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Perspectives in Cell Culture

Managing Serum Requirements for Cell Culture

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Culturing mammalian cells has not changed in almost 50 years. Typically, a synthetic basal medium is chosen to meet the environmental and nutritional requirements of a given cell line. Components, such as amino acids, vitamins, inorganic salts, and a carbon source such as glucose are commonly found in the classical basal media formulation. These basal formulations normally will not support cell growth alone, but must be further supplemented with animal serum, usually Fetal Bovine Serum (FBS) at a concentration of 5–20%.

What is Serum?

Whole blood is collected, refrigerated, and allowed to slowly clot. After centrifugation and removal of the clot, serum is the liquid that remains. Although serum is vitally important to the growth of cells cultured in a classical basal medium, it has never been fully characterized. There are over 1,000 different components[1] found in serum, including proteins, electrolytes, lipids, carbohydrates, hormones, enzymes, and other miscellaneous undefined constituents. Serum also supplies growth factors, nutrients for proliferation and differentiation, factors for binding to and inactivating toxic compounds such as proteases and free radicals, and attachment factors. In addition, serum also regulates cell membrane permeability and serves as a carrier for lipids, enzymes, micronutrients and trace elements into the cell. Finally, albumin, fetuin and other bulk proteins found in serum minimize non-specific absorption to cultureware and bioreactor surfaces, as well as influence the physical properties of the culture system, such as pH (sera are a source of natural buffers), shear stress, viscosity, osmolality and gas delivery rates.[2]

Why Review Serum Requirements and Usage?

Serum is absolutely necessary for growth and proliferation of most cell lines grown in a classical media formulation. However, serum has some characteristics that may require scientists to re-think their serum usage and management.

Production of Serum

Producing serum is costly and not efficient. FBS in particular must be sourced from areas that are free of bovine spongiform encephalopathy (BSE), foot-and-mouth disease, and other highly infectious diseases. One report estimates that only 7–9% of cattle are pregnant at time of slaughter. Each fetus, depending upon its age, will yield between 0.2 and 0.5 liters of blood, half of which will be serum. This means between 44 and 144 head of cattle must be slaughtered to obtain one liter of FBS.[3] Blood is collected using cardiac puncture and processed under refrigerated conditions to harvest the serum. After that, there is a quality control procedure to ensure that the serum meets raw material specifications before it is further processed.

Variability

All sera represent an undefined mixture in which composition varies from one lot to the next. Sensitive cell culture methods designed to collect quantitative experimental data may be seriously affected by the variability of the constituents in sera. For example, hormones are known to exert profound effects at concentrations in the pico- and nanogram ranges, effects that may be pervasive without respect to cell type,[4] yet serum hormone concentrations vary from lot to lot. In Honn, et al. (1975), four metabolites were evaluated in terms of their variability and possible toxicity to cell cultures.

Analysis of uric acid, urea, total bilirubin and creatine concentrations indicated a 2- to 6-fold variation in sera samples tested. Cholesterol has been reported as important as an attachment factor for increased plating efficiency,[5] however, cholesterol levels varied in different lots of FBS tested (29–165 mg/dl), suggesting that reliance upon different lots of sera supplements for consistent plating efficiency is tenuous. The concentration of glutamate in serum may also be toxic to cultured neuronal cells. Table 1 demonstrates the average and the range of values for 37 sera parameters from multiple pooled lots of FBS.[6]

Infectious Agents

Animal serum has the potential for carrying infectious agents, such as mycoplasma, viruses and prions, which may pose a risk to the cell lines being grown, or even the recipients of downstream processed therapeutic agents. For example, the presence of Bovine Viral Diarrhea Virus (BVDV) in FBS can interfere with animal vaccine production. The need for a defined culture system is essential for those engaged in clinical trials of biologicals intended for human therapy and *in vivo* diagnosis. Research and manufacturing applications involving gene and cell therapy as well as tissue engineering applications have accelerated efforts to eliminate all constituents of animal origin from these culture systems. Culture media manufacturing practices, including the use of animal by-products used in the production of media raw material components (secondary sourced material) and equipment sanitization procedures, are scrutinized to prevent inadvertent contamination of nutrient media.[7]

High Levels of Serum Proteins

Although beneficial in most respects, serum proteins can negatively influence the cell culture environment. Fibroblasts or other unwanted cells may overgrow the culture.* Downstream purification of proteins can be more difficult when presented with high levels of exogenous serum proteins. Levels of serum protein, which are present at approximately 5 mg/ml when using 10% serum supplementation, may introduce problems in the isolation of cell products present in µg/ml amounts. Residual proteins can be antigenic and can reduce antibody yield due to the presence of neutralizing antibodies. For the culture of hybridomas, it is not recommended that sera other than fetal bovine be used because of the high concentration of immunoglobulins in post-natal serum.

