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Eukaryotic Cell Culture

KNOW YOUR CELLS. When you start a series of experiments, someone in the lab may provide you with a tube or flask of cells, with brief instructions about the care of the cells. It isn't as simple as that. The cells may be easy to maintain, but the more you know about the cells, the more finely attuned you are to the cells' quirks, the quicker and more clear the interpretation of results will be. Look up the references for the cells. Speak to people who have used the cells, and ask for advice. Most importantly, monitor the cells constantly, until *you* are the expert on their growth.

TYPES OF CULTURES AND CELL LINES

Cell cultures are described in two ways:

- Origin of the cells.
- Manner of growth.

Classification by Origin

TABLE 1. Categories of Cell Cultures Based on Origin

	Origin	Similarity to original tissue	Ease of maintenance	Doublings
Primary cells	Animal tissue, fetal or adult	Representative	Difficult	0–1
Finite cell lines	Animal tissue, usually fetal	Representative	Difficult	Fetal: 20–80; adult tissue: very limited
Continuous cell lines	Spontaneous transformation of primary or finite cell lines	Not very representative; cells are less differentiated	Easy	Indefinite, with selection for higher growth rate
Transformed cell lines	Tumor tissue, spontaneous transformation in vitro of continuous cell line, or in vitro transformation with whole virus or virus DNA	Not very representative; less differentiated than parent	Easy	Indefinite, with selection for higher growth rate
Hybridomas	Fusion of antibody-secreting B cells and malignant myeloma cells	Not representative of either cell type, but not intended to be!	Difficult	Limited

Primary cells are cells isolated from animal or plant tissue and cultured.

Once a cell has divided it becomes a finite cell line, with the potential to become immortalized.

Repeated passage of a *finite cell line* derived from normal cells is done in a way to select for faster-growing variants that might become a *continuous cell line*. This is considered to be spontaneous transformation.

The origin of the primary cell or finite cell line will influence the growth pattern. If a cell is a fibroblast, it will have the adherent growth typical of fibroblasts. However, once the cell has been transformed, the growth pattern may no longer be typical of the original primary cell.

Continuous cell lines have a higher growth rate, a higher cloning efficiency, increased tumorigenicity, and more variable chromosome complement than finite cell lines.

Continuous cell lines are often manipulated to become transformed cell lines expressing a particular and needed phenotype.

The more differentiated the cell line, the slower it will grow.

Some commonly used cell lines

Cell line	Cell type and origin	Adherent or suspension growth
3T3	Fibroblast (mouse, embryo)	Adherent
BHK21	Fibroblast (Syrian hamster, kidney)	Suspension
MDCK	Epithelial cell (dog, kidney)	Adherent
HeLa	Epithelial cell (human, adenocarcinoma)	Suspension or adherent
PtK1	Epithelial cell (rat kangaroo, kidney)	Adherent
L6	Myoblast (rat, skeletal muscle)	Adherent
PC12	Chromaffin cell (rat, adrenal pheochromocytoma) Neural cell studies	Adherent
Sf9	(Ovary, fall armyworm) Baculovirus infection	Suspension
SP2	Plasma cell (mouse, myeloma) Fusion for hybridoma	Suspension

Transformed cells have been changed from normal cells to cells with many of the properties of cancer cells.

Some of these cell lines have actually been derived from tumors, or are transformed spontaneously in culture, by mutation. Cells can be deliberately transformed by a chemical or by a tumor-inducing virus. Such a virus carries a gene which induces either the errant or overproduction of a cell protein needed for growth, or the production of an aberrant protein needed for growth. No matter how transformation occurred, the result is a cell with altered functional, morphological, and growth characteristics, some of which are listed here:

- Growth to high cell density
- Lower requirement for growth factors and serum
- More anchorage independence
- Ability to proliferate indefinitely

Adherently cultured transformed cells are usually highly anchorage independent, and adhere lightly even to tissue culture dishes. Wash the cells very carefully, as the loose monolayer can be inadvertently aspirated away.

Hybridomas secrete monoclonal antibody into the media, often at a high enough concentration that the cell supernatant can be used directly for hybridizations.

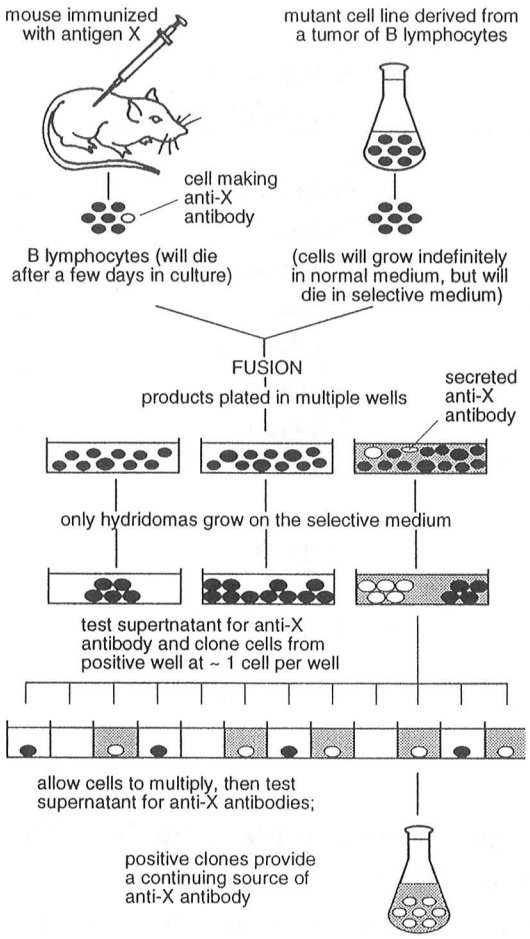

FIGURE 1.

Preparation of hybridomas that secrete monoclonal antibodies against a particular antigen (X). The selective growth medium used contains an inhibitor (aminopterin) that blocks the nonbiosynthetic pathways by which nucleotides are made. The cells must therefore use a bypass pathway to synthesize their nucleic acids, and this pathway is defective in the mutant cell line to which the non-B lymphocytes are fused. Because neither cell type used for initial fusion can grow on its own, only hybrid cells survive. (Modified, with permission, from Alberts et al. 1994.)

Classification by Manner of Growth

Cells are classified by the way they grow in liquid culture, or in semisolid medium. Growth characteristics are functional descriptions only, and are dependent on the origin of the cells.

Suspension and adherent growth are properties of the cell as well as of the culture conditions. Some cells can be manipulated to grow either way.

Suspension or adherent growth.

The first and most practical way cell growth is described is in terms of how the cells do in liquid culture. This information is always given with the cell line and, with origin of the cells, constitutes the major definition of the cell line.

- **Suspension cells** grow suspended in the growth medium. They are able to survive and proliferate without attachment to the culture vessel. Cells cul-

tured from *blood, spleen,* or *bone marrow,* especially *immature* cells, tend to grow in suspension. Cells in suspension look like little balls. The *advantages* of suspension growth are the *large numbers* of cells that can be achieved, and the *ease of harvesting.*

- **Adherent cells** grow in a monolayer, attached to the surfaces of the culture vessel. Cells that derive from *ectodermal* or *endodermal* embryonic cell layers tend to grow adherently. This includes fibroblasts and epithelial cells. Cells grown adherently have various shapes, but are, generally, flattened: The same cells grown in suspension would then become rounded. The advantage of adherent growth is the ability of the cells to adhere and spread on surfaces such as coverslips, making *microscopy, hybridizations,* and *functional assays* more easily performed.

Anchorage-dependent and anchorage-independent growth.

A subdivision of adherent growth is anchorage-dependent versus anchorage-independent growth. Although anchorage independence is measured in soft agar (in which the cells are imbedded/suspended), it can also have an effect on culture in tissue culture dishes. Anchorage dependence and independence are properties of the cell and cannot be altered by growth conditions.

- **Anchorage-dependent cells** require attachment to the surface for cell proliferation.

- **Anchorage-independent cells** do not require attachment for cell proliferation. This is often a property of transformed cells. Growth of anchorage-independent cells in tissue culture dishes looks more haphazard than the growth of anchorage-dependent cells, with the cells only loosely applied to the surface.

OBSERVING CELLS

It must become second nature to you to look at every culture of cells, macroscopically and microscopically, whenever you take out a flask to subculture or use for an experiment. If your cells aren't healthy, your experiments won't work reproducibly.

As you remove the culture vessel from the incubator, note:

- The color of the medium. There is a pH indicator in most media (see below, under "Medium") that turns yellow if it is acid or purplish if it is alkaline. Medium too basic or acidic can indicate contamination, an overgrown culture, a dead culture, or faulty CO_2 measurement or delivery.

- Any cloudiness in the medium, which could indicate contamination or a grossly overgrown culture.

- Clumped cells (suspension culture) or peeling cells (adherent culture).

If you have a flask or dish of cells, look at it with an inverted microscope under 40X power, before you even put it in the hood. Make it your routine to place any flask from the incubator directly on the microscope.

Adherent cells.
You should see a fairly regular arrangement of cells that appear to be relatively flat on the surface of the plate. Each type of cell has a characteristic shape in culture: round, triangular, squarish, elongated. The pattern of growth may be like a cobblestone street, or in swirls, or there may be random growth, with some cells seemingly growing on top of each other.

Within the individual cells you may see a darker round shadow of the nucleus, with the even darker nucleoli present. Sometimes the nucleus is so large that very little cytoplasm can be seen. Cells in cell division may appear as spheres, sometimes in pairs: Hourglass shaped (mitotic) cells with obvious alignment of the condensed chromosomes might even be seen.

Suspension cells.
Suspension cells are spheres. Even in a suspension culture, some cells may adhere lightly to the flask: These may not move when the flask is gently moved and may appear to be slightly flattened or triangular. On top of those cells you can see the round, apparently floating cells. They may appear granular, but you will not be able to see the nucleus or other organelles, even under 100X.

