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# Animal tissue culture principles and applications

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## Summary

Animal cell culture technology in today's scenario has become indispensable in the field of life sciences, which provides a basis to study regulation, proliferation, and differentiation and to perform genetic manipulation. It requires specific technical skills to carry out successfully. This chapter describes the essential techniques of animal cell culture as well as its applications.

## What you can expect to know

This chapter describes the basics of animal cell culture along with the most recent applications. The primary aim is to progressively guide students through fundamental areas and to demonstrate an understanding of basic concepts of cell culture as well as how to perform cell cultures and handle cell lines. This chapter gives insights into types of cell culture, culture media and use of serum, viability assays, and the translational significance of cell culture.

## History and methods

### Introduction

Cell culture is the process by which human, animal, or insect cells are grown in a favorable artificial environment. The cells may be derived from multicellular eukaryotes, already established cell lines or established

cell strains. In the mid-1900s, animal cell culture became a common laboratory technique, but the concept of maintaining live cell lines separated from their original tissue source was discovered in the 19th century. Animal cell culture is now one of the major tools used in the life sciences in areas of research that have a potential for economic value and commercialization. The development of basic culture media has enabled scientists to work with a wide variety of cells under controlled conditions; this has played an important role in advancing our understanding of cell growth and differentiation, identification of growth factors, and understanding of mechanisms underlying the normal functions of various cell types. New technologies have also been applied to investigate high cell density bioreactor and culture conditions.

Many products of biotechnology (such as viral vaccines) are fundamentally dependent on mass culturing of animal cell lines. Although many simpler proteins are being produced using rDNA in bacterial cultures, more complex proteins that are glycosylated (carbohydrate-modified) currently have to be made in animal cells. At present, cell culture research is aimed at investigating the influence of culture conditions on viability, productivity, and the constancy of post-translational modifications such as glycosylation, which are important for the biological activity of recombinant proteins. Biologicals produced by recombinant DNA (rDNA) technology in animal cell cultures include anticancer agents, enzymes, immunobiologicals [interleukins, lymphokines, monoclonal antibodies (mABs)], and hormones.

Animal cell culture has found use in diverse areas, from basic to advanced research. It has provided a model system for various research efforts:

1. The study of basic cell biology, cell cycle mechanisms, specialized cell function, cell–cell and cell–matrix interactions.
2. Toxicity testing to study the effects of new drugs.
3. Gene therapy for replacing nonfunctional genes with functional gene-carrying cells.
4. The characterization of cancer cells, the role of various chemicals, viruses, and radiation in cancer cells.
5. Production of vaccines, mABs, and pharmaceutical drugs.
6. Production of viruses for use in vaccine production (e.g., chicken pox, polio, rabies, hepatitis B, and measles).

Today, mammalian cell culture is a prerequisite for manufacturing biological therapeutics such as hormones, antibodies, interferons, clotting factors, and vaccines.

### Development of animal cell culture

The first mammalian cell cultures date back to the early 20th century. The cultures were originally created to study the development of cell cultures and normal physiological events such as nerve development. Ross Harrison in 1907 showed the first nerve fiber growth in vitro. However, it was in the 1950s that animal cell culture was performed at an industrial scale. It was with major epidemics of polio in the 1940s and 1950s and the accompanying requirement for viral vaccines that the need for cell cultures on a large scale became apparent. The polio vaccine from a de-activated virus became one of the first commercial products developed from cultured animal cells (Table 14.1).

### Basic concept of cell culture

Tissue culture is in vitro maintenance and propagation of isolated cells tissues or organs in an appropriate artificial environment. Many animal cells can be induced to grow outside of their organ or tissue of origin under defined conditions when supplemented with a medium containing nutrients and growth factors. For in vitro growth of cells, the culture conditions may not mimic in vivo conditions with respect to temperature, pH, CO<sub>2</sub>, O<sub>2</sub>, osmolality, and nutrition. In addition, the cultured cells require sterile conditions along with a steady supply of nutrients for growth and sophisticated incubation conditions. An important

factor influencing the growth of cells in culture medium is the medium itself. At present, animal cells are cultured in natural media or artificial media depending on the needs of the experiment. The culture medium is the most important and essential step in animal tissue culture. This depends on the type of cells that need to be cultured for the purpose of cell growth differentiation or production of designed pharmaceutical products. In addition, serum-containing and serum-free media are now available that offer a varying degree of advantage to the cell culture. Sterile conditions are important in the development of cell lines.

Cells from a wide range of different tissues and organisms are now grown in the lab. Earlier, the major purpose of cell culture was to study the growth, the requirements for growth, the cell cycle, and the cell itself. At present, homogenous cultures obtained from primary cell cultures are useful tools to study the origin and biology of the cells. Organotypic and histotypic cultures that mimic the respective organs/tissues have been useful for the production of artificial tissues.

### How are cell cultures obtained?

There are three methods commonly used to initiate a culture from animals.

#### **Organ culture**

Whole organs from embryos or partial adult organs are used to initiate organ culture in vitro. These cells in the organ culture maintain their differentiated character, their functional activity, and also retain their in vivo architecture. They do not grow rapidly, and cell proliferation is limited to the periphery of the explant. As these cultures cannot be propagated for long periods, a fresh explanation is required for every experiment that leads to interexperimental variation in terms of reproducibility and homogeneity. Organ culture is useful for studying functional properties of cells (production of hormones) and for examining the effects of external agents (such as drugs and other micro or macro molecules) and products on other organs that are anatomically placed apart in vivo.

#### **Primary explant culture**

Fragments exercised from animal tissue may be maintained in a number of different ways. The tissue adheres to the surface aided by an extracellular matrix (ECM) constituent, such as collagen or a plasma clot, and it can even happen spontaneously. This gives rise to cells migrating from the periphery of the explant. This culture is known as a primary explant, and migrating cells are known as outgrowth. This has been used to analyze the growth characteristics of cancer

**TABLE 14.1** Milestones in cell cultures and microfluidics.

1878	Claude Bernard	Established that a physiological state of the cell similar to the live cell can be maintained even after the death of the organism.
1907	Harrison	Cell entrapment and frog embryo nerve fiber growth in vitro.
1912	Alexis Carrel	Initiated tissue culture of chick embryo heart cells using embryo extracts as cultural media passaged for a reported period of 34 years.
1913	Steinhardt, Israeli, and Lambert	Grew vaccinia virus in fragments of guinea pig corneal tissue.
1916	Rous and Jone	Used trypsin to suspend attached cells in culture.
1927	Carrel and Rivera	First viral vaccine against chicken pox.
1948	Sanford, Earle, and Likely	Single-cell culture using microscale glass capillaries
1949	Enders, Weller, and Robbins	Polio virus grown on human embryonic cells in culture.
1952	Gey	Establishment of continuous cell line from a human cervical carcinoma (HeLa cells).
1955	Eagle	Established nutrient requirements of cells in culture and defined culture media for growth.
1956	Little Field	HAT (hypoxanthin, aminopterin, thymidine) medium introduced for cell selection.
1961	Hayflick and Moorhead	Studied human fibroblasts (WI-38) and showed finite lifespan in culture.
1965	Ham	First serum-free HAMS's media.
1975	Kohler and Milstein	First hybridoma secreting a monoclonal antibody.
1977	Genetech	First recombinant human protein: somatostatin.
1979	Terry, Jerman, and Angell	Gas chromatography system using silicon-etched microchannels
1985	Collen	Recombinant tissue plasminogen activator (tPA) in mammalian cells. Human growth hormone produced from recombinant bacteria was accepted for therapeutic use.
1986	FDA approval	First monoclonal antibody was approved by the FDA for use in humans (Orthoclone OKT3).
1986	Genetech	First recombinant protein commercialized (interferon alpha-2a).
1989	Amgen	Erythropoietin (EPO) recombinant protein produced in CHO cells available commercially.
1992	Manz, Harrison, Verpoorte, Fettingner, Paulus, Ludi, and Widmer	Micro-machined glass chip electrophoresis for separating molecules
1992	FDA approval	First genetically engineered recombinant blood-clotting factor used in the treatment of hemophilia A.
1996	Wilmut	Production of transgenic sheep (Dolly) through nuclear transfer technique.
1997	Hadd, Raymond, Halliwell, Jacobson, and Ramsey	Microchip device for enzyme assay
1998	Duffy, McDonald, Schueller, and Whitesides	Creation of microfluidic systems using PDMS (polydimethylsiloxane)
2000	Panaro, Yuen, Sakazume, Fortina, Kricka, Wilding	Lab-on-chip system (bioanalyzer)
2002	Cloneaid	Claimed to produce cloned human baby named EVE.
2003	Zheng	Protein crystallization chip
2004	FDA approval	First antiangiogenic monoclonal antibody that inhibits the growth of blood vessels or angiogenesis (for cancer therapy).
2005	Sanford, Earle, and Likely	Single-cell culture in open microfluidic systems
2005	Birch	Reported antibody titers at an industrial scale of 5 g/L and more.
2009	Nathalie Cartier-Lacave	Combined gene therapy with blood stem cell therapy, which may be a useful tool for treating fatal brain disease.
2011	Melanie Welham, David Tosh	1 M molecule treatment causes stem cells to turn into precursors of liver cells.
2012	Willison and Klug	Single-cell and single-molecule proteomics utilizing nanospace technology
2012	Maria Blasco	First gene therapy successful against aging-associated decline in mice.

cells in comparison to their normal counterparts, especially with reference to altered growth patterns and cell morphology.

### Cell culture

This is the most commonly used method of tissue culture and is generated by collecting the cells growing out of explants or dispersed cell suspensions (floating free in culture medium). Cells obtained either by enzymatic treatment or by mechanical means are cultured as adherent monolayers on solid substrate.

Cell culture is of three types: (1) precursor cell culture, which is undifferentiated cells committed to differentiate; (2) differentiated cell culture, which is completely differentiated cells that have lost the capacity to further differentiate; and (3) stem cell culture, which is undifferentiated cells that go on to develop into any type of cell.

Cells with a defined cell type and characteristics are selected from a culture by cloning or by other methods; this cell line becomes a cell strain.

### Monolayer cultures

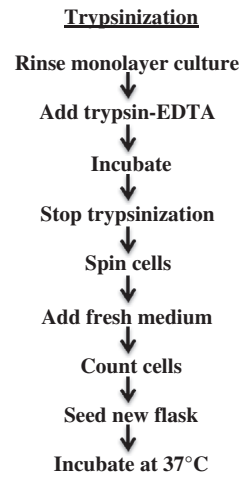
The monolayer culture is an anchorage-dependent culture of usually one cell in thickness with a continuous layer of cells at the bottom of the culture vessel.

### Suspension cultures

Some of the cells are nonadhesive and can be mechanically kept in suspension, unlike most cells that grow as monolayers (e.g., cells of leukemia). This offers numerous advantages in the propagation of cells.

### Cell passage and use of trypsin

Passaging is the process of subculturing cells in order to produce a large number of cells from pre-existing ones. Subculturing produces a more homogeneous cell line and avoids the senescence associated with prolonged high cell density. Splitting cells involves transferring a small number of cells into each new vessel. After subculturing, cells may be propagated, characterized, and stored. Adherent cell cultures need to be detached from the surface of the tissue culture flasks or dishes using proteins. Proteins secreted by the cells form a tight bridge between the cell and the surface. A mixture of trypsin-EDTA is used to break proteins at specific places. Trypsin is either protein-degrading or proteolytic; it hydrolyzes pepsin-digested peptides by hydrolysis of peptide bonds. EDTA sequesters certain metal ions that can inhibit trypsin activity, and thus enhances the efficacy of trypsin. The trypsinization process and procedure to remove adherent cells is given in [Flowchart 14.1](#).



**FLOWCHART 14.1** Trypsinization of adherent cells.

### Quantitation

Quantitation is carried out to characterize cell growth and to establish reproducible culture conditions.

