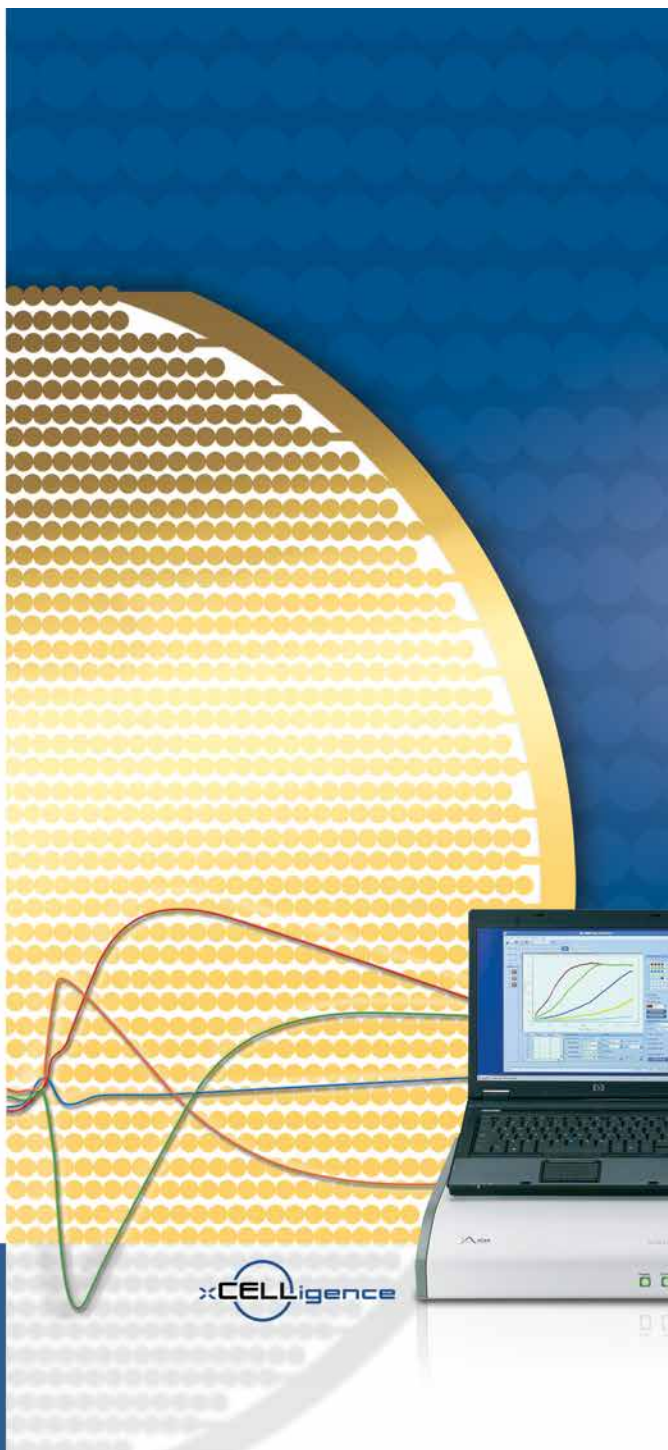ACEA
Biosciences, Inc.

xCELLigence **System**

Application Note No.7/January 2013

Culture and monitoring of animal cells
Basic techniques

For life science research only.
Not for use in diagnostic procedures.

xCELLigence

Culture of animal cells
Basic techniques

Introduction

Since the beginning of the twentieth century, the culture of tissue and cells has commonly been used in the laboratory. Cultures became more widely used after the introduction of antibiotics, which allow long-term propagation, and the development of defined media, which provide a controlled environment.

