creative bioscience solutions

EndoTrap®

Standard Application Protocol

Endotoxin removal systems – Chromatography systems for column or batch mode

Hyglos changed the way to remove endotoxins from aqueous solutions

EndoTrap eliminates common critical issues connected with present methods e.g. ultrafiltration, ion exchange chromatography or two phase extraction. **The protocol of EndoTrap is user friendly, yields rapid results as it is designed as a flow-through system and does not need training or special equipment.** EndoTrap is applicable for the downstream process as it is also available as 50% slurry. Furthermore the EndoTrap resin can be performed on fully automated liquid chromatography systems, too. Therefore EndoTrap connects the research and the production field.

EndoTrap is manufactured by Hyglos GmbH, Germany and is provided for research and bio-manufacturing use only.

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Introduction

EndoTrap is an affinity matrix for the efficient removal of bacterial endotoxins from solutions. EndoTrap can be employed both in batch or chromatography mode. Non specific binding of proteins to EndoTrap is extremely low, delivering a mass yield which typically exceeds 95%. The EndoTrap system can be reused at least three times (in general 10 times) without loss of endotoxin removal efficiency!

EndoTrap blue and EndoTrap red have similar characteristics. The products are different basically in their buffer compatibilities.

The most important differences between both EndoTrap-family products:

	EndoTrap® blue	EndoTrap® red
▪ pH (buffer)	pH 4-9	pH 6-9
▪ Ionic strength	up to 600 mM NaCl	up to 250 mM NaCl ***we recommend < 100 mM NaCl***
▪ Suitable with EDTA and other Calcium chelators containing buffers	No	Yes
▪ Customer specific equilibration buffer has to be enriched with calcium	Yes	No
▪ PBS can be used as equilibration buffer	Yes, when enriched **freshly** with 50-100 µM Ca^{2+}!	Yes
▪ Suitable for subsequent endotoxin detection with EndoLISA®	No	Yes

General specifications of EndoTrap®

Ligand	EndoTrap blue or EndoTrap red respectively
Binding capacity	2.000.000 EU/ml resin (each cleaning step theoretically yields a **two log reduction** of LPS)
Binding constant for LPS	Kd = 5 x 10^{-8} M
Support matrix	Highly cross-linked 4% agarose, spherical beads
Void volume	0.3 to 0.5 ml
Mean particle size	90 µm
Max. flow rate	0.2-1 ml/min
Max. pressure	3 bar, 43 psi, 0.3 MPa (when using automated systems)
Temperature stability	Regular use between 4°C and room temperature (20°C)
Storage	At 2-8°C in regeneration buffer (RB blue or RB red) supplemented with 0.02% sodium azide. **Do not freeze!**
Shelf live	EndoTrap is stable until the stated expiry date when stored correctly

Kit components of EndoTrap®

Prepacked columns		
	EndoTrap 1/1	**EndoTrap 5/1**
	1 x 1 ml column, 125 ml equilibration Buffer, 125 ml regeneration Buffer	5 x 1 ml column, 250 ml equilibration Buffer, 125 ml regeneration Buffer
Resin (50% slurry)		
	EndoTrap 10	**EndoTrap 50**
	20 ml resin (50% slurry), 250 ml equilibration buffer, 250 ml regeneration buffer	100 ml resin (50% slurry), 125 ml 10x equilibration buffer 125 ml 10x regeneration buffer
Material not provided		
	Storage buffer	
	Please add **0.02% sodium azide** to the necessary volume of regeneration buffer (RB)	

EndoTrap will be delivered at room temperature. Afterwards please store EndoTrap at 2-8°C.

Storage

EndoTrap is supplied as **prepacked columns** (EndoTrap 1/1 or 5/1, 1 ml column material) or as **50% slurry** (EndoTrap 10, 50 or 100) in regeneration buffer (RB) supplemented with 0.02% sodium azide. EndoTrap is stable until the stated expiry date when stored correctly. Regenerated EndoTrap matrix should be stored at 2-8°C in regeneration buffer (RB) supplemented with 0.02% sodium azide.
You can also use 20% ethanol as storage buffer; however, the storage time will then be only 4 weeks.
Do not freeze!

Which buffer systems are compatible with EndoTrap®?

