Automation of human umbilical cord-derived mesenchymal stem cell culture for clinical applications

Automation of Human Umbilical Cord-Derived Mesenchymal Stem Cell Culture for Clinical Applications

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Abstract

Mesenchymal stem cells (MSCs) are more desirable than embryonic stem cells (ESCs) for clinical applications, mostly due to reduced ethical concerns, ease in expansion, cellular plasticity, and reduced potential for tumorigenesis. They have the capability to replicate as undifferentiated cells or to differentiate into bone, cartilage, fat, muscle, tendon and marrow stromal therefore, they hold great potential for cell therapies. However, for MSCs to be successfully commercialised, well-defined, reproducible and scalable manufacturing processes need to be developed since the transition of these studies from the laboratory to industrial scale processes with consistent outputs is a major challenge. Since the cells themselves may be the final product so the quality of the manufactured cells needs to be ensured throughout the entire bioprocess.

In this doctorate, the development of a robust MSCs expansion process using an automated platform was investigated. Since it was identified that there was a lack of in-depth knowledge of the automated MSC culture process, the study focused on in-depth characterisation of the automated system and the also on the automated expansion methods. These findings were used to aid the development of a novel robust automated MSC expansion process. The manual expansion process was used as the baseline process. This study used model cell lines for the intial characterisation and process development work to ensure the aim and the objectives can be achieved in a cost effective manner. However, the developed work was later tested and enhanced using MSCs. In order to demonstrate clinical relevance, the developed process was tested with early passage MSCs and analyses were performed on the cells produced to illustrate that they were capable of meeting the requirements set by the International Society of Cellular Therapy (ISCT). The results obtained with the final MSC expansion work demonstrated that there was no significant difference in terms of yield between the automated and the manual process ($p > 0.05$) and the yields obtained compared well with those reported in literature for the static manual cell culture processes. Most importantly, cells from both processes were able to retain their immunophenotype, multipotency and ability to attach to tissue culture plastic. This study also demonstrated that Stem Pro xeno-free medium can support the growth and expansion of MSCs.

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Abbreviations

FGF	Fibroblast growth factor
FBS	Foetal Bovine Serum
PBS	Phosphate buffered saline
DMEM	Dulbecco's Modified Eagle's Medium
iPSC	Induced pluripotent stem cell
HOS	Human osteosarcoma
HDF	Human dermal fibroblast
GMP	Good manufacturing practice
ISCT	International Society for Cellular Therapy
HFEA	Human Fertilisation Embryology Authority
ICM	Inner cell mass
FDA	Food and Drug Administration
MEF	Mouse embryonic fibroblasts
ESC	Embryonic stem cell
MSC	Mesenchymal stem cell
BM-MSC	Bone marrow-derived mesenchymal stem cell
UC-MSC	Umbilical cord-derived mesenchymal stem cell
UKSCB	UK Stem Cell Bank
DMSO	Dimethyl sulfoxide
MSA	Measurement System Analysis
SAP	Systems Applications and Products
HOS	Human Osteosarcoma

Chapter 1. Introduction

This chapter provides the definition of cell therapy and describes the significance of cell culture automation in this area. Also, the appeal of umbilical cord-derived mesenchymcal stem cell (UC-MSC) as a stem cell candidate for cell therapy applications is discussed. Then the challenges and complexities of cell culture automation are outlined. Next, the key technical questions are specified. The final section details the aim and objectives of the study.

1.1 Cell Therapy and the Need for Cell Culture Automation

Cell therapy is the direct application of cells to prevent, treat or attenuate illness and it involves a variety of disciplines including stem cell biology, immunology, tissue engineering, molecular biology, biomaterials, transplantation biology, and regenerative medicine (Humes, 2003). Cell therapies are being investigated for a wide range of applications to treat diseases such as acute and chronic graft-versus-host disease (GVHD), diabetes, neurological disorders orders, and acute liver failure and cirrhosis (Volarevic *et al*., 2014). At present, there are over 1800 open clinical trials exploring the safety and therapeutic efficacy of cells in human patients (www.clinicaltrials.gov). The growing number of clinical trials is a reflection that stem cell therapy has the potential to offer novel and effective treatments for a wide range of unmet medical conditions. For these therapies to be successful, they must be able to prove their safety and efficacy to the regulators and also must be 'commercially viable', that is the ability to treat enough patients with reasonable cost to justify the investment (Dodson and Levine, 2015).

Cell based therapies can be generally categorised as (i) those derived from a patient's own cells (autologous therapy) and (ii) those derived from a donor's cells (allogeneic therapy). Over the last decade, there has been a steady increase in the development of autologous cell products due to lesser risks associated with immune rejection (Smith, 2012). For allogeneic therapies the biggest challenge is tissue incompatibility as cells derived from a single individual and administered to others will likely result in tissue rejection. Overcoming rejection using long-term immunosuppression carries high risk of mortality from infection and oncogenesis (Smith, 2012).

For autologous therapies, due to the relatively small volume of cell required (and due to the cells from the same patient required), the most practical method of increasing the scale of production is through horizontal methods (scale-out) and through replication of unit operation to increase the batches (Hourd *et al*., 2014). The traditional small volume cell production methods are largely manual and typically they are, expensive, labour intensive and involve high degrees of process variability (Kempner, 2002). Although these manual methods may be suitable for research and early stage clinical trials, for cell commercialisation and for autologous cell therapies, closed and small batch processing (scale-out) systems with reduced manual intervention are required, and if possible, manual intervention should be eliminated altogether (Kempner, 2002). An automated flask-based cell culture platform is one such system. This system is capable of replicating manual operation by using robotic arms to culture cells in aseptic chambers and is also capable of small batch processing.

The automated cell culture method offers numerous benefits including, significant reduction in the number of operators required, reduced dependence on skilled labour,

improvement in quality (Kempner, 2002). All these are crucial in the development of a cost-effective cell therapy process. In the area of stem cell therapy, cell culture automation is even more beneficial due to the sensitive nature of the stem cells as even a subtle change of culture method can result in rapid deterioration or phenotypical change to the cells. At present, the most widely used automated cell culture platform for stem cell culture is the Compact Select robotic flask handling platform (Thomas *et al.*, 2009; Thomas *et al.*, 2007; Liu et al., 2010). This platform consists of a robotic arm in a clean processing environment adjacent to an incubator. The system can carry out most cell processing activities on barcode-tracked adherent cell culture flasks with few deviations from conventional manual processing protocols (Liu *et al*., 2010).

1.2 MSC as a Stem Cell Candidate

In the past (Wei *et al.*, 2013), the focus of the public was always on pluripotent stem cells, mainly due to the potency ascribed to them. In particular, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) received more attention than adult stem cells. The ethical issues related to ES cell isolation (Robertson, 1999), promoted the development of induced pluripotent stem (iPSCs), but similar to the ESCs, one key property of iPSCs that may seriously compromise its utility is its potential for teratoma (non-cancerous tumour) formation (Zhang *et al*., 2012).

Due to the limitation of using ESCs and iPSCs in the clinic, more interest has developed in mesenchymal stem cells (MSCs), which are free of both ethical concerns and teratoma formation (Wei *et al.*, 2013). They possess self-renewal ability and multilineage differentiation into not only mesoderm lineages, such as chondrocytes, osteocytes and adipocytes, but also ectodermic cells and endodermic cells

(Lindenmair *et al*., 2012). The number of clinical trials on MSCs has been rising since 2004 and these cells have made greater progress in clinical trials (Wei *et al*., 2013). Hence, it is more likely that MSC will be the main candidate for cell therapy applications in future.

These cells can be easily isolated from bone marrow, adipose tissue, placenta, and the umbilical cord and can be successfully expanded *in vitro* (Lindenmair *et al*., 2012). In the past, the main source of MSCs was bone marrow, but recently umbilical cord (UC) has emerged as a promising source. Umbilical cord is an attractive source because the collection of MSCs from UC is a noninvasive process and these cells also possess remarkable immunomodulatory properties (Nagamura-Inoue and He, 2014).

1.3 Challenges Associated with Cell Culture Automation

There are several challenges of introducing automation to the traditional manual cell culture process. The costs of purchasing, installing, and maintaining these automated cell culture machines are high, and the purchase cost of each machine itself can be within the range of £500, 000 to £1, 000,000 (Storrs, 2013). In order to recover the investment made, the sales potential of the commercialised product needs to be high. It is believed that the sales potential will be high due to cell therapies targeting many unmet medical needs (Mount *et al.*, 2015). If the scale of production does not keep up with the market demand, sales potential will be restricted, and thus, reimbursement is not secure (Malik and Durdy, 2015). In order to ensure reimbursement is secure, it is crucial that each production lot is capable of achieving the targeted cell yield, both in terms of quality and quantity, so that no additional production runs or machines are required.

Many researchers have shown that direct translation of the manual work to the automated process may not result in similar results (Thomas *et al.,* 2009; Thomas *et al*., 2007; Liu *et al*., 2010), therefore to obtain similar or better yield (both in terms of quality and quantity), optimisation work is almost always necessary. As the process scale is considerably larger when the automated platform is used, the cost of optimisation experiments can be significantly high, therefore it is not always possible to run optimisation experiments with stem cells. Many researchers choose to run experiments with a model cell line, so, there is always a possibility that not all the work carried out with model cell lines are completely applicable to stem cells. For this reason, it is crucial to always test the end or developed process with stem cells as this will confirm that the developed process is indeed applicable for stem cells.

In addition to the complexities and challenges mentioned above, another issue that further complicates cell culture automation is the difficulty faced in distinguishing biological variations from other source of variations (Molloy, 2003). Sources of bioprocess variation can be divided into four main categories: (i) biological factors, (ii) raw materials and consumables, (iii) operational inputs (measurements, methods, personnel, and equipment) and (iv) environmental conditions (Hutchinson, 2014). Even today, stem cell science has not been completely understood, therefore, it is difficult to identify the real source of variation and understand which variation has the biggest effect on production quality as not all variations have negative effect on the cells.

Another issue is that the development of a suitable Good Manufacturing Practices (GMPs) compliant process for clinical-grade production of MSCs is necessary to deliver the biological product to the market. This can only be achieved by better

defining the variables of a production process such as the culture reagents and the expansion system used. The current standard procedure for *in vitro* culture of MSCs is based on adding DMEM with foetal bovine serum (FBS) (Muller *et al.*, 2006), but the use of a component like FBS in the large-scale expansion of MSCs leads to variability in cell growth characteristics. The serum proteins found in FBS have the potential to initiate xenogeneic immune responses (Shahdadfar *et al.*, 2005; Dimarakis and Levicar, 2006), and this can affect the safety of the patients who receive MSCs (Heiskanen *et al.* 2007).

The ill defined nature of serum-based media is not desirable for clinical applications as it places a heavy burden on researchers to provide well-defined data on all the components used in their study. The use of serum-free media can lead to a better consistency of large scale production by reducing the variation between batches. There are various serum-free media available on the market (discussed in Chapter 2), but the StemPro MSC culture medium is currently the only FDA-cleared medium. Various researchers have demonstrated that MSCs from some sources exhibit increased proliferation rates when cultured in StemPro media (Agata *et al*., 2009; Chase *et al*., 2010; Dos Santos *et al*., 2011).

A critical review of the recent publications on this area also identified that most automation studies have just demonstrated the applicability of automation for cell cultures, but did not carry out a comprehensive study of the automated cell culture process (further discussed in the Chapter 2). This highlights the need for a comprehensive process automation study. In order to develop a robust automated process, more knowledge of the automated process and also information regarding its

capability is required. This can only be achieved through a comprehensive process development work with the aid of a relevant statistical process control (SPC) tool such as the process capability analysis. Process capability is usually studied to assess the robustness of the process. A robust process is described as a process that is not significantly influenced by variations in process inputs (e.g., raw material lot), process variables (e.g., pipetting speed, etc), and environmental variables (e.g., ambient temperature and humidity) (Seyhan *et al*., 2010).

This study was carried out with the aid of Minitab statistical software; industry standard software (www.minitab.com). This tool is currently widely used by more than 100 industries including British Petroleum, Ford, Accenture, Bridgestone, and Thales. The real appeal of this software is that Minitab can be combined with the Systems Applications and Products (SAP) system. This is an enterprise resource planning (ERP) software that allows organisations to manage business operations by allowing integration of data from different aspects of the business (go.sap.com). The results and the developed analyses can be downloaded or transferred *via* SAP, enabling the user to take full advantage of the benefits of the SPC methods while staying within the required corporate framework.

After the completion of the above, all the results obtained from the process capability study will be used to develop a fully automated robust UC-derived MSC culture (with better defined process parameters and with reduced variations). The study will be repeated with cells obtained from another umbilical cord to demonstrate the 'robustness' of the protocol developed. The study will also attempt to evaluate if the

UC-MSC culture can be automated using xeno-free cell culture reagents as ultimately this will ensure better success of cell therapy (further discussed in the Chapter 2). Within this context, the key technical questions that the work addresses include:

1. Is there any significant difference between the manual and the automated process?
2. If there is a significant difference between the two processes, is the difference observed when the work is repeated with another cell line? If a difference is observed, is it due to biological variation or is it due to the cell expansion process used?
3. Are the measurement devices used capable of producing the same results repeatedly and consistently when process parameters remain the same? If there are variations, can they be reduced?
4. How capable and stable are both processes? Can stability and capability be further improved through process development work if required?
5. Is it possible to develop a robust UC-MSC culture expansion process?
6. Can xeno- and serum-free culture conditions be considered for automated UC-MSC cell culture process?
7. Can the Compact Select be applied to the allogeneic cell therapy area?

Questions 1 and 2 will be addressed in Chapter 4, questions 3 and 4 in Chapter 5, and questions 5 to 7 in Chapter 6 of the thesis, where the results of the analyses are discussed.

1.4 Aims and Objectives of the Project

The aim of the doctoral project is to investigate the possibility of developing a robust automated umbilical cord-derived mesenchymal stem cell (UC-MSC) expansion process that is capable of producing clinically relevant product. This can facilitate successful commercialisation of this stem cell therapy. Several model cell lines will be used to develop and optimise the automated cell culture process. In order to achieve this goal, a set of objectives has been identified which form the basis of each of the following chapters. The main hypothesis intended to be tested is that the automated process yield is comparable with the manual equivalent in terms of product (cells) quantity and quality.

Chapter 2 presents an introduction to the background of the research. A general introduction to cell therapy, regenerative medicine and MSCs are provided. The key challenges in this area are also detailed in this chapter. The remainder of the Chapter 2 focuses on some of the widely used cell expansion platforms in this area, including some of the commonly used automated platforms. Finally the benefits of selecting the Compact Select for this doctoral study are highlighted.

In chapter 3, the materials and methods used throughout the doctoral study are detailed. The key procedural differences between the 2 cell expansion methods; the manual and the automated process are highlighted in this chapter. The specific experimental methods and the statistical analyses used to meet the objectives of the study are detailed here.

The results of the initial study to compare and highlight the differences between

established manual and automated process using a model cell line are presented in Chapter 4. This work was carried out to assess how much work was necessary to transfer the manual MSC culture process to the automated platform. Several analyses were performed on the products harvested from both processes to evaluate the effect of the bioprocessing on the cells. The analysis is taken a step further in this chapter by repeating the work carried out using HDF cell line.

Chapter 5 presents the work to study some of the factors contributing to the findings obtained in the previous chapter. This chapter's main objective is to thoroughly characterise the automated cell expansion process. This was necessary to identify the main factors that have a significant effect on the productivity of the process. The information generated from the characterisation work was used to develop a robust automated MSC cell expansion process.

The final study to demonstrate the clinical applicability of the protocol developed is presented in Chapter 6. In this chapter, several analyses were carried out to evaluate the quality of the cells produced. The work carried out with xeno-free medium for the culture of MSCs is also detailed here. The results obtained were compared to those available in the literature.

Chapter 7 summarises the main contribution of this work and presents suggestion for future work. Finally, some of the example protocols (automated) used and statistical reports obtained are provided in the appendices.

Chapter 2. Background to Research

2.1 Introduction

This chapter describes the background against which this doctoral project is set. Cell therapy is an area that is gaining wide recognition by organisations worldwide due to its potential to treat many conditions that cannot be treated by small molecules or biologics. However, without overcoming the hurdles associated with manufacturing and regulatory considerations, it is unlikely that the full potential of cell therapy will be realised.

The right technology/system to be employed for stem cell expansion and differentiation depends on the type of therapy intended; whether it is an autologous therapy or an allogeneic therapy. Since it is believed that autologous therapies have better chance of gaining regulatory approval and entering the market, the focus of the study will be on meeting the cell demands of autologous therapies. With allogeneic therapies, immunological rejection will always be a concern, therefore, expensive immunosupressants may be required (Forbes and Rosenthal, 2014). While the side effects of immunosuppressive drugs can be minimised by careful monitoring and dose reduction, some substantial risks such as renal dysfunction and increased cancer risks still remain (Watson and Dark, 2012). In addition, the use of immunosupressants will ultimately result in additional cost burden for healthcare payers.

In this study, automated systems were selected to be the best production technology for commercialisation of autologous therapies. This is because only the automated systems offer scaling-out of cells to produce large batch volumes with minimal variations

between the physical and chemical properties of cells from one batch to another (greater reproducibility between batches). There is also less risk of process cross-contamination or mix-ups with this technology (Soares et *al*., 2014; Chandra *et al*., 2012; Thomas *et al.,* 2007). Although bioreactors are known to be capable of large-scale cell manufacture, they are better suited for allogeneic therapy applications, where lot sizes of billions of cells are required (Egloff and Castillo, 2012).

In order to demonstrate that the chosen production technology is capable of meeting the demands of the autologous therapy, the study will aim to demonstrate that the MSC culture process can be automated. The study will also aim to demonstrate that the yield from the automated process is capable of meeting the desired quality (described in Section 2.4.3) and quantity specification (based on the manual equivalent and also on the available literature). The use of automation in cell therapy is still relatively new, therefore, most studies so far have focused more on application of automation for stem cell manufacture, but considerably less on developing a robust automated and transferable protocol. For autologous therapies, where starting material can be scarce, greater emphasis should be placed on developing a robust automated protocol, as yield or quality variation might directly result in the failure of treatment. Therefore, there is a strong need for a comprehensive process automation (process development) study for this emerging industry and this provides the motivation for this research.

In Section 2.2, an introduction to regenerative medicine and cell therapies is provided. Section 2.3 focuses on key challenges in commercialisation of cell therapies and factors that can influence the success of such therapies. In Section 2.4, common types of cells used in Regenerative Medicine are described. In this section, reasons for selecting

MSCs for this study are discussed. Additionally, some of the challenges associated with the MSC manufacture are also detailed. In Section 2.5, appropriate manufacturing technologies for cell therapies are described for both the autologous and allogeneic therapies. In Section 2.6, the potential and the challenges for both the autologous and allogeneic therapies are discussed. In Section 2.7, widely used automated platforms are discussed and the key reasons for selecting the Compact Select automated platform are provided. In Section 2.8, alternative cell manufacturing technologies are discussed. Finally a summary of the chapter is provided in Section 2.9.

2.2. Regenerative Medicine and Cell Therapies

Regenerative medicine is simply described as replacement or regeneration of human tissues or organs, for the restoration or establishment of normal bodily functions (Mason and Dunhill, 2008). There are many definitions of cell therapy, but one commonly used definition describes cell therapy as the process of administering human beings with cells to treat, prevent or to diagnose a disease through the pharmacological, immunological or metabolic action of its cells or tissues (EMA, 2015).

There are two main types of cell therapies; autologous and allogeneic (Cheng, 2009). In autologous therapies, the cells are harvested from a patient and are expanded *in vitro* culture to large quantities and then returned back to the same patient. In allogeneic therapies, the cells are harvested from a single or many donors and are expanded *in vitro* to cater for many patients (Cheng, 2009).

The regulatory framework for cellular products has classified autologous and allogeneic therapies as either biologics (US) or medicinal products (EU). In the US,

autologous and allogeneic therapies are regulated by the FDA's Center for Biologics Evaluation and Research under Title 21 of the Code of Federal Regulations (CFR) Part 1271 as Human Cells, Tissues and Cell and Tissue-based Products (HCT/Ps) (Hourd *et al.*, 2013). The FDA is responsible for regulating cell-based therapies and compliance with the current Good Manufacturing Practice (cGMP) requirements (Hourd *et al*., 2013). In Europe, autologous and allogeneic therapies are regulated under the EMA's Advanced Therapy Medicinal Product (ATMP) Regulation. The EMA requires a cell therapy product that is classified as an ATMP to comply with GMP requirements for medicinal products (European Commission, 2007). In the UK however, the Medicines and Healthcare products Regulatory Agency (MHRA) is the regulatory authority for UK manufacturers or importers of ATMPs. The autologous and allogeneic therapies are facing major regulatory challenges due to the lack of regulatory harmonisation globally (lack of homogenous regulations between Europe and US, and also between Europe and the member states for autologous and allogeneic therapies). Thus, initiatives to harmonise the regulations for cell therapy products globally is imperative for the successful commercialisation of autologous and allogeneic therapies (Pearce *et al.*, 2014).

2.3 Key Challenges in Commercialisation of Cell Therapies

There are a number of challenges associated with commercialisation of cell therapies, and these challenges are separated into three categories: (i) pre-market, (ii) post-market and (iii) manufacturing challenges (Dodson and Levine, 2015). Pre-market challenges are mainly associated with the struggles faced by the cell therapy industry to secure funding during the development phase of therapies. The regulatory hurdles faced by the cell therapy industry are also considered to be pre-market challenges (Butler, 2008).

Post-market challenges are mainly associated with securing and maintaining a reimbursement level that is capable of exceeding the cost of production. In addition, getting the physicians and patients to adopt new cell therapies can be considered a significant post-market challenge (Dodson and Levine, 2015). Finally, manufacturing challenges are associated with the production and distribution of the cell therapies to the physicians and patients. The manufacturing challenges appear early in the research process and continue through the entire lifespan of a cell therapy product. Amongst the predominant manufacturing challenges are: (i) scaling up production and (ii) addressing distribution logistics (Lau *et al.*, 2008; Sipp and Turner, 2012).

Scaling up of production processes to produce a large number of cells is important to meet patient demand for successful therapies. However, scaling up of production processes is limited by factors including lack of tools and poor scientific understanding of key manufacturing issues; that is having the correct tests, tools and understanding to ensure the cells produced in scale up share the same characteristics as the original cells used in pre-clinical and clinical studies (Hourd *et al*., 2014). Scaling up of production processes remains a key bottleneck in the cell therapy industry, and it is imperative to address this challenge in order for cell-based therapies to become a market and success (Dodson and Levine, 2015).

Addressing distribution logistics is another key challenge that is faced by the cell therapy industry (Dodson and Levine, 2015). Cell therapies contain living cells that will not remain viable at ambient temperature over a prolonged period of time. Therefore, these therapies require more advanced and complex distribution strategies than typical small molecule or biologics therapeutics. Many cell therapy companies are

focusing on ways to transport effectively these treatments to patients, and overcome the distribution challenges (Jones *et al.,* 2012). Amongst the efforts include shipping the products both frozen and thawed, and placing the manufacturing facilities near the end-users (to reduce the shipping distance) (Dodson and Levine, 2015).

This section discusses the three key challenges related to pre-market, post-market and manufacturing faced by the cell therapy industry. These challenges should be addressed for the successful commercialisation of cell therapies. The following section will discuss the types of cells that are used in cell therapies, and in particular those used in regenerative medicine.

2.4 Cells used in Regenerative Medicine

Cells used in regenerative medicine are mostly stem cells. It is now accepted that a stem cell must fulfil three main criteria. Firstly, it must be capable of self-renewal in order to maintain the stem cell population, whilst maintaining its undifferentiated state. Second, it must be capable of multilineage differentiation, which is the ability to differentiate to multiple cell lines (Weissman *et al*., 2000; Placzek, *et al*., 2009) and the third is the *in vivo* functional reconstitution of the tissues (Weissman *et al.*, 2000).

There are various types of stem cells, and they all have their respective advantages and limitations, therefore, if they were to be considered for stem cell therapies, they need to be carefully selected. For example, some types of stem cells may have greater multilineage differentiation potential, but may not be able to tolerate rigorous processing steps (discussed in the following section). These stem cells can be broadly categorised into 3 main types; embryonic, adult, and induced pluripotent stem cells

(iPS). All these types are being actively explored because scientists believe that stem cells can be used to treat a wider range of diseases such as, Diabetes Mellitus, Graft-versus-Host-Disease (GVHD), Crohn, and Parkinson.

2.4.1 Embryonic stem cells (ESCs)

Embryonic stem cells (ESCs) obtained from blastomers of the cleavage stage embryo or inner cell mass of pre-implantation blastocysts (Figure 2.1) can differentiate into cell types of all 3 lineages (ectoderm, mesoderm, and endoderm) thus showing pluripotent potential (Lovell-Badge, 2001; Placzek *et al.*, 2009). These cells can be cultured in the undifferentiated state on the mouse embryonic fibroblast (MEF) feeder layer. When they are cultured in suspension as 3D cell aggregates they are known as the embryoid bodies (EB), ESCs can differentiate into specialised cells when exposed to suitable induction conditions (Gepstein, 2002).

Scientists believe that ESCs have infinite expansion potential unlike adult stem cells, which are only capable of differentiating into a few different types of cells and possess a limited number of generations *in vitro* (Stenderup *et al.*, 2003). However, stem cells are known to develop abnormal teratoma formation (Thomson, 2007), and this makes this research challenging.

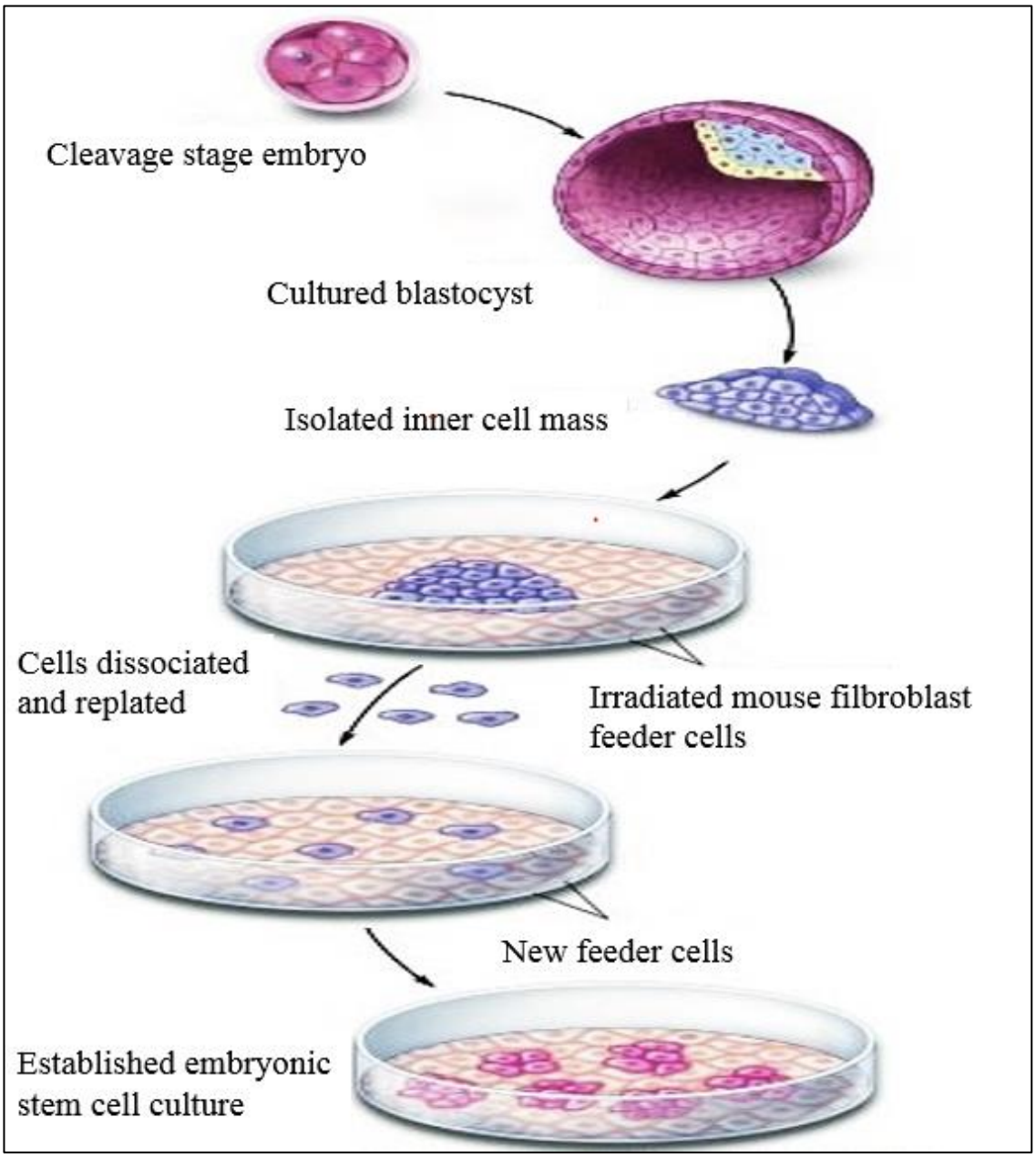


Figure 2.1 *The derivation of ESC lines (Winslow, 2001). Embryonic stem cells (ESCs) are obtained from blastomers of the cleavage stage embryo or inner cell mass of pre-implantation and are cultured in the undifferentiated state on the mouse embryonic fibroblast (MEF) feeder layer.*

Embryonic stem cell research can revolutionise the paradigm of medical practises, but there are also ethical issues associated with this type of research. People who oppose ESC research have 2 objections; that destroying human embryos means destroying a human life, and that the extraction of human embryos for research decrees them

"objects of utility rather than of inherent value" (Robertson, 2001; McLaren, 2001). Opponents object to the destruction of the embryos because they believe that killing embryos is equivalent to abortion. They argue that "life begins at conception and hence killing an embryo is equivalent to the killing of a human being" (Robertson, 2001).

The derivation of all human ESC lines required destruction of embryos until Robert Lanza's team demonstrated otherwise (Chung *et al*., 2006; Klimanskya *et al.,* 2006). These authors removed single blastomeres from the embryos using a biopsy technique. These biopsied embryos were then grown to the blastocyst stage and frozen. The blastomeres were cultured by an approach that mimicked the ICM niche. The success rate of this technique was similar to that of traditional human ESC derivation techniques that required embryo destruction (Klimanskya *et al.,* 2006).

Despite the success of this derivation method, many still argue that this method is flawed (Hudson *et al*., 2006). The proponents of this method believe that the biopsied embryos will not survive freezing and if the embryos were to be used *for in vitro* fertilization (IVF), the success rate of the IVF procedure will be significantly reduced. The prospective parents usually only have about 30% chance of conceiving from any given IVF cycle (www.nhs.co.uk), and this may be even further reduced if the embryo's viability is affected through biopsy or freezing.

Although Robert Lanza's work showed great potential, it still did not demonstrate that the biopsied embryo was fully functional. It would have been a breakthrough if it was demonstrated that the biopsied embryo could be used to produce a fully functional

human being. Until further work is done to demonstrate that taking a biopsy was not damaging to the embryo, the ethical issue still remains.

Embryonic stem cell cultures are complex due to the cells' dependence on serum, feeder layers, and growth factors that are not well characterised (Amit *et al*., 2003; Thomson, 2007). The feeder layers introduce batch-to-batch variability and prevent standardisation of protocols across laboratories and this makes bioprocessing development a challenge (Amit *et al*., 2003). The use of feeder layers can also potentially introduce a xeno-contamination into the cell culture process. Since mouse ESC lines are better understood and characterised than human ESC lines, they are used for early bioprocess development, as they can be cultured without the presence of feeders. However, there might be differences in "basic biochemical processes, the kinetics of differentiation, and gene expression" (Thomson, 2007). In addition to that, only few human ESC lines are currently available, so only a small percentage of the genetic diversity of the population are represented (Thomson, 2007).

Many organisations studying human ESCs are now moving on to other cell culture process such as the MSC and the iPS cell culture processes, as they are now aware of the difficulties involved in ESC expansion. Geron Corporation (USA), a human ESC research organisation, recently announced that it would not continue its stem cell programme due to clinical trial failures and funding issues (Geron, 2013). This company started the world's first clinical trial using human ESCs. These organisations have realised that human ESC culture processes have to be better understood before these cell lines should be considered for clinical trials.

2.4.2 Induced pluripotent stem cells

The use of human *in vitro* fertilisation (IVF) embryos to obtain human ESC lines is deemed by some as unethical, and as such, the scientific community is looking for alternative strategies for generating pluripotent cells. In 2006, Takashaki and Yamanaka (2006) produced groundbreaking work by reprogramming differentiated somatic cells to a pluripotent state.

One of the most critical steps of generating induced Pluripotent stem (iPS) cells is the use of viral vectors for reprogramming mature differentiated cells into pluripotent cells. The initial human iPS cells were produced by transducing adult fibroblasts with retroviral vectors that expressed OCT4, SOX2, KLF4, and c-Myc reprogramming transcription factors (Takahashi and Yamanaka, 2006). Retroviral vectors have the potential to activate endogenous oncogenes when iPS cells generated by this method are transplanted into the human body (Nienhuis *et al.*, 2006). A safer method may be to deliver reprogramming proteins into somatic cells, instead of using genes to induce iPS cells, but the efficiency is significantly reduced with this method (Zhou *et al.*, 2009; Kim *et al.*, 2009). Among the 4 transcription factors reprogrammed by Takahashi and Yamanaka (2006), only c-Myc is an oncogene. In order to reduce the tumourgenecity of iPS cells, Nakagawa *et al.* (2008) used the other 3 factors to induce the human iPS cells. Although the authors reported decreased efficiency in iPS derivation, no tumour was observed in progeny mice 100 days after birth without c-Myc.

Although these methods have yielded positive outcomes, a higher efficiency in iPS derivation is required for it to be considered a good source of stem cells. In addition to that, it is crucial to identify which iPS cell lines are safe before they can be

considered for therapeutic applications (Sun *et al*., 2010). Despite the promising potential of these cells, the risks-versus-benefits analysis for such cell therapies is still not fully understood, as there are still major limitations that continue to complicate their clinical translation (Neofytou, *et al*., 2015).

2.4.3 Mesenchymal stem cells (MSCs)

Human mesenchymal stem cells (MSCs) are multipotent adult stem cells, which are found in various tissues and organs, including, adult bone marrow, adipose tissue, the synovium, amniotic fluid, cord blood, and umbilical cord that can proliferate as undifferentiated cells (Table 2.1).

Table 2.1 *Main sources of MSCs for cell therapy.*

Source	**References**
Adult Peripheral Blood	Kuznetsov *et al.*, 2001
Adipose Tissue	Zuk *et al.*, 2002
Bone Marrow	Pittenger, 1999
Cord Blood	Jager *et al.*, 2009
Dermis	Young *et al.*, 2001
Muscle	Wada *et al.*, 2002
Pericyte	Crisan *et al.*, 2008
Periosteum	Wakitani *et al.*, 1994
Placenta	Fukuchi *et al.*, 2004
Synovial Membrane	Harvanova *et al.*, 2011
Trabecular Bone	Noth *et al.*, 2002
Umbilical Cord	Nekanti *et al.*, 2010

These MSCs can differentiate to lineages of adipocytes, chondroblasts, and osteoblasts (Baksh *et al*., 2007). It has also been shown that these cells can also differentiate into neuronal cells and hepatocytes (Seo *et al*. 2005, Dalous *et al*., 2012). These cells are a better alternative than human ESCs because they can retain their differentiation ability *in vivo* and do not easily form teratomas (Kuroda *et al*., 2010).

The current work with human ESCs is hindered by difficulties in producing enough cells in a reproducible manner for therapeutic development. Although adult stem cells such as MSCs are not as versatile as human ESCs in terms of differentiation and proliferation potential, they have some distinct advantages over ESCs. They do not require feeder-layers to maintain them in the undifferentiated state, and their cell fate can be better controlled, making them a more attractive choice for cell therapies (Liu *et al.*, 2011).

Although MSCs are classified as adult stem cells, they are more attractive than other types of adult stem cells due to their remarkable immunomodulatory properties. Mesenchymal stem cells from some sources, particularly from cord blood and the umbilical cord have demonstrated immunosuppressive properties. The most significant results on the immunosuppressive effects of MSCs have been observed in the treatment of acute Graft-Versus-Host-Disease (GVHD) disease. When GVHD patients who were steroid-resistant were infused with MSCs, the patients showed significant improvement (Ghannam *et al.*, 2010).

Until recently, bone marrow has been the main source of human MSCs, however aspirating bone marrow from the donor is invasive and is a painful process, therefore,

obtaining a willing donor can be difficult. In addition to that, the whole process of aspirating bone marrow involves significant hospital costs as anaesthetics and a hospital stay are required. The proliferation and differentiation capacity of the human bone marrow MSCs (BM-MSCs) decline when the number of *in vitro* passages increases (Bieback *et al*., 2004) and with increasing donor's age, the number of adult MSCs that can be obtained from bone marrow decreases considerably (Nekanti *et al*., 2010). These issues have limited the clinical application of BM- MSCs, and have encouraged scientists to look for other sources of human MSCs. Some of the promising sources include, cord blood, umbilical cords, and placenta. Umbilical cords are particularly attractive as several reports have shown that they are more primitive, proliferative, and immunosuppressive than their adult counterparts (El Omar *et al*., 2014).

Consistent positive research findings have encouraged scientists to consider this type of cell for research. They have also been named as a one of the cell sources in the recent human MSC clinical trials (FDA, 2013). The recent work done by De Araujo Farias *et al*. (2013) have reaffirmed that the UC-MSCs are indeed greatly valuable to the field of stem cell research by demonstrating that bone tissue can be created from these cells. These cells are excellent choice for transplantation as they have low cell immunogenicity (Weiss *et al.,* 2008). The self-renewal capacity, multilineage differentiation potential, and immunosuppressive properties of UC-MSCs make them an attractive and promising tool for regenerative medicine. Thus, they were chosen for this study. Although UC-MSCs are an attractive candidate for regenerative medicine applications, there are still some challenges associated with the use of these cells for cell therapy. The next section is dedicated towards discussing some of these challenges.

2.4.4 Challenges and complexities of UC-MSC culture

Mesenchymal stem cells are receiving a lot of attention due to their potential therapeutic applications for unmet medical needs. Umbilical cord-derived MSCs are particularly attractive because they can be collected relatively easily since they are considered to be a medical waste. Their pluripotency and differentiation potential have been well demonstrated, but there are still some uncertainties regarding the influence of *in vivo* aging (donor age) on the characteristics of the cells. A comprehensive study by Huang *et al.* (2013) demonstrated that MSCs from young group (19 to 25 years) exhibited a higher rate of proliferation and osteogenic differentiation capability, but less adipogenic capability than MSCs from an older group (29 to 35 years). Another study carried out by Alrefaei *et al*., 2015 also demonstrated that the proliferation potential of these cells decreased with increasing donor's age. This demonstrates the importance of considering donor's age prior to translation of these cells to a cell bank or to a clinic.

In addition to the challenges associated with biological variation, there are several types of technical challenges associated with UC-MSC culture. These are challenges associated with the *in vitro* culture of MSCs. They include challenges that are faced during the (i) cell extraction, (ii) cell characterisation and (iii) cell expansion process.

Cell Extraction

The most common cell extraction procedure involves cutting the umbilical cords into small tissue pieces after removing the arteries and veins and then using either (i) enzymatic digestion or (ii) explant culture to isolate the cells. If the enzymatic method

is selected, the isolation protocol involves digestion of the tissue with enzyme such as Trypsin and Collagenase I or II, followed by filtration and or centrifugation (Fu *et al*., 2006; Lindenmair *et al.,* 2014). If the explant culture approach is selected, the umbilical tissues pieces are soaked in an appropriate MSC culture medium at 37 °C and is placed in traditional cell culture incubator. Then the adherent cells will start to grow out of the tissue after approximately 10 days resulting in a confluent culture after two weeks (Majore *et al*., 2010).

Based on the currently available data, it cannot be concluded which method is the most suitable method as the findings are contradictory. Some authors have reported that the cell obtained via the enzymatic isolation method were able to exhibit higher proliferation rates (Salehinejad *et al*., 2012), but there are also contradictory reports that claim that cells isolated *via* the explant method exhibit better proliferation rates also (Hua *et al*., 2014).

For this study, the enzymatic isolation method was selected because this is the most commonly used. Additionally, isolation using enzymatic method is preferable as less waiting time is required to obtain cells from the cord (Lindenmair *et al*., 2014). The type of enzyme employed and the duration of the enzymatic treatment is critical, as prolonged exposure and exposure to harsh chemicals can degrade the extracellular matrix and cell membrane, and consequently, prevent the cells from adhering to the culture substrate when plated (Can and Karahuseyinoglu, 2007). Therefore, this is one of the critical steps that can affect the success of the MSC expansion process.

Another significant challenge in this area lies in obtaining enough cells for expansion from every umbilical cord. Findings by Maslova *et al*. (2012) demonstrated that it was not possible to obtain mesenchymal stem cells from every umbilical cord. In contrast, Secco *et al.* (2008) have shown that it was possible to obtain mesenchymal stem cells from all 65 human umbilical cords used. Maslova *et al*. (2012) also commented that 30 of their samples were contaminated by bacteria and fungi, therefore, it is likely that the researchers have not established the most robust and efficient cell isolation and culture protocol. The authors might have assumed that heterogeneity between the umbilical cords was a result of biological variation, but the heterogeneity issue might have been caused by human operator error.

Cell Characterisation

One of the biggest challenges in the study of human mesenchymal stem cells is to be able to characterise the population at different stages of proliferation and different experimental conditions. There is insufficient information on the markers that can define the cell types as MSC (Riekstina *et al.,* 2009). Due to the lack of a definitive set of surface markers that would determine the identity of human MSC, several analyses are needed to determine the characteristics of the population. For this reason, the International Society of Cellular Therapy (ISCT) proposed a set of standards to define human MSCs for both laboratory-based scientific investigations and for pre-clinical studies (Dominici *et al.,* 2006). "Firstly, the MSCs must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks. Secondly, 95% of the MSC population must express CD105, CD73 and CD90, as measured by flow cytometry. Additionally, these cells must lack expression (less than 2% positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II. Thirdly, the cells

must be able to differentiate to osteoblasts, adipocytes, and chondroblasts under standard *in vitro* differentiating conditions" (Dominici *et al.*, 2006). Most analyses require sacrificing cells, and therefore, require large amounts of cells to collect statistically significant data. Considering the limited number of mesenchymal stem cells obtained after isolation, it is a major challenge to acquire enough data throughout the cell expansion phase.

Cell Expansion

There are several complexities and challenges associated with the UC-MSC expansion process. One big challenge lies in shifting towards xeno-free cell culture media and to eliminate the use of FBS altogether during cell manufacture. The traditional MSC cell culture medium, Dulbecco's Modified Eagle's Medium (DMEM) was originally modified from Minimum Essential Medium (MEM) (Eagle, 1955) and this medium is commonly used to culture various cell lines. This medium formulation is chemically defined and contains no animal-derived products, but is uusally supplemented with 10 to 20 % (v/v) Foetal Bovine Serum (FBS) to increase productivity of the culture (Jung *et al.*, 2012).

Foetal bovine serum contains growth factors that improve cell attachment and proliferation, and other factors that bind and inactivate toxic products such as proteases and free radicals (Mannello and Tonti, 2007). The composition of each lot of FBS also depends on the quality of the animal used, and consequently, it can have an impact on the performance of the cell expansion process (Nekanti *et al.*, 2010). The addition of different lots of FBS to the medium may cause difficulties in the interpretation and reproducibility of results (Stute *et al*., 2004). In addition, FBS adds to the risk of

contamination from viruses, bacteria, prions, mycoplasma, and fungal agents (Mannello and Tonti, 2007), and screening for these entities before use, adds on to the process costs. In order to meet the regulatory and long-term safety requirements for cell-based therapies, better defined xeno-free culture media are required as animal serum containing media are ill-defined in nature (Chase *et al.*, 2012).

In order to overcome the limitations with the use of FBS, autologous sera and pooled thrombocyte lysate (PTL) can be used as alternative xeno-free medium supplements, but there are still many concerns regarding lot-to-lot variability and their consistent availability (Doucet *et al*., 2005; Muller *et al*., 2006; Gottipamula *et al*., 2012). Since serum is widely considered as an ill-defined component in cell culture media, many chemically defined serum-free media have been developed. These media do not need to be supplemented with FBS in order to support cell expansion (Chase *et al.*, 2010). Some of the commercially available media include StemPro (Life Technologies, USA), MesenGro (System Biosciences, USA), and MesenCult (Stemcell Technologies, UK). However, amongst all the media, StemPro MSC xeno-free medium from Life Technologies (USA) is the only medium that is currently FDA-cleared (Life Technologies, 2013).

The ill-defined nature of serum-based media is not desirable for clinical applications as it places a heavy burden on researchers to provide well-defined data on all the components used in their study. The use of serum-free-media can lead to a better consistency of large-scale production by reducing the variability between batches. Preliminary studies carried out by some researchers demonstrate that MSCs exhibit increased proliferation rates when cultured in StemPro medium (Agata *et al*., 2009;

Chase *et al*., 2010; Dos Santos *et al*., 2011). Since, shifting towards xeno-free media has numerous benefits, this study will also focus on investigating if UC-MSC culture processes can be automated using StemPro xeno-free MSC culture medium as using xeno-free medium will ultimately ensure better success for cell therapy purposes. Despite the promising potential of the StemPro xeno-free medium, there are certain disadvantages associated with the use of this medium. At present, one of the main issues associated with the use of this medium is its high cost (approximately £160 per bottle). Therefore, if this medium is used for cell expansion, media wastage should be brought to an absolute minimum. Another significant disadvantage is that, although there are reports that xeno-free media were found suitable for the isolation and expansion of MSCs to maintain their multipotent differentiation capacity, there is also contradicting evidence that xeno-free media do not support the isolation of MSCs (Crapnell *et al.*, 2013). This study will also investigate the potential of the StemPro xeno-free medium in supporting the isolation of MSCs from the UCs.

Another significant challenge in this area is that, while MSCs have rapid proliferation ability, they were found to lose their potency during sub-culture and at higher passage numbers (Chen *et al.*, 2014). Additionally, while early passage MSCs have demonstrated an enhanced ability to differentiate in to chondrocytes, adipocytes and osteocytes, cells at higher passage numbers demonstrated reduced differentiation potential (Chen *et al*., 2006). Since the biggest difficulty actually lies in determining when cells start showing signs of senescence, some authors have suggested using only cells from passage 2 to passage 6 cells for cell therapy purposes (Bonab *et al*., 2006). Hao *et al.* (2013) claimed that cells can be expanded up to passage 30 before they start senescing, but the work carried out by Mediana *et al*. (2015) demonstrate that cells can

start senescing as early as passage 9. Since stem cell science is still not completely understood (Lindenmair *et al*., 2012; Dalous *et al.*, 2012), it is difficult to assess if these differences are only due their biological characteristics or if cell bioprocessing techniques (isolation, expansion and cryopreservation) play a significant role. Although, it is desirable to extend the cultures for few more passages to maximise cell yield, inconsistent findings make it hard to determine if it is safe to expand these cells up to several more passages.

2.5 Defining the Appropriate Manufacturing Technology for Cell Therapies

Over recent years, cell therapies have emerged as a novel treatment approach and achieved a level of medical and commercial success. By 2008, the cell therapy industry had achieved worldwide sales of $410 million, with the potential market in the United States for these therapies exceeding 100 million people (Dodson and Levine, 2015).

Cell therapies have been identified as a potential treatment for a wide range of diseases including diabetes, neurodegenerative diseases, heart disease, musculoskeletal disorders and spinal cord injury (Reisman and Adams, 2014). The potential of cell therapies as possible treatment for a wide range of diseases is also observed through the growing number of cell therapy clinical studies. One study identified that there are around 2000 cell therapy products (a mix of autologous and allogeneic therapies) in clinical trials, and that most of the products were either in Phase I or II of the trials (Culme-Seymor *et al*., 2012). An analysis by Li *et al.* (2014) focusing specifically on stem cell therapies identified that the most common cell therapies in clinical trials are targeted at cardiovascular, neurological and liver diseases, cancer and bone conditions

(Li *et al*., 2014). The number of cell therapy products in clinical studies clearly indicates that cell therapies could potentially emerge as a novel treatment to a cure wide range of diseases and conditions (Parson, 2006).

Presently, the two main manufacturing technologies that can be considered for cell therapy processing at a commercial scale are bioreactors and automated platforms (Hourd *et al*., 2014). The choice of manufacturing technology to be employed purely depends on the type of cell therapy; whether it is an allogeneic or autologous therapy. There are two important criteria that must be considered before selecting an appropriate technology for a cell therapy product: (1) the growth, integrity and quality of the cells must be preserved and controlled, and (2) the cells should be harvested and recovered without disrupting them (Bartel and Borton, 2013).

For autologous therapies, the cells are harvested from a patient, cultured and expanded *in vitro,* and then returned to the same patient (Malik, 2012). This therapy is used for the repair of tissues in areas such as cardiovascular disease, diabetes, bone repair and spinal cord injuries (Atala and Allickson, 2015). Amongst the cell therapies that have gained marketing approval include Carticel for the treatment of cartilage defects, Epicel for the treatment of deep dermal burns and Provenge for the treatment of advanced prostate cancer (Dodson and Levine, 2015).

