

# Serum-free hybridoma culture: ethical, scientific and safety considerations

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**Despite considerable progress in the development of cell culture techniques, including the development of the serum- and protein-free media that now routinely support hybridoma and mammalian cell growth, fetal bovine serum (FBS) supplemented media are still commonly used: a practice that raises ethical, scientific and safety concerns. The use of FBS in hybridoma culture media is examined here, with regards to the development and production of monoclonal antibodies (mAbs), and it is our recommendation that researchers adopt serum-free cell culture methods to reduce animal use in this area.**

## Introduction

Cell culture techniques remain largely unchanged since the work of Henry Eagle in 1955 [1]. Eagle identified the specific nutritional requirements of two mammalian cell lines, such that the omission of a single essential nutrient resulted in the early death of the cultures. Eagle's minimum essential medium (MEM) contained a mixture of the amino acids, vitamins, cofactors, carbohydrates and salts necessary to support cell growth. This medium was supplemented with dialyzed horse serum, the absence of which caused the cells to degenerate and die in culture. Most basal cell media cannot support the growth of mammalian cells by themselves, and it has been common practice to supplement cell culture media with animal sera [2]. Today, serum additives are typically provided by fetal bovine serum (FBS), which is capable of supporting the growth of a variety of cell types. The availability and ease of storage of FBS [3], together with its rich content of growth factors and low gamma-globulin content in comparison with other animal sera [2,4], have led to its adoption as the standard medium supplement.

Although cell culture has proved immensely valuable from an ethical viewpoint (i.e. it has replaced many procedures that formerly used live animals), this technology could be enhanced by the complete removal of animal serum from the culture medium. Furthermore, the elimination of FBS from hybridoma media provides numerous technical and safety benefits, including easier purification of monoclonal antibodies (mAbs) from culture supernatants, reduced risk of certain contaminating microorganisms and decreased reagent cost.

## Ethical considerations of serum use

In recent years, FBS production methods have come under scrutiny because of animal welfare concerns: FBS is harvested from bovine fetuses taken from pregnant cows during slaughter and is commonly obtained by means of cardiac puncture without anesthesia. Should the fetuses be conscious during the bleeding procedure, which can occur anywhere from 5–35 min after the death of the mother [5], it is probable that they are exposed to pain and/or discomfort. This issue was addressed most recently at a workshop in Utrecht, The Netherlands [6], which proposed 13 recommendations, including those aimed at reducing animal suffering and a reduction in, or elimination of, the use of FBS, both of which would help to address animal welfare concerns.

## Scientific considerations of serum use

Supplementation of culture media with FBS introduces several variables into research protocols that should otherwise be tightly controlled. FBS is chemically undefined with a high between-batch variability [7], leading to an unpredictable cell culture system in which cell growth and/or experimental results might not be reproducible. This variability can be reduced by screening multiple batches of FBS to maintain consistency and reproducibility throughout research protocols. The commonly accepted criteria indicate that the test lot should be equal to, or exceed, 80% efficacy of the lot currently in use [8], and although this might alleviate some intra-assay variability, it raises concerns about variability between laboratories.

Fetal bovine serum has a high protein content, which can be problematic when culturing hybridoma cells for mAb production and, often, additional downstream processing steps are required for the purification of mAbs from supernatants containing FBS. For example, FBS contains ~100 mg of immunoglobulin per liter [8], and this concentration of exogenous proteins can exceed the concentration of the mAb product by between five- and several thousand-fold. Moreover, the cost of serum is high. The average cost of FBS is difficult to establish because of its fluctuation with market changes, but most US producers charged, on average, between \$115 and \$135 per liter in the year 2005 ([http://www.ams.usda.gov/LSMnpubs/pdf\\_monthly/pharm.pdf](http://www.ams.usda.gov/LSMnpubs/pdf_monthly/pharm.pdf)). Although FBS is added to the cell culture medium at a concentration of between 5% and 15%, it can still account for up to 70% of the cost of overall medium formulation [9]. Stoll *et al.*

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reported an 80% reduction in reagent cost when replacing serum-containing medium with a serum- and protein-free medium [10].

Although significant work has gone into standardizing or circumventing these technical problems, a more scientifically robust system can be achieved by eliminating FBS from cell culture media altogether.

### Safety considerations of serum use

The inclusion of animal sera in cell culture media is a potential source of contaminants, including prions, viruses and mycoplasmas. A report by Wessman and Levings [11] indicated that between 20% and 50% of commercial FBS was virus-positive. The high endotoxin content of FBS also raises questions regarding the suitability and safety of its continued use as a cell growth promoter. The use of FBS requires the elimination of microorganisms, particularly when the end products are used as therapeutic agents for humans and, in recent years, this has been a principal driving force for the development of serum-free media. For these reasons, serum-free media are frequently used for the production of therapeutic proteins. Despite advances in this area, limited progress has been made in eliminating serum from the media of the established cell lines used for research purposes, and contamination by potential human pathogens remains a serious safety concern.

