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On farm manipulation of bioactives

Bioactives impact on **p**harmaceutical and **d**iagnostic **productivity**

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Executive summary

FBS has traditionally been used as a media supplement for mammalian cell culture as it provides a rich source of growth factors, extracellular matrix proteins, fatty acids, lipoproteins, carrier proteins, antioxidants and buffering capacity. Since the 1980s there has been a growing trend to remove FBS from culture media due to potential contamination with adventitious agents, lot to lot variation, complicated downstream processing, high cost, and regulatory burden. Despite this FBS is used in the manufacture of most biologics produced in mammalian cells approved prior to circa 2000, including six to seven of the current top ten biologics by revenue. FBS will not be removed from these products as a new licence would be required, which, due to the regulatory burden and need for further clinical trials, would be extremely costly.

To reduce the opportunity for contamination due to FBS the FBS used for biologics manufacture undergoes extensive screening for adventitious agents. In addition FBS sourced from countries with a low risk for BSE are preferred. Australia and New Zealand are recognised as the safest sources of serum available in commercially viable volumes and have been assessed for BSE risk by the European Food Safety Authority (EFSA) as “highly unlikely” (GBR I). Generally for industrial uses FBS is sourced from Australia, New Zealand or the US, despite the fact that the US has had cases of BSE and has been assessed for BSE risk by the European Food Safety Authority as “likely but not confirmed or confirmed, at a lower level” (GBR 3). In addition on 19 Nov 2007 the US will open its borders with Canada to allow free movement of cattle. Canada is considered to have a higher risk for BSE than the US. This may result in more biologic manufacturers moving away from US sourced serum and provides an opportune time for Australian suppliers to gain market share.

Feedback from industry regarding interest in an improved FBS for supporting the production of biologics (i.e. through “on farm” manipulation) is that industry is always vigilant regarding issues relating to bioprocess improvement and cost reduction, however the regulatory burden would need to be low.

A significant market for FBS, though smaller than the biopharmaceutical market, is the production of live, attenuated viral (LAV) vaccines. Merck produces a number of LAV vaccines that require FBS-supplemented media; two of these, RotaTeq and Zostavax, are potential blockbusters. Merck currently use US-sourced FBS and calf serum for viral vaccine production. Senior Merck personnel have indicated interest in GBR1-sourced FBS and FBS with improved performance.

The research market is a substantial consumer of FBS, however, the supply and regulatory concerns of the industrial sector are not shared by the research sector where cost and performance are paramount. As a result this sector is more likely to purchase serum from cheaper sources than Australia, such as Mexico, France, South America, Central America and Canada. While this also means it is easier for researchers to switch to a better product they would need to see substantial benefits to use a more expensive product.

There is very little information available in the literature on the potential impact of variations in the bioactive content of FBS on biologic production. Information is available on the mode of action of many identified bioactives. Growth factors are probably the most important group. The major growth factors that would be of interest for further investigation are insulin like growth factors (IGFs) and their binding proteins, fibroblast growth factors (FGFs), transforming growth factors (TGFs), platelet derived growth factor PDGF) and epidermal growth factor (EGF) . In particular b-FGF plays an important role in the growth of a number of cell types and could be a lead candidate biomarker for assessing the quality of FBS through on farm manipulation of bioactives.

Contents

1 Introduction

1.1 Purpose and Description

The following is an extract from the Agreement, 13th June 2007, between Meat and Livestock Australia and Uniquest Pty. Ltd.

Background

The red meat bioactives industry in Australia is estimated to be worth $200mpa. MLA is aiming to increase the profitability of the red meat industry by supporting the growth of the bioactives industry. Our strategy is to facilitate growth by a combination of identifying new opportunities, identifying new technologies and co-investing in R&D with potential value adders. Our priority is to maximise the share of the added value that can be realised by the red meat producers and processors.

The largest sector of the Australian red meat bioactives industry currently is blood products, valued at $100mpa, which includes serum, plasma, fatty acids and growth factors. The greatest potential for significant impact on the red meat industry in the short to medium term is therefore seen to lie in the growth of this sector.

The highest value blood products are those used by the pharmaceutical industry for the manufacture of therapeutic and diagnostic chemicals. This is an expensive and capital intensive production process and it is known that there is natural variation in the efficacy of sera in these applications. The conventional solution by serum manufacturers to this variability is to combine many batches of blood in order to “even-out” the variation. The opportunity exists however to take advantage of this natural variation, and even to exploit it, in order to create products of greater value to the pharmaceutical and diagnostic manufacturers.

It is envisaged that genetic and physiological differences between the donor animals which underlie this variation could be exploited to the benefit of the whole value chain, with producers and processors able to make the biggest contribution and therefore claim a significant share of this benefit. It is recognised that only be developing products of real value to the pharmaceutical and diagnostic manufacturers can profitability of the red meat bioactives industry be developed. Before investing in the development of this capability, it is necessary to objectively assess both the potential for impact and the likely value of the impact on pharma manufacturers.

1.2 Objectives

The following is an extract from the Agreement, 13th June 2007, between Meat and Livestock Australia and Uniquest Pty Ltd.

The project will identify the potential impact of variations in the bioactives content of blood sera on the productivity of recombinant antibody manufacturers. It will involve the following activities:

- Identify the key technical parameters seen by manufacturers of recombinant proteins in mammalian systems as being the best measure of a growth mediums performance. E.g. cell growth rate, protein productivity per fermenter volume, yield, maximum cell density etc. This will probably require contacting the manufacturers for their views.
- Identify from the technical and patent literature the biochemical moieties which are known to affect the parameters of importance to manufacturers, including those which are known to be responsible for variation in performance from batch-to-batch of media

- Determine the potential for compounds identified within Project 1 above (Nature and Nurture) to impact on parameters of importance to manufacturers of recombinant proteins in Mammalian systems. (This will involve liaising with researchers in Project 1).
- Work with the researcher in Project 3 to identify the compounds/bioactives of economic significance to manufacturers
- Identify from the literature the significance of medium components to downstream processing in mammalian cell culture for recombinant protein production. E.g. Effects on filterability, chromatographic or ion exchange.

1.3 Scope

This study provides information on key growth factors in foetal bovine serum (FBS), suppliers of FBS and consumers of FBS. In addition it covers the relevant regulatory bodies governing the use of FBS in USA, Europe and Australia.

1.4 Methodology

Initially a literature review was conducted which covered relevant scientific journals, books, newsletters, company websites and catalogues. In addition the “Biopharma” website, which contains information on registered biopharmaceuticals in US and Europe, was purchased and reviewed.

Areas covered by the literature review included:

1. Biologics industry
2. FBS use in biotechnology
3. Key measures of a growth mediums performance in mammalian cell culture
4. Factors that impact on the quality of FBS with respect to cell culture
5. Effects of medium components on downstream processing in mammalian cell culture
6. FBS Market
7. Regulatory issues

The literature review provided information which was used to further contact relevant companies, institutions and universities to gain insight into the FBS market and its use by both the biopharmaceutical and research industries.

2 FBS use in Biotechnology

2.1 Definition of Biologics

The following terms have all been used in the pharmaceutical literature:

- Biologic
- Biopharmaceutical
- Products of pharmaceutical biotechnology
- Biotechnology-sourced products
- Biotechnology medicines

Historically in the pharmaceutical industry, ‘biologic’ refers to medicinal products derived from blood, vaccines, toxins and allergen products. Other biotechnology-derived products such as

antibiotics, plant metabolites and some hormones are not classified as biologics. The term 'biopharmaceutical' was coined in the 1980's to define proteins that were made by recombinant DNA technology (which includes hybridoma technology for monoclonal antibody production).

More recently the term incorporates nucleic acids used for gene therapy and antisense technology and includes proteins used for in vivo diagnostic purposes. Definitions as to what constitutes a biologic and biopharmaceutical can make it difficult to compare approvals reported by different sources. A recent survey of the industry suggests the following definition for a biopharmaceutical: "Biopharmaceuticals are pharmaceuticals inherently biological in nature due to their method of manufacture; specifically those using biotechnology and involving the use of live organisms" (Genetic Engineering and Biotechnology News; August, 2006). Today, the general consensus is that a "Biologic" and "Biopharmaceutical" are interchangeable terminology, but a biologic might incorporate some other products such as live attenuated vaccines.

2.2 Global biologics overview

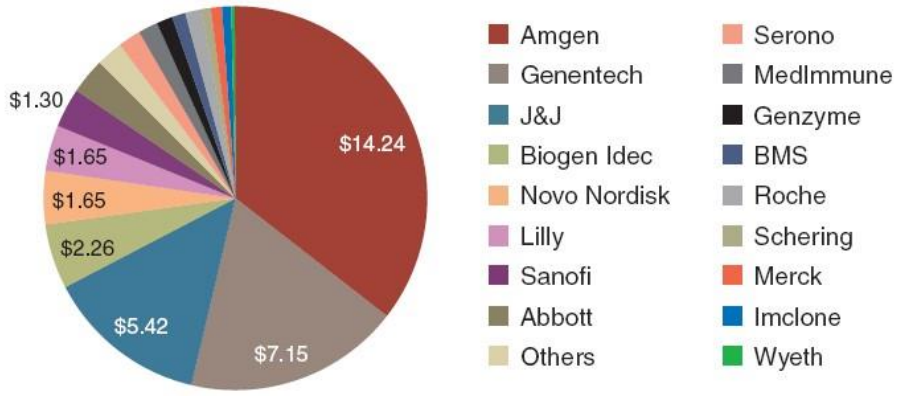
Global Pharmaceuticals and Biologics Sales were $US 650 and 63 billion respectively in 2006. Growth in revenues from Global Pharmaceuticals and Biologics was 7% and 20% respectively[1].

There are about 250 biologics approved for 380 indications, and more than 300 are in clinical trials targeting more than 200 diseases, including major disease indications such as cancer, Alzheimer's, heart disease, diabetes, multiple sclerosis, AIDS and arthritis.[2] The biologics industry is growing strongly in an environment where revenue from pharmaceuticals is decreasing. The industry is at present dominated by the large, specialist biotechnology/biopharmaceutical companies as shown in Figure 1, however big pharma are showing an increasing interest in adding biotechnology-derived products to their development pipeline for the following reasons;

- The number of pharmaceutical new molecular entities (NME) is in decline while biologic NME are increasing (

Figure 2). Biologics account for 50% discovery stage candidates and 20% applications for approval (Figure 3)

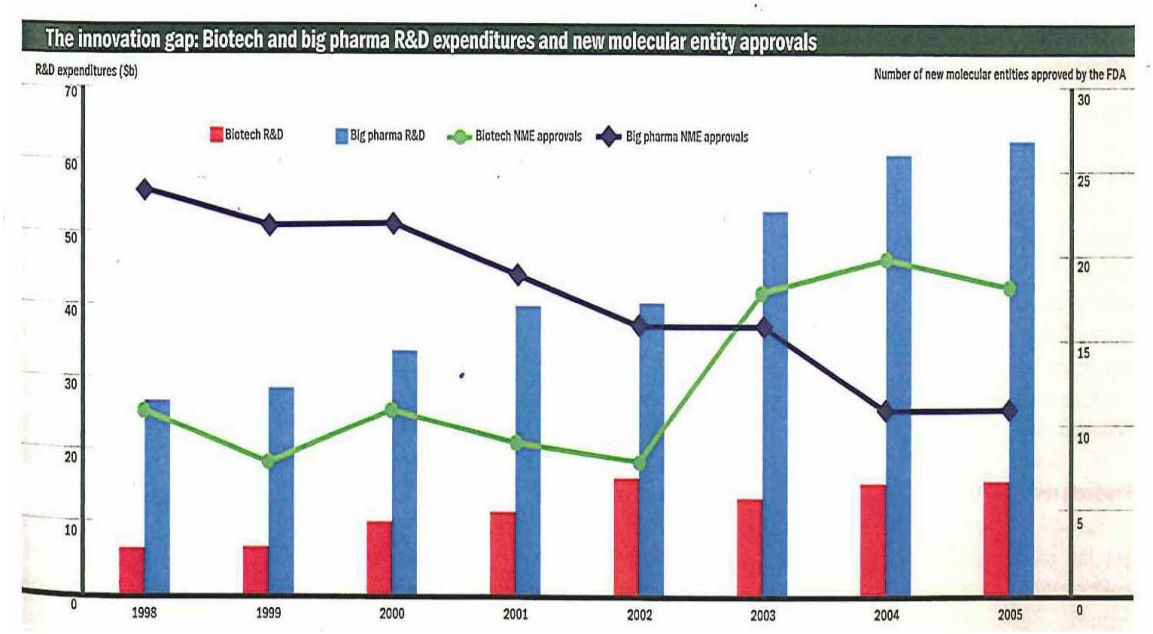
- Many drugs will be coming off patent in the near future, so Big Pharma are diversifying into biologics.
- The "herd mentality" operates at the level of Big Pharma. For example, Wyeth receive 37% of their revenue from biologics, whereas Pfizer (the largest Big Pharma with respect to revenue) derive 10%. In response, Pfizer have an active program for expansion into biologics, and now have 17 biologics in their clinical development pipeline (personal communication, Pfizer Australia).



	2005 US Sales ($ billions)	2006 US Sales ($ billions)	2005 growth (%)	2006 growth (%)
Amgen	11.79	14.24	22	21
Genentech	4.37	7.15	97	64
Johnson & Johnson	5.21	5.42	-2	-9
Biogen Idec	1.99	2.26	14	13
Novo Nordisk	1.38	1.65	32	20
Lilly	1.82	1.65	-1	4
Sanofi	0.98	1.30	46	33
Abbott	0.86	1.21	57	41
Serono	0.87	0.78	13	17
Astra Zenica	0.67	0.73	14	9

Figure 1 Top 18 companies responsible for the majority of revenue in biologics in 2006 (Figure taken from Aggarwal, 2007[3])

Figure 2 Biotech and Big Pharma R&D expenditures and NME approvals



- Approvals include only new molecular entities and include label approvals, new formulations and combinations.
- Certain drugs partnered between biotech and big pharma companies are counted in both groups.
- Big pharma is defined as the 15 largest global pharmaceutical companies by market cap.

(Source: Ernst and Young, Beyond Borders, 2006) [1]

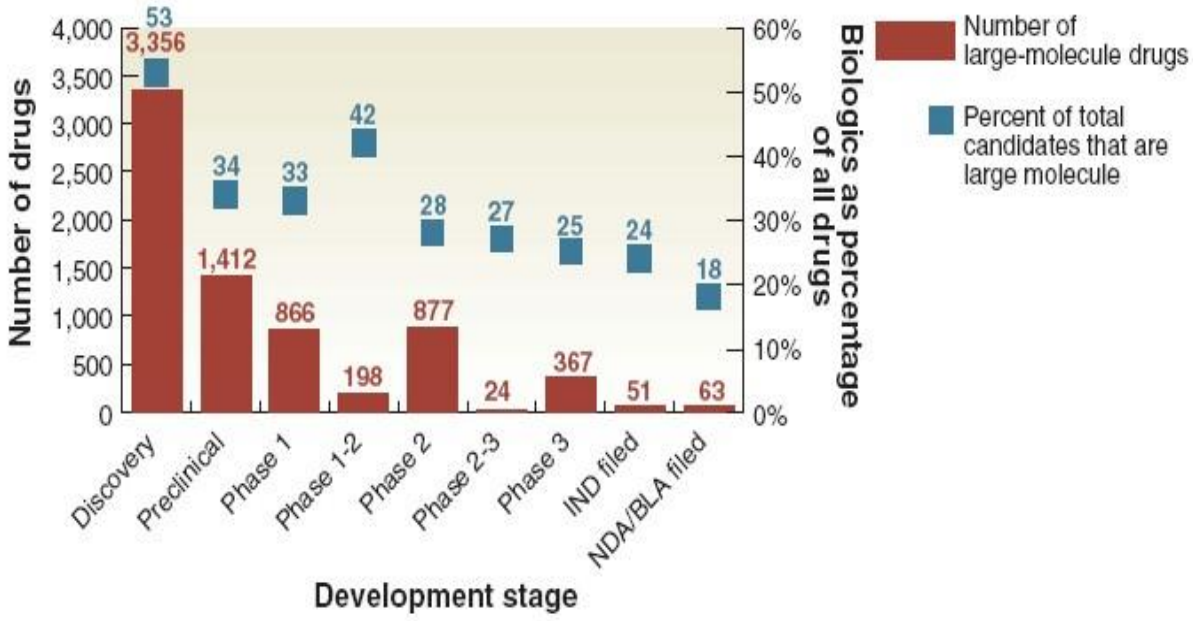
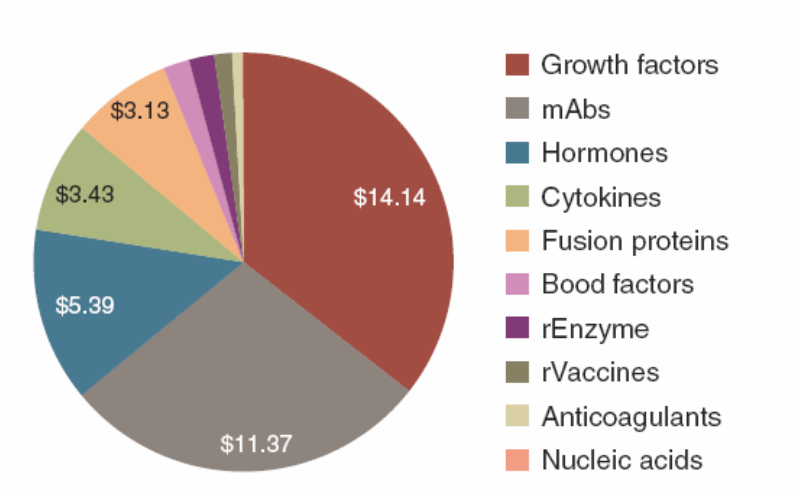


Figure 3 Proportion of biologics in various stages of development as a percentage of total drugs
(Figure taken from Lawrence, 2006)[3]



	2005 US Sales ($ billions)	2006 US Sales ($ billions)	2005 growth (%)	2006 growth (%)
Growth factors	12.11	14.14	10	17
mAbs	8.29	11.37	38	37
Hormones	4.51	5.39	24	19
Cytokines	3.11	3.43	4	10
Fusion proteins	2.81	3.13	36	11
Blood factors	0.74	0.78	11	5
rEnzymes	0.58	0.76	18	31
rVaccines	0.40	0.56	-10	40
Anticoagulents	0.34	0.34	-1	-1
Nucliec acids	0.06	0.02	-	-

Figure 4 Top ten categories of biologic drugs in terms of $US sales and growth rates for 2005 and 2006 (Figure taken from Aggarwal, 2007) [3]

Aggarwal[4] has categorised biologics into ten different groupings, as shown in Figure 4. The largest market is for growth factors, for which there are 11 brands. Erythropoietin (EPO) is the biggest selling biopharmaceutical, with four brands of EPO occurring in the top biopharmaceuticals according to revenue (Table 1). EPO has four brand names, Epogen (Amgen), Procrit (J&J) and Aranesp (Amgen) and Epogin/NeoRecormon. Both Epogen and Procrit are produced in adherent Chinese hamster Ovary (CHO) cells using roller bottle production systems. Both use FBS-supplemented media for production. Aranesp is a recombinant EPO with two extra engineered N-glycosylation sites to increase serum half life. As shown in

Table 7, Aranesp is reported as containing unspecified bovine-derived components for cell culture, however it is not specified that these components are from FBS. Amgen's patent protection for EPO is starting to expire in various countries, and there are many commercial entities worldwide with EPO follow-on (biosimilar) products in their pipeline. In August 2007, the EMEA approved a biosimilar EPO for Sandoz and two partner companies. The new EPO will be launched soon in Germany and the UK, at a price discount of 25%-30%. Thus the market share for EPO will continue to fall for the established brands, while biosimilar EPO products (likely to be produced in serum-free media) will gain market share.

