



AcroPrep™ 24-well Filter Plates with Depth & EKV Media or EKV Media for Clarification of Mammalian Cell Cultures

Summary

Centrifugation and filtration have been widely accepted as techniques required for clarifying complex cell cultures to recover extracellular proteins such as monoclonal antibodies (mAbs). These steps can be time consuming and costly for labs growing their cultures in 24-well culture plates. This document offers an alternative to the use of centrifugation/filtration/flocculation to clarify mammalian cell cultures and describes assessment of Pall's newly developed AcroPrep 24-well clarification and sterilization plate and AcroPrep 24-well sterilization plate for clarification of mammalian cell cultures (2 to 26 x 10⁶ cells/mL) and recovery of proteins present in the supernatant.

Introduction

In the pharmaceutical industry, there has been an increasing trend to develop and release biologics such as mAbs as therapeutic agents for pathologies including cancer and autoimmune diseases.

In recent years, biopharmaceutical manufacturing processes have demonstrated major improvements in the quality and recovery of mAbs, which to an extent has been associated with culturing the expressing cell lines at high cell densities. This has generated a great challenge in clarification and further downstream processing, highlighted in the need to remove a large amount of biomass and increased levels of contaminating cell debris generated during cell culture and harvesting.⁽¹⁾

Attempts are being made to reduce costs by continuous optimization of the individual steps such as increasing the yield of antibodies per volume of culture. Traditionally, centrifugation and a combination of filtration techniques such as cross-flow or tangential-flow filtration (TFF), and depth filtration (DF), have been widely accepted as techniques required for clarifying complex suspension cell cultures. Recent advances in single-use bioreactors have gained widespread acceptance in cell culture applications because they speed the production of biomolecules. Harvest and clarification operations remain largely dependent on centrifugation, TFF, and DF; all techniques that have yet to be widely adapted to single-use implementation.

Flocculation combined with depth filtration⁽⁴⁾ has been slow to gain acceptance in the manufacturing of mAbs, where mammalian cell culture processes such as Chinese hamster ovary (CHO) cell cultures, are typically utilized. Many flocculants are systematically evaluated with respect to their mechanism of action, impact on downstream processing and product quality, and potential disadvantages. The purpose of diatomaceous earth (DE) for the clarification of mAb samples dedicated for analytical purposes, is to aid the separation of complete cells and coarse debris; while suspended particles are removed by microfiltration at the same time. The DE method (flocculation) reduces the processing time by more than half with the wetted-DE staying porous, preventing blockage over the complete filtration.⁽²⁾

Centrifugation can be applied to cultures with high levels of solids. However, product recovery can be low due to high levels of solid particulates leading to large pellet volumes. Additionally, cell disruption from shear forces generated during centrifugation can further decrease the efficiency of harvest and clarification and potentially cause product damage and /or entrapment.

Depth filters are advantageous because they remove contaminants in single-use format, reducing the need for cleaning. However, depth filters are currently unable to handle high-solids cultures and therefore often are used in series with centrifugation. While TFF can handle high solids loading, this technique can exhibit poor yield due to a combination of excessive product dilution and cell lysis caused by shear forces during processing of dense feed streams.

The conventional method for mammalian cell line development usually involves a series of shake flasks for screening the cell lines prior to scale-up. The scale-up process often takes a significant amount of time because of the need to balance the complex interactions involved in the development of mammalian cell culture parameters. At present, there are a number of commercial small scale systems available with controllable microbioreactors that help speed up the development process by mimicking lab-scale bioreactors and allow several cell cultivations in parallel under different conditions.⁽³⁾

Regardless of the cell line development path taken, the use of 24-well culture plates for growth is often a pivotal part of these processes. Growth in a 24-well plate presents its own set of challenges during clarification and sterilization of the proteins of interest. To help streamline upstream process that utilize the 24-well form factor for cell growth, Pall Laboratory has expanded their product offerings to include an AcroPrep 24-well filter plate which combines cell clarification and sterile filtration functions. This document details the integration of Pall's newly developed AcroPrep 24-well clarification and sterilization filter plate and AcroPrep 24-well sterilization filter plate for clarification CHO and HEK293T cell cultures (2 to 26×10^6 cells/mL) and recovery of proteins present in the supernatant.

