of nitrogen by leaky ampoules, which will then explode violently on thawing. There is also a greater likelihood of transfer of contamination between ampoules and the buildup of contamination from outside, carried in when material is introduced and concentrated by the constant evaporation of the liquid nitrogen in the tank. With the introduction of improved insulation and reduced evaporation, vapor-phase storage is preferable. It also eliminates the risk of splashing when the liquid nitrogen boils when something is inserted and reduces evaporation of nitrogen into the room air. There is, however, a gradient in the temperature from the surface of the liquid nitrogen up to the neck of approximately 80°C, from −190°C to around −110°C in gas phase storage [Rowley & Byrne, 1992], although the design and composition of the racking system may help eliminate this gradient.

Some freezers have the liquid nitrogen located within the wall of the freezer and not in the storage compartment. It is replenished by an automatic feed with high and low level controls (Fig. 19.7*d*), and evaporated nitrogen is released via a relief valve. This has the advantages of gas-phase storage, with the added advantage of a lower consumption of liquid nitrogen and elimination of the temperature gradient. However, the nitrogen level is not visible and cannot be measured by a dipstick, so complete reliance has to be made on electronic monitoring (*see* below). In addition any blockage of nitrogen flow within the freezer wall can be very difficult or even impossible to eliminate, so it is essential that the liquid nitrogen be filtered and that steps be taken to ensure that no water, or water vapor, enters the system, as ice can also block nitrogen flow.

Δ ***Safety Note.*** Biohazardous material *must* not be stored in the liquid phase and teaching and demonstrating should also not be done with liquid-phase storage. Above all, if liquid-phase storage is used, the user must be made aware of the explosion hazard of both glass and plastic and must wear a face shield or goggles.

Monitoring and replenishing liquid nitrogen. The investment in the contents of a nitrogen freezer can be considerable and must be protected by a strict monitoring regime and electronic liquid level alarms. When the nitrogen is in the storage compartment, the level should be monitored at least once per week with a dipstick, and the level recorded on a chart. This should be done even if automatic filling is employed, as these systems can fail. Where the liquid nitrogen is totally enclosed, refilling is automatic based on level controls in the wall of the freezer but must still be backed up, preferably with two independent temperature recorders, both of which should sound an alarm, one if the temperature rises above −170°C and one above −150°C.

If liquid nitrogen storage is not available, the cells may be stored in a conventional freezer. The temperature in this freezer should be as low as possible; little deterioration has been found at −196°C [Green et al., 1967], but significant deterioration (5–10% per annum) may occur at −70°C.

19.3.7 Freezing Cultured Cells

The following protocol is based on the optimal conditions for most cultured cell lines but variations in the freezing rate or preservative may be required for some cells.

PROTOCOL 19.1. FREEZING CELLS

Outline

Grow the culture to late log phase, prepare a high concentration cell suspension in medium with a cryoprotectant, aliquot into ampoules, and freeze slowly (*see* Fig. 19.8).

Materials

Sterile or aseptically prepared:

- ❑ Culture to be frozen
- ❑ If monolayer: D-PBSA and 0.25% crude trypsin
- ❑ Growth medium (serum improves survival of the cells after freezing; up to 50%, or even pure, serum has been used. If serum is being used with serum-free cultures, it should be washed off after thawing)
- ❑ Cryoprotectant, free of impurities (*see* above): DMSO in a glass or polypropylene vial, or glycerol, fresh, and in a universal container
- ❑ Syringe, 1 to 5 mL, for dispensing glycerol if used (because it is viscous)
- ❑ Plastic ampoules, 1.2 mL, prelabeled with the cell line designation and the date of freezing

Nonsterile:

- ❑ Hemocytometer or electronic cell counter
- ❑ Canes or racks for storage (racks may already be in place in the freezer)
- ❑ Insulated container for freezing: polystyrene box lined with cotton wool or plastic foam insulation tube (*see* Fig. 19.2) or controlled rate freezer if available (*see* Fig. 19.5)
- ❑ Protective gloves, nitrile

Procedure

1. Make sure the culture satisfies the criteria for freezing (*see* Table 19.1), and check by eye and on microscope for:
   - **(a)** Healthy appearance (*see* Section 12.4.1).
   - **(b)** Morphological characteristics (*see* Section 15.5.1).
   - **(c)** Phase of growth cycle (should be late log phase before entering plateau (*see* Section 12.4.3).
   - **(d)** Freedom from contamination (*see* Section 18.3).
2. Grow the culture up to the late log phase, and if you are using a monolayer, trypsinize and count

the cells (*see* Protocol 13.2). If you are using a suspension, count and centrifuge the cells (*see* Protocol 12.3).

3. Resuspend at 2×10^6 to 2×10^7 cells/mL.
4. Dilute one of the cryoprotectants in growth medium to make freezing medium:
   (a) Add dimethyl sulfoxide (DMSO) to between 10% and 20%

   Δ ***Safety Note.*** DMSO can penetrate many synthetic and natural membranes, including *skin* and rubber gloves [Horita & Weber, 1964]. Consequently any potentially harmful substances in regular use (e.g., carcinogens) may well be carried into the circulation through the skin and even through rubber gloves. DMSO should always be handled with caution, particularly in the presence of any toxic substances.

   or
   (b) Add glycerol to between 20% and 30%.
5. Dilute the cell suspension 1:1 with freezing medium to give approximately 1×10^6 to 1×10^7 cells/mL and 5% to 10% DMSO (or 10–15% glycerol). It is not necessary to place ampoules on ice in an attempt to minimize deterioration of the cells. A delay of up to 30 min at room temperature is not harmful when using DMSO and is beneficial when using glycerol.
6. Dispense the cell suspensions into prelabeled ampoules, and cap the ampoules with sufficient torsion to seal the ampoule without distorting the gasket.
7. Place the ampoules on canes for canister storage (*see* Figs. 19.2, 19.6*a*, *b*, 19.7*a*), or leave them loose for drawer storage (*see* Figs. 19.6*c*, *d*, 19.7*b*–*d*).
8. Freeze the ampoules at 1°C/min by one of the methods described above (*see* Section 19.3.4). With the insulated container methods, this will take a minimum of 4 to 6 h after placing them at −70°C if starting from a 20°C ambient temperature (*see* Fig. 19.1), but preferably leave the ampoules in the container at −70°C overnight.
9. When the ampoules have reached −70°C or lower, check the freezer record before removing the ampoules from the −70°C freezer or controlled rate freezer, and identify a suitable location for the ampoules.
10. Transfer the ampoules to the liquid N_2 freezer, preferably not submerged in the liquid, placing the cane and tube into the predetermined canister or individual ampoules into the correct spaces in the predetermined drawer. This transfer must be done quickly (<2 min), as the ampoules will reheat at about 10°C/min, and the cells will deteriorate rapidly if the temperature rises above −50°C.

   Δ ***Safety Note.*** Protective gloves and a face mask should be used when placing ampoules in or near liquid nitrogen.

11. When the ampoules are safely located in the freezer, complete the appropriate entries in the freezer index (*see* Tables 10.2, 19.3).

19.3.8 Freezer Records

Records should provide (1) an inventory showing what is in each part of the freezer, (2) an indication of free storage spaces, and (3) a cell strain index, describing the cell line, its designation, its origin, maintenance details, and freezing procedures, what its special characteristics are, and where it is located. This record may be kept on a conventional card index, but a computerized database will give superior data storage and retrieval, and can also print labels. This type of data can be provided by separate tables within the same database used for the provenance of the cell line (Tables 19.2, 19.3; *see also* Sections 11.3.11, 12.7, 12.8). Material stored on disks or tape must have backup copies on disk or tape or must have a hard-copy printout.

Using a computerized database requires that the curator of the freezers manages this database. If entries are to be made by users, user stocks can have both read and write access, whereas seed stock and distribution stock should be accessible only to the curator. Alternatively, the whole file can be read only and updated by the curator from paper entries on cards or a logbook.

19.3.9 Thawing Stored Ampoules

When required, cells are thawed and reseeded at a relatively high concentration to optimize recovery. The ampoule should be thawed as rapidly as possible to minimize intracellular ice crystal growth during the warming process. This can be done in warm water, in a bucket, or in a water bath, but if the ampoule has been submerged in liquid nitrogen during storage, the warming bath must be covered in case the ampoule has leaked and inspired liquid nitrogen, when it will explode violently on warming (Fig. 19.9).

The cell suspension should be diluted slowly after thawing as rapid dilution reduces viability. This gradual process is particularly important with DMSO, with which sudden dilution can cause severe osmotic damage and reduce cell survival by half. Most cells do not require centrifugation, as replacing the medium the following day will suffice for a monolayer or dilution for a suspension. However, some cells (often suspension-growing cells) are more sensitive to cryoprotectants, particularly DMSO, and must be centrifuged after thawing but still need to be diluted slowly in medium first.

CHAPTER 21

Cytotoxicity

21.1 VIABILITY, TOXICITY, AND SURVIVAL

Once a cell is explanted from its normal in vivo environment, the question of viability, particularly in the course of experimental manipulations, becomes fundamental. Previous chapters have dealt with the status of the cultured material relative to the tissue of origin and how to quantify changes in growth and phenotypic expression. However, none of these data is acceptable unless the great majority of the cells are shown to be viable. Furthermore many experiments carried out in vitro are for the sole purpose of determining the potential cytotoxicity of compounds being studied, either because the compounds are being used as pharmaceuticals or cosmetics and must be shown to be nontoxic or because they are designed as anticancer agents and cytotoxicity may be crucial to their action.

New drugs, cosmetics, food additives, and so on, go through extensive cytotoxicity testing before they are released for use by the public. This testing usually involves a large number of animal experiments, although in Europe these experiments will be subject to new legislation [Cox & Chrisochoidis, 2003], introduced in 2009 for topical application and in 2013 for systemic application. There is much pressure, both humane and economic, to perform at least part of cytotoxicity testing in vitro. The introduction of specialized cell lines and interactive organotypic cultures, and the continued use of long-established cultures, may make this a reasonable proposition.

Toxicity is a complex event in vivo, where there may be direct cellular damage, as with a cytotoxic anticancer drug, physiological effects, such as membrane transport in the kidney or neurotoxicity in the brain, inflammatory effects, both at the site of application and at other sites, and other systemic effects. Currently it is difficult to monitor systemic and physiological effects in vitro, so most assays determine effects at the cellular level. Definitions of cytotoxicity vary [Kroemer et al., 2009], depending on the nature of the study and whether cells are killed or phenotypically altered. In addition cells may die by necrosis (*see* Fig. 12.1), apoptosis (*see* Plate 17*c*, *d*), self-digestion (*autophagy*); may cease proliferation (*cytostasis*); and/or may become terminally differentiated (e.g., cornification) [Galluzi et al., 2009]. Whereas demonstrating efficacy in an anticancer agent assay may require a *cytocidal* effect (cell killing), demonstrating the lack of toxicity of other pharmaceuticals may require a more subtle analysis of specific targets such as an alteration in gene transcription, cell signaling, or cell–cell interaction including those effects that may give rise to an inflammatory or allergic response.

Most assays oversimplify the events that they measure and are employed because they are cheap, easily quantified, and reproducible. However, it has become increasingly apparent that they are inadequate for modern drug development, which requires greater emphasis on specific molecular targets and precise metabolic regulation. Gross tests of cytotoxicity are still required, but there is a growing need to supplement them with more subtle tests of metabolic pathway regulation and signaling. Perhaps the most obvious of these tests is the induction of an inflammatory or allergic response, which need not imply cytotoxicity of the allergen and is still one of the hardest results to demonstrate in vitro.

Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications, Sixth Edition, by R. Ian Freshney

The traditional approach to cytotoxicity has been to concentrate on cell growth or survival. Cell growth is generally taken to be the regenerative potential of cells, as measured by clonal growth (*see* Protocol 20.10), net change in population size (e.g., in a growth curve; *see* Protocols 20.7, 21.8), or a change in cell mass (total protein or DNA) or metabolic activity (e.g., DNA, RNA, or protein synthesis; MTT reduction). Other aspects will be considered later (*see* Section 21.6).

21.2 IN VITRO LIMITATIONS

It is important that any in vitro measurement can be interpreted in terms of the in vivo response of the same or similar cells, or at least that the differences that exist between in vitro and in vivo measurements are clearly understood.

