PRINTERIA

Lab Protocols

Wet Lab

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Making competent cells

Making chemically competent cells

Materials:

- Plate with LB agar
- SOB
- LB
- CCMB80 buffer
- Ice bucket
- 50 ml Falcon vials
- 1.5ml microcentrifuge tubes
- 250 ml flasks
- We are using 10G cells

CCMB80 Buffer preparation (from iGEM) :

- 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

Protocol:

Day 1

Streak the cells on a plate with LB agar and incubate overnight at 37°C.

Day 2

Preculture: pick an isolated colony from the plate and inoculate 50 ml of LB with it. Incubate overnight at 37°C shaking the culture at 150-250 rpm.

Day 3

1. Inoculate 50 ml of SOB medium with 1 ml of the preculture and grow at 30°C to an OD600nm of 0.6 shaking the culture at 150-250 rpm.
2. Fill an ice bucket with ice. Pre-chill a 50 ml Falcon vial in ice.
3. Transfer the culture to the falcon vial.
4. Centrifuge at 3000g at 4°C for 10 minutes.
5. Decant supernatant into waste receptacle
6. Gently resuspend in 50 ml of ice cold CCMB80 buffer
7. Incubate on ice for 20 minutes

8. Centrifuge again at 3000G at 4°C. Decant supernatant into waste receptacle, and bleach before pouring down the drain.
9. Resuspend cell pellet in 6.25 ml of ice cold CCMB80 buffer.
10. Incubate on ice for 20 minutes. Prepare for aliquoting
11. Aliquot 50 µl into chilled 1.5ml microcentrifuge tubes
12. Store at -80°C indefinitely.

Safety considerations:

- Wearing laboratory coat, gloves and goggles is necessary to follow this procedure.
- All the manipulations of bacteria are carried out in a laminar flow cabinet.
- Work surfaces should be decontaminated with 70% alcohol.
- All materials that have been in touch with cells will be autoclaved.

Making electrocompetent cells

Materials:

- Plate with LB agar
- LB
- MilliQ water
- Glycerol at 10%
- Ice bucket
- 50 ml Falcon vials
- 1.5ml microcentrifuge tubes
- 250 ml flasks
- We are using 10G cells

Protocol:

Day 1

Streak the cells on a plate with LB agar and incubate overnight.

Day 2

Preculture: pick an isolated colony from the plate and inoculate 50 ml of LB with it. Incubate overnight at 37°C.

Day 3

1. Inoculate 5 ml of the preculture per liter of LB
2 Incubate and grow at 37°C with 250 rpm to an OD600nm of 0.6.
3. Split the culture in 50 ml falcon vials.
4. Incubate these tubes on ice for 15-60 minutes
5. Centrifuge at 2000g at 4°C for 10 minutes
6. Decant the supernatant and resuspend in 50 ml of MilliQ water.
7. Centrifuge at 2000g at 4°C for 10 minutes
8. Decant the supernatant and resuspend in 50 ml of glycerol at 10%
9. Centrifuge at 2000g at 4°C for 10 minutes
10. Decant the supernatant
11. Resuspend in 500 µl of glycerol lat 10%
12. Aliquot 50 µl in 1.5 ml tubes. Flash freeze and store at -80°C

Safety considerations:

- Wearing laboratory coat, gloves and goggles is necessary to follow this procedure.
- All the manipulations of bacteria are carried out in a laminar flow cabinet.
- Work surfaces should be decontaminated with 70% alcohol.
- All materials that have been in touch with cells will be autoclaved.

Making Mix and Go competent cells

From [Zymo Research]

Materials:

- Plate with LB agar
- SOB
- LB
- Wash buffer
- Competent buffer
- Dilution buffer
- Ice bucket
- 50 ml Falcon vials
- 1.5ml microcentrifuge tubes
- 250 ml flasks
- We are using 10G cells

Protocol:

Day 1

Streak the cells on a plate with LB agar and incubate overnight at 37°C.

Day 2

Preculture: pick an isolated colony from the plate and inoculate 50 ml of LB with it. Incubate overnight at 37°C shaking the culture at 150-250 rpm.

