People's Democratic Republic of Algeria
Ministry of Higher Education and Scientific Research

University of 8 Mai 1945, Guelma. Algeria

Faculty of Life and Natural Sciences, Earth and Universe Sciences
Department of Biology
Course for students of Master I Applied Immunolgy

Cell Culture

Prepared by

Dr. Awatif BOUMAZA
PhD. Cellular and Molecular Toxicology

========== 2024 ===========

Contents

Chapter I: Introduction to Cell Culture

1. Historical background ……..………………………………………………………...5
2. Definitions 6
2.1. What is Cell Culture? 6
2.2. Tissue culture 6
2.3.Organ culture 6
2.4. Primary Culture 6
2.5. Cell Line or secondary culture 7
2.6. Cell Strain 7
2.7. Finite vs Continuous Cell Line 7
2.8. Culture Conditions 7
2.9. Cryopreservation 7
2.10. Passaging 7
2.11. Trypsination 8
3. Types of cultured Cells 8
3.1. Primary cells 8
3.2. Transformed cells 8
3.3. Self-renewing cells 8
4. Cell morphology types 8

Chapter II: Cell Culture Laboratory and Safety

1.Safety in cell culture laboratory………………………………………………………………11
1.1.Biosafety Levels ……………………………………………………………………………11
1.2.Safety Equipment……………………………………………………………………………12
1.2.1.Safety Data Sheet (SDS) ……………………………………………………………………12
1.2.2.Personal Protective Equipment (PPE) ………………………………………………………13
1.3.Safe Laboratory Practices ………………………………………………………………...14
2.Cell Culture Equipment ……………………………………………………………………15
2.1.Basic Equipment for cell culture …………………………………………………………16
2.1.1.Clean Benches ……………………………………………………………………………16
2.1.1.1.Types of laminar flow cabinets …………………………………………………………16
2.1.2.Cell culture hood …………………………………………………………………………17
2.1.2.1.Classes of cell culture hoods ……………………………………………………………18
2.1.2.2.Air-Flow Characteristics of Cell Culture Hoods …………………………………………..19
2.1.2.3. Components/ Parts of Laminar flow hood (biosafety cabinets) ……………………………..19

2.1.2.4. Principle/Working of Laminar flow hood ……………………………………………………..20
2.1.2.5.Procedure for running the laminar flow cabinets ………………………………………………20
2.1.2.6.Cell Culture Hood Layout ……………………………………………………………………21
2.1.2.7.Precautions …………………………………………………………………………………...22
2.1.2.8.Biosafety cabinets vs clean benches …………………………………………………………23
3.3. Incubator …………………………………………………………………………………………24
3.3.1. Types of Incubators ……………………………………………………………………………25
3.4.Storage ……………………………………………………………………………………………25
3.5. Refrigerators ……………………………………………………………………………………..25
3.6. Freezers …………………………………………………………………………………………26
3.7.Cryogenic Storage ………………………………………………………………………………..26
3.8.Cell Counter ……………………………………………………………………………………...26

Chapter III: Cell Culture and Asepsis

1.Cell culture sterility ………………………………………………………………………28
2.Aseptic techniques …………………………………………………………………………28
2.1.Sterile Work Area …………………………………………………………………………28
2.2.Good Personal Hygiene ……………………………………………………………………29
2.3.Sterile Reagents and Media ………………………………………………………………..29
2.4.Sterile Handling ……………………………………………………………………………...29
3. Sterilization methods ………………………………………………………………… .30
3.1.Définition …………………………………………………………………………………..30
3.2.Importance of Sterilization …………………………………………………………………...30
3.3. Methods ……………………………………………………………………………………31
3.3.1.Physical Sterilization ………………………………………………………………………..31
3.3.2. Chemical Sterilization ……………………………………..…………………………..33
4.Cell culture contamination ……………………….………………………………………34
4.1.Quarantine and receipt of animal cell lines ……………………………………………35
4.1.1.Accessioning scheme ……………………………………………………………………..35
4.1.2.Management of cell lines in quarantine ……………………………………………..35
4.1.3.Quarantine laboratory design ……………………………………………………………..38
4.2.Microbial quality control …………………………………………………………………..38
4.2.1. Sources of contamination ……………………………………………………………38
4.2.2.Tests for bacteria, yeasts and fungi ……………………………………………………39
4.2.3.Tests for mycoplasma ……………………………………………………………………39
4.2.4.Virus testing ……………………………………………………………………………39
4.2.4.1.Sources of viral contamination ………………………………………………………40
4.3.Eradication of contamination ………………………………………………………42
5.Authentication ………………………………………………………………………………...42

Chapter IV: Cell Culture Media and Techniques

1.Culture media 45
1.1.Basic components in culture media 45
1.2. Natural media... 45
1.3. Artificial media 46

2.Animal cell culture *in vitro* 47
2.1.Basic concepts 48
2.2.Methods for Obtaining Cells 48
2.2.1.Organ culture 48
2.2.2.Primary explant culture 49
2.2.3.Cell culture 49
2.3.Freezing Cells and Thawing Cryopreserved Cells 50
Freezing Cells 50
Thawing Cryopreserved Cells 50
2.4.Cell passage and use of trypsin 51
2.5.Quantitation 52
2.6.Cell viability 54
3.Animal cell growth *in vitro*: 54
3.1.cycle and evolution 54
3.2.Behavior of cells *in vitro* 55

Chapter V: Cell Culture Applications

1. Model Systems in Health and Disease 57
2.Drug Development and Drug Testing 57
3.Virology and Vaccine Production 57
4.Tissue Regeneration and Transplantation 57
5.Genetic Engineering and Gene Therapy 58
*6.*Key limitations 58
6.Discrepancies between Cellular Environments *in vitro* and *in vivo* 58
7.Techniques histologiques 59
8.Techniques cytologiques 59
9.Techniques immuno-histochimiques 59
References 64

CHAPTER I
Introduction to Cell Culture

1. Historical background

Tissue culture as a technique was first used almost 100 years ago to elucidate some of the most basic questions in developmental biology. At the present time, animal and human cell cultures are significant tools widely used in many branches of life science. Different variants of cell culture found application in modelling diseases, IVF (*in vitro* fertilization) technology, stem cell and cancer research, monoclonal antibody production, regenerative medicine and therapeutic protein production. All those different scientific approaches would not be possible without some crucial discoveries that had been made over the centuries from Aristotelian spontaneous generation doctrine through Pasteur's experiments and Carrel's cell culture to large-scale cultures for therapeutic proteins production and vision of the future of regenerative medicine and *in situ* bioprinting of wounds. The main milestones in cell cultures are summarized in Figure 1. (1,2)

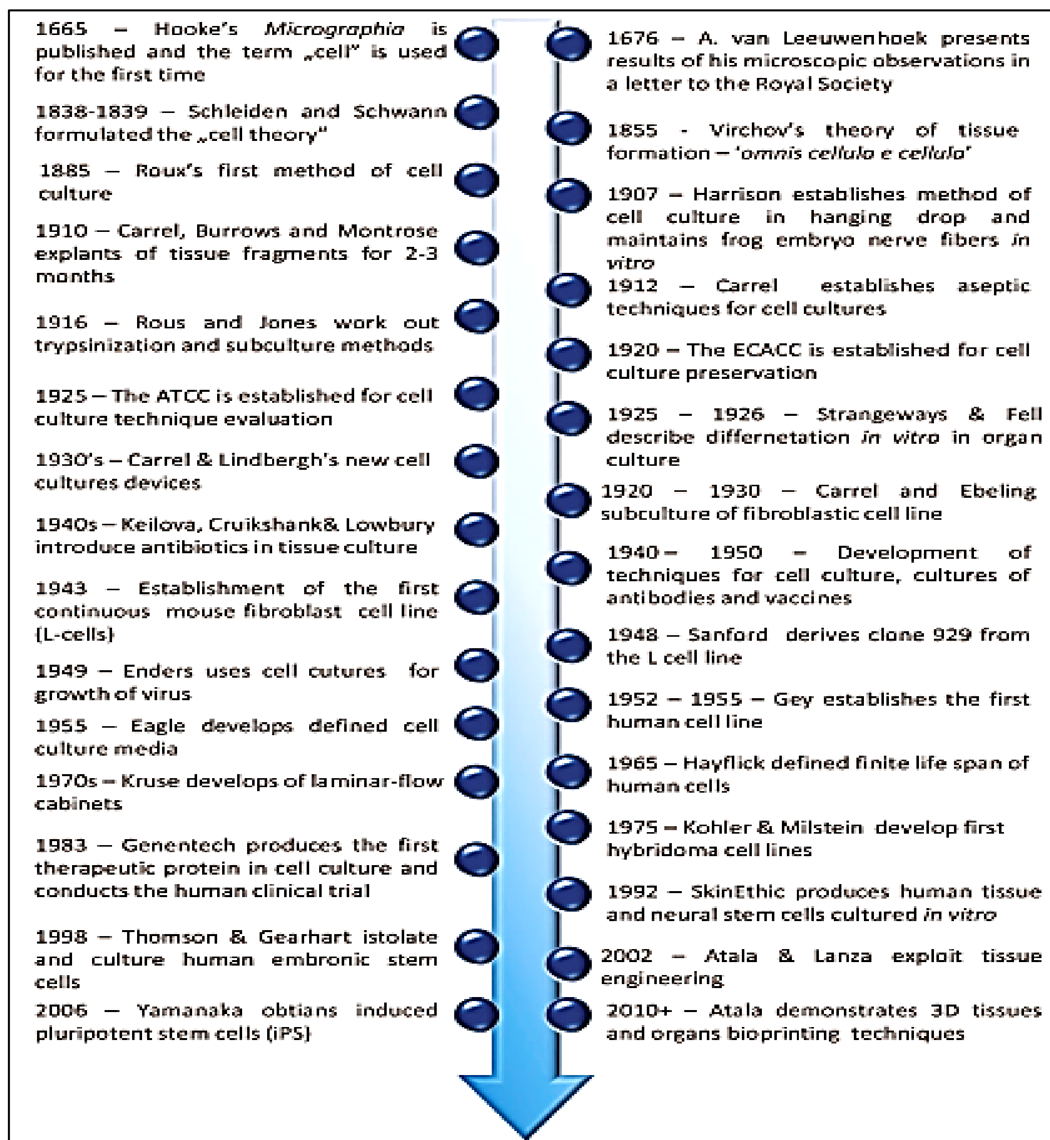


Figure 1. Timeline: key milestone in cell cultures (2)

2. Definitions

Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (e.g., metabolic studies, aging), the effects of drugs and toxic compounds on the cells, and mutagenesis and carcinogenesis. It is also used in drug screening and development, and large scale manufacturing of biological compounds (e.g., vaccines, therapeutic proteins). The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells.

2.1. What is Cell Culture?

Cell culture refers to laboratory methods that enable the growth of eukaryotic or prokaryotic cells in physiological conditions. Cells can be removed from an animal or plant for their subsequent growth in a favorable artificial environment. The cells may be obtained from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has already been established (3).

2.2. Tissue culture is the growth of tissues or cells in an artificial medium separate from the parent organism. This technique is also called micropropagation (3).

2.3.Organ culture is the cultivation of either whole organs or parts of organs *in vitro*. It is a development from tissue culture methods of research, as the use of the actual *in vitro* organ itself allows for more accurate modelling of the functions of an organ in various states and conditions. A key objective of organ culture is to maintain the architecture of the tissue and direct it towards normal development. In this technique, it is essential that the tissue is never disrupted or damaged. It thus requires careful handling. The media used for a growing organ culture are generally the same as those used for tissue culture. (2)

2.4. Primary Culture: Primary culture refers to the stage of the culture after the cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate (i.e., reach confluence). At this stage, the cells have to be subcultured (i.e., passaged) by transferring them to a new vessel with fresh growth medium to provide more room for continued growth. H4. Primary culture can consist of the culture of a complex organ or tissue slice, a defined mixture of cells, or highly purified cells isolated directly from the organism. (3)

2.5. Cell Line or secondary culture: After the first subculture, the primary culture becomes known as a cell line or subclone. Cell lines derived from primary cultures have a limited life span (i.e., they are finite), When a primary culture reaches confluency it must be removed from its current medium and transferred to a fresh growth medium. This ensures that the cells stay at an optimal density for continued growth and proliferation. Due to the providing of survival factors continuously, cells can acquire an indefinite lifespan, and cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population. (3)

2.6. Cell Strain If a subpopulation of a cell line is positively selected from the culture by cloning or some other methods, this cell line becomes a cell strain. A cell strain often acquires additional genetic changes subsequent to the initiation of the parent line. (3)

2.7. Finite vs Continuous Cell Line: Normal cells usually divide only a limited number of times before losing their ability to proliferate, which is a genetically determined event known as senescence; these cell lines are known as finite. However, some cell lines become immortal through a process called transformation, which can occur spontaneously or can be chemically or virally induced. When a finite cell line undergoes transformation and acquires the ability to divide indefinitely, it becomes a continuous cell line. (1,3)

2.8. Culture Conditions: culture conditions vary widely for each cell type, but the artificial environment in which the cells are cultured invariably consists of a suitable vessel containing a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, and gases (O_2, CO_2), and regulates the physico-chemical environment (pH, osmotic pressure, temperature). Most cells are anchorage-dependent and must be cultured while attached to a solid or semi-solid substrate (adherent or monolayer culture), while others can be grown floating in the culture medium (suspension culture) (2)

2.9. Cryopreservation: If a surplus of cells is available from subculturing, they should be treated with the appropriate protective agent (e.g., DMSO or glycerol) and stored at temperatures below –130°C (cryopreservation) until they are needed (2).