21.2.1 Pharmacokinetics

The measurement of toxicity in vitro is generally a cellular event. For example, it would be very difficult to recreate the complex pharmacokinetics of drug exposure in vitro, and between in vitro and in vivo experiments there usually are significant differences in exposure time to and concentration of the drug, rate of change of the concentration, drug metabolism (activation and detoxification), tissue penetration, clearance, and excretion. Although it may be possible to simulate these parameters—for example, using multicellular tumor spheroids for drug penetration or timed perfusion to simulate concentration and time ($C \times T$) effects—most studies concentrate on a direct cellular response, thereby gaining simplicity and reproducibility.

21.2.2 Metabolism

Many nontoxic substances become toxic after being metabolized by the liver; in addition many substances that are toxic in vitro may be detoxified by liver enzymes. For in vitro testing to be accepted as an alternative to animal testing, it must be demonstrated that potential toxins reach the cells in vitro in the same form as they would in vivo. This proof may require additional processing by purified liver microsomal enzyme preparations [McGregor et al., 1988], coculture with activated hepatocytes [Guillouzo & Guguen-Guillouzo, 2008] (*see also* Appendix II: Hepatocytes), or hepatoma-derived cells such as Hep-G2 or HepaRG (*see* Section 22.2.8). Coculture may use transfilter 3-D culture or 2-D cellular microarrays [Khetani & Bhatia, 2008]. Genetic modification of the target cells with the introduction of genes for metabolizing enzymes under the control of a regulatable promoter [Macé et al., 1994] has also been used.

21.2.3 Tissue and Systemic Responses

The nature of the response must also be considered carefully. A toxic response in vitro may be measured by changes in cell survival (*see* Protocol 21.3) or metabolism (*see* Section 21.3.4), whereas the major problem in vivo may be a tissue response (e.g., an inflammatory reaction, fibrosis, kidney failure) or a systemic response (e.g., pyrexia, vascular dilatation). For in vitro testing to be more effective, models of these responses must be constructed, perhaps utilizing organotypic cultures reassembled from several different cell types and maintained in the appropriate hormonal milieu.

It should not be assumed that complex tissue and even systemic reactions cannot be simulated in vitro. Assays for inflammatory responses, teratogenic disorders, and neurological dysfunctions may be feasible in vitro, given the right tissue-engineered models and a proper understanding of cell–cell interaction and the interplay of endocrine hormones with local paracrine and autocrine factors.

21.3 NATURE OF THE ASSAY

The choice of assay will depend on the agent under study, the nature of the anticipated response, and the particular target cell. In vitro assays can be divided into five major classes:

(1) *Viability*. An immediate or short-term response, such as increased and uncontrolled membrane permeability or a perturbation of a particular metabolic pathway correlated with cell proliferation or survival.
(2) *Survival*. The long-term retention of self-renewal capacity (5–10 generations or more).
(3) *Metabolic*. Assays, usually microtitration based, of intermediate duration that can either measure a metabolic response (e.g., dehydrogenase activity; DNA, RNA, or protein synthesis) at the time of, or shortly after, exposure. Making the measurement two or three population doublings after exposure is more likely to reflect cell growth potential and may correlate with survival.
(4) *Genotoxicity and Transformation*. Survival in an altered state (e.g., one or more genetic mutations with resultant alterations in growth control or malignant transformation).
(5) *Irritancy*. A response analogous to inflammation, allergy, or irritation in vivo; as yet difficult to model in vitro, but may be possible to assay by monitoring cytokine release in organotypic cultures.

21.3.1 Viability

Viability assays are used primarily to measure the proportion of viable cells after a potentially traumatic procedure, such as primary disaggregation, cell separation, or cryopreservation, rather than to look at a long-term cytotoxic response.

Most viability tests rely on a breakdown in membrane integrity measured by the uptake of a dye to which the cell is normally impermeable, such as Trypan Blue, Erythrosin, Naphthalene Black (*see* Plate 17*a*), or propidium iodide (*see* Fig. 20.7), or the release of a dye normally taken up and retained by viable cells (e.g., diacetyl fluorescein or Neutral Red), or the release of lactate dehydrogenase by leaky cells

grown until they are quite large ($>1 \times 10^3$ cells), when the growth of larger colonies tends to slow down; smaller, but still viable, colonies tend to catch up with these larger colonies. For *colony sizing*, stain the cultures earlier, before the growth rate of larger colonies has slowed down, and score all of the colonies.

Solvents. Some agents to be tested have low solubilities in aqueous media, and it may be necessary to use an organic solvent to dissolve them. Ethanol, propylene glycol, and dimethyl sulfoxide have been used for this purpose, but may themselves be toxic to cells. Hence the minimum concentration of solvent should be used to obtain a solution. The agent may be made up at a high concentration in, for example, 100% ethanol, then diluted gradually with BSS and finally diluted into medium. The final concentration of solvent should be <0.5% and a *solvent control* must be included (i.e., a control with the same final concentration of solvent but without the agent being tested).

Take care when using organic solvents with plastics or rubber. It is better to use glass with undiluted solvents and to use plastic only when the solvent concentration is <10%.

Although calculating the plating efficiency is one of the best methods for testing cell survival rates, it should be remembered that plating efficiency only applies to the clonogenic component of the cell population, which may not be representative of the whole cell population. The question does not arise if controls plate with 100% efficiency; in practice, however, control plating efficiencies of 20% or less are more likely and the response is being measured in a subset of the total cell population.

21.3.3 Assays Based on Cell Proliferation

Cell counts after a few days in culture can also be used to determine the effect of various compounds on cell proliferation, but at least in the early stages of testing, a complete growth curve is required (*see* Protocols 20.7–21.9) because the interpretation of cell counts at a single point in time can be ambiguous (*see* Fig. 20.10, day 7). Growth curve analyses, using cell counting, are feasible only with relatively small numbers of samples, as they become cumbersome in a large screen, although automating growth curves in multiwell plates using image analysis (Incucyte; Chip-Man; *see* Section 20.9.3; Figs. 20.11, 20.12) can make this feasible.

In cases where there are many samples, a single point in time—such as the number of cells three to five days after exposure—can be used. The time should be selected as within the log phase, and preferably mid-log phase, of control cells. Any significant effect should be backed up with a complete growth curve over the whole growth cycle or by an alternative assay, such as a survival curve by clonogenic assay (*see* Protocol 21.3) or MTT assay (*see* Protocol 21.4).

21.3.4 Metabolic Cytotoxicity Assays

Plating efficiency tests are labor intensive and time-consuming to set up and analyze, particularly when a large number of samples is involved (although this can be automated; *see* Section 26.4), and the duration of each experiment may be anywhere from two to four weeks. Furthermore some cell lines have poor plating efficiencies, particularly freshly isolated normal cells, so a number of alternatives have been devised for assaying cells at higher densities (e.g., in microtitration plates; *see* Section 21.3.5). None of these tests measures survival directly, however. Instead, the net increase in the number of cells (i.e., the growth yield; *see* Section 21.3.3), the increase in the total amount of protein or DNA, or continued metabolic activity, such as the reduction of a tetrazolium salt (MTT or XTT) to formazan or the synthesis of protein or DNA, is determined. Survival in these cases is defined as the retention of metabolic or proliferative ability by the cell population as a whole some time after removal of the toxic influence. However, such assays cannot discriminate between a reduction in metabolic or proliferative activity per cell and a reduced number of cells, and therefore any novel or exceptional observation should be confirmed by clonogenic survival assay.

21.3.5 Microtitration Assays

The introduction of multiwell plates revolutionized the approach to replicate sampling in tissue culture. These plates are economical to use, lend themselves to automated handling, and can be of good optical quality. The most popular are 96-well microtitration plates or *microplates* (*see* Plate 17*b*), each well having 28 to 32 mm^2 of growth area, 0.1 or 0.2 mL medium, and up to 1×10^5 cells. Microtitration offers a method by which large numbers of samples may be handled simultaneously, but with relatively few cells per sample. With this method, the whole population is exposed to the agent, and viability is determined subsequently, usually by measuring a metabolic parameter such as the ATP or NADH/NADPH concentration. Assay kits are available (*see* Appendix II: Cytotoxicity Assays).

The end point of a microtitration assay is usually an estimate of the number of viable cells, if the assay is done after the removal of the toxin. Although this result can be achieved directly by cell counts or by indirect methods, such as isotope incorporation, cell viability as measured by MTT reduction [Mosmann, 1983] is widely used as the endpoint [Cole, 1986; Alley et al., 1988]. MTT is a yellow water-soluble tetrazolium dye that is reduced by live, but not dead, cells to a purple formazan product that is insoluble in aqueous solutions. However, a number of factors can influence the reduction of MTT [Vistica et al., 1991]. The assay described in Protocol 21.4, provided by Jane Plumb of the Cancer Research UK Centre for Oncology and Applied Pharmacology, University of Glasgow, Scotland, UK, has been shown to give the same results as a standard clonogenic assay [Plumb et al., 1989] (*see also* Section 21.3.6). It illustrates the use of microtitration in the assay of anticancer drugs, but would be applicable, with minor modifications, to any cytotoxicity assay.

PROTOCOL 21.4. MTT-BASED CYTOTOXICITY ASSAY

Principle

Cells in the exponential phase of growth are exposed to a cytotoxic drug. The duration of exposure is usually determined as the time required for maximal damage to occur, but is also influenced by the stability of the drug. After removal of the drug, the cells are allowed to proliferate for two to three population-doubling times (PDTs) in order to distinguish between cells that remain viable and are capable of proliferation and those that remain viable but cannot proliferate. The number of surviving cells is then determined indirectly by MTT dye reduction. The amount of MTT-formazan produced can be determined spectrophotometrically once the MTT-formazan has been dissolved in a suitable solvent.

Outline

Incubate monolayer cultures in microtitration plates in a range of drug concentrations (Fig. 21.5). Remove the drug, and feed the plates daily for two to three PDTs; then feed the plates again, and add MTT to each well. Incubate the plates in the dark for 4 h, and then remove the medium and MTT. Dissolve the water-insoluble MTT-formazan crystals in DMSO, add a buffer to adjust the final pH, and record the absorbance in a plate reader.

Materials

Sterile:

- ❑ Growth medium
- ❑ Trypsin (0.25% + EDTA, 1 mM, in PBSA)
- ❑ MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma), 50 mg/mL, filter sterilized
- ❑ Sorensen's glycine buffer (0.1 M glycine, 0.1 M NaCl adjusted to pH 10.5 with 1 M NaOH)
- ❑ Microtitration plates (Iwaki)
- ❑ Pipettor tips, preferably in an autoclavable tip box
- ❑ Petri dishes (non–TC-treated), 5 cm and 9 cm or reservoir (Corning)
- ❑ Universal containers or tubes, 30 mL and 100 mL

Nonsterile:

- ❑ Plastic box (clear polystyrene, to hold plates)
- ❑ Multichannel pipettor
- ❑ Dimethyl sulfoxide (DMSO)
- ❑ DMSO dispenser (optional); such as Labsystems Microplate Dispenser (Cat No 5840 127, from Thermo Fisher; *see also* Fig. 4.7)
- ❑ ELISA plate reader (Molecular Devices, with SOFTmax PRO; *see also* Fig. 4.7; Appendix II: Plate Readers)
- ❑ Plate carrier for centrifuge (for cells growing in suspension; *see* Appendix II: Microtitration Plate Centrifugation)

Procedure

Plating out cells

1. Trypsinize a subconfluent monolayer culture, and collect the cells in growth medium containing serum.
2. Centrifuge the suspension (5 min at 200 *g*) to pellet the cells. Resuspend the cells in growth medium, and count them.
3. Dilute the cells to 2.5 to 50×10^3 cells/mL, depending on the growth rate of the cell line, and allowing 20 mL of cell suspension per microtitration plate.
4. Transfer the cell suspension to a 9-cm Petri dish, and, with a multichannel pipette, add 200 μL of the suspension into each well of the central 10 columns of a flat-bottomed 96-well plate (80 wells per plate), starting with column 2 and ending with column 11, placing 0.5 to 10×10^3 cells into each well.
5. Add 200 μL of growth medium to the eight wells in columns 1 and 12. Column 1 will be used to blank the plate reader; column 12 helps maintain the humidity for column 11 and minimizes the "edge effect."
6. Put the plates in a plastic lunch box, and incubate in a humidified atmosphere at 37°C for 1 to 3 days, such that the cells are in the exponential phase of growth at the time that drug is added.
7. For nonadherent cells, prepare a suspension in fresh growth medium. Dilute the cells to 5 to 100×10^3 cells/mL, and plate out only 100 μL of the suspension into round-bottomed 96-well plates. Add drug immediately to these plates.