Day 3

1. Use 0.5 ml of fresh, overnight E. coli culture grown in LB to inoculate 50 ml ZymoBroth™ or SOB medium in a 500 ml culture flask. Shake culture vigorously (150 - 250 rpm) at the appropriate temperature* until the OD600nm is 0.4 - 0.6. Buffer Preparation Prior to Harvesting the Cells… The Wash and Competent Buffers are provided as 2X stock solutions. They need to be diluted to 1X by adding an equal amount of Dilution Buffer. To prepare 5 ml of 1X Wash Buffer: Add 2.5 ml Dilution Buffer and 2.5 ml of 2X Stock Wash Buffer. To prepare 5 ml of 1X Competent Buffer: Add 2.5 ml Dilution Buffer and 2.5 ml of 2X Stock Competent Buffer. Please keep these freshly prepared 1X Buffers ice cold. These 1X Buffers are good for 2 days at 0-25°C. It is important that each step of the following procedure should be done on ice or at 0-4°C.
2. Transfer the culture from Step 1 to ice. After 10 minutes, pellet the cells by centrifugation at 3,000 - 3,700 rpm (i.e., 1,600 - 2,500 x g) for 10 minutes at 0 - 4°C.

3. Remove the supernatant and resuspend the cells gently in 5 ml ice-cold 1X Wash Buffer. Re-pellet the cells as in Step 2.
4. Completely remove the supernatant and gently resuspend the cells in 5 ml ice-cold 1X Competent Buffer.
5. Aliquot (on ice) 0.1 ml of the cell suspension into sterile microcentrifuge tubes. Cells are now ready for transformation with DNA or can be stored below -70°C for transformation at a later time.

Safety considerations:

- Wearing laboratory coat, gloves and goggles is necessary to follow this procedure.
- All the manipulations of bacteria are carried out in a laminar flow cabinet.
- Work surfaces should be decontaminated with 70% alcohol.
- All materials that have been in touch with cells will be autoclaved.

Transformations

Heat shock

Materials:

- Aliquots of chemically competent cells
- Ice bucket
- Soc
- Plates with LB agar and the appropriate antibiotic

Protocol:

1. Chill on ice an aliquot of 50 µl of chemically competent cells
2. Add to 1-5 µl of DNA to the aliquot
3. Incubate on ice for 30 minutes
4. Incubate at 42°C for 45 seconds
5. Incubate on ice for 5 minutes
6. Add 950 µl of SOC
7. Incubate at 37°C for 1 hour
8. Plate the cells with the appropriate antibiotic.

Safety considerations:

- Wearing laboratory coat, gloves and goggles is necessary to follow this procedure.
- All the manipulations of bacteria are carried out in a laminar flow cabinet or in the presence of an ignited Bunsen burner. If using the burner do not use gloves.
- Work surfaces should be decontaminated with 70% alcohol.
- All materials that have been in touch with cells will be autoclaved.

Electroporation

Materials:

- Aliquots of electrocompetent cells
- Ice bucket
- Soc
- Electroporation cuvettes
- Electroporator
- Plates with LB agar and the appropriate antibiotic

Protocol:

1. Chill on ice an aliquot of electrocompetent cells
2. Add 1-5 µl of DNA
3. Place the cells and the DNA in the electroporation cuvette
4. Place the electroporation cuvette in the electroporator
5. Electroporate the cells with 1500 V
6. Add 700 µl of SOC to the cuvette
7. Place the content of the cuvette in a tube and incubate at 37°C for 1 hour
8. Plate the cells with the appropriate antibiotic

Safety considerations:

- Wearing laboratory coat, gloves and goggles is necessary to follow this procedure.
- All the manipulations of bacteria are carried out in a laminar flow cabinet or in the presence of an ignited Bunsen burner. If using the burner do not use gloves.
- Work surfaces should be decontaminated with 70% alcohol
- All materials that have been in touch with cells will be autoclaved.

