Technical
User Guide

EtoxiClear®

Product Code: 3250

INTRODUCTION

Endotoxin are lipopolysaccharides (LPS) derived from the cell membrane of Gram-negative bacteria and are often found as impurities in final pharmaceutical products. Endotoxin elicit a variety of pathophysiological effects when administered systemically and the removal of residual endotoxin to below the threshold level for recombinant proteins produced in E.coli continues to be a major challenge for the biopharmaceutical industry.

According to pharmacopoeia the maximum permitted level of endotoxin for intravenous products is 5 EU (endotoxin units) per kg adult per hour, where 1 EU is equivalent to approximately 0.1 – 0.2 ng of endotoxin.

The removal of endotoxin is more challenging when associated with large protein molecules. While several methods adapted for specific products have been described for the removal of endotoxin from proteins, the methods based on selective adsorption are the most effective for the removal of LPS from final product.

EtoxiClear® provides cost effective and efficient endotoxin removal, using a high performance synthetic ligand affinity chromatography adsorbent, providing superior endotoxin removal from processing solutions and final products.

The adsorbent has a high dynamic binding capacity (> 1 x 10^6 EU/mL packed adsorbent); it has low protein binding (typical recoveries of the product > 90%) and this allows for up to 4 log clearance of the residual endotoxin present in the sample.

The affinity ligand is covalently coupled to 6% cross-linked, near monodisperse agarose beads (PuraBead® 6XL) to provide a sodium hydroxide stable adsorbent with excellent selectivity for endotoxin.

Properties of EtoxiClear®:

LIGAND:	Synthetic chemical ligand (proprietary)
ADSORBENT APPEARANCE:	White
MEAN PARTICLE SIZE (µM):	100 ± 10 µm
MATRIX:	Highly cross-linked 6% near monodisperse agarose (PuraBead® 6XL)
DYNAMIC BINDING CAPACITY:	>1,000,000 EU/mL of adsorbent*
RECOMMENDED OPERATIONAL FLOW RATE:	Up to 200 cm/hr (dependent on column size)
CHEMICAL STABILITY:	All commonly used aqueous buffers and co-solvents
OPERATING PH:	pH 4.0 to pH 8.0 (intermittent)
PH STABILITY:	Long term (3 months) pH 3 to pH 12
CLEANING / SANITIZATION:	0.5 - 1.0 M NaOH, 25°C
RECOMMENDED STORAGE CONDITION†:	2-30°C, 20% ethanol : 80% 0.1 M NaCl (v/v)

* Loading at 120 cm/h, 5-minute residence time (EtoxiClear® 5 mL Column)

Important Notes

- It is good practice to filter all protein samples and chromatography buffers prior to use.
- Removal of residual endotoxin from the final protein product solution may require process optimization to identify optimal buffer conditions.
- EtoxiClear® can be operated across a wide pH range (pH 4.0 – pH 8.0) for endotoxin binding applications.
- It is recommended that the endotoxin removal is carried out using aseptic techniques and for fraction collection use pyrogen free tubes/containers.
- The final protein solution should not be frozen before endotoxin removal. Freeze-thawing increases the formation of endotoxin micelles which can be more difficult to remove.
- For optimum performance of EtoxiClear® it is recommended that a maximum bed height of 10cm and a minimum residence time of 5 minutes are used.
- Below the pH of the isoelectric point (pI) of the target protein the net charge on the protein is positive and polyanionic (negatively charged) endotoxin molecules will interact strongly with the protein molecule. Consequently, the effective removal of endotoxin from the final product could be impaired at pH values below the pI of the target protein.
- Although EtoxiClear® has low non-specific binding, it does have a anionic functional group which may act as a very weak ion exchanger. This could lead to a slight binding of target protein which can be minimized by increasing the ionic strength of the buffer by the addition of salt (up to 0.3 M NaCl).
- Do not use ammonium or Tris based buffers. These buffers have shown to reduce the efficiency of endotoxin clearance with EtoxiClear®. Other buffers to avoid include MES and HEPES.
- Increasing the concentration of the buffer salt (i.e. from 10 mM to 100 mM sodium phosphate) can improve the target protein recovery without having a detrimental effect on endotoxin clearance.
- If required, endotoxin clearance can be optimized by decreasing the flow rate (increasing residence time) used during sample loading.

