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# The Art of Animal Cell Culture for Virus Isolation

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## 1. Introduction

In virology, cell culture usually refers to the *in vitro* growth and manipulation of cells from a tissue obtained from a multicellular organism. The term “cell culture” is often used interchangeably with “tissue culture”. Cell culture remains integral with virology, as viruses are obligate intracellular parasites that require replication within a living cell to produce copies of themselves (i.e., to form progeny virions). Both animal and plant cells are propagated in cell cultures. The only other practical alternatives to cell culture are to propagate the viruses in susceptible animal or plant hosts. This review covers only cell culture for animal viruses. Since the literal meaning of tissue culture is the culturing of tissue pieces, i.e. explant culture, the term “cell culture” is used in this review instead of “tissue culture”.

Cell cultures can be prepared from unicellular cells (e.g., white blood cells) or from a piece of “tissue”. We define tissue as an aggregate of similar cells forming a definite kind of structural material with a specific function in a multicellular organism. Tissues are first dissociated into smaller pieces by mechanical disruption (such as by cutting into smaller pieces using scissors). Next, the tissue pieces are subjected to treatment with agents that disrupt the extracellular matrix that holds cells together. The cell-dissociating agents usually are proteolytic enzymes such as collagenase and trypsin (to digest proteins) in combination with cation chelators such as ethylenediaminetetraacetic acid (EDTA) that bind, or chelate, the  $\text{Ca}^{2+}$  and other divalent cations on which cell-cell adhesion depends. The cells are then gently teased apart, suspended in cell growth medium and placed in sterile growth vessels. In the past, glass bottles or petri dishes were used. Indeed, in the context of virology, the term “*in vitro*” originally referred to cells grown “in glass”; this term was used to contrast them experiments carried out on cells grown in glass vessels as opposed to experiments using living organisms. Nowadays, polystyrene (plastic) vessels are most commonly used for cell culture. A majority of cell culture is still performed using techniques wherein the cells are grown in two dimensions. Newer technologies are available that permit cell growth in three dimensions;

using such techniques, it has been able to induce some cells to differentiate into forms that are not observed during two-dimensional growth. Such technology is still relatively new in the field of virology, and are not discussed in this review.

Since tissues are generally composed of different cell types, a heterogeneous population of cells is usually isolated during the first ("primary") attempt to isolate cells for cell culture. Therefore, subsequent efforts are made to separate the cells into the various types, with the goal of obtaining homogenous populations of cells. The genetic uniformity of a batch of cells can be attained through a process termed "cloning", wherein one cell is isolated and allowed to proliferate to form a "colony" or "clone". All the cells in a colony derive from a common ancestor, and are thus "clones" of each other.

Cells obtained from tissues tend to be "adherent", meaning they attach to the growing surface (of the flask or other vessel) then spread out to form a monolayer. On the other hand, white blood cells settle but do not adhere, and are maintained as "suspension" cultures. As such, they are usually constantly stirred by a spinning magnet in a type of growth vessel termed "spinner flask". Adherent cells growing as flat monolayers are generally of two types: fibroblast and epithelial. Fibroblasts are the most common cells of connective tissue, and synthesize collagen and the extracellular matrix. Epithelial cells line the cavities and surfaces of structures throughout a body, and also form many glands. In culture, they tend to appear rectangular and pack into tight monolayers that look like "brick pavements".

The art of cell culture for virus isolation has entered a renaissance in recent years. Significant improvements have been made in the quality of available reagents, plasticware, and in basic methodologies. For example, disposable plasticware has largely supplanted the use of glass vessels. This has reduced the costs of culturing cells in many ways: (a) glass bottles do not have to be washed and sterilized between uses, (b) cell culture plasticware can be purchased ready-made with growth surface coatings or electrostatic "treatments" that promote cell attachment to the growing surface, (c) plasticware is inherently safer in that it is relatively shatterproof. Other sterile disposable plasticware has significantly reduced work burdens. For example, disposable pipettes have made obsolete the task of washing glass pipettes, and the subsequent tasks necessary to prepare them for virology work. In the past, such tasks required the use of multiple detergent and acid washes, which created potential biological and chemical hazards, and required large amounts of distilled water. Apart from improvements in materials used for virology work, many new instruments are available that simplify cell culture technology and at the same time improve precision between experiments. For example, various cell counters are now available that make cell counting easy and reproducible in an intra- and inter-laboratory manner. Moreover, various new or engineered cell lines and primary cells are available for the propagation of viruses once considered very difficult to study *in vitro*. Unfortunately, along with material improvements and technological progress, less emphasis is usually placed on teaching the art of cell culture compared to molecular technology relevant to virus detection. This is a recipe for disaster, as cell culture is neither simple nor a thoughtless process. To paraphrase recent statements

by a colleague, “All cells cultured *in vitro* are angry; they are outside of their normal environment and maintained under artificial conditions, surrounded by physiologically incorrect concentrations of all things important to their well-being. No wonder it is so difficult to have well-behaved cell cultures”! Without adequate training and preparation, cell culture as an art and science becomes sloppy, and data generated by such practices are questionable. We have often not been able to repeat the results of others, and they have not been able to repeat ours, due to a difference in the cells used in our experiments. In some cases, the cells are not what they should be, in other cases, the cells are contaminated with adventitious agents that confound the results, and sometimes, the cells have changed, either through differentiation or genetic instability.

Due to the number of different disciplines now engaged in cell culture, the terminology used to describe the work varies substantially. This makes it difficult to communicate effectively using language salient to virology. For this review, eight key terms used for cell culture work and their definitions are presented in Table 1. The definitions given in Table 1 are from the Society for In Vitro Biology (SIVB), as in reference [1]. We strongly recommend adaption of SIVB terminology for inter-laboratory communications of cell culture work. Their definitions are well-thought out, and intuitively understandable.

In this chapter, we provide a review of some contemporary cell culture issues relevant to virus isolation. Some practical guidelines for virus isolation and the maintenance of cell lines are provided. The information we present should provide useful insights for virologists, and may be a useful review of some forgotten principles of virus isolation. This review is not meant to be comprehensive, as each topic would require substantial and detailed treatment, historic and contemporary. It should be noted that the terms “cell strain” and “cell line” (Table 1) are sometimes used interchangeably by other authors. Others also define “cell strain” as a culture of a single type of cell, and “cell line” as an *immortalized* culture of a single type of cell.

## 2. Basic validation of animal cells used for the isolation of viruses

Cell lines or primary cell cultures derived from vertebrates and invertebrates are used for the isolation of animal viruses. Animal cell lines can be purchased from well-known suppliers, including those listed in Table 2, and primary cells from suppliers such as those listed in Table 3. Alternatively, primary cell cultures can be established *de novo* from live animal cells, tissues or organs. Cells for virology work can also be obtained from university collections or from individual laboratories. In general, the best practice is to obtain cells at the lowest possible population doubling level (Table 1) from reputable suppliers that can provide documentation relevant to traceability. A problem we encounter repeatedly is that a majority of academic research laboratories totally lack or do not have an adequate “Quality System” or “Quality Practices” process, and traceability is problematic. For example, it has been nearly impossible to estimate the population doubling level (“true age of the culture”) of cells obtained from most research laboratories due to inconsistencies not only in record keeping but also due to lack of standardization of practices. The major problem encountered with cells that have been

highly passaged is that their phenotype can be quite different from their progenitors. This often makes it difficult to reproduce experiments performed in past years using the cells.