Customer specific buffers may be used for equilibration and endotoxin binding. Below you find summarized what to consider if you prefer using your own buffer:

EndoTrap® blue	EndoTrap® red
Endotoxin removal with EndoTrap blue works effectively in the pH range of 4-9 and in presence of NaCl concentrations in the range of 50-600 mM. Buffers like **HEPES, TRIS, MOPS, MES,** and **PIPES** may be used. Citrate buffers and chelators of divalent cations (like **EDTA**) **have to be avoided**. Do not forget: **Customer specific buffers must contain 50-100 µM Ca^{2+}** or Mg^{2+}!! Always add Ca^{2+} (e.g. $CaCl_2$) freshly to your customer specific buffer especially when using **PBS buffer**. Otherwise phosphate and Ca^{2+} form an insoluble complex and will precipitate. For **DNA application** we recommend following buffer composition: 10 mM Tris-HCl, 1 mM $CaCl_2$, 300 mM NaCl, pH 8.0	Endotoxin removal with EndoTrap red works effectively in the pH range of 6-9 and in presence of NaCl concentrations in the range of 50-250 mM. Buffers like **HEPES, PBS, TRIS, MOPS, MES, PIPES** and also **Citrate, Acetate, Glycine** and **Carbonate buffers** may be used. You can also use EndoTrap red with chelators of divalent cations (like **EDTA**). With a **"classical" PBS buffer** (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) you would get a LPS removal rate of ~97% after each cleaning step. To improve this performance we recommend diluting your PBS buffer 1:2 with endotoxin free water; due to our knowledge you will get LPS removal rates of ~99% with a "half-concentrated" PBS buffer (5 mM Na_2HPO_4, 0.9 mM KH_2PO_4, 68.5 mM NaCl, 1.35 mM KCl, pH 7.4). Therefore p**hosphate buffers with NaCl concentrations below 100 mM** are recommended when using EndoTrap red.

Comparison of the specifications of EndoTrap® blue and EndoTrap® red

	EndoTrap® blue	EndoTrap® red
Tested substances which can be applied onto the column	▪ proteins ▪ peptides ▪ antibodies ▪ **plasmid DNA**	▪ proteins ▪ peptides ▪ antibodies
Regeneration buffer (endotoxin concentration < 0.02 EU/ml)	Regeneration buffer "blue" (based on "**HEPES buffer**", pH 7.5)	Regeneration buffer "red" (based on "**Phosphate buffer**", pH7.4)
Equilibration buffer (endotoxin concentration < 0.02 EU/ml)	Equilibration buffer "blue" ("**HEPES buffer**", pH 7.5 **enriched with 0.1 mM $CaCl_2$**)	Equilibration buffer "red" ("**Phosphate buffer**", pH 7.4 **with 80 mM NaCl**)
If you want to use your **own buffer** for equilibration (instead of the kit included equilibration buffer)	We tested EndoTrap blue successfully with **HEPES, Borate, TRIS, MOPS, MES, PIPES** when 50-100 µm Ca^{2+} were freshly added.	We tested EndoTrap red successfully with **PBS, HEPES, Borate, TRIS, MOPS, MES, PIPES, Citrate, Acetate, Glycine** and **Carbonate buffers**
pI of applied proteins	pI from 5-9	pI from 5-9
pH (buffer)	pH 4-9	pH 6-9
Ionic strength	up to 600 mM NaCl	up to 250 mM NaCl ***we recommend < 100 mM NaCl***
Recommended working concentration of applied substances	1-10 mg/ml	1-10 mg/ml
Recommended sample volume	up to 50 ml	up to 50 ml
Tested substances which **do not interfere** with the performance of EndoTrap*	up to 10 mM DTT (Dithiothreitol) 0.005% Tween20® max. 0.005% NaDOC max. 0.5 M GdnHCl 10% DMSO 20% Isopropanol 20% Methanol 20% Ethanol 10% Glycerol / Glycerin 0.5 M urea (up to 2 M at pH 7 possible) 300 mM Imidazol	DTT not tested max. 0.005% NaDOC 20% DMSO 20% Isopropanol 40% Methanol 20% Ethanol 20% Glycerol / Glycerin 1 M Urea 300 mM Imidazol
Tested substances which **interfere** with the performance of EndoTrap and therefore have an **inhibitory effect** for the binding to LPS*	▪ > 10 mM NaOH ▪ SDS and other detergents ▪ Citrate ▪ EDTA, and other Calcium chelators (EGTA, HEDTA, NTA) ▪ Ammoniumsulphate	▪ > 250 mM NaCl ▪ SDS, Tween20 and other detergents ▪ GdnHCl ▪ Ammoniumsulphate
Tested kinds of LPS (bacteria strain)	▪ *Escherichia coli* K12, R1, R2, R3, R4 ▪ *Salmonella enterica* ▪ *Citrobacter freundii* ▪ *Citrobacter amalonaticus* ▪ *Citrobacter koseri* ▪ *Pseudomonas aeruginosa* ▪ *Pseudomonas stutzeri* ▪ *Enterobacter aerogenes* ▪ *Enterobacter asburiae* ▪ *Enterobacter cloacae* ▪ *Aeromonas hydrophilia*	▪ *Escherichia coli* K12 ▪ *Salmonella enterica* ▪ *Citrobacter freundii* ▪ *Pseudomonas aeruginosa* ***for*** ▪ ***Klebsiella pneumoniae*** ▪ ***Serratia marcescens*** ***we recommend EndoTrap red!***