### Hemocytometer

Cell counts are important for monitoring growth rates as well as for setting up new cultures with known cell numbers. The most widely used type of counting chamber is called a hemocytometer. It is used to estimate cell number. The concentration of cells in suspension is determined by placing the cells in an optically clear chamber under a microscope. The cell number within a defined area of known depth is counted, and the cell concentration is determined from the count.

### Electronic counting

For high-throughput work, electronic cell counters are used to determine the concentration of each sample.

### Other quantitation

In some cases, the DNA content or the protein concentration needs to be determined instead of the number of cells.

### Reconstruction of three-dimensional structures

Cells propagated as a cell suspension or monolayer offer many advantages but lack the potential for cell-to-cell interaction and cell–matrix interaction seen in organ cultures. For this reason, many culture methods that start with a dispersed population of cells encourage the arrangement of these cells into organ-like structures. These types of cultures can be divided into two basic types.

### ***Histotypic culture***

Cell–cell interactions similar to tissue-like densities can be attained by the use of an appropriate ECM and soluble factors and by growing cell cultures to high cell densities. This can be achieved by (a) growing cells in a relatively large reservoir with adequate medium fitted with a filter where the cells are crowded; (b) growing the cells at high concentrations on agar or agarose or as stirred aggregates (spheroids); and (c) growing cells on the outer surface of hollow fibers where the cells are seeded on the outer surface and medium is pumped through the fibers from a reservoir.

### ***Organotypic culture***

To simulate heterotypic cell interactions in addition to homotypic cell interactions, cells of differentiated lineages are re-combined. Co-culturing of epithelial and fibroblast cell clones from the mammary gland allows the cells to differentiate functionality under the correct hormonal environment, thus producing milk proteins.

## Types of cell culture

### **Primary cell culture**

These cells are obtained directly from tissues and organs by mechanical or chemical disintegration or by enzymatic digestion. These cells are induced to grow in suitable glass or plastic containers with complex media. These cultures usually have a low growth rate and are heterogeneous; however, they are still preferred over cell lines as these are more representative of the cell types in the tissues from which they are derived. The morphological structure of cells in culture is of various types: (1) epithelium type, which are polygonal in shape and appear flattened as they are attached to a substrate and form a continuous thin layer (i.e., monolayer on solid surfaces); (2) epitheloid type, which have a round outline and do not form sheets like epithelial cells and do not attach to the substrate; (3) fibroblast type, which are angular in shape and elongated and form an open network of cells rather than tightly packed cells, are bipolar or multipolar, and attach to the substrate; and (4) connective tissue type, which are derived from fibrous tissue, cartilage, and bone, and are characterized by a large amount of fibrous and amorphous extracellular materials.

### ***Advantages and disadvantages of primary cell culture***

These cultures represent the best experimental models for *in vivo* studies. They share the same karyotype as the parent and express characteristics that are not

seen in cultured cells. However, they are difficult to obtain and have limited lifespans. Potential contamination by viruses and bacteria is also a major disadvantage.

Depending on the kind of cells in culture, the primary cell culture can also be divided into two types.

### ***Anchorage-dependent/adherent cells***

These cells require a stable nontoxic and biologically inert surface for attachment and growth and are difficult to grow as cell suspensions. Mouse fibroblast STO cells are anchorage cells.

### ***Anchorage-independent/suspension cells***

These cells do not require a solid surface for attachment or growth. Cells can be grown continuously in liquid media. The source of cells is the governing factor for suspension cells. Blood cells are vascular in nature and are suspended in plasma and these cells can be very easily established in suspension cultures.

### **Secondary cell culture**

When primary cell cultures are passaged or subcultured and grown for a long period of time in fresh medium, they form secondary cultures and are long-lasting (unlike cells of primary cell cultures) due to the availability of fresh nutrients at regular intervals. The passaging or subculturing is carried out by enzymatic digestion of adherent cells. This is followed by washing and re-suspending of the required amount of cells in appropriate volumes of growth media. Secondary cell cultures are preferred as these are easy to grow and are readily available; they have been useful in virological, immunological, and toxicological research.

### ***Advantages and disadvantages of secondary cell culture***

This type of culture is useful for obtaining a large population of similar cells and can be transformed to grow indefinitely. These cell cultures maintain their cellular characteristics. The major disadvantage of this system is that the cells have a tendency to differentiate over a period of time in culture and generate aberrant cells.

## Cell line

The primary culture, when subcultured, becomes a cell line or cell strain that can be finite or continuous, depending on its lifespan in culture. They are grouped into two types on the basis of the lifespan of the culture.

## Finite cell lines

Cell lines with a limited number of cell generations and growth are called finite cell lines. The cells are slow growing (24–96 hours). These cells are characterized by anchorage dependence and density limitation.

## Indefinite cell lines

Cell lines obtained from in vitro transformed cell lines or cancerous cells are indefinite cell lines and can be grown in monolayer or suspension form. These cells divide rapidly with a generation time of 12–14 hours and have a potential to be subcultured indefinitely. The cell lines may exhibit aneuploidy (Bhat, 2011) or heteroploidy due to an altered chromosome number. Immortalized cell lines are transformed cells with altered growth properties. HeLa cells are an example of an immortal cell line. These are human epithelial cells obtained from fatal cervical carcinoma transformed by human papilloma virus 18 (HPV18). Indefinite cell lines are easy to manipulate and maintain. However, these cell lines have a tendency to change over a period of time.

## Commonly used cell lines

Nowadays, for the production of biologically active substances on an industrial scale, a mammalian cell culture is a prerequisite. With advancements in animal cell culture technology, a number of cell lines have evolved and are used for vaccine production, therapeutic proteins, pharmaceutical agents, and anticancerous agents. For the production of cell lines, human, animal, or insect cells may be used. Cell lines that are able to grow in suspension are preferred as they have a faster growth rate. Chinese hamster ovary (CHO) is the most commonly used mammalian cell line.

When selecting a cell line, a number of general parameters must be considered, such as growth characteristics, population doubling time, saturation density, plating efficiency, growth fraction, and the ability to grow in suspension. Table 14.2 shows some of the commonly used cell lines.

## Advantages of continuous cell lines

1. Continuous cell lines show faster cell growth and achieve higher cell densities in culture.
2. Serum-free and protein-free media for widely used cell lines may be available in the market.
3. The cell lines have a potential to be cultured in suspension in large-scale bioreactors.

The major disadvantages of these cultures are chromosomal instability, phenotypic variation in relation to the donor tissue, and a change in specific and characteristic tissue markers (Freshney, 1994).

## Growth cycle

The cells in the culture show a characteristic growth pattern, lag phase, exponential or log phase, followed by a plateau phase. The population doubling time of the cells can be calculated during the log phase and plateau phase. This is critical and can be used to quantify the response of the cells to different culture conditions for changes in nutrient concentration and effects of hormonal or toxic components. The population doubling time describes the cell division rates within the culture and is influenced by nongrowing and dying cells.

## Phases of the growth cycle

The population doubling time, lag time, and saturation density of a particular cell line can be established and characterized for a particular cell type. A growth curve consists of a normal culture and can be divided into a lag phase, log phase, and plateau phase.

### Lag phase

This is the initial growth phase of the subculture and re-seeding during which the cell population takes time to recover. The cell number remains relatively constant prior to rapid growth. During this phase, the cell replaces elements of the glycocalyx lost during trypsinization, attaches to the substrate, and spreads out. During the spreading process, the cytoskeleton reappears; its reappearance is probably an integral part of the process.

### Log phase

This is a period of exponential increase in cell number and growth of the cell population due to continuous division. The length of the log phase depends on the initial seeding density, the growth rate of the cells, and the density at which cell proliferation is inhibited by density. This phase represents the most reproducible form of the culture as the growth fraction and viability is high (usually 90%–100%), and the population is at its most uniform. However, the cell culture may not be synchronized, and the cells can be randomly distributed in the cell cycle.

**TABLE 14.2** Commonly used cell lines and their origins.

Cell line	Origin	Organism
H1, H9	Embryonic stem cells	Human
HEK-293	Embryonic kidney transformed with adenovirus	Human
HeLa	Epithelial cell	Human
HL 60	Human promyelocytic leukemia cells	Human
MCF-7	Breast cancer	Human
A549	Lung cancer	Human
A1 to A5-E	Amnion	Human
ND-E	Esophagus	Human
CHO	Ovary	Chinese hamster
Vero	Kidney epithelial cell	African green monkey
Cos-7	Kidney cells	African green monkey
3T3	Fibroblast	Mouse
BHK21	Fibroblast	Syrian hamster
MDCK	Epithelial cell	Dog
E14.1	Embryonic stem cells (mouse)	Mouse
COS	Kidney	Monkey
DT40	Lymphoma cell	Chick
S2	Macrophage-like cells	Drosophila
GH3	Pituitary tumor	Rat
L6	Myoblast	Rat
Sf9 and Sf21	Ovaries	Fall Army worm( <i>Spodoptera frugiperda</i> )
ZF4 and AB9 cells	Embryonic fibroblast cells	Zebrafish

### **Plateau phase**

The culture becomes confluent at the end of the log phase as growth rates during this phase are reduced, and cell proliferation can cease in some cases due to exhaustion. The cells are in contact with surrounding cells, and the growth surface is occupied. At this stage, the culture enters the stationary phase and the growth fraction falls to between 0% and 10%. Also, the constitution and charge of the cell surface may be changed, and there may be a relative increase in the synthesis of specialized versus structural proteins.

### **Monitoring cell growth**

The animal cell culture can be grown for a wide variety of cell-based assays to investigate morphology, protein expression, cell growth, differentiation,

apoptosis, and toxicity in different environments. Product yields can be increased if monitoring of cell growth is managed properly. A number of factors affect the maximum growth of cells in a batch reactor. Regular observation of cells in culture helps monitor cell health and the stage of growth; small changes in pH, temperature, humidity, O<sub>2</sub>, CO<sub>2</sub>, dissolved nutrients, etc., could have an impact on cell growth. Monitoring the rate of growth continuously also provides a record that the cells have reached their maximum density within a given time frame.

### **Characteristics of cell cultures**

Animal cell cultures show specific characteristics and differ from microbial cultures. The important characteristics of the animal cell are slow growth rate, requirement of solid substrata for anchorage-dependent cells, lack of a cell wall (which leads to



fragility), and sensitivity to physiochemical conditions such as pH, CO<sub>2</sub> levels, etc. Some of the fundamental bioprocess variables are as follows:

### Temperature

Temperature is one of the most fundamental variables as it directly interferes with the growth and production processes. On a small scale, thermostatically controlled incubators can be used to control temperature. However, cell cultures grown on a large scale in bioreactors require more sensitive control of temperature. Different bioreactors use different methods to maintain the temperature of the cell culture. Temperature in a bioreactor is maintained by a heat blanket and water jacket with a temperature sensor.

### pH

pH of the culture medium can be controlled by adding alkali (NaOH, KOH) or acid (HCl) solution. Addition of CO<sub>2</sub> gas to the bioreactor, buffering with sodium bicarbonate, or use of naturally buffering solutes help maintain the pH of the culture. A silver chloride electrochemical-type pH electrode is the most commonly used electrode in the bioreactor.

### Oxygen

Dissolved oxygen is the most fundamental variable that needs to be continuously supplied to the cell culture medium. It is consumed with a carbon source in aerobic cultures (Moore et al., 1995). Diffusion through a liquid surface or membranes is one of the methods for providing dissolved oxygen to the medium.

proliferation rapidly and accurately is the important requirement in many experimental situations that involve in vitro and in vivo studies. The cell number determination can be useful for determining the growth factor activity, concentration of toxic compound, drug screening, duration of exposure, change in colony size, carcinogenic effects of chemical compounds, and effects of solvents (such as ethanol, propylene, etc.).

The assays to measure viable cells (viability assays) are as follows:

1. [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT)/MTS/resazurin assay.
2. Protease marker assay.
3. ATP assay.

