Bacterial transformation

Materials

- Competent cells
- Water bath
- Incubator
- Petry dishes with their respective antibiotics

Procedure

1. Thaw 100 µL of chemically competent bacteria on ice
2. Add 1 µL of plasmid (10ng/uL)
3. Put on ice for 30 minutes
4. Heat shock at 42°C for 45 seconds
5. Return on ice for 5 minutes
6. Add liquid LB to 1 mL
7. Regenerate at 37°C for 30 minutes
8. Centrifuge cells and resuspend in 100 µL of liquid LB
9. Plate cells and resuspend in 100 µL of liquid LB
10. Let grow overnight at 37°C

Miniprep

Materials

- RNase A Solution (10mg/ml)
- Solution I
- Solution II
- Solution III
- Wash Solution
- Elution Buffer (Milli Q water)
- EZ-10 Column
- Overnight liquid culture

Procedure

1. Add 1.5 - 5mL overnight culture in the tube and centrifuge at 12,000rpm for 2 minutes. Drain the liquid completely. For low copy number plasmid, see the protocol on the following page.
2. Add 100µl of Solution I
3. Add 200µl of Solution II to the mixture and mix gently by inverting the tube 4-6 times and then keep at room temperature for 1 minute. To prevent contamination from genomic DNA, do not vortex. A homogenously blue suspension should also be observed. If the suspension contains uneven blue color, or white/brownish cell clumps, continue mixing carefully.
4. Add 350µl of Solution III and mix gently. Incubate at room temperature for 1 minute. A fluffy white material forms and lysate should become less viscous. If visual lyse

has been added in step 3, the suspension should be mixed until all traces of blue has gone and lysate becomes colorless

5. Centrifuge at 12,000rpm for 5 minutes.
6. Transfer the above supernatant (step 6) to the EZ-10 column. Centrifuge at 10,000rpm for 2 minutes.
7. Discard the flow-through in the tube. Add 750µl of Wash Solution to the column, and centrifuge at 10,000rpm for 2 minutes.
8. Repeat wash procedure in step 8.
9. Discard the flow-through in the collection tube. Centrifuge at 10,000rpm for an additional minute to remove any residual Wash Solution.
10. Transfer the column to a clean 1.5ml microfuge tube. Add 50µl of Elution Buffer into the center part of the column and incubate at room temperature for 2 minutes. Centrifuge at 10,000 rpm for 2 minutes.
11. Store purified DNA at -20ºC. **Note:** It is extremely important to add the Elution Buffer into the center part of the column. Incubating the column with the Elution Buffer at higher temperature (37ºC to 50ºC) may slightly increase the yield especially for large (>10,000bp) DNA Plasmids. Prewarming the Elution Buffer at 55ºC to 80ºC may also slightly increase elution efficiency.

Bacterial Glycerol Stocks for Long-term Storage

Materials

- Filtrated glycerol 50%
- Overnight liquid culture

Procedure

1. Follow the steps for Inoculating an Overnight Liquid Culture.
2. After you have bacterial growth, add 500 µL of the overnight culture to 500 µL of 50% glycerol in a 2 mL screw top tube or cryovial and gently mix.
3. ***Note:*** *Make the 50% glycerol solution by diluting 100% glycerol in dH20.*
4. ***Note:*** *Snap top tubes are not recommended as they can open unexpectedly at -80°C.*
5. Freeze the glycerol stock tube at -80°C. The stock is now stable for years, if it is kept at -80°C. Subsequent freeze and thaw cycles reduce shelf life.
6. To recover bacteria from your glycerol stock, open the tube and use a sterile loop, toothpick or pipette tip to scrape some of the frozen bacteria off of the top. Do not let the glycerol stock unthaw!
7. Grow your bacteria overnight at the appropriate temperature. Growth conditions, including copy number and growth temperature, can be found on your plasmid's information page. The next day you will be able to start an overnight culture for plasmid DNA prep the following day.

