathione-Sepharose beads:

1. 50 μL of Glutathione-Sepharose bead slurry was taken in an Eppendorf tube and was mixed with 200 μL cold PBS + 0.2 μL of 1 M PMSF (working concentration 1 mM) + 2 μL of 10% v/v Triton X-100 (working concentration 0.1% v/v).
2. The above tube was centrifuged at 2000 rpm for 3 mins at 4°C. The supernatant was discarded.
3. The above wash step was performed two more times.
4. The washed beads were kept resuspended in 100 μL cold PBS + 1 μL of 10% v/v Triton X-100 (working concentration 0.1% v/v), on ice.

Sample preparation:

5. One of the cell pellets (corresponding to 50 mL culture) was resuspended in 1.85 mL cold PBS + 2 μL of 1 M PMSF (working concentration 1 mM) + 4 μL of 0.5 M EDTA (working concentration 1 mM) + 2 μL of 1 M DTT (working concentration 1 mM) + 40 μL of 50X Protease Inhibitor Cocktail (PIC) (working dilution 1X) + 150 μL of 20 mg/mL lysozyme (working concentration 1.5 mg/mL) and kept on ice for 10 mins.
6. The resuspended cell suspension was sonicated for 4 cycles of 1 min total pulse on time, with 1 sec on, 2 sec off cycles.
7. 200 μL of 10% v/v Triton X-100 (working concentration 1% v/v) was added to the whole cell lysate and incubated at 4°C for 30 mins on a rotating stand.
8. The whole cell lysate was transferred to an Eppendorf tube and centrifuged at 14000 rpm for 30 mins at 4°C. The supernatant was taken out carefully and transferred to a tube labelled TCE and kept on ice, while the pellet was resuspended in 1 mL cold PBS + 1 μL of 1 M PMSF (working concentration 1 mM) + 20 μL of 50X Protease Inhibitor Cocktail (PIC) (working dilution 1X). 10 μL was aliquoted from the resuspended pellet in a tube labelled **S2**.
9. 10 μL of the TCE sample was aliquoted into a tube labelled **S1**.
10. The rest of the TCE sample was mixed with the Glutathione-Sepharose beads and incubated at 4°C for 2 hrs on a rotating stand.
11. The above tube was centrifuged at 1000 rpm for 2 mins at 4°C. The supernatant was transferred to an Eppendorf tube. 10 μL from this tube was aliquoted into a tube labelled **S3**.
12. The beads were washed thrice with 200 μL cold PBS + 2 μL of 10% v/v Triton X-100 (working concentration 0.1% v/v) as before. They were then kept resuspended in 100 μL cold PBS + 1 μL of 10% v/v Triton X-100 (working concentration 0.1% v/v).
13. 15 μL was aliquoted from the above tube into a tube labelled **Nb beads**, 4 μL of 5X Laemmli buffer was added, and boiled at 95°C for 7 mins.

14. 2.5 μL of 5X Laemmli buffer was added to tubes S1, S2 and S3. They were also boiled at 95°C for 7 mins.

A. SDS PAGE of purified GST-GFP Nb:

Sample preparation:

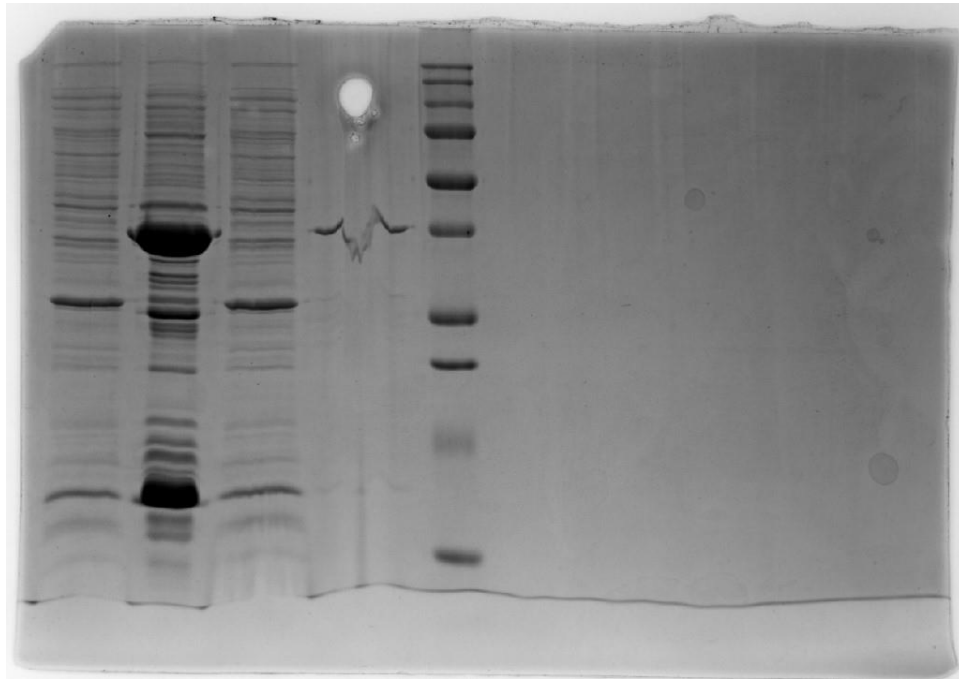
1. The boiled samples were centrifuged at 14000 rpm for 7 mins.
2. The supernatants were loaded according to the following scheme (4 μL of marker was loaded; gel: 4% stacking, 15% resolving, 1 mm gel):

