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The Complete Guide To

CELL CULTURE

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What's Inside

Introduction to CELL CULTURE

What is cell culture?

Cell culture is the growth of cells from an animal or plant in an artificial, controlled environment. Cells are removed either from the organism directly and disaggregated before cultivation or from a cell line or cell strain that has previously been established.

Cell culture applications

Cell culture:

- Is a major consistent and reproducible tool in molecular and cellular biology ***(Figure 1)***.
- Helps to study normal cell homeostasis, cell biochemistry, metabolism, mutagenesis, diseases, and compound effects.
- Is a model system for diseases and drug screening.

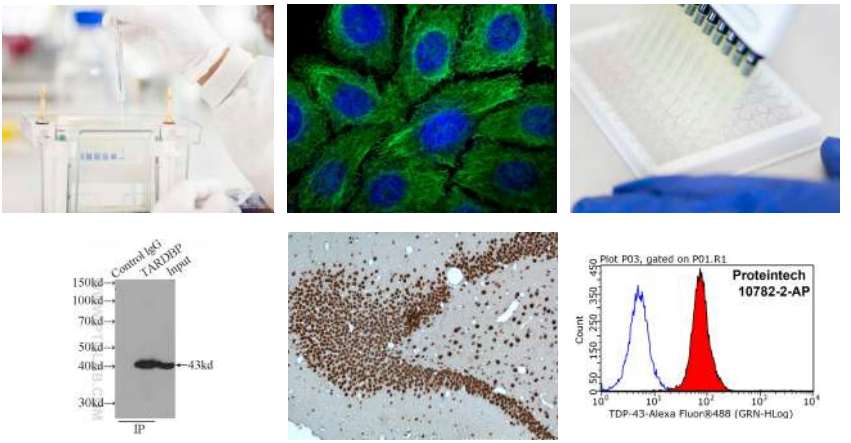

Figure 1. *Cell culture laboratory applications: western blot, ICC/IF, ELISA, IP, IHC, and FACS analysis*

Basic cell culture equipment

The specific equipment of a cell culture laboratory depend on the type of research conducted, however, all cell culture laboratories have the same common equipment being free from pathogenic microorganisms *(Figure 2)*.

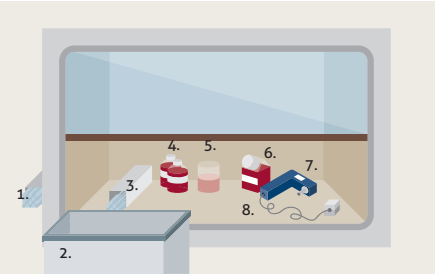

Figure 2. *Basic cell culture equipment.*

The extended list below is corresponding to the equipment and supplies for the majority cell culture laboratories, that allows the work to be more efficient and accurate.

- 70% ethanol antiseptic
- Aspiratory pump
- Autoclave
- Cell counter
- Cell culture flasks
- Cell culture-grade Petri dishes
- Cell culture-grade tubes of various sizes
- Cell culture hood
- Cell culture microwell plates
- Cell incubator
- Centrifuge
- Fridge/freezer
- Gloves
- Media, sera, cell media additives
- Pipettes of various capacities
- Serological pipettor
- Sterile filters
- Waste container
- Water bath

**Please note: The specific cell culture equipment depends on the cell type and aim of the study*

Cell culture safety

Work in a cell culture laboratory is associated with different risk factors and hazards such as toxins or mutagenic reagents. The human/animal material may contain viruses and other dangerous biological agents. Therefore, while manipulating human/animal material it is important to follow general safety guidance for laboratory practices.

1. Wash hands when entering and before leaving the laboratory.
2. Wear safety clothes (gloves, closed shoes, lab coat).
3. No eating, drinking, smoking.
4. No or low aerosol creation.
5. Decontamination of all surfaces before and after the experiment.
6. Work in accordance with the facility guidelines.
7. Dispose of all waste in an appropriate way.
8. Restricted access to authorized personnel only.
9. Avoid using sharp objects.
10. Always clearly label all samples.
11. Report all incidents to the safety officer.

