How to use *fluffy* testers for determining mating type and for other applications.
David Perkins

Background

While *mat A* and *mat a* wild types or other fertile strains of known mating type can be used to determine the mating type of segregants or newly acquired strains, the highly fertile aconidiate mutant *fluffy* (*fl*) is superior to wild type when used as the female parent in tests. *fluffy* strains form abundant perithecia more quickly and the perithecia are more readily observed, especially in small slants where wild type strains produce overlying macroconidia that may obscure perithecia and ascospores. Absence of macroconidia in fertilized *fluffy* cultures permits ejected ascospores to be shot to an opposing surface without interference.

The usefulness of *fluffy* testers is not limited to determining mating type. They have been important mainly in two categories of experiments -- those that require examining the unordered asci and ascospores that are ejected from the perithecium, and those that involve determining whether perithecia are fertile or barren (Perkins *et al.* 1989). Using *fluffy* testers to observe the presence and patterns of mutant or aborted ascospores has expedited the detection and diagnosis of chromosome rearrangements (Perkins 1974) and of Spore killer elements (Turner and Perkins 1993). *fluffy* testers have been used to study mutations that are expressed in the sexual phase (e.g. *per-1*, Perkins 1988), to demonstrate the occurrence of barrage reactions (Griffiths 1979, Perkins 1988; see Fig. 37 in Perkins *et al*. 2001), to show that repeat induced point mutation (RIP) occurs in long segmental duplications (e.g., Perkins *et al.* 1997), and to examine the effects of meiotic silencing (Shiu *et al.* 2001). *fluffy* testers have also been used extensively in determining the species status of wild-collected isolates (Turner and Perkins 1993, 2001; Dettman *et al.* 2003). See: *How to determine mating type*, *How to recognize and diagnose chromosome rearrangements*, *How to distinguish a Spore killer from alternative causes of ascospore abortion*, *How to demonstrate barrage in Neurospora*, *How to obtain uncontaminated progeny from crosses incolving per-1, How to determine the species of wild-collected isolates.*

The *fluffy* mutation was discovered and its high fertility was noted by Lindegren (1933). Other aconidiate strains are known that might be used in a similar way. For example, Smith (1962) used the *N. crassa* mutant *spray* for mating type tests on plates. However, no strains have proved superior to *fluffy* in fertility and convenience.

Procedure

Scoring individual strains as *mat A* or *mat a* is conveniently carried out using *mat A* and *mat a fluffy* strains as protoperithecial parents and fertilizing with the strain to be scored. Tests are made on the surface of agar crossing medium, either on lawns in petri plates or on slants in culture tubes. For plate tests on synthetic cross medium, plates are inoculated and incubated at 25°C until protoperithecia are visible microscopically (~ 4 days). Plates with lawns ready to fertilize can be stored at 5°C for several weeks before use.

Conidia or mycelia from strains to be tested are spotted on the surface at marked positions. Conidial scatter is minimized if a loop of sterile water is used to pick up conidia from a slant of the culture to be tested, taking care that loose conidia do not adhere to the loop. Alternatively, loopfuls of a small suspension of conidia may be used. A convenient method (used by R. L. Metzenberg) is to grow strains to be tested in 1 ml of liquid medium in 10 × 75 mm tubes. Once cultures have conidiated, the tube can be vortexed to make a suspension. In absence of conidia, a needle can be used to rub mycelia onto the surface of the tester Thirty spots are easily accommodated on each plate. Parallel tests on both *fl A* and *fl*

a provide assurance against false-negative tests, infertility, or spotting errors. Perithecia become visible in 2 or 3 days at 25ºC.