To successfully and efficiently scale-out autologous processes, manufacturers have to identify the manufacturing technology that is most robust; minimal variations between batches, greater reproducibility of cells without cross-contamination, cost-effective and able to produce cells without affecting the quality, efficacy and safety of these cells

(Jones *et al*., 2012). Thus, based on this basis, the best manufacturing technology for commercialisation of autologous therapies was identified to be automated platforms (further discussed in the Section 2.7). Automated systems can be a solution to support scale out (to handle large volume of batches), and can enable running several cultures in parallel to supply large volumes of cells for commercialisation.

For allogeneic therapies, cells obtained from a single donor or multiple donors are expanded to provide treatments to large number of patients (Malik, 2012). In contrast to autologous therapies, allogeneic therapies are more suited for large-scale manufacturing. Thus, scale-up (increasing manufacturing output by increasing the volume or number of cells processed for each batch) through more surface area is used in allogeneic therapy production (Hourd *et al*., 2014). To date, the commercialised allogeneic therapies include Apligraf for the treatment of venous leg ulcers and diabetic foot ulcers, Dermagraft for the treatment of diabetic foot ulcers, Osteocel for the treatment of bone regeneration as part of spinal surgery and Prochymal for the treatment of Graft-versus-host disease (GVHD) (Dodson and Levine, 2015).

To efficiently scale-up allogeneic processes, manufacturers have to identify the manufacturing technology that is most appropriate. This technology should be able to control the physiochemical parameters of the cells, minimise the changes in cell surfaces, reduce the shear stress level experienced by the cells and able to monitor the cell density (Egloff and Castillo, 2013). Presently, the bioreactor systems are the most ideal technology to be employed for allogeneic process scale-up (to scale-up adherent cells) (Egloff and Castillo, 2013). There are many configurations of bioreactor systems currently available such as hollow-fibre membrane bioreactors, microcarrier based

bioreactors, fixed or fluidised bioreactors and rotating wall bioreactors (Rodrigues *et al*., 2011). However, only the stirred tank and the hollow fibre bioreactors are discussed, as these are the most established bioreactors used for MSC expansion. The bioreactor systems are described in greater detail in Section 2.8.

2.6 Autologous or Allogeneic Therapies?

Autologous therapies have a favourable safety and risk profile due to the patient-specific nature (Trainor *et al.*, 2014). The use of an autologous therapy also reduces the chances of immune rejection and disease transmission (Atala and Allickson, 2015). Although autologous therapies have wide applications in many therapeutic areas, there are a number of limitations associated with this therapy. The large-scale production of cells for this therapy is challenging (Bartel and Borton, 2013). This is because for autologous therapies, a separate batch must be produced for each patient. Therefore the production for autologous therapies can only be scaled-out (increasing the manufacturing line or unit operation to increase the number of batches) instead of scaled-up (increasing manufacturing output by increasing the volume or number of cells processed for each batch) (Hourd *et al.*, 2014). The manufacturing process is also often complex; an error in the manufacturing process for autologous therapies results only in a failed treatment for a patient (Bartel and Borton, 2013).

The major drawbacks of allogeneic therapies include the possibility of immune rejection and disease transmissions. This is because the patients receiving this type of therapy receive cells from donors (Malik, 2012). For example, the most documented allogeneic therapy is hematopoietic stem cell transplants, in which 30–70% are

reported to result in GVHD (when the patient's immune system rejects the cells from the donor) (Garnett *et al*., 2013). In addition, extensive testing and history reviews required for this therapy increases the complexity of allogeneic therapies (Trainor *et al.*, 2014). If there weren't any drawbacks associated with immune rejection (Garnett *et al*., 2013), allogeneic therapies may the capacity to offer greater commercial opportunities. These therapies can potentially enable the treatment of many patients from the same cell bank in an off-the-shelf manner. Compared to production of patient-specific products (autologous therapies), batch production (allogeneic therapies) offers the opportunity for significant cost saving. However, the full potential of allogeneic therapies can only be realised if the hurdles associated with immune rejection are overcome.

2.7 Cell Culture Automation

For autologous therapies, cells need to be manufactured specifically for an individual patient using his or her own cells. Each patient receives an individual product batch, which needs to be manufactured, tested, and released. So thousands to tens of thousands of batches can be made for each indication every year. Given the nature of these therapies, the production scale remains the same for each batch. Thus, scale-up is not required and automation (scale-out method) is the ideal expansion system for meeting the demands of autologous cell-therapy manufacturing.

Automated platforms for cells culture expansion can be classified broadly into two categories; (i) automated platforms for adherent-type cell cultures and (ii) automated platforms for suspension-type cell cultures. Since most stem cells are anchorage-dependent cells (Illouz *et al*., 2011), flask or plate-based automated platforms are

used for cell expansion. These platforms are capable of growing and maintaining cells in well plates, roller bottles, and T-flasks. Generally, most of the automated systems for adherent cells on the market can maintain, expand, and harvest multiple cell lines (Kempner, 2002).

Figure 2.2 *The Freedom Evo automation platform. The automation platform based on Freedom EVO (Tecan, Switzerland) includes: Clean air cabinet (1), Robotic manipulator arm for moving automation-friendly cell-culture flasks (RoboFlask, Corning) (2), Liquid-handling arm with steel tips (3), Image-based cellular analyser (Cellavista, Roche) (4), Flask flipper module (5), Robotic shaker (6), Centrifuge (7) (Franscini, et al., 2011).*

These automated cell culture systems are capable of manufacturing up to 100 billion cells, making this system attractive for cell therapies (Rowley *et al*., 2012). Automation

offers significant advantages over manual cell culture. Manual cell therapy protocols are laboratory based and labour intensive. They require highly skilled personnel and weeks to months to harvest sufficient quantities of stem/progenitor cells from the isolated tissues. These manual procedures are expensive and can result in high phenotypic and yield variability between different trials and institutions. Automation can achieve high process reproducibility and there are currently several automated cell culture platforms on the market. These platforms have the potential to provide cost-effective, large-scale expansion of stem cells with consistent phenotype for clinical use and improved operational safety.

At present, there are over 20 automated cell culture systems on the market. It will not be possible to review every system, therefore, only three widely used systems will be discussed in this thesis (Table 2.2). Terstegge *et al*. (2007) were the first to demonstrate that human ESC culture could be automated. The CellHost (Hamilton, USA) automated cell culture system enables automated culture of ESCs in SBS-standard well plates. Another automated cell culture platform, Compact Select (Tap Biosystems, UK) was also shown to be an effective system for culture of adherent cells; BM-MSCs (Thomas *et al*., 2007), and human ESC lines, HUES7, and NOTT1, (Thomas *et al.*, 2009). The main components of this expansion system are described in the Material and Methods section (Chapter 3).

There is another system with additional functions; Freedom Evo (Tecan, Switzerland). Freedom Evo (Figure 2.2) has the capability to measure cell confluence during growth (*in-situ* cell assessment) and also perform centrifugation (Franscini, *et al.,* 2011). Despite having all these additional attractive features, the suitability of Freedom Evo

for stem cell culture cannot be determined as the system has only been used for one primary culture (Franscini, *et al*., 2011), and an attempt to expand bone marrow-derived MSC culture using the automated platform resulted in failure as cells were contaminated after one passage (Scott, 2009).

The price ranges quoted for the automated systems are estimates (Storrs, 2013), and if additional component or customization is required, these prices can vary significantly. Most of these system manufacturers offer the flexibility of modifying an existing system (customisation) to better suit customer requirements. For example, the price range quoted for the Compact Select is not inclusive of the plating system, and if this additional component is required, an addition of £550,000 is required.

Although in terms of capital cost, there is no cost advantage of choosing the Compact Select as the cell expansion system (Table 2.2), there is still a consumable cost advantage because this system is compatible with many ranges of flask sizes and with flasks from many suppliers (Table 2.3). Additionally, in terms of production capacity, only the Compact Select is capable of producing cells in the quantities of billions; as required for cell therapies, because it allows the use of T175 flasks, both in multilayer (many layers to a flask) and single layer format. These factors make this system a very attractive choice for MSC expansion.

Table 2.2 *Commonly used automated (Adherent) cell expansion systems.*

Automated Cell Culture Systems and Manufacturers	**Price Range (Capital Cost) (£)**	**Advantages**	**Disadvantages**	**Compatible Culture Vessels**	**References**
CellHost (Hamilton, US)	650,000	1. Has been used for stem cell expansion 2. Reduced cleaning requirement since this system operates without tubing	1. No centrifuge 2. Only well plate-based 3. Not capable of bulk dispensing of liquid 4. No live monitoring of cell confluence	Multiwell plates	Terstegge *et al*., 2007
Compact Select (TAP Biosystems, UK)	400,000 to 650,000 (without the advance plating module)	1. Has been used for stem cell expansion 2. Compatible with wide range of flask sizes and types (including multi-layer flasks) 3. Allows flask swirling and shaking to remove strongly attached cells	1. No centrifuge 2. No live monitoring of cell confluence	Flasks and multiwell plates (if advance plating module is installed)	Thomas *et al.,* 2009 Thomas *et al.,* 2007 Liu *et al*., 2010
Freedom Evo 150 (Tecan, Swizerland)	650,000	1. Allows flask tapping to remove strongly attached cells 2. Allows live monitoring of cell confluence	1 Only compatible with Corning RoboFlask 2. Stem cell expansion potential not well demonstrated	Flasks (Corning RoboFlasks only)	Franscini, *et al*., 2011

Table 2.3 *Flask formats compatible with the Compact Select automated platform.*

Type of Culture Vessel	Format	Manufacturers
Flask	Single layer: T75 (75 cm^2)	BD, USA Corning, USA
	Single layer: T175 (175 cm^2)	BD, USA Corning, USA Nunc, USA
	Triple layer: External of T175 (175 cm^2 per layer)	Nunc, USA
	Multi layer (10 layers): External of T175 (175 cm^2 per layer)	Corning, USA

For these reasons, the Compact Select was chosen as the cell expansion platform for this study. Good manufacturing practice (GMP) quality is defined by both the European Medicines Agency (EMA) and the US Food and Drug Administration as a requirement for clinical-grade cells that can provide optimal defined quality and safety in cell transplantation (Unger *et al.*, 2008). None of the stem cell automation work with the Compact Select has ever resulted in the cells losing their "stemness", therefore it can be concluded that the Compact Select is a safe choice for stem cell expansion work. The Compact Select has also been shown to be successful at preventing contamination when the GMP version of the Compact Select passed the "sterile fill" runs (Chandra *et al*., 2012), therefore this demonstrates that the use of this system will not raise any additional safety or quality concerns.

While, it has been demonstrated that stem cells expanded using the automated system can retain their "stemness", it was also shown that the use of Compact Select can result in lower cell yield (Liu *et al.*, 2010; Thomas *et al*., 2007). Compact Select and many other automated platforms do not have a built-in-centrifuge. This makes these systems not ideal for applications that require centrifugation, such as differential centrifugation for blood processing, but for adherent cell cultures, researchers have shown that cells can be expanded without using centrifugation (Liu *et al.*, 2010; Thomas *et al.*, 2009). While not having a centrifuge may have resulted in some differences in terms of growth and yield, this is not proven and is merely speculation at this stage.

2.8 Alternative Systems for Cell Expansion

In this section, the alternative systems for cell expansion of allogeneic therapies, particularly allogeneic therapies using MSCs, are discussed. The most common alternative systems for MSC cell expansion at a commercial scale include stirred tank and hollow fibre bioreactor systems.

2.8.1 Bioreactor system-stirred tank

The predominant bioreactor system that is typically used for commercialisation of allogeneic MSC therapies is the stirred bioreactor system. Other types of bioreactors such as the flow perfusion bioreactor system and the rotating wall bioreactor system have been used to expand MSCs, but the most widely used bioreactor system for MSCs expansion is the stirred bioreactor system. The benefits of this system have been demonstrated in a number of studies, and they include efficient mixing that creates a more favourable environment for cell expansion, full monitoring and control of process

parameters such as pH and dissolved oxygen, and flexibility in operating the culture in different feeding modes such as batch, fed-batch or continuous (Dos Santos *et al.*, 2010; Schop *et al.*, 2010; Kehoe *et al.*, 2010).

In stirred bioreactors, adherent cells are usually expanded on carrier systems such as scaffolds or microcarriers (Reichardt *et al.*, 2013). The use of scaffolds for MSC culture has been more focused on tissue engineering studies targeting MSC differentiation, rather than MSC expansion alone. For MSC expansion alone, microcarriers are usually the preferred carrier system and their potential in MSC expansion has been well demonstrated (Dos Santos *et al.*, 2010 and Schop *et al.*, 2010). Recently, some groups have even demonstrated the application of xeno-free media in microcarrier-based systems resulting in much higher cell yields than when using conventional medium choices with FBS (Heathmann *et al*., 2015; Dos Santos *et al.*, 2014).

There has been an increasing trend in using microcarriers to expand and differentiate adherent cells in large-scale stirred bioreactors (Szczypka *et al*., 2014). Since the microcarriers can break in high shear stress caused by the increased agitation in stirred tank bioreactors, not all of these reactors are suitable to be used with microcarriers. Thus, there has been an increasing trend in the development of microcarrier-based bioreactor systems that have reduced agitation to cultivate stem cells (Serra *et al*., 2011). In this bioreactor system, the surface on which the cells grow is in the form of microbeads. These microbeads are suspended in the culture medium of a stirred tank bioreactor.

Microcarriers provide a large surface area for cell attachment in the controlled culture conditions of the stirred tank bioreactors (Liu *et al*., 2014). Amongst the most common commercialised microcarriers include the solid, spherical or disc-shaped particles made of cellulose, polystyrene or dextran, all which provide a large surface for cell attachment (Zuhlke *et al.,* 2003). The other category is porous microcarriers that are typically made of materials such as collagen or gelatin (Storm *et al.,* 2010). These type of microcarriers purport to protect cells from high shear stress and also provide the necessary environment to aid in stem cell differentiation (Liu *et al*., 2014).

The advantage of employing microcarrier-based bioreactor systems is that they are well characterized from an engineering standpoint and they offer a significantly larger surface area per unit volume of bioreactor compared to other culture systems such as T-flask (Storm *et al.,* 2010). Bioreactor systems also provide a tighter control of culture conditions for cell expansion (as the microcarriers are maintained in suspension), and allow process conditions to be optimised to enhance cell growth using fed-batch or perfusion techniques. This system also allows easier process sampling and scale-up (Serra *et al*., 2011). Microcarriers-based bioreactors facilitate large-scale cell expansion and differentiation. Such systems also provide a promising technology for allogeneic therapies that require high volume batches with more than hundreds of billion cells per batch (Liu *et al*., 2014; Rodrigues *et al*., 2011).

The next section discusses the hollow-fibre type bioreactor, but compared to the microcarrier-based stirred tank bioreactors, the potential of these bioreactors for the MSC culture is less demonstrated. It should be noted that this technology is relatively new, therefore, it may take several years to realise the full potential of this type of

bioreactors.

2.8.2 Bioreactor system-hollow fibre

Hollow-fibre bioreactors are two-compartment systems consisting of a hollow-fibre bundle enclosed in a shell with ports for flow of medium in the intracapillary and/or extracapillary spaces (Rodrigues *et al.*, 2011). This system offers an increased surface area for cell culture to maximise adherent cell expansion in a minimal amount of space. However, this system has limitations in terms of process scale up or cell monitoring (Safinia *et al.*, 2005). One of the most common hollow fibre bioreactor system employed for MSC expansion is the Quantum® system (Terumo BCT, USA). This system consists of a disposable bioreactor that contains around 11, 500 hollow fibres with a surface area of 2.1 square meters (Lechanteur *et al*., 2014).

The system is fully automated where all the typical culture manipulations such as cell seeding and cell harvest in the hollow fibre bioreactor are managed by the computer-controlled systems that direct medium and gas exchange through the hollow fibre bioreactor. The primary advantage of the Quantum® system is that it allows production of cells according to GMP by offering a completely closed cell culture environment (Rojewski *et al*., 2013). However this system does not allow *in-situ* monitoring of cells, and cell confluence is estimated based on glucose consumption and lactate generation (Lechanteur *et al.*, 2014; Rojewski *et al*., 2013).

As stated earlier, the stirred tank and the hollow fibre bioreactors are the systems that are used for MSC expansion. Although other types of bioreactor such as the flow

perfusion and the rotating wall have been used to expand MSC, these are not the common and established systems for MSC expansion.

2.9 Conclusion

This chapter has highlighted the need for a robust manufacturing system that is capable of producing a clinically relevant product in sufficient numbers, quality and quantity required for cell therapy purposes. This chapter has also discussed two main classes of therapies; autologous and allogeneic therapies and the potential of success for both the classes of therapies. Since it was deemed that autologous therapies have a better chance of clinical and commercial success at present, automation was selected to be the best production technology for the manufacture of stem cells.

Compact Select is currently being used in many major pharmaceutical companies including GlaxoSmithKline, Merck, Pfizer, Bristol-Myers Squibb, AstraZeneca and Pfizer (www.tapbiosystems.com). Although in terms of capital cost, there is no cost advantage of choosing Compact Select as the cell expansion system, in terms of production capacity, only Compact Select is capable of producing cells in the quantities of billions; as required for cell therapies, because it allows the use of T175 flasks, both in multilayer and single layer formats. This makes this automated platform appealing for most major pharmaceutical companies as ultimately the success of a process greatly depends on its throughput. Although most automation work carried out with the Compact Select has resulted in either quality variation or yield reduction, most of the work done was not comprehensive and further work is necessary to understand the true cause of these variations.

This chapter also discussed some of the stem cells used in the cell therapy area, specifically, in the area of regenerative medicine. Although MSCs can be used for a wide range of treatments, the real appeal lies in its potential to meet unmet needs such as GVHD and Parkinson's diseaseThese cells are a good choice for transplantation since they have low cell immunogenicity, therefore, the possibility of immune rejection after transplantation is greatly reduced. Umbilical cord-derived MSCs are more attractive than BM-MSCs because the collection of MSCs from umbilical cords is a not an invasive process.

Despite the clear potential of MSCs, there are also several hurdles in the area of MSC manufacture that must be overcome in order for these therapies to be successful. This chapter has highlighted several challenges in the area of MSC expansion. While it is not possible to overcome all these challenges, it is certainly possible to overcome some challenges; especially the ones associated with the use of serum-containing media and the use of sub-optimal culture protocols. Therefore, the study will attempt to investigate the possibility of developing a robust automated umbilical cord-derived mesenchymal stem cell (UC-MSC) expansion process that is capable of producing clinically relevant product. The study will also aim to demonstrate the application of xeno-free media for the automated MSC culture. The next chapter will describe the material and methods used for the studies conducted in this doctorate.

Chapter 3. Material and Methods

3.1 Introduction

As indicated in the preceding chapters, stem cell therapies are a method of treatment for a variety of major unmet medical needs. This has sparked many research organisations' interest to focus on developing stem cell therapies to meet the needs of clinical therapies. At present one of the biggest hurdles in this area is the ability to manufacture cells in required quantity and quality (in a reproducible manner) for clinical therapies. Therefore, there is a pressing need for the development of such robust manufacturing processes. The experimental framework detailed in this chapter represents a novel attempt of developing a robust automated MSC culture process to meet the needs of clinical therapies.

The materials, general methods, analytical and experimental procedures used throughout the doctoral study are described in this chapter. This chapter is divided into 4 sections. Section 3.2 details the general cell culture materials and methods used in the study. The methods include general automated and manual experimental procedures. The manual cell culture procedures are based on the available cell culture manual at the Centre of Biological Engineering, Loughborough University, United Kingdom. The general automated procedures are based on the existing automated protocol readily available in the Compact Select software (example protocols are attached in Appendix I). The analytical methods used are detailed in Section 3.3. The experimental designs used are described in Section 3.4. These designs refer to the specific experimental methods and procedures developed and used to meet the objectives of the study.

3.2 General Cell Culture Materials and Methods

The general cell culture material and methods used are described in this section. This section is divided into 5 sections. Section 3.2.1 details the processes used; the manual and the automated process. Section 3.2.2 details the cell seeding procedure. Section 3.2.3 details the method used to isolate MSCs from the umbilical cord. Section 3.2.4 and 3.2.5 detail the passaging and cryopreservation method used for the study, respectively.

Three different cell types were used for this research project. An anchorage-dependent human osteosarcoma (HOS) TE85 cell line, an immortalised human dermal fibroblast (HDF) cell line, and a mesenchymal stem cell (MSC) line were used in this study. Cells for the MSC studies were isolated from fresh human umbilical cords by a colleague using the enzymatic digestion method (as described in section 3.2.3). The cords, cell culture protocols, and the cell isolation protocols were obtained from Future Health Technologies (FHT), UK.

Throughout the study, all the experiments were conducted aseptically. The water used for the preparation of aqueous solutions was filtered using the Milli-Q Direct Water Purification System (Millipore, USA). The resistivity of the solution produced at 25 ^{0}C is 18.2 MΩ. The automated process tubes and other relevant equipment were autoclaved using the Systec VX-95 autoclave (Systec, Germany). The cell culture methods described in this section are used for all of the cell lines employed in this study unless otherwise stated. The volumes of media, detachment enzyme, and PBS used depended on the size of the flask/vessels used (detailed in Table 3.1). Throughout

this study, confluent cell images were acquired using an Eclipse TS100 inverted microscope (Nikon, Japan).

Table 3.1 *Detachment enzyme, cell culture medium, and PBS volumes required for different sizes of culture vessels.*

Size of Culture Vessel	Growth Area (cm^2)	Detachment Enzyme Volume (mL)	Cell Culture Medium Volume (mL)	PBS volume (mL)
12 well plate	3.8	0.5	1	1
6 well plate	9.5	1	2	2
T25 flask	25	1	5	2
T175 flask	175	10	40	10

For HOS and HDF cell cultures, Dulbecco's Modified Eagle Medium DMEM (Life Technologies, UK) was used. This medium was supplemented with 10 % (v/v) foetal bovine serum (FBS) (Fisher Scientific, UK) and 2 mM UltraGlutamine (Fisher Scientific, UK). For MSC culture low-glucose DMEM with glutamax (Life Technologies, UK) was supplemented with 10 % (v/v) pre-screened foetal bovine serum (FBS; Fisher Scientific, UK) and 1% Penicillin Streptomycin (PenStrep; Life Technologies, UK). The DMEM medium with supplements (hereafter will be referred to as growth medium) was stored at 2 – 8 °C and used within one month of preparation.

Only when two different media are compared (Chapter 5 and Chapter 6), the DMEM medium with supplements will be referred to as the FBS-containing DMEM medium. For serum-free experiments discussed in Chapter 5 and 6, the medium was prepared

according to the manufacturer's instructions. The preparation of the Life Technologies StemPro® MSC Xenofree medium (hereafter referred to as StemPro) involved thawing the 5 mL StemPro supplement overnight at 2-8 °C and then adding this to 500 mL basal medium, followed by 5 mL of Glutamax (Life Technologies, UK).

3.2.1 Process description

The manual cell culture process (using human operator) was conducted in a biological safety cabinet, and the flasks were incubated in a 37^0C and 5% CO_2 controlled Heracell 150 Incubator (Thermo Scientific, USA). The cells were grown in polystyrene tissue flasks (BD Biosciences, UK) and the sizes of these flasks ranged from 25 to 175 cm^2. The automated cell culture processes were conducted using the Compact Select robotic system by selecting protocols that are defined using Extensible Markup Language (XML).

The automated cell culture process was conducted using the Compact Select (TAP Biosystems, UK) automated cell culture system. The Compact Select is a fully automated cell culture system that incorporates a small six-axis robotic arm that can process 130 T175 flask and plate incubators, controlled at 37 ^{o}C under 5% CO_2 and relative humidity of about 95 %. The flasks used were bar-coded for identification and cell process tracking. Two flask de-cappers and flask holders, automated medium pumping and an automatic cell counter (Cedex, Roche Innovatis AG, Germany) are integrated within a high-efficiency particulate air (HEPA) filtered cabinet to ensure sterility (Figure 3.1).

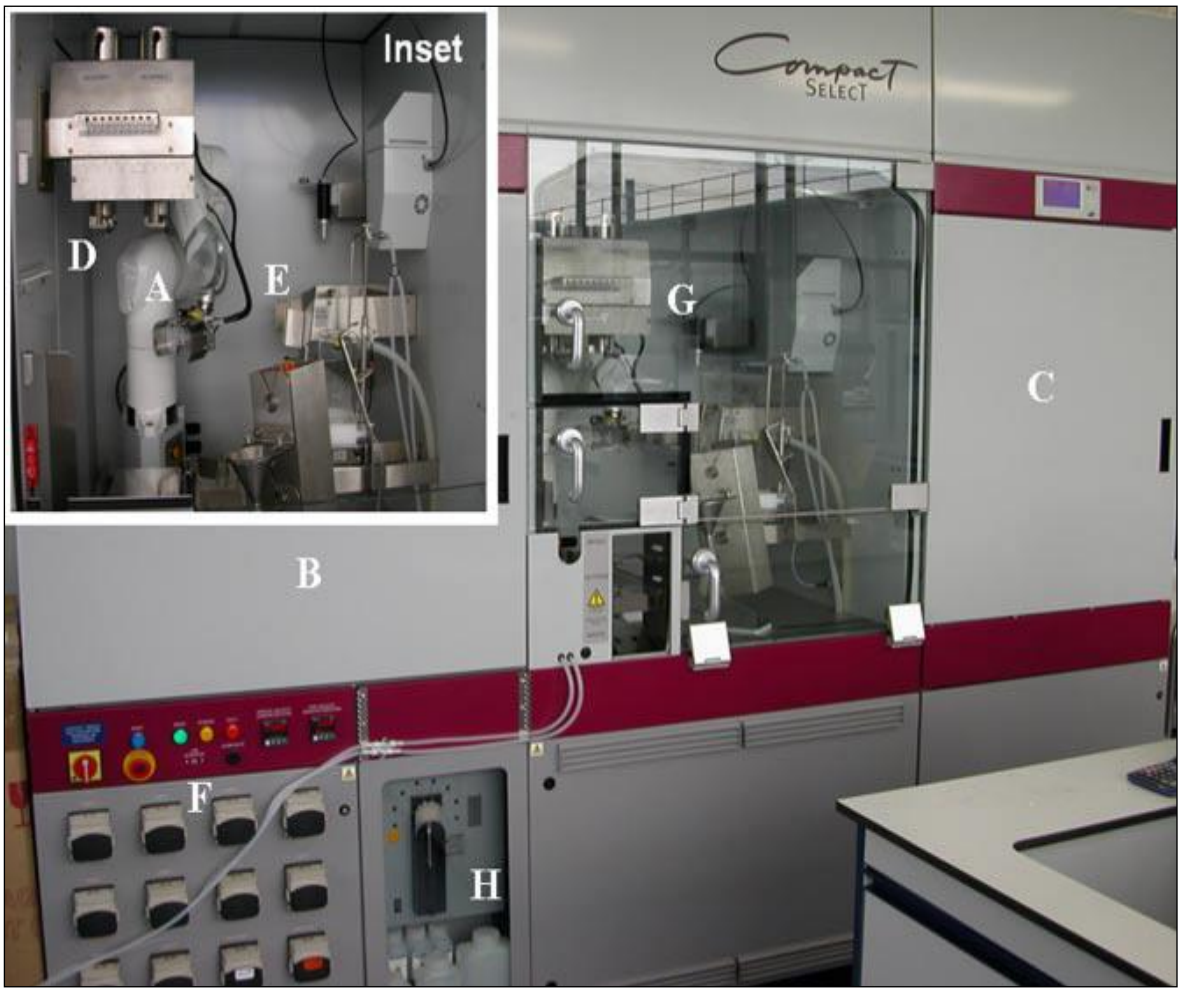


Figure 3.1 *Features of the Compact Select. Major processing components are labeled: A, Robot arm; B, Flask incubator; C, Plate incubator; D, Flask decappers; E, Flask holders; F, Media pumps; G, Pipette head; H, Cedex cell counter (Thomas, et al., 2007).*

The automated system can be divided into 4 main sections; (i) input, (ii) setup, (iii) system processing, and (iv) output (Figure 3.2). The input refers to the flasks, cell line details, protocols, and liquid required for the automated process run. These inputs are required for the setup and processing of the automated system. Before the automated run is initiated, the machine is usually prepared for use (set-up) by ensuring a sufficient number of pipette tips, T175 flask and adequate volume of reagents are loaded aseptically. In addition, sterile plastic tubing is also connected to allow for reagents to

be pumped and this is aided by the peristaltic pump system. The outputs refer to the processed flasks, process waste, and data obtained from the processes.

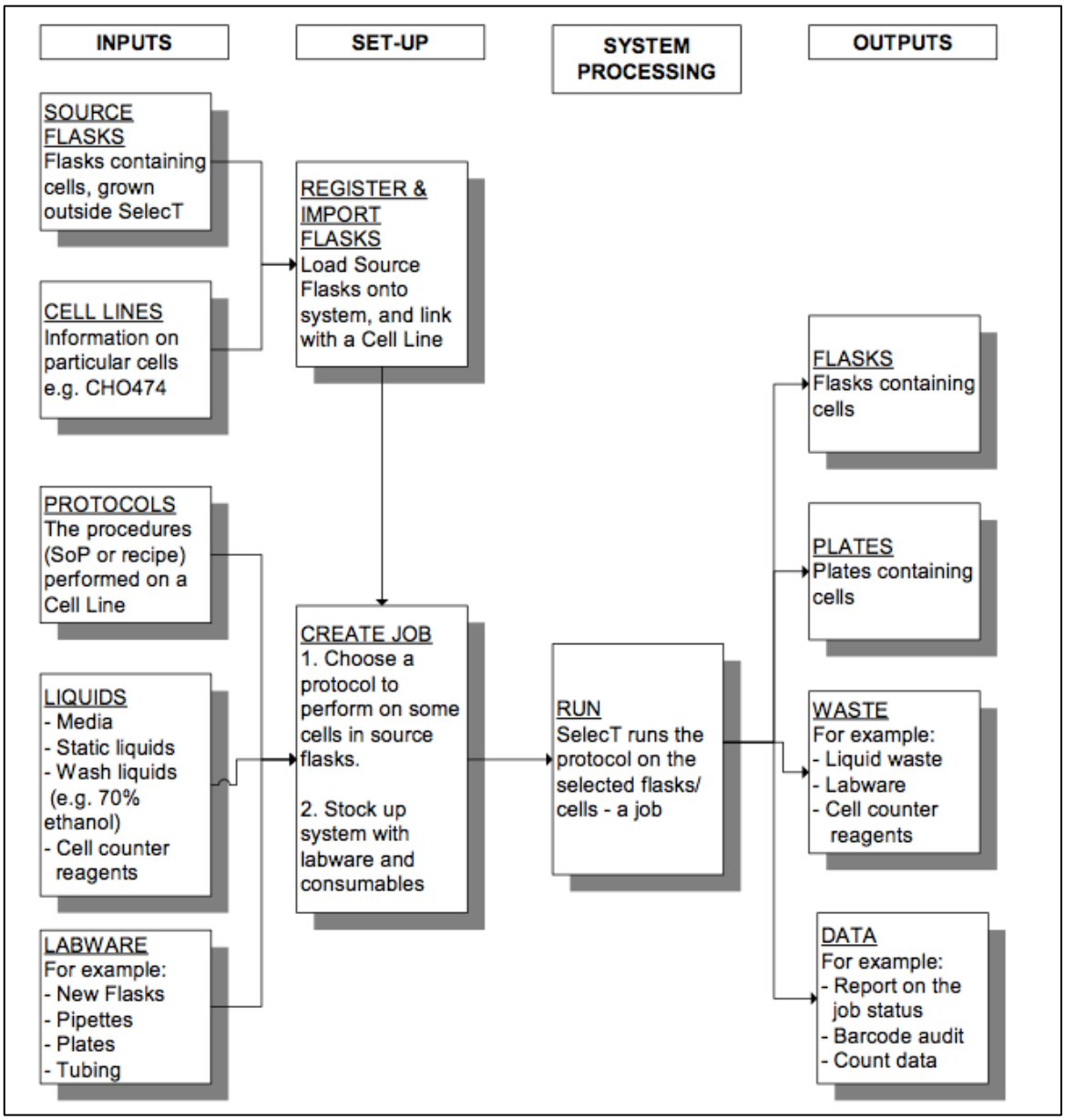


Figure 3.2 *Compact Select process overview (Tap Biosystems, 2010).*

Although specified in the Figure 3.2, the Compact Select used for the study does not carry out any cell culture tasks in the well plate format. Table 3.2 illustrates some of the programming terms (key functions) utilised by the XML protocol. This table also

describes how the automated functions compare with the manual process steps in order to carry out the intended task.

Table 3.2 *General automated key functions and how they compare with the manual cell culture steps.*

Automated Processing Step (XML Language)	**Function and Description**	**Comparison with the Manual Process Steps**
Fetch	Process of taking a flask out of the incubator	Identical
Dispense	Addition of an amount of liquid from the nozzle	Liquid is pipetted into the culture vessel
Store	Puts the currently held flask into the incubator	Identical
Pour	Process of pouring off waste medium into the waste chamber	Generally, waste is aspirated from the flask using a pipette
Dispose	Flasks are removed from the processing area	Identical
Shake	Holding the flask and moving it side to side, and is usually done to get the cells off the surface after the addition of detachment enzyme	Gentle tapping of the flask
Swirl	Holds the flask (horizontal) and moves it in a rotary tipping motions to spread the liquid over the lower surfaces of the flask.	Identical
Putdown	Flasks are placed in a slanted position on the flask holder	Flask is usually placed vertically or horizontally on the cell culture surface
Dump	Pours the liquid waste into the waste chamber	Liquid is usually aspirated out using a pipette
Incubate	Placing the flask in the incubator	Identical
Count	Identical	Identical
Mix	The contents of the flask are mixed by aspirating and dispensing a fixed volume of liquid using pipettes. This is done while flask is in slanted position on the flask holder	Identical, but the process is done while the flask is kept in a vertical position
Pipette	Medium is aspirated from one flask to another with a pipette	Identical

To ensure that correct volumes of reagent were dispensed throughout the automated process, calibration step was performed prior each Compact Select run. Briefly, the plastic tubing was primed and a small volume of reagent was dispensed into a T175 tissue culture flask containing liquid (pre-weighed). The flask was then exported (ejected from the system) and the contents were weighed on digital scales to determine the volume of reagent dispensed (1 mL of reagent is assumed to weighs 1 g). This value was then entered into the Compact Select software to calibrate the peristaltic pump system. This allowed the adjustment of the subsequent dispensing steps accordingly

3.2.2 Cell seeding procedure

A cryovial containing cells stored in liquid nitrogen was removed and thawed in 37 ^{0}C water bath. The contents of this cryovial were then transferred to a 15 mL conical tube, and 9 mL of growth medium was slowly added to the tube. This tube was then centrifuged at 220 x g for 5 minutes. After the removal of the supernatant, 10 mL of growth medium was added to the conical tube and the contents of the tube were mixed using a pipette. The cells were then counted using a Cedex cell counter (procedure described in Section 3.3.3.1). Cells were seeded at desired concentration in to the cell culture flasks pre-filled with warmed (up to 37 ^{0}C) growth medium. These flasks were then kept in the cell culture incubator until further manipulation. The volume of the pre-warmed medium used depended on the size of the flask/vessel used (detailed in Table 3.1). This procedure applies to all cell lines used for this study.

3.2.3 Isolation of MSCs from umbilical cord tissues

Sections of cord tissue (5-12 cm long) were shipped from the cord bank at ambient temperature, in secure shipping containers, in phosphate-buffered saline (PBS; Life Technologies, UK) without Ca^{2+} and Mg^{2+} solution, inside sterile, sealed 50 mL tubes, to Loughborough University. The PBS solution should not contain either Ca^{2+} or Mg^{2+} as these ions can interfere with the cord digestion process. These cord tissues were then removed from tubes with sterile forceps and positioned on trays. The remaining cord blood was squeezed from the cord by pressing the blunt edge of a sterile scalpel along the length of the cord. The cord tissues were placed in a Petri dish with PBS and 1% PenStrep.

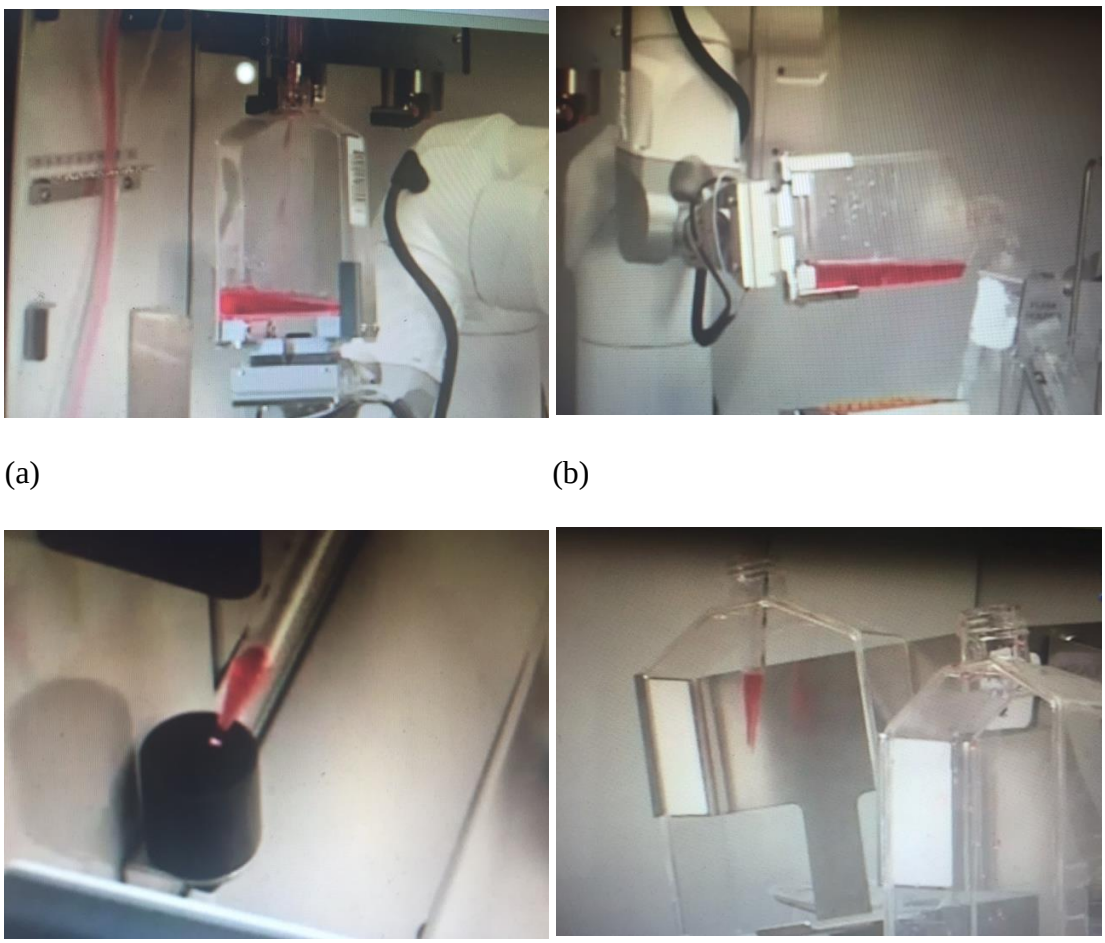
Each slice was chopped up into fine fragments (1-2 mm) using a scalpel. The fragments from each slice were then placed in individual 15 mL centrifuge tubes. Each slice was digested for 18h with collagenase (AMS Biotechnology Limited, USA) solution. Upon completion of the digestion, fragments were filtered through a 100 μm cell strainer into 50 mL tubes. The remaining tissue fragments were squeezed with the forceps to aid cell release after filtration. After filtration, 0.5 mL FBS and 3 mL DMEM were added to the suspension through the cell strainer to release the remaining cells on the strainer and to dilute the suspension. The cell suspension obtained after filtration and dilution was seeded into flasks containing cell culture medium. All culture flasks were incubated at 37°C and 5% CO_2. After 48 hours, the culture flasks were removed from the incubator, and spent medium containing dead cells and the extracellular matrix were aspirated and discarded. After washing the cell culture surface of the flasks with PBS, the complete MSC growth medium was added. The flasks were then returned to the incubator.

3.2.4 Cell passaging procedure

Following medium aspiration, the adherent flask surface was washed with PBS solution to remove any remaining factors from FBS or growth medium that can interfere with detachment enzyme activity. For HOS and HDF cell cultures, trypsin-EDTA (Life Technologies, UK) was used as the detachment enzyme. For MSCs, Tryple-Express (Life Technologies, UK) was used as the detachment enzyme. After washing, detachment enzyme solution was added, and the flasks were incubated for 5 minutes (if trypsin-EDTA was used) or for 10 minutes (if Tryple-Express was used).

Subsequently, enzyme activity was neutralised by adding the growth medium (volume equal to the volume of detachment enzyme). The cell suspension was the collected in a 50 mL conical tube and was centrifuged at 220 x g for 5 minutes, following which, the supernatant was aspirated off to leave the cell pellet, which was then re-suspended in 10 mL of growth medium and mixed by pipette. Viable cells were then counted using a Cedex cell counter (by taking 1 ml sample). Cells were then seeded at desired concentration into cell culture flasks, pre-filled with warmed (up to 37 ^{0}C) growth medium according to the size of the cell culture vessel used (Table 3.1). These flasks were then kept in the cell culture incubator until further manipulation.

However, for the automated culture the procedure described above had to be slightly modified because the automated platform did not come with a centrifuge. For the automated cell culture process, after the incubation was complete, fresh growth medium (containing FBS) was pumped into the flask to neutralise and dilute the enzyme (Figure 3.3 a).



(a) (b)

(c) (d)

Figure 3.3 *Automated passaging method. Figure (a) refers to neutralisation of the detachment enzyme, (b) pooling of cell suspension into another T175 flask prior to mixing and counting, (c) cell count using 1mL of sample and (d) re-seeding of cells into a new flask.*

Since the automated process did not allow the transfer of medium to a conical tube (prior to cell seeding/counting) as to how it was done for the manual cell culture, the cell suspension was left in a T175 flask or was pooled into a separate T175 flask if many flasks were processed (Figure 3.3 b) before the cells were mixed and counted (Figure 3.3 c). Then, the cells were re-seeded into a new T175 flask (Figure 3.3 d). Additionally, Compact Select used 10ml pipettes instead of 1000 uL tips for cell mixing and usually for the manual culture, for mixing, 1000 uL tips were used. In terms of processing times, there were also some differences between the two processes. The

automated platform required more time than the manual operator to process a similar number of flasks (Table 3.3) due to the linear nature of the process. The times were computed based on incubation time of 5 minutes.

Table 3.3 *A comparison between the time taken for each unit operation in the manual and the automated process.*

Process	**Time Required (minutes)**	
	Manual	**Automated**
Medium change for 1 flask	1	2 to 3
Medium change for 4 flask	1 to 2	4
Passaging 1 flask	12	20
Passaging 4 flasks	13-15	30

3.2.5 Cell cryopreservation procedure

Cells were harvested according to the same passaging procedure as described in Section 3.2.4 and were counted using a Cedex cell counter. Cells were then centrifuged at 220 g for 5 minutes and were re-suspended at the desired density in the standard freeze medium (10 % (v/v) dimethyl sulfoxide (DMSO; Sigma Aldrich, UK) with 90 % (v/v) FBS), and aliquoted into 1 mL cryovials (Corning Incorporated, USA). Vials of cells were placed in a Mr. Frosty freezing container (Nalgene, USA), and transferred to a -80 °C freezer for 48 hours, and then the cells were moved into liquid nitrogen for long-term storage.

3.3 Analytical Methods

Section 3.3 is divided into 6 parts. Section 3.3.1 describes the statistical analyses used for the study. Section 3.3.2 details the method used to calculate the cell growth rate and the cell yield coefficients. Section 3.4.3 details the method used to perform cell counts. Section 3.3.4 details the method used to record metabolite concentration in the growth medium. Section 3.3.5 describes the alkaline phosphatase assay carried out for HOS cells. The next section, Section 3.3.6 described the MTT assay carried out for MSCs. The penultimate section, Section 3.3.7 describes the flow cytometry analysis carried out for the MSCs. The final section, Section 3.3.8 describes the differentiation protocol that was used in this study to differentiate MSCs into specific cell types.

3.3.1 Statistical analyses

Three main types of statistics were used for this study: 2-tailed independent T-test (3.3.1.1), measurement system analysis (MSA) (3.3.1.2) and process capability analysis (3.3.1.3). These analyses were carried out using Minitab (Minitab, Ltd, USA) V17 software.

3.3.1.1 T-test

For each experimental condition, a sample size of 9 (n=9) was used unless otherwise stated. Statistical analyses were carried out using the 2-tailed independent T-test. Differences were considered significant when $p < 0.05$. The independent T-test was used when there were only 2 unrelated groups to compare. This test was performed assuming that the variances in the population are equal.

3.3.1.2 Measurement system analysis (MSA)

There are several techniques that can be employed to carry out MSA study. These include type 1 Gauge study (to measure repeatability and bias) and Gauge Bias and Linearity (Gauge B&L) study.

To assess the performance of the Cedex Cell Counter, type 1 Gauge study was carried out (Section 5.2.1, Chapter 5). A sample size of 25 (n=25) was used. This study is usually carried out by repeatedly measuring a reference part to assess the repeatability (precision) and bias (accuracy) error of a measurement system. For type 1 Gauge analysis, Cgk is used to assess precision and accuracy. Cgk refers to the distance to the closest control limit divided by 3 multiplied by standard deviation. Its value would be more than 1 if the gauge precision and accuracy were suitable for the given tolerance (Khan, 2013). If the bias is not significant, the P value for bias should be more than 0.05.

To assess the performance of the pipetting system (across a range of volumes), Gauge B&L study was then carried out (Section 5.2.2, Chapter 5). This gauge assesses the bias and the linearity (accuracy) over its operating range (Khan, 2013). A total sample size of 10 (n=10) was used. When a Gauge B&L study is carried out, Minitab displays a graph of the best-fitted line of the biases across the reference values. A positive bias indicates that the gauge over-estimates, meanwhile a negative bias indicates that the gauge under-estimates. If the bias is not significant, the P value for bias should be more than 0.05.

3.3.1.3 Process capability analysis

Process capability analysis was carried out using capability assistant (Minitab V17). The results are discussed in Section 5.4.3, Chapter 5. The process yields recovered for 5 passages from both the manual and the automated culture process were used to calculate capability statistics and allow comparison of the processes. In order to calculate capability statistics, nominal design specifications for cell yield were generated, based on a clinical and an experimental rationale. The nominal lower specification limit, based on the minimum therapeutic requirement per infusion (FDA, 2013), was set to 1×10^6 cells. Since the cell therapies require at least 6×10^8 cells to meet the minimum cell requirements per patient (discussed in Section 6.3.1), MSC yield has to be at least 2.8×10^6 cells per flask (assuming cells can only be expanded to a maximum of 5 passages using a seeding density of 8.75×10^5 cells and a population doubling value of 3). The nominal upper specification limit, based on the requirement to control over-confluence and its affect on cell state and other quality parameters, was set to 4.4×10^6 cells (Rowley *et al.*, 2012). The specification was applied to both processes in the same way to allow process comparison. The key operating parameters, such as seeding density, critical raw material and consumable batches were kept the same for both the manual and automated process runs.

In order to carry out this analysis, 2 critical assumptions have to be considered (Khan, 2013), otherwise the results obtained might be highly unreliable: i) the distribution of the process must be considered normal and (ii) the process must be in statistical control. In order to investigate if the distribution of the processes is normal, normality test (Anderson-Darling) was carried out. The Anderson-Darling test was used to

compare the empirical cumulative distribution function of the sample data with the distribution expected if the data were normal. It was considered significant for test of normality when *p* was less than 0.05.

In order to investigate if the process was within statistical control, Xbar-R control charts were used. The mean observations were plotted on Xbar-R control chart to assess whether the process was in statistical control. Control charts were based on probability theory and supported by Minitab, to detect non-random patterns in the data and the occurrence of special causes (ASTM, 2008). Process capability analysis was carried out after confirming the critical assumptions were valid. The Cp index was calculated according to the following equation:

$$C_p = \frac{USL - LSL}{6\sigma} \quad \textbf{(3.1)}$$

The Cpk indices were calculated according to the following equation:

$$Cpk = \min\left(\frac{USL - \mu}{3\sigma}, \frac{\mu - LSL}{3\sigma}\right) \quad \textbf{(3.2)}$$

where *USL* is the upper specification limit, *LSL* is the lower specification limit, σ is the estimated process standard deviation and μ is the process mean (ASTM, 2008).

Cp refers to the process capability to the specification range and it does not relate the location of the process with respect to the specifications. Values of Cp exceeding 1.33 indicate that the process is adequate to meet the specification. Values of Cp below 1.00 indicate the process is not capable of meeting specifications (Wooluru *et al*., 2014).

Cpk considers process average and evaluates the process spread with respect to where the process is actually located. If the characteristics or process variation is centered between its specification limits, the calculated value for Cpk is equal to the calculated value of Cp. Generally, a Cpk value greater than 1.33 indicates that a process is capable in the short term (Wooluru *et al.*, 2014).

