

Protocol for the Electroporation of *Neurospora* Spheroplasts

Gregory O. Kothe and Stephen J. Free- Department of Biological Sciences, SUNY/Buffalo, Buffalo NY 14260

A protocol for electroporating *Neurospora* strains is described. This protocol uses spheroplasts for transformation rather than germinated conidia with enzyme-weakened walls. High efficiency transformation is obtained with this system even with batches of Novozyme234 which worked poorly in the PEG based protocol.

The transformation of fungal cells is critical for an understanding of how they regulate transcriptional activity. PEG-mediated transformation procedures have been developed and extensively used to study the molecular biology of fungi. In using a PEG-mediated transformation procedure (Vollmer and Yanofsky 1986. *Proc. Nat. Acad. Sci. USA* **83**:4869-4873), we found that when we changed the lot of Novozyme 234 used to create *Neurospora* spheroplasts, our transformation efficiency dramatically decreased. In response to the difficulty in finding a lot of Novozyme 234 which gave adequately high transformation frequencies with the PEG-mediated transformation system, we have developed an electroporation procedure which gives high efficiency transformation even with lots of Novozyme 234 which worked poorly in the PEG-based system. Our electroporation system differs from the system described by Chakraborty, Patterson and Kapoor (1991 *Can. J. Microbiol.* **37**:858-863) in that we use spheroplasts for the transformation while they used germinated conidia whose cell walls had been weakened by a -glucuronidase treatment. We find that our procedure using spheroplasts gives a higher transformation frequency and is more reproducible than the germinated conidia procedure. An electroporation system similar to ours was mentioned by Arganoza, Ohnberger, Min and Akins (1994 *Genetics* **137**:731-742), but the procedures to prepare the cells and to perform the electroporation were not given in detail.

The electroporation protocol described below has allowed us to generate large numbers of transformants and was developed as part of an insertional mutagenesis experiment. We have been able to generate between 10,000 and 50,000 transformants per 10⁹ spheroplasts. The number of transformants is only limited by how many conidia are used, how much DNA one has and how many times one is willing to push buttons on the electroporation apparatus. We have used the procedure to transform spheroplasts successfully with pRAL-1, pCNS43 and pBARGEM7-KS1 plasmids. The procedure is as follows:

- 1) To obtain large numbers of conidia, grow the strain of interest on appropriately supplemented Vogel's agar medium (2% agar). We use four 500 ml Erlenmeyer flasks with 100 ml of medium in each for each transformation experiment. Four flasks are usually sufficient to give 10⁹ conidia from which 10,000 to 50,000 transformants can be generated. We grow the *Neurospora* at 30°C for 7 to 10 days. It is important to have large numbers of young healthy conidia.
- 2) Harvest the conidia into 400 ml of appropriately supplemented Vogel's liquid medium by pouring 100 ml of medium into each of the four flasks, suspending the conidia and filtering the

medium through sterile cheese cloth into sterile 500 ml Erlenmeyer flasks. We generally put 200 ml of medium into each 500 ml Erlenmeyer flask. Allow the conidia to germinate during a 4 to 6 hour incubation at 30°C in a shaking incubator (shaking at 200 rpm). At least 80% of the conidia should germinate during this incubation and you want to collect the germinated conidia before the germ tubes are longer than twice the diameter of the conidia.

3) Collect the germinated conidia by centrifugation at 4,000 rpm in a Sorvall HB4 rotor (2,500 x g) for 5 min. We use four sterile 30 ml Corex tubes and have to centrifuge four times in order to collect all the germlings into four pellets. Wash each pellet once with 25 ml of ice cold sterile water and once with 25 ml of ice cold 1 M sorbitol. Centrifuge at 4,000 rpm for 10 min to collect the cells between the wash steps.

4) Resuspend the four germling pellets in a total of 30 ml of 1 M sorbitol and transfer 15 ml of germlings into each of two sterile 100 mm Petri dishes. Add 15 ml of filter sterilized 5 mg/ml Novozyme 234 in 1 M sorbitol to each Petri dish. Place the Petri dishes in a shaking 30°C incubator shaking at 50 rpm and allow the Novozyme 234 to digest the cell walls for 45 -60 min. We often follow the digestion by examining the germlings under the microscope.

