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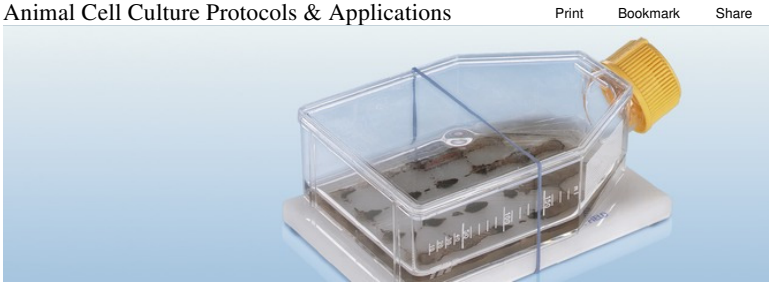
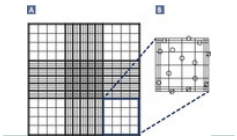
Animal Cell Culture Protocols & Applications



Protocols and useful hints for the successful culture of animal cells



This section provides useful hints for culturing animal cells (i.e., cells derived from higher eukaryotes such as mammals, birds, and insects). It covers different types of animal cell cultures, considerations for cell culture, and cell culture protocols.

Animal cell cultures

Depending on their origin, animal cells grow either as an adherent monolayer or in suspension.

Adherent cells are anchorage-dependent and propagate as a monolayer attached to the cell culture vessel. This attachment is essential for proliferation — many adherent cell cultures will cease proliferating once they become confluent (i.e., when they completely cover the surface of cell culture vessel), and some will die if they are left in this confluent state for too long. Most cells derived from tissues are anchorage-dependent.

Suspension cells can survive and proliferate without being attached to a substratum. Hematopoietic cells (derived from blood, spleen, or bone marrow) as well as some transformed cell lines and cells derived from malignant tumors can be grown in suspension.

Primary cells, finite cultures, and continuous cell lines differ in their proliferative potential (see below). Different cell types vary greatly with respect to their growth behavior and nutritional requirements. Optimization of cell culture conditions is necessary to ensure that cells are healthy and in optimal condition for downstream applications.

Extensive information on culturing cells can be found in reference 1.

Primary cell cultures

Primary cell cultures come from the outgrowth of migrating cells from a piece of tissue or from tissue that is disaggregated by enzymatic, chemical, or mechanical methods. Primary cultures are formed from cells that survive the disaggregation process, attach to the cell culture vessel (or survive in suspension), and proliferate.

Primary cells are morphologically similar to the parent tissue. These cultures are capable of only a limited number of cell divisions, after which they enter a non-proliferative state called senescence and eventually die out. Adherent primary cells are particularly susceptible to contact inhibition, that is, they will stop growing when they have reached confluency. At lower cell densities, however, the normal phenotype can be maintained. Primary cell culture is generally more difficult than culture of continuous cell lines.

Primary cell cultures are sometimes preferred over continuous cell lines in experimental systems. Primary cells are considered by many researchers to be more physiologically similar to in vivo cells. In addition, cell lines cultured for extended periods of time can undergo phenotypic and genotypic changes that can lead to discrepancies when comparing results from different laboratories using the same cell line. Furthermore, many cell types are not available as continuous cell lines.

Finite cell cultures

Finite cell cultures are formed after the first subculturing (passaging) of a primary cell culture. These cultures will proliferate for a limited number of cell divisions, after which they will senesce. The proliferative potential of some human finite cell cultures can be extended by introduction of viral transforming genes (e.g., the SV40 transforming-antigen genes). The phenotype of these cultures is intermediate between finite cultures and continuous cultures. The cells will proliferate for an extended time, but usually the culture will eventually cease dividing, similar to senescent primary cells. Use of such cells is sometimes easier than use of primary cell cultures, especially for generation of stably transfected clones.

Continuous cell lines

Finite cell cultures will eventually either die out or acquire a stable, heritable mutation that gives rise to a continuous cell line that is capable of unlimited proliferative potential. This alteration is commonly known as in vitro transformation or immortalization and frequently correlates with tumorigenicity.