Gel setup:

Sample	S1 5 μL	S2 5 μL	S3 5 μL	Nb beads 19 μL	Stained marker
Lane no.	1	2	3	4	5

3. The gel was run at 100 V while the dye front was in the stacking gel, and at 150 V while it was in the resolving gel.
4. After the gel had run (dye front reaching the bottom), it was taken out from the apparatus and the stacking gel was cut off. The gel was transferred to a box containing MilliQ water.
5. The water was then drained off, and Coomassie Blue staining solution was added to cover the gel. Staining was done for 5 mins on a shaker.
6. The Coomassie Blue stain was drained off, and destaining was done for 3 hrs. The gel was then imaged.

Gel results:



Lane 2 showed a large blob around 37 kDa (expected size of GST-GFP Nb), indicative of a large amount of protein in the pellet. Sonication was probably not complete.

Lane 4 showed a prominent band around 37 kDa but was distorted due to the presence of an air bubble higher up in the gel. There were no other contaminating bands too, which together suggested that the nanobody and nothing else had indeed bound to the beads.

A. Purification of C2 using GST-GFP Nb linked Glutathione-Sepharose beads:

Sample preparation:

1. One of the C2 cell pellets from 24 July, B (corresponding to 50 mL culture) was resuspended in 2 mL cold 50 mM HEPES (pH 8.0) + 2 μ L of 1 M PMSF (working concentration 1 mM) + 40 μ L of 50X Protease Inhibitor Cocktail (PIC) (working dilution 1X) + 120 μ L of 25 mg/mL lysozyme (working concentration 1.5 mg/mL) and kept on ice for 30 mins.
2. The resuspended cell suspension was sonicated for 3 cycles of 1 min total pulse on time, with 1 sec on, 2 sec off cycles.
3. The whole cell lysate was transferred to two Eppendorf tubes and centrifuged at 14000 rpm for 30 mins at 4°C. The supernatant was taken out carefully and distributed to two tubes labelled TCE and kept on ice, while the pellets were each resuspended in 1 mL cold 50 mM HEPES (pH 8.0) + 1 μ L of 1 M PMSF (working concentration 1 mM) + 20 μ L of 50X Protease Inhibitor Cocktail (PIC) (working dilution 1X). 10 μ L was aliquoted from one of the resuspended pellets in a tube labelled **S2**.
4. 10 μ L of one of the TCE samples was aliquoted into a tube labelled **S1**.
5. The rest of the TCE samples were mixed with 70 μ L of the prepared GST-GFP Nb linked Glutathione-Sepharose bead slurry from 29 July, A, and incubated at 4°C for 2 hrs on a rotating stand.
6. The above tubes were centrifuged at 1000 rpm for 2 mins at 4°C. The supernatants were transferred to Eppendorf tubes. 10 μ L from one of these tubes was aliquoted into a tube labelled **S3**.
7. The beads were washed thrice with 1 mL cold 50 mM HEPES (pH 8.0) as before.
8. The beads were then kept resuspended in 40 μ L cold 50 mM HEPES (pH 8.0) in a tube labelled **Nb beads**. 12 μ L of 5X Laemmli buffer was added and boiled at 95°C for 7 mins.
9. 3 μ L of 5X Laemmli buffer was added to tubes S1, S2 and S3. They were also boiled at 95°C for 7 mins.
10. The uninduced cell pellet was resuspended in 100 μ L cold 50 mM HEPES (pH 8.0). 30 μ L of 5X Laemmli buffer was added and boiled at 95°C for 7 mins.