3.3.2 Cell growth rate and yield coefficients

Cell growth rate is used as an indicator of culture performance. The rate of cell growth and the yield coefficients were calculated using the method described by Doran (1995). During the growth (exponential) phase, rate of cell growth, rx is described by the equation where μ is the specific growth rate and x is the viable cell concentration:

$$\boldsymbol{r_x = \mu x} \qquad \textbf{(3.3)}$$

In a closed system, where growth is the only process affecting cell concentration, $r_x = dx/dt$ and integration of Equation 3.3 gives an expression for x as a function of time. If μ is constant we can integrate directly with initial condition $x = x_o$ at t=0 to give:

$$x = x_o e^{\mu t} \qquad \textbf{(3.4)}$$

where x_o is the viable cell concentration at time zero. Equation 3.4 represents exponential growth. Taking natural logarithms:

$$\boldsymbol{lnx = lnx_o + \mu t} \qquad \textbf{(3.5)}$$

According to Equation 3.5, a plot of ln x versus time gives a straight line with slope μ. For growth rate calculations, the time that culture is in lag or stationary phases is not taken into consideration, and x_o and x are considered as points in the culture when the cells enter and exit the exponential growth phase respectively (determined from the linear region of a plot of lnx against t).

Doubling time, t_d is defined as the time taken for the cell population to double, or when x is equal to $2x_o$. By making this substitution in Equation 3.5, the following doubling time equation is obtained:

$$\mathbf{t_d = \frac{ln2}{\mu}} \qquad \textbf{(3.6)}$$

Yield coefficients allow nutrient requirements and production characteristics to be quantified (Doran, 1995). Yield coefficient can be defined as:

$$\boldsymbol{Y_{FG} = \frac{-\Delta F}{\Delta G}} \qquad \textbf{(3.7)}$$

where Y_{FG} is the yield factor, F and G are substances involved in metabolism, ΔF is the mass or moles of F produced, and ΔG is the mass or moles consumed.

3.3.3 Cell density and viability assessment

The cell counts (both manual and automated cell counts) for the doctoral study were performed using Cedex Cell Counter (Roche Innovatis, Germany), unless otherwise mentioned (Section 3.3.3.1). This was mainly to limit variation that can arise as a result of using different counting methods. Usually, for each experiment run, at least 3

additional counts with either the haemocytometer or the NucleoCounter (Chemometec, Denmark) are performed to ensure the results obtained using Cedex are reliable. These methods are described in Section 3.3.3.2 and Section 3.3.3.3, respectively.

3.3.3.1 Cedex cell counter

Trypan Blue dye exclusion test was used to determine the number of viable cells present in a cell suspension and is based on the principle that dead cells do not possess intact cell membranes that can exclude certain dyes (Strober, 2001). The Cedex system is an automated cell counter which integrates a liquid handling unit that mixes a defined sample volume with Trypan Blue (Sigma Aldrich, UK) in a mixing chamber (www.roche.com). This method is used in conjunction with digital image recognition to determine cell density, viability, diameter, and aggregate rate of the cell suspension. Stained samples are then passed through a flow cell where images are captured and analysed. The recognition software installed is capable of differentiating cells from debris, protein clumps and other pollutants allowing for reliable results to be obtained. The stained cell suspension is scanned and 30 images (up to 1000 particles are counted per image) are analysed per sample. Each sample was of 1 mL in volume, and at least 3 cell counts were performed each time. Before a set of cell counts was carried out a control substance (double-distilled water) was run through the system to confirm the absence of foreign particles within the flow cell.

3.3.3.2 Haemocytometer (manual count)

Cell suspensions were mixed using a pipette prior to sampling to ensure homogeneity of the suspension. Independent 0.02 to 0.5 mL samples were transferred to in 1.5 mL

Eppendorf tubes. Trypan Blue was then added to each sample, the ratio in volume of cell suspension to dye used for haemocytometer measurements was 1:1. At least a minimum of 5 minutes was allowed for the stain to penetrate the cells. The stained cell suspension was then loaded into a haemocytometer.

The traditional counting method uses a haemocytometer. A haemocytometer is a specialised microscope slide that contains grid lines that identify the chamber areas that can be used for counting. Each of these corner regions is 1 mm x1 mm in dimension and is divided into 16 small squares in a 4 x 4 array (Louis and Siegel. 2011). The volume of each of these four corners is 0.1 mm^3 or $1x10^{-4}$ mL (Louis and Siegel. 2011). The dye stained cell suspension was loaded into the edge of the chamber by pipetting 10 µL into each side of the chamber. The number of cells in each of the 4 quadrants were counted and the average count per quadrant was taken. It has to be multiplied by 10^4 to obtain the number of cells per mL in the sample applied to the haemocytometer (Louis and Siegel. 2011):

$$Total\ number\ of\ cells = 10^4\ x\ 2\ x\ sample\ dilution \quad (3.8)$$

$$Percentage\ Viability\ (\%) = \frac{Number\ of\ live\ cells}{Total\ number\ of\ cells}\ X\ 100 \quad (3.9)$$

3.3.3.3 NucleoCounter cell counter

The Nucleocounter system is a cell counting device based on the detection and counting of fluorescently labelled nuclei (www.chemometec.com). The viability is determined by a subtraction of the total cell number value from the non-viable count.

To obtain a non-viable count, a sample was withdrawn from the cell suspension using the NucleoCassette (Chemometec, Denmark). This was then run on the NucleoCounter and the reading obtained would be the non-viable count as viable cells would be able to exclude the propidium iodide stain (contained within the NucleoCassette).

To obtain the viable cell counts, the cell suspension was first mixed using a vortex mixer. Then, 100 μL of cell sample was transferred to a fresh eppendorf tube; 100 μL of NucleoCounter Buffer A (Chemometec, Denmark) was added to permeabilise the cell suspension, mixed, and then left for 30 sec. 100 μL of NucleoCounter Buffer B (Chemometec, Denmark) was then added to stabilise the solution, mixed and then left for 30 sec. The resulting mixture was then loaded into a NucleoCassette for the measurement of total cells. Finally, the number of viable cells was calculated by subtracting the number of dead cells in suspension from the total cell concentration.

3.3.4 Quantification of metabolite concentrations

Metabolites from 1mL spent medium collected at different time points during the culture were measured using a Nova BioProfile Flex Bioanalyser (Nova Biomedical Order Services, USA). The device provided readings for the concentration of glucose, glutamate, lactate, glutamine, ammonium, sodium, potassium, and calcium. Table 3.4 summarises the measurable range of various parameters for each sample analysed.

To evaluate the reliability of the bioanalyser readings obtained, 9 repeated measurements were performed and the maximum percentage deviations (standard deviation multiplied by 100) from the average values were calculated. The highest

maximum percentage deviation from the average value was calculated to be 8.4% for all the metabolites measured.

***Table 3.4**: Summary of the Bioprofile Flex Analyser tolerance limits.*

Parameter	Lower Limit	Upper Limit	Units
Glutamine	0.20	6.00	mmol/L
Glutamate	0.20	6.00	mmol/L
Glucose	0.00	15.00	g/L
Lactate	0.20	5.00	g/L
Ammonium (NH_4^+)	0.20	25.00	mmol/L
Sodium (Na^+)	40.00	220.00	mmol/L
Potassium (K^+)	1.00	25.00	mmol/L
Calcium (Ca_2^+)	0.10	10.00	mmol/L

3.3.5 Alkaline phosphatase assay

This assay was carried out for HOS cells using a fluorometric alkaline phosphatase (ALP) kit (Abcam, UK). The assay was conducted according to the manufacturer's instructions. The cells (1 x 10^5 cells) were first centrifuged at 220 x g for 3 minutes to remove insoluble materials. They were then added to the 110 µL ALP assay buffer and were mixed. The mixed cells were added to each well of a 96-well plate. Methylumbelliferyl phosphate disodium (MUP) 0.5 mM substrate solution of 20 µL was added to each well. The cells in the well plate were then incubated for 30 minutes at 37 ^{0}C, and the reaction was stopped by adding 20 µL of stop solution to each well containing cells. The fluorescence intensity was measured at Excitation/ Emission

360/440 nm using a fluorescence plate reader (BMG Labtech, Germany). Enzyme activity was calculated from the angular coefficient of the linear slope obtained from ALP standard Abcam solution, and was expressed as 4-methyumbelliferon generated per volume of sample (mU/mL). All the experiments were performed independently at least 3 times.

3.3.6 Flow cytometry

Flow cytometric analysis was carried out for MSCs (Chapter 6) according to the manufacturer's instructions of the BD StemflowTM human MSC kit (BD Biosciences, UK). The cell surface markers were analysed using each dye-conjugated antibody included in the kit. Flow cytometry analysis was performed on a Guava Flow Cytometer (Merck Millipore, UK). This analysis was carried out for MSCs to determine the cell fate after bioprocessing.

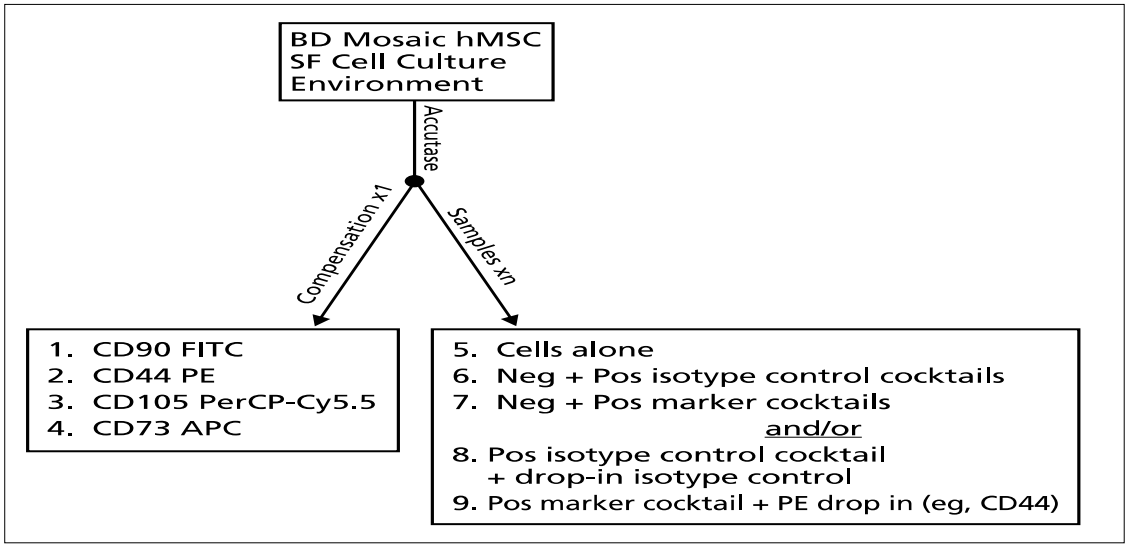


Figure 3.4 *Workflow schematic of the BD Stemflow human MSC Analysis Kit.*

MSCs were disassociated from the flask with Accutase cell detachment solution (Life Technologies, UK). Approximately, $4x10^6$ cells were fixed using BD Cytofix fixation buffer for 20 minutes, washed twice, re-suspended, and incubated for 10 minutes at room temperature using BD wash buffer. Then, 100ul cell suspension was stained through incubation at room temperature for 30 min with the fluorescent dye-conjugated antibodies against positive marker CD73, CD90, CD105, CD44, CD166, or the negative marker cocktail containing equally mixed PE-conjugated antibodies against CD11b, CD19, CD34, CD45 and HLA-DR, or each isotype control antibody. After staining, the cells were washed with PBS, and analysed using Guava Flow Cytometer. The expression of each marker or cocktail markers was determined by subtracting each fluorescent value from the value of each control antibody. The procedures carried out are summarised in Figure 3.4.

3.3.7 MTT assay

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell proliferation assay based on mitochondria activity was carried out using MTT Cell Growth Kit (Chemicon, US) according to the manufacturer's instructions. Cells were seeded at 1.25×10^4 cells per well in 6-well plates, and were incubated for 4 days, during which the culture medium was replaced. Wells were washed twice with PBS before 10 μL MTT (0.5 mg/mL) was added, and were incubated for 4 hours. Then 100 μL isopropanol with 0.04 N HCl was added to the culture medium to neutralise the alkaline solution. Absorbance was measured at 570 nm using a microplate reader (BMG Labtech, UK). All the experiments were carried out independently at least 3 times.

3.3.8 Differentiation assays

To confirm that MSCs maintain their multipotency after being cultured using T-Flasks, cells were exposed to the UC-MSC differentiation protocols to induce adipogenesis, osteogenesis, and chondrogenesis (Chapter 6). Negative controls were MSCs that were not exposed to the differentiation media. The protocols were carried out according to the manufacturer's instructions (Life Technologies, UK). All the experiments were performed independently at least 3 times.

For osteogenic differentiation, cells were plated at 5×10^3 cells per cm^2 tissue culture surface area and cultured overnight in MSC culture medium. Cells were then fed with the Osteogenesis Differentiation Medium (Life Technologies, UK), which was replaced every 3 to 4 days for 2 to 3 weeks. At the end of the feeding schedule, cells were stained with Alizarin Red (Life Technologies, UK) to assess calcium deposition. Cells were removed from culture, rinsed in PBS and were fixed with 4% paraformaldehyde (PFA; Sigma Aldrich, UK), rinsed with one wash of PBS, followed by one wash of distilled water, stained with 2% Alizarin Red solution (Sigma Aldrich, UK), rinsed thrice with distilled water and viewed using an Eclipse TS100 inverted microscope. All the experiments were performed independently at least 3 times.

For adipogenesis differentiation, cells were re-plated at 1×10^4 cells per cm^2 tissue culture plate and cultured overnight in MSC culture medium. Cells were then fed with the Adipogenesis Differentiation Medium (Life Technologies, UK), which was replaced every 3 to 4 days for 2 to 3 weeks. At the end of the feeding schedule, cells were stained with Oil Red (Sigma Aldrich, UK) to assess lipid formation. Cells were removed from culture, rinsed in PBS containing calcium and magnesium (Life

Technologies, UK) fixed in 4% paraformaldehyde (Sigma Aldrich, UK), rinsed with one wash of PBS containing calcium and magnesium followed by one wash of distilled water, stained with 2% Oil Red solution, rinsed three times with distilled water and viewed using an Eclipse TS100 inverted microscope (Nikon, Japan). All the experiments were performed independently at least 3 times.

For chondrogenesis differentiation, detached cells were pelleted at 100 × g for 5 to 10 minutes in a centrifuge. These cells were then re-suspended in MSC growth medium, (DMEM-FBS medium for the traditional cell culture process or StemPro medium for serum-free cell culture process) to generate a concentration of 1.6×10^7 cells per mL. Micro mass cultures were generated by seeding 5 μL droplet of cell solutions in a center of a well plate. Micro mass culture was cultivated for 2 hours in the incubator. The Chondrogenesis Differentiation medium (Life Technologies, UK) was added to the micro mass culture. This differentiation medium was replaced every 3 to 4 days for 2 to 3 weeks. At the end of the feeding schedule, cells were stained with Alcian Blue (Sigma Aldrich, UK) to assess the synthesis of proteogylcans. Cells were removed from culture, rinsed in PBS containing calcium and magnesium (Life Technologies, UK), fixed in 4% paraformaldehyde, rinsed with one wash of PBS containing calcium and magnesium followed by one wash of distilled water, stained with 2% Alcian Blue solution for 5 minutes, rinsed three times with distilled water and viewed using an Eclipse TS100 inverted microscope (Nikon, Japan). All the experiments were performed independently at least 3 times.

3.4 Experimental Design

The experimental procedures used for Chapter 4, 5, and 6 are described in Section 3.4.1, 3.4.2, and 3.4.3, respectively.

3.4.1 Experimental procedures: Chapter 4

The study discussed in Chapter 4 was carried using a permanent and transformed HOS (method described in Section 3.4.1.1) and an immortalised HDF (method described in Section 3.4.1.2) cell line. HOS cells used for the experiments were from passage 40 meanwhile HDF cells used were from passage 6.

3.4.1.1 HOS cells

Human osteosarcoma cells recovered from cryopreservation were expanded in culture according to procedures described in the Section 3.2 and 7.5 x 10^5 cells (per flask) were seeded into 9 T175 flasks. Three T175 flasks were sacrificed and the spent medium samples were analysed every 24 hours starting from time point 0 to 72 hours. Both manual and automated cell culture experiments were performed in triplicate (total n = 9). Statistical analysis was carried out using the 2-tailed independent T-test.

3.4.1.2 HDF cells

Passage 4 HDF cells recovered from cryopreservation were expanded in culture according to procedures described in Section 3.2 and 8.75 x 10^5 cells (per flask) were seeded into 12 T175 (175 cm^2) flasks and were cultivated. Three flasks were sacrificed and the spent medium samples were analysed every 24 hours starting from time point 0 to 96 hours. Seeding densities of 8.75 x 10^5 cells per T175 flask (5 x10^3 cells per cm^2 of flask surface) were chosen, unless otherwise stated. This work was performed in

triplicate (total n = 9). Statistical analysis was carried out using the 2-tailed independent T-test.

3.4.2 Experimental procedures: Chapter 5

The work discussed in Chapter 5 were carried out using HDF cells and MSCs. HDF cells used for these experiments were from passage 6 meanwhile MSCs used were from passage 7.

3.4.2.1 Performance of the Cedex cell counter

The method described here refers to the study discussed in Section 5.2.1. To investigate the performance of the Cedex cell counter, 9 flasks with HDF cells were passaged and the cell suspensions were collected in three 50 mL conical tubes. Cell samples (each 1ml) were taken from each conical tube and were counted using the Cedex cell counter, the NucleoCounter (Sartorius Stedim, France), and the haemocytometer. The counts were compared with one another. The cells were taken from the same cell pool to ensure to minimise variations. This work was performed in triplicate (total n = 9). Statistical analyses were carried out using 2-tailed independent T-test and also Type 1 Gauge analysis (MSA).

3.4.2.2 Performance of the liquid dispensing system

The method described here refers to the study discussed in Section 5.2.2. In order to calculate the pipetting accuracy, an in-house method was developed which involved pipetting a known volume of DMEM liquid (without FBS) into a flask containing liquid (with a known mass in grams). The flask was weighed after the flask was

pipetted with a desired volume. The volume was determined by assuming the density of the liquid was 1 g/mL (density of water) at standard temperature and pressure of 298K and 1 x 10^5 Pascal, respectively. Volumes dispensed were from 1 to 10 mL using a volume interval of 1ml. A total of 10 repeat measurements were taken for each volume interval (n=10). Statistical analysis was carried out using Gauge B&L (MSA) study.

3.4.2.3 Cell harvesting protocol for cells

The method described here is used to carry out the study discussed in Section 5.3.1. In order to investigate the difference between the automated and the manual cell harvesting process, 12 flasks were each manually seeded (by human operator) with 8.75 x 10^5 HDF cells. Then, 6 flasks were placed in the traditional (manual process) cell culture incubator and the remaining in the automated cell culture incubator.

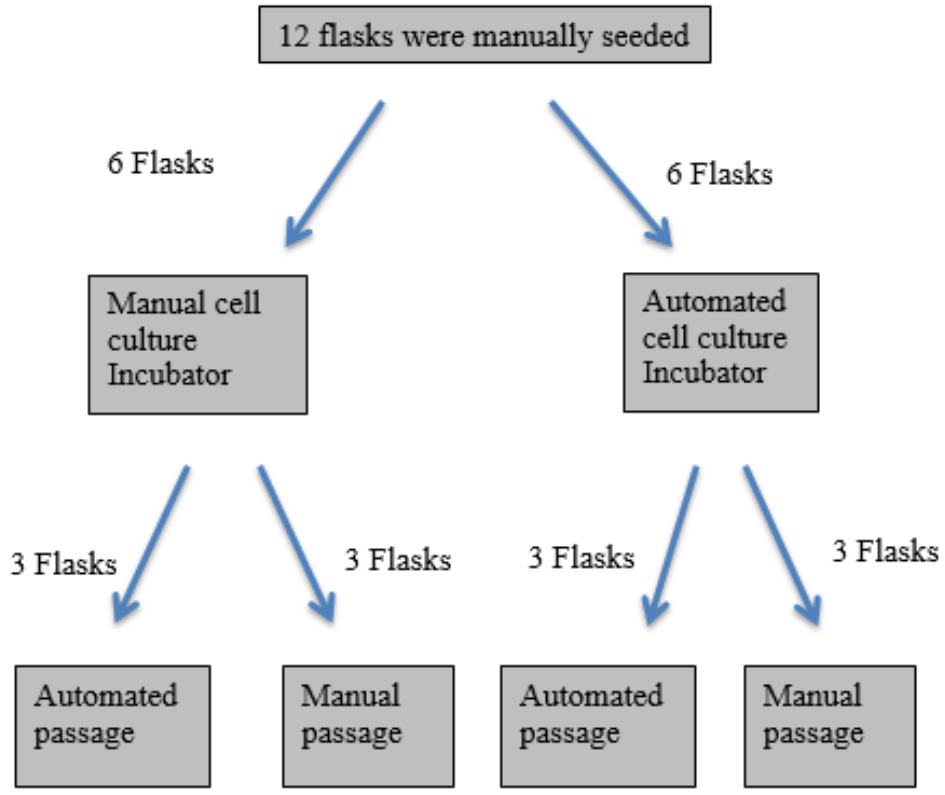


Figure 3.5 *The experimental design used to assess the manual and the automated HDF cell harvesting steps.*

After 24 hours, 3 flasks from the manual cell culture incubator were then transferred to the Compact Select to be passaged, and the remaining 3 flasks were passaged manually (human operator). Similarly, 3 flasks from the automated cell culture incubator were passaged using the Compact Select and 3 flasks were manually passaged. Figure 3.5 shows the experimental design used to assess the performance of both processes. All harvested cells were counted immediately using a Cedex cell counter. This experiment was performed in triplicate (total n=9).

In order to assess the MSC harvesting process, similar experimental design was employed, but there were several modifications. One major difference was the detachment enzyme used. Tryple-Express was used as the detachment enzyme. In order to investigate the difference between the automated and the manual cell harvesting process, 16 flasks were each manually seeded (by human operator) with 8.75×10^5 MSCs. Then 8 flasks were placed in the traditional (manual process) cell culture incubator and the remaining in the automated cell culture incubator.

After 48 hours, 4 flasks from the manual cell culture incubator were then transferred to the Compact Select to be passaged, and the remaining 4 flasks were passaged manually (human operator). Similarly, 4 flasks from the automated cell culture incubator were passaged using the Compact Select and the remaining 4 flasks were manually passaged. Figure 3.6 shows the experimental design used to assess the performance of both processes. All harvested cells were counted immediately using a Cedex cell counter. The method described here is used to carry out the study discussed in Section 5.4.1.

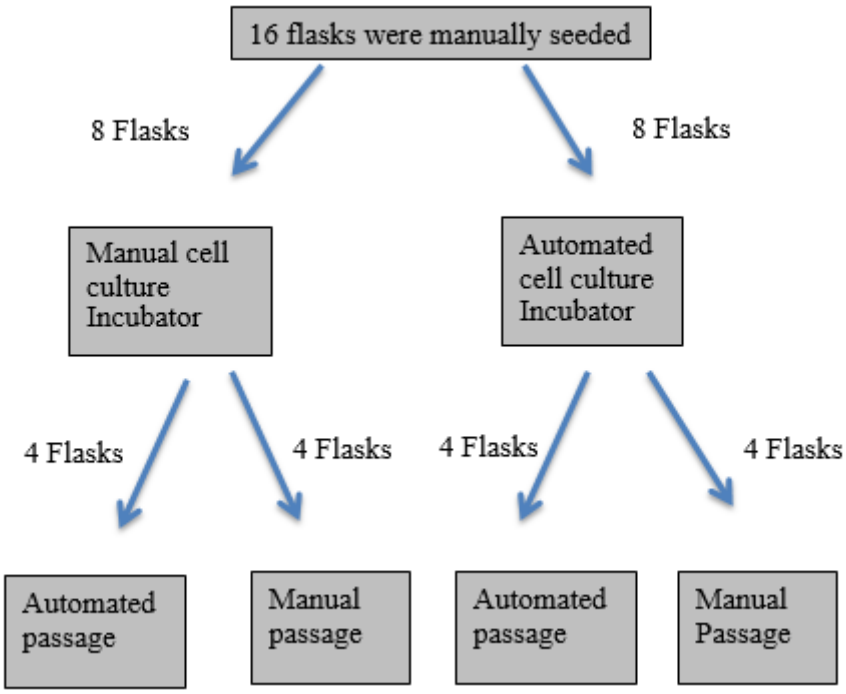


Figure 3.6 *The experiment design used to assess the manual and the automated MSC harvesting steps.*

3.4.2.4 Cell seeding protocol

The method described here was used to carry out the study discussed in Section 5.3.2. The next part of the work focused on evaluating the Compact Select's seeding process. A flask was seeded with 9 x 10^6 cells, and the automated cell culture seeding process was imitated with the flask placed in a slanted position (Figure 3.7).

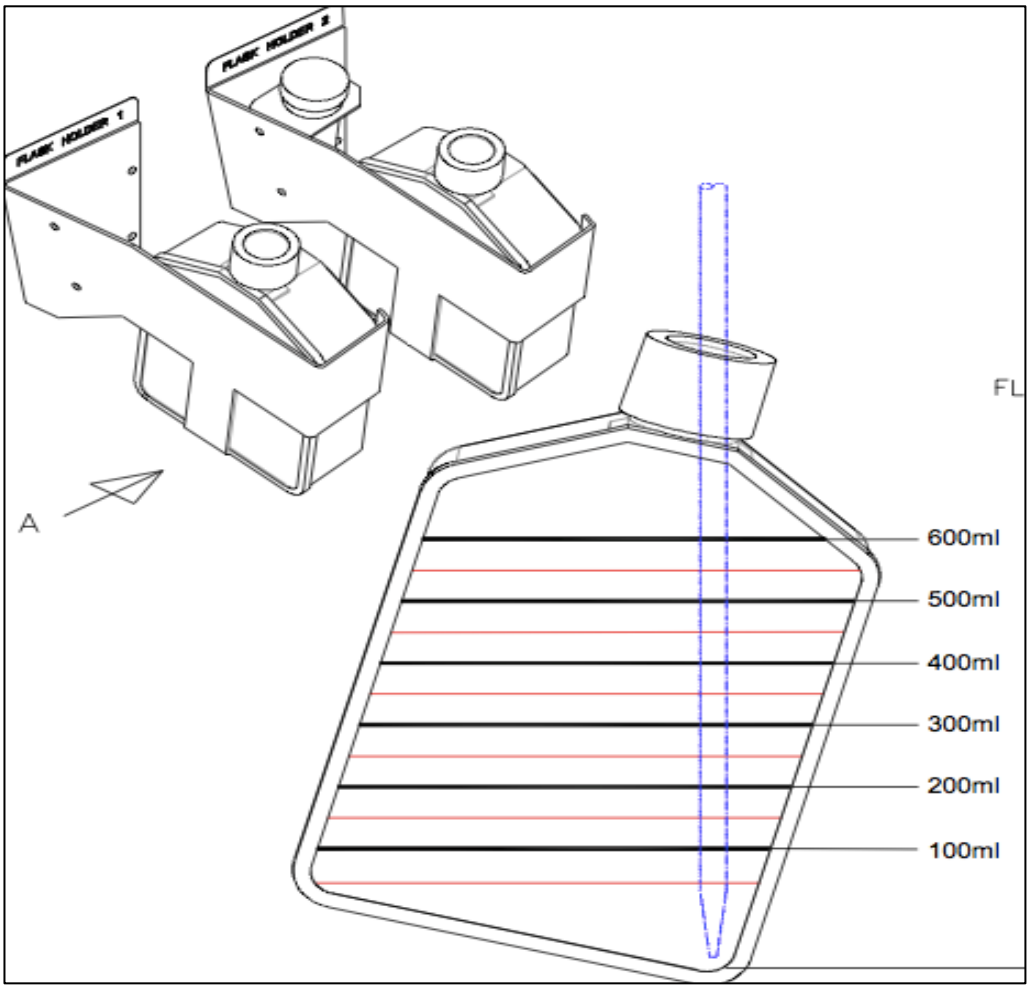


Figure3.7 *Flask position during the automated seeding process.*

This 'mother flask' (the flask were the cells are seeded from) was seeded with a larger concentration of cells, instead of the usual seeding density of $8x10^5$ cells per flask, to mimic the passaging process as closely as possible. Every 10 minutes, cell samples were taken from the cell suspension, and the concentrations of cells remaining in the cell suspension were determined using the Cedex cell counter (Figure 5.3). The cell suspension was mixed before a sample was taken to ensure that the sample was taken from a homogenous suspension.

3.4.2.5 Automated protocol modification study

The method described here is used to carry out the study discussed in Section 5.4.2. To adapt the complete manual MSC culture process to the Compact Select, 4 iterations of the automated protocol were written using XML programming. Figure 3.7 provides a simplified schematic of these automated protocols. The original protocol is labeled as protocol a, and the new iterations are labeled as protocols b, c, d and e.

The main difference between protocol (a) and the rest of the protocols developed is that, for the rest of the protocols (b to e), the flask was incubated without the detachment enzyme as the detachment enzyme was poured off as soon as the cells become immersed with the enzyme. The main difference between protocol (b) and protocols (c and d) is the incubation time employed. The main difference between protocol (d) and protocol (d) is that for protocol (e), an additional shaking step was introduced (discussed in Section 5.4.2, Chapter 5). To evaluate these protocols, 4 flasks were each seeded with 8.75×10^5 MSCs and were passaged after 3 days using each protocol (total n =4). This work was continued for a total of 2 passages. The cell yields obtained were compared. Statistical analyses were carried out using the 2-tailed independent T-test.

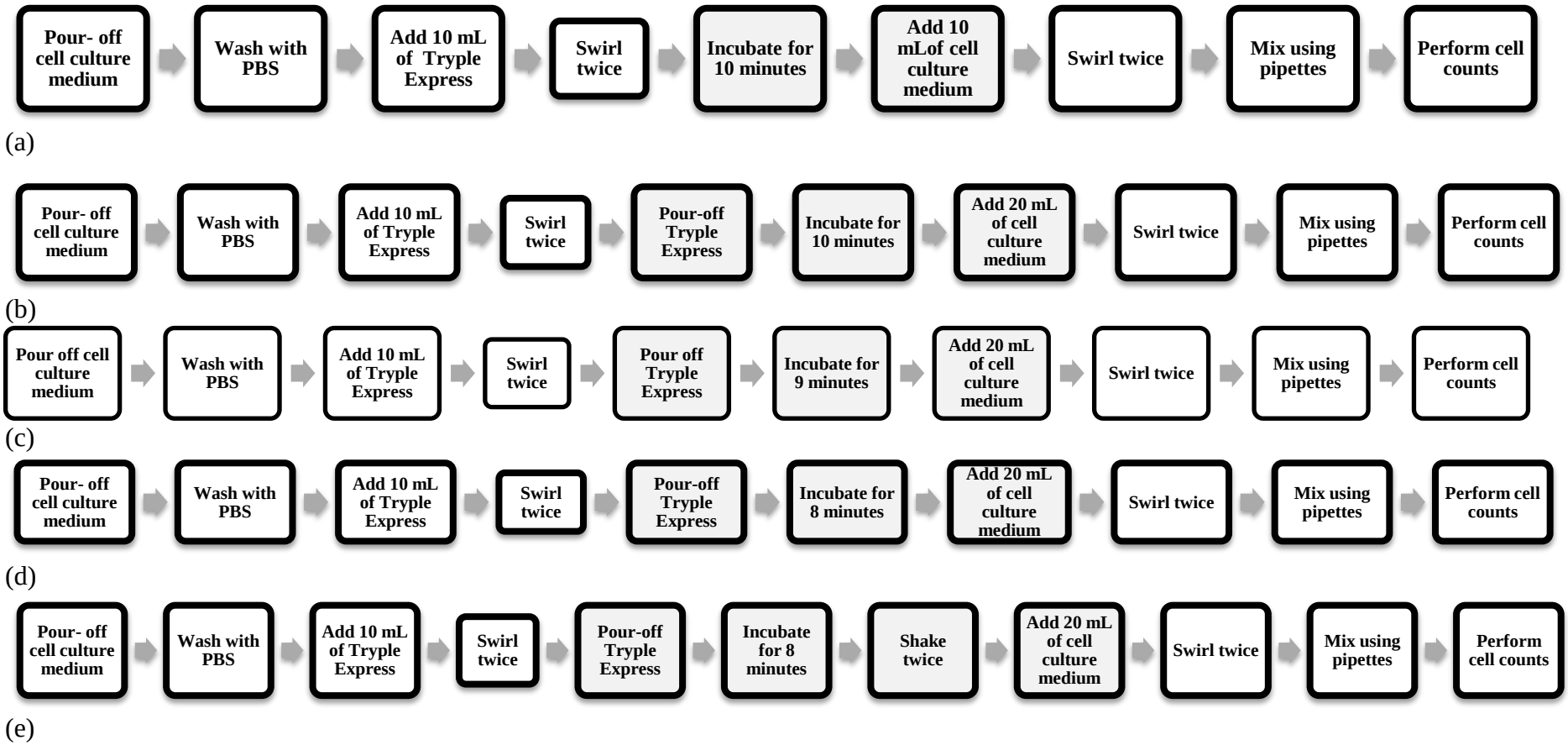


Figure 3.8 *Simplified schematic of the automated protocols tested. Protocol (a) refers to the original protocol meanwhile protocols (b) to (e) refer to the new iterations. The main differences are highlighted in grey.*

3.4.2.6 Assessing the suitability of the optimised automated MSC expansion protocol

The method described here is used to carry out the study discussed in Section 5.4.3. The yield obtained from the automated process was compared with the yield from the manual process. MSCs from passage 7 were used for the analysis and they were expanded for a total of five passages, up to passage 12 using Protocol (e) developed in the preceding section. Cells were seeded at 8.75 x 10^5 cells per T175 flask into 4 flasks (for each process) and were passaged after 3 days (for all passages). Statistical analyses were carried out using the 2-tailed independent T-test and process capability analysis.

3.4.2.7 Xeno- and serum-free MSC culture process (manual process)

The method described here is used to carry out the study discussed in Section 5.5. In order to investigate if the MSCs could be cultured in a serum-free medium, the StemPro medium was used. This work was conducted with T25 flasks only. Mesenchymal stem cells from passage 7 were cultured up to passage 10 using a seeding density of 5 x 10^3 cells/cm^2 per flask into 4 T25 flasks. Statistical analysis was carried out using the 2-tailed independent T-test.

3.4.3 Experimental procedures: Chapter 6

The MSCs were obtained from all sections of the human umbilical cords. All the experiments described in this chapter were performed in triplicates and were repeated with

cells from another umbilical cord (n = 6). All the analyses (flow cytometry, MTT, and differentiation assays) were carried out at passage 5 as it was not feasible to analyse cells at every passage due to high reagent cost and limited cell availability. Flow cytometry, MTT, and differentiation protocols are detailed in Sections 3.3.6, 3.3.7, and 3.3.8, respectively. Throughout this experiment, only a seeding density of 8.75×10^5 cells (5×10^3 cells/cm^2) was used.

Cells were isolated from a human umbilical cord according to the protocol described in Section 3.2.3. Cells from a single cord were split into 2, and cultured either in FBS-containing DMEM or StemPro medium until the first passage. Cells were expanded using the methods described in Section 3.2.4, but few modifications were made for the automated cell culture protocol based on the outcomes of the study carried out in the Chapter 5, Section 5.4.3. Protocol described as protocol (e) in Section 3.4.2.5 was selected for the automated process run.

Cells cultured in StemPro medium (passage 0) did not survive, therefore, the flask was discarded, but at passage 2, some cells that were split into FBS-containing DMEM medium were placed in both StemPro Medium and FBS-Containing DMEM. After passage 2, 3 flasks with StemPro medium, and 3 flasks with FBS-containing DMEM were simultaneously placed in the automated and the manual cell culture incubator, respectively, and were expanded according to methods described in Chapter 3. Cells were cultured until passage 5 via both the manual and automated cell culture methods. Several analyses were then performed on these cells. These include cell growth and viability (Section 3.3.2 and

Section 3.3.3), metabolite (Section 3.3.4), MTT (Section 3.3.7), flow cytometry (Section 3.3.6), and differentiation (Section 3.3.8) analyses.

Chapter 4. Results and Discussion: Comparing the Manual with the Automated Process

4.1 Introduction

The aim of the doctoral project was to investigate the possibility of developing a robust automated umbilical cord-derived mesenchymal stem cell (UC-MSC) expansion process that is capable of producing clinically relevant product in order to facilitate successful commercialisation of this stem cell therapy. To achieve this aim, several objectives were identified (Chapter 1). The objective of the study described in this chapter was to compare and highlight the differences between established manual and automated cell culture processes.

The manual cell culture process will provide the baseline to which results can be compared. The availability of baseline data is always critical for performance evaluation, as it is not possible to evaluate changes without reliable data on the situation before the intervention began. To evaluate the suitability of manual cell culture as the baseline process, it will be first confirmed if the baseline process data are within the acceptable range. For this reason, the cell doubling values of HOS cells from the automated and manual process runs will be compared with the literature (Liu *et al*., 2010).

In this study, a permanent and transformed Human Osteosarcoma (HOS) and an immortalised Human Dermal Fibroblast (HDF) cell line were selected as model cell lines because stem cells are known to be more sensitive to process variability than established

cell lines (Thomas *et al*., 2009) and thus, may present a greater technical challenge for preliminary studies. If primary cell lines are to be used, it will not be possible to distinguish biological variations from process variations. HOS and immortalised HDF cells are stable *in vitro* (Majeska *et al.,* 1980; Ouellette *et al*., 2008) with limited variation in doubling time or viability between passages (viability always above 98%). This means it will be possible to distinguish biological variation from process variation. In contrast, the MSCs (stem cells) have been reported to senesce and differentiate *in vitro* culture after less than 9 passages (Placzek, 2009; Abbasalizadeh *et al*., 2012).

The approach of using a model cell line for experimental work has another significant advantage in terms of cost. These cells are relatively inexpensive to culture as they do not require feeder layers or additional growth factors other than the ones found in Foetal Bovine Serum (FBS) to sustain their growth. In addition, HOS and immortalised HDF cells exhibit unlimited proliferation *in vitro* (Di Fiore *et al*., 2009; Miki and Rhim, 2007) so they can be expanded to quantities suitable for experimental needs with just a limited amount of starting material (approximately $1x10^6$ cells). If primary cell lines are used for preliminary experiments, instead of the ones chosen in this study, typically, they can only be expanded up to few passages as they start senescing relatively early (Miki and Rhim, 2007), therefore, more starting material will be required to make up the same quantity of cells required.

The key questions that will be addressed in this chapter are:

1. Are there significant differences between the manual and the automated process, in terms of process yield and the cell quality obtained?
2. If there are significant differences between the two processes, are the differences observed when the work is repeated with another another cell line? If differences are observed, are they due to biological variation or are they due to the cell expansion process used?

These are important key questions that must be addressed to assess how much further work is necessary to fully transfer the MSC culture process to the automated platform. Without these preliminary data, any effort in automating MSC culture process can result in a costly failure. Cell analyses (described in the Chapter 3) will be performed primarily to understand the impact of the processes (manual and automated) on the cells. The experiment performed with the HOS cells was later repeated with the HDF cells. The experimental methods are detailed in the Section 3.4.1, Chapter 3.

4.2 Manual Vs. Automated Process Runs

This section presents the analyses to evaluate and compare the manual cell culture process with the automated cell culture process for both the HOS and HDF cells. Section 4.2.1 and 4.2.2 describe the work carried out with the HOS and the HDF cells, respectively.

4.2.1 Human osteosarcoma (HOS) cells

Passage 40 HOS cells recovered from cryopreservation were expanded in culture according to procedures described in the Section 3.2. 7.5 x 10^5 cells were seeded into each

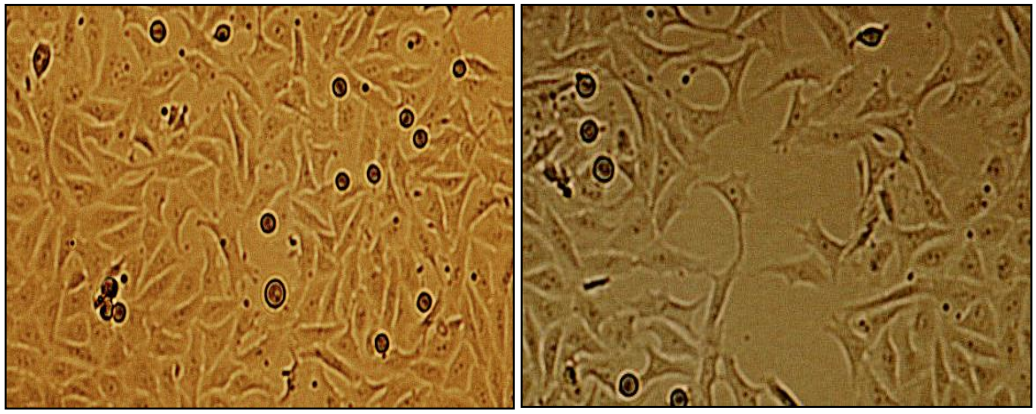
T175 flask (total flasks were 9 T175 flasks). Three T175 flasks were sacrificed and the spent medium samples were analysed every 24 hours starting from time point 0 to 72 hours. Both manual and automated cell culture experiments were performed in triplicates and were repeated twice (n = 9). For the automated cell culture process, the protocol was created to mimic the manual process as closely as possible (detailed in Chapter 3).

In order to compare the two processes (manual and automated), a number of important parameters were identified and analysed. These parameters include (a) cell morphology, (b) growth curve, (c) doubling time and growth rate (d) cell metabolites and (e) ALP assay.

The results discussed in Section 4.2.1 and the 4.2.2 are the results obtained for successful cell cultures. It should also be noted that while contamination (bacterial) was never observed for the HOS and the HDF cells cultured via the automated method, contamination was observed (several times) for the cells cultured manually. During the initial stages of the study, the cell cultures were carried out in a laboratory, where bacterial cells were also being cultivated, therefore, this was suspected to be the main reason why contamination was occurring. Although the sterility of the cell culture hood, incubator, and the consumables were always ensured prior to the manual culture, it was not possible to prevent contamination on every occasion. When the manual cell culture was transferred to a laboratory where only mammalian cell cultures were allowed, then the frequency of contamination was reduced, but never eliminated entirely.

(a) Cell morphology

Figure 4.1 compares the morphology of cells cultured *via* the manual and automated processes. All experiments performed with the HOS cells resulted in a successful culture, with no observable problems with attachment or proliferation, but it can be seen that there were more adherent cells in the manual process flasks (Figure 4.1). The cells in the manual process flasks appear smaller and more tightly packed than the cells in the automated process flasks, but this is to be expected when there are more cells per flask (Brattain *et al.*, 1981). The images of the confluent cells in the manual process flasks look similar to the images recorded by Liu *et al.* (2010).



(a) **(b)**

Figure 4.1*: Representative phase-contrast microscope images of the HOS cells obtained from (a) manual and (b) from the automated cell culture process after 72 hours of culture (n=9). Magnification: x40 was used.*

(b) Growth curve

Figure 4.2 shows the growth profiles of cells cultured for 72 hours. This figure shows that number of cells per flask increased from 7.5 x 10^5 to 5.24 x 10^6 for the manual process, but only up to 3.04 x 10^6 for the automated process. Cedex cell counts based on the trypan

blue exclusion method revealed that cell viability was always above 98% for all cell counts performed (for cells obtained from both the manual and the automated process). The average cell number obtained for the manual process was significantly different than the average obtained for the automated process after
72 hours ($p < 0.001$). However, the automated cell yield obtained through using this automated protocol compared well with the cell yield obtained by Liu *et al*. (2010). These authors used a similar cell line and cell culture system for automated processing.

Only small variations between the samples were observed as indicated by the error bars for the data obtained for both manual and automated cell culture processes (Figure 4.2). It is also important to note that there were only small variations between the error bars for the manual cell culture as there was only one human operator. In the area of biology, especially in the area of cell culture, operator-dependent variation is one of the biggest issues, therefore, process automation can be useful in reducing these variations. Liu *et al*. (2010) have confirmed that process automation using the Compact Select can reduce process variation caused by human error.

The cell population in a batch culture typically has the following phases of development: (i) lag phase, (ii) logarithmic or exponential growth phase, (iii) stationary phase and (iv) death phase (Doran, 1995). Lag phase occurs because upon inoculation of cells into fresh medium, the cells may take some time to acclimatise to the growth environment (Doran, 1995). In the exponential phase, the cells have adjusted to their new environment and are able to multiply rapidly, therefore, there is a steady exponential increase in cell number

density with time. In stationary phase (if there is no addition of nutrients to the culture), nutrient depletion and toxic or inhibitory by-products build-up occur. During this period since the cell division ceases, the growth rate is equal to the death rate (Doran, 1995).

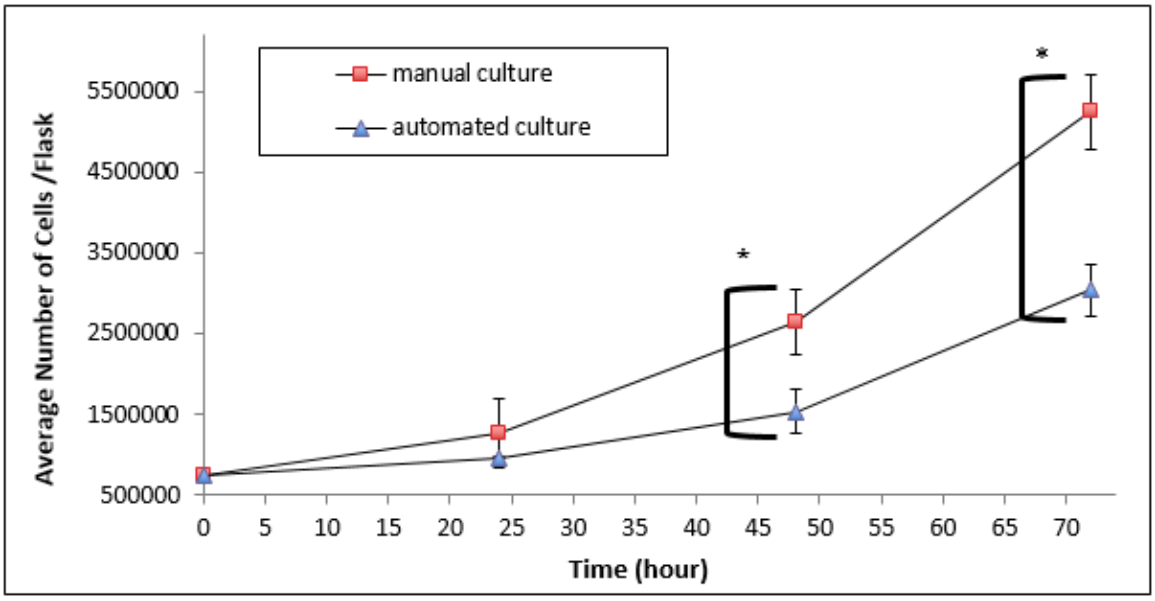


Figure 4.2 *Average number of viable HOS cells obtained from the manual and automated cell culture experiments over 72 hours of culture. Each T175 flask was seeded with 7.5 x10^5 cells. Error bars represent standard deviation of data (n=9). Asterisks (*) indicate significant difference, $p < 0.001$*

From the Figure 4.2, only the lag and the log phases can be observed, despite the image of cells demonstrating that the flask (manual culture) was already confluent on day 3, especially in the manual process flasks (Figure 4.1). This is not an abnormal scenario for the cancer cells as they have cancer elevated apoptotic threshold; that is increased ability to resist apoptosis (Klein, 2004).

(c) Growth rate and doubling time

In the subsequent section, the growth curve obtained was able to show the general growth pattern (lag and log phases) for the cells cultured via the manual and the automated process. The cell growth rates and doubling times are also calculated as they provide more information as to how the cells behave when they are their maximum growth potential (logarithmic phase) and by knowing these values, data obtained from this experiment can be compared with literature. The method of obtaining these values is detailed in Section 3.3.2, Chapter 3.

The average cell doubling time of 25.2 hours (with a maximum growth rate, μ_{max} value of 0.0274 hr^{-1}) obtained for the manual cell culture was within the range of the doubling times quoted in the literature (Kuettner *et al*., 1978; Clover and Gowan, 1994), but the average cell doubling time of 35.7 hours (μ_{max} value of 0.0194 hr^{-1}) obtained for the automated cell culture was not within this range. Initially, it was believed that the cells were taking a longer time to adapt to the culture conditions when the cells were cultured using the automated process, however further investigation revealed that this was not true (Chapter 5).

(d) Metabolite analysis

Comprehensive analysis of metabolites in cells at specific time and conditions is defined as "metabolomics". Metabolomics is an emerging field in human biology that is capable of providing quantitative data these data are vital information that can be used to solve many important questions related to human disease diagnosis, prognosis, and therapeutic

development (Djukovic *et al*., 2013). In order to obtain these quantitative metabolite data, spent media were analysed to measure the glutamine, glucose, lactate, and ammonium concentrations (method described in Chapter 3).

Glutamine and glucose are the main sources of energy for most mammalian cells and their passages through the glycolysis pathway result in the production of waste products such as lactate and ammonia (Takagi *et al.*, 2000; Gorfien *et al.*, 2003; Yuneva *et al.*, 2007; Wellen *et al*., 2010). As it has been claimed that metabolite accumulation and depletion can affect the cell growth and the productivity (Sellick *et al.*, 2011; Ahn and Antoniewicz, 2012), it was crucial to understand if metabolite accumulation in the cell culture media was the reason for reduced cell growth.

