Effects of Cellular Apoptosis on Productivity of a Mammalian Cell Culture Process

by

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Abstract

Apoptosis, programmed cell death, is a hot topic in recent research due to the potential applications to various areas by regulating its pathway. In industrial large scale animal cell culture processes, research on how to regulate or predict the apoptotic pathway and understanding what signals the apoptotic cascade has lead to a new opportunity to enhance process robustness, improve final performance including productivity, and eventually, reduce production costs. Current industrial cell culture processes normally involve a high cell density process in a large-scale bioreactor as a suspension culture that proliferates the cells beyond their optimal growth conditions. Under these conditions, apoptosis will be triggered, and consequently, cell viability will be decreased, and the chance for product degradation by the release of intracellular proteases and glycosidases will increase. Therefore, characterizing which culture conditions will induce apoptosis during a particular cell culture process can be a valuable tool to optimize cell viability and possibly productivity. Since the conventional method for cell count and viability measurement does not differentiate the cells in early to mid-stage apoptosis from the normal cells, it would be difficult to understand the effect of early stage apoptosis. This study elucidates the correlation between the culture conditions and apoptosis during a mammalian cell culture process and its effects on the productivity using real-time apoptotic assays for accurate cellular growth and death profiles. Apoptosis induced by low pH, glucose and glutamine limitation, lactate toxicity and Camptothecin has been shown to significantly increase the yield and specific productivity most likely due to release of product during secondary necrosis at the culmination of the apoptosis pathway.

Table of Contents

List of Tables

List of Figures

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1. Introduction

1.1. Background

Apoptosis is an important factor influencing viability, cell density, and productivity in a cell culture process. Understanding how the rate of apoptosis is affected by the process conditions is useful to improve process performance and to develop a real-time process monitoring methodology for troubleshooting purposes. Cell death occurs by either necrosis or apoptosis. Necrosis involves disruption of membrane integrity and is caused by severe physical or chemical damage to the cell. The cell swells and bursts osmotically releasing its contents into the culture (Mazur et al., 1999). On the other hand, apoptosis, or programmed cell death, is a sophisticated biochemical response to non-lethal stimuli, which allows for cell self-destruction of unwanted cells. Figure 1 is a schematic diagram showing necrosis versus apoptosis in a cell.

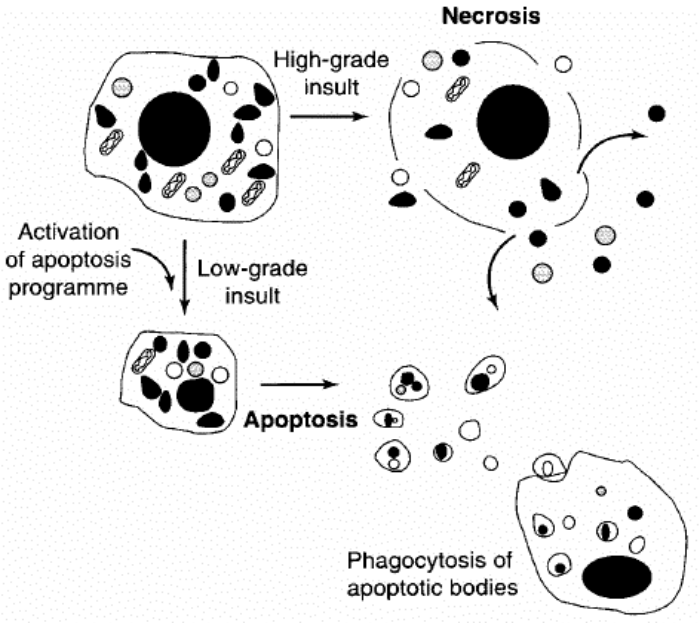


Figure 1. Necrosis versus Apoptosis

Cell death occurs in two ways: necrosis or apoptosis. Necrosis is caused by severe damage causing the cell to swell and burst. Apoptosis occurs in response to non-lethal stimuli, which triggers a biochemical cascade resulting in characteristic morphological changes and self-destruction. (Figure from Cotter and Al-Rubeai, 1995)

The term “apoptosis” was first noted in 1972 in a paper by Kerr, Wyllie and Currie to define the highly regulated morphology and biochemistry of programmed cell death different from that of necrosis (Kerr et al., 1972). The process of eliminating the DNA damaged, superfluous, or unwanted cells is characterized by nuclear chromatin condensation, cytoplasmic shrinking, and DNA fragmentation between nucleosomes into approximately 180 base pairs (Wyllie, 1980 reviewed by Hengartner, 2000). Fairly early in the pathway, translocation of the phospholipid phosphatidylserine (PS) and intracellular proteins to the cell surface occurs. This change is important since the exposure of the extracellular PS facilitates phagocytosis by macrophages in multicellular organisms (Fadok et al., 1992 reviewed by Hammill et al., 1999). When the membrane phospholipid becomes externalized, endonucleases then destroy the cell’s DNA and the cytoskeleton is restructured before the cell body collapses into membrane-bound apoptotic bodies in a process called blebbing**.** There is some indication in apoptotic B-cell lymphoma that apoptosis in cells at the stage of PS externalization and chromatin condensation and cleavage can be reversed and that loss of membrane asymmetry precedes the commitment to cell death (Hammill et al., 1999; Vaughan et al, 2002; Simak et al., 2002).

The complicated apoptosis process is controlled by a number of proteins including caspases (cysteine-containing aspartate-specific proteases) and can differ significantly based on the cell lines, culture conditions and stimuli (Al-Rubeai and Singh, 1998; Fussenegger et al., 2000). Under normal conditions, caspases are present as inactive proteins called procaspases or zymogens. Once triggered by stress or damage, a

cascade of events occurs resulting in programmed cell death (Hengartner, 2000), (see Figure 2).

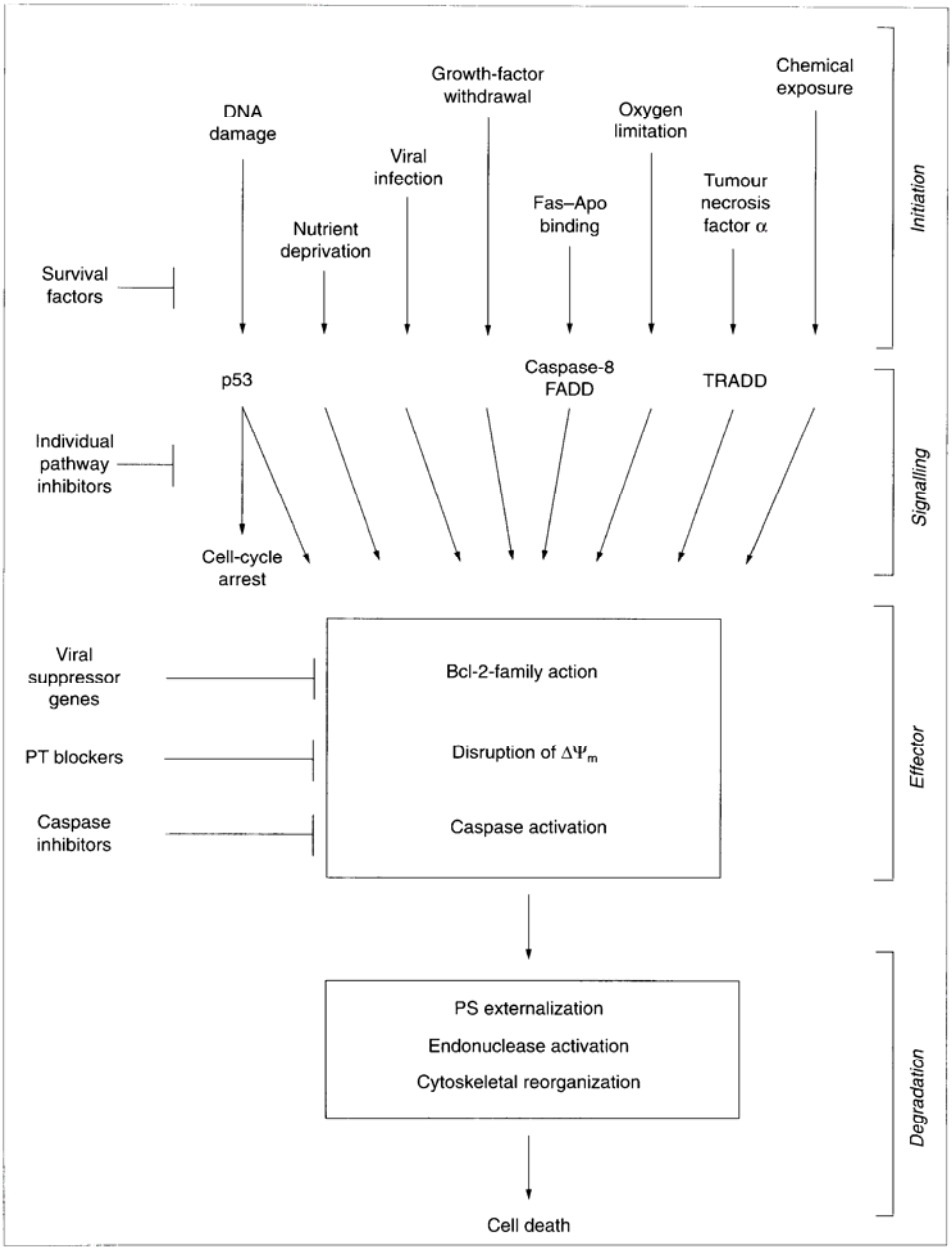


Figure 2: Apoptotic Cascade

The apoptotic cascade is comprised of four main phases: initiation, signaling, effector, and degradation. The pathway is initiated by a stimulus, which signals certain receptors on the cell surface. These signals initiate the Bcl-2 family of survival factor proteins, releases mitochondrial proteins, and activate effector or executioner caspases. The cascade ends with the degradation phase followed by either phagocytosis *in vivo* or secondary necrosis *in vitro*. Throughout the pathway there are a number of ways the cascade is regulated including survival factors, caspase inhibitors and individual pathway blockers (noted on left). (Figure from Mastrangelo and Betenbaugh, 1998)

There are two apoptotic pathways culminating in cell death: the death receptor pathway and the mitochondrial pathway (Green, 2000), (see Figure 3). Recent research has suggested a third pathway culminating in the endoplasmic reticulum causing the activation of caspase-12, however little is known at this time (Donovan and Cotter, 2004).

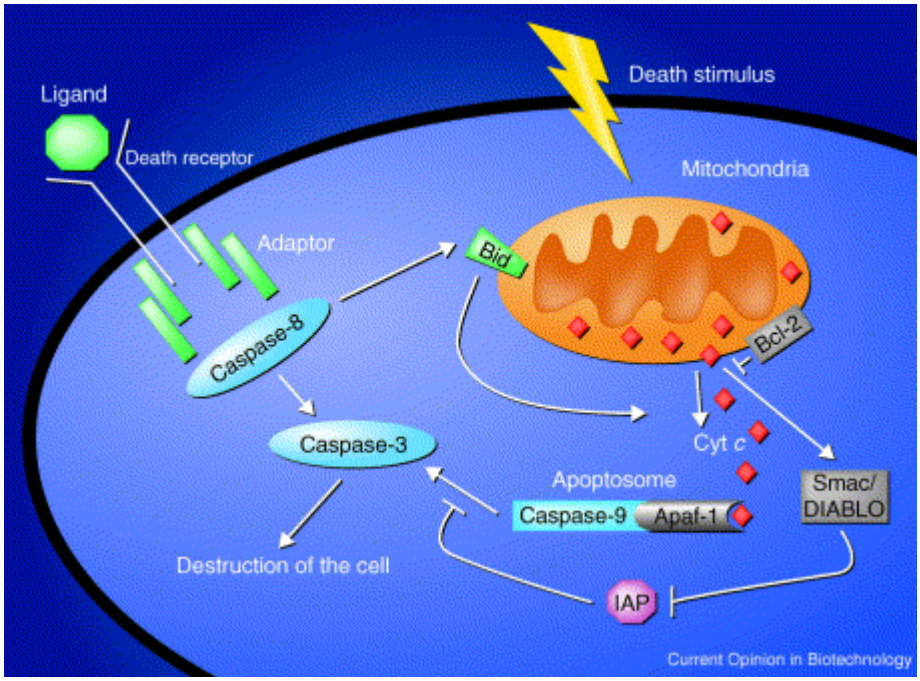


Figure 3: Apoptosis Pathway

There are two main apoptotic pathways in the cell: receptor mediated and mitochondrial. When a cell encounters an external stimulus, the receptor-mediated pathway is triggered causing a ligand to bind to a death receptor and the activation of an initiator caspase such as caspase 8. The mitochondrial pathway is normally triggered by internal stresses up regulating certain Bcl-2 pro-apoptotic proteins (i.e. Bid), which causes mitochondria permeability and the release of apoptotic proteins such as *Smac*/DIABLO[1], Apaf-1 and Cyt c. Cyt c binds to Apaf-1, which then activates caspase 9. Both caspase-8 and the apoptosome Caspase-9/Apaf-1 complex can trigger an executioner caspase such as caspase-3 leading to the amplification of executioner caspases and cell destruction. IAP and Bcl-2 survival proteins are important for regulation of the process. The S*mac*/Diablo complex is needed to inhibit the IAP family, which inactivates caspases. (Figure from Laken and Leonard, 2001)

[1] Abbreviations: *Smac*/Diablo, second mitochondrial activator of caspases/direct inhibition of apoptosis protein binding protein with low pI; Apaf-1, apoptosis protein activating factor-1; Cyt c, cytochrome c; IAP, inhibitor of apoptosis proteins

The death receptor pathway is activated when the cell is exposed to an external stimulus, in most cases, that launches the cascade by activating the zymogen procaspase-8 via the FAS/FADD death domain receptor. Caspases are activated by cleavage after the C-terminal aspartic acid residue and in turn continue the cascade by cleaving other enzymes at their active site cysteine with a specificity determined by four residues on the N-terminal end of the cleavage site. The procaspase-8/death receptor complex in turn activates the initiator caspases, caspase-2, 8, and 10, which triggers procaspase-3. Procaspase-3 with the help of caspase-8 and the apoptosome Caspase-9/Apaf-1 complex triggers the activation of caspase-3, an executioner or effector caspase.

As the levels of executioner/effector caspases increase, the active caspase-8 also cleaves pro-apoptotic *Bid*, a *Bcl-2* protein. This in turn causes the mitochondria to release cytochrome c which complexes to *APaf-1*, a cytosolic protein, catalyzing procaspase-9 cleavage thereby producing initiator caspase-9. An additional mitochondrial released protein, *Smac*/DIABLO, regulates the cascade by blocking the inhibitors of apoptosis protein (IAP) family, which bind to inactivate caspases. The *Smac/DIABLO* regulation is necessary to promote caspase-9 activation (Laken and Leonard, 2001). The pathways of the initiator caspases converge here to amplify additional executioner caspases (caspase-3, 6, 7). The *Bcl-2* family of survival factor proteins has pro-apoptotic and anti-apoptotic roles and it is these proteins that regulate the cytochrome c and apoptosis-inducing factor (AIF) release into the cytosol and additional caspase activation. It is during this phase that apoptosis reversal is possible (Donovan and Cotter, 2004, Lukovic et al., 2003). The relative ratio of death suppressor

and death inducer *Bcl-2* proteins determines the fate of the cell (Korsmeyer, 1995 reviewed by Al-Rubeai and Singh, 1998).

The cascade of caspase receptor-mediated events ends with the degradation phase and the formation of apoptotic bodies followed by cell death either through phagocytosis *in vivo* or secondary necrosis *in vitro* (Mastrangelo and Betenbaugh, 1998). The apoptotic pathway is regulated at various places in the cycle by different proteins. For example, apoptosis triggered by DNA damage from irradiation or drugs used for cancer chemotherapy has been found to be dependant on p53, the tumor suppressor gene, which is the protein that senses DNA damage and halts replication (Kerr, 1995). The p53 protein is a key player in both growth arrest and apoptosis, forcing "bad" cells to commit suicide after DNA damage. p53 has been shown to be a negative regulator of anti-apoptotic *Bcl-2* expression and is a key factor in cell cycle mediated apoptosis (Meikrantz and Schlegel, 1995; Fussenegger and Bailey, 1998).

1.2. Importance and Applications of Apoptosis

Interest in apoptosis research has increased considerably recently for a number of reasons including development of therapeutic treatments, cell culture technology development, metabolic engineering of mammalian cells and gene therapy. Programmed cell death is important in a number of normal processes including metamorphosis of insects and amphibians, development of the human nervous system, the immune response and homeostasis in various organisms (Vaux, 1993; Lee et al., 2000). Understanding the wide variety of stimuli that can induce or inhibit apoptosis is beneficial to research to develop therapeutic treatments to numerous diseases. This includes cancer for failing to respond to apoptotic signals and Parkinson's and Alzheimer's diseases characterized by

excessive apoptotic activity of neurons (Thompson, 1995 and Haass, 1999 reviewed by Fussenegger et al., 2000).

Apoptosis is detected almost exclusively in proliferating cells in cell culture processes and is particularly evident after periods of rapid cell growth (Fussenegger and Bailey, 1998; Meikrantz and Schlegel, 1995). This phenomenon is the main cause of cell death in biopharmaceutical animal cell culture processes including those for baby hamster kidney (BHK), insect, Chinese hamster ovary (CHO), and mouse myeloma (NSO) cells; and therefore presents an opportunity for process optimization (Al-Rubeai and Singh, 1998). The current industrial cell culture processes mostly involve a high cell density process in a large-scale bioreactor as a suspension culture that proliferates the cells beyond the optimal growth conditions. Mild stimuli to the cells during a cell culture process caused by deprivation of nutrients (Singh et al., 1994), growth hormones and oxygen (Al-Rubeai and Singh, 1998) in the culture medium, sudden pH change, mechanical stress by agitation, or accumulation of cellular metabolites can cause apoptosis (Perreault and Lemieux, 1993; Arden et al., 2004). During scale-up, there are many factors that contribute to cell damage and apoptosis, such as hydrodynamic shear and bubble damage caused by gas sparging (Marks, 2003). In addition, the equipment design can have a significant effect on the presence of key nutrients, oxygen transfer, concentration of toxic metabolites, and shear stress. There can also be issues with pH and dissolved oxygen (DO) gradients with inadequate mixing, all causing increased chance for apoptosis.

Since the productivity of a protein of interest often depends on the final cell density and the specific productivity per cell, loss of actively growing and producing cells

by apoptosis during the production stage will cause a reduction of overall product yield. Mercille and Massie found that apoptosis accounts for the abrupt decrease in viability observed in late exponential phase of batch cultures (Mercille and Massie, 1994). Dying cells release proteases and other components through secondary necrosis, which can lower the product yield through degradation and change in the product quality. Thus, higher product consistency and quality can be achieved by employing well-defined and reproducible culture conditions and by minimizing the release of intracellular proteases and glycosidases.

The most widely used methods for cell viability measurement using trypan blue dye or optical probes do not detect the cell population in the early stages of apoptosis. However, real-time monitoring of apoptotic events during a cell culture process could offer an opportunity to alter cell culture conditions much earlier to maintain cell viability and productivity or aid in process optimization during development stages (Vaughan et al, 2002). When parameters such as temperature, dissolved oxygen, agitation, nutrient concentration, or pH levels fluctuate enough to trigger an apoptotic cascade during a cell culture process, the early detection of apoptotic events may allow for correction of the process conditions before irreversible damage has occurred to the culture (Hammill et al., 1999). It would also be beneficial to determine which conditions the cell culture is sensitive to and to add additional process control to minimize apoptosis levels during process characterization.

Another application of apoptosis monitoring during a cell culture process is to prevent the cascade of events by using apoptosis-suppressing chemical additives, such as protease inhibitors and zinc ions, to block key effectors (Cohen and Al-Rubeai, 1995). In

one such instance, suramin, a growth factor inhibitor and anti-tumor agent shown to inhibit apoptosis during the exponential growth phase, was added to a CHO cell culture process, which was observed to protect cells in serum-free culture where apoptosis is normally present due to nutrient limitation (Zhanghi et al., 2000). Insulin and transferrin as well as caspase inhibitors are also now being investigated in this role. In addition, antioxidants, such as Vitamin E, have been shown to prevent or at least delay apoptosis induced by free radicals; and aurintricarboxylic acid and N-acetylcysteine (Laken and Leonard, 2001) has been used to inhibit DNA cleavage, allowing for extended protein production in cells (Mastrangelo and Betenbaugh, 1998; Fussenegger and Bailey, 1998).