Drug addition

8. Prepare a serial fivefold dilution of the cytotoxic drug in growth medium to give eight concentrations. This set of concentrations should be chosen such that the highest concentration kills most of the cells and the lowest kills none of the cells. Once the toxicity of a drug is known, a smaller range of concentrations can be used. Normally three plates are used for each drug to give triplicate determinations within one experiment.

9. For adherent cells:
   - **(a)** Remove the medium from the wells in columns 2 to 11. This can be achieved with a hypodermic needle attached to a suction line.
   - **(b)** Feed the cells in the eight wells in columns 2 and 11 with 200 μL of fresh growth medium; these cells are the controls.
   - **(c)** Transfer the drug solutions to 5-cm Petri dishes, and add 200 μL to each group of four wells with a four-tip pipettor.
   - **(d)** Add the cytotoxic drug to the cells in columns 3 to 10. Only four wells are needed for each drug concentration, such that rows A through D can be used for one drug and rows E through H for a second drug.
10. For nonadherent cells, follow steps 9b–d but prepare the drug dilution at twice the desired final concentration; add 100 μL of diluted drug or control medium to the 100 μL of cells already in the wells.
11. Return the plates to the plastic box, and incubate them for a defined exposure period.

Growth period

12. At the end of the drug exposure period, remove the medium from all of the wells containing cells, and feed the cells with 200 μL of fresh medium. Centrifuge plates containing nonadherent cells (5 min at 200 g) to pellet the cells. Then remove the medium, using a fine-gauge needle to prevent disturbance of the cell pellet.
13. Feed the plates daily for 2 to 3 PDTs.

Estimation of surviving cell numbers

14. Feed the plate with 200 μL of fresh medium at the end of the growth period, and add 50 μL of MTT to all of the wells in columns 1 to 11.
15. Wrap the plates in aluminum foil, and incubate them for 4 h in a humidified atmosphere at 37°C. Note that 4 h is a minimum incubation time, and plates can be left for up to 8 h.
16. Remove the medium and MTT from the wells (centrifuge for nonadherent cells), and dissolve the remaining MTT-formazan crystals by adding 200 μL of DMSO to all of the wells in columns 1 to 11.
17. Add glycine buffer (25 μL per well) to all of the wells containing DMSO.
18. Record absorbance at 570 nm immediately, because the product is unstable. Use the wells in column 1, which contain medium and MTT but no cells, to blank the plate reader.

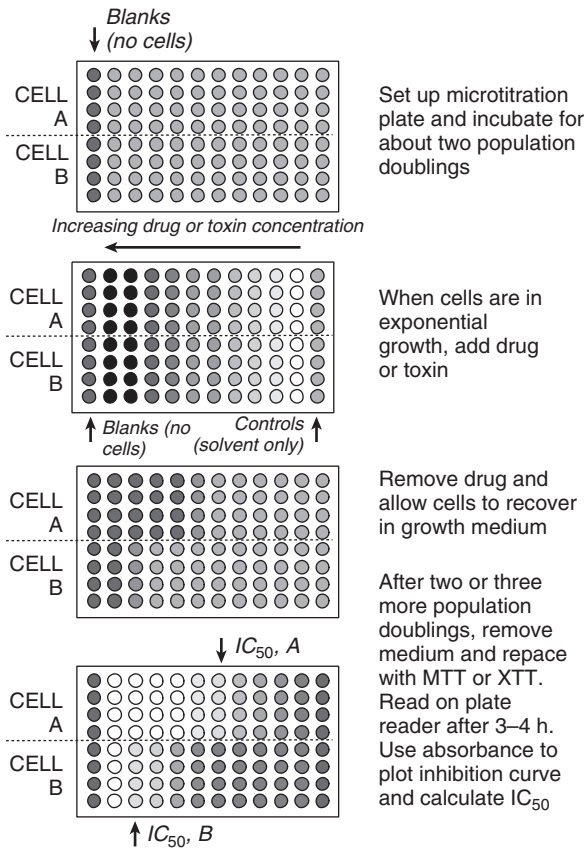

Fig. 21.5. Microtitration Assay. Stages in the assay of two different cell lines exposed to a range of concentrations of the same drug and then allowed to recover before the estimation of survival by the MTT reaction (*see* Protocol 21.4). The far left column has no cells and can be used as a blank to set the plate reader. This array is applicable when using plate sealers, when all wells are equivalent; however, with lids, there is a risk of an edge effect, probably due to evaporation, and it is better to leave the far left and far right columns blank (i.e., with medium only, as in Protocol 21.4), and some users leave the top and bottom rows blank as well. (*See also* Plate 17*b*).

Analysis of MTT assay

(1) Plot a graph of the absorbance (*y*-axis) against the concentration of drug (*x*-axis).

(2) Calculate the IC_{50} as the drug concentration that is required to reduce the absorbance to half that of the control. The mean absorbance reading from the wells in columns 2 and 11 is used as a control (columns 1 and 12 when plate sealers are used as in Fig. 21.5). The absorbance values in control columns should be the same. Occasionally they are not, however, and this is taken to indicate uneven plating of cells across the plate.

(3) The absolute value of the absorbance should be plotted so that control values may be compared, but the data can then be converted to a percentage-inhibition curve (Fig. 21.6) to normalize a series of curves.

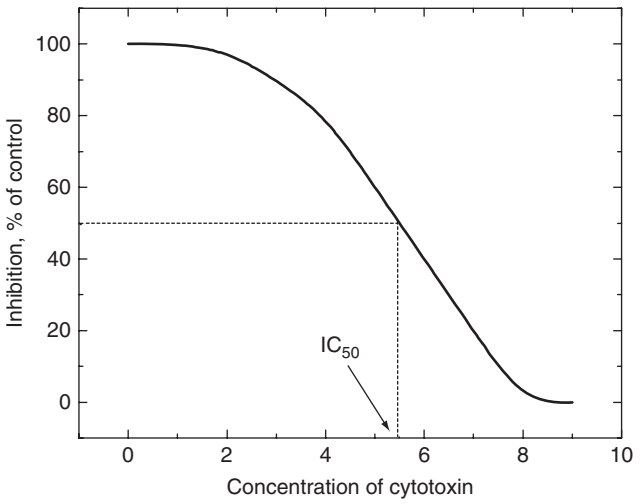

Fig. 21.6. Percentage Inhibition Curve. Test well values are calculated as a percentage of the controls and plotted against the concentration of cytotoxin. Typically a sigmoid curve is obtained, and ideally the IC_{50} will lie in the center of the inflexion of the curve.

Variations in MTT Assay

Other applications. A similar assay has also been used to determine cellular radiosensitivity [Carmichael et al., 1987b]. MTT can be used to determine the number of cells after a variety of treatments other than cytotoxic drug exposure, such as growth factor stimulation. However, in each case it is essential to ensure that the treatment itself does not affect the ability of the cell to reduce the dye and absorbance remains linear with cell number.

Duration of exposure. As with clonogenic assays (*see* Protocol 21.3), some agents may act more quickly, and the exposure period and recovery may be shortened. The cells must remain in exponential growth throughout (*see* Sections 12.4.3, 20.9.2), and the cell concentration at the end should still be within the linear range of the MTT spectrophotometric assay. When using a cell line for the first time, parallel plates should be set up for cell counts to generate a growth curve (*see* Protocol 21.4) and for MTT-formazan absorbance to ensure that absorbance is proportional to the number of cells. If the growth curve shows that the cells are moving into the stationary phase or the absorbance is nonlinear when plotted against cell concentration, shorten the assay and proceed directly to step 14 of Protocol 21.4.

Duration of exposure is related to the number of cell cycles that the cells have gone through during exposure and recovery. Cell cycle time will influence the choice between a short-form and long-form assay (Figs. 21.7, 21.8). With rapidly dividing cells, not only will the cell density increase more rapidly during exposure but, in addition, the response to cycle-dependent drugs will be quicker. When first trying an assay, it may be desirable to sample on each day of drug exposure and recovery. If a stable IC_{50} is reached earlier, then the assay may be shortened.

End point. Sulforhodamine, a fluorescent dye that stains protein, can also be used to estimate the amount of protein (i.e., cells) per well on a plate reader with fluorescence detection [Boyd, 1989]. It stains all cells and does not discriminate between live and dead cells. Labeling with [^{3}H]thymidine (DNA synthesis), [^{3}H] leucine [Freshney et al., 1975] or [^{35}S]methionine [Freshney & Morgan, 1978] (protein synthesis), or other isotopes can be substituted for MTT reduction. Quantitation is achieved by microtitration plate scintillation counting on a specially adapted scintillation counter (Perkin Elmer) or by preparing an autofluorogram and reading it on a densitometer [Freshney & Morgan, 1978].

In practice, it may not matter which criterion is used for determining viability or survival at the end of an assay; it is rather the design of the assay, such as duration of drug exposure and recovery, phase of the growth cycle (cell density, growth rate, etc.), that is more important. In a short assay with no or minimal recovery period, the endpoint must measure only viable cells (e.g., MTT), but in a longer assay the end point measures the difference between wells that have increased and those that have not, or have even decreased. In a monolayer assay, at least, nonviable cells will have been lost, and the increase or decrease relative to control wells is what is measured; whether by MTT, sulforhodamine, or isotope incorporation into DNA or protein becomes less important.

Handling. A variety of automated instruments are available to reduce the handling time required per sample, including autodispensers, diluters, cell harvesters, and programmable plate readers (*see* Fig. 4.7; Appendix II: Microtitration Equipment).

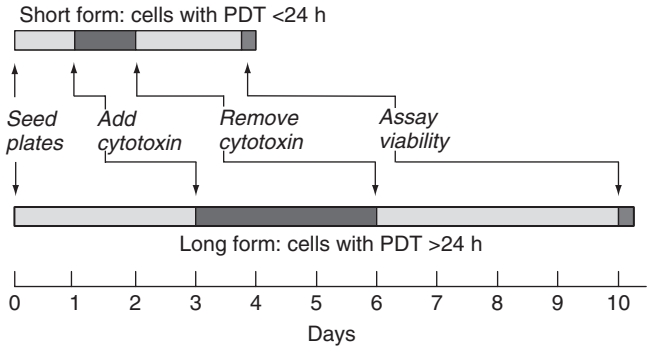

Fig. 21.7. Assay Duration. Pattern for short-form and long-form assays. The upper diagram represents an assay that is suitable for cell with a PDT < 24 h, and the bottom diagram represents an assay that is suitable for cells with a PDT > 24 h, although intermediate time scales are also possible.

TABLE 23.1. Primary Antibodies for Characterizing Pluripotent Stem Cell Markers

Name	Species	Company	Catalog number	Working dilution
NANOG	Goat polyclonal	R& D Systems	AF1997	1:20
OCT4	Goat polyclonal	Santa Cruz Biotechnology, Inc.	sc-8629	1:150
SSEA1	Mouse monoclonal	DSHB	MC-480	1:20
SSEA3	Rat monoclonal	DSHB	MC-631	1:20
SSEA4	Mouse monoclonal	DSHB	MC-813-70	1:20
PGC surface marker	Mouse monoclonal	DSHB	EMA-1	1:20
hTERT	Mouse monoclonal	Novocastra	NCL-L-hTERT	1:50
TRA-1-60	Mouse monoclonal	Chemicon, Ltd.	MAB4360	1:50
TRA-1-81	Mouse monoclonal	Chemicon, Ltd.	MAB4381	1:50

Source: Reprinted from Turnpenny & Hanley, 2007.

evidence for stem cell plasticity seems convincing, not least by the proof that adult cells such as epidermal keratinocytes and dermal fibroblasts can be reprogrammed to behave like pluripotent stem cells (*see* Section 23.3.5). It may be possible that stem cells found in many tissues of the adult acquire their new potency from the microenvironment into which they are transplanted, rather than that were already pluripotent in their native site.

Markers that have been used to confirm pluripotent stem cell identity include NANOG, OCT4, SSEA1, 3, and 4, PGC, hTERT, TRA-1-60, TRA-1-81 (Table 23.1) and alkaline phosphatase.

23.3.4 MSCs from Human Bone Marrow

One of the sites that has been exploited most extensively is the bone marrow from which can be derived not only hematopoietic stem cells but also mesenchymal stem cells (MSCs) with the potential to differentiate into a number of cell types including adipose, muscle, cartilage, bone [Hofmann et al., 2006; Gregory & Prockop, 2007], and cardiomyocytes [Kawada et al., 2004]. Protocol 23.5 for the cultures of MSCs and the following introduction was contributed by C. Gregory and D. Prockop, Texas A & M Health Science Center, Institute for Regenerative Medicine, 5701 Airport Road, Module C, Temple, TX; it is adapted from Gregory & Prockop [2007], and is reproduced here with their agreement.

