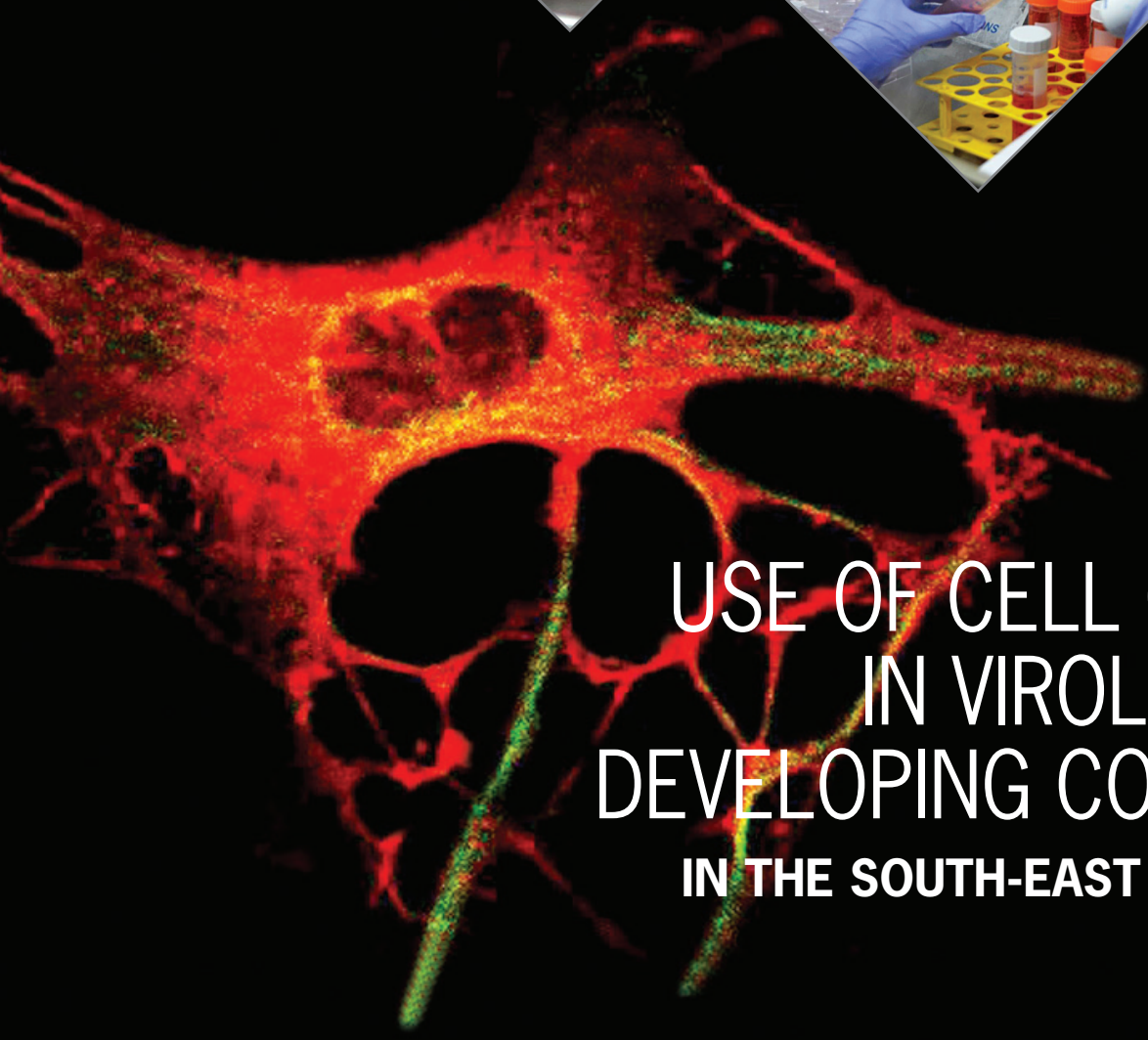
World Health Organization
Regional Office for South-East Asia



USE OF CELL CULTURE IN VIROLOGY FOR DEVELOPING COUNTRIES

IN THE SOUTH-EAST ASIA REGION

World Health Organization
Regional Office for South-East Asia

USE OF CELL CULTURE IN VIROLOGY FOR DEVELOPING COUNTRIES

IN THE SOUTH-EAST ASIA REGION

Suggested citation.Use of cell culture in virology for developing countries in the South-East Asia Region. New Delhi: World Health Organization, Regional Office for South-East Asia; 2. Licence: CC BY-NC-SA 3.0 IGO.

Cataloguing-in-Publication (CIP) data. CIP data are available at http://apps.who.int/iris.

Sales, rights and licensing. To purchase WHO publications, see http://apps.who.int/bookorders.
To submit requests for commercial use and queries on rights and licensing, see http://www.who.int/about/licensing.

FOREWORD

Viral diseases have assumed great importance in the recent past for individual, community and global health. During the past four decades, around 40 new pathogens have been discovered, half of which are viruses. Some of these viruses have brought about a paradigm shift in medical sciences and public health. These include the human immunodeficiency virus, hepatitis C virus, and a few more.

Few of these viruses have triggered alarm bells globally through epidemics and pandemics. During the current millennium Severe acute respiratory syndrome (SARS), influenza H5N1, influenza H1N1, Middle East Respiratory Syndrome (MERS) Coronavirus, Ebola and Zika Virus have disrupted life in several parts of the world with conspicuous implications on human health, animal health and the global economy due to travel and trade restrictions.

The International Health Regulations (2005) call for all countries to build adequate capacity to combat such public health emergencies of international concern thus obviating mortality, morbidity and economic losses. One such capacity indicator is reliable diagnosis of viral infections.

A few years back WHO had published simple guidelines on how to establish a virology laboratory in developing countries. There have been many technological advances since then. Newer genetic-based methods have become available. However, isolation of viruses and their characterization continues to remain as the gold standard in virology.

Recognizing the need for a comprehensive document on cell culture-based isolation techniques along with strengthening quality system and the need for and care of equipment and biosafety, this document has been developed by a group of renowned regional virologists.

I am sure that this document will be of immense use to our Member States in establishing virological diagnostic services in consonance with the International Health Regulations (2005), thus contributing to global health security.

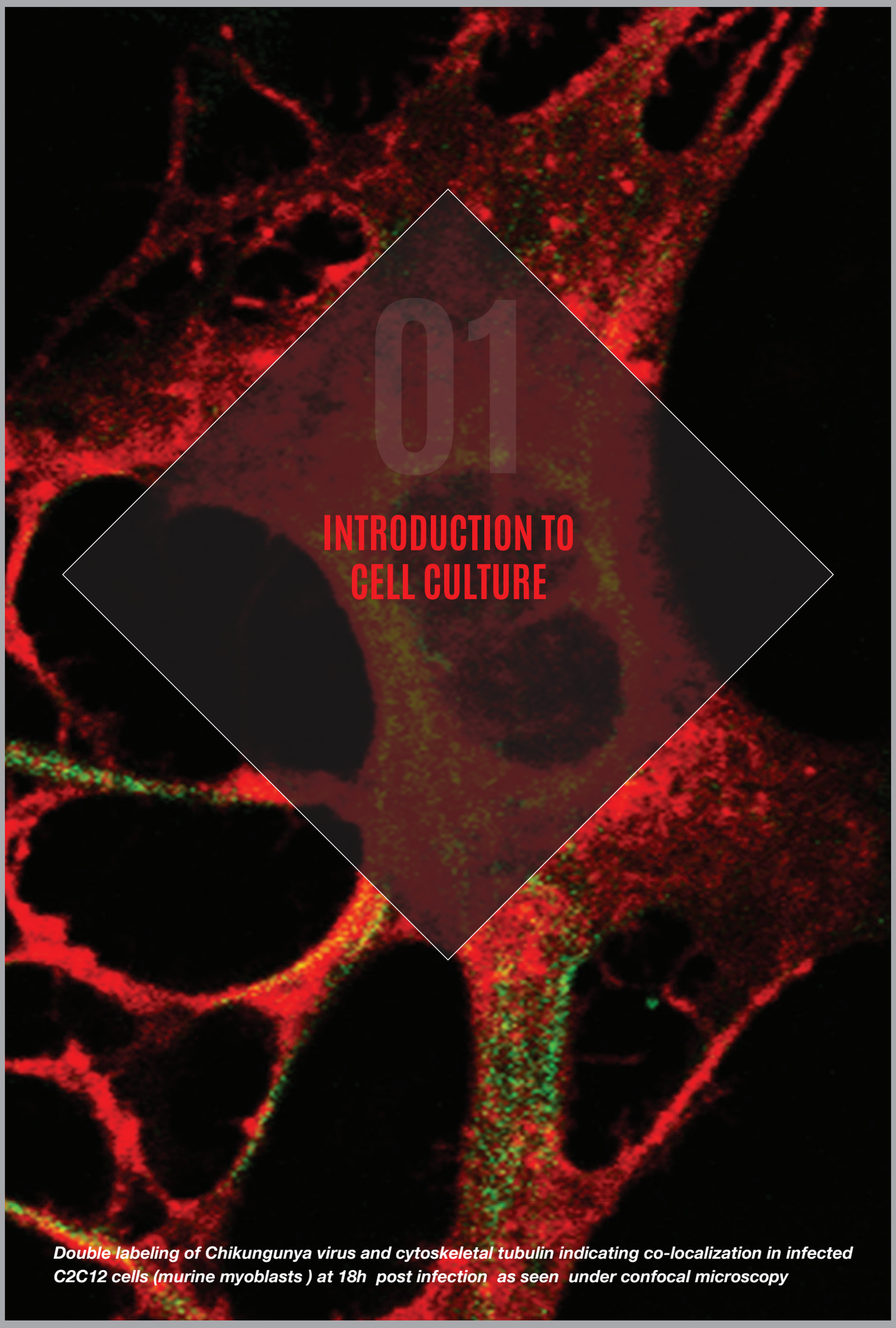
Dr Poonam Khetrapal Singh
Regional Director

CONTENTS

ABBREVIATIONS

2D	two dimensional
3D	three dimensional
ACDP	Advisory Committee on Dangerous Pathogens(guidelines)
ATCC	American Type Culture Collection
BHK	baby hamster kidney
BSA	bovine serum albumin
BSC	biosafety cabinet
BSL	biosafety level
BSS	balanced salt solution
BVDV	bovine viral diarrhoea virus
CHO	Chinese hamster ovary
CMC	carboxy methyl cellulose
CPE	cytopathic effect
CSF	cerebrospinal fluid
DMEM	Dulbecco's Modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxy ribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
EBSS	Earle's balanced salt solution
ECACC	European Collection of Authenticated Cell Cultures
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
EQAS	external quality assessment scheme
FBS	fetal bovine serum
FCS	fetal calf serum
FDA	United States Food and Drug Administration
GLP	good laboratory practice
GMEM	Glasgow's Minimum Essential Medium
GMO	genetically modified organism
GMT	good microbiological technique
HBSS	Hank's balanced salt solution
HDC	human diploid cell(s)
HEK-293	human embryo kidney 293
Hep B	hepatitis B
Hep C	hepatitis C
HEPA	high-efficiency particulate arrestor
Hepes	N-(2-hydroxyethyl) piperazine N´- (2-ethanesulfonic acid)
HIV	human immunodeficiency virus
HPV	human papillomavirus
HSV	herpes simplex viruses
ID50	inhibitory dose 50
IFA	immunofluorescence assay
IQAS	internal quality assessment scheme
IVF	in vitro fertilization
JEV	Japanese encephalitis virus
LAI	laboratory-acquired infection
LCMV	lymphocytic choriomeningitisvirus
mAbs	monoclonal antibodies
MDBK	Madin–Darby bovine kidney (epithelial cell line)
MDCK	Madin–Darby canine kidney (cell line)
MEM	minimum essential medium
MERS	Middle East respiratory syndrome
NCCS	National Centre for Cell Science
NIMHANS	National Institute of Mental Health And Neuro Sciences
NP	nasopharyngeal
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pfu	plaque-forming units
PI	post-infection
PM	Pitman-Moore
PVRV	purified vero rabies vaccine

QA	quality assurance
RPMI	Roswell Park Memorial Institute (medium)
SARS	severe acute respiratory syndrome
SFM	serum-free media
SOP	standard operating procedure
STR	short tandem repeat(s)
SV40	Simian vacuolating virus 40
TCID50	tissue culture infective dose
tPA	tissue plasminogen activator
TSE	transmissible spongiform encephalopathies
VERO	Verda reno - African green monkey kidney epithelial cells
VTM	viral transport medium
WHO	World Health Organization



01

INTRODUCTION TO CELL CULTURE

Double labeling of Chikungunya virus and cytoskeletal tubulin indicating co-localization in infected C2C12 cells (murine myoblasts) at 18h post infection as seen under confocal microscopy

01

INTRODUCTION TO CELL CULTURE

It has been over six decades since the discovery by Enders and his co-workers that polioviruses could replicate in cell cultures of non-neural origin. This has stimulated extensive use of cell cultures for propagation of a number of human and animal viruses. Consequently, cell culture is an indispensable tool in modern-day medicine with innumerable applications. Cell culture involves a complex of processes of cell isolation from their natural environment (in vivo) and subsequent growth in a controlled environmental artificial condition (in vitro). The term "cell culture"denotes growing of cells in vitro under conditions where the cells are no longer organized into tissues. This should not be confused with the term "tissue culture", which denotes maintenance and growth of tissues in a way that allows differentiation and preservation of the architecture and/or function. In cell culture, cells derived from specific tissues or organs are cultured as short-term, mid-term or long-term established cell lines, which are widely used for research and diagnosis, particularly in the case of viral infection, because pathogenic viral isolation depends on the availability of permissible cell cultures.

Cell culture provides the optimum setting for the detection and identification of numerous pathogens of humans, which is achieved via virus isolation in the cell culture as the "gold standard" for virus discovery. Despite technological advancements in the past two decades, starting with monoclonal antibody development to molecular techniques for rapid detection of viral infections, the need and utility of cell cultures has not diminished. Indeed, they are used in monitoring specificity and sensitivity of all newer rapid tests. A combination of virus isolation in cell culture and molecular methods is still critical in identifying viruses that were previously unrecognized. The influenza A pandemic of 2009 is a classic example of how cell culture was used in conjunction with molecular techniques for rapid diagnosis and identification of a novel recombinant. However, isolation of viruses using cell cultures is not appropriate for routine daily results, and specialized laboratories should rely on their own ability to use viruses as controls, perform complete investigations when needed, and store representative clinical strains whenever possible. By maintaining their ability to conduct viral culture, these laboratories could also provide strains for future vaccine development and human isolates for research purposes.

Cell culture needs a continuous commitment of energy and resources for professional use. Therefore, the key decision to be made is whether there will be an ongoing requirement of culture facilities or whether it might be economical to collaborate with an established laboratory or subcontract work for short durations. In case of a clear and accepted requirement to set up a cell culture facility, several basic issues need to be considered. A number of important conditions must be satisfied for successful establishment of a cell culture facility. These include the availability of an appropriate laboratory infrastructure and equipment, cell culture media, vessels and reagents,

It has been over six decades since the discovery by Enders and his co-workers that polioviruses could replicate in cell cultures of non-neural origin. This has stimulated extensive use of cell cultures for propagation of a number of human and animal viruses.

standardized protocols and trained sufficient human resources.

The establishment and use of cell culture also needs people with a high degree of expertise and knowledge of appropriate technology. This is because all cell lines are not equal, and the ability of a given virus to grow is quite unpredictable. This manual provides the basic guidelines related to all these aspects of cell culture. It is intended to provide the minimum requirements for establishing a cell culture laboratory for use in the context of diagnostic virology. It is not intended to serve as a comprehensive laboratory manual on cell culture.

1.1 Scope

These guidelines cover key aspects of establishing a cell culture laboratory for diagnostic virology in developing countries and address issues pertaining to policy and programme, infrastructure, human resources, technologies available and high quality systems. These guidelines do not describe the technique for processing of specimens because every laboratory has to develop its own standard operating procedures (SOPs). Moreover, standard textbooks are available for these techniques.

1.2 Guideline development process

The WHO Regional Office for South-East Asia commissioned the Department of Neurovirology, National Institute of Mental Health and Neuro Sciences (NIMHANS), Bengaluru, India, to develop the first draft guidelines using a team of experts in the country as well as from the region. The objective was to assist developing countries in establishing practical facilities for cell culture within diagnostic virology settings based upon a programme approach and available scientific evidence.

The draft guidelines were first reviewed by WHO and subsequently by two eminent reviewers from the region. Based on their recommendations, the guidelines were revised by NIMHANS.

The guidelines also provide information for the procurement of equipment, cell lines and products for use by cell culture laboratories. However, the information is only suggestive and WHO does not specifically endorse these products.

1.3 Guidelines development team

WHO wishes to acknowledge the support provided by the various experts in drafting, reviewing and finalization of the guidelines.(Annex)

02

PRINCIPLES OF CELL CULTURE

Filtration of growth medium required for cell culture

02

PRINCIPLES OF CELL CULTURE

2.1 Definitions

The term *"cell culture"* denotes in vitro growth of cells under conditions where the cells are no longer organized into tissues. This should not be confused with the term *"tissue culture"* which denotes maintenance and growth of tissues in a manner that allows differentiation and preservation of the architecture and/or function. Therefore, *cell culture* can be broadly defined as the removal of cells from an animal or plant tissue followed by their growth in a favourable synthetic milieu that simulates in vivo conditions. Removal of cells from the tissue may be followed by enzymatic or mechanical disaggregation procedures before cultivation. Besides, cells may also be derived from an already established cell line or strain.

Primary cell culture: It is a culture initiated 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 *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 numerous 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. If not, the term line will suffice.

Cell strain: This term is used for a cell line that is derived either from a primary culture or a cell line by *the selection or cloning of cells having specific properties or markers*. The properties or markers that are distinct to a cell line must be present consistently during all subsequent cultivation.

Passage: It is the transfer or transplantation of cells, with or without dilution, from one culture vessel to another. It is understood that at 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 "*subculture*". The passage refers to the number of times the cells have been subcultured or passaged. In descriptions of this process, the split ratio or dilution of the cells should be stated so that the relative cultural age can be ascertained and the characteristics of the cells remain unaltered.

2.2 Types of cell culture

2.2.1 Primary explantation: Cells isolated from donor tissue may be maintained by myriad techniques. An outgrowth of cells, from a plasma clot, or an extracellular matrix constituent, such as collagen, is generally observed when a tissue fragment adheres to the surface either spontaneously or augmented by mechanical means. Such a culture is termed "*primary explant*" and the migrating cells are known as the outgrowth. Cells are primarily selected on the basis of their ability to migrate from the explant and for their ability to proliferate upon subculture.

Upon mechanical or enzymatic disaggregation, suspension of cells/ aggregates comprise a proportion of cells capable of attaching to a solid substrate

Continuous cells grow rapidly; they have a higher cell density and aneuploid chromosome number. They differ in phenotypes from the donor tissue and stop expressing certain tissue-specific genes.

to form a monolayer. Cells within the monolayer, which are proficient in proliferation, are then selected at the first subculture. Subsequently, outgrowth from a primary explant may give rise to a cell line.

Explant culture is preferred where only small fragments of tissue are available or the fragility of the cells impedes survival following disaggregation. Differentiated cells found in a tissue have limited ability to proliferate. This is a genetically determined occurrence termed senescence. Hence, differentiated cells do not contribute to the formation of a primary culture, unless supplemented with conditions to promote their attachment and preserve their differentiated status. These cell lines, therefore, are known as finite.

2.2.2 Organotypic culture: Following disaggregation, the dispersed cells evidently lose their native histological characteristics as flattening of the explant due to cell migration and varying degree of necrosis.

Retention of the inherent histology and its associated differentiated properties may be enhanced at the air–medium interface, where gas exchange is optimized and cell migration minimized. Such a culture, known as "*organ culture*" may survive normally up to 3 weeks, but cannot be propagated further.

An alternative strategy for organ culture is the amplification of the cell stock by generation of cell lines from specific cell types and subsequent recombination in organotypic culture. Such an approach has witnessed limited success for the skin.

2.2.3 Continuous cell culture: Certain cell lines grow into immortal ones through a process called transformation, which can either occur spontaneously or is chemical or viral- induced. Transformation of a finite cell line, which acquires the ability to divide indefinitely, results in the formation of a continuous cell line.

Continuous cells grow rapidly; they have a higher cell density and aneuploid chromosome number. They differ in phenotypes from the donor tissue and stop expressing certain tissue-specific genes. Continuous cells acquire the ability to proliferate indefinitely, either through genetic mutations or artificial modification; they contain homogeneous genotypic and phenotypic characteristics.

For example, the widely used Vero lineage was isolated from epithelial cells of the kidney of an African green monkey (*Chlorocebussp.*) by Yasumura and Kawakita at Chiba University in Japan. The word "Vero" is an abbreviation of *verda reno*, which means "green kidney" in Esperanto.

2.2.4 Adherent and suspension culture: Cells can be grown in culture in two basic ways: as monolayers on an artificial substrate (i.e. adherent culture) or free-floating in the culture medium (suspension culture).

Most cells derived from vertebrates, except haematopoietic and a few other cell lines, are anchorage-dependent. Therefore, these cells require a specifically treated surface that allows cell adhesion and proliferation. Substrate materials that have undergone specific treatments to allow growth of cells are termed as substrates treated by tissue culture.

Other cell lines, especially commercially available insect lines, grow well in anchorage-independent suspension culture. Such cells can be maintained in

culture flasks that are not treated by tissue culture. However, with the proliferation of cells, the culture volume to surface area is increased, which hinders adequate gas exchange. Therefore, suspension cultures demand that the medium should be agitated to ensure proper aeration.

2.3 Essential components of cell culture

2.3.1 Substrates and matrices: One prerequisite of in vitro cell culture is an extracellular matrix or substrate which provides in vivo-like environment. Normal cells in tissues thrive amidst a three-dimensional (3D) environment, bordered by other cells, membranes, fibrous layers and adhesion proteins.

Presently, cells are cultured on flat plasticware which culminate into artificial two-dimensional (2D) sheets of cells. Much research has and continues to be devoted to the evolution of extracellular matrices or substrates that closely mimic the normal physiological conditions. The preference of a negatively charged substrate has been eliminated with the availability of plastics that are positively charged, which also provides some cell selectivity. Irrespective of the net charge of the synthetic substrates, it has been hypothesized that cells secrete matrix products that adhere to the substrate and provide ligands for the interaction of matrix receptors such as integrins. Therefore, recent techniques involve treatment of the substrate with a matrix product, such as collagen type IV, fibronectin or laminin, to promote cell adhesion facilitating better attachment.

2.3.2 Media: Cell culture media are complex combinations of salts, carbohydrates, vitamins, amino acids, metabolic precursors, growth factors, hormones and trace elements. The medium also helps maintain the pH and osmolality in a culture system, which is crucial for the growth and proliferation of cells. The pH in media is maintained by one or more buffering systems; CO_2/sodium bicarbonate, phosphate and HEPES being the most common. Often, phenol red, a pH indicator, is supplemented to the medium for colorimetric monitoring of pH variations. As different cell lines have different growth requirements, a wide range of media formulations are available commercially to aid the growth of each cell type.

2.3.3 Animal sera: Sera from fetal and calf bovine sources are a rich source for amino acids, proteins, vitamins (particularly fat-soluble vitamins such as A, D, E and K), carbohydrates, lipids, hormones, growth factors, minerals and trace elements. Additionally, serum acts as a buffering agent in the culture medium, inactivates proteolytic enzymes, increases medium viscosity (thereby reducing shear stress during pipetting or stirring), and conditions the growth surface of the culture vessel. Although the exact composition of sera is still undetermined, it is known to stimulate cell growth. Earlier, animal serum was a major source of mycoplasma contamination of tissue culture cells. Nowadays, commercially available sera are filtered through several 0.1μm pore (or smaller) filters to remove mycoplasma. Basal media supplemented with animal sera in specified concentrations is known as complete growth media. However, some cells are known to prefer serum-free media for growth.

2.3.4 Antibiotics and antimycotics: Addition of antibiotics and/or antimycotics is a precautionary measure (though not mandatory) to check microbial contamination. They are also added to cell culture media as a prophylactic to prevent contamination. Routine use of antibiotics or antimycotics for cell culture is not recommended as they inhibit normal cell growth. Since animal cells are eukaryotic in

origin, use of antimycotics may interfere with the normal cell metabolism.
The American Type Culture Collection (ATCC) does not recommend the use of antibiotics and/or antimycotics unless the cell line is irreplaceable as the process is lengthy and there is no guarantee that contamination will be eliminated.

2.4 Equipment

2.4.1 Cell culture hood: A clean working environment, free from dust and other airborne contaminants, is absolutely essential for cell culture. The cell culture hood ensures an aseptic work area, by maintaining a constant, unidirectional flow of high-efficiency particulate air (HEPA)-filtered air over the work area, besides permitting the containment of infectious splashes or aerosols generated during culture procedures. The air flow may be horizontal, blowing parallel to the work surface, or vertical, blowing from the top of the cabinet onto the work surface. Three kinds of cell culture hoods, designated as class I, II and III, are available to cater to varying research and clinical needs. Ideally, uninfected and infected cell cultures need to be handled in separate (dedicated) biosafety cabinets.

2.4.2 Culture vessels: Understanding the growth requirements of the cultures help in selecting the best culture system. There are four basic culture systems:

- Stationary monolayer cultures which are grown in undisturbed flasks, dishes and multiwell plates.
- Moving monolayer cultures which are grown primarily in roller bottles.
- Stationary suspension cultures which are grown without agitation in untreated dishes and flasks.
- Moving suspension cultures which are grown in mechanically stirred vessels (spinner flasks), bioreactors or fermentors.

The yield for monolayer cultures is limited by the area of treated growth surface available. Approximately, 0.5×10^5 cells/cm^2 to 1×10^5 cells/cm^2 of treated surface is a typical yield for confluent continuous mammalian cell lines. The total cell yield for suspension cultures is determined by the working volume of the vessel. In stirred systems, cell concentrations can easily reach between 1×10^6 cells/ml and 2×10^6 cells/ml of medium.

2.4.3 Flasks: The first glass flasks were developed by Alexis Carrel in the 1920s followed by Harry Earle who developed the widely used glass T-flasks in the 1940s. Disposable plasticware products, which have largely supplanted the use of glass vessels, are available in many sizes and shapes to suit the various needs in laboratories. This has reduced the costs of culturing cells in many ways: (i) glass bottles do not have to be washed and sterilized between uses; (ii) cell culture

TABLE 2.1

Description	Growth area (cm^2)	Recommended working volume (mL)	Cell yield 10^6*
T-25	25	5 to 10	2.5 X 10^6
T-75	75	15 to 25	7.5 X 10^6
T-150	150	30 to 50	15.0 X 10^6
T-175	175	35 to 60	17.5 X 10^6
T-225	225	45 to 75	22.5 X 10^6

* Cell line dependent. Based upon a density of 1 x $10^5/cm^2$.

plasticware products can be purchased ready-made with growth surface coatings or electrostatic "treatments" that promote cell attachment to the growing surface; (iii) plasticware is inherently safer in that it is relatively shatterproof. A list of the commonly used flasks is given in Table 2.1.

2.4.4 Cell culture dishes: Cell culture dishes offer the best economy and access to the growth surface; thereby making them the vessels of choice for cloning or other manipulations such as scraping that require direct access to the cell monolayer. Culture dishes are used with incubators that control CO_2 and humidity.

2.4.5 Multiwell plates: Originally designed for virus titration, multiwell plates have now become popular owing to their convenience in handling and significant savings in space, media and reagents when compared to an equal number of dishes. Plates must be used with incubators that control humidity and CO_2 levels.