A. SDS PAGE of purified GST-GFP Nb:

Sample preparation:

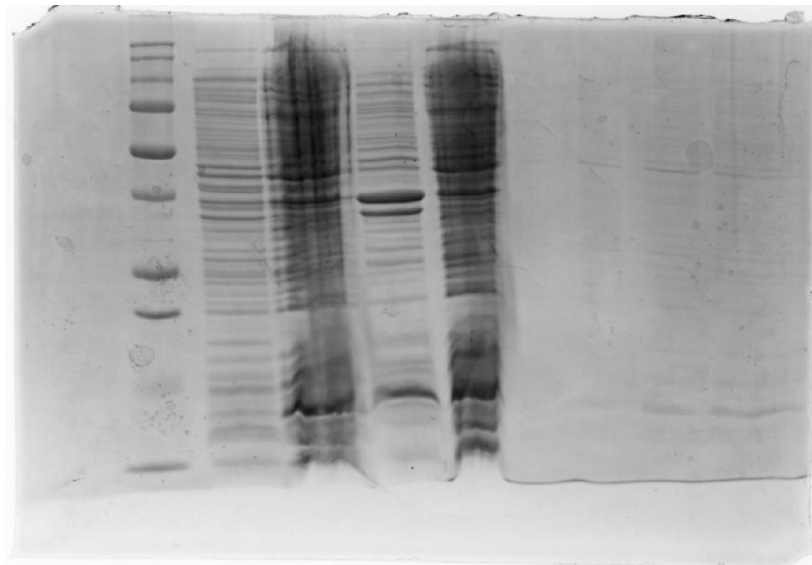
1. The boiled samples were centrifuged at 14000 rpm for 7 mins.
2. The supernatants were loaded according to the following scheme (4 μ L of marker was loaded; gel: 4% stacking, 15% resolving, 1 mm gel):

Gel setup:

Sample	-	Stained marker	Uninduced 5 μ L	S1 5 μ L	S2 5 μ L	S3 5 μ L	Nb beads 2 μ L	Nb beads 5 μ L	Nb beads 10 μ L	Nb beads 20 μ L
Lane no.	1	2	3	4	5	6	7	8	9	10

3. The gel was run at 100 V while the dye front was in the stacking gel, and at 150 V while it was in the resolving gel.
4. After the gel had run (dye front reaching the bottom), it was taken out from the apparatus and the stacking gel was cut off. The gel was transferred to a box containing MilliQ water.
5. The water was then drained off, and Coomassie Blue staining solution was added to cover the gel. Staining was done for 5 mins on a shaker.
6. The Coomassie Blue stain was drained off, and destaining was done for 2 hrs. The gel was then imaged.

Gel results:



Lanes 4 and 6 showed prominent bands around 75 kDa compared to the uninduced sample, but there were a lot of other bands as well. The pellet in Lane 5 did not show this band, hence sonication seemed to be proper.

The Nb bead lanes did not show any prominent bands around 75 kDa, but there was a prominent band just above 37 kDa, which might have been the GST-GFP Nb. Overall, there was no visible evidence of presence of C2 attached to the nanobody in these samples.

A. C2(2.0)-pETMCN secondary culture (continued from 30 July, C):

Subculturing:

From the C2(2.0) primary culture, 2 mL was added to 98 mL Terrific broth + 100 μ L of 50 mg/mL Kan (working concentration 50 μ g/mL) + 10 μ L of 1 M $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (working concentration 0.1 mM) (1:50 secondary culture).

1. The above flask was incubated at 37°C, with shaking at 180 rpm for around 1.5-2 hrs, until OD_{600} reached about 0.5.
2. From the above flask, a 1 mL uninduced sample was taken, after which it was induced at 0.5 mM IPTG.
3. The flask was incubated at 16°C overnight with shaking at 180 rpm.

On 1 August 2021:

4. The flask contents were centrifuged at 4500 rpm for 20 mins at 4°C to pellet the cells. The supernatants were discarded, while the pellets were stored at -20°C.

A. Transforming BL21(DE3) with SpyDock plasmid:

Strain to be transformed: *E. coli* BL21(DE3), made chemically competent using CaCl₂.

1. 2 tubes were taken, and each was filled with 100 µL of competent cell suspension. The following components were then added:
 - Tube SpyDock: 2 µL of SpyDock plasmid
 - Tube NC: 2 µL miniprep elution buffer
2. The tubes were kept on ice for 30 min after addition of DNA.
3. Heat shock at 42°C was given for 1.5 min.
4. The tubes were returned to ice, and kept there for 5 min.
5. The cells were allowed to recover by adding 1 mL LB broth to each tube and incubating the tubes at 37°C, with shaking at 190 rpm for 1 hr.
6. The tubes were taken out and centrifuged at 4000 rpm for 3 min to pellet the cells.
7. 0.9 mL supernatant media was taken out from each tube. The cell pellet was resuspended in the remaining media.
8. The cells were then plated on LB + Amp agar (spread using 8-10 glass beads per plate), and the plates were incubated at 37°C for 14 hrs.

A. Purification of C2(2.0) using Ni-NTA beads:

Preparing Ni-NTA beads:

1. 100 μ L of Ni-NTA bead slurry was taken in an Eppendorf tube and was mixed with 400 μ L HEPES wash buffer.
2. The above tube was centrifuged at 2000 rpm for 2 mins at 4°C. The supernatant was discarded.
3. The above wash step was performed two more times.
4. The washed beads were kept resuspended in around 100 μ L of HEPES wash buffer, on ice.

