0. Luria-Bertani (LB) Broth

Material

- 10 g tryptone
- 10 g NaCl
- 5 g extract yeast
- 1 L distilled water
- 25 mg/mL (stock solution) chloramphenicol

Equipment

- 1 L duran bottle
- Magnetic stirrer
- Autoclave
- Analytical balance
- spoon

Procedure

- Mix the tryptone, NaCl, and extract yeast in the bottle.
- Add the distilled water and stir until homogenized.
- Autoclave for 30 minutes.
- Let it cool and add 1 µL / 1 mL of 25 mg/mL chloramphenicol or any other proper antibiotics. However, the media can be stored without antibiotic at 4^{o}C.

1. Terrific Broth (TB)

Material

- 1.2 % tryptone
- 0.5% glycerol
- 2.4% yeast extract
- 1 L distilled water
- Proper antibiotic
- Phosphate buffer (0.17 MKH_2PO_4, 0.72 M K_2HPO_4)

Equipment

- 1 L and 250 mL bottle with cap
- Magnetic stirrer
- Autoclave
- Analytical balance
- spoon

Procedure

- Add 900 mL of deionized water to 24 g of yeast extract, 12 g of tryptone, and 5 mL of glycerol.
- Shake or stir until the solutes have dissolved and sterilize by autoclaving for 20 min at 15 psi (1.05 kg/cm^2).
- In seperate bottle, dissolve 2.31 g of KH_2PO_4 and 125.4 g of K_2HPO_4, dissolve in water to a final volume of 100 mL and autoclave for 15 min at 121°C.
- Allow the solution to cool to ~60°C and add 100 mL of sterile phosphate buffer.
- Store TB at room temperature; it will keep for at least 1 year

2. LB agar

Material

- 10 g tryptone
- 10 g NaCl
- 5 g extract yeast
- 15 g agar
- 1 L distilled water
- 25 mg/mL (stock solution) chloramphenicol

Equipment

- 1 L duran bottle
- Magnetic stirrer
- Autoclave
- Analytical balance
- spoon

Procedure

- Mix the tryptone, NaCl, extract yeast and agar in the bottle.
- Add the distilled water and stir until homogenized (it will not be totally dissolved).
- Autoclave for 30 minutes.
- Wait until the temperature ±55^{o}C.
- Add 1 µL / 1 mL of 25 mg/mL chloramphenicol or any other proper antibiotics.
- Spread the agar into sterile petri dish. Store the unused LB agar at 4^{o}C.

3. Cloning with restriction enzyme

➢ Double digestion

Material

The DNA Ligase was ordered from New England Biolabs

- 2 µL of 10x reaction buffer
- Vector DNA
- Insert DNA
- Restriction enzymes, 1 µL each (for example XpeI and SpeI)
- Nuclease-free water (adjust with total volume as 50 µL)

Equipment

- 1.5 µL eppendorf tube
- Pipette
- Pipette tips
- ice and its box
- heating block
- vortex
- centrifugation

Procedure

- Mix all the materials but add the enzyme as the last in the eppendorf tube.
- vortex gently (around 3 seconds).
- spindown for 10 seconds .
- Incubate at 37ºC for 45 minutes.
- Inactivate the reaction at 80ºC for 20 minutes.

➢ Ligation

Material

The restriction endonucleases used were ordered from New England Biolabs

- 2 µL of 10x reaction buffer
- Vector DNA (its amount was calculated here)
- Insert DNA (its amount was calculated here)
- Nuclease-free water (adjust with total volume as 20 µL)
- 1 µL of T4 DNA Ligase
- Ice cube

Equipment

- 1.5 µL eppendorf tube
- Pipette
- Pipette tips
- Ice box
- heating block
- vortex
- centrifugation

Procedure

- Put all the material and mixing product on ice.
- Mix all the materials (no ice) as ordered in the eppendorf tube.
- vortex gently (around 3 seconds).
- Incubate at room temperature for 10 minutes.
- Inactivate the reaction at 65ºC for 10 minutes.

