

TOWARDS A PRACTICAL FAIL-SAFE SYSTEM OF MANAGING POIKILOTHERMIC VERTEBRATE CELL LINES IN CULTURE

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SUMMARY

A series of 13 integrated precepts is used in a system for managing continuous laboratory propagation of poikilothermic vertebrate cell lines. The precepts are listed in sequence of application and discussed.

Tissue culture began in the pre-antibiotic era, and rigid adherence to strict aseptic technique was the only route that assured success. Almost embarrassing demands of technique, laboratory clothing, and even of laboratory demeanor were made of students and of practitioners at that time. Tissue culture had at least some characteristics of a cult, and the disciples were relatively few in number.

In the course of about three decades, the original esoteric techniques have evolved so that tissue culture has become a laboratory routine to a multitude of practitioners (1-5). With this progress there has developed a casual attitude towards tissue culture and an attendant occurrence of mishaps (6). Even with the use of antibiotics, contamination by bacteria or molds is an ever present threat, although these contaminants are usually grossly visible and demonstrable with ease. Mycoplasmal contamination also occurs and is more insidious, for it may not be readily apparent. Isolation or demonstration of mycoplasmas presents greater problems, and their presence in cell cultures may have disastrous consequences (6-8). Cell cultures may also be contaminated with protozoa (6) or by other cell lines. There have been gross misidentifications (6, 9, 10), and complete "wipe outs" of cell stocks from one or another cause.

To varying degrees, contamination, loss of identification, mixed or misidentified cultures, and even total destruction of stocks are continuing threats to successful cell culture.

We have the impression that, at least in culture of fish cell lines, the incidence of laboratory

disasters is growing. Many of these disasters need not have occurred; they could have been prevented by a methodical system of culturing. Rather surprisingly, we could find no published "how-to-do-it" instructions for the serious new cell culturist. Standard references do give advice on preventing contamination, but they do not include routines whereby cells may be reliably propagated with minimal problems.

A systematic approach to cell culture can minimize nearly to the vanishing point the frequency and the effect of catastrophes. Such an approach goes beyond merely "keeping" or "growing" cell cultures. Systematic culture demands, and results in, continuous propagation of clean cultures of correctly identified cells.

By using much of the experience and methods of others, we have developed and modified a system which has enabled us to establish amphibian and fish cell lines and to maintain them in an antigenically pure condition, free from microbial contamination, for 12 years. We make minimal claims to foresight. Good fortune has permitted modification of routines and the correction of mishaps and near catastrophes before they became fatal for the cell lines as biological entities.

Our operations are small and are usually restricted to lower vertebrates. We have close contact with many laboratories of similar size, research interests, and problems. Our purpose is to describe a system which has worked well for us. We hope that it will be of value to others and that others will make improvements on it.

SEQUENTIAL STEPS OF CULTURE MANAGEMENT

1. *Determine the degree of discipline needed.* The first step is to define the kind and degree of discipline needed for the particular project to be undertaken. The need for cell cultures may be seasonal or otherwise restricted, e.g. an activity which may be pursued during only part of an academic year. If the cultures to be used are readily available, e.g. from the Animal Cell Culture Collection of the American Type Culture Collection or from commercial sources, a practical approach is to obtain certified starter cultures, employ them for the period of planned work, and discard them. In such cases, a modified, less stringent management is then required for short-term culture.

2. *Separate the propagation of stock cultures and of working cultures.* Propagation of stock cultures should be an entirely separate operation from routine working or production cell culture. For this discussion, *stock cultures* are defined as those cells which are capital sources that are propagated to maintain their lineage. Routine *working* or *production cultures* are derived from stock cultures and are those that are used in routine applications. Laboratories should not carry cells on a routine basis to serve both as stock cultures and as working cultures. We suggest that stock cell culture maintenance should be a first priority task and that it be carried out independently of other laboratory duties.

