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1. 1% Agarose gel

Materials for a 100mL gel

- 1.00g of agarose
- 100mL of 1X TAE buffer
- 10µL of SYBR Safe DNA Gel Stain (ThermoFisher Scientific, no. S33102).

Steps

- Weigh agarose in an Erlenmeyer flask.
- Add 1X TAE buffer and heat up the mixture in the microwave oven so that the agarose has completely dissolved.
- Cool down the solution and add SYBR Safe DNA Gel Stain (ThermoFisher Scientific, no. S33102).
- Pour the liquid into a casting tray, place a comb (or two combs) in the tray and let the gel solidify.
- Once solidified, place the gel in a gel electrophoresis box, remove the combs and cover the gel with 1X TAE buffer.
- Load a DNA ladder (used: ThermoFisher Scientific GeneRuler DNA Ladder Mix, ready-to-use, no. SM0333) and the samples (see: sample preparation) into the wells.
- Connect the electrodes to a power supply (negative electrode is the end where the samples are loaded and positive electrode is the other end of the gel towards which the samples run).
- Run the gel electrophoresis at 120V.

Sample preparation

- Add 1µL of 6X DNA Loading Dye (used: ThermoFisher Scientific DNA Gel Loading Dye (6X), no. R0611) in 5µL of DNA sample.
- Mix by pipetting up and down, then and pipette 5µL of mix into a well.

2. *E.coli* DH5α chemically competent cells

Materials

- autoclaved $CaCl_2$ solution (ice cold)
- autoclaved $CaCl_2$ + 20% glycerol solution (ice cold)
- sterile 1,5 and 2ml Eppendorf tubes
- 50ml and 15ml Falcon tubes
- LB
- overnight cell culture

Steps

1. Grow DH5α cells in LB overnight at 37°C with shaking at 220rpm.
2. The following day, prepare Erlenmayer flask with 100ml of LB medium each.
3. Add 0,5 ml of the overnight culture each flask and let the culture grow for 2,5 hours or until OD600 is around 0.5.
4. Incubate the cultures on ice for 30min.
5. Divide cultures into 50ml Falcon tubes
6. Centrifuge at rpm4000 at 4°C for 10min.
7. Discard supernatant
8. Carefully resuspend cells in 5ml of 0,1M CaCl2.
9. Add CaCl2 to obtain volume of 25ml
10. Repeat steps 6-10 twice
11. After the final centrifugation discard supernatant
12. Resuspend cells in 1ml of ice cold 0,1M CaCl2+20% Glycerol
13. Pipette cells into Eppendorf tubes - 100µl into each
14. Store cells at -80°C

3. V. natreigens electrocompetent cells

Materials

- LB media + V2 salts
- electroporation buffer (680mM sucrose, 7,0mM K_2HPO_4 , pH=7) - sterile filtered a day before
- overnight culture of *V. natriegens*
- tubes chilled in dry ice
- Two 250ml Erlenmayer flasks

Steps

1) Leave electroporation buffer and eppendorf tubes in the cold room. You can also leave eppendorf tubes in the dry ice.
2) Measure 100mL of LB media + V2 salts in Erlenmayer flasks
3) Add 0,5ml of overnight culture
4) Let the culture grow at 220rpm and 37°C to OD600 0.5.
5) Divide culture 50ml tubes
6) Incubate on ice for 45min.
7) Centrifuge cells at 4000rpm for 20min at 4°C.
8) Discard supernatant
9) Resuspend pellets in 5ml of electroporation buffer.
10) Combine two tubes to increase the volume to 35ml with electroporation buffer.
11) Mix by inverting a few times and centrifuge at 4000rpm for 15min at 4°C.
12) Discard supernatant
13) Resuspend cells in 5ml and then fill the to 35ml.
14) Repeat steps 11-13 three times (resuspending, fill-up, inverting, centrifuge, discarding supernatant)

15) After the final wash, carefully remove supernatant and gently resuspended cells in 0,5ml of electroporation buffer.
16) Measure OD600
17) Dilute the sample with electroporation buffer if needed to achieve OD 16.
18) Allocate cells into the chilled tubes from step 1, 80µl per tube.
19) Store cells in -80°C.

4. Digestions with DTU Biobuilders protocol

We used the protocols, provided at the BioBrick tutorial Meetup by iGEM DTU, for our digestions.

