DIYbio Collaboration Protocols

Generating $CaCl_2$ Competent Bacteria- done at Hackspace

Before Starting

Materials Needed:

Pour minimal media plates.
5x M9 salts
Prepare 100ml LB per strain.
Prepare 50ml ice cold 0.1M $CaCl_2$ / 15% glycerol per strain
Pre-chill eppendorf tubes

Minimal media plates *(Already prepared)

In 50 ml Falcon:

Reagent	**Volume (ul)**
20% (w/v) D-glucose	1000 (1ml)
2mg ml-1 thiamine	50
1M CaCl2	5
1M MgSO4	100
melted Bacteriological Agar solution (≤50°C)	39000 (39ml)
5x M9 salt solution* ***(Must be added LAST as they cause precipitation)***	10000(10ml)

0.1M CaCl2 / 15% glycerol* (Already prepared)
In 50 ml Falcon;

Reagent	**Volume (ml)**
1M CaCl2	**5**
100% glycerol	**7.5**

5x M9 salts in 500ml dH20 *(Already prepared)

Reagent	**Mass (g)**

Na2HPO4	32
KH2PO4	7.5
NaCl	1.25

Day 1	
1	Streak cells on **minimal agar plate**. Incubate 37°C overnight.
Day 2	
2	Pick a colony into 5ml **LB** + 100µl 1M **$MgSO_4$**
3	Incubate 37°C in shaker overnight.
Day 3	
4	Inoculate 100ml **LB** in pre-warmed conical with 1ml of the 5ml **O/N culture** from Day 2.
5	Incubate 2hrs in 37°C shaker until the cells at early log phase of growth curve (A_{600} ~ 0.3)
6	Transfer to chilled, sterile 50ml Falcon tube and incubate on ice 10min
7	Cf 3300g 5min in benchtop RmT. Cf.
8	Resuspend in 10ml ice cold 0.1M $CaCl_2$ / 15% glycerol and incubate on ice 30min
9	Centrifudge 3300g 5min in benchtop RmT. Cf.
10	Resuspend in 1ml ice cold 0.1M **$CaCl_2$ / 15% glycerol**. Transfer 100µl aliquots to pre-chilled, pre-labelled eppendorf tubes. Store -70°C.

Extracting Genomic DNA from Gram-Negative Bacteria -using Generation Column Capture Kit (Qiagen)

Notes: Gram-negative bacterial cultures can be used either fresh or frozen. Typically,an overnight culture contains 1–3 x 109 cells per ml. Due to the small genome size of Gram-negative bacteria, up to 3 x 109 cells may be applied to the column for DNA purification. Thus, culture can either be used directly, or, if necessary, concentrated by centrifuging. To concentrate, pellet 1 ml of overnight culture at 13,000–16,000 x g for 1 min. Remove the supernatant, leaving 200 µl residual fluid. Thoroughly suspend the pellet in the residual fluid by pipetting up and down 10 times. Place the sample on ice for immediate use or store frozen at –80°C. Frozen bacterial samples should be thawed and equilibrated to room temperature (15–25°C) before beginning the procedure.Thus, culture can either be used directly, or, if necessary, concentrated by centrifuging. To concentrate, pellet 1 ml of overnight culture at 13,000–16,000 x g for 1 min.

Before Starting
Before Starting: **Preheat block or bath to 99°C.** **Materials Needed:** 2 Capture column 2 Waste collection tube DNA Purification solution 1 DNA Elution Solution

1	For each sample to be processed assemble the following: 1 Capture Column contained in a blue Waste Collection Tube, 1 additional blue Waste Collection Tube and 1 clear DNA Collection Tube. Label tubes.
2	Add 200 µl well-mixed sample to the Capture Column by gently touching the center of the matrix with the pipet tip during dispensing.

3	Incubate at room temperature to allow the DNA to adsorb. Incubate for at least 1 min or up to 1 h. The sample may not completely saturate the column.
4	Add 400 µl DNA Purification Solution 1. Incubate 1 min at room temperature. The solution may begin draining into blue Waste Collection Tube during the incubation.
5	Centrifuge 10 s at 2000–12,000 x g. A waste volume of 600 µl will be collected in the blue Waste Collection Tube.
6	Transfer the Capture Column to the second blue Waste Collection Tube.
7	Add an additional 400 µl DNA Purification Solution 1.
8	Incubate 1 min at room temperature.

9	Centrifuge 10 s at 2000–12,000 x g. A waste of volume of 400 µl will be collected. The matrix should be white or nearly white.
10	Add 200 µl DNA Elution Solution 2; no incubation is required.
11	Centrifuge 10 s at 2000–12,000 x g to collect an additional 200 µl waste. The matrix containing the purified DNA should be white.
12	Transfer the Capture Column to clear DNA Collection Tube and discard the second blue Waste Collection Tube containing 600 µl waste solution.
13	Add 100 µl DNA Elution Solution 2.
14	Incubate at 99°C for 10 min. Position tube so that the Capture Column is completely contained within the heat block to ensure proper heating.

