# Monoclonal Antibody Purification Using UNOsphere SUPrA™ Media

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## Introduction

The first step in purification of an important class of therapeutic proteins, the polyclonal or monoclonal antibodies (mAbs), is their capture from plasma or tissue culture supernatants. Protein A–based media are by far the most common class of affinity products used for this purpose. They bind with high affinity to the Fc region of most subclasses of antibodies and are one of the standard tools used in antibody capture and purification. In combination with ion exchange and ceramic hydroxyapatite chromatography, protein A–based media have been successfully used in the large-scale purification of numerous licensed mAb drugs.

UNOsphere SUPrA affinity chromatography medium is a protein A–based resin developed by Bio-Rad Laboratories, Inc., based on the proven UNOsphere™ matrix. UNOsphere SUPrA media can be integrated in a workflow of mAb purification in combination with UNOsphere Q media and CHT™ ceramic hydroxyapatite, as described in this tech note. The results demonstrate that UNOsphere SUPrA media in combination with the subsequent polishing steps delivers highly pure mAb1 and mAb2.

## Monoclonal Antibody Purification Workflow

The two antibodies were purified by using a workflow consisting of three steps: capturing the antibodies with UNOsphere SUPrA media, intermediate polishing with UNOsphere Q ion exchange resin, and final polishing using CHT ceramic hydroxyapatite.

### Purification of mAb1

mAb1 was obtained from Chinese hamster ovary (CHO) cell culture supernatant at a starting titer of 1 mg/ml.

### Capture by UNOsphere SUPrA Media

The separation of mAb1 was conducted on a BioLogic DuoFlow QuadTec™ 10 system (Bio-Rad). A 1 ml cartridge (0.3 x 4 cm) packed with UNOsphere SUPrA media was equilibrated with 10 column volumes (CV) of 1x PBS. A CHO cell culture filtrate containing mAb1 was loaded onto the cartridge and washed with 20 CV of the same buffer. The captured mAb1 was eluted at a linear velocity of 300 cm/hr at low pH using 100 mM glycine, pH 3.0. Pooled mAb1 fractions were incubated for 1 hr at pH 3.0 (to mimic a virus deactivation step) and then adjusted to pH 7.8 using 10 mM sodium phosphate, pH 8.9.

### Intermediate Polishing by UNOsphere Q Ion Exchange Chromatography

A 1 ml cartridge (0.3 x 4 cm) packed with UNOsphere Q media was equilibrated with 10 CV of 10 mM sodium phosphate, pH 7.8. mAb1 eluted from UNOsphere SUPrA media, pH 7.8, was loaded onto the cartridge at a linear velocity of 300 cm/hr. The cartridge was then washed with 10 CV of equilibration buffer. mAb1 in the flow-through fractions was collected, and the remaining bound proteins were removed from the column by 10 CV of 1 N NaOH.

### Final Polishing by CHT Ceramic Hydroxyapatite Chromatography

A 1 ml column (0.35 x 2.7 cm) packed with ceramic hydroxyapatite media (CHT Type I, 40 µm) was equilibrated with 10 CV of 10 mM sodium phosphate, pH 6.8 (buffer A). mAb1 collected from the flow-through fractions of UNOsphere Q media chromatography was adjusted to pH 6.8 with 20 mM sodium phosphate, pH 4.0, applied to the column at a linear velocity of 300 cm/hr, and washed with 15 CV of buffer A. Elution of mAb1 was performed with a 25 CV salt gradient formed between buffer A and buffer B (10 mM sodium phosphate, 1 M NaCl, pH 6.8). The column was stripped by 15 CV of 500 mM sodium phosphate, pH 6.5 (buffer C), at the end of the run.

### Purification of mAb2

mAb2 was purified from a mouse myeloma (NSO) cell culture supernatant at a starting concentration of 0.03 mg/ml. The tissue culture supernatant was adjusted to 1.5 M NaCl by adding solid NaCl (88 g/L) and 5 mM EDTA.