Materials

- Restriction enzymes
- Buffer
- DNA (digestion reaction should contain **100 ng** plasmid DNA)
- H20 (reaction should have a **total volume** of **10 µl**.)

Steps

1. While keeping the tubes on ice as much as possible, make the digestion reaction.

2. Incubate the tube at 37 °C for 60 min.

3. Heat inactivate the restriction enzymes by heating the tubes to 80 °C for 20 min

5. Ligations with DTU Biobuilders protocol

We used the protocols, provided at the BioBrick tutorial Meetup by iGEM DTU, for our ligations.

Materials

- Digestion reaction
- 10X T4 DNA Ligase Buffer
- T4 DNA Ligase
- H2O

Reaction should have total volume of 20 ul.

Steps

1. Make one ligation reaction in a PCR tube containing the things in table 1.
2. Incubate for 60 min at 37 °C
3. Heat inactivate the ligase by heating the reaction to 80°C for 20 min
4. Store the reaction on ice until they are used for transformation.

6. Ligation Protocol WITH T4 DNA Ligase

Please see the NEB website for supporting information on this protocol.

Materials

- 10X T4 DNA Ligase Reaction Buffer
- T4 DNA Ligase
- Vector DNA (4kb)
- Insert DNA (1kb)
- Nuclease-free water

Set up the T4 DNA Ligase Reaction

Note: T4 DNA Ligase should be added last. The table shows a ligation using a molar ratio of 1:3 vector to insert for the indicated DNA sizes. *Use NEB calculator to calculate molar ratios.*

1. Thaw the T4 DNA Ligase Buffer and resuspend at room temperature.

Tip: Alicuote the 10x buffer less concentrated so when thawing, the DTT gets soluble more easily.

2. Set up the following reaction in a microcentrifuge tube on ice:

Component	**Volume (µl)**
10X T4 DNA Ligase Buffer	
Vector DNA: 10-100 ng	
Insert DNA: ng	
Nuclease-free water	
T4 DNA Ligase	
Total	20

3. Gently mix the reaction by pipetting up and down and microfuge briefly.
4. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes. For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours.
5. Heat inactivate at 65 degrees C for 10 minutes.
6. Chill on ice and transform 1-5 µl of the reaction into 50 µl competent cells.

   Use 25 uL DH5α cells, and add 2 uL of reaction mixture.

7. Transformation of chemically competent cells

We used the protocols, provided at the BioBrick tutorial Meetup by iGEM DTU, for all our chemical transformations.

Materials

- Competent cells
- Ligation reaction
- Non-digested plasmid for positive control
- Plates with desired antibiotics
- Liquid SOB media

Steps

1. Thaw the competent cells ***while still on ice*** for 5-10 minutes.
2. Add DNA to the 3 competent cells tubes as follows:
    a. 2 µl ligation mix to one tube
    b. 1 µl A1 plasmid for positive control to another tube
    c. 1 µl H_2O for negative control to the third tube.
3. Incubate transformations on ice for 25 - 30 min
4. Heat shock at 42 °C for 45 seconds (yes, 42 °C)
5. Add 970 µl room temperature SOB
6. Add the 30 µl transformation mix to the 1,5 mL eppendorf tubes with SOB
7. Incubate at 37 °C for 1 - 2 hours in shake incubator
8. Spin down the cells in the centrifuge at 6,800 g for 3-4 min
9. From the centrifuged cell tubes, discard 800 µL of the

supernatant, and resuspend in

the last 200 µL.

11. Spread 100 µL of each transformation on plates with desired antibiotic concentration.

16. Spread additional 100 µl of negative control transformation on a LB plate 17. Incubate plates at 37 °C overnight

8. Electroporation and transformation of *Vibrio natriegens*

Materials

- Competent cells
- Plasmid DNA
- Electroporation cuvettes
- Aliquots of recovery medium (LB + v2 salts + 680 mM sucrose) preheated to 45 °C
- 1.5mL tubes
- Agar plates (LB + v2 + desired antibiotic)

Steps

1) Take a desired amount of chemically competent cells aliquotes
2) Let them thaw on ice for about 10min
3) Add 1µl of the plasmid DNA (100ng/µl) to the cells and gently mix them, while keeping them on ice all the time
4) Transfer the DNA suspension to a chilled electroporation cuvette (we recommend to cool them down in freezer room before you start the experiment)
5) Electroporate the cells at 0,9kV
6) Immediately recover cells in 500 µL of preheated recovery medium and then transfer the mixture into a 1,5 ml tube
7) Incubate cells at 37 °C for 1.5 h
8) Put agar cells for preheating in the incubator at 37 °C
9) Centrifuge cells at 3000x g for 1.0
10) Remove most of the supernatant (so that you have about 50µl left)
11) Resuspend cells in the remaining media

12) Plate the mixture on the prewarmed agar plates, containing appropriate antibiotic
13) Incubate the plates at 37°C for a few hours or overnight for colonies to appear.

