Cloning the *norV* promoter and *nrfA* gene into the iGEM Foundation Biobrick Standard to optimise *E. coli* for the conversion of nitric oxide (NO) to ammonia.

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Material and methods

Experiment 1 – competent cell production

5ml (1:20) of Top10 *E. coli* cells were inoculated into 100ml of LB stock solution. They were left to grow at 37^{o}C. Cells were stopped from growing when OD600 was between 0.4 and 0.45.
2Cells were placed in the cold room for 10mins.
3. Cells were harvested at 3000rpm, 4^{o}C for 8 mins.
4. Pellet was gently resuspended in chilled 100mM $CaCl_2$, 25ml.
5. Incubated on ice for 10mins.
6. Harvested cells at 3000rpm, 4oC for 8 mins.
7. Resuspended pellet in 2ml of 100mM CaCl2, 30% (v/v) glycerol and divided into 100ul aliquots.
8. Store at -80^{o}C

Experiment 2 – competent cell efficiency/transformations

1. Competent cells on ice. 2.0ml centrifuge tubes were labelled appropriately.
2. 1µl of DNA was then pipetted into its corresponding tube.
3. 50µl of competent cells were then pipetted into each tube.
4. Incubate on ice for 30 minutes.
5. Cells were then put into a 42°C water back, for 1 minute. Then placed back onto ice for 5 minutes.
6. 200µl of SOC media was added to each tube, and incubated at 37°C for 2 hours. Agar plates were labelled in triplicate, with the concentration of DNA which was added to each.
7. 70µl from each tube was pipetted onto the appropriate plate, the mixture was spread evenly across the plate.
8. Plates were incubated between 14 and 16 hours, overnight, at 37°C.

Experiment 3 – colony PCR

1. Preparation of primer working stock solution - 2µl of primer and 18µl of water.
2. Suspension of 2 separate colonies into 50µl of water, to act as NrfA and norV template.

Eppendorf	Buffer	dNTP's	Primer-F	Primer-R	DNA template	Taq polymerase	Water
	Volume of component in each eppendorf (µl)						
1 (1)	5	5	1.5	1.5	0.1	0.5	36.4
2 (1)	5	5	1.5	1.5	1	0.5	35.5
3 (1)	5	5	1.5	1.5	0.1	0.5	36.4
4 (1)	5	5	1.5	1.5	1	0.5	35.5
5 (2)	5	5	1.5	1.5	0.1	0.5	36.4
6 (2)	5	5	1.5	1.5	1	0.5	35.5
7 (2)	5	5	1.5	1.5	0.1	0.5	36.4
8 (2)	5	5	1.5	1.5	1	0.5	35.5

Figure 1: **A table showing the contents of Eppendorf's used in colony PCR in order to obtain norV and NrfA**. Eppendorf's 1-4 were carried out with colony 1 and 5-8 were carried out with colony 2. Numbers 1, 3, 5 and 7 were for norV, whilst 2, 4, 6 and 8 were for NrfA.

Section of program	Time (minutes)	Temperature (°C)
Initial	15	95
Main cycle 39x		
Initial denaturation	0.5	94
Annealing	0.5	50
Extension	3.5	72
Final extension	20	72

Figure 2: **Table showing the stages of the colony PCR process with the time (minutes) and temperature (ºC) of each cycle.**

Experiment 4: Diagnostic agarose gel electrophoresis

Agarose gel of concentration $1.5g100ml^{-1}$, 1µl of cyber safe stain adder per 50ml of gel. A total volume of 20µl was loaded into lanes on gel, consisting of 3µl of DNA/ladder (1kb) and 3.5µl of dye and 13.5µl of 1x TAE buffer.

Experiment 5: PCR purification

1. Addition of binding buffer to each PCR product (1:1 ratio).
(2. Addition of isopropanol to norV PCR product. Due to small size of the DNA.)
3. Transfer each to GeneJET purification column, centrifuge for 60 seconds at 12,000 rpm. Discard flow through.
4. Addition of 700µl of wash buffer. Centrifuge for 60 seconds at 12,000 rpm. Discard flow through.
5. GeneJET purification column was recentifugued for 60 second at 12,000 rpm. Discard flow through. Put column into 1.5ml Eppendorf.
6. 50µl of elution buffer was added to each tube. Centrifuge for 60 seconds at 12,000 rpm. Keep product at -20ºC.