### Serum-free cell culture

In light of the availability of serum-free media, the continued use of serum in cell culture media appears to be mainly based on historical practice rather than scientific need. The advantages of serum-free media are widely recognized: it provides a more defined, controlled cell culture environment and avoids animal welfare issues. Serum-free cultivation of divergent, established cell types has been reviewed [3,12], but little effort has been made to adapt existing cell lines to serum-free media. Many companies have designed serum-free media to support specific cell types, in addition to general-purpose serum-free media for the culture of human, primate, murine and insect cells.

In 2003, scientists with expertise in serum-free *in vitro* methods were convened for a workshop in Utrecht to discuss the replacement of FBS as it applies to *in vitro* methodologies. Following this, a report titled *The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture* [6] recommended the establishment of a public database containing information on the different cell types, and the composition of their serum-free media, to encourage the application of serum-free cell culture. Projects to create searchable databanks for commercially available serum-free media have also been initiated (<http://www.focusonalternatives.org.uk/>) [13,14]. Despite the development of numerous serum-free media formulations for the culture of various cell types, this has still to be universally embraced in the laboratory. A recent review of animal cell cultures identified serum-free media as an important development that needs to be addressed to ensure the future success of animal cell culture processes [15]. Because of the ethical, scientific and safety

concerns inherent in animal serum use, we emphasize the need to eliminate FBS from the cell culture medium of mAb-producing hybridoma cells.

### Serum-free hybridoma cell culture

The first experiments that completely eliminated serum from mammalian cell cultures were conducted in the laboratory of Ham [16]. Much of the early work to determine the optimal media requirements for cells in culture was performed with hematopoietic cells, particularly hybridomas. Because these cells are anchorage-independent, their nutritional requirements are less stringent than cells that adhere. Current hybridoma technology was pioneered by the work of Kohler and Milstein [17], in which murine antibody secreting cells were immortalized by fusing them with myeloma cells. The resulting cells produced virtually unlimited quantities of homogenous and specific antibodies, and the combined properties of cultured hybridoma immortality and the high specificity of the resulting mAbs have led to widespread use of mAbs [18]. It might be useful to note that hybridoma technology involves two steps: the development of the hybridoma and the subsequent production of mAb from an established hybridoma. This discussion focuses on the production of mAbs from an established hybridoma cell line using *in vitro* technologies. We do not support the *in vivo* production of mAbs using the mouse ascites method. This entails injecting the mAb-secreting hybridomas into the peritoneal cavity of mice, where they proliferate. As a consequence, fluid, which contains a high concentration of mAbs, is produced in the abdomen, and this is periodically withdrawn for the isolation of the mAbs. This practice is banned in several countries, and *in vitro* mAb production is used instead [19]. Although we support this approach, current *in vitro* methods of mAb production still fall short of animal welfare concerns because of their reliance on FBS.

The most commonly used media formulations for hybridoma culture are Dulbecco's modified Eagle's medium (DMEM) and RPMI-1640 [20], and these basal media are frequently supplemented with FBS. Chang *et al.* [21] reported the first successful use of a serum-free medium for hybridoma cultivation. In this study, five hybridoma cell lines were cultured in serum-free medium for more than three months, and continued to secrete mAbs at levels achieved in the presence of FBS. Subsequent to this, similar experiments in other laboratories have reported the adaptation of numerous established hybridoma cell lines with no cessation of mAb production [22–26]. In addition, substantial improvements in the homogeneity of antibody-containing supernatants using serum-free and protein-free media have been described. Despite these early successes with serum-free media, current hybridoma culture systems still need to be refined and optimized. A starting point for this would be the elimination of serum for all established hybridoma cell lines; however, to date, there has been a demonstrable lack of effort to implement these advances and replace serum in hybridoma culture media.

Recent developments in serum-free media formulations enable a gradual adaptation of hybridomas and other

transformed cell lines to serum-free media. Stoll *et al.* [10] adapted an IgA-secreting hybridoma cell line to grow in two commercially available, protein-free (and serum-free) media. Kreutz *et al.* [27] adapted a hybridoma cell line, which produced IgG, to grow in serum-free media in three passages. Other studies of mAb-secreting hybridomas adapted to grow in serum-free media have also been described [28–30]; in these, the adaptation was implemented over several passages, with increasing proportions of serum-free media replacing serum-containing media. This approach works for mAbs produced in low-density cultures or for use in research applications where mAb production is on a small scale (<1 g). For commercial applications and larger volumes of mAbs, these modifications should not be ignored simply because serum supplementation is an established practice for cell culture, for example, Moro *et al.* [31] carried out large-scale production of mAbs for human therapeutic use by cloning the hybridomas directly in serum-free medium.

Following on from these trials, we now briefly describe a specific example of how mAbs produced in serum-free media were used in the development of a diagnostic ELISA kit for human insulin. We hope that all the examples cited in this discussion serve as a guide for the elimination of serum from established hybridoma cell lines, and the subsequent use of the mAbs in research applications.