There are many commercially available endotoxin detection tests/kits available, however, if a chromogenic based (LAL) test is used (e.g. Associates of Cape Cod) it is recommended to include Glucashield® buffer. This renders the reagent insensitive to (1→3)-ß-D-glucan interference, which may be present in the sample, by effectively blocking the Factor G pathway of the endotoxin clotting cascade.

COLUMN PACKING

EtoxiClear® is supplied in 20% ethanol : 80% 0.1 M NaCl (v/v). The adsorbent can be packed using a saline solution (e.g. 0.1 M NaCl) or an equilibration buffer (e.g. 50mM sodium phosphate, 0.1 M NaCl, pH 7.2). Before commencing the column pack, consult the relevant manufacturer's instructions for the selected column. The method described below describes the packing of EtoxiClear® into axial columns.

Please note: it is recommended that the bed height of the column pack should be ~10 cm for optimum performance, as taller bed heights have shown to significantly add back pressure and shorter bed heights can affect the optimum performance of EtoxiClear®.

1. Assemble the column and remove air from the dead spaces by flushing the end piece and adaptor with packing solution (0.1 M NaCl solution) then close the column outlet.

2. Allow all materials to equilibrate to the temperature at which the chromatography process is to be performed.

3. If required to obtain a fixed bed height (i.e. for larger column packs), it is recommended to determine the slurry percentage. For example, weigh a sample of the complete slurry, drain away the preservative and re-weigh the adsorbent. The final weight over the total weight will determine the slurry percentage.

4. Carefully pour the adsorbent slurry into the column in a single continuous step. Pouring the adsorbent down the side of the column helps to prevent air becoming trapped within the adsorbent bed.

5. Allow the adsorbent to settle in the column leaving a dead volume of packing solution above the adsorbent bed.

6. Ensure the top adaptor is free from air by pumping the packing solution through. Attach the (open) top adaptor to the top of the column and adjust the adaptor to just above the bed, tighten the adaptor (seal) and attach to the workstation. Open the column outlet and apply a flow to the bed.

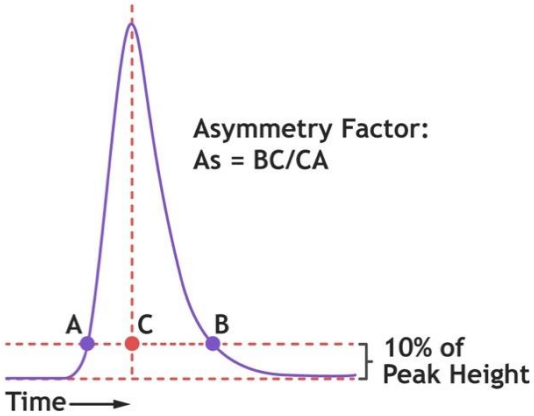
7. EtoxiClear® should be packed in the column until a pressure spike is observed, but not exceeding 3 bar (0.3 MPa) across the column. This should be achieved by incrementally increasing the flow rate (i.e. commence flow at ~ 0.5 bar then 0.5 bar pressure increments every 0.5 CV; for smaller columns, or if pressure cannot be accurately determined, commence flow at 100 cm/h then increase the flow rate by 100 cm/h increments).

8. Mark where the adsorbent has compressed to under the last pressure / flow condition, then lower the top adaptor to that mark.

