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EndoLISA®: a novel and reliable method for endotoxin detection

A new test for the sensitive detection of endotoxin has been developed, based on a lipopolysaccharide-selective, precoated microplate and a factor C–based detection reagent and presented in a complete kit format. The selective capture of lipopolysaccharide (LPS) is achieved using a phage-derived receptor protein exhibiting high affinity and high specificity for the conserved core region of LPS. After binding of sample-LPS to the microplate as the first stage of the assay, the original sample matrix is washed off, thereby eliminating potentially interfering components. In the second stage of the assay, LPS is detected by factor C in a process whereby the principal receptor of the *Limulus* amoebocyte coagulation cascade reacts with a fluorescence substrate. The new endotoxin test EndoLISA has a detection range from 0.05 EU/ml up to 500 EU/ml.

Endotoxins are heat-stable breakdown products of Gram-negative bacteria. Chemically, endotoxin is LPS, the main constituent of the outer cell membrane[1]. LPS triggers severe physiological reactions, and therefore detection of endotoxin contamination is mandatory in pharmaceutical production and highly relevant in life science and medical research. For years, the *Limulus* amoebocyte lysate (LAL) assay was the only practical method for direct detection of endotoxin[2]. More recently, a similar assay based on recombinant factor C has been introduced[3], avoiding the use of blood from the endangered population of horseshoe crabs. However, both test formats suffer from their status as homogeneous assays that can be materially disturbed (inhibited or stimulated) by many possible constituents of the sample. To overcome this basic limitation, samples must be diluted in order to lower the concentrations of interfering components. For the same reason, spike controls are required in order to prove the validity of a test result. In the face of these drawbacks, the scientific community is constantly seeking improved methods for endotoxin detection[4].

Solid phase–based test format and its advantages

ELISA techniques are well established in biochemical analysis and diagnostics. They are sensitive, robust and reliable. Nevertheless, an ELISA-based method for endotoxin detection is not available on the market because no one has succeeded in developing antibodies with both broad specificity for LPS and sufficient affinity, owing to the fact that LPS is a very heterogeneous substance class.

We have selected a bacteriophage receptor protein targeting the conserved core region of LPS. As in a classical ELISA, the target analyte (LPS) is selectively bound to the surface of the precoated test plate, allowing the sample matrix to be eliminated through a washing step. Subsequently, the LPS is detected by its intrinsic capacity to activate the zymogen form of factor C, which finally generates a fluorescence signal by conversion of a substrate. Using this ELISA-like principle, EndoLISA exhibits clear advantages over the established homogeneous detection methods, including: fewer false-positive results induced by, for example, β-glucan, proteases or phospholipids, fewer false-negative results caused by inhibitory constituents of the sample,fewer invalid results necessitating re-testing, less interference in complex samples, and therefore higher sensitivity; and broad dynamic range.

Application data and discussion of results

EndoLISA has a measurement range of four orders of magnitude, from 0.05 EU/ml to 500 EU/ml (**Fig. 1**). This is possible because fluorescence is used as the detection mode and because the sigmoid standard curve can be approximated by a four-parameter curve-fitting algorithm. The specified sensitivity limit is 0.05 EU/ml.

It is important that any alternative endotoxin detection method has a good correlation with the established method (the gold-standard LAL assay). Therefore, we compared various types of LPS over a broad concentration range (**Fig. 2**). The results for different LPS species indicated that EndoLISA has a linear correlation with the LAL assay over the entire measurement range ($R^2 = 0.91$).

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APPLICATION NOTES

Table 1 | Highest tolerated concentrations of substances for valid LPS spike recovery, a comparison between EndoLISA and LAL assay.

	Substance	Solvent	*EndoLISA*	*LAL assay*
Buffer/pH	Acetate (pH 4.0)	100 mM NaCl	50 mM	12.5 mM
	Acetate (pH 5.0)	100 mM NaCl	100 mM[a]	12.5 mM
	MES (pH 6.0)	100 mM NaCl	100 mM[a]	5 mM
	Potassium phosphate (pH 7.2)	100 mM NaCl	100 mM[a]	50 mM
	Imidazole (pH 7.4)	Water	500 mM	40 mM
	HEPES (pH 7.5)	100 mM NaCl	100 mM[a]	100 mM[a]
	Sodium borate (pH 9.0)	100 mM NaCl	100 mM[a]	50 mM
Salt	NaCl	Water	1 M	0.5 M
	KCl	Water	1 M	0.25 M
Chaotropic agent	Urea	Water	6 M	0.5 M
	Guanidinium chloride	Water	1 M	0.05 M
Organic solvent	Methanol	Water	20%[a]	5%
	Ethanol	Water	30%	0.5%
	2-Propanol	Water	20%	0.2%
	DMSO	Water	10%	2%
Detergent	SDS	Water	0.05%	0.001%
	CTAB	Water	0.004%	0.0001%
	Zwittergent 3-14	Water	0.02%	0.005%
	Tween 20	Water	2%	0.1%
	Triton X-100	Water	0.02%	0.005%
Chelator	EDTA (pH 8.0)	Water	0.4 mM	0.4 mM
	Citrate (pH 7.5)	Water	10 mM	10 mM
Protease inhibitor	Benzamidine	Water	100 mM[a]	0.1 mM
	PMSF	2-Propanol	5 mM	<0.05 mM
Antibiotic	Rifampicin	Methanol	3.5 mg/ml	0.04 mg/ml
	Chloramphenicol	Ethanol	3.5 mg/ml	0.1 mg/ml

[a]Highest concentration tested.