1. Materials and Methods

1.1 Materials/Equipment

1.1.1 Consumables

- Adhesive Plate Foil (-80 °C to 130 °C): VWR, P/N: 391-1275.
- AcroPrep 24-well filter plates with collection plates and lids, from Pall:
 - AcroPrep 24-well clarification and sterilization filter plates with Seitz® Cellulose K700P prefilter and 0.2 µm rated validated sterilizing-grade Supor® EKV membrane with built-in 0.65 µm MachV asymmetric prefilter layer, P/N: 97026 (8 pack) and 97016 (2 pack).
 - AcroPrep 24-well sterilization filter plates with 0.2 µm Supor EKV membrane, P/N: 97027 (8 pack) and 97017 (2 pack).
 - All AcroPrep 24-well filter plates, collection plates and lids used have undergone γ -irradiation at 17.4-30 kGy.
- Reverse Osmosis Water

1.1.2 Organisms

- CHO cells, S-line (suspension culture), sourced from internal cell culture collection
- HEK293T (adherent cells for suspension culture), sourced from internal cell culture collection

1.1.3 Equipment

- WPA Biowave II UV-Vis Spectrophotometer, (Biochrom♦).
- Eppendorf 5810 centrifuge with deep well-plate rotors A-2-DWP or A-2-DWP-AT.
- Multi-well Plate Vacuum Manifold: Pall, P/N: 5017.
- The cell density and viability were determined with the NucleoCounter♦ NC-200♦ automated cell counter and disposables Via1-Cassette♦, Chemometec, P/N 941-0012.

Other standard equipment commonly available in biology laboratories was used to generate the data in this document.

1.2 Methods

1.2.1 AcroPrep 24-well filter plates processed under vacuum or centrifugation

Centrifuge Processing

The AcroPrep 24 well filter and collection plates were centrifuged at 1,000 x g in an Eppendorf 5810 centrifuge with rotor A-2-DWP or A-2-DWP-AT.

Vacuum

The AcroPrep 24 well plates were processed using a Multi-well Plate Vacuum Manifold (Pall, P/N 5017), with vacuum at 15 inHg.

1.2.2 CHO cell culture

The CHO cell cultures were grown under sterile conditions after reviving from a cell bank stock (passage 8) in Hyclone[♦] ActiSM[♦] adaptation medium (Cytiva) supplemented with 0.87 g/L of UltraGlutamine[♦] I Supplement (Lonza Bioscience), at the following growth conditions: 37 °C, 135 rpm, and 8% CO₂. The cells were passaged every 2-3 days in the ActiSM adaptation medium for 1 week before continuing culture under the same conditions in Gibco[♦] Dynamis[♦] medium (Thermo Fisher Scientific) supplemented with 0.87 g/L of UltraGlutamine I Supplement and puromycin (1.25 mL/L medium, 12.5 µg/mL). At passages 19 to 22, the cells were harvested in Dynamis medium supplemented with only UltraGlutamine I Supplement. The cells had a density of ~ 2 - 3 x 10⁶ cells/mL and a viability > 98%.

1.2.3 High CHO cell density culture ('artificial')

To reach a high cell density of ≥ 26 x 10⁶ cells/mL, 2 - 3 x 10⁶ cell/mL CHO cells were centrifuged at 1,000 x g for 10 min. The appropriate volume of supernatant was removed to resuspend the cells to a final density of 2.6 x 10⁷ cells/mL. CHO cells were used and concentrated on the day of harvesting. Because of the number of tests to be performed, different CHO cell populations at different passage levels (19 to 22) were used to perform the testing with fresh concentrated CHO cells culture. The high CHO cell density culture was used with the AcroPrep 24-well clarification and sterilization filter plates to remove the cells from media.

1.2.4 HEK293T cell culture

The HEK293T cell cultures were grown under sterile condition, from a cell bank stock (stored in liquid nitrogen at 'passage 8') of adherent cells, thawed in FreeStyle[♦] 293 Expression Medium (Thermo Fisher Scientific, P/N 12338018) supplemented with 0.1% of Pluronic[♦] F 68 non-ionic surfactant (Thermo Fisher Scientific, P/N 24040032) at the following growth conditions: 37 °C, 135 rpm, and 5% CO₂. The cells were passaged every 2-3 days in FreeStyle 293 expression medium supplemented with 0.1% of Pluronic surfactant for 2 weeks under the same growth conditions until very little or no cell aggregation was observed under a microscope. The cells were harvested at passages 13 & 14. The cell density in suspension culture was ~ 2 - 4 x 10⁶ cells/mL with a viability > 98%.

The HEK293T cell culture was processed using the clarification and sterilization plate. The supernatant of the HEK293T culture was processed with the sterilization plate.

