REF gene Pre-trial protocols

Performing reactionary gene cutting

1. Preparation of growth substrate according to the protocol (see attached below).
2. Distributing the platform into two Erlenmeyer flasks - volume 200 ml.
3. To one Erlenmeyer flask we add ampicillin - lμ 0.2.
4. To the second flask we add chloramphenicol - lμ 0.2.
5. Preparing the medium for cutting the gene:

H2O-34μl
Buffer- 5μl
Plasmid- 7μl
Ecor1- 2μl
pst1- 2μl

5. 5 minutes – 80°.
6. 20 minutes – 37°.

Preparation of electrophoresis gel

1. Into the Erlenmeyer flask:

0.5g TAE 50μl + Agaroz
60 sec. in the microwave

2. 5 minutes cooling
3. Add 2.5 μl SYBR SAFE
4. Pour into working trays
5. Cutting the gel together with the DNA

Cleaning the DNA from the gel

1. Weigh an Eppendorf vial
2. Weigh vial with gel
3. Calculate how much a cube of gel alone weighs
4. Add μl380 NIT to the gene vial at the beginning

5 – 10 minutes – 50°

5. Place in the column

Centrifuge - 11000 RPM, 0.5 minute
Washing - μl700 of Buffer NT3

2 reps

6. Place column without liquid into the centrifuge for 2 minutes
7. Put the column into a new vial and give it 30 µl Elution Buffer
8. Label the vial E + P RFP and wait a minute
9. Put it in a nanodrop

Ligation

1. Add appropriate amount of deionized H2O to sterile mL tube.
2. Add 2 µL ligation buffer to the tube. Vortex buffer before pipetting to ensure that it is well-mixed Remember that the buffer contains ATP so repeated freeze thaw cycles can degrade the ATP, thereby decreasing the efficiency of ligation.
3. Add appropriate amount of insert to the tube.-
4. Add appropriate amount of vector to the tube.-
5. Add 0.5 µL ligase.-
6. To ensure you add only 5 µL, just touch your tip to the surface of the liquid when pipetting.-
7. Let the 20 µL solution sit at 22.5ºc for 30 minutes.-
8. Denature the ligase at 65ºc for 20 minutes.-
9. (ng vector x ratio x bp insert) / (pb vector) =ng insert

Transformation

1. Through a tube of chemically competent DH5-alpha cells by removing them from the 80 and placing the tube on ice for 20 min.-
2. Gently remove 50ul of cells into a clean 1.5ml pre-chilled labeled tube for each transformation.
3. Add 5ulfrom the ligation mix into each tube and mix by swirling the tip in the tube.
4. Incubate the tubes on ice for 30min.
5. Transfer the tubes to a 42ºc water bath for exactly 1min and place back in ice for additional 5min.
6. Resuspend the cell in 1ml of SOC medium and incubate at 37ºc for 1 hour shaking at 250 rpm.
7. Plate 1ul and 100ul of the cells on LB agar plates supplemented with the appropriate antibiotics and incubate overnight at 37ºc.

LB Agar Plates

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1. Weigh 17.5 grams of LB Agar powder, add into sterilized Pyrex container.

2. Fill with sterile water up to 500 ml mark.

3. Cap loosely and tape with autoclave tape.

4. Autoclave until it reaches 120 °C.

5. Once it reaches 120 °C count 15 min (try to keep the temp at 120 °C during those 15 minutes) and then turn down the temperature until it reaches 0°C.

6. Take the container out and wait until it is cool enough that it doesn't hurt to touch.

If starting from solid agar in the fridge, heat in microwave (cap unscrewed) 3min on high, 5 min on 30% (till it melts completely) Wait till cool enough so that you can touch it for a minute (60 degree Celsius)

7. Add appropriate volume of Amp or Kan (1000X). For 500 mL of LB Agar, add 500 uL of each antibiotic needed.

8. Swirl to mix, use pipette to transfer 20 mL into each plate or simply pour it carefully in the plate. Try to get the formation of bubbles.