Iliac crest bone marrow aspirates are generally preferred for the isolation and expansion of hMSCs, although MSC-like cells have been recovered from trabecular bone [Sakaguchi et al., 2004, synovium Sakaguchi et al., 2005], adipose tissue [Zuk et al., 2002], and even exfoliated teeth [Miura et al., 2003]. A 2-mL bone marrow aspirate is adequate for the production of enough MSCs for most applications. The bone marrow can be stored in heparinized blood drawing tubes charged with 3 mL αMEM and stored on ice for up to 8 h prior to processing. Longer incubations at 4°C decrease the initial rate of propagation of MSCs and are therefore discouraged.

Human bone marrow is initially processed by enriching for the nucleated component of the bone marrow that contains the hematopoietic and mesenchymal stem cells by Ficoll-mediated discontinuous density gradient centrifugation. The bone marrow is then cultured on 15-cm tissue culture plates or, after recovery on tissue culture plates, the cells can be expanded in Nunc Cell Factories (*see* Protocol 26.2) with frequent washes and media changes. The non-adherent hematopoietic component of the culture is gradually washed away over a few days resulting in an exclusively adherent MSC culture.

PROTOCOL 23.7. MSC PRODUCTION FROM HUMAN BONE MARROW

Outline

Fractionate bone marrow aspirate on Ficoll–Paque and seed the nucleate fraction into Petri dishes. Wash to remove hematopoietic cells and subculture when 60% confluent.

Reagents and Materials

Sterile or aseptically prepared:

- ❑ Bone marrow aspirate, 2 mL collected into 3 mL α-MEM
- ❑ Complete culture medium (CCM): a-MEM without ribonucleosides or deoxyribonucleosides, with 4 mM glutamine, 20% hybridoma qualified FBS, 100 U/mL penicillin, 100 g/mL streptomycin
- ❑ D-PBSA
- ❑ Trypsin/EDTA: porcine trypsin, 0.25% in PBSA with 1 mM EDTA
- ❑ Hanks's balanced salt solution without calcium or magnesium (HBSS)
- ❑ Ficoll-Paque
- ❑ Polypropylene centrifuge tubes, 15 mL and 50 mL
- ❑ Plastic tissue culture Petri dishes 15 cm diameter

- ❑ Plastic micro-pipettor tips for dispensing 10 to 1000 L

Nonsterile:

- ❑ Trypan Blue solution in 0.85% saline
- ❑ Microcentrifuge
- ❑ Refrigerated benchtop centrifuge with swinging bucket rotor
- ❑ Water bath set to 37°C
- ❑ Improved Neubauer hemocytometer
- ❑ Pipettors, Eppendorf P10, P20, P100, and P1000 or equivalent

Procedure

1. Uncap the drawing tube of bone marrow and transfer to one 50 mL centrifuge tube. Make the volume up to 25 mL with room temperature HBSS.
2. To another 50 mL centrifuge tube, add 20 mL Ficoll-Paque and gently overlay the 25-mL cell solution on to the Ficoll. The interface between the HBSS and the Ficoll should not be disrupted.
3. Centrifuge at 1800 *g* for 30 min at room temperature with the brake off.
4. After centrifugation, collect the white cell layer at the interface of the Ficoll and HBSS and transfer to a fresh 50-mL centrifuge tube.
5. Make the volume of the interface cell suspension up to at least 3 volumes with HBSS and centrifuge at 1000 *g* for 10 min at room temperature with the brake on. Repeat wash.
6. Suspend the cell pellet in 30 mL CCM pre-warmed to 37°C.
7. Add 10 L of the cell suspension to 10 L of Trypan Blue and assess the viability with a hemocytometer; viability should be above 80%.
8. Transfer the 30-mL cell suspension to a 15-cm diameter tissue culture Petri dish and culture in a humidified incubator under 5% CO_2 for at least 15 h.
9. Remove the Petri dish from the incubator and remove the medium.
10. Add and remove 20 mL pre-warmed D-PBSA to wash the monolayer.
11. Repeat the wash procedure 3 times.
12. Replace with 30 mL of fresh pre-warmed CCM.
13. Repeat this wash and medium replenishment every second day for 6 days.
14. After 6 days, examine the monolayers with an inverted microscope. Adherent, fibroblast-like colonies of MSCs should be clearly visible in the Petri dish (Fig. 23.4*a*, *b*). In some cases there may be signs of hematopoietic contamination, but these cells will be depleted upon passaging the cells. When the culture is 50% to 60% confluent, proceed to subculture.

Subculture of MSCs from human bone marrow

15. Inspect the MSC cultures. If the cultures consist of small, adherent, spindle-shaped fibroblastoid cells that are approximately 60% confluent (see Fig. 24.3*d*), proceed. If the monolayer is sparse (see Fig. 24.3*c*), continue to wash and replenish medium as described above.
16. Trypsinize the monolayer as follows:
    - **(a)** Wash the monolayer with 20 mL of pre-warmed PBSA and add 5 mL of trypsin/EDTA.
    - **(b)** Place the plate at 37°C for 2 min, and then inspect the monolayer at 10× magnification. The adherent cells should be in the process of detaching from the plastic substratum.
    - **(c)** Replace plate at 37°C for 2 min; then inspect again. Repeat inspection until 90% of the MSCs have detached from the plastic.
    - **(d)** Add 5 mL of CCM, transfer the 10 mL suspension to a 15 mL conical tube, and centrifuge for 10 min at 500 *g*.
    - **(e)** After the centrifugation, remove the supernate from the cell pellet and resuspend the pellet in 1 to 2 mL warm PBSA per tube. If necessary, combine multiple resuspended pellets for a single cell count.
17. Add 10 L of the cell solution to 10 L of Trypan Blue and count with a hemocytometer. An adequate concentration for the cell suspension should be between 2 to 5×10^5 cells per mL with a viability > 80%.
18. Suspend the MSCs at a concentration of 7×10^3 (for a final density of 50 cells per cm^2) to 1×10^4 cells/mL (for a final density of 100 cells per cm^2) in pre-warmed CCM. The cells should be plated at low density to maintain the rapidly self-renewing, multipotential phenotype.
19. Prepare the appropriate number of plates by adding 25 mL of pre-warmed CCM to each of the 15-cm plates.
20. Seed the plates by adding 1 mL of the suspension prepared in step 18. Slide the plates from side to side, do not swirl, to distribute the cells evenly. Replace the plates in the incubator.
21. After 2 to 3 days of culture, inspect the plates and make an assessment of morphology. The MSCs should adopt a small, spindle-shaped morphology with frequent refractile doublets (e.g., *see* Fig. 24.3*b*). This is the sign of a healthy culture of MSCs.

22. Aspirate the medium from the plates, wash the MSCs with 20 mL pre-warmed PBSA, and replace with 25 mL of fresh pre-warmed CCM.
23. The number of subsequent expansion plates per passage is limited only by the number of cells available to seed the plates. The volumes quoted in the protocol above are suitable for a single 15-cm plate of MSCs. This can be expanded proportionally to accommodate multiple plates where necessary.

23.3.5 Induced Pluripotent Stem Cells

Further evidence of plasticity in the generation of stem cells comes from the rapidly accumulating body of data demonstrating that adult cells can be converted to pluripotent stem cells by altering gene expression, by transfection [Nakagawa et al., 2007], which can be reversible using an excisable lentivirus construct [Sommer et al., 2010], a piggyback transposon [Kaji et al., 2009; Woltjen et al., 2009], retroviral infection [Aasen et al., 2008; Woltjen et al., 2009], adenoviral reprogramming [Stadfeld et al., 2008], transfection of microRNAs [Judson & Blelloch, 2009], or chemical manipulation [Huangfu et al., 2008; Lin et al., 2009]. Alterations in DNA methylation and histone acetylation [Kondo & Raff, 2004; Hrzenjak et al., 2006; Keenen et al., 2008; Boheler, 2009] are implied by most of the methods induced by the expression of transcription factors such as c-Myc, Klf4, Oct4, and Sox2.

The following introduction and Protocol 23.8 for reprogramming human dermal fibroblasts, using lentiviral infection to introduce the reprogramming factors, has been contributed by S. Sullivan, R. Jones, and G. P. Davey, School of Biochemistry and Immunology and Institute of Neuroscience, Trinity College, Dublin 2, Ireland.

Currently much interest surrounds the generation and use of human-induced pluripotent stem (iPS) cells for applications relevant to human therapy [Nishikawa et al., 2008]. In the long term these may include human cell replacement therapies, but in the short term such cells will be used primarily as sources of disease- or patient-tailored cells for drug screening (assaying activity, toxicity, efficacy) and for disease modeling (in this context defined as the process by which underpinnings of disease are investigated by in vitro models of disease using stem cell derivatives).

Successful reprogramming of human dermal fibroblasts depends on several key parameters, including avoidance of microbial contamination such as mycoplasma, high

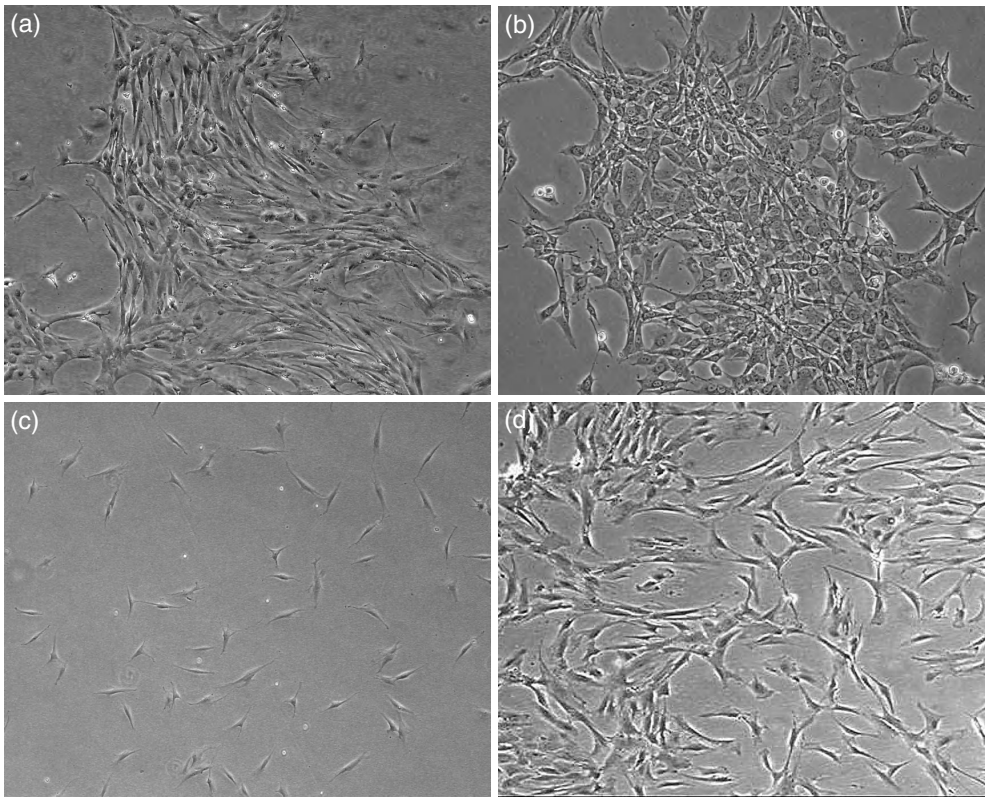

Fig. 23.4. Bone Marrow-Derived MSCs. (*a*, *b*) Colonies formed after plating of whole bone marrow mononuclear cells. Morphology and optimal passaging density of MSCs. (*c*) An early passage culture of MSCs. (*d*) A monolayer at the appropriate density for passage. (From Gregory & Prockop, 2007).

METHODS OF STERILIZATION

Sterilization is the inactivation of all microbes, including endospores, and can be achieved by mechanical means, heat, chemicals, electromagnetic radiation, or sonic energy (Table 1.1). When using heat, it may be either moist heat or dry heat. Traditionally, moist heat under pressure is provided by autoclaving, and dry heat is applied with ovens (see Table 1.1). However, the dead microbes and the associated cellular debris remain, making even sterilized material a potential hazard.