Rodent primary cell cultures form continuous cell lines relatively easily, either spontaneously or following exposure to a mutagenic agent. In contrast, human primary cell cultures rarely, if ever, become immortal in this way and require additional genetic manipulation to form a continuous cell line. However, cell cultures derived from human tumors are often immortal.

Continuous cell lines are generally easier to work with than primary or finite cell cultures. However, it should be remembered that these cells have undergone genetic alterations and their behavior in vitro may not represent the in vivo situation.

Safety and handling considerations for animal cell culture

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Legislation and regulatory guidelines

Before undertaking any work with human or animal tissue (e.g., to establish a primary cell culture), it is necessary to ensure that the nature of the work conforms to the appropriate medical-ethical and animal-experiment legislation and guidelines. It may be necessary to seek approval from the relevant regulatory authorities and/or individuals.

Safety considerations and biohazards

When working with potentially hazardous material, it is important to be aware of the possible risks associated with both the material and the experimental protocol. All cell cultures are considered a biohazard because of their potential to harbor an infectious agent (e.g., a virus).

The degree of hazard depends on the cells being used and the experimental protocol. Primary cell cultures in particular should be handled carefully as these cultures have a high risk of containing undetected viruses. Although commonly used cell lines are generally assumed to be free of infectious agents, care should still be exercised when working with these cell lines as it is possible that they contain infectious agents, such as latent viruses. Cell cultures used to study specific viruses should be assumed to have the same degree of hazard as the virus under study.

We recommend handling all material as potentially infectious to ensure the safest possible working environment. Work should be performed in an approved laminar flow hood using aseptic technique, and the creation of aerosols should be avoided (see Handling cell cultures). After the work is complete, all waste media and equipment (i.e., used flasks, pipets, etc.) should be disinfected by autoclaving or immersion in a suitable disinfectant according to institutional and regional guidelines.

Handling cell cultures

Adherence to good laboratory practice when working with cell cultures is essential for two reasons: first, to reduce the risk of exposure of the worker to any potentially infectious agent(s) in the cell culture, and second, to prevent contamination of the cell culture with microbial or other animal cells (see Aseptic technique and minimization of aerosols).

Aseptic technique and minimization of aerosols

Aseptic technique and the proper use of laboratory equipment are essential when working with cell cultures. Always use sterile equipment and reagents, and wash hands, reagent bottles, and work surfaces with a biocide or 70% ethanol before beginning work.

Creation of aerosols should be avoided — aerosols represent an inhalation hazard, and can potentially lead to cross-contamination between cultures. To avoid aerosols, use TD (to deliver) pipets, and not TC (to contain) pipets; use pipets plugged with cotton; do not mix liquids by rapidly pipetting up and down; do not use excessive force to expel material from pipets; and do not bubble air through liquids with a pipet. Avoid releasing the contents of a pipet from a height into the receiving vessel. Expel liquids as close as possible to the level of liquid of the receiving vessel, or allow the liquid to run down the sides of the vessel.

Proper use of equipment can also help minimize the risk of aerosols. For example, when using a centrifuge, ensure the vessel to be centrifuged is properly sealed, avoid drops of liquid near the top of the vessel, and use centrifuge buckets with caps and sealed centrifuge heads to prevent contamination by aerosols.

Laminar flow hoods

For the most efficient operation, laminar flow hoods should be located in an area of the laboratory where there is minimal disturbance to air currents. Avoid placing laminar flow hoods near doorways, air vents, or locations where there is high activity. Hoods are often placed in dedicated cell culture rooms.

Tips:

- Keep laminar flow hoods clean, and avoid storing equipment inside the hood.
- Before starting work, disinfect the work surface of the hood as well as the outside of any bottles (e.g., by wiping with 70% ethanol), and then place everything needed for the cell culture procedure in the hood.
- Arrange equipment, pipets, waste containers, and reagent bottles so that used items are not placed near clean items, and avoid passing used items over clean items.
- Place used items (e.g., pipets) in a container inside the hood, and disinfect or seal before removing from the hood.

Contamination

The presence of microorganisms can inhibit cell growth, kill cells, and lead to inconsistent results. Contamination of cell cultures can occur with both cell culture novices and experts.

