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Evaluation of the OSCAR™ system for the production of monoclonal antibodies by CHO-K1 cells

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ABSTRACT

Biopharmaceutical production of complex recombinant protein therapeutics currently relies on mammalian cells. The development of high-yielding stable cell lines requires processes of transfection, selection and adaptation. With several technologies available, selection has been most frequently based on dihydrofolate reductase or glutamine synthetase systems, which can be very time-consuming. Due to the pressure to reduce development costs and speed up time to market, new technologies are emerging, as the promising OSCAR™ expression system that could provide more rapid development of high-yielding stable cell lines than the traditional systems. However, further evaluation of its application in a wider range of cell types and media is still necessary. In this study, application of OSCAR™ for the transfection of a CHO-K1 cell line with a monoclonal antibody was evaluated. OSCAR™ was reasonably fast and simple, without negative impact on cell growth characteristics. However, minigene selection was critical, with only pDWM128 working for the cell line assessed. Initial relatively high levels of production decreased significantly in the first few weeks of passing, remaining relatively stable although with low yield thereafter. The results suggest that more work is required to develop methodologies and prove that OSCAR™ has significant value to the bioproduction industry.

1. Introduction

Therapeutic proteins have assumed an increasing importance in the last years, which is corroborated by their significant market growth (Durocher and Butler, 2009; Li and d'Anjou, 2009). They are applied in a wide range of areas that include treatment of diseases such as cancer, diabetes, anaemia, haemophilia, allergies, and blood clotting disorders, as well as research applications (Bhopale and Nanda, 2005). For their commercial production, several culture systems can be used, but for larger and more complex proteins mammalian cells are most commonly used (Andersen and Reilly, 2004; Chu and Robinson, 2001; Melton et al., 2001; Mohan et al., 2008) due to their ability to perform in vivo correct refolding and post-translational modifications (Werner et al., 1998), which guarantee a proper biological function of the protein.

In order to maximize the protein yield in mammalian cells, gene expression systems are routinely used that allow for a selection/amplification to obtain relatively high expressing stable cell lines for use in manufacture, e.g. the dihydrofolate reductase (DHFR) and the glutamine synthetase (GS) systems (Bebbington et al., 1992; Page and Sydenham, 1991; Wurm, 2004). Although exploiting different metabolic pathways (nucleotide metabolism for DHFR and glutamine metabolism for GS) (Lonza, 2012; Wurm, 2004), both methods require multiple rounds of amplification after transfection through the use of increasing concentrations of specific drugs (methotrexate in DHFR and methionine sulphoximine in GS) (Andersen and Reilly, 2004; Chusainow et al., 2009; Lonza, 2012). This results in time-consuming development and high costs associated with the use of specialized media and toxic chemicals.

Recently, a novel expression system known as OSCAR™ has been developed at the University of Edinburgh (Melton et al., 2001). This system relies on partially disabled minigene vectors that encode for hypoxanthine phosphoribosyltransferase (HPRT), essential for purine synthesis via the normal cellular salvage pathway (Barnes et al., 2001; Melton et al., 2001). HPRT-deficient mammalian cells transfected with one of these minigenes and a gene of interest are placed in a selective hypoxanthine aminopterin thymidine (HAT) medium that blocks de novo purine synthesis. This makes cell survival reliant on the salvage pathway using a disabled HPRT enzyme (Melton et al., 2001). Since large amounts of

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this enzyme are required for cell survival, selection and amplification in the OSCAR™ system takes place in a single step which potentially provides a time advantage over the traditional DHFR and GS systems. Additionally to being more expedite, OSCAR™ is also said to provide cells with higher (7-fold) stable (Melton et al., 2001) expression yields at lower (7-fold) costs of goods (Nicholson, 2012) due to the absence of specialized media and toxic chemicals, that are required by traditional systems. Nevertheless, the recentness of OSCAR™ demands for a further evaluation of its applicability in a wider range of cell types and media, as well as its scalability and performance in bioreactor cultures (Costa et al., 2010).

In the present work, the application of OSCAR™ for the transfection of a CHO-K1 cell line expressing a monoclonal antibody (mAb) was evaluated. This covered the impact of the expression system on cell growth characteristics, the levels and stability of mAb production achieved, as well as the ease of the implementation/execution of the methodology.