Most inverted microscopes only have a 40X lens as the highest power lens. If you want to observe your cells at higher magnification (if, for example, you want to check for bacterial contamination) you can remove a sample, and either fix and stain it or make a wet mount, and observe it under oil at 100X on an upright compound microscope (See Chapter 16).

You must learn to recognize what is normal for your cells. When you have a new cell line, look at as many cultures as you can, so you will be able to define for yourself the look of a "normal" culture. Consult with someone in the lab for even minor questions about the cells.

OBTAINING CELLS

Have a **hepatitis B vaccine** if you are working in a lab that deals with primary human cells, and especially, with blood cells. Even if you are not isolating the cells yourself, any spill or aerosol could be a risk.

Primary Cells

Primary cells are difficult to obtain. An animal must usually be killed and dissected, the cells isolated and cultivated. It is time consuming and fraught with possible mistakes. For many cell types, the numbers obtained are small, and the cells are short-lived. But primary cells are the closest approximation to the "real" thing, and if you really need them (and think hard about that), the trouble is worth the results.

You cannot assume someone will give primary cells to you. Often a lab won't have enough for their own experiments. Request primary cells with consideration of the difficulty of obtaining them, and do not be offended if you are refused.

- **Ask someone in the lab or department** who works on the cells. For a first experiment, ask whether you could get any extra cells from the next isolation he performs. Offer to help in any way.
- **Ask another investigator** who works on the cells. Unless this is someone you already have a good relationship with, this can be tricky. If the primary cells are vital to the experiments and are particularly difficult to obtain, you may want to officially *collaborate* with the procurer of the cells. Check with the head of the lab first, because a collaboration can have undesirable political ramifications.

A request letter should

Introduce yourself and your lab (briefly!)
Introduce your project (briefly!)
Say how you heard of the investigator's cells (briefly)
Say what you will do with the cells (briefly but honestly)
Give your address and phone number
End with mucho thanks

A request letter can

Offer a collaboration

If you do not hear from the investigator in a few weeks, follow up with an E-mail or phone call. Requests for cells are generally pretty low priority, and such letters often stay on the bottom of the pile, so don't take a lack of answer personally.

- Even if someone else provides you with the cells, **observe the procedure** at least once. Things change, and you may have to do it yourself in a hurry one day. It is also good to know as much about the cells as possible, and to show interest and appreciation to the provider of the cells.

A collaborator has a responsibility to supply the cells, but she then becomes a part of the project and an author on any publications resulting from experiments with the cells.

- **Hospitals and medical schools** are a good source for certain human tissue and cells, but you will have to find a contact to help you obtain the samples. Many **blood banks** may provide partially isolated blood cells for a fee.

Obtain as much information as you can about the maintenance of the cells.

- Most likely, you will have to **isolate the cells yourself.** Don't just get a protocol and try: If possible, ask someone who has done the isolation to show you.
- There are several **companies** that can provide primary cells, at a fairly steep price: This is worth it for a one-time experiment, when it doesn't make sense to lay out a huge expenditure on the isolation of a difficult or new cell type. Call your purchasing department for the numbers of those companies and, if possible, ask the company for the names of scientists who have used the cells and would be willing to talk to you.

Watch the isolation by the provider of the protocol with your protocol in hand, noting down anything that isn't clear to you or wouldn't be remembered if you were alone. Cell isolations are notoriously dependent on the trivial details—such as how long you shake the tube, which centrifuge was used, how high to fill the plate—and you want to get as much of that detail as possible.

Continuous Cell Lines

If a cell line grows well, it is usually simple to obtain cells. If it doesn't grow well, you may have the same difficulty getting the cells as you would obtaining primary cells.

Treat cells as infectious agents. *Cells may harbor a variety of viruses and other organisms. Observe the same precautions you would for infectious agents. No mouth pipeting! Wear gloves! Be careful!*

- Never forget that cell lines experience **phenotypic drift** with continued culture, and that the same cell line cultured in two different labs could well have different characteristics. If you are obtaining cells for particular experiments, get the cells from the people doing those experiments.
- If you are using the same cells as the rest of the lab members, **ask someone for a frozen vial.** This is preferable to cells obtained after a split, as the passage number will be smaller, and the cells more likely to run to type. If you take cells from a

split for a quickie experiment, the likelihood is that you will never get an earlier batch, and will regret it down the line.

- One of the most common and dependable sources for cell lines is the **American Type Culture Collection** (ATCC). This is a private, nonprofit organization that collects, preserves, and distributes (for a small fee) cultures of human and animal cells, as well as microorganisms, viruses, DNA probes, and plants. The cultures are guaranteed to be viable and contamination-free, and will be replaced if there is any problem in culturing them. The catalog contains not only the lists and descriptions of the cell lines, with references, media and freezing media formulations, and useful notes, but also has useful general information and tips.

You must reference the source of the cells in all publications and seminars.

- There are other **service organizations,** usually dealing with more limited cell types, who will provide cells free or cheaply. Some investigators have NIH grants to maintain certain strains and to provide those cells to others for research.

If the investigator obtained his own cells from ATTC or another commercial source, you should not bother him for the cells, but should get them from ATCC yourself. The only exceptions are if money is really scarce for you, or if the investigator has derived his own cell line or subgroup from the ATCC line.

- Look in the **Materials and Methods** section of the best papers dealing with the cells you want, and find the source of the cells used for the experiments. Note any particulars given, such as passage number or media used, as you will want to replicate conditions as much as possible. If the cells originated with that investigator, contact him for the cells.

PROTOCOL

Culturing Frozen Cells

If you go to a lab to pick up the cells yourself, bring some dry ice in an ice bucket in which to transport the cells.

Background

Eukaryotic cells are usually frozen in medium with serum and a freezing additive, and are stored in liquid nitrogen at –196°C. If ordered commercially, they will arrive in a vial or ampoule on dry ice. Frozen cells must be thawed rapidly and cultured immediately to maximize viability. To remove the freezing additive the medium is changed after 24 hours.

Frozen cells should be cultured as soon as possible. If you cannot culture the cells immediately, store them in liquid nitrogen.

Materials

- Culture medium, warmed to 37°C
- Culture vessel
- 70% ethanol in a small beaker
- 1-ml and 10-ml pipet, pipettor and tips

Procedure

1. Hold the vial in a 37°C water bath and agitate it rapidly. It will thaw in about 1 minute.
2. Drop the thawed vial in a beaker containing 70% ethanol and place it in a laminar flow hood for all manipulations.
3. Open the ampoule or tube. Do not allow ethanol to drip inside the vial.
4. Transfer contents of ampoule to culture vessel and immediately add warmed medium.
5. Replace cap and incubate for 24 hours.
6. Replace medium with fresh medium.
7. Incubate for 2–3 days or as indicated in the directions for those cells.

Cells are usually frozen in 1 ml volume, and are added to 10 ml of fresh medium.

*The freezing medium can also be removed **before** culture by spinning down the thawed cells and resuspending them in fresh medium. This is only necessary if the cells are particularly sensitive to the freezing additive or if you won't be there the next day to change the medium.*

Ampoules are made of glass and are under pressure, and there is a very real chance that the ampoules can explode. Always open ampoules in a biosafety cabinet: If none is available, wear eye and face protection. You should wear eye and face protection whenever you are manipulating samples in liquid nitrogen.

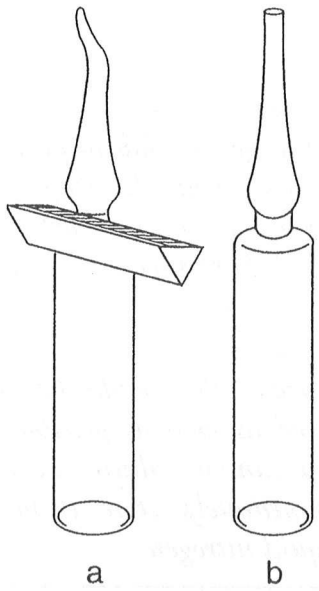

FIGURE 2.

Types of ampoules. Standard ampoules (*a*) must be nicked on the neck with a glass file that has been dipped in ethanol: After making a 1/8th score on one side, wrap the ampoule in gauze or a paper towel, hold the base firmly in the left hand and snap the top off with the right hand. Prescored ampoules (*b*) usually have a band around the neck, and can be wrapped and snapped open directly.

CELL MAINTENANCE

Routine Maintenance of Cell Lines

In order to maintain the cells, they must be:

- **Fed** (supplied with fresh medium). During growth, the medium will be depleted of needed factors and must be replaced.

Feeding and passaging are accomplished at the same time: It would be difficult to do one without the other.

- **Split** (passaged, the cell number reduced). During growth, the number of cells in the culture will increase beyond the capacity of the vessel and medium to sustain them.

- **Frozen.** Whenever you obtain or generate a cell line, aliquots of it must be frozen away. These aliquots are a backup for you should the cells phenotypically drift or all become contaminated.

Rule 1. Look at your cells! Never split, experiment with, or freeze cells without looking at the culture macroscopically, and on an inverted microscope. By constant observation, you will not only find contamination before it becomes a major problem, and be aware when a cell line has started to drift by noting morphological alterations, but you will "know" the cells, know when to split, when to experiment, and when to dump the experiment.

Feeding and Passaging

The faster the cells grow, the more often they must be fed and split. When you obtain the cells, find out:

- Are antibiotics used?
- How are they grown?
- What medium works best?
- Do the cells require serum?
- How often should the cells be fed and split?

Antibiotics

Antibiotics are used standardly in many labs but should not be needed if aseptic technique is being properly done. The half-life of many antibiotics is quite short at 37°C (see Table 2), so there often isn't as much antibiotic as the investigator believes.

With valuable cells or cells prone to contamination because of a lot of manipulation, it is sometimes too nerve-wracking for some investigators not to include antibiotics in the medium.

Antibiotics for cell culture are usually obtained as a dehydrated powder in a sterile vial. Rehydrate with sterile water or solvent. It is not necessary to filter-sterilize such a packaged antibiotic.

