

Changing medium and passaging cell lines

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Cell lines are widely used in biomedical research. This protocol describes the methods used routinely to change the medium and passage the cells. Medium changes keep the cells healthy by providing fresh nutrients, while cell passage or splitting is required to maintain cells in exponential growth. Despite the simplicity of the methods used, each cell line has idiosyncracies. Whether working with one or several cell lines, there is no substitute for knowledge of their needs, including the range of phenotypes and growth patterns under different physical and nutrient conditions. Given the necessary care and attention, most cell lines are easy to maintain and grow.

INTRODUCTION

Fifty years ago, maintenance of cell lines was arduous. Tissue culture medium had to be made from base constituents, serum was collected from the abattoir, there were no tissue culture plastics and sterility was maintained on the open bench without laminar flow hoods or antibiotics. However, as described in this protocol, many of the issues that researchers faced 50 years ago still need to be considered today to obtain reproducible and meaningful data.

Each and every cell line has slightly different requirements, phenotype and behavior, and there is no substitute for familiarity with the cell line being used. The culture of cell lines demands knowledge and expertise in a number of areas, including aseptic technique, phase microscopy and familiarity with the morphology of the cells at a range of magnifications. Good cell culture practice demands an acceptance that cell lines are living entities and an attitude that puts the well-being of the cells ahead of the convenience of the investigator.

A cell line is defined as a cell culture following its first passage¹. Cell lines may either be continuous (immortal) or finite (limited lifespan), but the culture methods are identical. This set of protocols will not include primary culture, for which separate protocols are needed for the isolation, culture and characterization of each cell type.

Most human continuous cell lines in routine use are derived from cancers. There are increasing numbers of human cell lines derived from normal embryonic or adult tissues and cancers that have been immortalized with viral genes or other recombinant DNA constructs. Embryonic stem cells can also be maintained in continuous culture. Rodent cell lines tend to transform spontaneously in culture, acquiring the property of continuous cell growth. The finite cell lines most frequently used are human diploid fibroblasts, which produce stable cultures that survive for approximately 50 generations.

Cell lines either grow as adherent cultures or in suspension. Slightly different maintenance methods are required, as reflected in the protocols.

Factors that need to be considered when culturing cell lines

Continuous culture and genome stability. Immortalization of normal human cells and spontaneous 'transformation' of rodent cells depend on the acquisition of genetic changes. The longer a cell

line is maintained in culture, the greater the chance that it will acquire one or more changes in genotype. Such changes may critically alter the characteristics of the cell line, but we can minimize genetic change and other variables influencing the cells by observing strict adherence to good cell culture practice. The most important means of minimizing such changes is to restrict the length of time that cell lines are grown in continuous culture. As a pragmatic rule of thumb, we advocate that a cell line should never be in continuous culture for more than 3 months. Beyond this limit, fresh cells should be obtained from the cell bank. If cells are continuously in culture for extended lengths of time, phenotypic and genotypic changes are likely to occur, and the chances of microbial contamination and cell line cross-contamination will increase.

Tissue culture medium. Tissue culture media are complex. Detailed information is available in chapter 8 of ref. 2. A relatively simple medium such as Eagle's MEM (minimal essential medium) contains amino acids, vitamins, salts and glucose. There are large numbers of different formulations and variants available from a range of commercial sources. The constituents may be sourced and prepared differently by each manufacturer and conform to different specifications and levels of purity, and the sources and chemical contaminants may change over time. Nevertheless, most commonly used cell lines are relatively undemanding and will grow in the same medium preparation regardless of the manufacturer. There are also many cell lines (e.g., HeLa) that will adapt readily to and grow in most types of media. A comparison of the culture requirements of two cell types is given in **Table 1**.

Each time a different formulation is used, the cells will need to adapt, and any change in culture conditions may favor cells carrying a genetic modification. Any genetic change that gives a cell a growth advantage can result in the rapid and complete displacement of the cells with the original genotype (as seen following cross-contamination). Adaptation to serum-free medium or drug resistance is likely to be accompanied by many genetic changes. Population dynamics indicates that where two populations share the same ecological niche and the same supply of nutrients, the fitter population will rapidly displace the weaker population, even where the growth advantage is small.

TABLE 1 | Two examples of cell cultures with widely differing features.

Feature	3T3 Swiss Albino mouse embryo fibroblasts	Human embryonic stem cells
Characteristics	Cells are highly sensitive to contact inhibition, and must be passaged at or before confluence to maintain a “non-transformed” state	Cells have an undifferentiated morphology and phenotype, which is considered important for them to retain their ability to produce cells of all three human germ layers. Careful observation of cultures is vital to ensure passage before widespread differentiation occurs
Growth medium	Recommended growth medium is DMEM (high glucose, 4 mM) with 10% newborn bovine serum. Note: Use of FBS fails to support appropriate growth and may alter the characteristics of the cells	Originally grown in DMEM with 10% FBS. A variety of serum-free media are being developed, for example using ‘Knock-Out’ DMEM with proprietary supplement ‘Serum Replacement’ and basic fibroblast growth factor. The colonies require special culture surface treatment—normally a feeder cell monolayer or a complex extracellular matrix preparation such as Matrigel
Passaging	Usually passaged at sub-confluence using 0.25% wt/vol trypsin. Careful handling of cells at harvest is required to avoid clumping and cells are seeded at low density (i.e., $3-5 \times 10^3 \text{ cm}^{-2}$)	Colonies of cells are dissected into fragments under a stereo-microscope and individually transferred to fresh feeder cells
Incubation conditions	5% CO ₂ in air at 37 °C	Usually cultured in 5% CO ₂ in air at 37 °C, but culture under low-oxygen conditions may help sustain an undifferentiated state

