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# Human Cell Culture Process Capability: A comparison of manual and automated production

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# Human Cell Culture Process Capability: A comparison of manual and automated production

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**Abstract** Cell culture is one of the critical bioprocessing steps required to generate sufficient human-derived cellular material for most cell-based therapeutic applications in regenerative medicine. Automated cell expansion is fundamental to the development of scaled, robust and cost effective commercial production processes for cell-based therapeutic products. This paper describes the first application of process capability analysis to establish and compare the short-term process capability of manual and automated processes for the in vitro expansion of a selected anchorage-dependent cell line. Estimates of the process capability indices (Cp, Cpk) have been used to assess the ability of both processes to consistently meet the requirements for a selected productivity output and to direct process improvement activities. Point estimates of Cp and Cpk show that the manual process has poor capability (Cp=0.55, Cpk=0.26) compared to the automated process (Cp=1.32, Cpk=0.25), resulting from excess variability. Comparison of point estimates, which shows that Cpk < Cp, indicates that the automated process mean was off-centre and that intervention is required to adjust the location of the process mean. A process improvement strategy involving an

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adjustment to the automated process settings has demonstrated in principle that the process mean can be shifted closer to the centre of the specification to achieve an estimated 7-fold improvement in process performance. In practice, the 90% confidence bound estimate of Cp (Cp=0.90) indicates that that once the process is centred within the specification, a further reduction of process variation is required to attain an automated process with the desired minimum capability requirement.

***Keywords*** *Process capability analysis, process transfer, continuous quality improvement, automated cell culture, regenerative medicine, tissue engineering, cell based therapy.*

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## Introduction

Regenerative medicine is a rapidly developing field that uses human cells or cell-based constructs as therapeutic products for a broad spectrum of clinical applications, predominantly aimed at degenerative conditions, organ failure, and tissue damage. Since the 1980's, research has significantly advanced the science of cell and tissue engineering research within the field of regenerative medicine but until recently there has been little focus on translation of this science from the laboratory bench into clinically and economically viable products (Mason and Hoare 2007; Mason and Dunnill 2008a). Growing efforts to commercialise these therapies are driving a need for scalable and robust manufacturing technologies to ensure these therapies are able to meet regulatory requirements, are economically viable at industrial scale production and are affordable (US Food and Drug Administration (USFDA) 1997; Directive 2004/23/EC; Williams and Sebastine 2005; British Standards Institute (BSI) 2006; Department of Trade and Industry (DTI) 2006; Commission Directive 2006/17/EC; Commission Directive 2006/86/EC; Advanced Therapy Medicinal Products (ATMP) Regulation 1394/2007; Mason and Dunnill 2008b; Lysaught *et al.* 2008).

The heterogeneity of human cell based medical products, in terms of complexity and the origin and type of cells, makes each manufacturing process unique. Moreover, the bioprocessing framework is distinctive because it involves critical steps for harvesting cells from the donor or patient, through cell expansion and tissue formation to the final step of implantation of the cell based product into the patient. Unlike biopharmaceutical product bioprocessing, the status of the living human cell is critical throughout all stages of the bioprocess for cell based

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products, making product consistency at least an order of magnitude more difficult to achieve from a bioengineering viewpoint (Mason and Hoare 2007; Kemp 2006). In practice, cell sources harvested from donors or patients are limited in quantity and therefore cell culture and population expansion is one of the critical bioprocessing steps required to generate sufficient cells (e.g. from 5.0 x $10^7$ to 5.0 x $10^9$ cells) for most cell therapeutic or tissue engineering applications (Palsson and Bhatia 2004). Current cell expansion approaches mainly involve laboratory-centred, labour intensive manual processes that require highly skilled personnel to perform meticulous aseptic manipulations over many days or even weeks. For most intended therapeutic applications, these manual processes lack the standardisation and reproducibility required for scaled, robust and cost effective commercial and regulatory compliant production (Vacanti 2006; Mason and Hoare 2007) . Many new developments in robot platforms and sensor technology however, are now making it possible to automate production processes for scaled-out and/or scaled-up approaches to expanding cell populations reproducibly and on a scale suitable for autologous or allogeneic cell based therapy. (Archer and Williams 2005; Kempner and Felder 2002; Thomas *et al.* 2008a). Several groups, including our own, have attempted to integrate automation into various aspects of human cell culture to address the issues of process scalability and reproducibility inherent in manual techniques but few have sought to use these platforms to evaluate process stability and capability (Kino-Oka *et al*. 2005; Joannides *et al*. 2006; Terstegge *et al*. 2007; Thomas *et al.* 2007 and 2009).

With growing expectations from regulatory agencies, better process understanding and control are likely to be key drivers in the manufacture of emerging cell-based products in order to minimise product quality variation, non-



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conformance and delays in product approval (BSI 2006, Vacanti 2006; Archer and Williams 2005; USFDA 2004a). As part of an early stage process transfer and continuous quality improvement programme, we have adopted a systematic approach to evaluate and compare the short-term stability and inherent variability of an existing manual process and a fully automated prototype process for the serial in vitro expansion of a selected anchorage-dependent cell line. We selected a permanent, transformed cell line (human osteocarcoma) as a model system to minimise process input variation from cell heterogeneity and allow a direct comparison of the cell expansion processes. Process capability analysis is applied to reliably assess and compare the behaviour of the processes in the state of statistical control. Estimates of the process capability indices, Cp and Cpk, are used to relate the process capability to the product requirements for a selected performance characteristic of both processes (cell yield) and provide a numerical measure of process robustness and production efficiency (American Society for Testing and Materials (ASTM) 2008). This analysis of process capability aims to improve understanding of the process and provide a scientific basis for determining the requirement for process adjustments that can result in process improvement in order to allow us to better specify and improve the potential quality performance of the automated system, its processes and its parts.

## Materials and methods

### General Culture Conditions

An anchorage dependent human Caucasian osteosarcoma (HOS) cell line was used in this study (Lot 01/J/066, European Collection of Cell Cultures (ECACC)).

