DEVELOPMENT OF REDUCED SERUM-FREE MEDIA FOR MRC-5 AND VERO CELLS USING DEFINITIVE SCREENING DESIGN

By

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Abstract

The purpose of this study was to rationally design animal component free, chemically defined serum free media (ACF-CD-SFM) for MRC-5 and Vero cells while adhering to the Quality by Design guidelines. This was achieved by using the Modified Vero Serum Free Medium (MVSFM) as the basal formulation and supplementing it with various combinations of growth factors (LONG® EGF, LONG® R^3 IGF-I, rTransferrin, bFGF, TGF-β3 and PDGF-AA), lipids (linoleic acid, cholesterol, and dexamethasone), lipid precursors (ethanolamine and phosphoethanolamine) and vitamins (all-trans retinoic acid, α-tocopherol and ascorbic acid). Media development was achieved by conducting a series of steps using different experimental methodologies with the end goal of satisfying the requirements of each cell line. MRC-5 and Vero cells were each cultured in specific media containing unique concentrations of supplements that were prepared according to the different statistical design methodologies.

The original objective was to create a SFM, however due to the stringent nutritious requirements of anchorage dependent cell lines, only a reduction to 0.5% FBS was achieved. For MRC-5 cells, the one-factor-at-a-time (OFAT) generated the Prototype + 0.5% FBS medium. The Definitive Screening Design (DSD) gave rise to the Delta 1 + 0.5% FBS, which was the optimum medium formulation for MRC-5 cells as it had comparable cell yields to DMEM + 10 % FBS. This result was confirmed by the Genetic Algorithms-Hill Climbing (GA-HC) method. In the case of Vero cells, the OFAT and the DSD confirmed that MVSFM + 0.5 % FBS was the most optimal formulation. The morphology in both media for both cell lines was comparable to that in DMEM-10% FBS. It was concluded that the DSD method successfully achieved a reduction of the serum concentration from 10% to 0.5% FBS.

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Dedication

I dedicate this thesis to all those curious minds who are trying to unravel the mysteries of nature and the universe through science.

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“I don’t study to know more but to ignore less”

- Sor Juana Inés de la Cruz

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List of Abbreviations

3T3	3-day transfer 3 x 10^5 cells
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
ACF	Animal component free
BALB	Bagg albino
bFGF	Basic fibroblast growth factor
CCL	Continuous cell lines
CD	Chemically defined
cGMP	Current good manufacturing practices
CHO	Chinese hamster ovary
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DoE	Design-of-experiments
DPBS	Dulbecco's phosphate-buffered saline
DSD	Definitive Screening Design
EGF	Epidermal growth factor
EMA	European Medicines Association
FST	Freestyle medium
FDA	Food and Drug Administration
FBS	Fetal Bovine Serum
GA	Genetic Algorithms
GPI	Glycosylphosphatidylinositol
GPEI	Global Polio Eradication Initiative
HC	Hill Climbing
HEK 293	Human Embryonic Kidney 293
HeLa	Henrietta Lacks
IGF- I	Insulin-like growth factor- I
IGFBPs	Insulin-like growth factor binding proteins
IMR-90	Institute of Medical Research- 90
ITS	Insulin Transferrin Selenium

L-EGF	LONG epidermal growth factor
L-R^3- IGF-I	LONG R^3 insulin growth factor- I
MβCD	Methyl-β-cyclodextrin
MCF-7	Michigan Cancer Foundation-7
MDCK	Madin Darby canine kidney
MEM	Minimal essential medium
M/P	MVSFM+5g/L Primatone
MRC-5	Medical Research Council- 5
MVSFM	Modified Vero serum free medium
OFAT	One factor at a time
PAT	Process Analytical Technology
PDGF	Platelet derived growth factor
PV	Process Validation
QbD	Quality by design
rTransferrin	Recombinant transferrin
SEM	Standard error of the mean
SF	Serum free
S-EGF	Standard epidermal growth factor
SFM	Serum-free media
TGFβ-3	Transforming growth factor β-3
TNF-α	Tumor necrosis factor- α
WHO	World Health Organization
WI-38	Wistar Institute- 38
wt	Wild type

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Chapter 1

Literature review

1.1 Introductory remarks

Serum is a vital component for cell culture medium as it provides critical nutritious components for growth, attachment, and proliferation. It is widely used in conjunction with basal media formulations for *in vitro* cell culture in the biopharmaceutical industry for vaccines and therapeutic protein production (Schröder *et al.,* 2004; Rorou *et al.,* 2014). Serum-dependent cell culture is widely employed in all areas of scientific research including regenerative medicine, tissue engineering, stem cell therapy, physiology, pharmacology, and toxicology (Taub, 1990; van der Valk *et al*., 2010). Serum, however, poses a number of problems due to its animal origin because it may contain contaminants such as bovine viruses (e.g. parvovirus), mycoplasma, bacterial endotoxins, and/or prions. The chemical composition of serum is highly variable and complex (Butler, 2005), and its quality can differ from batch to batch.

The constant demand imposed by regulatory and health agencies for higher quality, safety, and consistency, has driven the industry to move away from serum. As a result, in recent years, many commercially available serum-free media (SFM) formulations have appeared as alternatives for some mammalian and insect cell lines (Brunner *et al.,* 2010), and especially for suspension cells of high industrial value such as CHO (Chinese Hamster Ovary) (Schröder *et al.,* 2004) and NSO cells (Mouse myeloma cells) (Gorfien *et al.,* 2000). A major challenge currently facing industry is to formulate consistent and robust media for anchorage-dependent cell lines used for vaccine production, such as MRC-5 (Medial Research Council-5) or Vero cells (Butler, 2013).

These cells have more stringent nutrition requirements such as growth or attachment factors that are not contained in common basal media formulations but are provided by serum. In order to improve these formulations, it is necessary to add the specific components required by each cell line for maintenance and proliferation at the optimum concentrations. Currently, there are few methods or standard protocols available to create or optimize SFM formulations that satisfy current Good Manufacturing Practices (cGMP).

The incorporation of new statistical methods into the field of industrial bioprocessing has spawned higher quality biopharmaceutical products with enhanced safety. Moreover, with the upsurge of more powerful computer algorithms, it has become possible to create faster and more reliable optimization methodologies that can be used for SFM formulation. To help the reader understand the process of SFM formulation for the anchorage-dependent cell lines MRC-5 and Vero cells, a discussion on the following will be presented: the history of media development, the different types of SFM available, the use of statistical design-of-experiments (DoE) as applied to SFM design and finally, the use of Definitive Screening Design (DSD) strategies that employ the use of curve-fitting and optimization algorithms.

1.2 Creation of a nutrient media for cell culture

1.2.1 Basal media formulations

The requirement for cell growth media came as a result of the development of *in vitro* cell culture systems at the end of the nineteenth century. Originally, it began with the use of isosmotic salt solutions. However, it was not possible to maintain viable cells for long periods of time (Eagle, 1955; Jayme *et al.* 1997; Ham, 1983). Thus, researchers resorted to the use of biological supplements such as: blood serum or plasma, tissue extracts, and other humoral fluids (Sato, 1983;

Taub, 1990; Bjare, 1992 and Jayme *et al.* 1997) that proved to be very effective for cell growth. The most popular supplement, serum, was originally used as a genuine medium to maintain hematopoietic cells in culture. It was soon realized that it provided excellent growth support for other cells (Fisher et al., 1958; Bjare, 1992). Despite the practicality of these fluids, there were major drawbacks of their use, such as high levels of variability and a lack of standardized growth medium within laboratories.

Thus, it became critical to create basal media formulations, which are defined as complex mixtures of amino acids, carbohydrates, salts, vitamins, hormones, and growth factors (Burgener and Butler, 2005). Some of the pioneers in this field were Morgan *et al.* (1950), who developed the M199 media, and Eagle who published a basal nutrient formulation that recognized the importance of amino acids for the *in vitro* culture of mouse fibroblast cells (Eagle, 1955). Their strategy to improve basal media formulations was to analyze the molecular components of serum. Eagle (1959) refined his formulation and created the minimal essential medium (MEM), which drastically reduced the serum concentration.

These advances led to the appearance of new media formulations created to satisfy the requirements of different cell lines as it became clear that nutritional requirements varied among cell type and origin. Examples of these formulations include: Ham's F10, Ham's F12 and the MCBD series of media (Jayme *et al.,* 1997; Freshney, 2010). Subsequent research focused on the analysis of spent fluids, conditioned media feeding, and trial and error supplementation. Some of these studies gave rise to formulations such as Dulbecco and Freeman's (1959) DMEM (Dulbecco's modified Minimal Essential Medium), a medium designed as an improvement over Eagle's MEM for use in viral production by increasing the amino acid concentrations by four times as designed in Eagle's formulation to achieve higher plaque formation.

In the 1950's foundations for systematic media development were established in parallel with the advances in the biochemistry field. During this period, attempts were geared towards analysing, purifying and isolating key components involved in the promotion of cell growth and proliferation (e.g. growth factors, vitamins) (Taub, 1990; Freshney, 2010). The knowledge obtained from these studies was used by Hayashi and Sato (1976), who aimed to replace serum by the addition of selected hormones that promoted growth and stimulated differentiation of specific cells (van der Valk *et al*., 2010) to create an adequate defined media. However, the results were not as satisfactory because some growth factors did not prove to be as effective as expected. This phenomenon was not well understood at the time (Taub, 1990), but it was possibly explained by positive and/or negative interactions between different growth factors and other components.

New approaches to SFM design emerged, which combined different media formulations to compensate for the lack of certain nutrients in basal media. The pioneer was Murakami who combined DMEM with Ham's F12 to create the DMEM/F12, and the RPMI media 1:1 with the DMEM/F12 mixture to create the RDF medium (Jayme *et al.,* 1997). Many basal media formulations were created with the common goal of maximizing cell proliferation and productivity *in vitro*, while largely reducing the amount of serum or other animal derived supplements. However, due to the complex growth requirements of eukaryotic cells, most of the current basal media formulations still require supplementation with 10% serum to achieve and maintain an appropriate cell yield.

1.2.2 Serum: Advantages and disadvantages

Serum, and specifically fetal bovine serum (FBS), is the most common supplement to basal media formulations for mammalian cell culture. Serum is the supernatant of clotted blood

commonly obtained from bovine or equine sources (Butler, 2004). This biological fluid provides the cells with critical metabolites, cytokines, attachment factors, protease inactivation as well as protection against toxins and shear stress. The complexity of serum derives from all the substances released into it by virtually every type of cell in the body and the modifications during the clotting process (Ham, 1981). The bulk of molecules found in serum are proteins. Although most of their precise roles have not been fully defined yet; it is known that proteins such as albumin, fetuin, and fibronectin act as carrier molecules for hormones, minerals, and fatty acids, and they play an important role in cell attachment and proliferation (Freshney, 2010). In brief, serum provides cells *in vitro* with all the necessary nutrients commonly supplied *in vivo* through the blood stream. In addition, blood not only carries nutrients, but also collects metabolic waste products throughout the body (Malda *et al.,* 2008). Serum provides cells *in vitro* with some protection from waste toxins and environmental stressors. This double function makes it an incredibly complete and efficient supplement for cell culturing.

Despite all the advantages of serum supplementation, it has become clear that it is not ideal for stem cell cultures, regenerative medicine, bioprocessing, and biomanufacturing purposes. Most efforts to reduce or eliminate serum supplementation from culture media have come up as a result of technical or economic concerns (Jayme and Smith, 2000). The biological complexity of serum, its composition, variability, interference with downstream and product purification, and its potential to cause allergic reactions are some of the few reasons to define the media composition chemically (Murakami, 1984; Bjare, 1992).

There are four major disadvantages of serum for mammalian cell culturing. The first disadvantage is batch-to-batch variability. The composition of serum is directly related to the age, health, diet, sex, environmental conditions, geographic place of origin, and species of the donor

animals (Murakami *et al.,* 1982; Jayme and Smith, 2000, Butler, 2015). As a result, the quality of the serum can critically affect the growth and productivity of the cells. Therefore, it is necessary to carefully select and test each batch to achieve adequate cell growth and viability.

The second disadvantage is that serum may be a source of potential adventitious contaminants such as prions, mycoplasma, bacterial toxins, and/or viruses (Bader *et al*., 1998). The highest risk is posed by the transmission of the bovine spongiform encephalopathy (BSE) prion through vaccines and other biologicals. This has prompted the disuse of serum at any stage of production (Asher, 1999). The same issue is also associated with other media constituents from animal origin. Thus, the rapid advancement in cell and tissue engineering as well as the rise in clinical trials for human therapeutics is driving the industry to eliminate all constituents of animal origin from cell culture (Jayme and Smith, 2000). In addition, the production of vaccines is tightly controlled by international regulations for good manufacturing practices (GMP-regulations), which limit the amount of serum constituents in the final product when manufacturing injectable vaccines, since allergic reactions could be provoked (Bjare, 1992).

The third problem is the cost and availability of serum. These are closely related to each other. The price of serum can range from $500 to $1,000 per liter and can account for a significant portion of the cost of the media (Burgener and Butler, 2005). Serum shortages can occur due to the variability of the meat and dairy market, as well as adverse environmental conditions that may affect the cattle and its health. Such circumstances can increase the price of serum, which in-turn increases the cost of media production in the pharmaceutical industry. The fourth and last disadvantage is negative publicity raised by animal rights activists, who are concerned about animal treatment when obtaining serum (Gstraunthaler, 2003). As a result, a strong shift away

from serum-supplemented media has occurred that has led to the development of SFM formulations.

1.3 Serum-free media

As described above, SFM have become increasingly important due to the higher demand in safety and quality standards in the pharmaceutical industry established by regulatory and health safety agencies. Most SFM are formulated from one or a combination of basal media plus the addition of supplementary nutrients. As part of the serum replacement process, alternative components must be added to the medium to provide the cells with the necessary growth requirements. The initial approach was to supplement with proteins such as insulin, transferrin, bovine albumin (Clark *et al.,* 1981), fetuin, and fibronectin (Siemensma *et al.,* 2010). Despite defining the medium composition, these substances are animal derived and inherently carry the same disadvantages that serum does. This is undesirable for large scale bioprocessing applications as well as for biopharmaceutical production.

1.3.1 Protein hydrolysates

Proteins are crucial for the growth and maintenance of mammalian cells. Therefore, hydrolysates have become a viable alternative for protein supplementation. Protein hydrolysates are peptide fragments, created from hydrolysis of source proteins from plants, yeast, or animal tissue. They can be generated by exogenous enzymatic proteolysis with pepsin or papain; by hydrolysis using strong acids; or by cell autolysis performing temperature or osmotic changes (Lobo-Alfonso *et al.* 2010). Their use was transferred from microbiology where they were

originally used for the culture of microorganisms. Protein hydrolysates have been used for several purposes in the biotechnology industry throughout time as depicted in Figure 1.1.

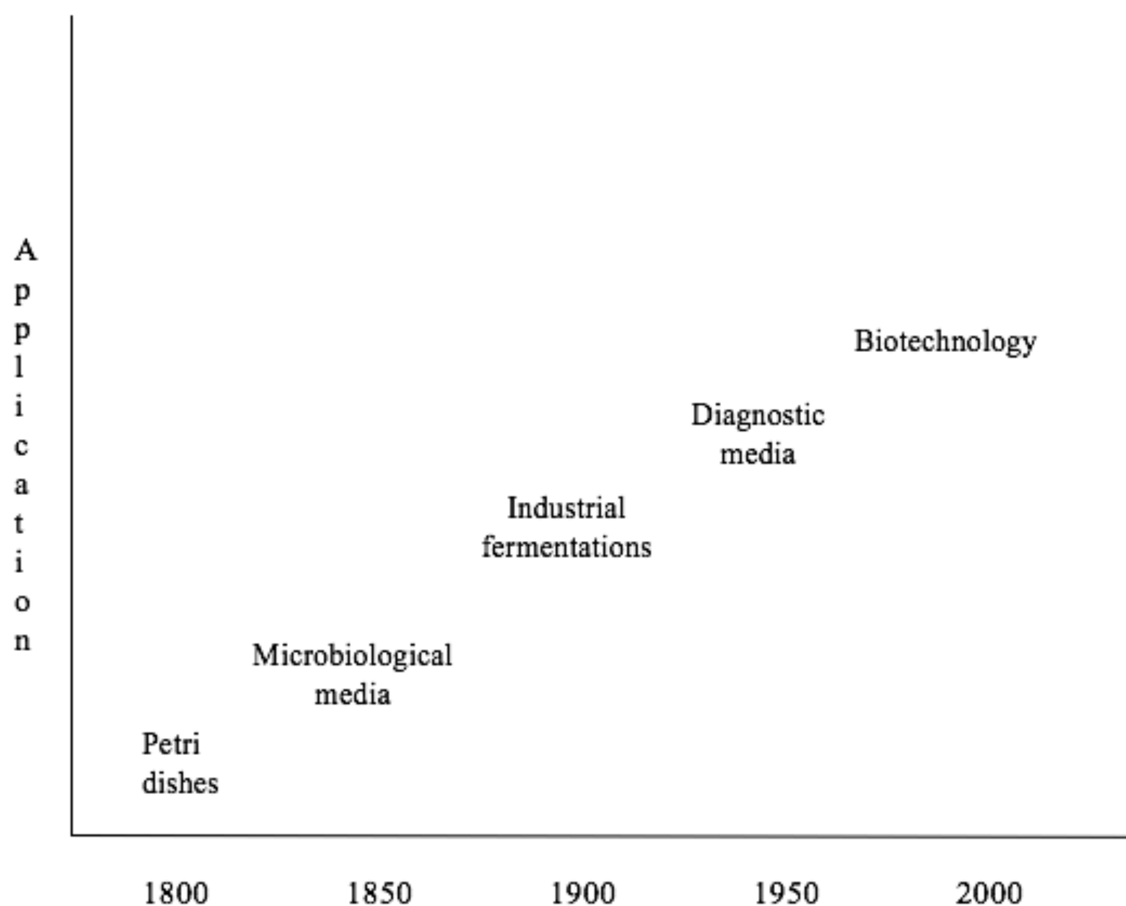


Figure 1.1 Use and applications of protein hydrolysates in the biotechnology industry throughout time. Modified from Pasupuleti *et al.,* 2010.

Protein hydrolysates became an obvious substitute for serum because they are a source of nitrogen, peptides, vitamins, free amino acids, micronutrients, carbohydrates, and fatty acids, including oleic, arachidonic, linoleic, and linolenic acid (Lobo-Alfonso *et al.,* 2010). Equally important is their ability to provide cells with some of the protection and buffering capacity conferred by serum. Several types of hydrolysates have been used for mammalian cell culture, and the first ones to be used were protein hydrolysates of bovine origin (peptones) such as Primatone™ RL and Lactalbumin hydrolysate (Siemensma *et al.,* 2010). However, due to their origin, they have the same pitfalls as serum and its derivatives. Therefore, it has been highly desirable to shift towards non-animal derived hydrolysates such as plant (e.g. soy, rice, wheat) and microbe-derived (e.g. yeast-extract) hydrolysates.

Plant and yeast hydrolysates are some of the most widely used supplements for media formulation, due to the fact that they provide peptides with specific amino acid sequences that act as growth, survival, or protection factors. Different studies have proved that these hydrolysates can be comparable to serum with even greater ability to stimulate growth and/or production and secretion of recombinant proteins (Burteau *et al.,* 2003; Sung *et al.,* 2004, Mosser *et al.,* 2012). They have been widely used across all cell types: from mammalian cells, including CHO (Ballez *et al.,* 2004; Mosser *et al.,* 2013; Richardson *et al.,* 2015), to insect cells (Ikonomou *et al.,* 2003; Kwon *et al.,* 2005). However, most of the SFM containing protein hydrolysates have been developed for CHO cells, as they are the "work-horse" for expression of recombinant proteins in mammalian cells, and there is a higher demand for their use in the industry. Fewer media formulations containing peptide hydrolysates have been created for anchorage-dependent cells, as has been the case for MDCK (Madin-Darby Canine Kidney) and Vero cells (Mazurkova *et al.,* 2008; Rourou *et al.,* 2014), because these cells have more stringent nutrition requirements. The

process of selection of a hydrolysate for any particular cell line is largely trial and error (Pasupuleti 2007) because cells may have different nutritional requirements depending on their type and origin. At the same time, certain plant hydrolysates are high in free amino acids, which may exert toxic effects (Burteau *et al.,* 2003).

Even though plant and yeast hydrolysates solve the major problem of serum (contamination with adventitious agents) they still lack a defined composition. Similar to serum, they also present lot-to-lot variability in their biochemical composition that results from several factors including the quality of the source protein and the method of hydrolysis. This variability has a direct impact on cell and/or product yield. In addition, the large undefined protein content can interfere with the downstream processing steps by clogging chromatography columns and adsorbing non-specifically to binding sites of the column matrices, reducing capacity and efficiency (Lobo-Alfonso *et al.,* 2010). Plant and yeast hydrolysates have provided a simple nutrient rich alternative to serum that has eliminated the risk of contamination with potential pathogenic agents. Their complex composition remains an issue to bioprocessing and prevents the definition of the media formulation in which they are included. One of the solutions has been to create protein-free media (PFM). This type of media is characterized by not containing supplemental polypeptide factors that would test positive using conventional protein assays. The use of PFM can ease the downstream processing (Jayme and Smith, 2000).

1.4 Chemically defined media

The main objective of any SFM formulation is to be chemically defined (CD). As its name implies, CD media is a type of SFM in which all chemical components are known. As a result,

batch-to-batch variability is greatly reduced and it is possible to achieve consistent production yields due to its known chemical formulation.

Similarly, CD media ease the downstream processing by reducing the number of unknown components that can negatively interfere with the purification process resulting in higher safety and quality of the final product. CD media can be formulated specifically for production, differentiation or proliferation (Freshney, 2010) by the addition or elimination of specific substances. However, most media formulations are proprietary, the component lists are not available, and little is known about their design methodology. The main challenge is to optimize the combination of nutrients that will maximize growth, proliferation, and production for each cell line, as some are more fastidious than others (Butler, 2015). This issue impacts largely on the cost and availability of CD-SFM formulations.

The ultimate goal is to achieve a CD animal-component free media (ACF) (Figure 1.2). In the past, this was one of the most challenging objectives to accomplish as most growth factors and proteins, which are integral to the cells' health and maintenance, were originally obtained from the purification of serum or other animal components (Jayme and Smith, 2000). With the advent of recombinant DNA technology, it is now possible to synthesize proteins from bacterial culture, eliminating the risk of contamination with adventitious agents from animal sources. This technology was at one time very expensive, but as the demand and technology has increased, prices have started to drop while the availability of more recombinant proteins and growth factors has increased.

The availability of CD media has increased over the past few years and its development has generally been focused on CHO and NSO (murine myeloma cells) for protein production (Gorfien *et al.,* 2000; Zhang and Robinson, 2005; Ma *et al.,* 2009). In the case of anchorage-

dependent cell lines that are mainly used for vaccine production, such as Vero, MRC-5, BHK-21 (Baby Hamster Kidney), and MDCK cells, there are few if any CD media available. This is due to their fastidious nature and their stringent growth requirements of these cell lines, making it costlier and more laborious to create a SFM.

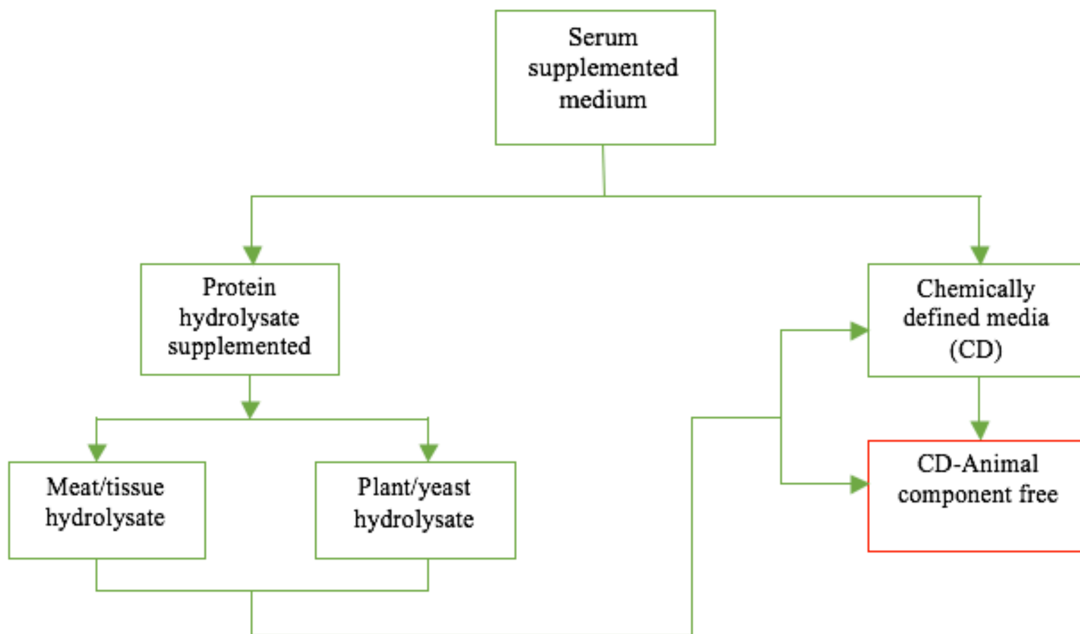


Figure 1.2 Evolution towards a chemically defined animal component serum free media formulation.

The availability of the media can also depend on the characteristics of the cells. Normal cell lines (e.g. MRC-5), are defined as those that do not significantly differ from cells in a healthy intact organism. They undergo senescence and have an unaltered diploid karyotype (Ham, 1981). Transformed cell lines (e.g. Vero, NSO or CHO cells), refer to those that exhibit malignant properties, cause malignant tumors in nude mice, can multiply without anchorage dependence (in suspension) and density-dependent growth inhibition. As the growth requirements for normal cell lines are more demanding than those of transformed cells, this complicates the design, production and formulation of CD-SFM for normal cells.

In order to find the optimal chemical composition for each cell line and its application, it is necessary to study the tissue of origin and the processes involved in the extra- and intracellular signaling pathways that regulate the proliferation and health of each cellular phenotype. Thus, it is necessary to test a wide range of mitogenic, growth stimulatory and nutritious components. Some are required for growth, cell division, maintenance, and proliferation of cells. The exploration of the different nutrients helps to understand how to supplement the basal media formulation that best suits the cell line. It is highly desirable that these components have minimal amounts of protein content to ease the downstream processing and that they do not originate from animal sources.

1.4.1 Defined media components

Mammalian cells, and particularly anchorage-dependent cells, require more nutritious compounds necessary for attachment and growth (Bjare, 1992). These obligatory substances have to be supplied in the nutrient medium. All the single defined components that are to be supplied to the media should not be derived or synthesized from any animal sources to satisfy the regulations in biomanufacturing. Lipids and amino acids, are commonly purified or derived from animal

source materials. However, efforts are being made to use plant-derived materials. As early as 1883, Ringer was the first to recognize the necessity for chemically defined media for cell culture (Jayme and Smith, 2000), this prompted an investigation on different media components that can be added to the basal media formulation of choice in order to create a CD-SFM. Below, a list is presented to give some insight into the different substances that play an important role in the growth regulation of anchorage-dependent cells.

1.4.1.1 Growth factors

As their name implies, growth factors play an important role in the differentiation and proliferation of cells. They are defined as hormone or hormone-like substances that regulate the growth of cells (Baserga, 1981). They are "non-nutritive substances that promote cellular multiplication but do not participate in the biosynthesis, metabolism or catalysis, but instead control proliferation in a regulative manner" (Ham, 1980; Shields, 1980). Growth factors exert a very complex interaction network that can vary depending on the origin of the cell (tissue and species specific). They can act synergistically, antagonistically, induce differentiation or interact with others in a positive or negative manner. For this reason, it is important to study and understand the growth factor interactions with each cell type and other media components.

As new technologies became less expensive and more widely used, the availability of recombinant growth factors has risen. Some of these now include growth factors that have been engineered to increase their potency and effectiveness on cell proliferation in SFM or to enhance and elicit cell differentiation. These engineered recombinant growth factors not only have an improved performance compared to regular growth factors but they are also produced in bacterial

or yeast hosts, thereby solving the issue of contamination with adventitious agents coming from animal sources.

Some examples of engineered recombinant growth factors are, LONG® R^3 Insulin-like Growth Factor- I (L-R^3-IGF-I), LONG® Epidermal Growth Factor (L-EGF) and recombinant human holo-transferrin (rTransferrin). L-R^3-IGF-I is an analog of insulin-like growth factor- I (IGF-I) produced in *Escherichia coli (E. coli)*. The mechanism behind L-R^3-IGF-I's enhanced activity are two modifications at the N-terminal extension peptide as depicted in Figure 1.3. First, there is an amino acid residue substitution of an arginine for glutamic acid at position three hence the R^3 nomenclature. Second, there is a fusion of a 13 amino acid N-terminal extension peptide, which is a portion of a growth hormone sequence. The overall result is a higher than 1000-fold reduced affinity to insulin growth factor binding proteins (IGBPs) compared to the native form of IGF-I (Repligen, 2016b).

L-EGF is a fusion protein which has a 53 amino acid extension peptide derived from the 46 N-terminal amino acids of porcine growth hormone. It can be produced at high yields in inclusion bodies in *E. coli* while retaining its biological activity *in vitro* (Xian *et al.*, 1996). Regarding cell culture applications, L-EGF was originally reported to have slightly lower potency in BALB/3T3 cells compared to the native EGF. However, synergistic effects have been observed with L-R^3 IGF- I in HEK 293, MDCK and cell lines (Repligen, 2016a).

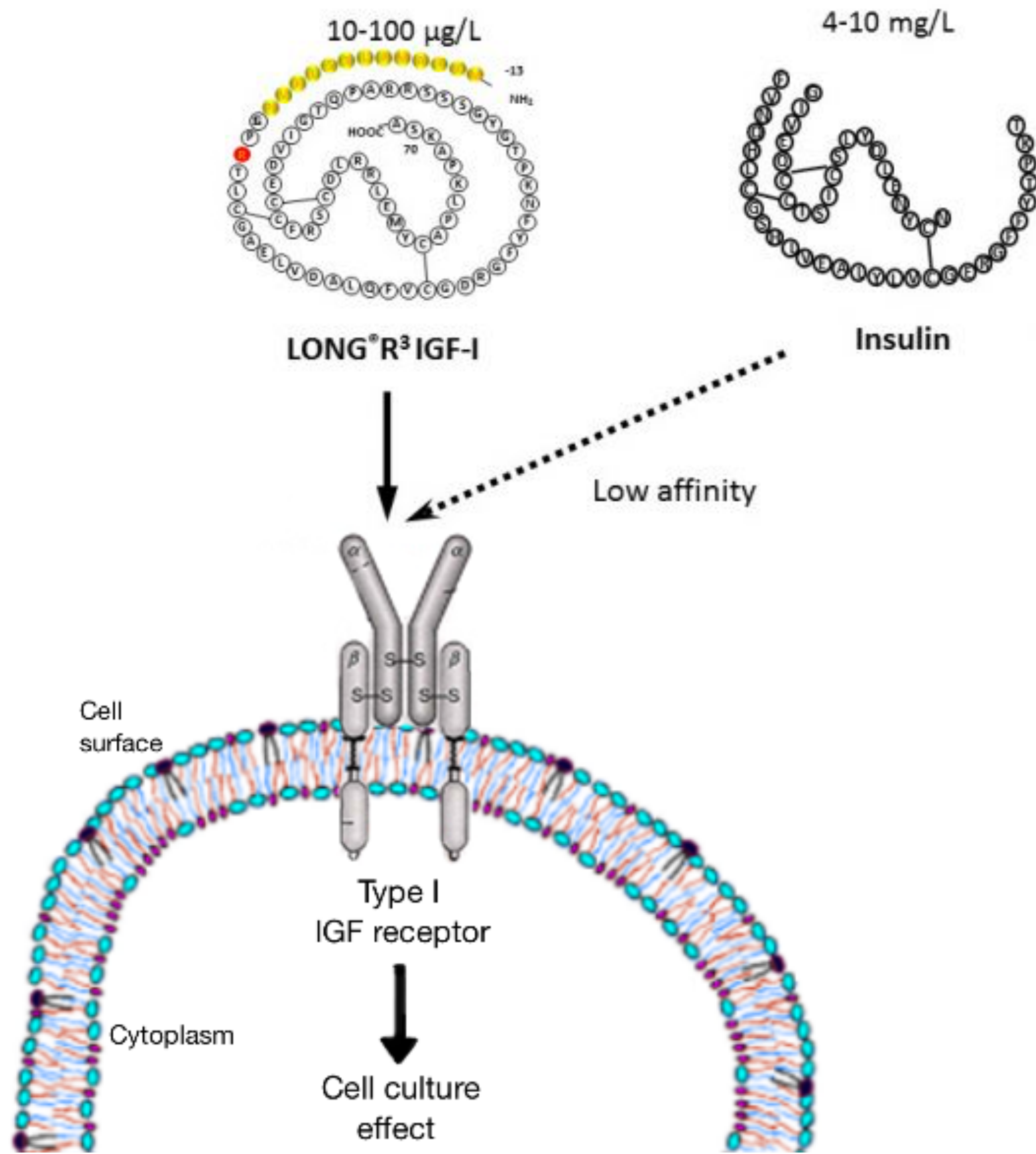


Figure 1.3 Structural differences from LONG® R^3 Insulin-like Growth Factor- I, which impact the binding affinity to the Type I IGF receptor compared to insulin. It should be noted that the concentration required to elicit a response is much lower in L-R^3-IGF-I compared to insulin. Modified from Repligen, 2016 b.

Recombinant transferrin (rTransferrin) is the recombinant version of the serum protein that is chiefly responsible for iron transfer and delivery to the cells (Penhallow *et al.,* 1986). Transferrin has been used to replace serum since Sato identified it as one of three key requirements for basal formulations. As such, it became part of the commonly used ITS (insulin-transferrin-selenium) supplement (Jayme *et al*., 1997). The main disadvantage of transferrin supplementation is that it is generally derived from bovine serum. This increases the risk of transmission of adventitious agents, complicating the creation of an animal-free SFM. Since iron is an essential cofactor for biochemical activities in mammalian cells (Arredondo and Núñez, 2005; Wang and Pantopoulos, 2011), its supplementation in animal-free SFM is generally provided by iron salts or chelators. Therefore, rTransferrin can be a viable alternative of iron supplementation for cell culture applications as it is produced in *Saccharomyces cerevisiae (S. cerevisiae)*. rTransferrin has been shown to be comparable to human-derived transferrin and better than iron salts in Vero and MDCK cell lines (Repligen, 2016c).

Other important mitogenic growth factors that have a positive impact on cell proliferation include platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and transforming growth factor-β (TGF-β). These growth factors have been proven to have a positive effect on cell growth. For example, TGF-β activates extracellular collagen accumulation in human lung fibroblasts; this effect is slightly enhanced by EGF (Fine and Goldstein, 1987). Moreover, when TGF-β is supplemented to the cell medium along with serum, ascorbate, and proline, it stimulates human dermal fibroblasts to proliferate and stratify up to 16 layers thick within the culture dish (Clark *et al.,* 1997). As well, it was found that the growth factors PDGF, EGF, IGF-1, IGF-2, TNF-α, TGF-β_1 and FGF-2 significantly stimulate proliferation of normal lung fibroblasts (Hertzel *et al.,* 2005).

1.4.1.2 Lipids

Lipids are important nutrients for mammalian cells as they are essential components of the cell membrane. They are “water-insoluble biomolecules, biosynthetically or functionally related to fatty acids and their derivatives” (Whitford and Manwaring, 2010). Lipids form a bilayer that is always in motion according to the fluid mosaic model from Singer and Nicholson (1972), where fatty acids, phospholipids, and cholesterol influence membrane fluidity. Lipids serve a highly important structural role as the receptor proteins are inserted or bound at the surface of the cell membrane (Savonnière *et al.,* 1996). As the membrane is the interface between the cell and the extracellular surroundings, the lipid organization and fluidity can impact the membrane proteins and their functions as well as the overall health of the cell. Hence, it is important to include lipids and/or their precursors into SFM formulations to maintain the overall health of the cells. Nevertheless, due to their hydrophobic nature, lipids and sterols have a low solubility in liquid medium, which is mainly composed by water.

Lipid precursors such as ethanolamine or phosphoethanolamine are miscible in water and are important for the biosynthesis of phospholipids. Ethanolamine, is a precursor for phosphatidylethanolamine present in glycosyl phosphatidylinositol (GPI) anchors which are important components of certain membrane proteins (Englund, 1993). Due to of their functional importance, the incorporation of these lipid precursors has become much more widely practiced in SFM formulations since it was originally introduced by Murakami (1982) who added ethanolamine as component to the ITS supplement. Equally important, is the role of sterols for the cell membrane. Mammalian cells contain cholesterol as its major sterol, which is indispensable for cell viability and proliferation (Ohvo-Rekilä *et al.,* 2002). Other synthetic sterols have been used to stimulate cell proliferation. Dexamethasone a corticosteroid, is capable of stimulating rat lung

fibroblast proliferation by upregulating the production of PDGF-α receptors. This in turn allows PDGF, a potent mitogen for mesenchymal cells, to stimulate proliferation of fibroblasts (Warshamana, 1998).

Cholesterol and other essential lipids, such as linoleic and linolenic acids (which are not water soluble), have been incorporated in media by dissolving in chemicals such as ethanol or methyl-β-cyclodextrin (MβCD) (Butler *et al.,* 1999; Christian *et al.*, 1997). However, there are different methods of lipid solubilisation, including the use of serum, serum extracts, albumins, emulsions, micelles, liposomes, and cyclodextrins. Out of these previous methods, only the last four allow the creation of an animal-free SFM (Whitford and Manwaring, 2010) and therefore their use is more desirable.

1.4.1.3 Vitamins

Vitamins are crucial components in basal and SFM formulations because they play a role as co-factors or as prosthetic groups for many enzymes and other cellular metabolism processes. The addition of these components is extremely important in cell culture because generally, cells cannot synthesize them. Media formulations that have a lack of vitamins lead to decreased cell growth, cell death or loss of productivity (Büntemeyer and Lehmann, 2001). Vitamins belonging to the Vitamin B group, which includes thiamin, riboflavin, niacin, pyridoxin, folic acid, cyanocobalamin, pantothenic acid, and biotin, are water-soluble and are predominantly incorporated directly into media formulations (Malda *et al.,* 2008). Less popular water-soluble vitamins, such as ascorbic acid (vitamin C) have been used in CMRL 1066, α-MEM, M199 and McCoy's 5A formulations (Freshney, 2010). This vitamin plays a key role in the maintenance of

the collagen networks by serving as a cofactor of the two enzymes lysil and prolyl hydroxylase that are essential to the collagen biosynthesis pathway (Boyera *et al.,* 1998).

In addition, water-insoluble vitamins such as vitamin A (retinoic acid) and vitamin E (α-tocopherol) have been used in SFM formulations, such as in the M199 medium, formulated by Morgan and coworkers (1950) or the SFM created by Cartaya, (1982). Retinoic acid has been shown to stimulate fibroblast growth and extracellular matrix production by human skin fibroblasts, as well as to potentiate the mitogenic effects of EGF (Harper and Borgoon, 1982; Varani *et al.,* 1990). Despite their importance for the cell metabolic processes, vitamins have the disadvantage of being very labile. They are generally sensitive to light and oxygen, which confers upon them low stability in media. An additional problem with, water-insoluble vitamins is they must be dissolved in organic solvents such as ethanol or dimethyl sulfoxide (DMSO) (Varani *et al*, 1990), which introduces unwanted compounds into the media formulation, potentially causing a negative impact in cell proliferation due to their toxicity.

1.5 Cell lines for vaccine production

Vaccines are biological preparations of antigenic components derived from, consisting of, or related to, a pathogen (Walsh, 2003). They stimulate the immune system to activate the humoral (antibody-producing) and cell-mediated immune responses of an organism, achieving long-term immunological protection (Levinson, 2010). Since their conception, vaccines have proven to be the most successful pharmaceuticals that have ever been created to control/prevent infectious diseases. They are considered a ground-breaking intervention in modern medicine (Nabel, 2013), due to their high effectiveness to prevent and stop the spread of disease.

Vaccines prevent around 2 to 3 million deaths world-wide every year (WHO, 2016) and improve lives by reducing the cost of health care while aiding domestic economies as they reduce disability and absence from work (Nabel, 2013). Many infectious diseases that were once feared due to their high fatality rates in human and animal populations are now rare or eradicated due to massive immunization campaigns implemented worldwide. As an example, smallpox was officially eradicated in 1980 and a current global effort is aiming to eradicate poliomyelitis. As part of the Global Polio Eradication Initiative (GPEI) from the World Health Organization (WHO), it was determined that it was crucial to produce a new generation of safer vaccines (Aylward and Tangermann, 2011; Thomassen *et al.,* 2013).

The main recommendation for the production of polio vaccines was to move away from primary monkey kidney cells and towards normal human diploid cells or Vero cells (WHO, 2004). Initially, normal diploid cells of human origin were used to replace primary cell culture. As a result, the normal human diploid cell line WI-38 was isolated at the Wistar Institute in the 1960's, which was used for the development of multiple vaccines including rabies (Barrett *et al.* 2009). Since WI-38 cells are not immortalized, they have a limited lifespan and currently there is a shortage of their supply for vaccine production. For that reason, an equivalent cell line, the MRC-5 cell line has been increasingly used as an alternative to replace WI-38 cells, due to its longer lifespan compared to the lung fibroblast cell line IMR-90 (Jacobs, 1976; Friedman and Koropchak, 1978; Griffiths *et al.,* 1982; Peetermans, 1992; Trabelsi *et al.,* 2012). However, the MRC-5 cell line suffers from the same problem as the WI-38 cell line, being that serial propagation of these cells results in senescence (Barrett *et al.*, 2009).

Initially, continuous cell lines (CCL) were not used for vaccine production, as they were considered to have tumorigenic potential. However, after extensive studies for potential oncogenic

properties, it was concluded that CCLs were acceptable substrates for vaccine production. Hence, the industry is moving towards immortal cell lines such as Vero, MDCK, PBS-1 and Per-C6® (Barrett *et al.*, 2009; Josefsberg and Buckland, 2012). Currently, GlaxoSmithKline, Merck and Sanofi use MRC-5 cells to manufacture hepatitis A and poliomyelitis virus vaccines. Vero cells are used for the production of poliomyelitis, rotavirus and smallpox vaccines (CDC, 2016).

1.6 Objectives of this thesis

The assumptions underlying this work are that, cell proliferation of MRC-5 and Vero cells under reduced or complete removal of FBS in MVSFM will be impacted negatively. Therefore, the replacement with growth stimulatory substances such as growth factors, vitamins, sterols, and lipid precursors will allow both cell lines to grow to equivalent cell yields equal to that of serum containing media.

This leads to the hypothesis that the addition of growth requirements (LONG® EGF, LONG® R^3 IGF-I, bFGF, TGF-β3, PDGF-AA, dexamethasone, cholesterol, linoleic acid, ethanolamine, phosphoethanolamine, rTransferrin, retinoic acid, α-tocopherol and ascorbic acid) to the basal medium MVSFM in CD-SF conditions will produce an equivalent cell yield by MRC-5 and Vero cells compared to when these cell lines are grown in serum-supplemented media (DMEM+10% FBS).

The overall objective of this project is to optimize a robust CD formulation using the basal medium MVSFM from Biogro Technologies designed by Burgener in 2000 for the growth of human MRC-5 and Vero cell lines using three different experimental designs and optimization methodologies: One-Factor-At-a-Time (OFAT), Definitive Screening Design (DSD) and Genetic

algorithms and Hill Climbing search (GA-HC) that would generate a suitable medium for its use in large-scale culture bioprocesses for viral vaccine production.

As part of the collaboration between Repligen©, Biogro Technologies, and the University of Manitoba, the bioactivities of three growth factors: i) LONG® Epidermal Growth Factor, ii) LONG®R3 Insulin-like Growth Factor-I, and iii) recombinant human transferrin were assessed in MRC-5 and Vero cells, individually using OFAT experiments by supplementing these substances to MVSFM along with a reduced amount of serum and measuring a final cell yield after reaching full confluence. Those growth factors and concentrations that had a positive influence in cell yield were subsequently used as a basis to decrease the serum concentration.