2.10. Passaging is the procedure of harvesting cells from a culture, transferring the cells to one or more culture vessels with fresh growth medium, and using those cells to start new cultures. It is also referred to as subculturing (2)

2.11.Trypsinization: is the process of cell dissociation using trypsin, a proteolytic enzyme which breaks down proteins, to dissociate adherent cells from the vessel in which they are being cultured. When added to cell culture, trypsin breaks down the proteins that enable the cells to adhere to the vessel. Trypsinization is often used to pass cells to a new vessel. When the trypsinization process is complete the cells will be in suspension and appear rounded (2)

3. Types of cultured Cells: Cells cultured in the lab can be classified into three different types: ***primary cells***, ***transformed cells***, and ***self-renewing cells*** (4).

3.1. Primary cells, such as fibroblasts obtained from skin biopsies and hepatocytes isolated from liver explants, are directly isolated from human tissue. Biomedical and translational research oftentimes relies on using these cell types since they are good representatives of their tissue of origin. However, there are stringent biosafety restrictions associated with handling these cell types. Furthermore, primary cells are generally characterized as "finite" and therefore rely on a continuous supply of stocks since their proliferation ceases after a limited amount of cell divisions and cell expansion is oftentimes impossible.

3.2. Transformed cells can be generated either naturally or by genetic manipulation. While the use of such immortalized cell lines leads to a cellular platform that generates fast growth rates and stable conditions for maintenance and cloning, their manipulated genotype may result in karyotypic abnormalities and nonphysiological phenotypes. On the other hand, standardized cell lines derived from human or nonhuman species or (e.g., *Chinese hamster ovary* (CHO), HeLa, *human umbilical vein endothelial cells* (HUVEC)) are oftentimes thoroughly characterized and may therefore be easier to set-up.

3.3. Self-renewing cells (*or stem cells*) include, for example, embryonic stem cells, induced pluripotent stem cells, neural and intestinal stem cells. These cells carry the capacity to differentiate into a diversity of other cells types, while their self-renewing property allows for long-term maintenance in vitro. Self-renewing cell types oftentimes act as physiologically relevant representatives of in vivo mechanisms.

4. Cell morphology types

In terms of growth mode cell cultures take one of two forms, growing either in suspension (as single cells or small free-floating clumps) or as a monolayer that is attached to the tissue culture flask. The form taken by a cell line reflects the tissue from which it was derived. For example, cell lines derived from blood (leukemia, lymphoma) tend to grow in suspension

whereas cells derived from solid tissue (lungs, kidney) tend to grow as monolayers. Attached cell lines can be classified as 1) endothelial such as BAE-1, 2) epithelial such as HeLa, 3) neuronal such as SH-SY5Y, or 4) fibroblast such as MRC-5. (2)

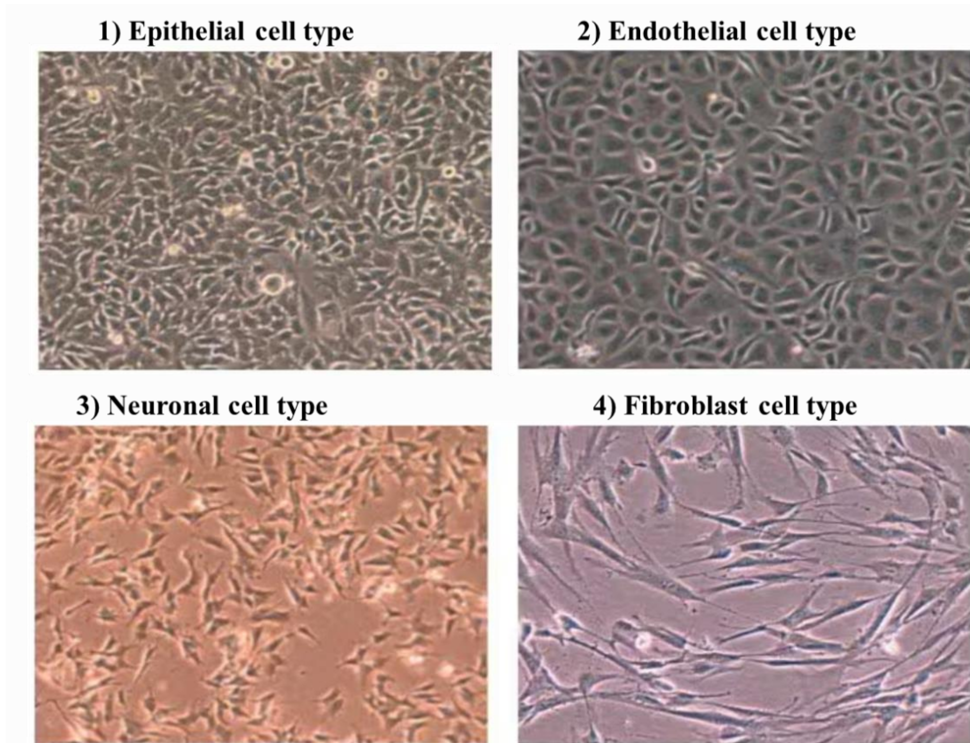


Figure 2. Examples of attached cell types. Cells are classified in 4 different cell type categories based on overall morphology 1) Epithelial 2) Endothelial 3) Neuronal or 4) Fibroblast (2)

CHAPTER II

Cell Culture Laboratory and Safety

The main characteristic of the cell culture laboratory, apart from those of a microbiological laboratory, is the maintenance of a high level of asepsis. The growth rate of cells in culture is much lower than that of the usual contaminants (bacteria, viruses, fungi, yeasts and mycoplasmas), so entry of the latter into the laboratory must be kept to a minimum. The ideal situation is to have a room that is isolated from the other activities, far from passage ways and dedicated exclusively to cell cultivation. The arrival of laminar flow cabinets reduced the need to isolate the working area but even so it is advisable to maintain a sterility gradient, from the outer environment to the inside of the flow cabinets, where the cultures are manipulated, and of the incubators. It is also necessary to facilitate access to the supply of different important equipment.

1.Safety in cell culture laboratory

In addition to the safety risks common to most everyday work places such as electrical and fire hazards, a cell culture laboratory has a number of specific hazards associated with handling and manipulating human or animal cells and tissues, as well as toxic, corrosive, or mutagenic solvents and reagents. The most common of these hazards are accidental punctures with syringe needles or other contaminated sharps, spills and splashes onto skin and mucous membranes, ingestion through mouth pipetting, and inhalation exposures to infectious aerosols.

The fundamental objective of any biosafety program is to reduce or eliminate exposure of laboratory workers and the outside environment to potentially harmful biological agents. The most important element of safety in a cell culture laboratory is the strict adherence to standard microbiological practices and techniques. (5)

1.1.Biosafety Levels

The regulations and recommendations for biosafety in the United States are contained in the document Biosafety in Microbiological and Biomedical Laboratories, prepared by the Centers for Disease Control (CDC) and the National Institutes of Health (NIH), and published by the U.S. Department of Health and Human Services. The document defines four ascending levels of containment, referred to as biosafety levels 1 through 4, and describes the microbiological practices, safety equipment, and facility safeguards for the corresponding level of risk associated with handling a particular agent. (3,6)

- ✓ **Biosafety Level 1 (BSL-1)**: BSL-1 is the basic level of protection common to most research and clinical laboratories, and is appropriate for agents that are not known to cause disease in normal, healthy humans.
- ✓ **Biosafety Level 2 (BSL-2):** BSL-2 is appropriate for moderate-risk agents known to cause human disease of varying severity by ingestion or through percutaneous or mucous membrane exposure. Most cell culture labs should be at least BSL-2, but the exact requirements depend upon the cell line used and the type of work conducted**.**
- ✓ **Biosafety Level 3 (BSL-3)** BSL-3 is appropriate for indigenous or exotic agents with a known potential for aerosol transmission, and for agents that may cause serious and potentially lethal infections**.**
- ✓ **Biosafety Level 4 (BSL-4)** BSL-4 is appropriate for exotic agents that pose a high individual risk of life-threatening disease by infectious aerosols and for which no treatment is available. These agents are restricted to high containment laboratories.

1.2.Safety Equipment

Safety equipment in a cell culture laboratory includes primary barriers such as biosafety cabinets, enclosed containers, and other engineering controls designed to remove or minimize exposure to hazardous materials, as well as personal protective equipment (PPE) that is often used in conjunction with the primary barriers.

The biosafety cabinet (i.e., cell culture hood) is the most important equipment to provide containment of infectious splashes or aerosols generated by many microbiological procedures as well as to prevent contamination of your own cell culture. (3,6)

1.2.1.Safety Data Sheet (SDS)

Safety Data Sheet (SDS) (Figure 3), also referred to as Material Safety Data Sheet (MSDS), is a form containing information regarding the properties of a particular substance. The SDS includes physical data such as melting point, boiling point, and flash point, information on the substance's toxicity, reactivity, health effects, storage, and disposal, as well as recommended protective equipment and procedures for handling spills.

Figure 3. Safety Data Sheet (SDS) poster [1]

1.2.2.Personal Protective Equipment (PPE)

Personal protective equipment (PPE) form an immediate barrier between the personnel and the hazardous agent, and they include items for personal protection (Figure 4). They are often used in combination with biosafety cabinets and other devices that contain the agents or materials being handled. (3,6)

- **Protective eyewear:** This encompasses goggles, face shields, and safety glasses, which are essential in protecting against chemical splashes and flying debris.
- **Protective clothing:** This includes lab coats, aprons, and gloves, which are important in shielding against chemical or biological spills and splashes. It is also crucial to choose the appropriate type of clothing for the specific task at hand, as different materials offer varying levels of protection. For example, cryogenic glove (thermal gloves) is designed specifically to handle nitrogen gas as they can withstand the temperature of -180°C.

Figure 4. Personal Protective Equipment in the Laboratory [2]

- **Respiratory protection:** This includes masks, respirators, and other devices designed to safeguard against hazardous fumes, pathogens, and particles. There are various types of respiratory protection aiming for specific use. It is important to choose the appropriate type of respiratory protection based on the specific hazards present in the laboratory. These are several examples of respiratory protection that you may find in the laboratory.
- **Footwear:** This includes closed-toe shoes and shoes with slip-resistant soles, which are essential in protecting against falling objects and spills.

1.3.Safe Laboratory Practices

The following recommendations are simply guidelines for safe laboratory practices, and they should not be interpreted as a complete code of practice. (7,8)

- Always wear appropriate personal protective equipment. Change gloves when contaminated, and dispose of used gloves with other contaminated laboratory waste.
- Wash your hands after working with potentially hazardous materials and before leaving the laboratory.
- Do not eat, drink, smoke, handle contact lenses, apply cosmetics, or store food for human consumption in the laboratory.

- Follow the institutional policies regarding safe handling of sharps (i.e., needles, scalpels, pipettes, and broken glassware).
- Take care to minimize the creation of aerosols and/or splashes.
- Decontaminate all work surfaces before and after your experiments, and immediately after any spill or splash of potentially infectious material with an appropriate disinfectant.
- Clean laboratory equipment routinely, even if it is not contaminated.
- Decontaminate all potentially infectious materials before disposal.
- Report any incidents that may result in exposure to infectious materials to appropriate personnel (e.g., laboratory supervisor, safety officer)

2.Cell Culture Equipment

The specific requirements of a cell culture laboratory depend mainly on the type of research conducted; for example, the needs of mammalian cell culture laboratory specializing in cancer research is quite different from that of an insect cell culture laboratory that focuses on protein expression. However, all cell culture laboratories have the common requirement of being free from pathogenic microorganisms (i.e., asepsis), and share some of the same basic equipment that is essential for culturing cells.

This section lists the equipment and supplies common to most cell culture laboratories, as well as beneficial equipment that allows the work to be performed more efficiently or accurately, or permits wider range of assays and analyses. Three types of equipment are used in cell culture (5):

A-Basic Equipment

- Cell culture hood (i.e., laminar-flow hood or biosafety cabinet)
- Incubator (humid CO_2 incubator recommended)
- Water bath
- Centrifuge
- Refrigerator and freezer (–20°C)
- Cell counter (e.g., Automated Cell Counter or hemacytometer)
- Inverted microscope
- Liquid nitrogen (N_2) freezer or cryostorage container
- Sterilizer (i.e., autoclave)

B-Expanded Equipment

- Aspiration pump (peristaltic or vacuum)
- pH meter
- Confocal microscope
- Flow cytometer

C-Additional Supplies

- Cell culture vessels (e.g., flasks, Petri dishes, roller bottles, multi-well plates)
- Pipettes and pipettors
- Syringes and needles
- Waste containers
- Media, sera, and reagents
- Cells

2.1.Basic Equipment for cell culture

2.1.1.Clean Benches

Laminar flow "clean benches" are not biosafety cabinets; these pieces of equipment discharge HEPA-filtered air from the back of the cabinet across the work surface toward the user, and they may expose the user to potentially hazardous materials. These devices only provide product protection. Clean benches can be used for certain clean activities, such as the dust-free assembly of sterile equipment or electronic devices, and they should never be used when handling cell culture materials or drug formulations, or when manipulating potentially infectious materials.

2.1.1.1.Types of laminar flow cabinets

Depending on the direction of movement of air, laminar flow cabinets are divided into two types:

a-Vertical laminar flow cabinet; in the vertical flow cabinets, the air moves from the top of the cabinet directly towards the bottom of the cabinet. A vertical airflow working bench does not require as much depth and floor space as a horizontal airflow hood which makes it more manageable and decreases the chances of airflow obstruction or movement of contaminated air downstream. The vertical laminar flow cabinet is also considered safer as it doesn't blow the air directly towards the person carrying out the experiments.

b-Horizontal laminar flow cabinet; in the horizontal laminar flow cabinets, the surrounding air comes from behind the working bench, which is then projected by the blower towards the HEPA filters. The filtered air is then exhausted in a horizontal direction to the workplace environment. One advantage of this cabinet is that airflow parallel to the workplace cleanses the environment with a constant velocity. The eluent air directly hits the operator, which might reduce the security level of this type of laminar flow cabinets.