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The HOS cells were cryopreserved, according to an established Standard Operating Procedure (SOP) and a master and working cell bank established to service the entire study (SOP031 2008). Cryopreserved cells recovered from the working cell bank were thawed according to an established SOP (SOP032 2008). The culture of HOS cells was conducted in 175-$cm^2$ T-flasks (T175 Nunclon, Nalgene Nunc, UK) using Dulbecco's modified Eagle's Medium (DMEM, Lonza, UK) supplemented with 2mM L-glutamine (Sigma-Aldrich, UK), 1% Non-Essential Amino Acids (NEAA) (Sigma-Aldrich, UK) and 10% Foetal Bovine Serum (Lonza, UK). The in vitro expansion of the HOS cell line was conducted in parallel monolayer cultures using both manual and automated protocols. A seeding density of 7 x $10^5$ cells/flask was used to achieve 70-80% confluence (i.e. the percentage of flask surface covered by cells) after 3 days of culture at 37$^o$C under an atmosphere of 5% $CO_2$ (v/v) and relative humidity of about 95%. Cells were continuously cultured for 9 days to achieve a nominal process target of 1 x $10^8$ cells. In order to keep cells at a sufficiently low density to stimulate growth, cells were passaged on day 3, day 6 and day 9. At each passage, spent medium was aspirated and cells detached from the bottom of the flask by exposure to a trypsin-EDTA (Sigma-Aldrich) solution (10 ml at 0.25% w/v) at 37$^o$C, 5% $CO_2$ (v/v) for 7 minutes. Flasks were split at a ratio of 1 to 4 after each passage. Manual and automated processes were carried out according to established SOPs (SOP012 2008; SOP043 2008) and maintained according to the guidance for good cell culture practice (Coecke *et al.* 2005). All final cell suspensions obtained from both automated and manual culture processes were tested and found negative for the presence of mycoplasma (Mycoplasma Experience Ltd, UK).

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## Instrumentation

The CompacT SelecT (The Automation Partnership, UK) is a fully automated cell culture platform which incorporates a small 6-axis anthropomorphic robotic arm that can access 90 x T175 flask and plate incubators, controlled at 37$^{o}$C under an atmosphere of 5% $CO_2$ (v/v) and relative humidity of about 95%. Flasks are bar-coded for identification and cell process tracking. Two flask decappers 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. The system allows the automation of seeding, feeding and other cell culture processes in order to maintain cell lines in standard T175 cell culture flasks (Figure 1).

INSERT FIGURE 1

## Manual HOS cell culture and passage protocol

Cells recovered from cryopreservation were seeded into one T175 flask. Once cells had attached to the bottom of the flask after 4 hours culture, the medium was replaced with fresh medium to remove cryoprotectant agents. After each passage, the cell suspension was centrifuged to remove the residual trypsin-EDTA. The collected cells were re-suspended in fresh medium and the concentration of viable cells for subsequent seeding determined by the trypan blue exclusion test on a haemocytometer under an optical microscope (Figure 1). On day 9, the cell suspension from each flask was pooled into one flask (nominally the production unit or lot), mixed by gentle swirling and the final concentration of viable cells measured by the trypan blue exclusion test to determine the total number of cells recovered (Figure 1).

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## Automated HOS culture and passage protocol using the CompacT SelecT

Previous studies by the authors have established the successful transfer of an existing manual HOS culture protocol to the fully automated culture process. The automated culture of HOS cells was developed to replicate the volume dependent (seeding volume, medium volume etc) and volume independent (seeding density, pH, temperature, mixing etc) operating conditions of the manual culture process as closely as possible. However, because the CompacT SelecT has no centrifugation capability, after each passage flasks were removed from the incubator and fresh medium pumped into the flask to neutralise and dilute the trypsin-EDTA. The cell suspension was mixed and a 1ml sample pipetted into the integrated Cedex® cell counter. After 9 days culture, the cell suspension from each flask was pooled into 1 flask (nominally the production unit or lot), mixed by repeat pipetting and the final concentration of viable cells measured by Cedex®, which uses the trypan blue exclusion test and automated digital image recognition to determine the total number of cells recovered (Figure 1).

## Experimental Framework

A previous measurement system analysis, comprising an established Gauge Repeatability & Reproducibility (Gauge R&R) methodology, verified that both the manual and automated measurement systems utilised to determine viable cell number introduce minor measurement error or bias and were capable of detecting product variation (Liu *et al*. 2007).

**Study 1:** Serial culture runs, involving the in vitro expansion of the anchorage-dependent HOS cell line, were conducted in parallel using both manual





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and automated (CompacT SelecT) protocols. Each process was used to generate 10 full-scale production units or lots of cells under operating conditions that were as identical as possible. The production units were generated over a 14 month 'long run' period so that substantial sources of variation in the response output could be captured, although some sources of variation were held constant i.e. selected key process operating parameters, such as seeding density, critical raw material, ancillary components and consumable batches. All process parameters were recorded in a Manufacturing Batch Record (MBR) for each production run to monitor deviations from the SOPs and 'out of specification' observations.

The process yields (total cell number of viable cells) recovered from the 10 production units or lots of cells generated from each of the manual and automated culture processes were used to calculate capability statistics and allow comparison of processes (ASTM 2008). To evaluate capability indices (Cpk, Cp), nominal design specifications for cell yield were generated based on a clinical and experimental rationale. The nominal lower specification limit, based on a putative minimum therapeutic requirement (Palsson and Bhatia 2004), was set to $5.0 \times 10^7$ cells and 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 $1.5 \times 10^8$ cells. The specification was applied to both processes in the same way to allow process comparison to be explored.

**Study 2:** A second study was carried out using an alternative automated process in which the settings of the passage process were adjusted in order to minimise the potential adverse effects of residual trypsin-EDTA on the processing of cells (based on a Cause and Effect analysis of the baseline automated process shown in Figure 2). The automated process was carried out as before except for the following: After each culture incubation, spent medium was removed from the



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flask and the cells washed with 30 ml of phosphate buffered saline (PBS) in order to remove any residual serum (from the medium). At each passage, a solution of 0.25% trypsin-EDTA (15 ml) was pumped into the flask, the flask swirled for 20 seconds to ensure an even coverage of enzyme across the cell sheet, and then the excess removed from the flasks. After 10 minutes at 37$^{o}$C, 5% $CO_2$ (v/v), the flasks were removed from the incubator and fresh medium pumped into the flask to neutralise and dilute the trypsin-EDTA. After 9 days culture, the cell suspension from each flask was pooled into 1 flask (nominally the production unit or lot), and the final concentration of viable cells measured as before.