Mix and Go

From Zymo Research

Materials:

- Aliquots of Mix and Go competent cells
- Ice bucket
- Soc
- Plates with LB agar and the appropriate antibiotic

Protocol:

1. Add 1-5 µl plasmid DNA to a tube of thawed Mix & Go cells on ice, mix gently for a few seconds (try to keep the added volume of DNA less than 5% of the total).
2. Add 400 µl of SOC
3. Incubate at 37°C for 1 hour
4. Plate the cells with the appropriate antibiotic.

Safety considerations:

- Wearing laboratory coat, gloves and goggles is necessary to follow this procedure.
- All the manipulations of bacteria are carried out in a laminar flow cabinet or in the presence of an ignited Bunsen burner. If using the burner do not use gloves.
- Work surfaces should be decontaminated with 70% alcohol
- All materials that have been in touch with cells will be autoclaved.

Testing the transformation efficiency

Protocol:

1 Transform 1 aliquot of competent cells with 1 ng of a control plasmid

2. Plate 5 µl, 50 µl and 300 µl of in plates with the appropriate antibiotic

3. Incubate overnight at 37°C

4. Count the colonies in the plate where you can find 30 to 300 isolated colonies

5. Use this equation to calculate the number of CFUs per microgram of DNA:

$$\frac{CFU}{\mu g} = \frac{Colonies\ in\ the\ plate \cdot (Volume\ of\ cells + Volume\ of\ SOC + Volume\ of\ DNA)}{Volume\ plated} \cdot + 1000$$

Equation 1. Estimation of the transformation efficiency

Lyophilization

Materials:

- Aliquots of 50 µl of cells
- Porous tape
- Freeze dryer
- Parafilm
- Silica gel

Protocol:

1. Keep the cells deep-frozen using liquid N2
2. Cover the aliquots with porous tape
3. Put the aliquots in the freeze dryer
4. Wait 2 days
5. Take the aliquots from the freeze dryer and quickly close them with parafilm and preserve them with silica gel

Safety considerations:

- Wearing laboratory coat, gloves and goggles is necessary to follow this procedure.
- All the manipulations of bacteria are carried out in a laminar flow cabinet or in the presence of an ignited Bunsen burner. If using the burner do not use gloves.
- Work surfaces should be decontaminated with 70% alcohol
- All materials that have been in touch with cells will be autoclaved.

Rehydration of freeze dried cells

Materials:

- Ice bucket
- Freeze dried cells
- MilliQ water
- Glycerol at 10%

Protocol:

From invivogen

1. Place the freeze dried cells in ice for 5 minutes
2. Resuspend the lyophil with the same volume of sterile water that the aliquot had before being lyophilized (we have also tried resuspending the lyophil with glycerol at 10%, getting better results)
3. Incubate on ice for 30 minutes

Safety considerations:

- Wearing laboratory coat, gloves and goggles is necessary to follow this procedure.
- All the manipulations of bacteria are carried out in a laminar flow cabinet or in the presence of an ignited Bunsen burner. If using the burner do not use gloves.
- Work surfaces should be decontaminated with 70% alcohol
- All materials that have been in touch with cells will be autoclaved.

Bacterial glycerol stock

Glycerinates are a preparation of cells with glycerol so as to have a long-term stock stored at -80°C

Materials:

- Glycerol 80%
- Overnight cell culture
- Cryoval

Protocol:

Final glycerol concentration is recommended to be 16%, so for a final volume of 1.8 mL it is necessary to add 360 µL of stock glycerol 80%

1. Put 1440 µl of the cell culture into the cryovial and add 360 µl of 80% Glycerol (cryoprotectant). Vortex the mix.
2. Store in the -80°C freezer

Safety considerations:

- Wearing laboratory coat and gloves is necessary to follow this procedure.
- All procedure must be carried out in sterilized conditions, so using the Bunsen burner.
- Work surfaces should be decontaminated with 70% alcohol.
- All materials that have been in touch with cells will be autoclaved.

Plasmid isolation: Miniprep

Plasmid DNA isolation was carried out using the commercial kit from Macherey-Nagel and so following the NucleoSpin®Plasmid EasyPure protocol.

