

Hints and precautions for the care, feeding and breeding of *Neurospora*.

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Recommended Citation

Perkins, D. D. (1986) "Hints and precautions for the care, feeding and breeding of *Neurospora*," *Fungal Genetics Reports*: Vol. 33, Article 12. <https://doi.org/10.4148/1941-4765.1588>

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Abstract

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feeding and breeding of *Neurospora*

Some of these notes describe what may be established routines in older laboratories, but are either unpublished or published in inaccessible places or scattered through the old literature, and thus may not be known to those just beginning to use *Neurospora*. Others maybe practices unique to our lab. The attempt here is to supplement compilations of methods such as Davis and de Serres (1970 *Methods in Enzymology* 17A:79-143) and the older Stanford *Neurospora Methods* (1963 *Neurospora Newsletter* 4:21-25). Hints and precautions regarding particular mutants or categories of mutants will also be found in individual entries in Perkins et al. 1982 *Microbiol. Rev.* 46:426-570 (referred to below as "Compendium"). Statements apply to *N. crassa* unless another species is indicated. *Neurospora Newsletter* is abbreviated N.N. All temperatures are °C.

1. Temperature; glassware. Crosses are usually made at 25°. Perithecia do not develop, or do so poorly, above 30°. We routinely make crosses on SC slants in 18 X 150 mm tubes, using foam or cotton plugs. Push-on metal or plastic caps should be avoided with cross tubes because they result in more rapid water loss and are more prone, to contamination during the extended incubation period. Crosses are made on petri dishes for specific purposes, as when shot asci are to be collected. But plates are more prone to contamination and desiccation than tubes, and less compact for incubation and storage.

2. Media and supplements. The synthetic crossing medium (SC) of Westergaard and Mitchell is most widely used. The need to adjust pH of SC to 6.5 can be avoided by substituting 0.7 g K₂HPO₄ and 0.5 g KH₂HPO₄ per liter for the monobasic salt in the original formula (1963 N.N. 4:2125). The formula in Davis and de Serres, p. 86, incorporates this change. The trace elements used for Vogel's medium N are suitable for SC at 0.1 ml per liter final volume; there is no need for a separate formula.

Russo, et al. (1985 N.N. 32:1011) have modified Vogel's Medium N for use as a crossing medium reducing NH₄NO₃ tenfold. The modified Vogel's can be made up as a 50X stock, whereas the Synthetic Cross formula of Westergaard and Mitchell allows only 2x.

High ammonium or amino nitrogen inhibits crossing. Keep amino acid, purine or pyrimidine supplements at a minimal level in crossing medium. If total amino acids do not exceed 0.3 mg/ml there is usually no problem with fertility. Usually the parent with the simplest requirements is preferred as female.

3. Use of per-1 to assure female parentage. Where the pedigree of a mitochondrial genome or plasmid is critical, and assurance is desired that only one parent is functioning as female, per-1 (type I) may be used. When per-1 is present in one parent, all perithecia should be black and no white perithecia should be present if the protoperithecial parent was per-1⁺, and vice versa if the protoperithecial parent was per-1.

4. False perithecia. in some single-mating-type strains, protoperithecia may enlarge and become pigmented so as to resemble small perithecia. These "false perithecia" are devoid of beaks, asci, and ascospores, and unfertilized strains exhibiting them remain completely sterile. The strains can develop normal perithecia upon fertilization with the opposite mating type. False perithecia are characteristic of single-mating-type cultures of *Neurospora tetrasperma* and of the Kirbyville, Texas population of *N. discreta*. False perithecia are also encountered sporadically in some *N. crassa* genotypes -- they are most commonly of mating type a. Some isolates of fl a (fluffy) genotype have tended to make false perithecia.

The false perithecia can be a nuisance for mating-type testing, and a cause of alarm if contamination is incorrectly suspected to be responsible. Ordinarily, continued complete sterility and failure to develop even after long incubation distinguish false from true perithecia, and there is no serious problem. False perithecia can be of serious concern, however, in crosses where legitimate true perithecia are barren as a result of duplications or of genes affecting meiosis and ascus development.

False perithecia are also an object of interest for those interested in sexual differentiation. For example, inactive mutants of mating type a show development of barren or false perithecia in abortive mating reactions with A or a testers (Griffiths et al. 1978 *Genetics* 88:239-254; 1982 *Can. J. Genet. Cytol.* 24:167-176).

