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METHODOLOGICAL RECOMMENDATIONS FOR THE SPECIAL WORKSHOP "CELL CULTURE AND HISTOGENESIS"

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For students of medicine, laboratory diagnostic, biochemistry, scientists who want to acquire practical and theoretical skills in culturing cells of higher eukaryotes.

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The main purpose of the methodological recommendations is to provide students with the opportunity to get acquainted with the methods of cell culture of higher eukaryotes, which are used at the present stage to acquire practical skills and gain new knowledge in conducting research in the field of Laboratory diagnostic, biochemistry, molecular and cell biology.

INTRODUCTION

The method of cell culture (method of "explantation" of cells) is one of the key methods of cell biology, which studies the features of proliferation, differentiation, migration, metabolic activity, and biochemical and molecular biological mechanisms of aging and death of a single cell and a population of genetically identical cells, for which all their properties are maintained in artificially created conditions in compliance with all the necessary rules of asepsis.

The technique of tissue culture was first proposed at the beginning of the last century [Harrison, 1907; Carel, 1912] to study the properties of animal cells free from the influence of the systemic microenvironment, characteristic of multicellular organisms both in normal and pathological conditions. In the initial stages, this technology was based on working with undispersed tissues. The first cloned strain was L929, which was obtained from mouse L-cells by capillary method [Sanford et al., 1948]. In the 1950s, for the analysis of viral plaques, Dulbecco [Dulbecco, 1952] described a method of obtaining monolayer cultures using trypsin, which is now widely used in the practice of cell cultivation. The first permanent human cell line HeLa was obtained and cloned by a group of authors [Gey et al., 1952, Puck and Marcus, 1955] using an irradiated cell feeder layer. In the 50s of the last century, there was a boom in the development of cell technologies: the development of rules for working with cell cultures, the establishment of technologies for the production of the necessary equipment (laminar boxes, carbon dioxide incubators, inverted microscopes, devices for cryopreservation of cells), as well as artificial media with growth-stimulating factors and sterile plastic dishes for cell incubation.

The most significant achievements obtained based on cultured cells are hybridoma technology - the production of monoclonal antibodies and their use for the diagnosis and treatment of the most common diseases of various etiologies, artificial insemination and reproductive medicine technologies, technologies related to the production and use of stem cells. Thus, cell cultures can be used in the following studies: a study of intracellular processes - DNA replication and transcription, protein synthesis and energy metabolism, drug metabolism; study of intracellular and membrane transport of substances; interaction with trophic substrates. Important aspects are the study of mechanisms of cell infection, cytotoxic effects, and ligand-receptor interaction. Also, using cultured cells, it is relevant to study intercellular interaction, morphogenesis, paracrine and autocrine effects, and kinetics of cell proliferation, for which both single-type cells and models of co-cultivation of different cell types are used. Organotypic models of cultured cells find their application in regenerative medicine and transplantation, including pathological conditions. Formation and secretion of cell products, creation of bioreactors and obtaining final products, production technologies, and obtaining targeted biologically activeproducts are integral parts of modern bioproduction.

The advantages of cell culture are the ability to accurately control the physicochemical environment (pH, temperature, osmotic and partial pressure, oxygen and carbon dioxide content), as well as biochemical and physiological conditions, which contributes to the repeatability and reproducibility of results, mechanization, and certification of research, saving reagents and time of certain works.

Cell culture very often replaces or complements existing models with

the use of animals. Often the use of cell culture is more convenient and cheaper compared to traditional experimental models using animals. Cell culture can represent with sufficient reliability the processes occurring in a certain type of cells in the body. In some cases, cell culture is indispensable, an example, in the case of the synthesis of chimeric proteins that require certain post-translational, such as glycosylation (R. Gouveia). The increasing use of recombinant therapeutic proteins in clinical practice, the transformation of antibodies into a powerful research tool, the gradual abandonment of animal models, and modern advances in stem cell research make cell culture a routine practice in many laboratories and encourages scientists to a more detailed understanding of the cellular processes that affect the cultivation of cells in artificial conditions. At the present stage of cell culture development, the question arises not only about the isolation and maintenance of certain cells, but also about the possibility of manipulating and modifying cells in culture for scientific or practical needs, such as the creation of recombinant or transgenic cell lines, modification of cells in the lines for artificial control of cell proliferation, increasing cell survival, reducing the associated metabolic products (lactate, ammonia, etc.), increasing productivity, regulation of post-translational modifications, etc. (M. Matascia, 2008,). Optimization of the cultivation process is due to several interrelated factors such as improvement of media and cultivation parameters (concentration of CO_2, glucose, growth factors, etc.) as well as modification of cells by inclusion or exclusion of certain genes. In addition, the study of morphological and kinetic markers of cell lines is necessary for the correct maintenance of culture, and timely response to any changes in the state of cell culture. It is known that under certain circumstances

cell culture can lose its inherent properties or die and, thus, lead to incorrect interpretation of the results of the experiment, and loss of valuable achievements and time.

Recently, very high hopes are placed on stem cells, which have a high potential for use in medical, genetic, applied, and fundamental research. The high proliferative and differentiation ability of stem cells makes them an extremely attractive object for development in the field of pharmacology, and personal and regenerative medicine (Stem cells and the future of regenerative medicine. National Academy Press Washington, D.C. 2003).

This manual contains guidelines for working with cell cultures of higher eukaryotes, including humans.

I. PREPARATION OF THE BOX ROOM, REAGENTS, AND DISHESFOR WORK WITH CELL CULTURES.

Lesson 1. Structure of the cell culture room, equipment, and safety.

The purpose of the lesson: familiarization with biosafety techniques and basic rules of work in the cell culture laboratory.

The basic rules of work in the laboratory for cell culture include:

1) safe handling of electrical, and gas equipment, as well as drainage;
2) all devices used for cell cultivation should be grounded, sockets and switches should be in a well-accessible and safe area;
3) the work of students and graduate students should take place only in the presence of responsible personnel;
4) the devices should be switched on and off only in agreement with the responsible personnel;
5) work in the culture laboratory should take place in the maximum permissible silence, without extraneous noise and distractions;
6) before starting to work under the laminar flow, the workplace should be equipped as conveniently as possible (chair height, access to utensils, automatic pipettes, gas or alcohol burner, etc;)
7) if questions arise during manipulations under the laminar flow, solve them only with the responsible personnel;

8) before starting work, you should find out what degree of biological safety is characteristic of a particular cell line or primary culture;
9) adhere to the rules and skills of sterile work as much as possible;
10) after finishing work, put the workplace in order and report on the work done.

When establishing a **laboratory for tissue culture, it is necessary to take into** account, first of all, the volume of work, the type of cultured cells or tissues, and the class of biological safety. In accordance with this, the laboratory is created.

Requirements for the premises of the cell culture laboratory:

1) sterile area, cleanliness, minimum movement of people, no through passage;
2) the location of the laboratory should be separate from the rooms where microbiological work and work with experimental animals are carried out;
3) the preparatory zone includes a sink, preferably divided into two blocks - for the primary processing of dishes, in which the cell cultivation was carried out, and a zone for successive washing (flowing, distilled, and deionized water) of dishes;
4) space for incubators;
5) refrigerators and freezers chambers for storage of reagents and cell culture media;
6) cabinets for storing sterile plastic and glassware;
7) freezers and equipment for cryopreservation and storage of biological material in liquid nitrogen.

Safety requirements for work in the cell culture laboratory

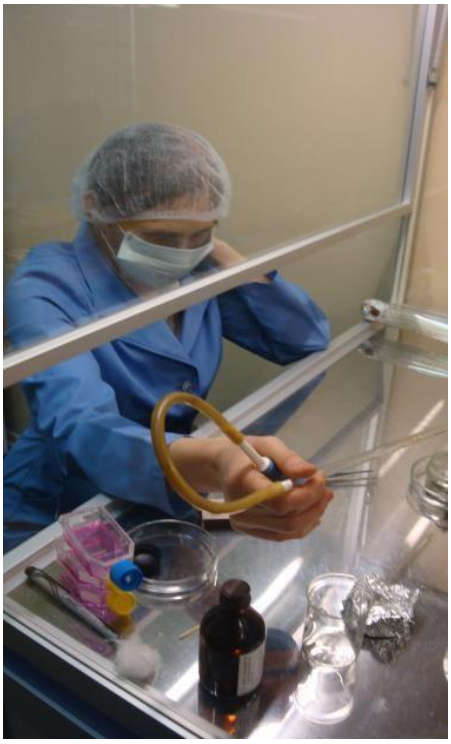
The cell culture laboratory is characterized by a number of additional features related to the specifics of cultivation. Permanent work is primarily associated with the biological hazards. As a rule, only well-trained personnel are allowed to work in the class of high-risk work (work with cell cultures contaminated with viral or bacterial infections, under the influence of high-risk substances - toxins, radioisotopes, etc. Therefore, an important step is the risk assessment before starting a particular study. The determination of the nature and extent of the potential hazard, how materials and equipment are used, and the degree and frequency of contact with the hazardous material must all be well justified and described. When working with cell cultures, there are risks associated with fire hazards (use of burners), and the threat of frostbite when working with liquid nitrogen. The main problem that comes to the fore in biomedical laboratories is the disproportion between excessive attention to the problems associated, for example, with genetic manipulation, and insufficient attention to the hazards of working with toxic substances, solvents, etc. It is important that biohazards in laboratory work are categorized [Relevant regulations of the Ministry of Health, Health and Biosafety Commission].

If a certain procedure to be carried out is associated with a risk for researchers, it is necessary to follow clear sequential manipulations based on standard operating procedures (SOPs), which are prepared before the start of work, taking into account all possible hazards.

The equipment used in the cell culture laboratory should have clear instructions for commissioning, preparatory phase, and shutdown after completion of work.

One of the important elements of creating a cell culture laboratory

is the availability of **basic equipment** (Fig.1):



A) - laminar flow cabinets equipped with horizontal and vertical laminar flow

B) Microscopes for in vivo observation of cells (light - Primo Star and inverted Axio Vision, Karl Ceiss)

Fig.1.Basic equipment of the cell culture room.

B) Carbon dioxide incubator for cell cultivation

D) Cryo equipment for storage cells in liquid nitrogen

Fig.1. (*Continued*)\ Basic equipment of the cell culture room.

The presented equipment can be divided into 4 groups

- A) instruments and equipment, that ensure work with cell cultures;
- B) devices for vital cell visualization (microscopes; video cameras);
- C) devices and equipment for cell cultivation;
- D) devices and equipment for cryopreservation and storage of cell cultures.

The sterility of work with cell cultures is achieved by conducting research in a box room. Creating the latter requires significant costs. An alternative**,** in this case, is the use of **laminar flow cabinets** or local sterile workstations. Technically, this

the problem is solved as follows - by blowing the workplace with a laminar flow of air, 1 dm^3 of which contains no more than 4 particles-pollutants with a size of 0.3-0.5 microns. Excessive air pressure in the working volume and special design measures exclude the possibility of air from the room to the location of the biological material. Laminar boxes are divided by the type of blowing into several groups: with horizontal flow of sterile air (type UO-BG - biological dedusting unit with horizontal flow). This type of laminar provides purification of the air supplied to the working area to the required level.

The main built-in components of the laminar flow cabinet are filters of fine and coarse air purification, such as ULPA, and HEPA (filtering capacity or sterility 99.999), with a pore diameter of exclusion up to 6 microns. The main purpose of the laminar box type BP-4-004 ("Box-R") is to work with potentially pathogenic cultures. It supplies sterile air, 1 dm^3 which contains no more than3 - 4 particles, the size of which is less than 0.3 microns. The device provides recirculation of the airflow, as a result of which the air from the working volume enters a special fine filter, from where part of it enters the surrounding space, and the rest passes through the fine filter again and enters the working volume again. Due to this design solution, the removal of material with pathogenic potential into the room is excluded.

A gas burner is supplied inside the laminar flow cabinet, which must have a well-regulated gas flow. Alcohol burners are also an alternative, which is used in most cases, as they are less accident-prone. In addition, laminar flow cabinets are equipped with ultraviolet lamps to sterilize the chamber before and after work. Periodic sterilization of the laminar flow box

is carried out with 70% alcohol solution, wiping all work surfaces, or in some cases, especially after contamination of cells by microorganisms, in formaldehyde vapor. This procedure is carried out after the end of the working day, preferably at the end of the working Sunday. A lid from the sterilizer is placed on the alcohol burner, and 5-10 ml of formaldehyde is poured into it, after which the burner is ignited and the laminar flow cabinet is tightly closed. After this procedure, the next day is well-ventilated. The location of the laminar flow cabinet should be carefully selected; usually, it is a corner of the room with good access to electricity, gas drainage, and drainage (in case of using automatic water suction liquids).

The second, no less important device is the **CO_2-incubator. The** main function of this device is to maintain the vital activity of cells outside the body**.** This is a thermostat with temperature control, with the maintenance of a humid atmosphere, and with the supply of carbon dioxide with a constant concentration to this chamber. For the cultivation of animal and human cells, the conditions to be observed in the CO_2 incubator are a temperature of 37^o C, 100% humidity in the chamber, and 5% CO_2. In this mode, cells, especially permanent cell lines can be cultured as long as you want. Periodically, the preventive shutdown of the CO_2-incubator is carried out, and the inner chamber is carefully washed. Laboratory thermostats for cell cultivation must meet certain requirements: to ensure high stability of the set temperature, to create a minimum temperature gradient across the useful volume, and to have a system of rapid temperature recovery after a short cooling. The inner surface of the thermostats should be made of biologically passive materials, that is, those that do not affect the viability of cells and are resistant to the components of nutrient media. Materials from which the internal and external parts of the thermostat and coatings were made

must be able to withstand decontamination with aqueous solutions of alcohol-rectification and sterilization by UV radiation. Modern models of these devices have a useful volume from 20 to 1400 liters. The internal volume is formed by polished plates of stainless steel or copper (inexpensive models). Usually, such thermostats have external and internal doors. The latter is made of transparent material, which allows you to observe the contents of the thermostat without disturbing the temperature regime. The temperature in the working chamber of the thermostats can be set in the range not exceeding the room temperature by 5-80^{o} C. Some models of thermostats have a built-in refrigerator, which allows you to set the temperature from -10^{o} C to 80^{o} C and maintain it in the required range when placing additional devices, such as rollers or shakers, in the thermostat. To maintain a constant pH of the culture medium and minimal evaporation during the incubation of cells, special devices were created - carbon dioxide incubators. By their design and main parameters they fully correspond to the above described thermostats. The main difference is the built-in system for the constant supply of a certain composition of the gas mixture in the useful volume and high relative humidity in it (Fig. 2). In the gas environment of the carbon dioxide incubator chamber, the content of oxygen and carbon dioxide is increased, which is regulated by a reducer connected to the cylinder (in particular, to carbon dioxide). As can be seen from Fig. 2, the reducer is equipped with two pressure gauges: the first one shows the pressure (in atmospheres) in the cylinder, the second one - at the outlet of the reducer, which provides a constant supply of the required percentage of carbon dioxide in the incubator chamber for cultured cells.