The evaluation of the interaction of the Repligen© growth factors with other recombinant growth factors, lipids and vitamins and their impact on cell proliferation (measured as cell/cm^2) were evaluated using the OFAT, DSD and GA-HC methodologies.

1.7 Media optimization using Quality-by-Design

Statistical experimental planning is a crucial step in bioprocessing and biomanufacturing given the complex nature of biological systems. Hence, the quality of the end-product depends on controlling variables that influence the manufacturing all throughout the production development (Mandenius *et al.,* 2009). The level of difficulty is increased in biotechnology related pharmaceuticals, as there are variables that are often unknown to the investigator. In order to achieve and maintain cGMPs during serum free media formulation, it is necessary to follow the recommendations provided by regulatory agencies. In order to achieve this, the national health regulatory agencies along with the pharmaceutical industry, and the academia began to incorporate the guiding principle of Quality-by-Design (QbD) (Mandenius *et al.,* 2009).

As such, both the Food and Drug Administration (FDA) and the European Medicines Association (EMA) have incorporated QbD in their Process Analytical Technology (PAT) and Process Validation (PV) regulatory frameworks for drug development and production to understand, control, and guarantee the quality of pharmaceutical products and the corresponding processes (Takahashi *et al.,* 2016, Riley and Li, 2011). These frameworks were aimed at incorporating mathematical models, sensor technologies, analytical techniques and design into the creation of new biopharmaceuticals (Mandenius *et al.,* 2009; Mandenius and Brundin, 2008). Some of the recommendations were the use and incorporation of Design of Experiments (DoE) into the early stages of biomanufacturing to develop knowledge about the bioprocess' interactions between the input variables and the resulting outputs to improve the performance of the commercial process (FDA. 2011).

DoE is a statistical methodology used to plan and organize one or a series of experimental tests. Thus, allowing the experimenter to make systematic changes to the input variables of the process or system (e.g. media formulation) according to the observed changes in an output response (Montgomery, 2013). Due to the nature of the media formulation process, in which many growth requirements are subject to testing and many variables are involved, it is highly important to use DoE to follow the QbD principles. Screening designs, a type of experimental designs, are usually performed during the early stages of the industrial process to determine which of the many factors (if any) have a significant effect on the biological system or process (Dougherty, 2013). Hence, screening designs are ideal for SFM development as they avoid experimental biases and reduce the number of experiments.

In contrast, one of the most commonly used techniques in biological experimentation is the one-factor-at-a-time (OFAT) experimental strategy. As its name implies, it consists of successively

varying each factor independently at different levels while keeping all other factors constant (Doughtery, 2013). This technique is only capable of determining how each factor affects the response variable. However, it does not consider any interactions between factors. The result is a saturated experimental design space that is inefficient, costly, time consuming, and the experimenter ends-up with a quasi-optimum value of the factors in question (Mandenius and Brundin, 2008).

Due to the capability of detecting effects while being able to study many factors (e.g. growth requirements) with a relatively small allocation of resources (SAS Institute, 2015) screening designs are ideal for SFM formulation. Traditionally, two-level fractional factorial designs (e.g Plackett-Burman) have been used for the design of SFM for many cell lines such as CHO cells, cytotoxic T lymphocytes and Vero cells (Jeon *et al*., 2010; Petiot *et al.,* 2010; Gonzalez-Leal *et al.,* 2011). In 2011, Jones and Nachtsheim created the definitive screening design (DSD). This methodology evaluates all factors at three levels (i.e. three different concentrations) and thus, it is capable of providing unbiased estimates of main effects allowing to determine full quadratic effects and some two-factor interactions (from 6 to 12 factors only). The DSD complies with the QbD guidelines and due to its efficiency and estimation capabilities, it is able to reduce design costs and time compared to OFAT or two-level factorial designs. The use of DSD as a tool may present the disadvantage of producing data that requires high statistical level of analysis. For that reason, data analysis can be performed by using GA to model and optimize the different variables (growth requirements) in a SFM. This technique has been used in bioprocessing to describe correlations and it is more adequate for describing biological processes than linear models (Takahashi *et al.* 2016).

Chapter 2

Materials and Methods

2.1 Cell lines and culture maintenance

MRC-5 (CCL-171™) and Vero (CCL-81™) cells were obtained from the American Type Culture Collection (ATCC). A working cell bank was established in 1 mL aliquots in Nalgene 2 mL cryogenic tubes, and stored in liquid nitrogen in DMEM+10% FBS with 10% DMSO (dimethyl sulfoxide).

Prior to use in experiments, cryovials containing cells were removed from liquid nitrogen storage and the cells and revived by rapid thawing in a 37˚C water bath. The cells were transferred to a 15 mL centrifuge tube and 5 mL of DMEM+10% FBS were added and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in 5 mL of DMEM+10% FBS and then transferred to T-75 flasks containing DMEM + 10% FBS, and incubated at 37˚C with 10% CO_2 in a humidified incubator. Thereafter, cultures were maintained in T-75 flasks, incubated at 37˚C with 10% CO_2 in a humidified incubator.

2.2 Subculture in T-flasks

2.2.1 Reagents and materials

All reagents used in cell culture were purchased from Gibco™ (Thermo Fisher Scientific, Waltham, Massachusetts, United States): Dulbecco's phosphate-buffered saline (DPBS) (Cat. 21600-010); TrypLE™ Express enzyme (Cat. 12604021); and Trypan blue solution 0.4% (Cat. 1520-061). Additional materials used in cell culture included individually wrapped serological sterile pipettes (Corning®, Sigma-Aldrich), and T-flasks (T-25 and T-75) (Corning®, Sigma-Aldrich).

2.2.2 Subculturing protocol

Cells were cultured in T-75 and T-25 flasks with working volumes of 20 mL and 5 mL of media, respectively. Cell cultures were passaged after reaching confluence. The subculturing protocol was performed as follows. The supernatant was removed and discarded. The monolayer of cells was washed with 5 mL or 1 mL of pre-warmed (37 ˚C) sterile DPBS for T-75 and T-25 flasks, respectively. TrypLE express recombinant trypsin was added in a ratio of 1 mL per 25 cm^2 (a total of 3 mL for a T-75 flask and 1 ml for a T-25). Cells were incubated for 5 minutes at 37˚C, then flasks were gently tapped to dislodge cells that remained attached onto the flask. TrypLE was neutralized using equal amounts of serum-free medium in which cells were to be subcultured. The cells were then transferred into a 15 mL centrifuge tube and centrifuged at 1500 rpm for 5 minutes. The supernatant was subsequently discarded, the cells were resuspended in the same total volume of media and thoroughly mixed to achieve a homogenous mixture, and a 0.5 mL sample was

transferred to a microcentrifuge tube and used for subsequent counting. Cells were inoculated in a new flask at a cell density of $1x10^4$ cells/cm^2.

2.2.3 Cell counting

Each sample was thoroughly mixed and two 50 µL sample were taken and diluted 1:2 with Trypan Blue Exclusion dye (Gibco™). The culture sample and dye were thoroughly mixed to achieve a homogenous mixture, and the samples were added to the slots of the Cedex XS Smart Slides (Roche Applied Science, Penzberg, Germany), which were subsequently placed in the Cedex XS Analyzer to obtain viable cell counts.

2.3 Cell culture media

2.3.1 Media suppliers

DMEM, powder, high glucose (Cat. 12100061) Gibco™, Thermo Fisher Scientific (Waltham, Massachusetts, United States) was dissolved in MilliQ ultrapure water as per manufacturer's instructions. Modified Vero serum-free media was prepared using the formulation provided by Biogro Technologies Inc. (Winnipeg, Canada) and subsequently supplemented with the different reagents described in Sections 2.3.2 to 2.3.4, below. Both of the above were filter sterilized using Steritop™ low protein binding 0.22 µm membrane filter (EMD Millipore, Darmstadt, Germany). FreeStyle™ 293 Expression Medium (Cat. 12338018) liquid medium was obtained from Gibco™, Thermo Fisher Scientific (Waltham, Massachusetts, United States).

Qualified, heat inactivated fetal bovine serum (FBS) Canada origin (Gibco™ Cat. 12484010) was used to supplement different media formulations as specified or for weaning purposes.

2.3.2 Media supplements and growth factors

The following media supplements were added to MVSFM. After addition, each media formulation was filtered sterilized. The following recombinant growth factors were kindly supplied by Repligen© (Waltham, Massachusetts, USA): LONG® Epidermal growth factor expressed in *E. coli*; LONG® R^3-IGF-I expressed in *E. coli*; Recombinant human transferrin (rTransferrin) expressed in *S. cerevisiae*. All other recombinant growth factors were all expressed in *E. coli* and were purchased from Sigma-Aldrich Corp. (St. Louis Missouri, USA), which included: Human epidermal growth factor (EGF); Human basic fibroblast growth factor (bFGF); Human platelet derived growth factor (PDGF-AA); and Human transforming growth factor beta 3 (TGF-β-3). The latter was reconstituted in a 5 mM citric acid with 0.1% bovine serum albumin (BSA) buffer for added stability.

2.3.3 Lipids, lipid precursors, vitamins and steroids

The following substances were purchased from Sigma-Aldrich Corp. (St. Louis Missouri, USA) unless specified. Linoleic acid (L1012) was prepared at a concentration of 1.93 µM by complexing it with a 20 mg/L solution of Methyl-beta-Cyclodextrin (MβCD) (C4951) to dissolve linoleic acid in water and mixed in a 37 ˚C incubated shaker platform for 30 minutes to achieve a homogenous solution. Ethanolamine (E0135) was diluted to a concentration of 1.66 M, O-phosphoethanolamine (P0503) and ascorbic acid (A4403) were both dissolved and vortexed

thoroughly in Milli-Q water in concentrations of 1 M and 0.1 M respectively. Dexamethasone (D8893) stock solution (20 µg/mL) was prepared by dissolving 1 µg in 1 mL of anhydrous ethanol (GreenField Specialty Alcohols Inc. Brampton, Canada) and subsequently adding 49 mL of sterile basal medium. The solution was then filter sterilized in a 0.22 µm Steriflip® (EMD Millipore, Darmstadt, Germany).

Cholesterol (C3045) was dissolved in MβCD (C4951) using a slight variation from the method described in Christian *et al.,* 1997. An 8 mM solution of MβCD was prepared in a glass tube using Milli-Q water. This solution was later complexed with powdered cholesterol to achieve an 8:1 molar ratio of MβCD to cholesterol (final cholesterol concentration of 1mM). The mixture was thoroughly vortexed for one minute and incubated overnight in a shaking 37˚C water bath. Subsequently, the solution was gently mixed to bring the dried cholesterol off the sides of the tube into the solution and incubated in a water bath sonicator for three minutes. This was repeated until a clear solution was obtained and most cholesterol crystals were dissolved. All-trans retinoic acid (R2625) and α-tocopherol (258024) were dissolved in absolute ethanol to make stock solutions of 6 mg/L and 0.44 M stock solutions. Both were dissolved in dark conditions and thoroughly vortexed to achieve a homogeneous mixture.

2.3.4 Polyamine supplement

The polyamine supplement was prepared by dissolving spermidine, spermine, putrescine and ornithine in MilliQ water to a concentration of 10 µM each. A 100 µM citrulline solution was prepared and mixed with the other stock solution. All reagents were purchased from Sigma-Aldrich Corp. (St. Louis Missouri, USA).

2.4 Cell culture for OFAT and DSD experiments

Cells were maintained and up-scaled in T-75 flasks. Subsequently, cells were inoculated at a cell density of $1x10^4$ cells/cm^2 into the different test media in T-25 flasks in triplicate and incubated 37˚C at 10% CO_2 until they reached confluence. Cells were counted using the subculturing protocol described in Section 2.2.2.

2.5 Statistical design and analysis

The cell count data was collected from the Cedex XS software (Roche Applied Science, Penzberg, Germany) and analyzed using JMP 12 (SAS Institute Inc.). Doubling times were calculated according to the following formula:

$$Doubling\ time = \frac{time * \log(2)}{\log(final\ concentration) - \log(initial\ concentration)}$$

Analysis of variance, Student's t-test, and Dunnett's test were used to evaluate the impact of the different factors on cell yield (Section 3.4.6). Statistical significance of doubling times was calculated performing a Student's t-test. A DSD outline was created by entering a maximum and a minimum concentration (level) for each factor of interest. This outline indicated the different levels for each growth stimulatory substance which were later used to produce the different media formulations. The cell counts obtained after testing each media formulations were used as response variables and analyzed using the JMP 12 software package to determine the different effects and interactions between factors.

2.6 Analysis using high performance computing for model regression and evaluation

The cell counts obtained from each of the media outlined by the DSD were analyzed using an algorithm developed at the IDEAS lab by Dr. Jose Juan Mijares Chan. The algorithm is based upon two optimization techniques that approximate the DSD model and find the optimal concentrations to maximize cell yield. The first technique, genetic algorithms (GA), falls under the umbrella of evolutionary computing techniques and it is known for its vast search coverage which propitiates a faster solution convergence (Mijares Chan, 2016). This technique was applied to perform a regression and approximate the DSD model's constants. Once the constants were defined, the "Hill-climbing Search" method was massively applied over different starting points. This technique was selected due its fast evaluation of local suboptimal solutions to match optimal media component concentrations with greater cell growth performance. The algorithm methodology used to process the data obtained using the DSD is outlined in Section 6.2.

Chapter 3

One Factor at a Time Optimization

3.1 Introduction

Development of an animal component-free, chemically defined (ACF-CD) serum free medium (SFM) formulation requires a controlled production process that can reduce: i) the risk of contaminants, ii) the protein load (to ease the purification process), and iii) the variability in the final product (Genzel *et al.,* 2006). For these reasons, the biopharmaceutical industry is constantly aiming to eliminate the use of animal-derived components (e.g. serum, trypsin, bovine serum albumin) during the entire production process, chiefly among cell culture media (Kluge *et al.,* 2013) by using recombinant growth factors and other proteins. Major progress has been made in large-scale production of monoclonal antibodies where it has been possible to use SFM formulations. However, traditional biopharmaceutical products, such as vaccines, still require the use of serum during the growth phase to ensure high cell densities and viral yields (Genzel *et al.,* 2004; Thomassen *et al.,* 2013). This has been especially challenging due to the stringent requirements of the cell lines used for vaccine production. Ideally, SFM should be used throughout the entire production process.

The process of developing an ACF-CD-SFM formulation for anchorage-dependent cells requires a series of strategic steps. The creation of a media is mostly based on empirical data (trial and error) as it is simply not feasible to create a SFM solely based on theory. Some information can be gathered from previous findings and previous work. Thus, it is of paramount importance to

select a basal media formulation. This must be done to satisfy the growth requirements and the individual needs of each cell line.

One of the SFM formulations that has been shown to be effective for anchorage dependent cells lines such as Vero and MRC-5 cells, is the MVSFM (Butler *et al.,* 2000, Liu *et al.* 2008), which was developed in the laboratory by Burgener (2000). Hence, the MVSFM was deemed the basal medium that would undergo further improvement and optimization with recombinant growth factors to establish a SFM for vaccine production in MRC-5 and Vero cells. The original MVSFM formulation created by Burgener (2000) is a CD-ACF-SFM specifically formulated for the Vero cell line. It is a low-protein content medium developed using recombinant growth factors to avoid the use of animal derived components. A variation of this medium was optimized for MRC-5 cells that contained Primatone®, enzymatic hydrolysate of meat protein.

The objective of this project was to create a CD-ACF-SFM for MRC-5 and Vero cells using MVSFM as the starting basal medium and supplement it with recombinant growth factors manufactured using non-animal sources, such as *E. coli* or *S. cerevisiae.* The recombinant growth factors produced by Repligen© satisfy this requirement and were used in this optimization process. In addition, these growth factors have modifications that are aimed to boost the performance of SFM due to their higher affinity and/or stability.

To perform the initial optimization and screening of substances that could improve MVSFM, the One-Factor-At-a-Time (OFAT) experimental approach was used. This is a traditional method that is still widely used in media optimization (Weuster-Botz, 2000; Montgomery, 2013). It entails selecting a starting point or baseline for each factor or variable and then changing each one over its range while fixing the others at the baseline levels (Chauhan *et al.,* 2007; Poorna and Kulkarni, 1995; Montgomery, 2013). This approach is suitable when few

parameters are compared or tested and it gives information about the impact of a single variable on a system. Its main advantage is that it requires a low amount of experimental planning and analysis compared to other experimental design approaches. However, it falls short when it comes to providing information regarding important experimental variables and the interactions between the different growth stimulatory substances contained in the media (Mandenius and Brundin, 2008).

3.2 OFAT experimental methodology

The OFAT optimization methodology was used to obtain initial data regarding the bioactivity and the individual impact of the three recombinant growth factors (L-EGF, L-R^3-IGF and rTransferin provided by Repligen©) on MRC-5 and Vero cells. The results were ultimately used to determine the concentrations of each growth factor in the optimized SFM formulations for each cell line. This methodology was selected because it is a reliable classical approach that has been widely used in SFM optimization and because only three variables (growth factors) were tested.

3.3 Comparison between recombinant S-EGF and LONG® EGF in MRC-5 and Vero cells

The data showed that there was no significant statistical difference in cell yield in MRC-5 when MVSFM was supplemented with S-EGF or L-EGF. As indicated in Table 3.1, the doubling-time of MRC-5 cells in S-EGF compared to the one on L-EGF, with L-EGF having a shorter doubling-time which was not significantly different. However, as it can be observed in Figure 3.1,

MRC-5 cells presented a slightly higher yield when the medium was supplemented with L-EGF compared to the one with S-EGF.

Both cell lines initially maintained in DMEM+10% FBS were subcultured in T-25 flasks in duplicate into MVSFM supplemented with a reduced serum concentration (1% FBS) with the addition of the S-EGF and L-EGF as stated above. The positive control was DMEM +10% FBS and the negative control was MVSFM + 1% FBS without EGF. Cells were counted at day four and passaged in the test media for two sequential passages to observe the long-term effect of growth factors in MRC-5 cells while cultured in reduced serum media. The cell yields of each consecutive passage were normalized against the positive control and averaged. Error bars were calculated as standard error of the mean (SEM).

When Vero cells were cultured in MVSFM supplemented with S-EGF or L-EGF (Figure 3.2), there was no statistical significant difference in cell yield as calculated using the Student's t-test. The doubling-time of MRC-5 cells grown in S-EGF and L-EGF differed by one hour (Table 3.2) which was not significantly different to each other according to the Student's t-test. The cells grown in L-EGF had the shortest doubling-time. The results indicated that both recombinant forms of EGF (S-EGF and L-EGF) had a similar effect on cell yield on each cell line. Nevertheless, in both cases, L-EGF had a shorter doubling-time and thus, L-EGF was deemed preferable for future use.

Table 3.1 Doubling time of MRC-5 cells in MVSFM + 1% FBS in the two forms of EGF and the positive control (DMEM + 10% FBS) n=2.

MVSFM + 1% FBS	Doubling-time (hours)	SEM (hours)
0 µg/L EGF	76.8	4.8
10 µg/L S-EGF	63.5	3.7
10 µg/L L-EGF	62.4	4.6
DMEM + 10% FBS	40.7	2.8

Table 3.2 Doubling-time of Vero cells in MVSFM + 1% FBS in the two forms of EGF and the positive control (DMEM + 10% FBS) n=2

MVSFM + 1% FBS	Doubling-time (hours)	SEM (hours)
0 µg/L EGF	39.3	1.6
10 µg/L S-EGF	33.6	2.0
10 µg/L L-EGF	32.6	2.7
DMEM + 10% FBS	26.1	0.8

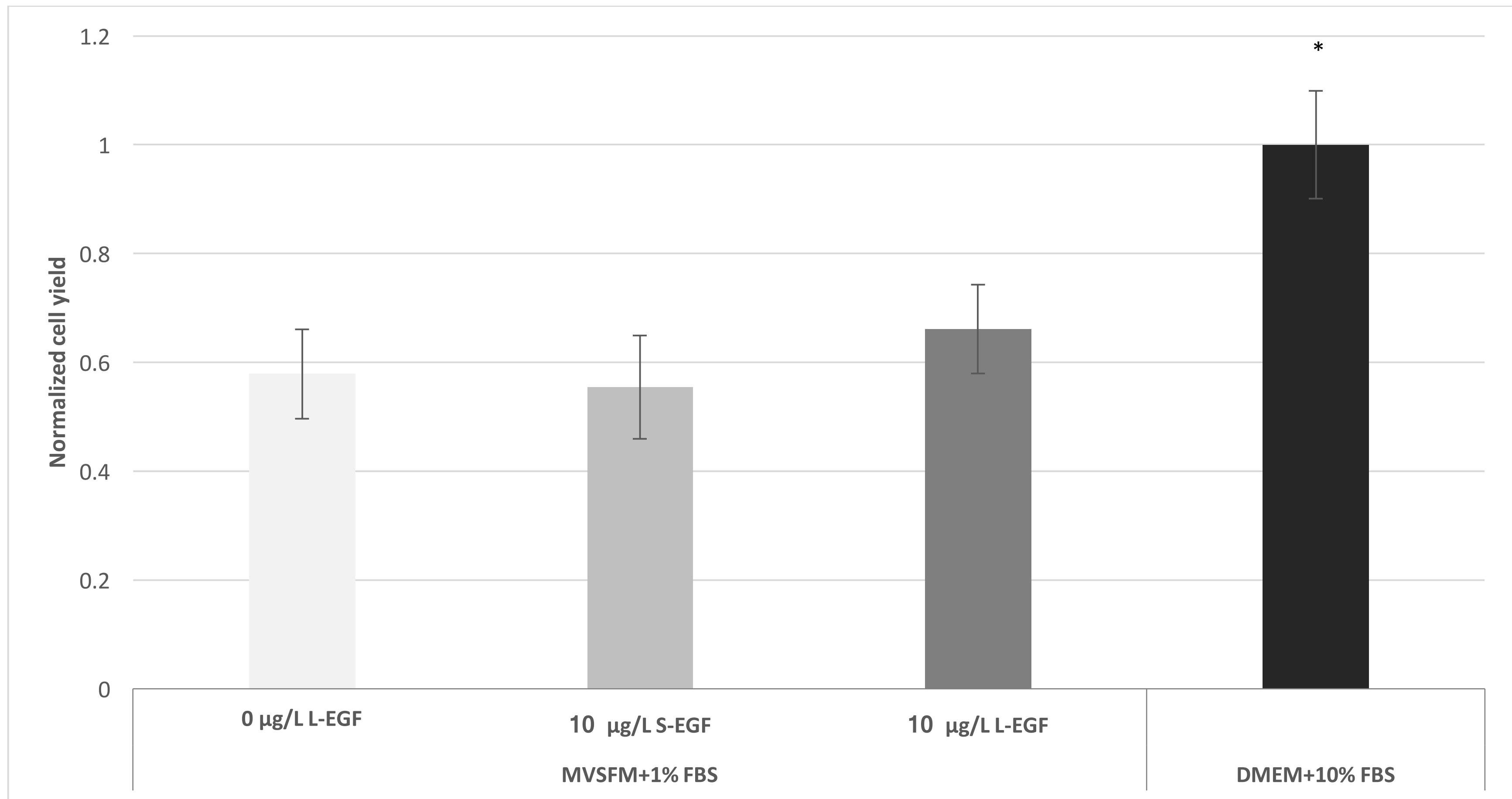


Figure 3.1 Normalized cell yield of MRC-5 cells grown in MVSFM+1% FBS (4 days). The effect of L-EGF was compared with S-EGF over the course of two passages (n=2). There was no significant difference between the two types of EGF. Cell yield was compared using Student t-test at $p < 0.05$. *, denotes significant statistical difference at $p < 0.05$ from all other values. Error bars are shown as SEM.

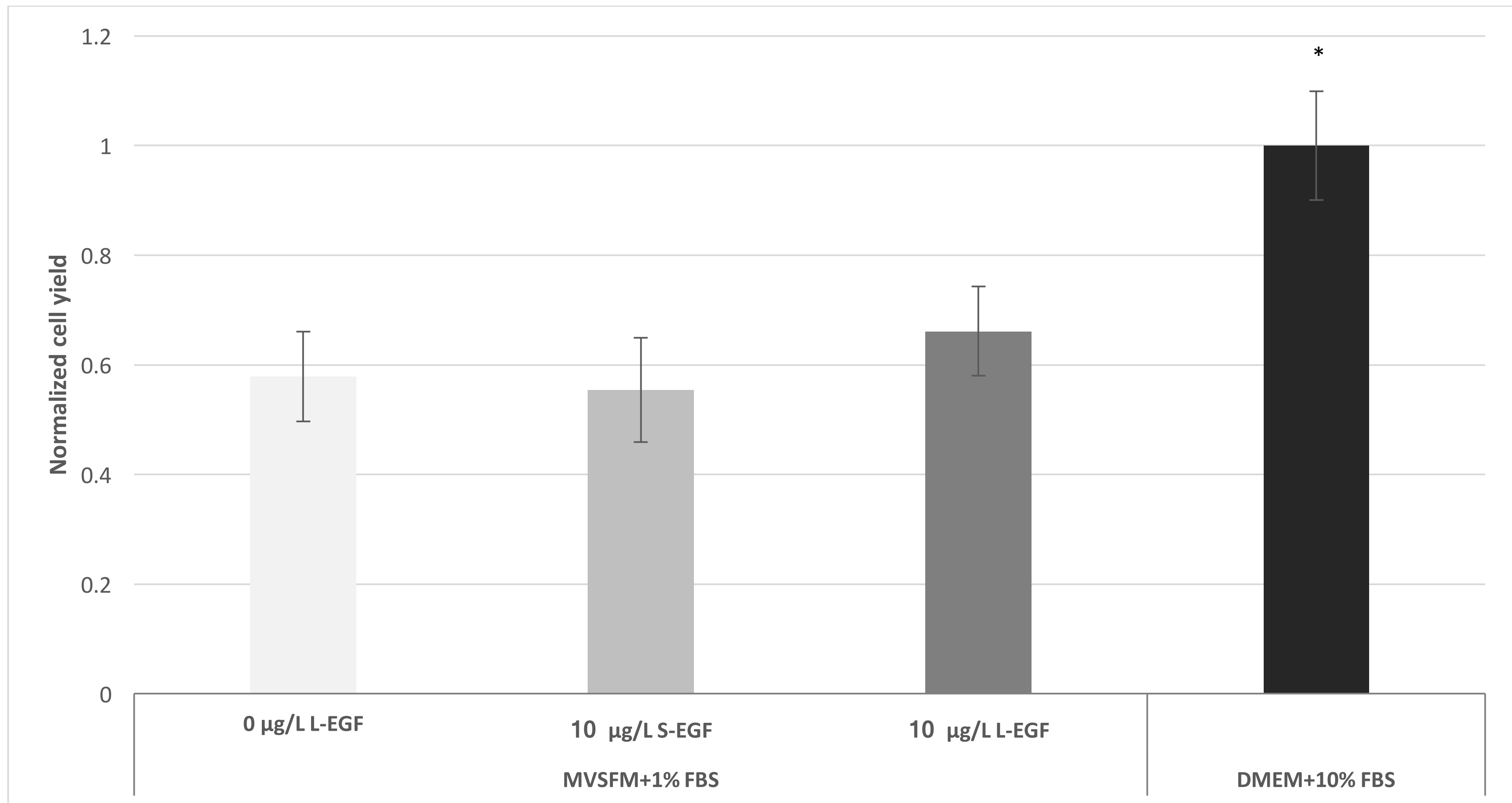


Figure 3.2 Normalized cell yield of Vero cells grown in MVSFM after 4 days. The effect of L-EGF was compared with S-EGF over the course of two passages (n=2). Cell yields were normalized to DMEM+10%FBS (positive control) and compared using Student's t-test at $p < 0.05$. *, denotes significant statistical difference at $p < 0.05$ from all other values. Error bars are shown as SEM.

3.3.1 Effect of S-EGF and L-EGF on MRC-5 and Vero cell yield

The data generated by this experiment indicated that there was no significant impact on cell yield or doubling-time when MRC-5 cells were grown on MVSFM + 1% FBS, with the addition of S-EGF or L-EGF. These recombinant growth factors differ in that L-EGF has an additional peptide extension, which protects the L-EGF fusion protein from proteolytic cleavage. Previous findings from Xian *et al.* (1996) demonstrate that when L-EGF was incubated in gastrointestinal flushings, native EGF was released. As such, the peptide extension could potentially be beneficial in a SFM formulation as it may prolong the stability of the growth factor during culture as well as increasing its shelf life. The resilience of L-EGF to proteolytic cleavage can be advantageous because it may protect the growth factor from trypsin cleavage during subculturing in a SF environment. As such, given that there was no statistical difference from the experimental results, that the doubling-times only differed by an hour, and the information provided by the literature, it was determined to replace the S-EGF (contained in the original MVSFM formulation) for L-EGF for subsequent optimized media formulations for both cell lines.

3.4 Optimization of L-EGF, L-R^3-IGF-I and rTransferrin in MVSFM for MRC-5 and Vero cells

After selecting L-EGF for the SFM formulation, the experimental concentrations of the growth factors provided by Repligen© were determined using literature values and those originally contained in MVSFM as a reference. The concentrations used are outlined in Table 3.3. All

experiments were performed in both MRC-5 and Vero cells to compare the impact that each growth factor exerted in each cell line.

Table 3.3 Concentrations of growth factors used in OFAT experiments using Vero and MRC-5 cell lines

Growth factor	Concentrations (µg/L)				
Long EGF	0	5	**10**	20	50
Long R^3 IGF-I	0	10	40	**80**	100
rTransferrin*	**0**	10		50	100

* Linoleic acid 0.54 mg/L incorporated only in the transferrin "media series"

Values bolded correspond to original values used in MVSFM

3.4.1 Optimization of L-EGF in MRC-5 cells

MRC-5 cells initially maintained in DMEM+10% FBS were subcultured in T-25 flasks in duplicate into the experimental media MVSFM supplemented with a reduced serum concentration (1% FBS) with the addition of L-EGF at the concentrations shown on Table 3.3. The positive control was DMEM +10% FBS and the negative control was MVSFM + 1% FBS without EGF. Cells were counted at day four and passaged in the test media for three sequential passages to observe the long-term effect of L-EGF in a reduced serum media as well as for statistical significance. The data analysis was performed as described in Section 3.3.

It was observed that there was no linear correlation between L-EGF concentration and cell yield. An increase in cell yield was observed at 5 µg/L compared to the negative control. However, L-EGF seemed to have a deleterious impact as the concentration of L-EGF increased up until 20 µg/L which was the lowest yield in the experiment. The highest cell yield was reached at 50 µg/L

(Figure 3.3), which was statistically significantly different from the negative control by the Student's t-test (Figure 3.4) and confirmed using Dunnett's test (both at $p < 0.05$). Similarly, the shortest doubling-time was achieved at the 50 µg/L L-EGF concentration as shown in Table 3.4, which was the significantly higher from the negative control according to the Student's t-test. Moreover, the doubling time of MRC-5 at this concentration was significantly higher than the positive control and therefore, it presented a lower cell yield. It was shown that L-EGF decreased the doubling time of MRC-5 cell yield as shown by one-way ANOVA (Figure 3.4). Thus, the concentration of L-EGF in MVSFM for MRC-5 cells was increased from 10 µg/L to 50 µg/L as it showed to be the most effective.

Table 3.4 Doubling-time of MRC-5 cells in MVSFM + 1% FBS in the different concentrations of L-EGF compared to the positive control (DMEM + 10 % FBS) n=3.

MVSFM + 1% FBS	Doubling-time (hours)	SEM (hours)
0 µg/L L-EGF	76.8	4.9
5 µg/L L-EGF	59.5	2.8
10 µg/L L-EGF	62.4	4.6
20 µg/L L-EGF	125.3	4.7
50 µg/L L-EGF	48.3	3.7
DMEM+10% FBS	40.7	2.8

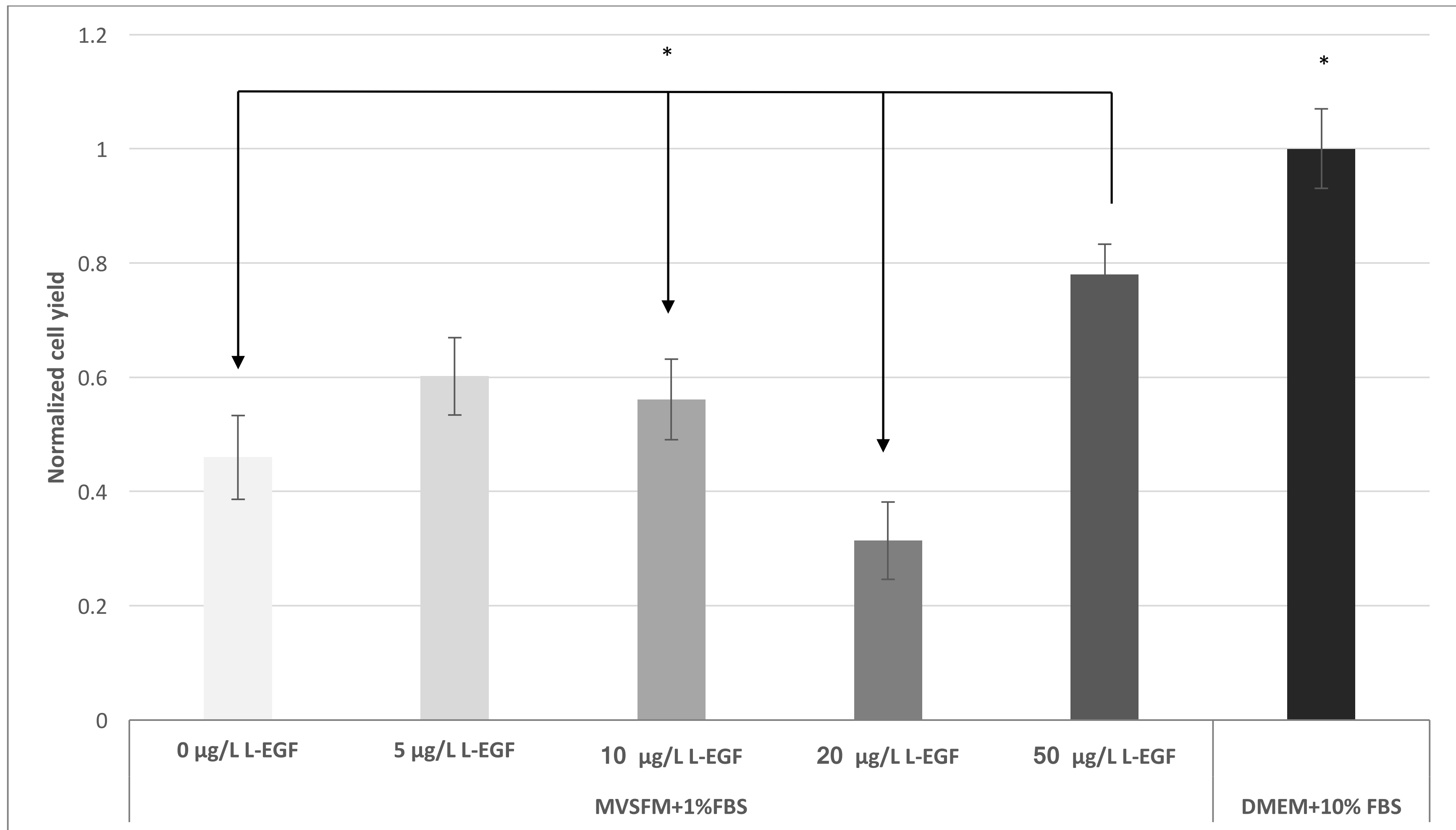


Figure 3.3 Normalized cell yields of MRC-5 cells in MVSFM+1% FBS using varying concentrations of L-EGF (n=3). Cell yields were normalized to the positive control DMEM+10%FBS. * denotes significant statistical difference at $p < 0.05$. Error bars are shown as SEM.

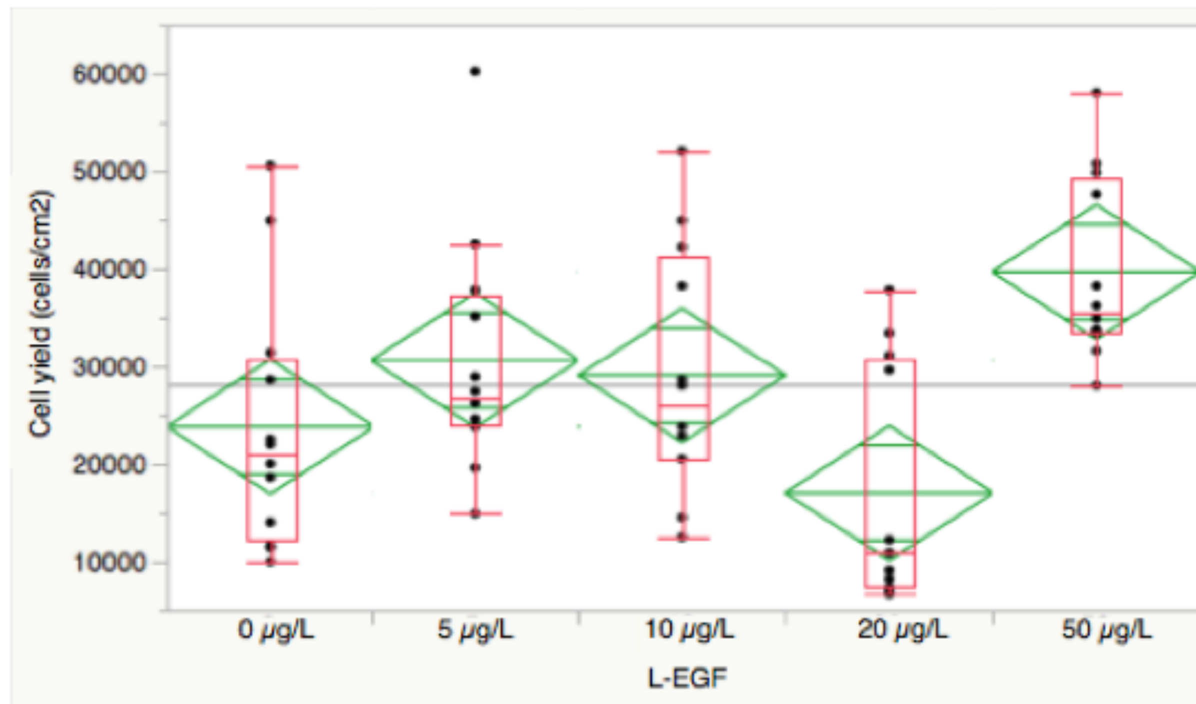


Figure 3.4 Box plot and mean diamonds of the cell yield of MRC-5 cells in MVSFM+1% FBS using varying concentrations of L-EGF (n=3). The middle line indicates the population mean.

3.4.2 Optimization of L-EGF in Vero cells

Vero cells initially maintained in DMEM+10% FBS were subcultured in T-25 flasks in duplicate into the experimental media MVSFM supplemented with a reduced serum concentration (1% FBS) and L-EGF at the concentrations shown on Table 3.3. The positive control was DMEM + 10% FBS and the negative control was MVSFM + 1% FBS without EGF. Cells were counted at day four and passaged in the test media for three sequential passages to observe the effect of L-EGF in a reduced serum media as well as for statistical significance. The data analysis was performed as described in Section 3.3.

Similar to the observations in MRC-5 cells, the correlation between the concentration of L-EGF and cell yield was not linear (Figure 3.5). The overall impact of L-EGF on Vero cell yield was evaluated performing a one-way ANOVA. As it was observed from the box plot (Figure 3.6), the highest group mean was obtained at 10 μg/L L-EGF. When the cell yield was compared to the negative control using Dunnett's test and Student's t-test (p-value = 0.0842), both tests showed that the 10μg/L concentration was not significantly different from the negative control.

Despite this result, the Student's t-test showed the cell yield for 10 μg/L was significantly different from the 5 μg/L concentration (Figure 3.5) and the doubling-time was the shortest one from all the experimental media (Table 3.5). This result although inconclusive, suggests that the original concentration value for the MVSFM formulation as described by Burgener (2000) was optimal. As a result, the concentration of L-EGF was maintained at the same value as the one in the original MVSFM formulation.

Table 3.5 Doubling-time of Vero cells in MVSFM + 1% FBS in the different concentrations of L-EGF compared to the positive control (DMEM + 10 % FBS). The n= 3 except for 10 µg/L (n=2).

MVSFM + 1% FBS	**Doubling-time (hours)**	**SEM (hours)**
0 µg/L L-EGF	39.3	1.6
5 µg/L L-EGF	41.3	2.5
10 µg/L L-EGF	32.6	2.7
20 µg/L L-EGF	35.6	1.5
50 µg/L L-EGF	34.2	2.9
DMEM+10% FBS	26.1	0.8

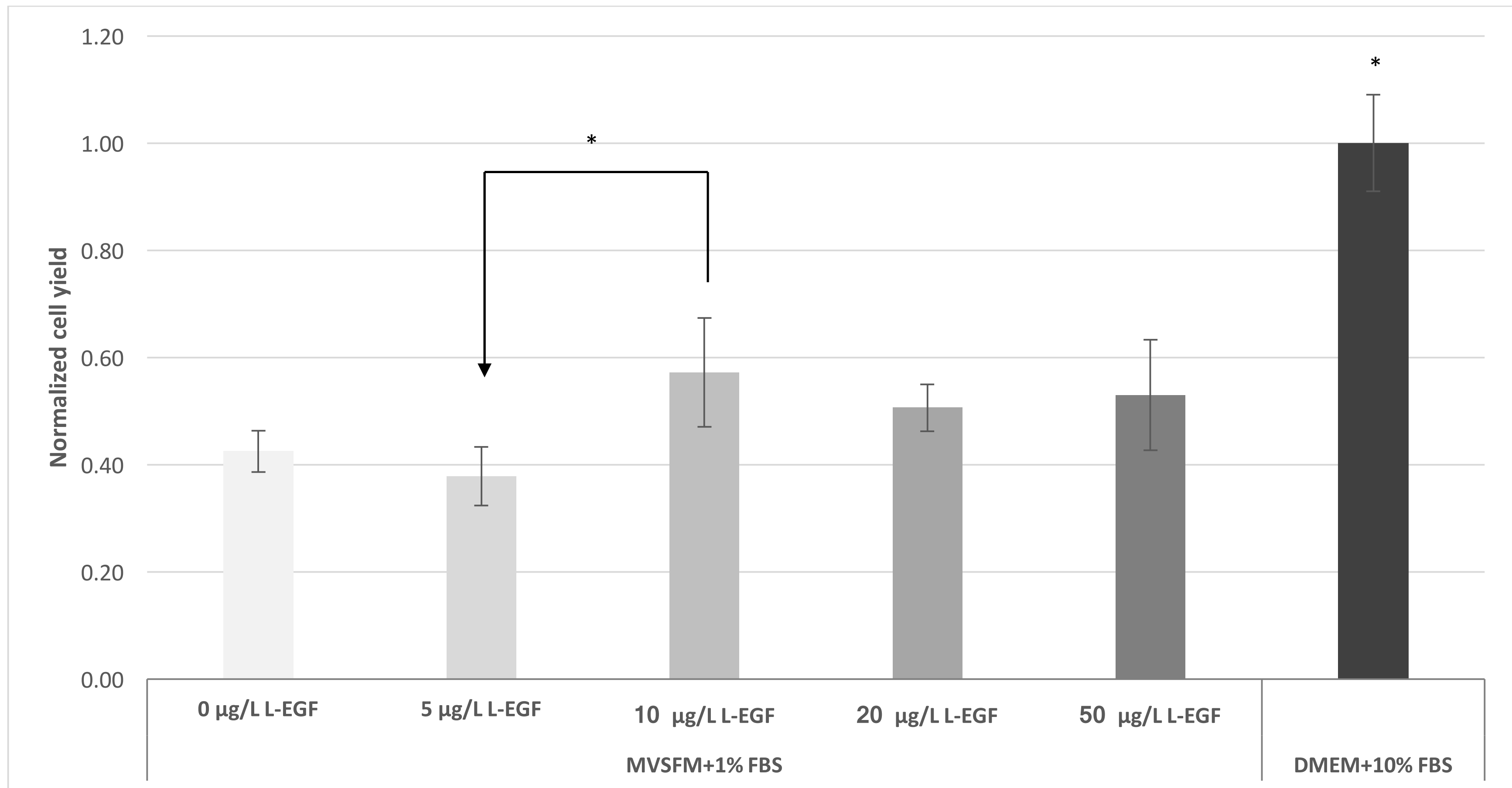


Figure 3.5 Normalized cell yield of Vero cells in MVSFM+1% FBS using varying concentrations of L-EGF. Cell yields were normalized to the positive control DMEM+10%FBS. * denotes significant statistical difference at $p < 0.05$. Error bars are shown as SEM (n=3) (10µg/L: n=2).

