**How to obtain progeny as random ascospore isolates.**
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## Background

In the early years, genetic analysis in Neurospora usually employed ordered asci (e.g., Houlahan *et al.* 1949, Barratt *et al.* 1954). This was probably inspired by the novelty of a haploid genetic system that allowed all products of individual meioses to be recovered, demonstrating the immediate results of Mendelian segregation, distinguishing first and second division segregation, and allowing centromeres to be mapped. Subsequently, ascus analysis was replaced for most purposes by random-ascospore analysis. Randoms were more convenient and efficient for building stocks, inbreeding, obtaining recombinannts, detecting linkage, and determining recombination frequencies (Perkins 1953). Linkage data based on random ascospore analysis were published using a format adapted from the classic compilation of maize data by Emerson *et al*. (1935) (Newmeyer 1957, Perkins 1959 and accompanying papers). See *How to use genetic methods for detecting linkage*.

Asci are still needed to provide critical information that cannot be obtained from random ascospores, e.g. regarding chromosome rearrangements, Spore killers, gene conversion, and genetic interference. For problems that require tetrad analysis, ordered asci can often be replaced by unordered, shot asci, which are much easier to isolate. See *How to obtain asci as unordered groups of ascospores ejected from the perithecium.*

## Procedures

Many variations exist in the methods used to obtain progeny from random ascospores. Which method is best in a particular situation will depend on the scope and aim of the experiment. Total isolation may be preferred when numbers are small and bias is to be avoided. Selective isolation is called for where the products of rare events are sought.

### *Isolate first, then heat shock***.**

Isolating ascospores directly to tubes is probably the easiest and most convenient procedure when small numbers of progeny are required (less than a few hundred). Because conidia are killed by the heat, there is no risk of contamination from airborne conidia. Also, possible bias from failure to detect late germinants is minimized. Isolation to small slants followed by heatshock in a 60°C water bath has been standard practice for thousands of conventional mapping and stock building crosses in the Perkins laboratory at Stanford. The standard procedure, first described briefly by Perkins (1959), is as follows:

A slab of 4% water agar about 2 cm × 5 cm is cut out of a prepoured plate and placed on a microscope slide. A loop of sterile water is used to collect ascospores, usually from the wall of a cross-tube. These are spread over the agar surface so that single spores are optimally spaced for isolation. The slide is placed on a white porcelain plate or other white surface and examined under 50 or 60× magnification with bright illumination from above. The black ascospores stand out in contrast to the white background. Spacing of spores on the slab should be such that well-

separated single ascospores are common. If they are too crowded, an additional loop of water is added and the loop is used to sweep surplus ascospores aside and increase the spacing between those that remain. If ascospores are too sparse, another loopful is added and spread. A flattened, sharpened platinum-iridium blade (in an 8.5 cm needle-holder) is then used to pick up well-separated ascospores and tube them, one by one, without ever touching the spore. (See *How to make tapered platinum-iridium needles and use them for ascospore isolation.*). Once an ascospore has been selected, the needle is inserted near the spore at an angle to the surface of about 30°. The needle is lifted slightly to fracture the agar along lines that encompass the ascospore. The blade is then maneuvered so that the dislodged piece (~0.25 mm diameter) adheres to it by surface tension as it is lifted out of the slab. The spore-bearing agar fragment is placed right-side-up on the surface of a slant in a 10 × 75 mm culture tube or on the surface of a sorbose plate The transferred piece is visible to the naked eye, confirming that the ascospore is no longer on the needle. With practice, 100 ascospores can be isolated in less than an hour.

The method works best when the agar substrate is rather stiff, and the surface on which spores are spread is not overtly wet. Medium with an agar concentration less than 4% does not fracture properly and the piece to be lifted out does not adhere well to the blade.
During manual isolation, the hand is steadied and tremor is minimized by bracing the heel of the hand on the corner of the microscope stage. First, while being viewed from the side, the needle blade is positioned well above the agar slab, ensuring that it will not hit the agar. Then, while viewed through the microscope, the needle is moved horizontally until the blade comes into the field of vision. The tip can then be safely lowered to the agar surface near the ascospore that is selected for isolation.

The platinum blade is flamed between transfers, using either a small bunsen burner or an alcohol lamp. By the time it has moved from the flame to the agar slab, a properly flattened blade will have cooled sufficiently to be ready for carving out the next piece of agar without melting it or activating the ascospore.

When ascospores are being isolated to 75 mm tubes, spare tubes with sterile plugs should be on hand, for use when a cotton plug is dropped, as is bound to happen.

Racks carrying 100 tubes are convenient. Each rack is labeled by affixing an adhesive paper label to the cotton plugs. Heat shock is by immersing the tubes to a depth of ~3 cm for 30 minutes in a 60°C water bath with a false floor. After being removed from the bath, tubes should never be returned to 60° because activated ascospores are hypersensitive to heat and will be killed.

Many variations are possible, such as using liquid medium in the tubes. If ascospores are germinated on a 4% agar plate, individual germlings can be picked up from the surface of the plate and tubed in similar fashion. If preferred, ascospores or germlings may be transferred to marked positions on sorbose plates rather than to tubes. Square plastic petri dishes with a grid are convenient but expensive. Ordinary round plates can be used effectively if placed over a paper grid that is marked with spaced numbers 1–26, or 1–16, or 1–37. Nichrome wire rather than platinum-iridium can be used to make a flattened blade for isolating spores, but it deteriorates rapidly because of oxidation, whereas platinum-iridium stays bright and clean.