It has also been shown that alleviating nutrient deprivation by feeding extra nutrients such as a single amino acid or glucose has rescued cultures from starvation-induced apoptosis (Perreault and Lemieux, 1993; Franek and Sramkova, 1996). In addition, an increase in metabolic byproduct levels such as lactate and ammonia from the catabolism of glucose and glutamine were found to decrease viability and specific productivity in BHK cells (Cruz, et al, 2000). Optimization of nutrient additives to reduce glucose and glutamine and supplementation with three amino acids (glutamic acid, aspartic acid and cystine) has been shown to increase cell viability and enhance productivity in fed-batch CHO cultures by reducing the byproduct levels. (Gorfien et al, 2003).

Furthermore, apoptosis induction is believed to be regulated by small changes in the intracellular environment such as pH since caspase activity is increased at acidic pH (Matsuyama et al., 2000 reviewed by Laken and Leonard, 2001). In another instance, regulating hydrodynamic stress at the low level has been shown to suppress the apoptotic

process where high agitation rates have increased the apoptotic levels, which suggest these are areas for process characterization and optimization (Dimmeler et al., 1996 reviewed by Al-Rubeai and Singh, 1998). In addition, metabolic engineering using anti-apoptotic survival genes has been reported to be effective in enhancing the survival properties under a wide variety of physiological stresses in bioreactors, such as nutrient and oxygen limitations, accumulation of toxic compounds and metabolites, and hydrodynamic stresses (Arden et al., 2004).

Understanding the various stress-induced pathways involved in apoptosis can aid in cell engineering design to block stress signals and improve protein production (Fussenegger, 2001). One example was based on *c-jun* antisense technology, which leads to proliferation control as well as enhanced resistance to apoptosis in Friend murine erythroleukemia (F-MEL) cells (Kim et al., 2000 reviewed by Laken and Leonard, 2001). Apoptosis has been shown to be cell-cycle specific in that most apoptosis occurs in transition from late stages of the G1 phase to the S phase of the cell cycle (Meikrantz and Schlegel, 1995). Arrest prior to this phase delays or blocks apoptosis until the cell cycle continues (Fussenegger and Bailey, 1998). Over expression of the *bcl-2* and *bcl-*x_L genes can inhibit the apoptosis cascade upstream protecting mammalian cells from stresses such as steroids, nutrient and serum deprivation, heat shock and irradiation (Vaux, 1993; Arden et al., 2004). The gene blocks the release of cytochrome c from the mitochondria, decreasing the levels of procaspase-9 activation, which halts executioner caspase activation and therefore arrests apoptosis (Fussenegger et al., 2000). In addition, cells that lack cytochrome c, Apaf-1 or caspase 9 are resistant stress-induced apoptosis (Reviewed by Green, 2000). By decreasing the apoptotic levels in the culture, the

cellular lifetimes were extended during the stationary phase of growth leading to increased product yield (Mastrangelo and Betenbaugh, 1998).

Because infinite proliferation beyond optimized cell density causes stress-induced apoptosis, a lot of recent research has focused on cell cycle-arrested cell technology. This technology has shown a significant increase in product productivity compared to the controls. Multicistronic expression systems allow for a rapid nonproductive cell growth phase to the optimal density followed by a proliferation-arrested production phase in which cells devote all of their energy to the production of the desired protein and decrease the chances of nutrient depleted apoptosis. One of the first examples was a temperature-sensitive CHO cell line in which lowering the temperature after the growth phase from 37°C to 30°C caused a growth arrest in the G1 phase and increased productivity by prolonging the production phase (Kaufmann et al., 1999; Moore et al., 1997). The decrease in temperature prolonged the cell viability by delaying apoptosis, not inhibiting the process. The temperature shift caused a significant increase in the sialylation of the protein, which is a desirable effect in protein therapeutic uptake *in vivo* (Kaufmann et al., 2001). However, the complex temperature regime was not attractive for industrial application and the temperature downshift altered protein expression causing posttranslational protein modifications. Chemicals, such as sodium butyrate, have also been used to cause growth arrest and subsequent apoptosis regulation. It has been used in conjunction with cells expressing *bcl-2* to increase the production phase and decrease apoptosis (Simpson et al., 1997; Arden et al., 2004).

Another example of the two phase metabolic engineering approach is the TET_{switch} proliferation-controlled production technology by Mazur and Fussenegger that

over expresses the cyclin-dependent kinase inhibitor gene, p27, which results in G1 phase cell-cycle-arrest in CHO cells (Mazur et al., 1998, 1999). Induction of the proliferation-inhibited production phase from the non-productive growth phase occurs with the decrease in tetracycline in the culture. This design allows for minimal levels of the antibiotic in the production medium, thereby avoiding complex and expensive downstream purification to clear it. Tetracycline degrades over time so the system is self-regulated and predictable in the bioreactor conditions. Productivity in these proliferation-inhibited cultures was as high as 10-15 times higher than normal cultures; and there were no significant changes to product quality (Kaufmann et al., 2001). Meents and Fussenegger further engineered the process into a large-scale compatible non-adherent serum-free process with increased production and decreased the use of animal serum, which is a current priority for regulatory agencies (Meents et al., 2002).

There are many advantages to decreasing or understanding apoptosis in a cell culture process including a higher consistency of the product due to well-defined and reproducible conditions. Another advantage would be a decrease in release of intracellular proteases and glycosidases so product quality is higher. Optimizing the culture conditions could possibly lower medium consumption, decrease costs and increase productivity since intracellular resources can then be devoted to protein production. Due to the highly redundant mechanisms in the apoptotic pathway, a mix of strategies for completely inhibiting apoptosis is most likely necessary including feeding schemes, chemical additives, metabolic engineering and survival gene expression (Arden and Betenbaugh, 2004). The induction rates and overall effects of apoptosis are cell line dependent (Singh et al., 1994), and numerous studies have shown that the apoptotic

effects on protein production are dependent on a number of factors including the particular anti-apoptotic proteins expressed, cell type, expression system, and the culture environment. Characterizing which conditions will have an influence on apoptosis in a particular cell culture process is valuable for enhancing robustness, optimizing cell viability and possibly increasing productivity.

1.3. Problem Statement and Hypothesis

Currently, most cell culture process conditions are developed based on optimizing cell density, viability and productivity without understanding the early stage apoptotic cell population. Since conventional analytical methods do not detect early stage apoptotic levels, when lower cell viability is detected, it may be already too late to reverse the apoptosis cascade to sustain high productivity. In order to understand the effect of the early stage apoptotic population on the final performance, a model mammalian cell line was evaluated for recombinant protein production during a cell culture process in bench scale bioreactors.

Various experiments were performed to determine which process parameters increase programmed cell death and minimize viability. The results were then evaluated to correlate the effects of apoptosis, the overall productivity of the cell culture process and the specific productivity per cell. When these parameters such as temperature, dissolved oxygen, agitation, nutrient concentration or pH levels fluctuate enough to trigger an apoptotic cascade, this information could allow the stress-induced process to be reversed without detrimental effects in a manufacturing setting if detected early enough. Camptothecin (CPT) was used as a positive control to induce apoptosis by causing DNA damage (Lisby et al., 1998; Shimizu and Pommier, 1997). CPT has been shown to

induce apoptosis in mammalian cells by inhibiting Topoisomerase I, which is an enzyme involved with DNA replication.

Apoptotic phase, cell density, viability, and productivity were monitored throughout the process, as well as, pH, dissolved oxygen, carbon dioxide levels, and metabolite concentration. A Guava® Technologies PCA-96 microcytometry system was used for apoptosis analysis. The following assays were used as real-time process monitoring for this study:

- Apoptosis:
    - ViaCount Assay: Cell density, viability determination, and apoptosis estimation
    - Nexin Method: Live, dead, early and late apoptotic cell discrimination
    - MultiCaspase Method: Viable, early to mid-apoptotic, late stage/dying and dead cells discrimination
- Reversed-Phase HPLC: Product titer
- Blood Gas Analyzer: pH, carbon dioxide (CO_2), and dissolved oxygen (DO)
- Cedex: Automated Cell density and viability measurement based on trypan blue methodology
- Nova BioAnalyzer: Metabolic concentration determination

1.4. Viability and Apoptosis Assay Methodology

Initial studies showed the Guava Personal Cell Analysis (PCA) system to be a convenient method that may be beneficial to process development as a tool to quickly optimize a cell culture process under compressed timelines for improved cell viability and decreased apoptosis with minimal analyst-to-analyst variability compared to other methods. Conventional methods of flow cytometry and microscopic analysis are expensive, time consuming and labor intensive which is unfavorable for modern

development and manufacturing cycles. The Guava PCA allows for many assays to be run throughout the cell culture process to better understand how the conditions and stages of the process influence apoptosis. The Guava assays allowed analysis of viable, apoptotic and dead cells as separate populations, whereas, trypan blue staining could only detect live and dead cell populations resulting in a more accurate lower viability for the ViaCount assay.

1.4.1. ViaCount Assay

The ViaCount assay determines the number of viable, mid/late apoptotic and dead cells in a sample based on uptake of two DNA-binding dyes with different cell permeability characteristics (Yokobata et al., 2003). The assay is based on detecting cell membrane changes associated with apoptosis. All cells take up the red dye in the ViaCount reagent so cellular debris is excluded. Because of breached membrane integrity, later stage apoptotic and dead cells allow the orange dye from the reagent to absorb allowing for discrimination of the populations. The conventional method for determining cell density and viability is trypan blue staining which can only detect live and dead cell populations. Since apoptotic cells are unable to reproduce, the Guava technology was designed to not consider these cells viable, whereas the trypan blue method does not separate out the apoptotic from viable thereby giving an elevated viable cell number.

1.4.2. Nexin Assay

The Nexin assay is based on the biochemical and physiological characteristics of the apoptosis pathway (Guava Nexin Kit Insert, 2003; Fishwild and Tran, 2004). An early characteristic of apoptosis is the translocation of the phospholipid

phosphatidylserine (PS) from the inner section of the plasma membrane to the cell surface as discussed earlier. This serves as a signal for the cells to be cleared by phagocytes of the immune system in a normal physiological setting. The Guava Nexin assay uses the dye Annexin V, which has a high affinity for PS to determine what percentages of cells in a population are undergoing early apoptosis.

Secondly, the cell impermeable dye 7-amino actinomycin D (7-AAD) is used to distinguish between viable and dead cells based on membrane integrity. It is excluded from healthy and early apoptotic cells, but is able to permeate later stage apoptotic and dead cells. This assay detects earlier stage apoptosis in addition to late stage so a higher number of apoptotic cells are detected than in the ViaCount method.

1.4.3. MultiCaspase Assay

The activation of the caspase cascade in most cases commits a cell to death by apoptosis; therefore caspase enzymes (cysteine-containing aspartate-specific proteases) play a pivotal role in the initiation and execution of the apoptotic cascade (Guava MultiCaspase Detection Kit Insert, 2003). The recognition of active caspase enzymes in a cell serves as a marker for mid and late stage apoptosis. The Guava Multicaspase assay uses a cell permeable inhibitor called sulforhodamine-valyl-alanyl-aspartyl-flouromethylketone (SR-VAD-FMK) to detect the cells that are expressing active caspases. The SR-VAD-FMK dye binds covalently to multiple caspases in the cell that have been activated during apoptosis. Similarly to the Nexin assay, this assay uses 7-AAD to distinguish between viable and dead cells.

1.4.4. Cedex Analyzer

The Cedex AS20 (Innovatis AG; Bielefeld, Germany) is an automated analyzer of cell density and viability measurement based on the conventional trypan blue dye

methodology[2]. The trypan blue method is the most currently used and widely accepted method for determining cell density and cell viability. Trypan blue enters a cell once its outer plasma membrane integrity becomes compromised upon cell death. This staining method distinguishes between viable cells, which exclude the dye due to intact outer membranes, and dead cells, which stain positive for trypan blue. However, trypan blue is not able to discriminate between healthy, viable cells and cells that are undergoing apoptosis.

2. Methods and Materials

2.1. Cell Line and Culture Conditions

An in-house recombinant mammalian cell line, growth medium and nutrient feed medium were used for all studies. Bench scale and scale-up reactors were equipped with calibrated dissolved oxygen, pH and temperature probes. Dissolved oxygen was controlled on-line through sparging with oxygen, and pH was controlled through additions of carbon dioxide or sodium bicarbonate. The seeding density for all studies was kept constant.

2.1.1. Study 1: Consistency Runs at Bench Scale and Scale-Up

Study 1 consisted of five bench scale runs and one pilot scale run in which the DO and temperature were controlled at 120 mmHg and 37ºC, respectively. The same batch medium and cells were used for both the bench scale and pilot scale runs to minimize process variation. The pH set point was 7.0 at the start of the culture and then maintained at a pH of 6.8 once the culture pH dropped after 3 days incubation due to lactate accumulation in the broth. Process samples were assayed daily for all six runs using the Blood Gas Analyzer, Cedex, and Nova Bioanalyzer for pH, carbon dioxide, DO, cell

[2] http://www.innovatis.com/uk/produkte/cedex/cedex_glob.html

density, viability, and nutrient and metabolite concentrations (data not presented). The ViaCount and Nexin apoptosis assays were run on Days 3, 6 and 9 for bench scale runs and 3, 6, 9 and 14 for the pilot scale run. The multi-caspase apoptosis assay was run on Day 6 only for all six runs and product titer was performed on Days 3, 6, 8, 10, 12 and 14. These runs were mainly used to determine assay conditions and gating specific for the cell type as described in the analytical section to follow.

2.1.2. Study 2: Base Concentration and pCO_2 Addition Runs

Study 2 consisted of eighteen-bench scale runs designed to mimic the pH and carbon dioxide profiles in the pilot scale process. At the start of the culture, the process was designed with the pH set at 7.0. pH decreased over time as the cells released carbon dioxide. Once the pH reached 6.8, it was maintained at the set point of 6.8 with base and carbon dioxide addition, which was added to the culture broth through a sparger. Because the carbon dioxide stripping was more efficient in the bench scale runs, the pH did not drop at the same rate as the scaled-up process (Marks, 2003). A new bench scale process was designed to more closely mimic the scaled-up process. The pH was programmed to drop to 6.8 during the first several days of the process at a rate of -0.007 pH units/hr and was used on all runs. The sparged carbon dioxide was added as a fixed percentage of the oxygen sparge rate from 0 to 20%.

In addition, the concentration of the base solution (0.1 – 0.5 M sodium bicarbonate) for pH control was varied to determine if unusually high osmolality was caused by overshoot between base and acid (in this case CO_2) addition during pH control in the bench scale runs. Lower base concentrations were tested to determine a more accurate addition to mimic that in the scaled-up process. The culture conditions for Study 2 are summarized in Table 1. All of the runs were assayed daily using the Blood

Gas Analyzer, Cedex, and Nova Bioanalyzer. The ViaCount and Nexin apoptosis assays and product titer were run on Days 3, 6, 8 10, 12 and 14. The MultiCaspase apoptosis assay was run on Days 6, 8, 12 and 14.

Table 1: Test Conditions for Study 2

Run #	**CO_2 Flow Rate** (% of O2 Flow Rate)	**Base Concentration (M)**
1	0	0.5
2	0	0.5
3	0	0.5
4	0	0.5
5	0	0.5
6	0	0.5
7	10	0.25
8	10	0.25
9	10	0.25
10	10	0.25
11	10	0.1
12	10	0.1
13	10	0.1
14	10	0.1
15	20	0.25
16	20	0.25
17	20	0.1
18	20	0.1

2.1.3. Study 3: Nutrient Starvation and Apoptosis Induction

Runs 1 through 3 from Study 2 were continued past Day 14 for Study 3 to increase the levels of apoptosis during the extended incubation in the reactors. The test conditions are summarized in Table 2. Reactor 1 was the control; and the nutrient feed was ended for Reactor 2 on Day 14. Reactor 3 was induced with a known apoptosis inducer, (S)-(+)-Camptothecin (CPT, Sigma-Aldrich Cat# C9911), to a final concentration of 5 mM. The CPT was dissolved in dimethyl sulfoxide (DMSO, Mallincrokrodt) and added to the reactor through a 0.2µm syringe filter on Day 14. The

reactors were sampled daily on the Blood Gas Analyzer, Cedex, Nova Bioanalyzer, ViaCount, Nexin, and MultiCaspase apoptosis assays and product titer.

Table 2: Test Conditions for Study 3

Run #	Additional Conditions Days 14-17
1	Control
2	Nutrient Starvation
3	Apoptosis Induction w/ 5 mM Camptothecin

2.1.4. Study 4: pH and DO Characterization Runs and Camptothecin Controlled Apoptosis Induction

Study 4 varied the pH and DO levels individually at five set points. Runs 9 and 10 were the control runs with conditions at pH 6.8 and 120 mmHg. The study conditions are summarized in Table 3. The pH was programmed to begin decreasing at a process time of 72 hours at a rate of -0.007 pH units/hr until the pH dropped to the test pH stated in Table 3. For the pH 6.9 and 7.0 conditions, the pH ramp was not implemented. The carbon dioxide was sparged at a flow rate of 10% of the oxygen sparge rate starting on Day 4 of the process in order to match the scale up process profile determined in Study 2. The base concentration used in the bench scale runs was 0.1 M sodium bicarbonate, which was shown to prevent increased osmolality from Study 2 (data not shown). In addition to the eighteen pH/DO reactors, Runs 19 and 20 were performed with the pH and DO center points and induced on Day 8 with CPT dissolved in DMSO through a 0.2 µm syringe filter to a final concentration of 1 µm. On Day 3 of the process, the reactor agitator malfunctioned in Run 20 resulting in no mixing for approximately 3 hours.

All of the runs were assayed daily using the Blood Gas Analyzer, Cedex, and Nova Bioanalyzer. The ViaCount assay was run on Days 3, 6, 8, 9, 10, 12 and 13, whereas the Nexin and MultiCaspase apoptosis assays were run on Days 8, 9, 10, 12 and

13. The product titer was run on Days 3, 6, 8, 10, 12 and 14 (Day 13 for Runs 19 and 20).

Table 3: Test Conditions for Study 4

	Run #	DO (mmHg)	pH setpoint (pH units)
Δ DO	1	60	6.8
	2	60	6.8
	3	100	6.8
	4	100	6.8
	5	140	6.8
	6	140	6.8
	7	180	6.8
	8	180	6.8
Controls	9	120	6.8
	10	120	6.8
Δ pH	11	120	6.6
	12	120	6.6
	13	120	6.7
	14	120	6.7
	15	120	6.9
	16	120	6.9
	17	120	7.0
	18	120	7.0
	19*	120	6.8
	20*+	120	6.8

*Reactor induced with 1 µM Camptothecin on Day 8
+Reactor agitator malfunctioned on Day 3 resulting in no mixing for approximately 3 hours.

2.2. Guava Assays

The following assays used the Guava Technologies PCA-96 system (Guava Technologies; Hayward, CA) (Guava Technologies, 2004; Mukwena et al., 2003)[3].

[3] The Guava assay methods were originally developed by Lindsey Holt and Todd Lumen, Internal Technical Report, Amgen Thousand Oaks, Cellular Process Development, December 2004

2.2.1. Guava ViaCount Assay

2.2.1.1. Procedure

Cells were stained with the ViaCount reagent (Guava Technologies, Cat# 4000-0040) depending on the cell concentration within 5 hours of sampling. Cell concentrations from $1x10^5$ to $1x10^6$ cells/ml were diluted 1:10 in staining solution (50 µl of cell solution and 450 µl of ViaCount reagent), cell concentrations from $1x10^6$ to $1x10^7$ cells/ml were diluted 1:20 in staining solution (20 µl of cell solution and 380 µl of ViaCount reagent), and cell concentrations over $1x10^7$ cells/ml were diluted 1:40 in staining solution (10 µl of cell solution and 390 µl of ViaCount reagent). The samples were then incubated at room temperature for 5-15 minutes before data acquisition on the Guava PCA-96 system (1000 cells counted/sample in triplicate). The system was calibrated daily using the recommended Guava Check application.

The Consistency Runs during Study 1 were used to set the intensity gates for the ViaCount assay for the cell line and assay conditions. The gates allow for discrimination between viable, mid-apoptotic and dead cells reproducibly. The gate details are summarized in Table 4 and Figure 4 shows a typical ViaCount dot plot with the gated lines for a Day 6 and Day 17 samples. The gates were set to position the live population in the upper left corner of the dot plot.