Sample preparation:

5. One of the cell pellets (corresponding to 50 mL culture) was resuspended in 2 mL HEPES sonication buffer and kept on ice.
6. 40 μ L of 50X Protease Inhibitor Cocktail (PIC) (working dilution 1X) and 150 μ L of 20 mg/mL lysozyme (working concentration 1.5 mg/mL) were added, and the tube was kept on ice for 30 mins.
7. The resuspended cell suspension was sonicated for 2 cycles of 1 min total pulse on time, with 1 sec on, 2 sec off cycles.
8. The whole cell lysate was divided into two Eppendorf tubes and centrifuged at 14000 rpm for 30 mins at 4°C. The supernatants were taken out carefully and transferred to tubes labelled TCE and kept on ice, while the pellets were resuspended in 1 mL NF H₂O. 40 μ L was aliquoted from the resuspended pellets in tubes labelled **S2**.
9. 40 μ L of TCE samples were aliquoted into tubes labelled **S1**.
10. The rest of the TCE samples were mixed with the Ni-NTA beads and incubated at 4°C for 2 hrs on a rotating stand.
11. The above tubes were centrifuged at 2000 rpm for 2 mins at 4°C. The supernatants were transferred to Eppendorf tubes. 40 μ L from these tubes was aliquoted into tubes labelled **S3**.
12. The beads were washed thrice with HEPES wash buffer as before.
13. 50 μ L of HEPES wash buffer and 30 μ L of 5X Laemmli buffer were added to these tubes (labelled **TCE**) and boiled at 95°C for 7 mins.
14. 12 μ L of 5X Laemmli buffer was added to tubes S1, S2 and S3. They were boiled at 95°C for 7 mins.
15. The uninduced cell pellet was resuspended in 1 mL NF H₂O. A 40 μ L aliquot was taken, and 12 μ L of 5X Laemmli buffer was added, and boiled at 95°C for 7 mins.

A. SDS PAGE of purified C2(2.0):

Sample preparation:

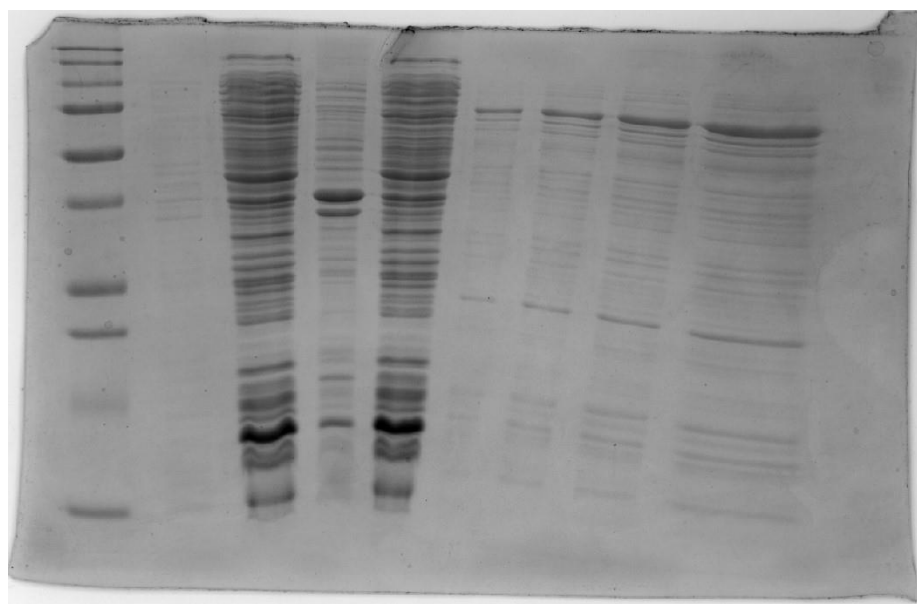
1. The boiled samples were centrifuged at 14000 rpm for 7 mins.
2. The supernatants were loaded according to the following scheme (4 μ L of marker was loaded; gel: 4% stacking, 15% resolving, 1 mm gel):

Gel setup:

Sample	Stained marker	Uninduced 5 μ L	S1 5 μ L	S2 5 μ L	S3 5 μ L	Beads 2 μ L	Beads 5 μ L	Beads 10 μ L	Beads 20 μ L	-
Lane no.	1	2	3	4	5	6	7	8	9	10

3. The gel was run at 100 V while the dye front was in the stacking gel, and at 150 V while it was in the resolving gel.
4. After the gel had run (dye front reaching the bottom), it was taken out from the apparatus and the stacking gel was cut off. The gel was transferred to a box containing MilliQ water.
5. The water was then drained off, and Coomassie Blue staining solution was added to cover the gel. Staining was done for 5 mins on a shaker.
6. The Coomassie Blue stain was drained off, and destaining was done overnight. The gel was then imaged.