4. Cloning with Gibson Assembly

➢ Fragment amplification

Material

- 5 µL of Q5 reaction buffer
- 2.5 µL of 10 mM dNTPs
- 1.5 µL of 10 mM forward primer
- 1.5 µL of 10 mM reverse primer
- Template DNA (variable)
- 5 µL of Q5 high GC enhancer
- To 25 µL of nuclease-free water
- 0.25 µL of Q5 high-fidelity DNA polymerase

Equipment

- 1.5 µL eppendorf tube
- Pipette
- Pipette tips
- Ice and its box
- PCR tubes
- Thermo cycler

Procedure

- Mix all the materials as ordered in the eppendorf tube.
- Put in the thermocycler

PCR Condition

- Initial denaturation: 98°C for 30 seconds
- 25 - 35 cycles at 72°C for 30 seconds
- Final extension at 72°C for 2 minutes
- Hold at 4°C

➢ Assembly

Material

- 0.02-0.5 pmols of PCR fragments
- 10 µL of gibson assembly master mix (2x)
- To 20 µL of nuclease-free water

Equipment

- 1.5 µL eppendorf tube
- Pipette
- Pipette tips
- Ice and its box
- heater block

Procedure

- Mix all the materials in the eppendorf tube.
- incubate at 50°C for 15 minutes.
- Store samples at -20°C.

5. Competent cell preparation

Material

- DMSO
- 5 M PIPES pH 6.7 (adjust with KOH or HCl)
- Inoue transformation buffer chilled to 0°C
- LB media
- 1.5 mL Microtubes

Equipment

- Ice & ice bucket
- Lab Timer
- liquid nitrogen
- 42°C water bath
- 18°C incubator
- Pipettes and Tips (10µl, 20µl, 200µl recommended)
- Pipettor
- Pipettes (10 mL, 50 mL)
- Microcentrifuge

Procedure

Day 1: Growing Bacterial Cultures

- Pick a single bacterial colony (2-3 mm in diameter) from a plate that has been incubated for 16-20 hours at 37°C.
- Transfer colony into 25 mL of LB broth in 250 mL flask.
- Incubate culture of 6-8 hours at 37°C with vigorous shaking (250-300 rpm).
- Inoculate three 500 mL flasks of 100 mL LB using the below volumes of this starter culture.
- Incubate all three flasks overnight at room temperature (18-22°C) with moderate shaking (180 rpm).

Flask	**Vol. of starter culture**
1	100 uL
2	20 uL
3	10 uL

- Day 2: Harvesting Cells and Freezing Competent Cells
- Read the OD600 of all three cultures. Continue to monitor every 45 min until reading is at 0.55
- Transfer the culture vessel to ice water bath for 10 min.
- Harvest cells by centrifugation at 3900 rpm for 10 min at 4°C in 50 mL falcon tube.
- Pour off medium and dry the tube (inverted) on paper towels for 2 min (use vacuum aspirator to remove any drops of remaining medium adhering to walls of the centrifuge bottle or trapped in its neck)
- Resuspend the cells gently (by swirling) in 32 mL of ice-cold (0°C) Inoue transformation buffer.
- Harvest cells by centrifugation at 2500 g for 10 min at 4°C.

- Pour off the medium and dry the tube on paper towels for 2 min (use vacuum aspirator to remove any drops of remaining medium adhering to walls of the centrifuge bottle or trapped in its neck).
- Resuspend cells GENTLY in 8 mL of Inoue transformation buffer (0°C).
- Add 0.6 mL of DMSO (swirl to mix bacterial suspension).
- Store on ice for 10 min.
- Quickly dispense 50 uL aliquots of suspensions into chilled, sterile microcentrifuge tubes (20 mL of suspension equals 400 tubes of 50 uL).
- Snap freeze competent cells in liquid nitrogen (store stock at -80°C).
- When needed, remove tube of competent cells from freezer, use immediately.
  Source: McClean Lab Protocol "The Inoue Method for Preparation of Competent *E.Coli* "Ultra-competent" Cells", Princeton.

6. Chemically competent cell transformation
   Material
   - Resuspended DNA to be transformed
   - 10 pg/µl positive transformation control DNA (e.g. pSB1C3 w/ BBa_J04450, RFP on high-copy chloramphenicol resistant plasmid. Located in the Competent Cell Test Kit.)
   - Competent cells (50µl per sample)
   - 1.5 mL microtubes
   - SOC or LB media (950µL per sample)
   - Petri plates with LB agar and antibiotic (2 per sample)

   Equipment
   - Ice and ice bucket
   - Lab timer
   - shaker heat block
   - Incubator
   - Sterile spreader
   - Pipettes and tips (10µl, 20µl, 200µl, 1000µl recommended)
   - Microcentrifuge

