Pittenger, T. H. Conidial plating techniques and the determination of nuclear ratios in heterokaryotic cultures.

The following information on techniques for plating conidia, together with a discussion of some of the problems involved in determining nuclear ratios in heterokaryons, is presented primarily for those investigators unfamiliar with these methods and for those who may wish to adapt them for other purposes. Although these methods are routinely used in our laboratory, they are not original with us. Many were developed in cooperation with K. C. Atwood and the reader is especially referred to the paper by Atwood and Mukai (1955 Genetics 40:438) for a discussion of methods of estimating nuclear ratios in heterokaryons.

Forced heterokaryons between auxotrophic strains are usually prepared on minimal slants or in growth tubes, simply by superimposing 7 to 10-day-old conidia from two compatible strains of the same mating type. Although dry conidia are usually used, suspensions of conidial mixtures are also satisfactory. If some control of the nuclear ratios is desirable, more elaborate methods previously described (Pittenger, Kimball and Atwood 1955 Am. J. Bot. 42:954) may be employed.

Heterokaryons between two genetically different kinds of nuclei produce macroconidia, some of which are heterokaryotic while others are homokaryotic. The relative frequency of these various classes of conidia can be found by plating on various types of media and a simple approximation of the relative proportions of the two

nuclear types can then be derived from these frequencies.

In making the initial suspension of conidia from a heterokaryotic culture for plating, a much larger sample of conidia than is actually needed is first removed to avoid any possibility of obtaining a nonrandom sample. One-week-old conidia are used whenever possible because of their higher viability. They are suspended in sterile distilled water (a saline solution is not needed for preparing suspensions of Neurospora conidia). Conidia may be removed with a sterile platinum spatula or an aspirator device (de Serres 1962 NN#1:10) is also very useful for this purpose. To get rid of conidial clumps and hyphal fragments, the suspension is first filtered through fine glass wool (Corning No. 3950) that has been placed in a small funnel and covered with aluminum foil for autoclaving. An aliquot of this suspension is then removed and diluted. With a little practice, it is simple to adjust by eye the conidial suspension so that it contains from $2-4 \times 10^6$ conidia/ml. (the proper dilution of a conidial suspension sealed in alcohol in a glass tube may be used as a standard for comparison). When 1 ml of this suspension is further diluted by a factor of 10^{-4}, 1 ml samples of the final dilution will contain from 200-400 conidia and such a concentration per plate is adequate for determining nuclear ratios in most heterokaryons. For dilution, culture tubes equipped with stainless steel closures (Bellco Glass, Inc.) are first sterilized; 9 ml of sterile distilled water is then added to each with one of the commercially available automatic pipetters (Palo Laboratory Supplies, Inc.). Two "Milk Dilution" bottles (Corning No. 1370) filled to the 99 ml mark are also very useful in making 10^{-4} dilutions when a large number of samples of the same suspension must be plated. In making dilutions in tubes, one of the vortex-type mixers (Vortex Jr. Mixer, Cole-Palmer) has been found to be extremely useful for mixing conidial suspensions.

We use an overplating technique rather than incorporating the conidia into the media. Using a syringe-type, hand-operated pipetter (B-D Cornwall No. 1270MH), 2 ml of a sterilized 1% solution of hot agar is dispensed into sterile 13 x 100 mm culture tubes equipped with aluminum or stainless steel closures which can be easily flipped off. These are then placed in a neoprene-coated test tube rack in a water bath maintained at approximately 45 ° C.

One ml samples of the conidial suspension are blown from a 1 ml blowout pipette into the tubes containing 2 ml of the 1% agar as forceably as possible, not only to remove all of the conidia from the pipette but also to insure adequate dispersal of the conidia in the overplating medium. The 3 ml is then poured onto the surface of a petri dish containing 20-25 ml of medium and tilted to spread the suspension evenly on the surface. Care should be taken to get all of the conidial suspension shaken out of the small tube and onto the plate. Performing this step properly will help in reducing plating errors. Usually 3 plates each of minimal and the two types of supplemented media are used. If there is reason to believe that the nuclear ratio of the heterokaryon is very disproportionate and if the heterokaryotic fraction is very low, it may be desirable to plate at more than one concentration in order to get a more accurate estimate of this fraction as well as of the minor nuclear component. Plates are incubated at 30° C for 3-4 days. When sorbose is used in the media, an incubation temperature of 25° C is usually not satisfactory because of poor viability of the conidia and a lessening of the paramorphic effect of the sorbose.

In preparing the plating media, we usually autoclave all of the components together in 1000-ml Erlenmeyer flasks (500 ml of medium/flask) for 15 minutes and pour them immediately after they are removed from the autoclave. Pouring the plates when the media are still hot helps to cut down on contamination and facilitates getting more uniform amounts of medium/plate, as compared to using media which have cooled considerably and are, consequently, more viscous. We have found that the use of absorbant discs, inserted into the lids of petri dishes to absorb excess moisture, drastically affects viability and size of colonies and must be avoided.