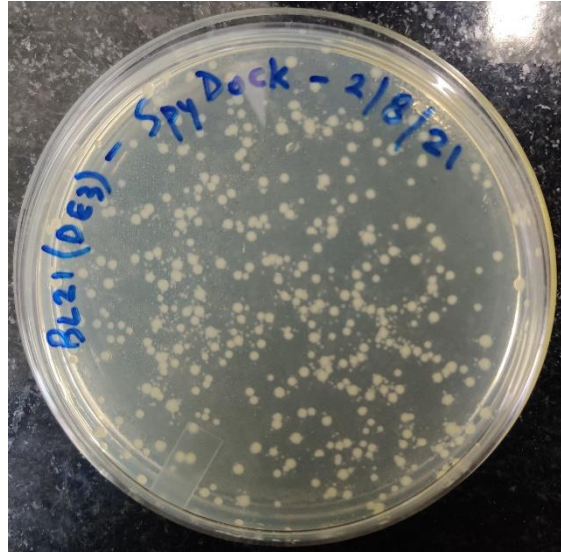
Gel results: (on 4 August 2021)



No prominent band could be observed just below 50 kDa (molecular weight of C2(2.0) was around 47 kDa).

A. Inoculation of BL21(DE3) transformed with SpyDock plasmid
(continued from 2 August, A):

1. The SpyDock plate from 2 August, A was taken out from 37°C and transferred to the hood at room temperature for inoculation. The picture of the plate is given below:



2. A colony from the plate was inoculated into a test tube containing 6 mL LB broth + 6 μ L of 100 mg/mL Amp (working concentration 100 μ g/mL). The plate was returned to 4°C.
3. The tube was incubated overnight at 37°C with shaking at 180 rpm.

A. SpyDock secondary culture (continued from 3 August, C):

Subculturing:

From the SpyDock primary culture, 2 mL was added to 98 mL LB broth + 100 μ L of 100 mg/mL Amp (working concentration 100 μ g/mL) (1:50 secondary culture).

1. The above flask was incubated at 37°C, with shaking at 180 rpm for around 2.5-3 hrs, until OD₆₀₀ reached about 0.5.
2. From the above flask, a 1 mL uninduced sample was taken, after which it was induced at 0.5 mM IPTG.
3. The flask was incubated overnight at 16°C with shaking at 180 rpm.

On 5 August 2021:

4. The flask contents were centrifuged at 4500 rpm for 20 mins at 4°C to pellet the cells. The supernatants were discarded, while the pellets were stored at -20°C.

A. Purification of SpyDock linked to Ni-NTA beads (continuing after 4 August, A):

Preparing Ni-NTA beads:

1. 100 μ L of Ni-NTA bead slurry was taken in an Eppendorf tube and was mixed with 400 μ L HEPES wash buffer.
2. The above tube was centrifuged at 2000 rpm for 2 mins at 4°C. The supernatant was discarded.
3. The above wash step was performed two more times.
4. The washed beads were kept resuspended in around 100 μ L of HEPES wash buffer, on ice.

Sample preparation:

5. One of the cell pellets (corresponding to 50 mL culture) was resuspended in 2 mL HEPES sonication buffer and kept on ice.
6. 40 μ L of 50X Protease Inhibitor Cocktail (PIC) (working dilution 1X) and 150 μ L of 20 mg/mL lysozyme (working concentration 1.5 mg/mL) were added, and the tube was kept on ice for 30 mins.
7. The resuspended cell suspension was sonicated for 4 cycles of 1 min total pulse on time, with 1 sec on, 2 sec off cycles.
8. The whole cell lysate was divided into two Eppendorf tubes and centrifuged at 14000 rpm for 30 mins at 4°C. The supernatants were taken out carefully and transferred to tubes labelled TCE and kept on ice, while the pellets were resuspended in 1 mL NF H₂O. 40 μ L was aliquoted from the resuspended pellets in tubes labelled **S2**.
9. 40 μ L of TCE samples were aliquoted into tubes labelled **S1**.
10. The rest of the TCE samples were mixed with the Ni-NTA beads and incubated at 4°C for 2 hrs on a rotating stand.
11. The above tubes were centrifuged at 2000 rpm for 2 mins at 4°C. The supernatants were transferred to Eppendorf tubes. 40 μ L from these tubes was aliquoted into tubes labelled **S3**.
12. The beads were washed thrice with HEPES wash buffer as before.
13. 50 μ L of HEPES wash buffer was added to each of these tubes (labelled **TCE**). 30 μ L aliquots were taken out from both tubes, 10 μ L 5X Laemmli buffer was added to each and boiled at 95°C for 7 mins.
14. 12 μ L of 5X Laemmli buffer was added to tubes S1, S2 and S3. They were boiled at 95°C for 7 mins.
15. The uninduced cell pellet was resuspended in 200 μ L NF H₂O. A 40 μ L aliquot was taken, and 12 μ L of 5X Laemmli buffer was added, and boiled at 95°C for 7 mins.