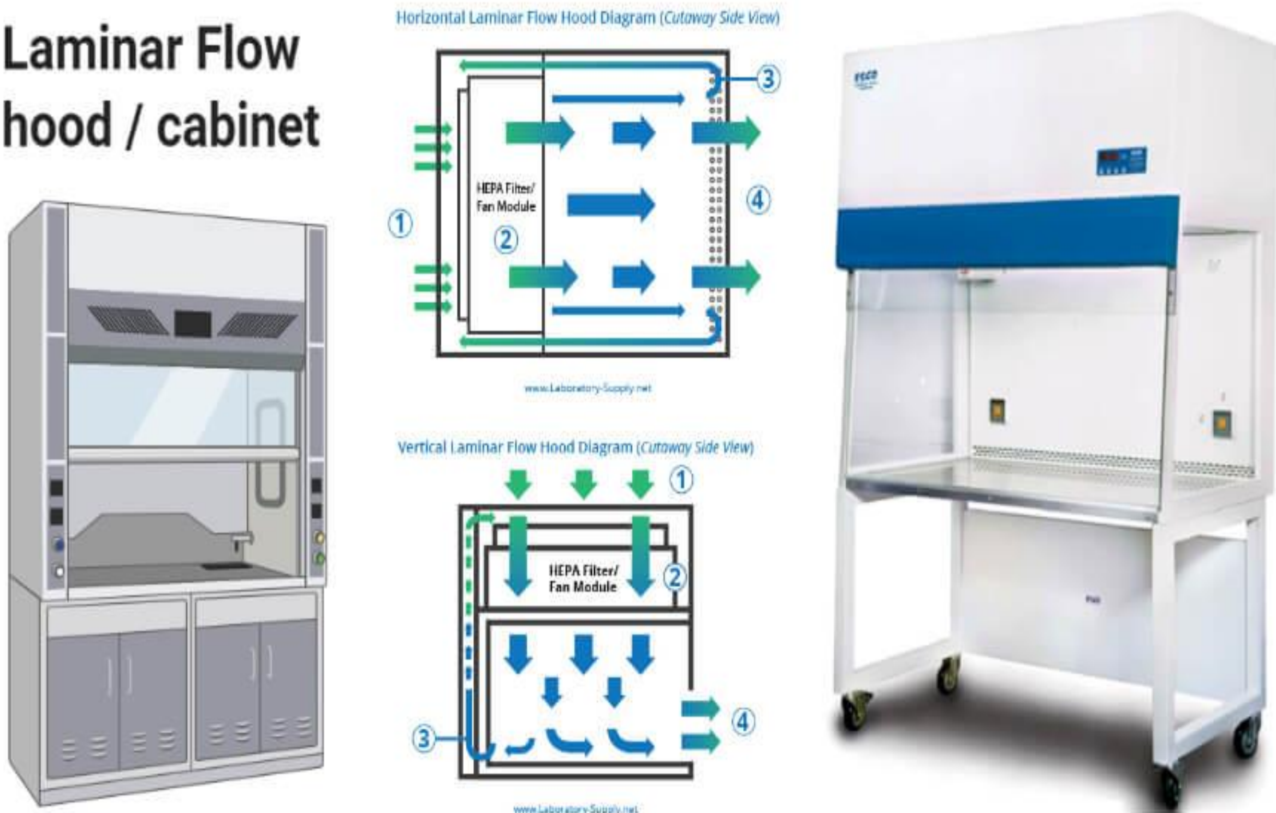


Figure 5 : Laminar flow hood/cabinet- Horizontal vs Vertical Laminar Flow Hoods [3]

2.1.2.Cell culture hood

The major requirement of a cell culture laboratory is the need to maintain an aseptic work area that is restricted to cell culture work. Although a separate tissue culture room is preferred, a designated cell culture area within a larger laboratory can still be used fort sterile handling, incubation, and storage of cell cultures, reagents, and media. The simplest and most economical way to provide aseptic conditions is to use a cell culture hood (i.e., biosafety cabinet).(8) The cell culture hood provides an aseptic work area while allowing the containment of infectious splashes or aerosols generated by many microbiological procedures. A Laminar flow hood/cabinet is an enclosed workstation that is used to create a contamination-free work environment through filters to capture all the particles entering the

cabinet. Three kinds of cell culture hoods, designated as Class I, II and III, have been developed to meet varying research and clinical needs. (5)

2.1.2.1.Classes of cell culture hoods

***Class I cell culture hoods**:* offer significant levels of protection to laboratory personnel and to the environment when used with good microbiological techniques, but they do not provide cultures protection from contamination. They are similar in design and air flow characteristics to chemical fume hoods (Figure 6).

Class II cell culture hoods are designed for work involving BSL-1, 2, and 3 materials, and they also provide an aseptic environment necessary for cell culture experiments. A Class II biosafety cabinet should be used for handling potentially hazardous materials (e.g., primate-derived cultures, virally infected cultures, and radioisotopes, carcinogenic or toxic reagents) (Figure 6).

Class III biosafety cabinets are gas-tight, and they provide the highest attainable level of protection to personnel and the environment. A Class III biosafety cabinet is required for work involving known human pathogens and other BSL-4 materials (Figure 6).

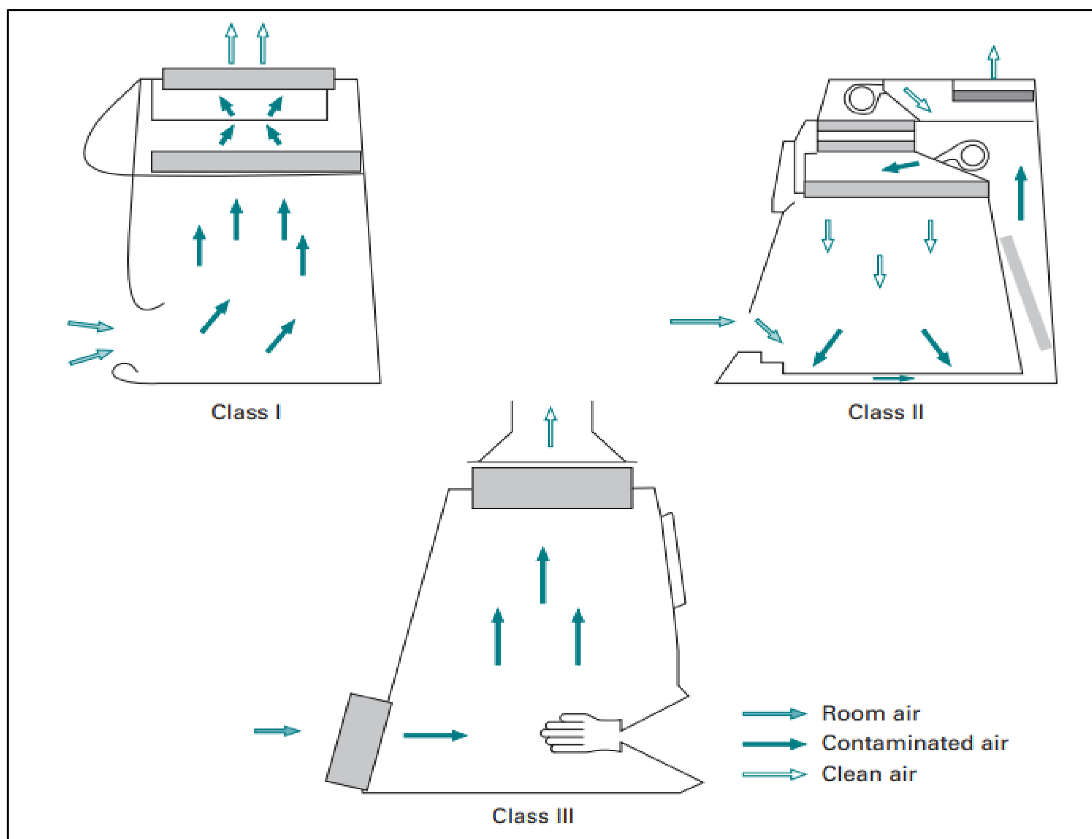


Figure 6. Schematic representation of tissue culture cabinets [4]

2.1.2.2.Air-Flow Characteristics of Cell Culture Hoods

Cell culture hoods protect the working environment from dust and other airborne contaminants by maintaining a constant, unidirectional flow of HEPA-filtered air over the work area. The flow can be horizontal, blowing parallel to the work surface, or it can be vertical, blowing from the top of the cabinet onto the work surface. Depending on its design, **a horizontal flow hood** provides protection to the culture (if the air flowing towards the user) or to the user (if the air is drawn in through the front of the cabinet by negative air pressure inside). **Vertical flow hoods**, on the other hand, provide significant protection to the user and the cell culture. (7)

2.1.2.3. Components/ Parts of Laminar flow hood (biosafety cabinets)

A laminar flow cabinet consists of the following parts (9)

a. Cabinet

- The cabinet is made up of stainless steel with less or no gaps or joints preventing the collection of spores.
- The cabinet provides insulation to the inner environment created inside the laminar flow and protects it from the outside environment.
- The front of the cabinet is provided with a glass shield which in some laminar cabinets opens entirely or in some has two openings for the user's hands to enter the cabinet.

b. Working station

- A flat working station is present inside the cabinet for all the processes to be taken place.
- Culture plates, burner and loops are all placed on the working station where the operation takes place.
- The worktop is also made up of stainless steel to prevent rusting.

c. Filter pad/ Pre-filter

- A filter pad is present on the top of the cabinet through which the air passes into the cabinet.
- The filter pad traps dust particles and some microbes from entering the working environment within the cabinet.

d. Fan/ Blower

- A fan is present below the filter pad that sucks in the air and moves it around in the cabinet.
- The fan also allows the movement of air towards the HEPA filter sp that the remaining microbes become trapped while passing through the filter.

e. UV lamp

- Some laminar flow hoods might have a UV germicidal lamp that sterilizes the interior of the cabinet and contents before the operation.
- The UV lamp is to be turned on 15 minutes before the operation to prevent the exposure of UV to the body surface of the user.

f. Fluorescent lamp

- Florescent light is placed inside the cabinet to provide proper light during the operation.

g. HEPA filter

- The High-efficiency particulate air filter is present within the cabinet that makes the environment more sterile for the operation.
- The pre-filtered air passes through the filter which traps fungi, bacteria and other dust particles.
- The filter ensures a sterile condition inside the cabinet, thus reducing the chances of contamination.

2.1.2.4. Principle/Working of Laminar flow hood

The principle of laminar flow cabinet is based on the laminar flow of air through the cabinet. The device works by the use of inwards flow of air through one or more HEPA filters to create a particulate-free environment. The air is taken through a filtration system and then exhausted across the work surface as a part of the laminar flow of the air. The air first passes through the filter pad or pre-filter that allows a streamline flow of air into the cabinet. Next, the blower or fan directs the air towards the HEPA filters. The HEPA filters then trap the bacteria, fungi and other particulate materials so that the air moving out of it is particulate-free air. Some of the effluent air then passes through perforation present at the

bottom rear end of the cabinet, but most of it passes over the working bench while coming out of the cabinet towards the face of the operator. The laminar flow hood is enclosed on the sides, and constant positive air pressure is maintained to prevent the intrusion of contaminated external air into the cabinet. (9)

2.1.2.5.Procedure for running the laminar flow cabinets

The procedure to be followed while operating a laminar flow cabinet is given below (9):

1. Before running the laminar flow cabinet, the cabinet should be checked to ensure that nothing susceptible to UV rays is present inside the cabinet.
2. The glass shield of the hood is then closed, and the UV light is switched on. The UV light should be kept on for about 15 minutes to ensure the surface sterilization of the working bench.
3. The UV light is then switched off, and a time period of around 10 minutes is spared before the airflow is switched on.
4. About 5 minutes before the operation begins, the airflow is switched on.
5. The glass shield is then opened, and the fluorescent light is also switched on during the operation.
6. To ensure more protection, the working bench of the cabinet can be sterilized with other disinfectants like 70% alcohol.
7. Once the work is completed, the airflow and florescent lamp both are closed and the glass shield is also closed.

2.1.2.6.Cell Culture Hood Layout

A cell culture hood should be large enough to be used by one person at a time, be easily cleanable inside and outside, have adequate lighting, and be comfortable to use without requiring awkward positions. Keep the work space in the cell culture hood clean and uncluttered, and keep everything in direct line of sight. Disinfect each item placed in the cell culture hood by spraying them with 70% ethanol and wiping clean (3, 5).

The arrangement of items within the cell culture hood usually adheres to the following right-handed convention, which can be modified to include additional items used in specific applications.

