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Optimization of the clone selection strategy: multi-step DoE for the optimization of media and feed for mAbs production

Experimental part carried out in the laboratory of
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Abstract

During the last 50 years the development of genetic engineering has allowed to modify organisms in order to make them express a protein of interest. Since more than 20 years, the demand of recombinant proteins for therapeutic needs is continuously growing. To be in line with the increasing demands of biologic molecules, the industry has to develop new tools to improve the titers of the cell lines used.

At Merck Serono in Vevey, the selection of the most productive clone consists of several steps. After transfection with the gene of interest, cells are tested in static and agitated condition and the clones giving the best titers are selected. Since the same platform parameters are used during the entire selection process, this raises the question whether the chosen clones are indeed the most performant ones or just the more adapted to those conditions. So, the aim of this work was to prove that, by changing the media composition, an increasing in titer may be achieved.

In this work, different clones of two cell lines, CHO-S and CHO-K1, were used. A sequentially aproach is adopted and design of experiments (DoE) are done according to the selections obtained after an accurate decision trees (DTs) analysis of a previous set of results.

At the beginning of this work, the results of a previous study conducted at Merck were analyzed with DTs. This experiment was performed with 5 different clones and 11 groups of AAs at different levels were tested. The analysis of the results obtained at WD14 led to the conclusion that the selections are clone specific and for this reasons it is not possible to find a recipe for the medium which is equal for every cell line.

The first experiment done was conducted with two different clones, P04 and T11, on the same plate and with the same platform condition, with the aim to evaluate the repetition errors. The repetition errors were not acceptable, as CVs were higher than 20%, due to two principal reasons: first of all, the grey silycon membrane was used on the plate; secondly, the plates used to measure the VCD were not coated. Indeed, in a previous study it was emerged that the grey membranes lead to an early cell death in the middle of the plate and that the T11 cells tend to adhere to the bottom of the guava plate if this is not coated.

Experiment 2 was split into two parts: the first one had the goal to evaluate the repetition errors in platform condition, while the second one was used to evaluate the effect of 3 of the 11 goups tested in experiment zero. In this case, two plates with equal conditions were evaluated and just one clone, P04, was considered. For the part regarding the repetition errors, better results were obtained compared to experiment 1, with CVs lower than 10%. Regarding the media blending part, it was found that the selections obtained on the titer lead to higher productivity than platform medium.

Experiment 3 was conducted on three plates, one for each clone (T15, T18 and T25), and different media and feed blending conditions were tested. It was chosen to test the groups that were found to be important in experiment zero. It was proved that, for all the clones, there were some conditions which performed better compared to the platform. In particular, it emerged that feed blending is not important compared to media blending.

Experiment 4 was performed with the same clones but, in this case, it was chosen to test also some conditions in which media and feed blending were combined. Moreover, since it was emerged from experiment 3 that K in the medium needs to be increased, it was decided to enrich the platform medium in potassium at 150% of the level in the standard medium. The results proved the importance of this salt showing that the productivity is slightly higher when the platform medium contains K than when it does not.

The last two experiments were performed in STs and had the goal to validate the results obtained in 96-DWPs. Since the STs allow to have less conditions to test, it was chosen to evaluate just one clone, T18. The results obtained at WD10 were analyzed and the importance of K was again highlighted.

In experiment 6 it was chosen to to try to fine tune the best level of K in both, the medium and the feed. The results obtained, verified the positive influence of potassium on the productivity but, unfortunately, this work was to short to be able to fine tune the best level of this salt.

Moreover, since it was proved that there are always some conditions which perform better than the platform, it was demonstrated that the clone selection strategy should be changed. In particular the best way to operate would be to do the first fed batch with 20 clones and 20 conditions for each clone in DWPs, in order to find the optimal environment. Then, only the best 10 clones should be selected with their own best media to be submitted in the second fed-batch to different feeding strategies. After this, 4 clones with their best combination of media and feeding strategies should go through the last fed-batch. Only the clone that obtains the best final titer must be selected in this last fed-batch. After this selection, media, feeds and other parameters may go through another round of optimization.

Sommario

Negli ultimi 50 anni lo sviluppo dell'ingegneria genetica ha permesso di modificare gli organismi con lo scopo di esprimere una proteina di interesse. Da oltre 20 anni la richiesta di proteine ricombinanti per esigenze terapeutiche è in continua crescita. Per essere in linea con la crescente domanda di agenti terapeutici, è necessario sviluppare nuovi strumenti per migliorare la produttività della linea cellulare utilizzata. Alla Merck Serono di Vevey, la selezione dei cloni più produttivi consiste di diversi step. In seguito alla tranfezione del gene di interesse, le cellule sono testate in condizioni statiche e dinamiche e i cloni più produttivi vengono selezionati. Dal momento che tutti questi step vengono svolti in condizioni standard, ci si pone la domanda se i cloni selezionati siano effettivamente quelli che producono meglio o se sono semplicemente quelli che meglio si adattano a quelle condizioni. Lo scopo di questo lavoro è quello di verificare che, cambiando le composizioni dei terreni di coltura, un aumento della quantità di anticorpi prodotta può essere raggiunto. In questo lavoro, vengono utilizzati diversi cloni di due linee cellulari, CHO-S e CHO-K1. Viene adottato un approccio sequenziale e gli esperimenti vengono designati secondo delle selezioni ottenute in seguito all'analisi dei risultati ottenuti in un precedente esperimento.

All'inizio di questo lavoro, i risultati di uno studio precedente condotto alla Merck sono stati analizzati con alberi decisionali (DTs). Questo esperimento è stato eseguito con 5 diversi cloni e 11 gruppi di amminoacidi a diversi livelli sono stati testati. L'analisi dei risultati ottenuti al WD14 ha portato alla conclusione che le selezioni sono specifiche per ogni clone e, per questo motivo, non è possibile trovare una ricetta per il medium che sia uguale per ogni linea cellulare.

Il primo esperimento fatto è stato condotto con due diversi cloni, P04 e T11, sulla stessa piastra e nelle stesse condizioni di coltura, al fine di valutare la ripetibilità. Gli errori ottenuti non erano accettabili, con coefficienti di variazione (CV) superiori al 20%, a causa di due ragioni principali: prima di tutto, è stata utilizzata la membrana in silicone grigia; secondo, le piastre utilizzate per misurare la VCD non erano state rivestite. Infatti, in uno studio precedente è emerso che le membrane grigie portano le cellule a una morte precoce al centro della piastra e che le cellule CHO-K1 tendono ad aderire al fondo della piastra, se questa non è rivestita.

L'esperimento 2 è stato suddiviso in due parti: la prima ha avuto l'obiettivo di valutare la ripetibilità nelle condizioni standard, mentre la seconda è stata utilizzata per valutare l'effetto di 3 degli 11 gruppi testati nell'esperimento zero. In questo caso, sono state valutate due piastre con condizioni uguali e solo il clone P04 è stato considerato. Per quanto riguarda la ripetibilità, sono stati ottenuti risultati migliori rispetto all'esperimento 1, con CV inferiori al 10%. Per quanto riguarda gli esperimenti in cui i mezzi di coltura sono stati mischiati, si è scoperto che le selezioni ottenute portano ad avere una produttività più elevata rispetto alle condizioni standard.

L'esperimento 3 è stato condotto su tre piastre, una per ogni clone (T15, T18 e T25), e sono stati sperimentati diversi mezzi di coltura e diversi nutrienti. Si è scelto di testare i gruppi che si sono rilevati importanti nell'esperimento zero. È stato dimostrato che, per tutti i cloni, esistono alcune condizioni che migliorano le performance rispetto alle condizioni standard.

L'esperimento 4 è stato compiuto con gli stessi cloni ma, in questo caso, si è scelto di combinare le tecniche di media blending e feed blending. In questo caso, è stato scelto di provare anche alcune condizioni in cui supporto e miscela di feed sono stati combinati. Inoltre, dal momento che è emerso dall'esperimento

3 che K nel medium deve essere aumentato, si è deciso di aggiungere al platform medium una quantità di potassio al 150% rispetto a quella presente nelle condizioni standard. I risultati hanno dimostrato l'importanza di questo sale mostrando che la produttività è nettamente superiore quando il K è aggiunto rispetto a quando non è presente.

Gli ultimi due esperimenti sono stati eseguiti in ST; l'obiettivo era di convalidare i risultati ottenuti in 96-DWPs. Poiché gli spin tubes consentono di avere meno condizioni per la prova, si è scelto di valutare solo un clone, T18. I risultati ottenuti al WD10 sono stati analizzati e l'importanza di K è stata nuovamente evidenziata.

Nell'esperimento 6 è stato scelto di cercare di trovare il livello ottimale di potassio. I risultati ottenuti dimostrano l'influenza positiva del potassio sulla produttività ma, purtroppo, questo lavoro è stato troppo breve per potere misurare la quantità ottimale di questo sale. Inoltre, dal momento che è stato dimostrato che ci sono sempre alcune condizioni che funzionano meglio di quella standard, si deduce che la strategia di selezione dei cloni dovrebbe essere modificata. In particolare il modo migliore per operare sarebbe quello di compiere la prima coltura fed-batch con 20 cloni e 20 condizioni per ogni clone in DWP, al fine di trovare l'ambiente ottimale. Quindi, solo i migliori 10 cloni dovrebbero essere selezionati per il secondo fed-batch, in cui i migliori terreni di coltura vengono combinati con diversi feed. Dopo di che, i 4 migliori cloni vengono selezionati per il terzo fed-batch con le loro migliori combinazioni di medium e feed. Solo il clone che ottiene il miglior titolo finale deve essere selezionato in questo ultimo fed-batch. Dopo questa selezione, i mezzi di coltura, i feed e altri parametri potrebbero essere ulteriormente migliorati.

Contents

Nomenclature

Ab	Antibody
Asn	Asparagine
ATF	Alternating Tangential Flow
CART	Classification and Regression Trees
CHO	Chinese Hamster Ovarian
CV	Coefficient of Variation
Cys	Cysteine
DNA	DeoxyriboNucleic Acid
DoE	Design of Experiment
DTs	Decision Trees
DWPs	Deep Well Plates
FDA	Food and Drug Administration
G	Group
G3A	Group 3 - Apolar
G3P	Group 3 - Polar
GS	Glutamine Synthetase
Ile	Isoleucine
IVC	Integral of the Viable Cell Density
K	Potassium
Leu	Leucine
mAb	Monoclonal Antibody

MB	Media Blending
Met	Methionine
MSX	Methionine Sulphoximine
OFAT	One Factor at Time
pAb	Polyclonal Antibody
PCD	Specific Productivity
PF	Platform Feed
PM	Platform Medium
Pyr	Pyruvate
SD	Standard Deviation
STs	Shake/Spin Tubes
TFF	Tangential Flow Filtration
Tyr	Tyrosine
VCD	Viable Cell Density
WD	Working Day

Chapter 1

Theorethical Background

1.1 Introduction

During the last 50 years the development of genetic engineering has allowed to modify organisms in order to make them express a protein of interest. After the discovery of recombinant DNA by Cohen and Boyer in 1973 [1], the potential of this technique was soon applied to the drug development field and, in 1978, the human insulin was synthesized by Genentech [87]. Since this first protein, a growing number of recombinant proteins is approved every year by the US food and drug administration (FDA). The number of new biological license applications has more than tripled in the last ten years. [42, 52] Since more than 20 years, the biopharmaceutical industry is a strongly growing branch of pharmaceutical industry, constituting the 28% [42] of the pharmaceutical market. It focuses on the production of drugs by employing genetically engineered bacteria, yeast and mammalian cells. [2]

Nowadays, the biopharmaceutical industry is facing a growing demand of recombinant proteins for therapeutic needs; the world-wide biologics market is projected to exceed USD390 billion by 2020 [53]. Monoclonal antibodies and related molecules have become a major therapeutic modality for indications in areas of inflammatory disorders, oncology, neurology and infection. [3, 4, 5, 6]

Many diseases cannot be treated with basic chemical molecules and need recombinant proteins; in Figure 1.1 the most important diseases which require recombinant proteins are reported. Many of them are monoclonal antibodies and their development has grown exponentially in the recent years.

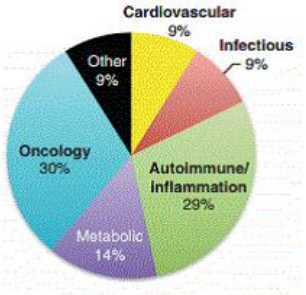

Figure 1.1: Principal diseases that need protein treatments (source: [87])

1.2 Monoclonal Antibodies (mAbs)

An antibody (Ab), is a glycoprotein belonging to the immunoglobulin family; it is an Y-shaped protein, as in Figure 1.2a, used by the immune system to neutralize pathogens such as bacteria and viruses; these pathogens are recognized due to the presence of an antigen on their membrane. [38]

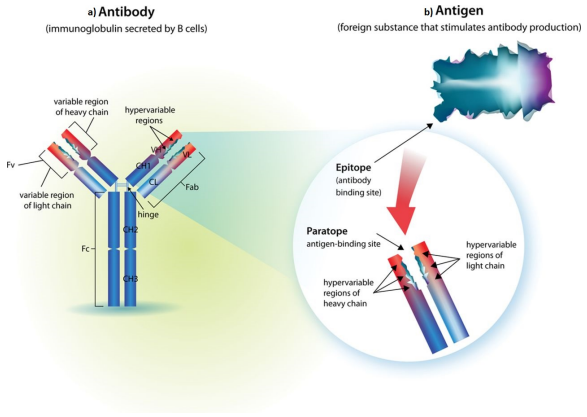

Figure 1.2: Typical structure of an antibody (a); mechanism of binding (b) (source: [38])

An antigen is a particular receptor composed of different epitopes that can be recognized by the antibody. An epitope is a sequence of three amino acids that can be detected by a specific paratope, a structure on the top of the antibody, which is composed by the three complementary amino acids. Each type of antibody contains a paratope that is specific for one particular epitope present on an antigen, allowing these two structures to bind together with precision. Using this binding mechanism, an antibody can tag a microbe or an infected cell for attack by other parts of the immune system, or can neutralize its target directly. [37]

The ability of an antibody to communicate with the other components of the immune system is mediated via a region located at the base of the "Y", which contains a conserved glycosylation site involved in these interactions. Antibodies are typically made of basic structural units each with two large heavy chains and two small light chains. Within the antibody structure, it is possible to identify two regions: the constant region (Fc), which does not change between the same family of antibody; and a variable region (Fv), which contains the binding site. Within the variable region, a hypervariable region is present; it is a very small region at the tip of the protein that allows millions of antibodies with slightly different tip structures to exist. This region contains the paratope, the antigen binding site (Figure 1.2b). This enormous diversity of antibody paratopes allows the immune system to recognize an equally wide variety of antigens.

Based on how many epitopes are recognized by an antibody, it is possible to divide them into two big categories: polyclonal antibodies (pAb) and monoclonal antibodies (mAb). [37]

MAbs are able to bind just one precise epitope and are then more specific; they are produced by a clone or genetically homogeneous population of fused hybrid cells, as shown in Figure 1.3; these antibodies are chemically and immunologically homogeneous.

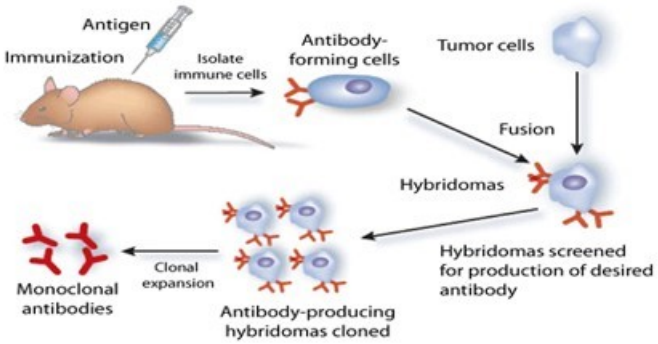

Figure 1.3: Production of monoclonal antibodies (source: [37])

They serve as experimental probes in cell biology, biochemistry, and parasitology, and are used in purification of biological substances and certain drugs. Because of their high specificity in binding to target antigens, they are used in the therapy of a wide variety of disorders, including Crohn disease, rheumatoid arthritis, rejection of transplanted organs, and neoplasms.[7] Monoclonal antibodies kill tumor cells by several mechanisms, including apoptosis and lysis mediated by complement and cytotoxic cells. Antibodies bind to the targeted cells and stimulate the recruitment of effector cells with the capacity for antibody-dependent cellular cytotoxicity (cf. Figure 1.4) or phagocytosis such as natural killer cells and monocytes/macrophages, respectively. These are all indirect mechanisms, mediated through the Fc region of the antibody [36].

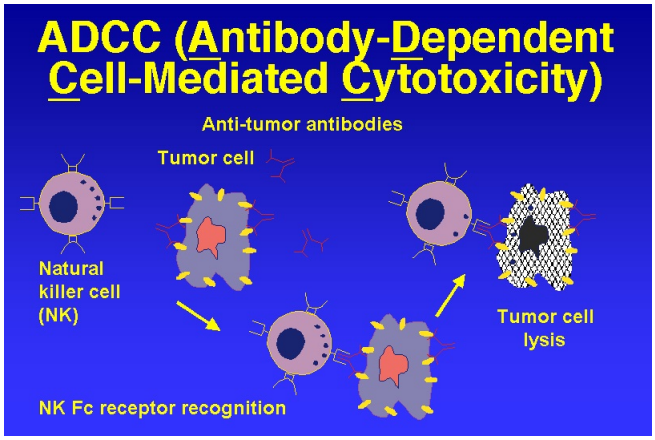

Figure 1.4: Antibody Dependent Cell-Mediated Cytotoxicity (ADCC) (source: [88])

Antibodies may also promote cell death via complement-dependent cytotoxicity, in which antibody binding to the target cell results in activation of the complement cascade, which leads to apoptosis [38]. They can also conduit radioisotopes or toxic agents linked to them, permitting the specialized delivery of therapeutic or diagnostic agents [39]. This last mechanism is shown in Figure 1.5.

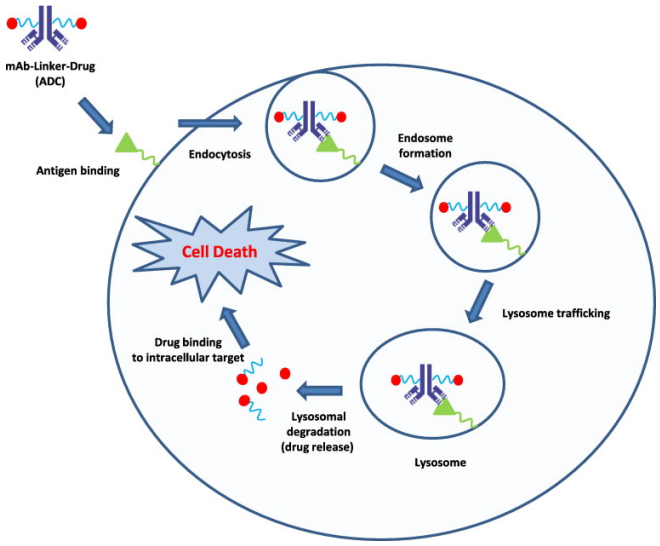

Figure 1.5: Mechanism of action of an antibody conjugated with a therapeutic agent (source: [89])

On the contrary, pAbs are able to recognize different epitopes and for that they are less specific. They are produced by B lymphocytes in response to an immense number of different antigens. [37]

1.2.1 Recombinant Proteins

Recombinant proteins are obtained after the transcription and the translation of a recombinant DNA fragment. In order to obtain this kind of molecules, it is necessary to clone the specific gene inside a vector that has to contain the signal needed for the beginning of both, transcription and translation. All the vectors must have one part specific for the organism, in which it will be introduced, to let the cells to select that gene and to replicate it.

After the insertion of the DNA sequence within the vector, the vector has to be put into a pre chosen cell type; the most used are bacteria and mammalian cells. The choice of the cell type depends on the type of protein that shall be obtained. Eukaryotic cells are widely used as host cells because they enable to study the behavior of the gene in a more physiological contest, due to the lack of post-translational changes in prokaryotic cells. (see Section 1.2.2)

The obtained proteins must be then purified and detected with specific techniques. This kind of proteins are used in both, research and pharmacological field, for this reason, they must be as similar as possible to the human ones.

The 60% of the 54 biologics approved from 2010 to 2014, is produced in mammalian cells [8] and, in particular, the 33% of all recombinant therapeutic proteins are produced in Chinese hamster ovarian cells. [9]The other 40% of the approved molecules are produced by non-mammalian systems, due to their low cost, complexity and their ease of control. [42]

1.2.2 The Importance of Glycosylation

Among the post-translational changes that take place into a recombinant protein, glycosylation is the most important one. [40, 41] Glycosylation is of particular interest due to the numerous discovered relationships between biological function and specific glycosylation structures. [59] N-linked glycosylation is essential for protein folding and influences several product-specific properties such as solubility, stability, enzymatic activity, immunogenicity, circulatory lifetime, charge variant distribution and therapeutic efficacy [35]. This can result in alterations of in vivo and in vitro monoclonal antibody properties. [59]

Moreover, alterations in glycosylation are linked with several pathologies. [60, 61]

The glycosylation variables are therefore considered as a critical quality attribute; obtaining uniform and consistent glycan patterns is becoming more and more important [35, 58]. However, most high throughput methodologies are focused on cell growth and productivity rather than on product quality, which is usually analysed during later process development stages in bioreactors. No high throughput measurments are available for glycosilation and the analytics is usually very tedious. Protein aggregation and fragmentation, which commonly occur during bioprocessing, are dependent on many factors including structural, environmental and processing factors [62, 63, 64]. These pose a risk in terms of loss of biological activity and immunogenicity. [62, 65, 66, 67, 68] These modifications are host cell line, clone and process dependent [69] and therefore, analyzing product quality early in development might be very useful to rapidly match the desired quality target product profile. [59]

1.3 CHO Cell Lines

The 40% of the 54 biologics approved from 2010 to 2014 is produced by non-mammalian systems, due to their low cost, reduced complexity and their ease of control. The other 60% is produced in mammalian cells [8] and, in particular, the 33% of all recombinant therapeutic proteins are produced in Chinese hamster ovarian cells. [9]

Amongst all the available mammalian cells, Chinese Hamster Ovarian cells (CHO cells) are the most widely used since 2000 [10, 42, 43]and for that they are very important for the modern biopharmaceutical industry [11]. CHO cells are currently dominating as a commercial production host because of their ease of use, strong regulatory track record, and safety profile.[12] Moreover, CHO cells have the capability to secrete proteins into the cell culture supernatant, which facilitates downstream processing. [13] At the end of the culture, in fact, it is sufficient to centrifuge the working volume and to collect the supernatant. By this way, cells do not have to be disrupted and the risk of changing the protein quality is less. Normally, the antibodies are collected from the supernatant by means of the affinity chromatography. With the use of protein-A on its stationary phase, in fact, this technique allows to pick up all the mAb in a very specific way.[44]

Since CHO cells have human-like post-translational modifications, they can avoid the immune reaction in the patient; [14, 15] in fact if a protein with different glycosylation is introduced in the human body, it would be recognized as foreign with negative consequences for the organism [16]. Different methods can be used to let these cells grow, but they are characterized by a different growth trend.

1.4 Culture Methods

Basically three processing methods are utilized: perfusion, fed-batch and batch. The difference between them is reflected in the way in which cells are fed.

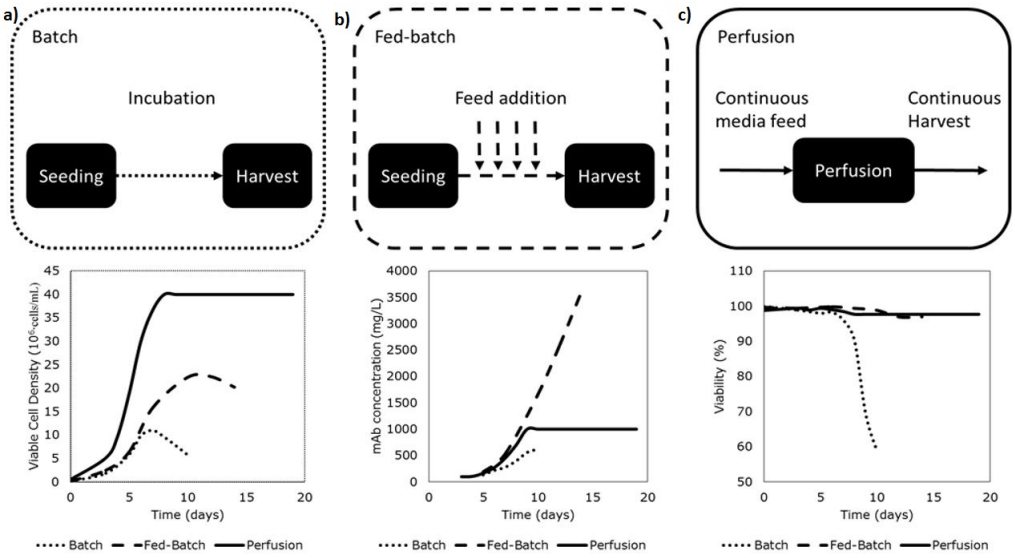

Figure 1.6: Comparison between the three culture methods

Batch culture, shown in Figure 1.6 a, is a closed system since at the start of the culture all the nutrients necessary for the cell growth are provided; no supplements are fed during the culture. Cell growth is here characterized by four phases:

1. during the lag phase the cells adapt to the medium in which they are and no increase of biomass is present;
2. during the log phase cells divide quickly with an exponential trend;
3. during the stationary phase a slowdown of the cells growth happens due to the reduction of nutrients;
4. during the death phase, cells start to die due to the shortage of nutrients.

This method is good to obtain quickly a high biomass but it cannot be applied for long time. Feeding is, in fact, favorable: there are some components, such as glucose and some amino acids, which are consumed in a fast way. By providing everything at the beginning of the culture, a particular attention must be paid on osmolality and oversaturation; if the quantity of glucose and amino acids is too high, the osmolality increases with a negative effect on the cells. Moreover, in this culture method, you have less control on what happens throughout the culture and, for this reason, it is the less used.

Fed-batch culture, represented in Figure 1.6 b, is a semi-open system since nutrients are added during the process but no medium is removed, so that the culture volume is increasing throughout the process. [21, 22]Based on cell growth patterns of fed-batch cultures, four culture phases are distinguished:

1. Start-up phase (L-Phase), from the inoculation day until the first day in which nutrients are added; it is close to a batch culture;
2. Exponential growth phase (G-phase): it lasts until the day in which the cultures reach the maximum cell density; biomass here increases in proportion with the quantity of nutrients putted within the culture;
3. Stationary phase (S-Phase), from the end of G-Phase until the culture viability is lower than 80%;
4. Death phase (D-Phase), is from the end of S-Phase until the end of the culture.

In this method is not the shortage of nutrients that determines the death of the cells, but there are other relevant factors that influence the growth slowdown, like the continuous production of toxic products that limit proliferation.

Perfusion culture, shown in Figure 1.6 c, is an open system in which fresh medium is continuously added while the old one is eliminated by two pump systems. It is divided into three phases;

1. in the first one (log phase) both of the pumps are not working and it is needed to achieve a great biomass (4 days). It is necessary to use some filters such as ATF and TFF which forbid to the cells to be removed together with the medium. This phase is like a batch culture;
2. during the start-up phase another increase of the number of cells; here both of the pumps start to work and fresh medium is added while the old one is removed (4 days);
3. the last phase is the stationary phase in which biomass is constant and both of the fluxes (the inner and the outer) do not change. Although biomass is constant, the cell age is not uniform. In fact, in order to maintain a steady state, a removal of cells is necessary [70]; this is the so-called bleeding stream and it is required to maintain a high viability [71]. With this process some young cells are removed, as well as some old cells. The duration of this phase is normally longer (12 days) than the others.

This method has different advantages:

- biomass is in a uniform physiological state;
- biomass production is constant in time;
- the residence time of the product is reduced, leading to less risk of aggregate formation;
- the produced antibodies stay less in contact with harvest product, and therefore, they have a more robust quality, since the process conditions are more uniform over time;
- the culture duration is much longer compared to fed-batch, so that the operation of cleaning and start-up are facilitated;
- there is the possibility to control the process setting the fluxes; in fact, by increasing the outer flux you terminate the culture, while by decreasing it you accumulate the medium. [17, 18, 19, 20]

On the contrary it features also an important disadvantage: the system is very sensitive and, since the process is quite long, the risk of contamination is quite high. Moreover, since the cells divide lots of time, there is the danger of having mutations in the cells; this factor is quite relevant due to transfected organism. Another important disadvantage is the high consuption of medium which is very costy.

The choice of the method depends on different factors, but the principal one is the metabolism phase in which the antibody is produced. If its production follow the cell growth it is called primary metabolism and the highest production will be in the log phase.

On the contrary, if the production start when the cell growth is ended, then it is called secondary metabolism, therefore the highest production will be in the stationary phase.

Both, fed-batch and perfusion processes, have been established at industrial scale. The benefits of fed-batch processing are fast development, simple format, ease of scale-up, and compatibility with large scale manufacturing of up to 20,000 L scale. [21, 22] Benefits of perfusion processing are high-volumetric output because of typically high-cell densities and short product exposure to cultivation conditions. The short product residence time makes perfusion technology particularly attractive for sensitive products, such as blood clotting factors or other proteins undergoing proteolytic attack or other forms of degradation under culture conditions. [18, 19, 20]

For relatively stable products such as monoclonal antibodies, fed-batch processing has become the dominant production technology at industrial scale. Fed-batch culture is currently the most common industrial process for CHO cell culture. For a fed-batch process volumetric productivity, product titer and quality are important outputs. These outputs are a function of viable cell density (VCD), specific productivity (q_P), and culture longevity, which in turn are highly influenced by the medium and feed composition. Selecting a suitable culture medium and feed platform is therefore pivotal for developing a fed-batch manufacturing process. [23]

1.5 Medium and Feed

The increasing demand for biopharmaceuticals produced in mammalian cells has led industries to enhance bioprocess volumetric productivity through different strategies. Among those strategies, cell culture media and feed development is of major interest. [24]

Nowadays different types of media are used; some are chemically defined medium, which means that they have a clearly defined composition; so that they have to be artificially composed. [25] The first chemically defined basal medium was the so called “Eagle’s Basal Medium” (BME). Other media were developed from this, like the Dulbecco’s Modified Eagle Medium (DMEM). Once the basal medium is prepared, some elements should be added due to the needs for most of the cells of additional supplements. [27] Chemically defined media typically contain 50-100 different components; each individual component can have an impact on different quantities, such as growth, viability, productivity and quality (aggregation, fragmentation, charge variants and glycan pattern). Missing just one essential component can have detrimental consequences on the performance of the cell culture. [76]

Some other media, instead, require serum and are called serum-based media; they are a natural mixture of vital component. Serum is a mix of growth factors, adhesion factors, hormones, transporters and mineral

element. It is often essential because it stimulates the growth of the cells, it provides the transport of different molecules and it stimulates the adhesion to the substrate; but, on the contrary, the use of serum is controversial for a number of reasons. First of all, the collection of fetal bovine serum (FBS), which is the most used, causes unnecessary suffering for the unborn calf [26]; secondly, seasonal and continental variations in the serum composition, produces considerable batch to batch variations. This causes phenotypical differences in cell cultures, resulting in variations of the results. Additionally, due to the risk of contamination, the use of animal products is strongly discouraged for production of new biological medicinal products [26]. However, different studies showed that the substitution of serum in many of the formulations resulted in a reduction of cell growth and productivity compared to those achieved with serum supplementation. [28, 29, 30]

Both types of media should satisfy central criteria:

1. To provide vitamins, ions and minerals to the cells
2. To maintain physiological parameters, such as high productivity and stability
3. Not to be toxic for cells

As well as for the media, the feed is chemically defined and it has to provide all the required supplements to the cells. Chemically defined basal media and feeds are currently the standard for CHO cell cultures. They should contain a balanced set of essential nutrients in a ratio that meets the demand of the cells for cell proliferation and production of the pharmaceutical protein. A feed is generally more concentrated than a basal medium to maximize culture volumetric productivity and product titer, as well as to avoid dilution effect. Studies have shown that the composition of culture media and feeds can influence cell growth, protein productivity [32], gene expression [33], product quality [13, 34, 35], and metabolism of lactate and ammonia. [33]

During the last years, several serum-free and chemically defined CHO-specific cell culture media have become available as well as some media systems that combine basal medium and feeds. [13]

1.6 Media Development

When defining a bioprocess for biopharmaceuticals production, the choice of a proper cell culture medium becomes a key factor. In recent years, research on cell culture media design has changed: definition of media for a wide range of cells and applications has shifted to defining specific formulations for specific cell lines, clone variants or process designs. [31]

Currently, process development of CHO cell culture usually starts with either commercially available or in-house recipes of culture media systems as a basis [32]. These media are designed based on different strategies to reach high volumetric productivities and product titers. [23] The optimal composition of a basal medium or a feed is highly dependent upon the basic type of CHO cell used, specific characteristics of the generated subclones, and type of product [13, 32]. Different cell line can be used and, since the glycosylation is cell line specific, it is important to choose the cell line depending on the desired recombinant protein quality. [77] Once the cell line is chosen, since different CHO cell types and clones

have different nutrient requirements, the medium and feed formulation and feeding strategies would have to be screened, designed and optimized [78]. Differences between clones derived from the same parental cell line may affect the choice of basal media and feeds in fed batch processes. For instance, some media are designed to boost cell growth to a high cell density [79], others are designed to extend the longevity of cells or to enhance cell specific productivity [56] as well as volumetric productivity. [23] Some others are, instead, designed to ameliorate the quality of the monoclonal antibodies. [51, 72]

Medium optimization is considered as one of the key contributors to the increase of titer. Over the last 10 years much progress was made in understanding cell nutrient requirements and the development of chemically defined media and feed formulations to better control cell metabolism. [36]

The culture medium has a significant impact on bioprocess performance, but high amino acid concentrations alone were not sufficient to ensure superior cell growth and high antibody production. [79] However, some key amino acids that were limiting in most media could be identified. Medium composition and development of an appropriate feed strategy is crucial, as excessive nutrient concentrations can be detrimental to cells causing toxicity and high osmolality. [37]

Different design approaches can be taken in order to define the best design. The objective of experimental design is to plan and conduct experiments in order to extract the maximum amount of information from the collected data in the smallest number of experimental runs, which means with a reduced experimental effort.

First of all, it is necessary to define a priori if it is wanted to test also the interactions among the factors or just one factor at time (OFAT). However, the traditional strategy used for medium development, relying on the variation of OFAT is laborious and time consuming [80]. Therefore, new technologies such as DoE and statistical analyses have been implemented. [81, 82, 83]

The basic idea is to change all relevant factors simultaneously over a set of planned experiments and then connect and interpret the results using mathematical models [84]. Different design strategy can be used, such as factorial designs, Plackett-Burman designs [85, 86], D-optimal designs, optimization design and mixture designs [56].

1.7 Clonal Cell Line Screening and Selection Process

To maximize productivity, stable cell lines that express the protein of interest must be established. At Merck Serono in Vevey, this process consists of several steps. It starts with the non-targeted integration of an expression vector which encodes the protein of interest and glutamine synthetase (GS). This enzyme provides a selective advantage to the recipient cells in the absence of glutamine in the environment. Transformed cells gain the ability of glutamine biosynthesis, an essential amino-acid for mammalian cell survival in culture. They will therefore grow while non-transfected cells will not. An additional selective pressure agent is added to select for the recipient cells with the highest GS activity, which are often the ones that amplified the incorporated plasmid. This compound is a glutamine synthetase inhibitor, methionine sulphoximine (MSX) [54].

At this stage, expression levels vary significantly between each protoclone. A wide screening assay is necessary to identify the most robust producers.

Cell lines are cultured in static conditions for several weeks and were ranked based on their capacity to grow and to produce the monoclonal antibody. The most promising candidates (around 60) are then transferred to agitated culture conditions and scaled-up in spin tubes. They are cultivated for a few weeks, so that they have time to adapt to the suspension phase before being evaluated in fed-batch with the standard conditions. The 15-20 best candidates were selected and a limited dilution was performed to ensure monoclonality. Another fed-batch culture followed by a stability study were then performed to evaluate productivity and robustness of the clones. At the same time, different fed-batch experiments were done to find the optimal environment and improve productivity, growth behavior and product quality.

Heterogeneity is a common observed phenomenon between different clones. Their behavior highly relies on culture environment (medium, feed and culture parameters). Thus, a change in these conditions may modify the ranking of the best producers.

Since the same platform parameters are used during the entire selection process, this raises the question whether the chosen clones are indeed the most performant ones or just the more adapted to those conditions. Some cell lines with a high productivity potential may be lost, because of a lower titer in the standard conditions than in more adapted ones. So, instead of relying on platform conditions, more variability shall already be introduced in the screening stage, to ensure better flexibility for later process development and anticipate limitation and other infavorable effects featured by a certain clone from early on.

The ideal approach to this issue would be to assess a maximum of conditions on a greater number of clones.

To do so, a modification of the process workflow has been considered and is being developed. Instead of scaling-up to cell culture in spin tubes, a transfer to 96-DWP would be preferred, as shown in Figure 1.7. In this high-throughput system, up to 80 fed-batches can be performed in parallel, allowing to test multiple culture conditions on several candidates at the same time.

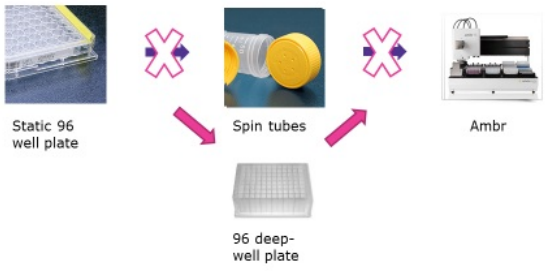

Figure 1.7: Modification of the process workflow

Culture media and feed can be optimized that way at an early stage of selection and cell lines evaluated in an environment which is adapted to them. [55]

One approach used for cell culture optimization is blending. This is an alternative to the laborious one factor at time strategy (OFAT) for testing different compounds, where only one component varies while all the other stay constant.

