



Research Center for
Pharmaceutical Nanotechnology
Tabriz University of Medical Sciences

RCPN

An Introduction to Cell Culture

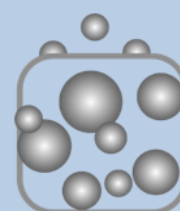
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Introduction

This handbook is intended as an introduction to cell culture basics, covering topics such as getting familiar with the requirements of a laboratory dedicated to cell culture experiments, laboratory safety, aseptic technique, and microbial contamination of cell cultures, as well as providing basic methods for passaging, freezing, and thawing cultured cells.

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Introduction to Cell Culture

What is Cell Culture?

In its simplest form, cell culture involves the dispersal of cells in an artificial environment composed of nutrient solutions, a suitable surface to support the growth of cells, and ideal conditions of temperature, humidity, and gaseous atmosphere. In such a system, a researcher can precisely measure the response of the cell's alterations in culture, prospective drugs, the presence or absence of other kinds of cells, carcinogenic agents, and viruses.

Classification of Cell Cultures

Primary Culture

- Cells when surgically or enzymatically removed from an organism and placed in suitable culture environment will attach and grow are called as primary culture
- Primary cells have a finite life span
- Primary culture contains a very heterogeneous population of cells
- Sub culturing of primary cells leads to the generation of cell lines
- Cell lines have limited life span, they passage several times before they become senescent
- Cells such as macrophages and neurons do not divide *in vitro* so can be used as primary cultures
- Lineage of cells originating from the primary culture is called a cell strain

Secondary Culture

When a primary culture is sub-cultured, it becomes known as secondary culture or cell line. Subculture (or passage) refers to the transfer of cells from one culture vessel to another culture vessel. This is periodically required to provide fresh nutrients and growing space for continuously growing cell lines. The process involves removing the growth media and disassociating the adhered cells (usually enzymatically). Such cultures may be called secondary cultures.

Cell Line

A cell line or cell strain may be finite or continuous depending upon whether it has limited culture life span or it is immortal in culture. On the basis of the life span of culture, the cell lines are categorized into two types:

- Finite cell lines - The cell lines which have a limited life span and go through a limited number of cell generations (usually 20-80 population doublings) are known as finite cell lines. These cell lines exhibit the property of contact inhibition, density limitation and anchorage dependence. The growth rate is slow and doubling time is around 24-96 hours.
- Continuous cell lines - Cell lines transformed under laboratory conditions or *in vitro* culture conditions give rise to continuous cell lines. These cell lines show the property of ploidy (aneuploidy or heteroploidy), absence of contact inhibition and anchorage

dependence. They grow either in a monolayer or in suspension (see below). The growth rate is rapid and doubling time can be 12-24 hours.

Types of cell culture

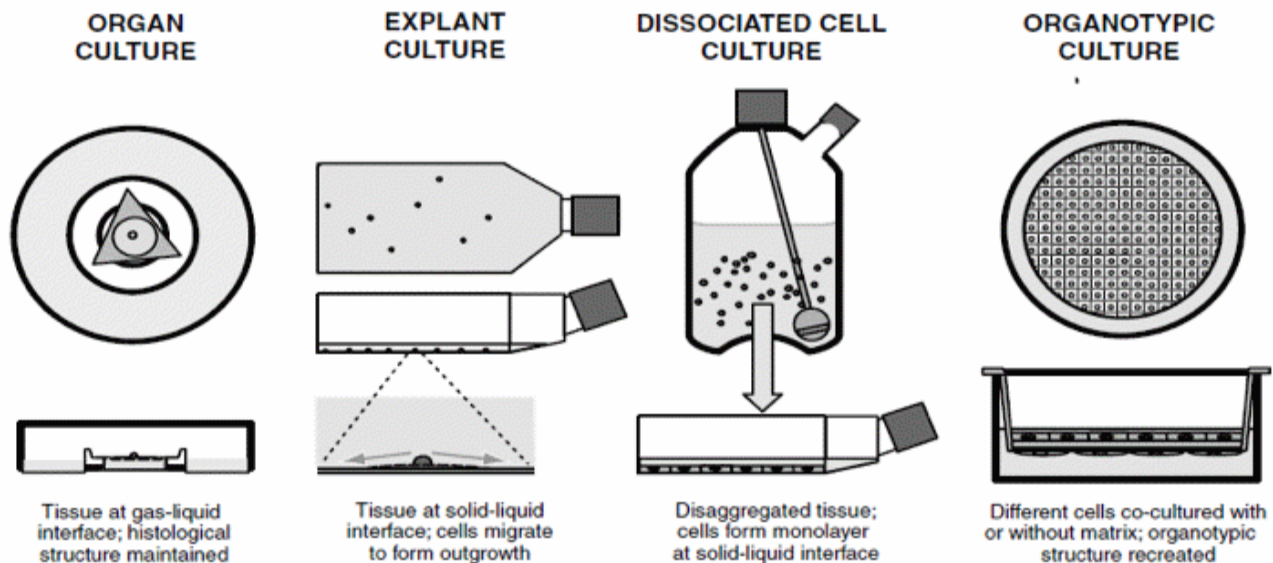


Fig1: Types of culture. Different modes of culture are represented from left to right. First an organ culture on a filter disk on a triangular stainless steel grid over a well of medium, seen in section in the lower diagram. Second, explants cultures in a flask, with section below and with an enlarged detail in section in the lowest diagram, showing the explants and radial outgrowth under the arrows. Third, a stirred vessel with an enzymatic disaggregation generating a cell suspension seeded as a monolayer in the lower diagram. Fourth, a filter well showing an array of cells, seen in section in the lower diagram, combined with matrix and stromal cells. (Cell culture basicshandbook, Invitrogen)

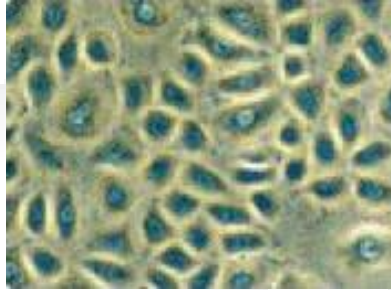
Record Keeping

When the sample arrives at the laboratory, it should be entered into a record system and assigned a number. This record should contain the details of the donor, identified by hospital number rather than by name, tissue site, and all information regarding collection medium, time in transit, treatment on arrival, primary disaggregation, and culture details, etc. [Freshney, 2002, 2005]. This information will be important in the comparison of the success of individual cultures, and if a long-term cell line is derived from the culture, this will be the first element in the cell line's provenance, which will be supplemented with each successive manipulation or experimental procedure. Such records are best maintained in a computer database where each record can be derived from duplication of the previous record with appropriate modifications. There may be issues of data protection and patient confidentiality to be dealt with when obtaining ethical consent.