2.4.6 Other equipment: Incubator (humid CO_2 incubator recommended), water bath, centrifuge, refrigerator and freezer (–20 °C), cell counter, inverted microscope, liquid nitrogen (N_2) freezer or cryostorage container, and autoclave.

2.5 Subculture

Cells require subculturing at regular intervals to maintain their exponential growth. In case of primary cultures, subculture involves the dissociation of the cells from each other and the substrate to generate a quantifiable single-cell suspension. Reseeding this cell suspension at a reduced concentration into a flask or dish generates a secondary culture, which can subsequently proliferate and is subcultured again to give a tertiary culture, and so on.

For an adherent monolayer culture, cells are ready to be subcultured when the monolayer is 70% to 90% confluent. While subculturing, the split-ratios and medium replenishment schedules for each cell type must be followed. A loosely attached cell gets dislodged by a sharp blow with the palm against the side of the flask. Other strongly adherent cell types require the digestion of their protein attachment bonds with proteolytic enzymes such as trypsin/EDTA. Mechanical forces such as scraping to dislodge the cells are preferred for remaining cell types.

Depending upon the cell type, suspension cultures are seeded at densities from 2×10^4 to 5×10^5 viable cells/mL and can attain densities of 2×10^6 cell/mL. If cells are seeded at too low a density, they undergo a lag phase of growth, either grow very slowly or die out completely. On the contrary, higher cell densities may lead to exhaustion of the nutrients in the medium leading to abrupt cell death.

2.6 Cryopreservation

Most cell cultures can be preserved for many years, if not indefinitely, at temperatures below –130 °C (cryopreservation), thereby eliminating the time, energy and materials required to maintain cultures not in immediate use. Cryopreservation also protects cells against phenotypic drift in the culture due to genetic instability and/or selective pressure.

Cryopreservation refers to the procedure of freezing cells slowly (generally –1 °C per minute) until they reach a temperature below –70 °C in a medium that includes a cryoprotectant. Cryoprotectant agents such as glycerol or dimethylsulfoxide (DMSO) allow water within the cell to escape by osmosis during the cooling process, thereby minimizing the formation of ice. Vials are subsequently transferred

Change of the pH of the medium may or may not be caused by fungal contaminants. The presence of fungal contamination can be distinguished from bacteria by the presence of filamentous structures in the suspension.

to a liquid-nitrogen freezer to maintain them at temperatures below –130 °C.

Recovery of cryopreserved cells is rapid and involves rapid thawing in a water bath at 37 °C. The cell suspension is then removed from the freeze-medium by gentle centrifugation and/or diluted with growth medium followed by seeding in a culture vessel in complete growth medium.

2.7 Contamination

Multiple risk factors contribute to contamination of cells in culture. The sources commonly implicated are other cell lines (cross-contamination), reagents, supplies such as pipettes and culture vessels, equipment such as tissue culture hoods and incubators, and laboratory personnel. The risk of contamination can be minimized or eliminated by proper precautions: using only reagents of known quality and sterility, quarantining new cell lines until they are tested to be free from contamination, performing routine maintenance and cleaning of all equipment and proper training of cell culture personnel. A large facility that deals with multiple cell cultures would ideally have a separate sterile cell culture room which would have dedicated equipment such as a biosafety cabinet for sterile work, scientific fridgerator for storage of reagents to be used on sterile cultures, cupboards for storage of sterile pipettes and an inverted microscope to monitor flasks for growth of cell culture monolayers.

2.7.1 Microbial contamination:

Bacterial contamination occurs within a few days and can be detected by the naked eye. However, slow-growing fastidious bacteria are difficult to detect. Distinct changes to the medium such as turbidity, presence of particles visible in suspension, and a rapid decline in pH (yellow colour, indicating acidity) indicate bacterial contamination.

Change of the pH of the medium may or may not be caused by fungal contaminants. The presence of fungal contamination can be distinguished from bacteria by the presence of filamentous structures in the suspension. On microscopic observation, yeast cells are larger than bacteria appearing round or ovoid, but may not appreciably change the pH of the medium. Microbacterial media, which can be used to test for bacterial and fungal contamination, include blood agar, thioglycollate broth, tryptic soy broth, BHI broth, Sabouraud broth, YM broth and nutrient broth with 2% yeast extract.

The use of antibiotics suppresses bacterial growth and thus masks contamination, which becomes very difficult to detect.

Mycoplasma contamination in cell lines is detected by direct (agarose and broth culture) and indirect methods. The fluorochrome DNA stain binds to the DNA of mycoplasma, thus allowing easy detection of the organisms using a microscope equipped with appropriate fluorescence optics. Some laboratories perform PCR-based mycoplasma testing, using commercially available kits.

2.7.2 Cellular cross-contamination:

The introduction of an undesired cell line to the preferred one is termed as cellular cross-contamination. This can sometimes lead to the replacement of the original cell with the contaminant, especially when the contaminant grows faster than the original.

Cell repositories now use DNA polymorphisms in addition to enzyme

polymorphisms, HLA typing and karyotyping to confirm the identity of their cell lines. Profiling of short tandem repeats (STR) by PCR amplification, followed by capillary electrophoresis, is one of the most reliable methods to study DNA polymorphism.

Cell cultures should be regularly tested to ensure the absence of contamination from both microorganisms as well as from other cell lines. If contamination is found, it is advisable to discard the culture and start fresh with a new stock. Prior to use for virus isolation or detection, cells should be "validated" for their intended purpose. A valid batch of cells implies that they have been tested and confirmed suitable for the isolation or detection of the target virus. Three questions must be answered during the validation process:

- Has authenticity been confirmed (are the cells what they should be)?
- Do the cells behave as expected before their intended use?
- If 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?

2.8 Three-dimensional cell culture

The major limitation of 2D cell culture lies in the fact that it does not take into account the in vivo environment where cells are naturally surrounded by other cells and extracellular matrix (ECM) in a 3D fashion. Therefore, experiments performed with cells grown in a 2D setting sometimes provide misleading and non-predictive data for in vivo responses. Failure of drugs during clinical trials, especially during the most expensive phase III, has been partially attributed to data collected from the 2D monolayer culture tests in which the cellular response to drug(s) is altered due to their unnatural microenvironment. Recent reports suggest that 3D cell culture systems, in contrast to 2D culture systems, represent actual microenvironment where cells reside in tissues more accurately.

Cells grow into 3D aggregates/spheroids using a scaffold/matrix or in a scaffold-free manner. Scaffold/matrix based 3D cultures can be generated by seeding cells on an acellular 3D matrix or by dispersing cells in a liquid matrix followed by solidification or polymerization. Scaffold-free 3D cell spheroids can be generated in suspensions by a number of ways such as the forced floating method, the hanging drop method or agitation-based approaches.

The 3D cell culture models are preferred over the traditional 2D monolayer cultures due to improved cell–cell interactions, cell–ECM interactions, and cell populations and structures that resemble in vivo architecture. Due to their increased applications in drug discovery, cancer cell biology, stem cell research, and many other cell-based analyses and devices, extensive research during the past decades has yielded a variety of 3D cell culture systems.

03

DESIGNING A CELL CULTURE LABORATORY AND THE REQUIREMENTS - INFRASTRUCTURE, EQUIPMENT, CELL CULTURE MEDIA, CONSUMABLES, HUMAN RESOURCE

Immunofluorescence staining of microtubule network of normal C2C12 cells (murine myoblasts) as seen under confocal microscope

03

DESIGNING A CELL CULTURE LABORATORY AND THE REQUIREMENTS – INFRASTRUCTURE, EQUIPMENT, CELL CULTURE MEDIA, CONSUMABLES, HUMAN RESOURCE

3.1 Introduction

Ideally, cell culture work should be conducted in a single-use facility which, if possible, should be separated into an area reserved for handling newly received material (quarantine area) and an area for material which is known to be free of contaminants (main tissue culture facility). However, several aspects that need to be considered while designing a tissue culture facility for use in a virology laboratory include the following:

- The number of users and type of users: virologists/microbiologists/geneticists
- The location would depend on whether it would be a stand-alone facility for a virology laboratory or a common facility for other users as well.
- The amount of space available
- The range and type of equipment
- Biosafety/biosecurity regulations of the country/state.

Depending on these factors the design of the laboratory, equipment, consumables, media and reagents should be planned. This chapter describes the basic principles which relate to all these aspects.

3.2 Layout of a cell culture laboratory

The standard accepted laboratory scheme for cell culture involves a general laboratory room, an anteroom (physically separating the general laboratory area from the cell culture room), and a specific, self-contained, cell culture room. It would be ideal to locate the virus culture room adjacent to the cell culture room though it must be physically separated from the clean cell culture facility. If this is not possible, work should be separated by time with all manipulations on clean material being completed before manipulations involving infectious material are undertaken. Different incubators should also be designated for different purposes. In addition, the work surfaces should be thoroughly cleaned between activities.

The minimum space requirement for the cell culture laboratory of a single-user facility would be a room that is 3 m x 3.05 m. All flooring, walls and other surfaces in the culture environment should be readily accessible, chemically resistant, non-adsorbent, and easy to clean. Shelves should not be used, and open flat surfaces should be minimized as these will need to be cleaned and can be a source for the collection of dirt and dust. Walls should be smooth skimmed and coated with epoxy paint. The floor should also be resistant and bonded to the wall so that there are no crevices or corners that cannot be cleaned, and any

The guidelines make recommendations regarding the laboratory environment including lighting, heating, the type of work surfaces and flooring and provision of hand washing facilities.

spillages can be easily isolated and cleaned. For storage of sterile pipettes and other dedicated equipment, it is preferable to use metal cabinets with glass doors that can be closed and cleaned easily.

Ventilation and airflow in the cell culture environment are critical to operation. At its simplest, there must be no disruption to the laminar airflow pattern in the biological safety cabinet and no undue circulation of dust and dirt that could occur with, for example, significant staff movement around the unit or location near drafts or vents. Ideally, there should be no openable windows; if so, they should be sealed to prevent drafts, insects and entry of dust. However, in a purpose-built facility, it is ergonomically and aesthetically desirable to provide a vent for natural light. When planning, one should also make provisions in case there is a need to fumigate the room or equipment. Sinks, coat hooks, and so on should all be kept in an anteroom and not in the culture room. It is also preferable that there is double door access to the room to ensure minimal contamination.

For most cell lines, the laboratory should be designated to at least category 2 based on the guidelines of the Advisory Committee on Dangerous Pathogens (ACDP, 1995)†. However, the precise category required is dependent upon the cell line and nature of the proposed work. The guidelines make recommendations regarding the laboratory environment including lighting, heating, types of work surfaces and flooring and provision of hand-washing facilities. In addition, it is recommended that laboratories should be run at air pressures that are negative to corridors to contain any risks within the laboratory.

† Advisory Committee on Dangerous Pathogens. Categorization of biological agents according to hazard and categories of containment, 4th edition. Sudbury, UK:Health & Safety Executive (HSE) books; 1995 (www.hse.gov.uk).

Ideally, there should be provision for temporary storage of small amounts of culture consumables next to the biosafety cabinet (BSC). However, cardboard can be a significant source of fungal spores, and large-scale storage in the culture area causes clutter. Consumables are best stored in a central location, and if cell culture is being undertaken on a larger scale, it is far more economical to bulk purchase supplies and distribute them as necessary to each laboratory. Provision also needs to be made for storing flammable materials, especially disinfectant alcohols, which are used for local disinfection and "swabbing" (disinfectant wiping of consumables and reagents as they enter the working space inside safety cabinets).

Regular training, SOPs and centralized management of all aspects of cell culture, particularly technique and safety, ensure that there is an economical, continuously monitored, high standard of operation. The functioning of vital equipment should be continuously monitored and recorded. Particular attention should be paid to the monitoring of environmental microbial levels throughout the facility. For example, "settle" plates should be periodically left in laminar flow cabinets to check for sterility, as well as in all laboratory areas to validate their cleanliness.

The microbial quality of cell stocks should also be monitored as part of this process. All cells coming into a facility, regardless of the source, especially if supplied by noncommercial sources, should be quarantined and initially

cultured in isolation from the general culture environment. Mycoplasma is the main microbial contaminant of concern because such contamination can go unnoticed for long periods and it is very easy to pick up and cross-contaminate stocks of cells. Ideally, a facility should have provision for routine Mycoplasma detection. Experience and expertise are required to do this job reliably, and where such expertise is not available in-house, commercial testing facilities should be used.

Industrial disinfectants such as Virkon and/or Tego need to be employed (at recommended dilutions) as part of routine cleaning of equipment and areas, in addition to diluted industrial methylated spirits or isopropyl alcohol, which is used for local disinfection and "swabbing".

3.3 Equipment for a cell culture laboratory

The specific requirements of a cell culture laboratory depend mainly on the type of work undertaken. However, all cell culture laboratories have the common requirement of being free from pathogenic microorganisms (i.e. asepsis), and share the same basic equipment that is essential for culturing cells.

Table 3.1 lists the equipment and supplies common to most cell culture laboratories, as well as beneficial equipment that allows for efficient performance of work.

TABLE 3.1: List of essential equipment required for establishing a tissue culture laboratory

Type of Equipment	Remarks
Cell culture Incubator	This should be a water-jacketed microprocessor-driven machine, with a digital display for temperature, humidity and CO_2 concentration. It is preferable to have a dual-chambered, stackable (one on top of another to save space) incubator with an alarm.
Clinical centrifuge	One unit for spinning small volumes and one unit for refrigerated centrifuge with multiple rotors for small tubes (15mL), large tubes (50mL) and plates (24well and 96 well plates)
Freezers (-20°C and -80°C)	One unit each of –20°C and –80°C freezers to store fetal calf serum, glutamine, antibiotics, conjugates and other reagents.
Hemocytometer	This is required for counting of cells.
Inverted microscope	This should preferably be with phase-contrast and fluorescence attachments.
Laminar flow hood	A biological safety cabinet class IIA is required for subculturing of cell cultutres.
Scientific refrigerator	A unit of 300–400 L capacity is required for storing media, serum and other solutions.
Liquid nitrogen tank	Its size would depend upon the number of users.
Micropipettes (p20, p200 and p1000)	At least two sets of three micropipettes each with dedicated sets for sterile and infected work.
Pipette pumps	Motorized pipette dispensers are required for dispensing medium, trypsin, serum and other reagents.
Sterilizing oven or autoclave	This is required for sterilization of glassware, filters, reagents, etc.
Vacuum pump	This is required for filtration of media.
Water bath	This is required for warming up of media and serum.
Water purification system:	This is required for preparation of cell culture media.

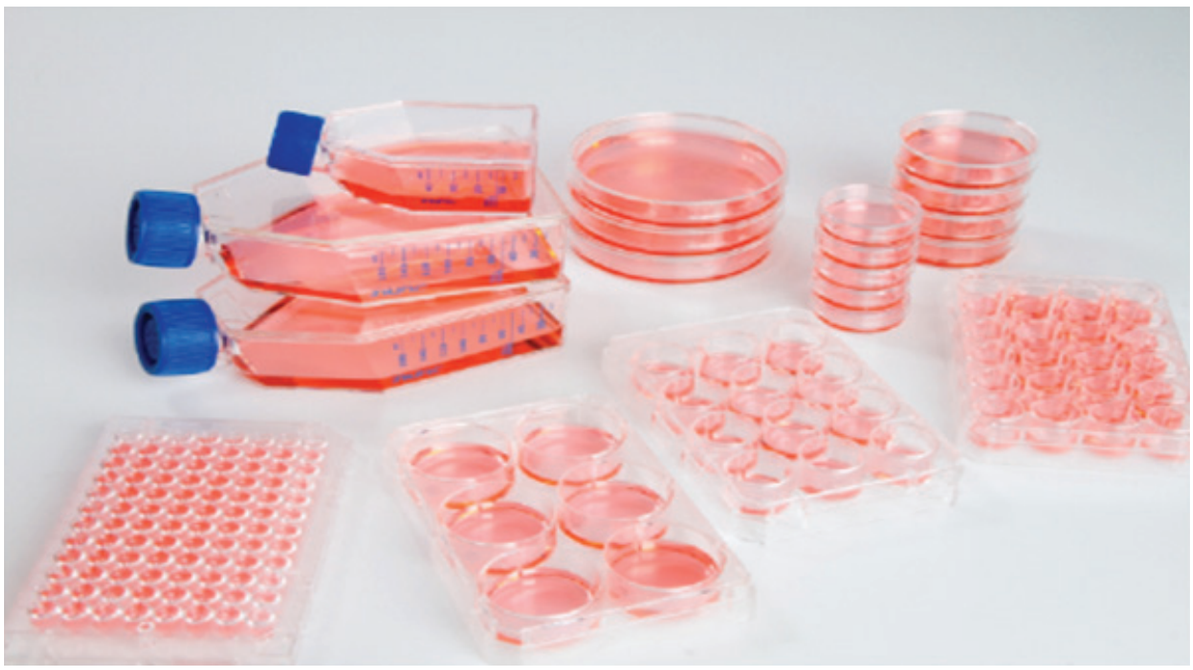


Figure 3.1: *Different types of cell culture flasks used in a virology laboratory*

Note that this list is not all inclusive; the requirements for any cell culture laboratory would depend upon the type of work conducted.

3.3.1 Cell culture vessels: In most laboratories, disposable polystyrene plastic vessels are used to grow anchorage-dependent cells (Figure. 3.1). The vessels are flat at the bottom to provide a surface for cell growth. The bottom surface of the culture vessels is coated by molecules, such as polylysine, laminin, gelatin, fibronectin etc. to mimic the natural ECM and allow cell attachment. Three types of culture vessels are commonly used for anchorage-dependent cells: flasks, dishes and multiwell plates. All three types are available in different sizes with different surface areas. The choice of the vessel depends upon the nature of the procedure and personal preference. Flasks with vented caps are preferred, since non-vented caps need to be loosened when the flasks are in the incubator to allow for exchange of gases (students have a hard time mastering this technique). Low-adhesion bacterial petri dishes (10cm) are used for the growth of embryoid bodies in the stem cell experiment.

3.3.2 Centrifuges: Centrifuges are used routinely in tissue culture as part of the subculture routine for most cell lines and for the preparation of cells for cryopreservation. By their very nature, centrifuges produce aerosols and thus it is necessary to minimize this risk. This can be achieved by purchasing models that have sealed buckets. Ideally, the centrifuge should have a transparent lid so that the condition of the load can be observed without opening the lid. This will reduce the risk for the operator being exposed to hazardous material if a centrifuge tube breaks during centrifugation. Care should always be taken not to overfill the tubes and to balance them carefully. These simple steps will reduce the risk of aerosols being generated. The centrifuge should be situated from where it can be easily accessed for cleaning and maintenance. Centrifuges should be checked frequently for signs of corrosion. A small bench-

top centrifuge with controlled braking is sufficient for most purposes. Cells sediment satisfactorily at 80–150 g. Higher gravitational forces may cause damage and promote agglutination of the cell pellet.

3.3.3 Incubators: Cell cultures require a strictly controlled environment for growth, for which an incubator provides the optimum environment. The incubator should be large enough, have forced-air circulation, and should have temperature control to within ±0.2 °C. Stainless steel incubators allow easy cleaning and provide protection against corrosion, especially if humid air is required for incubation. Although the requirement for aseptic conditions in a cell culture incubator is not as stringent as that in a cell culture hood, frequent cleaning of the incubator is essential to avoid contamination of cell cultures. Specialist incubators are used routinely to provide the optimum growth conditions, such as temperature, degree of humidity and CO_2 levels in a controlled and stable manner. Generally, incubators can be set to run at temperatures in the range of 28 °C (for insect cell lines) to 37 °C (for mammalian cell lines) and set to provide CO_2 at the required level (e.g. 5–10%). Some incubators have the facility to control the O_2 levels as well. Copper-coated incubators are also now available. These are reported to reduce the risk of microbial contamination within the incubator due to the microbial inhibitory activity of copper. The inclusion of a bactericidal agent in the incubator water trays will reduce the risk of bacterial and fungal growth. However, there is no substitute for regular cleaning.

The incubator should be large enough, have forced-air circulation, and should have temperature control to within ±0.2°C.

3.3.4 Inverted microscopes: Inverted microscopes are used to observe the cells in culture. Most inverted microscopes used for cell culture are equipped with 4x, 10x, 20x and sometimes larger magnification objective lenses. At least one inverted microscope is essential for a cell culture laboratory (Figure 3.2). Some microscopes are equipped with a photo tube and can

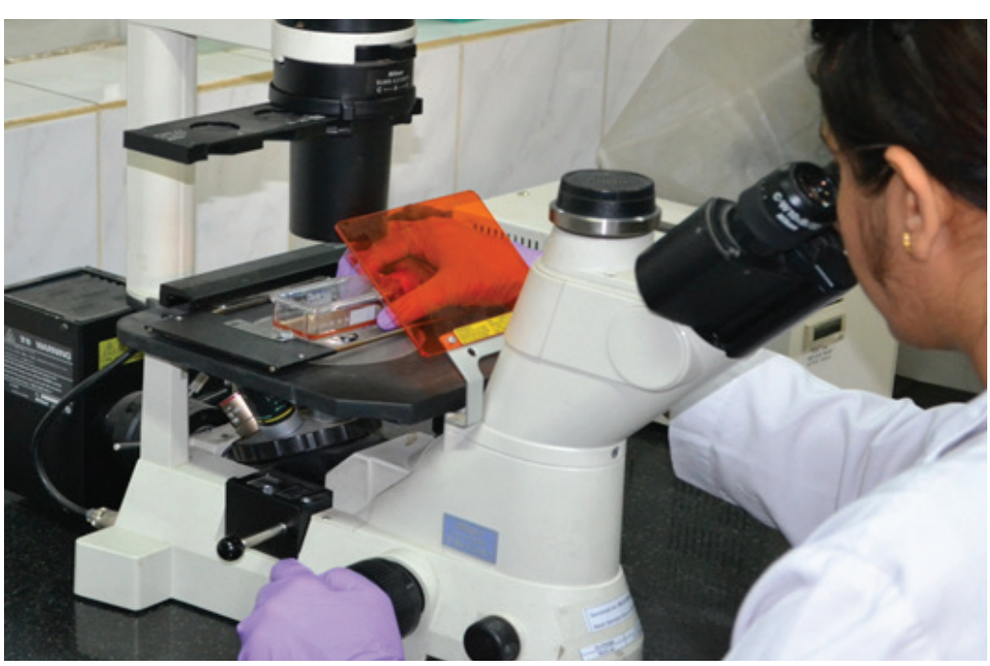


Figure 3.2: *An inverted microscope used to observe cells in a T-25 flask*

be connected to a camera for taking pictures of the experiments. Fluorescent inverted microscopes are inverted microscopes that are used to observe cells and molecules which have been labelled with fluorophores. Fluorescent microscopes are equipped with filters that will separate the absorbed light from the emitted fluorescent light. A set of filters are mounted on a block, called a filter cube. Fluorescent microscopes usually have several filter cubes with different sets of filters appropriate for observing different fluorophores. Fluorescent microscopes are connected to a high-intensity light source (usually a xenon arc or a mercury vapour lamp). (Refer to the manufacturer's manual for proper care and use of the fluorescent microscope.)

3.3.5 Freezers: Most cell culture reagents can be stored at –5 °C to –20 °C; therefore, an ultradeep freezer (i.e. a –80 °C freezer) is optional for storing most reagents. A domestic freezer is a cheaper alternative to a laboratory freezer. While most reagents can withstand temperature oscillations in an auto-defrost (i.e. self-thawing) freezer, some reagents such as antibiotics and enzymes should be stored in a freezer that does not auto-defrost.

3.3.6 Haemocytometers and Coulter counter: A haemocytometer is a device invented by Louis-Charles Malassez (1971) to count cells. It is required in a cell culture laboratory to enumerate cells prior to sub-culture. It is advisable to have multiple hemocytometers, one dedicated to each laboratory. If the haemocytometer coverslips are lost or broken, regular microscope coverslips, large enough to cover the chambers, may be used. An alternative way to count cells is to use a Coulter counter. (Refer to the manufacturer's instructions for care and use of the coulter counter.)

3.3.7 Laminar flow hoods (microbiological safety cabinets): A laminar flow hood is probably the most important piece of equipment for cell culture since, when operated correctly, it will provide a clean working environment for the product, while protecting the operator from aerosols (Figure 3.3). In these cabinets, operator and/or product protection is provided through the use of HEPA (high efficiency particulate air) filters. The level of containment provided varies according to the class of cabinet used. Three kinds of laminar flow hoods, designated as class I, II and III, have been developed to meet varying research and clinical needs. *Class I* cell culture hoods offer significant levels of protection to laboratory personnel and to the environment when used with good microbiological techniques, but they do not provide protection to cultures from contamination. In design and air flow characteristics, they are similar to chemical fume hoods.

Class II cell culture hoods are designed for work involving BSL-1, 2 and 3 materials, and they also provide an aseptic environment necessary for cell culture experiments. A class II BSC should be used for handling potentially hazardous materials (e.g. primate-derived cultures, virally infected cultures, radioisotopes, carcinogenic or toxic reagents). This category of hoods is recommended for use in a cell culture laboratory.

Class III BSCs are gas-tight, and they provide the highest attainable level of protection to personnel and the environment. A class III BSC is required for work involving known human viral pathogens and other BSL-4 materials.

Additionally, laminar flow hoods are also selected on the basis of air-flow characteristics. Since the hoods protect the

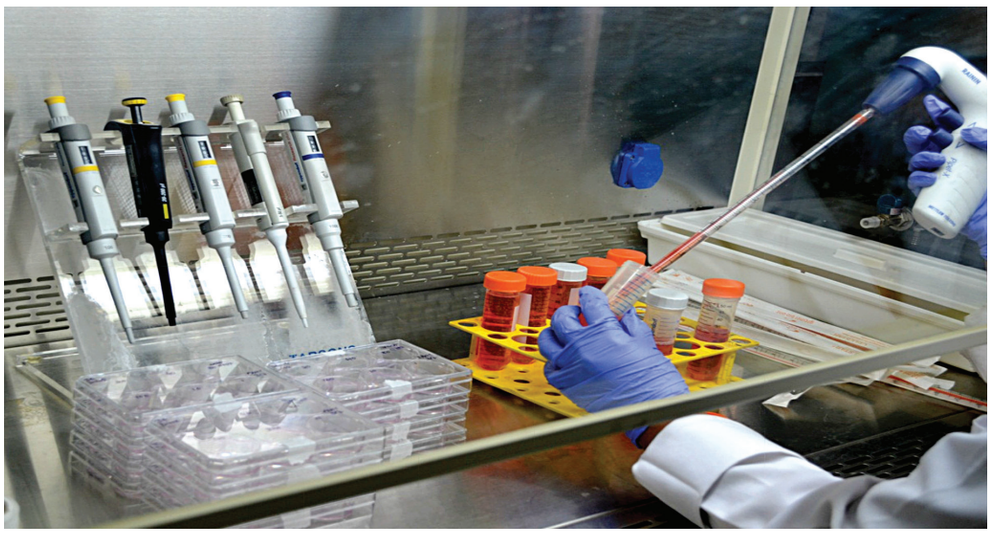


Figure 3.3: *Subculture of cell line in a biosafety cabinet*

working environment from dust and other airborne contaminants by maintaining a constant, unidirectional flow of HEPA-filtered air over the work area, the flow of air in the hood can be *horizontal*, blowing parallel to the work surface, or it can be *vertical*, blowing from the top of the cabinet onto the work surface. A horizontal flow hood provides protection to the culture (if the air-flow is towards the user) whereas a vertical flow hood provides significant protection to the user and the cell culture. It is preferable to always use a vertical flow hood for cell culture. The horizontal flow hood is generally used for media preparation in a virology laboratory.