Procedure

- Resuspend DNA in selected wells in the Distribution Kit with 10µl dH20. Pipet up and down several times, let sit for a few minutes. Resuspension will be red from cresol red dye.
- Label 1.5ml tubes with part name or well location. Fill lab ice bucket with ice, and pre-chill 1.5ml tubes (one tube for each transformation, including your control) in a floating foam tube rack.
- **Thaw competent cells on ice:** This may take 10-15 min for a 260µl stock. Dispose of unused competent cells. Do not refreeze unused thawed cells, as it will drastically reduce transformation efficiency.
- **Pipette 50µl of competent cells into 1.5ml tube:** 50µl in a 1.5ml tube per transformation. Tubes should be labeled, pre-chilled, and in a floating tube rack for support. Keep all tubes on ice. Don't forget a 1.5ml tube for your control.
- **Pipette 1µl of resuspended DNA into 1.5ml tube:** Pipette from well into appropriately labeled tube. Gently pipette up and down a few times. Keep all tubes on ice.
- **Pipette 1µl of control DNA into 2 ml tube:** Pipette 1µl of 10pg/µl control into your control transformation. Gently pipette up and down a few times. Keep all tubes on ice.
- **Close 1.5ml tubes, incubate on ice for 30 min:** Tubes may be gently agitated/flicked to mix solution, but return to ice immediately.
- **Heat shock tubes at 42°C for 45 sec:** 1.5ml tubes should be in a floating foam tube rack. Place in water bath to ensure the bottoms of the tubes are submerged. Timing is critical.
- **Incubate on ice for 5 min:** Return transformation tubes to ice bucket.
- **Pipette 950µl SOC media to each transformation:** SOC should be stored at 4°C, but can be warmed to room temperature before use. Check for contamination.
- **Incubate at 37°C for 1 hours, shaking at 200-300rpm**
- **Pipette 100µL of each transformation onto petri plates** Spread with sterilized spreader or glass beads immediately. This helps ensure that you will be able to pick out a single colony.
- **Spin down cells at 6800g for 3 mins and discard 800µL of the supernatant. Resuspend the cells in the remaining 100µL, and pipette each transformation onto petri plates** Spread with sterilized spreader or glass beads immediately. This increases the chance of getting colonies from lower concentration DNA samples.
- **Incubate transformations overnight (14-18 hr) at 37°C:** Incubate the plates upside down (agar side up). If incubated for too long, colonies may overgrow and the antibiotics may start to break down; un-transformed cells will begin to grow.
- **Pick single colonies:** Pick single colonies from transformations: do a colony PCR to verify part size, make glycerol stocks, grow up cell cultures and miniprep.

- **Count colonies for control transformation:** Count colonies on the 100µl control plate and calculate your competent cell efficiency. Competent cells should have an efficiency of $1.5x10^8$ to 6 x 10^8 cfu/µg DNA.

7. Competent cell test kit

   Materials
   - 70% ethanol
   - Ice
   - Competent cell aliquot(s)
   - Agar plates with chloramphenicol
   - SOC media

   Equipment
   - Paper towels
   - Lab marker / Sharpie
   - 1.5 mL microcentrifuge tubes
   - Container for ice
   - Competent Cell Test Kit
   - 42°C water bath (or hot water source and thermometer)
   - 37°C Incubators (oven and shaker)
   - Sterile glass beads or sterile cell spreader
   - Pipettor
   - Pipette tips

   Procedure
   - Clean your working area by wiping down with 70% ethanol.
   - Thaw competent cells on ice. Label one 1.5 mL microcentrifuge tubes for each transformation and then pre-chill by placing the tubes on ice.

     Do triplicates (3 each) of each concentration if possible, so you can calculate an average colony yield.
   - Spin down the DNA tubes from the Competent Cell Test Kit/Transformation Efficiency Kit to collect all of the DNA into the bottom of each tube prior to use. A quick spin of 20-30 seconds at 8,000-10,000 rpm will be sufficient. *Note:* You should resuspend the DNA in each tube with 50 µL dH2O.
   - Pipet 1 µL of DNA into each microcentrifuge tube.
   - Pipet 50 µL of competent cells into each tube. Flick the tube gently with your finger to mix.
   - Incubate on ice for 30 minutes.

Pre-heat water bath now to 42°C. Otherwise, hot water and an accurate thermometer works, too!