The choice of basal salts (e.g., Vogel's, Fries' or Westergaard and Mitchell's) for preparing plating media is somewhat arbitrary since no one type is best for all experimental purposes and for all strains. Primarily, conidial viability, size and morphology of colonies, the time needed to form countable colonies, and the type of sugars and other supplements used are all involved in selecting the proper plating medium. Colonies usually come up faster and are larger on Fries' and Vogel's than on the medium of Westergaard and Mitchell. Higher growth rate can be an advantage, but if the size of the colony is too large, this can be a disadvantage when large numbers are present on a single plate. Apparently, the presence of tartrate and citrate, respectively in the first two salt solutions causes discoloration of the media during autoclaving and we have found that sucrose hydrolysis is apparently inhibited by these compounds during autoclaving. Colonies from some strains may also conidiate earlier on these media. Data on viability and colony size with various salt and sugar mixtures have

been reported previously by Brockman and de Serres in their paper on sorbose toxicity (1963 Am. J. Bot. 50: 709).

In our own experience with the three basal salts and a variety of combinations of sugars, we have found that conidial viability is about the same if 1% sorbose is used with either 0.01% glucose, 0.05% glucose + 0.05% fructose, 0.005% glucose + 0.1% sucrose, or 0.1% sucrose + 0.2% glycerol. We have found that a medium containing 0.01% glucose, 1% sorbose and Westergaard and Mitchell's salts solidifed with 2% agar is satisfactory for most of our purposes. Germination is high on this medium and small, compact, easily-countable colonies ca. 1 mm in diameter are formed after 4 days of incubation at 30° C. Colonies do not conidiate readily and a large number of colonies of this size can easily be counted on a single plate. We have also found that viability of some strains is better on this medium when certain complete supplements or mixtures of several growth factors are used.

Colonies are counted after 4 days of incubation at 30° C, but in some cases, and especially with certain sugar mixtures, a significant proportion of the colonies may appear later than this and, consequently, the best time for counting needs to be individually determined. An automatic colony counter similar to the model manufactured by the New Brunswick Scientific Co. is a real aid to counting.

The number of colonies present on the minimal and supplemented plates is then used to determine the nuclear ratio present in the heterokaryon. We have been using the approximation proposed by Atwood and Mukai (1955 Genetics 40: 438). For this calculation, one needs to know not only the average number of nuclei per conidium, $\bar{n}$, but also the proportion of heterokaryotic conidia and homokaryotic conidia of the two components of the keterokaryon. It is troublesome to stain nuclei and to determine the value of $\bar{n}$ for every heterokaryon and we have been using a value of 2.5. Several years ago Atwood and I determined the average number of nuclei per conidium in over 33,000 individual conidia. Among this group were 46 hetero-karyons grown on minimal medium. The average nuclear number in the heterokaryons was 2.56. However, the formula is not particularly sensitive to changes in $\bar{n}$ and an estimated value can be used in many cases. For example, the value of the two nuclear components (a and b) of a heterokaryon can first be estimated by using a $\bar{n}$ of 2 and then estimated again, using the same plating data, by substituting a $\bar{n}$ value of 3. The values obtained will not differ from one another by much more than 1% if the values of a and b are somewhat proportionate, but if a and b are very disproportionate, the estimated differences will be significantly less than 1%.

The use of this formula is fairly straightforward, but there are circumstances where the nuclear ratios calculated from plating data may be in some error. Such a situation is well illustrated by the arg + meth, amyc heterokaryon studied by Atwood and Mukai. Because of the amycelial phenotype, the meth, amyc homokaryons are morphologically distinguishable on the methionine-supplemented plates from the hetero-karyotic colonies and they were able to compare the number of heterokaryotic colonies on the minimal and methionine plates. The number of heterokaryons was always greater on minimal than on methionine. They were able to make a more realistic estimate of the nuclear ratios by making certain assumptions regarding viability and applying a correction factor. Later, they were able to show that this inhibition could be atttibuted to the use of DL-methionine and could be relieved by using smaller concentrations of L-meth-ionine.

In the absence of morphological markers, this type of inhibition may go undetected unless one specifi-cally tests for it, but one will often get some indication of this by also using doubly-supplemented or com-plete medium and comparing colony counts on this medium with the two singly-supplemented plates. On the other hand, some types of inhibition are more readily observed, such as cases where the number of colonies found on one type of supplemented medium is significantly less than on minimal. These cases usually turn out to be situations where both homokaryotic and heterokaryotic conidia are inhibited, but to different degrees. Such inhibition has been observed with a variety of mutants in different heterokary-otic combinations. Since many growth factors are known to have an inhibitory effect on germination or growth, one should continually bear in mind that this type of inhibition phenomenon will prevent one from obtaining meaningful estimates of nuclear ratios.

If one is using a certain combination of mutant markers for the first time, it may be well to validate the basic assumptions in the plating techniques. One of the simplest ways is to transfer colonies from supplemented plates to minimal slants (care should be taken to transfer as little supplemented medium as

as possible) and the ones that grow and mature on minimal are judged to be heterokaryotic. These can then be compared with the numbers found on minimal plates. - - - Department of Agronomy, Kansas State University, Manhattan, Kansas.