The MTT assay allows simple, accurate, and reliable counting of metabolically active cells based on the conversion of pale yellow tetrazolium MTT. Nicotinamide adenine dinucleotide in metabolically active viable cells reduces tetrazolium compounds into brightly colored formazan products or reduces resazurin into fluorescent resorufin (Fig. 14.1). MTT and resazurin assays are widely used, as they are inexpensive and can be used with all cell types. The protease marker assay utilizes the cell-permeant protease substrate glycylphenylalanyl-aminofluorocoumarin (GF-AFC). The substrate, which lacks an aminoterminal blocking moiety, is processed by aminopeptidases within the cytoplasm to release AFC. The amount of AFC released is proportional to the viable cell number. This assay has better sensitivity than resazurin and the cells remain viable; thus, multiplexing is possible. The ATP assay is the most sensitive cell viability assay. It is

## Cell viability

The number of viable cells in the culture provides an accurate indication of the health of the cell culture (Stacey and Davis, 2007). Trypan blue and erythrosin B determine cell viability through the loss of cellular membrane integrity. Both these dyes are unable to penetrate the cell membrane when the membrane is intact, but are taken up and retained by dead cells (which lack an intact membrane). Erythrosin B stain is preferred over Trypan blue as it generates more accurate results with fewer false negatives and false positives.

### Cytotoxicity

The toxic chemicals in the culture medium affect the basic functions of cells. The cytotoxicity effect can lead to the death of the cells or alterations in their metabolism. Methods to access viable cell number and cell

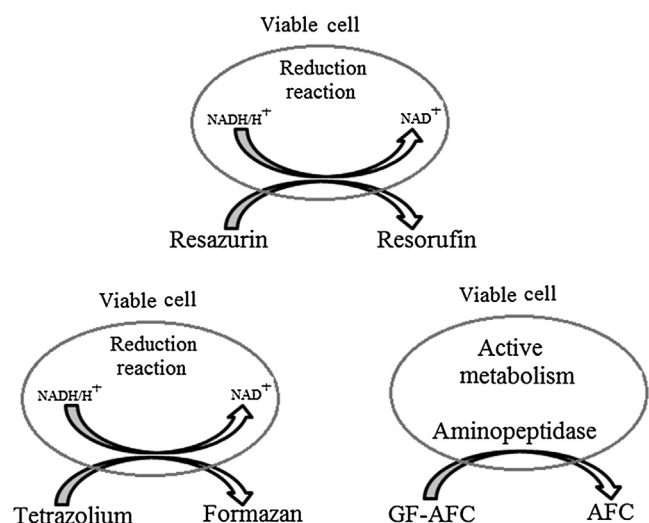
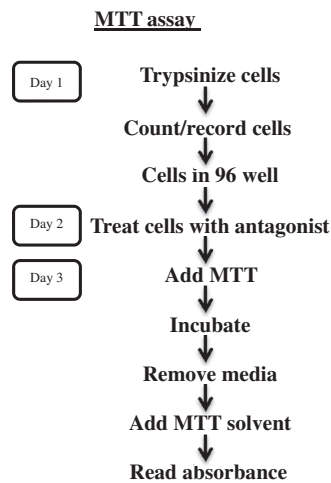


FIGURE 14.1 Schematic summary of biochemical events in different viability assays.



FLOWCHART 14.2 MTT assay.

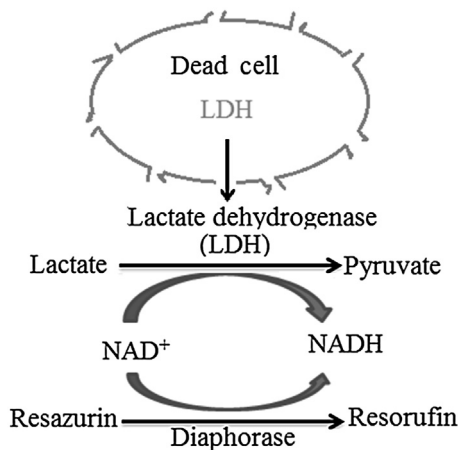


FIGURE 14.2 Principle of the LDH release assay.

measured using the beetle luciferase reaction to generate light. The MTT assay and procedure is given in [Flowchart 14.2](#).

Assays to detect dead cells are as follows:

1. Lactate dehydrogenase (LDH) release.
2. Protease release.
3. DNA staining.

The viable cells in culture have intact outer membranes. Loss of membrane integrity defines a “dead” cell. The dead cells can be detected by measuring the activity of marker enzymes that leak out of dead cells into the culture medium or by staining the cytoplasmic or nuclear content by vital dyes that can only enter dead cells. LDH is an enzyme that is present in all cell types. It catalyzes the oxidation of lactate to pyruvate in the presence of co-enzyme NAD<sup>+</sup>. In the damaged cells, LDH is rapidly released. The amount of released

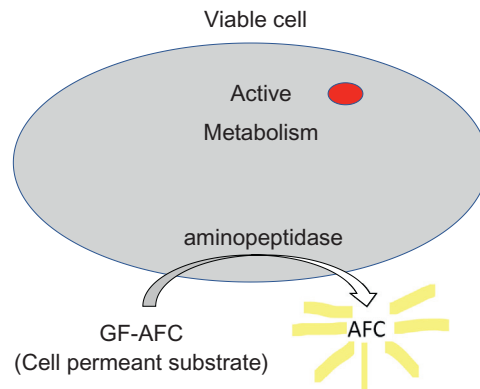


FIGURE 14.3 Principle of the luminescent protease release assay.

LDH is used to assess cell death ([Fig. 14.2](#)). This assay is widely used but has limited sensitivity as half-life of LDH at 37 °C is 9 hours.

The protease release assay is based on the intracellular release of proteases from the dead/compromised cell into the culture medium. The released proteases cleave the substrate to liberate aminoluciferin, which serves as a substrate for luciferase ([Fig. 14.3](#)) and leads to the production of a “glowtype” signal ([Cho et al., 2008](#)).

## Hayflick’s phenomenon

Hayflick limit or Hayflick’s phenomena is defined as the number of times a normal cell population divides before entering the senescence phase. Macfarlane Burnet coined the term “c limit” in 1974. [Hayflick and Moorhead \(1961\)](#) demonstrated that a population of normal human fetal cells divide in culture between 40 and 60 times before stopping. There appears to be a correlation between the maximum number of passages and aging. This phenomenon is related to telomere length. Repeated mitosis leads to shortening of the telomeres on the DNA of the cell. Telomere shortening in humans eventually makes cell division impossible, and correlates with aging. This explains the decrease in passaging of cells harvested from older individuals.

## Culture media

One of the most important factors in animal cell culture is the medium composition. In vitro growth and maintenance of animal cells require appropriate nutritional, hormonal, and stromal factors that resemble their milieu in vivo as closely as possible. Important environmental factors are the medium in which the cells are surrounded, the substratum upon which the

cells grow, temperature, oxygen and carbon dioxide concentration, pH, and osmolality. In addition, the cell requires chemical substances that cannot be synthesized by the cells themselves. Any successful medium is composed of isotonic, low-molecular-weight compounds known as basal medium and provides inorganic salts, an energy source, amino acids, and various supplements.

### Basic components in culture media

The 10 basic components that make up most of the animal cell culture media are as follows: inorganic salts ( $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$ ,  $\text{K}^+$ ), nitrogen source (amino acids), energy sources (glucose, fructose), vitamins, fat and fat soluble component (fatty acids, cholesterol), nucleic acid precursors, growth factors and hormones, antibiotics, pH and buffering systems, and oxygen and carbon dioxide concentrations.

Complete formulation of media that supports growth and maintenance of a mammalian cell culture is very complex. For this reason, the first culture medium used for cell culture was based on biological fluids such as plasma, lymph serum, and embryonic extracts. The nutritional requirements of cells can vary at different stages of the culture cycle. Different cell types have highly specific requirements, and the most suitable medium for each cell type must be determined experimentally. Media may be classified into two categories: (1) natural media and (2) artificial media.

#### Natural media

Natural media consist of naturally occurring biological fluids sufficient for the growth and proliferation of animal cells and tissues. This media useful for promoting cell growth are of the following three types:

1. Coagulant or clots: Plasma separated from heparinized blood from chickens or other animals is commercially available in the form of liquid plasma.
2. Biological fluids: This includes body fluids such as plasma, serum lymph, amniotic fluid, pleural fluid, insect hemolymph, and fetal calf serum. These fluids are used as cell culture media after testing for toxicity and sterility.
3. Tissue extract: Extracts of liver, spleen, bone marrow, and leucocytes are used as cell culture media. Chicken embryo extract is the most common tissue extract used in some culture media.

#### Artificial media

The media contains partly or fully defined components that are prepared artificially by adding several nutrients (organic and inorganic). It contains a balanced salt solution with specific pH and osmotic

pressure designed for immediate survival of cells. Artificial media supplemented with serum or with suitable formulations of organic compounds supports prolonged survival of the cell culture.

The artificial media may be grouped into the following four classes: serum-containing media, serum-free media, chemically defined media, and protein-free media.

### Serum

The clear yellowish fluid obtained after fibrin and cells are removed from blood is known as serum. It is an undefined media supplement of extremely complex mixture of small and large molecules and contains amino acids, growth factors, vitamins, proteins, hormones, lipids, and minerals, among other components (Table 14.3).

#### Advantages of serum in cell culture medium

1. It has basic nutrients present either in soluble or in protein-bound form.
2. It provides several hormones such as insulin and transferrin. Insulin is essential for the growth of nearly all cells in culture and transferrin acts as an iron binder.
3. It contains numerous growth factors such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- $\beta$ ), epidermal growth factor (EGF), and chondronectin. These factors stimulate cell growth and support specialized functions of cells.
4. It supplies protein, which helps in the attachment of cells to the culture surface (e.g., fibronectin).
5. It provides binding proteins such as albumin and transferrin, which helps transport molecules in cells.
6. It provides minerals such as  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Zn}^{2+}$ , etc., which promote cell attachment.
7. It increases the viscosity of the medium, which provides protection against mechanical damage during agitation and aeration of suspension cultures.
8. It provides appropriate osmotic pressure.

#### Disadvantages of serum-containing medium

1. Expensive: Fetal calf serum is expensive and difficult to obtain in large quantities.
2. Variation: Batch-to-batch variation occurs in serum, and there is no uniformity in composition of serum. This can affect growth and yields and can give inconsistent results.
3. Contamination: Serum medium carries a high risk of contamination with virus, fungi, and mycoplasma.

**TABLE 14.3** Serum components, their composition, and role in animal cell culture.

Component	Probable function
<b>Protein and polypeptide</b>	
Albumin	Major binding and buffering agent, antioxidant, transporter of insoluble molecules
Transferrin	Iron chelator and transporter
Alpha, beta, and gamma globulin fractions	Bind to iron and iron carrier and prevent infection
Regulatory proteins	Regulate gene expression
<b>Growth factors</b>	
Epidermal growth factor (EGF)	Proliferation and differentiation
Fibroblast growth factor (FGF)	Proliferation and differentiation
<b>Hormones</b>	
Insulin	Glucose and protein metabolism
Transferrin	Incorporation of iron by cells

4. Cytotoxic and inhibiting factors: The serum itself may be cytotoxic and may contain inhibiting factors, which in turn may inhibit cultured cell growth and proliferation. The enzyme polyamine oxidase in serum reacts with polyamines such as spermine and spermidine to form cytotoxic polyamino-aldehyde.
5. Downstream processing: The presence of serum in culture media may interfere with isolation and purification of culture products. Additional steps may be required to isolate cell culture products.
3. They decrease variability from batch to batch and improve reproduction between cultures.
4. Downstream processing of products from cell cultures in serum-free media is easier.
5. They reduce the risk of microbial contamination (mycoplasma, viruses, and prions).
6. Serum-free media are easily available and ready to use. They are also cost-effective when compared with serum-containing media.