Disinfection is the process that inactivates most or all microorganisms, with the exception of endospores and some acid-fast bacilli. Disinfectants can be subcategorized as high-level disinfectants, which kill all microorganisms with the exception of endospores with an exposure time of less than 45 min; intermediate-level disinfectants, destroy the majority of bacteria and viruses but fail to inactivate endospores; and low-level disinfectants, kill the majority of vegetative bacteria, some fungi, and some viruses typically with exposure times of less than 10 min [60]. Disinfectants are used on inanimate objects and can be sporostatic but are typically not sporicidal [45]. Antiseptics, a subset of disinfectants, destroy or inhibit the growth of microorganisms within or present on living tissues and are often referred to as biocides [45].

Steam sterilization and dry heat inactivation of infectious microbes can be monitored through the use of biological indicators or by chemical test strips that turn color upon having met set physical parameters. Usually, the spores of species of *Geobacillus* or *Bacillus* are used in either a test strip or suspension, as these organisms are more difficult to kill than most organisms of clinical interest [4]. Growth of the spores in liquid media after the cycle of sterilization is complete indicates the load was not successfully processed and should not be considered sterile.

In the days following September 11, 2001, letters containing the spores and cells of *Bacillus anthracis* were mailed to several news media offices and 2 U.S. senators resulting in the death of 5 people and the infection of 17 others. This introduced a pathogen into locations where methods

TABLE 1.1
Routine Methods of Sterilization[a]

Method [Ref.]	Temperature	Pressure	Time	Radiation (Mrad)
Dry heat [26]	150°C–160°C 302°F–320°F		>3 h	
Dry heat [26]	160°C–170°C 320°F–338°F		2–3 h	
Dry heat [26]	170°C–180°C 338°F–356°F		1–2 h	
Moist heat[a]	135°C 275°F	31.5 psig	40 min	
Boiling—indirect [26]			1 h	
Boiling—direct[b]			2 min	
Radiation—cobalt 60[c] [26]	Ambient		Hours	2–3
Radiation—cesium 137[c] [26]	Ambient		Hours	2–3
Electronic accelerators[d] [26,32]			<1 s	2.5
Ozone [25,69]		See cited references	See cited references	
Sonic energy [15,51]	Ambient or in combination with heat or pressure	See review by Chemat et al. [15]		

[a] Steam under pressure (i.e., autoclave).
[b] Boiling point of liquids—e.g., cumene (isopropylbenzene) 152°C (306°F).
[c] Dependent on curies in source.
[d] Electrostatic (Van de Graaff), electromagnetic (Linac), direct current, pulsed transformer.

of safe handling and eradication of such pathogens were not defined nor anticipated. Further complicating matters was that the microbes contained within the packaging had been optimized for airborne dispersal resulting in the substantial contamination of the built environment necessitating isolation and/or closure of government buildings.

As a consequence of the anthrax attacks of 2001, a number of laboratories have conducted experiments on the potential dispersal, detection, and eradication of organisms spread by these unconventional methods. Lemieux et al. [41] conducted experiments with simulated building decontamination residue (BDR) contaminated with 10^6 spores of *Geobacillus stearothermophilus* to simulate *B. anthracis* contamination. A single cycle did not effectively decontaminate the BDR. Only autoclave cycles of 120 min at 31.5 pounds per square inch gauge (psig)/275°F and 75 min at 45 psig/292°F effectively decontaminated the BDR. Two standard cycles at 40 min and 31.5 psig/275°F run in sequence were even more effective. The authors state that the second cycle's evacuation step probably pulled condensed water out of the pores of the materials, allowing better steam penetration. It was found that both the packing density and material type of the BDR significantly impacted the effectiveness of the decontamination process.

When materials made of unusual substances and/or densities are to be sterilized, the method of sterilization must be monitored and adapted according to the material. Common strategies include alterations of placement and/or lower packing densities within the autoclave. Other common methods of sterilization of problematic materials and substance include gases such as ozone or ethylene oxide, radiation, or less common electronic accelerators (see Table 1.1).

Solutions containing heat-labile components require a different approach. Filtration is generally the most accepted and easiest method. The Food and Drug Administration (FDA) and industry consider 0.22 μm filters to be sterilization grade based on logarithmic reductions of one of the smaller bacteria *Brevundimonas diminuta*, a non-lactose-fermenting environmental gram-negative rod [11]. Usually, an incubation period of at least 48 h is required for colony development of *B. diminuta* before the colonies are large enough to be viewed for counting. Griffiths et al. [28] describe a Tn5 recombinant method in which *B. diminuta* can be augmented with the genes for bioluminescence (luxABCDE) and fluorescence (*gfp*). Use of the modified microbes enabled detection within 24 h of incubation by either bioluminescence or fluorescence. They state that this method may aid in preventing quality control backlogs during filter-manufacturing processes.

A 0.22 μm filter will adequately sterilize the majority of heat-labile solutions used for molecular biology and microbiology. Labile tissue culture reagents might also require to be certified free from *Mycoplasma* contamination. A 0.1 μm filter is used to remove *Mycoplasma* from tissue culture solutions [71] (Table 1.2). Today, with the widespread availability of PCR machines, routine testing for *Mycoplasma*, *Acholeplasma,* and *Ureaplasma* via the amplification of sentinel genes has become the established method of choice for highest-sensitivity detection of contamination of filtered liquids by these microbes. Detection limits of less than 1 colony-forming unit (CFU)/mL are easily achieved within a few hours. This has transformed filtration-based sterile product release; rather than waiting on results from a 28-day biological culture test, manufacturers can now easily rely on this established methodology taking less than a few hours to complete.

TABLE 1.2
Filter Sterilization

Size (μm)	Purpose
0.1	Mycoplasmal removal
0.22	Routine bacterial removal
0.45	Plate counts of water samples
>0.45	Removal of particulates, some bacteria, yeast, and filamentous fungi

The removal of contaminants from air may be necessary in the case of fermentation, drug manufacturing, or chemical reactions requiring some form of gas. Various types of filters are available for the removal of organisms from air. One such filter is the Aerex 2 (Millipore). It can withstand 200 steam-in-place cycles at 293°F (145°C), can resist hydraulic pressures of 4.1 bard (60 psid), and has the ability to retain all phages when challenged with the Φ-174 bacteriophage at 29 nm in diameter and 10^7–10^{10} bacteriophages per cartridge [22]. In this particular scenario, the operator must assess their needs for how critical the product sterility (i.e., drugs versus topical cosmetics) must be, as well as the ability of the product to withstand pressure, heat, flow rate, and other parameters of the manufacturing process, and then choose a filter accordingly.

In some cases, a filter is used to recover bacteria from dilute solutions such as environmental water samples. The organisms of significance whose retention on the filter is most important should determine the filter pore size. The smaller the filter pore size, the slower the sample flow rate and throughput. Standard methods define an acceptable recovery rate for filter recovery of bacteria as being 90% of the number of bacteria (CFU) recovered from the same sample by the spread plate method under typically aerobic conditions. In one study performed by Millipore, filtration with a 0.45 μm pore-size filter provided 90% recovery of 12 microorganisms used, ranging in size from *B. diminuta* to *Candida albicans* (Table 1.2).

It is important to note that all filter sterilization is relative. Filter integrity for sterilization is usually done by a bubble test to confirm the pore size of the manufactured filter [11,31]. The bubble point is based on the fact that liquid is held in the pores of the filter (usually membrane) by surface tension and capillary forces, and the bubble pressure detects the least amount of pressure that can displace the liquid out of the pores of the filter [31]. While a 0.22 μm filter will remove most bacteria, it will not remove viruses, *Mycoplasma*, prions, and other small contaminants. Every sample type and the level of permissible substances in the filtrate must be assessed on a case-by-case basis. For example, filter sterilization of water or solutions used in atomic force microscopy might still allow sufficient numbers of small viruses and other particulates to pass through the filter and impede sample interpretation; under such conditions, ultrapure water may be needed. In addition, it should be noted that even though a filter has removed the microorganisms, it is still possible that any toxins that they produced may still be present.

ULTRASONIC ENERGY

Ultrasonic energy in the range of 20–500 kHz makes use of physical and chemical phenomena induced by an interaction of the ultrasonic energy with the structures and chemical constituents within the microbial cell. Principally, the sonic energy generates a wave front within the liquid medium. Alternating regions of compression and expansion occur causing pressure changes resulting in cavitation and bubble formation. During the expansion phase within the wave front, the expanding bubbles reach a state where they implode resulting in rapid condensation. The condensed molecules collide violently with the subsequent formation of a high-pressure shock wave of approximately 50 MPa resulting in a localized temperature of 5500°C [20]. These rapid changes in localized pressure and extremes of temperature serve to inactivate the microorganisms with the temperature being the main contributor to the cells' inactivation [20].

In a study of the use of high-energy ultrasound in the range of 24 kHz over 1 h where the nominal energy administered was applied at 1500 W/L or 5400 kJ/L, the mean killing observed for typical gram-negative bacteria found associated with wastewater such as total coliforms, fecal coliforms, and *Pseudomonas* spp. was 99.5%, 99.2%, and 99.7%, respectively [21]. Gram-positive microbes were found to be more recalcitrant to the sonic inactivation using this power density (W/L) and frequency (kHz) of energy where the mean inactivation observed for *Clostridium perfringens* and fecal streptococci was 66% and 84%, respectively. Sonic inactivation of microbes was found to follow first-order kinetics with respect to the bacterial population and was not affected significantly by the medium in which they were suspended [21]. The addition of 5 g/L of TiO_2 particles generally

Physical Components

Although researchers usually tend to focus on ingredients that can be added to a liquid medium, it should be remembered that manipulating the physical environment that the culture is placed in for temperature, oxygen tension, and even pressure may offer some microorganisms a competitive advantage in reproduction. The levels of these agents will be determined by which organism the technician is trying to isolate.[12] Thermophiles, such as *Thermogladius shockii*, for instance, can be enhanced by growing cultures at temperatures as high as 80°C–95°C.[13]

Antimicrobial Agents

Enrichment for specific groups or species of microorganisms can not only be achieved by defining conditions that will preferentially allow them to grow but can also be achieved through the use of agents that will inhibit the growth of competing organisms. In some instances, fast-growing organisms can be inhibited, to give fastidious or slower-growing organisms an advantage in a particular sample. An interesting clinical consequence of the use of antimicrobial agents as a method of enrichment is the observation of resistant organisms in patients who have been hospitalized. Hui et al.[14] have found that previous antibiotic exposure of nosocomial patients using mechanical ventilation can lead to an increase in antibiotic resistance.

PRESERVATION OF MICROORGANISMS

Introduction

Many laboratory test procedures require the use of microorganisms as reagents, while advances in biotechnology have resulted in the creation of engineered microorganisms. In both instances, to obtain consistent results, these organisms must be preserved in a manner that will allow for their genetic stability and long-term survival. Preservation of microorganisms can be accomplished by a variety of methods. These can include subculturing them, reducing their metabolic rate, or putting them into a state of quasisuspended animation.[15] The chosen method will usually depend on which organism one is trying to preserve. Despite the introduction of newer methodologies, many of the techniques that are used have not changed much since the previous editions of this book. Most still involve the use of drying, lyophilization, or storage in freezing or subfreezing temperatures.

Methods

Serial Subculture

This is a simple method in which the cultures are periodically passed in liquid or agar media. Some cultures can be stored on agar media in sealed tubes and survive for as long as 10 years.[16] This method has been used extensively for fungi but usually requires storage under mineral oil.[15] Despite its simplicity and applicability for cultures that cannot survive harsher preservation methods, serial subculture is not a very satisfactory method. In our hands, we have had problems with contamination, culture death, and the unintended selection of mutants. This has been particularly difficult in cases in which we were trying to develop new products for the identification of microorganisms. It was imperative to periodically confirm the identity of the organisms being used in the database.

