Proteus NoEndo™ µ (Micro) Spin Column Kits

User Guide

Introduction

The Proteus kits are designed for simple, rapid removal of endotoxin from a wide range of protein solutions. Proteus spin columns replace lengthy and expensive chromatographic methods such as phase separation and FPLC®.

There are four versions of the Proteus kits; the Proteus NoEndo™ µ (Micro), the Proteus NoEndo™ M (Mini), the Proteus NoEndo™ S (Standard) and the Proteus NoEndo™ HC (High Capacity) spin column kits which are suitable for different endotoxin loads. The Proteus NoEndo™ µ columns are ideal for low endotoxin loads (300-500 EU/ml) where the sample volumes are less than 600 µl. The Proteus NoEndo™ µ columns incorporate a proprietary SelfSeal™ membrane technology that combines the benefits of batch incubation with the speed of a spin column.

Large numbers of samples can be processed at the same time. There is negligible hold-up volume; ensuring high solute recovery with minimal nonspecific absorptive losses. Protein samples purified using Proteus spin columns may be used for a wide range of laboratory procedures such as biopharmaceutical preparations for proteins, antibodies and vaccines.

Unique Features of the Proteus NoEndo™ Spin Columns:

- Endotoxin levels as low as 0.03 EU/ml with typical protein recoveries > 95%.
- Proteus NoEndo™ kits are designed to eliminate tedious chromatographic steps normally associated with other affinity chromatography methods.
- The unique SelfSeal™ membrane in a column offers high speed, high capture efficiency and high reproducibility.
- These spin columns are rapid endotoxin removal columns which require minimal preparation time. Loose resin is marketed towards the traditional chromatography market.
- The spin columns allow multiple and parallel rapid purifications. This is ideal for rapid screening and method development.
- The kits offer a standardised method for high grade removal of endotoxin from a broad spectrum of monoclonal antibodies and proteins. All components of the kits will be fully traceable.

- The provision of a disposable spin column is ideally suited to GMP production where current user requirements and price sensitivities demand regeneration of the affinity matrix using harsh treatments.

Contents of Proteus NoEndo™ Kit with NoEndo™ Resin:

Proteus Kit code:	NoE2Micro	NoE24Micro	NoE100Micro
• Proteus NoEndo™ vials NoEndo™ resin	1 x 0.2 ml	1 x 2.4 ml	1 x 10 ml
• Proteus spin column (600 µl capacity in a fixed angle rotor)	2	24	100
• 2.2 ml centrifuge tubes	4	48	100
• Protocol Card	1	1	1

Additional Materials Required:

- 0.2 µm syringe filters for clarification (We recommend Proteus mini-clarification spin columns from Protein Ark Ltd. Order code # GEN-MSF500 (100 pcs, 0.2 µm low protein-binding PVDF membrane)).
- Low endotoxin pre-equilibration buffer (PBS recommended).
- 2.2 ml centrifuge tubes.
- A fixed angle rotor in a microfuge capable of handling 2.2 ml centrifuge tubes (diameter 11 mm).
- Quartz cuvettes for UV absorbance measurements.
- UV/VIS spectrophotometer.
- Pyrogen-free test tubes, pipettes and buffer for Endotoxin Assay.

Note: The preferred rotor is a fixed angle rotor in a microfuge. For optimal performance with a fixed angle rotor, ensure that the orientation of the spin column in the rotor is the same for sample binding and washing.

Chemical Compatibility:

Compatible with:	0.5M NaOH PBS buffers Detergents, Urea, guanidinium chloride Water miscible solvents (e.g. 20% ethanol)* Stable between pH 3-13 10 mM ETDA Stable to 2-40 ˚C, recommended storage temperature 2-8 ˚C Operational Flow Rates : 200 cm/hr
Incompatible with:	Oxidising agents Strong acids All Tris buffers All Glycine buffers Most buffers with amine groups

* NOTE: Water miscible organic solvents such as ethanol should be introduced at incremental concentrations up to the desired concentration otherwise bed movement in packed columns may occur. The same is true when exchanging from an aqueous organic solvent to an aqueous solution.

Storage Conditions:

- Remove the Proteus NoEndo™ resin vials from the kit and store it at 2-8˚C. There is no need to place the rest of the kit in a refrigerator or cold room. Do not freeze the resin vials or store them at room temperature. Freezing the suspension will damage the agarose beads. The resin is pre-swollen and defined. Proteus spin columns are stable for up to 2 years at 2-8 ˚C from the date of manufacture. The expiration date is recorded clearly on the outside of the pack. All resin is stored in 20% v/v ethanol containing 0.1M NaCl.
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Technical support:

Contact the Protein Ark technical support and sales centre for assistance:

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Disclaimer:

- This product is for research use only and is not intended for use in clinical diagnosis. No claims beyond replacement of unacceptable material or refund of purchase price shall be allowed.