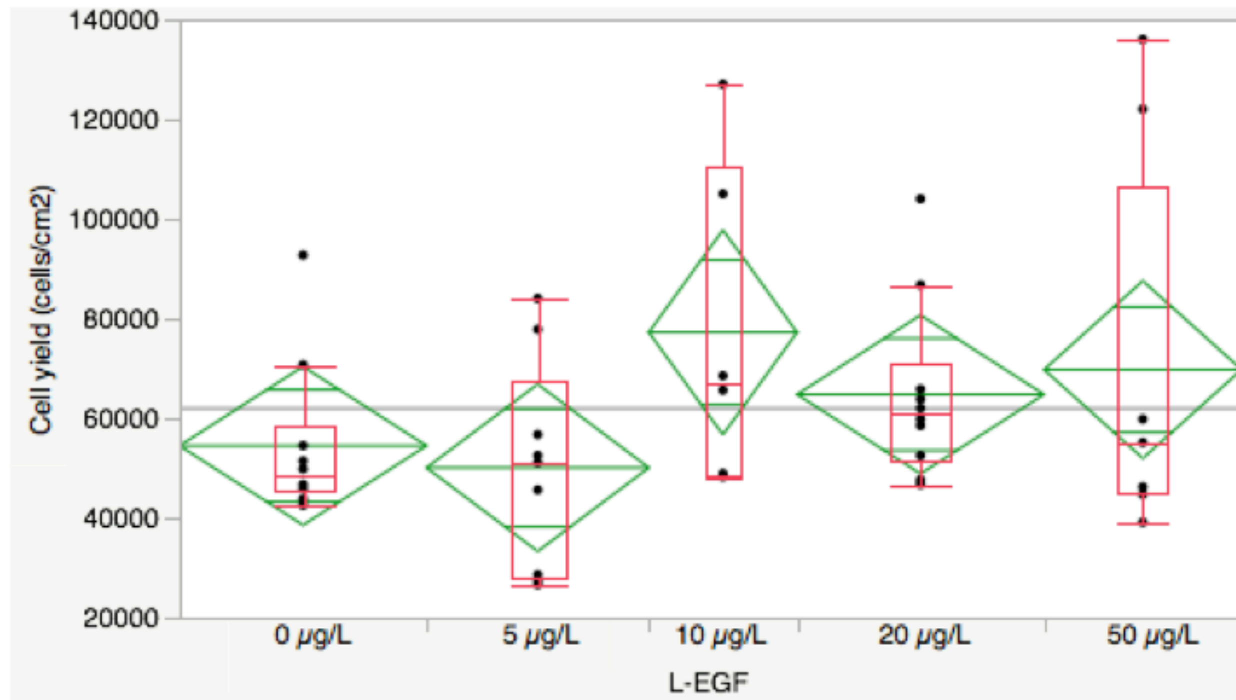


Figure 3.6 Box plot and mean diamonds of the cell yield of Vero cells in MVSFM+1% FBS using varying concentrations of L-EGF (n=3) (10µg/L: n=2). The middle line indicates the population mean. It can be observed that 10µg/L concentration was the highest group mean of all, although it was not significantly different from the negative control.

3.4.3 Effect of L-EGF on MRC-5 and Vero cells in OFAT experiments

The concentration ranges of L-EGF used in this experiment were based upon the original concentration of EGF used in MVSFM, as well as the literature value found in Hetzel *et al.* (2005), and were the mid-point concentrations between the negative control (0 µg/L), the original value (10 µg/L), and the one used in the aforementioned study (50 µg/L). This concentration was selected as Hetzel *et al.* (2005) determined that the highest cell yield achieved by normal human lung fibroblast cells was induced by the addition of 50 µg/L of EGF. In this study the cell yield was slightly higher at a concentration of 3 µg/L EGF, which exerted a similar effect to the 10 µg/L as shown using OFAT experiments, and the cell yield increased greatly at 50 µg/L EGF. The results using OFAT experiments with MRC-5 cells agree with this study. MRC-5 cell proliferation was induced by EGF. This result is supported by a study by Throm *et al.,* (2009), who found that collagen production in fibroblasts had a bimodal response to increasing concentrations of EGF, having a direct impact on attachment and consequently on cell proliferation.

In the case of Vero cells, the optimal EGF concentration was shown to be 10 µg/L. This result is consistent with the results of Burgener (2000), who also reported the optimal concentration of EGF to be 10 µg/L. However, the Student's t-test revealed that there was no statistically significant difference between this concentration and the negative control. This likely occurred because one 10 µg/L sample was lost and there was a larger sample size for the negative control. Due to the fact that both results agreed, this matter was not further investigated.

It is widely known that EGF can stimulate mitogenesis in EGF-responsive cells, and secretion of non-collagenous proteins in fibroblasts (Goldstein *et al.,* 1989; Kelley, 1992 and Hetzel *et al.*, 2005; Throm *et al.,* 2009). The results show that overall, both cell lines are responsive

to L-EGF and that this growth factor was capable of stimulating cell growth as described in the literature. However, the proliferative effect of L-EGF was concentration-dependent and the optimal concentrations differed for both cell lines.

3.4.4 Optimization of L-R^3-IGF-I in MRC-5 cells

LONG-R^3-IGF-I (L-R^3-IGF-I) was originally used in the MVSFM medium formulation, and thus, was not compared to any equivalent form of this growth factor. MRC-5 cells initially maintained in DMEM+10% FBS were subcultured in T-25 flasks in duplicate into the experimental media MVSFM supplemented with a reduced serum concentration (1% FBS) with the addition of L-R^3-IGF-I at the concentrations outlined on Table 3.3. The positive control was DMEM +10% FBS and the negative control was MVSFM + 1% FBS without L-R^3-IGF-I. Cells were counted at day four and passaged in the test media for three sequential passages to observe the long-term effect of L-R^3-IGF-I in a reduced serum media as well as for statistical significance. The data analysis was performed as described in Section 3.3.

The data from this experiment displayed a similar trend to that observed with L-EGF; that is, there was no linear correlation between cell yield and L-R^3-IGF-I concentration. The Dunnett's test shows that only 10 μg/L was significantly different from the control, no other statistical differences were observed (Figure 3.7). As shown in Table 3.6, the shortest doubling-time was achieved at this concentration; however, it was significantly different from the positive control. One-way ANOVA analysis confirmed that at least one of the concentrations (10 μg/L) differed from the others (Figure 3.8). The Student's-t test suggested that both the 10 and 80 μg/L concentrations were significantly different from the negative control at $p < 0.05$.

L-R^3-IGF-I did not have as positive an impact on MRC-5 cell yield as L-EGF. The highest cell yield obtained with L-R^3-IGF-I was approximately 60% (Figure 3.8) of the positive control, whereas the one obtained with L-EGF was about 80% of the positive control (see Figure 3.3). Thus, L-R^3-IGF-I seemed to have a lower positive impact compared to L-EGF in MRC-5 cell yield. As a result, the concentration of L-^{3}-IGF-I was decreased to 10 μg/L in MVSFM for MRC-5 cells.

Table 3.6 Doubling-time of MRC-5 cells in MVSFM + 1% FBS in the different concentrations of L-R^3-IGF-I compared to the positive control (DMEM + 10 % FBS) n=3.

MVSFM + 1% FBS	**Doubling-time (hours)**	**SEM (hours)**
0 μg/L L-R^3-IGF	96.7	3.7
10 μg/L L-R3-IGF	58.1	4.9
40 μg/L L-R3-IGF	78.8	5.3
80 μg/L L-R3-IGF	62.4	4.6
100 μg/L L-R3-IGF	69.9	4.1
DMEM+10% FBS	40.7	2.8

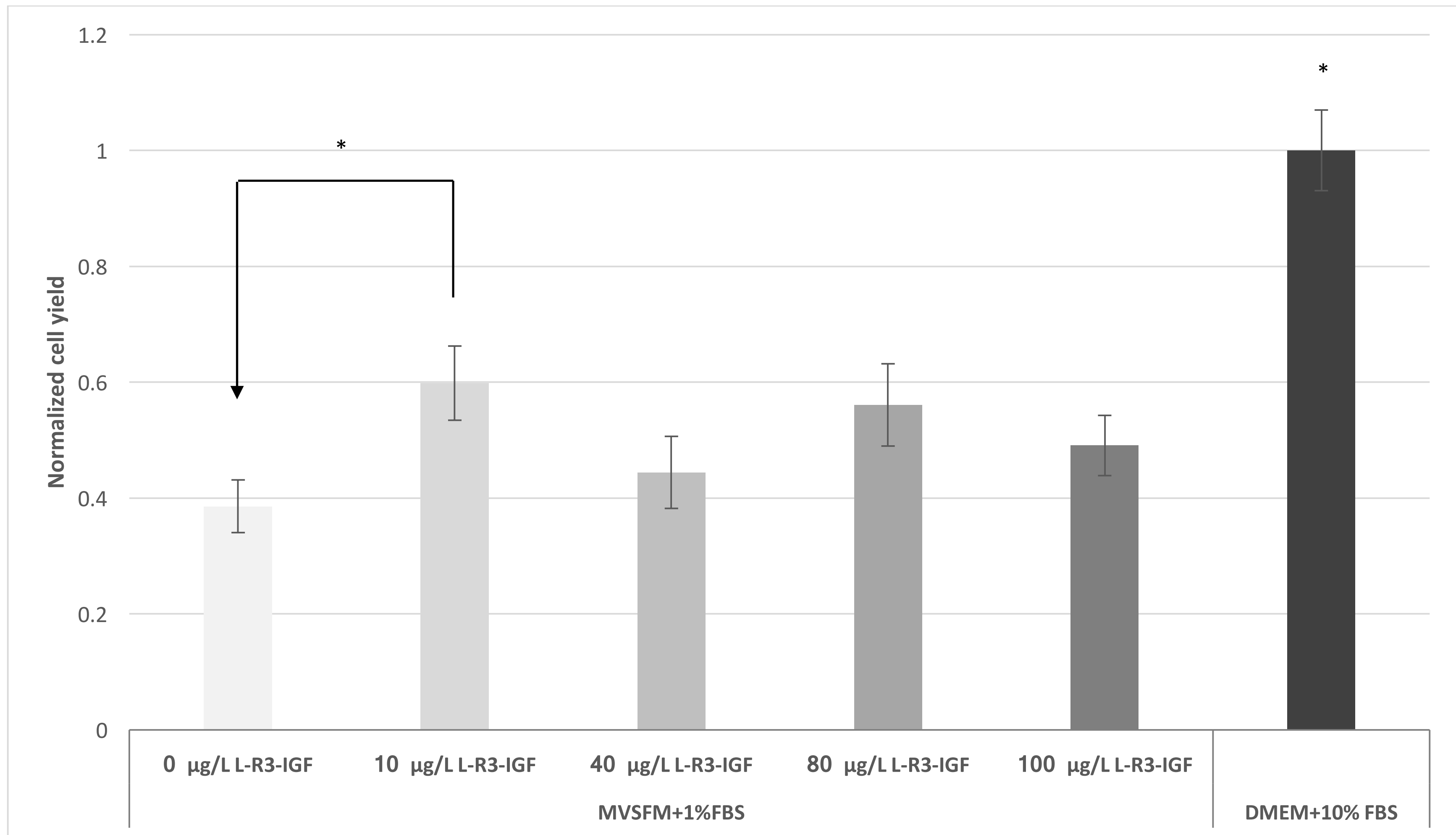


Figure 3.7 Normalized cell yield of MRC-5 cells in MVSFM+1% FBS using varying concentrations of L-R^3-IGF. Cell yields were normalized to the positive control DMEM+10%FBS. *, denotes significant statistical difference at $p < 0.05$ as determined by Dunnett's test. Error bars are shown as standard error of the mean SEM (n=3).

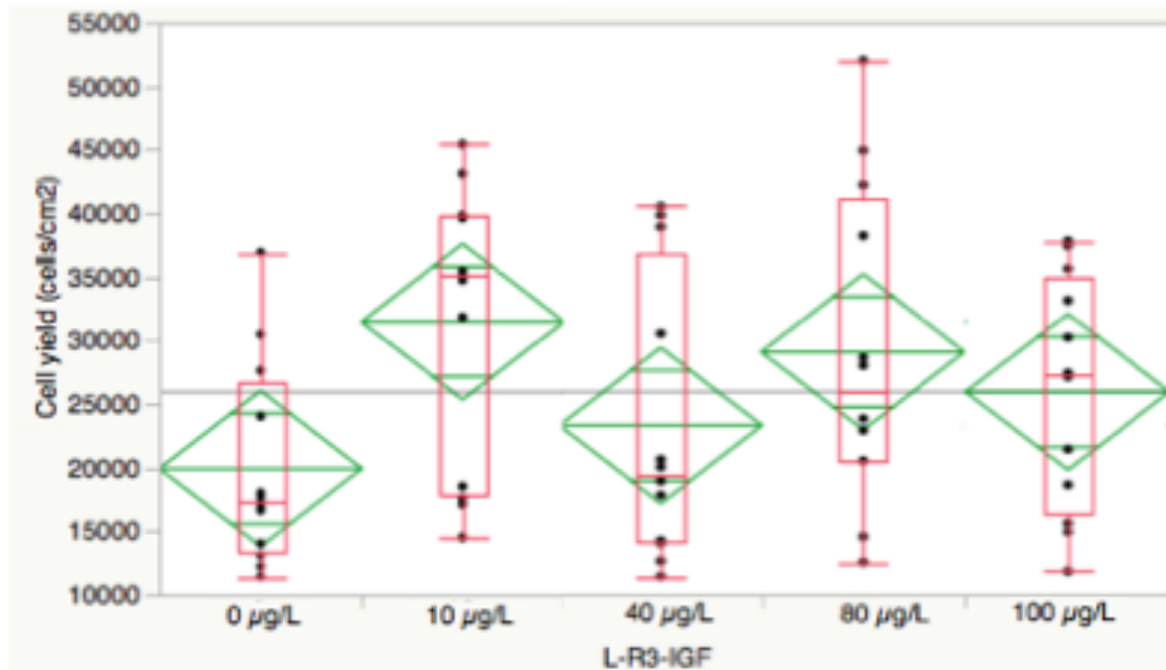


Figure 3.8 Box plot and mean diamonds of the cell yield of MRC-5 cells in MVSFM+1% FBS using varying concentrations of L-R^3-IGF-I. The middle line indicates the population mean. The null hypothesis of the ANOVA was rejected and 10 μg/L was found to be significantly different from the negative control by Dunnett's test. The Box Plot also shows that the 80 μg/L concentration was significantly different from the negative control at $p < 0.05$, as indicated by the Student's t-test (n=3).

3.4.5 Optimization of L-R^3-IGF-I in Vero cells

Vero cells initially maintained in DMEM+10% FBS were subcultured in T-25 flasks in duplicate into the experimental media MVSFM supplemented with a reduced serum concentration (1% FBS) with the addition of L-R^3-IGF-I at the concentrations outlined on Table 3.3. The positive control was DMEM +10% FBS and the negative control was MVSFM without L-R^3-IGF-I. Cells were counted at day four and passaged in the test media for three sequential passages to observe the long-term effect of L-R^3-IGF-I in a reduced serum media as well as for statistical significance. The data analysis was performed as described in Section 3.3.

Vero cells displayed a positive linear correlation between the increase in L-R^3-IGF-I and cell yield up to 80 µg/L, which was the concentration at which highest cell yield was achieved. After that, the cell yield decreased significantly at 100 µg/L as seen in Figure 3.9. It was observed that the impact of L-R^3-IGF-I on Vero cell yield was comparable to the one elicited by L-EGF as the highest cell yield obtained was 60% of that of the positive control in both cases (see Figure 3.5 and 3.9).

One-way ANOVA showed that at least one of the concentration levels was significantly different than the population mean (Figure 3.10). When 80 µg/L was compared to the negative control (0 µg/L) by the Student's t-test and Dunnett's test it was found that it was significantly different at $p < 0.05$. This concentration displayed the shortest doubling-time compared to all the other experimental media but it was longer than the positive control (Table 3.7), which was significantly different according to the Student's t-test. This result confirmed the values of the original concentration for L-R^3-IGF-I in MVSFM as optimized previously by Burgener (2000).

Table 3.7 Doubling-time of Vero cells in MVSFM + 1% FBS in the different concentrations of L-R^3-IGF-I compared to the positive control (DMEM + 10 % FBS). The n= 3 except for 80 µg/L (n=2).

MVSFM + 1% FBS	**Doubling-time (hours)**	**SEM (hours)**
0 µg/L L-R^3-IGF	44.8	2.3
10 µg/L L-R3-IGF	43.3	2.0
40 µg/L L-R3-IGF	38.4	3.8
80 µg/L L-R3-IGF	32.6	2.7
100 µg/L L-R3-IGF	39.8	3.0
DMEM+10% FBS	26.1	0.8

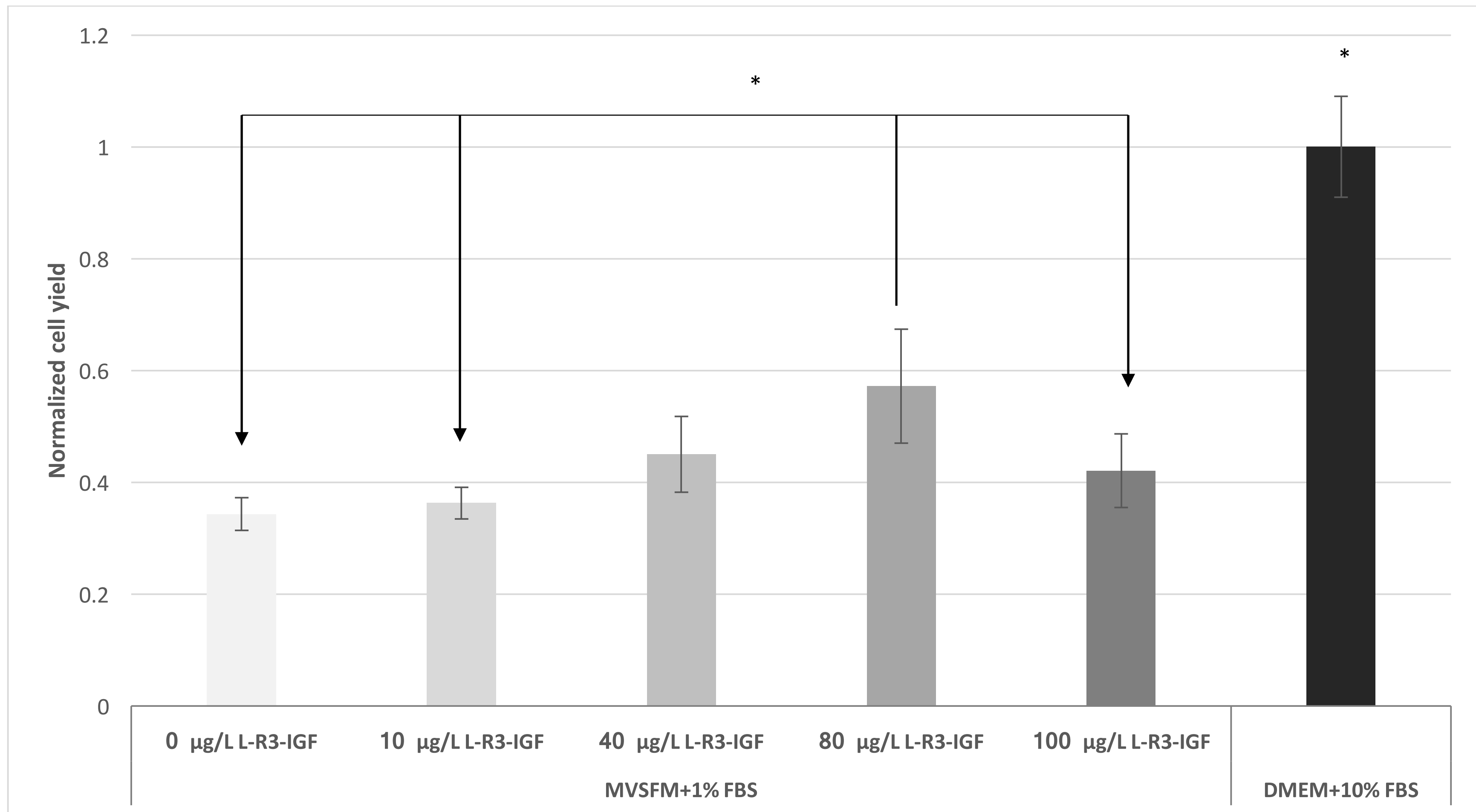


Figure 3.9 Normalized cell yield of Vero cells in MVSFM+1% FBS using varying concentrations of L-R^3-IGF-I. Cell yields were normalized to the positive control DMEM+10%FBS. *, denotes significant statistical difference at $p < 0.05$. Error bars are shown as SEM (n=3).

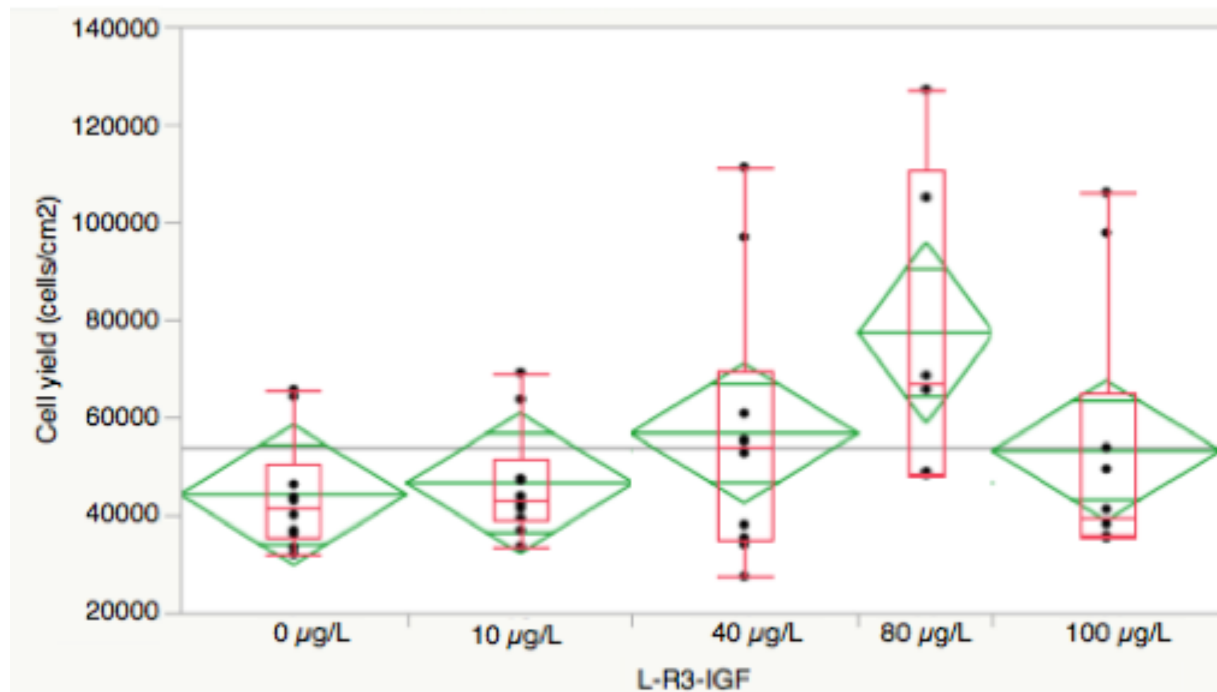


Figure 3.10 Box plot and mean diamonds of the cell yield of Vero cells in MVSFM+1% FBS using varying concentrations of L-R^3-IGF-I (n=3) (10µg/L: n=2). The middle line indicates the population mean. The null hypothesis of the ANOVA was rejected and 80 µg/L was found to be significantly different from the negative control.

3.4.6 Effect of L-R^3-IGF-I on MRC-5 and Vero cells in OFAT experiments

Insulin-like Growth Factor-I is a peptide that induces a mitogenic response, inhibits programmed cell death (apoptosis), and promotes cell proliferation and differentiation. IGF-I can be inhibited by different IGFBPs, which render it inactive and inhibit DNA synthesis (Kelley, 1992). The analog, L-R^3-IGF-I has proven to be a substitute for IGF-I as it is 20-fold more potent because it has little affinity for IGFBPs, which make it a more suitable supplement in a SFM (Yateman *et al.,* 1992). For this reason, it was chosen to optimize MVSFM for MRC-5 and Vero cells.

The results demonstrated that in the case of MRC-5 cells, two different concentrations of L-R^3-IGF-I elicited the same effect on MRC-5 cells: 10 μg/L and 80 μg/L. The latter result agrees with the optimized concentration reported by Burgener (2000), whereas the former was subjected to further testing. Since the Student's t-test revealed that both concentrations were significantly different from the negative control, a Dunnett's test was used for hypothesis testing in order to elucidate which concentration was the most optimal for MRC-5 cells. The Dunnett's tests allows for multiple testing of the treatment samples with a single control group by using narrower confidence intervals and reducing the error rate in the hypothesis testing (Lawrence, 2011). The Dunnett's test showed that the 10 μg/mL was statistically different from the negative control and thus, was the optimal concentration for MRC-5 cells. On the other hand, from a cost-effective standpoint, the 10 μg/mL was preferred to 80 μg/L in order to create a SFM. This concentration is slightly lower than the one reported by Yateman *et al*., (1992), where they showed that the effective range of stimulation of IGF-I in human dermal fibroblasts was 20-100 μg/L.

The results for Vero cells agreed with the previous findings of Burgener (2000) and were not subject to change. This experiment showed that MVSFM was optimal for Vero cells, and because Burgener used the OFAT experimental methodology for his previous work, the current work is consistent with these previous findings. As such, it was concluded that L-R^{3-} IGF-I has an effect on cell proliferation in both cell lines, as previously shown in other cell lines such as BALB/c 3T3 and WI-38 (Kelley, 1992; Pietrzkowski *et al.,* 1992). However, as observed in the previous experiment, the concentration differs in MRC-5 cells, which are fibroblasts, and Vero cells, which are of epithelial origin.

3.4.7 Optimization of rTransferrin-linoleic acid complex in MRC-5 cells

The rTransferrin used in these experiments was iron saturated (holo-transferrin) and ready for iron-delivery in cell culture. To test the effect of rTransferrin in MRC-5 cells, it was complexed with linoleic acid to enhance its bioactivity, as it is a carrier molecule that may interact with lipids in the membrane. The concentration of linoleic acid used was 0.54 μg/L, similar to the one contained in the Biogro CHO medium. Linoleic acid was solubilized using 20 mg/L of MβCD which was then thoroughly vortexed to complex with rTransferrin.

MRC-5 cells initially maintained in DMEM+10% FBS were subcultured in T-25 flasks in duplicate into the experimental media MVSFM supplemented with a reduced serum concentration (1% FBS) with the different concentrations of rTransferrin outlined in Table 3.3. The positive control was DMEM +10% FBS. Two negative controls were prepared, the first one was MVSFM + 1% FBS with no rTransferrin and no linoleic acid and the second one was MVSFM + 1% FBS

with linoleic acid but without rTransferrin. Cells were counted at day four and passaged in the test media for three sequential passages to observe the long-term effect of rTransferrin and linoleic acid in a reduced serum media and to obtain statistical significance. The data analysis was performed as described in Section 3.3.

As it can be observed in Figure 3.11, there was no significant effect on the cell yield of MRC-5 between the tested concentrations of rTransferrin. Figure 3.12 shows all the mean diamonds overlap with the population grand mean showing no statistical difference. This result was confirmed by a one-way ANOVA. The media containing: 0 µg/L rTransferrin + 0 mg/L linoleic acid and that containing, 0.54 mg/L linoleic acid and 50 µg/L rTransferrin showed very similar doubling-times ranging from 62.4 hours, to 67 hours. The longest doubling-time was obtained using 10 µg/L rTrasferrin and the shortest one was at 100 µg/L rTrasferrin. The variation was of 18.6 hours between the fastest and shortest doubling-time (Table 3.8) but the Student's t-test showed that there was no significant difference between the doubling times. Hence, it was concluded that rTransferrin did not have any significant effect on MRC-5 cell yield and therefore, it was not added into the optimized media formulation.

Table 3.8 Doubling-time of MRC-5 cells in MVSFM + 1% FBS in the different concentrations of rTransferrin and 0.54mg/L linoleic acid complex compared to the positive control (DMEM + 10 % FBS) n = 3.

MVSFM + 1% FBS	**Doubling-time (hours)**	**SEM (hours)**
0 µg/L transf 0 mg/L linol	62.4	6.6
0.54 mg/L linol	65.4	6.4
10 µg/L transf + linol	74.4	6.5
50 µg/L transf + linol	67	3.8
100 µg/L transf + linol	55.8	5.8
DMEM+10% FBS	40.7	4.8

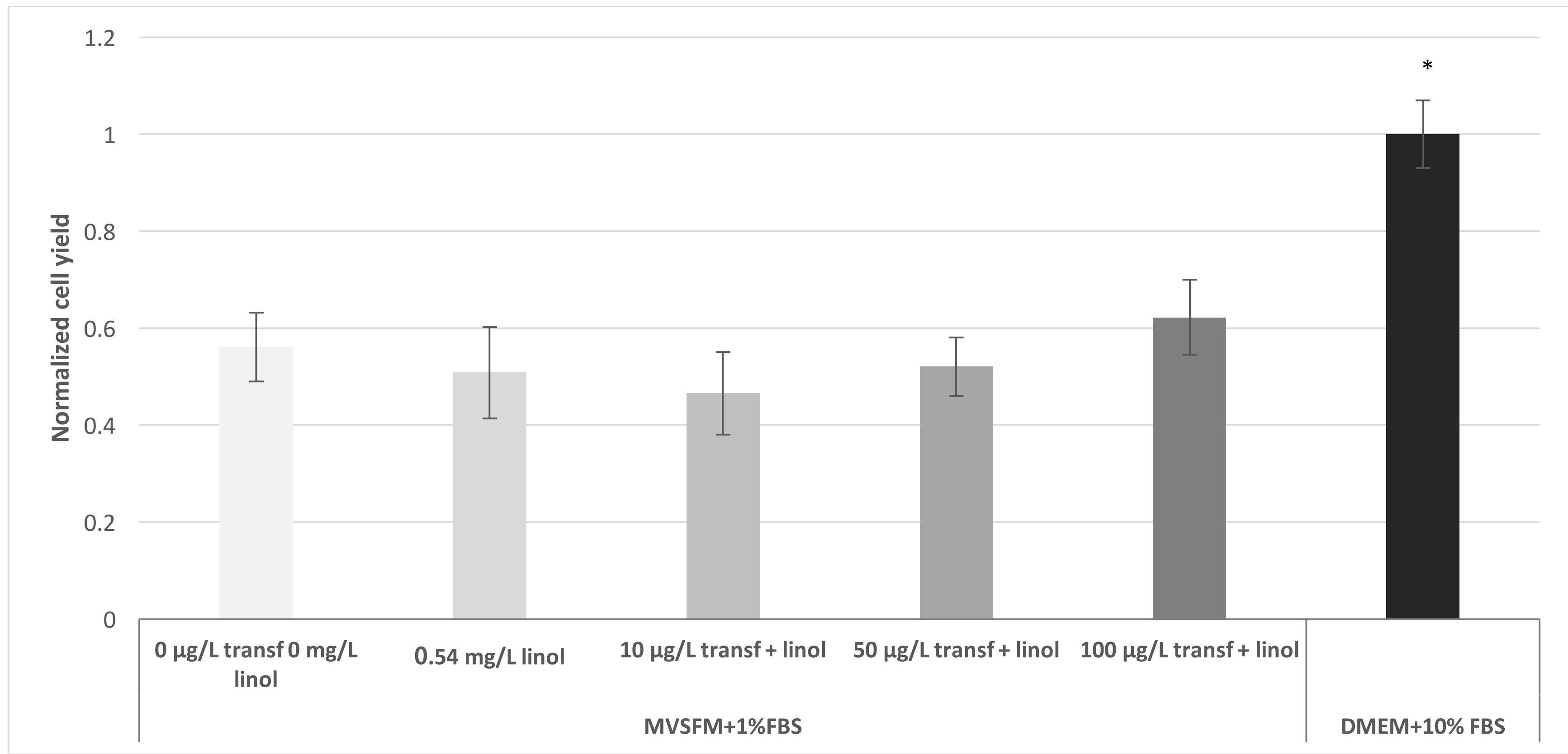


Figure 3.11 Normalized cell yield of MRC-5 cells in MVSFM+1% FBS using varying concentrations of rTransferrin with a constant amount of linoleic acid (0.54 mg/L). Cell yields were normalized to the positive control DMEM+10%FBS. *, denotes significant statistical difference at $p < 0.05$. Error bars are shown as SEM (n=3).

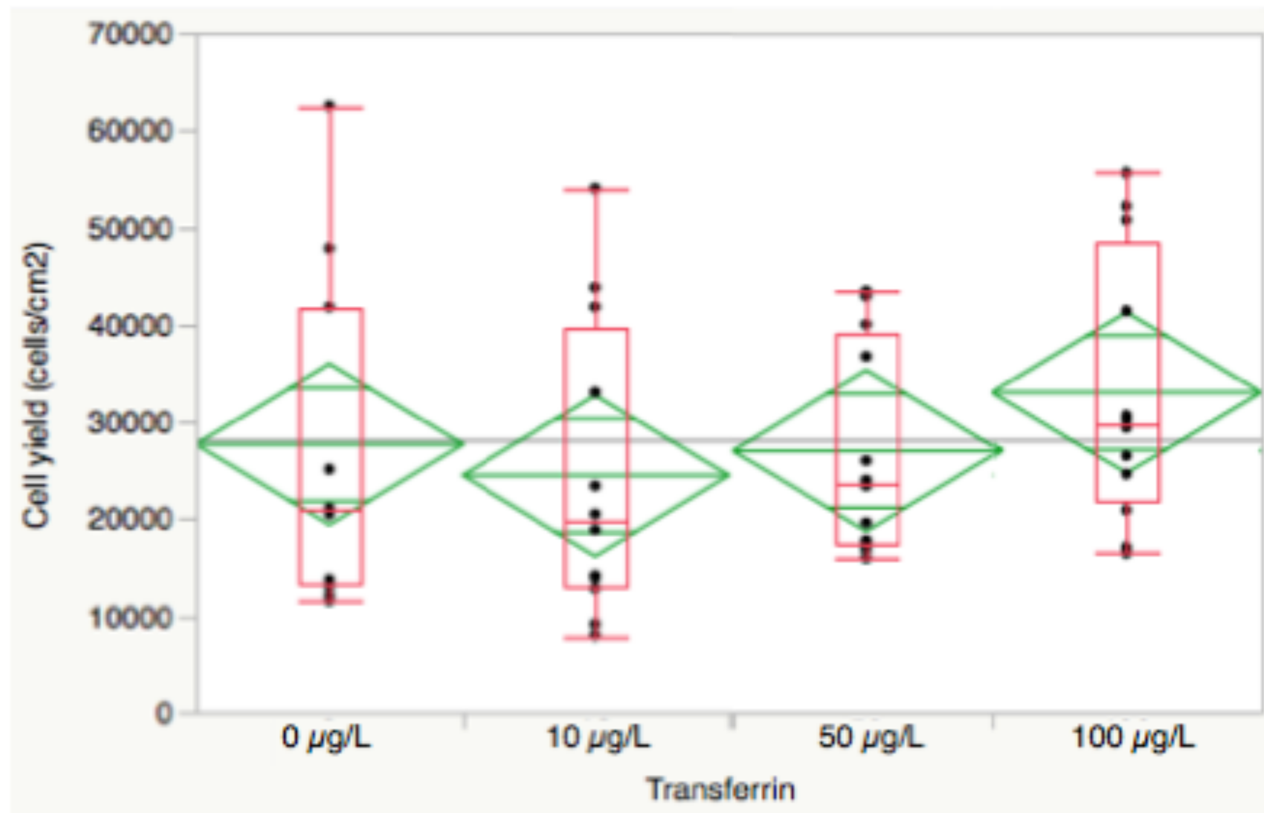


Figure 3.12 Box plot and mean diamonds of the cell yield of MRC-5 cells in MVSFM+1% FBS using varying concentrations of rTransferrin. The null hypothesis from ANOVA was not rejected meaning that there is no difference between concentrations (n=3).

3.4.8 Optimization of rTransferrin-linoleic acid complex in Vero cells

Vero cells initially maintained in DMEM+10% FBS were subcultured in T-25 flasks in duplicate into the experimental media MVSFM supplemented with a reduced serum concentration (1% FBS) with the addition of the different concentrations of rTransferrin outlined in Table 3.3 into the media. The positive control was DMEM +10% FBS. Two negative controls were prepared, the first one was MVSFM + 1% FBS with no rTransferrin and no linoleic acid and the second one was MVSFM + 1% FBS with linoleic acid but without rTransferrin. Cells were counted at day four and passaged in the test media for three sequential passages to observe the long-term effect of the rTransferrin and linoleic acid in a reduced serum media and for statistical significance. The data analysis was performed as described in Section 3.3.

Similar, to the result obtained with MRC-5 cells, it was noted that rTransferrin did not elicit any effect on Vero cell yield (Figure 3.13). There was no significant difference observed in cell yield throughout the concentration ranges of rTransferin tested on Vero cells. This result was confirmed by a one-way ANOVA (Figure 3.14). For this reason, rTransferrin was not incorporated into the optimized formulation for Vero cells. The doubling-times show low variability across the different concentrations of rTransferrin (from 32.6 to 38.7 hours) and they were not significantly different from each other as shown by the Student's t-test ($p < 0.05$). The most frequent doubling-time was 35 hours which indicates that rTransferrin did not elicit any impact on Vero cell yield (Table 3.9).

Table 3.9 Doubling-time of Vero cells in MVSFM + 1% FBS in the different concentrations of rTransferrin and 0.54mg/L linoleic acid complex compared to the positive control (DMEM + 10% FBS) (n=3).

MVSFM + 1% FBS	**Doubling-time (hours)**	**SEM (hours)**
0 µg/L transf 0 mg/L linol	32.6	2.7
0.54 mg/L linol	35.1	1.6
10 µg/L transf + linol	35.7	2.8
50 µg/L transf + linol	35.5	2.1
100 µg/L transf + linol	38.7	3.2
DMEM+10% FBS	26.1	0.8

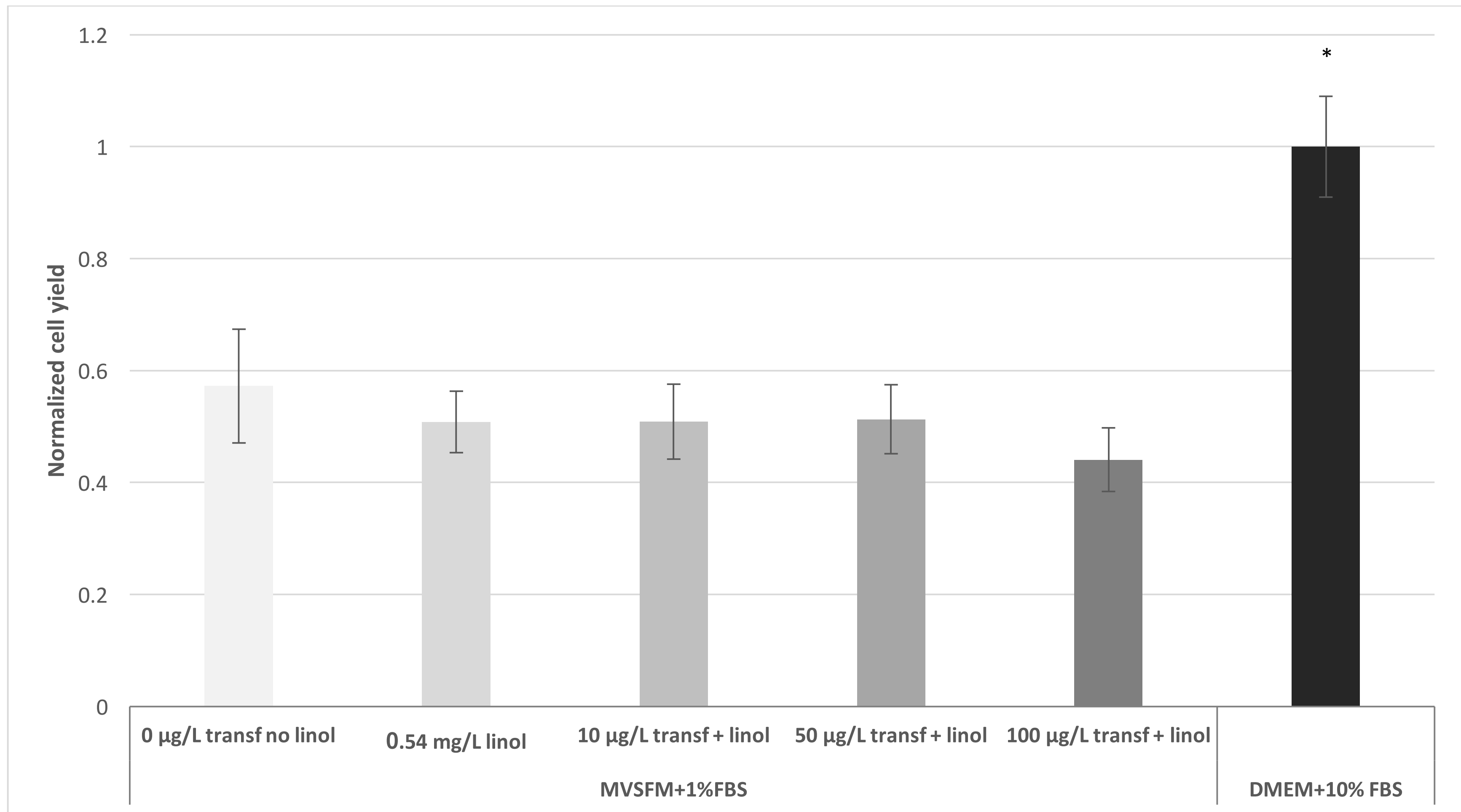


Figure 3.13 Normalized cell yield of Vero cells in MVSFM+1% FBS using varying concentrations of rTransferrin (transf) with a constant amount of linoleic acid of 0.54 mg/L. Cell yields were normalized to the positive control DMEM+10%FBS. *, denotes significant statistical difference at $p < 0.05$. Error bars are shown as SEM (n=3).