Blending uses design of experiments (DoE) to combine solutions and obtain new formulations with a wide range of concentrations. The effect and dose of many compounds can be tested that way in the medium or in the feed. Analytical tools are then used to predict the ideal mix for increasing cell line performance.[56]

Chapter 2

Materials and Methods

2.1 Cell Culture

2.1.1 Cell Line

Clonal cell lines derived from Chinese Hamster Ovary (CHO) cell line were used:

- CHO-S cell line expressing one monoclonal antibody (mAb A). Just one CHO-S clone was used in this study: P04. However, in experiment zero (see Section 2.5), also another clone was used, P05.
- CHO-K1 cell line expressing another monoclonal antibody (mAb B) . 4 CHO-K1 clones were considered: T11, T15, T18, T25

2.1.2 Media

Different media were used depending on the stage of the experiment. A proprietary chemically defined expansion medium was used to keep cells in culture. Unlike the production medium, it contains methionine sulphoximine (MSX), a glutamine synthetase inhibitor, to maintain a selective pressure. Two different proprietary chemically defined production media (Medium 1 and Medium 2) were used to promote monoclonal antibody expression. They are both platform media but one, Medium 2, does not contain sodium cloride ($NaCl$). During their preparations different components were added before the filtration, depending on the different experiments performed.

2.1.3 Cell Thawing

The different cell lines used in this project were stored in a frozen state at -80°C in a freezing solution containing DMSO. The cell vials (1.5 ml) were first thawed in a 36.5 °C water bath before the content was transferred in 30 ml of expansion medium and centrifuged at 200 g for 5 minutes. The supernatant was then discarded and the cells resuspended in 10 ml of fresh expansion medium. Viable cell density (VCD) was measured using the ViCELL™ XR cell counter (Beckman-Coulter), and expansion medium was added to adjust the density at 0.3×10^6 VCs/ml.

2.1.4 Cell Expansion

Cells were cultured in a 30 ml working volume at defined conditions (36.5°C, 80% humidity, 5% CO_2 and 320 rpm) within spin tubes. Spin tubes are small 50 ml capacity bioreactor tubes, shown in Figure 2.1. They contain openings in the cap that allow optimal gas exchange. The cells were then maintained in the exponential phase of growth. They were diluted in fresh expansion medium every two (0.3×10^6 VCs/ml) or three days ($0.2 \times 10^6 mioVCs/ml$) for at least 14 days before the beginning of a fed-batch experiment and for a maximum of 45 days.

Cell viability and density were assessed using the Vi-CELL™ XR cell counter. This device uses the trypan blue dye exclusion method in an automated way.

Figure 2.1: Spin tubes (source: [90])

2.2 Experiments Performed in 96-Deepwell Plates

96-deepwell plates (DWPs), shown in Figure 2.2, are normally used to screen many conditions in parallel. Each well is considered as a small 450µL working volume bioreactor. The device is composed by a plate and a three parts lid. The DWPs are covered with a silicon layer, a piece of permeable sealing membrane, and a steel lid: between them, a plastic film is presented.

Figure 2.2: Example of a 96-DWP (source: [91])

The steel part is perforated letting the gas exchange. The sterility is maintained thanks to a perforated silicon membrane and a plastic film that prevents the evaporation and the entry of pathogenic organisms, even allowing the gas exchange. To operate these experiments, the Biomek® FX (Beckman-Coulter) was used. It is a robotic platform, represented in Figure 2.3, able to pipette small liquid volumes (<250µL) in an automated way.

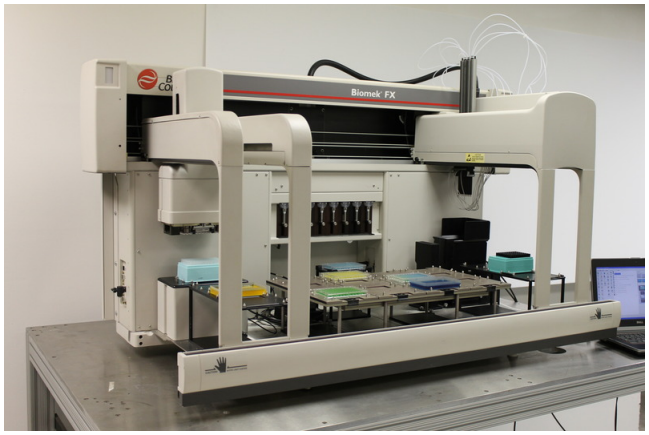

Figure 2.3: Biomek® FX (Beckman-Coulter)

The day before seeding, DWP were filled with 450 µL of media; then they were placed in the incubator for equilibration overnight.

At working day zero (WD00) cells were inoculated at a concentration of 0.2 viable million cells/ml into production medium for a total volume of 450 µL per well. The seeding method included first the removal of medium, in order to keep a working volume of 450 µL.

The cells were maintained at defined conditions (36.5°C, 90% humidity, 5% CO_2 and 320 rpm), inside

a shaker incubator. Every two (WD05-07-12-14) or three days (WD03-10), aliquots of 30-40µL were collected to evaluate viable cell density and viability of each culture using the Guava® EasyCyte™ Plus Flow Cytometry System (Merck Millipore). This device is reported in Figure 2.4.

Figure 2.4: Guava® EasyCyte™ Plus Flow Cytometry System (Merck Millipore)

The experiments performed in 96-DWPs are the first four.

2.2.1 Feeding and IPCs (In-Process Controls)

Feeding consisted in three different solutions:

- a glucose solution (400g/L)
- a proprietary chemically defined feed (Feed 1)
- an alkaline solution containing L-Cysteine and Tyrosine

Feed was added on WD03, WD05, WD07 and WD10 for a 14 days experiment according to Table 2.1.

WD	Solution 1		Solution 2		Glucose added	Feed 1 added
	Name	Volume	Name	Volume		
03	Feed 1 diluted	40 µl/ml	Mix GCT	40 µl/ml	4 g/l	3%
05	Feed 1	60 µl/ml	Mix GCT	40 µl/ml	4 g/l	6%
07	Feed 1	60 µl/ml	Mix GCT	40 µl/ml	10 g/l	6%
10	Feed 1	60 µl/ml	Mix GCT	40 µl/ml	10 g/l	6%

Table 2.1: Feed Protocol in 96-DWP

Due to stability problem of the solution and to low volume needed to be added in each well of the plate, the glucose solution and the alkaline one are first mixed together with water reverse osmosis (WRO) according to Table 2.2 :

WD	Solution 1		Solution 2		Solution 3	
	Name	Volume	Name	Volume	Name	Volume
03	FEED Cys/Tyr	1.875 ml	FEED Glucose	12.5 ml	WRO	35.625 ml
05	FEED Cys/Tyr	3 ml	FEED Glucose	10 ml	WRO	27 ml
07	FEED Cys/Tyr	3 ml	FEED Glucose	25 ml	WRO	12 ml
10	FEED Cys/Tyr	3 ml	FEED Glucose	25 ml	WRO	12 ml

Table 2.2: Mix of Cys/Tyr and Glucose solutions

Before each feed addition, a sample of 30-40µL was collected for viable cell density, viability and productivity analysis. Table 2.3 summarizes the timing for the IPC and feed.

WD	VCD/Viability	Feed	Titer
03	X	X	
05	X	X	
07	X	X	
10	X	X	X
12	X		X
14	X		X

Table 2.3: IPCs and feed timing

Viability and viable cell density were evaluated using the Guava® EasyCyte™ Plus Flow Cytometry System (Merck Millipore), which allowed to determine viability and absolute cell count. Samples were diluted into a dilution buffer to obtain a density between 0.1 and 0.5×10^6 cells/ml in each well.

Titer was quantified by the Octet QK (Pall ForteBio), which is shown in Figure 2.5. It uses Protein A sensors. Each sample was diluted 10 times in a dilution buffer (PBS pH = 7.4, BSA 0.1 g/L, Tween 20 at 1%) on WD10 and 20 times on WD12 and at WD14. Regeneration buffer was glycine 1.5 M and neutralization buffer was the same dilution buffer.

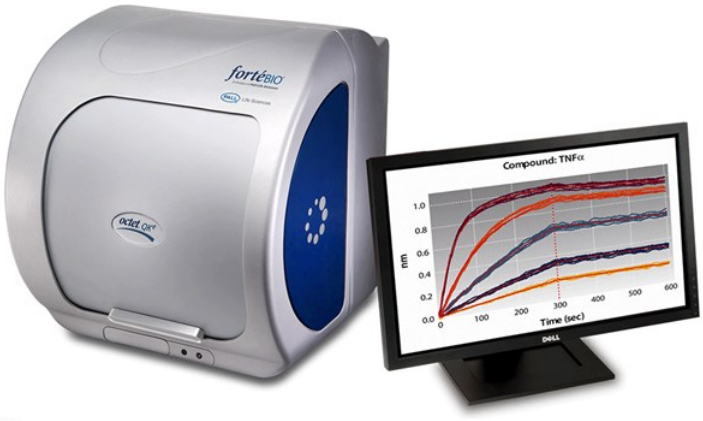

Figure 2.5: Octet QK (Pall ForteBio)

The volumes sampled for each IPC are represented in Table 2.4.

WD	Guava			Octet		
	Buffer [µl]	Sample [µl]	Dilution factor	Buffer [µl]	Sample [µl]	Dilution factor
03	72	8	10			
05	76	4	20			
07	116	4	30			
10	156	4	40	180	20	10
12	156	4	40	190	10	20
14	156	4	40	190	10	20

Table 2.4: Dilution protocol for Guava and Octet analysis

2.2.2 Media Blending Design

The design of the experiments done were made using MATLAB R2015b and Design Expert. In order to have a good set of experiments the design was always made according to the selections obtained after an accurate DTs analysis of a previous set of results (See Section 2.4.2) . The design was, indeed, driven by rational considerations following the results obtained until a certain moment, which was usually WD14. Only when the following experiment had to be started on the WD14 of the previous experiment, the design was done according to the results obtained at WD10 or WD12, based on the required time to set the experiment.

2.3 Experiments Performed in Shake Tubes (ST)

Shake tubes are the same tubes that are used for cells expansion. For a fed-batch experiment conducted in these small bioreactors, the working volume is 30 ml. The day before the start of the experiment, media are blended manually under the laminar flow hood; STs are then put into the incubator overnight for equilibration.

At WD00, cells are inoculated at a density of 0.2 million of viable cells/ml. A quantity of medium was first removed in order to maintain a working volume of 30 ml.

The experiments conducted in STs are the fifth and the sixth one.

2.3.1 Sampling and IPC

At days 03, 05, 07, 10, 12 and 14 probes were taken manually under the laminar flow hood. The quantity removed was less than 2.5 ml.

To determine the viable cell density the ViCells was used. Starting from WD05, samples were diluted in PBS buffer. The dilution factor was different according to the WD. In Table 2.5 the different volumes of buffer and sample are reported.

ViCell		
WD	Buffer [µl]	Sample [µl]
03	0	600
05	300	300
07	400	200
10	450	150
12	450	150
14	450	150

Table 2.5: Dilution factor for the ViCell

Samples were then centrifuged for 5 minutes at 2000 rpm and the supernatant was filtered with a syringe filter unit (0.22 µm). The filtered solutions were then used for the other analysis. Table 2.6 summarizes the timing for IPC.

WD	ViCells	Nova	Octet
03	X		
05	X	X	
07	X	X	
10	X	X	X
12	X	X	X
14	X	X	X

Table 2.6: Time schedule for IPC analysis

2.3.2 Sampling and Feeding

In addition to cell density and titer, the metabolites' profiles was calculating using the Nova Bioprofile 100+ (Nova Biomedical, Waltham, MA). These analysis had the goal to measure the concentration of glucose, and hence, to calculate the quantity of the secondary feed. This allows to avoid glucose overfeeding. The protocol for the feeding strategy is reported in Table 2.7.

WD	Solution 1		Solution 2		Solution 3	
	Name	Volume	Name	Volume	Name	Volume
03	FEED Cys/Tyr	1.38 µl/ml	FEED Glucose 400g/L	4 g/l	Feed 1	29 µl/ml
05	FEED Cys/Tyr	2.76 µl/ml	FEED Glucose 400g/L	7 g/l	Feed 1	58 µl/ml
07	FEED Cys/Tyr	2.76 µl/ml	FEED Glucose 400g/L	10 g/l	Feed 1	58 µl/ml
10	FEED Cys/Tyr	2.76 µl/ml	FEED Glucose 400g/L	10 g/l	Feed 1	58 µl/ml
12	FEED Cys/Tyr	N/A	FEED Glucose 400g/L	7 g/l	Feed 1	N/A

Table 2.7: Feed protocol in ST

According to Nova results, the difference between the wanted and the calculated quantity is fed.

The experiments conducted in shake tubes had the goals to validate the results obtained in the 96DWP.

2.4 Statistical Data Analysis

2.4.1 Box Plot

For this work, it was chosen to represent all the results with box plots. A box plot is a graphical representation of the distribution which is represented by the mean and four quartiles. In a typical box plot different part can be seen. Figure 2.6 shows an example of box plot, in which four regions are visible:

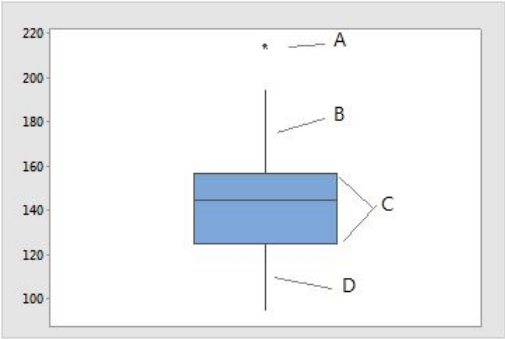

Figure 2.6: Example of Box Plot

- A: outlier (*): observation that is beyond the upper or lower whisker. It is a value that is more than 1.5 times the interquartile range away from the top or bottom of the box;
- B: upper whisker: represents the upper 25% of the distribution (excluding outliers);
- C: interquartile range box: middle 50% of the data;
- D: lower whisker represents the lower 25% of the distribution (excluding outliers);

Boxplots can help the reader in understanding the distribution of a set of data and in comparing several distributions.

2.4.2 Decision Trees

Decision tree (DT) is a systematic and automated tool providing a hierarchical order of binary decisions on the input variables. [75] It separates the output variables into two subgroups which are as heterogeneous as possible. DTs attempt to find a strong relationship between input values and target values in a group of observations that form a data set. When a set of input values is identified as having a strong relationship to a target value, then all of these values are grouped in a bin that becomes a branch on the DT. These groupings are determined by the observed form of the relationship between the bin values and the target. The root node of tree corresponds to the entire data space. Partitions of the space are associated with descendants of the root node. The leaves of the tree, or terminal nodes, correspond to subspaces which are not further partitioned. DTs can accomodate categorical and continuous types of input values with the porpouse of forming the DT. [74]

The basis for each partition is represented by a split of node into two nodes: the left node and the right one. Splits consist of evaluating, for each case in the node, one condition (for example Factor1 $>$ 150) . If the condition is true, the case "goes" to the left, else it "goes" to the right. All the possible decision criteria from a Z matrix are screened to find a decision criterion which is returning two subgroups. Each terminal node contains members of one class only. To classify a new case, imagine a "drop" that “falls” through the tree; starting from the root node, the case (drop) follows a "path" determined by the splitting rules and "falls" into a terminal node. The label of that node is assigned to the data point. The CART (Classification and Regression Trees) program implements such a tree-structured classification procedure by quantifying maximal class separation and selection of splits. [73]

As the resulting subgroups significantly decrease in size, such an analysis si very prone to overfitting and a cross-validation procedure (called pruning for DTs) is needed . For general conclusions, it is therefore important to prune (cut back) the tree to a level, which is likely to be universally valid. [72] In this work, 10-fold cross-validation was applied; however, before concluding it was decided to evaluate the results with different K-fold cross-validation and, in some cases, it was chosen to take another value of K due to better selections. All the selections were obtained with decision trees analysis.

2.4.3 Evaluation of Repetition Errors

In all the experiments performed in this work, some conditions were replicates in order to estimate the repetition errors. This quantity was calculated in two different ways.

The first one is based of the coefficient of variation (CV) and it is computed with Equation 2.1:

$$CV = \frac{SD(x)}{M(x)} \tag{2.1}$$

Where$SD(x)$ is the standard deviation, $M(x)$ is the mean and x is a vector containing the replicate results.

The CV shows the extent of variability in relation to the mean of a set of data. The more the variability is, the higher is the standard deviation and so the CV. On the contrary, if the variability of the data is low, the standard deviation will be small, leading to a low CV. The CV is usually expressed as a percentage.

The second one is more insightful as it does not consider a single measure using the standard deviation but it is capable to distinguish good and bad repetition experiments. It is explained with Equation 2.2:

$$Err(x) = \frac{\mid x_i - x_{i-1} \mid}{M(x)} \tag{2.2}$$

Where$M(x)$ is the mean and x is a vector containing the replicate results. The repetition errors measured with this equation are visualized with a box plot; hence the mean is considered to evaluate it. As mentioned before in this work both equations were considered and a graphic representation of the repetition errors is reported in Chapter 3 for every experiment.

2.4.4 Decision Variables

In this work, three variables were taken into account as the most important. The first one is the titer [mg/ml] which represent the quantity of mAb produced by the cells. As mentioned before, it is measured with the Octet. The second important variable is the IVC (integral of the viable cell density) which is calculated starting from the VCD (viable cell density) according to Equation 2.3:

$$IVC_{WD_i} = IVC_{WD_i - WD_{i-1}} + \frac{(VCD_{WD_i} + VCD_{WD_i - WD_{i-1})}}{2} \times (WD_i - WD_{i-1}) \tag{2.3}$$

Finally, the third quantity is the PCD (specific productivity) and it is computed according to Equation 2.4:

$$PCD_{WD_i} = \frac{Titer_{WD_i} - Titer_{WD_i - WD_{i-1}}}{0,5 \times (VCD_{WD_i} - VCD_{WD_i - WD_{i-1}})} \tag{2.4}$$

It correlates the quantity of protein producted to cell density, and for that it can be useful to compare different experiments.

Due to the fact that titer is measured starting from WD10, PCD results are available only for WD12 and 14, so it can be used just in the end of experiment. Moreover, since it is calculated from the titer and the VCD results, the PCD error comprends the errors of both the two quantities; this means that it can be more noisy compared to the errors calculated on IVC and titer.

2.5 Starting Point of the Experiments

The point of start of this master project is an experiment conducted in 2016 in the laboratory of Bio Process Sciences (BPS) at Merck Serono in Vevey [46]. The aim of this experiment was to see if media blending could generate diversity in the growth and titers of different clones.

Actually, all the steps of the clone selection process described in section 1.7 are perfomed in the platform medium; but nothing can assure that those selected clones are indeed the best producers. In fact, if the environment is changed, it can be possible that some clones produce better than the selected ones.

In order to see if this hypothesis was true, 160 conditions on 5 different clones were tested. The cells were two CHO-S clones (P04 and P05) and three CHO-K1 clones (T11, T15 and T18). 47 components were grouped in 11 groups according to the literature and to the previous experiments. Figure 2.7 summarizes the different groups and their levels.

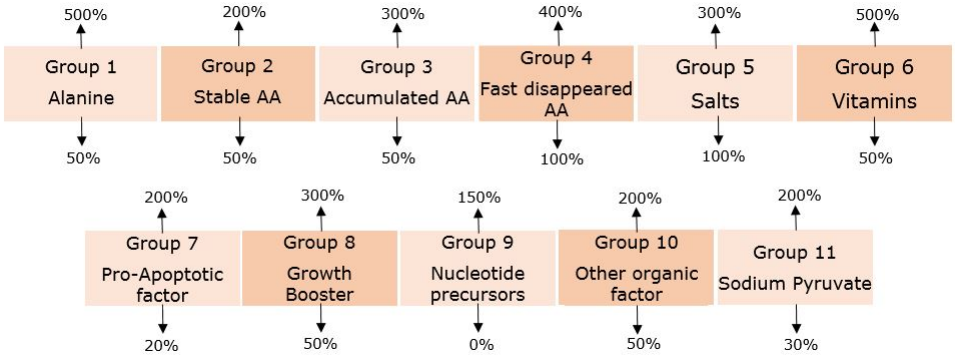

Figure 2.7: Different groups with their respective levels

Consumption data were available for amino acid and were used to identify four of the eleven groups. For the other factor, since no experimental data were available, it was decided to group them according to the results obtained in the literature. [48, 49, 50]

Since every group is composed of different molecules, the concentration was expressed in percentage of the concentration in the platform medium.

The first group (Figure 2.7, Group 1) consisted of only one amino acid that has a different profile from all the others (alanine). It accumulated from the beginning of the experiment for the first week, and was imported into the cell during the second part of the fed-batch. Since the profile is very atypical and the explanation of this is not known, a large range of concentrations was tested (50-500%).

Many amino acids profiles stayed constant during all fed-batch. They were apparently at the equilibrium in between consumption and feeding. Those were grouped in the second group (Figure 2.7, Group 2). A small decrease or increase compared to the platform medium was tested (50-200%).

Another group of amino acids (Figure 2.7, Group 4) was not detected any more after the first three days of experiment, they were probably not fed enough or accumulated in the cell. In all cases, they were not detected in the medium. It was logical to test only an increase in this group, so a range from 100 to 400% was tested.

The opposite profile was also observed for some amino acids (Figure 2.7, Group 3): an accumulation in the extracellular medium. They may be secreted from the cells, or maybe the feeding rate exceeds the consumption rate, which creates a balance in favour of the accumulation. The low level was 50% to test whether it would beneficial to have less accumulation.

Regarding the other components, such as vitamins and trace elements, they are not monitored during fed-batches and no information on their consumption is available. Literature was used to group these molecules. The salts that have an osmotic role, such asKCl and NaH_2PO_4, and that can be involved in transport and many other cellular functions, were grouped together (Figure 2.7, Group 5). This group was only increased compared to the platform media (100-300%).

Amongst vitamins, 3 could have a pro-apoptotic role (Figure 2.7, Group 7) and were grouped to test a lower concentration (20-200%).

Other vitamins (Figure 2.7, Group 6) were grouped and a large range was tested as their effect was

unknown (50-500%).

Some nucleotide precursors (Figure 2.7, Group 9) were traditionally added in the medium to boost the growth of the cells transfected [47]. However, since it is not relevant to supplement these molecules, only a decrease is evaluated (0-150%).

One compound could be growth booster (Figure 2.7, Group 8) and so it was tested to see if higher concentrations could increase productivity (150-300%).

The other organic compounds (Figure 2.7, Group 10) were grouped and a range of 50-200% concentration was set.

Finally, sodium pyruvate was considered as a group (Figure 2.7, Group 11).

All remaining components such as trace elements and some additional molecules were not classified. Their concentration stayed the same as in the platform medium (100%).

The composition of the 11 groups is reported in Table 2.8.

Group	Components	Group	Components
G1	Alanine	**G4**	Asparagine
G2	Arginine		Methionine
	Cysteine	**G5**	Phosphate
	Histidine		Potassium Chloride
	Leucine	**G6**	D-Biotin
	Proline		Niacinamide
	Threonine		Pyridoxine
	Tryptophan		Thiamine
	Tyrosine		Vitamin B12
	Valine	**G7**	Folic Acid
G3	Aspartic Acid		D-Pantothenic Acid
	Glutamic Acid		Riboflavin
	Glycine	**G8**	Putrescine
	Isoleucine	**G9**	Hypoxanthine
	Lysine		Thymidine
	Phenylalanine	**G10**	Ethanolamine
	Serine		Choline Chloride
G11	Pyruvate		Myo-Inositol

Table 2.8: Composition of the 11 groups

The design of experiment was made with Design Expert. The 160 conditions were equal for all the five clones.

The data analysis of this experiment was part of the work so that the results will be reported in Section 3.1.

2.6 Description of the Experiments Done

The experiment conducted in this work are designed according to the results obtained in the previous experiment described in section 2.5. The only experiment that is not related to the reference one is the

first one. 6 experiments were performed in this work. The first four were conducted in 96-DWPs; the last two in STs.

2.6.1 Experiment 1

The first experiment was done in order to test the replicate errors. All the devices that are used in fact, can be subjected to unpredictable variables that can lead to an increase of the errors. It is hence necessary to test equal conditions different times in order to see if the devices are reliable. The first experiment was performed with two different clones within one plate:

- one CHO-S clone (P04)
- one CHO-K1 clone (T11)

The total 80 experiments were done with platform medium and platform feed (40 replicates for each clone). The medium and the feed used in this experiment were respectively Medium 1 and Feed 1.

2.6.1.1 The Variability of 96 DWP Culture System

As shown in Section 3.2, different membrane and different guava plate coating strategy leads to an important variability in the results.

With the goal to reduce this variability, a study was conducted at Merck: different coating method and different membrane were tested.

Plates coated by using BSA-contained solution (Octet Buffer solution) were tested [45] and the different coating methods were compared. First, 180 µL of Octet buffer were transferred into each well of different Guava plate by using the Biomek; then plates were put in corresponding place for different experiments based on the information in Table 2.9. After that, the buffer solution was removed and plates were dried under the laminar flow until no liquid was observed in each well (white small crystals could be seen in the bottom of the wells).

	Coating Time / Temperature	Washing by WRO	Drying Time
1	1 min / Room Temp., Laminar flow hood	No	10 min
2	5 min / Room Temp., Laminar flow hood	Yes	10 min
3	30 min / Room Temp., Laminar flow hood	No	10 min
4	30 min / 37°C, Incubator	No	10 min
5	60 min / 37°C, Incubator	No	10 min

Table 2.9: Different coating method

Five spin tubes of K1 cells were then prepared and their VCDs were tested by using ViCell. After that, cells in one spin tube were poured into a flat plate under laminar hood and transferred into a coated guava plate. VCD was then measured by Guava device. The maximum, the minimum and the average values of VCD were cataloged and compared with the results obtained with the ViCell. The results of this study are reported in Section 3.2.1.1.

Regarding the different type of membrane, a comparative experiment was carried out by using two different silicon layers, reported in Table 2.10. [45] Two different types of cells were tested for 14 days cultivation under the standard procedure. VCD and viability of different plate during the process were recorded.

Parameters	Dark gray	White
Size holes in silicon layer	1.5 × 5 mm	1.5 × 5 mm
Exchange of headspace air	1 mL / min	1 mL / min
Evaporation rate (30°C, 50% humidity)	22 µL/well/day	22 µL/well/day
Remark	Hard[1]	Soft

Table 2.10: Different silicon membrane

The results of this study are reported in Section 3.2.1.2. Based on the results obtained on the study here described, it was chosen to perform all the experiments with the same type of membrane on the lid, the white and soft one. Regarding guava plate coating, it was decided to keep the one with the best results, which is the number 5 in Table 2.9.

2.6.2 Experiment 2

Since the grey membrane was used in the first experiment, and since the guava plates were not coated until the WD07, it was not easy to confirm the reliability of the devices. Hence, the second experiment was split into two parts: one has the aim to test the replicability; the other one has the goal to test the groups that were considered significant.

It was decided to use the P04 clone. Two plates with equal conditions were tested; in particular, in each plate 35 experiments were done with platform medium and platform feed. The other 45 were dedicated to the media blending. According to the results obtained in the experiment 0 (see Section 3.1) the groups that were important for the clone P04 were the fourth (fast disappeared amino acids), sodium pyruvate and the group of the stable amino acids.

Since the salts group (G5) seemed to have a positive effect on all the other clones, it was chosen to include it in the design; in particular, due to the presence of just two salts, a split into potassium chloride and sodium phosphate was made.

Moreover, due to the lack of some amino acids in stock, it was decided not to test the group 2; this choice was taken also because it should have been necessary to prepare media from scratch in order to test concentration lower than 100%; since the preparation of media from scratch is time consuming, it was decided to avoid it. The G4 was split into Asparagine (Asn) and Metionine (Met).

The solutions were prepared starting from Medium 1. Before its filtration, the different aliquotes of media were enriched in some components. Five stock solutions were then prepared, each with a component at 250% level. Due to dilution factor, it was possible to test just one component at time at the maximum level. For that, the design of this experiment was divided as follow:

- 35 experiments to test replicability (platform medium and platform feed)
- 5 experiments with each component at 250% level

- 5 experiments with each component at 200% level
- 5 experiments with each component at 150% level
- 9 experiments with two components at 175% level
- 9 experiments with 3 components at 150% level
- 12 experiments with 150 units randomly distributed across 5 components

The entire design of the 45 media blending conditions is reported in Appendix A.

2.6.3 Experiment 3

Experiment 3 was done with another type of cells because the CHO-S cells did not respond well to the environment changing. Among the available CHO-K1 clones, it was chosen to use three clones with completely different behavior also based on the experiment 0. Based on the experiments conducted at Merck [51] and on the results obtained (confidential data are not reported), the chosen clones were:

- T18: among all the clones, this is the one with the best growth profile and the best productivity
- T15: among all the clones, this is the one with an average behavior
- T25: among all the clones, this is the one with the worst growth profile and the worst productivity

Three plates with equal conditions were run in parallel and each plate was devided as follow: 5 replicates with platform medium and platform feed; 45 media blending to test the effect of the most significant group; 30 feed blending to see if benefits are presented compared to the media blending. The selections obtained in the previous study are reported in Table 2.11 for T15 and T18.

	T15			T18		
Group	G3	G5	G8	G2	G5	G8
TITER	$\geq$133.4	$\geq$157.0			$\geq$146.9	$<$169.9
IVC			$\geq$77.9	$<$186.5	$<$186.5	$\geq$150

Table 2.11: Selections obtained for T15 and T18

Regarding the T25, no selection are available. The design of the media blending experiments is reported in Appendix B.

The design of the feed blending experiments is reported in Appendix C.

2.6.3.1 Media Preparation

For this experiment, five stock solutions were prepared starting from Medium 2. The advantage of using the Medium 2 instead of the Medium 1 is that the first one does not contain any sodium chloride ($NaCl$). This permits to adjust the osmolality every time before filtering. After adding all the required amino acids, the osmolality and the pH were measured and adjusted. The goal was to have all the solutions at

300 mOsm and at pH 6.85. In experiment 2, media were prepared starting from Medium 1; in this case, however, just one component was added in each solution and at a concentration of 250%. This permitted not to have a consistent increasing in osmolality. On the contrary, in experiment 3, the solutions are more concentrated and the number of components to add is higher.

All the solutions were prepared at a concentration that allows to have the highest level of all the components, even when all the five were blended. All the solutions, except the one contained the amino acids of the G2, were then prepared at 850% level. Since the highest level of G2 was 200%, the stock solution was prepared at 600% level.

The corresponding calculation is reported below:

$$C_f \times V_f = \sum_1^N C_n \times V_n \tag{2.5}$$

Where:

C_f and V_f are the final concentration and the final volume, respectively; C_n and V_n are, respectively, the concentration and the volume of each solution present as part of the final one. N is then the number of the single solutions.

So in our case, the final concentration is 200 for the G2 solution, and 250 for all the others. If all the five solutions are blended, there will be one part at a high concentration which has to be calculated and four part at a concentration of 100.

So Equation 2.5 becomes:

$$250 = 0,2 \times C_n + 0,8 \times 100 \tag{2.6}$$

$$C_n = \frac{170}{0.2} = 850$$

Analogously:

$$200 = 0,2 \times C_n + 0,8 \times 100 \tag{2.7}$$

$$C_n = \frac{120}{0.2} = 600$$

2.6.3.2 Feed Preparation

Regarding the feed, the same counting was done. So in order to be able to achieve the highest level (150%) even when all the solutions are blended, the stock solutions must be at a level of 350%.

In this case, however, the solubility of some amino acids gave some problem. Despite it was checked before starting the preparation of the solutions, some critical amino acids were not soluble in the feed. In order to solve this problem, some solutions were warmed in the incubator at 36°C.

For two amino acids, Leucine (Leu) and Isoleucine (Ile), even an increase in temperature was not sufficient. For this reason, it was chosen to prepare two solutions of WRO enriched with Leu and Ile respectively.

Due to solubility limitations, the solutions were prepared at the maximum level possible, that is 300% for Leu and 350% for Ile.

For this reason all the conditions in which Leu (G2) and Ile (G3) were present, were modified. In particularly, with the Biomek, a 96-DWP was filled with the blended feed based on the design. After that 45 µl were removed from each well, and other 45 µl of a water solution was added as followed:

- 45 µl of WRO were added in those wells in which both, G2 and G3, were not presented
- 45 µl of a water solution containing Leu at 300% was added in those wells in which G2 was presented
- 45 µl of a water solution containing Ile at 350% was added in those wells in which G3 was presented
- 45 µl of a water solution containing Ile at 175% and Leu at 150% was added in those wells in which both, G3 and G2, were presented

By doing that, all the feed was diluted at 90% level and the design was modified for Leu and Ile. In particularly, when both, Leu and Ile, were present, their level became 105 and 107.5 respectively. When just one was present, the level of Leu became 120, while the one of Ile became 125.

2.6.3.3 Cysteine Tyrosine Solution

In G2, two critic amino acids were present: cysteine (Cys) and tyrosine (Tyr). Regarding the feed, these two amino acids are usually fed in a different solution (see section). So, in order to achieve 150% level of these components, it is necessary to increase their quantity in the Cys/Tyr solution. However, during the preparation of this solution, a precipitation happened as soon as the quantity of Cys passed the 100%. moreover, a solubility limitation of the Tyr, let to achieve maximum 120% level.

Due to these issues, it was decided to keep the cys at 100% level ant the Tyr at 120% level.

2.6.3.4 Potassium in the Feed

Since potassium (K) is not normally fed, the question about how to define the 100% level arose. Two were the possible strategies:

1. Consider the 100% as the quantity of K present in the medium
2. Assume that the ratio between the 100% in the feed and the 100% in the medium is the same that for an analog compound. Since the analog compound is phosphate (PO_4), that has a ratio $PO_4^{feed}/PO_4^{medium}$ of 13.8, the 100% for K becomes the quantity of K in the medium multiplied for 13.8.

At the end, based on some discussions, it was thought that the most logical thing was to assume the 100% level as the one present in the medium.

Since K is not fed, it is supposed that the quantity of K in the medium is enough for all the 14 days of experiment. Since it was demonstrated that for K1 cell line this is not true, it was decided not to increase too much the quantity of K that was fed.

2.6.4 Experiment 4

Analogously to experiment 3, experiment 4 was conducted with the same clones and with the same modality.

Three plates were then seeded. In this case, however, it was decided to investigate in a combination of media blending and feed blending. So, this time, the plate was distributed as follow:

- 20 conditions in which just media blending was done
- 56 conditions in which both, the feeds and the media, were blended
- 4 replicated conditions with platform medium and platform feed

Since it was performed right after experiment 3, the design was done based on the results at WD10 instead of on WD14.

One important question to mark, is that based on the result obtained in experiment 3 (see section), K seems to have an important role. In fact, as mentioned before, it is not present in the platform feed; because of this, cells affected this limitation. For this reason, the level of K in the platform medium was increased at 150%.

The design of the experiment 4 is reported in Appendix D and in Appendix E.

Regarding the medium and the feed, the same solutions of the experiment 3 were used. So, also in this case, the feed was diluted. However, from the results obtained in the experiment 3 (see section), it was noticed that G3 in the feed didn't have a special benefit. For this reason the feed was diluted at 90% but in this case just the Leu changed its level at 120% instead of 150%.

2.6.5 Experiment 5

Starting from the experiment 5, the main goal changed. The goal of experiments performed in 96-DWP, was basically to test different environments in order to obtain a set of conditions for each clone that were better than the platform one.

On the other hand, for the last two experiments, the goal was to validate the results obtained in 96-DWP. For these two experiments, it was chosen to work with STs, due to their better reliability. Moreover, due to a limitation in the number of conditions, it was not possible to test different clones. For this reason, just one clone was chosen: T18. This clone was considered to be the best one for different reasons; first of all, it is the most stable one, in the meaning that, in all the experiments performed, it had an acceptable replicate error. (see Chapter 3)

In fact, also in experiment 0 [46], 16 conditions were replicates two times; and, by analysing the coefficient of variations (CV), it emerged that for T15 they were higher than for T18.

Secondly, T18 is the clone that respondes better to environment changing and that produces more. T25 was priorly excluded due to its low productivity and growth.

To validate the results obtained in 96-DWP, 36 conditions were design based on the results previously obtained. Of these 36, 24 were dedicated to validate the selections obtained, the other 12 were used to test different groups that were not considered in 96-DWP.

The design of the experiment is reported in Appendix F.

In the 12 more conditions, it was chosen to split the G3 which was composed of 7 amino acids. The split was done based on the nature of the amino acids: 4 apolar amino acids were separated from the 3 polar ones. Secondly, since in the selections of T18 also G6 was present, it was decided to introduce also the vitamins group in the design.

2.6.5.1 Preparation of the Media and the Feed

Also in this case, all the solutions were prepared starting from the Medium 2, which let to adjust the osmolality at the end before filtering. For this design, 5 different group were presented. So, based on Equation 2.5 in paragraph 2.6.3.1, the concentration of all the groups are reported in Table 2.12.

Group	Concentration
Group 3P	600
Group 3A	600
Group 5 (K)	1100
Group 5 (PO_4)	600
Group 6	1100

Table 2.12: Concentrations of all the groups

Regarding the feed, only K was added at a concentration of 200%, assuming again the quantity of K in the medium as the 100% level.

2.6.6 Experiment 6

For this experiment, 48 different conditions were tested. In this case, there were three goals:

- redo some conditions of the experiment 5, that encountered early cell death due to glucose limitation
- tuning the best level of K
- validate the results obtained in 96-DWP

The design of the experiment is reported in Appendix G.

Due to glucose limitations obtained in the experiment 5 (see Section 3.7), the feed protocol was changed as in Table 2.13:

WD	Solution 1		Solution 2		Solution 3	
	Name	Volume	Name	Volume	Name	Volume
03	FEED Cys/Tyr	1.38 µl/ml	FEED Glucose 400g/L	4 g/l	Feed 1	29 µl/ml
05	FEED Cys/Tyr	2.76 µl/ml	FEED Glucose 400g/L	7 g/l	Feed 1	58 µl/ml
07	FEED Cys/Tyr	2.76 µl/ml	FEED Glucose 400g/L	12 g/l	Feed 1	58 µl/ml
10	FEED Cys/Tyr	2.76 µl/ml	FEED Glucose 400g/L	10 g/l	Feed 1	58 µl/ml
12	FEED Cys/Tyr	N/A	FEED Glucose 400g/L	7 g/l	Feed 1	N/A

Table 2.13: Changing in the feed protocol

Even if the NOVA analysis was in program for WD10, it was decided to feed anyway 10 g/l in each shake tube. This decision was taken because, from the first results of the NOVA, it seemed that some tubes were already at the limit. For that, it was decided not to have any dead time and to risk to have an overfeeding of glucose.