The second largest market is for monoclonal antibodies. There are 26 mAbs approved in the USA with 95% of revenue being derived from seven mAbs[3]. Table 1 shows that four of the top ten biologics according to revenue are mAbs; Remicade, Rituxin, Herceptin and Avastin. The two main disease indications where mAbs have application are cancer and inflammation. If the mode of action for mAbs is through antibody-dependent cellular cytotoxicity (ADCC) or compliment directed cytotoxicity (CDC), these mAbs are produced in CHO or NS0 myeloma cells, since appropriate glycosylation is required for antibody effector function. Although there are efficient mammalian cell expression systems now available which can achieve 1-2 g/L mAb productivity in serum-free media with no animal-derived components, products which have been licensed prior to the development of serum free media formulations (circa 2000) are produced in media supplemented with serum, usually FBS for enhanced growth performance and less bovine immunoglobulin (compared to new born calf and adult sera) which simplifies downstream processing. For example Remicade and Rituxan are both produced in media which is likely supplemented with FBS. Another biologic used to treat inflammation is Enbrel, which is classed as a fusion protein (p55 TNF receptor fused to a human IgG1 constant region). Enbrel is the largest selling biologic globally, and is produced in CHO cells in media which is likely supplemented with FBS (media is quoted as containing bovine-derived materials sourced from BSE/TSE-free countries only) (Appendix A).

Figure 5 shows the suitability of various classes of biologics for development as biosimilars, with first generation biosimilars showing no improvement over their brand name product, while second generation biosimilars will be improved versions. It has been suggested that the brand name products for more complex biologics such as mAbs are somewhat less threatened than the simple biologics such as cytokines and growth factors, as their mode of action is more complicated[3]. However a Rituxan biosimilar was launched in April 2007 in India by Dr Reddy for the Indian market under the brand name Reditux. This is the first reported mAb biosimilar globally, and demonstrates that biosimilars will be developed in the short term future in countries such as India and China. As well as Dr Reddy, Zenotech are conducting clinical trials on another Rituximab biosimilar for the Indian market. India is becoming a leading player in the development of biosimilars with a number of companies developing a range of biosimilars for the sizeable Indian market [5]

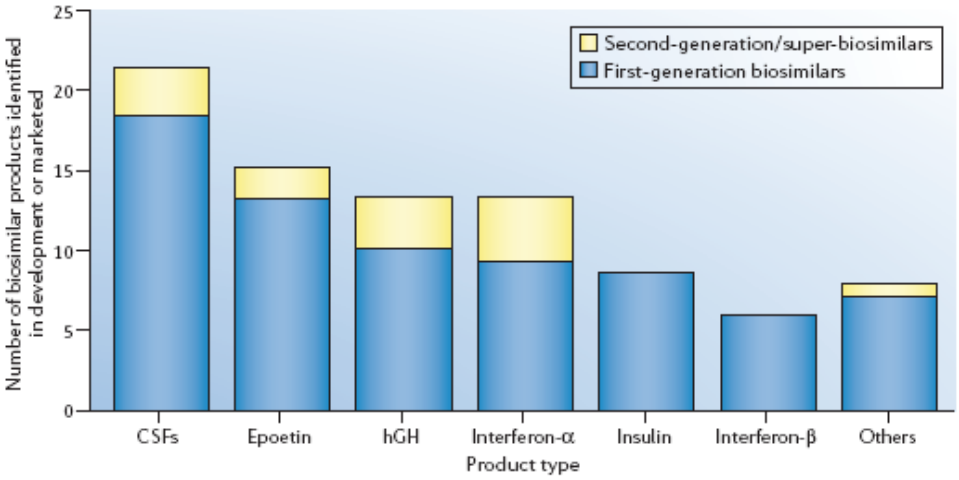


Figure 5 Suitability of class of biopharmaceutical for biosimilar development. Ease of biosimilar development influenced by ease of competition, existing markets, ease of product development and characterisation and varying level of patient support (Figure taken from Belsey et al. 2006) [8]

The development of biosimilars in the USA is impeded at present since there is no clear regulatory pathway for approval unlike the EMEA in Europe[6]. However legislation was introduced in the US House of Representatives and the US Senate in 2007 which will simplify the path to approval for biosimilars on a case-by-case basis.

Table 1 Top ten biopharmaceuticals by global sales
Data taken from Lawrence, 2006[3]

Product/company	Type	2005 $ billion	2006 $ billion
Enbrel (etanercept)/Amgen/ Wyeth	Recombinant fusion protein; soluble TNF receptor linked to IgG1	3,657	2,087
Remicade (infliximab)/ Centocor	Chimeric mAb; anti-TNF-alpha	3,477	2,042
Aranesp (darbepoetin alfa)/ Amgen	Recombinant erythropoietin with two additional N-glycosylation sites	3,273	1,948
Rituxan (rituximab)/ Biogen-Idec/Genentech	Chimeric mAb; anti-CD20	3,154	1,917
Procrit (erythropoietin)/Amgen	Recombinant erythropoietin	3,324	1,594
Herceptin (trastuzumab)/ Genentech	Humanized mAb; anti-HER-2	1,629	1,480
Neulasta (PEG-filgrastim)/ Amgen/Dompec Biotech	Recombinant methionyl human granulocyte colony stimulating factor (Filgrastim) conjugated to monomethoxypolyethylene glycol	2,288	1,309
Epogen (erythropoietin)/Amgen	Recombinant erythropoietin	2,455	1,217

Avastin (bevacizumab)/ Genentech	Humanized mAb; anti-vascular endothelial growth factor	1,264	1,134
Epogin/NeoRecormon (Roche)	Recombinant erythropoietin	1,710	898

2.3 Trends in the industry

The main production systems for recombinant protein biopharmaceuticals are mammalian cells and bacteria. Approximately 60-70% of biopharmaceuticals are produced in mammalian cells[7]. Prior to around 2000, most recombinant biopharmaceuticals were produced in basal media (containing salts, lipids and other specific additives) supplemented with FBS. Circumstances that have driven pharmaceutical/biopharmaceutical companies to serum-free and defined media for production of biopharmaceuticals (i.e. animal derived supplement-free cell culture) include:

1. The risk to supplies of animal derived supplements such as FBS, which may be brought about by fresh outbreaks of pathogens that have the potential to contaminate serum; for example bovine spongiform encephalitis (BSE), foot and mouth disease (FMD).
2. Batch to batch variability; batch variability is a function of animal breed, the feed given to the source animal, time of the year
3. Pressure from regulatory bodies, which have the responsibility of risk management and perceive the removal of animal-derived substances from cell culture media to decrease the risk to end users.
4. The increasing prevalence of biosimilars. It is estimated that US$157 billion (around 40%) of 2005 brand sales will be exposed to generic competition by 2015.[8]
5. Cos
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Since the development of BSE, regulatory bodies have become increasingly concerned about the presence of animal-derived supplements in sera. In response to this concern, biopharmaceutical/biotechnology companies have developed proprietary serum free or fully defined media, while cell culture media suppliers have developed commercially available serum free of fully defined media. As a result, recombinant protein biopharmaceuticals can now be produced in media free from animal-derived supplements. Accordingly, mammalian cell production systems are designed using vectors and host cells that are able to propagate in serum free or fully defined media (e.g. Lonza have developed the GS system for both myeloma (NS0) and Chinese hamster ovary cells (CHO); both these cell lines are propagated in fully defined media).

Further to point 1 above, big pharma are acutely aware of the risks inherent in using FBS from the perspective of supply constraints due to pathogen outbreaks. For example, Merck manufacture a number of live attenuated viral vaccines in cell lines such as MRC-5 in media supplemented with FBS. For some time, Merck have conducted an in-house R&D program associated with developing cell hosts that are capable of growing in defined media free of animal-derived supplements. The development of these serum-free virus production systems is part of a risk management contingency plan, so that in the event that serum supplies are threatened, a transition may be made to alternative production systems in a timely manner. The rationale for maintaining the status quo with viral production systems, which now use bovine serum, include:

1. Replacing serum-supplemented media with a defined media would necessitate the laying down of new master (MCB) and working cell banks (WCB), as existing MCB and WCB would not be adapted to serum-free conditions.

2. Since a new GMP bioprocess would need to be developed, the product would need to undergo comparability studies or clinical trial testing. Demonstration of equivalence through physico/chemical testing (as is applied to recombinant protein biopharmaceuticals) may not be relevant, although some demonstration of support and maintenance of cell lines would be required.

3. The development of new production systems with associated clinical trials may open the market to competition and loss of market share, although vaccines are a small sector of the overall biologics market,

Although vaccines are a small sector of the overall biologics market, there is a significant opportunity for marketing of FBS. Vaccines are divided into recombinant subunit vaccines (rVaccines), live attenuated and inactivated viral vaccines. The rVaccines are cloned subunits of pathogens and viruses, and may be regarded similarly to recombinant protein biologics, where the trend is for production using CHO or NS0 myeloma (and potentially other hosts) in serum-free media with no animal-derived supplements. Live, attenuated virus (LAV) vaccines are produced cell lines such as Vero cells and MRC-5. These include vaccines such as Zostavax, Rota Teq, Meruvax II (Merck) etc as detailed in Table X. The MRC-5 cell line is a human, fetal lung fibroblast cell line, and has been traditionally used for the production of LAV. The cell line is a fastidious and has a defined senescence, unlike cell lines such as CHOK1 and NS0 used for recombinant protein biologics production. The MRC-5 cell line requires FCS for growth, and purification of some of enveloped LAVs can be difficult due to their lability. As a result, relatively crude purifications are carried out on LAV, where cells are lysed and the supernatant containing the LAVs is clarified by filtration.

3 Key parameters for measuring growth medium performance and biopharmaceutical productivity

3.1 Current research associated with increasing recombinant protein biopharmaceutical productivity

In order to evaluate the potential market for a FBS product enriched in bioactives for the production of recombinant protein biopharmaceuticals and live, attenuated vaccines, it is important to review the current research associated with increasing recombinant protein productivity of mammalian cell lines. However, prior to discussing the currently available technologies aimed at increasing protein product production in mammalian cells, it is first necessary to define the cell system parameter that is to be increased. Usually, productivity of a mammalian cell culture system is measured by the volumetric product yield. This measurement is a function of the cell specific production rate (qP) and the integral of viable cell concentration (calculated as the area under a viable cell density (VCD) growth curve and expressed as viable cell time per unit volume)[9]:

Volumetric product yield = **cell** qP × integral of viable **cell** concentration

3.2 Strategies aimed at improving recombinant protein production

Various strategies have been applied to mammalian cell culture in an attempt to increase recombinant protein production efficiency. The effect these strategies have on VCD and recombinant productivity is diagrammatically represented in Figure 6.

3.2.1 Feed Optimisation

For fed batch systems, the most common approach is to rapidly accumulate and maintain viable cell density. For the most part, this has been achieved through optimisation of media and feeding regimes.[10 11 12] Although such methods will increase VCD over culture time (and hence improve volumetric product yield) the specific productivity per unit biomass is unlikely to improve dramatically. Thus, this technique is aimed at achieving maximal cell densities, rather than altering the cells capacity for recombinant protein synthesis.

3.2.2 Inhibition of apoptosis

Another approach has been to inhibit apoptotic cell death in an attempt to extend VCD over time. Common approaches to achieve this include: the expression of the anti-apoptotic proteins bcl-2 or bcl-XL[13]; use of caspase inhibitors[14]; and the expression of HSP70.[14] These techniques may increase volumetric protein production by maintaining VCD and extending culture time (cf. feed optimisation).

3.2.3 Biphasic cell culture strategies aimed at improving protein production

An alternative approach to increasing production efficiency has been based on the inverse relationship between cell proliferation rate and cell specific protein product production rate[15]. In contrast to feed optimisation and apoptosis inhibition which use a standard log type growth curve throughout the culture time, strategies that alter cell proliferation use a 'biphasic' cell culture strategy[9]. In the first phase of this approach, cells are grown to between mid and late exponential phase. At this point cell proliferation is halted and the second (protein product production) phase begins.

One of the most specific means of halting cell proliferation is the inducible expression of cell cycle kinase inhibitors such as $p21^{CIP1}$ which arrests cells in G1-phase by inhibiting the activity of the cyclin E-dependant kinase cdk2[16]. In practice, induction of $p21^{CIP1}$ in a biphasic CHO cell culture has resulted in increased cell specific production and a corresponding increase in cell size without an increase in volumetric yield.[17] In this situation, biomass per unit volume continues to grow over the culture time, even though it is not partitioned into new cells. This results in larger cells whose increased cell specific productivity is counteracted by a corresponding decrease in VCD. Furthermore, since the ratio of biomass to protein product had not changed, the specific production rate per unit biomass remains unchanged.

3.2.4 Cell Engineering

Advances in media formulations and optimisation of production bioprocesses have seen the industry standard for monoclonal antibody productivity increase to over a gram per litre. Longer term, further significant increases are likely to require innovations such as increasing cell-specific productivity through host cell engineering.

As an example of cell engineering, a novel approach towards increasing the mAb synthesis capacity of CHO cells by utilizing the transactivators ATF6 and XBP1 (common to both plasma cells and the unfolded protein response (UPR)) to simultaneously up regulate multiple ER-associated proteins[18]. By globally up-regulating the protein synthetic/processing machinery of the cell, increases in recombinant protein activity are possible[19]. Specifically, this can be achieved by expressing the active forms of transcription factors ATF6 and XBP1 in CHO cells and analyzing the extent to which multiple foldases are up regulated, which have the follow-on effect of increasing recombinant protein expression[20]. As previously mentioned, monoclonal antibodies are a major class of biopharmaceuticals and account for about 30% of biopharmaceuticals in development. Such mAbs are commonly produced on an industrial scale using CHO and NS0 myeloma cells. Consequently, the engineering of these cells in order to make them more efficient at producing mAbs or other proteins is of major commercial interest.

3.2.5 Lowered culture temperature

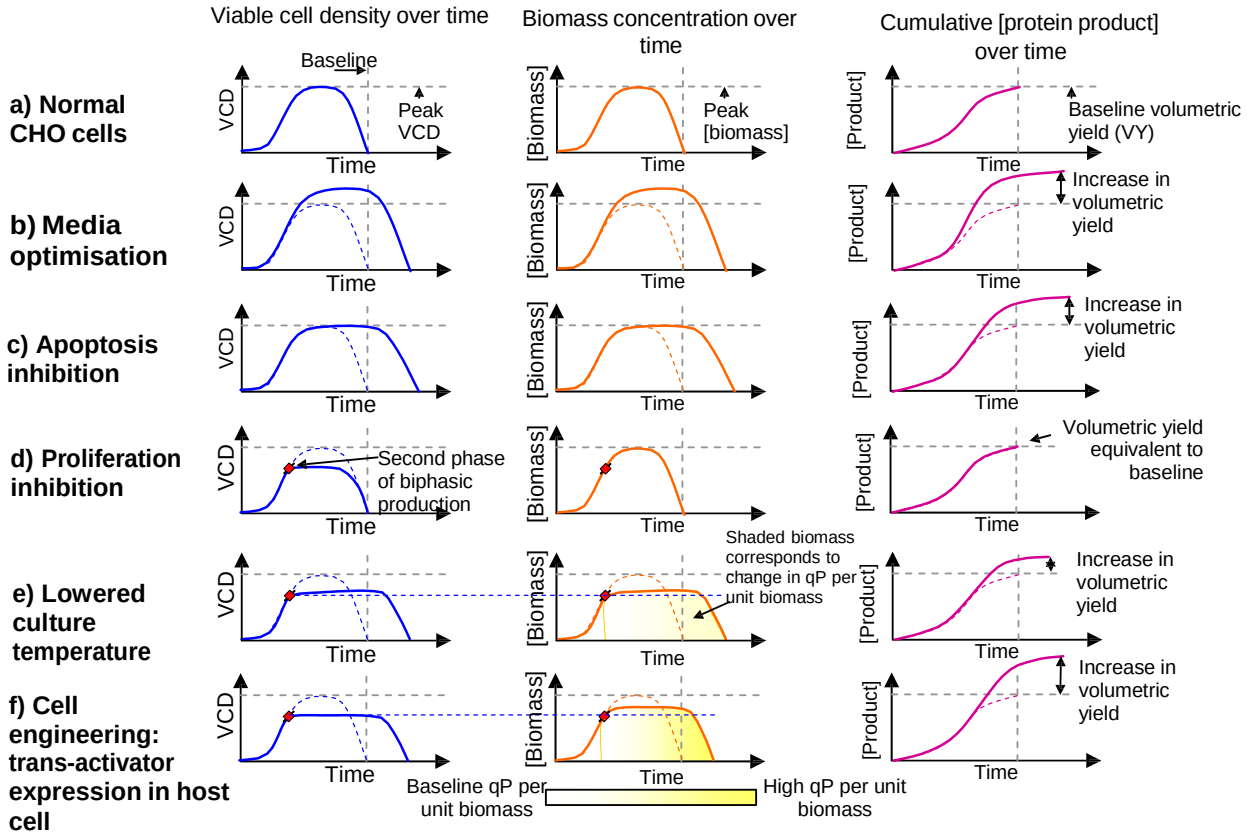
Lowering culture temperature may decrease the maximal VCD attained, but may also have the effect of extending the life of the culture, thus increasing volumetric yield. Lowered culture temperature may also impart some stress on cells, that may also result in expression of chaperone (stress) proteins, and have an effect on the capacity of the cells to secrete proteins (i.e. increases in qP). Research associated with culturing at decreased temperature is in preliminary experimental stages at present.

3.2.6 Summary

In summary, the consensus of opinion is that improvements in media formulation can lead to incremental increases in productivity. Accordingly the availability of a high grade FBS with enhanced concentration of bioactives (enhanced FBS supplement) could lead to increases in productivity through increases in cell density and biomass. If a FBS preparation was found to have a decreased concentration of apoptotic factors, increases in productivity through prolonged cell culture are feasible. Potential increases in specific productivity due to higher concentrations of growth factors etc. would need to be investigated experimentally.

For the longer term, research associated with increasing recombinant protein productivity in mammalian cells is focussing on cell engineering techniques to create superior hosts capable of being propagated in serum-free media, with an enhanced capacity to process and secrete recombinant proteins, resulting in increases in cell-specific productivity.

Figure 6 Models of existing strategies aimed at increasing CHO cell productivity. The diagrams are simplified representations of ideal cell culture behaviour in response to various manipulations aimed at increasing protein product yield. Diagram (f) is an estimate of the growth kinetics of CHO cells expressing transactivators. The dotted graph plots in each diagram represent the baseline behavior of normal CHO cells as shown in (a).