2. Materials and methods

2.1. Cells, plasmid and minigenes

The CHO-K1 cell line CCL-61 was obtained from the American Type Culture Collection (ATCC) and the HPRT-deficient cells CHO-K1 TG-2 were kindly provided by Edinburgh University.

For cell transfection, the monoclonal antibody CAB051 gene/plasmid was kindly provided by Biotecnol SA (Lisbon, Portugal), and the HPRT minigenes pDWM131, pDWM129 and pDWM128, linearized with BamHI, were provided by Edinburgh University.

2.2. Selection and validation of HPRT-deficient clones

CHO-K1 cells were seeded in petri dishes at 5×10^4 cells/ml in Dulbecco's Modified Eagle's Medium (DMEM, D6546, Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS, F7524, Sigma–Aldrich) and 200 mM L-glutamine (G7513, Sigma–Aldrich) (hereafter named growth medium). Cells were grown overnight at 37 °C, 5% CO_2 and the medium replaced by growth medium supplemented with 20 μg/ml 6-thioguanine (A4882, Sigma–Aldrich). Cells were further incubated for 14–15 days, after which individual clones of HPRT-deficient cells were selected and isolated using either cloning rings (Z370789, Sigma–Aldrich) or cloning disks (Z374458, Sigma–Aldrich), according to the manufacturer's procedure. Briefly, the bottom part of the cloning rings was coated with silicone grease (Z273554, Sigma–Aldrich), and then placed over individual colonies. Cells within the ring were detached using trypsin (T4049, Sigma–Aldrich), transferred to 24-well plates (5530300, Frilabo, Portugal) and expanded to 6 well plates (5530500, Frilabo, Portugal), 25 cm^2 T-flasks (5520100, Frilabo, Portugal) and 75 cm^2 T-flasks (5520200, Frilabo, Portugal). For their turn, the cloning disks were soaked in trypsin and placed directly over individual colonies. After 5–10 min the disks were removed and transferred to 24-well plates and expanded as described for the cloning rings.

The clones isolated were validated for HPRT-deficiency and mutation stability. For this, they were seeded in petri dishes (900095, Frilabo, Portugal) at 1.3×10^5 cells/ml and grown in growth medium supplemented with hypoxanthine aminopterin thymidine (HAT, H0262, Sigma–Aldrich) (hereafter called HAT medium) at 37 °C, 5% CO_2. A control was performed with the parental CHO-K1 cells in the same conditions. Clones were validated by their inability to grow in this medium.

2.3. Transfection

The HPRT-deficient clones validated and the CHO-K1 TG-2 cells were seeded in growth media into petri dishes at 1.3×10^5 cells/ml and incubated overnight at 37 °C, 5% CO_2, for transfection by calcium phosphate co-precipitation.

The CAB051 gene/plasmid was co-transfected with each of the three linearized HPRT minigenes. For this, 1 ml of 1× HEPES-buffered salt solution (HBS) was added to a DNA mixture of 20 μg HPRT minigene and 20 μg CAB051 plasmid (less than 100 μl DNA). Then, 62 μl of 2 M calcium chloride ($CaCl_2$, 202940, Sigma–Aldrich) was added, drop by drop, to the mixture and left for 45 min at room temperature until a precipitate was formed. The precipitate was slowly dropped over the cells on the petri dishes, after medium-removal. The plates were left at room temperature for 20 min with frequent spreading of the precipitate by tilting. Growth medium was added and the cells were incubated overnight at 37 °C, 5% CO_2.

Cells were then seeded, at known concentration, into 24 well plates (at least two plates for each transfection) with growth medium to provide an average of 1 HAT-resistant colony per well. After 24 h, the medium was replaced by HAT medium, to allow the selective growth of the transfected cells. Cells were then incubated for 3–4 weeks, with medium changes after the first 2–3 days and thereafter every 10–14 days.