Antifungal agents amphotericin B (Fungizone) and Mycostatin (Nystatin) are not recommended for routine use, because they can affect the membrane permeability of all eukaryotic cells.

Two standard antibiotics for cell culture

Gentamicin 5–10 mg/ml stock, final 50 µg/ml
Penicillin (10,000 units)/**Streptomycin** (10 mg/ml) stock, final 100 units/ml and 100 µg/ml

Aliquot in 1-ml tubes and store at –20°C. Add 1 ml of either solution to 99 ml of medium. If you use large quantities of media, make up 5-ml aliquots to add to 500-ml bottles of medium.

Culture Vessels

The type of vessel you use will depend on the kind of cells and the volume of cells you need. Most labs employ flasks and dishes that can be adapted to both adherent and suspension cultures, but there are a variety of containers for large-scale and specialized cell culture.

Most tissue culture containers are disposable, made of polystyrene, and have been radiation-sterilized. Glass containers are occasionally used, but the ease of use of disposable containers has made them the universal choice.

Untreated plastic is usually fine for suspension cells, but most adherent cells grow better on treated plastic. Labware companies sell "treated" dishes and flasks for tissue culture: The treatment (a permanent modification of the polystyrene surface with, for example, plasma or amino functional groups) varies from company to company. Cells may prefer the plates from a particular company, so it is not a trivial act to switch culture dish sources.

Plates may be treated with a protein that works nonspecifically, perhaps by supplying a positive charge. An example of this is poly-D-lysine.

Some cells require adherence to a particular substrate for differentiation or the expression of certain functions. This is usually a component or a mixture of components of the extracellular matrix, such as collagen, fibronectin, and laminin.

Dishes can be treated in the lab by adding a suspension of the material in solution to the dish to cover the surface, incubating the dish to promote attachment, pouring off the excess solution, and washing the surface with buffer or medium.

TABLE 2. Antibiotics Used in Cell Culture Medium

Antibiotic	Effective against	Working[a] concentration	Stock solution	Half-life in media (37°C)	Structural class	Mechanism of action
Benzylpenicillin (Penicillin G)	Gram-positive bacteria	100 units/ml (~100 µg/ml)	10,000 units/ml	2 days	Penicillins	Inhibits cell wall synthesis
Streptomycin	Gram-negative bacteria	100 µg/ml	10 mg/ml	4 days	Aminoglycosides	Inhibits translation (30S subunit)
Kanamycin	Gram-negative bacteria	100 µg/ml	10 mg/ml		Aminoglycosides	Inhibits translation (30S subunit)
Tetracycline	Broad-spectrum bacteria	10–50 µg/ml	5 mg/ml		Aminoglycosides	Inhibits translation (30S subunit)
Gentamicin (Gentamycin)	Broad-spectrum bacteria, mycoplasma	50 µg/ml	5 mg/ml	15 days	Aminoglycosides	Inhibits translation (30S subunit?)
Lincomycin	Mycoplasma, Gram-positive bacteria	50 µg/ml	5 mgl/ml			Inhibits translation (50S subunit)
Tylosin	Mycoplasma Gram-positive bacteria	10 µg/ml	5 mg/ml		Macrolide	Inhibits translation (50S subunit)
Amphotericin B (Fungizone)	Fungi, yeast	2.5 µg/ml	250 µg/ml	4 days	Polyene	Alters membrane permeability
Nystatin (Mycostatin)	Fungi, yeast	20 units/ml	2000 units/ml		Polyene	Alters membrane permeability

(Reprinted, with permission, from Harlow and Lane 1988.)

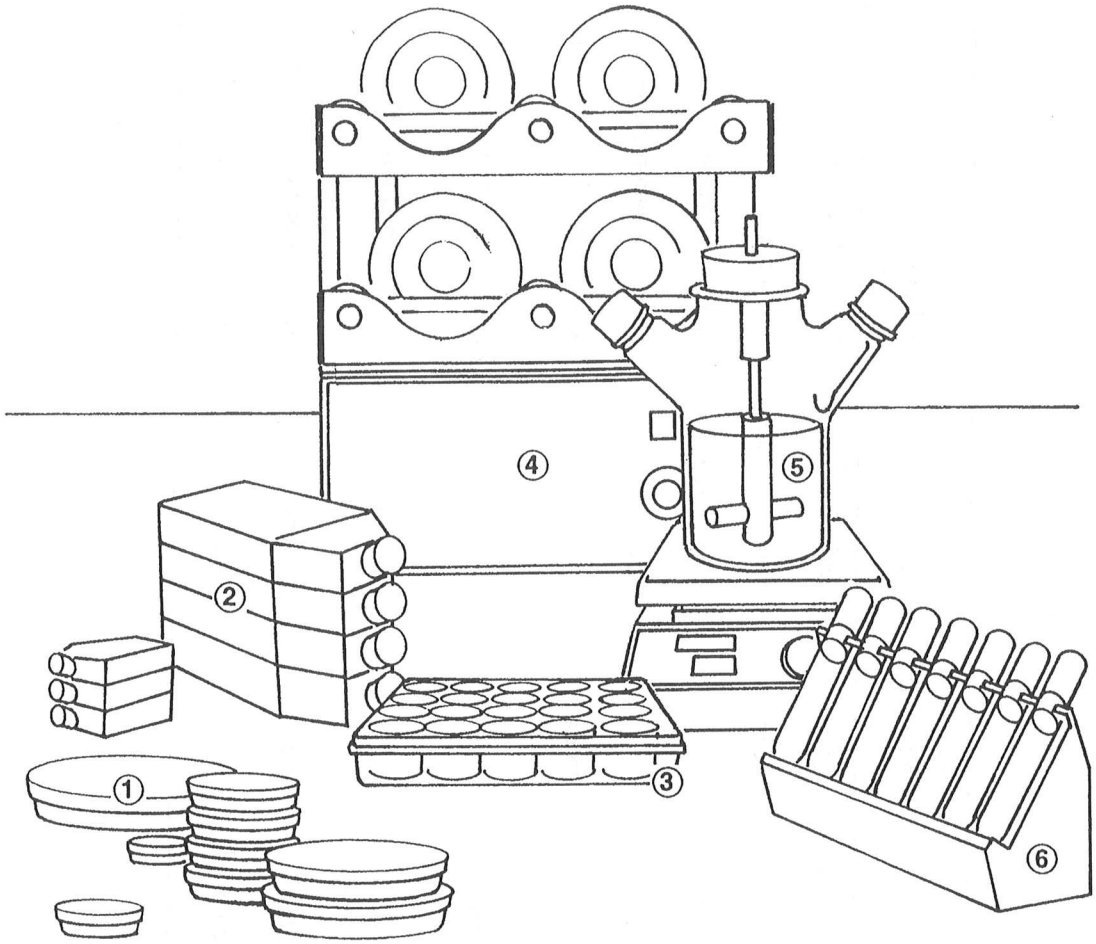

FIGURE 3.

Cell culture containers. **Key:** (1) *Dishes.* Generally used for adherent cells, most are treated to maximize attachment of cells. The 100-mm size, which holds 10 ml, should not be confused with bacterial dishes of the same size, as bacterial dishes are not treated for cell attachment. The 60-mm size holds 4–5 ml, and the 35-mm, 1–2 ml. (2) *Flasks.* Available with straight or canted necks: Straight necks minimize sloshing, which is good for suspension cultures, and canted necks permit easier access to the culture surface, which is useful when manipulating adherent cells. However, either neck type can be used for either adherent or suspension cultures. 25 cm^2 (50 ml), 75 cm^2 (250 ml), 175 cm^2 (750 ml) are common sizes. (3) *Multiple-well tissue culture plates.* A standard size of 86 x 128 mm is divided into 6-, 12-, 24-, 48-, or 96-well sizes, and is compatible with automatic diluters and plate readers. These are used for hybridoma and monoclonal antibody work, titrations, toxicity testing, and any experiment that requires a comparison of different cell treatments. They can be used for adherent and nonadherent cells. Plates are usually purchased treated, and some sizes are available with flat or round-bottomed wells: Most instruments require flat-bottomed wells. (4) *Roller bottles.* Usually used for maximum yield. Adherent or suspension cells can be grown in roller bottles. Can be used in an open or closed system. (5) *Spinner bottles.* Designed to spin gently, without subjecting cells to harsh mixing. Used for suspension and microcarrier cell cultures, as well as for insect cultures. Different gas mixtures can be added, and the bottles can be used in an open or closed system. (6) *Tubes.* For adherent cells. Round bottoms allow adherence on all parts of the tube. Leighton tubes have one side flattened, permitting microscopic observation.

Cell Medium

Commercially prepared medium has an expiration date, which should be nonrigidly adhered to. Use expired medium for cell washes.

Most labs either buy the medium *already prepared and bottled,* or as a *powder that must be rehydrated and filter sterilized.* Of course, the latter choice is less expensive, and especially makes sense if the medium is being used in bulk.

Cell media look alike, since most contain phenol red or another dye as a pH indicator. But they are not alike, so don't just use what medium is available. Check the *formulation* you need, and order it from a company that guarantees its media to be mycoplasma-free.

Medium with a phenol red pH indicator will look

Lemon yellow below	pH 6.5
Yellow at	pH 6.5
Orange at	pH 7.0
Red at	pH 7.4
Pink at	pH 7.6
Purple at	pH 7.8

Most cells grow best when medium is around pH 7.4.

Yellowish medium is *acidic* and can indicate
- An overgrown culture
- Bacterial contamination
- Too much CO_2 in the incubator

Purplish medium is *alkaline* and can indicate
- A sparse and non-growing culture
- Mold contamination
- Too little CO_2 in the incubator

Be careful when adding anything directly to cells, especially compounds dissolved in DMSO, ethanol, or methanol. These compounds are toxic to cells at high concentrations, and you should shake or stir the container while adding the compound to dilute the compound as quickly as possible.