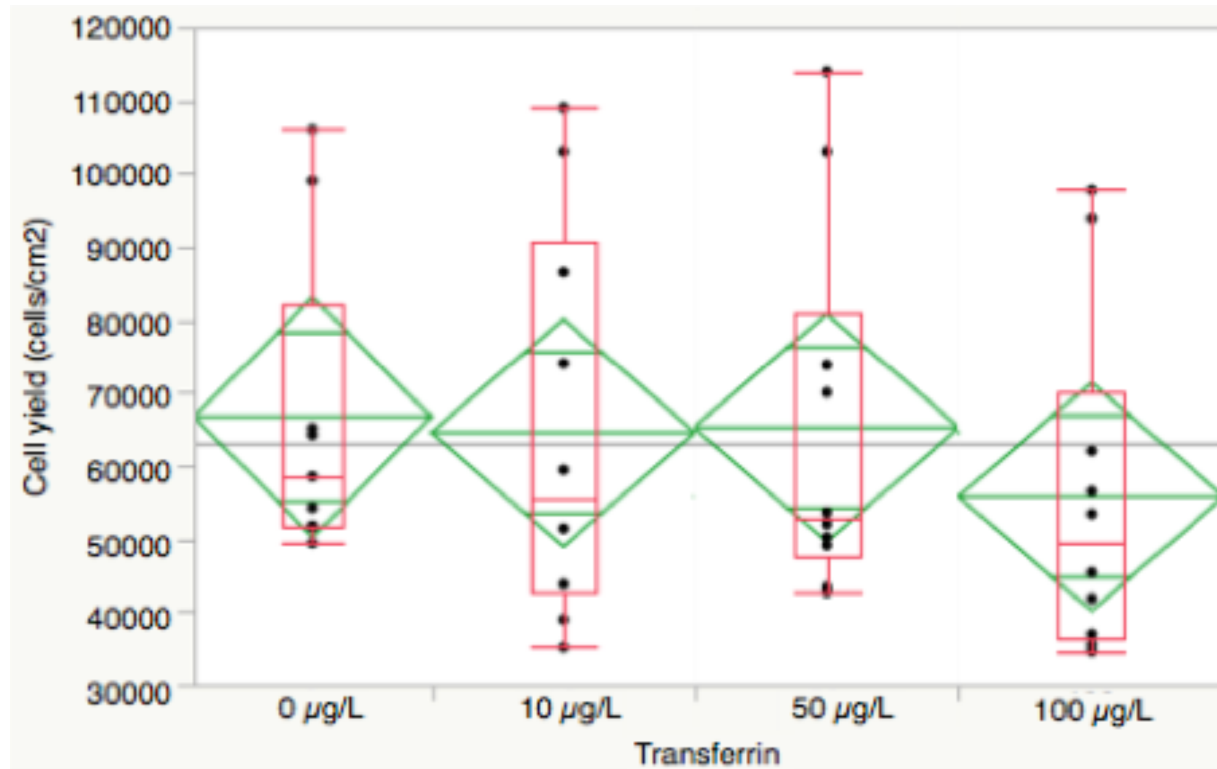


Figure 3.14 Box plot and mean diamonds of the cell yield of Vero cells in MVSFM + 1% FBS using varying concentrations of rTransferrin (n=3). The null hypothesis of the ANOVA was not rejected, indicating that there is no difference between concentrations.

3.4.9 Comparison between rTransferrin and $FeSO_4$

To further investigate why there was no effect observed on cell yield after using rTransferrin in either cell line, the experiment was revised. A novel MVSFM was formulated without the presence of $FeSO_4$, as contained in the original formulation (3.6 μM), to evaluate the sole effect of rTransferrin. In addition, linoleic acid may have interfered with its bioactivity; thus, it was eliminated from the test formulation.

MRC-5 cells initially maintained in DMEM+10% FBS were subcultured in T-25 flasks in duplicate into the experimental media MVSFM supplemented with a reduced serum concentration (1% FBS) with the different concentrations of rTransferrin outlined in Table 3.3 without linoleic acid. The positive control was DMEM +10% FBS. Two negative controls were prepared, the first one was MVSFM + 1% FBS with no rTransferrin and no $FeSO_4$ and the second one was the original MVSFM + 1% FBS containing 3.6 μM $FeSO_4$. Cells were counted at day four and passaged in the test media for two sequential passages. Error bars were calculated as standard error of the mean (SEM).

One-way ANOVA analysis showed that at least one concentration differed from the others (result not shown). A Student's t-test showed that the original MVSFM + 1% FBS (containing 3.6 μM $FeSO_4$) was significantly different to 100 μg/L of rTransferrin as shown in Figure 3.15. In this experiment, original MVSFM had the shortest doubling-time and 100 μg/L of rTransferrin had the longest doubling-time (Table 3.10) and they were shown to be significantly different from each other according to a Student's t-test. This result shows that the cells proliferated faster in the presence of 3.6 μM $FeSO_4$ than in any of the media containing rTransferrin. Thus, it was clear that rTransferrin did not elicit any overall effect in cell yield as there was no statistical significant

difference between MVSFM+1% FBS containing 3.6 μM $FeSO_4$ and the negative control without rTransferrin or $FeSO_4$. This may have occurred due to the fact that MVSFM was still supplemented with 1% FBS that contains serum transferrin, which is fulfilling the cell's iron requirements and/or due to the fact that MVSFM is based on DMEM which contains 0.1 mg/L ferric nitrate nonahydrate ($Fe(NO_3)_3 \cdot 9H_2O$). This medium component could not be eliminated from MVSFM and thus, it was determined that rTransferrin was not necessary in MVSFM.

Table 3.10 Doubling-time of MRC-5 cells in MVSFM + 1% FBS in the different concentrations of rTransferrin without $FeSO_4$ compared to MVSFM (with 3.6 μM $FeSO_4$) and the positive control (DMEM + 10 % FBS) n=2.

MVSFM + 1% FBS	**Doubling-time (hours)**	**SEM (hours)**
0 μg/L transferrin	37.1	1.8
10 μg/L transferrin	36.5	1.8
50 μg/L transferrin	37.4	1.3
100 μg/L transferrin	40.4	2.2
3.6 μM $FeSO_4$	34.1	1.1
DMEM+10% FBS	25.2	1.8

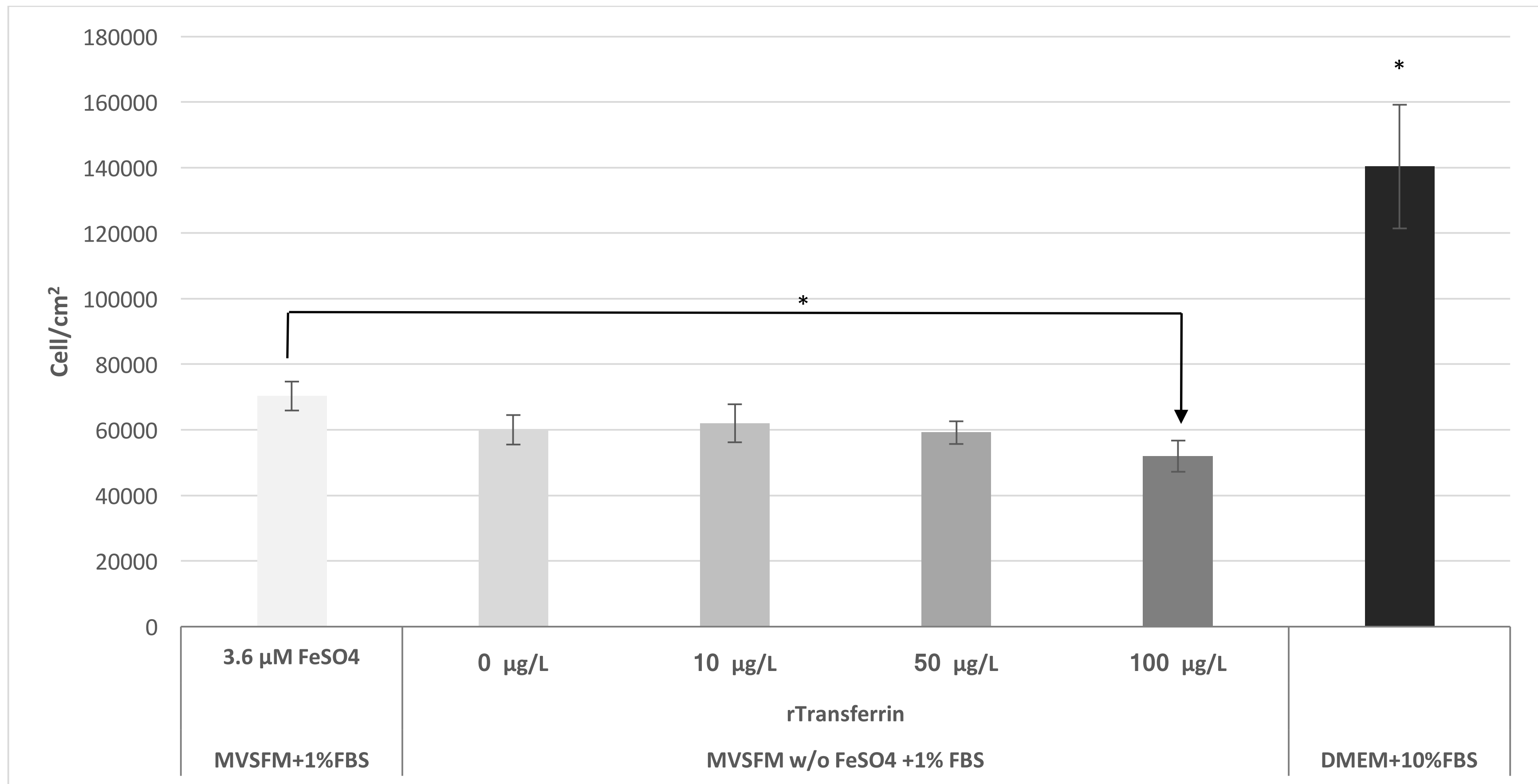


Figure 3.15 Cell yield of MRC-5 cells in MVSFM + 1% FBS using varying concentrations of rTransferrin without the addition of linoleic acid. The cell yields were compared with MVSFM originally supplemented with 3.6 µM $FeSO_4$ as a control (n=2). *, denotes a statistical significant difference at $p < 0.05$. Error bars are shown as SEM.

3.4.10 Effect of rTransferrin on MRC-5 and Vero cells in OFAT experiments

Transferrin is a protein that has been identified as a key protein for iron-delivery, which makes it essential for cell proliferation. Historically, cell culturists have relied upon this protein, along with insulin, selenium, and other growth factors, to support the growth of several cell lines (i.e. breast epithelium MCF-7, rat neuroblastoma, human diploid fibroblasts) in absence of serum (Bottenstein and Sato, 1979; Barnes and Sato, 1979; Kan and Yamane, 1984). For this reason, rTransferrin was tested in MVSFM along with linoleic acid in order to promote cell growth and proliferation of both MRC-5 and Vero cells. Linoleic acid was added because previous findings reported by Or *et al.* (1992) showed that the proliferative effect of transferrin was only observed after complexing it with linoleic acid. Since MVSFM did not contain transferrin as part of the formulation, four different concentrations that were similar to those used in the lab on previous studies and literature values from Freshney (2010), were used. The results show that rTransferrin complexed with linoleic acid did not elicit any effect on either cell line. This does not agree with the literature results aforementioned, which report that transferrin promotes cell growth.

As MVSFM contained 3.6 µM of $FeSO_4$, it was hypothesized that the iron was supplied to the cells by this source rather than by rTransferrin, which may be the reason that rTransferrin did not elicit cell growth. In order to reduce extraneous variables, linoleic acid was also eliminated in a subsequent experiment. Hence, an experiment was performed in which both, $FeSO_4$ and linoleic acid were eliminated from the basal medium to test the impact of rTransferrin on MRC-5 cells. The results from this experiment also showed no impact of rTransferrin in MRC-5 cell yield, confirming the previous result. The basal medium formulation contained $Fe(NO_3)_3 \cdot 9H_2O$, which could not be eliminated. Thus, it was concluded that $Fe(NO_3)_3 \cdot 9H_2O$ was supplying the cells with their iron requirements and thus rTransferrin was not necessary for either cell line.

3.5 Morphologic analysis in 1% FBS in all MVSFM

Throughout this experiments, cells were constantly observed under the microscope. Some morphological changes were observed after being passaged into a reduced concentration of serum in the media compared to the positive control (DMEM+10% FBS). Overall the changes were similar in all test media with reduced serum (1% FBS).

Morphologically, MRC-5 cells cultured in MVSFM+1%FBS (in all test media) were observed to become more elongated and with more cytoplasmic extensions (Figure 3.17C) compared to those in DMEM+10% FBS (Figure 3.17A). Few cells presented vacuolation (results not shown), this morphological change is typical of unhealthy cells that is due to the inadequacy of the medium (Freshney, 2010). In addition, vacuolization is a sign of autophagic cell death that was likely caused by serum starvation (González-Polo *et al.,* 2005). The cells cultured in low serum media became larger and adopted irregular shapes. Such morphological changes are typical of the senescent-like phenotype in normal human lung fibroblast as previously shown by Place and colleagues (2005), and Wistrom and Villeponteau (1990). After achieving confluence, cells started to regain their normal spindle-like shape when they became confluent (Figure 3.16D). However, it was still possible to observe some cytoplasmic enlargement compared cells grown in the positive control media (Figure 3.16B).

In the case of Vero cells, the morphology changed from a polygonal square shape when grown in DMEM+10%FBS (Figure 3.17A) to a more elongated, thinner, branched morphology, spindle-like shape when passaged into a reduced serum media (Figure 3.17C). This result was similar to those of Adams and colleagues (2015) where Vero cells presented similar morphological changes when they were put under nutritional stress. Once cells reached confluence, they started to regain their original square shape (Figure 3.17D); however, they did not achieve the same confluence level as those grown in the positive control grown in DMEM+10% FBS (Figure 3.17B).

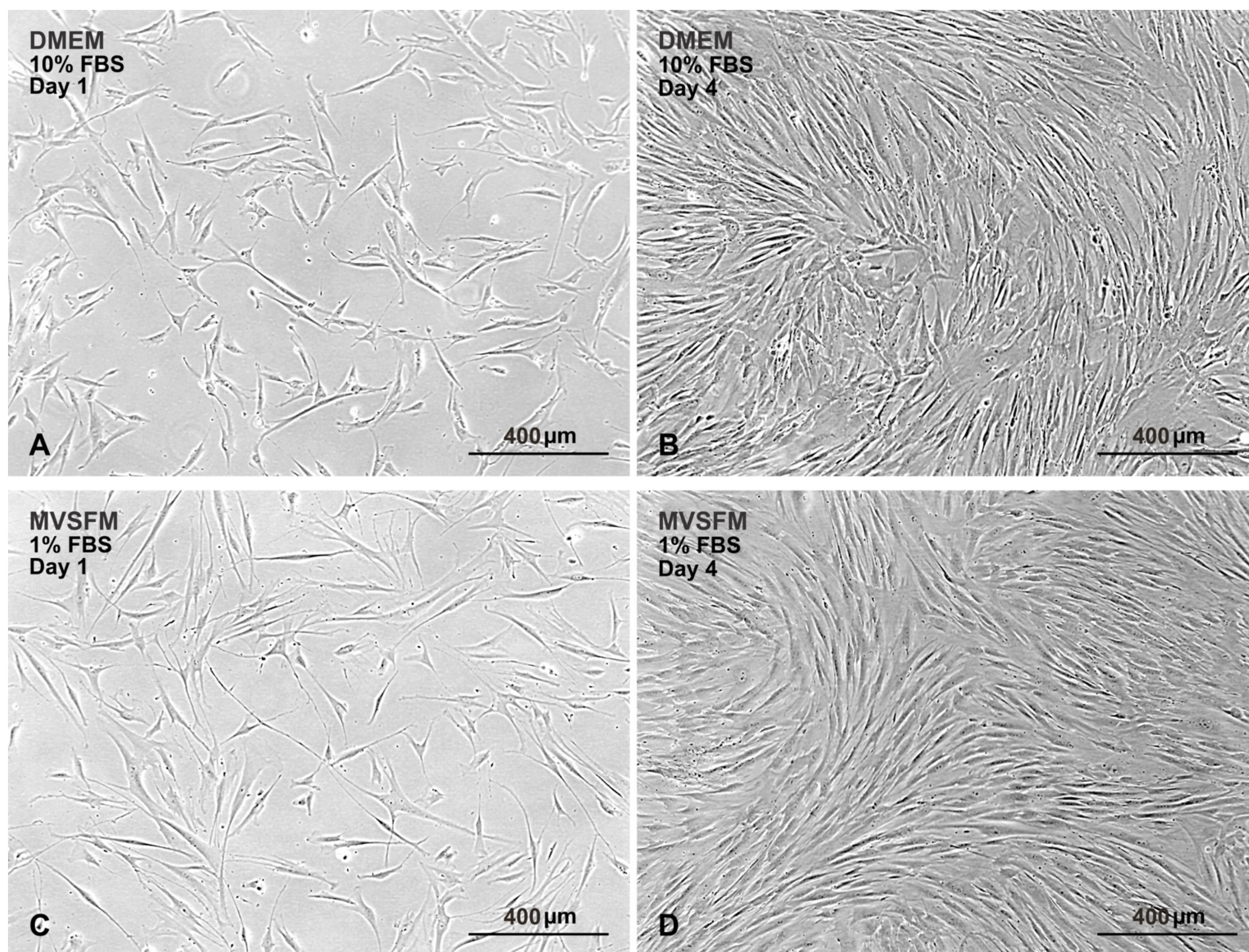


Figure 3.16 Photomicrographs contrasting the morphology of MRC-5 cells cultured in MVSFM+1%FBS and DMEM+10%FBS at low and high confluence. (A) shows the regular morphology of MRC-5 cells grown in 10% FBS at a low confluency. The cells present their regular spindle-like shape whereas in the case of those grown in MVSFM + 1% FBS (C) cells are enlarged and present various cytoplasmic extensions and an irregular stellate morphology. Once they reached confluence, cells at reduced serum remained enlarged (D) compared to the positive control (B). These morphological changes were observed throughout all OFAT experiments in all the test media. Phase contrast images are shown at 100x magnification.

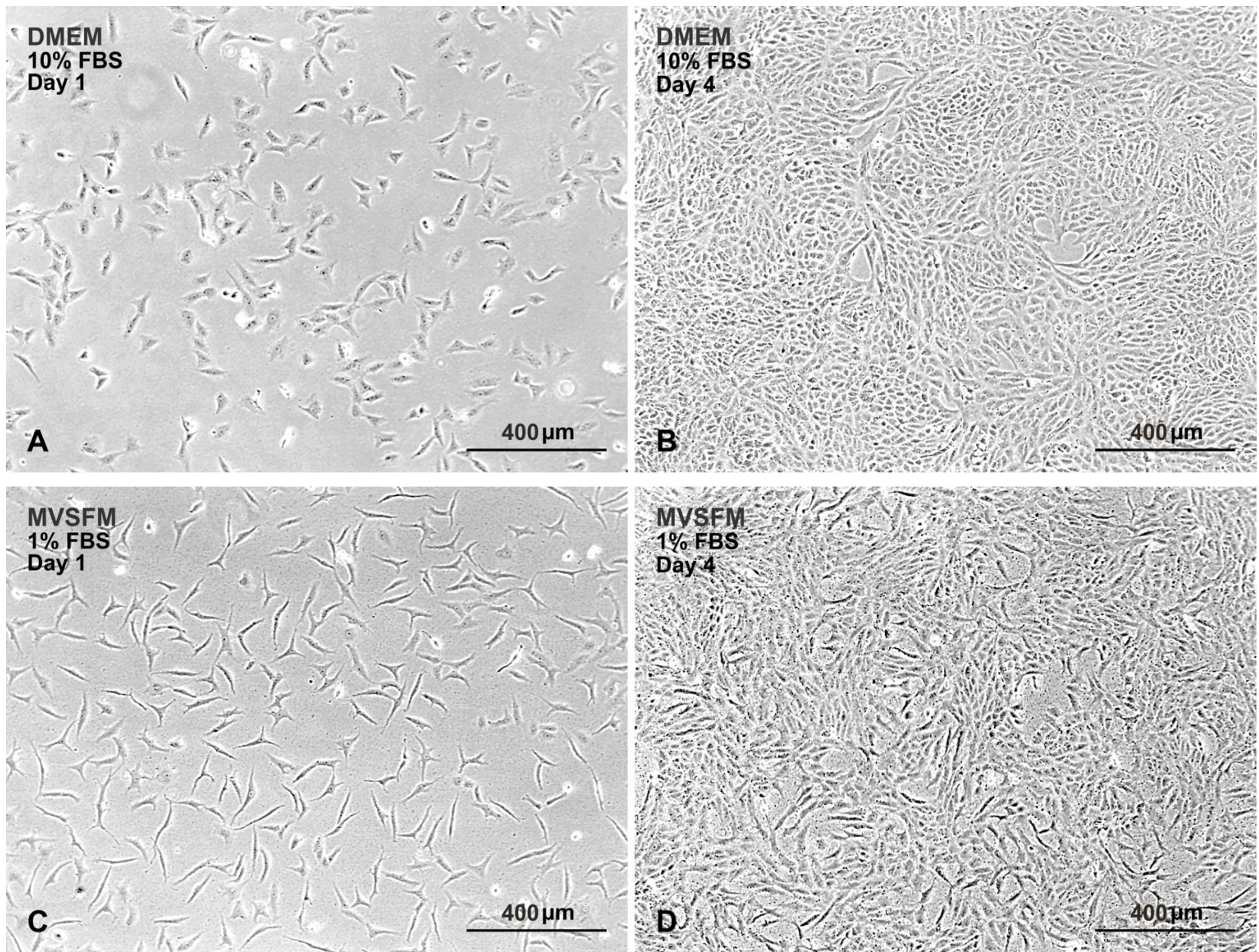


Figure 3.17 Photomicrographs contrasting the morphology of Vero cells in MVSFM+1%FBS and DMEM+10%FBS at low and high confluence. (A) depicts Vero cells grown in DMEM+10%FBS which present a square shape typical of epithelial cells in low confluency. Vero cells presented an elongated morphology after being cultured in MVSFM+1%FBS (C) and where slightly branched. Once they reached confluence, they regained their characteristic square shape (D) compared to those grown in DMEM+10% FBS (B), however as depicted in D, some cells remained elongated and maintained a spindle-like shape while in the case of DMEM+10% FBS they retained their characteristic polygonal shape throughout the culture. Phase contrast images are shown at 100x magnification.

3.6 Results from OFAT experiments

Throughout the OFAT experiments described in this chapter, a general decrease in cell yield was observed over each consecutive passage, despite the fact that cells were passaged into the same medium. The same trend was observed for both cell lines. Thus, cell yields were normalized to the positive control to obtain an average from the three experiments. This led to the conclusion that cells required adaptation into the optimized SFM media to obtain a steady cell yield over consecutive passages by gradually reducing the amount of serum.

In both cell lines, L-EGF and the S- EGF had the same effect on cell yield. Thus, S-EGF was omitted from the subsequent optimization experiments and only L-EGF was assessed. Of the three growth factors tested, only L-EGF and L-R^3-IGF-I had an impact on cell yield, which was positive or negative depending on the concentration. None of the growth factors presented a linear correlation. One of the assumptions was that there was an interaction between growth factors and that cell yield was not concentration dependent. As such, it was determined that the interaction between growth factors would be subject to further investigation. rTransferrin, did not elicit an effect on the cell lines tested either when alone or when complexed with linoleic acid. The result was confirmed when $FeSO_4$ was eliminated from the MVSFM formulation. This led to the conclusion that rTransferrin should not be added to the optimized media formulation because no major impact was observed and because the inorganic sources of iron were a more cost-effective option. Finally, cell morphology changed slightly when cells were cultured in a medium with a ten-fold serum percentage reduction. As such, it was determined that the cells required adaptation into the media with a low serum concentration.

It was observed that the growth factor requirements for MRC-5 cells were different from those of Vero cells. The OFAT experimental design showed that for MRC-5 cells the optimal concentration of L-EGF should be increased to 50 µg/L and the concentration of L-R^3 IGF should be decreased to 10 µg/L

from the MVSFM formulation originally designed by Burgener (2000), which in turn is more cost-effective. In the case of Vero cells, the OFAT optimization led to the confirmation of the previous results obtained by Burgener (2000). The highest relative cell yield, which was obtained with 10μg/L L-EGF, was not significantly different from the negative control, but was the same concentration that gave the optimal cell yield as published by Bugener (2000). Thus, it was assumed that this concentration confirmed Burgener previous finding. In the case of cell morphology, Vero cells were able to recover their original morphology after achieving confluence whereas MRC-5 cells did not, even after reaching the same state.

The final optimized concentrations obtained from the OFAT experimental design in each cell line is presented in Table 3.11. Since the OFAT experimental design does not detect interactions between different variables in a system (growth stimulatory substances required for each cell line), it was concluded that a different optimization methodology that could satisfy those requirements should be used for further experimentation. The medium shown below was tested in MRC-5 and used for adaptation as described in Chapter 4.

Table 3.11 Concentrations of growth factors optimized using the OFAT experimental design for MRC-5 and Vero cells.

Cell line	Media name	L-EGF	L-R^3-IGF	rTransferrin/ Linoleic acid
MRC-5	Prototype	50 μg/L	10 μg/L	0 μg/L
Vero	MVSFM	10 μg/L	80 μg/L	0 μg/L

3.7 Discussion

The results showed that two different optimized media formulations were obtained from the OFAT experiments for each cell line. In the case of Vero cells, the concentrations of growth factors were the same as those reported by Burgener (2000). However, in the case of MRC-5 cells, the optimized concentrations changed from 10 µg/L to 50 µg/L for L-EGF and from 80 µg/L to 10 µg/L for L-R^3-IGF-I. These results show that the requirements for each cell line were different and that the same concentrations may elicit different responses with respect to cell proliferation, as previously reported by Barnes and Sato (1980) and Ham (1983).

Furthermore, growth factors have a complex network of interactions in cells both between themselves and with other molecules and receptors. Due to the fact that two different concentrations of L-R^3-IGF-I elicited the same effect in MRC-5 cells, it was decided to further investigate if this was due to interactions with other growth factors contained in the medium formulation. One of the suspected interactions was between L-EGF and L-R^3-IGF-I, as it is widely known that they interact to stimulate cell proliferation (Stiles *et* al., 1986; Goldstein *et al.,* 1989, Pietrzkowski *et al.,* 1992; Qureshi *et al*., 1997). Other studies have reported mitogenic effects in diabetic ulcer fibroblasts produced by interactions between bFGF and IGF-I (Loots *et al.,* 2002), which leads to the notion that bFGF may have played a role in the observed results. Other growth factors known to promote cell growth and proliferation in fibroblasts could also conceivably influence the growth of MRC-5 cells, and they needed to be tested. These growth factors included: PDGF, TGF-β3, and dexamethasone (Kelley, 1992; Brenner *et al.,* 2001; Hertzel *et al.,* 2005). Some of these growth stimulatory substances have been shown to act on cell proliferation in a dose-dependent manner.

Given the known interactions, and because all the growth factors were tested OFAT, while the other variables were maintained at fixed values, the Definitive Screening Design (DSD) method was

used to evaluate interactions between growth factors and to provide a better optimization. OFAT experimentation methods require a large number of runs with multiple concentrations, which is very costly, and does not provide information regarding interactions between growth factors. The advantage of the DSD is that it can provide with information about the effect of factors as well as their interactions, in a comparatively lower number of runs. Thus, the DSD method was deemed the ideal tool to optimize the medium for MRC-5 cells.

However, before proceeding to use the DSD experimental approach, it was necessary to adapt the cells to low serum medium as it was observed that the cell yield decreased with every passage when the cells were transferred from medium containing 10% FBS to 1% FBS. As well, it was noted that morphological changes, such as elongation and size-expansion of the cytoplasm, occurred in cells transferred to low serum medium as compared to the cells cultured in the control medium (DMEM + 10 % FBS). These observations suggested that one of the factors that played a role in the decreased cell yield was the nutritional deficiency caused by serum starvation, which may have led to apoptosis and reduced cell yield, as reported by Kulkarni and McCulloch (1994). For these reasons, cells were adapted to low serum media.

Chapter 4

Media evaluation: Adaptation to Serum Free

4.1 Introduction

To perform further optimization experiments using the Definitive Screening Design (DSD), it was necessary to achieve stable cell yields throughout passages. As concluded in the previous chapter, cells required adaptation to lower serum concentrations to obtain a reliable cell yield in a SFM. Thus, both cell lines were weaned into a low serum concentration medium before proceeding to statistical design experiments. In the case of MRC-5 cells, the newly optimized media (Prototype, see Table 3.11) was tested and compared to other media formulations that had proven to successfully grow cells in SFM conditions. These media formulations are listed in Table 4.1.

The objective was to find the optimal media that could support MRC-5 cell growth at low serum concentration. In the case of Vero cells, MVSFM was selected as the medium of choice to adapt this cell line due to the fact that the OFAT experiments confirmed the results obtained by Burgener (2000). Thus, adaptation was performed in MVSFM and no other media were compared. Following the media evaluation, the DSD experiments were going to be performed using the most adequate medium resulting from this experiments as the basal medium formulation.

Table 4.1 Media used to adapt cells and source where it was obtained.

Media	Cell line	Author
MVSFM+ 5g/L Primatone	MRC-5	Burgener, 2000
Freestyle ™ 293		Gibco®, used by Biaggio et al., 2015
Prototype		Ureña-Ramírez, 2014
MVSFM	Vero	Burgener, 2000

MVSFM+ 5 g/L Primatone was designed in the Butler lab specifically for MRC-5 cells. However, the animal origin of the meat hydrolysate Primatone was deemed undesirable due to possible contamination with viruses or prion particles. The Freestyle medium is a commercially available medium specifically designed for HEK 293 cells. It was used by Biaggio (2015) to wean SK-Hep-1, HepG2, and HKB-11 cells to serum free conditions.

4.1.1 Adaptation of MRC-5 cells to MVSFM + 5 g/L Primatone medium

MRC-5 cells were cultured in T-75 flasks at a seeding density of $1x10^4$ cells/cm^2. Cells were originally grown in DMEM + 10% FBS which was chosen as the starting medium for adaptation. Subsequently, they were transferred to MVSFM + 5g/L Primatone by passaging the cells into progressively decreasing concentrations of serum as outlined in Table 4.2. This was achieved by serially subculturing the cells until near confluence, and transferring them until the next lower concentration. The cells were maintained in any one concentration of FBS until a consistent cell yield was achieved. The days at which the cells were passaged were dependent on their growth rate which was determined by daily microscopic examination. Doubling-times were calculated to compare the growth rate in the media containing a decreasing concentration of serum throughout every passage.

When MRC-5 cells were adapted using MVSFM + 5g/L Primatone, cells cultured in the medium containing 7.5% FBS showed a greater cell yield and a lower doubling-time than DMEM + 10% FBS (Figure 4.1 and Table 4.2). The increase in cell yield in a lower concentration of serum could have been possibly due to the increase in the level of nutrients. However, the doubling time at 7.5% FBS concentration was not significantly different to the positive control as shown by the Student's t-test.

Table 4.2 Doubling-time of MRC-5 cells in MVSFM+ 5g/L Primatone (M/P) n=3.

Passage #	Media	Doubling-time (hours)	SEM (hours)
8	DMEM+10%FBS	50.5	0.9
9	7.5% FBS M/P	41.9	2.7
10	5% FBS M/P	58	3.3
11	1% FBS M/P	59.7	3.8
12	1% FBS M/P	119.2	3.5
13	0.5% FBS M/P	340.3	4.7

In the medium containing 5% FBS, and in the first passage of the medium containing 1% FBS, the doubling-time of the cells was slightly above the doubling-time observed in the medium containing DMEM+10% FBS (approximately 50 hours), although there was some variation, as shown by the error bars in Figure 4.1. The doubling times were significantly different from the positive control only at passage 12 and 13 which were during the second incubation at 1% FBS and at 0.5% FBS. When cells were grown in these conditions, they showed a great increase in doubling-time (about two and seven times greater). It was concluded that the growth at a lower percentage of serum was not supported in this media formulation. The other disadvantage of this media is that it contains components of animal origin, which is undesirable.

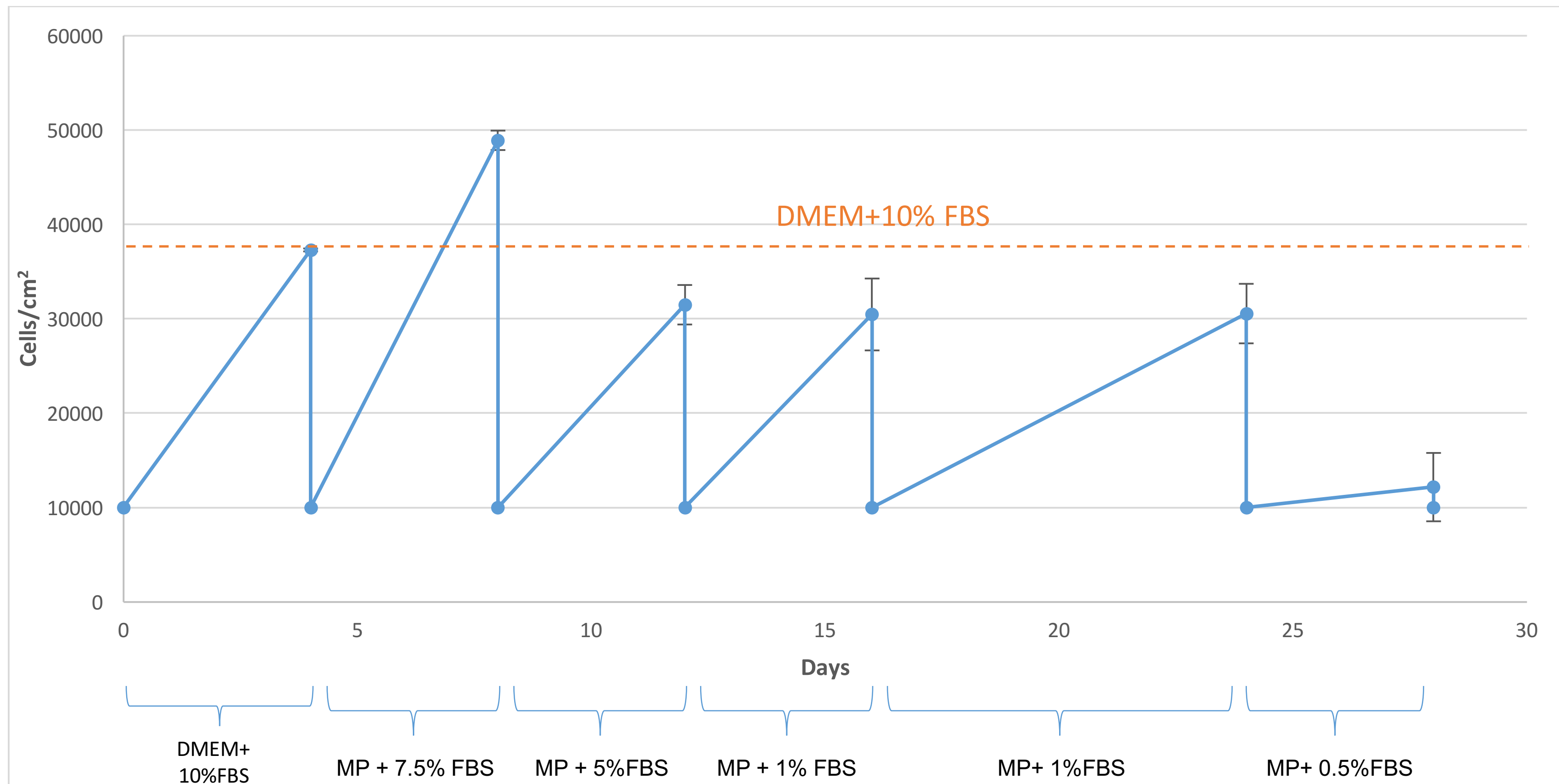


Figure 4.1 Serial subculture of MRC-5 cells in MVSFM + 5g/L Primatone (MP) media over the course of 28 days. Cells were adapted into media containing lower serum concentration by progressively decreasing the serum concentration at each successive passage. Cells did not survive after the first 0.5% FBS passage. Error bars are shown as SEM (n=3).

After growing MRC-5 cells in MVSFM + 5g/L Primatone at 0.5% FBS, the cells were unable to become confluent by day four as shown in Figure 4.2A compared to cells cultured in DMEM + 10% FBS (Figure 4.2B). In low serum concentration, the cells seemed to have spread-out and enlarged. While some of them kept their typical fibroblast shape, some cells became multinucleated, and presented an extremely large flattened cytoplasm and had irregular stellate morphology. The presence of vacuolation became more prevalent as the serum concentration was decreased as pointed in Figure 4.2A. These changes were triggered by serum starvation as described in Section 3.2. These characteristics were progressively more evident as the serum concentration was decreased. In contrast, MRC-5 cells grown in DMEM+10% FBS maintained their spindle-like shape throughout the culture time (Figure 4.2B), no vacuolation or cytoplasmic fibrous extensions were detected.

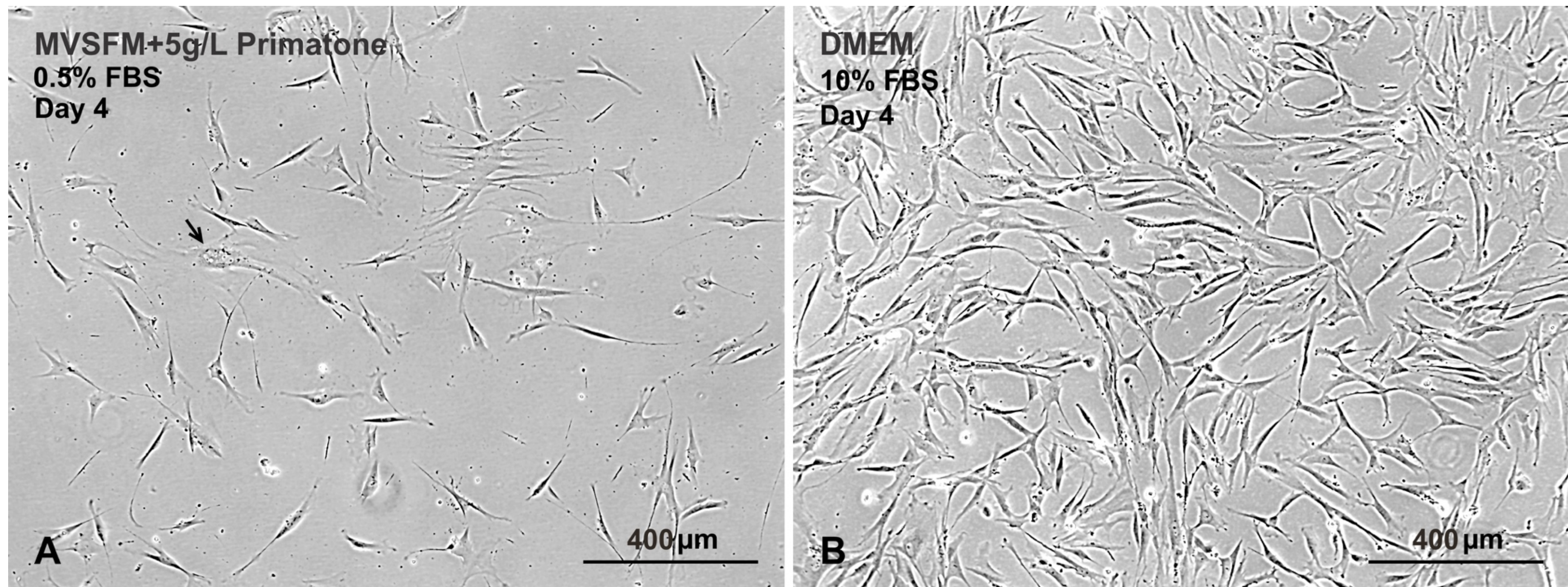


Figure 4.2 Photomicrographs contrasting the morphology of MRC-5 cells cultured in MVSFM+5g/L Primatone + 0.5% FBS and DMEM+10% FBS both on day 4. It can be observed in (A) that cells did not reach confluence at this time point. Most cells present an enlarged flattened cytoplasm with various cytoplasmic filaments. The arrow shows vacuolation in one of the cells. (B) shows the regular spindle-like morphology of MRC-5 cells cultured in DMEM+10% FBS. Phase contrast images are shown at 100x magnification.

4.1.2 Adaptation of MRC-5 to Freestyle™ 239 medium

MRC-5 cells were cultured in T-75 flasks at a seeding density of $1x10^4$ cells/cm^2. Cells were originally grown in DMEM + 10% FBS which was chosen as the starting medium for adaptation. Subsequently, they were transferred to Freestyle™ 239 medium by passaging the cells into progressively decreasing concentrations of serum as outlined in Table 4.3. The methodology was performed as described in section 4.1.1.

When MRC-5 cells were grown on Freestyle™ 239 medium, the doubling-time decreased by 10 hours when the medium was supplemented with 7.5% FBS, this decrease was statistically significantly different compared to DMEM+10% FBS. When the medium was supplemented with 5% FBS, the doubling time decreased by 5 hours compared to MRC-5 cells grown in the positive control (Table 4.3), but it was not significantly different. When the serum concentration was decreased to 1% FBS, there was a dramatic drop in cell yield and the doubling-time radically increased (by four times) compared to the positive control.

In an attempt to adapt cells to 1% FBS, they were passaged consecutively for three times at that concentration. The doubling-time was 139 hours and then increased two-fold at the second passage in 1% FBS, and decreased by approximately half on the third passage (Figure 4.3). The difference between all the three different passages at the same concentration of serum was significantly different as per the Student's t-test. At 1% FBS concentration, the MRC-5 cells underwent a radical morphological change. The cells presented a flattened cytoplasm, showed a highly irregular stellate shape with many cytoplasmic filament extensions (Figure 4.4A). The morphological change was presented since the cells were passaged into the Freestyle medium for the first time. Cells were very enlarged when compared to cells cultured in DMEM + 10% FBS

which were completely confluent at day five (Figure 4.4B). The morphological changes may have occurred due to the composition of the media which may contain substances that prevented the attachment of cells and that affected the morphology of the cells. These changes occurred rapidly following the attempted adaptation to 5% FBS media along with a decreased cell growth and cell yield. Thus, this commercial medium was deemed inadequate for MRC-5 cells as it was designed specifically to support HEK-293 in suspension culture.

Table 4.3 Doubling-time for MRC-5 cells in Freestyle 293 medium (n=3).

Passage #	Media	Doubling-time (hours)	SEM (hours)
7	DMEM+10%FBS	35.3	1.5
8	7.5% FBS Freestyle	25.3	0.9
9	5% FBS Freestyle	29.8	3.5
10	1% FBS Freestyle	139.4	1.4
11	1% FBS Freestyle	264.1	1.6
12	1% FBS Freestyle	171.6	1.4

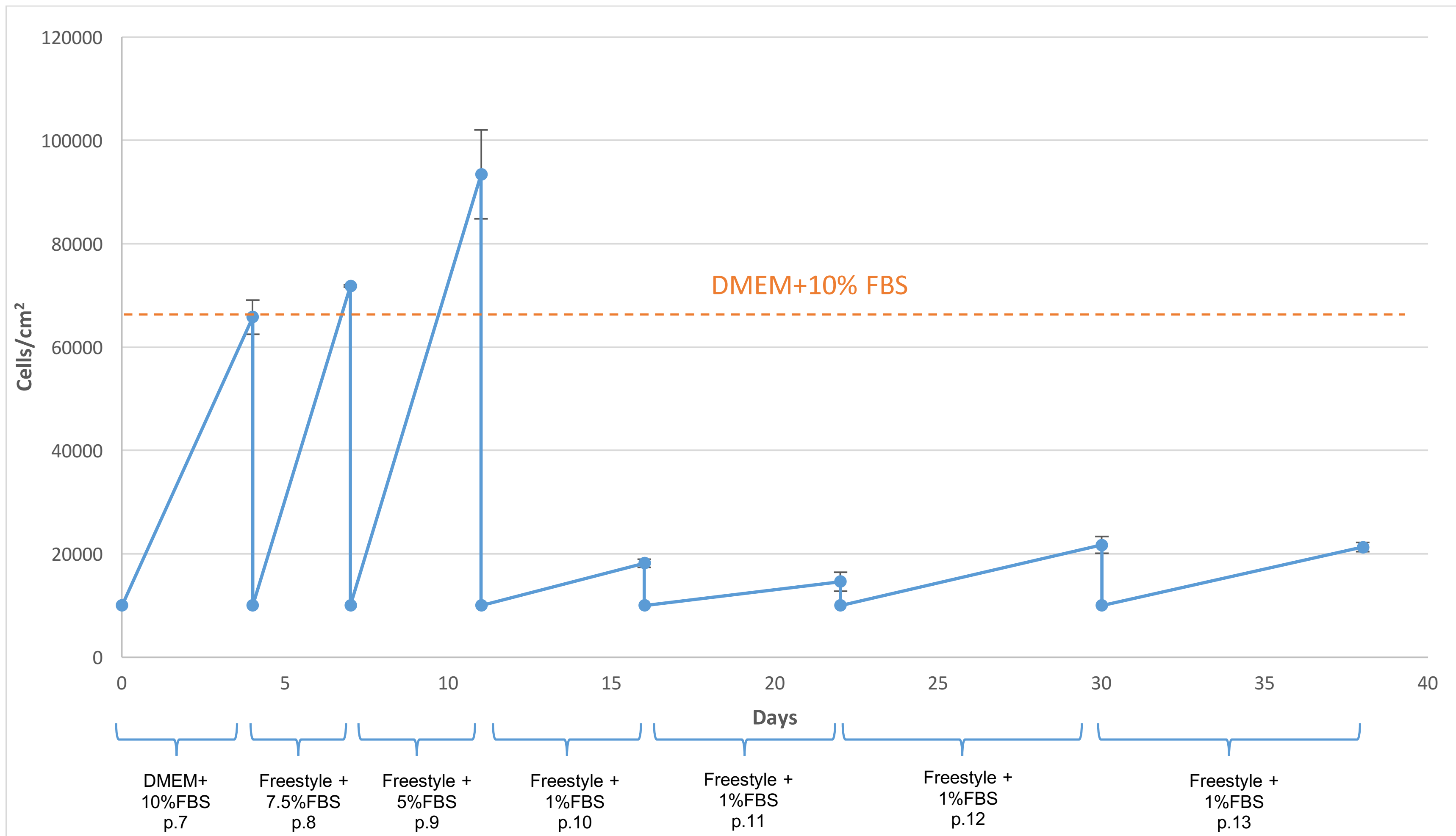


Figure 4.3 Serial subculture of MRC-5 cells in Freestyle media over the course of 37 days with a sequential decrease in the percentage of serum in the media. MRC-5 cells did not survive after the second passage in media containing 0.5% FBS. Error bars are shown as SEM (n=3).