The average researcher can do relatively little to standardize tissue culture medium. It is good practice to use only one manufacturer, and ideally only one batch, for a particular set of experiments. For comparative purposes, it is important to use the same formulation of medium throughout an experiment to eliminate variables. Likewise, when using several cell lines, it is essential to grow all the cells in the same medium to exclude any influence of the medium as a variable. If the conditions to be used differ from those under which the cells were prepared by the cell bank, a secondary bank can be produced. The secondary bank should use cells grown for a minimum of five passages in the new conditions, as the effects of adaptation are not evident immediately.

Serum. Serum is a complex mixture of biological materials and can be highly variable. Detailed information is available in chapter 8 of ref. 2. Serum may be of calf, human or horse origin, but the most frequently used serum is fetal calf serum. Serum provides factors that are missing from tissue culture media, including factors that promote cell growth and adhesion.

Most of the points concerning medium are equally applicable to serum. Each batch of serum will be different and each time a different batch of serum is used, the cells will adapt to the different conditions. Because batches of serum are a finite resource, the only way in which variation can be minimized is to purchase batches of serum that are as large as possible, aiming for a minimum of 12 months supply.

Batches of serum can differ dramatically in their ability to support the growth of cell lines. To screen a new batch, a large number of (we recommend at least ten) batch samples should be acquired from a range of suppliers and tested against the most fastidious cell lines in the laboratory using clonogenic assays. Both colony-forming ability and colony size should be compared for each batch. Serum suppliers understand the need for batch testing and will usually hold the batch for the minimum period required (about 4–6 weeks) to allow comparisons to be made.

Many cell lines can be adapted to or maintained in serum-free medium. Where cells are being used to produce products for

human use, the absence of serum is important to reduce immunological reactions and the possibility of microbial or viral contamination.

Trypsin (adherent cultures only). Various proteolytic enzymes are used to detach cells from the substrate, of which the most frequently used is trypsin. Purified trypsin from a commercial source is used in most laboratories at a concentration of 0.25% (wt/vol) in 0.5 mM EDTA (disodium ethylenediaminetetraacetic acid, also known as versene, a chelating agent) dissolved in calcium- and magnesium-free phosphate-buffered saline. Increasing the trypsin concentration does not necessarily accelerate detachment, and paradoxically diluting the trypsin can accelerate cell removal. Optimum activity of trypsin is achieved at 37 °C, so prewarmed trypsin may speed up cell detachment. Long incubation times and high trypsin concentrations are unhelpful, and tend to damage the cells, causing them to stick together.

Trypsin is derived from cows or pigs and can vary between batches, but generally is reliable if used fresh. If it is necessary to avoid animal products, a trypsin equivalent derived from microbial sources can be used, called TrypLE (Invitrogen).

If it is desirable to avoid trypsin altogether, for example for serum-free cultures, many commercial sources can provide alternatives (e.g., cell dissociation buffer from Invitrogen), although in our hands these are less effective than trypsin at detaching cells. Alternatively, the cells can be detached manually using a ‘rubber policeman’—a cell scraper either home made, for instance by creating a right angle in a Pasteur pipette, or bought commercially.

There are many applications of cell lines for which trypsin should be avoided, such as proteomic studies. Trypsin will strip proteins from the cell surface and will continue to act as a proteinase until it is neutralized by an inhibitor such as serum. Consequently, cells should not be left in trypsin for longer than is necessary.

Substratum (adherent cultures only). Manufacturers use various procedures for preparing a charged surface on the plastic to encourage cell attachment. As far as we know, there is little



difference in the ability of tissue culture plastics from different manufacturers to support the growth of adherent cells, except where the surface of the plasticware has undergone specific treatment. But, every time a different substratum from a different manufacturer is used, the cells may adapt, potentially promoting phenotypic and genotypic changes. It is sensible to use one supplier and grow all cells for comparative purposes on the same type of tissue culture plastic.

Temperature. Most cell lines are derived from animal tissues at 37 °C and are adapted to grow at this temperature. For routine maintenance, it is necessary to transfer cells between the incubator temperature of 37 °C and the ambient temperature, usually 20–25 °C. These relatively abrupt temperature changes can cause stress to cells, leading to changes in transcription and ultimately the protein profile of the cells. Temperature changes should be minimized, and particular care should be taken where facilities are shared, as incubator temperatures may drop when incubator doors are opened frequently. It can take up to 30 min for the temperature to recover and consequently the average temperature in the incubator during periods of heavy use can be well below 37 °C.

Routine maintenance involves adding constituents freshly taken from the freezer (such as trypsin and serum) and from the refrigerator (medium). To minimize cell stress, it is important to raise the temperature of these components at least to room temperature.