A. SDS PAGE of purified SpyDock:

Sample preparation:

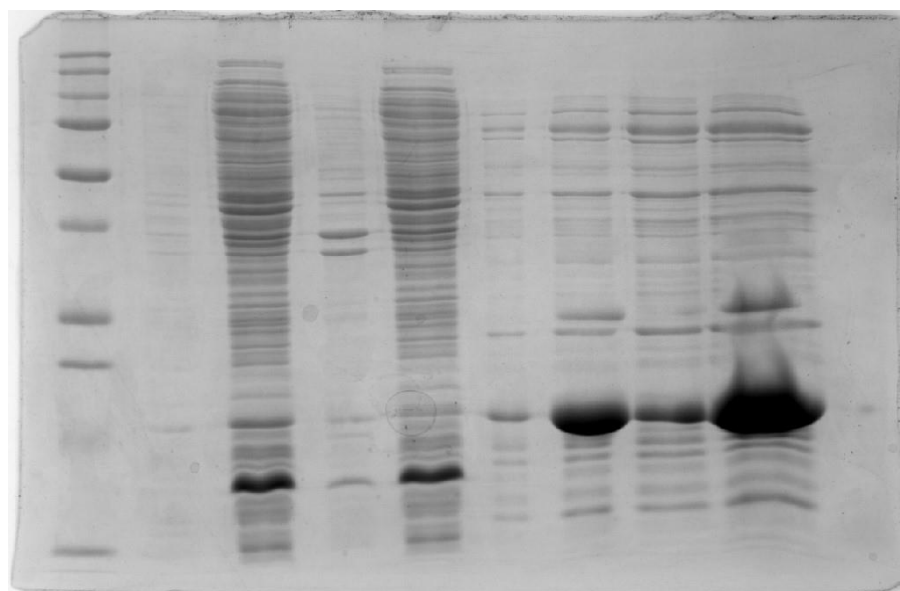
1. The boiled samples were centrifuged at 14000 rpm for 7 mins.
2. The supernatants were loaded according to the following scheme (4 μ L of marker was loaded; gel: 4% stacking, 15% resolving, 1 mm gel):

Gel setup:

Sample	Stained marker	Uninduced 5 μ L	S1 5 μ L	S2 5 μ L	S3 5 μ L	Beads 2 μ L	Beads 5 μ L	Beads 10 μ L	Beads 20 μ L	-
Lane no.	1	2	3	4	5	6	7	8	9	10

3. The gel was run at 100 V while the dye front was in the stacking gel, and at 150 V while it was in the resolving gel.
4. After the gel had run (dye front reaching the bottom), it was taken out from the apparatus and the stacking gel was cut off. The gel was transferred to a box containing MilliQ water.
5. The water was then drained off, and Coomassie Blue staining solution was added to cover the gel. Staining was done for 5 mins on a shaker.
6. The Coomassie Blue stain was drained off, and destaining was done overnight. The gel was then imaged.

Gel results: (on 6 August 2021)



A prominent band was visible around 15 kDa, which likely corresponds to SpyDock (around 13 kDa). Purification was thus considered successful.

A. Purification of SpyDock linked to Ni-NTA beads (continuing after 4 August, A):

Preparing Ni-NTA beads:

1. 100 μ L of Ni-NTA bead slurry was taken in an Eppendorf tube and was mixed with 400 μ L HEPES wash buffer.
2. The above tube was centrifuged at 2000 rpm for 2 mins at 4°C. The supernatant was discarded.
3. The above wash step was performed two more times.
4. The washed beads were kept resuspended in around 100 μ L of HEPES wash buffer, on ice.

Sample preparation:

5. One of the cell pellets (corresponding to 50 mL culture) was resuspended in 2 mL HEPES sonication buffer and kept on ice.
6. 40 μ L of 50X Protease Inhibitor Cocktail (PIC) (working dilution 1X) and 150 μ L of 20 mg/mL lysozyme (working concentration 1.5 mg/mL) were added, and the tube was kept on ice for 30 mins.
7. The resuspended cell suspension was sonicated for 4 cycles of 1 min total pulse on time, with 1 sec on, 2 sec off cycles.
8. The whole cell lysate was divided into two Eppendorf tubes and centrifuged at 14000 rpm for 30 mins at 4°C. The supernatants were taken out carefully and transferred to tubes labelled TCE and kept on ice, while the pellets were resuspended in 1 mL NF H₂O. 40 μ L was aliquoted from the resuspended pellets in tubes labelled **S2**.
9. 40 μ L of TCE samples were aliquoted into tubes labelled **S1**.
10. The rest of the TCE samples were mixed with the Ni-NTA beads and incubated at 4°C for 2 hrs on a rotating stand.
11. The above tubes were centrifuged at 2000 rpm for 2 mins at 4°C. The supernatants were transferred to Eppendorf tubes. 40 μ L from these tubes was aliquoted into tubes labelled **S3**.
12. The beads were washed thrice with HEPES wash buffer as before.
13. 50 μ L of HEPES wash buffer was added to each of these tubes (labelled **TCE**). 30 μ L aliquots were taken out from both tubes, 10 μ L 5X Laemmli buffer was added to each and boiled at 95°C for 7 mins.
14. 12 μ L of 5X Laemmli buffer was added to tubes S1, S2 and S3. They were boiled at 95°C for 7 mins.
15. The uninduced cell pellet was resuspended in 200 μ L NF H₂O. A 40 μ L aliquot was taken, and 12 μ L of 5X Laemmli buffer was added, and boiled at 95°C for 7 mins.

A. SDS PAGE of purified C2(2.0):

Sample preparation:

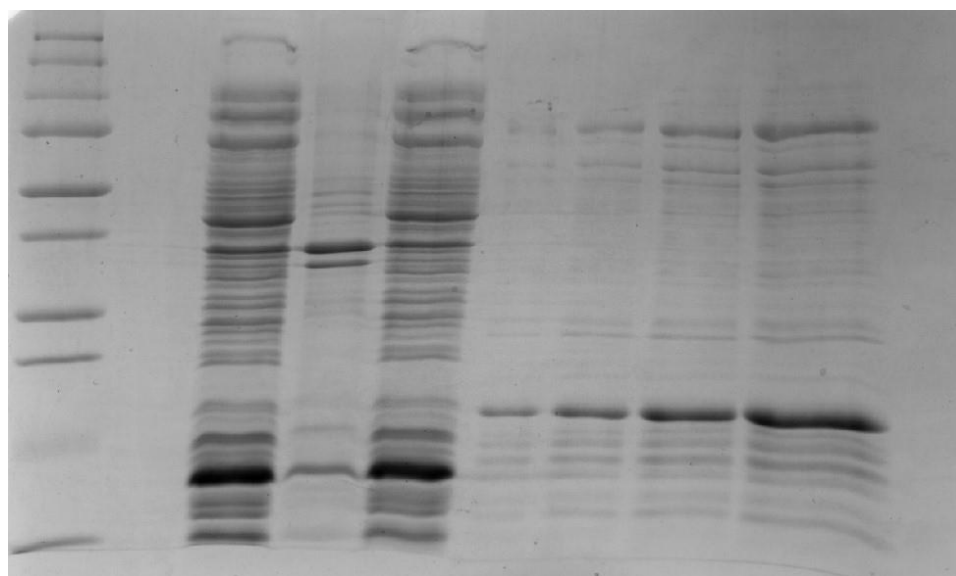
1. The boiled samples were centrifuged at 14000 rpm for 7 mins.
2. The supernatants were loaded according to the following scheme (4 μ L of marker was loaded; gel: 4% stacking, 15% resolving, 1 mm gel):

Gel setup:

Sample	Stained marker	Uninduced 5 μ L	S1 5 μ L	S2 5 μ L	S3 5 μ L	Beads 2 μ L	Beads 5 μ L	Beads 10 μ L	Beads 20 μ L	-
Lane no.	1	2	3	4	5	6	7	8	9	10

3. The gel was run at 100 V while the dye front was in the stacking gel, and at 150 V while it was in the resolving gel.
4. After the gel had run (dye front reaching the bottom), it was taken out from the apparatus and the stacking gel was cut off. The gel was transferred to a box containing MilliQ water.
5. The water was then drained off, and Coomassie Blue staining solution was added to cover the gel. Staining was done for 5 mins on a shaker.
6. The Coomassie Blue stain was drained off, and destaining was done for 3 hrs. The gel was then imaged.

Gel results:



Although a prominent band could be observed around 15 kDa, which confirmed the presence of the SpyDock attached to the beads, no prominent band could be observed just below 50 kDa (molecular weight of C2(2.0) was around 47 kDa).

A. Transforming BL21(DE3) with clones C1,8 and C2,7:

Strain to be transformed: *E. coli* BL21(DE3), made chemically competent using CaCl_2 .