* Please consider that the indicated concentrations and substances refer to the performance of EndoTrap. Some of these substances which can be used during the cleaning with EndoTrap are **not suitable for the LAL test**. EndoLISA® endotoxin detection assay can be used as an alternative method.

Protocols

Chromatography is traditionally done in two modes: discontinuous (batch mode) and continuous (column mode) chromatography. EndoTrap can be used either in batch or column mode. In general endotoxin removal of high endotoxin levels is more practical in the column mode. Batch mode may be used for small volumes or to increase contact time. **However, parameters such as pH, ionic strength, temperature, contact time etc. might have to be optimized for each application to obtain maximum endotoxin removal with minimum loss of product.**

If you want to pack the columns (small / large plastic columns) by yourself we provide a protocol "Procedure for packing gel into a column".

We also provide a protocol for HPLC / FPLC automated systems ("Application Protocol for Pilot Scale") for customers who prefer using their liquid chromatography systems.

Please inquire for our special protocols at www.hyglos.de!

Precautions

! EndoTrap is supplied / stored with sodium azide, which is a poison. The toxicological properties of this product have not been fully investigated. At the low concentrations of sodium azide in this product no risk of poisoning is expected with normal use. Still avoid ingestion, inhalation and skin contact. For further information see the EndoTrap Material Safety Data Sheet.

! All used materials like containers or pipette tips and buffers must be endotoxin free. Glass ware is preferred, as endotoxins can be removed by heat treatment (200°C, 4 h or 250°C, 1 h).

! Empty columns and funnels are delivered **not endotoxin free**. In order to exclude any co-contamination with LPS insert them in at least 1 M NaOH over night (6-12 h). Afterwards wash them with endotoxin free water and let them air dry. For details please inquire our protocol "Procedure for packing gel into a column".

! Buffers should be prepared from endotoxin free materials and endotoxin free water.

! Buffers, resin and sample should have the same temperature (4-20°C) during the cleaning steps.

! When using EndoTrap columns, all buffers including equilibration buffer EB and regeneration buffer RB should be **degassed** prior to use. When using EndoTrap gel slurry, degas slurry prior to use – see FAQs for details.

! EndoTrap 10x buffers have to be 1:10 diluted with endotoxin free water.

! Avoid proteases and organic solvents.