Samples from the wells with colonies were taken to assess antibody production by enzyme-linked immunosorbent assay (ELISA), as described below. Cell concentration was also determined by direct cell counting with a hemocytometer, to allow the calculation of the specific production rate (productivity) of each clone, expressed as pg/cell/day, according to Eq. (1). Clones with the highest values were expanded into 25 cm^2 flasks under selective conditions.

$$\text{Productivity} = \frac{C_{\text{antibody}}/C_{\text{cell}}}{t_{\text{culture}}} \quad (1)$$

where C_{antibody} is the antibody production in pg/ml, C_{cell} is the cell concentration in cell/ml, and t_{culture} is the time of culture in days.

The stability of production levels of the selected clones was assessed periodically for several weeks, as well as after a period of two years (with the culture subjected to periods of continuous passage and periods of cryopreservation), following the same methodology described above.

2.4. Cell growth characteristics

An evaluation and comparison of the growth characteristics of the original and transfected (selected clones 18 and 32) CHO-K1 cells was performed by determination of the cell doubling time (t_D). For this, cells were seeded at 1×10^5 cells/ml into 6-well plates and allowed to grow at 37 °C, 5% CO_2 for 2, 4, 6, 10, 24 and 48 h before direct cell counting with a hemocytometer and trypan blue staining. The t_D value was determined according to Eq. (2):

$$t_D = (t_2 - t_1) \times \frac{\log(2)}{\log(C_2/C_1)} \quad (2)$$

with C_1 and C_2 being the cell concentration at times t_1 and t_2, respectively, assuming a constant growth rate.

2.5. Antibody quantification by ELISA

Samples were analyzed for antibody productivity by ELISA following an optimized procedure described in SOP 2008-01 ANL from Biotecnol SA. Briefly, 96-well plates (9018, CoStar) were coated with capture antibody overnight and then blocked for 45 min at room temperature. Dilutions of the samples were added, as well as a standard of known concentration and a quality control, and