Potential contamination routes are numerous. For example, cultures can be infected through poor handling, from contaminated media, reagents, and equipment (e.g., pipets), and from microorganisms present in incubators, refrigerators, and laminar flow hoods, as well as on the skin of the worker and in cultures coming from other laboratories.

Bacteria, yeasts, fungi, molds, mycoplasmas, and other cell cultures are common contaminants in animal cell culture. To safeguard against accidental cell culture loss by contamination, we recommend freezing aliquots of cultured cells to re-establish the culture if necessary (see Freezing and viability staining of cells).

Microbial contamination

The characteristic features of microbial contamination are presented in the table Characteristic features of microbial contamination. The presence of an infectious agent sometimes can be detected by turbidity and a sharp change in the pH of the medium (usually indicated by a change in the color of the medium), and/or cell culture death. However, for some infections, no turbidity is observed and adverse effects on the cells are not easily observed.

Cell cultures should be routinely evaluated for contamination. Mycoplasmal infections are one of the more common and difficult-to-detect infections; their detection and eradication are described in further detail below.

Characteristic features of microbial contamination

Characteristic	Bacteria	Yeast	Fungi
Change in pH	pH drop with most infections	pH change with heavy infections	pH changes sometimes
Cloudy medium: Under microscope (100–400x)	Shimmering in spaces between cells; rods or cocci may be observed	Round or ovoid particles that bud off smaller particles	Thin filamentous mycelia; sometimes clumps of spores

Mycoplasmal infection — detection

Mycoplasmas are small, slow-growing prokaryotes that lack a cell wall and commonly infect cell cultures. They are generally unaffected by the antibiotics commonly used against bacteria and fungi. Furthermore, as mycoplasma do not overgrow cell cultures and typically do not cause turbidity, they can go undetected for long periods of time and can easily spread to other cell cultures. The negative effects of mycoplasmal contamination include inhibition of metabolism and growth, as well as interference with nucleic acid synthesis and cell antigenicity. Acute infection causes total deterioration of the cell culture, sometimes with a few apparently resistant colonies that may, in fact, also be chronically infected. There are two main approaches to detect mycoplasma — Hoechst 33258 staining (1, 3) and mycoplasma-specific DNA probes. Alternatively, a PCR-based, mycoplasma-testing service is offered by the ATCC or other organizations on a fee-for-service basis.

Mycoplasmal infection — eradication

The best action to take with a culture containing chronic mycoplasmal infection is to discard it by either autoclaving or incineration. Only if the cell culture is absolutely irreplaceable should eradication be attempted. This process should be performed by experienced personnel in an isolated hood that is not used for cell culture, preferably in a separate room. Elimination of mycoplasma is commonly achieved by treatment with various commercially available antibiotics such as a quinolone derivative (Mycoplasma Removal Agent), ciprofolxacin (Ciprobay), enrofloxacin (Baytril), and a combination of tiamulin and minocycline (BM-Cyclin). Treatment procedures and appropriate antibiotic concentrations can be found in the suppliers' instructions and in references 1 and 3.

Cross-contamination of cell lines

Cross-contamination of one cell culture with fast-growing cells from another culture (such as HeLa) presents a serious risk. To avoid cross-contamination, only use cell lines from a reputable cell bank; only work with one cell line at a time in the hood; use different pipets, bottles of reagents, and bottles of media for different cell lines; and check cells regularly for the correct morphological and growth characteristics.

Cell culture conditions

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Media and serum

The choice of cell culture medium is extremely important, and significantly affects the success of cell culture experiments. Different cell types have highly specific growth requirements, and the most suitable medium for each cell type must be determined experimentally. Common basal media include Eagle minimal essential medium (MEM), Dulbecco's modified Eagle medium (DMEM), RPMI 1640, and Ham F10. These contain a mixture of amino acids, glucose, salts, vitamins, and other nutrients, and are available either as a powder or as a liquid from various commercial suppliers.

