Investigation of Largescale Production and Quantification Methodology of Monoclonal Antibodies (MAbs)

By Catherese Fahey B.Sc

A thesis submitted for the degree of MSc

Dublin City University

The research work described in this thesis was carried out under the supervision of

Professor Martin Clynes, Dr. Elizabeth Moran &
Dr. Donnacha O Driscoll

National Cell and Tissue Culture Centre,
School of Biological Sciences.

2002

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award MSc is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: _____ ID No: 96923892

Candidate

Date: 22-01-03

Abstract

The unrivalled specificity with which an antibody binds to the antigen that has stimulated its production, forms the basis for the development of a multi-million euro biotech industry based on mammalian cell culture.
However, the large-scale production of monoclonal antibodies, (MAb) is faced with a number of major problems. These include the poor yield of hybridised cells and the low productivity of MAb in culture.

This thesis investigates aspects of the latter of these problems by attempting to design optimised culture conditions for growth and antibody production for a number of hybridoma producing cell lines namely the hybridoma producing anti-topoisomerase II-α MAb, 4/2D (IgG_1), the hybridoma producing MAb 1/11C (IgG_1), the hybridoma producing MAb to a novel complex of cytokeratin 6 and 9, 5C3 (IgG_1) and the hybridoma producing anti-mdr-1 MAb 6/1C (IgG_1).

Assay techniques such as Radio Immunodiffusion and ELISA were investigated to determine their reliability in quantifying monoclonal antibody in cell culture supernatant. The effect of commercially available serum free and protein free media on growth characteristics and productivity of the hybridoma producing cell lines were also examined. Finally, the environmental aspects of cell culture conditions (temperature and mixing) were considered.

A new system for quantifying MAb in supernatant was evaluated. This system, involving nephelometry (a turbidity-based system) was compared with the commonly used methods of ELISA and RID. The main advantage of such a system is that it gives real time analysis of antibody productivity whereas the RID method takes 2-3 days and the ELISA takes approximately 4 hours complete.

After evaluation on small-scale runs, a number of 10L fermentation runs were designed and executed to examine MAb production in two of the hybridoma producing cell lines 4/2D and 5C3 at large scale.

As a result of varying temperature and agitation, the culture conditions of 37 C and 100rpm were found to be the most efficient for cell growth and antibody productivity in all cases except the culture of 4/2D in PFHM II. In this case, the most efficient culture conditions in terms of MAb production were found to be 34 C and 200rpm. This result highlights the fact that each cell line has its own customised culture conditions.
Using the optimised culture conditions at large scale, it was found that quantities (mg/L) of antibody similar to those observed at small scale were being produced. The main difference however was that peak antibody productivity was occurring at day 3 and then decreasing thereafter. The exception to this was the culture of the hybridoma producing MAb, 5C3 in protein free conditions. Not only were antibody quantities increasing throughout the culture but also the levels of antibody produced were 10-15 fold in excess of the other cell line in both SSM and PFM. The results are discussed in detail and recommendations are made for future work.

Acknowledgements

I would like to sincerely thank Professor Martin Clynes for his help, guidance and encouragement during the course of this thesis.

I would like to also thank Dr. Liz Moran and Dr. Donnacha O Driscoll for their help and guidance throughout the course of this masters.

A special thanks to the immunology lab, Liz, Annemarie, Mick, Aine and Helena for their friendship and patience over the last two years. Thanks to Annemarie and Liz for answering all my questions no matter how stupid they were!. Thanks to Aine, Annemarie and Jason for the detailed proof reading! Thanks to Rob for all his help with computer problems and also to Yvonne, Carol and Mairead for all their help over the years.

I would also like to thank everyone in Archport who helped me out with my project. A special thanks to Cathal O Grady and Dave who taught me everything I know about largescale fermentation. To Paul Barham for all his help and guidance with ELISA"s. Thanks to Cathal Elliot, Anne Hannigan, Irene Cleary and Eunan McGlinchey for the help and guidance with all validation aspects of the project.

To the occupants of the office C217; thanks for the laughs and putting up with me under those many stressful days! Irene thanks for the use of your desk. Thanks to Jason, Eadaoin and Aisling for keeping me sane although it was touch and go there for a while!

Finally I would like to thank my friends and family for their constant love and support.

This thesis is dedicated to my mother
and the memory of my father

Table of Contents

Section 1 Introduction

1.1 The Industry

The unrivalled specificity with which an antibody binds to the antigen that stimulated its production forms the basis of its use. If monoclonal antibodies are produced against an antigen unique to a particular type of cell, these antibodies should selectively bind only at the surface of these cells. In this so called "magic bullet" effect, binding of the antibody could potentially trigger destruction of a particular cell, be it cancer cells, virally infected cells or microbial cells at the site of an infection (Walsh, 2001; Little *et al,* 2000).

By the end of 2001, 13 monoclonal antibody based therapeutic agents had gained regulatory approval by the FDA (Table 1.1). A further 59 are currently undergoing evaluation. The most common of these are in the area of cancer treatment (32 products).

Table 1.1 FDA approved therapeutic antibodies (adapted from Borrebaeck, 2001)

Product	Specificity	Indication	Year Approved
Orthoclotne OKT®3 (muromonab-CD3)	CD3	Acute kidney transplant rejection	1986
ReoPro® abcixiMAb	GpIIb/IIIa	Prevention of blood clotting	1994
Rituxan® (rituxiMAb)	CD20	Non Hodgkin's lymphoma	1997
Zenapax (DaclizuMAb)	CD25 (IL2R)	Acute kidney transplant rejection	1997
Remicade (InflixiMAb)	TNFα	Crohn's disease	1998
Simulect® (basilixiMAb)	CD25 (IL2R)	Acute kidney transplant rejection.	1998
Synagis® (palivizuMAb)	RSV	Respiratory syncytis virus	1996
Thymoglobulin®	Polyclonal abs	Acute kidney transplant rejection	1998
Herceptin (TrastuzuMAb)	Her2	Metastatic breast cancer	2000
CroFab®	Snake Venom	Rattlesnake antidote	2000
Campath® (alerntuzuMAb)	CD52	Chronic lymphocytic leukaemia	2001
Mylotarg® (gemtuzuMAbozogamicin)	CD33	Acute Myeloid leukaemia	2000

Due to the increased interest in the development of monoclonal antibodies (Mabs), the large-scale manufacturing sector of the industry is expanding rapidly. Volumes of commercialised antibodies are expected to increase from a production volume of 257kg in 1999 to 558kg in 2002. It is expected within the next year or two, that the average volume of monoclonal antibody produced by an individual manufacturer will increase by 20%, (from present averages of 75kg to 100kg by the end of 2002). Currently there are approximately 235 MAbs in development and by 2010, it is expected that 100 of these will be commercialised (Dutton, 2001; Kelley, 2001; Reichert, 2001).

Approximately half of the $5 billion annual turnover of the biotech industry is based upon large-scale mammalian cell culture processes. Table 1.2 shows examples of the revenue growth rates expected for some of these products over a five-year period.

Currently Ireland plays host to approximately 31 biotech companies (Bioresearch Ireland, 2001). Among them is the successful indigenous company Trinity Biotech plc, (formed in 1992) that specialises in the development, manufacture and marketing of diagnostic products which utilise immunoassay technologies. Trinity Biotech markets over 120 products through distributors in 80 countries worldwide. Numerous international companies have set up business in Ireland in the last few years many of which have experienced significant revenue growth over the past five years. Amongst these companies are Genzyme, Genemedics and Wyeth.

Table 1.2 Antibody and IgG fusion proteins presently on the market and expected growth figures for 2005.

Estimated Sales ($ million)	**2000 $**	**2005 $**
OKT3	30	35
Panorex	43	69
ReoPro	418	500
Rituxan	427	778
Zenapax	21	43
Simulect	30	70
Synagis	427	650
Remicade	371	900
Enbrel	652	1225
Herceptin	270	559
Myelotarg	20	144
Campath	0	110
Total	**$2710**	**$5803**
(Plus in Development		*$7383)*

1.1.1 Antibodies

When the body is challenged against a foreign substance (antigen), it responds by producing high affinity binding proteins, called antibodies (Mader, 1996). These proteins vary in terms of function, cell surface density, tissue distribution, specificity, avidity and isotype (Breedveld, 2001).

Antibodies are Y shaped molecules, which are composed of a basic four-chain (two heavy and two light chains) structure linked together by disulphide bonds (Figure 1.1). When the sequence of an antibody is examined closely it is clear that there are regions of extensive homology and regions of dissimilarity. The latter region is called the variable region and the homologous area is known as the constant region. The variable region is the critical site for antigen binding and is the distinctive feature that is unique to each individual antibody molecule (Soloski, 2000).

The main site of attachment to the epitope on the antigen-binding sites is called the complementarity-determining region (CDR). Different isotypes of immunoglobulins are defined by the structures of immunoglobulin CDR regions (Breedveld, 2000). There are five different classes of antibody: IgG, IgM, IgA, IgE and IgD. The most common and useful immunoglobulin is IgG due to its'
long biological half-life (>20 days) and its'
ability to interact with the human immune system (van Dijk & van de Winkle, 2001).

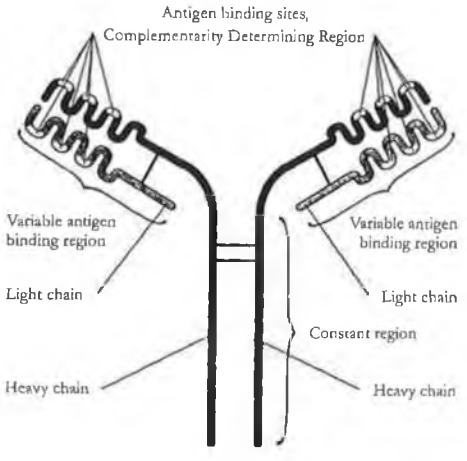


Figure 1.1 Structure of an antibody molecule

1.1.2 Antibody mechanism of action

Antibodies operate via a number of potential mechanisms. The most commonly known method of destruction is by blocking or sterically hindering the function of the target antigen. A prime example of an antibody operating like this is in the use of anti-tumour necrosis factor α (TNF-α) MAb. This MAb is used in the treatment of rheumatoid arthritis and Crohn's disease. The MAb binds and blocks the action of the pro-inflammatory cytokine (Glennie & Johnson, 2000). Similarly, MAb can prevent cell-cell interactions by blocking receptor ligand interaction. Antibodies may also kill cells by activating the complement cascade at the cell surface, with consequent lysis; or by binding to receptors on the surface of specialised effector cells, (such as phagocytes or killer cells) and triggering phagocytosis or antibody-dependent cell-mediated cytolysis (Gorter & Meri, 2000).

With the human immune system in mind, researchers wanted to replicate the operation of the immune system outside the human body. Here they could control the steps that were occurring and they could harvest the production of a single source of specific antibody. It was clear that every plasma cell derived from a B cell secretes antibody against a specific antigen. They believed that if a way could be found of harvesting these B cells and culturing them *in vitro*, then they could possibly develop therapeutics against a number of diseases and thus develop diagnostic kits for quicker identification of disease. This eventually, led to the creation of monoclonal antibodies.

1.1.3 Monoclonal Antibodies

In 1975, George Kohler and Cesar Milstein published a paper describing their new method for producing monoclonal antibodies (Kohler & Milstein, 1975). This paper described fusing two types of cells to form a hybridoma. The partner cells used were an antibody secreting B cell and a myeloma cell (continuously dividing B cell tumour). The resulting hybridoma combined the properties of each constituent, continuously secreting a single type of antibody when cultured in the ascities fluid in a mouse abdomen. This new method had a profound effect on immunology as it provides a powerful new method to search for cancer antigens due to their ability to recognise

minute differences between molecules and so scientists could at last produce defined antibodies in sufficient quantities to put antibody-based therapies to use. In 1984, Kohler and Milstein received the Nobel Prize for their discovery.

1.1.4 Monoclonal Antibody Production

To produce MAbs, mice are immunised by injection with the antigen to which the MAb is required. When an immune response has been raised, the B cells are harvested from the rodent, usually the spleen, and these are fused with immortal myleoma cells by fusion of the cell membranes with polyethylene glycol (PEG). This fusion procedure results in the production of immortalised hybrid cells or hybridomas. The remainder of the process is then one of isolation and production of hybridoma cells, which have retained the ability of the B cell to produce the desired antibody and the growth characteristics of the myleoma cell. The process of MAb production can therefore be considered in three parts: immunisation, fusion and selection of the required hybridoma (McCardle, 1998). In Figure 1.2 a pictorial representation of the procedure involved in MAb production can be seen.

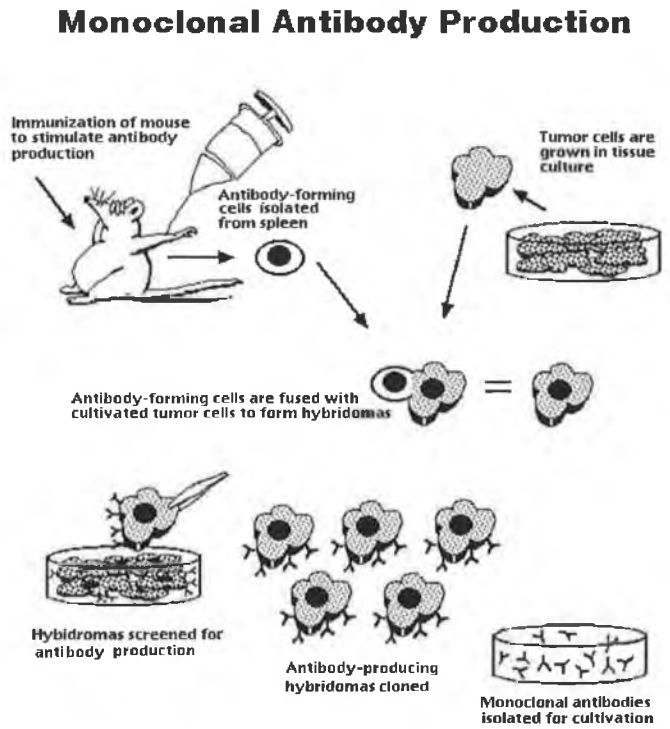


Figure 1.2 Monoclonal Antibody Production.

1.1.5 Humanised Antibodies

Murine monoclonal antibodies although widely used in the area of clinical diagnostics, have not had the amount of success originally expected in human therapy. Two major problems in the therapeutic use of murine monoclonal antibodies were identified at an early stage. Firstly, the specificity associated with monoclonal antibodies for their therapeutic targets did not always trigger the appropriate therapeutic response. Secondly, when murine antibodies were identified to work *in vivo*, the human immune system recognised them as foreign and a human anti-murine antibody immune response (HAMA) occurred (King, 1998). The OKT3 antibody used in the prevention of organ rejection overcame this problem by administering only single shot or via multiple use within a short period of a week to ten days before the antibody response developed. This antibody achieved FDA approval in 1986 (Clark, 2000a; Sgro, 1995; Goldstein *et al.*, 1985).

In an attempt to reduce and even eliminate the immunogenicity of murine monoclonal chimeric antibodies, recombinant DNA technology was applied. These methods involved splicing the gene sequence that codes for the mouse variable regions (at the antigen-binding site, the Complementary Determining Region) to the constant regions of a human antibody (Clark, 2000b; Jones, 1986). The resultant hybrid structures were more than 70% human and they contained the desired antigen-binding specificity of the parent murine monoclonal but were also less immunogenic when administered to humans.

The generation of human MAbs has proven to be problematic. Firstly, it is not appropriate to immunise humans for particular antigens; hence a suitable source of immune lymphocytes is not readily available. To overcome this problem, a system involving *in vitro* immunisation of human B cells was developed. The major drawbacks with these techniques were that MAbs generated are often of the IgM subclass, with low to intermediate affinities for the antigen (King, 1998). Although reports exist from the early 90's attempts at using human myeloma cell lines were problematic (Nilsson, 1971). In recent times Karpas *et al.* (2001) described a human myeloma cell line suitable for the generation of human monoclonal antibodies a technology, which was a major step away from the use of murine cell lines.

Humanised antibodies are also prepared by antibody engineering. In this instance, the antigen binding CDR's are grafted into human V region framework regions. The resultant antibodies are entirely human in nature except for these short sequences and should therefore have the same characteristics as a native human antibody (Walsh, 2001; Glennie & Johnston, 2000; Little *et al.*, 2000; Berkower, 1996). Hundreds of fully humanised antibodies have been produced using this technology. Clinical trials have been carried out on some but to date no significant immune response has been elicited.

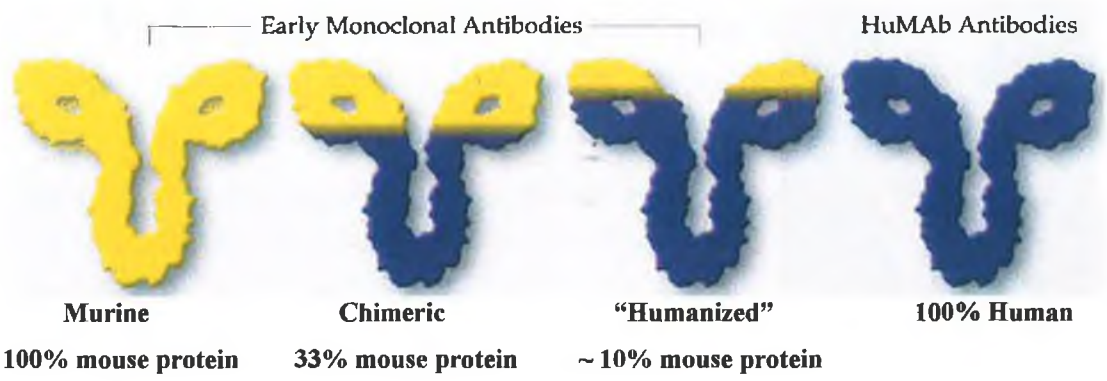


Figure 1.3 Evolution of antibodies

An alternative approach to the generation of human MAbs is the genetic manipulation of mice to disable the production of mouse immunoglobulin and to functionally replace them with human antibody producing genes. These *transgenic* mice like the "xenomouse" developed by Abgenix (CA, US) can then produce MAbs in response to immunisation to a variety of diverse antigens. A lot of success has been achieved with such technology, several high affinity antibodies have been generated (Fishwild *et al.*, 1996, Green, 1999, Yang *et al.*, 2001). MAbs from Xenomouse ™ animals have been shown to have therapeutic potential and both *in vitro* and *in vivo*, and appear to have the pharmacokinetics of normal human antibodies based on clinical trials. Thus this Xenomouse ™ technology should be possible to generate high affinity, fully human MAbs.

1.1.6 Applications of monoclonal Antibodies

Monoclonal antibodies have a large role to play in the diagnosis and treatment of life threatening diseases. Such as cancer and autoimmune disease. In recent years, major advances have been made in the areas of drug discovery, diagnosis and delivery to the target organ (Reff & Heard, 2001; Govindan *et al.,* 2000; Khaw, 1999; Cragg *et al.,* 1999)

1.1.7 Diagnostics

Monoclonal antibodies have been used in the design of sensitive detection assays such as Enzyme Linked Immunosorbent Assays (ELISAs). These tests have been used to detect normal and auto antibody levels, determine the presence and levels of auto antigens, viral/bacterial and other environmental antigens as well as assessing the levels of normal components in bodily fluids e.g. pregnancy testing kits (Borrebaeck, 2000). MAbs have also made an impact on the isolation and purification of molecules. In some applications a given MAb can be coupled to an insoluble surface and used to affinity purify a molecule of interest. This approach allows a several-fold purification to be performed in one step.

Cancer Imaging

Radioimmunodetection (RAID) is another diagnostic method that takes advantage of tumour specific or tumour associated radiolabeled monoclonal antibodies or other biological molecules, which bind to malignant tissue to diagnose the extent of disease in cancer patients.

RAID was introduced into clinical practice in an effort to provide a more accurate means of determining the extent of the malignant process in cancer patients. Two researchers, Pressman and Keighly (1948) were the first to demonstrate the use of radiolabelled antibodies in the targeting of a specific organ or tissue. They showed that the immunoglobulin fraction containing antibodies to normal rat kidneys could be

radiolabelled with ^{131}I (Iodine-131) and used to specifically target the kidneys *in vivo*. This work suggested the potential of antibodies for tumour imaging and therapy.

Initially however, a number of problems were encountered with this new technology. There was a lack of specific targets associated with various tumours used in *in vivo* trials and appropriate radiolabels for tagging the available antibodies. However, recent advances in monoclonal antibodies and improved radiolabeling methods have enabled investigators to image a host of cancers related to the breast (Burak *et al.*, 1998), lung, cervix and pancreas (Gold *et al.*, 2001).

Radioimmunotherapy has advantages over other typically used methods such as the computed tomographic scans (CT). CT scans depend on a change in size and architecture of organs. Radiolabeled monoclonals have the ability to detect abnormal cells at an early stage because of the expression of tumour antigens. Once cancer is diagnosed, accurate staging is important in deciding the most appropriate therapy. Later, during follow up surgery, levels of tumour antigens such as carcinoembyronic antigen for colon cancer or prostate specific antigen in prostate cancer, may indicate reoccurrence of the cancer long before conventional methods can reliably identify the disease material (Berkower, 1996).

1.1.8 Quantifying Monoclonal Antibodies

Radial Immunodiffusion (RID)

Radial Immunodiffusion evolved by Mancini *et al.*, (1965) and Fahey & McKelvey (1965), is a classical diagnostic method to determine the levels of IgG subclasses. This assay is widely used and is very easy to perform. However, when monitoring large and frequent samples the relatively long incubation times (approximately 72 hours) are a disadvantage.

The method involves antigen diffusing rapidly from a cylindrical well through an agarose gel containing an appropriate monospecific antibody. Antigen-antibody complexes, are formed, which under the correct conditions will form a precipitation ring. The ring size increases until equilibrium is reached between the formation and

breakdown of these complexes this point is termed "completion". A linear relationship exists between the square of the ring diameter and the antigen concentration. By measuring the ring diameters produced by a number of samples of known concentration, a calibration curve can be constructed. The concentration of the antigen in the unknown sample may be determined by measuring the ring diameter produced and reading it off the calibration curve.

ELISA (Enzyme Linked Immunosorbent Assay)

The basis of the ELISA method is that enzymes can be attached to antibodies without the loss of activity. The subsequent addition of a suitable substrate leads to colour development, which, can then be quantitated spectrophotometrically. Enzyme detection has become the standard method for most laboratory immunoassays and the most commonly used is the 'sandwich' ELISA. This involves immobilising an antigen to a solid support and incubating with the sample-containing antibody (primary antibody). After binding of the primary antibody, excess enzyme labelled secondary antibody is added. Commonly used enzymes are horseradish peroxidase (HRP) and alkaline phosphatase (AP). These convert substrate to product with a high turnover rate to allow the generation of a large amount of coloured product, which can be quantified (Crowther, 2001; King, 1998).

Nephelometry

Nephelometry is used to determine IgG concentrations in cell culture supernatants. The MININEPH (Binding Site, UK) used in this work is an endpoint nephelometer that uses the measurement of light scattered by an antibody/antigen complex to determine protein concentrations.

The light source is a diode laser that emits at 670nm. The focused light passes through a cuvette containing the reaction mixture, where the antibody/antigen complexes cause light to be scattered. The scatter is proportional to the amount of antigen/antibody complexes that have formed, and is detected by a photodiode.

Advantages of nephelometry are the relatively short incubation time and ability to measure small concentrations of antibody. Another advantage is that nephelometry assays may be readily automated and are therefore suitable for the routine measurement of IgG subclasses in large numbers of samples (Baker *et al.*, 2002).

1.2 Large Scale Mammalian Cell Culture

1.2.1 Introduction

The increased demand for animal cell derived products has stimulated the development of newer and more efficient apparatus and processes, in which manufacturers are able to harvest higher concentrations of cells.

With regards to the production of monoclonal antibodies, there are two major bottlenecks that must be overcome, these include the poor yield of hybridised cells and the low cellular productivity of monoclonal antibodies in culture (Kundu, 1998). These problems can be overcome by improving the design of available methods of culturing cells at large scale and involves the use of more efficient high-density culture methods.

The theory behind scale up is to take a process, producing a product at small scale and to increase the scale of production using a variety of means (Chisti, 1993; Resiman, 1993). It should be noted that when dealing with scale up it is important to adhere as closely as possible to the parameters established in the initial production phase. A typical scale up procedure normally involves taking a 1ml vial of cells from the working cell bank (WCB) and scaling up to a maximum of 30,000 litres depending on the requirements of the process (Freshney, 1994). This is usually achieved by one of two methods:

- Increasing the number of units already in operation
- Increasing the size of the culture system without increasing the number of vessels involved (McGlinchey *et al.*, 1998).

The latter method is probably the most commonly used when a high concentration of product is required.

Scaling up a process can be fraught with problems and there are a number of criteria, which must be taken into consideration. Firstly, mammalian cell lines are classified as either anchorage-dependent or anchorage-independent based on their growth characteristics in culture. Cells that can grow in suspension are easily scaled up through to production level using fermentation vessels of increasing size.

Anchorage-dependent cells tend to prove more difficult to scale-up. They require a surface for attachment in order to proliferate. Due to this, they are normally grown in hollow fibre reactors or in stirred tank reactors using microcarriers which act as a substratum for growth.

Mixing rates and aeration are major parameters that affect the growth of cells in culture (Section 1.2.4 and 1.2.5). In a stirred tank reactor cells require constant agitation for purposes of acquiring appropriate quantities of oxygen and nutrients. The main aim of mixing and aeration is to achieve non-turbulent, streamlined bulk flow patterns within the culture fluid so that mechanical stress damage on the cells is minimised (Griffiths, 1988).

The choice of medium used in culture is another critical parameter in the growth of cells, antibody productivity and downstream processing (Section 1.2.2).

1.2.2 Choice of Medium

1.2.2.1 Serum-supplemented Medium

Serum is defined as the *"black box"* of mammalian cell culture. It is composed of a complex mixture of more than 100 poorly defined biomolecules with different growth promoting and inhibiting activities. These components include hormonal factors, attachment factors, growth factors, proteins, lipids, and inorganic trace elements (Jayme, 1991; Griffiths, 1987). The exact composition is not entirely known and its constituents can vary in concentration from batch to batch. Serum is considered to be a very important component of any culture medium typically constituting 5–10% (v/v) of the growth medium for cells. There are a number of disadvantages when culturing cells in serum.

- Serum may contain selective inhibitors, bacterial toxins, lipids and polyamine oxidases, which form cytotoxic polyaminoaldehydes upon reaction with polyamines such as sperimidine (Cartwright & Shah, 1995).
- Serum screening is necessary due to the large batch to batch variation. This is a costly and time-consuming process. Also one serum type will not satisfy all cell types due to the different cellular requirements of cells depending on their origin either in the body, species specificity or depending on whether cells are normal or transformed. Separate screening has to be carried out for each cell line.
- The supply of serum is not always guaranteed. In times of biological drought and disease, social or economic downturns, it may be difficult to obtain a particular serum from a particular country. This may also result in an increase in cost of other serums due to the increased demand (Mizrahi & Lazar, 1988).
- Serum also tends to interfere with the production of monoclonal antibodies, resulting in more and complicated downstream processing steps. Often affinity column chromatography, gel filtration and ion exchange steps are required. These steps are time consuming and labour intensive, with extra purification steps required to remove traces of serum, leading to a costly downstream process and reductions in final MAb yield (Cartwright & Shah, 1995).
- Outbreaks of disease such as CJD and foot and mouth in livestock has been making it harder to source and use foetal calf serum safely.

Because of these reasons, regulatory authorities do not allow the use of serum during the harvesting of human therapeutic proteins.

1.2.2.2 Serum-free Medium

Serum-free Media (SFM) consist of basal media supplemented with a series of serum-free additives. The most commonly used basal media are: Dublecco's modified Eagle's medium (DMEM), Hams F-12, Roswell Park Memorial Institute (RPMI-1640) and Iscove's Modified Dubleccos Medium (IMDM). These can be used in combination with each other or singly. Differences have been observed between basal media in terms of the growth promoting effects, hence careful consideration should be taken when choosing a basal media (Kitano *et al.*, 1986; Murakami *et al.*, 1982a).

Each cell line will have different requirements for cell growth and productivity. Generally, additives are divided into the following groups:

Energy Sources

Carbohydrates are the main energy source for cultured cells with glucose, glutamine and glutamax (alanine-glutamine dipeptide) being the most commonly used. The concentration of specific carbohydrate sources used can vary in the range 5-20mM. When added at a high concentration, it is rapidly converted to lactate, which lowers the pH to inhibitory levels. Glutamine is another important energy source. The amino acid L-glutamine is sometimes added in conjunction with glucose to reduce the degree of conversion of glucose to lactate (Mizrahi and Lazar, 1988). Other sources that may be used include fructose (Mochizuki *et al.,* 1993) sucrose, maltose, galactose and mannose.

Amino Acids

Animal cells have a requirement for non-essential amino acids such as arginine, lysine, cystine and histidine. Other amino acids are often added to compensate for a particular cell lines' inability to make them. Blasey *et al.* (1989) found that hybridoma cells instantly started dying once amino acids were limited. When L-glutamine was depleted both cell growth and MAb production was halted.

Vitamins

Vitamins of the B group are necessary for cell growth and replication. Many vitamins act as co-factors. The vitamins most commonly added to basal media include biotin, choline, folic acid, pantothenic acid, thiamine, inositol and ethanolamine (Kovar & Franek, 1985; Murakami *et al.,* 1982b).

Lipids

Lipids, especially unsaturated fatty acids, are well known nutrients of serum-free media for animal cell cultivation *in vitro*. Requirements for these substances are mainly associated with their role as membrane components. In certain cases they are linked

directly with the control of cell growth (Kovář, 1987). Typical examples of lipids in culture media include the fatty acids linoleic acid, cholesterol and lipoproteins (Darfler, 1990b; Sato, 1984).

Transporter Proteins

Typical transport proteins include transferrin and albumin. Transferrin functions in acting as an iron transporter. The functions of iron include, acting as a co-factor in enzymatic reactions and it is closely associated with DNA synthesis and cell growth (Kovář and Franěk, 1987; Kovář and Franěk, 1984). However, in the development of protein-free media it is desirable to find an alternative to transferrin because of its high cost and the difficulty in removing it from culture media during downstream processing. The effect of replacing transferrin with ferric chloride ($FeCl^{3+}$) on two hybridoma cell lines was examined (Sanfelieu *et al.,* 1994). The results showed comparable cell growth characteristics and antibody productivity in one cell line. In the other cell line, growth was less but MAb production was similar to the control media containing transferrin. In another study Metcalf *et al.* (1994) examined the effect of replacing transferrin with 2-hydroxy-2,4,6-cycloheptarin-1-one (tropolone). This showed that tropolone could also replace transferrin in a large-scale culture system.

Hormones and Growth Promoters

Growth factors are termed mitogenic polypeptides capable of promoting DNA synthesis in resting cells and keeping cycling cells from entering the resting phase (G_0) (Mizrahi & Lazar, 1988). The most well know examples of growth promoters include insulin and hydrocortisone. Insulin is considered essential in the serum-free culture of hybridoma cells. The functions served by insulin include the stimulation of uridine and glucose uptake and the syntheses of RNA, protein and lipid. Insulin is generally added to the culture medium in the concentration range 5-10μg/mL (Zhou & Hu, 1997; Martial *et al.,* 1994; Glassy *et al.,* 1988; Kovář, 1986b). Other factors such as NGF (nerve growth factor) and EGF (epidermal growth factor) are added depending on the cell line.

Trace Elements

This group generally includes additives such as iron, zinc, copper and selenium. Many function as enzyme co-factors. Selenium is thought to have a special function in protecting cells from the toxic effects of peroxides (Kovář, 1988; Kovář & Franek, 1986). Their role in cell culture cannot be underestimated. Cleveland *et al.* (1983) developed a protein-free medium (PFM) replacing insulin, transferrin, albumin, and liposomes with the trace elements Al, Ag, Ba, Br, Cd, Co, Cr, F, Ge, I, Rb, Zr, Cu, Mn, Ni, Sn, Zn, Mo, V, Fe, Si, and Se. Immunoglobulin production was comparable for all cell lines tested between serum supplemented medium (SSM) and protein free medium with ten out of the eleven cell lines having comparable growth rates.

Numerous serum-free media have been developed, which have the ability to maintain the growth of mammalian cell lines in culture. Selected reviews, which deal with the development of SFM include the following: (Chua *et al.,* 1994; Simonson *et al.,* 1994; Chen *et al.,* 1993; Federspiel *et al,* 1991; Lang, *et al.,* 1991; Mariani *et al.,* 1991; Merten and Litwin, 1991; Morrow, 1990; Blasey & Winzer, 1989; Clark *et al.,* 1989; Wolfe *et al.,* 1988; Shacter 1987; Tharakan *et al.,* 1986a; Kovář and Franěk, 1986; Kawamoto *et al.*, 1986; Tharakan *et al.*, 1986b). Commercially available serum-free media differ widely in terms of their protein concentration, price and ability to maintain the growth and productivity of specific hybridoma cell lines. In the commercial arena, two other types of specific serum-free media exist:

1.2.2.3 Protein-free Medium (PFM)

This medium does not contain any quantifiable levels of protein. The development of protein-free media is a major step forward in the research and development of hybridoma culture and MAb production. Protein-free media allows easier purification of antibody because the culture supernatant is devoid of any background external macromolecules (Fran k and Dolníková, 1991, Tarleton, 1991). Numerous investigators have developed protein-free media replacing protein-based additives with trace elements, vitamins and other agents (Voigt and Zintl, 1999; Nagira *et al.,* 1995; Bertheussen, 1993; Darfler, 1990a; Cleveland *et al.,* 1983).

1.2.2.4 Chemically Defined Medium (CDM)

The use of chemically defined medium for the cultivation of hybridoma cells provides several advantages over the classical serum-supplemented medium. The use of defined media allows a quantitative study of the influence of each individual constituent and its concentration on cell growth and on monoclonal antibody synthesis and secretion. Other advantages include a reduction in the risk of contamination from protein-based products and higher antibody purity (Stoll *et al.,* 1996; Schneider, 1989)

1.2.2.5 Advantages of Serum-free Media

- Serum-free media reduces the risk of viral and endotoxin contamination often associated with serum.
- A growth media which eliminates any uncertainty regarding the identity and concentration of its constituents, is a major advantage when attempting to obtain product approval from the relevant regulatory authority.
- It has been shown that cells adapted to grow in SFM can have a product secretory capacity more than that obtained in SSM (Darfler, 1990a; Clark *et al.,* 1989; Tharakan *et al.,* 1986a)
- Monoclonal antibody purification is more efficient and economical compared to the separation of MAb cultured in SSM or ascitic fluid. In excess of 90% purity can be obtained (Graf *et al.*, 1991). Large volumes of supernatant can be applied to the chromatographic columns due to the low protein concentration of the serum-free media.

1.2.2.6 Disadvantages of SFM

- The process of adapting cells to serum-free media from serum-supplemented media is a slow time consuming process. It involves taking cells cultured in SSM and *"weaning"* them into SFM over a number of passages, slowly reducing the quantity of serum been used.
- In some cases the cost of adding the various growth factors and hormones required may result in the cost of the media being greater than that of SSM.

- SFM lacks the protection against shear forces previously obtained from SSM. This leads to difficulties in maintaining cell viability in scale up (Ramirez & Mutharasan, 1992; Ozturk & Palsson, 1991b).
- Cells cultured in SFM are less tolerant to changes in the environment such as pH and carbon dioxide fluctuations. Ultrapure reagents such as water are required and pH and temperature need to be carefully controlled.
- Cells in SFM are extremely fastidious and there is no universal SFM for growth thus identifying the optimum medium for a particular cell line can be time consuming (Simonson *et al.,* 1994; Barnes & Sato, 1980).

1.2.3 Culture Vessels

1.2.3.1 Static Culture Flasks

These flasks range in size from 12.5cm^2 to 175cm^2. They provide a substratum for growth of anchorage-dependent cells and they can maintain the growth of anchorage independent cells. They are generally used in the early stages of scale-up to inoculate roller or spinner bottles.

1.2.3.2 Roller bottles

In the beginning, the manufacturing of animal cell derived products was primarily carried out using glass roller bottles. Since then animal cell bioreactors have evolved extensively.

The effort behind the development of these bioreactors was stimulated by the desire to overcome some potential problems in scaling up of animal cell processes. Among the major drawbacks of the roller bottle systems are its labour-intensive nature, utilisation of valuable space and most importantly the unavailability of pH and dissolved oxygen control. On the other hand, the roller bottle system is very flexible as it allows rapid expansion or reduction in the production capacity. Hybridoma cells which are anchorage-dependent, can be grown on roller bottle apparatus. The major limiting factor however has been the fact that a maximum of 10^6cell/ml only, can be maintained in a healthy state within the culture. The MAb produced must then be concentrated by

methods such as ammonium sulphate precipitation. This method of growing hybridoma necessitates the use of large quantities of media, serum and ammonium sulphate.
An adaptation of the commonly used roller bottle method is the bubble chamber dialysis roller apparatus. This system involves the growth of cells inside dialysis tubing within a modified roller bottle. The roller bottle is modified to enable harvesting of the produced antibody. The cells within the dialysis tubing are maintained in suspension by an oscillating bubble. The media is stored on the outside of the tubing where nutrients may diffuse into the cells. Monoclonal antibody yields up to 0.5g during 20 days of culture have been reported (Pannell & Milstein, 1992).

1.2.3.3 Spinner Flasks

Spinner flasks are miniature bioreactors which are used in the scale-up of cells. They generally range in size from 50ml to 1L. They facilitate the growth of both anchorage independent and anchorage-dependent cells. Anchorage dependent cells require the addition of microcarriers for optimum growth. Spinner flasks need to be siliconised prior to use to prevent attachment of cells to the glass surface. The flasks consist of a glass vessel with two side arms and a top cap. The side arms facilitate the addition and removal of media and cells. Suspended from the top cap are a glass rod and a magnetic stirrer. Mixing occurs when the flask is placed upon a specific magnetic base, which can control the spinning speed of the magnetic stirrer. Spinner flasks provide excellent vessels for the scale-up of cells to 10L-bioreactor scale.

1.2.3.4 Stirred Tank Bioreactors

For producing large quantities of both anchorage-dependant and anchorage independent animal cells, stirred tank bioreactors (STB) and adaptations have become the method of choice. STB's have the capability to support the growth of high biomass cell culture fermentations in a sterile environment. STB's can be modified to provide *in vitro* cells with adequate supplies of aeration, agitation, temperature and pH. This is accomplished through computer operated control systems, which allow real time monitoring and control of the system (Lütkemeyer *et al*, 2000). The provision of services such as sterilised compressed air, water supply comprising both hot and chilled sources of

water, facilities for the drainage of waste products/exhaust gases and addition of fresh products are necessary to carry out efficient mammalian cell culture fermentation.

Traditional impeller driven apparatus such as the stirred tank bioreactors are available in a range of sizes from 1L up to tens of thousands of litres. Other types of bioreactors exist such as airlift reactors, in particular the draft tube internal-loop airlift devices, which are known to be one of the largest reactors for free suspension cultures (Meisenholder, 1999; Grima *et al.*, 1997). Airlift bioreactors are the preferred means of growing cells at large scale because this system provides a relatively mild and homogenous field of shear stress compared with the stirred tank reactor and bubble column fermenters. In some systems the turbulence has a focus and dissipates with distance from the impeller or sparger, having more of a damaging effect on the shear sensitive mammalian cells.

Smaller bioreactors are generally composed of a glass reactor vessel with a stainless steel head-plate. Larger bioreactors are usually composed of stainless steel 316, which is very expensive as it contains corrosive resistant material necessary for mammalian cell culture. For the production of animal cell derived products and MAb, the biotechnology industry takes advantage of the size of these bioreactors for the production of their products. However, there are a number of criteria which tend to have an effect on the growth of the cells and these must be considered at every stage in the development of a new process.

1.2.4 Aeration

The capability of a cell culture to propagate can be limited if oxygen is not supplied efficiently throughout the system. The aeration and agitation system should ensure that oxygen delivery is not a growth-limiting factor. Cellular functions utilising oxygen include cell maintenance, respiratory oxidation for growth and substrate oxidation into metabolic products (Bailey & Ollis, 1986). Oxygen consumption rates of animal cell cultures are low compared to bacterial cell cultures. This is due to their lower growth rates and cell densities. However, animal cells are more sensitive to vigorous mixing and gas sparging. Therefore, maintaining a sufficient oxygen supply to cell cultures is a principal consideration in large-scale and high-density mammalian cell cultures.

The most common and efficient means of delivering oxygen to the cells is via a process know as sparging. Sparging is described as the process of bubbling air or another gas e.g. through a relatively deep pool of the culture broth, usually through a device called a sparger which is normally located at the bottom of the bioreactor (Chisti, 2000).

However, mammalian cells lack the outer cell wall associated with bacterial and yeast cell; which leaves them more sensitive to mechanical agitation and aeration via sparging (Hu & Peshwa, 1991). The bubbles that form from the sparging effect carry oxygen up through the suspension culture dispersing air to the cells. The adverse effect of this method is that cells can attach to the bubbles and thus are drawn up to the liquid-gas interface (Reiter & Blüml, 1994; Jöbses *et al.,* 1991). At this point the bubbles escape into the gas phase leaving the cells on the surface of the suspension culture where they cannot escape the foam that forms as a result of escaping bubbles breaking through a thin film of serum and proteins (Handa-Corrigan *et al.,* 1988). In addition, cells may be subjected to hydrodynamic forces as a bubble approaches the air liquid interface as Figure 1.3 demonstrates.

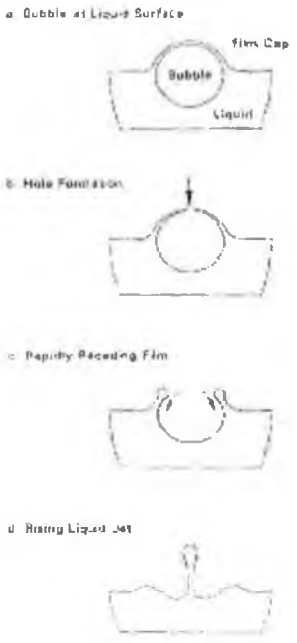


Figure 1.4 The hydrodynamic mechanism of bubble rupture at the air liquid interface. **A)** When a bubble approaches the air liquid interface, its upper part protrudes out of the interface, forming a hemispherical liquid film cap. Its lower part depresses the liquid below the interface **B)** Before the bubble collapses, the rupture starts at the thinnest apex of the film cap where a hole is formed and the liquid film thins to a critical thickness **C)** When this high-speed flow reaches the liquid layer surrounding the bubble cavity it pushes the liquid to flow inward under the bubble cavity **D)** When this symmetric flow meets at the bottom of the bubble cavity, a stagnation point is created and high pressure is resulted. This high-pressure results in bubble break up.

The high speed induced by bubble rupture is enough to severely damage animal cells. The high speed flow creates intense hydrodynamic stresses in liquid close to the bursting bubbles. The shear stress is estimated to be in the region of $95N/m^2$ in the receding film and $200\text{-}300N/m^2$ in the boundary layer surrounding the bubble cavity. Hybridomas are said to be sensitive to shear stresses beyond $1\text{-}5N/m^2$ (Jöbses *et al.*, 1991).

In addition, bubble escape can also lead to foam formation. Foaming results in a layer of bubbles forming over the surface at the top of the culture media. This prevents gas gaining entry into the culture media via headspace aeration. In addition, cells will attach to this layer of bubbles during mixing. This results in an increase in cell death and loss of productivity in the culture (Jobses *et al.*, 1991; Handa-Corrigan *et al.*, 1989). Bubble damage can also occur in agitated non-sparged bioreactors due to the entrainment of air through the culture fluid surface. In experiments on surface aerated cultures it has been found that cell damage begins when cell entrainment is initiated at stirring speeds between 150–200rpm (Kunas *et al.*, 1990).

In order to combat the adverse effects of sparging, other techniques must be employed to transfer oxygen to the cells. Common methods employed include:

- Headspace oxygenation
- External oxygenation
- Direct oxygenation using gas permeable silicone tubing or hydrophobic membranes

The simplest method of bubble-free oxygenation is transfer of oxygen from the headspace. This method is widely used in small-scale applications such as T-flasks and spinner flasks, at large scale production if foaming occurs gas delivered into the headspace will not be able to pass through the foam effectively thus reducing the gas supply to the cells.

Silicon aeration tubing enables bubble-free oxygenation of animal cells in culture. To prevent the formation of bubbles, the reactor headspace is connected to gas input tubes, which automatically adjust the pressure between the gas in the membrane and the liquid. The major problem with using silicone tubing for direct oxygenation is that the diffusion rate of oxygen through the tubing is very low and large amounts of tubing (approximately 10 metres for a 10L bioreactor) are required for dense cell culture systems. Scalability of such a process design would be a major problem involving huge quantities of materials. External oxygenation involves refreshing culture medium outside the culture vessel with new oxygen. This is usually achieved using an external arm from the bioreactor into which medium from the culture vessel can be pumped and fresh oxygen is supplied before being pumped back into the reactor vessel.

1.2.5 Agitation

The aim of agitation is to obtain uniform culture conditions in the bioreactor in order to obtain maximal gas transfer to the cells, to avoid gradients of nutrients, temperature and pH and to keep the cells in suspension. In a stirred tank reactor mixing is achieved using an impeller. There are a number of different types of impeller available which are specific for particular processes. The most commonly used impeller in mammalian cell culture is the marine impeller, which together with its angled blades enables efficient axial pumping capacity. Axial flow impellers produce higher flow per unit input characteristics compared to radial flow impellers. Thus they produce lower shear conditions suitable for the cultivation of animal cells compared to radial flow impellers. This ensures complete mixing of the reactor medium and improves refreshment of the upper most layer of liquid to achieve higher oxygen transfer rates without sparging.

Low agitation rates result in clumping. Clumping of cells can give rise to inaccurate cell counting. The cells inside of the clump are being starved of nutrients and air, which results in their early death and reduces overall antibody productivity. However, excessive agitation results in increased physical forces being exerted on the animal cells. Since animal cells are quite large (10-100μm) and lack an outer protective cell wall, they are rendered quite fragile. Therefore, large hydrodynamic forces are liable to result in the death of cells early on in the cell cycle (Elias *et al.*, 1995).

Attempts have been made to quantify detrimental hydrodynamic forces in terms of shear stress (τ), shear rate (γ) and turbulence intensity. Shear has two components. Shear stress is the force per unit area acting on a body and shear rate, is a measure of how the velocity changes as we move away from that body (Doran, 1995). They are related to each other by the following equation:

$$\tau = \mu\gamma \qquad \text{(Equation 1)}$$

μ = viscosity of the solution (Nsm^{-2})
τ = shear stress (Nm^{-2})
γ = average shear rate (s^{-1})

Shear can be visualised as liquid flow lines moving at different speeds and directions. Such velocity fluctuations occur in turbulent eddies. The smaller the eddy and the greater the velocity fluctuation then the greater will be the level of shear. There are two forms of shear:

- Localised shear, which occurs around objects moving in the culture media e.g. impeller and bubbles
- Shear in the bulk liquid arising from turbulence within the reactor. As the level of turbulence increases, the eddy size will decrease and the level of shear will increase

The type of fluid flow is important when looking at the effect of agitation on the growth of hybridoma cells. There are three main types of flow (Figure 1.4):

- Laminar
- Transient
- Turbulent

Laminar flow tends to be unidirectional and is visualised as "flow lines". There is very little movement between the fluid lines, which results in inefficient mixing of cells, culture media and oxygen in the bioreactor. Transient flow is a state that exists in between laminar and turbulent flow. In this state, fluid flows randomly between laminar and turbulent flow resulting in poor mixing of the culture components. During turbulent flow the fluid flow lines breakdown to form eddies. These eddies carry the fluids away from the fluid lines leading to a mixing of materials throughout the reactor. Turbulent flow conditions are required for good mass and heat transfer and are hence the most desired mixing state (Scragg, 1991).

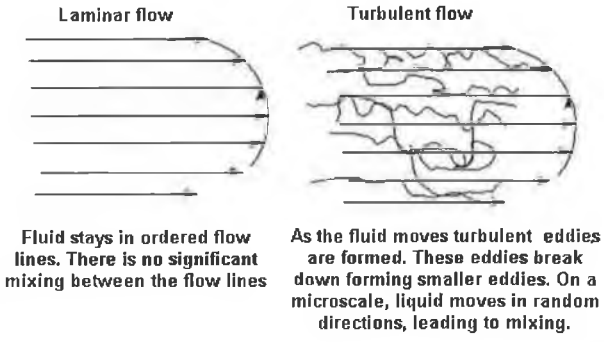


Figure 1.5 Diagrammatic representations of laminar and turbulent flow.

Along with turbulent flow conditions come the hazard of eddies. Eddies are best described as whirlpools; hence, the liquid rotates instead of following distinct flow lines. These whirlpools are unstable and continuously breakdown in a phenomenon referred to as a turbulence "cascade" (Aloi & Cherry, 1996). Eventually eddies breakdown to a size where they have insufficient energy to form smaller eddies. The smallest eddy size that is found is defined by the "Kolomogrov eddy size (ε)". Under normal stirring conditions the average size of the turbulent eddies is considerably larger than the average cell diameter. The resultant effect is that the cells are able to "ride" between eddies and thus are not affected by shear forces. However, at increased stirring speeds the Kolomogrov eddy size decreases resulting in collision with and damage to the cells.

1.2.5.1 Reducing the effects of Shear

Foetal Bovine Serum (FBS) and other common surfactants such as Pluronic F-68 are routinely used in addition to the culture media because they show protective effects against excessive agitation (van der Pol *et al.*, 1992; Velez *et al.*, 1986). Their protective nature has been related to surface active properties, which affect bubble-gas formation and bubble burst characteristics (Ozturk & Palsson, 1991b; Oh *et al.*, 1989, Handa-Corrigan *et al.*, 1989). Michaels *et al.* (1991) concluded that the protective effect afforded by FBS was both physical and biological where as that afforded by Pluronic F-68 was physical only. However, it is unknown if the FBS yields a significant protective effect under conditions of excessive turbulent shear. Others have reported Pluronic F-68 as having a fast acting biological effect, by interacting directly with the hybridoma cell membranes modifying the plasma membrane and making it more shear resistant (Ramirez & Mutharasan, 1992; Jobses *et al.*, 1991).

Other methods commonly used to avoid the foaming effect include liquid oxygen vectors such as perfluorocarbons (Lowe *et al.*, 1998; Deshusses *et al.*, 1997), surface aeration and the use of bubble free oxygenation through microporous or diffusion tubing made of silicone rubber (Chisti, 2000).

A number of studies have demonstrated no significant effect of high-speed impeller agitation on hybridoma cultures in the absence of sparging. Oh *et al.* (1989)

successfully cultured hybridoma cells at 450rpm in RPMI (+10% FBS). Kunas & Papoutsakis (1990) attributed decreased hybridoma growth rates at agitation levels in excess of 800rpm, to interactions of cells with Kolmogoroff microscale eddies of approximately 13μm in diameter. This is in agreement with the findings of Smith & Greenfield (1992) where in a study using serum-supplemented media cultures agitated at 200rpm yielded 26μg/mL, where as agitation at 600rpm yielded 27μg/mL. Growth characteristics showed a shorter growth period and decreased cell viabilities early in the culture.

High levels of viable cells decline at higher agitation rates in serum-free cultures. These have been linked to high metabolic rates in the exponential growth period and subsequent nutrient limitations (Kunas & Papoutsakis, 1990). Apparent exponential growth rates (μ_{app}) were independent of agitation conditions implying that instantaneous death events which are typical of physical disruption, did not result from cell interactions with turbulent eddies. The turbulence intensity however was sufficient to have subtler biological interaction with the cells resulting in increased glycolysis rates. This increase in glycolysis was required to provide energy either to satisfy a deficiency in other energy production pathways or to increase overall energy production. Also increased energy may be required for maintenance of internal cell structures or repair of membranes that are affected by turbulent fluid forces. Increased rates of viable cell decline were precipitated by limitation of glucose and glutamine rather than essential amino acids as found with SSM (Passini and Gouchee 1989; Lee *et al.,* 1988).

It has been proven that at increased agitation speeds there is a corresponding increase in the number of dead and damaged cells (Dodge & Hu, 1985). For example, it has been reported that the decreased cell viability at 200rpm resulted in a lower monoclonal antibody concentrate (140μg/ml) than in T-flasks (250μg/ml) (Lee *et al.*, 1988). In addition, the components of damaged cells are usually secreted into the cell culture suspension, which results in a reduction of the intrinsic growth rate of the cells. This in turn can affect the quality and quantity of product that is reaped from the cell culture. Furthermore, if the damage includes lysis of the cells the increased protein in the medium makes product purification difficult (Aloi & Cherry, 1995).

To date in the pharmaceutical industry, there have been numerous examples of large-scale operation of stirred tank bioreactors employing suspension cultures or modifications thereof for increased and more efficient production of cells and their products. It has been observed that agitation and sparging have had a more adverse effect on smaller scales than on larger scales. Therefore, excessive sparging and agitation should be avoided at smaller scales. However at larger scales successful employment of many relatively large-scale bioreactors demonstrates that agitation and aeration are not the biggest obstacles to overcome.

1.2.6 Growth Limitations

The major growth limiting factors associated with mammalian cell cultures are:

- Ammonium and lactate inhibition
- Depletion of energy sources, (glucose and glutamine)
- Amino acid deficiency
- Exhaustion or inactivation of growth factors
- Lipid, vitamin and trace element deficiency

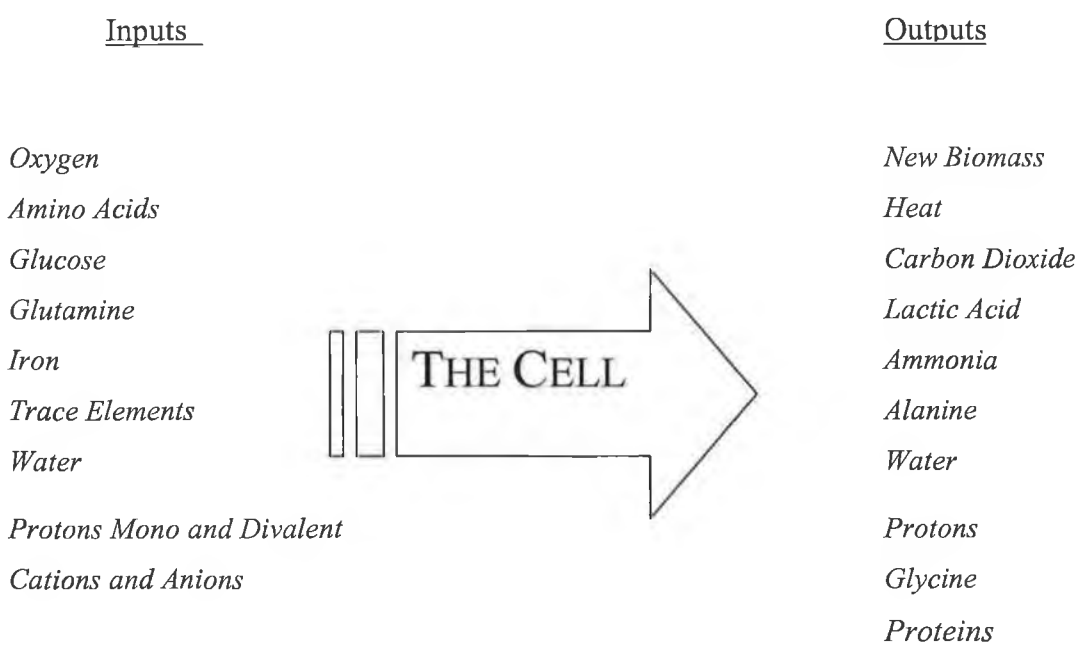


Figure 1.6 A cell and its environment (Fu & Barford, 1994; Spier, 1992).

Glucose and the amino acid L-glutamine have been proven to be extremely important in various proliferation and production mechanisms in culture (Jenkins *et al*, 1992). Glutamine is thought to be the more important of the two for the efficient culture of hybridoma cells. Ozturk & Palsson (1991a) observed that hybridoma cultures ceased to grow upon exhaustion of glutamine and that glutamine could substitute for depleted glucose but not *vice versa*.

The by-products of these energy sources are growth and product inhibitory if allowed to accumulate above a certain limit (Ljunggren & Häggström 1995; Kromenaker & Srienc, 1994; Omasa *et al.*, 1992; Reid & Greenfield, 1990). Accumulation of lactate and ammonia destroys the buffering capacity of the medium and therefore shifts the culture to a lower and undesirable pH environment. This increase in lactate and ammonia alters the cell growth rate and specific productivity of antibody in hybridoma culture (Chen *et al.*, 2000; Bibila & Robinson, 1995). In order to try and control the build up of these metabolic waste products, attempts have been made to supply glucose to the culture in a controlled manner such as using fed-batch routines. Maximisation of the fed-batch process is achieved by periodic supplementation of the culture with nutrients that are quickly consumed or exhausted. Hence the concentration of glucose can be maintained at a lower but appropriate level. This technique has been successful in keeping the lactate and ammonia concentrations below inhibitory levels.