Ordering Information

Kits	Quantity	Product Code
Proteus NoEndo™ µ (Micro) 2 column kit (Contents – 2 spin columns, 4 centrifuge tubes, 1 x 0.2 ml resin vial)	2 µ spin columns	GEN-NoE2Micro
Proteus NoEndo™ µ (Micro) 24 column kit (Contents – 24 spin columns, 48 centrifuge tubes, 1 x 2.4 ml resin vial)	24 µ spin columns	GEN-NoE24Micro
Proteus NoEndo™ µ (Micro) 100 column kit (Contents – 100 spin columns, 200 centrifuge tubes, 1 x 10 ml resin vial)	100 µ spin columns	GEN-NoE100Micro

Loose Resin	Quantity	Product Code
NoEndo™ Resin (25 ml loose resin)	25 ml NoEndo™ resin	GEN-NoE025ML
NoEndo™ Resin (100 ml loose resin)	100 ml NoEndo™ resin	GEN-NoE100ML

Accessories	Quantity	Product Code
Proteus Mini Clarification Spin Columns	100 pack	GEN-MSF500
<u>Empty spin/batch columns</u> Proteus '1-step batch' Midi spin column pack	 8 pack	 GEN-1SB08
<u>Empty FPLC columns</u> Proteus 1 ml FliQ column Proteus 5 ml FliQ column Proteus 10 ml FliQ column Proteus 20 ml FliQ column	 1 column 1 column 1 column 1 column	 GEN-FliQ1 GEN-FliQ5 GEN-FliQ10 GEN-FliQ20
<u>Empty scalable columns</u> 10 ml Single step column with bottom frit 25 ml Single step column with bottom frit 50 ml Single step column with bottom frit 100 ml Single step column with bottom frit	 10 pack 10 pack 10 pack 10 pack	 9452086-10 9452088-10 9452090-10 9452092-10

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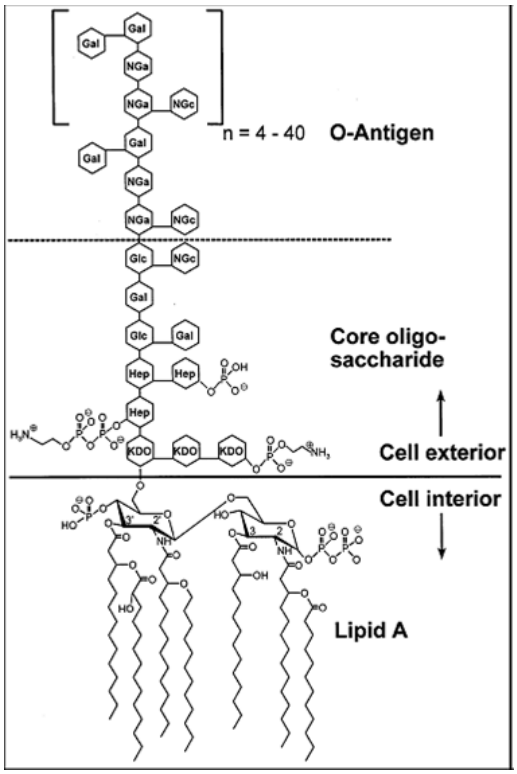
NoEndo™ Resin:

Proteus NoEndo™ μ (Micro) spin columns are designed specifically for the NoEndo™ resin. The NoEndo™ resin is specifically designed with a polyamine chemical synthetic ligand which has high selectivity for endotoxin. This ligand is immobilized on to nearly mono-disperse 6% agarose beads using established chemical methods. It is highly effective in binding and removing endotoxin from a variety of fluids such as water, aqueous solutions and biopharmaceutical preparations such as proteins, vaccines and antibiotics.

The SelfSeal™ Advantage:

The NoEndo™ μ (Micro) spin columns incorporate our proprietary SelfSeal™ membrane technology. The coated membrane is specially formulated to prevent any sample from leaking into the collection tube on an orbital mixer. Batch incubation can be performed at 4^0C and at room temperature. In a centrifuge, the membrane pores dilate and the eluate, free of endotoxin, passes into the collection tube. The contact time is maximized to ensure maximum endotoxin depletion and without losses of protein, antibody or domain antibody.

