

Transformation using protoplasts

Introduction

The following transformation protocol is based on resistant markers (e.g. hygromycinB, phleomycin) and is suited for gene disruption by targeted integration. 5-10 transformants/ μg DNA are obtained and about 10 % of the integration events occur at the homologous site. This procedure is derived from the methods of Tilburn *et al* . (1983) and Mallardier *et al* . (1989).

Materials

- **MM** (Minimal medium) : glucose, 10 g ; ammonium tartrate 0.92 g ; KCl, 0.52 g ; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.52 g ; KH_2PO_4 , 1.52 g ; trace elements solution (Cove, 1966) 1 ml ; H_2O 1 l ; pH adjusted to 6.8 with NaOH. Autoclave 30 min at 105°C.
- **MMS** : Minimal medium with 1M saccharose and 1 % oxoid agar (Unipath, England). Autoclave 30 min. at 105°C.
- **Soft agar** : MMS with 0.35 % oxoid agar. Autoclave 30 min at 105°C.
- **MS** : 1M sorbitol in 10 mM MOPS buffer pH 6.5. Filtrate on 0.45 μm filter.
- **MSC** : MS with 10 mM CaCl_2 . Filtrate on 0.45 μm filter.
- **PEG 60 %** (w/v) : PEG 4000 or 6000 (Merck) in MSC. Filtrate on 0.45 μm

Equipment

- 50°C water bath
- Microman pipettor (Gilson)
- Microcentrifuge

Procedure

1. Wash the pellet of protoplasts (see above protocol) twice in MSC and resuspend in MSC to get 5 to $25 \cdot 10^7$ protoplasts/ml.
2. In a 1,5 ml microcentrifuge tube mix 200 μl of the protoplast suspension ($1 \cdot 10^7$ protoplasts) and 5-20 μl of TE containing 1-5 μg of transforming DNA (linear fragment).
3. Using a special pipettor for viscous solutions add 50 μl of 60 % PEG. Gently homogenize by pipeting up and down. Incubate 20 min (or more) on ice.
4. Add 500 μl of 60 % PEG, homogenize by pipeting up and down. Incubate 20 min at room temperature.
5. Microcentrifuge 5 min at room temperature at 13.000 rpm. Take off the PEG. The protoplasts stick to the wall of the tube. Microcentrifuge 1 min more to pellet the protoplasts, take off the remaining PEG.

6. Resuspend the protoplasts in 200 μ l MSC, transfer in 4 ml sterile plastic tube, add 3 ml of soft agar MMS and spread onto MMS plates. After agar solidification, incubate at room temperature overnight for the expression of the HygromycineB^R or Phleomycin^R gene.
7. The following day, add 3ml of soft agar MM containing Hygromycin B (200 μ g/ml) or phleomycin (20 μ g/ml) and incubate until transformants appear.
8. Growing colonies are replicated with a toothpick on selective medium (MM+HmB or phleo) and then single colonies corresponding to the growth of an uninucleate conidium are isolated.
9. Southern blot hybridization analysis of the stable HmB^R /Phleo^R colonies determinates which transformants have integrated the disrupted gene at the homologous site.

Timetable

day 1 : Fungal culture overnight/18h

day 2 : Protoplast production 2-3h, transformation 2h

day 3 : Overlay of selective medium

Tips and general comments

1. Germinated spores give more competent protoplasts than hyphae. Protoplasts isolated from hyphae vary in size and organelle constitution and have low regeneration rates.
2. Quicker are made the protoplasts, more competent they are. Protoplast formation should not exceed 90 min.
3. The hydrolytic enzymes present in crude enzyme preparation (Glucanex or Novozyme) vary from batch to batch and its concentration should be tested before starting the transformation experiment. Fincham (1989) reported that depending on the batch of enzyme the frequency of transformants may slump 100 fold with *Neurospora crassa*.
4. For each transformation experiment, at least 1.10^7 protoplasts are needed. If fewer protoplasts are used , the frequency of transformants decreases.

Additional Information Provided By Raquel Lopez

When overlaying protoplast suspension slide buffer slowly down side of tube.

Protoplasts appear as a very dusty whitish interface

Use MSC to make dilutions for counting cells, not water.

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References

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