ASCOSPORES. OBTAINING PROGENY

5. Ripening of ascospores. Ascospores are unripe when first shot, even though fully black. For good germination, ascospores should be aged 7-10 days at 25°-30° (not 34°) after shooting begins, before isolation.

6. Rehydration of ascospores. Ascospores from old cross tubes that are desiccated should be rehydrated before heatshock to avoid poor germination (Strickland and Perkins N.N. 20:34-35). This is most likely to be a problem with crosses in small tubes. Rehydration can be accomplished conveniently by adding water to the cross tube or by holding isolated ascospores overnight on moist fresh medium before heatshock. If ascospores are isolated to slants prior to heatshock, fresh tubed medium gives better germination than medium that has partially dried down.

7. Ascospore viability and longevity. For ascospore maturation, some auxotrophs require supplementation of crossing medium even though they are heterozygous, recessive, and used as fertilizing parent. This is true of pan-2 (not pan-1), spe-1, and some nic, cys and met genes (see Compendium entries).

N. crassa ascospores may be stored in sterile water sealed in small vials without appreciable loss of viability for at least a year at room temperature and 18 months at 4° (B.R. Smith 1973 N.N. 20:34). Mature ascospores of N. crassa also remain viable in ordinary cross tubes and can be germinated after many months storage at 5°. In contrast, germination of N. tetrasperma ascospores is reported to decline after 19 days following simultaneous inoculation of A + a or after 11 days following inoculation of conidia from an (A + a) culture (Howe et al. 1966 Genetics 54:293-302).

When N. crassa perithecia become desiccated before all the asci within them have been shot, ascospores remain viable in the unextruded asci and show high germination months later when rehydrated and heatshocked. Intact linear asci may be extruded singly after perithecia that have been dried in this way are placed in liquid. Crosses made in 10 X 75 mm culture tubes often dry down before the perithecia have emptied their contents (P. St. Lawrence), and are conveniently stored for future use.

8. Heatshock. For heatshock, a 60° water bath is preferable to a hot-air oven. (Greater latent heat, better heat transfer, more stable). Allow at least 30 minutes at 60° to assure killing of vegetative cells with a water bath, double this for dry air. Cover the bath to assure killing of conidia on tube walls.

Never remove ascospores from 60° and then return to 60°. Once activated, ascospores are vulnerable to killing by heat.

9. Spontaneous germination. Some genotypes result in spontaneous ascospore germination (for example, the unpigmented ascospores of per-1 Type I). A significant fraction of ascospores carrying fl (fluffy) germinate spontaneously, at least in some crosses. This becomes apparent when ascospores are aged after isolating tetrads. Off-ratios may result if fl ascospores break dormancy before heatshock and are therefore killed.

10. Suspension and dilution of ascospores. When suspending ascospores for dilution series, add 0.1% Difco agar to increase viscosity and slow sedimentation; this concentration remains liquid at room temperature. For over-layering, 0.6%, 0.8% or 1% agar is used, and this solidifies.

11. Germination on surface of plates. It is convenient for some purposes to germinate ascospores after they have been spread on the surface of pre-poured 3% or 4% agar plates containing minimal medium N or other, appropriately supplemented, medium. Percent germination can be determined easily and quickly. Most auxotrophs and some morphological mutants can be distinguished from their wild counterparts, and counted or isolated selectively. Ascospores are taken by loop from a cross tube to a drop of water in the middle of the 3% agar plate; the desired number (about 200 per plate) can be obtained by adding or removing spores while examining the drop at 40-60 X magnification under the dissecting microscope. Spores are distributed evenly over the plate, using a sterile glass spreader. Do not spread to the extreme edge. Avoid scratching the surface. If the germinating ascospores are to be scored but not picked, ascospores may be suspended in 0.15% agar and spread in narrow parallel streaks with a Pasteur pipette (Maling 1959 Stanford thesis).

Plates are heatshocked 30-40 min above the surface of a 60° water bath with lids off, and with the bottom of the dish just touching the water. The bath should be covered. Alternatively, lids may be kept on if the plate being heat-shocked is placed on a partially submerged water-filled container such as a petri dish, just above the surface of the 60° water bath. (Allow 60 min in a 60° hot-air oven.)

In the absence of sorbose, inspection and isolation of germlings must be timed carefully because fast-growing germinants will overgrow the plate. Rough estimates of timing: 18 to 23 hours incubation at 20°, 15 to 16 hours at 25°, 5 to 7 hours at 34°, 11 to 12 hours at 39°. (39° is above the optimum.)

