Agrobacterium Transformation and Competent Cell Preparation

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Agrobacterium Transformation

Materials:
Gene-Pulse Cuvettes, 0.2cm (BIO-RAD #1652086)
LB
Spectinomycin
Rifampicin
LB plates with antibiotic

Protocol:
1. Dilute plasmid to 15 ng/µL (if the [salt] is too high, the cells will be killed by the electrical impulse)
2. Mix 2 µL diluted plasmid to 50 µL GV3101 agrobacterium in a 1.5mL tube
3. Transfer mixture into a cuvette that has been chilled for 20 min @ -20°C by adding to the side & then tapping down to eliminate air bubbles
4. Set the electroporator to 2.00 V (leave all other settings at default: 200 Ω , capacitance extender 250 µFD, capacitance 25µFD)
5. Snap cuvette into proper orientation, push in & hold both pulse buttons until beep (may take a few seconds) the time constant should read about 4.7
6. Add 1mL LB or SOC to cuvette & pipette up & down to mix, transfer 1 ml microfuge tube
7. Incubate at 28°C (cells will die at 37°C) for 2-3 hours before plating ~70 µL on LB+spectinomycin+rifampicin plates
8. Wrap plates with micropore & incubate at 28°C for 2-3 days, it really takes at least 2 days, even at 28°C so don't be disappointed if you don't see colonies the next day, be patient
9. Make liquid cultures from colonies for PCR testing

Agrobacterium Competent Cell Preparation

Materials
LB plates with 30 µg/ml Gen (2 plates is enough)
2 1 L culture flasks
2 sterile 500 ml centrifuge bottles
1.5 - 2 L sterile dH_2O (should be cold)
50 ml sterile10% glycerol in dH_2O (should be cold)

Protocol:
Note: The Agrobacterium we use is GV3101, which has both resistance to Rif (10µg/ml, in genome), Gen (30 µg/ml, in helper plasmid) This protocol is often done with any antibiotics but you can add Gen to plates and starter liquid cultures to be one the safe side.

1. From a frozen glycerol stock grow a 20 ml culture of Agrobacterium overnight at room temperature
2. The following morning dilute the culture 64,000 fold by adding 0.5 µl of culture to 2 ml of H_2O making a **4000 fold** dilution. From the 4000 fold dillution take 0.5 µl and add to 2 ml H_2O making a **16,000 fold** dilution, from the 16,000 fold dilution take 0.5 µl and add to 2 ml H_2O making a **64,000 fold** dilution
   Note: 0.5 µl of culture into 50 ml H_2O makes a 100,000 fold dilution and you can use 10 µl of this on a plate
3. Add 5 µl of the 64,000 fold dilution to an LB plate and thoroughly spread the culture on the plate, incubate at room temp or 28°C for 1 - 3 days
4. Once you have single colonies on a plate, pick a colony and start a 20 ml overnight culture in LB with Gen at 28°C , you can start this overnight culture early in the day as Agro grows slow and you want a thick culture for the next day. Make sure to take LB out of the fridge so that it is at room temperature for the 500 ml cultures the next day
5. Inoculate 2 500 ml flasks (use 1 liter flasks) of LB with 9 ml of the overnight culture, do not use antibiotic here, Make sure to start these cultures early in the day (if possible < 8:00am) as Agro grows very slowly, a culture started around

8:00am will be ready hopefully by 3:00pm

6. Grow cells to an $OD_{600\,nm}$ of 0.5 - 1
7. Precool Centrifuge with 500 ml bottle adaptors to 4°C
8. When cells are ready to harvest chill flasks on ice for 15 - 30 minutes
9. Centrifuge at 4000 g in bottles for 15 minutes at 4°C
10. Remove as much of the supernatant as possible and resuspended the pellet in each bottle in 500 ml of ice cold water, use graduations on side of centrifuge bottle to measure volume
11. Centrifuge at 4000 g for 15 min
12. Remove supernatant and resuspended the pellet in each bottle in 250 ml of ice cold water
13. Centrifuge at 4000 g for 15 min
14. Remove supernatant and resuspended the pellet in each bottle in 10 ml ice cold 10% glycerol
15. Combine contents of bottle into 1 50 ml Falcon tube (It will stand up the centrifugation force)
16. Centrifuge at 4000 g for 15 min
17. Remove supernatant and resuspend the pellet in 2 - 3 ml ice cold 10% glycerol. The cell concentration should be about 1 - $3*10^{10}$ cells/ml
18. Store in 1.5 ml microfuge tubes in 50 µl aliquots, freeze in LN_2, store in -80°C, cells are good for at last 6 months

Adapted from protocol by Sanjay

Agrobacterium Transformation Reagents

LB Liquid, 200 ml
In 200 ml dH_2O
+ 5 g LB Broth (Difco, Luria-Bertani)
Autoclave for 20 min

LB Plates, 200 ml
In 200 ml dH_2O
+ 5 g LB Broth (Difco, Luria-Bertani)
+ 3 g Agar (Fishger, BP 1423-500)
Autoclave for 20 min, swirl immediately, allow to cool to touch
Pour plates (≈ 35 ml) label, store at 4°C

4 mg/ml Ampicillin, 10 ml
In 10 ml of sterile dH_2O
+ 0.04g Ampicillin (Roche 70043429)
Filter sterilize using 0.45 µm syringe filter (Millipore Millex HA SLHA033SS)
Aliquot into sterile 1.5 ml microfuge tubes and store at -20°C

10 mg/ml Spectinomycin, 10 ml
In 10 ml sterile dH_2O
+ 0.1 g Spectinomycin
Filter sterilize using 0.45 µm syringe filter (Millipore Millex HA SLHA033SS)
Aliquot into sterile 1.5 ml microfuge tubes and store at -20°C

34 mg/ml Rifampicin, 10 ml
In 10 ml sterile dH_2O
+ 0.34 g Rifampicin
Filter sterilize using 0.45 µm syringe filter (Millipore Millex HA SLHA033SS)
Aliquot into sterile 1.5 ml microfuge tubes and store at -20°C in the dark, rifampicin is light sensitive

Antibiotic	**Stock (mg/ml)**	**Working (µg/ml)**	**µl of Stock in 5 ml**	**10 ml**	**20 ml**	**200 ml**
Ampicillin	10	50	12.5	25	50	500
Spectinomycin	10	100	50	100	200	2,000
Rifampicin*	34	150	22	44	88	882
Gentamici**n**	10	30	15	30	60	600

* light sensitive: store stock solutions and plates in the dark

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10% Glycerol, 200 ml
In 180 ml dH_2O
+ 20 ml glycerol
Autoclave for 20 - 30 min, cool and store in cold room