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Formulation of a defined medium to maintain cell health and viability *in vitro*

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Abstract. The first step in formulating a defined medium is to conduct a thorough search of the scientific literature. If a defined medium formulation is located that might be compatible with the intended cell system, a pilot study should be carried out to evaluate the general performance of the medium. Depending on the initial data obtained from this study, individual components of the medium and their concentrations may need to be manipulated (added/subtracted, increased/decreased) to obtain the desired results. Also, sometimes the basal medium or proportions of basal media must be changed. Because the formulation of a defined medium is a circular process, alteration of the basal medium type or ratio of basal media will necessitate redoing all of the previous addition/subtraction and optimization steps. Revalidation must also be done if vendors of components are changed or whenever different cells or cells of other ages are used in the system. This paper presents a brief procedure for formulating a defined media and an overview of the application of two defined media in muscle cell culture.

Key words: Cell culture, Defined medium, ITT and ITT-CF, Satellite cell

1. Introduction

Animal cells are routinely harvested and successfully maintained *in vitro* through their incubation in complex nutrient mixtures referred to as 'media'. The minimum constituents of basal cell culture media include salts, amino acids, fatty acids, nucleic acid precursors, vitamins, sugars and other metabolic factors obligate for cell survival [19, 20]. Successful formulations that support cell cultures and contain identifiable substances of known concentration above that provided by the basal medium are referred to as being 'defined' or 'chemically defined' media. Although defined medium and chemically defined medium are fundamentally related, they are formally different terms. A defined medium refers to a medium in which each added component is identified and its precise concentration is known, whereas a chemically defined medium describes a medium in which each added component is not only specified and of known concentration but additionally has a known chemical structure (personal communication, W.I. Schaeffer; SIVB Terminology Committee).

Since the cell culture medium is one of the fundamental components of all cell culture systems [10], it seems appropriate to include in this issue a paper on formulation of defined media. A few years ago, numerous researchers gained recognition for their contributions in formulating defined media [3–5, 12, 13, 21]. Today, however, anyone using cell cultures as mechanistic models probably uses a defined medium for the application of treatments (Table 1). A defined medium exists for nearly every cell culture operation and cell type [6, 8].

Defined media may be designed to cause a culture of cells to undergo a physiological transition, *in vitro*, [30] or may function to simply maintain the health and viability of cultured cells [3, 9]. In the former case, a medium may induce cells to either proliferate or differentiate, whereas the latter case may require the addition of a specific treatment to the defined medium in order to effect a cellular response. Since subsequent batches of defined media are identical in composition, variations in culture conditions are minimized. The use of such media enables experiments involving treatments to be carried out with a level of precision unattainable with traditional undefined media formulations. Devising a proper defined medium formulation is often imperative for generating repeatable and reliable data from a cell culture system. It is for this reason that the formulation process demands careful and constant consideration [1].

Recent evidence suggests that profound physiological transitions may occur through the use of very simplistic defined media [17, 30]. For example, myogenic satellite cells are enticed to differentiate and fuse when exposed to a defined treatment medium consisting of insulin, transferrin and thyroid hormone [30]. In this paper we present several guidelines and steps to follow in order to successfully formulate a defined medium applicable to one's

Table 1. Defined media development for muscle satellite cells

Animal	Reference	Function	Basal media	Major components
Rat	Allen et al., 1985	Proliferation, differentiation	DMEM[a]/MCDB[b] 104	Insulin, FGF[c], fetuin, linoleic acid, dexamethasone
Sheep	Dodson and Mathison, 1988	Maintenance	McCoy's 5A	Transferrin, myoinositol, $CaCl_2$, sodium pyruvate
Human	Ham et al., 1988	Proliferation	MCDB 120	EGF[d], insulin, dexamethasone, BSA[e], fetuin
Turkey	McFarland et al., 1991	Proliferation	McCoy's 5A	Insulin, FGF, fetuin, BSA, dex amethasone
Pig	Doumit et al., 1993	Proliferation	MEM[f]/MCDB 110	BSA, insulin, transferrin, dex amethasone, fetuin
Fish	Venkateswaran et al., 1995	Maintenance	Leiboveitz's L-15	Transferrin, myoinositol, $CaCl_2$, sodium pyruvate
Sheep	Vierck et al., 1995	Differentiation	DMEM/F-12	Insulin, transferrin, triiodothyronine
Horse	Erickson et al., 1998	Differentiation	DMEM/F-12	Insulin, transferrin, triiodothyronine, corticosterone, FGF