3. *Use binary propagation of stock cultures.* Culture management should be approached so that risk and responsibility are divided. This is a basic principle, and it should apply even if the work is a one-man operation. Management of cultures should be carried out by two people, or as though by two people (Fig. 1). Two separate sets of cultures should be maintained, each with its own media, solutions, records, and schedules of subculturing. Dichotomy or duplication, although one person may carry out all operations, provides a first back-up system to prevent catastrophe for an entire culture lineage. To realize the full benefits, the two systems should be completely separate and handled entirely independently of each other.

4. *Obtain serum annually and test it prior to purchase.* Serum is used in virtually all media for lower vertebrate cells, and fetal bovine

serum is used more than any other; it has some advantages, but it is not without some disadvantages (6-8, 11, 12). Bovine serum may contain any of several kinds of microbial contaminants including viruses, but the latter have not yet been proven to be a problem in lower vertebrate cells, possibly due to species specificity and to the usual comparatively cool temperatures of incubation. There is a growing awareness and a concern for the prevalence of mycoplasmas in sera used in culture media (6-8, 11, 12). Newer, larger volume testing by commercial producers will undoubtedly reduce the incidence of mycoplasma contamination. In efforts to minimize the risk, some investigators have found that heating serum at 55 to 60°C for 30 min will inactivate mycoplasmas if such are present (8). In addition to the above, and like other sera, the quality of fetal bovine serum varies from lot to lot, even though it may be obtained from a single supplier.

The widespread use of fetal bovine serum is almost traditional in lower vertebrate cell culture, and it is based in part on the fact that it has almost universally given satisfactory results. Undoubtedly, inertia is also a factor, for a majority of cell culturists have been reluctant to explore the feasibility of using serum from other kinds of animals.

Our cell culture work is almost exclusively applied in fish virology. Fetal bovine serum supports good growth of fish and amphibian cells, but in addition no lot to date that we have tested has shown neutralizing activity against the fish viruses with which we work. Some lots of human serum and of calf serum have shown neutralizing or inhibitory activity against a fish virus.

Upon the advice of others, we use and recommend the following system of buying serum. Our total needs for a year are estimated, and that quantity is ordered with the provision that it be a single lot and tested with the recently introduced large sample screening for mycoplasmas (7). The lot is put on reserve (most suppliers willingly provide this service), and a sample is sent to us for testing. We compared the sample with the previous year's lot for ability to support good fish cell growth. In the past, the comparison has been with at least three cell lines which we carried through several subcultures. (Present

trials indicate that comparative plating efficiency will provide a quicker and more sensitive test, and for the next purchase we will use that method.) Secondly, we test the serum for freedom from inhibitors of several of the viruses we work with. If in both tests the sample proves satisfactory, purchase and shipment of the lot is authorized. Upon receipt, it is dated and stored at -20°C . This serum is used both in the preparation of media for stock cultures and for routine media used in day-to-day production cultures.

5. Use only tissue culture quality water for all solutions. High quality water is essential for preparation of media and other tissue culture solutions. Our own needs are met by deionized water (mixed bed resins) that is doubly distilled in borosilicate glass apparatus. This water is used to hydrate powdered medium, because cells respond unfavorably to water of lesser quality.

U.S.P. water for injection is also appropriate for use in tissue culture. The water is sterile, pyrogen-free, and available through pharmacies in liter bottles. Small laboratories may find it advantageous and economical to buy the water needed for tissue culture media and solutions.

6. Insure sterility in the preparation of media and solutions. Media and solutions are prepared carefully to prevent contamination (13). A few preparations such as Versene solution and certain media prepared from powders are sterilized by autoclaving. Complete media, medium components, and solutions which cannot be autoclaved are passed through $0.45\text{-}\mu\text{m}$ and then $0.22\text{-}\mu\text{m}$ synthetic membrane ultrafilters (e.g. Millipore, Gelman, etc.) as essential decontamination steps.