The tested substances were dissolved in the respective solvents, and dilution series of the samples were prepared in water or 0.1 M NaCl and subsequently spiked with 5 EU/ml LPS (*Escherichia coli* O55:B5). EU/ml values and spike recovery (%) were calculated. The validity criterion of spike recovery was 50–200%. CTAB, cetyl trimethylammonium bromide; DMSO, dimethylsulfoxide; MES, methyl ethyl sulfonate; PMSF, phenylmethylsulfonylfluoride; SDS, sodium dodecyl sulphate.

To prove the robustness of EndoLISA, we tested various substance classes commonly used in biological buffer systems (**Table 1**). The results show that, compared to the LAL assay, EndoLISA is more permissive of high-salt conditions and also tolerates higher concentrations of chaotropic agents and organic solvents. Even in the presence of 6 M urea, the validity criterion was achieved. EndoLISA is functional in a pH range between pH 4 and pH 9 and at buffer concentrations of up to 100 mM. Moreover, detergents are known to affect the micelle structure of LPS and in doing so to modulate the activation of factor C[5]; very low concentrations of detergent enhance this activation, whereas higher concentrations cause inhibition. As shown, however, EndoLISA tolerates between 4 and 50 times higher detergent concentrations than the LAL assay.

Bivalent cations are essential for the functionality of both the LAL assay and EndoLISA. Therefore, chelating agents such as EDTA or citrate must be diluted below a certain concentration level or, in EndoLISA, can simply be neutralized by the addition of magnesium.

The above results indicate that in the EndoLISA test the negative influence of sample constituents is substantially smaller than for the LAL assay. Because of its solid phase–based test format, EndoLISA is more robust and can be applied to complex sample matrices without extensive dilution.

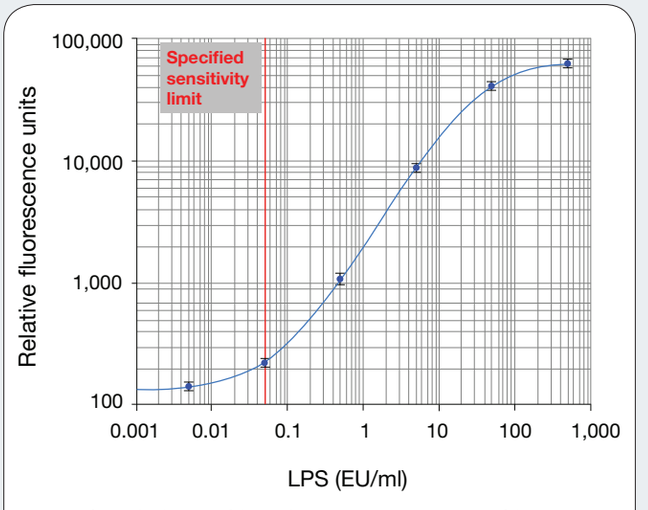

Figure 1 | Standard curve of the EndoLISA test: concentrations of the LPS standard are plotted against the relative fluorescence signal. EU, endotoxin unit.

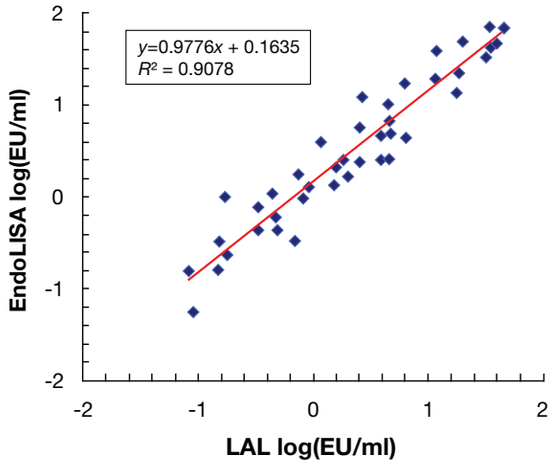

Figure 2 | Correlation plot of EndoLISA versus LAL assay. Multiple LPS preparations and dilutions from different sources, including LPS of mutant strains, were compared. The tested LPS samples were *E. coli* O55:B5, *E. coli* O111:B4 (phenol extract and EDTA extract), *E. coli* O128:B12, *E. coli* O45, *E. coli* K235, *E. coli* EH 100 (Ra mutant), *E. coli* J5 (Rc mutant), *E. coli* F583 (Re mutant), *Salmonella enterica* serotype Minnesota (wild type and Re mutant), *S. enterica* serotype Enteritidis, *S. enterica* serotype Abortus equi, *S. enterica* serotype Typhimurium, *Klebsiella pneumoniae*, *Serratia marcescens* and *Pseudomonas aeruginosa* serotype 10. Individual dilutions of the LPS samples were prepared in water. The coefficient of correlation (R^2) was determined by linear regression.

Perspectives

At present there are some areas in which endotoxin detection is not possible or requires cumbersome sample preparation methods. One such area is the detection of endotoxin in human body fluids, such as blood and serum or plasma[6]. One of our short-term goals is to evaluate EndoLISA for direct measurement in such clinical samples and also to provide protocols for diagnostic applications.

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