Other avenues followed involved the manipulation of metabolic pathways. For example in the case of lactate, lactate dehydrogenase (LDH) has an isozyme form and functions as a tetramer and converts cellular pyruvate to lactate. By removing this gene it aids in reducing the build up of lactate in the culture medium (Chen *et al.,* 2000). Gene targeting is a powerful tool for inhibition of gene expression on a permanent basis, or for systematic alteration of the mammalian genome. Another method used to reduce ammonia build up in culture involves the enzyme GS (glutamine synthase), which is not normally found in hybridoma cultures.

The incorporation of an immobilised glutamine synthase (GS) within hybridoma cell cultures slows down the accumulation of ammonia and replenishes glutamine by reversing the metabolism of glutamine (Okeson & Riley, 2001).

1.2.7 Temperature

The most frequently used temperature for the cultivation of mammalian cells is 37°C. However, it has been shown that cultivation at lower temperature results in slower growth and enhances specific productivity in cells (Suzuki & Ollis 1989; Reuveny, 1986). It is thought that the reason for this effect is related to the hybridoma cell cycle. The hybridoma cell cycle comprises four consecutive phases: DNA synthesis (S-phase), a gap (G_2-phase), a mitotis phase and a second gap (G_1-phase). An additional phase identified as the resting phase G_0, occurs when cells in the G_1 phase are no longer committed to enter the S phase. Synthesis and secretion of immunoglobulins by different cell lines have been reported to be restricted to the late G_1 and S phases. Hence, MAb productivity could be improved by methods which favour accumulation of cells in the G_1 phase. A decreased specific growth rate is reflected in a prolonged G_1 phase. Since MAb synthesis is assumed to take place in the G_1 phase, a prolonged stay of the cells at this stage may be responsible for the enhanced antibody productivity levels (Ramírez & Mutharasan, 1992; Suzuki, 1990).

Blomkolk & Gray (1994) reported highest cell density and MAb yield from non-agitated cultures at 37°C. In agitated culture, the highest cell yield was achieved at 34°C, with an increased lag phase recorded. The highest MAb productivity was achieved at 34°C and 37°C. At the upper scale of the temperature range, (39°C) Blomkolk & Gray reported no growth. This contrasts with the findings of Mutharasan & Sureshkumar (1991). They found that maximum cell density was recorded at 33°C, maximum cell yield at 35°C and maximum specific antibody yield at 38°C. The difference between maximum cell density and cell yield is that density pertains to the stage in the growth curve that most cell growth was recorded. On the other hand, cell yield is related to the total number of cells produced over the whole growth period.

The sharp contrast between the results obtained between the two researchers at the upper end of the temperature range 38°C and 39°C, has been attributed to the fact that 38°C is the upper limit for growth and small changes in temperature above this, may have had a serious detrimental effect on the growth and productivity of the cells.

1.3 Cell Culture Methods

Depending on the equipment available, it is possible to perform batch, fed-batch or continuous perfusion cultures. The choice of system and the operation mode allows the production of short or long term cultures, low or high density of cells and high or low concentrations of product. The following is a brief description behind each of the most common methods of producing the desired products and their applications in industry.

1.3.1 Batch Cultures

A batch culture is a closed system in which cells are inoculated into a quantity of nutrient medium contained within a suitable vessel. Cells are cultured to death and the antibody harvest is collected at a specific time point.

Traditionally, animal cells have been cultivated in batch culture either as suspension or monolayers. In a batch reactor, cells are inoculated into a system that contains the right nutrients, the required temperature, pH and dissolved oxygen levels. As the cell numbers increase, nutrients are consumed and waste products accumulate (Katinger & Scheirer, 1985). This process has an adverse effect on the environment of the culture which usually leads to a cessation of cell metabolism and the production of the desired product.

Simplicity in process control and operation, resulting in process consistency, low contamination rate and low operating costs are some of the advantages of batch culture (Tovey, 1985). On the other hand, the low product titre and productivity are major disadvantages associated with batch culture (Moro *et al*, 1994). The desired product is generally secreted by viable cells, hence product formation is directly proportional to the integrated viable cell concentration with time. Therefore, a high viable cell density maintained for a long time is required for maximum efficiency (Xie and Wang, 1997).

1.3.2 Fed-batch Culture

In fed-batch systems, nutrients are kept at appropriate concentrations by controlled feeding. Waste products and cells are not withdrawn. The culture is fed at intervals with fresh medium and there is a corresponding increase in the volume of the culture. As a result the overall cell densities and cell yields are increased per litre of media (Tovey, 1985).

Fed-batch is ideal for the production of substances such as MAb's, where the synthesis is maximal after the cells have reached the stationary phase. For example, it has been reported that a fed-batch culture operated for 550h reached a total cell concentration of nearly 5 x 10^7cell/ml and a peak viable cell concentration of over 1.5 X 10^7cells/ml (Hu & Aunins, 1997). Cell culture longevity in fed-batch culture of hybridomas is often limited by elevated medium osmolarity caused by repeated nutrient feeding. The use of a hypoosmolar medium can delay the onset of severe cell death resulting from the increased osmolality of the culture and in return increase the MAb productivity (Ryu & Lee, 1999).

If this system was to be modified to allow the addition of nutrients and removal of waste products, the resultant effect would be an increase in culture time and an improvement in the cell densities produced. This type of system is termed perfusion culture of cells.

1.3.3 Perfusion culture

Perfusion culture provides an *in vivo* like environment where suspended cells are retained by filters or are immobilised on beads. Perfusion cultures have been described as being advantageous for non-propagating cells, when cells of a limited life span are used and when the kinetics of product formation are typically non-growth related as is in the case of MAb's. The main aim of perfusion cell culture is to maintain the cells in the ideal culture environment for a longer period of time in order to increase productivity. The essential parameter in perfusion culture is the rate at which medium is replaced. By intensifying the replacement rate there is an increased provision of nutrients, which in turn leads to an increase in the viable cell density (Mercille *et al.*,

2000). A number of mechanical devices have been developed increasing the efficiency of perfusion cultures of animal cells with fresh medium. Such devices include membrane filtration systems, dialysis reactors, devices which use gravitational settling (Wen *et al.,* 2000), spin filters (Deo *et al.,* 1996; Yabannavar, 1992) and ultrasonic filters (Bierau, 1998; Gaidi *et al.*, 1996). The major limitation with the type of systems mentioned here are that they are closed systems, where small amounts of waste products are retained and this ultimately leads to the demise of the culture. In order to overcome this problem, the use of an open system such as a continual-flow culture system is necessary.

On reviewing the current processes used in large-scale cell culture production of recombinant proteins and antibodies it is clear that the industry has converged on using suspension cultures in stirred tank bioreactors with some form of controlled feeding system like batch or fed-batch (Chu & Robinson, 2001).

1.3.4 Hollow Fibre Bioreactors

Originally developed in 1972, the hollow fibre bioreactor (HFB) was designed to mimic the *in vivo* capillary system (Knazek *et al.,* 1972). The reasoning behind this was to try and maintain a more physiological environment for culturing cells with regard to nutrient supply, metabolic waste removal, and pH control while providing a stable microenvironment without shear (Jackson *et al.*, 1996).

A typical HFB system is composed (Figure 1.6) of a gas exchange unit, a bioreactor composed of capillary membranes made of cellulose, a media supply and waste bottles. The factors that may cause variations in the operation of hollow fibre bioreactors include pH, temperature, IC (Intracapillary) recirculation rate, bioreactor composition gas mixture, harvest rate and IC media feed rate (Czirbik, 1996; Lowrey, 1993).

Hollow fibre bioreactors vary from supplier to supplier in terms of their arrangement and composition of the hollow fibres. The fibre material may be composed of one of a range of materials including cellulose, acetate, cuprammonium, rayon and polyethylmethanacrylate (PAMA). In general it has been found that for adherent cell lines cuprammonium and cellulose are largely undesirable due to their hydrophobic

nature. Typically, the membranes will have a molecular weight cut off less than or equal to 45-50kDa. This is to prevent loss of immunoglobulin (IgG), which is approximately 150kDa (Lipman & Jackson, 1998).

Also included in a HFB are pH probes, dissolved oxygen probes and media reservoirs for the extracapillary space (ECS) and Intracapillary space (ICS) (Dhainaut *et al.*, 1992).

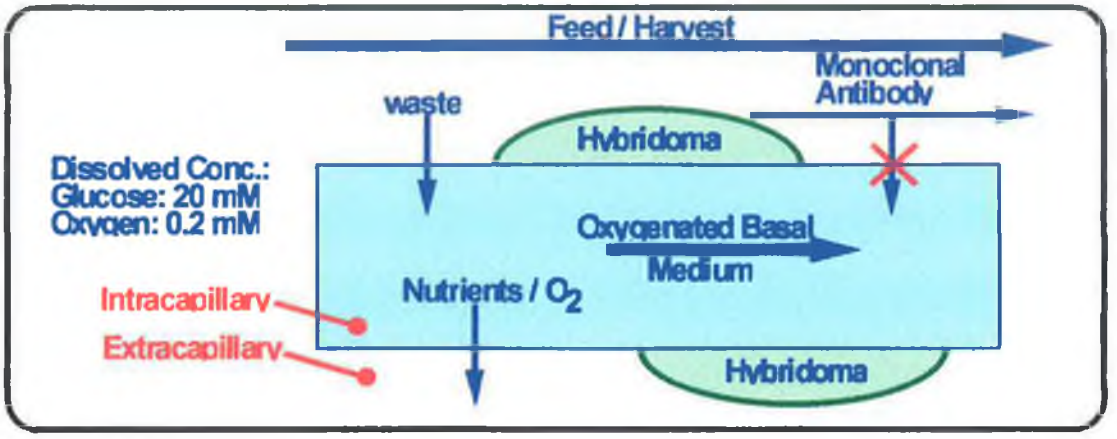


Figure 1.7 Diagrammatic representation of the production of monoclonal antibody in a hollow fibre bioreactor.

The ECS of the hollow fibre reactor is used for cell growth. The ECS is both compartmentally and physiologically separate from the intracapillary space. Cells are provided with nutrients from the culture medium, which is continuously circulating through the ICS. Small molecular weight metabolic waste products diffuse from the ECS to the ICS, while secreted immunoglobulins can be retained in the ECS for periodic collection.

Oxygen is provided by aerating the ICS stream. Recycle flow is analogous to blood flow through capillaries embedded in tissue hence the configuration of a hollow fibre bioreactor mimics, to a certain extent the human *in vivo* system. The two fibre-free regions at either end of the cartridge are used to introduce cells and fresh media into the ECS and to harvest the protein product.

Hollow fibre bioreactors have been reported to consistently produce concentrations of products in the range 0.7-2.3mg/ml (Altshuler *et al.*, 1986). Dhainout *et al.,* (1992) reported a total culture harvest of approximately 3.0-3.1g over a 25 day culture period. Piret & Cooney (1990) using an ultrafiltration hollow fibre bioreactor, have reported MAb production in excess of 17mg/ml. This system provides advantages over a conventional hollow fibre bioreactor as it retains growth factors and selectively concentrates high molecular weight protein products, which reduces the need for extensive and sometimes invasive downstream processing.

This system also offers a support medium for the growth of many cell types. The continuous perfusion system enables the constant supply of essential nutrients and removal of metabolic waste products necessary for optimal production (Omasa *et al.,* 1995). Cells are protected from shear, which is often a common fault found in standard stirred tank reactor systems where aeration via sparging can lead to damage of the sensitive cell membrane of an animal cell. Typical cell densities in the HFB can reach in the range of approximately 10^7-10^8cells/ml and cell viability and production can be maintained for extended periods.

The ability to frequently harvest the antibody from the bioreactor during production decreases the potential for degradation, which normally results from prolonged exposure to cellular proteases in the culture media (Graner *et al.,* 1999; van Erp *et al.*, 1991b). Downstream processing of the products from hollow fibre bioreactors has been made easier with the successful introduction of serum-free and protein-free media to cells (Dhainanut *et al*, 1992; van Erp, *et al.*, 1991a; Heifetz *et al.*, 1989; Klerx *et al.*, 1988). This important breakthrough has led to a decrease in downstream processing and production costs. HFB's also have their disadvantages. They have the potential for microbial contamination and mechanical failure, therefore technical expertise is required to avoid these complications. In addition, the materials and equipment used for production of MAbs in hollow fibre bioreactors are very expensive (Jackson *et al.*, 1996).

1.4 Validation

During the past 50 years, there has been an increasing awareness of the safety concerns surrounding the manufacturing of medical products. This is due to the occurrence of a number of tragic accidents in the past; for example in the 1930's in the USA a mistake was made in the formulation of children's syrup and in Europe in the 1960's the thalidomide tragedy led to the death of a number of people. These and other similar tragedies have contributed to the establishment of regulatory agencies such as the Food and Drug Administration (FDA) in the USA.

These regulatory agencies established guidelines and standards for industry which they have to adhere to. The growing awareness for the high levels of safety required and the resultant higher quality standards also led to several technical improvements in the manufacture of pharmaceuticals. In line with this there have also been improvements in the organisation of manufacturing processes leading to the implementation of Good Manufacturing Practice (GMP). The aim of GMP is to clearly define the system that is used to manufacture a specific product in a reproducible and documented manner, in order to ensure the highest possible quality, safety, and efficacy of the product. This can only be achieved if all the components and steps involved in the manufacturing process are thoroughly documented and validated. Today, the establishment of manufacturing processes for new biopharmaceuticals is only possible under GMP conditions (Hesse & Wagner, 2000).

The term validation describes the provision of documented evidence that assures a high degree of confidence that a system or process performs its intended function accurately and reliably, is under control and at all times reproducible (FDA, 1997). Validation is an important process for a number of reasons; firstly, it leads to an assurance of confidence in procedures and methods carried out by a lab. Secondly, it results in a more efficient system of documentation and recording within the lab, which makes the process of transferring methods or scale-up much easier in the long term. It also acts as a bench mark for comparison of methods in different studies and laboratories. Included in a labs overall validation policy should be documented information on all aspects of production processes, cleaning procedures, analytical methods, in process control test procedures, computerised systems and persons responsible for design, review, approval

and documentation of each validation phase. Prior to starting process validation activities appropriate qualification of critical equipment and ancillary systems should be completed and critical documentation collected as described below. It is also advised that the product life cycle described in Figure 2.6 is used as a guide when a new process is initiated.

1.4.1 Standard Operating Procedures

Standard operating procedures are a written tool, which are important for a number of reasons. Firstly, they define how a task is to be performed to achieve a specified outcome, and they standardise the way a task is to be performed to minimize variation. They also form the basis for training task performers, provide an informational tool that supports the performers, and, of course to fulfil a regulatory compliance. There are a number of different types of SOP's including administrative tasks, which are generally rule based such as who has permission to enter a particular area. Cognitive tasks are procedures relating to decision making activities for instance reviewing decision making activities and motor tasks which generally refer to tasks such as setting up a fermenter (Vesper, 2001).

1.4.2 The product life cycle

The product life cycle (Figure 2.6) is an essential guide for ensuring that the user is completing each step of the validation process before moving onto the next section. The following explanations will define what is involved at each step in the product life cycle.

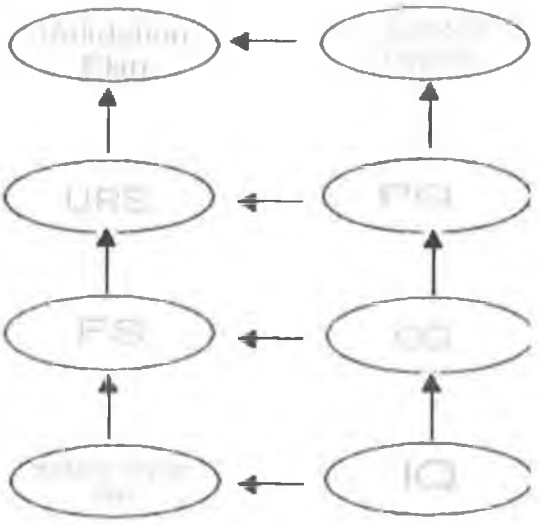


Figure 1.8 The Product Life Cycle.

User requirement specification (URS) This specification defines the intended functionality of the process. It should always be completed first as it can be used as a request for tender. This specification ascertains to what degree, quality has been built into the product. This is combined with the functional specification, which in the end produces a system specification.

- **Functional specification (FS)** The functional specification describes the operational and performance functionality of the system. It provides information regarding hardware and software to comply with the URS. It should always be completed prior to designing a process. Once the system becomes operational it acts as a version-controlled description of the system. FS should always include the following details:
    - System Attributes
    - Operational environment
    - Maintenance requirements
    - Other Specifications
    - Training Requirements
    - Reference documents
    - Appendices

- **Hardware and Software design** the design specification defines how the functionality described in the functional specification will be implemented. It usually contains a description of the following:
    - System design Overview
    - System description.
    - Input/Output instrumentation
    - Hardware operating environment
    - Electrical supplies

Qualification of the process is usually carried out by conducting the following activities, individually or combined:

- **Design Qualification (DQ)** Included in this section is documented verification that the proposed design of the facilities, equipment, or systems ARE suitable for their intended purpose (EMEA, 2000).

- **Installation Qualification (IQ)** This section contains documentation that ensures that all the system components are present and correctly installed and are capable of operating consistently within established limits and tolerances in compliance with the appropriate design and user requirements (EMEA, 2000; FDA, 1987). Typical documentation found in this section include:
    - System Overview
    - Installation Methodology
    - Description of all connections, e.g. piping and electrical
    - SOP's
    - Testing documentation
    - References

- **Operational Qualification (OQ)** This section contains information pertaining to the verification that the equipment or systems installed or modified, perform as intended throughout the anticipated operating ranges. Typical documentation included in this section includes:

- System Overview
- Responsibilities
- Testing Methodology
- Testing Structure

The OQ report describes conclusions of the testing and makes recommendations regarding implementation of system (FDA, 1987).

- **Performance Qualification (PQ)** This section includes documented verification that the equipment and ancillary systems, as connected together, can perform effectively and reproducibly based on the approved process method and specifications. This section will also include discrepancies found during testing (EMEA 2000; FDA, 1997).

1.4.3 Process Validation of a MAb Production Procedure

When undertaking process validation a manufacturer must evaluate all the factors that may affect product quality and ensure that they can repeatedly produce the desired level and quality of biological product. These factors vary considerably from process to process and may include all or some of the following criteria; raw materials, personnel, facilities and equipment. Process validation studies should be commenced early on in the research and development (R&D) stage. At this time extensive studies should be carried out on the product in order to chemically, physically and electrically characterise it. All aspects of the product, which impact on safety and effectiveness, should be considered. Examples of these aspects include performance, reliability and stability. Acceptable ranges or limits should be established for each characteristic to set up allowable variations (FDA, 1987).

An example of the steps undertaken to define set points and limits and ensure product quality are included in the following scenario: A cell line producing a recombinant protein at an optimum pH of 7.5 is chosen. The proven acceptable ranges from a validation study may vary between pH 7.1-7.9. A control parameter may then be selected between pH 7.3-7.7, providing 0.2pH units of process "safety" outside this range. As these criteria are being decided a series of physio-chemical and functional

assays are carried out to ensure that the product produced at any of the pH values within this range are of acceptable quality (Moran *et al.*, 2000; Federal Register, 1998).

A typical Process Validation Protocol would require the developer to include the following information (PDA, 1999):

- Short description of the process.
- Summary of the critical processing steps to be investigated.
- Finished product specification for release.
- In-process controls proposed with acceptance criteria.
- Additional testing carried.
- Sampling Plans.
- Departmental functions and responsibilities.
- Proposed timetable.

There are four different types of manufacturing process validations in the pharmaceutical industry prospective, concurrent, retrospective validations, and revalidation. The type used depends on the particular process and what stage it is at.

Prospective Validation This validation includes considerations that need to be made before a product is introduced or when there is a change in the manufacturing process which may affect the product characteristics, such as uniformity and identity. The following are described as the key elements of prospective validation.

- *Equipment* - Installation (Installation Qualification) and process (Performance Qualification)
- *Revalidation* – when there is a change in the process
- *Documentation* – It is essential to have documented evidence of each step in the validation program (EMEA, 2000; FDA, 1987)

Retrospective Validation This validation document provides evidence based on review and analysis of historical information. This method of validation is useful when there is a stable process with a large historical database. The main objective of this type of process validation is to support confidence in the process (EMEA, 2000)

Concurrent Validation This evaluates the process based on information generated during the actual implementation of the process. Typical situations in which one would use concurrent validation are (EMEA, 2000):

- When a step of the process is modified.
- The product is made infrequently.
- A new raw material is introduced in to a process/system.

Revalidation A manufacturing process would have to be revalidated when changes are made to critical components such as raw materials, changes or replacements in equipment and changes in the production facility (EMEA, 2000).

Validation is very important to ensure that strict guidelines and controls are implemented. For example, European sera have been banned from use in the production of biopharmaceuticals due to the known presence of transmissible spongiform encepha-lopathies (TSE's). However, serum that is certified to be from New Zealand is free from these contaminating agents. In 1994, 30,000 litres of so called New Zealand serum was sold world wide when only 15,000 litres had been produced. This is where vendor certification alongside quality control testing with validation elements, such as assay cross-validation, change controls and other contract procedure, to provide the maximum possible safety and quality level of raw materials comes into action (Hesse & Wagner, 2000).

1.4.4 Analytical Method Validation

"Validation of an analytical method is the process by which it establishes, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical applications"

(USP Chapter 1225)

Validation is an important process, which ensures that a procedure is fit for the purpose intended in that it ensures confidence that the correct results are obtained. It also identifies crucial aspects that are susceptible to change and can affect results. The following list details the parameters that should be considered when carrying out a validation procedure on an analytical method (ICH guidelines, 1994; ICH guidleines, 1995; Chow, 1997).

- Specificity – The ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically, these might include impurities.
- Accuracy – This is the closeness of agreement between the value, which is accepted either as the conventional or true value or an accepted reference value, and the value found.
- Precision – The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the homogenous sample under the prescribed conditions. Precision is often termed *inter assay variation.*
- Repeatability – This expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed *intra-assay precision.*
- Reproducibility – This expresses the precision between laboratories.
- Limits of detection – This is the lowest amount of analyte in a sample, which can be detected but not necessarily quantified to an exact value.
- Measurement range – The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which, it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.
- Limitations of the method (e.g. types of sample that are unacceptable).

- Linearity – of an analytical procedure is its ability to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample.

By carrying out these procedures and following guidelines set by the ICH, one can ensure that a validation method is robust and fit for the purpose intended.

1.5 Aims of Thesis

- It was the aim of this thesis to adapt a number of hybridoma cell lines to protein-free/serum-free culture conditions and to scale their productivity up to a 10L fermenter under optimised culture conditions. This involved looking at the effect of a number of commercially available serum-free and protein-free media on the growth and productivity of four hybridoma producing cell lines namely 4/2D, 5C3, 1/11C and 6/1C. Cell lines which adapted to and performed most efficiently in culture, were chosen for further scale up and optimisation studies.

- Optimisations studies involved measuring the environmental aspects of cell culture conditions (i.e. temperature and mixing) on the growth and productivity on two of the chosen hybridoma producing cell lines, 4/2D and 5C3. The most efficient culture conditions were chosen and used in the batch culture of these cell lines at 10L. The ability of the hybridoma cell lines to maintain growth and productivity at large scale was determined.

- A series of hollow fibre operations were performed using the hybridoma cell line 5C3, both in serum-free and serum-supplemented culture conditions. Antibody productivity from this system and cost efficiency were compared with that achieved in the 10L bioreactor.

- For the purpose of this thesis, it was necessary to use a method of quantifying MAb from culture supernatant that was efficient, reliable and easy to operate. Nephelometry was the method chosen. The main work involved validation of the nephelometer in order to ensure that the procedure was fit for the purpose intended in that it ensured confidence that the correct results were obtained when assaying the test samples. This validation work involved carrying out assays for precision, accuracy, reproducibility and specificity.

- The initial validation on the bioreactor and control systems, which involved carrying out both an Installational and Performance Qualification were performed.

Section 2 Materials and Methods

Addresses of suppliers are provided in Appendix III.

2.1.1 Water

Ultrapure water was used in the preparation of all media and solutions. This water was purified to a standard of 12-18ΩM/cm resistance by a reverse osmosis system (Millipore Milli-RO 10 Plus and Maxima UF water system, ELGA).

2.1.2 Glassware

Most solutions pertaining to cell culture and maintenance were prepared and stored in sterile glass bottles. All glassware including these bottles, used for cell culture work were soaked in a 2% (v/v) solution of RBS (Chemical Products) for at least one hour after which, they were scrubbed and rinsed several times in tap water. The glassware was washed in an industrial dishwasher, using Neodisher detergent (an organic, phosphate-based acid detergent), and rinsed twice with UHP. The materials were finally sterilised by autoclaving as described in Section 2.1.3.

2.1.3. Sterilisation

Water, glassware and all thermostable solutions were sterilised by autoclaving at 121°C for 20 minutes at 15p.s.i. Thermolabile solutions were filtered through a 0.22μm sterile, low protein-binding filter (Millipore, Millex-GV SLGV025BS). Large volumes, (up to 10 L) of thermolabile solutions were filter sterilised through a micro-culture bell filter (Gelman, 12158).

2.1.4. Media Preparation

The basal medium used during routine cell culture was prepared according to the formulations shown in Table 2.1. 10X media were added to sterile ultrapure water, buffered with $NaHCO_3$ and adjusted to a pH of 7.45-7.55 using sterile 1.5M NaOH and 1.5M HCl. The medium was then filtered through a sterile 0.22μm bell filter (Gelman,

G1423S) and stored in 500ml bottles at 4°C or in the case for large scale culture in 10L sterile bags until the expiry date recorded on each individual 10X medium container was reached (up to three months from production date). Prior to use, 100ml aliquots of basal media were supplemented with 2mM L-glutamine (Gibco 25030-024) and 5-15% foetal calf serum (FCS). This was then used as routine culture medium. This was stored for up to 2 weeks at 4°C after which time fresh culture medium was prepared. Hybridoma cells were grown in a basic basal medium, the commercially available DMEM. DMEM with Glutamax I (Glutamax is L-Amyl-L-Glutamine, high glucose concentration - 4.5mg, Gibco 61965-026) supplemented with 10% heat inactivated FCS (Myoclone, Gibco 10082-147) was used in these experiments. This was further supplemented for hybridoma growth and hybridoma cloning with 1% Penicillin streptomycin (Gibco, 15070-022).

Table 2.1 Preparation of basal media

	DMEM (1X) (Gibco 52100-039)
Ultrapure H_2O	10 L
7.5% $NaHCO_3$ (Riedel-de Haën, 31437)	49.3ml

2.2.1 Cell culture procedures

All cell culture work was carried out in a class II down-flow re-circulating laminar airflow cabinet (Nuaire Biological Cabinet). Strict aseptic technique was adhered to at all times. The laminar flow cabinet was swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all items brought into the cabinet. Each cell line was assigned specific media and waste bottles. At any time, only one cell line was used in the laminar air-flow cabinet, and upon completion of work with any given cell line the laminar air-flow cabinet was allowed to clear for at least 15 minutes. This was to eliminate any possibilities of cross contamination between the various cell lines. The cabinet itself was cleaned weekly with industrial disinfectants (Virkon, Antec

International or TEGO, TH Goldschmidt) as were all incubators used in the culture of cell lines and hybridomas. These detergents were alternated every month.

2.2.2 Subculturing cell lines

During routine subculturing or harvesting of adherent cell lines, cells were removed from their flasks by enzymatic detachment. Waste medium was removed from the cells which were then rinsed with pre-warmed trypsin-versene/EDTA (TV) solution (0.25% (w/v) trypsin (Gibco 043-05090), 0.01% (w/v) EDTA (Sigma) solution in PBS (Oxoid, BR14A). This ensured that any naturally occurring trypsin inhibitor in residual serum was deactivated. Fresh TV was then placed in the flask and incubated until the cells were seen to have detached (2-10 minutes). The TV solution was deactivated by the addition of pre-warmed basal medium containing serum. The entire solution was then transferred, to a sterile universal tube (Sterilin, 128a) and centrifuged at 1000rpm (Allegra^{TM} 6KR Centrifuge, Beckman, USA) for 5 minutes. The resulting pellet was then re-suspended in pre-warmed growth medium, cells counted and tissue culture flasks re-seeded at the required density.

Hybridoma cells are loosely adherent. Cells were passaged by tapping the flask lightly and/or gentle pipetting with a 10ml sterile pipette (Elkay, Ireland). Cell suspensions were pooled and centrifuged at 1000rpm for 5 minutes. The cell pellet was then re-suspended in culture medium and a cell count performed (Section 2.2.4) and the cells re-seeded at the required density. Cells were grown in 5% CO_2.

2.2.3 Sequential adaptation of hybridoma cells to protein-free and serum-free conditions

The cells were inoculated at double the normal seeding density, approximately $2x10^5$ cells/ml in a 75:25 (v/v) mixture of serum-supplemented: serum-free/protein-free medium. The culture was monitored until the density reached 1 x 10^6cells/ml. At this stage cell were subcultured in to a 50:50 (v/v) mixture of serum-supplemented: serum-free/protein-free medium. Again, the culture was monitored until it reached $1x10^6$cells/ml and subcultured in a 25:75 (v/v) mixture of serum-supplemented: serum-

free/protein-free medium. When the culture reached $1x10^6$cells/ml it was sub-cultured in to a 100% serum-free/protein-free medium.

2.2.4 Cell counting

Cells were trypsinised, pelleted and re-suspended in media as described in section 2.2.2. Cell counting and viability were carried out by using trypan blue (Gibco, 15250) dye exclusion technique. An aliquot of Trypan blue was added to a single cell suspension at a ratio of 1:2. After 3 minutes incubation at room temperature, a sample of the mixture was applied to the chamber of a Neubauer haemocytometer over which a glass cover slip had been placed. Cells in the 4 outer corner grids of the chamber were counted microscopically, an average per corner grid was calculated with the dilution factor taken into account and the final number multiplied by 10^4 to determine the number of cells per ml. Non-viable cells were those, which stained blue while viable cells excluded the trypan blue dye and remained unstained.

2.2.5 Cell freezing

Cells of various passage numbers were frozen and cryo-preserved to serve as master stocks. Vials could then be thawed and cultured for study. Cells to be frozen were harvested in the log phase of growth and counted as described in Section 2.2.4. The pellets were re-suspended in foetal calf serum (pre-cooled to 4°C) and an equal volume of freezing medium (DMSO (Sigma, D-5879)/serum 1:9 (v/v)) was added drop-wise to the cell suspension to give a final concentration of at least $5x10^6$ cells per ml. 1.5ml of the cell suspension was quickly placed in a cryovial (Greiner 122278), which was placed in the vapour phase of liquid nitrogen container for 2.5-3.5hr. After this, the cyro-vials were stored in liquid nitrogen until required.

2.2.6 Cell thawing

The cryopreserved cells were removed from the liquid nitrogen and thawed at 37°C. Immediately prior to removal of a cryovial from the liquid nitrogen, a sterile universal tube containing growth medium was prepared for the rapid transfer and dilution of thawed cells (thus reducing the exposure time to DMSO, which is toxic at room

temperature). Following the addition of the thawed cell suspension to the growth medium, the suspension was centrifuged at 1,000rpm for 3 minutes, after which the pellet was re-suspended in fresh growth medium. A viability count was carried out (Section 2.2.4) and the thawed cells were placed in tissue culture flasks with a quantity of medium and allowed to attach over-night. The following day the cultures were re-fed with growth medium to remove any residual DMSO.

2.3.1 Hybridoma cell lines

Details of the cell lines used for the experiments detailed in this thesis are provided in Table 2.2. Cell lines were maintained in $25cm^2$ (Costar 3056), $75cm^2$ (Costar 3376) or $175cm^2$ (Nunclon, NUNC 178883) tissue culture flasks at 37°C and fed every 2-3 days. Confluent hybridoma cell lines were grown for at least 6 days with no change of growth media when antibody was being harvested.

Table 2.2 Source description and media requirements of cell lines used in experiments described in this thesis.

Cell line	Subtype	Cell type description	Basal Medium	Source
6/1C	**IgG_1**	Hybridoma producing anti-mdr-1 MAb	DMEM (Glut I)*	NCTCC (Moran *et al.*, 1997)
4 2D	**IgG_1**	Hybridoma producing anti Topoisomerase II-alpha MAb	DMEM (Glut I)*/SFM/PFM*	NCTCC (Moran *et al.*, 1996)
1/11C	**IgG_1**	Hybridoma producing MAb (unknown kidney antigen)	DMEM (Glut I)*/ SFM/PFM*	NCTCC (Moran *et al.*, 1998; Lalor, 2001)
5C3	**IgG_1**	Hybridoma producing MAb to a novel complex of cytokeratin 6 and cytokeratin 9	DMEM (Glut I)*/SFM/PFM*	NCTCC (Larkin, 2002)

*Indicates other media were used in conjunction with this cell line and are described in section 2.3.2.

6/1C

The anti- mdr-1 MAb (IgG_1) producing clone 6/1C was produced following a combination of *in vivo* and *in vitro* immunization regimes in Balb/c mice with a synthetic 12-amino acid peptide that corresponds to amino acids 21-32 of P-glyocprotein (P-170).

P-Glycoprotein is an integral plasma membrane protein involved in the active efflux of cytotoxic materials from the cell and is consistently associated with multidrug resistance in cultured cell lines selected for multidrug resistance and in certain tumours (Moran *et al.*, 1997).

4 2D

The anti-topoisomerase II-α antibody (Clone 4/2D) was produced against a synthetic amino acid peptide conjugated to BSA. The peptide was chosen from the entire Topo II-α amino acid sequence for its apparent specificity to Topo II- α. The isotype of this antibody is IgG_1. Antibodies specific for topoisomerase II- α or topoisomerase II-β are valuable tools for investigating the exact physiological roles played by each isoform. Monoclonal and polyclonal antibodies have been utilised to quantitate and visualise the level of each of the enzymes within the nucleus of specific cells (Robinson *et al.*, 1993).

1/11C

The antibody producing hybridoma 1/11C (IgG_1) was generated employing a novel approach i.e. using dewaxed formalin fixed paraffin embedded archival tissue as an immunogen (Moran *et al.,* 1998). The use of dewaxed formalin fixed paraffin embedded archival tissue was employed in an attempt to identify novel antigens with possible diagnostic and/or prognostic value. Antibodies directed against archival normal kidney were generated as proof of principle that this method was feasible (Lalor, 2001).

5C3

As with the 1/11C, 5C3 (IgG_1) was also generated using dewaxed formalin fixed embedded archival tissue as an immunogen. In this case, formalin fixed paraffin embedded breast tumour tissue was employed as an immunogen. Internal sequencing revealed that the 5C3 reactive antigen was recognising a novel complex of human cytokeratin 6B and cytokeratin 9. Cytokeratin 6 has been detected in malignant and benign tumours. This cytokeratin in a complex with cytokeratin 5 has been previously detected in invasive breast cancers (Otterbach *et al.*, 2000).

2.3.2 Hybridoma Media

DMEM:	with Glutamax I supplied as a 1X stock (Gibco, 61962-026).
PFHM II:	protein-free media supplied as a 1X stock (Gibco, 12040-051)
UltraDoma:	protein-free media supplied as a 1X stock (Biowhittaker, 12-723F)
HyQADCFMab:	protein-free medium supplied as a 1X stock (HyClone SH30043.02)
CD Hybridoma:	protein-free chemically defined medium supplied as a 1X stock (Gibco, 11279).
HTM20CTH:	protein-free mediun supplied as a 1X stock (JRH, 14610)
PFM:	protein-free media, supplied as a 1X stock (Sigma, S2897)
HyQ®SFX-MAb:	serum-free medium supplied as a 1X stock (HyClone SH30206)
Hybridoma SFM:	serum-free low protein medium supplied as a 1X stock (Gibco, 12045).
Ex-CELL™620:	serum-free medium supplied as a 1X stock (JRH, 14620)
SFM:	serum-free media, supplied as a 1 X stock (Sigma, H4281)
TCH:	serum supplement supplied as a 1X stock (Celox, 2010026)
OptiMab:	protein-free supplement supplied as a 1X stock (Gibco, 11910) OptiMAb is a defined protein-free concentrate. The product consists of alternate carbon sources, a monoclonal antibody inducer, and essential nutrients which, have been shown to be significantly depleted during the high-density culture phase. In addition, this product has been formulated to shift culture

osmolarity into a range known to be optimum for antibody production (Mercille & Maisse, 1998; Jayme, 1991).

DMEM: with Glutamax II, supplied as a 1X stock (Gibco, 041-21661M) Glutamine is well known for its vital role in cellular metabolism, both as an energy source and as a precursor for nucleic acid synthesis. However its instability in culture media, which involves spontaneous deamination to pyrrolidone carboxylic acid (PCA) and ammonia makes it an unfavorable energy choice in culture medium. Glutamax II is a heat stable derivative of glutamine. Glutamax II used in conjunction with hybridoma cells, is a poor stimulator of cell division and growth due to its inability to travel through the cell especially the mitochondrial membrane. For this reason, the optimum time to add Glutamax II to the cells is when the culture is reaching the plateau phase of growth thus enhancing antibody production in the maximum number of cells (Hosoi *et al.*, 1988).

2.3.3 *Mycoplasma* analysis of cell lines

Mycoplasma examinations were carried out routinely (every 3 months) on all cell lines used in this study.

2.3.3.1 Indirect staining procedure for *Mycoplasma* analysis

Mycoplasma negative NRK cells (Normal Rat Kidney fibroblasts) were used as indicator cells for this analysis. The cells were cultured with supernatant (harvested from test cell lines after 2-3 days confluency) from test cell lines for 4 to 5 days, fixed and stained with Hoescht 33258 fluorescent stain (bisbenzamide) which binds specifically to DNA, thus it would stain the nucleus of the cell and any *Mycoplasma* infection, which can be seen as small fluorescent bodies in the cytoplasm of the NRK cells. Positive controls consisted of NRK cells infected with *Mycoplasma* enriched supernatants.

2.3.3.2 Direct staining procedure for *Mycoplasma* analysis

The direct procedure for *Mycoplasma* involved inoculating test samples onto an enriched *Mycoplasma* culture broth (Mycoplasma broth base, Becton-Dickinson, 255420) to optimise the growth of any contaminants and incubated at 37°C for 48h. Samples of this broth were then streaked onto plates of *Mycoplasma* agar base and incubated for 3 weeks at 37°C and 5% CO_2. Plates were checked microscopically every 7 days for the growth of small oval shaped colonies, which were indicative of *Mycoplasma* infection.

2.4 Antibody Detection

2.4.1 Mouse IgG ELISA

The purpose of the Mouse-IgG ELISA, was the quantatitive determination of mouse IgG in mouse hybridoma supernatants and ascities (Figure 2.1). The Elisa plates were coated with an anti mouse IgG (Fc specific), F (ab') fragment of goat antibody (Sigma M0284), 2μg/ml per well in 100 μl in carbonate buffer (1.378g $NaHCO_3$ and 0.382g Na_2CO_3 dissolved in 160ml distilled H_2O, the pH is calibrated to 9.5 $^{+/-}$0.1 with NaOH and finally the buffer is brought to a final volume of 20mls) and incubated overnight at 4°C. The plates were washed once in TBST (Tris Buffer Saline 0.1% (v/v) Tween 20 (Merck)) and then incubated with blocking buffer (1% (w/v) BSA (Sigma A3803), 0.1% Sodium Azide, PBS) for 1.5 hours at room temperature. The plates were then washed in 1%. 100μl of standards and sample was added to the antibody-coated plates and incubated at 37°C for 1 hour. This solution was then discarded and the plates washed 3 times in wash buffer, 1 % TBST. 100μl of secondary antibody, alkaline-phosphatase-linked rabbit anti mouse immunoglobulin, IgG (Sigma, diluted 1/2000 in TBST) was added to each well and incubated at 37°C for 1 hour. The secondary antibody was then removed and the plates washed three times in TBST as before. Plates were then incubated with the substrate solution (1mg/ml p-nitrophenyl phosphate (PNPP, Sigma N-9389) in 0.1M glycine, 0.001M $MgCl_2$, 0.001M $ZnCl_2$, pH 10.4) at 37°C for 0.5–1 hour or until a yellow colour appeared in the wells. The reaction was stopped by the addition of 1M NaOH, which also enhanced colour. Absorbencies were read on a Titerex ELISA plate reader at 405 nm. Positive reactivity was determined by comparing the supernatant containing wells with the standard curve.

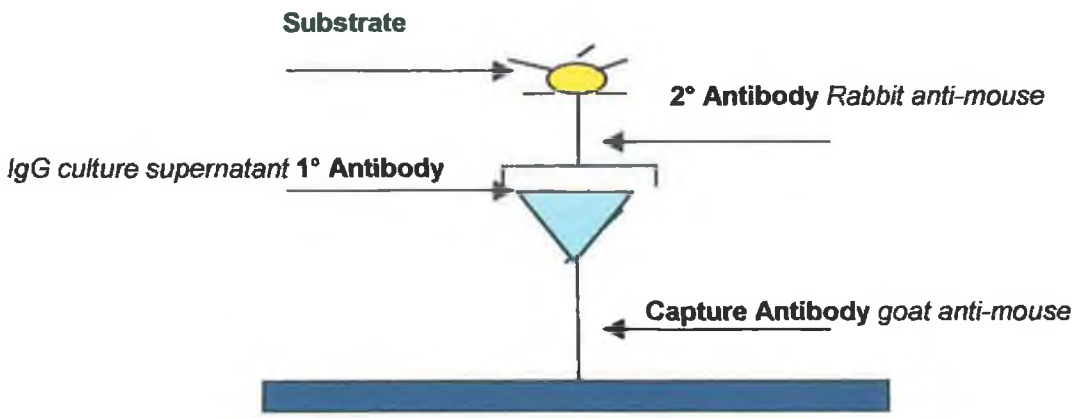


Figure 2.1 Representation of a Sandwich ELISA.

2.4.2 Nephelometry

The MININEPH (The Binding Site, UK) was used to determine IgG concentrations in cell culture supernatants. The MININEPH is an endpoint nephelometer that uses the measurement of light scattered by an antibody/antigen reaction to determine protein concentrations.

The light source is a diode laser that emits at 670nm. The focused light passes through a cuvette containing the reaction mixture, where the antibody/antigen complexes cause light to be scattered. The scatter is proportional to the amount of antigen/antibody complexes that have formed, and is detected by a photodiode (Figure 2.2).

The User Prepared Chemistry mode (UPC) was the program used. A stirring bar was initially added to the cuvette (ZK501.R). 10µl of the sample (neat or diluted) was carefully added to the bottom of the cuvette. The cuvette was then placed into the cuvette chamber. Using an electronic pipette 400 µl of reaction buffer (SN041) and 40µl of antiserum (PC272) are added to the cuvette. The assay settings included a blank time of 15 seconds and a read time of 900 seconds. Measurements of scatter were recorded at regular intervals to generate a curve of concentration versus scatter from which unknown samples may be determined.

The standard used in the generation of the standard curve was that used in conjunction with the RID plates (G272.3) (Section 2.4.3). The standard had a neat concentration of 1200 mg/L. The control used in these experiments was the c-myc MAB (IgG_1). Controls were examined at varying dilutions to ensure sample results lay in the linear region of the graph.

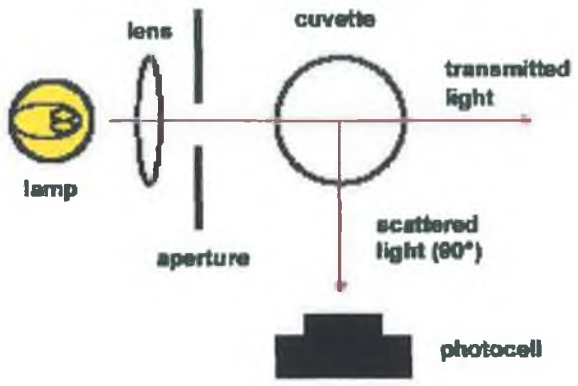


Figure 2.2 Representation of nephelometric process.

Validating the nephelometer involved performing inter and intra variation assays, along with determining the linearity of the standard curve, the specificity and accuracy of the assay.

The intra assay variation involved measuring a sample from the same specimen for approximately ten separate assays and comparing the average %CV (Coefficient of variation) of all the samples. The inter variation assay involved assaying samples from the same specimen in triplicate over four separate working days and comparing the results by measuring the average %CV.

The linear range and upper/lower limits of detection of the standard curve were determined by carrying out a range of standard curves and identifying these parameters The linear part of the curve was the region from which sample concentrations could be determined.

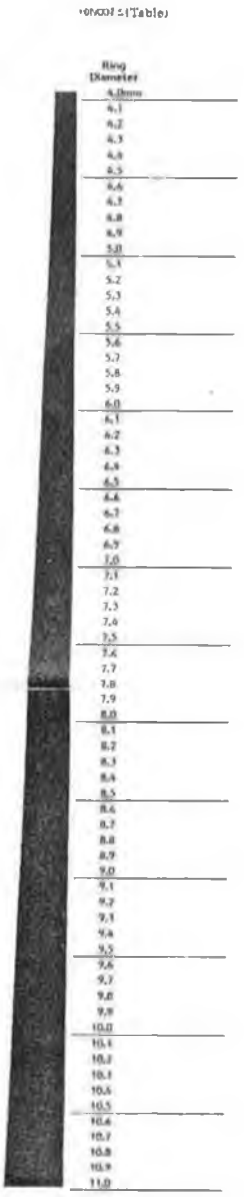
The specificity of the assay was determined by employing a variety of standard IgG and IgM samples and by measuring their concentration on the nephelometry. Results obtained were compared to their actual concentrations. The accuracy of the standards

was determined by comparing the theoretical value with the actual value (Section 2.10.4).

2.4.3 NanoRID

Radial Immunodiffusion (RID) using the NANORID detection kit (The Binding Site, GT272.3) is a technique that is routinely used for measuring the concentrations of various soluble antigens in biological fluids. It is principally derived from the work of Mancini *et al.,* (1964) and Fahey & McKelvey (1965). The method involves antigen diffusing rapidly from a cylindrical well through an agarose gel containing an appropriate monospecific antibody. Antigen-antibody complexes are formed, which under the appropriate conditions will form a precipitation ring. The diameter of the ring can be measured and compared to a list of RID references (Figure 2.3).

Calibrator and unknown samples of 10µl were added to each of the wells and allowed to diffuse for 72 hours.



Diameter of Ring	IgG
4.5	0.35
4.6	0.49
4.7	0.64
4.8	0.79
4.9	0.95
5.0	1.10
5.1	1.26
5.2	1.43
5.3	1.59
5.4	1.76
5.5	1.94
5.6	2.11
5.7	2.29
5.8	2.48
5.9	2.66
6.0	2.85
6.1	3.04
6.2	3.24
6.3	3.44
6.4	3.64
6.5	3.84
6.6	4.05
6.7	4.26
6.8	4.48
6.9	4.70
7.0	4.92
7.1	5.14
7.2	5.37
7.3	5.60
7.4	5.83
7.5	6.07
7.6	6.31
7.7	6.55
7.8	6.80
7.9	7.05
8.0	7.30
8.1	7.56
8.2	7.81
8.3	8.08
8.4	8.34
8.5	8.61
8.6	8.88
8.7	9.16
8.8	9.43
8.9	9.72
9.0	10.0
9.1	10.3
9.2	10.6
9.3	10.9
9.4	11.2
9.5	11.5
9.6	11.8
9.7	12.1
9.8	12.4
9.9	12.7
10.0	13.0
10.1	13.3
10.2	13.7
10.3	14.0
10.4	14.3
10.5	14.7
10.6	15.0
10.7	15.3
10.8	15.7
10.9	16.0
11.0	16.4

Figure 2.3 RID Reference table for mouse Immunoglobulins. Concentrations in mg/L (Adapted from The Binding Site Ltd product information sheet)

2.5 MAb Purification

2.5.1 Purification

The NAb™ Protein L Spin Kit (Pierce, 20530) was used to purify IgG from culture supernatants. Initially protein L beads were used but it appeared that the antibody was not binding, so the beads were changed and protein G beads were used.

0.2ml of immobilised Protein G gel slurry was washed by adding 0.3ml of PBS, mixing gently and then spinning down at maximum speed using a centrifuge (14000rpm). 0.4ml of supernatant was added to the protein G beads and gently mixed for 30 minutes. Non-specifically bound antibody was removed using 0.4ml of PBS and spinning for 1 minute. This was repeated three times.

The protein of interest was removed by adding 0.4ml of elution buffer and mixing for 5 minutes. Eluate was collected by spinning at maximum speed for 1 minute. This was repeated three times. Eluted samples were neutralised by adding immediately 40µl of 1M sodium phosphate, pH 8.0.

2.5.2 Dialysis

Culture supernatants were transferred to a Slid-a-lyser dialysis cassette (Pierce, 66406) and dialysed against two changes of PBS (10 L) overnight at 4°C.

2.5.3 Isotype analysis

The mouse MAbs were isotyped using an isotyping kit specific for mouse MAbs, i.e. Isostrip Mouse Monoclonal Antibody Isotyping Kit (Boehringer Mannheim, 1493027). The isotyping strip bears immobilised bands of goat anti-mouse antibodies corresponding to the common mouse antibody isotypes and to kappa and lambda light chains. The development tube also contains anti-mouse lambda and kappa chain antibodies immobilised on latex beads, which bind to all mouse monoclonal antibodies regardless of isotype. Upon addition of the test strip to the development tube containing the test antibody/latex bead complex, the complex travels up the strip until it binds to

the goat anti-mouse antibody specific for the monoclonal isotype. Results appear as a blue band in two sections corresponding to the subclass and light chain type.

Hybridoma supernatants were diluted 1/100 and ascities 1/20,000 in PBS and incubated for up to 10 minutes at room temperature. Once the positive control bands were suitably developed, the black strip and the base of the isotyping strip was removed to prevent any further band development.

Mouse MAbs were also istoyped using an ELISA based mouse MAb isotyping kit, (The Mouse Typer®Sub-Isotyping Panel, BIO-RAD, 172-2055).

For the ELISA method, ascities was diluted 1/100 and 1/1000 in carbonate buffer, pH 9.5. 100µl of this ascities solution was added to each well of a chosen column and plates were incubated for 1 hour at 37°C and then overnight at 4°C. Mouse immunoglobulins were included as a positive control. Plates were washed with wash buffer (0.1% (v/v) Tween 20 in PBS (phosphate buffer salts)). Non-specific sites were blocked with 1% (w/v) BSA in PBS for 1 hour at 37°C. The blocking buffer was then discarded and washed as before. 100µl of appropriate rabbit-anti-mouse immunoglobulin (i.e. anti-IgG 1, 2a, 2b, 3, anti-IgM, anti-kappa, and anti-lambda chain etc.) from the kit was added to each row (each column containing immobilised test antibody or standards). Plates were incubated at 37°C for 1 hour. Plates were then washed as previously described. 100µl of goat anti-rabbit immunoglobulin-alkaline phosphatase conjugated antibody diluted in blocking buffer was then added to each well and incubated at 37°C for 1.5 hours. Following the washing, 1 mg/ml p-nitrophenyl phosphate (PNPP, Sigma 104-0) in substrate solution was added to each well and incubated for 0.5-1 hour at 37°C. Colour enhancement was achieved by the addition of 0.2M NaOH. Plates were read on a Titerex ELISA plate reader at 405nm.

2.6 Immunocytochemical Analysis

Table 2.3 Source description and media requirements of cell lines used in immunocytochemical experiments described in this thesis.

Cell line	Cell type description	Basal Medium	Source
DLKP	Human non-small-cell lung carcinoma.	ATCC	NCTCC Law *et al.*, 1992
DLKPA	Human Non-small-cell lung carcinoma. Drug-selected (Adriamycin)	ATCC	NCTCC Clynes *et al.*, 1992
Zr-75-1	Human ductal breast carcinoma	RPMI-1640 (Gibco, 52400-025)	ECACC Engel *et al.*, 1978

NCTCC: National Cell and Tissue Culture Centre, Dublin City University, Glasnevin, Dublin 9, Ireland.

ECACC: European Collection of Animal Cell Cultures, Salisbury, Wiltshire, SP4 OJG.

ATCC: basal media consists of a 1:1 mixture of DMEM and Hams F12.

RPMI 1640: RPMI 1640 media supplied as a 1X stock (Gibco, 52400-025).

2.6.1 Preparation of cytospins

Glass slides were coated with poly-l-lysine (Sigma, P-8920) prior to all immunocytochemical procedures (Huang *et al.*, 1983). Glass slides were washed in 0.5% Tween 20, rinsed thoroughly, immersed in 70% IMS for 10 minutes and dried at 37°C. Approximately 10µl of poly-l-lysine was applied to one end of slides and spread into an even film over whole surface of slides. Coated slides were stored at RT until required.

Cells from actively growing cultures were trypsinised (Section 2.2.2), washed 3 times for 10 minutes in PBS and diluted to a final concentration of $1x10^6$cells/ml in PBS. Cytospins were allowed to air dry over night. Approximately 100µl aliquots of cell suspension were spotted directly onto coated slides, air-dried for 20 minutes after which

excess liquid was tapped off. Again, slides were allowed to air-dry overnight. All slides were foil wrapped and stored at –20°C until required.

2.6.2 Immunocytochemistry

All immunocytochemical studies on cell lines were performed according to the method of Hsu *et al.,* (1981) using an avidin-biotin horseradish peroxidase (HRP) conjugated kit (ABC) plus an appropriate secondary antibody.

Cytospin preparations were fixed for optimal time in ice-cold acetone (6/1C: 1 minute; 5C3: 4 minutes, 4/2D: 10 minutes; 1/11C: 4 minutes) and allowed to air dry for at least 15 minutes prior to immunostaining. Endogenous peroxidase activity was quenched by placing cytospins in 0.6% (v/v) H_2O_2/methanol. All slides were blocked for non-specific staining with 20% (v/v) normal rabbit serum for 20 minutes. Primary antibodies were applied to each sample optimally diluted in 1% TBST (Tris Buffer Saline 0.1% (v/v) Tween 20 (Merck)) (dilutions ranged from neat, 1 in 2 and 1 in 10). Primary antibodies were incubated overnight at 4°C or for 2 hours at RT, which was followed by a 30 minute incubation with biotinylated rabbit anti-mouse IgG (1/300 dilution in TBS/ 0.1% (v/v) Tween 20). Finally, Vectastain Elite ABC reagent (HRP conjugated)(Vector Laboratories, UK PK-7100) was applied for 25 minutes and the peroxidase substrate 3'-3-diaminobenzidine tetrahydrochloride (DAB) peroxidase substrate kit (Vector Laboratories, UK SK-4100) was then applied for 5-7 minutes. All incubations were carried out at RT and slides were washed after each incubation in 3 changes of (TBS/0.1% (v/v) Tween 20 over 15 minutes). Cells were lightly stained with haematoxylin, differentiated in 1% (v/v) acid alcohol and 'blued' in Scott's tap water. Following dehydration in graded alcohol, slides were cleared in xylene and mounted in DPX (BDH, UK). Negative control slides in which primary antibody was replaced by control mouse immunoglobulins (I-2000, Vector Laboratories, UK) (used within the manufacturers recommended concentration range) were included in all experiments.

2.7 SDS PAGE

2.7.1 Gel electrophoresis

Proteins were separated by SDS-PAGE (Laemmli *et al.*, 1970). Gels were prepared as outlined in Table 2.4 and poured into clean 10cm x 8cm gel casting cassettes (Mighty Small™ SE 245, Hoefer, US) which consisted of one glass plate and one aluminium plate separated by 0.75cm^2 plastic spacers. The resolving gel was poured first and allowed to set. A layer of saturated isobutanol (50ml isobutanol: 5ml dH_2O) was gently layered over the resolving gel to prevent drying out. When the resolving gel was set the layer of isobutanol was washed off with several changes of dH_2O. The stacking gel was then poured and a comb was fitted allowing the formation of wells for sample loading. Once the gels had set (at RT) they were wrapped in tinfoil and stored at 4°C if not used immediately. Approximately 15-30µg of protein was applied to each well of the polyacrylamide gel. Pre-stained molecular weight markers (New England Biolabs, UK) were also loaded onto the gel for the determination of the molecular weight of unknown protein samples. Gels were run at 250 volts and 45 milliamps. (Atto power pack, Atto Corp., Japan) for 1-1.5 hr or until the bromophenol blue line reaches 1cm from the end of the gel (values were halved if only one gel was being run), with 1X Tris/Glycine/SDS running buffer (Biorad, 161-0732). When the gel had run fully, electrophoresis was stopped. The gel was then placed in Coomassie stain for approximately 10 minutes and destained until the protein bands become visible. The composition of destain was: methanol 30%, glacial acetic acid 10%, H_2O 60%.