Three full-scale production units or lots of HOS cells were generated using the automated protocol with adjusted process settings. The data for this short verification run was acquired over a 1 month period. All process runs were carried out using process operating conditions that were as identical as possible to those used in Study 1.

INSERT FIGURE 2

**Statistical analysis:**

Process capability and statistical control parameters were evaluated using Minitab v15 software (Minitab Ltd, UK). It was not feasible to group measurements into subgroups because long cycle times (9 days) and operational expense limited the production volume to a small run of one product unit per process run. Process capability indices and control charts were therefore calculated using a sample consisting of individual observations. The individual observations (total cell number for each production unit) were plotted on an individuals moving range chart (I-MR chart) to assess whether the process was in statistical control and





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estimate process standard deviation. Control charts were subjected to a set of decision rules based on probability theory and supported by Minitab, to detect non-random patterns in the data and the occurrence of special causes (ASTM 2008; Nelson 1984; Montgomery 2005). Normal probability plots were used to confirm the data were normally distributed. The Anderson-Darling test (AD) was used to compare the empirical cumulative distribution function of the sample data with the distribution expected if the data were normal. A p-value of <0.05 was considered significant for tests of normality. The Cp index was calculated according to the following equation: $Cp = \frac{USL - LSL}{6\sigma}$. The Cpk index was calculated according to the following equation; $Cpk = \min\left(\frac{USL - \mu}{3\sigma}, \frac{\mu - LSL}{3\sigma}\right)$; where *USL* is the upper specification limit, *LSL* is the lower specification limit, σ is the estimated process standard deviation, μ is the process mean (ASTM 2008). Lower 90% confidence bounds were calculated for all capability estimates (smallest value for the process index that can be claimed with a stated confidence of 90%) (ASTM 2008). Cell yields were expressed as process mean +/- Standard error of the mean (SEM) and a Students t-test used to compare the cell population mean output.

## Results

**Study 1:** The cell population growth (total number of cells) in the manual culture reached an average of 1.3 x $10^8$ cells after 3 passages (9 days), which was close to the expected target of 1.0 x $10^8$ (based on a calculated doubling time of 20-24 hrs) and more than twice the cell growth achieved by the automated culture process (average of 6.0 x $10^7$ cells) (Table 1). Images of the HOS cells cultured by the manual or automated culture protocols showed that they appeared healthy post-





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passage and were morphologically similar (Figure 3). The viability data (determined by Trypan blue exclusion both by the Cedex® instrument in the CompacT SelecT and manually) for the cultured HOS cells indicated that neither process had an effect on cell survival. The mean live cell population in the 'granddaughter' flasks after 3 passages was 98% in the manual process and 95% in the automated process.

INSERT TABLE 1

INSERT FIGURE 3

Analysis of the I-MR control charts and histograms for the individual observations (Figure 4) showed that both the manual and automated processes were in statistical control over the course of the study (i.e. observations varied randomly around the centre line between the control limits and exhibited no trends or patterns in the plotted points) and that the data was normally distributed (i.e. normal probability plots showed that plotted points were close to a straight line and within the 95% confidence interval for both the manual (AD = 0.429; $p<0.245$) and automated (AD = 0.188; $p<0.870$) process data). This confirmed the validity of the data and satisfied the assumptions critical for the reliable estimation and interpretation of the process capability indices according to the ASTM standard guideline (ASTM 2008).

INSERT FIGURE 4

The behaviour of the process in the state of statistical control (indicating consistency of process output) was used to reliably describe its capability in terms





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of the proportion of the process output that is within the product specifications. Two short-term capability indices (Cp, Cpk) were calculated to provide a numerical summary that relates the process spread (the 6-σ variation) to the specification spread (Cp) and also considers the location of the process mean within the specification limits (Cpk) (ASTM 2008). Collectively, the Cp and Cpk indices were used to indicate the potential of the process to produce conforming material and to signal the requirement for process location adjustments and/or process variation reduction (ASTM 2008).

The process mean for the manual cell culture process (1.3 x $10^8$ cells) was located close to the centre of the nominal specification, but the capability histogram (upper panel of Figure 5) for the manual process data showed poor potential capability (Cp <1.0) resulting from excess variability. The process mean for the automated cell culture process (6.0 x $10^7$ cells) was poorly located within the nominal specification limit, but the capability histogram (middle panel of Figure 5) showed that the automated process performed with less variability than the manual process, resulting in adequate potential capability (Cp>1.0). Comparison of the point estimates for the Cp indices indicated that, relative to the specification limits, the automated process (Cp=1.32) could achieve better capability than the manual process (Cp=0.55) if the process can be centred.

INSERT FIGURE 5

The point estimate for the Cpk index of the automated process (Cpk=0.25) was lower than its corresponding Cp index (Cp=1.32). Comparison of the point estimates (Cp>1.0, Cpk<1.0), indicated that the automated process is capable, but

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not centred and not performing within the specification limits The point estimate for the Cpk index of the manual process (Cpk=0.26) was also lower than its corresponding Cp index (Cp=0.55). Comparison of the point estimates (Cp<1.0, Cpk<1.0), indicated that the manual process is not capable and not performing within the specification limits (ASTM 2008).

The Cpk indices of <1.00, indicated that a substantial part of the output of both processes falls outside the specification limit. Figure 5 shows that the Cpk indices for both processes were similar but had differing proportions of conforming units (proportion of the specification used by the process). Comparison of the CpL and CpU values (Figure 5) indicated that the process distribution is located closer to the lower specification limit for the automated process (CpL<CpU; Cpk=CpL) and closer to the higher specification limit for the manual process (CpL>CpU; Cpk=CpU). Analysis of the histograms showed that about 23% of the process output falls outside the lower specification limit for the automated process (equivalent to a predicted fall out of about 225,000 non-conforming parts per million (ppm)). For the manual process, about 21% was shown to fall outside the higher specification (predicted fall out of about 214,000 non-conforming ppm) but a small proportion (about 1%) also falls outside the lower specification (predicted fall out of about 6620 non-conforming ppm).