### Serum-free media

The use of serum in culture media presents a safety hazard and source of unwanted contamination for the production of biopharmaceuticals. As a number of cell lines can be grown in serum-free media supplemented with certain components of bovine fetal serum, the development of this type of medium with a defined composition has intensified in the last few decades. Eagle (1959) developed a “minimal essential medium” composed of balanced salts, glucose, amino acids, and vitamins. In the last 50 years, considerable work has been carried out to develop more efficient culture media to meet the specific requirements of specific cell lines.

#### **Advantages of serum-free culture media**

1. Serum-free media are simplified, and the composition is better defined.
2. They can be designed specifically for a cell type. It is possible to create different media and to switch from growth-enhancing media to differentiation-inducing media by altering the combination and types of growth factors and inducers.

#### **Disadvantages of serum-free media**

1. Growth rate and saturation density attained are lower than those compared to serum-containing media.
2. Serum-free media prove to be more expensive as supplementing with hormone and growth factors increases the cost enormously.
3. Different media are required for different cell types as each species has its own characteristic requirements.
4. Critical control of pH and temperature and ultra-purity of reagent and water are required as compared to serum-containing media.

### Chemically defined media

These media contain pure inorganic and organic constituents along with protein additions like EGFs, insulin, vitamins, amino acids, fatty acids, and cholesterol.

### Protein-free media

These media contain nonprotein constituents necessary for the cell culture. The formulations of DME, MEM, RPMI-1640, ProCHO TM, and CDM-HD are

examples of protein-free media. They promote superior cell growth and facilitate downstream purification of expressed products.

### Characterization of cell lines

The characterization of cell lines is important to ensure the quality of cell-derived biopharmaceutical products. It helps in determining the cell source with regard to its identity and the presence of other cell lines, molecular contaminants, and endogenous agents. The characterization of mammalian cell lines is species-specific and can vary depending on the history of the cell line and type of media components used for culturing.

Mammalian cell line characterization can be done in four ways:

1. Identity testing.
2. Purity testing.
3. Stability testing.
4. Virological safety testing.

#### **Identity testing**

Identity testing can be carried out by isoenzyme analysis. The banding pattern of the intracellular enzyme (which is species-specific) can be determined by using agarose gels. DNA fingerprinting and karyotyping, and DNA and RNA sequencing are alternative methods to identity testing.

#### **Karyotyping**

Karyotyping is important as it determines any gross chromosomal changes in the cell line. The growth conditions and subculturing of a cell line may lead to alteration in the karyotype; for example, HeLa cells were the first human epithelial cancer cell line established in long-term culture, and they have a hypertriploid chromosome number (3n1).

#### **Purity testing**

Bacterial and fungal contamination of cell lines occurs due to impure techniques and source material. The occurrence of contaminants can be tested by a direct inoculation method on two different media. Mycoplasma infection is the contamination of cell cultures/cell lines with mycoplasmas, and it represents a serious problem. Detection by microscopy is not adequate and requires additional testing by fluorescent staining PCR, ELISA assay, autoradiography, immunostaining, or microbiological assay.

#### **Stability testing**

Characterization and testing of cell substrate (cell line derived from human or animal source) is one of the most important components in the control of biological products. It helps to confirm the identity, purity, and suitability of the cell substrate for manufacturing use. The substrate stability should be examined at a minimum of two time points during cultivation for production. In addition, genetic stability can be tested by genomic or transcript sequencing, restriction map analysis, and copy number determination (FDA guidelines, 2012).

#### **Viral testing assays**

Virus testing of cell substrate should be designed to detect a spectrum of viruses. Appropriate screening tests should be carried out based on the cultivation history of cell lines. The development of characteristic cytopathogenic effect (CPE) provides an early indication of viral contamination. Some of the viruses of special concern in cell production work are human immunodeficiency virus, human papilloma virus, hepatitis virus, human herpes virus, hantavirus, simian virus, sendai virus, and bovine viral diarrhea virus. For detection of viruses causing immunodeficiency diseases and hepatitis, detection of sequences by PCR testing is adequate. Cells exposed to be serum or bovine serum albumin require a bovine virus test. Some of the viral testing assays are XC plaque assays, S + L-focus assay, reverse transcription assay. XC plaque assay is utilized to detect infectious ecotropic murine retroviruses. S + L-focus assay is used to test cells for the presence of infectious xenotropic and amphotropic murine retroviruses that are capable of interacting with both murine and nonmurine cells. Real-time (RT) assays such as real-time fluorescent product-enhanced reverse transcriptase (FPERT) assay and quantitative real-time for fluorescent product-enhanced reverse transcript (QPRT) assay detect the conversion of an RNA template to cDNA due to the presence of the RT template when retrovirus infection is present in the cell line.

### Advantages of animal cell culture

1. Physiochemical and physiological condition: Role and effect of pH, temperature, O<sub>2</sub>/CO<sub>2</sub> concentration, and osmotic pressure of the culture media can be altered to study their effects on the cell culture (Freshney, 2010).

2. Metabolism of cell: To study cell metabolism and investigate the physiology and biochemistry of cells.
3. Cytotoxic assay: Effect of various compounds or drugs on specific cell types such as liver cells can be studied.
4. Homogenous cultures: These cultures help study the biology and origin of the cells.
5. Valuable biological data from large-scale cell cultures: Specific proteins can be synthesized in large quantities from genetically modified cells in large-scale cultures.
6. Consistency of results: Reproducibility of the results that can be obtained by the use of a single type/ clonal population.
7. Identification of cell type: Specific cell types can be detected by the presence of markers such as molecules or by karyotyping.
8. Ethics: Ethical, moral, and legal questions for utilizing animals in experiments can be avoided.

### Disadvantages of animal cell culture

1. Expenditure and expertise: This is a specialized technique that requires aseptic conditions, trained personnel, and costly equipment.
2. Dedifferentiation: Cell characteristics can change after a period of continuous growth of cells in cultures, leading to differentiated properties compared to the original strain.
3. Low amount of product: The miniscule amount of mAB and recombinant protein produced followed by downstream processing for extracting pure products increases expenses tremendously.
4. Contamination: Mycoplasma and viral infection are difficult to detect and are highly contagious.
5. Instability: Aneuploidy chromosomal constitution in continuous cell lines leads to instability.

In addition, this system cannot replace the complex live animal for testing the response of chemicals or the impact of vaccines or toxins.

### Ethical issues

Despite considerable progress in the development of cell culture techniques, the potential biohazards of working with animal and human tissues presents a number of ethical problems, including issues of procurement, handling, and ultimate use of material. In most countries, biomedical research is strictly regulated. Legislation varies considerably in different countries. Research ethics committees, animal ethics committees for animal-based

research, and institutional research boards for human subjects have a major role in research governance.

Some guidelines for the use of experimental or donor animals include assurances of proper conditions for housing animals and minimal pain or discomfort to any animal that is put to death or operated upon. These guidelines apply to higher vertebrates and not to lower vertebrates such as fish or other invertebrates.

### Use of fetal bovine serum in animal culture of media

Fetal bovine serum (FBS)-supplemented media are commonly used in animal cell cultures. In recent years, FBS production methods have come under scrutiny because of animal welfare concerns. FBS is harvested from bovine fetuses taken from pregnant cows during slaughter. The common method of harvesting the fetus is by cardiac puncture without any anesthesia. This practice of harvesting FBS is inhumane as it exposes the fetus to pain and/or discomfort. In addition to moral concerns, numerous scientific and technical problems exist with regard to the use of FBS in cell culture. Efforts are now being made to reduce the use of FBS and replace it with synthetic alternatives.

In the case of human tissues, some considerations that need to be addressed are as follows (Freshney, 2011):

1. Consent: Patient's and/or relative's approval of tissue use.
2. Project summary: Explanation of the project, including the purpose, outcome, and medical benefits of the research.
3. Permission requests: Paperwork regarding possible use of the tissues.
4. Ownership: Establishment of ownership with regard to cell lines and their derivatives.
5. Patent issues: Commercial use of the tissues.

### Translational significance

In biomedical research, the use of animal and human cell cultures has become beneficial for diverse applications. It provides indispensable tools for producing a number of products, including biopharmaceuticals, mABs, and products for gene therapy. In addition, animal cell cultures provide adequate test systems for studying biochemical pathways, intra- and intercellular responses, pathological mechanisms, and virus production. Some of the applications of animal cell culture are discussed below.

**TABLE 14.4** Recombinant hepatitis B vaccines in eukaryotic cells.

Trade name	Manufacture	Recombinant protein	Expression host
Sci B-Vac	SciGen	HBsAgS, M, and L protein	Mammalian cells (CHO)
GenHevac B	Pasteur-Merieux Aventis	HBsAg S and M protein	Mammalian cells (CHO)
Enivac HB	Panacea Biotec Ltd	HBsAg S protein	Yeast ( <i>P. pastors</i> )
Revac-B	Bharat Biotech International Ltd	HBsAg S protein	Yeast ( <i>P. pastors</i> )

### Antiviral vaccines

Animal cell culture technology has played an important role in the development of viral vaccine production. The establishment of cell culture technology in the 1950s and the consequent replacement of live animals for the development of antigens have led to considerable progress in bioprocess technology. With the advent of DNA technology, molecular manipulation of viruses has led to the development of a recombinant vaccine against hepatitis B virus (HBV) and several others potential vaccines that are in the final phase of clinical trials. [Table 14.4](#) lists recombinant hepatitis B vaccines in eukaryotic cells.

### Viral particles production by cell culture

Viral particle production by cell culture differs from the production of molecules such as proteins, enzymes, and toxins by bacteria or animal cells. The product formation may not be related to the development or growth of a cell and may occur through secondary metabolic pathways, unlike virus production, which does not result from secondary metabolic pathway. Virus production occurs after the viral infection directs cell machinery to perform viral particle production.

Two stages are involved in viral production:

1. Cell culture system: This requires the development of an efficient system for conversion of the culture medium substrate in the cell mass.
2. Virus production: This phase differs from the infection phase and has different nutritional and metabolic requirements. A number of immortalized cell lines are used for the industrial production of viral vaccines. [Table 14.5](#) gives the cell lines used for vaccines.

### Production of virus-like particles

Most of the existing classical vaccines for viral disease are either altered or chemically inactive live viruses. However, incomplete inactivation of a virus or

**TABLE 14.5** Cell lines used for viral vaccine production.

Cell lines	Origin
BHK-21	Kidney (hamster)
Vero	Kidney (African green monkey)
MDCK	Kidney (cocker spaniel)
CHO	Chinese hamster
Hela	Epithelial cells (human)

reversion of an attenuated strain can risk infection in vaccinated individuals. Viruses with segmented genomes with a high degree of genetic exchange can undergo re-assortment or recombination of genetic material with viruses of different serotypes in the vaccinated host, which can result in the production of new variants of the virus. Moreover, some live virus vaccines are teratogenic; for example, Smithburn neurotropic strain (SNS) ([Smithburn, 1949](#)) and MP12-attenuated ([Caplen et al., 1985](#)) vaccine strains of the Rift Valley fever virus. A new type of vaccine that does not present the typical side effects of an attenuated or inactivated viral vaccine has been made possible with the development of rDNA technology. Virus-like particles (VLPs) are highly effective as they mimic the overall structure of the virus; however, these particles lack the infectious genetic material. Capsid proteins can aggregate to form core-like particles in the absence of nucleic acids. These spontaneously assembled particles are structurally similar to authentic viruses and are able to stimulate B-cell-mediated immune responses. In addition, VLPs stimulate a CD4-proliferative response and cytotoxic T-lymphocyte response ([Jeoung et al., 2011](#)).

VLPs resemble and mimic virus structure and are able to elicit a strong immune response without causing harm. The major advantage of VLPs is their simplicity and nonpathogenic nature. They are replication-deficient as they lack any viral genetic information, thus eliminating the need for inactivation of the virus. This is important as inactivation treatments lead to epitope modifications ([Cruz et al., 2002](#)). As the structural

morphology of VLPs is similar to the virus, the conformational epitopes presented to the immune system are the same as for the native virus particles. The immune response/antibody reactivity in the case of VLPs is significantly improved as VLPs present conformation epitopes more similar to the native virus. VLPs also induce a strong B-cell response. For broader and more efficient protection, it is possible to adapt one or more antigens to the multimeric protein structure. Another advantage offered by VLPs is that they significantly reduce vaccine costs as these can elicit a protective response at lower doses of antigen.

