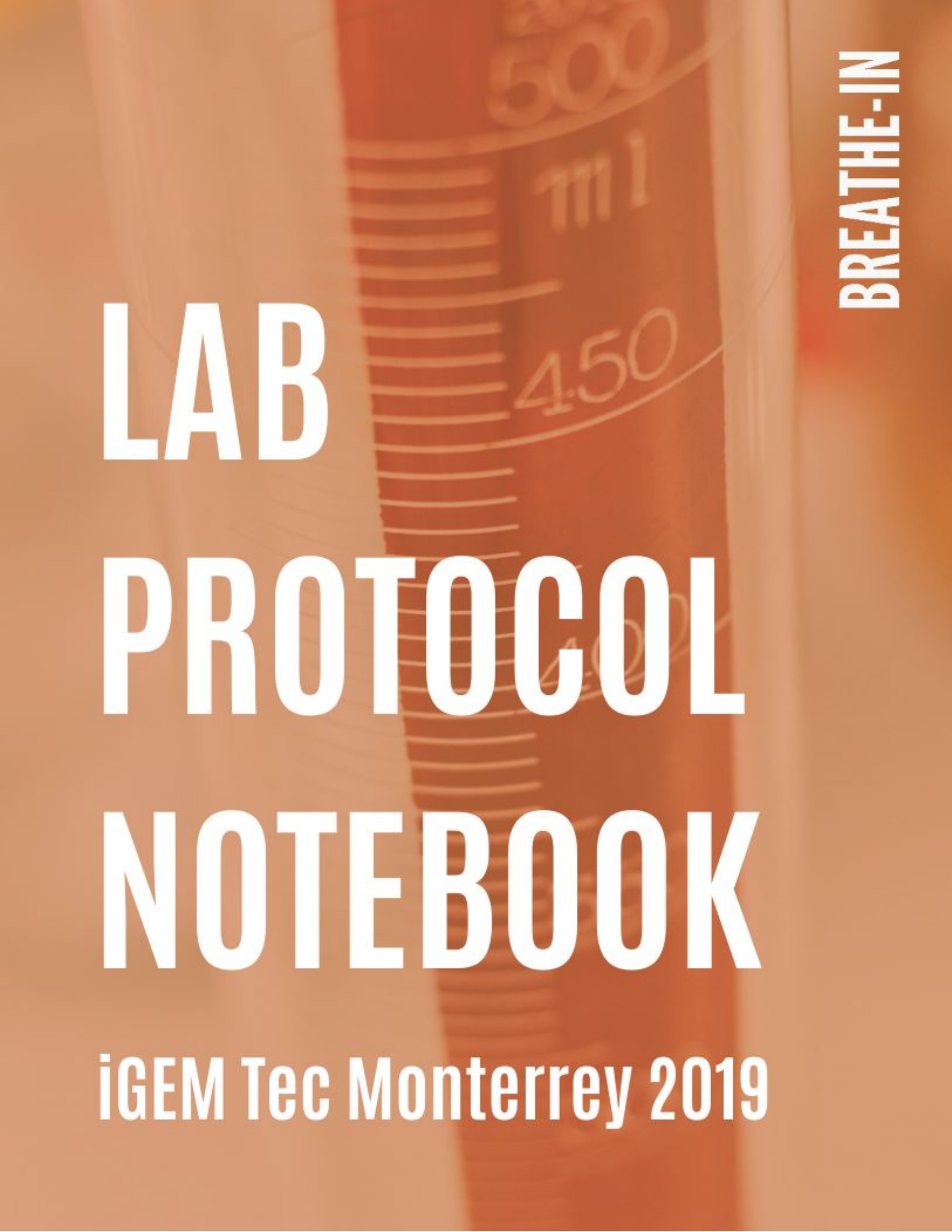

BREATHE-IN

LAB PROTOCOL NOTEBOOK

iGEM Tec Monterrey 2019

Index

PCR amplification

Materials:

- ❏ PCR tubes (200 µL)
- ❏ Milli-Q water
- ❏ NEB Master MIx
- ❏ Forward and reverse primers (10 uM)
- ❏ DNA template

Equipment:

- ❏ Thermocycler
- ❏ Ice bucket

Protocol:

