Effect of Pluronic F-68, 5% CO₂ Atmosphere, HEPES, and Antibiotic-Antimycotic on Suspension Adapted 293 Cells

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Abstract: The influence of different parameters upon cell culture of serum-free adapted 293 cells including the surfactant Pluronic F-68, carbon dioxide (5% CO₂ atmosphere), buffer HEPES and antibiotic-antimycotic has been explored. A defined serum-free medium (SFM) formulated without human or animal origin components from Invitrogen was used to grow the suspension adapted 293 cells. For all cell culture parameters cell density and viability of the suspension adapted 293 cells were monitored. The results indicated that the PF68 concentrations ranging from 0.05% to 0.2% can be used in the culture of the suspension adapted 293 cells since no negative effect upon either cell density or viability was detected. This will minimize the formation of aggregates during cell culture. It was demonstrated that neither the cell density nor the viability of the suspension adapted 293 cells were affected by the 5% CO₂ atmosphere at the inoculation cell densities evaluated. The use of the buffer HEPES in the cultivation of suspension adapted 293 cells did not cause negative effects upon cell density and viability. The addition of HEPES makes more robust the culture to pH fluctuations. The antibiotic-antimycotic can be used when needed at concentrations of up to 50 IU/ml for the culture of this particular cell line, with no apparent effect upon cell growth. The results obtained will contribute to a basic understanding of the 293 cell culture in the 293 SFM II and to the process development of their culture in bioreactors for the expression of different products of biotechnology interest.

Key Words: 293 cells, Serum free medium, Cell density, Cell viability, Suspension.

INTRODUCTION

Gene therapy is increasingly viewed as a novel form of drug therapy and a source of new therapeutic products for pharmaceutical companies [1,2]. Adenoviral vectors are currently the most utilized viral vectors in gene therapy industry [3]. The use of adenoviral vectors for gene therapy applications provides some advantages such as the high efficient transfection of a wide variety of cell types, including non-dividing cells in vitro and in vivo [4]. In addition, the capacity for insertion of foreign DNA is up to 37 kb when more than one region of the adenovirus genome is removed [5,6]. The adenoviral vectors also can be produced in high titers; i.e., 10¹¹ plaque forming units per milliliter–pfu/ml [7].

The human embryonic kidney (HEK) 293 cell line is a good host for the replication of most of the human adenoviruses and is currently used almost exclusively for packaging adenoviral vectors [8,9]. The 293 cells are used to isolate and propagate E1 mutants and to develop adenovirus as helper-independent cloning and expression vectors in mammalian cells [10,11]. The genes transfected into this cell line are often efficiently transcribed in transient expression assays [12,13]. This cell line has the ability to support the replica

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Carlsbad, CA. The 293 cells were grown in suspension in 293 SFM II from Invitrogen.

**Cell Conservation**

The originally attached cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) obtained from Sigma-Aldrich Ltd, UK, and supplemented with 10% heat-inactivated (56°C, 30 min) fetal calf serum (hiFCS; Mycoplex, PAA Laboratories). The cell line was subcultured every 3 to 4 days following digestion at room temperature with 0.5 ml trypsin/EDTA (Sigma-Aldrich Ltd, UK). The 293 cells were adapted to grow in suspension 293 SFM II (Invitrogen). The suspension adapted 293 cells were subcultured every 48h by centrifugation and resuspended in fresh medium to maintain cell density at 2 × 10^5 cells/ml. All cultures were maintained at 37°C in an incubator with a 5% CO₂ atmosphere. Cultures growing in suspension were incubated under same conditions and agitated at 100rpm. Cell densities were determined using the standard hemocytometer trypan blue exclusion method and reported as cells/cm² or cells/ml for attached and suspended cells, respectively. The viability reported as a percentage was determined by comparing the total number of cells to the number of viable cells. The results shown for the effect of F-68, 5% CO₂ atmosphere, HEPES and antibiotic-antimycotic represent the average of the experiments carried out in triplicates.

**RESULTS AND DISCUSSION**

**Effect of Pluronic F-68 upon 293 Cells**

The non-ionic surfactant Pluronic F-68 or PF68 has been used previously to avoid damage caused by agitation and sparging of mammalian and insect cells [22,23]. According to the protocol available for the cultivation of suspension adapted 293 cells in 293 SFM II provided by Invitrogen, the addition of the PF68 to such culture was not necessary because a surfactant is part of the formulation of the medium. The surfactant, should avoid the decrease in cell viability when scaled-up at bioreactor scale in agitated cultures. Previous studies in mammalian cells have shown that the interaction between PF68 and the cell membrane depended on the PF68 concentration varying from 0.05% to 0.2% (v/v) [24,25]. The concentrations of PF68 evaluated in this study included 0.05%, 0.1% and 0.2% (v/v).