Materials

Equipment

- Microcentrifuge
- Vortex mixer
- Manual pipettors

Consumables

- NucleoSpin®Plasmid EasyPure kit
- 1.5 mL microcentrifuge tubes
- Pipette tips

Protocol:

1 . Use 2–10 mL of a saturated E.coli culture, pellet cells in a microcentrifuge for 30s at > 12,000 x g. Discard the supernatant and remove as much of the liquid as possible.

2. Cell lysis

Add 150 µL Buffer A1. Resuspend the cell pellet completely by pipetting up and down.

Add 250 µL Buffer A2. Mix gently by inverting the tube 5 times. Incubate at room temperature.

Add 350 µL Buffer A3. Mix thoroughly by inverting the tube.

3. Clarification of lysate

Centrifuge for 3 min at full speed (> 12,000 x g).

4. Bind DNA

Place a NucleoSpin® Column into a Collection Tube and decant the supernatant onto the column. Centrifuge for 30 s at 1,000–2,000 x g. Discard flow-through and place the spin column back.

5. Wash and dry silica membrane

Add 450 µL Buffer AQ. Centrifuge for 1 min at full speed. Discard the flow through.

6. Elute DNA

Place the NucleoSpin® Column in a 1.5 mL microcentrifuge tube and add 50 µL Buffer AE. Incubate for 1 min at room temperature (18–25 °C). Centrifuge for 1 min at full speed.

Pro-tip 1: pre-heat the Buffer AE to 50 °C prior to the elution step as high temperature reduces ethanol carry-over.

Pro-tip2: increase the incubation step after adding Buffer AE to the column, as more incubation time increases the final DNA concentration.

Safety considerations:

- Wearing laboratory coat, gloves and glasses is necessary to follow this procedure.
- All materials in touch with bacteria will be disposed into waste autoclavable bags.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed to check restriction assays and PCR results.

Materials

Equipment

- Electrophoresis comb
- Electrophoresis tray
- Voltage source
- Microwave

Consumables

- Agarose powder
- SB buffer
- Red Safe DNA Loading Dye
- Orange DNA Loading Dye (6X) - Thermo / Gel Loading Dye Purple (6X)- NEB
- O'Gene Ruler Mix (weight ladder) - Thermo

Protocol:

1. Prepare Agarose gel

It is necessary to adjust the amount of agarose to get the desired gel concentration. To separate medium size fragments, the agarose gel is used in a concentration of 1% w/v.

- Weight 0.8 g of agarose powder in an erlenmeyer flask.
- Measure 80 mL of SB (1X) and add it to the erlenmeyer.
- Put the mix in the microwave until the agarose is completely dissolved.
- Let the mix cool down till it can be added to the electrophoresis tray
- Allow the solution to set.

2. Running Agarose Gel

- When agarose gel is solidified, remove the comb and cover the gel with SB (1X)
- Load the molecular weight ladder with RedSafe dye into the extreme wells (4 µl in each)
- Add ⅙ Volume of loading buffer mixed with RedSafe dye to each DNA sample
- Load the DNA samples in the remaining wells
- Run the gel with the required voltage. It depends on the voltage range of the electrophoresis buffer (V/cm) and the DNA sizes to separate.
- Visualize the DNA fragments using a UV transilluminator.

Pro-tip: if desired better band visualization, add the RedSafe Loading Dye 2000X directly to the agarose gel solution (Volume to add = Agarose solution volume / 2000).

Safety considerations:

- Wearing lab coat, gloves and goggles is totally required during the procol.
- Ethidium bromide is routinely used to stain DNA in gels. However, it is a potential toxic mutagen, so we avoid using it. As a substituent, we use the RedSafe DNA loading dye, which is a non-carcinogen safe buffer.
- Used agarose gels are disposed into cytotoxic waste container.

DNA gel extraction

Gel extraction of DNA band was carried out using the NucleoSpin® Gel and PCR Clean-up kit and so following the commercial protocol from Macherey-Nagel.