Sterility testing of all media and prepared solutions is an absolute necessity. We test a 2% sample of all solutions and media which we pre-

A. Medium preparation

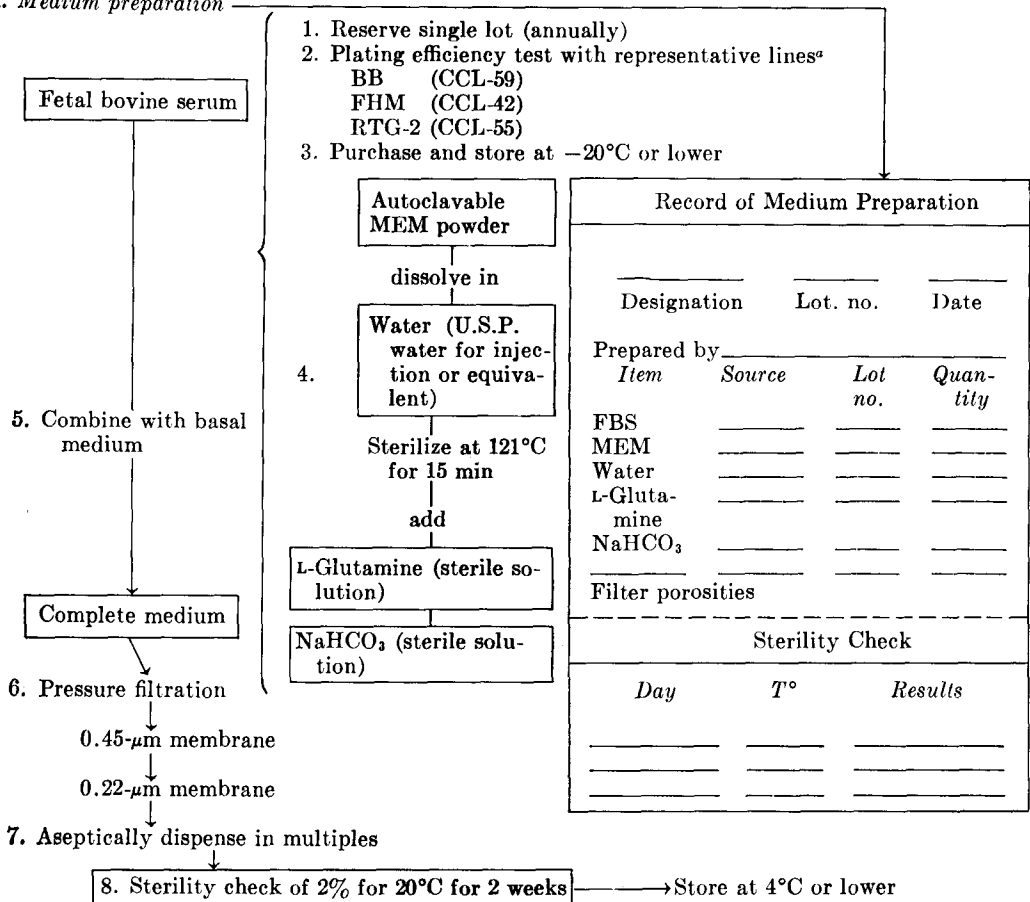
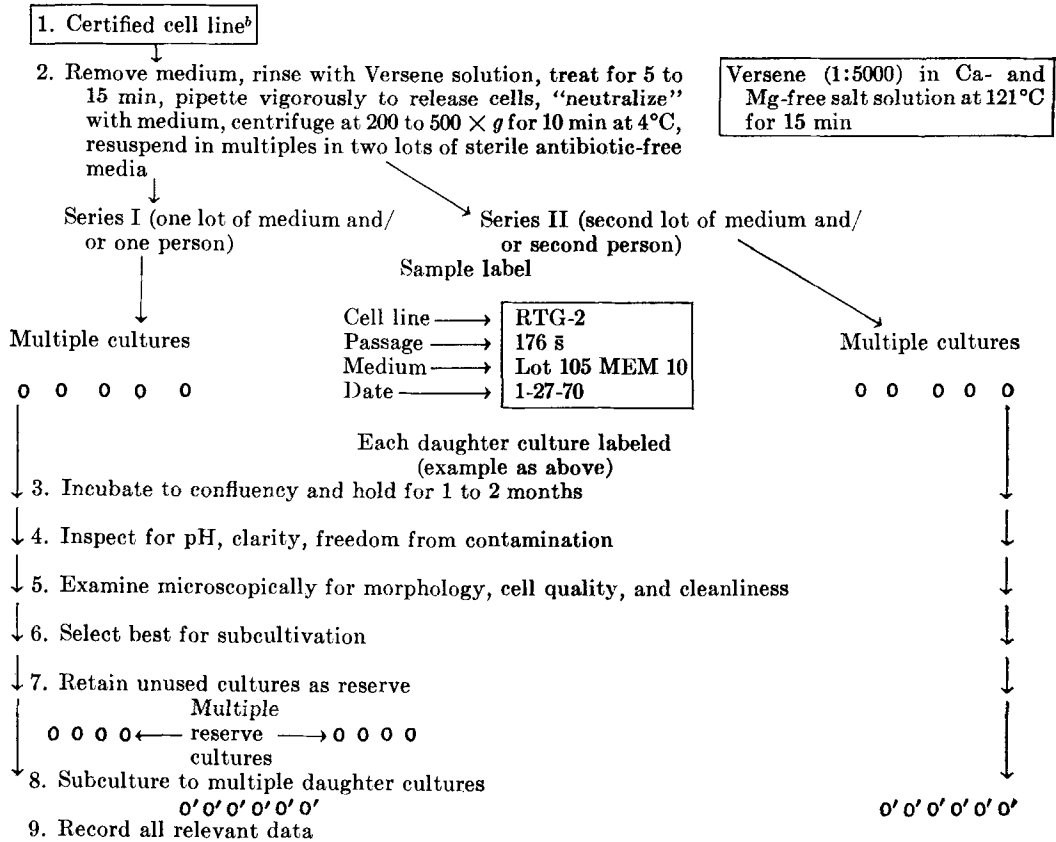