The effect of the different concentrations of PF68 upon cell density and viability of suspension adapted 293 cells was determined in vented T-25 flasks with a working volume of 10 ml. The initial cell concentration was 1.8 × 10^5 cells/ml for all flasks. The flasks were incubated at 37°C in a 5% CO₂ atmosphere. Samples were analysed daily for 6 consecutive days to determine the cell density and viability. As can be seen in Fig. (1) the density of the suspension adapted 293 cells was similar for all of the PF68 concentrations analysed compared to the control during the culture. This result correlated with those reported previously for insect cells [22,23,25] where concentrations of 0.05%, 0.1% and 0.2% did not affect the cell density. Similarly, the PF68 did not have any significant effect upon cell viability of the cell line (Fig. 2). These results indicated that any of the PF68 concentrations ranging from 0.05% to 0.2% can be used in the culture of the suspension adapted 293 cells. The addition of this surfactant can reduce the damage to the cells growing in suspension in 293 SFM II with agitation at bioreactor scale.

![Fig. (1). Effect of Pluronic F-68 upon cell density of suspension adapted 293 cells growing in SFM. Concentrations of Pluronic F-68 (PF68) used included 0.05%, 0.1% and 0.2% (v/v) was compared with the flask control. (●) flask control without addition of PF68, (■) 0.05% v/v PF68, (▲) 0.1% v/v PF68, (×) 0.2% v/v PF68.](image1)

![Fig. (2). Effect of Pluronic F-68 upon cell viability of suspension adapted 293 cells growing in SFM. Concentrations of Pluronic F-68 (PF68) used included 0.05%, 0.1% and 0.2% (v/v) was compared with the flask control. (●) flask control without addition of PF68, (■) 0.05% v/v PF68, (▲) 0.1% v/v PF68, (×) 0.2% v/v PF68.](image2)

**Effect of a 5% CO₂ Atmosphere Upon 293 Cells**

Oxygen supply is one of the major problems in large-scale of high density mammalian cell cultures. From a technical point of view, the most convenient method of supplying sufficient oxygen to a large-scale suspension cell culture is by sparging air. However, in many cases significant cell damage associated with cell-bubble interactions has been observed [26-28].

CO₂ is one of the products of aerobic metabolism, which can accumulate to toxic or metabolism–altering levels [29,30]. This compound can be removed from the culture medium by aeration. Usually, cell cultures are incubated under a 5% CO₂ atmosphere with appropriate NaHCO₃ concentration in the media to achieve a desired pH [31]. In small scale cultures, this atmosphere is controlled in the incubator.
whilst at larger scales the cultures are normally sparged with air supplemented with 5% CO₂. In some large-scale protocols for growing 293 cells in suspension, the gas is supplied to control the pH during the culture [32-34]. In this study, the effect of a 5% CO₂ atmosphere upon the cell density and viability of the suspension adapted 293 cells was evaluated, since previous studies showed the capacity of 293 cells to grow at normal rate in the absence of aeration [21].

To do this, the suspension adapted 293 cells were grown in T-150 flasks with a working volume of 100 ml in 293 SFM II. The initial concentration of cells was 2 × 10⁵ cells/ml, which corresponded to the initial concentration conventionally used in bioreactors for such cell line. Both flasks were incubated at 37°C in a 5% CO₂ atmosphere. The cap of the control flask was vented in order to permit gas diffusion. This was made possible by a 0.22 µm porous diameter filter integrated into the cap. The non-vented cap of the other flask was tightly sealed and wrapped in Parafilm. Cell growth and viability were monitored every 24 h for six consecutive days without medium replacement.