4 Key factors impacting on the quality of FBS for cell culture

FBS is a complex, undefined cell culture protein supplement, and is effective in supporting the growth and proliferation of a myriad of cell lines and primary cell types. As shown in Table 2, FBS contains many known proteins and certainly, a number of as yet unidentified serum proteins. Table 2 is by no means exhaustive and there are other known factors that have been shown to be important for cell growth and proliferation under certain physiological conditions. Importantly, there are also many unknown factors in FBS that no doubt would have an effect on cell growth and proliferation. The HUPO (Human Proteome Organisation) Human Plasma Proteome Project (PPP) has identified 3020 plasma proteins (where identification was made with two or more peptides)[21]. Considering the bovine plasma proteome would contain homologues of the majority of human proteins, the FBS proteome remains a somewhat uncharacterised cell culture supplement.

The growth of eukaryotic cells is influenced by a variety of molecular entities, not the least of which are the broad family of regulatory proteins termed growth factors (GF). Major GF that have been identified to influence growth include IGFs (and its binding proteins), FGFs, TGFs, PDGF and EGF. Table 3 shows a list of growth factors important for cell growth and their target cells.

A recent study by Zheng et al.[22] compared three batches of FBS from two different suppliers for their ability to support the growth of adult retinal pigment cells (ARPE-19). The study showed that one of the batches (FBS-II) resulted in a higher growth rate compared to those of the other two batches (FBS-1a and FBS-1b), and attempted to identify the factors by an LC-MS proteomic analysis approach. The samples of FBS were depleted of high abundance proteins prior to proteomic analyses. Interestingly the total number of proteins identified in the samples was approximately 90, a fraction of the total number of proteins known to be present in FBS. IGFII and IGFBP2 were identified in all three batches of FBS. Five proteins that were identified in FBS-II, but were not identified in FBS 1a or 1b include TGF1, b-FGF, glial growth factor (GGF), prepro-insulin-like growth factor I and IGFBP4. The authors contend that these factors may be responsible for the enhanced performance of FBS-II with respect to growth rate.

A subsequent experiment by Zheng et al. attempted to identify which factors were depleted from the media after prolonged culture of ARPE-19 (0-8 days), with the rationale that depleted factors may be involved in cell growth and spreading. Proteins that were depleted in culture included TGF1, b-FGF, glial growth factor (GGF), prepro-insulin-like growth factor I, IGFBP2 and IGFBP4. All these factors have been reported in the literature to be associated with cell growth/cell spreading. As well as identification of FBS-derived proteins, an analysis was also undertaken of the conditioned media (supplemented with FBS-II) between 0-8 days of culture to identify secreted host cell proteins. The authors contend that the identification of various ECM proteins such as collagens and keratins were an indication of cell growth. The data showed an increase in the concentration of these proteins over the 8 days; however there were no comparisons between cells cultured in media supplemented with FBS-1a or 1b.

In summary, this study is a recent study, using modern proteomic analysis techniques, and attempts to quantify the ability of various batches of FBS to support enhanced cell growth rates. The data show that a selection of factors known to influence the growth rate of a number of cell types are detected in one batch of FBS, and hypothesise that their presence is responsible for enhanced ARPE-19 growth rate. The scarcity of reports in the literature associated with detailed analysis of FBS may be due to the following:

1. The latest techniques for detailed proteomic analysis are now LC-MS based (not gel based), and are evolving rapidly. Mass spectrometers that were state-of-the-art 2-3 years ago (e.g.

Q-Star) are being superseded by instruments with enhanced sensitivity capabilities, such as the LTQ-FT (Thermo) (personal communication, Dr Mark Raftery, Biomedical Mass Spectrometry Facility, UNSW). Furthermore, new reagents such as iTRAQ isobaric tags, used in conjunction with LC-MS, have made possible the quantification of the differences between specific proteins in several samples against a control.

2. There is more interest in characterising the proteome of human plasma, principally for biomarker discovery. As previously mentioned, the majority of human proteins identified in human plasma will have their bovine homologue, and interest should perhaps focus on the function of newly discovered proteins in the human plasma proteome, which may then be extrapolated to its bovine counterpart.

4.1 Cell Culture

The culture of mammalian cells has been carried out for over 100 years, since R.G. Harrison demonstrated in 1907 the growth of frog neural tube tissue on frog lymph.[23] Initial attempts were carried out in serum or other biological fluids. In 1955 Eagle developed Eagle's minimum essential medium[24], consisting of 13 amino acids, 8 vitamins, 6 ionic species, and dialyzed serum. Since then many formulations have been developed including numerous serum-free formulations.

For mammalian cell culture there are various sub-classes of culture media. Classical or basal media, such as Dulbecco's modified eagle's medium, Ham's medium and medium 199, require serum supplementation. Reduced-serum media is based on modifications to basal media which allow a lower level of serum supplementation; these may include supplementation with growth factors or trace elements. Serum-free media do not require serum but may be supplemented with proteins, protein fractions or lipids which may not be completely defined. Protein-free media while more defined than serum-free media may still contain supplements from animal origins. Chemically defined media are protein-free and contain no extracts or components of unknown composition, removing lot-to-lot variability and increasing product consistency.

Despite the inherent disadvantages of a culture media supplemented with an undefined, complex animal-derived component such as serum it is still widely used for mammalian cell culture including the production of viruses, culture of stem cells and many molecular studies. FBS is one of the most effective serum supplements for cell growth due to its high content of embryonic growth factors while maintaining low levels of immunoglobulin's.

Important components for mammalian cell culture provided by FBS are shown in Table 2.

Table 2 Important components of FBS for mammalian cell culture

Component	Function
Albumin (major protein component of serum)	Provides buffering capacity; protection against shear; transport of fatty acids, steroids and fat soluble vitamins; neutralization of toxic compounds such as metal ions.
Growth factors	Modulators of cell growth
Trace elements – including ion, zinc and selenium	Essential for cell growth

Metal transporters – including transferrin and ceruloplasmin	provide transport for metals
Protease inhibitors - including alpha-1-antitrypsin and alpha-2-macroglobulin (both broad spectrum)	Prevent proteolysis of cells and secreted proteins
Attachment factors – including fibronectin, fetuin and laminin	Involved in binding of anchorage dependent cells to the substratum
Polyamines – including putrescin, ornithin and spermidine	Required for cell growth, ion channel modulators
Lipids – including cholesterol, linoleic acid and steroids	Involved in energy production, cell membrane structure, and signalling
Vitamins	Required for essential metabolic reactions

Growth factors are among the most important modulators of cell growth. The potential of these growth factors to promote accelerated growth and prolong culture life (i.e. reduce apoptosis) is of major interest to biopharmaceutical companies. The level of growth factors can vary between different batches of FBS resulting in different growth rates in cell culture. In addition different cell types require different growth factors. This is one of the major reasons each batch of FBS needs to be tested under specific cell culture conditions to determine its performance for that process. An overview of polypeptide growth factors and major target cells is shown in Table 3.

Table 3 Growth factors and their target cells (table extracted from Walsh) [25]

Growth Factor	**Major target cells**
Interleukins	Various, mainly cells mediating immunity and inflammation.
Interferon-γ	Mainly lymphocytes and additional cells mediating immunity and inflammation.
Colony stimulating factors	Mainly haematopoietic stem cells
Erythropoietin	Erythroid precursor cells
Thrombopoietin	Mainly megakaryocytes
Neurotrophic factors	Several, but mainly neuronal cell populations.
Insulin	Various
Insulin-like growth factors	A very wide range of cells found in various tissue types.
Epidermal growth factor	Various, including epithelial and endothelial cells and fibroblasts.
Platelet-derived growth factor	Various, including fibroblasts, glial cells and smooth muscle cells.
Fibroblast growth factors	Various, including fibroblasts, osteoblasts, and vascular endothelial cells
Transforming growth factors -α	Various

Leukemia inhibitory factor	Mainly haematopoietic stem cells.

It is becoming increasingly clear that Fibroblast Growth Factors (FGF) are important for the growth and propagation of a wide range of cell types. FGFs are a family of 20 different proteins (FGF1-20) with mitogenic, chemotactic and angiogenic properies. FGFs are a component of the ECM and are bound to the ECM protein heparin and other ECM glycosaminoglycans. One of the functions of FGF in the ECM is to promote wound repair. FGF-1 and FGF-2 (also termed acidic (a)-FGF and basic (b)-FGF respectively) do not contain a signal sequence for secretion. There are four separate FGF receptors (55-72% homology), and different receptors are found on a variety of cell types. As previously described, the study of FBS performance by Zheng et. al. identified b-FGF in FBS-II but not in FBS-1a or 1b. Furthermore, b-FGF has been found to be an important factor for ES and MSC cell growth, for example.

From the literature which describes known growth factors that have significant effects on the growth of various cells, candidate biomarkers for assessing the quality of FBS resulting from on farm manipulation of bioactives could include IGF-II and associated binding proteins IGFBP2 and IGFBP4, EGF, FGFs and TGF-β. In particular, there seems to be anecdotal evidence that b-FGF plays an important role in the growth of a number of cell types, and consequently could be a lead candidate biomarker for assessing the quality of FBS through on farm manipulation of bioactives.

4.2 FBS quality control

The EMEA “Note for guidance on the use of bovine serum in the manufacture of human biological medicinal products” (Appendix B) discusses and highlights the following:

- the preparation of batches
- what should be covered by the certificate of analysis
- which tests should be performed for adventitious agents (these include tests for: bacterial and fungal sterility; presence of molliculites; presence of general viral contaminants; and presence of specific viruses)
- appropriate tests for toxicity-cell growth assays and viral inactivation requirements.

Manufacturers of biopharmaceuticals require a reputable serum supplier with direct access to sources from approved countries. Suppliers must have comprehensive documentation for each batch of serum and allow audits at every stage of production. Further information on regulatory requirements can be found in Section 7.

Users often test a number of batches of FBS with their specific cell line under their specific culture conditions before selecting which batch to purchase. The supplier will then keep the selected batch (up to 3000L), or a specified volume, in reserve for the particular customer. In general if a batch of FBS is rejected by one customer it will be accepted by a different customer.

5 Effects of medium components on downstream processing in mammalian cell culture

Downstream processing of recombinant protein derived from mammalian cells is defined as all unit operations performed after the termination of fermentation. The aim of downstream processing is to remove contaminants derived from the media and media supplements as well as adventitious agents that derive from the host cell or from the production process. Recovery of recombinant protein biopharmaceuticals requires downstream processing that includes unit processes such as filtration, ultra/diafiltration, various modes of chromatography and viral removal/reduction processes, to yield a high level of purity. Harmful substances such as viruses, nucleic acids, endotoxins and foreign proteins must be reduced to levels specified by regulatory authorities. Processes and procedures employed in the downstream processing of recombinant biopharmaceuticals are shown in Unit Process

	Procedure
Cell Lysis	Mechanical Homogenisation
Clarification	Centrifugation Microfiltration
Protein refolding	Mixed tank
Product concentration	Ultrafiltration Precipitation
Buffer exchange/adjustment	Dialysis Diafiltration or Dilution
Purification	Chromatography Two-phase extraction
Product formulation	Buffer formulation Lyophilisation Packaging

Figure 7.

Unit Process		**Procedure**
Cell Lysis	→	Mechanical Homogenisation
Clarification	→	Centrifugation Microfiltration
Protein refolding	→	Mixed tank
Product concentration	→	Ultrafiltration Precipitation
Buffer exchange/adjustment	→	Dialysis Diafiltration or Dilution

Purification → Chromatography
Two-phase extraction

Product formulation → Buffer formulation
Lyophilisation
Packaging

Figure 7 Unit processes and procedures used in the downstream processing of recombinant protein biopharmaceuticals

All recombinant protein biopharmaceuticals and their production systems are intrinsically different and therefore require the development of specific downstream processes. Downstream processing can be costly, and the product must be recovered at high purity with as few unit processes as possible. Figure 8[26] shows the relationship between concentration of biotechnology-sourced products in the starting material and cost. Biopharmaceuticals are located in the lower regions of the left-right downward trend line and signifies their increased cost largely a result of the extent of downstream processing required to achieve the high level of purity required for therapeutic proteins.

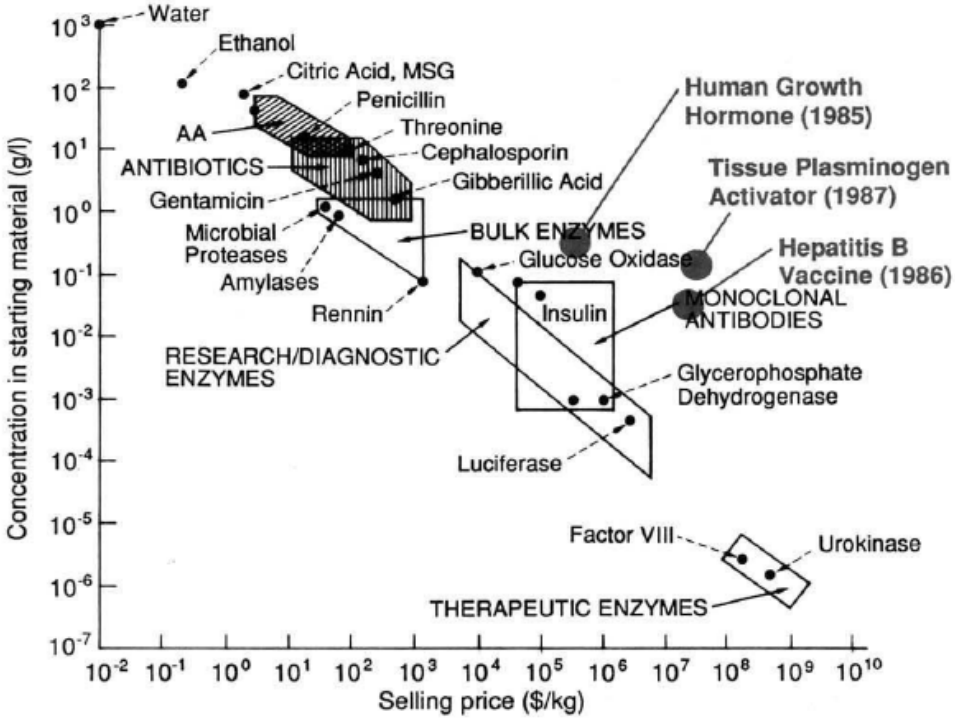


Figure 8 Concentration in starting material (pre-purification) of various biotechnology-sourced substances and their selling prices
(Figure taken from Knight, 1989)[26]

Contaminants are variable in nature and originate from different sources. For example, viral contamination can occur from cell lines that contain endogenous retroviral sequences (ghost particles), use of contaminated reagents or improper handling of the cell line. Therefore it is extremely important to ensure raw materials such as FBS are virus free before use in culture media. Endotoxins are ubiquitous, and it is essential not only to minimise their addition to the culture medium but also to remove them during downstream processing. Table 4 shows some typical impurities in protein pharmaceuticals, their detection and target levels

Table 4 Typical impurities in protein biopharmaceuticals, their detection and target levels.

Impurity	Detection Methods	Target Levels[a]
Endotoxin	Limulus Amoebocyte Lysate Tests (LAL ELISA)	5 Eu/kg/h
Host cell proteins	Host cell protein-specific ELISA ; SDS-PAGE ; identification by mass spectrometry where warranted	Parts per million (ppm)
Media proteins derived from FBS or defined protein additives.	SDS-PAGE; Bovine Ig Elisa; BSA-specific ELISA; identification by mass spectrometry where warranted.	ppm
Monoclonal antibodies used in immunoaffinity and other defined production proteins	SDS-PAGE; antigen-specific ELISA	ppm
DNA	Hybridisation assays; ELISA or DNA binding protein assays.	<10-100 g/dose
Infectious agents	Reverse transcriptase assays; cell culture cytopathic effects (CPE); electron microscopy	Eliminated or inactivated
Product variants[b] – deamidation products; oxidation products; amino acid substitutions. Aggregates Proteolytic products Highly conserved or homologous host cell species proteins	Isoelectric focussing; HPLC; HPLC tryptic mapping; mass spectrometry Gel permeation chromatography SDS-PAGE; HPLC Monoclonal antibody-based immunoassays	Dependent on product complexity and ability to resolve product variants from native product.

[a]Values presented represent the range of established levels or values which have been published for particular r-DNA derived protein biopharmaceuticals
[b]Target levels for variants must be examined on a case-by-case basis for each product. Generally variants >0.5% of total final product should be characterised.

FBS is an undefined media supplement and consists of many proteins. Table 5 shows the twenty most abundant proteins in human plasma, which account for >97-98% of the total protein mass (it is assumed that FBS would have a similar profile of abundant proteins), this group of proteins includes growth factors, hormones and other regulatory proteins.

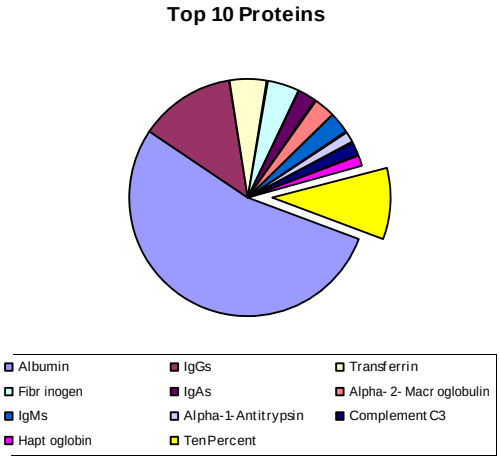


Figure 9 shows that just 10 proteins make up 90% of the total protein mass, with albumin and immunoglobulins making up 70-75% of total protein mass. Although the total number of proteins in serum is probably greater than 1000 when the 20 most abundant proteins are removed there is a mere 1-2% of the total protein mass remaining. The protein concentration range in serum has been estimated to span up to 13 logs.[27]

Table 5 Twenty most abundant proteins in human serum

Albumin	Apolipoprotein A1
IgGs	Apolipoprotein A2
Transferrin	Apolipoprotein b
Fibrinogen	Acid-1-Glycoprotein
IgAs	ceruloplasmin
Alpha-2-Macroglobulin	Complement C4
IgMs	Complement C1q
Alpha 1-Antitrypsin	IgDs
Complement C3	Prealbumin
Haptaglobin	Plasminogen

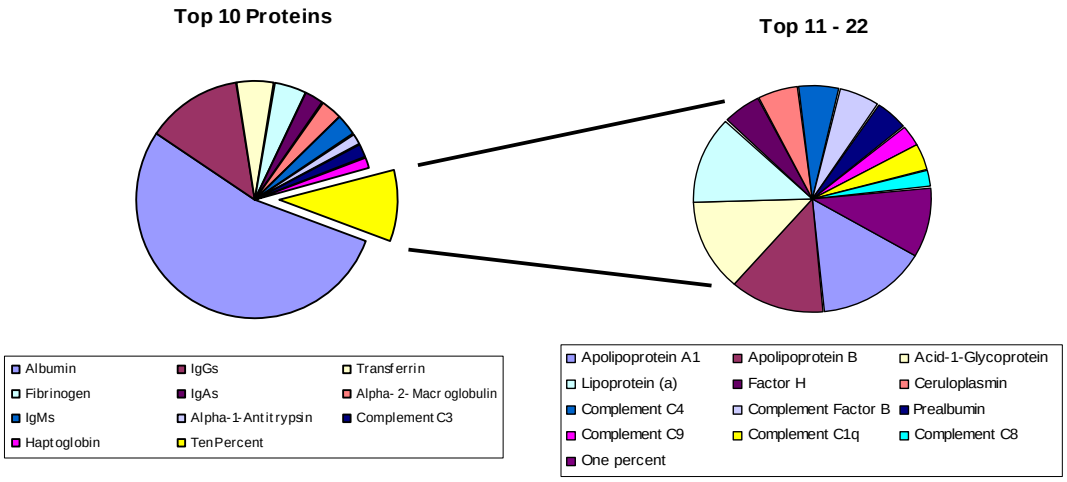


Figure 9 Relative abundance of proteins in human plasma

For therapeutic proteins, there are strict guidelines set down by regulatory bodies regarding the admissible levels of contaminants. Protein contaminants are derived from the cell host (i.e. either secreted by viable cells or released by non-viable cells into the media), or from proteinaceous cell culture supplements. These may be protein supplements such as albumin, transferrin and IGF-1 (defined media) or FBS (undefined media). A concentration of 10% FBS corresponds to 4-8 g/L FBS protein in the supernatant, while host cell protein may be between 0.1-0.4 g/L. As shown in

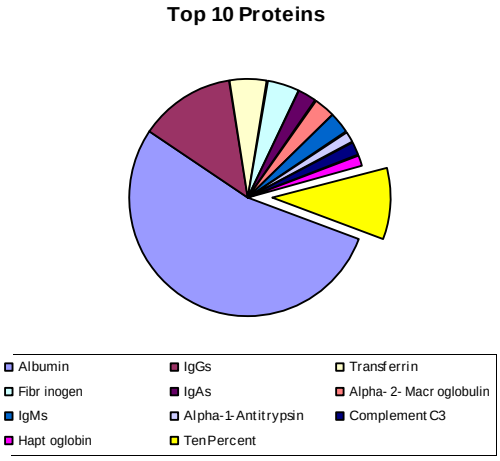


Figure 9, the most abundant protein in serum is albumin, which by nature is a carrier protein and thus non-specifically binds a variety of proteins. Bovine serum albumin (BSA) has a pI of 4.7 and a molecular weight of approximately 65 Da.