Figures 4.3(a) to (d) show the depletion of glutamine and glucose and the accumulation of lactate and ammonium in cultures over 72 hours for cells cultured manually and in the automated system. Figure 4.3 (a) shows that glutamine concentration in the manual process flasks decreased from 1.97 to 0.33 mmol/L and decreased from 1.92 to 1.07 mmol/L in the automated process flasks. Figure 4.3 (d) shows that glucose concentration in manual process flasks decreased from 5.57 to 1.35 mmol/L and decreased from 5.63 to 3.21 mmol/L in the automated process flasks (Ikebe and Suzuki, 2014).

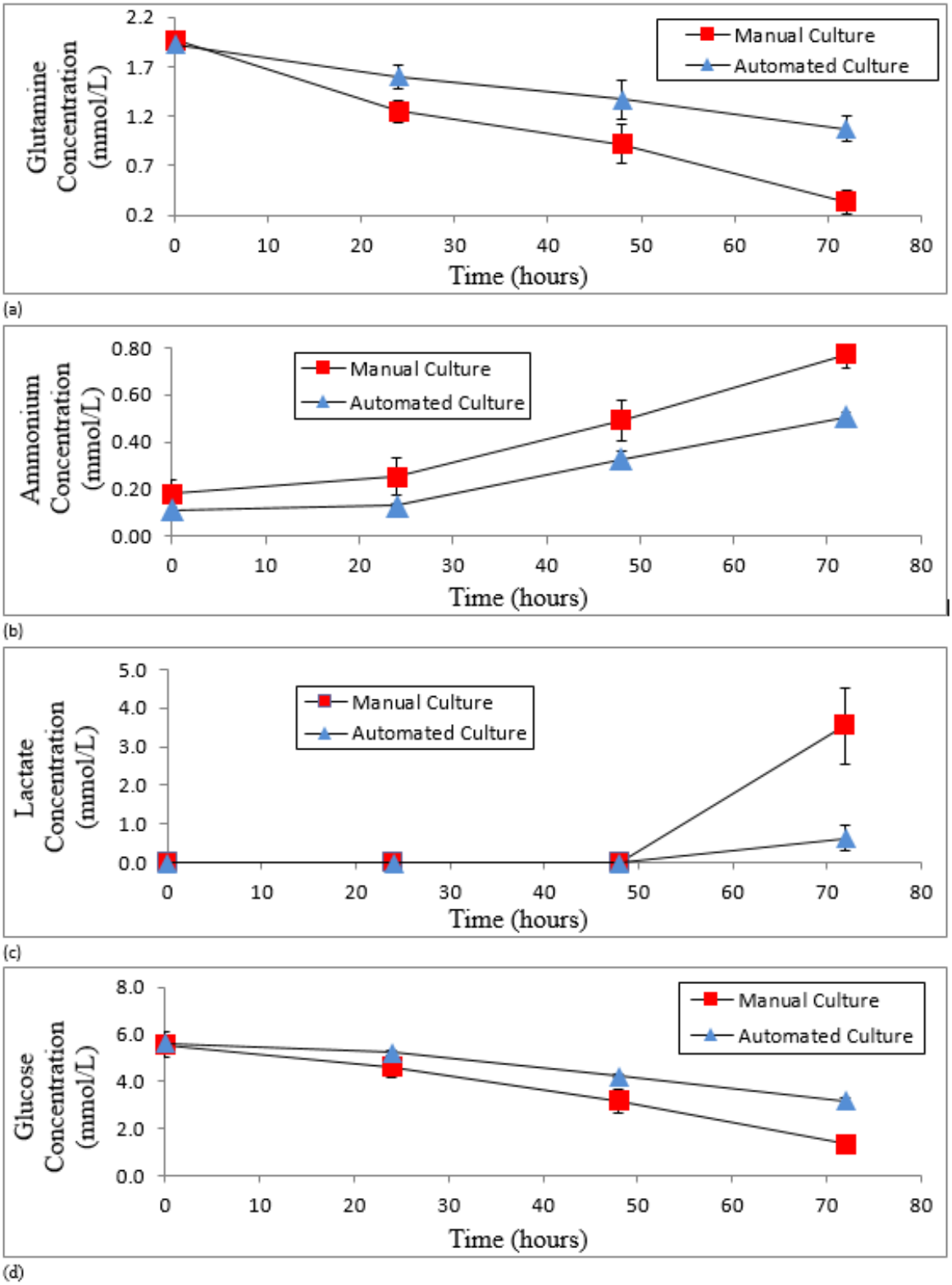


Figure 4.3 *Average glutamine (a), ammonium (b), lactate (c), and glucose (d) concentrations in spent media obtained from manual and automated cell culture experiments over 72 hours. Error bars represent standard deviation of data (n=9).*

The levels of ammonium slowly accumulated over 72 hours for both processes. Figure 4.3 (b) shows that the ammonium concentration in manual process flasks increased from 0.18 to 0.77 mmol/L and increased from 0.12 to 0.51 mmol/L in automated process flasks. It can also be noticed that the initial starting concentrations of ammonium for both culture processes were not similar. This may have been contributed by insufficient mixing before the samples were measured or could be due to increased spontaneous glutamine degradation in a particular batch/lot of growth medium as this can result in increased ammonium production. This is a common occurrence and can happen due to temperature or pH variation, and sometimes even when glutamine is stored properly.

Lactate was excreted only towards the end of the experiment for both the manual and automated cell culture processes (Figure 4.3 c). Lactate concentration values were 3.53 mmol/L for the manual process and 0.62 mmol/L for the automated process. It was reported that, lactate is usually toxic to cell growth only at a lactate concentration above 28 mmol/L in the growth medium (Ozturk and Palsson, 1991). However, Ozturk and Palsson (1990) reported that the inhibition of cell growth by ammonia plays a much more important role as even concentrations of 2 to 10 mmol/L can inhibit cell growth by up to 50 %. This is clearly not the case here, as the concentrations were considerably lower than the inhibitory concentrations.

In the automated cell culture flasks, the glucose and glutamine concentration remained higher than that observed in the manual process cell culture flasks, and as a result, the concentration of lactate and ammonium produced remained much lower. It would be

expected that any increase in productivity would significantly increase the demands on the cell for increased energy metabolism. In order to confirm this assertion, cell specific (per cell basis) glucose and glutamine consumption and lactate and ammonium production rates (pmol/cell/hour) were calculated (Figures 4.4a to 4.4d).

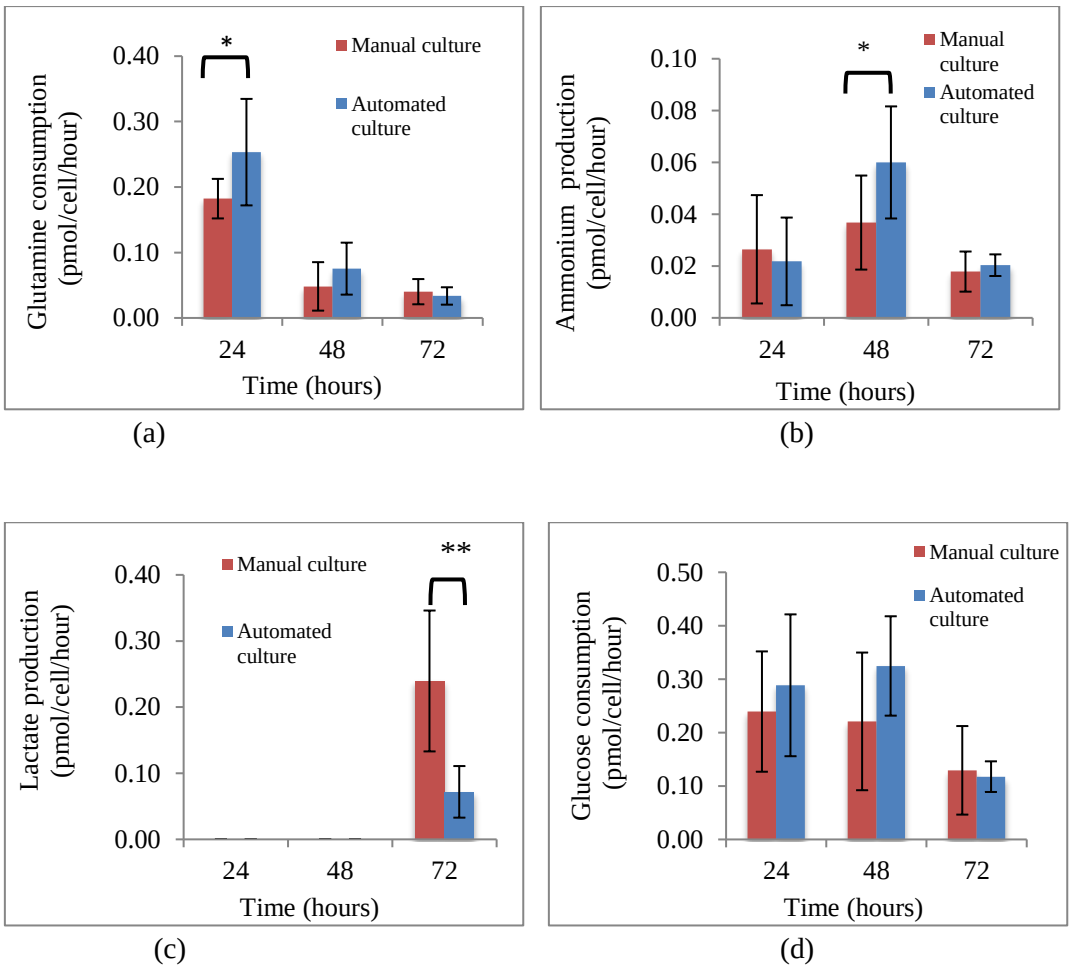


Figure 4.4 *Average glutamine (a) and ammonium (b), lactate (c), and glucose (d) consumption or production rates (pmol/cell/hour) in spent media obtained from manual and automated cell culture experiments over 72 hours. Error bars represent standard deviation of data (n=9). Asterisks (*) indicate significant difference, $p < 0.05$ and (**) indicate $p < 0.001$*

Figure 4.4 (a) shows that the cell specific glutamine consumption rates (pmol/cell/hour) were significantly different between the manual and the automated process only during the intial 24 hours. The consumption rates were also the highest on this day for both proceses. It has been reported that during stress, cells consume more glutamine (Yuneva, *et al.*, 2007) and it is believed that this was due to the cell detachment process prior to re-seeding. It has been shown that the detachment process can cause significant stress to cells (Neumann *et al.*, 2010) since this can cause significant damage to the cells and contribute to alteration of gene expression (Chaudhry, 2010). The glutamine consumption rate for day 1 was even higher for the cells in the automated process flasks. Since these cells were manually cultured prior to transfer, it was likely that the sudden change in passaging methods (differences between the manual and the automated processes are detailed in chapter 3) cells may have caused additional stress to the cells. Figure 4.4 (a) shows that were no differences in glutamine consumption rates on subsequent days.

In terms of glucose consumption, there were no significant differences ($p > 0.05$) in consumption rates at any time points between the two processes (Figure 4.4 d). It could be seen that glucose consumption rate was the lowest on the last day of culture and this is to be expected since metabolic demands are usually lower when cells reach the end of the exponential phase (Doran, 1995). In terms of ammonium production rates, it was observed that there were only significant differences ($p < 0.05$) on day 2 for both processes (Figure 4.4b). It was possible that more ammonium was produced either due to excess intake of glutamine or spontaneous degradation of glutamine. It is now widely accepted that even the slightest change in storage or experimental conditions can result in ammonium

production in culture solution (Jagusic *et al.,* 2016).

As discussed earlier, lactate was only produced during the last day of the experiment (Figure 4.4c). Lactate production values of 0.24 and 0.07 pmol/cell/hour obtained for the manual and the automated cultures, respectively were significantly different ($p < 0.001$). Although HOS cells in the manual process flask produced greater lactate, the actual lactate concentration in the flasks (discussed earlier) were far below inhibitory concentration of 28 mmol/L in the growth medium (Ozturk and Palsson, 1991).The calculation of glucose and glutamine consumption rates per cell, (apart from glutamine consumption data for day 1) confirmed the assertion that greater consumption of metabolites in the manual process flasks are due to greater cell numbers in the flasks.

The apparent yield coefficient values for ammonium produced from glutamine values of 0.37 mmol/mmol for the manual culture and 0.47 mmol/mmol for the automated culture were within the range of the values between 0.17 to 1.5 mmol/mmol quoted in the literature for a wide range of mammalian cells (Miller *et al*., 1988; Ozturk and Palsson 1991; Harigae *et al.,* 1994; Acosta *et al.,* 2007; Siegwart *et al*., 2008).

The yield coefficients for lactate produced from glucose of 0.85 mmol/mmol for the manual culture and 0.27 mmol/mmol for the automated culture illustrate that more lactate was produced in the manual cell culture flasks per mole of glucose consumed. The average yield coefficients of lactate from glucose obtained for the manual cell culture process were within the range of 0.47 to 2.00 mmol/mmol quoted in literature for most mammalian cells

(Aslankaraoglu, *et al.,* 2003; Helmlinger *et al.,* 2002; Winkenwerder, *et al.,* 2003), but these literature values are not specific to HOS cells as they are not available.

(e) ALP analysis

In order to further assess the cell quality and the effect of bioprocessing on the cells, an ALP assay was done on the cells harvested from both the automated and manual cell culture flasks according to the procedure described in Section 33.5, Chapter 3. HOS cells are cancer cells, and at present there are no specific analytical tests for this type of cell to confirm phenotype (Bielack *et al*., 2012). Because the skeletal ALP isoenzyme is a product of osteoblasts, correlations have been shown between skeletal ALP activity and the rate of bone formation, both *in vitro* and *in vivo*. The measurement of skeletal ALP activity in serum has been suggested as a useful index of the rate of bone formation and also a convenient biomarker for prognosis of osteosarcoma (Ren *et al*., 2015; Farley and Baylink, 1986). Higher ALP levels are correlated with healthier HOS cells (Ren *et al*., 2015)

Enzyme activity calculated from the angular coefficient of the linear slope obtained from the standard solution (Abcam, UK), is expressed as 4-methyumbelliferon generated per volume of sample (mU/mL) (Yoo *et al.,* 2012). An average value of 1.29 ± 0.33 mU/mL was obtained for cells from the manual process, and a value of 1.33 ± 0.29 mU/mL was obtained for cells from the automated process. There was no statistical difference in the average ALP values obtained for both processes ($p > 0.05$).

4.2.2 Human dermal fibroblasts (HDF) cells

Experimental work using the HOS cells addressed the first key question that was outlined at the start of this chapter. Using HOS cells, it was demonstrated that there was a significant difference between the manual and the automated process, in terms of process yield (cell numbers). In terms of quality (cell viability), there was no significant difference between the products of the two processes. Since yield is a very important parameter and will significantly impact the success of clinical therapy (discussed in Chapter 2), it is important to understand if this difference is observed when a different cell line is use. For this reason the experiment performed in Section 4.2.1 was repeated using HDF cell line.

Similar to the work carried out with HOS cells, all the experiments described in this chapter with HDF cells were performed in triplicates, and were repeated twice (n = 9). Third passage HDF cells recovered from cryopreservation were expanded in culture according to procedures described in Section 3.2, Chapter 3. 8.75×10^5 cells were seeded into each T175 flask (total of 12 flasks were used) and were cultivated (procedure detailed in the Chapter 3). Flasks were sacrificed and the spent medium samples were analysed every 24 hours starting from time point 0 to 96 hours.

Since HDF cells are not cancer cell lines, they cannot escape contact inhibition. Contact inhibition is a type of cell arrest that occurs when cells reach a high cell density. Generally, cells stop proliferating until the culture is split and re-plated (Leontieva *et al*., 2014). If the cells are seeded at a low seeding density, it might take longer for them to reach the desired

cell expansion factor. In order for the cell growth to be optimal, the selected seeding density must be neither too low nor too high.

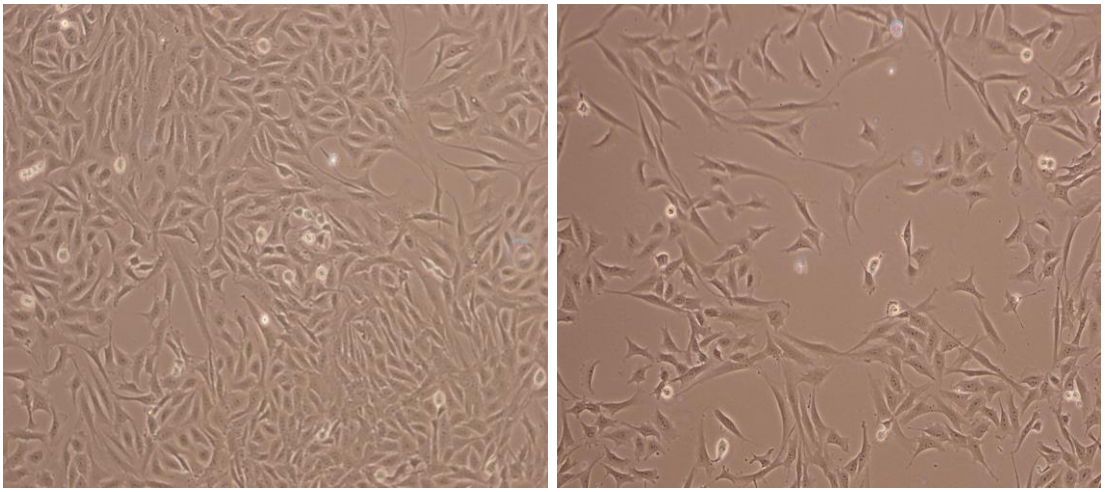
Before proceeding with the work to compare both the processes, a preliminary study was carried out to determine a suitable cell seeding density. Then, similar to the work conducted earlier, in order to compare the two processes (manual and automated), a number of parameters were analysed. These parameters included (a) cell morphology, (b) growth curve, (c) doubling time and growth rate, (d) cell metabolites and (e) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay. For HDF cells, cell quality (proliferation potential) was measured by MTT assay.

The preliminary experiments carried out to determine the suitable seeding density demonstrated that after 3 days of culture, the HDF cells seeded at 1×10^4, 5×10^3, and 2.5×10^3 cells/cm^2 had an average population doubling time of 41.7 ± 2.3, 38.4 ± 1.3, and 38.5 ± 1.5 hours, showing that cells at a lower density have a higher doubling rate than that at a higher density. Although more population doublings occurred at lower seeding densities, there was no significant difference between using 5×10^3 and 2.5×10^3 cells/cm^2 ($p > 0.05$), therefore, a seeding density of 5×10^3 cells/cm^2 was chosen. This density allowed more cells to be obtained without a significant reduction in the doubling rate. This seeding density is also the recommended seeding density in the literature (Herbert *et al*., 1997; Lama *et al*., 2012).

(a) Cell morphology

All experiments performed with HDF cells resulted in a successful culture, with no observable problems with attachment or proliferation, but it can be seen that there were more adherent cells in the manual process flasks (Figure 4.5). Both flasks contained spindle-shaped cells, although there were fewer cells in the automated process flasks. The spindle-shaped morphology was more apparent in the automated process flasks as they were less tightly packed (lesser cells). The spindle-shaped morphology demonstrated by these cells was similar to the morphology reported in literature (Hematti, 2012). These cells appear similar to the MSCs and are also plastic adherent like the MSCs (Chapter 6). Other authors have reported similar findings and this is not surprising as fibroblasts are one of the products of MSC differentiation (Hematti, 2012).

When the experiments were performed with HOS cells, even with high degree of flask confluence, cell viability was observed to be close to 100% (for both the manual and the automated process). In contrast, for the experiments performed with HDF cells, during the last sampling point (96^{th} hour), cell viability decreased to a range of 80-85% in the manual process flasks and to a range of 85-93% in the automated process flasks. In the manual process flasks, since the confluence level was higher, it was likely that more cells started losing their viability due to cell arrest associated with contact inhibition. HDF cells are not cancer cell line so they cannot escape contact inhibition.



(a) **(b)**

Figure 4.5*: Representative phase-contrast microscope images of the HDF cells obtained from (a) manual and (b) from the automated cell culture process after 96 hours of culture (n=9). Magnification x40 was used.*

Although the cell viability was higher in the automated process flasks than in the manual equivalent during the last sampling point, there was still some decrease in cell viability. It was believed that this was due to cells having less cell-to-cell contact in the automated process flasks. Cell-to-cell contact is required for cells to maintain their viability (Wei *et al*., 2011) and it can be seen in Figure 4.5 that there were lesser cell-to-cell contacts in the automated process flasks. While having too much of contact can result in cell death, having insufficient cell-to-cell contact can also result in cell death (Gerard and Goldbeter, 2014).

(b) Growth Curve

Figure 4.6 shows the growth profiles of cells cultured for 96 hours. Throughout the culture process (until 96 hours), there was a significant difference in average cell numbers

obtained from manual and automated process ($p < 0.001$). This figure shows that number of cells per flask increased from 8.75×10^5 to 3.20×10^6 for the manual process, but only up to 2.50×10^6 for the automated process for the first 72 hours. It can be seen that after 72 hours, the average number of viable cells decreased for both processes. Figure 4.6 shows that at the 96th hour, the number of cells per flask decreased to 2.84×10^6 for the manual process and from 2.34×10^6 cells per flask for the automated process. While the reduction is significant for the cells cultured manually ($p < 0.05$), this was not the case for the cells cultured *via* the automated process ($p > 0.05$).

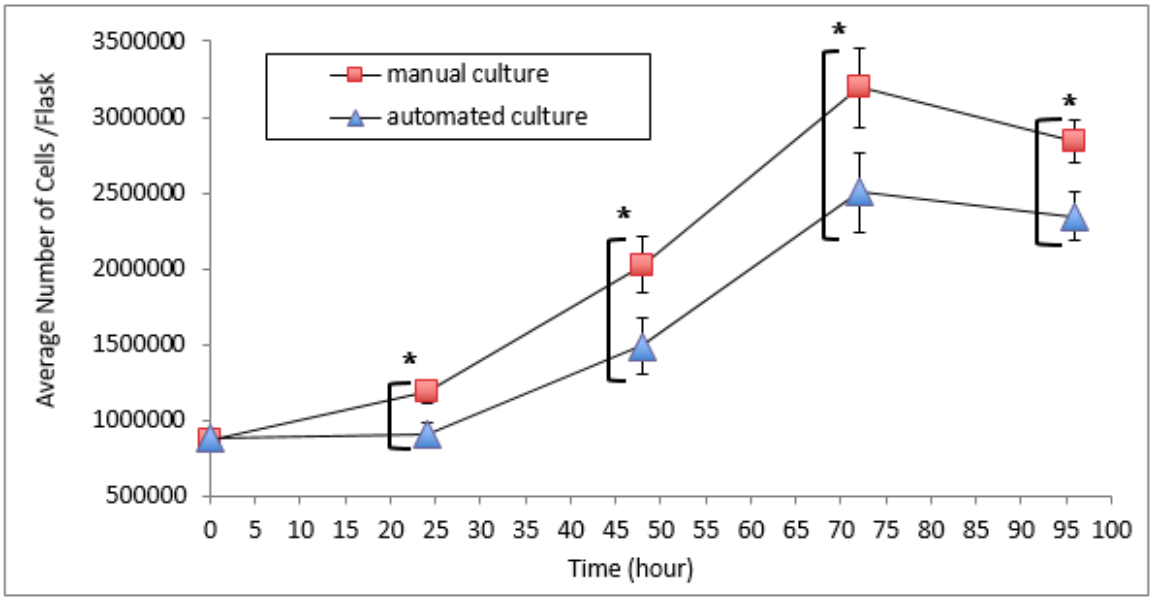


Figure 4.6 *Average number of viable HDF cells obtained for manual and automated cell culture experiments after HDF cells were cultured for 96 hours. Each T175 flask was seeded with 8.75 x10^5 cells. Error bars represent standard deviation of data (n=9). Asterisks (*) indicate significant difference, $p < 0.001$*

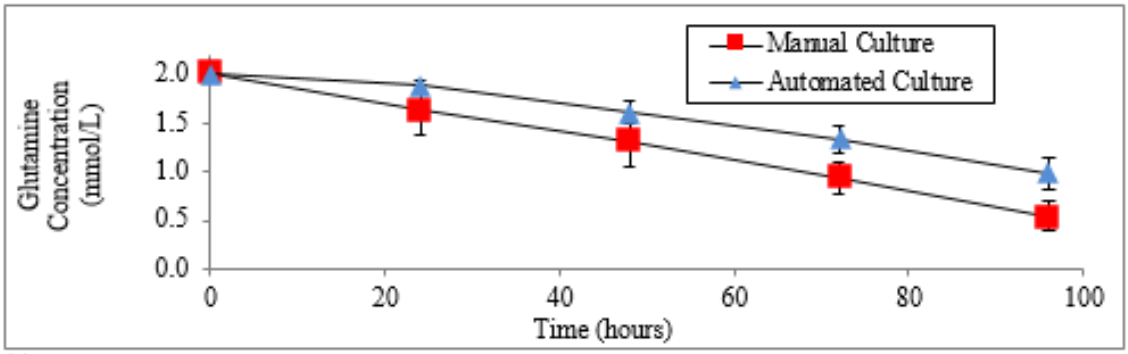
Initially the experiment was only conducted for 72 hours, but since no stationary or death phase was observed, the experiment was continued for 24 more hours. Only then, a decline in average viable cell numbers was observed. The experiment performed in the Section 4.2.1 was repeated using HDF cells mainly to confirm that the automated process yield is lower than the manual process yield even when a different cell line was used.

(c) Growth rate and doubling time

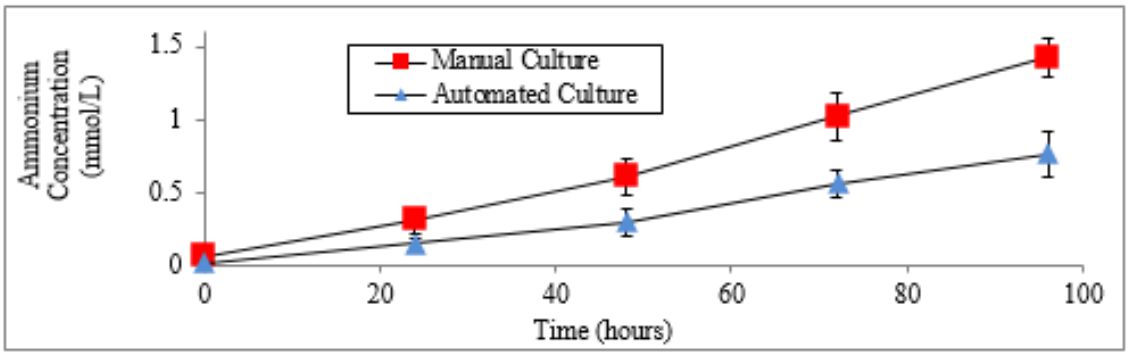
The average doubling time of 37.7 hours (with a maximum growth rate, μ_{max} value of $0.0184hr^{-1}$) obtained for the manual cell culture process was close to the average doubling time of 34 hours quoted in the literature (Ji *et al*., 2012), but the average doubling time of 45.6 hours (μ_{max} value of $0.0152hr^{-1}$) obtained for automated cell culture was considerably higher. This is an indication that cells may have been taking a longer time to double when they were cultured *via* the automated method.

(d) Metabolite analysis

Figures 4.7 (a) to (d) show the depletion of glutamine and glucose and the accumulation of lactate and ammonium in cultures over 96 hours for both the manual and automated HDF cell culture flasks. Figure 4.7(a) shows that the glutamine concentration in the manual process flasks decreased from 2.02 to 0.54 mmol/L, and decreased from 2.01 to 0.98 mmol/L in the automated process flasks. Figure 4.7(d) shows that glucose concentration in the manual process flasks decreased from 5.57 to 1.00 mmol/L, and decreased from 5.71 to 2.52 mmol/L in the automated process flasks.



(a)



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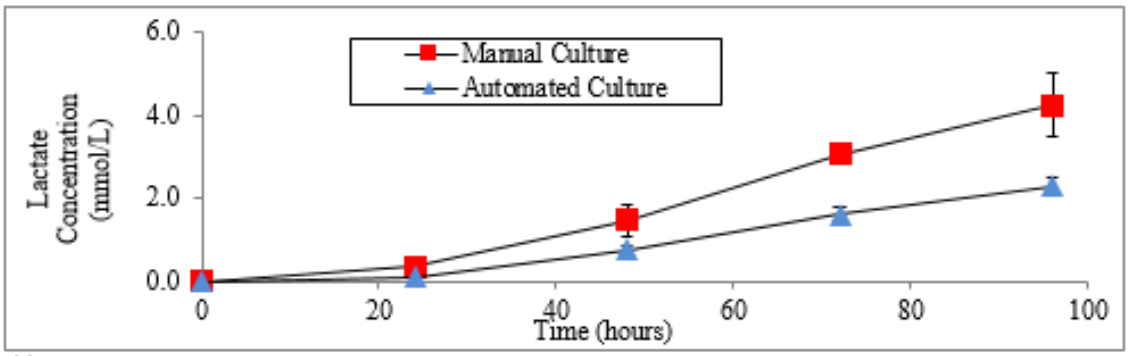
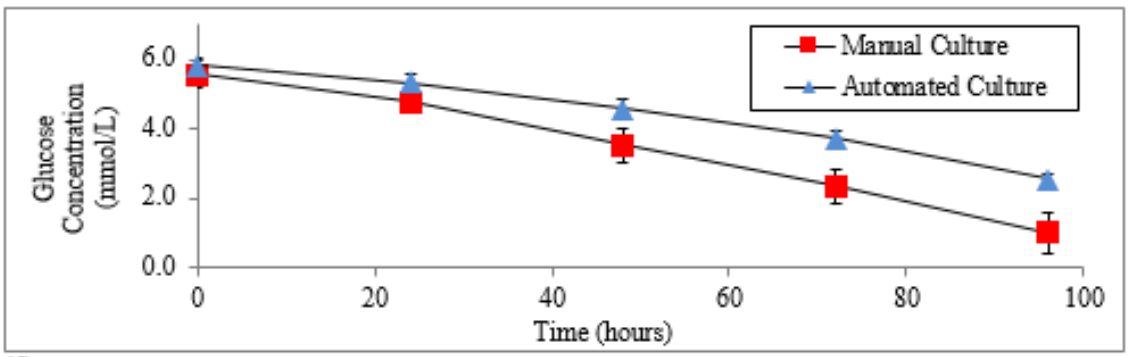


(c)



(d)

Figure 4.7 *Average glutamine (a), ammonium (b), lactate (c), and glucose (d) concentrations in spent media obtained from manual and automated cell culture experiments over 96 hours. Error bars represent standard deviation of data (n=9).*

The levels of ammonium slowly accumulated over 96 hours for both processes. Figure 4.7 (b) shows that the ammonium concentration in the manual process flasks increased from 0.07 to 1.43 mmol/L, and increased from 0.02 to 0.77 mmol/L in the automated process flasks. Similar trend was observed for lactate. The level of lactate in the flasks slowly accumulated over 96 hours for both experiments (Figure 4.7c). The final lactate concentration was 4.20 mmol/L for the manual process, and 2.60 mmol/L for the automated process. As discussed previously in the Section 4.2.1, lactate is usually toxic to cell growth only at a concentration above 28 mmol/L in the growth medium, but the inhibition of cell growth by ammonia is more significant as even concentrations of 2 to 10 mmol/L can inhibit cell growth by up to 50 % (Ozturk *et al.*, 1991). This was clearly not the case here, as the concentrations were considerably lower than the inhibitory concentrations.

The trend graphs above showed that the glucose and glutamine concentration remained higher than that observed in the manual process cell culture flasks, and as a result, the concentration of lactate and ammonium produced remained much lower. Usually, the increase in productivity increases the demands on the cell for increased energy metabolism. In order to evaluate if this was true, cell specific glucose and glutamine consumption and lactate and ammonium production rates (pmol/cell/hour) were calculated (Figures 4.8a to 4.8d).

The glutamine consumption rates per cell were the highest on the first day of the culture and this pattern was also observed for the HOS cells (Figure 4.8a). This strengthens the

earlier argument that the cell detachment process may have caused greater stress levels, resulting in the cell consuming more glutamine when they were initially seeded. It can be seen that cell specific glucose and glutamine consumption rates were higher for the automated cultures compared to the manual equivalent during the last day of culture ($p < 0.001$).

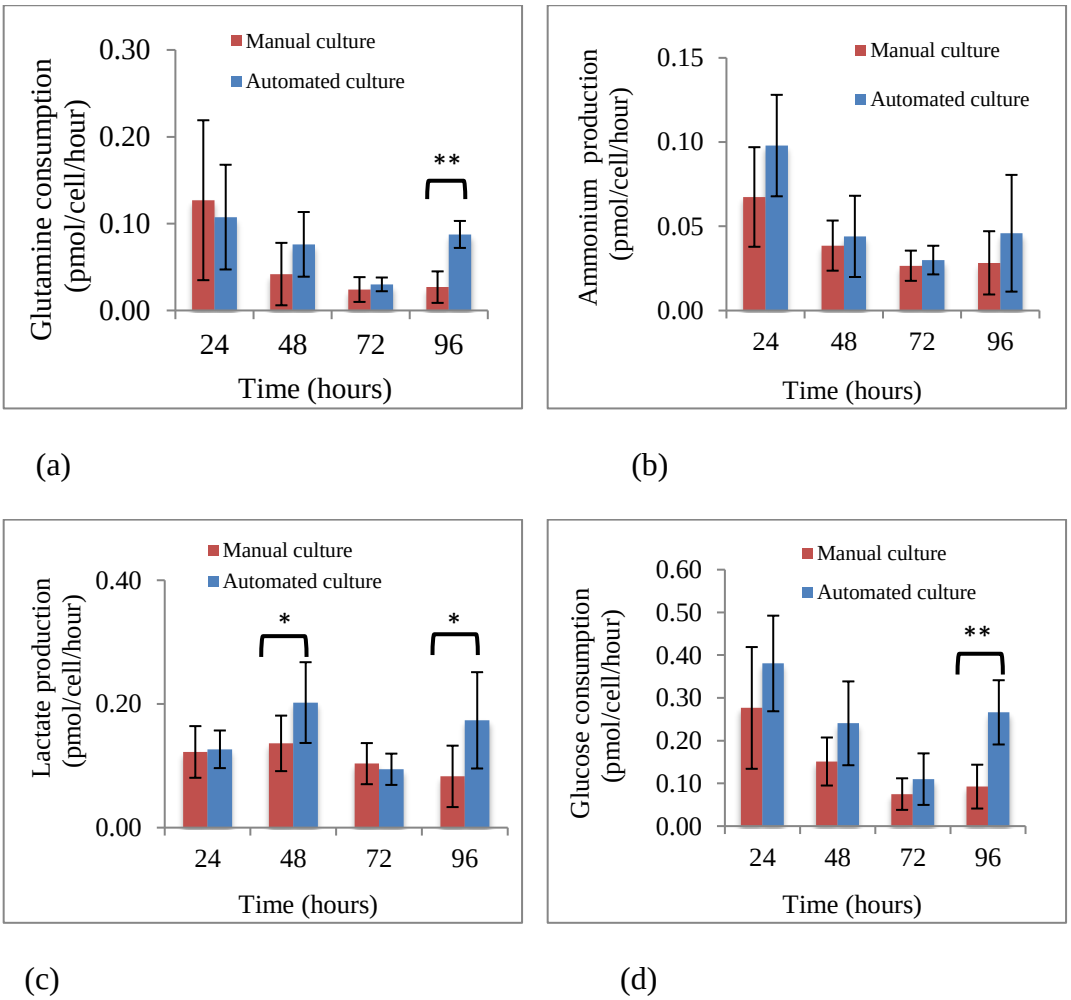


Figure 4.8 *Average glutamine (a) and ammonium (b), lactate (c), and glucose (d) consumption or production rates in spent media obtained from manual and automated cell culture experiments over 96 hours. Error bars represent standard deviation of data (n=9). Asterisks (*) indicate significant difference, $p < 0.05$ and (**) indicate $p < 0.001$*

Generally, towards the end of the culture, cell metabolic demands are lower (Doran, 1995), so glucose consumption rate per cell decreases, however sustained exposure to higher levels of glucose (per cell) can contribute to glucose-induced glucose uptake (Busik et al., 2002). It was likely this scenario contributed to the increase in glucose uptake towards the end of the experiment for the automated culture. It was proven later (discussed in Chapter 4) that lesser cells than programmed were seeded than in the automated process flasks due to a "pipetting error". Therefore, each cell in the automated process flasks was exposed to a higher concentration of metabolites than the cells in the manual process flasks.

Figure 4.8 (c) shows that the there were significant differences in cell lactate production rates between the automated and the maual process during Day 2 and Day 4 of the experiment ($p < 0.05$). Although the total lactate concentrations in both process flasks were below inhibitory concentrations (discussed earlier in this chapter), higher cell specific consumption of lactate in the automated process flasks further indicates that these cells may have consumed higher levels of glucose than necessary due to prolonged exposure to increased concentrations of glutamine. For over three decades, industries have controlled glucose levels in the cell culture media to limit the supply of glucose to the in order to reduce lactic acid production (Freund and Croughan, 2018).

In terms of production rates, it can also be observed that the cell specific ammonium production rates were also the highest on day 1, however the total concentrations in the flask (discussed earlier) were below inhibitory concentrations of 2mmol/L (Figure 4.8b). There were also no differences in ammonium production rates between the two processes ($p > 0.05$). The calculation of glucose and glutamine consumption rates per cell, (apart

from the consumption data for day 4) confirmed the assertion that greater consumption of metabolites in the manual process flasks are due to greater cell numbers in the flasks.

The average yield coefficient for ammonium produced from glutamine of 0.93 mmol/mmol for the manual culture, and 0.72 mmol/mmol for the automated culture were within the range of values of 0.46 to 1.5 mmol/mmol quoted in the literature for a wide range of mammalian cell types (Ozturk and Palsson 1990; Harigae *et al*., 1994; Miller *et al*., 1998; Acosta *et al.,* 2007). The yield coefficient for lactate produced from glucose was 0.94 mmol/mmol for the manual culture, and 0.51 mmol/mmol for the automated culture are within the range of 0.47 to 2 mmol/mmol quoted in literature for many mammalian cells (Aslankaraolu, *et al.,* 1990; Helmlinger *et al.,* 2002; Winkenwerder, *et al.,* 2003), but these values are not specific to this cell line as values specific to HDF cells are not available.

(e) MTT assay

In order to further assess the cell quality and the effect of bioprocessing on the cells, an (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay was done on the cells harvested from both the automated and manual cell culture flasks according to the procedure described in Section 3.3.5. In order to assess the cell proliferation potential based on the mitochondrial activity, MTT assay was conducted using the MTT Cell Growth Kit (Chemicon, USA). The MTT assay technology is widely used as evidenced by thousands of published articles (Riss *et al*., 2013). Viable cells with active metabolism can

convert MTT into a purple colored formazan product so dead cells do not have the ability to convert MTT into formazan (Riss *et al*., 2013).

Average MTT absorbance values of 0.32 ± 0.05 and 0.28 ± 0.08 were obtained for cells from the manual process and automated process, respectively There was no significant difference in the average MTT absorption values obtained from both processes ($p > 0.05$). This demonstrates that the quality of the cells was not affected when the cells were cultured using the automated platform.

4.3 Overall Assessment

As discussed earlier in this chapter, the maximum cell yields obtained for the manual processes were significantly higher ($p < 0.001$) than the maximum cell number obtained for the automated process for both cell lines. The HOS and the HDF cell growth rates and doubling times obtained for the cells cultured manually, compared well with the literature (Ji *et al*., 2012; Kuettner *et al*., 1978; Clover and Gowan, 1994), In contrast, for both the HOS and the HDF cells, when they were cultured via the automated method, the doubling values and the growth rates were not within the range of the values quoted in the literature, despite using the same seeding density (Ji *et al*., 2012; Kuettner *et al*., 1978; Clover and Gowan, 1994).

Cells cultured via both processes were able to demonstrate their ability to attach to the cell culture flasks and also viability close to 100 % (except during the death phase for HDF cells). The ALP and MTT assays carried out for HOS and HDF cells, respectively,

demonstrate that the cells harvested from the automated process flasks were of similar quality with the cells harvested from the manual process flasks. This indicates that quality was not the contributing factor for reduced cell yield. Metabolite analysis for both the cell lines has demonstrated that the manual and the automated cultures display a similar pattern of glutamine and glucose utilisation throughout the culture. Therefore, metabolite depletion was not the reason for reduced cell growth in the automated process flasks. If metabolite accumulation was the reason for reduced cell growth, the manual cell culture yield (average viable cells/flask) should have been lower than the automated cell culture yield since the ammonia concentration in the manual cell culture flasks were higher than the concentration recorded for the automated cell culture process. If nutrient depletion (glucose and glutamine) was the reason for reduced cell growth, there should have been fewer cells in the manual process flasks, but this was clearly not the case. Therefore, it can be concluded that nutrient depletion and accumulation were not the reasons why there were fewer cells in the automated process flasks. This study has shown that the metabolites consumption rates were generally similar for both processes, but it was not understood why the growth rates were significantly higher for the cells cultured manually.

Many other authors have also reported that the cell culture yield obtained for the automated cell culture process was considerably lower than the cell yield obtained for similar manual cell culture processes (Thomas *et al*., 2007; Liu *et al*., 2010; Zhao *et al.*, 2011). The authors suspected that the difference in growth profile and rates were due to methodological differences (cell passaging protocols employed) between the two

processes. Comprehesive studies were required to identify why there were differences between the manual and the automated process (previously discussed in the Chapter 1).

It is important to know that in order to meet the cell requirements for cell therapy, cells need to be produced in large quantities; it can be anything between 1×10^8 to 1×10^{12} cells for a minimum of 10 patients (Nekanti *et al*., 2010). At present, going by the HOS doubling times obtained in this initial study, and also by using the same seeding density used for the experiments discussed here, it would take at least 4 days to obtain 1×10^8 cells if the cells were manually cultured, and 6 days if they were cultured using the automated process. If the automated process performance does not improve, a minimum of extra two days will be required to meet the same expansion factor, and this will contribute to increased process costs. In addition, as described in Chapter 1, the success of autologous therapies greatly depend on the capability of the manufacturing systems to produce enough material (cells) to meet the cell demands.

A process that is capable of producing good quality cells in high yields is deemed as an efficient process and efficient processes are needed for the commercial success of a product. Therefore, a better process output is necessary, and methods to achieve this should be investigated. As described in Chapter 1, one way of improving process performance is by utilising a statistical process control tool; process capability analysis. Process capability analysis is not a new technique, but it is new in the area of cell culture, as only 1 cell culture automation study has so far employed this technique to improve the process performance (Liu *et al.,* 2010). This analysis reveals how well the manufacturing

process meets the specifications and provides insights into how to improve the process and sustain the improvements and it allows users to summarise process capability in terms of meaningful percentages and metrics (Khan, 2013). Assessing process capability before and after making process changes can be valuable as it verifies that improvements have been made (Khan, 2013).

It is clear that improvements have to be made to the automated process, therefore using process capability analysis will allow verification (based on numerical values) that a process is capable and stable. The study carried out in the next chapter will assess the performance of the system (process capability) and will focus on making improvements wherever possible. In order to make improvements, studies will be carried out using HDF cells as the model cell line. Justification for selecting HDF cells for the subsequent studies will be provided in the Chapter 5.

4.4 Conclusion

The study carried out in this chapter was able to address one of the key technical questions raised earlier in this chapter and ultimately, was able to meet the objective of the study. The study demonstrated that there was a significant difference in yield between the automated and the manual process, and also that the difference was observed when the work was repeated with another cell line. While the results from this chapter were able to confirm that the cell output of the automated process was significantly different from the manual process, irrespective of the cell line used, the results were not able to establish the factors that were contributing to this difference.

At present (based on Chapter 4 findings), it was still unknown if the difference was just due to the biological variation or if it was just due to the the cell expansion process used. However, since using both HDF and HOS cells resulted in similar findings, it was unlikely that the results were due to the biological variation of cells alone. The factors that can contribute to a lower process yield are biological variations (unique cell behaviour), differences in raw materials and consumables, operational inputs (measurements, methods, personnel, and equipment) and environmental conditions (temperature, humidity and pH). In order to have a fair comparison, the cell source and the cell passage numbers were the same for both processes. Raw materials (cell culture media, detachment enzyme and PBS solution) were purchased in large batch sizes and they were kept the same for both processes. Similar types of flasks were used for both processes, but the pipettes were not similar as Compact Select is only compatible with 10 mL pipettes instead of 1000 uL tips for cell mixing and for manual 1000 uL tips were used. Environmental conditions were controlled since cells were kept in incubators and utilised the same cell culture medium, therefore were rather consistent. In terms of operational inputs, these processes were carried out by one operator and the cell counts were performed using one counter. However, when the cells were cultured manually, it was impossible to ensure that all the steps were identical as these greatly depended on the skill of the operator and the endurance for repetitive work.

Hence, the objective of the next chapter is to study some of the key factors contributing to these differences and to identify the factors that have large impact on cell growth. This study will be carried out with the aid of process capability analysis tool; a tool for

statistical process control (SPC). Process capability analysis will also investigate how capable the process is and will help determine the suitability of automation for the expansion of MSCs. Without ensuring that the process is stable and also that it is capable of performing as it is designed to, it will not be possible to develop a robust automated protocol.

The study described in this chapter provided fresh impetus to conduct further studies to evaluate the potential of automation for the culture of MSCs. Cell therapies carry higher manufacturing risks than most small molecule pharmaceuticals. The success of an autologous therapy greatly depends on the capability of the manufacturing process. An error in the production of an autologous cell therapy directly translates into a failed treatment for a patient. The finished product must meet the quality and the quantity (cell yield) specification, as they are the fundamental manufacturing requirements (Hampson *et al.*, 2008).

Based on the preliminary studies carried out this in chapter, it is clear that the automated cell expansion system has only been able to meet one important requirement; that is the quality requirement. Therefore, one of the main focus of the next chapter will be in improving the automated process yield. This will be one of the determining factors of the suitability of the automation platform for MSC expansion. As identified in Chapter 2, for cell therapies, and most importantly for autologous cell therapies, since the starting material is scarce, the manufacturing system must be capable of producing enough cells to meet the minimum cell demands.

Chapter 5. Results and Discussion: Automated Process Development Work

5.1 Introduction

The study discussed in the previous chapter demonstrated that there was a significant difference in yield between the automated and the manual process. This study also demonstrated that the difference was observed when the work was repeated with another cell line (with the HDF cell line). This finding (lower yield for automated process) is consistent with the findings of others authors who have used the same automated platform for their studies (Thomas *et al*., 2007; Liu *et al*., 2010; Zhao *et al*., 2011). Additionally, Thomas *et al*. (2009) have even reported cell quality variation during the automated cell culture, but our findings did not demonstrate any significant quality issues.

The work discussed in the previous chapter confirmed that the output of the automated process was significantly lower than that of the manual process (irrespective of the cell line used), but the factors that were contributing to this difference were not established. The authors who reported similar findings speculated that the differences may be due to methodological differences between the 2 processes. So far, no comprehensive study has been carried out to characterise the automated cell culture process. The focus of most automation studies so far has been mainly on demonstrating the applicability of automated platform for the culture of cells. This clearly highlights the need for a comprehensive study of the automated cell culture process. Without comprehensive knowledge of the automated process, it is unlikely that a robust mesenchymal stem cell (MSC) culture process can be

developed. Therefore, the objectives of the study discussed in this chapter are: (i) to study some of the factors contributing to the growth difference and to identify the factors that have the largest impact on cell growth and (ii) to use the knowledge gained from (i) towards developing a robust automated MSC culture protocol.

In order to achieve the objectives listed out, few key questions that will be addressed in this chapter are:

- Are the automated platform's analytical devices (pipetting and cell counter) used capable of producing the same results consistently when process parameters remain the same?
- How capable and stable are both processes (manual and the automated)? Can these processes produce consistent outputs?

Most manufacturing processes start off being manual as they are developed in a laboratory-based environment, but these processes are seldom scale-up friendly and require significant process development and optimisation efforts to get them ready for large scale production. These should be done at early stages as once the process is locked down, a lot of work and expenses will be incurred if changes were required (Kamani *et al.*, 2014).

For the study discussed in this chapter, MSCs were not used for the initial stages of process characterisation work, as these studies require a large number of MSCs. This was not possible in terms of stem cell availability and operational costs. MSCs have a limited life span *in vitro* before they senesce so they can only be used for few passages before they are

replaced with a new batch of cells in order to meet the quantities required for the experiment. This is not ideal as cells from new batch or source can also contribute to biological variations.

Human osteosarcoma cell line was a relevant model for the study carried out in Chapter 4 because these cells were easy to culture and they exhibited less biological variation in culture, therefore, it was easier to evaluate the influence of the process on the yield obtained. While HOS cells were suitable for the study carried out in Chapter 4, HDF cells were chosen instead for the subsequent work, as they are a believed to be a better representative of MSCs. Human dermal fibroblast cells are cells that exhibit normal cell characteristics such as contact inhibition and most importantly, these cells are a normal (non-cancerous) product of MSC differentiation (Lee *et al.*, 2010).