9. The column is now packed and ready to use.

COLUMN EFFICIENCY TEST

1. Test the column at a flow rate of 100 cm/h.
2. Attach the column to an equilibrated workstation.
3. Commence flow for 1 CV (i.e. to equilibrate and obtain baseline).
4. Inject 2% to 5% CV of a 2% acetone or 2 M NaCl solution.
5. Continue flow until a UV (or conductivity) peak is observed and the trace has returned to baseline (1 to 1.5 CV).
6. End run and determine the asymmetry factor:



7. EtoxiClear® is an affinity adsorbent, therefore an asymmetry factor for an acceptable pack is between 0.8 and 1.6. The recommended plate count for an acceptable pack is ≥ 2000 N/m.

OPERATING INSTRUCTIONS

Note: The following recommendations are not prescriptive and thorough investigation of these parameters at small-scale is recommended for process optimisation, including flow rate optimisation to improve binding capacity/ resolution or decrease processing times.

1. Attach the column directly to the liquid chromatography system, ensuring that the tubing connecting the system to the column is primed with equilibration buffer. Recommended equilibration buffers are PBS or 50mM sodium phosphate, 0.1M NaCl, pH 7.2. Other equilibration buffers are as follows (including 0.1M NaCl):

   • sodium acetate

   • sodium citrate

2. **Note:** Although the adsorbent has been manufactured in a controlled environment, we recommend that the column is treated with a depyrogenation step prior to use.

3. Clean by flushing the liquid chromatography system and column with 0.5M NaOH at 60 cm/hr. Stop the flow and hold in 0.5M NaOH for ≥16 hours (overnight) at room temperature. Flush the NaOH out of the system and column using depyrogenated equilibration buffer (e.g. PBS or 50mM sodium phosphate, 0.1M NaCl, pH 7.2) at 60 cm/hr until the eluent pH is equivalent to the equilibration buffer entering the column.

4. Apply the protein solution/sample onto the column at the recommended operational flow rate of 120 cm/hr (≥ 5 minute residence time is recommended) and collect the flow through in endotoxin free collection vessels.

5. If required, wash the column with equilibration buffer at 120 cm/h to flush out and recover the remaining target protein with at least 1 CV or until a UV baseline is achieved.

6. The EtoxiClear® column can be regenerated by passing 2 CV of 0.5 M NaOH through the column, followed by an overnight hold in 0.5 M NaOH (> 16 hours). Note: EtoxiClear® is stable to exposure of sodium hydroxide 0.5 M to 1 M NaOH, though regeneration and re-use of the adsorbent is dependent on the nature of the sample applied to the column and will require validation by the end-user.

7. Post overnight hold, flush the column with a further 1 CV of 0.5 M NaOH.

8. Wash out the sodium hydroxide solution with equilibration buffer (50 mM sodium phosphate, 0.1 M NaCl, pH 7.2) until the eluent matches the pH of the equilibration buffer entering the column.

9. For storage, place the column into 20% ethanol: 80% 0.1 M NaCl solution and store at 2 –30 °C.

ORDER INFORMATION

Gel Slurry

Code	Description	Pack Size
3250-00010	EtoxiClear®	10 mL
3250-00025	EtoxiClear®	25 mL
3250-00100	EtoxiClear®	100 mL
3250-00500	EtoxiClear®	500 mL
3250-01000	EtoxiClear®	1000 mL

We also offer a range of larger pack sizes for supply of bulk resins into cGMP development and manufacturing scale processes.

EtoxiClear® is available in a carefully selected range of disposable columns sizes with bed volumes of 5 mL and 50 mL both with a standard bed height of 10 cm, suitable for process development applications and polishing steps.

Disposable Column Format

Code	Description
4251-00005	EtoxiClear® 5 mL Column
4251-00050	EtoxiClear® 50 mL Column

In addition, Astrea Bioseparations can offer column packing services. For more information on this or any other supply related matters, please do not hesitate to contact us at sales@astrea-bio.com

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Issue Date: 13 Dec 2022
CCR Number: N/A – trademarks updated
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