The procedures described above were adapted from methods devised by Mary Mitchell and used both for detecting new mutants (Lein et al. 1948 PNAS 34:435-442) and for identifying recombinants, mapping, etc. (Mitchell et al. 1952 PNAS 38:569-580; 1954 PNAS 40:436-440; 1959 Genetics 44:847-856). Appearance of germlings was illustrated by Mary Mitchell in Fig. 4.5 of Wagner and Mitchell 1955, 2nd ed., reproduced here as Fig. 1.

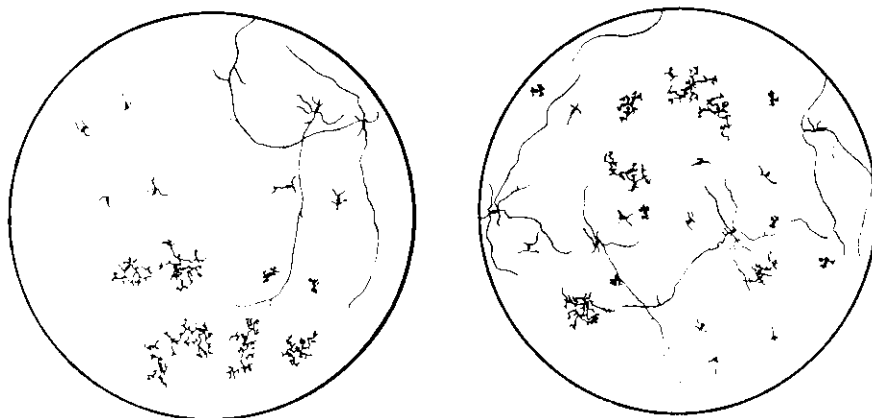


Figure 1. Camera lucida drawing of germinating ascospores showing the segregation of two genes in a cross of col-4 x pyr-2. Spores were isolated on minimal agar medium in petri dishes, heated at 60° for 30 minutes for activation, and incubated at 25° for 18 hours. This nutritional mutant grows sufficiently on minimal medium to permit identification of pyr-2 col-4 double mutants. One plate shows segregations in two asci with spore pairs isolated in order. From top to bottom the two asci show, respectively, genotypes pyr, pyr, col, col; and wild, pyr, col-pyr, col. The other plate shows results from plating spores from the same cross at random. Genetic analyses can be made directly using either procedure, or by transferring germinated spores to appropriately supplemented culture tubes for further testing. Much larger numbers can be observed conveniently using the random method. Reproduced with permission from R.P. Wagner and H.K. Mitchell, 1955. Genetics and Metabolism, 2nd edition. J. Wiley and Sons, Inc. N.Y.

12. Microscope and illumination. For most manipulations and observations (ascospore isolations; inspection of spores, perithecia, etc.), an excellent general-purpose microscope is the Stereo Zoom 7 with base, Bausch & Lomb Cat. No. BV1070 with 10X oculars (1984 price \$1417). This provides 10X-70X magnification. Illumination from above is best for isolating ascospores, with a white plate on the microscope stage to maximize contrast of the viable black spores and distinguish them from spores that are unripe or defective. Lighting from below using the frosted reflector is best for determining the percent of defective, hyaline ascospores when scoring chromosome aberrations. A balance of light from above and below is used for examining shot groups of eight ascospores and classifying them according to numbers that are black or nonblack (Perkins 1974 Genetics 77:459-489). Fixed-focus illuminators with snouts adapted for convenient use with the Stereo Zoom 7 are available from American Optical (A0 #363H, \$94) and B & L (Nicholas illuminator, B & L 31-33-05-28, \$100). Lighting intensity of both is just adequate for most purposes. Where more intense light is desired at high magnification, a focusable illuminator may be preferred, such as A0 653H (\$182) or B & L 31-33-60-40 (\$220).

13. Tools for manual isolation and transfer. Isolating and transfer needles are readily formed from 70% platinum 30% iridium wire .020 inch diameter, obtainable from Engelhard Industries, 700 Blair Rd., Cataret NJ 07008. (Obtain quote. Quantity purchase is more economical because there is a minimum tooling charge for each order. Platinum-iridium can be beaten and cut to make blades or needles are sharp, flat and thin, as desired.

14. Use of glass spreader for ascospores. If ascospores are being spread on agar with a glass spreader, use a separate spreader for each cross. Alcohol flaming is inadequate to kill all adhering ascospores (Newmeyer et al. 1971 N.N. 18:13).