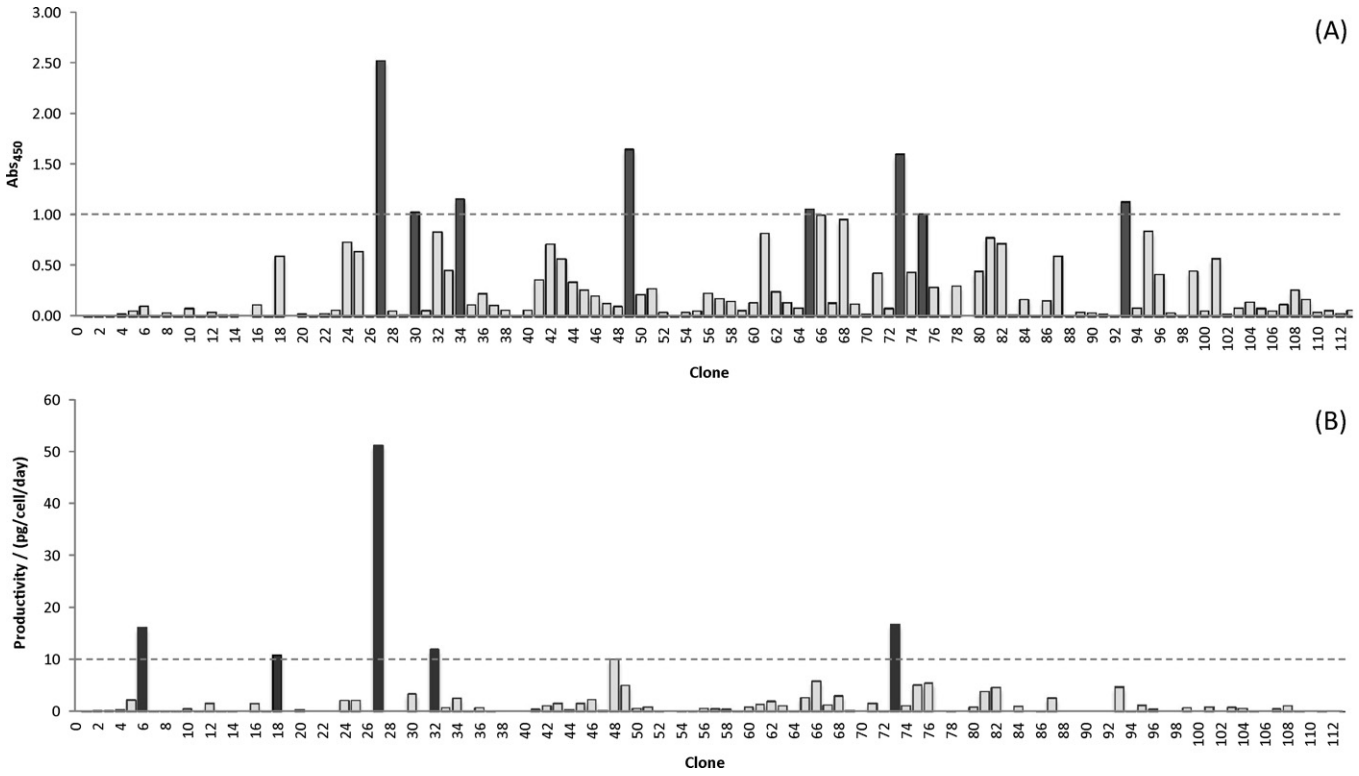

Fig. 1. Antibody production evaluated by two approaches for initial clone selection after transfection: (A) absorbance at 450 nm, and (B) cell productivity. The interrupted line denotes the cutoff limit established for clone selection, and a darker color highlights the selected clones.

the plates were incubated for 2 h at 37 °C. The detection antibody was then added and the plates incubated for 2 h at room temperature, after which a 3,3′,5,5′-tetramethylbenzidine (TMB, T0440, Sigma–Aldrich) substrate solution was added and allowed to react for 10 min, at room temperature. After stopping the reaction, the absorbance was read at 450 nm, and the antibody production was determined.

2.6. Statistical analysis

Statistical analysis of clone productivity and stability data was performed with the Statistical Package for the Social Sciences (SPSS) software, using one-way analysis of variance (ANOVA) with Bonferroni test, with a confidence level of 95%.

3. Results and discussion

3.1. Analysis of the two central phases of OSCAR™ technology

In the first phase of OSCAR technology, consisting of selection of HPRT-deficient clones, two methodologies for clone isolation were assessed: cloning rings and cloning disks. Comparing both in terms of procedure, cloning disks proved to be simpler and fastest. However, concerning efficiency, cloning rings were clearly superior, allowing the recovery of a much higher number of cells. Therefore, in any study similar to the present one, where the amount of cells isolated is very low, it is crucial to use a methodology that maximizes cell recovery, which makes cloning rings the best choice.

All the clones isolated were successfully validated as HPRT-deficient and suitable to use in the second phase (transfection).

Regarding transfection, three HPRT minigenes with different degrees of expression disability were evaluated for CHO-K1 co-transfection with CAB051 plasmid, in order to determine which would provide cells with better production. The results obtained showed that transfection of the CHO-K1 cell line was successful only with the most disabled minigene – pDWM128, since there were no cell colonies appearing when using the other minigenes.

The fact that the OSCAR™ expression system enables the selection of different minigenes clearly has advantages and limitations. On the positive side, it makes OSCAR™ potentially adaptable to the requirements of different cell lines, and allows tailoring of the system to the desired levels of expression. On the negative side, it implies more lab work in order to evaluate all minigenes. However, as soon as more and more cell types are tested with this system, it will be possible to know beforehand the applicability of each minigene to different cell lines, cell clones of the same cell line, and products and/or plasmids transfected. The construction of a database would be very useful, and the suggestion is here made.

3.2. Level and stability of clone productivity

Several clones (113) were obtained at the end of the transfection procedure. These clones were assessed by ELISA for the selection of the most productive. The initial selection was typically based on clones' absorbance, A_{450}, because it is the fastest approach. Another approach consists on determining productivity which, by measuring cell and antibody concentrations, becomes more labor intensive but also more accurate. The results obtained with both approaches are depicted in Fig. 1.