**Study 2:** Differences in cell population growth profile were observed between the automated and manual protocols in study 1 (Table 1). After implementing the adjustment to the automated passage process, a short verification run of 3 production units was conducted to demonstrate an affect on process performance. Results showed that the new passage protocol was effective for detaching cells from the surface of the flask and forming a single cell suspension at the end of the treatment. Images of the HOS cells cultured by the





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adjusted automated culture protocol showed that they appeared healthy post-passage and were morphologically similar to the cells cultured in both the manual and baseline automated processes (Figure 3). The viability data for the cultured HOS cells indicated that the process did not affect cell survival. The mean live cell population in the 'granddaughter' flasks after 3 passages was 95%.

The cell yield obtained from the adjusted automated process reached an average of 7.3 x $10^7$ cells, which was closer to the nominal target of 1.0 x $10^8$ cells and more than 1.2 fold (22%) higher than the that of the baseline automated process (6.0 x $10^8$ cells), resulting in a shift in the mean process yield closer to centre of the specification (Figures 4 & 5). Assuming that the process mean adjustment has no effect on process variability (i.e. Cp=1.32), a shift in process mean to 7.3 x $10^7$ cells would achieve an estimated Cpk of 0.61 (lower 90% confidence bound Cpk= 0.41). This would yield a predicted fallout of the process of 34,537 non-conforming ppm, representing about a 7-fold improvement in process performance.

## Discussion

The transition to robust, scaled culture and expansion of human cells represents one of the most significant bioengineering challenges that need to be met for cell based products to become safe, effective and affordable therapeutic modalities of regenerative medicine. Automated platforms have the potential to provide the operational stability and control to allow the scalable processing and population expansion of cells at the volumes required for cell based therapeutic products for human use, including products for clinical trials and cell repositories (Palsson and Bhatia 2004; Archer and Williams 2005). Previously, we have described the

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process transfer of a number of bench scale manual cell culture protocols to a completely automated cell culture platform (CompacT SelecT); including processes for the in vitro expansion of primary bone marrow-derived mesenchymal stem cells, primary umbilical cord-derived progenitor cells, and human embryonic stem cells (Thomas *et al*. 2007, 2008a, 2009). Several other groups are also beginning to integrate automation into various aspects of human cell culture to address the issues inherent in conventional manual techniques (Kino-Oka *et al*. 2005; Joannides *et al*. 2006; Terstegge *et al*. 2007). Until now, these studies have sought to demonstrate equivalent performance in terms of selected mean process output parameters and have not attempted to quantify and analyse inherent process variability relative to product specifications or requirements, referred to as process capability.

Process capability analysis approaches have been widely used in mature industry sectors such as the electronics, pharmaceutical and medical devices sectors, typically within quality-by-design frameworks and continuous quality improvement programmes (USFDA 2004b; Yang and Liu 2005; Pearn and Wu 2005; Liu *et al*. 2008; Cogdill and Drennen 2008). In accordance with current regulatory drivers under Good Manufacturing Practice and the new European Union Tissues and Cells Directives (ATMP Regulation 1394/2007/EC), these approaches, as part of a quality function early in development, are likely to be equally important to gaining better process understanding and control of emerging cell based therapy products and their manufacturing processes, particularly to minimise product quality variation, non-conformance and delays in product approval.

This study has demonstrated the application of process capability analysis to establish and compare the short-term process capability of a manual and a



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prototype automated culture process for the in vitro expansion of a selected anchorage-dependent cell line. The results of the short-term process capability analysis indicated that the manual process had poor potential capability (Cp<1.0) resulting from excess variability. The automated process was poorly located within the specification limits but was less variable than the manual process, resulting in a higher potential capability (Cp>1.0). The two processes had similar Cpk indices but were centred differently and had differing proportions conforming, which shows that the Cpk index only communicates process performance relative to one of the two specification limits. Similarly, the Cp value alone is not sufficient to communicate the proportion conforming. It is therefore necessary to compare values for both Cp and Cpk in order to determine the current proportion conforming. A comparison of point estimates, which showed Cpk < Cp, indicated that the automated process mean was off-centre and that intervention is required to adjust the process location by increasing the process mean. If the process mean is centred, Cpk=Cp=1.32, almost all of the process measurements fall inside the specification limits, representing an improvement of several orders of magnitude in process performance.

Overall, comparison of the point estimates for the Cp and Cpk indices indicated that, relative to the specification limits, the automated process could achieve better capability than the manual process if the process mean can be shifted closer to the centre of the specification (equivalent to about a 1.7 fold shift in process mean). This indicated that intervention is required to adjust the process location by increasing the process mean until Cpk=Cp=1.32. Assuming that the specification accurately reflects the customer requirement and that the process is properly centred with a normal distribution, this process would produce an estimated 78 non-conforming ppm (i.e. 39 ppm outside both the LSL and HSL

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limits), representing an improvement of several orders of magnitude in process performance.

However, in practice point estimates of capability indices are subject to sampling error as a function of sample size (ASTM 2008; Montgomery 2005). The 90% confidence bound estimate of Cp for the automated process was Cp=0.90, which means that for a sample size n=10, we can only be 90% confident that the Cp index is not less than or equal to 0.90. Even though the point estimate of Cp for the automated process met the minimum requirement (Cp ≥ 1.0) for a capable process (Pearn and Chen 1999; Montgomery 2005), the 90% confidence bound value (Cp=0.90) suggests that we are unable to conclude with 90% confidence that the process meets this minimal requirement. Put another way, to be 90% confident that the capability index was ≥1.00, based on a sample size n=10 we would need to achieve a capability index of ≥1.5, which is clearly higher than the observed point estimate for Cp (Cp=1.32). This indicates that, even with an adjustment to the process mean to properly centre the process, intervention is required to reduce process variation in order to achieve a minimum acceptable Cp ≥ 1.0 i.e. the ability to produce more than 99.73% lots within the specification.

Since the process capability analysis showed that the automated process was in control but not capable of meeting specifications without centring, an investigation to identify assignable causes for the bias was conducted using quality tools such as process mapping and cause and effect (C&E) analysis. The C&E matrix shown in Figure 2 was constructed to define and relate the key process steps and process input variables to the key outputs (cell yield) using a process map as the primary source. The high-level fishbone diagram (Figure 2) identifies the sub-processes or process steps, categorised as neutralisation, sub-culture, pooling and passage, as the main sources of interdependent variation. It





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was used to identify the relationships among the various parameters (inputs) and characteristics (outputs) from implicit knowledge in the first instance (Thomas *et al*. 2007; Thomson 2007). The limbs on the branches record the causes or the input variables that could be contributing to the observed effect.