High concentration of contaminating immunoglobulins can make it difficult to isolate the monoclonal antibody of choice. Calf serum is reported to have an IgG concentration between 1–25 mg/ml, whereas the concentration in untreated FBS generally ranges from 50 to 300 µg/ml.[10]

Manufacturers may add, at considerable cost, various treatment steps to the production of FBS to lower the concentration of antibody further, obtaining IgG concentrations less than 5 µg/ml. Treated sera reduce the need for prescreening sera lots for IgG and BVD antibody levels.

Classes of GIBCO™ Media

The potential for introducing adventitious contaminants decreases as the culture system is better defined.
Note: Other manufacturers' definitions may vary.

Classical Basal Medium

Basal formulation requiring serum supplementation between 5% and 10%.

Reduced Serum Medium

Basal formulation enriched with nutrients and animal-derived factors to reduce supplemental serum requirements.

Serum-Free Medium (SFM)

Basal formulation containing protein factors that do not require serum to support cellular function. Proteins may or may not be animal derived.

Protein-Free Medium (PFM)

Basal formulation that does not contain any proteins (may contain plant or yeast hydrolysates).

Chemically-Defined Medium (CDM)

Basal formulation that is protein-free and is comprised solely of biochemically-defined low molecular weight constituents.

From: Jayme, D.W. and Smith, S.R. (2000) *Cytotechnology*, **33**:27–36.

* Dialyzed serum, when used in conjunction with media supplemented with D-valine rather than L-valine has been described as a means to control fibroblast overgrowth.[8,9]

Stimulation or Inhibition of Growth

Factors can be present in any given lot that either stimulate or inhibit the desired culture response of specific cell types. Inhibition of growth and/or cellular function can be related to the level of specific serum constituents, such as amine oxidases.[11] The life span of diploid cells has also been shown to vary with different serum batches.[12]

Unwanted Induction of Differentiation

Factors in sera may also induce differentiation of progenitor cells, which may be counterproductive to the scientist's intent. Even when cultured under serum-free conditions, quenching the action of trypsin with a serum-supplemented medium may cause differentiation, because of high concentrations of calcium typically found in FBS.

Managing Serum Requirements

Increasingly, scientists are becoming concerned with the cost of serum, the need to reduce variability in experiments, and the regulatory issues governing biological products. Several strategies are presented below to assist the scientist in managing serum requirements.

Pre-qualifying and Reserving Sera

One way that scientists can overcome the problem of lot-to-lot variability is by pre-qualifying and reserving sera for their specific applications. Sera matching programs exist that allow for lots with similar performance and biochemical specifications to be purchased as replacements for sera lots that have worked well for specific applications in the past. Lot-matching programs help scientists target potentially suitable sera lots for their own applications, however, many scientists will also pre-qualify samples to further ensure that the proper lot of sera has been chosen.

Selected Sourcing of Sera

Sourcing sera from selected countries (United States, Mexico, Australia, Canada, New Zealand, and the Central American countries) has allowed scientists to address various regulatory requirements that restrict import and export of biologic material across international borders as a means to minimize risks associated with infectious agents. Sera should be supplied from countries meeting applicable USDA importation requirements. Sera from countries where Bovine Spongiform Encephalopathy (BSE) is not known to exist is commonly offered from sera manufacturers.

New Zealand, in particular, is an ideal source for animal sera. New Zealand is geographically isolated and economically dependent on agriculture. Its animal products are under the protection of the Ministry of Agriculture and Fisheries (MAF) of New Zealand and benefit from the safeguards and regulations intended to protect animal health. The Office International des Epizooties (OEI) publishes a list of serious diseases (e.g., BSE, foot-and-mouth disease, bluetongue) defined as follows: "Transmissible diseases which have the potential for very serious and rapid spread, irrespective of national borders, which are of major importance in the international trade of livestock and livestock products". None of the diseases on this list are known to occur in New Zealand.[13] However, New Zealand-sourced FBS represents only a small percentage of the sera available on the market.

Reduced Serum Media

Another strategy is to use a reduced serum medium. When using a medium allowing for a reduction in serum concentration, the turnover of valuable reserved lots is greatly reduced, thus significantly increasing the availability of the lot (see Table 2 for a list of reduced serum media). In addition, changing sera lots less frequently will often reduce experimental variability that could affect certain cell-based assays. For example, a scientist conducting a toxicity assay completed entirely with one lot of serum will not have the concern that between-lot variability has affected the integrity of the data.