1.2.5 Clarification of HEK293T cell culture by centrifugation

Cells were removed from an aliquot of the HEK293T culture at a viability > 98% and a cell density of 4 x 10⁶ cells/mL, pelleted by centrifugation for 5 min at 700 x g. The supernatant was then recovered and filtered using the AcroPrep 24-well sterilization filter plate. The turbidity, pH, and protein recovery were assessed before and after filtration of the cell-free extract.

1.2.6 **Hold-up volume / Time-through / pH / Conductivity / Turbidity / Optical density**

To assess hold-up, the volume of filtrate per well (Downstream) was compared to the initial volume, 5 mL, of CHO cells (Upstream). The difference represents the hold-up volume, which is the sample volume that cannot be recovered after filtration. The smaller this volume is, the more sample that is recovered.

To determine processing time, 5 mL/well aliquots of high-density CHO cell culture were processed with the AcroPrep 24-well filter plate (Upstream). Aliquot volumes of 6 and 7 mL/well of HEK293T cell suspension were processed under centrifugation or vacuum, respectively. The AcroPrep 24-well filter plate (Upstream) and the collection plate (Downstream) were used with the Pall Multi-well Plate Vacuum Manifold at 15 inHg or with the Eppendorf 5810 centrifuge at 1,000 x g. The time for the mammalian cultures to filter through the AcroPrep 24-well filter plate was recorded.

Determining pH/Conductivity of the mammalian cell cultures before and after filtration was carried out for several wells in various plates. The pH and conductivity variation could reflect the release of molecules from the filters to the downstream samples. The measured conductivity reading can be used to make assumptions on the quality of the process. The conductivity is expected to be similar before and after filtration, if no relevant release of plate/filter constituents is observed.

Turbidity/optical density of the mammalian cell culture (Upstream), before filtration, was determined by dilution in the growth media to record measurements at a wavelength of 600 nm.

The filtered samples (in collection plate) were not diluted and several wells were pooled to have 10 mL solution for turbidity measurements at 600 nm. Turbidity and optical density are an indication of the removal of cells and cell debris by the filtration step.

1.2.7 **Protein recovery**

An aliquot of the initial mammalian cell culture (Upstream) was spiked with Gamma-globulin (IgG) as a control.

Initial total protein (Pi)

The Pi (Upstream) in the mammalian cell culture needed to be measured in a cell-free culture to avoid interference with the detection method (fluorometry/Qubit and interferometry/Octet).

To remove the cells from the culture, several methods were used:

- Spin down a small volume of concentrated cultures at 1,000 x g for 5 minutes, with or without filtration through a 0.2 µm Nylon filter.
- Addition of a small amount of Diatomaceous earth (D.E.) to a small volume of concentrated CHO cell culture followed by centrifugation at 1,000 x g for 5 minutes, and/or filtration through a 0.2 µm filter.

Total protein (Pt) recovery

The total protein (Pt) concentration was determined on an Invitrogen[♦] Qubit[♦] 4 Fluorometer (Thermo Fisher Scientific) using the Qubit Protein Assay Kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

The recovery of total protein was determined by comparing the quantity of proteins in the samples filtered using the AcroPrep 24-well filter plates (Downstream, in the collection plate) and the Pi in the cell-free sample.

Immunoglobulin G (IgG) recovery

The concentration of the IgG spiked in the CHO cell cultures was assessed by label-free bio-layer interferometry (BLI) on a FortéBio Octet instrument (Sartorius) with protein A Biosensors, which allowed specific quantification of IgG.

The recovery of IgG in the filtrated samples (downstream, in the collection plate) was assessed and compared to the initial IgG concentration in the free-cell samples.

Results and Discussion

24-well Clarification and Sterile Filtration plates

The data in Table 1 are the average of the data collected with the high CHO cell density cultures. Table 2 shows a summary of the data collected with the HEK293T cell cultures and the supernatant.

Table 1

Parameters recorded for the CHO cell culture with a concentration of 26×10^6 CHO cells / mL and processing with the clarification and sterile filtration plate.