The differences in process mean output for the manual and automated processes can be traced to the observed differences in cell population growth profile, attributed to key operational parameter differences between the protocols. The similarity in population growth of cells in manual and automated culture up to the first passage suggested that the observed difference in subsequent cell population growth, culminating in lower cell output in the automated system, may be attributable to operational parameter differences introduced at the passage point. Despite minor non-linear operating parameter differences from the manual process (such as the modes of dispensing and removing medium, mixing cell suspensions etc), the most obvious such difference is the absence of a centrifugation step to remove residual trypsin from the harvested cell suspension before subsequent passage in the automated process. Instead, the automated process relies on neutralisation of trypsin through the addition of culture medium containing 10% FBS, which is known to contain trypsin inhibitors. Previous investigations of trypsin contamination and cell population growth have suggested that non-optimised automated passage protocols can result in incomplete neutralisation of the trypsin, leaving sufficient residual trypsin (typically 1–4%) to inhibit cell adhesion and cell growth and therefore contribute to the differences in cell yield observed between automated and manual culture protocols (Cruz *et al*. 1997; Thomas *et al*. 2007). Based on this hypothesis, a short trial verification run, comprising three production lots of cells, was manufactured using an alternative automated process in which the settings of the passage process were adjusted in

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order to eliminate residual trypsin activity and any adverse effect on the subsequent processing of cells. In practical terms, the adjusted process produced on average about 1.2 fold more cells than the baseline automated process, shifting the process mean closer to the centre of the specification, and resulting in a 7-fold improvement in process performance based on a point estimate of Cpk.

The results of this short verification run indicated that the trypsinisation sub-process may be a critical source of process variability and in principle, demonstrates that by making changes to the process settings, the process mean of the automated process can be shifted towards the centre of the specification to improve the actual capability of the automated process. However, further studies, based on statistically designed experiments (DOE), are required to find the optimal settings for the cell dissociation (trypsinisation) sub-process variables in order to further increase the process mean and reduce the overall process variability for long-term improvement (Thomas *et al*. 2008b).

Overall, this study demonstrates the potential process capability of an automated, scalable system as a platform suitable for the production of anchorage dependent cells in sufficient volumes for regenerative medicine applications. The need for detailed understanding of processes, process optimisation and process standardisation, is highlighted by the observed differences in cell population growth profile, attributed to key operational differences between the automated and manual protocols. The cell dissociation step in particular has important implications for the efficient scale-up and automation of bioprocesses for anchorage dependent cells.

The next phase of our systematic approach will target an improvement strategy leveraging key process variables, firstly to centre the automated process between the specification limits and then to reduce variation. The implementation

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of this focus is to create a process with Cp>1, although ultimately the improvement target will depend on a company's quality focus, attitude to risk, marketing plan and their competitor position.

In this study we selected a permanent, transformed cell line (HOS) as a model system to compare the short-term process capability manual and automated culture processes. Primary cells, such as embryonic stem cells (ESC), are known to be more sensitive to process variability than established cell lines and are likely to present a greater technical challenge for the scaled production of consistently safe, efficacious and genetically stable primary cellular preparations for successful applications in Regenerative Medicine.

Many studies have highlighted the sensitivity of primary cells, such as ESC, to variations inherent in manual handling procedures (Thomson 2007; Thomas *et al*. 2007 and 2009; Veraitch *et al* 2008). These studies also reveal how manual protocols for ESC cultivation could be improved by automating critical sub-processes to control variation in parameters such as cell density measurement, fluid flow, shear forces, pH and temperature. Further systematic process development studies will target other more clinically important anchorage-dependent cell types, aimed at translating features that are critical to quality from the perspective of patients to the features that are critical to quality in the automated manufacturing process (Mason and Dunnill 2008a and 2008b). The accumulated process understanding obtained during these studies will provide opportunities for further risk mitigation and allow a framework to be established to determine the design space which will define the critical unit control parameters that can be varied to consistently produce a cell based product with the desired specification.

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References

ATMP Regulation (EC) No. 1394/2007 of the European Parliament and of the Council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Reg (EC) No 726/2004. Official Journal of the European Union, 10.12.2007, L324: 121–137

Archer R, Williams DJ (2005) Why tissue engineering needs process engineering. Nat. Biotechnol 23(11): 1353-1355

ASTM (2008) Standard Practice for Process and Measurement Capability Indices. ASTM International E2281-08

BSI (2006) Publicly Available Specification 83 (PAS 83), Guidance on codes of practice, standardised methods and regulations for cell-based therapies

Coecke S, Balls M, Bowe G, Davis J, Gstraunthaler G, Hartung T, Hay R, Merten O-W, Price A, Schechtman L, Stacey G, Stokes W (2005) Good Cell Culture Practice: ECVAM Good Cell Culture Practice Task Force Report 2, ATLA 33: 261-287

Cogdill RP, Drennen JK (2008) Risk-based Quality by Design (QbD): A Taguchi Perspective on the Assessment of Product Quality, and the Quantitative Linkage of Drug Product Parameters and Clinical Performance. J Pharm Innov 3: 23-29

For Peer Review

Commission Directive 2006/17/EC of 8 February 2006 implementing Directive 2004/23/EC of the European Parliament and of the Council as regards certain technical requirements for the donation, procurement and testing of tissues and cells. Official Journal of the European Union 2006, 9.2.2006, L38/40 – L38/52: 40–52

Commission Directive 2006/86/EC of 24 October 2006 implementing Directive 2004/23/EC of the European Parliament and of the Council as regards traceability requirements, notification of serious adverse reactions and events, and certain technical requirements for coding, processing, preservation, storage and distribution of tissues and cells. Official Journal of the European Union, 25.10.2006, L294: 32–50

Cruz HJ, Dias EM, Moriera JL, Carrondo MJT (1997) Cell-dislodging methods under serum free conditions. Appl Microbiol Biotechnol 47: 482-488

Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards on quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of tissues and cells. Official Journal of the European Union 2004, 7.4.2004, L102/48 – L102/58: 48–58

DTI Global Watch Mission Report (2006) Advanced cell and tissue therapies – a mission to the USA

Joannides A, Fiore-Hériché C, Westmore K (2006). Automated mechanical passaging: a novel and efficient method for human embryonic stem cell expansion. Stem Cells 24: 230-235