1. Firstly, the primers annealing temperature was determined using the NEB online calculator tool. But throughout the protocol this temperature was changed to suit better our parts and reach maximum efficiency.
2. Add the compounds in the following order in a PCR tube:

Compound	50 µL Reaction
Milli-Q water	18 µL
Forward Primer	2.5 µL
Reverse Primer	2.5 µL
DNA Template	2 µL
NEB Master Mix (Keep it on Ice)	25 µL

3. Gently mix the PCR reactions and transfer the tubes to a thermocycler.
4. Thermocycling Configuration for our parts:

Step	Temperature	Time
Initial denaturalization	98 °C	3 min
34 cycles	98 °C 68 °C (Aligning) 72 °C	30 secs 30 secs 40 secs
Final extension	72 °C	3min
Hold	4 °C	∞

PCR clean-up (Purification)

*This protocol was adapted from the Quick-Start Protocol: QIAquick® PCR Purification Kit-QIAquick® PCR & Gel Cleanup Kit

Materials

- ❏ QIAquick® PCR Purification Kit
- ❏ DNA PCR products (50 µl)
- ❏ 1.5mL Eppendorf tubes
- ❏ QIAquick column with collection tube

Equipment

- ❏ Microcentrifuge (all centrifugation steps are carried out at 13,000 rpm)

Protocol for a 50 µl PCR reaction:

1. Add 250 µl of Buffer PB, 50 µl of DNA PCR products and 1 µl of pH indicator to a 1.5 ml Eppendorf tube and mix well.
2. The mixture should be color yellow: this indicates a pH ≤7.5 in which the adsorption of DNA to the membrane is efficient. If the color is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix.
3. To bind DNA, apply the mixture to the QIAquick column with collection tube and centrifuge for 30–60 s. Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 750µl Buffer PE to the QIAquick column and centrifuge for 30–60 s. Discard flow-through and place the QIAquick column back into the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml Eppendorf tube.
7. To elute DNA, add 50 µl Buffer EB to the center of the QIAquick membrane and centrifuge the column for 1 min.

Digestion with restriction enzymes

Materials:

- ❏ DNA samples
- ❏ Restriction enzymes:
  - ❏ EcoRI, SpeI, PstI, XbaI, BsaI, BsmBI, Esp3I
- ❏ NEB Enzyme Buffer (Depends on the efficiency of enzymes)
- ❏ Nuclease-free water
- ❏ PCR tubes (0.2 ml)

Equipment

- ❏ Thermocycler
- ❏ Ice bucket

Protocol:

5. Measure the nucleic acid concentration with an analytical instrument, such as a microvolume spectrophotometer (Nanodrop).
6. Add the compounds in the following order in a PCR tube:

Compound	50 µl Reaction
Nuclease-free Water	to 50 µl
NEB Enzyme Buffer	5 µl
DNA sample	2 µg
Restriction enzyme	1 µl

7. Incubate at 37˚C for 1 hour.
8. Inactivate the enzyme at 65°C or 80°C (depending on enzyme) for 20 mins.

***Notes:**

- The Enzymes and Buffer should be thawed on ice and kept in the ice bucket .
- Pro Tip: In case of using two enzymes add the first one and incubate for 1 hour, then add the other enzyme and incubate another 1 hour. Lastly, inactivate the enzymes at the proper temperature.

Agarose gel electrophoresis

Materials:

- ❏ Agarose Powder Benchmark
- ❏ TAE Buffer Accuris
- ❏ SmartGlow DNA stain 5X
- ❏ DNA sample
- ❏ 1 kb DNA Ladder NEB

Equipment:

- ❏ Benchmark InstaView Electrophoresis system with LED Transilluminator

Protocol:

1. Prepare a 1% agarose gel by mixing 0.25 gr of agarose powder with 25 mL TAE buffer and blend until mixture is clear (if necessary heat in the microwave in 15 seconds intervals)
2. Pour the agarose into the electrophoresis tray and insert the comb with the desire wells in place, avoiding bubbles.
3. After the gel to solidify remove the comb.
4. Put the gel in the electrophoresis chamber with 200 mL of TAE Buffer.
5. Mix 5 parts of SmartGlow Stain (15 µl) with one part of DNA sample (3 µl) and carefully add this to each well.
    a. Remember to add the 1 kb DNA Ladder with the SmartGlow Stain to compare the sizes of the bands.
6. Run the gel 90 mins at 100 V.
7. Visualize the DNA fragments in the LED transilluminator in the Instaview System.

