Technical Tip

October 2012

Culture of Animal Cells – Basic Techniques

1 Introduction

The culture of tissue and cells is commonly been used in the laboratory. Cultures become more widely used after the availability of defined cell media, which provide a controlled environment.

There are main advantages to using cell culture assays:

- Control of the environment
- Characterization and homogeneity of the samples
- *In vitro* modeling of in vivo conditions
- Economy, scale and mechanization of culture
- Avoid animal experiments

Contamination by microorganisms remains a major problem in tissue culture. Bacteria, mycoplasma, yeast, and fungi may be introduced via many sources, *e.g.* lab personnel, the atmosphere, work benches, solutions, instruments, or imported biological material.

2 Aseptic Techniques

To minimize the risk of contamination, follow these 5 rules:

1. Always check the cells carefully before handling (by eye and on a microscope).

   Become familiar with the indicators of abnormal cell growth.

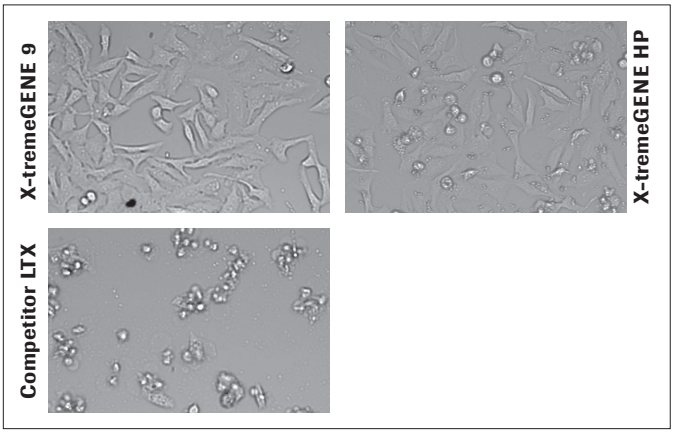


Figure 1: Morphology of HeLa cells after transfection. Cells look much healthier after transfection using X-tremeGENE Reagents than with the competitor reagent LTX.

2. Whenever possible, maintain cultures without antibiotics for at least part of the time, to reveal cryptic contamination.
3. Check sterility of all reagents before use.
4. Use dedicated media and reagents; do not share with other cell lines.
5. Maintain a high standard of sterility at all steps.

Mycoplasma contamination, which may slow cell growth, cannot be checked under a regular microscope. To confirm or rule out such contamination, use a mycoplasma test (*e.g.* Roche Applied Science Mycoplasma PCR ELISA Kit).

Environment

- There should be a laminar flow hood in the room dedicated to cell culture, and this hood should be used for all culture manipulations and storage of all equipment. The hood must be placed away from traffic or equipment that might generate air currents (*e.g.*, centrifuges, refrigerators and freezers).

  Always carefully clean the hood before and after your procedure. Remove all unneeded items.

- It is crucial to always keep the work surface clean and tidy. To achieve this, follow these 5 rules:

  1. Use 80% ethanol to clean the surface before starting.
  2. Place and keep on this surface only the items required for your procedure.

     This will reduce the possibility of contact between sterile and non-sterile items and facilitate culture manipulations.

  3. Clear space in the center of the bench, not just the front edge.
  4. Avoid spills. If they happen, immediately clean the area.
  5. Remove everything when you are done, and again clean the work surface.

- Reagents and media obtained from commercial suppliers will already have undergone strict quality testing. Most of the bottles are wrapped in polyethylene. The wrapping should be removed outside the hood. Unwrapped bottles should be cleaned with 80% ethanol whenever they are removed from the refrigerator or from a water bath.

  Regularly clean the refrigerator, the incubator and the water bath to avoid growth of mold or fungi.

- Imported cell lines should always be quarantined before being incorporated into your main stock. Do not perpetually use antibiotics; they will suppress some contaminants, but will not eliminate them.

Handling

- Use 80% ethanol to clean the work surface before and after your procedure or after any spills.
  Ethanol should be used to clean bottles, vessels or other items before they are introduced into the hood.

  Vessels in the incubator should not be in direct contact with the racks. Use a tray to store your vessels. This will reduce the possibility of introducing contaminants and spilling medium.

- Special care should be taken with caps. Use deep screw caps in preference to stoppers.
- When working on an open bench, flame glass pipettes and necks of the bottles before and after each use.
- Always use the pipettes which are best adapted your procedure; regularly clean them and check their calibration. Use a multi-channel pipette instead of a single pipette if you are working with multiwell plates. This will reduce both the time required to perform the procedure and the probability of contamination.
- Prepare as many reagents and equipment as possible in advance, to reduce the time the cultures are kept out of the incubator.

3 Culture Vessels

Most vertebrate cells cultured *in vitro* grow as monolayers on an artificial substrate. The choice of this substrate is crucial to cell adhesion. Although spontaneous growth in suspension is restricted to hemopoietic cell lines, rodent ascites tumors, and a few other selected cell lines, many transformed cell lines can be made to grow in suspension and become independent of the surface charge on the substrate. However, most normal cells need to spread out on a substrate to proliferate, and inadequate spreading due to poor adhesion or overcrowding will inhibit proliferation.

Substrates

- Glass is now rarely used. However, it has several advantages. It is easily washed without losing its growth-supporting properties and can be sterilized with either dry or moist heat.
- Single-use sterile polystyrene vessels provide a simple, reproducible substrate for culture. They also have superior optical properties and offer a flat growth surface, providing uniformly distributed and reproducible monolayer cultures.
  As manufactured, polystyrene is hydrophobic and does not provide a suitable surface for cell attachment, so tissue culture plastics are treated via corona discharge, gas plasma, g-irradiation, or chemicals to produce a charged, wettable surface.

  Treated products can vary in quality from one manufacturer to another. Therefore, test samples from several sources to determine which gives the best growth rate and plating efficiency for cells that are currently used in the laboratory. Perform these tests in appropriate medium containing no serum, a half-optimal concentration of serum, and an optimal concentration of serum.