Table 4: ViaCount Gates

Viability Discriminator		Apoptosis Discriminator	
X Intercept	Line Angle	X Intercept	Line Angle
0.21	30.3	0.91	29.6

Fig. 4A: Day 6

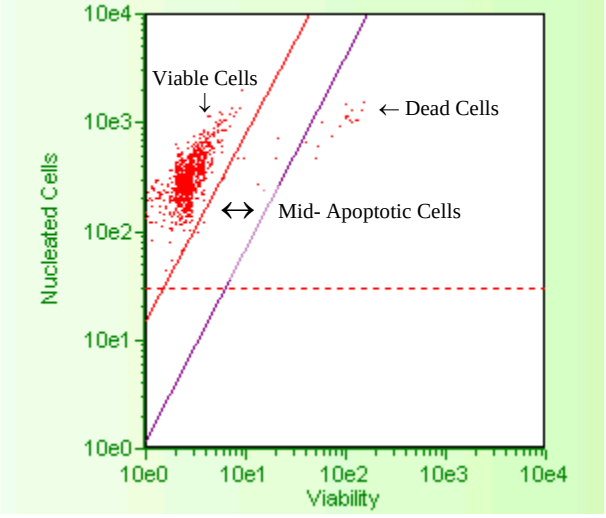


Fig. 4B: Day 17

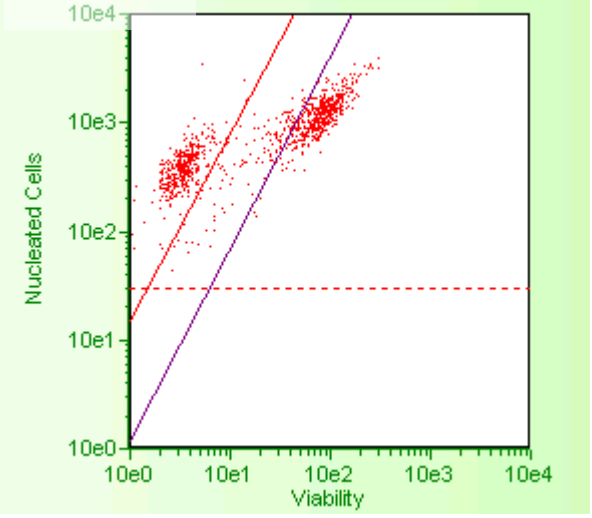


Figure 4: ViaCount Assay Dot Plot Examples

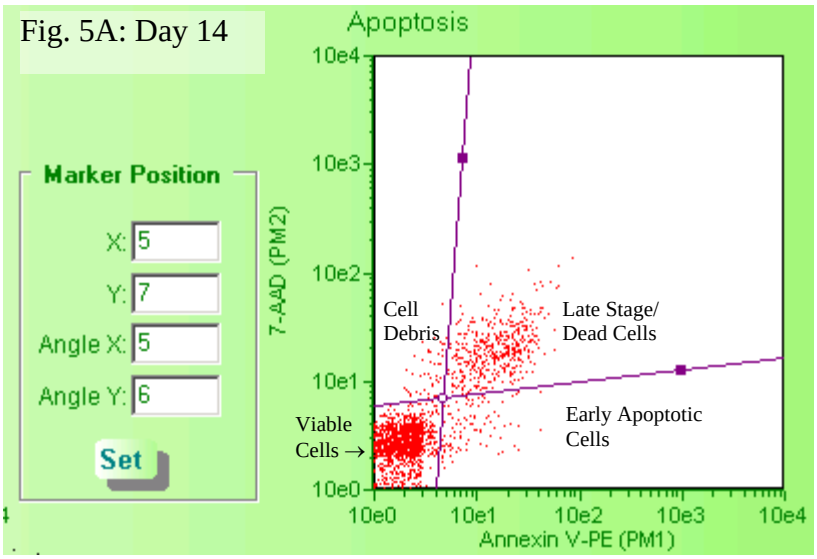
Cells were assayed using the Guava ViaCount method at different time points throughout the culture, shown here are Days 6 (A) and 17 (B). The gates were set during the control runs for this cell line to position the viable (live) cells at the upper, left corner of the dot plot. The different stages determined by the method are labeled in Figure A. There is an increased number of mid-apoptotic and dead cells in the Day 17 sample.

2.2.2. Guava Nexin Assay

2.2.2.1.Procedure

The cell samples were diluted with Phosphate Buffered Saline (PBS, Gibco; Grand Island, NY) to concentrations between $2x10^5$ and $1x10^6$ cell/ml in a total volume of 50 µl within 5 hours of sampling. The cells were spun in a microcentrifuge for 3 minutes at 500xg, after which 45 µl of the supernatant was removed and discarded. Each cell pellet was resuspended in a working solution consisting of 150 µl 1X Nexin Buffer (prepared from 10X Nexin Buffer, Guava Technologies, Cat# 4200-0060), 5 µl Annexin V-PE (Guava Technologies, Cat# 4700-0040), and 5µl Nexin 7-AAD (Guava Technologies, Cat# 4000-0060). The cells were incubated on ice for 20 minutes, shielded from light. After incubation, the total sample volume was brought to 350 µl by adding 185 µl of 1X Nexin Buffer before acquisition on the Guava PCA-96 system (2000 cells counted/sample, flow rate setting medium). The Nexin intensity gates were set to

position the live population in the lower left corner of the dot plot. The angles of the gates were then positioned to divide the dot plot into four quadrants. Each quadrant of the dot plot contains a distinct population of cells that is dependent on the presences and intensity of cellular stains per cell. The Nexin intensity gates needed to be adjusted slightly from assay to assay due to the nature of the assay and to properly discriminate the four populations under analysis. Examples of the Nexin Dot Plot are shown in Figure 5.



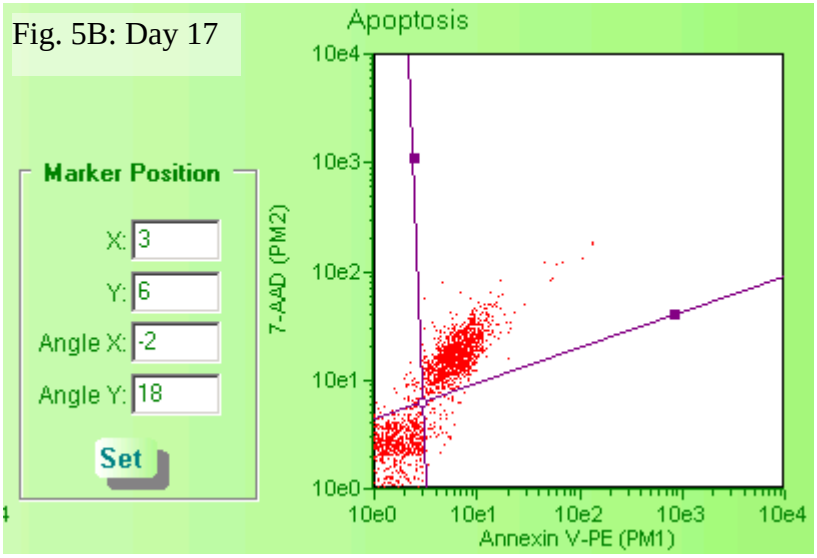


Figure 5: Nexin Assay Dot Plot Examples

Cells were assayed using the Guava Nexin method at different time points throughout the culture, shown here are Days 14 (A) and 17 (B). The gates are set to position the viable (live) cells at the lower, left corner of the dot plot. The quadrants are labeled in Figure A to show the different stages the Nexin method identifies. There is an increased number of Late Stage/Dead Cells (upper right) in the Day 17 sample.

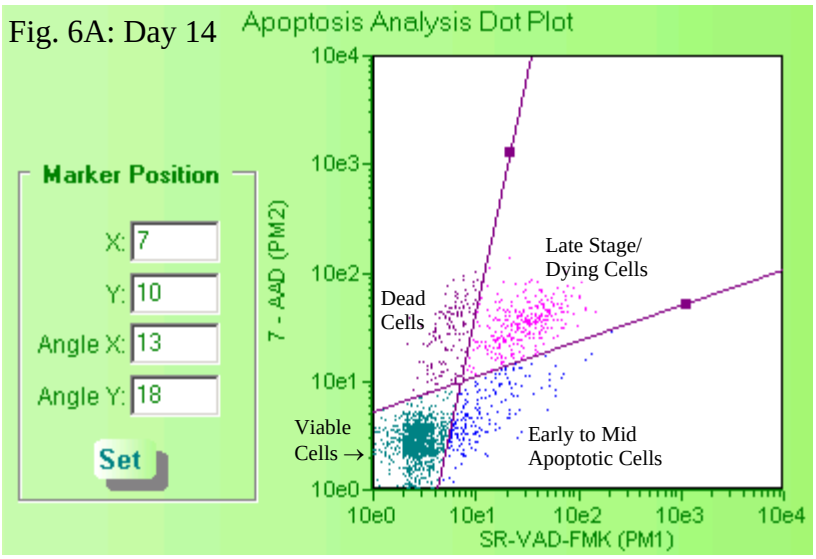
2.2.3. Guava MultiCaspase Assay

2.2.3.1.Procedure

Prior to staining, $5x10^4$ cells from each sample were brought to a total volume of 100 µl with 1X Apoptosis Wash Buffer (prepared from 10X Apoptosis Wash Buffer, Guava Technologies, Cat# 4200-0162) within 4 hours of sampling. To each sample, 5 µl

of 20X SR-VAD-FMK (prepared from SR-VAD-FMK reagent, Guava Technologies, Cat# 4100-0212) was added. The samples were incubated in a 37ºC/5% CO_2 incubator for 1 hour, shielded from light, and were mixed once by gently vortexing during that period. Following incubation, the cells were washed three times with 1 ml of 1X Apoptosis Wash Buffer and centrifuged at 300 to 400xg in a microcentrifuge for 5 minutes to pellet the cells. The samples were then resuspended in 100 µl of 1X Apoptosis Wash Buffer and each stained with 5 µL of Caspase 7-ADD (Guava Technologies, Cat# 4000-0064) for 10 minutes at room temperature, shielded from light.

Before acquisition on the Guava PCA-96 system, 200 µl of 1X Apoptosis Wash buffer was added to bring the sample volume to 305 µl. Data was acquired within 15 minutes after adding the 7-AAD dye. The MultiCaspase intensity gates were set each assay run and were not consistent from assay to assay due to the nature of the assay similar to that in the Nexin assay. Examples of the MultiCaspase Dot Plot are shown in Figure 6.



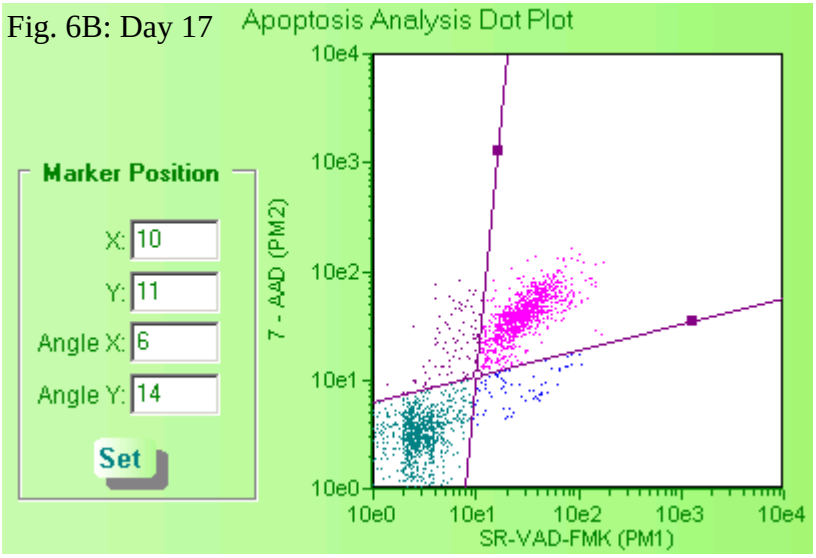


Figure 6: MultiCaspase Assay Dot Plot Examples

Cells were assayed using the Guava MultiCaspase method at different time points throughout the culture, shown here are Days 14 (A) and 17 (B). The gates are set to position the viable (live) cells at the lower, left corner of the dot plot (teal green). The quadrants are labeled in Figure A to show the different stages the MultiCaspase method identifies. There is an increased number of Late Stage/Dying Cells (pink) in the Day 17 sample.

2.3. Other Analytical Methods

2.3.1. Cedex Analyzer

2.3.1.1.Procedure

A daily sample from the bioreactor was mixed gently and 1000 µl of the sample was added to a Cedex AS20 cup and analyzed per manufacturer's recommended method. Samples were diluted 1:2 in PBS for Days 8 through 17.

2.3.2. Blood Gas Analyzer

2.3.2.1.Procedure

The Chiron CIBA-Corning 248 Blood Gas Analyzer (Chiron Diagnostics; Halstead, Essex, UK) was used to measure real time pH, carbon dioxide, and oxygen levels[4]. The values are used to calibrate the bioreactor controllers on a daily basis as needed. A daily sample from the bioreactor was removed with a 3 ml syringe and immediately analyzed on the Blood Gas Analyzer following the manufacturer's recommended method.

2.3.3. Nova BioAnalyzer

2.3.3.1.Procedure

The Nova Biomedical BioProfile 100 Plus (Nova; Waltham, MA) was used to monitor the progress of the bioreactor runs by determining the consumption and production of key metabolites, growth limiting nutrients and waste products[5]. The Nova analyzes pH, glutamate, lactate, glutamine, glucose, sodium, potassium, ammonium, and osmolality. A daily sample from the bioreactor was removed with a 3 ml syringe and analyzed on the Nova BioAnalyzer following the manufacturer's recommended method.

[4] http://www.gmi-inc.com/Categories/bloodgas.htm
[5] http://www.novabiomedical.com/bioprofile/bpanalyzers.html

2.3.4. Reversed-Phase HPLC

2.3.4.1.Procedure

The concentration of recombinant protein was determined by applying samples to a reversed-phase column on a High Performance Liquid Chromatography (HPLC) system. Bioreactor samples were centrifuged for 5 minutes at 2000 rpm and supernatant was removed and frozen at -30ºC until all samples from the study were taken and then assayed by an Agilent 1100 HPLC system. The titer samples were run by the Analytical Sciences group in Process Development.

3. Results & Discussion

3.1. Conventional Trypan Blue versus Guava Method for Determining Viability

Since the rate of apoptosis and the stimuli signaling the pathway can vary from cell line to cell line, this study looked at a model mammalian cell line to determine the effects certain conditions have on cell viability. Figure 7 shows Runs 1 through 3 from study 3 in which the reactors were extended to 17 days. The Figure shows the viable cell density and viability for each run as measured from the conventional trypan blue method compared to the Guava ViaCount method.

As seen in Figure 7, for the first 8 days of the culture when viability was high the two methods readings were very similar. In all three runs, a small dip in the ViaCount viable cell density is seen around Days 8 through 10 at which time the two methods start to show different results indicating apoptosis is occurring in the cultures. The viable cell density for all 3 runs started to decrease on Day 14. Run 3 viable cell density dropped significantly after the addition of CPT on Day 14 due to the excessively high concentration of CPT added. The concentration most likely caused necrosis in some cells in addition to apoptosis over time. Nutrient starvation was used in Run 2 to try and induce apoptosis; the viable cell density decreased steadily until Day 16, however the viability remained high compared to the other two reactors.

On Day 16, additional nutrients were added to Run 2 to keep the viability high to determine the affects of different levels of apoptosis on the productivity (to be discussed later). At this time, the viable cell density remained stable, but the viability started to decline steadily based on the ViaCount method. By the end of Day 17, the two viability methods showed significantly different results; the trypan blue method indicated a 27% higher viability than the ViaCount result for Run 1 and 18% and 17% for Runs 2 and 3,

respectively. Based on this data, the ViaCount method appears to provide more representative results of the actual viability in the process when apoptosis is present in the culture.

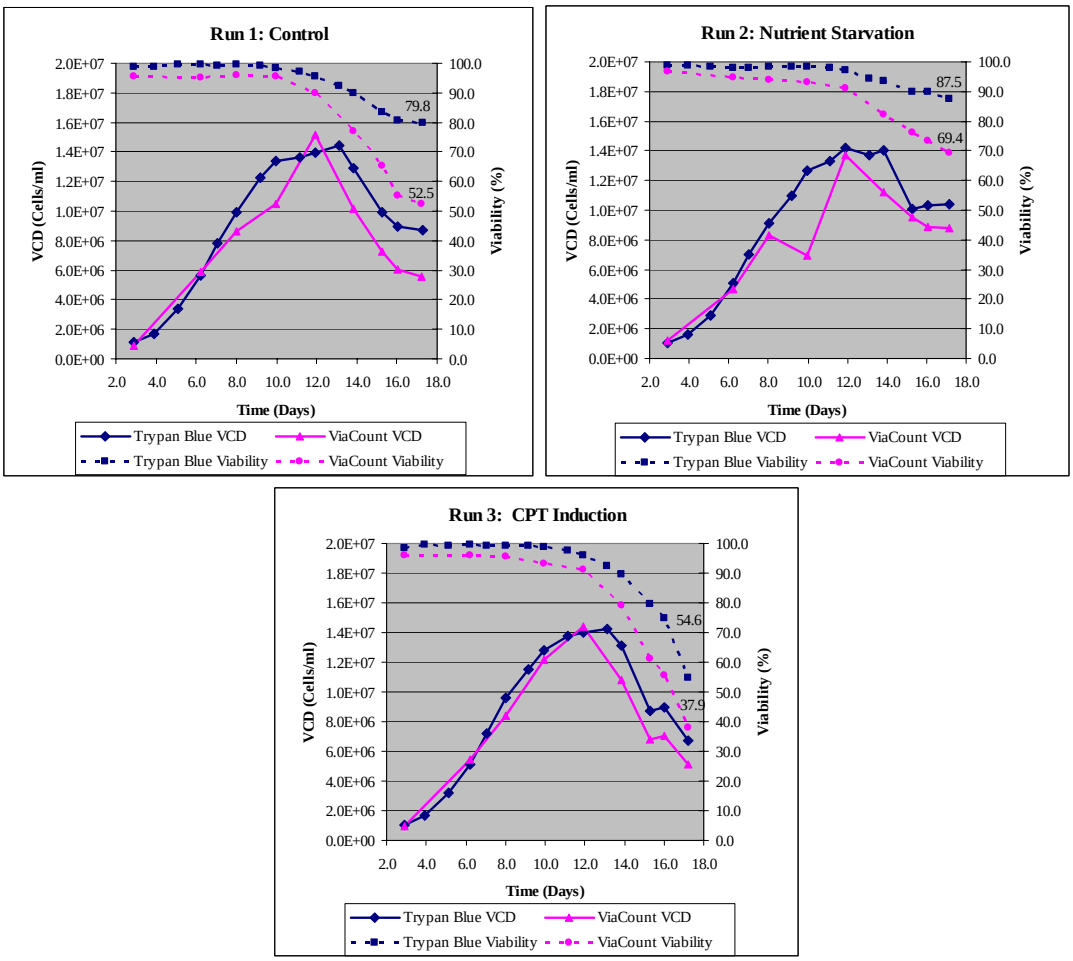


Figure 7: Viable Cell Density and Variability over Time with Apoptosis Induction

Growth curves of cells from Study 3 with apoptosis induction at Day 14 by nutrient starvation (Run 2) and CPT induction (Run 3). The culture was continued through Day 17. The viable cell density (solid line) and viability (dotted line) for the conventional trypan blue method using the Cedex instrument (blue) are compared to the Guava ViaCount method (pink) for the three runs. The two methods are similar through Day 8 of the process when viability was high and start to differ with increased apoptotic levels during later stages. The end viability at Day 17 is noted on the right with notable differences between the two assays due to apoptosis levels, which are not detected in the conventional trypan blue method.

In addition to the ViaCount method, two other apoptosis assays were used to determine various stages of the apoptosis process in the culture. Table 5 summarizes the

different levels of viability using these two methods in addition to the ViaCount and trypan blue methods for Day 17 of Runs 1 through 3. The Nexin assay shows the percentage of cells in early stages of apoptosis whereas the MultiCaspase assay provides data for the early/mid and late/dying stages of the culture.