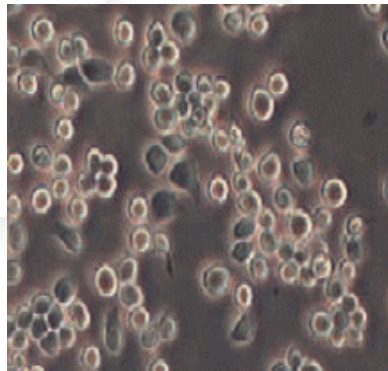
Morphology of Cells in Culture

On the basis of morphology (shape & appearance) or on their functional characteristics. They are divided into three:

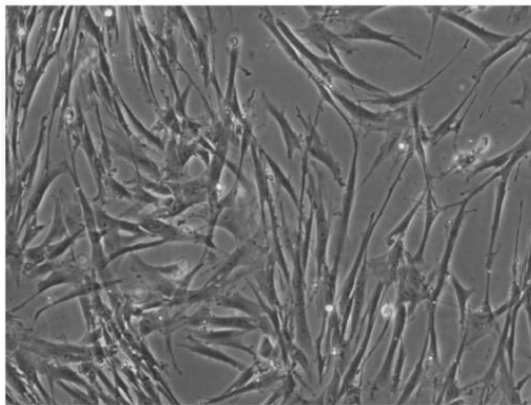
- Epithelial like-attached to a substrate and appears flattened and polygonal in shape



- Lymphoblast like- cells do not attach remain in suspension with a spherical shape



- Fibroblast like- cells attached to an substrate appears elongated and bipolar



Contact Inhibition

- When cells contact each other, they cease their growth.
- Cells arrest in G0 phase of the cell cycle
- Transformed cells will continue to proliferate and pile upon each other

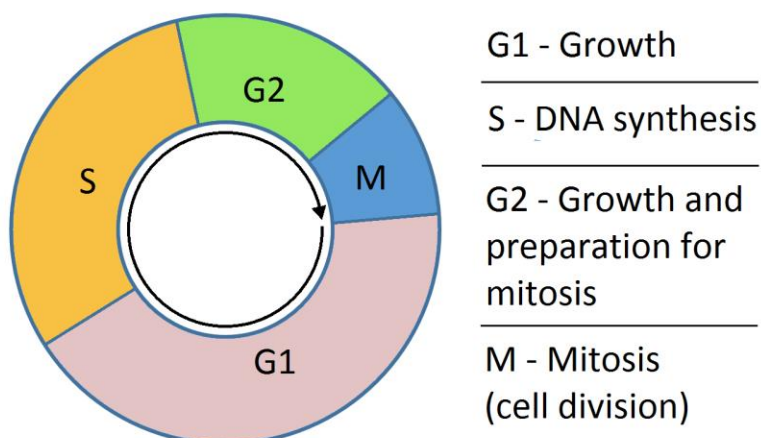


Fig2: cell cycle sample

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 (O₂, CO₂), and regulates the physicochemical 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).

Cell Culture Laboratory

Safety Aspects of Cell Culture

Risk Assessment

The main aim of risk assessment is to prevent injury, protect property and avoid harm to individuals and the environment. In many countries the performance of risk assessment is a legal requirement. Consequently risk assessments must be undertaken prior to starting any activity. The assessment consists of two elements:

1. Identifying and evaluating the risks.
2. Defining ways of avoiding or minimizing the risk.

For animal cell culture the level of risk is dependent upon the cell line to be used and is based on whether the cell line is likely to cause harm to humans. The different classifications are given below:

Table1. Classifications of risk levels in cell culture

Low risk	Non human/non primate continuous cell lines and some well characterized human continuous lines.
Medium risk	Poorly characterized mammalian cell lines.
High risk	Primary cells derived from human/primate tissue or blood.
	Cell lines with endogenous pathogens (the precise categorization is dependent upon the pathogen) – refer to ACDP guidelines, for details [†] .
	Cell lines used following experimental infection where the categorization is dependent upon the infecting agent – refer to ACDP guidelines, for details.

For most cell lines the appropriate level of containment is Level 2 requiring a class 2 microbiological safety cabinet. However, this may need to be increased to containment Level 3 depending upon the type of manipulations to be carried out and whether large culture volumes are envisaged. For cell lines derived from patients with HIV or Human T-Lymphotropic Virus (HTLV) Level 3 containment is required.

Containment is the most obvious means of reducing risk. Other less obvious measures include restricting the movement of staff and equipment into and out of laboratories. Good laboratory practice and good bench techniques such as ensuring work areas are uncluttered, reagents are correctly labeled and stored, are also important for reducing risk and making the laboratory a safe environment in which to work. The risk of exposure to aerosols or splashes can be limited by avoiding rapid pipetting, scraping and pouring. In addition, it is recommended that people working in laboratories where primary human material is used are vaccinated against Hepatitis B. Staff training and the use of written standard operating procedures and risk assessments will also reduce the potential for harm. Cell culture training courses covering the basics of tissue culture safety.

Operational standards

Training

- PI is to provide training, First aid and Post Exposure Protocol.
- PI is to identify the location of spill control material and safety equipment including eyewash and shower available on-site.
- All personnel must be trained in the Safe Work practices or supervised.

Access Control and Biosecurity

- Doors to laboratories where the work is done must not be left open
- Restrict entry to laboratory staff, animal handlers, maintenance staff and others on official business.
- A U of M WHIP sign is required with appropriate contact info, Containment level 2 and biohazard logo.
- Refer to Basic Lab Biosecurity Plan in the Biosafety Guide. (available in RCPN website and laboratories)

PPE

Wear the PPE as prescribed by your PI or as found below. Refer also to biosafety Guide.

Shoes

Wear close toed and heel shoes in the lab at all times.

Lab coats

- A clean lab coat for changing should be available at all times.
- No bare legs and arms are preferred i.e. long pants & socks, lab coat with long sleeves with cuffs and buttoned up.
- If a known or suspected exposure occurs to any clothing, decontaminate clothing before laundering E.g. autoclaving lab coats or treating spill with bleach. (Unless laundering facilities are within the containment laboratory and have been proven to be effective in decontamination).
- A back closing gown may be preferred or be required for some work. E.g. Work in a BSC depending on the specific hazards of your cell line, spill clean-up.

Face Protection

- Wear safety glasses for all bench work with the biological materials.
- For spill cleanup outside of the BSC wear face protection (full face shield or safety glasses and mask).

Gloves

- Wear gloves (e.g., latex, vinyl, co-polymer) for all procedures that might involve direct skin contact with potentially infectious material.
- Inspect gloves for tears and punctures before putting them on.
- Have gloves available in sizes required by lab personnel. Nitrile is preferred due to fewer allergy issues. Nitrile gloves will not maintain their integrity when punctured, thus identifying a potential exposure sooner.
- Do not touch contaminated surfaces with bare hands when removing your gloves.
- Wash your hands immediately after removing gloves.
- Remove your gloves when leaving the laboratory and before touching clean surfaces in the lab like phones, computer, light switch, door handles and reference books.
- Decontaminate your gloves with other laboratory wastes before disposal.
- Wear gloves if you have dermatitis or other lesions on the hands and you have indirect contact with potentially infectious material.

Biohazards

Viruses pathogenic for humans are one of the most likely biohazards presented by cell cultures. Where infection with an agent pathogenic for humans is known or suspected, the cell culture should be handled at a containment level appropriate for the agent concerned. Other potential biohazards should also be considered. These relate to components of the cell culture medium, other adventitious agents (e.g. contaminating mycoplasmas), and cell products, some of which may be biologically active molecules with pharmacological, immunomodulating or sensitizing properties. In addition, the generation and use of modified cells, for example, hybrids, transformed cells and cells containing recombinant DNA can be hazardous. These procedures could potentially result in the appearance of modified or reactivated viruses, novel fusion/hybrid proteins (especially in cross-species hybrids) and the expression of viral or cellular oncogenes. Laboratory workers should never culture their own cells. *In vitro* transformation or genetic modification could result in malignant disease or expression of an unusual pharmacologically active protein if they were to be accidentally inoculated into the donor. Therefore, human cells should be obtained from individuals having no association with the experimental work.