Background:



Endotoxin are the predominant lipids found in the outer membrane of gram negative bacteria. We know that sub-nanogram levels of endotoxin can trigger immune responses and alter the function of many different cell types and that removal of endotoxin is one of the most difficult downstream processes during protein or antibody purification. Gram negative bacteria are widely used as vectors for the manufacture of recombinant peptides and proteins. Much work has been spent over the years optimizing *E. coli* as an expression host for proteins from higher organisms. As a result, it is generally recognized that the first attempt to express a recombinant protein uses *E. coli* as the expression host. Phage display, which utilizes Gram negative bacteria, is increasingly used for the manufacture of monoclonal antibodies eg Morphosys, Germany. Recombinant viruses and viral vectors are also currently being developed for therapeutic applications including vaccination, gene therapy e.g. to treat conditions such as heart disease, diabetes, muscular dystrophy and cancer 'virotherapy'. These final products are always contaminated with endotoxin. Contaminating pyrogens such as lipopolysaccharides or endotoxin present in gram negative bacteria need to be removed from protein, antibody and viral vectors. This makes the final product suitable for animal studies, cell cultures and cell based assays.

Endotoxin are of great concern in the medical device and pharmaceutical industries. If the final product is not certified to be free of endotoxin, many experiments may fail. Efficient and cost-effective removal of endotoxin from R & D preparations is extremely challenging. Endotoxin removal for research into animal studies, transplantation, gene therapy, stem cell technologies, cell sorting and other mammalian cell treatments is vital. Endotoxin concentrations as low as 0.3-0.4 EU/ml can induce pyrogenic shock in mammals.

Many commercially available products are unable to remove endotoxin satisfactorily, or require time-consuming incubation steps. It is well documented that there is widespread dissatisfaction with all known commercial methods to remove endotoxin. Typically, the target protein binds as efficiently to the matrix as the endotoxin and cannot be recovered easily, or both endotoxin and protein elute in the flow-through fractions.

Incumbent technologies include:

1. *Affinity-based methods e.g. Polymyxin B-agarose resin.*
   Weaknesses: Poor binding capacity. Very expensive resin. Unreliable. Known to bind target proteins non-specifically.

2. *Non-affinity-based methods e.g. Ion exchange chromatography and ultrafiltration.*
   Weaknesses: Empirical design of experiments and prior knowledge of key physical properties of target proteins e.g. isoelectric point, molecular weight is required and often difficult to ascertain. Also requires access to both anion and cation exchange technologies.

3. *Detergent-based methods e.g. Phase separation using Triton X-114.*
   Weaknesses: Resultant Triton X-114 resides in the sample. It is very difficult to remove TX-114. Also requires instrumentation to heat to 42˚C and for subsequent detergent removal (with inevitable protein losses). Methodology is long-winded and unreliable. The detergent is relatively expensive and would add a significant cost to a manufacturing process. It may also affect the activity of the target protein.

The diversity in the number of methods to remove endotoxin indicates a dilemma in endotoxin removal. Often, endotoxin removal requires more than 1 of the above methodologies used in combination. Each of the above procedures address the problems associated with endotoxin removal in completely different ways. However, none of them has broad applicability. Let's take ultrafiltration as an example. For small proteins, such as myoglobin (*M*r ~ 18,000), ultrafiltration can be useful to remove large endotoxin aggregates, but not monomeric endotoxin which has a molecular weight between 10 and 20 kDa. With large proteins, such as immunoglobulins, ultrafiltration would not be effective for removing endotoxin aggregates. Usually, the procedures employed for endotoxin removal are unsatisfactory when you assess the following criteria: selectivity, adsorption capacity and recovery of the target species.

High cost has also severely dented widespread use of current endotoxin capture methods. In many cases, complete endotoxin removal is only achieved with large losses in protein yields. More importantly, strong selectivity is required as protein samples are often concentrated to between 5-30 mg/ml, and there is significant evidence showing that endotoxin do bind to proteins. Consequently, reduction or removal of endotoxin to less than 0.3 EU/ml sample (1 ng/mg; 10 EU/mg) is a very challenging task. Note that the term EU describes the biological activity of an endotoxin. E.g. 100 pg of the standard endotoxin EC-5 and 120 pg of endotoxin from E. coli O111:B4 have activity of 1 EU.

New fields in science increasingly demand that their raw ingredients are certified endotoxin free, as dictated by European Pharmacopoeia and FDA regulations (Gorbet and Sefton, 2005; Petsch et al, 2000). Many laboratories are forced to allocate resources to run parallel production lines that are endotoxin-free. Successful exploitation of an effective endotoxin removal kit will free up vital resources. Anything that reduces laboratory costs and inconvenience will, therefore, attract considerable interest

General Considerations for selecting Optimal Binding Conditions for the Proteus NoEndo™ kits:

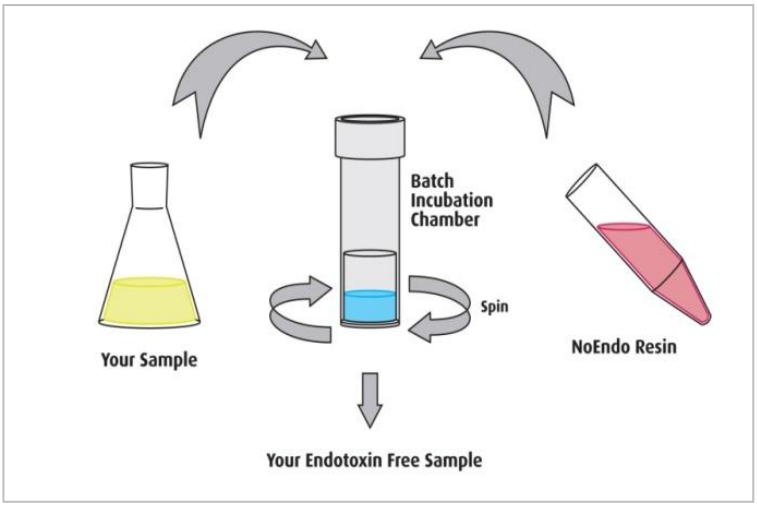
All samples should be filtered just prior to loading even if they have been filtered several days before using the sample. Aggregation/precipitation of proteins is common during storage and repeated freeze-thawing. Protein solution should not be frozen before endotoxin removal. Freeze–thawing increases the formation of endotoxin micelles which can be more difficult to remove.

No Ethanol during Batch Incubation:

Wash the resin with low endo PBS before batch incubation with the sample. This is detailed in the accompanying protocol.

Ethanol dilates the pores in the membrane. Removing the ethanol will ensure no leakage of the sample into the collection tube during the batch incubation (step # 3).

Endotoxin Removal Protocol:



Recommended Protocol:

PRE-EQUILIBRATION

1. Pipette up to 200 µl NoEndo™ resin slurry (50% NoEndo slurry) into the batch incubation chamber of the spin column barrel. Wash the resin at 13,000 x g for 20 sec.

2. Pre-equilibrate the NoEndo™ µ spin column with 600 µl equilibration buffer by centrifuging the spin column at 13,000 x g for 20 sec. It is critical that you repeat this step one more time with a further 600 µl fresh equilibration buffer.

NOTE: If using one spin column, ensure that the spin column is counterbalanced with a unit of equal weight (adjusted with distilled water).

SAMPLE LOADING

3. Transfer the spin column barrel to a fresh 2.2 ml centrifuge tube and load your required volume of filtered sample. The maximum sample volume is 600 µl. Close the lid and vortex for 15 sec to mix the sample and the NoEndo™ resin. Repeat the vortexing every 15 min for 1 hour. To achieve final endotoxin loads, < 0.1 EU/ml, a further batch incubation of 1 hour may be required.

NOTE: If the sample needs filtering, use a Proteus Mini Clarification spin column (Protein Ark Cat # GEN-MSF500, 0.2 µm pore size, 100 pc).

4. After batch incubation, centrifuge the column at 13,000 x g for 20 sec and collect the eluate. Spin times may need to be increased if the sample is not correctly filtered.

NOTE: If using one spin column, ensure that the spin column is counterbalanced with a unit of equal weight (adjusted with distilled water).

PURIFIED SAMPLE

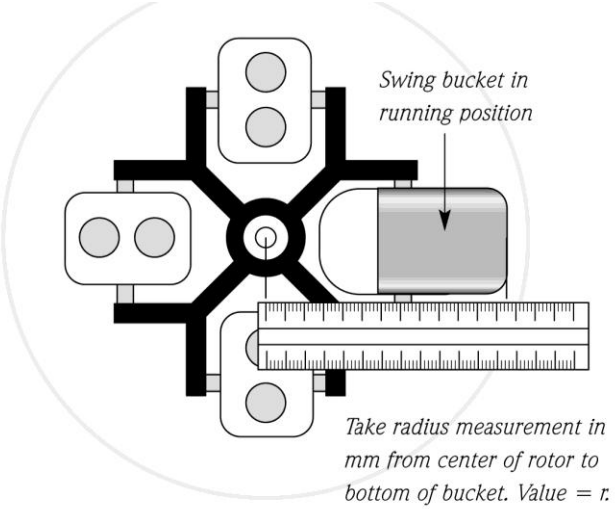
5. The eluate contains the target protein, largely depleted of endotoxin, and is now ready for further downstream analyses.