Inoculating a Liquid Bacterial Culture

Materials

- Liquid LB
- LB agar plate with the strain of interest

Procedure

1. Prepare liquid LB.
2. When ready to grow your culture, add liquid LB to a tube or flask and add the appropriate antibiotic to the correct concentration.
3. Using a sterile pipette tip or toothpick, select a single colony from your LB agar plate.
4. Drop the tip or toothpick into the liquid LB + antibiotic and swirl.
5. Loosely cover the culture with sterile aluminum foil or a cap that is not airtight.
6. Incubate bacterial culture at 37°C for 12-18 hours in a shaking incubator.

   Note: *Some plasmids or strains require growth at 30°C. If so, you will likely need to grow for a longer time to get the correct density of bacteria since they will grow more slowly at lower temperatures.*

7. After incubation, check for growth, which is characterized by a cloudy haze in the media (see right).

   Note: *Some protocols require bacteria to be in the log phase of growth. Check the instructions for your specific protocol and conduct an OD600 to measure the density of your culture if needed.*

   Note: *A good negative control is LB media + antibiotic without any bacteria inoculated. You should see no growth in this culture after overnight incubation.*

PCR

Materials

- SapphireAmp® Fast PCR Master Mix
- Primers (forward and reverse).
- Plasmid vector to act as our template.
- Nuclease-free distilled water to make the volume up to 50µL.Overnight liquid culture
- Agarose gel 1%

Procedure

1. Mix together
    - 1 µl of primer 1 (forward)
    - 1 µl of primer 2 (reverse)
    - 1 µl of plasmid (10 ng)
    - 9.5 µl of MilliQ
    - 12.5 µl of Master Mix (SapphireAmp®)

2. Use the following cycles
    - 1:00 at 98°C
    - 0:10 at 98°C
    - 0:15 at 58°C (change according to primers Tm)
    - 0:05 at 72°C (5 seconds for 1 kb below 5 kb fragment, 10 seconds for 1 kb above 5 kb fragment)
    - Repeat 30 times (35 cycles for PCR on colonies)
    - 1:00 at 72°C
    - ∞ at 12 °C

3. For the gel verification mix together (Charge in an agarose gel of 2%)
    - 5 µl of PCR product
    - 5 µl of Loading buffer (green one is better)
4. 100 watts for 30 minutes

Gibson assembly

Materials

- Isothermal assembly reaction mix
- Reaction master mix
- Insert(s)
- Plasmid
- H20

Procedure

1. Mix 10uL of the reaction master mix with 0.2 uL of the insert
2. Add the 1uL of the plasmid
3. Add 7.8 uL of water to have a final volume of 20uL
4. Incubate 30 min at 50°C and it is ready

Afterwards, proceed to transform the final product

1. Thaw 100 µL of chemically competent bacteria on ice
2. Add 5 µL of each insert (guanosine and theophylline) with different ratios (0.2, 0.4 and 0.6)
3. Put on ice for 30 minutes
4. Heat shock at 42°C for 45 seconds
5. Return on ice for 5 minutes
6. Add liquid LB to 1 mL
7. Regenerate at 37°C for 30 minutes
8. Centrifuge cells and resuspend in 100 µL of liquid LB

9. Plate cells and resuspend in 100 µL of liquid LB

10. Let grow at 37°C overnight

Triple-Bacterial transformation

Materials

- Competent cells
- Water bath
- Incubator
- Petry dishes with their respective antibiotics

Procedure

1. Thaw 200 µL of chemically competent bacteria on ice
2. Add 1 µL of plasmid (100ng) of each of the three plasmids
3. Put on ice for 30 minutes
4. Heat shock at 42°C for 1-minute seconds
5. Return on ice for 5 minutes
6. Add liquid LB to 1 mL
7. Regenerate at 37°C for 1 hour
8. Centrifuge cells and resuspend in 100 µL of liquid LB
9. Plate cells and resuspend in 100 µL of liquid LB
10. Let grow at 37°C overnight

Competent cells (XL1 Blue)

Materials

- 100% DMSO (fume hood)
- Dry ice/ethanol
- Liquid nitrogen
- Nitrogen resistant tubes (Nunc cryo tubes, or safe locks)
- TBjap
    - TBjap for 250 mL:
    - Pipes 0.755 g at 10 mM
    - $CaCl_2$ 0.555 g at 15 mM
    - NCl $_4$.66g at 250 mM
    - Adjust pH 6.7
    - $MnCl_2$ 2.72g at 55 mM