Some cells require the addition of other components to the prepared medium. L-Glutamine is a common addition, as some cells will fairly quickly exhaust the glutamine in the medium. Filter-sterilized and frozen aliquots can be thawed and added to the medium at the time of use.

Cell culture medium contains many heat-labile components, so you should store the media in the cold. However, medium should be warmed to 37°C before being added to cells: Never shock the cells by the addition of cold medium. But don't leave your bottle of medium in a 37°C water bath until you get around to feeding your

cells, because many ingredients, such as some antibiotics, have a shorter half-life at 37°C. Warm medium 10 minutes before use. Better yet, remove and warm only what you will need.

Even on slow-growing cells, the medium must be changed regularly, because the cells still will metabolize and may exhaust some of the medium components.

It is convenient to buy or make medium in 500-ml bottles. If you are using a 500-ml bottle a week, then add the serum and other labile components to this bottle. Don't forget to only have (or remove) enough medium in the bottle so you can add the other components for a final 500-ml volume.

If you will be adding 10% serum and 5 ml of antibiotics, set up bottles of medium with 445 ml of medium. Add the serum and antibiotic aliquots only as you need fresh medium.

Serum

Serum supplies needed growth factors and nutrients. Some cells, particularly transformed cells, have a very low serum requirement of around 0.5%. Some cell lines have been "trained" to survive in medium with low serum. As the needed components for serum are defined, more and more cells can be cultured with supplements and individually added components. This is a fortunate situation for you if your cells can be cultured without serum.

Serum is very expensive. *Always aliquot and freeze serum, and add it to medium just before use. Store unused portions of thawed aliquots in the refrigerator, where it will be fine for several weeks.*

These are the serum variables you must consider:

- **The percentage of serum.** Most cells require 5–20% in the medium for good growth.
- **The type of serum.** Some cells like horse serum. The standard for tissue culture cells is calf serum. Some cells require the more expensive fetal calf (also known as fetal bovine) serum, and some cells (usually human) require serum of their own species, the most expensive serum proposition of all.
- **Whether or not the serum is heat-inactivated.** Serum is subjected to heat to inactivate components such as complement.

To heat-inactivate serum

Thaw the frozen serum at room temperature. This may take 5 or 6 hours for a 500-ml bottle of serum. Incubate the thawed serum at 65°C for 30 minutes. Aliquot the serum. Aliquot for the percentage of serum and the amount of medium you usually use. Freeze and store. Thaw aliquots in a 37°C water bath as needed.

- **From where you should obtain the serum.** Ask someone in the lab, or who has worked on the cells, as particular cells only tolerate certain serum. There is great competition among companies selling serum, but this is not the place to save money!

Serum varies from lot to lot. Many labs assay lots of serum for their ability to support cell growth and function and buy bulk quantities of the best lot. There is also company-to-company variation, so always check with someone in the lab for the tried and tested best source of serum for your cells.

How to split cells

Each cell line grows at its own rate and will require splitting at an individual schedule. The density at which a cell grows profoundly affects its physiology, so you must take pains to maintain cells at a healthy density and to always use cells grown to the same density for experiments.

Cell cultures should be split so that they are seeded to a defined density. In theory, this means that the cells should be counted every time they are split. In practice, cells are often split at a particular ratio of old-to-new medium—say, at 1:4 (10 ml of cells are added to 30 ml of medium). Well, it is sloppy, but it does work for some cells. If you split your cells this way, check them before and after splitting sometime so you know what your cell numbers are. Always record the split ratio.

It is convenient to split cells twice a week. Most cell lines have a doubling time of approximately 24 hours, and so will grow to the original number in just a few days. You could split to a lower number of cells, but too sparse a culture won't grow. You could just wait and split once a week, but the cells will stop growing at high density and may not be as healthy a culture.

Common mistakes in culturing cells

- Letting stock cultures overgrow because you don't have time to split them on the proper day
- Using cultures that are obviously the wrong density for an experiment instead of waiting another day or splitting the cells again
- Using old media instead of making fresh media

Cells growing in a monolayer must be dislodged from the culture vessel and put into a single cell suspension. Since cells secrete extracellular matrix components to which they will bind tightly, and may be attached to each other by Ca^{++}-dependent receptor-ligand interactions, it can be difficult to do. Dislodging can be done physically, by scraping, but this does cause cell injury. Generally, cells are loosened by enzymatic degradation of the cell adhesion and extracellular matrix components with trypsin. Particularly strongly adherent cells are also treated with EDTA for chelation of Ca^{++} and with varied proteinases to digest the matrix.

Use the gentlest treatment that works on your cells.

TABLE 3. Cell Dissociation for Transfer or Counting; Procedures of Gradually Increasing Severity

1.	Shake-off	Mitotic or other loosely adherent cells
2.	Trypsin[a] in PBS (0.01–0.5% as required, usually 0.25%, 5–15 min)	Most continuous cell lines
3.	Prewash with PBS or CMF, the 0.25% trypsin[a] in PBS or saline-citrate	Some strongly adherent continuous cell lines and many cell lines at early passage stages
4.	Prewash with 1 mM EDTA in PBS or CMF, then 0.25% trypsin[a] in citrate	Some strongly adherent early passage cell lines
5.	Prewash with 1 mM EDTA, then EDTA 2nd rinse, and leave on, 1 ml/5 cm	Epithelial cells, although some may be sensitive to EDTA
6.	EDTA prewash, then 0.25% trypsin[a] with 1 mM EDTA	Strongly adherent cells, particularly epithelial and some tumor cells (*note:* EDTA can be toxic to some cells)
7.	1 mM EDTA prewash, 0.25% trypsin[a] and collagenase,[a] 200 units/ml PBS or saline-citrate or EDTA/PBS	Thick cultures, multilayers, particularly collagen-producing dense cultures
8.	Scraping	All cultures, but may cause mechanical damage and usually will not give a single cell suspension
9.	Add dispase (0.1–1.0 mg/ml) or pronase (0.1–1.0 mg/ml) to medium and incubate until cells detach	Will dislodge most cells, but requires centrifugation step to remove enzyme not activated by serum. May be harmful to some cells

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[a]Digestive enzymes are available (Difco, Worthington, Boehringer Mannheim, Sigma) in varying degress of purity. Crude preparations, e.g., Difco trypsin 1:250 or Worthington CLS grade collagenase, contain other proteases that may be helpful in dissociating some cells but may be toxic to others. Start with a crude preparation and progress to purer grades if necessary. Purer grades are often used at a lower concentration (mg/ml) as their specific activities (enzyme units/g) are higher.

PROTOCOL

Splitting Adherent Cells

1. Aspirate medium from the cell monolayer.
2. Gently add warm (37°C) PBS or culture medium without serum. Pipet the medium onto a wall of the culture vessel, not onto the cells, to avoid dislodging loosely adherent cells.
3. Aspirate the PBS or medium wash from the monolayer.
4. Add 0.25% trypsin/PBS, just enough to cover the cells when the culture vessel is tilted.
5. Aspirate the trypsin, almost immediately: Leave it on the cells only 10–30 seconds before removing it.

Cells are washed to remove traces of serum, which inhibits trypsin. You can use old medium without serum for washes.

6. Incubate the cells at room temperature for 5–15 minutes, checking every couple of minutes to see whether the entire monolayer slides when you tilt the culture vessel. (You can see this macroscopically.) When it does, you can end the incubation. Alternatively, you could incubate the cells at 37°C for 5 minutes.
7. Add fresh medium (the same volume you removed) and pipet vigorously to break cell clumps. Check on the inverted scope to be sure you have a single cell suspension and pipet until you do.
8. Remove 1 ml of cells to a microfuge tube.
9. Count the cells.
10. Figure out the dilution you must make to get to the recommended seeding concentration.
11. Remove that quantity of cells to a fresh culture flask.
12. Add the calculated amount of fresh medium (which has been warmed to 37°C).

Example:

If you have counted the cells and they are at 1.0×10^6 cells/ml, the desired seeding concentration is 10^5 cells/ml. The final volume in the flask will be 10 ml. You want to dilute the cells at 1:10. Add 1 ml of cell suspension to 9 ml of medium.

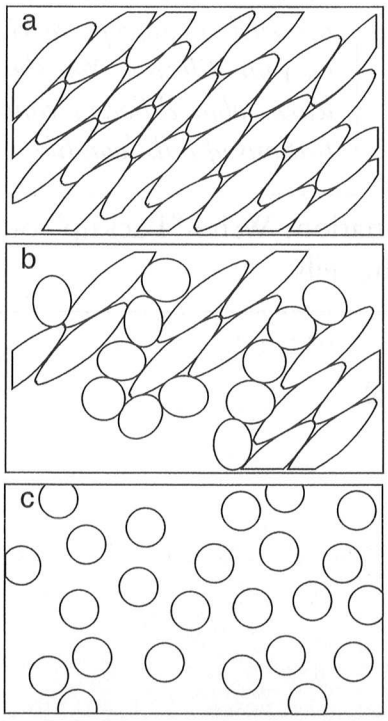

FIGURE 4.

Dissociation of adherent cells. The cells of the monolayer (*a*) appear flat and of low contrast. As cells are released from the substratum they will still be clumped together (*b*). Quite quickly after this, all cells are released from the surface, most are single cells (*c*), and fresh medium can be added.

13. Rotate the flask gently to be sure the cells are dispersed evenly over the surface.
14. Place the cells in the incubator. Be sure the cap is loose.

If you are using 100-cm plates for culturing your cells, be sure you are using tissue culture dishes, and not bacterial culture dishes. Tissue culture dishes are treated to promote cell adherence; cells will not stick to bacterial dishes.

PROTOCOL

Splitting Suspension Cells

1. Gently shake the culture flask so you can get an even suspension of cells. Remove 1 ml of cells to a microfuge tube.
2. Count the cells. Don't forget to look at the cells while you are counting, to check for the general condition.
3. Figure out the dilution you must make to get to the recommended seeding concentration.
4. Remove that quantity of cells to a fresh culture flask.
5. Add the calculated amount of fresh medium (which has been warmed to 37°C).
6. Place the cells in the incubator. Be sure the cap is loose.
7. Also incubate the original flask, without adding medium. Whenever you split cells, retain one of the original flasks as a backup in case of contamination. Write "Backup" on the flask, and discard that flask when you next split cells.