Proteus NoEndo™ µ (Micro) Spin Columns Easy-To-Read Protocol:

Fraction	Volume	Step	RCF	Spin Time
Wash	-	0.1 ml NoEndo™ Resin	13,000 g	20 sec
Pre-equilibration #1	600 µl	Low Endo PBS	13,000 g	20 sec
Pre-equilibration #2	600 µl	Low Endo PBS	13,000 g	20 sec
Sample Loading	Up to 600 µl	Batch Incubate	-	1 hour
Final Eluate	-	Sample	13,000 g	20 sec

Appendix:

How to convert rpm to g force (RCF) for the MIDI spin column using a swing bucket rotor



It is important that the Proteus spin columns are centrifuged at the correct speeds. Use of higher speeds than those indicated may damage the resin matrix and result in reduced performance. Many centrifuges display only rpm. See the diagram to enable accurate conversion between rpm and RCF (g force). This formula will work on any rotor providing an accurate measurement is taken from the center of the rotor to the bottom of the swing bucket at its open position (when the bucket is rotated through 90° in its running position).

$$RCF = 1.12 \times r\left(\frac{rpm}{1000}\right)^2$$

Eg. 500 g corresponds to 1630 rpm when the radius (r) = 170 mm

Product Performance:

Working with 50 µl and 100 µl NoEndo Resin Bed Volumes

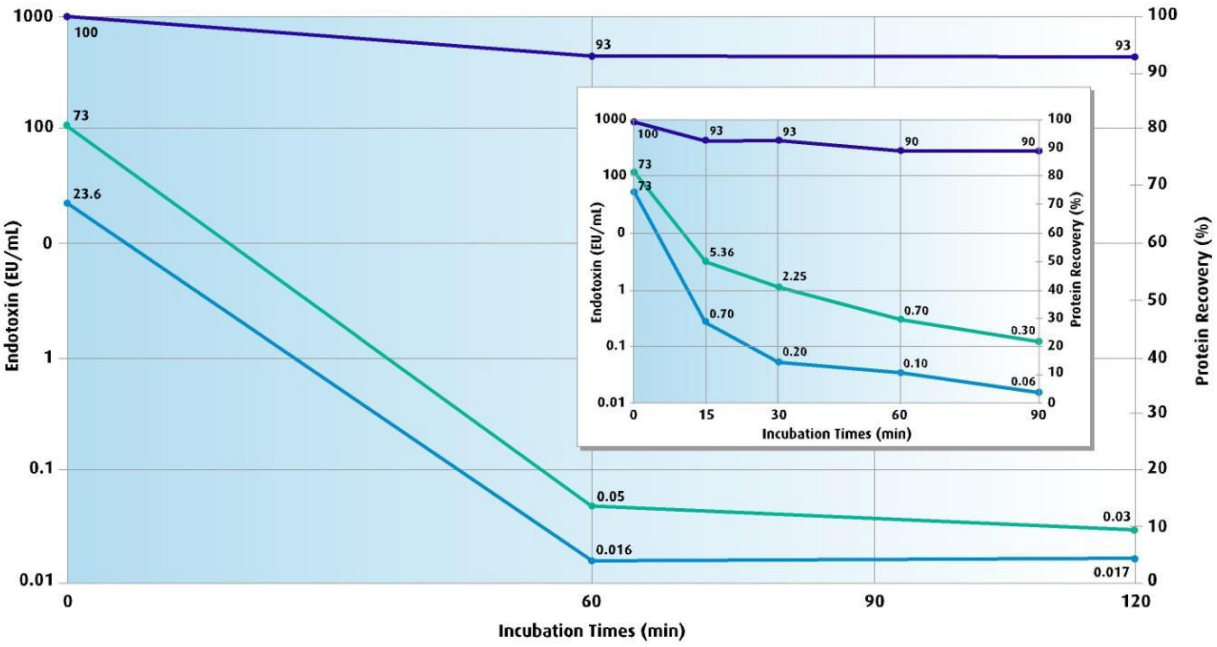


Figure 1: The Proteus NoEndo™ µ spin columns were challenged with rabbit IgG (1mg/ml) spiked with *E.coli* lysate. The Proteus NoEndo™ µ spin columns were loaded with 50 µl and 100 µl NoEndo™ resin. Endotoxin data was generated using the Charles River Endosafe-PTS assay. Protein recoveries in excess of 90% and 4 log reductions in endotoxin were observed.