Aseptic techniques required while working with cell culture

To be successful in cell culture, it is essential to remain a contamination free environment (bacteria, fungi etc) Aspetic techniques ensure that no microorganisms enter the cell culture. Cell culture steriity is ensured by set of procedures.

Handling	Reagents/Media	Workplace
Slow/careful handling.	Pre-sterilation of all reagents/ equipment.	Cell culture hood works properly
Sterilization of all items before starting.	No contamination in reagents (expiration date, appearance normal).	Frequent de-contamination (hood, fridge etc)
Sterile pipettes		Work area: sterile and tidy
No touching of sterile items to non-sterilized surfaces		

Table 1. Aseptic techniques required while working with cell culture.

Cell culture environment

Cell culture is an amazing tool that allows for easy controlling and manipulation of all physiochemical and physiological cell factors, such as, temperature, osmotic pressure, pH, gas, hormones, and nutrients.

Media	pH	Temperature	CO_2
Contains nutrients, growth factors, and hormones. Sera source of growth, tipids,hormones.	Average pH for mammatian cells is pH 7.4.	Depends on body temperature of host. Mammalian cell lines 36-37°C. Insert cell lines 27-30° C.	Controlled by media. Organic or CO_2 bicarbonate buffer systems are popular. Can impact pH. 4-10% CO_2 is most common.

Table 1. Cell culture environment.

Media supplements for cell culture

Media supplements help to optimize cell growth for specific applications depending on the chosen tissue or cell type. The advantages of using media supplements such as growth factors or cytokines are that they may improve cell viability and growth and keep cells healthier for longer (Table 3). For example, fibroblast growth factor (FGF) plays important roles in diverse biological functions in vivo and in vitro and can maintain cell culture over the weekend as Proteintech thermostable FGF (HZ-1285) does not require media changes every day *(Figure 3)*.

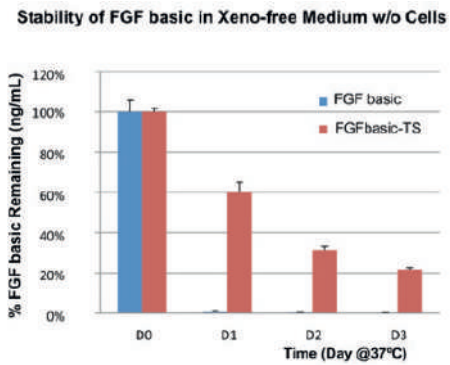

Figure 3. *The stability of FGFbasic-TS and FGF basic (E. coli-derived) in xeno-free, chemically defined cell culture media at 37˚C. The protein concentration was determined by ELISA each day for 3 days. After one day of incubation at 37˚C, FGF basic was undetectable, while FGFbasic-TS was present at levels of 60%, 35%, and 20% of its starting concentration at days 1, 2, and 3.*

Growth factors play an essential role to maintain the *in vitro* culture growth. Depending on the environment, certain cells can give rise to a variety of lineage-specific cell types. Table 3 contains a summary of the key growth factors needed for normal cell growth, metabolism, cell development in culture and their differentiation process.