15	Centrifuge 20 s at 2000–12,000 x g immediately following heating step.
16	Purified DNA will be released from the Capture Column. The 100 µl DNA solution should appear clear. The purified DNA is ready for analysis.

PCR (Polymerase Chain Reaction)
Primer dilution

- When primer is ordered, they come as dried DNA. This dried DNA has to be first re-suspended in dH2O and the concentration will be 100pmol/ul.
- How much volume of dH2O to add to achieve 100pmol/ul is given on the sheet by the company when you receive the primers.
- This then need be further diluted to 1pmol/ul, this is done by taking 10ul of the primer solution and adding 90ul of dH2O.

Before Starting

Materials Needed:
Autoclaved H2O
5 x Phusion HF Buffer
10mM dNTP Mix
10mM Forward Primer
10mM Reverse Primer
DNA Template
DM50 (optional)
Phusion DNA Polymerase

Amplification of plasmid backbones; PSB1C3 (Cholorphenicol Backbone),

PCR Components	Volume for single run (µl)	Volume (µl)
H2O	31	Master Mix
5 x Phusion HF Buffer	10	
10mM dNTP Mix	1	
10mM Forward Primer	2.5	
10mM Reverse Primer	2.5	
DNA Template	1	
DM50	1.5	
Phusion DNA Polymerase	0.5	
Total Volume	**50**	

PCR conditions	Temp (C)	Time
Initial Denaturation 1 cycle	95	30s
30 Cycles Denaturation Annealing Extension	95	10s
	55	25s
	72	1.5 min
Final Extension 1 cycle	72	10 min
Hold 1 cycle	4	∞

Plasmid Backbone	Expected Size (bp)	Concentrations after nanodrop (ng/ul)
PSB1C3 (Chloramphenicol)	2070	*Write concentrations here, required for calculating ligation volumes*

PCR Amplification of Marine Genes

	ANF	ANF	ANF (Negative control)	PSB1C3 (as positive control)
dH2O	32.5	32.5	32.5	36.5
Phusion polymerase	0.5	0.5	0.5	0.5
5x Gotaq Buffer	10	10	10	10
dNTPs	1	1	1	1
Forward primer with prefix/suffix (1pmol/ul)	2.5	0	0	0
Reverse primer with prefix/suffix (1pmol/ul)	2.5	0	0	0
Forward primer without prefix/suffix (1pmol/ul)	0	2.5	2.5	
Reverse primer without prefix/suffix (1pmol/ul)	0	2.5	2.5	
Indolifex gDNA (1ng/ul)	1	1	0	0
PSB1C3 template	0	0	0	1
PSB1C3 Forward primer(10mM)	0	0	0	1
PSB1C3 Reverse primer (10mM)	0	0	0	1
Total volume	50	50	50	50

- You should always get a result for the positive control, if they are no bands when you run the gel afterwards, this would mean that there was something wrong either with your pipetting, polymerase or conditions used
- Negative control should have no bands on gel, any bands is due to contamination
- Sometimes, you can also see bands corresponding to below your DNA ladder, this might be a primer band, → added too much primer

Gel Electrophoresis

Before Starting

Materials Needed:
Agarose
1x TAE Buffer
Loading Buffer
Hyper Ladder I (DNA ladder)
Ethidium Bromide
Nitrile Gloves
Pipette and tips
Electrophoresis chamber + Power supply
Gel casting tray and comb

Preparing 1% Agarose Gel	
1	Weigh 1.5g of agarose and put it into a 500ml conical flask
2	Measure 150ml of 1x TAE Buffer and add this to the conical flask (total gel volume depends on the size of the gel casting tray)
3	Heat for approx 1 min in the microwave. Swirl, heat again for 30s until the agarose dissolves. (Do not let the solution boil for long periods –may boil out of the flask)
4	Cool solution under running cold water until it is not painful to touch (around 50-55C)
5	Add 20 ul of 500ug/ul Ethidium Bromide to the solution and swirl to mix.
6	Seal the ends of the gel casting tray with rubber dams or tape and position the gel comb in the casting tray.
7	Pour the solution into a sealed casting tray in a slow steady stream, ensuring there are no bubbles
8	Let the gel cool until it is solid.
8	Carefully pull the combs out and remove the rubber dams or tape
Loading the Gel	
7	Add 1 part loading buffer to five parts of loading sample
8	Position the gel tray in the tank
9	Add enough TAE buffer so that there is about 2-3mm of buffer over the gel
10	Pipette 5 ul of Hyperladder I into the first well
11	Pipette your loading sample into the consecutive wells
Running the Gel	
12	Place the lid on the gel box.

13	Connect the electrode wires to the power supply, make sure the positive and negative electrodes are correctly connected (Run to Red)
14	Turn on the power supply to 100 volts, set timer to1.25 hr
15	Check to make sure that the current is running, there should be bubbles at the end of the tank,
16	When the run is complete, turn off the power and disconnect the wires from the power supply.
17	Remove the lid of the electrophoresis chamber.
18	Using gloves, carefully remove the tray and gel.