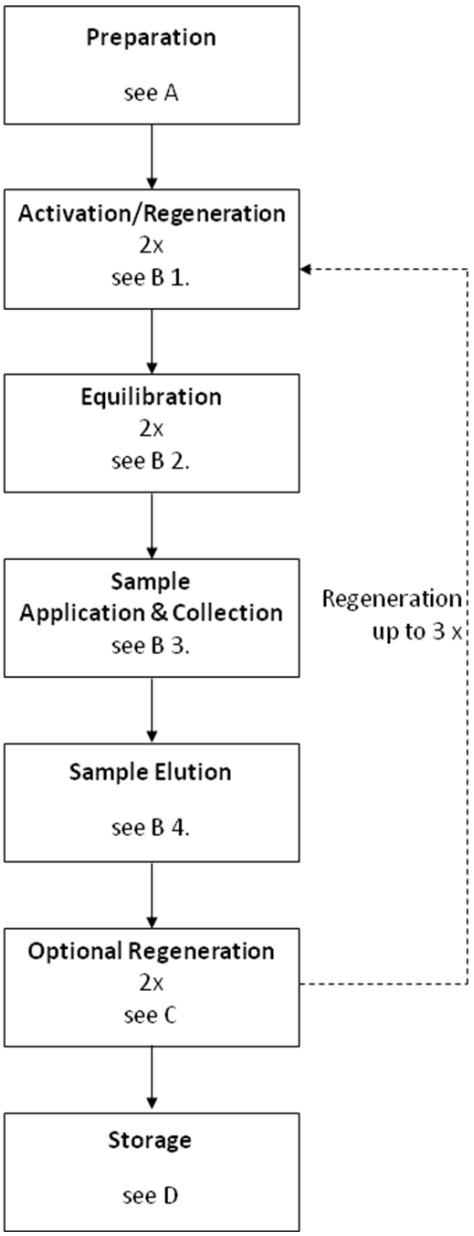
! **<u>If you use your customer specific buffers and EndoTrap blue:</u>**
Buffers used for endotoxin removal with EndoTrap blue need to contain **50-100 µM Ca^{2+}**.

Definition: **column volume** = used settled EndoTrap resin (e.g.: "ready-to-use" 1 ml column = 1 ml)
gel volume = used settled EndoTrap resin (after removing storage buffer from gel slurry)

Protocol - column mode

Preparation

- To use a **prepacked column** place the column in a suitable holder and remove the **top cap** first. This prevents air bubbles from being soaked up. Next remove **bottom cap**. Allow the storage solution to drain from the column. [~ 8 min] The flow stops automatically when the solution reaches the upper disc. Make sure to **never** let the EndoTrap resin run dry!



Activation and endotoxin removal

1. Fill up the column with **regeneration buffer** (RB)♦ [this corresponds to ~3 ml or **3 column volumes**] and let the column drain out completely. Repeat this step. [~12 min]
2. Fill up the column with **equilibration buffer** (EB)[1] or customer specific buffer [this corresponds to ~3 ml or **3 column volumes**] and let the column drain out completely. Repeat this step. [~12 min]
3. Apply your **sample** (either in **equilibration buffer** (EB) or in customer specific buffer) onto the column and start collecting your fractions (depending on the applied sample volume) immediately. Applied sample elutes directly after the column void volume (0.3-0.5 ml). The column can be constantly filled up, until the whole sample (up to 50 ml) is completely filled in. Afterwards let the sample drain completely from column. [flow rate: 0.2-1 ml/min]
4. In order to elute your entire sample, apply extra **equilibration buffer** (EB) or customer specific buffer (e.g. 1 ml or 1 column volume), let the column drain out and collect the flow through completely. [As substances pass through the column at different rates, it is important to test each fraction for the sample concentration. This can be done for example by measuring the optical density of the flow through fractions.]

Regeneration

- Fill up the column with **equilibration buffer** (EB) or customer specific buffer [this corresponds to 3 ml or **3 column volumes**] and let the column drain out completely. Repeat this step. [~ 12 min]
- Continue with **step 1** of "Activation and Endotoxin removal".

Storage

- If you want to store the column, close the column with the bottom cap and apply 1 ml or 1 column volume of **regeneration buffer** (RB) supplemented with **0.02% sodium azide** and store at 2-8°C (shelf life until the indicated expiry date).

  You can also use 20% ethanol as storage buffer; however, the storage time will then be only 4 weeks.