SDS-Page gel electrophoresis

Materials:

- ❏ Stacking solution
- ❏ Resolving solution
- ❏ Buffer 1X Tris-Glycine
- ❏ Type II Purified Water
- ❏ Ethanol
- ❏ Electrophoresis chamber

Gel preparation protocol:

1. Prepare the separation gel (10%). Mix in the following order:*After adding TEMED and APS to the SDS-PAGE separation gel solution, the gel will polymerize quickly, so add these two reagents when ready to pour.*
2. Pour gel, leaving ~2 cm below the bottom of the comb for the stacking gel. Make sure to remove bubbles.
3. Layer the top of the gel with isopropanol. This will help to remove bubbles at the top of the gel and will also keep the polymerized gel from drying out.
4. Remove the isopropanol and wash out the remaining traces of isopropanol with distilled water.
5. Prepare the stacking gel (4%). Mix in the following order:
6. Pour stacking gel on top of the separation gel.
7. Add combs to make wells. In ~30 min, the stacking gel should become completely polymerized.
8. Clamp gel into apparatus, and fill both buffer chambers with gel running buffer according to the instructions for the specific apparatus.
9. Load samples and molecular mass protein markers into wells for separation by electrophoresis.

Gel running protocol:

1. Place gel into the tank and remove the comb.
2. Fill inner chamber to the top with 1X running buffer and outer chamber to the half.
3. Heat the tubes with samples at 95˚C for 10 minutes.
4. Pipette ___ uL of your sample (que más le echamos??) and ___ uL of the ladder in the wells.
5. Let run at 40V for approximately 45 minutes or until the blue line almost reaches the bottom of the gel.

6. Increase the voltage to 120V and let it run fr 1-2 hrs.
7. When electrophoresis has finished, remove gel from glass plates.
8. Wash with type II water and leave on orbital shaker for 10 minutes inside a small container with distilled water to ⅔. Do this 3 times.
9. Add solution of Coomassie blue, acetic acid and ethanol to the half of the container.
10. Leave shaking overnight.
11. When gel color background is optimal, rinse with type II water.

DNA gel extraction

*This protocol was adapted from Promega's Wizard® SV Gel and PCR Clean-Up System

Materials

- ❏ Wizard® SV Gel and PCR Clean-Up System Kit
- ❏ Electrophoresis gel
- ❏ Resuspended DNA to be transformed
- ❏ 1.5mL Eppendorf tubes
- ❏ SV Minicolumn with Collection Tube
- ❏ Free-nuclease water

Equipment

- ❏ 60˚C water bath
- ❏ Microcentrifuge

Protocol

1. Excise DNA band from gel and place the gel slice in a 1.5ml eppendorf tube.
2. Add 10µl Membrane Binding Solution per 10mg of gel slice. Vortex and incubate at 50–65˚C until gel slice is completely dissolved.
3. Insert SV Minicolumn into Collection Tube.
4. Transfer dissolved gel mixture to the Minicolumn assembly. Incubate at room temperature for 1 minute.
5. Centrifuge at 16,000 × g for 1 minute. Discard flow through and reinsert Minicolumn into Collection Tube.
6. Add 700µl Membrane Wash Solution. Centrifuge at 16,000 × g for 1 minute and discard flow through and reinsert Minicolumn into Collection Tube.
7. Repeat Step 6 with 500µl Membrane Wash Solution. Centrifuge at 16,000 × g for 5 minutes and discard flow through.
8. Centrifuge the column assembly for 1 minute with the microcentrifuge lid open to allow evaporation of any residual ethanol.
9. Carefully transfer Minicolumn to a clean 1.5ml eppendorf tube.
10. Add 50µl of Nuclease-Free Water to the Minicolumn. Incubate at room temperature for 1 minute. Centrifuge at 16,000 × g for 1 minute.
11. Discard Minicolumn and store DNA at 4˚C.