Moreover, in this experiment, it was decided to analyse also the amino acids presented in the culture. A sample of 1 ml was taken at WD00, 03, 05, 07, 10, 12 and 14 from each shake tube; it was centrifuged at 2000 rpm for five minutes and then filtered. It was then stored at -20°C for a Biacore analysis.

Another important question to mark is that in this experiment it was decided to move three samples into another incubator with the temperature set at 33°C instead of 36°C. This shift in temperature was done at WD05.

Chapter 3

Results

3.1 Results of Experiment Zero

As mentioned in Section 2.5, the results of this experiment constitute the point of start of this thesis work. The overall distribution concerning the titer at WD12 is reported in Figure 3.1.

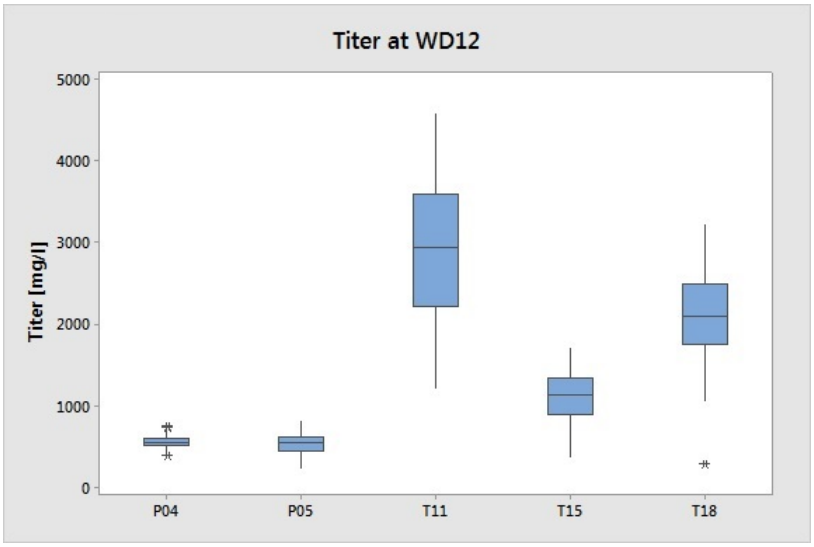

Figure 3.1: Experiment 0: Box Plot of the titer at WD12 for all the five clones

As you can see from the boxplot, the obtained ranges for the titer of all the K1 clones (T11, T15 and T18) are really wide; hence, there are some environments that stimulate more the productivity of the cells. Regarding the S clones (P04 and P05), the ranges are less wide because they respond less to the environmental changing.

Figure 3.2 shows the distribution concearning the IVC at WD12.

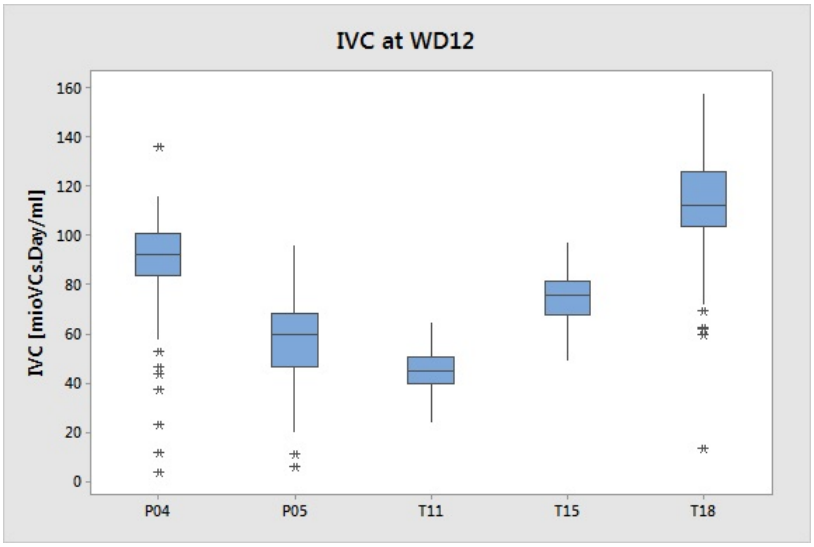

Figure 3.2: Experiment 0: Box Plot of the IVC at WD12 for all the five clones

In this case, the ranges are less wide for K1 clones (T11, T15 and T18) compared to the ones of titer, but wider for S clones (P04 and P05). This means that for K1 clones it is less difficult to improve the production of mAbs than to increase the VCD, as well as the IVC. Instead for S clones, it seems to be easier to improve cell growth than titer.

The entire data set of the results was then analyzed with DTs analysis, so that different selections for the titer and for the IVC are obtained. Figure 3.3 shows the DTs obtained on titer at WD14 with K=7.

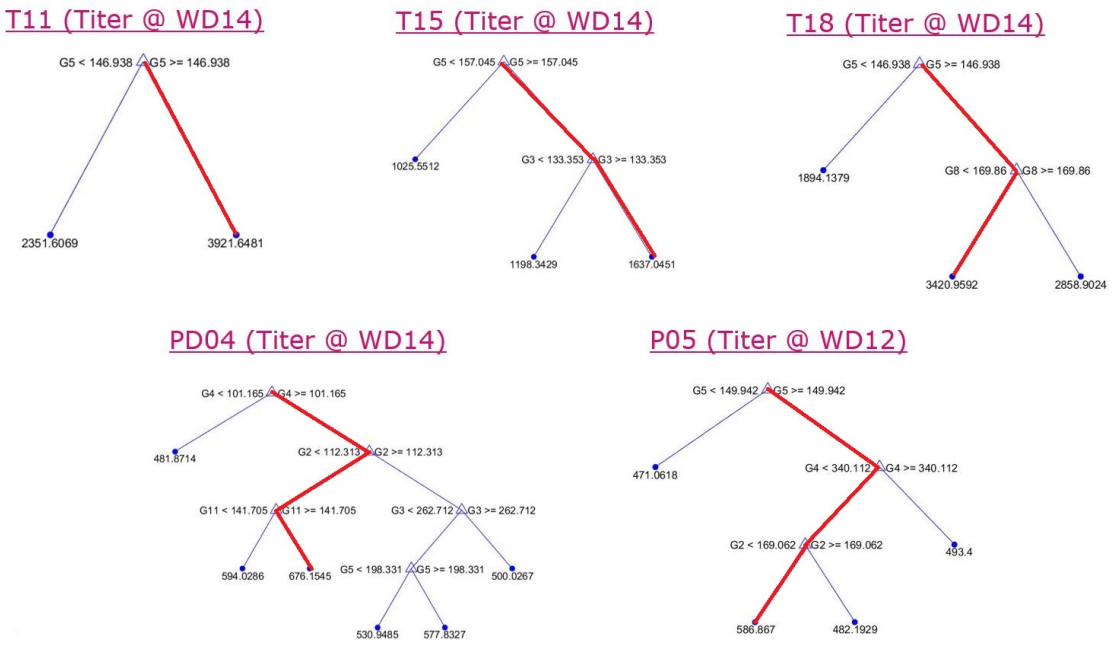

Figure 3.3: Experiment 0: DTs results on titer at WD14

The selection pathways towards maximal titer are highlighted in red and they are summarized in Table 3.1.

TITER					
Group	T11	T15	T18	P04	P05
G2				<112.3 (2)	<169.1 (3)
G3		>133.4 (2)			
G4				>101.4 (1)	<340.1 (2)
G5	>146.9 (1)	>157 (1)	>146.9 (1)		>149.9 (1)
G8			<169.9 (2)		
G11				>141.7 (3)	

Table 3.1: Experiment 0: Selections obtained for the titer at WD14

Each column corresponds to one decision tree analysis. For each clone, 1 to 3 significant selections were found. An important conclusion can be taken by looking at the selections; they are clone specific and, for that, it is not possible to find a media formulation which is the best for all the clones. This means that there are some groups which are important for some clones, but not for the others. For example, for the K clones, group 5 is dominating, while for S clones, G2 and G4 dominate, as they were chosen for all of them. This selection difference is present also within clones of the same family; for example, regarding the titer, the group 11 is important for P04 but not for the P05. The same conclusion can be taken by looking at the selections obtained for the IVC at WD14, which are shown in Table 3.2.

IVC					
Group	T11	T15	T18	P04	P05
G1			≥96.4 (2)		
G2	>78.6 (1)		<186.5 (1)	<157.6 (2)	<159.3 (1) ≥123.5 (3)
G4				≥101.2 (1)	≥101.2 (2) < 257.2 (5)
G5	≥147.4 (2)		≥150 (4)		
G6	<400.2 (3)		<452.4 (3)		
G8		≥77.9 (1)			
G9			≥70.5 (5)		≥91.3 (4)

Table 3.2: Experiment 0: Selections obtained for the IVC at WD14

As well as for the titer, those selections are obtained after a DT analysis with K=7. Also in this case the selection are clone specific and there are some groups, such as G1, G8 and G9 which are important just for one clone.

Another important question to mark, is that in some cases, the selections for the titer and for the IVC are slightly different between them. For example G2, which for K clones is not relevant regarding the titer, becomes important in the selections for the IVC. On the other hand, there are some groups, such as G4, which are important in both, the titer and the IVC.

As explained in Section 2.5, 16 conditions were replicates two times; the repetition errors were evaluated in terms of CV for every clone. The results for the K1 clones are reported in Table 3.3.

T11		T15		T18	
Titer	IVC	Titer	IVC	Titer	IVC
16%	3%	26%	1%	2%	9%
15%	0%	20%	1%	1%	7%
5%	2%	17%	4%	26%	21%
2%	8%	16%	6%	12%	3%
51%	34%	6%	7%	40%	11%
29%	31%	4%	4%	12%	9%
4%	0%	44%	2%	22%	29%
15%	5%	13%	13%	3%	14%
4%	5%	66%	51%	3%	1%
0%	1%	5%	1%	5%	1%
12%	18%	43%	8%	14%	11%
37%	6%	81%	11%	5%	4%
55%	19%	11%	1%	6%	5%
22%	18%	12%	14%	11%	16%
28%	5%	4%	3%	3%	3%
1%	1%	73%	29%	11%	3%

Table 3.3: Experiment 0: CVs of all the replicates experiment performed with CHO-K1 at WD14. Each row represents one replicate experiment.

It was chosen to report just the CVs results of WD14 of the titer and of the IVC. It is possible to point out that the CVs for the titer are normally higher than the one of the IVC. This can be explained by the

fact that normally the titer results have wider ranges compared to the IVC. For this reason it is easier to have an higher standard deviation in the results of the titer. Another things that can be highligted is that the T18 clone seems to be the most stable one. In fact it has just 5 CVs higher than 20%, while the other clones have 8 high CVs.

The results for the S clones are instead reported in Table 3.4.

P04		P05	
Titer 12	IVC 14	Titer 12	IVC 14
6%	5%	28%	43%
0%	15%	6%	16%
1%	5%	10%	4%
2%	8%	10%	7%
8%	0%	17%	6%
23%	44%	4%	N/A
1%	14%	2%	3%
0%	6%	2%	5%
2%	5%	18%	3%
5%	16%	1%	19%
7%	5%	1%	3%
6%	29%	7%	3%
1%	4%	15%	1%
6%	16%	1%	8%
8%	4%	3%	1%
13%	8%	13%	16%

Table 3.4: Experiment 0: CVs of all the replicates experiment performed with CHO-S at WD12. Each row represents one replicate experiment.

In this case, it was chosen to consider the results of the titer at WD12 and of the IVC at WD14 due to the fact that no results of titer at WD14 were available. For this cell line, the CVs are in general lower than for CHO-K1. This is quite understandable because as mentioned before, CHO-S cells responds less to the environmental changing. This means that for these clones the ranges of both, the titer and the IVC, are not so wide, leading to smaller standard deviation and so to smaller CVs.

3.2 Results of Experiment 1

As mentioned in Section 2.6.1, experiment 1 was conducted with two clones, P04 and T11, on the same plate and with the same platform condition. The goal was to test the replicate errors.

3.2.1 Overall Results

The VCD evolution of experiment 1 is reported in Figure 3.4.

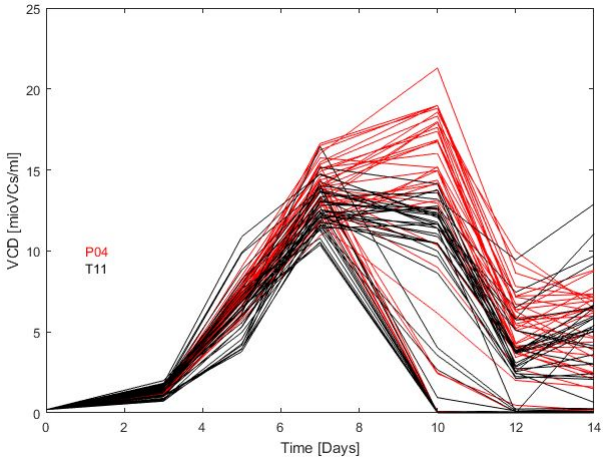

Figure 3.4: Experiment 1: VCD evolution. black lines correspond to the clone T11, while the red ones to the clone P04

At WD03 the range of the results is really small, so that the replicability is high. At WD05 the range is wide for T11 clone, due to the fact that the guava plate was not coated before the analysis (See Section 3.2.1.1) . Expecially after WD07 the high variability of VCD, with CV more than 40%, was observed for those wells having the same conditions. At WD10, in fact, T11 cells started to die. A possible reason can stand in two problems that were found in a previous research at Merck [45].

Firstly, the VCD was decreasing with time during the Guava test, as shown in Figure 3.5.

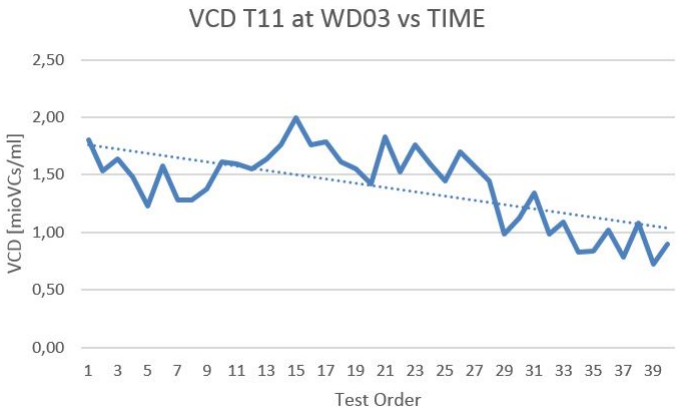

Figure 3.5: Experiment 1: Downward trend of VCD. The wells are numbered according to the test order: since the Guava device reads the plate by rows, A1 corresponds to 1, A2 to 2 and so on. Here just the results obtained on T11 clone are reported.

In the T11 samples (40), the VCD decreases based on the test order. In particular, the wells with the T11 clone are numbered according to the test order. A1 is then 1, A2 is the number two and so on. The downward trend means that in those wells analyzed at the beginning, such as the first two rows, the VCD is higher than in those wells analyzed at the end. This is not true for the other 40 samples in which P04

clone was tested. The results relative to P04 clone are reported in Figure 3.6.

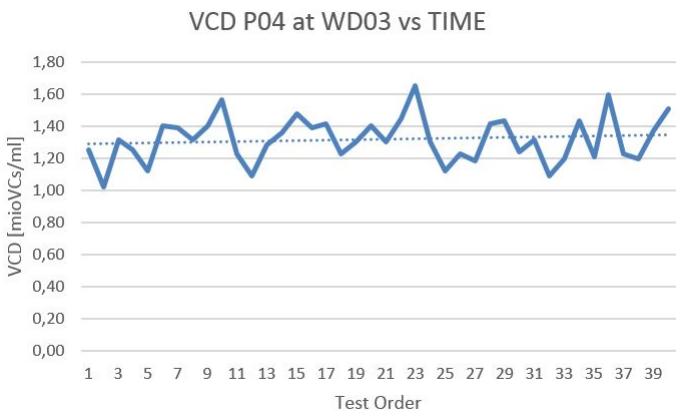

Figure 3.6: Experiment 1: Measurment of VCD at WD03 of P04 clone. The wells are numbered according to the test order: since the Guava device reads the plate by rows, A1 corresponds to 1, A2 to 2 and so on. Here just the results obtained on P04 clone are reported.

In this case the measurement is more constant in time. This difference is due to the fact that K1 clones tend to adhere to the bottom of the plate, while S clones not. To test an entire plate in the Guava device, almost 40 minutes are required; this interval time is sufficient to let the T11 cells to adhere.

Secondly, cell viability began to decrease significantly from WD07 to WD10 in those wells located in the center of the plate. Figure 3.7 reports the measurement of viability at WD07.

Viability at WD07										
	1	2	3	4	5	6	7	8	9	10
A	99,12	94,05	93,64	94,33	98,55	93,69	97,14	94,46	99,19	97,23
B	98,12	95,53	96,13	93,46	94,18	95,52	95,94	99,06	98,65	98,74
C	93,40	97,42	94,46	99,01	98,48	97,17	98,65	95,99	97,18	95,09
D	94,81	96,45	97,17	98,79	99,33	91,41	92,31	98,60	95,49	99,06
E	98,29	93,55	98,43	99,01	89,77	91,54	98,15	98,57	93,73	98,89
F	94,54	98,16	98,53	100,00	98,32	97,30	92,26	97,92	93,77	98,68
G	93,71	95,90	98,02	93,45	97,17	99,10	93,33	98,03	94,55	93,86
H	96,12	97,10	98,18	98,87	98,58	94,58	97,64	97,35	95,26	98,90

Figure 3.7: Experiment 1: Cell viability at WD07

In this figure the viability results are highlighted with different tonality of pink; the more it is dark, the higher is the viability. In this case the worst results are present in wells D06, E05 and E06, with a viability which is around 90%

Figure 3.8, instead, shows the viability results at WD10.

Viability at WD10										
	1	2	3	4	5	6	7	8	9	10
A	96,44	82,73	83,26	85,00	96,10	83,47	95,77	86,99	96,69	88,84
B	95,95	79,37	70,26	0,00	25,97	64,60	81,64	89,40	96,19	97,82
C	81,42	91,36	17,18	15,83	15,22	81,02	86,28	0,00	5,97	90,38
D	85,65	70,71	81,89	0,00	0,00	0,00	0,00	0,69	0,00	95,30
E	96,43	72,24	84,23	0,00	0,00	0,00	0,00	0,00	0,00	96,24
F	79,24	92,34	96,00	48,60	64,68	78,11	0,40	74,89	0,44	94,97
G	89,35	81,40	92,11	27,09	87,24	97,27	81,19	97,12	76,47	88,26
H	83,91	85,50	94,65	97,27	96,57	87,30	97,55	89,18	84,75	96,51

Figure 3.8: Experiment 1: Cell viability at WD10

In this case, almost all the wells disposed in the middle of the plate have a viability which is 0%. The reasons why there is this drop in viability are not known, but it is possible that the wells in the middle are less oxygenated. In fact, when the silicon membrane is put in the lid, it is a bit squeezed and for this it is possible that the holes in the middle of the plate become smaller than the ones disposed on the edges. For this reason a study was conducted in order to reduce the variability (see Sections 2.6.1.1). The results of this study are reported in Paragraphs 3.2.1.1 and 3.2.1.2.

The overall distribution of the IVC is reported in Figure 3.9

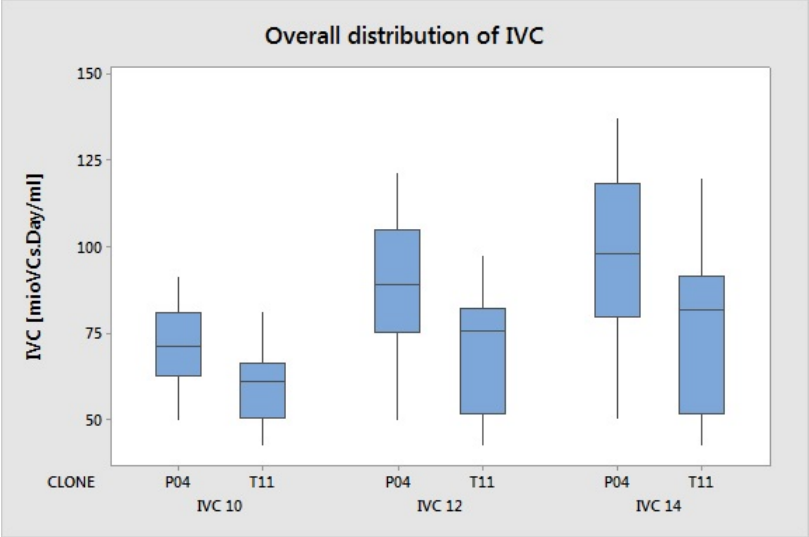

Figure 3.9: Experiment 1: IVC distribution of both the clones

For each WD, the IVC distribution of each clone is shown. The IVC for clone P04 is slightly higher due to the fact that T11 started to die in the middle of the plate from WD10. Moreover the plate was coated only from WD07, hence all the results for T11 clone of VCD are not reliable until WD07. In fact, before WD07, T11 cells adhered on the bottom of the plate, and, therefore, they were not detectable by the device. Another important question to mark is the range of IVC. This is wide for both the clones leading to a high repetition errors.

The overall distribution of the titer is, instead, reported in Figure 3.10.

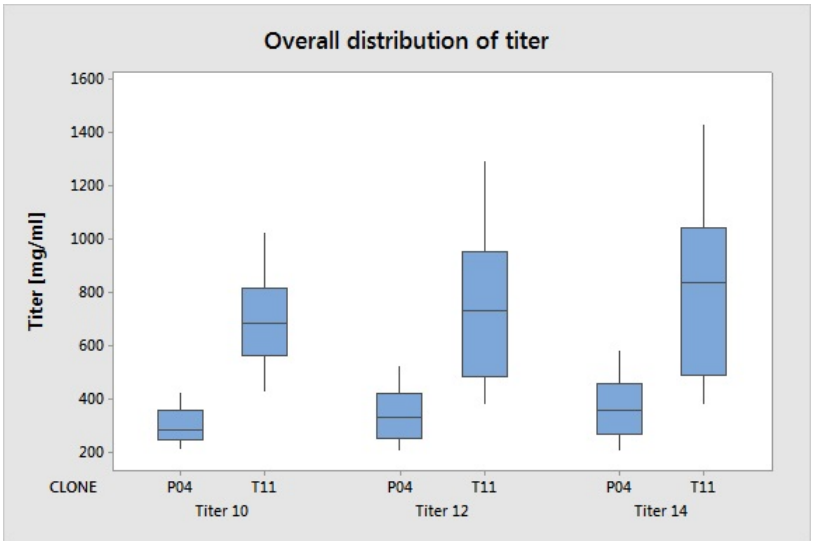

Figure 3.10: Experiment 1: Titer distribution of both the clones

As shown, T11 has a slightly higher production compared to P04 clone. However, the ranges of titer are quite wide, expecially for T11, even if the conditions were equal. This means that the repetition errors are quite high (see Section 3.2.2).

3.2.1.1 Coating Guava Plate

As mentioned in Section 2.6.1.1, a study was conducted in order to test if coating the Guava plate has some beneficial results for clones K1. The results of the different strategies are shown in Table 3.5

	Non-coated plate	1min, RT	5min, RT	30min, RT	30min, 37°C	60min, 37°C
ViCell (mioVCs/mL)	1.82	3.18	3.04	3.05	1.88	1.88
MAX (mioVCs/mL)	2.54	4.17	2.23	3.98	2.62	2.97
MIN (mioVCs/mL)	1.11	1.51	0.67	2.37	1.55	2.03
AVE (mioVCs/mL)	1.78	2.87	1.59	3.33	2.23	2.41
DEV	0.29	0.69	0.36	0.3	0.21	0.2
CV	16%	24%	23%	9%	9%	8%

Table 3.5: Results of the different coating method

In the first rows the different strategies are reported. In particular the time and the temperature in which the plate stays with the buffer inside are shown. In the first column, the different measured quantities are listed (see Section 2.6.1.1). The quantity considered significant to compare the results is the coefficient of variation calculated considering all the wells on the same plate.

CV was significantly decreased from 24% to 9% with the increasing coating time from 1 min to 30 min. With respect to temperature, there was no significant difference in variability (both 9% of CV) between Room Temp and 37°C (both at 30 min). Similar CV result was observed when the coating time was

extended to 60 min. Since the best results were obtained with the last coating method (60 min at 37°C), in all the following guava analysis with CHO-K1 cells, it was decided to use this technique.

In Figure 3.11 it is report the guava counting at WD07 after coating the plate.

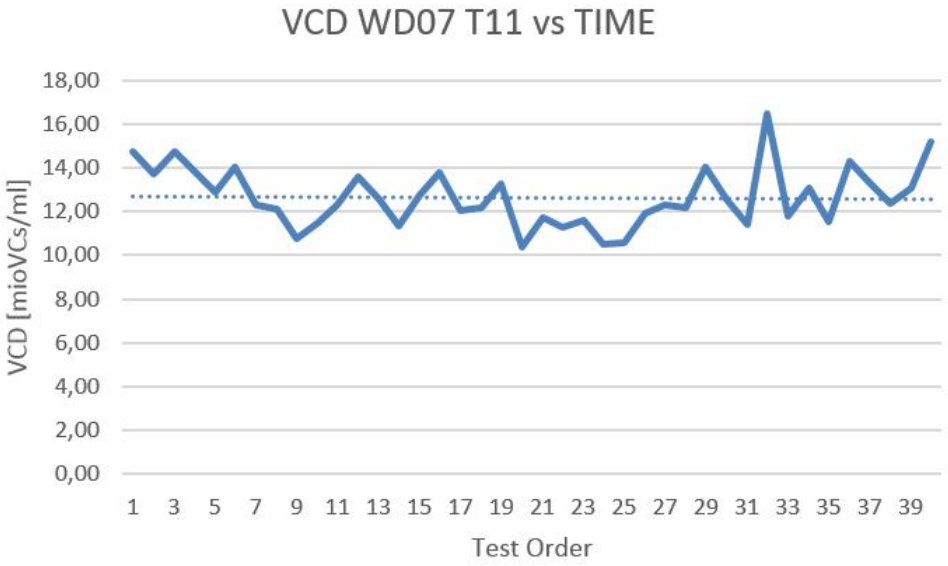

Figure 3.11: Experiment 1: Guava counting at WD07 after coating the plate

As shown, by coating the guava plate, the measurement become more constant in time also for T11 clone.

3.2.1.2 Changing Silicon Layer

The other study that was conducted to reduce the variability was the one which is explain in Section 2.6.1.1 that consider different silicon layer. The results obtained in this study are reported in Figure 3.12:

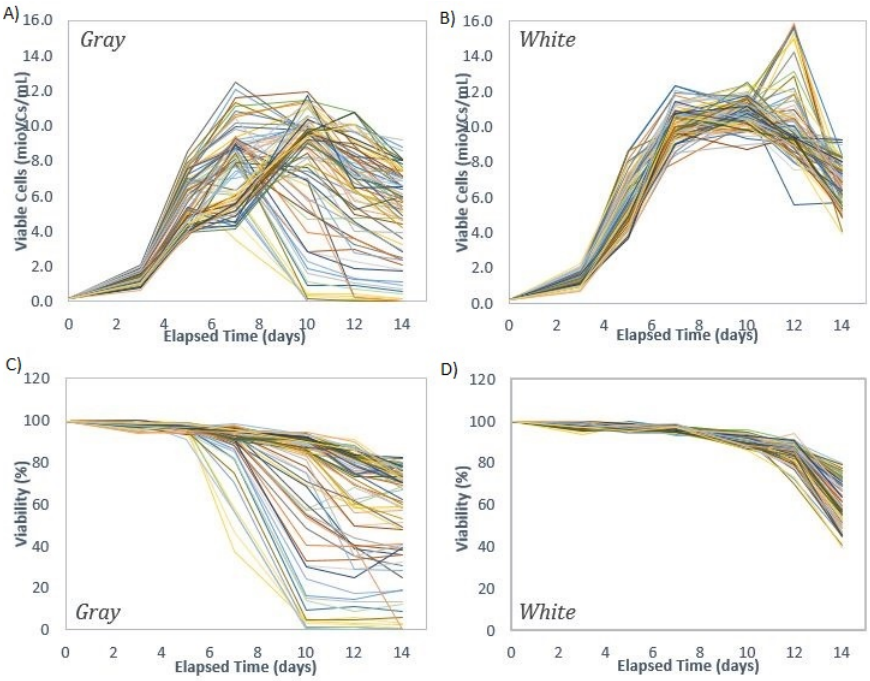

Figure 3.12: Comparison between white and gray membrane

In Figure 3.12B and 3.12D the data of the fed batch performed with white membrane are reported. Due to some feeding limit at WD10, the VCD of some wells (which had higher VCD at WD07) fell slightly. Regarding the viability, good replicability results were observed. Furthermore, the viability of 71 out of 80 wells were higher than 80% on WD12 and the average viability on WD was still 63%.

On the other hand, the plate with gray layer (cf. 3.12A and 3.12C) had clearly worse results. A wide distribution is present already at WD07. The CV rose up sharply from 23% on WD03 to 47% on WD10, then ended at 56% on WD14. Similarly, the viability of some wells went down dramatically since WD07.

Since it was demonstrated that among the membrane, the best one is the white one, for all the followed experiments it was decided to use this one.

3.2.2 Repetition Errors

As shown in Section 3.2.1, the ranges of both, the titer and the IVC, are wide, which means that the repetition errors are significant. In terms of CV the results are reported in Table 3.6.

	P04		T11	
WD	Titer	IVC	Titer	IVC
10	21%	17%	24%	17%
12	28%	24%	37%	24%
14	30%	27%	39%	29%

Table 3.6: Experiment 1: CV results

The CVs are not acceptable, since in most of the cases they are higher than 20%. This can be explained by the early cell death on the middle of the plate.

For that it was chosen to consider just the conditions in the border. The results are then reported in Table 3.7.

	P04		T11	
WD	Titer	IVC	Titer	IVC
10	13%	9%	17%	10%
12	14%	10%	23%	10%
14	14%	10%	21%	12%

Table 3.7: Experiment 1: CV results on the wells disposed on the border

By excluding the conditions located in the middle of the plate, the results slightly improve. This is due to the fact that the not attendible results, such as all the wells in which the cells died, are excluded.

However, by considering the calculation in Equation 2.2, different results are obtained. In Figure 3.13 the distributions of the repetition errors for P04 is reported.

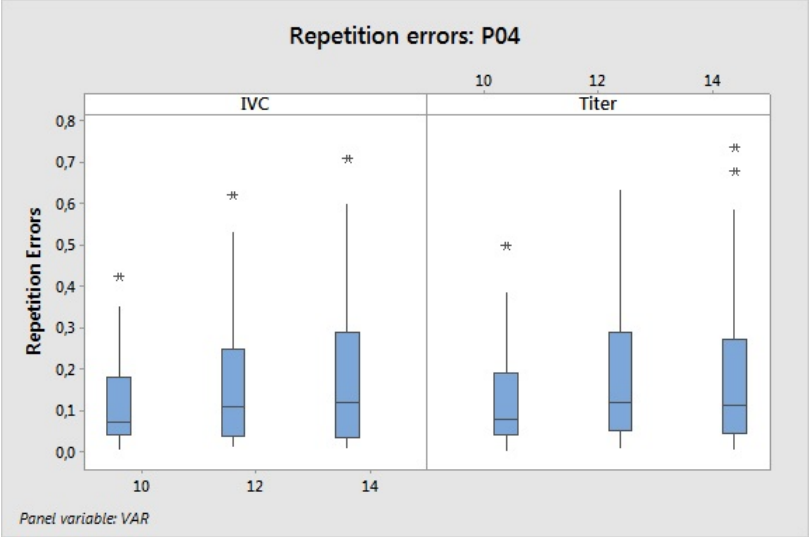

Figure 3.13: Experiment 1: Repetition Error distribution for P04

The repetition errors of the IVC increase in time and it is acceptable just at WD10. The repetition errors of the titer are instead more constant in time and there is just an increase in the error between WD10 and WD12. As for the IVC it is acceptable only at WD10, where the 75% of the errors are less than 20%. In Figure 3.14, instead, the distributions of the repetition errors for T11 is shown.

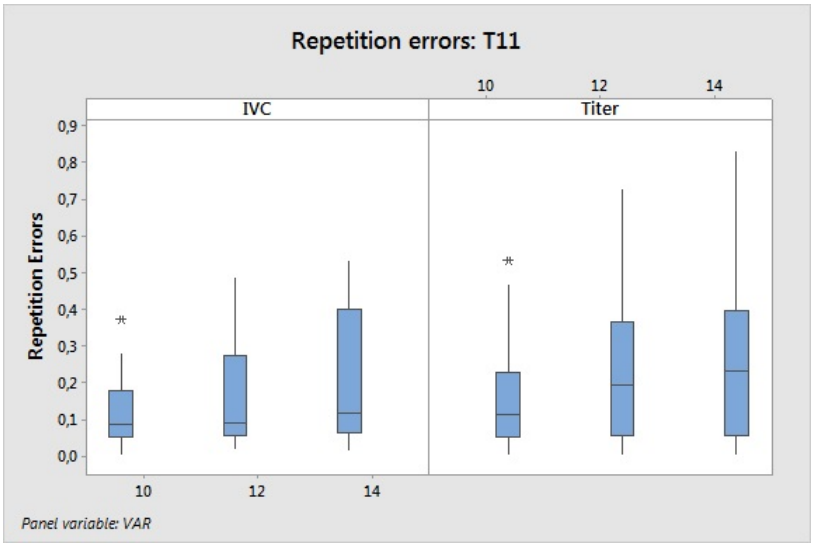

Figure 3.14: Experiment 1: Repetition Error distribution for T11

Also for this clone, the same trend is found. So, regarding the IVC the repetition errors increase with the time, while for the titer, the errors is more constant in time, with an between WD10 and WD12. Also in this case the errors are acceptable only at WD10.

As for the CVs, it was chosen to evaluate the results by excluding the wells located in the middle of the plate. Figure 3.15 shows the results for P04.

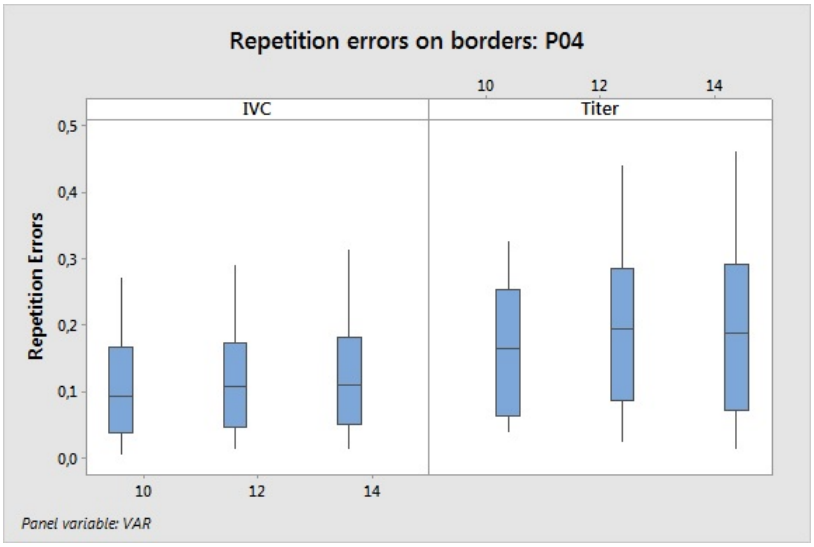

Figure 3.15: Experiment 1: Repetition Error distribution for P04 considering only borders

In this case, the errors concearning the IVC becomes smaller and acceptable in any time. Regarding the titer instead, the improvement consists only in removing the outliers. The same results are obtained for T11 clone. They are reported in Figure 3.16.

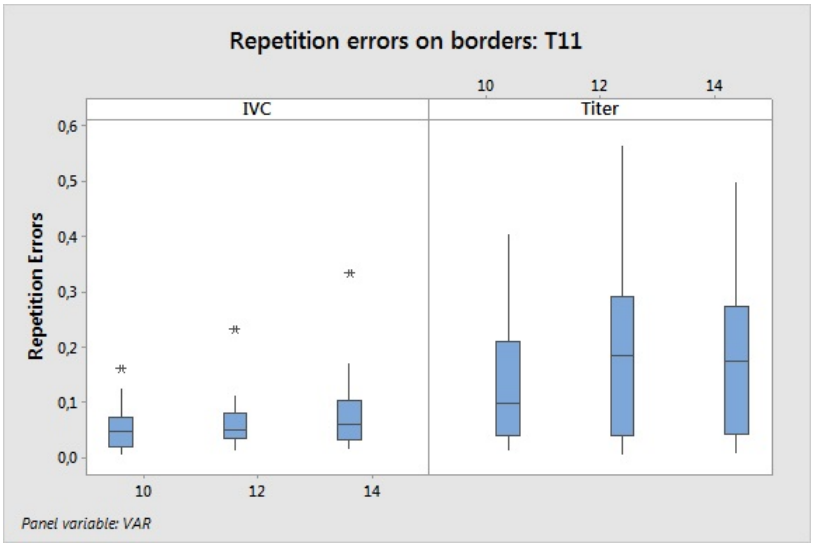

Figure 3.16: Experiment 1: Repetition Error distribution for T11 considering only borders

Although the IVC repetition errors improved, the ones relative to the titer have not ameliorated by considering only the wells on the borders.

3.2.3 Comparison on Clone T11

A comparison with the results obtained on experiment zero was done. It was decided to consider only the conditions disposed on the border in order to exclude those results which are not reliable. The comparison on the titer is reported in Figure 3.17.

