

## Protoplast Isolation and Transformation

1. Start an overnight culture of spores of the appropriate strain to be transformed by putting Triton-X on an older, sporulating plate and sterilely scrape off conidia. Place conidia in 250 ml PDB(U) medium in incubator at 37C, shaking at 150 rpm.
2. The following day, prepare enzyme solution as follows and keep stirring slowly:

	[Stock]	20ml
DH <sub>2</sub> O		17 ml
10 mM NaPO <sub>4</sub> pH5.8	0.2 M	2 ml
20 mM CaCl <sub>2</sub>	1.0 M	0.4 ml
B-glucorinidase (105 u/ml)		0.2 ml
Novozym 234		100 mg
1.2 M NaCl		1.4 g

3. Vacuum filter the overnight culture through a sterile Buchner funnel and Miracloth filter into a previously weighed 125 ml Erlenmyer flask.
4. Weigh out 2.0 g of mycelia into the flask using a sterile spatula.
5. Filter 20 ml of enzyme solution/g of tissue into the flask through a 0.45  $\mu$ m filter.
6. Shake for 3 hrs at 120 rpm.
7. Filter through a premade tube with cheesecloth and wire mesh into a 50 ml conical sterile tube and top off with 50 ml of Protoplast Resuspension Buffer.
8. Centrifuge at "4" for 5 min using a tabletop centrifuge.
9. Pour off supernatant and resuspend in 50 ml Protoplast Resuspension Buffer. Spin again as in step 8.
10. Resuspend pellet in 1.0 ml Resuspension Buffer. Dilute a small sample (5  $\mu$ l) 1:100 or 1:1000 to count protoplasts.
11. Dilute all protoplasts to  $1 \times 10^8$ /ml.
12. Add 10  $\mu$ g of DNA to be transformed to transformation tube.
13. Add 100  $\mu$ l of diluted protoplasts ( $10^7$ ).
14. Add 50  $\mu$ l of 25% PEG, mix by tapping and incubate on ice for 15 min.

15. Add 1.0 ml 50 % PEG, mix by tapping and incubate at room temperature for 15 min.
16. Place DNA/protoplast mix on petri plate in small drops spread across the plate using a disposable pipet.
17. Add 9 ml of regeneration medium (that will sustain growth of recombinants) and swirl plates. Once plates solidify, incubate face up at 37C.

(+) control protoplasts (w/PEG additions) plated on PDA(U)

(-) control protoplasts (w/PEG additions) plated with MLS (regeneration medium)

#### Protoplast Resuspension Buffer

	Stock	100 ml
0.85 M KCl	3 M	28.4 ml
0.1 M CaCl <sub>2</sub>	1 M	10 ml
Di H <sub>2</sub> O		61.6 ml

#### PEG Solution

	Stock	50 ml
0.6 M KCl	3 M	10.0 ml
50 mM CaCl <sub>2</sub>	1 M	2.5 ml
10 mM Tris (pH8)	1 M	1.0 ml
PEG 8000 (for 25 %)		12.5 g
PEG 8000 (for 50 %)		25.0 g

Calibrate a small beaker by filling to 50 ml and marking glass at the meniscus. Empty and add ingredients. Fill to mark with di H<sub>2</sub>O. Filter sterilize.

#### MLS agar (Regeneration Medium)

	200 ml
Czapecks' Broth	7 g
0.4 M NH <sub>4</sub> SO <sub>4</sub>	10.57 g
Agar	2 g
1.0 M sucrose	68.5 g

For +ve control, add 0.01 M uracyl