

How to make heterokaryons in *Neurospora crassa*. Anthony Griffiths

Background

When hyphae of two different strains of *N. crassa* encounter each other on their substrate there is a tendency for their cell walls and membranes to fuse forming a common cytoplasm. Nuclei of the two strains can then mingle to form a heterokaryon.

Heterokaryons are useful for a variety of applications such as;

1. Testing complementation
2. Testing for internuclear gene interaction
3. Testing for dominance
4. The specific locus test for mutations at a single locus
5. Isolating mutants in genes affecting heterokaryon formation.

Genetic aspects of making heterokaryons

If the two strains used are genetically heterokaryon-compatible they will form a relatively stable heterokaryon. If there is genetic incompatibility, an adverse cellular reaction occurs which can lead either to death or to very poor growth of the heterokaryon. Key genes in determining whether or not a stable heterokaryon will form are the heterokaryon incompatibility (*het*) genes. Generally, when making heterokaryons it is important to use component strains that have identical alleles for all the *het* genes. Furthermore, since the mating type locus, *Mat*, acts a *het* locus prospective heterokaryotic components should also have the same mating type. If component strains are derived from the same progenitor such as Oak Ridge wild type, then it can be assumed that the *het* alleles are all identical. In some cases the *het* allele genotype is listed in the FGSC stock descriptions. Strains of dubious ancestry can be assessed for their *het* genotype using standard *het* testers available from the FGSC.

Some examples of compatible combinations are as follows, where the letters c, d and e represent some of the key *het* genes.

C D e Mat-A plus *C D e Mat-A*

c D E Mat-a plus *c D E Mat-a*

Some examples of incompatible combinations are

C D E Mat-a plus *C D e Mat-a*

C D E Mat-A plus *c d e Mat-A*

C D E Mat-a plus *C D E Mat-A*

Useful strains for making heterokaryons on a routine basis contain mutant *Mat* idiomorphs that have null het-incompatibility function. These are designated *Mat-A^m* or *Mat-a^m*. Most of these alleles also confer sterility to that genotype, so if a compatible heterokaryon involving a nucleus with a *Mat* mutation (such as *Mat-a* plus *Mat-A^m*) is crossed, only the other nucleus (in this example the one carrying *Mat-a*) will participate in the cross. The mutant *Mat* strains are useful as “helpers” to support the growth and fertility of sickly strains.

The following examples of compatible combinations illustrate the use of *Mat* mutants in making heterokaryons.

C d e Mat-a plus *C d e Mat-A^m*

C D E Mat-A plus *C D E Mat-a^m*

Most applications of heterokaryons require forced heterokaryons. These involve the union of compatible strains that have different auxotrophic mutations. Hence on minimal medium neither strain can grow but because of complementation the heterokaryon can. Hence if strains are combined on minimal medium, a growing colony is almost certainly a heterokaryon. In addition to being easy to select, forced heterokaryons have the advantage of being more stable since both nuclear types are needed for continued growth. (Unforced heterokaryons can break down into their component strains by chance segregation of the nuclear components into different regions of the culture.) Letting *aux* represent a recessive auxotrophic mutation, a forced heterokaryon can be designated

aux-1 aux-2⁺ plus *aux-1⁺ aux-2*

A forced heterokaryon produces macroconidia of three types, shown below. The proportion of the latter two homokaryotic genotypes should reflect the nuclear ratio in the heterokaryon, which is not necessarily 1:1.

Heterokaryotic
aux-1 aux-2⁺
aux-1⁺ aux-2

Hence the nuclear components of a heterokaryon are easily recoverable by plating the conidia. Individual colonies must be tested for genotype. Certain recessive markers incorporated into the heterokaryotic component genotypes are convenient for announcing a single homokaryotic component. The morphological mutant *cot-1* and the cycloheximide resistance mutant *cyh-1* are examples of the many possible mutations that can be used in this regard.

“Triaryons” (more accurately, three component heterokaryons) are useful for some applications. The component genotypes must be designed for three way complementation, for example:

aux-1 aux-2 aux-3⁺
aux-1 aux-2⁺ aux-3
aux-1⁺ aux-2 aux-3

Procedure

The choice of forcing markers is crucial. The auxotrophic alleles must be tight, resulting in no significant growth on minimal medium. If they were leaky, this would obscure heterokaryon formation. As forcing markers, nucleotide or amino acid requiring auxotrophs (*e.g.* adenine, leucine) work better than auxotrophs with requirements for vitamins and other compounds required in much smaller amounts. Presumably this is because the macromolecular monomers and their chemical relatives are required at high concentrations in the cell. Hence the use of auxotrophic requirements of this type forces the two components into a stronger mutually dependent association as a heterokaryon. However, some tight vitamin-requiring alleles do make good forcing markers when combined. Generally the least desirable combination is of one nucleotide/amino acid marker and a vitamin marker because the difference in concentrations needed for complementation often results in a lopsided heterokaryon with a nuclear ratio significantly different from 1:1. However, if a lopsided nuclear ratio is desired, then this is one way of accomplishing it. Another way of trying to produce a lopsided ratio is to mix the conidia in proportions that favor the desired majority. This often gives the desired result in the first heterokaryotic colony formed, but as this culture grows it finds its natural nuclear ratio based on required levels of nutrients.

There are two basic methods of making heterokaryons.

1. Co-inoculation of dry conidial samples.

This is the easiest approach. From the two component strains, simply obtain small, approximately equal conidial samples using an inoculation tool, and stab them together in one spot on a plate or on a slant. If the strains are aconidial, wisps of fresh mycelium can be used instead. In general it is best to use small inocula because the mass of every inoculum represents a small supply of nutrients that can promote non-heterokaryotic growth. As a control, always stab the component strains individually on a separate plate or slant.

For most routine work, regular agar is adequate. However, it does contain low levels of nutrients, and these can be a problem in some cases. For leaky forcing markers, or for cleaner results, it is best either to use commercially purified agar or to “wash” some regular agar.

(Agar is washed as follows. Cover some agar powder with tap water and let it stand overnight. Decant and add fresh water a total of three times over three days. Next suspend overnight in distilled water. Decant and put the agar into a nylon grape-pressing bag. Squeeze out as much water as possible by hand. If a wine press is available, squeeze

the bag in the press. Re-suspend in acetone overnight in a fume hood. Wearing rubber gloves, squeeze again manually or if possible in the press. Spread the squeezed-out agar on trays in the fume hood to dry, which takes several days. Stir it occasionally. Grind the dried agar in a food blender to make a powder that will more easily dissolve when making up medium.)

If, after double inoculation, a colony appears, then it is likely that a heterokaryon has formed. To confirm this, there are two main tests:

- a) Under a dissection microscope, cut off a single hyphal tip with a sterile blade, and put the tip onto fresh minimal medium. Only a true heterokaryon should grow. (Note: the tip cannot be cut too short or all the cytoplasm will leak out and a false negative will be produced. Cut several tips.)
 - b) Plate some conidia at low density on minimal medium and look for fast-growing colonies, which must have developed from presumptive heterokaryotic conidia. Isolate a sample.
2. Co-inoculation of drops of conidial suspensions.

This method has two advantages. It is possible to control the size of the inoculum, and also, since conidial suspensions are used, this is the technique of choice for the preparation of large numbers of heterokaryons.

The minimal medium is poured into tubes and allowed to set unslanted. One or two drops (or a specific number of microlitres) of a suspension of each component strain are dropped into the tube. The conidia initially form a mixed "monolayer" on the surface of the agar. The component strains should be inoculated into separate tubes alone, to act as controls.