# Package Insert for EndoTrap® HD

**Chromatography resin for endotoxin removal**

## Introduction

EndoTrap HD is based on hydrophilic, dimensionally stable affinity matrix with excellent pressure/flow characteristics. Non-specific binding of proteins to EndoTrap HD is extremely low, delivering a mass yield which typically exceeds 98%. The EndoTrap HD system can be reused up to ten times without loss of endotoxin removal efficiency!

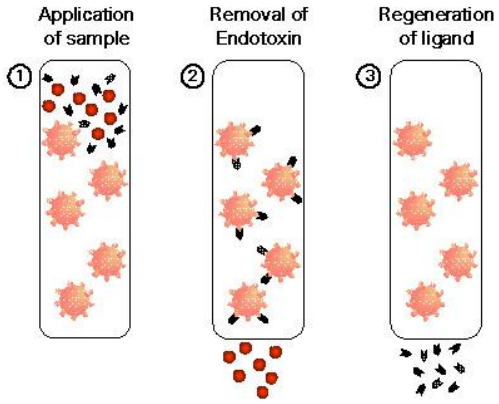
EndoTrap eliminates common limitations connected with present methods for LPS clearance as ultrafiltration, ion exchange chromatography, or two phase extraction.

- Specific LPS capture from complex mixtures
- Broad pI range for target proteins
- Broad pH/buffer range
- Compatible with commonly used buffer additives and high salt conditions

EndoTrap HD has been especially optimized for application in biomanufacturing processes. It can be used in **early or late biomanufacturing process step**:

- Robust and chemically defined matrix
- High LPS binding capacity
- Excellent chromatographic characteristics
- Low ligand leakage
- High reusability
- Leakage ELISA available
- A Regulatory Support File (RSF) is provided on request.

## Principle




**Principle of EndoTrap HD:**

1. Endotoxin-contaminated proteins and aqueous solutions are applied
2. Endotoxin is captured, proteins elute
3. Regeneration of ligand by using regeneration buffer

## EndoTrap® HD package size

| | | | |
|---|---|---|---|
| **Package sizes** | | | |
| | **EndoTrap HD 10** | **EndoTrap HD 50** | **EndoTrap HD 250** |
| | 10 ml settled resin | 50 ml settled resin | 250 ml settled resin |
| **Bulk** | | | |
| | **EndoTrap HD bulk** | | |
| | on request | | |
| **Materials not provided** | | | |
| | **Buffer required for operation** | | |
| | All buffers required for operation of EndoTrap HD have to be prepared by the customer. | | |

# Package Insert for EndoTrap® HD

**Chromatography resin for endotoxin removal**

## Precautions

- ! Equilibration and sample buffer used for endotoxin removal with EndoTrap HD have to contain **at least 0.1 mM free $Ca^{2+}$**.
- ! EndoTrap HD is supplied / stored with ProClin™ as preservative. For further information see the EndoTrap HD Material Safety Data Sheet.
- ! All materials used in the process, like containers or pipette tips and buffers, must be endotoxin free. Glass ware is preferred, as endotoxins can be destroyed by heat treatment (200 °C, 4 h or 250 °C, 1 h).
- ! Buffers should be prepared from endotoxin free materials with endotoxin free water.
- ! Buffers, resin and sample should have the same temperature (4-35 °C) during the processing steps.
- ! Avoid proteases (see page 8) and organic solvents.

## Specifications

| | | |
|---|---|---|
| LPS Binding Ligand | EndoTrap® HD ligand | |
| | Protein structure: | homo-trimer |
| | Molecular weight: | 150 kDa (trimer) |
| | Dissociation constant: | $K_D$ = 5 x $10^{-8}$ M |
| | Isoelectric point: | 8.52 |
| Bead Matrix | Hydrophilic, cross-linked methacrylic polymer | |
| | Particle size range: | 40 – 90 µm |
| | Exclusion limit: | 5000 kDa (globular proteins) 1000 kDa (PEG) |
| | Mean pore diameter: | 1000 Å |
| EndoTrap® HD | Immobilized ligand: | approx. 4 mg/ml |
| | Binding capacity: | > 5 x $10^6$ EU/ml resin (1 EU = 100 pg LPS) |
| | Operating pH range: | pH 5 – 10 |
| | Operating flow rate: | maximum 800 – 1000 cm/h |
| | Operating pressure: | up to 3 bar is recommended (maximum pressure drop on column is 7 bar) |
| | Temperature stability: | 4 – 35 °C |
| | Ligand leakage: | < 20 ng/ml (from 10 mg/ml BSA) |
| | Shipping condition: | ambient temperature |
| | Shelf life: | EndoTrap HD (unused material) is stable until the stated expiry date when stored correctly (at 2-8 °C). |