[a] Dulbecco's Modified Eagle's Medium.
[b] Molecular, Cellular and Developmental Biology Department at the University of Colorado.
[c] Fibroblast growth factor.
[d] Epidermal growth factor.
[e] Bovine serum albumin.
[f] Minimum Essential Medium.

research needs. We also discuss the use of two (similar) defined media ITT [30] and ITT-CF [17] on cultures of ovine satellite cells as an example to illustrate that small additions to a basal defined medium can cause significant changes in the overall growth of a culture. The ITT and ITT-CF illustration also demonstrates that the formulation of a defined medium may be used to induce the desired cellular state for a particular research focus (i.e. maintenance, proliferation or differentiation); [17, 29, 30].

2. Materials

A. Plastic cultureware
1. Tissue culture dish, 15 cm, Cat. No. 168381.[1]
2. Tissue culture plates, 24 well, Cat. No. 143982.[1]

B. Media, reagents and chemicals
1. Pig skin gelatin, Cat. No. G-1890.[2]
2. Trypsin, Cat. No. T-4549.[2]
3. Ethylenediamine tetraacetic acid (EDTA), Cat. No. E-5134.[2]
4. HEPES, Cat. No. H-9136.[2]
5. Pantothenate, Cat. No. P-5155.[2]
6. Biotin, Cat. No. B-4639.[2]
7. Insulin, Cat. No. I-1882.[2]
8. Triiodothyronine, Cat. No. T-6397.[2]
9. Transferrin, Cat. No. T-8027.[2]
10. Basic fibroblast growth factor (FGF), Cat. No. 40060.[3]
11. Hydrocortisone, Cat. No. H-0888.[2]
12. Giemsa stain, Cat. No. GS-1L.[2]
13. Horse serum (HS), Cat. No. 16050-122.[4]
14. Penicillin-streptomycin, 10,000 units of penicillin G and 10,000 μg of streptomycin sulfate per ml in 0.85% saline, Cat. No. 15140-122.[4]
15. Gentamicin, 10 mg/ml gentamicin sulfate, Cat. No. 15710-015.[4]
16. Dulbecco's Modified Eagle's Medium (DMEM), Cat. No. 12800-017.[4]
17. Ham's F-12 nutrient mixture, Cat. No. 21700-075.[4]
18. Formaldehyde, Cat. No. 2106-03.[5]
19. Methanol, Cat. No. A412-4.[6]

3. Procedures

A. Steps involved in the formulation of a defined medium (Figure 1)
1. Search the scientific literature for existing media that might be compatible with the intended cell system.
2. If defined medium formulations are located, screen them in the intended cell system as a pilot study.
3. Depending on the results of pilot study, add or subtract individual components of the culture media and monitor cellular responses to the altered formulations.