There are four 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.
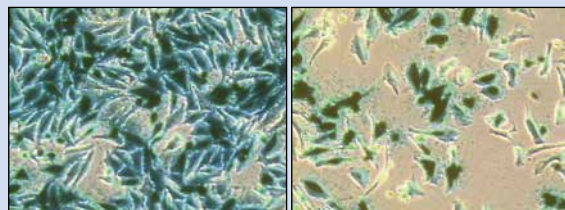
Aseptic Techniques

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

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.

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 6 rules:

1 Start with a clear surface.
2 Use 80% ethanol to clean the surface before starting.
3 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.

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

(A) FuGENE® HD Transfection Reagent　　　**(B) Reagent L**

Figure 1: FuGENE® HD Transfection Reagent delivers effective transfection with low cytotoxicity. HeLa cells (ATCC® CCL-2TM) were transfected with β-gal expression vector using (A) FuGENE® HD Transfection Reagent or (B) a transfection reagent (Reagent L) from another supplier, then stained 24 hours later.

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.

- 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.

- Biological material
  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.

Culture Vessels

Most of 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.
- Plastics
  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, γ-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, polytetrafluoroethylene (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 (hydrophobic) 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
Any type of flask or Petri dish can be used 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 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.

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			
10 cm^2	2	10	1×10^6
25 cm^2	5	25	2.5×10^6
75 cm^2	25	75	7.5×10^6
175 cm^2	75	175	1.8×10^7
225 cm^2	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. The CIM-Plate 16 allows the study of invasion and migration with the xCELLigence System and the E-Plate insert allows the study of cell-cell interactions.

Treated surfaces

■ Matrix coating (*e.g.* collagen 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.

Matrigel 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.
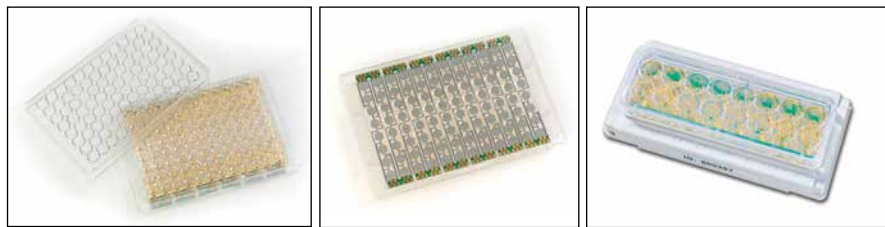
Media and Supplements

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:

Physico-chemical features

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

Epidermal cells should be maintained at pH 5.5. Phenol red may be added to the medium to provide an indicator of pH changes.

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 mosmol/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 mosmol/kg for mice). The temperature recommended for most human and warm-blooded animal cell lines is +37°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% CO_2.

- complete media
  Complete medium contains all necessary constituents and supplements such as: amino acids, vitamins, salts, glucose, organic supplements, hormones and growth factors, and antibiotics.
- 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 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.

Subculture (Passage) and cell lines

Subculture produces a more homogeneous cell line. 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 extinction.

- 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 xCELLigence System allows you to check the growth characteristics of your cells (Figure 2).
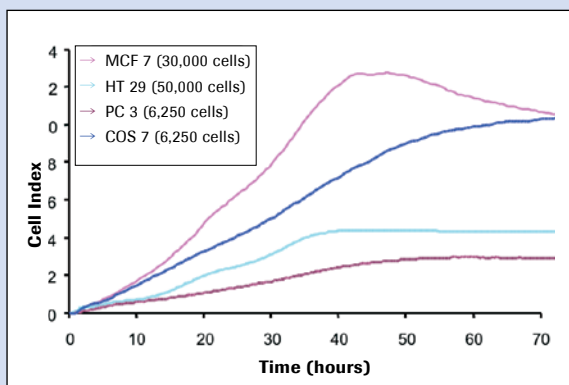
Figure 2: Cell proliferation curves of 4 different cell lines as recorded on the RTCA SP Instrument.