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. For more information, see Cell Culture Hood.

Disinfection

Methods designed for the disinfection/decontamination of culture waste, work surfaces and equipment represent important means for minimizing the risk of harm. Always wear appropriate personal protective equipment (PPE) such as gloves and eye protection when using concentrated forms of disinfectants. The selected gloves should protect against the substance being handled. Manufacturers' charts will help to identify the best gloves for the work.

The major disinfectants fall into four groups and their relative merits can be summarized as follows:

- Hypochlorites (e.g., Sodium Hypochlorite)
- Good general purpose disinfectant
- Active against viruses
- Corrosive against metals and therefore should not be used on metal surfaces e.g. centrifuges
- Readily inactivated by organic matter and therefore should be made fresh daily

Note: When fumigating a cabinet or room using formaldehyde all the hypochlorites must first be removed as the two chemicals react together to produce carcinogenic products.

- Phenolics
- Alcohol (e.g. Ethanol, Isopropanol)

Effective concentrations: 70% for ethanol, 60-70% for isopropanol

Their mode of activity is by dehydration and fixation. Effective against bacteria. Ethanol is effective against most viruses but not non-enveloped viruses

- Isopropanol is not effective against viruses
- Aldehydes (e.g. Formaldehyde)

Aldehydes are irritants and their use should be limited due to problems of sensitization should only be used in well ventilated areas.

Formaldehyde is used to fumigate laboratories. The formaldehyde is heated in a device so it will vaporize and all exposed surfaces are coated with the disinfectant.

Generally the use of aldehydes for disinfection and fumigation purposes can be hazardous. Check local regulations and with your safety advisor.

Waste Disposal

Any employer has a 'duty of care' to dispose of all biological waste safely in accordance with national legislative requirements. Given below is a list of ways in which tissue culture waste can be decontaminated and disposed of safely. One of the most important aspects of the management

of all laboratory-generated waste is to dispose of waste regularly and not to allow the amounts to build up. The best approach is 'little and often'. Different forms of waste require different treatment.

- Tissue culture waste (culture medium) – inactivate for at least 2 hours in a solution of hypochlorite (10,000ppm) prior to disposal to drain with an excess of water.
- Contaminated pipettes should be placed in hypochlorite solution (2500ppm) overnight before disposal by autoclaving and incineration.
- Solid waste such as flasks, centrifuge tubes, contaminated gloves, tissues, etc., should be placed inside heavy-duty sacks for contaminated waste and incinerated.
- If at all possible waste should be incinerated rather than autoclaved.
- Waste from specially licensed laboratories e.g. those handling genetically modified level 3 (GM3) organisms require specific treatment and tracking.

Did You Know?

Any employer has a 'duty of care' to dispose of all biological waste safely in accordance with national legislative requirements

Cell Culture Equipment

Basic Equipment

- Cell culture hood (i.e., laminar-flow hood or biosafety cabinet)
- Incubator (humid CO₂ incubator recommended)
- Water bath
- Centrifuge
- Refrigerator and freezer (–20°C)
- Cell counter (e.g., CountessR Automated Cell Counter or hemacytometer)
- Inverted microscope
- Liquid nitrogen (N₂) freezer or cryostorage container
- Sterilizer (i.e., autoclave)

Expanded Equipment

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

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

Aseptic Work Area

The human skin harbors a naturally occurring and vigorous population of bacterial and fungal inhabitants that shed microscopically and ubiquitously. Most unfortunately for cell culture work, cell culture media and incubation conditions provide ideal growth environments for these potential microbial contaminants.

Every item that comes into contact with a culture must be sterile. This includes direct contact (e.g., a pipette used to transfer cells) as well as indirect contact (e.g., flasks or containers used to temporarily hold a sterile reagent prior to aliquoting the solution into sterile media). Single-use, sterile disposable plastic items such as test tubes, culture flasks, filters, and pipettes are widely available and reliable alternatives to the laborious cleaning and sterilization methods needed for recycling equivalent glass items. However, make certain that sterility of plastic items distributed in multiunit packages is not compromised by inadequate storage conditions once the package has been opened.

Flame sterilization is used as a direct, localized means of decontamination in aseptic work at the open bench. It is most often used (1) to eliminate potential contaminants from the exposed openings of media bottles, culture flasks, or test tubes during transfers, (2) to sterilize small instruments such as forceps, or (3) to sterilize wire inoculating loops and needles before and after transfers. Where possible, flame sterilization should be minimized in laminar-flow environments as the turbulence generated by the flame can significantly disturb the sterile air stream.

Materials

- Antibacterial soap
 - 70% ethanol or other appropriate disinfectant
 - 95% ethanol
 - Clean, cuffed laboratory coats or gowns
 - Latex surgical gloves
 - Clean, quiet work area
 - Shallow discard pans containing disinfectant
 - Bunsen burner or pilot-activated burner
1. Frequently disinfect gloved hands with 70% ethanol while doing aseptic work.
 2. Dispose of gloves by autoclaving after use. Do not reuse. Bag and autoclave single-use laboratory coats after use. Bag, autoclave (if necessary), and wash other laboratory coats within the laboratory facility or send out for cleaning at a laundry certified for handling biologically contaminated linens.

Never take laboratory clothing home for washing.

Cell Culture Hood

The cell culture hood provides an aseptic work area while allowing the containment of infectious splashes or aerosols generated by many microbiological procedures. Three kinds of cell culture hoods, designated as Class I, II and III, have been developed to meet varying research and clinical needs. 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.

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.

Class II

Biosafety cabinet should be used for handling potentially hazardous materials (e.g., primate-derived cultures, virally infected cultures, radioisotopes, carcinogenic or toxic reagents).

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.

Use of the horizontal laminar-flow clean bench

Laminar-flow cabinets (hoods) are physical containment devices that act as primary barriers either to protect the material being manipulated within the hood from worker generated or environmental sources of contamination, or to protect the laboratory worker and laboratory environment from exposure to infectious or other hazardous materials that are present within the hood. Cell culture applications utilize two types of laminar flow hoods: (a) the horizontal-flow clean bench (described here) and (b) the biological safety cabinet (see Alternate Protocol). Both types of hoods use a high-efficiency particulate air (HEPA) filter and blowers that generate a no mixing stream of air.

The horizontal laminar-flow clean bench is used to provide a near-sterile environment for the clean (i.e., no contaminating) handling of nonhazardous material such as sterile media or equipment. Because the air stream pattern directs the flow of air within the hood directly back to the hood operator and the room (Fig. 1.3.1), horizontal flow hoods are never to be used with infectious agents or toxic chemicals.