Terms	Definitions <sup>a</sup>
Adventitious	Agents which contaminate cell cultures.
Cell line	A cell line arises from a primary culture at the time of the first successful subculture. The term cell line implies that cultures from it consist of lineages of cells originally present in the primary culture. The terms finite or continuous are used as prefixes if the status of the culture is known. The term "continuous line" replaces the term "established line".
Cell strain	A cell strain is derived either from a primary culture or a cell line by the selection or cloning of cells having specific properties or markers. In describing a cell strain, its specific features must be defined. The terms <i>finite</i> or <i>continuous</i> are to be used as prefixes if the status of the culture is known. If not, the term <i>strain</i> will suffice.
Passage	The transfer or transplantation of cells, with or without dilution, from one culture vessel to another. It is understood that any time cells are transferred from one vessel to another, a certain portion of the cells may be lost and, therefore, dilution of cells, whether deliberate or not, may occur. This term is synonymous with the term "subculture".
Passage number	The number of times the cells in the culture have been subcultured or passaged. In descriptions of this process, the ratio or dilution of the cells should be stated so that the relative cultural age can be ascertained.
Population doubling level	The total number of population doublings of a cell line or strain since its initiation <i>in vitro</i> . A formula to use for the calculation of "population doublings" in a single passage is: number of population doublings = $\text{Log}(N/N_0) \times 3.33$ where: N=number of cells in the growth vessel at the end of a period of growth. $N_0$ =number of cells plated in the growth vessel. It is best to use the number of viable cells or number of attached cells for this determination. Population doubling level is synonymous with "cumulative population doublings".
Population doubling time	The interval, calculated during the logarithmic phase of growth in which, for example, $1.0 \times 10^6$ cells increase to $2.0 \times 10^6$ cells. This term is not synonymous with "cell generation time".
Primary cell culture	A culture started from cells, tissues or organs taken directly from organisms. A primary culture may be regarded as such until it is successfully subcultured for the first time. It then becomes a "cell line". A primary culture may contain multiple types of cells such as fibroblasts, epithelial, and endothelial cells.

<sup>a</sup>Published in reference [1] and also available though: [http://www.sivb.org/edu\\_terminology.asp](http://www.sivb.org/edu_terminology.asp).

**Table 1.** Key cell culture terms.

Acronym	Entity
ATCC	American Type Culture Collection, USA
DSMZ	German Collection of Microorganisms and Cell Cultures, Germany
ECACC	European Collection of Cell Cultures, United Kingdom
ICLC	Interlab Cell Line Collection, Italy
LCRB	Japanese Collection of Research Bioresources, Japan

**Table 2.** Sources of animal cell lines for virology.

	Company
1	Allcells
2	Asterrand
3	ATCC
4	Cell Applications, Inc.
5	Invitrogen
6	Lifeline Cell Technology
7	Lonza
8	QBM Cell Science
9	ReachBio
10	Science Biosystems
11	ScienCell Research Laboratories

**Table 3.** Partial list of suppliers of primary cell cultures.

Whereas the terminology differs between laboratories and in different countries, many virologists use terminology coined by the ATCC and refer to continuous (previously termed “established”) cells as either: CCL (Certified Cell Lines), CRL (Certified Repository Lines), HB (Hybridomas), TIB (Cell Lines in Tumor Immunology Banks), and HTB (Cell Lines in Tumor Cell Banks). In general, cells designated as CCL by the ATCC are the most thoroughly characterized and have been certified by the National Institutes of Health American Cell Culture Collection Committee [2].

Prior to use for virus isolation or detection, cells should be “validated” for their intended purpose. A valid batch of cells implies they have been tested and confirmed suitable for the isolation or detection of the target virus. Three general questions must be asked and answered during the validation process:

- a. Has authenticity been confirmed (are the cells what they should be)?
- b. Do the cells behave as expected before we use them?
- c. When the task is virus isolation (or propagation): Will a wild-type virus similar to the target virus effectively infect the cells, replicate in them, and form progeny virions? If the task is virus detection: Will a wild-type virus similar to the target virus effectively infect the cells and form a virus-encoded product that can be detected by immunochemistry, PCR, or other relevant methods?

Ideally, each laboratory would have the capability of fully “characterizing” each cell line prior to its use for virus isolation. Nominally, this would include some sort of authentication of host cell species, verification that the cells are free of microbial contaminants, confirmation of cell phenotype and growth kinetics, and importantly, the cells should be tested using a contemporary wild-type isolate of the virus that is targeted for detection or isolation. In practice, in-house cell authentication is usually neither cost-effective nor practical for small diagnostic laboratories. For routine/general virology work, we have found that the following practices to be useful for the validation of cells for virus isolation:

- a. Obtain cells from a reputable source or laboratory – Resources such as the ATCC provide a certificate that details authenticity, and that the cells are not contaminated with mycoplasma, bacteria, or fungi. Furthermore, cell lines obtained from the ATCC are currently confirmed for species identity using a Cytochrome C subunit I (COI) PCR assay, and Short Tandem Repeat profiling (STR) [3], a PCR-based DNA profiling method for intraspecies identification. Particular attention should be focused on cells obtained through inter-laboratory transfer, as many are cross-contaminated with different cells or have been misidentified altogether [4]. Our experience is they are also often contaminated with adventitious agents.
- b. Use a pay-for-service provider to verify cell identity. Whereas we do not necessarily endorse any, examples of commercial service providers that authenticate animal cells are given in Table 4. Many universities also have excellent cell-authentication services.

1	ATCC
2	Bio-Synthesis, Inc.
3	CellBank Australia
4	DNA Diagnostics Center
5	ECACC
6	Genetica DNA Laboratories, Inc.
7	IDEXX RADIL
8	i Life Discoveries
9	Johns Hopkins Genetic Resources Core Facility
10	LGC Standards
11	MicroSynth
12	Orchid Cellmark
13	SeqWright

**Table 4.** Partial list of cell-identification service providers.

- c. Observe the cell morphology at low and high cell densities, and verify they conform to expectations. Aberrant cell shapes may be an indication that the wrong cells were obtained, or that the cells are contaminated with mycoplasma or other infectious agents.
- d. Verify the cells are mycoplasma-free. We have obtained mycoplasma contaminated cells from even the most prestigious laboratories; in one instance, 15/20 cancer cell lines were contaminated, and upon genetic analysis, five different mycoplasma species were uncovered in the cells. Each laboratory should have a mycoplasma test, suitable for the

detection of a wide variety of mycoplasma, of which there are over 100 species. Many PCR tests for mycoplasma have been described, and various kits are available commercially. We have found through experience that PCR tests should be carefully evaluated, as many of the older tests do not detect some of the more recently discovered mycoplasma species. In concert with PCR, mycoplasma isolation is recommended as a second test, especially if the PCR tests have not been updated. We specifically recommend culturing cells for a minimum of two weeks in the absence of antibiotics prior to performing mycoplasma tests, as some mycoplasma species are inhibited but not killed by antibiotics added to cell growth media. It is a good practice, however, to isolate any incoming cell lines and automatically treat as if they contain mycoplasma through the incorporation of plasmocin in the growth medium. Some of the other non-PCR based tests for mycoplasma include detection of live organisms using the Barile culture method [5], staining of cell cultures with fluorescent dye Hoeschst 33258 [6], and use of Plasmotest (InvivoGen), which is a cell-based assay. Plasmotest is performed using HEK-Blue™ -2 cells, which are HEK293 cells that are engineered with multiple genes from the toll-like receptor 2 (TLR2) pathway. In the presence of mycoplasma, HEK-Blue-2 cells secrete embryonic alkaline phosphatase, which then reacts with a specific substrate in the cell media to produce a blue color.