L-Glutamine. L-Glutamine is routinely added to tissue culture media, either as a separate supplement or incorporated into the basal medium. To add glutamine as a separate item, it should be dissolved in distilled water at an appropriate concentration (e.g., at 100× the working concentration), sterilized through a 0.22 μM filter and stored at –20 °C. Alternatively, it can be bought as a 100× working solution (200 mM) and 5 ml is added to a 500 ml bottle of medium.

Glutamine has a half-life of about 1 week at 37 °C and about 3 weeks at 4 °C (see ref. 3). Consequently, if a cellular function is supported by or needs L-glutamine, fresh medium should be added accordingly. However, the breakdown of glutamine releases ammonia, and therefore medium with glutamine added should not be stored for long periods. One way to overcome this issue is to use the stabilized dipeptide of L-glutamine, sold as Glutamax, which is much more stable, to the extent that the medium can be autoclaved.

L-Glutamine can provide energy through the citric acid cycle. However, most tissue culture media contain large amounts of glucose, and consequently the addition of L-glutamine may not be necessary for many cell lines.

Buffers and pH. Most mammalian cells grow at pH 7.2–7.4. Phenol red is usually added to medium and ranges in color from yellow at pH 6.5 to orange at pH 7.0 to red at pH 7.4 to pink at pH 7.6 and purple at pH 7.8.

Most tissue culture media contain bicarbonate, necessitating a 5% carbon dioxide atmosphere to maintain the pH at 7.4. HEPES, an organic buffer, is often added to medium to reduce the dependence on bicarbonate.

Antibiotics. With correct working practice, antibiotics should not be used for the routine maintenance of cell lines. In the presence of

antibiotics, contamination may be suppressed, but could alter the phenotype or genotype of the cells. Antibiotics are toxic and can alter the biochemistry of the cells. If an infection is not obvious, because it has been suppressed but not eliminated by antibiotics, all other cultures in the laboratory are at risk.

Labeling of cultures. Every container should be clearly labeled with the name of the cells, the passage number, the date the container was seeded and dates of medium changing. Inadequate or incorrect labeling of flasks or other tissue culture containers can result in cross-contamination and misleading data.

For each experiment, a minimum set of data needs to be recorded to enable reproducibility and comparability of cell culture experiments. These data include the source of the cells, their passage level, the culture conditions and evidence of the most recent Mycoplasma test.

Medium changing. In cell culture, nutrients become depleted and metabolic products increase in concentration. The latter may be toxic to the cells. How frequently the medium needs to be changed depends on the cell line and the type of medium. Cell lines grown in media with essential growth factors that can be depleted quickly (e.g., some serum-free and stem cell media) may need fresh medium every 1–2 d. For most continuous cell lines in conventional medium, two medium changes a week will maintain exponential cell growth.

It is important to relate the changing of the medium to the health of the cells and the timing of the experiment. The cells rapidly utilize the nutrients and release metabolites into the medium, resulting in a lowering of pH. The pH is accompanied by yellowness of medium containing pH indicator. The cell characteristics will change over time and according to confluence and nutrient conditions. Therefore, for optimal growth conditions and experimental reproducibility, the cells should be grown a standard number of days after being passaged and their medium changed 24 h before the cells are used for experimental purposes.

Passaging. The frequency of passaging (transfer between flasks with or without cell dilution) depends on the growth rate of the cell line and the seeding density at passage (split ratio). A typical split ratio is between 1:3 and 1:8 once a week. The cells can take much longer to resume exponential growth if they are split at higher dilution ratios. It should be remembered that passaging will initially result in a loss of cells. The proportion of cells lost is variable and depends on the cell line, the expertise of the operator and the plating efficiency of the cell line (the proportion of cells that reattach).

Ideally the cells should always remain in exponential growth. There are usually three growth phases in a cell flask: (a) a lag period as the cells adapt to the new conditions; (b) a phase of fast (exponential) growth; and (c) a plateau phase after the cells have completely covered the growing surface (are confluent). Because the biochemistry of confluent cells can be different from that of exponentially growing cells, for most purposes cells are harvested or passaged before they become confluent. Some cell lines can be kept as confluent cultures for long periods, whereas others tend to detach when they reach confluence. Some cell lines, particularly those derived from normal tissues such as human diploid fibroblasts, may be contact inhibited at confluence.

Quality control. The frozen stocks of every cell line in the laboratory should be DNA profiled to authenticate their origin against published profiles and provide a record of their genotype. DNA profiling is inexpensive and can be done commercially (e.g., Laboratory of the Government Chemist (LGC) or The Doctors Laboratory (TDL) in the UK) providing a guarantee of the provenance of the cells for as long as the frozen stocks survive.

The frozen stocks of every cell line in the laboratory should also be tested for Mycoplasma species, using testing regimes that will yield highly sensitive and specific results. Mycoplasma contamination can alter many biochemical processes. Mycoplasmas are the smallest free-living, self-replicating organisms (0.2–2 µm in diameter). Contamination with one of the many Mycoplasma species from human, cattle or pig sources is a frequent and usually occult event.

Safety and precautions. The major hazard related to the culture of continuous cell lines are the remote possibilities of accidentally implanting transformed cells in the operator or infection as a result of pathogens in the cells or one of the animal products. In general, cell cultures are not considered to be hazardous. However, there is always the possibility that the cells are infected with an organism that will multiply and be a hazard to laboratory workers. Consequently, it is important to know the history of any cell line taken into the laboratory. Ideally, sources of human cell cultures should be tested for the most frequent serious human blood-borne pathogens such as HIV 1 and 2, HTLV I and II and hepatitis B and C.