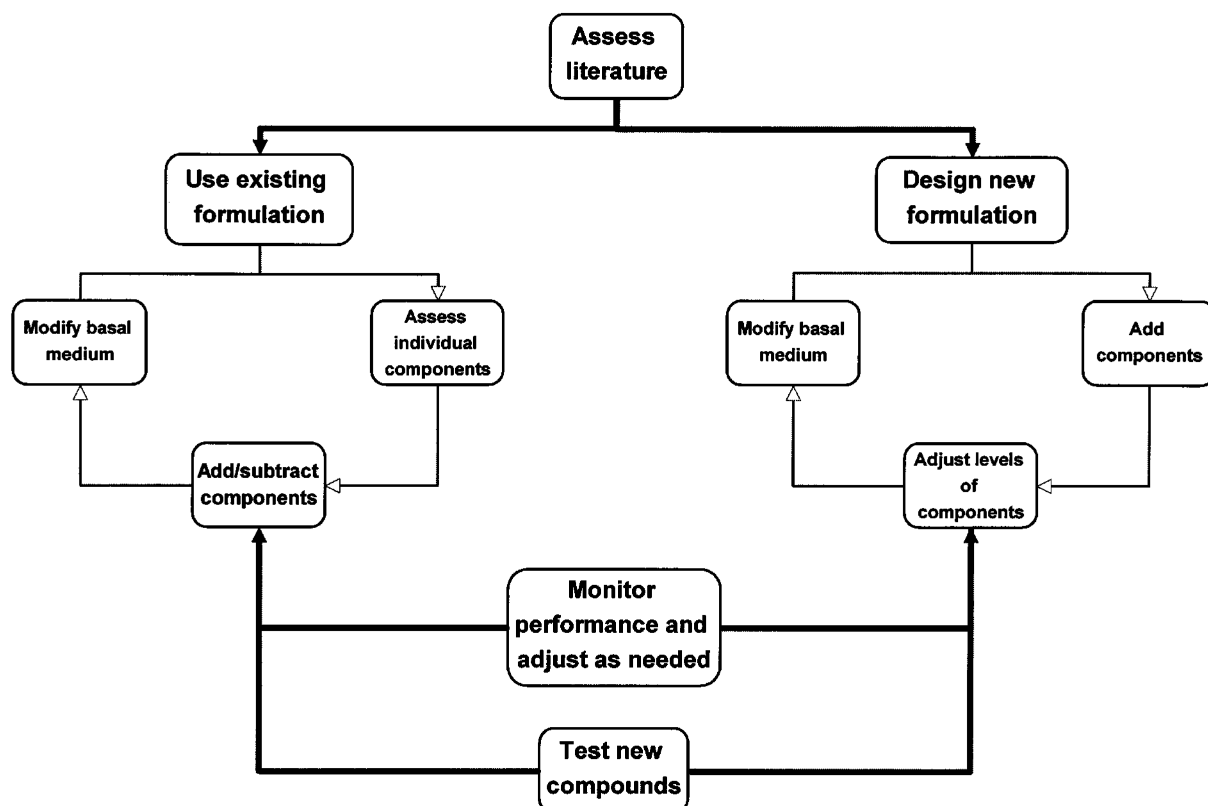

Figure 1. Flow chart illustrating pathways for devising a new defined media or adapting an existing media to suit one's intended cell culture system.

4. If the desired cellular response is not attained during the addition/subtraction steps [3, 12, 13], modify the basal media [9, 14, 25] and redo the previous addition and subtraction steps.
5. Once a formulation is derived that promotes the desired cellular response, carry out experiments involving treatment applications.
6. Monitor the formulated media to ensure the continuation of the desired cellular response.
7. Revalidate the medium if lot numbers of components or other physical parameters (i.e. substratum, plasticware) change.

B. Preparation of reagents for testing defined media ITT and ITT-CF in cell culture
1. Preparation of PBS
   a. Dissolve 10.0 g of NaCl, 0.25 g of KCl, 1.44 g of Na_2HPO_4 in 900 ml of glass distilled H_2O.
   b. Adjust pH to 7.08 with HCl/NaOH.
   c. Bring volume to 1000 ml in a volumetric flask.
   d. Place the PBS into a 1 liter bottle and autoclave for 60 min.
2. Trypsin solution
   a. Prepare PBS/EDTA by adding 0.168 g of $Na_2EDTA \cdot 2H_2O$ to 1 liter of PBS.
   b. Sterilize by autoclaving 60 min.
   c. Add 10 ml of sterile trypsin to 90 ml of sterile PBS/EDTA.
3. Preparation of DMEM
   a. Dissolve a 1 liter package of DMEM and 3.7 g $NaHCO_3$ in 900 ml of glass distilled water.
   b. Adjust the pH to 7.08 with HCl/NaOH.
   c. Bring the volume to 1000 ml in volumetric flask.
   d. In a laminar flow hood, filter sterilized the DMEM by passing the medium through a 0.22 μm filter into a 1 liter sterile bottle.
4. Hams F-12 medium
   a. Dissolve a 1 liter package of Hams F-12 and 1.176 g $NaHCO_3$ in 900 ml of glass distilled water.
   b. Adjust the pH to 7.08 with HCl/NaOH.
   c. In a laminar flow hood, filter sterilize the Hams F-12 by passing the medium through a 0.22 μm filter into a 1 liter sterile bottle.
5. DMEM-10% HS medium
   Prepare 100 ml of serum-containing medium by adding 10 ml of HS, 1 ml of Pen/Strep, and 0.5 ml of Gentamicin to 88.5 ml of DMEM.
6. Defined medium ITT [7, 17, 29, 30]
   Prepare 50 ml of DMEM:Hams F-12 in a 1:1 ratio. Remove 2.59 ml to allow for the addition of the following components:
   a. Insulin: 0.5 μm, 25 μl of 10^{-3} M stock.
   b. Triiodothyronine: 0.2 nM, 67.5 μl of 0.1 μg/ml stock.

c. Transferrin: 10 μg/ml, 0.25 ml of 2 mg/ml stock.
d. HEPES: 15 mM, 0.5 ml of 1500 mM stock.
e. Pantothenate 17 μm, 0.5 ml of 1700 μM stock.
f. Biotin 33 μm, 0.5 ml of 3300 μM stock.
g. Pen/Step: 0.5 ml.
h. Gentamicin: 0.25 ml.