## Operating EndoTrap HD

| | |
|---|---|
| Column dimension[1] | The column dimension to be applied in a process depends largely on sample composition and volume. The following constraints have to be considered:<br>1. Endotoxin content of the sample<br>2. Time-on-the-column (minimum time required for exchange)<br>3. Volume to be processed (flow rate) |
| | **1. Endotoxin content of the sample**<br>- To achieve best results, total LPS units applied should not exceed 30-50% of the maximum column capacity (5 x $10^6$ EU/ml resin). |
| | **2. Time-on-the-column**<br>- Time-on-the-column should be 30 seconds minimum. *Example: A 10 ml column should be processed with a maximum flow rate of 20 ml/min.* |
| | **3. Volume to be processed**<br>- In order to process a certain volume in a certain time, the column dimension (diameter vs. length) must allow a reasonable flow rate<br>- Flow rate could be in the range of 60 – 840 cm/hr<br>- Column size ratio should be between 1:1 and 1:3 (diameter:length) |

1 For examples see table 1 “Column dimension”.

# Package Insert for EndoTrap® HD

## Chromatography resin for endotoxin removal

## Operating EndoTrap HD – continued

! To ensure **low ligand leakage** we recommend starting the protocol with a regeneration step followed by an equilibration step, therefore the concentration of leached ligand in fractions should be in the range of 300 pg/ml to 10 ng/ml.

! Our experiments showed that the **first column volume** of sample has a higher ligand leakage than the rest of the purified sample. To ensure the lowest ligand concentration in your sample we recommend collecting the first column volume separately.

! When applying **concentrated sample solutions** (e.g. > 5 mg/ml) the concentration of leached ligand could be higher than 10 ng/ml in the very first fraction.

! Leakage of minute amounts of ligand is a matter of fact for all affinity materials. We recommend analysing the leakage of the LPS-binding ligand with our EndoTrap® Leakage ELISA (Article No. 800033).

| | | |
|---|---|---|
| **Packing procedure** | Use equilibration buffer for column packing. Packing velocity should be in the range of 800 – 1000 cm/hr. The operating pressure should not exceed 3 bar (43.5 psi). | |
| **Equilibration**[1] | Equilibration buffer should be identical with the sample buffer used for the process. Equilibration buffer has to contain 0.1 mM $Ca^{2+}$ (e.g. $CaCl_2$). | |
| | Protocol: | - Pre-equilibrate the column with 3 column volumes equilibration buffer plus 1 M NaCl<br>- Equilibrate the column with 3 column volumes equilibration buffer. |
| | Flow rate: | 600 – 840 cm/hr |
| **Endotoxin removal**[1] | EndoTrap HD works under a broad range of conditions, there are nearly no limits regarding pH, ionic strength and additives. | |
| | Sample buffer: | Customer defined |
| | Volume: | Customer defined |
| | Flow rate: | 60 – 840 cm/hr |
| **Regeneration** | EndoTrap HD can be regenerated under mild conditions by complexing $Ca^{2+}$ with EDTA at increased ionic strength. | |
| | Regeneration buffer: | 20 mM Tris, 1 M NaCl, 2 mM EDTA, pH 8.0 |
| | Volume: | 6 column volumes |
| | Flow rate: | 300 – 840 cm/hr |
| **Cleaning in Place (CIP)** | CIP should remove tightly bound, precipitated or denatured substances from the purification system. | |
| | CIP buffer: | 20 mM Tris, pH 8.0<br>supplemented with 6 M Urea or 2 M Gua/HCl |
| | Protocol: | - Clean the column with 6 column volumes CIP buffer.<br>- Wash immediately with at least 5 column volumes of equilibration buffer. Use reversed flow direction. |
| | Flow rate: | 600 – 840 cm/hr |
| **Sanitisation**[2] | Sanitisation reduces microbial contamination of the resin to a minimum. | |
| | Sanitisation buffer: | 0.1 M Acetic acid + 20% Ethanol |
| | Protocol: | - Incubate the column with sanitisation buffer for 2 – 12 hours |

1 For compatible substances and limitations see table 2 “Equilibration buffer / Customer defined buffer”.
2 For compatible substances see table 7 „Sanitation tests“.