As indicated in Fig. (3) the cell density of the 293 cells in both flasks increased during the first 72h of incubation. Without media replacement, after 72h cell concentration decreased due to the absence of nutrients in the medium and the accumulation of metabolites during the culture. Cell viability was higher than 70% during the first 96h of culture in both flasks (Fig. 4) but there was no significant difference between the viability profiles under the conditions established. The results obtained from this experiment showed that neither the cell density nor the viability of the suspension adapted 293 cells were affected by the 5% CO₂ atmosphere. However, this experiment was carried out with an initial cell density of 2 × 10⁵ cells/ml and may be distinct with different initial cell densities. This characteristic can be exploited for culturing the suspension adapted 293 cells in the bioreactor without sparging, since this may cause cell damage. Additionally, the inclusion of PF68 in the medium formulation may decrease the cell damage caused not only for agitation but also for sparging. The protocol proposed for the culture of suspension adapted 293 cells in the bioreactor involved growing them for 72h. At this time the optimum cell concentration for infection is reached. The infected cells were grown in fresh medium in the bioreactor for a further 72h before harvesting. The cell growth and post infection time coincided with the 72h stage at which the cells can grow without aeration. Without the CO₂ supply to the culture of suspension adapted 293 cells, pH control became more difficult, especially because of the low buffering capacity of the 293 SFM II determined previously [35]. For this reason it was necessary to investigate the addition of a more robust buffer system to the medium without impacting upon the cell density or viability of the suspension adapted 293 cells.

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Effect of HEPES Upon 293 Cells

According to the current protocols available for growing 293 cells, the pH of the medium is controlled by the addition of either 1M NaOH or NaHCO₃ [34,36]. However, the suspension adapted 293 cells can be particularly susceptible to the addition of such compounds. Furthermore, the addition of such compounds in relatively high amounts alters the composition of the medium affecting the osmolality and consequently the cell growth. For this reason it was necessary to investigate an alternative way for buffering the pH in the system without affecting the cell growth.

The N-2-hydroxyethyl-piperazine-N’-2-ethanesulfonic acid or HEPES has been described as one of the best buffers for biological research [37]. HEPES has been used previously in cell culture usually in the range of 10 to 25 mM with no toxic effects upon cells [32]. At most biological values of pH the HEPES molecule is zwitterionic, and is effective as a buffer system at pH values from 6.8 to 8.2. In this study two different concentrations of HEPES including 10 and 25 mM were used to determine the effect of the addition of such a compound upon cell density and viability of suspension adapted 293 cells grown in 293 SFM II. Three T-25 flasks with 10 ml working volume were seeded with 2 × 10⁵ cells/ml and hermetically sealed by using a non-vented cap tightly sealed and wrapped with Parafilm. The cell density...
and viability of the suspension adapted 293 cells were monitored every 24 h and compared with the flask control.

As shown in Fig. (5) the addition of HEPES to the concentrations analyzed did not result in significant difference in the cell density of suspension adapted 293 cells. The cell density increased during the first 72h in all cases as it was expected according to previous profiles obtained for the same cells under standard conditions. In the flask supplemented with 25 mM HEPES that increase was extended to 96 h but this was still a lower value than that obtained for 10 mM in 72h. Fig. (5) illustrates the comparison of viability of suspension adapted 293 cells at the HEPES concentrations studied. HEPES did not show any significant difference in cell viability during the time of culture.

![Viability of suspension adapted 293 cells](image)

**Fig. (5).** Effect of HEPES upon cell viability of suspension adapted 293 cells growing in SFM. Control flask (♦) without HEPES; Buffered cultures with HEPES (■) 10mM HEPES, (▲) 25mM HEPES.

At the end of the culture the cell viability in the control was lower than that in the supplemented cultures. This was attributed to the HEPES buffer capacity maintaining cell viability above 40% during the seven days of culture. In terms of cell viability, no significant differences were observed between 25 mM and 10 mM. According to the results obtained here, the addition of HEPES buffer in the cultivation of suspension adapted 293 cells did not show negative effect upon either cell density or cell viability. From both HEPES concentrations studied, the 25 mM was selected for the supplementation of the 293 SFM II in the bioreactor. With this condition established for growing the suspension adapted 293 cells, the amount of NaHCO₃ necessary to maintain the pH in the bioreactor during the culture process can be decreased. The addition of HEPES would also buffer the 293 SFM II during the culture in the bioreactor without CO₂ sparging.

**Effect of Antibiotic-Antimycotic Upon 293 Cells**

The production of adenoviral vectors in larger scale (bioreactor) requires many different steps such as the pumping of the medium, inoculation of the bioreactor, harvest of cells for infection, pumping of cells resuspended in small amount of fresh medium, infection with adenoviral vectors, completion of total working volume with fresh medium and harvest of infected cells. All of these steps required the connection, disconnection and/or reconnection of tubing from different inlets and outlets increasing the risk of contamination. Although good laboratory practices (GLP) were maintained at all times, the contamination risk had to be minimized if not eliminated. For this reason the use of antibiotics and/or antimycotics at this scale could be helpful.