### **Vaccines based on virus-like particles**

The FDA has approved VLP-based vaccines for HBV and HPV. The HBV vaccine was approved in 1986 and the HPV one in 2006 (Justin et al., 2011). To generate immunogenic VLPs, the S gene is cloned and expressed in a eukaryotic expression host such as yeast or mammalian cells (e.g., CHO cell line). The mammalian cell culture allows easy recovery because the cells are able to secrete the antigen HBsAg. The two companies producing CHO-based vaccines are the French-based Pasteur-Merieux Aventis (Gene Hevac B) and the Israeli-based SciGen (Sci-B-Vac). The Gene Hevac B vaccine contains the HBsAg S protein and M protein, whereas Sci-B-Vac contains the M and L proteins.

### **Human papilloma virus vaccine**

Viruses of the Papillomaviridae family are known to induce lesions and warts and also cause cervical cancer. Fifteen strains of Papillomaviridae are known to cause cervical cancer. HPV-16 is considered a high-risk HPV type as the risk of cancer may be higher than for other high-risk HPV types. The two virally encoded proteins of HPV are L1 and L2. L1 is the main capsid protein that forms the outer shell of the virus. L2 is found in the interior of the viral particle and is less abundant. The recombinant L1 VLP is able to induce neutralizing antibodies in animals. Gardasil (the first HPV vaccine) was approved by the FDA in 2006. This vaccine is manufactured by Merck and Co., Inc. Ceravarix, another HPV vaccine (manufactured by Glaxo Smithkline), was approved by the FDA in 2009. It uses the Trichoplusia ni (Hi-5) insect cell line infected with L1 recombinant baculovirus (Jiang et al., 1998; Wang et al., 2000).

A number other VLP-based vaccines are in clinical trials. These include the anti-influenza A M2-HBcAg VLP vaccine (Clarke et al., 1987), two antimalarial vaccine nicotine-Q $\beta$  VLPs (Maurer et al., 2005), and an anti-AngIIQ $\beta$  VLP. The VLP production in mammalian cell lines and Baculo cell lines of viruses infecting humans and other animals is summarized in Table 14.6.

## **Recombinant therapeutic proteins**

Proteins play a major role in carrying out biochemical reactions, transporting small molecules within a cell or from one organ to another, formation of receptors and channels in membranes, and providing frameworks for scaffolding. The number of functionally distinct proteins in humans far exceeds the number of genes as a result of post-translational modifications. These modifications include glycosylation, phosphorylation, ubiquitination, nitrosylation, methylation, acetylation, and lipidation. The changes in protein structure as a result of mutation or other abnormalities often lead to a disease condition. Protein therapeutics offer tremendous opportunities for alleviating disease. The first therapeutic from recombinant mammalian cells was human tissue plasminogen, which obtained market approval in 1986. At present, 60%–70% of all the recombinant therapeutic proteins are produced in mammalian cells.

### **Main therapeutic proteins**

The main therapeutic proteins can be divided into seven groups (Walsh, 2003):

1. Cytokines
2. Hematopoietic growth factors
3. Growth factors
4. Hormones
5. Blood products
6. Enzymes
7. Antibodies

Most of the proteins have complex structures and undergo chemical modification to insure full biological activity. Protein post-translation modifications (PTM) can happen in several ways. The most widely recognized form of PTM is glycosylation, which involves extensive sequence processing and trimming in the Golgi apparatus and endoplasmic reticulum. Eukaryotic cells are capable of carrying out this type of modification and are thus preferred in biopharmaceutical processes. Hamster, baby hamster kidney (BHK), and CHO cells are often the host cells of choice as glycosylation patterns generated from these cells are more similar to human patterns. Table 14.7 lists various therapeutic proteins produced in animal cell lines.

### **Cytokines**

Cytokines are proteins of the immune system that play a central role in immune response. Cytokines are produced as a result of immune stimulus by various white blood cells. Interferons (IFNs) were the first family of cytokines to be discovered and used as biopharmaceuticals.



**TABLE 14.6** VLP production in mammalian and baculo cell lines of viruses infecting humans and other animals.

Virus	Expression system	Cells	Recombination protein	Reference
SIV	B/IC	Sf9	Pr55 gag envelope protein	Yamshchikov et al. (1995)
Ebola	Human cells	HEK293T	VP40 and glycoprotein	Licata et al. (2004)
Rota virus	B/IC	Sf9	VP2, VP6, VP7	Parez et al. (2004)
SARS-CoV	Baculovirus-insect cell system	Sf21	E and M proteins	Ho et al. (2004)
SARS	Insect cell culture system	Sf-9	S protein, E protein, and M protein	Mortola and Roy (2004)
Marburg and Ebola virus	Mammalian cell culture system	293T cells	VP40 and glycoproteins	Swenson et al. (2005)
AAV	Insect cell culture system and mammalian cell culture system	Sf-9 and HEK 293	VP1, VP2, and VP3	Aucoin et al. (2007)
HIV-1	Baculovirus-insect cell expression system	Sf-21	HIV gag proteins like GagTN and GagRT	Pillay et al. (2009)
Influenza A	Baculovirus expression system	Sf-9	H1N1 protein, M1 protein, etc.	Krammer et al. (2010)
MERS-CoV	Recombinant baculoviruses (rBVs)	Sf9	Spike (S), envelope (E), and membrane (M) protein	Wang et al. (2017)
ZIKV	CHO	HEK293	rH7 antigens	Chen et al. (2019)

AAV, adeno-associated virus; *rBVs*, recombinant baculoviruses; *ZIKV*, Zika virus.

## Applications of interferons

IFN $\alpha$  is used for treatment of hepatitis, and more recently has been approved for leukemia and other types of cancers. IFN $\beta$  is used for treatment of multiple sclerosis and is marketed under the names Avonex, Belaseron, and Rebif. IFN $\gamma$  is used for the treatment of chronic granulomatous disease. Interleukin is another kind of cytokine that helps regulate cell growth, differentiation, and motility and is used as a biopharmaceutical. The recombinant form of IL-2 is used for the treatment of renal cell carcinoma.

### Growth factors

Growth factors are proteins that bind to receptors on the surface of cells to activate the cells for proliferation and or differentiation. The different types of growth factors are TGF, insulin-like growth factor, and (EGF. The primary sources of PDGF are platelets, endothelial cells, and the placenta. Two isoforms of this protein are present in the human body and both of these have one glycosylation site and three disulfide bonds. Examples of growth factors used as biopharmaceuticals are the following:

1. Osigraft/Eptotermin alfa (bone morphogenetic protein) is used for the treatment of tibia fractures, is grown commercially in CHO cells, and was first approved in 2001 in Europe.

2. InductOS/Dibotermin (bone morphogenetic protein) is used for tibia fractures and in spinal surgery; it is also commercially grown in CHO cells. This product was first approved in Europe in 2002.

### Hormones

Insulin, glucagon, gonadotropins, and growth hormones are the most well-known therapeutic hormones. The first biopharmaceuticals that obtained approval by regulatory agencies were insulin and recombinant human growth hormones. These were produced in microbial cells. The commercial recombinant forms of the gonadotropin family of hormones are Gonal-F, Luveris, Puregon, and Ovitrelle. All these are produced using CHO cells and are used for treating female infertility.

### Therapeutic enzymes

A number of recombinant therapeutic enzymes are expressed in mammalian cells. Tissue plasminogen activator (tPA) is a thrombolytic agent involved in dissolving blood clots. Recombinant tPA is commercially known as Alteplase and Tenecteplase, which are used for the treatment of acute myocardial infraction.

Fabry disease, a genetic metabolic disorder, is characterized by a lack of enzyme  $\alpha$ -galactosidase A. Fabrazyme (approved in 2001) is a recombinant

**TABLE 14.7** Various therapeutic proteins produced in animal cell lines.

Cell line	Therapeutic protein	Potential application	Product name	Approval (FDA)
CHO	tPA	Acute myocardial infraction	Activase	1987
BHK	Factor VIII	Hemophilia A	Kogenate FS	1993
Sp2/0	Immunoglobulin GI	Rheumatoid arthritis, Crohn's disease	Remicade	1998
BHK	Factor VIIa	Hemophilia A + B	Novo Seven	1999
HEK-293	Activated protein C	Severe sepsis	Xigris	2001
Hybridoma CHO	Immunoglobulin G2a	Non-Hodgkin's lymphoma	Bexxar	2003
CHO	Immunoglobulin GI	Colorectal cancer	Avastin	2004
CHO	Humanized IgG	Cancer	Perjeta	2012
CHO	Humanized IgG1; DM1	Cancer	Kadcyla	2013
CHO	Humanized IgG4 $\kappa$	Cancer	Keytruda	2014
CHO	Human IgG4 $\kappa$	Cancer	Opdivo	2014
NS0	Humanized IgG1	Cancer	Empliciti	2015
CHO	Human IgG1 $\kappa$	Cancer	Darzalex	2015
CHO	Humanized IgG1 Fab	Hemostasis	Praxbind	2015
CHO	Human IgG1	Cancer	Bavencio	2017
CHO	Humanized IgG1/ $\kappa$	Asthma	Fasenra	2017
CHO	Human IgG1 $\lambda$	Psoriasis	Tremfya	2017
CHO	Glycosylated IgG1	Multiple sclerosis	Ocrevus	2017
CHO	Human recombinant IgG1	Rheumatoid arthritis	Kevzara	2017

$\alpha$ -galactosidase A and is produced by genetically modified CHO cells.

### **Blood coagulation factors**

Hemophilia A is caused by the lack of blood-clotting factor VIII, hemophilia B is caused by deficiency of factor IX, and hemophilia C by lack of factor XI. Factor VIII and IX are proteins. The first recombinant factor VII products were Recombinate and Kogenate, which were expressed in CHO and BKH cells, respectively. Recombinant factor FIX is commercially sold as BeneFIX and is produced in recombinant CHO cells.

### **Antibodies**

Therapeutic antibodies are used in the treatment of cancer, cardiovascular disease, infections, and autoimmune diseases. In 2004, the antibody Avasin (Bevacizumab) was approved for the treatment of metastatic colorectal cancer. This antibody acts as an inhibitor of vascular endothelial growth factor. Zenapax, another commercially available antibody, is used during

prophylaxis for preventing the rejection of transplanted organs. This is commercially grown in the NSO cell line and was approved for human use in 1997.

## **Gene therapy**

### **Importance of cell culture in gene therapy**

Gene therapy involves the insertion, removal, or alteration of a therapeutic or working gene copy to cure a disease or defect or to slow the progression of a disease, thereby improving the quality of life. The human genome map was the first major step toward a new way of addressing human health and illness. Gene therapy holds great promise, however, the task of transferring genetic material into the cell remains an enormous technical challenge and requires *ex vivo* cell cultivation and adaptation from the lab to a clinically relevant state. The development of animal cell culture technology is imperative for advances in gene therapy.

Monogenic diseases caused by single gene defects (such as cystic fibrosis, hemophilia, muscular

dystrophy, and sickle cell anemia) are the primary targets of human gene therapy.

The first step in gene therapy is to identify the faulty gene. This is followed by gene isolation and generation of a construct for correct expression. Integration of the gene followed by delivery of the genetic material in vivo or ex vivo is crucial to the success of gene therapy. In in vivo therapy, the genetic material is introduced directly into the individual at a specific site, and in ex vivo treatment, the target cells are treated outside the patient's body. These cells are then expanded and transferred back to the individual at a specific site. The ex vivo technique involves gene therapy in the cultured cells, which are expanded and subsequently transferred to the targeted tissue.

### Clinical correlation

A number of clinical studies and trials for gene therapy have already been approved and are being conducted worldwide. From 1989 up to the present, about 500 clinical studies have been reported; 70% of these studies are intended for cancer treatment.