Typically, monoclonal antibody manufacturers use a three column process for purifying monoclonal antibodies from mammalian cell culture supernatants that consists of 1. Protein A (or G) affinity chromatography; 2. anion exchange chromatography for DNA and endotoxin removal); 3. Gel filtration chromatography for polishing and sizing (i.e. removal of aggregates). Two orthogonal steps for viral inactivation/removal are also included. For cell culture supernatants containing FBS an initial Protein A or Protein G step may be performed to principally remove albumin, some immunoglobulins and other FBS proteins, and to achieve a high purification factor for the target monoclonal antibody. It is not uncommon to include an initial ion exchange capture step to remove liquid volume prior to Protein A or G affinity chromatography. BSA presents a significant challenge to remove using anion exchange chromatography, as at a pH above 4.7, BSA will bind to the matrix. Alternatively cation exchange may be used as a capture step provided the target protein has a pI significantly above 4.7, whereby albumin will not bind to the matrix (4.7<buffer pH<target protein pI).

Another major contaminant in FBS is the immunoglobulins, which belong to a large group of related glycoproteins that make up approximately 20% of the serum proteins. All immunoglobulins share the basic structure of 2 identical heavy chains joined by disulfide bonds to 2 identical light chains. Both the heavy (H) chains and the light (L) chains are divided into constant and variable regions. The constant regions have similar amino acid composition between all the immunoglobulin classes while the variable regions encompasses about 110 amino acids characterized by a high degree of sequence variability The heavy chain, based on the amino acid sequence, determines the classes of an immunoglobulin. There are 5 types of H-chains that correspond to the following immunoglobulin classes: IgG, IgA, IgM, IgD, and IgE. IgG is further subdivided into 4 subclasses with ~95% homology. There are 2 subclasses of IgA. IgG and IgA exist in serum as monomers consisting of a single 4-polypeptide unit. IgM exists in serum as a pentamer. IgA may also polymerize to form polymers containing 2-5 structural units. Protein A and G bind immunoglobulins from many different species to varying degrees, as shown in Table 6. Bovine immunoglobulins bind more strongly to Protein G compared to Protein A.

Table 6 Binding of immunoglobulins from different species to protein A and G

SPECIES	PROTEIN G	PROTEIN A
Human	++	
IgG1	++	++
IgG2	++	++
IgG3	++	-
IgG4	++	++
Rabbit	++	++
Cow	++	+
Horse	++	-
Goat	++	+
Guinea pig	+	++
Sheep	++	-
Dog	+	++
Pig	++	++
Rat	-	-
Mouse	+	+
Chicken	-	-

++ strong

+ medium binding

- weak

Isoelectric focussing analyses of FBS show bovine immunoglobulins span a range of pI from approximately 4-9. The combination of Protein A and G binding to bovine immunoglobulins, together with the broad range of bovine immunoglobulin pI (which impacts on ion exchange chromatography) presents a challenge to their removal for mAb products.

In summary, the major cost involved in the production of recombinant protein biopharmaceuticals is downstream processing. The greater the number of unit processes, generally the greater the cost. Thus one of the aims of manufacturers is to decrease cost through simplifying downstream processing while maintaining product quality. The use of low protein, serum-free media reduces the protein contaminant load and the complexity of the protein contaminants, simplifying the downstream processing. Although advantages gained in downstream processing through eliminating FBS may be a significant driving force for the trend to defined media for mammalian cell culture, other factors such as securing long term supplies of BSE- (and other pathogen) free FBS are paramount. The risk of fresh outbreaks of BSE and other pathogens in countries that are at present free of these pathogens is perceived as a serious risk by the industry, and is perhaps the primary incentive for biopharmaceutical companies to exclude animal-derived supplements from their mammalian cell production bioprocesses (personal communication, Merck, Genzyme, Amgen).

6 FBS Market

6.1 Introduction

The FBS market is currently split fairly evenly between the research market and the industrial market ($US 150 million each). The research market will become relatively more important as FBS continues to be progressively removed from human and veterinary products. The industrial market includes the production of biologics and diagnostics. The market for diagnostics is relatively small and is not burdened by the same regulatory considerations as those required for biologics. The industrial market requires serum sourced from regions which have the highest safety rating (see section 8 on regulatory issues) whereas the academic market often uses cheaper serum sourced from regions such as Mexico, France, South America, Central America and Canada.

According to the International Serum Industry Association (ISIA) (2006) total FBS use is in excess of 750 000L, while other serum is in excess of 1 000 000L. However FBS accounts for 85% of $350 million dollars worth of serum sold annually. That would amount to 750 000L / $297.5 million or $397/L for FBS and 1 000 000L / $52.5 million or $53/L for ABS.

The Business Communications Co[28] estimates that sera sales in the US were $339 million in 2006 and will grow at a compound annual growth rate of 3.5%. In 2000 they estimated 1999 sera sales to be $174 million[29], amounting to a compound annual growth rate of 10% between 1999 and 2006. It has been estimated that raw FBS is worth $200/L while raw ABS is worth $5-8/L, with sterile FBS $400/L and clarified ABS $10/L (personal communication, Rick Clements, Bovogen).

6.2 Industrial Market

MLA purchased the reference book and website access to BIOPHARMA: Biopharmaceutical Products in the U.S. Market[30]. This database has been used to construct a table listing all the products within the database that claim to use any bovine materials during manufacture and are currently registered and marketed in the US or Europe,

Table 7. Further information on each of these products can be found in Appendix A. The Biopharma product entry number included in

Table 7 and Appendix A tables can be used to cross reference the data in the tables and to find further information about these products on the Biopharma website, www.biopharma.com.

From

Table 7 it can be seen that only a limited number of companies are using FBS in their cell culture. Only 3 companies claim to be using FBS: Merck; Genzyme; and Biomarin. In addition Amgen, Genzyme and Cytogen have products which may be using FBS. Transkaryotic Therapies and Genentech have products in which they use FBS for the construction of the Master Cell Bank (MCB) or Working Cell Bank (WCB) but not for large scale cell culture. Genzyme, Amgen, Curis, J & J, and CSL all have products which are reported to use materials of bovine origin however further clarification is not supplied.

Amgen's erythropoietin (EPO) Epogen, also marketed as Procrit by Ortho/J&J, with a combined 2005 market value of over $5 billion is not reported in the database as using bovine components however it also uses FBS for culture of CHO cells. Amgen is actively trying to remove FBS from all products. There may be other registered products not covered in

Table 7 that use FBS if that information was not covered in the Biopharma database. The products in

Table 7 that definitely use FBS are Cerezyme produced by Genezyme, Aldurazyme produced by Biomarin and 6 vaccines produced by Merck. Genzyme's Cerezyme is produced in cells that are grown to confluence in serum based media then transferred to serum-free media. Cerezyme had a $1billion market in 2006. Biomarin's Aldurazyme had a $94 million market size in 2006 and is produced in 2-3% serum. Total market size for Merck vaccines was not covered in the database however Merck has 2 vaccines that are potential blockbusters (>$1 billion/yr sales), Rotateq and Zostavax. Rotateq is grown in Vero cells and therefore may require only low levels of serum supplementation however Zostavax is produced in MRC-5 human diploid fibroblast cells and is probably supplemented with 10% FBS. The market value of the other 4 vaccines is relatively low. Merck is known to use FBS, newborn calf serum and calf serum for vaccine production (personal communication).

Products that may use FBS include Amgens Enbrel, Genzymes Fabrazyme and Cytogens ProstaScint. Enbrel is produced in CHO cells and was first registered in 1998 and would therefore most likely use FBS. It had a market size of over $4 billion in 2006. Fabrazyme is also produced in CHO cells, was first registered in the EU in 2001 and had a market size of $359 million in 2006. ProstaScint has only a small market, $9.1 million in 2006, and possibly uses FBS for cell expansion but not in the production media.

There are also a number of products reported as using animal origin material for cell culture. These include BMP-7 from Curis/J&J, Aranesp from Amgen, J&Js Remicade and CSLs Streptase. BMP-7 and Aranesp are both produced in CHO cells and were first registered in 2001. Remicade is produced in SP2/0 murine myeloma and was first registered in 1998. Streptase was first registered in 1977. As these products were first registered in 2001 or earlier they are likely to use FBS. Aranesp and Remicade are both blockbuster products with market sizes close to $4 billion.

Table 7 Biopharmaceutical products, registered and marketed in the US or Europe and manufactured with bovine components.
Compiled from www.biopharma.com

Biopharma product entry #	Company	Product	FBS	Unspecified, for cell culture	Calf/newborn serum	FBS, only for MCB and/or WCB	Plasma	BSA	Transferrin	lipoprotein	Thrombin	Tissue digest	Adenosine deaminase	Aprotinin (lung)	Lung surfactant	Pituitary extract	Testes extract	Gelatin/ collagen	CBER/FDA BSE list 2005
167	Genzyme	Cerezyme	Y	Y														Y	
187	Biomarin	Aldurazyme	Y																
491	Merck	Attenuvax	Y															Y	
504	Merck	Mumpsvax	Y															Y	
531	Merck	RotaTeq	Y																
533	Merck	Meruvax II	Y																
564	Merck	Varivax II	Y															Y	
565	Merck	Zostavax	Y															Y	
163	Genzyme	Fabrazyme	Y?	Y?															
267	Amgen	Enbrel	Y?	Y															
312	Cytogen	ProstaScint	Y?					Y	Y										
109	Curis	BMP-7		Y														Y	
143	Amgen	Aranesp		Y															
264	J & J	Remicade		Y															
624	CSL	Streptase		Y														Y	
145	Novo Nordisk	Novoseven			Y														
517	Sanofi Pasteur	IPOL			Y														
520	GlaxoSmithKline	Poliovirus vaccine inactivated			Y														
794	Wellcome	Wellferon			Y?														
162	Transkaryotic Therapies	Replagal			Y?	Y		Y	Y										
652	Advanced Biohealin	Dermagraft																	
942	ONY	Infasurf			Y														
115	Genentech	Rituxin				Y													
920	Mylan Labs	Thrombin-JMI					Y												
117	Genentech	Zevalin				Y													
245	MedImmune	Synagis						Y	Y	Y									
147/ 152	Baxter/Wyeth	Recombinate/ Bioclate						Y											
463	GlaxoSmithKline	Havrix						Y											
464	Merck	VAQTA						Y											
900	Cryolife	BioGlue						Y											
240	J & J	ReoPro									Y								
243	Lonza	Xigris									Y								
923	Angiotech	Vitagel									Y								
562	Crucell	Vivotif Berna										Y							
605	Enzon	Adagen											Y						
713	Talecris Biotherapeutics	Trasylol												Y					
737	Baxter	Tisseel Kit VH												Y					
941	Abbott	Survanta													Y				
651	Organogenesis	Graftskin														Y			

Biopharma product entry #	Company	Product	FBS	Unspecified, for cell culture	Calf/newborn serum	FBS, only for MCB and/or WCB	Plasma	BSA	Transferrin	lipoprotein	Thrombin	Tissue digest	Adenosine deaminase	Aprotinin (lung)	Lung surfactant	Pituitary extract	Testes extract	Gelatin/ collagen	CBER/FDA BSE list 2005
617	Amphastar	Amphadase															Y		
471	Sanofi Pasteur	Fluzone																Y	
489	Sanofi Pasteur	JE-VAX																Y	
659	Integra LifeSciences	Integra Artificial Skin																Y	
529	Chiron	RabAvert																Y	
567	Sanofi Pasteur	Live-YF-Vax																Y	
808	Baxter	FloSeal																Y	
626	Abbott	Abbokinase																Y	
108	Wyeth	INFUSE																Y	
455/ 459	Sanofi Pasteur	ActHIB/ OmniHIB																	Y
507	Sanofi Pasteur	Pertussis toxoid																	Y

6.3 Research Market

The predominant use for FBS at present in the research market is for culture of primary cell lines and culture of both embryonic (ES) and various adult stem cells. Since much work is being carried out on stem cell research, it is appropriate to analyse the current themes in stem cell culture.

The elucidation of a defined media that will support the growth of human embryonic stem (hES) cells in an undifferentiated form will be a major breakthrough in hES cell biology[31]. The development of hES cell-based therapies is contingent on the expansion of ES cells followed by the directed differentiation of cells down specific mesodermal, endodermal and ectodermal differentiation pathways. Although ES cells have traditionally been cultured in FBS-supplemented media, the trend is to move away from the use of animal-derived supplements for the same reasons as outlined in section 3.3.

There are a number of reports in the literature associated with the maintenance of pluripotency and self renewal of ES cells in an undifferentiated state (Appendix C). Growth factors that recur in reports as being important for pluripotency and self renewal include b-FGF, IGF-II, TGF-β, noggin and Activin A[32 33 34 35 36 37]. In a recent study by Ludwig et al.[19] b-FGF was found to be the most important factor, and its removal decreased total cell number and percentage of cells expressing ES cell markers. Caldwell et al.[38] have reported that cells isolated from the embryonic, neonatal, and adult rodent central nervous system divide in response to EGF and b-FGF.

Mesenchymal stem cells (MSC) are receiving much attention due to their relative ease of isolation for an adult stem cell (either from bone marrow or placenta), and due to their demonstrated plasticity. The differentiation capability of MSC into bone (osteoblasts),[39] cartilage (chondrocytes),[40] and cardiac tissue[41] has been reported. Recently it has been shown that bone marrow-derived MSC cultured with BMP-2 and b-FGF were able to enable spinal fusions in rabbits with severed spinal cords[42].

6.4 Contract Manufacturers

The four largest contract manufacturing organisations (CMO's) for mammalian cell culture in order of size are Boehringer Ingelheim, Lonza, Celltrion and Diosynth Biotech. The rapid increase in the number of mAbs being approved over the past 5 years has necessitated a concomitant increase in manufacturing capacity. Whereas cytokines and growth factors are administered in relatively small doses, mAbs for the treatment of cancer, for example, are required in gram quantities per patient. A number of smaller CMOs have recently been established to fill the gap in global manufacturing capacity[43]. CMOs are not only executing biologics production contracts for small start-up biotechnology companies for Phase I and II clinical trials, but are entering into longer term contracts with the established biologics manufacturers such as Genentech. Services extend from manufacturing according to client instructions using an established cell line through to cell line development, bioprocess development and manufacturing. Some CMOs have their own proprietary expression systems such as Lonza Biologics.

Lonza Biologics, with Boehringer Ingelheim, are the two largest CMOs globally. Lonza does not use FBS in any products in any of their facilities (personal communication). They have a total production capacity of 50 000L in Spain and 93 000L in the USA. In addition there is an R & D centre in UK and 4 x 20 000L fermenters under construction in Singapore. Lonza believe they are the leading contract manufacturer of monoclonal antibodies and recombinant proteins

worldwide, and that they are, or will become, the largest CMO worldwide. They claim to have more than 60 customers, including at least half of the top 20 pharmaceutical companies. Using their proprietary Glutamine Synthetase (GS) expression system, production levels have reached an upper limit of 5 g/L.[26]

Boehringer Ingelheim was the largest mammalian cell culture facility in Europe in 2004, with a capacity of 180 000L in Biberach Germany, which is to be expanded in 2007. Amgen and Medimmune are two of their customers. Celltrion in South Korea is the largest CMO in Asia with a production capacity of 50 000L which is currently being increased to 230 000L. The extra 180 000L is due to come on line in 2010. They announced in 2006 that they would provide Bristol-Myers Squibb with 50 000L + 60 000L capacity. Diosynth Biotechnology signed a multiyear contract in June 2003 to produce a commercial product for Pfizer. They have a capacity of 27 000L.

CMOs are independent organisations and enter into contracts with clients for the production of biologics. The CMO has responsibility for regulatory compliance with respect to manufacturing and delivering a product which meets product purity specifications and clears release assays. For these reasons CMOs will select product development and manufacturing pathways that trigger the least regulatory burden. CMOs will therefore not be likely to use FBS-supplemented serum unless specifically instructed by clients. Since CMO clients also endeavour to adopt best practice and minimise regulatory burden, it is therefore unlikely that CMOs would be a significant target for the marketing of FBS.

6.5 FBS suppliers

A list of FBS suppliers and their products has been constructed based on web searches and emails/phone calls to many of the identified providers,

Table 8 FBS suppliers and their products

Company	Website	FBS Use			Bovine sources
		therapeutic	IVD	research	
Australian Suppliers and Major International suppliers					
Sigma Aldrich (CSL, SAFC, JRH, JRS)	www.sigmaaldrich.com	Y	Y	Y	AUS, NZ, US, SA, USDA approved
Moregate biotech	www.**moregatebiotech**.com	Y	Y	Y	AUS
Hyclone (Thermofisher, Irvine Scientific)	www.hyclone.com	Y	Y	Y	AUS, NZ, US, USDA approved
Lonza (Cambrex, BioWhhittaker)	www.lonza.com	Y	Y	Y	AUS (not in catalogue), US, USDA approved
Paa laboratories	www.paa.com	Y	Y	Y	AUS, US, Can, USDA approved
Invitrogen (Gibco, Life Technologies)	www.invitrogen.com	N	Y	Y	US, USDA approved including Aus, NZ, Mexico and Central
Bovogen	www.bovogen.com	pending	Y	Y	Aus, NZ

* Other suppliers					
Animal Technologies	www.animaltechnologies.com	N	N	Y	USDA approved, Central American
Atlanta biologicals	www.atlantabio.com				
BioChemed	www.biochemed.com				US
Biochrom	www.biochrom.de	N	Y	Y	AUS, US, SA
BioWest, LLC	www.biowest.net				Aus, USA, Canada, SA, Central America, Denmark, France
Caissonlabs	www.caissonlabs.com	N	Y	Y	US, USDA approved
Equitech	www.equitech-bio.com	N	N	Y	AUS, US, Mexico
Gemini Bio-Products	www.gembio.com				US, NZ, USDA approved
ICPbio	www.**icpbio**.com	Y	Y	Y	NZ
Kraeber	www.kraeber.de	N			SA
Mediatech, Inc (part of cellgro)	www.cellgro.com	N	N	Y	US, USDA approved
PromoCell	www.PromoCell.com	N			AUS, US quality, EU quality, USDA quality
Quality Biological	www.qualitybiological.com	N	N	Y	US, USDA approved
Sera Labs International, Ltd	www.seralab.co.uk		Y	Y	Aus, NZ, US, EU grade, USDA grade
Valley Biomedical	www.valleybiomedical.com	N	Y	Y	US, ?