Table 2.4 Preparation of electrophoresis gels

Component	Resolving Gel (12.5% Acrylamide)	Stacking Gel (5% Acrylamide)
Acrylamide/Bis-acrylamide (code A-3574, Sigma, USA)	6.08ml	0.84ml
Distilled H_2O	4.95ml	2.84ml
1.5M Tris/HCL, pH 8.8 (code 161-0798, Bio-Rad, USA)	3.75ml	----------
0.5 M Tris/HCL, pH 6.8 (161-0799, Bio-Rad, USA)	----------	1.25ml
10% SDS (code 161-0416, Bio-Rad, USA)	150µl	50µl
10% Ammonium Persulphate (code A-3678, Sigma USA)	60µl	20µl
TEMED (code T-9281, Sigma, USA)	10µl	5µl

2.7.2 Protein Concentration Determination

Microtitre plate method

Protein concentration was determined by the BCA method (Smith *et al.,* 1985) using a kit obtained from Pierce (Pierce, 23225). Protein samples (crude cell lysates) were diluted to a final volume of 100µl in PBS. BSA protein standards of known concentration were also prepared from a 2mg/ml stock solution in PBS in borosicilliate test tubes. Negative controls consisted of PBS only. 100µl of the BCA reagent (prepared according to the manufacturers instructions) was added to each protein sample, mixed and incubated at 60°C for 30 minutes. Following a brief cooling period, samples were transferred to plastic cuvettes (Elkay) and the absorbence read on a spectrophotometer (Titertek, Multiscan Plus) at 562nm. A standard curve of absorbance versus protein concentration of the BSA standards was constructed. The protein concentration of the test samples was then calculated.

2.8 *In Vitro* Propagation of Hybridoma Cells

2.8.1 Hollow Fibre Bioreactor

All experiments with hollow fibre cultures were performed in the cellmax artificial capillary module (Cellco Inc, 400-011). Both serum-supplemented and the protein-free medium PFHM II were used during the course of these experiments.

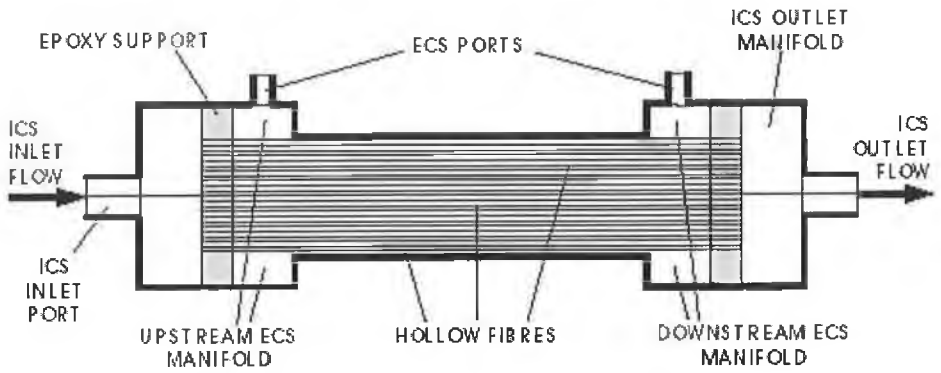


Figure 2.4 Longitudinal section through a typical hollow fibre cartridge. The terms upstream and downstream indicate axial position with respect to the bulk ICS flow. ICS = Intracpaillary space, ECS = Extracapillary space. (Adapted from http://www.iam.ubc.ca/~marek/Chapter1.pdf)

The cellmax system comprises of a pump station and artificial capillary module. The pump station compresses the pump tubing on the artificial capillary module forcing the culture medium to flow through the artificial capillary cartridge. The artificial capillary module is comprised of an artificial cartridge, oxygenation tubing, and a positive displacement pump on a plastic-flow-path stand. It is connected to a medium reservoir bottle by a short length of silicon tubing. Medium is oxygenated during its passage through the coils of silicone tubing on the flowpath.

A pre-culturing step was initially performed. This involved draining the ECS of fluid by flushing pre-warmed medium through the cartridge approximately five times (Figure 2.4). The inlet and outlet tubes from the artificial capillary cartridge were connected to

a bottle containing 100ml of pre-warmed culture medium. This system with medium flowing through the cartridge was set up at a high flow rate for 48 hours. At least two hours prior to inoculation, the system was refreshed with pre-warmed medium.

Cells were inoculated at a density of $5x10^7$cells/ml directly into the artificial capillary module via the side port tubing. The outlet and inlet tubing were closed with slide clamps to prevent ultrafiltration during inoculation. The flow rate of medium through the cartridge was set to low to enable maximum attachment of the cells to the hollow fibres.

The following settings were used for the operation of the Cellmax: 5% CO_2, temperature 37°C, for the first seven days flow-rate was set at 5ml/min to enable an environment where cells could attach to the hollow fibres. On subsequent harvests, depending on the degree of antibody production the flow-rate was increased to 25ml/min and 50ml/min. Each run in the hollow fibre lasted for 21 days. Supernatant was harvested every two days after day 7 for cell viability and productivity analysis. Medium was replaced at each harvest. Glucose and lactose analysis was performed using a glucose sensor kit (G2, MediSense).

2.8.2 Spinner Flask Culture

2.8.2.1 Spinner flask suspension culture

500ml and 1L glass spinners from Bellco (New Jersey) and 10L fermenter vessels from FT Applikon (Holland) were used for scale-up and growth of MAbs. Before glass vessels could be used for cell culture experiments they needed to be treated to prevent cells adhering to the glass.

After sterilisation flasks were rinsed with pre-warmed medium and incubated at 37°C ready for use. Hybridoma cells in the exponential phase of growth were collected in the manner described in Section 2.2.2. Cell counts were carried out using the method described in 2.2.4. A total working volume of 50ml of medium containing cells diluted to the required density were inoculated into the spinner. Flasks were incubated at 37°C on a magnetic stirrer spinner unit (Bellco).

Sampling was carried out in the laminar flow cabinet making sure to keep the agitator rotating to ensure a representative sample from the spinner flask was been obtained. A 1ml sample was taken and centrifuged at 1000rpm for 5 minutes. The pellets were resuspended in 10ml of the appropriate media. Cell counts were carried out as described in section 2.2.3. Cells were re-seeded at the desired inoculation density using fresh media.

2.8.2.2 Depyrogenation

The glass vessels were steeped in 0.5M NaOH overnight to remove any pyrogens that may have attached to the vessel. The NaOH was removed and the vessel was washed thoroughly with purified H_2O. Prior to siliconisation the vessel was dried overnight at 37°C.

2.8.2.3 Siliconisation of Spinner Flasks:

A small amount (approximately 10-15ml in the case of the 500 spinner flasks, 20-25ml in the case of the 1L flask and 60-75ml in the case of the 10L vessel) of 2% 1,1,1-dimethyldichlorosilane solution (BDH 331644V) was added to the flasks, which were then rotated by hand to ensure even coating of all the surfaces (including the agitator). This procedure was carried out with care in the fume hood. The flasks were allowed to air-dry for approximately 1 hour after which time they were rinsed three times in UHP. A small amount of UHP water was left in the spinner flask after the final rinse and the two side arms caps were not fully tightened to prevent steam ventilation during autoclaving. Flasks were then loosely covered with tin foil and sterilised at 121°C at 1bar absolute pressure for 20 minutes.

2.8.3 10 L Bioreator

2.8.3.1 System Background

The ADI Bio Controller ADI 1060, the ADI 1035 Bioconsole and the ADI 1040/25 were used in unison for the process control of the 10L Applikon bioreactor. The purpose of the ADI 1060 was to control and monitor mammalian cell culture systems in a controlled environment where pH, temperature, dissolved oxygen and mixing can be controlled.

The ADI 1035 Bioconsole was an actuator console that was used in combination with the ADI 1060 Bio Controller. It supports gas flow regulation, stirrer speed, pH control, dissolved Oxygen control, level control, temperature control, and liquid addition/withdrawal via pumps.

The ADI 1040/25 functions by connecting the ADI 1060 Bio Controller with the bioreactor and their actuator consoles. The measured sensor signals were amplified in the 1040/25 and were transmitted to the Bio Controller through serial communication. Depending on deviation from the set point, a controller output value was sent to an analog or digital output that would activate the actuator in the ADI 1035 in order to decrease the deviation from the set point. The control system is shown below (Figure 2.5) and outlines the roles played by each component of the process system. The associated 10L Bioreactor was a glass stirred tank reactor.

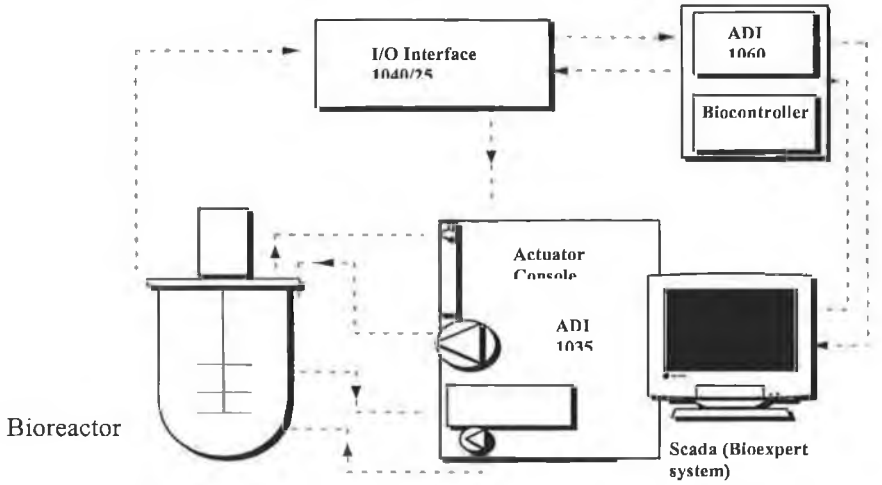


Figure 2.5 Schematic overview of the Applikon "bioprocess" system.

2.8.3.2 Preparation of the glass vessel

As per sections 2.8.2.2 and 2.8.2.3.

2.8.3.3 Preparation of the headplate

(See appendix IV for head-plate description). The ports in the bioreactor are numbered from 1 to 19. The parts associated with the head-plate are mounted in accordance with the diagram in appendix IV. All silicone O-rings were inspected for damage and, if necessary were replaced. When bioreactor parts were mounted in the head-plate, 75cm of C-flex tubing was connected to the bioreactor lid (see Figure X) in the following ports; 2 (a -c), 5 (a - c), 10,12, and 13. A Whatman filter gas exhaust system was added to the C-flex tubing at the following ports; 2a and 10. A Millex FG 50 filter was attached to the c-flex tubes on ports 2 (b+c) and 5(a, b,+ c). The rest of the tubes were folded at the end and tied off with a cable ties

All tubes, which were connected to the bioreactor lid, were secured with the aid of cable ties. This also applied to the connectors and the glassware connected to the silicone tubing.

2.8.3.4 Integrity Testing

2.8.3.4.1 Aeration tubing Test:

The vessel was filled with 10L of water ensuring that all silicon aeration tubing and connections were completely covered. While the baskets were in the water filled bioreactor, air was passed through the tubing. Leakage was signified by the visualisation of bubbles arising from any point in the tubing. A pressure holding gauge test using the Sartocheck was carried out. A digital pressure gauge was attached to one end of the air tubing and air was pumped into the tubing until a 0.6bar pressure was obtained. The other end of the tubing was clamped off. The pressure was not allowed to drop more than 250mbar over 15 minutes to enable successful completion of the test.

2.8.3.4.2 Bioreactor Testing:

The lid of the bioreactor was clamped tightly to the glass vessel. An integrity test was initiated using the Sartocheck by passing air under pressure (0.5bar) into the system ensuring that the pressure drop over 15 minutes was less than 50mbar.

2.8.3.5 Calibration

2.8.3.5.1 Temperature

A water bath was heated to 37°C. The bioreactor temperature probe was removed from its holder and was inserted into the bath until it recorded a stable temperature. A digital thermometer was used to measure the temperature of the water. The temperature recorded on the digital thermometer was entered into the ADI 1060 as the calibrated value for the temperature probe.

2.8.3.5.2 pH

A sample of media was removed aseptically from the bioreactor. The pH was determined using a calibrated pH meter. The value obtained is entered in to the ADI 1060 as the exact pH value of the medium.

2.8.3.5.3 Dissolved Oxygen Concentration

In order to generate a calibration curve to determine the dissolved oxygen concentration at any point during the culture, two points were chosen, an upper and lower limit. Initially nitrogen was gassed into the system. This resulted in a drop of dissolved oxygen concentration to approximately zero. Zero was entered as the first point of the curve in the ADI 1060. Air or oxygen was then gassed into the system until a stable reading of 100% was obtained. At this stage 100 was entered as the upper point of the calibration curve in the ADI 1060.

2.8.3.6 Inoculation

The vessel was filled with 10L of the appropriate medium and supplements. The contents were allowed to adjust to the required pH, temperature and dissolved oxygen levels for a period of approximately 2 hours. The appropriate inoculation density of cells was added as soon as the process conditions had stabilised. Sterility checks were carried out after the addition of each substance to the culture media.

2.8.3.7 Sampling

10ml samples were taken daily to calculate cell number, viability, and glucose/lactate concentrations. Using a welder, a sterile sample bag was connected to the outlet tubing from the culture medium and the sample medium was extracted and analysed.

2.8.3.8 Product Collection

At the end of a culture run, the agitator was switched off in order to allow the cells to settle out of suspension. The remaining culture medium was aseptically siphoned into 1L glass bottles. The medium was subsequently spun down and the supernatant frozen at $-20^{\circ}C$ until required.

2.9 10 L Validation

2.9.1 Performing an Installation Qualification (IQ)

The purpose of this IQ was to provide documented evidence that the Applikon 10L Bioreactor and control units were installed as per design intent and to the manufacturer's recommendations where applicable.

The IQ was the documentation process, which verified that the physical components of a system have been installed according to design specifications. The IQ also served as a final equipment audit prior to equipment operation. The installation of the system was verified by reviewing the equipment installed and using the check sheets provided in this protocol to document that the system components conformed to design specifications. In addition, where applicable, engineering documentation were reviewed and compared to the installed system.

The following list incorporates the headings used and explanations for each step in the validation process. These are only an outline, a full description is given in Appendix VII.

Specification & Documentation Review

This section identified a list of all specification and purchase documentation associated with the bioreactor and verifies that this documentation was complete and accurately describes the installed equipment.

Engineering & Safety Documentation Review

This section identified a list of all engineering documentation associated with the bioreactor and verifies that this documentation was complete and accurately describes the installed equipment.

Equipment List

A list of all installed process and ancillary equipment based on information from the purchase orders and equipment specifications were included here.

Instrument List

A complete list of instruments designated *critical or non- critical* was included in this section.

Critical instruments pertain to those whose performance will affect the operation of the system or the quality attributes of the process material.

Non-critical instruments were those provided for information or convenience purposes only. The information provided included an ID number, manufacturer, location, and description of each item.

Utilities Verification

A complete list of utilities supplied to the equipment based on equipment specifications.

Materials of construction % Lubricants list

A complete list of materials of construction of equipment components and lubricants used was included in this section.

Control of System Hardware

A complete list of major control hardware including installed boards and communications cabling based on information from purchase orders and equipment specifications. Verification that the items have been installed as specified was included. Indication of hardware identification, location, manufacture and description for each item as was applicable was also included.

Input/Output List/Loop check Documentation

Verification of existence and adequacy of loop checks performed by the installing contractor.

Setpoints & Software Parameters

Documented set points and other operating parameter data installed in the control panel or on local instrumentation

Software Documentation

The revision of installed software was recorded. Installation location, including hardware model Number, serial Number, manufacturer, reference specifications, printed copy of annotated applications code (ladder logic etc.), including date and version, software functionality flowcharts and documentation of any modifications made to the software.

Factory Acceptance Testing

Listed all audit and testing documentation including vendor audit report, test procedures and results of any testing performed on the system, including commissioning and Factory Acceptance Tests (FAT). Verified completeness of documentation and satisfactory resolution of any deviations or failed tests.

Standard Operating Procedures

Listed all applicable SOP's including procedures for operation, cleaning, maintenance, change control and calibration. Verified that these were in place, that they accurately describe the applicable equipment and that they were implemented by appropriate personnel. Training records should be available for all personnel operating the bioreactor.

Installational Qualification Discrepancy Summary

Documented any discrepancies or variations noted during the execution of the IQ protocol. Included the resolution of these items and/or any item outstanding that would require resolution in the future.

Once each section in the Validation Qualification has been completed it needs to be checked and signed off by at least three personnel. In this case these were the fermentation manager, the validation supervisor and the QA manager.

2.9.2 Operational Qualification

The purpose of the Operational Qualification (OQ) was to demonstrate that the Applikon 10L Bioreactor and control units operated according to the design intent and manufacturers recommendations.

The Performance Qualification provides documented evidence that the Applikon 10 L bioreactor and Control Units consistently function in accordance with the design specification and were effective in maintaining the growth of mammalian cells over a period, free from contamination. The PQ was the testing process, which evaluates the effectiveness and consistency of the Applikon 10 L bioreactor and Control Units during routine production as defined by the relevant Standard Operating Procedures (SOP's). The PQ verified that the equipment will consistency meet its predetermined specifications. The 10L bioreactor and Control Units were challenged during the PQ stage to determine the ability of the system to maintain adequate culture conditions.

Configuration Table ADI 1060

This section involved recording the sensor card settings for each of the following functions:

- pH
- DO_2
- Digital Output
- Analog Output
- Serial Communications

These were default values and should not change over the course of the fermentation run.

The next part of an operational qualification involved looking at the temperature, pH, DO_2, and level controls and how they reacted when asked to perform a function.

Temperature

To examine the ability of the temperature control system to maintain temperature at a constant the following test was set up and results were recorded.

Cultivation Mode

Control Temperature inside bioreactor at 37°C during cultivation, with a stirrer speed of 100rpm for at least 3 hours.

To test the response of the temperature control system to various commands, the following experiment was initiated and results were recorded.

Test Mode

- The temperature controller on ADI 1060 was initiated.
- "Stepresponses" were created by changing the temperature setpoint using a profile as seen in Table 2.5.
- The criteria for overshoot during cooling and heating were +/-2°C and within an hour after reaching the set-point the temperature must be stable (+/- 0.2°C).
  Periodical data was recorded.

Table 2.5 List of temperature "stepresponses"

SETPOINT CHANGE	DATE	SIGN INSPECTOR	CHECK: PASS/FAIL
37°C → 31°C			
31°C → 35°C			
35°C → 37°C			
37°C → 32°C			
32°C → 31°C			
31°C → 37°C			

pH

To test the response of the pH control system to various commands the following experiment was initiated and results were recorded (Table 2.6)

Test Mode

- The pH control on the AD I1060 was initiated.
- The pH simulator (setpoint 7.0 pH) was connected to the system.
- The value on CO (control output) was measured.

Table 2.6 List of pH commands

SET POINT CHANGE	CO/VALUE (CO %)	CHECK ACTUATOR PASS/FAIL	SET POINT CHANGE	CO/ PUMP BASE	CHECK ACTUATOR PASS/FAIL
4			4		
7			5		
8			6		
9			9		

DO_2

To test the response of the DO_2 control system to various commands the following experiment was initiated and results were recorded (Table 2.7).

Test Mode

- The DO controller in the ADI 1060 was initiated.
- A DO-simulator (setpoint 25%) was connected.
- The CO (control output) was checked.

Table 2.7 Dissolved Oxygen control values.

SIMULATOR	AIR VALVE (CO%)	CHECK ACTUATOR PASS/FAIL	STIRRER SPEED (CO%)	CHECK ACTUATOR PASS/FAIL
0				
50				

2.10 Statistical Analysis

All data was expressed as the mean of the number of replicates being analysed +/- standard deviation (sd)

Samples from the suspension culture of the hybridoma producing cell lines 4/2D and 5C3 (Section 3.4, 3.5, 3.6, 3.7, 3.8, 3.9) were recorded in duplicate and readings shown on graphs were an average of the two readings.

2.10.1 Specific Growth Rate

The growth of cells in batch suspension culture may be represented by the following equation:

$$\frac{dV}{dt} = \mu V \qquad \text{(Equation 1)}$$

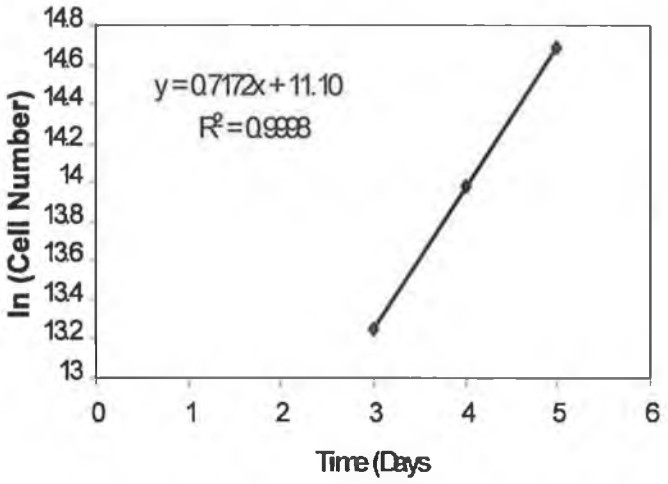
V = viable cell density (cells/mL)

μ = specific growth rate (1/t)

t = time (d)

The equation (1) above implies that the increase in the cell population (dV) at a certain time interval was proportional to the amount of biomass present (V). The symbol μ was the specific growth rate and represents the growth rate of a unit amount of cells.

If μ was constant, then equation (1) may be rearranged:



$$\frac{1}{V}\frac{dV}{dt} = \mu \qquad \text{(Equation 2)}$$

Integrated this gives:

$$\ln V = \ln V_0 + \mu t \qquad \text{(Equation 3)}$$

Where V_0 was the viable cell concentration (cells/ml) at time = 0.

The plot of ln V versus time will be a straight line with a slope of μ, the specific growth rate. It was important to remember that this equation holds true only when μ was constant i.e. $\mu = \mu_{max}$. The maximum specific growth rate, $\mu = \mu_{max}$, occurs during the period of exponential growth.

Sample Calculation

Calculation of the specific growth rate

Days	Cell No. (x10^6 cells/ml)	Ln (Cell number)
3	0.57E+06	13.25
4	1.18E+06	13.98
5	2.38E+06	14.68

u = u(max)= 0.7172d^{-1}
Vo = 6.60E+04 *(Starting conc. of cells)*

Figure 2.8 Sample calculation for the determination of specific growth rate μ.

1. A normal growth curve was tri-phasic with a maximum growth rate between the lag phase and the onset of the stationary phase (days 1-4).
2. Calculate the natural log (ln) of each viable count for days 1-4 and plot against time (days).
3. The slope of the best-fit linear regression line was equivalent to the specific growth rate and the intercept at the y-axis was equivalent to the natural log of the starting concentration of cells, V_0. In the example shown $\mu = \mu_{max} = 0.7172\ d^{-1}$ and $V_0 = 6.6 \times 10^4$ cells/ml.
4. If sufficient data was available within the time periods 0-1 days (lag or adaptation phase and days 4-6 (stationary phase), then these growth rates can be determined in a similar fashion.

2.10.2 Doubling Times/Number of Generations

The doubling time (t_d) simply represents the time taken for a cell population to double in number i.e. to proliferate from V_0 to $2V_0$ cells/ml. By substituting these into equation (3) we get:

$$\ln 2V_0 = \ln V_0 + \mu t_d \qquad \text{(Equation 4)}$$

and rearranging:

$$\frac{\ln 2}{\mu} = t_d \qquad \text{(Equation 5)}$$

The number of doublings or generations, *n*, may be calculated by:

$$n = \frac{1}{\ln 2} \ln \frac{V_f}{V_0} \qquad \text{(Equation 6)}$$

Where V_0 was the starting viable cell density and V_f the final cell density. If calculating the number of generations for the lifetime of a cell in culture, simply total the number of generations calculated at each sub-culture.

Sample Calculation:

Using data from the example shown above, the doubling time was determined as follows:

$$t_d = \frac{\ln 2}{0.7172} = 0.996\ d^{-1}\ or\ 23.19\ hours \qquad \text{(Equation 7)}$$

The number of generations, *n,* using measured data yields:

$$n = \frac{1}{\ln 2} \ln \frac{0.579 x 10^6}{0.66 x 10^5} = 3.127 \qquad \text{(Equation 8)}$$

2.10.3 Standard Deviation

Standard deviation tells how spread out numbers were from the average, calculated by taking the square root of the arithmetic average of the squares of the deviations from the mean in a frequency distribution.

It was calculated as follows:

$$\sigma = \frac{\sqrt{\sum (X - \mu)^2}}{N}$$

X = Sample.

μ = Mean.

N = Total Number of Samples.

Coefficient of Variance (CV):

$$CV = \frac{Standard\ Deviation}{Mean}$$

2.10.4 Accuracy

This is the closeness of agreement between the value, which is accepted either as the conventional or true value or an accepted reference value, and the value found. It is determined as follows:

$$[\frac{Theoretical\ \mathrm{Re}sult - Actual\ \mathrm{Re}sult}{Theoretical\ \mathrm{Re}sult}] \quad *100$$

Section 3 Results

3.0 Hybridoma 6/1C

3.1 Culturing anti-mdr-1 producing hybridoma, 6/1C in serum free and protein free media

A seven-day growth curve and subsequent passaging assays of the cells were carried out to monitor growth characteristics and antibody productivity of the cell line, 6/1C.

The 7-day growth curve was performed to identify the time of maximum cell density and viability of the cells when harvesting cells for scaling up purposes. It was also used to compare the growth characteristics of the cell line in each of the different media in order to identify which media supports the most efficient growth of the cells. The three-day passaging assay looked at the ability of the cells to achieve consistent cell density levels. This is important when looking at the stability of cell lines in culture over time.

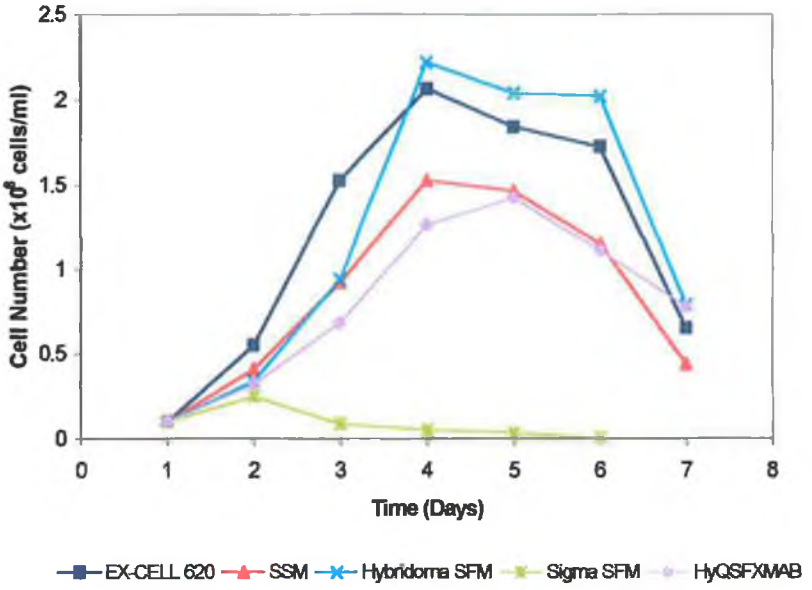


Figure 3.1 Shows comparison of the growth curves of the cell line 6/1C in **SSM** and **SFM** growth media. The cell line was cultured in six different media for a period of 7 days and cells counts were taken daily. Samples on day 6 were retained for further analysis of antibody productivity.

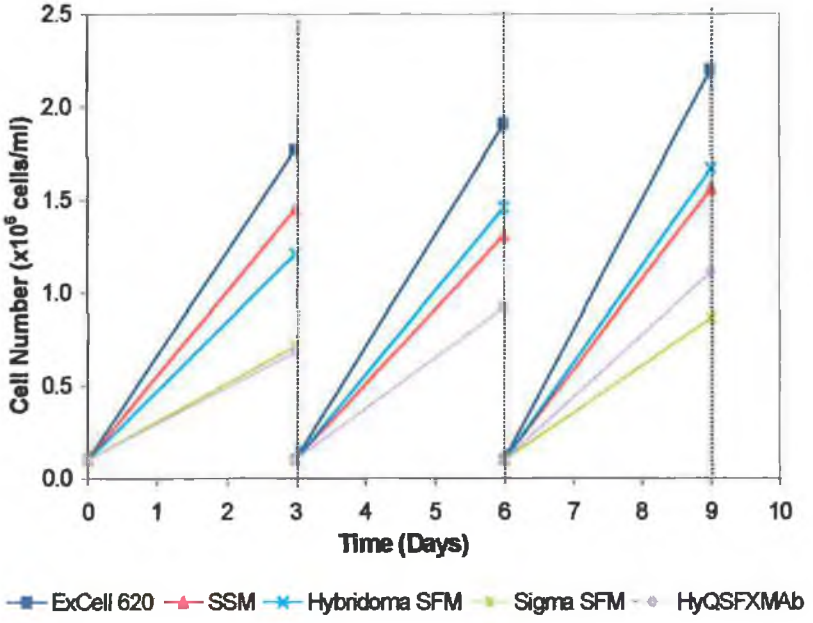


Figure 3.2 Shows comparison of the growth rates of the cell line 6/1C in SSM and SFM during routine passaging. Cells were re-seeded at $1x10^5$cells/ml and fed with fresh medium every three days.

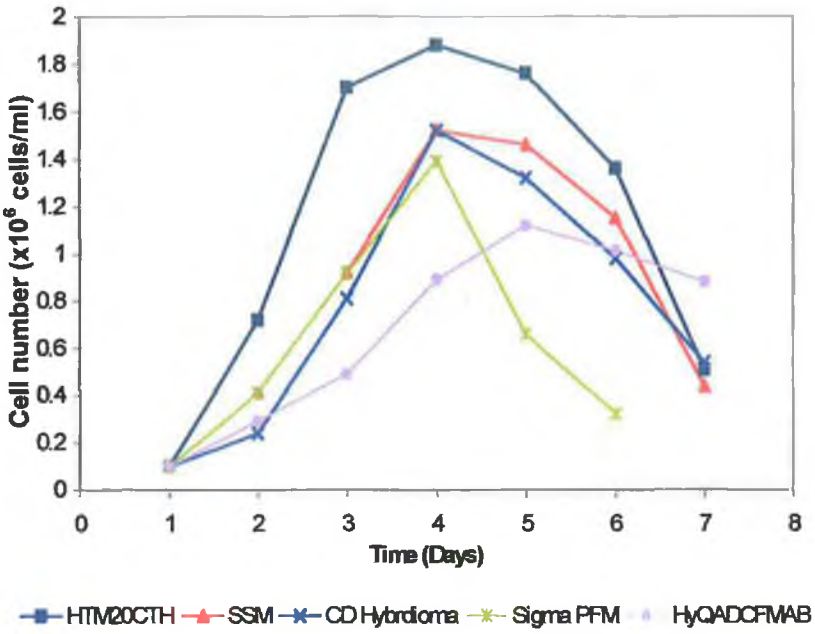


Figure 3.3 Shows comparison of the growth curve of the cell line 6/1C in SSM and PFM growth media. The cell line was cultured in six different media for a period of 7 days with cells counts taken daily. Samples on day 6 were retained for further analysis of antibody productivity.

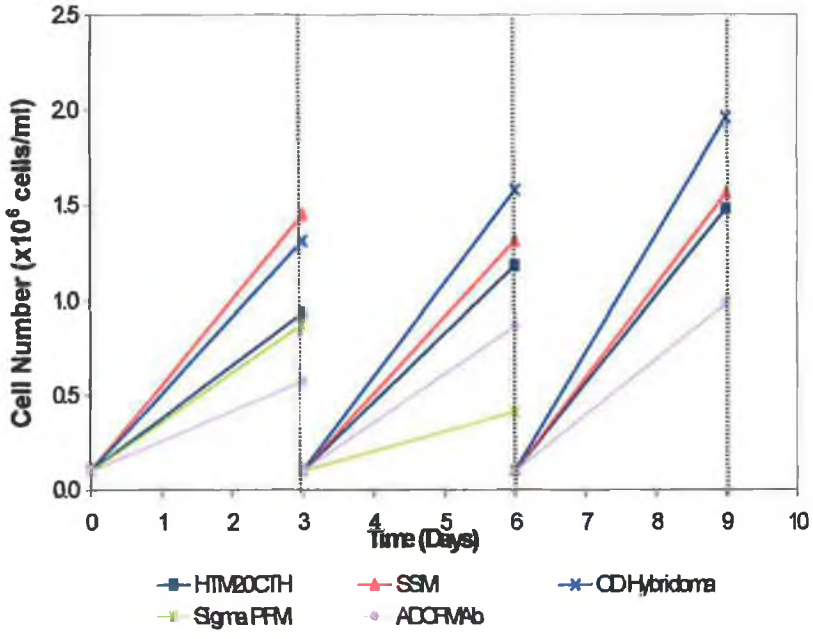


Figure 3.4 Shows comparison of the growth rates of the cell line 6/1C MAb in SSM and PFM during routine passaging. Cells were re-seeded at $1x10^5$ cells/ml and fed with fresh medium every 3 days.

Note: For clarification in Figure 3.2 2nd passage data for Sigma in SFM is superimposed on data for HyQSFXMAb hence it is not to completely visible to the eye. The same applies to Figure 3.4 where Sigma PFM is superimposed behind HyQADCFMAb in the 3rd passage.

The results in Figures 3.1-3.4 indicate that the media, which best sustains the growth of the 6/1C cell line are, in the case of SFM, ExCell 620 and Hybridoma SFM. In terms of the performance of the PFM, both HTM20CTH and CD Hybridoma, achieved higher and similar cell densities respectively, to that achieved with the positive control, SSM. The rest of the media were able to sustain some growth of the 6/1C cell line except for the Sigma supplied SFM, which yielded very poor results in terms of growth.

Cell density in each of the media peaked at day 4 with the exception of the serum free HYQSFX-MAb and the protein free ADCFMAb, both from Hyclone, which achieved maximum cell density at day 5.

In the stationary phase, multiple passage growth assays ExCELL 620, Hybridoma SFM, CD Hybridoma media all performed better in comparison to the serum-supplemented control (DMEM supplemented with 10% FCS). In addition over the assay growth periods the maximum cell density achieved for the cell line in each of the media was consistent.

Overall, the media that performed well in terms of growth were the ExCell 620, Hybridoma SFM and CD Hybridoma media.

3.2 Antibody Detection

3.2.1 ELISA

The ELISA method has proven to be unreliable and inaccurate as a method for quantifying antibody concentrations throughout the course of this work. The standard curve developed was reliable and reproducible but the results obtained for quantifying anti mdr-1, antibody from 1/11C cell line, antibody from 5C3 cell line and anti topisomerase II-α, (4/2D) antibody production in various media and in the ascites across a range of dilutions was never linear or reproducible. This was considered unusual as there were four cell lines producing varying quantities of antibody, and in the case of the antibody 5C3 large amounts of antibody were been produced in culture. This antibody was detected by other means but detection using the ELISA method yielded minimal quantities of antibodies (ranging 0.06-0.027 ng/ml).

In addition, the anti-topoisomerase II-α, 42D ascites did however yield results indicating the presence of antibody in the samples but the results were never linear across the range of dilutions used (range 100-400ng/ml). Optimisation of the ELISA would have needed more work to determine the linear range.

To rule out the possibility that a component of the serum was interfering with the assay reading or the antibodies ability to bind two standard curves were developed, the first standard curve was diluted out in washing buffer and a second was diluted out in DMEM containing 10% FBS and 1% P/S (Table 3.1).

Table 3.1 Shows comparison of the standard concentrations of IgG diluted in washing buffer (1% TBST) and DMEM containing 10% FBS to test for the presence of interfering substances. Absorbance was read at 405nm.

Standard Conc. (ng/ml) (In washing buffer)	*Absorbance (OD)*	*Standard Conc. (ng/ml) (In SSM)*	*Absorbance (OD)*
200.43	0.720	170.36	0.693
101.28	0.604	67.156	0.534
52.15	0.482	36.081	0.396
24.52	0.300	17.658	0.247
12.55	0.195	8.134	0.131

From a subsequent paired t test carried out on this data, it was concluded that the calculated t value of 6.11 is greater than the known table value of 2.132. This allows one to reject the null hypothesis at the p = 0.05 level of significance. One can conclude that the results obtained comparing the standard concentrations of IgG in washing buffer and in serum supplemented DMEM were significantly different from each other. Hence, the medium and the presence of serum may have been interfering with the readings from the assay (Appendix V sample calculation).

An attempt was made to remove the interfering substances in the supernatant by dialysis and then to purify it using the Protein-L spin kit. However, any antibody that was there appeared to be lost in this purification step.

Table 3.2 Examining the effect of dialysis, purification and concentration on the detection of MAb in the supernatant using the ELISA method.

Sample Dilution anti-mdr-1 (6/1C) in SSM	*Dialysis (OD)*	*Protein L-Spin (OD)*	*Concentrated sample (OD)*
Neat	0.042	0.046	0.260
1 in 2	0.027	0.010	0.136
1 in 10	0.021	0.017	0.039

3.2.2 Radial Immunodiffusion (RID) Assay

Typically, the presence of IgG in sample supernatant on a RID plate is signified by the formation of a white precipitin ring. This precipitin ring is due to the formation of antibody-antigen complexes. In the case of each of the anti-mdr-1 monoclonal antibody containing supernatants, no precipitin rings were formed indicating the absence of detectable quantities of antibody in the samples. Controls supplied with the RID kit yielded positive results validating the performance of the kit.

3.2.3 Isotyping

Positive IgG_1 bands were visualised in the positive ascites control (mdr-1 ascites). The bands obtained in the SSM containing mdr-1 were very faint and no bands were recorded for any of the protein free and serum free medium in which the 6/1C cell line was cultured. In order to investigate the presence of IgG in the supernatants, a Mouse IgG isotyping kit based on an ELISA method was used. This test was very specific as to the types of IgG present in the samples. The positive control of mdr-1 ascites yielded a colour change in each of the following wells; IgG_1, IgG_{2A}, IgG_{2B}, IgG_3, IgA (Kappa). This is indicative of an antibody that is not cloned out properly. Generally, one would expect to see a single dominant antibody species in this case IgG_1. Analysis of the mdr-1 in cell culture supernatant yielded a positive result for IgM in both the cases of SSM and PFM, which may be the reason no precipitin rings were identified as the RID kit was specific for IgG.

3.2.4 Nephelometry

The nephelometry results obtained for quantifying anti-mdr-1 MAb were low in comparison with those achieved by the 6/1C cell line in previous tests performed in our laboratory. Typically, values of 30-35mg/L were obtained in the past. However these measurements were carried out using RID, therefore a true comparison cannot be made between these results (Moran *et al*, 1998 Unpublished).

Table 3.3 Nephelometric analysis of antibody production by the anti-mdr-1 (6/1C) cell line when cultured in various serum-supplemented, protein free and serum free medium. Samples were measured after 6 days of cell growth. (For standard curve, refer to Section 3.10.3).

Sample (Medium)	*Antibody Concentration (mg/L)*	*Standard Deviation σ*
DMEM (+10% FBS)	15.56	1.44
HTM20CTH (10X Conc.)	12.27	0.60
EX-CELL 620 (10X Conc.)	8.17	1.16
Hybridoma SFM (10X Conc.)	7.90	1.69
CD Hybridoma (10X Conc.)	12.01	2.28
TCH (10X Conc.)	14.10	0.342
Sigma SFM (10X Conc.)	7.40	0.78
Sigma PFM (10X Conc.)	6.13	0.58

3.2.5 Immunocytochemical analyses of anti-mdr-1 MAb from the hybridoma cell line 6/1C supernatant using the positive control cell line, DLKPA.

Figure 3.5(A) anti-mdr-1 MAb (6/1C) in ascites (+control) (1/50 dilution)

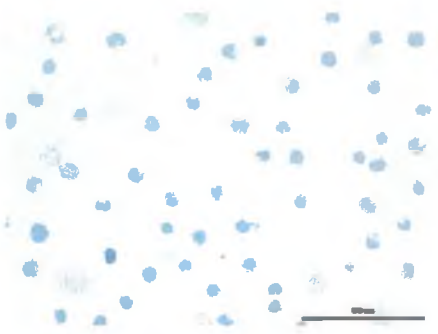


Figure 3.5(B) anti-mdr-1 MAb (6/1C) in ExCell620 (10X conc.) (1/2 dil.)

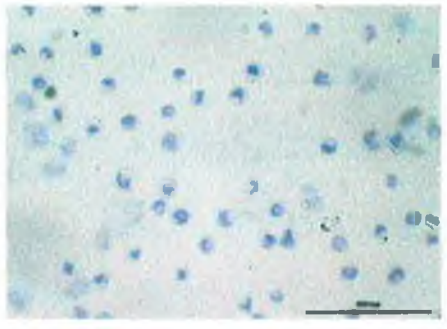


Figure 3.5(C) anti-mdr-1 MAb (6/1C) In Hybridoma SFM (10x conc.) (1/2 dil.)

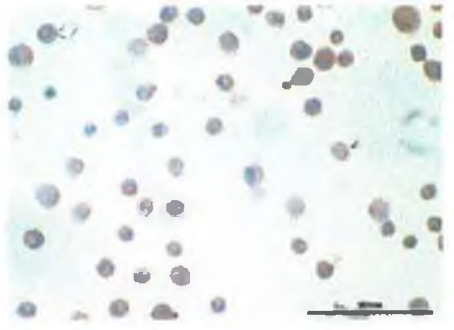


Figure 3.5(D) anti-mdr-1 MAb (6/1C) In SSM (1/2 dil.).

Figure 3.5(E) Negative control (PBS).

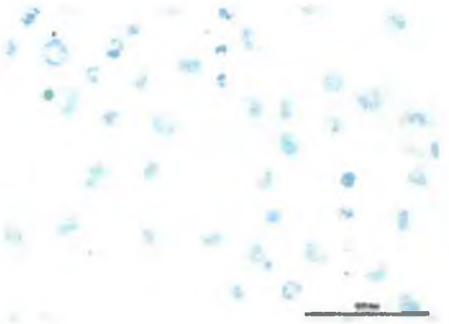


Figure 3.5(F) anti-mdr-1 MAb (6/1C). In HTM20CTH (10x conc.) (1/2 dil.).

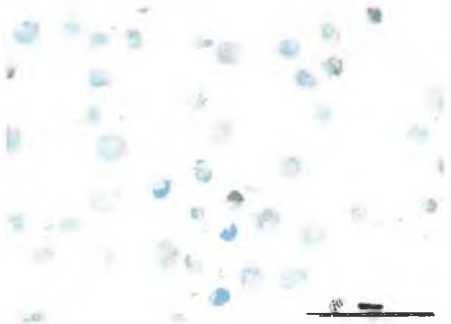


Figure 3.5(G) anti-mdr-1 MAb (6/1C) In CD Hybridoma medium (10X conc.) (1/2 dil.)

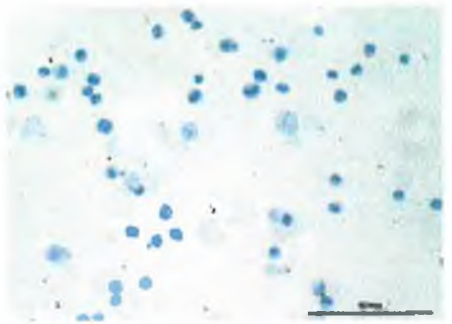


Figure 3.5(H) anti-mdr-1 MAb (6/1C) In Sigma PFM (10x conc.) (1/2 dil.).

Analysis of the immunocytochemical staining of DLKPA cells for the presence of anti-mdr-1 (6/1C) antibody in the following samples yields staining of the plasma membrane. In each of the following samples; ExCell 620 **(B),** Hybridoma SFM **(C),** HTM20CTH **(F),** and Sigma PFM **(H)** no staining of the plasma membrane was observed hence the results are in agreement with those obtained using RID and isotyping methods reiterating the point that IgG_1 not being produced in the cell culture supernatant.

Positive staining was however obtained from the samples cultured in CD Hybridoma **(G).** The staining was not as strong as that obtained for the positive control (ascites produced using 6/1C cell line diluted 1/50) or the mdr-1 cells in serum-supplemented medium.

Overall, antibody production for the mdr-1 cell line shown above was below the values typically achieved in this laboratory in the past and in papers published on the subject (35-50 mg/L) (Moran *et al.,* 1998 Unpublished; Bibila & Robinson, 1995). In addition, when the mdr-1 cell line was sent for commercial analysis, it was rejected due to lack of antibody productivity.

3.3 Growth Medium Supplements

The aim of this experiment was to characterise the growth and antibody productivity of the cell line, 6/1C in three supplemented media, namely OptiMAb, TCH and Glutamax II.

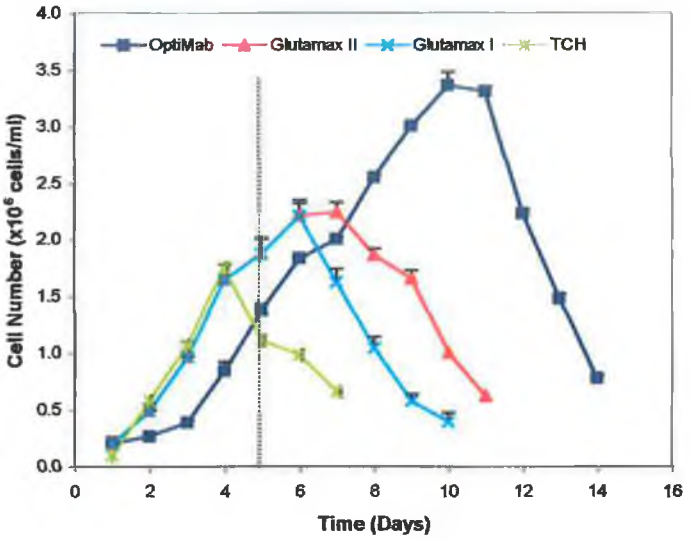


Figure 3.6 Comparison of the ability of DMEM supplemented with OptiMab and Glutamax II to sustain the growth of the hybridoma anti-mdr-1 MAb (6/1C) over time. (Control: serum supplemented DMEM with Glutamax I).

(..... Indicates when Glutamax II only was added to the culture)

3.3.1 Immunocytochemical analyses of anti mdr-1 MAb from the hybridoma cell line 6/1C supernatant using the positive control cell line DLKPA.

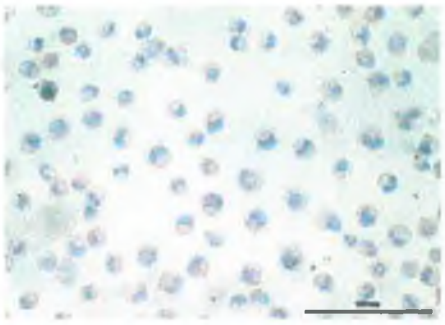


Fig 3.7(A) anti-mdr-1 MAb (6/1C) in in DMEM containing Glutamax I (1/2 dil.).

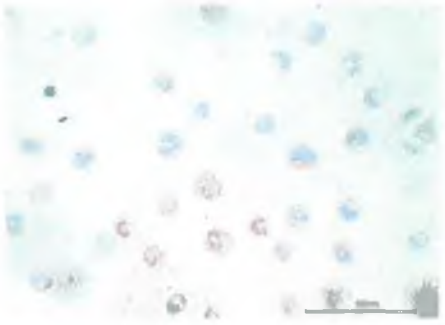


Figure 3.7(B) anti-mdr-1 MAb (6/1C) in DMEM containing Glutamax II (1/2 dil.).

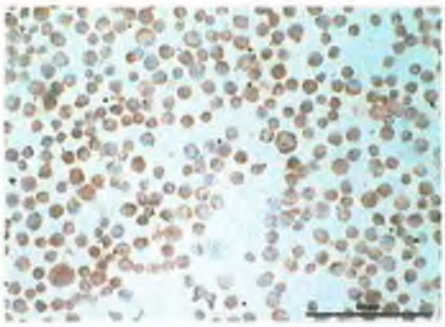


Figure 3.7(C) anti mdr-1 MAb (6/1C) in DMEM containing OptiMab (1/2 dil.).

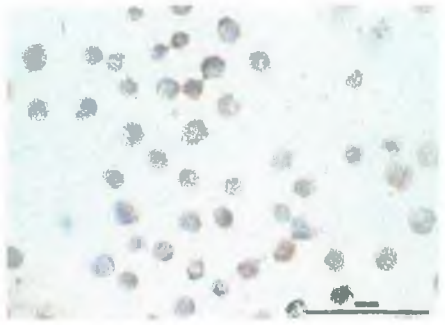


Figure 3.7(D) anti-mdr-1 MAb (6/1C) TCH (10x conc.) (1/2 dil.)

Immunocytochemical (Figure 3.7 A-D) results show positive mdr-1 staining of the plasma membrane for each of the three samples used here. The supernatant from the OptiMab supplemented culture yielding very strong positive staining further backing nephelometric results obtained in Table 3.4. Strong positive staining was also obtained in the presence of the TCH **(D)** supplement.

3.3.2 Nephelometric analysis

Table 3.4 Antibody concentrations obtained in the various DMEM supplemented media. (For standard curve, refer to Section 3.10.3).

Sample	Antibody Concentration (mg/L)	Standard Deviation σ
DMEM Glutamax I	15.56	1.44
DMEM + Glutamax II	19.21	2.01
DMEM +OptiMab	22.23	1.32

From Figure 3.6 the presence of the supplement OptiMab yielded a large increase in cell number and an increased exponential phase of growth in comparison to the control medium serum supplemented DMEM, and the other test medium Glutamax II supplemented DMEM. Feeding the cells with Glutamax II on day 5 only extended the culture in stationary phase for one day.

Immunocytochemical analysis shows strong staining of the plasma membrane for each of the supplemented samples.

The nephelometric results (Table 3.4) show increased antibody productivity for the culture of cells in medium supplemented with OptiMab.

However, the quantities of antibody achieved throughout the course of this work are lower than that previously produced by this cell line and other cell lines both in our laboratory and in published data. Typical quantities of antibody produced from hybridoma cell lines are approximately 50-60 mg/L. With this particular cell line in previous work concentrations in the range 34-54 mg/ml were obtained in standard static culture flasks (Moran *et al.*, 1998 Unpublished; Bibila & Robinson, 1995). Work carried out by an independent commercial company on the cell line 6/1C concluded that low levels and in some cases no antibody was being produced in culture. For this

reason, it was decided that it was not feasible to carry out further scale up and optimisation studies on this cell line.

Three new cell lines were chosen from the laboratory stocks, their adaptation to protein free conditions and subsequent scale up to large scale is detailed in the next sections 3.4, 3.5 & 3.6.

3.4 Hybridoma 4/2D

3.4.1 Culture of anti-Topoisomerase II-α producing cell line 4/2D in serum supplemented and protein free conditions

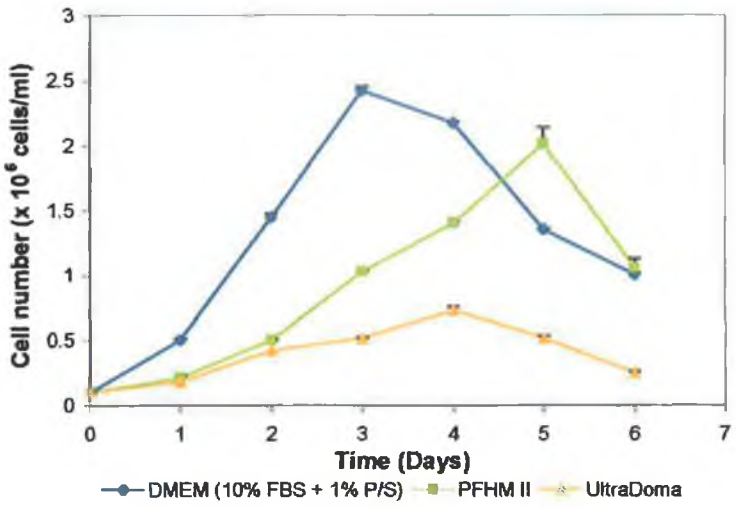


Figure 3.8 Shows comparison of the growth of the hybridoma producing cell line 4/2D in two protein free media versus the control medium serum supplemented DMEM. Samples were taken daily for cell viability analysis and on day 6 for antibody concentration determination.

3.4.2 Antibody Analysis

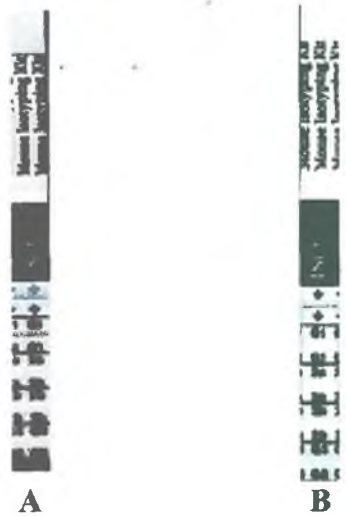


Figure 3.9 Isotyping analysis of **A)** MAb 4/2D in SSM, **B)** MAb 4/2D in PFM

Table 3.5 RID and nephelometric analysis of the hybridoma producing anti-Topoisomerase II-α MAb in serum supplemented and protein free conditions. N= 3 (For nephelometric standard curve refer to Section 3.10.3, RID sample calculation refer to Section 2.4.3)

Medium	*RID (mg/L)*	*Standard Deviation σ*	*Nephelometer (mg/L)*	*Standard Deviation σ*
DMEM (+10% FBS)	3.84	3.64	22.5	1.45
PFHM II	1.10	4.12	19.5	0.97
UltraDoma	1.685	5.92	9.2	1.07

Figure 3.9 shows isotyping for MAb 4/2D in SSM and PFM media. Figure 3.9A shows a strong positive band indicating the presence of IgG_1. Isotyping on the PFM sample yielded positive bands for IgG_{2A} and IgG_3.

The results in table 3.5 show the difference in results obtained between the two quantitative methods used to determine antibody concentrations. It was very difficult to make an accurate determination of results from the RID assay as it was difficult to read the small precipitate bands making it less objective than nephelometry. The nephelometric assay would be deemed more sensitive to measuring MAb in culture supernatant.

3.4.3 Immunocytochemical analyses of anti-topoisomerase II-α MAb from the hybridoma cell line 4/2D supernatant on the positive control cell line, DLKPA.

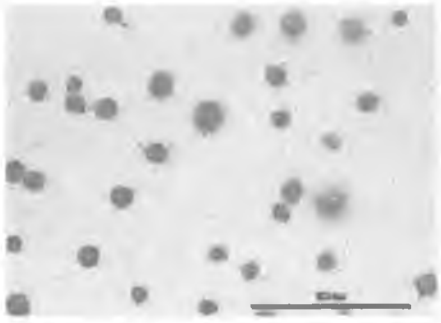


Figure 3.10(A) anti-topo II-α (4/2D) in SSM (1/2 dil.)

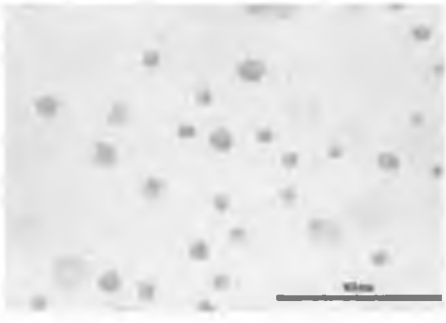


Figure 3.10(B) anti-topo II-α (4/2D) in PFHM II (1/2 dil.).

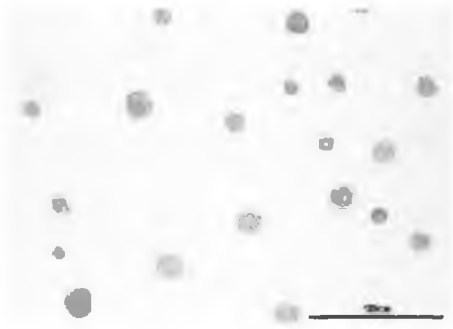


Figure 3.10(C) anti-topo II-α (4/2D) cultured in UltraDoma (½dil.)

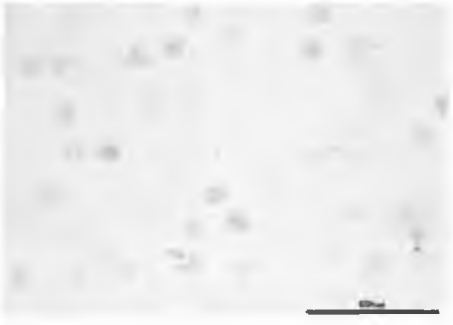


Figure 3.10(D) Negative control

Immunocytochemical analysis of the supernatant from the hybridoma producing anti-Topoisomerase II-α cell line, 4/2D in both serum supplemented and protein free culture (Figures 3.10 (A-D)) conditions showed positive staining for antibody in all of the cases.