The first product designed for gene therapy was Gendicine, a medication produced by Shenzhen Sibiono Genetech, China. Gendicine is used for head and neck carcinoma treatment. The tumor 4 suppressing gene p53 in recombinant adenovirus expresses protein p53, which leads to tumor control and elimination. SBN-cel is a cell line that was subcloned from the human embryonic kidney (HEK) cell line 293 and has been used for the production of Gendicine.

## Biopesticides

In recent years, biopesticides have gained importance due to increased concerns about agrochemicals and their residues in the environment and food. Biopesticides provide an effective means for the control of insects and plant disease, and they are environmentally safe. The biological control of insect pests by another living organism (in order to suppress the use of pesticides) is an age-old practice. Presently, a number of biological controls are being used as biopesticides. With the high cost of chemical-based pesticides and the development of resistance to multiple chemical pesticides, baculoviruses are one of the most promising biocontrols for insect pests and have been increasingly used effectively against caterpillars worldwide. However, the major impediment in the development of baculoviruses as biopesticides is the high cost and small volumes of in vitro methods. Development of an in vitro production process for

large quantities of baculoviruses at comparable costs to chemical pesticides will help provide insect control that is safe, efficacious, cost-effective, and environmentally safe.

### Baculovirus production in animal cell culture

A number of factors are important for a successful commercial production of bioinsecticides:

1. Large-scale production of viruses at competitive costs.
2. Economic production of viruses (i.e., low cost for the media and running the culture).
3. Effective cell line with high virus per cell productivity.
4. With passage of the virus into cells, there is a loss of virulence and an increased risk of mutant formation; this should be avoided.
5. The quality of the polyhedral produced in the cell culture should be comparable to those obtained from caterpillars.

The insect baculovirus cell system offers a number of advantages. It produces recombinant proteins that are functional and are immunologically active, as it is able to make post-translational modifications. The recombinant system uses a powerful promoter polyhedron.

### Cell lines for biopesticide production

The most commonly used cell lines in biopesticide production are the Sf21 and Sf9 cell lines, which are derived from ovarian tissues of the fall army worm (*Spodoptera frugiperda*). Sf9 cells show a faster growth rate and higher cell density than Sf21 cells and are preferred. High Five cell lines (designated BTI-Tn-5BI-4) established from *Trichoplusia ni* embryonic tissue are also being used.

### Viral mutant formation in cell culture

The continuous culturing of cells for virus production leads to virus instability and the so-called passage effect. This can result in a decrease of virulence and polyhedral production and a variety of mutations. All these changes affect commercial production in vitro. Two types of mutations are commonly seen in continuous passaging of cell cultures for viral productions: (1) defective infective particles (DIPs) and (2) few polyhedral (Fp) mutations.

Fp mutations are characterized by (1) reduced polyhedral, (2) enhanced production of BV, and (3) lack of

occluded virions in polyhedra. All these factors reduce the infectivity of the target pest.

Spontaneously generated Fp mutants have been reported in AcMNPV (*Autographa California* nucleopolyhedroviruses) (Wood, 1980), *Galleria mellonella* nucleopolyhedroviruses (GmMNPV) (Fraser and Hink, 1982), and *Helicoverpa armigera* nucleopolyhedroviruses (HaSNPV) (Chankraborty and Reid, 1999).

DIP mutations are the formation of DIPs. They occur due to serial passaging for long periods, which results in a decrease in the filtering of infectious virus. DIPs have been reported in a number of animal virus systems and in baculovirus systems. DIP formation can be avoided by low multiplicity of infection. This minimizes the probability of the defective virus entering the cell with an intact helper virion.

### Monoclonal antibodies

The majority of antibodies available on the market today are produced in animal cell cultures (Van Dijk and Van de Winkle, 2001). Animal cells are preferred because they are capable of glycosylation and structural conformation, which is essential for a drug to be productive. Hybridoma technology has been the most widely used method for small- and large-scale production of mABs. However, these antibodies have limited therapeutic applications since they produce an adverse immune response on repeated use.

A number of cell lines are now being used for the production of recombinant antibodies. The CHO lines are the most commonly used. Other cell lines used are marine myelomas NSO, Sp 2/0, HEK-93, and BHK.

A number of factors influence the production of mABs. For a high concentration of mAB production, the cell line should have high productivity. For high protein productivity, it is important that the selected cell line be productive in order to avoid large reaction volumes and the high cost of protein purification. Cell lines with the capacity to grow without anchorage offer an advantage in terms of scaling up the process; it is much simpler than with those designed for the growth culture of anchorage-dependent cells. Sp2/0 and NSO cell lines can grow naturally in suspension; other cells such as CHO and BHK can be easily adapted to this form of cultivation.

### Stem cells

Stem cells are unspecified cells that have the potential to differentiate into other kinds of cells or tissues and become specialized cells. The two characteristics that define stem cells are their ability of self-regenerate

and to differentiate into any other cells or tissues. These cells have the capability to renew themselves to form cells of more specialized function. In recent years, stem cell research has been hailed as a major breakthrough in the field of medicine. This property of turning a cell into any other specialized function cell has made researchers believe that stem cells could be utilized to make fully functional, healthy organs to replace damaged or diseased organs.

### Culturing embryonic stem cells in the laboratory

Human embryonic stem cells (hESCs) are grown on nutrient broth. These cells are traditionally cultured on mouse embryonic fibroblast feeder layers, which allows continuous growth in an undifferentiated stage. The mouse cells at the bottom of the culture dish provide a sticky surface to which the cells can attach. In addition, the feeder cells release nutrients into the culture medium. Researchers have now devised animal-free culture systems for hESCs and have used human embryonic fibroblasts and adult fallopian tube epithelial cells as feeder layers (in addition to serum-free mediums).

More recently, methods to subculture embryonic cells without the feeder layer have been developed. Martigel from BD Biosciences has been used to coat the culture plate (Hassan et al., 2012) for effective attachment and differentiation of both normal and transformed anchorage-dependent epithelioid and other cell types. This is a gelatinous protein mixture isolated from mouse tumor cells.

### Microfluidics three-dimensional culture

A major milestone in the biological sciences was the establishment of the tissue culture technique that can both maintain and propagate the growth of living cells under sterile in vitro conditions. Traditional cell cultures, which are two-dimensional (2D), are grown as monolayer cultures on a flat and rigid surface. Since their development, several advancements have been made to improve cell culture media as well as the biological materials used for culturing. The improvements have proven valuable for cell-based study due to their amalgamation of various modern analytical techniques, such as fluorescence, electrochemistry, and mass spectroscopy. 2D cell culture does not provide an adequate in vivo environment, where other cells surround the cells in a three-dimensional (3D) ECM (Edmondson et al., 2014). Cells under in vivo conditions both produce and continuously consume oxygen nutrients and other molecules, and such dynamic distributions are not mimicked in conventional 2D cell cultures. Moreover, 2D cell cultures fail to recapitulate

the highly complex 3D environment, function, and physiology of living tissues, the multitudinous regulatory interactions from surrounding tissue cells, the ECM, and other systemic factors that lead to nonpredictive data of an *in vivo* response (Li et al., 2012). The limitations of 2D cell culture systems have recently become more evident. Recent standard protocol advances in the fields of quantitative and system biology and imaging technology have allowed analysis of individual cells and observation of live individual cells growing in a natural physiological 3D environment. Cells cultured in a 3D model system more closely mimic *in vivo* conditions. Thus, unlike 2D cell cultures, which can sometimes cause misleading and nonpredictive data of *in vivo* responses, 3D systems are realistic for translating study findings. Compared to the 2D cell culture system, the 3D cell culture system provides a physiologically relevant and closer biomimetic environment, promotes better cell differentiation, and improves cell function (Edmondson et al., 2014). The 3D culture system holds great promise for applications in various fields, such as cancer cell biology, stem cell research, drug discovery, and various cell-based analyses and devices. While this culturing model offers state-of-the-art technology for facilitating drug development and numerous other applications, several hurdles remain before a universal, standardized, and validated system can be established (Sung et al., 2014). Recent developments in the transition from 2D to 3D cell cultures indicate promising applications for many industries; however, the cost of automation and easy-to-use readout systems are still key concerns.

The 3D cell culture system has provided a powerful tool that mimics a highly complex and dynamic *in vivo* environment, and it has gained greater momentum with the integration of microfluidic technology. Microfluidics is a technology characterized by the manipulation of fluids at the micron-scale for the improvement of diagnostics and cell culture research. It uses microfluidic devices to manipulate fluids in the small capillaries or microchannels. Microfluidics is a science of manipulating, mixing, monitoring, and analyzing minute volumes of fluids or gases on the surfaces of chips and microfluidic chips. This technology is ideal because it recreates the microenvironment of the vasculature and has become a powerful tool in cell culture research. It encompasses knowledge of the biological sciences, chemistry, physics, and engineering applications (Xu and Attinger, 2008). The microfluidic 3D cell culture model also allows precise spatial control over the gradients and medium exchange. It not only mimics but also promotes several biologically relevant functions not seen in the 2D cell culture. Furthermore, it has been increasingly used to generate high-throughput cell culture models and has shown

considerable promise for improving diagnostics and biological research (El-Ali et al., 2006).

Notably, microfluidic cell cultures are potential candidates for next generation cell analysis systems. Several 3D-based cell culture approaches have been created to provide a better biomimetic microenvironment for cells than those of 2D cultures. In addition, crucial liquid handling steps, including cell loading, nutrient supply, and waste removal—under physiologically relevant conditions—can be performed with real-time microscopy (Xu et al., 2014). Numerous microfluidic devices have been developed to not only provide nutrients and oxygen continuously for cell proliferation but also to investigate several characteristics of a dynamic 3D cell culture, such as differences in concentration, temperature gradients, and shear force conditions on cell transport and cultivation. Numerous microfluidic platforms for 3D cell culturing have been developed and based on the substrates used for microdevice fabrication, including glass/silicon-based, polymer-based, and paper-based platforms. Polydimethylsiloxane (PDMS)-based microdevices are the predominant form of microfluidic 3D cell culture systems because they are economical and allow permeability of O<sub>2</sub>, which is vital in cell proliferation. To provide an *in vivo*-like environment that resembles living tissues, several natural polymers, such as collagen, fibrin, and agarose, have been used to fabricate microfluidic devices (Li et al., 2012).

### **Applications**

Microfluidics technology has emerged as a viable and robust platform for tissue engineering—a multidisciplinary field aimed at replacing and repairing damaged and diseased tissues and/or organs and developing *in vitro* models to mimic physiological conditions. Successful clinical applications include the development of organ-on-a-chip technology—a microfluidic perfusion device for regenerative medicine—and a chip-based platform for the culture of cells and toxicological studies.

### **Organ-on-a-chip technology**

Scientists currently rely on *in vitro* cell culture platforms and *in vivo* animal models to study biological processes and develop therapeutic strategies, although informative have significant shortcomings (Ziółkowska et al., 2011). *In vitro* platforms may not simulate the intricate cell–cell and cell–matrix interactions that are vital to regulating cell behavior *in vivo* (Guillouzo & Guguen-Guillouzo, 2008). Organ-on-a-chip devices could offer biological relevance and be a requisite for high-throughput applications. An organ-on-a-chip is a microfluidic cell culture device comprising a microchip with continuously perfused chambers that are

infused with living cells that are arranged to mimic the 3D tissue microenvironment and physiology (Ghaemmaghami et al., 2012). These chips have the potential to significantly impact drug discovery and toxicity testing (Ghaemmaghami et al., 2012). The simplest functional unit of organ-on-a-chip devices consists of a single, perfused microfluidic chamber that is composed of a single type of cultured cell. These systems are utilized for studying organ-specific responses, chemical responses, such as drugs or toxins, and physical stimuli. In a complex system, two or more independently perfused parallel microchannels are connected by porous membranes to recreate interfaces between different tissues.

### **Tissue models on a chip**

Numerous tissue models have been developed to mimic in vivo biological processes. On-chip tissue models include those for the liver, kidney, lungs, intestines, muscle, fat, and blood vessels as well as models of tumors.

