Procedure for preparing and transforming spheroplasts of *Neurospora crassa*.

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Background: The following procedure is derived from several previously published works (Schweizer, M. et al. 1981; Akins and Lambowitz 1985; Orbach et al. 1986; Vollmer and Yanofsky 1986; Selitrennikof and Sachs 1991). The procedure is designed to produce a large number of spheroplasts to be stored frozen at -80°. We have found spheroplast transformation especially useful for rescuing mutants using cosmid libraries since high transformation frequencies can be obtained using uncut cosmids. Rescue strategies may involve sib selection (*eg*: Akins and Lambowitz 1985) when the chromosomal location of the gene of interest is not known, or a chromosome walking approach using the ordered cosmid information from the genome project if the approximate chromosomal location is known. Spheroplasts may also be used for routine transformations with plasmids and linear DNA.

Production and harvesting of conidia. For strains that produce conidia in amounts comparable to wild type, start with five conidia flasks. These are 250 ml Erlenmeyer flasks each containing 50 ml of solid Vogel's medium and plugged with cotton or foam plugs. They are inoculated with the desired strain in the center of the agar and placed in a 30° incubator for one to two days; until the mycelium begins to climb up the sides of the flask. They are then removed to a well lit room and allowed to conidiate for four to seven days at room temperature. Do not allow conidia to become much older than this or germination rates will decline.

To harvest the conidia prepare about 200 ml of autoclaved distilled water and allow it to cool to room temperature. Prepare a Buchner funnel (about 7 to 10 cm diameter) with two to three layers of wetted cheesecloth laid across the filtering surface, then wrap it in aluminum foil and autoclave. Using sterile technique, distribute the sterile water to the five conidia flasks, replace the plug, and vigorously swirl the water in the flask to suspend the conidia. Allow the "dust" of conidia to settle for about one min, then pour the suspended conidia through the cheesecloth on the Buchner funnel which is set up to drain into the bottle originally used for the sterile water.

The conidia should then be counted using a microscope and haemocytometer. Make appropriate dilutions to give a reasonable number of conidia to count. This is usually a 1/100 to 1/1000 dilution. The conidia should be used to inoculate a culture within 24 hours of being harvested – and the sooner the better.

Preparation of spheroplasts: Prepare two 2 liter baffled flasks each containing 500 ml of liquid Vogel's medium and inoculate these with the harvested conidia to a final concentration of between 5×10^6 to 1.5×10^7 conidia/ml. (If the concentration is much higher, the germination time is extended.) Incubate at 25°C to 30°C with gentle shaking (about 200 rpm). After 2.5 to 3 hr, use sterile technique and remove a few microliters of the culture. Examine these microscopically using a haemocytometer. Count the total number of conidia. Then count again, but only count the germinated conidia. (A conidium is considered germinated when a small protuberance is observed.) The goal is to achieve 80% germination. Continue the procedure of counting every 30 min until at least 80% germination is achieved. Do not continue past this, as spheroplasting efficiency will be reduced. The time required to achieve 80% germination varies

from strain to strain. Wild types are usually ready in 4 to 5 hr at 25°, or 3 to 3.5 hr at 30°, but slow-growing mutants can take up to nine hours. Note that some mutant strains may never reach 80% germination. Thus, some judgment may be required to decide when enough germination has occurred versus having those that have germinated become too old. We have made spheroplasts successfully from cultures that have achieved only 50-60% germination.

Once the 80% germination level has been reached, harvest the conidia in sterile bottles for a Sorvall GSA rotor (or Beckman JA14) at 5,000 rpm for 10 min at 4°. Remove the supernatant carefully by aspiration. The pellets are soft and easily disturbed. Keep the samples on ice.

Wash the conidia once with cold sterile distilled water and once with cold sterile 1M Sorbitol removing the supernatant by aspiration each time. Resuspend the final pellet in about 6 to 9 ml of cold 1M sorbitol. Determine the concentration of conidia using a haemocytometer. The maximum concentration should be 1×10^9 conidia/ml. Dilute with 1 M Sorbitol if necessary.

Transfer the suspension to a sterile 250 ml Erlenmeyer flask. Add 8 to 15 mg of Lysing Enzymes per ml of suspension. The amount of Lysing Enzymes used depends on the activity of the lot being used (see below). Incubate at 30°C with gentle agitation (100 rpm) for 30 to 60 min. The extent of spheroplasting should be monitored every 15 min as described in the next paragraph. (NOTE: As it relates to the final frequency of transformation obtained, there is considerable variation in the quality of the Lysing Enzymes from different suppliers, and even among different lots of enzyme from the same supplier. Therefore, once a good supply is identified it is advisable to accumulate a large stock for future use. It survives quite nicely at -20° for years. The last good batch of Lysing Enzymes we obtained was from Sigma, catalogue number L1412, lot number (69H1557). This was obtained in about 2003, so it is doubtful that any of this lot is still available. Hopefully, the succeeding lots will be as effective.)

Monitor the extent of spheroplasting by removing two 10 µl aliquots and placing them about 1 cm apart on a microscope slide. Add 10 µl of 10% SDS to one drop and examine both microscopically. The untreated sample should look like normal conidia while the SDS-treated sample will have some normal conidia and some “ghosts” which represent lysed spheroplasts. Count ghosts as a percentage of the total. The desired level of spheroplasting is >90%. Once this level is reached the reaction should be processed as described below to prevent reaching the protoplast stage (no cell wall left).