# Package Insert for EndoTrap® HD

## Chromatography resin for endotoxin removal

| | | |
|---|---|---|
| **Storage** | Unused resin can be stored in the container. Ensure that the container is densely closed.<br>EndoTrap HD is delivered in 20 mM $Na_2HPO_4$, 150 mM NaCl, 2 mM EDTA, pH 7.4, 2.5 ppm ProClin™ | |
| | Unused material: | at 2-8 °C as supplied |
| | Regenerated material: | at 2-8 °C in regeneration buffer supplemented with 2.5 ppm ProClin™ or 0.02% Na-Azide |
| | Note: | **Do not freeze!** |
| **Scaling-up** | After optimizing at laboratory-scale, the process can be scaled up. For this purpose some parameters have to be changed while others remain constant. | |
| | - Select bed volume according to required LPS binding capacity. | |
| | - Select column dimension so that high flow rates can be used.[1] | |
| | - Select linear flow rate during sample application to ensure that contact time is not shorter than that established in the small scale study. | |
| | - Keep sample concentration and gradient slope constant. | |

## Supplementary Information

### Column dimension

| **Resin volume** | **10 ml** | **50 ml** | **250 ml** | **250 ml** |
|---|---|---|---|---|
| **Column dimension** | 1.6 cm x 5 cm | 3 cm x 7 cm | 5 cm x 12 cm | 6 cm x 9 cm |
| **Max. flow rate [ml/min]** | 20 ml/min | 100 ml/min | 275 ml/min | 396 ml/min |
| **Max flow rate [cm/hr]** | 600 cm/hr | 840 cm/hr | 840 cm/hr | 840 cm/hr |
| **Time-on-the-column** | 0.5 min | 0.5 min | 0.9 min | 0.6 min |
| **Process volume** | 1.2 liter/hr | 6 liter/hr | 16.5 liter/hr | 23.8 liter/hr |

**Table 1, Column dimension:** We recommended a column size ratio between 1:1 and 1:3 (diameter:length). The maximum flow rate should not exceed 800-1000 cm/hr.

### Equilibration buffer

| | | |
|---|---|---|
| **Equilibration buffer*** | The column should be equilibrated with the same buffer which is used for sample, pH and different additives can be adjusted to the concentrations indicated in this table. | |
| | pH: | 5-10 |
| | Ionic strength: | 50-1000 mM NaCl |
| | Calcium conc.: | 0.1-10 mM $Ca^{2+}$<br>We recommend to add $Ca^{2+}$ (e.g. $CaCl_2$) freshly to your customer specific buffer (= equilibration and sample buffer). |
| | Possible additives: | up to 10 mM DTT (Dithiothreitol)<br>0.005% Tween20<br>max. 0.005% NaDOC<br>max. 0.5 M GdnHCl<br>10% DMSO<br>20% Isopropanol<br>20% Methanol<br>20% Ethanol<br>10% Glycerol<br>0.5 M Urea<br>300 mM Imidazol |
| | Interfering substances: | > 10 mM NaOH<br>SDS<br>Citrate<br>ETDA, and other Calcium chelators<br>Ammoniumsulphate |

**Table 2, Equilibration buffer / Customer defined buffer.***Some of the substances may interfere with the LAL assay.

1 See table 9 „Pressure / flow comparison“.

# Package Insert for EndoTrap® HD

## Chromatography resin for endotoxin removal

## Samples to be applied

| | | |
|---|---|---|
| **Sample to be applied to EndoTrap HD** | All kind of complex biological solutions and purified components can be processed on EndoTrap HD. | |
| | Sample materials: | proteins, peptides, anitbodies, plasmid DNA |
| | Sample concentration: | 1-20 mg/ml |

**Table 3, Samples to be applied.**

## Tested LPS sources

| | | |
|---|---|---|
| **Evaluated spectrum of EndoTrap HD towards various LPS sources** | *Escherichia coli* K12, R1, R2, R3, R4 | *Pseudomonas stutzeri* |
| | *Salmonella enterica* | *Enterobacter aerogenes* |
| | *Citrobacter freundii* | *Enterobacter asburiae* |
| | *Citrobacter amalonaticus* | *Enterobacter cloacae* |
| | *Citrobacter koseri* | *Aeromonas hydrophila* |
| | *Pseudomonas aeruginosa* | |