Table 5: Summary of Day 17 Cell Viability using Different Analytical Methods

Cell Viability Method	Run #	Distribution of Cell Population (%)				
		Live (Non-Apoptotic)	Early Apoptosis	Mid Apoptosis	Late Apoptosis	Dead
MultiCaspase	1	52.4	4.2		37.2	6.2
	2	66.1	4.6		22.3	7.0
	3	40.2	3.3		50.2	6.3
Nexin	1	35.5	2.5	56.4 (5.7% Nuclear Debris)		
	2	54.0	3.0	38.8 (4.2% Nuclear Debris)		
	3	30.7	3.2	64.0 (2.2% Nuclear Debris)		
ViaCount	1	52.5	18.6			28.9
	2	69.4	12.2			18.4
	3	37.9	10.8			51.3
Trypan Blue	1	79.8				21.2
	2	87.5				12.5
	3	54.6				46.4

3.2. Effects on Productivity with Increased Run Time, CPT Apoptosis Induction, and Nutrient Feed Starvation

The three runs were analyzed for protein titer to determine the affect apoptosis had on the productivity. Although the viability and viable cell density was highest in the nutrient feed starved culture in Run 2, the overall recovery was lowest among the three runs, (see Table 6). Run 1 and Run 3 had an 11% and 30% increase in productivity respectively compared to Run 2. Run 3, with the lowest viability and viable cell density, had the highest productivity. Furthermore, the specific productivity was highest in Runs 1 and 3 with the increased apoptotic levels and lowest viability. Figure 8 shows the effects of specific productivity over time for the three runs. This most likely occurred at

the culmination of the apoptosis cascade resulting in product being released when secondary necrosis transpired.

Table 6: Total Protein Yield after Various Levels of Apoptosis Induction

Run #	Normalized Total Protein (mg)
1: Control	1966
2: Nutrient Starvation	1772
3: CPT Induction	2304

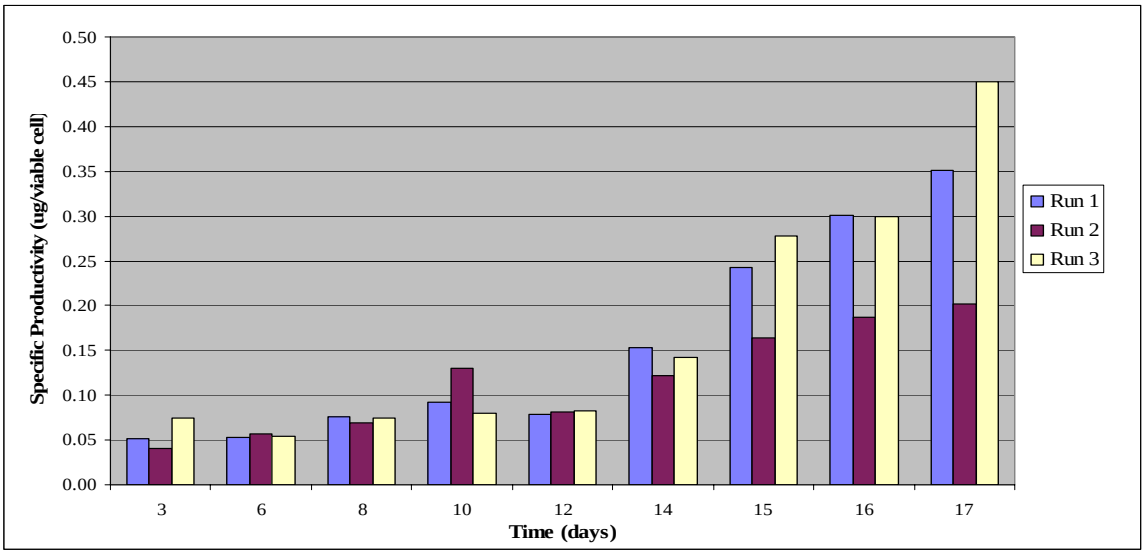


Figure 8: Effects on Specific Productivity with Various Levels of Apoptosis

This figure shows the specific productivity over time for the three runs in Study 3. The specific productivity was similar through Day 14 when the conditions changed to vary the apoptotic levels in the cultures. Run 3 with the lowest viability and viable cell density, had the highest specific productivity by Day 17 followed closely by Run 1 which also had high apoptotic levels.

3.3. pH, DO Levels, Apoptosis Induction Earlier in Process, and Agitation Issues

DO and pH levels were characterized in Study 4. Figures 9 and 10 show the viable cell density and viability over the duration of the culture for the varying DO levels. A shift in the viable cell density was found in all 18 runs of this study on Day 9 and increased again by Day 10. A small shift in viability was also seen suggesting stimulus was introduced during this time. Based on statistical analysis discussed later, this is most

likely due to nutrient deprivation in the higher cell densities in the culture. The total and specific productivity for these runs are summarized in Figure 11. The DO levels had minimal effect on the specific productivity of the cells; however the specific productivity was highest in the 180 mmHg, which had the lowest viable cell density. Figure 12 shows the total and viable cell count and normalized total protein of each DO condition over the duration of the process. The viable cell density and total protein yield are highest in the lower DO conditions. This is consistent with literature findings that showed that low DO levels in reactors prolong cell viability thereby increasing the productivity (Reuveny et al., 1986).

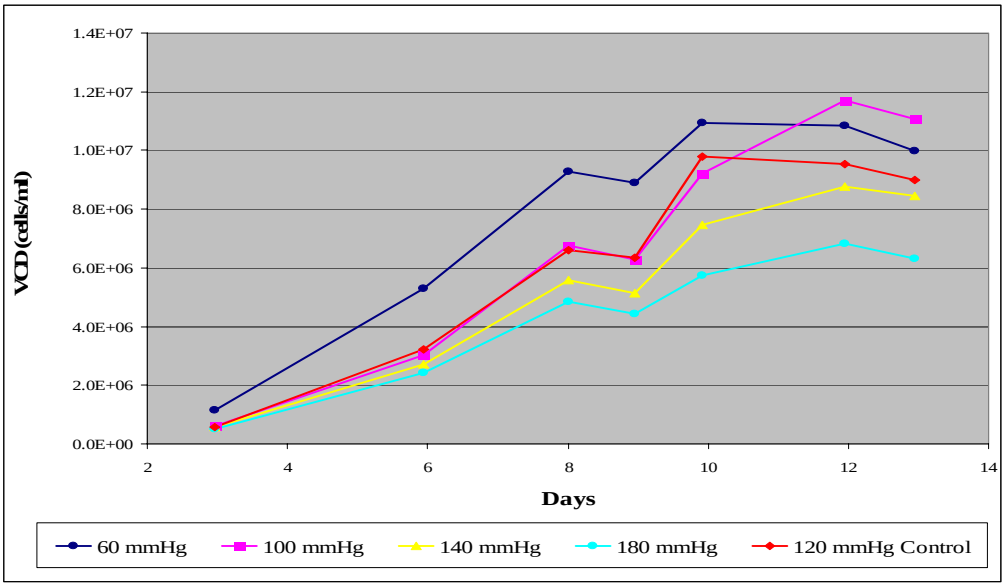


Figure 9: Viable Cell Density Profiles at Various DO Levels

Viable cell density profiles of cells cultured at various dissolved oxygen levels from Study 4. The viable cell density was determined by the Guava ViaCount method throughout the 14-day culture.

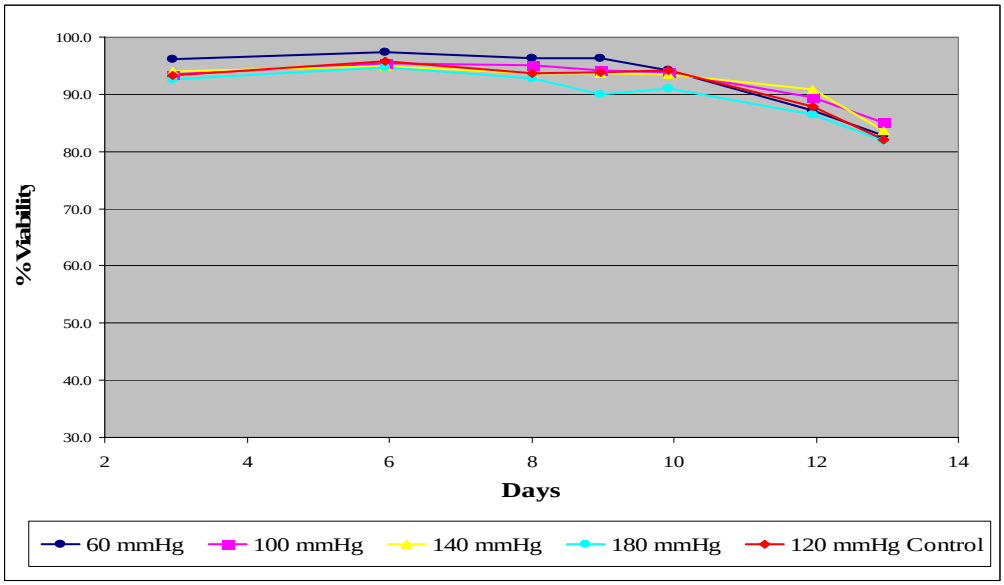


Figure 10: Cell Viability Profiles at Various DO Levels

Cell viability (% live) profiles of cells cultured at various DO levels from Study 4. The viability was determined by the Guava ViaCount method throughout the 14-day culture.

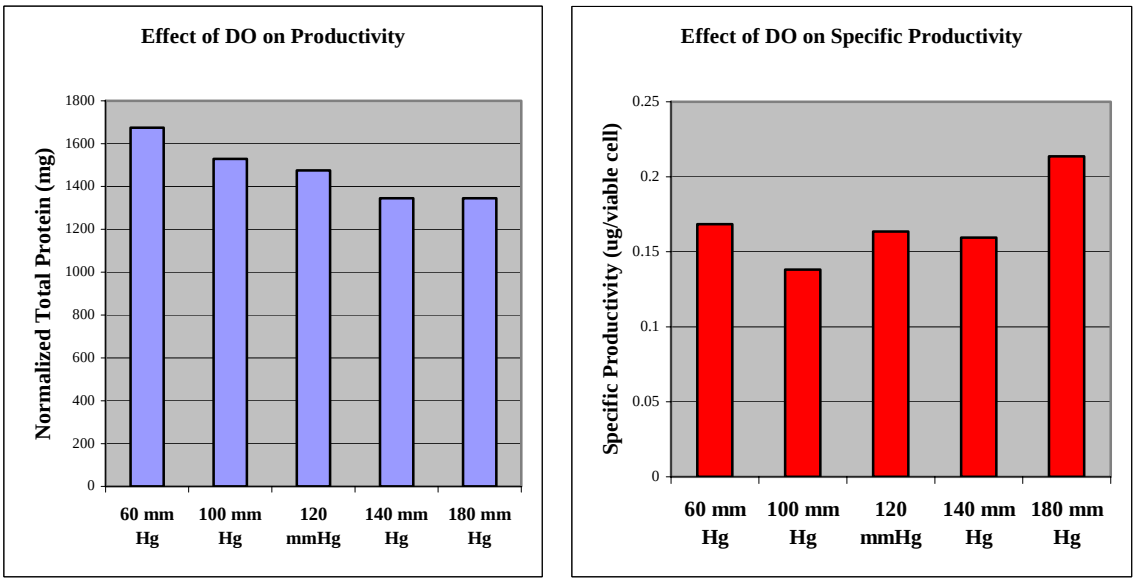


Figure 11: Total Protein Yield and Specific Productivity for Varying DO Levels

Figure 11 shows the effects of different DO levels in the culture on productivity (blue) and specific productivity (red) at the end of the 14-day cultivation. The specific productivity was highest in the 180 mmHg, which had the lowest viable cell density. Productivity decreases as the DO levels increase.

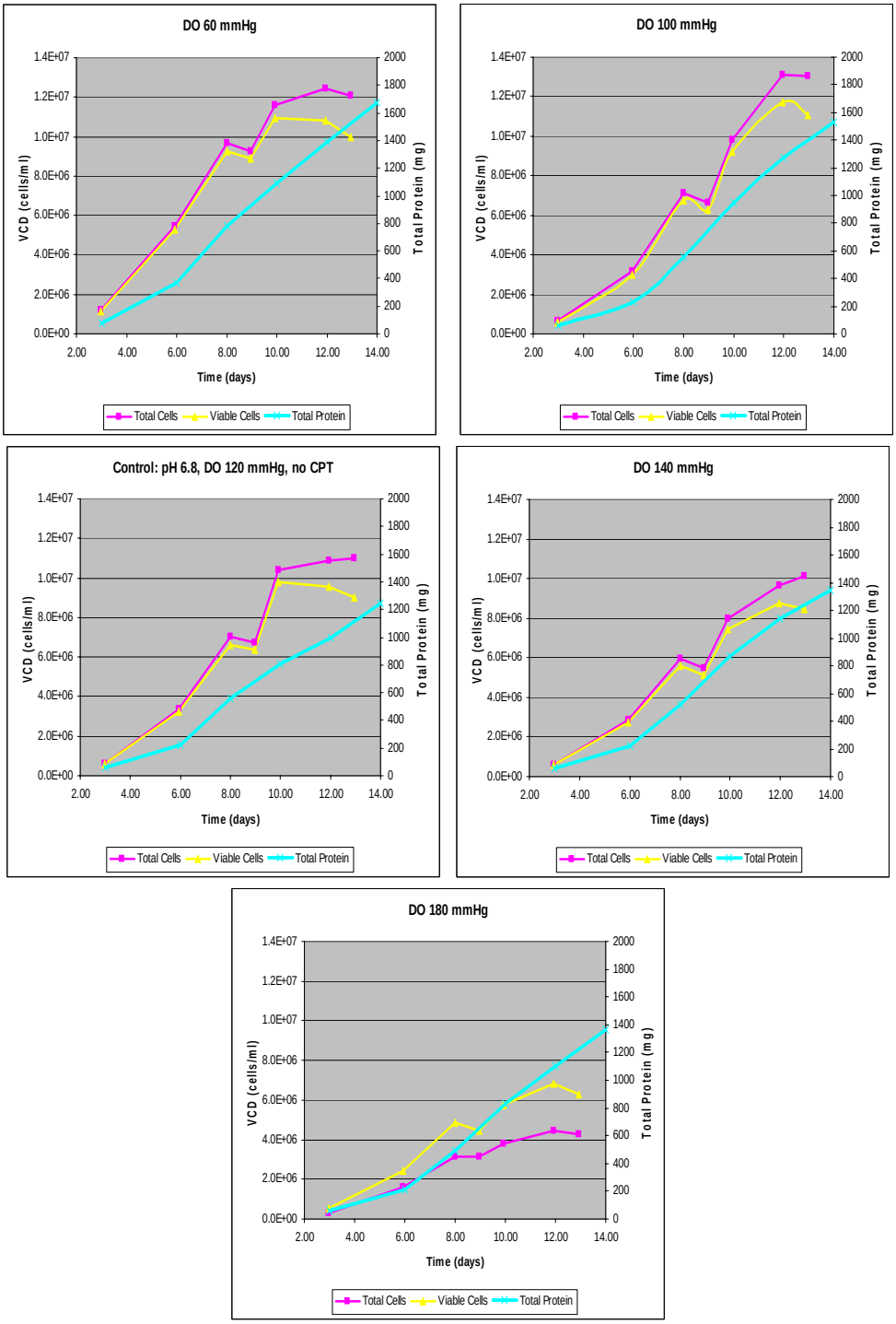


Figure 12: Viable Cell Density and Total Productivity as a Function of Time at Various DO Set Points

Growth profiles of cells cultured with various DO set points from Study 4 for the 14-day culture period. The total cell density (pink) and the viable cell density (yellow) were measured by the Guava ViaCount assay. The normalized total protein is noted in aqua.

Figures 13 and 14 show the viable cell density and viability over the duration of the culture for the varying pH levels. The specific productivity for these runs is summarized in Figure 15. Interestingly, the pH 6.6 condition had a high specific productivity result and increased levels of apoptosis in the culture indicating that secondary necrosis may have occurred. This is consistent with literature findings that apoptosis induction is believed to be regulated by small changes in the intracellular environment such as pH since caspase activity is increased at acidic pH (Matsuyama et al., 2000 reviewed by Laken and Leonard, 2001). Figure 16 shows the total and viable cell count and normalized total protein of each pH condition over the duration of the process. The pH level had a significant effect on the viable cell density, however the viability was not too different between the 5 runs with the exception of the pH 6.6 condition which had a significantly lower viability earlier in the process compared to the other runs.

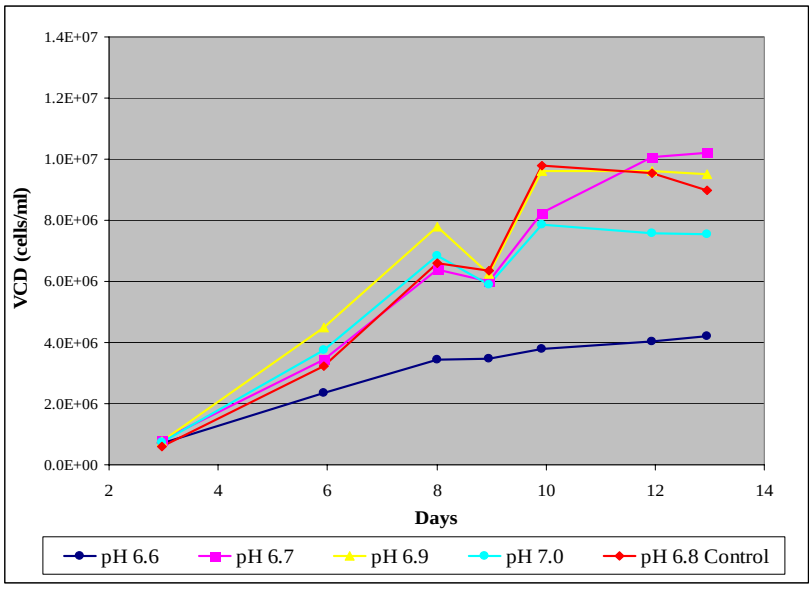


Figure 13: Viable Cell Density Profiles at Various pH Levels

Viable cell density profiles of cells cultured at various pH levels from Study 4. The viable cell density was determined by the Guava ViaCount method throughout the 14 day culture.

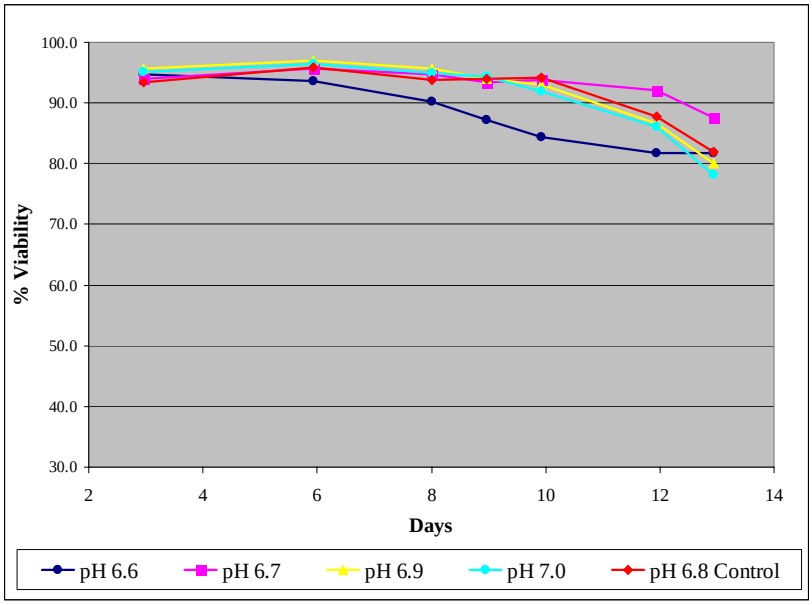


Figure 14: Cell Viability Profiles at Various pH Levels

Cell viability (% live) profiles of cells cultured at various pH levels from Study 4. The viability was determined by the Guava ViaCount method throughout the 14-day culture.