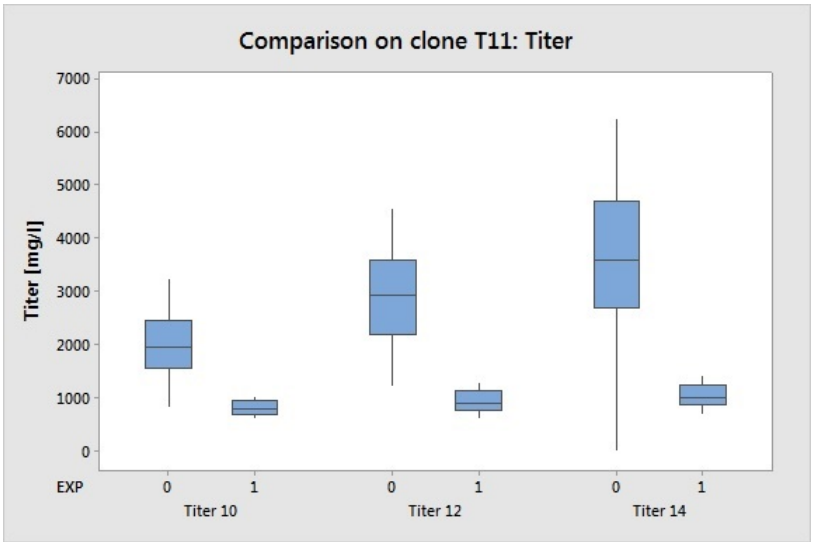

Figure 3.17: Comparison on clone T11: titer

The results obtained with media blending in exp 0 are slightly better then the results obtained with the

platform conditions in exp 1. This means that, regarding the clone T11, it respondes in a significant manner to the environmental changing; this leads to a clear possibility to improve consistently the mAb production in this clone.

The comparison on the IVC was not done due to non reliable data of VCD until WD07 [1].

3.3 Results of Experiment 2

As described in Section 2.6.2, this experiment was performed with one clone, P04, on two plates. Both plates were divided into two parts, one with the goal to test the repetition errors and the other aiming to test different components. The conditions were the same across the plates.

3.3.1 Overall Results

The VCD evolution is reported in Figure 3.18.

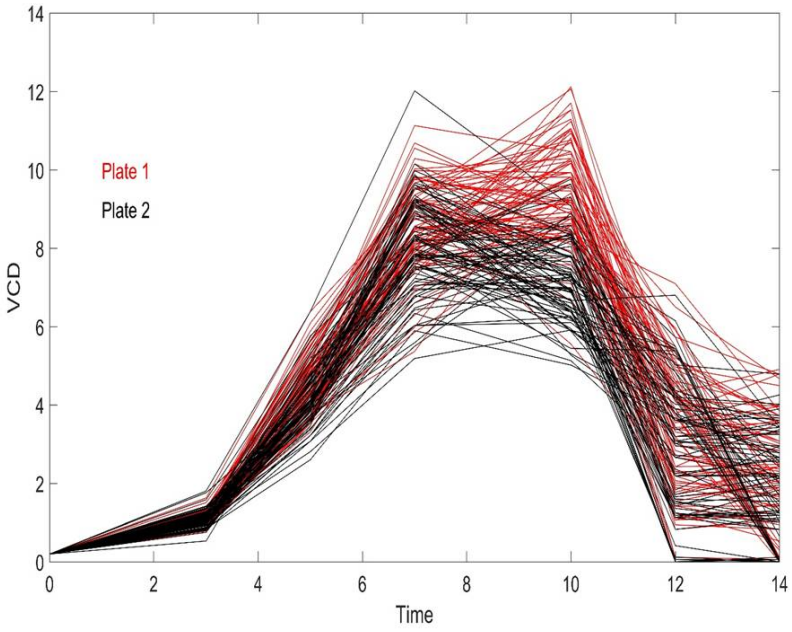

Figure 3.18: Experiment 2: VCD evolution. Red lines are referred to plate 1, while black ones to plate 2. The conditions were equal on the two plates.

It is possible to see some differences between the two plates. In particular the evolutions are similar untill WD07 but, starting from WD10, an early cell death in the second plate is visible. It is not clear the reason why the cells in the second plate started to dye. However, at WD12 and WD14 a similarity is obtained.

The IVC evolution is reported in Figure 3.19.

[1] Until WD07 the guava plate was not coated so, the results on the bottom edge are not reliable.

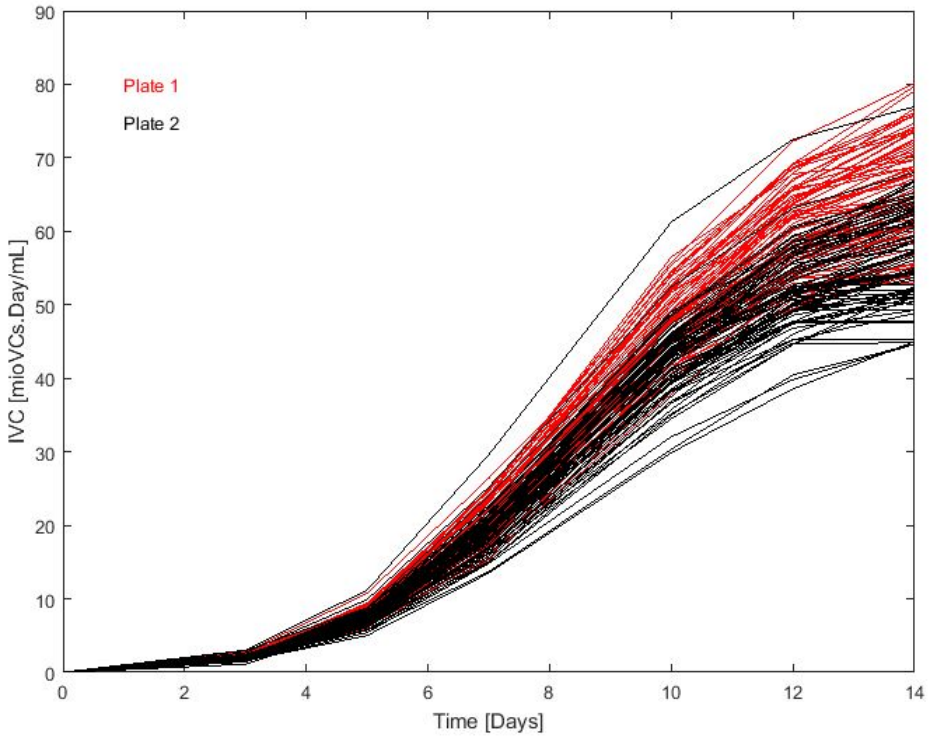

Figure 3.19: Experiment 2: Evolution of IVC. Red lines are referred to plate 1, while black ones to plate 2.

It is evident that, due to the early cell death in plate two, the same behaviour of VCD is found in IVC. But, in this case, also at WD12 and WD14 the differences are significant due to the fact that IVC is a quantity that depends on the evolution in time of the VCD.

This can be shown more clearly with a box plot. In Figure 3.20 the distribution of IVC in both the plates is shown.

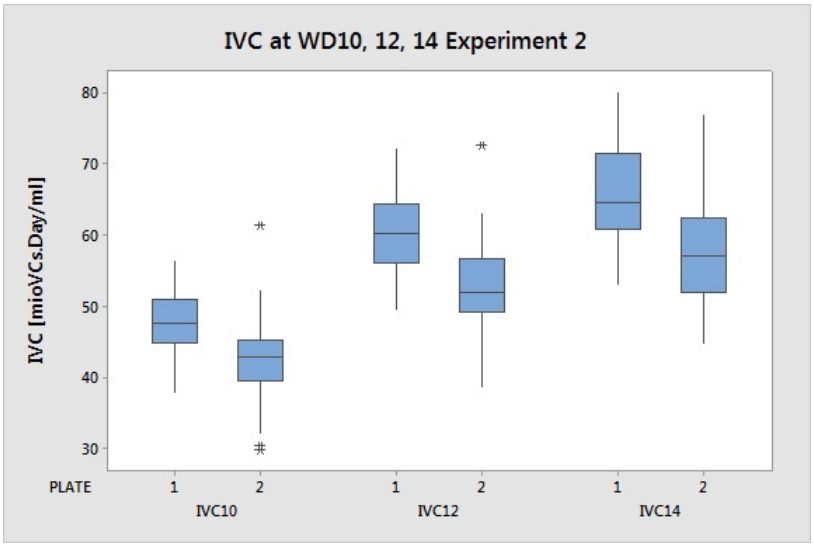

Figure 3.20: Experiment 2: Distribution of the IVC

Each boxplot represents the IVC distribution at a precise WD (10, 12 or 14) and in a precise plate (1 or 2). The distribution is slightly lower in plate 2 compared to plate 1, as it was already clear by seeing the time evolution of the IVC.

The overall distribution in time of the titer is, instead, reported in Figure 3.21.

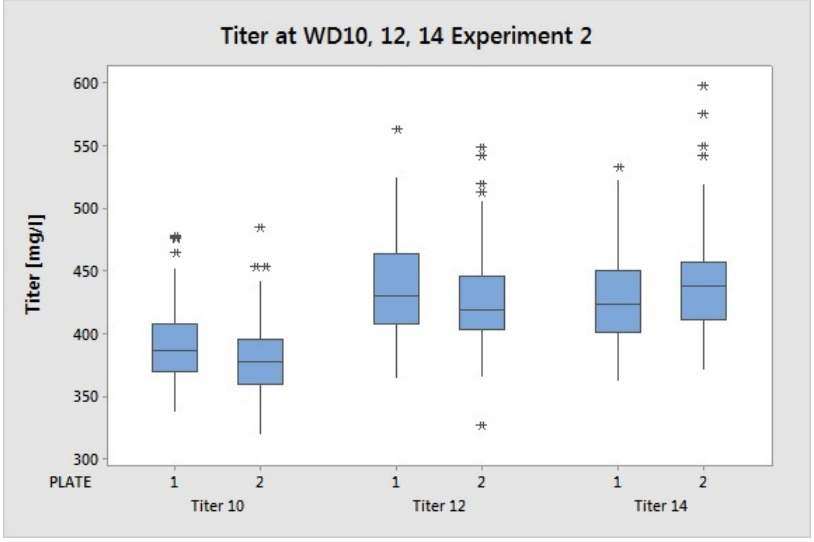

Figure 3.21: Experiment 2: Evolution in time of the titer distribution

In this case, the range is similar across the plates. One question that can be marked here, is that, normally, in a fed-batch culture, the titer distribution should increase in time; here this trend is observed in plate 2, where the cells started already to dye at WD10, but not in plate 1. This is explained by the fact that VCD drops significantly in this plate at WD12, so that there are few viable cells left at WD14 that are

still producing.

3.3.2 Repetition Errors

Figure 3.22 shows the distribution of the repetition errors calculated on the IVC in those wells performed with platform medium.

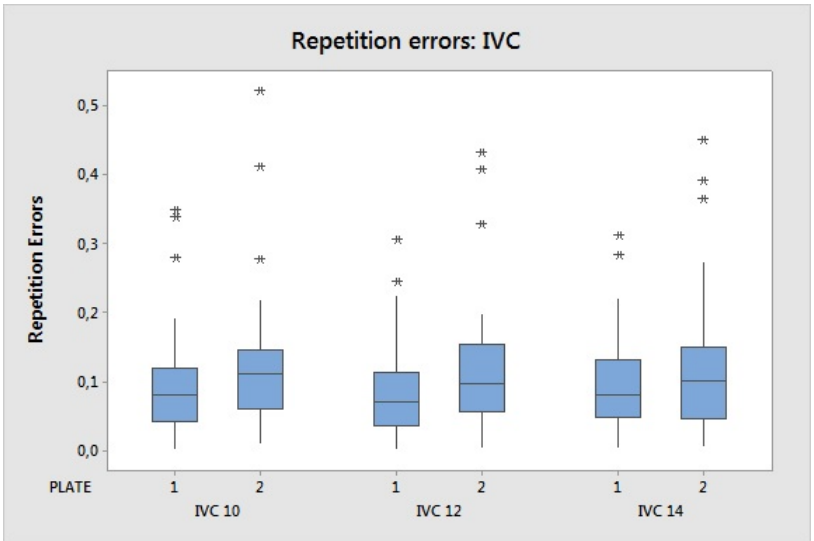

Figure 3.22: Experiment 2: Repetition Errors of the IVC in both the plate

As expected, the errors in the plate two is higher compared to the one in plate one. This is due to the early cell death in the plate two. One positive point is that during the time the repetition error stays constant and lower then 15%.

By looking at the repetition errors of titer, shown in Figure 3.23, the same trend is not seen.

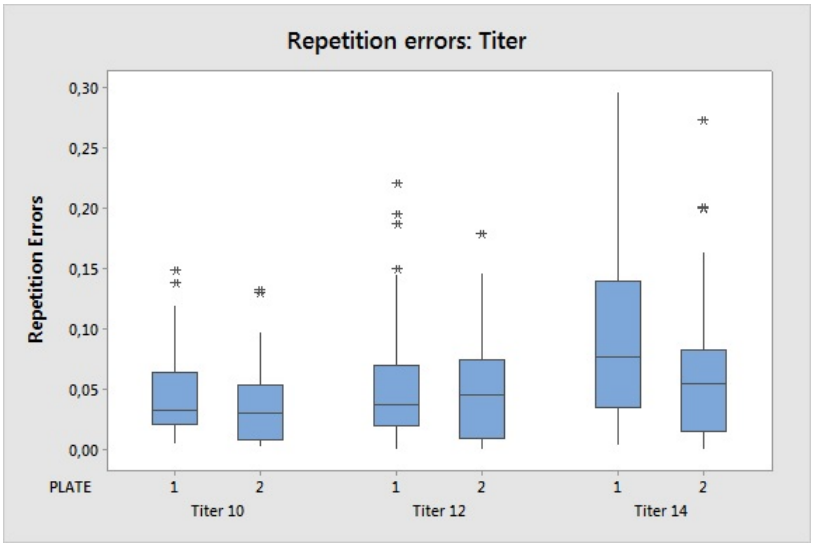

Figure 3.23: Experiment 2: Distribution of repetition errors calculated on the titer

In particular, the repetition errors is comparable between the plates and stay constant at WD10 and WD12. However, at WD14, the error in the first plate is slightly higher than the one in the second plate, but remains acceptable ($< 20\%$).

Since the two plates have the same conditions, it is possible to calculate the repetition errors also across the plate. Figure 3.24 shows the distribution of the repetition errors calculated on the titer.

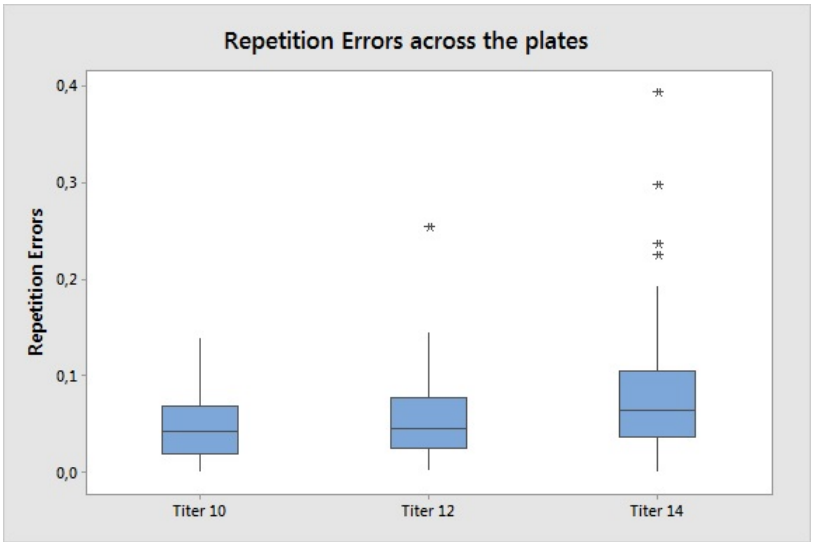

Figure 3.24: Experiment 2: Titer repetition errors across the plate

The errors are acceptable, due to the fact that the 75% of the errors are lower than 10%. By excluding the outliers, the errors are always lower than 20% and it is quite constant in time.

Figure 3.25 shows the distribution of the repetition errors calculated on the IVC.

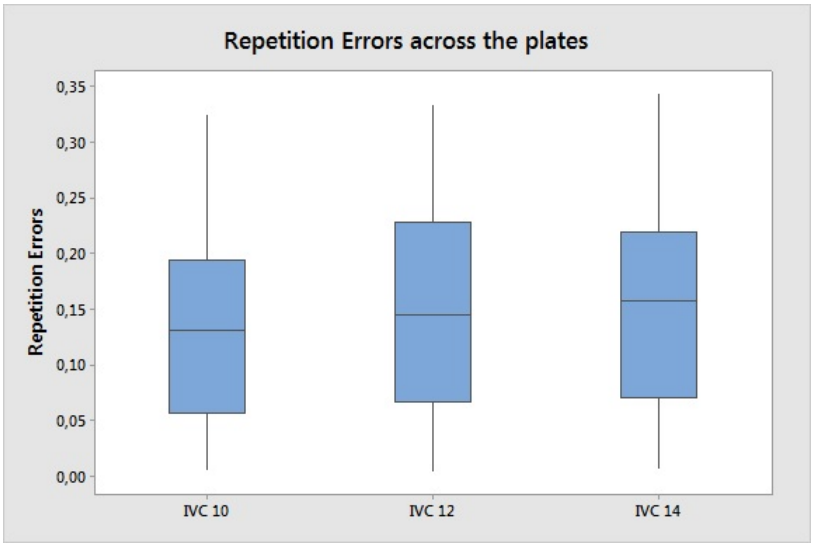

Figure 3.25: Experiment 2: IVC repetition errors across the plate

In this case, the errors are higher due to the cell death in the second plates. It rest constant in time but it is not acceptable due to the fact that the 75% of the repetitions errors are higher than 20%.

The errors were also calculated in terms of CVs both in the same plate and across the plates. Table 3.8 shown the results calculated on the same plate.

	IVC 10	IVC 12	IVC 14
Plate 1	8%	7%	8%
Plate 2	12%	11%	11%
	Titer 10	Titer 12	Titer 14
Plate 1	6%	7%	9%
Plate 2	5%	6%	7%

Table 3.8: Experiment 2: CVs of the replicates conditions

As expected the CVs are higher in plate two concearning the IVC. But, however, the results are positive, since all the CVs are lower than 13%.

Figure 3.26 shows the results obtained by calculating the CVs across the plates.

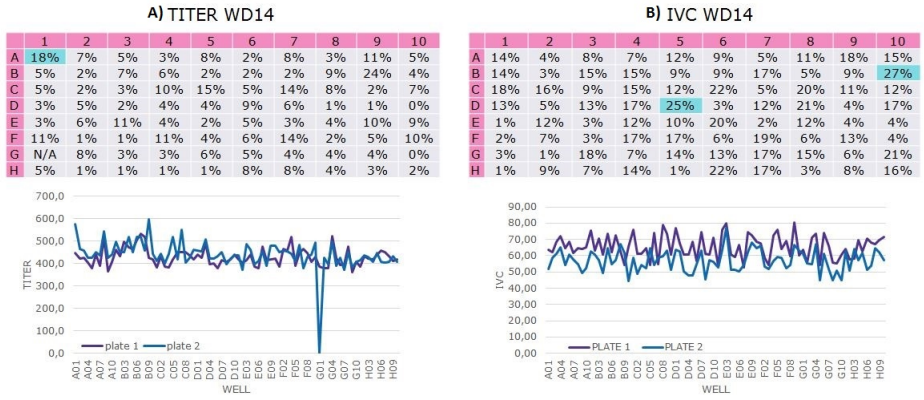

Figure 3.26: CVs across the plates in Experiment 2 at WD14

The two tables show the CVs calculated in each well concearning the titer (cf. Figure 3.26A) and the IVC (cf. Figure 3.26B). The graphs under the two tables show the trend of the titer and the IVC in the two plate. The more the two lines are overlapped, the lower is the deviations and so the CVs. As expected, also in this case, the CVs for the IVC are higher than the ones for the titer. The CVs distribution is generally smooth for titer, while there is more deviation around the mean for IVC. However, the CVs calculated on the IVC are still acceptable, since most of them are lower than 20%.

3.3.3 DTs Analysis on Media Blending Results

As described in Section 2.4.2, the results of media blending are then analyzed in order to see which were the best conditions. The DTs obtained on IVC with K=10 is reported in Figure 3.27

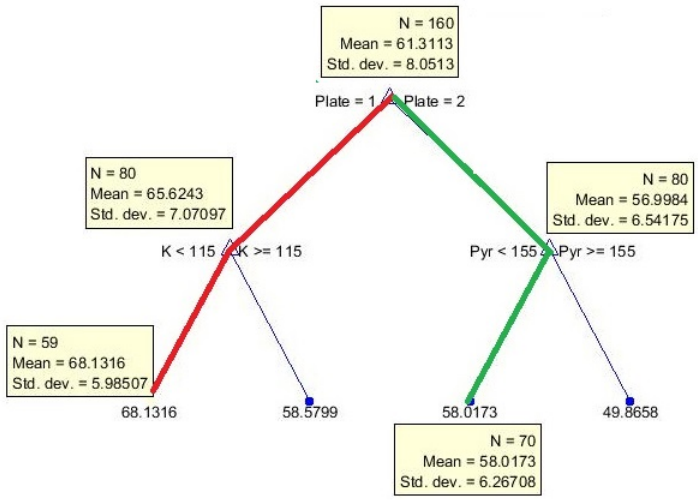

Figure 3.27: Experiment 2: DT analysis on IVC at WD14. The red pathway is the one that maximize the results in plate 1 while the green one is the one that leads to the best results in plate 2. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

The major driver here is the plate, because the cells survived more in the first plate. This means that none of the factors seems to have a clearer effect across the plates than the deviation in the plates itself. This is might be due to the fact that DWPs are not fully reliable combined with the fact that the chosen clone might not be the most responsive. The particular thing that can be noticed is that the selections are different across the plate; in fact, in plate 1, the most important component is K, while in the second one pyruvate (Pyr) seems to be more important. Another observation that can be taken is the fact that the mean in plate 1 is slightly higher compared to the one in plate 2.

The DTs with K= 10 obtained on titer is instead reported in Figure 3.28.

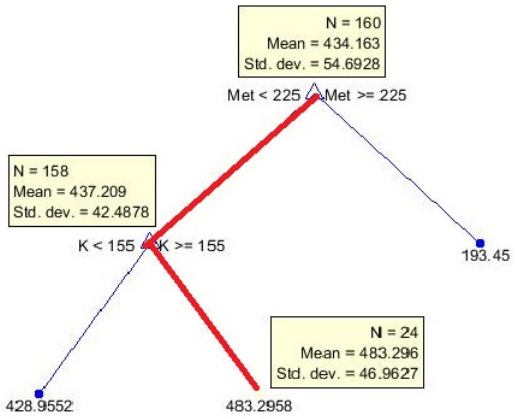

Figure 3.28: Experiment 2: DT analysis on Titer at WD14. The red pathway is the one that maximize the production of mAb. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

Regarding the titer, the major driver is not anymore the plate, but methionine (Met) and K are the two components which leads to the best results. Met was found to be important for this clone also in experiment zero, where the selection was Met $>$ 101.2 for both, the IVC and the titer. With this experiment its importance was confirmed and an upper limit for the range was found. Regarding K, one could think that it is a minor decision. However, this decision was already present when the number of cross validation was 5, which means that it is not negligible. Only 24 of the 160 experiments fulfil all the selections.

If a comparison is done between IVC and titer results, it is possible to notice that the selection on potassium is actually different. To obtain high IVC, in fact, potassium needs to be less than 115%, while to have a better titer it has to be higher than 155%.

Another interesting comparison is the one between the platform conditions and the media blending ones. Figure 3.29 shows this comparison on IVC at WD14.

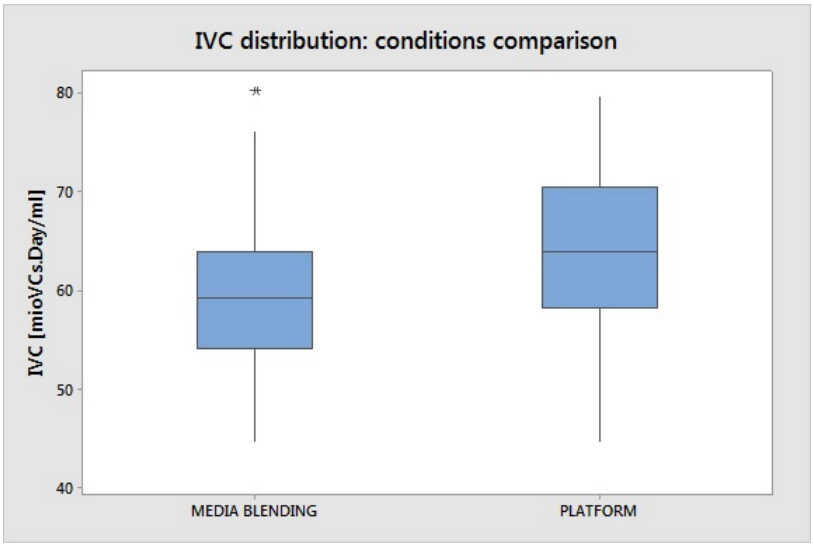

Figure 3.29: Experiment 2: Conditions comparison on IVC

In this case, platform conditions seem to have better results.

Figure 3.30 shows the comparison between media blending conditions and platform conditions on titer at WD14.

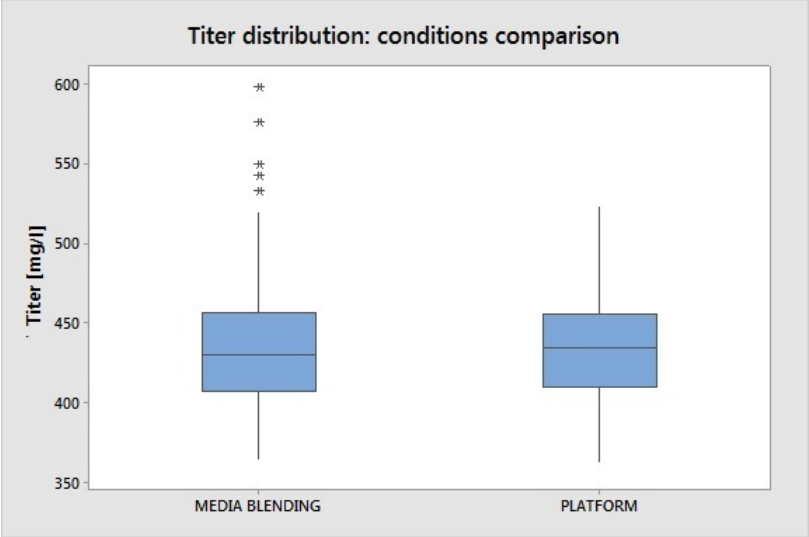

Figure 3.30: Experiment 2: Conditions comparison on titer

Concearning the titer instead, no differences can be noticed between platform medium and media blending. This means that this clone does not respond well to the environmental changing. But if only the selections obtained are considered, the results changes. Figure 3.31 shows the comparison between the platform conditions and those media blending conditions which were selected as the most important ones at WD14.

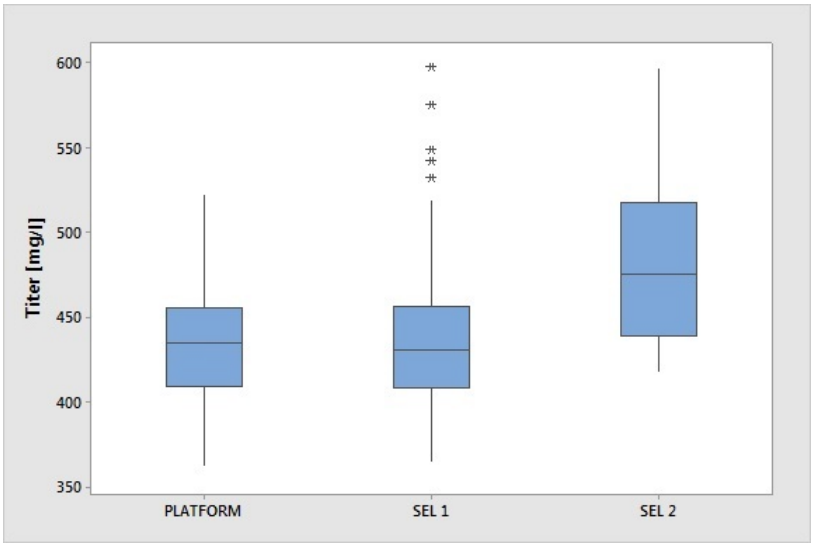

Figure 3.31: Experiment 2: Representation of the selections obtained on titer at WD14. SEL 1 is the first selection, which in this case is Met<225. SEL 2 is the second selection, which is K>155

This figure shows that although the results are not slightly improved with the first selection (Met<225), after the second one (K>155) they are consistently higher. This is another prove of the fact that the second selection is not negligible in this case but it is indeed important.

3.3.4 Comparison on Clone P04

A comparison on the results obtained in all the experiments performed with clone P04 was done. Figure 3.32 shows the distribution of titer in all the experiments at WD10 and WD12. The comparison on titer at WD14 was not possible since no data of experiment zero were available.

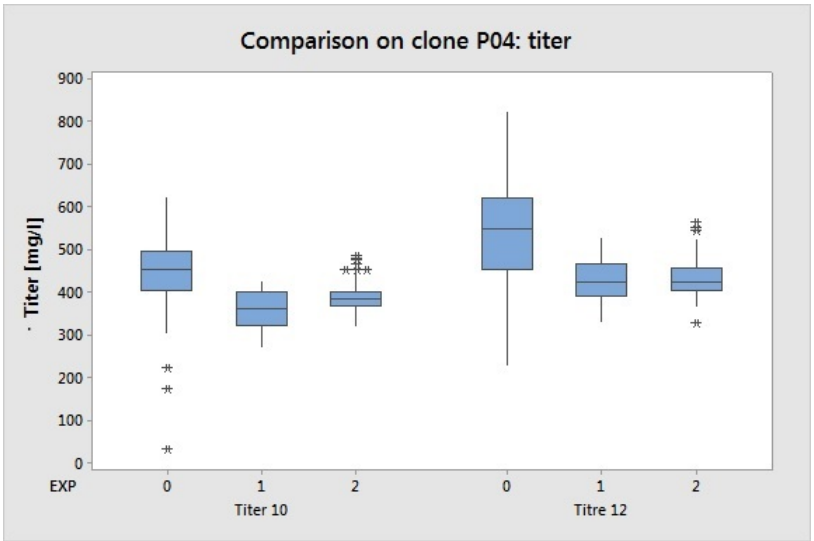

Figure 3.32: Comparison on clone P04: titer

The results obtained in experiment zero are slightly better than the ones in experiment 1 and 2. This can maybe be explained by the differences in age of the cells and in the modality of media preparation. In experiment zero, in fact, the cells were 14 days old, while in experiment 1 and 2 the cells were respectively 21 and 35 days old. Moreover, the media used in experiment zero were prepared from scratch. This means that the solutions are more homogeneous compared to the one prepared starting from powder medium.

The comparison can be made also on the IVC. As for clone T11, the results of the experiment 1 are omitted due to non reliable data. Figure 3.33 shows the comparison between experiment zero and experiment two.

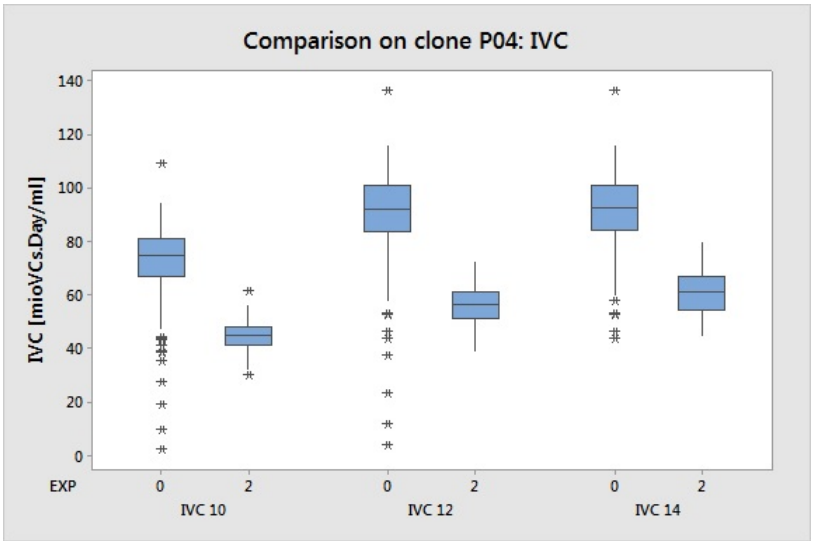

Figure 3.33: Comparison on clone P04: IVC

Also regarding IVC, the results in experiment zero are slightly higher than in experiment 2, much more than the titer is. The positive aspect is that in experiment 2 the negative outliers are removed.

3.4 Results of Experiment 3

As described in Section 2.6.3, this experiment was performed with three different clones, T15, T18 and T25. The VCD evolution is reported in Figure 3.34.

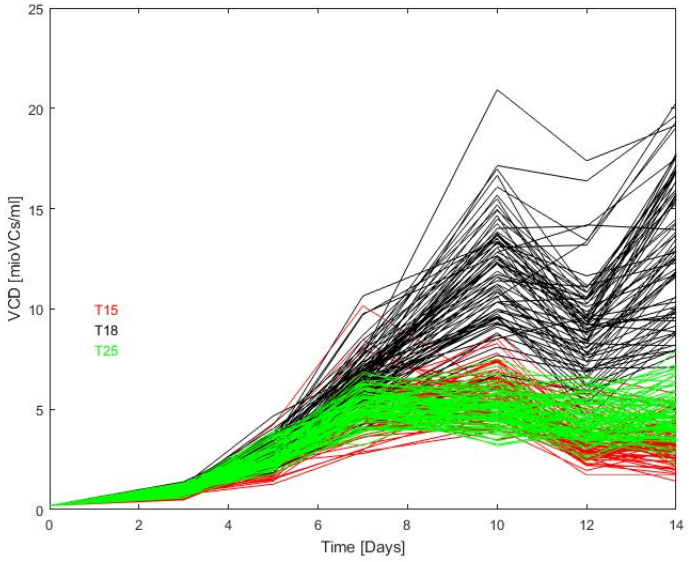

Figure 3.34: Experiment 3: VCD evolution. As explained in the legend, the red lines are related to the clone T15, the black ones to the T18 while the green ones to the T25 clone.

By looking at the graph, it is evident that clone T18 is the one that grows more and with the largest variability. This means that it seems to respond most to different conditions in the medium. Clone T15 has an intermediate peak of growth between T18 and T25, but it seems that the cells die before (WD12) in comparison to T25 clone. Moreover, a strange phenomenon can be seen: in most of the cases, the VCD is lower at WD12 than at WD14. This could be due to two different reasons: firstly it can evidence a clearly counting problem with the Guava device; secondly it is possible that during the sampling with the Biomek the taken volume was incorrect. It was then chosen to consider the results at WD12 not reliable. In Figure 3.35 the IVC evolution is reported.

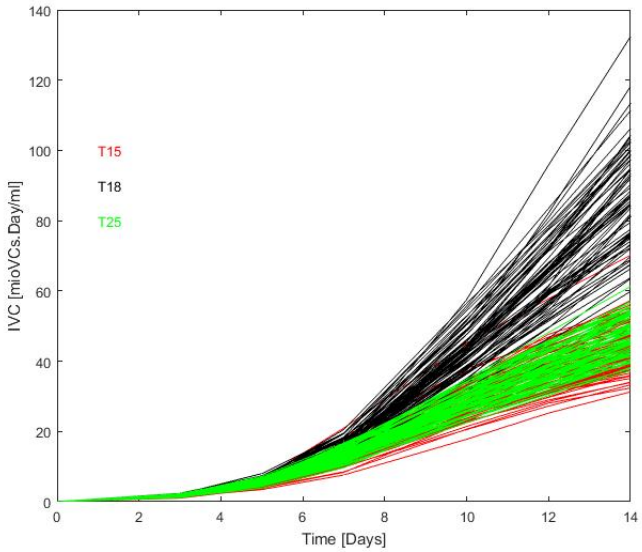

Figure 3.35: Experiment 3: IVC evolution. As explained in the legend, the red lines are related to the clone T15, the black ones to the T18 while the green ones to the T25 clone.

Again it is clear that clone T18 is the one with an higher growth compared to the others. Clones T15 and T25 have an IVC profile which is comparable.

Regarding the titer, instead, the distribution for different clones at WD14 is reported in Figure 3.36.

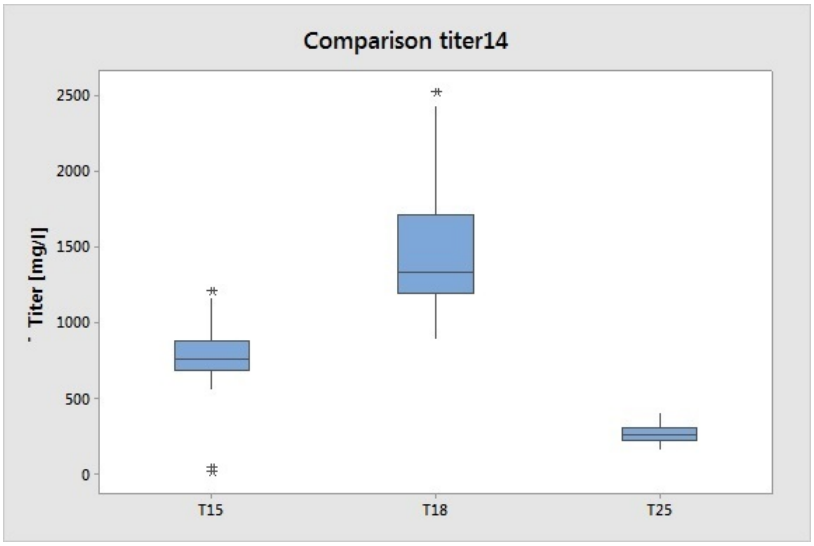

Figure 3.36: Experiment 3: Comparison of titer on WD14

It is evident that the clone T15 produces better than the T25 one, even if their IVC are comparable. Clone T18 is the one that produces the highest quantity of mAbs.

However, even though the clone T18 is the best producers, the clone with the best specific results seems to

be T15. In fact, by looking on the comparison on specific productivity (PCD) in Figure 3.37, the results obtained for T15 are slightly higher.