Kemp P (2006) Cell therapy – back on the up-curve (interview). Regenerative Medicine, 1(1): 9-14

For Peer Review

Kempner ME, Felder RA (2002) A review of cell culture automation. JALA 7(2): 56-62

Kino-Oka M, Ogawa N, Umegaki R, Taya, M (2005) Bioreactor design for successive culture of anchorage-dependent cells operated in an automated manner. Tissue Engineering 11: 535-545

Liu CZ, Han ZW, Hourd PC, Czernuszka JT (2008) On the process capability of the solid free-form fabrication: a case study of scaffold moulds for tissue engineering. Proc. IMechE Part H: J Engineering in Medicine 222: 377-391

Liu, Y., Hourd, P. and Williams, D.J., 'Measurement System Capability: A Comparison of Manual and Automated Cell Counting Systems, Tissue Engineering 13(7), 2007, 1722

Lysaught MJ, Kaklenec A, Deweerd E (2008) Great Expectations: Private sector activity in Tissue Engineering, Regenerative Medicine and Stem Cell Therapeutics. Tissue Engineering Part A 14(2): 305-315

Mason C, Dunnill P (2008a) The crucial linkage required between regenerative medicine bioprocesses and clinicians. Future Medicine 3(4): 435-442

Mason C, Dunnill P (2008b) The strong financial case for regenerative medicine and the regen industry. Future Medicine 3(3): 351-363

Mason C, Hoare M (2007) Regenerative Medicine Bioprocessing: Building a conceptual framework based on early studies. Tissue Engineering 13(2): 301-311

Montgomery D (2005). Introduction to Statistical Quality Control, 5th edition, Hoboken NJ, Wiley

Nelson LS (1984). The Shewart Control Chart- Tests for Special Causes. Journal of Quality Technology 16(4): 237-239

For Peer Review

Palsson BO, Bhatia SN (Ed) (2004) Tissue Engineering, Chapter 1: 1-17, Pearson Prentice Hall, NJ, US

Pearn WL, Chen KS (1999) Making decisions in assessing Process Capability index Cpk. Quality and reliability Engineering International 15: 321-326

Pearn WL, Wu C-H (2005) An effective modern approach for measuring high-tech product manufacturing process quality. Journal of the Chinese Institute of Industrial Engineers l22(2): 119-133

Standard Operating Procedure SOP012 (2008) Manual culture of Osteoblast-like cell line. Healthcare Engineering Department, Loughborough University

Standard Operating Procedure SOP043 (2008) Automated culture of Osteoblast-like cell line. Healthcare Engineering Department, Loughborough University

Standard Operating Procedure SOP031 (2008) Cryopreservation and storage of mammalian cell lines. Healthcare Engineering Department, Loughborough University

Standard Operating Procedure SOP032 (2008) Resuscitation of cryopreserved mammalian cell lines. Healthcare Engineering Department, Loughborough University

Terstegge S, Laufenberg I, Pochert J, Schenk S, Itskovitz-Eldor J, Endl E, Brustle O (2007) Automated maintenance of embryonic stem cell cultures. Biotechnol Bioeng 96: 195–201

Thomas RJ, Chandra A, Liu Y, Hourd PC, Conway P, Williams DJ (2007) Manufacture of human mesenchymal stem cell population using an automated cell culture platform. Cytotechnology 55: 31-39

Thomas RJ, Chandra A, Hourd PC, Williams DJ (2008a) Cell Culture Automation and Quality Engineering: A Necessary Partnership to Develop Optimized

For Peer Review

Manufacturing Processes for Cell-Based Therapies. Journal of Association of Laboratory Engineering:152-158

Thomas RJ, Hourd PC, Williams DJ (2008b) Application of process quality engineering techniques to improve understanding of the in vitro processing of stem cells. Journal of Biotechnology 136: 148-155

Thomas RJ, Anderson D, Chandra A, Smith N, William D, Young L, Denning C (2009) Automated, scalable culture of human embryonic stem cells in feeder free conditions. Biotechnol Bioeng 102(6): 1636-1644

Thomson H (2007) Bioprocessing of embryonic stem cells for drug discovery. Trends in Biotechnology 25(5): 224-230

US Federal Drug Administration (USFDA) (1997) Proposed approach to regulation of cellular and tissue based products. DN 97N-0068.

USFDA (2004a) Innovation or stagnation: Challenges and opportunity on the critical path to new medical products

USFDA (2004b) Pharmaceutical cGMPs for the 21st Century — A Risk-Based Approach. Final Report, Department of Health and Human Services, U.S Food and Drug Administration

Vacanti CA (2006) History of tissue engineering and a glimpse into its future. Tissue Engineering 12(5): 1137-1142

Veraitch FS, Scott R, Wong J-W, Lye GJ, Mason C (2008) The impact of manual processing on the expansion and directed differentiation of embryonic stem cells. Biotechnology and Bioengineering 99(5): 1216-1229

Williams DJ, Sebastine IM (2005) Tissue Engineering and Regenerative Medicine: Manufacturing Challenges. IEE Proc. Nanobiotechnol 152(6): 207-210

For Peer Review

Yang X, Liu CR (2005) Investigating Machining impact on fatigue variance: A machining quality perspective. Transactions of the ASME 127: 492-502

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## LEGENDS FOR FIGURES AND TABLES

**Figure 1:** Comparison of key features of the automated (left panel) and manual (right panel) cell culture systems. Specific features of the CompacT SelecT are shown. This automated cell culture platform can simultaneously manipulate 2 x T175 flasks and house 90 x T175 culture flasks in a robot accessible incubator. All culture processes are carried out within a sterile class II environment and require no manual intervention. The inset shows an enlarged image of the manipulation chamber and pipette head. Major processing components are labelled: A, Robot arm (inset); B, Flask incubator; C, Plate incubator; D, Flask decappers (inset); E, Flask holders (inset); F, Media pumps; G, Pipette head; H, Cedex® automated cell counter.

**Figure 2:** Cause-and-effect diagram for investigation of the low average viable cell yield obtained using the automated cell culture system. The quality improvement team identified potential causes of the problem, categorised within the primary sub-processes. Informed by differences between the manual and automated culture systems, the most likely causes were identified (indicated by open circle symbol).