3.4.4 SDS PAGE analysis

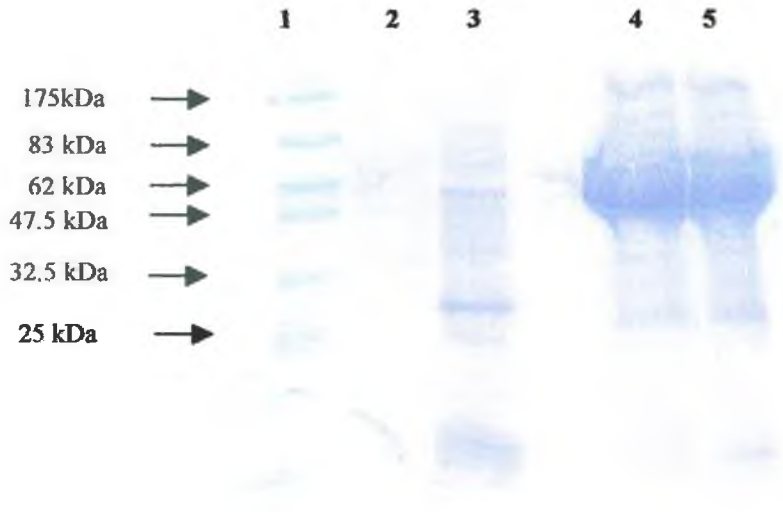


Figure 3.11 SDS PAGE (reducing gel) analysis of the hybridoma producing anti-Topisomerase II-α MAb (4/2D) cultured under protein free conditions and serum supplemented conditions. **Lane 1:** Markers, **Lane 2:** 4/2D in protein free medium (unconc.), **Lane 3:** 4/2D in protein free supernatant (conc. 10X.), **Lane 4:** 4/2D in serum supplemented medium (conc. 10X), **Lane 5:** 4/2D in serum supplemented medium (unconc.). Samples were seperated on a 12.5% SDS PAGE.

SDS-Page analysis of the supernatant from the anti-topoisomerase II-α MAb in protein free supernatant yielded clear bands at the 25 and 55kDa position in the concentrated supernatant at the expected molecular weights. Supernatant from the serum supplemented samples showed a band at ~55-70kDa and another band at 25kDa.

3.4.5 Post-purification Analysis using the NAb™ Protein L Spin Kit

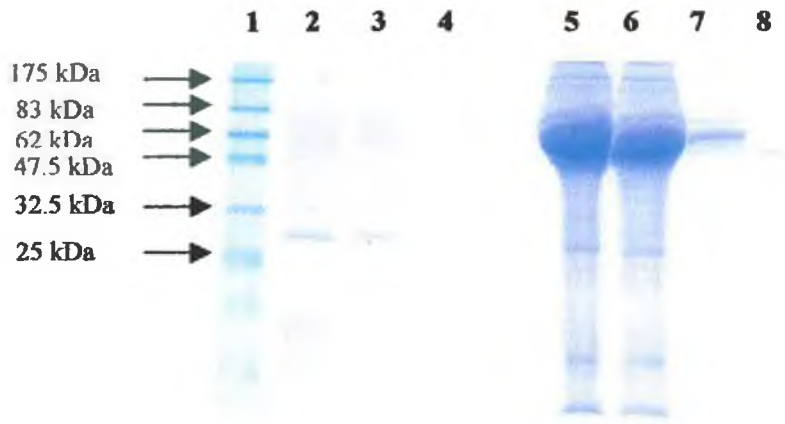


Fig 3.12 SDS PAGE (reducing gel) analysis following purification of the topoisomerase II-α MAb cultured in protein free and serum supplemented conditions (Section 2.5.1). **Lane 1:** Markers, **Lane 2:** protein free supernatant, **Lane 3:** Eluted protein free sample, **Lane 4:** Unbound protein free supernatant after purification, **Lane 5:** Serum supplemented supernatant, **Lane 6:** Unbound serum supplemented supernatant after purification, **Lane 7:** Serum supplemented wash after purification, **Lane 8:** Eluted serum supplemented sample. Samples were separated on 12.5% SDS PAGE.

3.5 Hybridoma 5C3

3.5.1 Culture of antibody producing cell line 5C3 in serum supplemented and protein free medium

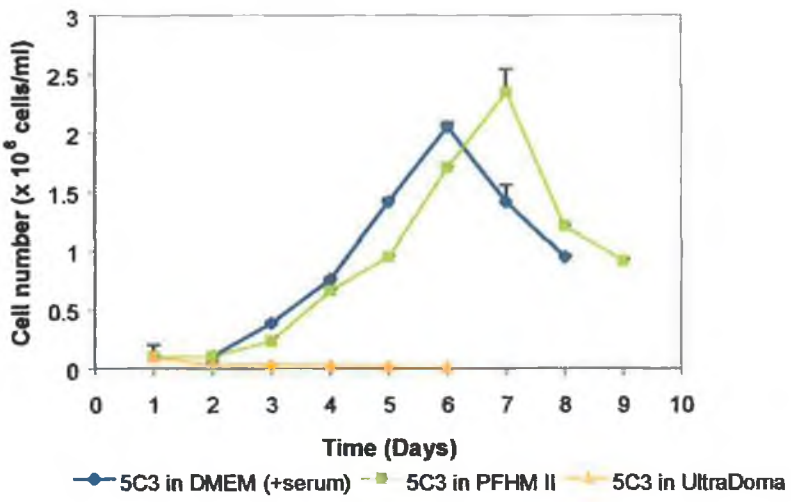


Figure 3.13 Shows comparison of the growth of the hybridoma producing 5C3 MAb in two protein free media against the control SSM. Samples were taken daily for cell viability analysis and on day 6-antibody concentrations were determined.

3.5.2 Antibody Analysis

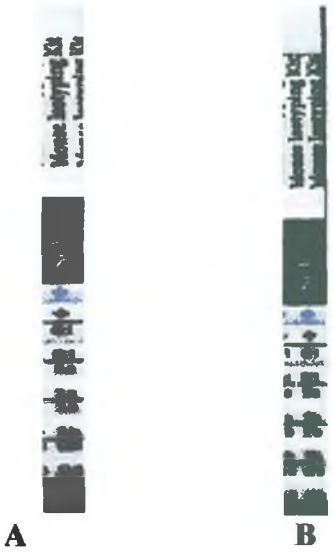


Figure 3.14 Isotyping analysis of **A)** MAb 5C3 in SSM, **B)** MAb 5C3 in PFM

Table 3.6 RID and nephelometric analysis of the antibody 5C3 in serum supplemented and protein free conditions. N = 3 (For nephelometric standard curve refer to Section 3.10.3, for RID sample calculation refer to Section 2.4.3)

Medium	*RID (mg/L)*	*Standard Dev. σ*	*Nephelometry (mg/L)*	*Standard Dev. σ*
DMEM (+10% FBS)	4.26	3.6	18.75	0.91
PFHM II	16.2	2.8	175.64	3.21
UltraDoma	1	2.73	2.79	0.548

Figure 3.14 shows isotyping for MAb 5C3 in SSM and PFM media. Figure 3.14A and B show strong positive bands indicating the presence of IgG_1 in both the SSM and PFM.

The results in table 3.6 show the difference in results obtained between the two quantitative methods used to determine antibody concentrations. It was very difficult to make an accurate determination of results from the RID assay, as it was difficult to read the small precipitate bands making it less objective than nephelometry. The nephelometric assay is a more sensitive method of measuring MAb in cell culture supernatant.

3.5.3 Immunocytochemical analyses of MAb from the hybridoma cell line 5C3 supernatant using the positive control cell line, ZR-75-1

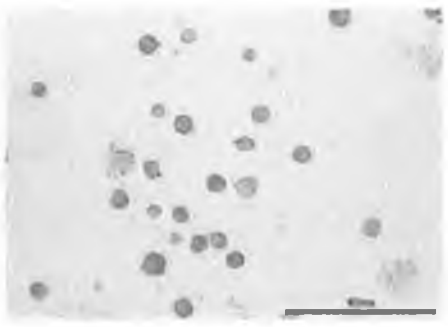


Figure 3.15 (A) 5C3 in SSM (1/2 Dil.)

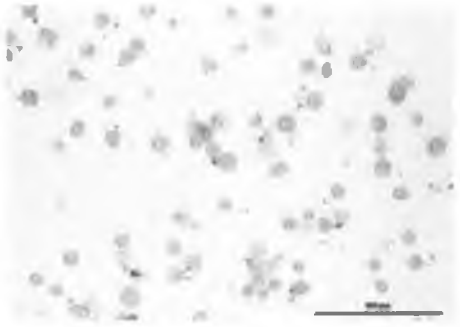


Figure 3.15 (B) 5C3 in PFHM II (1/2 Dil.)

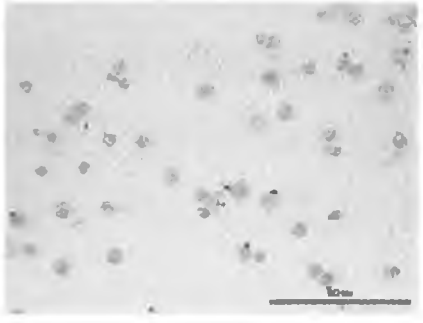


Figure 3.15 (C) Negative control (PBS)

Immunocytochemical analysis of supernatant from the hybridoma cell line, 5C3 cultured in the protein free (PFHM II) and serum supplemented medium yielded positive staining indicating the presence of antibody.

3.5.4 SDS PAGE Analysis

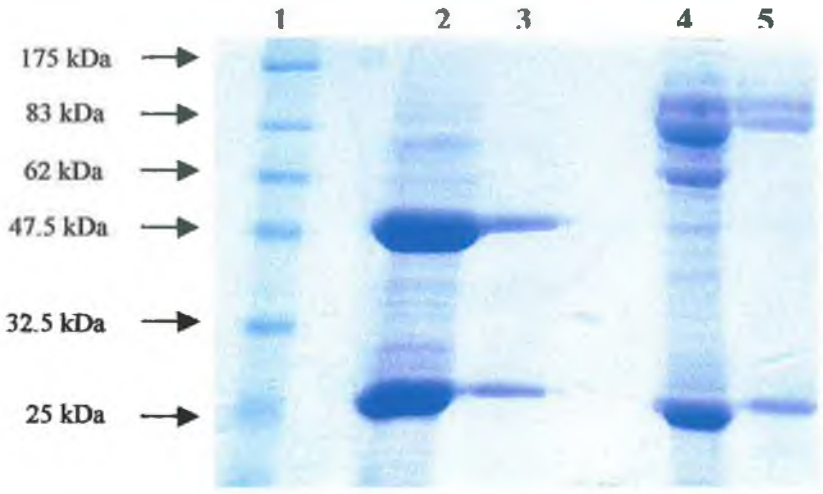


Figure 3.16 SDS PAGE (reducing gel) analysis of antibody produced by cell line 5C3 cultured under protein free conditions. **Lane 1:** Markers, **Lane 2:** 5C3 in Protein free medium (conc. 10X), **Lane 3:** Protein free supernatant (unconc.), **Lane 4:** 5C3 in serum supplemented medium (conc. 10X), **Lane 5:** 5C3 in serum supplemented medium (unconc.). Samples were seperated on 12.5% SDS-PAGE.

From the SDS-PAGE analysis the results indicated the presence of heavy and light chains in the serum-supplemented and protein free supernatants. The aggregates formed in the serum-supplemented samples were probably as a result of the presence of BSA in serum, which has a molecular weight of approximately 68kDa.

3.5.5 Post-purification Analysis using the NAb™ Protein L Spin Kit

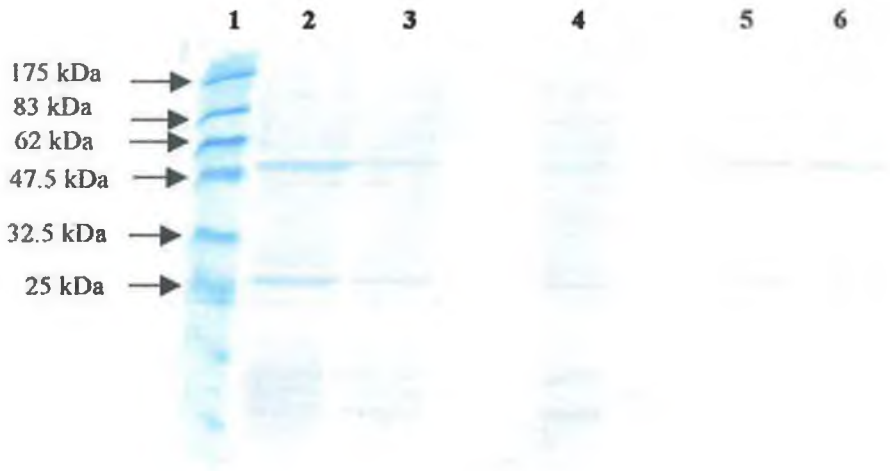


Figure 3.17 SDS PAGE (reducing gel) analysis following purification of the hybridoma producing MAb 5C3 cultured under protein free conditions (Section 2.5.1). **Lane 1:** Markers, **Lane 2:** Protein free supernatant, **Lane 3:** Unbound protein free supernatant after purification, **Lane 4:** Protein free wash after purification, **Lane 5:** Elution protein free sample (1), **Lane 6:** Eluted protein free sample (2). Samples were seperated on a 12.5% SDS-PAGE.

3.5 Hybridoma 1/11C

3.6.1 Culture of antibody producing cell line 1/11C in serum supplemented and protein free media

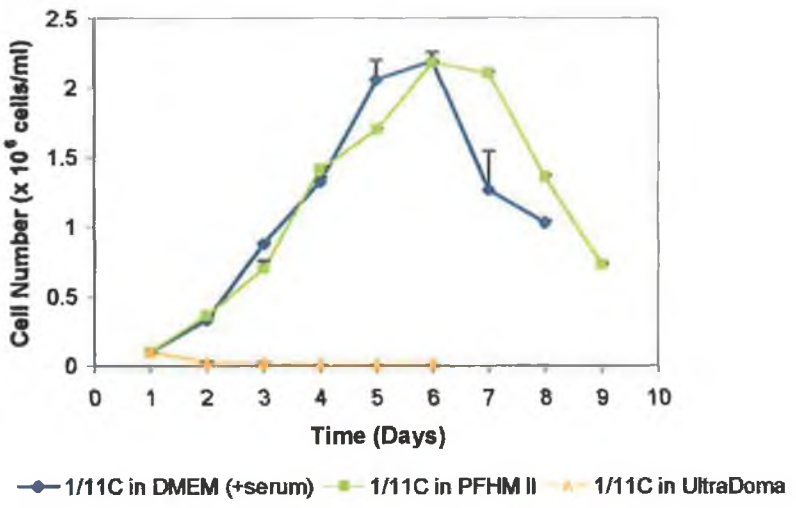


Figure 3.18 Shows comparison of the growth of the hybridoma producing MAb 1/11C in two protein free media against the control SSM. Samples were taken daily for cell viability analysis and on day 6 for antibody concentration determination.

3.6.2 Antibody Analysis

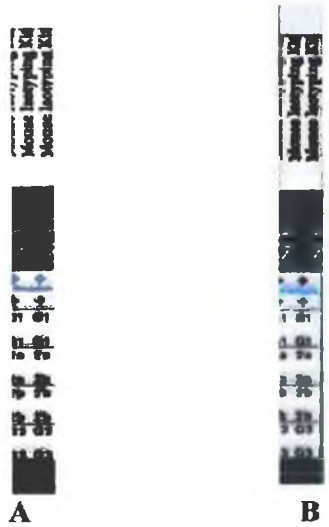


Figure 3.19 Isotyping analysis of **A)** MAb 1/11C in SSM, **B)** MAb 1/11C in PFM

Table 3.7 RID and nephelometric antibody analysis of the hybridoma MAb 1/11C in serum supplemented and protein free conditions. (For nephelometric standard curve refer to Section 3.10.3, RID sample calculation refer to Section 2.4.3)

Medium	*RID (mg/L)*	*Standard Dev σ*	*Nephelometry (mg/L)*	*Standard Dev σ*
DMEM (+10% FBS)	1.94	2.11	8.12	0.58
PFHM II	4.7	1.68	6.4	1.11
UltraDoma	1	3.24	1.89	0.8

Figure 3.19 shows that the isotyping analysis of the 1/11C antibody yielded no positive indication of the presence of IgG instead faint bands indicative of IgM were observed. Further analysis using the ELISA based mouse MAb isotyping kit (The Mouse Typer®Sub-Isotyping Panel) resulted in positive IgM bands being formed.

The results in table 3.7 indicate the difference in results obtained between the two quantitative methods used to determine antibody concentrations. It was very difficult to make an accurate determination of results from the RID assay, as it was difficult to read the small precipitate bands making it less objective than nephelometry. The nephelometric assay is a more sensitive method of measuring MAb in cell culture supernatant.

3.6.3 Immunocytochemical analysis of MAb from the hybridoma cell line 1/11C supernatant using the positive control cell line, DLKPA.

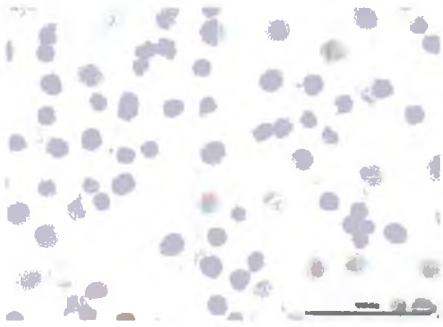


Figure 3.20(A) MAb 1/11C in SSM (1/2 dil)

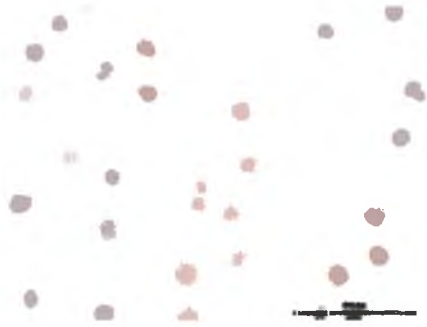


Figure 3.20(B) MAb 1/11C in PFHM II (1/2 dil)

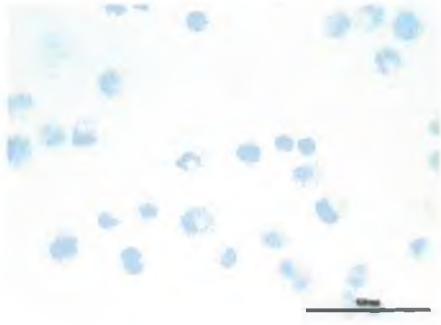


Figure 3.20(C) Negative control (PBS)

In contrast to the results obtained from the isotyping, RID and nephelometric analysis of the hybridoma producing MAb 1/11C cell line in SSM for antibody, immunocytochemical analysis yielded strong positive staining indicative of the presence of antibody.

Overall, the medium UltraDoma was unable to sustain the growth of the cell lines sufficiently, throughout the course of this experiment. Hence, the protein free medium PFHM II, which performed similarly to the positive control of serum supplemented DMEM was selected for further studies (Table 3.8).

As a consequence of the higher antibody and cell density levels achieved in the hybridoma producing cell lines 4/2D and 5C3 in both serum free and protein free medium these cell lines were chosen for comparison studies in further scale up experiments.

Table 3.8 Effect of different media on the growth of the hybridoma producing cell lines 4/2D, 5C3 and 1/11C.

	SSM	**UltraDoma**	**PFHM II**
4/2D	+++	+	++
1/11C	++	-	++
5C3	++	-	+++

3.7 Scale up and process optimisation for the production of anti-topoisomerase II-α antibody (4/2D)

Monoclonal antibody production was scaled from static flasks to a 10L-bioreactor using $75cm^2$, $150cm^2$ static flasks, 250ml and 1L spinner flasks as intermediaries for cell production. In addition, 250ml spinner flasks were also used to perform process optimisation experiments with special attention given to temperature and agitation optimisation.

3.7.1 Agitation

The aim of this experiment was to determine the most efficient agitation rate in terms of antibody productivity and cell growth in which, to produce MAb from the hybridoma producing cell line 4/2D both in SSM and PFM conditions. See section 1.2.5 for a detailed discussion on the effects of agitation.

Cells were cultured at 50, 100 and 200rpm. The effects of these agitation rates on growth and antibody productivity are shown in Figure 3.24.

At 50rpm in the serum-supplemented culture of the 4/2D cell line, clumping was observed. Culture at 100 rpm yielded cell densities in excess of $2.70x10^6$cells/ml. Antibody productivity was enhanced over that achieved at 50rpm but was very similar to levels achieved at 200rpm. Agitation at 200rpm resulted in a peak growth of $2.38x10^6$cells/ml, one day earlier than culture at 100rpm; cell viability rapidly declined after this stage (Appendix II).

The most efficient agitation rate for the growth of cells from the hybridoma producing cell line 4/2D in protein free medium was 100rpm. However, in terms of the concentration of antibody produced it is clear that agitation of 200rpm yielded the best results (Figure 3.24 C & D). This may be related to the lower specific-growth rate observed under these conditions.

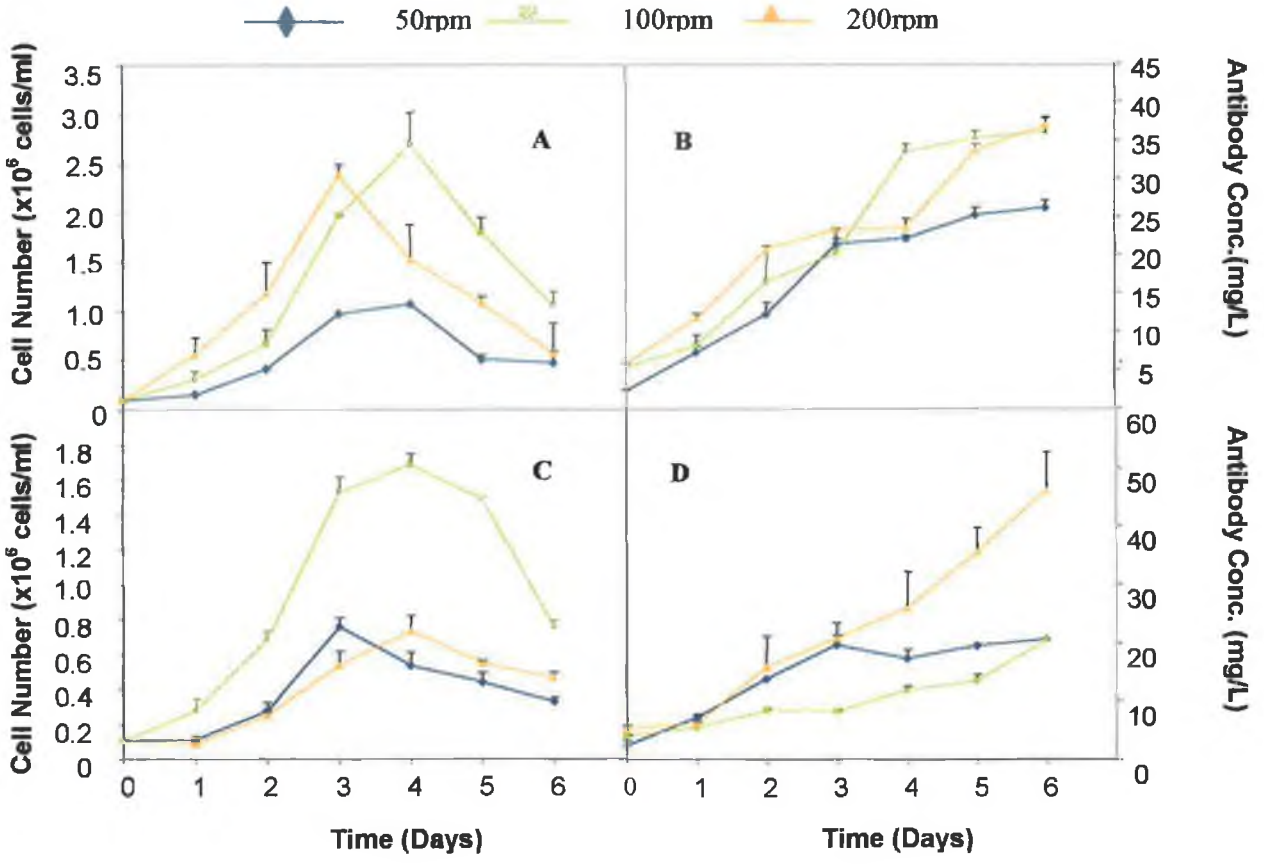


Figure 3.24 Shows cell growth and antibody productivity data from 4/2D cells grown in SSM (A & B), PFM (C & D) at varying rates of agitation.

Table 3.9 Tabulated specific growth rates, doubling time and number of generations, data for the culture of 4/2D in SSM and PFM at varying rates of agitation. Each run was carried out in triplicate therefore data shown is an average of three (CV's < 10%).

	SSM			*PFM*		
Agitation Rate (rpm)	**Specific Growth Rate μ**	**Doubling Time (td)**	**Number of generations *n***	**Specific Growth Rate μ**	**Doubling Time (td)**	**Number of generations *n***
50	*0.4624*	*18.20*	*4.37*	*0.987*	*16.80*	*4.56*
100	*0.672*	*24.75*	*2.92*	*0.6735*	*24.66*	*4.06*
200	*0.6936*	*23.98*	*2.12*	*0.5432*	*30.60*	*2.35*

In summary, the results shown here suggest that the large-scale agitation was most efficient in the case of SSM at 100rpm and PFM 200rpm

3.7.2 Temperature

The aim of this experiment was to determine the most efficient temperature in terms of antibody productivity and cell growth in which, to produce MAb from the hybridoma producing cell line 4/2D in both SSM and PFM conditions. See section 1.2.7 for a detailed discussion on the effects of temperature.

Growth characteristics for the culture of the 4/2D cell line in serum-supplemented medium at 34°C and 37°C were almost identical, yielding maximum cell densities in the range 2.7–2.8x10^6cells/ml. Antibody yields were however reduced at 34°C where a final concentration of approximately 27mg/L was achieved compared to 37 mg/L at 37°C. In contrast to this, growth at 39°C was very poor yielding a maximum cell density of approximately 0.3x10^6cells/ml and an antibody concentration of 12mg/L. Specific growth rate, doubling time and generation values were all similar for culture at 34°C and 37°C. However, culture at 37°C yielded better antibody productivity.

The protein free culture of the hybridoma producing 4/2D cell line yielded maximum cell density when cells were cultured at 37°C. A two-fold increase in cell number was observed over that at 34°C. Antibody productivity did not however yield the same trend. Final antibody concentration at 34°C was approximately 10 mg/L greater than that achieved at 37°C.

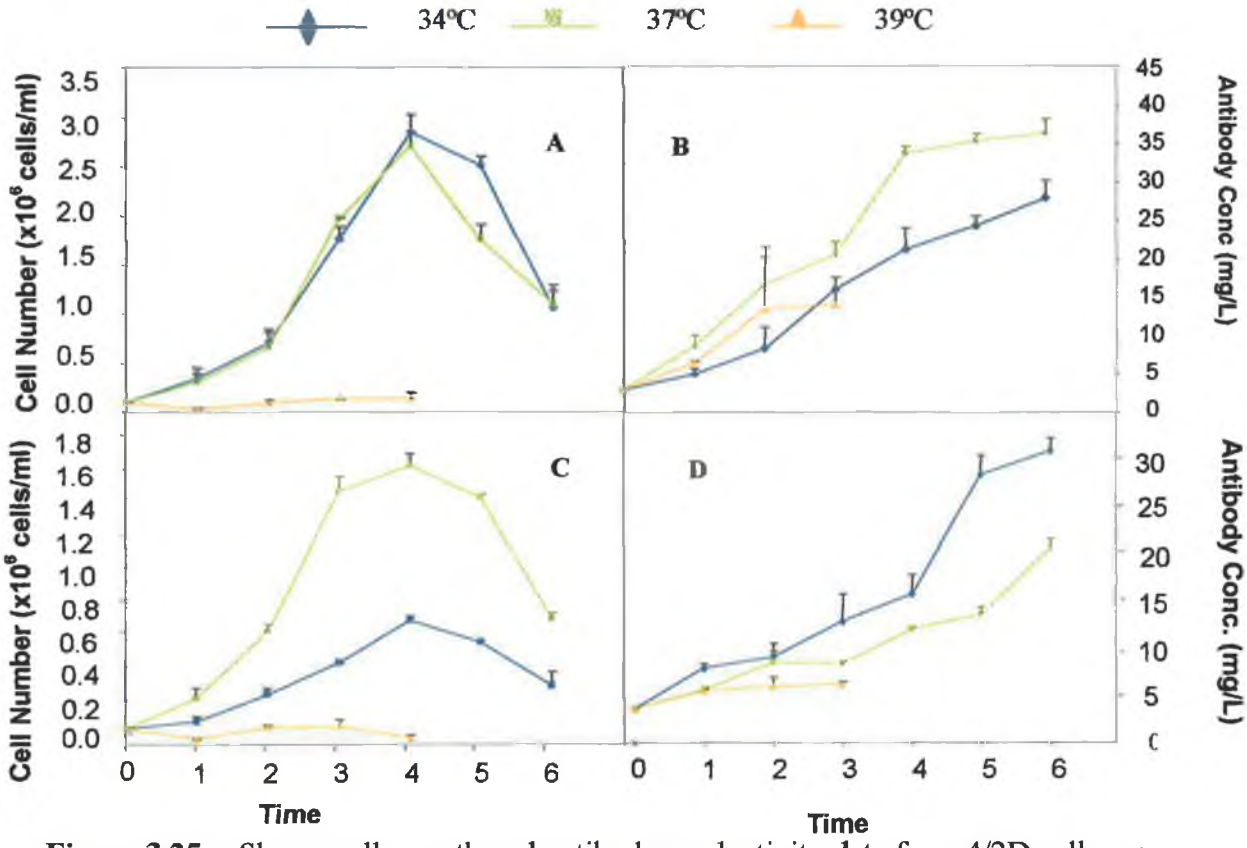


Figure 3.25 Shows cell growth and antibody productivity data from 4/2D cells grown in SSM (A & B), PFM, (C & D) at varying degrees of temperature.

Table 3.10 Tabulated specific growth rates, doubling time and number of generations, data for the culture of 4/2D in SSM and PFM at varying rates of agitation. Each run was carried out in triplicate (average of three with CV's < 10%).

	SSM			*PFM*		
Temperature (°C)	**Specific Growth Rate μ_{app}**	**Doubling Time (td)**	**Number of generations *n***	**Specific Growth Rate μ_{app}**	**Doubling Time (td)**	**Number of generations *n***
34°C	*0.6958*	*23.90*	*2.49*	*0.2139*	*77.75*	*0.462*
37°C	*0.672*	*24.75*	*2.92*	*0.6735*	*24.66*	*4.05*
39°C	Not enough data to compile Results			*0.6198*	*26.83*	*2.76*

In summary, maximum cell growth and antibody production was achieved in the serum-supplemented cultures at 37°C and 100rpm. In the protein free culture, the maximum cell growth was achieved at, 37°C and 200rpm but antibody production reached its highest level when the cells were cultured at 34°C.

The optimum process conditions described here were then used to culture cells at large-scale (10L bioreactor).

3.7.3 10L Fermentation Run

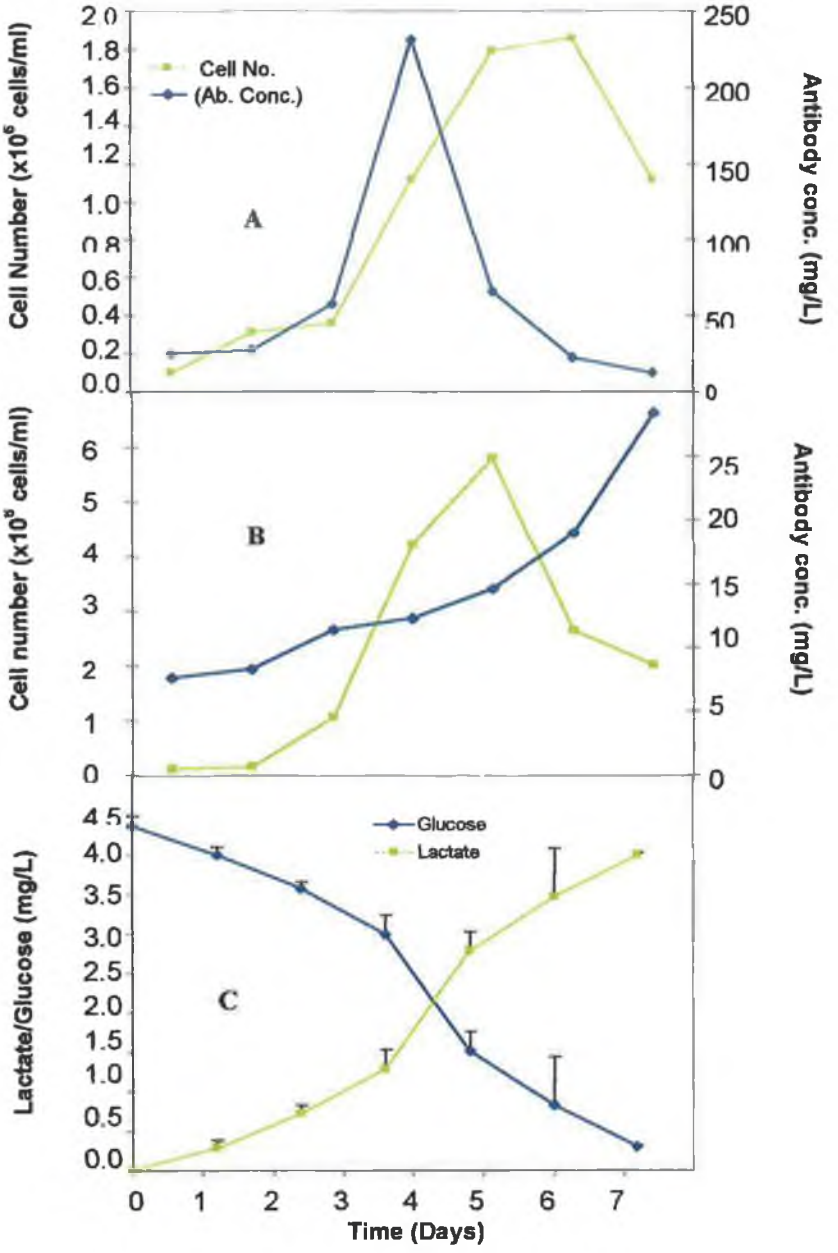


Figure 3.26 Shows cell growth and antibody production characteristics for 4/2D cultured in SSM at the 10L-batch scale. **A)** Batch 1, **B)** Batch 2. **C)** Average glucose versus lactate concentrations. Samples were analysed daily for antibody, lactose, glucose and cell viability.

The two runs performed are shown here as the results are very different and there would have been a huge difference in standard deviation if they were compared.

Table 3.11 Tabulated specific growth rates, doubling time and number of generations data for the culture of 4/2D in SSM.

	Specific Growth Rate μ_{app} (d^{-1})	**Doubling Time (td)**	**Number of generations** *n*
Run 1	*0.80*	*20.70*	*2.63*
Run 2	*0.4071*	*40.09*	*1.29*

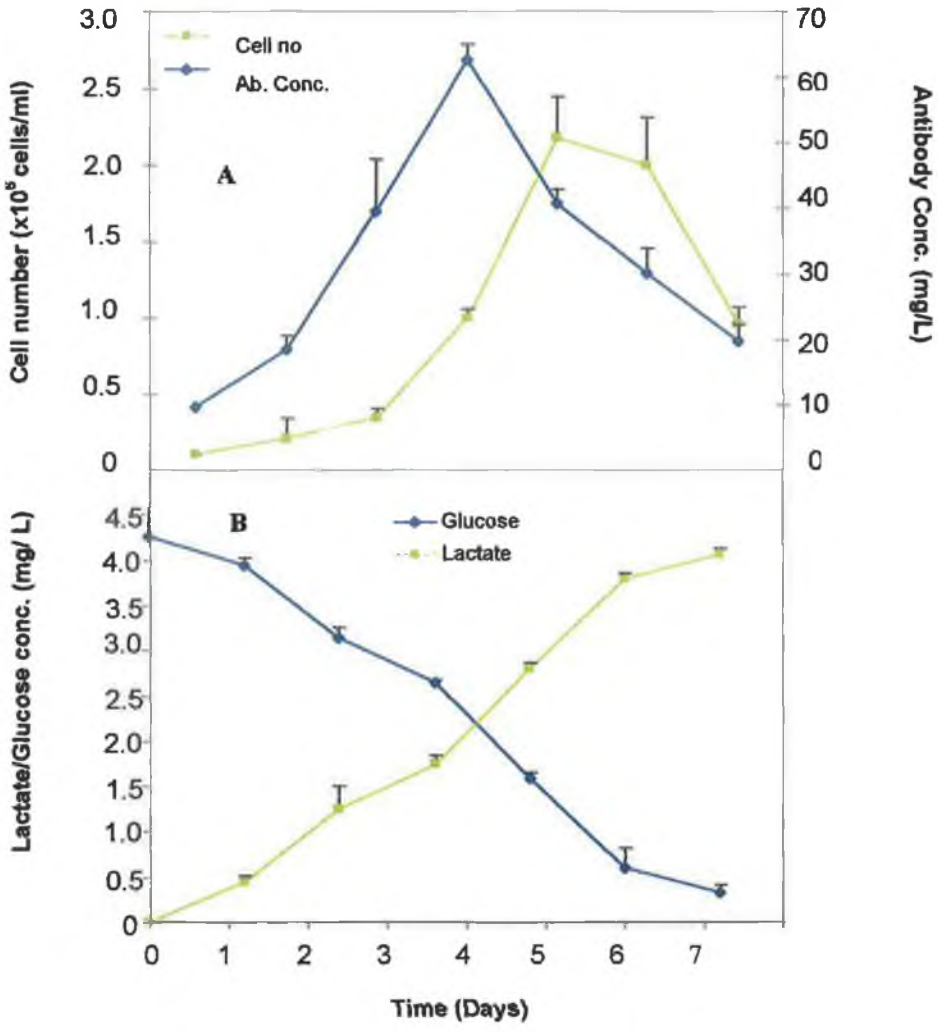


Figure 3.27 Shows cell growth and antibody production characteristics for **4/2D** cultured in PFM at the 10L-batch scale. **A)** Cell growth and antibody productivity **B)** Glucose versus lactate concentrations. Samples were analysed daily for antibody, lactose, glucose and cell viability.

Table 3.12 Tabulated specific growth rates, doubling time and number of generations data for the culture of 4/2D in PFM.

Run	Specific Growth Rate μ_{app}	Doubling Time (td)	Number of generations *N*
1	*0.7071*	*16.97*	*4.29*
2	*0.6698*	*19.42*	*3.46*

During the initial culture of the 4/2D antibody in serum-supplemented culture, antibody production peaked at day three to a high level and then dropped away towards the end of the culture. When this culture was repeated the high quantities of antibody achieved at day three were not repeated. However, the cell density achieved were a lot higher and there was no decline in antibody levels. A third run was due to be performed but due to commercial constraints on the equipment and laboratory space, it was unable to be carried out. The reasons behind why antibody levels maybe decreasing in culture are discussed in Section 4.4.

With regards to the culture of the hybridoma producing cell line 4/2D in PFM there was very little variation between the two batch culture results obtained in terms of specific growth rates, doubling times and number of generations. In comparison to the culture of the cell line 4/2D in SSM, antibody productivity also peaked at day 3 and a drop in antibody levels was observed after this time. The highest quantity of antibody was similar to that achieved at optimum conditions in the spinner flask.

3.8 Scale-up and process optimisation for production of antibody from hybridoma 5C3

3.8.1 Agitation

The aim of this experiment was to determine the most efficient agitation rate in terms of cell growth and antibody productivity in which to produce MAb from the hybridoma cell line 5C3 in SSM and PFM culture conditions. See section 1.2.5 for a detailed discussion on the effects of agitation.

Comparable cell densities were achieved in both SSM and PFM conditions at 100rpm. Agitation at 200rpm under protein free conditions was deleterious to growth and antibody productivity. In the presence of serum however comparable levels of antibody were produced at 200 and 100rpm suggesting the protective effect of serum at higher agitation rates.

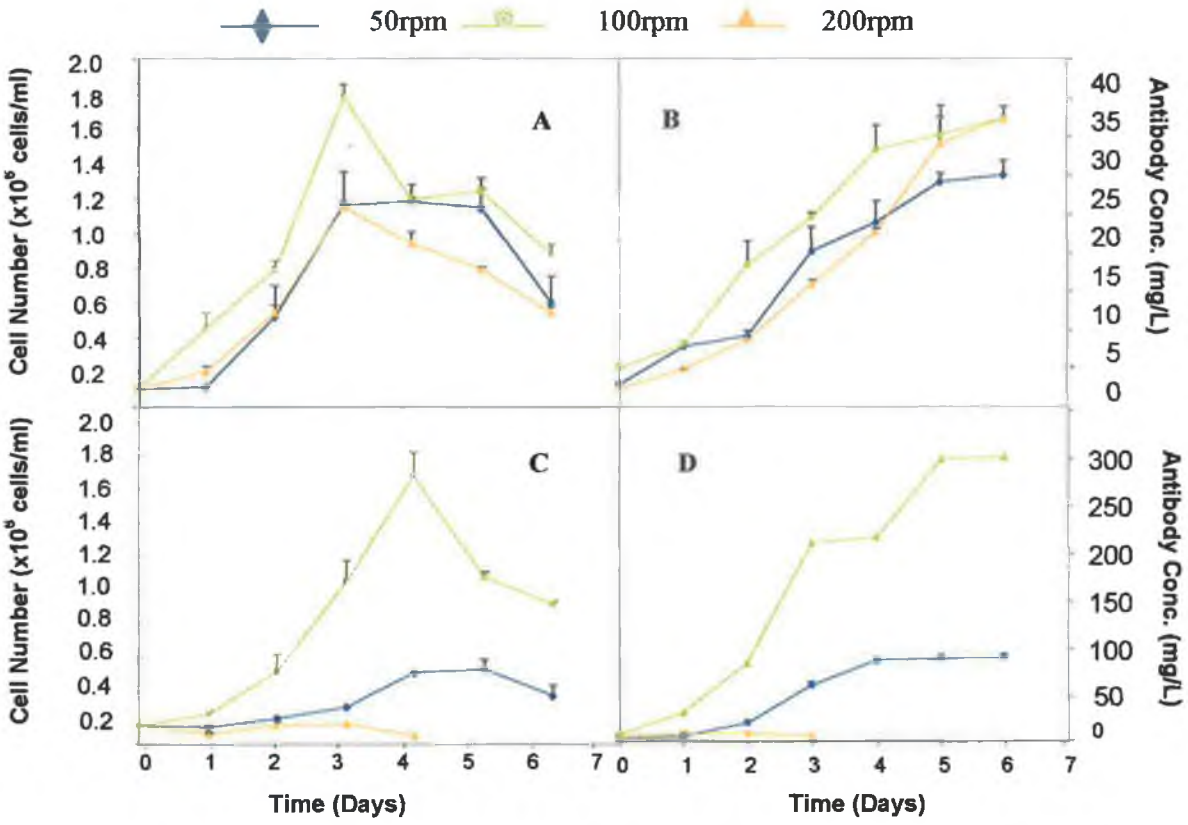


Figure 3.28 Shows cell growth and antibody productivity data for 5C3 cells grown in SSM (A & B), PFM (C & D) at varying rates of agitation.

Table 3.13 Shows tabulated specific growth rates, doubling time and number of generations, data for the culture of 5C3 in SSM and PFM at varying rates of agitation. Each run was carried out in triplicate (average of three with CV's < 10%).

	SSM			*PFM*		
Agitation Rate (RPM)	**Specific Growth Rate μ_{app}**	**Doubling Time (td)**	**Number of generations *n***	**Specific Growth Rate μ_{app}**	**Doubling Time (td)**	**Number of generations *(n)***
50	*0.6090*	*27.13*	*2.90*	*0.3680*	*45.12*	*1.88*
100	*0.4450*	*37.37*	*1.32*	*0.6660*	*24.95*	*2.47*
200	*0.4257*	*39.00*	*2.36*	*Cells dead*	*Cells dead*	*Cells dead*

In summary the agitation rate, which yielded the maximum cell density for 5C3, cells both in serum supplemented and protein free culture conditions, was 100rpm.

3.8.2 Temperature

The aim of this experiment was to determine the most efficient temperature in terms of cell growth and antibody productivity in which to produce MAb from the hybridoma cell line 5C3 in SSM and PFM culture conditions. See section 1.2.7 for a detailed discussion on the effects of temperature.

Maximum cell density and antibody productivity was achieved at 37^{0}C for both SSM and PFM cultures. Culture at 39^{0}C was deleterious to cell growth in SSM and PFM culture.

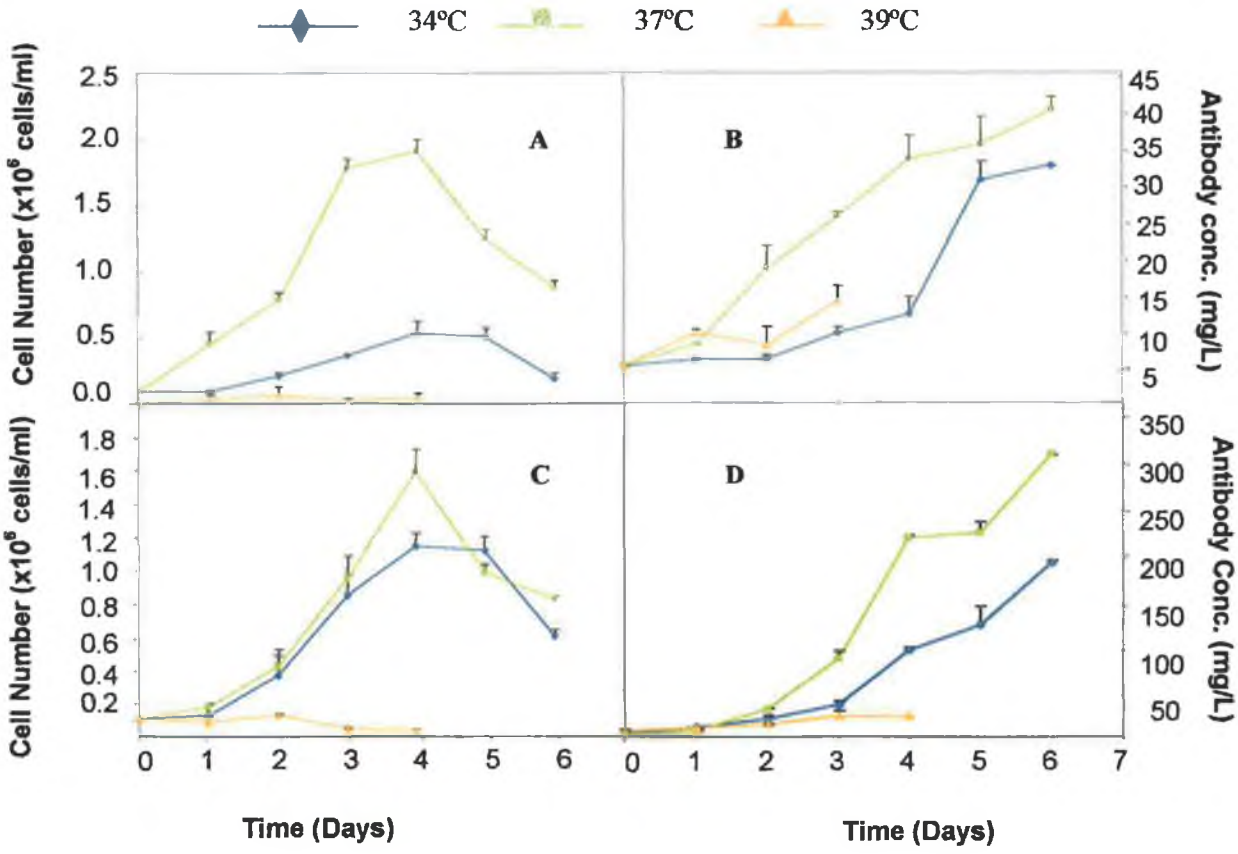


Figure 3.29 Shows cell growth and antibody productivity data for 5C3 cells grown in SSM (A & B), PFM (C & D) at varying degrees of temperature.

Table 3.14 Shows tabulated specific growth rates, doubling time and number of generations, data for the culture of 5C3 in SSM and PFM at varying degrees of temperature. Each run was carried out in triplicate (average of three with CV's < 10%).

	SSM			PFM		
Temperature (°C)	**Specific Growth Rate μ_{app}**	**Doubling Time (td)**	**Number of generations *n***	**Specific Growth Rate μ_{app}**	**Doubling Time (td)**	**Number of generations *n***
34°C	*0.4735*	*35.10*	*2.89*	*0.575*	*28.92*	*3.042*
37°C	*0.4450*	*37.37*	*1.320*	*0.6660*	*24.94*	*3.786*
39°C	*Not enough Data to Compile results*					

In summary, to obtain maximum cell growth and antibody productivity the 5C3 cells should be grown at 37°C and 100rpm.

The optimum process conditions described here were then used to culture cells at large-scale (10L bioreactor).

3.8.2 10L fermentation run

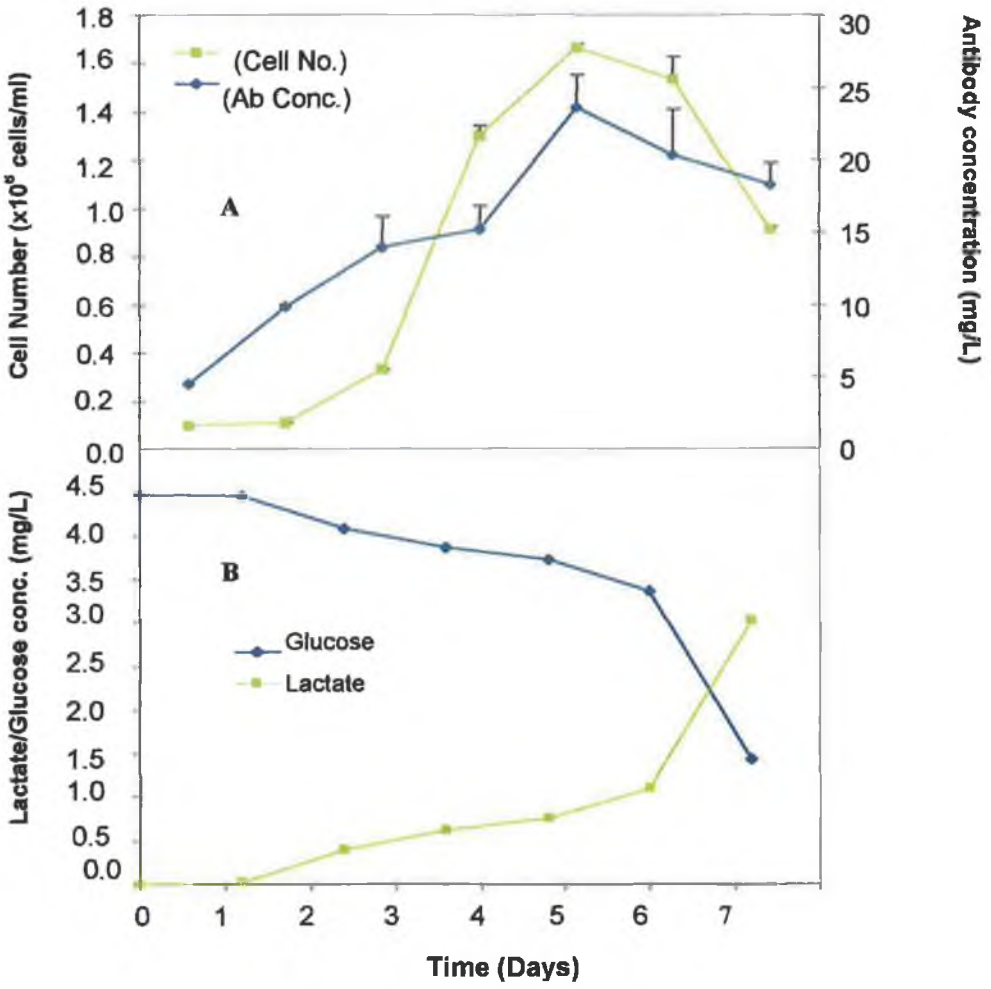


Figure 3.30 **A:** Characterising the growth rates and antibody concentration of 5C3 cultured in SSM, **B:** Average glucose versus lactate cultured in SSM at the 10L-batch culture scale.

Table 3.15 Specific growth rates, doubling time and number of generations. Data for the culture of 5C3 in SSM. (Only 1 run was carried out)

Agitation Rate (rpm)	Specific Growth Rate μ_{app} (d^{-1})	Doubling Time (td)	Number of generations *N*
Run 1	*0.8199*	*20.20*	*4.75*

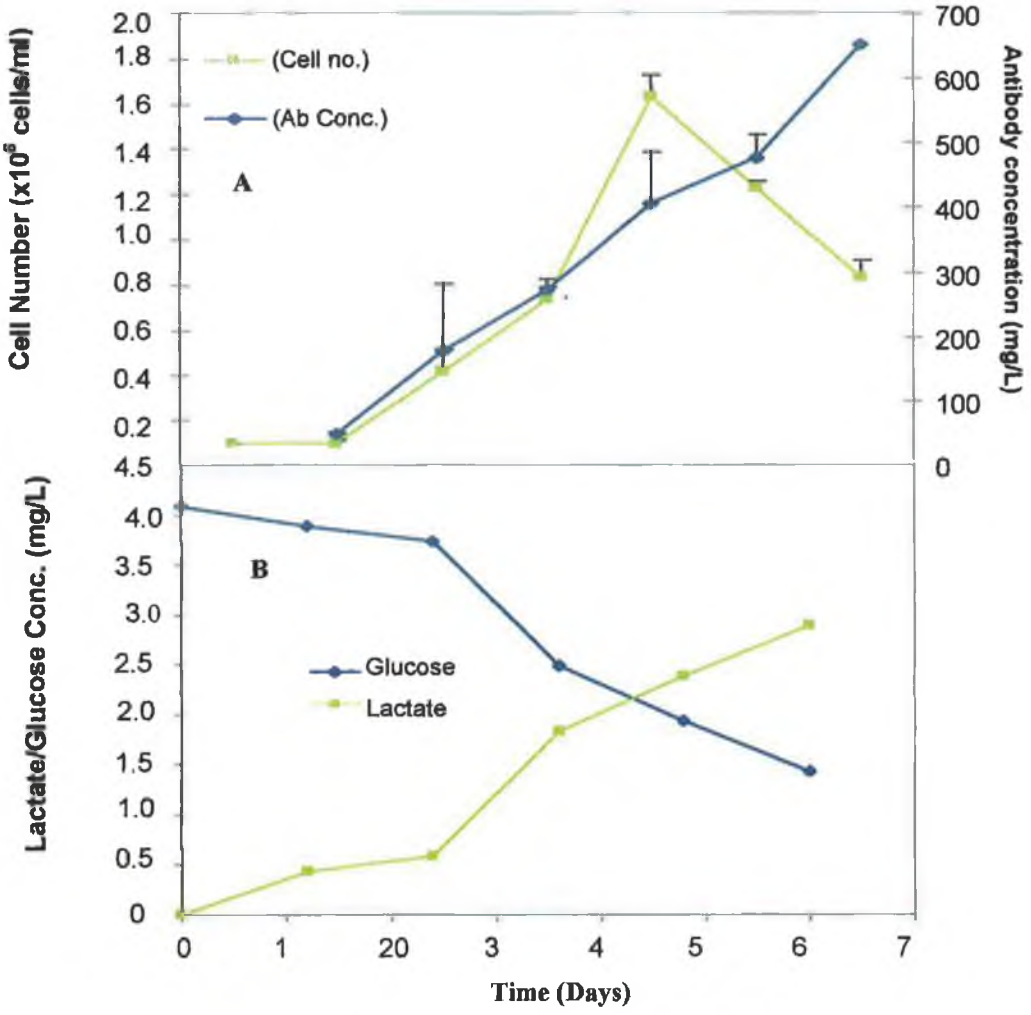


Figure 3.31 **A:** Characterising the growth rates and antibody concentration of 5C3 cultured in PFM, **B:** Average glucose versus lactate concentrations cultured in PFM at the 10L-batch culture scale.

Table 3.16 Tabulated specific growth rates, doubling time and number of generations. Data for the culture of 5C3 in PFM.

Temperature (°C)	Specific Growth Rate μ_{app} (d^{-1})	Doubling Time (td)	Number of generations *N*
Run 1	*0.7724*	*21.50*	*2.97*
Run 2	*0.5565*	*29.90*	*2.50*

3.8.3 Comparison of antibody production in each of the cell lines

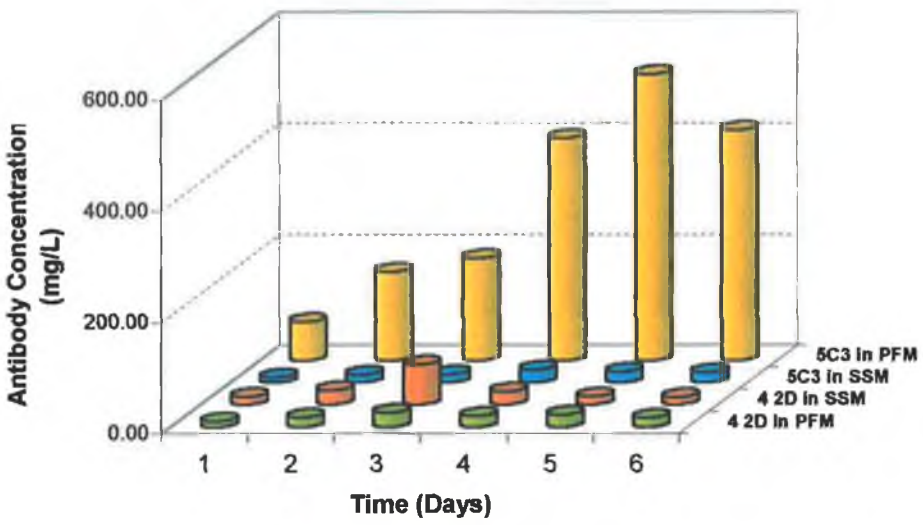


Figure 3.32 Shows the comparison of antibody concentration levels achieved with 4/2D and 5C3 in SSM and PFM

Table 3.17 Shows the comparison of antibody production and its cost for each cell line used in serum supplemented and protein free conditions. Antibody concentrations as determined on last day of the culture.
Calculations for costing can be found in the Appendix V.