- Heat-shock the cells by placing into the water bath for 45 seconds (no longer than 1 min). Be careful to keep the lids of the tubes above the water level, and keep the ice close by.
- Immediately transfer the tubes back to ice, and incubate on ice for 5 minutes.
- Add 950 µL of SOC media per tube (SOC can be replaced with LB), and incubate at 37°C for 1 hour shaking at 200-300 rpm.

  Prepare the agar plates during this time: label them, and add sterile glass beads if using beads to spread the mixture.
- Pipet 100 µL from each tube onto the appropriate plate, and spread the mixture evenly across the plate. Incubate at 37°C overnight or approximately 16 hours. Position the plates with the agar side at the top, and the lid at the bottom.
- Count the number of colonies on a light field or a dark background, such as a lab bench. Use the following equation to calculate your competent cell efficiency. If you've done triplicates of each sample, use the average cell colony count in the calculation.
  a. Efficiency (in cfu/µg) = [colonies on plate (cfu) / Amount of DNA plated (ng)] x 1000 (ng/µg)
  b. *Note: The measurement "Amount of DNA plated" refers to how much DNA was plated onto each agar plate, not the total amount of DNA used per transformation. You can calculate this number using the following equation:*
  c. Amount of DNA plated (ng) = Volume DNA added (1 µL) x concentration of DNA (refer to vial, convert to ng/µL) x [volume plated (100 µL) / total reaction volume (1000 µL)]

8. Plasmid Purification of high-copy plasmid DNA from E. coli (Macherey-Nagel)

   Material
   - Buffer A1
   - Buffer A2
   - Buffer A3
   - Buffer AW
   - Buffer A4
   - Elution buffer (AE)

   Equipment
   - Eppendorf tube
   - Micro centrifugation
   - Heating block

Procedure

- Cultivate and harvest bacterial cells Use 1–5 mL of a saturated E. coli LB culture.
- Pellet cells in a standard benchtop microcentrifuge for 30 s at 11,000 x g.
- Discard the supernatant and remove as much of the liquid as possible.
- Resuspend the cell pellet completely by vertexing or pipetting up and down. Make sure no cell clumps remain before addition of Buffer A2! Attention: Check Buffer A2 for precipitated SDS prior to use. If a white precipitate is visible, warm the buffer for several minutes at 30–40°C until precipitate is dissolved completely. Mix thoroughly and cool buffer down to room temperature (18–25 °C).
- Add 250 µL Buffer A2. Mix gently by inverting the tube 6–8 times. Do not vortex to avoid shearing of genomic DNA.
- Incubate at room temperature for up to 5 min or until lysate appears clear.
- Add 300 µL Buffer A3. Mix thoroughly by inverting
- Place a NucleoSpinR Plasmid / Plasmid (NoLid) Column in a Collection Tube (2 mL) and decant the supernatant.
- Pipette a maximum of 750 µL of the supernatant onto the column. Centrifuge for 1 min at 11,000 x g. Discard flowthrough and place the NucleoSpinR Plasmid / Plasmid (NoLid) Column back into the collection tube.
- Repeat this step to load the remaining lysate.
- Load supernatant 11,000 x g, 1 min and Wash silica membrane. Recommended: If plasmid DNA is prepared from host strains containing high levels of nucleases (e.g., HB101 or strains of the JM series), it is strongly recommended performing an additional washing step with 500 µL Buffer AW, optionally preheated to 50 °C, and centrifuge for 1 min at 11,000 x g before proceeding with Buffer A4. Additional washing with Buffer AW will also increase the reading length of DNA sequencing reactions and improve the performance of critical enzymatic reactions.
- Discard flow through and place the NucleoSpinR Plasmid / Plasmid (NoLid) Column back into the empty collection tube. Optional: Add 500 µL AW 11,000 x g, 1 min
- Add 600 µL A4 11,000 x g, 1 min 6 Dry silica membrane.
- Centrifuge for 2 min at 11,000 x g and discard the collection tube. Note: Residual ethanolic wash buffer might inhibit enzymatic reactions. 11,000 x g, 2 min 7 Elute DNA.
- Place the NucleoSpinR Plasmid / Plasmid (NoLid) Column in a 1.5 mL microcentrifuge tube and add 50 µL Buffer AE. Incubate for 1 min at room temperature. Centrifuge for 1 min at 11,000 x g. Note: For more efficient elution procedures and alternative elution buffer (e.g., TE buffer or water).