Adjusting the Sample Volume/Resin Bed Volume Ratio

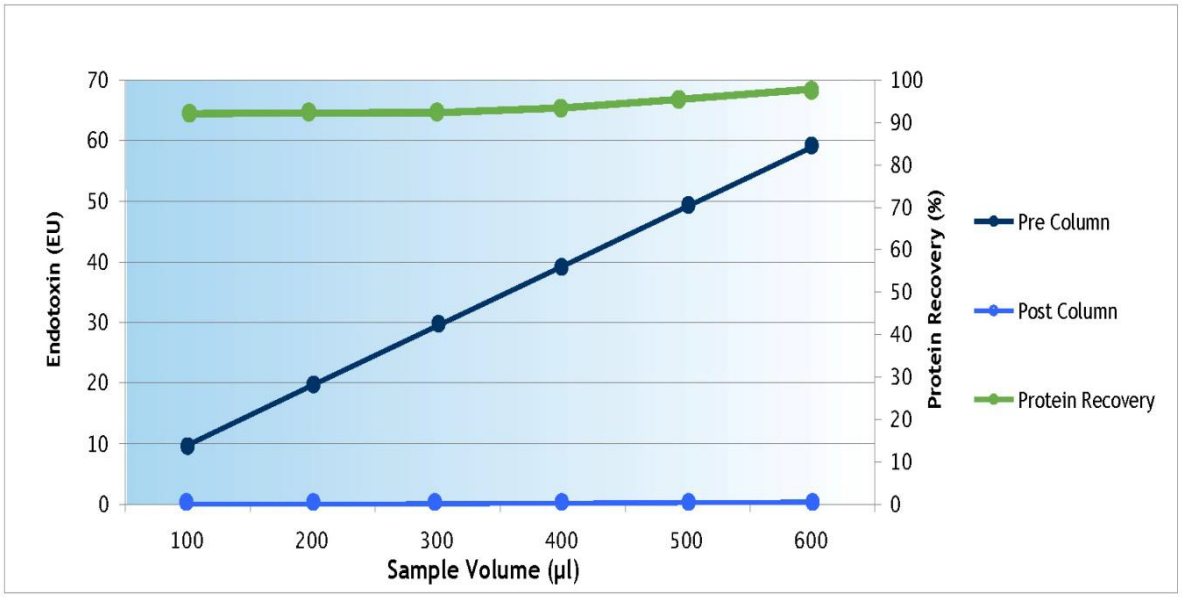


Figure 2: The Proteus NoEndo™ µ spin columns containing 50 µl resin were challenged with rabbit IgG (1mg/ml) spiked with *E.coli* lysate. Endotoxin data was generated using the Charles River Endosafe-PTS assay. Protein recoveries in excess of 90% and 4 log reductions in endotoxin were observed.

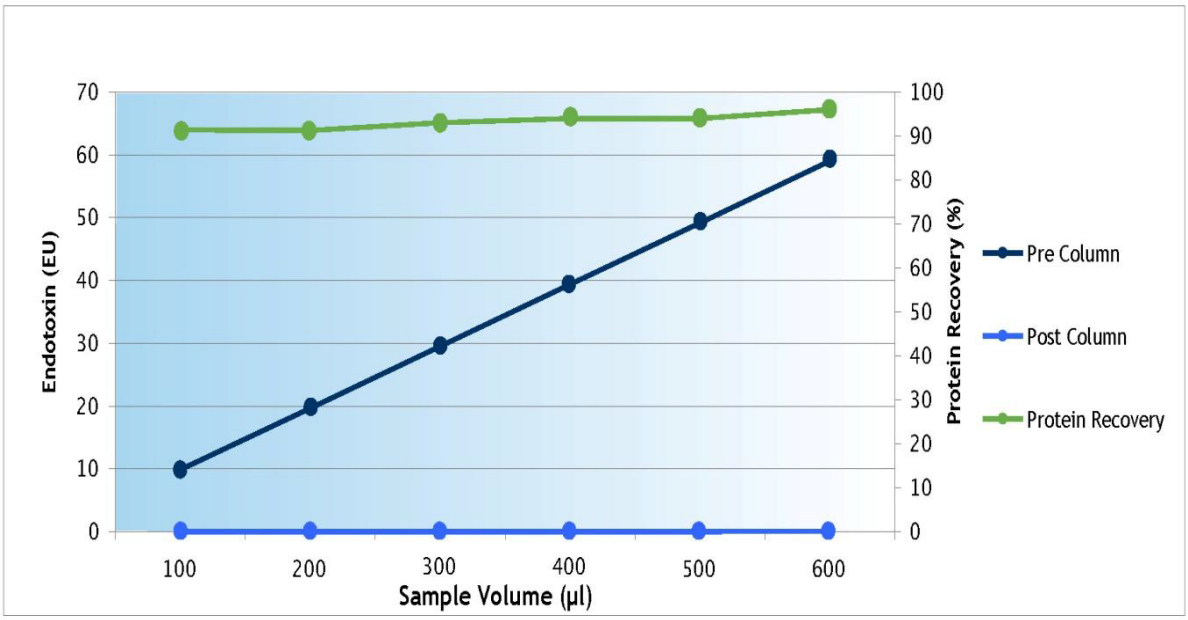


Figure 3: The Proteus NoEndo™ µ spin columns containing 100 µl resin were challenged with rabbit IgG (1mg/ml) spiked with *E.coli* lysate. Endotoxin data was generated using the Charles River Endosafe-PTS assay. Protein recoveries in excess of 90% and 4 log reductions in endotoxin were observed.