One of the major advantages of using HDF cells is that they have many molecular markers (CD105, CD73 and CD90) and morpholology (spindle shaped) that are similar to MSCs (Ishii *et al*., 2005) (Table 5.1). The absence of certain negative markers (CD14, CD19, CD34, CD45 and HLA-DR) for both cell types indicate they are rather similar in terms of marker expression. Therefore, it is possible that the majority of the work conducted with these cells to characterise the automated cell culture process can be applied for the automation of the MSC culture process. However, in order to reduce the risks of developing an irrelevant automated process (without real applicability for the MSC culture), some of the protocols developed with HDF cells were tested with MSCs (when they became available). While there are many similarities between these cell lines, there

are also some distinct differences between MSCs and HDF cells. Since MSCs are often referred to as multipotent cells they are capable of differentiating into mesodermal cell types, but since HDF cells are terminally differentiated cells they do not differentiate into these cell types (Alt *et al.,* 2011).

Table 5.1 *Biological characteristics of MSCs and HDF cells*

Biological Characteristics	HDF cells	MSCs	References
Morphology	Spindle-shaped Morphology	Spindle-shaped Morphology	Ishii *et al*., 2005
Surface marker expression	Presence of positive surface marker expression (CD105, CD73 and CD90)	Presence of positive surface marker expression (CD105, CD73 and CD90)	Alt *et al.,* 2011
	Absence of negative surface marker expression (CD14, CD19, CD34, CD45 and HLA-DR)	Absence of negative surface marker expression (CD14, CD19, CD34, CD45 and HLA-DR)	Ishii *et al*., 2005
Differentiation potential	Mesodermal cell types	None, since these are terminally differentiated cells	Kundrotas *et al.,* 2012

This chapter is divided into 7 sections. Section 5.2 discusses the results carried out to evaluate the performance of this measurement device and additionally, few other key components of the automated performance. Section 5.3 discusses the work carried out to evaluate the key methodological differences between the 2 processes. Section 5.4 discusses the work carried out to develop the automated MSC culture protocol. This section also

discusses the capability of the developed process. Section 5.5 discusses the applicability of xeno- and serum-free cell culture media for the culture of UC-MSCs. An overall assessment is provided in the Section 5.6 and finally a summary is provided in the Section 5.7.

5.2 Evaluating the Performance of Key Components

The results discussed in the preceding chapter (Chapter 4) demonstrated that the automated process yield was lower than the manual equivalent. This created the need for some investigation work to identify the contributing factors to this issue as the success of cell therapy largely depends on cell numbers. Frequently, process characterisation includes identification of important key performance indicators and also establishing the acceptable ranges for operational parameters (Rathore *et al*., 2007). As a first step towards better understanding the automated process, the key components of the automated system were thoroughly characterised.

The automated process is a complex system, which is designed to carry out various functions such as cell mixing, counting, washing, passaging, and pipetting/seeding (Liu *et al*., 2010). Some of key components of the automated system are the Cedex cell counter (Roche, Switzerland) and the pipetting system. The cell counter measures cell concentration (number of cells in 1 mL of sample) and the disposable pipette (controlled by the automated pipette head) can dispense a liquid volume between 0.1 to 10 mL. Section 5.2.1 and 5.2.2 discuss if these systems are capable of producing the same results consistently when process parameters remain unchanged (first technical question).

Measurement system analysis (MSA) is frequently used to control the quality and to improve the performance of equipment or processes (Erdmann *et al.,* 2010). For quality improvement work, it is standard practice to assess the reliability of measurements before performing any analyses. Usually, for industrial statistics the important aspects of the quality of are accuracy and precision. Accuracy of the measurement system depends on the amount of bias of the system. Bias is the difference between the average of multiple measurements on the same object and the reference value and is usually reduced by re-calibrating the measurement equipment. The extent to which bias is constant over time is called stability, and the extent to which bias is constant over the measured range is called linearity (Erdmann, *et al.,* 2010).

Precision relies on measurement spread, which is the standard deviation of repeated measurements on the same object. Precision is divided into repeatability and reproducibility. If all circumstances (e.g. measurement instrument and person) are kept the same for each of those repeated measurements, this variation is identified as repeatability. Variation due to varying circumstances (e.g. different operators, different measurement devices, or different environmental conditions) is called reproducibility (Erdmann, *et al*., 2010).

Gauge Repeatability and Reproducibility (Gauge R&R) analysis is one of the common MSA tools to assess precision, but since the Cedex cell counter utilises automated cell counting method (no varying circumstances), type 1 Gauge study is used instead of Gauge R&R to carry out repeatability (precision) and bias (accuracy) assessment. In order to

evaluate the performance of the automated pipettes, a Gauge Bias and Linearity (Gauge B&L) study is carried out to assess values over its operating range. Gauge B&L study determines whether the gauge is measuring accurately and precisely. This study assesses linearity (how accurate your measurements are through the expected range of measurements) and bias (how well your measurements compare to a reference value).

5.2.1 Performance of the Cedex cell counter

The Cedex system has a liquid handling unit that mixes a defined volume of cell sample and Trypan blue solution in a mixing chamber. Once mixed, the Trypan blue-stained samples are then pumped into a flow cell where microscopic images of cells are captured and analysed by the instrument's image-processing software. The automated counting system reduces operator error in handling large number of samples and up to 30 images per sample can be rapidly analysed by the software (www.roche.com).

A study by Huang *et al.*, 2010 was able to demonstrate that Cedex cell counter was capable of providing measurements that were both accurate and precise (for cell densities ranging from 3.13×10^5 to approximately 1.0×10^7 cells/mL). Accuracy and precision levels of more than 95.3 recovery and relative standard deviation (RSD) of less than 5 %, respectively were obtained. However, when the authors tested another Cedex cell counter, some differences were found. The authors suggested calibration to be performed whenever deviation from the expected value was observed. For this reason, it is important to compare the cell counts obtained with the cell counts from another device.

In order to assess this, cell samples were taken from the same conical tube and were counted using the Cedex cell counter, the NucleoCounter (Sartorius Stedim, France), and the haemocytometer (manual counts). The counts were compared with one another. The cells were taken from the same cell pool to minimise variations (experimental procedures are described in Chapter 3, Section 3.4.2.1). Error bars in Figure 5.1 illustrate that the average number of cells obtained from Cedex cell counter was not significantly different from the average value obtained from the NucleoCounter ($p > 0.05$), and neither is it significantly different from the average value obtained for the manual (Haemocytometer) counts ($P > 0.05$). Based on the cell counts obtained, it was decided that re-calibration was not required, but throughout the doctoral study, the Cedex cell counts obtained were always compared with cell counts from other devices to ensure the counts obtained were reliable.

Figure 5.1 was able to demonstrate that the Cedex cell counts were comparable with the manual and the Nucleocounter cell counts. In order to assess the accuracy and precision of the Cedex cell counter, type 1 gauge study was carried out. Since a reference value was required for the analysis, similarly to the procedure used earlier, cell samples were taken from a conical tube and 3 cell counts were initially performed using NucleoCounter. Subsequently, 25 cell counts were performed using Cedex cell counter (using cell samples from the same conical tube). Then, type 1 gauge analysis was performed using Minitab and a run chart was obtained (Figure 5.2). The complete Minitab report is attached in the appendices (Appendix II).

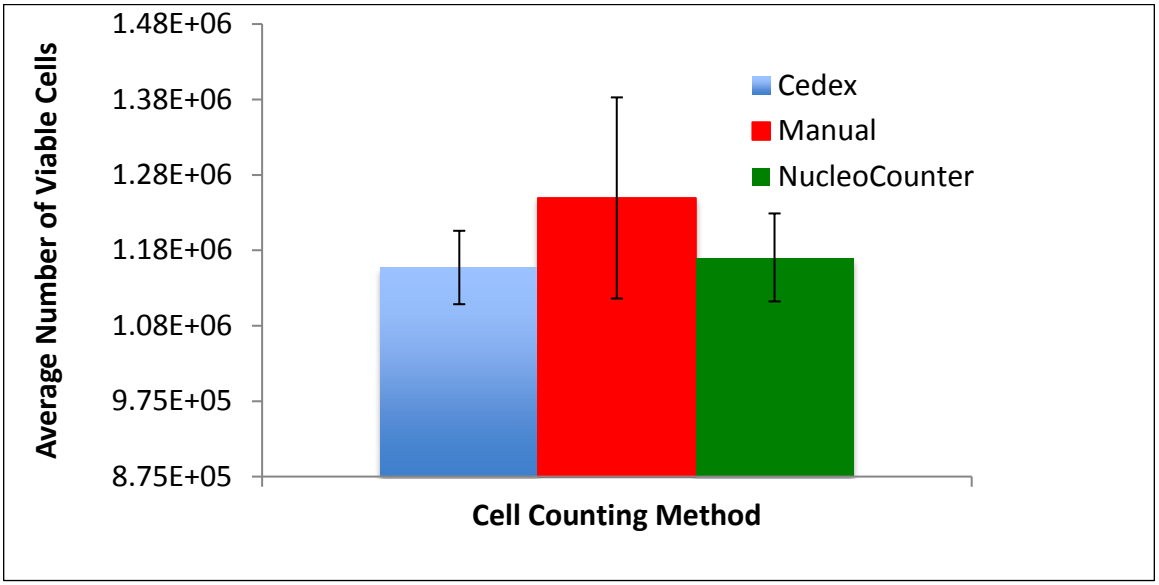


Figure 5.1 *Comparison of the cell counting methods. Error bars represent the standard deviation of the data (n=9).*

From Figure 5.2, it can be observed that there is no issue with bias (p value obtained for bias > 0.05). The C_{gk} value obtained is also more than 1 (statistical method detailed in chapter 3) and this demonstrates good precision and accuracy for the tolerance value selected (tolerance of < 10% was selected). This tolerance level is generally acceptable across most industries (Chen *et al*., 2010). The results from this analysis show that the Cedex cell counter used for this study is capable of demonstrating accuracy and precision so it was decided that re-calibration at this point was not necessary. Although manual counting with a haemocytometer is commonly used due to its low cost, this procedure is time consuming, and is subject to inter-user variation depending on the level of expertise of the analyst (Cadenna-Herrera, *et al*., 2015). Therefore, for this study, Cedex cell counter was used.

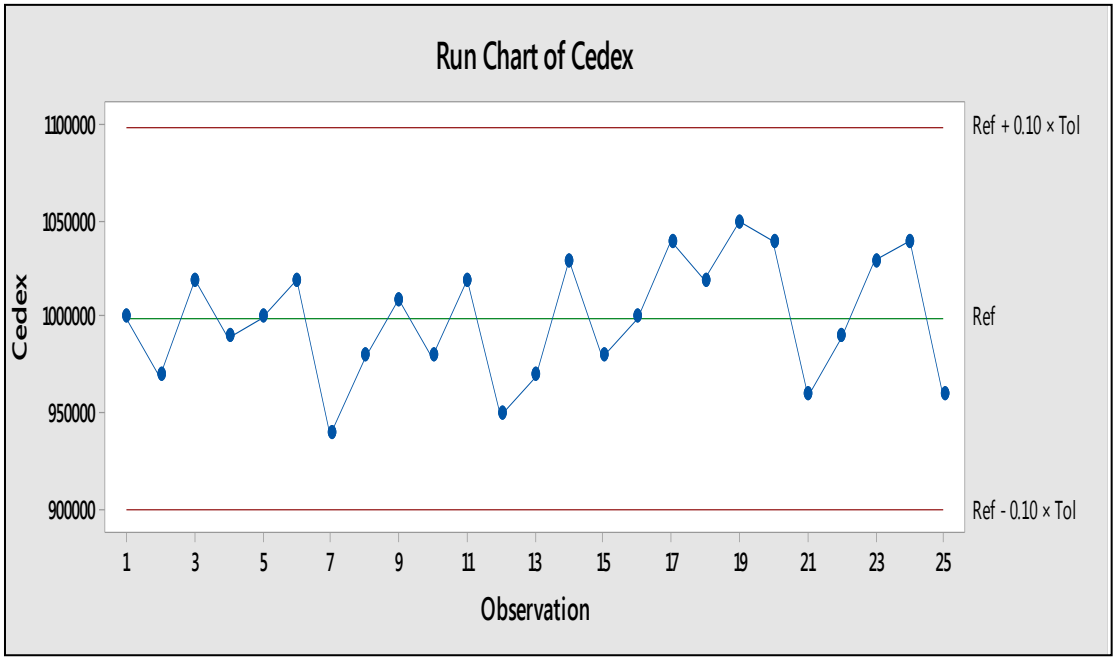


Figure 5.2 *Cedex run chart for type 1 gauge study. Samples were taken from the cell pool (n=25).*

5.2.2 Performance of the liquid dispensing system (pipette)

Automation of a process alone does not necessarily guarantee good performance, as the quality of the automated process mainly depends on the liquid dispensing (pipetting) system (Haney, 2008). As liquid handling procedures involve multiple pipetting steps, small errors in pipetting can accumulate over the course of the expansion process. Thus, verifying the performance of liquid handling procedures is a critical step in improving the performance of the automated process (Haney, 2008).

The precision of the automated pipetting process can be influenced by 3 main factors: low dispensed volumes, liquid viscosity, and absence of liquid in the receiving flask (Haney, 2008). The simplest validation method to evaluate the precision is to have the robot

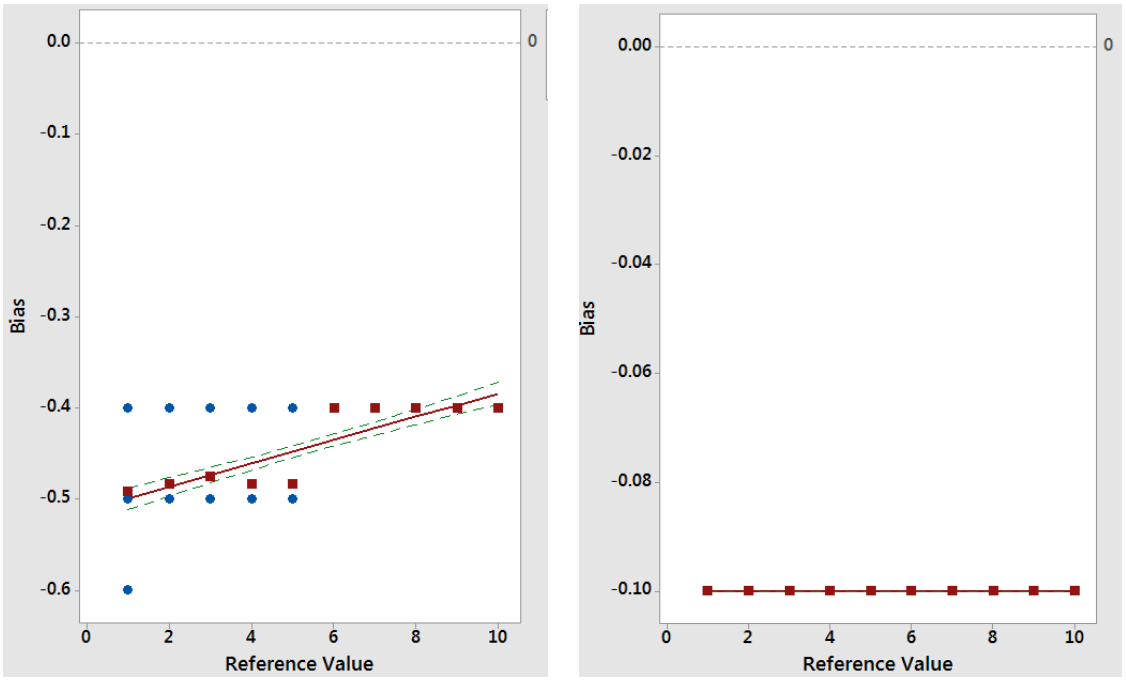
dispense a fluorescent dye into a well plate, and analyse it using a spectrometer (Haney, 2008). However, this method cannot be employed for the existing system, as the Compact Select used at Loughborough University does not have the ability to dispense liquid into well plates. Accuracy is commonly assessed gravimetrically because of the relationship between the volume of the liquid dispensed and its weight, which is defined for liquids of a known density (Haney, 2008).

To evaluate the pipetting performance, an in-house method was developed which involved pipetting a known volume of liquid into a flask containing liquid with a known mass in grams (method described in Chapter 3 Section 3.4.2.2). Gauge B&L study was carried out to determine whether your gauge (pipette) is dispensing accurately. It was observed that for all volumes dispensed between 1 to10 mL (using a volume interval of 1 mL), there was at least 0.4 mL difference between the programmed and actual dispensing volume. The measurements were repeated multiple times (n=9) and for all repeats, similar deviation was observed. In order to assess linearity (how accurate the measurements are through the expected range of measurements) and bias (how well the measurements compare to a reference value) a Gauge B&L study was carried out.

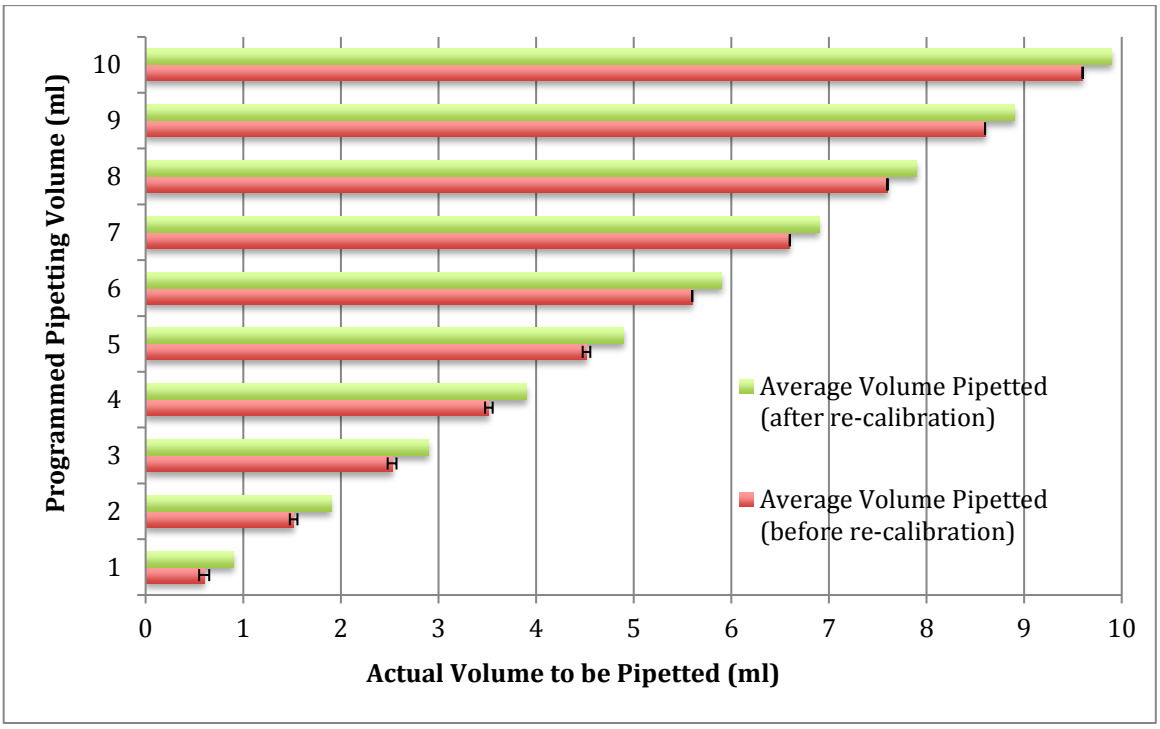
When a Gauge B&L study is carried out, Minitab displays a graph of the best-fitted line of the biases across the reference values. For pipetting volumes taken before re-calibration, bias is present and significant ($p < 0.05$ for all the pipetting volumes). The average bias is -0.44 mL (Figure 5.3 a). So, on average, the volume dispensed is lower than the reference value. The bias decreases with increasing pipetting volumes, as identified by the positively

sloped line in the scatterplot. A positive bias indicates that the gauge over-estimates, meanwhile a negative bias indicates that the gauge under-estimates. The inconsistency of the bias across dispensing volumes between 1 to 5 mL (Figure 5.3 a) indicates that the measurement system also has linearity problems.

For pipetting volumes taken after re-calibration, bias is present and significant ($p < 0.05$ for all the pipetting volumes). The average bias is -0.1 mL (Figure 5.3 b). So, on average, the volume dispensed is still lower than the reference value despite several attempts of re-calibrating this instrument. The bias does not increase with the increasing pipetting volumes, as identified by a linear line on the plot (Figure 5.3 b). The consistency of the bias across dispensing volumes between 1 to 10 mL indicates that the measurement system no real linearity problems. Overall, the system's real issue is gauge bias, therefore this is not a real problem as long as the Compact Select protocol can be adjusted to compensate for this bias. Additionally, the error bars in Figure 5.3 (c) demonstrate that the pipetting system has good precision.



(a) (b)



(c)

Figure 5.3 *Gauge B&L graphs: graph (a) shows bias obtained before calibration and graph (b) shows bias obtained after calibration. Figure (c) shows the actual pipetting values before and after re-calibration (n=10). Error bars represent the standard deviation of the data (n=9).*

From these data, it can be concluded that out of all the two key variables (Cedex Counter and the pipetting system) of the Compact Select, only the pipetting system was contributing to differences in cell growth. In order to ensure the Compact Select pipetting system pipettes the desired amount, and not 0.1 mL less, the XML script (automated protocol) was adjusted to seed 0.1 mL more to compensate for the pipetting error of 0.1 mL. While this work was able to demonstrate that the one of the key components was less accurate, further evidence was required to evaluate if this was the main cause that contributed to lower automated process yield.

5.2.3 Improving the yield of automated HDF cell culture process

The work discussed in the previous section demonstrated that the pipetting system was always dispensing incorrectly (0.1 mL less). This may have been the cause of reduced automated process yield, but this has to be confirmed, as there might be other issues in addition to this problem. In order to evaluate if the solution discussed in Section 5.2.2 solves the problem associated with lower automated process yield, the automated cell culture experiment discussed in Chapter 4 was repeated (pipetting volume was re-adjusted), but without taking samples every 24 hours. Cells were harvested after 72 hours (to get the maximum yield as at 96^{th} hour sampling point, cell death occurs). The cell yield obtained highlights another underlying problem.

The yield obtained for the automated cell culture process of 2.89×10^6 cells was still significantly lower ($p < 0.05$) than 3.2×10^6 cells obtained for the manual HDF culture in Chapter 4 (Figure 5.4). However, it was significantly higher than the yield of 2.5×10^6

cells obtained from the previous automated culture ($P < 0.05$) in Chapter 4. This was a cause of concern, as this meant that there were other problems, other than the ones that had been identified and subsequently rectified in the preceding section (Section 5.2.2). The next section (Section 5.3) focuses on discussing some of the work carried out to identify these factors.

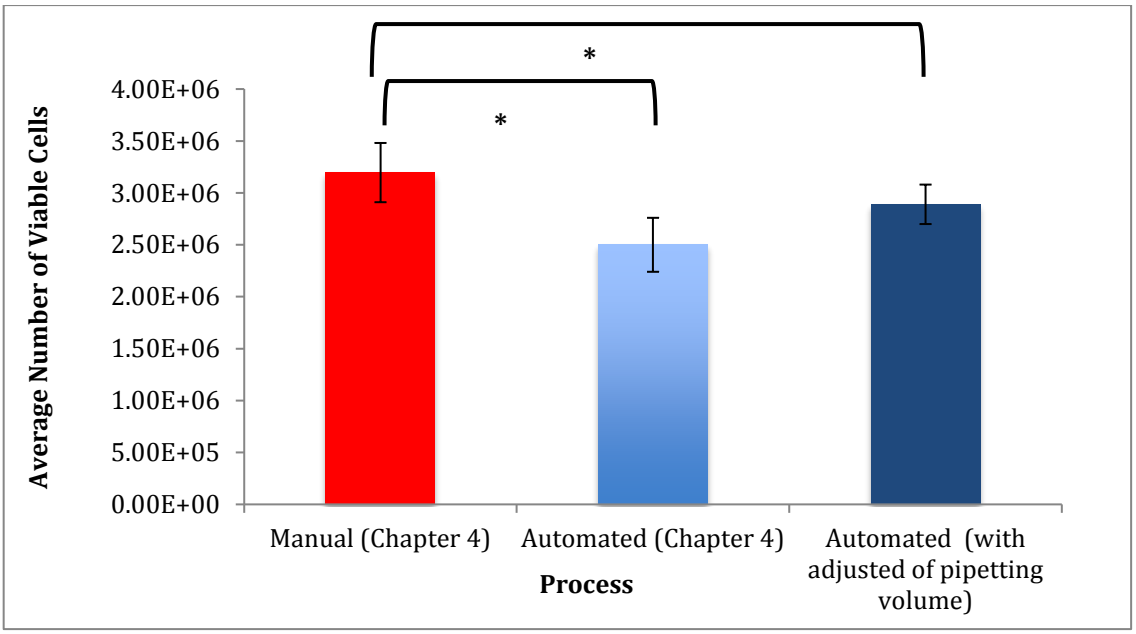


Figure 5.4 *Average viable cells recovered after 72 hours of culture (n=9). Error bars represent the standard deviation of the data (n=9). Asterisks (*) indicate significant difference, $p < 0.05$.*

5.3 Automated Vs. Manual: Methodological Differences

So far all the work discussed in this chapter focused on evaluating the performance of the measurement systems. While this is important, it is also necessary to understand if there the methodological differences between the processes have any real impact on the process yield. It was suspecte that methodological differences may be the reason why lower yield is obtained for the automated process (Liu *et al*, 2010; Thomas *et al*., 2007), so it is

important to investigate if this indeed true. Therefore, this section will now discuss the key differences between the processes and the also the effect these differences have on the yield. The manual and the automated cell culture protocols used are the standard protocols that were available in the laboratory (Chapter 3).

As described in Chapter 3, most of the process steps of the Compact Select automated passaging protocols are similar to that of manual passaging protocols. However, these two culture methods differ in few key process steps:

(i) **Harvesting process:** During the manual cell culture, once the dissociation enzyme has been added and the cells have been incubated for a given amount of time, the suspension is transferred to a conical tube. The cell suspension is centrifuged and the supernatant is aspirated in order to isolate a cell pellet. Then fresh medium is added to the conical tube and cell are counted and subsequently seeded into new flasks. However, during the automated cell culture process, fresh medium containing Foetal Bovine Serum (FBS) is pumped into the flask to neutralise and dilute the enzyme and then the suspension is then mixed. Then, 1 mL sample is taken and counted using the Cedex cell counter.

(ii) **Re-seeding**: Since the automated process does not allow the transfer of medium to a conical tube (prior to cell seeding/counting) as to how it is done for manual cell culture, the cell suspension is left in a T175 flask (or pooled into a separate T175 flask if many flasks are processed) before cells are mixed, counted and re-seeded into new T175 flasks. Additionally, Compact Select uses 10ml pipettes instead of 1000uL tips for cell mixing and usually for the manual culture, for mixing, 1000uL

tips are used.

Section 5.3.1 discusses the work carried out to compare the manual and the automated harvesting protocols. Section 5.3.2 discusses the work carried out to better characterise the automated seeding process.

5.3.1 Cell harvesting protocol

In order to investigate the difference between the automated and manual cell harvesting processes, flasks were manually seeded with HDF cells and were placed in both the manual and automated cell culture incubators (experimental procedures are described in Section 3.4.2.3, Chapter 3). Flasks kept in each incubator were subjected to (i) manual passaging and (ii) automated passaging after 24 hours. This experiment is designed in this way to evaluate the differences that can be observed due to the harvesting methods employed and the incubators used. Since it has been reported that temperature fluctuation (due to the opening and closing of the incubator) can contribute to growth variation (Quinn, 2014), it was necessary to rule out that this could have contributed to reduced automated process yield.

Figure 5.5 shows the average number of viable cells obtained for 2 passages when they were cultured using the automated and the manual harvesting method. The average number of cells obtained for the manual process (passage) of 1.44×10^6 was not significantly different from the average of 1.40×10^6 obtained for the automated process (passage) for cells cultured in the manual incubator ($p > 0.05$). The average number of cells obtained for

the manual process of 1.37 x 10^6 was also not significantly different from the average of 1.41 x 10^6 obtained for the automated process for cells grown in the automated incubator ($p > 0.05$).

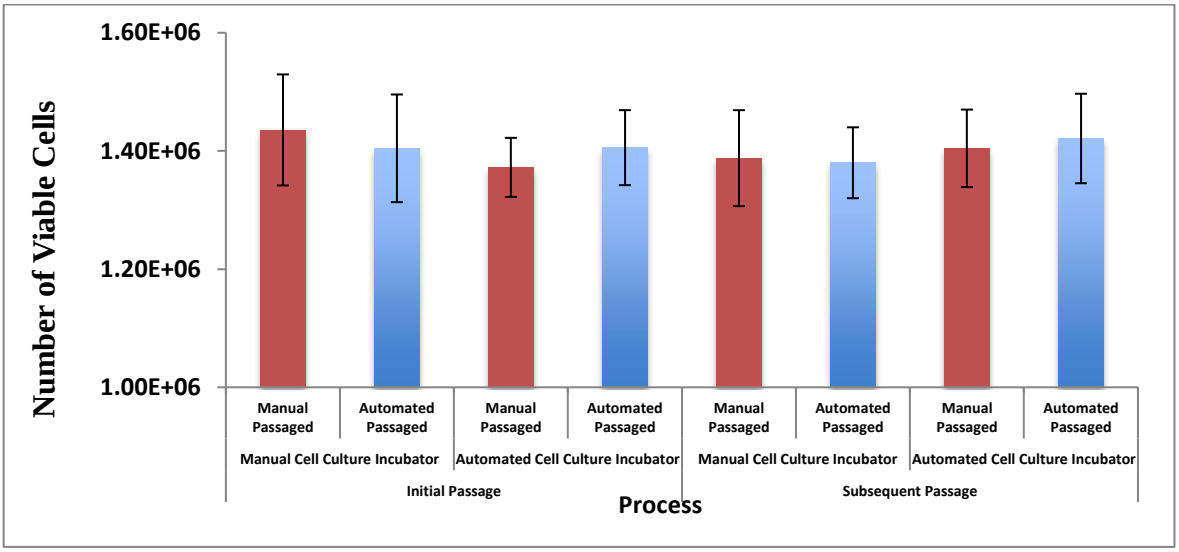


Figure 5.5 *Average number of viable HDF cells obtained through manual and the automated harvesting processes. Cells were seeded at 8.75 x 10^5 cells per T175 flask and were harvested after a day. This work was repeated for an additional passage. Error bars represent the standard deviation of the data (n=9).*

The automated cell culture passaging protocol involves prolonged exposure to trypsin-EDTA (detachment enzyme) as there is no centrifugation to completely remove the trypsin-EDTA from the growth medium. Huang *et al*. (2010) reported that increased or prolonged exposure to trypsin-EDTA can reduce cell growth. This work was continued for one more passage to understand the long terms effects of leaving residues of trypsin-EDTA in the growth medium.

Even for the subsequent passage, the average number of cells obtained for the manual

process was not significantly different than that of the automated process for cells grown in either incubators ($p > 0.05$). Most importantly, there was no significant difference in the average number of cells obtained between two passages ($p > 0.05$). The cell viability was above 90% throughout the cell culture process (for both passages). This indicates that the cell harvesting method (protocol) employed is suitable for the culture of HDF cells as no growth inhibition due to the detachment enzyme was observed. In addition, the results also confirm that the selection of incubator has no influence on the process yield.

The work carried out in this section demonstrated that the automated cell harvesting method did not impact the cell growth of HDF cells or cause any cell losses. Since the automated harvesting process did not contribute to lower automated process yield, more investigation work is required to identify the underlying cause of this problem. The next section discusses the work carried out to better characterise the automated seeding process.

5.3.2 Cell seeding protocol

As indicated earlier, since the automated process does not allow the transfer of medium to a conical tube (prior to cell seeding/counting), the cell suspension is left in a T175 flask before cells are mixed, counted and re-seeded into new T175 flasks. In order to investigate if the procedures significantly impact the yield of the processes, flasks were seeded with 9 x 10^6 cells each, and the automated cell culture seeding process was imitated with the flask placed in a slanted position (procedure is detailed in Section 3.4.2.4, Chapter 3). This flask was seeded with a larger concentration of cells, instead of the usual seeding density of 8.75 x10^5 cells per flask, to mimic the passaging process as closely as possible. Usually, during

the passaging process, cells are pooled from several flasks to one flask therefore the concentration of cells in the 'mother flask' will be considerably higher. Every 10 minutes, cell samples were taken from the cell suspension, and the concentrations were determined using the Cedex cell counter (Figure 5.6). The cell suspension was mixed before a sample was taken to ensure that the sample was taken from a homogenous suspension.

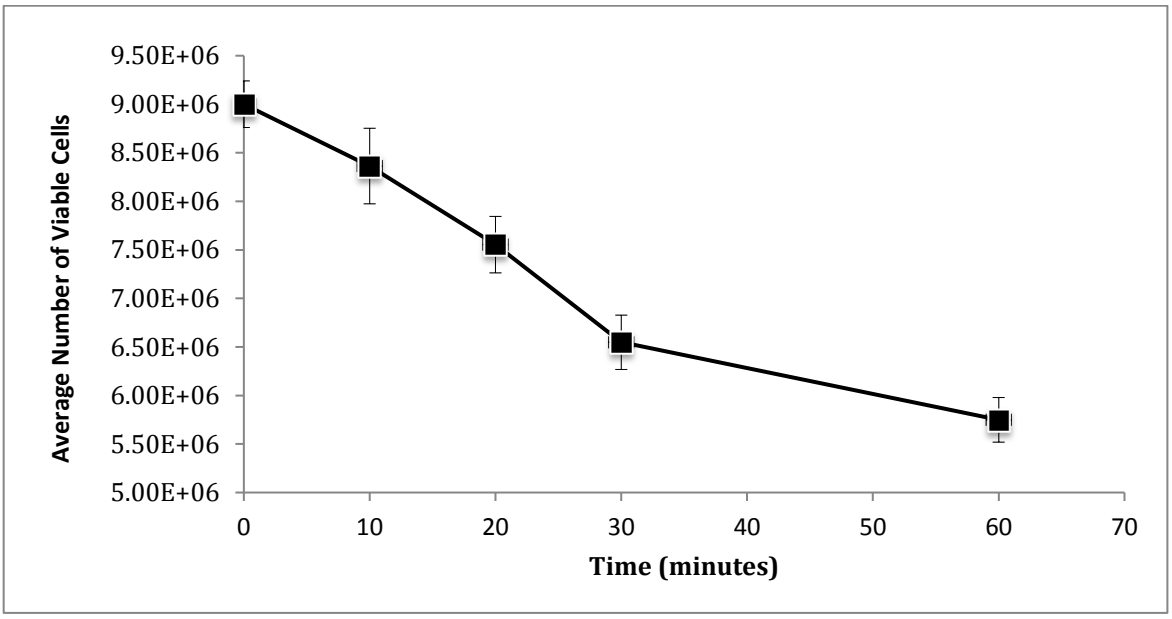


Figure 5.6 *Average numbers of viable HDF cells remaining in the growth medium at each time point. Cells were seeded at 9 x10^6 cells per T175 flask. Error bars represent the standard deviation of the data (n=9).*

From Figure 5.6 it can be seen that the concentration of cells in suspension decreased over time. The cell concentration decreased at a higher rate during the first thirty minutes, but slowed down during the last 30 minutes (cell viability remained above 90%). These rates (cell loss) were determined by drawing tangents on the graph, and by finding the slopes. These rates were determined to be approximately 8.17 x 10^4 cells per minute for the first

30 minutes, and 2.67 x 10^4 cells per minute for the last 30 minutes. As these rates (cell loss) were rather high, especially for the first 30 minutes, it was crucial to understand why this was happening so that this loss could either be minimised or prevented (the estimated time to complete the automated process runs are provided in Table 3.3, Chapter 3).
To understand and identify the source of the problem, this flask was then examined. It was identified that cells were attaching to all surfaces of the tissue culture flask, including the non-treated surfaces. Usually, the attachment to the non-treated surfaces is not noticeable during the automated process run because flasks are processed in a closed chamber and they are ejected out of the processing system into a waste container after the process run. In order to observe the surface thoroughly to detect attached cells, the medium was discarded, and the surface was washed with PBS solution to ensure that these cells were clearly visible.

As discussed earlier (Chapter 3), for the automated system, cells are pooled in a flask instead of a conical tube. Although cells also attach to the conical tube during the manual cell culture process, this rate of loss is negligible. This is mainly because conical tubes have considerably smaller surface area compared to a T-flask. Some automated systems come with cell scrappers to remove strongly attached cells. However, for the Compact Select, other methods have to be considered to prevent cell attachment. This ideal method will be the one that will allow the removal of cells form the cell culture surface without affecting the cells' quality.

This experiment confirmed that cell loss was one of the main reasons why lower cell yield was obtained for the automated HDF cell culture. The most important question now is that

if this undesired attachment occurs for the MSCs. In order to confirm this matter, this experiment was repeated with MSCs (when these cells became available). Experimental results illustrated that the MSCs did not demonstrate any significant attachment behaviour to the flask within 60 minutes. This was confirmed through the initial and the end cell count after 60 minutes. A total of $9 \pm 0.29 \times 10^6$ passage 7 cells were pooled into one flask and approximately $8.7 \pm 0.26 \times 10^6$ cells were obtained after 60 minutes ($p > 0.05$). The mixing employed was sufficient, as the cell concentrations remained constant throughout the 60 minutes. During another separate analysis, it was observed that the cells only started attaching to the surface, approximately 8 hours after seeding.

Although this is beyond the scope of this doctoral study, several methods of preventing cell attachment were investigated (data attached in Appendix IV). This work managed to identify one particular surface, which was able to completely prevent cell attachment; Ultra-Low attachment flask (Corning Incorporated, USA). The only drawback was that, at the present time, this surface was not produced in the T175 format, but it is anticipated that this will change if there is a greater demand for these flasks. For any experiments with strongly adhering cells such as the HDF cells, flasks such as the Corning flasks can be used to prevent cell attachment, but for the MSCs this was not required, as they do not attach to the surface of the flasks during the seeding process. For stem cell expansion process, the use of additional surfaces may complicate the expansion process as cell interaction with the surface material can influence cellular behavior and phenotype (McCorry, *et al.,* 2016). Even in terms of cost, this is no desirable as this may cause significant increase in the process cost. Therefore, the inclusion of additional coating material to the cell culture surface should be avoided whenever possible.

The findings reveal the HDF cell attachment characteristics are different from the MSCs although it was reported (Alt *et al.,* 2011) that they express similar surface marker expression (CD90, CD73, CD105) and morphology. This finding stresses the importance of testing some of the developed work with MSCs. The next section, Section 5.4 discusses the work carried out to develop and optimise the automated MSC culture process.

5.4 Development of the Automated MSC Culture Process

As described earlier, the first set of development work was achieved with HDF cells (Section 5.3). Since MSC availability was one of the main limitations of the doctoral study, cells used for the studies discussed in this chapter (development studies) were cells at passage 7. This is mainly because cells were required in large quantities (> 50 million) for the investigation studies (Section 5.4 and Section 5.5), therefore, cells had to be expanded to this quantity to meet the requirements of these studies. However, unlike MSCs derived from bone marrow, UC-derived MSCs are known to be capable of retaining their MSC characteristics even until passage 15 (Chen *et al*., 2014; Majore *et al*., 2013).

Trypsin-EDTA is commonly used protease enzyme to dissociate cell monolayers into single-cell suspensions during serial passages. For the MSC culture, trypsin-EDTA was not used as the detachment enzyme. Instead, Tryple Express (Life Technologies, USA) was used based on recommendation from Future Health Technologies (FHT), UK. For stem cells, generally Tryple Express is used, as it is known to be gentler on the cells (Pacifici and Peruzzi, 2012). Additionally, it is also free of animal-derived component. Tryple Express is a recombinant trypsin-EDTA like proteolytic enzymes produced from bacterial

fermentation (Heng *et al*., 2009). The manufacturer has claimed that the proprietary protease used in it strongly resembles trypsin-EDTA, but the manufacturer has kept the exact formulation confidential (www.thermofisher.com).

The work in the next section (Section 5.4.1) discusses the work carried out to compare the manual with the automated MSC harvesting process. This work is similar to the work conducted with the HDF cells in Section 5.3.1. This work (with MSCs) is done to assess the suitability of the existing automated cell culture passaging protocol for the culture of MSCs and to gauge if further process development is required. In addition, the previous protocol (used for the HDF) was developed to be used with trypsin-EDTA as the detachment enzyme, therefore the introduction of a new detachment enzyme may require the protocol to be re-adjusted in order to maximise cell yield.

5.4.1 Automated MSC harvesting method

The objective of this experimental work described here is to understand if there is any significant difference between the manual and the automated MSC harvesting process. The experiments performed with HDF cells enabled the limitations of the automated cell culture system to be understood, and allowed improvements to be made (e.g, improve the pipetting performance). However, it was not clear whether all the work conducted to optimise the HDF cell culture process was applicable to the MSC culture. In order to assess that, the experiment conducted in Section 5.3.1 was repeated with MSCs (experimental procedures are described in Section 3.4.2.3, Chapter 3).

Figure 5.7 shows the average number of viable cells obtained for 2 passages when they

were cultured using the automated and the manual harvesting method. The average number of cells obtained for the manual process (passage) of 2.66 x 10^6 was not significantly different from the average of 2.58 x 10^6 obtained for the automated process (passage) for cells cultured in the manual incubator ($p > 0.05$). The average number of cells obtained for the manual process of 2.59 x 10^6 was also not significantly different from the average of 2.63 x 10^6 obtained for the automated process for cells grown in the automated incubator ($p > 0.05$). The cell viability was above 90% throughout the cell culture process.

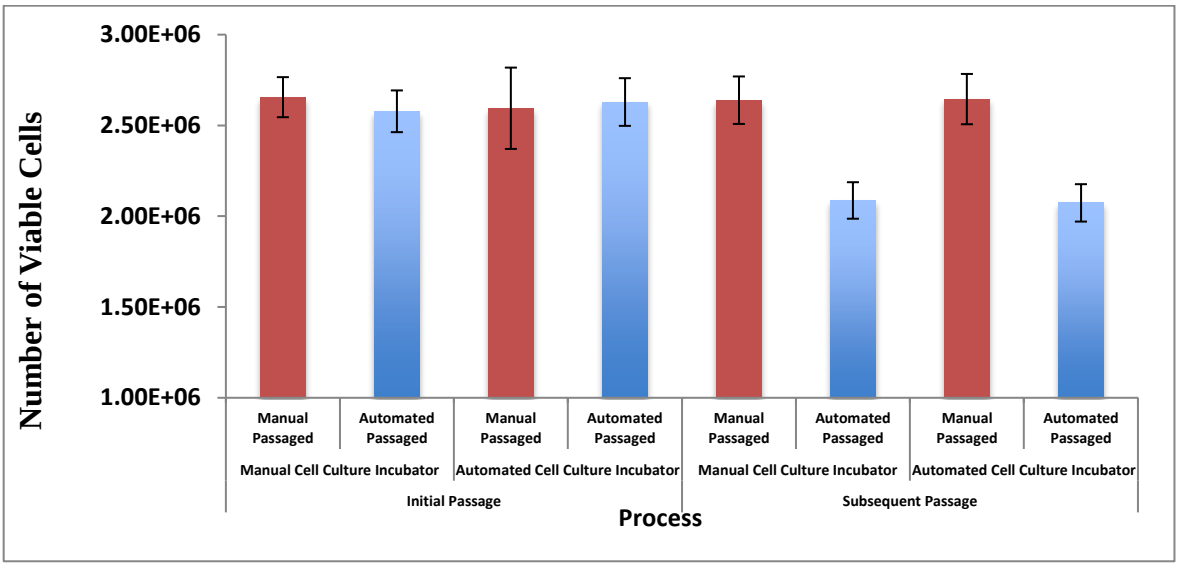


Figure 5.7 *Average number of viable MSCs obtained through the manual and the automated harvesting processes. Cells were seeded at 8.75 x 10^5 cells per T175 flask and were harvested after 2 days. This work was repeated for an additional passage. Error bars represent the standard deviation of the data (n=4).*

This work was then continued for one more passage to evaluate the long terms effects of leaving residues of Tryple-Express detachment enzyme in the growth medium. The average number of cells obtained for the manual process of 2.64 x 10^6 was significantly different higher than the average of 2.09 x 10^6 obtained for the automated process for cells

grown in the manual incubator ($p < 0.05$). The average number of cells obtained for the manual process of 2.65×10^6 was also not significantly higher than the average of 2.07×10^6 obtained for the automated process for cells grown in the automated incubator ($p < 0.05$). The viability of the cells was between 75 to 85 % during this passage. In contrast to the results obtained for the initial passage, the average number of viable MSCs obtained for the manual cell culture process was significantly higher than that of the automated cell culture process. The average cell yield obtained for the automated process for the subsequent passage was significantly lower than the yield obtained for the previous passage for cells grown in either incubator ($p < 0.05$).

When the manual process yield for the cells grown in the manual cell culture incubator is compared with the manual process yield for the cells grown in the automated cell culture incubator, there is no significant difference in the yield obtained for either passage ($p > 0.05$). This is the same case when the automated process yields are compared between the cells grown in 2 different incubators ($p > 0.05$). This work has shown that the choice of incubator used has no real influence on the growth of MSCs.

In contrast to the results obtained with the HDF cells (Section 5.3.1), the MSCs exhibited a decrease in cell viability during the subsequent passage when this passaging protocol was employed. The traditional method of automated cell culture passaging involves leaving the detachment enzyme with the cells in the incubator until the cells detach. However, the findings above have illustrated that this method reduces the viability of the MSCs. To avoid leaving detachment enzyme with the cells after the passaging step, the existing

protocol should ideally be modified, but it has to be first investigated if this is possible. The investigation and development work carried out are discussed in Section 5.4.2.

5.4.2 Automated process modification study

Since leaving the detachment enzyme in the growth media (after cell detachment) resulted in loss of cell viability for MSCs, an automated cell culture protocol that enables the removal of the detachment enzyme from the growth after cell detachment was required. Since no such protocol existed, detailed investigation work was required to assess if this could be developed. Most importantly, this automated protocol should be capable of producing cells in quantities comparable with the existing manual process.

The automated MSC culture process is not "completely automated" as the automated process operates based on the written protocol. This protocol uses XML script that has to be written by an operator. Therefore, several iterations were required to develop a protocol that was capable of producing cells with optimum quantity and quality. Some of the common functions used in the XML script are provided in Table 3.2, Chapter 3.

The original automated protocol used was not suitable for the MSC culture as this caused the reduction of cell yield and loss of cell viability of the cells during the subsequent passage (Section 5.4.1). Therefore, this created the need for protocol modification. It was important to investigate if it was possible to detach the cells without leaving the detachment enzyme in the growth. If this can be achieved, it will be mandatory to test the protocol with MSCs to confirm the suitability of the protocol developed because this was

not attempted previously by the automation scientists (Liu *et al.,* 2010; Thomas *et al*., 2007).

In order to develop a suitable protocol, 4 iterations of the automation protocol were written and tested in the Compact Select using MSCs. The protocols are detailed in Section 3.4.2.5, Chapter 3. The original protocol is labeled as protocol a, and the new iterations are labeled as protocols b, c, d and e. The cell yields obtained were compared with the cell yield obtained using the original protocol, protocol (a). The main difference between protocol (a) and the rest of the protocols developed is that, for the rest of the protocols, the flask is incubated without the detachment enzyme as the detachment enzyme is poured off as soon as the cells are become immersed with the enzyme. The summary of the main differences between the protocols is provided in Table 5.1.

Results obtained (Figure 5.8) illustrate that the protocol (e) is the protocol that allows the highest yield to be obtained. This protocol employs shorter enzymatic exposure time than protocol (b) and (c), but employs similar enzymatic exposure time as protocol (d). It is believed that shorter enzymatic times reduced effects associated with cell dehydration, therefore, there is minimal cell death when protocol (d) and (e) are employed. The real difference between protocol (d) and protocol (e) is that, for protocol (e), an additional shaking step is implemented. This additional step was required because when the detachment enzyme is poured off before the flasks are placed in the incubator, only a thin layer of the enzyme remains. Therefore, slight agitation (shaking) was necessary to aid the detachment of the cells of the surface.

Table 5.1 *Summary of the main differences between protocols developed for MSC culture.*

Protocol	**Removal of enzyme right after enzyme addition**	**Incubation (time)**			**Additional Shaking Step**
		8	9	10	
A (Original)				X	
B	X			X	
C	X		X		
D	X	X			
E	X	X			X

While protocol (a) was suitable for MSC culture for the initial passage, leaving the detachment enzyme together with cells resulted in loss of cell viability for the subsequent passage (viability was between 75 to 85%). In order to prevent this from happening, several protocols were developed. In terms of the cell yield obtained, only protocol (e) is capable of the producing the highest yield consistently for 2 continuous passages. Figure 5.8 shows that an average of 3.35×10^6 cells and 3.33×10^6 cells were obtained for the initial and the subsequent passage, respectively, by using protocol (e). There was no significant difference in the yield obtained between the initial and the subsequent passage ($p > 0.05$) when this protocol was employed. In terms of cell viability, cell viability was always above 90% for the cells cultured using this protocol. This indicated that this protocol did not have any adverse effect on the cells cultured.