7. Defined medium ITT-CF [27, 29]
Prepare 50 ml of DMEM:Hams F-12 in a 1:1 ratio. Remove 3.66 ml to allow for the addition of the following components:
a. Insulin: 850 nM, 42.5 μl of a 10^{-3} M stock.
b. Triiodothyronine: 0.2 nM, 67.5 μl of a 0.1 μg/ml stock.
c. Transferrin: 10 μg/ml, 0.25 ml of 2 mg/ml stock.
d. Hydrocortisone: 50 ng/ml, 50 μl of 50 μg/ml stock.
e. FGF: 100ng/ml, 2.5 ml of 2 μg/ml stock.
f. Pen/Step: 0.5 ml.
g. Gentamicin: 0.25 ml.

C. Ovine satellite cell culture procedure
1. Non-transformed ovine satellite cell strain I_1 was cloned from a parental satellite cell culture originally isolated from cells obtained from the semimembranosus and semitendinosus muscles of a black-faced wether lamb [11, 26].
2. Aliquots of this satellite cell strain, stored in liquid nitrogen, were quickly thawed and suspended in DMEM-10% HS.
3. Satellite cells were plated in pig skin gelatin-coated 15-cm dishes and incubated at 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2 for 24 h.
4. After 24 h, DMEM-10% HS media was removed, satellite cells were rinsed three times with sterile DMEM, and fresh DMEM-10% HS was provided.
5. Satellite cells were grown to 70–75% confluence and then detached from cultureware with trypsin/EDTA [23].
6. Satellite cells were plated at 100 nuclei/mm^2 on pig skin gelatin-coated 24-well plates and maintained in DMEM-10% HS.
7. After a 24 h attachment period in DMEM-10% HS, culture medium was changed to either ITT or ITT-CF.
8. After 120 h, ITT and ITT-CF media were removed, and satellite cell cultures were fixed in formalin/methanol.
9. Assessment of cell culture growth
a. Satellite cells were stained with geimsa [8].
b. The number of satellite cells present within 10 randomly chosen fields of view of each culture well were counted at 400× magnification on a phase-contrast Nikon Diaphot microscope [2].
c. Satellite cell proliferation was quantified as an increase in total nuclei number.
d. Differentiation of satellite cells was characterized by fusion of three or more nuclei [3].

4. Results and discussion

Our primary goals in formulating a defined medium are twofold. From a physiological perspective, a defined medium may be designed to maintain the health and viability of cells without the introduction of any growth variables [9]. Alternatively, a defined medium may be developed to produce definite physiological transitions of cells such as proliferation and differentiation *in vitro* [17, 30]. Defined media formulations are traditionally complex to produce and require exacting laboratory skills and a substantial investment of both time and materials. The steps involved in deriving a defined medium may take either little or considerable time, depending on whether a defined medium already exists that is applicable to one's research needs. With this in mind, the first step in the formulation of a defined medium is to assess the scientific literature and determine if a medium is available that might be used directly or adaptable for use with the intended cell type (Figure 1).

When searching the literature for defined media formulations which might be compatible with the intended cell culture system, the focus should first be directed towards finding media that have successfully supported the cell type of your intended system. As specific cell types are often relatively homologous across species, it may also prove beneficial to look for media used with the intended cell type even if the cells were derived from a different species.

If one or more defined medium formulations are located which appear to be compatible with the intended cell culture system, these media should initially be screened during a pilot study to evaluate the general performance of the medium. Depending on the initial data obtained from this study, it may be necessary to assess each individual component of the media [3]. Individual components of the medium and their concentrations may need to be manipulated (added/subtracted, increased/decreased) to obtain the desired response (i.e. proliferation, differentiation, quiescence) from the cultured cells.

If the desired cellular response is not attained with these addition/subtraction steps, it may become necessary to modify the basal medium. Basal media solutions routinely include Eagle's Minimum Essential Medium (MEM) or Dulbecco's Modified Eagle's Medium (DMEM) [15, 16]. Ham's defined nutrient mixtures F12 and F10 are regularly included in basal media solutions [18, 19]. Because basal media components are commercially available, their

modification for an intended application may involve changing the concentrations used or switching to a different basal media formulation.