5 mL concentrated CHO cell culture at 26 Million cells / mL Upstream

| | Upstream Culture | Downstream Filtrate (Vacuum: 15 inHg) | Downstream Filtrate (Centrifugation: 1,000 x g) |
|------------------------------------|-------------------------|----------------------------------------------|--------------------------------------------------------|
| Processing Time | – | 20.2 ± 6.3 min | 15 min |
| Hold-up Volume (trapped in filter) | – | 300 – 450 µL | 400 – 450 µL |
| pH | 7.2 | 7.3 | 6.8 |
| Conductivity (µS/cm) | ≈ 10,100 | ≈ 9,200 | ≈ 9,800 |
| Turbidity (NTU) | ≈ 1,900 - 2,600 | ≈ 1.8 | ≈ 2.4 |
| Optical Density at 600 nm | ≈ 18 - 19 | 0 | 0 |
| Total Protein Recovery (%) | – | 98.3 ± 8.2 (78 wells from 14 plates) | 95.4 ± 11.4 (79 wells from 14 plates) |
| IgG Recovery (%) | – | 91.3 ± 11 (77 wells from 14 plates) | 85.0 ± 6.9 (34 wells from 12 plates) |

Table 2

Parameters recorded for the HEK293T cell cultures at $2 - 4 \times 10^6$ cells/mL and processing with the AcroPrep 24-well clarification and sterile filtration plate or the AcroPrep 24-well sterile filtration plate.

| Step | Initial Culture | Clarification | Filtration Supernatant (0.2 µm filter) | |
|-------------------------------------------------------------|----------------------------------------------|-------------------------------------------------------|----------------------------------------------------------------------|-------------------------------------------------------------|
| Plate | – | 24-well Depth + EKV | 24-well EKV | |
| Material (HEK 293T cell culture/supernatant) | HEK293T at 2 – 4 Million cells/mL (Upstream) | HEK293T cell culture at 2 Million cells/mL (Upstream) | Supernatant of HEK293T cell culture at 4 Million cells/mL (Upstream) | |
| Sample | Upstream culture | Downstream filtrate (Vacuum: 7 mL/15 inHg) | Downstream filtrate (Vacuum: 7 mL / 15 inHg) | Downstream filtrate (Centrifugation: 6 mL, 1,000 x g/5 min) |
| Processing Time | - | 4.3 ± 0.4 min | 2.7 ± 0.7 min | 5.0 min |
| pH | 7.1 – 7.6 | | 7.2 | |
| Conductivity (µS/cm) | ≈ 10,836 | | ≈ 10,906 | |
| Turbidity (NTU) | ≈ 81 – 226 | | ≈ 0.91 | |
| Optical Density at 600 nm | ≈ 1.3 – 2.0 | | 0.001 | |
| Total Protein Recovery (%) (Max of 5.4 mg total protein) | – | 101.2 ± 0.4 | 101.2 ± 1.0 | 99.4 ± 1.0 |

The data show that the AcroPrep 24-well filter plates behave similarly when used under vacuum or centrifugation, with respect to parameters such as media pH, conductivity, optical density, and protein recovery.

The pH & Conductivity of the samples are in a similar range before and after filtration through the AcroPrep 24-well filter plates for both the CHO cells culture (Table 1) and the HEK293T culture (Table 2), indicating that none or negligible amounts of filtering media material are released downstream to the filtered samples. The removal of cells can also influence the pH and conductivity but the effect was not observed here.

The turbidity and optical density showed that after filtration of the mammalian cell cultures or supernatant of the HEK293T culture, the filtrates obtained with both AcroPrep 24-well filter plates contained clarified media with minimal breakthrough.

For the protein recovery, we can see that the overall total protein recovery from the CHO or HEK293T cell cultures was greater than 95% after filtration when both AcroPrep 24-well filter plates were used.

Moreover, the IgG recovery was lower than for the total proteins when the CHO cell cultures were used.

The issue came likely from the differences in the methods of detection, as well as several issues in removing the cells in the initial concentrated CHO cell cultures without damaging the CHO cells that could have released intracellular molecules and cellular debris that would create inconsistency in the data.

Conclusion

The aim of this work was to assess the efficiency of the AcroPrep 24-well clarification and sterilization filter plates and the 0.2 µm sterilization filter plates, when used with mammalian cell suspensions.

High-density CHO cell suspension, artificially concentrated, and HEK293T cell cultures were used with the AcroPrep 24-well filter plates under vacuum and in centrifugation. The data obtained suggests that both processes perform equivalently. The suitability of the AcroPrep 24-well filter plate for the clarification of mammalian cell cultures, in particular for high density CHO cells (up to 26×10^6 cells/mL) and HEK293T cells (up to 4×10^6 cells/mL), was demonstrated. Total protein recovery (from ≈ 5 to 10 mg initial total proteins) was determined to be $> 95\%$ with the AcroPrep 24-well filter plates regardless of which plate type was used.

References

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