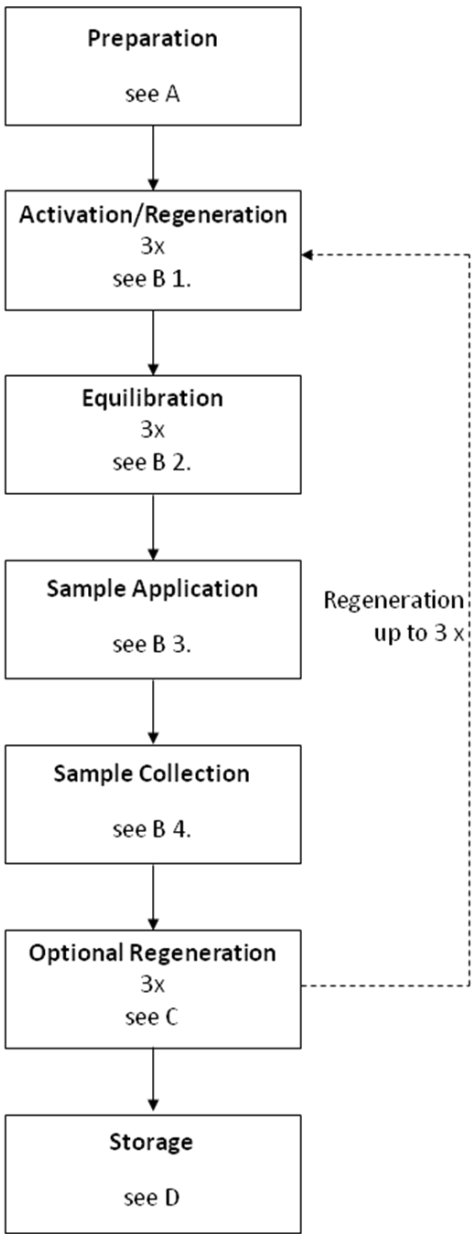
♦ The regeneration substance is NOT (sodium) deoxycholate! DOC would have cytotoxical effects on cell culture and also influence the cell growth and the morphology of the cells. It is reported that DOC induces DNA damage.

[1] **Equilibration buffer "blue" (EB):** 20 mM Hepes, 150 mM NaCl, **0.1 mM $CaCl_2$**, pH 7.5
Equilibration buffer "red" (EB): 10 mM Na_2HPO_4, **80 mM NaCl**, pH 7.4

Protocol - batch mode

Preparation

- All centrifugation steps should be carried out at ~ 1.200 x g for 5 min (bench top centrifuge)! Several contact times should be tested to determine the one best suited for endotoxin removal. **We recommend using a rate of 2:1 – 10:1 between sample and resin volume.**
- Remove storage buffer from gel slurry by centrifugation and discard the supernatant.



Activation and Endotoxin removal

1. Add 2 gel volumes of **regeneration buffer** (RB)♦ mix by gently shaking the tube for 5 sec; centrifuge, and discard the supernatant. Repeat this step twice.
2. Add 2 gel volumes of **equilibration buffer** (EB)[1], or customer specific buffer, mix by gently shaking the tube for 5 sec; centrifuge and discard the supernatant. Repeat this step twice.
3. Add the sample (either in **equilibration buffer** (EB) or in customer specific buffer) and incubate for ~ 30 min. Gently rock or rotate the tube while incubating.
4. Centrifuge at ~ 1.200 x g for 5 min (bench top centrifuge) and transfer the supernatant (= sample) to an endotoxin free tube.

Regeneration

- Resuspend the EndoTrap gel pellet in 2 gel volumes of **equilibration buffer** (EB) or customer specific buffer, mix by gently shaking the tube for 5 sec; centrifuge and discard the supernatant. Repeat this step twice. Continue with **step 1** of "Activation and Endotoxin removal".

Storage

- Resuspend the EndoTrap gel pellet in 1 gel volume of **regeneration buffer** (RB) supplemented with 0.02% sodium azide and store at 2-8°C (shelf life until the indicated expiry date). You can also use 20% ethanol as storage buffer; however, the storage time will then be only 4 weeks.

Optional steps (column / batch mode)

Endotoxin Detection (e.g. LAL assay from Cambrex):
- Check for endotoxin removal using an appropriate assay.
- If the LPS contamination is still too high, perform a second LPS removal step.

Optional polishing step:
- Combine the fractions and filtrate the solution over 0.2 µm membranes (e.g. Pall Supor® [PES, Polyethersulfone]) to ensure sterility.

Sample Recovery Rate:
- Measure the sample concentration with appropriate methods (e.g. BCA™ Protein Assay from Pierce or measure the absorption at 280nm)

[1] **Equilibration buffer "blue" (EB):** 20 mM Hepes, 150 mM NaCl, **0.1 mM $CaCl_2$**, pH 7.5
Equilibration buffer "red" (EB): 10 mM Na_2HPO_4, **80 mM NaCl**, pH 7.4

