# New Advanced Protein-Free, Animal Component-Free Medium for Recombinant Protein Expression in Adherent CHO Cell Cultures


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## Abstract

The expression of recombinant proteins has increased in importance in both research and pharmaceutical manufacturing applications. As more and more recombinant proteins are being used as therapeutic agents, the methods employed in their production are coming under increasing regulatory scrutiny. One of the areas of regulatory concern is the presence of animal-derived components in the media used to grow cells for recombinant protein expression. A CHO Protein-free Animal Component-free Medium (Sigma C5467) was previously developed to support suspension culture and to achieve the desired recombinant protein expression. In order to extend the usage of this medium to the adherent cell culture systems, such as roller bottles and fluidized bed bioreactors, we recently further developed a CHO Protein-free Animal Component-free Attachment Medium (Sigma C8730). Our data indicates that recombinant CHO cells growing in T-flasks or roller bottles with this newly developed attachment medium exhibit similar cell growth and a higher level of recombinant protein expression as compared to cells grown in DME/F12 supplemented with 10% fetal bovine serum. Moreover, CHO cells growing in suspension culture with Sigma C5467 medium can be directly transferred to T-flasks or roller bottles with C8730 medium for a short-term adherent culture without a pre-adaptation step. Besides CHO cells, various cell lines including HEK293, Vero and C127 can be maintained as adherent cultures in the Sigma C8730 medium with some modification and supplementation with appropriate recombinant growth factor(s). This observation strongly indicates that this new medium might be a good basal medium for any customers' specific cell line grown as adherent cultures. Finally, the performance of this medium (Sigma C8730) for CHO single cell colony isolation for the selection of new recombinant protein expressing cell lines is contrasted with serum-containing media commonly used for this procedure.


## Introduction

Chinese Hamster Ovary (CHO) cells are one of the most frequently used cell lines for the expression of recombinant proteins that require post-translational modification to express full biological function. CHO cells used for large-scale production of recombinant proteins are typically grown in suspension cultures using animal serum-supplemented medium. However, animal serum presents several well-documented problems for a biopharmaceutical manfacturers of therapeutic agents. This has led to the development of serum-free media, many of which contain proteins and/or protein hydrolysates from animals and plants. Since pharmaceutical and biopharmaceutical companies have developed many recombinant proteins as therapeutic agents, the methods used in their production are coming under increasing regulatory scrutiny. One of the main areas of concern is inclusion of animal-derived components in medium to grow cells for recombinant protein expression. Consequently, this has led to the development of media that are completely protein-free and animal component-free.

Sigma-Aldrich Corporation has developed a series of serum-free, protein-free animal component-free and chemically defined media for suspension culture of CHO cells to meet the various needs of biopharmaceutical industry. A CHO Protein-free Animal Component-free Medium (PF-AF)(Sigma C5467) has been developed to support the suspension culture of CHO cells and to achieve the desired recombinant protein expression. This CHO PF-AF medium contains recombinant human insulin, plant hydrolysates, and proprietary iron chelators. All other components are also of non-animal origin, including amino acids, vitamins, fatty acids and surfactants.

In order to extend the usage of this medium to adherent cell culture systems, we recently developed a CHO Protein-Free Animal Component-Free Attachment Medium (Sigma C8730). This medium contains no pluronic and extra-cellular matrix (ECM) proteins, such as collagen, fibronectin and vitronectin, which are usually obtained from animal origin. Currently, the culture of adherent cells in roller bottles and fluidized bed bioreactors with serum containing media is a commonly used technique for the production of recombinant proteins, and other clinical reagents in many biopharmaceutical companies. In this report, the performances of the new attachment medium (Sigma C8730) on adherent cultures of CHO cells and the expression of recombinant protein are presented. Similar cell growth and a higher level of recombinant protein expression in CHO PF-AF attachment medium is achieved as compared to that in DME/F12 supplemented with 10% fetal bovine serum (FBS) for attached CHO cells growing in T-flasks or roller bottles. In addition to CHO cells, various cell lines, such as HEK293, Vero and C127, can be maintained as adherent cultures in Sigma C8730 medium with some further modification and supplementation of appropriate recombinant growth factor(s). Finally, Sigma C8730 medium may be used for single cell colony formation for the development of new recombinant protein expressing CHO cell clones.

## Materials and Methods

Sigma-Aldrich Corporation (St. Louis, MO) supplied all chemicals, media and solutions unless otherwise stated.