Basal media are usually supplemented just before use with serum, L glutamine, and antibiotics and/or fungicides to give complete medium (also called growth medium). Serum is a partially undefined material that contains growth and attachment factors, and may show considerable variation in the ability to support growth of particular cells. Fetal calf serum (FCS) is the most frequently used serum, but for some applications, less expensive sera such as horse or calf serum can be used. Different serum batches should be tested to find the best one for each cell type. L-glutamine is an unstable amino acid that, with time, converts to a form that cannot be used by cells, and should be added to medium just before use. Antibiotics and fungicides can be used as a supplement to aseptic technique to prevent microbial contamination. The working concentration of commonly used antibiotics and fungicides is provided in the tables Commonly used antibiotics for animal cell culture and Commonly used fungicides for animal cell culture. Some cell types, particularly primary cells, require additional supplements (e.g., collagen and fibronectin, hormones such as estrogen, and growth factors such as epidermal growth factor and nerve growth factor) to attach to the cell culture vessel and proliferate.

Media, serum, and supplements should be tested for sterility before use by incubation of a small aliquot at 37°C for 48 hours. If microbial growth has occurred after this incubation, the medium or supplement should be discarded.

Commonly used antibiotics for animal cell culture

Antibiotic	Working concentration	Effective against	Stability at 37°C
Penicillin	50–100 U/ml	Gram-positive bacteria	3 days
Streptomycin	50–100 µg/ml	Gram-negative bacteria	5 days
Kanamycin	100 µg/ml	Gram-positive and gram-negative bacteria; mycoplasma	5 days
Gentamycin	5–50 µg/ml	Gram-positive and gram-negative bacteria; mycoplasma	5 days

Commonly used fungicides for animal cell culture

Antibiotic	Working concentration	Effective against	Stability at 37°C
Nystatin	100 U/ml	Yeasts and molds	3 days
Amphotericin B	0.25–2.5 µg/ml	Yeasts and molds	3 days

Incubation conditions

The incubation conditions used to culture cells are also important. Cell cultures should be incubated in an incubator with a tightly regulated temperature (e.g., a water-jacketed incubator) and CO_2 concentration. Most cell lines grow at 37°C and 5% CO_2 with saturating humidity, but some cell types require incubation at lower temperatures and/or lower CO_2 concentrations.

Cell culture vessel

The choice of growth vessel can influence the growth of adherent cells. Sterile, disposable dishes and flasks that have been treated to allow attachment of animal cells to the growing surface are available commercially.

Cell banking

For some cell cultures, especially those that are valuable, it is common practice to maintain a two-tiered frozen cell bank: a master cell bank and a working cell bank. The working cell bank comprises cells from one of the master bank samples, which have been grown for several passages before storage. If future cell samples are needed, they are taken from the working cell bank. The master cell bank is used only when absolutely necessary. This ensures that a stock of cells with a low passage number is maintained, and avoids genetic variation within the cell culture.

Culture instability

The growth rate of cells that have been repeatedly subcultured may sometimes unexpectedly decrease, and the cytotoxicity of, for example, a transfection process may unexpectedly increase. This instability can result from variations in cell culture conditions, genomic variation, and selective overgrowth of constituents of the cell population. We recommend using cells with a low passage number (<10 splitting cycles). To safeguard against instability in continuous cell lines, avoid senescence or transformation in finite cell lines, and maintain consistency in transfection experiments, we recommend creating cell banks by freezing aliquots of cells to recall into culture if and when necessary.

Essential protocols for animal cell culture

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Maintaining cell cultures

Establishment and maintenance of animal cell cultures require standardized approaches for media preparation, feeding, and passaging (or subculturing) of the cells. Cultures should be examined regularly to check for signs of contamination and to determine if the culture needs feeding or passaging.

The cell culture protocols below have been adapted from the following sources: *Culture of Animal Cells; a Manual of Basic Technique* (1), *Current Protocols in Molecular Biology* (4), and *Cells: A Laboratory Manual* (2). These protocols are examples of methods for general cell culture, and have not been rigorously validated and optimized by QIAGEN. There are many alternative protocols in current use.

IMPORTANT: Potentially biohazardous materials (e.g., cells, culture medium, etc.) should be sterilized before disposal, and disposed of according to your institution's guidelines.