#### Capture by UNOsphere SUPrA Media

A 5 x 50 mm (3.9 ml) column packed with UNOsphere SUPrA media was equilibrated at a flow rate of 300 cm/hr in 50 mM sodium phosphate, pH 7.0, containing 1.5 M NaCl, 2 M urea, and 5 mM EDTA. Following sample application, the column was washed to baseline with equilibration buffer and then with 50 mM HEPES, pH 7.0. The mAb was eluted at low pH with 0.1 M sodium acetate and 0.1 M arginine, pH 3.8.

#### Intermediate Polishing by UNOsphere Q Ion Exchange Chromatography

The protein A eluate was titrated to pH 8.0 with 2.5 M Tris, pH 8.5, and diluted with one volume of water. This material was then applied to a 5 x 50 mm (3.9 ml) column packed with UNOsphere Q media, equilibrated with 50 mM Tris, 0.11 M NaCl, pH 8.0, and followed with more equilibration buffer until the absorbance returned to the baseline level.

#### Final Polishing by CHT Ceramic Hydroxyapatite Chromatography

Flowthrough from UNOsphere Q media was applied to a column (5 x 50 mm, 3.9 ml) packed with CHT Type I, 20 µm, and equilibrated in 10 mM sodium phosphate, pH 7.0. The column was then developed with a linear 40 CV gradient to 1.0 M NaCl.

#### Analysis of Purifed Proteins

Protein fractions were analyzed by SDS-PAGE using Criterion™ Tris-HCl 4–20% linear gradient gels (Bio-Rad) stained with Bio-Safe™ Coomassie stain (Bio-Rad) or Flamingo™ fluorescent gel stain (Bio-Rad). Mass spectrometry analysis of gel-purified proteins was carried out with an autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics). Size-exclusion (SEC) high performance liquid chromatography (HPLC) was carried out with a Bio-Sil® SEC 400-5 column (Bio-Rad).

## Results

#### Assessment of Protein Purity

Reducing SDS-PAGE profiles are shown for mAb1 and mAb2 in Figure 1. The staining patterns indicate a high degree of purity for both proteins. Some residual bands are still visible in mAb1 profiles. Mass spectrometry analysis (data not shown) demonstrated that they are IgG fragments or IgG half-molecules that were not fully reduced during sample preparation. A similar staining pattern was observed for mAb2. Although these bands were not formally analyzed, their mobility on the gel suggests that they are also IgG related.

#### Clearance of Aggregates

One consistent feature of combining CHT ceramic hydroxyapatite chromatography with protein A capture is the remarkable ability to clear higher-order structures, as shown in Figure 2.

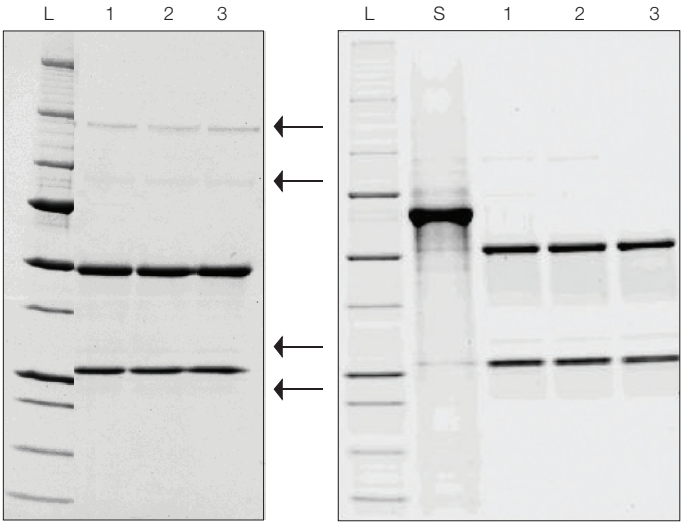


**Fig. 1. SDS-PAGE analysis of mAb1 (left panel) and mAb2 (right panel) chromatographic fractions.** Samples from different chromatographic steps were run on Criterion Tris-HCl 4–20% linear gradient gels; mAb1 was stained with Coomassie stain; mAb2 was stained with Flamingo stain. L, Precision Plus Protein™ unstained standards; S, cell culture supernatant; lanes 1, 2, and 3 show UNOsphere SUPrA media, UNOsphere Q media, and CHT ceramic hydroxyapatite media chromatographic pools, respectively. The arrows point to four bands identified by mass spectrometry as IgG half molecules or fragments.