3.3.8 Liquid nitrogen. Maintenance of cultured cells for any prolonged period requires that cell stocks can be kept well below the glass temperature of water (approximately –60 °C). In practice, *liquid nitrogen storage* is often a convenient general storage environment for stocks of cultured cells. However, electrical freezers operating at –120 °C can perform a similar function. Liquid nitrogen boils at –196 °C, and because the resultant nitrogen gas is an asphyxiant in high concentrations, proper handling and ventilation procedures are necessary. Proper, inherently safe methods for transport and storage must be used to get the liquid gas into the cell storage containers. As the storage vessels usually require topping up at least once a week (more regularly as the containers age), the route from the gas delivery/production area to the laboratory must be as short as possible and the surface and equipment appropriate for the transport involved. Storage of cells by multiple users for prolonged periods necessitates careful inventory management to ensure that cells are maintained optimally and economically. It is good practice to have two independently stored stocks of cells: one vessel containing master stocks of important cells and the other, in the laboratory environment, containing working stocks.

3.3.9 Pipettes and pipette aids: Individually-wrapped, sterile plastic serological pipettes of different sizes can be purchased. Alternatively, glass pipettes can be washed, plugged with cotton, sterilized and reused. Serological pipettes require the use of electrical or manual pumps to draw and release liquid. Micropipettes are used to transfer small volumes of liquid between 1 to 1000 μLs. A set of micropipettes should be kept in clean boxes exclusively

The culture medium is the most important component of the culture environment, because it provides the necessary nutrients, growth factors and hormones for cell growth, as well as regulates the pH and the osmotic pressure of the culture.

for cell culture procedures. Micropipettes need to be calibrated routinely to ensure precise measurements. Micropipette tips are kept in colour-coded, sterile boxes and must be sterilized before use.

3.3.10 Plasticware and consumables: Almost every type of cell culture vessel, together with support consumables such as tubes and pipettes, are commercially available as single-use, sterile packs. The use of such plasticware is more cost–effective than recycling glassware;it enables a higher level of quality assurance and removes the need for validation of cleaning and sterilization procedures. Plastic tissue culture flasks are usually treated to provide a hydrophilic surface to facilitate attachment of anchorage-dependent cells.

3.3.11 Refrigerators: For small cell culture laboratories, a refrigerator (preferably one without an auto-defrost freezer) is an adequate and inexpensive piece of equipment for storing reagents and media at 2–8 °C. For larger laboratories, a cold room restricted to cell culture is more appropriate. Make sure that the refrigerator or the cold room is cleaned regularly to avoid contamination.

3.3.12 Sterilizing oven or autoclave: All of the glassware, glass pipettes, pipette tips and tubes used for cell culture must be sterilized. Most of the materials can be purchased already sterilized and disposed of after use. A cheaper alternative is to purchase the material in bulk, non-sterile, and sterilize them on site, as needed. The tubes and glassware may be washed, rinsed with pure water and sterilized for re-use. A small- or medium-sized oven or autoclave is sufficient to sterilize the material required. A sterilizing oven or an autoclave must be operated and maintained following the manufacturer's instructions. A less expensive alternative is to use a kitchen pressure cooker for sterilizing smaller items.

3.3.13 Water purification system: In addition to rinsing the glassware, purified water is used for filling the water bath and the water container in the incubator. If used to make solutions and reagents for the cell culture, ultrapure water should be used. Most solutions and media used in cell culture are already prepared for purchase to obviate the need for an ultra purification system.

3.3.14 Water bath: A 37°C water bath is required for a cell culture facility to warm up the media and other reagents used for cells. The warm water in the water bath is the ideal environment for the growth of microorganisms and contaminants. Therefore, the water bath needs to be cleaned and the water replaced with fresh, distilled water routinely.

3.4 Cell culture media and reagents

The culture medium is the most important component of the culture environment, because it provides the necessary nutrients, growth factors and hormones for cell growth, as well as regulates the pH and the osmotic pressure of the culture. Although initial cell culture experiments were performed using natural media obtained from tissue extracts and body fluids, the need for standardization and media quality, as well as an increased demand, led to the development of chemically defined media. The three basic classes of media are *basal media, reduced-serum media and serum-free media*, which differ in their requirement

TABLE 3.2: Some examples of commonly used cell culture media and their uses

Media type	Examples	Uses
Balanced salt solutions	PBS, Hanks' BSS, Earle's salts DPBS HBSS EBSS	Form the basis of many complex media
Basal media	MEM	Primary and diploid culture
	DMEM	Modification of MEM containing increased level of amino acids and vitamins. Supports a wide range of cell types including hybridomas
	GMEM	Glasgow's modified MEM was defined for BHK-21 cells
Complex media	RPMI 1640	Originally derived for human leukaemic cells. It supports a wide range of mammalian cells including lymphocytes, hybridomas
	Iscoves DMEM	Further enriched modification of DMEM, which supports high-density growth
	Leibovitz L-15	Designed for CO_2-free environments
	TC 100 Graces insect medium Schneider's insect medium	Designed for culturing insect cells
Serum-free media	CHO HEK293	For use in serum-free applications
Insect cells	Serum-free insect medium	Specifically designed for use with Sf9 insect cells

for supplementation with serum. A list of commonly used media in a cell culture laboratory is given in Table 3.2.

3.4.1 Serum is vitally important as a source of growth and adhesion factors, hormones, lipids and minerals for the culture of cells in basal media. In addition, serum also regulates cell membrane permeability and serves as a carrier for lipids, enzymes, micronutrients and trace elements into the cell. However, using serum in media has a number of disadvantages including high cost, problems with standardization, specificity, and variability, and unwanted effects such as stimulation or inhibition of growth and/or cellular function on certain cell cultures. If the serum is not obtained from a reputable source, contamination can pose a serious threat to successful cell culture experiments.

3.4.2 Basal media: The majority of cell lines grow well in basal media, which contain amino acids, vitamins, inorganic salts, and a carbon source such as glucose, but these basal media formulations must be further supplemented with serum.

3.4.3 Reduced-serum media: Another strategy to reduce the undesired effects of serum in cell culture experiments is to use reduced-serum media. Reduced-serum media are basal media formulations enriched with nutrients and animal-derived factors, which reduce the amount of serum required.

3.4.4 Serum-free media (SFM) circumvents the issues associated with the use of animal sera by replacing the serum with appropriate nutritional and hormonal formulations. SFM formulations exist for many primary cultures and cell lines, including recombinant protein producing lines of Chinese hamster ovary (CHO), various hybridoma cell lines, the insect lines Sf9 and Sf21 (*Spodoptera*

TABLE 3.3: Commonly used reagents in a cell culture laboratory

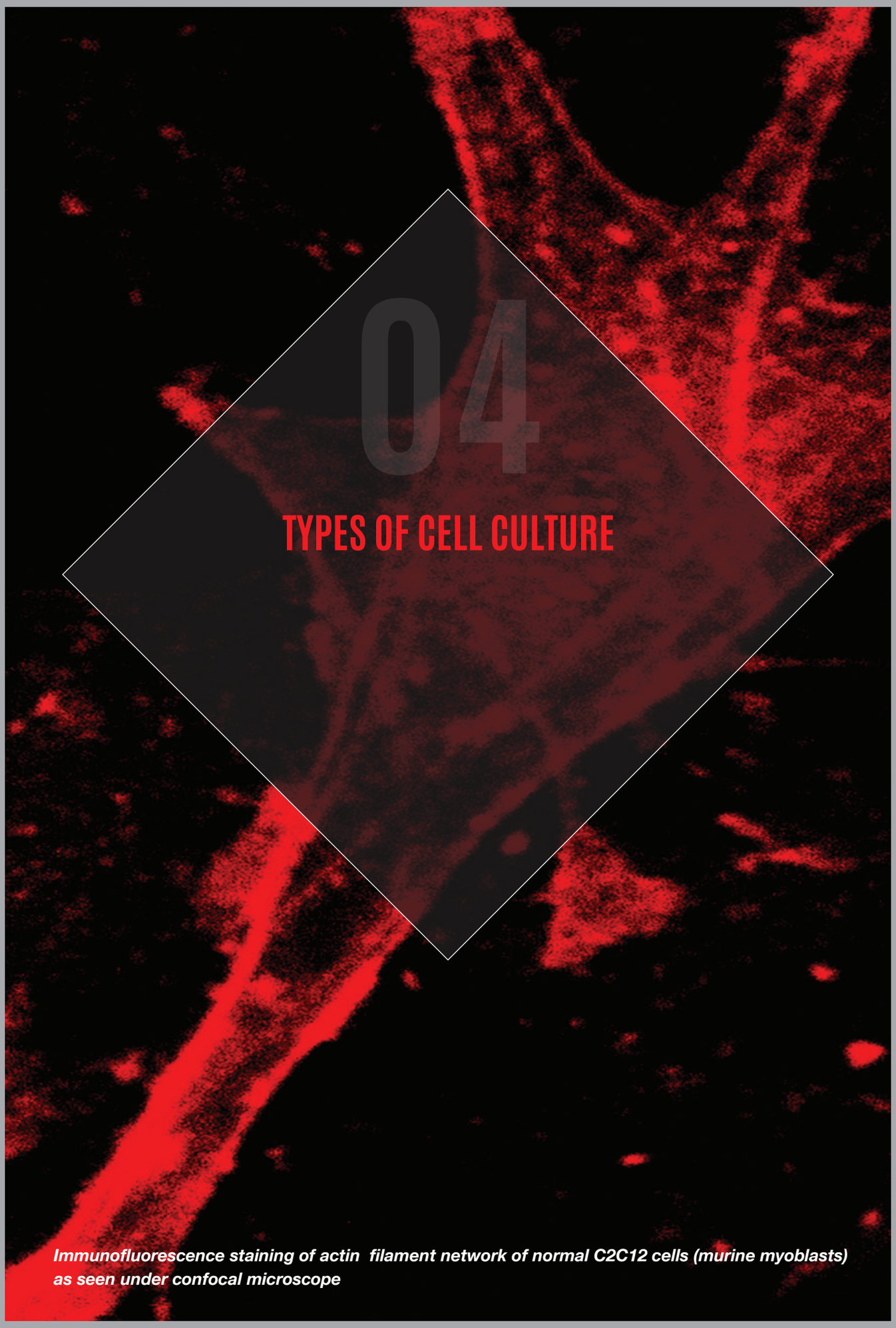
Reagent	Remark
Crystal violet	Required for vital staining of cells. Used to stain virus titration and plaque titration plates.
Dimethyl sulfoxide	Essential reagent used for the cryopreservation of cells in liquid nitrogen. It acts as a cryoprotectant.
PBS	Buffer used for dilutions of cell culture reagents and for washing cells prior to any staining procedure.
Fetal bovine serum	Essential nutrient required for cell growth in vitro.
Gelatin	Used as a coating agent to enhance attachment of cells on plastic/glass surfaces.
Methanol /4% paraformaldehyde/acetone	These are reagents used to fix cells to the flask/plate/slide prior to staining. Essential for fixation of cells prior to immunostaining.
Non-essential amino acids	A mixture that is available commercially and can be added as a supplement for certain type of cell culture.
Penicillin–streptomycin	Essential ingredients that need to be added to cell culture media. Prevents bacterial contamination of media.
Trypan blue	A dye that is used for staining dead cells and used for counting cells in a haemocytometer.
Trypsin	An enzyme that is used for disrupting and detaching cells from adherent surfaces at the time of subculture of cells.

frugiperda), and for cell lines that act as hosts for viral production, such as HEK-293, VERO, MDCK, MDBK, and others. One of the major advantages of using serum-media is the ability to make the medium selective for specific cell types by choosing the appropriate combination of growth factors.

Apart from media, the cell culture laboratory also requires a number of other chemicals and reagents which need to be procured form commercial sources. These reagents are required for a variety of purposes and their list along with their utility is summarized in Table 3.3.

3.5 Human resource

The human resource required for a cell culture laboratory would depend on the range of activities and the type of work undertaken. Nevertheless, a dedicated laboratory technologist is essential for a cell culture laboratory. The technologist must be a science graduate and should have undergone training for a minimum period of three months in an established cell culture laboratory.



04

TYPES OF CELL CULTURE

Immunofluorescence staining of actin filament network of normal C2C12 cells (murine myoblasts) as seen under confocal microscope

04

TYPES OF CELL CULTURE

Cell culture refers to the removal of cells from an animal or plant and their subsequent growth in a favourable artificial environment. Cell cultures can be prepared from unicellular cells (e.g. white blood cells) or from a piece of "tissue". Tissue can be defined as an aggregate of similar cells forming a definite kind of structural material with a specific function in a multicellular organism. The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been established. Generally, cell cultures are broadly classified divided into two types based on the system used for growing them in the laboratory: (i) monolayers on an artificial substrate (i.e. adherent culture) or (ii) free-floating in the culture medium (suspension culture). The basic differences between adherent cells and suspension culture are depicted in Table 4.1.

The majority of cells derived from vertebrates, with the exception of haemopoietic cell lines and a few others, are anchorage-dependent and have to be cultured on a suitable substrate that is specifically treated to allow cell adhesion and spreading (i.e. tissue-culture treated). However, many cell lines can also be adapted for suspension culture. Similarly,

TABLE 4.1: Differences between adherent cells and suspension cells

Adherent cells	Suspension cells
Appropriate for most cell types, including primary cultures.	Appropriate for cells adapted to suspension culture and a few other cell lines that are nonadhesive (e.g. haematopoietic).
Requires periodic passaging, but allows easy visual inspection under inverted microscope.	Easier to passage, but requires daily cell counts and viability determination to follow growth patterns; culture can be diluted to stimulate growth.
Cells are dissociated enzymatically (e.g. TrypLET™Express, trypsin) or mechanically.	Does not require enzymatic or mechanical dissociation.
Growth is limited by surface area, which may limit product yields.	Growth is limited by concentration of cells in the medium, which allows easy scale-up.
Requires tissue-culture treated vessel.	Can be maintained in culture vessels that are not tissue-culture treated, but require agitation (i.e. shaking or stirring) for adequate gas exchange.
Used for cytology, harvesting products continuously, and many research applications.	Used for bulk production, batch harvesting, and many research applications.

TABLE 4.2: Some examples of adherent cell lines used in diagnostic virology

Name	Species and tissue of origin	Morphology
MDCK	Dog kidney	Epithelial
Vero	African Green monkey kidney	Epithelial cells
MRC-5	Human lung	Fibroblast
Hep G2	Human liver	Epithelial cells
BHK-21	Syrian hamster kidney	Fibroblast
HEK-293	Human kidney	Epithelial cells
A549	Human lung	Epithelial cells
HeLa	Human cervix	Epithelial cells
HEp-2	Human HeLa contaminant	Epithelial cells
CHO	Chinese hamster ovary	Fibroblast

TABLE 4.3: Some examples of suspension cell lines used in diagnostic virology

Name	Species and tissue of origin	Morphology
SF9 (mixed)	*Spodoptera frugiperda*	Epithelial
HL60	Human leukaemia	Lymphoblastoid-like
NS0	Mouse myeloma	Lymphoblastoid-like
U937	Human histocytic lymphoma	Lymphoblastoid
Namalwa	Human lymphoma	Lymphoblastoid

most of the commercially available insect cell lines grow well in monolayer or suspension culture. Cells that are cultured in suspension can be maintained in culture flasks that are not tissue-culture treated, but as the culture volume to surface area is increased beyond which adequate gas exchange is hindered (usually 0.2–0.5 mL/cm^2), the medium requires agitation. This agitation is usually achieved with a magnetic stirrer or rotating spinner flasks. Some examples of adherent and suspension cell lines commonly used in virology laboratories as listed in Tables 4.2 and 4.3 respectively.

4.1 Types of adherent cells

Adherent cells are usually classified on the basis of the number of cycles the cells can be maintained through subculture in a laboratory. This is referred to as passage level. Based on the number of passages, cells can be classified into the following types:

4.1.1 Primary cell culture

Primary cell cultures are obtained straight from an excised animal tissue, subjected to enzymatic digestion and cultures either as an explant culture or following dissociation into individual cell suspension under artificial culture conditions in culture medium. These type of cultures are initially heterogeneous but later become dominated by fibroblasts. The isolated primary cells are of two types: adherent cells (anchorage-dependent cells) (Table 4.2) and suspension cells (anchorage-

independent cells) (Table 4.3). Adherent cells require attachment to the surface for the growth and most are derived from tissues of organs (e.g. kidney). Suspension cells do not require attachment for growth and are derived cells of blood system (e.g. lymphocytes). The primary cultures can be maintained only for a finite period of time and require intensive labour for preparation, although they retain many of the differentiated characteristics in vivo but have not been passaged. Once it is passaged, it will become a cell line and no longer remain primary. The commonly used primary cells are epithelial cells, fibroblasts, keratinocytes, melanocytes, endothelial cells, muscle cells, haematopoietic and mesenchymal stem cells.

4.1.2 Continuous cell lines

Continuous cell lines are subpopulations of the original primary tissue and capable of continual growth if they are supplied with nutrients and culture conditions. These consist of a single cell type that can be serially propagated for either limited number (~25–30) or indefinitely under artificial culture conditions and medium. Cell lines of a limited number of passages are usually diploid and maintain some degree of differentiation. Such cell lines senesce after a limited number of cycles of division, and thus master banks need to be prepared to maintain them for long periods.

Continuous cell lines, which can be propagated indefinitely, generally have this ability because they have been transformed into tumour cells. The advantage of transformed cell lines is limitless availability but they cannot retain the original in vivo characteristics. Most of these cell lines are archived in cell banks such as ATCC in the USA and the European Collection of Authenticated Cell Cultures (ECACC). Some of the continuous cell lines which are commonly used in research laboratories are Vero, MDCK, HEK-293, BGM, HEp-2, A549, etc.

Continuous cell lines, which can be propagated indefinitely, generally have this ability because they have been transformed into tumour cells.

4.1.3 Mixed cell cultures

Traditionally, enormous care is taken in cell culture laboratories to avoid cross-contamination of cell lines. However, in recent times mixed cultures are being used for increasing the yield of virus isolation. In this mixed cell culture method, different types of cells are grown as a monolayer in one vial so that it is advantageous to isolate viruses from clinical specimens that are likely to have more than one virus in them. This reduces the number of cell cultures that need to be inoculated in the diagnostic virology laboratory. Subsequent identification of the type of virus growing in the culture is carried out using immunofluorescent or molecular assays. For example, MRC-5 and A549 cell lines can be used as mixed monolayer in a vial for diagnosis of CMV, HSV and adenoviruses. Similarly, R-Mix cell is another cell line used for isolating a variety of viral respiratory pathogens. This is a combination of A549 and mink lung cells in a shell vial for isolation of parainfluenza virus types 1, 2 and 3; influenza virus type A; influenza virus type B; and respiratory syncytial virus.

4.1.4 Genetically engineered cells

Genetically engineered cells also referred to as "*transgenic cells*" offer the possibility of improving virus growth in cell culture, thereby enhancing better detection of viruses in cell culture. Although various approaches are available, the two common applications of cell engineering for diagnostic virology are: (i) engineering of susceptible cell lines to over-express virus receptors, and (ii) 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 β-galactosidase assays without the need for antibodies. An example of a cell line that has been engineered for effective isolation of a virus is L20B, used for the isolation of poliovirus. L20B cells are in fact derived from mouse L cells, and were engineered to over-express the poliovirus receptor. An example of genetically modifying cells to express reporter genes in response to virus infection is the ELVIS HSV system available for the detection and typing of human herpes simplex viruses (HSV) 1 and 2. The ELVIS HSV system uses BHK cells engineered to express β-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 β-galactosidase with a colorimetric substrate. Formation of a blue colour 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.

05

APPLICATIONS OF CELL CULTURE IN MEDICINE AND ALLIED SCIENCES

Carbon-dioxide incubator used in a cell culture laboratory

05

APPLICATIONS OF CELL CULTURE IN MEDICINE AND ALLIED SCIENCES

Animal cell culture technology has advanced significantly over the past few decades and is now generally considered a reliable, robust and relatively mature technology. At present, animal cell culture is an indispensable tool for several research streams. A number of assorted fields in biology use cell cultures to answer fundamental research questions. These include stem cell biology, in vitro fertilization (IVF) technology, cancer cell biology, monoclonal antibody production, recombinant protein production, gene therapy, vaccine manufacturing, novel drug selection and improvement (Figure 5.1).

Established continuous cell lines and primary cell cultures are major tools used in cellular and molecular biology, as they provide excellent model systems for studying the normal physiology and biochemistry of cells (e.g. metabolic studies, ageing), the effects of drugs and toxic compounds on the cells, and mutagenesis and carcinogenesis. The consistency and reproducibility of results obtained from using a batch of clonal cells in experiments have been further utilized for drug screening and development (coupled with advances in recombinant DNA technology), and large-scale manufacturing of biological compounds (e.g. vaccines, therapeutic proteins). To address the increased demands and challenges, intense research in this arena has led to the evolution of several technological advancements in terms of the methods adopted to culture cells in vitro. The emergence of three-dimensional (3D) cell culture systems is one such advancement made to replicate in vivo environment and thereby the normal physiological conditions more accurately. Newer cell culture strategies have also widened the fields of application of cell culture. Above all, the widespread concern regarding the extensive use of animals for laboratory experiments has necessitated the increase in preference to utilize animal cell cultures wherever possible. An overview of applications of cell culture in various spheres is presented below.

5.1 Virus isolation and identification of viruses from clinical specimens

Isolation and identification of viruses using cell culture is widely followed in the field of diagnostic virology. The primary purposes of viral cultivation are:

- To isolate and identify viruses in clinical specimens
- To prepare viruses for vaccines
- To do detailed research on viral structure, multiplication cycles, genetics and effects on host cells.

Infection of cells by cultivable viruses is often characterized by changes in the cell morphology, which can be observed under inverted light microscope. These changes are collectively called cytopathic effect (CPE) and may include rounding/ detachment from flask, syncytia/fusion of cells, shrinkage, increase refractivity, aggregation, loss of adherence, cell lysis/ death. The CPE for each virus-cell line is highly specific and is itself a primary tool

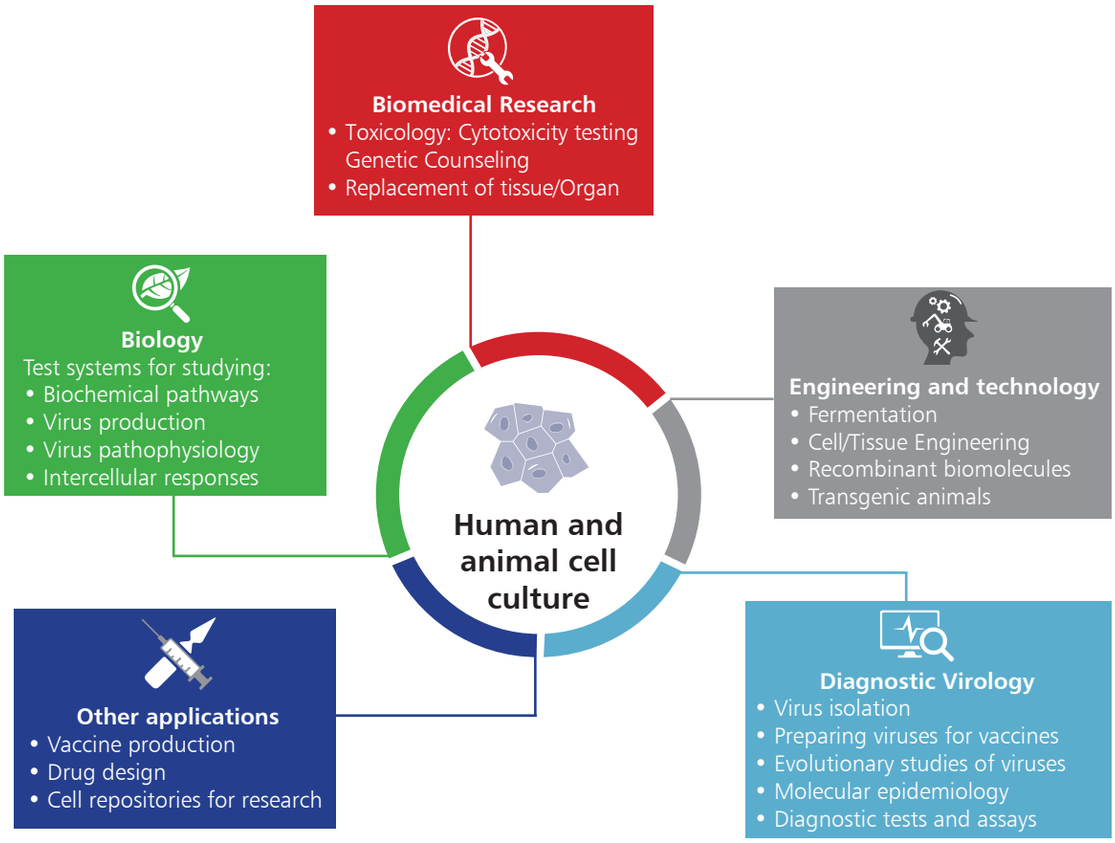


Figure 5.1: *Applications of human and animal cell culture in medicine and research*

to identify viruses. However, definitive identification would require other methods (use of specific antibodies or PCR) for confirmation. Since these reagents can be very expensive with a short shelf-life and because some viruses are not cultivable, many diagnostic virology laboratories are using PCR for more rapid identification of pathogens in patient's samples. The amount of virus (titre) in infected cell culture fluid can be quantified (Figure 5.2) by tissue culture infective dose (TCID50) or by plaque-forming units (pfu).

5.2 Vaccine production

Cell culture has been extensively exploited in the area of vaccine production for a prolonged period. The production of virus-based vaccines in large-scale cultures of animal cells in the early 1950s was the first industrial application of animal cell culture technology. Numerous vaccines that are available today worldwide have been generated in animal cell cultures. Few examples include vaccines for polio, measles, mumps, rubella and chickenpox. The inactivated rabies vaccine for human use was first prepared in cell culture in 1964. Subsequently, the human diploid cell (HDC) strain WI-38 was a suitable substrate for the propagation of the Pitman-Moore (PM) strain of fixed rabies virus but a low titre of virus production was the major drawback in this cell line. Further experiments led to the development of a human rabies vaccine purified from Vero cells. The resulting vaccine is known as the purified Vero cell rabies vaccine.

5.3 Production of monoclonal antibodies

Medical applications for monoclonal antibodies (mAbs) are extensive: diagnostic

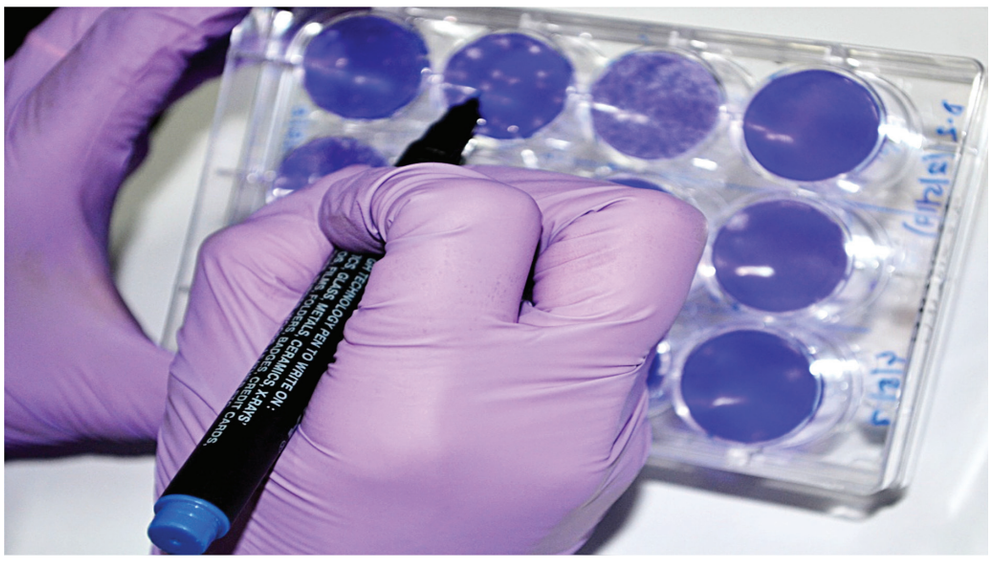


Figure 5.2: *Counting plaque-forming units for quantifying amount of virus in the infected culture fluid*

tools, therapies for various cancers, rheumatoid arthritis and cardiovascular conditions. A range of bio-therapeutics is currently dependent on cell culture methods for large-scale manufacturing of both commercial use and clinical studies. The robust implementation of this technology requires optimization of a number of variables, including (i) cell lines capable of synthesizing the required molecules at low operating cost; (ii) culture media and bioreactor culture conditions that meet quality specifications; (iii) appropriate indicators that provide information on process control; and (iv) good understanding of processes for smooth scale-up. At present, mAbs are one of the fastest growing products of pharmaceutical industry, with widespread applications in biochemistry, biology and medicine. As the market demand for mAbs is increasing, there is significant interest in developing proper models for mammalian cell culture processes for the biopharmaceutical industry. For mAb production, various mammalian cell lines are usually exploited, such as murine myeloma (NS0), murine hybridomas, Chinese hamster ovary (CHO), and PER.C6 human cells.