Fig.2.Reducer to carbon dioxide cylinder

It is also known that the cultivation of certain types of cells is more efficient with constant stirring. Structurally, these devices consist of a platform on which containers with cultivated cells are fixed and a drive mechanism that provides circular or reciprocating movement of the platform. When equipping CO_2 -incubators with such platforms, the possibility of changing gas flows in the chamber environment should be taken into account.

The next equally important device is **an inverted microscope (**Fig. 1, B), which is based on the principle of "invertedness", as a result of which cells attached to the bottom of a plate, vial, or Petri dish can be observed in the field of view. Using an inverted microscope, you can also observe cells growing in suspension. Most of the modern inverted microscopes are equipped with video cameras and digital cameras, which allow to carry out live imaging of cells at all stages of their cultivation, and microscopes have special software (e.g. AxioVision, Carl Zeis), which allow calculainge the area, staining intensity, size, and other parameters

of cultured cells, as well as constantly monitor the intensity of cell spreading on the substrate in a specific period of time.

The fourth group of basic equipment is devices for cryopreservation and storage of cells. These are freezers (kelvinators) with temperatures down to -70^{o} C, as well as Dewar tanks filled with liquid nitrogen. Usually, only personnel after special training is allowed to work with liquid nitrogen, taking into account all safety rules when handling reagents that can cause frostbite. To create cell banks equipped with cryo equipment, it is necessary to have a separate room where no other devices, especially heating devices, should be stored.

Among the auxiliary necessary equipment, first of all, there are autoclaves and dry heat ovens, bidistillers and deionizers for water purification, devices for ultrafiltration of media and buffers used for cell cultivation, tabletop centrifuges, multi-well spectrophotometers, automatic liquid aspirators, water baths, pH meters, laboratory magnetic stirrers, automatic varipipipettes for different volumes, hemocytometers, as well as a wide range of biochemical, molecular biological, and chromatographic instruments necessary for the equipment of the culture laboratory for a particular research area (Fig.3).

According to the rules of sterile work, the culture unit should be divided into several zones - a box in which there is a laminar flow cabinet, CO_2 - incubator, inverted microscope, as well as a refrigerator for storing the necessary media for cell cultivation and a pre-box room with additional equipment.

Dry oven and autoclave (20 l)

Semi-industrial autoclave (50 l)

device for preparation of deionized water

Tabletop centrifuge

Multi-well spectrophotometer

pH meter

Fig.3.Auxiliary equipment when working with cultured cells

Outside the box with laminar flow and CO_2-incubator in two separate rooms should be placed an autoclave and a dry heat cabinet for sterilization of solutions and dishes, as well as cryo equipment, which includes automated systems for cryopreservation of cells, as well as dewars for filling and storage of liquid nitrogen and dewars equipped with special numbered cassettes for long-term storage of cell lines, primary cultures, re-seeding strains, stem cells, clones, etc.

Rules of sterile work:

Remember: entrance to the cell culture room is restricted to specially trained personnel only. First of all, you need to have a change of protective clothing (gowns, caps, masks) and shoes (Fig.4). It is desirable to have overalls made of natural fabrics.

Fig.4. Training of personnel to work in sterile conditions

Before starting work, turn on the ultraviolet radiation in the cabinet-laminar flow (15-20 minutes), then the laminar air flow (REMEMBER - during direct work ultraviolet

is disconnected).

Before working in the laminar flow it is necessary to treat hands with cotton wool dipped in alcohol. After this procedure, you should not put your hands outside the laminar flow unit. If this happens, the procedure should be repeated. The use of sterile gloves is not always safe, as the use of ethanol and a burner in the laminar flow can lead to a fire.

The room in which sterile work with cells is carried out is not visited by strangers; it is not ventilated, but equipped with air conditioning. The walls and floors should be covered with a material that is well-washed with disinfectant solutions. It is desirable that the room is divided into two halves, the smaller one with the laminar, microscope, and CO_2 - incubator, and the larger part with other necessary equipment. The room with the laminar flow should be entered with a change of clothes and shoes. Since the work with cells has a rather high cost of basic andauxiliary means, to admit a specialist to work, it is necessary to instruct him/her, as well as to go through all the stages of preparatory work, and especially work with gas burners in the laminar flow cabinet.

STERILE WORK is not a set of methods, but rather a certain style of work. Under "style" in these circumstances it is understood that the quality of work, in this case, is determined not by "maximum", but by
"minimum achievements". It is not difficult to work in such a way that there is no pollution, the only problem is that you should always work this way (regardless of your mood and condition).

The rules of sterile work are almost identical for bacteria and eukaryotic cells. The only difference is that bacteria are usually more tolerant to their violation; purification of a valuable bacterial culture is a much simpler task than purification of a eukaryotic culture; the cost of a dead bacterial culture is much less than a lost eukaryotic culture.

The basic rule is to try to organize the work so that "clean" and "dirty" solutions and instruments are as far away from each other in time and space as possible. First, you should prepare sterile media, pour them into tubes and cups, close the stock solutions and take them out from under the laminar flow, and only then introduce the cell cultures under the laminar flow and manipulate them. It should be remembered that the main source of contamination during manipulations in the laminar flow cabinet is the hands of the experimenters, which are wiped with an alcohol solution before work.

A container with germination of cantankerous microorganisms is a gap in sterile work. It is extremely important in this case to localize the problem.

It is IMPOSSIBLE to work with bacteria, yeast, and cells of higher eukaryotes in one laminar flow tank. As a rule, separate rooms are used. If there is a need for one employee to work with these objects, the sequence of work is as follows - cells, bacteria, yeast.

Stationary in the laminar flow box can be, cases with sterile pipettes, boxes with autoclaved plastic tips, the necessary amount of plastic dishes, sterile water, saline, and phosphate-salt buffer. It is unacceptable that culture media, serum, and protein solutions are in the laminar flow box. A separate refrigerator must beallocated for these components. Before use, ready-made media are heated in a thermostat.

The sequence of work with cultured cells:

1. Preparation of media andreagents for cultivation.
2. Selection of the necessary amount of media required for a certain stage of work.
3. Cell transplantation and cryopreservation.

4. Seeding of cells in plates of different grades for short-term experiments.
5. Manipulation of cells in tablets.

If several specialists work in the box laminar, you should follow the work schedule. Each of the workers must leave a perfectly clean workplace.

Control questions:

Basic safety rules in the laboratory for cell culture.

Groups of basic equipment for working with cell cultures.

How is a local sterile space created?

Technical characteristics of laminar flow cabinets.

What is a CO_2 -incubator?

Structure, temperature, and gas conditions of the CO_2 -incubator.

Principle of construction of inverted microscope.

Auxiliary equipment for cultural boxing.

What devices ensure the maintenance and long-term storage of cultured cells?

What are the basic principles of sterile work?

*Class 2.*Preparation of solutions for cell culture.

Most cell culture media are supplied by companies specializing in the production of sterile media and growth-stimulating solutions. In early studies, natural media based on tissue extracts and natural body fluids such as chicken embryo extract, serum, lymph, etc. were used for cell cultivation. With the proliferation of cell lines, the need for a large number of media has led to the introduction of media with chemically precisely defined composition based on biochemical analysis of natural body fluids. Eagle's basic Eagle's medium [Eagle, 1955] and minimal Eagle's medium (MEM) [Eagle, 1959] were widely used with various additions of calf sera, human sera, protein hydrolysates, and embryonic extracts. By that time many permanent cell lines (L929, HeLa, and others) had already been obtained and it became clear that these media are suitable for most cell lines. Nowadays, a number of modified media have been developed (for example, RPMI 1640 medium was developed for lymphoblastic cell lines), or modification of media for specific cultivation conditions (Leibovitz's L15 medium was developed for cell cultivation in the absence of $C0_2$ and $NaHC0_3$, [Leibovitz, 1963]). In the late 40s and early 50s, most cells were grown in plasma or fibrinogen clots in the presence of tissue extracts. In his classic work, Eagle investigated the nutrient requirements of the obtained cell lines, as a result of which he was able to achieve cell multiplication in a medium of a certain composition containing a mixture of amino acids, vitamins, salts, and carbohydrates, as well as small amounts of bovine and human serum, with 27 environmental factors identified as essential for cell growth. They form the basis of the nutrient medium,

known as Igla basic medium. Of the 20 amino acids that were part of the medium, 13 were essential, and the rest could be synthesized from other carbon sources. Removal of any of the 7 vitamins led to the development of symptoms of vitamin deficiency. When cells were cultivated in BSI, the medium had to be frequently renewed, so it was replaced by Eagle's minimal medium (EMI), in which the concentration of nutrients was increased, which ensured continuous cell growth in culture without replacing the medium.

Today, the most popular media are Dulbecco's DMEM [Dulbecco & Freeman, 1959] or RPMI 1640 [Moore et al., 1967], in which serum is added. Serum-free media are also now used in industrial technology in order to simplify the process and reduce the risk of contamination of cell cultures by accidental infections. A popular compromise for many laboratories is a mixture of complex media such as Ham's F12 medium [Ham, 1965] with other media containing increased concentrations of amino acids and vitamins such as DMEM (Table 1).

In most cases, commercial liquid culture media are used for cell cultivation (Fig.5).

Fig.5 Liquid media for cell culture (Sigma, USA).

Table 1.

Content of components (mM) in culture media MEM, DMEM, RPMI-1640, F-12

Components (1)	**MEM**	**DMEM**	**RPMI-1640**	**F-12**
Amino acids				
L-arginine				$1,0 \times 10^{-4}$
L-asparagine	$6,0 \times 10^{-4}$	$4,0 \times 10^{-4}$	$1,1 \times 10^{-3}$	$1,0 \times 10^{-3}$
L-aspartic acid1,	$1,0 \times 10^{-4}$			5×10^{-4}
L-cysteine2,				0×10^{-4}
L-cystine1.	0×10^{-4}	2.0×10^{-4}	1.1×10^{-4}	
L-glutamic acid1,	$1,0 \times 10^{-4}$			4×10^{-4}
L-glutamine	$2,0 \times 10^{-3}$	$4,0 \times 10^{-3}$	$2,1 \times 10^{-3}$	$1,0 \times 10^{-3}$
Glycine		$4,0 \times 10^{-4}$	$1,3 \times 10^{-4}$	$1,0 \times 10^{-4}$
L-histidine	$2,0 \times 10^{-4}$	$2,0 \times 10^{-4}$	$9,7 \times 10^{-5}$	$1,0 \times 10^{-4}$
L-oxyproline			$1,5 \times 10^{-4}$	
L-isoleucine	$4,0 \times 10^{-4}$	$8,0 \times 10^{-4}$	$3,8 \times 10^{-4}$	$1,0 \times 10^{-5}$
L-leucine	$4,0 \times 10^{-4}$	$8,0 \times 10^{-4}$	$3,8 \times 10^{-4}$	$1,0 \times 10^{-4}$
L-lysine HC1	$4,0 \times 10^{-4}$	$8,0 \times 10^{-4}$	$2,2 \times 10^{-4}$	$2,0 \times 10^{-4}$
L-methionine	$1,0 \times 10^{-4}$	$2,0 \times 10^{-4}$	$1,0 \times 10^{-4}$	$3,0 \times 10^{-5}$
L-phenylalanine	$2,0 \times 10^{-4}$	$4,0 \times 10^{-4}$	$9,1 \times 10^{-5}$	$3,0 \times 10^{-5}$
L-proline			$1,7 \times 10^{-4}$	$3,0 \times 10^{-4}$
L-serine		$4,0 \times 10^{-4}$	$2,9 \times 10^{-4}$	$1,0 \times 10^{-4}$
L-threonine	$4,0 \times 10^{-4}$	$8,0 \times 10^{-4}$	$1,7 \times 10^{-4}$	$1,0 \times 10^{-5}$
L-tryptophan	$4,9 \times 10^{-5}$	$7,8 \times 10^{-5}$	$2,5 \times 10^{-5}$	$1,0 \times 10^{-5}$
L-tyrosine	$2,0 \times 10^{-4}$	$4,0 \times 10^{-4}$	$1,1 \times 10^{-4}$	$3,0 \times 10^{-5}$
L-valine	$4,0 \times 10^{-4}$	$8,0 \times 10^{-4}$	$1,7 \times 10^{-4}$	$1,0 \times 10^{-5}$
Vitamins				
P-aminobenzoic acid7,				$7,3 \times 10^{-6}$

Biotin			$8,2 \times 10^{-7}$	$3,0 \times 10^{-8}$
Choline chloride	$7,1 \times 10^{-6}$	$2,9 \times 10^{-5}$	$2,1 \times 10^{-5}$	$1,0 \times 10^{-4}$
Folic acid	$2,3 \times 10^{-6}$	$9,1 \times 10^{-6}$	$2,3 \times 10^{-6}$	$2,9 \times 10^{-6}$
Myo-inositol	$1,1 \times 10^{-5}$	$4,0 \times 10^{-5}$	$1,9 \times 10^{-4}$	$1,0 \times 10^{-4}$
Nicotinamide	$8,2 \times 10^{-6}$	$3,3 \times 10^{-5}$	$8,2 \times 10^{-6}$	$3,3 \times 10^{-7}$
D-pantothenate Ca	$4,2 \times 10^{-6}$	$1,7 \times 10^{-5}$	$1,1 \times 10^{-6}$	$2,0 \times 10^{-6}$
Pyridoxal HCl	$4,9 \times 10^{-6}$	$2,0 \times 10^{-5}$		$3,0 \times 10^{-7}$
Pyridoxine HCl			$4,9 \times 10^{-6}$	$3,0 \times 10^{-7}$
Riboflavin	$2,7 \times 10^{-7}$	$1,1 \times 10^{-6}$	$5,3 \times 10^{-7}$	$1,0 \times 10^{-7}$
Thiamine	$3,0 \times 10^{-6}$	$1,2 \times 10^{-5}$		$1,0 \times 10^{-6}$
Vitamin B12			$3,7 \times 10^{-9}$	$1,0 \times 10^{-6}$
Antioxidants				
Glutathione			$3,0 \times 10^{-6}$	
Inorganic salts				
$CaC1_2$	$1,8 \times 10^{-3}$	$1,8 \times 10^{-3}$		$3,0 \times 10^{-4}$
COP1	$5,3 \times 10^{-3}$	$5,3 \times 10^{-3}$	$5,3 \times 10^{-3}$	$3,0 \times 10^{-4}$
$MgS0_4$	$8,1 \times 10^{-4}$	$8,1 \times 10^{-4}$	$8,1 \times 10^{-4}$	
NaCl	$1,2 \times 10^{-1}$	$1,1 \times 10^{-1}$	$1,0 \times 10^{-1}$	$1,3 \times 10^{-1}$
$NaHC0_3$	$2,6 \times 10^{-2}$	$4,4 \times 10^{-2}$	$4,2 \times 10^{-3}$	$1,4 \times 10^{-2}$
$NaH_2 P0_4$	$1,0 \times 10^{-3}$	$9,1 \times 10^{-4}$		
$Na_2 HP0_4$			$5,6 \times 10^{-3}$	$1,0 \times 10^{-3}$
Trace elements				
$CuS0_4 - 5H_2 0$				$1,6 \times 10^{-8}$
$Fe(N0_3)_3 - 9H_2 0$			$2,5 \times 10^{-7}$	
$FeS0_4 -7H_2 0$				$3,0 \times 10^{-6}$
$ZnS0_4 - 7H_2 0$				$3,0 \times 10^{-6}$
Antioxidants				
Glutathione			$3,0 \times 10^{-6}$	