* This is not a comprehensive list as there are many small suppliers, including university departments and research institutes.

7 Regulatory

When animal-derived components are used in the biopharmaceutical industry the user must demonstrate that the risk of transmitting adventitious agents has been mitigated. Guidelines for this are found in both the US and European regulations, CFR and EDQM/EMEA respectively. Where the animal derived-component is of ruminant origin, such as FBS, it is necessary to also demonstrate that the risk of transmitting BSE has been mitigated. This is typically addressed by the Note for guidance on minimizing the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products, EMEA/410/01 Rev. 2 (Appendix D). Both US and European regulations recommend removal of animal products whenever possible.

Australian- and New Zealand-origin FBS is recognised as the safest serum available in commercially viable volumes. Generally for industrial purposes serum is sourced from Australia, New Zealand or the USA. For research purposes these sera are often substituted for a cheaper source such as Mexico, France, South America, Central America and Canada.

USDA is proposing to re-open the Canadian border to cattle older than 30 months on November 19 2007. This could have a significant negative impact on the US FBS market as Canada has a

higher BSE risk than the US. Currently only cattle less than 30 months of age can be imported from Canada to the US. BSE is thought not to occur in cattle younger than 30 months. Canada confirmed its tenth case of BSE since 2003 in May 2007.

7.1 United States Department of Agriculture (USDA)

USDA-Grade FBS is produced from raw materials originating only from USA or countries certified to be free of BSE, FMD and Rinderpest.

USDA publishes a list of countries in which BSE exists and a list of countries presenting an undue risk for BSE. USDA also publishes a list of countries free of FMD and Rinderpest. These lists (updated in January 2007) have been attached in Appendix E. USDA grade FBS is produced from serum originating in USA or those countries appearing on the FMD and Rinderpest free list and not appearing on either of the BSE affected lists, Table 9.

Table 9 Countries from which USDA grade FBS can be sourced

Australia	Iceland
Bahama Islands	Jamaica
Barbados	Mexico
Bermuda	Namibia
Belize	New Caledonia
Channel Islands	New Zealand
Chile	Nicaragua
Costa Rica	Panama
Dominican Republic	Panama Canal Zone
El Salvador	Papua New Guinea
Estonia	Territory of St Pierre and Miquelon
Fiji	Tobago
Greenland	Trinidad
Guatemala	Trust Territories of Pacific Islands
Haiti	USA
Honduras	

7.2 European Agency for the Evaluation of Medicinal Products (EMEA) and Therapeutic Goods Administration (TGA) Australia

Australian regulations tend to follow the EU. The TGA has compiled a list of European Union guidelines adopted in Australia, http://www.tga.gov.au/docs/html/euguide/euad_bio.htm. The following two guidelines are of interest for the EU and Australia with respect to the use of FBS for production of biologics:

1. Production and Quality Control of Monoclonal Antibodies Guidelines (pp.237-261 of Rules 1998 (3A) – 3AB3a)

In section 2.2 (pg241) it states: "Bovine sera should be checked and found negative for potentially dangerous viruses (at least bovine diarrhoea virus, infectious bovine rhinotracheitis and parainfluenza 3). In addition, bovine sera and other bovine derived biologicals should comply with the requirements in the Note for Guidance Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products.

2. Note for Guidance Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products (EMEA/410/01 REV 2 (Appendix D))

Section 3.2.1.1 relates to geographical sourcing of bovine materials and states:

"There are currently two organisations involved in the assessment of the BSE status of a specified country or zone. Firstly the Organisation Internationale des Epizooties (OIE) lays down the criteria for the assessment of the status of the countries in the chapter of the International Animal Health Code on BSE. OIE also provides a list of notified BSE cases worldwide. Secondly the European Commission Scientific Steering Committee (SSC) has established a system for classifying the countries according to their BSE risk (GBR) ".

Table 10 describes the GBR levels. In 2003 the responsibility for providing advice to the EU on BSE safety was transferred from the SSC to The European Food Safety Authority (EFSA). The EFSA website (www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178656289049.htm) provides a list of countries and their GBR level of risk (Appendix F). Those countries with GBR I or GBR II are listed in Table 11.

Table 10 Levels of GBR and corresponding prediction of risk

GBR Level	Presence of one or more cattle clinically or pre-clinically infected with the BSE agent in a geographical region/country
I	Highly unlikely
II	Unlikely but not excluded
III	Likely but not confirmed or confirmed, at a lower level
IV	Confirmed, at a higher level

Section 6.3 provides the detail for regulatory requirements for Bovine Blood Derivatives. This sections provides insight into collection of blood from abattoirs and also states that "blood must be sourced from countries classified GBR I and GBR II unless otherwise justified". It is of interest to note that USA is classified GBR III (likely but not confirmed, or confirmed at a lower level) and yet USA sourced serum is still widely used for the production of biopharmaceuticals. Japan is one of the few countries that will not accept products produced with US sourced FBS.

In addition the EMEA published the “Note for guidance on the use of bovine serum in the manufacture of human biological medicinal products” (Appendix B) in 2003 in which it was stated that “efforts should be made to reduce the use of bovine serum or to replace its use with material of non-animal origin”. In 2005 they published the “Revised guideline on requirements and controls applied to bovine serum used in the production of immunological veterinary medicinal products” (Appendix G) in which it was stated “Attempts should be made to reduce the use of bovine serum in the production of immunological veterinary medicinal products and whenever possible the use of non-ruminant materials is preferred”.

Table 11 Countries with BSE risk of GBR I (highly unlikely) or GBR II (unlikely but not excluded) as assessed by EU SSC or EFSA and their USDA status for BSE and FMD/Rinderpest

* I – BSE is highly unlikely, II – BSE is unlikely but not excluded

Country	EU GBR risk* Assessment	USDA BSE risk assessment	USDA FMD and Rinderpest Status
Australia	I	Absent	Absent
Iceland	I	Absent	Absent
New Caledonia	I	Absent	Absent
New Zealand	I	Absent	Absent
Panama	I	Absent	Absent
Argentina	I	Absent	Present
Paraguay	I	Absent	Present
Singapore	I	Absent	Present
Uruguay	I	Absent	Present
Vanuatu	I	Absent	Present
Costa Rica	II	Absent	Absent
El Salvador	II	Absent	Absent
Nicaragua	II	Absent	Absent
Norway	II	Undue risk	Absent
Sweden	II	Undue risk	Absent
Botswana	II	Absent	Present
Brazil	II	Absent	Present
India	II	Absent	Present
Kenya	II	Absent	Present
Mauritius	II	Absent	Present
Namibia	II	Absent	Present
Nigeria	II	Absent	Present
Pakistan	II	Absent	Present
Swaziland	II		Present

From Table 11 it can be seen that only Australia, Iceland, New Caledonia, New Zealand and Panama receive the lowest risk assessment for BSE from the EU and the US and are free of FMD and Rinderpest.

8 Conclusion

Opportunities exist to increase the profitability of the Australian red meat industry by supporting the growth of the bioactives industry by value adding to FBS by on farm manipulation or by increasing FBS market share.

Producing a better quality FBS would require not only a thorough understanding of the potential for on farm manipulation but also extensive research to identify the lot-to-lot variations in key serum proteins and the impact of those variations on biologic production. The lead candidate for further investigation would be b-FGF. Other potential candidates include IGFs and their binding proteins, FGFs, TGFs, PDGF and EGF.

There is a timely opportunity for Australia to increase its FBS market share due to industry fear of US-sourced serum presenting a higher risk for BSE after it opens its border to Canadian cattle on November 19, 2007.

9 Recommendations

- Further research should focus on b-FGF as the lead biomarker candidate for assessing the quality of FBS
- Second line potential biomarker candidates should include IGF-II, IGFBP2, IGFBP4, EGF and TGF-<
- Contact industrial manufacturers, such as Merck, Johnson & Johnson and Genzyme, currently using US-sourced FBS to discuss changing to Australian-sourced FBS.
- Undertake a comprehensive proteomic analysis of FBS using state-of-the-art techniques such as multidimensional protein identification technologies (MuDPIT), which incorporates liquid chromatography interfaced with mass spectrometry. The analysis will identify factors not previously known and may potentially lead to the characterisation of new factors present in serum that are important in cell growth.

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11 Appendices

11.1 Appendix A- Approved Biopharmaceuticals requiring bovine products

Compiled from www.biopharma.com
This table covers products within the biopharma database (updated September 2007) that are listed as using bovine components. There may be some registered products that use FBS or other bovine components that have been omitted if that information was not available in this database.
Ref # refers to the product entry number in the Biopharma database

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
Wyeth	108	Bone Morphogenic Protein-2, rDNA (Dibotermin alfa - INFUSE Bone Graft)	July 2 2002	Medical Device PMA	none	bovine collagen	Spinal fusion and tibia fractures, stimulates new bone formation	CHO	surgically implanted	* column chromatography * ultrafiltration * viral filtration	$308 million 2006
Curis Inc	109	Bone Morphogenic Protein-7, rDNA (eptotermin alpha - Osteogenic Protein-1 - BMP-7)	Jan 17 2001	Medical Device HDE	US for collagen	bovine collagen and unspecified animal-derived materials used for cell culture	Alternative to autograft, stimulates new bone formation	CHO	surgically implanted	* ultrafiltration * viral filtration * column chromatography * < irradiated	
Genentech	115	CD20 Mab, rDNA (Rituximab - Rituxan)	26 Nov 1997, 3 June 1998 in EU	Biologic BLA	none	MCB and WCB are frozen in 95% FCS.	relapsed or refractory low-grade or follicular, CD20 positive, B-cell non-Hodgkin's lymphoma	CHO	IV	* Affinity and ion exchange chromatography * viral inactivation	$3.97 billion 2005
Biogen / Genentech	117	CD20 Mab, rDNA/Y-90 & In-111 radioconj (Zevalin)	19 feb 2002	Biologic BLA		(same Mab as Rituxan, #115 above, that has been radiolabelled)	Patients not responding to Rituxan (see #111)	CHO	IV infusion	* Affinity and ion exchange chromatography * viral inactivation	$30 million 2005

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
Amgen	143	EPO, darb-, rDNA (Darbepoetin alfa - Aranesp)	17 Sep 2001	Biologic BLA		Unspecified materials of animal origin in cell culture	Anemia associated with chronic renal failure	CHO	IV or SC injection	* chromatography * filtration	$4.12 billion 2006 Projected $4.03 billion 2007
Novo Nordisk	145	Factor VIIa, rDNA (Coagulation Factor VIIa (Recombinant) – NovoSeven)	23 Feb 1996 EU 25 Mar 1999 FDA	Biologic BLA	BSE free source	New-born calf serum. 30ng/mg bovine IgG in final product	bleeding episodes in hemophilia	BHK	injection	* ion exchange * immunoaffinity chromatography	$818 million 2005 $993 million 2006
Baxter/Wyeth	147/152	Factor VIII, rDNA/Baxter (Antihemophilic Factor (Recombinant) – Recombinate). Also marketed as Bioclate by ZLB Behring (#152)	10 Dec 1992	Biologic PLA		<1ng BSA/dose	hemophilia A	CHO	IV injection	* ion exchange and size exclusion chromatography * immunoaffinity chromatography	$530 million 2006
Transkaryotic therapies	162	Galactosidase, alfa-, rDNA (Agalsidase alfa – Replagal)	Approved in at least 28 countries worldwide but cannot be approved in US until 2010	Biologic BLA		Bovine calf serum, transferrin and BSA. FBS used for development of the MCB and WCB.	Fabry disease, enzyme replacement therapy	Human fibroblast	IV infusion	* column chromatography * filtration	$95 million 2005, $118 million 2006

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
Genzyme Corp	163	Galactosidase, beta rDNA (Agalsidase beta – Fabrazyme)	24 Mar 2003 FDA 3 Aug 2001 EU	Biologic BLA	Serum from US or NZ	Serum and/or other unspecified bovine-derived media components	Fabry disease, enzyme replacement therapy	CHO	infusion	* chromatography	$305 million 2005 $359 million 2006
Genzyme	167	Glucocerebrosidase, rDNA (Imiglucerase - Cerezyme; beta-Glucocerebrosidase)	23 May 1994	Drug NDA	BSE/TSE free countries	Cells cultured to confluence in serum based media then transferred to serum free media. Unspecified bovine source materials for cell culture. Gelatin as stabilizing agent	Gaucher disease, enzyme replacement therapy	CHO	Infusion	Not stated	$933 million 2005 $1.01 billion 2006
Biomarin	187	Iduronidase, rDNA (laronidase - Aldurazyme)	30 April 2003	Biologic BLA	BSE free	FBS 2-3%	Hurler, Hurler-Scheie and Scheie forms of Mucopolysaccharidosis I (MPS I)	CHO	IV infusion	*chromatography *DNA removal *viral filtration	$ 94million 2006
J & J	240	Platelet Mab, rDNA (Abciximab – ReoPro)	22 Dec 1994	Biologic BLA		Bovine thrombin (it is unclear where/why this is used)	adjunct to percutaneous coronary intervention for the prevention of cardiac ischemic complications. Prevents blot clots	Sp2/0	Bolus then infusion	* viral inactivation and removal * MAb digestion *column chromatography	$363 million 2004 Projected $378 million 2007

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
Lonza	243	Protein C, activated, rDNA (Drotrecogin alfa (activated) – Xigris)	12 Nov 2001	Biologic BLA		Thrombin, used to activate product by cleaving inactive precursor	severe sepsis in adult patients who have an especially high risk of dying	HEK 293	infusion	* filtration * viral inactivation * capture (unspecified)* * back-end purification * TFF * activation * viral filtration * chromatography	$215 million 2005 Projected $365 million 2007
MedImmune	245	RSV MAb, rDNA (Palivizumab – Synagis)	June 19 1998	Biologic BLA	US or Canada, BSE/TSE free	BSA, transferrin and lipoprotein	prevention of serious lower respiratory tract disease	NSO (murine myeloma cell line)	IM injection	* microfiltration * 3 stage chromatography * Acid filtration * nanofiltration * add final buffer (pH 6) * sterile filter (0.22um) * lyophilise	$1.06 billion 2005 $1.07 billion 2006 Projected $1.12billion 2009 but then will be replaced by MEDI-524 Manufactured by MedImmune in Frederick, MD and as CMO by Boehringer Ingelheim in Biberach, Germany

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
J & J	264	TNF Mab, rDNA (Infliximab – Remicade)	24 Aug 1998 FDA	Biologic BLA	BSE/TSE-free countries only	Media contains bovine-derived materials	Crohn's disease, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, ulcerative colitis, inflammatory bowel disease, Behcet's disease	SP2/0	IV infusion	* affinity and ion exchange chromatographt * solvent-detergent viral inactivation * nano- or ultrafiltration	$3.76 billion 2005
Amgen	267	TNF Receptor-lgG Fc,rDNA (Etanercept-Enbrel)	2 Nov 1998	Biologic BLA	BSE/TSE-free countries only	Media contains bovine-derived materials	Rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, plaque psoriasis	CHO	SC injection	*chromatography *ultrafiltration *viral inactivation *viral filtration	$4.47 billion
Cytogen Corp	312	Prostate Mab radioconj. (Capromab Pendetide – ProstaScint)	28 Oct 1996	Biologic BLA		Transferrin and BSA. FBS is possibly used in cell expansion media but is not used in production media	radiodiagnostic imaging of prostatic tissues	Hybridoma	IV infusion	* filtration, clarification and concentration * chromatography *sterile filtration	$7.4 million 2005 $9.1 million 2006

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
Sanofi Pasteur	455/ 459	Haemophilus b Vaccine (PRP-T) (Haemophilus b Conjugate Vaccine (Tetanus Toxoid Conjugate) – ActHIB) Also marketed as OmniHIB (#459) by GlaxoSmithKline	March 1993	Biologic PLA	On the 2005 CBER/FDA list "of vaccines using bovine-derived materials from countries on the USDA's BSE list."	None stated	Prevention of HiB disease				
GlaxoSmithKline	463	Hepatitis A Virus Vaccine/GSK (Hepatitis A Virus Vaccine, Inactivated – Havrix)	22 Feb 1995, originally approved in Switzerland 1991	Biologic PLA		< 1 ng of bovine serum albumin/pediatric dose In 2005 it was removed from CBER/FDA "Current list of vaccines using bovine-derived materials from countries on the USDA's BSE list."	Prevent Hepatitis A	MRC-5 human diploid fibroblasts	IM	* sterile filtration * ultrafiltration * column chromatography * inactivated with formalin	$371 million 1997

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
Merck	464	Hepatitis A Virus Vaccine/Merck (Hepatitis A Virus Vaccine, Inactivated – VAQTA)	29 Mar 1996	Biologic PLA		BSA	Prevent Hepatitis A	MRC-5 human diploid fibroblasts	IM	* HPLC * inactivated with formalin	
Sanofi Pasteur	471	Influenza Vaccine/Sanofi (Influenza Virus Vaccine – Fluzone)	16 Sep 1947	Biologic PLA		Gelatin 0.5% as stabilizer	immunization against influenza disease caused by influenza virus types A and B	Chick embryos	injection		Supplies about half the US market. Supplied 90% of the US public sector influenza vaccine market for 1005/06 season
Sanofi Pasteur	489	Japanese Encephalitis Virus Vaccine (Japanese Encephalitis Virus Vaccine Inactivated - JE-VAX)	10 Dec 1992	Biologic PLA		Each dose contains 500ug gelatin	active immunization against Japanese encephalitis virus	infected mouse brains	SC injection		
Merck	491	Measles Virus Vaccine (Measles Virus Vaccine Live – ATTENUVAX)	21 Mar 1963 Generally replaced by M-M-R II	Biologic PLA		Each dose contains 10mg bovine gelatin and <1ppm FBS.	Prevent measles virus infection and disease	Chick embryos	SC injection		Generally replaced by M-M-R II (entry # 493)