Cell thawing

1. Heat a water bath to 37°C, and warm the growth medium into which the cells will be plated.
2. Add prewarmed growth medium to an appropriately sized cell culture vessel.
3. Remove a vial of frozen cells from liquid nitrogen, and place in the water bath until thawed.
   IMPORTANT: Wear protective goggles and gloves when thawing vials that have been stored in liquid nitrogen. Vials may

explode when removed from liquid nitrogen.
IMPORTANT: Proceed to step 4 as soon as the cells have thawed. Do not allow the cells to warm up before transferring them into growth medium.
4. Wash the outside of the vial with 70% ethanol or another suitable disinfectant.
5. Slowly pipet the thawed cell suspension into the cell culture vessel containing prewarmed growth medium. Swirl the vessel gently to mix the cells with the medium.
Note: Immediate removal of DMSO may sometimes be necessary, especially for suspension cells, primary cells, and sensitive cell types. For such cell types, pipet the thawed cell suspension into a sterile centrifuge tube containing prewarmed medium, centrifuge at 200 x *g* for 2 min, aspirate the supernatant, resuspend the cells in fresh growth medium, and then transfer to an appropriate cell culture vessel.
IMPORTANT: Thoroughly mix the cells in the cell culture vessel to ensure even distribution of the cells throughout the vessel.
6. Incubate cells overnight under their usual growth conditions.
7. The next day, replace the growth medium.

Trypsinizing cells

Trypsinization is a technique that uses the proteolytic enzyme trypsin to detach adherent cells from the surface of a cell culture vessel. This procedure is performed whenever the cells need to be harvested (e.g., for passaging, counting, or for nucleic acid isolation).

1. Aspirate the medium and discard.
2. Wash cells with PBS (phosphate-buffered saline) or HBSS (Hanks balanced salt solution) (see tables 1x PBS and 1x HBSS), aspirate, and discard. Repeat.
The volume of PBS or HBSS should be approximately the same as the volume of medium used for culturing the cells.
3. Add enough warmed 1x trypsin–EDTA solution (see table 1x trypsin–EDTA solution) to cover the monolayer, and rock the flask/dish 4–5 times to coat the monolayer.
4. Place the flask/dish in a CO_2 incubator at 37°C for 1–2 min.
5. Remove flask/dish from incubator and firmly tap the side of the flask/dish with palm of hand to assist detachment.
If cells have not dislodged, return the flask/dish to the incubator for a few more minutes.
IMPORTANT: Do not leave cells in 1x trypsin–EDTA solution for extended periods of time. Do not force the cells to detach before they are ready to do so, or clumping may occur.
Overly confluent cultures, senescent cells, and some cell lines may be difficult to trypsinize. While increasing the time of trypsin exposure may help to dislodge resistant cells, some cell types are very sensitive to trypsin and extended exposure may result in cell death. In addition, some cell lines will resist this treatment and will produce cell clumps.
6. Once dislodged, resuspend the cells in growth medium containing serum.
Use medium containing the same percentage of serum as used for growing the cells. The serum inactivates trypsin activity.
7. Gently pipet the cells up and down in a syringe with a needle attached to disrupt cell clumps.
If pipetted too vigorously, the cells will become damaged. Ensure that pipetting does not create foam.
8. Proceed as required (e.g., with passaging, freezing, nucleic acid isolation, etc.).

1x PBS

Composition
137 mM NaCl
2.7 mM KCl
4.3 mM Na_2HPO_4
1.47 mM KH_2PO_4

1x HBSS

Composition
5 mM KCl
0.3 mM KH_2PO_4
138 mM NaCl
4 mM $NaHCO_3$
0.3 mM Na_2HPO_4
5.6 mM D-glucose

1x trypsin–EDTA solution

Composition
0.05% (w/v) trypsin
0.53 mM EDTA
Dissolve trypsin and EDTA in a calcium- and magnesium-free salt solution such as 1x PBS or 1x HBSS*

Passaging cells

Many adherent cell cultures will cease proliferating once they become confluent (i.e., when they completely cover the surface of cell culture vessel), and some will die if they are left in a confluent state for too long. Adherent cell cultures therefore need to be routinely passaged, that is, once the cells are confluent, a fraction of the cells need to be transferred to a new cell culture vessel. Suspension cells will exhaust their culture medium very quickly once the cell density becomes too high, so these cultures similarly require regular passaging.

IMPORTANT: Although regular passaging is necessary to maintain animal cell cultures, the procedure is relatively stressful for adherent cells as they must be trypsinized. We do not recommend passaging adherent cell cultures more than once every 48 h.