Accidental inoculation of a cancer cell line in a needle led to the growth of a tumor⁴ and it is well known that cancers can be transferred during transplantation (e.g., ref. 3). The risk is liable to be greater for individuals who are immunodeficient, and therefore such persons should consider carefully and in consultation with their occupational health department whether they should be working with cell lines and, if so, what extra precautions might be taken.

Hantavirus and lymphocytic choriomeningitis virus have been transmitted to laboratory workers from rodent cell cultures⁵. Hepatitis B has been transmitted from a contaminated liquid nitrogen container⁶. At least one fatal viral infection has been reported⁷. Cell lines clearly have the potential to harbor viruses. For example, HIV may be present in Kaposi's sarcoma cells and Epstein–Barr virus in lymphoma or nasopharyngeal cancer cells.

A further perceived risk from animal-based tissue culture products is bovine spongiform encephalopathy (BSE). Consequently many laboratories, and especially those that might develop products for use in humans, use serum from countries where BSE has not been diagnosed. However, a potentially much greater but so far unquantified risk is that the cells or animal products carry viruses that are pathogenic to humans. As viral tests are limited, there is always a potential risk.

Genetic manipulation can activate infectious agents or result in new agents by recombination. There are recommended procedures for working with genetically modified organisms. The minimum requirement for handling human cell lines is containment level 2. It is recommended that wherever possible cells should be manipulated within a Class II Microbiological Safety Cabinet. Procedures that create aerosols (e.g., centrifugation, vortexing) should be contained. Disposal of laboratory waste should not expose anyone

to infection and prevent contamination. All infected waste should be made safe, for example, by autoclaving, before incineration. References to some of the national guidelines on good cell culture practice can be found in various publications^{8,9}.

The use of body fluids or cells derived from laboratory staff for research procedures is not recommended. The use of blood or tissue from laboratory staff for the development of transformed cell lines is prohibited, as the person concerned would have no immunity against the cells. Risks of exposure to contaminated cells or animal products can be minimized by avoiding aerosol production and the use of 'sharps' or glass and maintaining the usual barrier precautions. The use of plastic Pasteur pipettes is preferred to glass.

Latex or equivalent gloves help to protect the operator from infection by the cell lines (a rare event) and the cell lines from infection by the operator (a common event). When using gloves, it is necessary to replace them periodically, as prolonged use can cause small holes in the gloves. Each time gloves are put on, they should be disinfected with 70% alcohol (vol/vol, in aqueous solution) or the equivalent, inside the Class II cabinet.

Cryopreservation and storage. A well-characterized cryopreserved master seed-stock or 'bank' from which all future cultures are derived is required (see cell banking protocol, in preparation). The ability to cryopreserve a batch of vials each containing identical preparations of cells (a cell bank) that can be characterized, safety tested and made available for use over years and even decades is a vital tool for reliable research and is critical to the development of safe and standardized cell-derived products and cell therapies.

Cryopreservation should be a straightforward process for most cell lines. However, it is poorly understood, and if certain technical aspects are not adhered to, a number of problems can arise such as poor recovery or complete loss of cultures, and altered characteristics due to loss of cells more susceptible to the stress involved in preservation. It is important to ensure that any culture due for preservation is fit for the purpose and in general cells of high percentage viability, and in the exponential phase of growth, are most suitable for cryopreservation. The preparation and cooling of cells should be as reproducible as possible to ensure reliable recovery of cells, and there are a variety of devices on the market from simple insulated containers (e.g., 'Mr Frosty', InVitrogen) to controlled rate freezers that can apply reliable cooling profiles to hundreds of vials of cells (e.g., Kryo-10, Planer Ltd.). It is particularly important to recover a sample of any cryopreserved stocks immediately following preservation in case there has been a dramatic loss of viability or contamination. The method of recovery is also very important. Rapid thawing at 37 °C and careful dilution of cryopreserved cells in prewarmed medium avoiding rapid dilution and physical stress (such as rapid pipetting) should promote optimum survival and growth of cells.

Overview of procedure

In the procedure below we describe the steps that are undertaken to successfully maintain exponentially growing cultures of cell lines by changing medium and splitting the cells. Cell lines grow either in suspension or attached to the substrate. While the procedure differs slightly for the two types of culture, the principles are the same.

BOX 1 | CELL BANKS

American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20108-1549, USA.

LGC Promochem, Queens Road, Teddington, Middlesex TW11 0LY, UK. (European agents for ATCC—from Europe you will automatically be sent to this website if you try to log into the ATCC website).

DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Inhoffenstrasse 7 B, 38124 Braunschweig, Germany.

ECACC. European Collection of Animal Cell Cultures, Health Protection Agency, Porton Down, Salisbury, Wiltshire SP4 0JG, UK.

Japanese Collection of Research Bioresources (JCRB) Cell Bank, National Institute of Biomedical Innovation, 7-6-8 Saito-Asagi, Ibaraki-shi, Osaka 567-0085, Japan.

RIKEN BioResource Centre, 3-1-1 Koyadai, Tsukuba Science City, Ibaraki 305-0074, Japan.