- A wide, clear work space in the center with your cell culture vessels
- Pipettor in the front right, where it can be reached easily
- Reagents and media in the rear right to allow easy pipetting
- Tube rack in the rear middle holding additional reagents
- Small container in the rear left to hold liquid waste

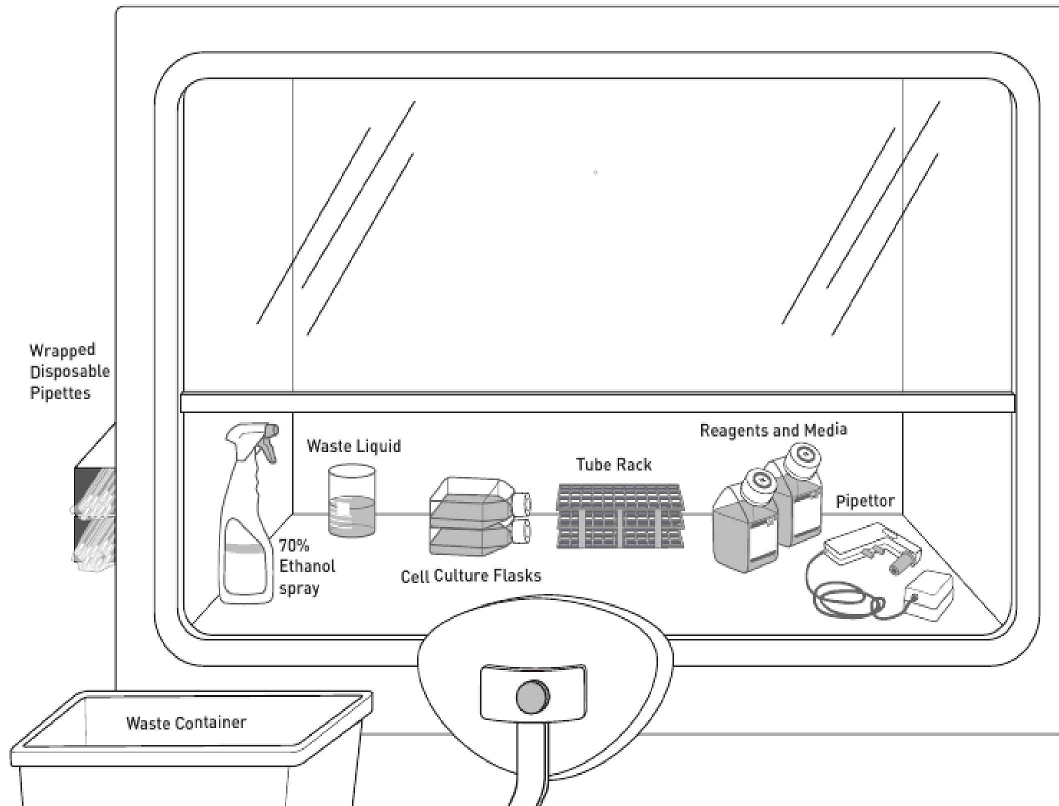


Figure 7. The basic layout of a cell culture hood for right-handed workers. Left-handed workers may switch the positions of the items laid out on the work surface. (3)

2.1.2.7.Precautions

While operating the laminar airflow, the following things should be considered (3,5):

1. The laminar flow cabinet should be sterilized with the UV light before and after the operation.
2. The UV light and airflow should not be used at the same time.
3. No operations should be carried out when the UV light is switched on.
4. The operator should be dressed in lab coats and long gloves.
5. The working bench, glass shield, and other components present inside the cabinet should be sterilized before and after the completion of work.

2.1.2.8.Biosafety cabinets vs clean benches

Many people don't understand the difference between biosafety cabinet and ultra-clean workbench, thinking that the two have similar uses. There are many misunderstandings. There are still many differences between an ultra-clean workbench and a biosafety cabinet. The difference between them concerns **principle** and **use**.

*A- **The Difference Between Biosafety Cabinet And Clean Bench In Working Principle***

- **Working principle of biosafety cabinet:** First of all, this part will define the two in principle. The biosafety cabinet is a negative pressure purification workbench, which can prevent the operator and the environment from being exposed to harmful aerosols generated during the laboratory process. It is a cage for infectious microorganisms. Correct operation of the biosafety cabinet can completely protect the operator and samples, and the working environment.
- **Working principle of clean bench**: The working principle of the ultra-clean workbench is to blow the purified air through the high-efficiency filter downward or horizontally through the work area to protect the samples. Since the work area is a positive pressure area, the airflow overflows through the operation window to only protect the samples, while the operators and the environment are not protected. If the operation contains any known or potential pathogenic aerosols, it will bring great danger to the operator.

B- The Differences While Using Biosafety Cabinet and Clean Bench

- **Special requirements for using a biosafety cabinet**

  1. Do not place objects and materials on window grilles. Cleaned, used, and virus-containing items should be placed separately. Keep unclean items away from openings as much as possible.

  2. Operators should try their best to wear long-sleeved threaded cuff work clothes and rubber gloves, and at least wash their hands with an antiseptic soap before and after work.

  3. Minimize the crossing of airflow in front of the window as much as possible. You can put all the items you need into the biosafety cabinet in an orderly manner before starting the machine, to reduce unnecessary entry and exit of arms.

  4. After preparation, the fan runs for 2-3 minutes to fully purify the air in the biosafety cabinet.

5. The tightness of the biosafety cabinet should be tested according to the frequency of use, generally not less than twice a year.

- **Special requirements for using ultra-clean workbenches**

1.Before starting the experiment, let the fan run for at least 5 minutes.
2.The ultra-clean bench does not protect the operator, so the operation of pathogenic bacteria and microbial agents that may cause harm to the human body should not be performed. The operator should keep in mind the airflow direction to prevent unsafe factors.

The biosafety cabinet is negative pressure. The air enters the machine from the external environment through the glass front window, and then the air flows upward and is discharged after passing through the high efficiency. Some are fully exhausted, and some are partially circulated. The ultra-clean workbench is positive pressure, and the clean air after high-efficiency filtration is blown from top to bottom, or blown horizontally, and finally enters the environment through the glass window. Which one is better? It depends on your requirement. In a word, the more applicable the better. If you want to buy biosafety cabinets, you can visit our website to know more. Drawell is a reliable biosafety cabinet manufacturer that you can trust.

2.1.3.Incubator

The purpose of the incubator is to provide the appropriate environment for cell growth. The incubator should be large enough for your laboratory needs, have forced- air circulation, and should have temperature control to within 37 ±0.2°C.

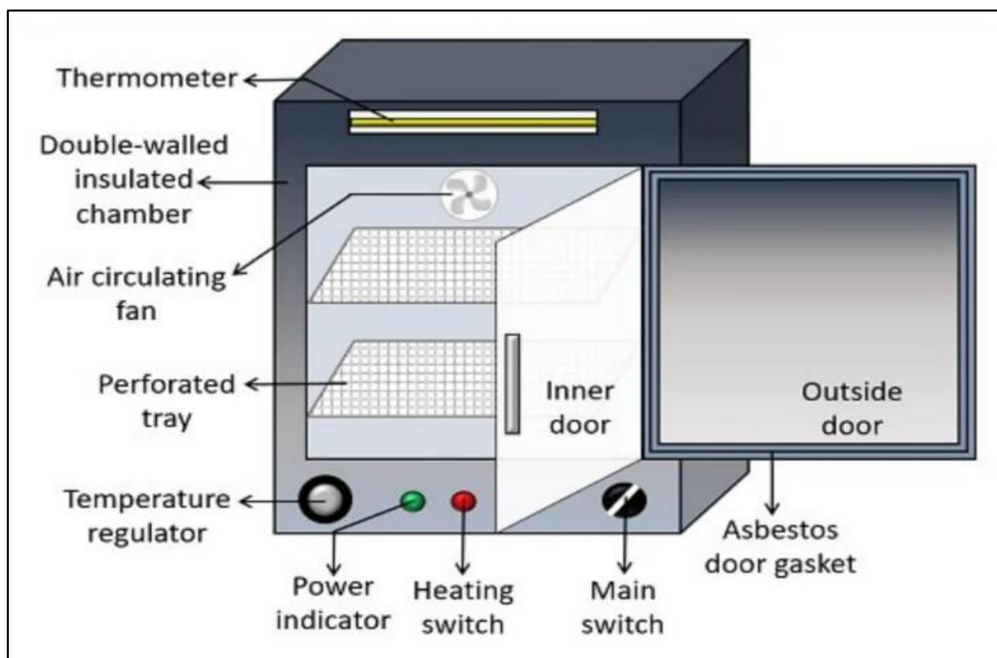


Figure 8: Parts of an Incubator [5]

Stainless steel incubators allow easy cleaning and provide corrosion protection, especially if humid air is required for incubation. Although the requirement for aseptic conditions in a cell culture incubator is not as stringent as that in a cell culture hood, frequent cleaning of the incubator is essential to avoid contamination of cell cultures (Figure 8). (9)

2.1.3.1.Types of Incubators

There are two basic types of incubators, **dry incubators** and **humid CO_2 incubators**. Dry incubators are more economical, but require the cell cultures to be incubated in sealed flasks to prevent evaporation.

Placing a water dish in a dry incubator can provide some humidity, but they do not allow precise control of atmospheric conditions in the incubator. Humid CO_2 incubators are more expensive, but allow superior control of culture conditions. They can be used to incubate cells cultured in Petri dishes or multi-well plates, which require a controlled atmosphere of high humidity and increased CO_2 tension (9).

2.1.4.Storage

A cell culture laboratory should have storage areas for liquids such as media and reagents, for chemicals such as drugs and antibiotics, for consumables such as disposable pipettes, culture vessels, and gloves, for glassware such as media bottles and glass pipettes, for specialized equipment, and for tissues and cells.
Glassware, plastics, and specialized equipment can be stored at ambient temperature on shelves and in drawers; however, it is important to store all media, reagents, and chemicals according to the instructions on the label.
Some media, reagents, and chemicals are sensitive to light; while their normal laboratory use under lighted conditions is tolerated, they should be stored in the dark or wrapped in aluminium foil when not in use (3,5).

2.1.5. Refrigerators

For small cell culture laboratories, a domestic refrigerator (preferably one without a autodefrost freezer) is an adequate and inexpensive piece of equipment for storing reagents and media at 2–8°C. For larger laboratories, a cold room restricted to cell culture is more appropriate. Make sure that the refrigerator or the cold room is cleaned regularly to avoid contamination (5).

2.1.6.Freezers

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

2.1.7.Cryogenic Storage

Cell lines in continuous culture are likely to suffer from genetic instability as their passage number increases; therefore, it is essential to prepare working stocks of the cells and preserve them in cryogenic storage (for more information, see Freezing Cells, page 37). Do not store cells in –20°C or –80°C freezers, because their viability quickly decreases when they are stored at these temperatures (3).

There are two main types of liquid-nitrogen storage systems, vapor phase and liquid phase, which come as wide-necked or narrow-necked storage containers. Vapor phase systems minimize the risk of explosion with cryostorage tubes, and are required for storing biohazardous materials, while the liquid phase systems usually have longer static holding times, and are therefore more economical.

Narrow-necked containers have a slower nitrogen evaporation rate and are more economical, but wide-necked containers allow easier access and have a larger storage capacity. figure

2.1.8.Cell Counter

A cell counter is essential for quantitative growth kinetics, and a great advantage when more than two or three cell lines are cultured in the laboratory.

The Countess® Automated Cell Counter is a bench-top instrument designed to measure cell count and viability (live, dead, and total cells) accurately and precisely in less than a minute per sample, using the standard Trypan Blue uptake technique. Using the same amount of sample that you currently use with the hemacytometer, the Countess® Automated Cell Counter takes less than a minute per sample for a typical cell count and is compatible with a wide variety of eukaryotic cells (9).

CHAPTER III

Cell Culture and Asepsis

The concept of sterility in cell culture is sometimes difficult to understand. Indeed, what is "sterile" does not contain any living organism. But a "sterile" cell culture in practice means a culture containing only the cells that we want to find there, and no other living organism.Anything that does not contain any cells must be sterilized. To do this, it is necessary to filter, in sterile conditions (that is to say under a flame or under a PSM), using a filter with a pitch of 0.22 µm. Autoclaving (passing at high temperature) of certain solutes is possible, but this technique cannot concern culture media, which contain proteins: autoclaving would cook them. This technique must therefore be reserved for chemical solutions that must be sterile.

1.Cell culture sterility

The biologist's fear is the contamination of cultures. Indeed, a cell whose culture is colonized by another organism is very disturbed, because the foreigner induces stress in it by diversion, because the contaminant proliferates more quickly and consumes nutrients from the environment, and stress by danger signals, which profoundly modify the reactions of a cell. The most common contaminants are bacteria from our skin: *E.coli* and *S.aureus*, as well as certain yeasts. Contamination by the Mycoplasma genus is also common. It happens that a cell culture becomes contaminated with another cell type. This is sometimes the case when the same person works on many lines at the same time in the same place. (8)

2.Aseptic techniques:

The aseptic techniques designed to provide a barrier between the microrganisms in the environment and the sterile cell culture, depends upon a set of procedures to reduce the probability of contamination from these sources. The elements of aseptic technique are a sterile work area, good personal hygiene, sterile reagents and media, and sterile handling.

2.1.Sterile Work Area

The clean area must be as clean as possible, by regular decontamination using a surface cleaner-decontaminant. As a precaution, it is also necessary to clean and decontaminate any surface entering the clean zone: vials, bottles, gloves, hands, etc. Successful cell culture depends heavily on keeping the cells free from contamination by microorganisms such as bacterial, fungi, and viruses. Non-sterile supplies, media, and reagents, airborne particles laden with microorganisms, unclean incubators, and dirty work surfaces are all sources of biological contamination. The simplest and most economical way to reduce contamination

from airborne particles and aerosols (e.g., dust, spores, shed skin, sneezing) is to use a cell culture hood. (8)

- The cell culture hood should be properly set up and be located in an area that is restricted to cell culture that is free from drafts from doors, windows, and other equipment, and with no through traffic.
- The work surface should be uncluttered and contain only items required for a particular procedure; it should not be used as a storage area.
- Before and after use, the work surface should be disinfected thoroughly, and the surrounding areas and equipment should be cleaned routinely.
- For routine cleaning, wipe the work surface with 70% ethanol before and during work, especially after any spillage.
- You may use ultraviolet light to sterilize the air and exposed work surfaces in the cell culture hood between uses.
- Using a Bunsen burner for flaming is not necessary nor recommended in a cell culture hood.
- Leave the cell culture hood running at all times, turning them off only when they will not be used for extended periods of time. (7)

2.2.Good Personal Hygiene

Wash your hands before and after working with cell cultures. In addition to protecting you from hazardous materials, wearing personal protective equipment also reduces the probability of contamination from shed skin as well as dirt and dust from your clothes. (9)

2.3.Sterile Reagents and Media

Commercial reagents and media undergo strict quality control to ensure their sterility, but they can become contaminated while handling. Follow the guidelines below for sterile handling to avoid contaminating them. Always sterilize any reagents, media, or solutions prepared in the laboratory using the appropriate sterilization procedure (e.g., autoclave, sterile filter). (9)

2.4.Sterile Handling

- Always wipe your hands and your work area with 70% ethanol.
- Wipe the outside of the containers, flasks, plates, and dishes with 70% ethanol before placing them in the cell culture hood.