  Although polystyrene is by far the most common and cheapest plastic substrate, cells may also be grown on polyvinylchloride (PVC), polycarbonate, polytetra-fluorethylene (PTFE; Teflon), Melinex, Thermanox (TPX), and a number of other plastics. The charged form of PTFE (hydrophilic) can be used for both regular monolayer cells and organotypic culture. The uncharged (hydro-phobic) form is suitable for macrophages and some transformed cell lines.

Choice of the Culture Vessel

To choose the most appropriate culture vessel for your assay, consider 6 points:

1. Mass of Cells Required:
   Cell yield is proportional to the available surface. Prepare small cultures in multiwell plates and use multiple replicates of each.

   Increasing the yield of cells growing in suspension requires simply increasing the volume of medium, as long as cells in culture are kept agitated and sparged with 5% CO_2 in air.

2. Type of Culture:
   Suspension or monolayer are used. Any type of flask or Petri dish can be taken when working with suspension cells. Stirrer bottles are used when agitation is needed to keep the cells in suspension.

   The rotational speed must be kept low (around 60 rpm), to avoid damage.

3. Atmosphere:
   Culture vented to the atmosphere or sealed
   Multiwell and Petri dishes have loose-fitting lids for easy access. These require a humid atmosphere and control of the CO_2 concentration.
   When venting is required, it is preferable to use flasks that have caps with permeable filters, as these allow CO_2 diffusion without risk of contamination.
4. Frequency of Sampling:
   If replicates must be processed in parallel, multiwell plates are ideal. If not, separate vessels should be used. Alternatively, some multiwell plates have removable wells that allow individual processing.
5. Analysis Required:
   For low-power microscopic observation of flasks, Petri dishes and multiwell plates, use an inverted microscope. If microscopy plays an important role in your study, it may be advantageous to use a chamber slide.
6. Cost:
   You must find the proper balance between cost and convenience. For example, Petri dishes are cheaper than flasks with an equivalent surface area, and are easier to examine and process.
   However, Petri dishes require humid, CO_2 -controlled conditions, and are also more prone to infection.

Recommended cell numbers for seeding of HeLa cells in different sizes of wells

Culture Vessel	Volume Recommended ml	Surface Area cm^2	Approximate Cell Yield for HeLa Cells
Multiwell Plates			
96-well plate	0.1	0.3	5×10^4
24-well plate	0.5	2	2×10^5
12-well plate	1	4	4×10^5
6-well plate	2	10	1×10^6
4-well plate	5	20	2×10^6
Petri Dishes			
3.5 cm diameter	2	9	9×10^5
6 cm diameter	5	21	2×10^6
10 cm diameter	12	55	5×10^6
Flasks			
25 cm^2	8-10	25	2.5×10^6
75 cm^2	12-15	75	7.5×10^6
175 cm^2	30-60	175	1.8×10^7
225 cm^2	60-100	225	2.2×10^7

Specialized System: Filter Wells

If the surface to which the cell is anchored is permeable, that surface may induce polarity in the cell by stimulating the basement membrane. Some manufacturers provide permeable supports in the form of disposable well inserts; these are available in many different sizes, materials, and membrane porosities.

Treated Surfaces

- Matrix Coating
  (*e.g.*, Roche collagen, laminin, or fibronectin)
  Poly-D-lysine can be used to coat the surface of plastic dishes. A concentration of 1 mg/ml is often used.

  Denatured collagen improves the attachment of many types of cells, *e.g.* epithelial cells.
  Undenatured collagen gel may be necessary for the expression of differentiated functions, *e.g.* neurite outgrowth from chick spinal ganglia.

  Diluting the concentrated collagen 1:10 with culture medium and neutralizing to pH 7.4 causes the collagen to gel, so dilution and dispensing must be rapid. It is best to incubate the gel with growth medium for a further 4–24 h before adding cells, to ensure equilibration between the gel and the medium.

  After this incubation, fibronectin (25–50 µg/ml), laminin (1–5 µg/ml), or both may be added to the medium.

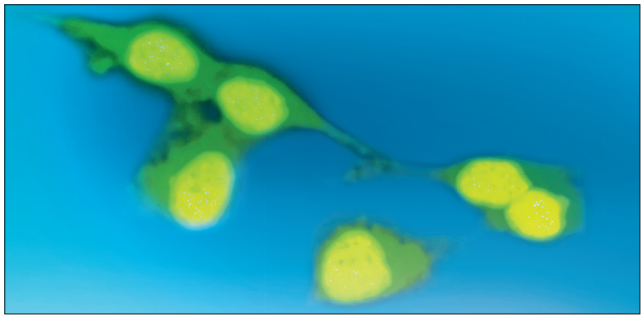
  Matrixgel contains laminin, fibronectin, and proteoglycans, with laminin predominant. Other matrix products include Pronectin F, laminin, fibronectin, vitronectin, entactin, and heparin sulfate.

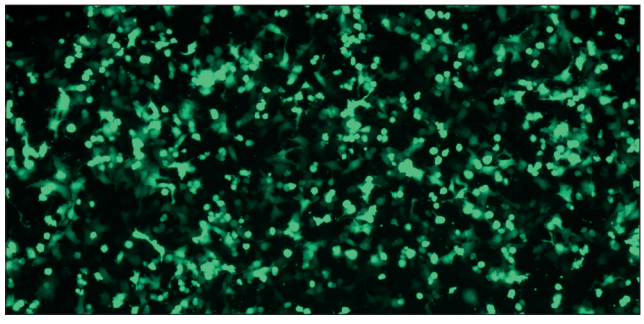
  Some matrix mixtures have been poorly characterized. If the goal of the study is examination of a cell mechanism, use such matrices only during intermediate stages. The final step must be done on a defined substrate.

  Extracellular matrix, derived from confluent monolayers of cells, can be used to provide the correct matrix for the culture of some specialized cells. A three-dimensional, matrix-like plasma is extensively used in tissue engineering.

- Feeder Layers
  Some cultures cannot be successfully grown with just a matrix coating. These more fastidious cells require support from living cells, particularly at low cell densities. This may be due to the release of metabolites or growth factors from the feeder cells.