Look at the cells on the inverted microscope to check on your dilution. You should learn what different concentrations of cells look like.

Example:

If you have counted the cells and they are at 2.3×10^6 cells/ml,
The desired seeding concentration is 5×10^5 cells/ml.
The final volume in the flask will be 10 ml.
($2.3 \times 10^6 = 230 \times 10^5$ so you can ignore the 10^5.)

23: 5 as 10 : x where x is the # ml of cells.

$x = 2.2$

Add 2.2 ml of cell suspension to 8.8 ml of medium.

PROTOCOL

Microscopic Count of Viable Cells

A sample of cells are mixed with *trypan blue*, a dye which is excluded by living cells, but stains dead cells a dark blue color. The cells are placed on a kind of gridded slide called a *hemocytometer*, and are counted manually under a microscope.

Materials

- Hemocytometer (improved Neubauer type), cleaned and dried each time. (Other hemocytometers can be used: the grid will look different.)
- Hemocytometer coverglass (reusable), cleaned and dried each time.
- 0.4% trypan blue (w/v) in PBS.
- Counter.
- Cells in suspension, either a suspension culture or a trypsinized adherent culture. Be sure adherent cells are trypsinized enough that they are single cells (see Fig. 4) and that you swirl the flask and take a representative sample.
- Pipettors, tips.
- Microfuge tubes.
- Phase contrast microscope, upright or inverted, with 10X objective.
- Sterile cell medium for dilutions.

Estimation of the number of cells is important in maintaining and freezing cell lines, and you should set yourself up so you can do it quickly and easily. Keep a hemocytometer and trypan blue stain ready.

A Coulter counter can also be used to count cells. Hemocytometer counting is more practical for single samples and has the added advantage of allowing you to actually look at the cells.

Procedure

1. Place the coverslip evenly on the middle of the hemocytometer.

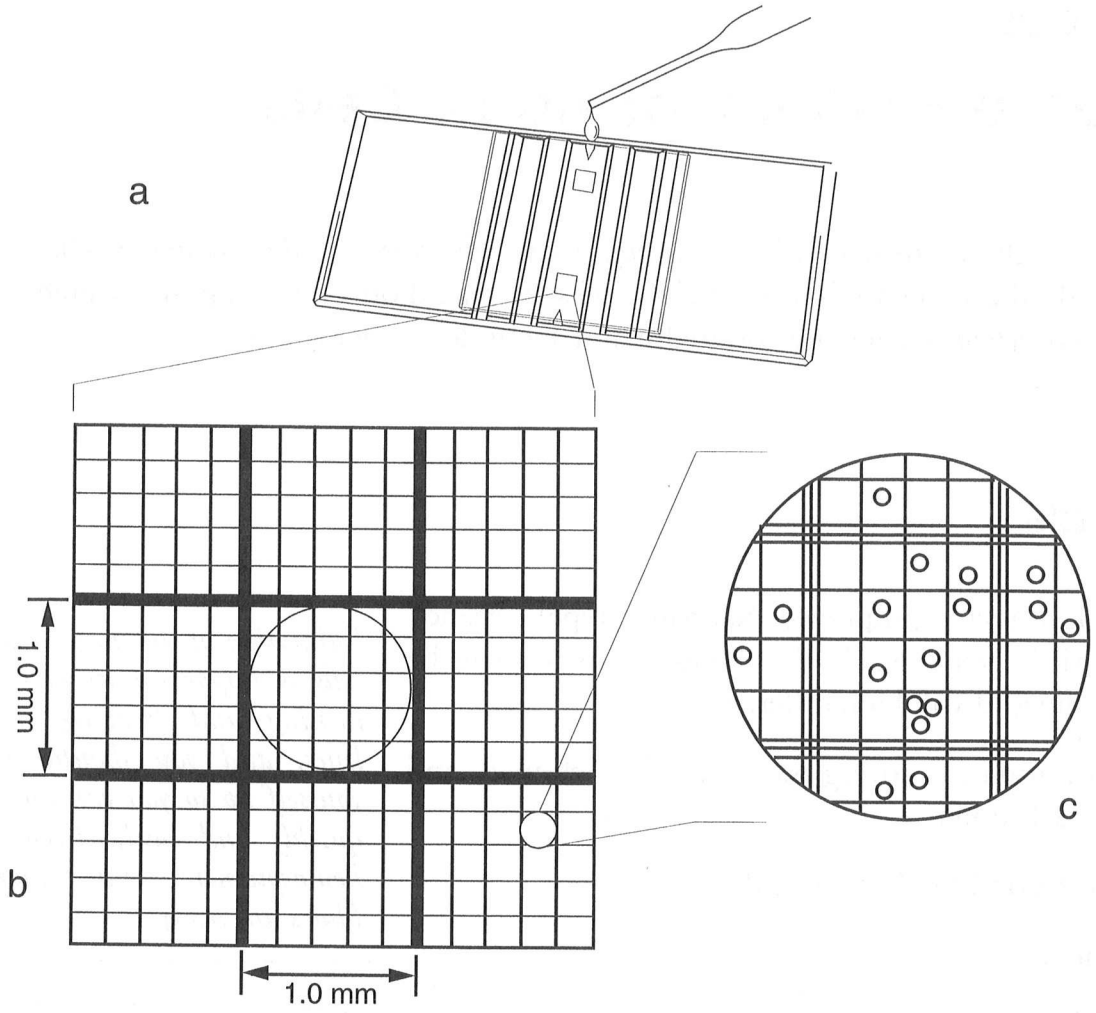

FIGURE 5.

Counting cells with a hemocytometer (Improved Neubauer). The hemocytometer is loaded with cells in both of the chambers on either side of the central trough (*a*). As you look at the slide with a 10X objective (100X total magnification), each 1-mm-square grid will take up the field of view. Each 1-mm square is divided into 25 smaller squares (*b*). Each of the 25 squares is divided into 20 smaller squares, to aid in counting small cells or sparse numbers of cells (*c*). It is the cell count for a 1-mm square that is used to calculate the cells/ml. (Modified, with permission, from Freshney 1994; copyright by Wiley-Liss, Inc.)

2. Remove 500 µl of cells and medium to a microfuge tube. If sample volume is low, take 100 µl.
3. Place 50 µl of the cells and 50 µl of the trypan blue solution in a microfuge tube. Tap the tube to mix.

Not having a well-suspended and well-mixed sample to add to the counting chamber is the major source of error in hemocytometer counting.

4. Remove 50 µl from the cell/trypan blue mix with a pipettor. Add 20 µl to each side of the coverslip by allowing a drop held at the end of the tip to be taken under the slide by capillary action. (If you have two different samples,

you can carefully load them one on each side.) Fill both sides of the chamber even if you only have one sample, or the cell count won't be accurate.

If you have fewer cells than 30, you could concentrate the cells by spinning an aliquot and resuspending in a smaller volume, but it usually isn't worth it. Make three independent counts to be able to best estimate your cell numbers.

5. Place the hemocytometer immediately on the stage of the microscope, and locate the grids in the hemocytometer at low power. You will only count cells in any one of the 1-mm-square areas, so move to a corner and up the magnification so that one entire grid takes up the field. You can count at low power, but it is more difficult to distinguish dead from living cells. If you are using an inverted microscope, drop the objective so you can get enough light—adjusting this will make a big difference.

6. Roughly count the cells in a 1.0-mm area, 25 boxes worth, to see if your cells need to be diluted or concentrated. Ideally, you should have between 30 and 300 cells/mm. If you have more, make a 1:5 or 1:10 dilution (For 1:10, mix 50 µl of cells from tube in step 2 with 450 µl of medium or buffer; mix and take 50 µl to add to 50 µl of trypan blue as in step 3.)

*To avoid counting cells twice, count only cells that lie on the top and left-hand-side lines of each box, and don't count cells that lie on the bottom and right-hand-side lines of each box. **Uniformity of counting**—counting the cells the same way every time—is the only way to get reproducible numbers.*

7. Count the viable cells in a 1-mm square. Dead cells will stain blue all over the cell, whereas viable cells will not stain (although they may have a blue rim or appear to be granular). It helps to have a counter with two channels, so you can count both dead and living cells and get a percentage of viable cells.

If your counts are very different from each other, say, over 20% different, you may not have resuspended the cells well enough or there may be clumps.

8. Count the cells in a total of three different 1-mm squares, and divide by three to get an average number of cells/1-mm square.

9. Calculate the number of cells per ml.

Example:

You have counts of 113, 99, and 118 (with an average of 110).

$110 \times 10{,}000 = 1.1 \times 10^6$ cells/ml

Since you mixed 50 µl of cells with 50 µl of trypan blue, your dilution factor is 2.

$2 \times 1.1 = 2.2 \times 10^6$

2.2×10^6 cells/ml is the number of cells in the original culture.

The average number of cells/1-mm square × 10,000 × dilution of sample = number of cells/ml in original sample.

10. Calculate the percentage of viable cells in the original culture. Divide the number of viable cells in three 1-mm areas by the total number of viable and nonviable cells in three 1-mm areas and multiply by 100.

A viability of less than 80–90% indicates an unhealthy cell population. The most likely explanation is that the cell culture is too dense, but it may also indicate contamination or a problem with the medium or serum.

FREEZING AND STORAGE OF CELLS

As cells grow the phenotype may change, or drift. Since it is important that your cells be predictable, you should freeze every cell line as soon as possible.

Unfortunately, it is still not possible to freeze and thaw most primary cells.

Before freezing cells

Check the requirements for freezing your particular cells.