FIG. 1. A flow chart for managed propagation of poikilothermic vertebrate cell lines.

B. Stock culture propagation protocol



Record of _____ cell line				Page _____		
Date	Pass	Source	Split ratio	Medium	Versene	
_____	_____	_____	_____	_____	_____	
_____	_____	_____	_____	_____	_____	
_____	_____	_____	_____	_____	_____	

10. Check annually for mycoplasmas

11. Expand appropriate stock cultures annually and use with antibiotics as source of fresh working stocks. Older working stocks to be discarded.

^a For virological application, each lot of serum is also tested for freedom from neutralizing activity against four fish viruses.

^b Or equivalent. Certified cell lines available from American Type Culture Collection, Rockville, Maryland 20852.

pare. Commercial laboratories test up to 10% of the products which they prepare and so, with the exception of serum, we cautiously trust the sterility of commercial products. We also keep alert for, and check, any turbid or otherwise suspicious commercial material.

Samples of cell culture medium containing serum are incubated for a minimum of 1 week and usually for 2 or more weeks before being cleared for use. Under our conditions, the princi-

pal sterility testing is carried out at 20°C, the temperature at which most of our cells are grown. We also test smaller quantities at 37°C. For simplicity, we use such sterile media as test media for testing other components or solutions, because we have found that some bacterial contaminants grow well therein and only poorly in bacteriological media.

Media for stock cultures are reserved exclusively for that purpose. Such media may be

safely kept at 4°C for up to 1 year without undue loss of nutrient qualities. We keep most of our stock culture media at -20°C and have safely stored them at that temperature for at least 2 years. Freezing in small lots is suggested, so that a batch of medium need not be repeatedly thawed and refrozen.

These procedures, plus the incubation of the cultured cells themselves, give continual monitoring of sterility. Other aspects of contamination control have been reviewed recently (13).

7. *Do not use antibiotics on stock cultures.* We follow with firm conviction the advice of cell culturists who for years have strongly advocated the maintenance of stock cell cultures on antibiotic-free media. This precept has been recommended since the early days of cell culture. Culture in antibiotic-free media facilitates more rapid detection of microbial contaminants and enforces a discipline of aseptic technique more effectively than if antibiotics are used.

8. *Disperse cultures with heat-sterilized Versene only.* Autoclaved Versene is used for dispersing stock cultures. Although dispersion with Versene only is less efficient than with Versene plus trypsin, the Versene solution has the advantage that it can be sterilized by heat.