1. 4 tubes were taken, and each was filled with 70 μL of competent cell suspension. The following components were then added:
 - Tube C2(2.0): 2 μL of C2 (2.0) MP (from July 27)
 - Tube C2,7: 2 μL of C2 clone, tube 7
 - Tube EV: 2 μL uncut empty pETMCN vector
 - Tube NC: 2 μL sterile water
2. The tubes were kept on ice for 30 mins after addition of DNA.
3. Heat shock at 42°C was given for 1.5 mins.
4. The tubes were returned to ice and kept there for 5 mins.
5. The cells were allowed to recover by adding 1 mL LB broth to each tube and incubating the tubes at 37°C , with shaking at 190 rpm for 1 hr.
6. The tubes were taken out and centrifuged at 4000 rpm for 3 mins to pellet the cells.
7. 0.9 mL supernatant media was taken out from each tube. The cell pellet was resuspended in the remaining media.
8. The cells were then plated on LB + Kan agar (spread using 8-10 glass beads per plate), and the plates were incubated at 37°C for 15 hrs.

A. Inoculation of transformants for primary culture from 13th August

1. The LB plates from the previous day were removed from 37° C and kept at 4° C.
2. 6 tubes were taken. 3 were filled with 6 mL LB while the other 3 were filled with 6 mL Terrific Broth. The 3 LB ones were labelled C2,7; C2(2.0) and EV. Similarly, the 3 Terrific Broth ones were labelled.
3. To each of the tubes, 0.6 µL of 1 M CoCl₂ and 6 µL of 50 mg/mL Kan (working concentration 50 µg/mL) were added.
4. The plates were brought out into the hood. From each of the plates (except the NC), one colony was inoculated into the corresponding LB tube, and another colony was inoculated into the corresponding Terrific Broth tube. The plates were kept back at 4° C.
5. The tubes were left to incubate overnight at 37° C and 190 rpm.

On 16th August 2021: The tubes had to be discarded as they were left to incubate for too long (no work happened at lab due to National Holiday on 15th August) and thus all the cells had settled down after overgrowth.

A. Inoculation of transformants from 13th August and untransformed bacteria for primary culture

8 tubes were taken and labelled and filled with 6 mL of corresponding media each and other components as follows: -

Tube	Supplements	Plate (2 colonies into each tube)
LB C2 II.0	0.6 µL of 1 M CoCl ₂ + 6 µL of 50 mg/mL Kan	C2 (2.0) from 13th August
LB C2,7	0.6 µL of 1 M CoCl ₂ + 6 µL of 50 mg/mL Kan	C2,7 from 13th August
LB pETMCN EV	0.6 µL of 1 M CoCl ₂ + 6 µL of 50 mg/mL Kan	EV from 13th August
LB NT	0.6 µL of 1 M CoCl ₂ + 6 µL of 50 mg/mL Kan	Untransformed BL21 (DE3) from Pradeep's plate
TB C2 II.0	0.6 µL of 1 M CoCl ₂ + 6 µL of 50 mg/mL Kan	C2 (2.0) from 13th August
TB C2,7	0.6 µL of 1 M CoCl ₂ + 6 µL of 50 mg/mL Kan	C2,7 from 13th August
TB pETMCN EV	0.6 µL of 1 M CoCl ₂ + 6 µL of 50 mg/mL Kan	EV from 13th August
TB NT	0.6 µL of 1 M CoCl ₂ + 6 µL of 50 mg/mL Kan	Untransformed BL21 (DE3) from Pradeep's plate

(The final working conc. of CoCl₂ was 0.001 M and for Kanamycin, it was 50 µg/mL.)

The tubes were left to incubate overnight at 37° C and 190 rpm.

A. Inoculation of primary culture from 17th August for secondary culture:

1. 8 tubes were taken and labelled and filled with 7mL of corresponding media and other components as follows:

Tube	Supplements	Primary Culture (140 µL into each tube)
LB C2 II-0	0.7 µL of 1 M CoCl ₂ + 7 µL of 50 mg/mL Kan	C2 II.0
LB C2,7	0.7 µL of 1 M CoCl ₂ + 7 µL of 50 mg/mL Kan	C2,7
LB EV	0.7 µL of 1 M CoCl ₂ + 7 µL of 50 mg/mL Kan	pETMCN EV
LB NT	0.7 µL of 1 M CoCl ₂ + 7 µL of 50 mg/mL Kan	NT
TB C2 II-0	0.7 µL of 1 M CoCl ₂ + 7 µL of 50 mg/mL Kan	C2 II.0
TB C2,7	0.7 µL of 1 M CoCl ₂ + 7 µL of 50 mg/mL Kan	C2,7
TB EV	0.7 µL of 1 M CoCl ₂ + 7 µL of 50 mg/mL Kan	pETMCN EV
TB NT	0.7 µL of 1 M CoCl ₂ + 7 µL of 50 mg/mL Kan	NT

2. The tubes were kept at 37° C and 190 rpm until OD₆₀₀ reached 0.5.
3. To each tube, 3.5 µL of 0.5 M IPTG was added for a working conc. of 0.25 mM.
4. The tubes were left to incubate at 16° C and 190 rpm overnight.