Inorganic salts				
$CaC1_2$	1,8 x10^{-3}	1,8 x10^{-3}		3,0 x10^{-4}
COP1	5,3 x10^{-3}	5,3 x10^{-3}	5,3 x10^{-3}	3,0 x10^{-4}
$MgS0_4$	8,1 x10^{-4}	8,1 x10^{-4}	8,1 x10^{-4}	
NaCl	1,2 x10^{-1}	1,1 x10^{-1}	1,0 x10^{-1}	1,3 x10^{-1}
$NaHC0_3$	2,6 x10^{-2}	4,4 x10^{-2}	4,2 x10^{-3}	1,4 x10^{-2}
NaH_2	1,0 x10^{-3}	9,1 x10^{-4}		
$P0_4$			5,6 x10^{-3}	1,0 x10^{-3}
Na_2 $HP0_4$				
Trace elements				
$CuS0_4$ - $5H_2$ 0				1,6 x10^{-8}
$Fe(N0_3)_3$ - $9H_2$ 0			2,5 x10^{-7}	
$FeS0_4$ -$7H_2$ 0				3,0 x10^{-6}
$ZnS0_4$ - $7H_2$ 0				3,0 x10^{-6}
Bases, nucleosides, etc.				
Hypoxanthine				3,0 x10^{-5}
Thymidine				3,0 x10^{-6}
D-glucose5,	6 x10^{-3}	2,5 x10^{-2}	1,1 x10^{-2}	1,0 x10^{-2}
Sodium pyruvate1,	0 x10^{-3}	1,0 x10^{-3}		1,0 x10^{-3}
Linoleic acid		3,0 x10^{-7}		
Lipoic acid				1,0 x10^{-6}
Phenolic red		2,7 x10^{-5}	4,0 x10^{-5}	1,3 x10^{-5}
	3,2 x10^{-5}			
Putrescine				1,0 x10^{-6}
CO_2	5%	10%	5%	2%

The shelf life of liquid media is limited. Most often, dry media are used, which are prepared as needed. A prerequisite for the preparation of cultivation media in the laboratory is the use of deionized, distilled water.

For a certain type of medium, it is necessary to make a suspension of a certain volume. Sometimes for cell cultivation, it is necessary to use multiply concentrated cultivation media, which are accordingly prepared using a larger suspension. Therefore, to prepare the medium from a dry suspension, it is necessary to dilute the suspension by gradually adding it to dissolve it in a certain volume. A magnetic stirrer is used for dissolution. When all components are completely dissolved and the pH of the medium is checked, the latter should be immediately filtered using 0.22-0.45 nm nitrocellulose filters to protect against microbial contamination.

Often not all components are present in the medium. For example, glutamine, sodium bicarbonate, glucose, and blood serum or other growth-stimulating factors and necessary components are added as needed.

Bovine embryonic serum (BES) is used as a growth-stimulating factor (Table 2).

The percentage of ETS in the medium for certain populations of cultured cells varies from 1 to 25%. Before use, ETS is inactivated by heating at 56^0 C for 40 minutes. Phenol red dye is used as an indicator of the pH of the media, which changes its color when the pH changes, and thus this indicator can be controlled.

Table 2

Components of fetal calf serum

Components (1)	**Concentration range(2)**
Proteins and polypeptides	40-80 mg/ml

Albumin	20-50 mg/ml
Fetuin	10-20 mg/ml
Fibronectin	1-10 mg/ml
Globulins	1-15 mg/ml
Protease inhibitor:α_1 -antitrypsin, α_2 -macroglobulin	0,5-2,5 mg/ml
Transferrin	2-4 mg/ml
Growth factors:	
EGF, PDGF, IGF-I, IGF-II, FGF, IL-1, IL-6	1-100 ng/ml
Amino acids	0,01-1,0 μM
Lipids	2,0-10,0 mg/ml
Cholesterol	10 μM
Fatty acids	0,1-1,0 μM
Linoleic acid	0,01-0,1 μM
Phospholipids	0,7-3,0 mg/ml
Carbohydrates	1,0-2,0 mg/ml
Glucose	0.6-1.2 mg/ml
Hexosamine	0,6 - 1,2 mg/ml
Lactic acid	0,5 -2,0 mg/ml
Pyruvic acid	20-10,0 μg/ml
Polyamines:	
Putrescine, spermidine	0,1-1,0 μM
Urea	170-300 μg/ml
Inorganic compounds:	0,14-0,16 mM
Chlorides	100 μM
Iron	10-50 μM
Potassium	5-15 mM

It should be remembered that sterilization of media, serums, and solutions

protease is carried out only ultrafiltration through nitrocellulose membrane filters.

Balanced salt solutions are often used for cultured cells (Table 3-6)

Table 3

Composition of Dulbecco's balanced phosphate-salt solution (Dulbecco's PBS, pH 7.2) without Ca^{2+} and Mg^{2+}

Components	**Mm-hmm.**	**g/l**	**mM**
COP1	74,55	0,2	2,68
IPS_2 $P0_4$	136,1	0,2	1,47
NaCl	58,44	8	136,9
NaH_2 $P0_4$ - H_2 0	138	2,2	8,06

Table 4

Composition of Dulbecco's balanced phosphate-salt solution (Dulbecco's PBS, pH 7.2) with Ca^{2+} and Mg^{2+}

Components	**Mm-hmm.**	**g/l**	**mM**
$CaC1_2$ (anhydrous)	111	0,2	1,8
COP1	74,55	0,2	2,68
IPS_2 $P0_4$	136,1	0,2	1,47
$MgS0_4$ - $7H_2$ 0	246,5	0,98	3,98
NaCl	58,44	8	136,9
Na_2 $HP0_4$ - $7H_2$ 0	268	2,2	8,06

Table 5

Composition of Earle's balanced salt solution (Earle's BBS)

Components	**Mm-hmm.**	**g/l**	**mM**
$CaC1_2$ (anhydrous)	111	0,02	0,18
COP1	74,55	0,4	5,37
$MgS0_4$ - $7H_2$ 0	246,5	0,2	0,81
NaCl	58,44	6,68	114,3
$NaHC0_3$	84,01	2,2	26,19
NaH_2 $P0_4$ - H_2 0	138	0,14	1,01
D-glucose	180,2	1	5,55
Phenolic red	354,4	0,01	0,03
Gas environment	5% CO_2		

Table 6

Composition of Hanks' balanced salt solution (Hanks' BBS)

Components	**Mm-hmm.**	**g/l**	**mM**
$CaC1_2$ (anhydrous)	111	0,14	1,3
COP1	74,55	0,4	5,37
$IPS_2\ P0_4$	136,1	0,06	0,4
$MgCl_2$ - $6H_2\ 0$	203,3	0,1	0,5
$MgS0_4$ - $7H_2\ 0$	246,5	0,1	0,4
NaCl	58,44	8	136,9
$NaHC0_3$	84,01	0,35	4,2
$Na_2\ HP0_4$ - $7H_2\ 0$	268,1	0,09	0,3
D-glucose	180,2	1	5,55
Phenolic red	354,4	0,01	0,03

To disintegrate adherent cells from the substrate, a trypsin solution (0.25%) prepared in a cell culture medium and EDTA solution (1 mM) prepared in Dulbecco's PBS containing no Ca^{2+} and Mg are used.$^{2+}$

Phosphate-salt buffers are sterilized by autoclaving or ultrafiltration, while culture media, serum, and trypsin solution are sterilized only by ultrafiltration through nitrocellulose filters (Fig. 6).

Thus, cell cultivation often uses buffer solutions that can be prepared independently.

Calculations for the preparation of phosphate-salt solutions are a necessary component in the preparation of students for the special workshop program "Cell culture and histogenesis".

Task for topic 1: ***make calculations for the preparation of buffers for culturing and washing cells.***

Option 1.

Perform calculations for the preparation of Hanks's BBS: volume - 15 ml, 2-fold solution; $CaC1_2$ in a 10% solution; glucose in a 20% solution

Option 2.

Perform calculations for the preparation of RBS with Ca^{2+} and $Mg^{2+:}$ volume -17ml; salt $Na_2HP0_4•7H_20$ is replaced by $NaH_2PO_4•12H_20$; salt KH_2P0_4 is replaced by $KH_2P0_4•7H_20$

Option 3.

Perform calculations for the preparation of Hanks' BBS: volume 25 ml, 3x, Ca^{2+} in a 20% solution, glucose in a 10% solution; $Na_2HP0_47H_20$ in 1M solution.

Option 4.

Make calculations for preparing VBS Earl: volume 185 ml; 4x, salt $MgS0_4•7H_20$ in 0.5 M solution, NaCl in 3M solution, salt $NaH_2P0_4•H_20$ replaced by $NaH_2P0_4•12H_20$.

Option 5.

Perform calculations for the preparation of RBS without Ca^{2+} and Mg^{2+}: volume - 12 ml; salt $Na_2HP0_4•7H_20$ is replaced by $Na_2HP0_4•5H_20$; KH_2P0_4 salt in 1.5 M solution; NaCl is contained in a 20% solution.

Option 6.

Make calculations for the preparation of BBS Hanks: volume - 132 ml; 0.7x buffer, KH_2P0_4 salt replaced by $KH_2P0_4•3H_20$; glucose in a 30% solution.

Option 7.

Perform calculations for the preparation of RBS with Ca2+ and Mg2+: volume - 11 ml, 3x; salt $MgS0_4$•$7H_20$ is replaced by $MgS0_4$•$2H_20$; salt KH_2P0_4 in a 1.5 M solution

Option 8.

Perform calculations for the preparation of RBS without Ca2+ and Mg2+: volume -110 ml; 3 times; KH_2P0_4 salt is replaced by KH_2P0_4•$7H_20$; NaCl salt in a 2.5 M solution

Option 9.

Make calculations for the preparation of Earl's BBS: volume 630 ml; NaCl in a 4.5 M solution, salt NaH_2P0_4•H_20 in a 2 M solution, glucose in a 5% solution

Option 10.

Make calculations for the preparation of BBS Hanks: volume -38 ml; 0.7x glucose in a 10% solution, $CaC1_2$ in a 2% solution for injections.

Option 11

Perform calculations for the preparation of RBS with Ca2+ and Mg2+: a volume of 220 ml of $CaC1_2$ in a 5% injection solution, $NaHCO_3$ and NaCl in 1.5 M solutions.

Option 12

Perform calculations for the preparation of Hanks's BBS: volume 340 ml, 2.5x, $CaC1_2$ in 1% solution; Na2HP04x7H20 was replaced by anhydrous NaCl in a 10% solution.

Option 13

Perform calculations for the preparation of RBS with Ca2+ and Mg2+: a volume of 1.45 l $CaC1_2$ in a 2% injection solution, NaCl in a 16% solution.

Anhydrous KN2P04 salt is replaced by KN2P04 x3H20 salt.

Option 14

Make calculations for preparing BBS Earl: volume 45.5 ml; 3.5 times solution, NaCl in 20% solution, $NaH_2P0_4•H_20$ salt in 2 M solution, $MgS0_4•7H_20$ salt replaced by $MgS0_4•2H_20$ salt, glucose – in 2.7 M solution.

Option 15.

Make calculations for the preparation of RBS with Ca2+ and Mg2+: volume - 140 ml; 1.5x solution, salt $Na_2HP0_4•7H_20$ in 1.2 M solution; NaCl in a 3.5 M solution, $CaC1_2$ in a 1.4 M solution.

An example of solving a typical problem for the preparation of the necessary dosages of certain phosphate-salt solutions for a given volume, the corresponding multiplicity and modification of the components

Perform calculations for the preparation of RBS without Ca2+ and Mg2+: volume - 530 ml; salt Na2HP04•7H20 is replaced by Na2HP04•2H20; salt KH2P04 is replaced by KH2P04•5H20.

Progress of problem solving

1) We calculate the required weight of $Na_2HP0_4•2H_20$ (g/l) instead of $Na_2HP0_4•7H_20$

M.m. $Na_2HP0_4•2H_20$ is 178. To prepare an 8.06 mM solution of Na2HP04•2H20, it is necessary to prepare a weight of 178x0.00806=1.43 g/l;

2) We calculate the required amount of $KH_2P0_4•5H_20$ (g/l) instead of KH_2P0_4.

M.m. $KH_2P0_4•5H_20$ is 226.1. To prepare a 1.47 mm solution of $KH_2P0_4•5H_20$, it is necessary to prepare a weight of 226.1x0.00147=0.33 g/l.

3) Taking into account the volume (530 ml), the content of all components of the solution, respectively, is:

CS1: 0.2x0.53=0.106 g/given volume

KH_2P0_4•$5H_20$: 0.33x0.53=0.175 g/given volume

NaCl: 8x0.53=4.24 g/given volume

Na_2HP0_4 • $2H_20$: 2.2x0.53=1.17 g/given volume.

Note: The progress of problem solving must be presented in writing, with a full description of all the components of the given solution and the personal signature of the performing student. Task conditions can be changed.

Question.

1. Why is it necessary to prepare phosphate-salt solutions from modified components (for example, instead of dry weight, concentrated solutions of certain components with a known molarity or percentage content are used)?

2. Why is it necessary to use multiple buffers (most often 2x buffers) when cultivating cells?

3. Describe the methods of sterilizing phosphate-salt buffers used for cell culture.

Preparation of media for filtration.

Nutrient media used for cell cultivation are sterilized by filtering through nitrocellulose sterile filters with a pore diameter of 0.22-0.45 microns. The filtration medium consists of a plastic or metal filter holder (Fig. 6) and the filter itself. The filtration sequence is as follows: the prepared culture medium is applied to the sterile membrane and pressed through syringe filter.