5) Using a wide bore pipet, transfer the spheroplasts into two sterile 30 ml Corex tubes and collect the cells by centrifugation in the HB4 rotor at 800 rpm (100 x g) for 5 minutes. Carefully remove the supernatant with an aspirator and gently resuspend the loosely packed spheroplast pellets in 25 ml of ice cold sterile 1 M sorbitol. Wash the spheroplast pellets three times by gently resuspending the cells in 25 ml of 1 M sorbitol, collecting the cells by centrifugation at 800 rpm and carefully aspirating the supernatant.

6) After the third wash, resuspend the spheroplasts in ice cold sterile STC (1.2 M sorbitol, 10 mM Tris-HCl pH 7.4, 10 mM CaCl₂). Centrifuge at 800 rpm for 5 min and remove the STC with an aspirator. Resuspend each pellet in 10 ml of STC and combine the pellets in one Corex tube. Centrifuge at 1,000 rpm (165 x g) for 5 min and carefully remove as much of the supernatant as possible with an aspirator.

7) At this point the spheroplasts are ready for electroporation. Place the Corex tube on ice and gently mix the loosely packed spheroplast pellet by pipetting up and down using a pipettor with a cut off tip (wide bore tip). You should have between 1 and 2 x 10⁹ spheroplasts in a volume of 1 to 2 ml. This will allow you to perform a number of electroporation experiments. Immediately before each electroporation put 80 µl of spheroplasts into a prechilled microfuge tube containing 3 to 6 µl of CsCl prepared DNA (use at least 1 µg of DNA). Mix by pipetting up and down several times with the wide bore pipet tip and transfer the spheroplast/DNA into a prechilled electroporation cuvette with a 0.2 cm gap (BioRad cuvette #165-2086).

8) Flick the spheroplasts to the bottom of cuvette with a downward motion of the wrist and tap the cuvette on a bench top until the spheroplasts are evenly distributed on the bottom of the cuvette. Place the cuvette into the slide chamber of a BioRad Gene Pulser apparatus and electroporate at 200 ohms, 25 microfaradays and 0.7 kilovolts (Field strength of 3.5 kV/cm). We get a time constant of 0.4 milliseconds under these electroporation conditions.

9) Following the electroporation, resuspend the spheroplasts immediately by adding appropriately 1 ml of sterile ice cold regeneration medium (1 M sorbitol, 2% glucose, 1 X Vogel's salts) with a Pasteur pipet and gently pipetting up and down in the electroporation cuvette. After mixing, the electroporation cuvette can be placed on ice until you have finished with all of your electroporations. Twenty to thirty transformations can be carried out with one batch of spheroplasts and we routinely get between 1,000 and 5,000 transformants in an individual transformation. The individual transformations are diluted in 10 ml of regeneration medium. We allow the spheroplast to regenerate their cell wall during a 24 to 36 h incubation at room temperature and then plate the cells out on a selective medium.

10) During a set of transformations, we often resuspend one of the transformations in 1 ml of sterile ice cold STC. These spheroplasts are added to 50 ml of molten (48°C) top agar (2% sorbose, 1 M sorbitol, 0.05% glucose, 0.05% fructose, 1 X Vogel's salts, 2% agar). We then add 10 ml of the spheroplast-containing top agar to each of 5 sorbose-selective plates (2% sorbose, 0.05% glucose, 0.05% fructose, 1 X Vogel's salts and the appropriate selective antibiotic). These plates are then incubated at 30°C. Transformant colonies will be visible in top agar under the dissecting microscope after 24 to 36 h. An approximate titer of transformants can be estimated by counting the number of colonies on the top agar plates.

11) Following the 24 to 36 h incubation in the regeneration medium, the cells are collected by centrifugation at 4,000 rpm in the HB4 rotor for 5 min. The cells are then resuspended in sterile water and plated on sorbose-selective plates at an appropriate titer as estimated from the top agar plates.

Acknowledgements: This work was supported by a grant from the University of Buffalo Foundation.

Last modified 7/25/96 KMC