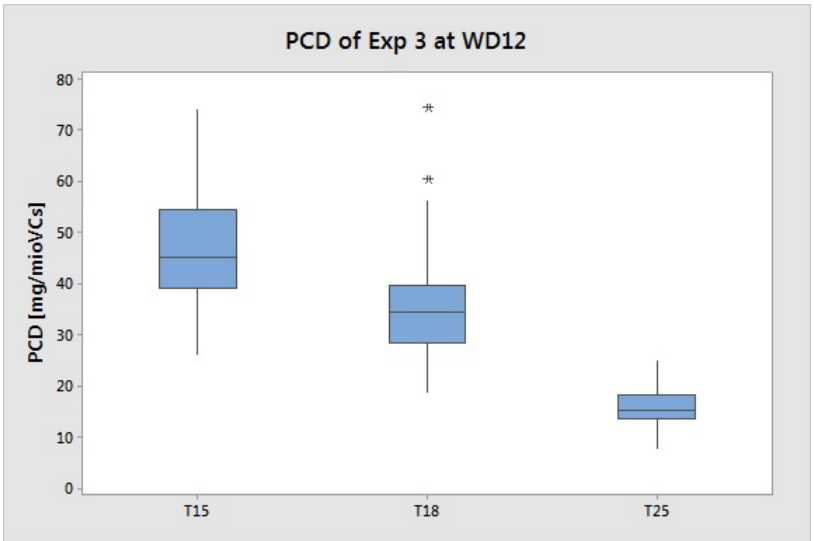

Figure 3.37: Experiment 3: Comparison between the clones on the PCD at WD12

This means that although for T15 the cells do not grow as for the T18, they are able to produce a quantity of mAb which is relatively higher.

3.4.1 Repetition Errors

As mentioned in Section 2.6.3, the platform condition was replicated 5 times in each clone in order to evaluate the repetition errors. The results on titer obtained in the T15 clone are reported in Figure 3.38.

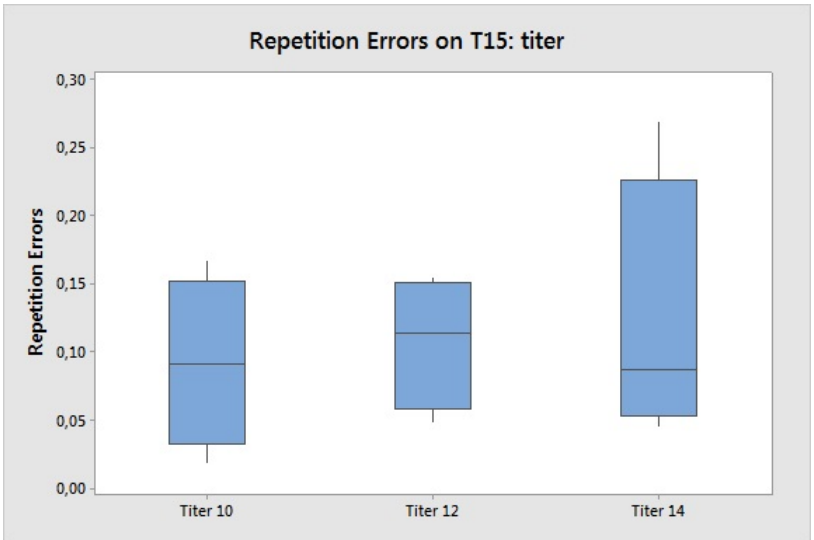

Figure 3.38: Experiment 3: Distribution of repetition errors calculated on titer (T15)

This picture shows the time evolution of the distribution of the repetition errors calculated on titer at differend WD, 10, 12 and 14. The repetition error is acceptable at WD10 and 12, as it is less then 20%. Then it becomes slightly higher at WD14, achieving a peak higher than 25%. However the mean rests under 10%, so it can be considered acceptable.

Figure 3.39 shows the repetition errors calculated, instead, on the IVC for clone T15.

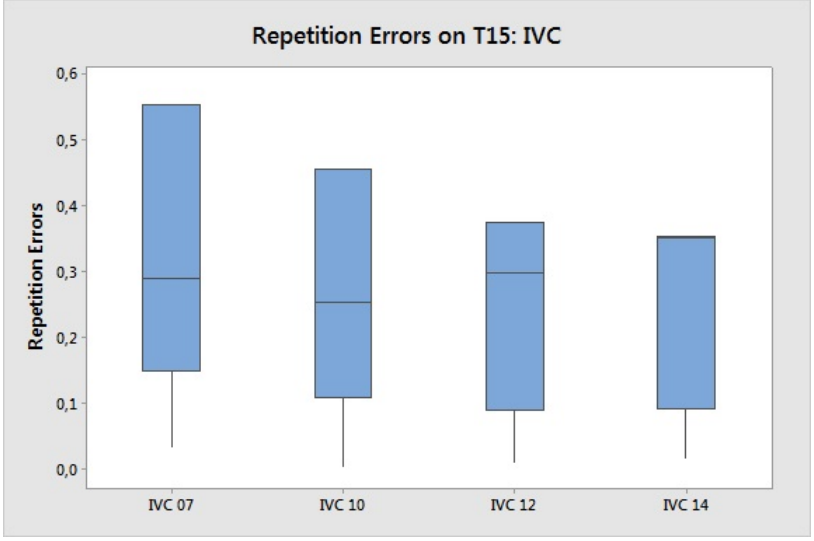

Figure 3.39: Experiment 3: Distribution of repetition errors calculated on IVC (T15)

The repetition errors on IVC seems to have a downward trend with the time. However, even at WD14, it remains not acceptable. This trend is visible also for T18 clone, shown in Figure 3.40.

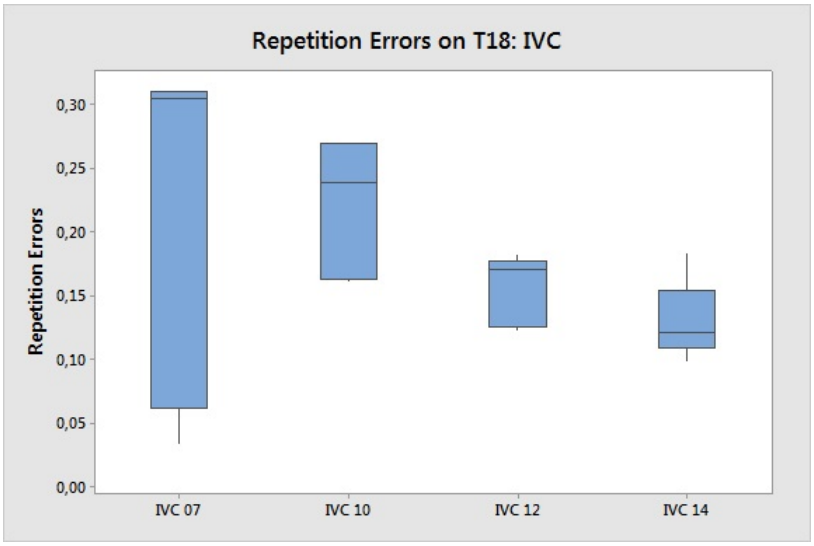

Figure 3.40: Experiment 3: Distribution of repetition errors calculated on IVC (T18)

In this case, even though the errors are quite high at WD07 and WD10, at WD12 and WD14 they become

acceptable. In Figure 3.41, the distribution of the repetition errors calculated on titer of T18 clone is shown.

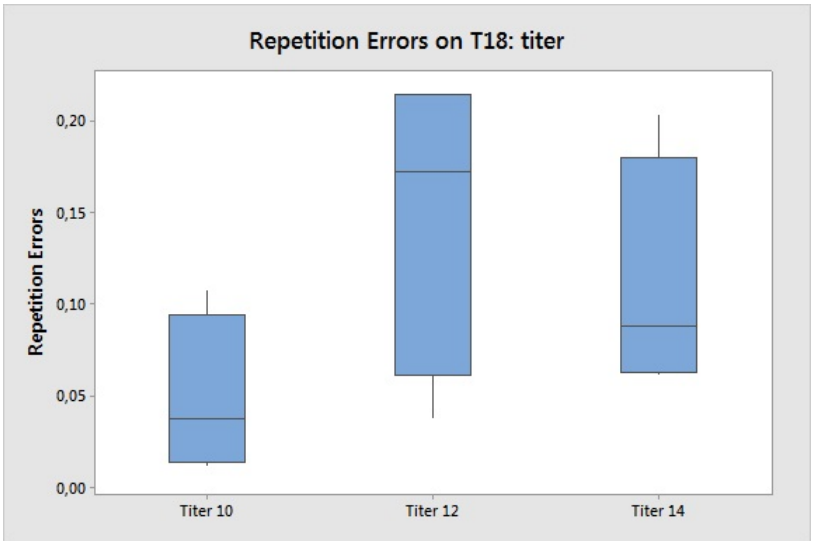

Figure 3.41: Experiment 3: Distribution of repetition errors calculated on titer (T18)

The repetition error is quite acceptable even if it is still high at WD12. For clone T25, the repetition error of the titer is shown in Figure 3.42

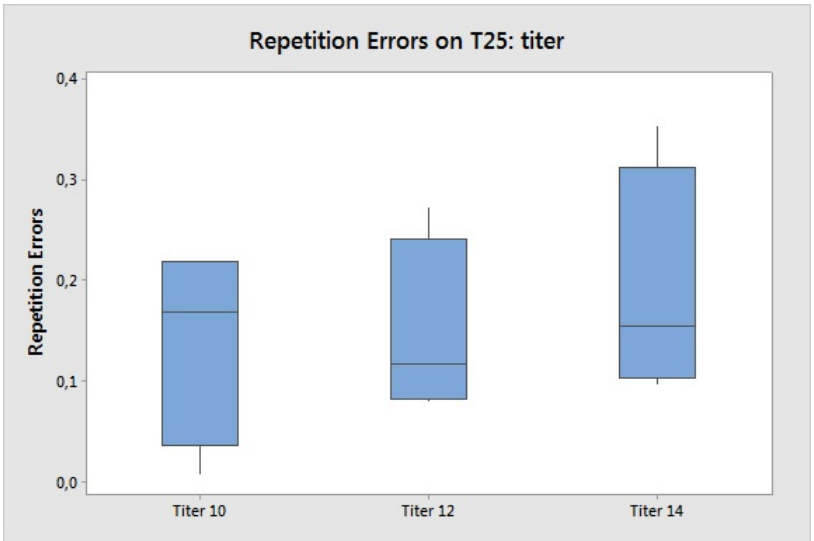

Figure 3.42: Experiment 3: Distribution of repetition errors calculated on titer (T25)

It seems to increase with the time and, expecially at WD14 is too high. Concearning the IVC, the results are reported in Figure 3.43.

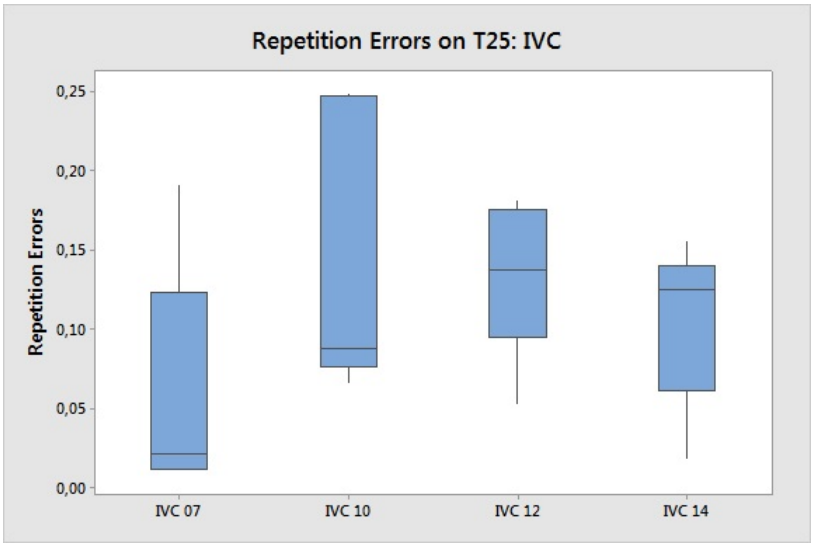

Figure 3.43: Experiment 3: Distribution of repetition errors calculated on IVC (T25)

In this case, execpt for the error at WD07, the same downward trend than the others two clones is observable. In this case it is always acceptable, except for WD10.

A conclusion can be taken: while the repetition errors calculated on titer tend to increase with the time, the ones calculated on the IVC have an opposite trend, diminishing in time. By comparing the errors obtained on the titer, T25 seems to be the most unstable clone, with a repetition error that is always unacceptable. The same comparison can be done also on the IVC. In this case the clone with the highest errors is the T15 clone.

3.4.2 DTs Analysis

The results of both, the titer and the IVC, were analyzed with DTs in order to find the best media composition. As shown at the beginning of this sections, the results are really different within the clones. This means that it is not possible to consider a DT analysis on the entire set of the results, because the major driver would be the clone. For this reason, the set of data was split into the three different clones and was analyzed separately. After an evaluation on the variation of K, it was chosen not to use the same K for all the different clones. In particular, the clones T15 and T18 were evaluated using K=10, while T25 with K=12. This decision was taken due to the fact that on clone T25, if K=10 was considered, in same cases no selections would have been found.

In this case, the results were evaluated at WD10, because the experiment 4 needed to be started on WD14 of the experiment 3.

Regarding the T15 clone, the selections obtained on the titer are reported in Figure 3.44.

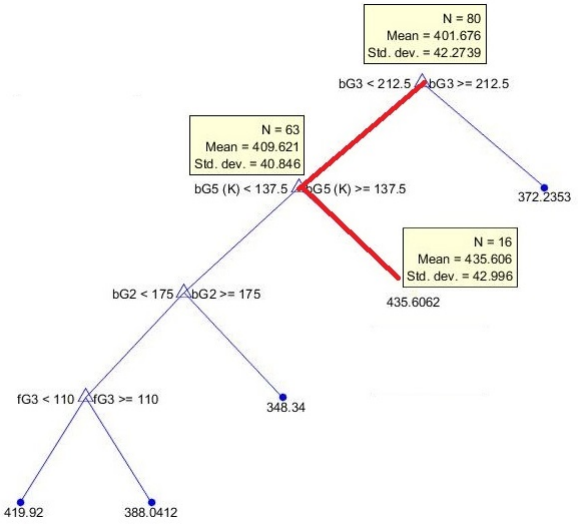

Figure 3.44: Experiment 3: DT analysis on titer at WD10 conducted on clone T15, considering K=10. When the group is preceded by a 'b' it means that it is contained in the medium, while if it is preceded by an 'f', it means that it is included in the feed. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

As shown in figure, the major drivers of this selection are the group G3 (accumulated AAs) and potassium added in the basal medium. 16 of the 80 experiments conducted with this clone have those characteristic, G3 in the medium less than 212.5 and K in the medium higher than 137.5. This means that 20% of the experiments are catalogated as the best results.

Figure 3.45 shows the same DT analysis made on the IVC at WD10 with K=10.

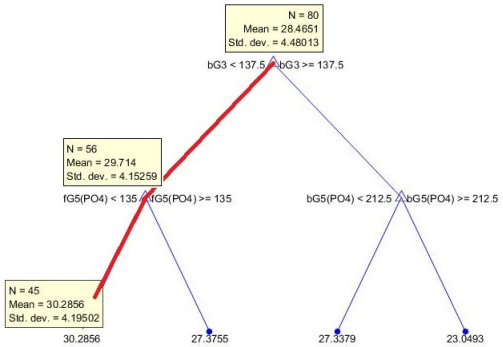

Figure 3.45: Experiment 3: DT analysis on IVC at WD10 conducted on clone T15, considering K=10. When the group is preceded by a 'b' it means that it is contained in the medium, while if it is preceded by an 'f', it means that it is included in the feed. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

As shown, the major drivers here are the group G3 in the medium and the phosphate in the feed. In this case, almost the 50% of the data are grouped in those selections. In this case, the selections obtained on

the IVC and on the titer are not completely different. In both of the cases, in fact, the major driver is the group 3 in the medium, with different selections. Although they are different, they are not in conflict with each other.

The same analysis was done on clone T18, with K=10 for both, the titer and the IVC. The results obtained on the titer are reported in Figure 3.46.

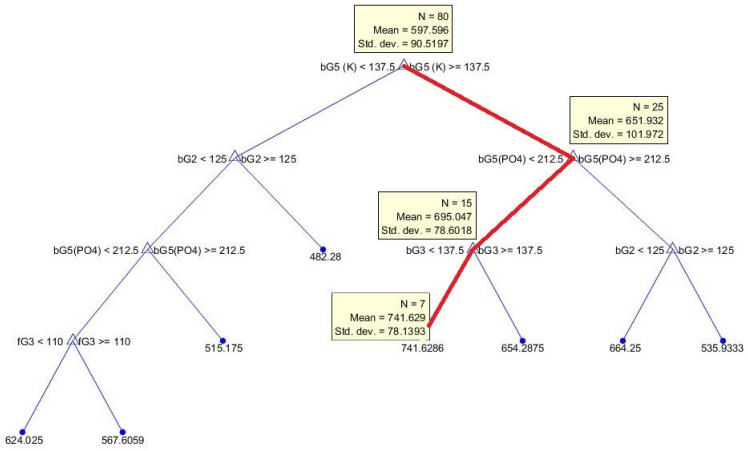

Figure 3.46: Experiment 3: DT analysis on titer at WD10 conducted on clone T18, considering K=10. When the group is preceded by a 'b' it means that it is contained in the medium, while if it is preceded by an 'f', it means that it is included in the feed. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

In this case, the feed does not seem to be important to achieve a better production. In fact, the three important selections obtained here are all based on components added into the medium. Potassium, phosphate and G3 seem to be the major drivers leading to the best productivity. However if the same analysis is performed considering K=7, the selection on G3 is not anymore present, which means that it is considered negligible. In this case, only a small part of the experiments, less than 10%, is included in these selections. Figure 3.47 shows the results of the same analysis done on the IVC at WD10.

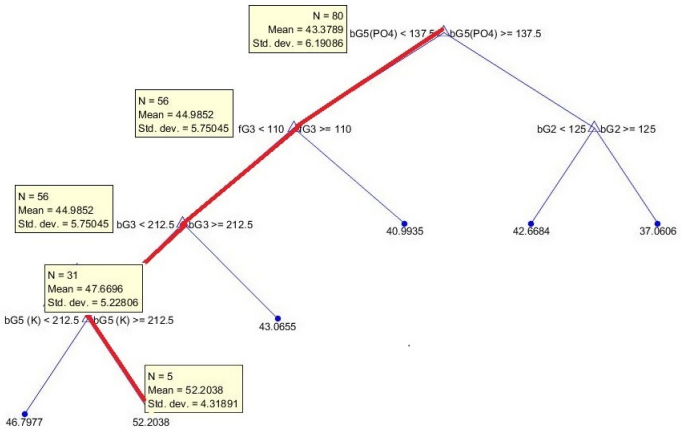

Figure 3.47: Experiment 3: DT analysis on IVC at WD10 conducted on clone T18, considering K=10. When the group is preceded by a 'b' it means that it is contained in the medium, while if it is preceded by an 'f', it means that it is included in the feed. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

Four selections are found here. Although only 5 experiments fulfil all the four best selections, the 38,75% of the experiments achieved the best results until the third selection. However, when the number of cross-validations is 5 or 7, only the first selection is present. When K=12, also the second selection appear but, since the second selection imply that G3 should not be added in the feed, it is possible to consider significative just the first one.

As mentioned early, for the clone T25, K was modified from 10 to 12 in order to have a better visualization of the selections. The results regarding the titer at WD10 are reported in Figure 3.48.

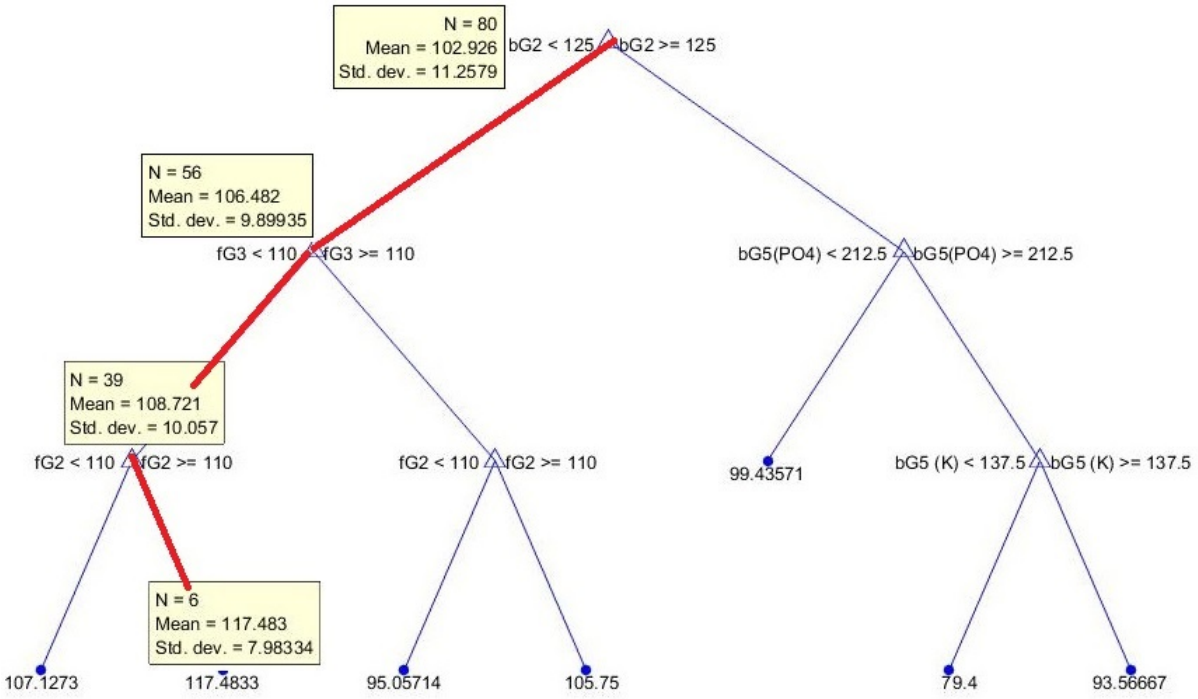

Figure 3.48: Experiment 3: DT analysis on titer at WD10 conducted on clone T25, considering K=12. When the group is preceded by a 'b' it means that it is contained in the medium, while if it is preceded by an 'f', it means that it is included in the feed. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

In this case, three selections were found as the most important ones and, almost the half of the experiments achieved the second selection. Moreover, here the feed seems to have an important role, due to the fact that 2 of the three selections are made on two groups presented in the feed, the G3 and the G2. However, the second selection means that G3 does not have to be added in the feed. Moreover, the most important selection obtained on the medium is G2<125; the G2 level in platform medium is already 100%, which is included in the first selection. It is then possible to conclude that in this case, the most important role is given to G2 in the feed. Also by analyzing the results with other values of K the same decisions are obtained, which means that no selection can be excluded.

The results obtained on the IVC are reported in Figure 3.49.

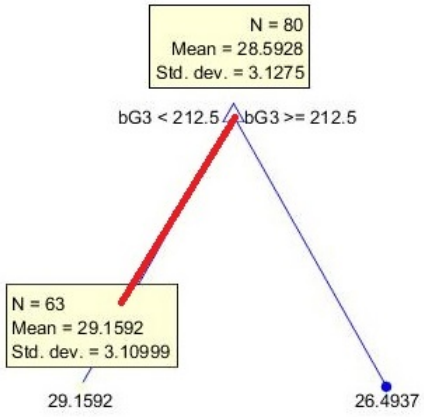

Figure 3.49: Experiment 3: DT analysis on IVC at WD10 conducted on clone T25, considering K=12. When the group is preceded by a 'b' it means that it is contained in the medium, while if it is preceded by an 'f', it means that it is included in the feed. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

In this case, just one selection was found and it separate the data set into two categories. The one in which G3 in the medium is lower than 212.5% comprehends almost the 79% of the results and it is catalogated as the best category.

An important conclusion can be taken; as for experiment zero, also in this case the selections are clone specific. There are some groups, such as G3 and G5 (which comprehends PO_4 and K), which are important for both the clones and for both the quantitities analyzed. Some others such as G2 which are important just for one clone, which in this case is T25.

3.4.3 Variation of the Selections with the Time

The results of the DTs analysis shown previously, are summarized in Table 3.9.

	IVC			Titer		
Group	T15	T18	T25	T15	T18	T25
G2_medium						<125 (1)
G3_medium	<137.5 (1)	<212.5 (3)	<212.5 (1)	<212.5 (1)	<137.5 (3)	
K_medium		>212.5 (4)		>137.5 (2)	>137.5 (1)	
PO_4_feed	<135 (2)					
PO_4_medium		<137.5 (1)			<212.5 (2)	
G3_feed		<110 (2)				<110 (2)
G2_feed						>110 (3)

Table 3.9: Experiment 3: Summary of DTs analysis at WD10

This table reports all the selections obtained for each clone on both, titer and IVC. The numbers in brackets in the table indicate the order of the selections. As mentioned before, the analysis were made

on WD10 due to the need to directly proceed on WD14 with the next experiment. For this reason it was decided to evaluate the variation of the selections with the time, in order to see if the selections at WD10 are equal to the one at WD12 and WD14.

The comparison for clone T15 is reported in Table 3.10.

	IVC			Titer		
Time	WD10	WD12	WD14	WD10	WD12	WD14
G3_medium	<137.5 (1)	<212.5(1)	<212.5(1)	<212.5 (1)	<137.5 (3)	<212.5 (2)
K_medium				>137.5 (2)	>137.5 (1)	>137.5 (1)
PO_4_feed	<135 (2)					
PO_4_medium					<212.5 (2)	<212.5 (3)

Table 3.10: Experiment 3: Comparison on the selections at different time for clone T15

By looking at the table, it is observable that, regarding the IVC, the selections do not change drastically. The major driver is always the group 3, but with different thresholds at WD12 and WD14 compared to WD10. Moreover, at WD10 there is one selection more that does not appear later in time. Regarding the titer, the same conclusion can be taken: the selections do not change significantly; however, what changes, is the order of the selections, expecially between WD10 and WD12. Table 3.11 reports the same comparison made on the selections of clone T18.

	IVC			Titer		
Time	WD10	WD12	WD14	WD10	WD12	WD14
G3_medium	<212.5 (3)		<212.5 (4)	<137.5 (3)		
K_medium	>212.5 (4)		>137.5 (3)	>137.5 (1)	>137.5 (1)	>137.5 (1)
PO_4_medium	<137.5 (1)	<212.5 (1)	<212.5 (2) <137.5 (5)	<212.5 (2)	<212.5 (2)	<212.5 (2)
G3_feed	<110 (2)	<110 (2)	<110 (1)			

Table 3.11: Experiment 3: Comparison on the selections at different time for clone T18

Also in this case the selections do not change drastically in time. For the IVC, the selections obtained at WD12 are comparable to the ones obtained at WD10, that present two less important selections more. At WD14 the selections change in order and not much in values. Regarding the titer, instead, the selections are equal at all the time, with just a less important selection more at WD10. Regarding the clone T25, instead, the results are reported in Table 3.12.

	IVC			Titer		
Time	WD10	WD12	WD14	WD10	WD12	WD14
G2_medium		<175 (6)		<125 (1)	<125 (1)	
G3_medium	<212.5 (1)	<212.5 (1)	<212.5 (1)			
K_medium		>137.5 (7)				>137.5 (1)
G8_medium		<137.5 (3)	<137.5 (3)			
PO_4_medium		<212.5 (4)	<212.5 (4)			<212.5 (2)
G3_feed		<110 (2)	<110 (2)	<110 (2)	<110 (2)	
G2_feed				<110 (3)		
G8_feed		<110 (5)	<110 (5)			

Table 3.12: Experiment 3: Comparison on the selections at different time for clone T25

For this clone, the selections do not change concearning the IVC. At WD12 the number of selections (7) is slightly higher than at WD10, where just one selection was found. At WD14 the number of selections is a little lower (5) but the order and the values are equal to those at WD12. Regarding the titer, instead, although the selections are equal at WD10 and WD12, at WD14 they completely change.

In conclusion, in this experiment, the selections do not change drastically in time, excepted for titer in T25 clone. So, for that, the selections obtained at WD10 are considered reliable. For this reason, a sequential approach can be thought. In particular, it si possible to design the subsequent experiment already on day 10, being able to do more experiments with the same cells and the same media.

3.4.4 Conditions Comparison

The results were also evaluated in order to see if the platform conditions had better results compared to the other conditions. The results regarding the IVC are reported in Figure 3.50 for clone T15.

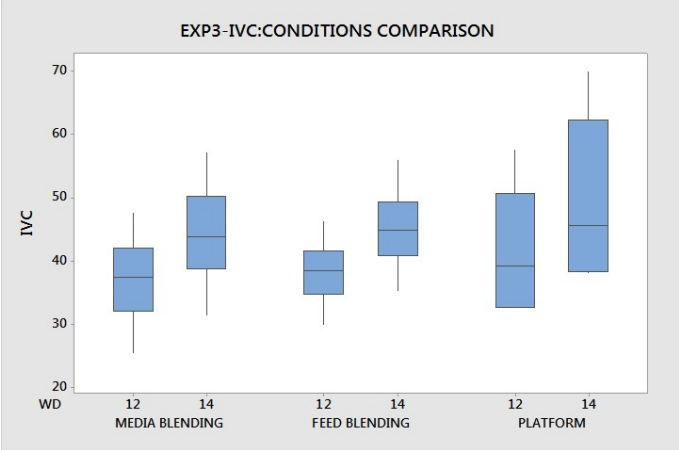

Figure 3.50: Experiment 3: Condition comparison on IVC on T15

The means of all the different conditions are equal but the third quartile is slightly higher for the platform condition. In Figure 3.51, the same distribution is reported for clone T18;

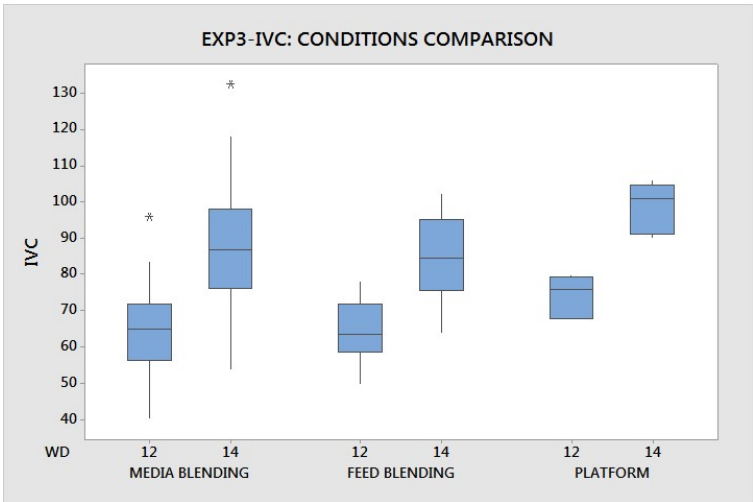

Figure 3.51: Experiment 3: Condition comparison on IVC on T18

In this case, instead, the platform condition is already higher at WD12, and, moreover, neither the means are comparable. Finally, for clone T25, the results are shown in Figure 3.52.

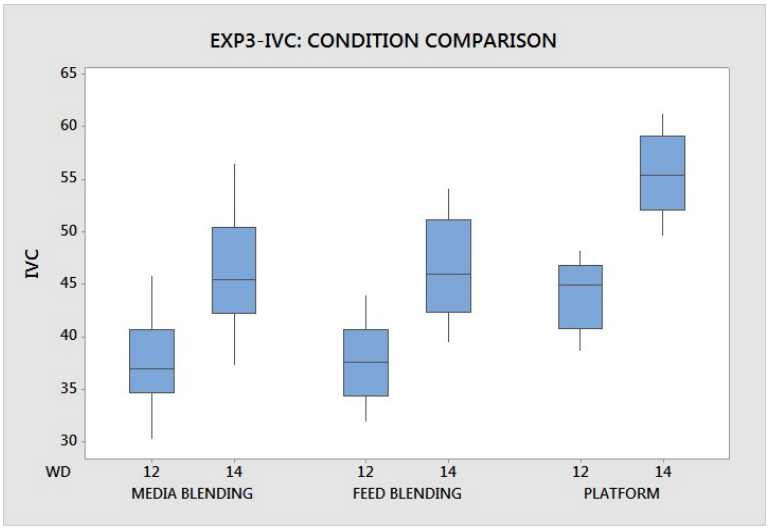

Figure 3.52: Experiment 3: Condition comparison on IVC on T25

The same results obtained for clone T18 are found. Also in this case, the platform condition is slightly better. In conclusion, regarding the IVC, for all the clones, platform medium and feed seem to have better results. Concearning the titer, instead, the results for clone T15 are reported in Figure 3.53.

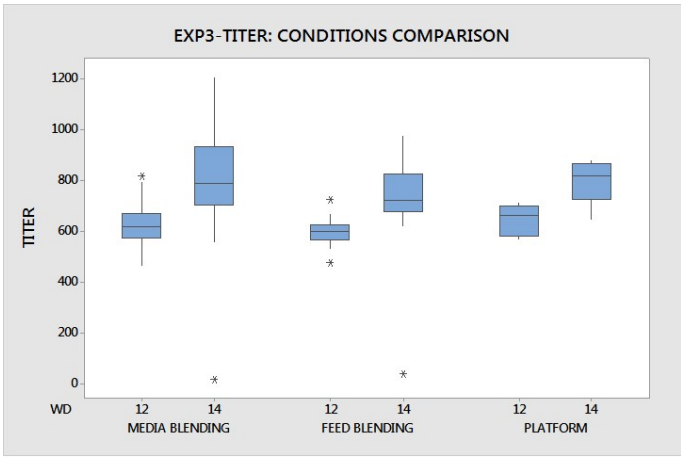

Figure 3.53: Experiment 3: Condition comparison on titer on T15

In this case, the platform conditions and the media blending ones seem to be comparable. In terms of mean, the platform conditions seem to be better but 75% of the results are higher when the media are blended. Feed blending, instead, is not relevant for this clone. Figure 3.54 shows the comparison between the platform medium and the selections obtained.

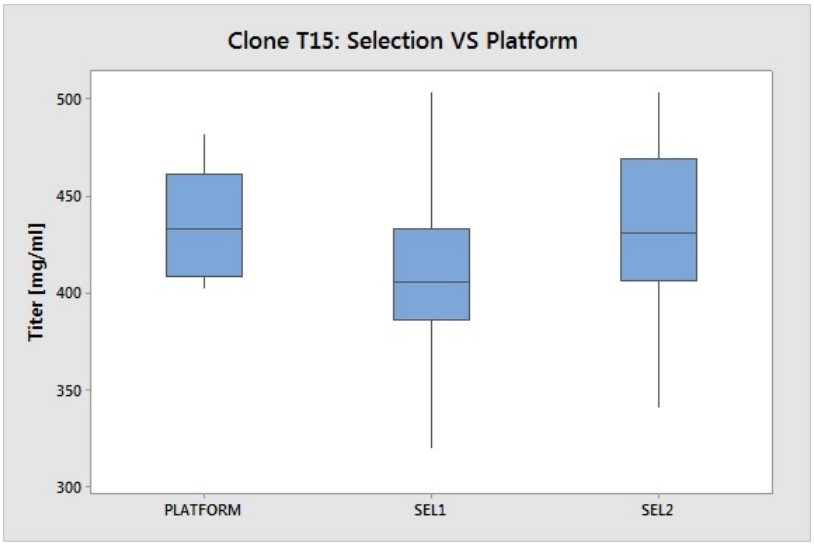

Figure 3.54: Experiment 3: Comparison between the results obtained in standard condition and the ones obtained with the selections (Clone T15)

In figure, the distribution of the titer results at WD10 is shown. When the first selection is applied, no beneficial effects are obtained compared to the platform. However, with the second selection, an increase in the results is obtained but the mean rest in line with the platform. This demonstrates that, although the results obtained with the selections are not slightly better than the one achieved with the platform, there is at least one condition in which the productivity is higher. In fact, the lines that represent the 75% of the results and the upper whisker are higher in the second selection. The comparison within the

conditions for the clone T18 is reported in Figure 3.55.

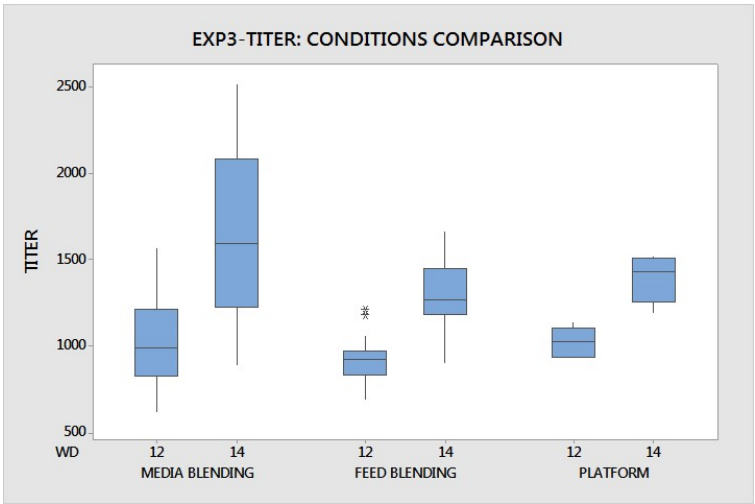

Figure 3.55: Experiment 3: Condition comparison on titer on T18

Here, media blending results are slightly higher than platform conditions. Feed blending, instead, do not seem to have beneficial effects on the titer. The same results can be represented in terms of selections, as in Figure 3.56.