- Avoid pouring media and reagents directly from bottles or flasks.
- Use sterile glass or disposable plastic pipettes and a pipettor to work with liquids, and use each pipette only once to avoid cross contamination.
- Do not unwrap sterile pipettes until they are to be used. Keep your pipettes at your work area.
- Always cap the bottles and flasks after use and seal multi-well plates with tape or place them in resealable bags to prevent microorganisms and airborn contaminants from gaining entry.
- Never uncover a sterile flask, bottle, petri dish, etc. until the instant you are ready to use it and never leave it open to the environment. Return the cover as soon as you are finished.
- If you remove a cap or cover, and have to put it down on the work surface, place the cap with opening facing down.
- Use only sterile glassware and other equipment.
- Be careful not to talk, sing, or whistle when you are performing sterile procedures.
- Perform your experiments as rapidly as possible to minimize contamination. (7,9)

3. Sterilization methods

3.1.Définition

Sterilization is defined as the process of destruction or elimination of all pathogenic microorganisms and organisms capable of giving rise to infection such as all spores, bacteria, fungi, etc., all disease-causing. It can be achieved by both physical, chemical, and other effective methods that inhibit growth or are free from food products, fluids, objects, solid materials, food packaging materials, raw materials, and other different products (9)

3.2.Importance of Sterilization

Imprtance of sterilization are given below (9):

- Sterilization is used to prevent the transmission of certain pathogenic disease-causing microorganisms into the body.
- It helps in sterile products to prevent contamination.
- Sterilization is an important process in research development laboratories.
- It prevents the contamination of instruments and areas in the pharmaceutical industry.
- It is used in the preparation process of cultures and other microbiology experiments.

- The sterilization process is used in the food industries such as canning, and high-pressure methods.
- Sterilization is used for the preparation of sterile dosage forms and sterility testing.

3.3. Methods

In order to completely eradicate microorganisms, sterilization techniques involve classification of sterilization which includes physical and chemical methods such as autoclaving (pressurised steam), chemical sterilization using ethylene oxide or hydrogen peroxide, radiation sterilisation using gamma rays or electron beams, dry heat (hot air), filtration using small-pore filters, and plasma sterilization are all necessary (low-temperature plasma). Depending on the material sensitivity and particular sterilizing requirements, each technique is used. (9, 10)

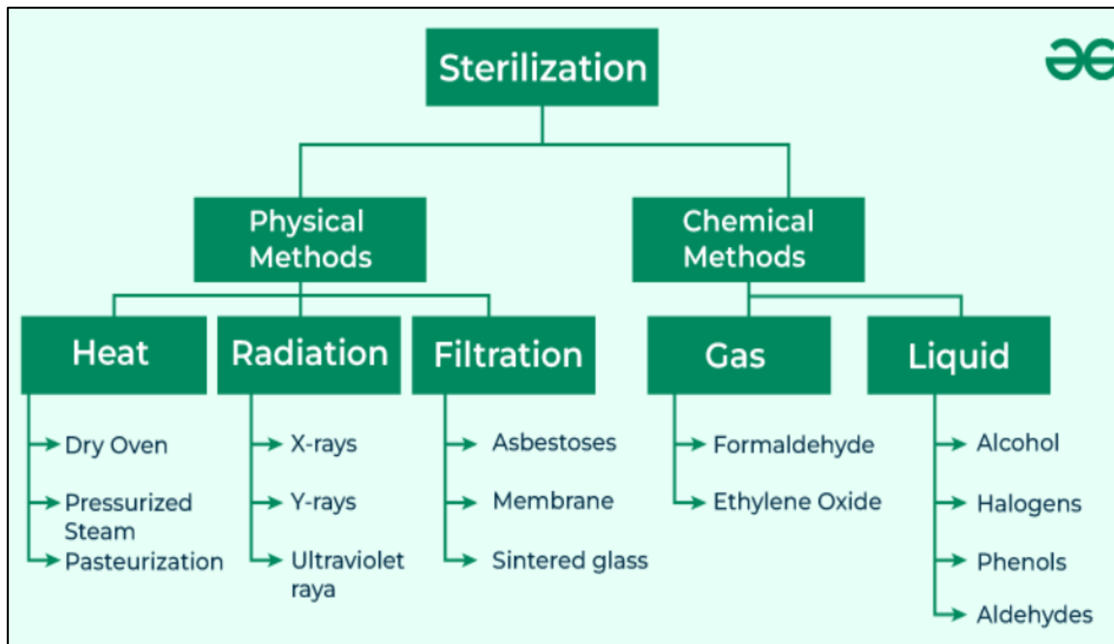


Figure 9: Sterilization methods [6]

3.3.1.Physical Sterilization

Physical sterilization is method of sterilization to destroy the microorganism by using physical methods like dry and wet heat, filteration and radiation. In these method wet or moist heat considerd to be the most effective method to sterilize the glasswares.

✓ **By Using Heat**

The most effective and reliable method of the sterilization process. There are two methods by using heat-dry heat and moist heat.

- **Dry heat**

- **Flaming** In this method heating instruments over the fire until they become hot in red. Instruments that are used such as point of forceps, Spatulas, inoculating loops, and Wires.
- **Incineration** It is a process that involves the combustion of organic substances contained in waste materials. Items such as contaminated cloth, animal carcasses, and pathological material. PVC and polythene can be dealt with.
- **Hot air oven** Hot air ovens are the electrical devices used for sterilization. The oven uses dry heat to sterilize items. Generally, they can be set at minimum to maximum temperature from 50˚C to 300˚C. The thermostat is present to control the temperature. This is the most widely used method of sterilization by dry heat.

- **Moist Heat**

Moist heat can be categorized into 3 groups (10):

- **Temperature below 100 C** Pasteurization of milk: This method is done by holding period at 63˚C for 30 minutes or 72˚C for 15-20 minutes followed by cooling. This method is not suitable for killing all spores.
- **Temperature at 100 C** Steam at atmospheric pressure is used to sterilize culture media. This is an inexpensive method. The principle of this method is first exposure kills vegetative bacteria and next exposure will kill vegetative bacteria that mature from the spore. It is intermittent sterilization by holding at a temperature of 100˚C for 20 minutes on three consecutive days.
- **Temperature above 100 C** An autoclave or steam sterilizer is an instrument that uses steam to sterilize equipment and other objects. This implies that all microscopic organisms, infections, parasites, and spores are inactivated. In any case, prions may not be annihilated via autoclaving at the regular 134˚C for 3 minutes or 121˚C for 15 minutes. It is suitable for the Items such as dressings, instruments, laboratory ware, media, and medical products.

✓ **Filtration**

Filtration assists with eliminating microorganisms from heat-labile fluids such as sera and solutions of antibiotics. Its working principle as viruses go through the normal filter channels, filtration can be used to obtain bacteria-free filtrates of clinical samples for virus isolation.

Types of Filters

- **Candle filters** These filters are used for the purification of water for industrial and drinking purposes. These are made under various grades of porosity.

- **Asbestos filters** are disposable and single usage. They tend to alkalinize filtered fluids. Their usage is less because of their carcinogenic property.
- **Sintered glass filters** These filters have low absorptive properties. They are brittle and costly.
- **Membrane filters** These filters are made of cellulose esters or other polymers. They are usually used for water purification and analysis, sterility testing, and preparation of solutions.

  ✓ **Radiation**

There are 2 types of radiation: Ionizing radiation & non-ionizing radiation

- **Non-ionizing radiation** In the non-ionizing method infrared is used for rapid mass sterilization of prepacked items such as syringes, and catheters. UV is used for disinfecting enclosed areas such as halls, operation theatres, and labs.
- **Ionizing radiation** In ionizing radiation, gamma rays and x-rays are used for sterilizing plastics, syringes, swabs, catheters, animal feeds, cardboard, oils, and metal foils.

3.3.2. Chemical Sterilization

Chemical sterilization is the method of sterilization to make surface free from microorganisms using certain chemicals like, chlorine, formaldehyde and ethanol. These chemical can interfere with the machinary of the microoganisms to terminate them. (10)

Using chemicals

- **Chemical agents:** The action of chemical agents are protein coagulation and Disruption of cell membrane resulting in exposure, damage, and loss of contents.
- **Chemical Alcohols:** Commonly used are Ethyl alcohol, and Isopropyl alcohol and must be utilized at concentrations of 60-90%. Isopropyl alcohol is used in the sanitization of the clinical thermometer. Methyl alcohol is viable against contagious spores, treating cabinets, and incubators. It is toxic and inflammable.
- **Aldehyde Formaldehyde:** It contains bactericidal and sporicidal and it has a great effect on viruses. It is used to preserve anatomical specimens and destroy anthrax spores on hair and wool.
- **Phenols:** These are acquired from the distillation of coal tar between 170˚ to 270˚C. The deadly impacts include- it can cause cell membrane damage, releasing cell contents, and causing lysis.

Gases

Types of gases used for sterilization are (7, 9, 10):

- **Ethylene oxide** works because of its alkylating of the amino, carboxyl, hydroxyl, and sulfhydryl groups in protein molecules and furthermore on DNA and RNA. It very well may be utilized on instruments such as heart-lung machines, respirators, dental equipment, books, and clothing.

- **Formaldehyde gas** This is generally utilized for fumigation of operational theatres and other rooms in clinics. Formaldehyde is formed by the addition of 150g of $KMnO_4$ to 280ml of formalin for each 1000cu.ft of room volume, in the wake of shutting the windows and different outlets. After fumigation, the doors ought to be sealed and left unopened for 48 hours.

- **Beta propiolactone (BPL)** is a result of ketene and formaldehyde with a boiling point of 163˚C. It has fat bactericidal activity yet is cancer-causing. It is equipped for killing all microorganisms and it is exceptionally dynamic against infections.

4.Cell culture contamination

For the cell culturist, two types of contamination require careful monitoring and constant vigilance: the contamination of cell cultures with **microbiological organisms** and the contamination of **one cell line with another**. Both forms of contamination are extremely prevalent and cannot be underestimated. Neither type can be eliminated, only controlled and managed to minimize the possibility of occurrence.

Contamination consequences can range from minor inconvenience (a flask of cells becoming contaminated with bacteria) to a major disaster (published results that may be invalid owing to cross-contamination of one cell line with another). Other types of contaminants, such as chemical contamination, may also cause problems (e.g., deposits of disinfectants or detergents on glassware; residues, impurities, and toxins in water, media or sera), but the common recurring problems are likely to be biological in origin. (10)

Routine observation is the first indicator of the overall health of a cell culture, not all contaminations are overt. An imperceptible contamination can become established before any gross indications become evident and remedial action can be taken. When a cell line is contaminated, procedures must be in place to dispose of the culture or eradicate the problem in a manner that is safe to other cell cultures in the laboratory and to the technical staff.

Cell culture collections and other bio-resource centres exist to provide quality- controlled cell lines, and recipients of their cell cultures must be assured of the contamination-free status

of the cell line. In contrast, where cell lines are being created *de novo* or being introduced into the laboratory from a source without quality control procedures in place, then quarantine culture facilities must be used. (10)

4.1.Quarantine and receipt of animal cell lines

All new cell lines brought into a cell culture area should be introduced into a quarantine laboratory specifically set aside and fully equipped for the purpose of handling unquail fied cell lines, with appropriately trained personnel (11)

4.1.1.Accessioning scheme

The introduction of cell lines into the cell culture area(s) of any organization should be a tightly controlled process. All the major cell culture collections and research organizations, non-profit and commercial, now have officers who are tasked with the sourcing, acquisition and addition of new cell lines to their collection, in a process known as accessioning or acquisition.

The process of accessioning not only covers the shipment of a cell culture from one organization to another but also includes obtaining, checking and archiving all attendant documentation describing provenance, culture conditions, biohazard risk, ownership (of intellectual property) and any restricted use.

The properties of the cell culture and its maintenance should be recorded on a formal document for evaluation and future reference. In addition, and prior to dispatch of the cell line from the source laboratory or supplier, all cell lines should be subject to a bio-hazard risk assessment by the accessioning representative.

The bio-hazard risk assessment will determine the appropriate level of containment to be used when handling the cell line in terns of bio-containment and genetic manipulation to ensure that the facility is accordingly accredited or licensed to handle the cell lines. Genetically modified cell lines should be carefully reviewed to assess the extent and the details of the genetic manipulation that has been performed (10)

4.1.2.Management of cell lines in quarantine

A flow diagram of the scheme used at European Collection of Authenticated Cell Cultures (ECACC) for the introduction of new cell lines into the collection is given in Figure 7. The main points are listed below. (11)

- All cultures should be handled in a quarantine laboratory separate from the main tissue culture area.

- Cultures should be in handled in a Class II Mirobiological Safety Cabinet (MSC) unless a higher level of containment is required. This will offer the operator protection, as the exact source of the cell line and its contamination status may not be known.