  This cellular interaction can modify the morphology, proliferation and differentiation of the target cells.





4 Media and Supplements

The development of cell culture led to an increasing demand for well-defined and adapted medium for cell lines that require specific conditions.
To select the appropriate medium for a given cell line, consider the following 4 things:

Physico-chemical Features

For most cell lines, the optimum pH will be between 7.0 and 7.4; HEPES is a strong buffer at this pH; typically 10-20 mM concentrations of HEPES are used for cell culture.

Human epidermal cells should be maintained at pH 5.5. Phenol red may be added to the medium to provide an indicator of pH changes (see Reference 1).

Cultures vary in their requirement for oxygen. Although atmospheric or lower oxygen tensions are preferable for most cell cultures, some organ cultures require up to 95% O_2 in the gas phase.
Most cultured cells have a fairly wide tolerance for osmotic pressure. As the osmolality of human plasma is about 290 mOsm/kg, it is reasonable to assume that this level is the optimum for human cells *in vitro*, although it may be different for other species (*e.g.*, 310 mOsm/kg for mice).
The temperature recommended for most human and warm-blooded animal cell lines is +37°C.

Birds have a higher body temperature. Avian cells should be maintained at +38.5°C.

Media Components

- Balanced Salt Solutions
  A balanced salt solution (BSS) is composed of inorganic salts and may include sodium carbonate and, in some cases, glucose.
  Commercial complete media will list which BSS formulation was used.

  Hanks' salts would imply the use of sealed flasks, whereas Earle's salts would imply a higher bicarbonate concentration compatible with growth in 5% CO2.

- Complete Media
  Complete medium contains all necessary constituents and supplements such as: amino acids, vitamins, salts, glucose, organic supplements, hormones and growth factors (*e.g.*, Roche Applied Science Insulin or hEGF), and antibiotics (*e.g.*, Roche Applied Science G-418 Solution).

- Serum
  Serum contains growth factors, which promote cell proliferation, as well as adhesion factors and antitrypsin activity. It is also a source of minerals, lipids, and hormones.

  Always check new batches of serum before use. The quality and the composition can vary greatly from batch to batch.

  Serum is inactivated by incubating it for 30 min at +56°C. Originally, heating was used to inactivate complements for immunoassays, but it may also have other, undocumented effects.

- Other Supplements
  In addition to serum, tissue extracts and digests have traditionally been used to supplement tissue culture media. The most common ones are amino acid hydrolysates (from beef heart) and embryo extract (chick embryo).

Selection of Medium and Serum

Information regarding the selection of appropriate medium for a given cell type is usually available in articles about the origin of the cell line. If information is not available, perform a simple cell growth experiment in multiwell plates with various commercially available media. It may be difficult to reproduce conditions from other laboratories because of variations in preparation or supplier, impurities present in reagents and water, and differences between batches of serum.

Serum-free Media

Using serum in a medium has a number of disadvantages: the physiological variability, the shelf life and consistency, the quality control, the specificity, the availability, the downstream processing, the possibility of contamination, the growth inhibitors, the standardization and the costs.

Using serum-free media and defined media supplements (Nutridoma-CS, Nutridoma-SP, Transferrin) offers three main advantages:

- The ability to make a medium selective for a particular cell type.
- The possibility of switching from growth-enhancing medium for propagation to a differentiation-inducing medium.
- The possibility of bioassays (*e.g.*, protein production) free from interference with serum proteins (easier downstream processing).

But serum-free media are not without disadvantages:

- It increases the number of media.
- It can lead to the selection of a sublineage that is not typical of the whole population.
- Cell proliferation is often slower.

5 Subculture (Passage) and Cell Lines

Subculture can produce more homogenous cell lines when combined with other contraints (*e.g.*, subcloning, selection). Additionally, after subculture, cells may be propagated, characterized and stored; this allows a much wider range of experiments.

Selection of a Cell Line

Apart from specific functional requirements, there are a number of general parameters to consider in selecting a cell line:

- Finite vs. Continuous
  Continuous cell lines are easier to maintain, grow faster, clone more easily, produce a higher yield per flask and are more readily adapted to serum-free medium.

  If a cell line transforms *in vitro*, it becomes a continuous cell line.

  Cell lines with limited culture lifespans are known as finite cell lines (finite cell cultures are formed after the first subculturing of a primary cell culture) and behave in fairly reproducible fashion; they grow a limited number of generations before senescing.

  To prepare your primary cell cultures, please check our tissue dissociation portfolio at
  www.collagenase.com
  www.roche-applied-science.com

- Normal or Transformed
  Is it important whether the line is malignantly transformed or not?

- Species
  Is species important? Nonhuman cell lines have fewer biohazard restrictions and have the advantage that the original tissue may be more easily obtainable.

- Growth Characteristics
  What do you require in terms of growth rate, yield, plating efficiency and ease of harvesting?
  You will need to consider the following parameters:

  1. population-doubling time
  2. saturation density
  3. plating efficiency
  4. growth fraction
  5. ability to grow in suspension

Tip: The passage number is an important factor to consider when developing an assay. The passage number can influence not only protein expression but also cell proliferation.

- Availability
  If you must use a finite cell line, are sufficient stocks available?
- Validation
  How well characterized is the cell line?

  Be sure to eliminate any possible cross-contamination.

- Phenotypic Expression
  Can the line express the right traits?
- Stability
  How stable is the cell line? Is it possible to clone it?
- Control Cell Line
  When using mutant, transfected, transformed, or abnormal cell lines, always grow a control cell line in parallel.

Maintenance

Once a culture is initiated, whether it is a primary culture or a subculture, it will need periodic medium changes. For example, HeLa cells are usually subcultured once per week. Other cell lines may be subcultured only every two, three or even four weeks (Figure 2).

- Modification of Cell Morphology
  Prior to use, cells should always be checked for any signs of deterioration, such as granularity around the nucleus, cytoplasmic vacuolation, or rounding of the cells with detachment from substrate. Such signs may imply that the culture requires a medium change or may indicate a more serious problem (inadequate or toxic serum/medium, microbial contamination or senescence of the cell line).