Materials

- 70% ethanol or other disinfectant
- Horizontal laminar-flow hood, certified for use
- Swabs (e.g., cheesecloth, paper towels)

- Pilot light-activated Bunsen burner

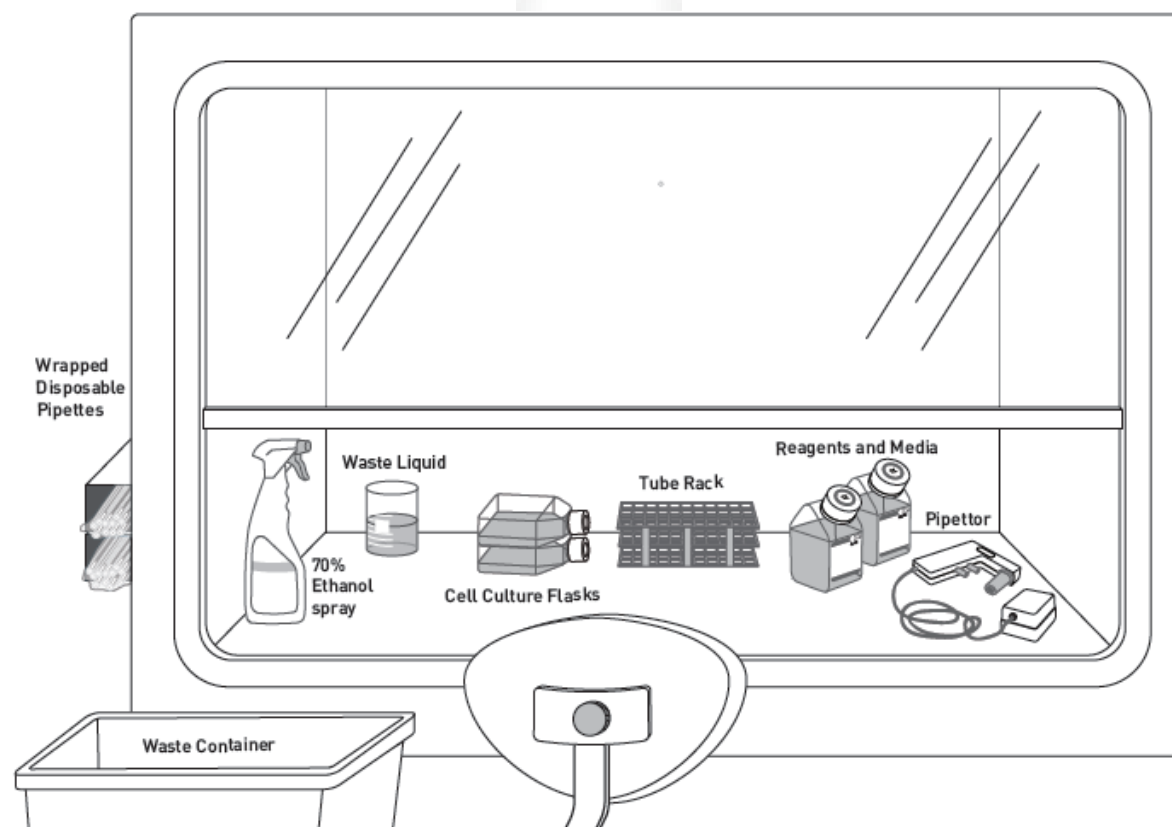


Fig3. The basic layout of a cell culture hood for right-handed workers. Left-handed workers may switch the positions of the items laid out on the work surface.(Cell culture basicshandbook, Invitrogen)

General principles of handling cell cultures: (Freshney; 2006)

- First and foremost, all supplies and reagents that come into contact with the cultures must be sterile (Phelan, 2007).
- Wash hands before and after handling any cell culture. Hand washing stations should be readily accessible within the laboratory.
- Handle only one cell line at a time. There are intrinsic risks of misidentification or cross contamination between cell cultures when more than one cell line is in use within the laboratory (Freshney, 2006).
- Handle continuous cell lines after the handling of short-term, finite cell cultures.
- Quarantine and handle under strict precautions all incoming cell lines until testing concludes the absence of mycoplasma. Alternatively, purchase cell lines from repositories which certify

that materials are mycoplasma-free prior to distribution.

- Avoid continuous long-term use of antibiotics within cell cultures. The overuse of antibiotics as prophylaxis may lead to cytotoxicity and pose an increased risk of covert mycoplasma contamination within the cell lines.
- Cultures should be inspected daily for signs of contamination. In addition, testing at regular intervals for mycoplasma should be conducted to ensure the purity and integrity of the culture.
- Promptly discard any contaminated cultures. Retention of these cultures poses a serious threat of cross contamination to other cultures in the laboratory. If clean-up of the contaminated culture is attempted, then any work with this culture should be reserved to the very end of the day to minimize transfer of the contamination.

Sterile Liquid Transfers

- Divide sterile solutions into small aliquots whenever possible. For instance, trypsin should be dispensed in single use quantities of 5 to 10 mL/tube. Ideally, reagents should be prepared in sufficient amounts to *only* meet the requirements for the number of samples that are being processed. Solutions which are left over should be discarded rather than reused. This reduces the chances for contamination and minimizes the consequences, if it does occur.
- **Always** use separate media bottles for every cell line. This important step reduces both the possibility of cross contamination with another cell line and limits the spread of contamination if the bottle of medium becomes contaminated.
- **Avoid** sharing bottles of media or other solutions with coworkers. Cross contamination and lack of accountability arise from sharing with others.
- Do not use the same pipette with different cell lines (Freshney, 2006). Never insert a pipette back into a bottle of medium after it has been used to feed a culture. This "double pipetting" saves on pipettes but can easily lead to widespread contamination by other cell lines or mycoplasma.
- Do not insert the non-sterile portion of adjustable pipettors into vessels containing cells or sterile solutions; it is not worth the risk of contamination.
- Use filtered pipette tips for aseptic transfers. Using unfiltered pipette tips to transfer cells or medium can result in contamination of the pipetting device and subsequent pipette tips.
- **Never** mouth pipette, even when nonhazardous substances are being transferred. Using a pipetting aid not only protects personnel but also reduces the risk of mycoplasma contamination of cultures with *M. orale* and *M. salivarium*.

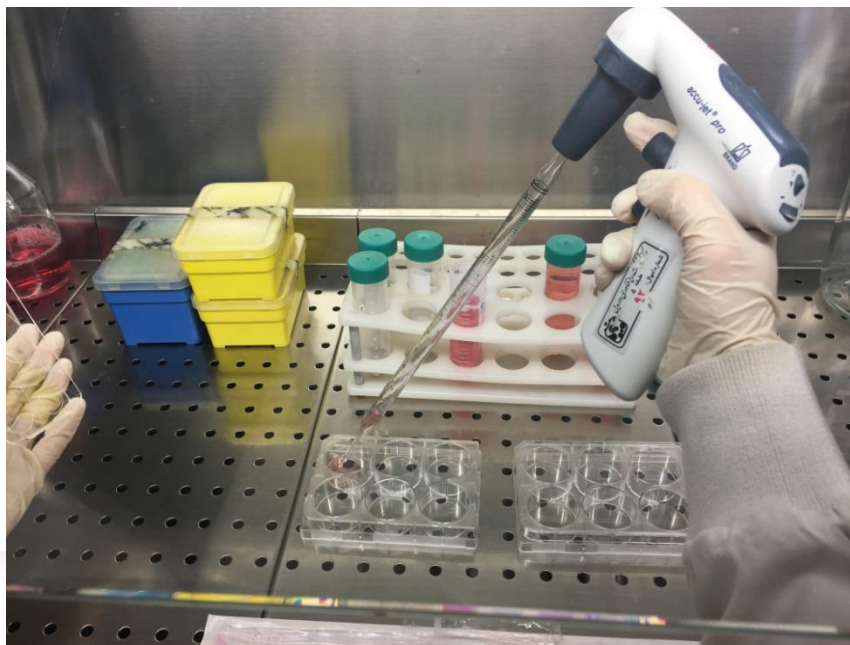


Fig4. Pouring is a risky means of transferring fluids. Try to find safer, more aseptic alternatives such as pumping.