Ligation

Materials:

- ❏ DNA samples (vector and insert)
- ❏ T4 DNA Ligase NEB
- ❏ T4 DNA Ligation buffer NEB
- ❏ Nuclease-free water
- ❏ PCR tubes (0.2 mL)
- ❏ Ice bucket

Equipment

- ❏ Thermocycler

Protocol:

1. Optimise the molar ratio for insert to vector (we used 1:5) using NEB calculator specific to the construct.
2. Add the compounds in the following order in a PCR tube:

Compound	20 µl Reaction
Nuclease-free water	Up to 20 µl
T4 DNA Ligase Buffer (10X) NEB	2 µl
Vector backbone	50 ng
Insert DNA	37.5 ng
T4 DNA Ligase (Keep it on Ice)	1 µl

3. Incubate at 4˚C overnight or 8-10 hours.
4. Heat inactivate at 80˚C for 20 minutes

***Notes:**

- The T4 DNA Ligase and Buffer should be thawed on ice.
- We recommend to aliquot small volumes of the Ligase Buffer in PCR tubes because it contains ATP which deactivates on exposure to heat.

Golden

For Golden Gate cloning we used the protocols provided by Boston University iGEM 2013 team:
http://2013.igem.org/wiki/images/6/61/MoClo_Level_0.xls
and http://2013.igem.org/wiki/images/4/4d/MoClo_Level_1_and_2.xls.
Once the last procedure is done, it's recommended that the products of this reaction are confirmed by blue-white screening. The protocol we used for this is provided by GoldBio:
https://www.goldbio.com/documents/1031/Blue%20White%20Screening%20of%20Bacterial%20Colonies%20using%20X-Gal%20and%20IPTG%20Plates.pdf

Competent Cells (*E. Coli*)

Electro (stbl4)

Material

- ❏ Sterile dH2O
- ❏ 50 mL LB
- ❏ Centrifuge Falcon tubes (50 mL)
- ❏ Overnight *E. coli* culture
- ❏ 20% glycerol
- ❏ PCR tubes (260 µl)

Equipment & Apparatus

- ❏ Centrifuge
- ❏ Spectrometer
- ❏ Incubator with shaker 37°C

Protocol

1. Inoculate 50 mL of LB with 10 µl of bacteria, put this in two falcons with 25 mL each.
2. Bacteria were grown at 37°C in a shaker (250 rpm) overnight.
3. In the spectrometer measure the OD600, when it reaches 0.3 chill the falcons on ice for 30 minutes.
4. Centrifuge at 3,000 rpm for 10 min at 4°C and discard the supernatant.
5. Wash the cells 3 times with one volume of cold sterile dH2O.
6. Wash the cells with cold 20% glycerol.
7. Distribute the cells in cold PCR tubes with 200 µl aliquotes.
8. Store competent cells at -80°C freezer.

Calcium (dh5alpha and BL21)

Materials

- ❏ SOB
- ❏ CCMB80 buffer
    - ❏ 10 mM KOAc pH 7.0 (10 ml of a 1M stock/L)
    - ❏ 80 mM CaCl2.2H2O (11.8 g/L)
    - ❏ 20 mM MnCl2.4H2O (4.0 g/L)
    - ❏ 10 mM MgCl2.6H2O (2.0 g/L)
    - ❏ 10% glycerol (100 ml/L)

- ❏ adjust pH DOWN to 6.4 with 0.1N HCl if necessary
    - ❏ adjusting pH up will precipitate manganese dioxide from Mn containing solutions.
- ❏ sterile filter and store at 4˚C