1. Harvest the cells, either by trypsinization (adherent cell cultures) or by centrifugation at 200 x *g* for 5 min (suspension cell cultures). Resuspend the cells in an appropriate volume of prewarmed growth medium containing serum.
The volume of medium used to resuspend the cells depends on the split ratio required (see step 2) and the size of the cell culture vessel. If too small a volume is used, it may be difficult to accurately pipet the desired volume to the new culture vessel.
Conversely, if too large a volume is used, the culture vessel may be too full following transfer of the cells.
Removal of trypsin may sometimes be necessary following harvesting of adherent cells, especially for primary and sensitive cell types. Centrifuge the cells at 200 x *g* for 5 min, carefully aspirate the supernatant, and resuspend the cells in an appropriate volume of prewarmed medium containing serum.
2. Transfer an appropriate volume of the resuspended cells to a fresh cell culture vessel containing prewarmed growth medium. Swirl the vessel gently to mix the cells with the medium.
IMPORTANT: Thoroughly mix the cells in the cell culture vessel to ensure even distribution of cells.
IMPORTANT: Some cell types will not survive if too few cells are transferred. We do not recommend high split ratios for primary cells, sensitive cell types, or senescent cultures.
For adherent cells, we recommend adding enough cells so that the culture takes approximately one week to reach confluence again. This minimizes the number of times the cells are trypsinized as well as the handling time required to maintain the culture.
When determining how many cells to transfer to the new cell culture vessel, it can be helpful to think in terms of how many cell divisions will be required for the culture to reach confluence again. For example, if half the cells are

transferred, then it will take the culture one cell division to reach confluency again; if a quarter of the cells are transferred then it will take 2 cell divisions, and so on. If a culture divides once every 30 h or so, then in one week it will undergo approximately 5 cell divisions. A split ratio of 1:32 (1:25) should therefore be appropriate for the cells to reach confluency in about one week. In step 1, resuspend the cells in 8 ml medium, and transfer 0.25 ml to the new cell culture vessel.

3. Incubate cells under their usual growth conditions.

Counting cells

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Cell counting using a hemocytometer

It is often necessary to count cells, for example, when plating cells for transfection experiments. One method for counting cells is to use a hemocytometer. A hemocytometer contains 2 chambers (see figure Counting cells using a hemocytometer). Each chamber is ruled into 9 major squares (volume of 0.1 mm^3 or 1 x 10^{-4} ml each). Cell concentration is determined by counting the number of cells within a defined area of known depth (volume).

This protocol is adapted from references 1, 2, and 4. It should be noted that there are many other protocols also in use.

1. Clean the surface of the hemocytometer with 70% ethanol or another suitable disinfectant, taking care not to scratch the surface of the central area. Dry with lens paper.
2. Clean the coverslip, wet the edges very slightly, lay it over the grooves and central area of the hemocytometer and gently press down.
   It is important that the coverslip is properly attached to obtain the correct chamber depth. The appearance of Newton's rings (bright and dark rings caused by interference in the air between the coverslip and the glass surface of the hemocytometer) will confirm that the coverslip is attached properly.
3. Harvest the cells, either by trypsinization (adherent cell cultures; see Trypsinizing cells) or by centrifugation at 200 x *g* for 5 min (suspension cell cultures). Resuspend the cells in an appropriate volume of prewarmed growth medium. At least 10^6 cells/ml are required for accurate counting.
   Tip: It may be necessary to centrifuge cells and resuspend in a smaller volume to obtain the desired cell concentration for counting. For adherent cells, it is important to produce a single-cell suspension after trypsinizing. Cell clumping will make counting difficult and inaccurate.
4. Mix the cell suspension sample thoroughly. Using a pipet, immediately transfer 20 µl to the edge of one side of the coverslip to fill one chamber of the hemocytometer. Repeat for the second chamber.
   The cell distribution should be homogeneous in both chambers. The cell suspension is drawn under the coverslip and into the chamber by capillary action.
   The cell suspension should just fill the chamber. Blot off any surplus fluid without disturbing the sample underneath the coverslip.
5. Transfer the slide to the microscope, and view a large square ruled by 3 lines using a 10x objective and 10x ocular. Count the total number of cells in 5 of the 9 major squares. Count cells that overlap the top and left border of squares but not those overlapping bottom and right borders. This prevents counting overlapping cells twice. If the cell density is too high, the cell suspension should be diluted, noting the dilution factor.
6. Repeat the counting for the second chamber to give a total of 10 squares.
7. Add the number of cells counted in all 10 squares together to give the number of cells in 1 x 10^{-3} ml.Multiply by 1000 to give the number of cells/ml.
   IMPORTANT: If the original cell suspension was diluted for counting, multiply by the dilution factor to obtain the number of cells/ml.
8. Clean the hemocytometer and coverslip by rinsing with 70% ethanol and then with distilled water. Dry with lens paper.