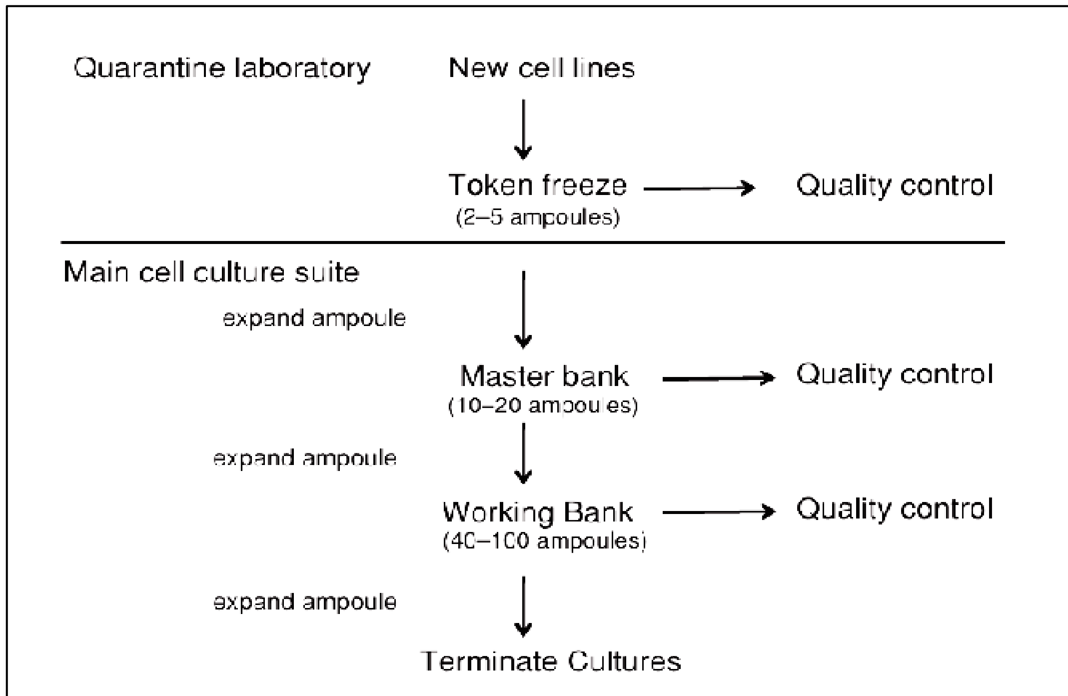


Figure 10. Hierarchical banking scheme for incoming cell lines as used at ECACC (10)

- An initial assessment of potential microbial contamination should be made immediately, (broth for bacteria; PCR-based or other rapid methodology analysis for mycoplasma).
- An assessment should be made of the cell type in the culture by examination and photographic documentation.
- A token freeze of between three and five ampoules should be made as soon as possible after receipt of the cell line.
- A complete set of quality control tests should be applied to the token freeze. These include a cell count and viability assessment, sterility tests (bacteria, yeast, fungi, mycoplasma), species identification and DNA characterization by restriction enzyme analysis or short tandem repeat (STR) PCR analysis.
- After satisfying the above conditions, an ampoule from the token freeze can be transferred to the main cell culture laboratory for the production of Master Cell Banks (MCBs) and Working Cell Banks (WCBs).

It is recommended that this approach be adopted for all cell lines received into the laboratory, irrespective of the source of the cell line.

Many laboratories routinely use antibiotics in their cell cultures. This practice is not recommended for a number of reasons. From a contamination detection or surveillance viewpoint, antibiotics can suppress bacterial contamination to a level that is undetectable by microscopic examination and can lead to the development and spread of antibiotic resistant microbial strains. In addition, although not eliminating any mycoplasma present, antibiotics, particularly the amino-glycosides, can reduce the level of infection below the level of detection of several currently used tests. It is essential that all quality control tests for microbial contamination be performed on cell cultures subcultured for at least two passages in antibiotic-free medium. (11)

4.1.3.Quarantine laboratory design

Optimal design criteria for the cell culture laboratory are described in Chapter I1. It is worth emphasizing that when establishing a cell culture laboratory, either within an existing facility or from new, an area (preferably an entire laboratory) should be set aside for quarantine purposes.

The quarantine laboratory should have the following features:

(i) It should be as far away from the main cell culture clean area as possible.

(ii) The air-handling system of the laboratory should operate under negative pressure with respect to the rest of cell culture area.

(iii) It should be self-contained with its own incubator, water bath, microscope, MSC, and so on.

(iv) Staff should have separate personal protective equipment (lab coats, along with disposable gloves, hairnets, shoe covers and face masks) for working in the quarantine laboratory.

(v) There should be an approved regimen in place for the routine and incident related cleaning of the equipment and fabric (walls, floors, doors, etc.), and the fumigation of the MSC. All staff working in the quarantine laboratory must be fully trained in the required procedures.

(vi) Protocols for the disposal of contaminated cultures and associated reagents should also be in place. (11, 10)

4.2.Microbial quality control

Microbial quality control is an essential part of all routine cell culture practice and should not be neglected. It comprises the testing of cell cultures and associated reagents for a variety of microorganisms.

4.2.1. Sources of contamination

Different microorganisms can contaminate cell cultures and come from different sources. These sources can be divided into three main categories:

- poor laboratory conditions
- personnel (particularly inadequately trained personnel)
- Non-quality-controlled cell lines.

Poor laboratory conditions are combated by the implementation of good laboratory conditions as described in Chapter II. It is important that all staff, in addition to having good aseptic technique while working in the MSC, are aware of the various possible routes and likely sources of contamination (Table 1) and are familiar with best practice techniques. (12)

Table 1. Common sources of microbial contamination (12)

Organism	Source
Bacteria	Clothing, skin, hair, aerosols (e.g. due to sneezing or pipetting), insecure caps on media bottles and culture flasks
	Air currents
	Humidified incubators
	Purified water
	Insects
	Plants
	Contaminated cell lines
Fungi (excluding yeasts)	Damp wood or other cellulose products, for example cardboard
	Humidified incubators
	Plants
Yeasts	Humidified incubators
	Operators
Mycoplasma	Contaminated cell lines
	Serum
	Media
	Operators

The most common sources of contamination are from laboratory personnel (particularly any with poor personal hygiene), and from cell lines received from external sources (although

serum can also be major source of mycoplasma). Wherever possible, obtaining cell cultures from a recognized cell culture collection is advisable.

4.2.2.Tests for bacteria, yeasts and fungi

When cell lines are cultured in antibiotic-free media, contamination by bacteria, yeasts or other fungi can frequently be detected by an increase in the turbidity of the medium and/or by a change in pH. Reagents added during the preparation of cell culture media, for example serum, glutamine or growth factors, or used in culture procedures, for example trypsin, can contribute to the risk of contamination. It is therefore good practice to set up quality control checks on culture media and reagents prior to use. The traditional method for the detection of bacteria and fungi involves microbiological culture using enrichment broths. (12)

4.2.3.Tests for mycoplasma

Mycoplasma infection of cell cultures was first observed by Robinson *et al.* in 1956. The incidence of mycoplasma-infected cultures has been found to vary between laboratories and arises from poor aseptic technique and insufficient training in good cell culture practices. (10) It is crucial that mycoplasma-free cultures and cell lines are used because the presence of mycoplasma can

- affect the rate of cell proliferation
- induce morphological changes
- cause chromosome aberrations
- influence amino acid and nucleic acid metabolism
- induce cell transformation.

Naturally, regulatory organizations insist that all cell cultures and additives used for the production of reagents for diagnostic kits as well as therapeutic agents are free of mycoplasma infection. (10)

4.2.4.Virus testing

There are three major concerns pertinent to the virus contamination of cell lines. These are (11):

(i) The safety of laboratory staff handling the cell lines and performing the procedures required for the reduction or elimination of the associated risk in experimental and other procedures.

(ii) The medicinal safety of any products derived from cells and their clinical application. This problem also impinges on regulatory matters.

(iii) The validity of any experimental data produced using contaminated cell lines.

4.2.4.1.Sources of viral contamination (11)

Potential sources of viral contamination are:

- the original tissue used to prepare the cell line
- other infected cultures
- growth medium
- serum
- other reagents (e.g. trypsin)
- laboratory personnel.

A-Tissue-derived contamination

The factors influencing the choice of starting material for cell line derivation should take into account the possibility of existing virus infection. The viruses potentially involved will depend on the species of origin, the tissue taken and the clinical history of the animal or patient. An evaluation of the viruses endemic to the population from which the tissue is isolated should be performed.

A risk assessment must be made of the incidence of viral infection in the population from which clinical specimens are derived and in the laboratory staff engaged in tissue processing. For example, 80–90% of the human population has experienced Epstein–Barr virus (EBV) infection and may still carry the virus in cells from their peripheral blood, lymph nodes or spleen (11). There is, therefore, a high risk of EBV contamination associated with human material. This is counterbalanced by the fact that most laboratory staff will also be EBV sero-positive (i.e. immune to reinfection). However, adequate precautions must be taken to ensure that sero-negative staff are not exposed to a risk of infection.

B-Contamination by other cultures in the laboratory

If there is inadequate quarantining and testing of incoming cultures (see Section inadequate segregation of different cultures, and poor practices and handling of cells in the laboratory, then the potential for a virally contaminated culture to cross-contaminate other cultures is clear.

C-Medium-derived contamination

The potential for basal media to pass on viral contamination to cells has been greatly reduced in recent years by the move amongst commercial media suppliers to source all components, as far as possible, from non-animal sources. However, cases of basal medium

contamination by Minute Virus of Mice (MVM) have been reported, probably due to poor rodent pest control during the shipping of powdered medium (11)

D-Serum-derived contamination

The risk of viral contamination of serum can be reduced (but not entirely eliminated) by careful sourcing – for example, from countries where certain viruses are not endemic and testing of the serum pool and/or bottled product. For most applications, BVDV, infectious rhinotracheitis virus and para-influenza-3 virus are probably the major concerns relating to bovine serum. It is known, for example, that 50–90% of cattle in the USA are infected with BVDV. Extensive testing of serum for the presence of viruses is performed by reputable suppliers, but levels of detection are limited by the sampling required and the sensitivity of the techniques employed. The level of contamination with viable virus particles can be considerably reduced by treating serum with one of a variety of methods, of which gamma-irradiation is probably the best in terms of both virus kill and maintenance of the serum's growth-promoting activity. (11)

E-Other reagents

Trypsin is widely used as a detachment agent in many cell culture protocols. It is usually of porcine origin and has been shown to be a potential source of porcine parvovirus. Other agents used in cell culture, and that are derived from a human or animal source (e.g. albumin, transferrin), must also be considered a potential source of viral contaminants from that species (11)

F-Personnel

Virus contamination originating from individuals performing cell culture has, over the years, proven difficult to document. However, the potential clearly exists, as a number of virus types, including rhinoviruses, rotaviruses and respiratory syncytial virus, have been shown to be extremely stable in aerosols, on workers' hands and on the surfaces (10, 11).

G-Transmissible spongiform encephalopathy

The risk of contamination of tissue culture cells with transmissible spongiform encephalopathy (TSE) agents as a result of the use of animal products as medium components has been considered to be low, in part because only a few (brain-derived) cell lines have been reported to be susceptible to TSE infection (12).

Nevertheless, in response to regulators' concerns, consideration must be given to the origin of FBS and other bovine sera. Most companies supplying bovine serum can supply

from source countries with no reported cases. In addition, the infectious agent (a prion) is likely to be present predominantly in neural tissue, which is beyond the blood–brain barrier, and there is also a species barrier to consider. With these facts in mind it is considered that serum poses little risk of transmitting BSE (10)

4.3.Eradication of contamination

If a cell culture is found to be contaminated with bacteria, fungi, mycoplasma or virus then the best method of elimination is to discard the culture. Fresh cultures can be obtained from seed stock, from a bio-repository or from the originator of that cell line. It is important to locate the source of the contamination (media, culture reagents, faulty safety cabinet, poor aseptic technique, etc.) to prevent a recurrence. As well as eliminating the contamination in any particular culture, it is important to eliminate the source of contamination by using completely new and tested media and reagents, cleaned and tested equipment and, where applicable, retrained staff. After any major contamination, the laboratory and MSC should, if possible, be fumigated according to the manufacturer's instructions (where applicable) and all surfaces swabbed with a suitable disinfectant. (10)

In the case of irreplaceable stocks it will be necessary to attempt to eliminate the contamination using antibiotics. This approach is possible for bacteria, mycoplasma, yeast and fungi. However, there are no reliable methods for the eradication of viruses from infected cultures. All elimination treatments must be performed in a facility separate from the main cell culture laboratory. It must be remembered that the majority of contaminating agents replicate faster than most cell cultures and so the chances of success are not high. Once the contaminant is identified and an antibiotic/antimycotic agent(s) has been chosen, the eradication process should be rigorously challenged to ensure that it is likely to be successful. Finally, all treated cultures must undergo a full regimen of quality control tests. At ECACC, after an eradication treatment we typically put treated cultures through a series of extended passages prior to retesting to confirm the absence of the contaminant. (10)

5.Authentication

Authentication of cell lines to confirm their identity and source of origin is an essential requirement in the management of cell stocks. Cross-contamination between cell cultures was first recognized in the late 1950s but has become more prevalent in recent times (10). Surveys of the scientific literature have suggested that up to 20% of published work based on the use of human cell lines specifies lines that are known to be misidentified (11,

12). This problem very often means that experimental data generated by the use of cross-contaminated or misidentified cell lines is invalid, and undermines the reliability of research based on its inclusion and use in future scientific endeavours (12). The ECACC and other culture collections have an obligation to raise awareness of this problem in the cell culture community and many bio-resource centres now actively post details of misidentified cell lines on their websites.

As more laboratories undertake authentication of their cell stocks, access to databases or libraries of authentication data is in demand. Bio-resource centres endeavour to make this information available, and the concept of having one super-database to house all available profiles has often been discussed. This will likely be explored in future years. Cross-contamination is most likely to occur via poor cell culture practices, such as handling more than one cell culture at a time. It is vitally important that cell lines, their growth media and additives, paperwork and identification (labelling) be segregated, either in time or in space. In addition, it is vital that all tissue culture plastic and other materials are removed from the MSC, which is then disinfected before work with the next cell line. It is poor cell culture practice to work with more than one culture at one time, use the same bottle of medium and components for more than one cell line, and not to clearly separate the paperwork and labels for each cell line. Failure to do so can result in a culture being cross-contaminated with another cell line. In the (somewhat unlikely) case where both cell lines in a contaminated culture repli- cate at the same rate, then their presence can be detected by differences in their mor- phology or determined by other criteria. If the contaminating cell line replicates faster than the host line, then within several population doublings the contaminating cell line will completely replace the original cell culture. (11)

Finally, where poor segregation of paperwork and labelling occurs a culture can be mislabelled. All of these problems are more likely to occur in cultures taken to high passage numbers or where slowly growing cultures are maintained for long periods of time. Cross-contamination may well go unnoticed in laboratories using many cultures with similar morphologies, for example fibroblast cultures. In order to avoid the problem at the outset, cell lines must be obtained from accredited and quality-controlled sources such as the original depositor or an established cell culture collection rather than being passed from one laboratory to another. It is important to emphasize that cell authentication is an essential part of quality assurance for both research and commercial use of cell cultures and should be of primary concern for everyone (12).