MATERIALS

REAGENTS

- Cells (see **Box 1** for a list of cell banks and their addresses)
- Selected cell culture medium (e.g., Sigma, InVitrogen, BioWhittaker) (see REAGENT SETUP)
- Selected batch-tested serum (typically fetal bovine serum (FBS)) (e.g., Sigma, InVitrogen, BioWhittaker)
- L-Glutamine
- Trypsin for detaching adherent cells. Trypsin is typically purchased as a 0.25% (wt/vol) solution in versene (EDTA) (e.g., Sigma, InVitrogen)
 - ▲ **CRITICAL** Always use a fresh aliquot of trypsin from the freezer for optimum activity.
- Tissue culture flasks; see REAGENT SETUP
- Sterile 9" Pasteur pipettes with and without plugs (without plugs for aspirating medium and with plugs for adding gases to culture containers)
 - ! **CAUTION** Plastic Pasteur pipettes are available, reducing the risk of injury and cell inoculation into the operator.
- Sterile 1, 5, 10 and 20 ml pipettes ! **CAUTION** Pipettes must be kept within the laminar flow hood throughout to maintain sterility. If a pipette touches the operator or the external surface of any other object within the cabinet, it should be considered contaminated and be discarded.

EQUIPMENT

- Certified Class II Biological Safety Cabinet (recirculating/top-vented)
 - ! **CAUTION** Laminar flow hoods that discharge sterile air horizontally, directly at the operator, can be dangerous for the operator, particularly if hazardous substances are present in the cabinet.
- Inverted microscope (e.g., Olympus, Nikon, Zeiss, Leitz)
- Vacuum source for aspiration, such as a foot-operated or a Venturi pump
 - ! **CAUTION** The aspirated medium must be collected in a suitable container for decontamination and disposal.
- 37 °C carbon dioxide incubator
- Indelible marker for labeling containers

REAGENT SETUP

Medium Continuous cell cultures are usually grown in a standard cell culture medium (e.g., DMEM or RPMI1640) supplemented with 5–20% FBS. For example, take a 500 ml bottle of medium (usually stored at 4 °C) and a 50 ml aliquot of serum (stored at –20 °C), thaw and mix.

Tissue culture flasks Cell lines are most frequently grown for routine purposes in tissue culture flasks, typically with a surface area of 25, 75 or 180 cm² (e.g., Sigma, InVitrogen), called T25s, T80s and T180s. There are also many other sizes and shapes of tissue culture container for a variety of purposes, and the protocols will need minor adaptation to cope with these.

PROCEDURE

Preparation ● TIMING 10–15 min

- 1| Prepare safety cabinet. Switch on and allow the cabinet to reach working airflow pressure for approximately 15 min before use. Swab working surface with disinfectant before use and between using different cell lines.
 - ! **CAUTION** Try to put as little as possible into the safety cabinet. Each object within the cabinet interrupts the air flow, making contamination more likely.
- 2| Warm medium. To avoid unnecessary stress to the cells, pre-equilibrate the medium in the incubator. If you are going to passage adherent cells, also thaw and warm an aliquot of trypsin.
 - ! **CAUTION** Water baths tend to harbor microbial contamination and so are best avoided for warming up medium.
- 3| Examine cells under an inverted phase microscope using low-power (×4) and high-power (×10 or ×20) objectives, noting morphology, viability and checking for signs of contamination such as cell death and plaques (cell-free areas in cell monolayers), cloudiness and/or yellow medium.
 - ▲ **CRITICAL STEP** It is usually necessary to check that the condenser is focused and the phase rings are correctly aligned before using the microscope. These steps are essential for optimal phase microscopy.
- 4| Transfer cells to laminar flow cabinet.
 - ! **CAUTION** The protocols are written for a right-handed person. Please reverse all instructions if you are left-handed.
 - ▲ **CRITICAL STEP** Never have more than one cell line in the cabinet. Clear the cabinet before introducing another cell line.
 - ▲ **CRITICAL STEP** Think about how you can maintain sterility at all times. Never take any risk with your aseptic technique.

Passaging and medium changing

5| If you are changing the medium of adherent cultures, follow option A. If you are changing the medium of suspension cultures, follow option B. If you are passaging adherent cultures, follow option C. If you are passaging suspension cultures, follow option D.

(A) Medium changing of adherent cultures ● **TIMING** 5 min after inspecting cells under the phase microscope

(i) Holding the culture flask in the left hand (at a 45° angle and with one corner planted firmly on the working surface) and the Pasteur pipette (connected to foot-operated Venturi pump or similar) held between the thumb and the first finger of the right hand and resting on the second finger, remove the cap with the little finger of the right hand (so that the inside of the cap is facing down and is not in contact with your hand and is not exposed to the air flow), insert a sterile pipette into the farthest, lowest corner of the flask and aspirate all the medium (see Fig. 1).

(ii) Replace the cap loosely and discard the pipette.

(iii) Take the bottle of medium and hold in the left hand at 45° or less (to avoid putting your hand or any other unsterilized object into the sterile laminar flow), insert the pipette, withdraw desired quantity of medium and replace cap loosely.

