

## The Endotoxin-free Separation of Protein Species via Asymmetrical Flow Field-Flow Fractionation (AF4)

The immunogenic potential of protein aggregates in biopharmaceuticals is currently the subject of numerous discussions. The presence—or—absence of immunogenicity is usually investigated in *in-vivo* studies. One important aspect in such investigations is the absence of endotoxins in the samples. AF4 is an advantageous preparative method for separating different aggregate species of proteins. The aim of this study was to establish a method to obtain endotoxin-free aggregate protein fractions that can later be utilized in *in-vivo* studies.

**Materials & Methods:** Fractionation of a native monoclonal antibody (MAb) and the same MAb after light exposure were carried out on a Wyatt Eclipse Separation System, composed of an analytical long-channel of 490  $\mu\text{m}$  in height, a DAWN-EOS multi-angle light scattering (MALS) detector and an ultraviolet (UV) detector, all connected to a fraction collector. The buffers and samples were prepared under aseptic conditions. The entire instrument was disinfected by rinsing with highly purified water (HPW) and ethanol 70% (V/V) before purging with running buffer.

**Results:** The MAb was stressed 48 hours by light exposure and subsequently analyzed by AF4/MALS. The chromatogram showed 3 main peaks using UV detection at 280 nm. By MALS detection these peaks were identified as fragments, monomeric protein and aggregates by their molecular weight. The collected fractions were tested to be free of endotoxins using a Limulus Amebocyte Lysate test. A benchmark was defined as 0.25 IU/ml, referring to the monograph "water for injections" in the *European Pharmacopoeia* 6.8. After establishing a meticulous cleaning program for the entire instrument, it was possible to keep the endotoxin levels far below the requested benchmark (<0.180 IU/ml).

**Conclusion:** This study shows that AF4 can be applied as an excellent preparative tool to generate samples for *in-vivo* studies since rather simple cleaning steps allow the elegant preparation of endotoxin-free protein fractions for preclinical research.

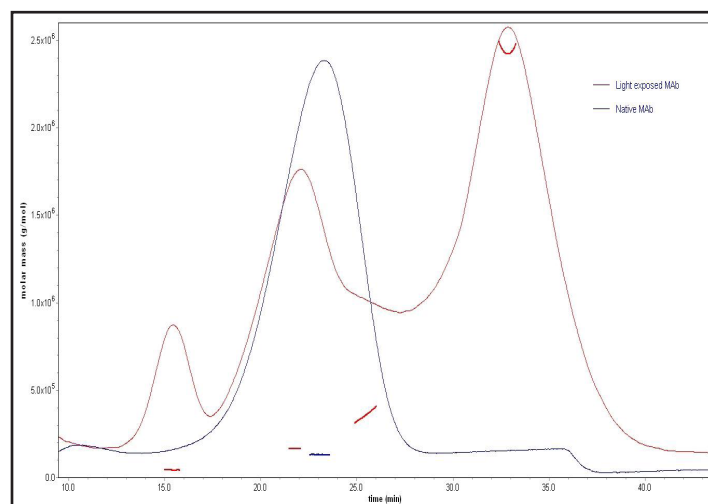


Figure 1. The three main peaks showing UV overlaid on the MALS signal.

Sample	Concentration of endotoxins
Fragments	< 0.100 IU/ml
Monomer	< 0.172 IU/ml
Aggregates	< 0.100 IU/ml

Table 1. Endotoxin levels in three collected protein fractions.

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