Materials

Equipment

- Microcentrifuge
- Heating block
- Vortex mixer
- Scalpel
- Manual pipettors

Consumables

- NucleoSpin® Gel and PCR Clean-up kit
- 2 ml microcentrifuge tubes
- 1.5 ml microcentrifuge tubes
- Pipette tips

Protocol:

1. Excise DNA fragment and solubilize gel slice

Take a scalpel to excise the DNA fragment from an agarose gel.
Determine the weight of the gel slice and transfer it to a 2 ml microcentrifuge tube. For each 100 mg of agarose gel <2 % add 200 µL Buffer NTI. For gels containing >2 %, double the volume of buffer.
Incubate the sample for 5–10 min at 50 °C and vortex briefly until the gel slice is completely dissolved.

Pro-tip 1: In this step, minimize the UV exposure time to avoid damaging the DNA.

Pro-tip 2: in order to increase the DNA yield, let the gel slice cool in the freezer previous to the addition of the NTI buffer.

2. Bind DNA

Place a NucleoSpin® 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 repeat again if it is necessary.

3. Wash silica membrane

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.

4. Dry silica membrane

Centrifuge for 1 min at 11,000 x g to remove Buffer NT3 completely.

5. Elute DNA

Place the NucleoSpin® Gel and PCR Clean-up Column into a new 1.5 mL microcentrifuge tube. Add 15–30 µL Buffer NE to and incubate at room temperature (18–25 °C) for 1 min. Centrifuge for 1 min at 11,000 x g.

Pro-tip 3: you can use milliQ water pre-heated to 50°C instead of Buffer NE

Safety considerations:

- Wearing laboratory coat, gloves and glasses is necessary to follow this procedure.
- Safety instructions provided with the commercial kit must be followed, as the NTI buffer contains guanidinium thiocyanate.

PCR clean-up

Purification of PCR products was carried out using the NucleoSpin® Gel and PCR Clean-up kit and so following the commercial protocol from Macherey-Nagel.

Materials:

Equipment

- Microcentrifuge
- Heating block
- Vortex mixer
- Manual pipettors

Consumables

- NucleoSpin® Gel and PCR Clean-up kit
- 1.5 ml microcentrifuge tubes
- Pipette tips

Protocol:

1. Adjust DNA binding condition

For very small sample volumes < 30 µL adjust the volume of the reaction mixture to 50–100 µL with water. Mix 1 volume of sample with 2 volumes of Buffer NTI.

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.

3. Wash silica membrane

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.

4. Dry silica membrane

Centrifuge for 1 min at 11,000 x g to remove Buffer NT3 completely.

5. Elute DNA

Place the NucleoSpin® Gel and PCR Clean-up Column into a new 1.5 mL microcentrifuge tube and add 15–30µL Buffer NE. Incubate at room temperature (18–25 °C) for 1 min. Centrifuge for 1 min at 11,000 x g.

Pro-tip: Buffer NE can be previously heated to 50°C in order to reduce ethanol carry-over.

Safety considerations:

- Wearing laboratory coat, gloves and glasses is necessary to follow this procedure.
- Safety instructions provided with the commercial kit must be followed, as the NTI buffer contains guanidinium thiocyanate

PCR - Q5 HF polymerase

From NEB

PCR reactions were performed in the domestication of DNA parts prior to the restriction-ligation assembly and as a step in the pBioBrickator compatible vector construction

Materials:

Equipment

- 250 µL tubes
- Manual pipettes and pipette tips
- Cold block / ice
- Thermocycler
- Pipettes and pipette tips

Consumables

- Sterile water
- 5X Q5 Reaction Buffer (NEB)
- Forward and reverse primers (10 uM)
- dNTP 2.5 mM
- Q5 High Fidelity polymerase (NEB)
- DNA template
- GC Enhacer (NEB) or DMSO 3%

Protocol:

PCR reactions with the Q5-HF polymerase were carried out following the New England Biolabs (NEB) protocol. For each PCR reaction, primers annealing temperature was determined using the New England Biolabs online calculator tool (NEB tm calculator).

1. Add all components in a 250 µL tube making up to a 25 µl reaction. If performing various PCR with different templates, a Master Mix is recommended to be done.
When doing a Master Mix always add Q5 enzyme last, then vortex the solution briefly and centrifugate before use.

    Pro-tip 1: use DMSO 3% or 5X GC enhancer as PCR additives when amplifying particularly difficult or high GC amplicons.