Apart from university testing facilities, service providers can also perform mycoplasma tests. Whereas we do not necessarily endorse any, examples of service providers that perform mycoplasma tests are listed in Table 5:

	ATCC
1	Bionique Testing Laboratories, Inc.
2	BioOutsource
3	Bioreliance
4	Charles River Laboratories
5	Clongen Laboratories, LLC
6	Invivogen
7	Lonza
8	Microtest Laboratories, Inc.
9	Minerva Biolabs
10	Mycosafe
11	Nucro Technics
12	Paragon Bioservices
13	Q Laboratories, Inc.
14	Quadscience
15	Wuxiapptech

**Table 5.** Partial list of mycoplasma testing services

- e. Evaluate the cells for contamination with agents other than mycoplasma [7]. Often times, laboratories assume that bacterial contaminants are easy to detect through the

color of the pH indicator dye in the growth medium. In particular, many growth media formulations incorporate phenol red, and a rapid conversion from red to yellow is used as evidence for the presence of bacterial contamination. Whereas the former is often true for bacteria that are fermenters or facultative anaerobes, it should be noted that obligate aerobes tend to make the media more basic. Moreover, some bacterial contaminants are missed during cursory inspection by microscopy because they are non-motile, replicate slowly, and are often present attached to the surfaces of the cultured cells. We have found *Propionibacterium acnes* (which is an aerotolerant anaerobe), various actinomycete species, and both gram positive and negative anaerobes in contaminated cell cultures. Often, the bacteria are resistant to antibiotics commonly used for cell culture, and worse, some are multiple-drug resistant and nearly impossible to eradicate.

- f. As a final validation step, verify the cells are suitable for the target virus. This is especially true for cancer cell lines, which are karyotypically abnormal or genetically unstable. Where possible, we examine the susceptibility of the cells to a contemporary, wild-type version of the target virus, as well as to a known laboratory strain of the same virus that serves as a reference standard. This comparison is made because viruses tend to mutate, and in the process, the affinity for the particular cellular receptor may change. Moreover, the quantity of cellular receptors on the cell may change, and this can affect the avidity of the virus for its receptor.

### 3. Complications arising from the use of primary cells for virus isolation

Primary cells (Table 1), which are non-immortalized cells taken directly from a living organism, are often used in clinical laboratories for the isolation of various viruses. For example, primary monkey kidney cells, which in the USA are obtained from rhesus or other macaques or from various African “green” monkey species, are used for the isolation of echo and other picorna viruses, and human parainfluenza and other paramyxoviruses. Primary cells are especially useful for diagnostic virology because some viruses are easier to isolate (or can only be isolated) in them. However, primary cells often harbor latent viruses that become reactivated once the cells are separated from kidneys and propagated *in vitro*, or, contain viruses that produce a persistent but subclinical infection of the host. The latter viruses may not cause significant (if any) pathology *in vivo*, where the cells exist in an environment with a functional immune system. But outside of the host and away from the immune system, the cells may be fully permissive and the same virus cause highly cytopathic effects (CPE). Unfortunately, some primary cells may also harbor viruses that can replicate in the host cells without causing easily recognized CPE, and also in the indicator cells used for their isolation (or detection) *in vitro*. Unwanted viruses in primary cells cause various complications relevant to the isolation of a target virus, including:

- a. They might quickly overtake a cell culture, reducing the chances of isolating the target virus.
- b. They may cause CPE identical to those of the target virus, thus causing a false positive preliminary assessment.

- c. They are obvious sources of contamination that complicate the isolation of the target virus in “pure” form.
- d. They may pose a biosafety risk to laboratory workers.

Noteworthy, primary cells can harbor contaminating agents other than viruses. For example, mycoplasma species are present in most animals, and are prevalent on the surfaces of the respiratory tract. Moreover, mycoplasma species exist as intracellular and extracellular varieties. For reasons not yet entirely clear, kidneys are “sanctuaries” for viruses. For this reason, we often hunt for new viruses in kidney cells sourced from exotic species (J. Lednicky, unpublished).

Some of the adventitious agents we have encountered in primary cells include:

- *Human cytomegalovirus* in human kidney cells
- *Lymphocytic choriomeningitis virus* in mouse kidney cells
- *Murine polyomavirus* in the kidneys of mice from a university rodent colony
- *Parainfluenza virus 5* (formerly *Simian virus 5*) in rhesus monkey kidney cells
- *Simian cytomegalovirus* in simian (various species) kidney cells
- Simian foamy retrovirus in rhesus and green monkey kidney cells

Primary cells also have a finite lifespan, and should be used with minimal passages *in vitro*. Otherwise, senescence of the cells can be mistaken for CPE caused by viruses. Various commercial suppliers currently provide primary cells from various tissues and species. These cells should be used judiciously for virus propagation or isolation. A common mistake is to assume that primary cells obtained from the suppliers are certified to be virus free. In reality, this is not the case. For example, the *donors* of primary human cells sold in the USA are examined (by serology) for antibodies to Hepatitis B and C viruses, and to HIV, following United States Food and Drug Administration (USFDA) guidelines, and if that information is not available, the *cells* are checked by PCR or other methods for the same viruses. [The USFDA is an agency of the United States Department of Health and Human Services responsible for protecting and promoting public health through the regulation and supervision of biopharmaceuticals, blood transfusions, cosmetics, dietary supplements, electromagnetic radiation emitting devices (ERED), food safety, medical devices, over-the-counter pharmaceutical drugs (medications), tobacco products, prescription, vaccines and veterinary products]. However, additional tests for other adventitious agents have not been mandated by the USFDA, and it may be impractical to check for the presence of many other agents with regard to cost and representative sampling reasons. Thus, commercially supplied human primary cells are sold with an advisory statement indicating the cells should be considered as potentially infected, and that biosafety practices be used when working with the cells.

Cell deterioration in primary cells due to improper cell growth media formulation can also be confused for CPE caused by viruses. It is important to maintain non-infected controls along with cells used for virus isolation for comparison. We have noted cell deterioration due to L-glutamine deficiency, and to improper dosage of antifungal agents in the growth media, among a few batches of commercially bought primary cells. Similarly, commercial

media formulations for primary human cells often include additives such as epinephrine, human recombinant epidermal growth factor, hydrocortisone, insulin, transferrin, and others; a mistake in the amount of some of these biomolecules added to the cell growth media can adversely affect cell viability.

Thus, primary cells are useful for the isolation of some viruses, but should be used with caution because: (a) they can contain adventitious agents, and (b) cell deterioration due to one of many different reasons can be mistaken for virus-induced CPE.

#### **4. Serum vs. serum-free cell culture media**

Serum has been a mandatory additive in cell growth media for much of the history of tissue culture, and is essential for cell growth, metabolism, and to stimulate proliferation ("mitogenic effect"). This is because serum in a complex mixture that provides (a) hormonal factors for stimulating cell growth and proliferation and promoting differentiated functions, (b) transport proteins carrying hormones (e.g. transcortin), minerals and trace elements and lipids, (c) attachment and spreading factors, and (d) stabilizing and detoxifying factors needed to maintain pH or to inhibit proteases either directly (e.g.,  $\alpha$ -antitrypsin inhibitor in serum is an important inhibitor of the protease trypsin), or indirectly, by acting as an unspecific sink for proteases and other (toxic) molecules [8].