- **Avoid** pouring sterile liquids from one vessel into another (Figure 1). The drop of liquid that usually remains on the lip of the vessel can easily form a liquid bridge between the non sterile outside and sterile inside of the vessel. This allows microorganisms from the outside to enter and contaminate the vessel and its contents, especially when pouring a second time. Pouring also increases the possibility of aerosol formation (Caputo, 1988). If pouring must be done, remove any liquid from the threads with a sterile alcohol wipe or gauze pads.
- **Avoid** spills or liquid bridges on the lips of dishes, bottles and flasks. They provide an easy access point for microorganisms into the vessel. Replace the caps of flasks that have wet threads or wipe dry with sterile alcohol wipes.
- **Clean up** any spills immediately and swab area with suitable disinfectants.

Working in hoods (Coecke et al., 2005)

Biological safety cabinets and laminar flow hoods provide containment and protection for the personnel, environment and cell cultures or products from biohazards and cross contamination during routine procedures. Many different types and classification of safety cabinets and hoods exist to meet the specific needs of any cell culture laboratory. Product selection will depend on the nature of the cell culture work and the biosafety level of the materials being used and processed. Horizontal laminar flow hoods should not be used for cell culture procedures. These biosafety hoods are designed to protect the work area only; air flow, and any contaminants it contains, is directed towards the operator (Coecke; 2005).

- Always have the biosafety cabinets certified at the time of installment and recertify if moved or repaired. It is also recommended to routinely test the quality of the airflow and filter integrity every 6 to 12 months.
- Biosafety cabinets may be equipped with germicidal UV lights for decontaminating work surfaces. However, the efficacy of UV lamps has been challenged. The UV light rays must directly strike a microorganism in order to destroy it. Over time, the UV output and germicidal capacity from the tube diminishes. Finally, there are safety concerns related to the exposure to UV light (Phelan, 2007). UV exposure is damaging to the eyes and skin, therefore, the UV light should *never* be on while the cabinet is in use.
- Biosafety cabinets and hoods should be turned on 15 minutes prior to use each day. Alternatively, keep hoods running 24 hours a day during the work week. Work surfaces should be wiped down with 70% ethanol, or other suitable disinfectant, before and after each use and between cell lines. Never use a flammable disinfectant, such as alcohol, if an open flame is in use nearby.
- Wipe down bottles and flasks with 70% ethanol or other suitable disinfectant before being placed in the cabinet.
- Wear a clean lab coat when working in a hood. This coat should be for hood use only and not be worn anywhere else in the laboratory.
- Limit people access to area around the hood while working. This reduces levels of airborne contaminants, unnecessary distractions and talking.
- Avoid unnecessary talking while working in the hood. Talking generates microbial laden aerosols that can then enter into the hood. Consider wearing a mask if talking is necessary or if you have a cold.
- Avoid moving materials in or out of the hood while work is in progress.
- Keep the hood work area clean and uncluttered. Do not use hoods as storage cabinets. Clutter makes it very difficult to clean the work surface properly and can disrupt the laminar flow around the work area.
- Do not use open flames, especially Bunsen burners, in laminar flow hoods. The heat plume from the flame will disrupt the moving curtain of filtered air provided by the hood and increase the risk of contamination. It is also a major safety issue. Serious hood explosions, fires and injuries have resulted from gas leaking from Bunsen burners or an open flame igniting alcohol used as a disinfectant.

- Doors in the culture area should be kept closed while hood is in use. Opening a door can create a back draft and disrupts laminar flow in hoods. Consider replacing traditional doors with sliding doors to eliminate this problem, especially in heavy traffic areas.

Preparation of cell growth medium

Before starting work check the information given with the cell line to identify what media type, additives and recommendations should be used.

Most cell lines can be grown using DMEM culture media or RPMI culture media with 10% Fetal Bovine Serum (FBS), 2 mM glutamine and antibiotics can be added if required (see table below).

Table2. General example using DMEM media

DMEM - Remove 50 ml from 500 ml bottle then add the other constituents.	450 ml
10% FBS	50 ml
2 mM glutamine	5 ml
100 U penicillin / 0.1 mg/ml streptomycin	5 ml

Check which culture media and culture supplements the cell line you are using requires before starting cultures. Culture media and supplements should always be sterile. Purchase sterile reagents when possible, only use under aseptic conditions in a culture hood to ensure they remain sterile.

Examples of the different media and their uses are given in Table 3 (see next page).

Table3. Different types of culture medium and their uses

Media Type	Examples	Uses
Balanced salt solutions	PBS, Hanks' BSS, Earle's salts DPBS HBSS EBSS	Form the basis of many complex media
Basal media	MEM	Primary and diploid culture
	DMEM	Modification of MEM containing increased level of amino acids and vitamins. Supports a wide range of cell types including hybridomas
	GMEM	Glasgow modified MEM was defined for BHK-21 cells
Complex media	RPMI 1640	Originally derived for human leukemic cells. It supports a wide range of mammalian cells including hybridomas
	Iscoves DMEM	Further enriched modification of DMEM which supports high density growth
	Leibovitz L-15	Designed for CO ₂ free environments
	TC 100 Graces insect medium Schneider's Insect medium	Designed for culturing insect cells
	CHO HEK293	For use in serum free applications

Serum free media	Ham F10 and derivatives Ham F12 DMEM/F12	Note: these media must be supplemented with other factors such as insulin, transferrin and epidermal growth factor. These media are usually HEPES buffered
Insect cells	Serum-Free Insect Medium 1	Specifically designed for use with Sf9 insect cells

Basic Constituents of Media

- Inorganic salts
- Carbohydrates
- Amino Acids
- Vitamins
- Fatty acids and lipids
- Proteins and peptides
- Serum
- Trace Elements

Each type of constituent performs a specific function as outlined below:

Inorganic Salts

The inclusion of inorganic salts in media performs several functions. Primarily they help to retain the osmotic balance of the cells and help regulate membrane potential by provision of sodium, potassium and calcium ions. All of these are required in the cell matrix for cell attachment and as enzyme cofactors.

Buffering Systems

Most cells require pH conditions in the range 7.2-7.4 and close control of pH is essential for optimum culture conditions. There are major variations to this optimum. Fibroblasts prefer a higher pH (7.4-7.7) whereas, continuous transformed cell lines require more acid conditions pH (7.0-7.4).

Regulation of pH is particularly important immediately following cell seeding when a new culture is establishing and is usually achieved by one of two buffering systems; (i) a “natural” buffering system where gaseous CO₂ balances with the CO₃/HCO₃ content of the culture medium and (ii) chemical buffering using a zwitterions called HEPES. Cultures using natural bicarbonate /CO₂ buffering systems need to be maintained in an atmosphere of 5-10% CO₂ in air usually supplied in a CO₂ incubator. Bicarbonate/CO₂ is low cost, non-toxic and also provides other chemical benefits to the cells.

HEPES has superior buffering capacity in the pH range 7.2-7.4 but is relatively expensive and can be toxic to some cell types at higher concentrations. HEPES buffered cultures do not require a controlled gaseous atmosphere.

Most commercial culture media include phenol red as a pH indicator so that the pH status of the medium is constantly indicated by the color. Usually the culture medium should be changed/replenished if the color turns yellow (acid) or purple (alkali).

Carbohydrates

The main source of energy is derived from carbohydrates generally in the form of sugars. The major sugars used are glucose and galactose, however, some media contain maltose or fructose. The concentration of sugar varies from basal media containing 1g/L to 4.5g/L in some more complex media. Media containing the higher concentration of sugars are able to support the growth of a wider range of cell types.