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
Merck	504	Mumps Virus Vaccine (Mumps Virus Vaccine Live – Mumpsvax)	28 Dec 1967, not approved by EU but approved in some EU countrie	Biologic PLA		Bovine gelatin, 14.5mg/dose, and FBS <1ppm/dose	Prevent mumps	Chicken embryo	SC		Generally replaced by M-M-R II (entry # 493)
Sanofi Pasteur	507	Pertussis Vaccine, acellular, conc./BIKEN (Acellular Pertussis Vaccine Concentrate (For Further Manufacturing Use) - pertussis toxoid)	20 Aug 1992 Used in DTaP/dTap vaccines: Tripedia	Biologic PLA	on 2005 CBER/FDA "Current list of vaccines using bovine-derived materials from countries on the USDA's BSE list."		Prevent whooping cough				$304 million 1999, for acellular pertussis vaccines in various combinations
Sanofi Pasteur	517	Poliovirus Vaccine (Poliovirus Vaccine Inactivated (Monkey Kidney Cell) - IPOL	21 Dec 1990	Biologic PLA	BSE/TSE free source	Newborn calf serum <1ppm in final dose, only used in initial culture (not used in large scale culture).	Prevention of poliomyelitis	Vero	SC	* clarification * filtration * chromatography * heat inactivation	$118 million 1999

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
GlaxoSmithKline	520	Poliovirus Vaccine (IPV)/GSK (Poliovirus Vaccine Inactivated)	13 Dec 2002	FDA approved only as component of Pediarix #441). Marketed separately or in combination in Europe	USDA-designated BSE free countries	Calf serum	Prevention of poliomyelitis	Vero		* clarifiacation * ultrafiltration * daifiltration * chromatography * formaldehyde viral inactivation	
Chiron	529	Rabies Vaccine/Chiron (Rabies Vaccine, Inactivated – RabAvert)	20 Oct 1997	Biologic PLA	Countries free of BSE	Polygeline (processed bovine gelatin) in culture medium, <12mg/dose	Pre- and post-exposure prophylaxis against rabies	Chicken embryo fibroblasts	IM injection	* viral inactivation * centrifugation	
Merck	531	Rotavirus Vaccine, rDNA/Merck (Rotavirus Vaccine, Quintavalent – RotaTeq)	3 Feb 2006	Biologic BLA		FBS Contains trace amounts of FBS in final formulation	Prevention of rotavirus	Vero	oral		

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
Merck	533	Rubella Virus Vaccine (Rubella Virus Vaccine Live - Meruvax II)	1979 for Meruvax II, Meruvax was approved on 9 June 1969	Biologic PLA		FBS, <1ppm in final dose	Prevent German measles	Human diploid lung fibroblasts	SC injection	* ultrafiltration * beta-propiolactone inactivation	Generally replaced by M-M-R II (entry # 431)
Crucell	562	Typhoid Vaccine/Berna (Typhoid Vaccine Live Oral Ty21a - Vivotif Berna)	15 Dec 1989	Biologic PLA		Bovine tissue digest in the culture medium	Protection against typhoid fever	Bacterial strain Ty21 a	oral		$16 million 2006 projected
Merck	564	Varicella Virus Vaccine (Varicella Virus Vaccine Live (Oka/Merck) - Varivax II)	17 Mar 1995	Biologic PLA		Bovine gelatin 8.9mg/dose, Trace quantities of FBS	Protection against chicken pox	MRC-5 Human diploid fibroblasts	SC		
Merck	565	Varicella Virus Vaccine/adult (Zoster vaccine live (Oka/Merck) – Zostavax)	25 May 2006 FDA and EU	Biologic BLA		15.58mg hydrolyzed gelatin/dose (presumably bovine, less likely porcine), Trace quantities of FBS	prevention of herpes zoster (shingles) in individuals 60 years of age and older	WI-38 Human diploid cell culture	SC		
Sanofi Pasteur	567	Yellow Fever Vaccine/Sanofi (Yellow Fever Virus Vaccine, Live - YF-VAX)	22 May 1953	Biologic PLA		Gelatin added as stabiliser	Prevent yellow fever	Chicken embryos	SC injection		

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
Enzon	605	Adenosine Deaminase, PEG-(Pegademase Bovine – Adagen)	21 Mar 1990	Drug NDA	Bse free countries	Adenosine deaminase (ADA) purified from calf intestine	Enzyme replacement therapy for ADA deficiency-type severe combined immunodeficiency (SCID)	NA	injection		$20 million in 2005 $25 million 2006
Amphastar Pharmaceutivals	617	Hyaluronidase, bovine/Amphastar (Hyaluronidase injection – Amphadase)	26 Oct 2004	Drug NDA biogeneric		Extracted from bovine testes	As an adjuvant to increase the absorption and dispersion of other injected drugs	NA	injected		
CSL	624	Streptokinase/Astra (Streptokinase – Streptase)	18 Aug 1977	Biologic PLA		Media from bovine source, gelatin	acute myocardial infarction	Beta-hemolytic streptococci bacteri a	IV	* precipitation * chromatography * adsorption	Reducing, being replaced by more advanced recombinant thrombolytic products
Abbott	626	Urokinase/Abbott (Abbokinase; Urokinase For Injection; Abbokinase Open-Cath)	16 Jan 1978	Drug NDA		Gelatin (does not state where/how this is used)	pulmonary embolism	neonatal human kidney cells (HNK)	intravenous or intracoronary infusion		2002 report expected $360 million by 2005. Now largely replaced by newer thrombolytic agents

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
Organogenisis	651	Skin, Cultured/Apligraf (Apligraf – Graftskin)	22 May 1998	Medical device PMA	Countries (US) free from BSE	Bovine collagen, bovine pituitary extract	non-infected partial and full-thickness skin ulcers and diabetic foot ulcers	Cultured from neonatal foreskin, fibroblasts and keratinocytes	Engrafted		
Advances Biohealing Inc.	652	Skin, Cultures/Demagraft (interactive Wound Dressing – Desmagraft)	28 Sep 2001	Medical device PMA	Countries (US) free from BSE	Bovine calf serum	Full thickness diabetic foot	Cultured from neonatal foreskin	Engrafted		
Integra LifeSciences	659	Skin/Integra (Integra Bilayer Matrix Wound Dressing)	1 Mar 1996	Medical device PMA		Bovine collagen	bilayer membrane sheets for temporary skin replacement. Integra BMWD for wounds and ulcers. Integra DRT for burns and scar contracture	NA			
Talecris Biotherapeutics	713	Aprotinin (Aprotinin Injection – Trasylol)	29 Dec 1993	Drug NDA		Aprotinin – derived from bovine lung tissue	prophylactic use to reduce perioperative blood loss	NA	Injection		$259 million 2005, $195 million 2006, market decreasing due to safer and cheaper alternatives

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
Baxter	737	Fibrin Sealant/Baxter (Two-Component Fibrin Sealant, Vapor Heated - Tisseel Kit VH)	1 May 1998	Biologic BLA		Aprotinin (#713)	as an adjunct to hemostasis in surgeries	NA	Applied topically		Projected $400 million annually
Wellcome	794	Interferon alfa-n1 (Interferon alfa-n1, Lymphoblastoid – Wellferon)	25 Mar 1999. Voluntarily withdrawn 1999, revoked 2000. Received UK approval in Mar 1986	Biologic PLA		Calf or horse serum	hairy cell leukemia and chronic hepatitis B	Namalva cell line (human leukocyte/lymphoblastoid cell line)		* immunoaffinity chromatography	Still manufactured and marketed in Japan as Sumiferon by Sumitomo Chemical Co.
Baxter	808	Thrombin-Collagen Gel (Gelatin Matrix Hemostatic Sealant - FloSeal Matrix Hemostatic Sealant – FloSeal)	29 Mar 2005	Medical device PMA	US	collagen	For surgical procedures as an adjunct to hemostasis when control of bleeding by ligature or conventional procedures is ineffective or impractical	NA	Extruded from syringe onto bleeding area		

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
Cryolife	900	Bovine Albumin Glue (CryoLife BioGlue Surgical Adhesive - BioGlue Surgical Adhesive)	Dec 1999	Medical device HDE and PMA	BSE free countries	BSA	help seal leaks around sutures (surgical stitches) or staples in large blood vessels such as the aorta or the femoral and carotid arteries.	NA	Sprayed on bleeding wounds		
Mylan Labs	920	Thrombin/GenTrac (Thrombin - Thrombin, Topical (Bovine Origin); Thrombin-JMI)	3 Feb 1986	Biologic PLA	USDA inspected and approved abbatoirs	Bovine plasma	an aid to hemostasis whenever oozing blood and minor bleeding from capillaries and small venules is accessible	NA	Spray on, or apply with sponge, or apply dry powder directly	During manufacture, bovine-derived prothrombin is fractionated from bovine plasma then activated by tissue thromboplastin (bovine) in the presence of calcium chloride and purified chromatographically.	$221 million 2005 $250 million 2006
Angiotech	923	Thrombin/Collagen/Plasma (Vitagel Surgical Hemostat)	13 Jun 2000. EU approval Sept 1998	Medical device PMA	US	Thrombin (#920) and collagen	Helps control bleeding	NA	Spray on		$7.5 million expected for 2006
Abbott	941	Beractant (Beractant Intratracheal Suspension – Survanta)	1 Jul 1991	Drug NDA		Bovine lung surfactant	prevention and treatment ("rescue") of Respiratory Distress Syndrome (RDS) (hyaline membrane disease) in premature infants	NA	intratracheal instillation	Organic solvent-extracted post-mortem bovine lung surfactants containing phospholipids, neutral lipids, fatty acids and surfactant-associated proteins	

Company	Ref #	Product	Date 1st approved	FDA class	Bovine source	Bovine components	Indication	Host Cell line	Administration	Purification	Market
ONY	942	Calfactant (Calfactant - Infasurf Intratracheal Suspension)	2 Jul 1998	Drug NDA	US	Newborn calves	prevention of Respiratory Distress Syndrome (RDS) in premature infants at high risk for RDS and for the treatment ("rescue") of premature infants who develop RDS	NA	intratracheal instillation	organic solvent extract of bovine lung surfactant obtained by postmortem cell-free bronchoalveolar lavage (washing) of the lungs of calves followed by solvent-extraction purification	

HDE - humanitarian device exemption
PMA - pre-market approval
NDA – new drug applications
BLA – biologics license application

MRC-5 –human diploid lung fibroblast
CHO – chinese hamster ovary
BHK – baby hamster kidney
SP2/0 – a transformed murine myeloma cell line
NSO – a murine myeloma cell line
VERO – African green monkey kidney cells
AGMK – African green monkey kidney
HEK 293 – a human embryonic kidney cell line
HNK human neonatal kidney cells

MCB – master cell bank
WCB – working cell bank

Approved Multivalent Vaccines requiring bovine products

Compiled from www.biopharma.com
This table covers multivalent vaccines within the biopharma database (updated September 07) that include a component vaccine which requires bovine material fro manufacture.

Company	Ref	Product	Date 1st approved	FDA class	Includes product #	Bovine components	Indication	Administration	Market
Sanofi Pasteur	431	DTaP Vaccine/Sanofi USA (Diphtheria & Tetanus Toxoids & Acellular Pertussis Vaccine Adsorbed - Tripedia)	20 Aug 1992	Biologic PLA	Diptheria and Tetanus Toxoid (#424). Acellular Pertussis vaccine concentrate (#507)	Gelatin (presumed bovine)from Diptheria and tetanus toxoid. Gelatin is presumed to have negligible risk for contamination with BSE/TSE. Due to Acellular Pertussis component it was listed in 2005 by CBER/FDA as being on the "Current list of vaccines using bovine-derived materials from countries on the USDA's BSE list."	Prevention of diptheria, tetanus and pertussis (whooping cough)		
GlaxoSmith Kline	439	DTaP-HBV, rDNA-Hib-IPV/GSK (Combined Diphtheria, Tetanus, acellular Pertussis, Hepatitis B, inactivated Poliovirus and Haemophilus influenzae type b vaccine - Infanrix Hexa)	23 OCT 2000 only in Europe		Trivalent Infanrix (DTaP) (#359), Inactivated poliovirus (IPV) (#520), Engerix-B (recombinant HBsAg vaccine) (#175), OmniHIB (Haemophilus b conjugate vaccine (tetanus toxoid conjugate)) (#459)		Prevention of diphtheria, tetanus, whooping cough, hepatitis B, poliomyelitis and disease caused by Haemophilus influenzae type b		

Company	Ref	Product	Date 1st approved	FDA class	Includes product #	Bovine components	Indication	Administration	Market
Glaxo S mith Kline	441	DTaP-HBV, rDNA-IPV/GSK (Diphtheria and Tetanus Toxoids and Acellular Pertussis Adsorbed, Hepatitis B (Recombinant) and Inactivated Poliovirus Vaccine Combined – Pediarix)	13 Dev 2002	Biologic BLA	Infanrix (DTaP vaccine (#433); itself a 3-component vaccine), Engerix-B (hepatitis B virus vaccine) (#175), and Poliovirus Vaccine (IPV)/GSK (not previously approved in the U.S.) (#520).	Calf serum	Prevention of diphtheria, tetanus, whooping cough,Hepatitis B and polio		
Sanofi Pasteur	443	DTaP-Hib Vaccine/Sanofi (Diphtheria & Tetanus Toxoids & Acellular Pertussis Vaccine Adsorbed plus Haemophilus influenzae type b (Hib) vaccine - TriHiBit; combination of Tripedia and ActHIB or OmniHIB; DTaP-Hib)	27 Sep 1996. Not approved in EU	Biologic	Tripdedia (#431), ActHIB (#455) or OmniHIB (#459)	uses bovine-derived materials from countries on the USDA's BSE list due to ActHIB and OmniHIB	Prevention of H. influenzae type b and/or diphtheria, tetanus and whooping cough		

Company	Ref	Product	Date 1st approved	FDA class	Includes product #	Bovine components	Indication	Administration	Market
Sanofi Pasteur	445	DTaP-Hib-Polio Vaccine/Sanofi (Diphtheria & Tetanus Toxoids & Acellular Pertussis Vaccine Adsorbed plus Haemophilus influenzae type b (Hib) vaccine plus Poliovirus Vaccine Inactivated (Human Diploid Cell) – Pentacel)	BLA pending, Marketed in Canada since 1998, also marketed in 8 other countries	Biologic BLA	Tripedia (#431), Poliovirus Vaccine Inactivated (#518), ActiHIB (#455) or OmniHIB (#459)	Bovine serum approx 50ng/dose Contains Tripedia and ActiHIB/OmniHIB which use bovine-derived materials from countries on the USDA's BSE list.	Prevention of Haemophilus influenzae type b, diphtheria, tetanus, whooping cough and poliomyelitis in a single injection at a single visit	IM	
Glaxo S mith Kline	466	Hepatitis A & B, rDNA Vaccine (Hepatitis A Inactivated & Hepatitis B (Recombinant) Vaccine – Twinrix)	11 May 2001	Biologic BLA	Hep A Havrix (# 463) and HepB Engerix (#175)		Prevention of Hep A and Hep B, probably also Hep D	IM	
Sanofi Pasteur	467	Hepatitis A & Typhoid vaccine (Viatim; Vivaxim; hepatitis A virus, inactivated plus Salmonella typhi purified Vi polysaccharide)	No FDA or EU approval. It is sold into individual EU countries		Avaxim (# 465), TYPHIM (#561)	<10ng BSA / dose	Prevention of hepatitis A and Typhoid	IM	

Company	Ref	Product	Date 1st approved	FDA class	Includes product #	Bovine components	Indication	Administration	Market
Merck	492	Measles & Mumps Virus Vaccine (Measles and Mumps Virus Vaccine Live - M-M-Vax)	18 Jul 1973	Biologic PLA	Attenuvax (#491), Mumpsvax (#504)	<14.5mg gelatin/dose, <1ppm FBS/dose	Prevention of measles and mumps		Generally replaced by M-M-R 11 (#493) or ProQuad (# 494) vaccines
Merck	493	Measles Mumps & Rubella Vaccine [Measles, Mumps and Rubella Virus Vaccine Live - M-M-R II vaccine	22 Apr 1971	Biologic PLA	Attenuvax (#491), Meruvax II (#533) and Mumpsvax (#504)	<1ppm FBS/dose, <14mg gelatin/dose	Prevention of measles, mumps and rubella		Not available
Merck	494	Measles Mumps Rubella & Varicella Vaccine (Measles, Mumps, Rubella and Varicella (Oka/Merck) Virus Vaccine Live – ProQuad)	2005	Biologic PLA	M-M-R 11 (#493), Varivax (#564)	<1ppm FBS/dose, <14mg gelatin/dose	Prevention of measles, mumps, rubella and chickenpox		
Merck	496	Measles & Rubella Virus Vaccine/Merck (Measles and Rubella Virus Vaccine Live - M-R-Vax II)	22 Apr 1971	Biologic PLA	Attenuvax (#491), Meruvax II (#533)	<14.5mg gelatin/dose, <1ppm FBS/dose	Prevention of measles and rubella		Generally replaced by M-M-R 11 (#493) or ProQuad (#494) vaccines

11.2 Appendix B Pluripotency Factor Summary

Reference and abstract for each article at bottom of document.