Supplement	Cat. No.	Involved in/used for:
Fibroblast growth factor (FGF)	HZ-1285	• Embryonic development, neuron differentiation, and the proliferation of cells of mesodermal origin and many cells of neuroectodermal, ectodermal, and endodermal origin.
Bone morphogenetic protein 2 (BMP-2) and bone morphogenetic protein 4 (BMP-4)	HZ 1128 and HZ-1045	• Bone formation and regeneration. • Uses as differentiation factor of pluripotent stem cells and promotes osteogenic differentiation of mesenchymal stem cells.
Hepatocyte growth factor (HGF)	HZ-1084	• Epithelial-mesenchymal transition. • Acts as a mitogen for many cell types including hepatocytes.
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	HZ-1002	• Promoting growth and differentiation of hematopoietic progenitor cells. • Used to support the in vitro colony formation of granulocyte-macrophage progenitors.
Activin A	HZ-1138	• Regulating cell proliferation. • Used to maintain stem cell pluripotency and self-renewal in vitro.
Beta-nerve growth factor (beta-NGF)	HZ-1222	• Differentiation and survival of neurons.
Stem cell factor (SCF)	HZ-1024	• Promoting survival, expansion, and differentiation of hematopoietic stem cells.
Transforming growth factor beta (TGF-beta)	HZ-1011 HZ-1092 and HZ-1090	• Regulating cell growth, proliferation, and differentiation. • Widely used, e.g., in undifferentiated embryonic stem cell culture and induced pluripotent stem cells.
Vascular endothelial growth factor (VEGF121 and VEGV165)	HZ-1204 and HZ-1038	• Angiogenesis and vasculogenesis. • Used in endothelial cell culture.
Wnt3a	HZ-1296	• Regulation of neuronal stem cell self-renewal. • Used in expansion of neural stem cells.

Table 3. Selected media supplements and their role in cell culture.

Cell culture WORKFLOW

Cell culture workflow in 4 simple steps

ISOLATE

- Separate fresh tissue of interest.
- Treat tissue with enzyme(s) (e.g., trypsin, collagenase, protease) and/or mechanically to isolate cells.
- Wash, count, and seed cells.

VERIFY

- Examine cells under a brightfield microscope to assess their growth state, attachment to culture vessels/flasks, and to check for any signs of infection.
- Monitor cells for the following days until they reach confluence.
- Verify isolated cell types by their morphology and expressed biomarkers.

CULTURE

- Passage cells to propagate the cell line.
- Make master and working cell banks.
- Immortalize cells if necessary.

INVESTIGATE

- Plan and execute experiments.
- Keep monitoring cell state and possible infections using a brightfield microscope.

Considerations before starting CELL CULTURE

Temperature	✓ 36–37°C for mammalian cells. ✗ Temperature variability >0.5°C in the incubator.
CO2 level	✓ 4–8% if the medium uses CO2-bicarbonate buffers to maintain optimal pH.
Health check	✓ Check cell culture on a regular basis – visually and under a brightfield microscope. ✗ A sudden change in medium color (when pH indicators are used) and/or clarity or a foul smell may indicate infection. ✓ A brightfield microscope with 20–60x magnification will allow you to spot developing bacterial/fungal infections. ✗ In case of infection decontaminate and discard cells and clean the working area. The removal of infection with antimicrobial agents should be used as a last resort if no cell banks of the cell line exist.
Viability check	✓ Check cell culture on a regular basis – visually and under a brightfield microscope. ✗ Sudden detachment/rounding up of adherent cells, excessive clumping of suspension cells, or the presence of cell debris is indicative of apoptosis.
Cell density	✓ Plate cells at 10–30% density during standard passaging. Plating cells too sparsely may impede their growth. ✓ Cells should be passaged at 80–90% density, toward the end of the logarithmic growth phase. ✗ Do not allow your cells to overgrow – this will slow down their recovery after passaging.

Table 4. Considerations before starting cell culture.

Cell types & culture CHARACTERISTICS

Working with the primary cell culture

Primary culture cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate (i.e., reach confluence). To allow growth of the culture to continue past this point, cells need to be subcultured by transferring them to a new culture dish with fresh growth medium. Primary cell culture: cell lines directly expanded from tissues. Unless they undergo an immortalization procedure, primary cells have a limited lifespan and usually reach senescence after 10–20 passages.

Working with the cell line culture

After the first subculture, the primary cells start to become a cell line or subclone. Cell lines derived from primary cultures have a limited lifespan as a consequence of being outside of their tissue niche. While cell culturing, those cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population over time.

Continuous cell culture: Cells are immortalized and can be grown for many passages with no significant loss of viability. Many are well-known cell lines with very defined characteristics *(summary Figure 4)*.