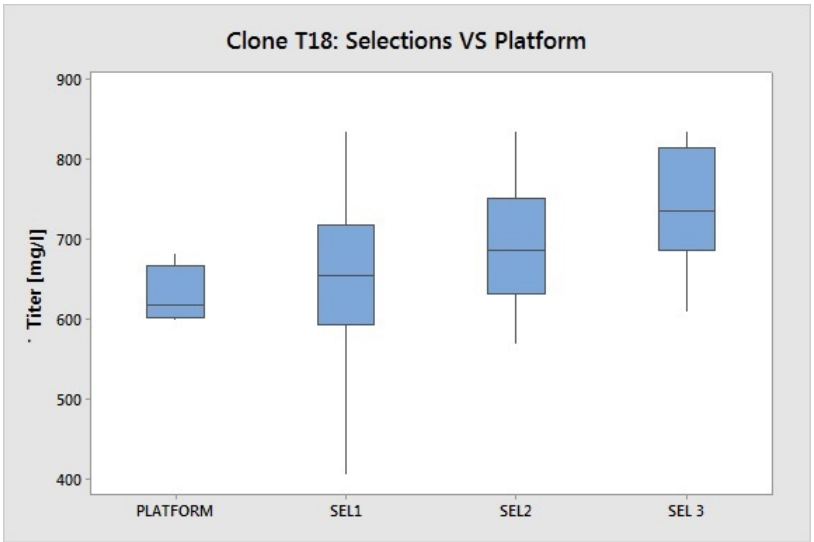

Figure 3.56: Experiment 3: Comparison between the results obtained in standard condition and the ones obtained with the selections (Clone T18)

This figure shows how the results obtained in standard condition can be slightly improved by applying the selections obtained with DTs analysis. Also in this case, then, it was verified that the productivity of a particular clone can be improved with DTs analysis. The results relative to the condition comparison on clone T25 are shown in Figure 3.57

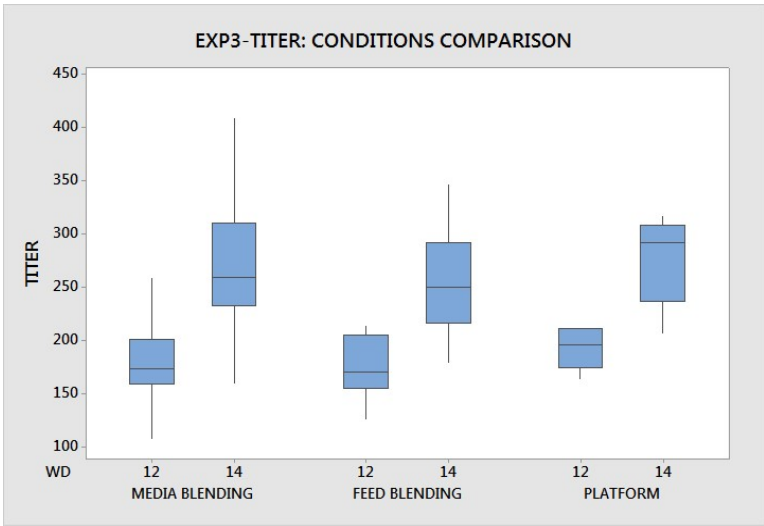

Figure 3.57: Experiment 3: Condition comparison on titer on T25

In this case, platform medium, media and feed blending are comparable. This means that this clone does not strongly respond to environmental changing, as clone T18. However, if all the selections obtained on this clone are applyed, the results can be slightly improved, as reported in Figure 3.58.

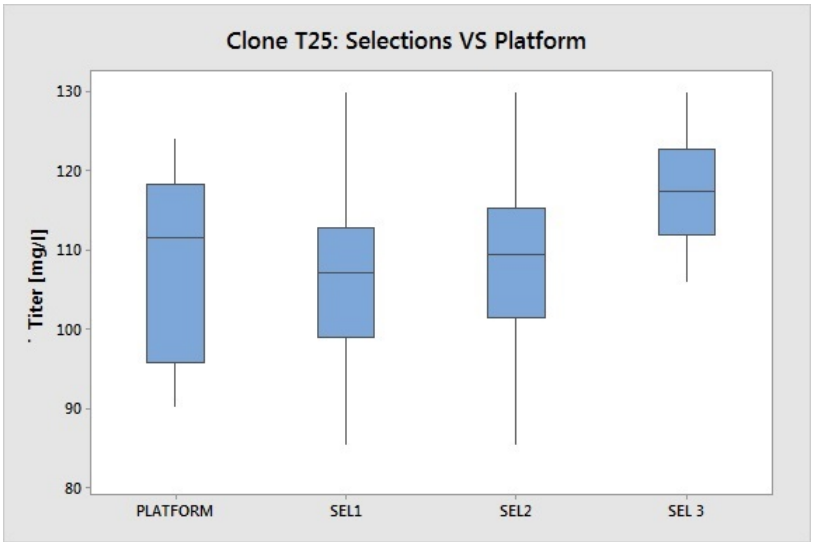

Figure 3.58: Experiment 3: Comparison between the results obtained in standard condition and the ones obtained with the selections (Clone T25)

Again, as for the other clone, it is proved that DTs analysis on the obtained results can let to achieve an higher productivity.

3.5 Results of Experiment 4

As described in Section 2.6.4, this experiment was performed with the same three clones of experiment 3, T15, T18 and T25. The design of this experiment was done according to the results obtained at WD10 of experiment 3.

The VCD evolution is reported in Figure 3.59.

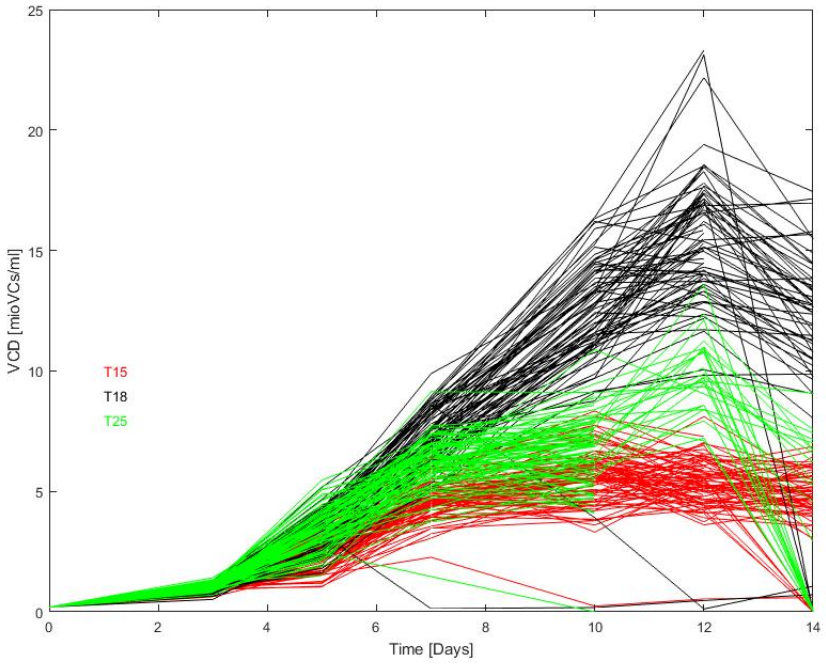

Figure 3.59: Experiment 4: VCD evolution in time. As explained in the legend, the red lines are related to the clone T15, the black ones to the T18 while the green ones to the T25 clone.

As shown in figure, the same results as experiment 3 were obtained. T18 is the clone that grows more, while T25 and T15 are comparable until WD10. After WD10 the T25 clone continue to grow, while T15 rest constant in time. By looking at the image it is possible to notice that some lines, expecially green line, are interrupted after WD10 or after WD12. This is due to some problems encountered with the Guava device at WD12 and WD14. Due to these problems, some results were lost; in particular 11 results of the first plate containing T15 and 22 results of the second plate containing T18 were lost at WD14; regarding the third plate, containing T25, only the results of 25 wells are complete. All the others are lost already at WD12. For this reason, the analysis on the IVC of T25 clones are done at WD10, while for the others two clones they were analyzed at WD12. It is possible to compare the VCD results obtained here, with the one obtained in experiment 3, reported in Figure 3.34. One difference from the experiment 3, is that is this case there is not a drop in VCD at WD12; moreover, regarding clone T25, an higher maximum value is obtained. In fact, while the peak achieved in experiment 3 was around 10 mioVCs/ml, in experiment 4 it reached a value of 15 mioVCs/ml. Also for clone T18 the results of the VCD are improved; however,

the achieved peak is quite the same (around 25 mio VCs/ml), but in this case, the number of wells who achieved this results are higher. No differences in terms of VCD are instead registered for clone T15. The IVC evolution is shown in Figure 3.60.

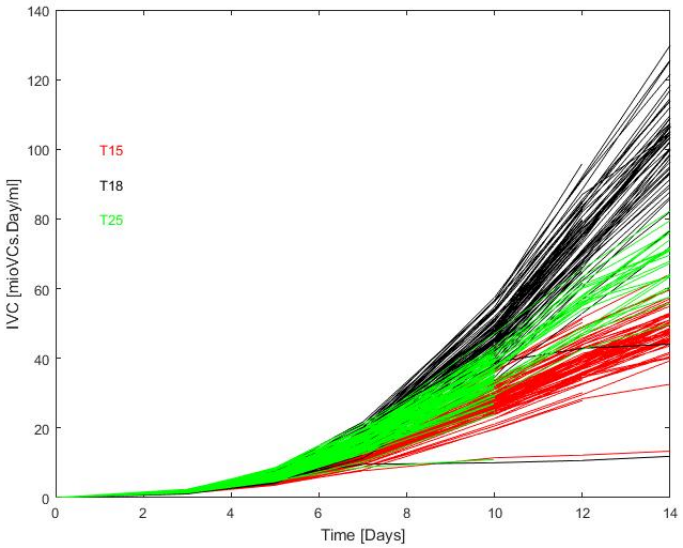

Figure 3.60: Experiment 4: IVC evolution in time. As explained in the legend, the red lines are related to the clone T15, the black ones to the T18 while the green ones to the T25 clone.

As mentioned already before, also in this case some lines are interrupted. Regarding the reliable data, T18 is the clone that has the higher IVC followed by T25 and T15. This is in line with the results obtained in the previous experiment. The same comparison as for the VCD can be done with Figure 3.35. As for the VCD, the results improved for clone T25. For the other two clones, instead, the range of IVC remained constant.

The comparisons on the titer and on the PCD between the clones are here omitted due to the fact that the results are analoguous to experiments 3 (cf. Section 3.4).

3.5.1 Repetition Errors

Also in this case, one conditions with platform feed and medium enriched in potassium, was replicated four times. This allows to compare the repetition errors obtained in the different clones. The repetition errors obtained on titer for clone T15 are reported in Figure 3.61.

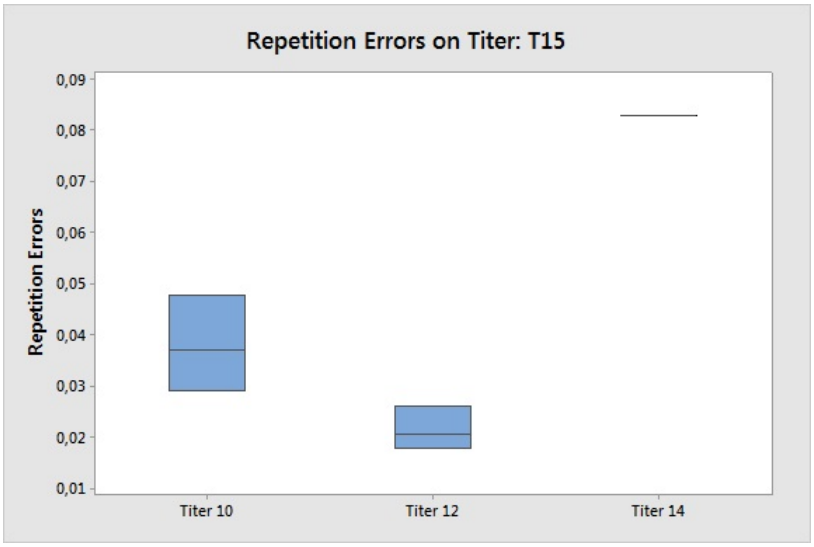

Figure 3.61: Experiment 4: Repetition errors obtained on titer for clone T15. At WD14 it is represented with just a line because of sampling problem. In particular, the quantity of culture that was sampled was not enough in some wells, so that the quantity of mAbs was not detectable in two of the four wells.

This errors is very low in any time, being less than 10%. At WD10 and WD12 the distributions are even lower than 5%. At WD14 it is represented with just a line due to sampling problems with the Biomek. In particular, the quantity of culture sampled was evidentely not enough in some wells and this led to have a 'Too Low' quantity of mAbs in some wells of the plate. It happened, in fact, that the tips of the Biomek did not achieved the right level of liquid and so, they did not aspirate the required quantity of culture for the analysis. For that, the titer was not detectable by the Octet. The same analysis on the replicates was done for clone T18, the results of which are shown in Figure 3.62.

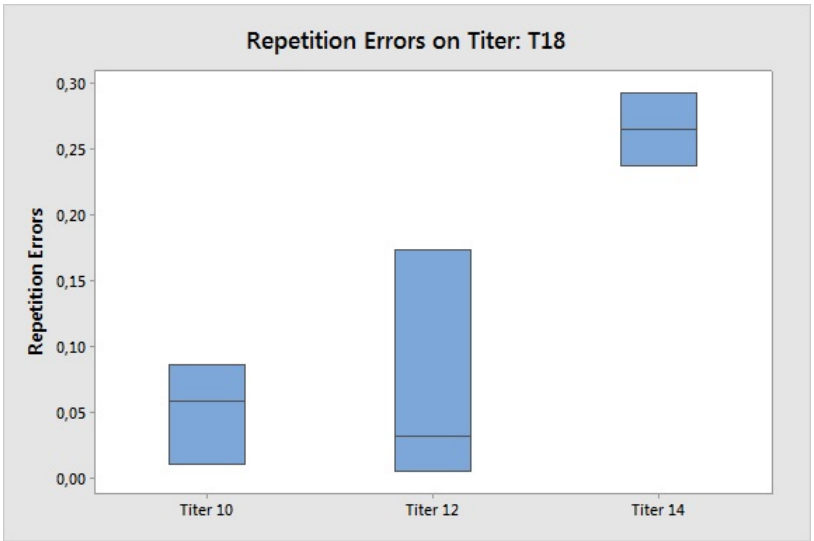

Figure 3.62: Experiment 4: Repetition errors obtained on titer for clone T18

In this case, the errors are higher compared to clone T15, but they are acceptable, excepted for WD14. At WD10 and WD12, in fact, the entire distributions are under 20% and, if just the mean is considered, it is even lower than 5% at WD12 and around 6% at WD10. At WD14, the errors slightly increase, becoming not acceptable. Finally, the results for clone T25 are reported in Figure 3.63.

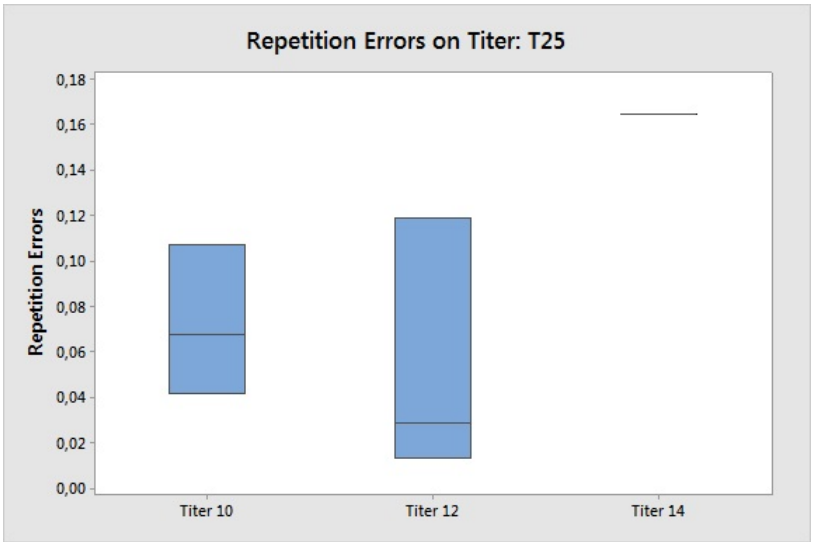

Figure 3.63: Experiment 4: Repetition errors obtained on titer for clone T25

Also in this case the errors are always acceptable, being always less than 20%. In terms of mean, at WD10 the error is 7% and it decrease at WD12 becoming 3%. At WD14 it is represented with just one line at a level of 17%. The analysis of the errors was done also on the IVC on all the clones. The results concearning the T15 clone are reported in Figure 3.64.

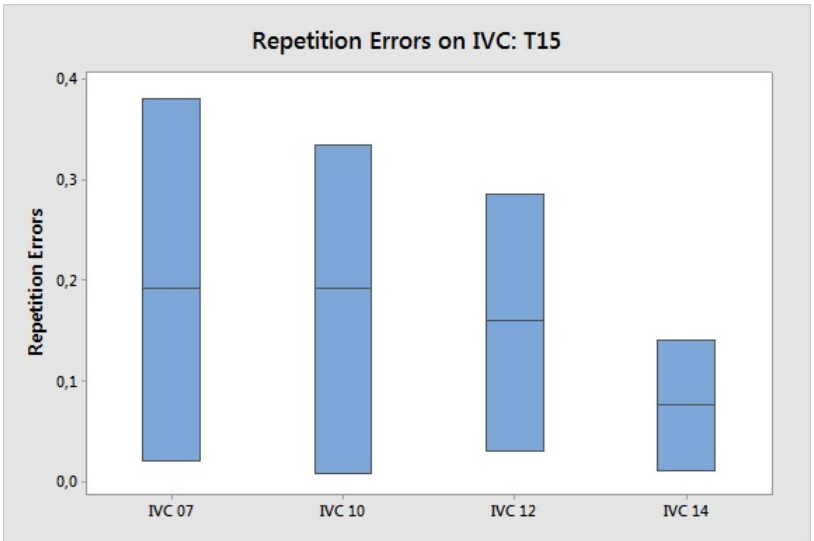

Figure 3.64: Experiment 4: Repetition errors obtained on IVC for clone T15

As in experiment 3, the errors have a deceasing trend with the time. In this case, the error becomes acceptable just at WD14, while in the rest of time is still high, with a third quartile which pass the 30%. Different results are obtained for clone T18; they are shown in Figure 3.65.

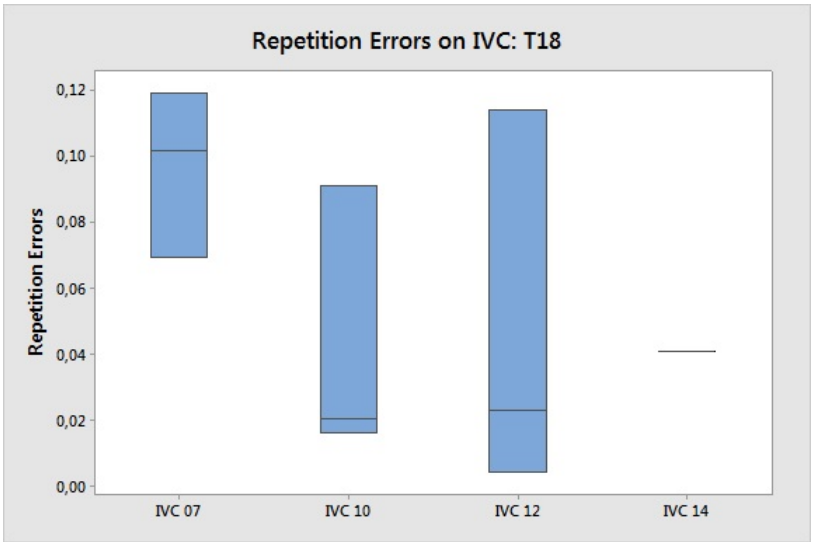

Figure 3.65: Experiment 4: Repetition errors obtained on IVC for clone T18

In this case, the repetition errors are slightly lower than for clone T15. Also in this case it seems to have a downward trend with the time, but it is less marked. Excepted for WD07, the errors are always less than 10% with the means at around 3% at WD10 and WD12. At WD14 it is represented with just one line due to loss of data; the only one reliable result is anyway around 4%. Regarding T25, the repetition results were available only at WD07 and at WD10. They are reported in Figure 3.66.

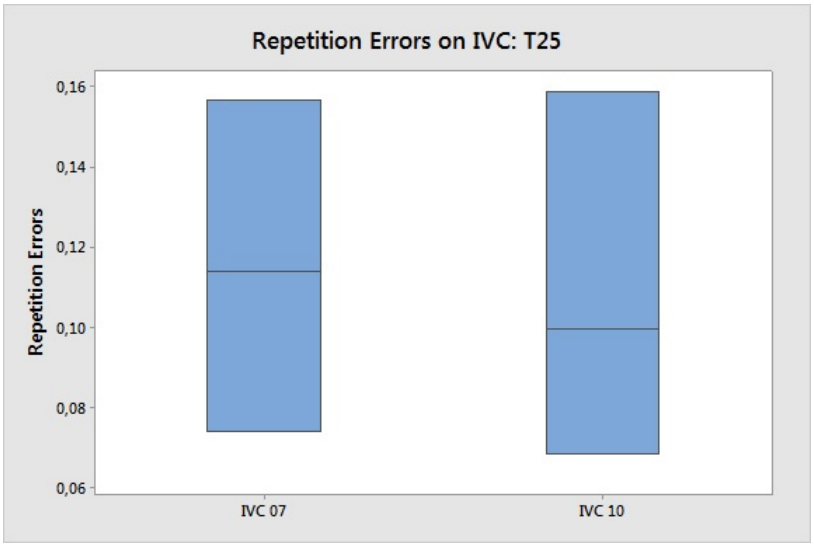

Figure 3.66: Experiment 4: Repetition errors obtained on IVC for clone T25

In this case, the errors are acceptable in both the WDs. The mean decreases from 11.5% at WD07 to 10% at WD10.

As for experiment 3, here it was confirmed that regarding the IVC, the repetition error tends to decrease with the time.

3.5.2 DTs analysis

As for the previous experiments (experiment 2 and 3), all the results were analyzed with DTs analysis. As mentioned already at the beginning of this section, the results of the IVC of clone T25 were analyzed at WD10, while all the other results were done based on the results at WD12, due to the fact that some results were lost at WD14 regarding both, the titer and the IVC. For clone T15, due to the fact that no selections were obtained at WD12 regarding the IVC, WD14 is considered. The results obtained on the IVC are reported in Figure 3.67.

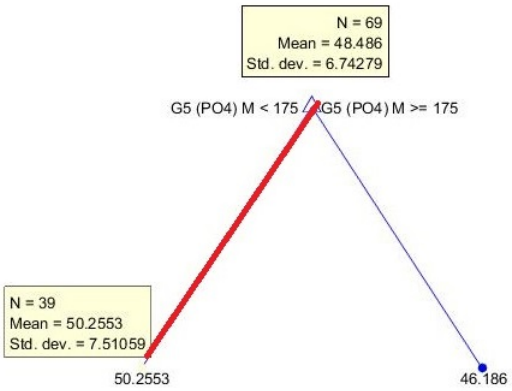

Figure 3.67: Experiment 4: DTs obtained at WD14 on the IVC of clone T15 considering K=7 When the group is followed by a 'M' it means that it is contained in the medium, while if it is followed by an 'F', it means that it is included in the feed. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

The results are obtained considering K=7. In the first node N is equal to 69 instead of 80 because as mentioned before some data were lost at WD14. As shown by the red pathway, just one selection is obtained on phosphate. With this selection, the data set is splitted into two categories; the one with PO_4 in the medium minor than 175% has better results in terms of IVC. Regarding T18, the selection on the IVC at WD12 are shown in Figure 3.68.

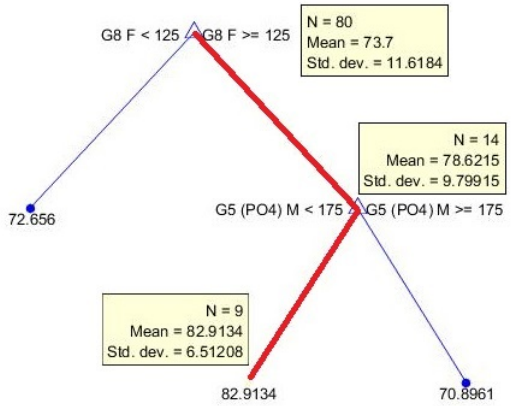

Figure 3.68: Experiment 4: DTs obtained at WD12 on the IVC of clone T18 considering K=12. When the group is followed by a 'M' it means that it is contained in the medium, while if it is followed by an 'F', it means that it is included in the feed. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

In this case, it was chosen to have K=12 due to the fact that K=10 did not lead to a selection on the IVC. The selection obtained here are two, one on the group 8 in the feed and the other on phosphate in the medium. In this case, just the 11% of the experiments achieved this level of the selection. As mentioned before, for clone T25, the considered results were the one at WD10, which are shown in Figure 3.69.

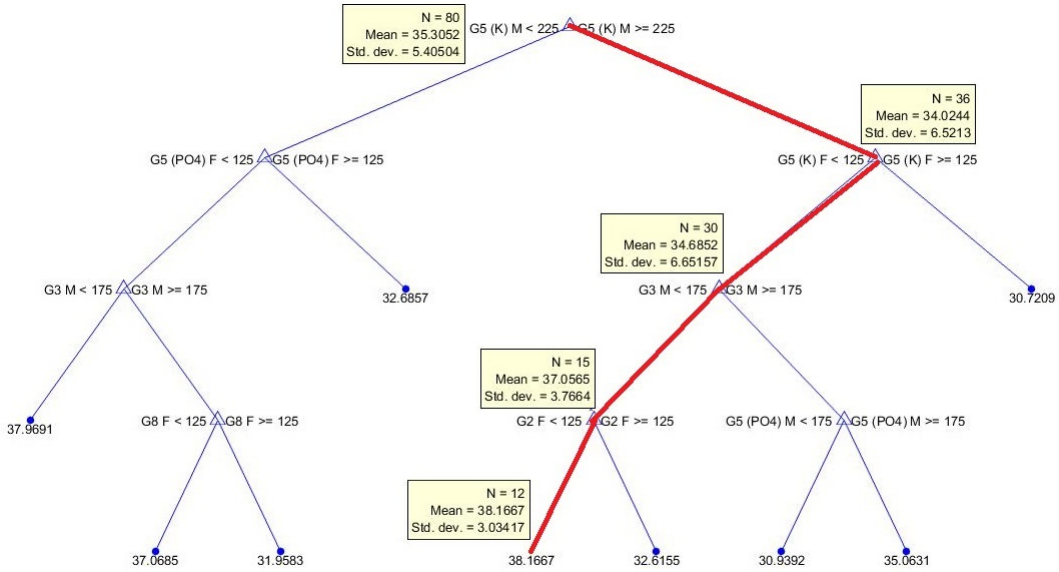

Figure 3.69: Experiment 4: DTs obtained at WD10 on the IVC of clone T25 considering K=10. When the group is followed by a 'M' it means that it is contained in the medium, while if it is followed by an 'F', it means that it is included in the feed. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

Four selections were found on the IVC of T25 clone. The major driver is K in the medium, which has to

be higher than 225%. Potassium in the feed do not seem to give beneficial effects on the IVC. K in the feed has, indeed, to be less than 125%, which means at the same level of the platform feed. The last two selections are less important and they are on the group 3 in the medium and the group 2 in the feed. The same analysis for the titer is reported in Figure 3.70 for clone T15.

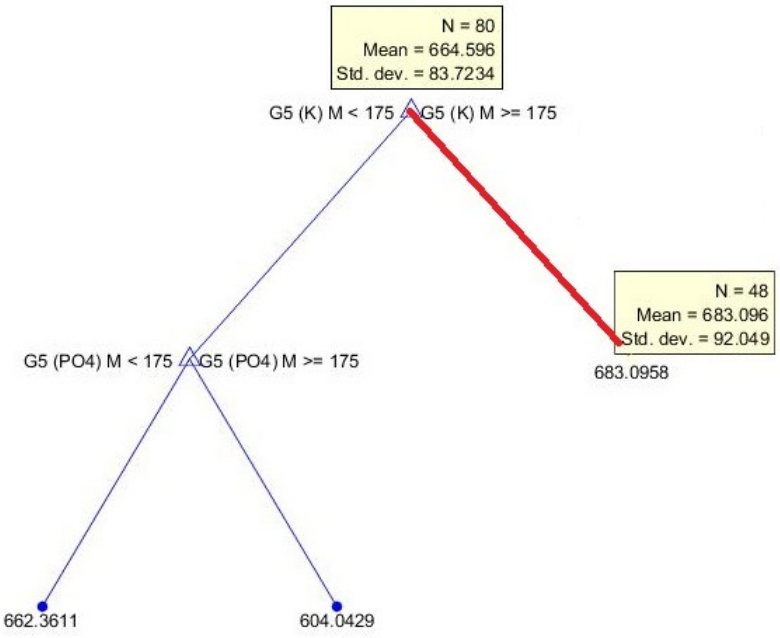

Figure 3.70: Experiment 4: DTs obtained at WD12 on the titer of clone T15 considering K=7. When the group is followed by a 'M' it means that it is contained in the medium, while if it is followed by an 'F', it means that it is included in the feed. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

In this case, the only factor which seems to be important for the titer is potassium in the medium. It has to be higher than 175% and 48 of the 80 experiments verified this condition. Also for clone T18 the most important component is K. The results are shown in Figure 3.71.

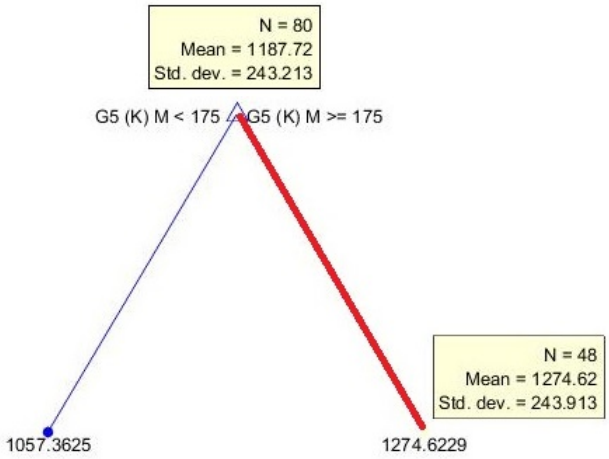

Figure 3.71: Experiment 4: DTs obtained at WD12 on the titer of clone T18 considering K=12. When the group is followed by a 'M' it means that it is contained in the medium, while if it is followed by an 'F', it means that it is included in the feed. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

The selection is the same as for clone T15. This means that this component is clearly important for all the CHO-K1 clones. Although it is not important for the IVC of clones T15 and T18, it is the major driver for the IVC of clone T25 as well as for the titer of T15 and T18. The same results are not obtained for clone T25 regarding the titer. They are shown in Figure 3.72.

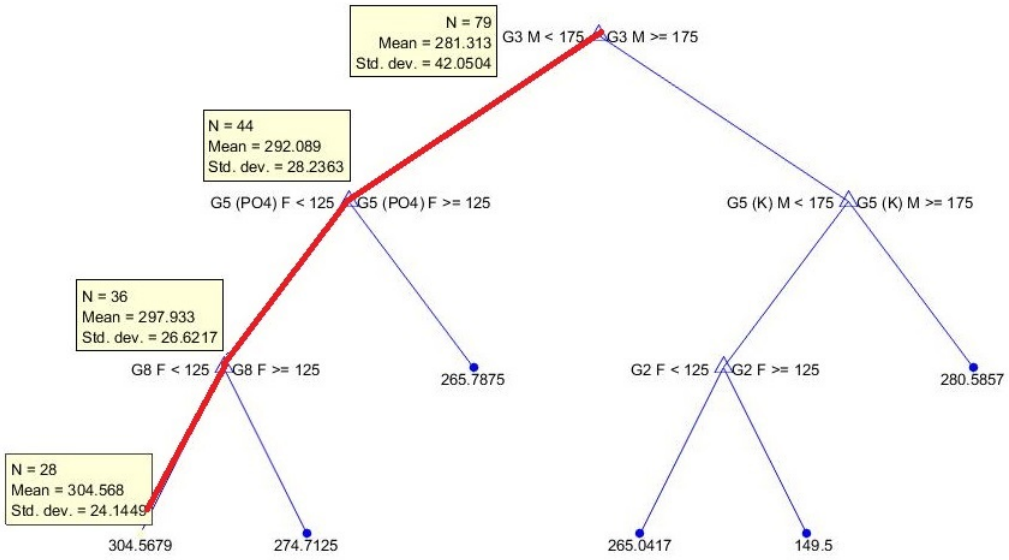

Figure 3.72: Experiment 4: DTs obtained at WD12 on the titer of clone T25 considering K=10. When the group is followed by a 'M' it means that it is contained in the medium, while if it is followed by an 'F', it means that it is included in the feed. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

In this case, in fact, three selections were found, but K does not appear as the most important. However, although it is not present in the left branch, it appears in the right one as the second selection. This means that it remains an important factor.

3.5.3 Variation of the Selections with the number of cross validation K

As mentioned in Section, different K were evaluated in order to find the one that was able to find some selections in all the analyzed quantities. While this analysis was being done, an important fact was seen. In some cases, the variation of K led to a variation in the selection pathway. In particular, this phenomenon came out for the clone T25, in both, the IVC and titer.

Table 3.13 summarizes the selections obtained for clone T25 when K was modified.

IVC 10		Titer 10	
K=5 K=7	K_m $<$ 225(1) PO_4_f $<$ 125(2)	K=10	G3_m $<$ 125(1)
K=10 K=12	K_m $>$ 225(1) K_f $<$ 125(2) G3_m $<$ 175(3) G2_f $<$ 125(4)	K=5 K=7 K=12	G3_m $>$ 125(1) K_m $>$ 175(2) G2_f $>$ 125(3)

Table 3.13: Comparison on the selections obtained with different values of K

The reported results are the ones concearning the IVC and the titer at WD10. The results at WD12 and 14 are omitted because no variations with K were seen. The evaluated values of K were 4: 5, 7, 10 and 12. As shown in table, concearning the IVC different selections are obtained. When K is equal to 5 or 7, potassium in the medium needs yo be less than 225%, while when K is equal to 10 or 12, it has to be higher than 225%. As mentioned before, for this clone it was chosen to evaluate the results of the IVC at WD10, due to loss of data from WD12. Since different selections were encountered, it was chosen to evaluate the available results at WD12 and WD14 in order to see if the selections obtained in those days were more in line with those obtained with a lower value of K or an higher one. Since at WD14, it was found that potassium needs to be higher than 175%, it was chosen to keep K=10.

Concearning the titer, two different selections were find for G3 in the medium. In particular, if K=10, G3 needs to be lower than 125%, while with all the other values of K, it has to be higher than 125%. In this case, since the results were evaluated at WD12 to obtained the selections, no importance was given to this difference. No variation in the selections are, in fact, present at WD12 and WD14.

3.5.4 Comparison with Experiment 3

Experiment 3 and experiment 4 were performed with the same clones. Theoretically, experiment 4 was designed starting from the results obtained at WD10 on experiment 3. The principal difference between the two experiments is that in experiment 4, also a combination between media blending and feed blending is considered. A comparison was done on both, the IVC and the titer. Figure 3.73 shows the comparison on titer at WD12 for each clone.

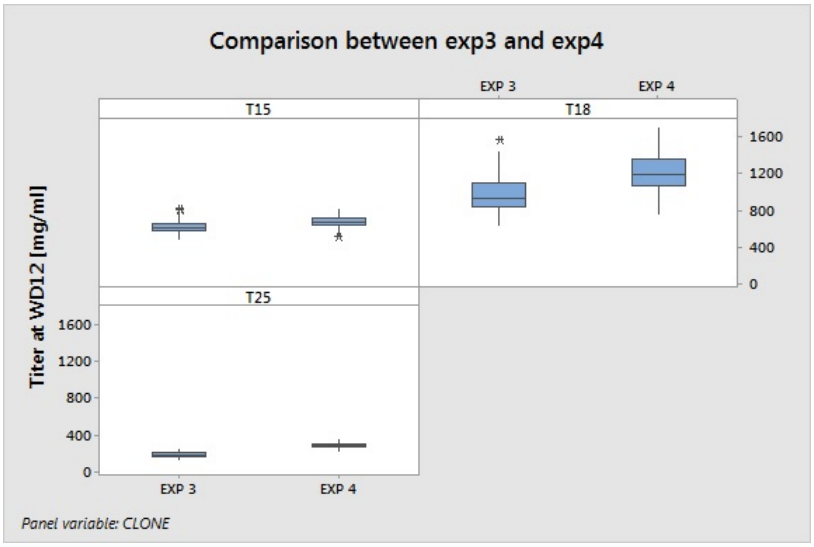

Figure 3.73: Comparison between the results of titer at WD12 for each clone

Each panel of the graph, corresponds to a different clone. For all the clones, the results improved from experiment 3 to experiment 4. This means that, even if the design was obtained starting from WD10, the obtained selections were reliable, as it was concluded in Section 3.4.3. Figure 3.74 reported the same comparison on IVC at WD10.

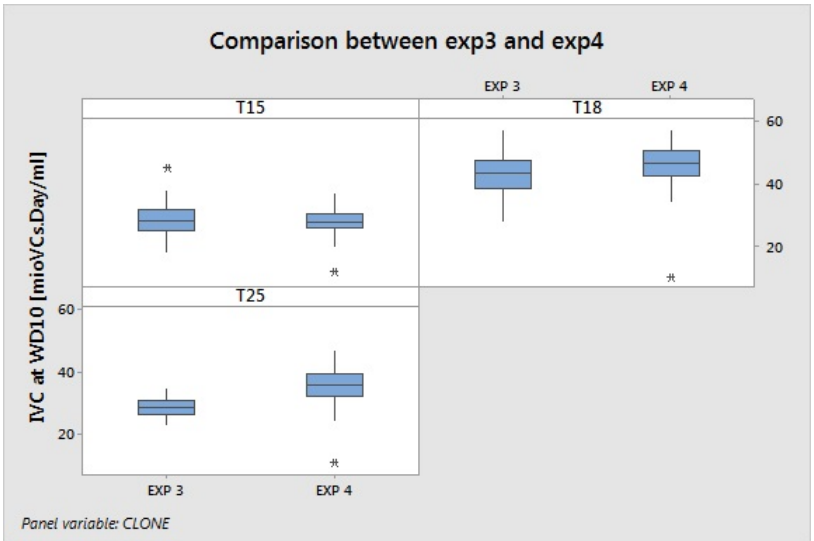

Figure 3.74: Comparison between the results of the IVC at WD10 for each clone

In this case, the results improved for clones T18 and T25, while they rested constant for clone T15. In order to understand by what this improvement of the results is caused, it was chosen to evaluate the differences in distributions among the different conditions. The comparison on titer for clone T15 is reported in Figure 3.75.