Tip: The passage number is an important factor to consider when developing an assay. The passage number can influence not only protein expression (Figure 3) but also cell proliferation (Figure 4).
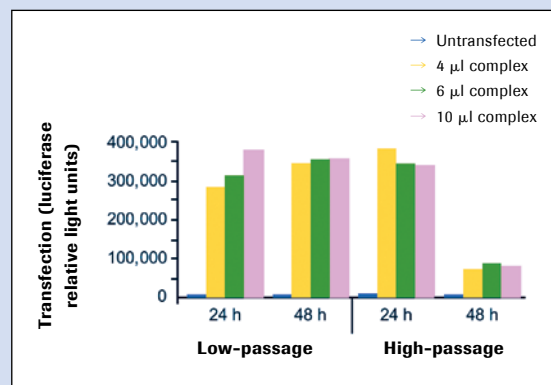
Figure 3: Influence of passage number on protein expression. Low- and high-passage number RAW 264.7 (ATCC® TIB-71™) cells were transfected with three different volumes of transfection complex and FuGENE® HD Transfection Reagent (see Biochemica No. 3, 2007, page 32).

A Passage #2
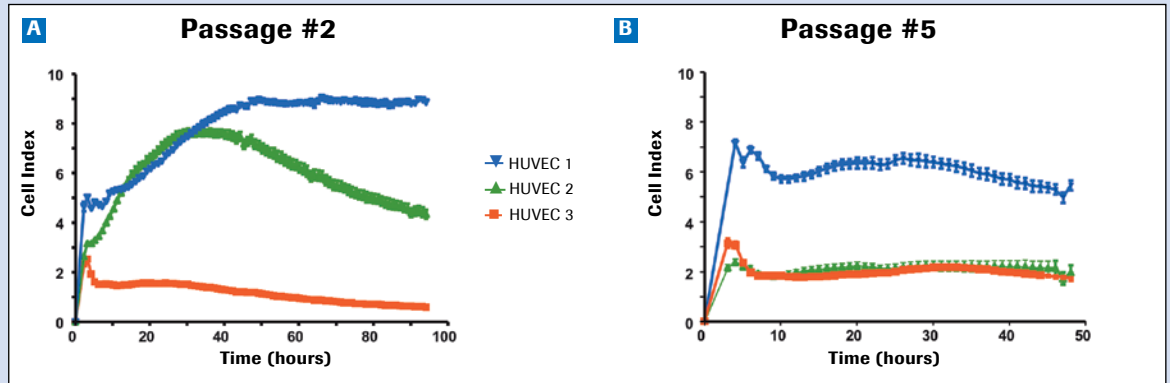
B Passage #5

Figure 4: Influence of origin and passage number on proliferation.
In this experiment human umbilical vein endothelial cells (HUVECs) were acquired from three different vendors and monitored with the xCELLigence System. The cells were cultured according to each supplier's instructions and microscopic examination indicated that these cells were morphologically indistinguishable (data not shown). The cells were seeded in wells of E-Plates 96 and adhesion, spreading, and proliferation were continuously monitored. As shown in Figure A, even though the cells are from the same tissue, significant differences in the kinetic pattern of adhesion and growth are observed. Cells obtained from Source 1 (HUVEC1) show a characteristic increase in Cell Index, which peaks at

~2 hours, followed by a very static lag phase that does not enter into an exponential growth phase. HUVEC obtained from Sources 2 and 3 both display a short lag phase followed by a proliferation phase. However, the growth rates of the two cell lines differ significantly, HUVECs from vendor 2 and 3 having a Cell Index doubling time of 17 hours ± 1 hour (for 5-25 hours after seeding) and 36 hours ± 1.7 hours (for 12-35 hours after seeding), respectively. Moreover, HUVECs show a dramatic shift in growth and adhesion kinetics in later passages. In as little as 4 cell passages, HUVECs from all the different suppliers fail to enter proliferative phase (Figure B).

■ 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 5).