Specifications:

Details	**NoEndo™ µ (Micro) Spin Column**
Typical *in situ* binding capacity per column	300-500 EU
Typical endotoxin binding capacity per ml	500-800 EU/ml
Minimum endotoxin levels tested post-column	<0.03 EU/ml
Typical endotoxin clearance after 1 hour incubation	3 log reduction
Typical endotoxin clearance after 3 hour incubation	4 log reduction
Maximum sample load volume	0.6 ml
Bed volume	0.01-0.1 ml loose
Resin	NoEndo™ resin
Bead size range	100 µm
Proteus matrix	Cross-linked 6 % agarose
Recommended working pH	4-8

Questions and Answers:

1. *What is the preferred rotor for the NoEndo™ spin columns?*
   The preferred rotor is a fixed angle rotor in a microfuge.

2. *Do I need to filter the buffers prepared in my laboratory?*
   It is good laboratory practice to filter all buffers.

3. *Do I need to pre-filter my sample before loading it on to a NoEndo™ spin column?*
   All samples should be filtered through a final 0.2 µm pre filter or a Proteus mini clarification spin column (GEN-MSF500) just before the sample loading step.

4. *What are the typical binding capacities of NoEndo™ µ (Micro) spin columns?*
   The endotoxin binding capacity will critically depend on the resin bed volume. For 0.1 ml resin volume, the typical endotoxin binding capacity is 300-500 EU if the desired final target endotoxin load is <0.03 EU/ml (after 1 hour incubation).

5. *How should I prepare my sample for the NoEndo™ spin column?*
   Many chromatographic procedures demand that the sample is pre-conditioned prior to loading. It is recommended that all samples are pre-filtered down to a 0.2 µm pore size.

6. *What is the maximum volume of solution I can load on to a spin column?*
   You can load up to 0.6 ml in a 300 fixed angle rotor in a microfuge.

7. *What is the recommended loading buffer?*

It is recommended that low-endotoxin PBS buffer be used. Extra care should be taken with buffers containing amines as these interfere with the resin's ability to capture endotoxin.

8. *What is the highest speed that I should spin the NoEndo™ µ (micro) spin column?*

There is no need to spin the devices at speeds greater than 14,000 x g.

9. *Is there a minimum spin speed for the NoEndo™ spin columns?*

There are no minimum speeds for NoEndo™ spin columns. The devices can be spun at speeds as low as 2,000 x g.

10. *Why are the sample loading steps for the NoEndo™ µ spin columns up to 3 hours?*

The NoEndo™ µ columns incorporate our proprietary SelfSeal™ membrane technology. The membrane is specially formulated to prevent any sample from leaking into the collection tube on an orbital mixer. In a centrifuge, the membrane pores dilate and the eluate, free of endotoxin, passes into the collection tube. The contact time is maximised to ensure maximum endotoxin depletion without losses of protein, antibody or domain antibodies.

11. *How can I detect endotoxin levels in my sample?*

There are many commercially available endotoxin detection instruments available in the market. However, based on test data reliability, use of kinetic chromogenic, turbidometric LAL assay and the Endosafe-PTS assay from Charles River Laboratories, Wilmington, MA, US, is recommended.

12. *Should I be concerned if the resin dry out during the centrifugal steps?*

The NoEndo™ resin is robust and partially dried resin rehydrate rapidly. There are no adverse effects upon the performance of the resin.

13. *Can I re-use the NoEndo™ Mini spin columns?*

These are disposable columns. Discard the resin after each use!

14. *How do I determine the protein recovery?*

Protein recovery can be determined by measuring the absorbance of the sample before and after using the Proteus NoEndo™ spin column.

15. *My protein sample contains glycerol. Will this affect the performance of the NoEndo™ spin columns?*

Glycerol does not impact upon the performance of the NoEndo™ column.