- Check the ATCC catalog for the formulations for freezing the particular cell type.
- Certainly, check with lab members for modifications of the recommended freezing medium and conditions, but be wary of this information because many lab members have never themselves consulted the ATCC catalog or any literature but have fallen back on general techniques that usually work, will probably work, but may not be maximum in effectiveness for these particular cells.
- If it is a new cell line, call the originator of the line (check the literature).
- If you can find no information for your cells, do use one of the basic recipes given below.

Be sure you have sterile freezing ampoules (cryotubes). These must be screw-cap plastic ampoules, made to resist severe cold. They may be flat or round bottomed, either of which is fine for freezing (but you may want the flat bottomed for centrifuging). Glass ampoules may also be used. *Don't use snap-cap microfuge tubes.*

Make sure you already have space for your cells in liquid nitrogen. Liquid nitrogen tanks are notoriously crowded, and you don't want to get there with your thawing cells, searching for a tower. Also, if you put your cells in the wrong place, someone may dump them.

PROTOCOL

Freezing Cells

1. Grow cells to log phase in the usual maintenance medium/serum.
2. Perform a viable count. Do not freeze down cells with greater than 20% dead cells.
3. Determine how many cells and ampoules you will need. Each ampoule will take 1×10^7 cells (or between 4×10^6 and 2×10^7 cells) in 1 ml of medium.

Too few cells, and the culture may never start to grow upon refreezing: too many, and the culture will be unhealthy.

Freeze cells down at a concentration at which, when they are diluted 1:10 at thawing, they will be at 5× the normal seeding concentration.

Example:
If the normal seeding concentration is 5.0×10^5 cells/ml, you want cells after thawing and resuspension in fresh medium at 2.5×10^6 cells/ml. Since the cells will be diluted 1:10, freeze cells down at 2.5×10^7/ml.

4. Prepare freezing medium, 1 ml/aliquot plus 10%. Freezing medium typically contains regular culture media, 10–20% serum, and 5–10% glycerol or DMSO. If you don't know what freezing medium to use, freeze the cells in 20% serum and 10% DMSO.
5. Resuspend the pellet in freezing medium by pipetting gently.
6. Dispense 1 ml per ampoule. Keep cells on ice.
7. Place the ampoules in a freezer box you have lined with paper towels. Put in your vials and place the box near the top of a freezer that is –60°C or less. Leave them there for 16 to 24 hours.

Label each ampoule, even if you are preparing a box of ampoules. Write the cell type, the passage number, number of cells, the date of freezing, your name. Do not use tape labels, which become brittle and break off in the cold. Write on the vial or ampoule with a permanent lab marker, one that doesn't wash off with ethanol.

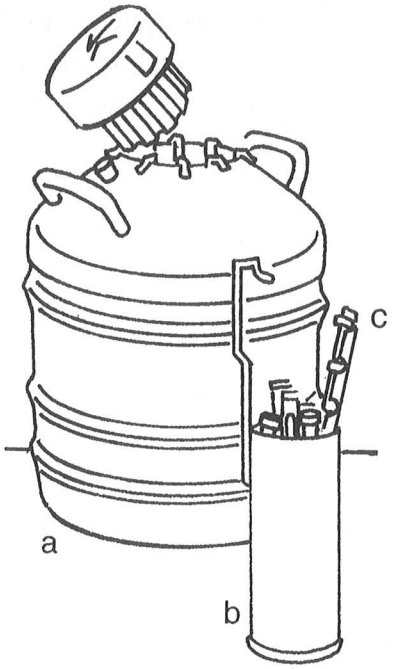

FIGURE 6.

Liquid nitrogen tank (*a*), canister (*b*), and canes (*c*). Remove the cap of the tank, and lay it aside. Grasp the hooked end of the canister and lift it up and out of the tank. Remove the appropriate cane from the canister, and snap out (or in) the tube. *Immediately* replace the cane, canister, and cap. Wear gloves and face protection, even to remove one tube.

8. Pour a bit of liquid nitrogen into an ice bucket, and place your tubes in here while you transport them to the liquid nitrogen freezer. If you can't get liquid nitrogen, place the vials on dry ice.

If you have a programmable freezer, cool the ampoules at –3°C/min until –60°C is reached.

9. Put the tubes in the appropriate place and immediately record their location in your files, and in a liquid nitrogen book, if one is kept.

Using liquid nitrogen

- Cells are kept either in a liquid nitrogen tank, which is filled with liquid nitrogen, or in a liquid nitrogen freezer, which is hooked up to a large tank of nitrogen. Tanks must be refilled manually every few weeks with more liquid nitrogen.
- Automatic fill tanks should be checked periodically to be sure they are working properly and that the tank is not empty.
- Wear heavy gloves when manipulating canes, racks, or boxes. Liquid nitrogen can cause bad burns.
- Wear, at least, latex gloves when removing a tube from a box or cane.

Don't open the tank or freezer if the liquid nitrogen is low. You want to keep the contents as cold as possible.

- Don't hang into liquid nitrogen tanks or freezers, or deeply breathe the vapor. Use your hand to "brush" away the vapor, and you will see the liquid nitrogen below.
- Protect your eyes with safety glasses when you do any manipulations with liquid nitrogen, because vials can suddenly break and shatter in the extreme cold.
- Never put loose vials in a tank or freezer. Each tube must be in the appropriate box or cane. Request space well in advance of the time you will need it from the person in charge.
- Don't disable the alarm. Check periodically to be sure no one else has disabled it.
- If you hear an alarm from a liquid nitrogen freezer or tank, immediately notify the lab. If it is the weekend or 3 A.M., first check the alarm panel to see if, indeed, the liquid nitrogen level or temperature is low. Then call the person in charge.

CONTAMINATION

Contamination happens, but it is usually a drag and can be a disaster. Good aseptic technique will prevent most problems, but remain vigilant always to detect contamination in its earliest stage. Look at every flask you take out of the incubator; in fact, get in the habit of running your eyes over all the flasks in the incubator, whenever you open the door.

If you see a contaminated flask belonging to someone else, notify that person immediately.

Contamination can be caused by bacteria, yeast, fungi, mold, mycoplasma, and by other tissue culture cells.

How to Recognize Contamination

Macroscopically. Look at the flask or dish of cells as you pick it up, and hold it to the light, checking for:

- **Cloudiness.** Even in a dense culture, the medium should be clear. Look for cloudiness in the medium or patches of cloudiness that move and shift with movement of the flask. Some molds may actually form colonies that float on the medium surface.

- **Medium color change.** The phenol red pH indicator in red medium will turn yellow in acidic conditions, magenta in alkaline conditions. A good bacterial infection will often turn the medium yellow, and fungal contamination may turn it hot pink.

Check the flask before throwing it away! A rapidly growing or overgrown cell culture might also turn the medium yellow, and a problem with the CO_2 level in the incubator could cause yellow (too much CO_2) or pink (not enough CO_2) medium.

- **Smell!** Obviously, don't open the flask and stick your nose in the flask. But many contaminants have a noticeable and characteristic odor that might even be detected as you open the incubator door.

Microscopically. Look at the flask or plate on low power (10x objective) first and then, more thoroughly, at higher power (40x objective, 400 total magnification) on an inverted microscope, scanning up and down with the focus knob to examine different "depths" of the medium. Look for:

To determine if a shape is a microorganism, look for regularity. There are occasionally mineral precipitates from the medium, or cell granular extrusions and debris, but these will be irregular in appearance.

- **Other organisms.** At low power, the mycelia of *fungi* will appear as long strands, often across the entire field. *Yeast* may also be visible at this magnification, and will appear to be smooth balls, sometimes with budding.

  At high power, bacteria may be seen. These may be rods, or cocci, singly or in chains or clumps. They may be associated with the cells, and be seen apparently within the cells. The cells may be lysed. There may be so many that the cells are obscured. There may be only one contaminant every two fields (which still counts as contamination!). They may be motile.

Don't confuse motility with Brownian motion, as all particles will gently vibrate back and forth. Cell granules may gently shake, and can resemble bacterial contamination.

- **Damaged cells.** An infection can kill cells: It can even cause every cell to lyse and/or cause a monolayer to lift off the plastic completely. More likely, the cells become more irregular looking (and smaller or larger), with a dark granularity visible even at low power.

Unless they are extremely rare and almost impossible to obtain, do not try to rescue contaminated cells by pulsing them with antibiotics! Throw the cells away!

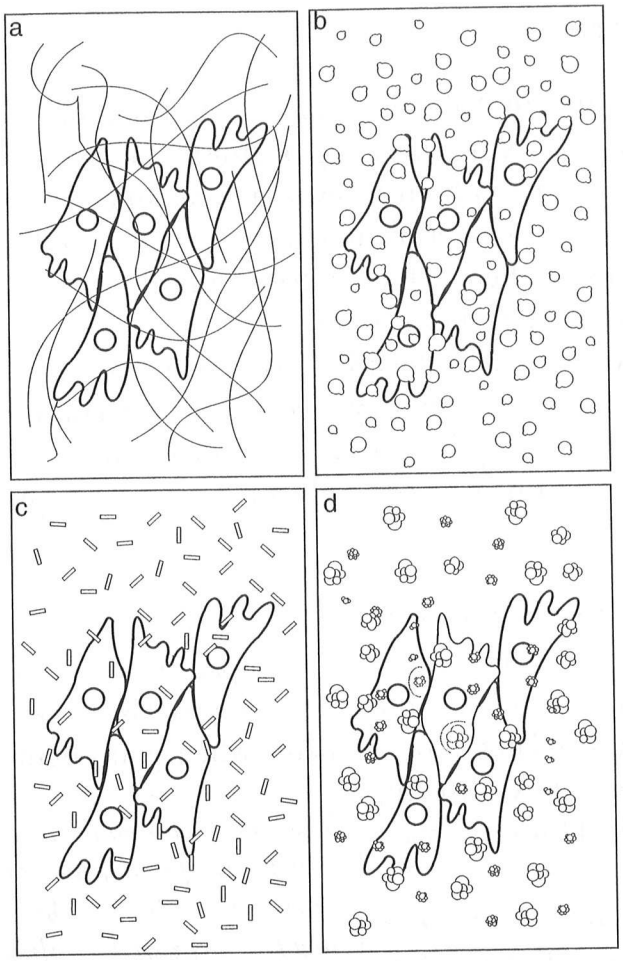

FIGURE 7.