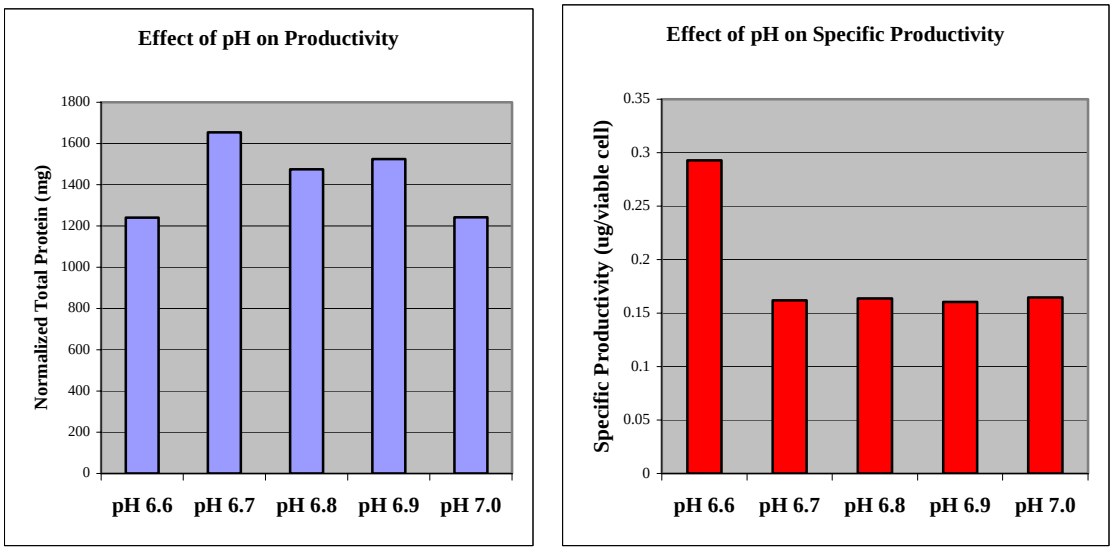


Figure 15: Total Protein Yield and Specific Productivity at Various pH Levels

Figure 15 shows the effects of different pH levels in the culture on productivity (blue) and specific productivity (red) at the end of the 14-day cultivation.

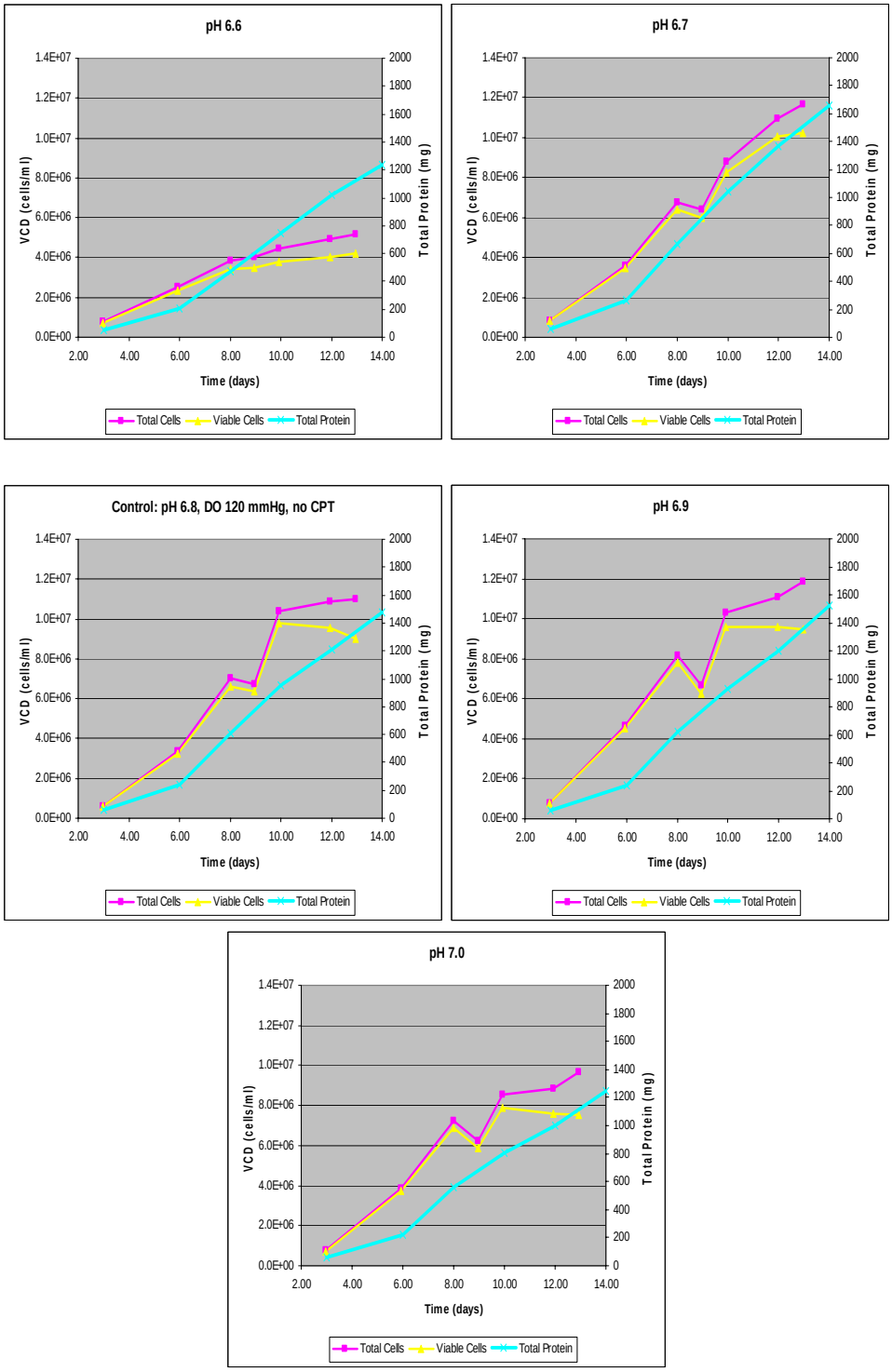


Figure 16: Viable Cell Density and Total Productivity as a Function of Time at Various pH Set Points

Growth profiles of cells cultured with various pH set points from Study 4 over the 14-day culture period. The total cell density (pink) and the viable cell density (yellow) were measured by the Guava ViaCount assay. The normalized total protein is noted in aqua.

Figures 17 and 18 show the viable cell density and viability over the duration of the culture with CPT induction on Day 8. The CPT induction had a significant effect on the viable cell density and viability in the two runs. The normalized total protein yield and specific productivity for these runs are summarized in Figure 19. Although the viable cell density was significantly lower in the CPT induced cultures, the yield was still similar to the control; therefore the specific productivity was significantly greater in these runs.

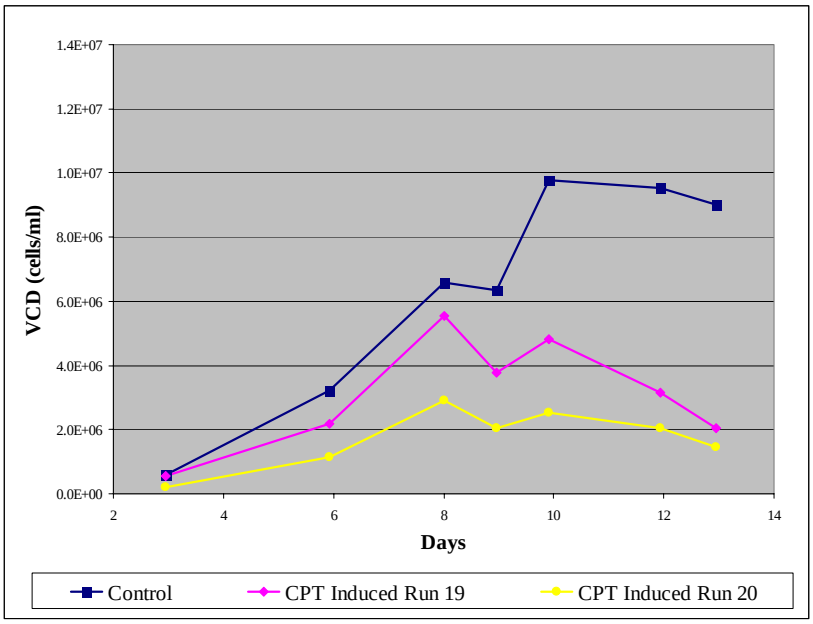


Figure 17: Viable Cell Density with CPT Induction

Viable cell density profiles of cells induced with CPT on Day 8 from Study 4. The viable cell density was determined by the Guava ViaCount method throughout the 14-day culture.

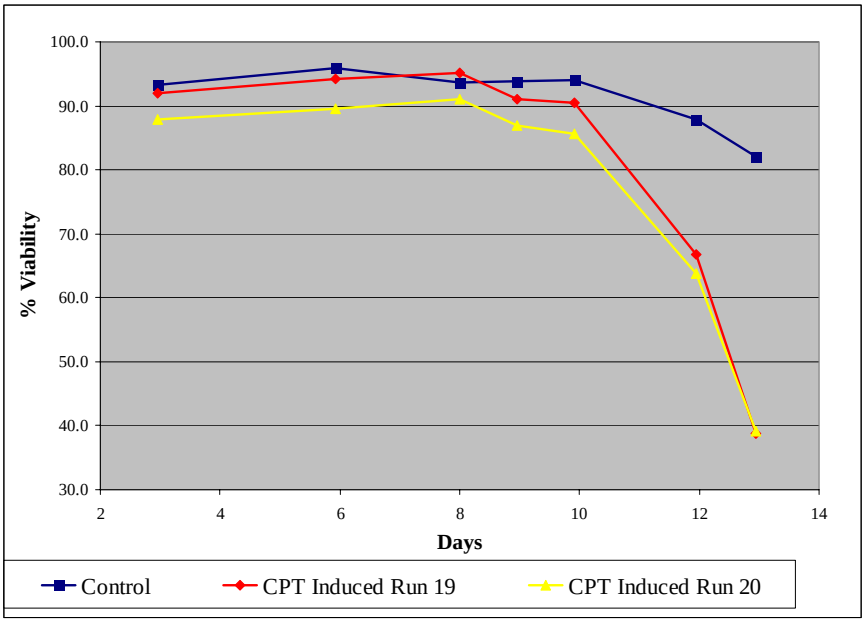


Figure 18: Cell Viability with CPT Induction

Cell viability (% live) profiles of cells induced with CPT on Day 8 Study 4. The viability was determined by the Guava ViaCount method throughout the 14-day culture. Run 20 started out with a lower viability due to the agitator malfunction on Day 3 and remained slightly lower throughout the cultivation.

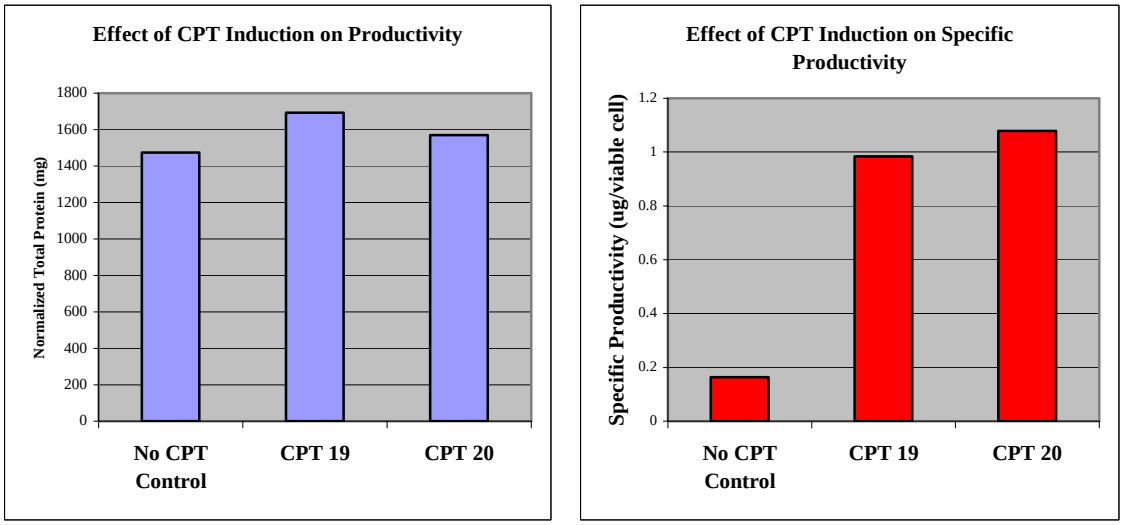


Figure 19: Total Protein Yield and Specific Productivity with CPT Induction

Figure 19 shows the effects of CPT induction in the culture on productivity (blue) and specific productivity (red) at the end of the 14-day cultivation. Final productivity was not affected.

3.4. Statistical Analysis

All of the data from the runs in Study 2 and 4 were statistically analyzed using JMP Software. The most significant factors affecting overall viable cell density for Study 4 were the addition of the CPT to the reactors and dissolved oxygen levels, (see Appendix 1). When both studies were included and the CPT parameter was removed, the most significant factor affecting the live cell numbers was pH. Next, the ViaCount viability response was analyzed and the most considerable effects came from CPT induction and decreased glucose levels in the culture, (see Appendix 2). Glucose can be a limiting substrate during abnormally elevated rates of glycolysis, such as in conditions of severe hydrodynamic shear stress and reduced dissolved oxygen concentration. These oxygen-depriving conditions alter the cell metabolism such that the main energy source occurs through glycolysis increasing the utilization of glucose and decreasing the utilization of glutamine (Mercille and Massie, 1994).

The total apoptotic response correlated with the metabolic levels such as lactate buildup, glucose and glutamine limitation, sodium and potassium concentrations, osmolality, and the viable cell density, (see Appendix 3). This is consistent with other findings that an effective inducer of apoptosis in cell culture processes has been found to be glutamine limitation followed by glucose limitation and ammonia and lactate toxicity (Singh et al., 1994; Mercille and Massie, 1994). The total normalized protein yield was most affected by the viable cell density and the CPT induction, (see Appendix 4). Glucose levels and CPT inductions had the most significant effect on the specific productivity response, (see Appendix 5). Figure 20 shows the response of specific productivity with an increase of viability. As the viability increases, the specific

productivity decreases suggesting that apoptosis and cell death levels play a significant role in productivity of the culture most likely due to secondary necrosis.

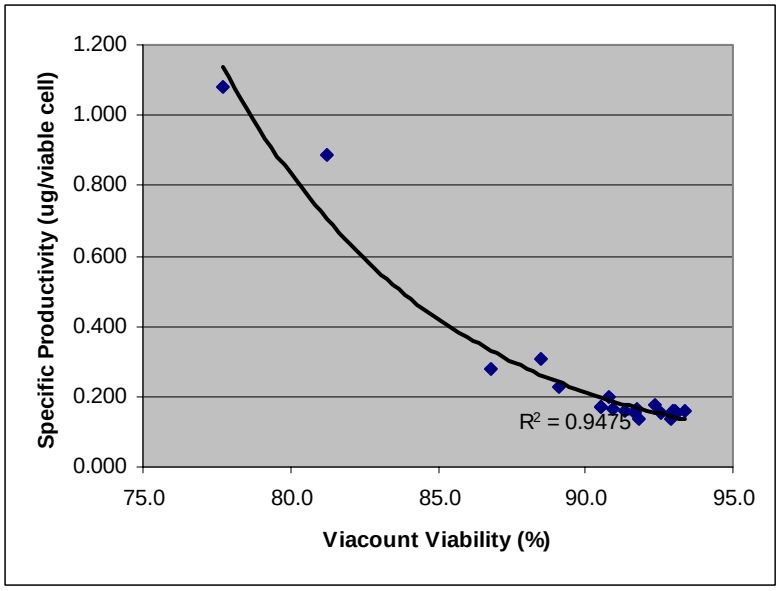


Figure 20: Specific Productivity versus ViaCount Viability

Figure 20 shows the response of specific productivity with the increase in cell viability. As the viability increases, the specific productivity decreases suggesting that apoptosis and cell death levels play a significant role in productivity of the culture.

4. Conclusion & Future Experiments

Results have shown that the Guava ViaCount method is more representative of actual viability levels in a cell culture process than the conventional trypan blue method. The ViaCount method was the simplest, most reproducible, and quickest of the three apoptosis assays. It also provides a more accurate cell density measurement compared to the others. The MultiCaspase assay results were slightly inconsistent since the assay is more sensitive and less robust than the other methods due to its complexity. The assay is time consuming with long incubation times, multiple wash steps, and signal stability was an issue. Because there is some overlap with the different stages of apoptosis, the results between the three Guava methods vary slightly. To get a true representation of the

various apoptotic levels in the culture all three methods should be run. For example, the MultiCaspase method reagent binds to many different caspases and earlier than the PS in Nexin, whereas the Nexin reagent only binds PS giving slightly lower levels of apoptosis.

The ViaCount method is recommended to determine viability and cell density more accurately over the trypan blue method, and it can be used for future optimization of the process. The information from all three assays is useful depending on the stage of apoptosis that is being investigated. The three apoptosis assays have been useful to determine the actual levels of viability in the process to establish which parameters have a significant affect on the viability, apoptotic levels, productivity and specific productivity of the model cell line. This information is useful for process development to allow for optimization by inhibiting or regulating apoptotic levels, and consequently, the possibility to increase the viable cell density and productivity.

Although apoptosis was not found to have a negative effect on total productivity in this particular cell culture process as hypothesized, the significant increase in the product yield and specific productivity are most likely due to release of product during secondary necrosis at the culmination of the apoptosis pathway. Another possibility is that specific productivities have been shown to increase in nutrient-poor medium or apoptotic conditions because the cells are growth arrested and the cell's resources can be used for protein production rather than cell proliferation. The overall protein production could be reduced, however, due to the lower cell density (Mastrangelo and Betenbaugh, 1998). The decrease in productivity and lower apoptotic levels in the nutrient feed deprived run compared to the other two runs in Study 3 could be due to a slower metabolism from the nutrient starvation rather than inductions of the apoptosis cascade.

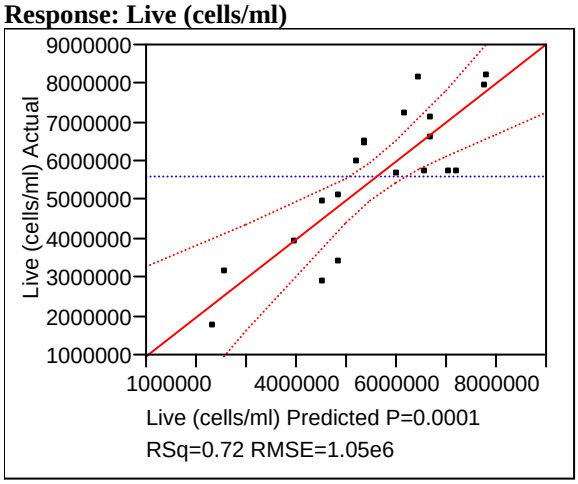
Although specific productivity has been shown to be elevated with increased apoptosis levels, this is most likely not a desired effect in an industrial cell culture process. Cells that undergo apoptosis or necrosis release proteases and other components into the culture thereby decreasing cell proliferation and increasing the chance for product degradation. In addition, the material released from secondary necrosis can cause production issues with downstream processing such as reducing the performance of tangential flow filtration processes. Thus, higher product consistency and quality could be achieved by employing well-defined and reproducible culture conditions and by minimizing the release of intracellular proteases and glycosidases. The effect on the quality of the product should be examined in future experiments as well as the level of cell lysis using lactate dehydrogenate as an internal marker for cell release. Material harvested from the higher apoptotic cultures could also be purified with downstream processes to determine the effect of increased specific productivity due to apoptosis. Understanding how these levels correspond to productivity and product quality will allow for better control of cell culture processes in industrial scale processes.

This study showed the correlation between the culture conditions and apoptosis during a mammalian cell culture process and its effects on the productivity using real-time apoptotic assays for accurate cellular growth and death profiles. This technology could be useful as a possible Process Analytical Technology (PAT) tool to monitor the manufacturing cell culture process real-time to detect cell culture quality issues early. PAT monitoring is becoming a hot topic in the industry and a desired technology by the regulatory agencies as a mode to ensure final product quality and build quality into the manufacturing process.

Another application could be as a process monitoring methodology for troubleshooting purposes. It would be beneficial to determine which conditions the cell culture is sensitive to and to add additional process control to minimize apoptosis levels during process characterization. Results from this study have shown that lower pH during cultivation decreases the viable cell density and increases apoptosis. This is consistent with literature findings that acidic pH in the intracellular environment can increase caspase activity in the apoptosis pathway (Matsuyama et al., 2000 reviewed by Laken and Leonard, 2001). Apoptosis was also significantly increased by glucose and glutamine limitation, lactate toxicity and other metabolite concentrations in the culture such as sodium and potassium. Alternatively, lower dissolved oxygen levels increased the viable cell density and prolonged cell viability in the reactors thereby increasing the productivity. Understanding what signals the apoptotic cascade and how to regulate it would provide a new opportunity to enhance process robustness, improve final performance including productivity, and, eventually, reduce production costs.