The use of an existing medium formulation as a basis for formulating a new medium for an intended cell culture system is in many ways similar to designing an entirely new medium. Both are somewhat circular processes, as culture media must be monitored and adjusted in order to elicit the desired cellular responses of one's particular research focus. When designing a new formulation, inevitably one begins by adding individual components such as growth factors to the basal medium and monitoring the responses of cells maintained in such formulations [3, 13]. Once a formulation is derived that promotes the desired response in the intended cell culture system, experiments involving treatment applications can be carried out.

When utilizing the defined medium in a research situation, the effects of the medium must be monitored in order to ensure that its continued application elicits its intended function. This is especially true when different lot numbers of components are used or when other physical parameters of the culture system, such as substratum or plasticware, are changed. Because the overall growth, phenotype and genetic stability of cultured cells are often contingent upon the medium in which they are maintained [22], its proper formulation and application are integral parts to the overall success of a particular research endeavor.

For several decades satellite cell research has incorporated the development of serum-free and defined media capable of supporting satellite cell growth *in vitro*. A historical timeline of this research is presented in Table 1. As indicated in this table, satellite cells from several species have been successfully maintained in culture within a variety of defined media. The precise function of these media may be variable, as some maintain satellite cells in a quiescent state while others induce states of cellular proliferation or differentiation. Experiments with satellite cells are often designed to discern specific factors which regulate proliferation and differentiation. Treatments can be easily applied by supplementing specific agents, such as growth factors, to the media [24]. If both treatment and controls utilize an identical defined medium formulated for maintenance and not growth of the cells, differences in cellular responses (i.e. proliferation or differentiation) can be attributed to the supplemented agent. Research carried out in this manner has yielded considerable insight into the factors which regulate proliferation and differentiation of satellite cell populations [3, 10, 14].

The formulation of a cell culture medium has a major influence on the overall health and viability of cells maintained *in vitro* and slight variations in media components may elicit different growth responses from an identical strain of cells. Such was the case with ovine satellite strain I_1 cells, shown in Figure 2, as they exhibited profound differences from one another when maintained in culture with either ITT or ITT-CF (Figure 2a, b). Although initially plated at equal densities, after 120 hours in these media the total numbers of cells were significantly greater in those cultures maintained in ITT-CF (Figure 3a). These cultures initially plated at 100 cells/mm^2 exhibited final densities of approximately 30 cells/mm^2 and 120 cells/mm^2 in ITT and ITT-CF respectively (Figure 3a). Because of these differences in cell number it appears reasonable to conclude that ITT does not support the mitotic division of ovine satellite cells whereas ITT-CF induces a modest degree of proliferation. Since cell numbers declined in cultures maintained in ITT these results also suggest that this media may have a detrimental effect on the overall health and viability of ovine satellite cells *in vitro*. Interestingly, despite a decrease in total cell numbers in cultures maintained in ITT, the relative proportion of differentiating satellite cells was found to be higher in these cultures when

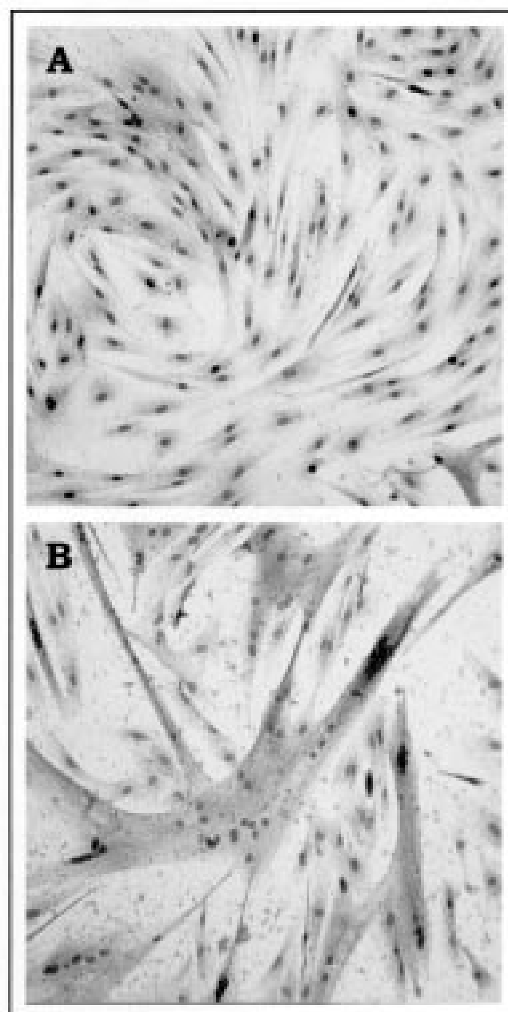

Figure 2. Ovine satellite cells maintained in defined media A) ITT-CF and B) ITT.