Fig. 1 clearly demonstrates the difference between the two methodologies for the selection of the most productive clones. The most noticeable difference is the higher number of clones that appear to be of interest in a selection based on absorbance values compared to a selection based on productivity levels. However, more relevant is the fact that different clones would be selected with each methodology. Setting as selection criteria absorbance values higher than 1 (clones 27, 30, 34, 49, 65, 73, 75 and 93) and productivities above 10 pg/cell/day (clones 6, 18, 27, 32 and

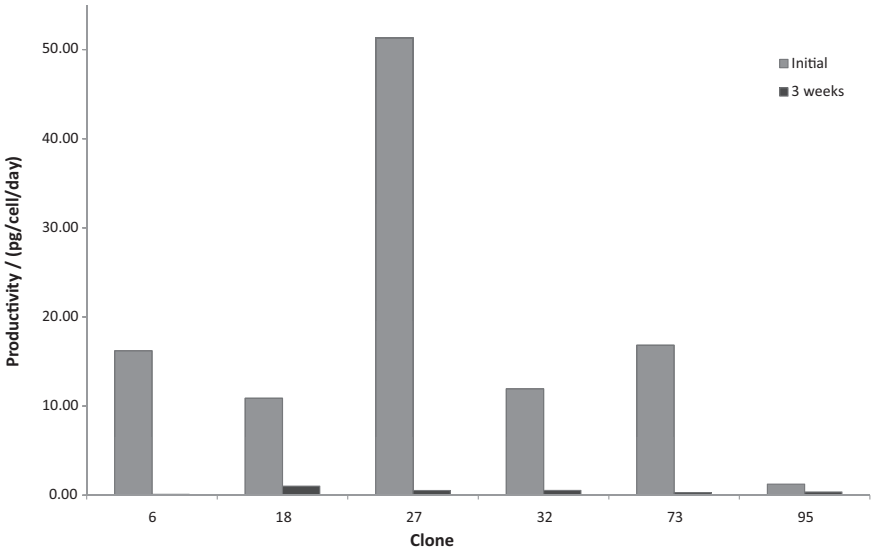

Fig. 2. Initial productivities of clones 6, 18, 27, 32, 73 and 95 and the corresponding productivities obtained after three weeks in culture.

73), there are only two clones selected by both approaches (27 and 73). Furthermore, in some cases, such as clone 6 and clone 93, the results of one methodology are in complete contrast with the other.

These results reveal a strong impact of the selection methodology on the outcome of the selection process. Therefore, although the analysis of productivity of each clone is less straight-forward and more time-consuming than the simple assessment of the absorbance values, the fact that it takes into consideration both cell and antibody concentrations makes it the preferred method to base clone selection. Considering these observations, the clones with productivities above 10 pg/cell/day were selected (6, 18, 27, 32 and 73) for further analysis. Additionally, one clone with lower productivity levels (clone 95) was also selected to evaluate and compare the stability of production in both lower- and higher-producing clones.

Production stability was evaluated by periodic monitoring of clones during 6 weeks, and additional measurement after two years. The first measurement was performed after 3 weeks of culture (passed three times a week) and the production levels obtained are shown in Fig. 2.

It is clear that a substantial decrease (between 70% and 99%) of production occurred in the period of only three weeks (Fig. 2).

Further evaluation of the evolution of productivity with time was performed, as presented in Fig. 3. It should be noted that since clones 6 and 73 have shown levels of production already very low at this stage, they were not considered in this evaluation.

Fig. 3 shows that the levels of production continue to decrease with time, although not so abruptly as the decrease observed in Fig. 2. Nevertheless, the productivity levels obtained after 6 weeks in culture still showed a significant ($p < 0.05$) reduction.

It should be stressed that the differences in productivity levels among the tested clones were attenuated over time (Fig. 3). Indeed, although clone 18 was clearly the highest-producer ($p < 0.05$) in the first weeks, at the end of the time in analysis all clones showed no significantly different ($p > 0.05$) levels of productivity.

To further assess production stability, clones 18 and 32 were selected (based on their superior cell proliferation) to be cultured during two years after which additional measurements of productivity were performed. The results obtained are shown in Table 1.

The productivity levels of clones 18 and 32 after 2 years were not significantly different ($p > 0.05$) from the productions obtained at the end of the previous period of analysis (6 weeks), indicating a tendency for stabilized levels of production.