- Replacement of the Medium
  Four factors indicate the need for the replacement of culture medium:

  1. Drop in pH
     Most cells stop growing as the pH falls from pH7.0 to pH 6.5 and start to lose viability between pH 6.5 and pH 6.0.

     As the pH drops, the indicator in the medium changes from red through orange to yellow.

  2. Cell Concentration
     High cell concentrations exhaust the medium faster than low concentrations.

3. Cell Type
   Normal cells usually stop dividing at high density due to cell crowding, growth factor depletion, etc. The cells arrest in the G1 phase of the cell cycle and deteriorate very little, even if left for two to three weeks (or longer).

4. Deterioration of Morphology
   This factor should be checked frequently. You should always be aware of the morphology since this may reveal the presence of contamination.

Criteria for Subculture

- Density of the Culture
  Cells should be subcultured prior to confluence.

  The ideal method for determining the correct seeding density is to perform a growth curve at different seeding concentrations. This allows you to determine the minimum concentration that will give a short lag period and early entry into rapid logarithmic growth.

- Exhaustion of Medium
  Medium requires periodic replacement. If the pH falls too rapidly, subculture may be required.

- Time since Last Subculture
  Routine subculture is best performed according to a strict schedule, so that reproducible behavior is achieved.

  It is essential to become familiar with the growth cell cycle for each cell line. Cells at different phases behave differently with respect to proliferation, enzyme activity, glycolysis and respiration, synthesis of specialized products, etc.

- Requirements for Other Procedures
  When cells require operations other than routine propagation (*e.g.*, increasing stock, changing vessel or medium), this procedure should ideally be done at the regular subculture time.

  Cells should not be subcultured while still in the lag phase; cells should always be taken between the middle of the log phase and the plateau phase (Figure 3), as determined during a previous subculture (unless experimental requirements dictate different timing).

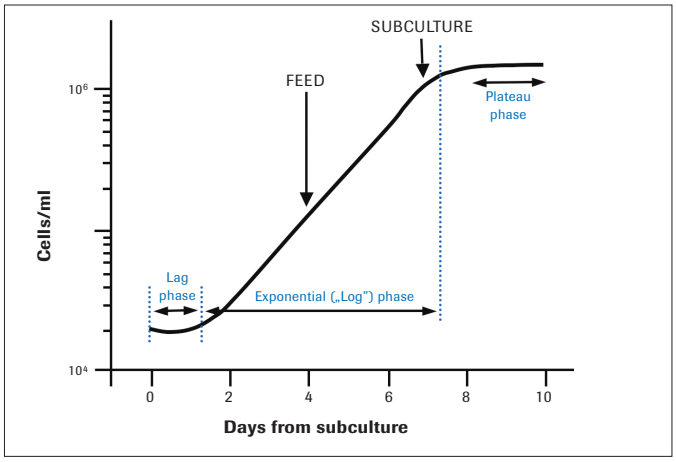


Figure 2: Growth curve and culture maintenance. Semilog plot of cell concentration versus time from subculture, showing the lag phase, exponential phase, and plateau, and indicating times which subculture and feeding should be performed.

6 Contamination

As previously mentioned, maintaining asepsis is still one of the most challenging tasks while culturing cells. Each of the many steps in the protocol offers a potential route for contamination.

Sources

The routes to contamination may be divided into 4 groups:

1. Technique:
   Manipulation of the different items (pipettes, bottles, culture vessels, etc.) management of the work place (dust, spills, clutter etc.) and the operator (clothes, hair, hands, breath).
2. Materials and Reagents:
   Solutions, glassware, instruments (*e.g.*, pipettes), culture vessels.
3. Equipment and Facilities:
   Room air (air conditioning), hoods, incubators, pumps.
4. Biological Matters:
   Imported material, dissection etc.

When starting with new reagents or material, always check the sterility and the quality of each before including them in your process.
Do not forget to regularly check the water, which is often a source of contamination.

Monitoring

To avoid contamination, we recommend:

- Examine your cells visually and with a microscope before each operation. Determine whether the morphology and growth of your cells are normal. If contamination is suspected, clear the hood and the bench and check each sample more carefully.
- Record the nature of the contamination when one occurs.
- When working with different cell lines in parallel, pay close attention to avoid cross-contamination.

Always suspect cross-contamination when a culture changes its appearance or phenotypic characteristics. For example, cells can start to pile up at high density in the plateau phase, when they are normally contact-inhibited. Alternatively, cells may start to grow faster or to reach a higher saturation density.

Eradication

The most reliable method of eradication is to discard the contaminated cultures and the material/reagents used to produce them.

- If only one culture is contaminated, discard that culture and the source material used.
- If the contamination is widespread, decontaminate the equipment and discard the stock solutions.

If you identify a microbial contamination, you should first check the potential roots or causes of the contamination: aseptic techniques used, the medium and reagents, the hood (*e.g.*, last filter/pressure check), the incubator, the refrigerator, the pipettes and other tools, the laboratory coats, the introduction of a new cell line, the quality of the water, the autoclave, plastic disposable items (pipettes, Petri plates, tips, etc.).
If the problem is affecting other people, check and decontaminate shared facilities (temperature, CO_2, humidity, new plastic disposables) and reagents (pH, improperly filtered water, new cell batch).

Microbial Contamination

Unless stocks are irreplaceable, you should discard cells and contaminated reagents rather than attempting decontamination. When decontamination is unavoidable, it should always be done by an experienced member of the team working in quarantine.

Protocol for Microbial Decontamination:

1. Collect the contaminated medium carefully.
   If possible, the organism should be tested for sensitivity to a range of individual antibiotics. If not, autoclave the medium or add hypochlorite.
2. Wash the cells in DBSS (Hanks BSS without bicarbonate, with Penicillin, Streptomycin, Amphotericin B and Kanamycin or Gentamycin). For monolayers, rinse the culture 3 times with DBSS, trypsinize, then wash the cells twice more in DBSS by centrifugation and resuspension. For suspension cultures, wash the culture five times (in DBSS) by centrifugation and resuspension.
3. Reseed a fresh flask at the lowest reasonable seeding density, depending on cell type.
4. Add high-antibiotic medium and change the culture every 2 days.
5. Subculture in a high-antibiotic medium.
6. Repeat Steps 1 to 4 for three subcultures.
7. Remove the antibiotics, and culture the cells without them for a further three subcultures.
8. Recheck the cultures (phase-contrast microscopy, Hoechst staining).
9. Culture the cells for a further two months without antibiotics, and check to make sure that all contamination has been eliminated.