Previously, human and horse serum, collected aseptically through venipuncture, was the source of serum for tissue culture. This was later supplanted by less expensive bovine serum sourced from blood taken from slaughterhouse bovines. The bovine blood is collected using somewhat crude methodology, the blood clotted, serum separated, then usually filtered using 0.1  $\mu\text{m}$  filters. Both calf and fetal bovine serum (FBS) are used for cell culture media. However, primarily because of its rich content of growth factors and its low  $\gamma$ -globulin (antibody) content, FBS has been adopted as the standard supplement of cell culture media [8]. Unfortunately, filtration using smaller pore filters is technically difficult due to the complex composition of serum. Moreover, mycoplasma and viruses are not always retained by the filters; mycoplasma presumably due to small size and their inherent flexibility, and viruses due to their small dimensions and often pleomorphic nature. Thus, serum itself has often been the source of mycoplasma and virus contamination [9-14]. Viruses that may be common contaminants of bovine calf or FBS include: bovine viral diarrhea virus (BVDV) [15-19], bovine polyomavirus [20, 21], bovine parvovirus [22-24] (J. Lednicky, unpublished), and bovine herpes viruses [25-28]. Inadvertent contamination of cultured cells by these serum-derived viruses has obvious repercussions not only with regard to data generation, but also because it exerts a toll on time wasted in the performance of laboratory work, and the costs thereof. And it is usually the case that problems are noted many months afterwards (in common language, the problems occur "downstream").

Whenever economically feasible, we suggest using gamma-irradiated low antibody FBS or calf serum for tissue culture media. Low antibody sera are desirable to reduce the chances of antibody neutralization of the target virus, thus improving chances of virus isolation.

Gamma-irradiation is generally performed after filtration, and acts as a secondary safeguard [29-31]. The irradiation process purportedly inactivates viruses and live microorganisms with minimal damage to product integrity [32, 33]. Gamma-irradiation should be performed using a validated process; we have occasionally encountered batches of gamma-irradiated sera replete with filamentous carbonaceous material that to the untrained eye may be mistaken for fungal mycelia. The presence of filamentous material is due to protein degradation resulting from improper handling during the gamma-irradiation process. A word of caution: We have noted that small DNA viruses such as parvo- and polyomaviruses in sera are not effectively inactivated by gamma-irradiation (Lednický and Wyatt, unpublished observations). Similar observations were recently published by others [34, 35]. Knowledge over the susceptibility of cell lines to bovine parvoviruses and polyomaviruses is relatively scant.

Another important consideration when purchasing calf serum or FBS is the level of endotoxin [36]. Failure of some cell culture attempts can sometimes be traced to the level of endotoxin in the sera. When presented with the choice, it is always best to purchase sera with the lowest possible endotoxin levels. Industry standards for serum sold at present specify  $< 10.0$  EU/ml, where  $1 \text{ EU/ml} \sim 0.1 \text{ ng/ml}$  (EU = endotoxin unit). Endotoxin is detected by the limulus amoebocyte lysate assay, which can detect down to  $0.01$  EU/ml.

Risks associated with the use of animal-sourced components in the culture milieu have led to the quest for protein-free, animal-free cell growth media. To-date, various cell lines have been adapted to grow in serum-free media. And numerous defined serum-free media formulations are available for some of the cell lines commonly used for virus isolation, such as for the African green monkey kidney cell line termed Vero, and for Madin Darby Canine Kidney (MDCK) cells. The use of serum-free cell growth media has been validated for the isolation or propagation of many viruses [37], including those used for vaccines. As an example, a chimeric parainfluenza virus type 3 - respiratory syncytial virus was propagated to 100-fold higher titers in Vero cells grown in serum free cell growth media than could be attained with a serum-containing media formulation [38]. Vero cells grown in serum-free media has been used for the production of reovirus [39]. MDCK cells grown in serum-free media was used for the production of Influenza H5N1 virus used as a vaccine [40]. Rabies virus used for vaccine production was grown to higher titers in Vero cells in serum free than in serum-containing cell growth media [41].

Thus, serum free media should be considered for virology applications that entail routine virus propagation or vaccine virus production. Less explored is the use of serum free media for the isolation of viruses from clinical specimens. To-date, many laboratories have not experimented with this option, probably because many serum-free media formulations are used without the addition of antibiotics and antifungals, or with their use at  $1/5$  to  $1/10$  the concentrations that would normally be used in serum-containing media. At lower concentrations, the antibiotics and antifungals might not effectively suppress contaminating microorganisms that are often present in clinical specimens. However, we have found most cells that have been adapted to growth in serum-free media do tolerate antibiotics. For

example, we have used MDCK cells in serum-free media with antibiotics for the isolation of influenza viruses. As there are now many different serum-free cell media formulations, it is likely that successful methodologies using these for diagnostic virology will be developed. A particularly important aspect of such work would be the reduction of costs for diagnostic virology laboratories in less privileged nations, since serum is expensive.

As pointed out above, serum is a complex mixture and lot to lot variation and inconsistency is common. This is because the source animals themselves differ, and their nutrition status, hydration, over-all wellbeing, etc. have direct effects on the quality of the serum. Hormone and vitamins levels in the sera can vary, and all things taken together, can have a significant impact on cell culture. Thus, another argument for using serum-free cell growth media, where possible, is there is potentially better process control. Prior to the development of serum-free cell growth media, many researchers would test multiple serum lots for a particular application, then purchase a large lot of the best performing batch of serum. This is still an advisable practice when serum must be used for long-term projects, and for high-throughput work, but imposes large cost and storage burdens.

## 5. Remediation of mycoplasma contamination

Mycoplasma are bacteria (class Mollicutes) that are among the most common and serious contaminants of cell cultures. There are two genera of mycoplasma that are relevant to cell culture, *Acholeplasma* and *Mycoplasma*, and they have several unique properties that distinguish them from other prokaryotes. In particular, they lack a cell wall, instead using sterols to maintain their plasma membrane. *Mycoplasma* require cholesterol or similar sterols derived from vertebrate hosts, which they incorporate into their cell membranes, whereas *Acholeplasma* grows in the absence of sterols (but incorporates them if present). Since they lack cell walls, mycoplasma are unaffected by antibiotics that interfere with murein (peptidoglycan) formation of cell walls, such as penicillin and other beta-lactam antibiotics. They are also resistant to streptomycin. In the early 1990's, it was estimated that about 15% of cell cultures in the USA were contaminated with mycoplasma [42]. The most likely sources of mycoplasma for laboratories engaged in cell culture are: (a) previously contaminated cell cultures (which can include new cultures from unknown sources or some obtained from cell banks), (b) laboratory equipment, media, reagents that came into contact with contaminated cultures, and sera used for cell cultivation [43], and (c) personnel involved in cell maintenance [44].

Because they are relatively small (0.15–0.3  $\mu\text{m}$ ), it is difficult to filter them out of suspension. Both intracellular and extracellular types of mycoplasma exist. Mycoplasma replicate relatively slowly as they spread through a cell culture. A few mycoplasma species comprise 95% of cell culture isolates: *Acholeplasma laidlawii*, *Mycoplasma arginini*, *M. fermentans*, *M. hominis*, *M. hyorhinitis*, *M. orale*, *M. pirum*, and *M. salivarium* [42, 44-46]. It should be noted that by definition, members of the genus *Mycoplasma* are restricted to vertebrate hosts. For this reason, many researchers assume plant-based materials are free of mycoplasma that infect cells derived from vertebrates. However, we suggest caution against such notions; as

contamination of plant-based media components by *A.laidlawii* has recently been reported [47]. The consequences of mycoplasma contamination of cultured cells may vary from subtle to severe. The overall effects of mycoplasma on a cell culture vary according to the mycoplasma species infecting the cells, the mycoplasma burden (titer), the type of cells infected, and the duration of the infection [48]. Mycoplasma attach to cell membranes in order to obtain nutrients, and in the process can damage the host cell's membranes, DNA and RNA, and intracellular organelles. Their presence can exert profound effects ranging from unexpected alterations of growth patterns and host gene expression to modulation of host metabolism (e.g., produce pH-altering metabolites), induction of chromosomal aberrations, depletion of media, alteration of product yields (such as virus titer), and alteration of cytokine production and other functions of cells of the immune system [44, 48-50]. It goes without saying that efforts must be exerted at *preventing* mycoplasma contamination of cell cultures, following good cell culture practices [44].