	10 L (mg)	***10 L Bioreactor € /mg***
5C3 in SSM	290	2.60
5C3 in PFM	2965	0.26
42D in SSM	190	3.95
42D in PFM	200	3.89

3.9 Culture of hybridoma cell line 5C3 in the hollow fibre bioreactor

In the experiments described here, the hybridoma producing cell line 5C3 was examined for its ability to produce monoclonal antibody both in serum supplemented and protein free media in a hollow fibre bioreactor. The results were compared with those obtained from culture of this cell line in static flasks, spinner bottles and the 10-L bioreactor.

The results in Figure 3.33 compare the percentage viability of the culture of 5C3 in protein free medium and serum-supplemented medium over a culture period of 21 days. The results show that all three runs performed had comparable viabilities but the culture of cells in serum-supplemented medium had a higher viability for a longer time. With such a result, it is generally expected that more antibody would therefore be produced, as cells were kept in a viable state for longer. However, as was observed in all experiments comparing the MAb production of 5C3 in serum supplemented and protein free medium this was not the case.

3.9.1 Serum supplemented versus protein free culture of hybridoma 5C3 in a hollow fibre bioreactor

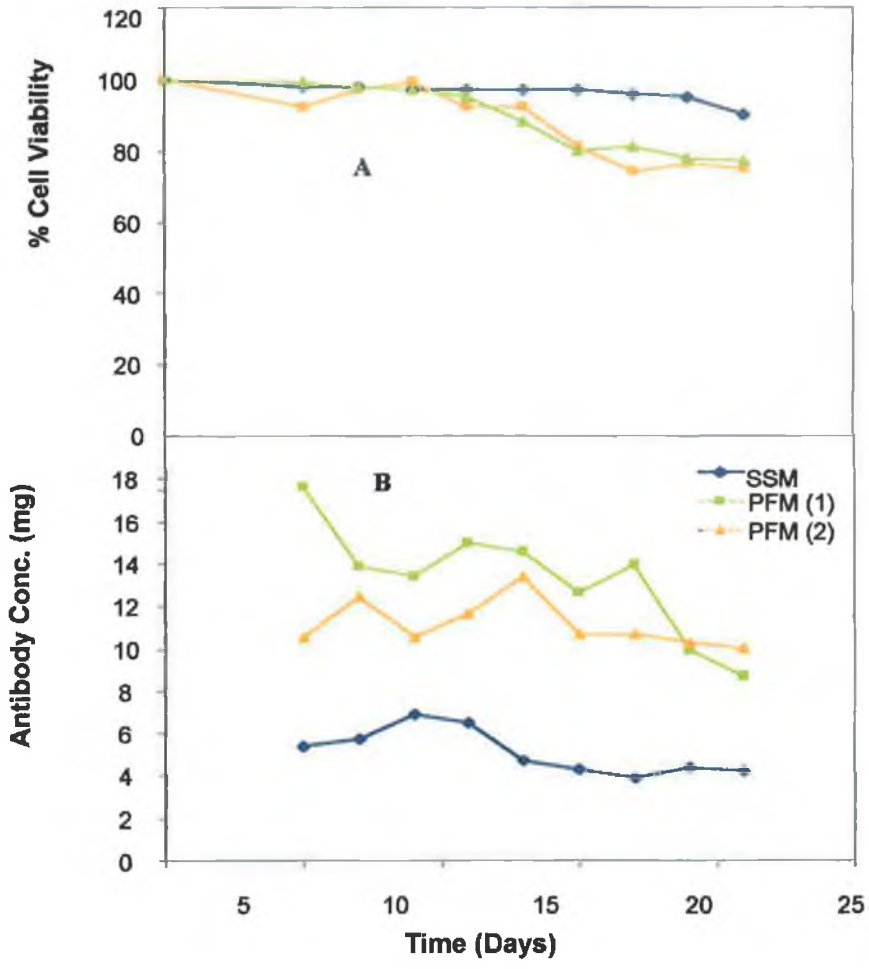


Figure 3.33 **A:** Shows the % viability of 5C3 cells cultured in SSM and PFM, **B:** Shows the antibody concentration of the 5C3 antibody cultured in SSM and PFM.

The culture period lasted 21 days with samples taken every two days for the determination of viability and antibody concentration. The difference between PFM (1) and (2) is that the first PFM run was carried out in a new hollow fibre cartridge. The second PFM run was performed in a cartridge that previously contained SSM medium and cells.

Table 3.18 Comparisons of the final antibody concentrations obtained from the culture of the 5C3-cell line in a hollow fibre bioreactor. The total volume harvested from the hollow fibre was 485mls. The culture period lasted 21 days.

	Total Antibody Produced (mg)
SSM	46
PFM (1)	119.47
PFM (2)	99.97

3.9.2 SDS-PAGE analysis

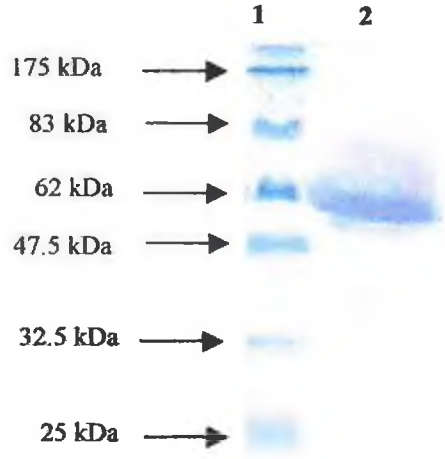


Figure 3.34 Shows SDS-PAGE analysis of MAb from hybridoma 5C3 produced in a hollow fibre bioreactor using serum supplemented medium **Lane 1:** Markers, **Lane 2:** Neat supernatant from SSM. Samples were separated on a 12.5% SDS-PAGE..

3.9.2 SDS-PAGE analysis

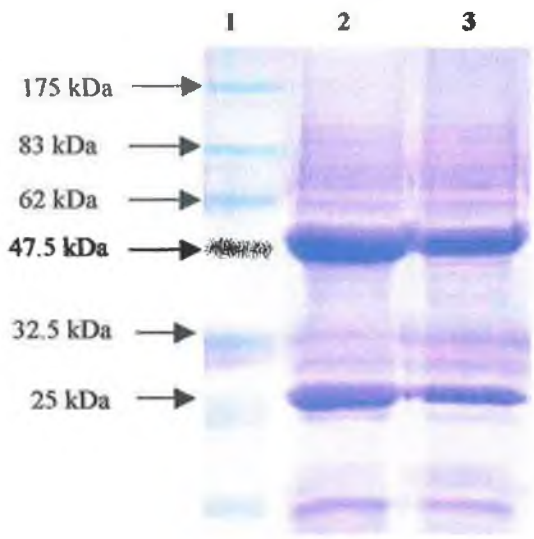


Figure 3.35 Shows the analysis of SDS PAGE of MAb from hybridoma 5C3 produced in a hollow fibre bioreactor with protein free medium. **Lane 1:** Markers, **Lane 2:** Neat supernatant from PFM (1), **Lane 3:** Neat supernatant from PFM (2). Samples were separated on a 12.5% SDS-PAGE.

The results achieved in these experiments show the superior quality and quantity of MAb produced from the 5C3-cell line under protein free conditions compared to serum supplemented conditions. How these results relate to antibody productivity in the 10L fermenter is discussed in section 4.5.

3.10 Analytical Validation of Nephelometric system.

For the purpose of this thesis, it was necessary to set up a method of quantifying MAb from culture supernatant that was efficient, reliable and easy to operate. This method was to replace the more commonly used ELISA and RID methods.

Nephelometry was the method chosen. The MININEPH (Binding Site, UK) used in this thesis was an endpoint nephelometer that used the measurement of light scattered by an antibody/antigen complex to determine protein concentrations (Section 1.1.8 & 2.4.2).

The first step required was to validate the nephelometer to ensure that the procedure was fit for the purpose intended in that it ensured confidence that the correct results were obtained when assaying the test samples.
Section 2.9.4 details the parameters that should be considered when carrying out a validation procedure on an analytical method.

For the purpose of this thesis, the following tests were carried out to test the validity of the assay (Section 2.4.2):

- **Inter-assay variation** – involved assaying samples in triplicate over four separate days and calculating the %CV (Section 2.9.4).
- **Intra-assay variation** – involved using the same sample carried out in approximately 10 assays and the results were compared (Section 2.9.4).
- The linear range of the standard curve was defined.
- The specificity of the assay (using IgG of various subtypes and IgM) was determined.

3.10.1 Inter-Assay Variation for Nephelometer

Table 3.19 *Shows Inter-assay variation.* The test sample used was supernatant from hybridoma 5C3 cultured in PFHM II. This experiment consisted of ten repeat assays at one analyte concentration (N=3)

Assay	*300 s*	*240 s*	*180 s*	*120 s*	*60 s*	*0 s*	*Average Scatter0*	*Standard Deviation*	*% CV*
1	558	591	587	581	604	599	591.67	8.43	**1.42**
2	595	613	590	590	599	594	596.83	8.61	**1.44**
3	499	499	492	501	482	498	495.17	7.14	**1.44**
4	459	456	474	485	486	487	476.00	11.97	**2.15**
5	548	559	563	592	581	567	568.33	15.82	**2.78**
6	592	591	605	617	618	620	607.17	13.23	**2.18**
7	497	508	513	520	511	517	511.00	8.17	**1.58**
8	592	588	598	603	605	615	600.17	9.70	**1.62**
9	436	464	466	457	451	439	452.17	12.58	**2.78**
10	537	547	544	561	567	555	551.83	11.21	**2.03**

This inter assay variation was performed to examine precision. By definition, the precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the homogenous sample under the prescribed conditions. The data in Table 3.19 measures the similarity between the %CV obtained for each assay carried out from the repeated assaying of a sample from the same source over the course of a day. By comparing the %CV between each assay very little variation was observed hence, validating the reliability of the instrument to reproduce results over the course of the day. In order to measure the overall precision of the assay the coefficient of variance of all ten assays was determined. This was achieved by obtaining the averages of each of the ten assays and finding their average and standard deviation. From this, a %CV of 10.2% was obtained. This is slightly outside the acceptable %CV of 10%.

3.10.2 Intra-Assay Variation

Table 3.20 *Shows intra-assay variation.* Test sample used was supernatant from hybridoma 5C3 cultured in PFHM II. These data represent the average coefficient of variation (CV) of three within-batch measurements (N=3).

Assay	*300*	*240*	*180*	*120*	*60*	*0*	*Average Scatter*	*Standard Deviation*	*% CV*
1	606.5	617	623.5	645.5	636.5	644	628.83	15.94	**2.54**
2	549.5	546	548	567.5	575	574.5	560.08	14.76	**2.64**
3	521	525.5	531.5	546	540.5	541.5	543.33	15.15	**2.84**
4	418	420	418	422	417.5	436	421.92	9.10	**2.22**

The inter-variation assay involved assaying samples from the same specimen in triplicate over four separate working days and comparing the results by measuring the %CV. Again, acceptable levels of %CV are less than 10. The intra-assay is often termed the repeatability assay. By definition, repeatability expresses the precision under the same operating conditions over a short interval of time. This assay verifies the ability of the nephelometer to reproduce results over time.

3.10.3 Determination of the linear range for the nephelometric assay

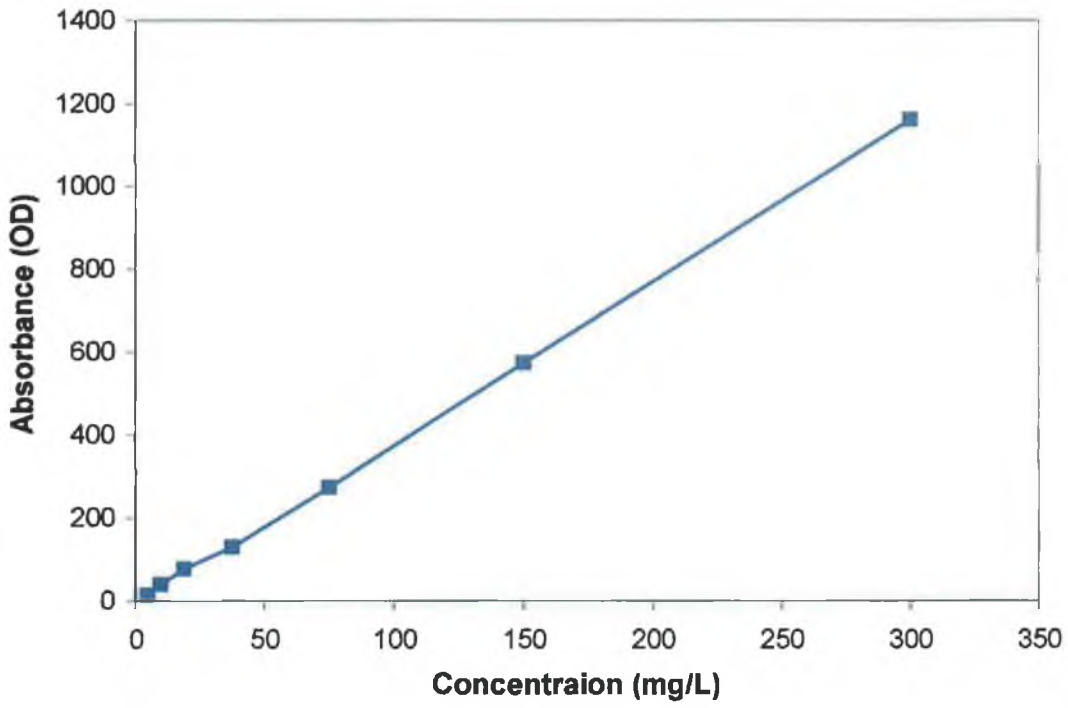


Figure 3.36 Standard Curve in the range 0-300mg/L for determining antibody concentrations using the nephelometer.

Initially the measurement range of the assay was determined. The standard used in the generation of the standard curve was that used in conjunction with the RID plates, an IgG antibody (G272.3) (Section 2.4.3). The standard had a neat concentration of 1200 mg/L. The control used in these experiments was the c-myc (IgG_1). Controls were examined at varying dilutions to ensure sample results lay in the linear region of the graph.

The range of an analytical procedure is the interval between the upper and lower concentration of analyte in the sample for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. In this case, it was found that although the nephelometer could measure samples at a

concentration of 1200mg/L but they were not stable over the assay length. At the lower end of the curve it was found that the lower limit of detection was 9.785 mg/L

By linearity, it is meant that the analytical procedure has the ability to obtain results that are directly proportional to the concentration (amount) of analyte in the sample. From this curve, it was found that the range of the linear range of the curve was between 9.375-300mg/L. A number of repeat standard curves were carried out over time to verify this and compare values for regressional coefficients (R^2) (Tables 3.21 & 3.22).

Table 3.21 Comparison of the regression coefficients obtained for standard curve assays performed over 7 days.

Day	*Regression coefficient (R^2)*
1	0.997
2	0.993
3	0.997
4	0.993
5	0.995
6	0.997
7	0.999

Table 3.22 Comparison of the regression coefficients obtained for standard curve assays performed weekly for a month.

Day	*Regression coefficient (Rr^2)*
1	0.997
7	0.999
14	0.993
21	0.996
28	0.996

The data above shows that repeated standard curves carried out over a month yielded very little variation. The regression coefficient is a measurement of the relationship between a dependent variable and an independent variable. The closer the calculated regression value is to one the more exact it is.

3.10.3 Determination of the specificity of the nephelometric assay

Table 3.23 Determination of the specificity of the assay – using IgG of various subtypes and IgM.

Antibody	*Subclass*	*Concentration (mg/L)*	*Nephelometric determined conc. (mg/L)*	*% Coefficient of Variance*	*Accuracy*
c-Myc	*IgG_1 (Kappa)*	200.00	202.11	3.092	1.05
Anti Cytokeratin No. 18	*IgG_1 (Kappa)*	20.00	25.33	4.985	26.65
Anti-Bromodeoxyuridine	*IgG_1 (Kappa)*	480.00	451.51	1.055	5.93
Ki-67	*IgG_1 (Kappa)*	265.00	245.19	2.29	7.47
AntiFodrin MAB1622	*IgG_1 (Kappa*	100.00	116.67	1.425	16.67
Negative IgM Control	*IgM*	100.00	25.35	135.6	74.65
P-glycoprotein (MDR)	IgG_{2b} (Kappa)	500.00	466.07	3.27	6.8
EIF-4E	IgG_{2b} (Kappa)	250.00	222.14	4.43	11.1

The purpose of this experiment was to determine the specificity and accuracy of the assay for varying subtypes and samples of IgG. Specificity is the ability to assess unequivocally the target analyte in the presence of components, which may be expected to be present. Typically, these might include impurities. Accuracy is the closeness of agreement between the value, which is accepted as either, the conventional or true value or an accepted reference value, and the value found.

The specificity and accuracy obtained with relation to the IgG samples of c-myc, Ki-67, anti-bromodeoxyuridine, p-glycoprotein was acceptable. Results obtained for anti-fodrin MAB1622 and the anti-cytokeratin No. 18 were not as specific or accurate as those obtained for the other IgG samples. In addition, the reactivity of the anti IgG sera with the IgM yielded results indicative of poor binding and specificity for each other (Bossuyt *et al.*, 2001).

From this, it can be concluded that nephelometry is not specific for IgM. In addition when determining a suitable control for the assay, a number different IgG standards of the specific isotype being tested for, should be analysed. This will avoid the use of a control, which has a high % inaccuracy, as observed in Table 3.23 whereby different degrees of accuracy were observed for each different control.

Section 4 Discussion

Obtaining higher yields of monoclonal antibodies (MAb) is a challenge in hybridoma technology. The lack of information and detailed knowledge of animal cells and their microenvironment gives rise to the vast differences of productivity *in vitro* and *in vivo*. If a MAb producing cell line maintained the same specific antibody production rate *in vitro* as they do *in vivo*, they would produce 720g of IgG per day (in a 10L bioreactor at a density of 10^7cells/ml). This is not the case however, as their actual production rate is approximately 0.1g of IgG per day (in a 10L bioreactor at a density of 10^7cells/ml) (Kundu *et al.*, 1998).

There are three main ways of increasing MAb production *in vitro:*

- Optimisation of culture conditions
- Increasing the scale of the culture
- Increasing the density of the culture

During the course of this thesis, each of these methods was examined. Hybridoma cells were adapted to grow successfully in protein-free medium and scaled up to a final production volume of 10L. Productivity and quality of antibody were compared with those obtained from utilising a high-density culture system, a hollow fibre bioreactor. In order to determine possible process conditions that may increase antibody productivity, growth of hybridoma cells using a variety of agitation and temperature conditions were also investigated.

4.1 Adaptation of hybridoma producing cell lines to protein-free and serum-free medium

The *in vitro* production of MAb in defined or low-protein cell culture medium is the prime objective for many biotechnology industries involved in the production of antibody therapeutics. The reason underlying this trend is that research has moved away from using serum-supplemented medium due to regulatory issues. These include the high protein load, which interferes with the purification and identification of antibody, the undefined composition of serum, the batch-to-batch variation in quality, the risk of contamination from viruses, mycoplasma and prions as well as the ethical questioning surrounding the production of foetal serum. As a consequence of these problems a lot of research work has been devoted over the years to searching for serum replacements (Chen *et al.*, 1993; Mochizuki *et al.*, 1993; Ramirez *et al.*, 1990; Blasey & Winzer, 1989; Shacter, 1987; Barnes & Sato, 1980). The result of this research has led to the availability of numerous commercially available serum-free, protein-free and chemically defined media.

For the purpose of this thesis, commercially available media have been used to demonstrate that a number of MAb producing cell lines, each exhibiting unique characteristics in culture, can be adapted to grow and secrete MAb in protein-free, chemically defined, and serum-free media.

All media purchased suggested an adaptation protocol, which involved either a sequential reduction in serum and a corresponding sequential increase in protein-free/serum-free medium or else a method of direct adaptation to the protein-free/serum-free medium. Both methods were examined. It was found that the direct adaptation method resulted in decreased cell viability; and visually the cells appeared to be in an unhealthy state. The second method, sequential adaptation gave the cells longer to adapt to the reduction of certain components normally found in serum (Sinacore *et al.*, 2000). Hence, higher cell viabilities were obtained.

4.1.1 Adapting hybridoma 6/1C to protein-free and serum-free conditions

Having completed the adaptation of the cell lines to each of the media, stationary growth phase assays were performed to monitor the growth of the cells during routine passaging. This assay is an effective way of monitoring the cells performance in a particular culture environment over time. Using this method rapid identification of any difficulties associated with the cell line reproducing its growth during repeated sub-culturing was possible, thus it was a good indicator of the life span of a cell line in a particular culture environment. In addition, a simple growth curve was performed whereby cells were monitored from lag phase through to the decline phase. This allows visualisation of the particular growth characteristics of the cell line in a particular culture environment. From the growth curve it is possible to pin point the time of maximum cell density and viability, which is important when harvesting cells for scale-up purposes.

From the results of these experiments (Figures 3.1, 3.2, 3.3, 3.4) it is clear that the serum-free media that performed best in terms of cell number in comparison to the serum-supplemented control, were Excell 620, Hybridoma SFM, the protein-free medium HTM20CTH and the chemically defined CD Hybridoma medium.

It is important when performing optimisation and actual cell culture processes that there is a system in place which can efficiently determine the quantity of antibody that is being produced in culture. Failure to ensure this type of system may result in loss of productivity due to the fact the antibody might reach its peak productivity on a certain date and may drop off after that due to the presence of proteases etc in the culture supernatant (Teige *et al.,* 1994; Kratje *et al.,* 1994; Enfors, 1992). Traditionally ELISA has been the method of choice for the quantification of monoclonal antibodies in cell culture. ELISA's can be designed in a variety of formats, the most commonly used include the sandwich, indirect and competitive assays (Crowther, 2001). For quantifying antibody in the work presented in this thesis the sandwich ELISA was performed.

Initial quantification of the anti-mdr-1 MAb 6/1C using the ELISA method for the cell line in serum-supplemented and various protein-free and serum-free media yielded

unsatisfactory results. The standard curve was reproducible but the positive control i.e. MAb produced in ascities never yielded linear results across the range of dilutions used. No positive results were obtained for anti-mdr-1 MAb 6/1C cultured in the various media both for normal and concentrated supernatants. During the course of this work there was problem with the supply of the particular serum being used i.e. Myoclone heat inactivated foetal bovine serum (Gibco). This serum had been continuously used in the culture of this anti-mdr-1 antibody, but its supply was halted during the course of this research work and a number of serums were supplied as alternatives. Myoclone had been used because it was pre-heat inactivated. All of the new serums that were supplied were not heat inactivated although the supplier assured us they were at the time of purchase. While it cannot be said that this was the reason why antibody production was decreased in the hybridoma cell line 6/1C, it can be speculated that the use of different serums may have caused some instability in the cells resulting in a decrease in the production of antibody. In order to examine the effect of serum on the quantification of the antibody, an ELISA was performed spiking an antibody of known concentration in to serum-supplemented medium and comparing results achieved with the results obtained from the standard curve (Table 3.1). T-test analysis of the results indicated that there was a significant difference between the results obtained. This result indicates that indeed the serum was having a significant effect on the quantification of results.

Other methods employed to determine the presence or quantify the presence of antibody 6/1C in supernatants yielded poor results. Presence of antibody was not detected on the RID plates, isotyping using the isostrips yielded a very faint IgG band for anti-mdr-1 MAb from cells cultured in serum-supplemented medium and no bands were detected for the samples cultured in the protein-free and serum-free media. Isotype analysis using the ELISA based mouse IgG, kit yielded very faint IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 results for the serum-supplemented medium and faint IgM results for the protein-free and serum-free media samples. The results obtained with the serum-supplemented samples indicate that a non-isotype specific capture antigen was used or that sample over loading may have occurred.

Nephelometric analysis of these samples yielded very small quantities of antibody. Results shown (Table 3.3) are well below the average expected antibody productivity level, which can depending on process conditions lie anywhere between 50–100μg/ml

(Kundu *et al.*, 1998; Bibila & Robinson, 1995; Reuveny *et al.*, 1986). In the case of hybridoma 6/1C, it typically produced between 35-50μg/ml IgG in previous studies in the laboratory (Moran *et al.,* 1998 unpublished data).

Immunocytochemical analysis is not a quantitative method. Instead, it determines the presence of antibody based upon staining of the plasma cell membrane of the positive control cell line DLKPA, in the case of hybridoma producing anti-mdr-1 cell line 6/1C.

Of the media, which induced the best growth characteristics of the hybridoma 6/1C in culture only the protein-free CD Hybridoma yielded positive staining as can be seen from Fig 3.5(G). On comparison of these results to those obtained when using nephelometric analysis, culture of this cell line in this medium yielded the highest levels of antibody.

In order to increase antibody productivity of the hybridoma producing cell line 6/1C, three commercially available supplements were employed. OptiMab is a defined protein-free concentrate, which consists of alternate carbon sources, a monoclonal antibody inducer and essential nutrients which, have been shown to be significantly depleted during the high-density culture phase (Mercille & Maisse, 1998). Glutamax II, the second supplement is a heat stable derivative of the amino acid glutamine. It is a poor stimulator of cell division and growth so the optimum time to add it to the culture is during late log phase of growth hence enhancing MAb production. TCH is a serum replacement that supports the growth of mammalian cells. As one can see from Figure 3.6, in terms of growth, OptiMab increased the exponential phase by 4 days and reached higher cell densities in the range of 3.5 x 10^6cells/ml. Immunocytochemical staining of supernatants taken following addition of all three supplements showed mdr-1 positive staining. Notably, strong staining was recorded in the OptiMab supplemented samples. Nephelometric analysis also detected an increase in the levels of antibody in the samples containing OptiMab and Glutamax II.

Due to the difficulty in detecting quantifiable antibody using the ELISA and RID systems and the inability of the isotyping kits to detect strong IgG bands, it was likely that further studies on the 6/1C hybridoma cells would prove difficult.

It appeared that the 6/1C cell line had a decreased rate of antibody productivity and in some cases had ceased antibody production altogether. In many of the adapted media, little or no antibody production was observed. It should also be mentioned that work carried out by an independent commercial company on the cell line 6/1C concluded that low level and in some cases, no antibody was being produced in culture. The reason why loss of antibody productivity was observed during the course of the work is not apparent, but a review of the literature suggests a number of possibilities.

As cells adapt to new environments such as the adaptation from serum-supplemented medium to protein-free/serum-free medium it was noted that the cells no longer attached to the surface of the flask. This may have been due to a possible attachment enhancement factor which may be present in serum-supplemented medium and absent from the serum-free/protein-free medium. Alternatively, it may have been change in the expression of genes involved in cell adhesion, extracellular matrix or cytoskeletal organisation in the new culture environment. In effecting such changes, the expression of regulatory elements controlling their expression may also have been altered. Cellular events such as the cell cycle and distribution of organelles are closely linked to cell adhesion, cytoskeletal structure and cell shape. It is possible their expression is also affected when the cells are adapted to suspension growth. If the expression of such genes is affected it is possible that other downstream genes such as those responsible for antibody production are also affected (Korke *et al.*, 2002). This is possible since stable production for antibody-producing cells is complex. Separate genes, which are integrated and regulated independently, encode heavy and light chains. Secretion of mature antibody into the supernatant depends on accurate assembly of these chains in the endoplasmic reticulum (Strutzenberger *et al.*, 1999; Martial-Gros *et al.,* 1999). In addition mycoplasma infection of hybridoma can compromise antibody production levels, however mycoplasma analysis was carried out on samples every 6 months and each time results were negative for contamination.

In summary, the inability of a cell line to secrete antibody could be due to one of the following factors:

- Genetic instability leading to modifications of the primary structure of IgG chains or a problem in the regulation of transcription/translation of mRNA (Hunkeler *et al.*, 1989).
- Defects in the assembly of the Heavy and Light chains – nutritional conditions and growth conditions can influence assembly factors (Gardner *et al.*, 1985).
- Improper post-translational modification; it has been shown that the level of glycolysation of the protein is dependent not only on the metabolic state of the cell but also on the binding supplements in the medium. The protein transport system can be blocked until a correct configuration is achieved (Martial *et al.*, 1994; Hendershot, 1990)

4.1.2 Adaptation of the hybridomas 4/2D, 5C3, and 1/11C to protein-free medium.

Three cell lines were adapted to new protein-free media (UltraDoma and PFHM II) that had been previously used and proven to work well in our laboratory (Larkin, 2002). Growth characteristics and productivity in culture were compared between the three cell lines. The three cell lines used were the hybridoma producing 5C3-cell line, the hybridoma producing 1/11C cell line and the hybridoma producing cell line 4/2D. These three cell lines were adapted to the new media using the sequential adaptation process (Section 2.2.3). The results indicated that all three cell lines adapted most favourably to the PFHM II medium (Figure 3.8, 3.13 and 3.18). It is also clear from the results obtained from nephelometry, immunocytochemistry and RID and SDS-PAGE that the cell lines with the highest antibody productivity were the hybridoma producing cell line, 4/2D and the hybridoma producing cell line, 5C3. Average production rates in static flask culture were 20μg/L for hybridoma 4/2D both in serum-supplemented and protein-free conditions. For hybridoma 5C3, average production in static flask culture was approximately 20μg/L in serum-supplemented medium and 180μg/L in protein-free conditions. Because quantifiable and reproducible quantities of antibody were being obtained in culture, these cell lines were chosen for further scale-up studies in the protein-free medium (PFHM II) and the serum-supplemented medium. It should be noted however that although SDS-PAGE was performed to determine the presence of antibody culture supernatants it is not an exact method as the presence of serum may interfere with the detection of bands. A more suitable method for the absolute detection

of IgG bands is that of Western Blotting. In addition, a positive IgG control should have been included in these gels along with media controls. Media controls contain the culture medium minus the cells or product. The bands that appear could then be compared to the samples containing supernatant enabling an exact determination of the antibody bands to be made.

4.2 Effects of agitation on monoclonal antibody production from the hybridoma 4/2Dand 5C3 cell lines.

The use of agitation as a means of maintaining uniform growth conditions in culture can, if used excessively, lead to physical damage of the cells. At large scale there is an increased demand to meet oxygen and nutrient requirements and to remove CO_2. The increased mass transfer requirement is met by an increase in the rate of agitation or by sparging air through the medium. These methods have the disadvantage of being potentially damaging to the cell. The damage can be as clear-cut as cell death or can manifest itself in a manner which results in a reduction of the intrinsic growth rate of cells, which can reduce the amount of product being formed. In addition, if damage results in lysis of the cells the increased non-specific protein in the medium makes the process of protein purification very difficult (Aloi & Cherry, 1996). On the other hand low agitation rates result in cell aggregation (clumping). This leads to inaccurate cell enumeration and reduced antibody productivity as cells inner most in the clump are starved of oxygen and nutrients and hence cells die early on in the cell cycle.

In the work presented in this thesis, three rates of agitation were examined, 50rpm, 100rpm and 200rpm. This range includes rates, which are generally described as low, normal and excessive.

As is shown in Figure 3.24, culture of the cell line 4/2D in serum-supplemented and protein-free media shows maximum cell density was achieved at 100rpm. Reduced cell growth was observed at 50rpm under both culture conditions. Culture at 100rpm in protein-free medium yielded a three-fold increase in cell density compared to that at 200rpm. Reduction in cell number at 200rpm was most likely as a result of the increased shear experienced by cells in protein-free medium, which lacks the protective nature of serum (Jöbses 1991, van der Pol *et al.*, 1992). However, an increase in

antibody production of 56% at 200rpm was recorded. This is unusual, as one would expect that in the absence of a shear protectant such as serum, the cells would be adversely damaged as was shown when the 5C3-cell line was cultured in the same medium. However this reiterates the point the all cell lines are different and productivity will vary in different culture environments.

Another noticeable characteristic concerning the culture of the line 4/2D in serum-supplemented medium is the fact that the maximum cell density under culture conditions of 200rpm was achieved one day earlier (Figure 3.24). The increased rate of cell growth in the exponential phase and of cell decline at 200rpm after maximum cell growth (Day 3) may be related to high metabolic rates in the exponential growth period and subsequent nutrient limitations (Smith & Greenfield, 1992). The increased agitation intensity may have been sufficient to have a subtler biological interaction with the cells resulting in an increase in the rate of glycolysis. Such increased rates of glycolysis at higher agitation rates may have been required to provide energy either to satisfy a deficiency in another energy production pathway or to increase overall energy production thus providing maintenance of internal structures or repair of membranes affected by turbulent fluid forces. To overcome such limitations in energy that occur after maximum cell density has been reached, the addition of energy sources such as glucose and glutamine in appropriate quantities and at appropriate times can be a solution (Abu-Reesh & Kargi, 1991; Dodge & Hu, 1985). Noticeably, there were comparable levels of 4/2D MAb produced at 200rpm and 100rpm in serum-supplemented medium. This trend has been observed previously by Smith and Greenfield (1992), where culture at 200rpm yielded 27mg/L and that at 600rpm yielded 26mg/L, suggesting no loss in cell activity at excessive rates of agitation.

The hybridoma cell line 5C3, cultured in serum-supplemented and protein-free medium (Figure 3.28) yielded similar results to the hybridoma cell line, 4/2D when cultured under various rates of agitation. Agitation at 100rpm yielded the highest cell densities and antibody concentration. Culture of cells at 200rpm in protein-free medium had a deleterious effect on the growth and antibody productivity suggesting that these cells cultured in protein-free conditions are extremely fragile in shear stress. Interestingly, there was a substantial increase in the quantity of antibody produced by the hybridoma producing cell line 5C3 in protein-free medium. Results obtained at 50rpm and 100rpm

were five and ten fold respectively more than that achieved in serum-supplemented conditions.

4.3 Effects of temperature on monoclonal antibody production from the hybridoma cell lines 4/2D and 5C3.

The set point temperature 37°C has been so widely accepted to be most efficient culture temperature for mammalian cells since the beginning of cell culture procedures, that temperature has received very little attention as a process optimisation variable. For this reason, the number of reports on attempts to optimise the temperature are limited in comparison to those obtainable for pH, dissolved oxygen concentration and medium composition.

Previous research work has investigated temperatures in the range 29°C to 42°C. Reuveny *et al.,* (1986) carried out work in the temperature range 28°C to 37°C with the view to prolonging higher cell viabilities in the decline phase.

It has been generally recognised that low culture temperatures suppress cell growth and enhances cell productivity. This is based on the reasoning that suppression of cell growth at low temperatures usually occurs in the G_1 phase, which is the phase during which antibody production occurs. However the effect of low temperature on production has been reported to be cell line dependent with reports of enhanced, (Kauffmann *et al.*, 1999; Furukawa & Ohsuye, 1998), decreased (Barnabē & Butler, 1992; Sureshkumar & Mutharasan, 1991), stabilised (Merten & Litwin, 1991) or unaffected (Chuppa *et al.*, 1996; Weidemann *et al.*, 1994; Bloemkolk *et al.*, 1994) specific production rates.

Reducing the temperature can, depending on the cell line, have many advantages over culture at the normally used temperature 37°C. Such advantages include a decrease in proteolytic activity (Kratje *et al.,* 1994; Tiege *et al.,* 1994; Enfors, 1992). This is essential if complex proteins prone to degradation are produced by cell lines with high proteolytic activity. In such cases, a reasonable decrease in the culture temperature might modulate the severity of any extra and intra cellular proteolytic attack.

In general, it has been observed that there has been a decrease in cell metabolic activities at lower culture temperatures. This is reflected by a decrease in the specific glucose uptake rate, the specific lactate production rate, the specific glutamine uptake rate and the specific CO_2 evolution rate. Reuveny *et al.,* (1986) and Sureshkumar, & Muthaeasan (1991) reported a 200% decrease in specific glucose uptake rate at 33°C. If uptake rates of other components are affected in a similar manner it can be expected that at lower temperatures less medium will be required for cultivation hence there would be huge savings in the cost of production at large scale.

The purpose of the experiments carried out here was to examine the effects of the culture temperatures 34°C, 37°C and 39°C on the growth of the hybridoma cell lines 4/2D and 5C3 in serum-supplemented and protein-free culture conditions.

Similar growth rates (Section 2.10.1 for calculation) and growth characteristics were observed between the culture of the hybridoma producing cell line 4/2D in serum-supplemented medium at 37°C and 34°C (Table 3.10). However, an increase in MAb productivity of 22.2% was observed.

Culture at 39°C had a deleterious effect on the growth and productivity of the 4/2D cell line (Figure 3.25). This result was in agreement with that reported by Blomkolk & Gray (1994) who observed that both cell growth and antibody productivity were reduced at 39°C.

Maximum cell density was obtained from the hybridoma producing cell line 4/2D at 37°C in protein-free medium. However, maximum antibody production occurred at 34°C. From Table 3.10 it is clear that the specific growth rate at 34°C is lower than that achieved at 37°C and 39°C. According to Suzuki & Ollis (1989), a decreased specific growth rate is reflected in a prolonged stay of the cells in the G_1 stage of the hybridoma cell cycle. Since monoclonal antibody, synthesis takes place in the G_1 phase it is not surprising that a prolonged stay at this stage ultimately led to higher concentrations of antibody in the supernatant.

Similar growth patterns and antibody production rates were obtained when the hybridoma producing cell line, 5C3 cells were cultured in serum-supplemented at 34°C

and 37°C (Table 3.14). There was an observed increase of 18.75% in antibody production at 37°C. Culture at 39°C appeared again to be stressful for antibody production.

Culture at 37°C yielded the most efficient results with regards to cell number and antibody production (Figure 3.29). The specific growth rate at 34°C was reduced ($0.5750d^{-1}$) compared to 37°C ($0.6660d^{-1}$). Nevertheless, no increase in antibody production was observed. This is in contrast to the result obtained when culturing the hybridoma producing cell line 4/2D in protein-free medium. With a reduced specific growth rate, antibody production was increased by 33%. In the case of 5C3 cultured in protein-free medium a reduction in antibody production of 38% was observed. This reiterates the point made earlier during this discussion that no two cell lines are the same hence one cannot assume that the same operating conditions will maximise productivity (Weidemann *et al.*, 1994).

In summary, the work carried out on agitation and temperature in spinner flasks has shown that the culture conditions of 37°C and 100rpm are the most efficient for cell growth and antibody production in each of the cell lines studied.

4.4 Production of monoclonal antibodies from hybridoma 4/2D and 5C3 in 10 L bioreactors

The increased demand for the production of larger quantities of vaccines and MAb based therapeutics in the last two decades has led to the development of larger and more efficient approaches to their production. These methods include a wide range and size of bioreactors and feeding strategies. Bioreactors, depending on the type can range in capacity from one to tens of thousands of litres. In the past three years, the volume of commercial antibody production has increased two fold (Dutton, 2001). Currently there are 235 monoclonal antibodies nearing clinical trials and by 2010 it is expected that 100 of these will be commercialised (Kelley, 2001). The importance of developing efficient production processes cannot be underestimated.

Along with increased demand for monoclonal-based therapeutics comes the increased need for control systems, which can monitor the culture environment of the cells. The ADI control system employed for the control of the 10L fermenter, in general proved very straightforward to operate and set up but was not without its operational problems. It was found in one early calibration run that if there happened to be a surge in electricity causing the unit to shut down it failed to retain the original settings at start up. This resulted in some cases, in the temperature and dO_2 concentration rising to very high levels. This problem needed to be rectified before a proper run was initiated as if this were to happen during a culture run, all cells and product would be lost. It turned out that an upgrade in software rectified the problem.

For the purpose of this thesis, two of the hybridoma producing cell-lines 4/2D and 5C3 were scaled up from a working cell bank to 10L scale. The main aim of this work was to show that scale up could be achieved without loss of cell activity and antibody productivity. The production strategy used was that of a simple batch culture, cells were grown for approximately six days and the product was harvested daily for further antibody analysis.

Duplicate runs of the culture of the hybridoma producing cell line 4/2D in serum-supplemented medium were performed. The initial run resulted in the surprising observation that maximum antibody production occurred at day three and antibody

levels dropped considerably thereafter (Figure 3.26). The maximum antibody concentration was approximately ten-fold greater than that achieved at the spinner flask stage and the subsequent run. The second run yielded results similar to those achieved in the smaller scale studies i.e. with maximum cell density occurring at day four and maximum antibody productivity occurring at day six.

The duplicate runs carried out for the culture of the 4/2D cell line in protein-free medium were very similar. It is clear that, from the data in Table 3.12, that specific growth rate, doubling times and the number of generations completed were almost identical. The observed growth and antibody production trends were also comparable to those achieved at the spinner flask scale.

Noticeably, however in comparison with the first batch run of the hybridoma producing monoclonal antibody 4/2D, in serum-supplemented medium, antibody production peaked at 72 hours after which there was again a decrease in antibody levels. This observation suggested that the assumption that the action of acid proteases present in the culture medium may be leading to the denaturation of the antibody (Teige *et al.,* 1994; Kratje *et al.,* 1994). To examine this suggestion further, cells at small scale were maintained in culture for five days after maximum antibody production was reached. The samples were analysed to determine antibody loss but no such decrease in antibody concentration was observed. It appeared that factors created in the bioreactor that were not encountered in the spinner flask were possibly causing this loss of antibody to occur for instance there may have been a large build up of proteases which resulted in the denaturation of the antibody. There is no evidence to suggest this is the case but in future work section 5.2 I have detailed experiments, which could be carried out to monitor the protease action.

As was characteristic of the culture of the hybridoma producing cell line 5C3, larger quantities of antibody were observed in the smaller spinner flask in protein-free medium, in some cases 30 fold that achieved with the same cell line in serum-supplemented conditions (Figure 3.30, 3.31). Smith & Greenfield (1992) observed similar results with a hybridoma cell line they cultured in PFHM II. They noticed an increase from approximately 27μg/ml in serum-supplemented medium to 180μg/mL in

protein-free medium. They attributed this dramatic difference to the nutrient composition of both media.

Table 4.1 below compares the various concentrations of amino acids present in both media. They suggest that the non-essential amino acid alanine which is present in high quantities in PFHM II and not in DMEM may have an important role to play in the increased concentrations of monoclonal antibody being detected. Alanine is produced in high concentrations in hybridoma cultures as a result of both glucose and glutamine metabolism and it is believed to contribute to cell metabolism as a non-toxic nitrogen sink for glutamate amino groups. Smith & Green (1992) have postulated that alanine may be included as an alanine-glutamine dipeptide, which stabilises glutamine against spontaneous degradation. However, this dipeptide more commonly known as Glutamax I is used in the serum-supplemented medium as the carbohydrate source and does not result in higher levels of antibody production.

From the Table 4.1 it is clear that nearly all of the other amino acids are present in higher concentrations compared to the basal medium DMEM. Hence it cannot be ruled out that one of these or a combination of these could be having an enhancing effect on the production of MAb from the hybridoma producing cell line 5C3.

In summary, high levels of antibody production where achieved when the hybridoma cell line, 5C3 was cultured in protein-free medium. Along with the reasonably pure nature of this antibody (Figure 3.16) due to its culture in protein-free medium it seems as if optimum conditions for production of such an antibody have been identified.

In serum-supplemented medium the levels of antibody produced are much lower than in protein-free conditions. This may be due to the interference of serum when reading samples using the nephelometer but the fact that poor results showed up on both RID analysis and the isostrip analysis (Figure 3.9), leads to the conclusion that culture of antibody 5C3 in serum-supplemented medium resulted in very low levels of antibody production.

However as discussed previously, growth of 4/2D in protein-free medium did not produce MAb in quantities comparable to the 5C3. It could be said that this hybridoma producing cell line 4/2D is simply a poor producer of monoclonal antibody.

Table 4.1 Amino acid concentrations in DMEM and PFHM II.

Amino Acid	**DMEM**		**PFHM II**
EAA	mg/L	mM	mM
L-Arginine	*84*	*1.00*	*1.24*
L-Cysteine	*48*	*0.40*	*0.14*
Glutamine(Glutamax)		*3.97*	*3.91*
L-Histidine	*42*	*0.20*	*0.37*
L-Isoleucine	*105.00*	*0.80*	*1.47*
L-Leucine	*105.00*	*0.80*	*2.52*
L-Lysine	*146.00*	*0.79*	*1.56*
L-Methionine	*30.00*	*0.20*	*0.45*
L-Phenylalanine	*66.00*	*0.40*	*0.77*
L-Theronine	*95.00*	*0.80*	*1.06*
L-Tryptophan	*16.00*	*0.078*	*0.27*
L-Tyrosine	*72.00*	*0.40*	*0.50*
L-Valine	*94.00*	*0.80*	*1.04*
NEAA			
L-Alanine	*0.00*	*0.00*	*5.89*
L-Asparagine	*0.00*	*0.00*	*3.34*
L-Aspartate	*0.00*	*0.00*	*0.10*
Glutamate	*0.00*	*0.00*	*1.02*
L-Glycine	*30.00*	*0.40*	*0.17*
L-Hydroxyproline	*0.00*	*0.00*	*0.19*
L-Proline	*0.00*	*0.00*	*3.18*
L-Serine	*42.00*	*0.40*	*2.86*

4.5 Antibody production from hybridoma 5C3 in a hollow fibre bioreactor

The artificial capillary cell culture provides an ideal environment for high-level monoclonal antibody production. Hybridomas cultured in artificial capillary systems generally secrete an increased amount of MAb in comparison to MAb production in conventional culture. This is because hybridomas cultured in hollow fibres are continuously bathed in a fresh supply of oxygen and nutrients from the circulating growth medium. At the same time lactate and growth-inhibitory secreted proteins diffuse out of the extracapillary space surrounding the cells and are diluted into the circulating medium. Hollow fibre bioreactors can consistently produce concentrated antibody depending on the type and size of the unit used. With some hybridomas, a single artificial capillary module can produce levels of MAb similar to ascities concentrations (~1mg/ml) (Beck *et al.*, 1990, Altshuler, *et al.,* 1986, Piret & Cooney, 1990).

The culture of hybridoma producing cell line 5C3 in the hollow fibre bioreactor, yielded a high concentration of antibody in the protein-free medium with an average of 5.52mg of antibody product being produced per day, compared to that provided in serum-supplemented medium of 2.19mg of antibody being produced per day (Figure 3.33).

If one examines the difference in antibody production between the first and second run in the protein-free medium, there is a decrease in the quantity of antibody produced in the second run (Figure 3.33, Table 3.18). The reason could be due to the fact that during the second run the cells were adapted from serum-supplemented conditions into protein-free conditions. If one looks at the results obtained for the culture of cells in serum-supplemented medium they were consistently well below that achieved in the presence of the protein-free medium (PFHM II). It seems that in the case of the serum-supplemented medium used in this work that something was hindering the production of antibody or may be interfering with its detection. Therefore, when the cells in the hollow fibre were weaned off serum-supplemented into protein-free culture conditions the presence of small traces of serum in the second protein-free culture could have caused the decrease in antibody quantified.

4.5.1 10 L Batch Fermenter Culture versus Hollow fibre Bioreactor

The hollow fibre bioeractor is the most commonly used method by which MAbs are cultured in projects where only gram quantities of antibody are required. However, when larger quantities are required, larger fermenter systems must be employed.

The main advantages of the hollow fibre bioreactor system over the 10L fermenter system are:

- Higher cell densities of 10^7–10^8 are obtained.
- The continuous supply of nutrients and removal of waste products ensures optimum growth conditions for the production of antibody.
- The batch removal of the product every two days (depends on each cell line) decreases the possibility of antibody degradation, which may occur due to prolonged exposure to cellular proteases (Reuveny & Lazar, 1989).
- The batch removal of the product ensures that dead cells and cellular debris are also removed, which results in increased space and new cell growth.
- The hollow fibre bioreactor used in this work was much easier to set up and operate compared to fermenters, which required a number of days for preparation and testing.
- The capital costs for the hollow fibre and associated equipment used in this work was much lower than fermenters and less space was required to house them.
- Fermenters on the other hand require a large area to house the fermenter itself and its associated controls (Lipman & Jackson, 1998).

Fermenters however, have their advantages over hollow fibre bioreactors. They are more stringently controlled in terms of pH and DO_2 concentrations *in situ*, which becomes more important as the culture period increases.

As can be seen from the Table 4.2 below the quantity of antibody produced in the fermenter far exceeds the alternative in the hollow fibre bioreactor. The table shows the cost per mg to produce antibody in each of the cell culture methods utilised throughout the course of this thesis.

Table 4.2 Comparing antibody production in the 10L fermenter and in a hollow fibre bioreactor (cellmax artificial capillary module, Cellco Inc.). Total volume used in hollow fibre bioreactor was approximately 4500 ml.

	10L (mg)	*HFB (mg)*
5C3 in SSM	290	45.97
5C3 in PFM	2965	116
4/2D in SSM	190	_____
4/2D in PFM	200	_____

Not surprisingly, the production of the 5C3 MAb in protein-free medium is the most cost effective of all methods. Its production (per mg) in the hollow fibre bioreactor is significantly more expensive than culture in the 10L fermenter. Interestingly the cost of production in the 10L fermenter is the same as that in the spinner flask. However, production of MAb in spinner flask at large scale could not be deemed feasible. Numerous units of flasks and associated magnetic stirrer bases would be required along with designated incubators. Spinner flasks are more prone to contamination due to the extra manipulations that must be carried out in the laminar flow cabinet and the movement of the flasks from the incubators to the flow cabinets.

Table 4.3 Shows comparison of the cost of production of two monoclonal antibody producing cell lines in serum-supplemented and protein-free medium by a variety of different methods.

Cell Line	*Static Flask € /mg*	*Spinner Flask € /mg*	*10 L Bioreactor € /mg*	*Hollow Fibre Unit € /mg*
5C3 in SSM	39.91	20.15	2.60	17.66
5C3 in PFM	0.44	0.26	0.26	0.313
4/2D in SSM	20.66	9.23	3.95	_____
4/2D in PFM	63.00	16.85	3.88	_____

4.6 MININEPH (Nephelometer)

The "MININEPH" is a bench-top laser nephelometer capable of performing a range of quantitative serum protein assays. The basis behind nephelometry is the ability to measure changes to analyte concentration in proportion to turbidity resulting from immune complex formation in solution. It is ideal for replacing assays currently performed by Radial Immunodiffusion (RID) and other manual techniques such as the ELISA.

The advantages of the nephelometer over currently used methods are shown in table 4.4.

Table 4.4 Comparison of the most frequently used assays for the determination of human IgG subclass levels (Adapted from Baker *et al.*, 2002).

Assay	*Sensitivity*	*Intra assay variation*	*Inter assay variation*	*Automation*	*Assay Time*
RID	ng/ml	Small	Small	No	48 --72 hours
Nephelometry	ng/ml	Very small	Very small	Complete	3-15 minutes
ELISA	ng/ml	Medium	Medium	Partly	3-4 hours

With assays completed in 3-15 minutes, this method is a logical choice for analysis of cell culture supernatants for the presence of antibodies. Real time analysis of the quantity of antibody will allow harvest of the antibody at peak times during fermentation runs. However as Table 3.23 shows nephelometry using the IgG anti sera is not specific for samples containing IgM. In such a case an assay using IgM anti serum will have to be employed and new assay criteria developed. Also in order to determine a standard a number would have to be analysed as the accuracy can vary from one to another (Section 3.10.3).

Throughout the course of this work, the nephelometer was used continuously as the method of choice for the quantification of antibody. As one can see from the results in section 3.10, validation work was carried out on the nephelometer to ensure that the procedure was fit for the purpose intended in that it ensured confidence that the correct results were obtained when assaying the test samples.

The acceptable coefficient of variance for the validation of analytical techniques is 10%. Validation work involved carrying out assays to verify the precision and reliability of the assay. The results obtained for the intra and inter variation assay showed very little variation (CV < 10%) between the results obtained when the sample was consecutively measured on ten separate occasions throughout a day (Inter Assay Variation, Table 3.18) and over a four day period (Intra Assay Variation, Table 3.19). This gave a reasonable indication of the repeatability of the instrument to yield similar results over time. However when the precision and reliability of the assay were considered it was found that the intra-assay variation was 10.2% and the inter assay variation was 15.97%. These large %CV could be explained by the fact that the system was manual and there may be a large pipetting error. This would endorse the bid for a fully automated system, which are available on the market and might reduce the error involved in the automated system.

The results obtained for repeated determination of the standard curve all have regression coefficient values of greater than 0.9990 (Figure 3.36 and Table 3.20). The linear range of the assay was also determined and found that the lower and upper limits of detection were 9.75 and 600mg/L respectively.

The accuracy and specificity of the assay was determined using a variety of IgG antibodies of varying subtypes of IgG and IgM, measuring, them against the standard curve and comparing them to their know concentrations. It can be seen from Table 3.22 that specificity and accuracy was not acceptable in each of the cases. In particular the results obtained for the anti-fodrin MAB1622 and the anti-Cytokeratin No. 18 were not as specific or accurate as those obtained for the other IgG samples. In terms of accuracy they was a difference of 16.67 and 26.65% between the know concentration of the standard and that obtained from nephelometric analysis. The results obtained for the IgM control, which had a coefficient of variance of 135.6 suggested that the specificity of the IgG anti sera did not include IgM.

Overall, as a method for quantifying MAb in supernatant the nephelometer appears to be the most efficient and reliable method used during the course of this work. RID analysis took three days and results were often difficult to read, ELISA was difficult to perform and often-yielded non-reproducible and non-linear results in our hands.

Advances in the area of nephelometry assay development are yielding fully automated 96-well plate assays allowing multiple samples to be analysed in a shorter time. In addition, because of these investigations, it is recommended to use a range of standards to validate the nephelometric assay.

Section 5 Conclusion and Future Work

5.1 Conclusions

1. The main findings of this thesis relate to the use of different hybridoma producing cell lines and their requirements for optimal cell growth and antibody productivity. It is clear that each cell line performs differently under different culture conditions. Therefore, it can be said that different cell lines cannot be cultured under the same process conditions. For example, the optimum conditions for the growth of the hybridoma producing cell lines 4/2D and 5C3 in serum supplemented and in the case of 5C3 in protein free medium were 100rpm and 37°C. The culture of the hybridoma 4/2D in protein free medium yielded higher antibody yields when agitated at 200rpm.

2. Another point noted was the reduction in antibody production of the 4/2D cell line after day 3 in the large-scale process. This was observed in one of two runs under SSM conditions. At small-scale antibody levels increased consistently throughout the 6 day culture period. Although no evidence is available, it is possible that protease inhibitors may have had a role, but this needs to be clarified with future work.

3. A number of methods were examined in the production of MAb namely the hollow fibre bioreactor, 10L fermenter and spinner flasks. These three methods were compared in detail in terms of antibody productivity. However, the cost of using each method must also be taken into consideration when developing a cost effective production process. In general, the production of MAb in protein free medium has proven much cheaper than culture in serum-supplemented medium. This is mainly due to the huge increase in serum prices observed in the last two years due to a shortage in high quality serum. The price of sera used in this work has almost doubled. In terms of the most cost efficient method of production, it was found that culture of each of the cell lines was cheaper in the 10L fermenter.

4. A new system of quantifying MAb, the nephelometer has been introduced into the laboratory. This method has proven to be a reliable and efficient means of quantifying antibody. It replaced the more commonly used methods of RID and ELISA, which are both unreliable and time consuming. For example, a

nephelometric assay takes 15 minutes to complete, RID takes three days and ELISAs approximately 5 hours.

5.2 Future Work

1. An investigation into why a drop in antibody production was observed at large scale past day 3 and as to why these trends were not observed in spinner flasks should be carried out. It has been reported in the literature that antibody loss can occur in culture due to the action of acid proteases (Teige, 1994: Kratje *et al.*, 1994). To prevent this from happening in the future it would be necessary to identify what factors are responsible for the development of the acid proteases and how it can be removed or reduced. According to the literature the traditional way to handle acid proteases is to run the culture at reduced temperatures (Enfors, 1992). In order to monitor protease activity in culture, an assay based on the measurement of trichloroacetic acid soluble peptides, which are released from isotypocally labelled protein substrates could be performed. Another assay based on characterisation with chromogenic substrates would involve testing culture supernatants with artifical chromogenic peptide substrates based on *p*-nitroanalide dihydrochloride conjugates. This will allow a partial characterisation of the protease because of the specificity of the derivatives to particular types of enzymes (Kratje *et al.*, 1994). By monitoring the protease action under different operating conditions, one will be able to determine the most appropriate environment in which to culture the cells with reduced protease action.

   Another explanation for the reduction in antibody could have been the decrease in glucose and increase in lactate concentrations, which would correspond with the decreases in antibody productivity. To examine this possibility further experiments looking at the effect of varying concentrations of lactate and glucose in culture medium on antibody productivity need to be examined.