CHAPTER IV

Cell Culture Media and Techniques

1. 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 are similar to their media *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. (13)

1.1.Basic components in culture media

The 10 basic components that make up most of the animal cell culture media are as follows: inorganic salts (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}), nitrogen source (amino acids), energy sources (glucose, fructose), vitamins, fat and fat soluble component (fatty acids, cholesterols), nucleic acid precursors, growth factors and hormones, antibiotics, pH and buffering systems, and oxygen and carbon dioxide concentrations. (13)

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. (13)

1.2. Natural media

Natural media consist of naturally occurring biological fluids sufficient for the growth and proliferation of animals cells and tissues. This media useful for promoting cell growth are of the following three types (14):

a.Coagulant or clots: Plasma separated from heparinized blood from chickens or other animals is commercially available in the form of liquid plasma.

b.Biological fluids: This includes body fluids such as plasma, serum lymph, amniotic fluid, pleural fluid, insect hemolymph, and foetal 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.

1.3. 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 (13).

The artificial media may be grouped into the following four classes: serum-containing media, serum-free media, chemically defined media, and protein-free media (14).

a.Serum-containing media

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 3) (14).

Table 3. Serum components, their composition, and role in animal cell culture (14)

Component	Probable function
Protein and polypeptide	
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
Growth factors	
Epidermal growth factor (EGF)	Proliferation and differentiation
Fibroblast growth factor (FGF)	Proliferation and differentiation
Hormones	
Insulin	Glucose and protein metabolism
Transferrin	Incorporation of iron by cells

b.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 foetal 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 (14).

c.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.

d.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.

2.Animal cell culture *in vitro*

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 (15)

2.1.Basic concepts

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_2, O_2, osmolality, and nutrition. In addition, the cultured cells require sterile conditions along with a steady supply of nutrients for growth and sophisticated incubation conditions (16)

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 (16).

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 (16).

2.2.Methods for Obtaining Cells

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

2.2.1.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* (16, 17).

2.2.2.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 cells in comparison to their normal counterparts, especially with reference to altered growth patterns and cell morphology (16, 17).

2.2.3.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 (17).

Cell culture is of three types (17):

a.Precursor cell culture, which is undifferentiated cells committed to differentiate;
b.Differentiated cell culture, which is completely differentiated cells that have lost the capacity to further differentiate; and
***c.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.

The cultured cells can form Monolayer cultures or Suspension cultures (17):

- ✓ ***Monolayer culture*** the monolayer cultures 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 culture:*** some of the cells are non-adhesive 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.

2.3.Freezing Cells and Thawing Cryopreserved Cells

2.3.1. Freezing Cells

When a surplus of cells becomes available during subculturing, they can be preserved at that passage through freezing with cryoprotective agents (e.g., glycerol or dimethyl sulfoxide (DMSO)) that prevent the formation of harmful extra- or intracellular crystals (16). To that end, cells are dissociated from the culture vessel and condensed. The cell pellet is resuspended in 1 mL of freezing medium (e.g., knockout serum replacement medium supplemented with 10% DMSO) and ~1 $\times 10^6$ cells are transferred into each cryovial. After 20–30 minutes, the cryoprotectant will have penetrated the cells. Cooled down overnight at −80°C at a controlled freezing rate of 1–2°C/min, the vials are then transferred to liquid nitrogen for long-term storage (16).

Note: While glycerol and DMSO are both suitable cyroprotective agents, handling of DMSO needs to be carefully monitored. In high (stock) concentrations, DMSO is toxic to personnel and cultured cells and therefore cannot be added to cells without prior dilution. This toxicity also affects cells in freezing medium containing 10% DMSO when left for several hours at room temperature, highlighting the need to transfer cells to −80°C for storage within 30 minutes. In general, chemically protective gloves should be worn to safeguard personnel from the hazards of DMSO and its solutes to easily penetrate membranes, including the skin (16, 17).

2.3.1. Thawing Cryopreserved Cells

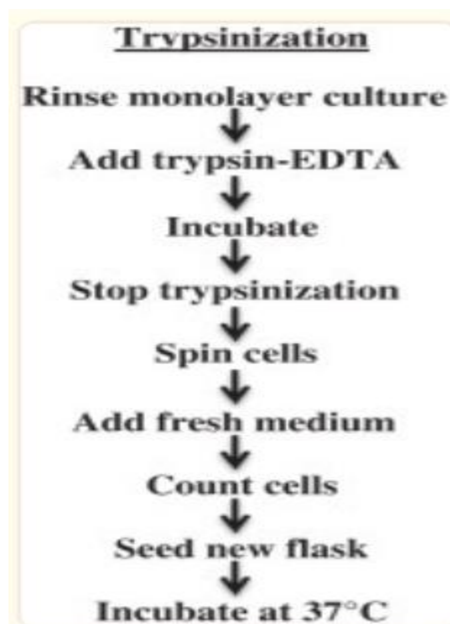
Most mammalian cells can be preserved in liquid nitrogen (<130°C) for numerous years since all biological processes are halted at these temperatures. To recover cells, 10 mL of complete medium is prewarmed in a water bath. After removing the frozen vial from liquid nitrogen, it is immediately placed into a 37°C water bath and gently swirled until two-thirds of the content are completely thawed. The vial is wiped with 70% ethanol and placed in a biosafety cabinet where 1 mL of the prewarmed medium is added in a drop-wise fashion to the partially thawed vial to minimize the osmotic stress imposed upon the cells when DMSO is diluted. The contents of the now completely thawed vial are transferred also in a drop-wise fashion to the remaining 9 mL of complete medium and centrifuged at 300 × *g* for 3 minutes. After aspirating the supernatant, the cell pellet can be washed once in medium to remove residual cryopreservatives. Cells are then resuspended in complete medium and transferred to a cell culture vessel. Cell attachment should occur within 24 hours. (16, 17).

Note: The viability of cells after cryopreservation is impacted by their ability to cope with the stressors of freezing and thawing. It is therefore recommended to perform the thawing process as swiftly as possible. When handling cryovials that have been frozen with glycerol as the cryoprotectant, the thawing process can be simplified by diluting the cryopreserved cells ten times directly into complete, prewarmed medium, avoiding the centrifugation and washing step (16)

2.4.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 the following Flowchart (16)



Trypsinization of adherent cells.

2.5.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. Cells can die in the process of culturing or during handling and passaging. When relying on a specific concentration of live cells to start a culture or needing a specific number of live cells for an assay it is important to distinguish between live and dead cells. Cell counting is also helpful when assessing growth rates.

Since cells are commonly cultured in the millions, the number of cells are first counted in a small volume and then extrapolated to the full cell volume. To achieve this, all cells are dissociated, pelleted, and evenly resuspended in a suitable medium volume.

In a 1:1 dilution with 0.4% trypan Blue, a small volume of the cell suspension is mixed in an Eppendorf tube. Trypan Blue dye permeates only nonviable cells that can therefore be excluded from the subsequent quantification (18). This occurs by loading 10 µL of the cell mixture in Trypan Blue onto a hemacytometer. Using an inverted microscope, phase contrast, and a magnification of at least 10X, all cells located in the four outer squares are counted. Viable cells contain a darker “halo,” while nonviable cells stain blue/black.

To determine the total number of viable cells, the number of cells found in all four squares is divided by 4 (to determine the average cell number in 1 mm^2), multiplied by 10^4 (to obtain the cell number per mL), multiplied by 2 (to account for the dilution factor of Trypan Blue) and multiplied by the initial medium volume of the entire cell suspension. The percentage of

viable cells can be determined by dividing the number of unstained cells by the total number of cells, and multiplying the ratio by 100. A healthy cell culture is characterized by 80–95% cell viability (18).

The hemacytometer is prepared by covering both counting chambers with a cover glass. Subsequently, 10 µL of a 1:1 cell suspension with 0.4 % Trypan Blue is loaded onto the filling notch of one of the counting chambers. Through capillary action, this volume will cover the grid that can be observed in an inverted microscope at magnifications of at least 10X.

The average of cells covering squares A–D determines the number of cells per mm^2. Viable cells in these squares are counted by excluding nonviable cells that appear black due to their absorption of Trypan Blue through their permeable cell membranes. Only cells overlapping with one of the outer horizontal and vertical borders should be included (18).

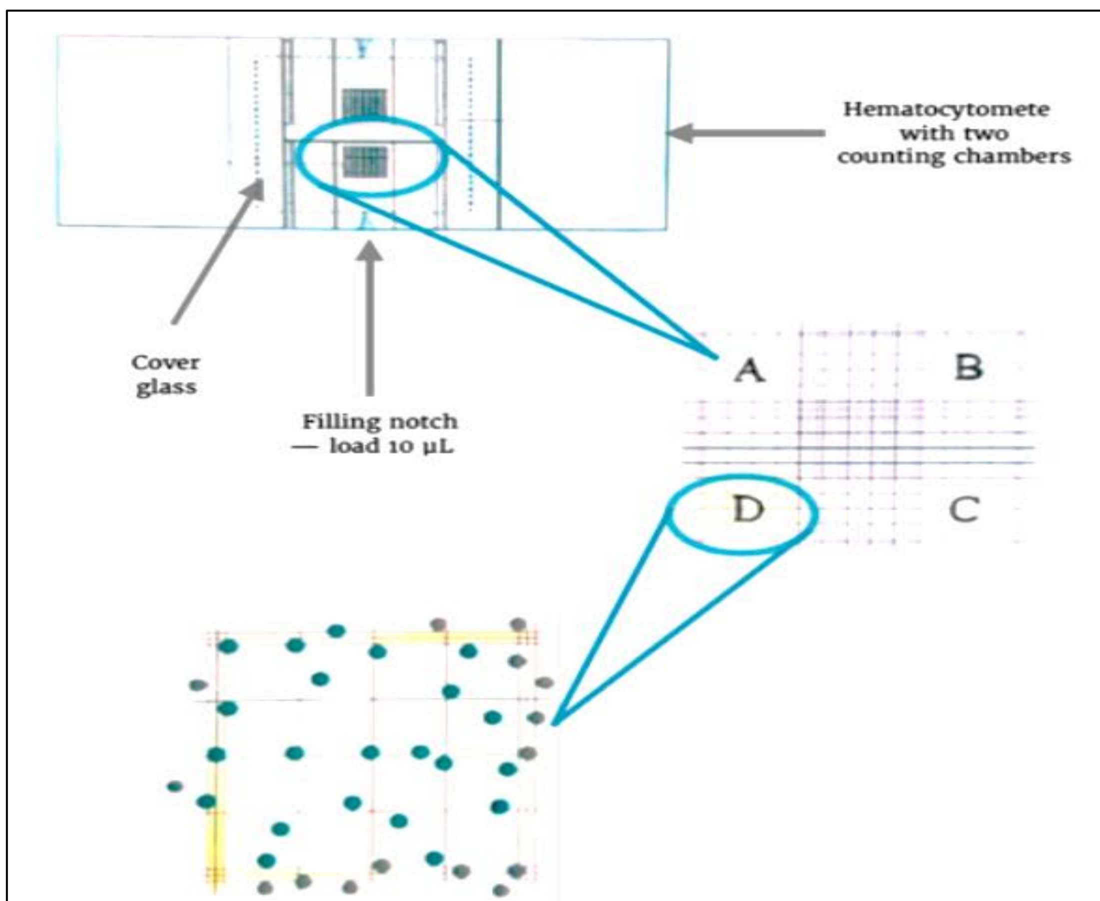


Figure 11: Cell quantification using Trypan Blue (18).

2.6.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 (18).

3.Animal cell growth *in vitro*:

3.1. Cycle and evolution

If you remove tissue from an embryo, dissociate it into a suspension of single cells, and plate them out onto a culture dish, a series of characteristic events occurs. Firstly, cells are in a lag phase, usually no more than 1-2 days in length, during which there is little or no increase in cell number. During this time, cells are "conditioning" the medium, undergoing internal cytoskeletal and enzyme changes and adjusting to the new medium. Secondly, the cells undergo a period of rapid division, so-called log phase growth. Then, as they approach confluency and form contacts with one another, their rate of division slows and they begin to express a program of differentiation characteristic of their tissue of origin. Muscle cells fuse and acquire cross-striation, epithelial cells from the kidney or gut become linked by junctional complexes and transport ions from one surface to another, heart cells begin to beat spontaneously (19).