If a cell culture is contaminated with mycoplasma, there are two remediation solutions: (a) Destroy (autoclave) the culture and dispose of it, and start with a new culture, or (b) Treat the culture with specific antibiotics or other biochemicals that are toxic to mycoplasma but safe for cells. Most commonly used cell culture antibiotics are not effective against mycoplasma contamination but other antibiotics have shown success in eliminating mycoplasma from cell cultures. In the near past, the following treatments were used to clear mycoplasma-contaminated cell cultures: (a) one to two week treatment with the fluoroquinolone Mycoplasma Removal Agent (MRA, from ICN Biochemicals [now MP Biochemicals]), (b) two week treatment with the fluoroquinolone ciprofloxacin (which is better known by many as Ciprobay), and (c) three rounds of sequential one-week treatment with BMCyclin (Roche), which contains a pleuromutilin and a tetracycline derivative [51, 52]. Prior to the use of the aforementioned mycoplasma eradication products, long-term treatments with tetracyclines were common. We and others have found such mycoplasma eradication treatments to be of limited efficacy. Instead, when necessary, we favor the use of plasmocin (InvivoGen) treatment (for a minimum of two weeks). Plasmocin is an antibiotic mixture that consists of a combination of a macrolide and a quinolone, and it is active against both intra- and extracellular mycoplasma. No permanent alterations were detected in eukaryotic cells treated with plasmocin [53], suggesting the product may be generally safe for most cell lines. This has been our experience so far with the many cell lines we have treated upon receipt as a prophylactic measure (J. Lednicky and D. Wyatt, unpublished). Plasmocure, a newer antibiotic mixture for the eradication of mycoplasma from cell cultures, is now also available from Invivogen; however we have not tested this product, and cannot comment on its efficacy.

One last important consideration is some cultures may be infected by more than one mycoplasma species [54; J. Lednicky and D. Wyatt, unpublished data]. Failure to rid a culture of mycoplasma may arise if one (or more) of the species in a contaminated culture is resistant to the antibiotics being used to eradicate the mycoplasma. Because mycoplasma biology varies among the species, it is not correct to generalize the effect of a certain

antibiotic or mixture of antibiotics for all (i.e., an antibiotic or antibiotic mix that works for the eradication of one may not work for a different mycoplasma species) [55].

In the last few years, we have not detected mycoplasma in cell lines that we have validated in our respective laboratories. And we have noted a decrease in the percentage of mycoplasma-contaminated cells obtained from reputable laboratories. However, we still frequently detect mycoplasma in virus preparations obtained from commercial suppliers, clinical laboratories, and other sources. Thus, it is advisable to assess virus stocks for mycoplasma contamination. This is particularly important for cell culture-produced virus stocks that are intended for animal studies. Especially so if the virus must be injected into the animals through intracerebral, subcutaneous, or intraperitoneal routes. In animal studies, it is rarely the case that researchers examine whether inter-laboratory discrepancies might be due to the presence or absence of mycoplasma in the challenge virus. Related to this, it is also worthwhile to verify that hybridoma antibodies used for animal studies are mycoplasma free.

## 6. Microscopy for cell culture

We have noted that many laboratories engaged in cell culture, including virology laboratories, are up to now only equipped with microscopes that use transmitted brightfield illumination. And many of these laboratories use microscope objectives that in combination with the eyepiece lenses produce a magnified image of only 200X or so. A big problem is that cells in culture appear virtually transparent when observed with an optical microscope under brightfield illumination. To improve visibility and contrast, analysts must then reduce the opening size of the substage condenser iris diaphragm, but this usually results in a serious loss of resolution and the introduction of diffraction artifacts. Moreover, subtle morphological changes are impossible to observe when cells are viewed using low-magnification using transmitted brightfield illumination. Thus, it is easy to miss telltale signs of cell deterioration or "stress", and also of CPE, when cultured cells are viewed using transmitted brightfield illumination at relatively low magnification. The images of cells photographed using transmitted brightfield illumination under low magnification tend to lack adequate resolution for teaching purposes and are less desirable for data capture. Indeed, laboratories that utilize transmitted brightfield illumination often resort to the use of various cell-staining procedures so that features of virus-infected cells can be more readily visualized. This adds costs and often presents biosafety hazards, and also kills the cells being studied. When cell staining must be used as an adjunct for microscopy, the following are useful:

1. May-Grünwald-Giemsa stain, used to visualize cytopathology. Stained infected cell monolayers are compared to those of non-infected controls. The May-Grünwald-Giemsa stain will differentially stain DNA nucleoproteins (red/purple) and RNA nucleoproteins (blue). The stained cells are also examined for the presence of syncytia or giant cells, inclusion bodies, and other viral CPE.

2. Modified Wright-Giemsa stain for white blood cells, suspension cells, or cells grown as monolayers on coverslips. Available commercially as Diff-Quick test, we have found this staining process useful for the detection of coxiella- or chlamydia-infected cells, viral inclusion bodies, and similar applications. Chromatin margination, cytoplasmic stranding, vacuoles, and the shape of viral inclusion bodies are easily visualized, giving clues on which virus may be in a cell.
3. Gimenez stain. This stain is useful for intracellular bacteria that stain poorly using a Gram stain. We have used this stain to detect bacteria growing attached to the outside of cells grown as monolayers in cell culture.

A better way to visualize cultured cells is to use an inverted microscope that is set up for phase contrast microscopy. With phase contrast, relatively thin objects such as flat cells become visible under the light microscope. Differences in cell structure are amplified during phase contrast, resulting in an image that can be regarded as an optical density map. Fine structures in the cells not easily detected by brightfield microscopy are clearly visualized, facilitating assessment of cell "condition", and CPE are easier to detect. Phase contrast microscopy is especially advantageous because living cells can be observed without being killed, "fixed" (preserved), and stained. We equip our phase contrast microscopes with objectives that provide a wide, flat view, and recommend magnification of up to at least 400X. Economical, high-quality digital cameras are now widely available and highly recommended for image capture. There are various microscopes on the open market that are affordable, rugged, and suitable for most virology applications using cells grown as monolayers. The authors of this manuscript use microscopes made by Leitz, Nikon, and Olympus in their laboratories. For high containment laboratories wherein face shields must be used [e.g., face shield that is a component of a powered air purifying respirator (PAPR) assembly], a focusing screen in LCD format is convenient. Otherwise focusing is difficult, especially when the face-shield is scratched from everyday "wear and tear", or has turned somewhat opaque from repeated decontamination with sodium hypochlorite or similar harsh decontaminating solutions).