Procedure

1. Inoculate Stratagene strain XL-1blue (from Epicurian comp. cells) for XL-1 blue cells: + 30 ug/mL tetracycline (stock: 5 mg/mL in EtOH)
2. Incubate at 18°C to an OD of 0.6 at 600 nm (will take approx. 24 hours) *GS3 rotor should be precooled
3. Place Erlenmeyer on ice for 10 min.
4. Pour culture in Sorvall GS3 centrifuge tube (use clear GS3 centrifuge tubes which have not been used for other plasmids before)
5. Prepare 100 mL of "TBjap" + 2mL of 100% DMSO on ice (in blue cap glass bottle)
6. Spin GS3 rotor (must be precooled) at 2500 g and 4°C, for 10 minutes

7. Discard supernatant completely and re-dissolve pellet in 80 mL of "TBjap" (Precooled, from above).
8. Keep suspension on ice (for 10 min)
9. Spin again for 10 min. at 2500 g and 4°C, (in precooled rotor)
10. Walk to the cold room
11. Discard supernatant and resuspend pellet in 18.6 mL of precooled "TBjap"
12. Add 1.4 mL of DMSO (->final conc, of 7%)
13. Let bacteria sit on ice for 10 min and prelabeled cryotubes on the lid ("C" = comp. cells)
14. Dispense suspension gently into cryotubes (200uL), freeze immediately in liquid nitrogen, (if sterilizing is important: put shortly in an ethanol/dry ice bath and back again into nitrogen)
15. Store immediately in a prelabeled Box at -70°C

Growth test

Materials

- LB Medium
    - NaCl (10 g/l)
    - Yeast Extract (5 g/l)
    - Peptone (10 g/l)
- Spectrophotometer
- Cuvettes

Procedure

Preculture

1. Inoculate a 50-ml flask containing 20 ml of LB with the strain.
2. Incubate overnight at 27°C in a shaking incubator. This should result in a stationary phase of approximately 10^9 CFU/ml.

Measurements

1. On the following day, use 100 µl of the prepared culture to inoculate 250 ml of LB in a 500-ml flask. Immediately remove an aliquot and incubate the flask at 37°C in a shaking incubator.
2. Set the blank value of the spectrophotometer using LB medium and measure the $OD_{600\ nm}$ of the aliquot.
3. Every hour removes an aliquot from the flask and measure the OD. If needed, dilute the aliquots with water to measure an OD between 0.1 and 1.

Analysis

1. Plot log (OD) against time (be careful to multiply the OD of diluted samples by their dilution factors).
2. Use the plot to determine the lag, exponential and stationary phases.
3. Use two points from the stationary phase to calculate the growth rate μ (in $time^{-1}$) as follows:

$$\mu = \frac{\ln OD_2 - \ln OD_1}{(t_2 - t_1)},$$

LB Medium

Materials

- Bacto-tryptone 10g
- Yeast extract 5g
- NaCl 5g
- NaOH 1N 1 mL
- Distilled water
- Autoclave

Procedure

1. Mix the 10g of bacto-tryptone, 5g of yeast extract, 5g of NaCl and 1mL of NaOH 1N in one liter of distilled water.
2. Mix well and dissolve by heating with frequent agitation.
3. Boil for one minute until complete dissolution.
4. Sterilize in autoclave at 121°C for 15 minutes.
5. **Note:** *The color of the prepared medium is clear amber, slightly opalescent.*

Petry dishes (LB + agar)

Materials

- Bacto-tryptone 10g
- Yeast extract 5g
- NaCl 5g
- NaOH 1N 1 mL
- Bacto-agar 15g
- Distilled water
- Autoclave

Procedure

1. Mix the 10g of bacto-tryptone, 5g of yeast extract, 5g of NaCl and 1mL of NaOH 1N and 15g of Bacto-agar in one liter of distilled water.
2. Mix well and dissolve by heating with frequent agitation.
3. Boil for one minute until complete dissolution.
4. Sterilize in autoclave at 121°C for 15 minutes
5. Wait until the medium is not too warm and add the antibiotics need it
6. Afterwards, put 20Ml of the medium in petri dishes under sterile conditions and let dry it
7. Finally store them at -80°C