### Cell Lines

CHO K1 cells (ATCC # CRL-61), HEK293 and Vero cells were obtained from the American Type Culture Collection (ATCC). CHO cell line 1 expressing a proprietary recombinant antibody was transferred from a customer to Sigma for custom medium development and optimization.

### Culture media

The media used in this study are CHO Protein-Free Animal Component-Free Medium (C5467) and DME/F-12 with 10% Fetal Bovine Serum (FBS).

### Cell Culture and Cell Growth Assays

Cells growing in DMEM/F12 + 10% fetal bovine serum were trypsinized and then washed once with HBSS. The cell pellet was resuspensed with Hank's Balanced Salt Solution (HBSS) and seeded into T-flasks or roller bottles at a 25% cell seeding density with Sigma C8730 attachment medium or other modified media. The speed for roller bottles is 0.5 rpm. In some cases, CHO cells growing in suspension culture with Sigma C5467 medium, were seeded directly into T-flasks with C8730 medium without re-adaptation to adherent culture.

During adherent culture, the spent medium samples were collected everyday for the analysis of recombinant IgG production. At the same time, the attached cells were trypsinized and counted by hemacytometer to determine cell growth and viability.

For single cell colony formation assays, 250 CHO cells were plated in a duplicate set of 100 mm culture plates with DME/F12 + 10% FBS. Two days later, the medium was replaced with C8730 on one set of plates. Then all cultures were incubated for 2 weeks or until the colonies could be visualized at 37°C with humidified air and 5% $CO_2$. Single cell colonies were stained by crystal violet.

### Quantitation of Recombinant Humanized IgG

The IgG secreted into the medium by CHO cell line 1 was measured by HPLC (Waters 2690 HPLC Millipore, MA) using a protein-G affinity column. The analysis is an affinity chromatography method, utilizing an analytical column packed with poly-flow through particles designed for very rapid mass transport. The protein-G has a high affinity for IgG under neutral conditions. The column does not retain other proteins such as albumin. The bound IgG is then quickly removed with an acidic solution. The amount of IgG in the subsequent peak is detected and quantitated by UV absorbance at 210 nm.

## Results

**Table 1. Inhibition of Pluronic F68 on cell attachment.**

| Pluronic mg/ml | Attached cells ( x $10^6$ cells) |
|---|---|
| 0 | 3.92 |
| 0.065 | 3.08 |
| 0.125 | 1.84 |
| 0.25 | 0.78 |
| 0.5 | 0.01 |
| 1 | ~0 |

CHO K-1 cells grown in DME/F12 + 10% FBS were re-seeded in T-25 flasks at a density of 25% with Sigma C8730 with different concentrations of Pluronic F68. After 4 days of culture, attached cells were trypsinized and counted.

**Adherent Cultures of CHO-K1 Grown in Sigma C8730 Medium on T-Flasks, Roller Bottles and Micro-Carrier Beads**

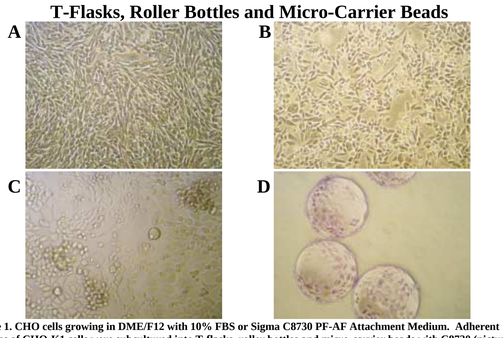


Figure 1. CHO cells growing in DME/F12 with 10% FBS or Sigma C8730 PF-AF Attachment Medium. Adherent cultures of CHO-K1 cells were subcultured into T-flasks, roller bottles and micro-carrier beads with C8730 (pictures taken on third day of culture). CHO adherent cultures in C8730 medium have similar cell morphology to cells growing in DME/F12 with 10% FBS. A) CHO cells in T-25 Flasks with DME/F12 with 10% FBS. B) CHO cells in T-25 flasks with C8730 C) CHO cells in Roller bottles with C8730 D) Micro-carrier beads with CHO cells in C8730, cells were visualized by crystal voilet staining.