(iv) Hold flask at 45° in the left hand, remove the cap with the little finger of the right hand, insert the syringe or pipette facing the roof of the flask (away from the cells) and gently add medium. Note that adherent cells tend to be grown in flasks with a surface area of 25, 75 or 180 cm². Typical volumes of medium for these flasks are 5, 15 and 30 ml, respectively.

(v) Gas if necessary. Replace the cap fully and label the flask with an indelible marker. Some flasks have a gas-permeable lid and for these the lid can be closed fully. If the cap is to be left partly open to allow the gas to enter from the incubator, fully close the cap and then open it 180°. If the incubator is not gassed, it will be necessary to gas the container before closing the lid. The correct gas mixture (usually 5% CO₂ in air) should be blown gently (so that it ruffles the surface of the medium) via a plugged Pasteur pipette into the container for approximately 10 s (count one thousand, two thousand, etc). Finally, the lid should be closed fully.

! CAUTION Bottled gas may not be sterile.

(B) Medium changing of suspension cultures ● **TIMING** 5 min after inspecting cells under the phase microscope

(i) Suspension cultures can be grown in tubes, flasks or roller bottles that are slowly rotated. If not already in a container suitable for centrifugation, transfer the medium containing the cells into a suitable sterile container and centrifuge at approximately 100g for 5 min at room temperature (20–25 °C). Never pour medium from a tissue culture container. Optimum times and speeds of centrifugation can vary between cell lines.

! CAUTION Centrifugation can damage cells. Avoid speeds greater than 100g.

(ii) Holding the tube in the left hand (at a 45° angle or less) and Pasteur pipette (connected to foot-operated Venturi pump or similar), remove the cap with the little finger of the right hand (so that the cap is facing down and is not in contact with your hand, nor is exposed to the laminar flow), insert the pipette toward the top of the medium and gently aspirate by slowly lowering the pipette until about half the medium is removed.

(iii) Replace the cap loosely, place the tube in a vertical holder and discard the pipette.

(iv) Take the bottle of medium and hold in the left hand at 45°, insert the pipette, withdraw the desired quantity of medium and replace the cap loosely.

(v) Hold the tube at 45° or less in the left hand, remove the cap with the little finger of the right hand, insert the pipette against the side of the tube and gently replace the amount of medium removed. Gently resuspend cells by drawing the medium containing the cells into and out of the pipette twice.

(vi) Gas if necessary (see Step 5A(v)). Replace the flask cap, tighten and label with cell name, passage number and date. Note that it is not essential to follow a strict protocol to maintain suspension cultures, as most cells growing in suspension can be maintained simply by diluting the culture by an appropriate amount, up to 100-fold, which effectively both changes medium and passages the cells.

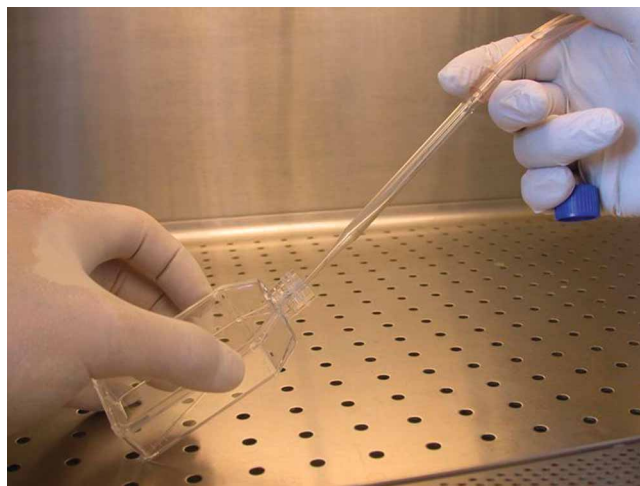


Figure 1 | Example of aseptic technique for removing medium from a tissue culture flask.

(C) Passaging adherent cultures ● TIMING 10–15 min after inspecting cells under a phase microscope

(i) Holding the flask in the left hand (at a 45° angle and with one corner planted firmly on the working surface) and Pasteur pipette in the right hand, remove the cap with the little finger of the right hand, insert the pipette into the farthest, lowest corner of the flask and aspirate all the medium (see **Fig. 1**).

(ii) Replace the cap loosely and discard the pipette.

(iii) Take the bottle of trypsin and hold in the left hand at 45° or less, insert a sterile 1 ml pipette and withdraw 1 ml trypsin for a T25 flask. The ratio of trypsin to growing surface should be 1:25—that is, 1 ml for a T25, 3 ml for a T75 and 7 ml for a T175—but the volume of trypsin is not important as long as the trypsin covers the cells and is removed again immediately after it has been added.

▲ CRITICAL STEP Cultures that do not contain serum will either need special handling to remove all traces of trypsin (usually by centrifugation after detachment) or be detached using some other non-enzymatic medium, for example, http://www.ebioscience.com/ebioscience/specs/antibody_00/00-4555.htm, http://www.hyclone.com/media/new_prod/hyqtase.htm, <https://catalog.invitrogen.com/index.cfm?fuseaction=viewCatalog.viewProductDetails&productDescription=303&CMP=LEC-GCMSSEARCH&HQS=13150%2D016>.