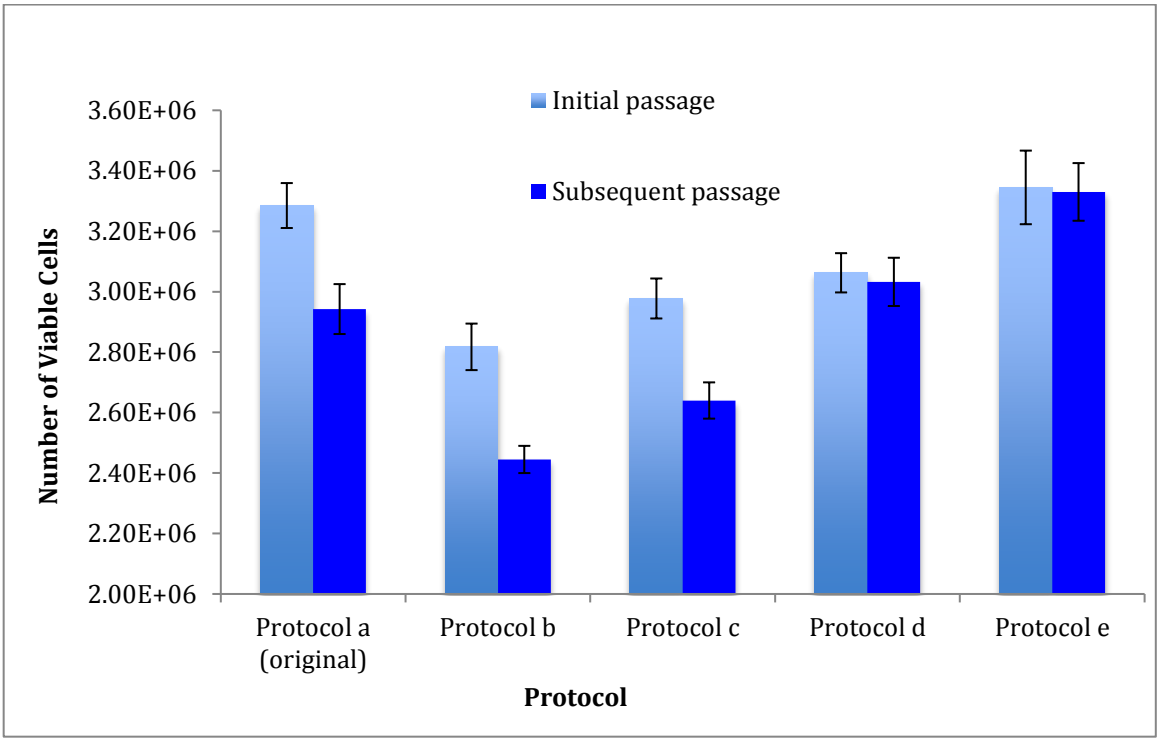


Figure 5.8 *Average number of viable MSCs obtained from using 5 different protocols. Cells from passage 7 were seeded at 8.75 x 10^5 cells per T175 flask and were harvested after 3 days. This work was repeated for an additional passage. Error bars represent the standard deviation of the data (n=4).*

Figure 5.8 illustrates that an average of 3.03 x 10^6 and 3.06 x 10^6 viable MSCs were obtained for the initial and the subsequent passage, respectively, by using protocol (d). Although the yield was not significantly different for 2 continuous passages ($p > 0.05$) when protocol (d) was employed, it was not possible to recover all cells. Cell remained highly viable (above 90%) therefore cell death was not a reason for obtaining lower cell yield. In order to confirm this, the discarded flasks were inspected, and cells were found still attached to the treated cell culture surface of the flask. Hence, this protocol was deemed not suitable for the culture of MSCs since not all cells were recovered during the harvesting process. It was then decided that an additional agitation step was necessary to

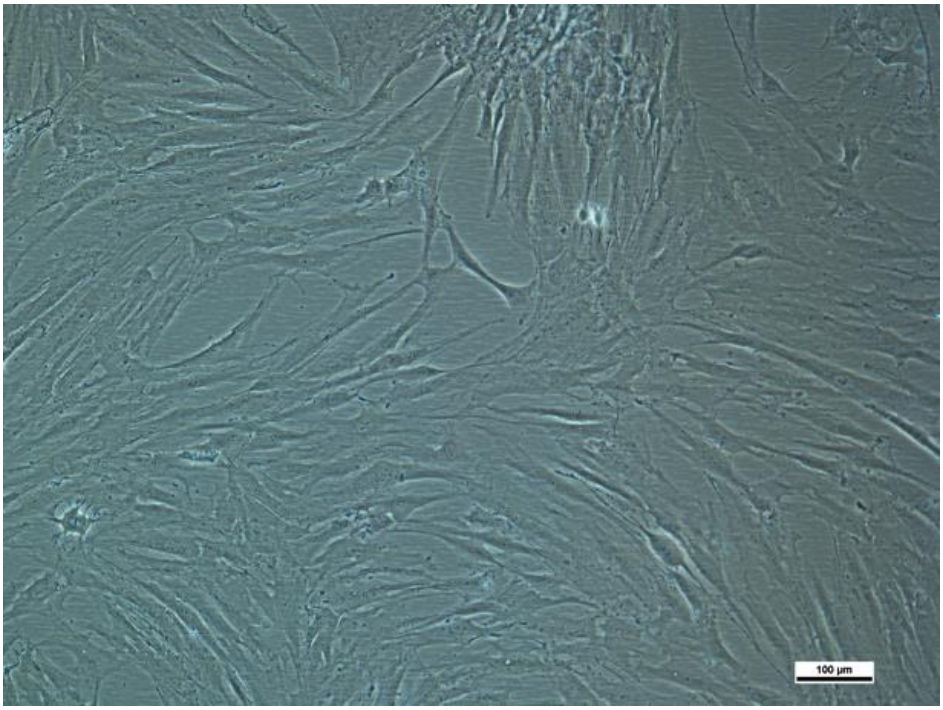
detach the cells from the bottom of the flask. Some preliminary investigation work revealed that the shaking step was the only automated step capable of performing the agitation procedure (key automated functions are listed in Section 3.2.1, Chapter 3). The cell viability and the yield were the lowest when the cells were cultured using protocol (b) and (c) for the initial and the subsequent passage (viability dropped below 75%). Figure 5.8 shows that an average of 2.82×10^6 and 2.98×10^6 viable MSCs were obtained for the initial passage by using protocol (b) and protocol (c), respectively. For the subsequent passage, an average of 2.45×10^6 and 2.64×10^6 viable MSCs were obtained using protocol (b) and protocol (c), respectively, and these values are significantly lower that the average viable MSCs obtained for protocol (d) ($p < 0.05$). The main difference between these protocols and protocol (d) is that these protocols employed a longer incubation time compared to protocol (d). This incubation time was suitable for the original protocol, protocol (a), but when the protocol was modified, this perhaps resulted in cell dehydration, as there was only minimal amount of liquid in the flask when the flask was placed in the incubator. While there was no significant difference between the protocol (c) and the protocol (d), there was a significant difference between protocol (b) and (d) in terms of cell yield; that is between the incubation time of 10 and 8 minutes ($p < 0.05$). Based on these findings, incubation time of 8 minutes was selected for future work as the ideal incubation time.

This work has shown for the first time that cells can be detached without incubating the flask containing the cells together with the detachment enzyme as long the cells are immersed completely in the enzyme before the enzyme if poured off. This way, even when

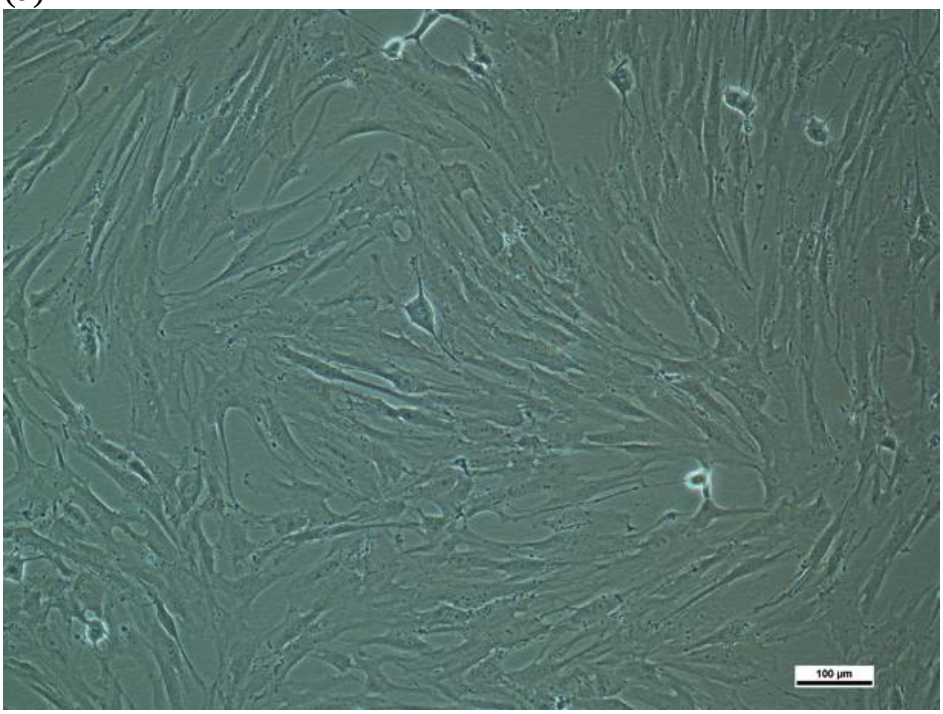
the medium was poured off, a thin layer of liquid remains. It was not possible to get rid of the liquid completely, but this amount was negligible as reflected by the cell viability and the yield obtained using protocol (e). Next, it was important to test the suitability of this protocol for the culture of MSCs over a longer period (5 passages). It was also be important to determine the stability and capability (process capability analysis) of this develop process so that the protocol can be further improved if it was necessary.

5.4.3 Assessing the suitability of the optimised automated MSC expansion protocol

The automated process developed in Section 5.4.2 was compared against the manual process. MSCs from passage 7 were used for the analysis and they were expanded for a total of 5 passages, up to passage 12 (detailed in Section 3.4.2.6, Chapter 3). All experiments performed resulted in a successful culture, with no observable problems associated with attachment or proliferation (Figure 5.9). These cell images are similar to the other MSC images (with spindle-shaped morphology) found in literature (Nekanti *et al.*, 2010). It was also observed that, in terms of morphology, these cells were similar to HDF cells (Chapter 4).



(a)



(b)

Figure 5.9 *Representative phase-contrast microscope images of MSCs at the final passage (passage 12). Images (a) and (b) are the images of the cells cultured using via the manual and the automated process, respectively. Cells were seeded at 8.75 x* 10^5 *cells/ per flask in T175 flask and were passaged after 3 days. Magnification x100 was used. The scale bars represent 100 um.*

The MSCs nutrient consumption and metabolite production were also assessed for a total of 5 passages to evaluate if there were significant differences in metabolite production and consumption pattern between these two processes. The metabolite data enabled the specific consumption rates (for glutamine and glucose pM/cell/hour) to be calculated. Media samples were taken at 0 hour and at the end of the cell culture process, that is after 72 hours and were analysed using a bioanalyser. The metabolite concentration profiles are presented in Figures 5.10.

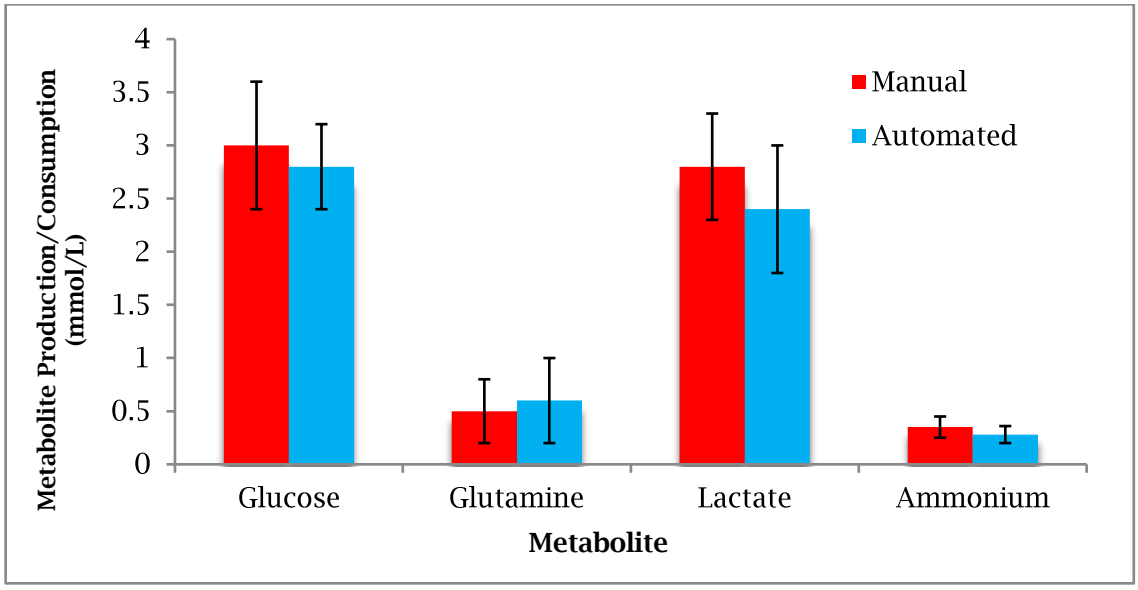


Figure 5.10 *Average glucose and glutamine consumption and ammonium and lactate production over 5 passages. Cells from passage 7 were passaged for a total of 5 passages. Error bars represent the standard deviation of the data (n=4).*

As expected, the concentrations of glucose and glutamine decreased while the concentrations of lactate and ammonium increased. The average initial concentrations of glucose and glutamine for the (manual and automated) FBS-containing DMEM cultures were 5.45 mmol/L and 1.93 mmol/L, respectively. The glucose consumed (in mmol/L) by

the cells cultured using the FBS-containing DMEM were 3.02 mmol/L for the manual process and 2.81 mmol/L for the automated process. The glutamine consumed by the cells cultured using the FBS-containing DMEM were 0.52 mmol/L for the manual process and 0.61 mmol/L for the automated process.

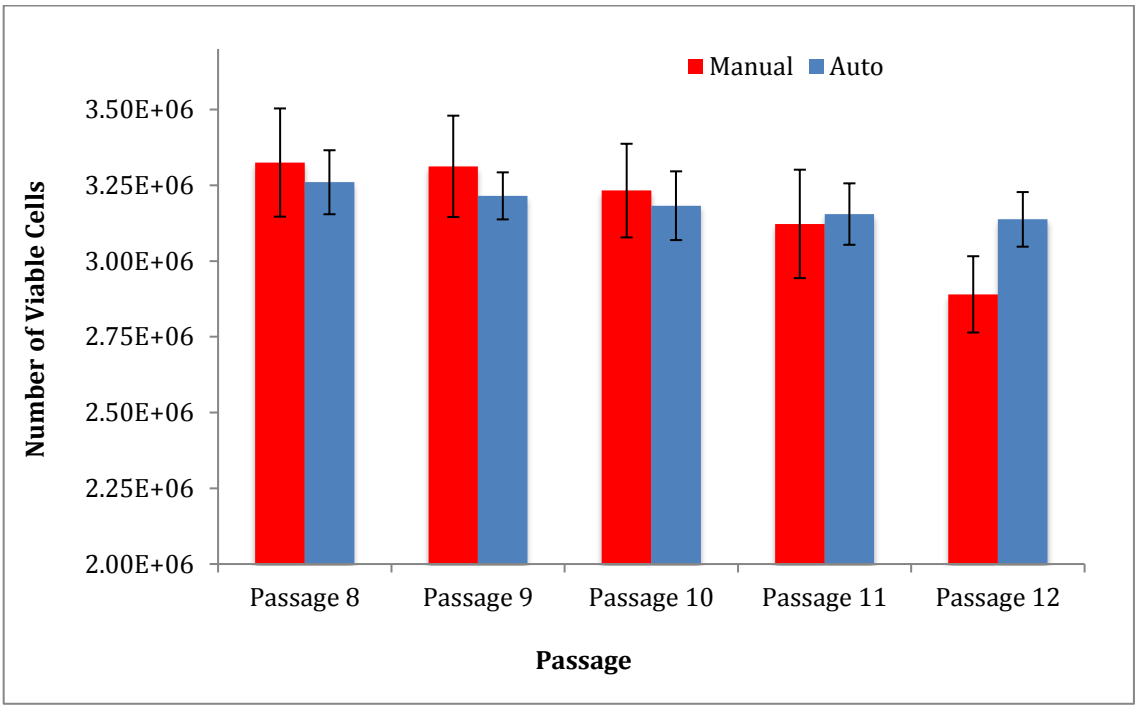


Figure 5.11 *Average number of viable MSCs obtained through manual and the automated cell culture process. Cells were seeded at 8.75 x 10^5 cells per T175 flask and were passaged after 3 days. Cells from passage 7 were passaged for a total of 5 passages. Error bars represent the standard deviation of the data (n=4).*

The average ammonium concentrations (and also the total production values as no ammonium was produced initially) at the end of the experiment (day 3) were 0.35 mmol/L for the manual and 0.28 mmol/L in the automated process flask. The average lactate concentrations at the end of the experiment were 2.83 mmol/L for the manual FBS-containing DMEM, 2.41 mmol/L in the automated process flask. These metabolites did not

reach the inhibitory concentrations reported in literature for the MSCs (Schop *et al*., 2009) of 20 mmol/L and 2 mmol/L for lactate and ammonium, respectively. Using these metabolite data, specific glucose and glutamine consumption values (pmol/cell/day) were calculated.

There was no significant difference in the glucose and glutamine consumption rates between the cells cultured *via* the manual and the automated process ($p > 0.05$). The specific glucose consumption rates for the manual and the automated process of 18.1 and 16.1 pM/cell/hour, respectively were within the range of values between 10 to 24 pmol/cell/hour quoted in literature (Lavrentieva *et al*., 2010; Dos Santos *et al*., 2011). The specific glutamine consumption rates for the manual and the automated process of 3.02 and 3.06 pM/cell/hour, respectively were within the range of values between 2 to 48 pmol/cell/hour quoted in literature (Lavrentieva *et al*., 2010; Dos Santos *et al*., 2011). No abnormal consumption trend was observed for either process.

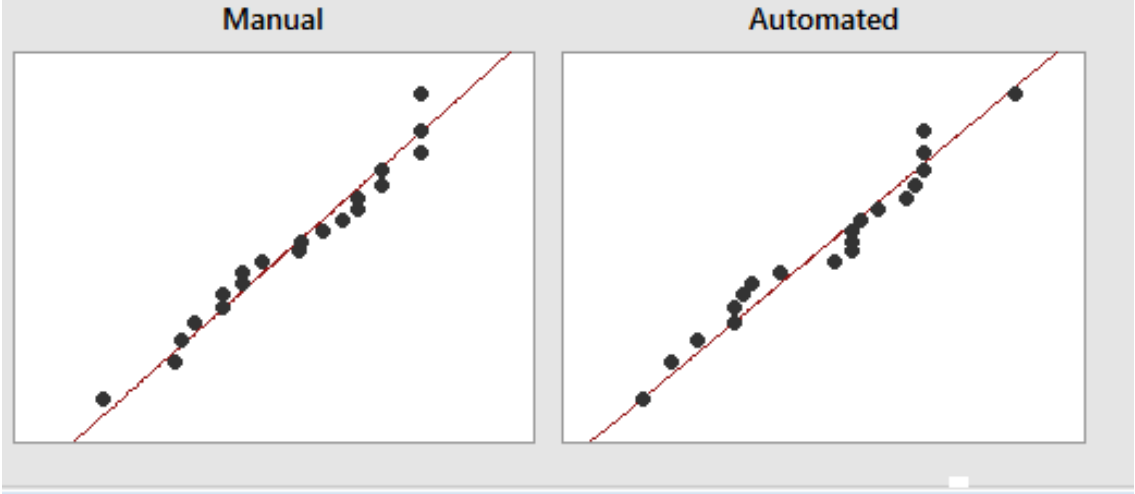
The average viable MSCs obtained for the manual culture were 3.33×10^6, 3.31×10^6, 3.23×10^6, 3.12×10^6 and 2.89×10^6 for passage 8, 9, 10, 11, and 12, respectively (Figure 5.11). The average viable MSCs obtained for the automated culture were 3.26×10^6, 3.22×10^6, 3.18×10^6, 3.16×10^6 and 3.14×10^6 for passage 8, 9, 10, 11, and 12, respectively (Figure 5.11). In terms of cell yield, there is no significant difference in the yield obtained between the 2 processes for all passages ($p > 0.05$), but the error bars demonstrate that there were smaller variation between the cell counts when they were cultured using the automated process (Figure 5.11). The population doubling (PD) values

obtained were between 3 to 4. The PD values obtained for the work discussed in Chapter 6 using early passage cells (passage 5) and same medium were between 4.5 to 5.0 PD, but this was to be expected when early passage cells were used (Majore *et al*., 2011).

As discussed in Chapter 1, Estimates of the process capability indices are used to relate the process capability to the product requirements for a selected performance characteristic (cell yield) of both processes and to provide a numerical measure of process robustness and production efficiency. When measuring process capability, Cp and Cpk are measures of short-term process capability and they are calculated using the estimates of the process standard deviations (Khan, 2013). Cp refers to the process capability to the specification range and it does not relate the location of the process with respect to the specifications, meanwhile Cpk considers process average and evaluates the process spread with respect to where the process is actually located. If the characteristics or process variation is centered between its specification limits, the calculated value for Cpk is equal to the calculated value of Cp (Wooluru *et al*., 2014).

For this study, Minitab's capability comparison assistant was used to carry out process capability analysis (statistical method is described in Section 3.3.1.3, Chapter 3). The advantage of using this capability assistant is that, this capability assistant allows two processes to be compared simultaneously. In order to carry out this analysis, 2 critical assumptions have to be considered (Khan, 2013), otherwise the results obtained might be highly unreliable: i) the distribution of the process must be considered normal and (ii) the process must be in statistical control. In order to investigate if the distribution of the

processes is normal, Normality test (Anderson-Darling) was carried out. The p-values above 0.05 obtained demonstrate the distribution is indeed normal (Figure 5.12).

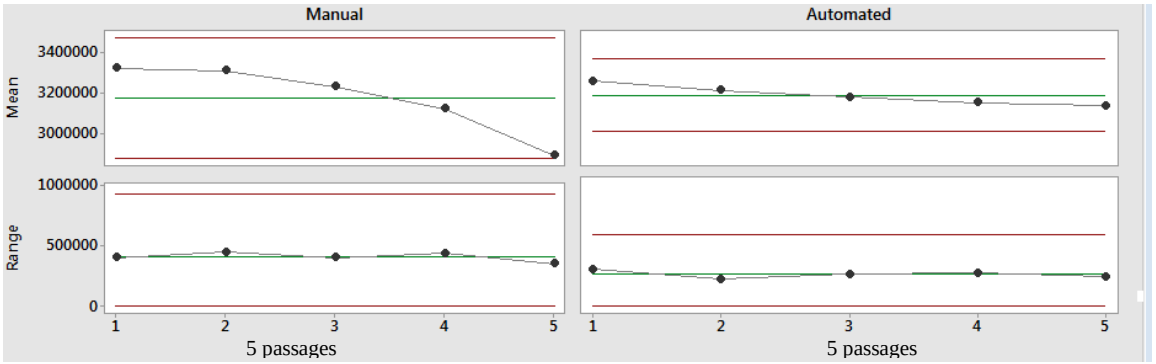


(a) P value: 0.530 (Pass) (b) P Value: 0.299 (Pass)

Figure 5.12 *Normality plots obtained for (a) manual process and (b) automated process. The spread demonstrates normal distribution as data points are very close to the theoretical line plotted. P value > 0.05 are obtained for both processes indicate normal distribution.*

In order to investigate if the process was within statistical control, Xbar-R control charts were used. The Xbar-R control charts obtained for the experimental data (Figure 5.13) confirm that both the manual and the automated processes were within statistical control. While both the processes were within the statistical control, the control charts show that that there was lesser shift in the mean for the automated process indicating a more stable process (Figure 5.13). This demonstrates that there is a greater reduction in the process mean for the manual process than in the automated equivalent. It is commonly known that cell proliferation potential decreases with increasing passage numbers, but it can be seen in the control charts that this is more obvious for the cells cultured manually. The data points

were within the 2 red lines and this indicated a stable process. Process capability analysis was then carried out, as both the critical assumptions were valid.



(a) (b)

Figure 5.13 *Control charts obtained for (a) manual process and (b) automated process for 5 passages (passages 8 to 12). The data points are within the 2 straight lines indicating a stable process.*

Figure 5.14 shows the spread of data for both the manual and the automated process. Both distributions were within specification limits. Process capability analysis (capability comparison analysis) revealed that there was no significant difference in means (average viable cell yield obtained) between the two processes ($P > 0.05$). The process means (average viable cells) for the manual and the automated cell culture process of 3.18×10^6 and 3.19×10^6 respectively, were located close to the centre of nominal specification. However, the more centred capability histogram (upper panel of Figure 5.14) for the automated process data illustrated better process capability as a result of lesser variations (lower standard deviation value). The standard deviation for the automated process was significantly lower than the standard deviation for the manual process ($p < 0.05$).

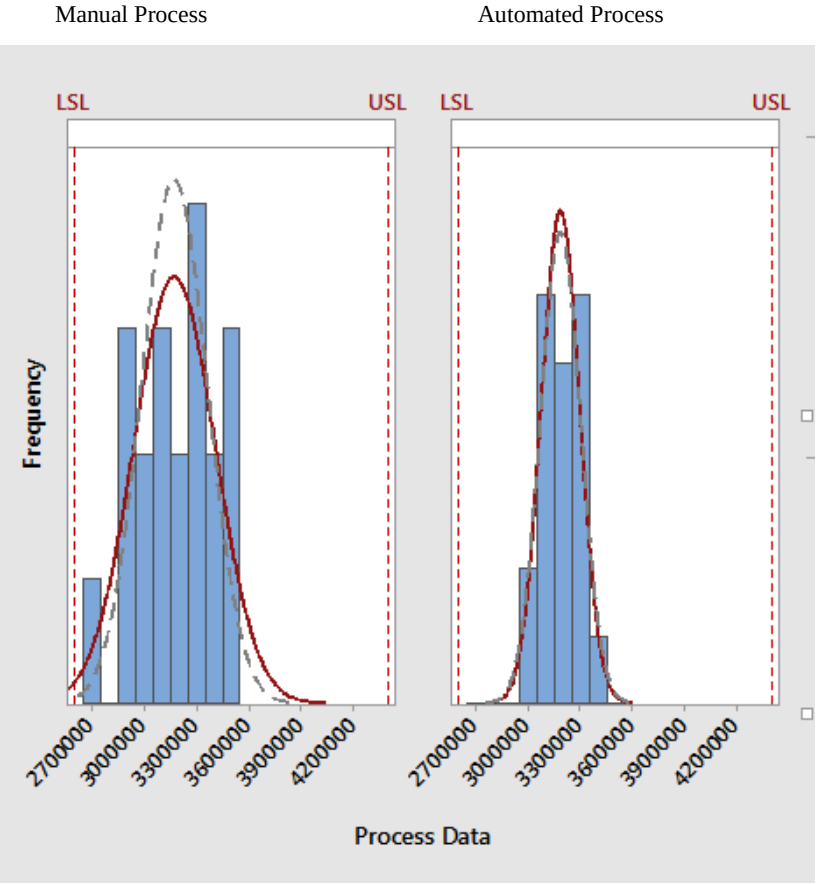


Figure 5.14 *Capability histogram obtained for both the manual and automated process.*

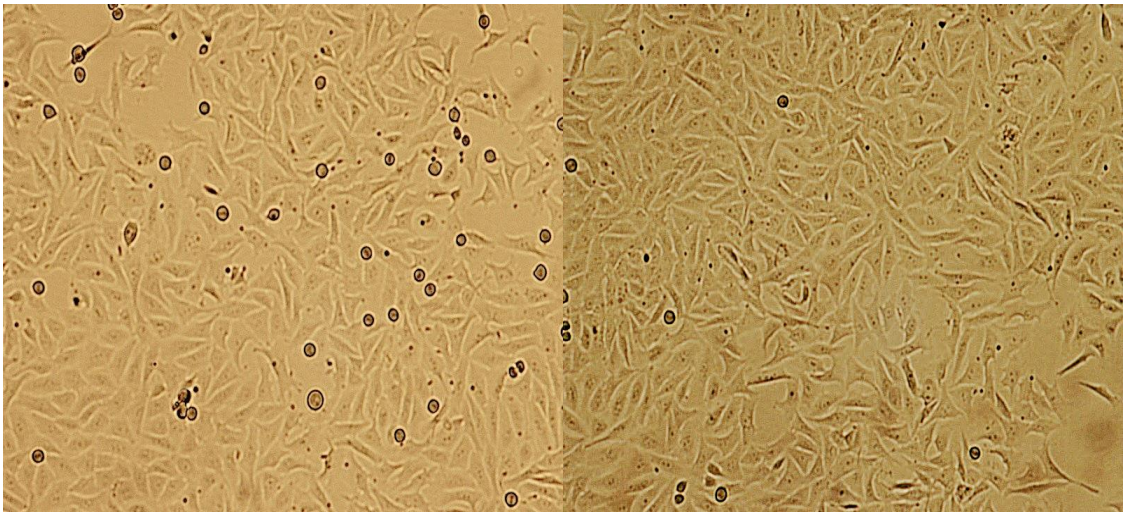
The Cp indices for both processes were above 1.33 and this indicated that both processes had high capability and were within the specification limits. However, the Cp index for the manual process of 1.58 was not similar to its corresponding Cpk index of 1.01. Also, the Cp index for the automated process of 2.60 was not similar to its corresponding Cpk index of 1.70. This indicated that both processes were not centered within the specification limits selected. This indicated that the process data distribution was not close to the mean (skewed to the left) and from the histogram (Figure 5.14) we can see that the data was closer to the lower specification limit (LSL). Higher Cpk index for the automated process

indicated that the automated process had better centering and from this we can deduce that automated process was more capable than the manual equivalent.

Process capability analysis demonstrated that in terms of yield, there was no significant difference between the manual and the automated process, but the automated process was capable of producing more consistent yields with lesser variations (Figure 5.13). In terms of stability and capability (based on the control charts, indices and the capability histogram), it can be seen that the automated process performs better than the manual process. Hence, based on the study carried out, it was concluded that the automated cell culture was capable of robust cell production.

5.5 Xeno- and Serum-Free MSC Culture Process (Manual Process)

In order to investigate if MSCs can be cultured in a serum-free medium, cells were cultured using StemPro medium. Due to the high cost of the StemPro medium, the experimental work (preliminary) was conducted with T25 (25 cm^2) flasks only. Cells from passage 7 were cultured up to passage 10 using a seeding density of 5 x 10^3 cells/cm^2 (total of 1.25 x 10^5 cells) per flask in T25 flask (described in Section 3.4.2.7, Chapter 3). All experiments performed resulted in a successful culture, with no observable problems associated with attachment or proliferation (Figure 5.15). The cell images are similar to the other MSC images (spindle shape morphology) found in literature (Nekanti *et al*., 2010).



(a) (b)

Figure 5.15 *Representative phase-contrast microscope images of passage 10 MSCs cultured manually in (a) FBS-containing medium, (b) StemPro medium. Cells were seeded at 5 x 10^3 cells/cm^2 per flask in 25cm^2 flask, and were passaged after 3 days for 3 consecutive passages. Magnification x40 was used.*

The average viable MSCs obtained for FBS-DMEM medium culture were 4.75 x 10^5, 4.64 x 10^5, 4.57 x 10^5 for passage 8, 9, and 10, respectively (Figure 5.16). The average viable MSCs obtained for the StemPro medium culture were 4.90 x 10^5, 4.61 x 10^5, 4.40 x 10^5 for passage 8, 9, and 10, respectively (Figure 5.16). The average viable cell numbers obtained for both cell cultures were not statistically different, although it was later shown (Chapter 6) that significantly more cells were obtained when the cells were cultured using StemPro medium ($p < 0.05$). The PD values obtained were below 4 PD, but the PD values obtained for the work discussed in Chapter 6 were between 4.5 to 6.5 PD. However, this is usually the case when late passage cells are used (Majore *et al*., 2011). It has been widely demonstrated that MSCs PD values decrease with the increasing passage number.

It was not understood why there was no statistical difference in the average number of cells obtained using the StemPro and the FBS-containing DMEM culture as the results obtain in Chapter 6 contradict with this finding. It is believed that the StemPro was less effective for MSC culture because the cells were exposed to FBS-containing DMEM for a longer time (7 passages). It was likely that the cells may have become more adjusted to the FBS-containing DMEM culture as they were cultured for 7 passages with FBS-DMEM medium. To avoid any potential suppression of cell proliferation capability, the work described in Chapter 6 demonstrates attempts to transfer MSCs to StemPro medium right after isolation of cells from umbilical cord tissues.

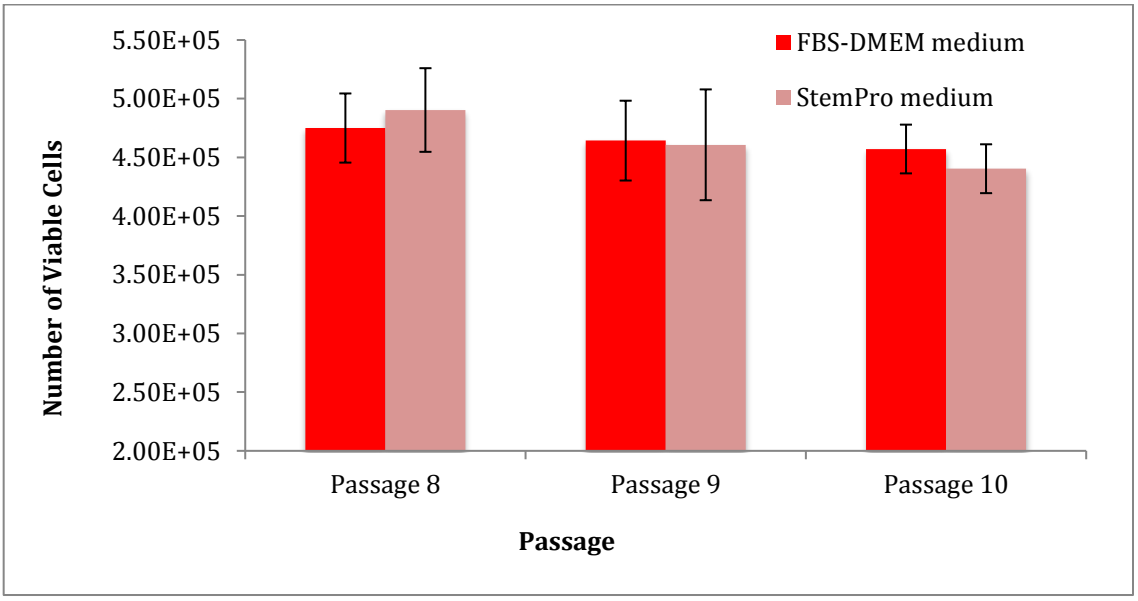


Figure 5.16 *Average number of viable MSCs obtained through FBS-DMEM and the StemPro medium culture. Cells were seeded at 1.25 x 10^5 cells per T25 flask. Cells from passage 7 were cultured for 3 consecutive passages. Error bars represent the standard deviation of the data (n=4).*

Results demonstrated that cells can be directly transferred to serum-free medium without any loss of cell viability (viability was above 90% for both the experimental conditions). All experiments performed resulted in successful cultures, with no observable problems with attachment or proliferation. These results were encouraging, as this meant that cells did not require weaning or any additional growth supplements to support cell growth in StemPro. This preliminary work demonstrates that cells can be easily transferred to the serum-free (StemPro) medium culture from the FBS-containing DMEM culture without any loss of viability.

While the preliminary results seem promising, the cost of the StemPro cell culture medium (approximately £160 per bottle) may pose a problem during large-scale cell manufacture since this is almost double the cost of using FBS-DMEM medium (approximately £70 per bottle including the cost of FBS). Since StemPro is the only synthetic medium for MSC culture that has obtained FDA clearance, there is no other choice, but to use this medium for cell culture until other alternative cheaper media obtain regulatory clearance. This illustrates that at the present time, the cost of automated manufacture will remain high due the high cost of the medium used.

5.6 Overall Assessment

The study discussed in Chapter 4 demonstrated that there was a significant difference in the yield between the automated and the manual process, and also that the difference was observed when the work was repeated with another cell line (with the HDF cell line). The authors who reported similar findings have speculated that the difference may be due to

methodological differences between the 2 processes (Thomas *et al*., 2007; Liu *et al*., 2010; Zhao *et al*., 2011). So far, no comprehensive study was ever carried out to characterise the automated cell culture process. This created the need for a comprehensive study on the automated process. To carry out this study, two different cell lines were used; HDF cell and the MSC. Part of the work developed with the HDF cells was re-tested with MSCs (when these cells became available) to confirm that they were indeed applicable for MSC expansion. The MSA study carried out demonstrated that although the machine was a validated system, the pipetting system was not accurately dispensing liquid (prior to re-calibration). The type 1 Gauge analysis (MSA) study carried out in this chapter to assess repeatability confirmed that the Cedex cell counter demonstrated both good precision and accuracy.

After the completion of the work to assess the performance of the key components of the automated platform, the key differences between the processes (methodology) were also studied. Two key differences were highlighted and they included the harvesting and also the re-seeding process. Through several experimental studies (using HDF cells), it was concluded that the harvesting process was not the reason why the cell yield from the automated process was lower. Not having a centrifuge did not pose a disadvantage. In addition to the pipetting discrepancy, the other factor contributing to reduced automated process yield (for HDF cells) was due to the unique characteristic of the cell line itself. It was found that the cells were attaching to all surfaces of the flask even before the seeding process was complete and this resulted in seeding less than programmed cells into the new flasks. Despite MSCs sharing similar properties with this cell line, the MSCs did not

demonstrate any significant attachment behavior during the cell seeding process. This is an important finding, as it was not previously known that cells attachment behavior (unspecific attachment) could result in a significantly lower cell yield. Although the culture of MSCs have always been identified as a highly complicated process (Karp and Teo, 2009), the automated culture of MSCs is significantly less complicated that that of the HDF cells as there is no need for additional coated flask to prevent cell attachment during the re-seeding stage (discussed earlier).

The work developed with HDF cells enabled a good understanding of the automated process, but every cell line is unique. Therefore, the work developed (protocol) had to be tested with MSCs. In contrast to the results obtained with the HDF cells, the MSCs exhibited a decrease in cell viability after a passage of culture when the original protocol was used. Since the automated platform contains no centrifuge, during the detachment process, the automated cell culture protocol (generic protocol) is developed in such a way that the detachment enzyme is neutralised with an equal amount of cell culture medium. However, early work carried out by Thomas *et al.,* 2008 confirmed that leaving the detachment enzyme in the cell culture results in inhibition of MSC growth. This indicates that protocol modification was necessary to increase the automated process yield and to prevent the loss of cell viability.

This original protocol was then modified to avoid leaving the detachment enzyme with the cells in the incubator throughout the incubation time because this resulted in the enzyme being brought forward to the new passage (when the cells were seeded into new flasks).

During the dissociation step, after applying the detachment enzyme, the enzyme was immediately poured off, but it was first ensured that the whole surface of the T-flask was first immersed with the enzyme. This flask with only a residual coating of enzyme was then incubated. In order to test if this method could result in good process yield, 4 new protocols were developed and tested with MSCs. Through series of work, the optimum incubation time of 8 minutes was selected, but some additional steps were included to maximise cell recovery. This work showed for the first time that cells could be detached without leaving the detachment enzyme in the flask throughout the incubation period.

The developed protocol was then tested with MSCs over a period of 5 passages. The manual process was used as the benchmark process. There was no significant difference between the manual and the automated process yield. In terms of metabolite data, there was also no significant difference between the two processes in terms of glucose and glutamine consumption rates. Ammonium and lactate concentrations for both the manual and automated process were below inhibitory levels. Process capability analysis using the data obtained for these runs was carried out using Minitab software. Both processes were robust, but the automated process demonstrated better process capability than the manual equivalent. This demonstrated that the automated protocol developed and optimised was indeed suitable for the automated culture of MSCs. Process capability analyses have been used in mature industry sectors such as the medical devices and electronic sectors, but have not been extensively used in cell therapy area (Liu *et al.*, 2010). The capability analysis carried out in this study is a novel attempt to provide a numerical measure of MSC culture process robustness.

Finally, to investigate if MSCs could be cultured in a serum-free medium, a small-scale manual study was carried out. MSCs were cultured in both StemPro (serum free) and DMEM-FBS medium. This work resulted in successful cultures, with no observable problems associated with attachment or proliferation. The yield obtained for the StemPro culture was not significantly different than the yield obtained for the DMEM-FBS culture. The results were promising as it indicated that serum- and xeno-free cell culture conditions can be considered for MSCs. For clinical therapies, to facilitate regulatory approvals, the use of animal-derived components should be avoided (discussed in Chapter 1). Although the cost of using xeno-free reagents may contribute to an increase in the process costs, the use of FBS can also cause a considerable increase in process costs since all biologically derived starting materials, including FBS need to be screened and or tested for the presence of adventitious agents if they were to be used in the cell manufacturing process. Therefore, the use of animal-derived component should be avoided if it was possible. This is also one of the advantages of using Tryple Express, instead of trypsin-EDTA as the detachment enzyme for the MSC manufacturing process as Tryple Express is derived from animal free source.

The objectives of this chapter are: (i) to study some of the factors contributing to the growth difference and to identify the factors that have large impact on cell growth and (ii) to use the knowledge gained from (i) towards developing a robust automated MSC culture protocol. The work carried out this chapter enabled these objectives to be met. The development of a robust automated method is mandatory to reduce waste during large-scale process runs. Many organisations are now looking for new methods to reduce waste

and to maximise profit, therefore are considering tools such as Lean Manufacturing, Six Sigma, and Kaizen. These tools are not necessarily easy or cost effective to implement during serial production stage as most processes would have been finalised and to make any minor process changes, significant amount of work or cost would be necessary (Kamani *et al.,* 2014). The creation of a robust protocol (such as the one developed) should be done at the development stage to reduce waste at a later stage. Material and components used should be thoroughly characterised at this stage (development), as it will be more complicated to introduce changes once a process has been validated.

The use of model cell line allowed the objectives of the chapter to be achieved in a more cost effective manner. There is also no real concern about the applicability of the work developed for MSC culture as the developed protocols were tested with MSCs and were "fine-tuned" to suit MSCs. The success achieved with the work carried out in this chapter provides the impetus to carry on with the work required to demonstrate that the automated process developed has real clinical applications (Chapter 6). In order to confirm that the developed automated MSC culture process can be used for clinical applications, additional data are required by the International Society of Cellular Therapy (ISCT) (detailed in Chapter 6). Ultimately, the work carried out can be used as a reference or guidance for many automation studies as most flask-based automated platforms operate in a similar manner. There might be some subtle differences, but some of the basic functions (pipetting, incubation, and cell counting) are common for all. Although most automated systems have been validated, the findings of this study have shown that it is still necessary to evaluate the performance of the automated system before use through MSA study.

5.7 Conclusion

The work discussed in this chapter was able to address the objectives and the technical questions listed earlier in the chapter. The investigation and the development studies carried out to meet the objective of this chapter enabled the limitations of the system to be identified. In addition to that, they also enabled a better understanding of the cell culture processes. Prior to this study, it was not known that cell loss was occurring due to the unique cell behaviour. It was also not known previously that the automated pipette was not pipetting the desired volume (although the automated platform was a validated system). This study allowed the creation of a robust and optimal automated cell culture protocol for the culture of MSCs. This study also confirmed that the centrifugation process was not necessary for the automated cell expansion (irrespective of the automated platform used). Compact Select is currently being used in many major pharmaceutical companies including GlaxoSmithKline, Merck, Pfizer, Bristol-Myers Squibb, AstraZeneca and Pfizer (www.tapbiosystems.com). Therefore, the work carried out has a wide applicability as the work carried out here not only demonstrates the applicability of the system for MSC culture, but also the capability (and the stability) of the system for MSC culture.

As identified in Chapter 1, the main aim of the doctoral project is to investigate the possibility of developing a robust automated MSC culture process that is capable of producing clinically relevant product in order to facilitate successful commercialisation of this stem cell therapy. In order to achieve this aim several studies were carried out based on the objectives that were listed out in Chapter 1. The work carried out to meet the objectives listed out in this chapter was able to demonstrate: (i) a robust automated MSC

culture protocol can be developed and (ii) MSCs can be cultured using xeno- and serum-free medium.

Since the main limitation of this doctoral study was the availability of MSCs and the cost of these cells and reagents, a significant part of the work had to be carried out with model cell lines and mid-passage MSCs. However, the work developed still needs to be tested with early passage MSCs to demonstrate the relevance of all the development work carried out so far. In addition, it has to be demonstrated that MSCs produced from the automated process run are capable of meeting the requirements set by the ISCT. Most automation studies carried out with MSCs so far are proof-of concept studies mainly to demonstrate MSCs can be automated. These studies have not carried out differentiation studies (Scott, 2009; Thomas *et al*., 2007). The greatest hurdle in clinical-scale stem cell expansion is to be able to maintain the phenotype and the viability of cells throughout the cell expansion process, therefore the demonstration of these characteristics (as required by the ISCT) are mandatory to prove their clinical relevance. The will be the objective of the studies discussed in the next chapter (Chapter 6).

Chapter 6. Results and Discussion: Automation of Mesenchymal Stem Cell (MSC) Culture Process

6.1 Introduction

As described in Chapter 2, MSCs can be isolated from many different tissue sources. For this study, umbilical cord-derived mesenchymal stem cells (UC-MSCs) were used. These cells can be acquired through non-invasive and painless collection procedures and in terms of cost they are the best option as they are extracted from clinical waste. Umbilical cords are also an ethically non-controversial source of MSCs. These cells have extensive expansion capabilities, are multipotent, and do not induce teratomas when delivered to the patient (Fong *et al.*, 2012). These factors make UC-MSCs an appealing choice of stem cells for this study.

As identified in Chapter 1, the main aim of the doctoral project was to investigate the possibility of developing a robust automated mesenchymal stem cell (MSC) culture process that is capable of producing a clinically relevant product in order to facilitate successful commercialisation of this stem cell therapy. In order to achieve this aim several studies were carried out based on the objectives that were listed out in Chapter 1. Throughout the study, the main limitations were the availability of MSCs, the cost of these cells and also the cost of reagents for MSC culture. Due to these reasons, the work had to be carried out with model cell lines and mid-passage MSCs. Therefore, the work developed still needs to be tested with early passage MSCs to demonstrate the relevance of all the development work carried out so far.

The studies carried out in the preceding chapter (Chapter 5) demonstrated that the development of a robust automated MSC culture expansion process was possible. The potential of the automated protocol developed was demonstrated for a total of 5 passages. The automated process demonstrated better capability and stability than the manual equivalent. However, to demonstrate the clinical relevance of the developed automated cell expansion process, the cells produced from this process must be capable of meeting the requirements set by the International Society of Cellular Therapy (ISCT).

As discussed in Chapter 5, the greatest hurdle in clinical-scale stem cell expansion is to be able to maintain the phenotype and the viability of cells throughout the cell expansion process, therefore the demonstration of these characteristics (as required by the ISCT) are mandatory to prove their clinical relevance. It is also detailed in the preceding chapter (Chapter 5) that HDF cells are very similar to MSCs (in terms of cell surface markers and morphology) and the main method of distinguishing them is by carrying out differentiation studies.

International Society of Cellular Therapy has proposed three criteria to define MSCs (Dominici *et al.*, 2006). First, MSC must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks. Second, 95% of the MSC population must express CD105, CD73 and CD90 and these cells must lack expression (less than 2% positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II (as measured by flow cytometry). Since the biological property that most uniquely identifies MSC is their capacity for tri-lineage MSC differentiation, the cells must be shown to

differentiate to osteoblasts, adipocytes and chondroblasts using standard in vitro tissue culture-differentiating conditions. Differentiation to osteoblasts can be demonstrated by staining with Alizarin Red or von Kossa staining. Adipocyte and chondroblast differentiation are usually demonstrated by staining with Oil Red O and Alcian blue, respectively. Most published protocols for such differentiations are similar, and kits for such assays are now commercially available.

The use of mid- and late-passage cells (passage 7 and above) was the best option in terms of cost for development work, but another important factor that facilitates regulatory approval and also ensures reproducibility is the ability to keep record of complete production history of a process. This is only possible if all process parameters are fully known and fully defined. This includes knowing the complete history of the cells used from the time of extraction to the last passage they are expanded to. For this reason, MSCs used in the study described in this chapter will be the cells obtained immediately after isolation from umbilical cord tissues. Whenever biological materials, especially mammalian cells are used for bioprocessing studies, it has to be demonstrated that the results are reproducible even when cells are obtained from a different source (in this case, a different umbilical cord). For this reason, the study discussed in this chapter will be using cells from two different umbilical cords. The cells will not be pooled to gauge if there are any significant biological variations. Flow cytometry analysis will be performed for cells from both the cords to understand how the cells from two cords differ in terms of surface marker expression levels.