■**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:

❶ drop in pH
Most cells stop growing as the pH falls from pH 7.0 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 to 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 as soon as they reach 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 prolife-ration, 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 5), as determined during a previous sub-culture (unless experimental requirements dictate different timing).
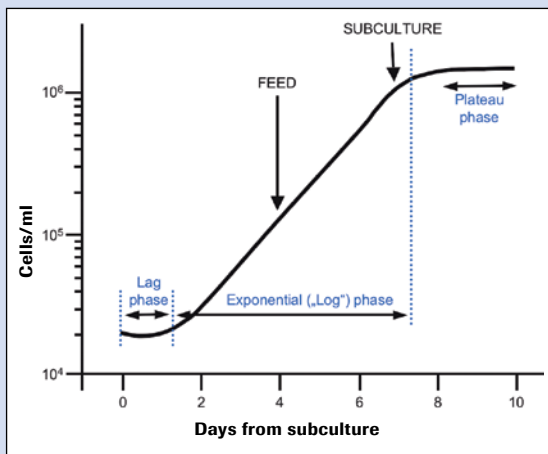
Figure 5: Growth curve and culture maintenance. Semilog plot of cell concentration versus time from subculture, showing the lag phase, exponential phase, and plateau, and indicating times at which subculture and feeding should be performed.

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...), management of the work place (dust, spills, clutter...) and the operator (clothes, hair, hands, breath).

Do not work in the cell culture room if you are sick. If the experiment cannot be postponed, use a mask.

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...

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 that you:
- 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.

Mycoplasma

Mycoplasma-contaminated cultures should be treated using, for example, BM-Cyclin 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.

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, and 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 2×10^6-2×10^7 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.

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.

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 1×10^6 cells/ml (can be estimated according to the culture vessel used, see "Culture Vessels" section above).
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 steps 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 mm².

Analysis

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

$$c = n/v$$

where 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 1×10^{-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, and the cell concentration is derived from the count.

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 a 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 6) formazan salts.

### Protocol for estimating cell viability by dye exclusion

1. Prepare cell suspension by trypsinization and resuspension in medium.
2. Take a clean hemocytometer 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. At 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.
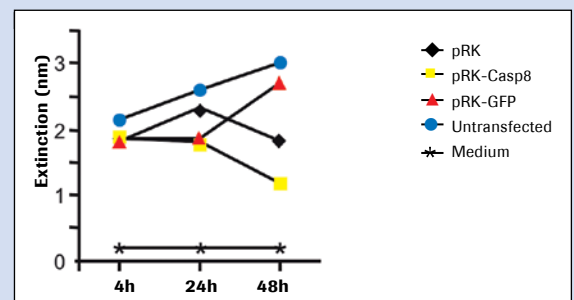
Figure 6: Colorimetric quantitation of cellular metabolism using WST-1 Cell Proliferation Reagent. Higher values mean higher metabolic activity. The data show that metabolic activity of cells transfected with pRK-Casp8 was strongly reduced 48 hours post transfection, suggesting that caspase-8 over-expression 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 (^{3}H-thymidine or bromodeoxyuridine, BrdU 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 nuclei microscopically. 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 7). 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 phase in turn is followed by the G2 (Gap 2) phase in which the cell prepares for re-entry 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.

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.
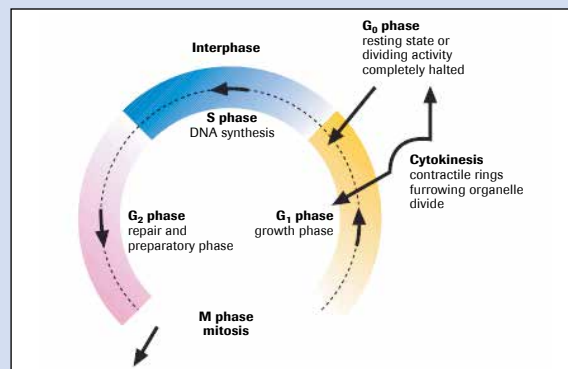
Figure 7: The Cell Cycle.
The cell cycle is divided into four phases: G1, S, G2, and M. Progression through the cycle is driven by cyclins interacting with CDC kinases and stimulated by nuclear oncogenes and cytoplasmic signals initiated by receptor kinase interaction with ligand. The cell cycle is arrested at restriction points by cell cycle inhibitors such as Rb and p53.