2. In order to try and optimise MAb production further, fed batch cultures at the 10L scale should be carried out. Batch cultures last only 6 to 7 days depending on the cell line. With the lead in time and cost involved in setting up fermentation runs at this scale it would be more economical to utilise a fed-batch regime. From the work

carried out in this thesis the growth, antibody productivity, glucose and lactate characteristics of the hybridoma producing cell lines 4/2D and 5C3 have been clearly determined. Using this information one can identify the appropriate times to harvest and re-feed each cell line. Figures 3.26, 3.27. 3.30 and 3.30 show the exact time after which lactate becomes inhibitory and glucose rate limiting. It is clear from these figures that this time line varies from cell line to cell line. Therefore, exact process conditions should be determined for each individual cell line.

3. Further investigation into why the hybridoma producing cell line 5C3 cultured in protein free medium yielded concentrations of MAb 10-15 fold in excess of that achieved in the serum supplemented control is required. In addition, an investigation into why similar results were not being obtained for the culture of the hybridoma producing cell line 4/2D in protein free conditions is necessary. In order to try to mimic the antibody productivity results achieved from the hybridoma producing cell line 5C3, it would be necessary to take a basal medium and add various growth factors or additives in an attempt to recreate the culture conditions that yielded such a positive result. With such a medium, it would be clear what it is comprised of and may reveal what exactly is having the positive effect on antibody productivity.

4. On reflection of this is work western blot analysis to give an exact determination of the presence of MAb in the cell culture supernatants. SDS-PAGE was used but interference particularly in the case of serum-supplemented samples leaves a question over the exact existence of IgG bands.

Section 6 Bibliography

Abu-Reesh, I & Kargi, G (1991). Biological responses of hybridoma cells to hydrodynamic shear in an agitated bioreactor, *Enzyme Micro. Technol* **13,** 913-919.

Aloi, E.L & Cherry, R.S (1996). Cellular Response to agitation characterised by energy dissapitation at the impeller tip, *Chemical Engineering Science* **51 (9),** 1523-1529.

Altshuler, G.L., Dziewulski, D.M., Sowek, J.A. and Belfort, G. (1986). Continuous hybridoma growth and monoclonal antibody production in hollow fibre reactors-separators, *Biotechnology and Bioengineering*, **28**, 646-658.

Bailey, J.E & Ollis, D.F. (1986). Biochemical engineering fundamentals, 2nd edn, New York McGraw-Hill.

Baker, K.N., Rendall, M.H., Patel, A, Boyd, P, Hoare, M., Freedman, R.B. and James, D.C. (2002). Parid monitoring of recombinant protein products: a comparison of current technologies, *TIBTECH*, **20 (4),** 149-156.

Barnabē, N & Butler, M (1992). Effect of temperature on Nucleotide pools and monoclonal antibody production in a mouse hybridoma, *Biotechnol. Bioeng.* **44**, 1235-1245.

Barnes, D & Sato, G. (1980). Methods for growth of cultured cells in serum-free medium, *Anal. Biochem.* **102**, 255-270.

Beck, T, Shueh, L, Sitlani, S and Menaker, M (1990). Monoclonal Antibody Production in the CELLMAX® Artificial capillary system, *FOCUS* **17 (1),** 1-5.

Berkower, I (1996). The promise and pitfalls of monoclonal antibody therapeutics, *Current Opinion in Biotechnology* **7**, 622-628.

Bethheussen, K. (1993). Growth of cells in a new defined protein-free medium. *Cytotechnology* **11**, 219-231.

Bibila, T.A. & Robinson, D.K. (1995). In pursuit of the Optimal Fed-Batch Process for Monoclonal Antibody Production, *Biotechnology Progress* **11,** 1-13.

BioResearch Ireland: Bio-Industry: A-Z of Irish Biotechnology: Biotech Companies [online] http://www.biores-irl.ie [Accessed 29-October-2001].

Bierau, H (1998). A comparison of intensive cell culture bioreactors operating with Hybridomas modified for inhibited apoptotic response, *J of Biotechnol.* **62**, 195-207.

Blasey, H.D. & Winzer, U. (1989). Low protein serum-free medium for antibody-production in stirred bioreactors. *Biotechnol. Lett.* **11 (7),** 455-460.

Bloemkolk, J., Gray, M., Merchant, F., Mosmann, T. (1994). Effect of temperature on hybridoma cell cycle and MAb production, *Biotechnol. Bioeng*, **40**, 427-431.

Borrebaeck, C.A.K (1988). Human MAbs produced by *in-vitro* immunisation, *Immunology Today* **9**, 355-359.

Borrebaeck, C.A.K and Carisson, R (2001). Human Therapeutic antibodies, *Current Opinions in Pharmacology* **1**, 404-408.

Borrebaeck, C.A.K (2000). Antibodies in diagnostics-from immunoassays to protein chips, *Immunology Today* **21,** 379-382.

Bossuyt, X, Vranken, G, Mariën, G and Blanckaert, N (2001). Monoclonal IgM: difficulties with correct measurement, *Ann Clin Biochem* **38**, 708-710.

Brass, J.M (1996). Quality Assurance after process changes of the production of a therapeutic antibody, *Pharmaceutica Acta Helevetiae* **71**, 395-403.

Breedveld, F.C (2000). Therapeutic monoclonal antibodies, *Lancet* **355**, 735-40.

Burak, W.E, DePalatis, L, Nines, R, Hitchcock, C, Hinkle, G, Houchens, D and Farrer, W.B (1998). Biodistribution and Localisation of Radiolabelled NR-LU-10 Fab fragment in Human Breast Cancer Xenographs, *Nuclear Medicine & Biology* **25**, 633-637.

Chen, K., Liu, Q., Xie, L., Sharp, P.A., Wang, D.I.C (2000). Engineering of a Mammalian Cell Line for Reduction of Lactate Formation and High Monoclonal Antibody Production, *Biotechnol. Bioeng.* **72**, 55-61.

Chen, Z., Ke, Y., and Chen, Y., (1993). A serum-free medium for hybridoma cell culture, *Cytotechnology* **11**, 169-174.

Chisti, Y (2000). Animal-cell damage in sparged bioreactors, *TIBECH* **18** 420-432.

Chisti, Y (1993). Animal Cell Culture in Stirred Bioreactors: Observations on Scale-up, *Process Biochemistry* **28**, 511-517.

Chow, S-C (1997). Pharmaceutical Validation and Process Controls in drug development. *Drug Information Journal* **31**, 1195-1201.

Chu, L and Robinson, D.K. (2001). Industrial choices for protein production by large-scale cell culture; *Current Opinion in Biotechnology* **12**, 180-187.

Chua, F., Oh, K.W.S., Yap, M., Teo, W.K., (1994). Enhanced IgG production in Erdf media with and without serum, *J.Immunol. Methods* **167**, 109-119.

Chuppa, S, Tsai, Y-S, Yoon, S, Shackleford, S, Rozales, C, Bhat, R, Tsay, G, Matanguihan, C, Konstantinov, K and Naveh, D (1996). Fermentor temperature as a tool for control of high-density perfusion cultures of mammalian cells, *Biotechnol. Bioeng.* **55 (2)**, 328-338.

Clark, S.A., Looby, D., and Griffiths, J.B., (1989). The adaptation of hybridoma cell lines to grow and secrete monoclonal in serum-free/defined medium. In: R.E. Spier., J.B. Griffiths., J. Stephenne and P.J. Croovy, (Eds.), Advances in Animal Cell Biology and Technology for Bioprocesses. Butterworth, London (European Society for Animal Cell technology, 9th meeting). 291-297.

Clark, M (2000a). Antibody humanisation: a case of the emperor's new clothes'? *Immunology Today* **21 (8)**, 397-402.

Clark, M (2000b). Antibody Reshaping [Online] http://www/path.cam.ac.uk/mrc7/reshaping/intro.html. [Accessed 22-August-00].

Cleveland, W.L., Wood, I., Erlanger, B.F., (1983). Routine large-scale production of monclonal antibodies in a protein free culture medium, *J. Immunol. Methods* **56**, 221-234.

Clynes M, Redmond A, Moran E, Gilvarry , (1992). Multiple drug resistance in variants of a

human non-small cell, DLKPA. *Cytotechnology;* **10,** 75-89.

Cragg, M.S., French, R.R., and Glennin, M.J. (1999). Signalling antibodies in cancer therapy, *Current Opinion in Immunol.,* **11** 541-547.

Crowther, J.R. (2001). The ELISA guidebook, 1st edn. Humana Press.

Czirbik, R.J., Rosen, S.M., Trunfio, D.M., Fischber, E.W., Palmer, S.M. (1996). Factors affecting antibody production efficiency in hollow fibre bioreactors, [online] http://www.devicelink.com/ivd/archive/97/07/007.html [Accessed 04-October-2001].

Darfler, F.J. (1990a). A protein-free medium for the growth of hybridomas and other cells of the immune system, *In Vitro Cell. Dev. Biol.* **26,** 769-778.

Darfler, F.J. (1990b). Preparation and use of lipid microemulsions as nutritional supplements for culturing mammalian cells, *In Vitro. Cell. Dev. Biol.* **26,** 779-783.

Deo, Y.M, Mahadevan, M.D. and Fuchs, R (1996). Practical considerations in-operation and scale up of spin-filter based bioreactors for monoclonal antibody production, *Biotechnol. Progress* **12 (1),** 57-64.

Deshusses, M.A. Chen, W., Mulchandani, A and Dunn, J.I. (1997). Innovative Bioreactors, *Current Opinion in Biotechnology* **8**, 165-168.

Dhainaut, F., Bihoreau, J.L., Meterreau, J., Lirochou, R., Vincentelly, R and Maignot, G. (1992). Continuos production of large amounts of monoclonal immunoglobulins in hollow fibres using protein free medium, *Cytotechnology* **10**, 33-41.

Dodge, T.C and Hu, W.S. (1985). Growth of hybridoma cells under different agitation conditions, *Biotechnol. Lett.* **8 (10)**, 683-686.

Doran, P.M. (1995). Bioprocessing Engineering Principles, 1st edn, Academic Press Ltd., London 24-28.

Dutton, G (2001). MAb Manufacturing Concerns, *Genetic Engineering News* **21 (14),** 1-5.

Elias, C.B., Rajiv, B.D., Milind, S.P., Jyeshtharaj, B.J. and Raghunath, A.M. (1995). Turbulent shear stress-effect on mammalian cell culture and measurement using laser doppler anemometer, *Chemical Engineering Science* **50 (15),** 2431-2440.

EMEA (2000). ICH Q7A, Good manufacturing practice for active pharmaceutical ingredients, CPMP/ICH/4106/00, London.

Enfors, S-O (1992). Control of in vivo proteolysis in the production of recombinant proteins, *TIBTECH* **10,** 310-315.

Engel LW, Young NA, Tralka TS, Lippman ME, O'Brien SJ, Joyce MJ (1978). Establishment and characterisation of three new continuous cell lines derived from human breast carcinomas. *Cancer Res* **38,** 3352-3364.

Fahey, J.L. and McKelvey, E.M (1965). Quantitative determination of serum immunoglobulins in antibody-agar plates, *J.Immunol.* **94**, 84-90.

FDA, (1987). Guideline on general principles of process validation. Centre for drug evaluation and research, Centre for biologics Evaluation and research and Centre for devices

and radiological health Food and rug administration; 1-13.

FDA, (1997). Points to consider in the manufacture and testing of monoclonal antibody products for human use. U.S Department of Health and Human Services, Centre for Biologics Evaluation and Research; 1-50.

Federal Register, (1998). Q6B Specifications: Test procedures and Acceptance criteria for Biotechnological Biological Products. **63,** 31507-31513.

Federspiel, G., McCullough, K.C. and Kihm, U (1991). Hybridoma antibody production in vitro in type II serum-free medium using Nutridoma-SP supplements, *J.Immunol. Methods* **145,** 213-221.

Fishwild, D.M., O'Donnell, S.L., Bengoechea, T., Hudson, D.V., Harding, F., Bernhard, S.L., Jones, D., Kay, R.M., Higgins, K.M., Schramm, S.R., Loberg, N (1996). High-Avidity human Ig_k monoclonal antibodies from a novel strain of minilocus transgenic mice, *Nat. Biotechnol.* **14**, 845.

Franěk, F., and Dolnikova, J., (1991). Hybridoma growth and monoclonal antibody production in iron-rich protein-free medium: effect of nutrient concentration, *Cytotechnology* **7,** 33-38.

Freshney, R.I. (1994). Culture of animal cells: A manual of basic techniques, 3rd edn, Wiley-Liss Inc, NY.

Fu, P and Barford, J.P. (1994). Methods and strategies for the process control and optimisation of monoclonal antibody production, *Cytotechnology* **14,** 219-232.

Furukawa, K and Ohsuye, K (1998). Effect of culture temperature on a recombinant CHO cell line producing a C-terminal α-amidating enzyme, *Cytotechnology* **26,** 153-164.

Gaida, T Doblhoff-Dier O, Strutzenberger, K, Katinger, H, Burger, W, Groschl, M, Handl, B, Benes, E (1996). Selective retention of viable cells in ultrasonic resonance field devices, *Biotechnology Progress* **12 (1)**, 73-76.

Gardner, J.S., Chiu, A.I.H., Maki, N.E., Harris, J.F., (1985). A quantitative stability analysis of human Mab production by heteromyeloma hybridomas, using an immunofluorescent technique, J.Immunol. Methods **85,** 335-346.

Glassey, M.C., Tharakan, J.P., and Chau, P.C., (1988). Serum free media in hybridoma culture and monoclonal antibody production, *Biotech. Bioeng* **32,** 1015-1028.

Glennie, M.J and Johnson, P.W.M (2000). Clinical Trials of Antibody Therapy, *Immunology Today* **21**, 403-410.

Gold, D.V, Cardillo, T, Goldenberg, D.M and Sharkey, R.M (2001). Localisation of pancreatic cancer with radiolabelled monoclonal antibody PAM4, *Critical reviews in Oncology/Haematology* **39,** 147-154.

Goldstein, G (1085). A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants, *The New England J. of Medicine* **313 (6)** 337-341.

Gorter, A and Meri, S (2000). Immune evasion of tumour cells using membrane-bound compregulatory proteins, *Immunology Today* **20**, (12) 576-582.

Govindan, S.V., Goldenberg, D.M., Hansen, H.J., and Griffiths, G.L. (2000). Advances in the use of monoclonal antibodies in cancer radiotherapy, *PSTT* **3 (3)** 90-98.

Graf, H., Rabaud, J.N., and Egly, J.M., (1991). Screening of various mammalian cell culture media to establish a downstream purification scheme, *J.Immunol. Methods* **139,** 135-144.

Graner, M.J., Poeschi, D.M., Conroy, M.J., and Hammer, B.E. (1999). Effect of Harvesting Protocol on Performance of a Hollow Fibre Bioreactor, *Biotechnol. Bioeng.* **65 (3),** 334-340.

Green, L.L. (1999). Antibody engineering via genetic engineering of the mouse: Xenomouse strains are a vehicle for the facile generation of therapeutic human monoclonal antibodies, *J. Immuno. Methods 231*, 11-23.

Griffiths, J.B. (1988). The cultivation of Animal Cells in Continuous-flow Culture. In: Spier, R.E. and Griffiths, J.B. (ed), Animal Cell Biotechnology Volume 3, 1st edn, Academic Press Inc, Florida, USA 179-215.

Griffiths, J.B. (1987). Serum and Growth factors in cell culture media – An introductory review, *Dev. Biol. Stan.* **66,** 155-160.

Grima, M.E , Chisti, Y, Murray, M-Y (1997). Characterization of shear rates in airlift bioreactors for animal cell culture, *J. Biotechnol.* **54,** 195-210.

Handa-Corrigan, A., Emery, A.N. and Spier, E (1989). Effect of gas-liquid interfaces on the growth of suspended mammalian cells: mechanisms of cell damage by bubbles, *Enzyme Microbiology Technology*, **11**, 230-235.

Heifetz, A.H Braatz, J.A., Wolfe, R.A., Barry, R.M., Miller, D.A., Solomon, B.H. (1989). Monoclonal Antibody Production in Hollow Fibre Bioreactors Using Serum-Free Medium, *Biotechniques* **7 (2),** 192-199.

Hendershot L.M., (1990). Immunoglobulin heavy chain and binding protein complexes are dissociated in vivo by light chain addition, *J. Cell. Biol.* **111**, 829-837.

Hesse, F and Wagner, R (2000). Developments and improvements in the manufacturing of human therapeutics with mammalian cell cultures. *TIBTECH* **18**, 173-180

Hsu SM, Raine L, Fanger H. (1981). Use of avidin-biotin-peroxidase comples (ABC) immunoperoxidase techniques. *J Histochem.* **29,** 577-580.

Hunkeler, N., Hu, W.S., Srienc, F (1989). Hybridoma subpopulations affecting culture productivity, Proceedings of the Annual American Chemical Society Meeting.

Hu, W-S and Aunins, J.G. (1997). Large-scale mammalian cell culture, *Current Opinion in Biotechnology* **8**, 148-153.

Hu, W-S and Peshwa, M.V (1991). Animal cell Bioreactors- Recent Advances and Challenges to Scale-Up, *The Canadian Journal of Chemical Engineering* **69**, 409-417.

Huang WM, Gibson SJ, Facer P, GN J, Polak JM (1983). Improved section adhesion for immunocytochemistry using high molecular weight polymers of L-lysine as a slide coating. *Histochemistry*; **77(2)** 275-279.

ICH Guidelines. Validation of Analytical Procedures Definitions and Terminology, ICH Q2A 111/5626/94.

ICH Guidelines. Validation of analytical Procedures and Methodology, ICH Q2BCPMP/ICH/2891/95.

Jackson, L.R., Trudel, L.S., Fox, J.G., and Lipman, N.S. (1996). Evaluation of hollow fibre bioreactors as an alternative to murine ascities production for small-scale monoclonal antibody production, *J Immunol. Methods* **189**, 217-231.

Jayme, D.W. (1991). Nutrient optimization for high-density biological production applications, *Cytotechnology* **5,** 15-30.

Jenkins, H.A., Butler, M and Dickson, A.J. (1992). Characterisation of glutamine metabolism in two related hybridomas, *J of Biotechnol.*, **23**, 167-182.

Jöbses, I., Martens, D and Tramper, J. (1991). Lethal Events during Gas Sparging in Animal Cell Culture, *Biotechnol. Bioeng.* **37**, 484-490.

Jones, P.T (1986). Replacing the complementarity-determining regions in a human antibody with those from a mouse, *Nature* **321,** 521-525.

Jorjani, P and Ozturk, S.S, (1999). Effects of cell density and temperature on oxygen consumption rate for different mammalian cell lines, *Biotechnol. Bioeng.*, **64 (3),** 349-356.

Karpas, A, Dremucheva, A, Czepulkowski, B.H. (2001). A human myeloma cell line suitable for the generation of human monoclonal antibodies, *PNAS* **98 (4),** 1799-1804.

Katinger, H and Scheirer, W. (1985). The cultivation of Animal Cells in Continuos-flow Culture. In: Spier, R.E. and Griffiths, J.B. (ed), Animal Cell Biotechnology Volume 1, 1st edn, Academic Press Inc, Florida, USA 167-191.

Kauffmann, H., Mazur, X., Fussenegger, M and Bailey, J.E. (1999). Influence of low temperature on productivity, proteome and protein phosphorylation of CHO cells, *Biotechnol. Bioeng.* **63 (5),** 573-582.

Kawamoto, T., Shintani, Y., Ichimori, Y., Tsukamoto, K., Sasai, S and Kida, M.,(1986). Development of a serum-free medium for growth of NS-1 mouse myeloma cells and its application to the isolation of NS-1 hybridomas, *Anal. Biochem.* **130,** 445-453.

Kelley, B.D. (2001). Biochemical engineering, Bioprocessing of therapeutic proteins, *Current Opinion in Biotechnol.* **12**, 173-174.

Khaw, B-A (1999). Antibodies as delivery systems for diagnostic functions, *Advanced Drug Delivery Reviews* **37** 63-80.

King, J.D. (1998). Applications and Engineering of Monoclonal Antibodies, 1st edn. Taylor and Francis Ltd, London EC4A 3DE.

Kitano, K., Shintani, Y., Ichimori, Y., Tsukamoto, K., Sasai, S and Kida, M. (1986). Production of human monoclonal antibodies by heterohybridomas, *Appl. Microbiol. Biotechnol.* **24,** 282-286.

Klerx, J.P.A.M., Verplanke, J.C., Bloak, C.G. and Twaalfhaven, L.C. (1988). In vitro production of monoclonal antibodies under serum free conditions using a compact and inexpensive hollow fibre cell culture unit, *J. Immunol. Methods* **111,** 179-188.

Knazek, R A; Gullino, P M; Kohler, P O; Dedrick, R L (1972). Cell culture on artificial capillaries: an approach to tissue growth in vitro, *Science*, **178 (56),** 65-66.

Kohler, G and Milstein, C (1975). Continuos cultures of fused cells secreting antibody of predefined specificity, *Nature,* **256,** 495-497.

Korke, R, Rink, A, Seow, T.K., Chung, C.M.M., Beattie, C.W., Hu, W-S, (2002). Genomic and proteomic perspectives in cell culture engineering, *J. Biotechnol.* **94,** 73.92.

Kovar, J & Franek, F., (1984). Serum-free medium for hybridoma and parental myeloma cell cultivation: a novel composition of growth-supporting substances, *Immunol. Lett.* **7,** 339-345.

Kovar, J & Franek, F., (1985). Hybridoma cultivation in defined serum-free media: growth promoting substances, I, Transferrin, *Folia Biologica (Praha)* **31,** 167-175.

Kovar, J & Franek, F., (1986a). Serum-free medium for hybridoma and parental myeloma cell cultivation, *Methods in Enzymology* **121,** 277-292.

Kovar, J. (1986b). Hybridoma cultivation in defined serum-free media: Growth-supporting substances III. Ethanolamine, vitamins, and other low molecular weight nutrients, *Folia Biologica (Praha)* **32,** 369-376.

Kovar, J.(1986c). Hybridoma cultivation in defined serum-free media: Growth supporting substances II. Insulin, other hormones, and growth factors, *Folia Biologica (Praha)* **32,** 301-310.

Kovar, J., (1987). Hybridoma cultivation in defined serum-free media: Growth supporting substances IV. Lipids and serum albumin, *Folia Biologica (Praha)* **33,** 377-384.

Kovar, J & Franek, F., (1987). Iron compounds at high concentration enables hybridoma growth in protein-free medium, *Biotechnol. Lett.* **9 (4),** 259-264.

Kovar, J., (1988). Hybridoma cultivation in defined serum-free media: Growth supporting substances V. Trace elements, *Folia Biologica (Praha)* **34,** 264-269.

Kratje, R.B., Lind, W and Wagner, R (1994). Evaluation of the proteolytic potential of in vitro-cultivated hybridoma and recombinant mammalian cells, *J. Biotechnol.* **32,** 107-125.

Kromenaker, S.J. and Srienc, F (1994). Effect of lactic acid on the kinetics of growth and antibody production in a murine hybridoma: secretion patterns during the cell cycle, *J. Biotechnol.*, **34,** 13-34.

Kunas, K.T. and Papoutsakis, E.T. (1990). The protective effect of serum against hydrodynamic damage of hybridoma cells in agitated and surface-aerated bioreactors, *J. Biotechnol.*, **15 (1-2),** 57-69.

Kundu, K.P., Prasad, N.S, Electricwala, S.E.,Varma, R. and Datta, D. (1998). Getting higher yields of monoclonal antibodies in culture, *Indian Journal of Physiol Pharmacol*, **42 (2),** 155-171.

Łabęcki, M (2001). Experimental and Theoretical studies of protein transport in Hollow Fibre Bioreactors for Mammalian Cell Culture: PhD thesis, The University of British Columbia, Vancouver, B.C., Canada [online] http://www.iam.ubc.ca/~marek/Chapter1.pdf [Accessed 31-November-2001].

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature Lond* 1970; **227,** 680-685.

Lalor, P (2001). Optimisation of the Methodology for the Isolation and Identification of an Unknown Antigen, MSc. thesis, Cranfield University, Silsoe, United Kingdom

Lang, A.B., Schuerch, U., and Cryz, S .J.Jr (1991). Optimization of growth and secretion of human monoconal antibodies by hybridomas cultured in serum-free media, *Hybridoma* **10 (3),** 401-409.

Larkin, A.M (2002). The expression of multiple drug resistance (mdr) protein in invasive breast cancer, PhD thesis, Dublin City University, NCTCC Dublin 9, Ireland.

Lee, M.G. Huard, T.K., Kaminski, M.S., Palsson, S.O. (1988). Effect of mechanical agitation on hybridoma cell growth, *Biotechnol. Lett.* **10 (9),** 625-628.

Lewis, R (1994). Monoclonal antibodies find utility in Cell Biology, *The Scientist* **8 (24)**, 16.

Lipman, N.S. & Jackson, L.R. (1998). Hollow fibre bioreactors: an alternative to murine ascities for small scale (<1 gram) monoclonal antibody production, *Research in Immunology*, **149 (6)**, 571-576

Little, M Kipriyano, S.M., Le gall, F and Moldenhauer, G (2000). Of mice and men: hybridoma and recombinant antibodies, *Immunology Today* **21 (8)**, 364-370.

Ljunggren, J and Häggström, L (1995). Specific growth rate as a parameter for tracing growth limiting substances in animal cell cultures, *J. Biotechnol.* **42** 163-175.

Low, K & Harbour, C (1985). Growth kinetics of hybridoma cells: (2): The effects of varying energy source concentrations, *Develop. Biol. Standard,* **60** 73-79.

Lowe, K.C, Davey, M.R., Power, J.B. (1998). Perfluorochemicals: their applications and benefits to cell culture, *TIBTECH* **16** 272-277.

Lowrey, D, Murphy, S and Randal, A.G. (1993). The effect of intracapillary media feed protocols on hollow fibre cell culture, *Biotechnol. Lett.*, **15 (10),** 1025-1030.

Lowrey, D, Murphy, S and Randal, A.G. (1994). A comparison of monoclonal antibody productivity in different hollow fibre bioreactors, *J Biotechnol.*. **36,** 35-38.

Lütkemeyer, D., Peggendorf, I., Schener, T., Zhag, J., Knoll, A., Lhmann, J. (2000). First Steps in Robot Automation of Sampling and Sample Management during Cultivation of Mammalian Cells in Pilot Scale, *Biotechnology Progress* **16,** 822-828.

Mader, S.M. (1996). Biology, 5th edn, The Times Mirror Higher Education Group, Inc, USA.

Mancini, G, Carbonara, A.O., Heremans, J.F. (1964). Immunochemical quantitation of antigens by single radial immunodiffusion, *Immunochem.*, **2,** 235-254.

Mariani, E., Mariani, A.R., Monaco, M.C.G., Lalli, E., Vitale, M., and Facchini, A. (1991). Commericial serum-free media: hybridoma growth and monoclonal antibody production, *J.Immunol. Methods* **145,** 175-183.

Martial, A., Dousset, B., Dardenne, M., Engasser, J.M., Nabet, P., and Marc, A (1994). Insulin utilization and kinmetic effect on hybridoma metabolism in batch and continuous culture, *J. Biotechnol.* **15 (2),** 195-203.

Martial-Gros, A, Sarin, K.K., Mukhopadhyay, A, Ghosh, S (1999). Feasibility studies of large scale production of human anti-tetanus toxoid monoclonal antibodies, *J. Biotechnol.* **67**, 205-216.

McArdle, J (1997). Alternatives to ascities production of monoclonal antibodies, Animal Welfare Information Center Newsletter, **8 (3-4)** [online] http://www.nalusda.gov/awic/newsletters/v8n3/8n3mcard.htm [Accessed 25-August-01].

McGlinchey, E and O'Driscoll, D (1998). Scale-Up of Animal Cell Cultures. In Clynes, M (ed), Mammalian Cell Culture, 1st edn, New York : Springer Lab Press 459-481.

McKenna, N (2001). Challenges in Scale-up of Antibody Production, *Gen. Eng. News,* **21 (14)**, 12-13.

Meisenholder, G (1999). Post-modern Culture: Maximising Cell Culture Output at every level, The Scientist **13 (14):21**; [online] http://www.the-scientist.com/yr1999/july/profile2_990705.html [Accessed 04-October-2001].

Mercille, S; Johnson, M; Lanthier, S; Kamen, A.A; Massie, B (2000). Understanding factors that limit the productivity of suspension-based perfusion cultures operated at high medium renewal rates, *Biotechnol. Bioeng,*, **67 (4)**, 435-450.

Mercille, S. and Maisse, B (1998). Apoptosis-resistant NS/0 EIB-19K myelomas exhibit increased viability and chimaeric antibody productivity under cell cycle modulating conditions, *Cytotechnology* **28 (1-3),** 189-203.

Merten, O.-W. and Litwin, J. (1991). Serum-free medium for fermentor cultures of hybridomas, *Cytotechnology* **5**, 69-82.

Metcalf, H., Field, R.P., Froud, S.J. (1994). The use of 2-hydroxy-2,4,6-cycloheptarin-1-one (tropolone) as a replacement for transferrin. InR.E. Spier, J.B. Griffiths and W. Bertholds (Eds.), Animal Cell Technology, Products of Today, Prospects for tomorrow. (European Society for Animal Cell Technology, 12th meeting) 88-90.

Mizrahi, A and Lazar, A, (1988). Media for cultivation of animal cells: an overview, *Cytotechnology* **1**, 199-214.

Mochizuki, K., Sato, S., Kato, M and Hasizume, S (1993). Enhanced production of human monoclonal antibodies by the use of fructose in serum-free hybridoma culture media, *Cytotechnology* **13**, 161-173.

Moore, A, Mercer, J, Dutina, G, Donahue, C.J., Bauer, K.D., Mather, P.J., Etcheverry, T., and Ryll, T (1997). Effects of temperature shift on cell cycle, apoptosis and nucleotide pool in CHO cell batch cultures, *Cytotechnology* **23**, 47-54.

Moran EB, McGowan ST, McGuire JM, Frankland JE, Oyebade IA, Waller W, Archer LC, Morris LO, Pandya J, Nathan SR, Smith L, Cadette ML, Michalowski JT (2000). A systematic Approach to the Validation of Process Control Parameters for Monoclonal Antibody Production in Fed-Batch Culture of a Murine Myleoma. *Biotechnol. Bioeng.* **69**, 242-254.

Moran E, Larkin A.M., Cleary I, Barnes C, Kennedy S.M., Kelehan P and Clynes, M (1998). Monoclonal antibodies raised to paraffin wax embedded archival tissue; feasibility study of their potential to detect novel antigenic markers, *J. of Immunol. Methods* **219** 151-159.

Moran E, Larkin A-M, Cleary I, Nugent W, O'Driscoll D, and O' Dea C (1998). Production of mdr-1-specific monoclonal antibody (6/1C) by six different methods and assessment of IgG levels obtained, quality of MAb, cost of production plus start-up and ease of operation of all methods, Unpublished.

Moran E, Cleary I, Larkin A.M., Nic Amhlaoibh, R, Masterson A, Scheper R.J., Izquierdo M.A., Center M, O'Sullivan F, and Clynes M (1997). Co-Expression of MDR-associated Markers, Including P-170, MRP and LRP and cytoskeletal proteins, in three resistant variants of the human ovarian carcinoma cell line, OAW42, *European J of Cancer* **33 (4)** 652-660.

Moran, E, Larkin, A.M, Woodman, A, Doherty, G and Clynes, M (1996). Antibodies generated to synthetic peptides specifically recognise their target antigens (P-170 and topoisomerase II-α) by immunochistochemical techniques and Western blotting, Cytotechnology, 19 (3) [Abstract].

Moro, A.M., Rodrigues, M.T., Gouvea, M.N., Silvestri, M.L., Kalil, J.E. and Raw, I. (1994). Multiparametric analyses and hybridoma growth on glass cylinders in a packed-bed bioreactor system with internal aeration. Serum supplemented and serum free media comparison for MAb production, *J.Immunol. Methods*. **176**, 67-77.

Morrow, J., (1990). Serum-free media spur development of monoclonal antibody technology, *Gen. Eng. News* **May 9**, 24.

Murakami, H., Edamoto, T., Nakamura, H., and Omura, H. (1982a). Growth of myeloma MPC-11 cells in serum-free growth factor supplemented medium,. *Agric.Biol.Chem.* **46 (7)**, 1831-1837.

Murakami, H., Masui, H., Sato, G.h., Sueoka, N., Chow, T.P. and Kano-Sueoka, T. (1982b). Growth of hybridoma cells in serum-free medium: ethanolamine is an essential component, *Proc.Natl.Acad.Sci.* U.S.A. **79**, 1158-1162.

Nagira, k., Hara, T., Hayashida, M., Osada, K., Masamobu, S., Sasamoto, K., Kina, K and Murakami, H., (1995). Development of a protein-free medium with iron salts replacing transferrin for a human-human hybridoma, *Biosci. Biotech. Biochem.* **59 (4)**, 743-745.

Oh, S.K.W., Nienow, A.W., Al-Rubeai, M, Emeneson, J (1989). The effect of agitation intensitywith and without sparging on the growth and antibody production of hybridoma cells, *J. Biotechnol.* **12**, 45-62.

Okeson, C.D. and Riley, M.R. (2001). Glutamine replenishment and ammonia removal in hybridoma cell cultures via immobilised glutamine synthase, *Biochem. Eng.* **9** 125-133.

Omasa T, Ishimoto M, Higashiyama K, Shioya S, Suga K (1992). The enhancement of specific antibody production rate in glucose and glutamine-controlled fed-batch culture, *Cytotechnology.* **8 (1)** 75-84.

Omasa, T., Kobayashi, M., Niskikawa, T., Shioya, S., Suga, K-I., Uemura, S-I., Kitani, W. and Imaiura, Y. (1995). Enhancement of Antibody Production by Growth Factor Addition in Perfusion and Hollow-Fibre Culture Systems, *Biotechnol. Bioeng.*, **48 (6),** 673-680.

Otterbach F, Bankfalvi A, Bergner S, Decker T, Krech R, Boecker W (2000). Cytokeratin 5/6 immunohistochemistry assists the differential diagnosis of atypical proliferations of the breast. *Histopathology* **3**, 232-40

Ozturk, S.S., and Palsson, B.O. (1991a). Growth, metabolic and antibody production kinetics of hybridoma cell culture. 1. Analysis of data from controlled batch reactors, *Biotechnol. Prog*, **7** 471-480.

Ozturk, S.S., and Palsson, B.O., (1991b). Examination of serum and bovine serum albumin as shear protective agents in agitated cultures of hybridoma cells, *J. Biotechnol.* **18**, 13-28.

Passini, C & Gouchee, C (1989). Response of a mouse hybridoma cell line to heat shock, agitation and sparging, *Biotechnol. Progress* **5**, 175-188.

Pannell, R and Milstein, C (1992). An oscillating bubble chamber for laboratory scale production of monoclonal antibodies as an alternative to ascitc tumours, *J. Immunol Methods* **146**, 43-48.

PDA, (1999). "Note for guidance on Process Validation" (CPMP/QWP/848/96 draft, Sept 1999)

Piret, J.M. & Cooney, C.L. (1990). Mammalian cell and protein distributions in ultrafiltration hollow fibre bioreactors, *Biotechnol. Bioeng* **36,** 902-910.

Pressman & Keighly (1948). The zone of activity of antibodies as determined by the use of radioactive tracers: The zone of activity of nephritoxic antikidney serum, *J.Immunol* **59** 141-146.

Ramírez, O.T., & Mutharasan, R., (1992). Effect of serum on the plasma membrane fludity of hybridomas: an insight into shear protective mechanism, *Biotechnol. Prog* **8**, 40-50.

Reff, M.E. & Heard, C (2001). A review of modifications to recombinant antibodies: attempt to increase efficiency in oncology applications, *Critical Reviews in Oncology/Haematology* **40** 25-35.

Reichert, J.M (2000). New biopharmaceuticals in the USA: trends in development and marketing approvals 1995-1999, *TIBTECH* **18**, 364-369.

Reid S & Greenfield, P.F (1990). Studies on Hybridoma and Insect Cells in Suspension Culture, *Aust. J. Biotechnol* **4 (2)**, 135-141.

Reisman, H.B. (1993). Problems in Scale-Up of Biotechnology Production Processes, *Critical Reviews in Biotechnol.* **13(3)** 195-253.

Reiter, M & Blüml, G (1994). Large-scale mammalian cell culture, *Current Opinion in Biotechnology*, **5**, 175-179.

Reuveny, S, Velez, D, Macmillan, J.D, and Miller, L (1986). Factors affecting cell growth and monoclonal antibody production in stirred reactors, *J. Immunol. Methods* **86**, 53-59.]

Reuveny S, & Lazar A. (1989). Equipment and procedures for production of monoclonal antibodies in culture, *Adv Biotechnol Processes*, **11** 45-80

Robinson R.G., Rapp L, Bowdish K.J., Graham M.A., Huff A.C., Coughlin S.A., (1993). Isolation and characterization of monoclonal antibodies to a recombinant human topoisomerase II polypeptide, *Hybridoma* **4**, 407-15

Ryu. J.S. and Lee, G.M. (1999). Application of hypoosmolar medium to fed-batch culture of hybridoma cells for improvement of culture longevity, *Biotechnol. Bioeng,* **62 (1)**, 120-3.

Sanfeliu, A., Damgaard, B., Cairo, J.J., Casas, C., Sola, C. and Godia, F. (1994). Low-level FCS adaptation and media developemnt for the culture of two hybridoma cell lines producing IgG and IgM monoclonal antibodies. InR.E. Spier, J.B. Griffiths and W. Bertholds (Eds.), Animal Cell Technology, Products of Today, Prospects for tommorow. (European Society for Animal Cell Technology, 12th meeting), Butterworth -Heinemann, 101-104.

Sato, J.D. (1984). Cholesterol requirement of NS-1 mouse myeloma cells for growth in serum-free medium, *Mol. Biol. Med.* **2,** 121-134.

Schneider, Y-J., (1989). Optimisation of hybridoma cell growth and monoclonal antibody secretion in a chemically defined, serum- and protein-free culture medium, *J.Immunol. Methods* **116,** 65-77.

Scragg, A.H. (1991). Bioreactors in Biotechnology, 1st edn. Ellis Horwood Ltd., Great Britian.

Sgro, C (1995). Review: Side effects of a monoclonal antibody muromonab CD3/orthoclona OKT3: bibliographic review, *Toxicology* **105** 23-29.

Shacter, E., (1987). Serum-free medium for growth factor –dependent and –independent plasmacytomas and hybridomas, *J.Immunol. Methods* **99,** 259-270.

Simonson, L., Morton, H. and Reedy, E. (1994). Hybridoma cell growth and monoclonal antibody yield in serum-free media, *Cytobios* **77,** 159-165.

Sinacore, M.S, Drapeau, D and Adamson, S.R. (2000). Adaptation of Mammalian Cells to Growth in Serum-Free Media, *Molecular Biotechnol.* **15,** 249-257.

Smith, C.G, and Greenfield, P.F (1992). Mechanical agitation of hybridoma in suspension cultures: Metabolic effects of FBS, Plurionic F68, and albumin supplements, *Biotechnol. Bioeng.* **40,** 1045-1055.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Prevenzano MD, Fujimoto EK, GoekeNM, Olson BJ, KlenkDC. Measurement of Protein using Bicinchronic Acid, *Anal. Biochem* 1985; **150**, 76-85.

Soloski, M.J (2000). Overview of the Science and Uses of Monoclonal Antibodies [Online] http://altweb.jhsph.edu/science/meetings/mab/solsokii.htm [Accessed July, 2001).

Spier, R.E. (1998). Animal and plant cell technology: A critical evaluation of the technology/society interface, *J. Biotechnol.* **65,** 111-125.

Stoll, T.S., Muhlethaler, K., von Stockar, U., and Marison, I.W., (1996). Systematic improvement of a chemically-defined protein-free medium forhybridoma growth and monoclonal antibody production, *J. Biotechnol* **45 (2),** 111-123.

Strutzenberger, K, Borth, N, Kunert, R, Steinfellner, W, Katinger, H (1999). Changes during subclone development and ageing of human antibody-producing recombinant CHO cells, *J. Biotechnol.* **69**, 215-226.

Sureshkumar, G.K. & Muthaeasan, R (1991). The influence of temperature on mouse-mouse hubridoma growth and monoclonal antibody production, *Biotechnol Bioeng* **37,** 292-295.

Suzuki E & Ollis, D.F. (1989). Cell cycle model for antibody production, *Biotechnol. Bioeng*, **37**, 292-295.

Tarleton, R.L (1991). Medium-scale production and purification of monoclonal antibodies in protein-free medium, *Biotechniques* **11 (5),** 590-593.

Teige, M., Weidemann, R and Kretzmer, G (1994). Problems with serum-free production of antithrombin III regarding proteolytic activity and product quality, *J. Biotechnol.* **34,** 101-105.

Tharakan, J.P. and Chau, P.C. (1986a). IgG production kinetics in serum free media, *Biotechnol. Lett.* **8 (8),** 529-534.

Tharakan, J.P., Lucas, A., and Chau, P.C,M (1986b). Hybridoma growth and antibody secretion in serum supplemented and low protein serum-free media, *J.Immunol. Methods* **94**, 225-235.

Tovey, M.G. (1985). The cultivation of Animal Cells in Continuos-flow Culture. In: Spier, R.E. and Griffiths, J.B. (ed), Animal Cell Biotechnology Volume 1, 1st edn, Academic Press Inc, Florida, USA 195-207.

Trinity Biotech website: http://www.trinitybiotech.com/javapg2.htm [Accessed 31-November-2001].

Van der Pol, L, Bakker, W.A.M, Tramper, J (1992) Effect of low serum concentrations on Growth, Production and shear sensitivity of hyridoma cells, *Biotechnol. Bioeng.* **40,** 179-182.

Van Dijk, M.A and van de Winkel, J.G.J (2001). Human antibodies as next generation therapeutics, *Current Opinion in Chemical Biology* **5,** 368-374.

Van Erp, R Adorf, M., van Sommersen, A.P.G., and Schönherr, D.W. (1991a). Monitoring of the production of monoclonal antibodies by hybridomas. Part I: Long-term cultivation in hollow fibre bioreactors using serum-free medium, *J. Biotechnol.* **20,** 235-248.

Van Erp, R., van Sommersen, A.P.G and Grobnau, T.C.S. (1991b). Monitoring of the production of monoclonal antibodies by hybridomas. Part II: Characterisation and purification of acid protease present in cell culture supernatant, *J. Biotechnol.*, **20**, 249-262.

Velez D, Reuveny S, Miller L and Macmillan, J.D. (1986). Kinetics of monoclonal antibody production in low serum growth medium, *J. Immunol Methods*, **86**, 45.52.

Vesper, J.L. (2001). Writing procedures that contribute to performance, *Biopharm* **August** 22-26.

Voigt, A and Zintl, F (1999). Hybridoma cell growth and anti-neuroblastoma monoclonal antibody production in spinner flasks using a protein-free medium with microcarriers, *J. Biotechnol.* **68**, 213-226.

Walsh, G (2001). Monoclonal Antibody-Based Therapeutics, *Biopharm* Europe March.

Wen Z-Y, Teng, X-W. and Chen, F (2000). A novel perfusion system for animal cell cultures by two step sequential sedimentation, *J. Biotechnol.* **79**,1-11.

Weidemann, R., Ludwig, A and Kretzmer, G (1994). Low temperature cultivation-Astep towards process optimisation, *Cytotechnology* **15**, 111-116.

Wolfe, R.A., Braatz, J.A., Miller, D.A., and Heiftz, A.h., (1988). A new serum-free medium for monoclonal antibody production, *Biotechniques* **6 (1),** 62-67.

Winter, G, and Milstein, C (1991). Man-made antibodies, *Nature* **349,** 293-299.

Xie, L and Wang, D.I.C (1997). Integrated approaches to the design of media and feeding strategies for fed-batch cultures of animal cells, *TIBTECH* **15,** 109-113.

Yabannavar, V.M., Singh, V and Connelly, N.V. (1992). Mammalian Cell Retention in a Spinfilter Perfusion Bioreactor, *Biotechnol Bioeng.* **40,** 925-933.

Yang X-D, Jia, X-C, Corvalan J.R.F, Wang P and Davis, C.G. (2001). Development of ABX-EGF, a fully human anti-EGF receptor monoclonal antibody, for cancer therapy, *Critical Reviews in Oncology/Haematology* **38,** 17-23.

Zhou, W., and Hu, W-S., (1997). Effect of insulin on a serum free hybridoma culture, *Biotechnol Bioeng.* **47 (2),** 181-185.

Appendix I

Abbreviations

ABC/HRP	Streptavidin/biotin-horseradish peroxidase conjugate
ADCC	antibody dependent cell mediated cytotoxicity
BSA	Bovine serum albumin
CDM	Chemically Defined Medium
CDR	Complementary Determining Region
cGMP	current Good Manufacturing Practice
CJD	Creutzfeldt-Jakob Disease
CT	Computed Tomographic
DAB	Diaminobenzidine
dH_2O	Deionised distilled water
DMEM	Dulbeccos Modified Eagles Medium
DMSO	Dimethyl sulfoximide
DQ	Design Qualification
EBV	Epstein Barr Virus
ECS	Extracapillary Space
EGF	Epidermal Growth Factor
ELISA	Enzyme linked Immunosorbent Assay
FBS	Foetal Bovine Serum
FDA	Federal Development Authority
$FeCl^3$	Iron Chloride
FS	Functional Specification
HAMA	Human-Anti-Murine antibody
HEPES	4-(2-hydroxethyl-)-piperazine ethane sulphonic acid
HFB	Hollow Fibre Bioreactor
ICS	Intracapillary Space
IMDM	Iscoves Modified Dubleccos Medium
IMS	Industrial methylated spirits
IQ	Installational Qualification
KDa	Kilo Dalton

MAb	Monoclonal Antibody
MCB	Master Cell Bank
MDR1	MDR-1 encoded P-glycoprotein (170kDa)
NF	National Formulary
NGF	Necrosis Growth Factor
Ni	Nickel
°C	degrees Celcius
OQ	Operational Qualification
PAGE	Polyacrylamide gel electrophoresis
PAMA	Polyethylmethanacrylate
PBS	Phosphate buffered saline
PEG	Polyethylene Glycol
PFM	Protein Free Medium
PQ	Performance Qualification
RAID	RadioImmunoDetection
RID	RadioImmunodiffusion
RNA	RibonucleicAssay
RP-HPLC	Reverse Phase High performance Liquid Chromatography
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulphate
SEC	Size Exclusion Chromatography
SFM	Serum Free Medium
Si	Silicon
SOP	Standard Operating Procedure
SSM	Serum Supplemented Medium
TBS	Tris buffered saline
TEMED	N,N,N,N,-tetramethyl-ethylenediamine
TNF	Tumor Necrosis Factor
Topo IIα	Topoisomerase IIα (170kDa)
Tris	Tris (hydroxymethyl) aminomethane
TSE	Transmissible spongiform encepha-lopathies
TV	Trypsin/versene

URS	User requirement Specification
USP	United States Pharmacopoeia
WCB	Working Cell Bank
ε	Kolmogrov eddy
%	Percentage
(v/v)	volume per volume ratio
µg	microgram
γ	average shear rate (s^{-1})
τ	average shear stress (Nm^{-2})
µ	Visocsity (Nsm^{-2})

Appendix II

Graphical Representation of the culture of 5C3 in the 10L bioreactor

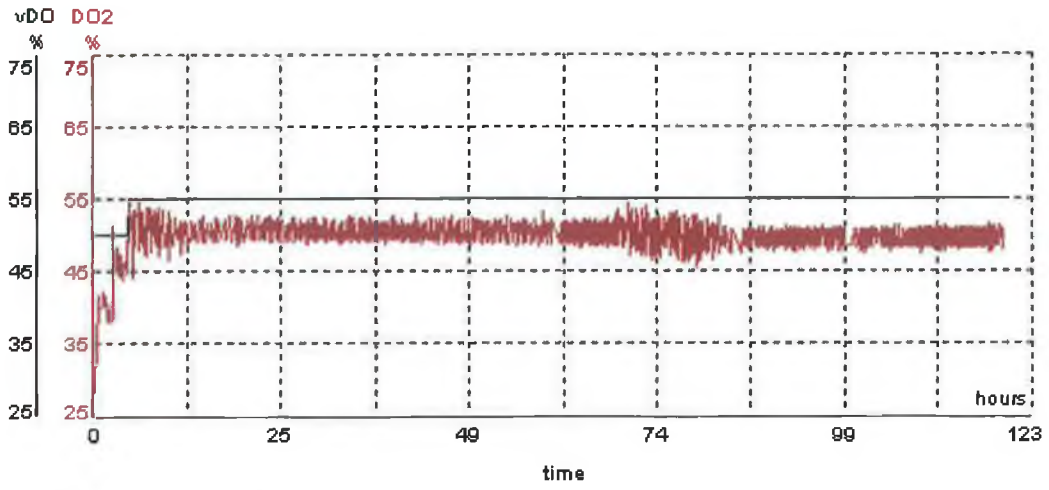


Fig 7.13 Graphical demonstration of set point dissolved oxygen concentration versus actual dissolved oxygen concentrations during the 10 L batch run of 5C3 cultured in PFHM II.

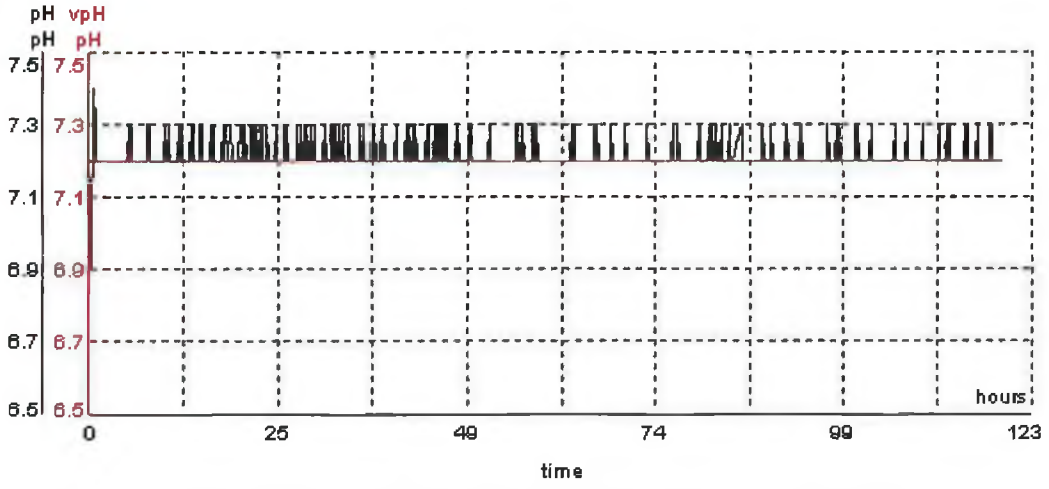


Fig 7.14 Graphical demonstration of set point pH versus actual pH concentrations during the 10 L batch run of 5C3 cultured in PFHM II.

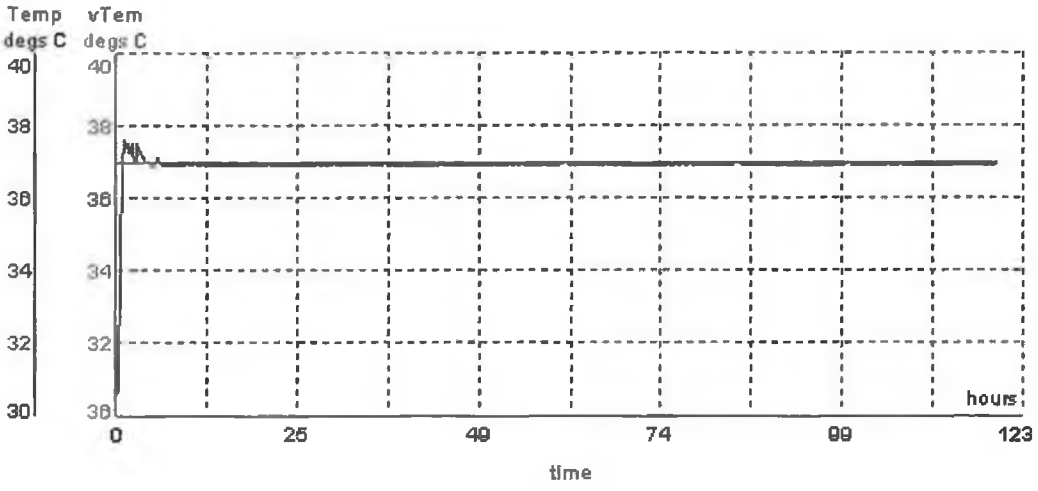


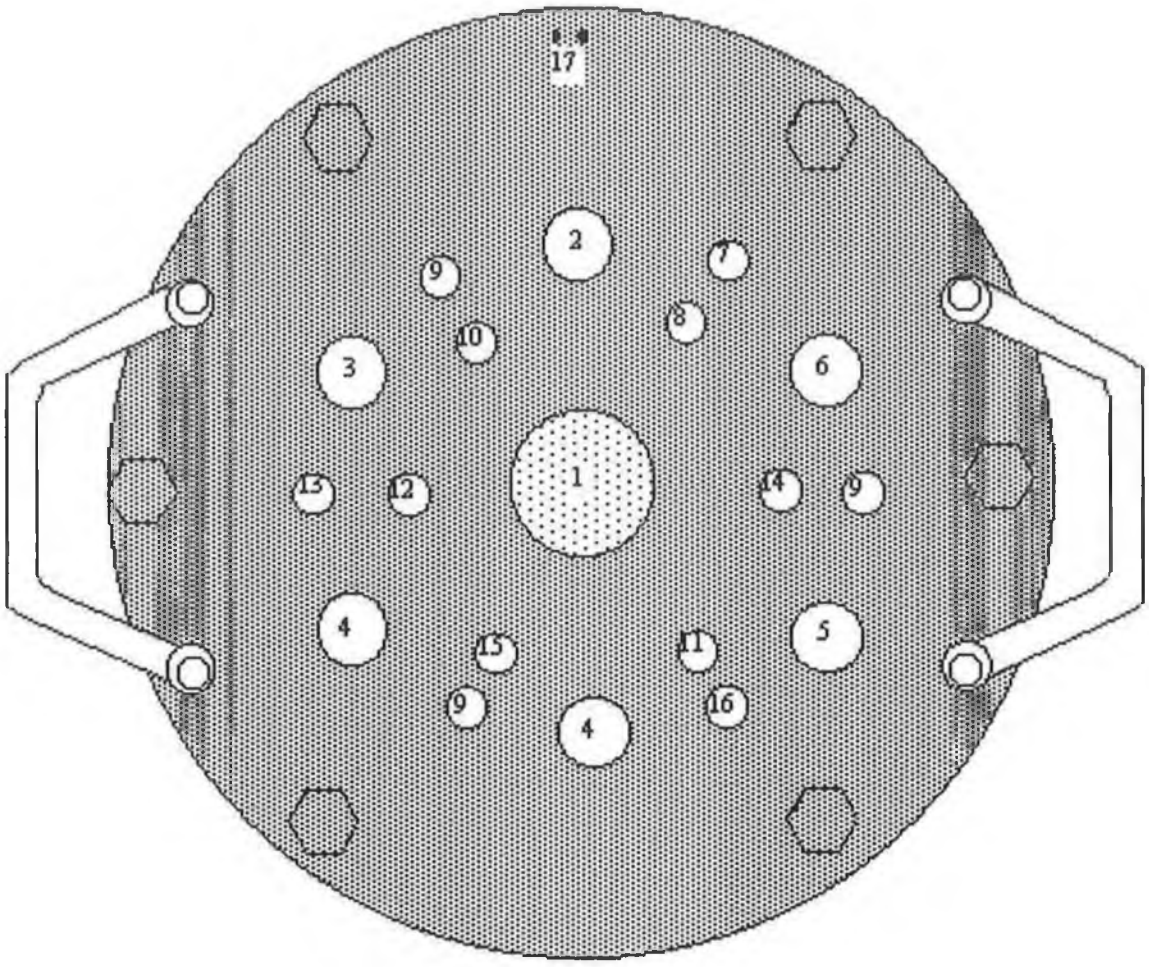
Fig 7.15 Graphical demonstration of set point temperature versus actual temperature during the 10 L batch run of 5C3 cultured in PFHM II.