Literature

1. Case Gould, M.J. (1984) **Endotoxin in Vertebrate Cell Culture: Its Measurement and Significance. In Uses and Standardization of Vertebrate Cell Lines**. (Tissue Culture Association, Gaithersburg, MD), 125-136.
2. Copeland et al. **Acute Inflammatory Response to Endotoxin in Mice and Humans.** Clin. Diagn. Lab. Immunol..2005; 12: 60-67.
3. Greisman SE, Hornick RB. **Comparative pyrogenic reactivity of rabbit and man to bacterial endotoxin.** Proc Soc Exp Biol Med 1969;131:1154-8
4. Raetz, C.R. (1990) **Biochemistry of Endotoxins.** Annu. Rev. Biochem. 59:129-170.
5. Rietschel ET, Brade H: **Bacterial endotoxins.** Scientific American 267 (2): 54-61, 1992.
6. Roslansky, P.F., Dawson, M.E. and Novitsky, T.J. Plastics (1991). **Endotoxins and the Limulus Amebocyte Lysate Test.** J. Parental Sci. Tech. 45:83-87.

Background Information

Principle of EndoLISA® endotoxin detection

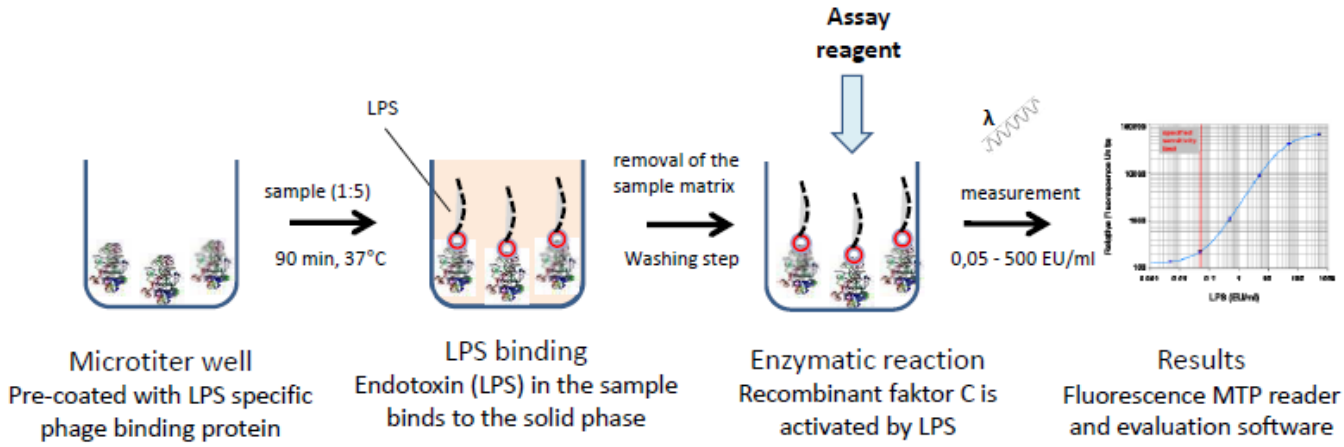
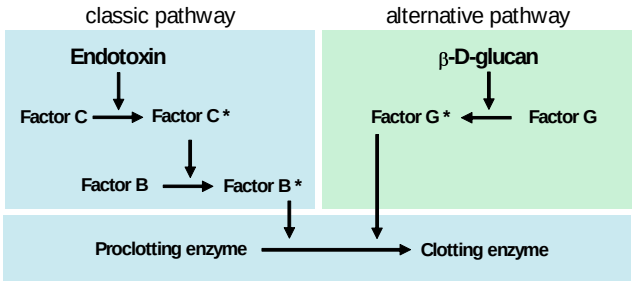


Fig. 5 **Principle of EndoLISA® test for endotoxin detection.** Endotoxin binds to the LPS specific phage binding protein of the pre-coated microtiter plate. Subsequently the sample matrix is removed by a washing step. The assay reagent contains recombinant Factor C (rFC) and a substrate. LPS binding activates the recombinant Factor C and the active form of the enzyme modifies the substrate and results in the generation of a fluorogenic compound. After addition of assay reagent to the samples, fluorescence detection is performed in a fluorescence reader. The endotoxin concentration of the samples is determined by standard curve analysis.