### Development of a novel insulin ELISA

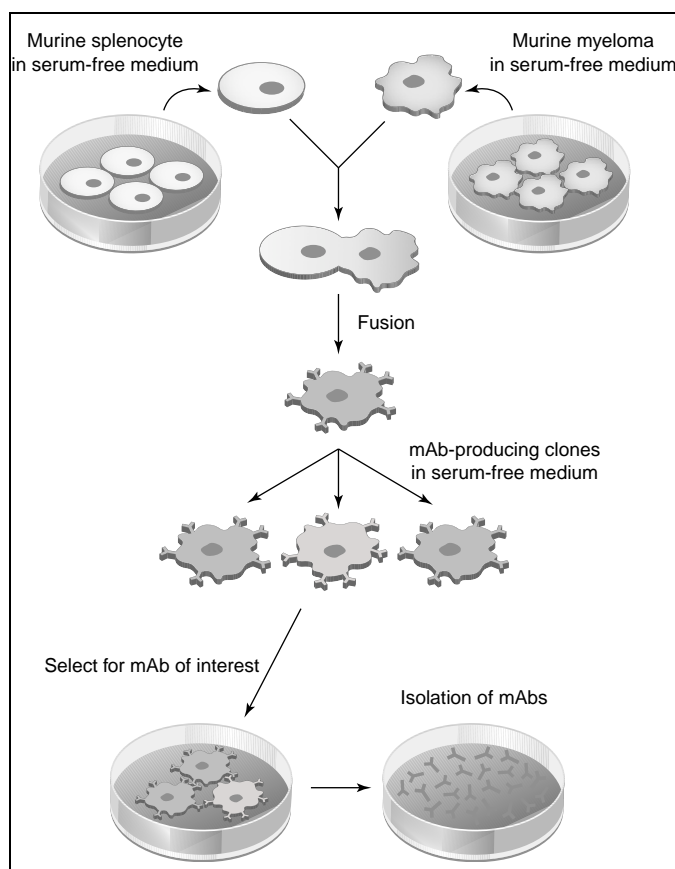
In the course of a clinical trial involving patients with type II diabetes, a highly specific and sensitive insulin ELISA that does not depend on the use of animal proteins was developed. Concerns for animal welfare led us to produce anti-human insulin mAbs without using the ascites method and with gradual elimination of FBS. Hybridoma cultures, producing paired anti-human insulin mAbs, were grown, *in vitro*, in the presence of FBS; however, the daughter cells were weaned off FBS, gradually, and finally cultured in serum-free media. The purified mAbs were then incorporated into an ELISA, in which BSA was replaced with human serum albumin (HSA) [M.S. Even *et al.*, unpublished data]. This novel insulin ELISA corresponds well with, and provides equal precision and reliability to, other methods currently used in clinical and research situations and is now commercially available for research purposes (<http://www.lincoresearch.com/products/ezhiasf-14k.html>).

We believe this method serves as a guide for the adaptation of existing hybridoma cell lines to serum-free media, and the subsequent use of the mAbs for the development of an immunoassay that is both ethical and scientifically valid. To our knowledge, only one other group has produced mAbs, *in vitro*: the hybridomas were grown in serum-supplemented media and then used in different immunological applications, including ELISA [32]. Although this study found serum-free conditions to be suitable for mAb production, the mAbs isolated from serum-free cultures were not evaluated for use in immunoassays.

### Concluding remarks

The replacement of serum-containing media with serum-free media provides important ethical and scientific advantages [33]. As cell culture techniques continue to improve, the need to switch to serum-free media has become imperative. The development of a mAb-producing hybridoma can be carried out without using FBS in the culture medium. Serum-free media are highly effective for the culture of parental cells (splenocytes and myelomas) before fusion and even during selection of hybridomas immediately after fusion [25,26,34]. The next step – production of mAbs from selected hybridoma cells – is also greatly improved by the elimination of serum from culture media. The result is a well-defined hybridoma culture system that can be replicated within and between laboratories and reduces animal use (Figure 1).

In consideration of the ethical mandate to incorporate non-animal methods in scientific research [35], we recommend that all established hybridoma cell lines be transferred to serum-free media. Online, searchable databases for serum-free media products, their applications and availability are available (<http://www.zet.or.at>



**Figure 1.** Process by which monoclonal antibodies (mAbs) can be produced from hybridomas in serum-free medium. Established cultures of mAb-secreting cells (splenocytes) can be adapted to grow in serum-free medium; the same process can be carried out with tumor cells (myelomas). The two cell types are fused together and the resulting hybridomas, which can also be maintained in serum-free medium, are screened for the production of mAbs of interest. Specific clones are then selected and expanded for production of sufficient mAbs. This method for mAb production is ethical, scientifically valid and eliminates safety concerns surrounding the use of FBS. Figure adapted from Access Excellence @ the National Health Museum (see: <http://www.accessexcellence.org/RC/VL/GG/monoclonal.html>).

and <http://www.focusonalternatives.org.uk>) and we urge researchers to access and contribute to these databases to help eliminate FBS from culture media and improve upon cell culture systems.

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