For cell cycle analysis, you can also use the xCELLigence System in combination with compounds known to characterize the different phases. Certain signatures of cell cycle arrest, such as mitotic arrest, can be determined with the xCELLigence System. Furthermore, the cells can be synchronized in S phase by pretreatment with 2 mM thymidine and then released. A very specific pattern can be observed as the cells progress through the cell cycle in synchrony.

Cytotoxicity

The indicators of cytotoxicity can vary, depending on the study performed. 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, for example, 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.

The **dose-response relationship** describes the biological effect induced by different concentrations of a substance (Figure 8). This curve should be determined whenever a new study is initiated, in order to fix the optimal conditions for the assay.
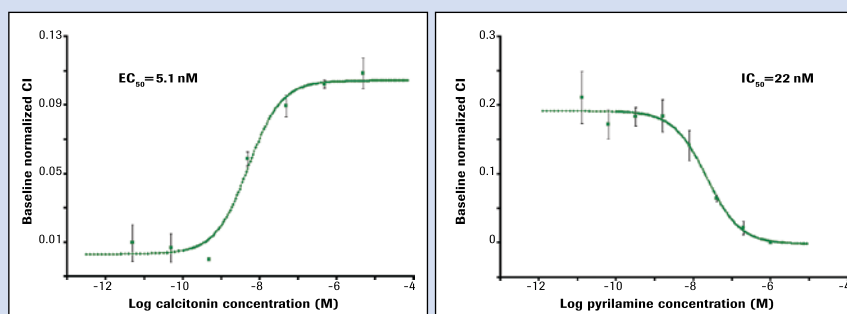
Figure 8: Dose-response curves obtained with the xCELLigence System RTCA Software. The **half-maximal effective concentration,** or EC_{50}, refers to the concentration of a compound which induces a response halfway between the baseline and the maximum. The EC_{50} represents the concentration of a compound 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.

CI = Cell Index

Ordering Information

Product	Cat. No.	Pack Size
xCELLigence RTCA DP Instrument	**00380601050**	**1 Bundled Package**
RTCA DP Analyzer	05469759001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
xCELLigence RTCA SP Instrument	**00380601030**	**1 Bundled Package**
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RTCA SP Station	05229057001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
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RTCA MP Station	05331625001	1 Instrument
RTCA Control Unit	05454417001	1 Notebook PC
E-Plate 16	05469830001	6 Plates
	05469813001	6 x 6 Plates
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	06324746001	6 x 6 Plates
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CIM-Plate 16	05665817001	6 Plates
	05665825001	6 x 6 Plates
E-Plate 96	05232368001	6 Plates
	05232376001	6 x 6 Plates
E-Plate VIEW 96	06472451001	6 Plates
	06472460001	6 x 6 Plates
E-Plate Insert 96	06465412001	1 x 6 Devices (36 16-Well Inserts)
E-Plate Insert 96 Accessories	06465455001	6 Units (6 Receiver Plates + 6 Lids)

References

1. R. Ian Freshney (2005). *Culture of animals cells. A manual of basic techniques, 5th edition* (ISBN: 0-471-45329-3).

2. R. A. Dixon and R. A. Gonzales (1994). *Plant Cell Culture. A Practical Approach, 2nd edition., Oxford University Press. Publication* (ISBN: 0-19-963402-5).

3. *Apoptosis and Cell Proliferation Manual, 3rd edition* (<https://www.roche-applied-science.com/sis/apoptosis/docs/manual_apoptosis.pdf>).

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

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Notes

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