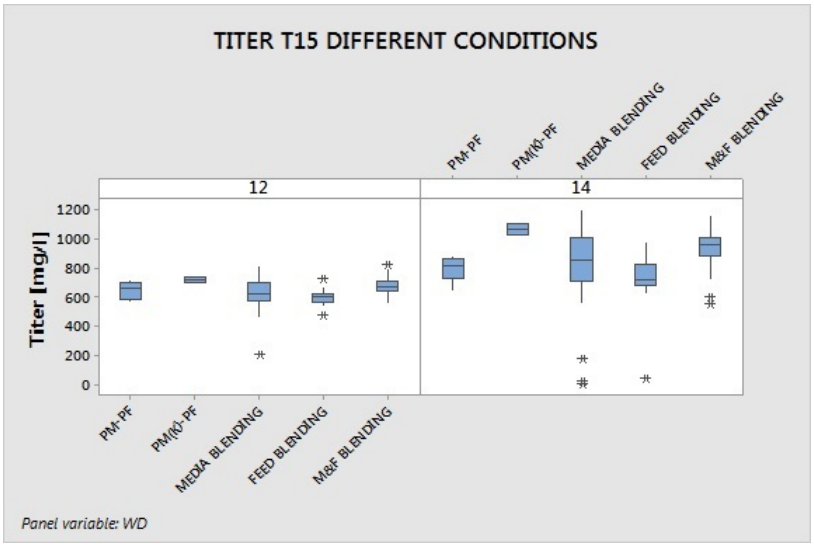

Figure 3.75: Condition comparison on titer for clone T15. PM-PF is referred to platform medium and platform feed; the K in the brackets means that the platform medium is enriched just of potassium. M&F blending, instead, means that both, the media and the feed, are blended

In this figure, the distribution obtained with the different conditions are reported. In particular, PM-PF is referred to platform medium and platform feed; the K in the brackets means that the platform medium is enriched just of potassium. M&F blending, instead, means that both, the media and the feed, are blended. By looking at the image, one important conclusion can be found. It is clear, in fact, that the results are better when potassium is included in the platform medium. The conditions in which just K is added seem to be better than all the other conditions. This is visible also in Figure 3.76.

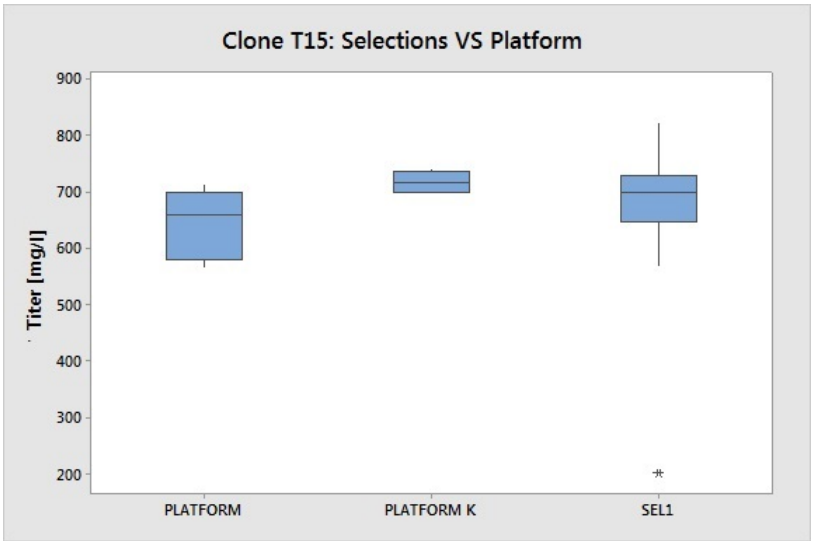

Figure 3.76: Comparison between the titer results obtained at WD12 in experiment 3 with platform condition, in experiment 4 with the platform medium enriched in K and with the selections (Clone T15).

In this figure, the results obtained at WD12 in experiment 3 with platform condition, in experiment 4 with the platform medium enriched in K and with the selections are compared. By looking at those distribution, it is evident that an enrichment of K in the medium is beneficial for the productivity of the cells. This phenomenon is present also in the IVC, the results of which are reported in Figure 3.77.

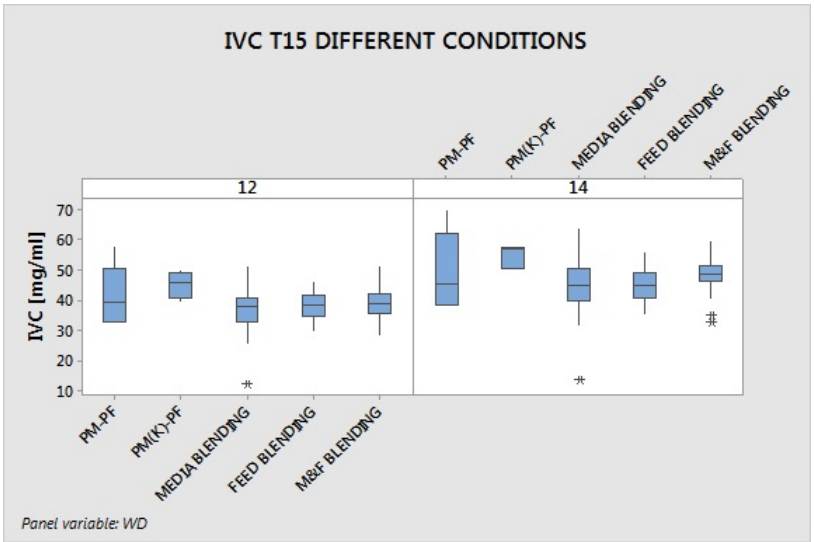

Figure 3.77: Condition comparison on IVC for clone T15. PM-PF is referred to platform medium and platform feed; the K in the brackets means that the platform medium is enriched just of potassium. M&F blending, instead, means that both, the media and the feed, are blended

In this case, however, the enrichment of potassium seems to be better than the platform medium just in terms of mean and, in general, the platform conditions are the ones that perform better. Concearning the IVC of clone T18, the same results can be seen. They are reported in Figure 3.78.

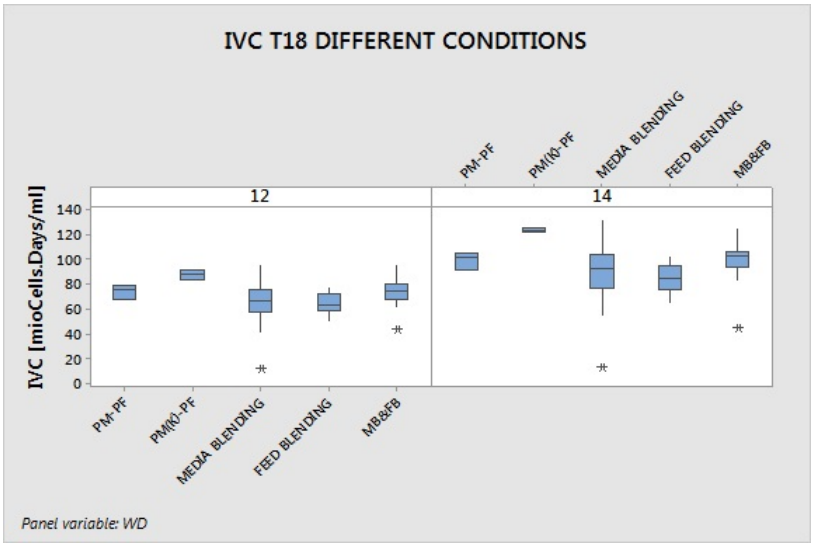

Figure 3.78: Condition comparison on IVC for clone T18. PM-PF is referred to platform medium and platform feed; the K in the brackets means that the platform medium is enriched just of potassium. M&F blending, instead, means that both, the midia and the feed, are blended.

Also in this case, potassium in the medium seems to be really important, leading to better results compared to all the other conditions. Also in the titer, the results of which are reported in Figure 3.79, the role of potassium is higlighted.

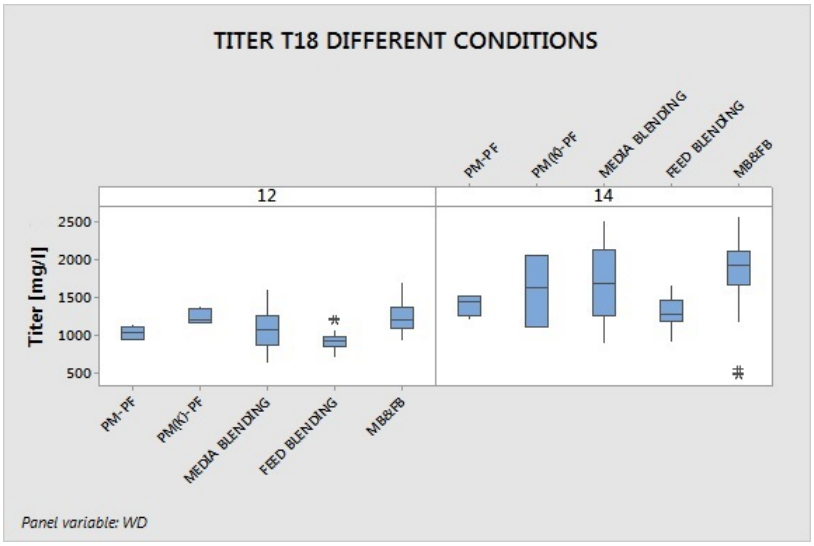

Figure 3.79: Condition comparison on titer for clone T18

In this case, however, although the platform medium enriched in potassium is better than the normal platform medium, it seems that the conditions in which both, the media and feed, are blended, are better at WD14. At WD12, instead, the performance obtained with platform medium (with potassium) and m&f blending are comparable. The importance of K can be seen also by looking at Figure 3.80.

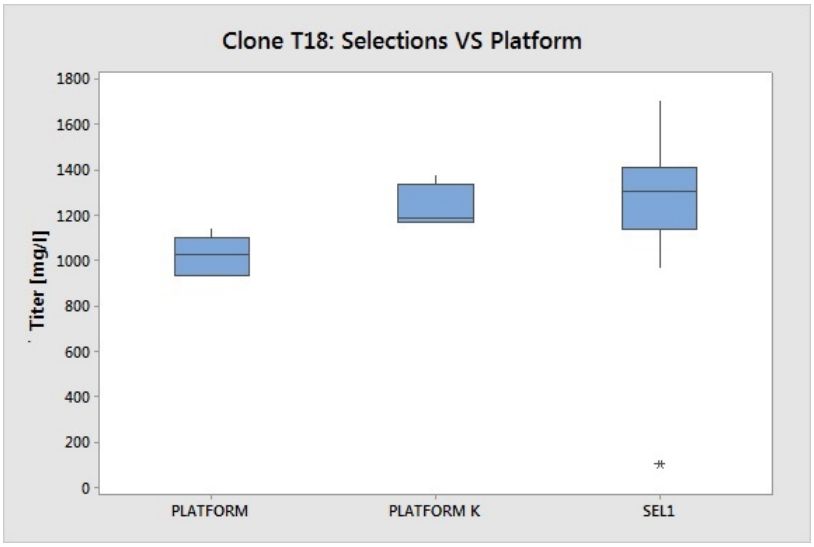

Figure 3.80: Comparison between the titer results obtained at WD12 in experiment 3 with platform condition, in experiment 4 with the platform medium enriched in K and with the selections (Clone T18).

In this case, differently from clone T15, the selection obtained leads to slightly better results compared to the platform enriched just in potassium. However, the importance of potassium is strongly highlighted, due to the fact that selection 1 is K $>$ 175. The results obtained for the titer in clone T25 are shown in Figure 3.81.

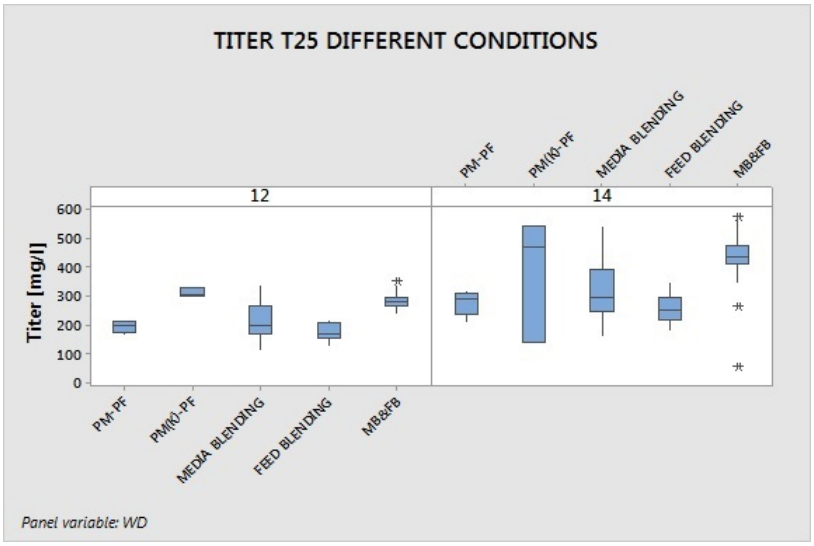

Figure 3.81: Condition comparison on titer for clone T25. PM-PF is referred to platform medium and platform feed; the K in the brackets means that the platform medium is enriched just of potassium. M&F blending, instead, means that both, the midia and the feed, are blended.

Also in this case, the importance of potassium can be noticed. The most important conditions are, indeed, the platform condition with the add of potassium and the combination of media and feed blending. This

is clearly evident also by looking at Figure 3.82.

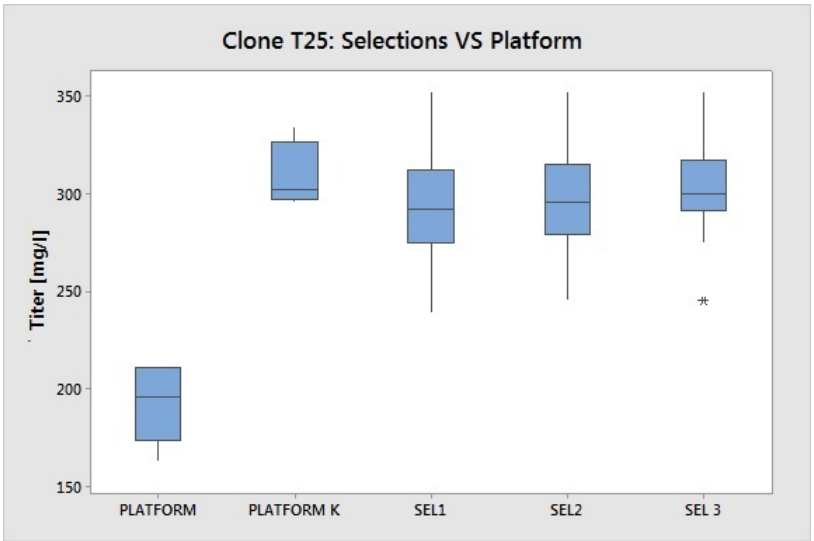

Figure 3.82: Comparison between the titer results obtained at WD12 in experiment 3 with platform condition, in experiment 4 with the platform medium enriched in K and with the selections (Clone T25).

In this case, although with the selections the results are not slightly improved compared with the platform enriched in K, it is demonstrated that this salt has a strong positive effect on this clone. By looking at the selections obtained in the previuous paragraph (cf. Figure 3.72), it seems that K is not important for this clone since it does not appear in the best selection pathway. However, it appears as a second selection in the right pathway. The hypothesis done before on the importance of K is then verified with this comparison between the two experiments. Moreover, T25 was the clone the VCD of which was sligthly improved. It is then possible that this is due to the presence of K in the medium. This is verified by looking at the comparison on the condition done at WD10 on the IVC. The results are reported in Figure 3.83.

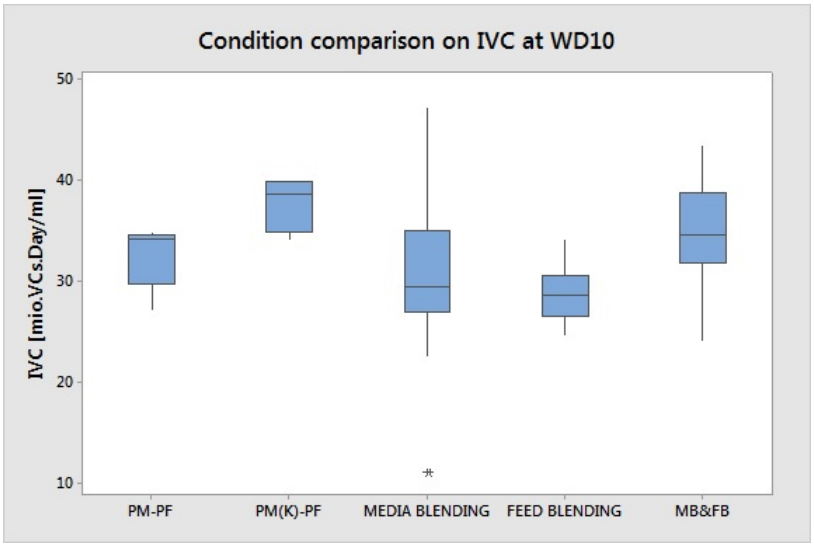

Figure 3.83: Condition comparison on IVC at WD10 for clone T25. PM-PF is referred to platform medium and platform feed; the K in the brackets means that the platform medium is enriched just of potassium. M&F blending, instead, means that both, the midia and the feed, are blended.

Again, the importance of potassium is proved. In conclusion, by comparing all the images, two important conclusions can be taken. First of all the role of potassium is clearly important and for that it is necessary to investigate more on this component. Moreover, it is not present in the platform feed; this means that the quantity in the medium should be enough for all the duration of the culture. It is clearly demostrated that this is not true. K needs to be added at least in the medium to ensure that the cells will not be affected by a lack of it. Secondly, it emerged that feed blending does not have an important role if it is not combined with media blending. This means that media blending is clearly more important than the feed blending and, for this reason, feed blending is not considered in experiments 5 and 6. The only component that it was decided to add in the feed, was potassium, due to its clear importance for this CHO-K1 cell line.

3.5.5 Choice of the Clone for Experiment 5

Due to the fact that experiment 5 was conducted in STs, it was not possible to test all the different clones that where tested in 96-DWPs, due to feasibility problem. For the choice of the clone, different things were evaluated, like the productivity and the stability. T25 was therefore excluded a priori for three different reasons. First of all, it is the clone that produces less; secondly, during experiment 4, the results of 55 wells were lost from WD12, so that less data were available. Finally, it was not studied in experiment zero, and therefore, no data and selections were available for T25.

Regarding the other two clones, T15 and T18, a study on the repetition errors in terms of CVs was done, in order to evaluate the most stable one. The results are reported in Table 3.14.

	T15			T18		
EXP	Titer 12	Titer 14	IVC 14	Titer 12	Titer 14	IVC 14
0	29%	26%	1%	4%	2%	9%
	22%	20%	1%	7%	1%	7%
	19%	17%	4%	23%	26%	21%
	6%	16%	6%	14%	12%	3%
	5%	6%	7%	26%	40%	11%
	1%	4%	4%	11%	12%	9%
	31%	44%	2%	15%	22%	29%
	16%	13%	13%	1%	3%	14%
	55%	66%	51%	2%	3%	1%
	6%	5%	1%	4%	5%	1%
	32%	43%	8%	7%	14%	11%
	71%	81%	11%	4%	5%	4%
	11%	11%	1%	5%	6%	5%
	13%	12%	14%	6%	11%	16%
	10%	4%	3%	0%	3%	3%
	65%	73%	29%	7%	11%	3%
3	10%	12%	27%	9%	10%	7%
4	3%	5%	7%	8%	32%	2%

Table 3.14: Comparison on the CVs obtained in the different experiments on clones T15 and T18.

In experiment 0, 16 were the replicates conditions and each of them was replicated two times. In the other two experiments, just one condition was replicated 5 times in experiment 3 and 4 time in experiment 4. By looking at the table, it is possible to notice that clone T15 has 17 CVs which are higher than 20%, while, for T18, only 8 CVs are major than 20%. For this reason, T18 was the chosen clone for experiment 5, as it was considered the most stable one. Moreover, by looking at the results obtained in 96-DWPs, it is evident that for clone T18, the variability in both, IVC and titer is really large. This means that it strongly responds to environmental changing, compared to T15.

3.5.6 Comparison on Clone T15

This clone was used in three experiments, experiment 0, 3 and 4. For this reason it was chosen to compare the results obtained in all of those experiments in order to see if they are in line. The results will be compared in terms of selections and distribution. Due to some sample problems in experiment 4, it was chosen to compare the results of the titer at WD12. The results are reported in Figure 3.84.

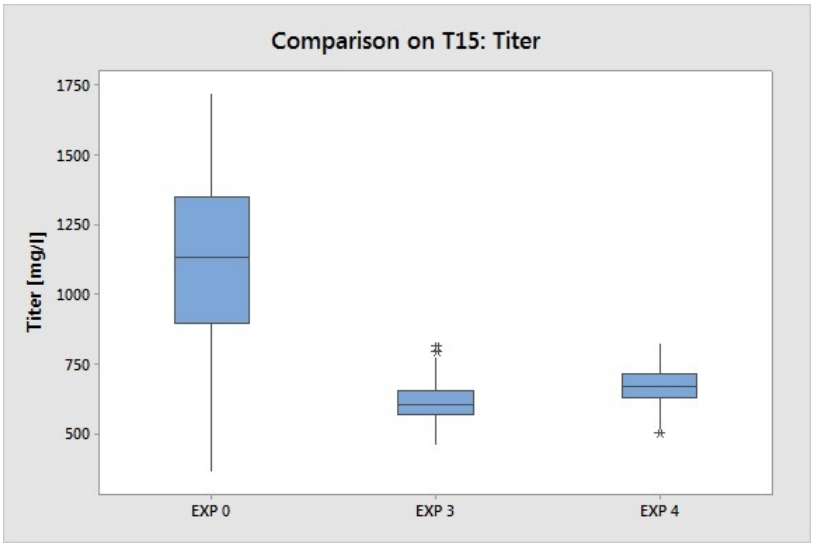

Figure 3.84: Comparison across the experiments on titer at WD12

By looking at the image, it is clear that the results obtained in experiment 0 are slightly higher compared to the other two. Again, as for clone P04, this can be explained by the different ways in which the media were prepared and by the differences in cell's age. Although the results were not improved passing from experiment 0 to the others, it is possible to notice that the results improved from experiment 3 to 4. As mentioned already in Section 3.5.4, this is due to the increase of potassium in platform medium from 100% to 150%. The same results are obtained by comparing the IVC at WD12. The results are shown in Figure 3.85.

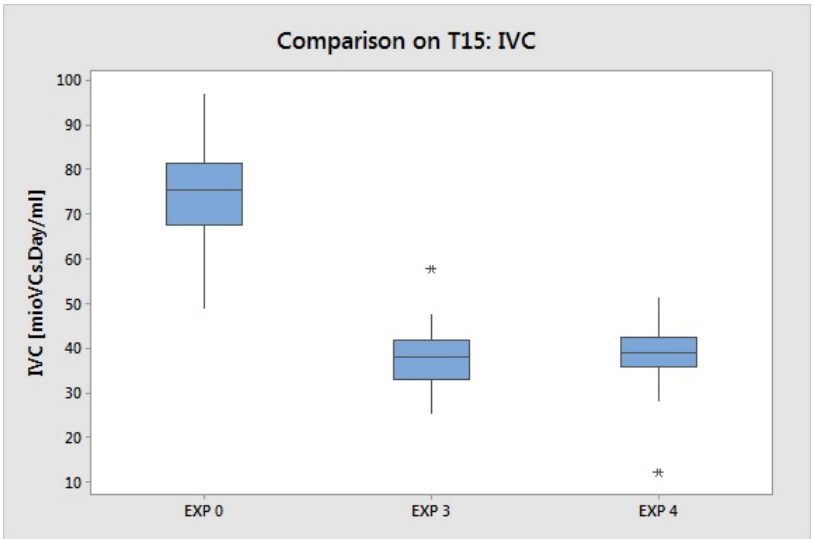

Figure 3.85: Comparison across the experiments on the IVC at WD12

Also in this case, it seems that the cells used for experiment 0 grow more compared to the ones used at

later stage. It is then important also to investigate into the way in which the cells are stored. In fact, experiment 0 was performed one year before experiment 3 and 4. This storage period can maybe influence the performance of the cells.

A comparison was done also on the selections obtained in the different experiments. All the selections obtained on this clone are summarized in Table 3.15.

	Titer			IVC		
EXP	0	3	4	0	3	4
G3	>133.353 (2)	< 212.5 (1)			< 137.5 (1)	
G5_K	> 157.045 (1)	> 137.5 (2)	> 175 (1)			
G5_PO_4						< 175 (1)
G8				> 77.9 (1)		
PO_4feed					< 135 (2)	

Table 3.15: Selections obtained on clone T15 in all the experiments

In the first column, all the groups found to be important for this clone are listed. The selections obtained on these groups are reported in the following three columns regarding the titer and in the last three columns regarding the IVC. Concearning the titer, the results are in line and it was found that the major drivers were always G3 and G5. Different results are, instead, found in the IVC. The important groups change across the experiment; in particular, G8, which was considered important in exp 0, was not found in exp 3 and 4. G3 was important only in experiment 3, while PO_4 was found to be important in both, exp 3 and exp 4, but in different places. In fact, the selections obtained on PO_4 are relative to the medium in exp 3 and to the feed in exp 4.

3.5.7 Comparison on Clone T25

The same comparison made on clone T15 was done on clone T25. In this case, just the comparison on the selections is reported due to the fact that this clone was not used in exp 0. The comparison between exp 3 and 4 in terms of distributions is already done in Section 3.5.4.

The selections obtained on this clone, are summarized in Table 3.16.

	Titer		IVC	
EXP	3	4	3	4
G3		< 175 (1)	< 212.5 (1)	< 175 (3)
G5_K				> 225 (1)
G2	< 125 (1)			
G8 feed		< 125 (3)		
G5_PO_4feed		< 125 (2)		
G2 feed	> 110 (3)			< 125 (4)
G3 feed	< 110 (2)			
G5_K feed				< 125 (2)

Table 3.16: Selections obtained on clone T25 in all the experiments

In this case the selections are different in both, titer and IVC. In particular, concearning the titer, no

important group for exp 3 were found in exp 4. Regarding the IVC, instead, only the selection on the G3 was confirmed and, in particular, the range of selection decreased from exp 3 to exp 4.

3.6 Results of Experiment 5

As described in Section 2.6.5, this experiment was performed in STs with the goal to validate the results obtained in 96-DWPs. In particular, from the experiments performed in 96DWPs on this clone, it was emerged the important role of potassium. For that, the principal aim of this experiment was to verified this importance. Secondly it was chosen to test also the group relative to vitamins and to split the group 3 into two smaller groups, one containing the polar amino acids (G3P) and one containing the apolar ones (G3A). The profile of growth of the cells is reported in Figure 3.86.

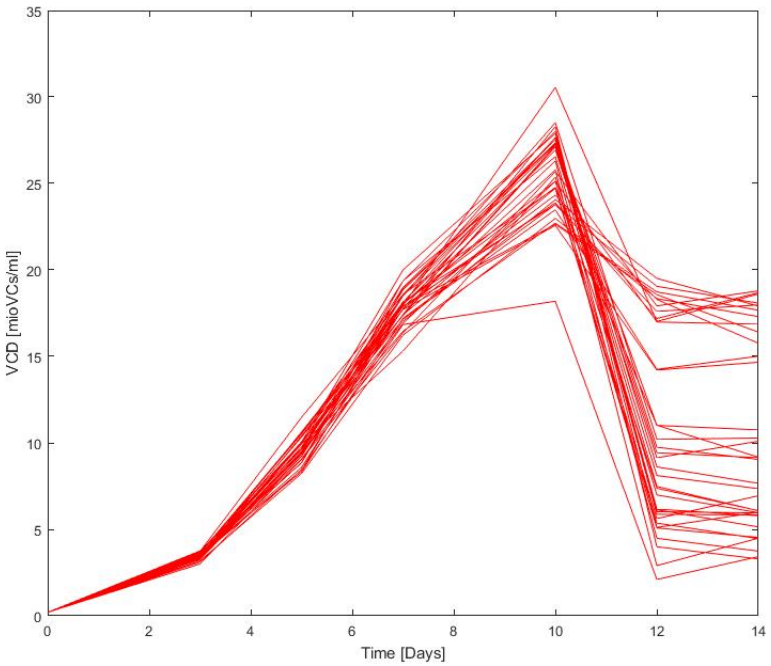

Figure 3.86: Experiment 5: VCD evolution in time

One important fact can be noticed. Until WD10, cells are fine and they grow well. Starting from WD12, most of the cells start to die. This is due to lack of glucose. In fact, in order to feed the cells, the feeding protocol reported in Table 2.7 in Section 2.3.2 was followed. The feeding protocol, however, can change in relation to the cell type and growth. In this case, T18 is the clone that grows more and for this reason, its glucose consumption is higher compared to the other CHO-K1 clones. The glucose profile is reported in Figure 3.87.

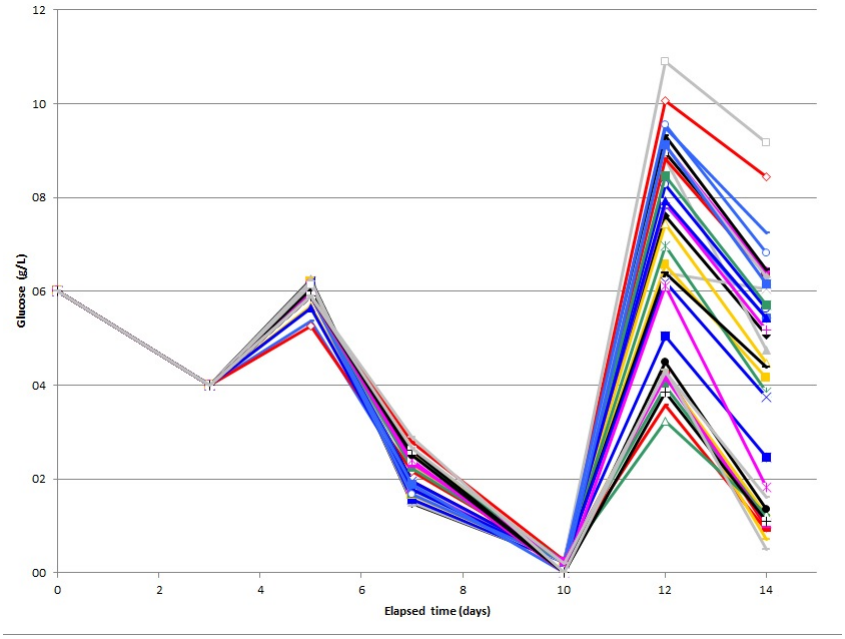

Figure 3.87: Experiment 5: measured quantity of glucose in the culture at different time. Each line represents the results obtained in the corresponding ST.

Each line present in this figure, represents one ST. By looking at the graph, it is quite clear that, until WD10, the measured quantity of glucose does not change too much across the ST; starting from WD12 the range of measurement is quite wide. This confirms that cells suffered from a lack of glucose at WD10. At WD10, in fact, most of the cells started to die and this explain why at WD12 the measured quantities of glucose are all different. In fact, in the STs in which the cells started to die, the consuption of glucose is less than in the other STs. For this reason it was chosen to consider the results obtained at WD10 as reliable.

3.6.1 Repetition Errors

Also in this experiment, a replicate condition was present. In particular, it was chosen to replicate the platform condition enriched in glucose four times. Since the results are attendible until WD10, it was chosen to analyze the repetition errors at WD07 and WD10 for the IVC and at WD10 concearning the titer. The repetition errors obtained on the IVC are shown in Figure 3.88.

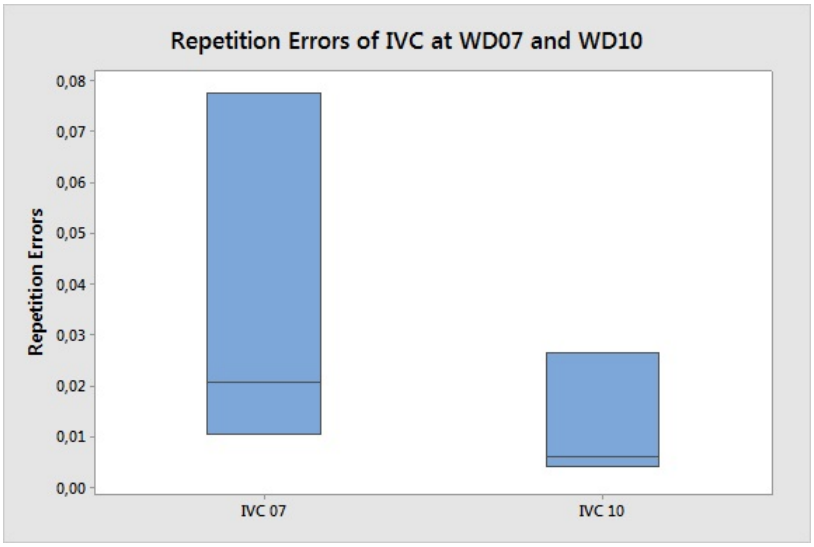

Figure 3.88: Experiment 5: Repetition errors concearning the IVC at WD07 and WD10.

The repetition errors are really low at both, WD07 and WD10. At WD07 it is a bit higher, reaching a maximum of almost 8%; however, the mean is around 2%. At WD10 the entire range is below 3%. Regarding the titer at WD10, the repetition errors are listed in Table 3.17.

Repetition Errors at WD10: Titer
0.03
0.06
0.04

Table 3.17: Experiment 5: Repetition errors concearning the titer at WD10

Also in this case, the errors are really low with a maximum of 6%. Also in terms of CV, the replicability is good. The values of CV are reported in Table 3.18.

	IVC 07	IVC 10	Titer 10
CVs	3%	1%	4%

Table 3.18: Experiment 5: CV calculated on titer at WD10 and on IVC at WD07 and WD10

The obtained values are really low, so that it is possible to affirm that the replicability in this experiment is high.

3.6.2 DTs Analysis

Also in this case the results were analyzed in order to understand which were the most important components. The results obtained on the titer at WD10 are reported in Figure 3.89.

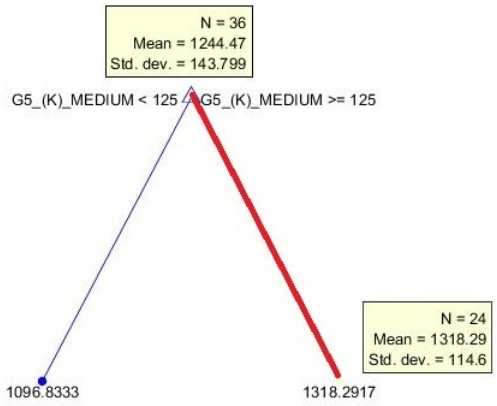

Figure 3.89: Experiment 5: DT analysis obtained on titer at WD10 considering K=10. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

The considered K is 10. As shown, the major driver is potassium, which has to be higher than 125%. This means that the 12 conditions in which just G6 and G3 were present, were not as good as the 24 conditions in which potassium was added. The importance of K can be seen also by looking at Figure 3.90.

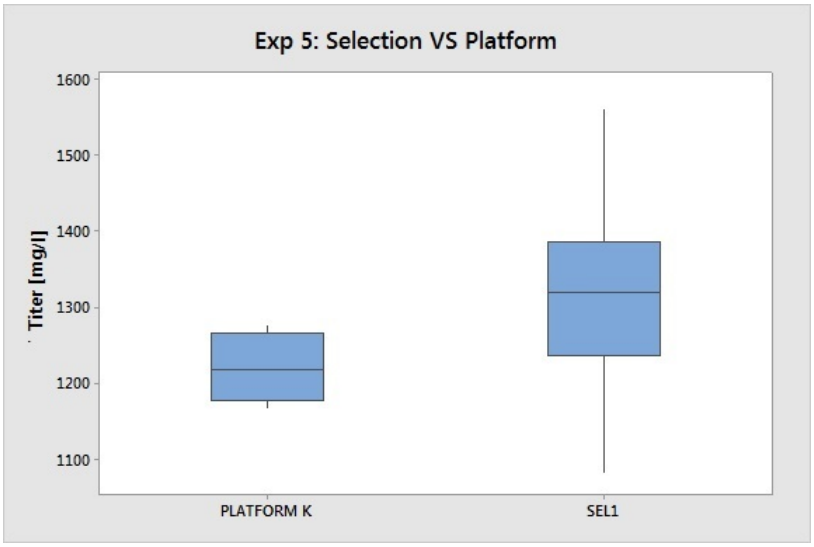

Figure 3.90: Experiment 5: Comparison between the selection obtained and the platform enriched in potassium

It shows the comparison between the results obtained at WD10 with the platform medium enriched in K (level 150) and with the selection media (K>125). It is then clear that potassium should be even higher than 150%. The same results were obtained concearning the IVC. They are reported in Figure 3.91.

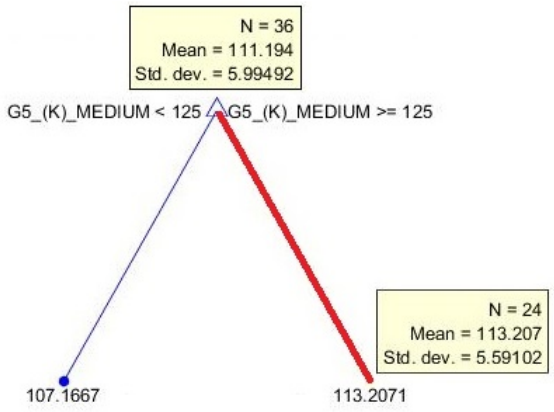

Figure 3.91: Experiment 5: DT analysis obtained on the IVC at WD10 considering K=10. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

This means that the importance of potassium is confirmed. In particular, the STs containing K, were the ones in which the cells were growing more and, therefore, in which the cells were consuming more glucose. Because of that, the cells started to die in those 24 STs, while the viability was still high for the other 12. This can be seen by looking at the selections obtained after WD10. They are reported in Table 3.19.

	IVC	TITER	
Group	WD14	WD12	WD14
G3A		$>$ 137.5 (1)	
G3P		$<$ 187.5 (2)	
G5	$<$ 125 (1)		
G6			$<$ 150 (1)

Table 3.19: Experiment 5: Selections obtained after WD10 on both, the IVC and the titer.

The selections concearning the IVC at WD12 are not reported due to the fact that no selections were obtained with different values of K. By looking at the table, it is clear that G6 becomes important just at WD14. This can be explained by the fact that the cells died in 24 STs containing G5 and, in some cases, also G3. Regarding the IVC, the selection changed at WD14 due to the fact that the cells in the medium enriched in potassium were died.

3.7 Results of Experiment 6

As mentioned in Section 2.6.6, this experiment had different goals. First of all it was important to test again some conditions of experiment 5 paying attention on the feeding of glucose in order to avoid early cell death. Secondly it was necessary to investigate in the best level of potassium in both, the medium and the feed. The VCD profile is reported in Figure 3.92.