Appendix III

List of Suppliers

- Applikon Ltd De Brauwweg 13, 3125 AE, Schiedern, Holland.
- Amersham International plc. Bucks HP7 9NA, UK.
- BDH laboratory Supplies, Poole, BDH15 1TD, England.
- Beckman Instruments (UK) Ltd, High Wycombe, Bucks HP12 4YH, UK.
- Bio Rad loboratories Ltd Hertz HP2 7TD, UK.
- Becton Dickinson UK Ltd, Between towns Road, Crowley, Oxford OX4 3LY, UK.
- Boehringer Manneheim, Roche Diagnostics Ltd. Bell Lane, Lewes, East Sussex BN7 1LG, UK.
- Cellon Ltd 204 route d'Arlon, L-8010 Strassen, Grand-Duche de Luxemberg.
- Costar Cambridge MA, USA
- Dako, 16 Manir Courtyard, Hughendon Avenue, High Wycombe, Bucks HP13 5RE, UK.
- Gelman, United Kingdom Pall Gelman Sciences, Brackmills Buisness park, Caswell Road, Northampton NN4 7EZ, UK.
- Gibco, Life Technologies Ltd, 3 Fountain Drive, Inchinnan Buisness Park Paisley PA4 9RF, UK.
- Hoefar. Hoefar Scientific Instruments, San Fransisco, USA.
- Lennox John F Kennedy Drive, Naas Road Dublin 12.
- Merck, Magna Park, Little-worth, Leicestershire, UK.
- NUNC A/S. Roskilde, Denmark.
- Nuaire, 2100 Fernbrook Lane, Plymouth, Minnesota 55447, USA.
- Pierce, Post Office Box 117, Rockford Illinois 61105 USA.
- Sigma Diagnostics, St.Louis, MO 63178 usa.
- Sigma, Sigma-Aldrich Company Ltd. Fancy Road, Poole, Dorest, BH12 4QH, UK.
- Sterlin Ltd, Middlesex TW148QS, UK.
- The Binding Site Ltd PO Box 4073, Birmingham, B29 6AT, England.
- Vector laboratories Ltd., 16 Wulfric Square, Bretton, Peterborough PE3 8RF, UK.
- Vector laboratories, Inc. Burlingame, CA 94010 USA.
- AGB Scientific Dublin Industrial Estate, Dublin 9.

Appendix IV
Reactor Headplate



1. Stirrer Motor
2. Triplet
3. Stopper
4. Ph Electrode
5. Triplet
6. O_2 Probe
7. Stopper
8. Level (Low)
9. Level (Medium)
10. Head Space Outlet
11. Basket Screws
12. Long Harvest Line
13. Sample Line
14. Stopper
15. Stopper
16. Basket Screws
17. Temperature Probe
18. Stopper
19. Level (High)

Appendix V

Sample Calculation

T-test

$$t = X_1 - X_2 \Big/ \sqrt{\frac{\sum d^2 - \frac{\sum (d)^2}{n}}{n(n-1)}}$$

t = t value

X_1 = Sample 1

X_2 = Sample 2

d = Difference (X_1 - X_2)

n = Sample Number

Subject	*Sample 1* X_1	*Sample 2* X_2	*Difference (d)*	*Squared difference* (d^2)
1	0.720	0.693	0.027	0.0007
2	0.604	0.534	0.070	0.0049
3	0.482	0.396	0.086	0.0074
4	0.300	0.247	0.053	0.0028
5	0.195	0.131	0.064	0.0041

$X_1 = 2.301$

$X_2 = 2.001$

d = 0.3

$d^2 = 0.09$

n = 5

Inserting the figures into the formula:

T = 6.11

Degrees of freedom = 4

The calculated value of $t = 6.11$ is greater than the table value of 2.132 allows one to reject the null hypothesis at the $p = 0.05$ level of significance and one can conclude that the results obtained comparing the standard concentrations of IgG washing buffer and in serum supplemented are significantly different.

Sample Calculation for costing the production of 1 mg of antibody from the culture of 5C3 in protein free conditions in a large-scale bioreactor

Costings for 4/2D in SSM

	Days post set-up	µg/ml	mg	Volume	Cost of medium €	Cost of Production €
10 L	6	19	190	10000	751.31	3.95
Spinner Flask	6	8.12	0.406	50	3.75	9.23
Static Flask	6	36.31	0.0363	10	0.75	20.66

Costings for 4/2D in PFM

	Days post set-up	µg/ml	mg	Volume	Cost of medium €	Cost of Production €
10 L	6	20	200	10000	777.4	3.89
Spinner Flask	6	46.015	0.2302	50	3.88	16.85
Static Flask	6	12.38	0.0124	10	0.78	63.00

Costings for 5C3 in SSM

	Days post set-up	µg/ml	mg	Volume	Cost of medium €	Cost of Production €
10 L	6	29	290	10000	751.31	2.60
Spinner Flask	6	37.22	0.1861	50	3.75	20.15
Static Flask	6	18.79	0.01879	10	0.75	39.91
HFB	21	10.32	42.92	4450	812.1	18.92

Costings for 5C3 in PFM

	Days post set-up	µg/ml	mg	Volume	Cost of medium €	Cost of Production €
10 L	6	296.46	2964.6	10000	777.4	0.26
Spinner Flask	6	294	14.7	50	3.88	0.26
Static Flask	6	175.64	1.76	10	0.78	0.44
HFB	21	28.08	115.855	4125	370.41	3.20

	Days post set-up	µg/ml	mg	Volume	Cost of medium €	Cost of Production €
10 L	6	296.46	2964.6	10000	777.4	0.26

2964.6 mg = 777.4

1 mg= X

Therefore 777.4/2964.6 = 0.26 (Cost of producing 1 mg)

Appendix VI

Viability Tables

Time (Days)	*Viability (%)*	*Viability (%)*	*Viability (%)*
	50 rpm	***100 rpm***	***200 rpm***
0	*100.00*	*100.00*	*100.00*
1	*93.75*	*95.72*	*95.19*
2	*82.10*	*94.38*	*95.24*
3	*84.10*	*93.06*	*89.64*
4	*77.73*	*96.23*	*66.51*
5	*53.45*	*78.59*	*51.74*
6	*43.64*	*61.22*	*33.45*

Table 7.1 Cell viability's for **4/2D** cells cultured in serum supplemented medium.

Time (Days)	*Viability (%)*	*Viability (%)*	*Viability (%)*
	50 rpm	***100 rpm***	***200 rpm***
0	*100.00*	*100.00*	*100.00*
1	*100.00*	*92.40*	*100.00*
2	*89.24*	*93.36*	*95.66*
3	*89.40*	*94.10*	*90.19*
4	*80.60*	*90.02*	*86.48*
5	*54.18*	*81.54*	*65.27*
6	*39.74*	*60.02*	*52.97*

Table 7.2 Cell viability's for **4/2D** in protein free medium.

Time (Days)	*Viability (%)*	*Viability (%)*	*Viability (%)*
	50 rpm	***100 rpm***	***200 rpm***
0	*100.00*	*100.00*	*100.00*
1	*100.00*	*99.43*	*95.29*
2	*96.71*	*97.37*	*94.92*
3	*95.52*	*97.04*	*89.45*
4	*87.02*	*94.35*	*74.53*
5	*70.48*	*87.76*	*71.32*
6	*39.28*	*74.92*	*58.84*

Table 7.3 Cell viability's for **5C3** cultured in serum supplemented medium.

Time (Days)	*Viability (%)*	*Viability (%)*	*Viability (%)*
	50 rpm	***100 rpm***	***200 rpm***
0	*100.00*	*100.00*	*100.00*
1	*76.06*	*97.37*	*75.69*
2	*74.05*	*95.93*	*68.12*
3	*81.32*	*97.12*	*No growth*
4	*59.42*	*84.00*	*No growth*
5	*41.09*	*69.92*	*No growth*
6	*39.28*	*43.58*	*No growth*

Table 7.4 Cell Viability's for **4 2D** cell cultured in protein free medium.

Time (Days)	*Viability (%)*	*Viability (%)*	*Viability (%)*
	34°C	***37°C***	***39°C***
0	*100.00*	*100.00*	*100.00*
1	*98.22*	*95.72*	*100.00*
2	*97.83*	*94.38*	*78.57*
3	*97.84*	*93.06*	*69.84*
4	*93.32*	*96.23*	*86.11*
5	*86.04*	*78.59*	*48.00*
6	*69.33*	*61.22*	*No growth*

Table 7.5 **4 2D** in serum supplemented medium.

Time (Days)	*Viability (%)*	*Viability (%)*	*Viability (%)*
	34°C	***37°C***	***39°C***
0	*100.00*	*100.00*	*100.00*
1	*100.00*	*92.40*	*8.26*
2	*93.27*	*93.36*	*2.91*
3	*92.82*	*94.10*	*No growth*
4	*85.62*	*90.02*	*No growth*
5	*78.24*	*81.54*	*No growth*
6	*54.35*	*60.02*	*No growth*

Table 7.6 Cell viability's for **4 2D** cultured in protein free medium.

Time (Days)	*Viability (%)*	*Viability (%)*	*Viability (%)*
	34°C	***37°C***	***39°C***
0	*100.00*	*100.00*	*100.00*
1	*100.00*	*99.43*	*93.91*
2	*97.59*	*97.37*	*83.84*
3	*95.50*	*97.04*	*80.89*
4	*89.90*	*94.35*	*78.33*
5	*76.46*	*87.76*	*No growth*
6	*53.14*	*74.92*	*No growth*

Table 7.7 Cell viability's for **5C3** cultured in serum supplemented medium.

Time (Days)	*Viability (%)*	*Viability (%)*	*Viability (%)*
	34°C	*37°C*	*39°C*
0	*100.00*	*100.00*	*100.00*
1	*94.10*	*97.37*	*6.42*
2	*97.00*	*95.93*	*7.41*
3	*95.56*	*97.12*	*1.50*
4	*87.99*	*84.00*	*1.48*
5	*69.33*	*69.92*	*No growth*
6	*53.34*	*43.58*	*No growth*

Table 7.8 Cell viability's for **5C3** in protein free medium.

Large Scale Culture:

Time (Hours)	*Viable cell count ($x10^6$ cells/ml)*	*Viability (%)*	*Antibody Concentration (mg/L)*	*Antibody Concentration (mg/L)*
0	*0.10*	*100.00*	***Batch 1***	***Batch 2***
1	*0.23*	*93.66*	*14.158*	*8.24*
2	*0.7*	*91.91*	*25.86*	*11.40*
3	*2.67*	*90.71*	*72.91*	*12.30*
4	*3.78*	*85.00*	*23.44*	*14.60*
5	*2.24*	*64.21*	*14.92*	*18.90*
6	*1.555*	*49.61*	*13.45*	*28.37*

Table 7.9 Average Viable cell count, % Viability and antibody concentration for **4 2D** cultured in serum supplemented medium.

Time (Hours)	*Viable cell count ($x10^6$ cells/ml)*	*Viability (%)*	*Antibody Concentration (mg/L)*
0	*0.10*	*100.00*	*9.76*
1	*0.20*	*100.00*	*18.47*
2	*0.34*	*96.75*	*39.72*
3	*0.99*	*91.36*	*62.64*
4	*2.19*	*91.63*	*40.89*
5	*1.99*	*80.33*	*30.14*
6	*0.96*	*58.03*	*19.72*

Table 7.10 Viable cell count, % Viability and antibody concentration for **4 2D** cultured in protein free medium.

Time (Hours)	*Viable cell count ($x10^6$ cells/ml)*	*Viability (%)*	*Antibody Concentration (mg/L)*
0	*0.1*	*100.00*	
1	*0.11*	*97.74*	*9.838*
2	*0.33*	*94.29*	*13.942*
3	*1.3*	*96.55*	*15.222*
4	*1.665*	*87.40*	*23.583*
5	*1.535*	*67.95*	*20.346*
6	*0.91*	*36.99*	*18.280*

Table 7.11 Viable cell count, % Viability and antibody concentration for **5C3** cultured in serum supplemented medium.

Time (Hours)	*Viable cell count ($x10^6$ cells/ml)*	*Viability (%)*	*Antibody Concentration (mg/L)*
0	*0.10*	*100.00*	
1	*0.10*	*100.00*	*48.78*
2	*0.42*	*96.59*	*177.28*
3	*0.74*	*90.11*	*273.20*
4	*1.64*	*86.51*	*405.95*
5	*1.23*	*68.89*	*475.35*
6	*0.83*	*32.73*	*652.45*

Table 7.12 Viable cell count, % Viability and antibody concentration for **5C3** cultured in protein free medium.

VII Installational Qualification

Installation Qualification

Applikon 10 L Bioreactor (BIO-4) and Control System

Pre-Execution	Signature	Title	Date
Prepared By: Catherese Fahey	C Fahey	Research Scientist	2/11/02
Approved By: Cathal O Grady	Cathal O Grady	Fermentation Manager	13/11/01
Approved By: Cathal Elliot	Cathal Elliott	Validation Supervisor	18/10/01
Approved By: Irene Cleary	Irene Cleary	QA Manager	21/1/02

Post-Execution	Signature	Title	Date
Prepared By: Catherese Fahey	C Fahey	Research Scientist	13/5/02
Approved By: Cathal O Grady	C Grady.	Fermentation Manager	10/6/02
Approved By: Cathal Elliot	Anne Lanning on behalf of Cathal Elliot	Validation Supervisor	29/7/02.
Approved By: Irene Cleary	Irene Cleary	QA Manager	5/8/02

Contents

1. Purpose:

The purpose of this Installation Qualification is to provide documented evidence that the Applikon 10 L Bioreactor and control units are installed as per design intent and to the manufacturer's recommendations where applicable.

2. Scope

The scope of the Installation Qualification, includes the Applikon 10 L Bioreactor and control units. All other aspects of the mechanical and electrical installation of the unit and their assembly are included.

3. System Description

The ADI Bio Controller ADI 1060, the ADI 1035 Bioconsole and the ADI 1040/25 are used in unison for the process control of the 10 L Applikon bioreactor. The purpose of the ADI 1060 is to control and monitor mammalian cell culture systems in a controlled environment where pH, temperature, dissolved oxygen and mixing can be controlled.

The ADI 1035 Bioconsole is an actuator console that is used in combination with the ADI 1060 Bio Controller. It supports gas flow regulation, stirrer speed, pH control, dissolved Oxygen control, level control, temperature control, and liquid addition/withdrawal via pumps.

The ADI 1040/25 functions by connecting the ADI 1060 Bio controller with the bioreactor and their actuator consoles. The measured sensor signals are amplified in the 1040/25 and are transmitted to the Bio Controller through serial communication.

Depending on deviation from the set point, a controller output value is sent to an analog or digital output that will activate the actuator in the ADI 1035 in order to decrease the deviation from the set point. The control system is shown below (Fig. 1) and outlines the roles played by each component of the process system. The associated 10 L Bioreactor is a glass stirred tank reactor.

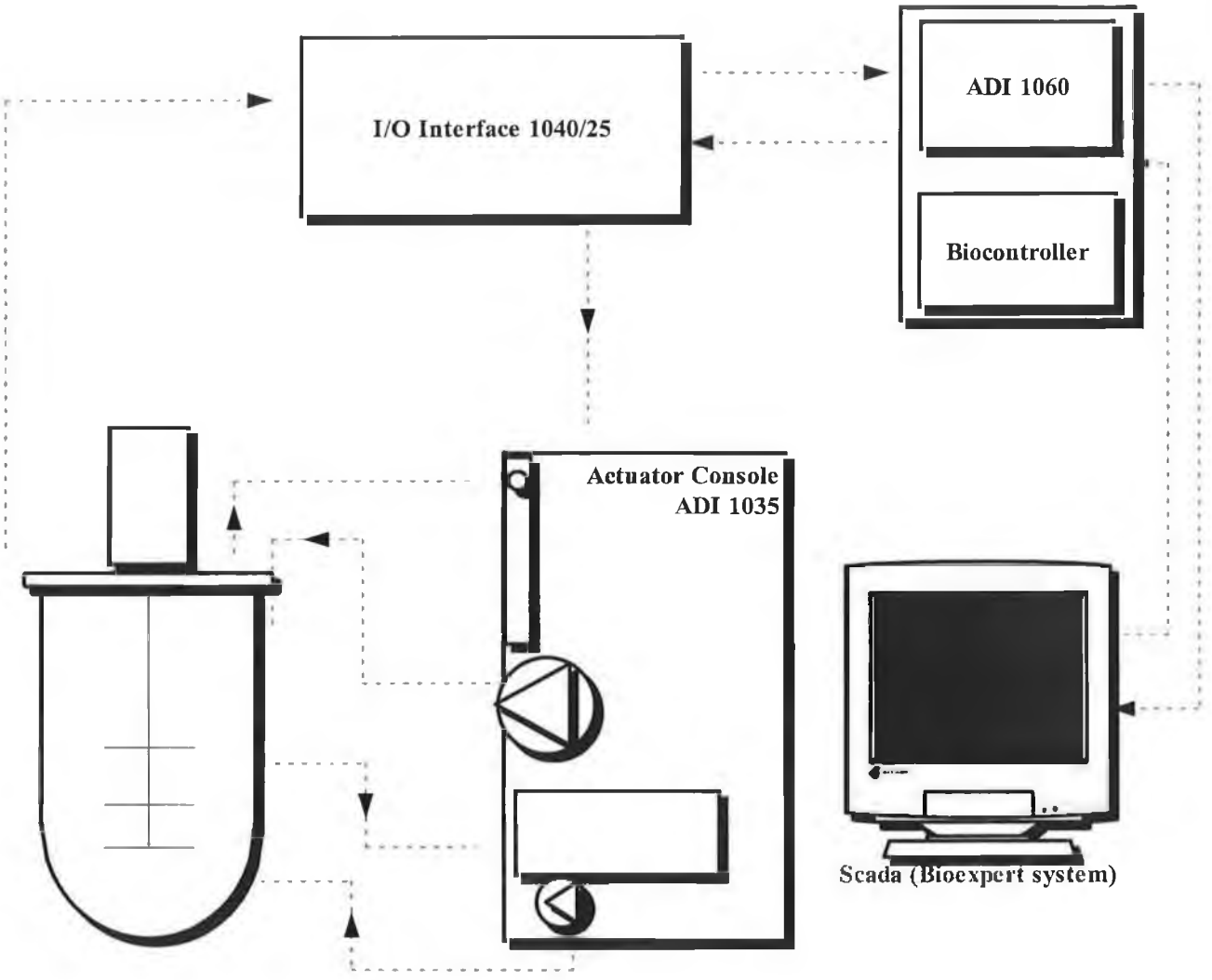


Fig 1: Schematic overview of the Applikon "bioprocess" system.

4. Pre Protocol Checks:

Have the appropriate representatives reviewed the Installation Qualification? Is the protocol approved and ready for execution?

Yes/No: _____ File Location: _____

Verified By: _____ Date: _____

Have Standard Operating Procedures have been drafted for the operation of the Bioreactor & Control System?

Yes/No: _____ File Location: _____

Verified By: _____ Date: _____

Detail relevant SOP numbers: _____

_____

All recommended spare parts on order?

Yes/No: _____ File Location _____

Verified By: _____ Date: _____

Comments:
Signed: _____ **Date:** _____
Approved By: _____ **Date:** _____

5.0 Installation Qualification:

The Installation Qualification is the documentation process, which verifies that the physical components of a system have been installed according to design specifications. The IQ also serves as a final equipment audit prior to equipment operation. The installation of the system will be verified by reviewing the equipment installed and using the check sheets provided in this protocol to document that the system components conform to design specifications. In addition, where applicable, engineering documentation will be reviewed and compared to the installed system.

5.1 Specification & Purchase Documentation Review

Reference Attachment A1

This section identifies a list of all specification and purchase documentation associated with the bioreactor and verifies that this documentation is complete and accurately describes the installed equipment.

5.2 Engineering & Safety Documentation Review

Reference Attachment A2

This section identifies a list of all engineering documentation associated with the bioreactor and verifies that this documentation is complete and accurately describes the installed equipment. The list should include the following documents: design specification, functional specification, P&ID's, electrical schematics, wiring diagrams, loop check documentation, equipment operation, maintenance manuals and spare parts lists.

List all safety valves, piping and ancillary equipment associated with this system, which need to be inspected for safety. Verification that inspection has been performed and is current must be recorded. (Include copies of this information in the validation file).

5.3 Equipment List

Reference Attachment A3.

A list of all installed process and ancillary equipment based on information from the purchase orders and equipment specifications are included here. All items should be verified to ensure installation as specified. Equipment tag number, model number, serial number, location, manufacture, description, for each item should be indicated. Verification that preventative maintenance routines have been established for major equipment is also included.

5.4 Instrument List

Reference Attachment A4

A complete list of instruments designated *critical or non- critical* is included in this section.
Critical instruments pertain to those whose performance will affect the operation of the system or the quality attributes of the process material.
Non-critical instruments are those provided for information or convenience purposes only. The information provided will include ID number, manufacturer, location, and description of each item.

These instruments should be uniquely identified and calibrated in accordance with Archport Ltd SOP's. Copies of calibration documentation should be included or an indication provided of where it is located.

5.5 Utilities Verification

Reference Attachment A5:

A complete list of utilities supplied to the equipment based on equipment specifications. The installation is inspected to verify that the correct utilities have been installed and that these meet specifications and equipment requirements.

5.6 Materials of Construction and lubricants list

Reference Attachment A6:

A complete list of materials of construction of equipment components and lubricants used is included in this section.

5.7 Control System Hardware

Reference Attachment A7

A complete list of major control hardware, including installed boards and communications cabling based on information from purchase orders and equipment specifications. Verification that the items have been installed as specified is included. Indication of hardware identification, location, manufacture, description for each item as is applicable. Also a system configuration drawing showing system architecture is included (if applicable).

5.8 Input/ Output List / Loop Check Documentation

Reference Attachment A8

Verify existence and adequacy of loop checks performed by the installing contractor. Persons performing checks in field and console or operator interface should sign documentation. Loop checks should be used on P & ID's. Include a reference as built copies of these in loop check documentation package.

5.9 Setpoints & Software Parameters

Reference Attachment A9:

Document set points and other operating parameter data installed in the control panel or on local instrumentation. Note the presence and adequacy of security measures against set point and software modification, if applicable.

5.10 Software Documentation

Reference Attachment A10:

The revision of installed software is recorded. Record the installation location, including hardware model No., serial No., manufacturer, reference specifications, printed copy of annotated applications code (ladder logic etc.), including date and version, software functionality flowcharts and documentation of any modifications made to the software.

5.11 Factory Acceptance Testing (FAT)

Reference Attachment A11:

List all audit and testing documentation including vendor audit report, test procedures and results of any testing performed on the system, including commissioning and Factory Acceptance Tests (FAT). Verify completeness of documentation and satisfactory resolution of any deviations or failed tests.

5.12 Standard Operating Procedures List

Reference Attachment A12.

List all applicable SOP's including procedures for operation, cleaning, maintenance, change control and calibration. Verify that these are in place, that they accurately describe the applicable equipment and that they are implemented by appropriate personnel. Training records should be available for all personnel operating the bioreactor.

5.13 Installation Qualification Discrepancy Summary

Reference Attachment A13.

Document any discrepancies or variations noted during the execution of the Installation Qualification protocol. Include the resolution of these items and/or any item outstanding that will require resolution in the future.

If changes to the system are required, their implementation will follow the standard operating procedures. All such changes must be documented in this attachment and copies of other documentation relating to such changes must be referenced in this protocol.

6. SUMMARY CONCLUSIONS

Signed_____ **Date**_____

Attachments

A1: Specification & Purchase Documentation Review

A2: Engineering & Safety Documentation Review

A3: Equipment List

A4: Critical Instrument List/ Non-Critical Instruments

A5: Utilities Verification

A6: Material of Construction/Lubricants List

A7: Control System Hardware

A8: Input/ Output List /Loop check Documentation.

A9: Set Points & Software Parameters

A10: Software Documentation

A11: Factory Acceptance Testing

A12: Standard Operating Procedures List

A13: Installation Qualification Discrepancy Summary

Archport Ltd Protocol No.: 2610-29-IQ-004		
ATTACHMENT : A1	Specification & Purchase Documentation Review	Pg 1 of 2

Doc. No.:	Rev./Issue No.	Date: 27/10/1998
Title: PRICES, TERMS AND CONDITIONS FOR PURCHASE OF 15L BIOREACTOR AND CONTROL UNITS.		Location: ATTACHED
Inspected by: CS	Date: 13/5/02	Discrepancy (Y/N): N

Doc. No.:	Rev./Issue No.	Date:
Title:		Location:
Inspected by:	Date: DA CS 13/5/02	Discrepancy (Y/N):

Doc. No.:	Rev./Issue No.	Date:
Title:		Location:
Inspected by:	Date: DA CS 13/5/02	Discrepancy (Y/N):

e:
"THE PURCHASE ORDER FORM COULD NOT BE LOCATED" CS 20/7/02.

Performed by: CFaley Date 13/5/02.

Reviewed by: Anne [illegible] Date 24/7/02

Archport Ltd Protocol No.: 2610-29-IQ-004		
ATTACHMENT : A2	Engineering & Safety Documentation Review	Pg 1 of 3

Doc. No.: V3UECE0051	Rev./Issue No.	Date: OCTOBER 1994
Title: AUTOCLAVABLE BIOREACTOR IS-20L, USER MANUAL HARDWARE SPECIFICATION		Location: ARCHPORT 5124
Inspected by: C Fahey.	Date: 13/5/02	Discrepancy (Y/N): N

Doc. No.: V3UECE0051	Rev./Issue No.	Date: OCTOBER 1994
Title: AUTOCLAVABLE BIOREACTOR IS-20L, USER MANUAL, DRAWINGS		Location: Archport 5124
Inspected by: C Fahey.	Date: 13/5/02	Discrepancy (Y/N): N

Doc. No.: V1UBCES013	Rev./Issue No. 3	Date: July 1998
Title: USER MANUAL STIRRER Speed CONTROLLER ADi 1032 FOR MOTOR P100-310		Location: ARCHPORT 5124
Inspected by: C Fahey.	Date: 13/5/02	Discrepancy (Y/N): N

ID No	Description	Inspection Frequency	Last date of inspection	Discrepancies (Y/N)
	N/A CF 13/5/02			

Performed by: C Fahey. Date 13/5/02.

Reviewed by: Aine [illegible] Date 25/7/02

Archport Ltd Protocol No.: 2610-29-IQ-004		
ATTACHMENT : A2	Engineering & Safety Documentation Review	Pg 2 of 3

Doc. No.: V1USCE1013	Rev./Issue No. 3	Date: November 1996
Title: BioConsole 1035 – Instrument Manual		Location: Archport Ltd 5124
Inspected by: C Fahey.	Date: 13/5/02	Discrepancy (Y/N): N

Doc. No.: V1USCE1013	Rev./Issue No. 3	Date: November 1996
Title: BioConsole 1035 – Drawings		Location: Archport Ltd 5124
Inspected by: C Fahey.	Date: 13/5/02	Discrepancy (Y/N): N

Doc. No.:	Rev./Issue No. 1.1X	Date: January
Title: ADI 1060 BioController – Engineer Manual		Location: Archport Ltd 5124
Inspected by:	Date: 13/5/02	Discrepancy (Y/N): N

ID No	Description	Inspection Frequency	Last date of inspection	Discrepancies (Y/N)
	N/A	CF 13/5/02		

Performed by: C Fahey. Date 13/5/02

Reviewed by: Alan [illegible] Date 23/5/02

Archport Ltd Protocol No.: 2610-29-IQ-004		
ATTACHMENT : A2	Engineering & Safety Documentation Review	Pg 3 of 3

Doc. No.: √ 1URCE0251	Rev./Issue No.	Date: JANURARY 2000
Title: INTERFACE 1040/25 – HARDWARE MANUAL		Location: ARCHPORT Ltd 5124
Inspected by: C Sales.	Date: 13/5/02	Discrepancy (Y/N): N

Doc. No.: √ 1URCE0251	Rev./Issue No.	Date: JANURARY 2000
Title: INTERFACE 1040/25 – DRAWINGS		Location: ARCHPORT Ltd 5124
Inspected by: C Sales	Date: 13/5/02	Discrepancy (Y/N): N

Doc. No.: √ 1URCE025516	Rev./Issue No. 1.4 X	Date: DECEMBER 1999
Title: INTERFACE ADI 1040/25 SERIAL COMMUNICATIONS MANUAL		Location: ARCHPORT Ltd 5124
Inspected by:	Date: 13/5/02	Discrepancy (Y/N): N

ID No	Description	Inspection Frequency	Last date of inspection	Discrepancies (Y/N)
	N/A	C Sales 13/5/02		

Performed by: C Sales Date 13/5/02

Reviewed by: Anne Jammy Date 25/7/02

Archport Ltd Protocol No.: 2610-29-IQ-004		
ATTACHMENT : A3	Specification & Purchase Documentation Review	Pg 1 of 5

Description	Identification	Discrepancies (Y/N)
Name :	BIOREACTOR	N
Supplier :	APPLIKON	N
Type :	15 L DISHED BOTTOM	N
Type of process :	Cell Culture PERMEATION	N
Equipment Manual :	V3UECE 0051	N
Manufacture. year :	1999	N
Equipment no. :	Z6110000 10	N
Archport Ltd information :	N/A	N
Serial number :	N/A	N
Facility :	ARCHPORT LABORATORY	N
Room location:	5124	N
Supplier order reference.no.	N/A	
External dimensions of equipment:	A3 PERDRAWING D3015-1-12.011	N
Internal dimensions of apparatus:	N/A	N
Preventive maintenance	N/A	N
Certificate :	N/A	

Comments:

N/A

B15/02

Performed by: _____ Date 13/5/02

Reviewed by: _____ Date 13/5/02

Archport Ltd Protocol No.: 2610-29-IQ-004		
ATTACHMENT : A3	Specification & Purchase Documentation Review	Pg 2 of 5

Description	Identification	Discrepancies (Y/N)
Name :	BIOCONTROLLER ADI 1010	N
Supplier :	APPLIKON	N
Type :	CONTROLLER	N
Type of process :	Cell culture fermentation	N
Equipment Manual :	BIOCONTROLLER Explorer MANUAL	N
Manufacture. year :	1999	N
Equipment no. :	25101000010	N
Archport Ltd information :	N/A	N
Serial number :	PU1355 1990821	N
Facility :	ARCHPORT EXPANSION	N
Room location:	5124	N
Supplier order reference.no.	N/A	N
External dimensions of equipment:	N/A	N
Internal dimensions of apparatus:	N/A	N
Preventive maintenance	N/A	N
Certificate :		

Comments:

N/A

13/5/02

Performed by: C Salas Date 13/5/02

Reviewed by: Dave Jennings Date 24/5/02

Description	Identification	Discrepancies (Y/N)
Name :	BIOCONSOLE 1035	N
Supplier :	APPLIKON	N
Type :	CONTROLLER	N
Type of process :	Cell culture fermentation	N
Equipment Manual :	V10SCE 1013	N
Manufacture. year :	1999	N
Equipment no. :	1035	N
Archport Ltd information :	N/A	N
Serial number :	P16460129	N
Facility :	ARCHPORT EXTENSION	N
Room location:	S124	N
Supplier order reference.no.	N/A	N
External dimensions of equipment:	N/A	N
Internal dimensions of apparatus:	N/A	N
Preventive maintenance Certificate :	N/A	N

Comments:

N/A 13-5-02

Performed by: C Foley Date 13/5/02

Reviewed by: Anne Lanning Date 24/5/02

EU DECLARATION OF CONFORMITY

The company Applikon Dependable Instruments B.V., Schiedam, The Netherlands, hereby certifies that the instrument:

ADI 1035 BIO CONSOLE

meets the requirements of the EU Directives 89/336/EEC (Electromagnetic Compatibility) and 73/23/EEC (Low Voltage).

SOURCE OF THE SPECIFICATIONS:

89/336/EEC:	EN 50081-1 (1992) EMC Generic emission standard. Residential, commercial and light industry. EN 61000-3-2 (1995) EMC Limits for harmonic current emissions (equipment input current ≤ 16A per phase). EN 61000-3-3 (1995) EMC Limits concerning voltage fluctuations and flicker for equipment having an input current up to and including 16 A per phase. EN 50082-1 (1992) EMC Generic immunity standard. Residential, commercial and light industry. EN 50082-2 (1995) EMC Generic immunity standard. Industrial environment (including table A.4).
73/23/EEC	EN 61010 Safety requirements for electrical equipment for measurement, control and laboratory use.

ing J. van Burg, President. 23.10.96

Applikon Dependable Instruments
De Brauwweg 13
3125 AE Schiedam
The Netherlands
Tel.: (31)(0)10-2983555
Fax.: (31)(0)10-4379648

Description	Identification	Discrepancies (Y/N)
Name :	INTERFACE ADT 100125	N
Supplier :	APPLIKON	N
Type :	CONTROLLER	N
Type of process :	Cell culture fermentation	N
Equipment Manual :	VIURCE0251	N
Manufacture. year :	2000	N
Equipment no. :	Z5/04C025D	N
Archport Ltd information :	N/A	N
Serial number :	P4108011	N
Facility :	ARCHPORT EXTENSION	N
Room location:	5124	N
Supplier order reference.no.	N/A	N
External dimensions of equipment:	N/A	N
Internal dimensions of apparatus:	N/A	N
Preventive maintenance	N/A	N
Certificate :		

Comments:

Performed by: [signature] Date 13/5/02

Reviewed by: [signature] Date 24/5/02

ification + Purchase Attachment A3
…mentation Review
17/10 ?

EU DECLARATION OF CONFORMITY

The company Applikon Dependable Instruments B.V., Schiedam, The Netherlands, hereby certifies that the instrument:

ADI 1040/25 INTERFACE

meets the requirements of the EU Directives 89/336/EEC (Electromagnetic Compatibility) and 73/23/EEC (Low Voltage).

SOURCE OF THE SPECIFICATIONS:

89/336/EEC	EN 50081-1 (1992) EMC Generic emission standard. Residential, commercial and light industry. EN 61000-3-2 (1995) EMC Limits for harmonic current emissions (equipment input current $\leq$ 16A per phase). EN 61000-3-3 (1995) EMC Limits concerning voltage fluctuations and flicker for equipment having an input current up to and including 16 A per phase. EN 50082-1 (1992) EMC Generic immunity standard. Residential, commercial and light industry. EN 50082-2 (1995) EMC Generic immunity standard. Industrial environment (including table A.4.1, A.4.2, A.4.3 and A.4.5).
73/23/EEC	EN 61010 Safety requirements for electrical equipment for measurement, control and laboratory use.

ing J. van Burg, President.

December 13th 1999

Applikon Dependable Instruments
3125 AE Schiedam
Tel.: (31)(0)10-2983555

De Brauwweg 13
The Netherlands
Fax.: (31)(0)10-4379648

applikon®
DEPENDABLE INSTRUMENTS

Archport Ltd Protocol No.: 2610-29-IQ-004		
ATTACHMENT : A3	Specification & Purchase Documentation Review	Pg 5 of 5

Description	Identification	Discrepancies (Y/N)
Name :	Stirrer Motor Assembly	........N..........
Supplier :	Applikon	........N..........
Type :	P100	........N..........
Type of process :	Cell culture fermentation	........N..........
Equipment Manual :	V IUBCE 5013	........N..........
Manufacture. year :	1998	........N..........
Equipment no. :	Z 51000001	........N..........
Archport Ltd information :	N/A	........N..........
Serial number :	P 151 79/19	........N..........
Facility :	ARCHPORT Ext.	........N..........
Room location:	S124	........N..........
Supplier order reference.no.	N/A	........N..........
External dimensions of equipment:	N/A	........N..........
Internal dimensions of apparatus:	N/A	........N..........
Preventive maintenance Certificate :	N/A	........N..........

Comments:

NA

Performed by: C Daly Date 13/5/02

Reviewed by: Anne Lanning Date [illegible]/7/02

ACHMENT A3
ification >
HASE Documentation
Review
29/7/02.

EU DECLARATION OF CONFORMITY

CE

The company Applikon Dependable Instruments B.V., Schiedam, The Netherlands, hereby certifies that the instrument:

ADI 1032/1 STIRRER SPEED CONTROLLER

meets the requirements of the EU Directives 89/336/EEC (Electromagnetic Compatibility) and 73/23/EEC (Low Voltage).

SOURCE OF THE SPECIFICATIONS:

89/336/EEC:	EN 50081-1 (1992) EMC Generic emission standard. Residential, commercial and light industry. EN 50081-2 (1993) EMC Generic emission standard. Industrial environment. EN 50082-1 (1992) EMC Generic immunity standard. Residential, commercial and light industry. EN 50082-2 (1995) EMC Generic immunity standard. Industrial environment (including table A.4.1, A.4.2 A.4.3 and A.4.5). EN 61000-3-2 (1995) EMC Limits for harmonic current emissions (equipment input current ≤ 16A per phase). EN 61000-3-3 (1995) EMC Limits concerning voltage fluctuations and flicker for equipment having an input current up to and including 16 A per phase.
73/23/EEC	EN 61010 Safety requirements for electrical equipment for measurement, control and laboratory use.

ing J. van Burg, President. 14.10.96

Applikon Dependable Instruments
3125 AE Schiedam
Tel.: (31)(0)10-2983555

De Brauwweg 13
The Netherlands
Fax.: (31)(0)10-4379648

Archport Ltd Protocol No.: 2610-29-IQ-004		
ATTACHMENT : A4	Instrument List	Pg 1 of 1

1 Critical Instruments

Component	Function	Type	Tolerance	Serial No.	Last Calibration Date	Discrepancies (Y/N)
Stirrer Speed Controller	Mixing	PICO	0-200RPM	ZS1000001		N
pH Meter	Measurement of Acid/Base levels	Fermprobe	0-14 pH UNITS	F635-B402DH		N
DO2 probe	Measurement of O2/CO2, pC levels.	Chypride	0-100%	Z7120470O2		N
Level Control	Detection of fluid level			Z7112K0001		N

Performed by: C Foley Date 23/5/02

Reviewed by: Dave Jennings Date 29/7/02

Archport Ltd Protocol No.: 2610-29-IQ-004		
ATTACHMENT : A4	Instrument List	Pg 1 of 1

2 Non-Critical Instruments

Component / Part No.	Function	Type	Range	Tolerance	Serial no.	Last Calibration Date	Discrepancies (Y/N)
....	....	....	....	....	....	....	....
....	....	....	....	....	....	....	....
....	....	....	....	....	....	....	....
....	....	....	....	....	....	....	....
....	....	....	....	....	....	....	....
....	....	....	....	....	....	....	....
....	....	....	....	....	....	....	....
....	....	....	....	....	....	....	....
....	....	....	....	....	....	....	....
....	....	....	....	....	....	....	....

NA

Performed by: C Foley Date 13/5/02

Reviewed by: [signature] Date 24/7/02

AJ 24/7/02

Archport Ltd

Protocol No.: 2610-29-IQ-004

ATTACHMENT : A5	Utilities Verification	Pg 1 of 1

Utility	Location / Source	Specified	Actual	Method	Verified by / Date
O_2 gas	5124	From Archport O_2 systems	As Specified	See Validation of gas system	CS 29/7/02
N_2 gas	5124	From Archport N_2 system	As Specified	See Validation of gas system	CS 29/7/02
CO_2 gas	5124	From Archport CO_2 system	As Specified	See Validation of gas system	CS 29/7/02
Air Gas	5124	From Archport Air System	As Specified	See Validation of gas system	CS 29/7/02
Power Supply	5124	220V	As Specified	See Validation of Electric System	CS 29/7/02

Performed by: C Sahey   Date 29/7/02

Reviewed by: Anne Manning   Date 29/7/02

Archport Ltd Protocol No.: 2610-29-IQ-004		
ATTACHMENT : A6	Material of Construction / Lubricant List	Pg 1 of 1

Equipment	As specified	As Supplied / Installed	Acceptable (Y/N)	Verified by
BIOREACTOR	BOROSILICATE GLASS	AS SPECIFIED	Y	CF
HEAD PLATE	STAINLESS Steel SS316	As Specified	Y	CF
STIRRER Assembly	STAINLESS Steel SS316	As Specified	Y	CF

Lubricant List:

Equipment	As specified	As Supplied / Installed	Acceptable (Y/N)	Verified by
SPECIAL GREASE FOR STIRRER ASSEMBLY U3LA1Z00031	SPECIAL GREASE	AS SUPPLIED	Y	CF

Performed by: C Foley Date 13/5/02

Reviewed by: [illegible] Date [illegible]

Archport Ltd Protocol No.: 2610-29-IQ-004		
ATTACHMENT: A7	Control System Hardware	Pg 1 of 1

Equipment ID #:
BIOCONTROLLER ADI 1060
Location:
ARCHPORT 5124
Description :
CONTROL UNIT
Manufacturer:
APPLIKON
Model # /Serial #:
2S1060010 / P433/5579821

Equipment ID #:
INTERFACE ADI 1060ES
Location:
ARCHPORT 5124
Description :
HARDWARE CONTROL UNIT
Manufacturer:
APPLIKON
Model # /Serial #:
2S1040025C / P4/080111

Equipment ID #:
BIOCONSOLE ADI 1035
Location:
ARCHPORT 5124
Description :
CONTROL UNIT
Manufacturer:
APPLIKON
Model # /Serial #:
1035 / P1L460/29

Performed by: [signature] Date 13/5/02

Reviewed by: [signature] Date [illegible]

Archport Ltd Protocol No.: 2610-29-IQ-004		
ATTACHMENT : A8	Input/Output List /Loop Check Documentation	Pg 1 of 1

Input Listing	Output Listing	As Supplied / Installed	Acceptable (Y/N)	Verified by

N/A

Loop Check	Certifice Available (Y/N)	As Supplied / Installed	Acceptable (Y/N)	Verified by

N/A

Performed by: C Fahey Date 13/5/02

Reviewed by: D. Cannon Date 29/5/02

National Cell & Tissue culture Centre Protocol No.: 2610-29-IQ-004		
ATTACHMENT : A9	Set Points & Software Parameters	Pg 1 of 1

Instrument/Equipment I.D.: BIOCONTROLLER ADI 1060 (Z510600010)		
Parameter/Setpoint: BIOS V4.51pg. Version 3.1		
Inspected by: CF	Date: 12/10/01	Discrepancies (Y/N) N

Instrument/Equipment I.D.: DAEWOO COMPUTER (Z12 B)		
Parameter/Setpoint: Bioexpert NT Version 2.25.091		
Inspected by: CF	Date: 12/10/01	Discrepancies (Y/N) N

Instrument/Equipment I.D.:		
Parameter/Setpoint: N/A CF 12/10/01		
Inspected by:	Date:	Discrepancies (Y/N)

Comments : N/A CF 12/10/01.

Document any discrepancies on attachment A13.

Performed by: C Foley Date 15/5/02

Reviewed by: Anne Flanagan Date 24/7/02

Archport Ltd

Protocol No.: 2610-29-IQ-004

ATTACHMENT : A10	Software Documentation	Pg 1 of 1

SOFTWARE TITLE	REF. No	VERSION	DATE PRINTED COPY	LOCATION INSTALLATION	MODEL No.	SERIAL No.	MANU FACTURER	SOFTWARE FLOWCHARTS
BIOEXPERT.NT DATA ACQUISITION + CONTROL PROG.	V1UP1 27122	2	JUNE 1998	ARCHPORT 5124	N/A	N/A	Applikon	N/A
ADI 1060 BIOCONTROLLER	N/A	3.1	JAN 1999	ARCHPORT 5124	N/A	N/A	Applikon	N/A

Any modifications ? N/A 13/5/02 CF

Performed by: C Foley Date 13/5/02

Reviewed by: Anne [illegible] Date 29/7/02

Archport Ltd Protocol No.: 2610-29-IQ-004		
ATTACHMENT : A11	Factory Acceptance Testing (FAT)	Pg 1 of 1

FAT Doc. No.:	Rev./Issue No.	Date:
Title: N/A		Location:
Inspected by: CS	Date:	Discrepancy (Y/N):

Comments:

N/A
CS

Performed by: R Bailey Date 13/5/02

Reviewed by: A Hanney Date 14/5/02

Archport Ltd

Protocol No.: 2610-29-IQ-004

ATTACHMENT : A12	Standard Operating Procedures List	Pg 1 of 1

Operating Procedure Title	SOP No.	Rev.	Date	Responsible Department	Verified by
Cell Culture Assembly of a 10L Bioreactor	1				
Cleaning and Siliconisation of a 10 L bioreactor and its components	2				
Preparation of accessories for a 10 L bioreactor culture system.	3				
Cell Culture standard procedure for counting cells	4				
Cell Culture Standard Procedure for calculating the kLA value for the 10L bioreactor	5				

Performed by: [signature] Date 13/5/02

Reviewed by: [signature] Date 29/7/02

Archport Ltd Protocol No.: 2610-29-IQ-004		
ATTACHMENT : A13	Installation Qualification Discrepancy Summary	Pg 1 of 1

Reference: _____

Discrepancy/Variation: _____

_____

Resolution: _____

_____

_____

Satisfactorily completed? (Y/N): _____

Signature Date

Reference: _____

Discrepancy/Variation: _____

_____

_____

Resolution: _____

_____

_____

N/A 13/5/02

Satisfactorily completed? (Y/N): _____

Signature Date

Reference: _____

Discrepancy/Variation: _____

_____

_____

Resolution: _____

_____

Satisfactorily completed? (Y/N): _____ _____

Signature Date

Performed by: _____ Date 13/5/02

Reviewed by: _____ Date 24/5/02

Appendix VIII ***Operational Qualification***

Note: Operational Qualification has not been completely signed off see discrepancy summary for reasons.

Operational Qualification
Applikon 10 L Bioreactor (Z611000010)

Pre Execution.	Signature	Title	Date
Prepared By: Catherese Fahey	CFahey	Research Scientist	10-July-2002.
Approved by: EUNAN MCGLINCHEY pp Cathal O Grady	ECMcGlinchey	Cell Culture Manager	30/7/02
Approved by: Anne Hannigan	Anne Hannigan	Validation Supervisor	30/7/02
Approved by: Irene Cleary	Irene Cleary	Validation Manager	30/7/02

Post Execution	Signature	Title	Date
Prepared By: Catherese Fahey	CFahey	Research Scientist	4-November-2002
Approved by: Cathal O Grady		Cell Culture Manager	
Approved by: Anne Hannigan		Validation Supervisor	
Approved by: Irene Cleary		Validation Manager	

Contents:

Page Number

1. Purpose:

The purpose of the Operational Qualification (OQ) is to demonstrate that the Applikon 10 L Bioreactor and control units operate according to the design intent and manufacturers recommendations.

2. Scope:

The scope of the Operational Qualification includes the Applikon 10 L Bioreactor and control units. All other aspects of the mechanical and electrical installation of the unit and its assembly are included.

3. System Description:

The ADI 1060 Bio Controller, the ADI 1035 Bioconsole and the ADI 1040/25 are used in unison for the process control of the 10 L Applikon bioreactor. The purpose of the ADI 1060 (Software version V1.3) is to monitor mammalian cell culture systems in an environment where pH, temperature, dissolved oxygen and mixing can be controlled.

The ADI 1035 Bioconsole is an actuator console that is used in combination with the ADI 1060 Bio Controller. It supports gas flow regulation, stirrer speed, pH control, dissolved Oxygen control, level control, temperature control, and liquid addition/withdrawal via pumps.

The ADI 1040/25 functions by connecting the ADI 1060 Bio controller with the bioreactor and their actuator consoles. The measured sensor signals are amplified in the 1040/25 and are transmitted to the Bio Controller through serial communication. Depending on deviation from the set point, a controller output value is sent to an analog or digital output that will activate the actuator in the ADI 1035 in order to decrease the deviation from the set point.

The associated 10 L Bioreactor is a glass stirred tank reactor.

4. Pre-protocol Checks:

Have QA representatives reviewed the IQ? Are ali critical discrepancy items resolved? Have outstanding discrepancy items been reviewed and have resolution actions put in place?

Yes/No: Yes — File Location: IQ 5124 ARCHIVE
Verified By: CSaley — Date: 4/11/02

Have Standard Operating Procedures been drafted for the operation of the 10 L Bioreactor & Control System?

Yes/No: Do — File Location: —
Verified By: CSaley — Date: 4/11-/02

Detail relevant SOP numbers: —

Has GMP training been provided to relevant maintenance and operational personnel? Have reports been completed and filed?

Yes/No: Do — File Location —
Verified By: CSaley — Date: 4/11/02

Is a maintenance program in place and has it been approved?

Yes/No: Do — File Location —
Verified By: CSaley — Date: 4/11/02

Are all utility connections live and sufficient to support normal operation? Is unit prepared for start up and where applicable has basic first charges of oil etc. have been added to commence OQ attributes.

Yes/No: Yes — File Location —
Verified By: CSaley — Date: 4/11/02

Comments:

Signed: CSaley — **Date:** 4/11/02

Approved By: _____ — **Date:** _____

5. Operation Qualification

5.1 Configuration Table ADI 1060

❑ Fill in the settings from the ADI 1060 controller in the tables below.

Sensor Cards

	PH
Low Alarm	2
High Alarm	12
P gain	100
I (time)	0
D (time)	0
Bias	0
Deadzone	0.10

TEMPERATURE	
Low Alarm	36
High Alarm	38
P gain	20
I (time)	45
D (time)	0
Bias	0
Deadzone	0

Performed by: CSeeley Date 4-11-02

Reviewed by: _____ Date _____

DISSOLVED OXYGEN	
Low Alarm	50
High Alarm	55
P gain	10
I (time)	0
D (time)	0
Bias	0
Deadzone	5

Digital Outputs:

OUTPUT	FUNCTION	LOW %	HIGH %	CYCLE TIME (SECS)	DOSE
1,3	O_2 Valve	0	100	20	— L
1,4	CO_2 Valve	0	100	5	— L
2,1	Base Pump	0	100	10	gal/min
2,3	Heating	0	100	30	0° C

Analog Outputs:

OUTPUT	FUNCTION	MIN VALUE (MA)	MAX VALUE (MA)
1	Stirrer	0	20
2	Feedpump	0	20
3	Feedpump 2	0	20
4	pH Recorder	0	20
5	Temp. Recorder	0	20
6	DO_2 Recorder	0	20

Serial Communications:

ADI 1040/25 COMMUNICATION SETTINGS					
RS 422	Baudrate	Parity	Stobit	Databit	Device
1	19200	No	1	7	N/A

HOST COMMUNICATIONS SETTINGS					
RS 485	Baudrate	Parity	Stobit	Databit	Device
1	19200	No	1	7	N/A

Performed by: MSabey. Date 4-11-02

Reviewed by: _____ Date _____

5.2 Temperature Control

5.2.1 Cultivation Mode

- ❑ Control Temperature inside bioreactor at 37^{0}C during cultivation, with a stirrer speed of 100 rpm for at least 3 hours.

PID Settings:

TEMPERATURE ^{0}C	
P	20
I	45
D	0
Bias	0

5.2.2 Test Mode:

- ❑ Start the temperature controller on ADI 1060.
- ❑ Create "stepresponses" In Bioexpert changing the temperature setpoint using a profile as mentioned in the table below.
- ❑ The criteria for overshoot during cooling and heating are 2^{0}C and within an hour after reaching the set-point the temperature must be stable (+/- 0.2^{0}C).
- ❑ Make a trend, record operator actions, alarms and periodical data. Report on the results.

SETPOINT CHANGE	DATE	SIGN INSPECTOR	CHECK: PASS/FAIL
37°C ⟶ 31°C	4-11-02	CGaley	Pass
31°C ⟶ 35°C	4-11-02	CGaley	Pass
35°C ⟶ 37°C	4-11-02	CGaley	Pass
37°C ⟶ 32°C	4-11-02	CGaley	Pass
32°C ⟶ 31°C	4-11-02	CGaley	Pass
31°C ⟶ 37°C	4-11-02	CGaley.	Pass

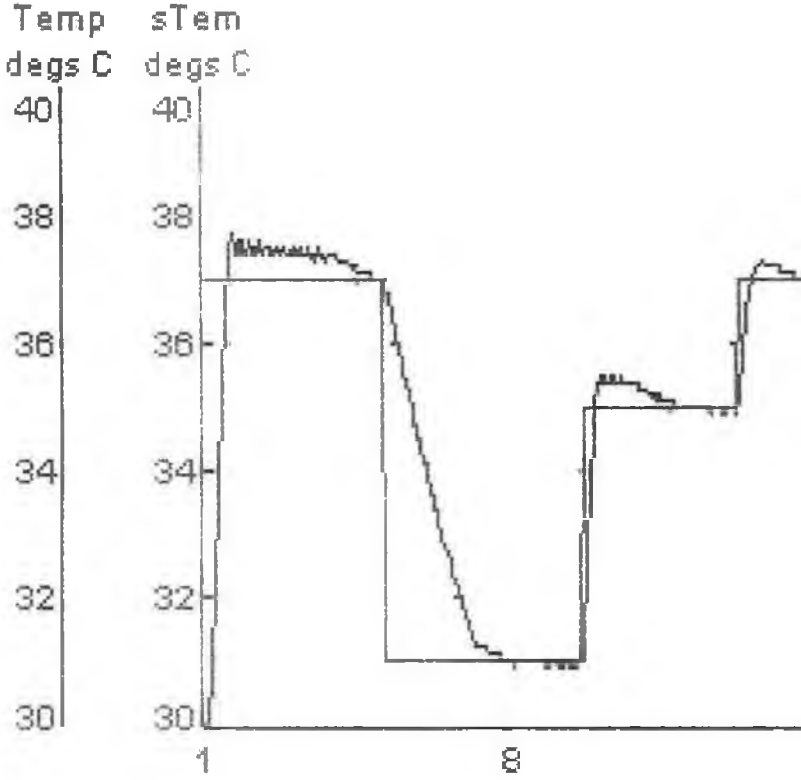
Comments:

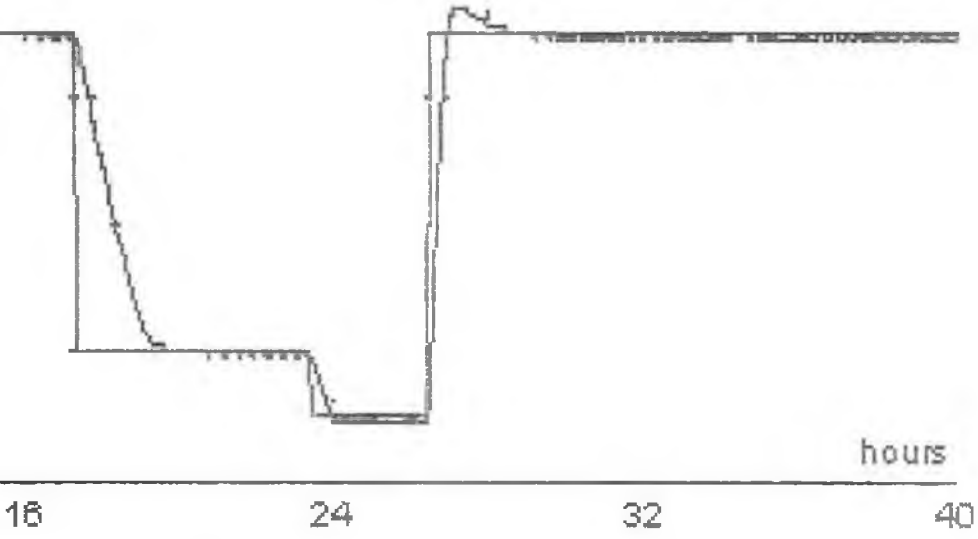
ALARM TEST WERE NOT CARRIED OUT HERE WILL BE PERFORMED IN PQ.
AFTER REACHING THE SETPOINT THE TEMP. WAS NOT STABLE WITHIN (+/- 2°C) 1 HOUR. STABILITY WAS OBTAINED AFTHIS TIME. TEST WILL HAVE TO BE REPEATED USING LIMITS OF +/- 0.5°C AS THE LIMITS +/- 0.2°C WERE TOO TIGHT FOR SOME OF THE SETPOINT CHANGES.