Table 1. Q5 PCR set up

Components	Volume (µl)
5x Buffer Q5	5
2.5mM dNTP	2
Primer FW 10 nM	1.25
Primer RV (10 nM)	1.25
DNA template 1 ng/µl	2
Q5 HF polymerase	0.25
Sterilized H2O	13.25
Total	25

2. Gently mix the PCR reactions and transfer the tubes to a thermocycler.
3. Thermocycling Conditions for a Routine PCR with Q5 - HF polymerase

Table 2. Thermocycler configuration for the PCR with Q5-HF DNA polymerase

Step	Temp	Time
Initial denaturalization	98°C	10 min
25 to 35 cycles	98°C 50-72°C (annealing T[a]) 72°C	5 -10 secs 10 to 30 secs 20 to 30 secs
Final extension	72°C	10-30 sec / kb
Hold	4 - 10°C	∞

Safety considerations:

- Wearing laboratory coat, gloves and glasses is necessary to follow this procedure.
- PCR reagents are classified as non-hazardous. Follow the specified handling and disposal considerations included in the safety data sheets provided by the manufacturer.

Colony PCR

Colony PCR is a method for determining the presence of insert DNA in a plasmid construct. This procedure was carried out in order to check the presence of a domesticated DNA part or transcriptional unit.

Materials:

Equipment

- 250 µL tubes
- Freeze block / ice
- Thermocycler
- Pipettes and pipette tips

Consumables

- Sterile water
- Standard Taq buffer (10X) NEB
- Primers forward and reverse (10 uM)
- dNTPs 2.5 mM
- Taq polymerase enzyme - NEB
- 5 µl diluted colony

Protocol:

Colony PCR protocol from New England Biolabs (NEB). For each PCR reaction, primers annealing temperature was determined using the New England Biolabs online calculator tool (NEM TM calculator).

1. Colonies are picked and pipetted down in 50µl of sterile PCR water. 5 µl are used as template for the PCR.

2. On ice, add all components in a 250 µL tube, making up to a 25 µl volume reaction. If checking the presence of a plasmid in many colonies, a Master Mix is recommended to be done by including all reagents except colonies.

Table3. Colony PCR set up

Components	Volume (µl)
10X React Buffer (Standard Taq)	2
dNTPs 2.5 mM	2
Primer Forward 10 uM	0.4
Primer Reverse 10 uM	0.4
Taq Polimerasa	0.1
Colony	5
Destillated H2O	10.1
Total reaction volume	20

2. Gently mix the PCR reactions and transfer the tubes to a thermocycler.
3. Thermocycling Conditions for a cPCR with Taq DNA pol.

Table 4. Thermocycler configuration for the PCR with Taq DNA polymerase

Step	Temp	Time
Initial denaturalization	98°C	10 min
25 - 35 cycles	98°C 50-72°C (annealing T^a) 72°C	5-10 secs 10 - 30 secs 20-30 secs
Final extension	72°C	1 min / kb
Hold	4 - 10°C	∞

Safety considerations:

- Wearing laboratory coat, gloves and glasses is necessary to follow this procedure.
- Colonies must be picked up in sterile conditions, so using the Bunsen burner or the laminar flow cabinet. If using the burner do not wear gloves.
- Consumables in contact with bacteria culture will be disposed into autoclavable bags.

Golden Braid assembly

Golden Braid assembly is a one-pot digestion/ligation reaction where one or more DNA inserts are assembled into a vector backbone using Type IIS endonucleases and T4 DNA ligase.
This procedure was carried out to create all our GB basic parts (Level 0) and transcriptional units (Level 1).