**Comparison of Cell Growth and IgG Production with Sigma C8730 and DME/F12 plus 10% FBS in T-Flask**

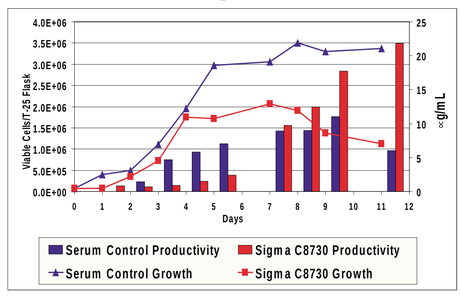


Figure 2. Cell growth and IgG production in CHO cell line 1 were compared under adherent culture conditions in Sigma C8730 Medium or DME/F12 with 10% FBS in T-flasks. Spent medium samples were collected everyday for analysis of IgG production. The attached cells were trypsinized and counted for cell growth. The data indicates cell growth in C8730 was not as high as DME/F12 with 10% FBS but production of recombinant IgG was much greater in Sigma C8730.

**Comparison of IgG Production of CHO Cell Line 1 in Roller Bottles and T-Flasks**

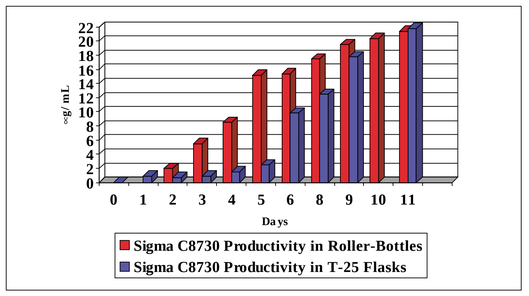


Figure 3. CHO cells growing in DME/F12 with 10% FBS were subcultured into T-flasks and roller bottles with Sigma C8730 PF-AF Attachment Media. Spent media samples were collected everyday for analysis of IgG production. The data indicates roller- bottle IgG production of the CHO cell line 1 was comparable to T-flask.

**Adherent Cultures of Various Cell Lines Grown in Sigma C8730 Medium**

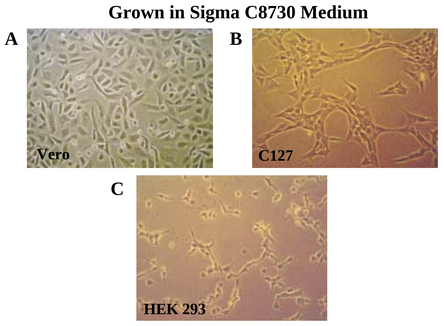


Figure 4. Adherent cultures of various cell lines by using Sigma C8730 medium. Vero, C127 and HEK 293 cells previously growing in serum-containing medium were subcultured directly into Sigma C8730 (pictures taken after 24 hours of incubation). Initial cell attachment in Sigma C8730 protein free medium indicate excellent adaptability to wide range of cell types. A) Vero cells, B) C127 cells, C) HEK 293 cells.

**One Step Conversion of Suspension Cultured CHO cells to Adherent Culture in Protein Free Medium**

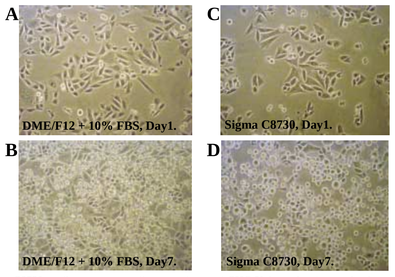


Figure 5. Sigma C8730 medium supports adherent cultures of CHO K-1 previously growing in suspension culture with Sigma C5467 medium. A) CHO cells adapted to Sigma protein-free medium (C5467) in suspension were transferred to DME/F12 with 10% FBS, 1-day culture. B) same as (A), 7-day culture. C) CHO cells adapted to Sigma protein free medium (C5467) in suspension were transferred to Sigma C8730 PF-AF medium, 1-day culture. D) same as (C), 7-day culture. CHO cells exhibit quick adaptability between Sigma protein free suspension medium and protein free adherent culture medium.