The development of a suitable Good Manufacturing Practices (GMPs) compliant process for clinical-grade production of MSCs is necessary to deliver the biological product to the market. In addition to knowing the complete history of the cells, even the variables of a production process such as the culture reagents and the expansion system used should be defined. The current standard procedure for *in vitro* culture of MSCs is based on adding DMEM with foetal bovine serum (FBS) (Muller *et al.,* 2006), but the use of a component like FBS in the large-scale expansion of MSCs leads to variability in cell growth characteristics. The serum proteins found in FBS have the potential to initiate xenogeneic immune responses (Shahdadfar *et al.*, 2005; Dimarakis and Levicar, 2006), and this can affect the safety of the patients who receive MSCs (Heiskanen *et al.* 2007).

The ill-defined nature of serum-based media is not desirable for clinical applications as it places a heavy burden on researchers to provide well-defined data on all the components used in their study. The use of serum-free-media can lead to a better consistency of large-scale production by reducing the variability between batches. There are various serum-free media available on the market (discussed in Chapter 2), but the StemPro MSC culture medium is currently the only FDA-cleared medium. Various researchers have demonstrated that MSCs exhibit increased proliferation rates when cultured in StemPro medium (Agata *et al*., 2009; Chase *et al*., 2010; Dos Santos *et al*., 2011). The preliminary experiments to test the applicability of xeno-free culture reagents in MSC culture (presented in Chapter 5) have yielded positive results in terms of cell viability. However, more data such as cell phenotype and cell differentiation potential are required in order to confirm that xeno-free medium is a suitable replacement for the traditional serum-

containing medium.

A critical review of the MSC culture automation work has revealed that most of the automation studies carried out with MSCs so far were not comprehensive studies and were done mainly to demonstrate that the MSC culture process is scalable. The study conducted by Scott (2009) is brief as his single attempt to expand bone marrow-derived MSC culture using the automated platform resulted in failure as cells were contaminated after one passage (Scott, 2009). The study conducted by Thomas *et al.* (2007) with bone marrow-derived MSCs demonstrated MSC culture can be automated for a limited number of passages. This study only used surface markery analysis (flow cytometry) to confirm cell phenotype and there was a difference in cell growth rate between the manual and the automated process.

In Chapter 5, it was demonstrated that the general automated cell detachment method (used by Thomas *et al.,* 2007) was not suitable for the MSCs, therefore the protocol was modified. The modified protocol demonstrated enhanced cell viability and was found to be more suitable for the MSCs. In addition, several other improvements were made to increase the cell productivity (methods are detailed in Chapter 3 and there results are discussed in Chapter 5). The work carried out in Chapter 5 allowed the development of a robust and an optimal automated cell culture protocol that can be used to achieve the aim of the doctoral study, but the success of the development work can only be confirmed by doing a comprehensive analysis that is capable of meeting the requirements of ISCT.

Thus, the objective of the study described in this chapter is to meet the aim of the doctoral study; that is to investigate the possibility of developing a robust MSC culture process for clinical applications. The experiment will be carried out using cells from two umbilical cords (side-by-side comparison). In addition, to the required analyses (as set by ISCT), additional analyses will be carried to better characterise the cell culture processes. The manual MSC culture process will be used as the baseline process.

The key questions that will be addressed in this chapter within this context are listed below;

- Can xeno- and serum-free culture conditions be considered for automated MSC cell culture processes?
- Can the Compact Select be applied to the allogeneic cell therapy area?

This chapter is divided into 5 sections. Section 6.2 discusses the experimental work carried out with MSCs. Section 6.3 is dedicated towards discussing the potential application of the Compact Select in the allogeneic cell therapy area. The main experimental findings are discussed and summarised in Section 6.4 and 6.5, respectively.

6.2 Automating MSC Culture

MSCs were obtained from all sections of the human umbilical cords. All the experiments described in this chapter were performed in triplicate and were repeated with cells from another umbilical cord (n = 6). All the analyses were carried out at passage 5 as it was not

feasible to analyse cells at every passage due to limited cell availability at early passages. The experimental used is detailed in Section 3.4.3, Chapter 3.

As previously discussed in Chapter 4, it was deemed that a seeding density of 5×10^3 cells/cm^2 was suitable for successful MSC culture. Although various authors have shown that a lower seeding density increases the doubling rates, extensive work done by Majore *et al*. (2011) demonstrated that when cells are seeded at higher concentrations ($> 4 \times 10^3$ cells/cm^2), cells can retain their MSC characteristics for longer. Throughout this experiment, only a seeding density of 8.75×10^5 cells or 5×10^3 cells/cm^2 was used.

Cells were isolated from a human umbilical cord according to the protocol described in Section 3.2.3, Chapter 3. Cells from a single cord were split into 2 and one part was cultured in FBS-containing DMEM and the other in StemPro until the first passage. Cells cultured in StemPro (passage 0) did not survive, therefore, the flask was discarded (representative cell images attached in Appendix V), but at passage 2, some cells isolated into FBS-containing DMEM were placed in both StemPro and FBS-Containing DMEM. After passage 2, 3 flasks with Stempro, and 3 flasks with FBS-containing DMEM were simultaneously placed in the automated and the manual cell culture incubator, respectively, and were expanded according to methods described in Chapter 3. Cells were cultured until passage 5 via both the manual and automated cell culture methods.

In order to compare the two processes (manual and automated), a number of important parameters were identified and analysed. These parameters include cell morphology

(Section 6.2.1), cell growth and metabolite analysis (Section 6.2.2), cell surface marker analysis (Section 6.2.3) and cell differentiation (Section 6.2.4). These analyses were carried out according to methods described in Chapter 3.

6.2.1 Cell morphology

All experiments performed resulted in a successful culture, with no observable problems associated with attachment or proliferation, but it can be seen in Figure 6.1 (day 3 of the culture), that there were more adherent cells in flasks containing StemPro. Flasks containing the cells that were cultured in FBS-containing DMEM appeared to be less tightly packed on the day 3 of the cell culture process, but this it to be expected when cells are less confluent (Jackson *et al.,* 2009). The cell-to-cell gap was also wider for these cells, and their spindle-shaped morphology was more evident as they were not too closely packed. These cell images were similar to the other MSC images found in the literature (Pereira *et al.,* 1995; Sugii *et al.,* 2010; Nekanti *et al.,* 2010). Although these cells demonstrate similar morphology to the mid-passage MSCs (Chapter 5), the images obtained here (Figure 6.1) show a greater degree of confluence than those images. For both studies, cell culture time of 3 days was employed. It was also observed that, in terms of morphology, these cells were similar to HDF cells (Chapter 5).

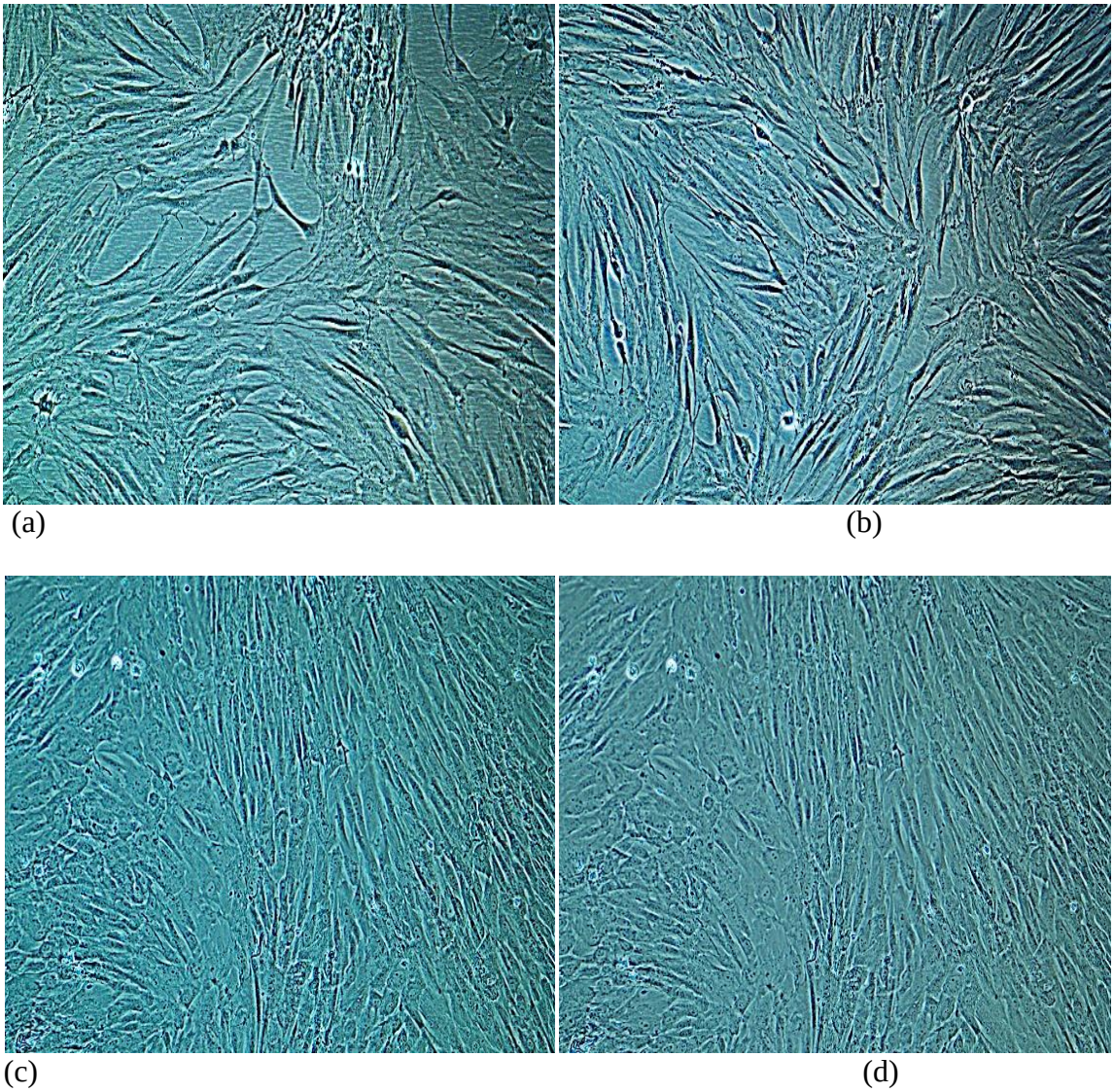


Figure 6.1 *Representative phase-contrast microscope images of MSCs at Passage 5. Images (a) and (b) are the images of the cells cultured using the FBS-containing DMEM via the manual and the automated process, respectively. Images (c) and (d) are the images of the cells cultured using the StemPro medium via the manual and the automated process, respectively. Cells were seeded at 5 x* 10^3 *cells/*cm^2 *per flask in T175 flask and were passaged after 3 days. Magnification x100 was used.*

6.2.2 Cell growth and metabolite analysis

Figure 6.2 shows the number of cells/cm^2 obtained over the 3 days by using the traditional FBS-containing DMEM seeding density was 2.32 x 10^4 for the manual cell culture process, and 2.38 x 10^4 for the automated cell culture process. This figure also illustrates that the number of cells/cm^2 obtained by using the StemPro MSC medium was 3.14 x 10^4 for the manual cell culture process, and 3.07 x 10^4 for the automated cell culture process. There was no significant difference in average cell numbers obtained from the manual and automated processes ($p > 0.05$), but there was a significant difference in cell numbers obtained by using two different types of media ($p < 0.05$).

Cells from 2 cords were not pooled in order to assess if there was any distinguishable cord-to-cord variation, but this study was not able to demonstrate any significant variation as shown by the range of the error bars (Figure 6.2), but it is important to note that only 2 umbilical cords were compared due to their limited availability. For the actual clinical use, MSCs are encouraged to be pooled as there has been claims that merging MSCs from different donors has been shown to generate higher and more stable suppression in both mixed lymphocyte cultures (MLC) and after phytohemaglutinin (PHA) stimulation (Samuelsson *et al.*, 2009). The authors (Samuelsson *et al.*, 2009) claimed that MSCs interact together to boost immunosuppressive functions, and cell pooling is possible with these cells as HLA-matching with the recipient is not required for immunosuppression. However, for experimental use, pooling is not recommended so that any adverse events observed can be traced back to the biological sample used.

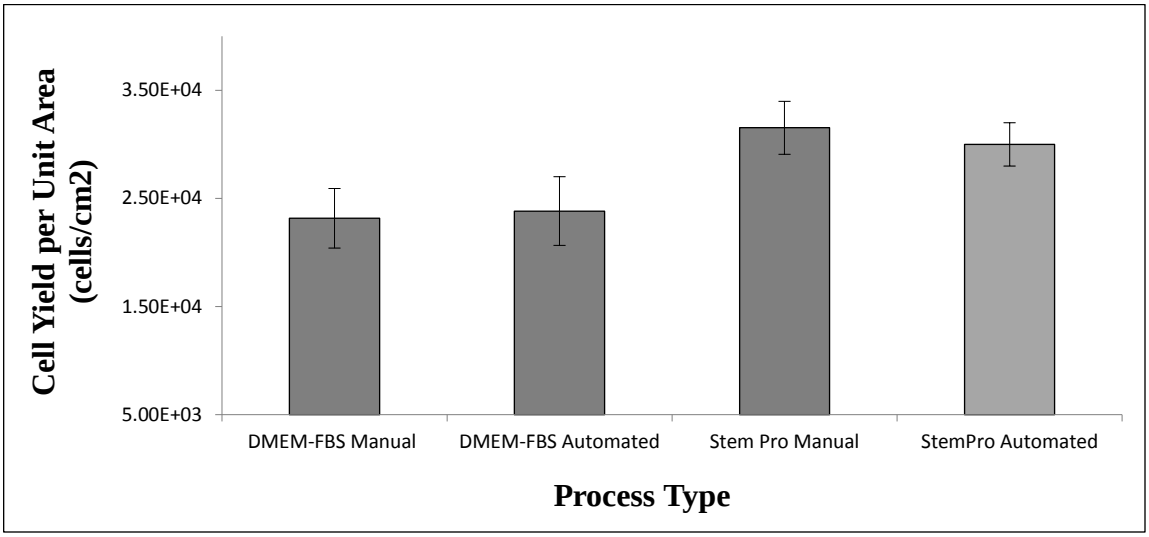


Figure 6.2 *compares the cell yield obtained after 3 days in culture when cells were seeded at 5 ± 0.06 x 10^3 cells/cm^2 in T175 flasks.*

The early passage cells reached confluence within the 3 days for all the experimental conditions, but the cells cultured in the FBS-containing DMEM were the least confluent of all. The average cell population doubling times for cells at passage 5 corresponded to 32.5 hours for the manual FBS-containing DMEM, 32.0 hours for the automated FBS-containing DMEM, 27.5 hours for the manual StemPro medium, and finally 27.1 hours for the automated StemPro cell culture processes. Cells thus exhibited a higher growth rate when they were cultured in StemPro and this consistent with the findings of the study carried out by Agata *et al.,* 2009) that used similar media for their UC-MSC culture process. The cell doubling times obtained for UC-MSCs were lower than the doubling times between 29.4 to 36.1 hours for passage 5 reported in the literature (Ren *et al*., 2016; Majore *et al.,* 2010) and this indicates that the processes used to expand MSCs were optimal.

Cells collected from the umbilical cord exhibited remarkable cell doubling potential as the doubling times reported in the literature for BM-MSCs are usually only between 38 to 42 hours (Suva *et al.*, 2004; Sarugaser *et al.*, 2005; Baksh *et al.*, 2007; La Rocca *et al.*, 2009). A recent study by Simoes *et al.* (*2*013) reported that when compared to adult sources such as adipose and bone marrow, the UC-MSCs displayed similar proliferation abilities; average folds increase of 7.4 for FBS-containing DMEM culture and 11.0 for StemPro medium culture. As the results presented here corroborated their findings, it is possible to conclude that the UC-MSCs have greater expansion potential than the BM-MSCs.

The work here has also shown that it was possible to obtain approximately 9 billion cells from the StemPro UC-MSC culture in 2 weeks of culture based on an initial (passage 0) seeding density of $8.75x10^5$ cells (5×10^3 cells/cm^2). This calculation was based on a fold increase value of 6.3 (i.e., 3.14×10^5 cells obtained per cm^2 ÷ by 5×10^3 cells seeded per cm^2). This fold increase value was obtained from the experimental data shown in Figure 6.2. If the BM-MSCs were to be used, using figures quoted by Simoes *et al.* (*2*013), and a similar seeding density as above, only 80 million cells can be obtained in 2 weeks. Thus, this highlights the potential of UC-MSCs for clinical applications.

Agata *et al.* (2009) reported that the StemPro MSC medium encouraged rapid growth of BM-MSCs at early passages, but caused the cells to senesce at passage 5 by gradually reducing the proliferation rate, therefore, it was necessary to investigate if the StemPro MSC medium had such effect on the cells. The authors also reported that the cells cultured in the traditional FBS-containing medium were able to retain their proliferation

capabilities, and continued to proliferate beyond passage 5. In order to assess the proliferation potential based on the mitochondrial activity, MTT assay was conducted using the MTT Cell Growth Kit (Chemicon, USA). This assay demonstrated that the cells cultured in StemPro medium were more metabolically active than cells cultured in FBS-containing DMEM (Figure 6.3). This indicated that the StemPro cell culture medium enabled an increase in the cell growth rate without reducing the mitochondrial potential of the cells. Therefore, this demonstrates that it is possible to culture MSCs beyond passage 5 using StemPro medium.

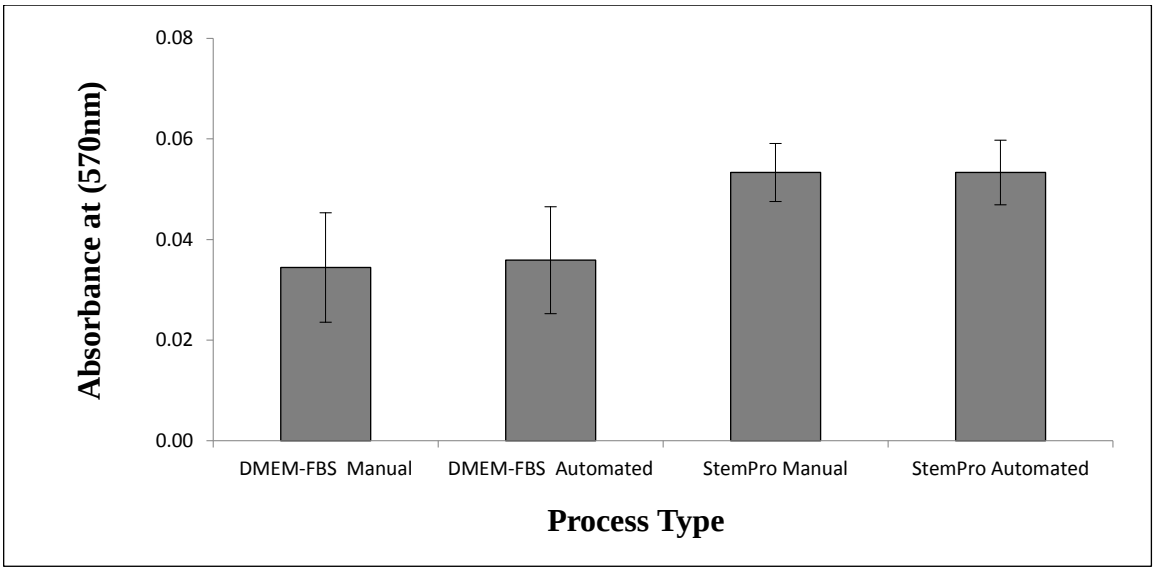


Figure 6.3 *Absorbance measured using (Chemicon, USA) MTT assay kit*

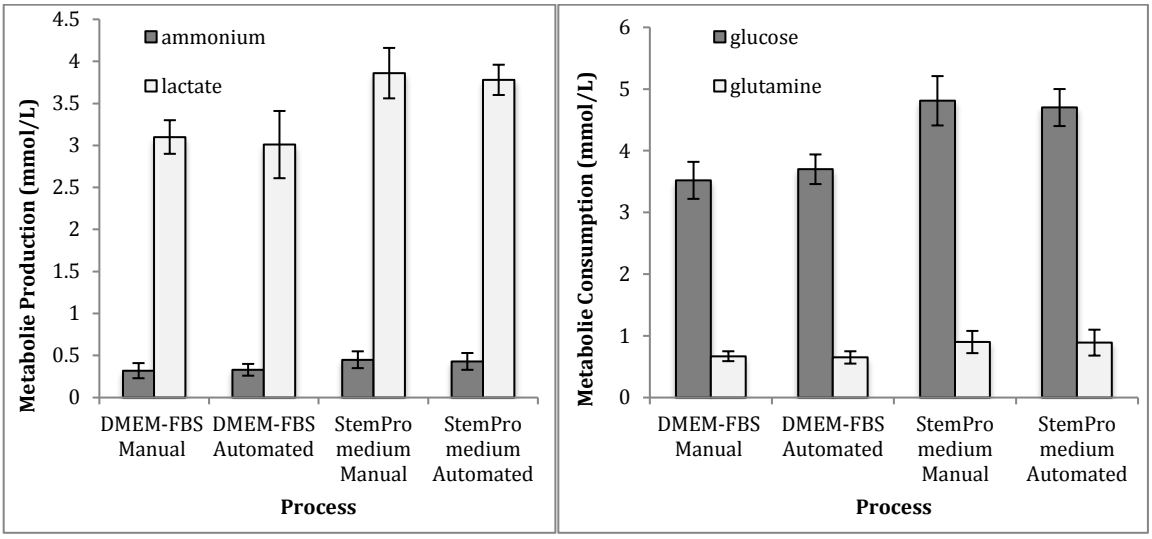
UC-MSCs can be isolated from various sections of the umbilical cord, such as the sub-amnion, Wharton's Jelly, and adventitia (Kim *et al*., 2013). In this study, the cells were obtained from the whole of the umbilical cord, and not just from the Wharton's Jelly. Nekanti *et al*. (2010) claimed that the cells from the Wharton's Jelly have higher proliferative rate than from other parts of the umbilical cord, but some researchers (Kita *et*

al., 2010; Stubbendorff *et al.,* 2013) disputed this by demonstrating that the cord-lining-derived cells have the highest proliferation rate. Another recent research finding (Mennan *et al.,* 2013) demonstrated that there was no significant difference between cells from any of these sections. It was believed that proliferation rate also depends on the umbilical cord source, such as the age and the weight of the donor therefore, it will be difficult to determine the accurate reason for differences in cell proliferation as there are too many variables involved.

Although various researchers have reported that UC-MSCs have higher proliferation rates than the other types of MSCs, the factors that govern the cell proliferation potential were not completely understood. Pietila *et al*. (2012) carried out a Transmission Electron Microscopy (TEM) analysis, and compared the UC-MSCs and BM-MSCs from a wide range of age groups (18 to 50 years). The authors reported that the mitochondrial-to-cytoplasm area ratio in UC-MSCs was higher than that of in the BM-MSCs, and also that the UC-MSCs showed more abundant rough endoplasmic reticulum (rER) than BM-MSCs. These findings may indicate the overall maturation levels of these cells and possibly can be used to determine the exact passage when cells are no longer suitable for clinical use (i.e. late passage cells that may have lost their stem cell characteristics, but so far only one study has made such claims. At present, early passage cells are preferred for clinical use to ensure that the cells are still maintaining all the desired functions.

In addition to all the analyses mentioned above, the MSCs nutrient consumption and metabolite production were also assessed to enable a better understanding of how different

culture conditions affect cellular metabolism. The metabolite data enabled the specific consumption rates (for glutamine and glucose pM/cell/hour) and the apparent yield values (lactate from glucose and ammonium from glutamine in mmol/mmol) to be calculated. Medium samples were taken at 0 hour and at the end of the cell culture process (after 72 hours) and were analysed using a bioanalyser. The metabolite concentration profiles are presented in Figures 6.4 a and 6.4 b.



(a) (b)

Figure 6.4 *(a) metabolite production in mmol/L and (b) metabolite consumption in mmol/L. Error bars represent the standard deviation of the data (n = 6).*

Similarly to the results obtained for the MSC cultured in the preceding chapter (Chapter 5), the concentrations of glucose and glutamine decreased while the concentrations of lactate and ammonium increased. Initially (at 0 hour), neither ammonium nor lactate was detected in the culture processes. The average initial concentrations of glucose and glutamine for the (manual and automated) FBS-containing DMEM cultures

were 5.52 mmol/L and 1.98 mmol/L, respectively. The average initial concentrations of glucose and glutamine for the manual and automated StemPro-containing medium cultures were 5.01 mmol/L and 2.02 mmol/L, respectively.

The average ammonium concentrations (and also the total production values as no ammonium were produced initially) at the end of the experiment (day 3) were 0.32 mmol/L for the manual FBS-containing DMEM, 0.33 mmol/L for the automated FBS-containing DMEM, 0.45 mmol/L for the manual StemPro medium, and finally 0.43 mmol/L for the automated StemPro medium. The average lactate concentrations at the end of the experiment were 3.10 mmol/L for the manual FBS-containing DMEM, 3.01 mmol/L for the automated FBS-containing DMEM, 3.86 mmol/L for the manual StemPro medium, and 3.78 mmol/L for the automated StemPro medium. These metabolites did not reach the inhibitory concentrations reported in literature for the MSCs (Schop *et al*., 2009) of 20 mmol/L and 2 mmol/L for lactate and ammonium, respectively.

The glucose consumed (in mmol/L) by the cells cultured using the FBS-containing DMEM were 3.52 mmol/L for the manual process and 3.71 mmol/L for the automated process. These consumption values are lower than the values obtained for the cells cultured using the StemPro medium; 4.81 mmol/L for the manual process, and 4.70 mmol/L for the automated process. The glutamine consumed by the cells cultured using the FBS-containing DMEM were 0.67 mmol/L for the manual process and 0.65 mmol/L for the automated process. These values are lower than the values obtained for the cells cultured using the StemPro medium; 0.90 mmol/L for the manual process, and 0.89 mmol/L for the automated process. Using these metabolite data, specific consumption values

(pM/cell/hour) and yield coefficients (mmol/mmol) were calculated (Table 6.1).

Table 6.1 *Specific consumption rates and yields obtained for the UC-MSCs.*

Process Type	**Specific Glucose Consumption**	**Specific Glutamine Consumption**	**Lactate Yield From Glucose**	**Ammonium Yield From Glutamine**
	pM/cell/hour		**mmol/mmol**	
Manual FBS-containing DMEM culture	12.0 ± 2.77	2.29 ± 0.65	0.88 ± 0.11	0.48 ± 0.09
Automated FBS-containing DMEM culture	12.3 ± 3.68	2.17 ± 0.67	0.81 ± 0.19	0.51 ± 0.14
Manual StemPro medium culture	12.1 ± 2.18	2.27 ± 0.91	0.80 ± 0.13	0.50 ± 0.09
Automated StemPro medium culture	12.4 ± 3.23	2.35 ± 0.89	0.81 ± 0.14	0.48 ± 0.11

The yield coefficients of lactate from glucose (between 0.81 to 0.88 mmol/mmol), and the yield coefficients of ammonium from glucose (between 0.48 to 0.51mmol/mmol) were not significantly different between the manual and the automated processes for both the culture media ($p > 0.05$). These are within the ranges of values quoted in the literature for mammalian cells between 0.47 to 2 mmol/mmol, and 0.17 to 1.5mmol/mmol for lactate and ammonium respectively (Ozturk and Palsson 1991; Winkenwerder *et al.,* 2003; Harigae *et al.,* 1994; Miller *et al*., 1998; Aslankaraoglu *et al.,* 1990; Helmlinger *et al.,* 2002;; Acosta *et al.,* 2007). The yield coefficients of lactate from glucose quoted in literature for the MSCs were considerably higher, and some were even higher than the

theoretical maximum of 2 mmol/mmol (Ahn *et al*., 2011), but these values were neither for the static T-flask MSC cultures nor for the UC-MSC cultures. There are very few metabolite studies carried out using the MSCs, and the MSC yield coefficients quoted in the literature are mostly for non-static culture of BM-MSC (Schop *et al.*, 2009; Dos Santos *et al*., 2011).

There was no significant difference in the glucose and glutamine consumption rates between the cells cultured via the manual and the automated process ($p > 0.05$). Neither was there any significant difference in the glutamine and glucose consumption rates between the cells cultured in the FBS and the StemPro medium ($p > 0.05$). The glucose and glutamine specific consumption rates between 12.0 to 12.4 pM/cell/hour and 2.17 to 2.29 pM/cell/hour, respectively, were also within the ranges of the values quoted in the literature for MSCs (Higuera *et al*., 2009; Lavrentieva *et al*., 2010; Dos Santos *et al*., 2011; Reichardt *et al.*, 2013).

6.2.3 Cell surface marker analysis (by flow cytometry)

There is no single surface marker to characterise MSCs (discussed in Chapter 2). Most studies generally follow the minimal criteria set by the ISCT. In order to determine the expression levels, flow cytometry analysis was carried out according to procedure described in Chapter 3. Cell surface marker expression of the CD73, CD90, CD105, CD19, CD34, CD 11B, HLA-DR, and CD45 was determined for all experimental conditions (Figure 6.5).

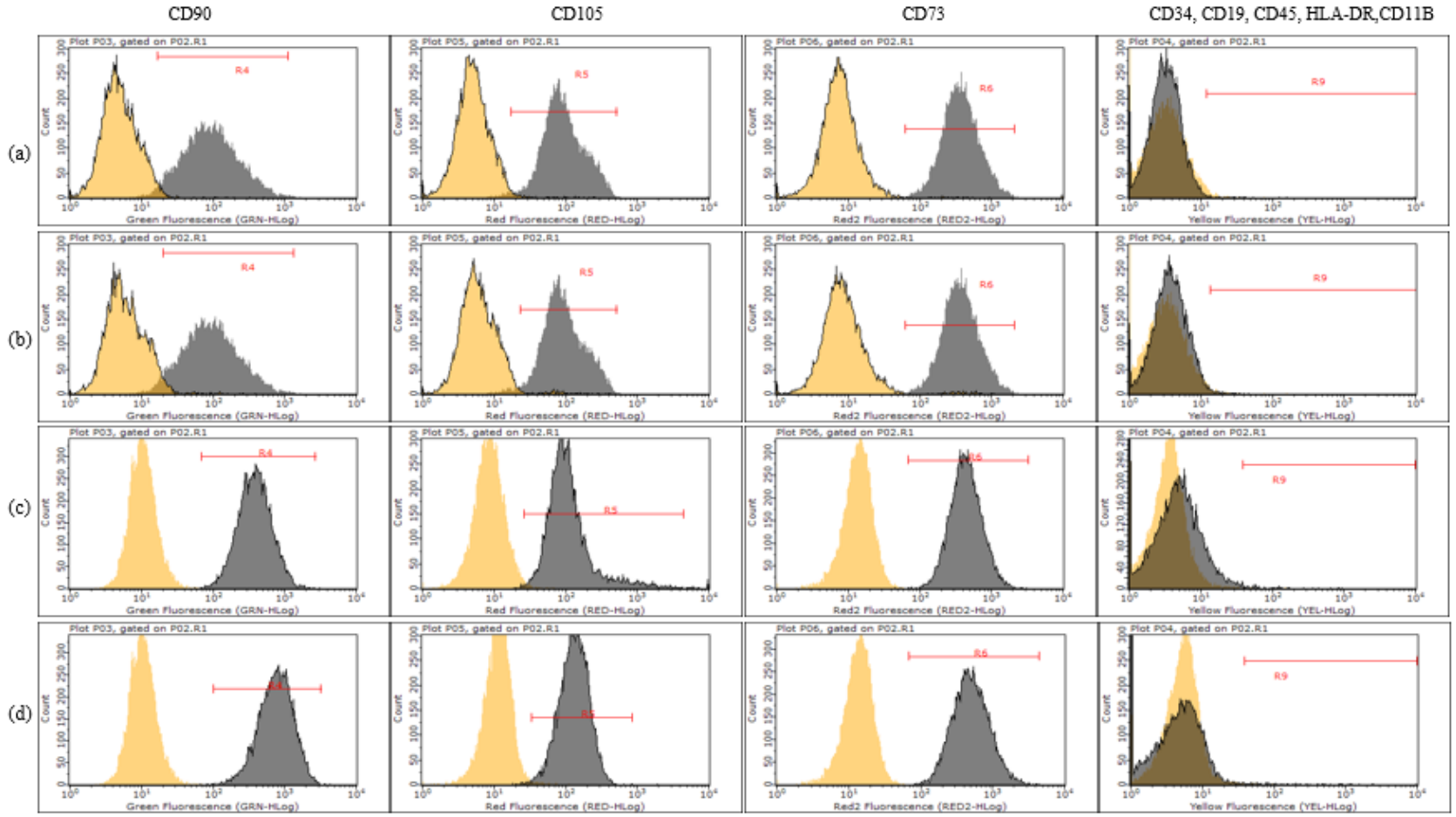


***Figure 6.5**: Phenotype analyses of MSCs at passage 5 by flow cytometry to compare surface marker expression of cells from (a) manual FBS-containing DMEM culture, (b) automated FBS-containing DMEM culture, (c) manual StemPro medium culture, and (d) automated StemPro medium cell culture. Flow cytometry images (a to d) are representative images of two independent biological samples. Isotype controls that illustrate unspecific binding are highlighted in yellow.*

The majority (> 95 % positive) of the MSCs were positive for the MSC surface markers CD73, CD90, and CD105 (Figure 6.6). The data demonstrated furthermore that there was little if any expression detectable of CD19, CD34, CD 11b, HLA-DR, and CD45 (negative markers) respectively, suggesting the absence of endothelial and hematopoietic cells types. This finding strongly agrees with data published by other research groups (Sarugaser *et al.*, 2005; Lu *et al.*, 2006), which reported that the majority of UC-MSCs express low levels of HLA-DR (class II antigen) compared with BM-MSCs. For this reason, they are also being widely being used for the treatment of GVHD, Crohn's disease and multiple sclerosis (Newman *et al*., 2009).

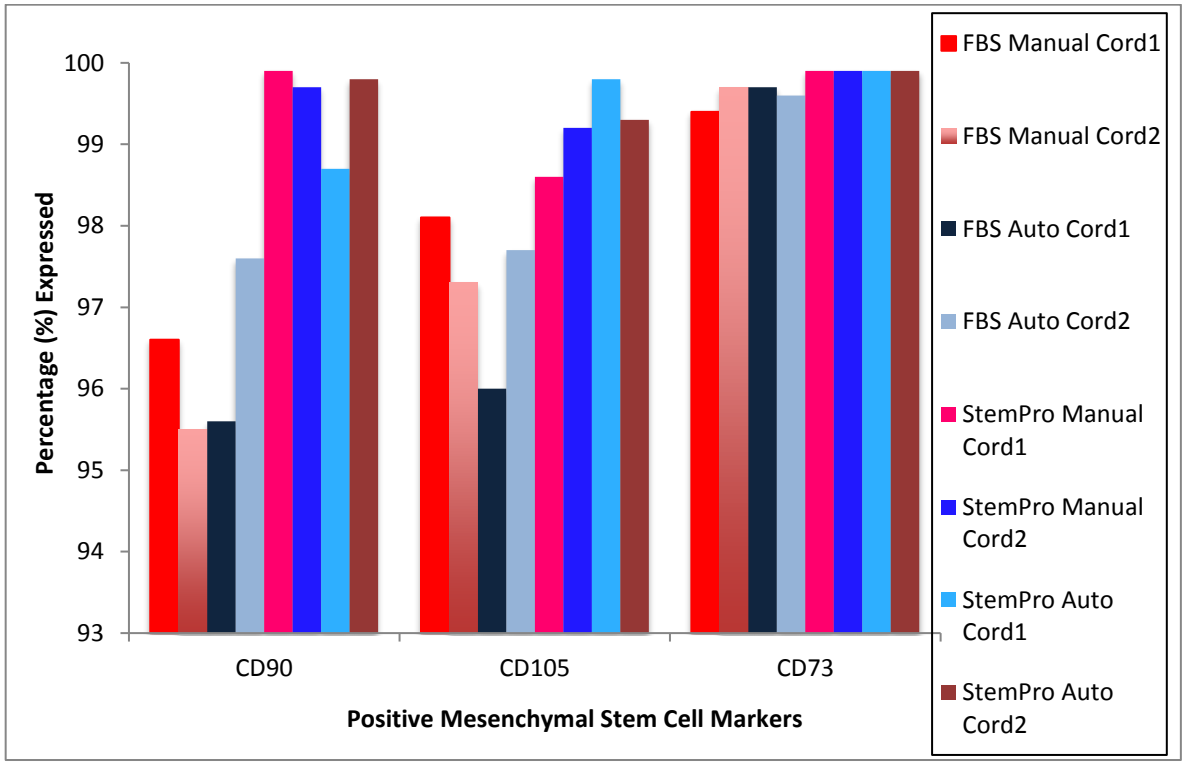


Figure 6.6*: Bar chart shows the percentage positive phenotypic expression of the MSCs from the 2 human umbilical cords at passage 5.*

Figure 6.6 clearly illustrates that there was a similar level of CD73 expression by cells harvested from all the different process conditions. Cells cultured using the StemPro medium for both the automated and manual processes expressed higher levels of CD90 and CD105 markers, but also expressed higher levels of the negative markers. This was not a cause of concern as these levels are within the acceptable range set by the ISCT; less than 2 % of the cells expressed CD14, CD19, CD34, CD45 and HLA-DR (Figure 6.7), thus meeting the criteria set by the ISCT (Dominici *et al.*, 2006).

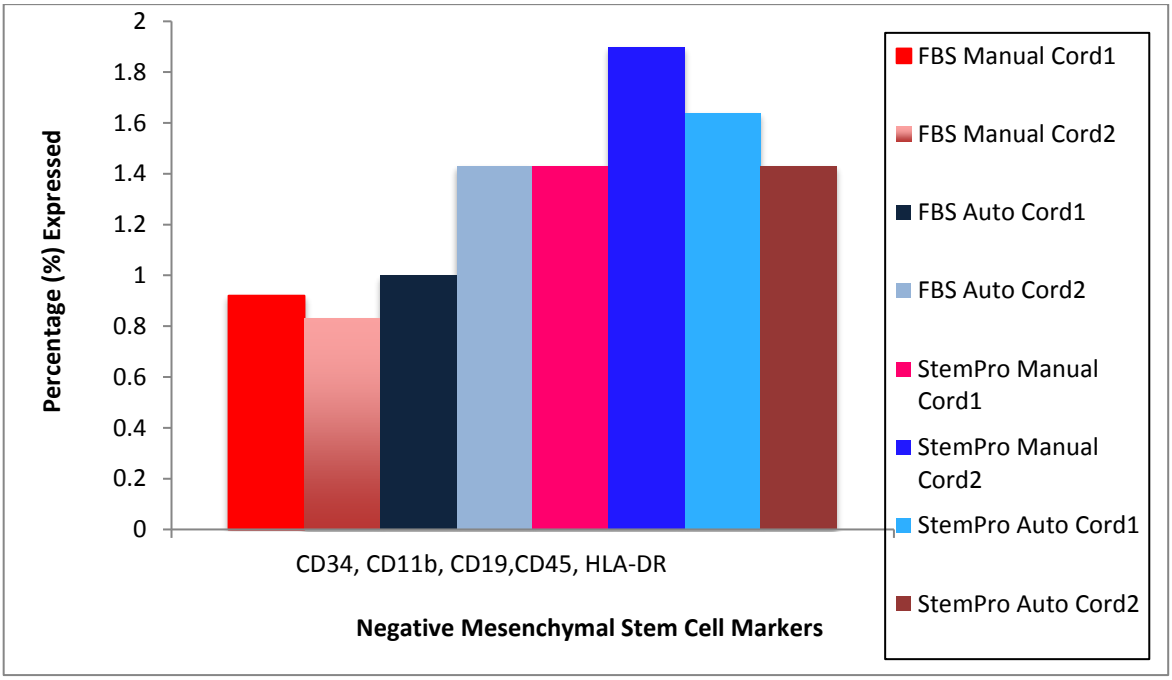


Figure 6.7*: Bar chart shows the percentage negative phenotypic expression of the MSCs from the 2 human umbilical cords at passage 5.*

The MSCs used for this study exhibited surface markers and plastic adherence as required by the International Society for Cellular Therapy (ISCT), but in order to confirm that these cells were indeed MSCs, differentiation study (into osteocytes, chondrocytes, and

adipocytes) was required (Dominici *et al*., 2006). As it has been reported that fibroblast cells are capable of expressing all the MSC surface markers, differentiation study is a prerequisite in every MSC study (Alt *et al*., 2011).

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6.2.4 Cell differentiation

The MSCs expanded in StemPro Medium and in FBS-containing DMEM at Passage 5 (for both manual and automated processes) were investigated for their *in vitro* differentiation capacity along adipogenic, osteogenic, and chondrogenic lineages (Figure 6.8). The differentiation work was carried out according to the methods described in Chapter 3 using commercially available StemPro differentiation kits (Life Technologies, UK).

All the cells were able to efficiently differentiate into adipocytes and osteocytes and chondroblasts as shown by Oil Red O, Alizarin red, and Alcian Blue staining, respectively (Figure 6.8). In addition, all induced samples revealed prominent chondrogenesis, as shown by dark blue staining of the chondrogenic pellet. The oil red staining for adipocytes is more prominent for cells cultured in StemPro medium than in FBS-containing DMEM

medium, but no detectable difference in staining for osteocytes and chondroblasts was observed between all samples. The images show that the MSCs used for this study were able to demonstrate multilineage differentiation capacity as required by ISCT.

In contrast to adipogenic and chondrogenic differentiation potentials, which were confirmed by several scientific groups, the statements regarding the osteogenic potential of UC-MSCs were different. While some investigators describe their osteogenic potential comparable to BM-MSCs (Diao *et al*., 2009; Hou *et al*., 2009), others show that UC-MSCs are poorly osteogenic, and only some of them are capable of undergoing osteogenic differentiation (Girdlestone *et al*., 2009; Majore *et al*., 2011). This is not the case with this study.

In this study, visible osteogenic staining was only observed after 21 days of osteogenic induction, but it was possible to observe chondrogenic and adipogenic staining in less than 12 days of induction. Although it took longer for the MSCs to differentiate into osteocytes, these cells still stained darkly when they were stained with alizarin red stain (Figure 6.8). Therefore, it was assumed that the delay in differentiation had no real impact on MSCs ability to differentiate along the osteogenic pathway as even the manufacturer's protocol recommended cells to be induced longer with osteogenic differentiation medium (up to 21 days) prior to staining with Alizarin red dye.

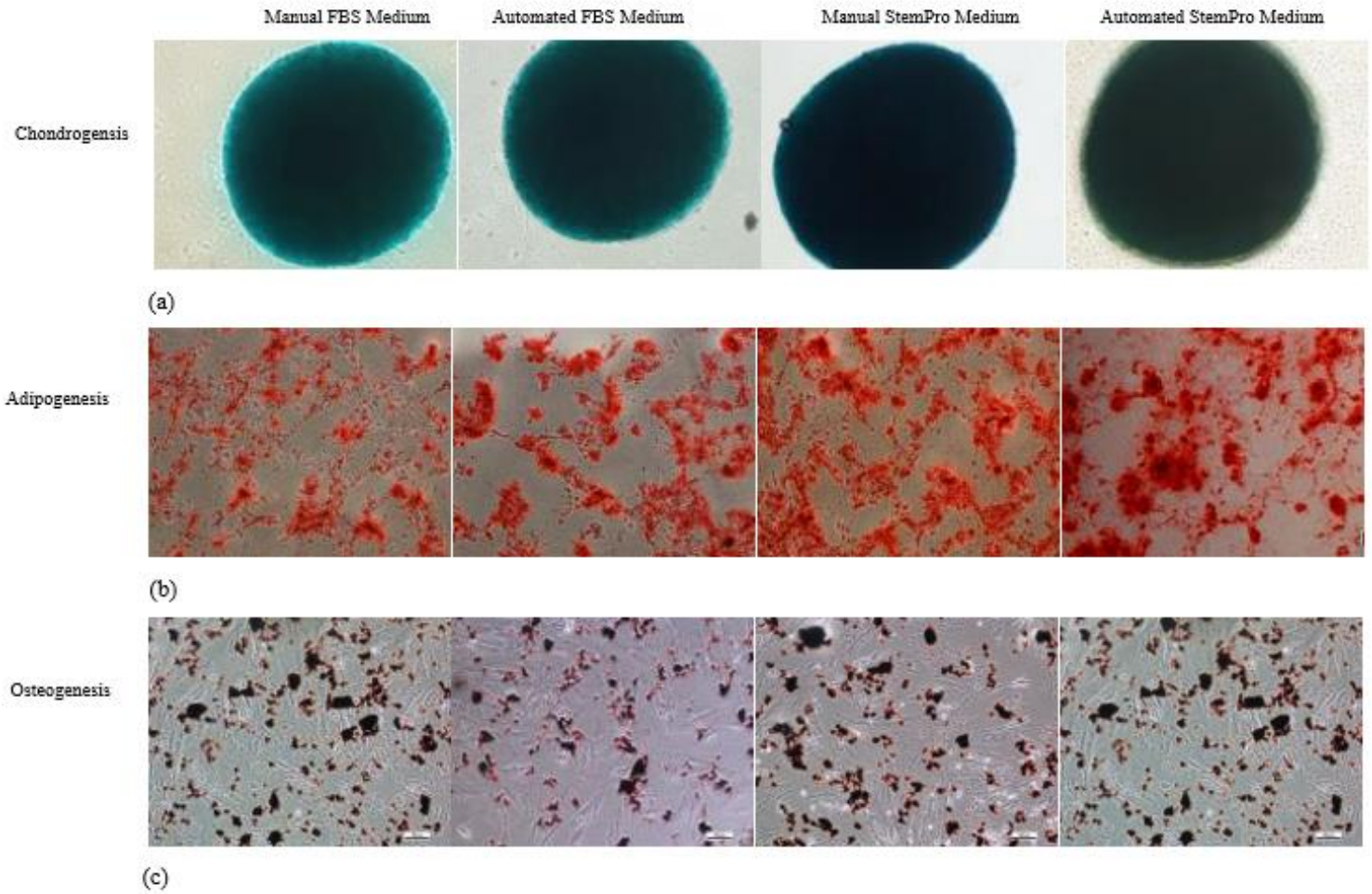

***Figure 6.8**: Differentiation capacity of MSCs at passage 5. MSCs derived after expansion, were induced to differentiate along (a) osteogenic (Alizarin Red staining), (b) adipogenic (oil red O staining) (c) and chondrogenic (Alcian blue staining) lineages. Images are representative images (magnification x10) from 2 independent biological samples.*

Although it was shown in the Figure 6.4 that cells cultured using the StemPro medium expressed higher levels of negative markers, this had no visible effect on the cell differentiation potential (Figure 6.5). The cells were still able to demonstrate MSC characteristics when they were cultured using StemPro medium. The results demonstrate that the StemPro medium has the potential to replace the traditional FBS-containing DMEM. If a method to isolate cells without using FBS can be developed, it will be possible for animal-derived products to be completely eliminated from the cell culture process.

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6.3 Application in the Allogeneic Cell Therapy Area

As discussed in Chapter 2, the Compact Select is mainly suitable for autologous therapies where only scale-out is required. However, the Compact Select can also be considered for small-scale allogeneic therapies if the cell demand is not significantly high per year (approximately billion cells per year), especially for drugs that have received Orphan drug status (targeting a smaller number of patients). In this section, a brief hypothetical evaluation will be carried out to assess whether it is possible to

consider Compact Select for the manufacture of cells for allogeneic cell therapies (based on one year's cell demand).

In this study, the Osiris Therapeutics' (Osiris, 2013) MSC therapy; Prochymal for the treatment of acute graft-versus-host disease (GvHD) was used as a test case. Prochymal is a stem cell product (allogeneic) that is stored frozen and infused through a simple intravenous line without the need to immunosuppress the recipient (Osiris 2013). It recently received orphan drug status from European Medicines Agency (EMA). GvHD was chosen as the target therapy because the cells required for treatment of this indication are within the middle range of cell demand (1.5×10^6 per kg of patient) of cells required for allogeneic therapies (FDA, 2013). This evaluation attempts to show that the process of automation can also be suitable for some allogeneic therapies, where the cell requirements are not too high.

Prochymal gained approved in Canada and New Zealand for the management of acute GvHD in children (Osiris, 2013) and also Orphan drug status. It is also available for adults and children in eight countries including the United States, under an Expanded Access Programme (Osiris, 2013). This makes Prochymal for GvHD indication a promising therapy.

The evaluation will be performed based on these assumptions;

1) Prochymal gains EMA approval. At present this drug is being considered by this agency, and has not received regulatory approval.

2) The MSCs for the project will be isolated from the donated human umbilical cords and not from the bone marrow. In the actual clinical study, the MSCs were actually obtained from bone marrow as this study was initiated in early 2007 when bone marrow was the main source of MSCs. Recent clinical studies are mostly using MSCs obtained from the umbilical cords (discussed in Chapter 5). Obtaining MSCs from the umbilical cords is cost effective as these cells are available in abundance as a waste product.