Storage at Low Temperatures

Some species are capable of being stored for long periods at 4°C–8°C on agar plates or on slants. Sorokulova et al. have found that the use of acacia gum in storage media will allow long-term storage of organisms even at room temperature.[17]

In our lab, we have been able to store cultures at −20°C for extended periods. We have found that this can be accomplished by growing the cultures in liquid media for 24–48 h and then mixing one part sterile glycerol to three parts culture. While this does not work for all organisms, it allows the use of a standard refrigerator for preserving cultures. Many laboratories store organisms at −70°C to −80°C by first mixing them with 10% glycerol.[18] Many cultures can be stored indefinitely in liquid nitrogen. A method for this can be found at www.cabri.org (accessed April 1, 2014). While the use of liquid nitrogen is a good method for storing microorganisms, for most laboratories, it requires the expense of constantly refilling a specialized thermos. Tedeschi and De Paoli in their review of cryogenic methods of preserving microorganisms point out that in this era of being able to manipulate the microorganism genome, it is important to pick a cryogenic preservation method that will preserve the genotype and phenotype of the original organism.[19]

Freeze-Drying

This is a widely used method in which a suspension of microorganisms is frozen and then subjected to a vacuum to sublimate the liquid. The resulting dried powder is usually stored in vials sealed in a vacuum. Many factors can affect the stability of the culture. These include the growth media, the age of the cultures, the phase of the culture, and the concentration of the organisms in the suspension.[15,20] The length of sample viability can vary substantially, but cultures surviving for up to 20 years have been reported.[21] Freeze-drying requires a cryoprotective agent to provide maximal stability. Usually, the organisms are suspended in 10% skim milk.[22]

Storage in Distilled Water

This was cited as a method of preservation of *Pseudomonas* species and fungi in the first edition of this book.[15] Recent studies have found that it is still an effective method of preserving fungi,[23] as well as a wide variety of bacteria, including *Pseudomonas fluorescens*, *Erwinia* spp., *Xanthomonas campestris*, *Salmonella* spp., *Yersinia enterocolitica*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus*.[24] Stationary-phase organisms grown on agar media and then suspended in 10 mL of sterile water were found to be stable when sealed with parafilm membranes and then stored at room temperature in the dark. Even greater stability could be obtained by suspending the organisms in a screw-capped tube with phosphate buffered saline at pH 7.2, containing 15.44 μM KH_2PO_4, 1.55 mM NaCl, and 27.09 μM Na_2HPO_4.[24]

Drying

Sterile soil or sand has been used for preserving spore-forming organisms by adding suspensions and then drying at room temperature. In addition, bacterial suspensions have been mixed with melted gelatin and then dried in a desiccator. Both methods have produced samples that are stable for long periods of time.[15] Recently, a method has been developed in which a microliter quantity of a bacterial suspension is mixed with a predried activated charcoal cloth–based matrix contained within a resealable system that can then be stored. Experiments with *E. coli* have found that viability of over a year at 4°C can be obtained.[25]

Recovery and Viability of Preserved Microorganisms

Several factors can affect the viability of microorganisms that have been stored by either freezing or drying. These can include the temperature at which microorganisms are recovered, the type and volume of the media used for recovery, and even how quickly microorganisms are solubilized in a recovery medium.[15] Once reconstituted, microorganisms should be evaluated for cell survival.

Usually, the losses of preservation can be overcome by initially preserving large numbers of microorganisms. In doing this, one must be careful to avoid two problems. The first is that if only a very small fraction of organisms is recovered, this can result in the selection of biochemically

distinct strains. The second is that if a culture has inadvertently been contaminated with even a few microorganisms, the preservation technique may lead to a selection for the contaminant.[26]

Drying and freeze-drying have been known to cause changes in several characteristics of preserved microorganisms. These can include colonial appearance and pathogenicity.[15] After revival from a frozen or dried state, many protocols usually advise subculturing the organisms at least two or three times in an attempt to restore any characteristics that may have been lost and to confirm the genetic identity of these organisms.[15]

REFERENCES

1. Aaronson, S., Enrichment culture, in: *CRC Practical Handbook of Microbiology*, O'Leary, W., ed., CRC Press, Boca Raton, FL, 1989, p. 337.
2. Keen, J., Durso, I., and Meehan, L., Isolation of *Salmonella enterica* and Shiga-toxigenic *Escherichia coli* O157 from feces of animals in public contact areas of United States zoological parks, *Appl. Environ. Microbiol.*, 73, 362, 2007.
3. Miles, K.I., Wren, M.W.D., and Benson, S., Is enrichment culture necessary for clinical samples? *Br. J. Biomed. Sci.*, 63, 87, 2006.
4. Ottesen, A.R., Gonzalez, A., Bell, R., Arce, C., Rideout, S., Allard, M., Evans, P. et al., Co-enriching microflora associated with culture based methods to detect salmonella from tomato phyllosphere, *PLoS One*, 8(9), e73079, 2013.
5. Al-Zeyara, S.A., Jarvis, B., and Mackey, B.M., The inhibitory effect of natural microflora of food on growth of *Listeria monocytogenes* in enrichment broths, *Int. J. Food Microbiol.*, 145, 98, 2011.
6. Murray, P., Rosenthal, K., and Pfaller, M., *Medical Microbiology*, 7th edn., Mosby, St. Louis, MO, 2012, p. 25.
7. Ohmura, N., Sasaki, K., Matsumoto, N., and Saiki, H., Anaerobic respiration using Fe^{3+}, S^0, and H_2 in the chemolithoautotrophic bacterium *Acidithiobacillus ferrooxidans*, *J. Bacteriol.*, 184, 2081, 2002.
8. Pant, P. and Pant, S., A review: Advances in microbial remediation of trichloroethylene (TCE), *J. Environ. Sci.*, 22(1), 116, 2010.
9. Wu, Y., Xia, L., Yu, Z., Shabbir, S., and Kerr, P.G., In situ bioremediation of surface waters by periphytons, *Bioresour. Technol.*, 151, 367, 2014.
10. Ünal, B., Perry, V.R., Sheth, M., Gomez-Alvarez, V., Chin, K.-J., and Nüsslein, K., Trace elements affect methanogenic activity and diversity in enrichments from subsurface coal bed produced water, *Front. Microbiol.*, 3(175), 1, 2012.
11. Johnson, D.B. and Hallberg, K.B., The microbiology of acidic mine waters, *Res. Microbiol*., 154, 466, 2003.
12. Ohmura, M., Kaji, S., Oomi, G., Miyaki, S., and Kanazawa, S., Effect of pressure on the viability of soil microorganisms, *High Press. Res.*, 27, 129, 2007.
13. Meyer-Dombard, D.R., Shock, E.L., and Amend, J.P., Effects of trace element concentrations on culturing thermophiles, *Extremophiles*, 16, 317, 2012.
14. Hui, C., Lin, M.-C., Jao, M.-S., Ne, R., Liu, T.-C., and Wu, R.-G., Previous antibiotic exposure and evolution of antibiotic resistance in mechanically ventilated patients with nosocomial infections, *J. Crit. Care*, 28, 728, 2013.
15. Lapage, S., Redway, K., and Rudge, R., Preservation of microorganisms, in: *CRC Practical Handbook of Microbiology*, O'Leary, W., ed., CRC Press, Boca Raton, FL, 1989, p. 321.
16. Antheunisse, J., Preservation of microorganisms, Antonie van Leeuwenhoek, *J. Microbiol. Serol*., 38, 617, 1972.
17. Sorokulova, I., Watt, J., Olsen, E., Globa, L., Moore, T., Barbaree, J., and Vodyanoy, V., Natural biopolymer for preservation of microorganisms during sampling and storage, *J. Microbiol. Methods*, 88, 140, 2012.
18. Cameotra, S.S., Preservation of microorganisms as deposits for patent application, *Biochem. Biophys. Res. Commun.*, 353, 849, 2007.
19. Tedeschi, R. and De Paoli, P., Collection and preservation of frozen microorganisms, *Methods Mol. Biol.*, 675, 313, 2011.
20. Morgan, C.A., Herman, N., White, P.A., and Vesey, G., Preservation of micro-organisms by drying: A review, *J. Microbiol. Methods*, 66, 183, 2006.
21. Miyamoto-Shinohara, Y., Sukenobe, J., Imaizumi, T., and Nakahara, T., Survival curves for microbial species stored by freeze-drying, *Cryobiology*, 52, 27, 2006.
22. Crespo, M.J., Abarca, M.L., and Cabañes, F.J., Evaluation of different preservation and storage methods for *Malassezia* spp., *J. Clin. Microbiol.*, 38, 3872, 2000.

4. ÎNSĂMÂNȚAREA MEDIILOR DE CULTURĂ OBȚINEREA CULTURILOR PURE CULTURI ȘI COLONII MICROBIENE

4.1. TEHNICI DE ÎNSĂMÂNȚARE

Însămânțarea constă în depunerea pe suprafața sau în profunzimea mediilor de cultură a microorganismelor din produsele patologice sau din alte surse (apă, sol etc).

Instrumentele folosite pentru efectuarea unei însămânțări sunt **ansele de însămânțare, pipetele Pasteur** sau **pipetele gradate**. Ansele sunt formate dintr-un fir de platină îndoit sub formă de buclă la o extremitate, montat pe un mâner de metal sau ebonită (Fig. 17).

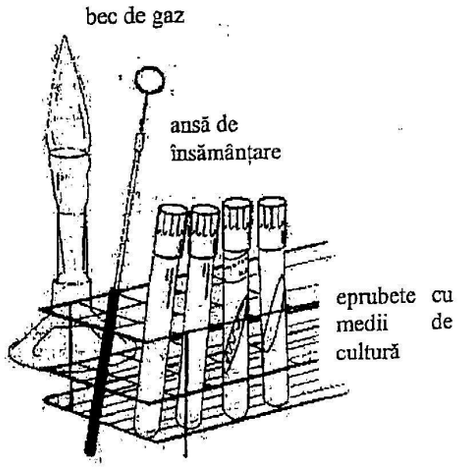

Fig. 17 – Materiale necesare însămânțării unei culturi microbiene

Operațiunea de însămânțare trebuie făcută cu toate precauțiile de asepsie, folosind medii de cultură și instrumente sterile, numai sub protecția unei flăcări, pentru a se evita introducerea în mediile de cultură a altor microorganisme decât cele prezente în produsul însămânțat. Deschiderea recipientelor în care se găsesc mediile și produsul de însămânțat se face cu mare atenție, în apropierea unei flăcări; gura recipientelor se flambează. De asemenea, trebuie sterilizate ansa sau pipeta cu care se efectuează însămânțarea atât înainte cât și după această operațiune. După sterilizare, este necesar ca ansa sau pipeta să fie lăsate să se răcească înainte de a le pune în contact cu produsul de însămânțat, deoarece temperatura ridicată distruge microorganismele.

Pentru realizarea condițiilor de asepsie, însămânțarea se face în încăperi lipsite de curenți de aer (sau chiar în boxe sterile), persoana nu vorbește, poartă bonetă, eventual și mască.

Conduita unei însămânțări (Fig. 18) este următoarea:

- instrumentul cu care se face însămânțarea se ține în mâna dreaptă cât mai aproape de extremitatea opusă celei care se introduce în recipient și se sterilizează în flacără; ansa se ține ca un creion iar pipetele se țin între degetul mare și mijlociu cu arătătorul aplicat pe orificiul prevăzut cu dopul de vată (Fig. 18-1);
- recipientul din care se prelevă sau în care urmează a fi depus materialul manipulat se ține de extremitatea inferioară în mâna stângă în așa fel încât conținutul și traiectul instrumentului să poată fi permanent controlat cu vederea;
- se scoate dopul recipientului cuprinzându-l între degetul mic și palma mâinii drepte; cât timp recipientele sunt fără dop se vor menține înclinate pentru a evita depunerea în interiorul lor a particulelor de praf sau a microorganismelor din atmosferă (Fig. 18-2);
- se sterilizează prin flambare gâtul și gura recipientului prin rotirea în flacără timp de câteva secunde;
- se introduce instrumentul de însămânțare și se face prelevarea materialului după care se retrage cu grijă pentru a evita atingerea pereților, flambându-se apoi gura și gâtul recipientului (Fig. 18-3, 18-4, 18-5);
- se lasă jos recipientul cu produs și se ia tot cu mâna stângă recipientul în care se face însămânțarea, deschizându-l în mod asemănător;
- se depune materialul de însămânțat în recipientul respectiv (eprubetă sau placă Petri) – Fig. 18-6;
- după terminarea operației se sterilizează ansa fără a se introduce direct bucla în flacără, căci urmele de lichid pot intra brusc în fierbere, stropind și contaminând suprafața din jur. Ansa se trece de mai multe ori prin flacără până când bucla și toată lungimea firului se înroșesc. Pipetele se introduc în cilindrul pentru pipete infecte; ele nu se flambează (Fig. 18-7).

Când recipientul cu produs și cel în care se face însămânțarea sunt eprubete, acestea se pot ține odată în mâna stângă, deschizându-le simultan (Fig. 18-8). Celelalte operații sunt identice.

Însămânțarea mediilor lichide

Dintr-o cultură sau dintr-un produs lichid se iau cu ajutorul pipetei Pasteur câteva picături care sunt introduse apoi, cu precauțiile expuse mai sus, în profunzimea mediului lichid.