Although there were substantial levels of aggregates in mAb2 eluted from the capturing step (Figure 2, left panel), the final CHT ceramic hydroxyapatite pool appears to be completely devoid of these unwanted materials (Figure 2, right panel).

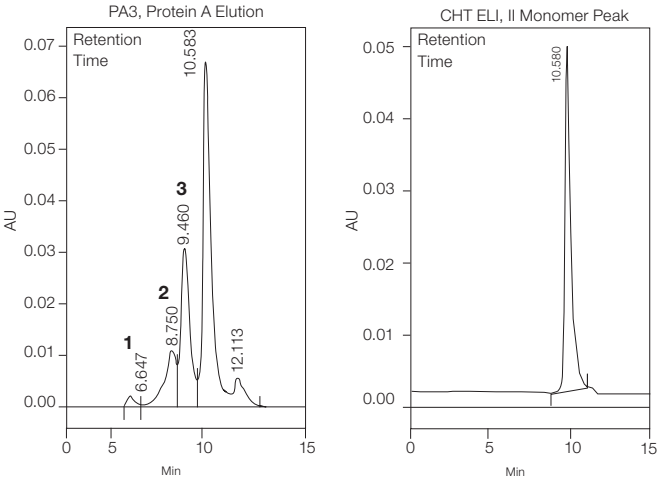


**Fig. 2. Removal of impurities by CHT chromatography.** Elution fractions from the capture (left panel) and final polishing (right panel) chromatographic steps were analyzed by SEC-HPLC to evaluate levels of contamination. The peaks corresponding to aggregates (peaks 1 and 2) and dimers (peak 3) are removed by CHT ceramic hydroxyapatite to yield a clean sample.

**Table 1. mAb1 and mAb2 impurity clearance data.**

| | Protein A, ng/mg | | Host Cell Proteins, ng/mg | | DNA, ng/mg | | Aggregate/Dimer Content, % | |
|---|---|---|---|---|---|---|---|---|
| **Sample** | **mAb1** | **mAb2** | **mAb1** | **mAb2** | **mAb1** | **mAb2** | **mAb1** | **mAb2*** |
| Cell culture supernatant | — | — | $3.5 \times 10^4$ | $1.4 \times 10^6$ | $>5 \times 10^3$ | $>1.6 \times 10^5$ | ND | ND |
| UNOsphere SUPrA fraction | 3 | ND | 175 | 197 | 18.6 | 19 | ND | 42 |
| UNOsphere Q fraction | 0.8 | 112 | <3 | 86 | 3.5 | 1.9 | ND | 40 |
| CHT ceramic hydroxyapatite fraction | <0.7 | <0.4 | <2 | 48 | 2.5 | 3 | <0.03/<0.03 | <0.1 |

–, Not applicable. ND, Not determined.
* Dimer content was not determined for mAb2.

### Clearance of Host Cell Proteins, Protein A, and DNA

UNOsphere SUPrA media show an excellent ability to clear host cell proteins and DNA (Table 1). Host cell protein clearance, on a per mg basis, was between 3 and 5 orders of magnitude. The DNA clearance was between 2 and 6 orders of magnitude. However, irrespective of the amount of contaminating protein A, CHT ceramic hydroxyapatite was capable of removing this impurity to below detectable levels. Finally, levels of impurity in the two mAbs were different at the final step. In particular, the level of host cell protein contaminants was higher for mAb2. The failure to clear all impurities to below the level of detection could be due to a variety of reasons, including insufficiently optimized protocols, different starting levels of impurity in the cell culture supernatants (Table 1), and different host cell impurities (mAb1 was produced in CHO cell culture supernatant and mAb2 in NSO cell culture supernatant).

## Conclusion

UNOsphere SUPrA affinity media can be used to remove impurities to extremely low levels. In a workflow comprising a capture step and two polishing steps, UNOsphere SUPrA, UNOsphere Q, and CHT ceramic hydroxyapatite media can provide mAbs of high quality and purity.



Information in this tech note was current as of the date of writing (2008) and not necessarily the date this version (rev A, 2008) was published.

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