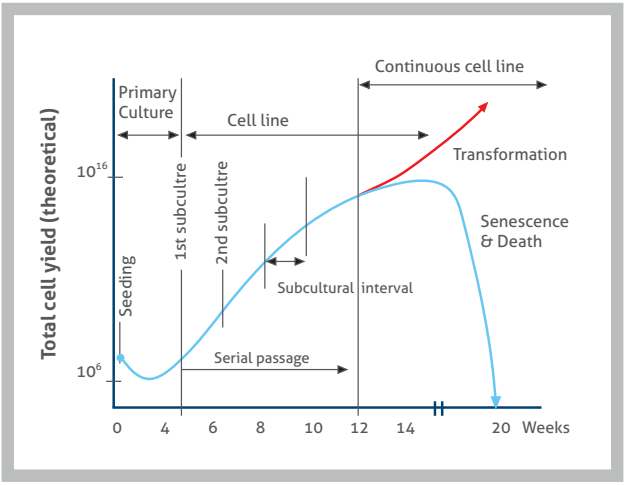

Figure 4. Primary cells culture vs. cell line culture.

Working with adherent cells

Adherent cells: types of cell lines that grow in the monolayer attached to the surface. While passaged (see standard passaging), a detaching agent (e.g., trypsin) needs to be used to detach them from the surface. They re-attach to the surface within a few hours upon plating. Based on their morphology, adherent cells can be divided into two main categories:

- Fibroblast-like: elongated shape, usually migrate on the dish
- Epithelial-like: polygonal shape, stationary, grow in patches

Suspension cells (lymphoblast-like): type of cell lines that grow in suspension and do not form monolayers on the surface. Cells form clumps, especially at high density *(summary Figure 5)*.

Working with cells in suspension

Figure 5. Mammalian cell lines differ in shape. Left: adherent fibroblast-like cells with an elongated shape; Middle: adherent epithelial-like with a polygonal shape; Right: suspension lymphoblast-like cells with a rounded shape.

Cell CONTAMINATION

Contamination of cell cultures is a serious risk to your samples. Consequently, safety measures, including aseptic techniques, should be put in place to actively prevent it and your samples should be regularly checked for any sign of infections. Cell contamination may severely affect their metabolism, growth, and viability, as well as decrease transfection efficiency and threaten the reproducibility of cell-based experiments.

Contamination in cell culture may be due to chemical and biological agents. Chemical contamination, such as the presence of endotoxins, detergents, or impurities of used media, sera, or other media supplements can be controlled by using trusted suppliers that guarantee rigorous batch testing. Biological contamination can arise from improper aseptic techniques and most often comes from the direct environment in the cell culture area. The most common biological contaminants are bacteria, fungi (yeast and mold), viruses, and mycoplasma *(summary Figure 6).*

Bacterial contamination:

Many bacterial strains can grow in commonly used media in mammalian cell culture. Due to rapid logarithmic growth, the signs of contamination are easily detectable within a few days of the initial contamination.

- Loss of medium clarity.
- Faster acidification of medium (medium turns orange/yellow if phenol red is present).
- A thin bacterial film appears on the surface of cell culture vessels/flasks.

Bacteria can be identified by a brightfield microscope check (20–60x magnification) as slowly moving, dark, round- or rod-shaped objects that are significantly smaller than mammalian cells.

Mycoplasma contamination:

Mycoplasma are very small (0.2um) bacteria characterized by their lack of a cell wall. Unlike standard bacterial contamination, mycoplasma often grow at a slow rate in infected cultures and can be present with no visible signs of infection such as loss of clarity or change of pH.

- Mycoplasma do not contain cell walls, so they cannot be seen with a standard brightfield microscope.
- Due to their slow growth rate, mycoplasma infection may develop for a long time without any signs.
- Mycoplasma infection often affects cell growth rates, metabolism, and can even lead to chromosome aberrations of samples.
- Routine testing using immunofluorescence with DNA stain, ELISA, or PCR is recommended to monitor possible infections

Fungal (yeast/mold) contamination:

Similar to bacterial contamination, yeasts can grow rapidly in mammalian culture media, leading to a loss of medium clarity, albeit at a slower rate.