❏ Detergent-free, sterile glassware and plasticware

❏ OD600nm spectrophotometer

Method

1. Ethanol treat all working areas for sterility.
2. Inoculate 250 ml of SOB medium with 1 ml vial of seed stock and grow at 20˚C to an OD600nm of 0.3. This takes approximately 16 hours. Room temperature will work. You can adjust this temperature somewhat to fit your schedule. **Aim for lower, not higher OD if you can't hit this mark.**
3. Fill an ice bucket halfway with ice. Use the ice to pre-chill as many centrifuge bottles as needed.
4. Transfer the culture to the centrifuge tubes. Weigh and balance the tubes using a scale.
5. Centrifuge at 3000g at 4˚C for 10 minutes.
6. Decant supernatant into waste receptacle..
7. Gently resuspend in 80 ml of ice cold CCMB80 buffer. Pro tip: add 40ml first to resuspend the cells. When cells are in suspension, add another 40ml CCMB80 buffer for a total of 80ml. Pipet buffer against the wall of the centrifuge bottle to resuspend cells. Do not pipet directly into cell pellet!
8. After pipetting, there will still be some residual cells stuck to the bottom. Swirl the bottles gently to resuspend these remaining cells.
9. Incubate on ice for 20 minutes.
10. Centrifuge again at 3000G at 4˚C. Decant supernatant into waste receptacle, and bleach before pouring down the drain.
11. Resuspend cell pellet in 10 ml of ice cold CCMB80 buffer.
12. If using multiple centrifuge bottles, combine the cells post-resuspension.
13. Incubate on ice for 20 minutes. Prepare for aliquoting.
14. Make labels for aliquots. Prepare dry ice in a separate ice bucket. Pre-chill tubes/plates on dry ice.
15. Aliquot into chilled tubes or into chilled microtiter plates
16. Store at -80˚C indefinitely.
17. Flash freezing does not appear to be necessary
18. Perform test transformations to calculate your competent cell efficiency

19. Thawing and refreezing partially used cell aliquots dramatically reduces transformation efficiency by about 3x the first time, and about 6x total after several freeze/thaw cycles.

https://parts.igem.org/Help:Protocols/Competent_Cells

Transformation protocol (*E. Coli*)

Heat shock

Materials

- ❏ Resuspended DNA to be transformed
- ❏ 10pg/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)
- ❏ 1.5mL Microtubes
- ❏ SOC Media (950µL per sample)
- ❏ Petri plates w/ LB agar and antibiotic (2 per sample)

Equipment

- ❏ Ice bucket
- ❏ 42˚C water bath
- ❏ 37˚C incubator
- ❏ Incubator with shaker 37°C
- ❏ Sterile spreader or glass beads
- ❏ Pipettes and Tips (10µl, 200µl, 1000µl recommended)
- ❏ Microcentrifuge

Preparations

- ❏ Agar + LB Petri plates
- ❏ SOC Media
- ❏ Water bath at 42˚C

Protocol

1. Add 50 uL of competent cells (from stock) to a 1.5 mL tube, keeping it in ice at all times.
2. Pipette 5 uL of DNA into the same tube and let sit in ice for 30 minutes.
    a. Positive control: add 5 uL of competent cell test
3. Heat shock tubes at 42˚C for 45 seconds
4. Thaw tubes on ice for 5 minutes.
5. Add 950 uL of SOC media to each tube.
6. Incubate at 37˚C for 1 hr, shaking at ___ rpm.
7. Centrifuge for 3 min at 14,000 rpm.
8. Discard 800 uL of supernatant and resuspend cells.

9. Pipette 100 uL onto petri plates and spread with sterile glass beads or spreader.
10. Incubate overnight at 37˚C.

https://parts.igem.org/Help:Protocols/Transformation

*Electroporation

Materials:

- ❏ Electrocompetent cells
- ❏ Positive transformation control DNA (Competent Cell Test Kit)
- ❏ SOC medium
- ❏ 1.5mL Eppendorf tubes
- ❏ Electroporation cuvettes
- ❏ Petri plates with LB+Agar and antibiotic

Equipment

- ❏ Ice bucket
- ❏ Electroporator
- ❏ 37˚C incubator
- ❏ Incubator 37°C with shaker
- ❏ Sterile spreader or glass beads

Protocol

1. Add 50 uL of electrocompetent cells (from stock) to a 1.5 mL tube, keeping it in ice at all times.
2. Pipette 5 uL of DNA into the same tube and let sit in ice for 30 minutes.
    a. Positive control: Add 5 uL of competent cell test
3. Introduce the 55 uL of cells with DNA in a electroporation cuvette (previously chilled).
    a. Negative Control: Add 50 uL of electrocompetent cells without DNA in a cuvette.
4. Insert the cuvette in the electroporator and choose the appropriate protocol:
    a. Bacterial: 2.5 kV for 1 pulse
5. Add 950 uL of SOC media to each tube.
6. Incubate at 37˚C for 1 hr, shaking at 250 rpm.
7. Centrifuge for 3 min at 14,000 rpm.
8. Discard 800 uL of supernatant and resuspend cells.
9. Pipette 100 uL onto petri plates and spread with sterile glass beads or spreader.
10. Incubate overnight (12-16 hours) at 37˚C.