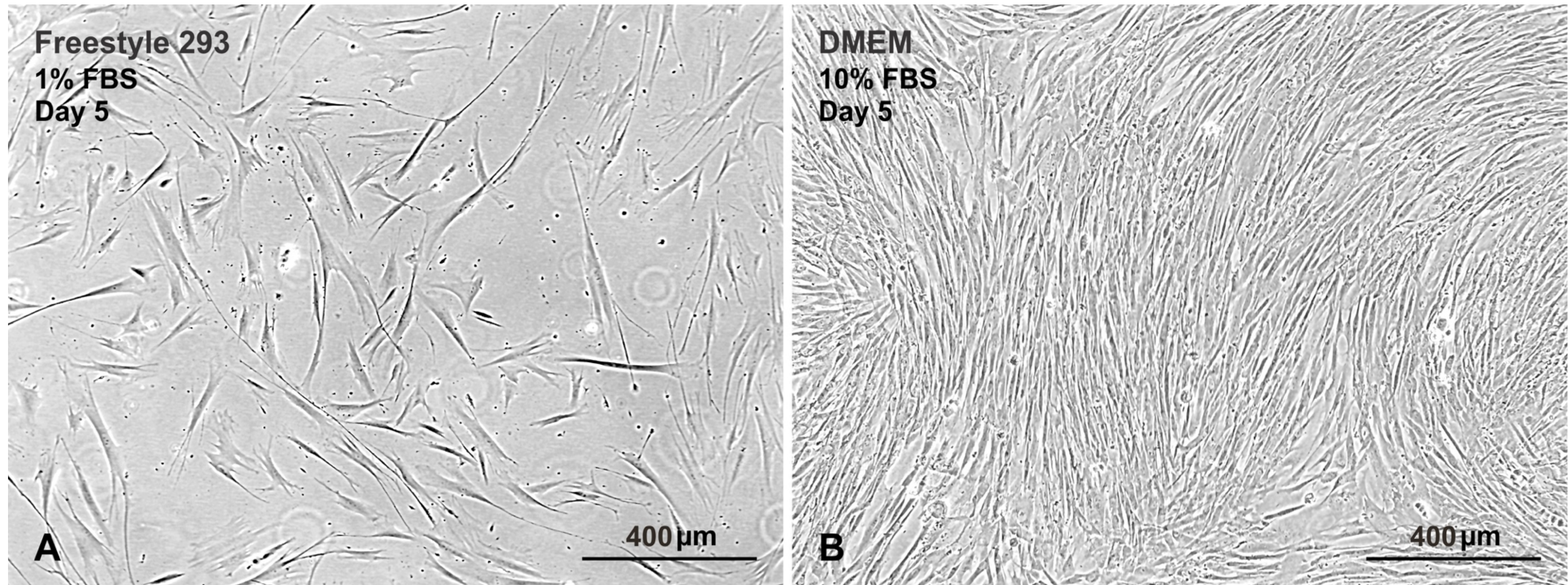


Figure 4.4 Photomicrographs contrasting the morphology of MRC-5 cells cultured in in Freestyle 293 media at 1% FBS and DMEM+10% FBS at day 5. Cells grown in Freestyle 293 (A) lost their spindle-like shape, they were shown to have more cytoplasmic extensions, they were greatly enlarged, and the shape of the cells was stellate and highly irregular as compared to the positive control (B). No vacuoles were observed. MRC-5 cells grown in Freestyle 293 (A) did not reach the same confluence level as those grown in DMEM+10% FBS (B) at the same time point. Phase contrast images are shown at 100x magnification.

4.1.3 Adaptation of MRC-5 to Prototype medium

MRC-5 cells were cultured in T-75 flasks at a seeding density of $1x10^4$ cells/cm^2. Cells were originally grown in DMEM + 10% FBS which was chosen as the starting medium for adaptation. Subsequently, they were transferred to Prototype medium (optimized using the OFAT method as seen in Chapter 3) by passaging the cells into progressively decreasing concentrations of serum as outlined in Table 4.4. The adaptation was performed as described in section 4.1.1.

After culturing MRC-5 cells in Prototype medium, some differences were detected compared to MVSFM + 5g/L Primatone and Freestyle 293 media. The doubling-time of the cells did not increase significantly when cultured in medium containing 7.5% or 5% FBS and were comparable to doubling-times (approximately 35 hours) observed in cultures containing DMEM + 10% FBS (Table 4.4). The doubling-times greatly increased when the cells were cultured in Prototype medium containing 1% and 0.5% FBS, similar to the previously tested media MVSFM+ 5g/L Primatone and Freestyle 293 (Tables 4.2, 4.3, and 4.4). However, in Prototype medium, the cells displayed sustained growth with more consistent cell yields in medium containing 0.5% FBS (Table 4.4 and Figure 4.5).

Table 4.4 Doubling-time of MRC-5 cells in Prototype medium (n=3)

Passage #	Media	Doubling-time (hours)	SEM (hours)
7	DMEM+10%FBS	35.3	1.8
8	7.5% FBS Prototype	36	0.5
9	5% FBS Prototype	36.3	1.6
10	1% FBS Prototype	82.8	1.9
11	0.5% FBS Prototype	92.8	2.6
12	0.5% FBS Prototype	97.2	2.3
13	0% FBS Prototype	169.2	1.3

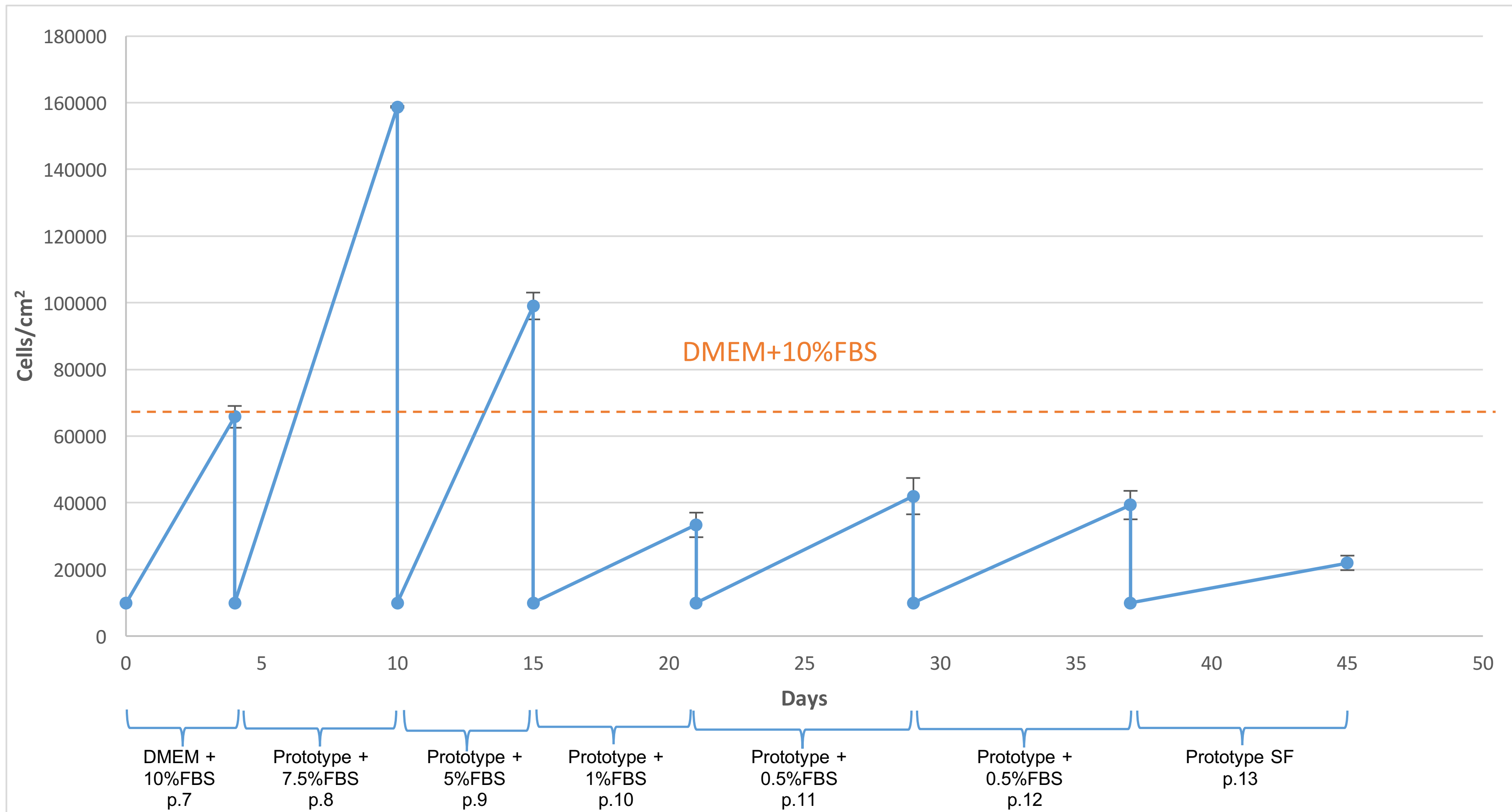


Figure 4.5 Serial subculture of MRC-5 cells in Prototype medium over the course of 37 days with a sequential decrease in the percentage of serum in the medium. This medium supported growth of MRC-5 cells up to one passage. Error bars are shown as SEM (n=3).

MRC-5 cells grown in Prototype + 0.5% FBS presented a flattened morphology, with enlarged cytoplasm and filamentous cytoplasmic extensions (Figure 4.6A), the morphological changes were not as pronounced as those observed when the cells were cultured in MVSFM+5g/L Primatone +0.5%FBS (Figure 4.2A) or Freestyle medium (Figure 4.4A). Cells cultured in Prototype + 0-.5 %FBS did not achieve the same level of confluence as the one achieved by DMEM+10% FBS (Figure 4.6B). When cells were grown in Prototype SF medium, the cells became extremely elongated forming striated parallel arrays and lost their spindle-like shape (Figure 4.7A). These morphological changes were due to nutritional starvation caused by the lack of serum and other substances critical for growth, proliferation and attachment.

The Prototype medium was the only one capable of sustaining one passage in the absence of serum (i.e. serum free conditions) despite the fact that radical morphological changes were observed (Figure 4.7A). However, the Prototype medium had the advantage of being chemically defined, compared to the one formulated by Burgner (2000). For these reasons, it was selected as the medium for subsequent DSD experiments.

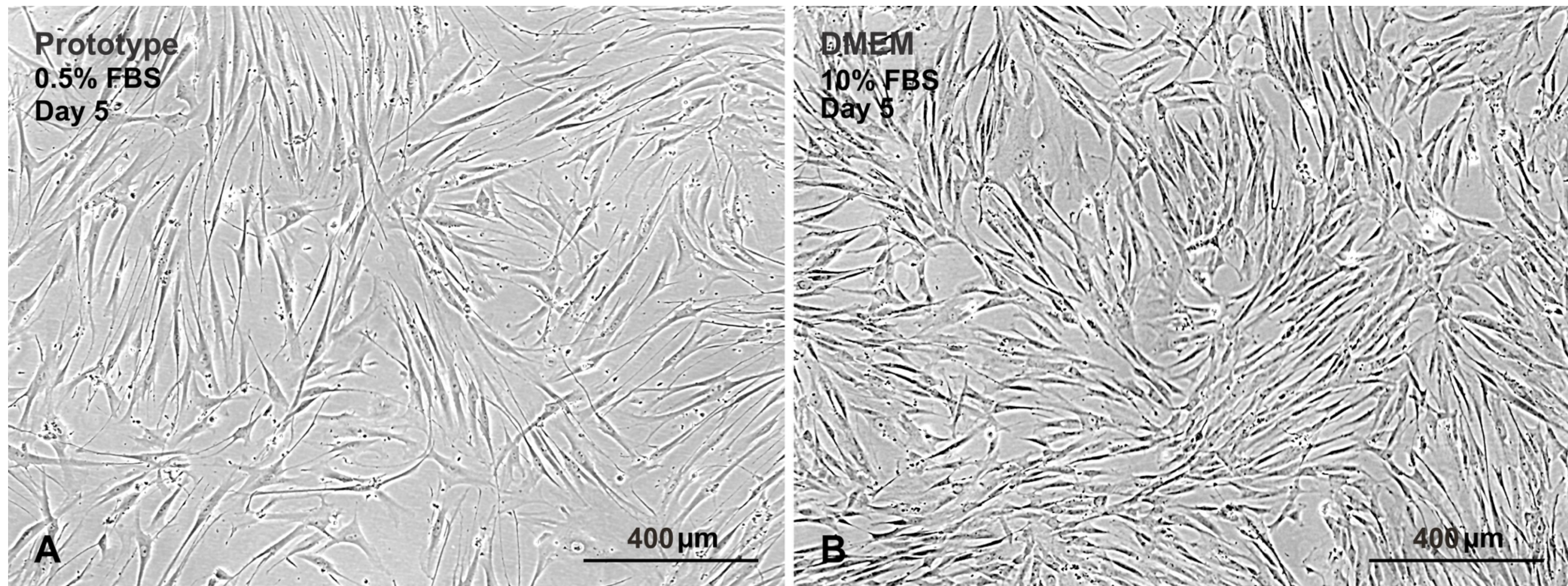


Figure 4.6 Photomicrographs contrasting the morphology of MRC-5 cells cultured in Prototype + 0.5% FBS and DMEM+10%FBS. Cells grown in Prototype + 0.5% FBS (A) displayed a flattened cytoplasm with few extensions and were much larger compared to those grown in DMEM+10% FBS (B). Cells grown in Prototype media did not display vacuolation and most retained their spindle-like shape compared to those grown in Freestyle media.

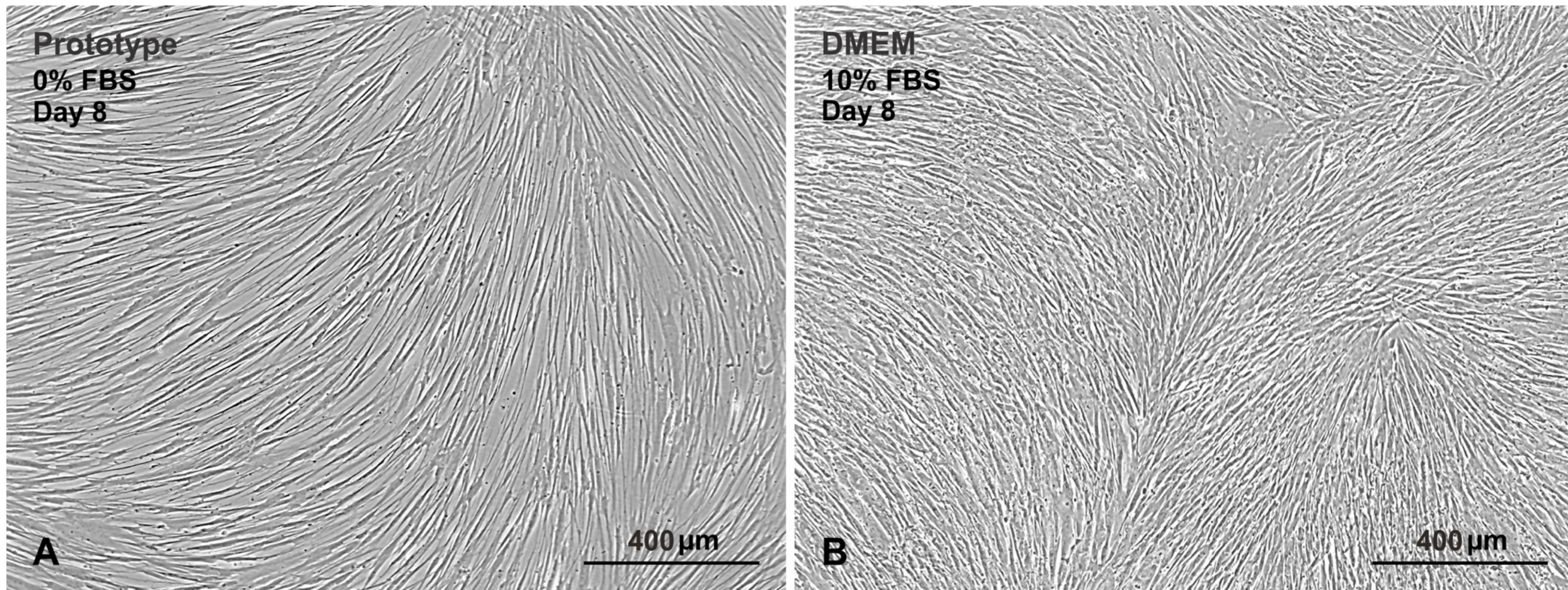


Figure 4.7 Photomicrographs contrasting the morphology of MRC-5 cells cultured in Prototype + 0% FBS ºand DMEM+10%FBS. MRC-5 cells in Prototype SF medium. Cells were extremely elongated due to serum starvation, and survived in Prototype SF medium for only one passage

4.2 Adaptation of Vero cells to MVSFM

Vero cells were cultured in T-75 flasks at a seeding density of $1x10^4$ cells/cm^2. Cells were originally grown in DMEM + 10% FBS which was chosen as the starting medium for adaptation. Subsequently, they were transferred to MVSFM by passaging the cells into progressively decreasing concentrations of serum as outlined in Table 4.5. Cells were adapted as described in section 4.1.1.

Vero cells were adapted to low serum media using MVSFM. This media was chosen (Table 4.1) due to its chemically defined composition and because of the previous results confirmed by OFAT experiments. Cells were weaned from serum-dependence using the same experimental setup as the one described in the section above. This experiment was conducted to compare the growth of Vero cells during adaptation to low serum to that of MRC-5 cells.

The main difference that was noted was when cells were adapted to 7.5% or 5% FBS, they never surpassed the cell yield obtained with DMEM + 10% FBS (Figure 4.8), as observed in all previous experiments performed with MRC-5 cells (Figures 4.1, 4.3 and 4.5). However, when Vero cells were cultured in progressively decreasing concentrations of serum, their doubling-times did not show such a large increase compared to MRC-5 cells. Vero cells grown at the lowest serum concentration (0.1% FBS) had approximately a two-fold increase in doubling-time compared to the 3-5 fold increase in doubling-time at the lowest serum percentage used (0.5% FBS) for MRC-5 cells. Moreover, Vero cells did not display any morphological changes during the adaptation process, compared to the OFAT experiments when the serum was reduced more drastically from 10% to 1% FBS. This experiment demonstrates that different cell lines have different nutritious requirements.

Table 4.5 Doubling-times in days and hours of Vero cells weaned down in MVSFM medium (n=3).

Passage #	Media	Doubling-time (hours)	SEM (hours)
8	DMEM + 10% FBS	17	0.3
9	MVSFM + 7.5% FBS	27.4	2.7
10	MVSFM + 5% FBS	27.2	0.4
11	MVSFM + 1% FBS	39.3	0.3
12	MVSFM + 1% FBS	29.1	1.0
13	MVSFM + 0.5% FBS	47.8	0.2
14	MVSFM + 0.25% FBS	39.3	0.5
15	MVSFM + 0.1% FBS	37.8	0.2

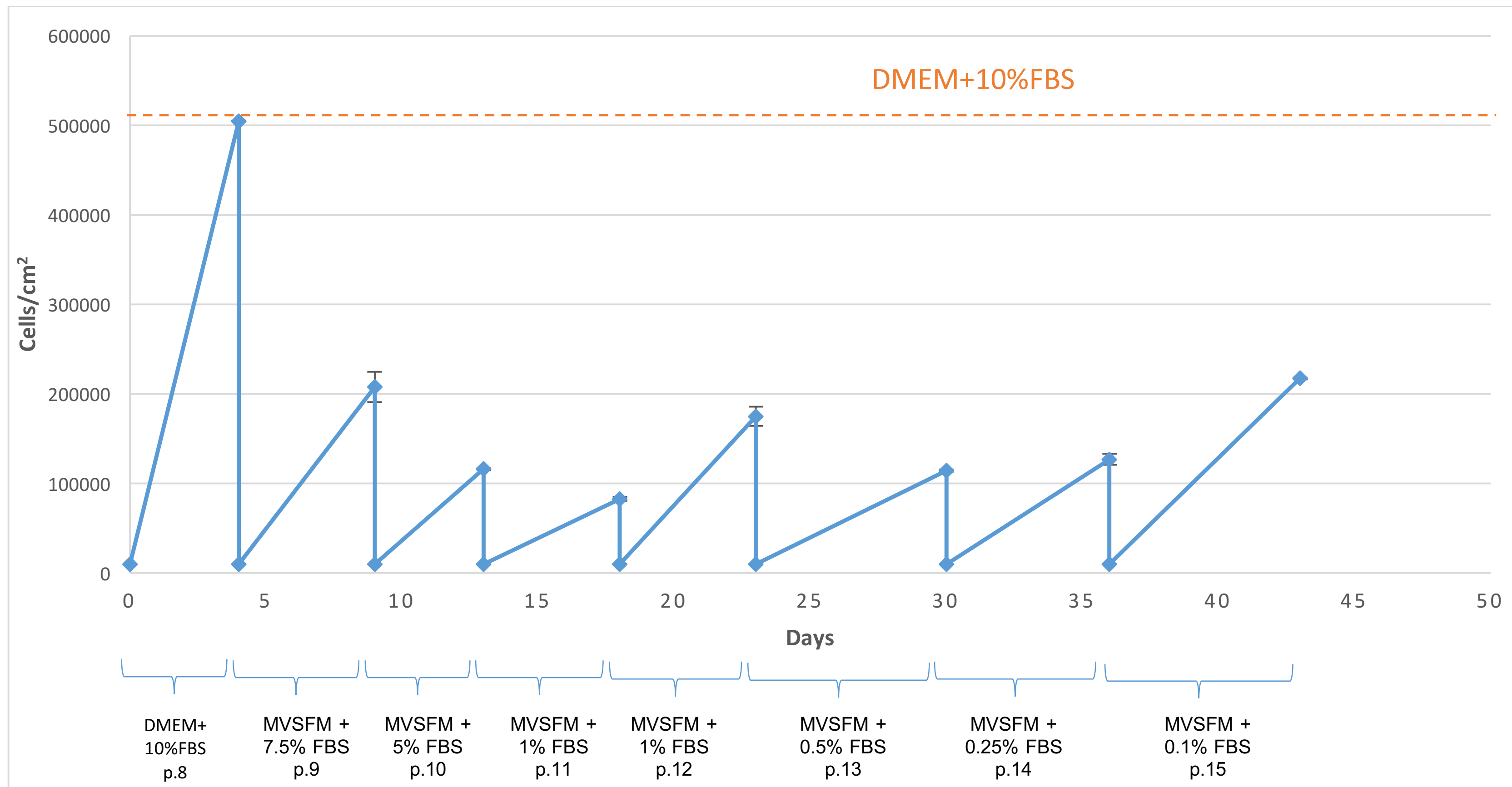


Figure 4.8 Serial subculture of Vero cells in MVSFM as a basal medium over the course of 43 days with a sequential decrease in the percentage of serum in the media. The cells never surpassed the cell yield of the positive control (DMEM + 10% FBS). Error bars are shown as SEM (n=3).

4.3 Discussion

The overall results demonstrated that the optimized medium Prototype proved to be more adequate for adapting MRC-5 cells to SFM compared to the two other media: MVSFM + 5 g/L Primatone and Freestyle™ 293. The Freestyle™ 293 medium was selected because it was shown to support the growth of SK-Hep-1, HepG2, and HKB-11 cell lines in SF conditions, as reported by Biaggio *et al.* (2015) who obtained positive results on serum free adaptation with the Freestyle medium which was designed for HEK 293 rather than for the above-mentioned cell lines. However, the data demonstrated that MRC-5 cells were negatively impacted by the reduction of serum in all media tested. The results presented here show that Prototype medium supported growth of MRC-5 cells in SF conditions, but only for a single passage.

Even prior to adaptation of cells to low serum conditions, the doubling-time of MRC-5 cells grown in DMEM + 10% FBS was variable. This could be due to differences in serum lot from one experiment to another. As well, there was some variability in doubling-time observed from passage to passage in some of the adaptation steps with all media tested when the same concentration of serum was used. This phenomenon was also observed in the adaptation studies performed by Burgener (2000), and may have been caused by a lack of nutritious and attachment factors, or deficiencies in protective proteins and/or lipids found in serum in the different media formulations tested.

This experiment determined that the Prototype medium + 0.5% FBS was the most optimum medium for MRC-5 cell adaptation to low serum. Cells cultured in this medium presented a more stable growth and the lowest doubling-time of all the media tested. This was likely caused by the fact that cells adapted their metabolism to this low serum medium in Prototype + 0.5 % FBS by

producing the necessary attachment factors and other proteins necessary for their survival (Burgener, 2000). It should be noted that the increased doubling time towards the end of the adaptation to a reduced serum formulation was likely caused by serum starvation. This conclusion was reached because a control culture of MRC-5 cells grew in DMEM+10% FBS for 20 passages, before showing signs of senescence. These cells originated from the same batch which was used to perform all the other adaptation experiments. This suggests that there was a limitation in the composition of SFM that prevented the growth of these cells beyond 15 passages rather than cells entering senescence at this point.

When Vero cells were adapted to low serum conditions in MVSFM, there was less impact on doubling-time and cell yield as the serum concentration was decreased. This may be because transformed cells have less stringent nutritious requirements to those in normal diploid cells such as MRC-5 cells.

Chapter 5

Definitive Screening Design

5.1 Introduction

Quality is of utmost importance when it comes to biomanufacturing. The industrial process requires time and cost-effective solutions that lead to a successful quality product. For that reason, it is clear that in order to design any media formulation, quality should be built into the product rather than tested or inspected (Shivhare and McCreath, 2010). Therefore, it is necessary to conduct proper planning of the experimental design such that the media formulation may be designed using a reduced number of experiments. The first step is to perform a process characterization in order to achieve the quality product of interest.

In the case of a serum free medium (SFM) formulation, the system to be evaluated is the cell line itself. Cells are complex dynamic systems with very intricate structures and very specific growth requirements. To date, despite technological advances and the availability of detailed information derived from omics studies, it has not been possible to define all the pathways and end-product outcomes produced by the molecular machinery within cells (Alberts *et al.,* 2008). Moreover, each cell line has specialized functions and slightly different biochemical machineries and pathways, depending on its tissue and species of origin. For all these reasons, it is impossible to define a cell in its entirety and its key growth requirements for growth and survivability. Anabolic pathways in the cell may be considered as the transformation of basic building blocks (nutrients) to biomass and/or macromolecular products (Mandenius and Brundin, 2008). Thus, it is not necessary to know all the details of the biochemical pathways in the cell to design a SFM; if

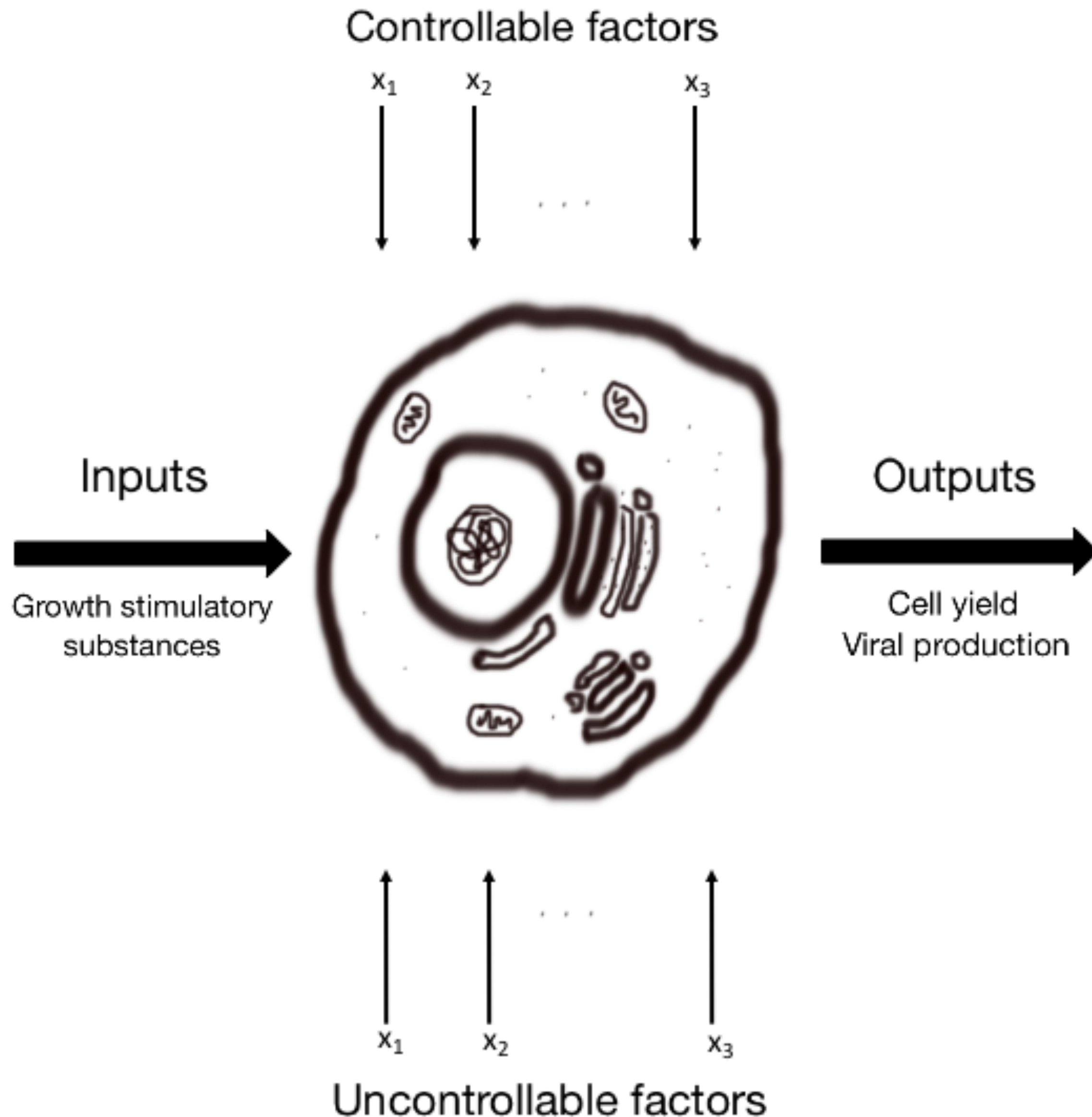


Figure 5.1 General model of a cell "System" in the design of a SFM formulation. The inputs to be evaluated are the Growth Stimulatory Substances. The output responses are Cell Yield, and Product Yield (protein production or virus titer). Some of the Controllable Factors include incubation temperature, percentage of CO_2, cell culture vessel, etc., whereas the Uncontrollable Factors may be nutrient degradation, variations in the equipment, etc. Modified from (Montgomery, 2013).

the cell can be described as a “System”, such as the one depicted in Figure 5.1.

Experimentation can provide information about how the “System” works. An experiment may consist of one or more tests where planned changes are made to the input variables of a process or system. As a result, changes in the output response(s) are observed, detected, and identified (Montgomery, 2013). In order to design such an experiment, it is necessary to define the input variables (factors) that will be applied to the system and the desired output response(s). In the case of the design of a SFM, the input variables are the growth stimulatory substances, while the output responses are the cell yield and/or product yield (protein production or viral titer produced).

The growth stimulatory substances (input variables) are prone to interact with each other through complex regulatory biochemical pathways in ways that may be beneficial or detrimental to the cell (Ham, 1981; Baird and Dunkin, 1986; Block *et al.,* 1996 Lebeche *et al.,* 1999; Hetzel, 2005). The outcome may range from a decrease or increase in cell yield and/or productivity, to changes in cell morphology due to cell differentiation or apoptosis (programmed cell death). These are the output parameters that may or may not be desired. Traditional experimental design methods use a less efficient OFAT approach that fails to consider interactions between factors (Rathore, 2009).

5.1.1 The Design of Experiments (DoE) approach

To create an optimal SFM for a specific cell line, statistical Design of Experiments (DoE) methods are the strategy of choice because they can provide understanding of the effects of multidimensional combinations and interactions of the various factors that influence the system or process (Rathore et al., 2007). DoE is a powerful methodology, which avoids experimental biases

and reduces the required number of experiments (Mandenius and Brundin, 2008). It can offer returns four to eight times greater than the cost of running the multiple experiments, plus reduce the time required to complete the analyses to a fraction of what it would take to run an OFAT experiment (Mandenius and Brundin, 2008; Shivhare and McCreath, 2010). The most appropriate DoE methods to tackle complex biological systems are factorial experiments, in which factors are varied together instead of one at a time (Montgomery, 2013).

Factorial DoEs facilitate the study of complex matrices of input and output parameters that may be interlinked or independent of each other. They facilitate elucidation of the main effects and determine interactions (Rathore *et al*., 2007), while providing reliable results in low numbers of experiments (Shivhare and McCreath, 2010). Factorial DoEs can evaluate the direction of the true optimum, whereas OFAT methods lead to quasi-optimum results. The use of factorial design was introduced into the chemical manufacturing control (CMC) review process in 2004 as a result of the pharmaceutical industry current Good Manufacturing Practice (cGMP) for the 21st Century Initiative (Rathore, 2009). The details have been described by different national bodies and worldwide industrial organizations, such as the International Conference on Harmonization (ICH) quality guidelines, the United States' Food and Drug Administration (FDA), and the European Medicines Agency (EMEA) (Mandenius and Brundin, 2008; Mandenius 2009; Rathore, 2009; Riley and Li, 2011).

5.1.2 Definitive Screening Design (DSD) experimentation methodology

The advantages of the DoE approach prompted the use of factorial Plackett-Burman designs (Liu *et al.,* 2007, Jeon *et al.,* 2010; Petiot *et al.,* 2010; Gonzalez-Leal *et al.,* 2011; Parent

et al., 2016), which are the most common factorial DoEs used in media development. However, newer and more efficient approaches have emerged. As an example, the Definitive Screening Design (DSD) can make better estimations regarding interactions between factors, namely: i) main effects of each factor; ii) two factor interactions, which are those between factors; and iii) quadratic interactions, which are those that occur when the factor interacts with itself.

The DSD can unambiguously identify main effects and quadratic interactions better than fractional factorial designs or the Plackett-Burman designs. This is achieved with a low number of runs compared to fractional factorial designs (Jones and Nachtsheim, 2011) paired with response surface modeling which is used to optimize (Erler, *et al.,* 2013). DSD can be paired-up with modelling techniques that can aid the optimization process, such as genetic algorithms, which have been recently used in the biopharmaceutical process to optimize viral production (Takahashi *et al*., 2016). These mathematical optimization methodologies can be combined with the DSD to create a SFM that that fulfills the QbD guidelines.

5.2 Definitive Screening Design for MRC-5 cells using growth factors

The observed lack of correlation between growth factor concentration and cell yield led to the hypothesis that concentration-dependent interactions had played a role MRC-5 cell yield, as demonstrated by the OFAT experiments. In addition, basic fibroblast growth factor (bFGF), included in the original MVSFM formulation at a concentration of 1µg/L, was not varied nor evaluated in any the OFAT experiments. This may have impacted the results obtained and caused some of the variations observed due to interactions.

To determine if Repligen's L-EGF and L-R^3 IGF-I interacted with other substances, either synergistically or antagonistically, a DSD was set up because it has proven to be a successful methodology to characterize the production of biomanufacturing of monoclonal antibodies, pDNA, vaccine formulation and other recombinant proteins (Ornek and Ramsey, 2012; Erler *et al.,* 2013 Tai *et al.,* 2015; Clark, 2016). To fulfill the requirements of a minimum number of factors in the experimental design, several growth factors were included in the evaluation in the same DSD experiment, including bFGF, TGF-β3, PDGF-AA, and dexamethasone, as listed in Table 5.1. The DSD experiment was used to perform factor screening in media and to evaluate the different substances and their interactions with respect to cell yield.

The DSD can assess the impact of factors in a low number of runs. It is capable of estimating main effects and some two-way interactions using three levels (concentrations), which allows it to estimate some quadratic effects in a single experiment using a low number of runs. These attributes in a DoE are crucial to the efficient design of a SFM, in a cost-effective manner, as it can give information about the "system" in early stages of the bioprocess or design. The DSD is described by Equation 5.1 (Jones and Nachtsheim, 2011):

$$\underbrace{\eta}_{\text{Response}} = \underbrace{\beta_0}_{\text{constant}} + \underbrace{\sum_{i=1}^{k} \beta_i x_i}_{\text{Main effects}} + \underbrace{\sum_{i=1}^{k} \beta_{ii} x_i^2}_{\text{Quadratic effects}} + \underbrace{\sum_{i=1}^{k-1} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j}_{\text{Combined effects}} \qquad \text{Equation 5.1}$$

Significant advantages of the DSD compared to other factorial designs are that: i) main effects are orthogonal and free of aliasing (i.e. it does not include the influence of other factors) with quadratic effects and two-way interaction effects; ii) no quadratic or two-way interaction

effect is fully aliased with another quadratic or two-way interaction effect; iii) the DSD can estimate every term of a full quadratic model (Ramsey and Stephens, 2014).

The number of runs (media formulations) in the experiment was dependent on the number of factors. For "k" factors (2k+1 if it is an even number and 2k+3 if it is an odd number), all factors are evaluated at 3 levels to estimate quadratic effects. The highest and the lowest concentrations were entered into the JMP software, which calculated the middle level as shown in Table 5.1. The selection of the concentrations was performed using previous OFAT experiments, literature values and cost effectiveness. In this experiment, six factors were used, leading to a total number or 13 media formulations as shown in Table 5.2.

Table 5.1 Concentrations (levels) used for DSD. Only the lower and the upper level were selected inputs. The middle level was calculated by the JMP 12 software.

Growth stimulatory substance	Lower level	Middle level	Upper level
Dexamethasone	0 μg/L	20 μg/L	40 μg/L
PDGF-AA	0 ng/L	250 ng/L	500 ng/L
bFGF	0 μg/L	5 μg/L	10 μg/L
TGF-β3	0 ng/L	375 ng/L	750 ng/L
LONG-EGF	0 μg/L	50 μg/L	100 μg/L
LONG-R^3-IGF-I	0 μg/L	50 μg/L	100 μg/L

Table 5.2 DSD outline created by the JMP 12 software using the values from Table 5.1.

Media name	Dexamethasone (μg/L)	bFGF (μg/L)	PDGF-AA (ng/L)	L-EGF (μg/L)	L-R^3-IGF-I (μg/L)	TGF-β3 (ng/L)
1	40	10	0	50	0	750
2	0	0	500	50	100	0
3	20	0	0	0	0	0
4	40	0	500	100	0	375
5	40	5	0	100	100	0
6	20	5	250	50	50	375
7	40	0	250	0	100	750
8	0	5	500	0	0	750
9	0	10	250	100	0	0
10	0	10	0	0	100	375
11	0	0	0	100	50	750
12	20	10	500	100	100	750
13	40	10	500	0	50	0

5.2.1 Culture of MRC-5 cells in DSD experiments

The experimental media formulations were prepared using MVSFM as a basal medium, without growth factors added, and supplemented with 0.5% FBS. All the different growth stimulatory substances were supplemented to the media using the concentrations detailed in Table 5.2. The 13 different experimental media formulations were subsequently filtered sterilized. The negative control was MVSFM + 0.5% FBS without growth factors and DMEM+10% FBS was used as the positive control.

MRC-5 cells were adapted to Prototype + 0.5% FBS after determining that this medium was the most adequate for MRC-5 cell growth at low serum concentrations as concluded in Chapter 4. MRC-5 cells were cultured in Prototype + 0.5% FBS and upscaled in T-75 flasks using the subculture protocol described in Section 2.2.2. Once the cell inoculum was large enough, the cells were transferred into T-25 flasks containing the different media formulations at a seeding density of $1x10^4$ cells/cm^2. Two independent replicate flasks were cultured for each media formulation. The media were changed twice over the course of the experiment. Cells were counted at day seven, which was the time point at which most of the cells were near confluent under all the experimental conditions. The experiment was repeated three times for statistical significance.

5.3 MRC-5 cell yield in DSD using growth factors

As shown in Figure 5.2, it was possible to observe a steady decrease in cell yield from the positive control (DMEM+10% FBS) until Medium 11. Subsequently, a plateau was seen within the group of media: 10, 2, 3, Negative control (no GFs), 7 and 9 which show a comparable cell yield. The Student's t-test at $p < 0.05$, identified four different statistically similar groups of DSD media.

Group 1 consisted of the positive control medium (DMEM + 10% FBS) and Medium 1. Medium 1 presented a cell yield of 126,000 cells/cm^2, which achieved a cell density of 91.9% of the cell density observed in the positive control medium (132,788 cells/cm^2). This was the highest cell yield of all the DSD experimental media (Figure 5.2). The doubling-time of the cells cultured in Medium 1 was one hour longer than the doubling-time of cells cultured in the positive control medium (Table 5.3) and they did not show a statistical significant difference.

Group 2 included Media 6, 12, 13, and 4, in which the cell yield ranged from 77% to 68% of the cell yield observed in the positive control medium. The greatest cell yield was achieved by MRC-5 cells in DSD Medium 6, although the yield of MRC-5 cells in Medium 6 was only about 15% of the cell yield observed in cultures containing Medium 1. There was a 9% difference in cell yield between Cultures in Medium 6 (highest yield of this group) and cultures in Medium 4 (lowest yield of this group), and the doubling-time ranged from 49.2 to 52.1 hours (Table 5.3).

Group 3 was composed of Media 8, 5, and 11, and as observed in Group 1 and 2 cultures, a steady decrease in MRC-5 cell yield was observed between Medium 8 to Medium 11 (Figure 5.2). Cell yields in Media 8, 5, and 11 ranged from 59%-50% of the cell yields observed in the positive control medium. As observed in Group 2 cultures, there was a 9% difference in cell yield between cultures in Medium 8 (highest yield of this group) and cultures in Medium 11 (lowest yield of this group), and the doubling-time ranged from 57.2 to 60.5 hours (Table 5.3).

Group 4 encompassed the DSD media that achieved the lowest cell yields and consisted of Media 10, 2, 3, the negative control (MVSFM + 0.5 % FBS, with no growth factors), 7 and 9 (Figure 5.2). Cell yields in this group were 38% to 33% of the yields observed in cultures in the positive control medium. A 12% decrease in cell yield was observed between cultures in Medium 11 (lowest cell yield in Group 3) compared with cultures in Medium 10 (highest cell yield in Group 4). However, within Group 4, cell yields were consistent, with only a 5% difference between cultures in Medium 10 (highest cell yield in Group 4) and cultures in Medium 9 (lowest cell yield in Group 4), and the doubling-times ranged from 70.5 to 77.0 hours (Table 5.3), representing the largest difference in growth rate of all the DSD media tested.