6 Contamination

Mycoplasma

Mycoplasma-contaminated cultures should be treated using BM-Cycline or tylosin at the manufacturer's recommended concentration (in place of the usual antibiotics) in DBSS and the collection medium. The culture must be checked again to make sure that all contamination has been eliminated (*e.g.* with Roche Applied Science Mycoplasma PCR ELISA Kit).

Protocol for Detecting Mycoplasma in Contaminated Cell Culture with Roche Applied Science Mycoplasma PCR ELISA Kit

1. Centrifuge 1 ml cell culture supernatant at approx. 200 x g, 10 min at + 15 to +25°C.
2. Centrifuge supernatant in a fresh microfuge tube at 13,000 x g, 10 min at +2 to +8°C.
3. Completely remove supernatant without touching the pellet. Resuspend pellet in 10 µl sterile double-dist. water.
4. Positive control: Transfer 10 µl positive control DNA into a tube.
   Negative control: Transfer 10 µl sterile double dist. water into a tube.
5. Add 10 ml lysis reagent to samples and controls.
6. Add 30 µl neutralization reagent.
7. Transfer 25 µl ready-to-use PCR mix to an amplification cup.
8. Add 15 ml sterile double dist. water.
9. Add 10 µl sample and controls.
10. Start PCR program.
11. Pipet 40 µl denaturation reagent into a tube.
12. Add 10 µl amplification product.
    Incubate 10 min at +15 to +25°C.
13. Add 450 µl hybridization reagent (freshly prepared).
14. Transfer 200 µl to microplate well.
    Incubate 3 h at +37°C (on a shaker at 300 rpm).
15. Wash with 3 x 250 µl washing buffer (1x).
16. Add 200 µl anti-DIG-POD, working dilution.
    Incubate 30 min at +15 to +25°C
    (on a shaker at 300 rpm).
17. Wash with 5 x 250 µl washing buffer (1x).
18. Add 100 µl TMB substrate.
    Incubate 20 min at +15 to +25°C
    (on a shaker at 300 rpm).
19. Add 100 µl stop reagent.
20. Determine absorbance at 450 nm with a reference wavelength at approx. 690 nm.

Protocol for Treating Mycoplasma-contaminated Cell Cultures with BM Cyclin

1. Remove culture medium from culture vessels by aspiration.
2. Add new culture medium containing BM Cyclin 1 (4 µl of stock solution/ml, final concentration 10 µg/ml).
3. Cultivate the cells for 3 days as usual.
4. Remove culture medium.
5. Add new culture medium containing BM Cyclin 2 (4 µl of stock solution/ml, final concentration 5 µg/ml).
6. Cultivate the cells for 4 days.
7. Repeat the above cycle twice.
8. Check for mycoplasma contamination (*e.g.*, with a DNA fluorochrome such as DAPI).

Viral Contamination

There are no reliable methods for eliminating viruses from a culture.

7 Cryopreservation

To protect the investment made in establishing your cell lines you will have to preserve them.

Why?
Preservation (*e.g.*, by freezing) helps guarantee the genotypic/phenotypic stability of your cells and protects your stock against any type of contamination. Other reasons for freezing a validated stock of cells include: avoiding senescence or transformation, saving time/materials that would otherwise be spent maintaining lines not in immediate use.

How?
The best way to preserve cells is to freeze them. Before starting, you must make sure that the culture satisfies the following criteria: free of contamination, healthy, proper morphological characteristics, proper phase of growth (late log phase before entering plateau).

Protocol for Freezing Cells:

1. Check the cells.
2. Grow the culture up to the late log phase.
3. Resuspend at 2 x 10^6-2 x 10^7 cells/ml.
4. To prepare freezing medium, dilute one of the cryoprotectants (10-20% dimethyl sulfoxide [DMSO] or 20-30% glycerol) in growth medium.
5. Dilute the cell suspension 1:1 with freezing medium.
6. Dispense the cell suspensions into vials and freeze them slowly (at 1°C/min) to avoid crystal formation (*e.g.* using freezing container or tubular foam pipe insulation or programmed, controlled-rate freezer).
7. When the samples have reached -70°C, transfer them to liquid nitrogen.

Protocol for Thawing Cells:

1. Take the ampoule from the liquid nitrogen.
2. When the ampoule has thawed, clean it with 80% ethanol.
3. Transfer the content to a culture vessel.
4. Add dropwise 1 ml of serum and then 9 ml of medium (or 10 ml if working in serum-free conditions).
5. Pellet the cells by centrifugation.
6. Discard the supernatant and resuspend cells in fresh growth medium.
7. Check the cells after 24 h.

When working with liquid nitrogen, always wear a face shield, as well as gloves and a closed lab coat.

8 Quantitation

Quantitation is used to characterize cell growth and to establish reproducible culture conditions.

Hemocytometer
The concentration of a cell suspension may be determined by placing the cells in an optically clear chamber under a microscope. The cell number within a defined area of known depth is counted and the cell concentration is derived from the count.

8 Quantitation

Protocol for Cell Counting Using a Hemocytometer (Specifically, an Improved Neubauer Hemocytometer)

1. Clean the surface of the hemocytometer with 80% ethanol.
2. Clean the coverslip and wet the edges. Press down in order to attach the coverslip properly to the slide.
3. Trypsinize the monolayer as usual and resuspend in medium to give an estimated concentration of 1 x 10^6 cells/ml (can be estimated according to the culture vessel used, see "Culture Vessels" section before).
4. Mix the suspension thoroughly to disperse the cells and transfer 1 ml suspension to a vial.
5. Mix the cells thoroughly, pipetting vigorously to disperse any clumps, then collect 20 µl.
6. Transfer the cell suspension immediately to the edge of the hemocytometer chamber, expel the suspension and let it be drawn under the coverslip by capillarity.