Protein and Concentration	Culture Method	Cell Lines	Culture period	Ref	KSR ?	Comments
Chemically defined media (CDM), standard hES media in DMEM-F12 (no whole serum but has BSA fraction V) with a) N2 supplements (1mM hTransferrin, 8.61uM hInsulin, 1mM progesterone, 1mM putrescine, 1mM selenite) or b) N2 supplements and B27 supplements (d-biotin, BSA, catalase, L-carnitine, HC1, corticosterone, ethanolamine HC1, D- galactose, glutathione (reduced), insulin, linoleic acid, progesterone, putrescine, sodium selenite, SOD, T3/Albumin complex, DL-a-tocopherol, DL-a-tocopherol acetate and transferrin). Both media supp with 2Ong/mL bFGF.	Feeder free on matrigel with dispase digestion	H1 and HSF6	17 passages over 5 months for N2-CDM. 27 passages over 5.5 months for B27/N2CDM	(Yao, Chen et al. 2006)	No	Karyotype normal (at p8 and 22). Teratomas done (p7 and 9).
bFGF (40ng/mL plus SR)	On matrigel in unconditioned hES cell media. Collagenase	H7 and H9	15+ passages	(Xu, Roster et al. 2005)	Yes	Cytogenetic analysis done at 15 passages but only on one cell line H7. They say cell density is critical for maintaining hESC.
Activin A (5ng/mL in non-CM)	Enzyme digestion using collagenase or trypsin.	H1 and 16 and 111T (aneuploid)	20 passages	(Xiao, Yuan et al. 2006)	Y e s	Found activin A induces Oct-4, nanog, nodal, Wnt 3, bFGF and FGF8 while suppressing BMP signals. Not too sure on their conclusions in the microarray. A lot of conflicting data. Teratomas at

Protein and Concentration	Culture Method	Cell Lines	Culture period	Ref	KSR?	Comments
						p10. Found Act A up-regulation in hES in non CM: presumed Act A expression inhibitors present in CM. Get Dravid article from here. Dravid states Wnt not sufficient for hES cell maintenance.
Activin (50ng/mL), KGF or FGF7 (SOng/mL) and 10mM nicotinamide.	Feeder free on laminin (2Oug/mL) using collagenase N.	HSF6	>20 passages	(Beattie, Lopez et al. 2005)	Yes	KGF affects proliferation. MC has an anti-apoptotic effect. Act A and TGF-B act through the same SMAD pathway (Smad2/3). BMPs a different one (Smad 1/4/5). Karyotype done but not stated when.
Activin (25ng/mL) in non-CM.	Feeder free on matrigel.	BGN1 BGN2 H1	4 days	(James, Levine et al. 2005)	Yes	They show TGFB/Act/Nodal acts through Smad2/3. Also show SMAD 2/3 activation is required for Wnt activation. Activin supported cells seemed to have a very different morphology to those in CM or those supported by activation of Wnt pathway by BIO. Karyotype normal but no clue as to when conducted. Cell apparently at high passage.
bFGF (40ng/mL) and Noggin (50Ong/mL)	Feeder free on matrigel in standard hES media with KSR	H1	7 passages	(Wang, Zhang et al. 2005)	Yes	NIH/3T3 cells failed to support hES. 3T3 cells transfected with Noggin did though. Noggin alone did not support hES for more than 2 weeks. Karyo done after only 5 passages.
Sphingosine-1 (10uM) and PDGF (20ng/mL)	A standard stem cell media	hES-2/3/4	hES-2 (16 passages), hES-	(Pebay, Wong et	No	Karyotyping hES-3 (p58), hES-2 (p6), hES-4 (p8). Teratomas at p6

Protein and Concentration	Culture Method	Cell Lines	Culture period	Ref	KSR ?	Comments
	without any serum. Feeder free on matrigel was conducted for short periods but was mostly on feeders for extended culture.		3 (83) and hES-4 (11)	al. 2005)		(hES3) and p4 (hES4).
HESCO contains 4 ng/ml bFGF, 16Oug/m1 insulin, 88 ug/ml transferrin, 100 ng/ml Wnt3a, 100 ng/ml April or BAFF, 2.5 mg/ml albumin, and 2.5x cholesterol lipid supplement.	On fibronectin in hE SCO medium. Passaged with collagenase or 0.0025% trypsm.	H9 and BG01	6 months (H9) and 2 months (BG01).	(Lu, Hou et al. 2006)	No but used prior to transfer	Cells were shifted from media with KSR. CD30 may be present? Karyo done at 6 months (H9) and 2 months (BG01).
Wnt. Used a GSK-3 inhibitor BIO (at 2uM and 5uM).	Feeder free using non-conditioned standard hES cell media.	HE BGN1, BGN2	3-7 days	(Sato, Meijer et al 2004) .	Yes	Wnt signalling inactivates GSK-3 which allows B-catenin to accumulate in the nucleus thus activating transcription of Wnt target genes. BIO blocks GSK-3. Karyo normal.
Activin/NodaFTGF-B he composition of CDM was 50% IMDM plus 50% F12 NUT-MIX, supplemented with 7 ug/ml of insulin, 15 ug/ml of transferrin, 450 uM of monothioglycerol and 5 mg/ml bovine serum albumin fraction V and Activin (lOng/mL) and bFGF (12ng/mL).	Feeder and serum free experiments for 10 weeks done without matrigel. A number of other experiments done with feeder or on matrigel.	H9, hSF-6	10 weeks in the absence of serum, feeders or matrigel. (Bullshit they had BSA)	(Vanier, Alexander et al. 2005)	No but used in cultures prior.	Cells were cultured prior to experiments using MEFs, collagenase digestion and KSR. There is some contradictory evidence about whether TGF-B supports hES or not. Karyotype done before experiments began?
IGF-II at 30 ng/mL in defined media (standard hES media).	Matrigel with collagenase IV digestion.	H9, HE hES3, CAl	IGF-II sustained hES undiff for 12	(Bendall, Stewart et al.	Yes.	IGF-II is produced by hES cell derived fibroblasts in response to bFGF. IGF-II has a direct role in

Protein and Concentration	Culture Method	Cell Lines	Culture period	Ref	KSR ?	Comments
See also Wang below			passages.	2007)		survival and self-renewal of hESC. Blocking IGFR impeded cell expansion (caused by increased apoptosis), blocking FGFR1 increased differentiation. Karyo mentioned in methods but nothing in results.
DC-HAIF consisted of DMEM/F12 (Invitrogen), 2% fatty acid-free Cohn's fraction V BSA (Serologicals), lx Nonessential amino acids, 50 U/ml Penicillin, 50 ug/m1 Streptomycin, 50 µg/ml Ascorbic Acid, 10 µg/ml bovine or human Transferrin, 0.1 mM VMercaptoethanol (all from Invitrogen), lx Trace Elements A, B & C (Mediatech), 10 ng/ml heregulin (Peprotech), 10 ng/ml Activin A (R&D Systems), 200 ng/ml LR3-IGF1 (JRH Biosciences), and 8 ng/ml FGF2 (Sigma or R&D Systems).	Dig Digested with disn On iase ' matrigel in MEF CM or non-CM on MEFs. For DC-HALF they used matrigel GF depleted.	HE BG01/2/3 , CyT49	collage 7-9 months for BG01 and 02. CyT49 for 3 months	(Wang, Schulz et al. 2007)	Yes but only on cultures prior to DC-HALF	Heregulin is a ligand for receptors ERB2/3 (EGFR family members). LR^3-IGF1 is an IGF-1 analogue (it is a recombinant IGF-1 that is not bound by IGFBPs). Blocking ERB2 inhibited proliferation and induced apoptosis. Karyotype of BG01 at p18 and BG02 at p26. Teratoma at 25 passages.

Cell Survival and Proliferation

Protein and Concentration	Culture Method	Cell Lines	Culture	Ref	KSR used?	Comments
KGF — See Beattie 2005 above						
Neurotrophins (NT3, NT4 and brain derived neurotrophic factor [BDNF] at 5Ong/mL)	On matrigel and on MEFs. Passaged by 0.05% trypsin.	H1 and H9	Clones derived in media with NTs then grown for 20 passages.	(Pyle, Lock et al. 2006)	Yes	NTs also increased proliferation. Possibly due to suppression of Apoptosis.
ROCK inhibitor Y-27632 (10uM)	Feeder layer and feeder free on Matrigel.	KhES-1/2/3		(Watanabe, Ueno et al. 2007)	Yes	ROCK = Rho associated kinase. Y-2 treatment improved cloning efficiency on MEF and matrigel but not collagen coated dishes.

Beattie, G. M., A. D. Lopez, et al. (2005). "Activin A maintains pluripotency of human embryonic stem cells in the absence of feeder layers." Stem Cells 23(4): 489-95.

To date, all human embryonic stem cells (hESCs) available for research require unidentified soluble factors secreted from feeder layers to maintain the undifferentiated state and pluripotency. Activation of STAT3 by leukemia inhibitory factor is required to maintain "stemness" in mouse embryonic stem cells, but not in hESCs, suggesting the existence of alternate signaling pathways for self-renewal and pluripotency in human cells. Here we show that activin A is secreted by mouse embryonic feeder layers (mEFs) and that culture medium enriched with activin A is capable of maintaining hESCs in the undifferentiated state for >20 passages without the need for feeder layers, conditioned medium from mEFs, or STAT3 activation. hESCs retained both normal karyotype and markers of undifferentiated cells, including Oct-4, nanog, and TRA-1-60 and remained pluripotent, as shown by the in vivo formation of teratomas.

Bendall, S. C., M. H. Stewart, et al. (2007). "IGF and FGF cooperatively establish the regulatory stem cell niche of pluripotent human cells in vitro." Nature 448(7157): 1015-21.

Distinctive properties of stem cells are not autonomously achieved, and recent evidence points to a level of external control from the microenvironment. Here, we demonstrate that self-renewal and pluripotent properties of human embryonic stem (ES) cells depend on a dynamic interplay between human ES cells and autologously derived human ES cell fibroblast-like cells (hdFs). Human ES cells and hdFs are uniquely defined by insulin-like growth factor (IGF)- and fibroblast growth factor (FGF)-dependence. IGF 1 receptor (IGF1R) expression was exclusive to the human ES cells, whereas FGF receptor 1 (FGFR1) expression was restricted to surrounding hdFs. Blocking the IGFII/IGF1R pathway reduced survival and clonogenicity of human ES cells, whereas inhibition of the FGF pathway indirectly caused differentiation. IGF-II is expressed by hdFs in response to FGF, and alone was sufficient in maintaining human ES cell cultures. Our study demonstrates a direct role of the IGF-II/IGF1R axis on human ES cell physiology and establishes that hdFs produced by human ES cells themselves define the stem cell niche of pluripotent human stem cells.

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is required downstream of WNT signaling, which we have previously shown to be sufficient to maintain the undifferentiated state of hESCs. Strikingly, we show that in ex vivo mouse blastocyst cultures, SMAD2/3 signaling is also required to maintain the inner cell mass (from which stem cells are derived). These data reveal a crucial role for TGFbeta signaling in the earliest stages of cell fate determination and demonstrate an interconnection between TGFbeta and WNT signaling in these contexts.

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Xu, C., E. Rosler, et al. (2005). "Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium." Stem Cells 23(3): 315-23.
Previous studies have shown that prolonged propagation of undifferentiated human embryonic stem cells (hESCs) requires conditioned medium from mouse embryonic feeders (MEF-CM) as well as matrix components. Because hESCs express growth factor receptors, including those for basic fibroblast growth factor (bFGF), stem cell factor (SCF), and fetal liver tyrosine kinase-3 ligand (F1t3L), we evaluated these and other growth factors for their ability to maintain undifferentiated hESCs in the absence of conditioned medium. We found cultures maintained in bFGF alone or in combination with other factors showed characteristics similar to MEF-CM control cultures, including morphology, surface marker and transcription factor expression, telomerase activity, differentiation, and karyotypic stability. In contrast, cells in media containing Flt-3L, thrombopoietin, and SCF, individually or in combination, showed almost complete differentiation after 6 weeks in culture. These data demonstrate that hESCs can be maintained in nonconditioned medium using growth factors.

Yao, S., S. Chen, et al. (2006). "Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions." Proc Natl Acad Sci U S A 103(18): 6907-12.
Chemically defined medium (CDM) conditions for controlling human embryonic stem cell (hESC) fate will not only facilitate the practical application of hESCs in research and therapy but also provide an excellent system for studying the molecular mechanisms underlying self-renewal and differentiation, without the multiple unknown and variable factors associated with feeder cells and serum. Here we report a simple CDM that supports efficient self-renewal of hESCs grown on a Matrigel-coated surface

over multiple passages. Expanded hESCs under such conditions maintain expression of multiple hESC-specific markers, retain the characteristic hESC morphology, possess a normal karyotype in vitro, as well as develop teratomas in vivo. Additionally, several growth factors were found to selectively induce monolayer differentiation of hESC cultures toward neural, definitive endoderm/pancreatic and early cardiac muscle cells, respectively, in our CDM conditions. Therefore, this CDM condition provides a basic platform for further characterization of hESC self-renewal and directed differentiation, as well as the development of novel therapies.

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pluripotency is poorly understood. To identify the signaling pathway molecules that maintain the proliferation of hESCs, we performed a microarray analysis comparing an aneuploid H1 hESC line (named HIT) versus euploid H1 hESC line because the H1T hESC line demonstrates a self-renewal advantage while maintaining pluripotency. We find differential gene expression for the Nodal/Activin, fibroblast growth factor (FGF), Wnt, and Hedgehog (Hh) signaling pathways in the H1T line, which implicates each of these molecules in maintaining the undifferentiated state, whereas the bone morphogenic protein (BMP) and Notch pathways could promote hESCs differentiation. Experimentally, we find that Activin A is necessary and sufficient for the maintenance of self-renewal and pluripotency of hESCs and supports long-term feeder and serum-free growth of hESCs. We show that Activin A induces the expression of Oct4, Nanog, Nodal, Wnt3, basic FGF, and FGF8 and suppresses the BMP signal. Our data indicates Activin A as a key regulator in maintenance of the stemness in hESCs. This finding will help elucidate the complex signaling network that maintains the hESC phenotype and function.

Xu, C., E. Rosler, et al. (2005). "Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium." Stem Cells 23(3): 315-23.

Previous studies have shown that prolonged propagation of undifferentiated human embryonic stem cells (hESCs) requires conditioned medium from mouse embryonic feeders (MEF-CM) as well as matrix components. Because hESCs express growth factor receptors, including those for basic fibroblast growth factor (bFGF), stem cell factor (SCF), and fetal liver tyrosine kinase-3 ligand (F1t3L), we evaluated these and other growth factors for their ability to maintain undifferentiated hESCs in the absence of conditioned medium. We found cultures maintained in bFGF alone or in combination with other factors showed characteristics similar to MEF-CM control cultures, including morphology, surface marker and transcription factor expression, telomerase activity, differentiation, and karyotypic stability. In contrast, cells in media containing Flt-3L, thrombopoietin, and SCF, individually or in combination, showed almost complete differentiation after 6 weeks in culture. These data demonstrate that hESCs can be maintained in nonconditioned medium using growth factors.

Yao, S., S. Chen, et al. (2006). "Long-term self-renewal and directed differentiation of human embryonic stem cells in chemically defined conditions." Proc Natl Acad Sci USA 10308): 6907-12.

Chemically defined medium (CDM) conditions for controlling human embryonic stem cell (hESC) fate will not only facilitate the practical application of hESCs in research and therapy but also provide an excellent system for studying the molecular mechanisms underlying self-renewal and differentiation, without the multiple unknown and variable factors associated with feeder cells and serum. Here we report a simple CDM that supports efficient self-renewal of hESCs grown on a Matrigel-coated surface over multiple passages. Expanded hESCs under such conditions maintain expression of multiple hESCspecific markers, retain the characteristic hESC morphology, possess a normal karyotype in vitro, as well as develop teratomas in vivo. Additionally, several growth factors were found to selectively induce monolayer differentiation of hESC cultures toward neural, definitive endoderm/pancreatic and early cardiac muscle cells, respectively, in our CDM conditions. Therefore, this CDM condition provides a basic platform for further characterization of hESC self-renewal and directed differentiation, as well as the development of novel therapies.

11.3 USDA lists of affected Countries for BSE, FMD and Rinderpest

Countries/Regions Affected with Bovine Spongiform Encephalopathy (BSE) Countries in which BSE exists 9 CFR 94.18(a)(1)

Austria	Japan
Belgium	Liechtenstein
Czech Republic	Luxembourg
Denmark	Netherlands
Finland	Oman
France	Poland
Germany	Portugal
Greece	Slovakia
Ireland (Republic of)	Slovenia
Israel	Spain
Italy	Switzerland
United Kingdom [includes Great Britain (England, Scotland, Wales, Isle of Man), Ireland, Northern Ireland, and the Falklands]	

Countries presenting an undue risk for BSE 94.18 (a)(2)*

Albania	Former Yugoslav Republic of Macedonia
Andorra	Monaco
Bosnia-Herzegovina	Norway
Bulgaria	Romania
Croatia	San Marino
Federal Republic of Yugoslavia	Sweden
Hungary	

*Import requirements less restrictive than those that would be acceptable for import into the United States and/or because of inadequate surveillance, present an undue risk of introducing bovine spongiform encephalopathy into the United States.

Countries Considered Minimal-Risk with Regard for BSE

94.18(a)(3)
Canada

Countries with Indigenous BSE Cases that May Export Whole Cuts of Boneless Beef.

9 CFR 94.27
Japan

Countries/Regions Free of Foot-And-Mouth Disease (FMD) and Rinderpest

9 CFR 94.1

*Austria	Iceland
Australia	*Northern Ireland
*Bahama Islands	*Republic of Ireland
Barbados	*Italy
*Belgium	Jamaica
Bermuda	*Japan
Belize (British Honduras)	*Luxembourg
Canada	Mexico
*Channel Islands	*Namibia (Region South of the Veterinary Cordon Fence)
*Chile	*Netherlands
Costa Rica	*New Caledonia
*Czech Republic	New Zealand
*Denmark	Nicaragua
Dominican Republic	*Norway
El Salvador	Panama
*Estonia	Panama Canal Zone
Fiji	*Papua New Guinea
*Finland	*Poland
*France	*Portugal
*Germany	*Spain
*Great Britain (England, Scotland, Wales, Isle of Man)	*Sweden
*Greece	*Switzerland
Greenland	Territory of St. Pierre and Miquelon
Guatemala	Tobago
Haiti	Trinidad
Honduras	Trust Territories of the Pacific islands
*Hungary	

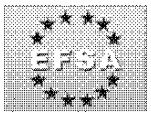
***Special categories CFR 94.11 (meat imports/land borders)**

***SPECIAL CATEGORY REGARDING RINDERPEST AND FMD BECAUSE, EVEN THOUGH THE COUNTRY/AREA HAS BEEN DETERMINED BY THE USDA TO BE FREE OF RINDERPEST AND FMD, ONE OR MORE OF THE FOLLOWING CONDITIONS OCCUR:**

(1) They supplement their national meat supply through the importation of fresh, chilled, or frozen meat of ruminants or swine from countries/regions that are NOT designated in Title 9, CFR, Part 94.1(a) (hereafter known as The Regulations) as free of rinderpest or FMD; or
(2) They have a common land border with countries/regions that are NOT designated in The Regulations as free of rinderpest or FMD; or
(3) They import ruminants or swine from countries/regions that are NOT designated in The Regulations as free of rinderpest or FMD under conditions less restrictive than would be acceptable for importation into the United States

Last Modified: January 21, 2007

11.4 APPENDIX D Geographical BSE risk assessment



European Food Safety Authority

TSE/BSE Risk Assessment Team of the SCIENTIFIC PANEL ON BIOLOGICAL HAZARDS

Geographical BSE Risk (GBR) assessments covering 2000-2006

List of countries and their GBR level of risk as assessed by the Scientific Steering Committee and the European Food Safety Authority (EFSA)[1]

This document covers an overview of the period 2000 to 2006 for the published results on BSE risk assessments carried out in all EU Member States and a number of third countries. All assessments of third countries are on the basis of a request submitted by the third country to the European Commission with the aim of establishing a trade relation on bovines and bovine products. The method used to assess the likelihood of BSE being present in a country is the Geographical BSE Risk Assessment (GBR). The GBR methodology assessment was developed by the Scientific Steering Committee (SSC) of the European Commission (EC) in 1998 (SSC, 23 January 1998) and formally adopted on 18 February 1999 (revised 23 April 1999). The method was further refined with the SSC opinions adopted 6 July 2000, 11 January and 7 November 2002.

All opinions and appended GBR reports from the SSC are published at the webs te of the European Commission: http://ec.europa.eu/food/fs/sc/ssc/outcome_en.html

In 2003, responsibility for carrying out the GBR assessments was transferred from the SSC to EFSA. Two mandates were received in order to re-assess a total of 19 countries (Argentina, Australia, Botswana, Brazil, Canada, Chile, Costa Rica, El Salvador, Namibia, New Zeeland, Nicaragua, Norway, Mexico, Panama, Paraguay, Swaziland, Sweden, USA, and Uruguay) and EFSA added a GBR assessment (Rep. of South Africa) under a self-tasking mandate.

The outcome of the assessments by the EFSA Scientific Expert Group on GBR can be found at: http://www.efsa.europa.eu/science/tse_assessments/catindex_en.html

The table in annex provides an overview on the outcome of all GBR assessment conducted by the former Scientific Steering Committee and by EFSA.
Countries highlighted indicate a country where positives cases have been detected by surveillance and laboratory diagnosis*.

[1] The (former) Scientific Steering Committee of the European Commission provided till 2003 scientific advice to the EC supporting legislation in the area of TSE and BSE. This responsibility was handed over to the European Food Safety Authority in 2003, providing from then onwards, independent, scientific advice in all areas of food and feed safety including TSE, supporting the EC legislation.

