

Removal of endotoxin from monoclonal antibodies using Vivapure™ centrifugal ion exchange membrane devices

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Introduction

Endotoxins are lipopolysaccharides present in the cell wall of most Gram-negative bacteria, and are frequently present as contaminants in protein solutions purified in research environments. Endotoxins have profound biological effects at low concentrations and thus they require removal prior to use of such preparations in biological cell-based assays.

The term EU is used to describe the activity of endotoxins, and typically the limit for endotoxin is set at 50 EU/mg for bioactive proteins destined for cell-based assays; limits may be lower for more demanding *in vivo* applications. Achieving this low level is often a challenge in biological research as endotoxins are robust molecules surviving extremes of temperature and pH that would normally denature proteins. Thus, it is often difficult to remove endotoxins from sensitive substances such as antibodies.

A variety of methods are already available for the removal of endotoxins such as anion exchange chromatography, ultrafiltration, hydrophobic interaction chromatography, sucrose gradient centrifugation and extraction with detergents (Petsch 2000). However, all of these methods have distinct disadvantages. For example ultrafiltration, although effective for removal of endotoxin from water, is less effective in the presence of proteins.

This is a result of the fact that large endotoxin aggregates are not so readily formed, leaving smaller monomeric units free to pass through the membrane with the protein being purified. The use of detergents for phase separation of endotoxin has been effective in some cases but requires the heating of the solution with the resultant possibility of denaturation of the protein. Additionally trace levels of detergent will remain with the purified protein, which in itself is not always desirable. Endotoxins are strongly negatively charged under conditions commonly encountered during protein purification (i.e. above ~pH 2 they will carry a negative charge).

This negative charge facilitates the use of anion exchange chromatography for their absorption from solution. Anion exchange matrices have positively charged groups on their surface (DEAE or quaternary amines), which are balanced with negative counter ions from the buffer system. A negatively charged endotoxin will displace these counter ions and bind to the matrix. Binding occurs when the salt concentration (ionic strength) of the mobile phase is low enough for the ionic groups on the endotoxin molecule to serve as counter ions for the positively charged groups on the stationary phase.

Under these conditions the endotoxin will bind to the stationary phase surface. This "exchange" of negative counter ions for negative molecules gives the matrix its name of anion exchanger. If this binding of endotoxin can be achieved under conditions at which the protein of interest carries a net positive charge (i.e. at a pH below its isoelectric point) then the protein will be repelled from the matrix and flow through with the mobile phase, in what is often termed negative chromatography mode (Figure 1).

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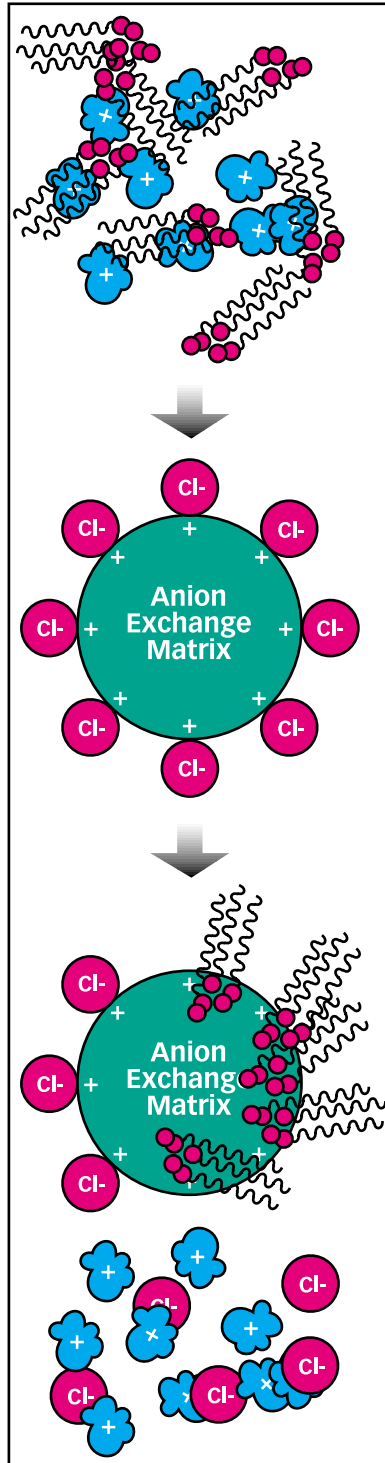
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However, this will often result in dilution of the protein several fold, which often then dictates an additional concentration step.

Additionally, packing small chromatography columns and maintaining them sanitary is time consuming and requires specialist knowledge and equipment. Centrifugal ion exchange membrane spin columns offer an alternative to traditional ion exchange chromatographic removal of endotoxin.

They also avoid the development of lengthy procedures and the use of expensive equipment and potentially could rapidly yield high levels of endotoxin-free protein. In this report we tested the use of centrifugal anion exchange membrane devices for the rapid removal of endotoxin from research grade antibody solutions required for cell-based activity investigations.



Mixture of positively charged protein and negatively charged endotoxin.

Anion exchange matrix - positive surface charge balanced with negative counter ions.

Endotoxin displaces counter ions and remains bound to solid phase.

Protein remains unbound and free to pass through the matrix unhindered.

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Absorption of endotoxin from a basic monoclonal antibody using Vivapure™ Mini Q spin columns.

Materials and Methods a)

The sample used in this study was 115 mg of monoclonal antibody in 1.3 ml of phosphate buffered saline, pH 7.2. This monoclonal antibody has an isoelectric point of 7.5. In order to minimise extraneous contamination of samples with endotoxin, all reagents and containers described below were supplied or prepared endotoxin free. Additionally, pH meter probes and magnetic stirrer bars were depyrogenated according to the manufacturers instruction or by soaking in 0.5M sodium hydroxide for 1 hour followed by extensive rinsing in water for irrigation.