**Figure 3:** A representative selection of images of HOS cells cultured using the baseline automated (centre panel) and manual cell expansion (left panel) protocols. The right panel shows the images of HOS cells cultured using automated process with the adjusted passage protocol settings. Images show cells in monolayer culture prior to passage 3. Cells are morphologically similar.

**Figure 4:** Plots of individual observations (I chart) and moving ranges (MR chart) for variables data from manual (upper panel) and automated (lower panel) culture processes. The I-MR chart for the automated process (lower panel)is split into two

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stages showing the individual observations and moving ranges for the first 10 production units (Study 1) and for the 3 production units (lots 11-13) generated from the adjusted automated protocol (Study 2). Tests for special causes for the variation confirmed that all points varied 'randomly around the centre line and were within the control limits.

**Figure 5:** Process capability histograms for the manual process data (upper panel), automated process data (middle panel) and adjusted automated process data (lower panel). Total number of harvested cells (cell yield) is shown on the y-axis. Dashed vertical lines indicate the lower specification limit (LSL) = $5.0 \times 10^7$ and the upper specification limit (USL) = $1.5 \times 10^8$. Solid arrow indicates process mean and the solid line represents the normal density function for within variance. Process and capability data is displayed in the boxes at the left side of each panel. Cp = process capability irrespective of process centring, Cpk = process capability accounting for process centring, CpU = process capability relative to the USL, CpL = process capability relative to the LSL.

**Table 1:** HOS cell population expansion (total number of cells/flask) at each passage, for cells grown using the manual and baseline automated culture processes (Study 1) and the automated process with adjusted passage protocol settings (Study 2). Mean total number cells/flask +/- SEM based on 3 passages for each of 10 production runs (Study 1). Mean total number cells/flask +/- SEM based on 3 passages for each of 3 production units generated from the adjusted automated protocol (Study 2). Automated culture process means significantly different ($p<0.05$) from manual culture process means (two sample t-test) is indicated by an asterisk.

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**Table 1**

| | Manual Culture | Automated Culture | Adjusted Automated Culture |
|---|---|---|---|
| | Mean cell number/flask +/- SEM | Mean cell number/flask +/- SEM | Mean cell number/flask +/- SEM |
| Passage 1 (Day 3) | $4.6 \times 10^6$ +/- $1.3 \times 10^6$ | $3.4 \times 10^6$ +/- $1.1 \times 10^6$ | $4.4 \times 10^6$ +/- $2.5 \times 10^5$ |
| Passage 2 (Day 6) | $8.0 \times 10^6$ +/- $2.1 \times 10^6$ | $4.2 \times 10^6$ +/- $1.7 \times 10^6$* | $5.3 \times 10^6$ +/- $1.0 \times 10^6$ * |
| Passage 3 (Day 9) | $1.3 \times 10^8$ +/- $4.2 \times 10^7$ | $6.0 \times 10^7$ +/- $1.6 \times 10^7$* | $7.3 \times 10^7$ +/- $9.0 \times 10^6$ |

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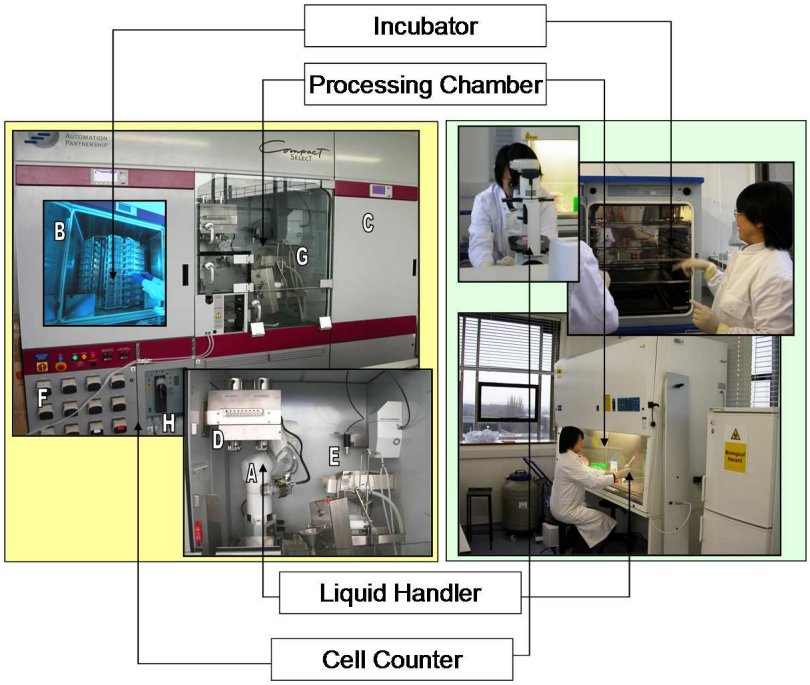


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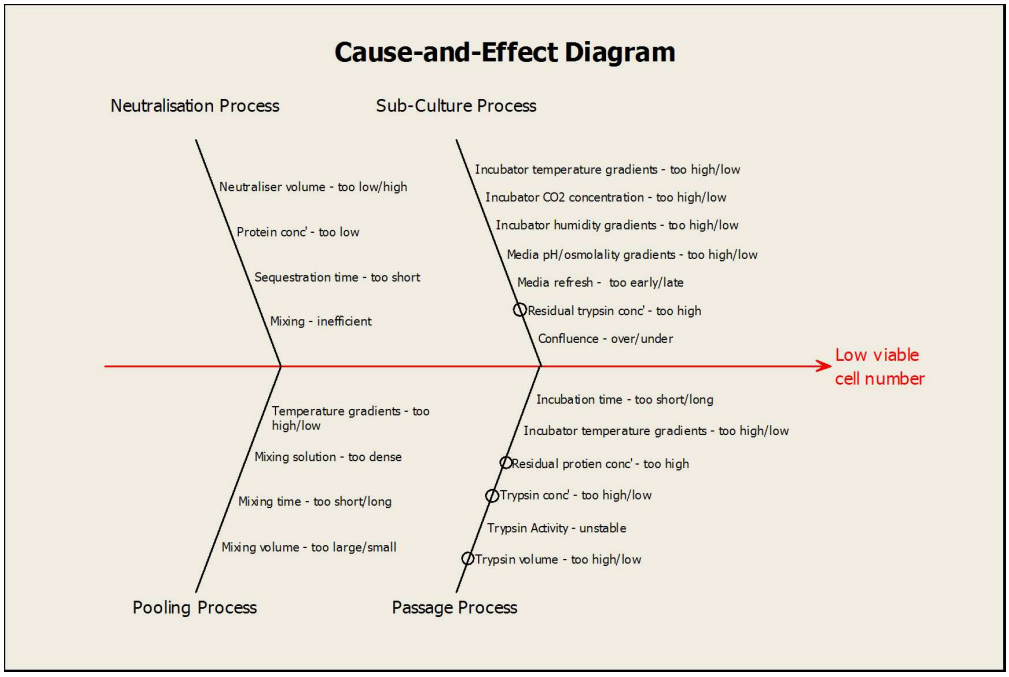