6.3.1 Target market and demand

The target market, where the product will be sold, was decided to be the United Kingdom (UK) because it is easier to penetrate the local market. Upon the identification of the geographical location of the target market, the next step was to identify the market size. The market size for the treatment of GvHD was calculated in order to determine the amount of MSCs needed to be produced per annum, and consequentially to determine the production scales of the manufacturing processes. As a first step, the number of patients with GvHD in the United Kingdom was identified as 2520 patients. This value is based on the UK population of 63 million (ONS, 2016), and also on the information provided by the Committee for Orphan Medicinal Products (COMP). COMP states that GvHD affects less than 0.4 in 10,000 people in the European Union (EU). Out of that value, only 12% suffer from acute GvHD might benefit from cell therapy (Dignan *et al*., 2012).

Upon the determination of the total number of patients that can be targeted through this therapy, the next step was to determine the total amount of MSCs required. MSC production is based on factors such as the market penetration capacity (market size),

the dosage requirement and the period of a treatment. Therefore, the equation to determine the cells needed per annum based on the market penetration capacity is shown in Equation 1:

Based on a market penetration capacity of 10 % (or 30 patients):

MSCs needed = $1.5 \times 10^6 \frac{\text{cells}}{\text{kg}} \times 75.8 \text{ kg} \times 30 \times 2 \times 4$ **Equation 1**

$= 27 \times 10^{10}$ cells required per year

$= 2.3 \times 10^{10}$ cells required per month

Assumptions Made:

1) Average body mass of people in the UK is 75.8 kg according to a league table of the world's 'fattest' nations from the London School of Hygiene & Tropical Medicine, (2013).
2) The MSC required per infusion are 1.5×10^6 cells (FDA, 2013). This value is an average of the cell range between 1×10^6 to 2×10^6 quoted on the FDA website (FDA, 2013).
3) MSCs are administered through intravenous infusion 2 times a week for a total of 4 treatments (FDA, 2013).
4) The 10% market penetration value chosen is considered to be reasonable as even the best selling drug Humira by Abbots Pharmaceuticals, USA for arthritis indication only has an average market penetration value of 20% (Krempa, 2012).

6.3.2 Flasks and media required

In order to determine the number of Compact Select robots required, the number of flasks required during the final expansion stage was determined (Equation 2):

Flasks needed for FBS-containing DMEM culture:

$= 2.7 \times 10^{10}$ cells / 4×10^{7} cells = 675 flasks per year **Equation 2**

Therefore, 675/12 = 57 flasks required per month

Flasks needed for StemPro medium culture:

$= 2.7 \times 10^{10}$ cells / 5×10^{7} cells = 540 flasks per year **Equation 3**

Therefore, 540/12 = 45 flasks required per month

Assumptions Made:

1) It was possible to obtain at least 2.32×10^{4} cells per cm^2 for FBS-containing DMEM culture for either the manual or the automated processes (Section 6.2). If the cells are cultured in Hyper Flasks (Corning, USA), approximately 4×10^{7} cells can be obtained from one flask (total surface area of 1720 cm^2).
2) It was possible to obtain at least 3×10^{4} cells per cm^2 for StemPro culture for either the manual or the automated process (Section 6.2). If the cells are cultured in Hyper Flasks (Corning, USA), approximately 5×10^{7} cells can be obtained from one flask (total surface area of 1720 cm^2).
3) The Compact Select is able to process a maximum of 130 flasks at a time (Chapter 3).

The evaluation above illustrates that only 1 Compact Select is required to produce the required quantity of cells per year. This calculation is based on the Compact Select being run 12 times throughout the year. Work done in this chapter demonstrated that it only takes 12-20 days for them to be expanded until passage 5. Therefore, it is possible for the Compact Select to easily achieve 12 runs a year. Therefore this brief analysis demonstrated that Compact Select can be used to manufacture cells for allogeneic cell therapies if the cell demands are not too high.

6.4 Conclusion

There was no significant difference in the average cell numbers obtained from the manual and automated processes ($p > 0.05$), but there was a significant difference in cell numbers obtained by using the two different types of growth media ($p < 0.05$). Cells exhibited a higher growth rate when they were cultured in StemPro medium. In contrast to the results obtained in Chapter 4 (for both the HDF and the HOS cell cultures), the results demonstrated that the choice of the manufacturing process has minimal or no impact on the end cell yield. The work carried out in the preceding chapter (Chapter 5) allowed the development of a robust process for the MSC culture. Through various test runs and protocol reiterations, the automated protocol was optimised to suit the MSCs. It was not possible to transfer the manual protocol directly to the automated process, as there were some distinct differences between the two processes (discussed in Chapter 2 and Chapter 4).

In terms of consumption rates (glucose and glutamine), there was no significant difference in consumption rates between cells from the manual and the automated process ($p > 0.05$). Also, there was no difference in the consumption rates between

cells cultured in FBS-containing DMEM and the StemPro medium ($p > 0.05$). Lactate and ammonium concentration recorded in all process flasks were below inhibitory levels reported in the literature. No abnormal metabolite accumulation or depletion was observed in any of the process flasks. The MSCs obtained from the manual and automated processes for both types of culture media exhibited the required surface markers and plastic adherence and multilineage differentiation capacity (osteocytes, chondrocytes, and adipocytes) as required by the ISCT.

This study was also able to address two of the key questions that were listed in Chapter 1. This study demonstrated that xeno- and serum-free culture conditions are suitable for both the manual and automated culture of MSCs. In addition, the hypothetical evaluation carried out demonstrated that the study with the Compact Select is also applicable for the allogeneic cell therapy area, and not just in the autologous cell therapy area, provided that the cell demand per year is reasonable (as demonstrated in this Chapter). Although the cell demand for GvHD is not within the lower range of cell demands of allogeneic therapies, based on its Orphan Drug designation, it caters for a smaller target market.

Although this study was able to demonstrate that xeno- and serum-free cell culture processes can be automated, it was not able to demonstrate that MSCs can be isolated in completed xeno-and serum-free conditions. A number of researchers, including Chase *et al.* (2010) have also shown that it was not possible to isolate MSCs without using FBS or xeno-free components, but one study by Yang *et al*. (2011) has reported that another commercially available xeno-free medium, MesenCult (STEMCELL Technologies, USA) is capable of supporting serum-free UC-MSC isolation. This

medium was not investigated for this study as no other studies have reported similar results. In addition, this medium is not FDA-cleared, therefore, without regulatory clearance, the use of this medium has no real significance to the field.

Although this study only shows data until passage 5, other researchers have shown that unlike BM-MSCs, UC-MSCs are capable of retaining their multipotency until at least passage 18 (Majore *et al*., 2011) or passage 24 (Peters *et al*., 2010) of culture before reaching senescence, provided that the same seeding density (5×10^3 cells/cm^2) is used throughout the culture. In MSC culture, cells are usually only grown for a few passages, most often until passage 5 or 6, if they are to be used to be for clinical trials (Redaelli *et al*., 2012). It has been reported that BM-MSCs start losing their multipotent characteristics after passage 5 when they are cultured *in vitro* (Wagner *et al.*, 2010), therefore, most scientists usually use low passage cells to maintain the efficacy of these cells. It is believed that when the potential of MSCs are more clearly established through rigorous gene and molecular studies, these cells can be expanded for a few additional passages before they are sent to the clinics. This is also a cost saving measure as there will be less manual work associated with the cell isolation and characterisation required.

In terms of clinical potential, it is believed that UC-MSCs will exceed the potential of BM-MSCs as there are already 4 studies in phase III of clinical trials, despite umbilical cords being a relatively new cell source. These cells are being considered for various indications including chronic spinal cord injury, knee articular cartilage injury, and haematologic malignancies (FDA, 2013). At present, there are no studies associated with UC- MSCs or BM-MSCs in Phase IV of the clinical trials. Some of the early

studies using BM-MSCs were initiated in mid-2007, but have not been able to successfully gain approval from the FDA. It is possible that, due to the remarkable potential of UC-MSCs, all future studies will be conducted using these cells.

The results (based on doubling times calculated) corroborate the belief that UC-derived cells proliferate markedly faster than MSCs harvested from bone marrow. Umbilical cord cells can be more rapidly expanded to quantities sufficient for cell based therapies or tissue engineering applications without the prolonged exposure to *in vitro* conditions which increases the risks of cell transformation or evolution of chromosomal abnormalities (Rubio *et al.,* 2005). The study has also demonstrated that the cells cultured in StemPro medium exhibit a higher doubling potential than the cells cultured in FBS-containing DMEM. The MTT absorbance assay conducted illustrated that cells cultured in StemPro medium expressed higher mitochondrial potential than the cells cultured in FBS-containing DMEM, indicating increased proliferation potential. Although the cells cultured in StemPro medium expressed higher levels of negative markers, these levels did not have any visible effect on the cells' differentiation potential.

At present, the only real drawback of considering StemPro cell culture medium for MSC culture is the cost of this medium. Although serum is an expensive cell culture reagent, the StemPro cell culture medium is even more expensive, therefore cheaper alternatives should be developed (discussed in Chapter 5). These alternatives should be capable of obtaining FDA-clearance as this can potentially facilitate regulatory approval of the cell products. At present, StemPro is the only xeno- and serum-free cell culture medium that has received clearance from FDA.

Ultimately, the study carried out in this chapter is able to demonstrate that the development of robust automated MSC processes for clinical applications is indeed possible. This was only made possible through a series of development work carried out in the previous chapters (Chapter 4 and 5). The main hypothesis tested was that the automated process yield was comparable with the manual process yield in terms of product (cells) quantity and quality (stated in Chapter 1). These results (Section 6.2.2 to 6.2.4) support the hypothesis that the automated process yield compares well with the manual process yield. In terms of cell growth, cell attachment, marker and differentiation properties, there were no obvious differences between the manual and the automated process yields.

The work carried out with model cell lines allowed for a significant reduction in the development cost. Although there are some differences between the automated and the manual cell culture processes, by using a suitable protocol and by better understanding the capacity of the automated process, process yield can be significantly increased (demonstrated in this chapter).

Chapter 7. Conclusions and Future Work

7.1 Introduction

Due to the limitation of using embryonic stem cells (ESCs) and induced pluripotent stem (iPS) cells in the clinic, more emphasis was placed on using mesenchymal stem cells (MSCs), which are free of both ethical concerns and teratoma formation (discussed in Chapter 2). MSCs possess self-renewal ability and multilineage differentiation into not only mesoderm lineages, but also ectodermic cells and endodermic cells. The number of clinical trials on MSCs has been rising since 2004 and these cells have made greater progress in clinical trials. Hence, it is more likely that MSC will be the main candidate for cell therapy applications in future.

However, the transition of these studies from the laboratory scale to industrial scale processes with consistent outputs (in terms of quantity and quality) still remains as a major problem faced in the area of cell therapy. Since the number of clinical trials involving the MSCs are rapidly rising, suitable good manufacturing processes (GMP) that are capable of producing reproducible generation of cell populations at the required scales to meet the commercial demands need to be developed. However, the complex characteristics of the manufacturing processes and the highly sensitive nature of these cells to, make the generation of standardised manufacturing processes a challenging task. This provided the impetus for a doctoral study to be carried out in this area of cell therapy. This chapter summarises the insights gained during this study.This chapter also provides recommendations and directions for future work that will further advance the understanding of these topics.

7.2 Overall Conclusion

The main focus of this work dedicated towards investigating if the development of a fully automated robust MSC culture that is capable of producing clinically relevant product was possible. Since one of the greatest hurdles in clinical-scale stem cell expansion is to be able to maintain the phenotype and the viability of cells throughout the cell expansion process, the demonstration of these characteristics, as required by the International Society of Cellular Therapy (ISCT) are mandatory to prove their clinical relevance.

To achieve the aim of the research, several objectives were identified. The objective of the study described in Chapter 4 was to compare the manual with the automated cell culture process (using model cell lines). This initial work was important to understand if there were any real differences between the 2 processes. Investigation revealed that there was a significant difference in yield between the automated and the manual process, and also that the difference was observed when the work was repeated with another cell line. In terms of quality, there was no significant difference between the two processes.

While the results discussed in Chapter 4 were able to confirm that the cell output of the automated process is significantly different from the manual process, irrespective of the cell line used, the results were not able to establish the factors that were contributing to this difference. A critical review of the recent publications on this area also identified that most automation studies have just demonstrated the applicability of automation for cell cultures, but did not carry out a comprehensive study of the automated cell culture process.

This highlighted the need for a comprehensive study on the automated cell culture process. In order to develop a robust automated process, more knowledge of the automated process and also information regarding its capability was required. This can only be achieved through a comprehensive process development work with the aid of relevant statistical process control (SPC) tool such as the process capability analysis. Therefore, the objectives of the work discussed in Chapter 5 was to (i) study some of the factors contributing to the growth difference and to identify the factors that have large impact on cell growth and (ii) use the knowledge gained from (i) towards developing a robust automated MSC culture protocol.

The gauge studies (measurement system analysis) carried out in Chapter 5 demonstrated that the pipetting system was not accurately dispensing liquid. Gauge studies carried out in this chapter to assess repeatability confirmed that the Cedex Cell Counter demonstrated both good precision and accuracy. The investigation studies carried out enabled the limitations of the system to be identified. These limitations were not identified in any other automation studies using the same manufacturing platform.

In addition to identifying the limitation of the automated cell processing system, 2 key differences between the automated and the manual process (methodology) were also identified and studied; the harvesting and also the re-seeding process (Chapter 5). In addition to the pipetting discrepancy, the other factor contributing to reduced automated process yield (for HDF cells) was due to the unique characteristic of the cell line itself. It was found that the cells were attaching to all surfaces of the flask even before the seeding process was complete and this resulted in cell loss. Despite MSCs

sharing similar properties with this cell line, the MSCs did not demonstrate any cell loss during the automated re-seeding process, as they did not attach to the surface of the flask during the entire duration of the seeding process.

The work developed with the HDF cells enabled a good understanding of the automated process, but to ensure that the developed process was applicable for MSC expansion, the work developed (protocol) was tested with MSCs (Chapter 5). In contrast to the results obtained with the HDF cells, the MSCs exhibited a decrease in cell viability after a passage of culture when the original protocol was used. Since the automated platform contained no centrifuge, during the detachment process, the automated cell culture protocol (generic protocol) was developed in such a way that the detachment enzyme was neutralised with an equal amount of cell culture medium. However, work carried out by Thomas *et al.,* 2008 confirmed that leaving the detachment enzyme in the cell culture results in inhibition of MSC growth.

The original protocol was then modified to avoid leaving the detachment enzyme with the cells in the incubator throughout the incubation time because this resulted in the enzyme being brought forward to the new passage (when the cells were seeded into new flasks). In order to test if this method could result in good process yield, 4 new protocols were developed and tested. Through series of work, the optimum incubation time of 8 minutes was selected, but some additional steps were included to maximise cell recovery. This work demonstrated for the first time that cells could be detached without leaving the detachment enzyme in the flask throughout the incubation time.

The developed protocol was then tested with MSCs (from passage 7) over a period of

5 passages. The manual process was used as the benchmark process. Process capability analysis was then carried out using Minitab software. Although process capability analysis is not a new technique, it is still a relatively new technique in the area of cell therapy and has not been used previously to assess the stem cell manufacturing process. This analysis was successfully used in this study to demonstrate the robustness of the MSC manufacturing processes. The developed automated process demonstrated enhanced capability and stability, and the yield of the automated process was not significantly different than that of the manual process. This demonstrated that the protocol developed was indeed suitable for the automated culture of MSCs.

The success achieved with the work carried out in Chapter 5 provided the motivation to carry on with the work required to demonstrate that the automated process developed had real clinical relevance (Chapter 6). Most automation studies carried out with MSCs so far were proof-of concept studies mainly to demonstrate MSC culture can be automated, therefore did no carry out differentiation studies. The demonstration of these characteristics are mandatory to prove their clinical relevance.

The use of mid-passage cells (passage 7 and above) was the best option in terms of cost for preliminary and development work, but another important factor that determines facilitates regulatory approval and also ensures reproducibility is the ability to keep record of complete production history of a process. This is only possible if all process parameters are fully known and fully defined and this includes knowing the complete history of the cells used from the time of extraction to the last passage they are expanded to. For this reason, MSCs used in the study described in Chapter 6 was obtained immediately after extraction. Whenever biological materials, especially

mammalian cells are used for bioprocessing studies, it has to be demonstrated that the results are reproducible even when cells are obtained from a different source and for this reason, the study discussed in this chapter also used cells from two different umbilical cords.

The work discussed in Chapter 6 demonstrated that there was no significant difference in the average cell numbers obtained from the manual and automated processes ($p > 0.05$), but there was a significant difference in cell numbers obtained by using two different types of media ($p < 0.05$). Cells exhibited a higher growth rate when they were cultured in StemPro medium. No abnormal metabolite accumulation or depletion was observed in any of the process flasks. The MSCs obtained from the manual and automated process for both types of culture media exhibited surface markers and plastic adherence and multilineage differentiation capacity (osteocytes, chondrocytes, and adipocytes) as required by the ISCT. The cells from both processes also exhibited remarkable cell doubling potential (doubling times between 27 to 32 hours) as the doubling times reported in the literature for BM-MSCs were usually only between 38 to 42 hours (detailed in Chapter 6).

The experimental findings demonstrated that UC-MSCs were a suitable choice of stem cells. This clearly highlights the potential of these cells as promising candidates for cell therapies. The MSC culture using the StemPro medium was a proof-of-concept that it was possible to move towards a xeno- and serum-free cell expansion process. The StemPro cell culture medium was suitable for MSC expansion as this medium enabled MSCs to exhibit increased proliferation potential. This knowledge will greatly benefit researchers who are considering the commercialisation of MSC therapies.

In addition, the hypothetical evaluation carried out demonstrated that the study carried out with Compact Select is also applicable in the allogeneic cell therapy area, and not just in the autologous cell therapy area, provided that the cell demand per year is reasonable (Chapter 6). Although the cell demand for GvHD is not within the lower range of cell demands of allogeneic therapies, based on its Orphan Drug designation, it caters for a smaller target market.

Although this study was able to demonstrate that xeno- and serum-free cell culture process can be automated, it was not able to demonstrate that MSCs can be isolated in completed xeno-and serum-free condition. A number of researchers, including Chase *et al*. (2010) have also shown that it was not possible to isolate MSCs without using FBS or xeno-free components, but one study by Yang *et al.* (2011) reported that another commercially available xeno-free medium, MesenCult (STEMCELL Technologies, USA) was capable of supporting serum-free UC-MSC isolation. This medium was not investigated for this study as no other studies have reported similar results. In addition, this medium is not FDA-cleared, therefore, without regulatory clearance, the use of this medium has no real significance to this study.

At present, the only real drawback of considering StemPro cell culture medium for MSC culture is the cost of the medium therefore cheaper alternatives should be developed (discussed in Chapter 5). These alternatives should be capable of obtaining FDA-clearance as this can potentially facilitate regulatory approval of the cell products. Although the cost of using xeno-free reagents may contribute to an increase in the process costs, the use of FBS can also cause a considerable increase in process costs since all biologically derived starting materials, including FBS need to be screened and

or tested for the presence of adventitious agents if they were to be used in the cell manufacturing process.

Ultimately, the study carried out in this chapter was able to demonstrate that the development of robust automated MSC process for clinical applications was indeed possible, provided the process parameters were well characterised. This was only made possible through a series of development work carried out in the previous chapters (Chapter 4 and 5). The work carried out with model cell lines allowed significant reduction in the development cost. Although there were some differences between the automated and the manual cell culture process, by using a suitable protocol and by better characterising the automated cell culture process, the automated process yield was significantly improved. There was also no real concern about the applicability of the work developed for MSC culture as the developed protocols were tested with MSCs and were "fine-tuned" to suit MSCs.

Crucial to the successful manufacture of any cell-based therapies on an industrial scale, is the fundamental understanding of the bioprocessing techniques and its impact on the cells. Where stem cells are to be used as a source for cell therapy, this understanding has to be extended not only to preventing cell loss, but also its impact on the cell expansion and differentiation. This study has shown that process automation was suitable for the MSC culture process, but this was only realised through a comprehensive optimisation study. The initial work with HDF and HOS cells made it possible to distinguish the biological variations from the experimental errors. This automation study also demonstrated that both automated and manual processes were not similar, and direct translation of manual cell culture process conditions to the

automated process can result in cell growth variations and reduced yield.

The work in this thesis demonstrated that it was possible to develop an automated manufacturing process with good process capability and stability. The developed manufacturing process was able to produce a product that was clinically relevant. Since Compact Select is currently being used in many major pharmaceutical companies including GlaxoSmithKline, Merck, Pfizer, Bristol-Myers Squibb, AstraZeneca and Pfizer, the work carried out here will be widely applicable. This work can aid companies towards developing reproducible manufacturing and cell culture processes. In addition, the reduction of process variation and the increase in yield make the automated expansion process more economically viable and more appealing for investors.

7.3 Future Work

The work done in this thesis is a contribution to the area of stem cell manufacture. The results obtained in this study provide a base for the development of a robust automated cell expansion process for the MSCs. Many parameters have been optimised in this study for the implementation of such a robotic system for cell expansion. Although the data collected in this thesis represent a substantial step forward in the development of MSC automated cell culture processes, this study is still in the development phase. Further studies will be required to contribute to a greater understanding of both the engineering and biological parameters controlling cell fate during the automated MSC cell expansion process.

MSCs have to be characterised at different stages of growth when cultured on the automated system. This research carried out here only presents the analyses at the

beginning and the end of the cell culture process. Although the analyses carried out were able to demonstrate the clinical relevance of the product, the limited availability of the biological material did not allow more studies to be carried out. If there had been any fluctuation in cell marker levels (at certain passages), it would not have been detected. However, since studies of the biology of MSCs are continuously improving and also because more specific markers for the characterisation of these cells are becoming available, in future, it may also be possible to carry out more analyses using fewer cells to achieve the same objective.

The development of a serum-free medium for the isolation of UC- MSCs will be an important step in studying human MSCs in a consistent and reproducible manner. The complete elimination of the FBS will allow better process consistency to be achieved. In order to establish a completely serum-free cell culture process, more biological studies have to be carried out to better understand parameters that influence the viability and the fate of the MSCs. At present, it is not completely known the exact component in FBS that supports the isolation of MSCs from umbilical cord, although the work to find animal-free alternatives for FBS started more than 30 years ago (Rauch *et al*., 2011).

In addition to carrying out differentiation studies, functionality studies of the differentiation potential and performance *in vivo* (animal testing) of the *in vitro* developed MSCs should be carried out. In addition to that, karyotype study should be carried out to assess if there had been any chromosomal abnormalities because this issue remains controversial. Although many studies have reported that MSCs are chromosomally stable (Ruan *et al*., 2014), others have reported abnormalities in

prolonged passages (Borgonovo *et al.,* 2014). These studies will further demonstrate the potential of MSCs, expanded using the procedures developed in this work, for use in regenerative medicine applications.

For this study, cells were cultured in a single-layered standard T-flask (automation compatible), but to meet larger cell demands, cells needs to be cultured in vessels that can produce greater number of cells. HyperFlask (Corning, USA) is a triple-layered flask (automation compatible) that offers 1720cm^2 growth area in a footprint of a traditional T175 (175 cm^2) flask. At present, it is not known if this flask is capable of producing the same quality of cells that a T175 flask can produce, but if this can be achieved, this will increase the production capacity of the automated cell culture process.

Cell culture automation promises great potential, but in order to realise its full potential, it has to meet most users' needs. There are automated systems on the market that have some significant advantages over the competitors. There are some systems, which allow temperature manipulation through out the cell culture process. This is useful, as some researchers have demonstrated that cells show different characteristics at different temperatures (Schop *et al*., 2009). Some systems also allow better sampling techniques (well plate applications), and this is useful for cell culture applications where only limited biological materials are available. At present, with the Compact Select, all these are not possible. It will be useful for cell culture scientists to also consider using other automated cell culture systems as one system will not be able to meet the needs of all applications. Additionally, manufacturers of these systems should work with end users to understand the limitations of the system developed and to

continuously improve the performance of these systems based on the limitations identified.

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Appendices

Appendix I: Automated Cell Culture Protocol: Examples

Protocol attached below (Figure A1) is a general automated media exchange protocol available in the Compact Select software.

```
<SelecT_Protocol>
  <properties>
    <description>Media change</description>
    <flaskingtime units="s">0</flaskingtime>
    <platingtime units ="s">0</platingtime>
  </properties>

  <steps>

    <fetch> <!-- default batch size is 1 -->

      <dump pause = "5s" />
      <dispense liquid= "cell wash"  volume = "20ml" />
      <swirl repeat ="5" speed = "100%" pause = "5s" capped
= "no" />
      <dump pause = "5s" />

      <dispense liquid= "calf serum"  volume = "40ml" />
      <dispense liquid= "medium b"  volume = "5ml" />
      <store /> <!—the source flask is no longer processed
after this -->

    </fetch>
  </steps>
</SelecT_Protocol>
```

Figure A1 *Media exchange protocol*

Protocol attached below (Figures A2 and A3) is the general automated cell harvest and seed protocol available in the Compact Select software:

```
<SelecT_Protocol>
  <properties>
    <description>Cell Harvest 5 flasks, pool to new flask,
count pool, dilute pool, seed 2 new flasks,  dilute pool,
create 10 plates.</description>
    <flaskingtime units="s">0</flaskingtime>
    <platingtime units ="s">0</platingtime>
  </properties>

  <steps>
    <new>
      <putdown name = "pool" />
      <!--pool 5 source flasks into 1 new flask called pool-
->
      <fetch maxrepeat = "5" interleave = "5">
          <dump pause = "2s" />
          <dispense liquid="cell wash" volume="10ml" />
          <swirl repeat  = "2" speed = "20%"  capped = "yes"
/>
          <dump pause = "2s" />
          <dispense liquid="trypsin" volume="10ml" />
          <swirl repeat  = "2" speed = "20%"  capped = "yes"
/>
          <incubate period = "5m" />
          <dispense liquid= "calf serum"  volume = "10ml" />
          <shake repeat = "3"
 speed = "75%"
 pause = "5s"
 capped = "yes" />
          <pour name = "pool" pause = "2s" />
          <dispose />
      </fetch>

      <!--count for updating to the database-->
      <count name = "pool" fromheight = "10mm" />

      <!--dilute the pool flask to make up total volume to
150ml-->
<pipette    fromliquid = "calf serum"
                  toname = "pool"
                  volume = "50ml"
                  fromheight = "10mm"
  toheight = "10mm"
                  aspiratespeed = "1ml/s"
                  dispensespeed = "0.76ml/s"
                  pause = "5s"
```

Figure A2 *Cell harvest and seed protocol*

```
                  newtip = "yes"/>

      <!--seed new flasks using 50ml from the diluted pool
flask-->
      <new repeat = "2">
        <putdown name = "new seed flask" />
        <pipette  fromname = "pool"
                  toname = "new seed flask"
                  volume = "50ml"
                  fromheight = "10mm" toheight = "10mm"
                  aspiratespeed = "1ml/s"
                  dispensespeed = "0.76ml/s"
                  pause = "5s"/>
        <pickup name = "new seed flask"/>
        <store passage = "yes"/>
      </new>

      <!--dilute the pool flask to make up total volume to
150ml-->
      <pickup name = "pool" />
      <dispense liquid="calf serum" volume="100ml" />

      <!--plate out into 10 plates throw pool flask away -->
      <plate  pause = "2s"
              plates = "10"
              numberofwells="384"
              volume = "50ul"
              emptystartcolumn = "1"
              emptyendcolumn = "1" />

      <dispose />
    </new>

  </steps>

</SelecT_Protocol>
```

Figure A3 *Cell harvest and seed protocol*

APPENDIX II: Type 1 Gauge Study Report

The report attached below (Figure A4) is the Type 1 Gauge Study report obtained for the Cedex cell counter using Minitab V17. The statistical and experimental procedures are described in Section 3.3.1.2 and Section 3.4.2.1, respectively in Chapter 3. The results obtained are discussed in Chapter 5 (Section 5.2.1).

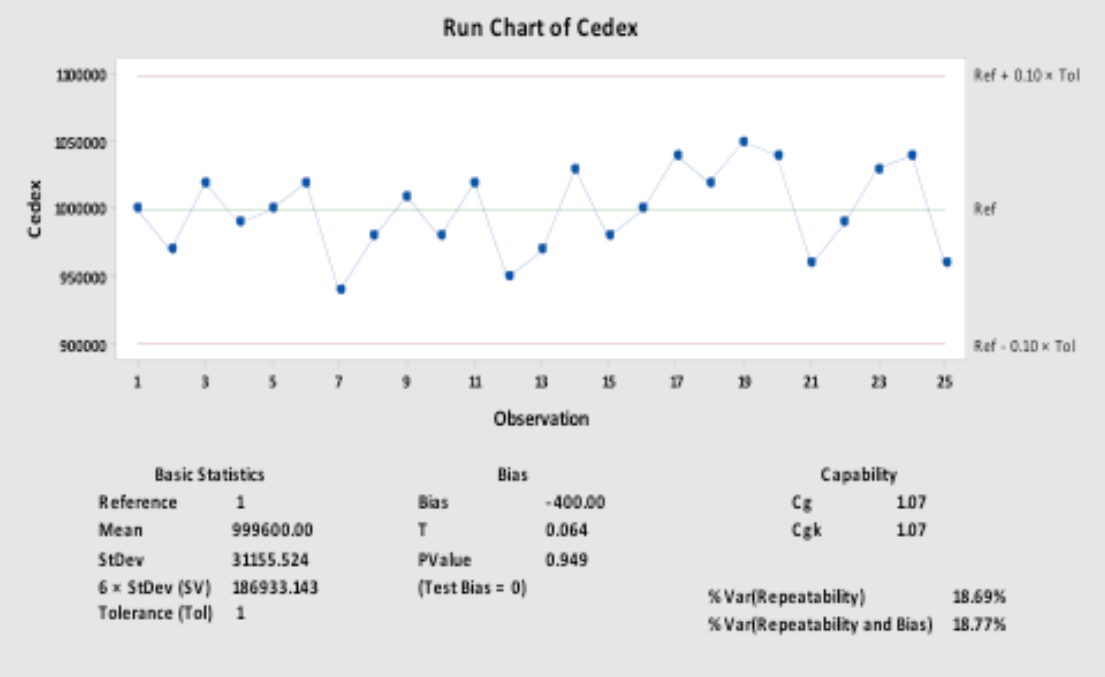


Figure A4 *Type 1 gauge run report*

Appendix III: Gauge Bias and Linearity (B&L)

The report attached below (Figure A5) is the Gauge Bias and Linearity report (before calibration) obtained from Minitab V17 for the study carried out to evaluate the performance of the automated pipettes. The statistical and experimental procedures are described in Section 3.3.1.2 and Section 3.4.2.2, respectively in Chapter 3. The results obtained are discussed in Chapter 5 (Section 5.2.2). The report obtained using data after re-calibration (Figure A6) is attached in the subsequent page.

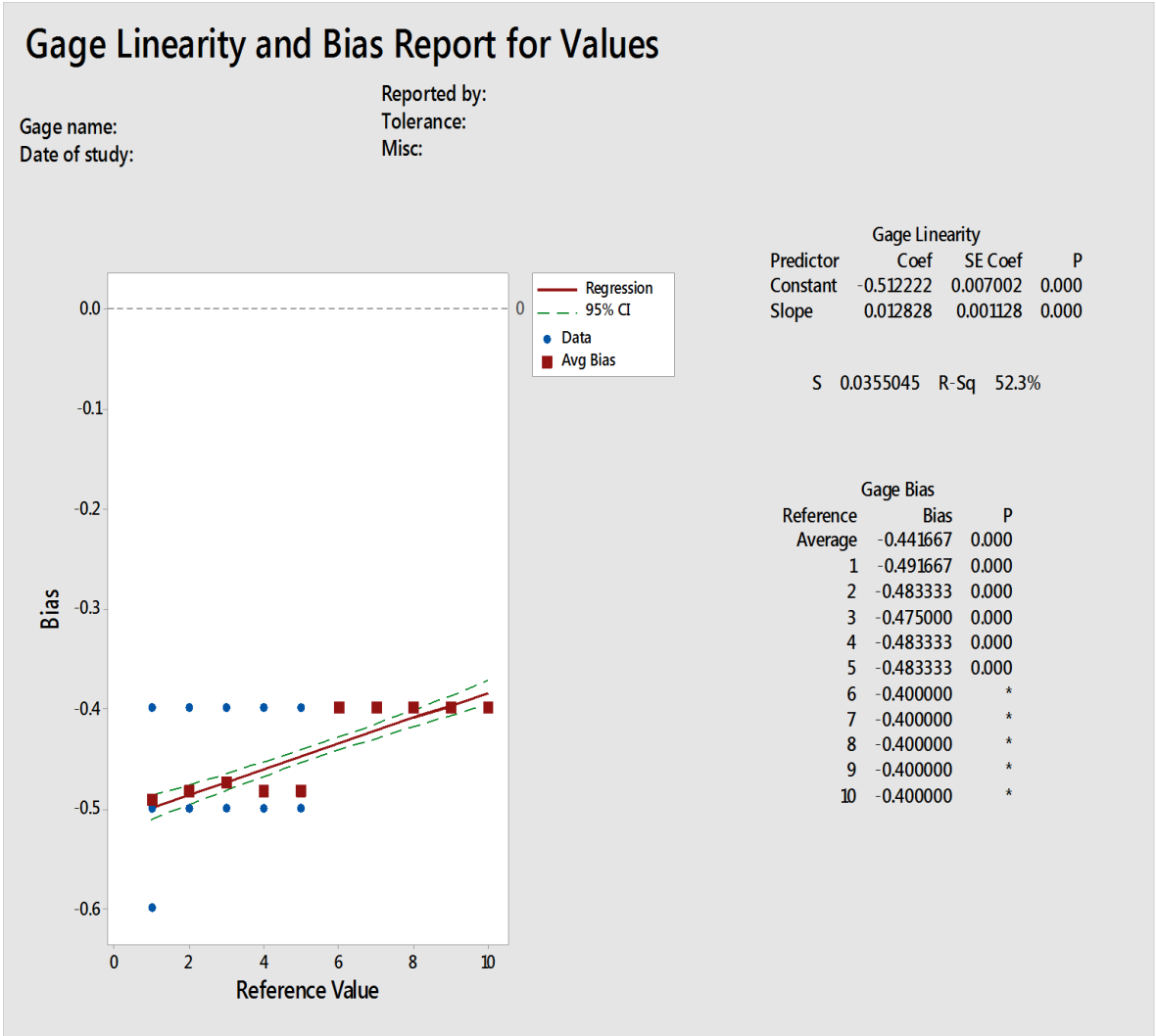


Figure A5 *Gauge Bias and Linearity report for data before calibration*

The report attached below (Figure A6) is the Gauge Bias and Linearity report (after calibration) obtained for the study carried out to evaluate the performance of the automated pipettes.

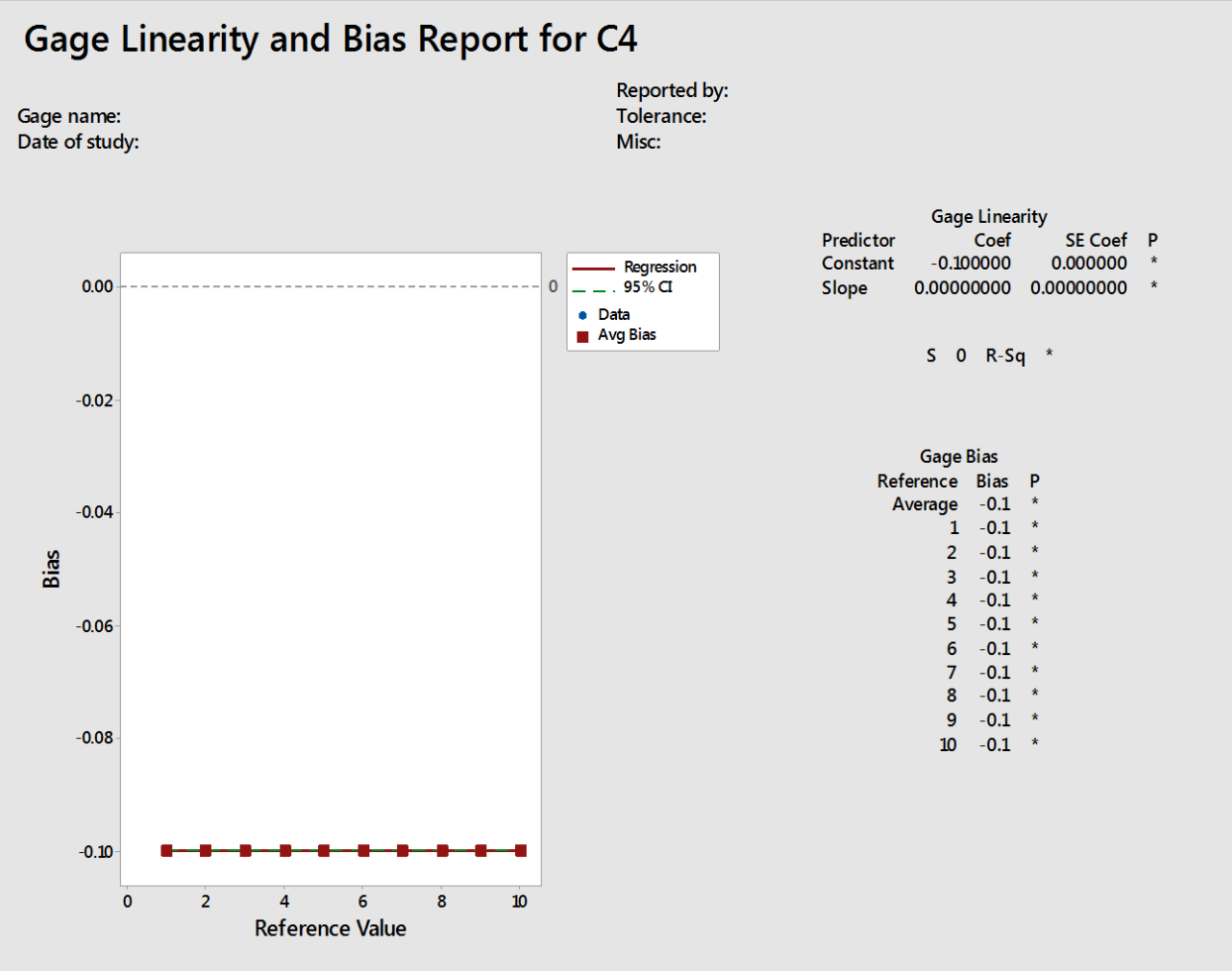


Figure A6 *Gauge Bias and Linearity report for data after calibration*

Appendix IV: Preventing Attachment of HDF Cells

The adsorption of cells onto surfaces can be influenced by various chemical and physical properties of the material surface layer including the surface roughness, wettability, electric charge, and presence of certain atoms (Heitz *et al.,* 2003). It is known that by adjusting the surface wettability, optimum protein adsorption or inhibition can be achieved (Bacakova *et al*., 2011). In order to find the ideal solution for the attachment issue mentioned in Section 5.3.2, various materials (that were commercially available) to prevent cell attachment were investigated.

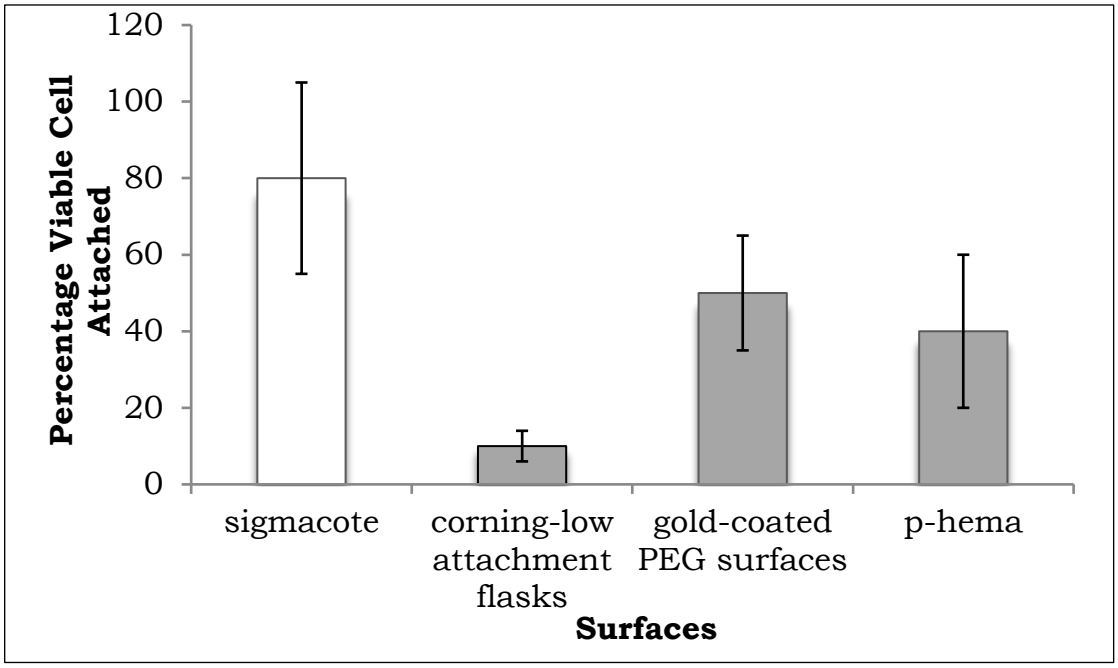


Figure A7 *The bar chart shows the percentage of HDF cells attached on various coatings over the percentage of the cells attached on standard T25 flask surface. Cells were seeded at* $1.5 \pm 0.10 \times 10^4$ *per* cm^2 *of culture surface. Error bars represent the standard deviation of the data (n=9). The shaded columns represent the hydrophilic surfaces.*

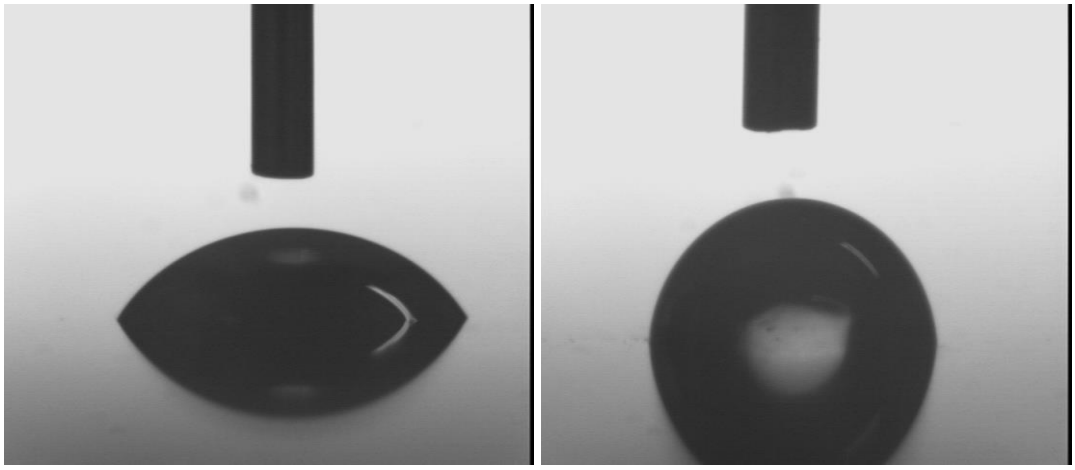
Some cell culture systems such as the Ultra-low attachment flasks (Corning Incorporated, USA) and gold-coated polyethylene glycol (PEG) well plates (Orla Chemicals, UK) came ready coated. Therefore, they did not require additional coating, but others including Sigmacote (Sigma Aldrich, UK) and Poly (2-hydroxyethyl methacrylate) (p-hema) (Sigma Aldrich, UK) had to be coated onto a surface. They were coated onto T25 flasks, instead of T175 as some of the coating materials were either expensive or were not available in T175 format. Cells were seeded at $1.5 \pm 0.10 \times 10^4$ cells/cm^2 into the respective flasks and well plates, and the number of cells attaching within the first 60 minutes was recorded. From Figure A7 it was clear that Corning ultra-low attachment flask was able to prevent at least 90 % of the cells from attaching. In order to understand the properties of the material that was preventing cell attachment, contact angle analysis was performed (Figure A8).

In order to investigate why different surfaces had varying levels of cell attachments, contact angle of these surfaces were measured using OCA 20 (Data Physics GMBH, Germany) equipment. If the material is water repelling (hydrophobic), a water drop on the surface of the material will have a contact angle greater than 90 degrees. It was believed that that the low adhesion materials should be super hydrophilic (contact angle $< 40^0$) in order for the protein to repel (Kazemzadeh *et al.,* 2013). Hydrophilic surface is known to strongly adsorb water and as a result, there is only a minimal surface tension when in contact with liquids containing biological material due to its similarity (Bacakova *et al.,* 2011).

As expected, the Corning low attachment flask surface was superhydrophilic (contact angle 30^0). All others surfaces were hydrophilic, apart from Sigmacote (contact angle

91^0). The contact angles for the rest of the hydrophilic surfaces were 45^0 and 56^0 for p-hema and PEG, respectively.

This experiment demonstrated that all the hydrophilic surfaces were effective at preventing cells from attaching to the flask/vessel surfaces. It is believed that the Sigmacote surface was ineffective in preventing cell attachment because it was a hydrophobic surface, although Lee *et al.*, 1998 reported otherwise for their studies (hybridoma cells) for their studies).



(a) (b)

Figure A8 *Images produced by the Contact Angle Analysis Software. Figures show the representative contact angle images of the (a) hydrophilic Corning's ultra-low attachment surface and (b) hydrophobic Sigmacote coated surface.*

References:

Bacakova, L., Filova, E., Parizek, M., Ruml, T. and Svorcik, V., 2011. Modulation of cell adhesion, proliferation and differentiation on materials designed for body implants. *Biotechnology Advances*, 29(6), 739-747.

Heitz, J., Svorcík, V., Bacáková, L., Rocková, K., Ratajová, E., Gumpenberger, T., Bäuerle, D., Dvoránková, B., Kahr, H., Graz, I. and Romanin, C., 2003. Cell adhesion on polytetrafluoroethylene modified by UV-irradiation in an ammonia atmosphere. *Journal of Biomedical Materials Research. Part A*, 67(1), 130-137.

Kazemzadeh, A., Ganesan, P., Ibrahim, F., He, S. & Madou, M.J., 2013. The Effect of Contact Angles and Capillary Dimensions on the Burst Frequency of Super Hydrophilic and Hydrophilic Centrifugal Microfluidic Platforms, a CFD Study. *PloS one*, 8(9).

Lee, G.M., Huard, T.K. and Palsson, B.O., 1988. Effect of anchorage dependency on growth rate and monoclonal antibody production of hybridoma cells. *Biotechnology Letters*, 10(5), 307–312.

Appendix V: MSC Images (Discarded flasks)

The images below (Figures A9 and A10) represent microscope images of MSCs cultured using StemPro medium (passage 0). Figures A9 and A10 are images of MSCs from Cord 1 and Cord 2, respectively on day 3 of culture. No adherent cells were observed in both flasks. The experimental procedures are described in Section 3.4.3, Chapter 3. The results obtained are discussed in Chapter 6.

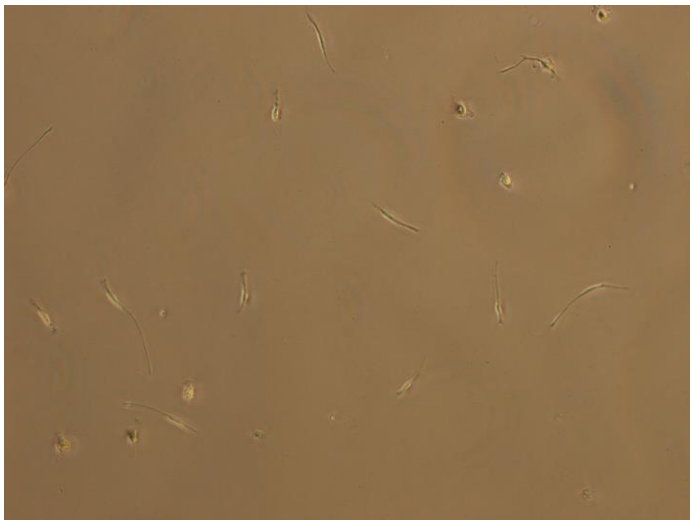


Figure A9 *MSCs from Cord 1*

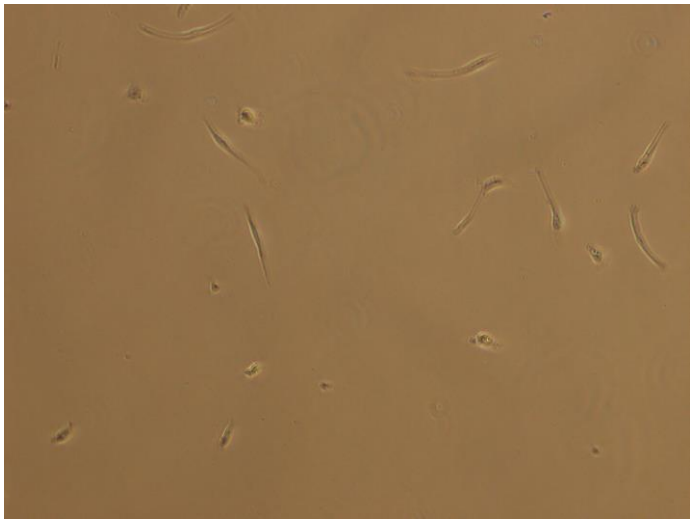


Figure A10 *MSCs from Cord 2*