9. PCR with PHUSION polymerase

Full protocol can be found here: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0012393_Phusion_HighFidelity_DNAPolymerase_UG.pdf

Add items in this order described in the pipetting table below. Use the amount of template DNA described in part 4.4. of the protocol (scale according to reaction volume you're using).

Component	**20 ul reaction**	**50 ul reaction**	**Final concentration**
H20	add to 20 ul	add to 50 ul	
5X Phusion HF Buffer*	4 ul	10 ul	1 x
10 mM dNTPs	0,4 ul	1 ul	200 uM each
Forward primer**	X ul	X ul	0,5 uM (umol/L)
Reverse primer**	X ul	X ul	0,5 uM (umol/L)
Template DNA	X ul	X ul	
DMSO (optional)***	(0,6 ul)	(1,5 ul)	(3 %)
Phusion DNA polymerase	0,2 ul	0,5 ul	0,02 U/ul

4.4 Template

General guidelines for low complexity DNA (e.g. plasmid,lambda or BAC DNA) are: 1 pg–10 ng per 50 µL reaction volume. For high complexity genomic DNA, the amount of DNA template should be 50–250 ng per 50 µL reaction volume.

PCR program.

Table 2. Cycling instruction

Cycle step	2-step protocol		3-step protocol		Cycles
	Temp.	Time	Temp.	Time	
Initial Denaturation	98 °C	30 s	98 °C	30 s	1
Denaturation	98 °C	5–10 s	98 °C	5–10 s	25–35
Annealing (see 5.3)	–	–	X °C	10–30 s	
Extension (see 5.4)	72 °C	15–30 s/kb	72 °C	15–30 s/kb	
Final extension	72 °C 4 °C	5-10 min hold	72 °C 4 °C	5-10 min hold	1

10. PCR with DreamTaq Green MasterMix

Full protocol can be found here:
https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0012704_DreamTaq_Green_PCR_MasterMix_K1081_UG.pdf

Steps

1. Gently vortex and briefly centrifuge DreamTaq Green PCR Master Mix (2X) after thawing.

2. Place a thin-walled PCR tube on ice and add the following components for each 50 µL reaction:

- DreamTaq Green PCR Master Mix (2X) 25 µL
- Forward primer 0.1-1.0 µM
- Reverse primer 0.1-1.0 µM
- Template DNA 10 pg - 1 µg
- Water, nuclease-free to 50 µL

Total volume 50 µL

3. Gently vortex the samples and spin down.

4. When using a thermal cycler that does not contain a heated lid, overlay the reaction mixture with 25 µL of mineral oil.

5. Perform PCR using the recommended thermal cycling conditions outlined below:

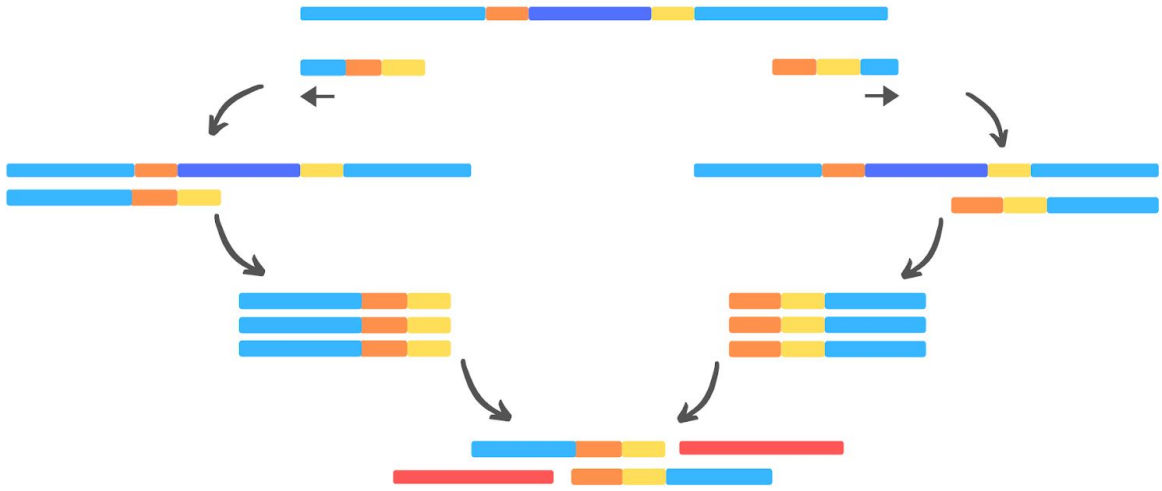
Step	Temperature, °C	Time	Number of cycles
Initial denaturation	95	1-3 min	1
Denaturation	95	30 s	25-40
Annealing	Tm-5	30 s	
Extension*	72	1 min	
Final extension	72	5-15 min	1