When sterilizing solutions by autoclaving, it is necessary to prepare cotton gauze swabs, which are used to close the containers with solutions (Fig.) It is advisable to close the container with foil or lacquered paper on top of the cotton gauze plug. The autoclaving container is filled with liquid to 2/3 of the volume. The autoclaving mode for each solution is set according to its characteristics. Most often, it is 1.5 atmospheres for 30-40 minutes.

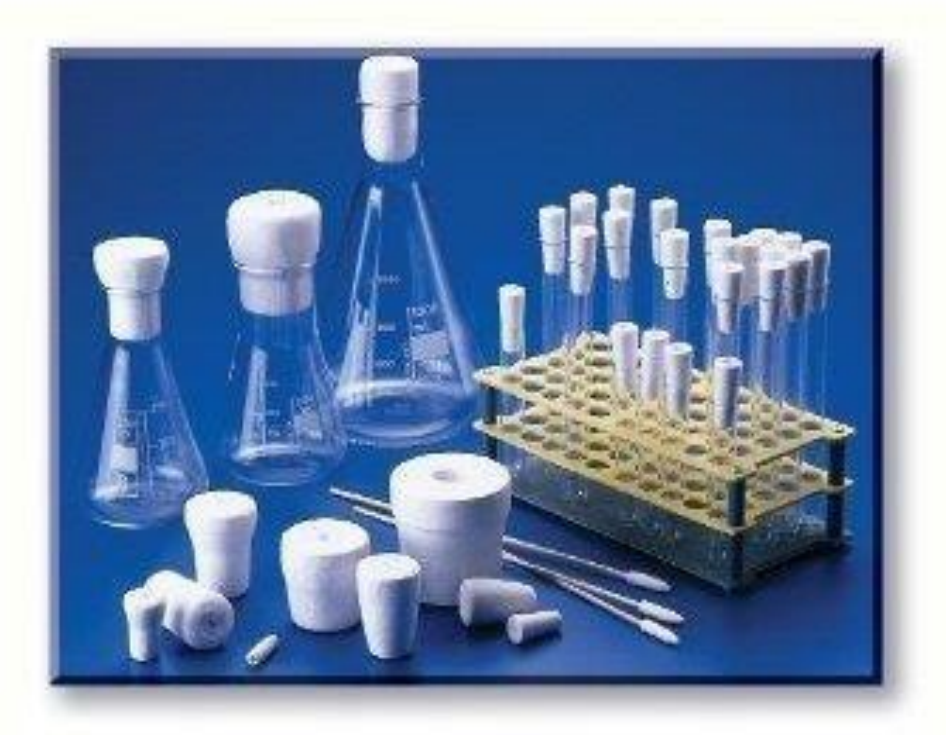


Figure 6. Cotton gauze swabs for autoclaving liquids.

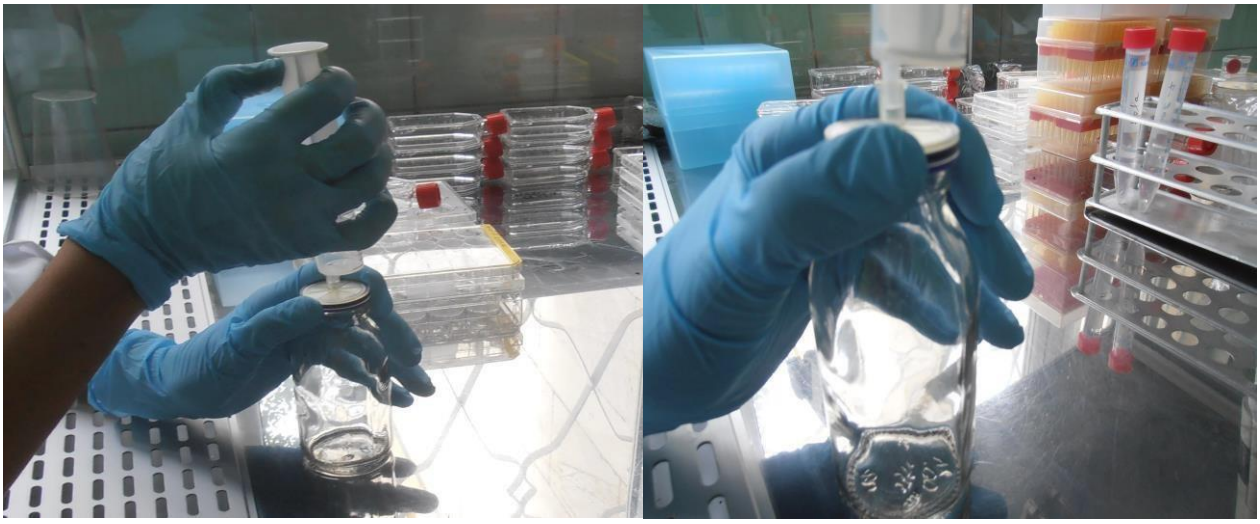


Figure 7. Sequence of ultrafiltration with using 0,22 micron nitrocellulose filters

After autoclaving, the containers with solutions are closed with a rubber corks that are sterilized by autoclaving or boiling. In addition, it is advisable to burn the corks, moistened with 70% alcohol solution.

Storage of cultivation media and phosphate-salt buffers is carried out at a temperature of $+4^0$ C. Storage of trypsin solution and fetal calf serum is carried out at -20^0 C, preferably in small aliquots necessary for cultivation.

Glutamine is added separately to the culture media, as this component can degrade in the complete nutrient medium. Therefore, glutamine can also be stored in small aliquots in frozen form.

Often a mixture of antibiotics is used to cultivate certain cell lines or primary cultures. For each specific

In this case, this mixture is specified in the culture conditions given in the certificate for cultured cells.

Control questions:

History of creation of cultivation media.

What components are used for cultivation media?

What components are used in cell culture to stimulate the growth of cell populations?

How to prepare media from dry suspensions? Methods of

sterilization of solutions for cultivation.

Which solutions were recommended to be prepared by ultrafiltration only?

Prepare an individual phosphate-salt solution (for example, calculate the necessary weights for the preparation of 2x Hanks' solution in a volume of 35 ml).

Practical task*: Calculate and prepare cotton swabs and autoclave individually prepared solution.*

Class 3. **Types of dishes for cell culture, preparation and sterilization of glassware.**

Cell culture dishes are divided into **glass** and **plastic** (Fig.8)

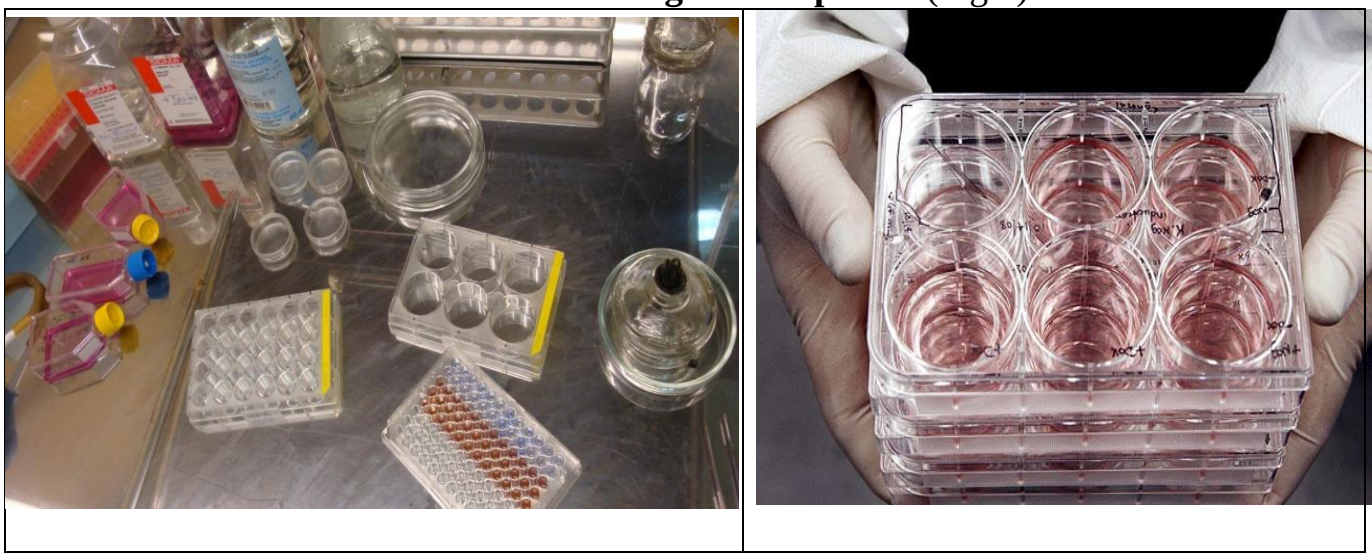


Figure 8. Dishes for cell culture: plates, Petri dishes (glass and plastic), glass dishes for media, vials; pipettes.

Plastic tableware is disposable and therefore cannot be sterilized. Depending on the tasks and main objectives of the scientific or production process, different types of dishes are used. The most popular are vials with different working surfaces (from 25 cm^2 to 500 cm^2) and Petri dishes with different diameters. Depending on the conditions of the experiment and the type of cultured cells (suspension and adherent), vials or cups with low and high adhesive properties are selected.

The most commonly used tablets are 6- 24 and 96

wells. 96-well dishes are of three types (flat-bottomed, V-shaped, and round-bottomed). For certain dishes, there are optimal rates of planting cells and the volume of culture medium used (Table 7).

Table 7.

Characteristics of plastic dishes used for cell cultivation.

Vial type	S (see^2)	V (ml)	Wednesday	C (cells)
Vial	24	50	7	$4,8x10^5$
vial	25	70	7	$5x10^5$
vial	75	250	25	$1,5x10^6$
vial	83	260	30	$1,6x10^6$
vial	175	800	68	$3,5x10^6$
Cup 35x10	9,6		2,5	$1,6x10^5$
Cup 65x15	21		6,0	$4,2x10^5$
Cup 100x20	58		16,0	$1,1x10^6$
96 well plate	0,32		0,2	$6x10^3$
24 hole tablet	1,88		1,5	$3,7x10^4$
12 hole tablet	3,83		2,0	$7,6x10^4$
6 hole tablet	9,4		3,0	$1,8x10^5$

Among another plastic crockery should be noted – tubes for cryopreservation of cells, centrifuge tubes for different volumes, disposable pipettes, etc. Especially necessary are nozzles for reusable filtration of solutions with replaceable filters (nitrocellulose with a pore diameter of 0.22 microns) and disposable filters, so-called syringe filters designed to filter a small number of solutions and buffers (fig., given in the previous section). In addition, prefilters with pore diameters from 0.45 μm are used for filtration

The most famous companies that are suppliers of plastic tableware are Nunc, Gibco, Falcon, Sarstadt, TPP, Cellstar.

Glassware - the main part of which belongs to the auxiliary dishes. These are dishes of various capacities, the main purpose of which is the preparation of solutions for cell cultivation. These dishes are specially prepared. Washing of dishes necessarily includes the procedure of soaking in special detergents or in a solution of potassium bichromate "chromovka" (93 g of potassium bichromate in rubber gloves and a respirator or cotton gauze bandage is well ground into a powdered mass in a mortar, the minimum required volume of water is added and 1 liter of sulfuric acid is added in portions). The dishes are washed clean in a certain detergent, preferably one that does not contain biological additives, rinsed in running water through a solution of "chrome", then washed well in running water, then rinsed in two or three portions of distilled and undistilled water. Sterilization of tableware is carried out in several ways:

- hot steam sterilization using autoclaving; for this purpose, glassware is covered with two or three layers of foil, and carefully wrapped in autoclaving paper. A special strip should be attached to the top of one of the bottles or Petri dishes to certify the sterility of the dishes. If the mode in the autoclave is observed (40 minutes, 1.5 atmospheres), then black transverse stripes appear on this strip, and this indicates the sterility of the dishes. Also subject to autoclaving are tips for automatic varipipettes, which are placed in special closed racks (Fig.
- sterilization with dry air using special "dry heat" cabinets (the mode is sufficient for sterilization at 150^{o} C for 2-3 hours or 180^{o} C for 40 minutes. For this purpose, the holes of glassware are again well closed with foil.

Glass pipettes of different volumes are often used for cell cultivation. Pipette preparation also includes the procedure of

thorough washing, for which cylinders are used for soaking pipettes and their treatment in detergents, followed by thorough rinsing in many changes of running and distilled water. Before sterilization, the blunt ends of pipettes are closed with cotton swabs, and placed in special cases, or each pipette is wrapped separately in the paper that can withstand sterilization at 180^{o} C. Sterilization modes are indicated above.

Remember: the quality of work with cell cultures depends on the degree of absolute sterility of the dishes.

Note: the dishes used for cell culture are never used for biochemical and molecular biological research. Immediately after sterilization, the dishes are also not used. It should be cooled to room temperature. Among the auxiliary dishes for which you can not follow strict sterilization rules are tubes in which biochemical manipulations with cells after their cultivation are carried out.

Since all manipulations with cells and culture media are carried out using sterile pipettes, you should practice this procedure very well.

Rules for working with sterile pipettes:

1) wipe the inner surfaces of the laminar box with an alcohol solution;
2) turn on ultraviolet irradiation and injection of sterile air in the box-laminar flow chamber for 30-40 minutes;
3) before start work it is necessary turn off the ultraviolet irradiation;
4) all manipulations in the box are carried out over a burner flame (alcohol or natural gas);
5) to ensure proper and safe operation in the laminar flow

it is necessary to carry out the correct pipette grip (Fig. 9)

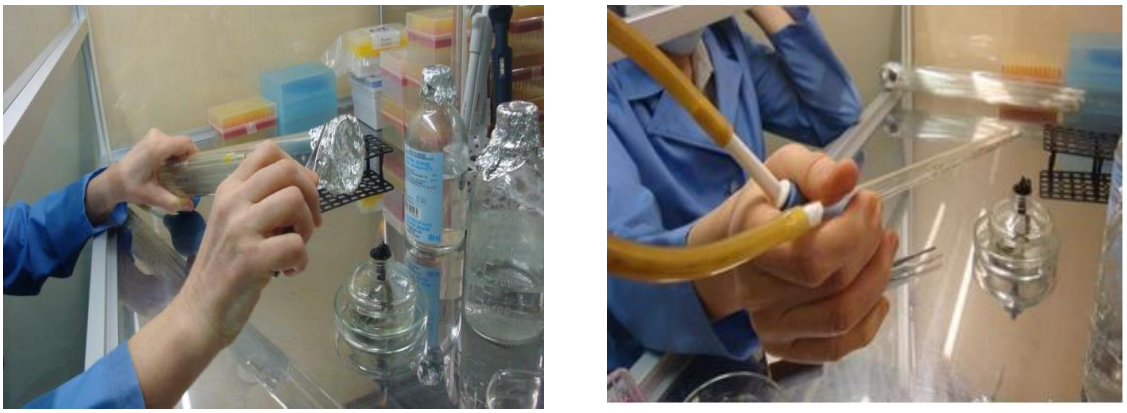


Figure 9. Preparation of sterile pipettes for work.