9. Allow plates to cool and solidify. Mark antibiotic on side of plate.

If LB Agar solidifies before being poured, microwave to liquefy again.

TSS

Preparation

To make 50 mL:

1. 5g PEG 8000
2. 1.5 mL 1M $MgCl_2$ (or 0.30g $MgCl_2$*$6H_20$)
3. 2.5 mL DMSO
4. Add LB to 50 mL

Filter sterilize (0.22 μm filter)

Notes

1. PEG 3350 can be used instead of PEG 8000. According to the original CT Chung paper, PEG 3350 produces better efficiency, and other sized PEGs can be used as well albeit with a slight loss in efficiency.

2. pH from original Chung CT paper calls for pH to be acidic (pH=6.5)... be sure to pH solution before sterilization.
3. If using non-chemically resistant filters (e.g., cellulose nitrate), add DMSO after sterilization. DMSO should be sterile in and of itself, so it may be wise to add it afterwards if you are unsure about the compatibility of your filters.
4. Store at 4˚C or -20˚C.

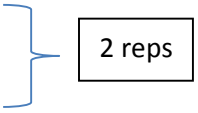
Electrophoresis gel:

1. Into the Erlenmeyer flask:
2. 0.5g TAE 50µl + Agaroz
3. 60 sec. in the microwave
4. 5 minutes cooling
5. Add 2.5 µl SYBR SAFE
6. Pour into a working tray

Cutting reactionary plasmid PET :(cutting reactions in µl100)

1. Enzyme restriction Xho1- µl5
2. Enzyme restriction NCo1- µl5
3. Plasmid PETTR µl20
4. Buffer FDgreen µl10
5. DDW µl60
6. Incubation for 20 min at 37 ° C
7. To remove the phosphates from the edges add µl2.5 FAST AP (phosphatase)
8. Incubate for 15 minutes at 37 ° C.
9. Then move to 80 ° C for 10 minutes to stop the action.
10. We load onto agarose gel
11. We cut the DNA together with the gel
12. We produce DNA according to the cleaning protocol - DNA from the gel:

- Weigh an Eppendorf vial
- Weigh vial + gel
- Calculate how much a cube of gel alone weighs
- Add µl380 NTI gene vial at the beginning
- 5-10 minutes at 50°
- Place in column
- Centrifuge - 11000 RPM, 0.5 minute
- Washing - µl700 of Buffer NT3



13. Place column without liquid in a centrifuge for 2 minutes.
14. Place the column in a new vial and give it 30 µl Elution Buffer.
15. Sample testing with a nanodrop device.

.**Plasmid prep**

1. Centrifuge, 4000 rpm for 10 minutes.
2. Prepare a bench and put the tubes there.
3. Input 150 microliter of Buffer A1.
4. Input 250 microliter of Buffer A2, flip six times.
5. Let it stay for two minutes.
6. Input 350 microliter of Buffer A3, flip six times.
7. Centrifuge on full rpm for 5 minutes
8. Put the tubes content into the double containers from the kit.
9. Centrifuge in 5000
rpm for one minute, dump liquids afterwards.
10. Input 450 microliter of Buffer AQ.
11. Centrifuge on full speed for one minute, dump the liquids.
12. Centrifuge on full speed for one minute.
13. Input 50 microliter of Buffer AE, wait for a minute.
14. Centrifuge on full speed for one minute.

Making a lysis buffer

1. 50 mM buffer phosphate pH 7.6 30 ml.
2. 300 mM NaCl 3.6 ml.
3. 0.2 mg/ml lysozyme 1.2 ml.

Exploding membranes

1. Put 300ulx12 of lysis buffer into 6+6 eppendorfs.
2. Do sonication.
3. Centrifuge on max speed for 20 minutes in 4c temp.