9. Glycerol aliquot

   Material

   - 100% sterile glycerol
   - Sterile nuclease-free water
   - Cultivated cells

   Equipment

   - Pipette
   - 1 mL pipette tips
   - Sterile scissor
   - -80ºC freezer

   Procedure

   - Add 20% of 100% sterile glycerol of the total volume of the stock.
   - Add 80% cell culture of the total volume into the same microtube.
   - Store all the samples in -80ºC freezer.

10. PCR product purification (Macherey-Nagel)

    Material

    - Buffer NT3
    - Buffer NTI
    - Buffer NE

    Equipment

    - Eppendorf tube
    - Micro centrifugation
    - Heating block
    - NucleoSpin® Gel and PCR Clean-up Column

Procedure

- Check if Wash Buffer NT3 was prepared accordingly. Adjust DNA binding condition For very small sample volumes < 30 µL adjust the volume of the reaction mixture to 50–100 µL with water. It is not necessary to remove mineral oil. Mix 1 volume of sample with 2 volumes of Buffer NTI (e.g., mix 100 µL PCR reaction and 200 µL Buffer NTI). Note: For removal of small fragments like primer dimers dilutions of Buffer NTI can be used instead of 100 % Buffer NTI.
- Add 2 vol NTI per 1 vol sample 2 Bind DNA.
- Place a NucleoSpin® Gel and PCR Clean-up Column into a Collection Tube (2 mL) and load up to 700 µL sample.
- Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube.
- Load remaining sample if necessary and repeat the centrifugation step. Load sample 11,000 x g 30 seconds.
- Add 700 µL Buffer NT3 to the NucleoSpin® Gel and PCR Clean-up Column.
- Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube. Recommended: Repeat previous washing step to minimize chaotropic salt carry-over and improve A260/ A230 values
- Add 700 µL NT3 11,000 x g 30 s + 700 µL NT3 11,000 x g 30 s 18
- Centrifuge for 1 min at 11,000 x g to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube. Note: Residual ethanol from Buffer NT3 might inhibit enzymatic reactions. Total removal of ethanol can be achieved by incubating the columns for 2–5 min at 70 °C prior to elution.
- Place the NucleoSpin® Gel and PCR Clean-up Column into a new 1.5 mL microcentrifuge tube (not provided).
- Add 15–30 µL Buffer NE and incubate at room temperature (18–25 °C) for 1 min. Centrifuge for 1 min at 11,000 x g. Note: DNA recovery of larger fragments (> 1000 bp) can be increased by multiple elution steps with fresh buffer, heating to 70 °C and incubation for 5 min.

11. DNA extraction from agarose gel (Macherey-Nagel)

Material

- Buffer NT3
- Buffer NTI
- Buffer NE

Equipment

- Eppendorf tube
- Micro centrifugation
- Heating block
- NucleoSpin® Gel and PCR Clean-up Column

Procedure

- Minimize UV exposure time to avoid damaging the DNA. Take a clean scalpel to excise the DNA fragment from an agarose gel. Remove all excess agarose.
- Determine the weight of the gel slice and transfer it to a clean tube. For each 100 mg of agarose gel < 2 % add 200 µL Buffer NTI. For gels containing > 2 % agarose, double the volume of Buffer NTI.
- Incubate sample for 5–10 min at 50°C. Vortex the sample briefly every 2–3 min until the gel slice is completely dissolved.
- Add 200 µL NTI per 100 mg gel 50°C 5–10 min.
- Place a NucleoSpin® Gel and PCR Clean-up Column into a Collection Tube (2 mL) and load up to 700 µL sample.
- Centrifuge for 30 s at 11,000 x g. Discard flow-through and place the column back into the collection tube. Load remaining sample if necessary and repeat the centrifugation step.
- Add 700 µL Buffer NT3 to the NucleoSpin® Gel and PCR Clean-up Column. Centrifuge for 30 s at 11,000 x g.
- Discard flow-through and place the column back into the collection tube. Recommended: Repeat previous washing step to minimize chaotropic salt carry-over and low A260/A230.
- Centrifuge for 1 min at 11,000 x g to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and the collection tube. Note: Residual ethanol from Buffer NT3 might inhibit enzymatic reactions. Total removal of ethanol can be achieved by incubating the columns for 2–5 min at 70°C prior to elution.
- Place the NucleoSpin® Gel and PCR Clean-up Column into a new 1.5 mL microcentrifuge tube (not provided). Add 15–30 µL Buffer NE and incubate at room temperature (18–25 °C) for 1 min. Centrifuge for 1 min at 11,000 x g. Note: DNA recovery of larger fragments (> 1000 bp) can be increased by multiple elution steps with fresh buffer, heating to 70°C and incubation for 5 min. See section 2.6 for detailed information.