5.4 Cancer research

Normal as well as cancer cells can be grown in vitro. Therefore, cell culture provides a broad scope to study and understand the various aspects of cancer biology. Further, the role of carcinogens and carcinogenic potentials of different agents have been extensively evaluated on the basis of cell culture experiments. On the other hand, the protective roles of various natural and synthetic substances against cancer have also been studied in cell culture systems.

5.5 Tissue culture and engineering

Cell culture forms a fundamental component of tissue culture and tissue engineering. Culturing and cryopreservation of mesenchymal stem cells is a major application of human cell culture in stem cell industry. Tissue engineering potentially offers dramatic improvements in low-cost medical care for hundreds of patients annually.

5.6 Genetic engineering

In 1986, human tissue plasminogen activator (tPA) became the first therapeutic protein from recombinant mammalian cells

The first and most readily observed effect following exposure of cells to toxicants is morphological alteration in the cell layer and/or cell shape in monolayer culture.

to obtain market approval. Today 60–70% of all recombinant protein pharmaceuticals are produced in mammalian cells. Like tPA, many of these proteins are expressed in immortalized Chinese hamster ovary (CHO) cells, but other cell lines, such as those derived from mouse myeloma (NS0), baby hamster kidney (BHK), human embryo kidney (HEK-293) and human retinal cells have gained regulatory approval for recombinant protein production.

5.7 Study of the effects of toxins and drugs

Cell culture is an essential prerequisite for determining the toxicity of therapeutic compounds and toxicology research. General toxicity tests can be carried out on many cell types (e.g. fibroblasts, HeLa and hepatoma cells). Parameters including vital staining, cytosolic enzyme release, cell growth and cloning efficiency are used as end-points to measure toxicity. Organ-specific toxic effects are tested using specialized cells by measuring alterations in membrane and metabolism integrity and/or in specific cell functions (e.g. glycogen metabolism in primary hepatocyte cultures, beating rate in mixed myocardial cells or myocytes, and phagocytosis in macrophages).

The first and most readily observed effect following exposure of cells to toxicants is morphological alteration in the cell layer and/or cell shape in monolayer culture. Gross modifications such as blebbing or vacuolization can be observed using light microscopy whereas fine ultrastructural modifications require analysis by transmission or scanning electron microscopy. Another indicator of toxicity is altered cell growth. The effect of chemicals on the capability of cells to replicate is used as an index of toxicity; the concentration of the substances at which 50% of the cells do not multiply is called the median inhibitory dose (IDso).

5.8 Production of high value therapeutics

Production of human proteins with high therapeutic potential that undergo post-translational modifications (glycosylation, carboxylation, etc.) cannot be successfully carried out in bacteria and yeasts. These organisms do not possess the machinery to perform post-translational changes. Hence, pharmaceutical proteins that do not require post-translational modifications can be produced by bacteria or yeasts, e.g. insulin, albumin, growth hormone. Mammalian cell cultures are extensively used for the production of compounds/therapeutics such as tPA, clotting factors (VIII and IX), and erythropoietin.

The past two decades have seen significant advances in cell culture technology that have increased the expression of recombinant proteins from 100 mg/L to several g/L. These advances have resulted from intensive research in cell line engineering, media development, feeding strategies, cell metabolism, better process understanding and their impact on product quality and scale-up.

5.9 Cell culture in three dimensions

Traditional cell culture, first developed by Wilhelm Roux in 1885, involves growing cells on flat plastic dishes (culture plates) or flasks. This is also known as 2D cell culture. The advancement of polymer technology witnessed a shift from glass to plastic as the preferred material for culture vessels. Cells are also cultured on biologically derived matrices such as collagen or fibrin, and more recently, synthetic hydrogels such as polyacrylamide gel in order to stimulate

phenotypes that are not expressed on conventionally rigid substrates.

Within a tissue, cells grow in a 3D microenvironment, where they interact with different cell populations. Therefore, the responses elicited by cells in response to an external stimulus or otherwise are different from that of cells grown in 2D systems. The extracellular matrix (ECM) is important in the survival, proliferation, differentiation and migration of cells in vivo, which is not taken into account for 2D cell culture. Variations in cell morphologies grown in 2D and 3D culture systems have also been reported. The scalability of 3D culture systems, with the capability for culturing 500 cells to millions of cells or from single dish to high-throughput low-volume systems, has increased its demand in industrial biotechnology. To address these lacunae of the existing 2D cell culture systems, 3D cell culture, also referred as "biology's new dimension" has come up in a major way during the past decade.

Owing to many advantages, 3D cell cultures have witnessed an increased demand in the fields of drug discovery, cancer biology, regenerative medicine and basic life science research.

06

USE OF CELL CULTURE IN A DIAGNOSTIC VIROLOGY LABORATORY

Double labeling of Chikungunya virus and cytoskeletal actin indicating co-localization in infected C2C12 cells (murine myoblasts) at 18h post infection as seen under confocal microscopy

06

USE OF CELL CULTURE IN A DIAGNOSTIC VIROLOGY LABORATORY

Cell culture involves a complex of processes of cell isolation from their natural environment (in vivo) and subsequent growth in a controlled artificial environment (in vitro). The discovery in the early 1900s that cells could be propagated in vitro provided virologists with an alternative to embryonated eggs and laboratory animals for isolation of viruses. Isolation and identification of human viral pathogens considered as the "gold standard" for virus detection can be achieved using cell culture. Viruses replicate to reach high titres within susceptible cells and can be identified by immunofluorescence tests using monoclonal antibodies or neutralization tests using reference sera or by molecular methods. The primary purpose of viral cultivation is to isolate and identify viruses in clinical specimens and to prepare viruses for vaccines. Replication of viruses in cell cultures also provides an isolate that can be used for additional studies such as antiviral susceptibility testing, serotyping, epidemiological evaluations as well as for detailed research on virus structure and replication and to study host cell virus interactions.

As early as 1913, vaccinia virus was grown in cell cultures, and in the 1930s both smallpox virus and yellow fever virus were propagated in cell cultures for the purpose of vaccine production. In the 1950s, polioviruses were replicated in cell cultures that were not of neural origin. However, although virus isolation in cell cultures was employed by research laboratories by the early 1960s, diagnostic services were limited to major medical centres. In the early 1970s, diagnostic virology services expanded dramatically, with the availability of chemically defined culture media, highly purified reagents and commercially prepared cell lines and a little later cell-dispensing equipment for preparing replicate cultures. Control of contamination facilitated with antibiotics and clean air equipment helped decrease the use of experimental animals for isolation of viruses from clinical specimen.

Diagnostic virology laboratories now can purchase their cell cultures from biological supply houses; however, some laboratories still prepare cell cultures in-house. Examples of well-known cell types in a diagnostic virology laboratory are African green monkey kidney (Vero) cells, Madin–Darby canine kidney cells (MDCK), human lung fibroblasts (MRC-5), human epidermoid carcinoma cells (HEp-2), human lung carcinoma cells (A549), Aedes albopictus gut epithelial cells (C6/36) and others (Figure 6.1). The number and types of cell culture tubes inoculated for each clinical specimen depend on the specimen source and the viruses suspected of causing a given disease.

The appropriate selection, collection, transport and processing of clinical samples are important for successful virus isolation (Table 6.1). Preservation of the viral infectivity until cell cultures can be inoculated is essential. Body sites and collection methods vary according to the

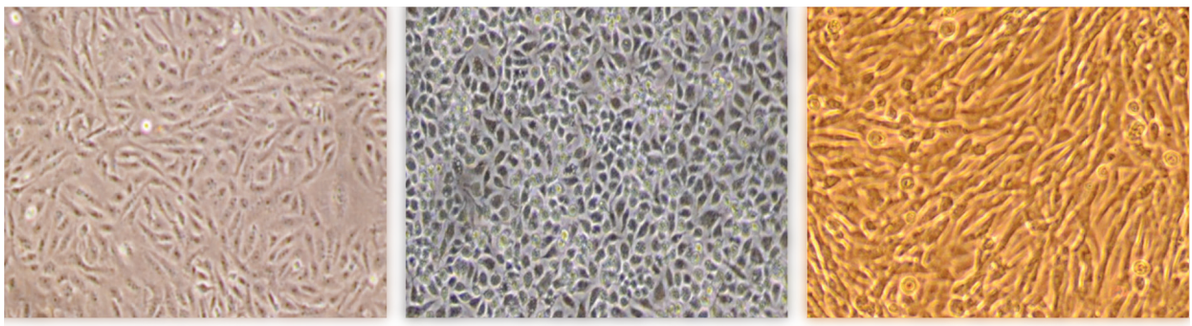


Figure 6.1: *Frequently used cell lines for isolating viruses from clinical samples under phase-contrast light microscope. (A) Vero cells (B) C6/36 cells (C) BHK-21 cells*

type of infection and viral aetiology. Clinical samples collected from body sites such as skin and the genital tract, are collected with a Dacron or polyester swab and placed in viral transport medium (VTM), most types of which contain antibiotics, a buffered salt solution, a proteinaceous substance (such as albumin, gelatin or serum), and a pH indicator. Respiratory tract samples including sputum, bronchial alveolar lavage specimens, nasopharyngeal (NP) washes, NP aspirates (NPA), NP swabs (NPS) and oropharyngeal swabs are collected in VTM. Specimens such as cerebrospinal fluid (CSF) and body fluids, which are expected to be free of microbial contamination, are collected in sterile containers. Keeping the samples cool (2–8 °C or on wet ice or ice packs) until cell culture inoculation helps preserve viral infectivity and increases the chances of virus recovery.

Certain sample types (e.g. respiratory samples) require clarification prior to inoculation into cell cultures. The transport medium tube is vortexed, the swab is discarded, the liquid medium is centrifuged, and the supernatant fluid is used to inoculate the cell cultures. The sample pellet can be used for antigen detection assays. Extensively contaminated clinical material such as stool may be liquefied in antibiotic-containing medium and filtered through a 0.22μm filter prior to inoculation into cell cultures.

The processed inoculum (0.2–0.3 mL) may be added to the cell culture tube, either simply or by adsorption inoculation. Adsorption inoculation allows more efficient adsorption of viral particles to the cells thus increasing the rate of virus recovery. Viral tube cultures are incubated for days to weeks depending on the specimen source and the suspected virus(es). Cell monolayers are screened by microscopic examination daily till the defined incubation period.

Growth of viruses in cell culture is usually detected by visualizing morphological changes in the cells, known as CPE. Degenerative changes in monolayer cells such as swelling, shrinking, rounding of cells to clustering, syncytium formation, loss of adherence and, in some cases, complete destruction of the monolayer provides evidence of viral presence (Figure 6.2). These changes are collectively called the cytopathogenic effect or CPE of the virus (Table 6.2).

TABLE 6.1: Collection guide by specimen type for virus isolation from clinical specimens

Specimen type	Specimen collection
Genital lesion swab skin lesion swab	Select non-crusted lesion with clear fluid and clean exterior with sterile saline. If desired, aspirate fluid with 26–30 gauge tuberculin needle and transfer to VTM, rinsing needle with VTM. Aseptically unroof lesion, blot fluid with swab and collect cells from base of lesion. Submit in VTM (with aspirate, if collected).
Miscellaneous body fluids	Collect at least 2 mL into a sterile container. Do not add VTM.
Nasal swab	Insert moistened swab into nostril, leave in place several seconds, and transfer to VTM. If both nares sampled, use separate swabs and combine into a single VTM.
Nasal wash	Inject up to 5mL physiological saline per nostril with patient's head tilted back. Bring patient's head forward with nostrils over sterile container to catch flowing saline. Transfer wash to VTM.
Nasopharyngeal (NP) swab	Insert moistened swab with flexible metal shaft into posterior nasopharynx. Rotate swab several times and leave in place for 10–15 seconds. Gently remove swab and submit in VTM. If both nares sampled, use separate swabs and combine them into a single VTM.
Nasopharyngeal aspirate	Use a fine catheter with bulb or suction trap. Insert catheter into nasopharynx, apply gentle suction, leave catheter in place for a few seconds, then gently withdraw. Flush tubing with 2–3mL VTM and transfer contents to original VTM vial.
Nasopharyngeal wash	Inject up to 5mL physiological saline into nostril while closing the other. Suction wash or have patient expel saline into sterile container. Transfer wash to VTM.
Rectal swab	Do not use lubricant. Insert culturette swab about 2 inches into the rectum, rotate swab to collect fecal material, and carefully remove swab from the rectum. Insert swab in sheath and crush ampoule to release transport medium.
Sputum	Early morning specimen preferred. If possible, patient should rinse mouth and gargle first. Collect 3–5mL deep cough expectorant into sterile screw-capped container. Submission with VTM is optional.
Stool (feces)	Collect 2–10g into a sterile leak-proof container. Do not use preservatives.
Stool swab	Collect stool, then thoroughly coat culturette swab with fecal material. Insert swab in sheath and crush ampoule to release transport medium.
Throat swab	Moisten swab with physiological saline and vigorously rub against tonsils and posterior pharynx. Submit swab on VTM.
Throat wash	Patient should clear mucus and post-nasal secretions from throat and mouth. Patient gargles for 30–60 seconds with 2–3mL physiological saline and spits wash into a sterile container. Transfer wash to VTM.
Tissue biopsy/ autopsy	Aseptically collect at least a pea-sized sample into VTM. Avoid cross-contamination with other collection sites. Autopsy specimens should be collected within 24 hours of death, if possible.
Tracheal aspirate	Use a fine catheter with suction trap. Insert catheter through endotracheal tube. Apply suction and withdraw catheter once specimen is collected. Flush catheter with 2–3mL VTM and transfer contents to original VTM vial.
Urethral swab	Insert fine aluminum shaft swab for 2–4cm into the urethra, carefully rotate swab three times, then remove gently. Submit swab in VTM.
Urine	Collect 2–15mL midstream, clean-catch (first morning void preferred) urine into sterile container. Do not add VTM. Transport to the laboratory within 24 hours.

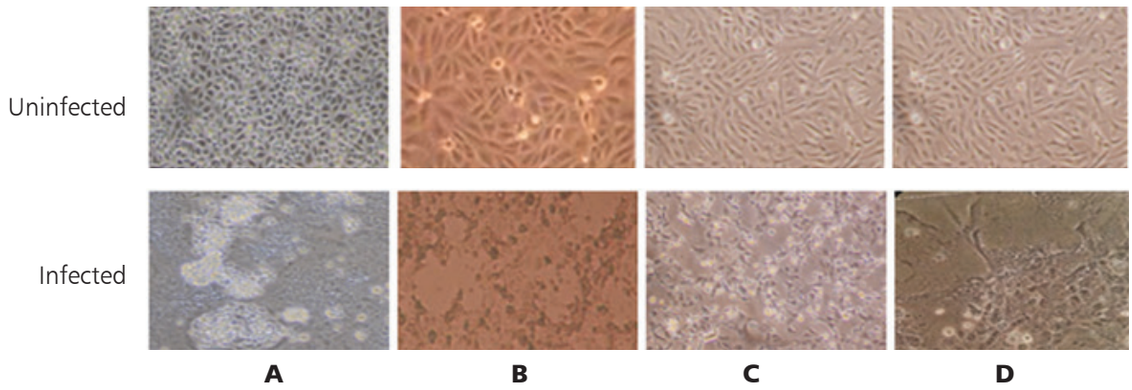


Figure 6.2: *Virus induced cytopathic effects in host cells. (A) Dengue virus infected C6/36 cells (B) Influenza virus infected MDCK cells (C) Chikungunya virus infected Vero cells (D) Measles virus infected Vero cells.*

TABLE 6.2: Cytopathic effect of medically relevant viral pathogens in cell cultures

Virus	Cell lines commonly used	Description of cytopathic effect observed	Final identification of isolates
Adenovirus	Fibroblasts, A549, RhMK	Grape-like clusters or "lacy"pattern observed 5–8 days post-infection (PI)	Immunofluorescence assay (IFA) for group, Neutralization for type, PCR
CMV	Fibroblasts	Foci of contiguous rounded cells, 10–30 days PI	CPE, PCR
Enteroviruses	Fibroblasts, RhMK	Small, round cells with cytoplasmic tails, 2–5 days PI	IFA for groups, Neutralization for type, PCR
HSV	Fibroblasts A549	Rounded large cells, 2–3 days PI Rounded large cells, 1–4 days PI	IFA, PCR
Influenza virus	MDCK	Undifferentiated CPE Cellular granulation, 4–8 days PI	IFA, PCR
Parainfluenzavirus	MDCK	Rounded cells with some syncytia, 4–8 days PI	IFA, PCR
Rhinovirus	Fibroblasts	Degeneration and rounding observed, 7–10 days PI	CPE, PCR
RSV	Fibroblasts RhMK HEp-2	Infrequent with granular degeneration Syncytia observed, 4–10 days PI Syncytia observed, 4–10days PI	IFA, PCR
VZV	Fibroblasts A549 HNK	Some CPE observed which include small, round cells 6–8 days PI Small round cells observed 6–8 days PI Small, round cells observed 6–8 days	IFA, PCR
JEV	C6/36	No CPE observed	IFA, PCR

When necessary, confirmatory testing of virus cultures positive by CPE can be carried out using immunofluorescence techniques that use monoclonal antibodies of known specificity with viral antigens expressed in the infected cells (Figure 6.3). However, immunofluorescence staining cannot be used to definitively identify all viruses

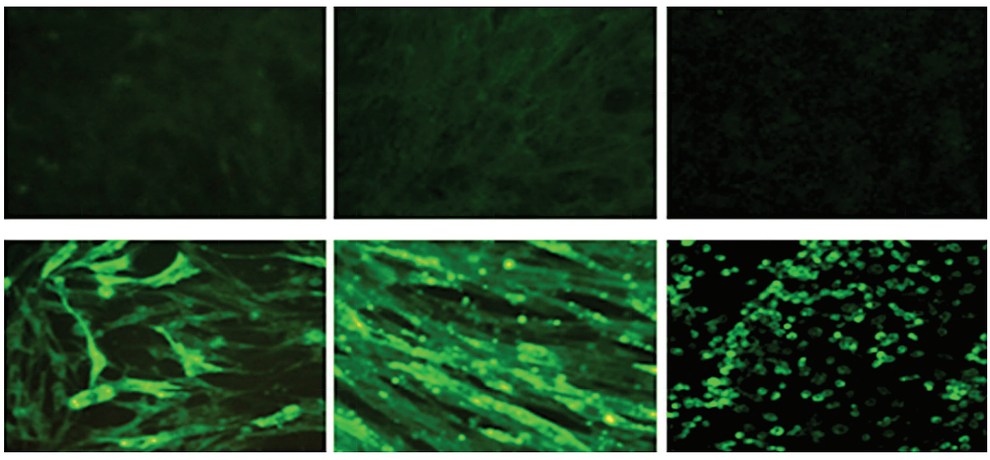


Figure 6.3: *Detection of virus antigen by immunofluorescence assay. Apple green fluorescence indicates the presence of Chikungunya virus in infected Vero cells (A), Rabies virus in infected BHK-21 cells (B) and Dengue-3 in infected C6/36 cells (C). No fluorescence observed in uninfected cells.*

such as coxsackieviruses, polioviruses and echoviruses of the "enterovirus" group, which are closely related and have numerous serotypes. Identification of enteroviral serotypes within the families requires confirmation by the neutralization method using reference sera. Neutralization testing is not routinely performed in most clinical laboratories and is generally reserved for reference laboratories. Identification of a viral isolate can also be achieved using molecular methods using virus specific primers and polymerase chain reaction.

Cell culture technology has also experienced innovative modifications, allowing virus isolation in cell culture to continue to make a significant contribution in viral disease diagnosis (Table 6.3).

The shell vial has become popular for containment of cell culture monolayers wherein the cell monolayer is grown on a coverslip that resides in the bottom of the vial. The small vials fit easily into a centrifuge for use in viral detection assays that involve centrifugation-enhanced inoculation of the monolayer. The shell vial culture method, first developed for CMV, dramatically decreases the time required for detection of viruses in cell culture. The method involves centrifugation of the specimen onto the cell culture monolayer grown on a coverslip which is incubated for 1-2 days, followed by fluorescent antibody staining of the cells, regardless of whether CPE is visible or not. HSV, influenza virus, mumps virus, various respiratory viruses, enteroviruses, adenoviruses, dengue virus and VZV have been isolated in shell vials.

Techniques involving combinations of different cell types grown together as a single monolayer in a vial and the application of various mAbs, each labelled with a different fluorochrome, have been applied for the detection of several viruses in the same vial. Culturing for the simultaneous detection of adenovirus, CMV and HSV in the same shell vial has been approached using a mixture of MRC-5 and A549 cells in the cell monolayer and staining with a cocktail of adenovirus, CMV and HSV antibodies, each raised in a different species. A mixture of monolayers of cells, selected on the basis of their capability of isolating different viruses causing respiratory tract infection are available commercially.

TABLE 6.3: Advantages and disadvantages of various cell culture methods for virus detection

Method	Advantages	Disadvantages
Cell cultures traditional method	Isolate wide variety of viruses (including unanticipated agents, mixed cultures); isolates can be used for additional studies: antiviral susceptibility testing, serotyping, and epidemiological studies	Expertise required to read CPE; long incubation period for some viruses, need for maintaining a variety of cell culture types in the laboratory
Shell vials with centrifugation/ pre-CPE stain	Short turnaround time for detection; occupies less space than tubes; some available as cryopreserved cells; may isolate viruses that replicate poorly or not at all in standard tube cell cultures; requires less expertise than tube cultures if pre-CPE staining is used	Reading stained preparations is labour-intensive; unanticipated agents may be missed when pre-CPE staining targets only one or a few viruses; isolates not available from fixed/stained vials
Co-cultivated cells	Same as for shell vials, support growth of a wider range of viruses, most results finalized in 2–3 days when pre-CPE staining is used	Same as for shell vials
Transgenic cells (ELVIS)	Same as for shell vials plus detection by colour change rather than application of mAbs, simplify identification because of specificity for a single virus	Targeted for detection of only a single virus group (HSV)

The application of transgenic cells in cell cultures involves the stable introduction of genetic elements into a cell such that when a particular virus enters this cell, a virus specific event is triggered that results in the production of an easily measurable enzyme (ELVIS for enzyme-linked virus-inducible system). The advantages and disadvantages of the different cell culture systems used for isolation / detection of viruses is explained in Table 6.3.

A recent modification of the traditional cell culture involves the use of genetically engineered cell lines such as the L20B, which is used for the isolation of polio viruses and Vero/SLAM for isolation of measles and rubella viruses. Another example of genetically engineered cell line uses genes that are transfected into indicator cell lines so that the cell line responds to a specific viral protein present in the specimen. Activation of the promoter triggers a reporter enzyme such as β-galactosidase that acts on a substrate to indicate the presence of the virus being sought. This approach has been most widely used for HSV and HIV.

No one cell culture type can support the growth of all medically relevant viruses; hence, virology laboratories should maintain several different cell culture types. The minimum requirements are Hela and/ or RD cells, L20B used for the isolation of enteroviruses, Vero and BHK-21 cell lines used for the isolation of herpes simplex viruses, MDCK and embryonated eggs for isolation of influenza viruses, HEp-2 for the isolation of RSV and an insect cell line such as *Aedes albopictus* C6/36, which is useful for isolation of a number of arboviruses. The type of cell lines that are required for a specific specimen is determined by the information provided by the ordering physician to the laboratory and by knowledge of the range of viruses that can usually be isolated from a given specimen type.

07

QUALITY IN A CELL CULTURE LABORATORY: DETECTION AND PREVENTION OF CONTAMINATION, MONITORING GOOD LABORATORY PRACTICES, TROUBLESHOOTING

Immunoflouresence staining of Rabies virus antigen in infected BHK 21 cells (baby hamster kidney cells) – under fluorescent microscope

07

QUALITY IN A CELL CULTURE LABORATORY: DETECTION AND PREVENTION OF CONTAMINATION, MONITORING GOOD LABORATORY PRACTICES, TROUBLESHOOTING

A quality system is a part of overall quality management that aims at consistency, reproducibility, traceability and efficaciousness of the laboratory services. Quality does not happen by chance but needs to be developed in an organization. Quality needs to be systematic and quality systems are the basis on which the quality of the product or service is built.

A quality system has five essential elements (Table 7.1).

TABLE 7.1: Five key elements of a quality system

1. Organizational management structure
2. Quality standards
3. Documentation
4. Training
5. Monitoring and evaluation

The outcome is significantly influenced by many pre-analytical, analytical and post-analytical factors.

A clinical virology laboratory should be designed in a manner so that biohazard risks to the laboratory personnel and the general public are minimized and that cell cultures are protected from environmental contamination.

7.1 Key elements of a quality system

7.1.1 Management commitment

Ensuring quality in any laboratory is the responsibility of all staff members. However, the commitment of top laboratory management to provide guidance and adequate financial resources makes their role critical. Quality costs but poor quality costs more. Quality has to be all pervasive and encompass human beings, equipment, processes, reagents and interpretation skills.

7.1.2 Quality standards

Quality standards are an integral part of the quality system. They are designed to help laboratories meet regulatory requirements, including local health regulations, and monitor laboratory functions, thereby ensuring laboratory safety and consistency of performance. In the absence of any defined standards, senior scientists should develop pre-agreed criteria or standards and share these with all staff members.

7.1.3 Documents

A variety of documents and their control are required by any quality system. All cell

The procedure manual used in the laboratory should be in sufficient detail so that an inexperienced technologist can perform the procedure without additional information.

culture procedures must be described in written standard operating procedures (SOPs). An SOP is one of the most important documents in a cell culture laboratory. Besides providing the complete details of how exactly a test or a procedure is carried out in a laboratory, the SOP also provides information on specimen collection, laboratory safety instructions, purpose and limitations of the procedure, turnaround times, interpretation of results, and above all, the line of authority.

The procedure manual used in the laboratory should be in sufficient detail so that an inexperienced technologist can perform the procedure without additional information. One copy of the manual should be readily available to bench personnel, and another copy should be stored separately in case of accidents. The document control system is required to ensure that the up-to-date document is used.

SOPs must be validated, reviewed regularly and modified, if necessary. The modified versions should be signed and dated by the laboratory director. The recent most version of SOPs should be available directly at the workplace. The old versions should be removed from the laboratory and should be archived, if required.

The records must be stored for long periods of time but should be available for prompt retrieval and safe archiving. Archiving of the source documents and other essential documents must be such that data are kept in an integer state and can neither be lost nor altered to achieve this goal.

Records of usage, maintenance and calibration should be kept in the laboratory and should be routinely monitored. Reports of the tests should be released only after proper scrutiny and documentation of the scrutiny with the signature and date by the laboratory supervisor.