Appendix 1: Statistical Analysis – Viable Cell Density Response

Response: Live (cells/ml)



Summary of Fit

RSquare	0.716921
RSquare Adj	0.663843
Root Mean Square Error	1052291
Mean of Response	5608178
Observations (or Sum Wgts)	20

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	3	4.48698e13	1.4957e13	13.5071
Error	16	1.77171e13	1.1073e12	Prob > F
C. Total	19	6.25869e13		0.0001

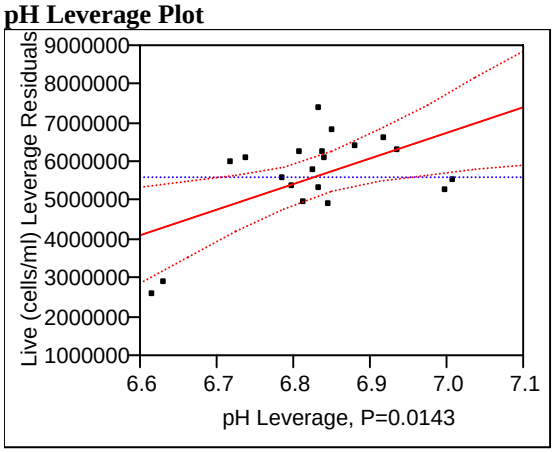
Parameter Estimates

Term	Estimate	Std Error	t Ratio	Prob>|t|
Intercept	-37119190	16394561	-2.26	0.0378
pH	6589660.3	2399439	2.75	0.0143
O2 (mmHg)	-30437.64	8516.174	-3.57	0.0025
CPT addition[no]	1705045.3	394895.4	4.32	0.0005

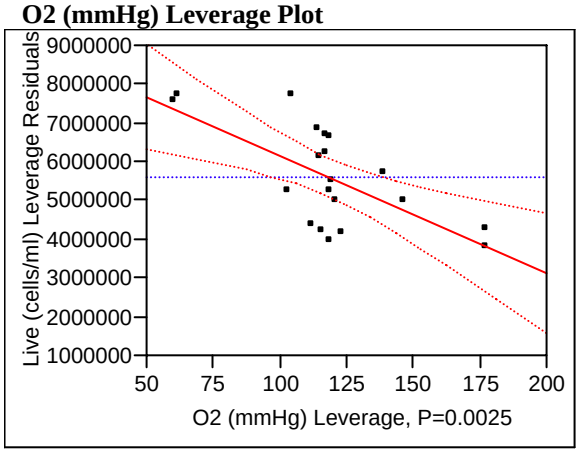
Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
pH	1	1	8.35176e12	7.5423	0.0143
O2 (mmHg)	1	1	1.4145e+13	12.7742	0.0025
CPT addition	1	1	2.06433e13	18.6426	0.0005

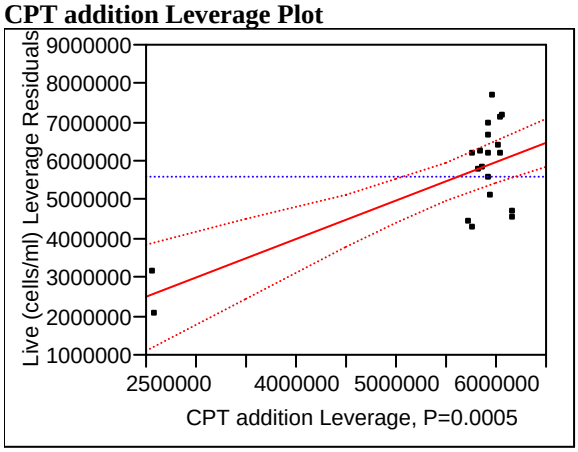
pH Leverage Plot



O2 (mmHg) Leverage Plot



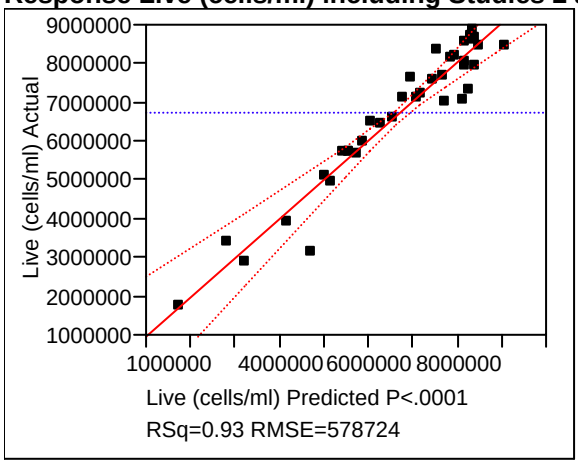
CPT addition Leverage Plot



Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
no	5949187.3	248200.53	5958359
yes	2539096.6	748745.08	2456547

Response Live (cells/ml) including Studies 2 and 4



Summary of Fit

RSquare	0.930679
RSquare Adj	0.901351
Root Mean Square Error	578723.6
Mean of Response	6761387
Observations (or Sum Wgts)	38

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	11	1.1691e+14	1.0628e13	31.7334
Error	26	8.70795e12	3.3492e11	Prob > F
C. Total	37	1.25618e14		<.0001

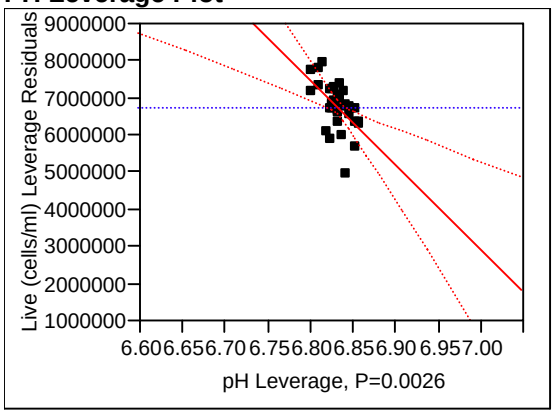
Parameter Estimates

Term	Std Error	t Ratio	Prob>|t|
Intercept	41468979	4.34	0.0002
pH	6752942	-3.34	0.0026
CO2 (mmHg)	10123.43	-0.30	0.7632
O2 (mmHg)	6462.66	0.74	0.4650
Gluc (g/l)	2687433	0.35	0.7290
Lac (g/l)	2160804	0.49	0.6289
Gln (mM)	625092	-2.17	0.0392
Glu (mM)	1381584	-0.94	0.3573
NH4+ (mM)	690184.9	-2.10	0.0453
Na+ (mM)	314573.4	1.53	0.1388
K+ (mM)	1063752	0.87	0.3940
Osm (mOsm/kg)	177829.3	-1.21	0.2359

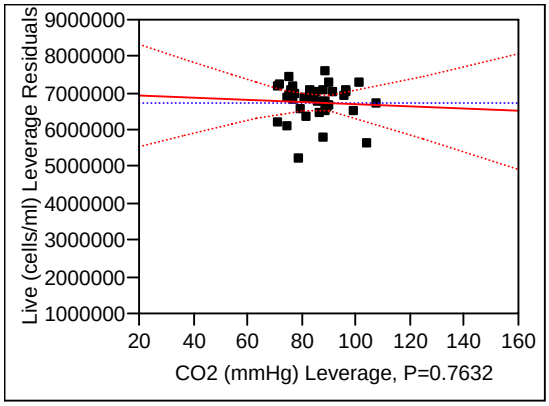
Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
pH	1	1	3.73023e12	11.1377	0.0026
CO2 (mmHg)	1	1	3.10503e10	0.0927	0.7632
O2 (mmHg)	1	1	1.84199e11	0.5500	0.4650
Gluc (g/l)	1	1	4.10792e10	0.1227	0.7290
Lac (g/l)	1	1	8.01219e10	0.2392	0.6289
Gln (mM)	1	1	1.57941e12	4.7158	0.0392
Glu (mM)	1	1	2.94212e11	0.8785	0.3573
NH4+ (mM)	1	1	1.48045e12	4.4203	0.0453
Na+ (mM)	1	1	7.81221e11	2.3326	0.1388
K+ (mM)	1	1	2.51662e11	0.7514	0.3940
Osm (mOsm/kg)	1	1	4.93045e11	1.4721	0.2359

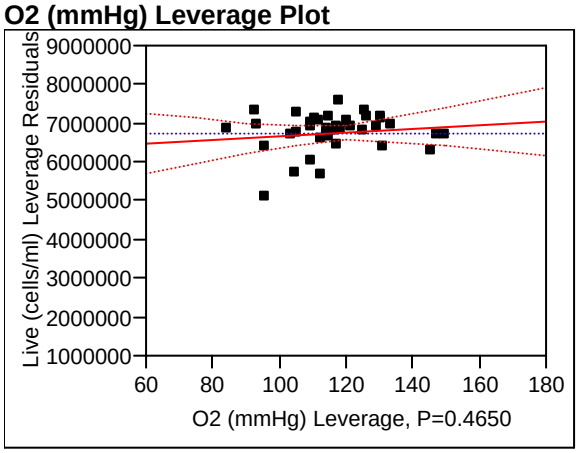
PH Leverage Plot



CO2 (mmHg) Leverage Plot

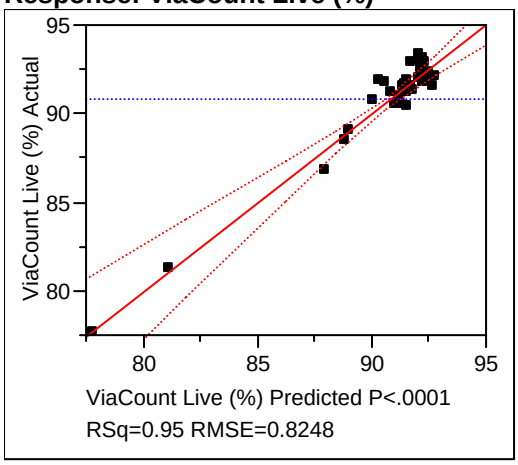


O2 (mmHg) Leverage Plot



Appendix 2: Statistical Analysis – Total Viability Response

Response: ViaCount Live (%)



Summary of Fit

RSquare	0.951543
RSquare Adj	0.925295
Root Mean Square Error	0.82483
Mean of Response	90.85505
Observations (or Sum Wgts)	38

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	13	320.63322	24.6641	36.2523
Error	24	16.32829	0.6803	Prob > F
C. Total	37	336.96150		<.0001

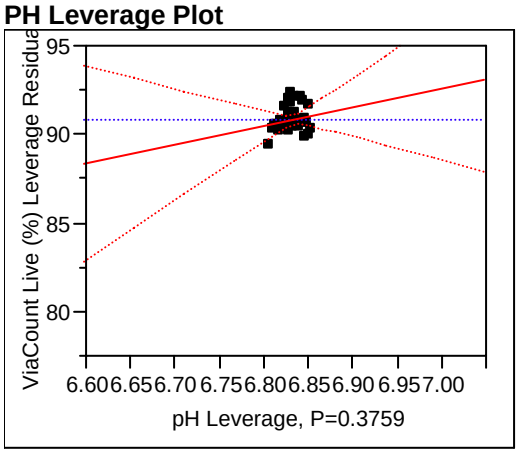
Parameter Estimates

Term	Std Error	t Ratio	Prob>|t|
Intercept	77.63687	0.70	0.4914
pH	11.50668	0.90	0.3759
CO2 (mmHg)	0.015966	-2.32	0.0289
O2 (mmHg)	0.010177	-1.35	0.1904
Gluc (g/l)	3.993405	-3.53	0.0017
Lac (g/l)	3.187788	-1.79	0.0856
Gln (mM)	0.972432	1.85	0.0768
Glu (mM)	2.142376	-2.21	0.0365
NH4+ (mM)	1.075727	1.88	0.0726
Na+ (mM)	0.470164	-0.50	0.6223
K+ (mM)	1.60696	-0.66	0.5144
Osm (mOsm/kg)	0.262061	0.39	0.7007
Live (cells/ml)	3.674e-7	0.25	0.8072
CPT Induction[n]	0.671531	8.16	<.0001

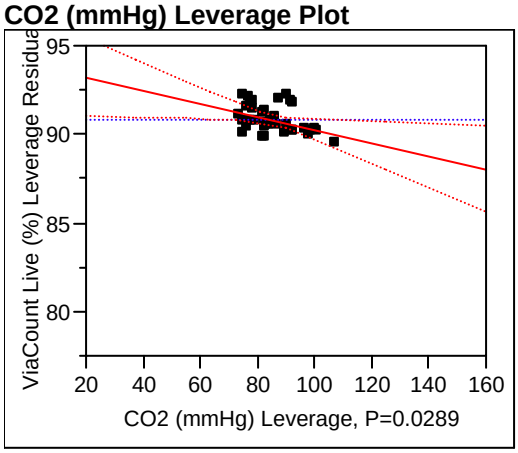
Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
pH	1	1	0.553918	0.8142	0.3759
CO2 (mmHg)	1	1	3.674840	5.4014	0.0289
O2 (mmHg)	1	1	1.235309	1.8157	0.1904
Gluc (g/l)	1	1	8.466409	12.4443	0.0017
Lac (g/l)	1	1	2.186981	3.2145	0.0856
Gln (mM)	1	1	2.326500	3.4196	0.0768
Glu (mM)	1	1	3.336935	4.9048	0.0365
NH4+ (mM)	1	1	2.398443	3.5253	0.0726
Na+ (mM)	1	1	0.169464	0.2491	0.6223
K+ (mM)	1	1	0.297940	0.4379	0.5144
Osm (mOsm/kg)	1	1	0.102931	0.1513	0.7007
Live (cells/ml)	1	1	0.041445	0.0609	0.8072
CPT Induction	1	1	45.312072	66.6016	<.0001

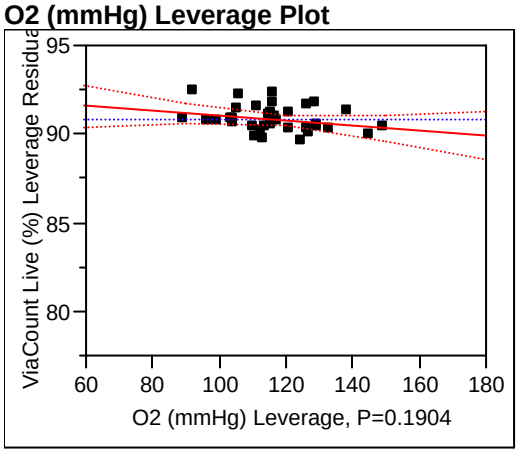
PH Leverage Plot



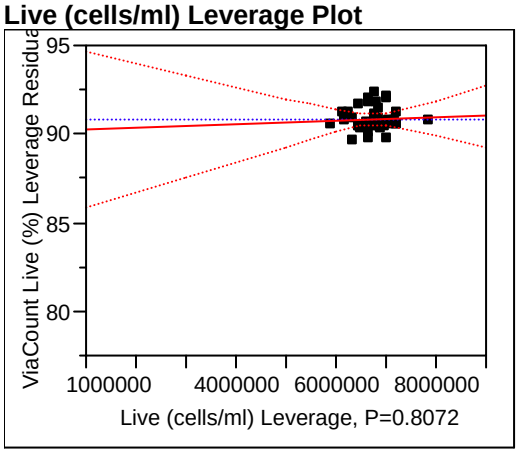
CO2 (mmHg) Leverage Plot



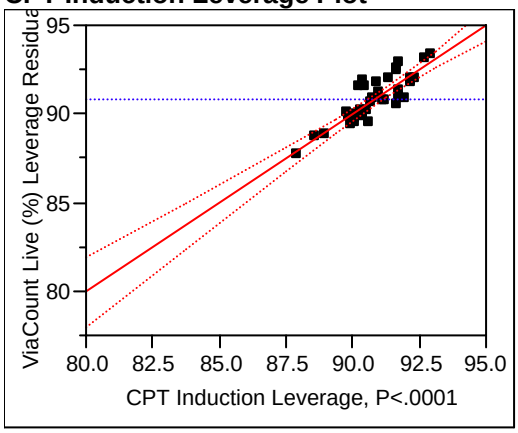
O2 (mmHg) Leverage Plot



Live (cells/ml) Leverage Plot



CPT Induction Leverage Plot

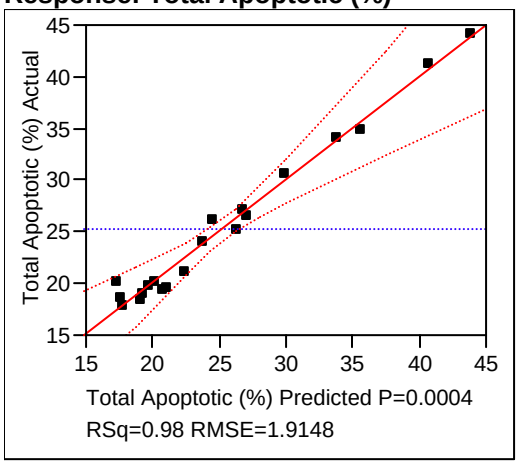


Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
n	91.431934	0.1513293	91.4871
y	80.471227	1.2793914	79.4786

Appendix 3: Statistical Analysis – Total Apoptotic Response

Response: Total Apoptotic (%)



Summary of Fit

RSquare	0.981197
RSquare Adj	0.940456
Root Mean Square Error	1.914832
Mean of Response	25.38245
Observations (or Sum Wgts)	20

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	13	1147.9694	88.3053	24.0838
Error	6	21.9995	3.6666	Prob > F
C. Total	19	1169.9689		0.0004

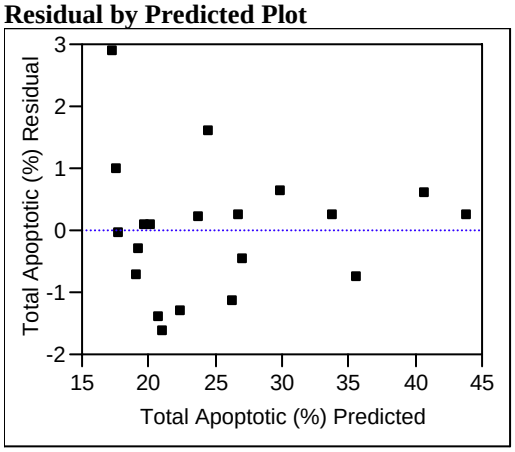
Parameter Estimates

Term	Std Error	t Ratio	Prob>|t|
Intercept	393.3158	3.28	0.0167
pH	48.31232	-2.02	0.0904
CO2 (mmHg)	0.05416	2.55	0.0437
O2 (mmHg)	0.040102	-0.36	0.7291
Glucose(g/l)	20.77028	4.43	0.0044
Lactate (g/l)	30.8474	4.21	0.0056
Glutamine (mM)	4.672703	-3.74	0.0096
Glutamate (mM)	15.46097	0.95	0.3773
Ammonium(mM)	5.764247	0.81	0.4498
Na+ (mM)	5.525022	3.73	0.0097
K+ (mM)	9.528369	3.89	0.0081
Osmolality (mOsm/kg)	2.850858	-3.89	0.0081
Cells x Time (e6cell-day)	0.54483	1.60	0.1597
Live (cells/ml)	0.000002	-4.64	0.0036

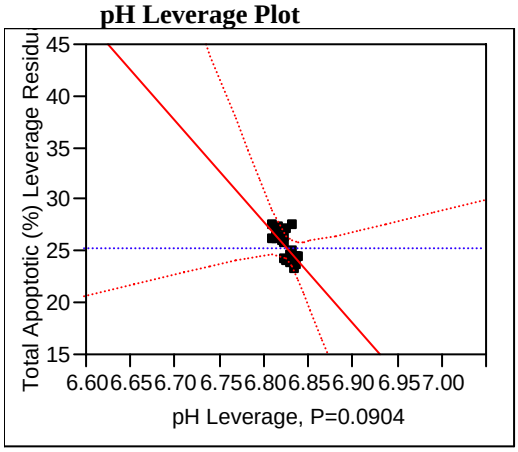
Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
pH	1	1	14.904237	4.0649	0.0904
CO2 (mmHg)	1	1	23.785720	6.4872	0.0437
O2 (mmHg)	1	1	0.482775	0.1317	0.7291
Glucose (g/l)	1	1	71.902072	19.6101	0.0044
Lacate (g/l)	1	1	64.899220	17.7002	0.0056
Glutamine (mM)	1	1	51.253336	13.9785	0.0096
Glutamate (mM)	1	1	3.331756	0.9087	0.3773
Ammonium (mM)	1	1	2.395305	0.6533	0.4498
Na+ (mM)	1	1	51.040204	13.9204	0.0097
K+ (mM)	1	1	55.515723	15.1410	0.0081
Osmolality (mOsm/kg)	1	1	55.359621	15.0984	0.0081
Cells x Time (e6cell-day)	1	1	9.439182	2.5744	0.1597
Live (cells/ml)	1	1	78.792605	21.4894	0.0036