Reduction of serum concentration in the culture system may be desirable for technical or economic reasons. Replacement of a basal medium with an enriched synthetic nutrient medium permitting reduced serum supplementation may be a convenient alternative, depending on the application. The most economical way of reducing serum is the use of a low serum-requiring medium that has been supplemented with other growth promoting factors, as opposed to a completely serum-free or even chemically-defined medium. Enriched media supplemented with reduced levels of FBS (or alternative serum) exhibit unique advantages by combining the economy of serum reduction with the broader range of cell culture applications supported by standard serum-supplemented media. Reduced serum media have also been useful for monolayer cultures, where a primary contribution of serum is to facilitate cell attachment.

Adherent cell lines may be maintained indefinitely in these growth factor-enriched media with a 50–80% reduction in serum requirement.[14] For some applications medium replenishment may be serum-free to reduce cost and serum artifacts after the cells have attached at the appropriate inoculation density.

Serum-Free Media

Serum-free media describe a class of media that do not require supplementation with serum. They may contain discrete proteins or bulk protein fractions. Successful serum-free media development result in commercially available media formulations requiring no serum additive for many relevant cell types. Formulations exist for the recombinant protein producing lines of Chinese Hamster Ovary (CHO), various hybridoma cell lines, the insect lines *Drosophila,* Sf-9, and Sf-21 (*Spodoptera frugiperda*), and for cell lines that act as hosts for viral production, such as 293, VERO, MDCK, MDBK, etc. (see Table 3 for serum-free alternatives). Serum-free media for virus production are also available free of all human- or animal-derived components. By using a serum-free medium, the scientist eliminates the need to pre-qualify sera lots entirely.

Protein-Free Media

Protein-free media are a subclass of serum-free media that are free of all proteins, but may contain plant or yeast hydrolysates. Protein-free media are particularly useful for isolating recombinant proteins from adherent or suspension cell culture (see Table 3 for a selection of protein-free media). *Other manufacturers' definitions of protein-free media may vary.*

Chemically-Defined Media

These media are another subclass of serum-free media that contain no proteins, hydrolysates, or components of unknown composition. These media are free of animal-derived components and all components have a known chemical structure. Chemically-defined formulations have been developed for suspension cultures, eliminating the need to address the cell attachment factor requirements for adherent cultures (see Table 3 for selection of chemically-defined media). *Other manufacturers' definitions of chemically-defined media may vary.*

Many lines, such as CHO and the various hybridoma cell lines, can be grown in either serum-free, protein-free, or chemically-defined media formulations. However, in contrast to serum-supplemented media, serum-free and chemically-defined formulations are generally highly specific and formulated for one or only a few specific cell types.

Summary

Cell culturists have traditionally relied on the benefits of animal sera, primarily FBS, to support their cell cultures in spite of the known disadvantages associated with the use of sera. As sera are biologically sourced, scientists are forced to consider:

- Cost
- Variability
- Infectious agents
- High levels of serum proteins

These issues have led scientists to explore options to manage sera including:

- Pre-qualifying and reserving sera
- Selected sourcing of sera
- Reduced serum media
- Serum-free, protein-free, or chemically-defined media

Using a combination of the above options allows more effective management of sera usage and requirements. For example, supply of geographically sourced FBS can be even more limited than that of standard sera products. Reducing the serum concentration will extend the lifespan of any given lot of serum, stretching the supply of geographically sourced material. The use of serum-free media formulations eliminate the concern that infectious agents may be introduced into the cell culture system from animal sera. Better-defined culture conditions will speed therapeutic and diagnostic development due to minimization of culture condition development. When eliminating or reducing serum concentrations in culture, advantages over classic culture methods are realized:

- Increased definition
- More consistent performance
- Easier purification and downstream processing
- Precise evaluations of cellular function
- Increased growth or productivity
- Better control over physiological response
- Enhanced detection of cellular mediators