7.1.4 Training

The cell culture laboratory should have a training plan for all its staff members. The objectives of training should be clearly delineated. It is essential to clearly understand the training needs of different categories of staff. The training programme and training material should be developed to meet these needs of staff members. Training should be imparted through experts who have substantial hands-on experience in all aspects of cell culture.

Post-training the staff members must be provided with an enabling environment so that skills acquired by them can be translated into quality cell culture work.

7.1.5 Assessment of quality system

A quality system can be assessed either through an onsite inspection (audit) or by sending known but undisclosed material to the laboratories for testing (quality assessment scheme). The latter can be done within an institute by internal staff (internal quality assessment scheme – IQAS) or through an external agency (external quality assessment scheme – EQAS).

7.2 Quality control checks in cell culture laboratories

Some of the areas where quality control checks are critical are briefly described below.

7.2.1 Tissue culture and media

Within a given cell line, there may be significant variations in sensitivity to virus isolation, which may depend on the particular cell line or clone and the passage number. Information on a particular cell line

should be recorded accurately including the source, type, passage number, confluency and cell condition. The loss of a cell line routinely passaged for use can lead to a severe disruption of workflow, and therefore provisions must be made for back-up cells in the event of contamination or laboratory accident. These back-up systems include:

- Freezing and storage of low passage cells at –70°C or liquid nitrogen tanks.
- Use of paired stock flasks in which a second flask is held as a back-up until the new flask displays good growth.
- Carrying of a parallel set of stock.
- Cells purchased commercially should be certified to be free from mycoplasma, fungal and bacterial contamination as well as adventitious viral agents such as SV40.

Other quality control procedures that may aid in minimizing the risk of contamination include the exclusion of laboratory personnel with infectious diseases from handling tissue culture, and separate laboratory apparel, reagents and glassware for tissue culture. Cell lines should be handled separately and the BSC should be decontaminated in between uses.

7.2.2 Media

Following filter sterilization, aliquots of the media should be taken and checked for bacteriological or fungal contamination. These samples should be examined daily for five days for freedom from any contamination. Aliquots of material such as fetal calf serum (FCS) and L-glutamine should also be examined for sterility. New lots of medium and FCS that have passed the sterility check should be monitored for their ability to support cell growth.

7.2.3 Equipment

Periodical calibration of all major laboratory equipment from certified agencies should be undertaken. Salient recommendations for routine laboratory maintenance and performance checks on equipment are given in Table 7.2.

TABLE 7.2: Laboratory equipment maintenance

Equipment	Action
Incubators	Daily temperature, CO_2 and humidity checks Weekly decontamination of interior
Safety cabinets	Daily air pressure check and cleaning of ultraviolet lamp Work surface should be decontaminated after each use Annual checks for air velocity and filter integrity and paraldehyde decontamination, as applicable
Microscopes	Daily cleaning of objectives and stage, log of lamp usage and annual overhaul
Refrigerators and freezers	Daily temperature check , periodic calibration Annual check of compressor and refrigerant levels
Water baths	Daily temperature checks Weekly decontamination
Refrigerated centrifuges	Weekly decontamination Annual inspection of motor, speed calibration and drive system
Autoclaves	Daily temperature check and monthly spore strip testing
Pipetting devices	Gravimetric volume check monthly, periodic calibration Annual overhaul

TABLE 7.3: Development of a quality system

Quality policy	⇨	**Mission statement**
Quality plan	⇨	**Implementation of policy**
Quality manual	⇨	**Policy, Plan and application of standards**
Procedures	⇨	**Development and application of SOPs**
Work instructions	⇨	**Methodology to carry out specific tasks**
Training of staff	⇨	**Implementation of quality system and used of SOPs**
Monitoring and evaluation	⇨	**Assessment of quality and correction process**

7.3 Development and implementation of the quality system

A quality system can be developed in a step-wise manner as shown in Table 7.3 and implemented as shown in Table7.4.

7.4 Avoidance of microbial contamination

Cell cultures have the potential of getting contaminated from other cell lines, inadequate laboratory conditions and poor application of aseptic techniques and good laboratory practice (GLP) by the staff. Thus, the use of cells and reagents of known origin and quality alone is not sufficient to guarantee quality of product. Quality must be ensured throughout the production process and also in the final product. Routine screening aids the early detection of contamination since all manipulations are a potential source of contamination.

The three main types of microbial contaminants in tissue culture are:

- Bacteria and fungi
- Mycoplasma
- Viruses.

TABLE 7.4: Key steps in implementing a quality system

Commitment of top management
• Motivation and support
• Allocation of resources
• Quality policy
• Selection of standard
⇓
Involvement of all laboratory personnel
⇓
Gap analysis (with reference to the selected standards
• Define target
• Develop a plan
• Delegate responsibilities to the team
⇓
Where to start?
• Technical areas
– IQC
_ External quality assessment (EQA)
• Documentation
– SOPs - development and implementation
> Equipment
> Test Procedures
– Pre-analytic and post-analytic
• Training on the standard
⇓
Audit
• Train internal auditor
• Re-evaluate gap between standard and status of laboratory
• Corrective action
• Preventive action
⇓
Management review
⇓
Continuous quality improvement (CQI)

7.4.1 Bacterial and fungal contamination

Bacterial contamination is generally visible to the naked eye and detected by a sudden increase in turbidity and colour change of the culture medium as the result of a change in pH. These contaminations dramatically reduce the life of cell culture. Daily microscopic observation of cultures will ensure early detection of contamination and enable appropriate early action. In addition, specific tests for the detection of bacteria and fungi should be used as part of quality control screening procedure.

7.4.2 Mycoplasma contamination

Mycoplasmas are widely present. There are five major species that are tissue culture contaminants, namely *Mycoplasma hyorhinis, M. arginini, M. orale, M. fermentans and Acholeplasmalaidlawii.*

The effects of mycoplasma infection are more insidious than those of bacteria and fungi, inducing several long-term effects. These include:

- Reduced growth rate
- Morphological cellular changes
- Chromosome aberrations
- Alterations in amino acid and nucleic acid metabolism.

Detection of mycoplasma contamination is often neglected. A possible reason is that mycoplasma contamination is difficult to detect requiring the use of specialist techniques. In the past, only specialist laboratories, such as culture collections, have performed these tests. However, diagnostic kits are now available and, though not perfect in results, can complement other screening processes.

7.4.3 Viral contamination

Some cell lines contain endogenous viruses. Few cell lines secrete virus particles or express viral antigens on their surface. Bovine serum is another potential source of bovine viral diarrhoea virus (BVDV) contamination. Use of infected serum will lead to contamination of cell lines with the virus. Contamination of cell lines with BVDV may cause slight changes in growth rate but since this virus is non-cytopathic, macroscopic and microscopic changes in the culture will not be detected. If possible, one should procure serum that is certified to be tested for BVDV.

7.4.4 Environmental monitoring

It is good practice to monitor the laboratory environment where cell cultures and their products are prepared. BSCs should be checked every 6 months to ensure that they are working efficiently. However, it is also advisable to monitor the number of contaminants within the cabinet by periodically placing open settle plates (blood agar bacteriological culture plates) on the cabinet work surfaces. Settle plates should be used to assess airborne microbial burden at selected points around the laboratory. Plates should be left open for a period of 4 hours. After this time, they should be covered, placed in sealed boxes and incubated at 32 °C and 22 °C for up to 7 days. At the end of this period, the plates should be examined for the presence of microbial growth.

7.4.5 Troubleshooting for contamination

Continuous use of antibiotics is unnecessary and can lead to the development of antimicrobial resistance. The resistant strains are difficult to eradicate and may require the use of more expensive and toxic antibiotics that may damage the cell cultures. In addition, the prophylactic use of antibiotics may mask a low level of contamination.

On detecting a contamination in cell culture, whether due to bacteria, fungi or mycoplasma, the recommended course

of action is to discard the culture and continue the work with earlier stocks that are known to be free of contaminants or obtain fresh stocks from a recognized source. However, if this is not possible, eradication of the contaminant may be attempted with the use of antibiotics.

Viral infections are difficult to remove from cell cultures. These intracellular parasites do not respond to antibiotic treatment and it is not possible to remove them by usual separation techniques. If virus-free stocks or a virus-free alternative is not available, then a thorough risk assessment should be undertaken before continuing work with the infected cell line. The assessment should be followed with appropriate actions and evidence generated for their success.

08

BIOSAFETY AND BIOSECURITY IN A CELL CULTURE LABORATORY

Subculture of cells inside a Biosafety cabinet

08

BIOSAFETY AND BIOSECURITY IN A CELL CULTURE LABORATORY

In recent past, biosafety and biosecurity (or biorisk) concerns have been discussed frequently on local, national and international platforms. Biorisk has increased especially with increase in innovations and applications of animal/human cells culture technology. This necessitates the requirement of a stringent biorisk assessment programme in each laboratory/biological industry. To achieve proper biorisk management, one needs to understand the type of experimentations involved, type of cell culture, source of cells, culture types, intrinsic properties of cell lines, common contaminants and any other inherently harbouring pathogens; hence, only case-by-case assessment will assure proper biorisk management by defining the containment level and reducing the risk. Traditionally, when people work with clean tissue culture they are more concerned about the contamination of culture from self and therefore adopt appropriate precautions to avoid contaminating the culture. However, the concept and the awareness of biorisk associated with the cells to human beings is relatively recent and of significant importance.

In the past, laboratory-acquired infections (LAIs) linked to handling of cell cultures and/or containing virus suspension have been under-reported. Many studies clearly indicate the risk involved with handling of cell cultures. Infection of laboratory workers with recombinant vaccinia viruses amplified in cell culture has been reported in 1986, 1991, 2003, 2004, 2006 and 2009 by different researchers. Later in 2012, the requirement of guidelines to ensure the safety of workers, handling vaccinia virus have been reviewed. Hence, a biorisk assessment while working with cell cultures has become an integral part of the biosafety establishment in animal/human cell culture laboratories.

8.1 Biosafety

Biosafety is defined as the set of guidelines, which describes the containment principles, technologies and practices that are implemented to prevent the unintentional exposure to pathogens and toxins, or their accidental release. The case-to-case risk assessment is the key procedure, which ensures the establishment of biosafety measures. Risk can be defined as "possibility of loss, harm or injury".

8.2 Biorisk

Biorisk is the probability or chance that a particular adverse event (in the context of this document: accidental infection or unauthorized access, loss, theft, misuse, diversion or intentional release), possibly leading to harm, will occur.

8.3 Biorisk assessment

Biorisk assessment is the process to identify acceptable and unacceptable risks, risks of accidental infection and laboratory biosecurity risks (risks of unauthorized access, loss, theft, misuse, diversion or intentional release) and their potential consequences. Risk assessment helps in keeping workers, workplace, community and environment safe.

The scope of achieving required biosafety in a tissue culture laboratory is easy in a new building, which is to be constructed in comparison with converting the existing building.

8.4 Laboratory layout

Infrastructure is the main requirement for distinguishing a tissue culture laboratory, which is required for maintaining aseptic conditions and adequate biosafety. However, fulfilment of good laboratory design requirements is the most neglected aspect. Many times, laboratories have been adapted for the purpose of tissue culture in settings that are not ideal. In such circumstances, the following practices can ensure biosafety and the required quality of work.

- Preferably conduct the work in a single-use facility.
- Identify a quarantine area by separating the area reserved for handling newly received material/ specimens, which have potential of contamination of mixed microorganism.
- Identify the main area for handling material, which is known to be free of contaminants.

8.4.1 If there is constraint of space

- Separate the activity by time and type of material, i.e. first handle the clean cell culture material followed by handling the "quarantined material".
- Label the quarantine material incubators with a biohazard label.
- Use different incubators for clean and quarantine material.
- Systematically clean the work surfaces between activities with appropriate disinfectant.

The scope of achieving required biosafety in a tissue culture laboratory is easy in a new building, which is to be constructed in comparison with converting the existing building. Alteration of structural design of the building, modification of ventilation, air conditioning is expensive and cumbersome; this limits the required degree of modification and hence often compromises biosafety.

When a new building is contemplated, there is more scope for integrated and innovative design and facilities that may be positioned for ergonomic and energy-saving reasons, rather than structural ones. The following points should be considered while designing a new facility for cell culture work.

8.5 Work surfaces, flooring and other requirements

At least biosafety level (BSL)-2 laboratory setup should be designated for handling most of the cell cultures. However, based on the risk assessment report on the nature of pathogen/samples handled or type of cells cultured or nature of work done in the proposed laboratory, the precise BSL can be decided. The following laboratory facilities should be designated as per standard guidelines:

- It is suggested that laboratories should be operated at air pressures that are negative to corridors to ensure the proper containment.
- Work surfaces including bench tops, walls and flooring should be smooth, monolithius walls and easy to clean, waterproof and resistant to a variety of chemicals.
- Provision for hand washing sink with constant water supply.
- Eye wash station for eye washing provision for emergency situation.
- Placements of laboratory equipment for safe handling convenience and as per workflow of the laboratory.
- Consumables: Single-use consumables will avoid cross-contamination due to repeated handling and exposure.

8.6 Care and maintenance of laboratory areas

To make sure safe and clean working environment in a cell culture laboratory, the following practices should be adopted:

- Manage all spills immediately.
- Clean work surfaces both inside and outside of the microbiological safety cabinet, the floors and all other equipment routinely.
- Clean humidified incubators to avoid fungal and bacterial growth in the water trays.
- Perform periodical calibration and maintenance of all major laboratory equipment with certified engineers. BSCs/laminar flow cabinet should be checked after every 6 months for safety functioning to protect product, user and environment.
- This activity confirms the required airflow and proper functioning of high-efficiency particulate air (HEPA) filters.

8.7 Waste disposal

To avoid exposure of infectious material to the laboratory personnel or the environment it is recommended that the laboratory :

- identify and develop correct disposal procedures.
- use correct disinfectants effective against the agents.
- all standard guidelines for waste management should be practised to reduce the risk.

8.8 Risk assessment of cell cultures

The main aim of risk assessment is to achieve maximum biosafety, prevent injury, protect property and avoid harm to individuals and the environment. The management of risks associated with animal/human cultures requires careful case-to-case assessment of (i) intrinsic properties of cell cultures;(ii) type of manipulation involved in the experiments;(iii) modification of genetic material; and (iv) unintentional and deliberate infection of cell lines with pathogenic microorganisms.

The risk assessment principles that are applicable to cell cultures harboring pathogens are exactly similar to those that apply to pathogens. The following parameters have to be line-listed and checked during assessment:

Cell cultures harbouring pathogens	Properties of pathogenic organism to be noted are	Disease parameters and therapy
• Pathogen characteristics • Pathogenicity of organism • Infectious dose • Mode of transmission • Host range • The epidemiology • Risk group • Possible reservoir and vector • Possible zoonosis • Stability and the persistence of the organism in the environment • Physicochemical properties	• Susceptibility to disinfectants • Physical inactivation • Resistance to antiviral compounds and sensitivity pattern • Resistance to antibiotics	• Availability of an effective prophylaxis • Availability of an efficient therapy • Reported case of laboratory-acquired infection (LAIs)

8.9 Intrinsic properties of cell cultures

Understanding the three intrinsic properties of cells is the key to ensuring the safe cell culturing practice in the laboratory. These are: (i) origin of cells/ pathogen species; (ii) cell type; (iii) tissue/ organ type (along with the culture status).

8.10 Origin of cells/ pathogens species

As a rule, the closer the genetic relatedness of cell culture handled in the laboratory, the higher the biorisk to the humans. Hence, human or primate cells may pose a high risk compared to cells of non-human origin. Similarly, the risk reduces with mammalian, invertebrate and avian cells in comparison with that of human or primate cells. Characteristically, pathogens have specific species barriers; sometimes few pathogens might cross this barrier. This may be the reason for almost 70% of the emerging infectious diseases being zoonotic in nature. Reports have been published showing clear evidence for many viruses crossing species barriers from animal reservoirs to humans. Hence the biorisk involved with infected animal/ human cell cultures should not be overlooked.

8.11 Cell type

Repeated sub-culture of cells into many passages affects the growth property and may lead to a series of mutations in the cells. Cells may also harbour endogenous viruses and transformed cell cultures with greater tumorigenic potential may become neoplastic in case of accidental or deliberate introduction into the human body.

To address the issues of cross-contamination of cell lines and/or a lack of proof of identity for the cell lines received from external sources, many conventional and new technologies being used are cytogenetic analysis, isoenzyme analysis, DNA fingerprinting, PCR and flow cytometry. The characterization will help in systematic risk assessment of the culture types:

- If primary cells obtained directly from fresh tissues ➔ first assess for risk based on the pathogen-carrying status of the animal species of origin and cell or tissue type
- If a long time is needed for characterization/detection of inherent contaminating agents ➔ reduce risk by avoiding obtaining cells from fresh tissue.

8.12 Type of manipulation

Different kinds of manipulations are routinely being done with cell cultures in biological/biomedical, diagnostic laboratories and related industries to develop a process, product or for detection of pathogens. Risk assessment in this situation depends on:

- the type of manipulating procedure; pipetting, vortexing, use of needles, animal handling for the conduct of in vivo experiments;
- availability of safety equipment;
- quantity of cell/virus culture; and
- personal protective equipment.

8.13 Deliberate infection of cell cultures with pathogenic microorganisms

For understanding the biology of pathogens, host pathogen interactions and immunological responses or oncogenicity studies and virus stock preparation for future research, deliberate in vitro infection of animal or human cell cultures is mandatory. In such cases, biorisk assessment depends on the risk group of infected pathogen as defined by WHO(2004).

Example: Cell cultured Japanese encephalitis virus (JEV) in laboratory may be assigned to risk group 2 because JEV belongs to the class of risk group 2 in a country like India where it is endemic. But bulk culturing of human immunodeficiency virus (HIV) may be assigned as risk group 3, even though HIV in India is classified as a risk group 2 virus. This is because high concentration of viral particles and risk of exposure is more in bulk culture; this can be categorized under risk group3, so BSL-3 laboratories and practices are required for handling such cultures. Knowledge of intrinsic and native properties of the infecting pathogen and natural history of the disease caused by the pathogen will help in the identification of potential risk. In the case of any accidental exposure of such cell cultures, prophylaxis and management of infection can be achieved to reduce the risk by providing effective treatment.

8.14 Risk with adventitious contaminating agents

Adventitious contamination/biological contamination is the most common

Reported viral contaminants of animal/human cell culture or tissues that can cause human diseases

In human tissues
• Hepatitis viruses: HBV, HCV, HDV, HEV, HGV
• Human retroviruses: HIV-1, HIV-2, HTLV-1, HTLV-2
• Herpes viruses: EBV, CMV, HHV-6, HSV-1, HSV-2
• Papovavirus: different HPV sequences, no virus production
• Rhinoviruses
• AAV-subtypes
• EBV
• Bovine herpesvirus 4 (BoHV-4)
• BVDV
In primate tissues
• Flaviviruses: Yellow fever virus, Kyasanur forest virus
• Filoviruses: Marburg, Ebola
• Simian haemorrhagic virus
• Rabies virus
• Hepatitis A virus
• Poliovirus
• Herpes viruses (herpes B virus and others)
• SV40 (nonpathogenic for humans)
• Simian immunodeficiency virus (infection but also disease in humans?)
• Monkey pox
• Simian foamy virus
• Retroviruses
In rodent tissues
• Lymphocytic choriomeningitisvirus (LCMV)
• Hantaan virus (haemorrhagic fever with renal syndrome)
• Monkey pox

The biological risk assessment of genetically modified organisms (GMO) depends mainly on the properties of hosts, vectors used, probability of adverse effect on public health and the environment in comparison with the wild type.

problem in cell culture laboratories/industries; many times this has very serious consequences. Depending on the ease of detection in cell cultures, biological contaminants can be divided into two subgroups: (i) bacteria and fungi easily detectable and relatively easy to prevent and to treat; (ii) viruses, prions, mycoplasma and cross-contaminating other mammalian cells, parasites are more difficult to detect and treat, hence they present potentially serious problems. Some contaminating *Mycoplasma* spp.belong to the class of risk group 2. Few reports that indicate harbouring of viruses in cell culture include rodent cell culture carrying Hantaan virus and primate cells harbouring Marburg virus with a broader host range, which can infect humans while handling and thus increase the biorisk.

Human and non-human primate cultures are also likely to harbour viruses that are highly pathogenic to humans. When handling freshly prepared primary cell cultures, adventitious contamination with parasite is more common if donor organism is infected with specific parasites. Even though most cell cultures are resistant to prion infection, the use of bovine-derived products as tissue culture supplements may also lead to the contamination with unconventional agents that cause transmissible spongiform encephalopathies (TSE). In such situations, the nature of the infecting pathogen is unknown; hence it is recommended to work at the minimum containment level-2 and autoclave contaminated cultures as well as all the material from clean tissue culture. HeLa cells carrying human papillomavirus (HPV) or cells known to be transformed by a pathogenic virus and human cell lines are not routinely screened for HIV, Hep B or Hep C.

8.15 Risk assessment of genetically modified cell cultures

The biological risk assessment of genetically modified organisms (GMO) depends mainly on the properties of hosts, vectors used, probability of adverse effect on public health and the environment in comparison with the wild type. Genetic modification leading to transgenesis demands a thorough evaluation of risk. For gene therapy, many viral vectors are created in vitro by required genetic modifications; for example, by replacing retroviral gag, pol, env genes with therapeutic genes retroviral vectors are created. A packaging cell is essential to further produce these vector particles. These cell lines provide all the viral proteins required for capsid production and the virion replication and maturation of the vector.

Special precautions should be taken during risk assessment of packaging cell lines, as there are chances of generation of replication-competent viruses due to homologous recombination between viruses, the replication-deficient viral vector and viral sequences present in the packaging cell. This may result in development of hazardous characteristics of the resulting virus population. Hence, assessment of these cell cultures depends on the following key points:

- risk group of the viral vector used
- infectivity
- host range spectrum
- integration capacity or insertional mutagenesis

- stability
- physiological role of the transgene, if expressed.

8.16 Biological risk management in cell culture laboratories

In conclusion, biorisk varies depending on the diversified applications of cell culture technology. Hence, it is essential to assess and manage the biological risk involved in handling of animal/human cell cultures. Performing risk assessment with cell culture would help in:

- determining the required containment level
- understanding the intrinsic properties of the cell culture
- knowing the potential for inadvertent or deliberate contamination with pathogens
- managing acquired risky properties as a result of genetic modification
- noting type of manipulation(s) explains the risk spectrum.

It is also important to follow key behavioural elements of the laboratory worker, which have a major impact on the biorisk management process. Some workers may have a low opinion of the safety programme and may take excessive risks while working. These are normally young workers and less aware of infectious risk.

The following biosafety measures should be practised in handling animal/human cell cultures

Biosafety measures	Benefits in reduction of biorisk
Develop: A biosafety manual and laboratory biosafety guidelines for the laboratory/facility	Addresses all biosafety issues in the laboratory
Training: All personnel must be trained in safe work practices or supervised	Confirms the common understanding of safety principles and practices
Prepare standard operating procedures (SOPs) for handling cell culture activities and provide SOP training to the staff	Ensures uniform flow of work practice among the staff and reduces the most common errors, hence reduces the risk
Practices of good microbiological techniques (GMT)	Ensures overall safety
Access control and biosecurity	Limits the unauthorized access to cell culture facility and secures the biological material
Immunization (e.g. hepatitis B personnel working with human and non-human primate cell lines)	Provides protection against inherently harbouring viruses in cell lines
Do not ever use defective culture vessels/flasks	Reduces contamination in the laboratory
Do not contaminate culture due to poor practice of aseptic technique	May contaminate with airborne pathogens
Treat each new culture used first time in the laboratory as potentially infectious	Reduces exposure of individual to unknown risk factors
Manage any culture fluid spills immediately with validated disinfectant	Minimizes the exposure time and neutralizes the spill
Work with one cell culture at a time	Avoids cross-contamination of two different cell lines
Disinfect the work surfaces between two handlings involving cell lines	Provides safe working area to the next person and avoids cross-contamination
Prepare separate aliquots of growth medium and FBS for each cell lines	Avoids cross-contamination of two cell lines and minimizes wastage of medium

Exposure control: minimize pouring actions and large volume handling procedures	Reduces cross-contamination and aerosol formation
Perform correct operation of biosafety cabinet (BSC) before and after cell culture activity	Sterilizes working area and ensures safety of the user
Limit the use of antibiotics, antifungals in growth media	Continuous use of antibiotics may lead to development of resistant organisms with slow-growing properties
Identify BSC to culture new cell lines till the appropriate tests are negative	Reduces the risk of exposure to inherent unknown pathogens present in cell lines
Frequently perform quality control of cell lines for probable contaminating pathogens	Increases confidence and ensures timely action in case of contamination
Use BSL-2 or higher containment levels for cell cultures obtained from unidentified sources	Provides required level of protection and ensures personal and environmental safety
Do not store laboratory clothing in contact with street clothing	Takes care of spread of infection to family members and community
• Strictly limit the use of needles, syringes and other sharp objects. • Use puncture-resistant sharps container • Replace glass with plastic where possible • Separate areas of paper work and report writing from biohazardous materials work areas	Controls accidental exposure
Develop post-exposure prophylaxis protocol and well-trained staff	Helps in performing first aid treatment to the exposed person

Note: Partially adapted from Pauwels et al., 2007.

8.17 Novel approaches in biological risk management of cell culture laboratories

Innovations and developments in the engineering sciences have led to many novel technologies to replace human beings with automated equipment. Many of these novel approaches provide safer working environment by reducing risk of exposure. Few examples are given below:

- Use of automated mechanical platforms for cell culture procedures.
- Use of liquid handling robot contained in BSC for long-term cell culture requirements.
- Development and use of non-infectious cell-based assay.

Thus, biorisk in the laboratory can be handled with overlapping safeguard measures.

09

CARE AND MAINTENANCE OF CELL CULTURE LABORATORY EQUIPMENT

Pipetting devices used in a cell culture laboratory

09

CARE AND MAINTENANCE OF CELL CULTURE LABORATORY EQUIPMENT

A large number of equipment is mandatory in any cell culture laboratory (Table 9.1). Proper performance of equipment is a prerequisite for obtaining quality results for any laboratory procedure. Only quality equipment should be purchased and installed and all these must also be maintained regularly and validated frequently to ensure their optimal functioning

TABLE 9.1: Suggested essential equipment for a cell culture laboratory

Equipment	Suggested number	Justification
Biosafety cabinets (BSC)	2	One for handling clean culture and the other for processing clinical specimens/ stock viruses
CO_2 incubators	2	One for uninfected cell cultures and the other for infected cell cultures
Incubator	1	To grow cells in controlled environment
Freezers	2	–20 °C and –70 °C for storage
Refrigerated centrifuge	1	To separate desired products without damaging their integrity
Autoclave	2	One for decontamination and the other for sterilization
Hot air oven	1	To sterilize glassware
Inverted light microscope	1	To observe cells growing in flasks, To detect CPE in infected cells
Fluorescent microscope with photography attachments	1	To detect antigens
Water purification/distillation system	1	To provide high-grade water suitable for tissue culture work
Micropipettes (100μL, 200 μL, 20 μL, etc.)	Multiple	
Multichannel pipettes: 8 and 12 channel pipettes (20–200 μL and 50–300 μL)	Multiple	
Liquid nitrogen containers	1–2	Prolonged storage
Glassware such as volumetric flasks, measuring cylinders, pipettes, conical flasks, reagent storage bottles (50 mL, 100 mL, 250 mL, 500 mL and 1000 mL)	Assorted	Medium and reagent preparation and storage

TABLE 9.2: Disinfectants for use in a cell culture laboratory

Characteristic	Description
Criteria for disinfectant	Broadly effective against a range of microorganisms Harmless (non-corrosive) to equipment components Harmless to laboratory workers
Avoid	Disinfectants that give rise to volatile organic chemicals since these can promote expression of heat shock and stress proteins(these include phenols, isoamyl alcohol, betamercaptoethanol). Avoid bleach-containing cleaners. Chlorine bleach and its derivatives with oxidizing activity corrode stainless steel and copper. Any strong smelling disinfectant.
Recommended disinfectant	Use quaternary ammonium disinfectant (2%) Follow this by wiping with 70% alcohol to remove any remaining traces of the disinfectant.