9. *Handle only one cell line at a time.* A basic rule of cell culture is that only one line be handled at a time. The reasons for this are to minimize human error, to minimize misidentification, and to prevent cross-contamination of cell lines with one another.

10. *Propagate all cell lines in multiple cultures.* All cell cultures, but especially stock cultures, should be kept in multiples of at least three or four, and in sealed vessels; tubes, or small flasks or bottles are convenient. When cultures are split, three or more subcultures are prepared. Small culture vessels are easily examined microscopically for cell quality and cleanliness, and they can be incubated in relatively small racks, trays, etc., and require comparatively little incubator space. Multiple tube cultures are particularly convenient, because many may be kept in a single rack. When subculture is required, the best appearing culture is chosen as stock for daughter cultures. The unused cultures provide reserve units and a second back-up system.

Concerning production cultures, in contrast to stock cultures, the practice of carrying cell lines

in multiple vessels is a routine procedure in most laboratories. It is important to keep at least one major culture vessel of each cell line in strict reserve as insurance against the occasional contamination. Contamination can then be sustained without causing a major disaster, and the reserve cultures can be used to reduce lost time. This practice saves the time required for growing up populations of comparable size from the (usually much smaller) stock cultures.

11. *Subculture stock cell lines at minimal frequency.* Subculture frequency should be consistent with convenience and with requirements of cell maintenance. Our active stock cultures of cells of lower vertebrates are usually incubated at 15°C and subcultured only three to five times a year. The more tolerant cultures may be kept at 4°C and subcultured less frequently.

If prolonged maintenance of stock cultures is required, low temperature storage (-85°C or lower) should be considered. The development, rationale, and methods of freezing tissue culture cells have been reported in the works of Evans and co-workers (14) and Coriell, Greene, and Silver (15). Storage of cells in the frozen state will usually provide secure maintenance; in order of decreasing dependability, liquid nitrogen, Dry Ice, or mechanical freezers are available. If frozen storage is not feasible, cells may be incubated at about 5°C. For cells of warm-blooded vertebrate origin, some authors have recommended incubation at about 28 to 30°C to prolong the culture generation time. Cells from lower vertebrates lend themselves particularly well to low temperature maintenance. Many poikilotherm vertebrate cell lines grow slowly and survive well at 15°C or lower.

12. *Maintain detailed records.* Complete record keeping is essential (Fig. 1). This includes identification labels on every culture vessel, and a permanent record indicating the cell line, the date of each subculture, the medium involved (kind and lot number), the number of subcultures made, and the outcome. In practice, notations of outcome concern aberrant behavior or contamination. The record keeping provides a second monitoring of the sterility of the solutions used with the cells.

A second set of records is kept for solutions prepared from components (Fig. 1). These records include manufacturers' lot numbers, and

the lot number of every component used to prepare the solutions.

Chemicals used in cell culture operations are kept in the cell culture work area, and used only by, or under supervision, of personnel trained in cell culture work. The risks of chemical contamination are thereby minimized.

13. *Follow a regular testing of stock cell lines for mycoplasmas.* Stock cultures should be tested at least once a year for mycoplasmas. Procedures for such testing have been published (7, 16), and mycoplasma media and components are available commercially. Detection of mycoplasmas is probably most reliably performed by personnel who are specifically trained and experienced; therefore, we prefer to have our testing done by an outside laboratory with such qualified personnel.

In addition to the above, we suggest that working or production cultures be discarded at yearly intervals and that new production cultures be derived from those which have been carried without antibiotics. We use this system because it provides a measure of assurance that we are working with cultures devoid of competing or confounding microorganisms.

CONCLUSIONS

The foregoing system has been developed and modified during the past 12 years of our work with amphibian and fish cell lines. It has enabled us to establish and to maintain poikilotherm cell lines in an axenic condition.

Adoption of the system by others will probably require more time and effort than is being presently expended in managing cultures. We think it likely, however, that over-all the system will actually save time and effort because it will minimize lost time due to avoidable mishaps in culturing cells. Even a single failure to meet scheduled work can more than offset the effort spent in properly managing cell cultures.

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