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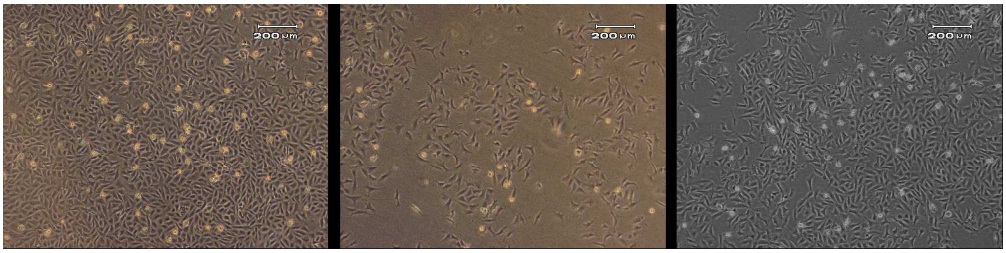


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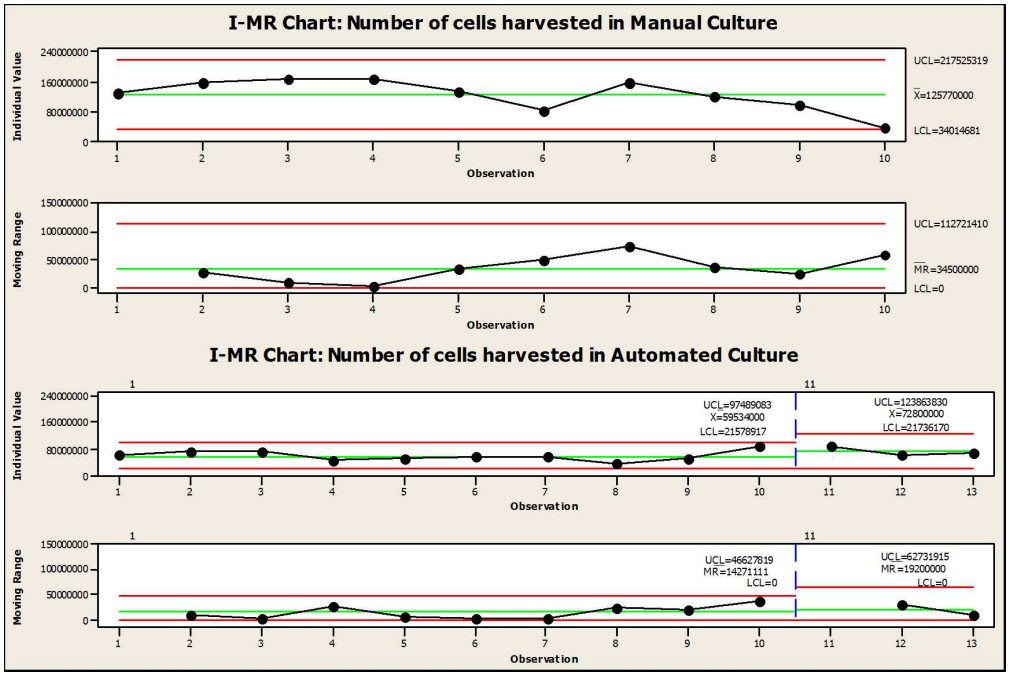


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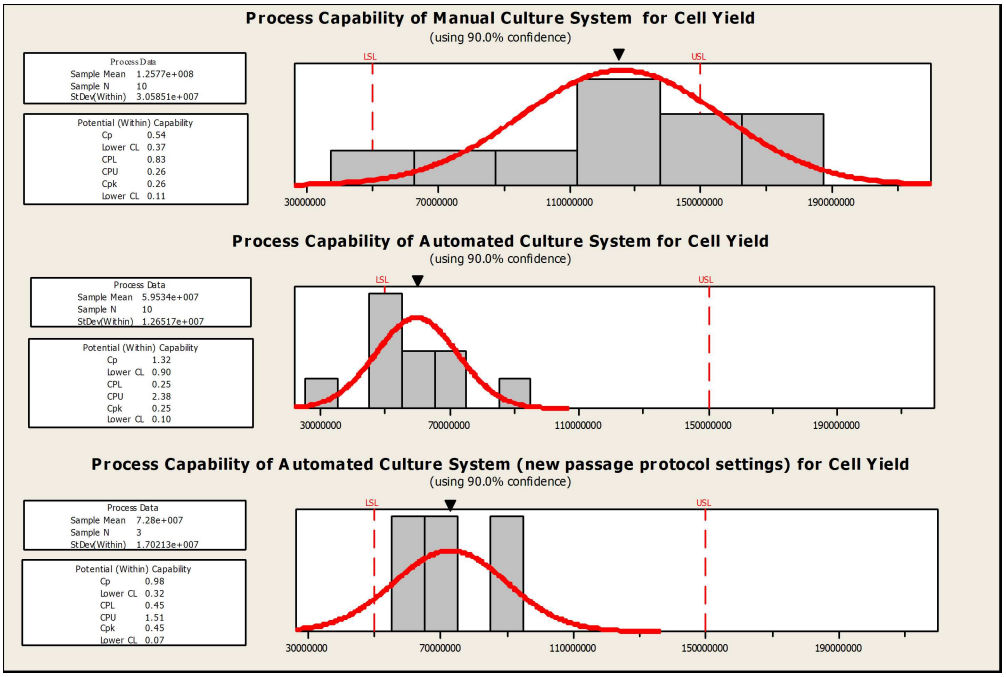


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760x507mm (96 x 96 DPI)