Competence and transformation (*Bacillus subtilis*)

Materials (one sample):

- ❏ Petri plates with *Bacillus subtilis* seed stock
- ❏ 2 Sterile petri plates
- ❏ 37˚C incubator with shaker
- ❏ 3 Sterile glass test tubes with cap
- ❏ *Bacillus subtilis* medium 1:
  - ❏ Bacillus salts 10X (250.0 µL)
  - ❏ Glucose 20% (62.5 µL)
  - ❏ MgSO4 1M 12.5 µL
  - ❏ Casamino acids 20% (2.5 µL)
  - ❏ Tryptophan 10 mg/mL (31.2 µL)
  - ❏ Histidine 10 mg/mL (31.2 µL)
  - ❏ Glycine 10 mg/mL (31.2 µL)
  - ❏ Sterile H2O (2079.0 µL)
- ❏ *Bacillus subtilis* medium 2:
  - ❏ Bacillus salts 10X (250.0 µL)
  - ❏ Glucose 20% (62.5 µL)
  - ❏ MgSO4 1M 12.5 µL
  - ❏ Casamino acids 20% (1.25 µL)
  - ❏ Tryptophan 10 mg/mL (3.1 µL)
  - ❏ Histidine 10 mg/mL (3.1 µL)
  - ❏ Glycine 10 mg/mL (3.1 µL)
  - ❏ Sterile H2O (2165.0.0 µL)

Method:

1. Pick 4 to 6 colonies from the petri plate and resuspend into one of the test tube containing 2.5 mL of the first medium.
2. Incubate at 37˚C and 300 rpm for 5 hours.
3. Make a dilution 1:10 of the culture with the medium number two (250 µL culture + 2.25 mL of medium)
4. Pass 1 mL to one tube (control) and 1 to another. Add 10 µL of ADN to the last one.
5. Incubate at 37˚C and 300 rpm for 1.5 hours.
6. Prepare 2 petri plates with agar LB and antibiotic according to its requirements.
7. Pipette 100 uL of the sample without DNA into a petri plate (control) and 100 uL of the transformated sample into another one and spread with sterile glass beads or spreader.
8. Incubate at 37˚C for 24 hours.

Glycerol stocks

Materials

- ❏ Glycerol
- ❏ Distilled water
- ❏ Tube with screw top

Protocol

1. Dilute pure glycerol with water to obtain a 50% glycerol solution.
2. Add glycerol solution to a screw top tube.

   Note: the proportion between glycerol solution and liquid bacterial culture should be 1:1.
3. Pipette a proportional amount of liquid bacterial culture to the tube.
4. Store at -80˚C.

Plasmidic DNA extraction (Miniprep)

Materials:

- ❏ Cell culture in LB medium
- ❏ 2 Eppendorf tubes
- ❏ Micropipette
- ❏ Miniprep Solutions
- ❏ Microcentrifuge
- ❏ Isopropanol
- ❏ Etanol 70%
- ❏ Nuclease free water
- ❏ RNase

Protocol:

1. Add 1 mL of your cell culture to an 1.5 mL microcentrifuge tube, centrifuge at 14,000 rpm for 30 seconds and discard supernatant, keeping pellet. (Optional: Repeat until there's no more culture left)
2. Add 200 mL of cell resuspension solution (_____) and resuspend pellet using a micropipette or vortex.
3. Keep in room temperature for 5-10 min.
4. Add 200 mL of neutralization solution and mix by inversion of the tube.
5. Keep in room temperature for 5-10 min.
6. Add 200 mL of Cell lysis solution and gently invert the tube a few times.
7. Keep tube in ice for 10 min.
8. Centrifuge tube at 14,000 rpm for 5 min.
9. Meanwhile add 1 mL of isopropanol to a new tube and label.
10. Add supernatant to new tube with isopropanol .
11. Incubate at -20˚C for approximately 1 hour. (Minimum time: 10 min, maximum time: 2 hr)
12. Spin down at 14,000 rpm for 10 min and discard supernatant.
13. Add 200 mL etanol 70% and resuspend pellet.
14. Centrifuge again at 14,000 rpm for 5 min and discard supernatant carefully.
15. Dry out pellet for 5 min in and incubator at 37˚C.
16. Add 20 uL of nuclease free water with RNase (10 ng/uL) and resuspend.
17. Run gel or store at 4˚C.