The decision to use antibiotics and/or antimycotics in order to prevent contamination is generally based on the particular needs of the process. Although it has been a common practice, their use in culture media has some disadvantages to be considered, such as masking of contaminants, which become apparent when the antibiotic is removed. Ideally, the use of antibiotics should be avoided but in cell culture especially at large scale it is sometimes necessary as explained above. Maintaining the culture free of contamination is very important especially when the culture is valuable and the preparation of the inoculum is time consuming such as during the manufacture of adenoviral vectors.

The type and concentration of antibiotics and/or antimycotics should be determined experimentally for each particular cell line. The supplemented cell culture must contain the minimum concentration of antibiotic possible in order to avoid contamination but without causing negative effects upon the cell line. In the particular case of the 293 cells, it has been recommended by Invitrogen to use antibiotic-antimycotic when necessary. This mixture of antibiotic-antimycotic contains penicillin and amphotericin B. The spectrum of microorganisms eliminated with such compounds is, for penicillin Gram-positive bacteria, for streptomycin Gram-positive and Gram-negative bacteria and for amphotericin B fungi and yeast. The recommended concentrations of such compounds for cell culture in SFM according to Invitrogen were 50-100 U/ml, 50-100 g/ml and 0.25-2.5 μg/ml, respectively.

In this study, the effect of different concentrations of the antibiotic-antimycotic upon cell density and viability of the suspension adapted 293 cells was evaluated. During the log phase and viability higher than 90%, cells were centrifuged and resuspended in fresh medium at a final cell density of 2 × 10⁵ cells/ml. The suspension was split into five vented T-25 flasks with 10 ml working volume. One flask was kept as a control and the antibiotic-antimycotic was added to the rest of the flasks at different concentrations including 25, 50, 75 and 100 IU/ml. The cultures were incubated at 37ºC in a 5% CO₂ atmosphere. Samples were taken every 24 h and cell density and viability were recorded. As shown in Fig. (6) the cell density of suspension adapted 293 cells control was similar in all the concentrations of antibiotic-antimycotic analyzed during the first 48 h of culture. After that time the cell density from the culture supplemented with 25 IU/ml of antibiotic-antimycotic presented a similar profile to that of the culture control. The cell density of the cultures supplemented with 75 and 100 IU/ml decreased dramatically after 72 h of incubation. These results showed that any concentration of antibiotic-antimycotic ranging from 25 IU/ml to 100 U/ml affected the cell density of the suspension adapted 293 cells during the first 48 h and only the lowest concentration showed similar cell density to the control culture.

The results presented in Fig. (7) show the viability of the suspension adapted 293 cells. The viability was maintained...
above 80% after 48h incubation for all the concentrations analyzed without medium replacement. After this time the viability decreased drastically especially at concentrations of 75 and 100 IU/ml of antibiotic-antimycotic. This decrease was partially due to the lack of nutrients and the accumulation of cell metabolites in the medium as shown in previous experiments. However, this effect was enhanced by the presence of the relatively high concentrations of the antibiotic-antimycotic. The results from this study showed that the antibiotic-antimycotic can be used at concentrations up to 50 IU/ml for the culture of this particular cell line, with no apparent negative effect upon the cell density and viability for 48h. From these results, the 293 SFM II medium supplemented with antibiotic-antimycotic was suitable for use in the bioreactor in order to avoid contamination from bacteria, fungi and/or yeast. According to previous findings, the time needed for cell growth prior to infection (48h) was the same as the post-infection time. Therefore, medium replacement during this period of time was not necessary.

**Fig. (6).** Effect of antibiotic-antimycotic upon cell density of suspension adapted 293 cells growing in SFM. Control: (■) culture without addition of antibiotic-antimycotic. Cultures with addition of antibiotic-antimycotic: (●) 25 IU/ml, (♦) 50 IU/ml, (▲) 75 IU/ml and (△) 100 IU/ml.

**CONCLUSIONS**

The results indicated that PF68 concentrations ranging from 0.05% to 0.2% can be used in the culture of the suspension adapted 293 cells since it did not show any negative effect upon either cell density or viability. Similarly, the addition of HEPES buffer in the cultivation of suspension adapted 293 cells did not cause negative effects upon either cell density or cell viability. The 5% CO₂ atmosphere was not limiting for the cell growth of 293 cells at the cell densities evaluated. The antibiotic-antimycotic can be used up to 50 IU/ml for the culture of the HEK 293 cell line, with no apparent negative effect upon the cell density and viability for 48h. The findings presented here provide valuable information for the cell culture of 293 cells growing in suspension in serum-free medium.

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**REFERENCES**