12. Colony PCR

Material

- 5 µL of Q5 reaction buffer
- 2.5 µL of 10 mM dNTPs
- 1.5 µL of 10 mM forward primer
- 1.5 µL of 10 mM reverse primer
- Template DNA (variable)
- 5 µL of Q5 high GC enhancer
- To 25 µL of nuclease-free water
- 0.25 µL of Q5 high-fidelity DNA polymerase

Equipment

- 1.5 µL eppendorf tube
- Pipette
- Sterile pipette tips
- Sterile glass tube
- Ice and ice box
- PCR tubes
- Thermo cycler

Procedure

- Wipe the bench with the alcohol
- Mix all the material for preparing master mix.
- Transfer 25 µL of master mix into PCR tube.
- Prepare 5 mL broth media + proper antibiotic in a glass tube or 15 mL falcon tube.
- Pick single colony with 10 µL pipette tips then dip it and stir at least three times (do not touch the side of the tube. Afterwards, put all the pipette tips, that was dipped in the PCR tube, into the broth media.
- Run the PCR.
- Analyze the PCR product using gel electrophoresis.
- Mark the positive result and cultivate the colony in the broth media accordingly.

PCR condition

- 95°C for 6 minutes (disrupt cells, separate DNA)
- Cycle 35 times:
  - 95°C for 30 s (melting)
  - 60°C (or whatever temperature is appropriate) for 30 s (annealing)
  - 72°C for X s (elongation)
- 72°C for 10 minutes (final elongation)
- 4°C forever
- For long amplicons, X = 1 minute + 2.5 s per 100bp
- For shorter amplicons, under ~1kb, this can be shortened judiciously.

13. Cultivation or starter culture in LB Broth

Material

- Target colony
- Proper antibiotic
- LB broth
- 70% alcohol

Equipment

- Sterile glass tube
- Inoculation loop
- Shaker incubator
- Sterile pipette tips
- Cotton cap that is covered with the aluminium

Procedure

- Wipe the bench with the 70% alcohol.
- Prepare the glass tubes and fill it with the 5 mL LB broth and such amount of proper antibiotic.
- Pick single colony with 1µL inoculation loop then dip it into the prepared media.
- Incubate for overnight at 37°C; 150 rpm. For plasmid purification purposes, the LB medium used can be up to 10 mL and make sure that OD_{600} >2.5.

14. 100 mg/mL Ampicillin stock solution

    Material

    - Ampicillin, sodium salt
    - Deionized water

    Equipment

    - Analytical balance
    - small spatula/spoon
    - weighing paper
    - 15 mL falcon tube

    Procedure

    - Weight 1 g ampicillin and dissolve in 10 mL sterile deionized water.
    - Filter the antibiotic with sterile syringe and millipore into 15 mL sterile falcon tube then store at 4^{o}C fridge
    - 100 mg/mL Ampicillin stock solution

15. 25 mg/mL Chloramphenicol stock solution

    Material

    - Ampicillin, sodium salt
    - 99.7% ethanol

    Equipment

    - Analytical balance
    - small spatula/spoon
    - weighing paper
    - 15 mL falcon tube

    Procedure

    - Weight 250 mg chloramphenicol and dissolve in 10 mL 99.7% ethanol.
    - Filter the antibiotic with sterile syringe and millipore into 15 mL sterile falcon tube then store at 4^{o}C fridge

16. VHb-GFP or VHb (in pSB1C3) expression in *Escherichia coli* BL21

Material

- VHb-GVP or VHb starter culture
- LB or TB broth
- 25 mg/mL chloramphenicol
- colonies from LB agar+chloramphenicol plate
- 0.6 M δ-aminolevulinic acid (ALA)
- 70% ethanol

Equipment

- Sterile 500 mL baffled flask
- Sterile 1 mL and 200 µL pipette tips
- 1 mL and 200 µL pipette
- Parafilm
- Sterile cotton cap covered with aluminium foil
- Shaker incubator

Procedure

- Wipe the bench with 70% ethanol and keep the bench under sterile condition.
- Transfer 100 mL of LB broth into the baffled flask (for 80% headspace).
- Add 100 µL of chloramphenicol, 33.3 µL of ALA (the have 0.2 mM of final concentration) and 1 mL of starter culture.
- Seal the cap with parafilm to avoid the air circulation through the cap.
- Incubate at 37^{o}C; 150 rpm for 26 hours

Note

For another parameter of the expression condition, it has been tried to induce CO at time zero. The CO was aerated for around 20 second. During the incubation, the incubator was set at dark condition.