4. And you get 6 tube sup and 6 tube pellet.

Expressing protein

1. Make starters.
2. Put 50x6 ul AMP and 500x6 ul starter into 6 cube tubes.
3. Put in a spectrophotometer on OD 600 looking for 0.6-0.8 results.
4. Put IPTG in tubes for 4 hours in 37c temp.
5. Then put 1ml from the tubes to eppendorfs.
6. Add 1ml 1% glu to the eppendorfs.
7. Centrifuge on max speed for 10 minutes.
8. Test in gel.

PCR Colony Phusion

1 Prepare tubes with 15 µl water and 4 colonies for each tube.

2 Prepare the master mix for 15 µl reaction:

H2O – 96 µl

PCR Mix – 180 µl

P1 – 18 µl

P2 – 18 µl

Colony – X

3 In new tubes insert 13 µl master mix and 2 µl colony and make 2 control tubes one positive with 15 µl of closed plasmid and one negative with 15 µl of water.

4 PCR config for 15 µl:

Cycle step	Temp	Time
Initial denaturation	95c	5 minutes
30 cycles	95c	30 seconds
	56c	30 seconds

	72c	1.45 minutes
Final extension	72c	5 minutes
Hold	4c	Infinite

Heat shock

1. Bath on 42.7c temp for 1 minute.
2. Put into ice.
3. Take 1ml LB and put into the tubes.
4. Put in the incubator for 30 minutes.

How to make a Page SDS

Bottom part –

1. H2O – 3.74 ml
2. 30% Acrylamide – 4.16 ml
3. 1.5M Tris pH 8.8 – 2 ml
4. 10% SDS – 0.1 ml
5. 10% APS – 0.05 ml
6. TEMED – 0.01 ml

Top part –

1. H2O – 2.9 ml
2. 30% Acrylamide – 0.66 ml
3. 1.5M Tris pH 8.8 – 0.4 ml
4. 10% SDS – 0.04 ml
5. 10% APS – 0.02 ml
6. TEMED – 0.01 ml

Ethanol Precipitation

1. Ligation mix – 20 µl
2. H2O – 50 µl
3. Glycogen – 2 µl
4. NaOH – 30 µl
5. Vortex for 20 seconds
6. Ethanol – 200 µl
7. Centrifuge max speed for 20 minutes on 4c temp
8. Extract all the water carefully after carefully taking the tube from the centrifuge.
9. Add 300 µl of Ethanol 70%
10. Centrifuge max speed for 5 minutes on room temp
11. Extract all the liquid carefully
12. We leave the tube open for 2 minutes to let the alcohol evaporate
13. Add 15 µl to the tube
14. Vortex
15. And you have dna without salt

Ni protocol

1. Transform the pETtr plasmids containing genes 1, 2 and 4 into BL21(DE3) (LB + Amp+ 1% glucose plates) electrocompetent cells (total 3 transformations)
2. Pick up a single colony and grow up an overnight starter with 1% glucose + Amp (3 ml) and incubate in a shaker incubator @ 37^0C 250 RPM.
3. In the next morning inoculate 50 ml LB culture 1:100 (LB + Amp) and grow up at 37^oC till OD_{600} = 0.6.
4. Add IPTG to a final concentration of 0.5mM and grow the culture for additional 4h.
5. Harvest cells by centrifugation and freeze pellet at -20^0C.
6. Lyse cells in lysis buffer (20ml 50mM Na Phosphate pH 7.6, 300mM NaCl, 0.2mg/ml lyzozyme, 10mM imidazole and EDTA free protease inhibitor) and sonication.

7. Clarify lysate by centrifuging the cells @ 12,000g @ 4^0C.
8. Load the clarified lysate onto a column containing pre-equilibrated 0.5ml NiNTA-agarose (NEB) (collect sample for analysis).
9. Wash 3 times with 5ml wash buffer (50mM Na Phosphate pH 7.6, 300mM NaCl, 50mM Imidazole, total 15ml) (collect samples for analysis).
10. Elute the protein with 0.5ml fractions of elution buffer (50mM Na Phosphate pH 7.6, 300mM NaCl, 250mM Imidazole).