Pour the contents of the flask into a sterile 50 ml capped disposable tube and spin at the lowest speed in a clinical centrifuge for 10 min. Remove the supernatant by aspiration. Wash the spheroplasts once with sterile 1 M Sorbitol and once with sterile MCS (10 mM MOPS, pH6.3; 50 mM $CaCl_2$; 1 M Sorbitol). Spheroplasts are fragile so the pellets should be suspended by gentle inversion and/or gentle stirring with a sterile glass rod or pipette tip. Suspend the final pellet in 6 to 8 ml of sterile MCS.

Determine the concentration of the sphaeroplasts suspension using a haemocytometer. A dilution series (1/10, 1/100, 1/1000) in 1 M Sorbitol will be required. Adjust the spheroplast concentration to between 5×10^8 to 5×10^9 sphaeroplasts per ml using sterile MCS. If the

concentration is already too low spin again and resuspend the sphaeroplasts in an appropriate volume of MCS.

For each ml of sphaeroplasts solution add 13 µl DMSO; 65 µl filter sterilized Heparin (5mg/ml); and 275 µl sterile PMC (40% PEG-4000; 10 mM MOPS, pH 6.3; 50 mM $CaCl_2$). Mix gently but completely. Aliquot the spheroplasts to sterile eppendorf tubes and store at -80º. Depending on future needs, the size of the aliquots should be varied. For example, 50 µl aliquots are good for testing viability and transformation frequency. Aliquots of 1 ml are useful for large transformation experiments using several different pools of cosmids or several different individual cosmids. Color-code the eppendorfs according to the volume they contain to make finding the appropriate tube easier once they are at -80º.

Determining viability of the spheroplasts. The following day thaw a small aliquot of sphaeroplasts on ice to determine the viable sphaeroplasts concentration. Prepare a dilution series (1/1000, 1/10,000, 1/ 100,000 and 1/1,000,000 dilutions) using sterile 1 M Sorbitol. Add 100 µl of each dilution to a separate tube containing 50 ml of top agar, mix well by inversion, and spread the 50 ml evenly over 5 viability plates. Incubate the plates 24 to 48 hr at 30ºC. Count the number of colonies on the viability plates and determine the sphaeroplasts viability. Viability should be greater than 1×10^7 per ml.

Viability Plates
20 ml 50x Vogel's
1 ml Biotin solution
1 ml Trace Elements
15 g Agar
850 ml dH_2O
Plus supplements, if required
Autoclave 20 min, add 100 ml sterile 10X Sugars

10X Sugars
200 g Sorbose
5 g Fructose
5 g Glucose
2 g myo-inositol
Add distilled H_2O to 1 litre, dissolve completely, autoclave 20 min

Top Agar
20 ml 50x Vogel's
182 g Sorbitol
1 ml Biotin solution
1 ml Trace Elements solution
15 g Agar
850 ml distilled H_2O
Plus supplements, if required.

Autoclave 20 min, add 100 ml sterile 10X Sugars, mix well, cool to 44º in a water bath before using. Left over top agar can be stored at room temperature under sterile conditions and reautoclaved, or melted with a Bunsen burner, when needed.

Transforming the spheroplasts. The number of sphaeroplasts used in a transformation depends on the amount of DNA required to maintain a sphaeroplast to DNA ratio of 6×10^6 to 3.5×10^7 viable spheroplasts per μg of DNA. For example, in a sib selection procedure using pools of about 250 cosmids, as much as 10 μg of DNA might be used. The amount of DNA used when transforming a single cosmid might be only 0.1 to 1 μg. To test the transformability of newly prepared spherolplasts choose a plasmid that contains a selectable drug resistance marker for *Neurospora* (eg, benomyl, hygromycin, bleomycin, etc) and use 1 μg in a test transformation.

Thaw spheroplasts on ice. Prepare the DNA to be transformed by placing the amount required in an eppendorf tube. The volume can be as high as 50 μl, but should not exceed 50% of the volume of the spheroplasts being transformed. Add 5 μl of heparin (5 mg/ml) and 2 μl of 50 mM spermidine·3HCl to the DNA, mix, and leave on ice for 15 min. Add the DNA to the spheroplasts. Mix gently and leave on ice for 30 min.

Lipofectin has been reported to increase transformation frequency by 3 to 10 fold (Selitrennikoff and Sachs 1991). We have not used this modification, but if maximal transformation frequencies are required, it may be useful. The protocol calls for 35 μg of lipofectin to be added for each ml of spheroplast suspension used following the above 30 min incubation. Incubation is continued for an additional 15 min.

Regardless of whether lipofectin is used or not, sterile PMC is then added to 10 times the volume of the spheroplasts plus DNA mixture. Mix gently and incubate at room temperature for 20 min. The transformation mixture is added to Top Agar (10 mL/plate) that contains the selectable antibiotic of choice. Mix gently but thoroughly, and layer on to plates made according to the "viability plate" recipe except that the plates should now also contain the chosen antibiotic. The plates are incubated at 30°C for 2 to 7 days.

Transformation frequencies depend on a number of factors including quality of Lysing Enzymes, the strain being transformed, the nature of the selection for transformants, and the size of the DNA used for transformation. Considering all these factors, transformation frequency with a 50 kb cosmid using selection for hygromycin resistance could give a frequency of 100 to 2000 transformants per μg of DNA. A 5 kb hygromycin resistance-containing plasmid could give as many as 10,000 colonies per ug of DNA.

References:

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