European Food Safety Authority

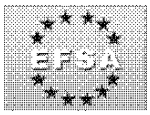
TSE/BSE Risk Assessment Team of the SCIENTIFIC PANEL ON BIOLOGICAL HAZARDS

Annex• Table with countries and GBR assessment and level of risk.

Levels of GBR and corresponding prediction of risk:

GBR level	Presence of one or more cattle clinically or pre- clinically infected with the **BSE** agent in a geographical region/country
I	Highly unlikely
II	Unlikely but not excluded
III	Likely but not confirmed or confirmed, at a lower level
IV	Confirmed, at a higher level

	Country name	Outcome and Year of Publication		
		Assessment by the SSC		Assessment by EFSA
1.	**Andorra**	III (12/2002)		
2.	**Albania**	III (03/2001)		
3.	**Argentina**	I (07/2000)	I (04/2003)	I (06/2005)
4.	**Australia**	I (07/2000)		I (07/2004)
5.	**Austria**	II (07/2000)	III (05/2002)	
6.	**Belarus**	III (04/2003)		
7.	**Belgium**	III (07/2000)		
8.	**Botswana**	I (02/2001)		II (02/2005)
9.	**Brazil**	I (03/2001)	I (04/2003)	II (06/2005)
10.	**Bulgaria**	III (06/2002)		
11.	**Canada**	II (07/2000)		III (07/2004)
12.	**Chile**	I (07/2000)	I (04/2003)	III (06/2005)
13.	**Colombia**	II (03/2001)		
14.	**Costa Rica**	I (05/2001)	I (04/2003)	II (02/2005)
15.	**Croatia**	III (06/2002)		



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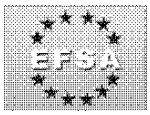
N°	Country name	Outcome and Year of Publication		
		Assessment the SSC		Assessment by EFSA
16.	Cyprus	III (03/2001)	III (04/2003)	
17.	Czech Republic	III (03/2001)		
18.	Denmark	III (07/2000)		
19.	El Salvador	I (06/2001)		II (02/2005)
20.	Estonia	III (03/2001)	III (04/2003)	
21.	Finland	II (07/2000)	III (05/2002)	
22.	Former Yugoslavian Republic of	III (04/2003)		
23.	France	III (07/2000)		
24.	Germany	III (07/2000)		
25.	Greece	III (12/2002)		
26.	Hungary	III (03/2001)		
27.	Iceland	I (06/2002)		
28.	India	II (03/2001)		
29.	Ireland	III (07/2000)		
30.	Israel	III (09/2002)		
31.	Italy	III (07/2000)		
32.	Kenya	II (05/2001)		
33.	Latvia	III (06/2002)		
34.	Lithuania	III (02/2001)	III (04/2003)	
35.	Luxembourg	III (07/2000)		
36.	Malta	III (09/2002)		



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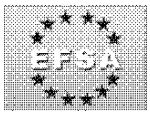
N°	Countryname'	Outcome and Year of Publication		
		Assessment by the SSC		Assessment by EFSA
37.	Mauritius	II (03/2001)		
38.	Namibia	I (02/2001)		II (02/2005)
39.	Mexico			III (07/2004)
40.	Netherlands	III (07/2000)		
41.	New Caledonia	I (03/2003)		
42.	New Zealand	I (07/2000)	I (11/2002)	I (05/2005)
43.	Nicaragua	I (02/2001)		II (02/2005)
44.	Nigeria	II (06/2001)		
45.	Norway	I (07/2000)		II (07/2004)
46.	Pakistan	II (03/2001)		
47.	Panama	I (06/2001)		I (06/2005)
48.	Paraguay	I (07/2000)	I (04/2003)	I (06/2005)
49.	Poland	III (03/2001)		
50.	Portugal	IV (07/2000)		
51.	Republic of South Africa			III (07/2004)
52.	Romania	III (05/2001)		
53.	San Marino	III (06/2002)		
54.	Singapore	I (03/2001)	I (03/2003)	
55.	Slovak Republic	III (03/2001)		
56.	Slovenia	III (05/2001)	III (09/2002)	
57.	Spain	III (07/2000)		
58.	Swaziland	I (02/2001)		II (02/2005)



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N°	Countryname'	Outcome and Year of Publication		
		Assessment by the SSC		Assessment by EFSA
59.	Sweden	II (07/2000)		II (07/2004)
60.	Switzerland	III (02/2001)		
61.	Turkey	III (06/2002)		
62.	United Kingdom	IV (07/2000)		
63.	Uruguay	I (01/2001)	I (04/2003)	I (06/2005)
64.	USA	II (07/2000)		III (07/2004)
65.	Vanuatu	I (06/2002)		

Date : 01.08.2006

11.5 Appendix E– EMEA Guidelines

European Medicines Agency
Veterinary Medicines and Inspections

London, 9 November 2005
Doc. Ref. EMEA/CVMP/743/00-Rev.2

COMMITTEE FOR MEDICINAL PRODUCTS FOR VETERINARY USE

(CVMP)

REVISED GUIDELINE ON REQUIREMENTS AND CONTROLS APPLIED TO BOVINE SERUM USED IN THE PRODUCTION OF IMMUNOLOGICAL VETERINARY MEDICINAL PRODUCTS

ADOPTED BY CVMP	10 October 2001
REVISED DRAFT AGREED BY IWP	June 2004
ADOPTION BY CVMP FOR RELEASE FOR CONSULTATION	14 June 2004
END OF CONSULTATION (DEADLINE FOR COMMENTS)	15 January 2005
AGREED BY IWP	June 2005
ADOPTION BY CVMP	13 July 2005
REVISION ADOPTED BY CVMP	9 November 2005
DATE FOR COMING INTO EFFECT	1 January 2006

This guideline replaces the revised guideline on Requirements and Controls applied to Bovine Serum (Foetal or Calf) used in the production of Immunological Veterinary Medicinal Products (EME A/CVMP/743/00-Rev. 1)

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REVISED GUIDELINE ON REQUIREMENTS AND CONTROLS APPLIED TO BOVINE SERUM USED IN THE PRODUCTION OF IMMUNOLOGICAL VETERINARY MEDICINAL PRODUCTS

TABLE OF CONTENTS

EXECUTIVE SUMMARY

This Guideline outlines the tests and inactivation treatments, which should be applied to bovine serum to ensure acceptable quality and to minimise the risks of transmitting infectious diseases. It should be noted that this Guideline is advisory by nature and should be read in conjunction with the requirements for substances of animal origin in the European Pharmacopoeia and relevant EU guidelines.

1. INTRODUCTION (BACKGROUND)

Starting materials of animal origin are necessary for the production of immunological veterinary medicinal products (IVMPs). Attempts should be made to reduce the use of bovine serum in the production of IVMPs and whenever possible the use of non-ruminant materials is preferred.

It is however recognised that materials of animal origin, including bovine serum are still essential ingredients of the cell culture media used in the production of many IVMPs. Different risks are associated with the use of such starting materials. Indeed, the nature and quality of the bovine serum used in the manufacturing process can profoundly influence the quality of the finished product. In consequence, it is strongly recommended that a risk analysis, taking into account the quality and properties of the serum batches and the impact of these sera on the quality of the finished product be conducted by the vaccine manufacturers prior to use. This should result in a number of batches of bovine serum being selected for the production of different types of vaccines.

2. SCOPE

The presence of extraneous agents in bovine serum certainly represents a major risk to the quality of the finished product. Therefore this Guideline will focus mainly on the risk due to extraneous agents Testing before and after inactivation may be carried out by the serum supplier, by the manufacturer, by a contract laboratory or by more than one of these. This Guideline is not intended to prescribe which parties should carry out the testing but rather to define the testing which should be done at each stage of processing and the relevant quality standards that apply. It is recognised that not all serum suppliers, contract laboratories or manufacturers will necessarily possess the expertise and facilities required to perform all of the testing specified. The tests should be carried out in accordance with GMP, GLP or ISO 9001 principles; it is the responsibility of the manufacturer to ensure that the testing is carried out to the required standard. It is therefore strongly recommended that the testing is performed by the manufacturers themselves. Where this is not practical, testing can be devolved to the serum supplier or a contract laboratory; but responsibility for providing the necessary data to demonstrate compliance with the relevant quality standards rests with the manufacturer. It is essential that all available information regarding the quality of the serum be known by the vaccine manufacturer to enable the manufacturer to conduct a risk assessment on the use of the serum. It is essential that PIMP manufacturers manage the potential risk posed by the use of bovine serum by using selected batches of bovine serum for the production of different types of vaccines. The selection of these serum batches must be based on a risk analysis taking into consideration the biological properties of the serum and the intended use of the wmp.

Considering the risk of pestiviruses in bovine serum, the highest risk will be with live and inactivated vaccines indicated for use in pestiviruses susceptible species (cattle, small ruminants and pigs). Of these, the greatest risk is associated with the use of live vaccines in pregnant pestiviruses susceptible females. Manufacturers should take into account that the production of the vaccine virus in pestivirus vaccines may be influenced by interfering BVD antibodies in the bovine serum used for production.

3. LEGAL BASIS

This Guideline concerns the application of Title II of Annex I to Directive 2001/82/EC as amended with a view to addressing the data requirements that need to be fulfilled for each new batch of bovine serum used in the manufacture of immunological veterinary medicinal products.

4. MAIN GUIDELINE TEXT

4.1. SOURCE

There are a range of bovine sera used by veterinary IVMP manufacturers (see also definitions):

- Adult Bovine Serum
- Calf Serum (under 12 months)
- New-Born Calf Serum (under 20 days)
- Foetal Bovine Serum
- Donor Bovine Serum (up to 36 months)

4.2. PREPARATION OF BATCHES

Products of animal origin should be prepared in a homogeneous manner, designated with a batch number.

4.3. ASSAYS AND CONTROLS TO BE CARRIED OUT EITHER BY THE VACCINE MANUFACTURER OR UNDER THEIR RESPONSIBILITY.

A number of samples from each batch of serum should be used for the following tests.

4.3.1. Bacterial and fungal sterility tests

The serum batch complies with the requirements of the tests for sterility of the European Pharmacopoeia Monograph. It is recommended to carry out these tests after filtration of the batch and before inactivation.

4.3.2. Tests for the presence of mollicutes

The serum batch complies with the requirements of the tests for the presence of mollicutes as described in the European Pharmacopoeia Monograph (tests for Mycoplasma) applied to the culture method. It is recommended to carry out these tests after filtration of the batch and before inactivation.

4.3.3. Tests for the presence of viral contaminants

4.3.3.1. General and Specific Tests

The combination of general and specific tests to be carried out should be capable of detecting viruses inducing viraemia and transplacental infection such as Bovine Adenovirus, BVD Virus (see below), Parvovirus, Bovine Respiratory Syncytial Virus, Reovirus, Parainfluenza 3, IBR and those responsible for diseases exotic to Europe (such as Bluetongue). Methods such as PCR and RT-PCR can usefully be used to increase the probability of detecting viral contaminants. If such tests are used they should be demonstrated to have a sensitivity and specificity at least equivalent to the conventional tests. In addition, the Manufacturer should be able to clarify whether or not any nucleic acid detected originates from infectious particles.

4.3.3.2. Tests to detect Bovine Viral Diarrhoea virus

These tests should be carried out, a first time, before the inactivation treatment to assess the infectious titre of Bovine Viral Diarrhoea Virus potentially present to ensure it is below the level that has been shown to be effectively inactivated in the validation tests for inactivation treatment. Secondary tests should be performed after the inactivation treatment at which time no virus should be detected in the final serum batch. These tests could be omitted if no virus is detected before inactivation treatment.

The serum to be tested for virus isolation is incorporated in a nutritive medium used for cultivation of bovine cells sensitive to Pestiviruses. After 3 passages of the cells inoculated, an immuno-cytochemical technique is applied to the cells with a reference serum monospecific (polyclonal or a pool of monoclonal antibodies) for BVD virus. It is recommended to perform such an immuno-cytochemical technique on microplates to increase the number of clones of cells, which are observed in the wells. If virus is isolated from the serum tested, it has to be titrated directly from the bovine serum batch to ensure it is below the level that has been shown to be effectively inactivated in the validation tests for inactivation treatment.

In addition to the tests recommended in this Guideline, other validated methods such as PCR and RTPCR can usefully be used to increase the probability of detecting viral contaminants. If such tests are used they should be demonstrated to have a sensitivity and specificity at least equivalent to the conventional tests. In addition, the Manufacturer should be able to clarify whether or not any nucleic acid detected originates from infectious particles.

Control cells are used for each test, cultivated with a bovine serum controlled and inactivated.

4.3.3.3. Tests to detect BVD antibodies

The samples are tested using a validated technique to detect BVD antibodies. The tests to detect BVD antibodies are important to be carried out to perform a pertinent risk assessment in regard to the potential impact of the presence of BVD antibodies in bovine serum on partial or complete neutralisation of possible virus present and on the validation process.

4.4. INACTIVATION TREATMENT

Due to the risk associated with the use of bovine serum in contaminating the finished tvmp, it is absolutely necessary, in addition to controls performed on each batch of serum, to inactivate the serum by validated and efficacious treatments for increased reduction of potential undetectable organisms. For validation, the rationale of the choice of the viral strains must be indicated, including representatives of different viral families (enveloped or naked viruses, DNA

or RNA viruses) and representatives of viruses with different degrees of resistance to various types of treatments. The following viruses may be used for the validation of the inactivation procedure: BVD virus, IBR virus, one of the bovine enteroviruses, bovine adenovirus, one of the Reoviruses (REO) and Porcine Parvovirus (PPV). A check for pestiviruses must be included.

The titration of the chosen viruses should be carried out (before inactivation treatment) after incubation at 37°C for 1 hour with the serum which will be submitted to the inactivation treatment. It is recommended to use gamma radiation as a means of obtaining a safe but biologically active product. In consequence, the validation study has to determine the consistency and efficiency of the process while maintaining the product performance.

For nactivation by irradiation, the validation study has to

1. determine the optimal temperature,
2. establish a standard packaging configuration,
3. establish a representative distribution of dosimeters capable of assessing the effective dose reached in the mass of the product whatever its position during the treatment,
4. set specific time limits in relation to the dose received,
5. determine the minimum and maximum radiation exposure or dose received by the product itself and
6. establish a radiation dose range that protects product integrity while maximising inactivation of microbial contaminants. The validation study must therefore demonstrate the actual dose received throughout the mass of the serum. Inactivation of the bottled serum batch with a minimum guaranteed dose of 30 kiloGray (kGy) is an efficacious treatment to inactivate most micro-organisms and/or viruses present (30 kGy is equivalent to 3 Mrad).

For inactivation by means other than the application of minimum of 30 kGy to the serum in each bottle, the validation studies undertaken must be suitable to demonstrate the extent to which the process to be applied is appropriate, effective and reproducible.

DEFINITIONS

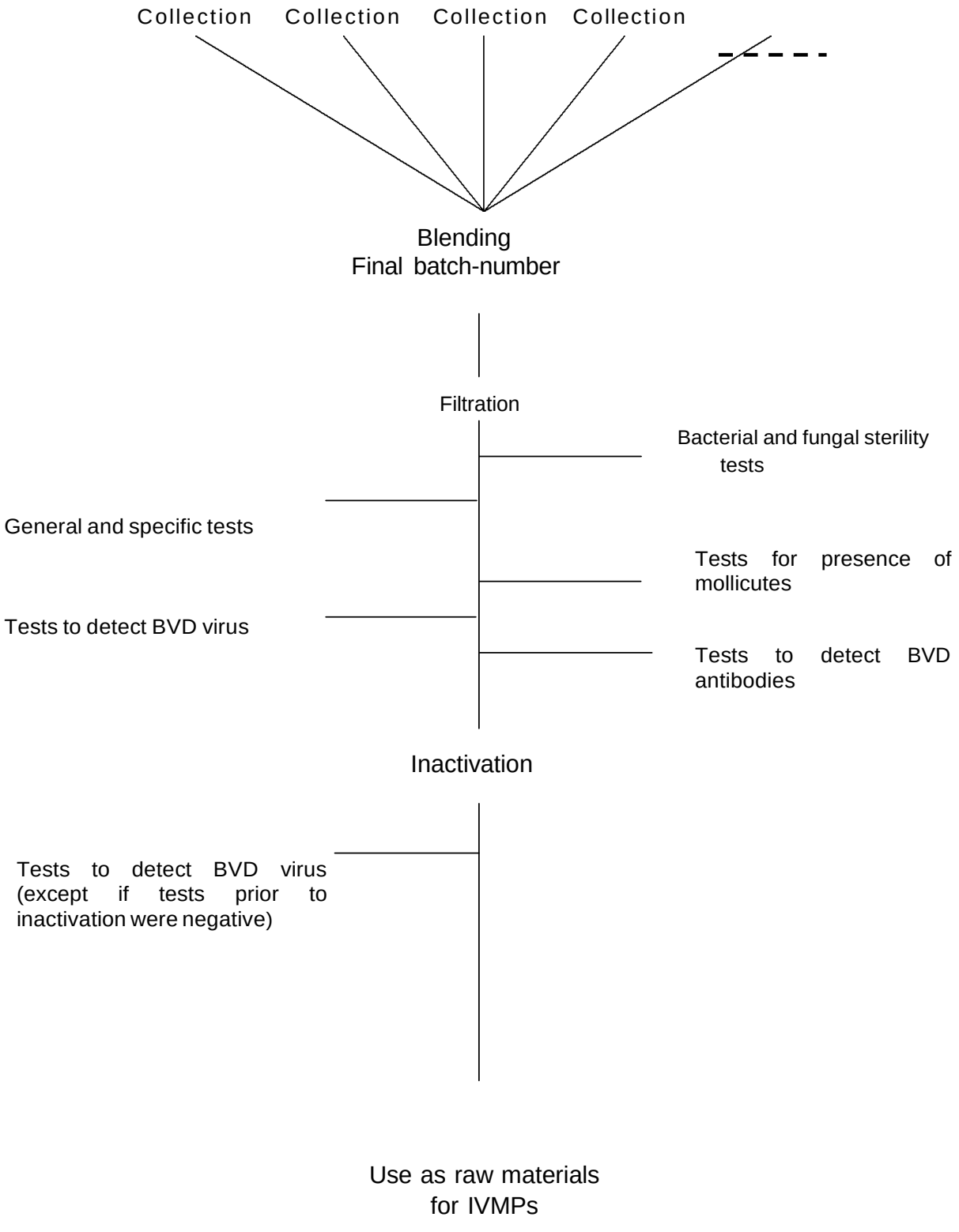
Adult Bovine Serum: bovine serum is derived from bovine blood collected post mortem from cattle that are declared fit for slaughter and for human consumption.
Calf Serum (under 12 months): produced in a similar way to Adult Bovine Serum.
New-Born Calf Serum (under 20 days).
Foetal Bovine Serum: obtained from foetuses from cattle declared fit for slaughter and for human consumption.
Donor Bovine Serum (up to 36 months): this is produced by repeat bleeding of donor animals from controlled standing herds. It is recommended that these herds are not vaccinated against BVD

REFERENCES

EMEA CPMP Note for Guidance on the use of bovine serum in the manufacture of human biological medicinal products (CPMP/BWP/1793/02)

Flow chart of tests to be carried out at the different stages of the process of production of Bovine Serum.

Tests to be carried out at the different stages of the process of production of Bovine Serum



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