

GAP Lab
Protoplast Isolation and Transformation of an *A. flavus* pyr mutant
Protocol

1. Start an overnight culture of 3357-5 by putting 1-3ml of Triton-X100 on an older, sporulating plate and scraping the whole plate using a glass spreader bar, add to 100ml of PDBU grow at: 200rpm and 28°C for 12-16hrs, 30°C 10-12h
2. The following day freshly prepare the enzyme solution
3. Pour the culture into 2 50ml conical tubes
4. Centrifuge the overnight culture at "6" for 10 minutes in the clinical centrifuge. (Around 8k RPM in swinging bucket)
5. Pour off supernatant and resuspend with sterile water.
6. Centrifuge at "6" for 12 minutes in the clinical centrifuge.
7. Pour off supernatant
8. Filter the enzyme solution using a .45 μ filter and add 15ml of the enzyme solution for each 5ml of tissue (each conical tube).
9. Shake for 3-4hrs at 70rpm at 30°C
10. Centrifuge at 200rpm for 1 minute in a swinging bucket rotor or at "2" for 1 minute in the clinical centrifuge.
11. Pipet the supernatant into a new 50ml conical tube and top off to 50ml with STC buffer, (use a 50ml serological pipet to slowly remove only the supernatant).
12. Centrifuge at 3000rpm for 5 minutes in a swinging bucket rotor or at "4" for 10 minutes in the clinical centrifuge
13. Pour off supernatant
14. Resuspend in 50ml STC buffer
15. Repeat step #12
16. Pour off supernatant leaving just a little STC in the bottom of each conical, be careful not to tilt the tube back up to soon or you will need to resuspend in STC and spin again.
17. Create 1:10 and 1:20 dilutions in STC
18. Using the hemocytometer, count the protoplasts present in five of the boxes
19. Calculate total number of protoplasts using the formula:
protoplasts x dilution x 50,000
20. If necessary dilute the protoplasts to $<1 \times 10^8$ /ml, generally the concentration is about 1×10^7 /ml
21. Add 100 μ l of diluted protoplasts to 1.5ml tubes (one with just protoplasts, one with just auxotrophic marker, and the rest with your constructs)
22. Add 1-10 μ g of each DNA to be transformed
23. Mix by gently pipeting up and down
24. Incubate on ice for 20 minutes
25. Add 1ml of 50% PEG
26. Mix by gently pipeting up and down
27. Incubate at RT for 20 minutes
28. Plate 200-300 μ l of protoplasts on the surface of recovery media
 - a. MLS
 - i. Protoplasts (negative control)
 - b. MLS+U
 - i. Protoplasts (positive control)
29. Stack the plates and incubate at 37°C for 2-4 days.
30. Pick colonies using sterile toothpick and transfer to 60mm PDB agar plates, grow 3-4days and isolate DNA using CTAB method if needed to pcr screen for knockout.

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Materials and Solutions

PDBU (100ml)

- 2.4g Potato Dextrose Broth
- 0.112g uracil
- 100ml diH₂O

Enzyme Solution (30ml)

- 25.5 ml 1.2M NaCl (7g in 100ml diH₂O)
- 3ml 0.2M NaPO₄
- 0.14g Lysing enzyme (4°C) Sigma #L-14122
- 0.08g Driselase (-20°C) Sigma #D-9515
- 300µl β-glucorinidase (4°C) Sigma #G-0876
- 0.08g BSA (4°C dry) Sigma #A2153-100G

50% PEG (100ml)

- 50g PEG (MW 4,000)
- 1ml 1M stock TrisCl (pH7.5)
- 1ml 1M stock CaCl₂
- Filter sterilize

STC Buffer (500ml)

- 109.32g Sorbitol
- 5ml 1M stock TrisCl (pH7.5)
- 5ml 1M stock CaCl₂
- 500ml diH₂O
- Autoclave and store at 4°C

MLS (500ml)

- 17.5g Czapeck's broth
- 26.43g 0.4MNH₄SO₄
- 5g agar

MLS+U

- 17.5g Czapeck's broth
- 26.43g 0.4MNH₄SO₄
- 5g agar
- 0.561g uracil