17. Harvesting

Material

- Expression Culture
- Lysis buffer pH 8.5

Equipment

- Centrifuge
- Eppendorf tubes/Falcon tubes
- Analytical balance

Procedure

- Weight out equal amount of expression culture in tubes
- Centrifuge 4700 rpm 30 min 4°C or until pellet has formed (depends on what centrifuge used)
- Discard supernatant
- Add lysis buffer and resuspend
- Lyse cells by sonication
- Centrifuge (Falcon tubes)
- Analyse protein concentrations of supernatant.

18. *E. coli* lysis

Material

- Cell pellet
- lysis buffer pH 8.5

Equipment

- Sonicator
- Proper size of beaker glass
- Ice and ice box
- 1 mL pipette tips
- vortex
- 2 mL eppendorf tube
- centrifugation

Procedure

- Resuspend the cell pellet with 2 mL lysis buffer per 1 g pellet.
- Mix thoroughly either with pipette (by pipetting up and down) or vortex.

- transfer the resuspence into appropriate size of clean glass beaker. Put the beaker on the ice even during the sonication.
- Sonicate the resuspence at 40% amplitude for 1 minute. The sonicator was set for 2 second on and 3 second off.
- Repeat the sonication at least for three cycles and make sure that the probe is kept cold before starting another cycle.
- Place the sonicated cells in eppendorf tube and centrifuge for at least 10 minutes; 15000 RCF and 8^{o}C.
- Proceed further for such analysis accordingly.

19. SDS-Page

Material

- 50 µL of supernatant from the cell lysis (sample)
- lysis buffer pH 8.5
- protein ladder
- SDS-page gel
- 2x loading buffer
- Staining buffer (0.2 % coomassie, 40% methanol, HAc 10%, Mill-Q-water)
- Destaining buffer
- 1x running buffer for SDS-Page

Equipment

- Power supplies
- Electrophoresis chamber
- 1.5 mL eppendorf tube
- 200 mL pipette
- Heat block
- 200 mL pipette tips
- Centrifugation

Procedure

➢ *E. coli* lysis

- **The supernatant for the sample can be obtained from the cell lysis as mentioned on number 19 of this protocol or can be prepared as mentioned below.**
- Transfer 1 to 2 mL expressed sample into eppendorf tube
- Check OD620
  - 1 OD_{620}-unit corresponds to approximately 0.5 g dw/L, and
  - 1 g dw/L corresponds to approximately 0.5 g total protein/L

- Centrifuge at 11000 rpm for 7 minutes
- Discard the supernatant
- Resuspend the pellet with Tris-HCl buffer pH 7.5
- Put the sample in dry ice for 2 minutes and then in water for other 2 minutes
    - Repeat the previous step
- Centrifuge at 11000 rpm for 7 minutes
- Transfer the supernatant into another eppendorf tube
- Resuspend the pellet with Tris-HCl buffer pH 7.5
- The supernatant is marked as soluble and insoluble for the pellet.

➢ Sample Loading Preparation
- Mix 20-40 ug protein with loading buffer (1:1), both insoluble and soluble sample
- Boil it (around 95^{o}C for 5-7 minutes)
- Load the sample into the gel accordingly. Load the ladder as much as 5 uL.

➢ Running Sample
- Set the chamber for SDS-Page
- Prepare the gel and read the manual of the gel brand
- Load the running buffer into the chamber through the cassette holder (in the middle)
- Run the first time at 100 V for 5 minutes, then increase the voltage to 150 and run it for 20 minutes or when then blue line reaches the black line in the bottom.

➢ Staining Sample
- Soak the gel with staining solution for at least 30 minutes.
- Remove the staining solution, then replace with destaining solution. Soak it for overnight.