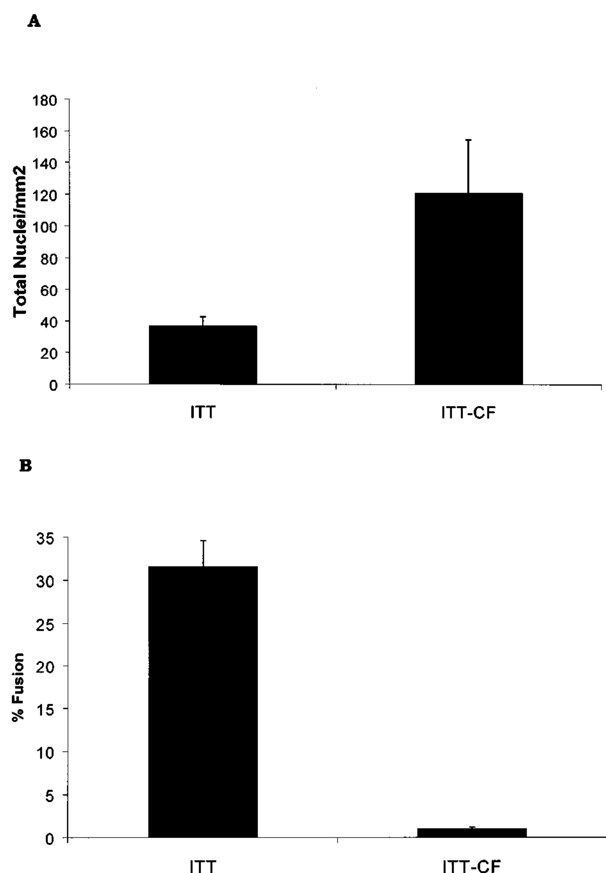

Figure 3. Effects of defined media on the proliferation and differentiation of ovine derived satellite cells. A) Total number nuclei in ovine satellite cell cultures maintained in ITT and ITT-CF. B) Relative percentages of fused (differentiated) ovine satellite cells maintained in ITT and ITT-CF.

compared to those sustained in ITT-CF (Figures 2b and 3b).

The results from the ovine satellite cell cultures clearly indicate that both the number of cells comprising a culture as well as the stages of cellular proliferation and differentiation can be altered through the formulation of the cell culture media. Knowledge of such differences in proliferation and differentiation potentials may be of extreme importance when determining whether to utilize a certain media for a given treatment application. For example, if one is interested in determining if a particular factor induces ovine satellite cells to differentiate, one might opt for the utilization of defined media ITT-CF. The rationale behind this choice being that since cells maintained in ITT-CF routinely exhibit minimal differentiation, if a treatment causes a significant number of cells to differentiate, one could reasonably conclude that this effect was a result of the treatment agent alone. Similarly, if one is interested in determining whether a particular factor enhances the mitotic division of ovine satellite cells, one might implement the ITT media. Since proliferation is not routinely observed in ITT, if modest increases in cell number are observed within a given treatment this effect could also be attributed to the treatment agent. Because ITT causes a decline in cell numbers, caution must be utilized when making inferences about such experiments as the overall health and viability of these cells might be compromised in this media.

The selection of a proper cell culture media is an integral component of any cell culture system. Its importance can not be overlooked or easily disputed, because the growth, phenotype, health and stability of cultured cells are dependent upon the media in which they are maintained. Devising a defined medium formulation for a particular cell culture system may involve a considerable commitment of time and material resources. However, as the viability of cultured cells and the quality of data obtained from a cell culture system are contingent upon the cell culture media, its proper formulation is imperative and warrants making the necessary investments. Furthermore, once properly formulated a defined media may play a key role in the overall success of a particular research application.

Notes on suppliers

1. Nunc Inc., Naperville, IL, USA
2. Sigma Chemical Company, St. Louis, MO, USA
3. Collaborative Biomedical, Bedford, MA, USA
4. Gibco BRL Life Technologies, Grand Island, NY, USA
5. J.T. Baker, Paris, KY, USA
6. Fisher Scientific, Fair Lawn, NJ, USA

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