(iv) Holding the flask in the left hand (at a 45° angle or lower to avoid laminar flow), remove the cap with the little finger of the right hand and insert the pipette and gently extrude trypsin onto the cells. Replace the cap loosely. The time taken for the cells to detach depends on the cell line and the temperature and activity of the trypsin. Trypsin can be added at 4 °C, at room temperature or pre-warmed at 37 °C. Higher temperature will detach the cells faster, but if the cells are loosely attached, they may detach before the trypsin has been removed.

▲ CRITICAL STEP Some highly adherent epithelial cells and some very confluent cultures are more difficult to detach and benefit from a pre-rinse with sterile phosphate-buffered saline to remove the serum (which neutralizes the action of the trypsin).

(v) Using a rocking motion, distribute the trypsin over the cells, covering the whole growing surface of the flask.

(vi) Holding the flask in the left hand (at a 45° angle and with one corner planted firmly on the working surface) and Pasteur pipette (connected to foot-operated or Venturi pump or similar), remove the cap with the little finger of the right hand (so that the cap is facing down and is not in contact with your hand, nor is in the laminar flow), insert the pipette into the farthest, lowest corner of the flask and aspirate all the trypsin.

(vii) Replace the flask cap securely. Transfer to a 37 °C incubator.

(viii) After an appropriate period (usually 2–3 min), depending on your experience with the cell line, inspect the flask under the phase contrast microscope at low power to confirm that the cells have rounded up. If not, return the cells to the incubator for the same period as before. If the cells have rounded up, sharply knock the flask on the bench on its base (the short side opposite the cap) to help detach the cells. Confirm the extent of detachment under the microscope. If cell detachment is incomplete, return the flask to the incubator for another 2–3 min and repeat the process. When detachment is complete, return the flask to the safety cabinet.

▲ CRITICAL STEP Some cells completely round up in trypsin, whereas others shrink, but can detach without fully rounding up. Having knocked the flask on the bench, it is important to check immediately under the microscope that the cells are in motion, as this observation confirms that the cells have detached.

Note: Some people do not remove the trypsin until after the cells are detached. They then add medium, centrifuge the cells (100g for 5 min at room temperature) and aspirate all the trypsin and medium before adding fresh medium and subculturing. This procedure adds an extra step, is unnecessary (unless there is a requirement to remove as much trypsin as possible) and exposes the cells to additional stress, including centrifuging.

(ix) Take the bottle of medium and hold in the left hand at 45°, insert the syringe or pipette, withdraw the desired quantity of medium and replace the cap loosely. If, for example, a T25 flask is being subcultured at 1:3, 15 ml of medium will be required. If you need to count cells, see **Box 2**.

▲ CRITICAL STEP For some applications, a set seeding density may be needed. In this case, it is necessary to undertake a viable cell count and seed accordingly (see **Box 2**).

(x) Transfer the required flasks, for example, three new T25 flasks, to the safety cabinet. Loosen caps.

(xi) Holding the flask containing cells in the left hand and pipette in the right hand, remove the cap with the little finger of the right hand, add all the medium and then aspirate all the medium back into the pipette.

(xii) Transfer all medium and cells to the new flasks in appropriate ratios. For example, transfer all medium and cells to the first new T25 flask. Remove two-thirds of the medium and cells. Replace the cap. Transfer half of the remaining medium and cells to each of the second and third flasks.

Note: The additional step of transferring all the medium to the first flask and withdrawing 10 ml is not essential, but can help to separate cell clumps and provide a more even distribution of cells in the three flasks.

▲ CRITICAL STEP Some people re-use the original flask. The original flask was trypsinized, which could alter the surface characteristics. There is also a greater risk that the flask is contaminated.

(xiii) Gas if necessary (see Step 5A(v)). Replace the flask cap, tighten and label with cell name, passage number and date.

BOX 2 | CELL COUNTING

Living cells exclude the vital dye Trypan blue, whereas dead or dying cells with a compromised plasma membrane take up the dye and turn blue. The cells can be counted using a hemocytometer to give an estimate of the cell concentration and percentage viability. Trypan blue is carcinogenic in mice at high concentration and may be teratogenic. It may be toxic if ingested or inhaled. The safety data sheets concerning Trypan blue should be consulted before use.

Method

1. Clean the hemocytometer chamber ('Neubauer improved') and a hemocytometer coverslip with 70% (wt/vol) alcohol/water. Dampen the edges of the coverslip and position by pressing and sliding the coverslip across the surface of the hemocytometer so that the coverslip becomes fixed to the chamber surface. Interference patterns (rainbow colors of Newton's rings) should be seen on either side of the central drainage channel. *Note:* Do not use ordinary slide coverslips, as these are thinner and prone to break and cause injury.
2. Add 0.1 ml of a 0.4% (wt/vol) solution of Trypan blue to 0.9 ml of cell suspension in a sterile tube and mix by inversion. *Note:* To ensure that the sample is representative, it is essential that the cell solution is thoroughly mixed before the sample is taken.
3. Draw up a small quantity of the stained cells using a fine glass or plastic pipette and immediately apply the tip to the open edge between the hemocytometer and the coverslip, allowing capillary action to draw the suspension completely under the coverslip. Repeat on the other side of the hemocytometer.
4. Examine under a microscope using as a minimum a $\times 10$ objective, focusing on the lines drawn on the hemocytometer.
5. Count and record the number of live and dead cells in as many large squares as required (there are nine large squares on each side, and the same number of squares should be counted on each side of the hemocytometer). For accurate cell counting, a minimum of 1,000 cells need to be counted and for an approximate estimate at least 100 cells should be counted. Calculate the average cell count per square. *Note:* Count cells overlapping only two of the four boundaries of each square.
6. Calculations:

To calculate the total number of cells per ml of suspension, multiply the average cell count per square \times the dilution factor (10 in this example) $\times 10^4$ to give the number of cells per ml in the suspension (remember to also multiply by the volume of the suspension to give the total cell number harvested). These calculations are repeated for the live, dead and total cell number and the viability index as a percentage is (the number of live cells divided by the total number of cells) $\times 100$.