Freezing and viability staining of cells

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For some cell cultures, especially valuable ones, it is common practice to maintain a two-tiered frozen cell bank: a master and a working cell bank. The working cell bank comprises cells from one of the master bank samples, which have been grown for several passages before storage. If and when future cell samples are needed, they are taken from the working cell bank. The master cell bank is used only when absolutely necessary. This ensures that a stock of cells with a low passage number is maintained, and avoids genetic variation within the culture.

1. Check that cells are healthy, not contaminated, and have the correct morphology.
2. Change the medium 24 h before freezing the cells.
   Adherent and suspension cell cultures should not be at a high density for freezing. We recommend freezing cells when they are in the logarithmic growth phase.
3. Adherent cultures: harvest the cells by trypsinization, resuspend in medium containing serum, centrifuge at 200 x *g* for 5 min, and then resuspend cells in freezing medium (see table Freezing medium) at a density of 3–5 x 10^6 cells/ml.
   Suspension cultures: centrifuge the cells at 200 x *g* for 5 min, and resuspend in freezing medium at a density of 5–10 x 10^6 cells/ml.
   IMPORTANT: Freezing medium containing DMSO is hazardous and should be handled with caution.
4. Transfer 1 ml of the cell suspension (approximately 3–5 x 10^6 adherent cells or 5–10 x 10^6 suspension cells) into each freezing vial. Label vials with the name of cell line, date, passage number, and growth medium.
   Tip: It may also be useful to note the cell density in the freezing vials before storing. This enables determination of the cell density that provides optimal recovery after thawing.
5. Place freezing vials in racks and transfer to a polystyrene box (with walls approximately 15 mm thick) lined with cotton wool. Store box in a –80°C freezer overnight.
   It is important that cells are frozen at a rate of 1°C/min. A controlled-rate freezing device can be used instead of the polystyrene box and cotton wool method.
6. The next day, quickly transfer the vials to a liquid nitrogen chamber, making sure that the vials do not begin to thaw.

Freezing medium

Composition
Growth medium (RPMI, DMEM, etc.) containing 10–20% FBS and 5–20% glycerol or DMSO

Viability staining

Trypan blue staining provides a method for distinguishing between viable (i.e., capable of growth) and nonviable cells in a culture. This staining method is based on "dye exclusion": cells with intact membranes exclude (i.e., do not take up) the dye and are considered viable.

1. Harvest the cells, either by trypsinization (adherent cell cultures) or by centrifugation at 200 x *g* for 5 min (suspension cell cultures). Resuspend the cells in an appropriate volume of pre-warmed growth medium to give a cell density of at least 10^6 cells/ml.
2. Add 0.5 ml 0.4% (w/v) trypan blue (see table Trypan blue) and 0.3 ml PBS or Hank's balanced salt solution (HBSS; see tables 1x PBS and 1x HBSS) to 0.1 ml of the cell suspension. Mix thoroughly, and let stand for 1–2 min.
   Alternatively, add 0.4 ml trypan blue directly to 0.4 ml of cells in growth medium.
   At least 10^6 cells/ml are required for accurate counting.
3. Count the stained and unstained cells using a hemocytometer (see Cell counting using a hemocytometer).
   Blue-stained cells are nonviable and unstained cells are viable.
   No. of viable cells/ Total no. of cells = % viability

Trypan blue

Component	Amount
Trypan blue	0.4 g

1x PBS or 1x HBSS	100 ml

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