Principle of Limulus amebocyte lysate (LAL) test for endotoxin detection

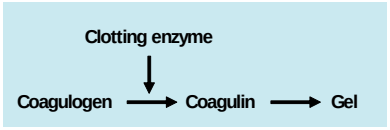
The protease cascade and rationale of traditional LAL assays are illustrated (**A**). Factor C, the first component in the cascade, is a protease zymogen that is activated by endotoxin binding. In this pathway, Factor B is activated by Factor C. An alternative pathway, the Factor G pathway, can be activated by ß-D-glucan binding. Downstream, Factor C and Factor G pathways individually activate a proclotting enzyme into a clotting enzyme. (**B**) The turbidimetric assay employs the native substrate, coagulogen, which can be cleaved into coagulin. Coagulin then begins to form a gel-clot, resulting in an increased turbidity. The chromogenic LAL test uses a synthetic chromogenic peptide substrate that can be cleaved by the clotting enzyme, resulting in a product that exhibits a yellow colour. The densities of the yellow colour (OD 405 nm) and turbidity (OD 340 nm) correlate with endotoxin concentration. (**C**) Specific inhibition of ß-D-glucan interference can be achieved by using recombinant Factor C (rFactor C) which acts to cleave a synthetic substrate after activation by endotoxins. Alternatively, certain compounds are used which specifically block activation of Factor G by ß-D-glucan.

A **Activation of coagulation cascade system in *Limulus* amebocytes**

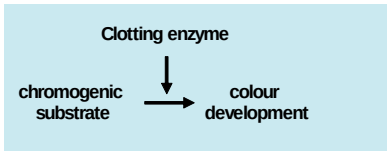


B **Endotoxin detection assays**

(kinetic) turbidimetric assay:

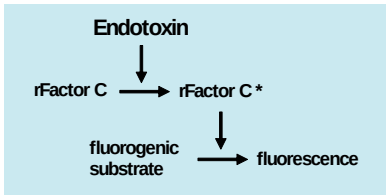


(kinetic) chromogenic assay:

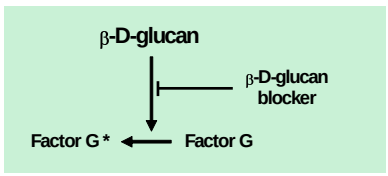


C **Inhibition of β-D-glucan interference**

recombinant Factor C:



blocking of β-D-glucan:



ß-Glucan Blocker blocks the glucan-sensitive G pathway in LAL, rendering the test more specific to endotoxin. Experiments have demonstrated the ability of **Factor C** to selectively recognize endotoxin and activate the protease cascade. To create an endotoxin-specific assay Factor C has been purified and cloned. When activated by endotoxin binding, recombinant Factor C acts upon the fluorogenic substrate in the assay mixture to produce a fluorescent signal in proportion to the endotoxin concentration in the sample.

EndoTrap® HD

For biomanufacturing process we recommend EndoTrap® HD. EndoTrap® HD has been especially optimized for application in biomanufacturing processes. It can be used in early or late biomanufacturing process steps. EndoTrap® HD is based on a hydrophilic, dimensionally stable affinity matrix with excellent pressure / flow characteristics. Also a Regulatory Support File can be provided. EndoTrap® HD is only available as slurry.

Workflow: EndoTrap® for endotoxin removal and subsequent endotoxin detection with EndoLISA®

As a complete workflow solution Hyglos recommends the combination of EndoTrap® red and EndoLISA®. The solid phase of EndoTrap® red differs from the solid phase of EndoLISA® and therefore this system is the perfect combination for a workflow including an endotoxin removal and an endotoxin detection step.

Hyglos Endotoxin Removal and Endotoxin Detection Service

Hyglos offers an endotoxin removal and also an endotoxin detection service.

For endotoxin detection we use a kinetic chromogenic LAL assay and an EndoLISA® endotoxin assay. Our classic **endotoxin detection service** includes:

- Preliminary screening (measurement of 3 sample dilutions, not necessary when the LPS concentration is known)
- Validation of standard curve
- Inhibition and enhancement tests of each sample
- Double testing of each sample
- Results within 2 weeks (with express service within 3 days!)

Use our expertise for your product: endotoxin removal service.

With this service best results were already achieved with even the most challenging products like tricky to handle antibodies and plant extracts.

Our **endotoxin removal service** includes:

- Removal of endotoxin with the adapted protocol
- Endotoxin detection before and after the removal steps as described above in the “endotoxin detection service”

Please inquire for our service.

For inquiries and technical support please contact:

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If you like to learn more about our products and services, please visit our website www.hyglos.de.