Experiment 6: miniprep on colony overnights

1. Cells centrifuged for 6 minutes at 4000 rpm. Supernatant discarded.
2. Pelleted cells resuspended in 250µl of Resuspension Solution, vortexed to ensure cells were resuspended completely. Transferred to Eppendorf tube.
3. Addition of 250µl of Lysis Solution to each Eppendorf. Mixed by inverting tube 6 times.
4. Addition of 350µl of Neutralisation Buffer to each Eppendorf, mixed by inverting 6 times.
5. Centrifuged for 5 minutes at 12,000 rpm.
6. Transferred supernatant to GeneJET spin column.
7. Centrifuged for 1 minute at 12,000 rpm and discarded flow-through.
8. Addition of 500µl of Wash Solution and centrifuge for 60 seconds at 12,000 rpm. Discarded flow-through.
9. Repeat step 8.
10. Discarded flow-through and centrifuged for another 60 seconds at 12,000 rpm.
11. Transferred GeneJET spin column to Eppendorf tube. Added 50µl of Elution Buffer, left to stand for 2 minutes. Then centrifuged for 2 minutes at 12,000 rpm.
12. Minipreps stored, at -20ºC

Experiment 7: agarose gel extraction

1. Cut out required bands. Used scalpel on UV light box.
2. Samples put into pre-weighed Eppendorf's and reweigh.
3. Addition of binding buffer in 1:1 volume to gel.
4. Incubated at 55ºC for 10 minutes, inverted every 2-3 minutes to aid process of gel dissolving.
5. Samples transferred to GeneJET purification column, centrifuged for 1 minute at 12,000 rpm for 60 seconds. Flow through discarded, then column was placed back into collection tube.
6. Addition of 700µl of wash buffer too each sample, centrifuged for 1 minute at 12,000 rpm for 60 seconds.
7. Centrifuged empty column, again.
8. Column was put into an Eppendorf tube, add 50µl of elution buffer. Centrifuged for 1 minute at 12,000 rpm.
9. Stored extracted DNA at -20ºC.

Experiment 8: Diagnostic restriction digest

Eppendorf	Restriction enzyme	Buffer H (x10)	Distilled water	DNA
	Volume of component (µl)			
1 (None)	0.0	0.8	4.2	3
2 (Prefix restriction enzyme)	0.2	0.8	4.0	3
3 (Suffix restriction enzyme)	0.2	0.8	4.0	3
4 (Both)	0.2 of each	0.8	3.8	3

Figure 3**: Table to show the components and there quantities required for diagnostic digests.**

- Diagnostic digests were carried out for 2.5 hours at 37ºC or on the bench overnight.

Experiment 9: Restriction digest

- 23µl of DNA
2µl of prefix restriction enzyme
2µl of suffix restriction enzyme
3µl of 10x buffer H (Roche)
- Restriction digests were either carried out overnight on the bench or at 37ºC for 2.5 hours.

Experiment 10: Ligation of plasmid (pSB1C3) with norV and NrfA

- Carried out overnight at 4ºC.
- Using T4 ligase and it's corresponding buffer

	Component volume added (µl)				
Ligation ratio (P:I)	norV	Buffer	Enzyme	Water	Plasmid
1:5	3.5	1.0	1.0	-	5.0
1:3	1.0	1.0	1.0	2.0	5.0
1:1	0.3	1.0	1.0	2.7	5.0
1:0	-	1.0	1.0	5.0	5.0

Figure 4**: Table showing components and quantities for ligation of norV (3.066ngµl^{-1}) into pSB1C3**

	Component volume added (µl)				
Ligation ratio (P:I)	NrfA	Buffer	Enzyme	Water	Plasmid
1:3 (1 & 2)	8.15	2.0	2.0	6.6	1.25
1:1	2.7	1.0	1.0	2.0	1.25
1:0	0	1.0	1.0	6.75	1.25

Figure 5: **Table showing components and quantities for ligation of NrfA (3.066ngµl^{-1}) into pSB1C3 (4ngµl^{-1})**