Do not overfill the chamber; this would change its volume.

7. Repeat step 5 and 6 to fill the second chamber, if available.
8. To count the cells, transfer the slide to the microscope stage.
9. Count the cells lying in the central area for both chambers.

To avoid counting the same cell twice, count only cells that lie on the top and left-hand lines of each square, but not those on the bottom or right-hand lines.
For routine subculture, attempt to count between 100 and 300 cells per mm^2.

Analysis

Calculate the average of the two counts, and derive the concentration of your sample using the formula:

$$c = n/v$$

"c" is the cell concentration (cells/ml),"n" is the number of cells counted, and "v" is the volume counted (ml). For the improved Neubauer hemocytometer, the depth of the chamber is 0.1 mm and the central area 1 mm^2; therefore v is 0.1 mm^3 or 1 x 10^{-4} ml. The formula then becomes:

$$c = n/10^{-4} \text{ or } c = n \times 10^4$$

Electronic Counting

For high throughput work, electronic cell counters can be used to determine the concentration of each sample.

Other Quantitation

In some cases, *e.g.* if the downstream application does not require this data, the number of cells need not be determined. However, the DNA content or the protein concentration should be determined.
The cell concentration is derived from the count.

9 Cell Viability, Cell Proliferation and Cytotoxicity

Cell Viability

Cell viability assays, *i.e.* determination of the number of healthy cells in a sample, are often useful when non-dividing cells (such as primary cells) are isolated and maintained in culture; this helps to determine optimal culture conditions for these populations.
The most useful and straightforward method for determining viable cell number is to stain the cells with a dye such as trypan blue and count them in hemocytometer (such as the Neubauer hemocytometer). The dye allows you to distinguish between healthy cells with uncompromised membrane integrity (unstained) and unhealthy ones (stained blue).
One can also measure metabolic activity by incubating cells with tetrazolium salts that are cleaved into colored, water insoluble (MTT) or water-soluble (XTT, WST-1, Figure 3) formazan salts. In addition, cell viability can be assayed using Roche´s easy-to-apply one-step Cell Viability Imaging Kit.

Protocol for Estimating Cell Viability by Dye Exclusion

1. Prepare cell suspension by trypsinization and resuspension in medium.
2. Take a clean homocytometer and fix the coverslip.
3. Add one drop of Trypan Blue to the cell suspension.
4. Load the suspension into the hemocytometer and count the cells as described above.

Application of Cell Proliferation Reagent WST-1 for the Measurement of Cellular Metabolism of HeLa Cells Transfected with a Caspase-8 Expression Plasmid*

Experimental Procedure

HeLa cells (ATCC® CCL-2™) were transfected with the expression vectors pRK5, pRK-GFP, and pRK-Casp8. 4, 24, and 48 hours after transfection, 10 µl WST-1 Cell Proliferation Reagent was added to each well. After 60 minutes of incubation at 37°C, the generated WST-1 formazan was quantitated in a spectrophotometer.

Results

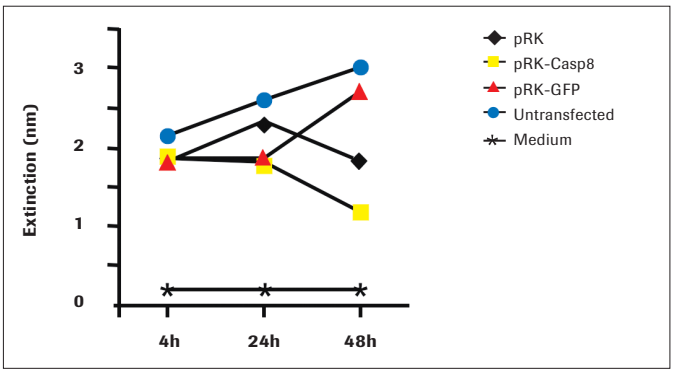


Figure 3: Colorimetric quantitation of cellular metabolism using WST-1 Cell Proliferation Reagent. Higher values mean higher metabolic activity. The data show that metabolic activity of cells transfected with pRK-Casp8 was strongly reduced 48 hours post transfection, suggesting that caspase-8 overexpression had a toxic effect on the cells.
* Data kindly provided by S. Adam, University of Kiel, Germany.

Protocol for Measuring Metabolic Activity

1. Culture cells in microplates (tissue culture grade, 96 wells, flat bottom) in a final volume of 100 µl/well culture medium in a humidified atmosphere (*e.g.*, 37°C, 5% CO_2).
2. Add 10 µl/well Cell Proliferation Reagent WST-1.
3. Incubate the cells for 0.5 to 4 h in a humidified atmosphere (37°C, 5% CO_2).
4. Shake thoroughly for 1 min on a shaker.
5. Using a background control as blank, measure the absorbance of the samples with a microplate (ELISA) reader at 420-480 nm.
   The reference wavelength should be more than 600 nm.

Cell Proliferation

An alternative way to determine the health of a culture is to perform a cell proliferation assay, *i.e.* to determine the number of dividing cells. One way of measuring this parameter is by performing clonogenic assays. In these assays, a defined number of cells are plated onto an appropriate matrix and the number of colonies that form are counted after a period of growth. Drawbacks to this type of assay are that it is tedious and it is not practical for large numbers of samples.

Another way to analyze cell proliferation is to measure *DNA Synthesis*. In these assays, labeled DNA precursors (^{4}H-thymidine or bromodeoxy-uridine, BrdU (*e.g.*, Roche Applied Science Cell Proliferation ELISA, BrdU (chemiluminescent) Kit) are added to cells and their incorporation into DNA is quantified after incubation. The amount of labeled precursor incorporated into DNA is quantified either by measuring the total amount of labeled DNA in a population, or by detecting the labeled nucleimicroscopically. Cell proliferation can also be measured using more indirect parameters. In these techniques, molecules that regulate the *Cell Cycle* (also called proliferation markers) are measured either by their activity (*e.g.*, CDK kinase assays) or by quantifying their amounts (*e.g.*, Western blots, ELISA, or immunohistochemistry).