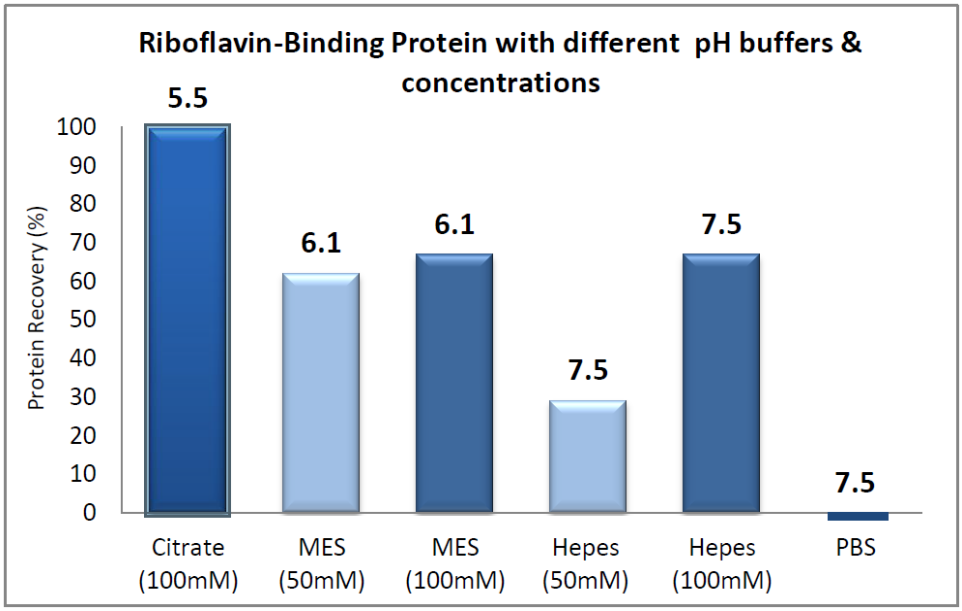
Troubleshooting Assistance

The eluate does not flow easily through the spin column

- Pre-filter the sample just before loading onto the NoEndo™ spin column to prevent the column being clogged with any sample-derived particulates.
- Increase the spin time or spin speed for the elution step.

The recovery of target protein is low

- Some proteins have high affinity for endotoxin.
- There may be interactions between the resin and the protein. If the protein is phosphorylated or acidic, pI of the target protein and the buffer pH needs to be considered. Ideally, the pH of the buffer should be 1 pH unit below the pI of the protein to ensure that the protein has a net positive charge. Data is presented below to show that recovery of an acidic and phosphorylated protein e.g. RBP can be improved by changing the buffer composition.



- The NoEndo™ resin has a cationic functional group which may act as a very weak anionic exchanger at pH values above the pI of the protein. This could lead to slight binding of the target protein which can be minimised by increasing the ionic strength of the buffer up to 0.5 M NaCl.
- Avoid Tris as Tris can cause lower protein recovery.
- The addition of EDTA (up to 20 mM) can enhance protein recovery without affecting endotoxin removal.

Poor resolution of the target protein

- The sample volume or concentration may be too large for the capacity of the resin. In this case, reduce the sample load or sample volume.
- The sample may also need to be filtered carefully.

High levels of endotoxin in my sample

- Avoid freeze-thawing as this increases the formation of endotoxin micelles which can be more difficult to remove.
- 0.1 M glycine can cause minor reductions in endotoxin removal.
- The presence of metal ions (Ca^{2+}, Mg^{2+} and Cu^{2+}) in the sample can hinder the removal of endotoxin.
- Up to 0.3 M NaCl is compatible with endotoxin removal.

Glossary:

Affinity Chromatography – Chromatographic separation based on a specific interaction between an immobilised ligand and a binding site on a macromolecule.

Bed volume – The total volume occupied by the chromatographic packed bed. It is also referred to as the column volume or CV.

Endotoxin – A heat-stable pyrogenic toxin present in the intact bacterial cell. Endotoxins are lipopolysaccharide complexes that occur in the cell wall.

EU/ml – A quantification of endotoxin levels relative to a specific quantity of reference endotoxin. 1 EU/ml is approximately equal to 0.1 ng/ml.

EU - The unit EU (endotoxin unit) describes the biological activity of LPS.

Freeze-thawing – A method that is sometimes used to break open cells by successive periods of slow freezing and thawing. Ice crystals are generated during the freezing stage, which disrupt the cells when they melt during thawing. The method, however, is slow and releases a limited amount of subcellular components.

Isoelectric point – The pH at which the protein has no net charge.

LAL - Limulus amebocyte lysate (LAL) is an aqueous extract of blood cells (amoebocytes) from the horseshoe crab, Limulus polyphemus. LAL reacts with bacterial endotoxin.

Micelles – These arelipid molecules that arrange themselves in a spherical form in aqueous solutions.

Monoclonal Antibodies – Monoclonal antibodies are monospecific antibodies that are the same because they are made by identical immune cells that are all clones of a unique parent cell.

Recombinant Protein -Proteins that result from the expression of recombinant DNA within living cells are termed recombinant proteins.

Turbidometric assay -A method for determining the concentration of a substance in a solution by measuring the loss in intensity of a light beam through a solution that contains suspended particulate matter.

Viral Vectors - Viral vectors are agents commonly used by molecular biologists to deliver genetic material into cells.

References

Persch, D., Anspach, F.B. (2000) 'Endotoxin removal from protein solutions', J Biotechnol, 76 (2-3):97-119.

Gorbet, M.V., Sefton, M.V. (2005) 'Endotoxin: the uninvited guest', Biomaterials, 26 (34):6811-7.

Magalhaes, P. et al. (2007) 'Methods of endotoxin removal from biological preparations', J Pharm Phram Sci, 10 (3):388-404.

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