Amino Acids

Amino acids are the building blocks of proteins. 'Essential' amino acids must be added to culture media as cells are not able to synthesize these themselves. The concentration of amino acids in the culture medium will determine the maximum cell density that can be achieved – once depleted the cells will no longer be able to proliferate.

In relation to cell culture, glutamine, an essential amino acid, is particularly significant. In liquid media or stock solutions glutamine degrades relatively rapidly. Optimal cell performance usually requires supplementation of the media with glutamine prior to use.

Adding supplements of non-essential amino acids to media both stimulates growth and prolongs the viability of the cells in culture.

Vitamins

Serum is an important source of vitamins in cell culture. However, many media are also enriched with vitamins making them consistently more suitable for a wider range of cell lines. Vitamins are precursors for numerous co-factors. Many vitamins, especially B group vitamins, are necessary for cell growth and proliferation and for some lines the presence of B12 is essential. Some media also have increased levels of vitamins A and E. The vitamins commonly used in media include riboflavin, thiamine and biotin.

Proteins and Peptides

These are particularly important in serum free media. The most common proteins and peptides include albumin, transferrin, fibronectin and fetuin and are used to replace those normally present through the addition of serum to the medium.

Fatty Acids and Lipids

Like proteins and peptides these are important in serum free media since they are normally present in serum e.g. cholesterol and steroids essential for specialized cells.

Trace Elements

These include trace elements such as zinc, copper, selenium and tricarboxylic acid intermediates. Selenium is a detoxifier and helps remove oxygen free radicals.

Freezing and Thawing Cells

Comments

- Cells should be free of contamination in the form of bacteria, yeast, or fungi.
- Mycoplasma testing should be performed prior to freezing.
- Freezing media depends on the cell line.

- One vial must be saved for testing the success of the freeze.

Adherent Cells

Materials

- Phosphate Buffered Saline (PBS)
- Trypsin/EDTA solution
- Tissue Culture Media
- Cold Freezing Media (usually 10% dimethylsulfoxide, DMSO)
- Labeled Cryovials (~3 per 100-mm plate for)
- 100-mm plate of confluent cells

Freeze Procedure

Pre-freeze

1. Check for bacterial, yeast, or fungal contamination under a microscope.
2. Test a sample for mycoplasma using specific kits.

Freeze

3. Trypsinize cells (standard protocol).
4. Re-suspend cells in media, transfer to a sterile centrifuge tube, centrifuge at 1000 RPM and 4°C for 3-5 min.
5. Remove supernatant with sterile Pasteur pipette.
6. Quickly re-suspend pellet by adding 1 ml freezing media per vial to be frozen.
7. Aliquot 1 ml freezing media plus cells per vial, and place on ice.
8. Freeze overnight at -80°C.
9. Transfer vials to liquid N₂ tank for indefinite storage.

Post-freeze

10. Remove a vial from liquid N₂ tank and follow the thaw procedure below to test the success of the freeze.

Thaw Procedure

1. Warm tissue culture media without selection antibiotics to 37°C, and label 100-mm tissue culture plate.
2. Remove vial from liquid N₂ tank and hold in 37°C water bath until sides are thawed but center remains frozen.
3. Gently pour cells into the plate. Do not shake the vial.
4. Add 9 ml warm media dropwise to the partially frozen cells.
5. Place plate in incubator.
6. Change media as soon as cells are attached (remove DMSO a.s.a.p.).

Suspension Cells

Materials

For Freeze

- 75 cm² T-flasks of cells in late log phase (~ 40 mL/T-flask)
- Cold freezing medium (usually contains 10% dimethylsulfoxide, DMSO)
- Labeled cryogenic vials (~5 per 40 mL volume of cells in late log phase)

For Thaw

- Cold tissue culture medium
- 25 cm² T-flask

Freeze Procedure

Pre-freeze

1. Check for bacterial, yeast, or fungal contamination under a microscope.
2. Test a sample for mycoplasma using specific kits.
3. When cells have reached late log phase, determine cell density using Coulter counter. Calculate total number of cells in flask, and determine amount of freeze medium needed. (Cells should be resuspended in freeze medium at 5,000,000 to 20,000,000 cells/mL.)

Freeze

4. Centrifuge cells in 50 mL Falcon tube at 1000g for 15 minutes.
5. While cells are spinning, make freeze medium (*e.g.*, 90% FBS, 10% DMSO). Label cryogenic vials with date, cell type, and user's initials.
6. Suction away supernatant from centrifuged cells and add freeze medium. Triturate cells until homogeneous.
7. Quickly aliquot 1 mL of freeze stock per cryogenic vial. Screw each vial closed.
8. Put vials into storage box and place box, insulated with paper towels, into Tupperware[®] container. Put entire container into -20°C freezer.
9. After 3 hours, transfer container to -80°C freezer and store overnight.
10. Next day, put cells into appropriate rack in liquid N₂ tank.

Post-freeze

11. Remove a vial from liquid N₂ tank and follow the thaw procedure below to test the success of the freeze.

Thaw Procedure

1. Slowly remove appropriate tray rack from liquid N₂ tank. Remove long safety pin and take out one vial from appropriate tray.
2. Put tray back in slot and put safety pin back in place. Return tray rack to liquid N₂ tank and cap tank again.
3. Rapidly thaw vial in 37°C water bath until only a small ice pellet remains. Spray down vial with ethanol, wipe, and place into hood.
4. Pipette contents of vial (~ 1 mL) into T25 flask.
5. Slowly add 4 mL of cold culture medium, at a rate of about 1 drop every 10 seconds, swirling occasionally. Add another 5 mL of culture medium.
6. Place flask in appropriate incubator.
7. Since freeze medium contains dimethylsulfoxide (DMSO), spin down cells after 6-12 hours and resuspend in fresh, prewarmed medium in new T25 flask.

Key Points

1. Most text books recommend washing the thawed cells in media to remove the cryoprotectant. This is only necessary if the cryoprotectant is known to have an adverse effect on the particular cell type. For example, some cell types are known to differentiate in the presence

of DMSO. In such cases the cells should be washed in media before being added to their final culture flasks.

2. The addition of the thawed cell suspension to culture medium effectively dilutes the cryoprotectant (e.g. DMSO) reducing the toxicity of the cryoprotectant. That is why it is important to add the thawed cell suspension to a larger volume of culture medium immediately after the ampoule has thawed; do not allow thawed ampoules to sit at room temperature for long periods.
3. Do not use an incubator or the palm of your hand to thaw cell cultures since the rate of thawing achieved is too slow resulting in a loss of viability. Use a water bath as described in the protocol above.
4. If a CO₂ incubator is not available gas the flasks for 1-2 minutes with 5% CO₂ in 95% air filtered through a 0.2µm filter.
5. For most cultures it is best practice to subculture before confluence is reached so that the cells are harvested during their log phase of growth and are at optimum viability ready for seeding into new flasks. Furthermore there are some specific cell types that must be sub cultured before confluence is reached in order to maintain their characteristics e.g. the contact inhibition of NIH 3T3 cells is lost if they are allowed to reach confluence repeatedly.
6. Some hybridomas may be slow to recover post resuscitation therefore start in 20% (v/v) FBS and 10% (v/v) hybridoma enhancement supplement in the appropriate medium.