Contaminants in an adherent cell culture under 400X power on the inverted microscope. (*a*) Mold; (*b*) yeast; (*c*) bacteria, small rods; (*d*) bacteria, clumps of cocci. Some of the cocci have been internalized by the adherent cells.

Suspension cultures are harder to observe microscopically than adherent cultures, especially at higher power.

- After you place the flask on the microscope stage, leave it for a minute to let the turbulence slow.
- Focus on the bottom of the flask, and look carefully for slightly adherent cells that may have attached microorganisms.
- Focus up through the flask, and whenever you have cells in focus, use your fine focus knob to examine the medium around the cells for contamination.

If you have a doubt about the presence of contamination

1. Make a wet mount or a stained slide (see Chapter 16). Centrifuge 1 ml of medium from the cells and resuspend in 50 µl of medium or buffer. Put on a slide and add a coverslip for a wet mount, or smear, fix, and stain with methylene blue or a Gram stain, and observe under high power.
2. Streak some of the cells/medium on a nutrient or blood agar plate, and incubate at 37°C for 3 days. Colonies? Contamination!
3. Remove 1 ml of cells/medium to a sterile microfuge tube and incubate for 3 days in the incubator. Observe for cloudiness, and make a wet mount.

Mycoplasma Contamination

Recognizing mycoplasma contamination. A difficult and ubiquitous problem! Mycoplasmas are the smallest self-replicating organisms, as small as 0.3 μm, and can generally not be seen with an inverted microscope. These bacteria have no cell wall, and are not susceptible to commonly used antibiotics. Investigators usually don't know their cells are infected until the viability decreases or the cells don't function as they should.

Without the aid of special stains, or unless the cells have been visibly damaged by a particularly heavy infection, the only way mycoplasma infection is suspected is that experiments stop working. The only way to detect and treat mycoplasma contamination is to routinely screen cells for it, a very simple procedure.

Preventing mycoplasma contamination. Use only media and serum that have been certified to be mycoplasma free. Most companies can make this guarantee. Only obtain cells that are guaranteed to be mycoplasma free. This is easy when ordering commercially, more difficult when you obtain cells from another investigator. Ask directly whether the cells have been checked for mycoplasma contamination.

The most common way in which mycoplasma are introduced to cells is through newly acquired, already infected cell lines. Test new cell lines for mycoplasma, and never freeze a culture until it has been checked.

Routine screening is a commitment, and investigators and labs who only employ cells for short-term experiments don't want to bother. Fine. They might never know that the reason they couldn't raise antibodies against a cell surface protein, or express a protein effectively, was that they were using mycoplasma-infected cells.

Screening for mycoplasma contamination. If the integrity of the cells is important to you, screen them routinely every 4–6 weeks. There are a number of ways to do this:

- **Fluorescent staining of mycoplasma** is the easiest, cheapest, and most common way to screen.
- **PCR detection using mycoplasma-specific primers** might be the easiest way if your lab is geared up for running gels and doing PCR. You can make your own primers, or order them (and the appropriate controls) from a company.

- **Send a sample away to one of the many companies that does mycoplasma testing.** The company may provide mailers, but this isn't as fast (although it is easier and more expensive) as doing it yourself.

And if you find mycoplasma contamination... Good luck.

- The best bet is to throw the cells out and start with another thaw.
- You could ignore it.
- There are methods to "cure" a culture of mycoplasma, but they are difficult and should only be used for irreplaceable cells. The best bet is to contact a company that manufactures kits for removal of mycoplasma. ICN and Boehringer-Mannheim have effective kits: Call and ask them for suggestions.

Most non-cell biology labs do nothing, on the "why fix it if it ain't (obviously) broke" belief. But mycoplasma contamination can cause changes in cell function that aren't obvious. It can cause chromosomal abnormalities, loss of the characteristic of interest, reduced capacity to support viral growth.

Cross Contamination

Cells can infect other cell cultures. This cross contamination is thought to be quite widespread, with many investigators inadvertently growing, using, and freezing contaminating cells instead of the intended one.

This is very preventable.

- **Obtain your cells from a reputable place,** or check the identity of the cells yourself. There are companies that can check this.
- **Don't ever work on more than one cell line at a time.** Don't even have flasks of different cells in the hood at the same time.
- **Never use the same pipet** for different cell lines.
- **Never use the same bottle** of medium or trypsin for the same cell line.
- **Never put a pipet back** into a bottle of medium after you have used it to pipet cells.

- **Use plugged pipets** for cell maintenance.

Check the cells for cross contamination if your cell line suddenly grows or functions differently than usual, and you have ruled out mycoplasma contamination. (Change can also be explained by mutation or drift.)

CO_2 INCUBATORS AND TANKS

CO_2 is used in an open culture system to regulate the pH of the cell medium. It is purchased as a cylinder of compressed gas and is dispensed into an incubator that can monitor and report the CO_2 content as well as maintain a set temperature.

CO_2 Incubators

- Never put any cells in an incubator unless you have checked with the other incubator users.
- Water in a stainless steel pan in the incubator prevents the cultures from drying out. Also, CO_2 detectors are accurate only in a humidified atmosphere. Keep the pan filled with sterile distilled water, and change the water once a week. Don't use bacterial growth inhibitors in this water, which may damage the stainless steel.
- Frequent cleaning of the trays that hold the cultures will help prevent contamination. Wipe down the trays with 70% alcohol at least weekly. Autoclave the trays monthly.

Never use Clorox or another chlorine bleach to clean trays. It is toxic to the cells.

- Minimize opening and closing the incubator door. Don't leave the door ajar while you carry your cells to the hood. Fluctuations of temperature and CO_2 levels aren't good for many cell lines.
- The incubator alarm will sound when the CO_2 runs low.
- Most CO_2 incubators are set at 37°C: Some may not be, so check. A buzzer or alarm will sound when the temperature rises above or falls below the setpoint. Reset the temperature. If the incubator needs to be repaired, a temporary incubator must be found.

Never change any setting on an incubator without consultation with all lab members.

- A thermometer is often kept on one of the trays in the incubator, to confirm the temperature readings of the incubator's temperature sensor. Be careful not to break the thermometer in the incubator! The most accurate thermometers are

mercury filled, and an incubator contaminated with mercury might never be able to support cell growth again. To accurately check the temperature, place the thermometer in a beaker of water in the incubator.

- A buzzer, but possibly an alarm, will sound when the water jacket needs water. This water jacket is needed to maintain the temperature. Fill it with a hose attached to deionized water (not distilled). Do not add antibacterial agents to the water. If the incubator does not have an alarm, look for condensation on the roof on the chamber, which indicates that the water in the jacket is low.

If the alarm goes off for a CO_2 incubator (indicating that the gas has run out) and you don't have another tank, ***don't open the incubator door!*** *The gas inside will last about a day if you don't open the door.*

- Most incubators are set on 5% CO_2, but the setting depends on the particular cells and the medium used.
- CO_2 readings on the incubator readout can be inaccurate. Occasionally, and whenever changes in medium color make you suspect a problem, you should determine the proper CO_2 percentage in the chamber with the use of a Fyrite gas analyzer. This instrument is the best way to get an independent CO_2 reading, but it must be done carefully to get accurate results.

Measurement of CO_2 levels with a Fyrite gas analyzer. The Fyrite (Bacharach, Inc.) uses a volumetric gas analysis method involving selective absorption of CO_2 in a chemical solution. The aspirator bulb pumps the gas sample into the analyzer and purges the measuring chamber of the previously analyzed sample.

Always store the Fyrite in the hard plastic carrying case. Don't be put off by the long directions. Analysis of the CO_2 concentration in an incubator will take about 30 seconds.

1. Hold the Fyrite upright and away from your face. Press the plunger momentarily to vent the tester.
2. Invert the Fyrite to drain the liquid from the top.
3. Turn the Fyrite upright, and allow the fluid to drain to the bottom.
4. Hold the Fyrite at eye level. Loosen the locknut at the rear of the scale. Slide the scale until the top of the fluid column lines up with the zero on the scale. Tighten the locknut.
5. Attach the open end of the rubber gas sampler hose to the sample port on the incubator. Do not attach the tube to the Fyrite tester at this time. Pump the aspirator bulb a few times to clear the air from the sampler line.

6. Hold the Fyrite upright, and place the rubber connector tip for the sampler tube over the plunger valve, and pump the aspirator bulb at plunger valve during the final squeeze.
7. Invert the Fyrite, and allow all the liquid to drain from the top. Turn upright, and allow all the liquid to drain to the bottom. Repeat once.
8. Momentarily hold the Fyrite at a 45° angle to allow the fluid droplets to drain to the bottom.
9. Hold the Fyrite upright. Allow the fluid a few seconds to stabilize. Determine the percent CO_2 from the level of the fluid column. A delay of 5–10 seconds in taking the reading may result in a slight error; a longer delay may result in a substantial error.
10. Repeat steps 6–10 until two consecutive readings agree.
11. Remove the Fyrite hose from the sample port to allow the chamber to breathe.

The dark red fluid floating on the top of the Fyrite solution is normal. It has been added to the solution to prevent excess foaming at the meniscus and does not indicate defective fluid.