### **Liver-on-a-chip**

Various chemicals and drugs, when administered over a long period, result in adverse effects and acute liver toxicity, known as hepatotoxicity (Gershell & Atkins, 2003). In vitro models used for identifying drug-induced liver toxicity have drastically limited utility. Therefore, efficient and reliable tools for testing liver toxicity are required. Microfluidics devices for liver tissue and cells that can maintain metabolic activity and can be used for drug discovery and toxicity studies have shown great potential for solving this problem.

Bioreactors with a perfused multiwell plate device were developed by Domansky et al. (2010) to recapitulate both the physiological and mechanical microenvironments of hepatocytes that can support both growth and functional integrity for up to 1 week. Khetani and Bhatia (2008) developed microscale cultures of human liver cells in a multiwell micropatterned co-culture system that can maintain phenotypic functions of liver cells for up to several weeks.

### **Tumor-on-a-chip**

A significant challenge for cancer research is the early detection and development of in vitro strategies for studying the role of drug-carrier design in tumor transport and therapies for targeting rapidly dividing cancer cells while leaving normal, healthy cells untouched. The microfluidics tumor-on-a-chip platform can be used for detecting circulating tumor cells (CTCs) in blood flow, which may lead to early diagnosis of cancer (Millner et al., 2013). A variety of designs for studying the microenvironment of microfluidic

devices that culture solid and liquid tumors were reviewed by Young (2013). Tatosian and Shuler (2009) developed a novel microfluidic system to study the multidrug resistance of cancer cells to chemotherapeutic combinations. Jang et al. (2011) fabricated a microfluidic device with an active injection system that produced 64 of 100 combinations of different chemical solutions at various concentrations and stored them in isolated chambers. To optimize system parameters for varied types of cancer cells while requiring minute amounts of reagents and cells, Jedrych et al. (2011) generated a microfluidics system for photodynamic therapy-based measurements. This system allows light-induced photosensitizers to be delivered to the carcinoma cells, which—on reaction with oxygen—produce a chemical toxin that is lethal to tumor cells.

## World Wide Web resources

1. <http://www.fda.gov>  
<http://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/vaccines/ucm202439.pdf>  
<http://www.fda.gov/BiologicsBloodVaccines/ApprovedProducts/ucm205541.htm>  
 The Food and Drug Administration (FDA or USFDA) protects and promotes public health through the regulation of all foods (except meats and poultry), the nation's blood supply, and other biologics (such as vaccines and transplant tissues). Drugs must be tested, manufactured, and labeled according to FDA standards before they can be sold or prescribed.
2. <http://www.promega.com>  
<http://www.promega.com/~ /media/files/products%20and%20services/na/webinars/mechanism%20of%20toxicitywebinar2.pdf?la=en>  
 Promega manufactures enzymes and other products for biotechnology and molecular biology.
3. <http://www.who.int>  
[http://www.who.int/biologicals/publications/trs/areas/vaccines/cells/WHO\\_TRS\\_878\\_A1Animalcells.pdf](http://www.who.int/biologicals/publications/trs/areas/vaccines/cells/WHO_TRS_878_A1Animalcells.pdf)  
 The World Health Organization (WHO) is a specialized agency that is concerned with international public health. It is affiliated with the United Nations and headquartered in Geneva, Switzerland. WHO ensures that more people, especially those living in dire poverty, have access to equitable, affordable care, so that they can lead healthy, happy, and productive lives.
4. <http://amgenscholars.com>

- <http://amgenscholars.com/images/uploads/contentImages/biotechnology-timeline.pdf>  
Amgen Scholars provides hundreds of undergraduate students with the opportunity to engage in a hands-on summer research experience at some of the world's leading institutions.
5. <http://monographs.iarc.fr>  
<http://monographs.iarc.fr/ENG/Monographs/vol90/mono90-6.pdf>  
The IARC monographs identify environmental factors that can increase the risk of human cancer. These include chemicals, complex mixtures, occupational exposures, physical agents, biological agents, and lifestyle factors.
  6. [www.iptonline.com](http://www.iptonline.com)  
<http://www.iptonline.com/articles/public/IPTFIVE76NP.pdf>  
IPTonline publishes "The Pharmaceutical Technology Journal," which is designed to provide information on the latest ideas, cutting-edge technologies, and innovations shaping the future of pharmaceutical research, development, and manufacturing.
  7. <http://www.aceabio.com>  
[http://www.aceabio.com/UserFiles/doc/literature/xcell\\_appnotes/RTCA\\_AppNote07\\_ACEA\\_LoRes.pdf](http://www.aceabio.com/UserFiles/doc/literature/xcell_appnotes/RTCA_AppNote07_ACEA_LoRes.pdf)  
ACEA Biosciences, Inc. (ACEA) is a privately owned biotechnology company. ACEA's mission is to transform cell-based assays by providing innovative and cutting-edge products and solutions to the research and drug discovery community.
- ## References
- Aucoin, M.G., Jacob, D., Chahal, P.S., Meghrou, J., Bernier, A., Kamen, A.A., 2007. Virus-like particle and viral vector production using the baculovirus expression vector system/insect cell system. *Methods Mol. Biol.* 388, 281–296.
- Bhat, S.M., 2011. *Animal Cell Culture Concept and Application*. Alpha Science International Limited, Oxford.
- Caplen, H., Peters, B.J., Bishop, D.H.L., 1985. Mutagen-directed attenuation of Rift Valley fever virus as a method for vaccine development. *J. Gen. Virol.* 66, 2271–2277.
- Chakraborty, S., Reid, S., 1999. Serial passage of a Helicoverpa armigera nucleopolyhedrovirus in Helicoverpa zea cell cultures. *J. Invertebr. Pathol.* 73, 303–308.
- Chen, T.H., Liu, W.C., Chen, I.C., Liu, C.C., Huang, M.H., Jan, J.T., et al., 2019. Recombinant hemagglutinin produced from Chinese Hamster Ovary (CHO) stable cell clones and a PELC/CpG combination adjuvant for H7N9 subunit vaccine development. *Vaccine* 37 (47), 6933–6941.
- Cho, M.H., Niles, A., Huang, R., Inglese, J., Austin, C.P., Riss, T., et al., 2008. A bioluminescent cytotoxicity assay for assessment of membrane integrity using a proteolytic biomarker. *Toxicol. Vitro* 22, 1099–1106.
- Clarke, B.E., Newton, S.E., Carroll, A.R., Francis, M.J., Appleyard, G., Syred, A.D., et al., 1987. Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein. *Nature* 330, 381–384.
- Cruz, P.E., Maranga, L., Carrondo, M.J.T., 2002. Integrated process optimisation: lessons from retrovirus and virus like production. *J. Biotechnol.* 99, 199–214.
- Domansky, K., Inman, W., Serdy, J., Dash, A., Lim, M.H., Griffith, L. G., 2010. Perfused multiwell plate for 3D liver tissue engineering. *Lab. a chip* 10 (1), 51–58.
- Eagle, H., 1959. Amino acid metabolism in mammalian cell cultures. *Science* 130, 432–437.
- Edmondson, R., Broglie, J.J., Adcock, A.F., Yang, L., 2014. Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors. *Assay. Drug. Technol.* 12 (4), 207–218.
- El-Ali, J., Sorger, P.K., Jensen, K.F., 2006. Cells on chips. *Nature* 442 (7101), 403–411. FDA guidelines. (<http://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/vaccines/ucm202439.pdf>2012).
- Fraser, M.J., Hink, W.F., 1982. The isolation and characterization of the MP and FP plaque variants of Galleria mellonella nuclear polyhedrosis virus. *Virology* 117, 366–378.
- Freshney, R.I., 1994. *Culture of Animal Cells: A Manual of Basic Technique*, 3rd ed. Wiley-Liss Inc, New York.
- Freshney, R.I., 2010. *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications*. Wiley, John & Sons, Inc, New Jersey.
- Freshney, R.I., 2011. *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications*. Wiley, John & Sons, Inc, New Jersey.
- Gershell, L.J., Atkins, J.H., 2003. A brief history of novel drug discovery technologies. *Nat. Rev. Drug. Discovery* 2 (4), 321–327.
- Ghaemmaghami, A.M., Hancock, M.J., Harrington, H., Kaji, H., Khademhosseini, A., 2012. Biomimetic tissues on a chip for drug discovery. *Drug. Discov. Today* 17 (3), 173–181.
- Guillouzo, A., Guguen-Guillouzo, C., 2008. Evolving concepts in liver tissue modeling and implications in vitro toxicology. *Expert. Opin. drug. Metab. Toxicol.* 4 (10), 1279–1294.
- Hassan, F., Ren, D., Zhang, W., Gu, X.-X., 2012. Role of c-Jun N-terminal protein kinase 1/2 (JNK1/2) in macrophage-mediated MMP-9 production in response to Moraxella catarrhalis lipooligosaccharide (LOS). *PLoS ONE* 7, e37912.
- Hayflick, L., Moorhead, P.S., 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* 3, 585–621.
- Ho, Y., Lin, P.H., Liu, C.Y., Lee, S.P., Chao, Y.C., 2004. Assembly of human severe acute respiratory syndrome coronavirus-like particles. *Biochemical and biophysical research communications* 318 (4), 833–838.
- Jang, Y.H., Hancock, M.J., Kim, S.B., Selimović, Š., Sim, W.Y., Bae, H., et al., 2011. An integrated microfluidic device for two-dimensional combinatorial dilution. *Lab. a Chip* 11 (19), 3277–3286.
- Jedrych, E., Pawlicka, Z., Chudy, M., Dybko, A., Brzozka, Z., 2011. Evaluation of photodynamic therapy (PDT) procedures using microfluidic system. *Analytica Chim. acta* 683 (2), 149–155.
- Jeoung, H.Y., Lee, W.H., Jeong, W., Shin, B.H., Choi, H.W., Lee, H.S., et al., 2011. Immunogenicity and safety of the virus-like particle of the porcine encephalomyocarditis virus in pig. *Virology J.* 8, 170.
- Jiang, B., Barniak, V., Smith, R.P., Sharma, R., Corsaro, B., Hu, B., et al., 1998. Synthesis of rotavirus-like particles in insect cells: comparative and quantitative analysis. *Biotechnol. Bioeng.* 60, 369–374.