Water

The water used for making media and washing glassware is a frequent source of chemical contamination and requires special care to ensure its quality. Traditionally, double or triple glass distillation was considered to be the best source of high quality water for cell culture media and solutions. Newer purification systems combining reverse osmosis, ion exchange and ultra filtration are capable of removing trace metals, dissolved organic compounds and endotoxins and are increasingly popular. However, these systems must be properly maintained and serviced to ensure continued water quality. Because of its aggressive solvent characteristics, highly purified water can leach potentially toxic metal ions from glassware or metal pipes, and plasticizers from plastic storage vessels or tubing. These contaminants can then end up in media or deposited on storage vessels and pipettes during washing and rinsing. Water used to generate steam in autoclaves may contain additives to reduce scale buildup in pipes; these potentially toxic additives can also end up on glassware.

Incubators

Cell cultures require a strictly controlled environment in which to grow.

Specialist incubators are used routinely to provide the correct growth conditions, such as temperature, degree of humidity and CO₂ levels in a controlled and stable manner. Generally, they can be set to run at temperatures in the range of 28°C (for insect cell lines) to 37°C (for mammalian cell lines) and set to provide CO₂ at the required level (e.g. 5-10%). Some incubators also have the facility to control the O₂ levels. Copper-coated incubators are also now available. These are reported to reduce the risk of microbial contamination within the incubator due to the microbial inhibitory activity of copper. The inclusion of a bactericidal agent in the incubator water trays will also reduce the risk of bacterial and fungal growth. However, there is no substitute for regular cleaning.

Centrifuges

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

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

Storage area

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.

Note: Do **not** store cells in -20°C or -80°C freezers, because their viability quickly decreases when they are stored at these temperatures.

Biological Contamination

Biological contaminants can be subdivided into two groups based on the difficulty of detecting them in cultures:

- Those that are usually easy to detect: Bacteria, molds and yeast;
- Those that are more difficult to detect, and as a result potentially more serious culture problems: Viruses, protozoa, insects, mycoplasmas and other cell lines.

Note: Ultimately, it is the length of time that a culture contaminant escapes detection that will determine the extent of damage it creates in a laboratory or research project.

Bacteria, Molds, and Yeasts

Bacteria, molds and yeasts are found virtually everywhere and are able to quickly colonize and flourish in the rich and relatively undefended environment provided by cell cultures.

Because of their size and fast growth rates, these microbes are the most commonly encountered cell culture contaminants. In the absence of antibiotics, microbes can usually be readily detected in a culture within a few days of becoming contaminated, either by direct microscopic observation. Or by the effects they have on the culture (pH shifts, turbidity, and cell destruction).

However, when antibiotics are routinely used in culture, resistant organisms may develop into slow growing, low level infections that are very difficult to detect by direct visual observation. Similar detection problems can occur with naturally slow growing organisms or very small or intracellular bacteria that are difficult to see during routine microscopic culture observation. These cryptic contaminants may persist indefinitely in cultures causing subtle but significant alterations in their behavior. By the time these cryptic contaminants are discovered, many experiments and cultures may have been compromised. Viruses

Due to their extremely small size, viruses are the most difficult cell culture contaminants to detect in culture, requiring methods that are impractical for most research laboratories.

Their small size also makes them very difficult to remove from media, sera, and other solutions of biological origin. However, most viruses have stringent requirements for their original host species' cellular machinery (may also be tissue specific) which greatly limits their ability to infect cell cultures from other species. Thus, although viruses may be more common in cell cultures than many researchers realize, they are usually not a serious problem unless they have cytopathic or other adverse effects on the cultures. Since cytopathic viruses usually destroy the cultures they infect, they tend to be self-limiting.

Thus, when cultures self-destruct for no apparent reason and no evidence of common biological contaminants can be found, cryptic viruses are often blamed. They are perfect culprits, unseen and undetectable; guilty without direct evidence. This is unfortunate, since the real cause of this culture destruction may be something else, possibly mycoplasma or a chemical contaminant, and as a result will go undetected to become a more serious problem.

Note: A major concern of using virally infected cell cultures is not their effects on the cultures but rather the potential health hazards they pose for laboratory personnel. Special safety precautions should always be used when working with tissues or cells from humans or other primates to avoid possible transmission of viral infection (HIV, hepatitis B, Epstein-Barr, simian herpes B virus, among others) from the cell cultures to laboratory personnel. Contact your safety office for additional assistance if in doubt as to appropriate procedures for working with potentially hazardous tissues, cultures or viruses.

Table 4: Common Problems Associated With Cell Culture

Problem	Possible cause	Potential solution
Cells difficult to remove from plastic	Enzyme solution too weak Inhibitor present in medium (for example, serum) Cells too confluent and enzyme cannot access cell-substrate interface	Higher concentration needed Cells require more careful washing Cells require trypsinisation at lower cell density
Cells not adhering readily to plastic	Cells too heavily treated with trypsin Insufficient serum or attachment factors Dissociating agent (for example, not inactivated fully) Mycoplasma contamination	Use less trypsin or treat for less time Add more Add serum or specific inhibitors Discard if infected
Suspension cells clumping together	Mycoplasma contamination DNA from lysed cells sticking cells together	Discard if infected Add DNase
Poor growth in culture	Absence or lower than normal levels of certain additives Contamination by bacteria, mycoplasma or fungi Discard if infected Cell density too low	Add missing components Discard if infected Increase density
Cell death/low viability	Incorrect pH Faulty media	Correct pH Correct preparation
Too acidic pH	CO ₂ content too high Contamination	Modify If infected, discard
Too basic pH	Insufficient CO ₂ Too few cells	Caps too tight Increase cell density

Trypsinising/passaging adherent cell lines

Materials required

- Trypsin/EDTA
- Sterile PBS
- Cell culture flasks
- 10 and 25ml pipettes
- 5% Virkon
- Trypan Blue/counting chamber (optional)
- Growth medium

Method

1. Put trypsin, growth medium and PBS into 37C incubator for 45-60 minutes to warm before starting.
2. Swab cabinet with 5% Virkon and 70% ethanol.
3. Decant off spent media from each flask to be split.
4. Add 25ml of warm PBS to each flask and wash monolayer gently. Decant off PBS into 5% Virkon. Add 5-10ml of trypsin to each flask. Lay the flask flat and wait for 1-2 minutes, or until most of the cells have rounded up (observe them under a microscope). Decant off excess trypsin into 5% Virkon, leaving about 1mL in the flask.
5. Incubate flask at 37C for 1-2 minutes.
6. Retrieve flask from incubator and knock the side of the flask against the palm of your hand a few times to dislodge the cells. The cells should come off easily - if not, reincubate the flask for a further 1-2 minutes.
7. Resuspend the cells from each flask in 5-10ml of warm growth medium, and divide into labeled flasks. [If a cell count is to be performed, add 10m l of cell suspension to 90m l of Trypan Blue and mix well. Add 10m l to a haemocytometer and do cell count. See below]
8. Add 25-50mL of warm growth medium to the cell suspension in each flask, gently mix, and put flasks in 37C incubator with 5% CO₂.

Determining cell number and viability with a Hemacytometer and trypan blue staining

Determining the number of cells in culture is important in standardization of culture conditions and in performing accurate quantization experiments. A hemacytometer is a thick glass slide with a central area designed as a counting chamber.