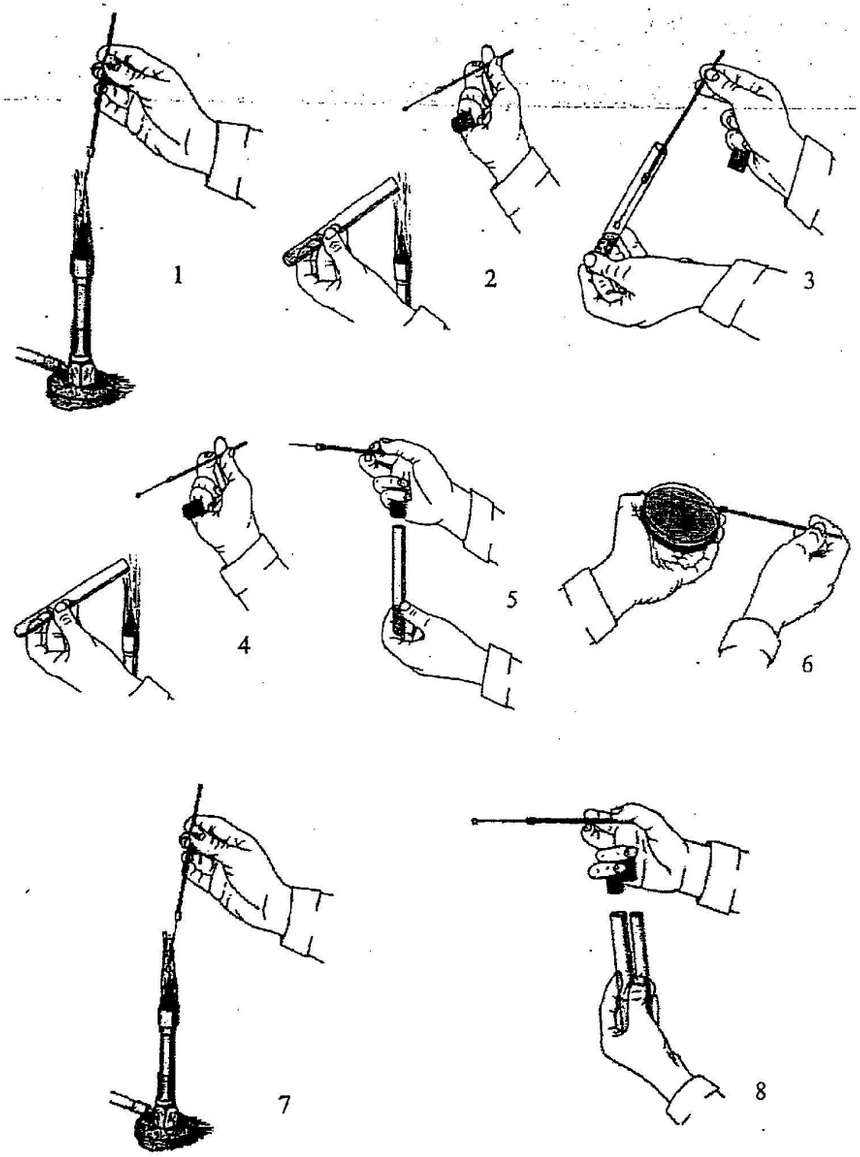

Fig. 18 – Tehnica însămânțării unei culturi microbiene cu ajutorul ansei

În cazul în care produsul de însămânțat este o cultură microbiană pe mediu solid sau un produs semisolid, se ia cu ansa o cantitate mică din acesta și se suspensionează în mediul de cultură, omogenizând amestecul cu ansa pe peretele tubului.

Însămânțarea pe suprafața mediilor solide

Atunci când trebuie însămânțat un produs sau o cultură din mediu lichid, se ia cu o pipetă Pasteur sau cu ansa o picătură din produsul respectiv, după care acesta se disemineză pe suprafața mediului de cultură din eprubete sau din cutiile Petri. Însămânțarea se poate face fie cu ansa descriind striuri (Fig. 19), fie cu o pipetă, prin înclinarea tubului sau a plăcii cu mediu în așa fel încât produsul să fie repartizat pe toată suprafața mediului.

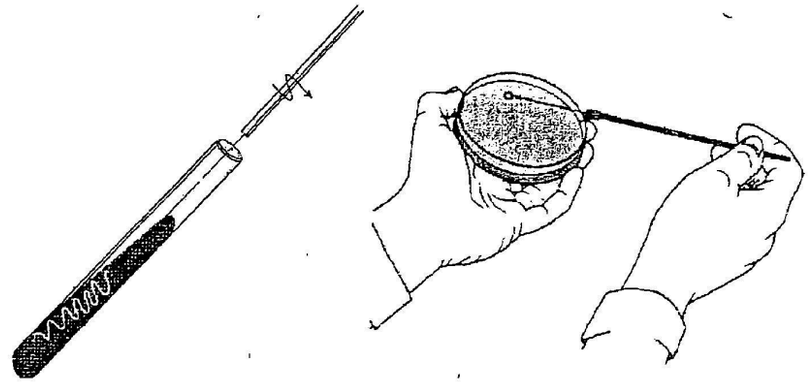

Fig. 19 – Însămânțarea prin descriere de striuri la suprafața mediilor solide

Însămânțarea în profunzimea mediilor solide

Însămânțarea în profunzimea mediilor solide se face prin încorporare sau prin înțepare.

Pentru însămânțarea prin încorporare, mediile (geloză, gelatină) se topesc în baia de apă și se răcesc la 45^0 C. Se introduce apoi o cantitate din produsul de însămânțat. Se omogenizează amestecul prin rularea tuburilor (eprubetelor) între palme, fără a se produce bule de gaz, după care mediile însămânțate sunt lăsate să se solidifice (Fig. 20).

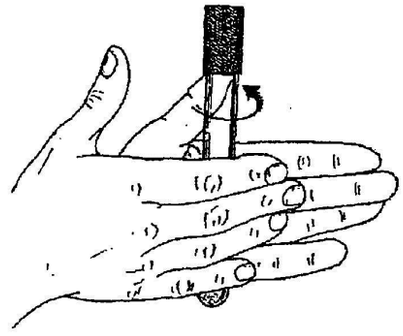

Fig. 20 – Omogenizarea amestecului prin rularea tubului între palme

Însămânțarea prin înțepare se efectuează cu ajutorul firului ansei după ce a fost încărcat cu produs (Fig. 21).

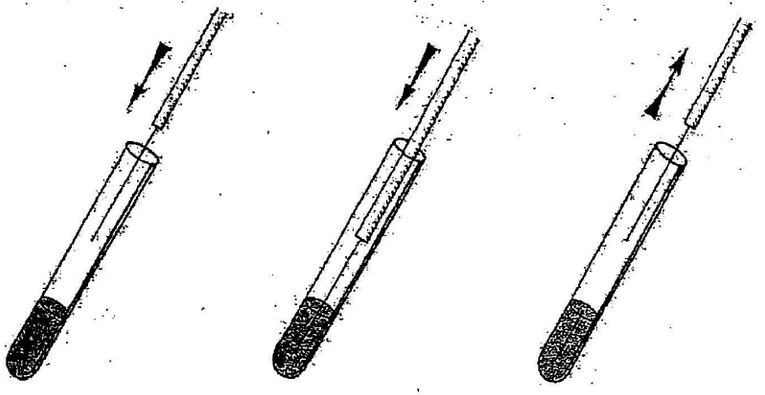

Fig. 21 – Însămânțarea mediilor de cultură prin înțepare

4.2. OBȚINEREA CULTURILOR PURE

O cultură pură conține o singură specie sau tulpină microbiană. Numai cu o cultură pură sunt posibile următoarele studii:

- morfologice (forma și mărimea celulelor);
- de cultură (caracteristicile de creștere ale culturii pe diferite medii);
- fiziologice și biochimice;
- de patogenitate (proprietatea microorganismelor de a produce boli la plante sau animale);
- serologice (studiul antigenelor celulare și a anticorpilor serici);
- genetice (variabilitatea, sexualitatea);
- bacteriofagi specifici.

Deși în natură se găsesc culturi pure, frecvența cu care se află într-un mediu este considerată foarte mică. O cantitate mică de sol fertil poate conține o varietate considerabilă de virusuri, bacterii heterotrofe și autotrofe, multe specii de drojdii, alge, ciuperci microscopice și protozoare. Aceste microorganisme pot exista ca saprofite, comensale, simbionte sau antagoniste.

Pentru izolarea unui anumit microorganism în cultură pură se pot folosi tipuri speciale de medii. În general, sunt folosite mediile de îmbogățire, selective sau diferențiale.

Agenții de îmbogățire și de selecție pot fi de natură chimică sau fizică.

Pentru izolarea microorganismelor termofile, mediul însămânțat se incubează la o temperatură ridicată (50^0 C sau mai mare).

Pentru izolarea microorganismelor halofile se adaugă în mediu NaCl 10%; pentru izolarea bacteriilor osmofile se adaugă o soluție concentrată de zaharoză. Multe bacterii plasmolizează în asemenea medii, în timp ce bacteriile halofile sau osmofile nu sunt afectate. De asemenea, pentru izolarea unor anumite tipuri de bacterii se utilizează o anumită valoare de pH a mediului, adăugarea de antibiotice specifice sau crearea condițiilor de anaerobioză.

Folosirea efectivă a acestor metode pentru izolarea unei culturi pure presupune cunoașterea caracteristicilor fiziologice și biochimice ale microorganismelor.

Tehnicile de izolare diferă în funcție de tipul de microorganisme: aerobe sau anaerobe.

Obținerea unor microorganisme aerobe în cultură se poate efectua prin mai multe metode:

- izolarea pe medii solide obișnuite, prin diluarea produsului cu obținerea de colonii izolate, pornind de la considerentul că o colonie provine din multiplicarea unei celule bacteriene;
- izolarea prin însămânțări pe medii speciale de cultură, favorabile dezvoltării numai a anumitor specii microbiene;
- izolarea bazată pe diferențele de comportare a bacteriilor față de agenți fizici, chimici și biologici;
- izolarea prin metode biologice, folosind animale de laborator.

4.2.1. Izolarea pe medii solide

Se realizează prin metoda diluțiilor succesive și prin metoda diseminării pe suprafața mediului.

Metoda diluțiilor succesive

Cultura sau materialul care conține microorganisme este diluată în apă sterilă, utilizând un anumit coeficient de diluare, de obicei egal cu 10. În acest fel, se obține o serie de diluții în care numărul celulelor scade în progresie geometrică. Pentru prepararea diluțiilor, apa sterilă este repartizată în eprubete uscate sterile, câte 9 ml în fiecare. Apoi 1 ml din suspensia inițială luat cu o pipetă sterilă este transferat în prima eprubetă cu 9 ml apă sterilă. Aceasta este diluția 1/10 (10^{-1}). Suspensia obținută este amestecată foarte bine cu ajutorul pipetei prin barbotare, ceea ce asigură o

14. DETERMINAREA SENSIBILITĂȚII BACTERIILOR LA ANTIBIOTICE (ANTIBIOGRAMA)

Determinarea sensibilității bacteriilor la antibiotice și chimioterapice este obligatorie pentru fiecare tulpină bacteriană, în vederea instituirii unui tratament corect.

Sensibilitatea bacteriilor față de antibiotice se testează "*in vitro*" punându-le în condiții optime și standardizate de cultivare (mediul de cultură, inocul, timp de incubare etc.) în prezența unor cantități descrescânde de antibiotic.

După modalitățile tehnice de realizare se deosebesc două metode de testare a acestei sensibilități:

- ✓ metoda difuzimetrică;
- ✓ metoda diluțiilor seriate.

14.1. METODA DIFUZIMETRICĂ KIRBY-BAUER

Tehnica Bauer adoptată de NCCLS (National Committee for Clinical Laboratory Standards) din SUA este larg utilizată în majoritatea țărilor. Este metoda uzuală pentru laboratoarele care testează un număr relativ mic de tulpini bacteriene cu creștere rapidă, fără diferențe semnificative a ratei de creștere de la tulpină la tulpină.

Principiul metodei

Prin depunerea discurilor (microcomprimatelor) cu antibiotice pe suprafața unui mediu solid însămânțat cu o cultură bacteriană, substanța antimicrobiană activă va difuza în mediu, prezentând o scădere constantă a gradientului de concentrație de la marginea microcomprimatului spre periferie.

După un anumit timp de incubație se vor contura două zone distincte: una în care creșterea microbiană este inhibată de concentrații de substanță antimicrobiană și o zonă de creștere, în care concentrația de antibiotic este prea mică pentru a inhiba creșterea.