***Heat shock, then isolate.***

In many laboratories, ascospores are routinely heat shocked and allowed to germinate before they are isolated. This avoids the effort wasted when nongerminants are tubed. Heat shocking prior to isolation will usually be preferred when numbers are large and accurate counts are needed, or when desired segregant types are phenotypically distinguishable under the microscope soon after germination, as may be true for some but not all morphological mutants and for certain auxotrophs on diagnostic media (Lein *et al*. 1948; see Wagner and Mitchell 1955, Fig. 29 [reproduced as Fig. 20 in Perkins *et al*. 2001]).

Again, there are many variations in the procedures used. A spreader may be used to distribute the ascospores over the surface of a prepoured plate, either before or after heat shock. Alternatively, prepoured plates may be overlayered with dilute agar medium containing heat-shocked ascospores.

*Spread on plate before activating.*

Plates are prepoured using minimal 3% or 4% agar medium with 1% sucrose (plus supplements if needed). Some laboratories prefer sorbose medium. A large drop of water is placed in the middle of the plate, and a loop with sterile water is used to take ascospores from the wall of a cross tube and place them in the drop. The drop is examined at 40 to 60× under a dissecting microscope to check the number of spores. About 200 ascospores per plate is optimal. If necessary, additional ascospores are added. (Alternatively, ascospores are placed in 0.5 ml sterile water in 10 × 75 mm tubes which are then poured into the plates.) The spores are distributed over the plate with a sterile glass spreader.

Plates are heat shocked, either for 1 hour in a 60°C oven or (preferably) 30 minutes in a covered water bath with the uncovered petri-dish bottom just touching the water to ensure heat transfer. Following heat shock, germlings can be isolated after 4 or 5 hours incubation at 34°C (or overnight at 19°C). Different morphologies and requirements can often be recognized soon after germination.

If a conventional glass spreader is used repeatedly, ascospores may not all be killed when the spreader is dipped in alcohol between uses and the alcohol is burned off (Newmeyer and Wallace 1971). This would be a problem only if the spreader is to be used for different batches of spores. Throw-away spreaders that present no such problems can readily be made anew using sterile long-nosed disposable pipettes (e.g., VWR Cat. No. 14672-380). This is done by making two (or three) bends successively in the thin nose of the pipette by approaching it to a small flame such as a bunsen burner pilot-light or an alcohol lamp.

*Activate before spreading or overlayering.*

The following description is adapted from Davis and de Serres (1970):

"Ascospores are suspended in sterile 0.1% agar (in water) to prevent settling, and are diluted appropriately in the same medium. [0.1% agar is viscous but does not gel at room temperture.] Hemacytometer counts are made to determine the proper dilution. Pipetting of suspensions should be done slowly to keep ascospores uniformly suspended. The ascospore suspension is heat-shocked in a covered water bath and plated on regular plating medium at a dilution roughly adjusted to minimize colony overlap after 18–20 hours growth at 25°C. Colonies demonstrably arising from single ascospores are isolated into tubes at this point. Care should be taken in the isolation procedure to avoid hyphae of adjacent colonies which lie out of focus within the agar."

“Hyphae that spread beneath the agar surface from neighboring colonies are best seen if isolating is done under 30× magnification or higher, with the plate on the stage of a dissecting microscope and with illumination from below and at a slight angle.”

Metzenberg (1989) has described the following method to prevent suspended ascospores from settling:

"It is often desirable to plate Neurospora ascospores for quantitative counts. This is made more difficult because they are so large and dense that they settle out of suspension very quickly. Often this is partly remedied by suspending them in 0.05% agar instead of water. In my experience, however, the agar often coagulates into small irregular masses, giving an unpredictable viscosity, and also a "noisy" field of view under the microscope. A 1% solution of polyvinyl pyrrolidone (PVP-360, Sigma Chemical Co.) provides a good viscosity and appears to be completely nontoxic. It is conveniently made up and autoclaved as a 10% solution (w/v), and then one part is added to about nine parts of ascospores suspended in water."

When large numbers are involved or accurate counts are needed, overlayering rather than spreading is preferred. The following procedure for plating ascospores in a shallow top layer is taken from several sources, including Newmeyer (1954), Murray (1960, 1963), and Catcheside and Austin (1971).

“Plates are prepoured with an underlayer (Vogel's medium with sorbose 0.5%, glucose 0.0125%, fructose 0.025%, agar 2%, and supplements as needed). Ascospores are suspended at the desired concentration in unsupplemented Vogel's medium with 0.1% sucrose, 0.5% or 0.6% agar. After heatshock, overlayering is with 2.5 ml to 5 ml spore suspension per plate. Before adding the overlayering, the prepoured plates are brought to 60°C so that ascospores will sink to the bottom of the overlayer for ease of observation. Rather than pipetting the overlayer, portions of the spore suspension may be distributed to 12 × 100 mm tubes (2.5 ml each) prior to heatshock, and poured from the tubes.”

In all the above, it should be noted that glass petri plates, used in early studies, are somewhat larger than the plastic plates now in use. Volumes may therefore need to be altered slightly.

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