LAB PRACTICE, HOUSEKEEPING

15. Avoid drafts. Make transfers in a draft-free room. Don't walk breezily past someone transferring Neurospora. We carry out all routine operations (transfers, isolations, platings, etc.) at bench or table in an open lab, taking care to work near a bunsen flame and to sterilize surfaces before and after use. Air circulation from air conditioning is shut off while transfers are being made in the laboratory. Except for manipulations involving massive transfers of conidia or opening profusely conidiating plates, we have never found it necessary to work in a special enclosure or transfer room. Where risk of conidial scatter is high, a nonventilated circulation-free tissue culture enclosure has been used (Labconco 11000, 1984 price \$1445). A small alcohol lamp can be used within the hood. Fume hoods through which air is circulating should be avoided; a regulation "biohazard hood" with its near-gale velocities would probably be a catastrophe.

16. Decontamination of work space and glassware. Wipe bench surfaces before and after use with 70% alcohol, or dilute phenol (Lysol), or dilute hypochlorite (household bleach). Autoclave all contaminated glassware and media before discarding or sending to dishwasher. Exceptions: Pipettes may be discarded into dilute hypochlorite.

17. Mite control. Infestation of cultures by mites can be a serious problem. Mites can pass by or through plugs and closures, carrying conidia and other contaminants. Don't leave cultures unnecessarily for long periods at room temperature, so as to risk mite infestation. For the same reason, don't bring soil, plants, vegetables, fruit or other possible sources of mites into the work area or into refrigerators where cultures are stored. Large slants, which retain moisture for weeks or months before drying down, are the most prone to become infested. My impression is that mites do not proliferate effectively in cultures that are completely dried. This is based on very limited experience.

If more than one different type of contaminant is found in a tube or plate, inspect for mites (about the size and shape of protoperithecia; use 20-40X magnification). If mites are found, autoclave or freeze cultures and accessories. Both eggs and adults are killed after 24 hours at -18° (Subden and Threlkeld 1966 N.N. 10:14). Clean infested area scrupulously and treat equipment, benches, shelves, incubators, refrigerators and surroundings with a miticide. Subden and Threlkeld recommend Kelthane. Because resistance may develop, Threlkeld (personal communication) suggests alternating Kelthane and Malathion. We have, in the past, cautiously used Lindane applied to incubators and benches in alcoholic solution (which leaves a residue), and have found it highly effective. Lindane has since been listed as a carcinogen (see Merck Index), but carcinogenicity data are said to be equivocal ("An Assessment of the Health Risks of Seven Pesticides...". N.R.C. Board on Toxicology, Nat. Acad. Press, 1982).

Freezing kills ascospores but not conidia. A method for using carbon dioxide to rid cultures of mites without killing ascospores has been described by Metzenberg (1985 N.N. 32:22).

18. Avoid saturated atmosphere in closed containers. Never enclose inoculated plates or tubes in a small airtight container. Condensate in a saturated atmosphere creates a film of moisture allowing mycelia to grow through plugs and out of petri dishes.

STOCKKEEPING

19. Avoid transcription errors. Read labels before and after transfer of stock cultures

20. Long-term preservation. Minimize vegetative transfer of "stem" stocks, to avoid acquisition of mutations and rearrangements. Subculture from the primary stock to make a working stock for fertilizing crosses, etc. The primary stock should promptly be put into suspended animation, such as silica gel (Perkins 1977 N.N. 24:16-17; see also N.N. 26:24-27), -20° deep-freeze (Perkins 1973 N.N. 20:33), cryopreservation (Jong, et al. 1979 N.N. 26:26), or lyophil (Barratt et al. 1959 Science 112:122-123).

21. Silica gel. Make certain that silica gel tubes are securely sealed to exclude moisture. Longevity requires sustained desiccation. Double-thickness parafilm is effective for cotton-plugged tubes if the film is stretched to render it adherent and to seal it tightly to the glass.

We decided to use plain 13 X 100 mm culture tubes with cotton plugs and parafilm seals for silica gel stocks, in preference to screwcap tubes. With the latter, we could not be confident that the seal was tight, and the constricted neck seemed to make pipetting more difficult.

