# Cell Culture

Subjects: Cell Biology

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The cultivation of cells in a favorable artificial environment has become a versatile tool in cellular and molecular biology. Cultured primary cells and continuous cell lines are indispensable in investigations of basic, biomedical, and translational research.

Keywords: contamination ; mycoplasma ; media ; GCCP ; DMEM

## 1. Introduction

Cell culture experiments are widely used in biomedical research, regenerative medicine, and biotechnological production. Due to restrictions on the use of laboratory animals by animal protection laws and the strict implementation of the 3Rs (**R**eplacement, **R**eduction, and **R**efinement) formulated by William Russell and Rex Burch to improve the welfare of animals, it can be expected that the general use of cell lines will further increase during the next years to substitute animal-based research [1]. However, it should be noted that cell culture experiments, when not properly conducted, are prone to errors. Therefore, it is essential that cell culture studies are performed with good cell culture practice (GCCP) to assure the reproducibility of in vitro experiments [2].

## 2. Classification of Cell Culture Types

Cell lines can be roughly classified into three groups, namely (i) finite cell lines, (ii) continuous cell lines, also known as immortalized or indefinite cell lines, and (iii) stem cell lines [2]. Finite cell lines are normally derived from primary cultures and have slow growth rates. As such, they can be grown for a limited number of cell generations in culture before finally undergoing aging and senescence, a process that is indicated by loss of the typical cell shape and enrichment of cytoplasmic lipids. Importantly, finite cell lines are contact-inhibited and arrested in the $G_0$, $G_1$, or $G_2$ phase after forming monolayers [3].

In contrast, continuous growing cell lines are typically obtained from transformed or cancerous cells, divide rapidly and achieve much higher cell densities in culture than finite cell lines. In some cases, these cell lines exhibit aneuploidy (i.e., one or more chromosomes being present in greater or lesser number than the others) or heteroploidy (i.e., having a chromosome number that is other than a simple multiple of the haploid number). They often can be grown under reduced serum concentrations, are not contact-inhibited, and might form multilayers. Stem cells are an undifferentiated or partially differentiated pluripotent cell type originating from a multicellular organism. These cells can be extended to indefinitely more cells of the same type or alternatively can be triggered under the right conditions to produce cells with specialized functions. As such, they can act as a kind of multipotent precursor for many different cell types.

In all cases, the growth of cells from various sources requires an artificial but controlled environment, in which sometimes highly specialized media, supplements, and growth factors are needed for proper cell growth. A cell type can either grow adherent (attached to a surface) requiring a detaching agent for passaging, or alternatively can be free floating in suspension. Adherent cells can be further divided into fibroblast-like cells having an elongated shape and epithelial-like cells characterized by a polygonal shape. Similarly, each cell culture can have unique properties in regard to morphology, viability, doubling time, and genetic stability and their handling and maintenance may require different media, culture conditions, and additives or processing agents including antibiotics, detachment solutions, or surface coating for cell attachment [2]. Non-adherent or suspension cells grow either as single cells or as free-floating clumps in liquid medium that do not require enzymatic or mechanical dissociation during passaging. However, in some cases, these cells demand shaking or stirring for adequate gas exchange and proper growth. Typical examples of non-adherent cells are hematopoietic cell lines derived from blood, spleen, or bone marrow that proliferate without being attached to a substratum. Nevertheless, also some adherent cell lines can be adapted to grow in suspension, which allows for more manageable cell culturing at larger scales with higher yields in special applications [4][5]. In addition, compared to adherent cells, cells grown in suspension are generally easier to handle. Exemplarily, when adherent cells should be analyzed by

analytical flow cytometry or fluorescence-activated cell sorting (FACS), the cells must first be detached from their substratum. However, enzymatic digestion or the usage of non-enzymatic cell dissociation buffers can result in the degradation of surface proteins, which might prevent their subsequent identification and cell separation in respective protocols. This makes it extremely challenging to use flow cytometry for phenotyping and characterization of adherent cells [6]. Trypsin, for example, is frequently used for detaching adherent cells. It time-dependently degrades most cell surface proteins by cleaving peptides after lysine or arginine residues that are not followed by proline [7]. This results in degradation of most surface proteins during cellular dissociation. Similarly, other enzymes such as extracellular matrix-specific collagenases, the serine protease elastase cleaving the peptide bond of C-terminal neutral, non-aromatic amino acid residues [8], the peptidase dispase hydrolyzing *N*-terminal peptide bonds of non-polar amino acid residues [9], and many other detachment agents provoke the significant break down of proteins.