- Relatively rapid progression from initial infection to a serious threat.
- pH is unchanged or slightly increases at later stages of the contamination, which can affect your experiments.
- Brightfield microscope check (20–60x magnification): rod-shaped or branched filamentous objects that become visible to the naked eye at later stages of contamination.

Many fungi can produce spores capable of surviving for a long time in a dormant state in hostile environments and, therefore, in the event of fungal contamination it is particularly important to decontaminate all working surfaces and incubators to prevent repeated contaminations.

Virus contamination:

Viral contaminations are not very common but can potentially pose a serious health threat to cell culture personnel.

- Viruses are hard to detect because they do not affect cell culture growth and cannot be seen with a brightfield microscope.
- Dedicated assays (e.g., ELISA, PCR) can be used to test for infections.

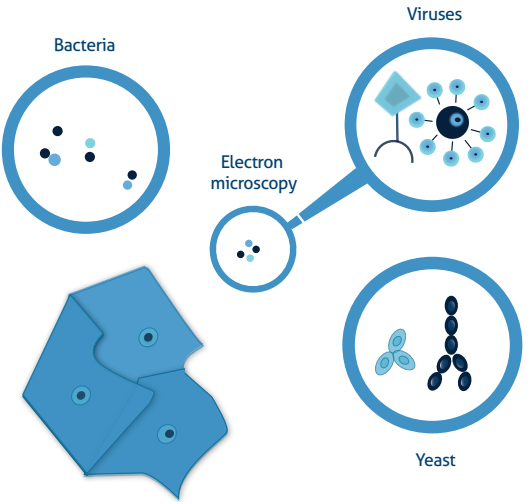

Figure 6. Cell culture contamination. Cells can be contaminated with bacteria (small, dark, rod-shaped that can form strings), yeast (elongated, branched structures, light with darker edges), or viruses (visible only with electron microscopy).

Cell culture PROTOCOL

Standard passaging (subculture):

Passaging refers to the diluting of cells that have reached high confluence to supplement cells with fresh medium to enable continuous culture propagation.

For mammalian cells, passaging should be performed when cells are toward the end of the logarithmic growth phase, so before they reach the stationary phase (Figure 3). For adherent cells that usually means reaching 80–90% confluence (there is still space for growth on a culture dish); for suspension culture, it is when cells start to clump and the culture becomes cloudy, especially if a dish is shaken slightly.

Passaging of cells at the stationary phase is not recommended because they tend to take longer to begin the logarithmic growth phase upon seeding. Additionally, the build-up of lactic acid in dense cultures may impact cell metabolism.

Adherent cells:

1. Remove medium.
2. Wash cells with calcium- and magnesium-free balanced salt solution (phosphate buffered saline, Hanks' balanced salt solution, etc...
3. Add detaching agent (e.g., trypsin). Incubate at 37°C until cells are fully detached from the dish (2–20 min depending on the cell line).
4. Resuspend cells in fresh medium, pipette thoroughly to obtain single cell suspension. If your medium does not contain serum, you need to inactivate detaching agents, e.g., by addition of trypsin inhibitors. Measure total number of cells (see: counting cells).
5. Plate cells onto a new dish at the desired cell density.

Suspension cells:

1. Collect cells with medium and briefly centrifuge (150–300 xg, 3–5 min) to pellet cells.
2. Remove medium and gently resuspend cells with a balanced salt solution. Briefly centrifuge (150–300 xg, 3–5 min) to pellet cells.
3. Remove salt solution and resuspend cells in fresh medium. Measure total number of cells (see: counting cells).
4. Plate cells onto a new dish at desired cell density.

It should be standard procedure to keep a record of the number of cell passages for all cell lines. For primary cell lines with a finite lifetime, it helps to monitor their viability and plan experiments before they reach senescence and cease dividing. For immortalized cells, it helps to monitor their age and design experiments up to a certain passage number.