**Table 4, tested LPS sources:** Efficiency of LPS removal has been tested for various gram-negative bacteria strains.

## Applications data

### Endotoxin removal from various buffer systems

| Buffer | Endotoxin removal efficiency [%] | BSA recovery rate [%] |
|---|---|---|
| 20 mM Acetat, pH 5.0 | 99.98 | 98.94 |
| 20 mM MES, pH 6.0 | 99.96 | 97.55 |
| 20 mM MOPS, pH 7.0 | 99.93 | 99.10 |
| 20 mM Hepes, pH 7.4 | 99.93 | 96.49 |
| 20 mM Tris, pH 8.0 | 99.93 | 96.45 |
| 20 mM Borat, pH 9.0 | 99.95 | 97.94 |
| 20 mM Borat, pH 10 | 99.99 | 98.16 |

**Table 5, Endotoxin removal from various buffer systems:** Column mode (1 ml column; flow rate: 1 ml/min): 10 ml BSA solution spiked with endotoxin (20 mg/ml, 600 EU/ml) was incubated in various buffers at different pH. All buffers contained 150 mM NaCl and 0.1 mM $CaCl_2$.

### Endotoxin removal at various salt concentrations

| Ionic strength | Endotoxin removal efficiency [%] |
|---|---|
| 20 mM Hepes, pH 7.4, 0.1 mM $CaCl_2$ | 84.10 |
| 20 mM Hepes, pH 7.4, 0.1 mM $CaCl_2$, 50 mM NaCl | 99.83 |
| 20 mM Hepes, pH 7.4, 0.1 mM $CaCl_2$, 150 mM NaCl | 99.98 |
| 20 mM Hepes, pH 7.4, 0.1 mM $CaCl_2$, 300 mM NaCl | 99.97 |
| 20 mM Hepes, pH 7.4, 0.1 mM $CaCl_2$, 600 mM NaCl | 99.96 |
| 20 mM Hepes, pH 7.4, 0.1 mM $CaCl_2$, 1 M NaCl | 99.87 |

**Table 6, Endotoxin removal at various salt concentrations:** Column mode (1 ml column; flow rate: 1 ml/min): 10 ml endotoxin spiked BSA (20 mg/ml, 600 EU/ml) was incubated in Hepes buffer (20 mM Hepes, pH 7.4; 0.1 mM $CaCl_2$) with increasing concentrations of NaCl.

### Sanitisation test

| Sanitisation buffer | Incubation time | Endotoxin removal efficiency [%] | Factor of reduction [CFU] | |
|---|---|---|---|---|
| | | | *Listeria* | *E.coli* |
| 0.1 M Acetic acid + 20% EtOH | 4 hours | 99.89 | $10^7$ | $10^7$ |
| 70% EtOH | 6 hours | 99.82 | $10^7$ | $10^7$ |
| 0.1 M HCl | 6 hours | 99.87 | $10^7$ | $10^7$ |

**Table 7, Sanitisation test:** Batch mode: Endotoxin removal of 1.5 ml endotoxin spiked BSA (20 mg/ml, 600 EU/ml) with 0.1 ml EndoTrap HD resin. The indicated sanitisation buffer provided 100% reduction of bacterial contamination ($10^7$ CFU incubated for indicated time). Endotoxin removal is not affected when resin is exposed to the same buffers for 24 hours.

# Package Insert for EndoTrap® HD

## Chromatography resin for endotoxin removal

### Endotoxin removal from various samples

| Sample | pI | Sample Volume [ml] | Sample Concentration [mg/ml] | EU before polishing [EU/ml] | EU after polishing [EU/ml] | LPS removal efficiency [%] | Sample Recovery [%] | Ligand Leakage [ng/ml] |
|---|---|---|---|---|---|---|---|---|
| BSA | 4.7 | 100 | 10 | 4984 | 0.3821 | > 99.99 | 106 | 3.22 |
| IgG | 8.3-8.9 | 100 | 10 | 20387 | 0.108 | > 99.99 | 95 | 1.13 |
| Lysozym | 10.7 | 100 | 10 | 11359 | 0.21 | > 99.99 | 101 | 11.6 |

**Table 8a, Endotoxin removal from various samples:** Column mode (10 ml column; flow rate: 3 ml/min): Endotoxin spiked sample was polished in Hepes buffer (20 mM Hepes, pH 7.4; 150 mM NaCl, 0.1 mM $CaCl_2$).