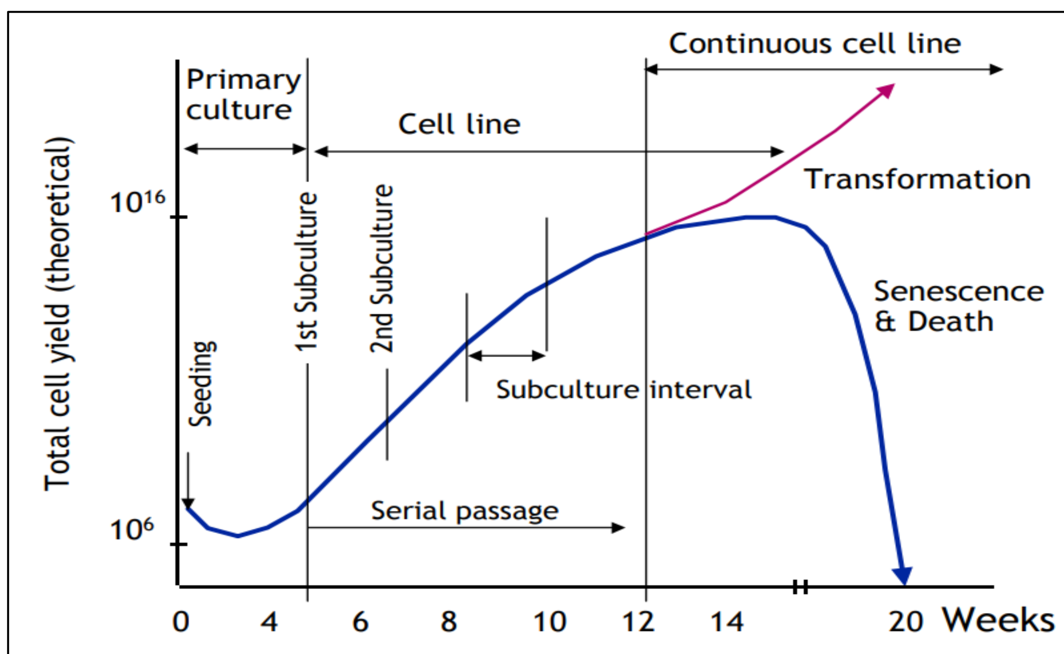


Figure 12: evolution and cycle of cell growth *in vitro* (19)

Cultures such as those just described are referred to as primary cultures, because they are prepared from cells taken directly from the animal. The cells divide or not (depending on what they are accustomed to), acquire differentiated characteristics, and ultimately die. For the next experiment, it's back to the animal again to obtain new tissue and prepare new culture. Alternatively, in the case of cells that divide in culture, it is possible to 'passage' or 'subculture' them by inducing them to detach from the substrate, 'splitting' them (i.e., diluting them several-fold in medium and replating them into new dishes), and allowing them to reenter log phase growth. However, the properties of the cultured cells often change gradually with passaging, as more rapidly dividing cell populations come to predominate and more 'differentiated' cells, which divide more slowly, are lost (19).

3.2.Behavior of cells *in vitro*

The behavior of cells *in vitro* depends on the cell culture type: adherent, suspension and feeder. Each has its own specific characteristic and behavior. These three types of cell cultures are also used for different purposes:

- ✓ *Suspension cultures* are often employed for the large-scale production of proteins, antibodies, and other biologics.
- ✓ *Adherent cultures* are used for studying cell behavior in a more natural microenvironment or for specific applications like tissue engineering.
- ✓ *Feeder cells* (*fibroblasts)* provide physical support to help the cell cultures adhere and grow. Let’s review what each of these cell cultures are and discuss their characteristics and behaviour (19).

CHAPTER V

Cell Culture Applications

1. Model Systems in Health and Disease

Cell culture is one of the most important techniques in cellular and molecular biology since it provides a platform to investigate the biology, biochemistry, physiology (e.g., aging) and metabolism of wild-type cells and diseased cells. The interaction and route of infection between wild-type cells and pathogenic agents (e.g., bacteria and viruses) can also be studied in specific cocultures.

Furthermore, immortalized cancer cell lines have given researchers insight into the biology of cancer and through the selective treatment of wild-type cells with UV radiation, viruses, and toxins, causative agents of tumorigenicity have been identified.

Finally, human-induced pluripotent stem cells (hIPSCs) have been derived from individuals with inherited disorders and differentiated toward the affected cell type in which the disease manifests. These hIPSC-derived somatic cells are suitable platforms for the studying molecular mechanisms of a disease in a dish (15)..

2.Drug Development and Drug Testing

Cell culture tools can also be applied to screen novel chemicals, cosmetics, and drug compounds for their efficacy and assess drug cytotoxicity in specific cell types. Detoxifying cell types such as hepatocytes and kidney cells are oftentimes of high interest for these purposes. When using cell co-cultures or diseased cells obtained from individual patients, it is also possible to screen for drugs to selectively target specific cell types (e.g., in cancer treatment), at doses that are nontoxic and with minimized side-effects for the patient. Furthermore, large-scale cells cultures can serve for the generation of genetically engineered proteins, antibodies, hormones. and biopharmaceuticals that can be isolated and used therapeutically (17, 19)..

3.Virology and Vaccine Production

Cell culture with mammalian cells offers a host for viruses to replicate, allowing researchers to study their growth rates, development, and conditions required for their infectious cycle. Furthermore, the attenuated viruses used in vaccines against polio, measles, chicken pox, rabies, and hepatitis B are raised in animal cell cultures. (11).

4.Tissue Regeneration and Transplantation

hIPSCs, embryonic stem cells, and adult stem cells have the capacity to regenerate and differentiate into specialized cell types that can be used as replacement tissues or organs. These

cell cultures are oftentimes performed in a 3D protein matrix that allows cells to self-organize into functional cell clusters (organoids).

5.Genetic Engineering and Gene Therapy

The expression of specific genes and their impact on cells can be studied by the introduction of new genetic material (e.g., DNA, RNA) into the nucleus of cultured mammalian cells. Similarly, the importance of genes in regulating specific pathways can be observed through silencing them. Oftentimes, viral vectors or specialized enzymes are used to carry out these tasks. Altering the genome of cells can also aid in restoring dysfunctional genes in patients.

The overarching benefit of using cell culture techniques to address these basic scientific and translational research questions is the homogeneity and reproducibility of data that can be generated using clonal cell lines. Studying an isolated, simplified cellular system in a well-defined and controlled environment limits the exposure of confounding effects inherent to an in vivo system and therefore allows for the generation of simplified but robust data sets (14, 17).

6.Key limitations

6.1.Discrepancies between Cellular Environments in vitro and in vivo

One of the pillars of cell culture research is the design of a defined cellular environment in which single variables can be manipulated in order to monitor cellular responses. To achieve this goal, the cellular environment in vitro is oftentimes oversimplified and relies, for example, on a single cell type cultured in a monolayer. However, data generated from such a cellular system does not truly phenocopy the intricate cellular interactions between different cell types and extracellular matrices of an in vivo environment (17).

To address this drawback, there is currently significant research into the design of cell cocultures that allow paracrine signaling between cells that cohabitate space in vivo, as well as bioartifical matrices that facilitate cellular growth in their native 3D orientation. The goal is the design of cellular systems that mimic the complexity of the multicellular in vivo niche, yet also allow standardization for cell culture assays (17)..

6.2.Discrepancies between Gene Expression in Primary Cells and Immortal Cell Lines

The oftentimes most relevant cell types for addressing translational research questions—primary cells—are in fact very difficult to isolate and culture *in vitro* due to their limited proliferation and functional capacity *ex vivo* (14).

To delay senescence, viral transfection of primary cells can sequester tumor-suppressor proteins, thereby extending the number of possible passages and allowing the emergence of

immortal cell lines. Although this facilitates their culture ex vivo, this technique also introduces the expression of carcinogenic genes. In addition, immortalized cell lines can acquire mutations during subculturing that can further interfere with the cellular phenotype and create a non-physiological cell culture system (14).

7.Techniques histologiques

8.Techniques cytologiques

9.Immunohistochemistry techniques

Immunohistochemistry (IHC) uses antibodies to detect antigens in a tissue sample. It's one lab technique a pathologist may use to check for signs of disease following a biopsy. IHC is commonly used to diagnose cancer, predict treatment response and determine likely outcomes (prognosis) of the disease.

9.1.Definition

Immunohistochemistry (IHC) is a lab technique pathologists use to look for signs of disease in a tissue sample. A pathologist uses lab tests to diagnose medical conditions.

As part of diagnosis, a healthcare provider may remove tissue and send it to a lab for testing. For example, they may remove part of a tumor and send it to a lab to test for cancer cells. This is called a biopsy. An IHC is just one method a pathologist may use to study the sample once it arrives at the lab (23)

IHC is the most common type of immunostaining. Immunostaining involves using antibodies and special markers to "label" parts of a tissue sample so they're easier for pathologists to identify.

The word "immunohistochemistry" provides clues about what's involved:

- ✓ ***Immuno*** means relating to your immune system. Your immune system detects substances in your body (called **antigens**). It makes antibodies to find and destroy antigens that don't belong, like pathogens (viruses, bacteria, fungi and parasites) and cancer cells. An IHC uses the antigen-finding properties of antibodies to detect and grab onto antigens in a tissue sample. Antibodies stain the sample so the pathologist can see the antigens they're attached to when viewed beneath a microscope.
- ✓ ***Histo means tissue***. An IHC examines a tissue sample
- ✓ ***Chemistry*** studies the tiny building blocks that make up all matter, including human tissue. An IHC uses a microscope to see otherwise invisible antigens that may indicate diseases

9.2. **When is immunohistochemistry performed?**

An IHC can be used to:

- ✓ Diagnose a condition: An IHC allows healthcare providers to diagnose conditions like cancer. It can help providers determine the type of cancer (for example, carcinoma, melanoma or sarcoma). It also allows healthcare providers to pinpoint the origins of cancer that's spread (metastatic cancer).
- ✓ Determine prognosis: An IHC can determine how high-risk, or aggressive, a cancer is. It also can help providers stage and grade cancer. This information can help providers determine the best options for treatment.
- ✓ Predict treatment response: An IHC can identify characteristics of tumor tissue that provide clues about how cancer may respond to treatment. For example, pathologists can identify breast and prostate cancers that are likely to grow in the presence of certain hormones, like estrogen and testosterone. These cancers may respond best to treatments that block these hormones (hormone therapy).
- ✓ Monitor treatment response: An IHC allows providers to monitor whether treatments are working to rid your body of the disease.

Researchers also perform IHC to develop new drug treatments. IHC helps researchers learn more about how the smallest parts of your body work, like your cells and the molecules inside them. IHC provides insight into how diseases affect these processes and what treatments can help (23).

9.3. What diseases can be diagnosed by immunohistochemistry?

Healthcare providers most commonly use IHC to diagnose cancer, but it can also diagnose other conditions, including Alzheimer's disease, Parkinson's disease and muscular dystrophy. It can identify pathogens that cause infection, too. The first successful IHC stain occurred in 1941, when researchers identified the bacteria associated with pneumonia (*pneumococcus*) in a tissue sample (23).

9.4. **How does the test work?**

An IHC uses antibodies to detect a target antigen in a tissue sample. The target antigen is a marker, indicating a specific disease is present. If the antibody recognizes the antigen, it will attach (bind) to it. The binding process is similar to a lock (antigen) and key (antibody). If the antibody binds to the antigen, the tissue sample will stain a certain color when viewed beneath a microscope.

9.4.1.Test steps

- A pathologist links an antibody to an enzyme that will react if the antibody binds to the target antigen.
- The antibody binds to the target antigen if it's present.
- The attachment causes the enzyme to react.
- The reaction causes the tissue sample to stain a certain color when viewed beneath a microscope.

For the results to be reliable, pathologists must accurately complete multiple steps.

9.4.2.Preparing the sample

Preparing the sample ensures it stains correctly. If an antigen is present, it stands out in colored segments against the background. To prepare the sample, pathologists:

1. Preserve the tissue. Tissue consists of cells that die over time. Preserving, or "fixing," the tissue slows the process. Fixation maintains the tissue's structure, so it stains effectively. One of the most common substances used to fix the tissue is formalin, a formaldehyde solution.
2. Ensure antigens are accessible. The fixation process can sometimes block parts of the antigen so the antibody can't bind to it. A process called antigen retrieval can re-expose the antigen's binding points so antibodies can attach.
3. Block similar structures where an antibody may bind. Sometimes, antibodies bind to substances similar in structure to the target antigen — but that aren't the same. .Pathologists block these structures beforehand so the antibody only attaches to the target antigen.

9.4.3. Selecting antibodies

Pathologists select antibodies known to bind to the target antigen. IHC uses either polyclonal antibodies or monoclonal antibodies.

- Polyclonal antibodies:A mix of different antibodies. These antibodies may attach to multiple binding sites on an antigen.
- Monoclonal antibodies: Identical copies of the same antibody. Monoclonal antibodies will only attach to a specific binding site on an antigen.

9.4.4.Detecting the antigen

Pathologists prepare the antibody to stain tissue containing the antigen.

Pathologists:

1. Link the antibodies to an enzyme. Examples of enzymes include horseradish peroxidase and alkaline phosphatase.
2. Add the antibody with the enzyme to the tissue sample.
3. View the sample underneath a microscope.
4. Look for staining that indicates the antigen is present.

The first successful IHC used a similar process. Instead of linking the antibody to an enzyme, the researchers linked it to a fluorophore. A fluorophore absorbs light and reflects it. The fluorophore stains the sample when viewed under a fluorescence microscope. This technique is now considered a different type of immunostaining called immunofluorescence.

9.5.What are the limitations of immunohistochemistry?

There aren't standard guidelines for each step in immunohistochemistry. Different labs use different techniques, which means results may vary.

Also, recent research has shown that not all antibodies available for IHC do what they're supposed to — that is, detect the target antigen in a sample. If there are problems with the antibodies, a test may give results that are false, including:

- **False-positive:** An IHC detects an antigen that *is not* present.
- **False-negative:** An IHC doesn't detect an antigen that *is* present.

Labs must have quality controls in place so that every step preserves the tissue and ensures a high-quality stain. To improve IHC accuracy, pathologists can test antibodies on tissue known to contain the target antigen to ensure it stains before testing an unknown tissue sample.

9.6.How accurate is immunohistochemistry?

When performed correctly and with quality controls in place, immunohistochemistry is a reliable method for cancer diagnosis. One study reports that IHC can accurately identify the primary location of metastatic cancer with 70% to 90% accuracy.

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