Nevertheless, the loss of production ability after 2 years, in comparison with the initial measurements, is considerable and,

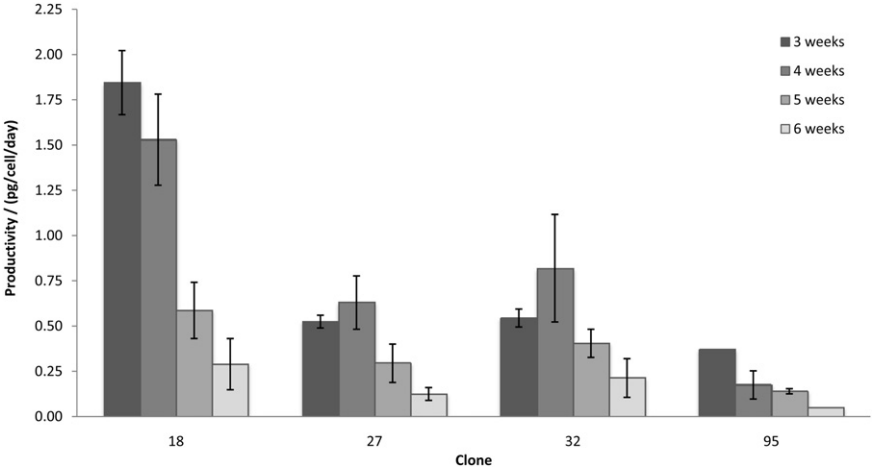

Fig. 3. Evolution of productivity levels of clones 18, 27, 32 and 95 during 6 weeks.

Table 1
Values of productivity obtained for clone 18 and clone 32 after two years in culture.

Cells	Productivity (pg/cell/day)
Clone 18	0.21
Clone 32	0.31

Table 2
Doubling times (t_D) of the original CHO-K1 cells and of the mAb-producing clones 18 and 32.

Cells	t_D (h)
CHO-K1	20.97 ± 3.76
Clone 18	21.86 ± 4.30
Clone 32	22.69 ± 0.98

therefore, in opposition to what has been observed in studies with other cell lines/proteins (Melton et al., 2001), the OSCAR™ methodology does not provide stability of high yield production when applied to the CHO-K1 cells producing the CAB051 mAb.

It should be noted that the clones with better levels of production and stability (18 and 32) would not have been selected if the absorbance method for clone selection had been used.

3.3. *Effect of transfection on cell growth characteristics*

The growth characteristics of CHO-K1 cells before and after transfection with OSCAR™ were evaluated. For this, the doubling times were determined for the original CHO-K1 cells and for the mAb-producing clones 18 and 32, after two years in culture, as shown in Table 2.

Using the OSCAR™ system a high gene copy number is generated, which is said not to compromise the growth rate of cells (Melton et al., 2001). This is a common problem after transfection, since the ability to produce something not natural to the cells can be a metabolic burden and therefore affect growth (Gu et al., 1992, 1995). In the present work the presence of the gene does not appear to significantly affect the doubling times of the transfected clones. This may be associated with the fact that the clones are not expressing at particularly high levels and therefore clone cells continue to grow as the parent cells.

4. Conclusions

The OSCAR™ technology appears as a possible alternative expression system to the more traditional methods. It is potentially faster for cell line development (by combining selection and amplification in one step) and to provide cells with higher and more stable expression yields, without significant impact on cell growth characteristics. In this study, its applicability for the expression of a monoclonal antibody in CHO-K1 cells was tested. Globally, the OSCAR™ technology proved to be reasonably fast and simple with mAb-producing cells obtained after 6–8 weeks. However, the selection of the minigene proved critical, and only the most disabled minigene (pDWM128) worked for the CHO-K1 cells assessed in this study. It was found that the growth characteristics of the cells are not hindered by the process. However, in this evaluation the relatively high expression level initially obtained, >10 pg/cell/day, rapid and sharply decayed in the first few weeks of culture, <1 pg/cell/day, and remained low but stable when evaluated over two years. Additionally, this study also suggests the strong impact of the methodology of clone selection on the outcome of the process, advising the use of productivity analysis, instead of simple comparison of the absorbance values of culture supernatants.

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