Cell counting

Despite the recent development of automated counters, the Neubauer chamber is still the most common method used for cell counting. A Neubauer chamber is used to perform a proper cell count, although the dimensions and volumes of each chamber may differ.

The necessary elements to perform a cell count *(Figure 7):*

a) Cellular dilution to measure
b) Neubauer chamber
c) Optical microscope
d) Cover glass
e) Pipette / micropipette with disposable tips

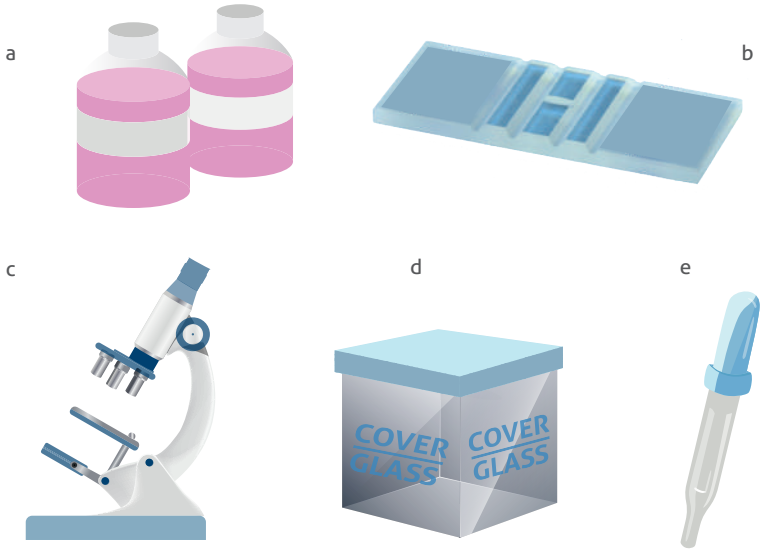

Figure 7. The equipment needed to perform a cell count with a Neubauer chamber

While performing the cellcount please remember:

- Avoid counting cells that are obvious cell debris vs. rounded cells
- When using trypan blue, count the cells that have excluded the dye
- Cell suspensions should be dilute enough so that the cells do not overlap each other on the grid
- Mix the cell suspension thoroughly before taking a sample to count
- Count the cells in selected squares
- Decide on a specific counting pattern to avoid bias

The formula for calculation of the cell concentration:

$$\text{Concentration (cel / ml)} = \frac{\text{Number of cells}}{\text{Volume (in ml)}}$$

- The number of cells is the sum of all cells counted in all squares
- The volume is the total volume of all the squares counted
- In case a dilution was applied, the final concentration needs to be converted to the original concentration before the dilution

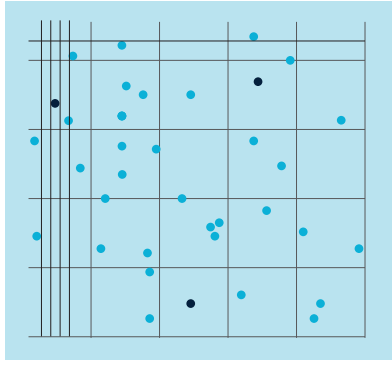

Figure 8. The equipment needed to perform a cell count with a Neubauer

An example of cell countingwith a Neubauer chamber:

- We counted the cells in four squares to obtain a reliable estimate of the number of cells.
- We obtained the following cell numbers: 54, 48, 52, and 60, which gave us a total of 214 cells.
- The area of one square in the Neubauer chamber used equals 0.01 cm2, while the depth of the chamber is 0.01 cm.
- Multiplying 0.01 cm2 by 0.01 cm gives 0.0001 ml total volume per one square.
- Using the above equation, we divide the total number of cells (214) by volume (0.0001*4 squares = 0.0004) to obtain a concentration of 535 000 cells per ml.