Double digestion

Materials

- DNA sample(s) in water or TE buffer
- 10x digestion buffer
- Restriction enzymes (EcoRI or SpeI or XbaI or PstI)
- DNA loading buffer (if electrophoresis is subsequent)

Procedure

1. Add the following substances in a sterile tube: DNA around 1ug 10x Digestion buffer 2uL, 1Ul of each enzyme, add water until 20uL
2. Incubate at recommended temperature (37.0 degrees) for 2 or 4 hours.
3. Incubate at 80°C for 1 hour to deactivate the enzymes
4. Take 2 to 5 uL of the digested sample, add loading buffer, and run it on the agarose gel to check the weight band.

Beta galactosidase assay (Using cuvettes)

Materials

- Reagents and chemicals
    - LB: 10 g bactotryptone, 5 g yeast extract, 10 g NaCl in 1 L of distilled water. Autoclave LB to sterilize and store at room temperature.
    - Antibiotic stock solutions: Ampicillin, Chloramphenicol, Spectinomycine, Kanamycin.
    - IPTG stock solution. To make a 100 mM stock concentration of isopropyl-β-d-thiogalactoside (IPTG) dissolve 0.238 g in 10 mL of distilled water, filter sterilize, aliquot, and store at −20°.
    - Z-buffer: 16.1 g $Na_2HPO_4 \cdot 7H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 0.246 g $MgSO_4 \cdot 7H_2O$, add distilled water to 1 L, adjust pH to 7.0 (if necessary). Do not autoclave. Immediately before using Z-buffer add β-mercaptoethanol (BME) to a final concentration of 0.27%.
    - 0.1% sodium dodecyl sulfate (SDS) in water.
    - Chloroform
    - ONPG stock solution: *O*-nitrophenyl-β-d-galactosidase (ONPG) is dissolved in Z-buffer (without BME) to a final concentration of 4 mg/mL and stored at −20°.
    - 1 M Na_2CO_3.
    - Arabinose in powder (0.2%)
- Equipment
    - Spectrophotometer
    - Centrifuge
    - Heated water bath
    - Micropipettes

    - Incubator
    - Vortex
- Material
    - Loops
    - Falcon tubes (5ml)
    - Tips for micropipettes
    - Cuvettes
    - Small glass test tube
-

Procedure

1. Pick single colonies from the transformation plates and use them to inoculate 3 mL of LB in an Eppendorf tube containing: 1.5 uL of IPTG (50uM), 33mg of Arabinose in powder. The antibiotics respectively: 1.5 uL of Spectinomycine, 3uL of Chloramphenicol, 3uL of Ampicillin and 1.5 uL of Kanamycin. The cultures will be put overnight in an incubator at 37°C under shaking conditions for 12-16 hours approximately.

2. Afterwards, use 50 µL of the overnight cultures to inoculate 3 mL of LB containing the antibiotics and IPTG (using the 50uM) and 0.2% arabinose.

3. Grow cultures at 37° until the cells reach an OD_{600} between 0.3 and 0.7 (0.5 is ideal). Typically, this takes between 2 and 3 hours.

4. Place the cultures on ice for 30 min.

5. Place 1 mL of the cultures in a cuvette to read and record the OD_{600} value.

6. Set up the assay tube (in duplicate) by placing 200 µL of the cultures in a small glass test tubes that contain 800 µL of Z-buffer (with BME). Be sure to set up a tube that will serve as a blank using 200 µL of LB.

7. Add 30 µL of 0.1% SDS and 60 µL of chloroform to each tube and vortex for 10 s.

8. Allow tubes and the 4 mg/mL ONPG solution to equilibrate at 28° in a water bath for 15–20 min.

9. Start the assays by adding 200 µL of the ONPG solution to each assay tube, be sure to record the time at which each of the assays was started. It is recommended to add ONPG to each tube at 5 s intervals.

10. Mix the tubes by gently shaking or vortex them at a low speed.

11. When the tubes turn yellow, stop the reactions by adding 500 µL of 1 M Na_2CO_3.

12. Record the time at which each reaction was stopped. The ideal range for the OD_{420} is between 0.6 and 0.9. It is important to try and stop each of the assays when they have reached the same OD_{420}.