* The recommended extension step is 1 min for PCR products up to 2 kb. For longer products, the extension time should be prolonged by 1 min/kb

6. Load 5-15 µL of PCR mixture directly on a gel.

11. Overlap extension PCR (OE-PCR)

Overlap extension PCR (OE-PCR) is a method to scarlessly join together PCR fragments by utilizing primers containing a 5'-end overlap. This makes the fragments act as primers for each other, combining them in PCR. Normal primer design rules should be followed when designing the 5'-overlaps.



Overlap extension PCR to delete a DNA sequence from the strand

Steps after primer design:

1) Prepare PCR mixture according to the PCR protocol optimized for your primers without primers & Instead of a template, add your amplified DNA strands. Use a large volume, 1/2 - 3/4 of the total PCR reaction. Use a molar ratio of 1:1.

2) Run PCR 15 cycles, using the annealing temperature of the homologous regions

3) remove tubes from PCR & add primers. Remember to set the total Volume correctly in the PCR machine for the next step!

4) PCR 30 cycles

5) Run gel of PCR products from step 4 and extract & purify correct band

6) Transform complete vector from step 5 into your vector

7) Plate the transformed cells

8) Pick colonies & verify deletion by PCR

9) Analyse product from Step 8 in gel

12. SDS page

Aim of SDS page is to separate proteins according to their size by electrophoresis using a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins.

Materials

- Protein samples
- 6X Loading dye
- Tubes
- Mini-PROTEAN® TGX Precast Protein Gel (Bio-Rad)
- Running buffer

Steps

1) Prepare 30ul of protein samples
2) Add 10ul of loading dye into the tubes containing protein samples
3) Heat up the tubes at 90°C for 3min, using heat block.
4) Use any kD Mini-PROTEAN® TGX Precast Protein Gel (Bio-Rad)
5) Remove the tape at the bottom of the precast gel cassette and assemble the cassette into the Mini-PROTEAN Tetra cell.
6) Add running buffer to the inner and outer chambers. Make sure that you fully fill the inner chamber.
7) Remove the comb from the gel.
8) Load 10ul of each sample and 7,5ul of protein ladder into the wells.
9) Run the gel at 150V for 35-60min, until the dye reaches the reference point marked on the gel.
10) Remove the cassette from the cell and remove the gel from the cassette using opening levers.
11) Wash the gel under the water.

13. Coomassie Blue Staining

Materials

- SDS page gel
- Staining container
- Destaining buffer

Steps

1) Place the washed gel obtained in SDS page into a staining container.
2) Cover the gel with water and live it on the orbital shaker, at room temperature for 30min in order to wash off the running buffer.
3) Remove the water and add staining buffer into the container, so that it covers the gel.
4) Next place the container back on the orbital shaker, at room temperature for 1 hour.
5) Remove the staining buffer from the container, save it, since it can be reused.
6) Add destaining buffer into the container.
7) Then place the container back on the orbital shaker for 2 hours.
8) Remover the destaining buffer, collect it in the staining buffer trash.
9) Add water to the container, so it fully cover the gel.
10) Leave the gel in water on orbital shaker overnight.
11) Check the gel.

14. Western blot IgG HC or LC detection

Protocol by Mari Piirainen, Aalto University, School of Chemical Technology. Some parts have been modified for our project.