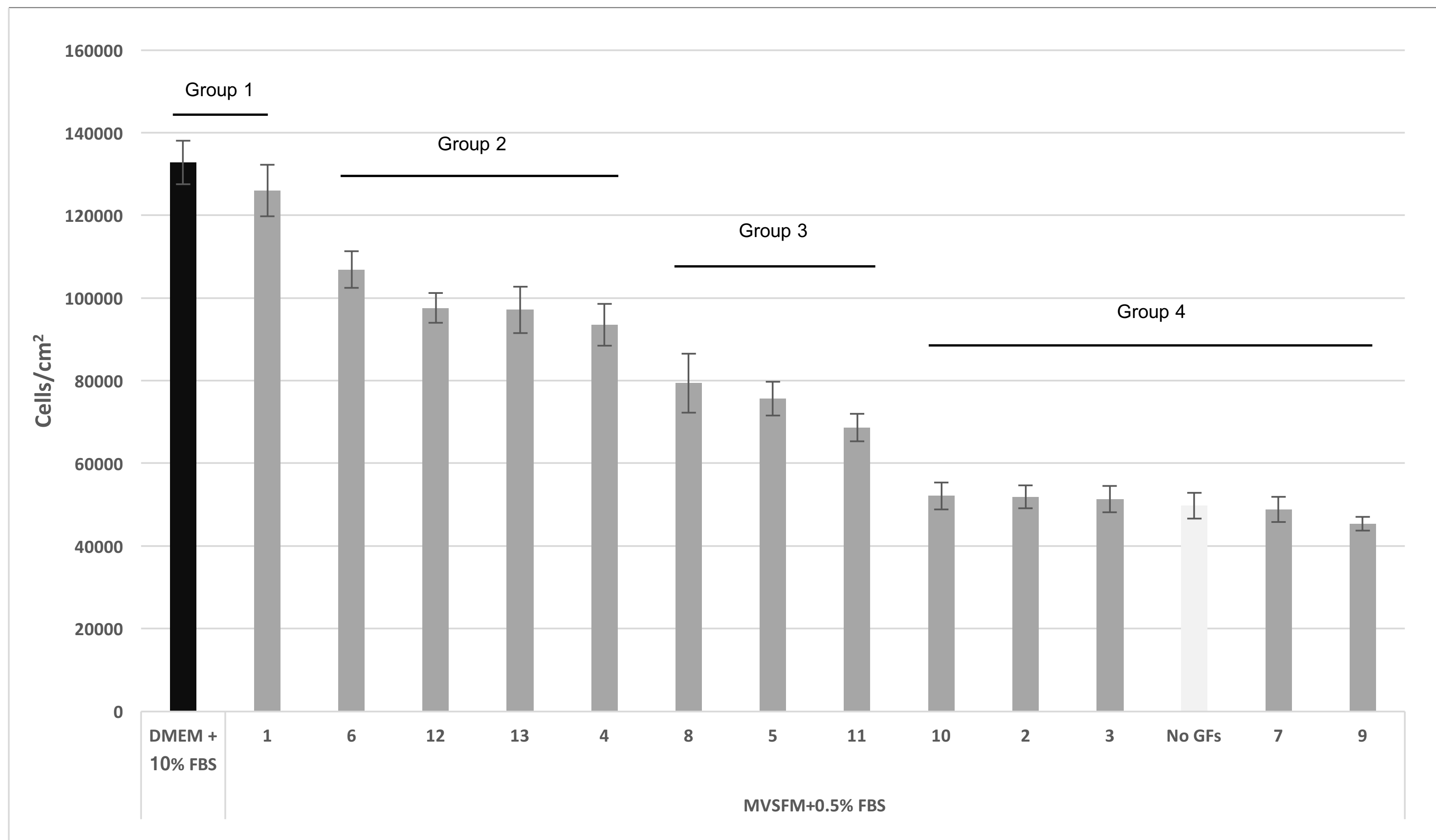


Figure 5.2 MRC-5 cell yields after seven days of growth in the DSD experimental media (Table 5.2) using MVSFM + 0.5% FBS as the basal medium. Cell yields of cultures in the DSD experimental media are displayed as dark gray columns. (n=6) The positive control medium (DMEM + 10% FBS) is indicated by the black column, and the negative control medium (MVSFM + 0.5% FBS, and no growth factors) is indicated by the light gray column. Error bars are shown as SEM.

Table 5.3 Doubling-times of MRC-5 cell cultures in all the DSD experimental media (n=6).

Media	Doubling-time (hours)	SEM (hours)
DMEM + 10% FBS	45.0	2.1
1 + 0.5% FBS	46.0	2.2
6 + 0.5% FBS	49.2	1.6
12 + 0.5% FBS	51.1	1.4
13 + 0.5% FBS	51.2	1.8
4 + 0.5% FBS	52.1	1.9
8 + 0.5% FBS	56.2	3.0
5+ 0.5% FBS	57.6	1.5
11+ 0.5% FBS	60.5	1.4
10 + 0.5% FBS	70.5	1.4
2 + 0.5% FBS	70.7	1.3
3 + 0.5% FBS	71.2	1.4
No GFs + 0.5% FBS	72.5	1.5
7+ 0.5% FBS	73.5	1.4
9+ 0.5% FBS	77.0	1.2

In conclusion, Medium 1 was the most suitable for the culture of MRC-5 cells. Both cell yield and the doubling-time were comparable to those observed by MRC-5 cells in DMEM + 10% FBS medium. The morphology of MRC-5 cells cultured in Medium 1, evaluated over the duration of the experiment, was almost identical to the morphology of the cells observed in cultures containing the positive control medium (Figures 5.3 A and B). Cells in both cultures were spindle-like in shape, were similar in size, did not display vacuolation as in other media formulations (data not shown), and their cytoplasms were not enlarged. The DSD experimental Medium 1 was renamed “Delta 1” and was selected for subsequent analysis and optimization.

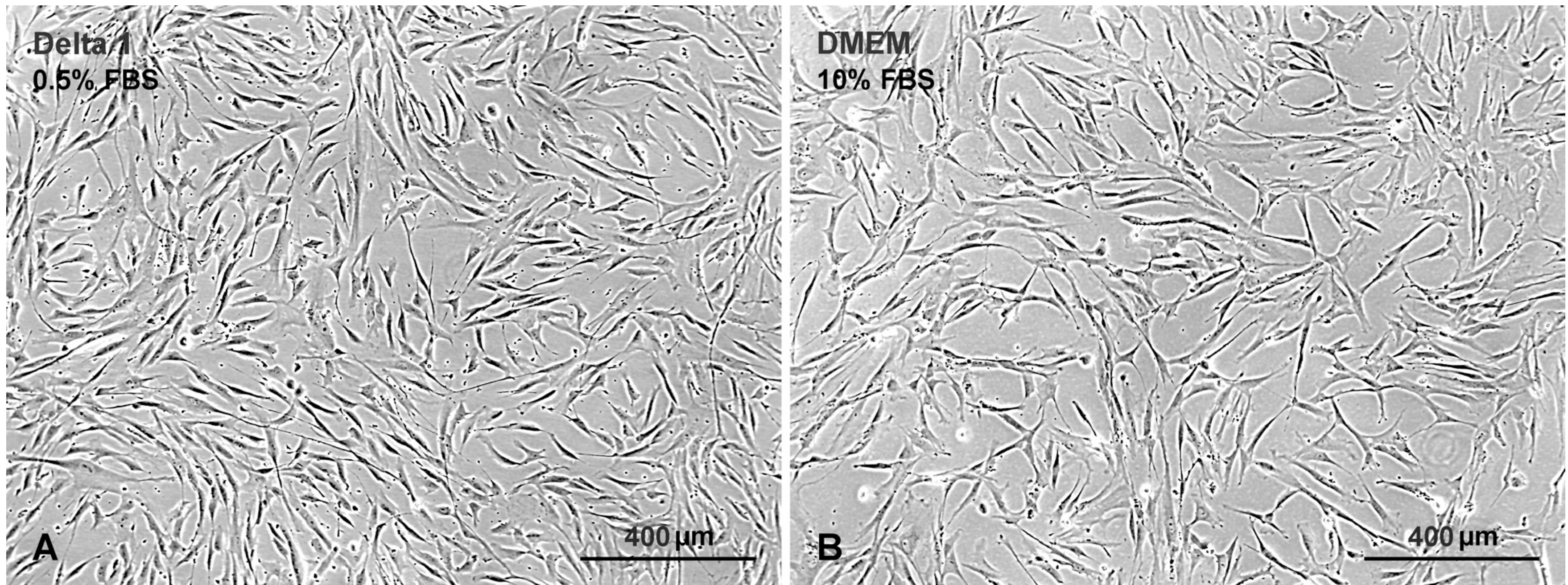


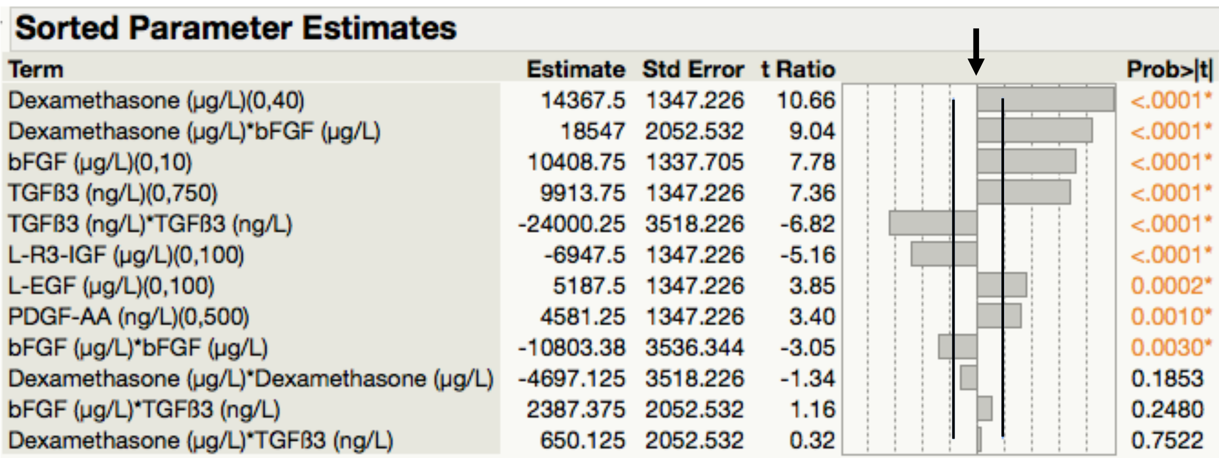
Figure 5.3 Morphology of MRC-5 cells grown in Delta 1 + 0.5% FBS on Day 4. A) Cells cultured in Delta 1 + 0.5% FBS medium presented a comparable shape to those grown in DMEM + 10% FBS medium; B) Cells cultured in both media presented a spindle-like shape.

5.3.1 Analysis of interactions of growth factors in MRC-5 cells

To determine which growth stimulatory substances played a positive role in the growth of MRC-5 cells, and which ones were detrimental to it, JMP 12 software was used to calculate effects from the different growth stimulatory substances on MRC-5 cell yield. The effects were evaluated using the Sorted Parameter Estimates report (Figure 5.4) where the bars to the right of the arrow show a positive effect on the cell yield of MRC-5, and those to the left show a negative effect. Only those bars which are past the black vertical lines have statistically significant effect on cell yield. The parameter estimates in the regression model (described by Equation 5.1) are related to the effect estimates (Montgomery, 2013).

These estimates are the model coefficients denoted by the β's from Equation 5.1 which describe the effects (main, combined, and quadratic) in the DSD model. This report gives a quantitative analysis of: i) the effects, described by the estimate column; ii) the standard error of the estimate; iii) the t-Ratio which is the ratio of the estimate to the standard error that follows a Student's t distribution, and tests whether the true value of the parameter is zero; and 4) the Prob |t|, which is the p-value for the test that the parameter value is zero against the two-sided alternative that it is not (JMP, 2016).

The analysis showed that all six growth stimulatory substances had a statistically significant impact on MRC-5 cell yield. It was shown that dexamethasone presented the highest positive main effect on MRC-5 cell yield with respect with the other growth factors. The summary of the effects with the type of effect is shown in Table 5.4 and the graphical representation is shown in the prediction profiler depicted in Figure 5.5.



Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Dexamethasone (µg/L)(0,40)	14367.5	1347.226	10.66	<.0001*
Dexamethasone (µg/L)*bFGF (µg/L)	18547	2052.532	9.04	<.0001*
bFGF (µg/L)(0,10)	10408.75	1337.705	7.78	<.0001*
TGFß3 (ng/L)(0,750)	9913.75	1347.226	7.36	<.0001*
TGFß3 (ng/L)*TGFß3 (ng/L)	-24000.25	3518.226	-6.82	<.0001*
L-R3-IGF (µg/L)(0,100)	-6947.5	1347.226	-5.16	<.0001*
L-EGF (µg/L)(0,100)	5187.5	1347.226	3.85	0.0002*
PDGF-AA (ng/L)(0,500)	4581.25	1347.226	3.40	0.0010*
bFGF (µg/L)*bFGF (µg/L)	-10803.38	3536.344	-3.05	0.0030*
Dexamethasone (µg/L)*Dexamethasone (µg/L)	-4697.125	3518.226	-1.34	0.1853
bFGF (µg/L)*TGFß3 (ng/L)	2387.375	2052.532	1.16	0.2480
Dexamethasone (µg/L)*TGFß3 (ng/L)	650.125	2052.532	0.32	0.7522

Figure 5.4 Sorted parameter estimates report showing the effects impacting MRC-5 cell yield. They were sorted by the absolute value of the t Ratio, showing the most significant effects (dexamethasone, bFGF and TGF-β3) at the top. A bar chart shows the t Ratio with vertical lines in black showing critical values for the p-value 0.05 significance level.

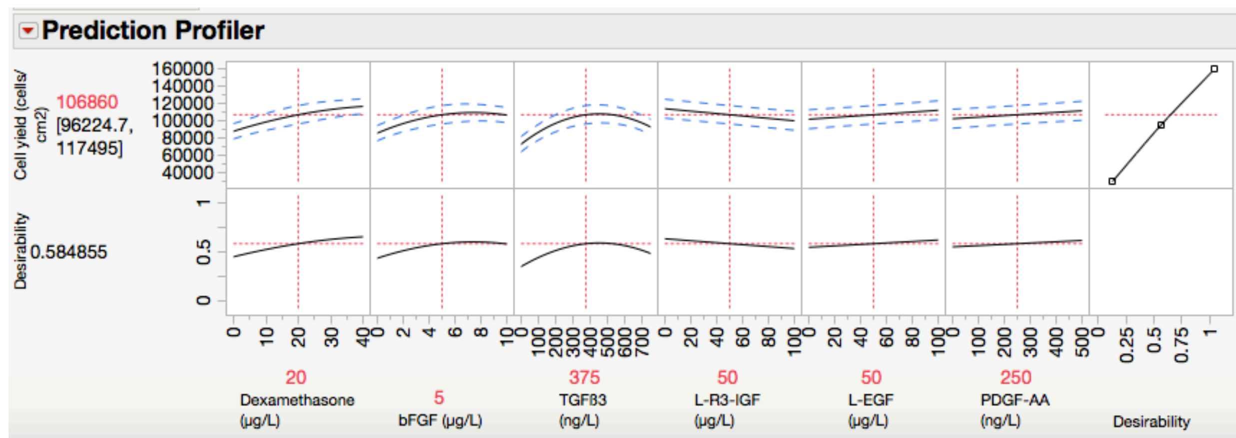


Figure 5.5 Prediction profiler traces of each factor. The importance of each growth factor can be assessed by the steepness of the prediction trace (the greater the slope of the trace, the greater the importance of the factor). The curvature terms denote the quadratic interactions between factors, in this case between dexamethasone, bFGF and TGF-β3.

Table 5.4 Significant effects in MRC-5 cell yield and their interactions

Effect	Factors	Impact
Main	Dexamethasone	Positive
	bFGF	Positive
	TGF-β3	Positive
	L- R^3-IGF-I	Negative
	L-EGF	Positive
	PDGF-AA	Positive
Quadratic	TGF-β3* TGF-β3	Negative
	bFGF*bFGF	Negative
Combined	Dexamethasone*bFGF	Positive

The curvatures of the first three factors (dexamethasone, bFGF and TGF-β3) were due to the combined and quadratic effects and their interactions. The only statistically significant combined effect was the one achieved between dexamethasone and bFGF. This interaction had a positive effect on MRC-5 cell yield. As the concentration of one increased, it would positively impact the effect of the other, ultimately enhancing cell yield.

Two quadratic effects were identified: bFGF*bFGF and TGF-β3* TGF-β3, both had a negative impact on cell yield even though both growth factors presented a main positive effect. In both cases the negative quadratic interaction was described by the function $y = -(x^2)$ giving it a negative parabola shape. At the same time, because each of these growth factors alone presented a positive main effect on cell yield, it skewed the curve to the right (Figure 5.5).

L-EGF, PDGF-AA, and L-R^3-IGF-I presented main effects with lesser impact on cell yield as observed by their slight steepness which was very close to zero (Figure 5.5). L-R^3-IGF-I was

the only growth factor that presented a main negative effect, represented as a linear function (Figure 5.5).

5.3.2 Results of DSD in MRC-5 cells using growth factors

The experiments determined that the Delta 1 medium was comparable to the positive control medium, and therefore, was the most suitable for the culture of MRC-5 cells. This medium contained MVSFM as the basal medium plus the growth stimulatory substances shown in Table 5.5, where L-EGF was the only growth factor provided by Repligen which had an impact on MRC-5 cell yield. According to the prediction profiler (Figure 5.5), dexamethasone, bFGF and TGF-β3 were the effects with the highest impact and interactions in regards to MRC-5 cell yield. It was concluded that the DSD method successfully screened and identified the factors and their interactions that played a role in MRC-5 cell yield.

Table 5.5 Concentrations contained in Delta 1 optimized using the DSD for MRC-5 cells.

	Delta 1 medium formulation (with MVSFM as a basal medium)			
Growth factors	Dexamethasone	bFGF	L-EGF	TGF-β3
Concentration	40 μg/L	10 μg/L	50 μg/L	750 ng/L

5.4 Medium evaluation: Serum free adaptation using Delta 1 in MRC-5 cells

As it was concluded that the Delta 1 was the most optimal medium for MRC-5 (see Table 5.5). Delta 1 was used to adapt the cells to determine if in the absence of serum this formulation was capable of sustaining an equivalent cell yield to cell cultures grown on the positive control

medium DMEM + 10% FBS. MRC-5 cells were cultured in T-75 flasks at a seeding density of $1x10^4$ cells/cm^2. Cells were originally grown in DMEM + 10% FBS, which was chosen as the starting medium for adaptation. In this adaptation experiment, due to the high positive impact of Delta 1 on MRC-5 cell yield, it was determined that the serum percentage could be decreased by half (i.e. Delta 1 + 5% FBS) in the first adaptation passage. Subsequently, the adaptation process continued by transferring the cells into progressively decreasing concentrations of serum as outlined in Table 5.6. This was achieved by serially subculturing the cells until near confluence, and passaging them to the next lower concentration. The cells were maintained in any one concentration of FBS until a consistent cell yield was achieved. The days at which the cells were passaged were dependent on their growth rate which was determined by daily microscopic examination. Doubling-times were calculated to compare the time of growth in the media containing a decreasing concentration of serum throughout every passage.

The yield of MRC-5 cells increased greatly in the first passage (Figure 5.6) and the doubling-time decreased from 28.8 to 22.4 hours in Delta 1 + 5% FBS medium (Table 5.6), this decrease was not significantly different. However, when the serum concentration was reduced from 5% to 1% (i.e. Delta 1 + 1% FBS) the doubling-time increased from 22.4 to 45.9 hours (Table 5.6). The decrease in serum concentration from 0.5%, 0.25%, and 0.1% further increased the doubling-times to 48.9, 55.6, and 108.1 hours, respectively. MRC-5 cells cultured in Delta 1 serum free medium grew extremely slowly, with a doubling-time of 359.5 hours which was not statistically comparable to the positive control. The growth curves and doubling-times revealed that the performance of MRC-5 cells in Delta 1 SFM was not equivalent to either DMEM + 10% FBS or Delta 1 + 0.5% FBS media as per the Student's t-test. For this reason, it was determined

that another model/method should be used to determine the optimized concentrations for the growth stimulatory substances used in this DSD experiment.

Table 5.6 Doubling-times of MRC-5 cells adapted to Delta 1 media (n=3)

Passage #	**Media**	**Doubling-time (hours)**	**SEM(hours)**
p.6	DMEM+10% FBS	28.8	0.2
p.7	Delta + 5% FBS	22.4	3.5
p.8	Delta + 1% FBS	45.9	1.0
p.9	Delta + 1% FBS	42.8	1.1
p.10	Delta + 0.5% FBS	48.9	2.7
p.11	Delta + 0.25% FBS	54.7	1.2
p.12	Delta + 0.25% FBS	56.5	1.5
p.13	Delta + 0.1%	108.1	0.3
p.14	Delta SF	359.5	0.2

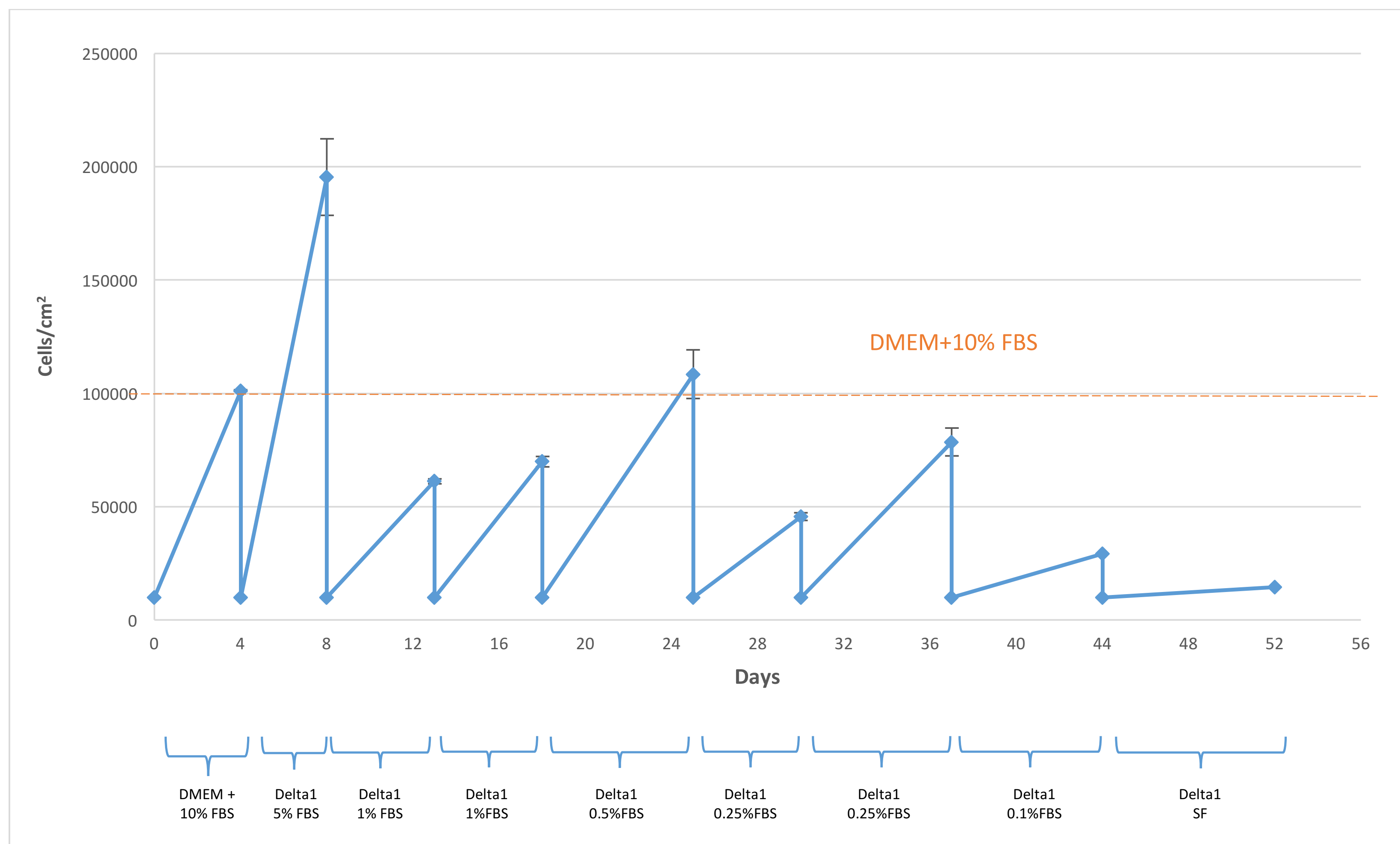


Figure 5.6 Serial subculture of MRC-5 cells in Delta 1 medium over the course of 52 days with progressive decreases in the percentage of serum in the culture medium. Delta 1 SF supported growth of MRC-5 cells up to one passage. Error bars are shown as SEM (n=3).

5.5 Definitive screening design for Vero cells

To optimize the medium for Vero cells and to determine impact of the growth stimulatory substances tested on MRC-5 cells on Vero cell yield, it was decided to use the same DSD method (Table 5.2) that was applied to MRC-5 cells, which would enable a comparison of the effects of the growth stimulatory substances in two different cell lines.

5.5.1 Culture of Vero cells for DSD experiments using growth factors

The experimental media formulations were prepared using MVSFM as a basal medium, without growth factors added, and supplemented with 0.5% FBS. All the different growth stimulatory substances were supplemented to the media using the concentrations detailed in Table 5.2. The 13 different experimental media formulations were subsequently filtered sterilized. The negative control was MVSFM + 0.5% FBS without growth factors and DMEM+10% FBS was used as the positive control.

Vero cells were adapted to MVSFM + 0.5% FBS after determining that this medium was the most adequate for Vero cell growth at low serum concentrations as concluded in Chapter 4. Vero cells were cultured in MVSFM + 0.5% FBS and upscaled in T-75 flasks using the subculture protocol described in Section 2.2.2. Once the cell inoculum was large enough, the cells were transferred into T-25 flasks containing the different media formulations at a seeding density of $1x10^4$ cells/cm^2. Two independent replicate flasks were cultured for each media formulation. The media were changed twice over the course of the experiment. Cells were counted at day seven. At

this time point most of the cells were near confluent under all the experimental conditions. The experiment was repeated three times for statistical significance.

5.6 Vero cell yield in DSD using growth factors

Although there was little variation in Vero cell yields among the experimental media formulations, the Student's t-test at $p < 0.05$, identified four major groups of DSD media (Figure 5.7). Group 1 consisted of the positive control medium and group 2: Medium 13, in which Vero cell yields were statistically different from cultures in all other media formulations. Even though the highest Vero cell yield of the DSD experimental media was observed in cells cultured in Medium 13, this yield was much lower, and statistically different from that observed in the positive control medium (Figure 5.7). The doubling-time Medium 13 was 33.5 hours compared to that of MVSFM which was of 31.2 hours which was larger and it was therefore determined that none of this media were as suitable for Vero cells as the one previously optimized using OFAT experiments. A possible reason for this result is that the different growth stimulatory substances have a different impact in Vero cells compared to MRC-5 cells. As such, either the concentration range or the factors themselves may have not necessarily been suitable for the Vero cell line.

Cell yields observed in the remaining experimental media formulations can be divided into three more subgroups: Group 3 encompassing the Negative control, Media 11, 10, 1, 9. Group 4 was comprised of media 8, 4, 2, 5, 12, and 7 (Figure 5.7). As it can be observed in Table 5.7, the time ranged from 35.1 to 37.4 hours which was only a variation of 2.3 hours which was not significantly different within the group. Group 5 was composed of cultures containing Media 6 and 3 which had doubling-times of 38.4 and 39.8 hours respectively. It was concluded that none

of the media formulations from the DSD had a significant impact on Vero cell yield compared to the one designed by Burgener (2000) which was confirmed using OFAT experiments (see Chapter 3).

Table 5.7 Doubling-times of Vero cell cultures in all the DSD experimental media (n=3).

Media	Doubling-time (hours)	SEM (hours)
DMEM + 10% FBS	29.5	3.9
13 + 0.5% FBS	33.5	1.3
No GFs + 0.5% FBS	34.8	1.5
11 + 0.5% FBS	35.1	1.2
10 + 0.5% FBS	35.1	2.0
1 + 0.5% FBS	35.2	1.7
9 + 0.5% FBS	35.2	1.6
8 + 0.5% FBS	36.2	1.4
4 + 0.5% FBS	36.4	1.4
2 + 0.5% FBS	36.6	1.4
5 + 0.5% FBS	36.8	1.5
12 + 0.5% FBS	37	1.7
7 + 0.5% FBS	37.4	1.6
6 + 0.5% FBS	38.4	0.8
3 + 0.5% FBS	39.8	0.3

5.6.1 Analysis of interactions of growth factors in Vero cells

After analysing the effects of the growth factors on Vero cells, the sorted parameter estimates report (Figure 5.8) revealed that only four factors had a significant impact on Vero cell yield. Those effects (represented as horizontal gray bars) to the right of the black arrow had a

positive effect whereas those to the left had a negative effect. Only those past the black vertical lines were statistically significant. In this experiment, the statistically significant effects for Vero cells were those shown in Table 5.8, where there were two growth factors eliciting a positive effect and two a negative.

From the six different growth factors tested, it can be observed that only bFGF, TGF-β3 and L-R^3-IGF-I had an effect on Vero cell yield. The largest impact on Vero cell yield was elicited by the combined effect of L-R^3-IGF-I * TGF-β3 (Figure 5.8). As observed from Figure 5.9, regarding the individual analysis of significant factors using the prediction profiler, bFGF displayed the largest effect in Vero cell yield. The rest of the factors did not appear to have a significant impact on Vero cell yield, as there was a very slight steepness or curvature in five out of the six factors used in this experiment. It was concluded that Vero had different nutritious requirements than MRC-5 cells. Further elucidation of the impact of growth factors on cell yield would require further DSD experiments in which the range of concentrations would be modified using information from the literature to obtain more meaningful results for this specific cell line.

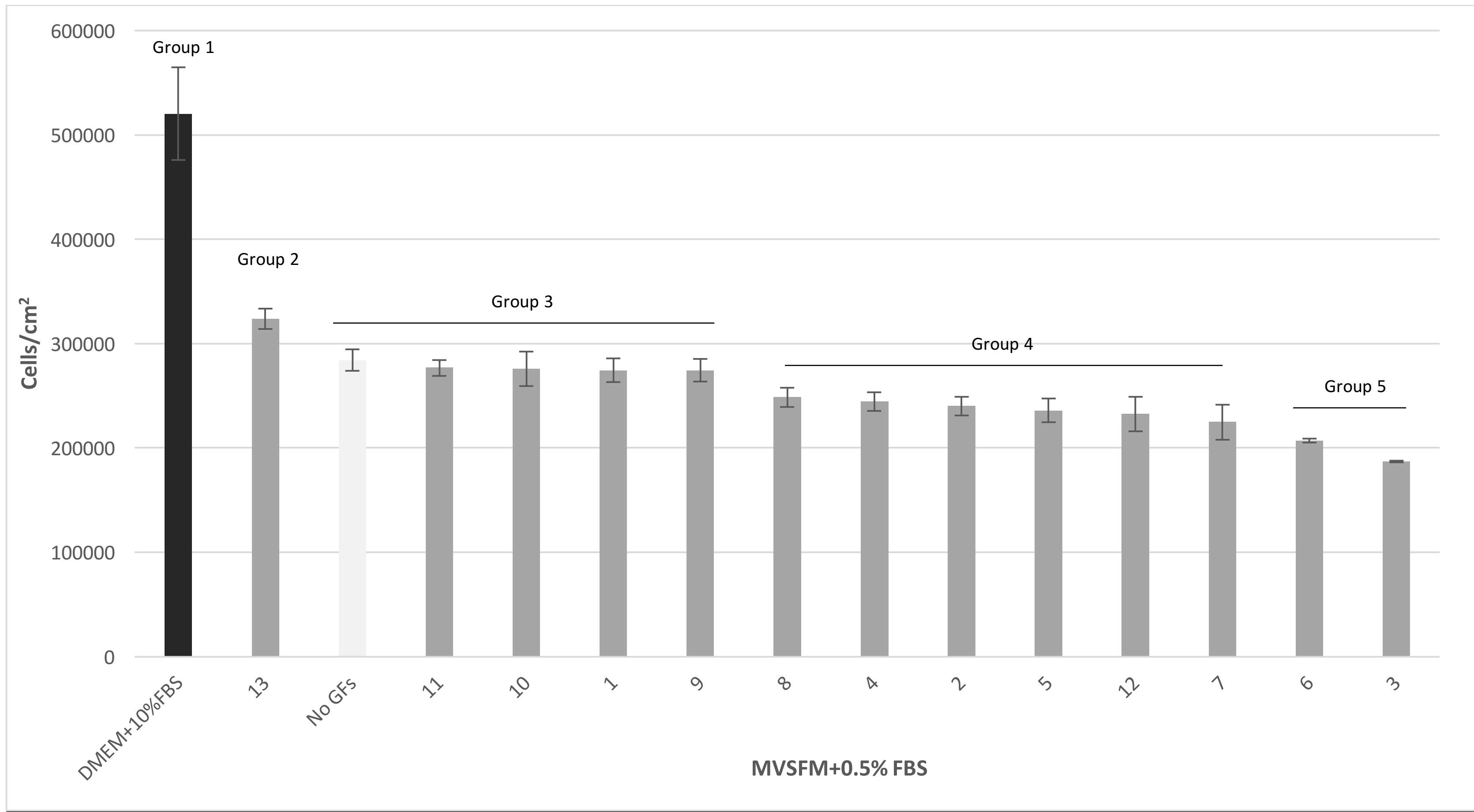
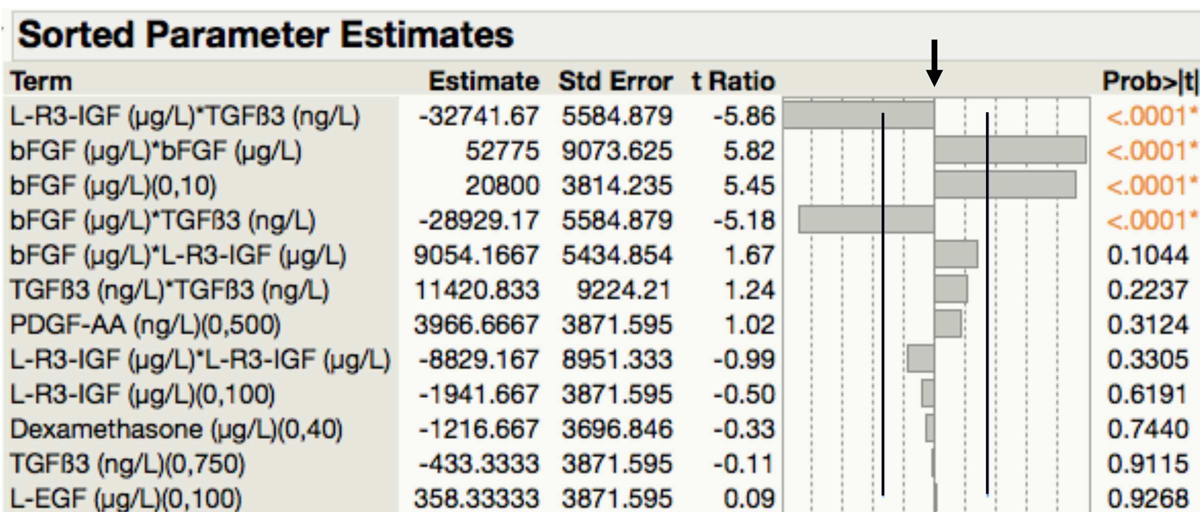


Figure 5.7 Cell yield of Vero cells after four days of growth in the experimental media outlined in Table 5.2 using MVSFM no GFs+ 0.5% FBS as a basal medium. The cell yields of the experimental medium are shown in dark gray. DMEM+10% FBS was used as the positive control (black) and MVSFM (No GFs) + 0.5% FBS was the negative control (light gray). Error bars are shown as SEM (n=3).



Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
L-R3-IGF (μg/L)*TGFß3 (ng/L)	-32741.67	5584.879	-5.86	<.0001*
bFGF (μg/L)*bFGF (μg/L)	52775	9073.625	5.82	<.0001*
bFGF (μg/L)(0,10)	20800	3814.235	5.45	<.0001*
bFGF (μg/L)*TGFß3 (ng/L)	-28929.17	5584.879	-5.18	<.0001*
bFGF (μg/L)*L-R3-IGF (μg/L)	9054.1667	5434.854	1.67	0.1044
TGFß3 (ng/L)*TGFß3 (ng/L)	11420.833	9224.21	1.24	0.2237
PDGF-AA (ng/L)(0,500)	3966.6667	3871.595	1.02	0.3124
L-R3-IGF (μg/L)*L-R3-IGF (μg/L)	-8829.167	8951.333	-0.99	0.3305
L-R3-IGF (μg/L)(0,100)	-1941.667	3871.595	-0.50	0.6191
Dexamethasone (μg/L)(0,40)	-1216.667	3696.846	-0.33	0.7440
TGFß3 (ng/L)(0,750)	-433.3333	3871.595	-0.11	0.9115
L-EGF (μg/L)(0,100)	358.33333	3871.595	0.09	0.9268

Figure 5.8 Sorted parameter estimates report showing the effects impacting Vero cell yield. Estimates are sorted by the absolute value of the t-Ratio, showing the most significant ones (L-R^3-IGF-I, bFGF) at the top. A bar chart shows the t-Ratio with vertical lines in black showing critical values for the p-value 0.05 significance level.

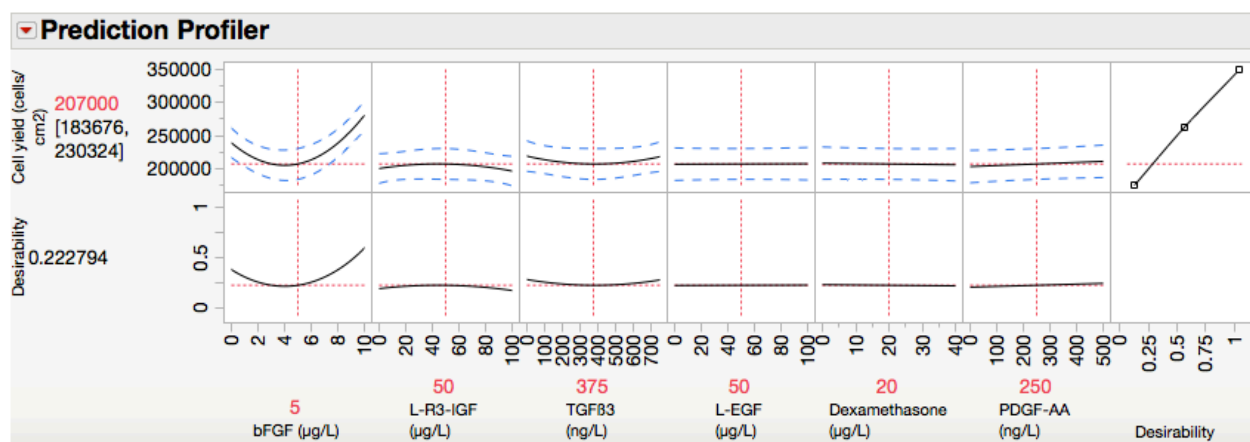


Figure 5.9 Prediction profiler traces of each factor. The importance of each factor can be assessed by the steepness of the prediction trace. The curvature terms denote the quadratic interactions between factors, in this case between, bFGF, L-R^3-IGF-I, and TGF β3 Definitive Screening Design of growth factors.

Table 5.8 Significant effects in Vero cell yield and their interactions.

Effect	Factors	Impact
Main	bFGF	Positive
Quadratic	bFGF*bFGF	Positive
Combined	L-R^3-IGF-I * TGF-β3	Negative
	bFGF* TGF-β3	Negative

5.1.1 Results of DSD in Vero cells using growth factors

It was concluded from this experiment that the best medium formulation achieved by the DSD methodology was Medium 13 which was composed of MVSFM as a basal medium with the addition of the growth factors listed in Table 5.9, but it was not comparable to either the positive control or achieved the doubling-time of MVSFM. The formulations for this media were similar in that they both contained bFGF, but Medium 13 had a 10-fold higher concentration of this growth factor, and they both contained L-R^3-IGF-I with MVSFM containing 30 µg/L more of this growth factor. Medium 13 contained dexamethasone and PDGF-AA but it did not contain L-EGF, which was included in MVSFM (Table 5.9). As a result, it was concluded that MVSFM was a more suitable medium for Vero cells and that the DSD was not able to find a medium more optimum than that obtained with the OFAT experimentation methodology. As such, a growth curves were used to better understand the growth kinetics of Vero cells in both DMEM+10% FBS and MVSFM+0.5% FBS.

Table 5.9 Comparison of the growth factor composition between Medium 13 and MVSFM.

	Dexamethasone	bFGF	PDGF-AA	L-R^3-IGF-I	L-EGF
Medium 13	40 µg/L	10 µg/L	500 ng/L	50 µg/L	-
MVSFM	-	1 µg/L	-	80 µg/L	10 µg/L

5.7 Growth curves of Vero cells MVSFM +0.5% FBS and DMEM + 10% FBS

As mentioned in the previous section, growth curves for Vero cells were performed to characterize the growth kinetics of this cell line in the medium that proved to be the most suitable for Vero cells. This was done to evaluate the growth of Vero cells in cultures containing MVSFM + 0.5% FBS compared to DMEM + 10% FBS. Vero cells were counted each day for eight days. Cell cultures were expanded in the medium of interest and subsequently inoculated into T-25 flasks at a seeding density of 1×10^4 cells/cm^2. In the case of the cells grown on MVSFM + 0.5% FBS, they were first adapted to that serum concentration and then expanded to generate sufficient cells to conduct the growth curve experiments. Three independent replicate experiments were performed for each medium type to ensure robust statistical analyses. Cells were counted each day as described in Section 2.2.3, and the flasks counted on the corresponding day were terminated and discarded.

There was a lag phase observed for cells in MVSFM + 0.5% FBS but cells in serum-based medium assumed a log phase growth from inoculation. Vero cells showed a log phase from day 1 (Figure 5.10). Vero cell yields were about 40% of the cell yields observed in cultures containing the positive control medium throughout the experiment, and both media reached the stationary phase at around day 6. At the end of the growth curve, the cell yield of Vero cells in MVSFM + 0.5% FBS reached 56% of the cell yield observed in cultures containing the positive control medium. It was concluded that MVSFM + 0.5% FBS medium was not equivalent to DMEM + 10% FBS.