- Justin, C., Masum, H., Perampaladas, K., Heys, J., Singer, P.A., 2011. Indian vaccine innovation: the case of Shantha Biotechnics. *Globalization Health* 7, 9.
- Khetani, S.R., Bhatia, S.N., 2008. Microscale culture of human liver cells for drug development. *Nat. Biotechnol.* 26 (1), 120–126.
- Li, X.J., Valadez, A.V., Zuo, P., & Nie, Z. (2012). Microfluidic 3D cell culture: potential application for tissue-based bioassays.
- Licata, J.M., Johnson, R.F., Han, Z., Harty, R.N., 2004. Contribution of Ebola virus glycoprotein, nucleoprotein, and VP24 to budding of VP40 virus-like particles. *Journal of virology* 78 (14), 7344–7351.
- Maurer, P., Jennings, G.T., Willers, J., Rohner, F., Lindman, Y., Roubicek, K., et al., 2005. A therapeutic vaccine for nicotine dependence: preclinical efficacy, and phase I safety and immunogenicity. *Eur. J. Immunology* 35, 2031–2040.
- Mello, I.M.V.G., Meneghesso da Conceicao, M., Jorge, S.A.C., Cruz, P.E., Alves, P.M.M., Carrondo, M.J.T., et al., 2008. Viral vaccines: concepts, principles, and bioprocesses. In: Castilho, L.R., Moraes, A.M., Augusto, E.F.P. (Eds.), *Animal Cell Technology: From Biopharmaceuticals to Gene Therapy*. Taylor & Francis Group, New York, pp. 435–458.
- Millner, L.M., Linder, M.W., Valdes, R., 2013. Circulating tumor cells: a review of present methods and the need to identify heterogeneous phenotypes. *Ann. Clin. Lab. Sci.* 43 (3), 295–304.
- Moore, A., Donahue, C.J., Hooley, J., Stocks, D.L., Bauer, K.D., Mather, J.P., 1995. Apoptosis in CHO cell batch cultures: examination by flow cytometry. *Cytotechnology* 17, 1–11.
- Mortola, E., Roy, P., 2004. Efficient assembly and release of SARS coronavirus-like particles by a heterologous expression system. *FEBS Lett* 576, 174–178.
- Parez, N., Garbarg-Chenon, A., Fourgeux, C., Le Deist, F., Servant-Delmas, A., Charpilienne, A., et al., 2004. The VP6 protein of rotavirus interacts with a large fraction of human naive B cells via surface immunoglobulins. *Journal of virology* 78 (22), 12489–12496.
- Smithburn, K.C., 1949. Rift Valley fever: the neurotropic adaption of virus and experimental use of this modified virus as a vaccine. *Br. J. Exp. Pathol.* 30, 1–16.
- Stacey, G.N., Davis, J., 2007. *Medicines from Animal Cell Culture*. John Wiley & Sons, Chichester.
- Sung, J.H., Srinivasan, B., Esch, M.B., McLamb, W.T., Bernabini, C., Shuler, M.L., et al., 2014. Using physiologically-based pharmacokinetic-guided “body-on-a-chip” systems to predict mammalian response to drug and chemical exposure. *Exp. Biol. Med.* 1535370214529397.
- Swenson, D.L., Warfield, K.L., Negley, D.L., Schmaljohn, A., Aman, M.J., Bavari, S., 2005. Virus-like particles exhibit potential as a pan-filovirus vaccine for both Ebola and Marburg viral infections. *Vaccine* 23 (23), 3033–3042.
- Tatosian, D.A., Shuler, M.L., 2009. A novel system for evaluation of drug mixtures for potential efficacy in treating multidrug resistant cancers. *Biotechnol. Bioeng.* 103 (1), 187–198.
- van Dijk, M.A., van de Winkel, J.G.J., 2001. Human antibodies as next generation therapeutics. *Curr. Opin. Chem. Biol.* 5, 368–374.
- Walsh, G., 2003. *Biopharmaceuticals – Biochemistry and Biotechnology*, 2nd ed. John Wiley and Sons, Chichester.
- Wang, C., Zheng, X., Gai, W., Zhao, Y., Wang, H., et al., 2017. MERS-CoV virus-like particles produced in insect cells induce specific humoral and cellular immunity in rhesus macaques. *Oncotarget* 8 (8), 12686.
- Wang, M.Y., Kuo, Y.Y., Lee, M.S., Doong, S.R., Ho, J.Y., Lee, L.H., 2000. Self-assembly of the infectious bursal disease virus capsid protein, rVP2, expressed in insect cells and purification of immunogenic chimeric rVP2H particles by immobilized metal-ion affinity chromatography. *Biotechnol. Bioeng.* 67, 104–111.
- Wood, H.A., 1980. Isolation and replication of an occlusion body-deficient mutant of the *Autographa californica* nuclear polyhedrosis virus. *Virology* 105, 338–344.
- Xu, J., Attinger, D., 2008. Drop on demand in a microfluidic chip. *J. Micromech. Microeng.* 18 (6), 065020.
- Xu, X., Farach-Carson, M.C., Jia, X., 2014. Three-dimensional in vitro tumor models for cancer research and drug evaluation. *Biotechnol. Adv.* 32 (7), 1256–1268.
- Yamshchikov, G.V., Ritter, G.D., Vey, M., Compans, R.W., 1995. Assembly of SIV virus-like particles containing envelope proteins using a baculovirus expression system. *Virology* 214 (1), 50–58.
- Young, E.W., 2013. Cells, tissues, and organs on chips: challenges and opportunities for the cancer tumor microenvironment. *Integr. Biol.* 5 (9), 1096–1109.
- Ziółkowska, K., Kwapiszewski, R., Brzózka, Z., 2011. Microfluidic devices as tools for mimicking the in vivo environment. *N. J. Chem.* 35 (5), 979–990.

## Further reading

- Castilho, L.R., Moraes, A.M., Augusto, E.F.P., 2008. *From Biopharmaceuticals to Gene Therapy*. Animal Cell Technology. Taylor & Francis Group, New York.
- Freshney, R.I., 2015. *Culture of Animal Cells: A Manual of Basic Techniques and Specialized Applications*, 7<sup>th</sup> Edition Wiley Blackwell, USA.
- Kolesnikova, S., Moiseeva, I., 2017. Prospects for the use of animal cell cultures in screening of pharmaceutical substances. *J. Phys.: Conf. Ser.* 784, 012028. Available from: <https://doi.org/10.1088/1742-6596/784/1/012028>.
- Luo, Tao, et al., 2019. Microfluidic single-cell manipulation and analysis: methods and applications. *Micromachines* 10 (2), 104.
- Naskalska, A., Dabrowska, A., Szczepanski, A., Milewska, A., Jasik, K.P., Pyrc, K., 2019. Membrane protein of human coronavirus n163 is responsible for interaction with the adhesion receptor. *J. Virology* 93 (19), e00355–19.
- Polat, A.N., Özlü, N., 2014. Towards single-cell LC-MS phosphoproteomics. *Analyst* 139, 4733–4749.
- Stacey, G.N., Davis, J., 2007. *Medicines from Animal Cell Culture*. John Wiley & Sons, Chichester.
- Verma, A.S., Singh, A., 2014. *Animal Biotechnology: Models in Discovery and Translation*, 1st Edition Academic Press, USA.
- Yu, F., Hunziker, W., Choudhury, D., 2019. Engineering microfluidic organoid-on-a-chip platforms. *Micromachines* 10 (3), 165.

## Glossary

- Antigen** Any substance that causes your immune system to produce antibodies against it.
- Aseptic** Free from pathogenic microorganism.
- Cell culture** To grow in vivo
- Cytotoxicity** The degree to which an agent has specific destructive action on certain cells.
- Differentiation** A change in a cell causing an increase in morphological or chemical heterogeneity.
- Immortalized** Changing a cell type with limited lifespan in vitro into a cell type with unlimited capacity to proliferate; sometimes achieved by animal cells in vitro or by tumor cells.
- In vitro** Cell growth outside the body, in glass, as in a test tube.
- In vivo** Cell growth in a living organism.



**Medium** A buffered selection of components in which an organism naturally lives or grows.

**Monolayer** A single layer of adherent cells on substratum.

**Passage** The process of passing or maintaining cells through a series of hosts or cultures.

**Primary culture** A culture initiated from an explant of cells, tissues, or organs in media conducive to their growth.

**Trypsinization** Use of the enzyme trypsin to remove adherence proteins from a cell surface.

**tPA** Tissue plasminogen activator  
**VLPs** Virus-like particles

## Abbreviations

<b>AcMNPV</b>	<i>Autographa California</i> nucleopolyhedroviruses
<b>BHK</b>	Baby Hamster kidney
<b>CD4</b>	Glycoprotein on the surface of Helper T cells that serve as a receptor for HIV
<b>CDM-HD</b>	Chemically defined medium
<b>CHO</b>	Chinese Hamster ovary
<b>CPE</b>	Cytopathogenic effect
<b>DIP</b>	Defective infective particle
<b>DME</b>	Dulbecco's modified eagle's media
<b>EDTA</b>	Ethylenediaminetetraacetic acid
<b>EGF</b>	Epidermal growth factor
<b>ELISA</b>	Enzyme-linked immunosorbent assay
<b>FBS</b>	Fetal bovine serum
<b>Fp</b>	Few polyhedral mutations
<b>FPERT</b>	Real-Time fluorescent product-enhanced reverse transcriptase assay
<b>GF-AFC</b>	Glycyl-phenylalanyl-amino-fluorocoumarin
<b>GmMNPV</b>	<i>Galleria mellonella</i> nucleopolyhedroviruses
<b>HaSNPV</b>	<i>Helicoverpa armigera</i> nucleopolyhedroviruses
<b>HBcAg</b>	Hepatitis B core antigen
<b>HBV</b>	Hepatitis B virus
<b>HEK</b>	Human embryonic kidney
<b>HeLA</b>	Established human epithelial cell line derived from cervical carcinoma
<b>hESCs</b>	Human embryonic stem cells
<b>Hi-5</b>	Cells (BTI-TN-5B1-4) derived from the parental <i>Trichopulsia ni</i> cell line
<b>HPV</b>	Human papilloma virus
<b>HPV18</b>	Human papilloma virus 18
<b>IFN</b>	Interferon
<b>IL-2</b>	Interleukin-2
<b>L1 VLP</b>	HPV with L1 major capsid protein
<b>LDH</b>	Lactate dehydrogenase
<b>mAB</b>	Monoclonal antibody
<b>MEM</b>	Minimum essential media
<b>MMT</b>	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
<b>MP12</b>	Strain invented by serial mutagenesis of RVF virus with Egyptian ZH501 and ZH548 strains
<b>MTS</b>	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
<b>PDGF</b>	Platelet-derived growth factor
<b>PTM</b>	Post-translation modifications
<b>QPERT</b>	Quantitative real time for fluorescent product-enhanced reverse transcript assay
<b>rDNA</b>	Recombinant DNA
<b>RT</b>	Real-time assays
<b>SNS</b>	Smithburn neurotropic strain
<b>STO</b>	Mouse embryonic fibroblast cell line
<b>TGF</b>	Transforming growth factor
<b>TGF-B</b>	Transforming growth factor beta

## Long answer questions

1. What are the components of serum and how do they help the cell culture?
2. What is the role of media in animal cell culture?
3. What are the advantages and limitations of animal tissue culture?
4. How can cell viability and cytotoxicity be tested in cell culture?
5. What is the role of cell culture in gene therapy and viral vaccines?
6. How can microfluidics revolutionize animal tissue culture?

## Short answer questions

1. What is the Hayflick effect?
2. What is the source of cells for primary monolayer cell culture?
3. Serum is one of the basic components of cell culture media (true/false)?
4. What was the first recombinant human protein?
5. What are the different phases of the growth curve?
6. Is the VLP-based HPV vaccine approved by the FDA?

## Answers to short answer questions

1. Limited replication capacity of cells in culture medium.
2. Organ/tissue of live animal.
3. False.
4. Somatostatin.
5. Lag phase, log phase, and plateau phase.
6. Yes, Gardasil (the first HPV vaccine) was approved by the FDA in 2006.

## Yes/no type questions

1. Are cells obtained directly from organs and tissues in primary cell culture?
2. Is secondary culture used for studying transformed cells?
3. Is identity testing a way to determine purity of culture?
4. Is IFN- $\alpha$  used for the treatment of multiple sclerosis?

5. Is Bevacizumab approved for the treatment of colorectal cancer?
  6. Does passage effect leads to an increase in the virulence of cultured viruses?
  7. Do stem cells can not differentiate into other kinds of cells?
  8. Microfluidic devices provide nutrients and oxygen for cell proliferation.
  9. Living cells are used in organ-on-a-chip microfluidic cell culture.
  10. Can embryonic cells be cultured without any feeder layer?
2. Yes—Secondary cultures are used in the study of transformed cells as these cultures maintain their cellular characteristics.
  3. No—For testing the purity, one should use fluorescent staining PCR or ELISA.
  4. No—IFN- $\beta$  is used in the treatment of multiple sclerosis.
  5. Yes—It is an inhibitor of vascular endothelial growth factor.
  6. No—Passage effect leads to viral instability.
  7. No—Stem cells can differentiate into other kind of cell types.
  8. Yes—Microfluidic devices also help in investigating characteristics of 3D cell culture.
  9. Yes—Chambers of organ-on-a-chip devices are continuously infused with living cells.
  10. Yes—Martigel from BD biosciences can be used to coat the culture plate.

### Answers to yes/no type questions

1. Yes—Mechanical, chemical, or enzymatic disintegration of tissues and organs is required in primary cell culture.