Therefore, several milder enzyme mixtures (e.g., Accutase, Accumax) or non-enzymatic cell dissociation reagents such as a mixture of ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid (NTA) chelating divalent metal cations have been introduced for routine cell passaging and manipulation of sensitive cells. These formulations are less toxic and preserve most epitopes for subsequent flow cytometry analysis [10][11].

More recently, the culturing of cells in a three-dimensional microenvironment has become the focus of researchers. These 3D cell cultures are either produced by culturing cells within a defined scaffold such as hydrogel or polymeric materials derived from extracellular matrix proteins or agarose or as self-assembly systems in which the cells grow in clusters or spheroids. It is well-accepted that these in vitro cell models offer the possibility to study cellular reactions in a closed system that better resembles the physiological situation than cell culture technologies that rely on two dimensions. As such, these models are particularly interesting for those studying aspects of cell-to-cell interactions, tumor formation, drug discovery, stem cell research, and metabolic interactions [12]. In comparison to 2D systems, 3D models have the potential to completely change the way drug efficacy testing, disease modeling, stem cell research, and tissue engineering research take place [12]. Finally, these systems will substantially decrease the use of laboratory animals in some research areas, which is a key aspect of the 3R principle [1].

## 3. Culture Media

Proper cell culture media are critical in the maintenance and growth of cell cultures and to allow the reproducibility of experimental results. Some cells additionally need non-essential amino acids (alanine, asparagine, aspartic acid, glutamic acid, glycine, proline, and serine) for effective growth and the reduction of the metabolic burden of cells.

The most common standard media used to preserve and maintain the growth of a broad spectrum of mammalian cell types are, for example, Dulbecco's modified Eagle medium (DMEM) and Roswell Park Memorial Institute (RPMI) media. Typically, these media contain carbohydrates, amino acids, vitamins, salts, and a pH buffer system (**Table 1**).

**Table 1.** Formulation of a Dulbecco's modified Eagle medium (DMEM) with high glucose.

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| **Inorganic salts/buffers:** $CaCl_2$: 0.2 g/L, $Fe(NO_3)_3 \times 9\ H_2O$: 0.0001 g/L, $MgSO_4$: 0.09767 g/L, KCl: 0.4 g/L, $NaHCO_3$: 3.7 g/L, NaCl: 6.4 g/L, $NaH_2PO_4$: 0.109 g/L |
| **Amino acids:** L-Arginine × HCl: 0.084 g/L, L-Glutamine: 0.584 g/L [1], Glycine: 0.03 g/L, L-Histidine × HCl × $H_2O$: 0.042 g/L, L-Isoleucine: 0.105 g/L, L-Leucine: 0.105 g/L, L-Lysine × HCl: 1.46 g/L, L-Phenylalanine: 0.066 g/L, L-Serine: 0.042 g/L, L-Threonine: 0.095 g/L, L-Tryptophan: 0.016 g/L, L-Tyrosine × 2 Na × 2 $H_2O$: 0.12037 g/L, L-Valine: 0.094 g/L |
| **Vitamins:** Choline chloride: 0.004 g/L, Folic acid: 0.004 g/L, *myo*-inositol: 0.0072 g/L, Niacinamide: 0.004 g/L, D-Pantothenic acid (hemicalcium): 0.004 g/L, Pyridoxal hydrochloride: 0.004 g/L, Riboflavin: 0.0004 g/L, Thiamine × HCl: 0.004 g/L |
| **Others:** D-Glucose: 4.5 g/L [2], Phenol red × Na: 0.0159 g/L [3], Pyruvic acid × Na: 0.11 g/L |

[1] In some formulations, L-Glutamine should be freshly added, [2] the concentration of D-Glucose in DMEM with low glucose is 1 g/L, [3] Phenol red has a weak estrogenic activity [13] and is therefore omitted in some DMEM formulations.