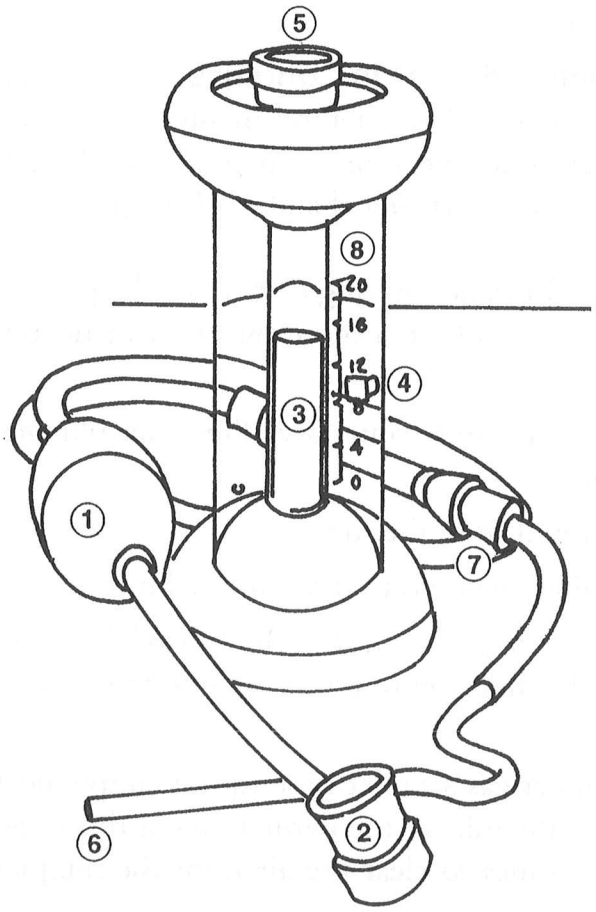

FIGURE 8.

Fyrite gas analyzer. **Key:** (1) *Aspirator bulb.* (2) *Connector tip.* (3) *Fluid.* (4) *Locknut.* (5) *Plunger.* (6) *Sampler hose.* (7) *Saturator filter.* (8) *Scale.*

Precautions for using the Fyrite

- The reagent used for the chemical absorption and measurement of gas in the Fyrite is potassium hydroxide that has been dyed red. Potassium hydroxide is corrosive and poisonous. If you get some on your skin, *flush with water.* Follow that washing with a rinse with vinegar: If your lab or your lunch doesn't happen to have vinegar, flush longer with water. If it gets in your eyes, flood with water and then wash with a 5% boric acid solution. Seek medical help.
- Do not invert the Fyrite when the plunger is depressed.
- Do not hold the Fyrite near your face when the top plunger is depressed.
- Always hold the Fyrite by the fins to prevent heat transfer from your hands.
- Always moisten the filter in the sampling tube before taking a sample. Failure to do so will result in inaccurate readings.
- For maximum accuracy, the Fyrite must be at ambient temperature. Do not store the Fyrite in a location subject to extreme temperatures, such as a windowsill.
- Check the strength of the Fyrite fluid whenever the instrument is used. After taking a reading with the Fyrite, do not vent the sample. Invert the Fyrite again, and take another reading. If there is an increase of 1/2% or more on the second reading, fluid replacement is necessary. Follow the manufacturer's directions. Fresh Fyrite fluid should be good for approximately 350 samples.
- With the Fyrite vented and in the vertical position, it should be possible to adjust the zero scale to the top of the fluid column. If this is not possible, fluid should be added or removed. To add fluid, hold the Fyrite upright and press the plunger. Add clean tap water a few drops at a time. To remove fluid, consult a manual or the manufacturer.

CO_2 Tanks

- A tank of compressed CO_2 is attached to the incubator and delivers CO_2 at a level determined by presetting the incubator.
- The tanks and attachments to the incubator look more complicated than they are. If you are careful, there is very little danger.
- CO_2 tanks are usually delivered to a central location at the institution, and are brought to the lab as needed. The tanks are rented, so return the empty tanks as

Be careful when using compressed gases. Carbon dioxide (and argon, helium, and nitrogen) are inert, colorless, odorless, and tasteless, but can cause asphyxiation and death in confined, poorly ventilated areas. They can cause severe frostbite to the eyes or skin. If treated carelessly, tanks of compressed gas can explode.

soon as possible to reduce costs. Returns are usually handled at the same central location as deliveries.

- The cylinder cap protects the cylinder valve from mechanical or weather damage. It should be removed from the cylinder only when the cylinder is supported and ready to be attached to the incubator.
- Many incubators have an automatic CO_2 tank switch, a small box that usually sits on top of the incubator. This system monitors the regulated CO_2 supply into the incubator, and will switch from the empty tank to a full one. It also sounds an alarm if all tanks are empty, and turns off the CO_2 supply during a power failure to prevent toxic CO_2 accumulation in the incubator.

The regulator

The gas in cylinders is at very high pressure; in fact, dangerously high pressure. The regulator reduces the pressure of the gas so it can be used safely.

- There are two basic types of pressure regulators, single stage and two stage. They look the same. The two-stage regulator will deliver a more constant pressure

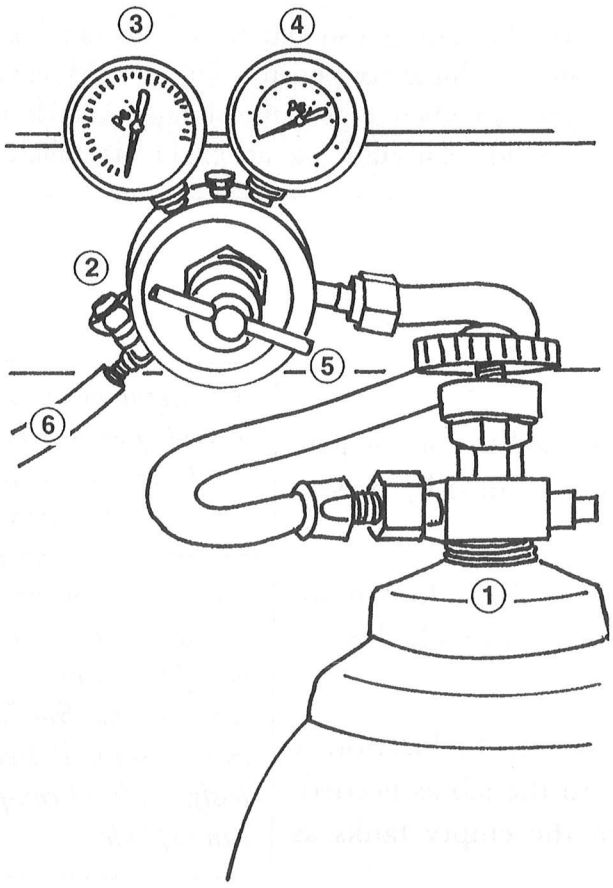

FIGURE 9.

A pressure regulator. **Key:** (1) *Cylinder valve of the gas tank.* (2) *Flow control valve.* (3) *Gauge for delivery pressure.* (4) *Gauge for tank pressure.* (5) *Pressure adjusting screw.* (6) *Tubing to incubator.*

under more stringent operating conditions than the single-stage regulator. You can't tell one from the other.

The gauge with the smaller numbers measures the flow, the one with the larger numbers measures the remaining pressure of the gas in the tank.

- Most regulators (whether single or two stage) will have two gauges. One measures the tank pressure, and one measures the flow or delivery pressure.
- Some regulators don't fit some cylinders: This is done to prevent interchange of equipment for incompatible gases. If the regulator doesn't fit, don't force it.

Precautions to take when using cylinders of compressed gases, including CO_2

- When storing or moving a cylinder, have the cap securely in place.
- When moving large cylinders, have them capped and strapped to a properly designed wheeled cart.
- All cylinders must be restrained by straps, chains, or a suitable stand to prevent them from falling.
- Do not expose cylinders to temperatures over 50°C.
- Never use a cylinder that cannot be properly identified: Don't rely on color to identify the contents.
- Use the appropriate regulator on each cylinder. Don't use a cylinder that has been altered or tampered with.
- Never lubricate, modify, or force a cylinder valve. Don't loosen or remove the safety plug or rupture disc.
- Rapid release of a compressed gas will cause an unrestrained hose to whip dangerously and may also build up a static charge that could ignite a combustible gas.
- Never bleed a cylinder completely empty; leave a slight pressure to keep contaminants out.
- When not in use, cylinder and bench valves should be tightly closed.
- Don't order a surplus of cylinders. They are a safety hazard and, in most places, there is a daily rental fee.
- Remove the regulators from empty cylinders and replace the protective caps. Mark the cylinder "Empty" and return to Purchasing, or to whomever will send it back to the company.
- Don't use damaged or corroded cylinders, or cylinders with a test date more than 5 years old stamped on the shoulder. Return them to the vendor.
- Some gases are flammable (acetylene, butane, ethane, hydrogen, methyl bromide, propane), some highly reactive (oxygen), some toxic (sulfur dioxide, ammonia, chlorine), so check for the hazard of the particular gas before using it.

- Tanks are sometimes attached to a manifold, a series of pipes and metal tubing that permits connection of several cylinders to a common supply line and regulator for a larger continuous flow of gas. Effectively, multiple (usually, up to four) tanks are regulated as one, preventing wear and tear of cylinder mounting, and saving time. However, since several incubators in the lab may be supplied with CO_2 from the same set of tanks, this dependence on one source can leave the laboratory vulnerable.

How to change CO_2 tanks on an incubator

1. Turn off the empty tank by screwing closed the cylinder valve.
2. Recap the empty tank and roll it or cart it out of the way.
3. Move the new tank into place, and strap it.
4. Attach the regulator to the cylinder valve outlet.
5. Turn the pressure-adjusting screw counterclockwise until it turns freely.
6. Open the cylinder valve slowly until the tank gauge on the regulator registers the cylinder pressure. Check that it is the expected value. If not, the valve may be leaking.
7. With the flow-control valve at the regulator outlet closed, turn the delivery-pressure adjusting screw clockwise until the required delivery pressure is reached (consult manual for incubator).

Control of flow can be regulated by means of a valve supplied in the regulator (the flow control valve) or by a supplementary valve installed in a pipeline downstream from the regulator. **The regulator itself should not be used as a flow control by adjusting the pressure to obtain different flow rates.** This defeats the purpose of the pressure regulator.

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