Performed by: CGaley Date 4-11-02

Reviewed by: _____ Date _____

11/6/02.




hours
16
24
32
40
time

Time h:m	Temp degs C	sTem degs C	Time h:m	Temp degs C	sTem degs C
c:///MONOCAL.frm					
0:30	28.6	37	1:02	36.8	37
0:31	28.8	37	1:03	37	37
0:32	29	37	1:04	37.1	37
0:33	29.3	37	1:05	37.2	37
0:34	29.5	37	1:06	37.4	37
0:35	29.7	37	1:07	37.5	37
0:36	30	37	1:08	37.6	37
0:37	30.1	37	1:09	37.7	37
0:38	30.3	37	1:10	37.7	37
0:39	30.6	37	1:11	37.7	37
0:40	30.8	37	1:12	37.6	37
0:41	30.9	37	1:13	37.6	37
0:42	31.2	37	1:14	37.6	37
0:43	31.5	37	1:15	37.5	37
0:44	31.8	37	1:16	37.5	37
0:45	32.1	37	1:17	37.5	37
0:45	32.3	37	1:18	37.4	37
0:47	32.5	37	1:19	37.4	37
0:48	32.9	37	1:20	37.4	37
0:49	33.2	37	1:21	37.4	37
0:50	33.5	37	1:22	37.4	37
0:51	33.8	37	1:23	37.5	37
0:52	34.1	37	1:24	37.5	37
0:53	34.4	37	1:25	37.6	37
0:54	34.8	37	1:26	37.6	37
0:55	35.1	37	1:27	37.5	37
0:56	35.4	37	1:28	37.5	37
0:57	35.6	37	1:29	37.5	37
0:58	35.9	37	1:30	37.4	37
0:59	36.1	37	1:31	37.4	37
1:00	36.4	37	1:32	37.4	37
1:01	36.6	37	1:33	37.4	37

Time h:m	Temp degs C	sTem degs C
1:34	37.4	37
1:35	37.4	37
1:36	37.5	37
1:37	37.5	37
1:38	37.6	37
1:39	37.6	37
1:40	37.6	37
1:41	37.5	37
1:42	37.5	37
1:43	37.5	37
1:44	37.4	37
1:45	37.4	37
1:46	37.4	37
1:47	37.4	37
1:48	37.4	37
1:49	37.4	37
1:50	37.4	37
1:51	37.5	37
1:52	37.5	37
1:53	37.6	37
1:54	37.5	37
1:55	37.5	37
1:56	37.5	37
1:57	37.4	37
1:58	37.4	37
1:59	37.4	37
2:00	37.4	37
2:01	37.4	37
2:02	37.4	37
2:03	37.5	37
2:04	37.5	37
2:05	37.5	37

START OF STABILITY TEST @37°C

2:06	37.5	37	2:41	37.4	37
2:07	37.5	37	2:42	37.5	37
2:08	37.5	37	2:43	37.5	37
2:09	37.4	37	2:44	37.5	37
2:10	37.4	37	2:45	37.5	37
2:11	37.4	37	2:46	37.5	37
2:12	37.4	37	2:47	37.4	37
2:13	37.4	37	2:48	37.4	37
2:14	37.4	37	2:49	37.4	37
2:15	37.4	37	2:50	37.4	37
2:16	37.5	37	2:51	37.4	37
2:17	37.5	37	2:52	37.4	37
2:18	37.5	37	2:53	37.4	37
2:19	37.5	37	2:54	37.4	37
2:20	37.5	37	2:55	37.4	37
2:21	37.5	37	2:56	37.5	37
2:22	37.4	37	2:57	37.5	37
2:23	37.4	37	2:58	37.5	37
2:24	37.4	37	2:59	37.5	37
2:25	37.4	37	3:00	37.4	37
2:26	37.4	37	3:01	37.4	37
2:27	37.4	37	3:02	37.4	37
2:28	37.4	37	3:03	37.4	37
2:29	37.5	37	3:04	37.4	37
2:30	37.5	37	3:05	37.4	37
2:31	37.5	37	3:06	37.4	37
2:32	37.5	37	3:07	37.4	37
2:33	37.5	37	3:08	37.4	37
2:34	37.4	37	3:09	37.5	37
2:35	37.4	37	3:10	37.5	37
2:36	37.4	37	3:11	37.5	37
2:37	37.4	37	3:12	37.4	37
2:38	37.4	37	3:13	37.4	37
2:39	37.4	37	3:14	37.4	37
2:40	37.4	37	3:15	37.4	37

3:16	37.3	37
3:17	37.3	37
3:18	37.4	37
3:19	37.4	37
3:20	37.4	37
3:21	37.4	37
3:22	37.4	37
3:23	37.4	37
3:24	37.4	37
3:25	37.5	37
3:26	37.5	37
3:27	37.5	37
3:28	37.4	37
3:29	37.4	37
3:30	37.4	37
3:31	37.4	37
3:32	37.4	37
3:33	37.3	37
3:34	37.3	37
3:35	37.3	37
3:36	37.4	37
3:37	37.4	37
3:38	37.4	37
3:39	37.4	37
3:40	37.4	37
3:41	37.4	37
3:42	37.4	37
3:43	37.4	37
3:44	37.4	37
3:45	37.4	37
3:46	37.4	37
3:47	37.4	37
3:48	37.4	37
3:49	37.4	37
3:50	37.4	37

3:51	37.4	37	4:26	37.1	37
3:52	37.4	37	4:27	37.1	37
3:53	37.3	37	4:28	37.1	37
3:54	37.3	37	4:29	37.1	37
3:55	37.3	37	4:30	37.1	37
3:56	37.3	37	4:31	37.1	37
3:57	37.3	37	4:32	37.1	37
3:58	37.3	37	4:33	37.1	37
3:59	37.3	37	4:34	37.1	37
4:00	37.3	37	4:35	37.1	37
4:01	37.3	37	4:36	37.1	37
4:02	37.3	37	4:37	37.1	37
4:03	37.3	37	4:38	37.1	37
4:04	37.3	37	4:39	37.1	37
4:05	37.3	37	4:40	37.1	37
4:06	37.2	37	4:41	37.1	37
4:07	37.2	37	4:42	37.1	37
4:08	37.2	37	4:43	37	37
4:09	37.2	37	4:44	37	37
4:10	37.2	37	4:45	37	37
4:11	37.2	37	4:46	37	37
4:12	37.2	37	4:47	37	37
4:13	37.2	37	4:48	37	37
4:14	37.2	37	4:49	37	37
4:15	37.2	37	4:50	37	37
4:16	37.2	37	4:51	37	37
4:17	37.2	37	4:52	37	37
4:18	37.2	37	4:53	37	37
4:19	37.2	37	4:54	37	37
4:20	37.2	37	4:55	37	37
4:21	37.1	37	4:56	37	37
4:22	37.1	37	4:57	37	37
4:23	37.1	37	4:58	37	37
4:24	37.1	37	4:59	37	37
4:25	37.1	37	5:00	37	37

End of Stability Test
@ 37°C

5:01	37	31
5:02	37	31
5:03	37	31
5:04	37	31
5:05	37	31
5:06	36.9	31
5:07	36.9	31
5:08	36.8	31
5:09	36.8	31
5:10	36.7	31
5:11	36.7	31
5:12	36.6	31
5:13	36.5	31
5:14	36.5	31
5:15	36.5	31
5:16	36.4	31
5:17	36.3	31
5:18	36.3	31
5:19	36.2	31
5:20	36.2	31
5:21	36.2	31
5:22	36.1	31
5:23	36	31
5:24	36	31
5:25	35.9	31
5:26	35.9	31
5:27	35.8	31
5:28	35.8	31
5:29	35.7	31
5:30	35.7	31
5:31	35.6	31
5:32	35.6	31
5:33	35.5	31
5:34	35.5	31
5:35	35.4	31

5:36	35.4	31	6:11	33.8	31	6:46	32.4	31
5:37	35.3	31	6:12	33.8	31	6:47	32.4	31
5:38	35.3	31	6:13	33.7	31	6:48	32.4	31
5:39	35.2	31	6:14	33.7	31	6:49	32.3	31
5:40	35.2	31	6:15	33.6	31	6:50	32.3	31
5:41	35.2	31	6:16	33.6	31	6:51	32.3	31
5:42	35.1	31	6:17	33.6	31	6:52	32.2	31
5:43	35.1	31	6:18	33.5	31	6:53	32.2	31
5:44	35	31	6:19	33.5	31	6:54	32.1	31
5:45	35	31	6:20	33.4	31	6:55	32.1	31
5:46	34.9	31	6:21	33.4	31	6:56	32.1	31
5:47	34.9	31	6:22	33.4	31	6:57	32	31
5:48	34.8	31	6:23	33.3	31	6:58	32	31
5:49	34.8	31	6:24	33.3	31	6:59	31.9	31
5:50	34.7	31	6:25	33.2	31	7:00	31.9	31
5:51	34.7	31	6:26	33.2	31	7:01	31.9	31
5:52	34.6	31	6:27	33.2	31	7:02	31.8	31
5:53	34.6	31	6:28	33.1	31	7:03	31.8	31
5:54	34.6	31	6:29	33.1	31	7:04	31.8	31
5:55	34.5	31	6:30	33	31	7:05	31.7	31
5:56	34.5	31	6:31	33	31	7:06	31.7	31
5:57	34.4	31	6:32	32.9	31	7:07	31.7	31
5:58	34.4	31	6:33	32.9	31	7:08	31.6	31
5:59	34.3	31	6:34	32.9	31	7:09	31.6	31
6:00	34.3	31	6:35	32.8	31	7:10	31.6	31
6:01	34.2	31	6:36	32.8	31	7:11	31.6	31
6:02	34.2	31	6:37	32.8	31	7:12	31.5	31
6:03	34.2	31	6:38	32.7	31	7:13	31.5	31
6:04	34.1	31	6:39	32.7	31	7:14	31.4	31
6:05	34.1	31	6:40	32.7	31	7:15	31.4	31
6:06	34.1	31	6:41	32.6	31	7:16	31.4	31
6:07	34	31	6:42	32.6	31	7:17	31.3	31
6:08	34	31	6:43	32.6	31	7:18	31.3	31
6:09	33.9	31	6:44	32.5	31	7:19	31.3	31
6:10	33.9	31	6:45	32.5	31	7:20	31.3	31

START OF STABILITY

3PL

07:21	31.3	31	07:56	30.9	31	08:31	31.2	31
07:22	31.2	31	07:57	30.9	31	08:32	31.2	31
07:23	31	31	07:58	30.9	31	08:33	31.2	31
07:24	31.1	31	07:59	30.9	31	08:34	31.2	31
07:25	31	31	08:00	30.9	31	08:35	31.2	31
07:26	31	31	08:01	30.9	31	08:36	31.2	31
07:27	31	31	08:02	30.9	31	08:37	31.2	31
07:28	31	31	08:03	30.9	31	08:38	31.2	31
07:29	31.1	31	08:04	30.9	31	08:39	31.1	31
07:30	31.1	31	08:05	30.9	31	08:40	31.1	31
07:31	31.2	31	08:06	30.9	31	08:41	31	31
07:32	31.2	31	08:07	30.9	31	08:42	31	31
07:33	31.2	31	08:08	30.9	31	08:43	31	31
07:34	31.2	31	08:09	31	31	08:44	31	31
07:35	31.2	31	08:10	31	31	08:45	31	31
07:36	31.2	31	08:11	31	31	08:46	31	31
07:37	31.2	31	08:12	31	31	08:47	31	31
07:38	31.2	31	08:13	31	31	08:48	31.1	31
07:39	31.2	31	08:14	31	31	08:49	31.2	31
07:40	31.2	31	08:15	31	31	08:50	31.2	31
07:41	31.1	31	08:16	31	31	08:51	31.2	31
07:42	31.1	31	08:17	31	31	08:52	31.2	31
07:43	31.1	31	08:18	31	31	08:53	31.2	31
07:44	31.1	31	08:19	30.9	31	08:54	31.2	31
07:45	31.1	31	08:20	30.9	31	08:55	31.2	31
07:46	31.1	31	08:21	30.9	31	08:56	31	31
07:47	30.9	31	08:22	30.9	31	08:57	31	31
07:48	30.9	31	08:23	30.9	31	08:58	31	31
07:49	30.9	31	08:24	30.9	31	08:59	31	31
07:50	30.9	31	08:25	31.1	31	09:00	31	31
07:51	30.9	31	08:26	31.1	31	09:01	31	31
07:52	30.9	31	08:27	31.1	31	09:02	30.9	31
07:53	30.9	31	08:28	31.1	31	09:03	30.9	31
07:54	31	31	08:29	31.1	31	09:04	30.9	31
07:55	31	31	08:30	31.1	31	09:05	30.9	31

09:06	31	31	09:41	31
09:07	30.9	31	09:42	31
09:08	30.9	31	09:43	31
09:09	30.9	31	09:44	31
09:10	30.9	31	09:45	30.9
09:11	30.9	31	09:46	30.9
09:12	30.9	31	09:47	30.9
09:13	30.9	31	09:48	30.9
09:14	30.9	31	09:49	30.9
09:15	30.9	31	09:50	30.9
09:16	31	31	09:51	30.9
09:17	31	31	09:52	30.9
09:18	31	31	09:53	30.9
09:19	31	31	09:54	30.9
09:20	31	31	09:55	30.9
09:21	31	31	09:56	31
09:22	31	31	09:57	31
09:23	31	31	09:58	31
09:24	31	31	09:59	31
09:25	31	31	10:00	31
09:26	31	31	10:01	31
09:27	31	31	10:02	31
09:28	30.9	31	10:03	31
09:29	31	31	10:04	31
09:30	31	31	10:05	31.2
09:31	30.9	31	10:06	31.4
09:32	30.9	31	10:07	31.6
09:33	30.9	31	10:08	31.9
09:34	31	31	10:09	32.2
09:35	30.9	31	10:10	32.4
09:36	31	31	10:11	32.7
09:37	31	31	10:12	32.9
09:38	31	31	10:13	33.2
09:39	31	31	10:14	33.4
09:40	31	31	10:15	33.7

31	10:16	33.9	35
31	10:17	34	35
31	10:18	34.2	35
31	10:19	34.4	35
31	10:20	34.5	35
31	10:21	34.7	35
31	10:22	34.8	35
31	10:23	34.9	35
31	10:24	35	35
31	10:25	35.1	35
31	10:26	35.2	35
31	10:27	35.2	35
31	10:28	35.3	35
31	10:29	35.3	35
31	10:30	35.4	35
31	10:31	35.4	35
31	10:32	35.4	35
31	10:33	35.5	35
31	10:34	35.5	35
31 END OF STABILITY @31°C	10:35	35.5	35
35	10:36	35.5	35
35	10:37	35.5	35
35	10:38	35.4	35
35	10:39	35.4	35
35	10:40	35.4	35
35	10:41	35.4	35
35	10:42	35.4	35
35	10:43	35.4	35
35	10:44	35.4	35
35	10:45	35.4	35
35	10:46	35.4	35
35	10:47	35.4	35
35	10:48	35.4	35
35	10:49	35.5	35
35	10:50	35.5	35

START OF STABILITY @ 35°C

10:51	35.5	35	11:26	35.4	35
10:52	35.5	35	11:27	35.4	35
10:53	35.4	35	11:28	35.4	35
10:54	35.4	35	11:29	35.4	35
10:55	35.4	35	11:30	35.3	35
10:56	35.4	35	11:31	35.3	35
10:57	35.4	35	11:32	35.3	35
10:58	35.4	35	11:33	35.3	35
10:59	35.4	35	11:34	35.3	35
11:00	35.4	35	11:35	35.3	35
11:01	35.4	35	11:36	35.3	35
11:02	35.5	35	11:37	35.3	35
11:03	35.4	35	11:38	35.3	35
11:04	35.4	35	11:39	35.3	35
11:05	35.5	35	11:40	35.3	35
11:06	35.5	35	11:41	35.3	35
11:07	35.4	35	11:42	35.3	35
11:08	35.4	35	11:43	35.3	35
11:09	35.4	35	11:44	35.3	35
11:10	35.4	35	11:45	35.2	35
11:11	35.4	35	11:46	35.2	35
11:12	35.4	35	11:47	35.2	35
11:13	35.4	35	11:48	35.2	35
11:14	35.4	35	11:49	35.2	35
11:15	35.4	35	11:50	35.2	35
11:16	35.4	35	11:51	35.2	35
11:17	35.4	35	11:52	35.2	35
11:18	35.4	35	11:53	35.2	35
11:19	35.4	35	11:54	35.2	35
11:20	35.4	35	11:55	35.2	35
11:21	35.4	35	11:56	35.1	35
11:22	35.4	35	11:57	35.1	35
11:23	35.4	35	11:58	35.1	35
11:24	35.4	35	11:59	35.2	35
11:25	35.4	35	12:00	35.2	35

12:01	35.2	35
12:02	35.1	35
12:03	35.1	35
12:04	35.1	35
12:05	35.1	35
12:06	35.1	35
12:07	35.1	35
12:08	35.1	35
12:09	35.1	35
12:10	35.1	35
12:11	35.1	35
12:12	35.1	35
12:13	35.1	35
12:14	35.1	35
12:15	35.1	35
12:16	35.1	35
12:17	35.1	35
12:18	35.1	35
12:19	35.1	35
12:20	35.1	35
12:21	35.1	35
12:22	35.1	35
12:23	35.1	35
12:24	35.1	35
12:25	35	35
12:26	35	35
12:27	35	35
12:28	35	35
12:29	35	35
12:30	35	35
12:31	35	35
12:32	35	35
12:33	35	35
12:34	35	35
12:35	35	35

12:36	35	35	13:11	35
12:37	35	35	13:12	35
12:38	35	35	13:13	35
12:39	35	35	13:14	35
12:40	35	35	13:15	35
12:41	35	35	13:16	35
12:42	35	35	13:17	35
12:43	35	35	13:18	34.9
12:44	35	35	13:19	34.9
12:45	35	35	13:20	34.9
12:46	35	35	13:21	34.9
12:47	35	35	13:22	35
12:48	35	35	13:23	34.9
12:49	35	35	13:24	35
12:50	35	35	13:25	35
12:51	35	35	13:26	35
12:52	35	35	13:27	35
12:53	35	35	13:28	35
12:54	35	35	13:29	35
12:55	35	35	13:30	35
12:56	35	35	13:31	35
12:57	35	35	13:32	35
12:58	35	35	13:33	35
12:59	35	35	13:34	35
13:00	35	35	13:35	35
13:01	35	35	13:36	35
13:02	35	35	13:37	34.9
13:03	35	35	13:38	34.9
13:04	35	35	13:39	34.9
13:05	35	35	13:40	34.9
13:06	35	35	13:41	34.9
13:07	35	35	13:42	34.9
13:08	35	35	13:43	34.9
13:09	35	35	13:44	34.9
13:10	35	35	13:45	34.9

35	13:46	34.9	35
35	13:47	35	35
35	13:48	35	35
35	13:49	35	35
35	13:50	35	35
35	13:51	35	35
35	13:52	35	35
35	13:53	35	35
35	13:54	35	35
35	13:55	35	35
35	13:56	35	35
35	13:57	35	35
35	13:58	35	35
35	13:59	35	35
35	14:00	35	35
35	14:01	35	37
35	14:02	34.9	37
35	14:03	35	37
35	14:04	35	37
35	14:05	35.1	37
35	14:06	35.2	37
35	14:07	35.4	37
35	14:08	35.5	37
35	14:09	35.6	37
35	14:10	35.8	37
35	14:11	35.9	37
35	14:12	36	37
35	14:13	36.2	37
35	14:14	36.3	37
35	14:15	36.4	37
35	14:16	36.5	37
35	14:17	36.6	37
35	14:18	36.6	37
35	14:19	36.7	37
35	14:20	36.8	37

END OF STABILITY @35°C

Start of stability @ 37°C

14:21	36.8	37	14:56
14:22	36.9	37	14:57
14:23	36.9	37	14:58
14:24	37	37	14:59
14:25	37	37	15:00
14:26	37	37	15:01
14:27	37.1	37	15:02
14:28	37.1	37	15:03
14:29	37.1	37	15:04
14:30	37.1	37	15:05
14:31	37.2	37	15:06
14:32	37.2	37	15:07
14:33	37.2	37	15:08
14:34	37.2	37	15:09
14:35	37.2	37	15:10
14:36	37.2	37	15:11
14:37	37.2	37	15:12
14:38	37.2	37	15:13
14:39	37.3	37	15:14
14:40	37.3	37	15:15
14:41	37.3	37	15:16
14:42	37.3	37	15:17
14:43	37.3	37	15:18
14:44	37.2	37	15:19
14:45	37.2	37	15:20
14:46	37.2	37	15:21
14:47	37.2	37	15:22
14:48	37.2	37	15:23
14:49	37.2	37	15:24
14:50	37.2	37	15:25
14:51	37.2	37	15:26
14:52	37.2	37	15:27
14:53	37.2	37	15:28
14:54	37.2	37	15:29
14:55	37.2	37	15:30

37.2	37	15:31	37.1	37
37.2	37	15:32	37	37
37.2	37	15:33	37	37
37.2	37	15:34	37	37
37.2	37	15:35	37	37
37.2	37	15:36	37	37
37.2	37	15:37	37	37
37.2	37	15:38	37	37
37.2	37	15:39	37	37
37.2	37	15:40	37	37
37.2	37	15:41	37	37
37.2	37	15:42	37	37
37.1	37	15:43	37	37
37.1	37	15:44	37	37
37.1	37	15:45	37	37
37.1	37	15:46	37	37
37.1	37	15:47	37	37
37.1	37	15:48	37	37
37.1	37	15:49	37	37
37.1	37	15:50	37	37
37.1	37	15:51	37	37
37.1	37	15:52	37	37
37.1	37	15:53	37	37
37.1	37	15:54	37	37
37.1	37	15:55	37	37
37.1	37	15:56	37	37
37.1	37	15:57	37	37
37.1	37	15:58	37	37
37.1	37	15:59	37	37
37.1	37	16:00	37	37
37.1	37	16:01	37	37
37.1	37	16:02	37	37
37.1	37	16:03	37	37
37.1	37	16:04	37	37
37	37	16:05	37	37

16:06	37	37	16:41	36.9	37	17:16	37	37
16:07	37	37	16:42	36.9	37	17:17	37	37
16:08	37	37	16:43	36.9	37	17:18	37	37
16:09	37	37	16:44	36.9	37	17:19	37	37
16:10	37	37	16:45	37	37	17:20	37	37
16:11	37	37	16:46	37	37	17:21	36.9	37
16:12	37	37	16:47	37	37	17:22	36.9	37
16:13	37	37	16:48	37	37	17:23	36.9	37
16:14	37	37	16:49	37	37	17:24	36.9	37
16:15	37	37	16:50	37	37	17:25	36.9	37
16:16	37	37	16:51	37	37	17:26	36.9	37
16:17	37	37	16:52	37	37	17:27	37	37
16:18	37	37	16:53	37	37	17:28	37	37
16:19	37	37	16:54	37	37	17:29	37	37
16:20	37	37	16:55	37	37	17:30	37	37
16:21	37	37	16:56	37	37	17:31	37	32
16:22	37	37	16:57	37	37	17:32	37	32
16:23	37	37	16:58	37	37	17:33	37	32
16:24	37	37	16:59	36.9	37	17:34	36.9	32
16:25	37	37	17:00	37	37	17:35	36.9	32
16:26	37	37	17:01	36.9	37	17:36	36.9	32
16:27	37	37	17:02	36.9	37	17:37	36.8	32
16:28	37	37	17:03	36.9	37	17:38	36.8	32
16:29	37	37	17:04	36.9	37	17:39	36.7	32
16:30	37	37	17:05	37	37	17:40	36.7	32
16:31	37	37	17:06	36.9	37	17:41	36.6	32
16:32	37	37	17:07	36.9	37	17:42	36.5	32
16:33	37	37	17:08	36.9	37	17:43	36.5	32
16:34	37	37	17:09	37	37	17:44	36.4	32
16:35	37	37	17:10	37	37	17:45	36.4	32
16:36	37	37	17:11	37	37	17:46	36.3	32
16:37	37	37	17:12	37	37	17:47	36.3	32
16:38	36.9	37	17:13	37	37	17:48	36.2	32
16:39	36.9	37	17:14	37	37	17:49	36.2	32
16:40	36.9	37	17:15	37	37	17:50	36.1	32

2nd of Stability @ 37°C

17:51	36.1	32	18:26	34.4
17:52	36	32	18:27	34.3
17:53	36	32	18:28	34.3
17:54	35.9	32	18:29	34.2
17:55	35.9	32	18:30	34.2
17:56	35.8	32	18:31	34.2
17:57	35.8	32	18:32	34.1
17:58	35.7	32	18:33	34.1
17:59	35.7	32	18:34	34.1
18:00	35.6	32	18:35	34
18:01	35.6	32	18:36	34
18:02	35.5	32	18:37	33.9
18:03	35.5	32	18:38	33.9
18:04	35.4	32	18:39	33.8
18:05	35.4	32	18:40	33.8
18:06	35.3	32	18:41	33.8
18:07	35.3	32	18:42	33.7
18:08	35.2	32	18:43	33.7
18:09	35.2	32	18:44	33.6
18:10	35.1	32	18:45	33.6
18:11	35.1	32	18:46	33.5
18:12	35	32	18:47	33.5
18:13	35	32	18:48	33.5
18:14	34.9	32	18:49	33.4
18:15	34.9	32	18:50	33.4
18:16	34.9	32	18:51	33.3
18:17	34.8	32	18:52	33.3
18:18	34.8	32	18:53	33.2
18:19	34.7	32	18:54	33.2
18:20	34.7	32	18:55	33.2
18:21	34.6	32	18:56	33.1
18:22	34.6	32	18:57	33.1
18:23	34.5	32	18:58	33.1
18:24	34.5	32	18:59	33
18:25	34.4	32	19:00	33

32	19:01	32.9	32	
32	19:02	32.9	32	
32	19:03	32.9	32	
32	19:04	32.8	32	
32	19:05	32.8	32	
32	19:06	32.7	32	
32	19:07	32.7	32	
32	19:08	32.7	32	
32	19:09	32.6	32	
32	19:10	32.6	32	
32	19:11	32.6	32	
32	19:12	32.5	32	
32	19:13	32.5	32	
32	19:14	32.4	32	
32	19:15	32.4	32	
32	19:16	32.4	32	
32	19:17	32.3	32	
32	19:18	32.3	32	
32	19:19	32.3	32	
32	19:20	32.3	32	
32	19:21	32.3	32	
32	19:22	32.3	32	
32	19:23	32.3	32	
32	19:24	32.2	32	
32	19:25	32.2	32	
32	19:26	32.2	32	
32	19:27	32.2	32	
32	19:28	32.2	32	
32	19:29	32.2	32	
32	19:30	32.2	32	
32	19:31	32.1	32	START OF STABILITY @ 32°C
32	19:32	32.1	32	
32	19:33	32.1	32	
32	19:34	32.1	32	
32	19:35	32.1	32	

19:36	32.1	32	20:11	32	32
19:37	32.1	32	20:12	32	32
19:38	32.1	32	20:13	32	32
19:39	32.1	32	20:14	32	32
19:40	32.1	32	20:15	32	32
19:41	32.1	32	20:16	32	32
19:42	32.1	32	20:17	32	32
19:43	32.1	32	20:18	32	32
19:44	32.1	32	20:19	32	32
19:45	32.1	32	20:20	32	32
19:46	32.1	32	20:21	32	32
19:47	32.1	32	20:22	32	32
19:48	32.1	32	20:23	32	32
19:49	32.1	32	20:24	32	32
19:50	32	32	20:25	32	32
19:51	32	32	20:26	32	32
19:52	32	32	20:27	32	32
19:53	32	32	20:28	32	32
19:54	32	32	20:29	32	32
19:55	32	32	20:30	32	32
19:56	32	32	20:31	32	32
19:57	32	32	20:32	32	32
19:58	32	32	20:33	32	32
19:59	32	32	20:34	32	32
20:00	32	32	20:35	32	32
20:01	32	32	20:36	32	32
20:02	32	32	20:37	32	32
20:03	32	32	20:38	32	32
20:04	32	32	20:39	32	32
20:05	32	32	20:40	32	32
20:06	32	32	20:41	32	32
20:07	32	32	20:42	32	32
20:08	32	32	20:43	32	32
20:09	32	32	20:44	32	32
20:10	32	32	20:45	32	32

20:46	32	32
20:47	32	32
20:48	32	32
20:49	32	32
20:50	32	32
20:51	32	32
20:52	32	32
20:53	32	32
20:54	32	32
20:55	32	32
20:56	32	32
20:57	32	32
20:58	32	32
20:59	31.9	32
21:00	32	32
21:01	32	32
21:02	32	32
21:03	32	32
21:04	32	32
21:05	32	32
21:06	32	32
21:07	32	32
21:08	32	32
21:09	32	32
21:10	32	32
21:11	32	32
21:12	32	32
21:13	32	32
21:14	32	32
21:15	32	32
21:16	32	32
21:17	32	32
21:18	32	32
21:19	31.9	32
21:20	31.9	32

21:21	31.9	32	21:56	32	32
21:22	31.9	32	21:57	32	32
21:23	31.9	32	21:58	32	32
21:24	31.9	32	21:59	32	32
21:25	31.9	32	22:00	32	32
21:26	32	32	22:01	31.9	32
21:27	32	32	22:02	32	32
21:28	32	32	22:03	31.9	32
21:29	32	32	22:04	31.9	32
21:30	32	32	22:05	31.9	32
21:31	32	32	22:06	31.9	32
21:32	32	32	22:07	31.9	32
21:33	32	32	22:08	31.9	32
21:34	32	32	22:09	32	32
21:35	32	32	22:10	32	32
21:36	32	32	22:11	32	32
21:37	32	32	22:12	32	32
21:38	32	32	22:13	32	32
21:39	32	32	22:14	32	32
21:40	32	32	22:15	32	32
21:41	32	32	22:16	32	32
21:42	32	32	22:17	32	32
21:43	32	32	22:18	32	32
21:44	31.9	32	22:19	32	32
21:45	31.9	32	22:20	32	32
21:46	31.9	32	22:21	32	32
21:47	32	32	22:22	32	32
21:48	32	32	22:23	32	32
21:49	32	32	22:24	31.9	32
21:50	32	32	22:25	31.9	32
21:51	32	32	22:26	31.9	32
21:52	32	32	22:27	31.9	32
21:53	32	32	22:28	31.9	32
21:54	32	32	22:29	31.9	32
21:55	32	32	22:30	32	32

22:31	31.9	32
22:32	31.9	32
22:33	32	32
22:34	32	32
22:35	32	32
22:36	32	32
22:37	32	32
22:38	32	32
22:39	32	32
22:40	32	32
22:41	32	32
22:42	32	32
22:43	32	32
22:44	32	32
22:45	32	32
22:46	32	32
22:47	32	32
22:48	32	32
22:49	32	32
22:50	31.9	32
22:51	31.9	32
22:52	31.9	32
22:53	32	32
22:54	31.9	32
22:55	32	32
22:56	32	32
22:57	32	32
22:58	32	32
22:59	32	32
23:00	32	32
23:01	32	32
23:02	32	32
23:03	32	32
23:04	32	32
23:05	32	32

23:06	32	32	23:41	31.7	31
23:07	32	32	23:42	31.7	31
23:08	32	32	23:43	31.7	31
23:09	31.9	32	23:44	31.6	31
23:10	31.9	32	23:45	31.6	31
23:11	31.9	32	23:46	31.6	31
23:12	31.9	32	23:47	31.5	31
23:13	32	32	23:48	31.5	31
23:14	31.9	32	23:49	31.4	31
23:15	31.9	32	23:50	31.4	31
23:16	32	32	23:51	31.4	31
23:17	32	32	23:52	31.3	31
23:18	32	32	23:53	31.3	31
23:19	32	32	23:54	31.3	31
23:20	32	32	23:55	31.2	31
23:21	32	32	23:56	31.2	31
23:22	32	32	23:57	31.2	31
23:23	32	32	23:58	31.1	31
23:24	32	32	23:59	31.1	31
23:25	32	32	24:00	31.1	31
23:26	32	32	24:01	31.1	31
23:27	32	32	24:02	31.1	31
23:28	32	32	24:03	31	31
23:29	31.9	32	24:04	31	31
23:30	32	32	24:05	31	31
23:31	31.9	31	24:06	31	31
23:32	31.9	31	24:07	31	31
23:33	31.9	31	24:08	31	31
23:34	31.9	31	24:09	31	31
23:35	31.9	31	24:10	31	31
23:36	31.9	31	24:11	31	31
23:37	31.8	31	24:12	31	31
23:38	31.8	31	24:13	30.9	31
23:39	31.8	31	24:14	30.9	31
23:40	31.7	31	24:15	30.9	31

START OF STABILITY @ 31°C

END OF STABILITY @ 32°C

24:16	30.9	31
24:17	30.9	31
24:18	30.9	31
24:19	30.9	31
24:20	30.9	31
24:21	30.9	31
24:22	30.9	31
24:23	30.9	31
24:24	30.9	31
24:25	30.9	31
24:26	30.9	31
24:27	31	31
24:28	30.9	31
24:29	30.9	31
24:30	30.9	31
24:31	30.9	31
24:32	30.9	31
24:33	30.9	31
24:34	30.9	31
24:35	30.9	31
24:36	30.9	31
24:37	30.9	31
24:38	30.9	31
24:39	30.9	31
24:40	30.9	31
24:41	30.9	31
24:42	30.9	31
24:43	30.9	31
24:44	30.9	31
24:45	30.9	31
24:46	30.9	31
24:47	30.9	31
24:48	30.9	31
24:49	30.9	31
24:50	30.9	31

24:51	30.9	31	25:26	30.9
24:52	30.9	31	25:27	30.9
24:53	30.9	31	25:28	30.9
24:54	30.9	31	25:29	30.9
24:55	30.9	31	25:30	30.9
24:56	30.9	31	25:31	30.9
24:57	30.9	31	25:32	30.9
24:58	30.9	31	25:33	30.9
24:59	30.9	31	25:34	30.9
25:00	30.9	31	25:35	30.9
25:01	30.9	31	25:36	30.9
25:02	30.9	31	25:37	30.9
25:03	30.9	31	25:38	30.9
25:04	30.9	31	25:39	30.9
25:05	30.9	31	25:40	30.9
25:06:00	30.9	31	25:41	30.9
25:07	30.9	31	25:42	30.9
25:08	30.9	31	25:43	30.9
25:09	30.9	31	25:44	30.9
25:10	30.9	31	25:45	30.9
25:11	30.9	31	25:46	30.9
25:12	31	31	25:47	30.9
25:13	30.9	31	25:48	30.9
25:14	30.9	31	25:49	30.9
25:15	30.9	31	25:50	30.9
25:16	30.9	31	25:51	30.9
25:17	30.9	31	25:52	30.9
25:18	30.9	31	25:53	30.9
25:19	30.9	31	25:54	30.9
25:20	30.9	31	25:55	30.9
25:21	30.9	31	25:56	30.9
25:22	30.9	31	25:57	30.9
25:23	30.9	31	25:58	30.9
25:24	30.9	31	25:59	30.9
25:25	30.9	31	26:00	30.9

31	26:01	30.9	31
31	26:02	30.9	31
31	26:03	30.9	31
31	26:04	30.9	31
31	26:05	31	31
31	26:06	30.9	31
31	26:07	30.9	31
31	26:08	30.9	31
31	26:09	30.9	31
31	26:10	31	31
31	26:11	31	31
31	26:12	31	31
31	26:13	31	31
31	26:14	31	31
31	26:15	31	31
31	26:16	31	31
31	26:17	31	31
31	26:18	30.9	31
31	26:19	30.9	31
31	26:20	30.9	31
31	26:21	30.9	31
31	26:22	30.9	31
31	26:23	30.9	31
31	26:24	30.9	31
31	26:25	30.9	31
31	26:26	30.9	31
31	26:27	30.9	31
31	26:28	30.9	31
31	26:29	30.9	31
31	26:30	30.9	31
31	26:31	30.9	37
31	26:32	30.9	37
31	26:33	31	37
31	26:34	31.1	37
31	26:35	31.3	37

END OF STABILITY @ 31°C

26:36	31.5	37	27:11	37.3	37
26:37	31.8	37	27:12	37.3	37
26:38	32.1	37	27:13	37.4	37
26:39	32.4	37	27:14	37.4	37
26:40	32.7	37	27:15	37.4	37
26:41	33	37	27:16	37.4	37
26:42	33.3	37	27:17	37.4	37
26:43	33.6	37	27:18	37.4	37
26:44	33.9	37	27:19	37.4	37
26:45	34.2	37	27:20	37.4	37
26:46	34.5	37	27:21	37.4	37
26:47	34.7	37	27:22	37.4	37
26:48	35	37	27:23	37.4	37
26:49	35.2	37	27:24	37.4	37
26:50	35.4	37	27:25	37.4	37
26:51	35.6	37	27:26	37.4	37
26:52	35.8	37	27:27	37.4	37
26:53	35.9	37	27:28	37.4	37
26:54	36.1	37	27:29	37.4	37
26:55	36.2	37	27:30	37.4	37
26:56	36 4	37	27:31	37.4	37
26:57	36.5	37	27:32	37.4	37
26:58	36.6	37	27:33	37.4	37
26:59	36.7	37	27:34	37.4	37
27:00	36.8	37	27:35	37.4	37
27:01	36.8	37	27:36	37.3	37
27:02	36.9	37	27:37	37.3	37
27:03	37	37	27:38	37.3	37
27:04	37	37	27:39	37.3	37
27:05	37.1	37	27:40	37.3	37
27:06	37.1	37	27:41	37.3	37
27:07	37.2	37	27:42	37.3	37
27:08	37.2	37	27:43	37.3	37
27:09	37.2	37	27:44	37.3	37
27:10	37.3	37	27:45	37.3	37

Stable at stability @ 37°C

27:46	37.2	37
27:47	37.2	37
27:48	37.2	37
27:49	37.2	37
27:50	37.2	37
27:51	37.2	37
27:52	37.2	37
27:53	37.2	37
27:54	37.2	37
27:55	37.2	37
27:56	37.2	37
27:57	37.2	37
27:58	37.2	37
27:59	37.2	37
28:00	37.2	37
28:01	37.1	37
28:02	37.1	37
28:03	37.1	37
28:04	37.1	37
28:05	37.1	37
28:06	37.1	37
28:07	37.1	37
28:08	37.1	37
28:09	37.1	37
28:10	37.1	37
28:11	37.1	37
28:12	37.1	37
28:13	37.1	37
28:14	37.1	37
28:15	37.1	37
28:16	37.1	37
28:17	37.1	37
28:18	37.1	37
28:19	37.1	37
28:20	37.1	37

28:21	37.1	37	28:56	37	37
28:22	37.1	37	28:57	37	37
28:23	37.1	37	28:58	37	37
28:24	37.1	37	28:59	37	37
28:25	37.1	37	29:00	37	37
28:26	37.1	37	29:01	37	37
28:27	37.1	37	29:02	37	37
28:28	37.1	37	29:03	37	37
28:29	37.1	37	29:04	37	37
28:30	37.1	37	29:05	37	37
28:31	37	37	29:06	37	37
28:32	37	37	29:07	37	37
28:33	37	37	29:08	37	37
28:34	37	37	29:09	37	37
28:35	37	37	29:10	37	37
28:36	37	37	29:11	37	37
28:37	37	37	29:12	37	37
28:38	37	37	29:13	37	37
28:39	37	37	29:14	37	37
28:40	37	37	29:15	37	37
28:41	37	37	29:16	37	37
28:42	37	37	29:17	36.9	37
28:43	37	37	29:18	36.9	37
28:44	37	37	29:19	36.9	37
28:45	37	37	29:20	37	37
28:46	37	37	29:21	37	37
28:47	37	37	29:22	37	37
28:48	37	37	29:23	37	37
28:49	37	37	29:24	37	37
28:50	37	37	29:25	37	37
28:51	37	37	29:26	37	37
28:52	37	37	29:27	37	37
28:53	37	37	29:28	37	37
28:54	37	37	29:29	37	37
28:55	37	37	29:30	37	37

29:31	37	37
29:32	37	37
29:33	37	37
29:34	37	37
29:35	37	37
29:36	37	37
29:37	36.9	37
29:38	37	37
29:39	36.9	37
29:40	36.9	37
29:41	37	37
29:42	37	37
29:43	37	37
29:44	37	37
29:45	37	37
29:46	37	37
29:47	37	37
29:48	37	37
29:49	37	37
29:50	37	37
29:51	37	37
29:52	36.9	37
29:53	37	37
29:54	37	37
29:55	37	37
29:56	36.9	37
29:57	36.9	37
29:58	36.9	37
29:59	36.9	37
30:00	37	37
30:01	37	37
30:02	36.9	37
30:03	37	37
30:04	37	37
30:05	37	37

30:06	37	37	30:41	37	37
30:07	37	37	30:42	36.9	37
30:08	36.9	37	30:43	37	37
30:09	37	37	30:44	37	37
30:10	37	37	30:45	37	37
30:11	37	37	30:46	37	37
30:12	36.9	37	30:47	37	37
30:13	36.9	37	30:48	36.9	37
30:14	36.9	37	30:49	37	37
30:15	36.9	37	30:50	36.9	37
30:16	36.9	37	30:51	37	37
30:17	37	37	30:52	36.9	37
30:18	36.9	37	30:53	36.9	37
30:19	36.9	37	30:54	37	37
30:20	37	37	30:55	36.9	37
30:21	37	37	30:56	36.9	37
30:22	37	37	30:57	37	37
30:23	37	37	30:58	36.9	37
30:24	37	37	30:59	36.9	37
30:25	37	37	31:00	37	37
30:26	37	37	31:01	37	37
30:27	37	37	31:02	37	37
30:28	37	37	31:03	37	37
30:29	37	37	31:04	37	37
30:30	36.9	37	31:05	37	37
30:31	37	37	31:06	37	37
30:32	37	37	31:07	37	37
30:33	36.9	37	31:08	37	37
30:34	36.9	37	31:09	36.9	37
30:35	36.9	37	31:10	36.9	37
30:36	36.9	37	31:11	36.9	37
30:37	36.9	37	31:12	36.9	37
30:38	37	37	31:13	36.9	37
30:39	37	37	31:14	37	37
30:40	36.9	37	31:15	36.9	37

31:16	37	37
31:17	37	37
31:18	37	37
31:19	36.9	37
31:20	37	37
31:21	37	37
31:22	37	37
31:23	36.9	37
31:24	36.9	37
31:25	37	37
31:26	36.9	37
31:27	36.9	37
31:28	36.9	37
31:29	36.9	37
31:30	37	37
31:31	36.9	37
31:32	37	37
31:33	36.9	37
31:34	37	37
31:35	37	37
31:36	37	37
31:37	37	37
31:38	37	37
31:39	37	37
31:40	37	37
31:41	37	37
31:42	36.9	37
31:43	36.9	37
31:44	37	37
31:45	36.9	37
31:46	37	37
31:47	36.9	37
31:48	36.9	37
31:49	37	37
31:50	37	37

31:51	37	37	32:26	36.9	37
31:52	37	37	32:27	36.9	37
31:53	37	37	32:28	37	37
31:54	37	37	32:29	37	37
31:55	37	37	32:30	37	37
31:56	37	37	32:31	37	37
31:57	37	37	32:32	37	37
31:58	37	37	32:33	37	37
31:59	36.9	37	32:34	37	37
32:00	36.9	37	32:35	36.9	37
32:01	36.9	37	32:36	36.9	37
32:02	37	37	32:37	36.9	37
32:03	37	37	32:38	36.9	37
32:04	37	37	32:39	36.9	37
32:05	36.9	37	32:40	36.9	37
32:06	36.9	37	32:41	36.9	37
32:07	37	37	32:42	36.9	37
32:08	37	37	32:43	37	37
32:09	37	37	32:44	36.9	37
32:10	37	37	32:45	36.9	37
32:11	37	37	32:46	37	37
32:12	37	37	32:47	37	37
32:13	37	37	32:48	37	37
32:14	37	37	32:49	37	37
32:15	37	37	32:50	37	37
32:16	37	37	32:51	37	37
32:17	37	37	32:52	37	37
32:18	36.9	37	32:53	36.9	37
32:19	37	37	32:54	36.9	37
32:20	36.9	37	32:55	36.9	37
32:21	36.9	37	32:56	36.9	37
32:22	36.9	37	32:57	36.9	37
32:23	36.9	37	32:58	36.9	37
32:24	36.9	37	32:59	36.9	37
32:25	36.9	37	33:00	36.9	37

33:01	36.9	37
33:02	36.9	37
33:03	37	37
33:04	36.9	37
33:05	37	37
33:06	37	37
33:07	36.9	37
33:08	37	37
33:09	36.9	37
33:10	37	37
33:11	37	37
33:12	36.9	37
33:13	36.9	37
33:14	36.9	37
33:15	36.9	37
33:16	36.9	37
33:17	36.9	37
33:18	36.9	37
33:19	36.9	37
33:20	36.9	37
33:21	36.9	37
33:22	36.9	37
33:23	36.9	37
33:24	36.9	37
33:25	36.9	37
33:26	36.9	37
33:27	36.9	37
33:28	37	37
33:29	37	37
33:30	36.9	37
33:31	36.9	37
33:32	36.9	37
33:33	36.9	37
33:34	36.9	37
33:35	36.9	37

33:36	36.9	37	34:11	36.9	37
33:37	36.9	37	34:12	36.9	37
33:38	36.9	37	34:13	37	37
33:39	36.9	37	34:14	36.9	37
33:40	36.9	37	34:15	37	37
33:41	36.9	37	34:16	37	37
33:42	36.9	37	34:17	37	37
33:43	36.9	37	34:18	37	37
33:44	37	37	34:19	37	37
33:45	37	37	34:20	37	37
33:46	36.9	37	34:21	37	37
33:47	36.9	37	34:22	37	37
33:48	36.9	37	34:23	37	37
33:49	37	37	34:24	37	37
33:50	37	37	34:25	37	37
33:51	37	37	34:26	37	37
33:52	36.9	37	34:27	37	37
33:53	36.9	37	34:28	37	37
33:54	36.9	37	34:29	37	37
33:55	36.9	37	34:30	37	37
33:56	36.9	37	34:31	37	37
33:57	36.9	37	34:32	37	37
33:58	37	37	34:33	37	37
33:59	37	37	34:34	37	37
34:00	36.9	37	34:35	37	37
34:01	36.9	37	34:36	37	37
34:02	36.9	37	34:37	37	37
34:03	37	37	34:38	37	37
34:04	36.9	37	34:39	37	37
34:05	37	37	34:40	37	37
34:06	37	37	34:41	37	37
34:07	37	37	34:42	37	37
34:08	37	37	34:43	37	37
34:09	37	37	34:44	36.9	37
34:10	36.9	37	34:45	36.9	37

34:46	37	37
34:47	37	37
34:48	36.9	37
34:49	36.9	37
34:50	37	37
34:51	37	37
34:52	37	37
34:53	37	37
34:54	37	37
34:55	37	37
34:56	37	37
34:57	36.9	37
34:58	36.9	37
34:59	36.9	37
35:00	36.9	37
35:01	36.9	37
35:02	36.9	37
35:03	36.9	37
35:04	36.9	37
35:05	36.9	37
35:06	37	37
35:07	36.9	37
35:08	36.9	37
35:09	36.9	37
35:10	36.9	37
35:11	37	37
35:12	37	37
35:13	37	37
35:14	37	37
35:15	36.9	37
35:16	37	37
35:17	36.9	37
35:18	36.9	37
35:19	37	37
35:20	37	37

35:21	36.9	37	35:56	37	37
35:22	36.9	37	35:57	36.9	37
35:23	36.9	37	35:58	36.9	37
35:24	36.9	37	35:59	36.9	37
35:25	37	37	36:00	36.9	37
35:26	37	37	36:01	36.9	37
35:27	36.9	37	36:02	36.9	37
35:28	36.9	37	36:03	36.9	37
35:29	36.9	37	36:04	36.9	37
35:30	36.9	37	36:05	36.9	37
35:31	36.9	37	36:06	36.9	37
35:32	36.9	37	36:07	37	37
35:33	36.9	37	36:08	37	37
35:34	36.9	37	36:09	37	37
35:35	36.9	37	36:10	37	37
35:36	36.9	37	36:11	37	37
35:37	36.9	37	36:12	36.9	37
35:38	36.9	37	36:13	37	37
35:39	36.9	37	36:14	36.9	37
35:40	36.9	37	36:15	37	37
35:41	36.9	37	36:16	36.9	37
35:42	36.9	37	36:17	36.9	37
35:43	36.9	37	36:18	36.9	37
35:44	36.9	37	36:19	36.9	37
35:45	36.9	37	36:20	36.9	37
35:46	36.9	37	36:21	36.9	37
35:47	36.9	37	36:22	36.9	37
35:48	36.9	37	36:23	36.9	37
35:49	36.9	37	36:24	36.9	37
35:50	36.9	37	36:25	36.9	37
35:51	36.9	37	36:26	36.9	37
35:52	36.9	37	36:27	37	37
35:53	36.9	37	36:28	37	37
35:54	36.9	37	36:29	37	37
35:55	36.9	37	36:30	37	37

36:31	37	37
36:32	37	37
36:33	37	37
36:34	37	37
36:35	37	37
36:36	37	37
36:37	36.9	37
36:38	37	37
36:39	36.9	37
36:40	36.9	37
36:41	36.9	37
36:42	36.9	37
36:43	36.9	37
36:44	36.9	37
36:45	36.9	37
36:46	36.9	37
36:47	36.9	37
36:48	36.9	37
36:49	37	37
36:50	37	37
36:51	37	37
36:52	37	37
36:53	37	37
36:54	37	37
36:55	37	37
36:56	36.9	37
36:57	37	37
36:58	37	37
36:59	36.9	37
37:00	36.9	37
37:01	36.9	37
37:02	36.9	37
37:03	36.9	37
37:04	36.9	37
37:05	36.9	37

37:06	36.9	37	37:41	36.9	37	38:16	36.9	37
37:07	36.9	37	37:42	36.9	37	38:17	36.9	37
37:08	37	37	37:43	36.9	37	38:18	36.9	37
37:09	36.9	37	37:44	36.9	37	38:19	36.9	37
37:10	37	37	37:45	36.9	37	38:20	36.9	37
37:11	37	37	37:46	36.9	37	38:21	36.9	37
37:12	37	37	37:47	37	37	38:22	36.9	37
37:13	37	37	37:48	37	37	38:23	36.9	37
37:14	37	37	37:49	37	37	38:24	36.9	37
37:15	37	37	37:50	36.9	37	38:25	36.9	37
37:16	37	37	37:51	37	37	38:26	36.9	37
37:17	36.9	37	37:52	37	37	38:27	36.9	37
37:18	36.9	37	37:53	36.9	37	38:28	37	37
37:19	36.9	37	37:54	36.9	37	38:29	36.9	37
37:20	36.9	37	37:55	37	37	38:30	37	37
37:21	36.9	37	37:56	36.9	37	38:31	36.9	37
37:22	36.9	37	37:57	36.9	37	38:32	37	37
37:23	36.9	37	37:58	36.9	37	38:33	37	37
37:24	36.9	37	37:59	36.9	37	38:34	37	37
37:25	36.9	37	38:00	36.9	37	38:35	37	37
37:26	36.9	37	38:01	36.9	37	38:36	37	37
37:27	37	37	38:02	36.9	37	38:37	37	37
37:28	36.9	37	38:03	36.9	37	38:38	36.9	37
37:29	36.9	37	38:04	36.9	37	38:39	36.9	37
37:30	37	37	38:05	36.9	37	38:40	36.9	37
37:31	36.9	37	38:06	36.9	37	38:41	36.9	37
37:32	36.9	37	38:07	36.9	37	38:42	36.9	37
37:33	37	37	38:08	36.9	37	38:43	36.9	37
37:34	36.9	37	38:09	36.9	37	38:44	36.9	37
37:35	36.9	37	38:10	37	37	38:45	36.9	37
37:36	37	37	38:11	37	37	38:46	36.9	37
37:37	37	37	38:12	37	37	38:47	36.9	37
37:38	36.9	37	38:13	37	37	38:48	36.9	37
37:39	36.9	37	38:14	37	37	38:49	36.9	37
37:40	37	37	38:15	36.9	37	38:50	36.9	37

38:51	37	37	39:26	36.9	37
38:52	37	37	39:27	36.9	37
38:53	37	37	39:28	36.9	37
38:54	37	37	39:29	36.9	37
38:55	37	37	39:30	36.9	37
38:56	37	37	39:31	36.9	37
38:57	37	37	39:32	36.9	37
38:58	37	37	39:33	36.9	37
38:59	36.9	37	39:34	36.9	37
39:00	37	37	39:35	36.9	37
39:01	36.9	37	39:36	36.9	37
39:02	36.9	37	39:37	36.9	37
39:03	36.9	37	39:38	36.9	37
39:04	36.9	37	39:39	36.9	37
39:05	36.9	37	39:40	36.9	37
39:06	36.9	37	39:41	36.9	37
39:07	36.9	37	39:42	37	37
39:08	36.9	37	39:43	36.9	37
39:09	36.9	37	39:44	36.9	37
39:10	36.9	37	39:45	36.9	37
39:11	36.9	37	39:46	36.9	37
39:12	36.9	37	39:47	36.9	37
39:13	37	37	39:48	36.9	37
39:14	37	37	39:49	36.9	37
39:15	37	37	39:50	36.9	37
39:16	36.9	37	39:51	36.9	37
39:17	37	37	39:52	36.9	37
39:18	37	37	39:53	36.9	37
39:19	37	37	39:54	36.9	37
39:20	36.9	37	39:55	36.9	37
39:21	36.9	37	39:56	36.9	37
39:22	36.9	37	39:57	36.9	37
39:23	36.9	37	39:58	36.9	37
39:24	36.9	37	39:59	36.9	37
39:25	36.9	37	40:00	37	37

5.3 pH control:

PID settings:

PH CONTROL	
P	100
I	0
D	0
Bias	0

5.3.1 Test Mode:

- ❑ Start the pH control on the ADI1060.
- ❑ Connect a pH simulator (setpoint 7.0 pH).
- ❑ Check the CO (control outputs), which are mentioned in table below and fill out the results.
- ❑ Check the correct function of the actuators.

Assume start pH > 10 Assume start pH < 4.

SET POINT CHANGE	CO/VALUE (CO %)	CHECK ACTUATOR PASS/FAIL	SET POINT CHANGE	CO/ PUMP BASE	CHECK ACTUATOR PASS/FAIL
10 CO4/10/02	100	Pass	4	100	Pass
9 CO4/10/02	100	Pass	5	100	Pass
8 CO4/10/02	100	Pass	6	100	Pass
7 CO4/10/02	100	Pass	9	100	Pass
10 CO 4 4-11-02	100	Pass	10	100	Pass

Comments: ON = 100 OFF = 0

Performed by: BBaley Date 4-11-02

Reviewed by: _____ Date _____

5.4 DO control

PID settings:

DO_2 CONTROL	
P	10
I	0
D	0
BIAS	0

5.4.1 Test Mode

- ❑ Start the DO controller in the ADI1060
- ❑ Connect a DO-simulator (setpoint 25%)
- ❑ Check the CO (control output and fill out the results in the table below).
- ❑ Check the right function of the actuator.

SIMULATOR	AIR VALVE (CO)% CR 4/11/02	CHECK ACTUATOR PASS/FAIL	STIRRER SPEED (CO%)	CHECK ACTUATOR PASS/FAIL
0	100	Pass	100rpm	Pass
50	0	Pass	100rpm	Pass

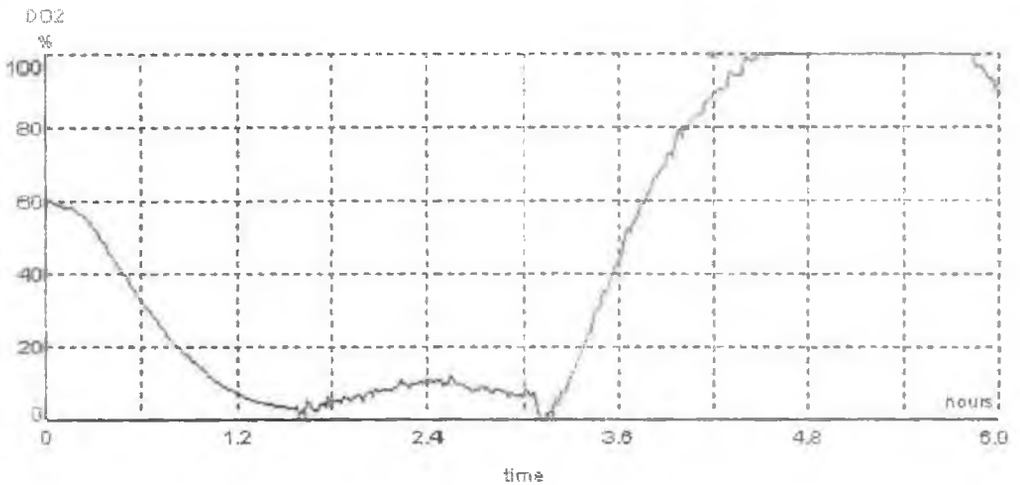
SIMULATOR	N_2 VALVE (CO%)	CHECK ACTUATOR PASS/FAIL
0	0	Pass
50	100	Pass

Comments: OP = 100 OFF = 0

CR 4/11/02

CS
4/11/02.

TRACE OF DO_2 CALIBRATION.



Performed by: _____ Date 4-11-02.

Reviewed by: _____ Date _____

5.5 Level Control:

5.5.1 Test Mode

- ❑ Setpoint level: contact
- ❑ Move the level detector on the vessel in and out the medium
- ❑ Check if the level card is reaching from contact to no contact showing on the ADI1060 screen and fill out the results in the table below.

LEVEL	READING SCREEN ADI1060	PASS/FAIL
Contact	Contact	Pass
No Contact	No Contact	Pass

Comments:

4/11/02

Performed by: Baley Date 4-11-02

Reviewed by: _____ Date _____

6.0 Discrepency Summary

THE ALARM TEST WAS NOT PERFORMED ON TEMPERATURE IN SECTION 5.2.

STABILITY OF TEMPERATURE WITHIN THE LIMITS +/- 0.2°C WITHIN 1 HOUR OF SETTING THE TEMPERATURE WERE NOT REACHED.

SOP FOR THE OPERATION OF THE 10L BIOREACTOR + CONTROL SYSTEM HAVE NOT BEEN COMPLETED.

GMP TRAINING HAS NOT BEEN PROVIDED TO RELEVANT MAINTENANCE + OPERATIONAL PERSONNEL.

THERE IS NO APPROVED MAINTENANCE PROGRAM IN PLACE FOR THE OPERATION OF THE SYSTEM.

Performed by: _____ Date 2/11/02

Reviewed by: _____ Date _____

7.0

Conclusion

THE DEVIATIONS ENCOUNTERED IN THIS OPERATIONAL QUALIFICATION MEANS THAT A NUMBER OF TESTS ARE REQUIRED TO BE REPEATED BEFORE FINAL SIGNING OFF CAN BE COMPLETED.

Performed by: _____ Date 6/11/02

Reviewed by: _____ Date _____