Materials:

Equipment

- Thermocycler
- 250 µL tubes
- Pipettes and pipette tips

Consumables

- Sterilized water
- BSA (10X)
- BsaI (NEB or Thermo) / Esp3I - BsmBI (Thermo)
- T4 DNA ligase buffer (Thermo)

Protocol:

Table 5. Reaction set-up for the Level 0 Golden Braid assembly

Reagents	Level 0
DNA part	75 ng
Universal Acceptor plasmid (BBa_P10500)	75 ng
TSII endonuclease (BsmBI)	1 µl
T4 DNA ligase	1 µl
BSA (10X)	1.5 µl
T4 DNA ligase buffer (Thermo)	1.5 µl
Sterilized H20	up to 15 µl

Table 6. Reaction set-up for the Level 1 Golden Braid assembly

Reagents	Level 1
DNA part	75 ng / each GB basic part
Destination vector	50 ng circularized plasmid 25 ng linearized plasmid
TSII endonuclease (BsaI)	1 µl
T4 DNA ligase	1 µl
BSA (10X)	1.5 µl
T4 DNA ligase buffer (Thermo)	1.5 µl
Sterilized H20	up to 15 µl

Reaction mix must be transferred to a thermocycler machine with program conditions indicated as follow:

Table 7. Thermocycler program conditions for the Golden Braid assembly

Time	Temperature
1 cycle - 10 min	37 °C
x 25 cycles	
3 min	37 °C
4 min	16 °C
1 cycle - 10 min	50 °C
1 cycle - 10 min	80 ° C

Safety considerations:

- Wearing laboratory coat, gloves and glasses is necessary to follow this procedure.

Gibson Assembly

Gibson assembly was performed for assembling a BioBrick compatible plasmid (BBa_K2656200) for the insertion of GB transcriptional units.

Materials:

Equipment

- Thermocycler
- 250 µL tubes
- Pipettes and pipette tips

Consumables

- Gibson Assembly Master Mix
- Deionized water
- DNA samples

Protocol:

Gibson reaction was carried out following the NEB protocol.

1. For the assembly of 2-3 fragments, set up the following reaction on ice:

Table 8. Gibson assembly set up

Total amount of fragments	0.02–0.5 pmols X µl
Gibson Assembly Master Mix	10 µl
Deionized H20	10 - X µl
Total Volume	20 µl

2. Incubate samples in a thermocycler at 50°C for 15 minutes when 2 or 3 fragments are being assembled.

Safety considerations:

- Wearing laboratory coat, gloves and glasses is necessary to follow this procedure.

Restriction enzyme digestion

Restriction enzyme digestions were performed for pBioBrickator compatible vector assembly, BioBrick modules digestion and restriction assays.

Restriction enzymes

EcoRI, XbaI (BioBrick prefix)	pUPD2+insert	BioBrick assembly
SpeI, PstI (BioBrick suffix)	pUPD2+insert	BioBrick assembly
BsaI, BsmBI	GB parts	Restriction analysis

Materials

Equipment

- Automatic pipettes and pipette tips
- Ice bucket
- 1.5 ml microcentrifuge tube
- Incubator (37°C)
- Heat block

Consumables

- Sterile water
- CutSmart Reaction buffer (10X) - NEB
- Restriction enzyme
- DNA sample

Protocol:

Digestion reactions were carried out following the NEB protocol.

1. The reaction components must be taken in a Ice bucket, so they are added in order (water, buffer, DNA and enzymes) to a microcentrifuge tube.

Table 9. Reaction set-up for a 30 µl final volume

DNA	1 ug
Restriction enzyme	1 µl

CutSmart Buffer (10X)	3 µl
H_2O	until 30 µl

Pro-tip: add 1500 ng of DNA instead of 1000 ng in order to have an exceed of template to be digest.

2. Mix gently and incubate 1 hour at 37°C.

3. Finally, deactivate the enzymes at the temperature indicated for each type.

EcoRI, XbaI, BsaI - 65 °C 20 min

BsmBI, PstI, SpeI - 80 °C 20 min

Safety considerations:

- Wearing laboratory coat, gloves and glasses is necessary to follow this procedure.
- Digestion reagents are classified as non-hazardous. Follow the necessary handling and disposal considerations provided by the manufacturer.

DNA ligase reaction

DNA ligase reactions were performed for vector construction (backbone-vector sticky ends ligation) and for BioBrick assembly of transcriptional units.

Materials

Equipment

- Automatic pipettes and pipette tips
- Ice bucket
- Microcentrifuge

Consumables

- Sterilized water
- T4 DNA ligase (NEB)
- T4 DNA ligase buffer (NEB)

Protocol:

Ligation reactions were done following the NEB protocol.

