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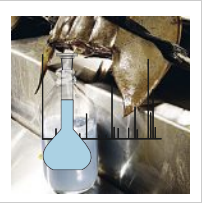

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A Novel Chemical Assay that Brings Endotoxin Analysis into the 21st Century

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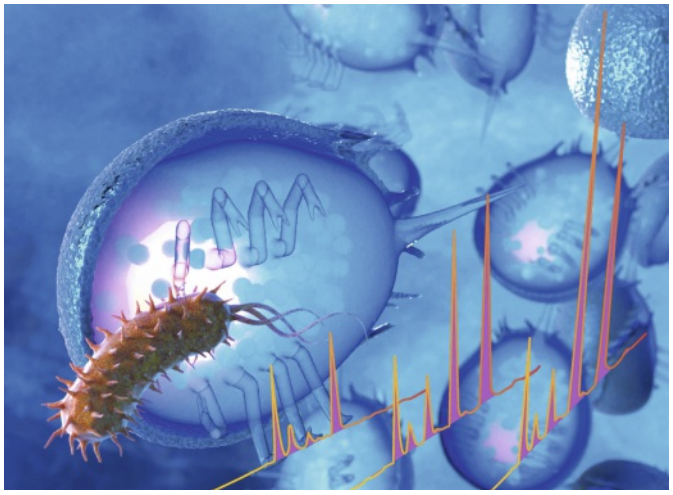
Endotoxins (ETs) cover, non-covalently bound, to about 75% the outer cell wall of all Gram-negative bacteria. These are ubiquitously found in our environment, *e.g.* in non-sterile pharmaceutical preparations and bioprocesses, nutrition, and at working places like butcheries or cotton fields. If ETs enter the human blood stream, they initiate at very low concentrations a strong immune response with potentially fatal outcome. In consequence, health authorities worldwide regulate strictly the maximal ET content in pharmaceutical preparations at very low levels, *e.g.* 0.25 EU (0.025 ng)/mL for water of injection.

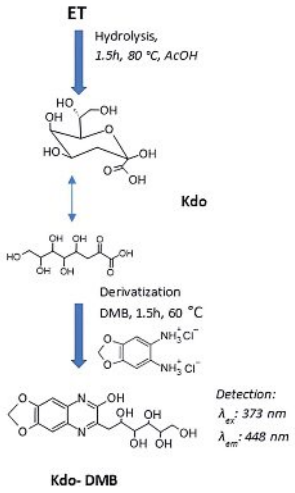
Today, ET testing is performed with biological assays; the gold standard is the 1977 FDA-accepted Limulus Amoebocyte Lysate (LAL) assay. However, for the widely used LAL test, the blood of horseshoe crabs (*Limulus polyphemus*) is needed, a marine species that is highly endangered. Those biological assays are very sensitive and specific to the complex, heterogeneous, and large molecular weight distribution of ET molecules. But they are expensive and show inherent poor reproducibility and recovery. Results are strongly dependent on sample handling and composition. That may lead to false negative test results as shown for certain ET-spiked monoclonal antibody (MAB) formulations, endangering patient safety.

In the framework of an ET-removal depth filter development project, our group established a reproducible, automatable, low-cost chemical ET assay. Every ET molecule contains one to three units of 3-deoxy-D-manno-oct-2-ulsonic acid (Kdo), which is almost only found in ETs. Kdo is quantitatively released by mild acidic hydrolysis. To reach sensitive detection, Kdo is derivatized with the fluorophore 1,2-diamino-4,5-methylenedioxybenzene-2 HCl (DMB) which recognizes specifically its α-keto-acid functionality. That circumvents matrix effects of, *e.g.*, neutral sugars from the ET molecule or proteins, present in complex bioreactor matrices. DMB increases Kdo's hydrophobicity, making it suitable for reversed-phase HPLC separation, *e.g.*, from sialic acids. The Kdo content is converted to ET content. LAL and Kdo-DMB-HPLC assay results show a strong correlation even in crude bioreactor ET samples.

The Kdo-DMB-HPLC assay opens the transition of endotoxin analytics to the 21st century.



Art picture of an *E. coli* bacteria cell in front of a horseshoe crab whose harvested blue blood is used to extract the enzymes needed to perform the conventional LAL assays. In the foreground on the right, an exemplary chromatogram is visible as it is obtained for Kdo-DMB separation from sialic acids in a Gram-negative bacterium bioreactor matrix.



Scheme of the Kdo-DMB-LC assay workflow. The first step is mild acidic hydrolysis of the ET molecule followed by derivatization of the released Kdo sugar acid with the fluorophore DMB. The mixture is separated by RP-(U)HPLC from matrix compounds, and Kdo-DMB is detected *via* fluorescence detection.

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