For sterile manipulations with culture media and cells, glass pipettes of various volumes are most often used. Their expediency is primarily due to the lowest risk of contamination of sterile material due to the large size of the sterile surface. However, after using the pipette for one procedure it should be moved to the tray with the waste material.

The sequence of the pipette preparation procedure is as follows:

A) remove the lid from the pencil case in the flame of vodka (this is done with the right hand). The lid after calcination over the flame is set aside to the right.

B) Holding the pencil case in the left hand, make sure that the tips of the pipettes are extended in the flame of the burner with light movements. After that, with thethumb of the right hand, grab the desired volume of the pipette (in the case of a glass pipette, this graduation is visible, in the case of a metal pipette, the graduation should be carried out on the blunt end of the pipette).

C) After that, you can carefully put aside the pencil case from your left hand, put on the pear, and work with the pipette, or you can intercept the pipette with your left hand closer to the blunt end, close the pencil case with your right hand, holding all its components over the flame, and then manipulate the

pipette

***Note*:** Do not forget that when manipulating the pipette, its sharp end should be directed towards the sterile airflow in the laminar flow, and in no case touch other objects under the laminar flow. If you have taken the last pipette out of the case, the case may not be closed.

B) After closing the case, a pear with an adapter from a medical tube is put on the blunt end of the pipette. The pipette is ready for use.

Control questions:

How are cell culture dishes divided?

By what parameters is plastic tableware divided?

Methods of washing glassware used for sterile work.

How should the dishes be prepared for sterilization? Whatmethods of sterilization do you know?

How are plastic tips for automatic pipettes sterilized?

Describe the sequence of preparation of the laminar flow box for work. Describe the sequence of the procedure for preparing a glass pipette for workin a laminar flow box.

***Practical task**: to carry out the procedure of pipette preparation for sterile manipulation.*

.

Activity 4. **Determination of the concentration of cultured cells by routine counting**

Using a hemocytometer is the easiest, cheapest, and most informative way to determine the number of cells. By observing the cells directly, it is possible to determine the morphology of the cells, microbial contamination of the cell suspension, the proportion of red blood cells in the preparation of lymphoid cells, as well as to determine the ratio of live and dead cells by excluding dyes (trypan blue, erythrosin B, nigrosin).

Goryaev chambers are used to assess the concentration of cells (Fig.10) and **Fuchs_Rosenthal.**

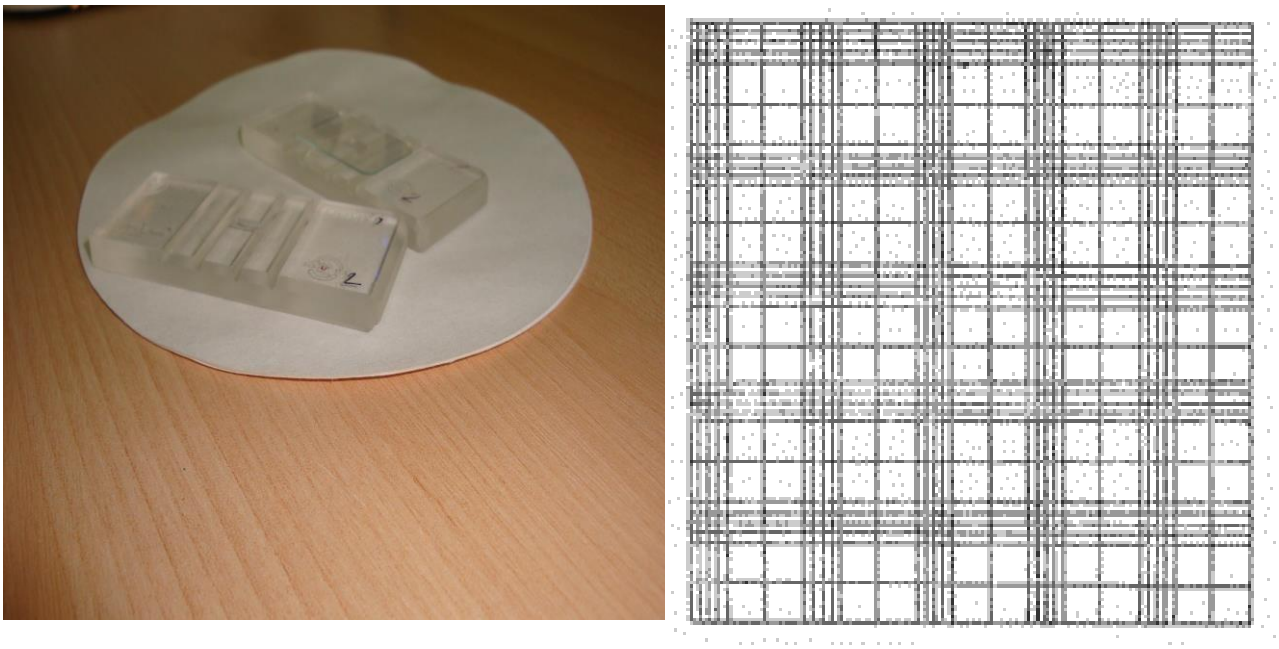


Fig.10. Goryaev camera

The cameras consist of thick slides with engraved grids on them. On both sides of the grid area, there are strips 0.1 mm higher than the grid areas, which are intended

for lapping the cover glass until the Newton rings appear. After rubbing the glass, a chamber is formed, closed on the sides and slits, which are used to fill the chamber with a suspension of cells. The principle of separation of the Goryaev chamber is as follows. It consists of

225 large squares with a side equal to 1/20 mm. Each of the large squares is divided into 16 small squares with sides equal to 1/400 mm.

So: the technical characteristics of the Goryaev camera

- gap 0.1 mm
- small square side 0.05±0.001 mm, area - 0.0025 mm^2 , volume - 0.00025 mm^3
- side of large square 0.2±0.0015 mm, area - 0.04 mm^2 , volume - 0.0004 mm^3 (μl)
- grid side 3±0.005 mm, grid area 9 mm^2 , chamber volume 0.9 mm^3 (μl)

Before the operation, the chamber slide and ground cover glass are well rinsed under tap water, rinse with distilled water and wipe dry. Then cover glass is rubbed against the camera until iridescent "Newton" rings appear (Fig.11)

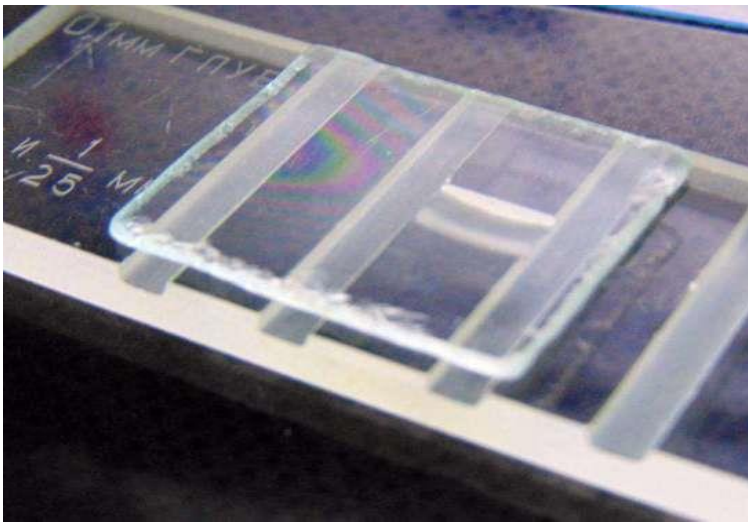


Fig.11. Preparation of Goryaev chamber for filling with hanging cells.

Preparation of cells for counting.

Primary cultures, or cells taken after cultivation, are centrifuged at 1000g for 10 minutes. Resuspend the pellet in 1 mlof phosphate-buffered saline or Hanks' solution. Take a 50 µl aliquot and add 50 µl of 0.4% trypan blue dye solution (to a final dye content of 0.2%). After staining for 5 min, mix the cells thoroughly and add them with a Pasteur pipette or an automatic pipette to a Goryaev chamber covered with a cover glass.Remove excess solution with filter paper.

Cell counting is carried out depending on the size and initial concentration of cells. Thus, if we are talking about determining the concentration of cells that are cloned by the method of limiting concentrations, then it should be calculated in the entire field of view, that is, in 225 large squares, red blood cells are countedin 80 small squares after their dilution (at least 200 times).

Progress of cell counting:

Count the cells under the x10 objective. If there is sufficient cell density, count in four sets of 16 small squares (in each corner of the central shaded area). Count first only the unshaded cells. Divide this number by 4 to get the average number of cells in $1mm^2$. Since the height of the chamber is 0.1 mm, the conversion factor for the hemocytometer is 10^4 .

So, the general formula for counting cells in a Goryaev chamber is as follows:

$$\mathbf{X=(a*4000*c)/b,}$$

where **X is the number of** cells in 1 mm^3 ; **a is the** sum of cells counted in a certain volume of the chamber; **b is the** number of small squares counted; **c is the** dilution of cellular suspension.

For example, the obtained average value of cells is 20 cells in $1mm^2$. Then $20x10^4$, is equal to the concentration of cells in the stained suspension, and taking into account the dilution by half with the dye - in the original suspension, the concentration of live cells is $2x20x10^4$, that is, equal to $4x10^5$ cells per 1 ml.

To determine the viability of the cells being analyzed, it is necessary to count the cells stained with trypan blue accordingly.

For example, the obtained average value of cells is 2 cells in $1mm^2$. Then $2x10^4$, is equal to the concentration of dead cells in the stained suspension, and taking into account the dilution by half with the dye - in the original suspension the concentration of dead cells is $2x2x10^4$, that is, equal to $0.4x10^5$ dead cells per 1 ml. To determine the percentage of dead cells in the suspension, it is necessary to determine the total concentration of cells, which will be $4.4x10^5$. then $4.4x10^5$ -100%, and $0.4x10^5$ -x, respectively, the proportion of dead cells in the suspension will be equal to 9%, and live cells - 91%.

If there is a need to determine the exact concentration (for example, when constructing a growth curve of the cell population), the cell counting procedure should be repeated several times, respectively concentrating the cell suspension or diluting it. To concentrate the cells, the procedure of centrifugation of cells with their subsequent dilution in a smaller volume of buffer or culture medium is used; to dilute the cell suspension; an aliquot is taken and diluted with a certain buffer or culture medium.

Determination of the exact concentration of cells is necessary for seeding, cloning of cells, and construction of the growth curve. The procedure of cell counting using trypan blue is introduced in the structure of preclinical studies.

For standardization determination concentration cells в within the framework of standard operating procedures create a protocol (SOP).

SOP 1

STUDY OF CELL CONCENTRATION BY INCLUSION OF THE VITAL DYE TRYPAN BLUE

1. Purpose

Determination of cell concentration and viability in suspension.

2. Distribution

In laboratory diagnostics, clinical practice.

3. Purpose

By incorporating trypan blue dye into dead cells, determine the cell concentration, and the ratio of live to dead cells in the suspension.

4. Object

Cells

5. Materials and equipment

Reagents

Incubation environment:

RPMI solution (10.2 g/l) +

ETS - fetal calf serum (10%) + 0.4% trypan blue

dye solution *Laboratory glassware*

96-well plates;

Penicillin vials for media.

Equipment

Centrifuge.

Pipettes.

Microscope.

Goryaev
camera.

Thermostat.

Materials.

Tips for pipette dispenser (50, 100, 200, 300 μl), sterile. Filter paper cotton No. 1.

6. Preparation of solutions

Preparation of trypan blue dye: dilute 400 mg of the dye in 100 ml of phosphate-buffered saline, pH 7.2 (or by increasing or decreasing the weight and volume of phosphate-buffered saline, respectively)

7. Preparation of samples for analysis

Pellet the cell suspension by centrifugation at 1000 g for 5 min. Dilute the cell pellet in a certain volume (1 ml).

8. Research protocol

А). The effluent concentration cells dilute by a factor of two, (3-4 dilution steps, Table 8) .

Б). Take an aliquot of cells (50 μl) from each dilution and add an equal volume of trypan blue dye.

В). Paint for 5 minutes.

Г). After that, add the cells to a pre-prepared Goryaev chamber (each concentration under study in at least 2 parallels).

Table8

Cell dilution scheme for counting in Goryaev chamber

А) Theoretical:

concentration/ml	**1:2**	**1:-4**	**1:8**	**1:16**
200 000	100 000	50 000	25 000	12 500

В) practical

Hole	1	2	3	4
A	100 000	50 000	28 000	13 000
B	100 500	52 000	25 000	12 000
M	100 250	51 000	26 500	12 500
m	1666,7	666,7	1000,0	333,3

9. Presentation of the results

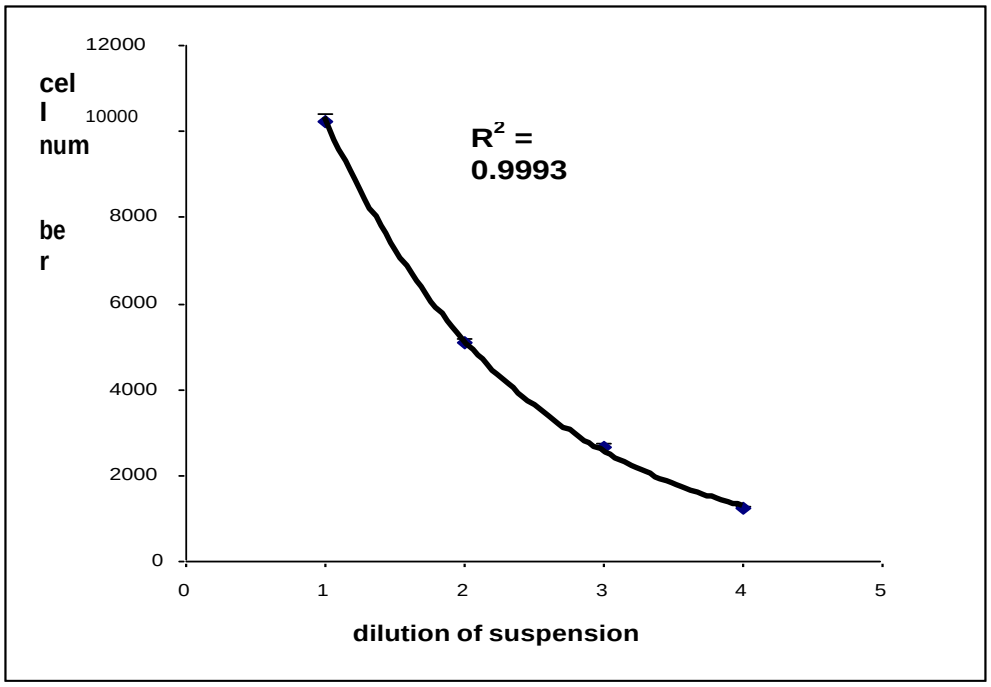


Fig.12. The curve of counting cells in the Goryaev chamber in the conditions of their successive 2-fold dilution.