**Single Cell Colony Forming Assays of CHO K-1 Cells in Sigma C8730 Medium and DMEM/F12 + 10% FBS**

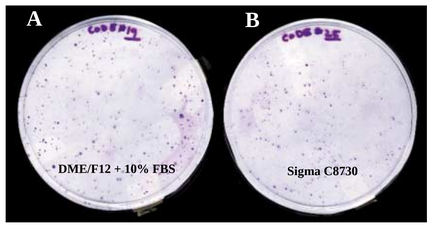


Figure 6. Single Cell Colony Forming Assays of CHO K-1 cells were performed in Sigma C8730 Medium and DMEM/F12 + 10% FBS. 250 CHO-K1 cells grown in serum containing medium were plated in duplicate sets of 100mm tissue culture plates with DME/F12 + 10% FBS. Culture medium was replaced with Sigma C8730 after two days of incubation on one set of plates. After 2 weeks of incubation, the single cell colonies were visualized by crystal violet staining. A) DME/F12 with 10% FBS. B) Sigma C8730 Medium. Pictures indicate comparable ability of C8730 and DME/F12 +10% FBS to support single cell colony growth.

## Discussion

Sigma C8730 CHO PF-AF attachment medium was designed to expand the applications of Sigma C5467 CHO PF-AF medium. In this presentation, we report the performance of Sigma C8730 medium on adherent cell cultures and recombinant protein expression. The first step in attachment medium development is to analyze the effect of surfactants on cell attachment. Pluronic F68, a surfactant used in Sigma C5467 PF-AF medium, prohibits the attachment of CHO cells on tissue culture treated flasks in a dose-dependent manner when cultured in the Sigma C8730 medium (Table 1). As the surfactant levels increase in concentration, cell attachment decreases. The results indicate that Pluronic F68 has a dramatic affect on attachment for media development.

Several different types of culture systems are available for adherent cell culture. We have tested a few of them with Sigma PF-AF C8730 medium. The new medium supports the adherent culture of CHO cell line 1 in T-flasks, roller bottles and micro-carrier beads with few detached cells (Fig. 1 B, C and D). CHO cells grown in C8730 medium show a smaller cell size and more epithelial cell-like morphology as compared to cells growing in serum containing medium (Fig. 1A). The ability to maintain attachment in a dynamic cell culture system will benefit many different applications.

Many CHO cells are being used for recombinant protein expression and currently use medium with serum. An experiment was initiated to compare CHO cultures grown in serum containing medium verses Sigma protein free attachment medium. Adherent cultures of CHO cell line 1 were seeded in T-flasks with Sigma C8730 medium and DME/F12 with 10% FBS. Cells reached a lower maximum density in C8730, as compared to cells grown in DME/F12 with 10% FBS. This can be attributed to adaptation of the cells to protein free media. The cells tend to detach from flasks and grow in suspension in late culture (Fig. 2). However a higher level of recombinant protein expression was observed in the culture with C8730. Additionally, Sigma C8730 medium supports similar adherent cell growth and IgG production of CHO cell line 1 cultured in roller bottles with a speed at 0.5 rpm, as compared with that in T-flasks (Fig. 3).

Sigma C8730 medium supports the attachment of various cell lines in addition to CHO cells (Fig. 4). However, further modifications of this medium, such as supplementation with appropriate growth factors, are required to promote the growth of those other attached cells.

CHO cells, previously grown in suspension with Sigma C5467 medium, can be directly transferred to T-flasks and then can be cultured with C8730 medium without pre-adaptation (Fig. 5). After 6-7 days of culture in Sigma C8730, some cells were found that detached from the surface of flasks and returned to growing in suspension.

Finally, single cell colony formation of CHO K-1 cells can be achieved by using Sigma C8730 medium after an initial 2 days of culture in serum containing medium (Fig. 6). This data strongly suggests that C8730 medium is useful for the selection of recombinant CHO cell clones after transfection. The utilization of C8730 instead of serum containing media to select recombinant protein expressing cell clones will help to quickly identify cell clones with maximum performance in C5467 protein free suspension medium without the need to go through a protein free adaptation procedure.

## Conclusion

- Sigma C8730 CHO medium can be used to support adherent cultures and recombinant protein expression in T-flasks, roller bottles and micro-carrier beads with CHO cells previously cultured in serum containing medium as adherent cultures.
- CHO cells grown in suspension with the Sigma C5467 medium, from which C8730 is derived, can be directly cultured into T-flasks or roller-bottles under adherent conditions with Sigma C8730 without any pre-adaptation procedure.
- Sigma C8730 CHO medium is useful for the selection of single cell colonies of recombinant CHO cell clones.
- Sigma C8730 CHO medium has the potential to support adherent cultures of a broad range of different cell lines.


## Acknowledgement

We would like to thank all that contributed to the research and development of the CHO media product line at the Sigma-Aldrich R&D St. Louis facility.


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