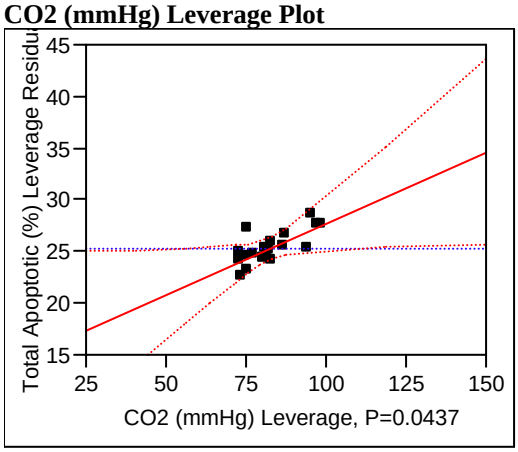
Residual by Predicted Plot



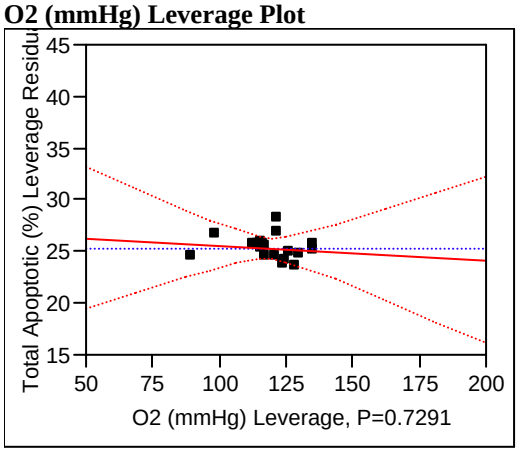
pH Leverage Plot



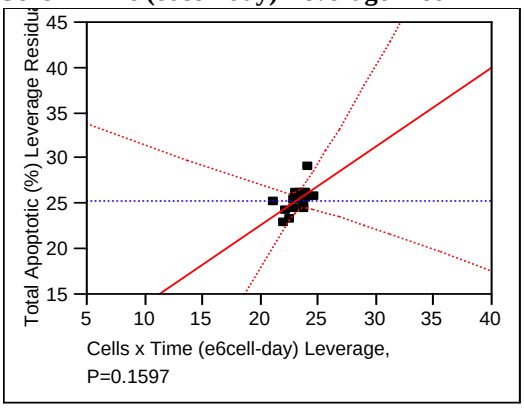
CO2 (mmHg) Leverage Plot



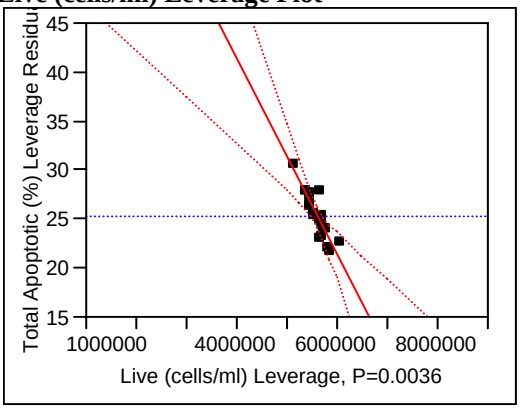
O2 (mmHg) Leverage Plot



Cells x Time (e6cell-day) Leverage Plot

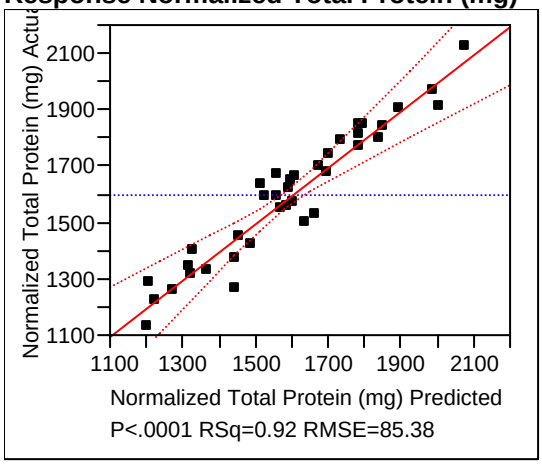


Live (cells/ml) Leverage Plot



Appendix 4: Statistical Analysis – Total Protein Response

Response Normalized Total Protein (mg)



Summary of Fit

RSquare	0.918137
RSquare Adj	0.868307
Root Mean Square Error	85.3803
Mean of Response	1597.377
Observations (or Sum Wgts)	38

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	1880446.2	134318	18.4254
Error	23	167665.3	7290	Prob > F
C. Total	37	2048111.5		<.0001

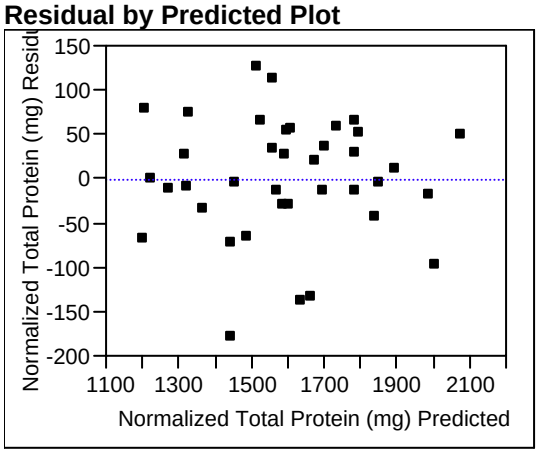
Parameter Estimates

Term	Std Error	t Ratio	Prob>|t|
Intercept	8339.06	1.02	0.3186
pH	1236.079	-0.83	0.4135
CO2 (mmHg)	2.043573	0.44	0.6666
O2 (mmHg)	1.12642	1.33	0.1967
Gluc (g/l)	480.8488	-0.75	0.4601
Lac (g/l)	382.7342	0.24	0.8114
Gln (mM)	107.2701	1.73	0.0975
Glu (mM)	236.0006	-1.21	0.2386
NH4+ (mM)	118.1159	1.03	0.3115
Na+ (mM)	49.6342	1.17	0.2556
K+ (mM)	178.5916	-0.40	0.6946
Osm (mOsm/kg)	27.94619	-0.78	0.4423
Live (cells/ml)	0.000038	4.20	0.0003
CPT Induction[n]	95.43862	-4.45	0.0002
Total Apoptotic (%)	7.323472	0.92	0.3683

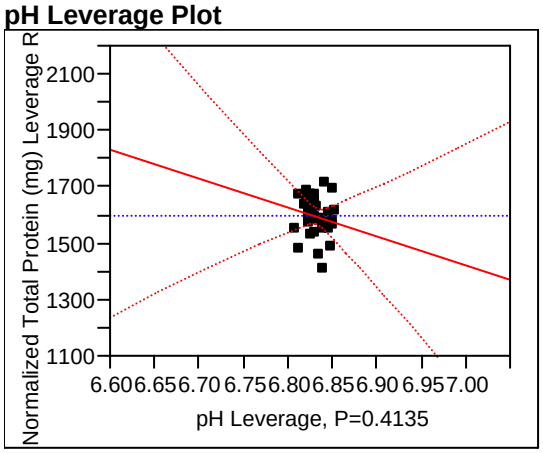
Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
pH	1	1	5055.49	0.6935	0.4135
CO2 (mmHg)	1	1	1388.11	0.1904	0.6666
O2 (mmHg)	1	1	12884.27	1.7674	0.1967
Gluc (g/l)	1	1	4114.58	0.5644	0.4601
Lac (g/l)	1	1	424.82	0.0583	0.8114
Gln (mM)	1	1	21757.03	2.9846	0.0975
Glu (mM)	1	1	10673.08	1.4641	0.2386
NH4+ (mM)	1	1	7806.06	1.0708	0.3115
Na+ (mM)	1	1	9911.36	1.3596	0.2556
K+ (mM)	1	1	1152.39	0.1581	0.6946
Osm (mOsm/kg)	1	1	4456.32	0.6113	0.4423
Live (cells/ml)	1	1	128527.21	17.6311	0.0003
CPT Induction	1	1	144623.39	19.8392	0.0002
Total Apoptotic (%)	1	1	6139.17	0.8422	0.3683

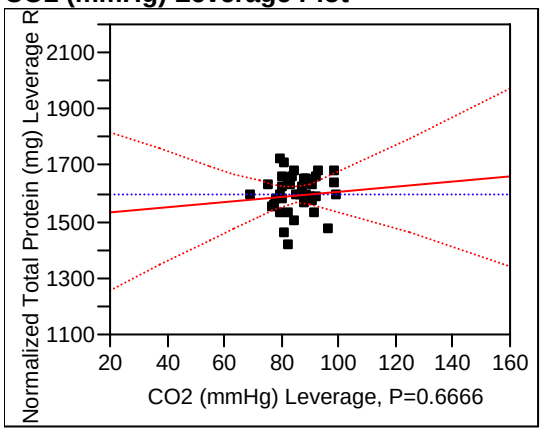
Residual by Predicted Plot



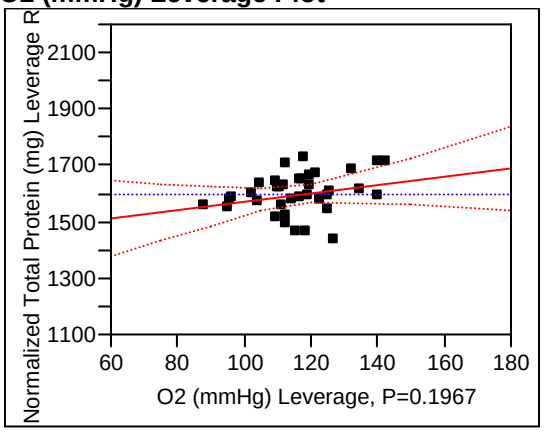
pH Leverage Plot



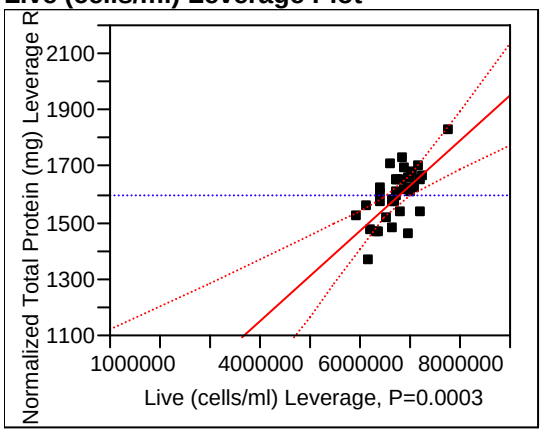
CO2 (mmHg) Leverage Plot



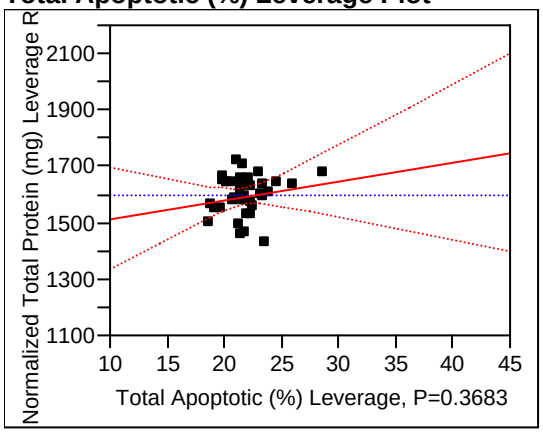
O2 (mmHg) Leverage Plot



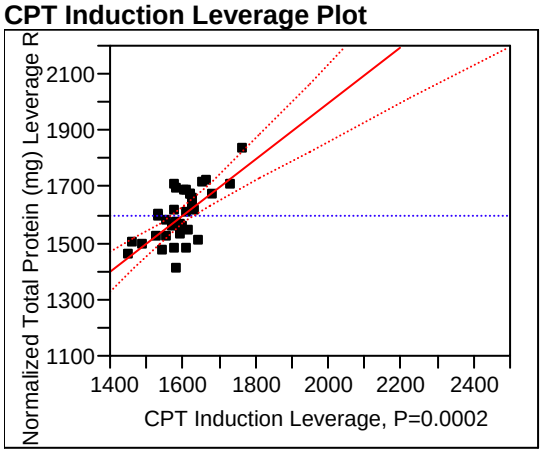
Live (cells/ml) Leverage Plot



Total Apoptotic (%) Leverage Plot



CPT Induction Leverage Plot

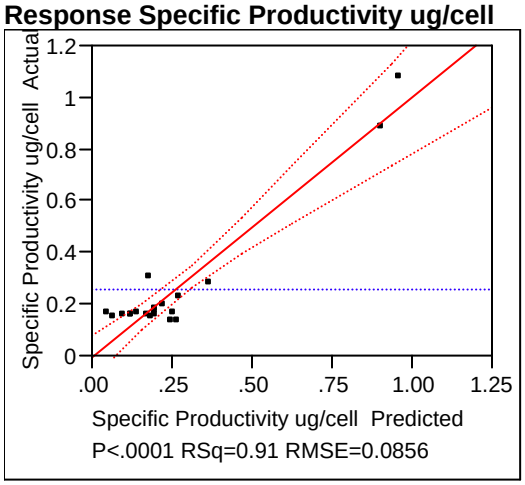


Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
n	1552.6300	17.11030	1592.05
y	2402.8196	181.36073	1693.33

Appendix 5: Statistical Analysis - Specific Productivity Response

Response Specific Productivity ug/cell



Summary of Fit

RSquare	0.90961
RSquare Adj	0.885507
Root Mean Square Error	0.08564
Mean of Response	0.260701
Observations (or Sum Wgts)	20

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	4	1.1070904	0.276773	37.7371
Error	15	0.1100135	0.007334	Prob > F
C. Total	19	1.2171039		<.0001

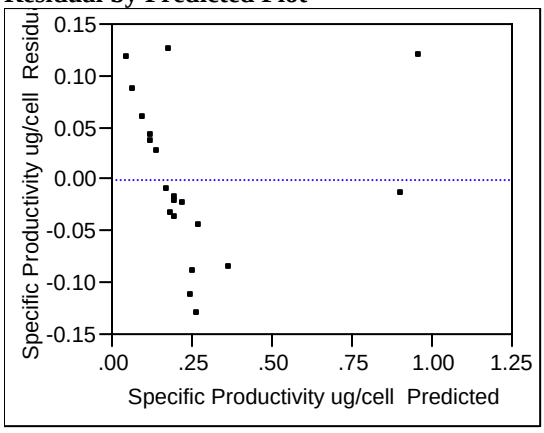
Parameter Estimates

Term	Std Error	t Ratio	Prob>|t|
Intercept	1.762433	0.15	0.8810
pH	0.244741	0.06	0.9541
CO2 (mmHg)	0.001105	-4.93	0.0002
Total Apoptotic (%)	0.003315	7.01	<.0001
O2 (mmHg)	0.000712	-2.90	0.0111

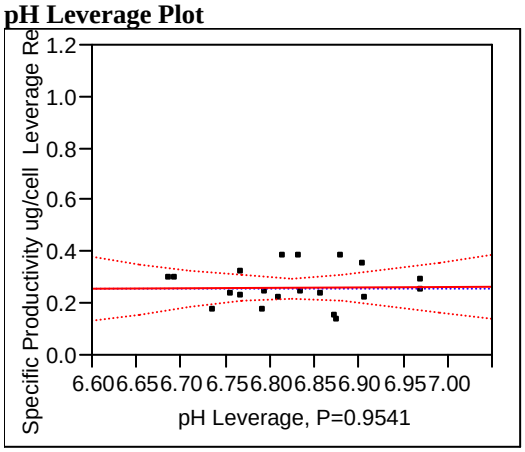
Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
pH	1	1	0.00002507	0.0034	0.9541
CO2 (mmHg)	1	1	0.17835510	24.3182	0.0002
Total Apoptotic (%)	1	1	0.36037538	49.1361	<.0001
O2 (mmHg)	1	1	0.06151744	8.3877	0.0111

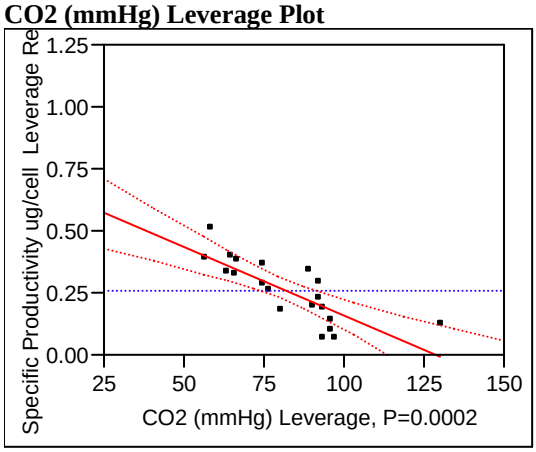
Residual by Predicted Plot



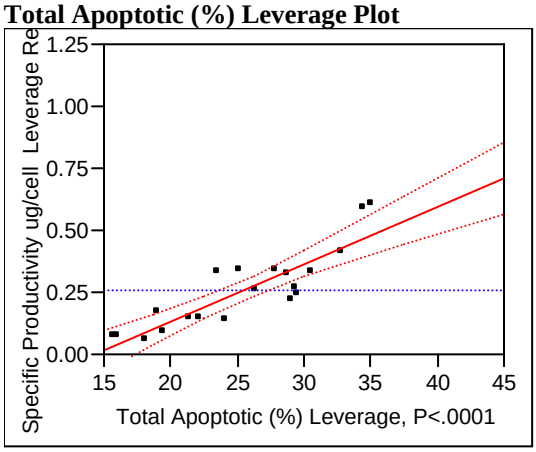
pH Leverage Plot



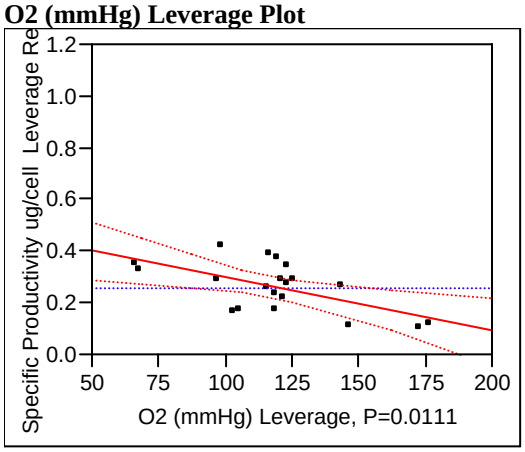
CO2 (mmHg) Leverage Plot



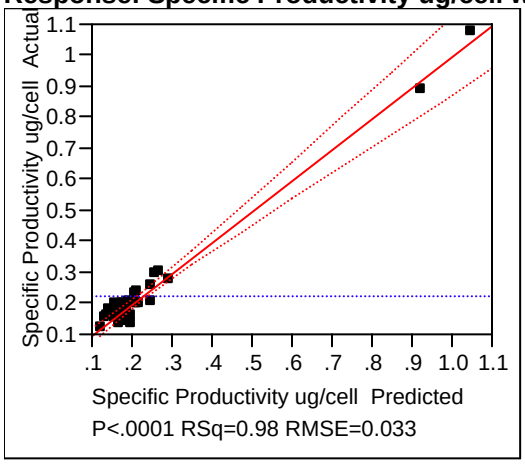
Total Apoptotic (%) Leverage Plot



O2 (mmHg) Leverage Plot



Response: Specific Productivity ug/cell with both Studies 2 and 4



Summary of Fit

RSquare	0.980606
RSquare Adj	0.968801
Root Mean Square Error	0.03298
Mean of Response	0.228901
Observations (or Sum Wgts)	38

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	14	1.2648873	0.090349	83.0671
Error	23	0.0250163	0.001088	Prob > F
C. Total	37	1.2899036		<.0001

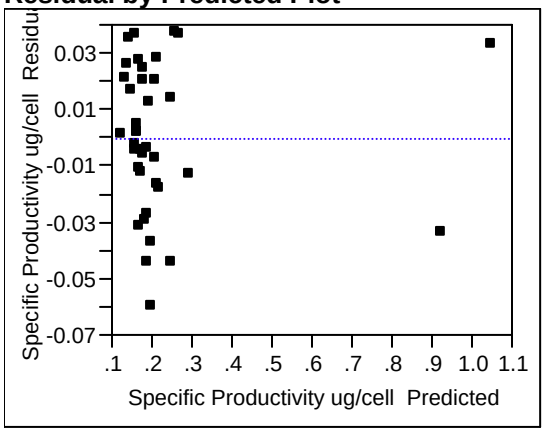
Parameter Estimates

Term	Std Error	t Ratio	Prob>|t|
Intercept	3.111373	0.69	0.5001
pH	0.465864	-0.85	0.4030
CO2 (mmHg)	0.000761	1.51	0.1450
O2 (mmHg)	0.000414	1.35	0.1899
Gluc (g/l)	0.175868	3.73	0.0011
Lac (g/l)	0.131577	2.12	0.0450
Gln (mM)	0.048734	-2.07	0.0501
Glu (mM)	0.095652	0.65	0.5228
NH4+ (mM)	0.043782	-1.41	0.1706
Na+ (mM)	0.018867	1.51	0.1439
K+ (mM)	0.06534	1.60	0.1242
Osm (mOsm/kg)	0.010489	-1.24	0.2286
Live (cells/ml)	1.543e-8	0.02	0.9857
Mid Apoptotic (cells/ml)	2.136e-7	-2.14	0.0435
CPT Induction[n]	0.028605	-13.76	<.0001