1. The exact proportion of insert and vector is calculated using the NEB ligation tool. Commonly, it is used a 3:1 insert:vector molar ratio. Once calculated, set up the following reaction on ice:

Table 10. T4 DNA ligase reaction set up

Insert and vector	ng specified by the molar ratio
T4 Ligase (NEB)	0.5 µl
T4 Ligase Buffer (10X)	1 µl
Distilled water	up to 10 µl

2. Gently mix the reaction and microfugate.
3. Incubate 20 minutes at room temperature.
4. Finally, deactivate the ligase at 65°C for 10 min.

Safety considerations:

- Wearing laboratory coat, gloves and glasses is necessary to follow this procedure.
- Ligation reagents are classified as non-hazardous. Follow the necessary handling and disposal considerations provided by the manufacturer.

Culture of E. coli

LB broth (Lennox) preparation

Materials:

- Vegetable origin peptone
- Yeast extract
- NaCl

Protocol:

1. Add 10g of peptone, 5g of yeast extract and 5g of NaCl for each 20g of LB that is been prepared.
2. Vigorous shake this mixture.

Liquid medium preparation

For 500 ml of medium:

Materials:

- LB
- Distilled water
- 500 ml autoclavable bottle
- Autoclave

Protocol:

1. Add 10g of LB to the bottle
2. Add 500 ml of distilled water to the bottle
3. Vigorous shake this mixture
4. Put the bottle into the autoclave and sterilize

Solid medium preparation

For 500 ml of medium:

Materials:

- LB
- Agar-agar
- Distilled water
- 500 ml autoclavable bottle
- Autoclave
- Petri dish
- 1000x antibiotic

Protocol:

1. Add 10g of LB and 10g of Agar-agar to the bottle

2. Add 500 ml of distilled water to the bottle
3. Vigorous shake this mixture
4. Put the bottle into the autoclave and sterilize
5. Let the bottle chill until it is at 50°C
6. Add 500 µl of the antibiotic the bottle
7. Extend 25 ml of the contents of the bottle in each petri dish.

Liquid medium culture

After plating transformed electrocompetent cells with a ligation product, single colonies are picked up and inoculated into liquid medium.

Materials

Equipment

- Shaker

Consumables

- Pipette tip or inoculating loop
- Culture tubes
- LB broth liquid medium
- Antibiotic (1000x)

Table 11. Antibiotic resistance of the used backbones

Vector backbone	**Antibiotic resistance**
BBa_P10500	Chloramphenicol
alpha GB destination vector	Kanamycin

Protocol:

Add 4 ml of LB broth and 4 µl of the antibiotic to a culture tube per colony that will be picked. A single colony is picked up and inoculated into the liquid medium with a pipette tip or inoculating loop, so this culture is then incubated overnight (12-16 h) at 37 ºC with vigorous shaking (220 rpm).

Safety considerations:

- Wearing laboratory coat and glasses is necessary to follow this procedure.
- Work surfaces should be decontaminated with 70% alcohol
- Bacteria inoculation must be done in sterile conditions, so using the Bunsen burner.
- Do not wear gloves when using the bruner.
- Consumable in contact with bacteria culture will be disposed into autoclavable bags.

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Petri dish culture

Cells are grown in solid agar medium in order to isolate specific colonies.
Agar petri dishes must contain the antibiotic for the resistance carried by the transformed strain. 100 µl of ChromoMax™ IPTG/X-Gal Solution (Fisher BioReagents) was also added to the agar plates when performing blue-white screening.

Materials:

- LB agar plates
- Sterile glass spreader or plating beads
- Bunsen burner

Protocol:
1. Spread the bacteria culture all over the plate using the glass spreader or plating beads. Dispose the plating beads after spreading.
2. Invert plates and incubate overnight at 37°C.

Safety considerations:

- Wearing laboratory coat and glasses is necessary to follow this procedure.
- Work surfaces should be decontaminated with 70% alcohol
- Bacteria spread must be done in sterile conditions, so using the Bunsen burner.
- Do not wear gloves when using the bruner.
- Consumable in contact with bacteria culture will be disposed into autoclavable bags.