Note: Sampling is crucial, both of the original cell suspension and of the stained cells. If in any doubt about the results, repeat the process until consistent results are obtained.

(D) Passaging suspension cultures ● TIMING 10–15 min after inspecting cells under phase microscope

- (i) If not already in a container suitable for centrifugation, transfer the medium containing the cells into a suitable sterile container and centrifuge at approximately 100g for 5 min at room temperature. Optimum times and speeds of centrifugation can vary between cell lines.
- (ii) Aspirate medium to the level of the cells as follows: holding the tube in the left hand (at a 45° angle) and Pasteur pipette (connected to foot-operated Venturi pump or similar), remove the cap with the little finger of the right hand (so that the cap is facing down and is not in contact with your hand, nor is facing the air flow), insert the pipette toward the top of the medium and gently aspirate by slowly lowering the pipette until all the medium is removed.
! CAUTION Be careful not to aspirate the cell pellet.
- (iii) Replace the cap loosely, place the tube in a vertical holder and discard the pipette.
- (iv) Take the bottle of medium and hold in the left hand at 45° or less, insert the pipette, withdraw the desired quantity of medium and replace the cap loosely.
- (v) Hold the tube at 45° or less in the left hand, remove the cap with the little finger of the right hand, insert the pipette against the side of the tube and gently add medium. Gently aspirate the cells into the medium and withdraw the contents of the tube. The tube may not be large enough to hold all the medium, in which case transfer the medium and cells to another container (e.g., a Universal) for gentle mixing (close the container and gently turn through 360° three times).
- (vi) Transfer medium and cells to appropriate number of tubes (original tube may be re-used, but can be a false economy as it is more likely to get contaminated than a new container).
- (vii) Replace the cap fully and ensure tubes are labeled.
- (viii) Gas if necessary (see Step 5A(v)). Return cells to the incubator. Note that it is not essential to follow a strict protocol to maintain suspension cultures, as most cells growing in suspension can be maintained simply by diluting the culture by an appropriate amount, up to 100-fold, which effectively both changes medium and passages the cells.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Problem	Possible causes	Solutions
Slow cell growth	Bacterial, fungal or viral contamination	Discard cells
	Mycoplasma	Test and discard positives
Change in growth pattern	Change in the composition of medium or serum or faulty equipment	Replace medium or serum. Check incubator temperature and humidity
Cloudy or yellow medium	Contamination	Discard cells
	Cells growing rapidly	Change medium or split cells
Cells detaching	Contamination	Discard cells
	No serum	Add serum
	Cells too confluent	Split cells
Change in phenotype	Cross-contamination	Discard cells
	Change in the composition of medium or serum	Replace medium or serum
No cells on growing surface	Cells dead	Discard and replace cells from frozen stocks
	Cells detached	As above
	Cells not adhered	Use tissue culture plastics ^a

^aPlasticware produced for other purposes is very similar in appearance to that used for mammalian cell cultures, but because the surface is not treated, the mammalian cells will not attach.

ANTICIPATED RESULTS

The aim of changing the medium and splitting the cells is to maintain healthy cells in exponential growth.

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- Schaeffer, W.I. Usage of vertebrate, invertebrate and plant cell, tissue and organ culture terminology. *In Vitro* **20**, 19–24 (1984).
- Freshney, R.I. (ed.) *Culture of Animal Cells* 4th edn. (Wiley Liss, New York, 2000).
- Southam, C.M. Homotransplantation of human cell lines. *Bull NY Acad. Med.* **34**, 416–423 (1958).
- Gugel, E.A. & Sanders, M.E. Needle-stick transmission of human colonic adenocarcinoma. *N. Engl. J. Med.* **315**, 1487 (1986).
- Lloyd, G. & Jones, N. Infection of laboratory workers with hantavirus acquired from immunocytomas propagated in laboratory rats. *J. Infect.* **12**, 117–125 (1984).
- Tedder, R.S. *et al.* Hepatitis B transmission from a contaminated cryopreservation tank. *Lancet* **346**, 137–140 (1995).
- Hummeler, K., Davidson, W.L., Henle, W., LaBocchetta, A.C. & Ruch, H.G. Encephalomyelitis due to infection with *Herpesvirus simiae* (Herpes B virus); a report of two fatal, laboratory-acquired cases. *N. Engl. J. Med.* **261**, 64–68 (1959).
- UKCCCR. UKCCCR guidelines for the use of cell lines in cancer research. *Br. J. Cancer* **82**, 1495–1509 (2000).
- Coecke, S. *et al.* Guidance on good cell culture practice. *Altern Lab Anim* **33**, 261–287 (2005).