Within the last few years, the concept of using digital inverted microscopes has generated much interest among virologists. We have tested the EVOS-x1 brightfield and phase contrast microscope (AMG, Germany) and find it easy to use. These microscopes have a high resolution LCD viewing screen that is useful for instruction purposes, as it can be simultaneously be viewed by many people. These microscopes are portable and have a relatively compact overall dimension that makes them facile to install within biosafety cabinets or class III gloveboxes. Focusing on the image by viewing the LCD screen is convenient in high-containment biosafety laboratories wherein work is performed using protective face masks such as work with PAPR.

## **7. Maintenance of virus-infected cell cultures over a few months**

The isolation of some viruses may take weeks to two months or more. There are many reasons for this. In many cases, the indicator cells are sub-optimal for high-titer replication

of the virus. Some viruses associated with lesions may be defective; for example, *Measles virus* (MeV) associated with subacute sclerosing panencephalitis may cause cytopathic effects without liberating virions [56 and references therein]. Other viruses cause persistent, sub-clinical infections in their natural hosts and may have evolved to replicate slowly. Thus, prolonged incubation, often up to two months, is required for the isolation of some polyomavirus JC virus variants that have an archetypal regulatory region [57; J. Lednicky, unpublished]. We have been able to isolate viruses considered “nearly impossible” to isolate *in vitro* by maintaining cell cultures for prolonged periods post-inoculation with clinical (or environmental) samples. A companion process to the long-term maintenance of cultures is the periodic performance of “blind passages” of the infected cell (discussed in more detail below).

The key to maintaining cells over many months is to slow down their metabolism and/or their population doubling time. This is performed on a case-by-case basis, since not all cells will respond the same way to any given protocol, and there may be variability within a given cell line, depending on age, passage history, etc. An important goal is to maintain the cells in a state relevant for the target virus. For example, would the target virus normally infect (and replicate in) cells that are mitotically active/dividing, as for parvoviruses? If so, the cells should not be allowed to become confluent. On the other hand, does the virus normally infect contact-inhibited “confluent” cells? Should the cells be terminally differentiated? Must the cells be rotated to create air and liquid interfaces as performed for the isolation of rhinoviruses using cells in roller bottles?

The three usual ways of maintaining cells for long-term observation are:

- a. Use growth medium with a reduced glucose concentration. For example, commercial Dulbecco’s Modified Eagle Medium (DMEM) is sold as “high” or “low” glucose formulations. Cells can be propagated in high glucose DMEM, then transitioned to low glucose DMEM after they are inoculated with virus.
- b. Switch from nutrient rich to traditional growth media formulations after the cells are inoculated with virus. For example, cells might be grown in DMEM, but once infected, maintained in Eagle’s Minimum Essential Medium (EMEM). As a rough measure, DMEM has about 4x the concentration of amino acids found in EMEM.
- c. Reduce the amount of serum in the cell growth medium after the cells have been inoculated with virus. For example, instead of 10% FBS, in many cases cell growth media with 1 – 3% serum can be used to re-feed cells. Furthermore, instead of FBS, calf serum can sometimes be used for the maintenance/re-feed media. This helps to slow down cell replication and metabolism because overall, calf serum has a lower concentration of growth promoting factors than FBS.

We have used the procedures above, solely or combinations thereof, for the isolation of various viruses. It is a good idea to validate growth media for each application; we have found that cell growth media are not necessarily equitable between manufacturers (i.e., all things kept equal, DMEM from supplier A may not work as well as DMEM from supplier B).

One technique that has worked well for the isolation of viruses that infect and replicate slowly in cells derived from African green monkeys is this: substitute Vero E6 for other cell lines derived from African green monkey cells. Vero E6 cells are more contact-inhibited than similar closely related cell lines, and can be maintained for long periods of times with minimal to no media changes. For example, we have been able to isolate human metapneumoviruses (CPE detected in 10 – 14 days) and other paramyxoviruses such as parainfluenza 4A and 4B viruses in Vero E6 cells maintained in serum-free media, with changes of the growth media every two weeks. This concept was popularized by Akibo *et al* [58] for the isolation of metapneumoviruses, whereas Vero E6 cells were previously thought less useful than tertiary monkey kidney (tMK) and the cell line LLC-MK2 for the isolation of those viruses.

## 8. Adventitious viruses in cell-lines

It is not uncommon to receive virus-contaminated cell lines from suppliers, and this is especially true for cells obtained through inter-laboratory transfer. One problem is that the cells may have become infected with bovine viruses (from serum) that replicate relatively slowly (i.e., the time it takes for them to complete a replication cycle and form progeny virions is higher than that of the cell population doubling time). These contaminating viruses are referred to as “adventitious” viruses (i.e., they are viruses that should not be present).

Many times, the adventitious viruses go unnoticed, and the deterioration of the cells is attributed to some type of “folklore” prevalent among cell culture practitioners. For example, we have encountered batches of cells that deteriorated when seeded at low densities, but not at high densities. Researchers who had been working with those particular cells did not question the cell propagation instructions provided with the cells. We discovered the reason the cells survived when seeded at high densities was because they were infected with parvovirus, and many parvoviruses require actively replicating cells to form progeny virions *in vitro*. Upon further investigation, we found that some cell lines that are available from commercial sources are packaged along with instructions that suggest that for propagation, no more than five cell passages (with seedings at high densities) should be attempted for “optimal” results. We surmise that a similar issue exists for those cells (that they are infected with parvo- or other viruses that require actively replicating cells)!

Apart from sera, contaminating viruses can also be traced to laboratory workers, to animal-sourced enzymes used for cell culture (such as porcine trypsin), and to other biologicals used for cell culture. A recent compilation of bovine and porcine viruses that may contaminate bovine serum and porcine trypsin is available in ref. 59. As new viruses are discovered, awareness of their possible presence in biologicals like sera and trypsin draws more interest and attention. For example, porcine trypsin has been traced as the source of *Torque teno sus virus* (TTSuV), a member of the family *Anelloviridae* that is a contaminant of many cell lines. Indeed, TTSuV was found in fifteen cell lineages, originating from thirteen different species,

and its presence in the cell lines probably traced to the use of porcine trypsin during the propagation of those cells [60]. Anelloviruses are small DNA viruses that replicate within the nuclei of infected cells, and CPE due to their presence have not been well described at present. Porcine circoviruses 1 and 2 (PCV1 and PCV2) have also been detected in cell lines including those used for vaccines, and have been traced to the use of porcine trypsin [61-63].

We recently traced a filtered amino acid supplement as the source of a contaminating reovirus, and learned from some industry colleagues they had made the same finding. However, as typical of these cases, the findings are not published and thus the information not widely disseminated. Reoviruses however can have wide host range and infect many different cell lines [60, 64].

In some cases, unusual bacteria, and even some single-celled eukaryotic microorganisms cause cell contamination problems that are attributed to viruses. This is because many people engaged in cell culture have little experience with the detection and identification of these types of organisms. We were once tasked with identifying a “virus” affecting some important in-house developed cancer cell lines, which turned out to be infected with chlamydia.

Adventitious viruses can confound research results in many unexpected ways. In one memorable event, cells were thought to have been transformed through a “hit and run” mechanism, as the transformed cells did not retain an oncogene that had been transfected into the precursor (non-transformed) cells. However, it was shown that the cells were infected with bovine polyomavirus, and were expressing its tumor protein genes (Lednický, unpublished observations). This dashed the investigators’ hopes for a patent application. It is also distressing when one performs electron microscopy and discovers that more than one virus is present in the specimen being viewed (or worse, only the wrong virus is visualized). Contaminated cell lines are a main reason gene expression studies can vary significantly between laboratories. Biopharma and the vaccine industry are by now very cognizant of the dangers posed by using contaminated cell lines, due to historic and recent problems caused by adventitious viruses.