Materials

- A freshly run SDS-PAGE gel
- Nitrocellulose membrane (Bio-Rad, 0.45 µm pore size)
- Transfer buffer (SDS-PAGE running buffer with 20 % methanol)
- Blotting device (Bio-Rad Trans-Blot Turbo)
- TBS-T (TBS with 0.1 % Tween-20)
- 5 % milk (w/v) in TBS-T
- 1 % milk (w/v) in TBS-T
- Detection antibody
- ECL detection kit: Thermo Scientific SuperSignal West Pico Chemiluminescent
- Chemiluminescence imaging device

Blotting

1. Cut nitrocellulose membrane to meet the size and shape of the separation gel (entire separation gel approx. 5.5 cm x 8.5 cm). Do not touch the actual membrane with your hands, use tweezers.
2. Soak the pieces of Whatman filter paper and the nitrocellulose membrane in transfer buffer.
3. After the SDS-PAGE run, remove the concentration gel and soak the separation gel in transfer buffer for a few minutes.
4. Set up the blotting layers on the platform in the order shown in the picture below. Roll out possible air bubbles with a tube. Two gels fit in one platform of

the blotting machine. Both blotting platforms can be used simultaneously, but the amount of gels per platform needs to be the same.

5. Blot for 30 min at 1.0 A, 25 V (Program name StandardSD, constant voltage)
6. If needed, do Ponceau S staining for the blotted membrane (see separate protocol).

Blocking and detection

1. By using tweezers, move the blotted nitrocellulose membrane to a container with 12 ml of 5 % milk in TBS-T. Block the membrane at +4 ˚C on a shaker platform overnight.
2. Dilute both antibodies in 15 ml 1 % milk in TBS-T. Incubate membrane in both antibody solutions (one at a time) at RT for 60 min on a shaker platform. His antibody: 1:4000 dilution = 3 µl per 15 ml, Sigma Anti-Mouse IgG detection antibody: 1:10 000 dilution = 1.5 µl per 15 ml.
3. Rinse membrane twice with TBS-T.
4. Wash membrane by incubating it in 15-20 ml TBS-T on a shaker platform for 5 minutes. Repeat for three times in total
5. Mix equal amounts of solutions 1 and 2 from the detection kit, and dilute the mix 1:2 in TBS-T. You need in total 1-2 ml per membrane.
6. Pipette the mixed detection solution evenly on the membrane. Incubate in detection solution for 5 minutes.
7. Detect the signals using Image reader

Method: Chemiluminescence

Sensitivity: standard or high

15. qPCR standards

Quantification of plasmids with qPCR requires standards, that have a known copy number of target gene(s). The protocol here is adapted from an article by Lee *et al*. (2006). Two sets of standards are required. However, as described in the article, it is also possible to use a single plasmid as a standard if it contains both of the genes required for quantification. In that case, amplification of both standards is done using the same template but different primers.

Here a housekeeping gene (*dxs*) from *Vibrio natriegens* is cloned into TOPO® TA cloning vector (Invitrogen). *Dxs* is often used for this purpose, because it is typically present only as a single copy in the genome. TOPO® TA vector backbone contains amp and kan resistance cassettes. The same plasmid can be used as a standard for quantifying the housekeeping gene and plasmids harboring amp or kan resistance cassette. Information of copy number of both genes is required, because plasmid copy number is calculated by dividing the copy number of target gene (here *bla*/*kanR*) with the copy number of housekeeping gene (here *dxs*).

Steps

1. Isolate genomic DNA from *Vibrio natriegens*' (or bacterium of choice) genome with e.g GeneJET Genomic DNA Purification Kit (Thermo Scientific) according to manual.

2. Amplify *dxs* gene from *Vibrio natriegens*' genomic DNA (or bacterium of choice) with PCR using **Taq polymerase**. Taq polymerase allows easy cloning into TOPO® TA cloning vector. Kit used: DreamTaq Green PCR Mix (2x) (Thermo Scientific™). A **50 ul** reaction was prepared.

Primers for amplifying a fragment of *Vibrio natriegens*' *dxs* gene were kindly provided by Marburg iGEM 2018 team.

dxs_Vibrio_F: 5'-GTT TTC AGC CGC TTC TAA CG-3'
dxs_Vibrio_R: 5'-TCG GTA AAG GCC GAA TAG TG-3'

Melting T of primers (Tm = 4 x (G+C) + 2 x (A+T)): +60 °C for both primers
PCR program according to kit manual. Amplicon size: **92 bp.**

3. Check product size on 1% agarose gel. Load all PCR product. Cut and purify the correct size band (92 bp). Kit used for purification: NucleoSpin® Gel and PCR clean-up (Macherey-Nagel).