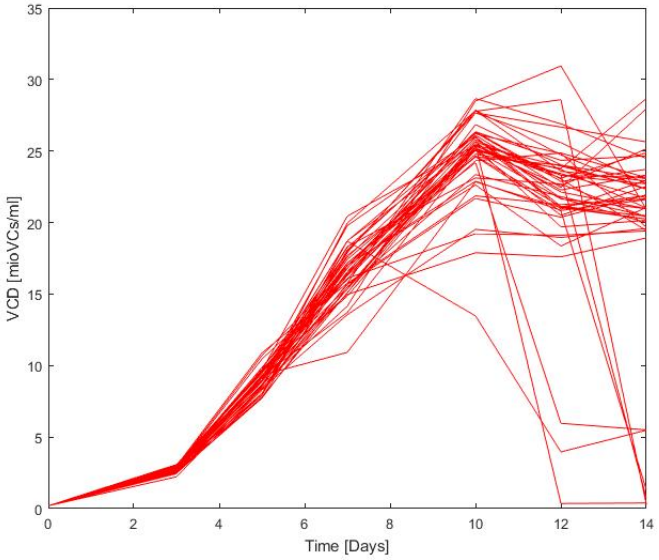

Figure 3.92: Experiment 6: VCD evolution in time

By looking at the graph, it is possible to see that 6 samples were lost. in particular one sample started to die already between WD07 and WD10, two samples between WD10 and WD12 and the last three in the last days of culture. By looking at the glucose profile in those sample it was noticed that again, the cause of this cell death is due to lack of glucose. The measured quantity of glucose in time is shown in Figure 3.93.

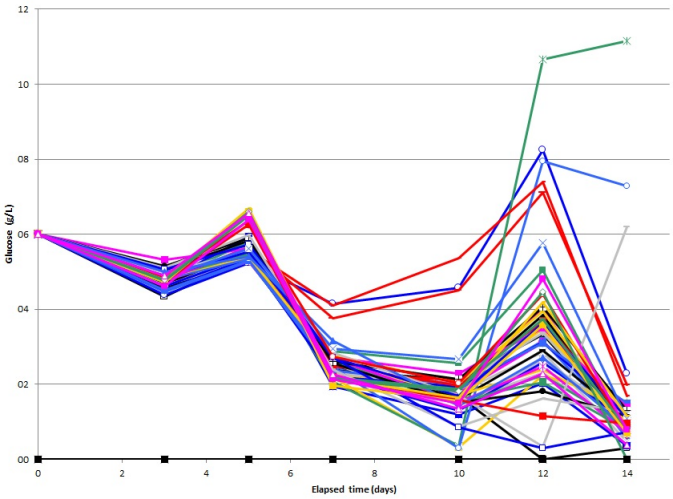

Figure 3.93: Experiment 6: evolution of the measured quantity of glucose. Each line represents the results obtained in the corresponding ST.

Different things can be noticed by looking at this picture. In particular, until WD05, the level of glucose is mostly the same in all the samples. Starting from WD07, 3 samples are characterized by a higher

level of glucose. These three samples are the one that were subjected to a shift in temperature. The decreasement of the temperature, therefore, let the cells to consume less glucose than normal; this means that it is possible to feed less glucose in those sample, allowing not to have a significant increase in osmolality. Apart of those three samples, other three samples stopped to consume glucose due to cell death. this means that at WD14, just the results of three sample are not attendible, even if 6 samples were subjected to cell death.

3.7.1 Repetition Errors

Also in this case, some conditions were replicated in order to evaluate the repetition errors. In particular, four conditions were replicated two times. The results concearning the IVC are reported in Figure 3.94.

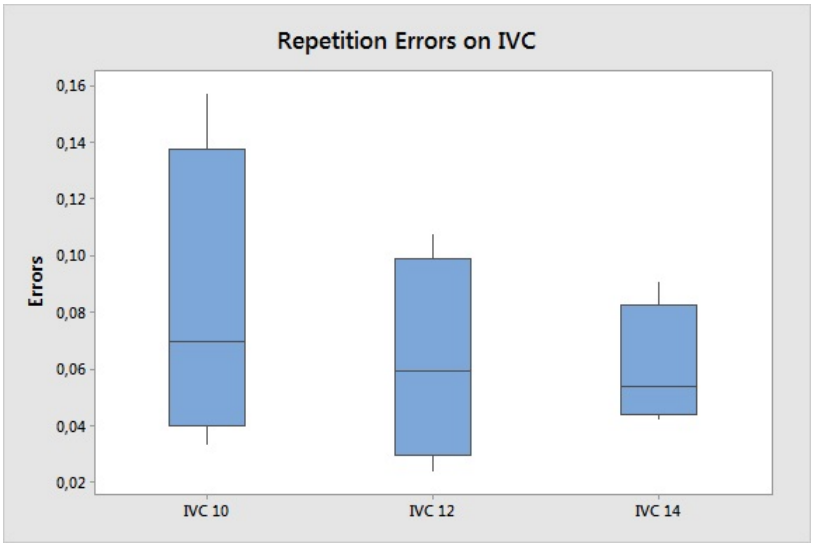

Figure 3.94: Experiment 6: Repetition errors on IVC

The repetition errors seem to decrease with the time and it is always acceptable, as it is lower than 20%. In terms of mean, it is always lower than 7%. The same cannot be said for the titer, the results of which are reported in Figure 3.95.

Figure 3.95: Experiment 6: Repetition errors on titer

In this case, in fact, the repetition errors are quite high and they become acceptable at WD14. This can be explained by the fact that the quantity of culture that needs to be sampled is really low, precisely 20 µl at WD10 and 10 µl at WD12 and WD14. Regarding the experiments performed in 96-DWPs, this quantity was sampled automatically with the Biomek. In experiment 5 and 6, instead, all the sampling are done manually under the laminar flow hood. For this reason, it is possible that some mistakes were done. This can explain the high variability in the results concearning the titer. In fact, the quantity sampled for the ViCell is slightly higher and, for that, it is easier to sample manually avoiding mistakes.

3.7.2 DTs Analysis

The selections obtained after DTs analysis were evaluated concearning both, the titer and the IVC. In this case, it was chosen to consider K=12 and the evaluated results are those at WD14 due to lower repetition errors.

Figure 3.96 shows the results on the IVC.

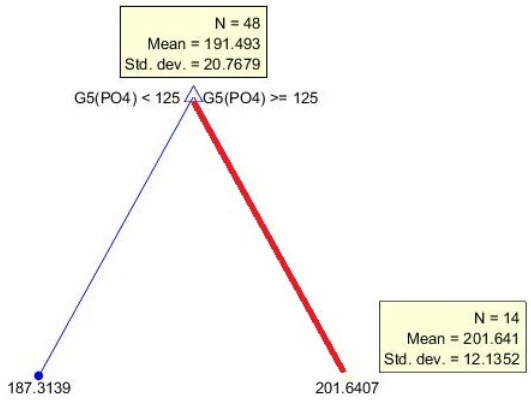

Figure 3.96: Experiment 6: DTs analysis on IVC at WD14. K was considered equal to 12. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

The important parameter for the IVC is phosfate, which has to be higher than 125%. Concearning the titer, instead, the major driver is potassium. The results are reported in Figure 3.97.

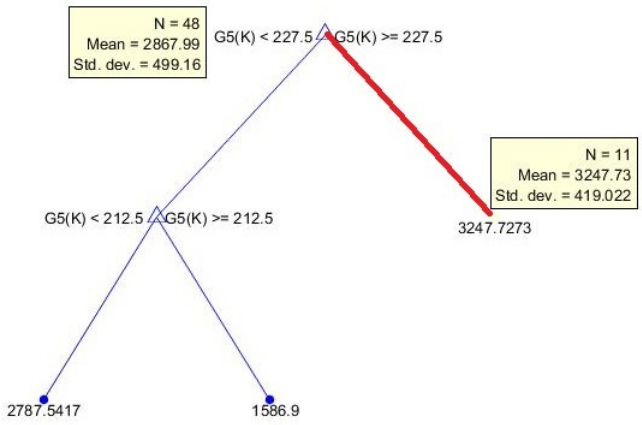

Figure 3.97: Experiment 6: DTs analysis on titer at WD14. K was considered equal to 12. The yellow boxes indicate the number of experiments (N) which have the selected characteristics, the mean and the standard deviation of those experiments.

It seems that, the most productive medium is the one that is enriched in potassium at a level which is at least the double of the platform medium. This confirms the results obtained in 96-DWPs in which it was marked the important role of potassium. Figure 3.98 shows the comparison between the selected conditions and the platform ones.

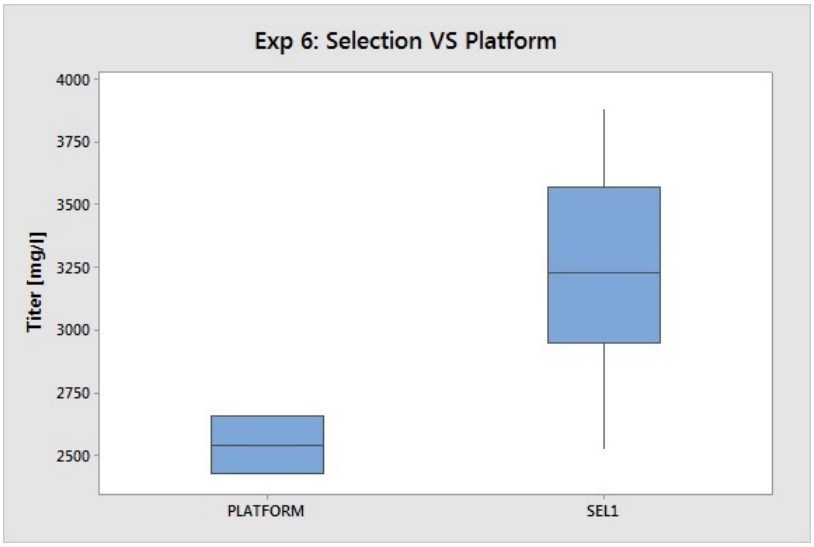

Figure 3.98: Experiment 6: Comparison between the selection obtained and the platform condition

This clearly highlights the importance of potassium in the medium. The titer obtained with the platform medium (one experiment repeated two times) is indeed slightly lower compared to the one obtained in the 11 selected conditions. Moreover, since the goal of this experiment was to fine tune the level of potassium in the medium, it was decided to analyse the increase in titer by varing just K in the medium. The results are shown in Figure 3.99.

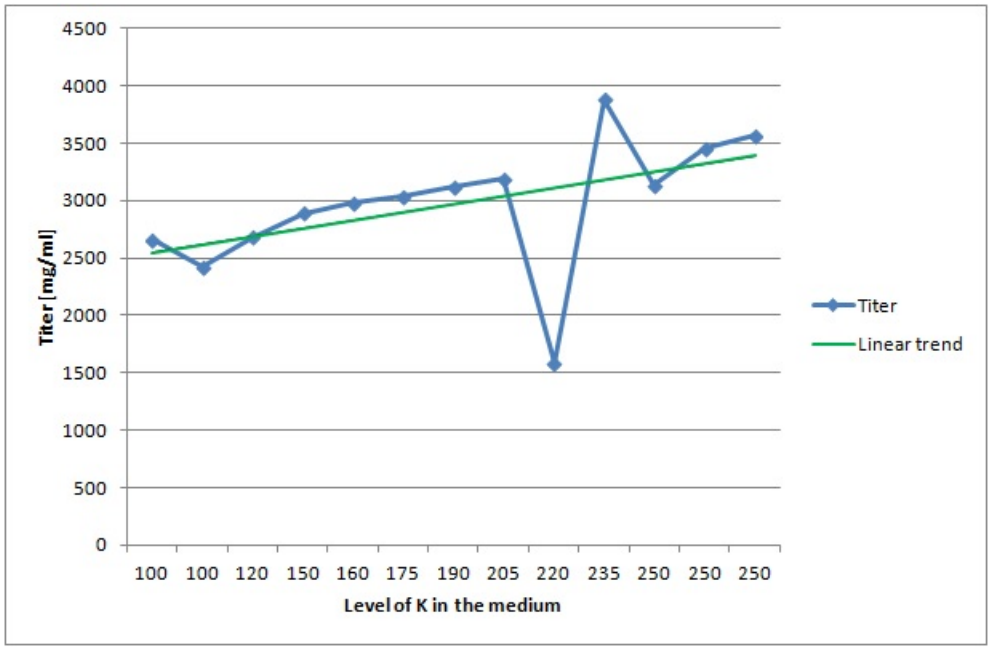

Figure 3.99: Experiment 6: titer in relation to the quantity of K in the medium.

By looking at the graph, it is evident the fact that by increasing K in the medium, titer increases. The

negative result obtained when K level is 220 is due to the fact that the cells died between WD10 and WD12. Moreover, the condition in which K is equal to 250% was replicated three times; for this reason, it was chosen to shift the temperature of one of those STs to 33°C at WD05. This corresponds to the first results obtained with K=250, which is down the linear trend line. This means that, in this case, no beneficial effects in shifting the temperature are reported concearning the titer. By the way, it seems that the best level of potassium is around 235%. However, this is just one results so it must be proved by repeating this experiment different time.

3.8 Overall Comparison: Clone T18

This clone was used in all the experiments of this work, except for experiment 1 and 2. For this reason it was chosen to evaluate the selections obtained and the improvement in the results among the experiments. Table 3.20 shows the selections obtained on titer and IVC for this clone.

	IVC					Titer				
Exp	0	3	4	5	6	0	3	4	5	6
G1						$>$ 96.4 (2)				
G2						$<$ 186.5 (1)				
G3		$<$ 212.5 (3)					$<$ 137.5			
G3 feed		$<$ 110 (2)								
G5_K	$>$ 146.9 (1)	$>$ 212.5 (4)		$>$ 125		$>$ 150 (4)	$>$ 137.5 (1)	$>$ 175		$>$ 227.5
G5_PO_4		$<$ 137.5 (1)	$<$ 175 (2)		$>$ 125		$<$ 212.5 (2)		$>$ 125	
G6						$<$ 452.4 (3)				
G8	$<$ 169.9 (2)									
G8 feed			$>$ 125 (1)							
G9						$>$ 70.5 (5)				

Table 3.20: Comparison on the selections obtained for clone T18

G5 was confirmed to be the most important group for this clone. In fact, both, potassium and phosphate, are always present in the selections. However, it was not confirmed the importance of the groups that were found in experiment zero, such as G1, G2, G6 and G9.

The comparison concearning the titer at WD14 are reported in Figure 3.100.

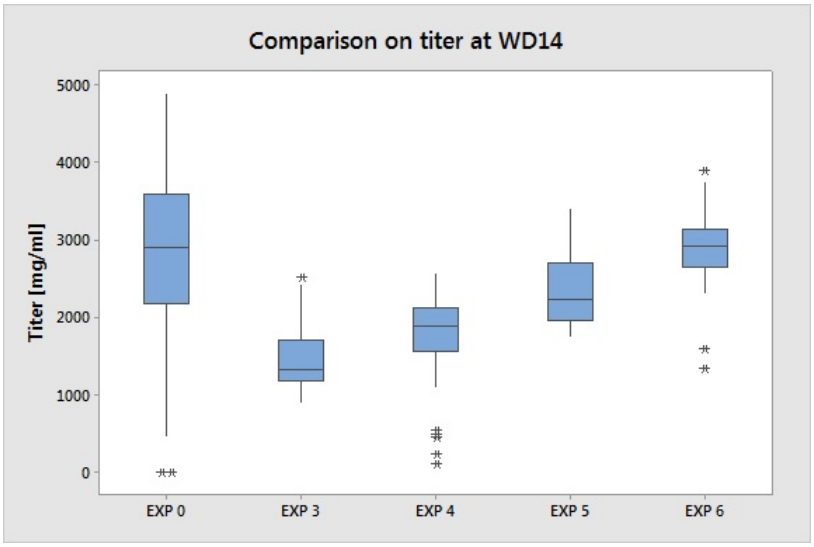

Figure 3.100: Comparison on the titer at WD14 in all the experiments performed with clone T18

It is clear that the results obtained in experiment 0 are better. However, the trend of all the other experiments performed is good. In fact, by analysing each experiment with DTs and by taking the best selections, an improvement in the results is obtained. In particular, experiment 6 achieved a level in the mean which is comparable to experiment 0. Moreover, the negative results obtained in experiment 0 were removed already in experiment 3 and, excluding the outliers, the lowest values in all the experiments performed, are higher than those in experiment 0. In fact, the lower whisker of all the experiments lies always in a range which is higher than the one in experiment 0.

Chapter 4

Conclusions

The aim of this project was to provide an improved clone selection method which comprehends media optimization; in particular, the hypothesis was that many clones with high productivity potential are discarded because they are not adapted to the platform. A modification of the environmental conditions from early on should provide an improved basis to find a high producer and simultaneously optimize the process. By varying the environmental conditions, the ranking of the clones could in fact be different. Normally, once the best clone is selected, a step of improvement of the culture conditions is made in order to fine tune the media and increase the productivity. Selecting the clone with its specific conditions earlier could reduce the duration of this fine-tuning step. In this project, it was decided to start from the data obtained from a preliminary study, which was called experiment 0. In this experiment, 47 components were divided into 11 groups and 160 media blending conditions were designed to be tested on 5 different clones. By analyzing the results obtained at WD14, it emerged that the selections are clone specific and that, for this reason, there is not a medium which is optimal for every clones. Not only a high diversity in the results was generated, but for each clone, some conditions allow to obtain better titers and growth than with the platform medium. 3 of the eleven groups were found to be important for CHO-S line while 5 for CHO-K1. In this work, different clones of these two cell lines were tested in different conditions, in order to see if there were some environments that stimulated more the productivity compared to the platform one. To do so, a sequential approach was adopted and design of experiments (DoE) were made according to the selections obtained after an accurate decision trees (DTs) analysis on a previous set of results.

In every experiment, an aliquote of culture was sampled at different WD in order to measure the viable cell density (VCD) and the titer.

Starting from the results obtained in experiment zero, 6 experiments were done sequentially.

The first experiment done was conducted with two different clones, P04 and T11, on the same plate and with the same condition, with the aim to evaluate the repetition errors.

The second experiment had two different goals: the first one was to evaluate the repetition errors in platform condition, while the second one was to test the effect of 3 of the 11 groups tested in experiment zero; in particular, the three groups tested were G5 (KCl and PO4), G4 (Asn and Met) and G11 (Pyruvate). In this case, two plates with equal conditions were evaluated and just one clone, P04, was considered.

The third experiment was proceed on three plates, one for each clone, and different media and feed blending conditions were tested. The platform condition was replicated 5 times on each plate in order to be able to compare them with the other conditions. It was chosen to test the groups that were found to be important for CHO-K1 cell line in experiment zero. This groups were: G2 (stable AAs), G3 (accumulated AAs), G5 (KCl and PO4) and G8 (growth booster).

The fourth experiment was performed right after experiment 3 with the same clones. In this case, it was chosen to test also some conditions in which media and feed blending were combined. Moreover, since it was emerged from experiment 3 that K in the medium needs to be increased, it was decided to enrich the platform medium in potassium at 150% of the level in the standard medium.

All of these experiment were conducted in 96-DWPs. However, due to validate the results obtained, it was chosen to use a different system. It was then decided to conduct experiment 5 and 6 in STs as they are more reliable than 96-DWP. They were both performed with just one clone, T18. The aim of these two experiment were different: first of all, it was necessary to validate the importance of potassium and to fine-tune the level of this salts. Secondly, it was decide to test the group containing the vitamins (G6) and to split G3 into two different groups based on chemical properties of the AAs. In particular, polar AAs were separated from the apolar ones.

The results obtained in the first experiment were not acceptable, as the repetition errors were too high, achieving a value which was around 35%. Two reasons were identified: first of all, the grey silicon membrane was used on the plate; secondly, the plates used to measure the VCD were not coated. Indeed, in a previous study it was emerged that the grey membranes lead to an early cell death in the middle of the plate. Moreover, it was found that cells tend to adhere to the bottom of the plate if this is not coated, leading to a not reliable results. When the membrane was changed and the Guava plate was coated (experiment 2), better results were obtained, reducing the repetition errors to 10%. Regarding the media blending part of the experiment 2, it was found that the selections obtained on the titer lead to higher productivity compared to platform medium. So it is demonstrated for this clone that there are some environments that stimulate more the production of mAbs.

However, it was seen that CHO-S cell line are not slightly influenced by environmental changing. This means that optimizing the media and the feed can be quite hard for this cell line. With experiment 3 and 4, it was proved that, for all the clones, there were always some conditions which performed better compared to the platform. In particular, it emerged that feed blending is not important compared to media blending. One important fact that emerged from this experiments, was the importance of potassium. The productivity is, indeed, slightly higher when the platform medium contains K. With the two experiments performed in STs, the importance of K was verified. No beneficial effects were, instead, obtained with vitamins nor by splitting G3. Unfortunately, this work was to short to be able to fine tune the best level of potassium. In conclusion, it was proved that there are always some conditions which perform better than the platform and, for this reason it was demonstrated that the clone selection strategy should be changed. The ideal approach would be to assess a maximum of conditions on a greater number of clones.

Ideally, a maximum of 20 different conditions to apply to 20 clones would be found. It may be mandatory to use a deep-well plate system to test those conditions on those clones (5 plates). However, 20 conditions are quite a few if the number of groups that need to be tested is high. A new design could be performed with a reduced number of groups. But, even by reducing the number of the groups, the minimum number

of conditions to test would be too high to be tested on 15-20 clones. In addition, when a group impacts the final titer, it is not known which compound of this group has an influence since all compounds are varied in the same proportions. It could be interesting to study the impact of each individual compound of a group to try to reduce the design. Of course, this cannot be done for all groups, but this could be investigated for the most influent ones, as it was done in this work for G3, G4 and G5.

Regarding G3, it was chosen to split it just at a later stage of the work and no conclusions were found about the important components.

G4 was found to be important for CHO-S clones and, since it was composed of just two amino acids it was more easy to investigate on the importance of the single amino acids. However, since CHO-S cells do not respond well to the environmental changing, it was not easy to determine if the importance of G4 was due to Met or Asn. Asn is not present in the selection obtained, while Met is. But this result needs to be verify with some other experiments and in some other systems, such as ST, as it was done for G5.

G5, which is composed of two salts, was found to be a really important group for all the clones in experiment zero. For this reason, it was chosen to split it, in order to see if the major driver was potassium or phosphate. Already in experiment 3 it emerged the importance of K. In particular, by enriching the platform medium with this component, the results improved drastically in all the analyzed clones, in both, titer and IVC. In fact, it was deduced that KCl was not present in sufficient amounts to ensure a correct productivity of the cells. Media blending identified K+ as limiting in the proprietary medium. This component is indeed not present in the proprietary feed and, for this reason, an accurate investigation should be done. It is important, therefore, to understand if an enrichment of K is required just in the medium or also in the feed. Since it is a salt, it does not have any solubility problem in the feed.

So, a step for the future is to fine tune the quantity of this component, which seems to be really important for the productivity of the cells. By looking at the selections obtained, it emerged that also phosphate is important, expecially for clones T15 and T18. In particular, in most of the experiments, it emerged that PO_4 has a beneficial effect on the IVC. For this reason, it is possible to conclude that salts are in general important for both, the productivity and the growth, of the cells and that a deeper investigation should be done on those components.

As mentioned before, in this work it was chosen to proceed in a sequential way, by designing an experiment starting from the results obtained in the previous one. But by doing that, the cells age change and so the results could be different. In particular, when the cells are getting older, their productivity can be reduced, leading to a variation in the results. In fact, the repetition errors sometimes were quite high. This can be attributed not only to the age of the cells but also to some problem in sampling. Indeed, although the Biomek is a reliable robotic system, sometimes it is possible that some air bubbles resting in the capillaries. Moreover, since it is the user who gave all the inputs to the machine, it is possible that sometimes the tips do not aspirate the right quantity of culture from the small wells of the 96-DWPs. For this reason, it would be necessary to elaborate a protocol for the Biomek, in which all the quantities that the user have to give as inputs are specified. In this work in fact, there were some sampling problems regarding the titer in some wells. Being all fed-batch experiments, the titer should always increase in time; in some cases it was seen that the measured quantity of mAb at WD14 was lower than the one at WD12; in the worst case, it was registered that in some wells, normally disposed on the edge of the plate,

the titer was even too low to be detected. This is clearly a sampling problem, in which the tips did not aspirate the right quantity of culture since they probably did not reach the right liquid level.

Another important question to be considered is that all the devices should be verified before the beginning of each experiment. In this work, some problems were encountered with the guava device. During the later stage of experiment 4, in fact, it seems that it stopped to count: the measured number of cells in each wells was, indeed, always zero. Normally this happens when the capillary of the device is obstructed and needs to be changed. However, in this case, it happened also when the capillary had just been changed. For this reason a high amount of data was lost and it is possible that the other results obtained where not reliable.

In this experiment, regarding CHO-K1 cell line, three completely different clones were chosen. This selection was done based on the productivity of the cells, by chosing the best producer (T18), the worst procucer (T15) and one clone with intermediate characteristics (T15). Considering also the platform clone would probably had been a better choice; this, in fact, would have proved that, compared to the selected clone, there are some other clones that produce better in other conditions. This can be the next step: compare the clone that is normally used in production with one or more clones that respond well to environmental changing in order to see if the initial hypothesis is verified.

Finally another important point to be studied is the feeding strategy. Nowadays, the feeding protocol is always followed and in same cases it needs to be changed to be adapted to a different cell line. But it would be interesting to investigate in different timing for the feeding in order to see if some beneficial effect are reported. For example, it is possible that a particular cell line needs a daily feeding. In this work, some problem with glucose were found for the clone T18; an increase in the quantity of fed glucose, can be deleterious for the cells if the osmolality raises significantly. In this case, therefore, it would be interesting to see if a daily feeding starting from WD07 is beneficial. In this way, the added quantity of glucose would be lower and no important variation in osmolality would be registered.

In conclusion an improvement in the clone selection strategy can be obtained in the follow manner. From right after the transfection to the three last fed-batches, there is a reduction from 10.000 clones to about 20. These progressive eliminations will of course be performed in the platform. It is only with a reduced number of clones that the media blending approach is feasible. Optimally, one would have to test the feeding strategies combined with the media blending. It is indeed possible that for a specific clone, one media could be optimal in the feeding platform, but another media performances could be enhanced by a different feeding strategy. However, the number of conditions at each step would become too high. For example, testing 20 media and 4 feeding strategies would give 80 condition per clone, leading to 20 plates to test.

Thus, the best way to operate would be to do the first fed batch with 20 clones and 20 conditions for each clone in DWP (5 plates in total). Different fed-batch experiments would been tested to find the optimal environment and improve productivity, growth and product quality. Then, the best 10 clones only will be selected with their own best media to be submitted in the second fed-batch to different feeding strategies. After this, 4 clones with their best combination of media and feeding strategies will go through the last fed-batch. Only the clone that obtains the best final titer will be selected in this last fed-batch. After this selection, the media, feeds and parameters may go through another round of optimization. Unfortunately, this work was too short to be able to improve all the selection strategy; but, on the other hand, it proved

the importance of media blending when an increase of the productivity wants to be achieved. For that, it pones the basis for a future study in which all the conditions, the clones and the feeding strategies will be planned in advance.

Appendix A

	K	PO_4	Asn	Met	Pyr		K	PO_4	Asn	Met	Pyr		K	PO_4	Asn	Met	Pyr
A01	100	100	100	175	175	**D03**	175	175	100	100	100	**F10**	130	130	160	100	130
A04	100	175	175	100	100	**D04**	100	175	100	100	175	**G01**	100	100	100	250	100
A08	200	100	100	100	100	**D06**	100	200	100	100	100	**G02**	100	100	100	100	250
A09	100	100	100	200	100	**D08**	100	150	150	100	150	**G03**	100	160	130	160	100
A10	100	130	160	100	160	**D09**	100	100	100	150	100	**G05**	130	130	130	100	160
B04	190	100	100	100	160	**E01**	175	100	175	100	100	**G07**	100	150	150	150	100
B06	160	100	130	130	130	**E02**	100	250	100	100	100	**G08**	250	100	100	100	100
B07	190	130	100	100	130	**E04**	150	100	100	150	150	**G10**	100	100	130	160	160
B09	160	100	100	190	100	**E07**	175	100	100	100	175	**H01**	100	175	100	175	100
C01	100	100	250	100	100	**F01**	100	100	150	100	100	**H02**	160	100	130	100	160
C02	160	100	130	160	100	**F02**	150	100	150	150	100	**H03**	130	100	130	160	130
C03	100	150	100	150	150	**F03**	150	100	100	150	150	**H05**	100	100	100	100	150
C05	150	100	100	100	100	**F06**	150	150	150	100	100	**H08**	100	100	150	150	150
C07	175	100	100	175	100	**F08**	150	100	150	100	150	**H09**	100	150	100	100	100
C10	100	100	100	100	200	**F09**	100	100	200	100	100	**H10**	100	175	175	100	100

Appendix B

	K	PO_4	$G3$	$G2$	$G8$		K	PO_4	$G3$	$G2$	$G8$		K	PO_4	$G3$	$G2$	$G8$
A2	250	100	175	150	100	**D1**	100	250	100	200	100	**F3**	100	100	100	150	100
A3	100	175	250	150	250	**D4**	100	100	100	200	100	**F7**	250	175	250	100	100
A4	100	250	175	200	175	**D6**	250	175	175	200	175	**F8**	100	100	100	100	250
A5	100	100	250	100	250	**D7**	100	100	100	200	250	**G1**	250	175	100	100	250
A7	250	250	100	200	250	**D9**	250	100	250	200	100	**G2**	250	100	250	100	250
A8	100	250	100	100	250	**D10**	175	175	100	200	100	**G3**	250	250	250	200	100
A10	100	175	100	100	100	**E1**	175	250	100	150	175	**G7**	250	250	100	100	100
B1	100	100	100	100	175	**E3**	175	100	250	100	175	**G8**	250	100	100	150	250
B7	175	250	175	100	250	**E4**	250	100	100	100	100	**G9**	100	250	100	200	250
B8	100	250	100	100	100	**E5**	175	100	175	200	250	**G10**	100	100	250	100	100
B10	250	100	100	200	175	**E8**	100	100	250	200	175	**H1**	250	250	175	100	100
C3	100	100	250	200	100	**E9**	250	100	100	100	250	**H3**	100	250	250	100	100
C4	250	100	250	200	250	**E10**	100	250	250	100	250	**H4**	100	250	250	200	250
C7	250	250	250	100	175	**F1**	175	100	100	100	100	**H8**	250	250	250	150	250
C9	175	250	250	200	100	**F2**	100	100	175	100	100	**H9**	250	250	100	200	100

Appendix C

	K	PO_4	$G3$	$G2$	$G8$		K	PO_4	$G3$	$G2$	$G8$
A6	100	100	100	150	100	**D8**	100	100	100	100	150
A9	120	100	150	150	120	**E2**	100	150	150	150	100
B2	150	150	100	100	150	**E6**	120	150	120	120	100
B4	120	120	150	100	120	**E7**	100	120	150	120	150
B5	150	100	100	100	100	**F4**	100	150	150	100	150
B6	100	150	100	100	100	**F5**	150	150	150	100	100
B9	150	100	150	150	100	**F6**	100	150	100	150	150
C1	100	100	100	100	120	**F9**	120	120	100	120	150
C2	100	100	150	100	100	**G5**	150	100	150	100	150
C5	100	100	100	120	100	**G6**	100	120	120	150	100
C6	150	100	100	150	150	**H2**	100	120	100	100	100
C8	120	150	120	100	150	**H5**	150	120	120	150	120
D2	100	150	120	120	120	**H6**	150	150	150	150	150
D3	100	100	120	100	100	**H7**	150	150	100	150	100
D5	120	100	100	100	100	**H10**	100	100	150	150	150

Appendix D

	Media			Feed					Media			Feed			
	K	PO_4	G3	K	PO_4	$G2$	$G8$		K	PO_4	G3	K	PO_4	$G2$	$G8$
A2	250	200	100	100	100	100	150	**E6**	150	200	100	100	100	150	100
A3	200	100	200	100	100	150	100	**E7**	250	200	200	100	100	100	150
A4	250	100	100	100	150	100	100	**E8**	150	100	200	100	150	100	100
A9	150	200	200	150	100	100	100	**E10**	150	200	200	100	100	150	100
A10	150	200	100	100	100	100	150	**F1**	250	100	150	100	150	100	100
B1	250	100	100	100	100	150	100	**F2**	200	100	200	150	100	100	100
B2	150	100	100	100	150	100	100	**F3**	200	150	100	100	100	150	100
B5	250	200	200	100	150	100	100	**F4**	250	200	100	100	100	150	100
B6	200	150	100	150	100	100	100	**F6**	200	150	100	100	150	100	100
B9	200	200	150	150	100	100	100	**F8**	200	200	150	100	150	100	100
B10	250	200	200	100	100	150	100	**F10**	150	200	200	100	150	100	100
C2	250	100	100	100	100	100	150	**G1**	200	200	150	100	100	100	150
C4	250	150	200	150	100	100	100	**G2**	250	200	100	100	150	100	100
C5	250	150	200	100	100	150	100	**G3**	150	150	150	150	100	100	100
C6	250	200	100	150	100	100	100	**G4**	150	200	100	150	100	100	100
C7	150	150	150	100	150	100	100	**G5**	250	100	200	100	100	150	100
C8	250	150	200	100	150	100	100	**G6**	250	100	150	150	100	100	100
C10	250	200	200	150	100	100	100	**G7**	150	100	100	150	100	100	100
D1	250	100	200	100	150	100	100	**G8**	150	100	200	100	100	100	150
D2	250	100	200	150	100	100	100	**G9**	250	150	200	100	100	100	150
D4	250	100	150	100	100	150	100	**G10**	150	150	150	100	100	150	100
D6	200	200	150	100	100	150	100	**H2**	150	150	150	100	100	100	150
D8	200	100	200	100	150	100	100	**H4**	200	150	100	100	100	100	150
D9	250	100	100	150	100	100	100	**H5**	250	100	150	100	100	100	150
D10	250	100	200	100	100	100	150	**H6**	150	200	100	100	150	100	100
E1	150	100	100	100	100	150	100	**H7**	150	100	200	100	100	150	100
E4	200	100	200	100	100	100	150	**H8**	150	200	200	100	100	100	150
E5	150	100	100	100	100	100	150	**H10**	150	100	200	150	100	100	100

Appendix E

	K	PO_4	$G3$		K	PO_4	$G3$
A1	150	200	100	**C9**	250	200	200
A5	250	100	200	**D3**	250	200	100
A6	150	200	200	**D7**	250	200	200
A7	150	200	200	**E2**	250	200	100
A8	250	100	200	**E3**	150	100	200
B3	250	200	100	**E9**	250	100	200
B4	250	200	100	**F5**	250	100	100
B7	150	200	200	**F7**	150	200	100
C1	150	200	200	**H1**	250	100	200
C3	250	100	100	**H9**	250	100	200

Appendix F

RUN	$G3P$	$G3A$	PO_4	K	$G6$	$K - Feed$	**RUN**	$G3P$	$G3A$	PO_4	K	$G6$	$K - Feed$
1	100	100	100	150	100	100	**19**	200	200	150	200	100	100
2	100	100	100	150	100	100	**20**	200	200	175	250	100	200
3	100	100	100	150	100	100	**21**	200	200	150	250	100	100
4	100	100	100	150	100	100	**22**	200	200	100	250	100	100
5	100	100	100	150	100	150	**23**	200	200	150	250	100	150
6	100	100	100	150	100	200	**24**	200	200	175	250	100	100
7	100	100	175	150	100	100	**25**	200	175	100	100	100	100
8	100	100	150	200	100	150	**26**	200	100	100	100	100	100
9	100	100	175	250	100	100	**27**	175	200	100	100	100	100
10	100	100	100	250	100	100	**28**	100	200	100	100	100	100
11	175	175	100	150	100	200	**29**	100	175	100	100	200	100
12	175	175	150	200	100	150	**30**	175	200	100	100	200	100
13	175	175	150	200	100	100	**31**	175	175	100	100	200	100
14	175	175	100	200	100	100	**32**	100	100	100	100	200	100
15	175	175	150	200	100	200	**33**	100	175	100	100	250	100
16	175	175	100	250	100	150	**34**	200	100	100	100	250	100
17	200	200	175	150	100	200	**35**	175	200	100	100	250	100
18	200	200	175	200	100	100	**36**	200	100	100	100	250	100

Appendix G

RUN	$G3P$	$G3A$	PO_4	K	$G6$	$K - Feed$	**RUN**	$G3P$	$G3A$	PO_4	K	$G6$	$K - Feed$
1	100	100	100	150	100	150	**25**	100	100	100	235	100	100
2	100	100	150	200	100	150	**26**	100	100	100	250	100	100
3	175	175	150	175	100	150	**27**	100	100	100	250	100	100
4	175	175	150	130	100	150	**28**	100	100	100	250	100	100
5	200	200	175	150	100	150	**29**	100	175	100	100	200	100
6	100	100	100	120	100	200	**30**	175	175	150	200	100	100
7	100	100	100	120	100	200	**31**	175	175	100	200	100	100
8	100	100	100	120	100	200	**32**	100	100	175	150	100	100
9	175	200	100	150	200	200	**33**	200	200	150	200	100	100
10	175	200	100	150	200	200	**34**	200	200	175	250	100	100
11	175	200	100	150	200	200	**35**	175	200	150	250	100	100
12	175	175	100	175	100	200	**36**	100	175	100	200	200	100
13	200	200	175	150	100	200	**37**	175	175	100	250	200	100
14	200	175	100	175	100	200	**38**	200	175	175	250	100	100
15	200	100	100	150	100	200	**39**	100	100	175	250	100	100
16	100	100	100	100	100	100	**40**	100	100	100	150	200	100
17	100	100	100	100	100	100	**41**	100	175	100	150	200	100
18	100	100	100	120	100	100	**42**	200	100	175	100	250	100
19	100	100	100	150	100	100	**43**	175	200	100	150	250	100
20	100	100	100	160	100	100	**44**	200	100	100	150	250	100
21	100	100	100	175	100	100	**45**	200	175	100	200	100	100
22	100	100	100	190	100	100	**46**	175	200	100	250	200	100
23	100	100	100	205	100	100	**47**	100	175	100	150	250	100
24	100	100	100	220	100	100	**48**	200	200	150	250	100	100

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