9.1 Prerequisites of equipment

The laboratory equipment should be designed to prevent or limit contact between the laboratory technologist and the infectious material. These should be crafted from materials that are impermeable to liquids and resistant to corrosion. The equipment should be free of sharp edges, burrs and unguarded moving parts. In addition, all equipment must be designed, constructed and installed to facilitate simple and smooth operation and provide for ease of maintenance, cleaning, decontamination using commonly available disinfectants (Table 9.2) and certification testing.

9.2 Positioning of equipment

The physical environment of a cell culture laboratory is critical for efficient performance and optimal life of the equipment. Walls of the cell culture laboratory should be smooth skimmed and coated with epoxy paint. The floor should be resistant and bonded to the wall so that there are no crevices or corners that cannot be cleaned, and any spillages can be easily isolated and cleaned. Sinks, coat hooks, etc. should all be kept in an anteroom and not in the culture room.

For equipment to function adequately, the laboratory area should be protected from air currents from windows or air-conditioning systems. The BSC must be located far from the laboratory air circulation zones in order to avoid air currents that could affect the curtain of air inside the cabinet. The floor on which the BSC is located must be flat and leveled. The free space around the cabinet recommended by the manufacturer must be ensured. Some of these prerequisites are shown in Table 9.3.

9.3 Care and maintenance of critical equipment

9.3.1 Biosafety cabinet

A BSC is probably the most important piece of equipment for cell culture since, when operated correctly, it provides a clean working environment for the product, while protecting the operator from aerosols. Cell culture requires a class II

TABLE 9.3: Prerequisites for installation of equipment in a cell culture laboratory

Action	Impact/action
Position	Ventilation, air flow, room temperature and direct sunlight can affect the temperature and humidity functions of equipment. Ensure protection.
Move	Lift equipment only by the sides of the bottom, never from front and back; do not ever lift equipment using the door.
Placement on the floor	Do not place any incubator directly on the floor. Instead, use a support stand to avoid impact of air movement that is created each time the door is opened and will sweep dirt, dust and contaminants directly into the incubator chamber.
Clearance around equipment	Adequate clearance to allow for ventilation and access to power cords and connectors including gas hookup
Avoid placement in damp and humid corners	This is to avoid fungal growth.
Eliminate cardboard storage in and around equipment	The cardboard can get wet and then breed fungi.
Post installation and prior to use	Clean and disinfect the interior of equipment as per recommendations in the manufacturers' manual.

cabinet that recirculates HEPA-filtered air and exhausts a portion of that air back into the room through a HEPA filter.

The maintenance of internal components must be done only by trained and qualified personnel. Personal protection must be worn to perform the routines. The BSC must be decontaminated before any maintenance work involving opening its surfaces or internal components is contemplated. Some of these tasks include changing of filters, conducting tests requiring access to the interior surfaces or exposure of the cabinet, prior to moving the cabinet to a different location and after a spill of a material containing high-risk agents.

Every week, decontaminate the work surface and the interior surfaces of the cabinet with 70% ethanol. Clean the front glass door and the surface of the ultraviolet lamp using a domestic cleaning solution. Verify the precision of the manometer's reading, indicating any fall in pressure through the HEPA filter.

Every month, clean the exterior surfaces, especially the front and the upper part using a piece of damp cloth to remove the dust, disinfect the surface of the lower compartment with 70% ethanol or a suitable disinfecting solution, and verify the state of the service valves.

The certification process should be initiated annually according to established norms. Annually, also check the intensity of the ultraviolet lamp with a radiometer and fluorescent lamp. Replace these, if necessary.

9.3.2 Incubator

Most mammalian cells require 37 °C for their growth. Insect cell cultures grow at 28 °C. There may also be a need for a regulated use of CO_2 and possibly other gas mixtures in the cabinet.

The centrifuge should preferably be located near the BSC and at an appropriate height. Vibrations caused by centrifuges should not damage other equipment, cause unnecessary noise, or allow the centrifuge to creep and potentially fall.

The incubator should ideally be located close to the laminar cabinet to reduce temperature changes, which could affect cultured cells. Cell culture incubators require the ambient temperature to be usually 5–10 °C colder than the target temperature. If the ambient temperature is too close to the target temperature, standard units can overheat, making temperature control in the room critically important for reliable operation.

Do not use an incubator in close proximity of flammable or combustible materials as components inside of this equipment could act as ignition sources during operation. Avoid spilling acid solutions inside the incubator. These cause the incubation chamber material to deteriorate. Avoid incubating substances generating corrosive vapours. Avoid placing receptacles on the lower cover which protects the heating elements. Use appropriate personal protective elements when using the incubator.

Incubators should be cleaned regularly, at least every 14 days and after any infectious material spill, using appropriate disinfectants. Disconnect the incubator before initiating the cleaning processes. Use non-abrasive cleaning agents: a piece of cloth dampened with mild detergent for cleaning easily reached interior and exterior surfaces. Avoid any contact between cleaning agents and electric elements. Wait until the incubator is dry before connecting it again.

Before performing any repairs, verify that the incubator has been decontaminated, is clean and *disconnected* from the electrical feed line.

9.3.3 Centrifuge

The centrifuge should preferably be located near the BSC and at an appropriate height. Vibrations caused by centrifuges should not damage other equipment, cause unnecessary noise, or allow the centrifuge to creep and potentially fall. All centrifuges used for cell culture and biological procedures must have some form of seal either above the rotor head or over the buckets to prevent aerosol leaking.

Do not carry out a technical intervention in a centrifuge if it has not been previously decontaminated.

Every month, verify that the external components of the centrifuge are free of dust and stains. Clean the rotor compartment using a mild detergent. Test that the connecting and adjustment mechanisms of the rotor are in a good condition. Lubricate as per manufacturer's recommendations. Check the locking/safety mechanism of the centrifuge's cover which is critical in ensuring safety of the operator. Recently manufactured centrifuges have sealed ball bearings, which do not require lubrication.

If the centrifuge is refrigerated, test the temperature by using an electronic thermometer. The temperature must not vary by more than ± 3 °C. Use a timer to examine the precision of the time controls. The time measured must not vary by more than ±10% of the programmed time.

Every six months, verify the state of the motor's brushes, if the centrifuge has a motor with brushes. Replace with new ones, if necessary.

9.3.4 Autoclave

An appropriately sized autoclave is important for uninterrupted cell culture work. Although appropriately sourced plastic consumables and pre-sterilized

reagents can eliminate the potential for contamination, there can still be huge cost savings by using specific pieces of glass equipment that can be repetitively autoclaved, used and recycled, particularly reagent and media bottles.

Two autoclaves are needed as clean and waste autoclaves to prevent cross-contamination. A backup autoclave obviates unanticipated downtime. The operational characteristics of the autoclave should also be regularly checked with spore strips to validate operations of the autoclave.

The autoclave demands daily supervision and continuous preventive maintenance due to its multiple components and systems. Before initiating the sterilization processes, ensure that the cold water, compressed air and vapour supply valves are open. Verify that the pressure from the vapour supply line is at least 2.5 bar. Test the condition of manometers and thermometers. Ensure that there are no vapour leaks in any of the systems functioning in the autoclave.

9.3.5 Dry oven

The maintenance required by a drying oven is simple. No complex routine maintenance is necessary. Before carrying out any maintenance routine on the oven, verify that it is at room temperature and disconnected from the electrical feed outlet.

9.3.6 Liquid nitrogen

Liquid nitrogen storage is often a convenient general storage environment for stocks of cultured cells. Liquid nitrogen boils at –196°C, and because the resultant nitrogen gas is an asphyxiant in high concentrations, proper handling and ventilation procedures are necessary. As the storage vessels usually require topping up at least once a week (more regularly as the containers age), the route from the gas delivery/production area to the laboratory must be as short as possible and the surface and equipment appropriate for the transport involved.

Storage of cells by multiple users for prolonged periods necessitates careful inventory management to ensure that cells are maintained optimally and economically.

9.3.7 Refrigerator

Refrigerators are generally not very demanding from the maintenance perspective. If connected to good quality electrical circuits and good ventilation flows around the unit, they can function for years without specialized technical service. The refrigeration circuit is sealed during manufacturing and does not have components requiring routine maintenance.

Cleaning the interior of refrigerator is important. Verify that the refrigerator's inner shelves are clean. These are generally made of rust-proof metallic mesh. Move the empty shelves towards the front. Dampen a piece of cloth with a mild detergent and apply by rubbing surfaces gently. Dry and place in their original position.

If the refrigerator has drawers, cleaning is done the same way. Empty the drawers and dismount from the adjustment devices. Remove them from the refrigerator. Once the shelves and drawers are dismounted, clean the interior walls of the refrigerator using a mild detergent. Dry before mounting the internal accessories. Apply a mild detergent with a damp piece of cloth to the drawers. Rub carefully. Dry the drawers and put them back on their mounts in the refrigerator.

Avoid using steel wool or other abrasive materials for cleaning the shelves and

drawers. Avoid using gasoline, naphtha or thinners, as these damage the plastic, the packing or the paint on the surfaces.

A door gasket in bad condition produces various problems in the functioning of cooling units. It allows humidity to enter, which condenses and freezes inside the evaporator, increases the time needed by the compressor for maintaining the selected temperature, affects the storage temperature and increases the operational costs.

Many modern refrigerators have automatic cycles for defrosting the evaporator in order to avoid frost accumulation. Some models do not have defrosting cycles and the process should be done manually every six months when the frost is more than 8 mm. Remove the contents of the compartments, disconnect the freezer, leave the door open, remove the water while it is accumulating in the compartments by using a sponge or a piece of absorbent cloth.

Never use sharp elements to remove ice or frost from the evaporator. Such an action can perforate the wall of the evaporator and allow the refrigerant gas to escape causing a serious defect.

9.3.8 Microscope

Microscopes are very vulnerable equipment and require simple but regular care and maintenance on a daily basis.Cover the microscope with a protective cover (of plastic or cloth). Ensure that it is kept in a well-ventilated place where the humidity and temperature are controlled. If it has a ventilated storage box equipped with a light bulb for humidity control, place the microscope inside, turn on the light and close the box.

Clean the immersion oil from the 100x objective. Use lens paper or, if not available, use medicinal type cotton. Clean the sample holders and the condenser. Place the light intensity control rheostat in the lowest position and then turn on the lighting system completely.

Remove dust particles from the microscope's body. Use a piece of cloth dampened with distilled water. Remove dust particles from the eyepieces, objectives and condenser. Use a rubber bulb for blowing air. Next, clean the lenses' surface with a lens cleaning solution. Do not apply this solution to lenses directly, but on lens paper and then rub their surfaces gently with the wet paper.

9.3.9 Pipettes

Pipettes are used very frequently and hence require frequent inspection in order to detect abnormal wear and tear or damage and/or to verify that they are in good working condition. Every day, inspect and verify that the pipette is clean. If dirt is detected, it must be cleaned using a suitable solvent or a mild detergent solution. Check the manufacturer's recommendation regarding the compatibility of the pipette with solvents to select the appropriate one.

Sterilize the pipette according to the manufacturer's indications. Some pipettes can be sterilized in an autoclave using a cycle of 121 °C for approximately 20 minutes. Some manufacturers recommend sterilizing the pipette using a 60% isopropanol solution and washing the components with distilled water, then drying and assembling.

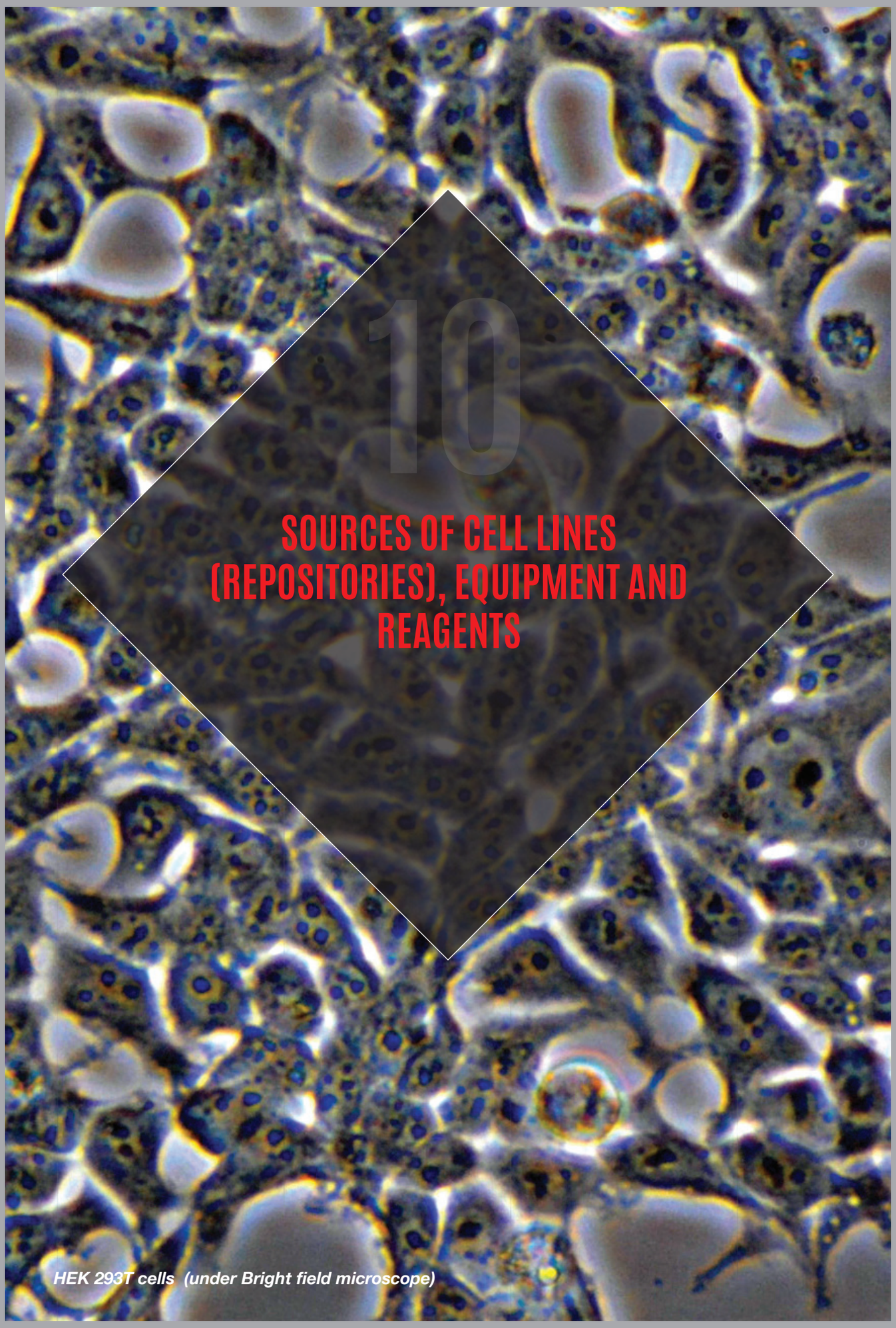
If a pipette has been used with harmful substances, it should be completely decontaminated before it is used in other procedures. A pipette used daily must be disassembled at biannual intervals. To do so, follow the procedure described by the manufacturer in the user manual.

Clean the O rings, the plunger and the inside of the cylinder before lubricating. If the internal components were contaminated accidentally, all the surfaces should be cleaned with a mild detergent and then with distilled water. Lubricate the plunger and piston with silicone grease. Always use the lubricant recommended by the manufacturer. Remove any excessive lubricant with absorbent paper. Assemble following the reverse process to that of disassembly.

Calibrate the pipette before using standardized procedures. The calibration method depends mainly on the volume the pipette handles. The smaller the volume range of the pipette, the more demanding and costly the calibration process is.

Summary

Given the critical nature of equipment in a cell culture laboratory and their high cost, it is imperative that apart from their careful selection, these equipment must be continuously and meticulously maintained through exacting care. This will prolong their life and optimize their functions.



10

SOURCES OF CELL LINES (REPOSITORIES), EQUIPMENT AND REAGENTS

HEK 293T cells (under Bright field microscope)

10

SOURCES OF CELL LINES (REPOSITORIES), EQUIPMENT AND REAGENTS

The cell culture system has become an integral part of virus research, as virus needs a host system for sustenance and replication. The availability of continuous cell lines derived from vertebrates and invertebrates have played an important role in virology and virtually replaced the use of animals. Cell lines are an important tool in virus research not only as a sensitive system for virus isolation and serological studies during outbreaks but also for large-scale propagation of viruses leading to development of vaccines and diagnostic reagents. The emergence of the recombinant DNA technology has emphasized the importance of cell lines as it has emerged as a clean system for production of immunobiologicals, enzymes, hormones, anticancer products, insulin, interferon and monoclonal antibodies. However, the major thrust area of cell culture application today has been vaccine and monoclonal antibody production due to their unique characteristics such as safety, ease in production, simpler downstream processing and cleaner end products. Cell culture-based vaccines are being produced effectively either by using conventional methods such as inactivation with chemicals or by using advanced techniques such as recombinant DNA technology, vector-based technology and virus attenuation. The cell culture system provides an additional advantage as the physiochemical conditions can be monitored and controlled according to the need, which is not provided by any other system.

10.1 Sources of cell lines

With the advancements made in cell culture techniques and the availability of a number of well-defined cell culture media, a number of continuous cell lines have been established from vertebrates and invertebrates ever since the establishment of the first vertebrate cell line in 1952.[1] Though most of the cell lines are of academic importance, a few have become useful tools for research in virology and biotechnology. Acquisition of a well-characterized cell line for research is the primary responsibility of the researcher to provide authenticated data using the cell line. The source of acquisition is therefore important to have a cell line, which is authenticated by a reliable source or agency. Authentication assures the origin of the cell line, growth parameters, media requirements and, more importantly, certifications regarding data on the tests conducted to confirm the absence of genetic drift, microbial contaminants especially mycoplasma and other adventitious agents. These contaminants are inherent to some organisms and are difficult to detect in cell cultures even after several passages.

Acquiring a cell line is easier today as several reputed cell banks or cell repositories across the globe provide most of the important cell lines on a commercial basis. Cell lines procured from such agencies are authenticated and are well-characterized and are certified free from contamination of mycoplasma and latent viruses. Cell lines also undergo tests for tumorigenicity.

American type culture collection ATCC is the premier institution in the USA involved in the acquisition, storage, authentication, management and distribution of biological materials, viz. reference microorganisms, cell lines etc.

Cell lines could also be acquired from other laboratories where the cell lines are in use either at a personal or an official level. However, despite the assurances from the donor, there is a potential hazard of mycoplasma contamination; these also need authentication for species origin. Presently, in-house development of a cell line is also possible due to the availability of technical knowhow, availability of a large plethora of media formulations and trained human resource. However, in-house development of a cell line is time-consuming, as it requires ample time for establishment and characterization of the cell line in addition to the time required to get ethical clearance/patient consent.

10.2 Major global suppliers of cell lines

10.2.1 American Type Culture Collection (ATCC)

American type culture collection ATCC is the premier institution in the USA involved in the acquisition, storage, authentication, management and distribution of biological materials, viz. reference microorganisms, cell lines etc. The mission of ATCC is to produce high-quality products to promote scientific research for the betterment of society. Apart from microorganisms and cell lines, the product range of ATCC includes certified fetal bovine serum (FBS), cell culture media and reagents, quality control strains, nucleic acids, proteins, cell extracts, etc. ATCC also provides assistance to scientists and organizations to get customized solutions for the management of biological materials. Biorepository management also includes authentication and characterization of biological materials, data management, off-site biorepository management, regulatory and legal compliance, intellectual property rights management, etc. (Further information and catalogues are available on the ATCC website www.atcc.org.) The products of ATCC can also be obtained through life science companies.

10.2.2 European Collection of Authenticated Cell Cultures (ECACC):

ECACC is based in London, UK and is recognized by the international scientific community for procurement, maintenance and supply of cell lines and associated products. ECACC has made formal agreements with other cell repositories, viz. ATCC, Cell Bank Australia, etc. to supply cell lines and other products listed in their catalogues. Since its establishment in 1985, ECACC has expanded enormously and now holds over 40,000 authenticated cell lines and strains. In addition to these two major cell banks/repositories, many other cell banks exist in other countries providing fully characterized cell lines. The major ones with large collection of cell lines are Cell Bank Australia; Deutsche Sammlung von Mickroorganismen und Zellkulturen, Germany; Riken Bio Resource Center Cell Bank, Japan; Coriell Biorepository, USA; Korean Cell Line Bank, South Korea; Bio Resource Collection and Research Center, Taiwan; Institute ZooprofilatticoSperimentale of Lombardy and Emilia-Romagna Bio-bank of Veterinary Resources, Italy. In India, the National Centre for Cell Science (NCCS), Pune maintains a cell repository of well-characterized cell lines procured from authorized cell repositories and fulfils the requirements of Indian scientists.

10.3 Equipment

Major equipment indispensable to a cell culture laboratory are listed below along with sources of procurement.

10.3.1 Biosafety cabinet

A biosafety cabinet (BSC) has become indispensable in today's cell culture laboratory to provide a clean area to handle sterile materials during management of animal cells and tissues. It is an enclosed workstation and acts as a primary barrier confining infectious agents (virus) during manipulation of the agent or infected clinical samples, thus giving protection to laboratory personnel and the immediate laboratory environment. It also helps in the containment of aerosol generated during different procedures, viz. centrifugation, grinding/blending, vortexing, sonication, vigorous shaking or handling of high concentrations of infectious agents. BSCs are available in different classes, viz. class I, class II (further divided into four categories, i.e. A1, A2, B1 and B2) and class III, which give varying levels of protection to the product and to the user from infectious agents (Table 10.1). Class II A2 is ideal for cell culture work as well as for handling moderately infectious agents as they protect the product, the personnel and the environment. It has HEPA filters at the air supply and exhaust points.

Source: several internationally reputed brands with hundreds of installations in world's leading laboratories are available in the market at affordable prices. Lab Congo, Thermo, NuAire, etc. are world leaders in BSC manufacturing and have networks in almost every country for supply and maintenance. Recently ESCO, Haier, etc. have also come up in the market with different models at reasonable prices.

10.3.2 CO_2 incubator

CO_2 incubators are indispensable in a cell culture laboratory where vertebrate cells are maintained. They come in different sizes and provide environment conducive to the growth of cells by maintaining the required temperature, humidity and CO_2 levels. The ones routinely used in cell culture laboratories are mid-sized models (140–180 L) that meet most of the technical specifications though a wide range of incubators from "personal" bench-top incubator (<40 L) to large-capacity incubators (>700 L) is available. Large CO_2 incubators (for production) may use steam generators or atomizers to control relative humidity levels but most research laboratory CO_2 incubators use humidity pans to produce humidity between 70% and 75% through evaporation of water. Maintaining a constant temperature and humidity in the incubator is critical for proper growth of cells. To maintain the chamber temperature to the set point, the incubators are available with two heating options: water-jacketed and radiant-walled. Although both heating systems are accurate and reliable, they have their advantages and disadvantages.

TABLE 10.1: Types of BSCs with different specifications

BSC	Face velocity (feet/sec)	Recirculation airflow %	Exhaust airflow %	Exhaust system
Class I	75	0	100	Hard duct
Class II A1	75–100	70	30	Exhaust to room/out
Class II A2	100	30	70	Exhaust to room/out
Class II B1	100	30	70	Hard duct
Class II B2	100	0	100	Hard duct
Class III	NA	0	100	Hard duct

Source: CO_2 incubators have been manufactured and marketed by several leading manufacturers based in the USA and Europe with manufacturing units in most of the developing countries. Thermo Fischer Scientific, Binder, Eppendorf (New Brunswick), NuAire, Shel lab, etc., are the market leaders in CO_2 incubator manufacturing and are reputed for their performance.

10.3.3 Inverted microscope

An inverted microscope is also indispensable in a cell culture laboratory for observation of cells and cell organelles. The inverted microscope differs from normal microscopes as it has the objectives below the platform pointing upwards so as to get a clear view of the cells in the culture vessel. A number of leading manufacturers in Europe and Japan (Leica, Zeiss, Nikon, Olympus, etc.) supply high-quality inverted microscopes of various specifications with excellent after-sales service.

10.3.4 Centrifuges

Low-speed centrifuges are needed for settling down cells from a cell suspension while high-speed centrifuges with refrigeration are needed for virus studies to remove cell debris from virus suspension. Ultracentrifuges are needed for pelleting viruses. Centrifuges with fixed angle rotors or swinging heads are available, which suit the requirements of cell culture and virology laboratories. Eppendorf (Germany), Hettich (Germany), Sorvall (USA), Thermo Fischer (USA), Hitachi (Japan), etc. are the market leaders along with a number of new manufacturers.

10.3.5 Water bath

Water bath is needed to thaw cell culture media and reagents, which were stored at 4 °C as well as to thaw cells during revival of cryopreserved cells. Water baths of varying specifications are manufactured and supplied by a number of reputed firms (Eppendorf, Thermo Scientific, Duran, GE Whatman, etc. in addition to local manufacturers.

10.3.6 Refrigerators (+4 °C)

Refrigerators are essential in a cell culture laboratory to store cell culture media and reagents. A number of reputed brands are available locally (Samsung, LG, Godrej, Kelvinator, etc.), and can serve the requirements of a cell culture laboratory.

10.3.7 Freezer (-20°C)

Freezers that maintain –20 °C temperatures are needed to store FBS, antibiotics and other reagents. Care should be taken to procure freezers from a reputed brand to maintain the required temperature.

10.3.8 Freezer (–80°C)

Freezers that maintain –80 °C temperatures are needed in cell culture laboratories where microorganisms are handled and stored for longer periods. Procurement of a–80 °C freezer needs special attention, as only a few brands are able to maintain the required temperature consistently especially in countries where the power supply is erratic. Thermo Scientific (USA), New Brunswick (USA), Revco (USA) and ESCO (Singapore) have the largest number of installations in leading laboratories both in developed and developing countries with consistent performance over the years. All these brands provide excellent technical support after installation.

10.3.9 Liquid nitrogen refrigerator and transport container

A liquid nitrogen refrigerator is essential in a cell culture laboratory to cryopreserve cells. Cryopreservation is a process where cells or whole tissues are preserved by cooling to sub-zero temperatures (–130 °C to −196 °C) in liquid nitrogen where biological activity, including the biochemical reactions that would lead to cell death, is effectively

stopped. Cryopreservation helps to have a validated stock of cells as reserve, which is not affected by any genetic drift, transformation or contamination. Liquid nitrogen refrigerators of varying capacities ranging from 26 to 110 L are ideal for laboratories whereas bigger ones cater to industrial needs.

10.3.10 Cell counter

A cell counter is also essential for a cell culture laboratory to determine the number of cells in cell culture studies. An automated cell counter is designed for high performance and accuracy and is slowly replacing the haemocytometer. Products from Thermo Fischer, Merck and other reputed manufacturers are being used in leading laboratories.

10.3.11 Autoclave

An autoclave serves a dual purpose in the laboratory, i.e. for decontamination of discarded equipment and consumables as well as for sterilization of laboratory materials (glassware, plasticware, media, etc.). During autoclaving, air is removed from the chamber and with pressurized steam (1.1 bar) the temperature and pressure is increased to the required levels (121 °C, 15–20 lb or more). Since pressure used is >0.5 bar, the autoclave assembly is classified as a pressure system and must be designed to a strict engineering standard. Horizontal as well as vertical models with varying capacities with high quality consistent performance along with biosafety features and documentation facility are available.