4. Measure concentration with e.g. NanoDrop.

5. Clone gel purified *dxs* PCR product into TOPO® TA cloning vector (Invitrogen) according to protocol. Reaction time may be extended to 30 min to increase cloning efficiency. In our experiments, 4 ul of purified PCR product was used (c= 57,8 ng/uL).

6. Transform the plasmid into chemically competent **DH5a.** Electroporation can also be used with electrocompetent cells. After heat shock, recover cells in SOB/SOC medium. Plate 10 and 100 ul on LB + amp (100 ug/ml) plates. Xgal can be used for blue/white screening of colonies containing the plasmid with insert. However, cloning efficiency with TOPO TA® is high, so correct transformants can also be screened with PCR using the same primers. Grow plates overnight at +37 C.

7. Inoculate 10 ml tubes with liquid LB + amp (100 ug/ml) medium with individual colonies from the transformation plates. Grow overnight at +37 C with 250 rpm shaking.
8. Purify plasmids from o/n cultures. Kit used: GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific). Elution volume= 50 ul.

9. Do PCR again using the purified TOPO plasmid + *dxs* insert as template. Same primers as earlier. This is to check that the insert is actually in the plasmid. Check product on 1 % agarose gel.

10. To remove genomic DNA or other carryover, load approximately **40 ul** of purified TOPO + *dxs* plasmid on 1 % agarose gel. Purify the fragment from gel.

11. Measure concentration with Qubit if possible.

12. Calculate copy number (copies/ul) of *dxs* from DNA concentration. See article (Lee *et al*. 2006) for equation how to calculate this.

13. Sequence TOPO + dxs plasmid with dxs_Vibrio_F: 5'-GTT TTC AGC CGC TTC TAA CG-3' and dxs_Vibrio_R: 5'-TCG GTA AAG GCC GAA TAG TG-3' primers.

14. Prepare a dilution series ranging from approximately 10^8-10^1 copies/ul of TOPO + *dxs* plasmid. These will be used as standards for qPCR. Adequate standards on a PCR strip in order, so that templates can be pipetted later using a multichannel pipette. Store at -20 C until use. Avoid freeze/thaw of standards and keep them at ice all times.

Reference:

Lee C., Kim J., Gu Shin S. & Hwang S. (2006). Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. Journal of Biotechnology (123): 273–280.

16. qPCR templates for plasmid copy number quantification

Template DNA for quantification of plasmid copy numbers should be total DNA extracted from bacterial cells. In literature, growth phase to which cells should be grown is either exponential or stationary. For references, see for example articles by Lee *et al.* (2006) and Tschirhart *et al.* (2019).

Steps

1. Transform bacterial cells with the plasmid you wish to quantify and plate of LB medium supplemented with antibiotics. Grow o/n until colonies appear.
2. Inoculate a starter culture (e.g. 5 ml of LB + antibiotic) with a single colony. Grow o/n.
3. Refresh approximately 300 ul of o/n starter culture in 5 ml of fresh LB + antibiotic supplemented medium. Prepare three replicates.
4. Grow cells to stationary phase (this seems to be a more common practice).
5. Extract total DNA using a kit (e.g. innuPREP Bacteria DNA Kit by Analytik Jena) or with a method described by Tschirhart *et al.* (2019).
6. Measure DNA concentration. Total DNA extracts can be normalized to for example 2 ug/ml as described by Lee *et al*. (2006). Or, templates can be diluted 1:100 as described by Tschirhart *et al.* (2019). In our experiment, 1:100 dilutions were used.
7. Store samples at -20 C until use.

References

Lee C., Kim J., Gu Shin S. & Hwang S. (2006). Absolute and relative QPCR quantification of plasmid copy number in Escherichia coli. Journal of Biotechnology (123): 273–280.

Tschirhart T., Shukla V., Kelly E.E., Schultzhaus Z., NewRingeisen E., Erickson J.S., Wang Z., Garcia W., Curls E., Egbert R. G., Yeung E. & Vora G. J. (2019). Synthetic Biology Tools for the Fast-Growing Marine Bacterium Vibrio natriegens. ACS Synthetic Biology 8(9): 2069-2079

17. PureFrac method from "A robust fractionation method for protein subcellular localization studies in Escherichia coli" by Malberhe *et al.*, with modifications for our project.