In recent years, we have helped various researchers as well as biotechnology companies identify adventitious viruses affecting their work. Among the adventitious viruses we have recently found are:

- *Bovine herpesvirus 4* (BoHV-4) in human HeLa and Hek293, and in canine MDCK cells. BoHV-4 has been reported by others to be capable of infecting cells of various different species, including human cells [65]. In MDCK cells, cells infected with some strains of BoHV-4 produce vacuoles at the cell to cell junctions, and this is often mistaken for “cell stress” due to nutrient depletion.
- BVDV in MDBK (Madin Darby Bovine Kidney) cells. This finding is consistent with the known biology of BVDV, and MDBK cells are often used for *in vitro* studies of BVDV. For example, see [66].
- *Mouse minute virus* (MMV) in Chinese hamster ovary (CHO) cells. MMV is a notorious contaminant of rodent cell lines important for the biopharmaceutical industry [67].

- Reovirus in various mammalian and insect cell lines. Electron microscopy was used to identify these viruses; follow-up tests were not performed to determine the viral species. Nevertheless, the presence of reoviruses in insect cell lines was a surprise to our industry clients, who experienced catastrophic losses of insect cell lines used for baculovirus-based technologies.
- Vesivirus in Crandell–Rees Feline Kidney (CRFK) cells. We have detected one instance of vesivirus contamination of CRFK cells, using electron microscopy.

NOTE: We have also experienced a few cases where researchers working with cells that were contaminated with adventitious viruses had attributed cell deterioration to poor technique (graduate students were often blamed).

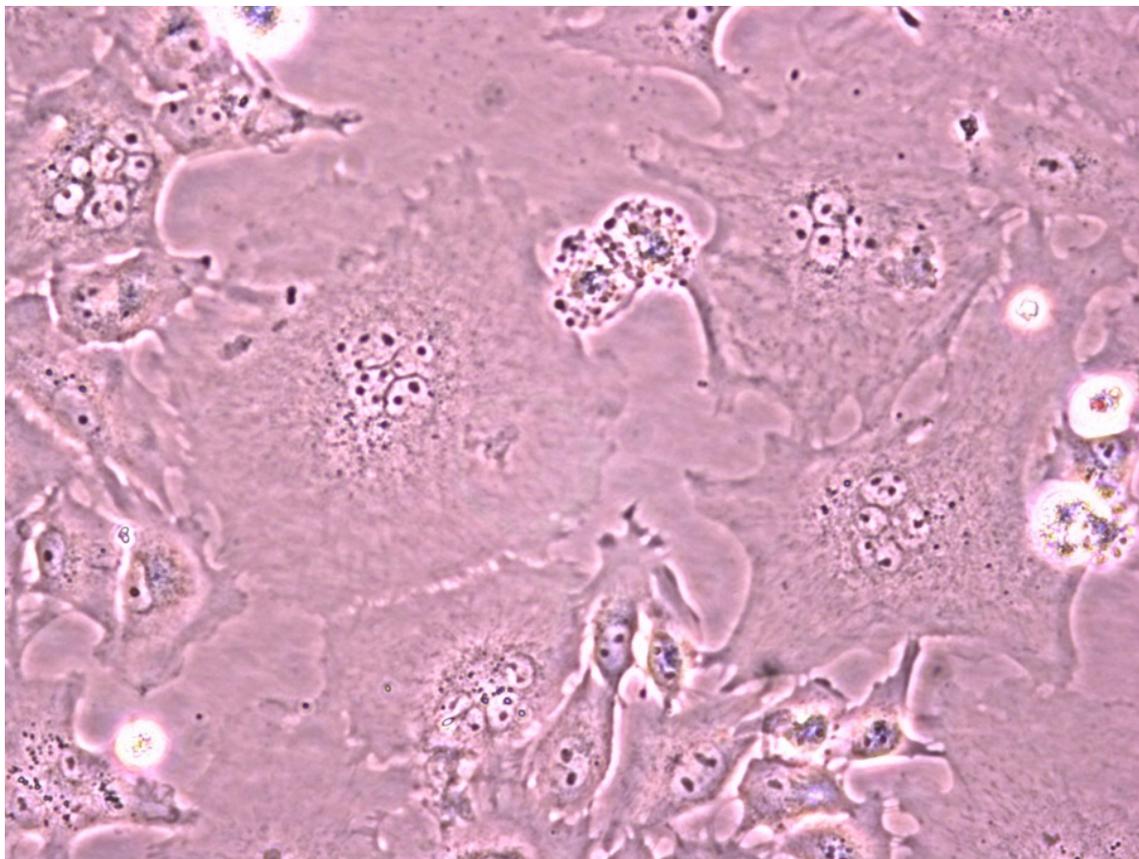
## 9. Engineered cell lines for virus isolation

As elegantly pointed out by Dr. Paul Olivo [68], rapid diagnostic assays based on direct detection of viral antigen or nucleic acid are used with increased frequency in clinical virology laboratories. Regardless, virus culture remains the only way to detect infectious virus and to analyze clinically relevant viral phenotypes, such as drug resistance. Growth of viruses in cell culture is costly, labor intensive and time-consuming and requires the use of many different cell lines. Transgenic technology offers the possibility of using genetically modified (“engineered”) cell lines to improve virus growth in cell culture and to facilitate detection of virus-infected cells. Whereas various approaches are available, the two common applications of cell engineering for diagnostic virology are: (a) engineering of susceptible cell lines to over-express virus receptors, and (b) genetically modifying cells so that they express a reporter gene only after infection with a specific virus, allowing for the detection of infectious virus by rapid and simple enzyme assays such as  $\beta$ -galactosidase assays without the need for antibodies.

Conceptually, just because a cell line is susceptible and permissive for a virus does not also mean that the optimal virus receptor is present on the cell surface. The number of viral receptors on the cell surface might also be suboptimal. If an authentic gene for the viral receptor is over-expressed (through genetic engineering), virus attachment and entrance into the cell should be improved. For that reason, we have engineered Vero E6 and other cells (such as Mv1 Lu, which are mink lung cells), that over-express canine signaling lymphocyte activation molecule (cSLAM), thought to be the major virus receptor of *Canine distemper virus* (CDV). Essentially the same rationale was used by others in their decisions to engineer cSLAM-over-expressing Vero-derived cell lines [69, 70]. Analogous to the experience of the other groups, we find that many CDV strains are detected within one day of infection of the SLAM-expressing cells vs. up to one month in conventional Vero cells. A photograph of Mv1-cSLAM cells showing syncytia within 16 hrs of infection by an American type 2 CDV strain is shown in the figure below. A related cell line that expresses human SLAM, Vero-hSLAM, is used for the isolation of MeV [71], a virus closely related to CDV and is also often difficult to isolate using conventional Vero cells.

Similarly we and others have engineered MDCK cells that over-express a sialyl transferase 1 (SIAT1) that catalyze the formation of  $\alpha$ -2,6 - linked sialic acid receptors recognized by human influenza A viruses. In our hands, most contemporary human influenza H1N1 and H3N2 viruses are detected earlier, and form higher virus titers in the engineered MDCK cells, than occurs in conventional MDCK cells (J. Lednicky and D. Wyatt, data to be presented elsewhere), in agreement with a recent report [72]. Apart from standard MDCK cells, we have also engineered additional cell lines that over-express SIAT1, and also, cell lines that over-express SIAT4 to catalyze the formation of  $\alpha$ -2,3 - linked sialic acid receptors (data and information to be presented elsewhere). With these SIAT1 and SIAT4 over-expressing cells, we have isolated influenza viruses that others were unable to, and also, as for [72], we have recovered viruses from frozen stocks that others had great difficulty with.