10.3.12 Water purification unit

Cell culture work requires highly purified, de-ionized water for preparation of cell culture medium and reagents. Earlier, distilled water either in metal containers or glass containers (borosilicate) was used to cater to the needs of cell culture. However, these practices were discontinued due to various technical difficulties (heavy maintenance, contamination, etc.) and inconsistency in water quality. With the advancement in technology, ultrapure water which is free from particulate matter, contaminants, proteases and endocrine disruptors, is made available to researchers by a combination of techniques, viz. membrane filtration, reverse osmosis, ultraviolet light treatment, etc. Merck Millipore (MilliQ systems) is the world market leader in water purifying technology and different models are available to suit laboratory and industrial requirements.

In addition to the above equipment, other minor equipment, viz. vortex mixture, weighing balance, magnetic stirrer, etc. are also needed in a cell culture laboratory. These equipment could be procured from the local market.

10.4 Cell culture media and reagents

Though studies to develop cell cultures were initiated in 1878, the non-availability of a nutritionally optimum cell culture medium delayed the successful establishment of a cell culture or a cell line till 1952. Despite employing different combinations of basal media and undefined animal products (serum, plasma, embryo extracts, peptones, etc.), the specific requirements of cells to proliferate and grow to a continuous cell line could not be met. The first nutritionally optimum cell culture medium for cultivating vertebrate cells was designed by Harry Eagle in 1955 as a minimum essential medium (MEM) by modifying his own basal medium developed in the early 1940s with high concentration of amino acids. Availability of MEM has broadened cell culture research by the development of newer cell lines from different tissues from vertebrates as well as the application of cell lines for various products of human

use. Given below is a list of important cell culture media used for vertebrate cultures.

10.4.1 Minimum Essential Medium with Earle salts (MEM)

MEM with Earle salt has become the backbone of vertebrate cell culture research ever since its development and has been used for various cell culture-based applications, viz. development of vaccines, diagnostic reagent and immunobiologicals. MEM has supported the replication and growth of a wide variety of cell lines and strains and has been modified by several scientists to suit their requirements. MEM is now commercially available from leading manufacturers, viz. ATCC, Invitrogen, Sigma Chemical Company, etc. in more than 50 combinations with varying concentrations of bicarbonate, calcium, magnesium, amino acids, buffers, etc. MEM is available both in liquid as well as powder form. The shelf life of the liquid medium is short while that of the powder form is higher if stored at specified temperatures. All these manufacturers have subsidiaries in almost all the developed and developing countries with good supply network. In addition, local companies (HI Media, India) are also manufacturing and marketing MEM with qualities at par with the market leaders.

10.4.2 Dulbecco's Modified Eagle's medium (DMEM)

DMEM is a variant of MEM modified by Dulbecco for culturing mouse embryo cells. It has roughly twice the concentration of amino acids and four times the concentration of vitamins, ferric nitrate and sodium pyruvate. The original formulation contained 1000 mg/L of glucose, but in the more commonly used variations this amount was increased to 4500 mg/L. DMEM and all its variants are also manufactured and marketed by ATCC, Invitrogen, Sigma, etc.

10.4.3 Leibovitz's L-15 medium

The L-15 medium is formulated for cell culture without CO_2 incubation. The standard sodium bicarbonate/CO_2 buffering system is replaced by a combination of phosphate buffers, free-base amino acids, higher levels of sodium pyruvate and galactose. The medium supports a wide range of human and monkey cell lines and is available from all the leading media manufacturers either in liquid or powder form.

10.4.4 RPMI 1640 medium (Roswell Park Memorial Institute)

This is a general purpose medium and has been used with a number of vertebrate cells, especially haematopoietic cells. RPMI 1640 is employed for both attached as well as suspension cultures and is also used for culturing of fresh human lymphocytes, hybrid cells, etc.

10.4.5 Ham's nutrient mixtures

Ham's F-10, F-12 and other modifications, originally designed for the growth of CHO cells, have been found useful in culturing human diploid cells (HDC) and white blood cells, rat hepatocytes, prostrate epithelial cells, etc. Ham's nutrient mixtures are found useful for growing a variety of cells both in serum-containing and serum-free formulations.

10.4.6 Grace's medium

The Grace's insect cell culture medium is the most widely used for cultivation of insect cell lines, which have become an important tool for expression of heterologous proteins.

10.4.7 Animal serum

Animal serum is indispensable for the proper growth of animal cells and, based on requirement, is provided at a concentration ranging from 5% to 20%. Though the exact composition of serum is unknown, it serves as a source for amino

acids, proteins, vitamins (particularly fat-soluble vitamins such as A, D, E and K), carbohydrates, lipids, hormones, growth factors, minerals and trace elements. Serum also buffers the culture medium, inactivates proteolytic enzymes, increases medium viscosity (which reduces shear stress during pipetting or stirring), and conditions the substratum for promoting cell attachment and proliferation. FBS is the most widely used animal serum for cell culture research due to its growth-promoting qualities. Procurement of FBS is very crucial as it might contain inherent contaminants such as mycoplasma and other latent viruses. It is therefore advisable to procure certified FBS only.

10.4.8 Antibiotics

To control or minimize the growth of bacteria, fungi and other microbial contaminants, the use of antibiotics is advised within the specified limits. However, in well-established cell culture laboratories, the use of antibiotics is minimized except when developing new cell cultures from host tissues or in isolation of viruses from clinical samples. A combination of penicillin and streptomycin is the most frequently used antibiotics in cell culture though other broad-spectrum antibiotics, viz. gentamicin etc. are also recommended. Antifungal agents are not used in routine cultures unless heavy fungal contamination occurs due to shifting of laboratories, equipment, etc. The most commonly used fungicides are Mycostatin and Nistatin. All the antibiotics are available commercially from all the reputed media manufacturers, viz. Invitrogen, Sigma, etc.

10.5 Other cell culture reagents

10.5.1 Phosphate-buffered saline (PBS): PBS is the most used biological saline in cell culture. It is generally used for washing cells during subcultivation as well as suspending the cells during other manipulations. PBS is available commercially; it can also be prepared in-house using the required chemicals.

To control or minimize the growth of bacteria, fungi and other microbial contaminants, the use of antibiotics is advised within the specified limits.

10.5.2 Trypsin: Trypsin is used in cell culture to dissociate attached cells from the substratum as well as from each other by catalysing the hydrolysis of peptide bond between carboxyl end of arginine/tyrosine and any other amino acid. Excessive exposure to trypsin is detrimental as it penetrates into the cells and affects the intrinsic properties of the cell. Trypsin activity can be stopped by the addition of serum (alpha-2 microglobulins). In serum-free formulations, trypsin activity is stopped by the addition of soybean trypsin inhibitor. Generally, trypsin is not used alone for cell dissociation. TPVG, a combination of trypsin, versene and glucose in phosphate-buffered saline is generally used for cell dissociation. TPVG is available commercially from all the reputed media manufacturers, viz. Invitrogen, Sigma, etc. Other enzymes used for cell dissociation are chymotrypsin, collagenase, dispase, pronase, etc.

10.5.3 Trypan blue: Trypan blue is a vital stain used to selectively stain dead tissues or cells. Since live cells are excluded from staining, this staining method is also described as a dye exclusion method. Trypan blue is available in powder and liquid form from media manufacturers.

10.5.4 Dimethylsulfoxide (DMSO): DMSO is a cryoprotective agent, which is used during cryopreservation of cells. DMSO serves several functions during the freezing process,viz. freezing point depression, greater dehydration and delay

in intracellular freezing that minimizes the solution effects. Greater recovery of cells is possible using DMSO as a cryoprotectant. DMSO is available commercially from all the reputed cell culture reagent manufacturers.

10.5.5 Carboxy methyl cellulose (CMC): CMC is used in various concentrations (1–4%) as an overlay for plaque assay during virus quantitation by limiting the spread of the virus. For better results, ultrapure CMC from a reputed manufacturer (Sigma, Invitrogen, etc.) is advised.

10.6 Plasticware for cell growth

Anchorage-dependent cells need a substratum for attachment and growth. The culture vessels not only provide an environment conducive for the cells to grow but also act as a barrier from contaminants. Polystyrene bottles and plates are treated with different collagen, laminin, gelatin poly-L-lysine, or fibronectin, etc.to make them hydrophilic. Routine maintenance of cultures is made in small culture vessels (25cm^2) while upscaling of cells is done in large flat-bottom vessels or in roller bottles. The other plasticwares used in cell culture work include culture dishes, multiwell plates, pipettes, etc. Since cells are highly sensitive to the substratum, high quality plasticware is a must for better growth of cells. Thermo Fischer, Nunc, Corning, Falcon, etc. have their network across the globe and they provide high-quality plasticware for all cell culture needs.

11-A

STANDARD OPERATING PROCEDURES FOR BASIC CELL CULTURE TECHNIQUES

Seeding of cells into a six well tissue culture plate

11-A

STANDARD OPERATING PROCEDURES FOR BASIC CELL CULTURE TECHNIQUES

Introduction

Cell lines will grow in tissue culture flask until the surface area is covered or the medium is depleted of nutrients. Contact inhibition is the cessation of cell motility that occurs when a cell culture growing in a flask reaches confluence. Once the growth reaches the maximum confluency, cells should be subcultured to prevent cell death. To subculture the cell line, cells should be detached from the substrate into a suspension. The degree of adhesion varies from cell line to cell line but in the majority of cases proteases, e.g. trypsin, are used to release the cells from the flask. However, this may not be appropriate for some lines where exposure to proteases is harmful or where the enzymes used remove membrane markers/receptors of interest. Such cells should be brought into suspension into a small volume of medium mechanically with the aid of cell scrapers.

A. Subculture of cell line

1.0 Purpose

This SOP describes the procedure for subculture of cell lines.

2.0 Equipment

- Cell culture hood (i.e. laminar-flow hood or BSC)
- Incubator (humid CO_2 incubator recommended)
- Water bath
- Centrifuge
- Refrigerator and freezer (–70°C)
- Cell counter
- Inverted microscope
- Liquid nitrogen freezer or cryostorage container
- Sterilizer (i.e. autoclave)

3.0 Aseptic techniques

Successful cell culture depends mainly on keeping the cells free from contamination by microorganisms such as bacterial, fungal, mycoplasma and viruses. Non-sterile supplies, media and reagents, airborne particles laden with microorganisms, unclean incubators and dirty work surfaces are all sources of biological contamination. Aseptic techniques to reduce the probability of contamination from these sources are a sterile work area, good personal hygiene, sterile reagents and media, and sterile handling.

All cell culture work is done in a BSC.

4.0 General

4.1. Bench and work areas should have as few items and pieces of equipment as possible.

4.2. Keep the access door for the culture room closed when the incubator door is open.

4.3. Pre-sterilize the hood by using ultraviolet light for 20 minutes.

4.4. Wipe down the hood's working surface with 70% ethanol before and after each use. It is encouraged to wipe down with 70% ethanol all items placed in the hood such as pipetting devices, bottles of pre-warmed media, aliquots, etc.

Successful cell culture depends mainly on keeping the cells free from contamination by microorganisms such as bacterial, fungal, mycoplasma and viruses.

5.0 Media preparation for cell culture

5.1 Dehydrated tissue culture media

a. Take 900mL of double distilled water.

b. Add the contents of one unit vial of dehydrated media to the water at room temperature with stirring until dissolved.

c. Rinse the vial with a small amount of distilled water to remove traces of powder and add to the above solution.

d. Add 2.2 g of sodium bicarbonate .

e. Adjust the pH if required between 7.1 and 7.4 using 1N HCl or 1N NaOH. Note that pH tends to rise during filtration and hence adjust it 0.2–0.3 units below the final desired pH.

f. Make up the final volume to 1000mL with distilled water.

Antibiotics

The following antibiotics can be aseptically added to a litre of the media:

Gentamycin (50mg/mL solution) 1.0 mL

Penicillin and streptomycin 10 000 units

5.2 Sterilization of tissue culture media

Sterilize the media by filtering through a sterile membrane filter (sterilized by autoclaving at 15 lb for 15 minutes and 121°C) of 0.22 micron or less porosity using positive pressure to minimize loss of carbon dioxide.

Sterility check

Add 0.5–1.0 mL of filtered media to a tube containing sterile thioglycolate broth and incubate at 37 °C for 48 hours. If the broth is clear after 48 hours, the media is sterile.

5.3 TVG (trypsin versene glucose)

TVG consists of the following components in 1 x PBS:

1. Trypsin 0.1%
2. Versene 0.2%
3. Glucose 0.05%

Stock solutions for TVG

A). 10x PBS

NaCl	80.00 g
KCl	2.00 g
Na_2HPO_4	14.42 g
KH_2PO_4	2.00 g
Dist.H_2O	upto 1 L

To prepare 1X PBS, add 100ml of 10X PBS to 900ml of D/W

B) 2% Trypsin

Trypsin	2.00 g
D/W	upto 100mL

Stir the above solution on a magnetic stirrer for 4hours or O/N at 4 °C. Sterilize by filtering through a sterile membrane filter of 0.22μ pore size. A sterility check can be done before using the solution.

C) 0.2% Versene

EDTA	200	mg
D/W	100	mL

Sterilize by autoclaving at 15 lb and 121 °C for 15 minutes.

D) 10% Glucose

Glucose	10.00	g
D/W	upto 100	mL

Sterilize by autoclaving at 15 lb and 121°C for 15 minutes.

E) 1% Phenol red

Phenol red	1.00	g
D/W	upto 100	mL

Preparation of working solution of TVG

Prepare 840mL of 1 x PBS and to this add 1.0 mL of 1% phenol red (indicator). Sterilize by autoclaving at 15 lb and 121 °C for 15 minutes. Cool this sterile solution and then add the following sterilized solutions to it:

Trypsin 2.0%	50 mL
Versene 0.2%	100 mL
Glucose 10.0%	5 mL

Do a sterility test as mentioned above before using this TVG.

Alternatively, these media can be procured in a ready-to-use liquid form

6 Subculturing

Most cells are anchorage-dependent and must be cultured while attached to a solid or semi-solid substrate (adherent or monolayer culture), while others can be grown floating in the culture medium (suspension culture). Split ratio and incubation time will vary for different cell lines. Further, the split ratio of the same cell type may vary sometimes from laboratory to laboratory.

6.1 Subculture of adherent cells

6.1.1 Discard the spent culture media from a confluent monolayer of the cell culture flask (e.g T25 flask).

6.1.2 Add 1 mL TVG (pre-warmed at 37 °C) to each T-25 flask.

6.1.3 Incubate the flask at 37 °C approximately for 2 minutes. Note that the actual incubation time varies with the cell lines used.

6.1.4 Observe the cells under a microscope for detachment.

6.1.5 Discard the trypsin.

6.1.6 Add 5mL of the complete growth medium to the flask and gently detach the cells from the surface with the help of a pipette.

6.1.7 Dilute cell suspension to the seeding density recommended for the cell line; pipette the appropriate volume into the new cell culture flasks.

6.1.8 Incubate at 37°C for 1–2 days.

6.2 Subculture of suspension cells

6.2.1 Swirl the flask to evenly distribute the cells in the medium.

6.2.2 From the sample, determine the total number of cells and percent viability using a haemocytometer, cell counter, and trypanblue exclusion.

6.2.3 Gently centrifuge the cell suspension at 100g for 5–10 minutes, and resuspend the cell pellet in a fresh growth medium.

6.2.4 Calculate the volume of media required to dilute the culture down to the recommended seeding density.

6.2.5 Aseptically add the appropriate volume of pre-warmed growth medium into the culture flask. Split the culture to multiple flasks, if needed.

6.2.6 Incubate at 37°C for 1–2 days.

7. Quality control

- Cells should be observed routinely for indications of instability or deterioration.
- Cultures should be examined both macro- and microscopically for presence of contamination.
- Cell cultures may be contaminated with bacteria, moulds, yeasts, mycoplasma, viruses and cells.
- Contamination may result from environment, inadequately sterilized culture media or solutions or poor aseptic techniques.

8. Measures to minimize contamination in a cell culture laboratory

- Media and other solutions should be tested for sterility before use.
- Serum purchased should be certified free from bacteria and mycoplasma.
- Cultures should be tested at least twice a year for mycoplasma and other contaminants.
- Contaminated cultures should be immediately discarded.
- Seed cultures should be purchased from supply houses which certify that their stocks do not contain cellular or microbial contaminants.
- Cultures from non-certified sources should be strictly isolated until sterility and verification tests are completed.
- Stock cultures should be handled infrequently and only by persons trained in tissue culture techniques.
- Mouth pipetting should never be used in a cell culture laboratory.
- The use of laminar flow cabinets with ultraviolet lamps for culture manipulations will minimize contamination from air-borne sources.

B. Cryopreservation of cell lines

Scope

This protocol is intended for the cryopreservation of cell line in the laboratory.

Introduction

Cell lines are cryopreserved to ensure long-term storage at low passages, to reduce the risk of contamination, to reduce the risk of cross-contamination with other lines, to minimize genetic change in continuous cell lines, and to avoid ageing and transformation. Cell lines that are not being used regularly should be stored to save staff time and consumables. The basic principle of successful cryopreservation and revival is a slow freeze and quick thaw. Cells should be cooled at a rate of –1°C to –3 °C per minute. Cells can be preserved indefinitely in the vapour phase of liquid nitrogen. For successful cryopreservation, the cell line should be healthy with a viability of >90% and no signs of contamination, and cells should be harvested during their logarithmic growth phase (this can be achieved by using pre-confluent cultures, i.e. cultures that are below their maximum cell density). Cryoprotectants such as 10% dimethyl sulfoxide (DMSO) or 2% glycerol must be used to reduce cell damage by the formation of ice crystals. To avoid contamination, sterile techniques are of crucial importance while working with a cell culture laboratory.

1.0 Purpose

This SOP describes the procedure for cryopreservation of cell lines.

2.0 Material and equipment

- Young confluent monolayer grown on tissue culture flask eg. T-75
- 0.25% Trypsin-EDTA
- Appropriate media (cold media)
- Dimethyl sulfoxide (DMSO)
- Fetal bovine serum(FBS)
- Pipettes
- 2mL cryovials
- Cryopreservation container
- Parafilm
- Cell culture hood (i.e. laminar-flow hood or BSC)
- Incubator (humid CO_2 incubator recommended)
- Water bath
- Centrifuge
- Refrigerator and freezer (–70 °C)
- Cell counter
- Inverted microscope
- Liquid nitrogen freezer or cryostorage container
- Sterilizer (i.e. autoclave)

3.0 Procedure for cryopreservation of cells

3.1 Prepare a freezing solution containing 100μLdimethyl sulfoxide (DMSO), 100μL MEM and 800μL FCS inside a clean BSC.

3.2 For adherent cells, gently detach cells from the tissue culture vessel following the procedure used during the subculture. Resuspend the cells in the complete medium.

3.3 Centrifuge at 2000 rpm for 5 minutes at 4°C.

3.4 Discard the supernatant and resuspend the pellet using freezing solution.

3.5 Aliquot in a 1.8 mL cryovial (labelled with the name of cell line, passage no. and date of cryopreservation).

3.6 Gradually freeze the cryovial from 0 °C to –70 °C at approximately 1 °C per minute using a controlled rate cryo-freezer or a cryo-freezing container such as "Mr Frosty".

3.7 Transfer the vial to liquid nitrogen.

Cell lines that are not being used regularly should be stored to save staff time and consumables. The basic principle of successful cryopreservation and revival is a slow freeze and quick thaw.

4. Safety precautions

1. Follow the biomedical waste disposal guidelines for disposal of used materials.

2. Wear safety equipment including apron, cryogloves and face shield while handling liquid nitrogen.

3. Staff should work in pairs when handling liquid nitrogen.

C: Revival of cell line

1.0 Purpose

This SOP describes the procedure for revival of cell lines.

2.0 Equipment

- Cell culture hood (i.e. laminar-flow hood or BSC)
- Incubator (humid CO_2 incubator recommended)
- Water bath
- Centrifuge
- Refrigerator and freezer (–70°C)
- Cell counter
- Inverted microscope
- Liquid nitrogen freezer or cryostorage container
- Sterilizer (i.e. autoclave)

3.0 Procedure for revival of cell lines

3.1. Retrieve cryovial from liquid nitrogen and immediately transfer to the water bath for thawing.

3.2. Transfer the vial into a BSC. Before opening, wipe the outside of the vial with 70% ethanol.

3.3. Add 1 mL of complete medium to the cryovial and slowly transfer the contents to a centrifuge tube containing 10mL of complete medium.

3.4. Centrifuge the cell suspension at approximately 2000rpm for 5–10 minutes. (The actual centrifugation speed and duration vary depending on the cell type.)

3.5. Aseptically decant the supernatant without disturbing the cell pellet.

3.6. Gently resuspend the cells in 5 mL of complete growth medium, and transfer them into a T-25 flask.

3.7. Incubate cells for an additional 48 hours to ensure complete revival, and periodically observe under microscope for morphological characteristics.

D: Detection of Mycoplasma in cell culture

1.0 Introduction

Mycoplasma is a small, round or filamentous prokaryotic organism, frequent and occult contaminant of cell cultures. Because of the small size, it can pass through filters used to prevent bacterial and fungal contamination and potentially spread to all the cultures in a laboratory. They are a problem because they can induce changes to the cell cultures (altered growth rates, morphological changes, chromosomal aberrations and altered cell metabolism). It is essential that all new cell cultures entering a laboratory and all cell banks are tested for the presence of Mycoplasma. It is recommended that two techniques be used, selected from a PCR-based method, indirect staining and an agar and broth culture.

2.0 Purpose

This SOP describes the different methods to detect Mycoplasma in cell lines in the laboratory.

3.0 Methods

3.1.1 Different methods to detect Mycoplasma in the laboratory

Sl No	Method	Description	Commercial kits
1	**Microbiological culture**	Growth in liquid medium Formation of typical small colonies on agar	In house In house
2	**Biochemical assays**	• Detection of adenosine phosphorylase activity (6-MPDR assay) • Enzymatic conversion of ATP to ADP by luciferase • Chromatographic detection of conversion of radioactively labelled uridine to uracil by mycoplasmal uridine phosphorylase	• MycoTect™ Kit (Gibco, Invitrogen) • MycoAlert™ Mycoplasma Detection Kit (Lonza) • Phased out due to involvement of radioactivity
3	**Immunological assays**	• Immunofluorescence • Enzyme linked immunosorbent assay (ELISA)	• Accurate Chemical & Scientific , ICN Biomedicals • Mycoplasma Detection Kit (Roche) -but this assay does not detect *M. fermentans*, Mycoplasma EIA Detection Kit (Stratagene)
4	**Molecular biology assays**	• Liquid hybridization assay • Autoradiography (dot-blot) with mycoplasma specific probes • Polymerase chain reaction (PCR), Reverse transcription PCR • PCR-ELISA	• Mycoplasma Tissue Culture NI (MTC-NI) Rapid Detection System (GenProbe) • Phased out due to involvement of radiography • Universal Mycoplasma Detection Kit (ATCC), MycoSEQ™ Mycoplasma Real-Time PCR Detection Kit (Life Technologies) • Mycoplasma PCR ELISA (Sigma-Aldrich)
5	**Microscopic detection assays**	• Direct DNA fluorescent staining (DAPI, Hoechst 33258) • Indirect DNA fluorescent staining with indicator cell line • Fluorescence in situ hybridization • Electron microscopy	• EZdetectTM DAPI Stain Kit (HiMedia Labs) • In-house (Ref: Sigma-Aldrich for procedure) • Mycoplasma Stain Kit (Sigma-Aldrich) • In-house procedure but labour and time intensive

3.1.2 Mycoplasma detection methods, their sensitivity, and advantages and disadvantages

S. No.	Methods	Sensitivity	Advantages	Disadvantages
1	Direct DNA stain(Hoechst,33258)	Low	Rapid, cheap	Can be difficult to interpret
2	Indirect DNA stain (Hoechst,33258) with indicator cells (e.g. 3T3)	High	Easy to interpret because contamination amplified	Indirect, hence more time-consuming
3	Broth and agar culture	High	Sensitive	Slow, may need expert interpretation
4	PCR	High	Rapid	Requires optimization
5	Nested PCR	High	Rapid	More sensitive than direct PCR, but more likely to give false-positives
6	ELISA	Moderate	Rapid	Limited range of species detection
7	Autoradiography	Moderate	Rapid	Can be difficult to interpret if contamination is low level
8	Immunostaining	Moderate	Rapid	Can be difficult to interpret if contamination is low level

3.1.3 Procedure for DNAfluorochrome staining method to detect mycoplasma

3.1.3. A: Reagents

4´, 6-diamidino-2´-phenylindole-dihydrochloride [DAPI] and Hoechst 33258 stain

B: Method

i. The cells are grown in vessels containing sterile coverslips.

ii. After growth for several days to approximately half-confluency, the coverslips are washed and stained with the fluorochrome.

iii. As mycoplasmas contain DNA, small dots or flecks of fluorescence can be seen concentrated on the cell surface, and in the surrounding medium and on the culture dish.

iv. In contrast, cell nuclei will appear as large fluorescent oval areas.

11-B

STANDARD OPERATING PROCEDURES FOR VIRUS ISOLATION USING CELL CULTURE

Seeding of cells into a six well tissue culture plate

11-B

STANDARD OPERATING PROCEDURES FOR VIRUS ISOLATION USING CELL CULTURE

Propose

This document provides procedure and guidance for viral isolation from human respiratory secretion and swabs. The viral isolation algorithm was developed from viral culture standard methods.

Principle

The relative importance of viral isolation as a diagnostic method is rapidly diminishing; however,it still remains necessary because it is the only technique capable of providing a viable isolate that can be used for antiviral susceptibility testing.An additional advantage is that in contrast to most antigen and nucleic acid detection methods, viral culture allows detection of multiple viruses, not all of which may have been suspected when the culture was requested.

Growth of viruses in cell culture is usually detected by visualizing morphological changes in the cells, known as CPE.
Cell cultures are typically viewed microscopically to detect CPE every one to two days for the first week of incubation. When necessary, confirmation can be achieved by scraping the infected cells from the walls of the tube or vessel in which they are growing and preparing a fluorescent antibody stain using monoclonal antibodies specific for the virus, or a neutralization test using specific antisera.

Equipment

- Tissue culture flask (T-75 flask)
- Tissue culture flask (T-25 flask)
- Disposable pipette size 1mL, 5mL, 10mL
- Centrifuge tube size 15 mL, 50 mL
- Beaker size 250 mL, 500 mL
- Millipore filter size 0.2 micron
- 96 well plate
- Cryotube size 2 mL
- Haemocytometer
- Glass bottle size 200 mL, 500 mL
- Inverted microscope
- Autoclave
- Incubator
- Refrigerator
- Freezer –20 °C, –70 °C
- Biosafety cabinet
- Water bath
- Refrigerated centrifuge
- Hair blower
- Slide 12 well

Reagents

- Minimum essential media (MEM)
  (Gibco BRL Cat. No. 41500-018)
- Fetal bovine serum (FBS)
  (Gibco BRL Cat. No. 26140-087)
- Bovine serum albumin fraction V (BSA)
  (Sigma Cat. No. A-4503)
- Trypsin Type II-S (from porcine pancreas)
  (Sigma Cat. No. T-7409)
- Trypsin acetylated type V-S (from bovine pancreas)
  (Sigma Cat. No. T-6763)
- L-glutamine minimum 99% (TLC)
  (Sigma Cat. No. G-3126)
- MEM vitamin solution
  (Gibco BRL Cat. No. 11120-052)
- d-biotin (vitamin H)
  (Sigma Cat. No. B-4501)
- Folic acid (vitamin M)
  (Sigma Cat. No. F-7876)

After the long serial propagation of cell culture, the susceptibility of cell cultures to viral infection normally decreases and may present with contaminating agents such as Mycoplasma spp.