Cell Cycle

The cell cycle is made up of four phases (Figure 4). In the M phase (M = mitosis), the chromatin condenses into chromosomes, and the two individual chromatids, which make up the chromosome, segregate to each daughter cell. In the G1 (Gap 1) phase, the cell either progresses toward DNA synthesis and another division cycle or exits the cell cycle reversibly (G0) or irreversibly to commit to differentiation. During G1, the cell is particularly susceptible to control of cell cycle progression; this may occur at a number of restriction points, which determine whether the cell will re-enter the cycle, withdraw from it, or withdraw and differentiate. G1 is followed by the S phase (DNA synthesis), in which the DNA replicates. S in turn is followed by the G2 (Gap 2) phase in which the cell prepares for reentry into mitosis. Checkpoints, at the beginning of DNA synthesis and in G2, determine the integrity of the DNA and will halt the cell cycle to allow either DNA repair or entry into apoptosis if repair is impossible. The Phospho Histone H3 Imaging Kit (Roche) is a convenient method for fast cell cycle analysis by quantification of mitotic cells.

Apoptosis, or programmed cell death, is a regulated physiological process whereby a cell can be removed from a population. Characterized by DNA fragmentation, nuclear blebbing, and cell shrinkage, apoptosis can be detected via a number of marker enzymes and kits (see Roche Applied Science products). Roche´s DNA Fragmentation Imaging Kit is a TUNEL assay-based method for accurate and fast quantitative fluorescence detection of apoptosis in medium to high throughput cellular workflows.

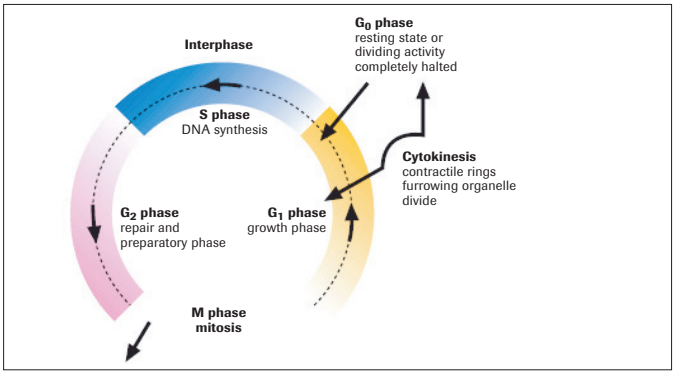


Figure 4: The Cell Cycle. The cell cycle is divided into four phases: G1, S, G2, and M. Progression round the cycle is driven by cyclines interacting with CDC kinases and stimulated by nuclear oncogenes and cytoplasmic signals initiated by receptor kinase interaction with ligand. The cell cycle is arrested at restriction points by cell cycle inhibitors such as Rb and p53.

Cytotoxicity

Cell viability and toxic effects can be assayed using Roche´s easy-to-apply one-step Cell Viability Imaging Kit. The indicators of cytotoxicity can vary, depending on the study performed (*e.g.*, Roche Applied Science Cytotoxicity Detection Kit^{Plus}, (LDH)). The cytotoxicity effect can lead to the death of the cells or just to an alteration of their metabolism.

This toxic effect can be initiated by addition of compounds or by addition of effector cells.

Demonstrating the lack of toxicity of a given compound may require subtle analysis of its interaction with specific targets, *e.g.* a study of its ability to alter cell signaling or to initiate cell interactions that would give rise to an inflammatory or allergic response.

To test the potential cytotoxicity of compounds/cells, consider the following parameters:

- Concentration of Compound
  A wide range of concentrations should be tested to determine the survival curve.
- Medium/Serum
  In some cases, the serum may have a masking effect and lead to an underestimation of the cytotoxicity effect.
- Duration of the Exposure
  The action of one compound can happen within a few seconds or over several hours.
- Cell Density
  For most of the assays, confluent cells are not used. However, if you want to study the endothelial barrier function, you will need confluent cells in order to see an effect.
- Colony Size
  Some agents are cytostatic, *i.e.* they inhibit cell proliferation but are not cytotoxic. During continuous exposure they may reduce the *size* of colonies without reducing the *number* of colonies. In this case, the size of the colonies should be determined by densitometry, automatic colony counting or counting the number of cells per colony with the naked eye.
- Solvents
  Some agents to be tested have low solubilities in aqueous media, and it may be necessary to use an organic solvent to dissolve them. Ethanol, propylene glycol and dimethyl sulfoxide have been used for this purpose, but may themselves be toxic to cells.

  The final concentration of solvent should be maintained as low as possible (<0.5 %) and a solvent control must always be included in the study. Be aware that some organic solvents are not compatible with plastics.

9 Cell Viability, Cell Proliferation and Cytotoxicity continued

The **Dose-response relationship** describes the biological effect induced by different concentrations of a substance (Figure 5). This curve should be determined whenever a new study is initiated, in order to fix the optimal conditions for the assay.