Another example of a cell line that has been engineered for effective isolation of a virus is L20B, used for the isolation of poliovirus, which is an enterovirus. L20B cells are derived from mouse L cells, and were engineered to express the poliovirus receptor. One advantage of using these cells over human cell lines such as Hep2 and RD for the isolation of polioviruses is that few human enteroviruses can complete their replication cycle in mouse cells [73]. Thus, it is possible to engineer cells normally not permissive for a particular virus into a permissive version.



**Figure 1.** Mv1Lu-cSLAM cells 16 hrs post-inoculation with lung homogenate from a dog with distemper including neurologic signs. Multinucleated cells, a sign of CDV infection, are evident. An American type 2 CDV variant was isolated. Original magnification at 400X.

For the genetic modification of cultured cells (to express a virus receptor), it is no longer necessary to first clone a cDNA of a particular virus receptor in many instances. Many companies now sell full-length cDNAs of human (and non-human) genes already cloned into plasmid expression vectors. Some of the new generation expression vectors are also highly active across species, thus eliminating the need to optimize enhancer/promoter sequences in the expression vector.

The second general approach for the engineering of cells for virus detection/isolation, that of modifying cells to express reporter genes in response to virus infection, is still relatively new but has already proven useful for diagnostic virology. For example, the ELVIS HSV system marketed by the company Diagnostic Hybrids for the detection and typing of human herpes simplex viruses (HSV) 1 and 2. The ELVIS HSV system uses BHK cells engineered to express  $\beta$ -galactosidase in response to HSV infection. After the engineered cells are inoculated with clinical specimen, a blue precipitate forms over the infected cells due to interaction of  $\beta$ -galactosidase with a colorimetric substrate. Formation of a blue color facilitates detection of infected cells, with the net effect of reducing the time from sample inoculation to virus detection from (typically) seven or more days to one to two days. A subsequent immunofluorescence assay using monoclonal antibodies specific to HSV 1 or 2 is used to type the virus.

## 10. Paradigms for virus isolation

We are often asked “What is the best combination of indicator cells to use for the (primary) isolation of viruses from clinical specimens?” Of course, this is not a question that has one correct answer. Indeed, there is no magic combination that is universally applicable. And variability exists according to the availability of reagents in various countries. The best answer to the question of which cells to use is to rely on the experience of the virologists working on site, and to use as wide a variety of indicator cells as possible for the isolation of unknown viruses. There are however some guidelines that are applicable across many applications:

- a. Many respiratory viruses replicate in the upper respiratory tract, and require temperatures lower than 37°C for optimal replication. For each type of indicator cell line (or primary cells), it is advisable to inoculate replicate cell cultures, and incubate one at 35° to 37°C, the other at 32° to 34°C.
- b. Cultures should not be considered negative for virus isolation if CPE are not detected. A second measure should be considered and well-thought criteria should be developed before rejecting a “negative” culture. For example, would CPE form if the cultures were held for a longer period of time? Examples of secondary evaluations include performance of hemagglutination (using spent culture media) or hemadsorption tests (performed directly on the infected cells), and electron microscopy. Hemagglutination and hemadsorption tests are best performed using red blood cells from two unrelated species (e.g., one mammalian and one avian species). Electron microscopy should be performed using material from spent media to detect liberated virions, and also, on a

sample of the infected cells (often, the number of liberated virions is too low to be easily visualized through electron microscopy, and virus infection is determined only by examining the infected cells themselves).

- c. For all work, non-infected cultures (negative controls) should be maintained and examined in parallel with any virus-infected cultures.
- d. Where economically feasible, new generation molecular tests should be used to assist in the identification of new viruses.
- e. Some viruses require “adaptation” prior to adequate replication in cultured cells and the formation of CPE. In the past, the process referred to as “blind culture” was performed when virus was suspected but CPE inapparent. A popular version of this method is to periodically remove samples from a culture of presumably infected cells, and to inoculate that into a new batch of cells. This process is repeated four times. An adjunct to former process is to split the infected cells (if confluent) into a larger flask or into several flasks and allow the cells to replicate. This may make CPE apparent if actively replicating cells are optimal for the detection of the CPE caused by a particular virus.
- f. The isolation of viruses from mosquitoes, ticks, etc., can be challenging. Many mosquito-borne viruses are best amplified to high titer in mosquito cell lines, where they often proliferate without causing CPE, prior to subculture in animal cells. Occasionally, it has also been necessary to inoculate newborn or suckling rodents (subcutaneously or intracerebrally) to obtain a high virus titer for the inoculation of cell cultures.
- g. During the primary isolation of virus from clinical or environmental specimens, many laboratories routinely filter specimens through a 0.45 $\mu$ m filter prior to inoculation of cell cultures. This filtration step is performed to remove bacteria, fungi, and other potential microbial contaminants, and non-living particulates. A problem with this filtration step is that many viruses are pleomorphic and some have long, filamentous forms that may exceed 0.45  $\mu$ m. This includes some influenza viruses, and morbilliviruses. Also, in clinical specimens, many viruses are attached to cellular and other debris, and are trapped by the filter. We recommend the inoculation of two batches of cells; one with a filtered aliquot, the other unfiltered, of the virus specimen. Unfortunately, it is not uncommon these days for bacteria in clinical specimens (such as normal flora that are contaminants of naopharyngeal swabs) to be resistant to penicillin and streptomycin; we prefer to use an antibiotic mixture that includes neomycin in addition to penicillin and streptomycin.
- h. When economically possible, we suggest inoculation of cells growing on a relatively large growing surface with specimen. The idea behind this is to effect a dilution of the inoculum over a wide area, reducing the concentration of toxic agents. For example, add a small inoculum (e.g. 100  $\mu$ l) to cells in a flask with a growing surface of 75 cubic centimeters (T75 flask). This is especially helpful when specimens must be pre-treated with a high concentration of antibiotics (such as when attempting the isolation of virus from a stool sample).

- i. A largely forgotten finding is that antibiotics can suppress the growth of some viruses. This concept is anti-dogmatic, but should always be considered when attempts at the isolation of a particular virus are not very successful. A recent example is described in [74].
- j. Some viruses remain cell-associated, as mentioned above, and for effective virus isolation, the virions must be liberated from the material they are attached to. This is one reason some clinical specimens designated for virus isolation are “vortexed” in the presence of glass beads prior to inoculation of the specimen onto cells. Instead of bead disruption, in some cases, freeze-thaw of the specimen can be used to dissociate the virions from the cells or debris they are attached to. Freeze-thaw cannot be used for herpesviruses and other viruses that are rapidly inactivated by such as process. Some viruses such as JC virus can be dissociated from cell debris by pretreatment of the specimen with neuraminidase. In any case, it is advisable to explore whether mechanical or biochemical options are available to improve virus isolation. However, for biosafety reasons, it is always best not to use procedures that create aerosols.

## 11. Closing remarks

In this article, we have discussed some of the art behind virus isolation through cell culture. Due to the complexities of cell culture, and the nature of the biomaterials used, it is not possible to consistently attain the same end results at all times. Moreover, viruses constantly mutate, and so the “rules of the game” can change. Therefore, the practice of cell culture for virus isolation is part art, part science, and part luck. Nevertheless, following principles mentioned in this manuscript, we have succeeded at maintaining cultured cells for long periods, and have isolated and propagated many “difficult” viruses.

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