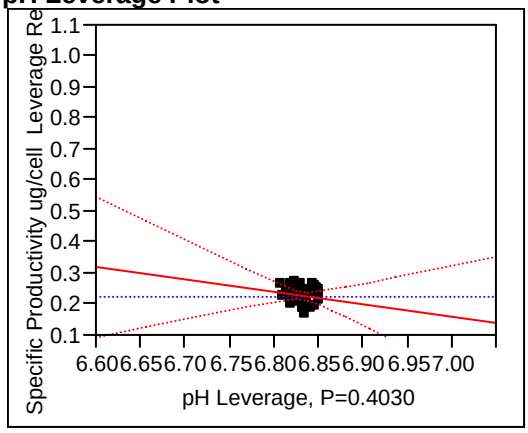
Effect Tests

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
pH	1	1	0.00078956	0.7259	0.4030
CO2 (mmHg)	1	1	0.00247605	2.2765	0.1450
O2 (mmHg)	1	1	0.00198489	1.8249	0.1899
Gluc (g/l)	1	1	0.01515846	13.9367	0.0011
Lac (g/l)	1	1	0.00488729	4.4934	0.0450
Gln (mM)	1	1	0.00464846	4.2738	0.0501
Glu (mM)	1	1	0.00045799	0.4211	0.5228
NH4+ (mM)	1	1	0.00217615	2.0008	0.1706
Na+ (mM)	1	1	0.00248989	2.2892	0.1439
K+ (mM)	1	1	0.00277021	2.5469	0.1242
Osm (mOsm/kg)	1	1	0.00166411	1.5300	0.2286
Live (cells/ml)	1	1	0.00000036	0.0003	0.9857
Mid Apoptotic (cells/ml)	1	1	0.00496354	4.5635	0.0435
CPT Induction	1	1	0.20594530	189.3464	<.0001

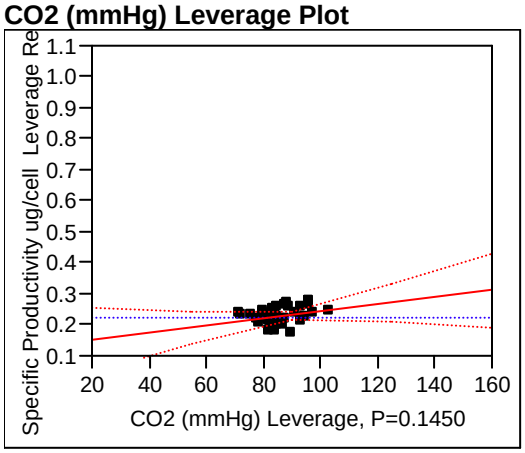
Residual by Predicted Plot



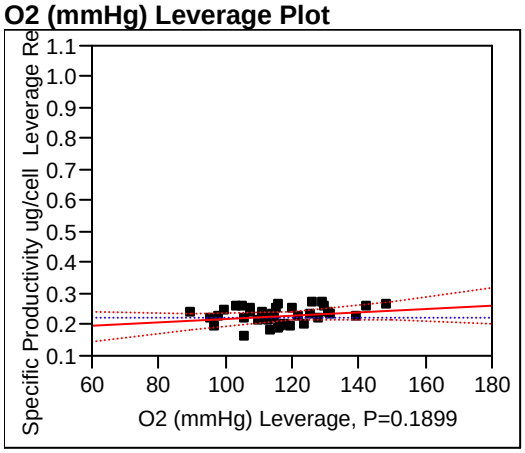
pH Leverage Plot



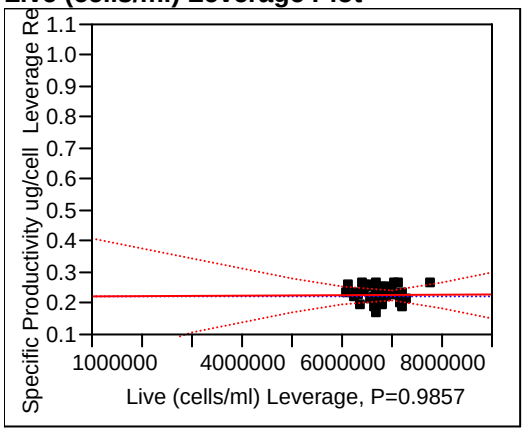
CO2 (mmHg) Leverage Plot



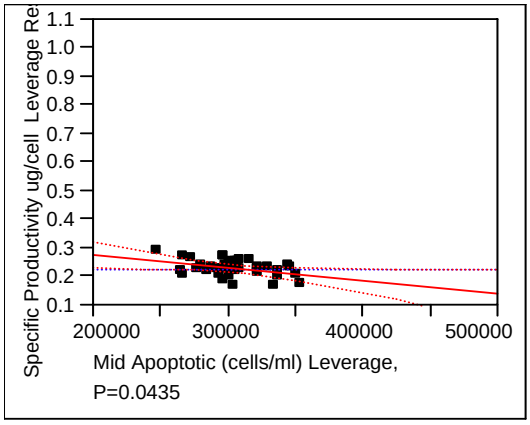
O2 (mmHg) Leverage Plot



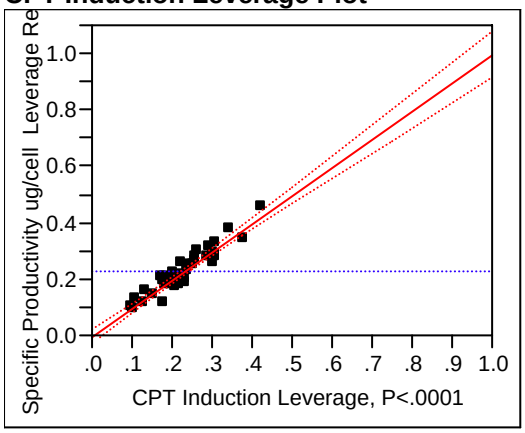
Live (cells/ml) Leverage Plot



Mid Apoptotic (cells/ml) Leverage Plot



CPT Induction Leverage Plot



Least Squares Means Table

Level	Least Sq Mean	Std Error	Mean
n	0.18746828	0.00613913	0.186946
y	0.97468680	0.05446172	0.984081

References

Al-Rubeai, Mohamed and Rabinder P. Singh, "Apoptosis in Cell Culture", *Current Opinion in Biotechnology*, 1998, V 9, pages 152-156

Arden, N., T. Nivitchanyong, and M. J. Betenbaugh, "Cell Engineering Blocks Cell Stress and Improves Biotherapeutic Production", *BioProcessing Journal*, 2004, V 3, pages 23-28

Arden, Nilou and Michael J. Betenbaugh, "Life and Death in Mammalian Cell Culture: Strategies for Apoptosis Inhibition", *Trends in Biotechnology*, 2004, V 22, pages 174-180

Cohen, J. John and Mohamed Al-Rubeai, "Apoptosis-Targeted Therapies: The 'Next Big Thing' in Biotechnology?" *Trends in Biotechnology*, 1995, V 13, pages 281-283

Cotter, Thomas G. and Mohamed Al-Rubeai, "Cell Death (Apoptosis) in Cell Culture Systems", *Trends in Biotechnology*, 1995, V 13, pages 150-155

Cruz, H.J, C.M Freitas, P.M Alves, J.L. Moreira and M.J.T. Carrondo, "Effects of Ammonia and Lactate on Growth, Metabolism, and Productivity of BHK Cells", *Enzyme and Microbial Technology*, 2000, V 27, pages 43-52

Dimmeler, S., J. Haendeler, V. Rippmann, M. Nehis, and A.M. Zeiher, "Shear Stress Inhibits Apoptosis of Human Endothelial Cells" *FEBS Letters*, 1996, V 399, pages 71-74 reviewed by Al-Rubeai, Mohamed and Rabinder P. Singh, "Apoptosis in Cell Culture", *Current Opinion in Biotechnology*, 1998, V 9, pages 152-156

Donovan, Maryanne and Thomas G. Cotter, "Control of Mitochondrial Integrity by Bcl-2 Family Members and Caspase-Independent Cell Death" *Biochimica and Biophysica Acta*, 2004, V 1644, pages 133-147

Fadok, V. A., D.Voelker, P.A. Campbell, J.J. Cohen, D.L. Bratton, and P.M. Henson, "Exposure of Phosphatidylserine on the Surface of Apoptotic Lymphocyes Triggers Specific Recognition and Removal by Macrophages", *Journal of Immunology*, 1992, V 148, pages 2207-2216 reviewed by Hammill, Adrienne K., et al., "Annexin V Staining Due to Loss of Membrane Asymmetry can be Reversible and Precede Commitment to Apoptotic Death", *Experimental Cell Research*, 1999, V 251, pages 16-21

Fishwild, Dianne M. and Kimvan T. Tran, "Robustness and Reliability of the Guava Nexin Apoptosis Screening Assay on the Guava PCA-96 System", Guava Technologies Application Note 2004

Franek, Frantisek and Karolina Sramkova, "Protection of B-Lymphocyte Hybridoma Against Starvation-Induced Apoptosis: Survival-signal Role of Some Amino Acids", *Immunology Letters*, 1996, V 52, pages 139-144

Fussenegger, Marin, “The Impact of Mammalian Gene Regulation Concepts on Functional Genomic Research, Metabolic Engineering, and Advanced Gene Therapies” *Biotechnology Progress*, 2001, V 17, pages 1-51

Fussenegger, Martin and James E. Bailey, "Molecular Regulation of Cell-Cycle Progression and Apoptosis in Mammalian Cells: Implications for Biotechnology", *Biotechnology Progress*, 1998, V 14, pages 807-833

Fussenegger, Martin, James E. Bailey, and Jeffrey Varner, “A Mathematical Model of Caspase Function in Apoptosis”, *Nature Biotechnology*, 2000, V 18, pages 768-774

Gorfien, Stephen, William Paul, David Judd, Lia Tescione, and David Jayme, “Optimized Nutrient Additives for Fed-Batch Cultures”, *BioPharm International*, 2003, V 16, pages 34-40

Green, Douglas R., “Apoptotic Pathways: Paper Wraps Stone Blunts Scissors”, *Cell*, 2000, V 102, pages 1-4

Guava MultiCaspase Detection Kit Insert, Revision date January 23, 2003 4600-0391 rev A

Guava Nexin Kit Insert, Revision Date October 24, 2002, 4600-0070 rev B

Guava Technologies Application Data Report: Amgen Process Development, Presented by Kurt Harris, June 9, 2004

Haass, C., “Apoptosis, Dead End for Neurodegeneration?” *Nature*, 1999, V 399, pages 204-207 reviewed by Fussenegger, Martin, et al., “A Mathematical Model of Caspase Function in Apoptosis”, *Nature Biotechnology*, 2000, V 18, pages 768-774

Hammill, Adrienne .K, Jonathan W. Uhr, Richard H. Scheuermann, “Annexin V Staining Due to Loss of Membrane Asymmetry can be Reversible and Precede Commitment to Apoptotic Death”, *Experimental Cell Research*, 1999, V 251, pages 16-21

Hengartner, Michael O. “The Biochemistry of Apoptosis”, *Nature*, 2000, V 407, pages 770-776

Kaufmann, Hitto, Xenia Mazur, Romina Marone, James E. Bailey, and Martin Fussenegger, “Comparative Analysis of Two Controlled Proliferation Strategies Regarding Product Quality, Influence on Tetracycline-Regulated Gene Expression, and Productivity”, *Biotechnology and Bioengineering*, 2001, V 72, pages 592-602

Kaufmann, Hitto, Xenia Mazur, Martin Fussenegger, James E. Bailey, "Influence of Low Temperature on Productivity, Proteome and Protein Phosphorylation of CHO Cells", *Biotechnology and Bioengineering*, 1999, V 63, pages 573-582

Kerr, J.F., A.H. Wyllie and A.R. Currie, "Apoptosis: A Biological Phenomenon with Wide Ranging Implications in Tissue Kinetics", *British Journal of Cancer*, 1972, V 26, pages 239-257

Kerr, John F. R., "Neglected Opportunities in Apoptosis Research", *Trends in Cell Biology*, 1995, V 5, pages 55-57

Kim, YH, A. Kitayama, M. Takahashi, E. Niki, and E. Suzuki, "Establishment of an Apoptosis-Suppressible, Cell-Cycle Arrestable Cell Line and its Application for Enhancing Protein Production of Serum-Free or Supplemented Culture", *Cytotechnology*, 2000, V 32, 125-134 reviewed by Laken, Haley A and Mark Leonard, "Understanding and Modulating Apoptosis in Industrial Cell Culture", *Current Opinion in Biotechnology*, 2001, V 12, pages 175-179

Korsmeyer, S. "Regulators of Cell Death", *Trends in Genetics*, 1995, V 11, pages 101-105 reviewed by Al-Rubeai, Mohamed and Rabinder P. Singh, "Apoptosis in Cell Culture", *Current Opinion in Biotechnology*, 1998, V 9, pages 152-156

Laken, Haley A and Mark Leonard, "Understanding and Modulating Apoptosis in Industrial Cell Culture", *Current Opinion in Biotechnology*, 2001, V 12, pages 175-179

Lee, Sang Won, Young-Gyu Ko, SookHee Bang, Key-Sun Kim and Sunghoon Kim, "Death Effector Domain of a Mammalian Apoptosis Mediator, FADD, Induces Bacterial Cell Death", *Molecular Microbiology*, 2000, V 35, pages 1540-1549

Lisby, Michael, B. Krogh, F. Boege, O. Westergaard, and B. Knudsen, "Camptothecins Inhibit the Utilization of Hydrogen Peroxide in the Ligation Step of Topoisomerase I Catalysis", *Biochemistry*, 1998, V 37, pages 10815-10827

Lukovic, Dunja, Akira Komoriya, Beverly Packard, and David Ucker, "Caspase Activity is not Sufficient to Execute Cell Death" *Experimental Cell Research*, 2003, V 289, pages 384-395

Marks, David M., "Equipment Design Considerations for Large Scale Cell Culture", *Cytotechnology*, 2003, V 42, pages 21-33

Mastrangelo, Alison J. and Michael J. Betenbaugh, "Overcoming Apoptosis: New Methods for Improving Protein-Expression Systems", *Trends in Biotechnology*, 1998, V 16, pages 88-95

Matsuyama, S, J. Llopsis, Q. Deveraux, R.Y. Tsien, and J.C. Reed, "Changes in Intramitochondrial and Cytosolic pH: Early Events that Modulate Caspase Activation During Apoptosis", *Nature Cell Biology*, 2000, V 2, pages 318-325 reviewed by Laken, Haley A and Mark Leonard, "Understanding and Modulating Apoptosis in Industrial Cell Culture" *Current Opinion in Biotechnology*, 2001, V 12, pages 175-179

Mazur, Xenia, Martine Fussenegger, Wolfgang A. Renner and James E. Bailey, “Higher Productivity of Growth-Arrested Chinese Hamster Ovary Cells Expressing the Cyclin-Dependent Kinase Inhibitor p27”, *Biotechnology Progress*, 1998, V 14, pages 705-713

Mazur, Xenia, Hans Eppenberger, James Bailey, and Martin Fussenegger, "A Novel Autoregulated Proliferation-Controlled Production Process Using Recombinant CHO Cells", *Biotechnology and Bioengineering*, 1999, V 65, pages 144-150

Meents, Heiko, Barbara Enenkai, Rolf Werner, Marti Fussenegger, “$p27^{Kip1}$-Mediated Controlled Proliferation Technology Increases Constitutive sICAM Production in CHO-DUKX Adapted for Growth in Suspension and Serum-Free Media” *Biotechnology and Bioengineering*, 2002, V 79, pages 619-627 presented by Wei-Shou Hu, “Metabolic Engineering of Mammalian Cells for Bioprocessing”, Cellular Bioprocess Technology Course, University of Minnesota, 2004

Meikrantz, William and Robert Schlegel, "Apoptosis and the Cell Cycle", *Journal of Cellular Biochemistry*, 1995, V 58, pages 160-174

Mercille, Sylvain and Bernard Massie, “Induction of Apoptosis in Nutrient-Deprived Cultures of Hybridoma and Myeloma Cells”, *Biotechnology and Bioengineering*, 1994, V 44, pages 1140-1154

Moore, Alison, J. Mercer, G. Dutina, C. Donahue, K. Bauer, J. Mather, T. Etcheverry, and T. Ryll, “Effects of Temperature Shift on Cell Cycle, Apoptosis, and Nucleotide Pools in CHO Cell Batch Cultures”, *Cytotechnology*, 1997, V 23, pages 47-54

Mukwena, N.T., F. Veraitch, S. Santhalingham, M. Al-Rubeai, and P. Goix, "At-line monitoring of Cell Cultures: Rapid Cytometric Evaluation of Cellular Physiology", Guava Technologies Application Note 2003

Perreault, Josee and Real Lemieux, “Essential Role of Optimal Protein Synthesis in Preventing the Apoptotic Death of Cultured B cell Hybridomas”, *Cytotechnology*, 1993, V 13, pages 99-105

Reuveny, S., D. Velez, J.D. Macmillan, and L. Miller, “Factors Affecting Cell Growth and Monoclonal Antibody Production in Stirred Reactors”, *Journal of Immunological Methods*, 1986, V 86, pages 53-59

Shimizu, T and Y Pommier, “Camptothecin-Induced Apoptosis in p53-Null Human Leukemia HL60 Cells and their Isolated Nuclei: Effects of the Protease Inhibitors Z-VAD-fmk and Dichloroisocoumarin Suggest an Involvement of both Caspases and Serine Proteases”, *Leukemia*, 1997, V 11, pages 1238-1244

Simak, Jan, Karel Holada, and Jaroslav G. Vostal, “Release of Annexin V-Binding Membrane Microparticles from Cultured Human Umbilical Vein Endothelial Cells After Treatment with Camptothecin” *BMC Cell Biology*, 2002, V 3, pages 11-20

Simpson, Nicholas H., Anne E. Milner, and Mohamed Al-Rubeai, "Prevention of Hybridoma Cell Death by bcl-2 During Suboptimal Culture Conditions" *Biotechnology and Bioengineering*, 1997, V 54, pages 1-16

Singh, R. P., M. Al-Rubeai, C. D. Gregory, and A. N. Emery, "Cell Death in Bioreactors: A Role for Apoptosis" *Biotechnology and Bioengineering*, 1994, V 44, pages 720-726

Thompson, C.B., "Apoptosis in the Pathogenesis and Treatment of Disease", Science, 1995, V 267, pages 1456-1462 reviewed by Fussenegger, Martin, et al., "A Mathematical Model of Caspase Function in Apoptosis", *Nature Biotechnology*, 2000, V 18, pages 768-774

Vaughan A.T.M., C.J. Betti, and M.J. Villalobos, "Surviving Apoptosis", *Apoptosis*, 2002, V 7, pages 173-177

Vaux, David L., "Toward an Understanding of the Molecular Mechanisms of Physiological Cell Death", *Proc. Natl. Acad. Sci.*, 1993, V 90, pages 786-789

Wyllie, A.H, "Glucocorticoid-Induced Thymocyte Apoptosis is Associated with Endogenous Endonuclease Activation" *Nature*, 1980, V 284, pages 555-556 reviewed in Hengartner, Michael O. "The Biochemistry of Apoptosis", *Nature*, 2000, V 407, pages 770-776

Yokobata, Kathy, Colleen Helsel, Larry Boyle, Jestine Ho, Katherine Gillis, and Dianne Fishwild, "Monitoring Apoptosis using Four Different Assays on the Guava PCA System", Guava Technology Notes, Revised 5 May 2003

Zhanghi, James A., Wolfgang Renner, James E. Bailey, and Martin Fussenegger, "The Growth Factor Inhibitor Suramin Reduces Apoptosis and Cell Aggregation in Protein-Free CHO Cell Batch Cultures" *Biotechnology Progress*, 2000, V 16, pages 319-325