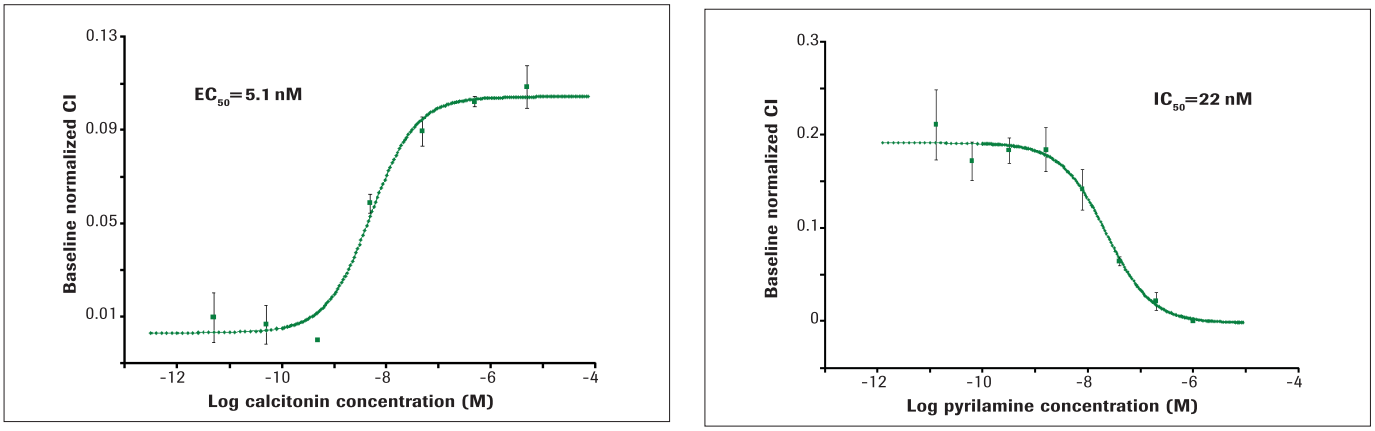


Figure 5: Dose-response curves.

The **half-maximal effective concentration**, or **EC_{50}**, refers to the concentration of a compound which induces a response halfway between the baseline and the maximum. The EC_{50} represents the concentration of a compound where 50% of its maximal effect is observed.

The **half-maximal inhibitory concentration**, or **IC_{50}**, is the concentration of a compound required to inhibit a process by half. IC_{50} represents the concentration of a compound that is required for 50% inhibition *in vitro*.

The **median lethal dose, LD_{50}** (abbreviation for "Lethal Dose, 50%"), or **LCt_{50}** (Lethal Concentration & Time) of a toxic compound is the dose required to kill half the tested population.

10 Ordering Infomation

Product	Catalog Number	Pack Size
Transfection		
X-tremeGENE HP DNA Transfection Reagent	06 366 244 001	0.4 ml
	06 366 236 001	1.0 ml
	06 366 546 001	5 x 1 ml
X-tremeGENE 9 DNA Transfection Reagent	06 365 779 001	0.4 ml
	06 365 787 001	1.0 ml
	06 365 809 001	5 x 1 ml
Gene Knockdown		
X-tremeGENE siRNA Transfection Reagent	04 476 093 001	1 ml (400 transfections in a 24-well plate)
	04 476 115 001	5 x 1 ml (2,000 transfections in a 24-well plate)
Reporter Gene Detection		
Anti-GFP	11 814 460 001	200 µg
Apoptosis		
Caspase 3 Activity Assay	12 012 952 001	1 kit (96 tests)
DNA Fragmentation Imaging Kit	06 432 344 001	1 kit (96 tests)

10 Ordering Infomation

Product	Catalog Number	Pack Size
Cell Viability & Cytotoxicity		
Cytotoxicity Detection Kit^{PLUS} (LDH)	04 744 926 001	1 kit (400 tests)
	04 744 934 001	1 kit (2,000 tests)
Cell Viability Imaging Kit	06 432 379 001	1 kit (5 × 96 tests)
Cell Cycle Analysis		
Phospho Histone H3 Imaging Kit	06 569 161 001	1 kit (5 x 96 tests)
Cell Proliferation		
Cell Proliferation Reagent WST-1	11 644 807 001	25 ml (2,500 tests)
Cell Proliferation ELISA, BrdU (chemiluminescent)	11 669 915 001	1 kit (1,000 tests)
Tissue Dissociation		
Liberase DL Research Grade	05 401 160 001	2 × 5 mg
	05 466 202 001	2 × 50 mg
Liberase DH Research Grade	05 401 054 001	2 × 5 mg
	05 401 089 001	2 × 50 mg
Liberase TL Research Grade	05 401 020 001	2 × 5 mg
Liberase TM Research Grade	05 401 119 001	2 × 5 mg
	05 401 127 001	2 × 50 mg
Liberase TH Research Grade	05 401 135 001	2 × 5 mg
	05 401 151 001	2 × 50 mg
Traditional Collagenase A	10 103 578 001	100 mg
	10 103 586 001	500 mg
	11 088 793 001	2.5 g
Traditional Collagenase B	11 088 807 001	100 mg
	11 088 815 001	500 mg
	11 088 831 001	2.5 g
Traditional Collagenase D	11 088 858 001	100 mg
	11 088 866 103	500 mg
	11 088 882 001	2.5 g
Traditional Collagenase H	11 074 032 001	100 mg
	11 074 059 001	500 mg
	11 087 789 001	2.5 g
Traditional Collagenase P	11 213 857 001	100 mg
	11 213 856 001	500 mg
	11 213 873 001	2.5 g
Traditional Collagenase / Dispase	10 269 638 001	100 mg
	11 097 113 001	500 mg
DNase I, Grade II	10 104 159 001	100 mg
Dispase I	04 942 086 001	10 x approx. 2 mg
Dispase II, Grade II	04 942 078 001	5 x 1 g
Papain	10 108 014 001	100 mg (10 ml)
Pronase	10 165 921 001	1 g
	11 459 643 001	5 g

10 Ordering Infomation continued

Product	Catalog Number	Pack Size
Antibiotics		
G-418 Solution	04 727 878 001	20 ml (1 g)
	04 727 894 001	5 x 20 ml (5 g)
Hygromycin B from *Streptomyces* sterile-filtered *Hygroscopicus*	10 843 555 001	20 ml (1 g)
Mycoplasma Detection and Elimination		
BM-Cyclin	10 799 050 001	37.5 mg (for 2 x 2.5 l medium)
Mycoplasma PCR ELISA	11 663 925 910	1 kit (96 reactions)
Growth Factors and Cytokines		
hGH ELISA	11 585 878 001	1 kit (192 tests)
Oncology Research		
TeloTAGGG Telomerase PCR ELISA^{PLUS}	12 013 789 001	1 kit (for up to 96 reactions)
Virus Research		
Reverse Transcriptase Assay, colorimetric	11 468 120 910	1 kit (200 tests)

For more products related to cell biology, please visit **www.roche-applied-science.com**

11 References

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(5) Lab FAQs, 3rd edition (<https://www.roche-applied-science.com/publications/print_mat/lab_faqs.pdf>)

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