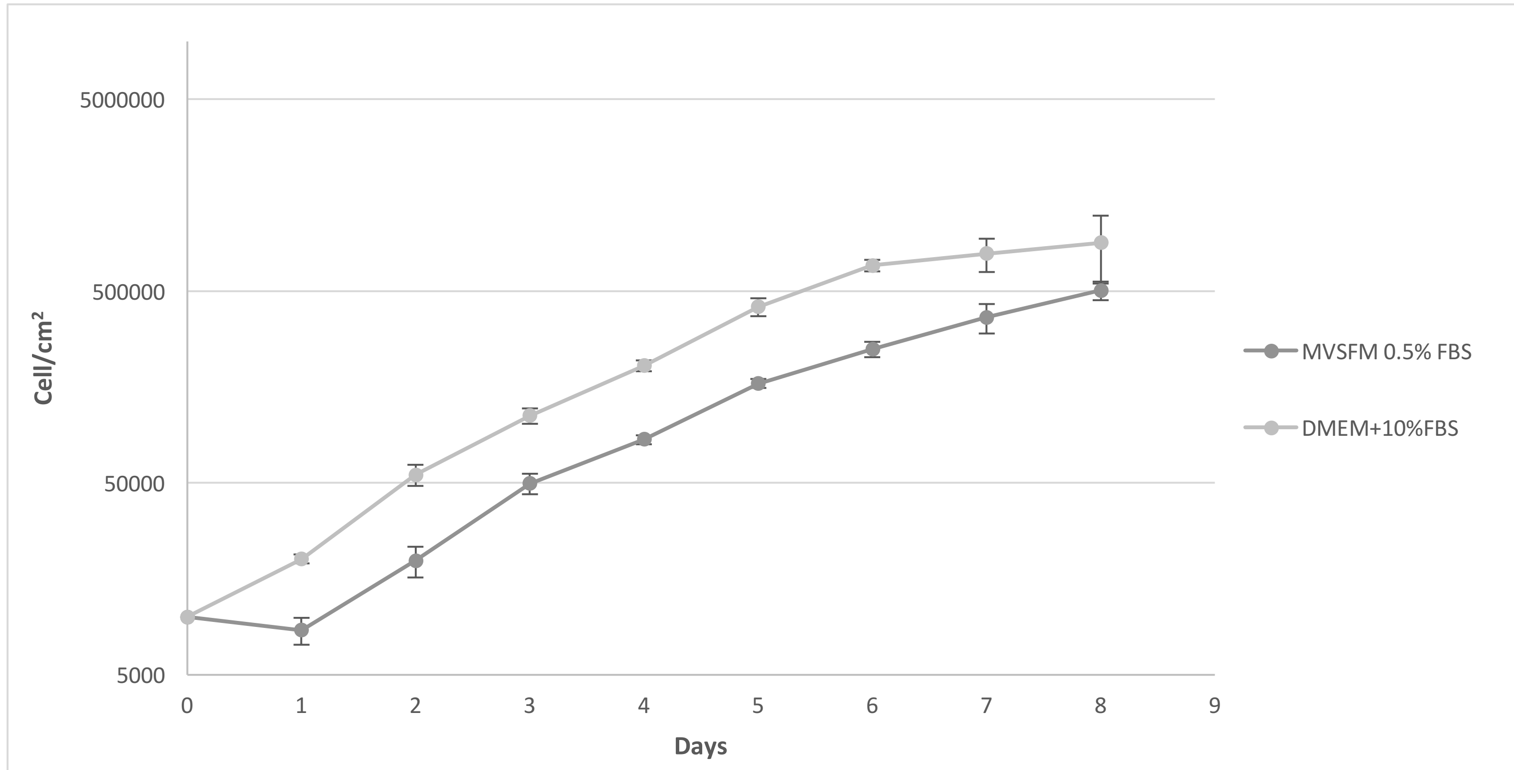


Figure 5.10 Logarithmic growth curves of Vero cells from cultures containing MVSFM + 0.5% FBS medium compared to the positive control medium. (DMEM+10% FBS). Error bars are shown as SEM.

5.8 DSD using Lipids and Vitamins for MRC-5 cells

The composition of the basal formulation of Delta 1 was reviewed to determine which growth stimulatory substances were lacking and which ones could be added to potentially stimulate the growth of MRC-5 in serum free conditions. The following requirements needed to be met: i) maintain a low protein composition; and ii) be non-animal derived. It was proposed that the substances that may be lacking in the medium were lipids and vitamins.

The DSD method was selected for this optimization process, as it had previously proven to be a useful tool to provide information regarding the importance of each factor. In addition, as serum had always been part of the formulation, it was clear that this was an extraneous variable that could be impacting the results of the DoE and it had to be removed in order to eliminate the error and confounding from the experiment. Three categories of substances were chosen for this DSD experiment: lipids, lipid precursors, and vitamins. These substances have shown to stimulate growth in fibroblasts. The concentrations tested were based upon literature values (Table 5.10).

Table 5.10 Concentrations of lipids and vitamins used for DSD matrix.

Growth stimulatory substance	Lower level	Middle level	Upper level
Ethanolamine	0 µM	50 µM	100 µM
Phosphoethanolamine	0 µM	100 µM	200 µM
Cholesterol	0 mg/L	5 mg/L	10 mg/L
Retinoic acid (Vit A)	0 µg/L	9 µg/L	18 µg/L
α-tocopherol (Vit E)	0 µM	25 µM	50 µM
Ascorbic acid (Vit C)	0 µM	100 µM	200 µM

Table 5.11 DSD outline for experiment using vitamins and lipids created by the JMP 12 software using the values from Table 5.10.

Media name	Ethanolamine µM	Phospho-ethanolamine µM	Cholesterol mg/L	Retinoic Acid µg/L	α-tocopherol µM	Ascorbic Acid µM
I	100	200	0	9	0	200
II	100	100	0	18	50	0
III	50	200	10	18	50	200
IV	50	0	0	0	0	0
V	100	0	5	0	50	200
VI	100	200	10	0	25	0
VII	0	0	10	9	50	0
VIII	50	100	5	9	25	100
IX	100	0	10	18	0	100
X	0	200	5	18	0	0
XI	0	200	0	0	50	100
XII	0	0	0	18	25	200
XIII	0	100	10	0	0	200

5.8.1 Culture of MRC-5 cells in Lipid and Vitamins DSD experiments

The experimental media formulations were prepared using Delta 1 serum free (See Table 5.5) as a basal medium. All the vitamins and lipids were supplemented to the media using the concentrations listed in Table 5.11. The 13 different experimental media formulations were subsequently filtered sterilized. The negative control was Delta 1 serum free and DMEM+10% FBS was used as the positive control.

MRC-5 cells were adapted to Delta 1 SF after determining that this medium was the most adequate for MRC-5 cell growth at low serum concentrations as concluded in Section 5.3. MRC-5 cells were cultured in Delta 1 SF and upscaled in T-75 flasks using the subculture protocol described in Section 2.2.2. Once the cell inoculum was large enough, the cells were transferred into T-25 flasks containing the different media formulations at a seeding density of $1x10^4$ cells/cm^2. Two independent replicate flasks were cultured for each media formulation. The media were changed twice over the course of the experiment. Cells were counted at day seven, which was the time point at which most of the cells were near confluent under all the experimental conditions. The experiment was repeated three times for statistical significance.

5.9 MRC-5 cell yield DSD using lipids and vitamins

As it can be observed in Figure 5.11, MRC-5 cells did not survive in media III, VII, VIII, IX and X as the cell yield was zero or very close to that value. These media were characterized for containing cholesterol and all-trans retinoic acid at different concentrations (Table 5.11). This may have occurred due to the combination of the ethanol used to dissolve the all-trans retinoic acid and dexamethasone along with the MβCD used to dissolve cholesterol, which may have been toxic to the cells. The other components were present in some of the formulations but not in others, and cell death was therefore attributed to the solvents used to dissolve cholesterol and retinoic acid.

Daily microscopic observation showed that in the above-listed media, the cells started to detach and die on day 3. Similarly, most MRC-5 cells died in media II, XII and I, which showed a negative doubling-time (data not shown) due to cell death. The aforementioned media contained all-trans retinoic acid.

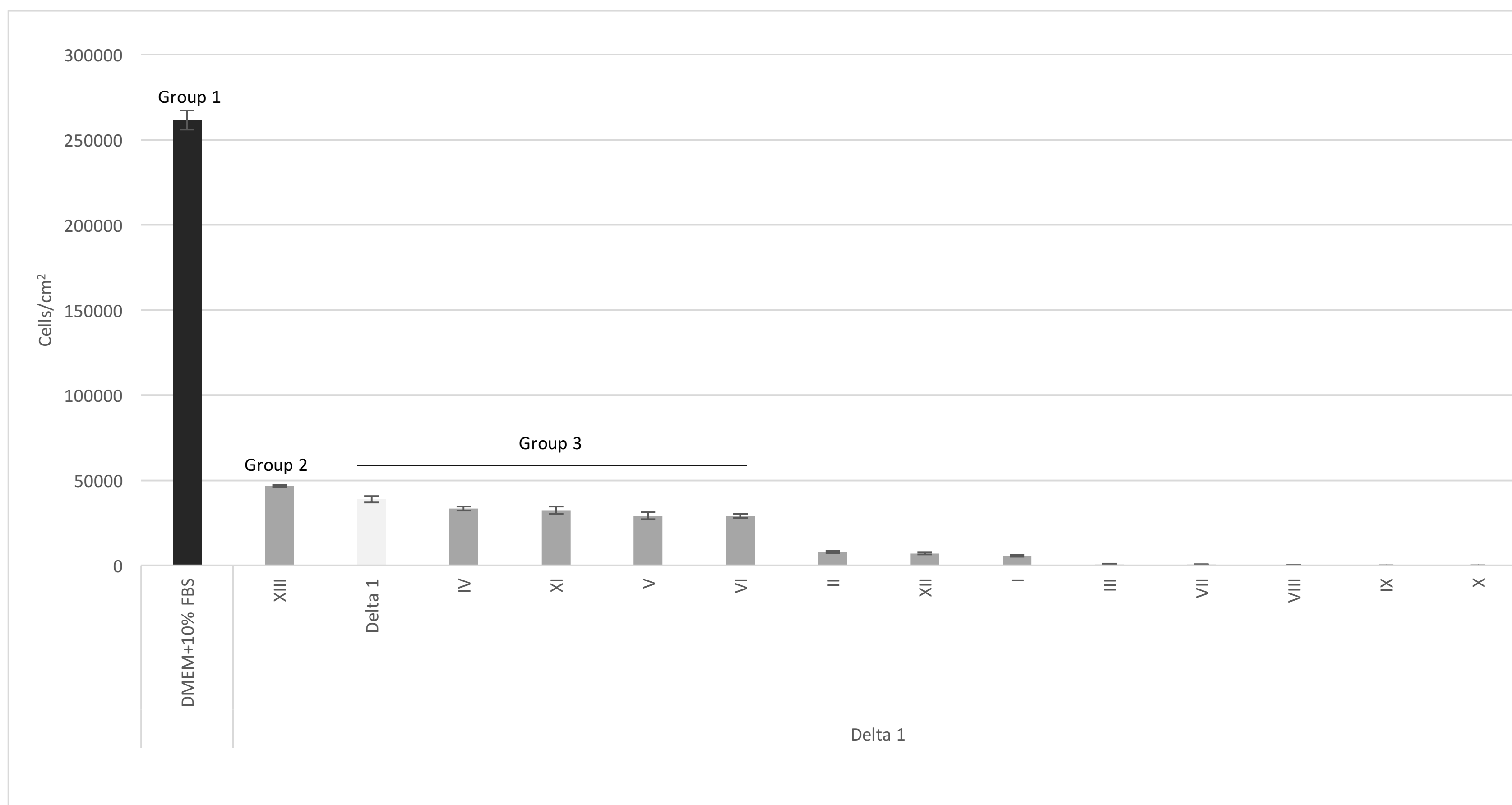


Figure 5.11 Cell yields of MRC-5 cells after seven days of growth in the experimental media outlined in Table 5.10. Delta 1 SF was the basal medium and the negative control (dark gray bar). Cell yields in the experimental medium are shown indicated as gray bars. Cell yields in positive control medium (DMEM + 10% FBS) are indicated by the black bar. Negative control (Delta 1) is indicated by the light gray bar. Error bars are shown as SEM.

Table 5.12 Doubling-time of MRC-5 cells in the experimental SF media formulations that supported cell growth.

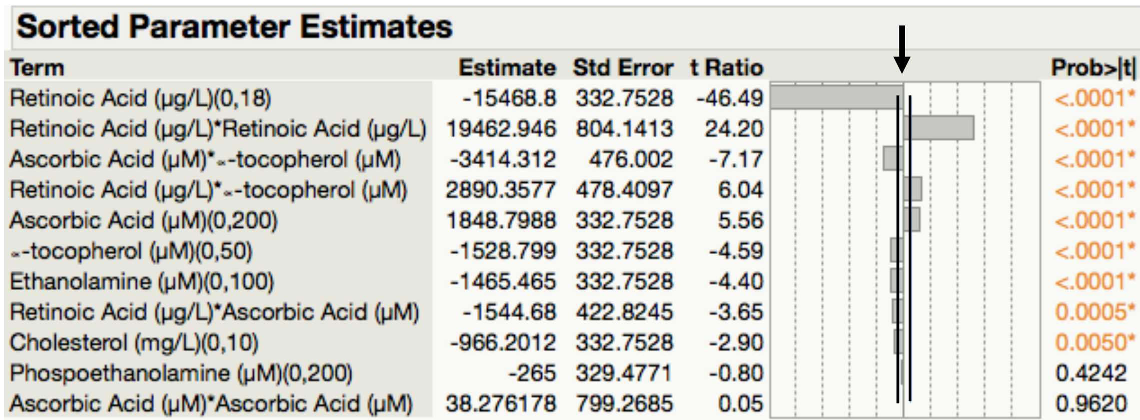
Media	Doubling-time (hours)	SEM(hours)
DMEM+10% FBS	35.68	1.0
XIII	75.62	0.2
Delta 1	85.60	0.8
IV	96.24	0.4
XI	99.02	0.7
V	108.44	0.6
VI	109.14	0.6

From the analysis performed using a Student's t-test on the media formulations that supported MRC-5 cell growth, only two statistically different groups were detected. The first one, composed only by Medium XIII, which achieved the highest cell yield of all the experimental media. The second group was constituted by Delta 1 SF, IV, XI, V and VI. The medium that supported the highest cell yield (46, 644 cell/cm^2 in seven days) and the shortest doubling-time (75.62 hours) of all the experimental media (Table 5.12) was named Gamma XIII (Figure 5.11). Although the cell yield in Gamma XIII medium was close to the cell yield in Delta 1 SF medium, the yields were significantly different from each other at $p < 0.05$ as determined by the Student's t-test. The second highest cell yield was achieved by the negative control medium, Delta SF. Cell yield in the Gamma XIII medium were not comparable to the positive control, DMEM + 10% FBS medium, which was approximately 5.6 times greater.

5.9.1 Analysis of interactions of lipids and vitamins in MRC-5 cells

The analysis of effects was performed for the DSD experiment using lipids and vitamins. The sorted parameter estimates (Figure 5.12) in which the effects (represented as horizontal gray bars) that had a positive effect were shown to the right of the black arrow whereas those that had a negative effect are shown the left. Figure 5.12 revealed that the main effect of retinoic acid and its quadratic interaction had the greatest statistically significant impact on MRC-5 cell yield, this can be observed in Figures 5.12 and 5.13.

Retinoic acid by itself as a main effect, had a very large negative impact. However, as a quadratic effect it was positive for MRC-5 cell yield. This resulted in a parabola pronouncedly skewed to the left (Figure 5.13). The prediction profiler also showed that all the other effects had almost no importance as the slopes of these lines presented little to no steepness, meaning that they had an almost null impact on cell yield as compared to all-trans retinoic acid.



Sorted Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Retinoic Acid (µg/L)(0,18)	-15468.8	332.7528	-46.49	<.0001*
Retinoic Acid (µg/L)*Retinoic Acid (µg/L)	19462.946	804.1413	24.20	<.0001*
Ascorbic Acid (µM)*-tocopherol (µM)	-3414.312	476.002	-7.17	<.0001*
Retinoic Acid (µg/L)*-tocopherol (µM)	2890.3577	478.4097	6.04	<.0001*
Ascorbic Acid (µM)(0,200)	1848.7988	332.7528	5.56	<.0001*
-tocopherol (µM)(0,50)	-1528.799	332.7528	-4.59	<.0001*
Ethanolamine (µM)(0,100)	-1465.465	332.7528	-4.40	<.0001*
Retinoic Acid (µg/L)*Ascorbic Acid (µM)	-1544.68	422.8245	-3.65	0.0005*
Cholesterol (mg/L)(0,10)	-966.2012	332.7528	-2.90	0.0050*
Phospoethanolamine (µM)(0,200)	-265	329.4771	-0.80	0.4242
Ascorbic Acid (µM)*Ascorbic Acid (µM)	38.276178	799.2685	0.05	0.9620

Figure 5.12 Sorted parameter estimates report showing the effects of lipids and vitamins on MRC-5 cell yield. They were sorted by the absolute value of the t-Ratio, showing the most significant effects (retinoic acid) at the top. A bar chart shows the t-Ratio with vertical lines in black showing critical values for the p-value 0.05 significance level.

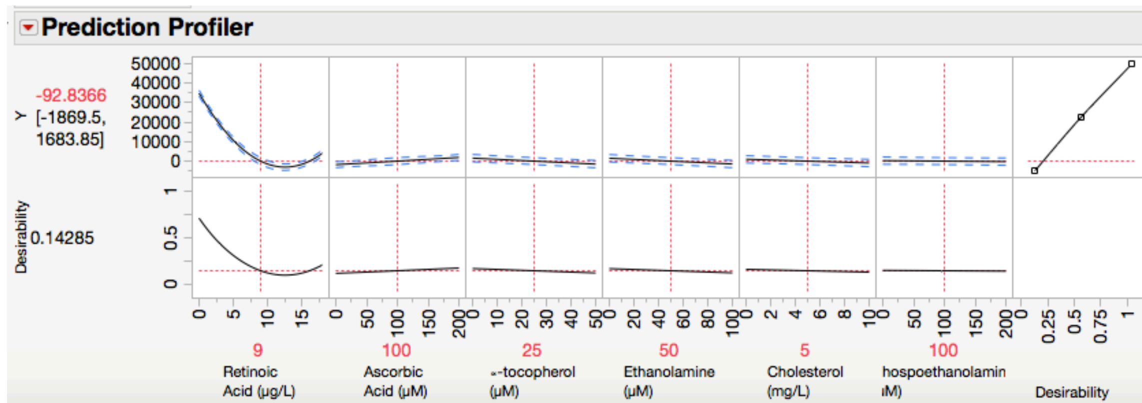


Figure 5.13 Prediction profiler traces of each factor (lipids and vitamins). The importance of each factor can be assessed by the slope of the prediction line. The curvature terms denote the quadratic interactions between factors.

5.9.2 Results of DSD using lipids and vitamins in MRC-5 cells

In this experiment, it was concluded that Gamma XIII that was composed of Delta 1 medium as a basal medium (see Table 5.5) with the addition of the supplements shown in Table 5.13 was the best SF medium formulated thus far, but it was not equivalent to Delta 1 + 0.5% FBS.

Table 5.13 Vitamins and lipids in Gamma XIII SF using Delta 1 SF as a basal medium.

	Gamma XII SF medium (with Delta 1 as a basal medium)		
Lipid/Vitamin	Phosphoethanolamine	Cholesterol	Ascorbic acid
Concentration	100 μM	10 mg/L	200 μM

It was concluded that Gamma XIII SF lacked other substances contained in serum that allow MRC-5 cells to grow in Delta 1 + 0.5 % FBS comparably to when grown in DMEM + 10 % FBS. As can be seen from the composition of Gamma XIII in Table 5.13, the cell yield of MRC-5 was not affected by ethanol which was used to dissolve the vitamins all-trans retinoic acid or α-tocopherol because these components were not part of the Gamma XIII formulation. Cholesterol was part of this formulation. Hence, it was concluded that MβCD was not toxic to the cells. Although dexamethasone was contained in the basal medium formulation (see Table 5.5, Delta 1 SF formulation) and this steroid was dissolved in ethanol (see Section 2.3.3), it was used at a very low concentration, and it did not kill the cells. This result led to the conclusion that the effect of vitamins and lipids included in this experiment was not as significant on the growth of MRC-5 cells in serum free conditions.

5.10 Growth curves of MRC-5 using Gamma XIII and DMEM + 10% FBS

Finally, growth curves of MRC-5 cells cultured in Gamma XIII and DMEM + 10% FBS media were conducted to characterize the kinetics of MRC-5 cell growth in the optimized CD-ACF-SFM formulations. The medium was changed every two days. As it can be seen in Figure 5.14, on day 3, the growth of MRC-5 cells in Gamma XIII SF medium was comparable to cell growth in DMEM + 10% FBS, when the cell density in Gamma XIII SF medium was 82% of the cell density achieved by MRC-5 cells growing in the positive control medium.

As previously seen in the growth curves of the optimized media that contained 0.5% FBS, there was a decrease in cell yield on day 2, which may be due to the lack of the necessary factors that promote attachment of cells. This would compromise the later stages of growth, as seen in Figure 5.14. The cells reached stationary phase at day 5, which could be due to a lack of key nutritious factors contained in serum required by MRC-5 cells which were available at low concentrations in Delta 1 + 0.5% FBS which was a more suitable medium for MRC-5 cells.

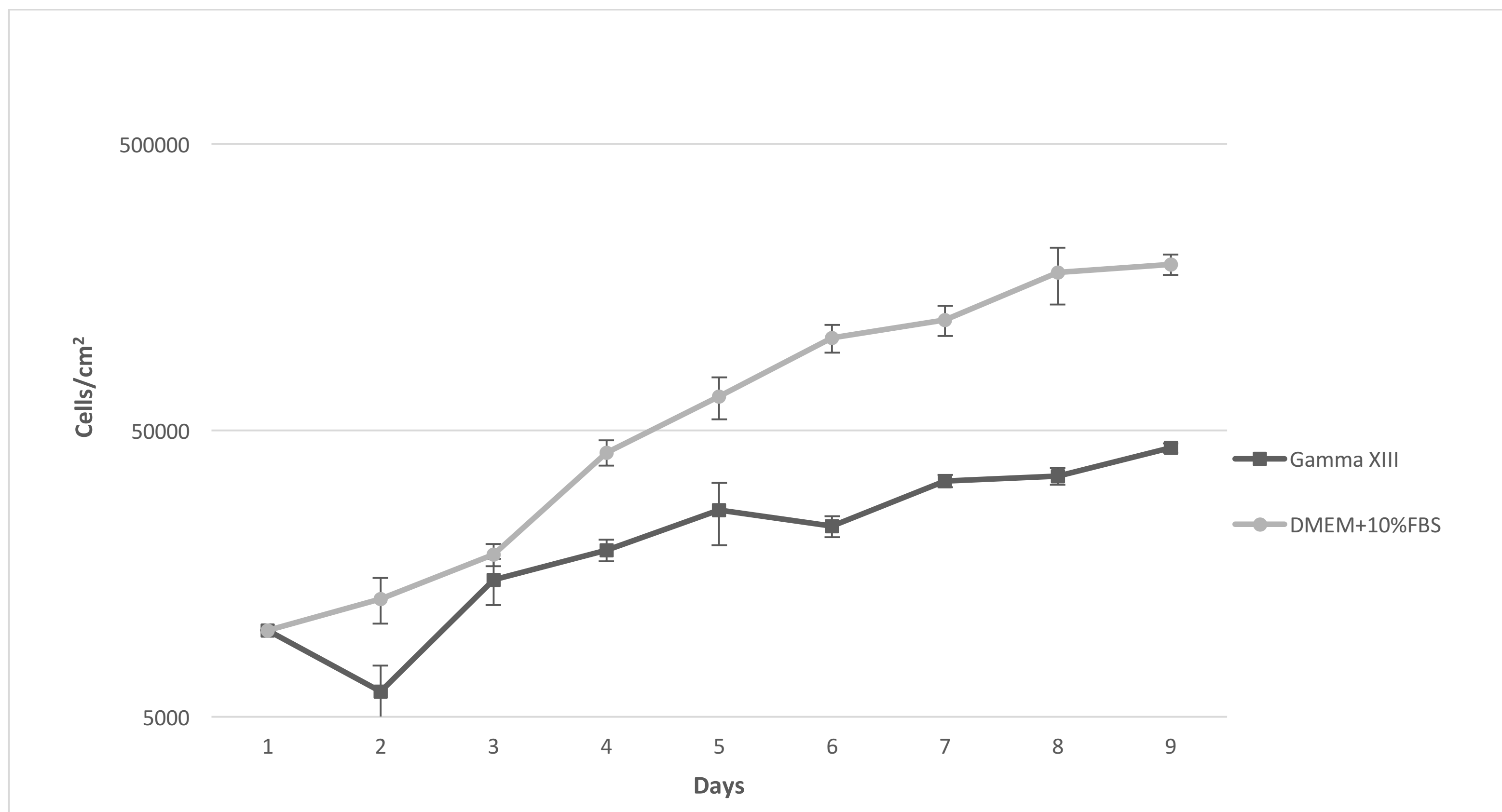


Figure 5.14 Growth curve of MRC-5 cells cultured in Gamma XIII SF medium (square) and the positive control (DMEM + 10% FBS) media (circle). The cell yields are expressed in cells/cm^2 (n=3).

5.11 Discussion

5.11.1 DSD experiment using growth factors

The overall results obtained from the DSD experiment on MRC-5 cells using L-EGF, L-R^3-IGF-I, bFGF, TGF β-3, PDGF-AA, and dexamethasone using MVSFM as a basal medium formulation with 0.5% FBS, show that in MRC-5 cells only one of the media was comparable to the positive control DMEM + 10% FBS. This medium, designated Delta 1 that was supplemented with 0.5% FBS, was capable of supporting cell growth of MRC-5 cells by maintaining cell yields, doubling-times, and morphology comparable to those observed in the positive control medium (DMEM + 10% FBS). Delta 1 medium contained 40 µg/L dexamethasone, 10 µg/L bFGF, 50 µg/L L-EGF and 750 ng/L TGF-β3. The DSD proved to be a very efficient technique to determine the main effects and interactions of the growth factors in Delta 1 + 0.5% FBS.

The growth factors contained in Delta 1 medium were also found to stimulate lung fibroblast proliferation in a study by Hertzel *et al.* (2005) that determined that IGF-I and PDGF had a positive impact on cell proliferation, which does not correspond to the results obtained in the current experiments. This could be due to the fact that Hertzel *et al.* (2005) used PDGF-BB as opposed to PDGF-AA. This isoform was selected for the current experiments because a previous study by Warshamana *et al.,* (1998) found that dexamethasone stimulated the activation of the PDGF-α receptor, which ultimately induces lung fibroblast proliferation mediated by PDGF-AA. However, the concentration range used in the current study (0- 500 ng/L) differed from the one used by Hertzel *et al.* (3-30 µg/L) due to the high cost of this recombinant growth factor. Several studies have demonstrated that L-R^3- IGF-I has cell proliferative effects, as discussed in Section

3.4. This experiments showed that this growth factor did not appear to have a significant impact on MRC-5 cell proliferation compared to the other growth factors contained in Delta 1 medium.

Analysis of Vero cell yield in the DSD experimental media with the same concentrations of growth factors, showed that this medium did not elicit the same effect this cell line, leading to the conclusion that the growth factor requirements for Vero cells are not the same as those of MRC-5 cells. This conclusion is supported by the fact that Vero cells are epithelial cells, whereas MRC-5 cells are fibroblasts. Throughout the experiment, Vero cell yields cultures were very consistent amongst the experimental media and almost invariable compared cell yields of MRC-5 cells grown in the experimental DSD media 1-13. For this reason, it was determined that this was not a transferrable experiment and that a different experiment should conducted to optimize Vero cells with growth factors and concentration ranges specific to the nutritious requirements of this cell line. It was concluded that MVSFM was a more adequate medium for this cell line because it had a faster doubling-time than any of the DSD experimental media.

The overall conclusion derived from the DSD with growth factors was: on MRC-5 cells, the result obtained suggests that at a concentration of 0.5% FBS, the addition of the growth stimulatory substances: dexamethasone, bFGF, L-EGF, and TGF-β3 at the concentrations shown in Table 5.5 which defined the Delta 1 formulation, produced an equivalent cell yield to that of the positive control. In the case of Vero cells, the DSD did not generate a medium compared to DMEM+10% FBS and MVSFM + 0.5 % FBS was found to be a more suitable medium for Vero cells.

5.11.2 DSD experiment using vitamins and lipids

Despite the previous success obtained using DSD with growth factors, this experiment was not able to obtain an optimized medium that was equivalent to the positive control after supplementing Delta 1 SF (the new basal medium) with lipids and vitamins. The addition of lipids has always posed a problem in SFM due to issues with solubilisation (Freshney, 2010), due to the fact that lipids and some vitamins are hydrophobic molecules that must be solubilized in ethanol or MβCD. The combination of these solvents may be toxic to the cells in SF, even at low concentrations, as the medium does not contain the protective proteins or factors normally found in serum.

Even those substances that are water soluble, such as ascorbic acid (vitamin C), and have proven to stimulate dermal fibroblast cell proliferation and collagen production in several studies (Hata and Senoo, 1989; Phillips *et al.,* 1994; Boyera *et al.,* 1998), did not have a significant positive impact on yields of MRC-5 cells in the current study, as shown by the sorted parameter estimates. The best media, which was designated as Gamma XIII (containing 100 µM phosphoethanolamine, 10 mg/L cholesterol, and 200 µM ascorbic acid) did not elicit an equivalent cell yield to that of the positive control medium (DMEM + 10% FBS) or that achieved by Delta 1 + 0.5% FBS. The cell yield of MRC-5 cells cultured in Gamma XIII had the highest cell yield of all CD-ACF-SF formulations and it showed to be statistically significantly different from Delta 1 SF. Gamma XIII did not contain any of the lipid insoluble molecules dissolved in ethanol, which was suspected to have a negative impact on cell yield. This extraneous variable was not tested using the DSD experimentation methodology, and therefore it was difficult to account for its effect in this experiment.

As Delta 1 was used as the basal medium formulation for this experiment and this medium was optimized using 0.5 % FBS, it was hypothesized that serum could be interacting with the growth factors included in the Delta 1 formulation, and that this was an extraneous variable. In order to counteract that, a blocking DSD experiment could have been performed in SF conditions to eliminate the errors and confounding effects of serum (Jones and Nachtsheim, 2016). It should be noted that the interactions and effects obtained from the models from the DSD may not reflect all possible interactions because they do not account for cell receptors and other signalling pathways that occur in the cell.

As was hypothesized, the elimination of serum was deleterious to yields of MRC-5 cells. Thus, this experiment concluded that a ACF-CD-SFM would require more steps for optimization in SF conditions because of the stringent nutritious requirements of MRC-5 cells, and that the DSD method is a useful tool as long as the factors being evaluated are provided in the concentration ranges that correspond to the nutritional requirements of the cell line of interest. However, Delta 1 + 0.5% FBS was the most optimum medium so far produced using the DSD.

Chapter 6

Genetic Algorithms and Hill-Climbing methods for Media Optimization

6.1 Introduction

Other mathematical methods that can enhance the bio-manufacturing processes have been recommended within the QbD guidelines through the PAT (Process Analytical Technology) such as tools for multivariate control of process data modelling over the course of technological development stages (Takahashi *et al*, 2016). Some of these mathematical models can lead to more optimized products and processes. The DSD, as any other DoE, is a mathematical technique that projects a surface solution function over a design space. In other words, the DSD is only a mathematical representation of the design space described by Equation 5.1 (see p.101) and it does not define a maxima or minima of the model, which are obtained by other search algorithms (Mijares Chan, 2016). Examples of search algorithms used by the JMP 12 software include Stepwise Regression analysis and Least Squares/Forward Stepwise evaluation. Since these algorithms perform a quick search using a low power processing hardware, the results generated may give a suboptimal solution.

Given that the Stepwise Regression search method is as vulnerable to falling into local minima within the design space as any other local search method, it was proposed that a robust and a global search technique should be introduced, such as genetic algorithms (GA) (Takahashi *et al*, 2016). While this technique improves the probability of finding the global minimum, it performs the search at a slower rate. In addition, it has the possibility to be performed in parallel

with other analyses. GAs have the capability of producing a well-fitted model that can be readily searched using methods such as stochastic Hill-Climbing compared to response surface modeling (Gulati *et al.,* 2010). This technique relies on random starting points and searches for minimum values locally. The search is repeated for a determined set of iterations, which in the end of the evaluation of the different optimizations can result in the best local minimum (Rudolf and Koppen, 1996). The DSD generated a medium (Delta 1 + 0.5% FBS) for MRC-5 cells that had produced a comparable cell yield to the positive control DMEM + 10% FBS.

The objective of this experiment was to determine if it was possible to generate a medium formulation for MRC- 5 cells that would produce a higher cell yield than Delta 1 + 0.5% FBS using the results derived from the DSD using growth factors by inputting them in the GAs optimization and stochastic Hill Climbing (HC) search.

6.2 Algorithm methodology

The optimization was performed in the IDEAS laboratory in the Departments of Computer Science by Dr. Jose Juan Mijares Chan. The model regression evaluation was performed using GA was 1000 generations or iterations. Each generation included the following operators: i) only in the first generation: A group of populations (10000) was defined, each gene in the population or parent represents a random coefficient (β_x) from Equation 5.1 (pg. 101); ii) selection, a group of genes per population are randomly selected by pairs for cross-over with another population. This is applied 90% of the time; iii) cross-over, using single point cross over, some genes are exchanged with the other population genes; iv) mutation, a random number is replaced randomly 5% of the time. This introduces a random effect on the populations; v) elitism this step compares the offspring with the parents, selecting only the best genetic content and replacing the parents on the

next generation. This ensures an elite solution every generation or at least not worse than the previous one. This is constrained by surveying if the populations show diversity, meaning they have a standard deviation greater than zero; vi) fitness function, in this step, the error of the model is compared against the observed data. This operator is used at the elitism evaluation distinguish the fitness of the offspring from the parents.

The optimization of the concentration for each growth stimulatory substance was performed using stochastic Hill Climbing using 1000 evaluations in parallel for 1000 iterations. Each iteration involved selecting 10,000 random points for the search where each starting point represented the value of the concentration of each factor (growth stimulatory substance) represented in Equation 5.1 as x_i. The resulting maximum cell yield was considered to be the next point. The current evaluation point was compared against the starting point, with the following criteria: if the evaluation point was greater than the maximum cell yield, the maximum cell yield was now equal to the resulting evaluation point result. After 10,000 iterations, the resulting locations represented the best local maxima of the algorithm.

6.2.1 Model result interpretation

Model fitting using GA was performed by comparing the optimized theoretical model to the experimental results from Section 5.3, which were the cell yields obtained from the different media formulations (Figure 5.2). In the beginning (generation 0), the error of the model versus the observed data was very large (in the order of magnitude of 10^4). As the algorithm evolved, the model with the best-fit was selected, and the error decreased significantly to 0.01 (Figure 6.1).

The GA optimization gave a mean $R^2 = 0.9836$ with respect to the model error, while the Stepwise Regression model used by the JMP software generated a mean $R^2 = 0.4941$. This means

that the model optimization performed using genetic algorithms was more robust than the Stepwise Regression model. The coefficients obtained using GA were used as the input for Equation 5.1 to solve for the variables (x_i) or concentrations from each growth factor.

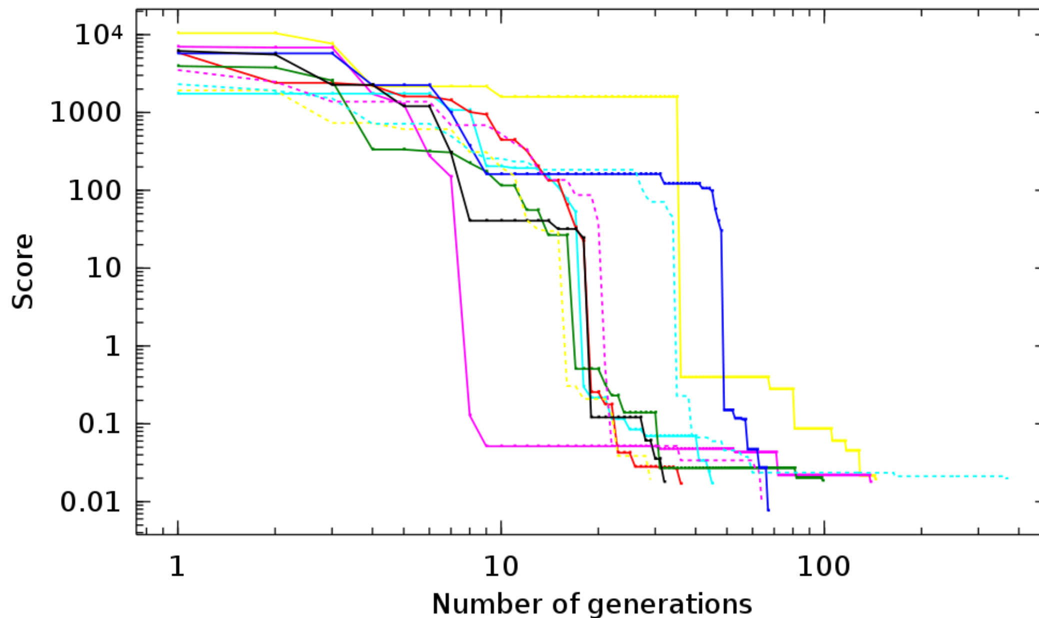


Figure 6.1 Coefficient optimization using parallel the Genetic Algorithm model over the course of 1000 generations. The coefficients were optimized using this method. The error score decreased with increasing number of generations. Each color represents one iteration.

Regarding the growth factor concentration optimization search using the HC methodology (Figure 6.2), the values of the optimized coefficients were used to solve for the values of the variables using Equation 5.1 to determine the optimized concentrations of growth factors using the results from the genetic algorithm. As the number of iterations increased, it was possible to obtain a growth close to 80% of the best value, as seen in Figure 6.2. The resulting first eight optimized concentrations obtained from the search were selected for subsequent validation by testing the media on MRC-5 cells. The values are shown in Table 6.1.

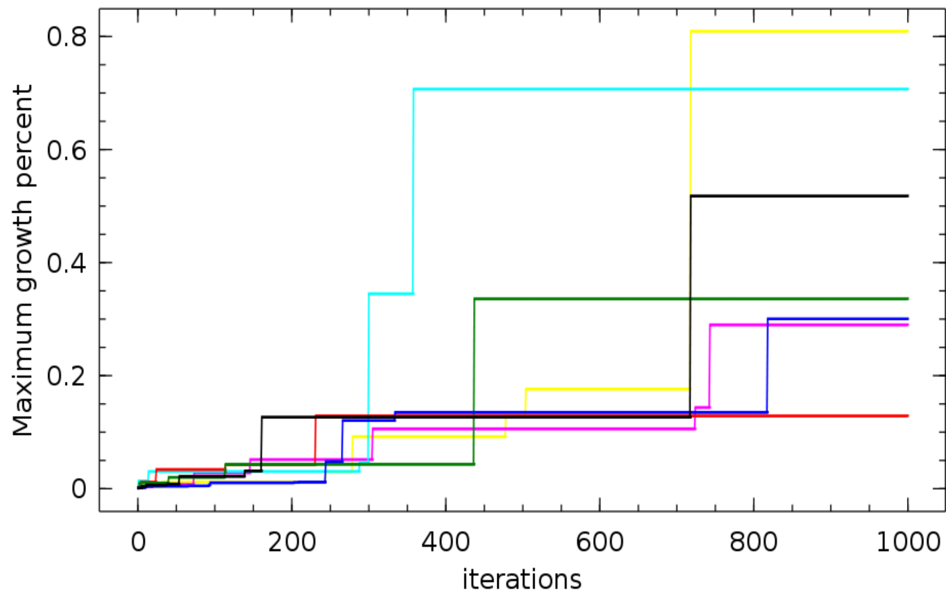


Figure 6.2 Hill Climbing stochastic search for optimized factor (growth stimulatory substances) concentrations that achieved maximized growth of MRC-5 cells. Each color represents one iteration.

6.2.2 Cell culture of model validation using MRC-5 cells

The experimental media formulations were prepared using MVSFM as a basal medium, without growth factors added, and supplemented with 0.5% FBS. All the different growth stimulatory substances were supplemented to the media using the concentrations detailed in Table 6.1. The eight different experimental media formulations were subsequently filtered sterilized. The negative control was MVSFM + 0.5% FBS without growth factors and DMEM+10% FBS was used as the positive control. Delta 1 + 0.5% FBS was used for comparison and to determine which media was most favourable.

Table 6.1 Outline of the top eight media formulations optimized using GA-HC methodology media series was dubbed GH. Delta 1 is included for comparison

Media	Dexamethasone (μg/L)	bFGF (μg/L)	PDGF-AA (ng/L)	L-EGF (μg/L)	L-R^3-IGF-I (μg/L)	TGF-β3 (ng/L)
GH1	24	0.6	0	7	22	548
GH2	14	0	0	12	79	542
GH3	7	0.2	0	0	68	0
GH4	1	0	63	6	60	0
GH5	3	3	57	4	60	712
GH6	2	3	0	68	60	132
GH7	22	4	0	16	0	0
GH8	39	5	0	2	24	0
Delta 1	40	10	0	50	0	750

For comparison to the DSD experiment (see Section 5.3), MRC-5 cells were adapted to Prototype + 0.5% FBS. Thus, they were cultured and upscaled in this medium in T-75 flasks using the subculture protocol described in Section 2.2.2. Once the cell inoculum was large enough, the cells were transferred into T-25 flasks containing the different media formulations at a seeding density of $1x10^4$ cells/cm^2. Two independent replicate flasks were cultured for each media formulation. The media were changed twice over the course of the experiment. Cells were counted at day seven, which was the time point at which most of the cells were near confluent under all the experimental conditions. The experiment was repeated three times for statistical significance.

6.2.3 Validation results using GH media in MRC-5 cells

After testing the eight different media formulations with MRC-5 cells listed in Table 6.1, it was concluded that none of the media were statistically comparable to DMEM + 10% FBS as it can be seen in Figure 6.3. In this experiment the Student's t-test (at $p < 0.05$) identified five different statistically similar groups within the experimental media, which were all supplemented with 0.5% FBS. Group 1 was composed of the positive control, DMEM+10% FS. Group 2 was composed of Delta 1 + 0.5% FBS and GH 8 media. These formulations showed the highest cell yield from all the media and the doubling-times differed by approximately two hours (Table 6.2), which were not significantly different according to the Student's t-test. The most notable difference in composition between these two media was the fact that Delta 1 did not have L-R^3-IGF-I whereas GH8 did not contain TGF-β3. None of them contained PDGF-AA.

Group 2 overlapped with Group 3 as it was composed of media GH8, GH6, GH7 and GH5. This group of media was mainly characterized for not containing PDGF-AA (Table 6.1). Within group 3 the doubling-times presented a range of 6 hours. Group 4 was composed of GH 1, GH3, GH2 and GH4, this group presented the large difference of 15.6 hours in doubling-time within the group. In this case, the doubling times are not statistically significant to each other except for GH4. Group 4 was mainly characterized by containing the lowest concentration of bFGF (Table 6.1). The last group was composed by the negative control meaning that all other media were significantly different from the negative control that had the largest doubling-time of 142.7 hours (Table 6.2).

Several differences were observed in this experiment. The doubling-time of the positive control (DMEM + 10% FBS) was not consistent with the one obtained in the DSD experiment, as it decreased by about 5 hours, meaning that the cells grew faster. On the other hand, MRC-5 cells

cultured in Delta 1 + 0.5% FBS did not show the same result as previously observed in Section 5.3, as the doubling-time increased by 3 hours. These discrepancies may have been due to differences in seeding density or incubation temperature variations.

In the case of the morphology of MRC-5 cells cultured in GH8 + 0.5% FBS, the cells presented some slight differences compared to those grown in DMEM + 10% FBS. Cells maintained their spindle-like shape and did not show any signs of vacuolation, but the cytoplasm was slightly enlarged (Figure 6.4).

Table 6.2 Doubling-times of MRC-5 cells cultured in the genetic algorithm optimized media (n=3).