Cell banks (cryopreservation)

Mammalian cell lines are suitable for long-term storage at low temperatures (below -130°C – usually in liquid nitrogen). Cell lines are routinely frozen to make and keep reference/parental cell lines, newly produced transgenic cell lines, keep stocks of primary and immortalized cells, and for shipping purposes. The viability of cell banks is dependent on the cryopreservation procedure employed when making them and on the proper storage conditions. It is good laboratory practice to create a number of master stocks at the lowest possible passage number for long-term storage together with a number of working stocks for general use in experiments.

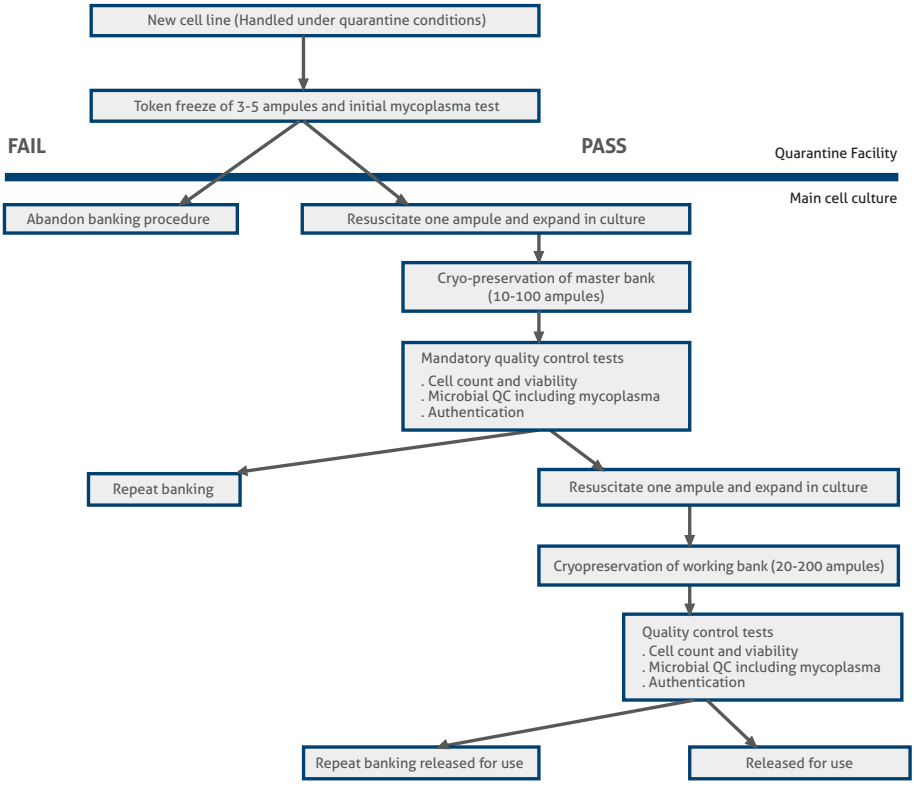

Figure 9.. Cell banking overview. chamber

Preparing cell banks

1. Grow cells toward the end of the logarithmic growth phase (~90% confluence). Ideally, use cells with the lowest possible passage number.
2. Passage cells as normal – detach adherent cells, collect and wash suspension cells (see: standard passaging).
3. Measure total cell number (see: counting cells).
4. Resuspend cells in freezing medium. In general, the freezing medium is routine culture medium supplemented with a cryoprotectant, an agent that prevents the formulation of ice crystals from the water present within cells that would destroy cell integrity. DMSO and glycerol are the two most commonly used cryoprotective agents. The exact concentration is cell-dependent and should be experimentally verified, but they are usually used at 5–10% (vol/vol) final concentration.

   Note: Prepare freezing medium before adding to cells because concentrated DMSO is toxic to cells.
5. Aliquot cells into cryovials (usually 106–107 cells/ml) and gently cool down to -80°C. The most efficient cooling rate is 1°C per 1 minute, which can be achieved by putting vials into a commercial cryofreezing container or by wrapping them in a thick layer of cotton wool and placing in a -80°C freezer.
6. After 1–3 days place cells in a liquid nitrogen dewar and store in liquid nitrogen or the gas phase of liquid nitrogen for long-term storage.