Table 1: Profile of Fetal Bovine Sera

Description	Average	Range	N
Endotoxin	0.356 ng/ml	0.008–10.0	39
pH	7.4	7.20–7.60	40
Inorganic Salts			
Calcium (Ca^{2+})	13.6/100 ml	12.6–14.3	43
Chloride (Cl^{-})	103 meq/L	98–108	43
Inorganic Phosphorous	9.8 mg/100 ml	4.3–11.4	43
Potassium (K^{+})	11.2 meq/L	10.0–14.0	43
Selenium	0.026 µg/ml	0.014–0.038	25
Sodium (Na^{+})	137 meq/L	125–143	43
Other Components			
Alkaline Phosphatase	255 mU/ml	111–352	43
Blood Urea Nitrogen	16 mg/100 ml	14–20	43
Creatine	3.1 mg/100 ml	1.6–4.3	43
Direct Bilirubin	0.2 mg/100 ml	0.0–0.5	43
Glucose	125 mg/100 ml	85–247	43
Hemoglobin	11.3 mg/100 ml	2.4–18.1	17
Lactate Dehydrogenase	864 mU/ml	260–1,215	43
Serum Glutamate Oxalacetate Transaminase	130 mU/ml	20–201	43
Total Bilirubin	0.4 mg/100 ml	0.3–1.1	43
Uric Acid	2.9 mg/100 ml	1.3–4.1	43
Steroids and Hormones			
Cholesterol	31 mg/100 ml	12–63	43
Cortisol	0.5 µg/ml	< 0.1–2.3	43
Follicle Stimulating Hormone	9.5 ng/ml	< 2–33.8	34
Growth Hormone	39.0 ng/ml	18.7–51.6	40
Leutinizing Hormone	0.79 ng/ml	0.12–1.8	38
Parathyroid Hormone	1,718 pg/ml	85–6,180	41
Progesterone	8 ng/100 ml	< 0.3–36	42
Prolactin	17.6 ng/ml	2.00–49.55	40
Prostaglandin E	5.91 ng/ml	0.5–30.48	37
Prostaglandin F	12.33 ng/ml	3.77–42.00	38
T3	119 ng/100 ml	56–233	41
T4	12.1 ng/100 ml	7.8–15.6	42
Testosterone	40 ng/100 ml	21–99	42
Thyroid Stimulating Hormone	1.22 ng/ml	< 0.2–4.5	40
Protein			
Total Protein	3.8 g/100 ml	3.2–7.0	43
Albumin	2.3 g/100 ml	2.0–3.6	43
Insulin	10 mU/ml	6–14	40

From: Price, P.J. and Gregory, E.A. *In Vitro*, Vol. 18, No. 6, 576-584, 1982.

Table 2: Reduced Serum Media

Application	Cells	Media
Standard basal formulations enriched with defined components which are normal constituents of serum and allow for a 50–90% reduction in serum supplementation	A wide range of cells routinely cultured in D-MEM and MEM	Advanced D-MEM and Advanced MEM
A modification of Eagle's Minimum Essential Medium. Ideal for use during cationic lipid transfections	A wide range of cells	Opti-MEM® I Reduced-Serum Media

Table 3: Serum-Free Media

Application: Recombinant Protein Production	Cells	Media
Mammalian	CHO	CD CHO Medium, CD CHO AGT™, CHO-S-SFM II
	HeLa, S3, 293-F, 293-H	CD-293 Medium, CD 293 AGT™, 293 SFM II, Freestyle™ 293 Expression Medium
Insect	Sf9, Sf21, Tn-368	Sf-900 II SFM
	BTI-TN-5B1-4 (High Five™)	Express Five® SFM
	Drosophila (D.Mel-2), Schneider S2	Drosophila-SFM

Application: Monoclonal Antibody Production	Cells	Media
Hybridomas	CD-Hybridoma Medium	CD Hybridoma AGT™, Hybridoma-SFM PFHM-II Protein-Free Hybridoma Medium

Application: Virus Production	Cells	Media
Virus Production	COS-7L, VERO, BHK-21, HEp-2	VP-SFM, VP-SFM AGT™
	293-F, 293-H	CD-293 Medium, CD 293 AGT™ 293 SFM II
	MDBK, MDCK, BHK-21, PK-15	OptiPro™ SFM

Application: Cellular Studies	Cells	Media
Dermatology	Keratinocytes	Defined Keratinocyte-SFM (without BPE) Keratinocyte-SFM (with BPE)
Hematology–Oncology	Lymphocytes (human primary lymphoid cells and transformed lymphoid lines)	AIM V® Media
Hematopoiesis	Hematopoietic Cells (bone marrow peripheral blood and neonatal cord blood)	StemPro®-34 SFM
	Peripheral Macrophages and Monocytes	Macrophage-SFM
Vascular Research	Endothelial Cells	Human Endothelial-SFM
Hepatic Research	Hepatocytes	Hepatozyme-SFM
Neurobiology	Neuronal Cells	Neurobasal™ Media, B-27 Supplements
Transgenic Mice	Embryonic Stem Cells (mouse)	KnockOut™ Serum Replacement KnockOut™ D-MEM

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