Cell culture

1. Inoculate 10 ml of LB supplemented with the appropriate antibiotic with a single colony
2. Grow preculture at 37°C at 220 rpm.
3. The next day, inoculate 25 ml of LB supplemented with the appropriate antibiotic.
4. Grow culture at 30°C with 220 rpm until OD_{600} = 0.5
5. Induce with 25 mM L-rhamnose, and reincubate at 30°C at 220 rpm.
6. Harvest cells at 3 h post-induction.
7. Normalize by calculating the amount culture to transfer to a new 50 ml tube: V = 8 / OD_{600} ml
8. Centrifuge for 15 min at 4500 g.

From here on, all liquids and tubes on ice, centrifugations performed at 4°C, resuspensions by pipetting gently up and down.

Periplasmic extraction

1. Resuspend the pellet in 850 µl PBS and transfer to a 1.5 ml microfuge tube.
1. Centrifuge for 3 min at 20,900g and carefully discard the supernatant.
2. Resuspend the pellet in 900 µl of Buffer 1 and incubate on ice for 5 min.
3. Centrifuge for 3 min at 20,900g and carefully discard the supernatant.
4. Resuspend the pellet in 400 µl of Mg-water (by pipetting for ≈ 30 sec) and incubate on ice for 2 min.
5. Centrifuge for 3 min at 20,900g.

6. Transfer the supernatant to a new 1.5 ml microfuge tube without disturbing the pellet and store at -20°C (periplasmic fraction)

The pellet can be frozen at -20°C and stored at this point.

Cell disruption

1. Wash the cells by resuspending the pellet in 1 ml of Buffer 2.
2. Centrifuge for 3 min at 20,900g and discard the supernatant.
3. Resuspend the pellet in 750 µl of Buffer 3 and immediately proceed to the next step.
4. Sonicate with the following cycle parameters while the sample is incubated in an ice bath: 2 sec sonication – 2 sec resting, for 4 minutes, amplitude 20%.
5. Centrifuge at max g for 30 min.
6. Transfer the supernatant to a new 1.5 ml microfuge tube and store at -20°C (cytoplasmic fraction)
7. Resuspend the pellet in 1 ml of Buffer 3 and store at -20°C (insoluble fraction containing membrane fragments and potential inclusion bodies)

Recipes

Mg-water

- Ultrapure sterile water
- 1 mM $MgCl_2$
- 100 µM N-Ethylmaleimide (we don't use due to purification system's requirements)

Buffer 1

- 100 mM Tris pH 8.0
- 500 mM sucrose

- 0.5 mM EDTA pH 8.0
- 1 Protease Inhibitor Cocktail tablet (per 50 ml)
- 100 µM N-Ethylmaleimide (we don't use due to purification system's requirements)

Buffer 2

- 50 mM Tris pH 8.0
- 250 mM sucrose
- 10 mM $MgSO_4$
- 1 Protease Inhibitor Cocktail tablet (per 50 ml)
- 100 µM N-Ethylmaleimide (we don't use due to purification system's requirements)

Buffer 3

- 50 mM Tris pH 8.0
- 2.5 mM EDTA pH 8.0
- 1 Protease Inhibitor Cocktail tablet (per 50 ml)
- 100 µM N-Ethylmaleimide (we don't use due to purification system's requirements)

Reference: Malherbe, G., Humphreys, D. P., & Davé, E. (2019). A robust fractionation method for protein subcellular localization studies in Escherichia coli. *BioTechniques, 66*(4), 171-178. doi:10.2144/btn-2018-0135

18. Fluorescence expression in Microtitre

Culture cells overnight (from either plate or glycerol stock) in 2 ml of LB medium.

1. Reculture the cells in the morning in a new culture tube. Add 20 µl overnight culture to 2 ml LB medium. Culture for 3 hours.
2. Dissolve the AHL stock in DMSO.
3. Start up the Synergy equipment, and set the protocol. Make sure the chamber is pre-heated to 37˚C.
4. After 3 hours of growth, add 90 µl of fresh LB medium together with the appropriate concentrations of AHL to a 96 well plate and then add 90 µl of cell culture.
5. Measure the OD600 together with the fluorescence (for GFP 485, 528) every 10 minutes for 6 hours.
6. After the experiment, normalize the GFP expression data by the OD600 cell density, so the data will include small differences in cell growth.

Protocol kindly provided by Christopher Jonkergouw.