10. Related documents and files

Provide a protocol for cell counting and present the counting curve as a graph.

11. List of references

1. Molecular Biology of the Cell, B. Alberts, D. Bray, J. Lewis, et al. "The World", 1987

2. Adams R. Cell Culture Methods for Biochemists, M. "Mir", 1983, 263

12. Terms and abbreviations

SOP - Standard Operating Procedure. A detailed, written instruction

to achieve reproducibility of each specific action. ETS - embryonic calf serum

13. **Developer *X***

To determine the correlation coefficient, compare the results of cell counting conducted by two researchers by Student's criterion (Table 9). Confidence limits for t with f - degrees of freedom (Petunin Y.I. Application of the theory of random processes in biology and medicine, Kyiv 1981, p.59)

Table 9

Student's criterion, confidence limits for t with f - degrees of freedom

f	Bilateral	limits	**f**	Bilateral	limits
	5%	1%		5%	1%
1	12,71	63,66	20	2,086	2,845
2	4,303	9,925	21	2,08	2,831
3	3,182	5,841	22	2,074	2,819
4	2,776	4,604	23	2,069	2,807
5	2,571	4,032	24	2,064	2,797
6	2,447	3,707	25	2,06	2,787
7	2,365	3,499	26	2,056	2,799
8	2,306	3,355	27	2,053	2,771
9	2,262	3,25	28	2,048	2,783
10	2,228	3,169	29	2,045	2,756
11	2,201	3,106	30	2,042	2,75
12	2,179	3,055	40	2,021	2,704
13	2,16	3,012	50	2,009	2,768
14	2,145	2,977	60	2	2,66
15	2,131	2,947	80	1,99	2,639
16	2,12	2,921	100	1,984	2,622

17	2,11	2,898	200	1,972	2,601
18	2,101	2,878	500	1,985	2,586
19	2,093	2,861	~	1,98	2,578
f	2,5%	0,5%	**f**	2,5%	0,5%
	Unilateral borders			Unilateral borders	

$f= g+h-2$; $t=(M_1 - M_2)/ \sqrt{m_1^2 + m_2^2}$

Tasks

Option 1.

The concentration of cells counted in the Goryaev chamber is $5.5x10^3$/ml, the percentage of dead cells is 7.4%, and the total volume of cells is 7.4 ml. Cells must be planted for cultivation at a concentration of $3.5x10^4$/ml of living cells. Describe the sequence of preparation of cell suspension for planting.

Option 2.

In 43 small squares of Goryaev's chamber, the number of cells is 32, of which 7 are stained with trypan blue. Calculate the concentration of cells and the percentage of dead cells (10 μl of 1% trypan blue dye was added to 40 μl of the cell suspension).

Option 3.

There are 8 cells in 5 large squares of Goryaev's chamber. When planting cells for cloning by the limiting dilution method, it is necessary to plant 1 cell in a volume of 25 μl, 50 μl, 100 μl, and 150 μl. Calculate the initial concentration of cells in the suspension and the dilution in which the cells should be seeded.

Option 4.

There are 2 cells in 13 small squares. Dead cells in the suspension were not recorded. What is the total number of cells in the suspension, the volume of which is 18.4 ml?

Option 5.

The concentration of cells in the suspension is $2.2x10^4$ cells. What is the average number of cells contained in 10 small squares, 3 large squares when diluting the suspension with trypan blue dye in 1.25; 1.5 to 2 and 2.5 times, respectively?

Option 6.

Macrophage suspension was diluted 2, 5, and 10 times. When diluted 5 times, the number of cells in 40 small squares was 12 cells. How many cells are contained in 3 large squares when diluting the suspension by 2 times; in 56 squares at a 10-fold dilution and what is the initial concentration of macrophages.

Option 7.

When cloning cells in a volume of 122 ml, the cell content was 1.3x102. What manipulations should be carried out in order to sow cells in a 12-well plate in 4 wells (the volume of each is 1.25 ml) at a concentration of $1.75x10^3$ cells/ml? When counting in Goryaev's chamber, how many cells will be contained in 72 small squares in 10 large squares and in 2 large and 5 small squares?

Option 8.

Solving a typical problem of counting cells in a Goryaev chamber.

There are 33 cells in 80 small squares. Of them, 3 cells are dead. What is the

concentration of the initial cell suspension if 50 μl of the suspension with the addition of 25 μl of trypan blue dye are used for counting?

According to the cell counting formula

X=(a*4000*c)/b,

a=33; b=80; c=1.5 times

Therefore, X=33x4000x1.5/80=2475 cells/in 1 mm3 (1 μl).

Accordingly, the total concentration in 1 ml = $2.475x10^6$ cells.

The percentage of dead cells can be calculated in 2 ways:

A) 3 cells out of the total number of 33 are 9.1% dead, i.e. the cell suspension contains 90.9% live cells and 9.1% dead cells

Control questions:

Tell about the main purpose of the

hemocytometer.Technical characteristics

of the Goryaev chamber.

How is the Goryaev chamber prepared for cell counting?

How is the cell suspension prepared for counting?

Determination of the percentage of

viable cells.What is the standard

operating procedure?

Prepare a protocol for determining cell concentration using the Goryaev chamberafter staining with trypan blue.

Practical task. Calculate the concentration of cells in several parallels (at least 2), and determine the average value and the standard deviation.Present the results in the form of a graph. Determine the correlation coefficient by Student's criterion in pairs between researchers who counted cells according to Table 9.

Activity 4.

MTT-colorimetric method of estimating the number of living cells by the activity of mitochondrial dehydrogenases of cells

The MTT test is used to evaluate the proliferative parameters of cultured cells, the activity of their mitochondrial dehydrogenases, as well as to determine the cytotoxic/cytostatic effect [Alley M.C. et al., 1988; Mosmann T., 1983]. The basis of the method is the ability of mitochondrial enzymes of a living cell to transform 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) - a yellow salt into a purple crystalline MTT-formazan. In order to use this method of assessing the proliferative parameters of each studied cell line, control determinations of dehydrogenase activity are carried out. To do this, cells are planted in successively decreasing concentrations determined by counting cells with trypan blue and cultivated for a certain period of time. At all stages of cultivation, a parallel routine cell count is performed using trypan blue, and optical indicators are determined when water-soluble (almost colorless, but yellowish in stock concentrations) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl -2-tetrazolium bromide, which is added to cultured cells and is transformed by dehydrogenases of living cells into purple formazan, which crystallizes inside the cell (Fig. 13). As shown in the photo, the color can be from light purple to pink.

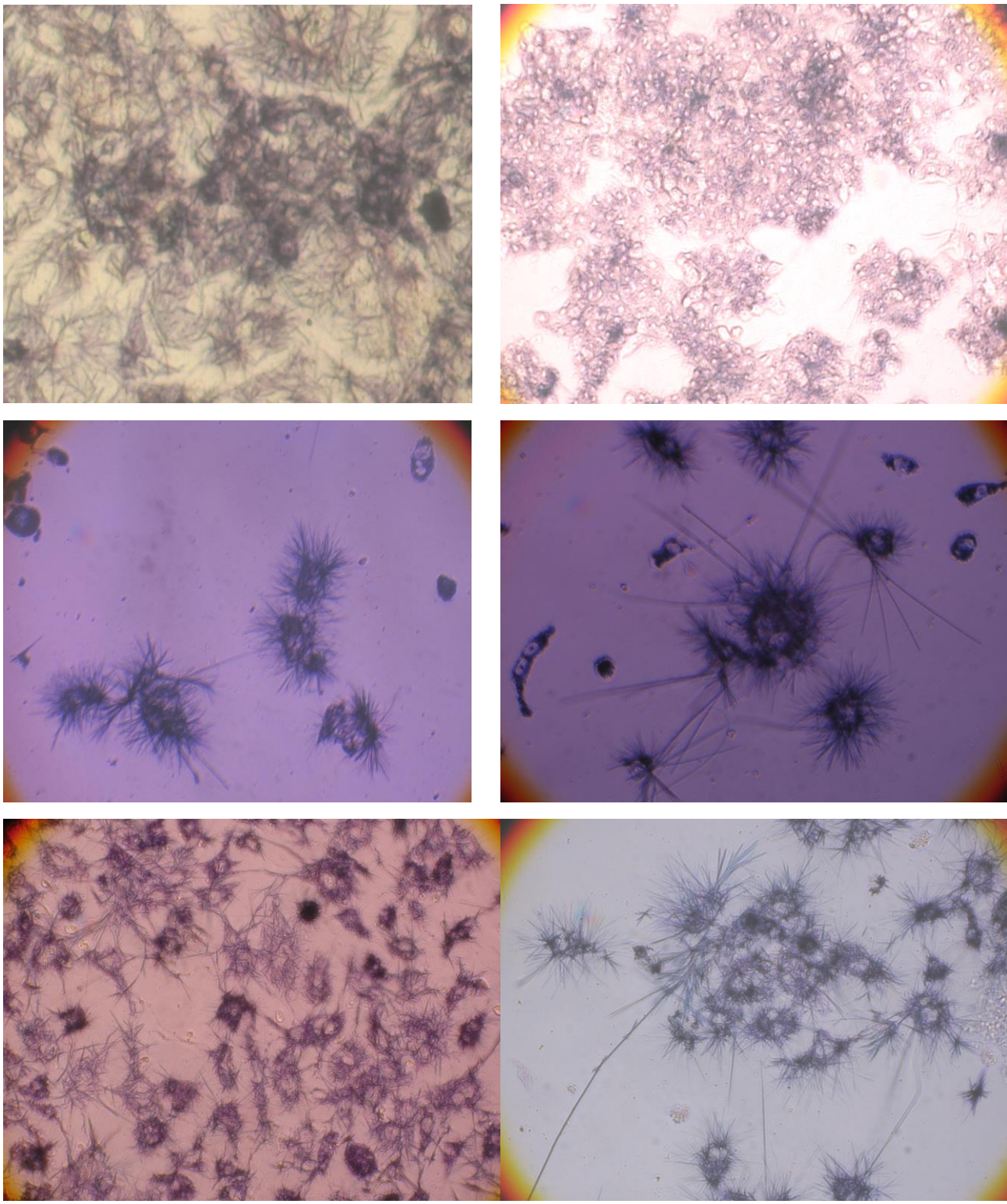


Fig. 13. Incorporation of water-soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-tetrazolium bromide into cells and its transformation into formazan crystals.

The transfer of formazan into a solution using suitable organic solvents, such as dimethylsulfoxide (DMSO) or isopropanol, and subsequent photometry

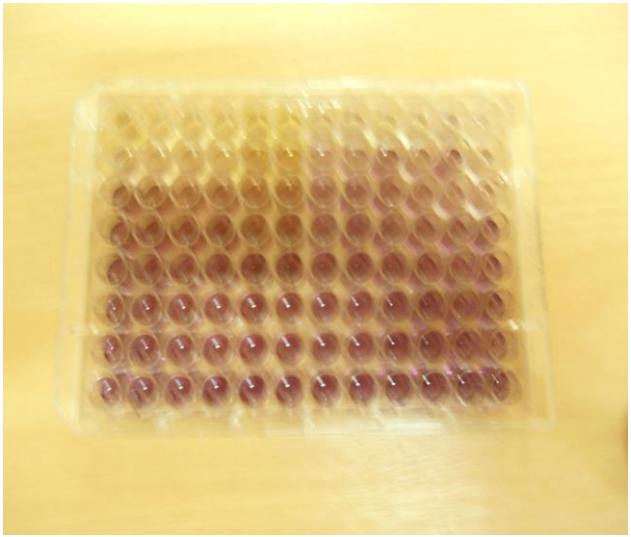
allow to accurately compare the change in optical density of the solution relative to the control with the change in the number of viable cells, and in cytotoxic studies to assess specific cell death that induced by one or another cytotoxic agent. The recovery of formazan is affected by the concentration of the dye in the incubation medium, and the optimal concentration varies widely for different cell lines, and the absorption spectrum of formazan depends on the pH and density of the cell suspension. Thus, in a suspension of higher density and with a critically low concentration of cells in the well, errors occur that lead to an overestimation of the results of chemosensitivity [Cherepovych et al., 2006]. Therefore, when using the MTT test on a large scale, for example, for screening potential therapeutic agents, or determining the cytotoxic effect, it is advisable, working with a certain culture, to determine the optimal concentration of cells in the control, at which there is no change in spectrophotometric indicators associated with large errors in parallel studies and, accordingly, not the reliability of the obtained results. In addition, it is known that dehydrogenase activity in living cells depends on the phase of the cell cycle. Thus, in the state of "proliferative rest" (G0), activation of catabolism enzymes was noted: phenylalanine hydroxylase, tyrosine, pyridine nucleosidase, lactic acid dehydrogenase, tricarboxylic acid cycle dehydrogenase, acid phosphatase, ATPase, and serine protease. Cells at rest are characterized by a significant decrease in respiratory activity. The rate of protein turnover in resting cells is generally more intense than in proliferating cells, although there is specificity for individual proteins and cell type. The main reason for accelerated protein renewal against the background of reduced synthesis is accelerated protein degradation.