The exact design of the hemacytometer may vary; the one described here is the Improved Neubauer from Baxter Scientific. The central portion of the slide is the counting platform which is bordered by a 1-mm groove. The central platform is divided into two counting chambers by a transverse groove. Each counting chamber consists of a silver footplate on which is etched a 3 × 3-mm grid. This grid is divided into nine secondary squares, each 1 × 1 mm. The four corner squares and the central square are used for determining the cell count. The corner squares are further divided into 16 tertiary squares and the central square into 25 tertiary squares to aid in cell counting.

Accompanying the hemacytometer slide is a thick, even-surfaced cover slip. Ordinary cover slips may have uneven surfaces that can introduce errors in cell counting; therefore, it is imperative that the cover slip provided with the hemacytometer is used in determining cell number.

Cell suspension is applied to a defined area and counted so cell density can be calculated.

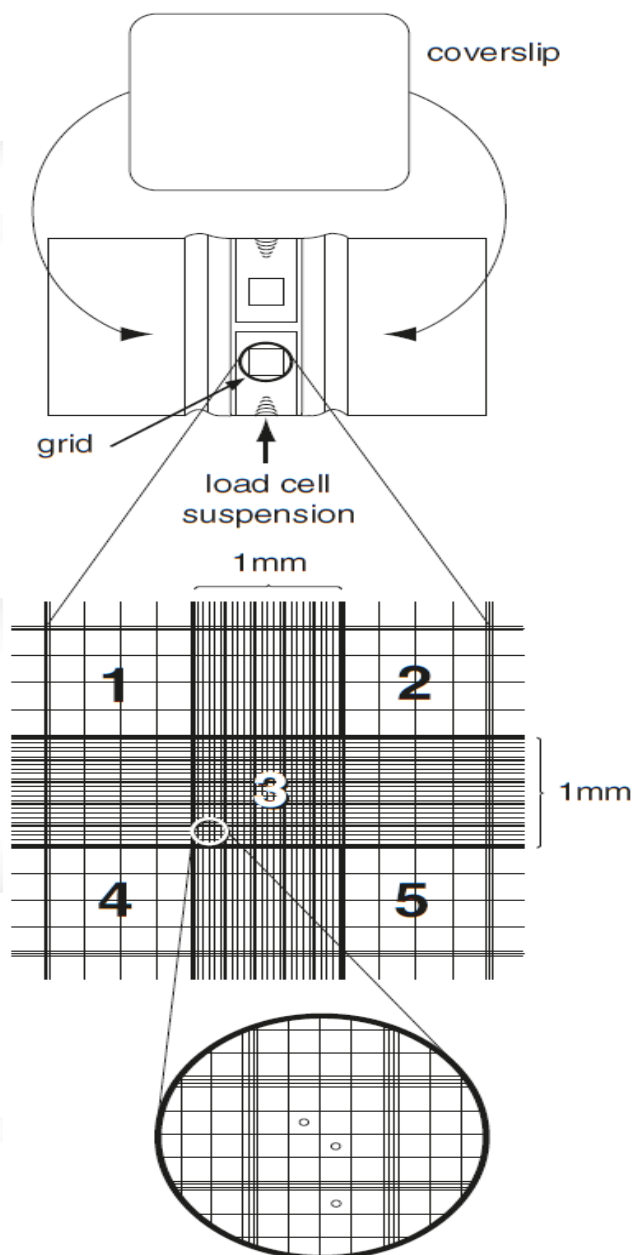


Fig5. Hemacytometers slide (Improved Neubauer) and cover slip. Cover slip is applied to slide and cell suspension is added to counting chamber using a Pasteur pipette. Each counting chamber has a 3×3 -mm grid (enlarged). The four corner squares (1, 2, 4, and 5) and the central square (3) are counted on each side of the hemacytometer (numbers added). (Cell culture basics, Invitrogen)

Materials

- 70% (v/v) ethanol
- Cell suspension
- 0.4% (w/v) trypan blue or 0.4% (w/v) nigrosin prepared in HBSS

- Hemacytometer with cover slip (Improved Neubauer, Baxter Scientific) Hand-held counter

Prepare hemacytometer

1. Clean surface of hemacytometer slide and cover slip with 70% alcohol. Cover slip and slide should be clean, dry, and free from lint, fingerprints, and watermarks.
2. Wet edge of cover slip slightly with tap water and press over grooves on hemacytometer. The cover slip should rest evenly over the silver counting area.

Prepare cell suspension

3. For cells grown in monolayer cultures, detach cells from surface of dish using trypsin (see Basic Protocol, steps 1 to 4).
4. Dilute cells as needed to obtain a uniform suspension. Disperse any clumps.

When using the hemacytometer, a maximum cell count of 20 to 50 cells per 1×1 mm square is recommended.

Load hemacytometer

5. Use a sterile Pasteur pipette to transfer cell suspension to edge of hemacytometer counting chamber. Hold tip of pipette under the cover slip and dispense one drop of suspension.

Suspension will be drawn under the cover slip by capillary action.

The hemacytometer should be considered no sterile. If cell suspension is to be used for cultures, do not reuse the pipette and do not return any excess cell suspension in the pipette to the original suspension.

6. Fill second counting chamber. Count cells
7. Allow cells to settle for a few minutes before beginning to count. Blot off excess liquid.
8. View slide on microscope with 100 \times magnification.

Position slide to view the large central area of the grid; this area is bordered by a set of three parallel lines. The central area of the grid should almost fill the microscope field. Subdivisions within the large central area are also bordered by three parallel lines and each subdivision is divided into sixteen smaller squares by single lines. Cells within this area should be evenly distributed without clumping. If cells are not evenly distributed, wash and reload hemacytometer.

9. Use a hand-held counter to count cells in each of the four corners and central squares. Repeat counts for other counting chamber.

Five squares (four corners and one center) are counted from each of the two counting chambers for a total of ten squares counted.

Count cells touching the 3 middle line of the triple line on the top and left of the squares. Do not count cells touching the middle line of the triple lines on the bottom or right side of the square.

Calculate cell number

10. Determine cells per milliliter by the following calculations:

Cells/ml = average count per square \times dilution factor $\times 10^4$

Total cells = cells/ml \times total original volume of cell suspension from which sample was taken.

The number 104 is the volume correction factor for the hemacytometer: each square is 1×1 mm and the depth is 0.1 mm.

Stain cells with trypan blue to determine cell viability

11. Determine number of viable cells by adding 0.5 ml of 0.4% trypan blue, 0.3 ml HBSS, and 0.1 ml cell suspension to a small tube. Mix thoroughly and let stand 5 min before loading hemacytometer.

Either 0.4% trypan blue or 0.4% nigrosin can be used to determine the viable cell number. Nonviable cells will take up the dye, while live cells will be impermeable to dye.

12. Count total number of cells and total number of viable (unstained) cells. Calculate Percent viable cells as follows:

$$\% \text{ viable cells} = \frac{\text{number of unstained cells}}{\text{total number of cells}} \times 100$$

13. Decontaminate cover slip and hemacytometer by rinsing with 70% ethanol and then deionized water. Air dry and store for future use.

Checking cells

1. Cells should be checked microscopically daily to ensure they are healthy and growing as expected. Attached cells should be mainly attached to the bottom of the flask, round and plump or elongated in shape and refracting light around their membrane. Suspension cells should look round and plump and refracting light around their membrane. Some suspension cells may clump. Media should be pinky orange in color.

2. Discard cells if:

- They are detaching in large numbers (attached lines) and/or look shriveled and grainy/dark in color.
- They are in quiescence (do not appear to be growing at all).

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Good luck!



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