22. Heterokaryons for preserving difficult stocks. Aconidiate stocks, which show low survival after freezing, can be homogenized and silica gelled, though this is considerably more laborious than preparing conidial suspensions. If aconidiate strains are heterokaryons compatible with Oak Ridge and have a phenotype appropriate for forcing, they can be preserved most easily as phenotypically wild-type conidiating heterokaryons in combination with a^ml ad-3B cyh-1 as a nonmating "helper" component (Perkins 1984 N.N. 31:41-42). Such heterokaryons can also be used to advantage for sheltering and preserving difficult genotypes. Because osmotic stocks are readily lost, os stocks should be replicated and monitored or carried in heterokaryons.

Auxotrophs such as inl and others affecting membrane components have short half-lives in absence of supplement but show good survival if supplemented. I doubt that their long-term survival on silica gel has been determined.

MUTAGENESIS, ENRICHMENT AND REPLICATION

23. Ultraviolet. For UV mutagenesis, commercially produced 20-watt germicidal lamps that fit small fluorescent fixtures are convenient and inexpensive. A new lamp may show violent fluctuations at first, becoming steady after a few hours use. Output gradually declines as the glass becomes increasingly opaque with use. At each use, the lamp should be allowed to warm up 10-15 min to get maximum 256 radiation. Monitoring with a UV meter is essential where precision of dosage is important. Do not expose eyes to UV light; wear protective goggles or ordinary glasses.

24. Chemicals. Chemical mutagens are potentially hazardous. Protocols for inactivating mutagens and for decontaminating glassware may be found in chapters by L. Ehrenberg and C.A. Wachtmeister 1977, pp. 401-418 in "Handbook of Mutagenicity Test Procedures" (Kilbey et al., eds.). Elsevier/N. Holland Press.

25. Filtration-enrichment. For filtration-enrichment, the method of Applegate et al., employing sorbose liquid medium, is recommended (1978 N.N. 25:17; Yoder 1979 N.N. 26:23-24). Avoid loss of surviving conidia in concluding steps by pelleting with dead conidia as carrier (D.E.A. Catcheside; described in Yoder 1979).

26. Replication. Protocols for replication, applicable to strains with wild type or cot morphology, are given by Nilheden et al. 1975 Hereditas 79:239-250 (see pp. 246-248); Littlewood et al., 1972 J. Bact. 110:1017-1021. For examples of replication using conidiating colonial strains see Schroeder 1970 MGG 107:291-304, DeLange et al. 1981 Genetics 97:247-259, Stadler et al. 1979 MGG 171:59-68, 1984 Mutat. Res. 127:39-47.

Plates for replication should be allowed to dry enough before use so that there is no condensate or water-film on the surface.

COLONIAL GROWTH

27. Sorbose. Sorbose (used to induce colonial growth) caramelizes when autoclaved if medium N or another medium containing NH4 is used. A brown breakdown product is produced. This does not usually seem to affect colonialization. But caramelization can be avoided by autoclaving a sorbose-glucose-fructose 20X solution separately from the medium and combining after sterilization (D.E.A. Catcheside), or more simply by substi-

tuting Synthetic Cross medium (which is NH₄-free) for Medium N (T. Legerton). The amount of sorbose that is needed depends on the medium. Formulas are given by Davis and de Serres, p. 88.

28. Use of cot-1. cot-1, which grows colonially above 30°, can be a valuable adjunct to sorbose (or can be used without sorbose) for platings. (see e.g. D.G. Catcheside 1966 Aust. J. Biol. Sci. 19:1039-1046; D.E.A. Catcheside 1970 *ibid* 23:855-865; Schroeder *et al.* 1980 pp. 55-62 in de Serres *et al.* (eds.), "Conference on DNA Repair and Mutagenesis in Eukaryotes").

29. Spot-testing on plates. Spot-testing to score requirements or resistance is accomplished routinely 25 spots per plate in 5 X 5 arrays, holding plates inverted to avoid scatter, and cooling the needle in medium or in sterile water (R.H. Davis). Basic spot-testing medium: 0.4% sucrose, 0.8% sorbose; incubation at 25° (Davis and de Serres, p. 88).

SOLUTIONS, MEDIA

30. Stock solutions of supplements. Concentrated solutions of amino acids and other growth factors can be kept conveniently for extended periods at 5° unsterilized, for addition to medium before autoclaving. Growth of microbial contaminants is prevented by periodic addition of a drop of chloroform to the stock solution. Riboflavin is bleached by visible light. Shield supplemented media from light, and keep stock solution in amber bottle.