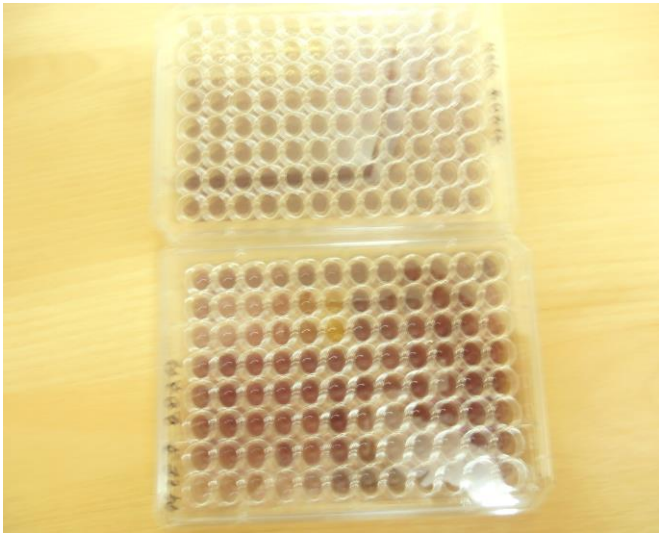
Therefore, in order to determine the number of living cells associated with the activity of mitochondrial dehydrogenases, 3-4 hours before the end of the cell incubation period, a pre-prepared 10-fold solution of MTT (Sigma, USA) is added to the wells of a 96-well plate (20 μl per 200 μl of incubation medium at the rate of 5 mg/ml phosphate-salt buffer) and incubated under the

same conditions for 3-4 hours. After centrifugation of the tablet (1500 g, 5 min) in a centrifuge equipped with a special holder, the supernatant is removed from each well using a semi-automatic aspirator or pipette. After that, 100 µl of dimethyl sulfoxide (Serva, Czech Republic) is added to each well to dissolve the formazan crystals (Fig. 6). The tablet is placed for mixing on a shaker with slow rotation for 10-15 minutes. Also, to increase the intensity of staining and accelerate the dissolution of formazan crystals, 10 µl of 0.25 M glycine is added to the wells at the rate of 100 µl of DMSO. The value of the optical absorption of the solution is measured using a multiwell spectrophotometer at a wavelength of 540-570 nm, with a reference wavelength of 620 nm (Fig. 14). The measurements are repeated 2-3 times, the obtained results are averaged (Fig.)

So, the MTT test allows you to detect a minimum of viable cells in one well of a standard 96-well culture plate.

The most important advantage of the MTT method is its speed and safety in comparison with classical methods of assessing the proliferative indicators of cells using radioisotope methods. The MTT method is recognized as promising for the study of individual chemosensitivity, although it requires technical improvement.





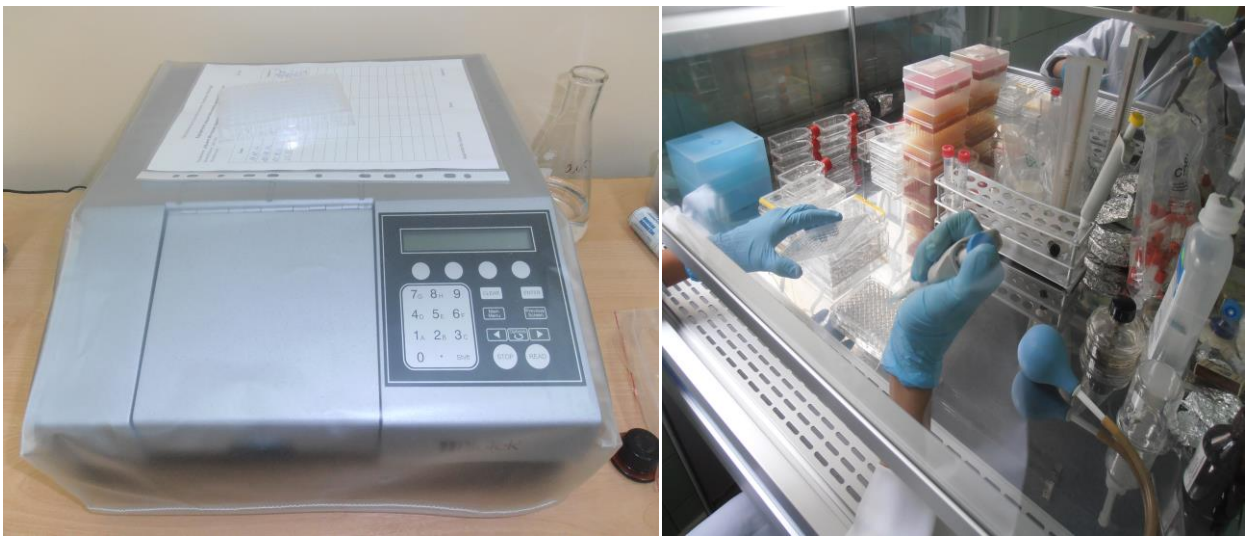


Fig. 14 Multiwell spectrophotometer ("reader") for automatic determination of colorimetric/spectrophotometric indicators in 96-well tablets. Addition of MTT to 96-well plates and staining after dissolution of formazan crystals

For example, when cells are seeded at different initial concentrations to construct a growth curve, the first step is to determine the optical absorption at at least three time points (the most optimal is 6 hours, which is considered "0" (2 hours of adaptation period after cell seeding and 4 hours of incubation with MTT is the minimum time for the inclusion of the dye in living cells). Also, depending on the known characteristics of cell population doubling (if we are talking about a certified cell line with known growth parameters), two or three more time points of cell incubation are chosen to construct a characteristic curve. In the given example , we are talking about an adhesive cell line with a doubling time of about 24 hours.

Table.10

The number of living endothelial cells (in extinction units) depending on their initial density and incubation period when cultured in complete nutrient medium (DMEM, 10% ETC)

Concentration of cells/ml ($x10^3$)	6 hour	24 hour	48 hour
	M±m	M±m	M±m
2,5		0,0235±0,0015	0,027±0
5	0,027±0,001	0,042±0,008	0,0685±0,0045
10	0,0385±0,0075	0,061±0,007	0,1095±0,0055
20	0,0535±0,0005	0,0915±0,0195	0,1455±0,0115
25	0,068±0,004	0,1315±0,0225	0,1925±0,0045
50	0,1005±0,0005	0,1395±0,0105	0,231±0,016
75	0,111±0,002	0,1765±0,0295	0,2975±0,0375
100	0,141±0,006	0,1725±0,0095	0,319±0,012
125	0,177±0,01	0,253±0,02	0,3155±0,0385
250	0,234±0,007	0,269±0,013	0,307±0
375	0,3475±0,0035	0,374±0,058	0,4585±0,0495
750	0,7265±0,0365	0,505±0,029	0,498±0,006

The course of work to determine the activity of mitochondrial dehydrogenases in the MTT test according to the standard operating procedure (SOP)

1. ***Appointment***

Study of the number of living cells, proliferative activity and activity of dehydrogenases.

2. ***Distribution***

Study of cytotoxic, mitogenic effect of substances.

3. ***Purpose***

consists in determining the parameters of colorimetric absorption in the wells at different concentrations of cells and at different times of their incubation

4. ***Object of research***

Cell line, or primary culture.

5. Materials and equipment

5.1. Reagents

• RPMI-1640 solution (Igla medium, 199);

• Blood serum of bovine embryos;

• Gentamicin;

• Physiological (0.9%) solution of sodium chloride (NaCl);

• Dimethyl sulfoxide (DMSO);

• Dye 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT);

• Ethyl alcohol 96%;

5.2. Laboratory dishes

• Camera Horyaev;

• Centrifuge tubes;

5.3. Equipment

• Medical centrifuge;

• pH meter

• Dispenser with an adjustable volume of 200-1000 μl;

• Dispenser with adjustable volume, 20 - 200 μl;

• Tablet spectrophotometer,

• CO2 incubator.

5.4. Materials.

• Tips for the dispenser, 200-1000 μl

• Tips for a dispenser, 50-300 μl

• Filter, sterile with a pore diameter of 0.22 μm, "Millipor".

• Marker.

• 96-hole flat-bottom tablet;

• Filter paper, Whatman No. 1;

• Tweezers;

• Scissors;

6. Preparation of solutions

• 100 mg of MTT is dissolved in 20 ml of phosphate-salt buffer, pH 7.2. Get a stock MTT solution (with a concentration of 5 mg/ml). Filter through filter paper, titrate in aliquots of 1-2 ml, store at -20 0C in a container protected from light.

• Buffered physiological solution (BSP), pH 7.4: BSP is prepared in deionized water based on:

7. Preparation of samples for analysis

• Obtain a cell suspension and dissolve it in complete RPMI-1640 culture medium at a concentration of 2×10^6/ml,

8. Research protocol.

• 100 μl of cell suspension at a concentration of 2 x 10^6/ml are introduced into

the wells of a flat-bottomed 96-well plate in 3 parallels of each sample and sequential titration of cells in decreasing concentrations is carried out. At the same time, spread the cells in the same concentrations for cell counting after staining with trypan blue

• Cells are incubated for 6, 24 and 48 hours at 370C in a CO2 incubator.

• 4 hours before the end of the incubation period, 10 μl of MTT solution is added **to** the cells incubated in 100 μl and incubated under the same conditions.

• After fixation of the reagent by living cells and its transformation into formazan crystals, the tablet is centrifuged in a special stand (tablet holder) at 1000g for 10 minutes, the supernatant is collected.

• Add 100 μl of DMSO to the stained cells and incubate for 15 minutes at 370C.

• After dissolving the dye in DMSO, measure the optical density (E) of the samples in the wells using a multiwell spectrophotometer at a wavelength of 570 nm.

• The obtained results are expressed in the form of growth curves, indicating the initial concentration of seeded cells, or the dependence of optical absorption of cells in colorimetric determination in a certain time interval depending on the concentration of cells in the well.

9. Presentation of results

• Research results are presented in the form of a report, which includes information on the data of mathematical and statistical analysis, a photomicrograph and a full description of the performed procedure.

10. Accompanying documents and files.

• In the accompanying documents, an electronic version of the report and a printed version with a mandatory graphic representation of the obtained results should be provided (the student’s report is given as an example)

11.Literature.

- Colangelo D., Guo H.Y., Connors K.M., Silvestro L., Hoffman R.M. Noncolorimetric measurement of cell activity in three-dimensional histoculture using the tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide: the pixel image analysis of formazan crystals // Anal Biochem. – 1992. – Vol. 205(1). - P. 8-13

- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assayas. J Immunol Methods 1983: 65: 55-63.

Conclusion: So, the MTT test is an indirect method of determining the proliferative indicators associated with the activity of dehydrogenases of living cells. In some cases, this method may raise questions about the artifactuality of the obtained data. However, it has a number of advantages that are not available for the traditional method of direct counting in the Goryaev chamber. In particular, the MTT test detects cells at dilutions that are unattainable by direct counting, although the results obtained are also somewhat questionable. The next advantage of the MTT test is the speed and volume of the measurement, because the direct method requires careful counting under a microscope, and the MTT test uses only optical indicators to determine the amount, which can be quickly calculated using special screening equipment.

Control questions:

1. What is the essence of the MTT-colorimetric method of research?

2. In which phase of the cell cycle is the activity of dehydrogenases the highest?

3. What parameters affect the recovery of formazan?

4. What color does 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-tetrazolium bromide have in aqueous solution and in formazan crystals?

5. What solvents are used to dissolve formazan crystals?

6. At what wavelength is extinction measured in the MTT test?

7. We recall what the standard operating procedure is.

8. List the equipment needed for colorimetric determination of cell viability in the MTT test.

9. How is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-tetrazolium bromide prepared, stored and used?

10. Perform calculations for 50 ml of single, 10-fold and 3.5-fold phosphate-salt buffer.

11. Present the sequence of research according to the protocol.

12. Draw a diagram of the experiment.

13. What conclusions can be drawn regarding the determination of proliferative indicators in the MTT test?

14. Carry out processing of the received data, statistical analysis by group and compare the data of routine counting in the Goryaev chamber and in the MTT test

Activity 5

Construction of a growth curve for cells of a permanent cell line .

Cell culture is primarily influenced by the microenvironment. The acceptability and reliability of using cultured cells as a model for studying physiological functions in vivo is constantly subject to criticism. Often, due to the adaptation of cultured cells to artificially created culture conditions, the latter do not show the phenotype they have in vivo.

As a result of the lack of cellular heterogeneity and three-dimensional architecture of tissues in cells in culture, intercellular and cell-matrix interactions are reduced. The culture lacks most of the humoral factors, and as a result, in the artificial microenvironment, non-specialized progenitor cells that spread, migrate and proliferate are favored. The influence of the microenvironment on culture occurs in the following directions:

1) the nature of the substrate on which the culture grows (dense such as plastic or other semi-liquid – collagen, gel, suspension);

2) degree of contact with other cells;

3) physico-chemical and physiological composition of the environment;

4) composition of the gas phase;

5) incubation temperature.

Provision of an appropriate microenvironment, including adhesion to the substrate, concentration of nutrients, hormones or growth factors, as well as intercellular interaction are the main conditions for the manifestation of specialized cell functions (Alberts et al., 2002). Most cells from dense tissues grow as an attached monolayer unless transformed to become substrate-independent. Therefore, after tissue disaggregation or subculture, cells need to attach to the substrate before division begins. Cell adhesion is mediated by specific cell surface receptors for extracellular matrix molecules. Therefore, often during the cultivation of cells, plastic is covered with molecules of the extracellular matrix. It was shown

that the following main classes of transmembrane proteins are involved in the processes of intercellular and cell-matrix adhesion: Ca2+-independent (cell adhesion molecules, CAM) and Ca2+-dependent cadherins are mainly involved between homologous cells. Two identical molecules of such a protein interact with each other [Rosenman & Galatin, 1991; Alberts et al., 2002, Cavallaro & Christofori, 2004].

Cell entry into the cell cycle is regulated by signals from the microenvironment. The low density of cells leads to the fact that, without close contacts, the cell enters an active state and divides. Conversely, at a high cell density, the proliferation of normal cells is inhibited and the division of transformed cells is not inhibited. Inhibition of proliferation is initiated by cell contacts and enhanced by cell crowding as a result of cell shape changes. as well as limitation of distribution on the substrate. Intracellular control is mediated by cyclins and cyclin-dependent kinases [Planas-Sliva & Weinberg, 1997; Reed, 2003], which are affected by signaling cascades activated by phosphorylation of the intracellular domains of receptors upon their binding to growth factors. Negatively acting factors, such as p53 proteins [Sager, 1992; Mcllwrath et al., 1994], pl6 [Russo et al., 1998] or Rb-gene expression products [Sager, 1992], stop the passage of the cycle at restriction points, or control points of the cycle. Communication between extracellular elements of cell cycle control (both positive, for example PDGF, and negative, for example TGF-β) and intracellular effectors is mediated by cell membrane receptors and biochemical signaling pathways, primarily those involving protein phosphorylation and the participation of secondary messengers such as cAMP, Ca2+ and diacylglycerol [Alberts et al., 2002].

That is why an important stage in the study of any population of cells disintegrated from the tissue, or already transferred into culture, is the determination of optimal conditions for their cultivation. The first step in studying any cell population (a line of genetically identical cells provided by the Cell Culture Bank or a primary culture explanted from a multicellular organism) is the construction of a growth curve.

Cell population growth curve

It is known from the literature that the development of any cell population has a phase character (Fig. 15). The growth of cell populations in culture is described by a curve [Adams R, 1983, Freshny, 2009], on which a number of typical areas can be distinguished: at the initial stage, cells do not grow, their number and biomass do not change - this is the so-called adaptive period, or lag phase (Fig. 1, I). During this period, the incubation medium is adapted to a specific cell population (conditioning). The duration of the lag phase is determined by the optimal concentration of planted cells, cultivation conditions and the composition of the cell incubation medium. After that, the cells enter the phase of exponential growth (II), when the majority of the cell subpopulation is involved in intensive proliferation, and doubles after a certain period of time characteristic of this population of cells.