For tests on slants, it is convenient to use 1 ml synthetic cross medium in 10 × 75 mm tubes. If large numbers are being prepared, a Pasteur pipette is used to introduce into each tube a small drop of a visibly turbid suspension of fresh *fluffy* mycelial fragments. Details for preparing the suspended *fluffy* inoculum are given by Taylor (1965). The *fluffy* slants are ready for testing after 4 days incubation at 25°C, and they can be stored at 5°C for at least 3 weeks before use. They are fertilized using a short, stiff platinum-iridium or nichrome needle hammered to form a flattened blade. The conidia or mycelia used for fertilizing should be rubbed across the surface of the slant, and if obscuring *fluffy* mycelia are present on the wall of the tube opposite the slant, these should be removed with a swipe of the inoculating needle. Tubes have the advantage that the possibility of conidial scatter is eliminated.

If asci are to be examined, as in scoring rearrangements or Spore killers, *fluffy* strains of Rockefeller-Lindegren (RL) genetic background (*fl*(RL) *A* and *a*, FGSC 6682, 6683) are preferred when the strains to be tested are of Oak Ridge (OR) background. This is because nonselective abortion of asci occurs in crosses between highly inbred strains such as *fl*(OR) *A* and *fl*(OR) *a*. In aborting asci, all eight ascospores shrink until they look like tiny bubbles. (See Raju *et al*. 1987, Perkins and Pollard 1989. See: *How to minimize bubble-ascus abortion.*) Abortion of "bubble" asci does not interfere with mating type determinations.

If f_1 strains are to be retained from the test crosses, *fl*(OR) testers (FGSC 4317, 4347) should be used rather than the *fl*(RL) testers, so as to provide progeny that are of OR heterokaryon-incompatibility genotype, which is preferred as the standard. (Note that ascospores containing *fluffy* often germinate spontaneously, without heat shock.)

fluffy testers are available not only for *N. crassa* but also for *N. intermedia*, *N. sitophila* (Table 1), and *N. tetrasperma* (see: *How to determine mating type*). However, the *N. crassa fluffy* testers can be used for preliminary determination of mating type even in interspecific crosses with these species. Mating reactions in interspecific crosses with *N. crassa fluffy* are usually clear, with perithecia developing on one of the mating type testers but not on the other, even though sexual development may not progress very far. *N. discreta* strains are an exception, failing to show an unambiguous reaction with either of the *N. crassa fluffy* testers.

Light is not essential for perithecial development of *N. crassa* laboratory strains. However, some homothallic Neurospora strains fail to form perithecia in constant dark or constant light (Raju 1981). Although the need for a light-dark cycle has not been demonstrated for heterothallic species, several labs attempt to simulate natural conditions by rigging incubators with lights timed to provide 12 hours light, 12 hours dark., both for crosses and for vegetative cultures.

***Maintenance of fluffy stocks*:** Vegetatively propagated *fluffy* stocks deteriorate occasionally. Subcultures acquire undesirable traits such as decreased fertility, delayed protoperitheciation, or production of large brown protoperithecia (false perithecia). For example, Veenhuizen and Kolmark (1986) noted lowered fertility of a *fluffy* tester and showed that it was due to a modifier. Fully fertile *fluffy* strains were recovered in progeny when their modified strain was crossed to wild type. The Stanford lab has also used crosses to rid *fluffy* strains of modifiers that were responsible for false perithecia and slow sexual development. Making a cross to extract the unmodified *fluffy* mutation should ordinarily be unnecessary, however, because duplicate stocks of the original, unmodified testers are already available. Well-behaved, unmodified *fluffy* stocks are maintained by FGSC in suspended animation. (It would be well for individual laboratories to do the same.) Aconidiate strains such as *fluffy* can be preserved on silica gel, though this is difficult. Cryopreservation is probably less laborious. *fluffy* strains do not survive freezing

at −20°C, but they can be simply and effectively preserved at lower temperatures. Storage of aconidial Neurospora strains by freezing mycelia at −80°C was investigated by Pounder and Bowman (1999). The following is adapted from their account:

"*fluffy* A (FGSC 4960), *fluffy* a (FGSC 4961), and *acon-3* (FGSC 5074) were grown on plates of Vogel's Minimal Medium with 2% sucrose for 3 days at room temperature to a confluent mycelial lawn. Approximately 1 cm square plugs were cut from the agar and placed into sterile 1.5 ml Eppendorf tubes. The tubes were placed in a −80°C freezer with no flash freezing and no glycerol or DMSO added. Agar plugs were retrieved from −80°C after 7 days to 9 months and plated onto the same medium as above. They grew to form a confluent lawn in 3 days. The recovered *fluffy* strains functioned normally in mating type tests. Whole agar slants with mycelia from the aconidial strains were frozen at −80°C. Pieces chipped from the frozen agar grew well on plates following storage of 1 month. Recovery from agar plugs and slants stored for 1 month (the only time tested) at −20°C was slower than from those stored at −80°C, with 3-7 days required for a confluent lawn of mycelia to grow. The recovery of aconidial strains after freezing under these conditions may be due to the larger amount of mycelia stored initially. Sufficient mycelia are present in the 1 cm plugs to allow recovery of the strains, providing a convenient alternative storage technique for aconidial strains."

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Table 1: *fluffy* testers in current use.

Genotype[a]	FGSC No.	Allele	Background	Applications
N. crassa[b]				
fl(OR) *A* *fl*(OR) *a*	4317 4347	P P	b_7 × OR b_7 × OR	Testing mating type, species, Spore killer. Detecting and scoring rearrangements in other than OR background.
fl(RL) *A* *fl*(RL) *a*	6682 6683	P P	b_6 × RL b_4 × RL	Testing mating type, species, Spore killer. Detecting and scoring rearrangements in other than RL background.
fl; Sk-2^K *A* *fl; Sk-2*^{K} *a*	3297 6137	P; K-Brunei P; K-Brunei	b_9 × OR b_{10} × OR	Identifying new killers and strains resistant to killing.
fl; Sk-3^K *A* *fl; Sk-3*^{K} *a*	3579 3580	P; K-Rouna P; K-Rouna	b_{10} × OR b_{10} × OR	Identifying new killers and strains resistant to killing.
fl; per-1 A *fl; per-1 a*	3311 3312	P; PBJl P; PBJ1	f_1 × OR f_1 × OR	Used as female to avoid conidial contamination when spontaneously germinating *per*$^-$ ascospores are isolated. Used to show maternal origin of perithecial walls in reciprocal crosses × *per*$^+$.
N. intermedia				
fl A *fl a*	5798 5799	P P	b_7, *crassa* → *intermedia* Shp b_7, *crassa* → *intermedia* Shp	Testing *mat*, species, Spore killers, rearrangements.
N. sitophila				
[*fl; Sk-1*^{K}][c]				
fl; Sk-1^S *A* *fl; Sk-1*^{S} *a*	4887 4888	P P	b_5, *crassa* → *sitophila* FGSC 1134, 3191.	Testing *mat*, species, Spore killers, rearrangements. (For efficient rearrangement tests use K × K or S × S.)

This table is adapted from Perkins *et al*. (1989).

[a] Unless otherwise indicated, strains of *N. crassa* and *N. intermedia* are sensitive to killing by either *Sk-2*^{K} or *Sk-3*^{K}.

[b] (OR) and (RL) signify strains backcrossed to Oak Ridge or to Rockefeller-Lindegren *N. crassa* wild types. See Perkins and Pollard.(1989). See *How to minimize "bubble-ascus" abortion in crosses for cytology***.**

[c] Satisfactory *fl; Sk-1*^{K} stocks are not available. In their place, morphologically wild-type *N. sitophila Sk-1*^{K} stocks P8085 A (FGSC 2216) and P8086 a (FGSC 2217) are recommended for use, on slants in 12 × 100 mm tubes. (Conidia may obscure perithecia in 10 × 75 mm tubes.)