Media	Doubling-time (hours)	SEM (hours)
DMEM+10%FBS	39.7	1.3
Delta1+0.5% FBS	49.3	1.4
GH8 +0.5% FBS	51.2	1.6
GH6 +0.5% FBS	54.1	1.0
GH7 +0.5% FBS	55.4	1.1
GH5 +0.5% FBS	57.4	1.4
GH1 +0.5% FBS	62.0	1.2
GH3 +0.5% FBS	67.4	1.3
GH2 +0.5% FBS	74.1	1.2
GH4 +0.5% FBS	77.6	1.0
No GFs +0.5% FBS	142.7	0.5

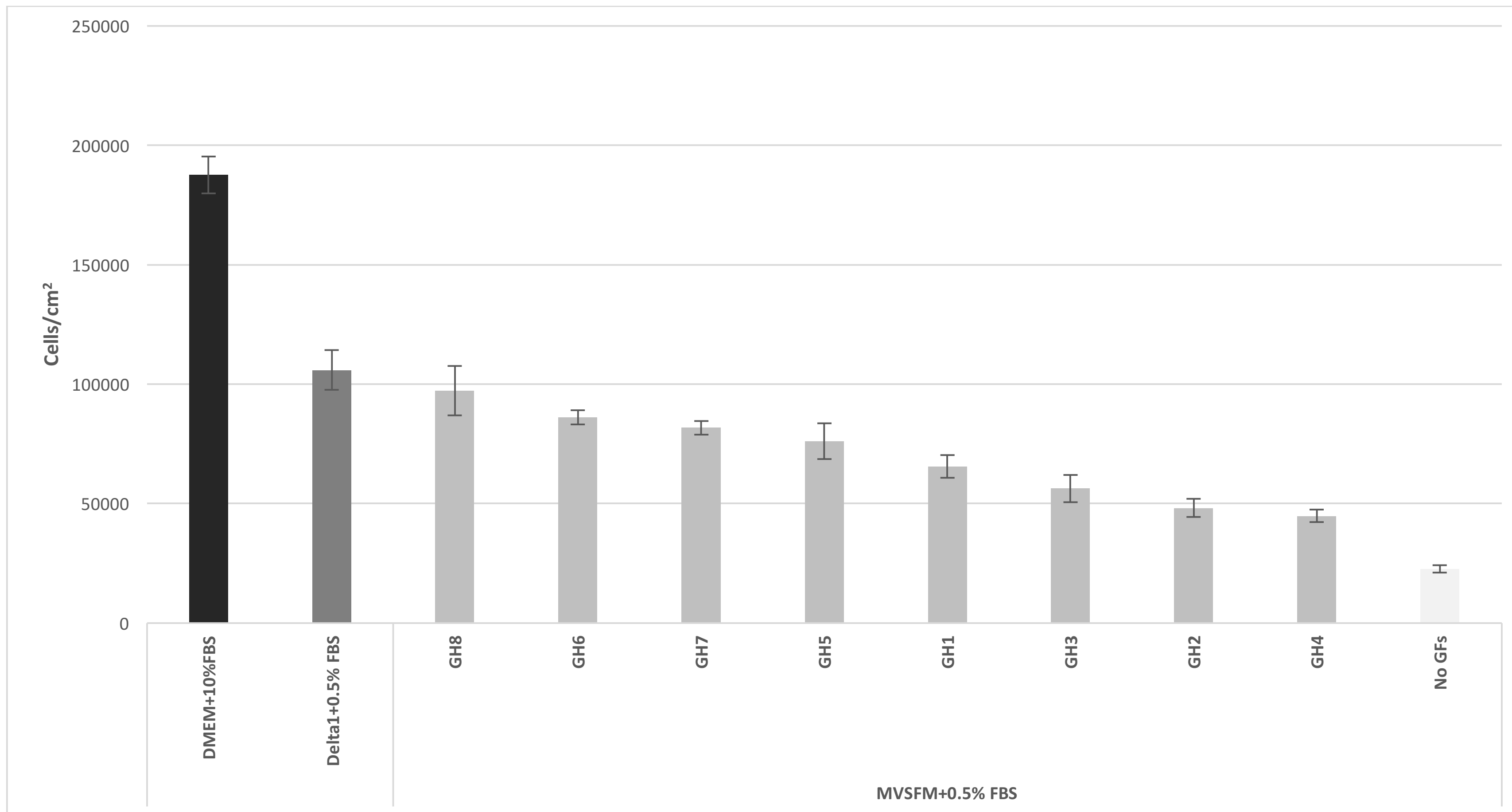


Figure 6.3 Cell yield of MRC-5 cells using the optimized media created using GA-HC method. MRC-5 cell yields in the experimental media are indicated by gray columns. The Delta 1 + 0.5% FBS medium is indicated by the dark gray column. The basal media formulation, which served as the negative control (MVSFM + 0.5% FBS with no growth factors) is indicated by the light gray column. The positive control medium (DMEM + 10% FBS) is indicated by the black column. Error bars are shown as SEM (n=3).

This experiment showed that despite the reduced calculated model error and the efficiency in the optimization technique, the GA-HC methods were not capable of finding a medium that could generate a higher cell yield than the one obtained with the DSD. This experiment confirmed that Delta 1 +0.5 % FBS was the most optimum medium for MRC-5 cells.

Table 6.3 Comparison of growth factor composition for the two best media.

Growth factors	Dexamethasone	bFGF	L-EGF	TGF-β3	L-R^3-IGF-I
Delta 1	40 μg/L	10 μg/L	50 μg/L	750 ng/L	-
GH8	39 μg/L	5 μg/L	2 μg/L	-	24 μg/L

However, since the cell yield of MRC-5 in Delta 1 + 0.5% FBS and GH8 + 0.5% FBS media were comparable, and did not show statistically significant differences to each other (as per the Student's t-test), it was decided to further investigate if GH8 + 0.5% FBS had a similar performance to Delta 1 + 0.5% FBS. The GH8 medium was of interest due to the fact that it was a cost-effective formulation compared to Delta 1. The GH8 medium contained the same growth factors as Delta 1 + 0.5% FBS except for TGF-β3. Some of the other differences were that GH8 it had L-R^3-IGF-I in the formulation, and it contained half of the concentration of bFGF as shown in Table 6.3. In addition, TGF-β3 is costlier than L-R^3-IGF-I. For those reasons, growth curves were performed to compare if the growth profile of MRC-5 cells were comparable using the GH8 and Delta 1 + 0.5 % FBS.

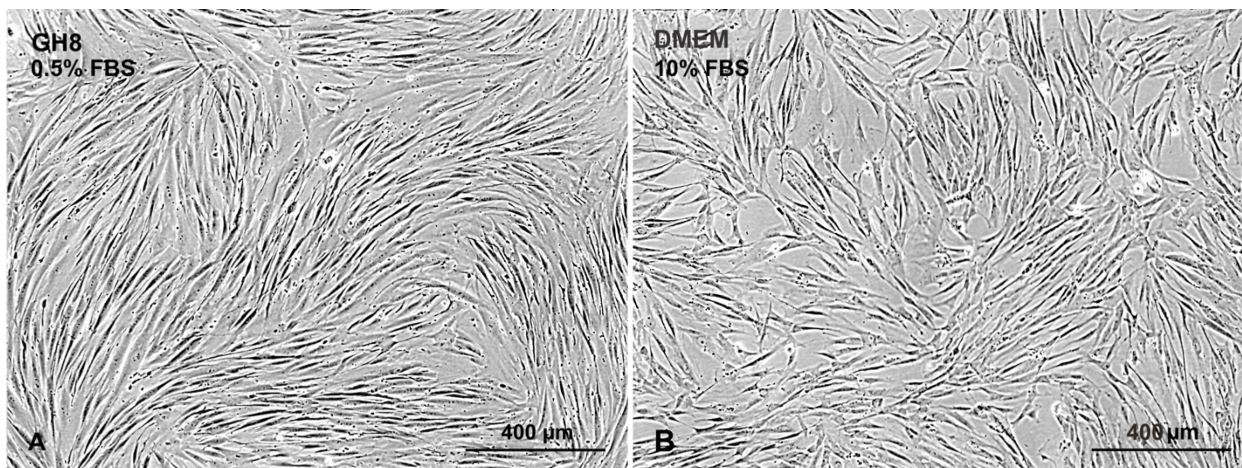


Figure 6.4 Photomicrographs contrasting the morphology of MRC-5 cells in GH8 + 0.5% FBS and DMEM+10% FBS on day 4. A depicts MRC-5 cells that are slightly flattened and presented an enlarged cytoplasm compared to the cells shown in B which were cultured in the positive control medium (DMEM + 10%FBS). Phase contrast images are shown at 100x magnification.

6.2.4 Growth curves of MRC-5 cells in the different optimized media

Growth curve experiments were performed to elucidate differences in growth profiles of MRC-5 cells in the different optimized media. The media tested were: i) the positive control (DMEM + 10% FBS); ii) the OFAT optimized medium (Prototype + 0.5% FBS); iii) the top medium from the DSD analysis (Delta 1 + 0.5% FBS); and iv) the top medium from the GA-HC optimization (GH8 + 0.5% FBS) The composition of these media is listed in Table 6.4.

MRC-5 cells were counted each day for eight days. Cell cultures were expanded in the medium of interest and subsequently inoculated into T-25 flasks at a seeding density of $1x10^4$ cells/cm^2. In the case of the cells grown at low serum concentrations, they were first adapted to media containing 0.5% FBS and then expanded to generate sufficient cells to conduct the growth

curve experiments. Three independent replicate experiments were performed for each media to ensure robust statistical analyses. Cells were counted each day as described in Section 2.2.3, and the flasks counted on the corresponding day were terminated and discarded.

The lowest yield was observed in cultures containing Prototype + 0.5% FBS medium (Figure 6.5). This was followed by cultures containing GH8 + 0.5% FBS medium, then by cultures containing Delta 1 + 0.5% FBS medium. The highest yield of MRC-5 cells was achieved in cultures containing DMEM+10% FBS (Figure 6.5). On day 1, cells grown in media containing 0.5% FBS showed a pronounced lag-phase compared to cultures containing the positive control medium, where no lag-phase was observed. Cell growth was equivalent in Prototype + 0.5% FBS and GH8 + 0.5% FBS media on day 1. Cells grown with Delta 1 + 0.5% FBS medium displayed slightly greater growth, but this was not statistically different from the other two media. On day 2, cells cultured in Delta 1 + 0.5% FBS medium reached an equivalent cell yield to that of the positive control, while the other two formulations continued to lag in growth. However, by day 2, it was clear that the cells had entered the exponential phase in all the media formulations.

During the exponential phase, the values of the cell yields in cultures containing Prototype + 0.5% FBS and GH8 + 0.5% FBS media were similar until day 5. By day 6, however, MRC-5 cells in GH8 + 0.5% FBS medium continued in the exponential phase, whereas growth of cells in cultures containing Prototype + 0.5% FBS began to decline and the cells began to enter stationary phase (Figure 6.5). On day 7, cells in GH8 + 0.5% FBS medium were in stationary phase. On day 8, cells in Prototype + 0.5% FBS medium reached a maximum yield of 55,066 cells/cm^2, while cells in GH8 + 0.5% FBS medium reached a maximum yield of 80,976 cells/cm^2.

Cells cultured in Delta 1 + 0.5% FBS medium displayed growth kinetics that were similar to that of the positive control medium (DMEM + 10% FBS). However, cells in Delta 1 + 0.5%

FBS medium grew slightly slower during the exponential phase (days 3, 4 and 5) than cells cultured in DMEM + 10% FBS medium and the cultured cells in the positive control medium achieved higher cell densities. By day 6, the densities of MRC-5 cells grown on Delta 1 + 0.5% FBS medium had comparable cell yields to cells grown in the positive control medium. By day 7, cells cultured in both Delta 1 + 0.5% FBS and DMEM + 10% FBS media had entered the stationary phase. The maximum yield of MRC-5 cells cultured on Delta 1 + 0.5% FBS medium was 148,586 cells/cm^2, while the maximum yield of MRC-5 cells cultured on DMEM + 10% FBS medium was 190,377 cells/cm^2.

This experiment showed that the growth kinetics of MRC-5 cells cultured in Delta 1 + 0.5% FBS medium were comparable to those cells cultured in DMEM + 10% FBS medium, especially during early and late exponential phase. It was concluded that GH8 + 0.5% FBS medium was not equivalent to Delta 1 + 0.5% FBS medium, and therefore, it was not used for subsequent optimization. MRC-5 cells cultured in GH8 + 0.5% FBS medium had a very similar performance to cells cultured on Prototype + 0.5% FBS, which was the least optimal formulation for MRC-5 cells. MRC-5 cells seemed to have required extra growth requirements for cell attachment in the early stages of growth in all optimized media, as suggested by the pronounced lag phase on day 1. It was concluded that the GA Hill-Climbing optimization methods did not lead to a better formulation. However, the growth curves confirmed that the Delta 1 + 0.5% FBS medium was the most optimum formulation for MRC-5 cells.

Table 6.4 Summary of optimized media supplemented with 0.5% FBS for MRC-5 cells using different statistical/mathematical methods.

Growth factor \ Media	MVSFM (Original medium)	Prototype (OFAT)	Delta 1 (DSD)	GH8 (GA-HC)
Dexamethasone	-	-	40 μg/L	39 μg/L
bFGF	1 μg/L	1 μg/L	10 μg/L	5 μg/L
PDGF-AA	-	-	-	-
L-EGF	10 μg/L	50 μg/L	50 μg/L	2 μg/L
L-R^3-IGF-I	80 μg/L	10 μg/L	-	24 μg/L
TGF-β3	-	-	750 ng/L	-

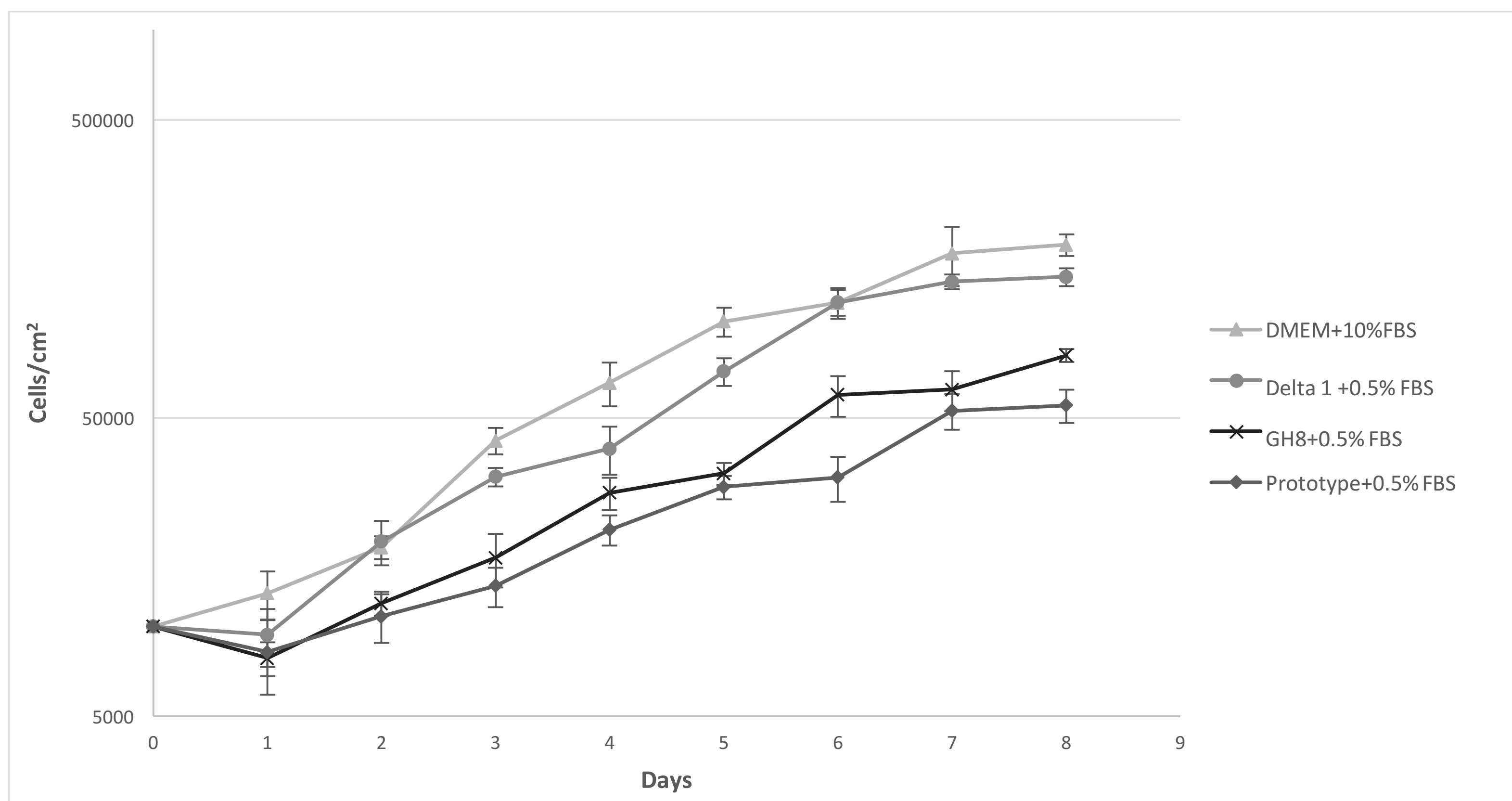


Figure 6.5 Logarithmic growth curves of MRC-5 cells in media containing low serum concentrations (0.5% FBS) derived from different optimization methodologies (specified in brackets). Cell growth in Prototype + 0.5% FBS medium is indicated by the diamond (OFAT); cell growth in Delta 1 + 0.5% FBS medium is indicated by the circle (DSD); cell growth in GH8 + 0.5% FBS medium is indicated by the cross (Genetic Algorithms and Hill Climbing); cell growth in the positive control medium (DMEM + 10% FBS) is indicated by the triangle. Cell yields are expressed in cells/cm^2. Error bars are shown as SEM (n=3).

6.3 Alternatives for SFM improvement: Polyamines supplement

An alternate approach to improve a SFM formulation is to use other media supplements that have been shown to promote cell growth and proliferation for other cell lines. Testing of supplements with known composition has the advantage of leading to a subsequent DSD optimization if the original supplement was proven to increase cell yield and production. A "polyamine supplement" that supported CHO cell growth and proliferation in a chemically defined media (CD Biogro CHO medium) was developed by Spearman *et al.,* (2016). Based on the successful use of polyamine for CHO cell culture, this supplement was evaluated for MRC-5 cell culture (Table 6.5).

Table 6.5 Concentrations used in polyamine/amino acid supplement.

Polyamine supplement	Final concentration in Delta 1
Spermidine	10 µM
Spermine	10 µM
Putrescine	16.2 µM*
Ornithine	10 µM
Citrulline	100 µM

*Delta 1 and MVSFM originally contained 6.2 µM putrescine

MRC-5 cells were tested in both Delta 1 + 0.5% FBS and Delta 1 SF media. A 10x supplement was prepared and added to the medium prior to its use. Cells were seeded at $1x10^4$ cells/cm^3 in T-25 flasks and the experiment was performed with three independent replicate flasks for each culture condition. DMEM + 10 % FBS medium was used as the positive control. Delta 1 + 0.5% FBS and Delta 1 SF media without polyamine supplements were used as negative controls.

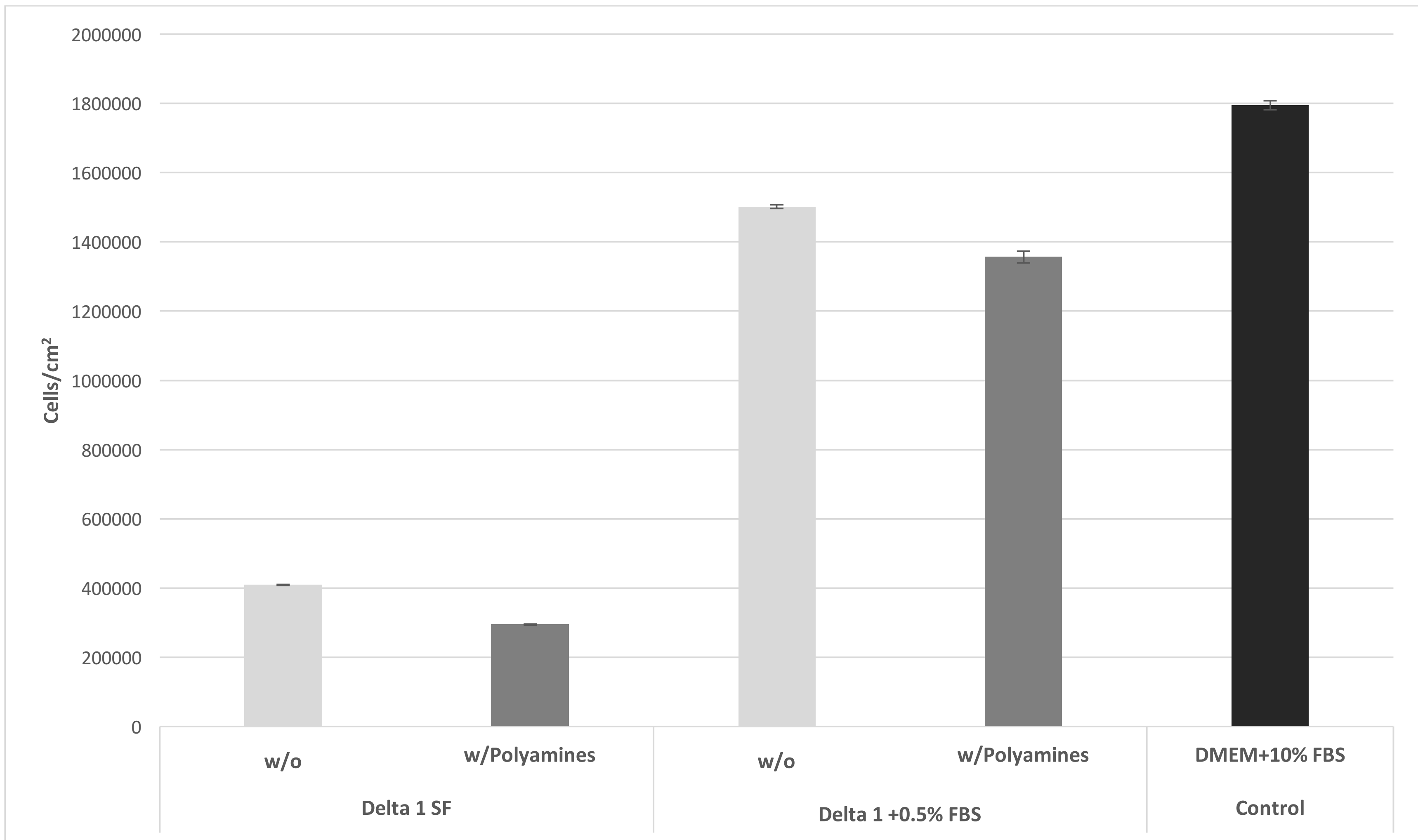


Figure 6.6 Growth of MRC-5 in Delta 1 + 0.5% FBS and Delta 1 SF media using polyamines supplements (dark gray bars). The media was tested against the corresponding media without supplements (light gray bars), and compared with growth of MRC-5 cells cultured in the positive control medium, DMEM+10% FBS (black bar). Error bars are shown as SEM (n=3).

Table 6.6 Doubling-times of MRC-5 cells in the different media supplemented with polyamines.

Media	Doubling-time (hours)	SEM (hours)
Delta 1 SF	22.4	0.2
Delta 1 SF+ Polyamines	24.6	0.2
Delta 1+ 0.5% FBS	16.6	0.3
Delta 1 + 0.5% FBS+ Polyamines	17.0	0.5
DMEM+10% FBS	16.0	0.5

As it can be observed in Figure 6.6, it was clear that the polyamine supplement was detrimental to the growth of MRC-5 cells. In both treatments, when polyamines were added, the cell yield decreased. The addition of polyamines induced a two-hour increase in the doubling-time of MRC-5 cells in Delta 1 SF medium, which was a significant increase as per the Student's t-test. In the case of Delta 1 + 0.5% FBS medium, the addition of polyamines to this medium caused a 1.6-hour increase in the doubling-time (Table 6.6). However, in this case, the increase in doubling-time was not significantly different.

In contrast, the Student's t-test showed that the cell yield of the cells cultured in Delta 1 + 0.5% FBS medium with and without polyamines were statistically significant from each other, whereas the cell yield of cells cultured in Delta 1 SF medium with and without polyamines were not statistically different. As such, there was no significantly deleterious effect on the growth of MRC-5 cells in Delta 1 SF as a basal media when polyamines were added. However, supplementation with polyamines did not improve the cell yield of MRC-5 cells, and for this reason it was not used as a supplement for further optimization.

The data derived from this experiment revealed that the polyamine supplement containing spermidine, spermine, putrescine, ornithine, or citrulline had deleterious impacts on yields of

MRC-5 cells. This experiment was performed to rapidly screen the effects of polyamines as growth stimulatory substances, which had previously been shown to increase yields of CHO cells (Spearman *et al.,* 2016). However, the results of the current experiment were consistent with data reported by Gahl *et al.* (1976) who reported that spermine and spermidine were detrimental to the *in vitro* growth of fibroblasts cells. This experiment demonstrated that certain supplements that are optimized for one cell line are not easily translatable for their use in SFM formulations for other cell lines.

6.4 Summary of results

The GA-HC optimization method concluded that the extraneous and inaccurate optimization results may be generated because some variables may interact with other factors in unknown ways. In addition, if many different factors need to be screened, they could be integrated into one single DSD experiment that could provide with more valuable information regarding the interactions of the growth factors used. One disadvantage is the large number of replicates and samples to be handled. However, the DSD provides “blocking” options that are able to separate experiments into blocks in order to reduce variability and confounding variables, but it is still able to estimate effects and interactions for all the factors tested (Jones and Nachtsheim, 2016).

The GA-HC optimization method could be improved by reintroducing information back to the algorithm regarding the validation or by providing the composition of the media that generated the highest cell yield. This would enable the algorithm to perform a more optimal search that could focused towards the area of the design space where the value of a more optimal concentration. In conclusion, the design of a serum free media requires a large amount of rational stages that can

yield an optimal medium for the cell line of interest. In addition, the results show that SFM that have been designed specifically for one cell line can rarely be transferrable to other cell lines as the requirements for each cell line are very specific.

6.5 Discussion

The cell yield values obtained from the DSD were used to optimize the medium using Genetic Algorithms and Hill-Climbing methodology to obtain higher cell yields than those obtained using the optimization algorithms provided by the JMP method. This experiments were performed because these optimization methodologies could have provided with a medium that could support the growth of MRC-5 cells in SF conditions.

Even though the theoretical model obtained using high level computing optimization methodologies resulted in a lower error than the one obtained using the JMP algorithms, these algorithms were not able to find a medium equivalent or better than Delta 1 + 0.5% FBS as it was confirmed later by the growth curve experiments. However, this methodology confirmed that Delta 1 + 0.5% FBS was the most optimum medium and that the DSD was a more adequate tool for this purpose. Future work could be directed towards improving the algorithms by providing feedback using the values obtained from the validation experiment and reducing the search area closer to that of the values from the Delta 1 + 0.5 % FBS medium, rather than searching for this value in the entire design space. Data from cell culture experiments could be used to train the algorithms to obtain higher yields.

Chapter 7

Discussion

7.1 Serum free media design for anchorage dependent cells

The objective of this project was to design a serum free medium formulation (SFM) for two anchorage dependent cell lines: MRC-5 cells and Vero cells. This project was focused on making use of the different experimental design methodologies that have been used in bioprocessing and biomanufacturing for SFM design and to improve the process production of biopharmaceuticals (Mandenius and Brundin 2008). Previously, Burgener (2000) designed MVSFM for Vero cells and it was reported to support the growth of Vero cells for reovirus and dengue production (Butler *et al.,* 2000 and Liu *et al.,* 2008). For this reason, MVSFM was chosen as the starting basal medium formulation that would be subjected to optimization using three mathematical/statistical approaches: one-factor at-a-time (OFAT), Definitive Screening Design (DSD), and Genetic Algorithms (GA)/Hill-Climbing stochastic search. The OFAT method was selected because it is a traditional method that was used by Burgener (2000) to design MVSFM; the DSD was selected because it is a new methodology (Jones and Nachtsheim, 2011) that can effectively screen and characterize the system in a few number of runs. The GA/Hill-Climbing stochastic search methods were selected because they can perform a design validation and optimization faster and efficiently using parallel processing.

The OFAT experimental approach was able to generate media formulations for both cell lines. In the case of MRC-5 cells, the concentration increased for L-EGF by 40 µg/L and the concentration of L-R^3-IGF-I decreased by 70 µg/L from the original MVSFM formulation (Table 3.11). This methodology confirmed the concentrations of MVSFM for Vero cells as this was the cell line in which this medium

was originally designed for. The OFAT approach was originally selected for this experiments due to the fact that only three Repligen growth factors were to be tested: L-EGF, L-R^3-IGF-I and rTransferrin. The experiments concluded that only the first two had an impact on cell yield on both cell lines, rTransferrin did not have an impact on cell yield and the inorganic sources of iron supplied in the basal media were already supplying the cells with the necessary iron in culture.

This methodology is advantageous in the design of a SFM for several reasons: i) it is a simple classical methodology widely used in all research fields; ii) it required very little planning and the data can be analysed using simple statistical techniques; iii) it allowed to test a wide range of concentrations which can be selected as desired. In this experiment, the OFAT methodology proved to be useful to optimize MVSFM for MRC-5 cells which was designated Prototype medium (see Table 3.11). This approach allowed to test for five different concentrations in the case of L-EGF and L-R^3-IGF-I, and four concentrations for rTransferrin. The OFAT experiments gave detailed information on what the cell yield of MRC-5 at each concentration of the three growth factors. The concentrations that yielded the highest amount of cells were selected and used in combination to create the most optimum media for both cell lines.

However, as its name implies, the OFAT method only evaluated one growth factor at a time, giving rise to inconsistencies in the correlation between concentration and cell yield. Therefore, there were three main disadvantages regarding this method when designing a SFM: i) it was not capable of detecting interactions between the different growth factors tested; ii) due to the fact that each factor was tested at a wide range of concentrations, there was a large number of experimental media to be tested; iii) there was a high variability within experiments that can lead to errors in the optimization. Thus, the OFAT approach is an inefficient and costly optimization methodology that only provides with partial information of the system (Czitrom, 1999). This led to the use of alternative optimization methodologies that could target the aforementioned disadvantages.

Previous studies have made use of Design of Experiments (DoE) in bioprocessing as well as in the optimization of SFM for various cell lines. DoE is used to minimize the variance in estimation of model parameters (in this case, the growth stimulatory substances concentrations) followed by the statistical analysis to filter out the actual values from the various errors that exist in the system (Kumar and Rathore, 2014). Thus, the DoE experimentation methodology is capable of finding an optimal concentration while reducing the error. As such, the method of analysis and its reliability directly depend on the design (Czitrom, 1999; Montgomery, 2013, and Kumar and Rathore, 2014). DoE has been shown to be far more efficient and effective than the traditional OFAT approach and that several designs previosuly used in SFM design and optimization (Jeon *et al*., 2010; Petiot *et al.,* 2010; Gonzalez-Leal *et al.,* 2011) such as fractional factorial designs and Plackett-Burman designs. However, newer methodologies that allow to screen and characterize the system have appeared. That is the case of the Definitive Screening Design (DSD) (Jones and Nachtsheim, 2011) which follows the Quality by Design (QdB) guidelines from the international health regulatory agencies. The DSD has become a popular in the characterization and processing development of monoclonal antibodies, pDNA, vaccine formulation and other recombinant proteins (Ornek and Ramsey, 2012; Erler *et al.,* 2013 Tai *et al.,* 2015; Clark, 2016).

This methodology provides with several advantages compared to the OFAT approach and to other experimental designs: i) the DSD can provide estimates of active main effects, two-factor interactions, and purely quadratic effects in effect-sparse systems (Erler *et al.,* 2013); ii) the number of experimental test runs is balanced to number of effects modeled (resolution) and iii) the efficient number of runs gives rise to a low-cost, timely solution as compared to the OFAT approach. Conversely, some of the disadvantages to the DSD are that i) it is important to have a carefully planned design to be able to screen for the factors in a range that will significantly impact the system in the design space; ii) it is necessary to conduct some post analysis of the results to achieve the optimization (Ramsey and Stephens, 2014),

therefore, previous statistical knowledge is necessary to perform these experiments, and iii) to accurately estimate main, combined and quadratic effects, it is necessary to have of six or more factors (Jones and Nachtsheim, 2011).

In this experiment, the DSD was used to perform the screening of six growth factors for MRC-5 cells having the Prototype medium was the basal medium. The DSD gave rise to an optimal medium for MRC-5 cells that was comparable to DMEM + 10% FBS. The DSD reduced the number of experimental media from approximately 70 to 13 compared to traditional parameter screening followed by response surface modeling (Erler *et al.,* 2013), reducing cost and time. It was able to efficiently identify and model the effects that impacted the cell yield of MRC-5 cells. The factors with the highest impact on MRC-5 cell yield were, dexamethasone, bFGF and TGF- β3 (Figure 5.5). The DSD experiment was performed with an addition of 0.5% FBS as a supplement to support cell growth and the medium that produced the highest cell yield was named Delta 1.

The same experiment was performed for Vero cells, using MVSFM as the basal medium. However, the DSD was not able to find a medium that generated a higher cell yield than MVSFM. Thus, it confirmed that the MVSFM was the most optimal medium created for Vero cells. This result was likely caused because the concentrations that were used could not elicit an impact in Vero cells and the concentration range should have been specifically planned for this specific cell line to have a design space that could optimize for a higher cell yield in Vero cells. Despite this result, the DSD was highly successful in generating an optimal medium for MRC-5 cells.

Thus, it was proposed to completely remove the serum from the formulation and perform a second DSD experiment using Delta 1 as the new basal medium and screening lipids and vitamins as the supplements. This experiment did not prove to be as successful in creating an animal component-free, chemically defined (ACF CD) SFM, which could be due to different reasons. First, the concentrations obtained for this experiment were carried out in dermal fibroblasts as opposed to lung fibroblasts, which

may have different nutritious requirements. Second, the combination of solvents (ethanol and MβCD) used to dissolve the hydrophobic lipids and vitamins in the aqueous solution may be toxic for the cells. The protective effect from the proteins contained in serum is lacking in ACF CD SFM formulation. This is one of the biggest challenges in the creation of ACF CD SFM formulations as cells require lipids and vitamins but the incorporation into the media can result in deleterious effects towards cell yield. Third, the concentrations used were obtained from literature values which used OFAT experiments, which may transfer errors associated with this methodology; thus, giving inaccurate values of optimal concentrations that elicit cell growth and proliferation. Fourth, the lipids and vitamins could be interacting with other substances previously included in the medium. As such, it might have been useful to use the blocking tools in the DSD in order to test all 12 growth stimulatory substances at the same time while being able to perform a reasonable amount of tests while reducing variability and error (Jones and Nachtsheim, 2016). Finally, the adaptation to low serum media in MRC-5 is slow and it may be deleterious to SFM design as this is a normal diploid cell line that triggers replicative senescence, which is characterized by growth arrest (Place *et al*., 2005, Augert and Bernard, 2013). As such, if the adaptation is successful, due to the time frame necessary for such process, cells might already be undergoing senescence and therefore the cell yield might not be as high when cultured in the SFM.

As it was concluded that Delta 1 + 0.5% FBS was the most optimal formulation. However, it was necessary to perform a validation testing on the DSD that could confirm the result and possibly provide with further optimization. The GA/Hill-Climbing stochastic search methodologies allow for both process validation as well as optimization. Commonly, response surface methodology is used for this purpose. However, GAs have been recommended by the QbD guidelines and they have the advantage of performing the search of the optimum solution in a large design space. Moreover, this methodology can be performed using parallel processing making uniform searches much faster by making use of more powerful computers (Kwok and Ahmad, 1997).

These methodologies showed that they were able to find an optimized solution and to reduce the error in the model compared to the stepwise regression search used by the JMP software. After the validation was performed and the optimized concentrations were tested, only the GH8 + 0.5% FBS medium yielded comparable cell growth to Delta 1 + 0.5% FBS. However, the growth kinetics of MRC-5 cells were studied by performing growth curves for both media (Figure 6.5). This experiment determined that the Delta 1 + 0.5% FBS was the most optimal medium for MRC-5 cells. As a result, one of the disadvantages of the GA-HC methodology is that it is necessary to re-enter the data back to the algorithm as feedback in order to improve it which prolongs the testing time and it makes it a costlier solution. Despite this disadvantage, once the algorithm is trained to perform the optimal search, it can be readily used for subsequent optimizations more efficiently and faster (Mijares Chan, 2016).

Other optimization strategy is to transfer other supplements that have proved to be useful in the creation of other CD SFM formulations for other cell lines. As such, the polyamines supplement originally designed for CHO cells (Spearman *et al.,* 2016) was tested in Delta 1 both at 0.5% FBS and SF conditions. The supplement showed that transferring other supplements may not be a straightforward solution for SFM formulation due to the widely different nutritious requirements from each cell line. Spermine and spermidine had previously shown to be deleterious to fibroblast cell proliferation (Gahl *et al.,* 1976) which could have affected MRC-5 cell yield. Similarly, the concentrations should be narrowed down to values that elicit cell growth and proliferation in that particular cell line. This was the case when the DSD experimental design originally created for MRC-5 cells was used for Vero cells. The growth factors may elicit the desired cell growth and proliferation effects but at different ranges that may fall out of the scope of the design space (Erler *et al.,* 2013). Therefore, in order to design an experiment, it is important to evaluate if those parameters before performing the experiment. In this case, preliminarily OFAT experiments aided by information from literature values can be useful to achieve a more meaningful result.

Commercially available serum free media such as the Freestyle medium which is a ACF CD SFM did not prove to be successful in the culture of MRC-5 cells under SF conditions. The Prototype, the Delta 1 and the Gamma XIII media were capable of supporting the growth of MRC-5 cells in SF conditions for at least one passage while Freestyle was only successful until 1% FBS. Other commercially available formulations for MRC-5 cells include plant protein hydrolysates such as the one created by Glaxosmithkline biologicals (Aerts *et al.,* 2011). Protein hydrolysates are undesirable because they pose the same issues as serum in that their composition is variable and can difficult the downstream processing, but plant sources make it animal component-free (ACF), which is advantageous.

The creation of a ACF-CD-SFM requires a series of experimentation and optimization steps that follows a rational approach. In the past OFAT experimentation was the main approach to design a SFM. However, as newer and more efficient statistical methodologies have appeared that have been recommended by the health regulatory agencies, the industry has started to move on to the use of DoEs and rapidly started to adopt the use of the newer methodologies such as the DSD that require lower runs to perform a screening and a validation at the same time. The incorporation of GA-HC validation and optimization methodologies still requires development to reach the capabilities of the DSD, however it can be customized which may be advantageous if more optimizations are going to be performed using the same algorithm. Thus far, the DSD delivered the best results as compared to the two other methodologies in the case of MRC-5 cells.

Using these three methods, it was possible to optimize different media formulations for MRC-5 cells. Starting from the MVSFM +1% FBS as the original medium formulation to the Prototype + 0.5 % FBS optimized using the OFAT approach, followed by the Delta 1+ 0.5 % FBS and the Gamma XIII SF optimized using DSD to the GH8 + 0.5% FBS using GA-HC search. From all of the aforementioned media formulations the Delta 1 + 0.5 % FBS proved to be the only one comparable to the standard serum-

supplemented medium, DMEM+ 10% FBS. In the case of Vero cells, the two different experimental approaches, OFAT and DSD proved the MVSFM was the best medium for this cell line.

7.2 Future work: Viral assay of adenovirus to determine media suitability for vaccine production

The production of a SFM for MRC-5 and Vero cells is of commercial importance for vaccine production. In this project it was possible to create the Delta 1 + 0.5 % FBS, a media formulation that was comparable to DMEM + 10 % FBS. A second media formulation that used Delta 1 as a basal medium, Gamma XIII, supported the growth of MRC-5 cells in SF conditions. In the vaccine production process, generally, cells are grown using a serum based medium followed by the use of a SFM for viral production (Thomassen *et al.,* 2013). To investigate the effectiveness of the optimized serum free media for vaccine production, it is necessary to evaluate viral production of MRC-5 cells compared to that in serum-supplemented medium (DMEM + 10% FBS).

Future work derived from this project can make use of the human adenovirus type 5 (Ad5) dl309 as a model for viral production in MRC-5 cells. This strain has been extensively used in vectors for use as viral vaccines, and for gene transfer vectors for gene therapy in place of wild type (wt) Ad5 (Bett *et al.* 1995). Viral production can be used as the response variable in a DSD. However, due to the high number or replicates required, the analysis can become an obstacle when using viral yields a response variable.

The importance of adenovirus production is derived from the fact that this virus is used for the biomanufacturing of gene therapy applications and viral oncolytics (Cyrstal *et al.,* 1995; Iyer *et al.,* 1999). The study of the kinetics of the viral infection by viral plaque assays is essential to determine virus titers produced by host cells using an optimized SFM compared to serum-containing media. Most

studies on adenovirus production have focused on HEK 293 cells as the host cell line (Lesch *et al.,* 2015; Shen *et al.,* 2012; Kamen and Henry, 2004; Iyer *et al.,* 1999). A potential viral strain to evaluate viral production by MRC-5 cells cultured in Delta 1 + 0.5% FBS, Delta SF, and Gamma XII SF, compared with virus production by MRC-5 cells in the serum-based media (DMEM + 10 % FBS) is Ad5 dl309 (Silva *et al*., 2016)

Chapter 8

Conclusion

The objective of this research was to create a SFM formulation for MRC-5 and Vero cells and to identify the growth stimulatory substances that played a role in cell proliferation and morphology, while following the cGMP and QbD guidelines outlined by the industry and health regulatory agencies. Three experimentation and optimization strategies were used, from which four different media were derived using MVSFM as the starting medium: In the case of the one-factor-at-a-time (OFAT), the Prototype + 0.5% FBS was produced, the Definitive Screening Design (DSD) gave rise to the Delta 1 + 0.5% FBS and the Gamma XIII SF, which used the Delta 1 as a basal medium, and Genetic Algorithms-Hill Climbing (GA-HC) method produced the GH8 + 0.5 % FBS. In the case of Vero cells, the OFAT and the DSD confirmed that MVSFM was the best formulation for this cell line.

OFAT experimentation methodology is time consuming, costly, and does not provide information regarding the interactions between the growth stimulatory substances being optimized in a SFM. In addition, this experimentation methodology does not follow the cGMP guidelines. As a result, the optimization can be partial and give ambiguous information regarding the bioactivity of each substance. Nonetheless, it can be advantageous if there is little information regarding the impact of the growth stimulatory substance on the cell yield for the cell line of interest, when few factors are tested and it requires low amounts of statistical analysis. After using this optimization methodology, it was found that MRC-5 cells required different concentrations of L-EGF and L-R^3-IGF-I compared to Vero cells. This methodology confirmed that the optimal

concentrations for Vero cells correspond to those reported by Burgener (2000). rTransferrin did not elicit a positive effect on cell yield for either MRC-5 or Vero cells, as $FeSO_4$ and $Fe(NO_3)_3$, are cheaper iron sources, supplied in the basal medium.

DSD was a useful tool in the screening of important factors when the growth stimulatory substances were provided in the SFM at concentrations appropriate for the cell line under investigation. In this case, an optimization the Delta 1 medium was obtained, and the growth factor dexamethasone, bFGF, TGF β-3, and to a lesser extent L-EGF, were found to have a positive impact on MRC-5 cell yields under reduced the serum concentration of 0.5% FBS which was comparable to that of serum based media DMEM + 10% FBS. These substances did not elicit the same effect on Vero cells. The lipids and vitamins that seemed to have a minor positive effect on MRC-5 cell yield were phosphoethanolamine, cholesterol, and ascorbic acid as determined by the DSD. The second DSD optimization led to the creation of the Gamma XIII medium. The GA-HC methodologies generated the GH8 + 0.5% FBS but it was not comparable to the Delta 1 + 0.5% FBS. This method can be useful in the case that many optimization iterations are to be performed as these algorithms can be trained to model the real response.

Extraneous variables in any experimental design can lead to erroneous analysis and optimization as well as confounding. Ethanol and MβCD used to solubilize lipids and vitamins as well as serum were all identified as extraneous variables. They should be eliminated from the initial experimental design. Moreover, blocking of the DSD can be used in order to generate a more accurate optimization by eliminating these variables and without having to do a large series of experiments. Throughout these experiments, it was shown that no two cell lines have the same nutritional requirements and require separate optimization processes when designing a SFM formulation. Moreover, supplements are not directly transferrable and may require additional

optimization, as it was the case with the polyamine supplements that were shown to have a positive effect on CHO cells, but had a negative effect on MRC-5 cells. Due to difficulties in adaptation to serum free media, complete removal of serum in SFM for MRC-5 and Vero cells negatively impacted cell proliferation.

It was concluded that Delta 1 + 0.5 % FBS was the best medium formulation for MRC-5 cells that had comparable cell yields with DMEM + 10 % FBS. And that MVSFM + 0.5% FBS was the best medium formulation for Vero cells. Future studies should be focused on the determination of the viral titer production in MRC-5 or Vero cells cultured in low serum or serum free media is sustained and/or comparable to titers obtained in these cells grown in standard serum-containing medium (DMEM + 10% FBS).

Chapter 9

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