13. Gently vortex the reaction tubes then allow them to sit at room temperature for 10–15 min to allow the chloroform and cell debris to settle.

14. Place 1 mL of the reaction into a cuvette and read and record the OD_{420} and the OD_{550} value.

15. β galactosidase activity is expressed as Miller Units which are calculated using the equation:
16. Miller units = $1000 \times [OD_{420} - (1.75 \times OD_{550})/t \times v \times OD_{600}]$

17. Where *t* is the total time of the reaction expressed in minutes and *v* is the volume of culture used in the assay (which for this protocol is 0.2 mL).

OD420	OD550	OD600	t (min)	v (ml)	MU	
				0.2	#VALUE!	

Beta galactosidase assay (Using 96 plate reader)

Materials

- Reagents and chemicals
    - LB: 10 g bactotryptone, 5 g yeast extract, 10 g NaCl in 1 L of distilled water. Autoclave LB to sterilize and store at room temperature.
    - Antibiotic stock solutions: Ampicillin, Chloramphenicol, Spectinomycine, Kanamycin.
    - IPTG stock solution. To make a 100 mM stock concentration of isopropyl-β-d-thiogalactoside (IPTG) dissolve 0.238 g in 10 mL of distilled water, filter sterilize, aliquot, and store at −20°.
    - Z-buffer: 16.1 g $Na_2HPO_4 \cdot 7H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 0.246 g $MgSO_4 \cdot 7H_2O$, add distilled water to 1 L, adjust pH to 7.0 (if necessary). Do not autoclave. Immediately before using Z-buffer
    - ONPG stock solution: *O*-nitrophenyl-β-d-galactosidase (ONPG) is dissolved in Z-buffer to a final concentration of 4 mg/mL and stored at −20°.
    - Arabinose in powder (0.2%)
    - Thermo Scientific B-PER Bacterial Protein Extraction Reagent

Procedure

1. Pick single colonies from the transformation plates and use them to inoculate 3 mL of LB in an Eppendorf tube containing: 1.5 uL of IPTG (50uM), 33mg of Arabinose in powder. The antibiotics respectively: 1.5 uL of Spectinomycine, 3uL of Chloramphenicol, 3uL of Ampicillin and 1.5 uL of Kanamycin. The cultures will be put overnight in an incubator at 37°C under shaking conditions for 12-16 hours approximately.

2. Afterwards, use 50 µL of the overnight cultures to inoculate 3 mL of LB containing the antibiotics IPTG (using the 50uM) and 0.2% arabinose.

3. Measure the OD of the cultures until they reach an OD_{600} between 0.3 and 0.7 (0.5 is ideal). Typically, this takes between 2 and 3 hours.

4. Take 200 µL of the cultures in a 1.5 mL Eppendorf tubes and centrifugate them at 4000 rpm for 5 minutes and discard the supernatant

5. Resuspend the cells adding 200 µL of Thermo Scientific B-PER Bacterial Protein Extraction Reagent and mix well. (Be sure to set up a tube that will serve as a blank using 200 µL of LB and the 200 µL of Thermo Scientific B-PER Bacterial Protein Extraction Reagent).

6. Take 50 µL of the previous mix step, put them in new 1.5 mL Eppendorf tubes, add 150 µL of 4 mg/mL ONPG, mix properly. Take in account the time when you set up the assay by adding the ONPG (It is recommended to add ONPG to each tube at 5 s intervals).

7. When the tubes turn yellow, stop the reactions by adding 200 µL of 1 M Na_2CO_3.

8. Record the time at which each reaction was stopped.

9. Afterwards place the samples in a 96-plate reader

10. Read and record the OD_{420} and the OD_{550} value.

11. β galactosidase activity is expressed as Miller Units which are calculated using the equation:
12. Miller units = $1000 \times [OD_{420} - (1.75 \times OD_{550})/t \times v \times OD_{600}]$

13. Where *t* is the total time of the reaction expressed in minutes and *v* is the volume of culture used in the assay (which for this protocol is 0.2 mL).

OD420	OD550	OD600	t (min)	v (ml)	MU	
				0.2	#VALUE!	