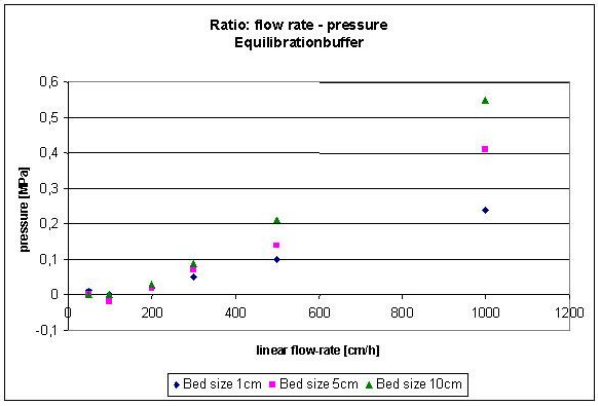
| Sample | pI | Sample Volume [ml] | Sample Concentration [mg/ml] | EU before polishing [EU/ml] | EU after polishing [EU/ml] | LPS removal efficiency [%] | Sample Recovery [%] | Ligand Leakage [ng/ml] |
|---|---|---|---|---|---|---|---|---|
| BSA | 4.7 | 1000 | 1 | 577 | 0.027 | > 99.99 | 96 | 0.573 |
| IgG | 8.3-8.9 | 1000 | 1 | 1317 | < 0.025 | > 99.99 | 98 | 0.334 |
| Lysozym | 10.7 | 1000 | 1 | 255 | < 0.025 | > 99.99 | 95 | 1.40 |

**Table 8b:** Column mode (10 ml column; flow rate: 10 ml/min): Endotoxin spiked sample was polished in Hepes buffer (20 mM Hepes, pH 7.4; 150 mM NaCl, 0.1 mM $CaCl_2$).

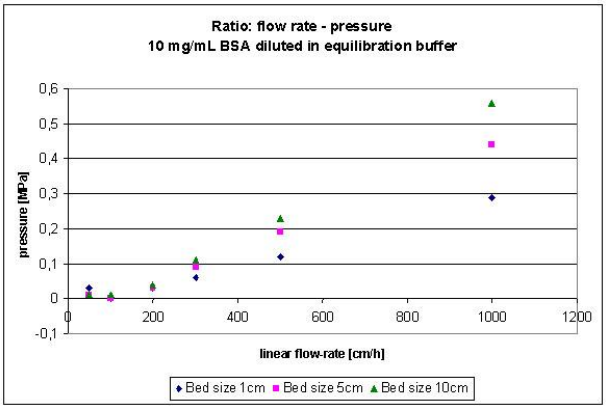
### Pressure / flow comparison

| | Bed size: 1 cm | | Bed size: 5 cm | | Bed size: 10 cm | |
|---|---|---|---|---|---|---|
| Flow rate [cm/h] | Pressure [MPa]: buffer | Pressure [MPa]: BSA | Pressure [MPa]: buffer | Pressure [MPa]: BSA | Pressure [MPa]: buffer | Pressure [MPa]: BSA |
| 50 | 0.01 | 0.03 | 0 | 0.01 | 0 | 0.01 |
| 100 | 0 | 0 | 0 | 0 | 0 | 0.01 |
| 200 | 0.02 | 0.03 | 0.02 | 0.03 | 0.03 | 0.04 |
| 300 | 0.05 | 0.06 | 0.07 | 0.09 | 0.09 | 0.11 |
| 500 | 0.1 | 0.12 | 0.14 | 0.19 | 0.21 | 0.23 |
| 1000 | 0.24 | 0.29 | 0.41 | 0.44 | 0.55 | 0.56 |

**Table 9, Pressure / flow comparison:** The pressure / flow comparison between buffer (20 mM Hepes, pH 7.4; 150 mM NaCl, 0.1 mM $CaCl_2$) and BSA (10 mg/ml dissolved in buffer). The pressure / flow data were determined in Millipore Vantage column (diameter 16 mm, height 250 mm) packed to a bed height as indicated using regeneration buffer as the mobile phase at 20 °C.