Restriction Digest for Ligation
Protocol from the Registry of Standard Biological Parts

	Insert	linearized plasmid backbone
DNA	250ng	250ng
dH2O	adjust to 16ul	adjust to 16ul
NEB Buffer 2	2.5ul	2.5ul
BSA	0.5ul	0.5ul
Enzyme 1	0.5ul EcoRI	0.5ul EcoRI
Enzyme 2	0.5ul SpeI	0.5ul PstI

- Incubate in thermo cycler
- Digest mixtures were incubated at 37 for 30mins
- Heat inactivated at 80C for 20mins

Ligation

Volume	
ANF digest	2
PSB1C3 digest	2
10 x T4 ligase buffer	2
T$ DNA ligase	1
H2O	13
Total	20

Incubated 10mins at room temperature
Heat inactivated at 80C for 20mins

Transforming CaCl2 Competent Bacteria: (Transforming by heat-shock)

Before Starting
Materials Needed: 15ml falcons Prepare LB agar plates with appropriate antibiotic Ice Bucket

Day 1	
	ON ICE: add 4 µL of your DNA to the surface of the still-frozen pellet of competent cells. Add 8ul to another still-frozen pellet competent cell. DO NOT DISTURB THE CELLS WITH YOUR PIPETTE TIP. The cells are supposed to thaw slowly on ice with the DNA solution slowly mixing.
1	Incubate ON ICE for 45 min.
2	Place the 1.5mL Eppendorf tube, containing the competent cells/DNA mix, in a 37°C water bath for 10min. (This is the 'heat shock')

3	Place the 1.5mL Eppendorf tube, containing the competent cells/DNA mix, back in your ICE BUCKET for 2min
4	Add 1.3ml LB media (NO ANTIBIOTIC) to the 1.5mL Eppendorf tube, containing the competent cells/DNA mix.
5	Transfer this approx. 1.5mL solution to a labelled 15mL Falcon tube.
6	Place this labelled 15mL Falcon tube into an Incu-shaker for 1 hour at 37°C. *The cells are supposed to repair their outer membranes and transcribe & translates genes encoded by the plasmid, in particular the antibiotic resistancegene, during this perioed*
7	Use a P1000 pipette to transfer the approx. 1.5mL solution from the 15mL Falcon tube back to a new, labelled 1.5mL Eppendorf tube.
8	Spin this tube for 2min at max speed in a benchtop centrifuge.

9	Remove all the supernatant with an pipette and tip
10	Resuspend the pellet with 100µL LB media
11	Spread 10µl of the resuspended cell solution onto a labelled selective nutrient agar plate.
12	Spread the remaining 90µl of the resuspended cell solution onto another, separate labelled selective nutrient agar plate.
13	Place the plates in a 37°C static incubator, leave overnight (alternatively a 30°C static incubator over the weekend if it's a Friday etc)
Day 2	

14	Pick a colony into 2ml selective LB. Incubate in 37°C in shaker overnight for MiniPrep the next day.

Miniprep: Plasmid DNA Extraction (Anachem kit)

Before Starting
Materials Needed: KeyPrep columns Collection tubes Solution 1 (S1) Solution 2 (S2) Neutralising Buffer (Buffer NB) Wash buffer Elution Buffer

1.Centrifugation	
	Pellet 1.5 of bacterial culture containing the plasmid by centrifugation at 6,000 x g for 2 min
2. Resuspension of pellet	
	Add 250ul S1 to the pellet and resuspend the cells completely by vortexing or pipetting

3. Alkaline lysis	
	Add 250ul of S2 and gently mix by inverting tube several times (4-6 times) to obtain a clear lysate. Incubate on ice or at room temperature for NOT longer than 5 min
4. Neutralisation	
	To neutralise the lysate, add 400ul of **Buffer NB** and **gently mix** by inverting the tube several times (6-10 times) until a white precipitate forms. Centrifudge at 14,000-16,000 *x g* for 10min
5. Loading to column	
	Transfer the **supernatant** to a column assembled in a clean collection tube. Centrifuge at 10,000 *x g* for 1 min. Discard flow through.

6. Column washing	
	Wash the column with 700ul **Wash Buffer** and centrifuge at 10,000 x g for 1 min. Discard flow through.
7.Column drying	
	Centrifuge the column at 10,000 x g for 1 min to remove residual ethanol
8. DNA elution	
	Place the column into a clean microcentrifuge tube. Add 50ul of **Elution Buffer** directly onto the centre of the column membrane and stand for 1 min. Centrifuge at 10,000 x g for 1 min to elute DNA. Store DNA at -20C.

Analytical Digest

- This confirms whether the gene has been inserted into the backbone

	Single digest (When one enzyme used)	Double digest (When two enzymes used)
Component	Amount (ul)	Amount (ul)
dH20	2.5	1.5
Buffer 1X	1	1

DNA template	5	5
BSA	0.5	0.5
Eco RI 1	1	1
Pst I 2	0	1

- Digest mixtures incubated at 37C for 2hrs then heat inactivated at 65C for 20mins
- Then load onto 1% agarose gel