Cu cât diametrul zonei de inhibiție este mai mare, cu atât germenele este mai sensibil, adică, cantitatea de antibiotic necesară inhibiției bacteriei testate (concentrația minimă inhibitorie = C.M.I.) este mai mică și invers. Există deci o relație invers proporțională între diametrul zonei de inhibiție și C.M.I. Aceasta se poate exprima grafic sub forma unei curbe de regresie pe o diagramă, cu ajutorul căreia se poate, cunoscând diametrul zonei de inhibiție, afla cu oarecare aproximație C.M.I.-ul respectiv.

Pentru tehnica Kirby-Bauer s-au putut măsura diametrele critice

care permit clasificarea germenilor studiați în "sensibili", "rezistenți" și "intermediari", în cazul unor infecții de gravitate medie și folosind antibiotice în doze medii.

Standardizarea condițiilor tehnice de lucru

Fiecare din componentele care concură la efectuarea antibiogramei pot influența diametrul zonei de inhibiție și deci criteriile de interpretare. Astfel, un mediu cu o concentrație mai mare de geloză va condiționa zone de inhibiție mai reduse și invers; un inocul prea bogat va determina un diametru de inhibiție mai redus și invers.

Condițiile tehnice referitoare la mediul de cultură (compoziție, pH), inocul, felul discurilor (microcomprimatelor), efectuarea testului, inocularea, trebuie precis standardizate.

Materiale necesare:

- plăci Petri cu agar Müeller-Hinton turnat în strat uniform de 4 mm grosime. Concentrația gelozei din mediu este de 1,5 - 1,7 %, pH-ul=7,2 - 7,4 măsurat înainte de turnarea în plăci (50° C). Acest mediu are o valoare nutritivă care permite dezvoltarea optimă a unei mari varietăți de germeni și nu conține inhibitori ai unor substanțe bacteriene;
- cultura bacteriană de cercetat și cultura martor de 18 ore. Este necesar ca inoculul din germenele de cercetat să fie reprezentativ, adică să cuprindă toate categoriile populațiilor microbiene, uneori heterogenă din punct de vedere al rezistenței. Pentru prepararea inoculului se va pleca de la 4-6 colonii identice, dezvoltate pe un mediu neselectiv;
- etalonul 0,5 din scara McFarland cu sulfat de bariu;
- tampoane de vată pe tijă de lemn;
- trusă cu discuri de antibiotice, comercializată;
- pensă sau repartitor automat de discuri;
- riglă gradată.

Tehnica de lucru:

- ➢ se însămânțează într-un tub cu bulion nutritiv cca. 5 colonii bine individualizate, reprezentative pentru bacteria testată; se ajustează turbiditatea corespunzătoare etalonului 0,5 McFarland; din momentul etalonării plăcile trebuie inoculate în minimum 15 minute;
- ➢ se procedează identic cu tulpina de referință (martor);
- ➢ se aduc discurile de antibiotice la temperatura camerei;

- se usucă suprafața agarului Müeller-Hinton (20-30 minute) la 37^0 C cu placa întredeschisă;
- se însămânțează plăcile; tamponul de vată se imersează în suspensia bacteriană etalonată; se scurge excesul de lichid prin rotirea tamponului de pereții eprubetei; se descarcă apoi tamponul în striuri paralele pe suprafața mediului, succesiv în trei direcții prin rotirea plăcii cu câte 60^0; în final cu vârful tamponului se parcurge circumferința plăcii; în acest fel inoculul este dispersat uniform;
- se lasă placa 3-5 minute, niciodată mai mult de 15 minute, pentru adsorbția inoculului;
- se depun discurile de antibiotic la distanță de 15 mm de marginea plăcii și 30 mm între centrele a două discuri vecine folosind o pensă sau un aparat automat de repartizare; ideal antibioticele se grupează pe familii;
- se incubează plăcile cu capacul în jos în funcție de bacterie (35^0 C pentru stafilococ, 37^0 C pentru restul bacteriilor); durata incubării este de 16-18 ore; este contraindicată așezarea a mai mult de două plăci una peste alta.

Citirea și exprimarea rezultatelor:

Se supun citirii numai plăcile care prezintă o cultură corespunzătoare din punct de vedere al purității și densității. Citirea se face cu ochiul liber, măsurând diametrul zonei de inhibiție în mm, de 2-3 ori în diferite direcții, cu ajutorul unei rigle gradate (Fig. 90).

Exprimarea rezultatelor se face prin transcripția directă a diametrului zonei de inhibiție în categorii de tulpini sensibile, rezistente sau intermediare (Tabelele VIII, IX).

14.2. METODA DILUȚIILOR SERIATE (DETERMINAREA C.M.I.)

Materiale necesare:

- eprubete sterile;
- pipete gradate sterile;
- mediu de cultură (bulion simplu, mediu Müeller-Hinton lichid);
- cultura în bulion de 18-20 ore a tulpinii de cercetat.

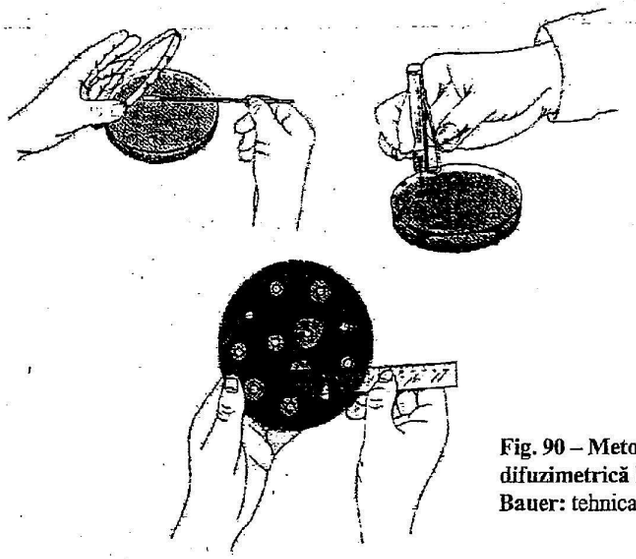

Fig. 90 – Metoda difuzimetrică Kirby-Bauer: tehnica de lucru

Tabel VIII – Aprecierea sensibilității la antibiotice funcție de diametrul zonei de inhibiție pentru o bacterie Gram pozitivă (*Staphylococcus* sp.)

Antibioticul	Concentrația de antibiotic/ micro-comprimat	Diametrul zonei de inhibiție (mm)			Echivalent C.M.I. (μg/ml)	
		R	I	S	R	S
Penicilină	10 unit	≤28	-	≥29	β lactamaze*	≤0,1
Oxacilină	1 μg	≤10	11-12	≥13	≥4	≤2
Ampicilină	10 μg	≤28	-	≥29	β lactamaze*	≤0,25
Meticilină	5 μg	≤9	10-13	≥14	≥16	≤8
Amoxicilină	20 μg	≤19	-	≥20	≥8	≤4
Cefalotin	30 μg	≤14	15-17	≥18	≥32	≤8
Vancomicină	30 μg	-	-	≥15	≥32	≤4
Gentamicină	10 μg	≤12	13-14	≥15	≥8	≤4
Kanamicină	30 μg	≤13	14-17	≥18	≥25	≤6
Tobramicină	10 μg	≤12	13-14	≥15	≥8	≤4
Eritromicină	15 μg	≤13	14-22	≥23	≥8	≤0,5
Tetraciclină	30 μg	≤14	15-18	≥19	≥16	≤4
Doxicilină	30 μg	≤12	13-15	≥16	≥16	≤4
Ciprofloxacin	5 μg	≤15	16-20	≥21	≥4	≤1
Nitrofurantoin	300 μg	≤14	15-16	≥17	≥128	≤32
Cloramfenicol	30 μg	≤12	13-17	≥18	≥32	≤8
Rifampicină	5 μg	≤13	17-19	≥20	≥4	≤1

R – tulpină rezistentă;
S – tulpină sensibilă;
I – tulpină intermediară;

β-lactamaze* - tulpinile penicilin-rezistente de *Staphylococcus aureus* produc β-lactamaze, fiind preferată testarea unor microcomprimate impregnate cu o concentrație de 10 μg penicilină în locul celor impregnate cu ampicilină; penicilina ar trebui utilizată pentru testarea susceptibilității tuturor antibioticelor din grupul penicilinelor β-lactamaz-labile cum ar fi: ampicilina, amoxicilina, carbenicilina, piperacilina.

Tabel IX – Aprecierea sensibilității la antibiotice funcție de diametrul zonei de inhibiție pentru o bacterie Gram negativă (*Escherichia coli* ATCC 25952)

Antibioticul	Concentrația de antibiotic/ micro-comprimat	Diametrul zonei de inhibiție (mm)			Echivalent C.M.I. (μg/ml)	
		R	I	S	R	S
Ampicilină	10 μg	≤13	14-16	≥17	≥32	≤8
Carbenicilină	100 μg	≤19	20-22	≥23	≥64	≤16
Amoxicilină	20 μg	≤13	14-17	≥18	≥32	≤8
Cefalotin	30 μg	≤14	15-17	≥18	≥32	≤8
Gentamicină	10 μg	≤12	13-14	≥15	≥8	≤4
Kanamicină	30 μg	≤13	14-17	≥18	≥25	≤16
Tobramicină	10 μg	≤12	13-14	≥15	≥8	≤4
Streptomicină	10 μg	≤11	12-14	≥15	-	-
Tetraciclină	30 μg	≤14	15-18	≥19	≥16	≤4
Doxicilină	30 μg	≤12	13-15	≥16	≥16	≤4
Acid nalidixic	30 μg	≤13	14-18	≥19	≥32	≤8
Cloramfenicol	30 μg	≤12	13-17	≥18	≥32	≤8
Nitrofurantoin	300 μg	≤14	15-16	≥17	≥128	≤32

Tehnica de lucru:

Se repartizează în eprubete sterile mediul de cultură în care se efectuează diluții succesive de antibiotic, în așa fel încât să se obțină în fiecare tub o concentrație precisă de antibiotic/ml.

Diluțiile trebuie să meargă de la cea mai mare concentrație a antibioticului posibilă de obținut în sânge, până la o concentrație inferioară C.M.I. a tulpinii martor.

Exemplu: penicilina se diluează, de obicei, în așa fel încât să se obțină următoarele concentrații de antibiotic/ml: 100 U.I.; 10 U.I.; 1 U.I.; 0,1, U.I.; 0,01 U.I.; 0,001 U.I.

Streptomicina și aureomicina se diluează astfel încât să existe la 1 ml mediu de cultură următoarele cantități: 100 μg; 10 μg; 1 μg; 01 μg; 0,01

μg și 0,001 μg.

În fiecare eprubetă, ca și în tubul martor (fără antibiotic) se însămânțează o cantitate constantă din cultura în bulion de 18 ore a tulpinii cercetate (în mod curent se pune în fiecare tub 1 sau 2 picături dintr-o diluție efectuată din cultura în bulion).

Se incubează tuburile la 37° C, timp de 20-24 ore.

Interpretarea rezultatelor:

Se face în raport cu dezvoltarea sau nedezvoltarea germenilor în mediu. Tubul martor trebuie să conțină o cultură corespunzătoare tipului cultural al speciei.

În primele tuburi din serie, în care cantitatea de antibiotic este mare, mediul, în cazul când bacteria este sensibilă, rămâne nemodificat. În tuburile următoare, cultura este slab dezvoltată, iar în ultimele tuburi, cultura va fi tot așa de bogată și cu același aspect ca și în tubul martor.

C.M.I. a antibioticului pentru tulpina testată este dată de concentrația de antibiotic din ultimul tub fără creștere evidentă a culturii (Fig. 91).

Metoda diluțiilor în mediul lichid dă informații precise privind C.M.I. a antibioticului. Este însă laborioasă și costisitoare.

Indiferent de metoda folosită, în testarea sensibilității la antibiotice a unei tulpini bacteriene se folosesc tulpini martor din colecție, care și-au păstrat nealterat spectrul natural de sensibilitate la antibiotice.

Exemplu:

- ✓ tulpina Oxford de *Staphylococcus aureus* (NCTC - 6571) în cazul testării bacteriilor Gram-pozitive;
- ✓ *Escherichia coli* (NCTC - 10418) în cazul testării bacteriilor Gram negative.

C.M.I. a tulpinilor martor trebuie să se reproducă (± o diluție) de la o determinare la alta.

Dacă se efectuează numărătoarea germenilor viabili din tuburile fără cultivare evidentă, se poate determina **concentrația minimă bactericidă (C.M.B.)** definită ca, cea mai mică concentrație de antibiotic care distruge în proporție de 99,9 - 100% bacteria din inoculul inițial.