Equilibration buffer




10 mg/ml BSA

## Reusability

| Cycle number | Endotoxin removal efficiency [%] | Endotoxin removal efficiency [log] |
|---|---|---|
| 0 | 99.9996 | 5 |
| 1 | 99.9994 | 5 |
| 2 | 99.9999 | 6 |
| 3 | 99.9990 | 5 |
| 4 | 99.9994 | 5 |
| 5 | 99.9991 | 5 |
| 6 | 99.9998 | 5 |
| 7 | 99.9990 | 5 |
| 8 | 99.9995 | 5 |
| 9 | 99.9984 | 4 |
| 10 | 99.9990 | 5 |

**Table 10, Reusability:** 100 ml BSA solution (10 mg/ml) was spiked with $10^6$ endotoxin units and endotoxin removal by EndoTrap HD (10 ml column) was measured. After each removal step the resin was regenerated with regeneration buffer and equilibrated with Hepes buffer (20 mM Hepes, pH 7.4; 150 mM NaCl, 0.1 mM $CaCl_2$) before starting the next round.

## Hints & Tricks

### What can I do if I am not satisfied with the performance of EndoTrap® HD?

Please consider the chemical characteristics of your sample before choosing one improvement step.

| Problem | EndoTrap® HD |
|---|---|
| **… bad sample recovery rate …** | |
| - due to **ionic interactions** | Increase the NaCl concentration of the equilibration / sample buffer. 150 – 250 mM NaCl should be sufficient. |
| - due to **interactions** with **lipopolysaccharides** | Hydrophobic interaction of your sample with LPS might be possible.As lipopolysaccharides form aggregates, it might be also possible that your sample arranged within these aggregates. It may help to disintegrate the aggregates or to reduce their size. For that purpose you can use Triethylamine (combined with 15 min ultrasonic treatment) or detergents.<br>Note: Detergents may interfere with endotoxin detection in the LAL assay. The final concentration of Triethylamine and Tween20 should not exceed 0.5% and 0.005%, respectively. |
| **… bad LPS removal rate …** | |
| - due to **depletion of calcium** | If you work with calcium binding proteins, ensure that your equilibration / sample buffer contains at least 0.1 mM free $Ca^{2+}$. |
| - due to interference with **buffer additives** | Chelators of divalent cations (like EDTA, EGTA, Acetat- or Citrate buffers) have to be avoided. |
| - due to limiting **contact time** | Time-on-the-column should be at least 30 seconds. |
| - due to limiting **LPS binding capacity** | To achieve best results, total LPS units applied should not exceed 30-50% of the maximum column capacity (5 x $10^6$ EU/ml resin). |
| **… bad Up-Scaling results …** | |
| - due to the **change of parameters** | Check, if parameters in “Operating EndoTrap HD” (page 2 & 3) like endotoxin capacity, time-on-the-column and volume to be processed become limiting. |

# Package Insert for EndoTrap® HD

**Chromatography resin for endotoxin removal**

## How should I work with proteases when using EndoTrap® HD?

Proteases may destroy the EndoTrap ligand during LPS removal. Please perform the cleaning steps at conditions where your protease is less active, e.g. 4°C, or change the buffer composition if possible.

Example: If you work with pepsin, we recommend to work above pH 6 since pepsin is an acidic protease.

## Protocols

Chromatography is traditionally done in two modes: discontinuous (batch mode) and continuous (column mode) chromatography. EndoTrap HD can be used either in batch or column mode. In general endotoxin removal of high endotoxin levels is more efficient in the column mode. Batch mode may be used for small volumes or to increase contact time.

Please inquire for our special protocols at www.endotrap.de.

| | | |
|---|---|---|
| EndoTrap®<br>Protocol: “Procedure for packing gel into a column” | Protocol how to pack gravity flow columns. | on request |
| EndoTrap®<br>Standard Application Note<br>(for R&D) | EndoTrap Standard Protocol<br>(column [gravity flow] / batch mode)<br>based on EndoTrap blue & EndoTrap red | on request |
| EndoTrap®<br>Application Protocol for Pilot Scale<br>(for R&D) | EndoTrap Protocol<br>for HPLC / FPLC automated systems<br>based on EndoTrap blue & EndoTrap red | on request |

**Gentaur Molecular Products**
**Voortstraat 49**
**1910 Kampenhout, Belgium**
**http://www.gentaur-worldwide.com**