20A. Reagent

1. 5x Electrode Buffer

- TRIS : 75 g
- Glycine : 360 g
- SDS : 25 g

Adjust the pH to 8.3 with HCl, add Mill-Q-water to 5 L final volume

1. Loading Buffer

- TRIS 0.5 M pH 6.8 : 3.3 mL
- SDS 10% w/v : 8 mL
- Glycerol : 4 mL
- Bromophenol blue 0.2% w/v : 1 mL (200 mg in 100 mL Mill-Q-water)
- DTT (dithiotreithol) : 2.62 g

Add Mill-Q-water to 20 mL

1. Staining Solution

- Coomassie 0.2% : 1 g
- Metanol 40% : 200 mL
- HAc 10% : 50 mL
- Mill-Q-water : 260 mL

1. Destaining Solution

- HAc : 10%
- Metanol : 40%

20. VHb analysis with spectrophotometry

Material

- Lyse buffer
- CO gas
- Sodium dithionate

Lysed cells

Equipment

- Spectrophotometer “Agilent Technology Cary 60 UV-Vis”

Procedure

21. GFP analysis using flow cytometry

Material

- 5-10 mL Luria Bertani (LB) media

- Proper colony
- Proper antibiotic
- PBS pH 7.4
- Shaker incubator
- 70 % ethanol

Equipment

- Glas tubes
- 1.5 mL Eppendorf tubes
- Spectrophotometer
- Cuvettes
- Flow cytometer “BD Accuri C6 plus”
- 1 µL inoculation loop
- Cotton lid covered in aluminium foil

Procedure

- Wipe bench with 70 % ethanol and work close to the flame.
- Take one colony with the 1 µL inoculation loop and add to the 5-10 mL LB media with proper antibiotic.
- Incubate in 37 °C at 150 rpm over night in the glas tube with cotton lid covered in aluminium foil.
- Take incubated sample and put 1 mL into 1.5 mL Eppendorf tubes.
- Discard supernatant.
- Resuspend with PBS until OD=1 is obtained with.
- Run it in the flow cytometer and analyse the data.

22. Lysis buffer pH 8.5 solution

Material

- Distilled water
- 50 mL of Tris-HCl 1M; pH 9.0
- 50 mL of NaCl 1 M
- 25 mL of glucose 1 M
- 50 mL of glycerol 100%
- 2 mL of EDTA 0.5 M
- 100 µL of ascorbic acid 0.1 M
- 1 M HCl solution
- 1 M NaOH solution

Equipment

- pH meter
- calibration solution
- Volumetric pipette
- Pipet filler
- 1 L volumetric flask
- Stirrer
- Magnetic stirrer

Procedure

- Mix the tris-HCl, NaCl, glucose, glycerol, EDTA, and ascorbic acid.
- Dilute into 900 mL distilled water.
- Adjust the pH to 8.5 either with HCl or NaOH, depends on the initial pH.
- Adjust the volume into 1 L with distilled water..

23. Phosphate Buffer Saline solution

Material

- 1 L distilled water
- 8 g of NaCl
- 0.2 g of KCl
- 1.44 g of Na_2HPO4
- 0.24 g of KH_2PO4
- HCl solution

Equipment

- pH meter
- Analytical balance
- spoon
- weighing paper
- 1 L volumetric flask
- stirrer
- magnetic stirrer

Procedure

- Weight the chemicals as much as mentioned above and transfer into the volumetric flask
- Add 800 mL distilled water.
- Adjust the pH to 7.4 with HCl.
- Add distilled water to a total volume of 1 liter.

24. Human hemoglobin expressions

Material

- 5 mL Luria Bertani (LB) media
- 500 mL LB media
- Proper colony
- Proper antibiotic
- 70 % ethanol
- Incubator with shaking function
- CO gas
- SDS-PAGE-gel
- 0.6 M δ-aminolevulinic acid (ALA)
- 0.1 mM IPTG

Equipment

- 1 µL inoculation loop
- Glas tube
- 2 L cultivation flask
- Incubator with shaker function
- Cotton lids covered in aluminium foil

Procedure

- Take one good colony and add to 5 mL LB media containing proper antibiotic in the glas tube to make the starter culture with the 1 µL inoculation loop and put in the incubator over night.
- Inoculate the 500 mL LB in the 2 L flask with the 5 mL starter culture and incubate in 37 °C at 150 rpm until OD>2. Induce with IPTG and ALA and incubate at 28 °C for 14 h.
- Harvest protein.

24. Up-scale fermentation protocol is found here