Thawing cells from banks

1. Transport frozen cell vial in low temperature (portable liquid nitrogen container or dry ice) to cell culture area.
2. Prepare a culture dish with pre-warmed medium.
3. Thaw cells rapidly (e.g., in a 37°C water bath).

   Note: Thawing cells rapidly ensures high cell viability.
4. Optional step to remove cryopreservant and non-viable cells: resuspend cells in medium and briefly centrifuge (150–300 xg for 3–5 min.). Resuspend cell pellet in fresh medium.
5. Transfer freshly thawed cells into a dish.

Cell transfection

Transfection of cells refers to the delivery of nucleic acid (DNA or RNA) into cultured cells. The most commonly used reagents are cationic lipids that can associate with nucleic acids to form positively charged complexes that allow interaction of DNA/RNA with the negatively charged cell membrane. That leads to the efficient entry of nucleic acids into cells via endocytosis.

Alternatively, nucleic acids can be delivered into cells by electroporation, co-precipitation of DNA with calcium phosphate, or polybrene/DMSO shock.

The delivered DNA does not usually integrate with the host genome. Therefore, DNA expression is generally transient. However, it is possible to engineer stable cell lines with transfected DNA stably integrated with the genome, but this requires a method of selecting cells with stable integration with a dedicated DNA vector design and antibiotic selection.

Cell culture TROUBLESHOOTING

My cells are not growing.

Check that the medium is recommended for the particular cell type.

Recommendations can be obtained from the biggest cell banks (ATCC and ECACC) and from previous scientific publications reporting use of the cell line.

Most cell lines require additional supplements for their growth. The most commonly used are serum (e.g., fetal bovine serum, concentration depends on the cell type, typically 5–20% (vol/vol)), glutamine, and non-essential amino acids (NEAA).

My adherent cells are not attaching to cell culture dishes.

Check the type of dishes that you use.

Many manufacturers provide dishes for suspension cultures with very hydrophobic surfaces that are not suitable for adherent cell culture. In this case, adherent cells do not attach to the surface and die.

Consider whether your cell line requires a special coating to improve cell adherence.

Most commonly used coating agents include poly-L-lysine (diluted in water), collagens (diluted in low molar acid solutions such as acetic acid or hydrochloric acid), and fibronectin (diluted in calcium- and magnesium-free PBS).

Specific guidelines are provided by manufacturers, but most commonly used protocols involve the preparation of a diluted coating agent, which is applied to dishes, followed by 1 h incubation at room temperature in the tissue culture hood and removal of excess agent by washing surfaces a few times with PBS.

My cells are regularly infected with mycoplasma or other pathogens.

Examine your aseptic procedures for possible methods of optimization.

Always work with cells under dedicated cell culture hoods and wear appropriate PPE, including gloves and a lab coat.

Regularly clean and decontaminate the working area. Use sterile tips and pipettes, sterilize any glassware and tools by autoclaving.

If needed, maintain two cell cultures, one with antibiotics and one antibiotic-free and examine both cultures regularly for any signs of infection.

Ideally, the regular use of antibiotics in routine cell culture should be limited because they can mask low-level infections, making them hard to detect, and provide selective pressure for developing antibiotic-resistant pathogens in your working area.

Consider microbiological testing of your working and master stocks to validate they are not contaminated.

I cannot recover any/ many cells from my cell banks.

Cells may not have been frozen or thawed properly; please follow our guidelines above.

Ensure that frozen vials are stored at the appropriate temperature (below -130°C) at all times.

Consider freezing more cells per one vial and seed freshly thawed cells at a higher density to allow logarithmic growth from the very beginning.

I have problems achieving efficient transfection of my samples.

The efficiency of cell transfection depends on many aspects such as general cell metabolic state and passage number. Also, some medium supplements, such as antibiotics or serum, can decrease transfection efficiency. In principle, transfection protocols should be optimized for every cell line, considering variables such as the selection of a transfection reagent, the ratio of DNA/RNA to transfection reagent, and cell density during transfection.

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