31. Inhibition by supplements. In designing media and supplementation, be alert to inhibitions due to competition for transport. Some of these are documented in Compendium entries for his, hom, ser-3, gua, arg, lys and leu. For example, if his⁻ or hom⁻ ascospores are germinated on organic complete medium containing casein hydrolysate, growth may not go beyond the germination tube.

32. Color-coding. Color-coding of diagnostic media is convenient and less error-prone than marking plugs, tubes, or plate lids. Grocery-store "U.S. Certified Food Color" (e.g., McCormick, Schilling or Co-op) is effective and is not inhibitory at concentrations giving pale colors.

33. Effect of medium on conidiation. Conidiation of wild type varies depending on substrate. Glycerol complete medium (no. 2 in Tatum *et al.* 1950 Am. J. Bot. 37:38-46) was developed to maximize conidiation. Difco Neurospora Medium (a rich complete) tends to inhibit conidiation. Factors affecting conidiation are considered by Turian 1964 Nature 202:1240 and by Davis and de Serres, p. 138.

HETEROKARYONS

34. Vegetative incompatibility. Wild populations of N. crassa are highly polymorphic for het genes at numerous loci, so that heterokaryon-compatibility is rare. Similarly, laboratory wild-types and marked stocks may be vegetatively incompatible with one another because of heterogeneous background, and inbreeding often is required in order to obtain het-compatible combinations. (See Mylyk 1975 Genetics 80:107-124; 83:275-284; Perkins 1975 *ibid* 80:87-105).

Strains of opposite mating types are het-incompatible with each other in N. crassa. The vegetative-incompatibility of opposite mating types (but not of other het genes) may be circumvented by introducing the recessive suppressor tol into both partners (Newmeyer 1970 Can. J. Genet. Cytol. 12:914-926), or by using a mutant mating-type allele. a^m33 has lost the het-incompatibility function but not ability to mate; a^m1 has lost both vegetative incompatibility and mating functions; these mutant mating types were induced in OR background (Griffiths *et al.* 1978 Genetics 88:239-254). a^m1 has proved useful as a helper component in heterokaryons (Perkins 1984 N.N. 31:41-42)

35. Counting nuclei. Nuclear counts in conidia (or hyphae) are readily made with a fluorescent microscope after staining with DAPI and Hoechst 33258 (Raju 1982 N.N. 29:24-26). Alternatively, conventional staining methods may be used (see e.g. Serna and Stadler 1978 J Bact. 136:341-351), but they are more complicated and time-consuming.

36. Cleaning up new mutants. Before being subjected to extensive genetic or phenotypic analysis, new mutant strains from mutant hunts or other sources should be monitored for the presence of (1) secondary mutations or modifiers (by crossing by standard wild type and recovering the single mutant as an f1), and (2) chromosome rearrangements (by examining ascospores in a cross of the mutant by fluffy or another standard tester).

37. Gene order and map distance. Whenever possible, establish gene order using 3-point data in preference to combining 2-point data from separate crosses. rec genes are polymorphic in stocks of different parentage, and these can result in local differences up to 10 or 20 fold in recombination in specific regions, depending on differences in rec genotype. (For example, see Catcheside 1973 Aust. J. Biol. Sci. 26, p. 1342).

Don't base gene order just on the 'distance' apparent in your cross from markers in a "standard" published map. There is no 'standard' map so far as distances are concerned, because the maps are based on crosses which were highly heterogenous with respect to rec genotypes. In contrast, gene order from 3-point or multiple-point crosses is reliable and doesn't depend on absolute values.

In calculating recombination values (map distances) from 3-point data, double cross-overs contribute to each component interval. One must add the double crossovers to the single crossovers to obtain the value for each segment.

38. Nomenclature and conventions. With new mutants, assign a unique allele (isolation) number to each occurrence. Allele numbers should be preceded by a letter or letters indicating your name or institution, e.g. CB1, CB2, etc. if your name were Charley Brown. Choose a combination not already preempted (see FGSC stocklist for list of prefixes in use. Allele numbers of markers should be recorded for all stocks maintained or sent to others. Do not confuse allele (isolation) number (e.g. CB513) with locus number (e.g., - in al-2), or with accession (stock or strain) number (e.g., FGSC no. 2583). Assign new locus numbers (e.g. al-4) only when allelism with previously established loci (al-1, al-2, al-3) has been excluded. For summary and references on nomenclature see Compendium pp. 427-428 and Barratt et al. 1965 N.N. 8:23-24. - - - Department of Biological Sciences, Stanford University, Stanford, California 94305