- Penicillin–streptomycin, stock solution (10 000 U/mL penicillin; 10 000 µg/mLstreptomycin ,Gibco BRL Cat. No. 15140-122)

Safety

Appropriate protective clothing is to be worn at all times. All work is to be undertaken in a biosafety cabinet. The handling of infectious viral agents is to be performed according to the standard laboratory safety practices (WHO Biosafety Manual); therefore, appropriate precautions must be taken at all times including:

- Working under a BSC.
- Always use proper personal protective equipment.
- Use sterile techniques at all times.
- Turn on the ultraviolet light in the BSC 10 minutes before working in the BSC and 10 minutes after working with the infectious material.
- Wipe any vessels and laboratory supplies with 70% ethanol before moving them inside the BSC.
- Any waste generated in the laboratory is packed in a biohazard bag and autoclaved at 121 °C +/- 1°C for 15 minutes. The bag should then be tightly sealed and kept separate from general waste.

Precaution

- Do not freeze and thaw virus seed many times as this may cause significant loss in infectivity
- Never process clinical specimens for virus isolation and laboratory-adapted virus strains at the same time.
- Never process virus culture from humans and from animal virus in the same laboratory.

Quality control

After the long serial propagation of cell culture, the susceptibility of cell cultures to viral infection normally decreases and may present with contaminating agents such as *Mycoplasma* spp. Therefore, the laboratory should follow the guidelines of cell providers for restriction on passage number. If necessary, when doing subculture from frozen cell stock, testing of cell sensitivity may be required using TCID 50 or quantitative PCR or other relevant methods. In case of unusual growth of cell line or colour of media, testing and treatment of *Mycoplasma* contamination in cell culture is also required.

I. Subculture of cell line

Procedure

1. Discard media fluid from a 80–100% confluent monolayer of cell line flask cultures (25 cm^2 or 75 cm^2 TC flask).

2. Wash cells with PBS 4 mL to 8 mL per flask.

3. Discard all PBS fluid into a sterile beaker.

4. Add 2–8 mL trypsin-EDTA solution (pre-warmed at 37 °C +/– 1°C) to each culture flask.

5. Leave the flasks in incubator at 37 °C +/– 1 °C for 5–10 minutes (depends on the cell type).

6. Pour off the trypsin-EDTA and leave 1–2 mL in the flask.

7. Tap flask to slough off the cell sheet.

8. Resuspend cells with 5–10 mL of the growth medium. Break up cell clumps by gently pipetting up and down.

9. Add the required amount of growth medium (depends on the split ratio

Type ofcell lines	Viruses susceptible	Split ratio	Incubation period (80–100% confluent)
MDCK	Influenza, parainfluenza,H5N1	1:5	3 days
HEp-2	RSV, adeno, HSV	1:5	3 days
Vero	HSV	1:5	3 days
LLC-MK2	Parainfluenza	1:5	3 days

Note: The split ratio and incubation time of the same cell type sometimes vary from laboratory to laboratory.

of each cell type) and distribute the diluted cell suspension to new flasks.

10. Label the TC flasks with passage number and date of passage.

11. Incubate at 37 °C +/-1 °C for 1–5 days. Time of incubation varies with differing cell types and degree of confluence.

II. Specimen handling

Procedure

Both respiratory secretion and swabs should not be kept at 4 °C for more than 48 hours. When isolation of the pathogens cannot be done within 48 hours, the samples need to be kept at –70 °C until isolation of the pathogens can be done. Keeping the samples at 4 °C for more than 48 hours will make isolation of the pathogens more difficult or impossible. Immediately after samples are taken, they need to be put on ice continuously until they are transferred to a 4 °C refrigerator. The samples kept at –70 °C need to be transported in liquid nitrogen or dry ice.

III. Specimen preparation and testing

Procedure

1. If the samples are swabs, centrifuge the nasopharyngeal/throat swabs at 8000 rpm for 20 minutes at 4 °C and keep the supernatants. About 1–1.5 mL of supernatant is obtained per swab. Use the supernatant as the inoculum material.

2. Observe 24–48-hour-old cell cultures for proper morphology and degree of confluence. Label TC flask with sample ID, date of inoculation and passage history.

3. Discard the old medium, wash the cells with PBS (–) once and pour off.

4. Add 0.2 mL of the inoculum material per 25 cm^2 TC flask (use duplicate flasks for each type of cell lines).

5. Keep the flasks in the incubator at 37 °C +/– 1 °C for 1 hour and shake the flask every 15 minutes.

6. Add 5 mL maintenance medium to each flask and keep the flask in the incubator at 37 °C +/– 1 °C.

Note: Some viruses such as human metapneumovirus normally grow very slowly and the laboratory needs to maintain virus-infected cell cultures over a few months. The long-term maintenance of cultures could be obtained by changing the media formula that reduce glucose/ nutrition/serum such as DMEM that has about 4 x the concentration of amino acids found in EMEM.

7. Observe each flask daily for CPE under the microscope. If CPE is observed, harvest the cells for IFA staining with specific monoclonal antibody.

8. For flasks with no CPE, aspirate the culture medium and use it as the inoculum material. Inoculate 0.2 mL of the culture medium in fresh cell line, following steps 1 to 7. If there is still no morphological change in the second round, the result is reconfirmed by IFA using pooled monoclonal antibodies; if it shows positive, the harvested cells will be further tested by IFA using specific monoclonal antibody, PCR or other relevant methods.

Result analysis

Examine and compare the cell monolayer in uninfected cell culture flasks and specimen-inoculated cell culture flasks. Degenerative changes in cell monolayer provide evidence of viral presence. The spectrum of change is broad, ranging from swelling, shrinking and rounding of cells to clustering, syncytium formation and, in some cases, complete destruction of the monolayer.

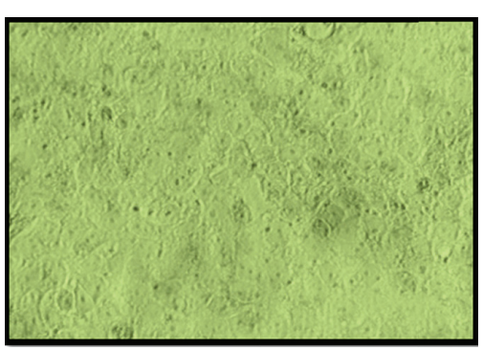


Figure 11.1: *Normal HEp-2 cells*

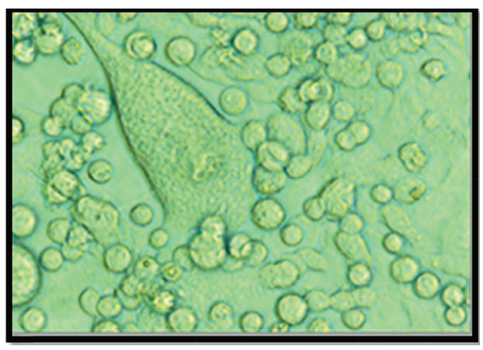


Figure 11.2: *Syncytium formation of RSV-infected HEp-2 cells*

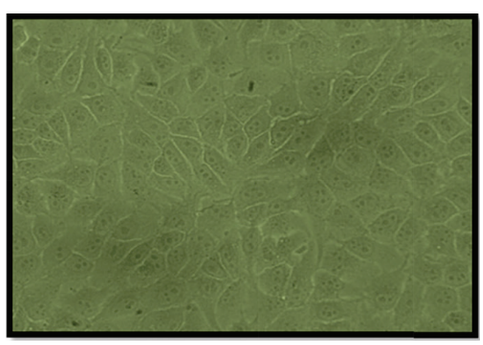


Figure 11.3: *Normal MDCK cells*

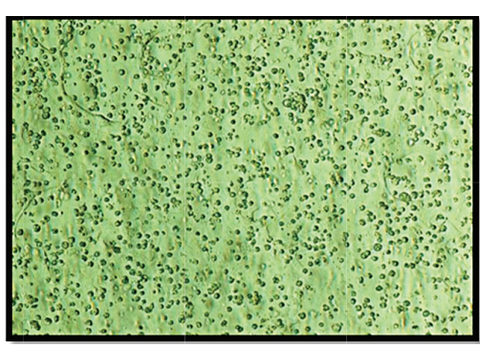


Figure 11.4: *CPE of influenza-infected MDCK*

Type of virus	Characteristic of CPE
Influenza type A	Vacuolation and cell degradation
Influenza type B	Vacuolation and cell degradation
RSV	Syncytium formation
Parainfluenza	**Moderate** cell degradation
Adeno	Grape-like clusters

Viral transport media (VTM)

1.0% BSA in MEM

10%BSA	100	mL
1xMEM	900	mL

Add penicillin, streptomycin and fungizone to the final concentration of 1000U/mL, 1000 μg/mL and 4 μg/mL. Filter with a 0.2 μm Millipore filter.

Reagents used for cell culture

1. PBS (–) without Ca++ and Mg++

   PBS(–) 10x

NaCl	160	g
KCl	4	g
Na_2HPO_4	23	g
KH_2PO_4	4	g
DW2	2000	mL

   Autoclaved at 121 °C ± 1 °C for 15 min (storable for 6 month)

2. PBS(–) (working solution)

PBS(–) 10x	200	mL
DW2	1800	mL

   Aliquot 500 mL per glass bottle and autoclaved at 121 °C ± 1 °C for 15 min, keep in 5 °C ± 3 °C refrigerator (storable for 6 months)

3. 1:5000 EDTA in PBS(–)

EDTA	0.4	g
PBS(–)	2000	mL

   Aliquot 500 mL per glass bottle and autoclaved at 121 °C ± 1 °C for 15 min, keep in 5 °C ± 3 °C refrigerator (storable for 6 months)

4. 1% trypsin solution

Trypsin (Sigma, T-7409)	10	g
Add DW2 to	1000	mL

   Filter with 0.2μm membrane filter and aliquot in 200 mL in sterile glass bottle, keep in –20 °C ± 5°C freezer (storable for 6 months)

5. 0.04% trypsin-EDTA solution

1% trypsin solution	20	mL
1:5000 EDTA in PBS(–)	500	mL

   Keep in 5 °C ± 3 °C refrigerator (storable for 6 months)

6. 10^5 unit/mL penicillin and 0.1 g/mLstreptomycin solution

Penicillin	5 000 000	unit
Streptomycin	5	g
Add sterile DW2 to	50	mL

   Aliquot 2 mL into each sterile tube, keep in –20 °C ± 5 °C freezer (storable for 6 months)

7. 1 mg/mL fungizone solution

fungizone	50	mg
Add sterile DW2 to	50	mL

   Aliquot 2 mL into each sterile tube, keep in 5 °C±3 °C refrigerator (storable for 6 months)

8. 0.1g/mL Kanamycin solution

Kanamycin	5	g
Add sterile DW2 to	50	mL

   Aliquot 2 mL into each sterile tube, keep in –20 °C ± 5 °C Freezer (storable for 6 months)

9. Trypsin-acetylated

Trypsin-acetylated	0.1	g
DW	100	mL

   Filter with a 0.2 μm Millipore filter. Store at –20 °C.

10. 0.04% trypsin-EDTA

EDTA	0.2	g
PBS(–)	1	L

    Autoclave at 121 °C for 15 minutes and let it cool to room temperature before adding 1% trypsin 40 mL using aseptic technique. Store at 4 °C

11. 3% L-glutamine

L-glutamine	3	g
DW	100	mL

    Filter with a 0.2 μm Millipore filter. Store at –20 °C.

12. 1x MEM

MEM	9.61	g
DW	1000	mL

Filter with a 0.2 μm Millipore filter. Store at 4 °C.

13. Stock folic acid

Folic acid	0.1	g
PBS(–)	100	mL

Filter with a 0.2 μm Millipore filter and place 1 mL in each tube. Store at –20 °C.

14. d-biotin stock solution

Biotin	0.1	g
PBS(–)	100	mL

Filter with a 0.2 μm Millipore filter and place 1 mL in each tube. Store at –20 °C.

15. 10% FBS in MEM (for growing cell culture)

MEM	90	mL
FBS	10	mL

Add penicillin and streptomycin to the final concentration of 100 U/mL and 100 μg/mL. Filter with a 0.2 μm Millipore filter. Store at 4 °C.

16. Maintenance medium

For MDCK cells

MEM	100	mL
17.5 % bovine serum	1.15	mL
Trypsin-acetylated	0.5	mL
MEM vitamin solution	0.1	mL
Folic acid stock solution	0.1	mL
Biotin stock solution	0.1	mL
3% L-glutamine	3.0	mL

Add penicillin and streptomycin to the final concentration of 100U/mL and 100 μg/mL. Filter with a 0.2 μm Millipore filter. Store at 4 °C.

17. For HEp-2 cells

MEM	98	mL
FBS	2	mL
3% L-glutamine	1.5	mL

Add penicillin and streptomycin to the final concentration of 100U/mL and 100 μg/mL. Filter with a 0.2 μm Millipore filter. Store at 4 °C.

Work sheet for respiratory viral isolation by cell culture

Name of laboratory .....

MDCK passage no. ..... Date of inoculation .....

HEp-2 passage no. ..... Date of inoculation .....

Analyst..... Date of final conclusion.....

No.	Sample ID / Lab. code	MDCK							HEp-2							Isolate code	Date collected
		1	2	3	4	5	6	7	1	2	3	4	5	6	7		
1																	
2																	
3																	
4																	
5																	
6																	
7																	
8																	
9																	
10																	

Results record:– = negative, ± = equivocal, = normal; + = 25% CPE; ++ = 50% CPE; +++ = 75% CPE; ++++ = 100% CPE, ND = not done ,Pn = perform next passage

12

REFERENCES AND FURTHER READING

12

REFERENCES AND FURTHER READING

1. Brown DW(1997). Threat to humans from virus infections of non-human primates. Rev Med Virol. 7(4):239–46.

2. Buehring GC, Eby EA, Eby MJ (2004). Cell line cross-contamination: how aware are mammalian cell culturists of the problem and how to monitor it?In Vitro Cell Dev Biol Anim. 40(7):211–15.

3. Butler DA, Scott MR, Bockman JM, Borchelt DR, Taraboulos A, Hsiao KK, Kingsbury DT, Prusiner SB (1988). Scrapie-infected murine neuroblastoma cells produce protease-resistant prion proteins. J Virol. 62(5):1558–64.

4 Cabrera CM, Cobo F, Nieto A, Corte´s JL, Montes RM, Catalina P, Concha A (2006).Identity tests: determination of cell line cross-contamination. Cytotechnology. 51(2):45–50. doi: 10.1007/s10616-006-9013-8.

5. Capes-Davis A, Theodosopoulos G, Atkin I, Drexler HG, Kohara A, Macleod RA, Masters JR, Nakamura Y, Reid YA (2010). Check your cultures! A list of cross-contaminated or misidentified cell lines. Int J Cancer.127(1):1–8. doi: 10.1002/ijc.25242. 127(1):1–8.

6. Cronier S, Laude H, Peyrin JM (2004). Prions can infect primary cultured neurons and astrocytes and promote neuronal cell death.ProcNatlAcad Sci USA. 101(33):12271–6.

7. Eagle H (1955). The specific amino acid requirements of a human carcinoma cell (Stain HeLa) in tissue culture. J Exp Med. 102(1):37–48.

8. Edmondson R, Broglie JJ, Adcock AF, Yang L.(2014). Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors.Assay Drug Dev Technol. 12(4):207–18. doi: 10.1089/adt.2014.573.

9. Freshney RI. Basic principles of cell culture.In: Freshney RI, Vunjak-Novakovic G, editors. Culture of cells for tissue engineering.Hoboken, NJ, USA: John Wiley and Sons, Inc; 2006. doi: 10.1002/0471741817.ch1

10. Freshney RI. Culture of animal cells: amanual of basic technique and specialized applications. Hoboken, NJ, USA: John Wiley and Sons; 2011.

11. Gey GO, Coffman WD, Kubicek MT (1952). Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res. 12:264–65.

12. Grace TD (1962). Establishment of four strains of cells from insect tissues grown in vitro. Nature. 195:788–9.

13. Grist NR. Diagnostic methods in clinical virology. 2nd edition. Oxford, England: Blackwell Scientific Publications; 1974.

14. Gugel EA, Sanders ME (1986). Correspondence. Needle-stick transmission of human colonic

adenocarcinoma. New Engl J Med. 315(23):1487

15. Hawkes RA. General principles underlying laboratory diagnosis of viral infections. In: Lenette EH, Schmidt N editors.Diagnostic procedures for viral and rickettsial infections.5thed.Amrican Public Health Association; 1979:3–139.

16. Herman P, Verlinden Y, Breyer D, Van Cleemput E, Brochier B, Sneyers M, Snacken R, Hermans P, Kerkhofs P, Liesnard C, Rombaut B, Van Ranst M, Van Der Groen G, Goubau P, Moens W (2004). Biosafety risk assessment of the severe acute respiratory syndrome (SARS) Coronavirus and containment measures for the diagnostic and research laboratories. Applied Biosafety.9(3):128–42.

17. Hodinka, RL, Kaiser L (2013). Is the era of viral culture over in the clinical microbiology laboratory?J ClinMicrobiol. 51(1):2–4. doi: 10.1128/JCM.02593-12.

18. Hudu, SA, Alshrari AS, Syahida A, Sekawi Z(2016). Cell culture technology: Enhancing the culture of diagnosing human diseases. J ClinDiagn Res. 10(3):DE01–5. doi: 10.7860/JCDR/2016/15837.7460.

19. Isaacs SN (2012). Working safely with vaccinia virus: laboratory technique and review of published cases of accidental laboratory infections.Methods Mol Biol. 890:1–22. doi: 10.1007/978-1-61779-876-4_1.

20. Langdon SP (2004). Cell culture contamination: an overview. Methods Mol Med. 88:309–17.

21. Leibovitz A (1963). The growth and maintenance of tissue-cell cultures in free gas exchange with the atmosphere. Am J Hyg. 78:173–80.

22. Leland DS,Ginoechio CC (2007). Role of cell culture for virus detection in the age of technology. ClinMicrobiolRev.20(1):49–78.

23. Lloyd G, Jones N (1986). Infection of laboratory workers with hantavirus acquired from immunocytomas propagated in laboratory rats. J Infect. 12(2):117–25.

24. Louz D, Bergmans HE, Loos BP, Hoeben RC (2005). Cross-species transfer of viruses: implications for the use of viral vectors in biomedical research, gene therapy and as live-virus vaccines. J Gene Med. 7(10):1263–74.

25. Matsuo Y, Nishizaki C, Drexler HG (1999). Efficient DNA fingerprinting method for the identification of cross-culture contamination of cell lines. Hum Cell. 12(3):149–54.

26. Moore GE, Gerner RE, Franklin HA(1967).Culture of normal human leukocytes. JAMA. 199(8):519–24.

27. Pauwels K, Herman P, Van Vaerenbergh B, Do Thi CD, Berghman L, Waeterloos G, Van Bockstaele D, Dorsch-Hasler K, Sneyers M (2007). Animal cell cultures: risk assessment and biosafety recommendations. Applied Biosafety. 12(1):26–38.

28. Van den Akker E, van der Vlugt CJB, Bleijs A, Bergmans HE (2013). Environmental risk assessment of replication competent viral vectors applied in clinical trials: potential effects of inserted sequences. Curr Gene Ther. 13(6):395–412.

Manuals

1. World Health Organization. Manual for the laboratory diagnosis and virological surveillance of influenza; 2011.

2. WHO Animal Influenza Training Manual, The National Training Course on Animal Influenza Diagnosis and Surveillance, Harbin China, 20–26May2001.

3. World Health Organization,Laboratory biosafety manual, 3rd edition, Geneva:WHO; 2004(http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/)

4. The Art of Animal Cell Culture for Virus Isolation, InTechOpen. Published on 17 October 2012 (https://www.intechopen.com/books/biomedical-tissue-culture/the-art-of-animal-cell-culture-for-virus-isolation)

5. Standard Operating Procedure of respiratory virus isolation by cell culture technique; SOP13-02-467, Thai National Influenza Center National Institute of Health, Department of Medical Sciences, Revised 9th 2016

6. Cryopreservation and Storage of Cells [website]. Sigma-Aldrich (http://www.sigmaaldrich.com/technical-documents/protocols/biology/cryopreservation-and.html, accessed 12 June 2017).

7. Manual of Respiratory Panel 1 Viral Screening and Identification kit; Millipore Corporation, USA Cat.No. 3105

Web links

1. European Collection of Cell Cultures (ECACC): http://www.ecacc.org.uk

2. American Type Culture Collection (ATCC): http://www.atcc.org

3. DSMZ – German Collection of Microorganisms and Cell Cultures:http://www.dsmz.de

4. Italian Cell Line Collection: http://www.iclc.it

5. http://www.sigmaaldrich.com/catalog/product/sigma/v7884?lang=en&region=GB

6. https://www.atcc.org/en/Products/Cells_and_Microorganisms/Cell_Lines.aspx

7. https://www.vanderbilt.edu/viibre/CellCultureBasicsEU.pdf

8. (https://www.atcc.org/~/media/PDFs/Culture%20Guides/AnimCellCulture_Guide.ashx)ATCC Cell Culture Guide

ANNEX

List of contributors

Dr Anita Desai, PhD, MAMS
Professor and Head
Department of Neurovirology
National Institute of Mental Health And
Neuro Sciences (NIMHANS)
Bengaluru 560029, India
Tel: +91 80 2699 5778
Fax: +91 80 2656 4830
Email: anitasdesai@gmail.com

Dr Arunkumar GovindaKarnavar, PhD
Head
Department of Virus Research
Manipal Centre for Virus Research (MCVR)
Manipal University
Manipal 576104, Karnataka, India
Tel: +91 820 2922718
Email: arun.kumar@manipal.edu

Dr Asha Abraham, MD
Professor
Department of Clinical Virology
Christian Medical College, Ida Scudder Road
Vellore 632 004
Tamil Nadu, India
Tel: +91-9994206483
Email:asha_ma@cmcvellore.ac.in

Dr D. T. Mourya, MSc, PhD, FNASc
Director and Scientist G
National Institute of Virology
20-A, Dr Ambedkar Road,
Pune 411001, India
Phone: +9120-26006201
Fax: +9120-26122669
Email: directorniv.gmail.com, dtmourya@gmail.com, mouryadt@hotmail.com

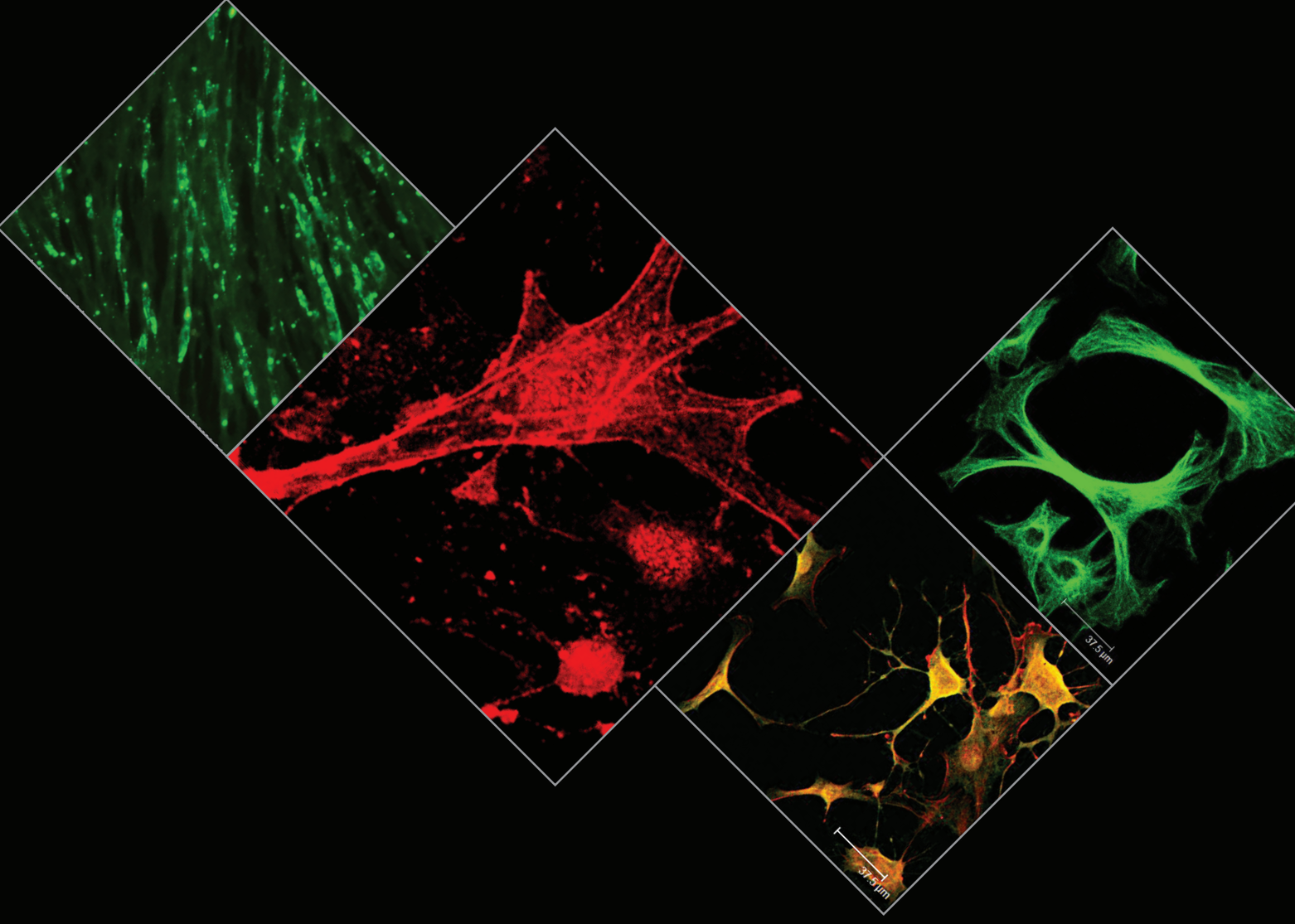
Dr Malinee Chittaganpitch, MSc
Chief of Respiratory Virus Section, Thai NIC
National Institute of Health
Department of Medical Sciences
88/7 SoiBamrasnaradura Hospital
Tivanon Road, Nonthaburi11000, Thailand
Tel: 662-9510000 ext. 98419
Fax: 662-5911498, 662-5915449
Email: malinee.c@dmsc.mail.go.th

Dr Rajesh Bhatia, MBBS, MD
Former Director
Communicable Diseases, WHO-SEARO
New Delhi 110002, India
Phone: +91 – 9810607349
Email: drrajesh.bhatia1953@gmail.com

Dr Vasanthapuram Ravi, MD,FAMS, FASc
Professor
Department of Neurovirology
National Institute of Mental Health
And Neuro Sciences (NIMHANS)
Bengaluru 560029, India
Tel: +91 80 2699 5126
Fax: +91 80 2656 4830
Email:virusravi@gmail.com

Dr Aparna Singh Shah, M.D.
Regional Adviser
Health Laboratory Services and
Blood Safety
WHO Regional Office for South-East Asia
WHO House, IndraPrastha Estate,
Ring Road, New Delhi-110 002
Tel: +91-11-23309632; 43040632
Fax: +91-11-23309195; 23705663
E mail: shahap@who.int

The photographs in this cell culture document have been contributed by Mr. Ayushman Ghosh and Ms. Amita Bhagat, Ph.D students in the Department of Neurovirology, NIMHANS, Bangalore.



World Health Organization
Regional Office for South-East Asia
World Health House
Indraprastha Estate,
Mahatma Gandhi Marg,
New Delhi-110002, India
Website: www.searo.who.int

ISBN: 978-92-9022-600-0