Four Vivapure™ Mini Q spin columns were washed sequentially with 0.5 ml of water for irrigation (WFI, Baxter), 0.5 ml of 0.5M sodium hydroxide, 2 x 0.5 ml of WFI and 0.5 ml Dulbecco's phosphate buffered saline, pH 7.2 (Gibco) by loading each solution into the device followed by centrifugation at 2,000 g for 5 minutes.

The monoclonal antibody sample was divided equally amongst the four Mini spin columns and centrifuged as above. The flow through from each column was then filtered through a 0.2 µm sterilising centrifugal filtration device (Corning, Costar Spin-X®, 2000 g for 5 minutes) and pooled. Residual monoclonal antibody was recovered from the Vivapure™ Mini columns by washing each twice with 0.5 ml of phosphate buffered saline as above, collecting and combining the washes.

The concentration of monoclonal antibody was measured in all samples using absorbance measurements at 280 nm and the known extinction coefficient of the protein. All volumes were estimated by weight assuming the density of the solutions to be 1 g/ml.

Endotoxin (EU) was measured using a kinetic turbidimetric assay (Charles River Endosafe®) following the manufacturers instructions. The test sample, endotoxin standards, positive and negative controls are mixed with an equal volume of Limulus Amoebocyte Lysate (LAL) reagent in a microtitre plate. The increase in optical density is measured over time in an incubating plate reader (Biotek). Quantitative endotoxin values of the test samples are determined by comparison with a standard endotoxin curve. Results are calculated using Charles River Endosafe® validated software, Endoscan-V.

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Results and discussion a)

High recovery of antibody was achieved, a mass balance of 92%, with very high clearance of endotoxin - 30 fold reduction in endotoxin (Table 1).

Table 1: Monoclonal antibody recovery and endotoxin level following purification using Vivapure™ Mini Q

Sample	Total antibody (mg)	Antibody recovery (%)	Endotoxin (EU)
Start material	115	-	3450
Vivapure™ Mini Q Flow through	93	81	112
Vivapure™ Mini Q Wash #1	11	10	ND
Vivapure™ Mini Q Wash #2	1	1	ND

ND = Not determined

Losses of protein are thought in part to be due to volume losses as a result of handling small volumes of concentrated protein solutions. The flow through product remained at constant concentration and was suitable for its intended use as endotoxin was reduced to 1.2 EU/mg from 30 EU/mg.

Absorption of endotoxin from an acidic monoclonal antibody using Vivapure™ Maxi H spin Q columns.

The monoclonal antibody sample was adjusted to pH 5.5 (i.e. below its pI) by the slow addition of dilute hydrochloric acid with constant mixing.

Residual monoclonal antibody was recovered from the Vivapure™ maxi columns by washing each with 15 ml of phosphate buffered saline adjusted to pH 5.5 as above, collecting and combining the washes.

Materials and Methods b)

The sample used in this study was 150 mg of monoclonal antibody in 48 ml of phosphate buffered saline, pH 7.2 (PBS). This monoclonal antibody has an isoelectric point of 6.0.

This was then divided equally amongst the four maxi spin columns and centrifuged as above. The flow through from each column was then pooled and adjusted back up to pH 7.2 with the addition of 0.5M sodium hydroxide.

The concentration of monoclonal antibody and endotoxin levels in all samples was measured as described above.

Four Vivapure™ Maxi H spin Q columns were washed sequentially with 17 ml of water for irrigation (WFI, Baxter), 17 ml of 0.5M sodium hydroxide and 3 x 17 ml of WFI and 17 ml Dulbecco's phosphate buffered saline, (Gibco, previously adjusted to pH 5.5 with the addition of concentrated hydrochloric acid) by loading each solution into the device followed by centrifugation at 500 g for 5 minutes.

The pH-adjusted pool was then filtered through a 0.2 µm sterilised filter (Millipore Stericup™) and stored at 4°C.

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Results and discussion b)

High recovery of antibody was achieved, a mass balance of 91%, with very high clearance of endotoxin – in excess of 286 fold reduction in endotoxin (Table 2).

Table 2: Monoclonal antibody recovery and endotoxin level following purification using Vivapure™ Maxi Q

Sample	Total antibody (mg)	Antibody recovery (%)	Endotoxin (EU)
Start material	150	-	45,500
Vivapure™ Maxi Q Flow through	125	83	159
Vivapure™ Maxi Q Wash	12	8	ND

ND = Not determined

Losses of protein are thought in part to be due to volume losses as a result of handling small volumes of concentrated protein solution. The flow through product was reduced in concentration slightly due to dilution on pH adjustment but remained suitable for its intended use as endotoxin was reduced to 1.3 EU/mg from 303 EU/mg.

Conclusions

Vivapure™ centrifugal anion exchange membrane devices proved highly effective in removal of contaminating endotoxin from research grade monoclonal antibody solutions. This clearance of endotoxin was maintained in a high conductivity buffer, PBS, preventing the need for any diafiltration into low salt buffers prior to the anion exchange.

Additionally we have demonstrated that this method is applicable to acidic proteins by simple pH adjustment prior to application to the charged membrane. In addition to the high protein recovery the starting concentration of the antibody solution was maintained obviating the need for any further processing.

This method is a trouble-free method for removal of endotoxin from small volumes of protein solutions and would allow for easy processing of multiple samples in parallel over a short period.

Vivascience offers the same devices with different volume capacities (up to 75 ml in Vivapure Mega devices), which would facilitate the use of this same technique to isolate reduced endotoxin fractions on a larger scale.

References

Petsch, D. and Anspach, F.B. (2000) Endotoxin removal from protein solutions. *Journal of Biotechnology*, 76, 97-119