Protein induction assay

Materials:

- ❏ Cell culture in LB medium
- ❏ IPTG 20mM stock
- ❏ 50 ml conical tubes
- ❏ 1l sterile matraz
- ❏ LB media

Equipment

- ❏ Incubator with shaker
- ❏ OD600nm spectrophotometer

Protocol:

Take one fresh colony of the desired organism and inoculate it in 5-10 ml lb media in a 50 ml conical tube.

Grow it at 30˚c O/N.

Make a 1:50 dilution on fresh media at your desired volume and grow at 37˚c until it reaches OD600 of 0.6

Induce with the desired IPTG concentration and grow it at 37˚c.

Cells were then centrifuged at 4400 RPM during 10 minutes three times. If storing was needed the the pellets were stored at -20˚c and supernatants at 4˚c.

Protein purification assay

The cellular proteins were extracted according to the rapid isolation method used by Wu et al (2009).

Materials:

- ❏ Buffer A
- ❏ Buffer B
- ❏ Ni-NTA Superflow columns
- ❏ Imidazole
- ❏ Ice

Equipment:

- ❏ Centrifuge(Thermo)
- ❏ Sonicator

Protocol:

1. The frozen cell pellet was thawed and resuspended in Buffer A (50 mM Tris–Base [pH 8.0], 150 mM NaCl, 10% [v/v] glycerol, 20 mM imidazole).
2. Then, the cells were lysed by sonication on ice and the lysate was exposed to pulses for 10 seconds, repeating themselves every 40 seconds, with an amplitude of 60%. The process was repeated for 75 minutes.
3. The retained supernatant was loaded onto a Ni-NTA Superflow column which was pre-equilibrated with Buffer A.
4. After loading, the Ni-NTA column was washed with Buffer A with 40 mM imidazole.
5. The column was equilibrated again with Buffer A and then eluted with Buffer B (50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 10% [v/v] glycerol, 500 mM imidazole).
6. The samples were then analysed using SDS-PAGE.

Fluorescence kinetic curve

1. Take one fresh colony and inoculate it in 5-10 ml lb media in a 50 ml conical tube.
2. Grow it at 30˚c O/N.
3. Make a 1:50 dilution on fresh media at your desired volume and grow at 37˚c until it reaches OD600 of 0.6
4. Make a 1:2 dilution on fresh media with the different iptg concentrations and the different controls to a 100µl final volume in 96 wells plate.
5. Measure the fluorescence in a microplate reader at the different times.
6. Grow at 30˚c between times in a shaker incubator. .

Bradford

*This protocol was adapted from Sigma Aldrich Bradford Reagent Technical Bulletin

Materials:

- ❏ Bradford reagent
- ❏ Ni-NTA 150 mM Imidazole Elution buffer
- ❏ Bovine Serum Albumin (BSA)
- ❏ 1.5 ml Eppendorf tubes
- ❏ PCR tubes (200 µl)
- ❏ 96 well Plate

Equipment:

- ❏ Microtiter

Protocol

*It is necessary to do duplicates or triplicates for each sample, in this protocol we did duplicates of all protein standards and unknown protein samples .

1. In PCR tubes prepare the protein standards of BSA diluted in Elution buffer with concentrations: 0, 125, 250, 500, 1000, 1500 and 2000 µg/ml.
2. Prepare the unknown sample(s) with an approximate concentration between 100–2000 µg/ml: we used a sample diluted 10 µl of unknown protein in 90 µl of Elution Buffer.
3. Add 250 µl of Bradford reagent to each well you will use, except for the blank wells.
4. Add 5 µl of the protein standards, 5 µl of unknown samples and 5 µl of diluted of unknown protein (1/10) to separate wells in the 96 well plate and mix well with Bradford reagent. Remember to make duplicates.
5. Add 5 µl of only Elution buffer to the blank wells, triplicate.
6. Let the samples incubate at room temperature for 5–10 minutes.
7. Measure the absorbance at 595 nm in the microtiter.
8. Plot the net absorbance vs. the protein concentration of each standard.
9. Determine the protein concentration of the unknown sample(s) by comparing the Net Abs 595 values against the standard curve.

***Notes:**

- The protein-dye complex is stable up to 60 minutes. The absorbance of the samples must be recorded before the 60 minute time limit.