In the logarithmic phase of cell population growth in the case of using a genetically identical cell line, when most cells synchronously enter the cycle phases, conditions are selected to determine the impact on the metabolism of proliferating cells. In particular, the cytotoxic/cytostatic effect of potential biologically active substances is investigated.

The exponential growth phase of the cell population is followed by the slow growth phase (III). During this period, the number of cells participating in mitosis gradually decreases, and when the resources of the microenvironment and substrate area are exhausted, as a result of contact inhibition, the growth of the cell population enters the plateau or stationary phase (IY).

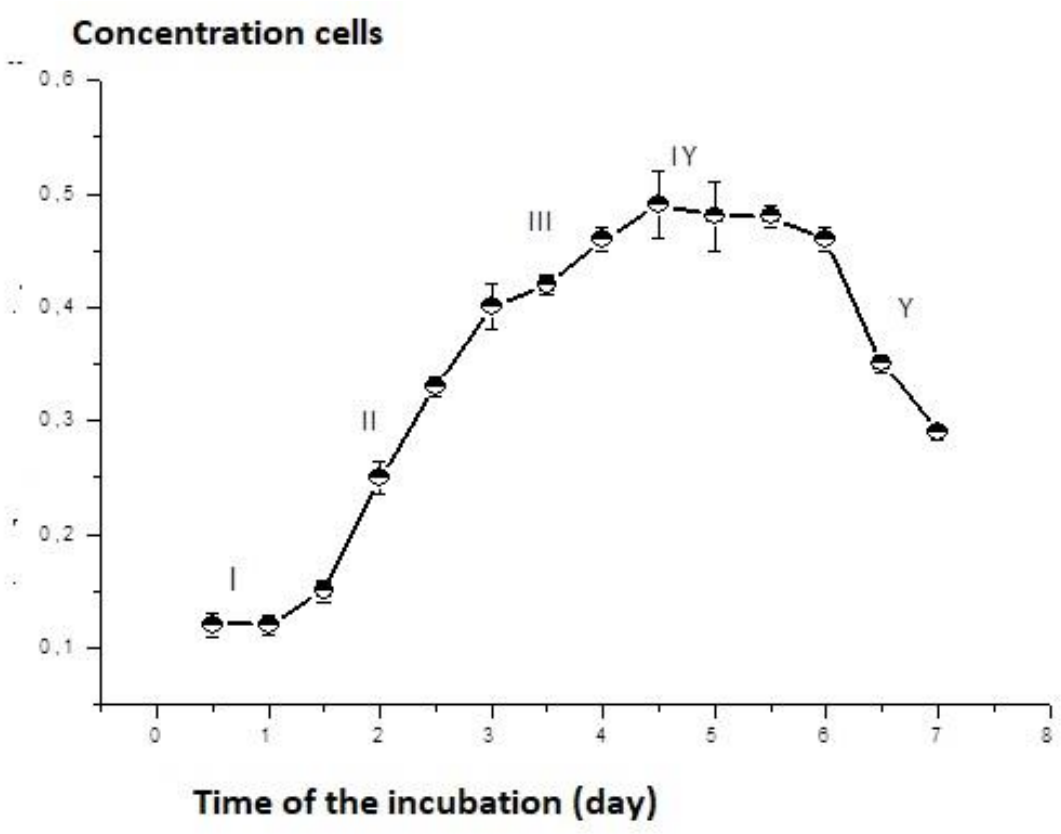


Fig. 15. Characteristic growth curve of the cell population in culture: I-lag phase; II-logarithmic phase; III-phase of slow growth; IY-stationary phase of growth; Y-phase of degradation

In this phase, the number of cells and their biomass almost does not change. If we extrapolate the phase of stationary growth to the phase of the cycle, we can assert the beginning of the period of "proliferative rest" for this population, which for most cells in the subpopulation is expressed in the G0/G1 transition. Transition of cells from this phase to proliferation is possible only with exogenous stimulation.

Then, when the resources of the environment are completely exhausted, there is a phase of cell population degradation (Y), during which most of the cells die. However, if the population of transformed cells is considered, then in this phase, under conditions of nutrient substrate deficiency, a subpopulation of cells with high clonogenic potential can be selected. Therefore, to study the influence of biologically active agents, it is reasonable to model the growth phases of cell lines, which are represented by genetically identical cells and, accordingly, under the

same conditions, the microenvironment can be synchronized in a certain phase of growth, which is the basis for determining cytotoxic/pro-proliferative, about -/anti-apoptotic effect on the cell subpopulation, as well as the ability of the cell population to differentiate in different directions (especially when it comes to stem cells) and their clonogenic potential.

Thus, to construct a growth curve, cell cultivation is carried out for at least 5-10 days (sometimes up to 2 months of cultivation is required) and growth parameters for this population are determined at all stages of cultivation.

1. The most widely used method is cell counting using the vital dye trypan blue, which is incorporated into dead cells.

2. Today, methods of colorimetric visualization of cells based on the inclusion of vital dyes by mitochondrial dehydrogenases are also widely used.

3. Cytofluorimetric analysis is also an important step, which allows, by including intercalating dyes in DNA, to determine both the level of apoptotic cells and the distribution of cells by cell cycle phases.

4. The radioisotope method, which is based on the inclusion of H3-thymidine in DNA during its replication. Today, these methods are given a secondary role due to various considerations, first of all, it is related to occupational safety and admission according to the first list of experimental works dangerous to human health.

The most widely used today is the MTT colorimetric method, which is based on the ability of mitochondrial enzymes of a living cell to reduce 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, Sigma) is a yellow salt in purple crystalline MTT formazan. However, the activity of dehydrogenases at different phases of the cell cycle is different, so this method also has certain limitations. Actually, the complex use of three of the four methods proposed above can be used to construct a growth curve at different stages of cell population development.

Construction of growth curve for primary culture and cell line

Thus, if a growth curve is required, the first step to be taken is to perform a routine cell count. using hemocytometers. The most common routine hemocytometer is the Goryaev chamber.

For this, cells were seeded in 24-well (in a volume of 1 ml) or 12-well (in a volume of 2 ml) plates with a concentration of 3.5-5.0x104 cells/ml and cultivated for different periods of time. At each stage, cells are counted, viability and concentration are determined (Table 11).

For example, the table shows the growth of the population of endothelial cells (a mouse endothelial cell line) and the primary culture of rat intestinal cells explanted by the trypsinization method. Both cultures were incubated for 8 days without changing the medium, in order to determine all stages of population growth.

Table 11

The concentration of endothelial cells during long-term cultivation: counting using a Goryaev camera after staining the cells with trypan blue

Time incubation cells	***«0»***	***12 hours***	***24 hours***	***36 hours***	***48 год***	***72 hours***
MAEC (alive)	3,5 x10^4	4,2 x10^4	7,3x10^4	9,8 x10^4	14,1 x10^4	18,2 x10^4
MAEC (dead)	1,7 x10^3	2,2 x10^3	2,8 x10^3	5,5 x10^3	6,7 x10^3	1,1 x10^4
% dead	4,6 %	4,9%	3,7%	5,3%	4,5%	5,7%
Time incubation cells	96 ***hours***	120 ***hours***	144 ***hours***	168 ***hours***	192 ***hours***	

MAEC (alive)	17,4 x104	16,4 x104	14,2 x104	11,1 x104	7,5 x104	
MAEC (dead)	1,3 x104	1,4 x104	2,2 x104	5,8 x104	8,4 x104	
% alive	7,2%	7,9%	13,4%	34,3%	52,8%	

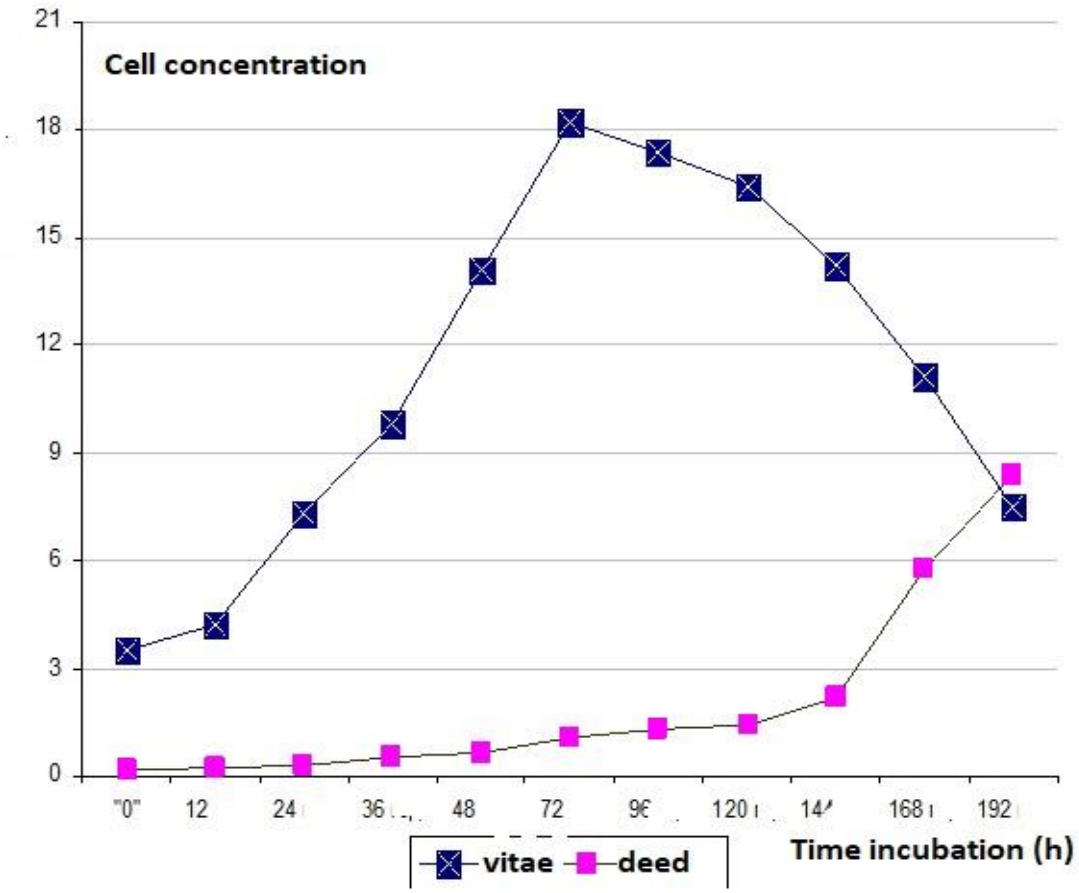


Fig. 16. Growth curve of endothelial cells during long-term cultivation without changing the medium.

As you can see, from the given data, the growth of endothelial cells is described by a characteristic curve, from which it can be seen that the doubling time is approximately 22±2 hours, which is indicated in the certificate of these

cells. However, without changing the medium, there is a gradual death of the cell population and an increase in the subpopulation of necrotic cells, and on the 8th day of cultivation, the concentration of the dead reaches about 50%.

Practical task:

Sow cells in successively decreasing concentrations with "step" 2 according to Table 12.

A) suspension culture (initial concentration $1x10^6$ cells/ml)

B) adhesive culture (initial concentration $2.5x10^5$ cells/ml)

Table 12

Cultivation scheme

Dilution (cells/ml)	1	1:2	1:4	1:8	1:16	1:32
Suspension culture (theoretical dilution)	$1x10^6$	$5x10^5$	$2,5x10^5$	$1,25x10^5$	$6,2x10^4$	$3,1x10^4$
M1	X1	X1:2	X1:4	X1:8	X1:16	X1:32
M2	X2	X2:2	X2:4	X2:8	X2:16	X2:32
M3	X3	X3:2	X3:4	X3:8	X3:16	X3:32
M±m	Σ (Xn/n) ±m					
Adhesive culture (theoretical dilution)	$2,5x10^5$	$1,25x10^5$	$6,2x10^4$	$3,1x10^4$	$1,6x10^4$	$8x10^3$
M1	X1	X1:2	X1:4	X1:8	X1:16	X1:32
M2	X2	X2:2	X2:4	X2:8	X2:16	X2:32
M3	X3	X3:2	X3:4	X3:8	X3:16	X3:32
M±m	Σ (Xn/n) ±m					

Carry out cell counting and correlation analysis between theoretical values and practical counting. Carry out statistical processing of the results. Present the results in the form of a table and graphs.

Literature

1. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. & Walter, P. (2002), Molecular biology of the cell, 4th ed, New York. Garland.
2. Cavallaro, U. & Christofori, G. (2004). Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. Natl Rev. Cancer 4:118-132.
3. Mcllwrath, A., Vasey, P., Ross, G. & Brown, R. (1994). Cell cycle arrests and radiosensitivity of human tumour cell lines:Dependence on wild-type p53 for radiosensitivity. Cancer Res.54:3718-3722.
4. Planas-Silva, M. D. & Weinberg, R. A. (1997). The restriction point and control of cell proliferation. Curr. Opin. Cell Biol.9:768-772.
5. Sager, R. (1992). Tumor suppressor genes in the cell cycle. Curr.Opin. Cell Biol 4:155-160.
6. Russo, A. A., Tong, L., Lee, J. O., Jeffrey, P. D. & Pavletich, N. P.(1998). Structural basis for inhibition of the cyclin-dependent kinase cdk6 by the tumour suppressor pl61NK4a. Nature 395: 237-243.
7. Rosenman, S. J. & Gallatin, W. M. (1992). Cell surface glycoconjugates in intercellular and cell-substratum interactions. Semin. Cancer Biol. 2:3 57-366.
8. Reed, S. I. Ratchets and clocks: the cell cycle, ubiquitylation and protein turnover. Nat. Rev. Mol. Cell Biol.-2003.- 4(11):855-864.

Control questions:

1. Name the differences between cells in a culture and in a multicellular organism.

2. The main phases of cell population growth in culture.

3. What factors are necessary for cell division?

4. What are the properties of growth factors (mitogens)?

5. What stages of exogenous stimulation of cell proliferation do you know?

6. Principles of building a growth curve for culture cells.

7. Remember how we count cells after staining with trypan blue.

8. Specify the technical characteristics of Goryaev's camera (From the previous section)

9. Principles of cell counting at high concentrations and in liquid culture.

10. What is a cell line and primary culture?

11. What is contact braking? How does this manifest itself in the cultivation of normal and transformed cells?

12. In your opinion, why is the growth of primary culture cells and cell line different?

13. Conduct a comparative analysis of cell counts by group with the same initial concentrations of seeded cells.

14. Explain how data is calculated?

15. What is the minimum number of parallel studies allowed in order to have statistically significant results?