Common media such as DMEM are available in a ready-to-use liquid form or alternatively in powdered media for easier storage and a longer shelf life. Moreover, many media can be obtained with different glucose concentrations (low or high

glucose) as well as in formulations with and without L-glutamine or alternatively with stabilized glutamine. Finally, they are sold with or without a pH indicator such as phenol red.

Importantly, basal media typically contain no proteins, lipids, hormones, or growth factors. Therefore, these media require supplementation with fetal bovine serum (FBS), or often referred to as fetal calf serum (FCS), commonly at a concentration of 5–20% (*v*/*v*). FBS is obtained from the blood of fetuses of healthy, pre-partum bovine dams. The final serum is depleted of cells, fibrin, and clotting factors by centrifugation of the clotted blood. It should be noted that FBS from different sources might differ in growth factor and hormone profiles, virus content, endotoxin load, osmolality, total protein and metal content, sugars, and final processing (e.g., filtration, testing for potential contaminations). Therefore, most scientists prefer to buy traceable FBS batches with reliable lot-to-lot consistency to obtain reproducible results during experimentation.

In addition, newborn calf serum (NBCS) obtained from calves less than 20 days of age, calf bovine serum (CBS) sourced from calves aged between 3 weeks to 12 months, and adult bovine serum (ABS) isolated from adult cows more than 12 months old are frequently used to supplement cell culture media. Some researchers routinely heat-inactivate the serum at 56 °C for 15–30 min to inactivate the complement and to destroy potential bacterial contaminants. However, in most cases this is not necessary and should be omitted because heat inactivation also reduces the concentration or biological activity of growth factors that are required for proper cell growth.

However, it should be noted that serum-containing media has a number of disadvantages. Serum is complex, has an indefinite composition leading to batch-to-batch variation, increasing the risk of contamination, and the use of serum is commonly associated with ethical concerns in terms of avoiding the suffering of fetuses and animals [14][15]. Therefore, the development of serum-free media (SFM) has become a research hotspot during the last decades [14]. In principle, SFM can be divided into five types, namely (i) common SFM, (ii) xeno-free medium containing human-source but no animal components, (iii) animal-free medium, (iv) protein-free medium, and (v) chemically defined medium [14][16]. All these media contain key components (e.g., energy sources, vitamins, amino acids, lipids, trace elements, and inorganic salt ions) and are often enriched with special supplements such as anti-shear protectants, nucleic acids, and other ingredients that are required to improve the culture performance for certain cell types or applications [14]. Unfortunately, certain companies and suppliers of SFM often provide incomplete or no information at all about the composition of their media. Therefore, researchers already started a decade ago to install an online serum-free online database for the interactive exchange of information and experiences concerning SFM [15].

Biological contamination arising from bacteria, yeast, fungi, and mycoplasma can be better prevented by the addition of antibiotics and anti-mycotics to cell culture media. Most of them act by either inhibiting cell-wall synthesis (e.g., penicillin), interfering with membrane permeability (e.g., amphotericin B), or by inhibiting protein synthesis by preventing the assembly of the bacterial initiation complex between mRNA and the bacterial ribosome (e.g., streptomycin). However, the routine usage of antibiotics might develop slow growing persistent/resistant bacterial contaminants that may cause subtle alterations of cell differentiation and behavior [17]. In addition, antibiotics such as penicillin, streptomycin, and gentamycin can significantly alter gene expression and regulation and could modify the results of studies focused on drug response, cell regulation, and differentiation [18][19]. For example, a concise review has recently highlighted numerous publications that have shown the impact of antibiotics and antimycotics such as penicillin/streptomycin, gentamicin, and amphotericin B on in vitro properties of cells including proliferation, differentiation, survival, and genetic stability [20]. Similarly, a comprehensive literature search has found a number of reported side effects that are induced by different antibiotics, again supporting the notion that antibiotic-free culture media are recommended when possible to ensure the reliability and reproducibility of cell culture findings [21]. Consequently, researchers should avoid the permanent use of antibiotics in cell culture and should better try to implement strict aseptic working conditions to prevent bacterial contaminants in cell culture.

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