A Time-Saving Recombinant Factor C (rFC) Endotoxin Test Including a Novel Microplate Pre-Coated with Control Standard Endotoxin (CSE) Concentrations and Positive Product Controls

PETRA SCHNEIDER, MARTIN VOGL, THOMAS UHLIG, HOLGER GRALLERT AND GREGORY DEVULDER • e-mail: gregory.devulder@biomerieux.com • bioMérieux SA, France

PDA Annual Meeting 2018 • Orlando, FL

BACKGROUND

The vast majority of endotoxin tests for pharmaceuticals and medical devices as well as their in-process intermediates and raw materials are microplate-based. They require tedious reconstitution and dilution steps for preparation of Control Standard Endotoxin (CSE) dilutions and Positive Product Controls (PPCs). These time-consuming manual handling steps can result in substantial variability and a significant rate of invalid results that demand repeat testing.

To address these issues as well as to reduce the time for microplate preparation, we have developed a ready-to-use microplate - the GOPLATE™ - embedding required CSE amounts in dried format. Thus, the conventional standard dilution has become completely obsolete, in turn the GOPLATE significantly lowers the risk for human error and cost-intensive test repetition. The GOPLATE is included in a complete test kit, **ENDOZYME® II GO.**

The new microplate is the first of its kind, ready-to-use and enables more than 50% reduction in handling-time as well as consistent standard curve and PPC accuracy.

RECOMBINANT HORSESHOE CRAB FACTOR C (rFC)

Recombinant Horseshoe Crab Factor C (rFC) is an exact synthetic copy of the endotoxin-sensitive enzyme naturally harboured by the blood of horseshoe crabs. Compared with Limulus amebocyte lysate (LAL) methods, endotoxin detection assays based on rFC offer:

Specificity, flexibility, lot-to-lot consistency and, importantly: a sustainable, animal-saving and secure source.

rFC tests are available as fluorescence end-point assays in 96-well microplate format and validated in exactly the same way as conventional methods according to pharmacopoeial Bacterial Endotoxin Testing criteria. The US FDA included rFC assays in the Guidance for Industry in 2012 and, in 2016, the European Pharmacopoeia followed suit. Recently, the Japanese Pharmaceutical and Medical Device Agency published a collaborative study demonstrating equivalence between rFC and LAL assays.

ENDOZYME® II GO - featuring the GOPLATE™

The new ENDOZYME II GO is the evolution of ENDOZYME II, using the same reagents in a more efficient way. The key component, the GOPLATE, is pre-filled with CSE for the standard curve 0.005 - 50 EU/mL and PPCs 0.5 EU/mL, all in duplicate replicates (Fig. 2.).

ENDOZYME II GO - fast workflow in only 3 steps:

Add water and samples to the dedicated wells.

Prepare and add the assay reagent.

Run the assay in a fluorescence reader for e.g. 20 - 60 minutes depending on the desired sensitivity (0.05 - 0.005 EU/mL).

Fig. 1. ENDOZYME II GO kit components.

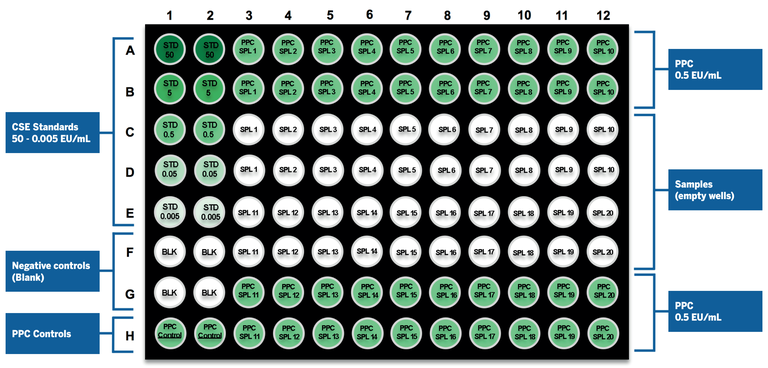

Fig. 1. Plate layout of the GOPLATE.

AIM OF STUDY & MATERIALS

The aim of this study was to compare the new rFC test ENDOZYME II GO with kinetic-chromogenic LAL tests in terms of inclusivity, interferences, precision, exclusivity and limit of quantification. To this end, three different lots of ENDOZYME II GO were tested as well as LAL kits from two different suppliers. Fluorescence and absorbance readers used in the study included a BioTek FLx800 and a BioTek Epoch2 coupled with Gen5 software (BioTek Instruments Inc, Winooski, VT).

RESULTS

INCLUSIVITY

Endotoxin levels of purified lipopolysaccharide samples from 14 different strains of Gram-negative bacteria and Reference Standard Endotoxin (RSE) were tested using the ENDOZYME II GO and LAL tests from two manufacturers in parallel. The rFC test correlated well to both LAL tests with correlation coefficients of 0.948 and 0.935, respectively (Fig. 3). This correlation was slightly higher than the correlation between the two LAL tests (r = 0.933).

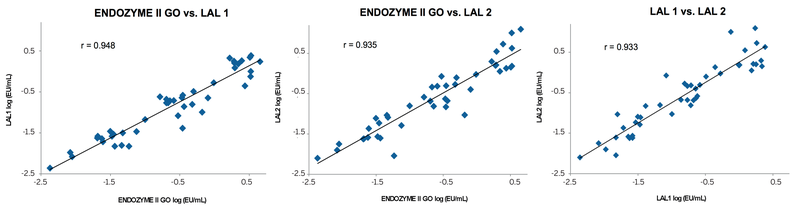

Fig. 3. Correlation of different endotoxin assays in response to 15 different LPS samples. Samples were measured in serial dilutions in single replicates (n = 44). Endotoxin values [EU/mL] were calculated from the respective standard curve and are plotted as logarithm. R values were calculated by Pearson correlation tests. A.) ENDOZYMEII GO vs. LAL1 B.) ENDOZYME II GO vs. LAL2 C.) LAL1 vs. LAL2

INTERFERENCE TESTING

For evaluation of interferences, different samples such as common excipients, proteins, organic solvent and culture medium were tested in duplicates without and with PPCs of 0.5 EU/mL. A PPC recovery of 50-200 % indicated a valid result. All three methods were comparable regarding tolerated concentrations of substances for valid PPCs. The rFC test yielded a higher rate of valid recoveries (Tab. 1.)

Category	Substance	Conc./ dilution	PPC recovery in ENDOZYME® II GO [%]	PPC recovery in LAL1 [%]	PPC recovery in LAL2 [%]
Common excipients	Sodium citrate	1 mM	112	161	92
		0.1 mM	106	161	118
	Dextrose	5%	80	134	92
		0.5%	95	165	108
	NaCl	500 mM	58	57	**1**
		50 mM	92	135	84
	Polysorbate 20	0.02%	77	61	50
		0.002%	101	69	**43**
	PBS	1x	82	96	**36**
		1:10	100	142	110
Proteins	MAB-33	0.1 mg/mL	117	**603**	**46**
		0.01 mg/mL	108	190	92
	HSA	1 mg/mL	94	**332**	138
		0.1 mg/mL	111	188	76
Organic solvent	Ethanol	1%	99	135	94
		0.1%	114	174	104
Culture medium	IMDM	100%	**35**	**214**	55
		10%	91	**262**	130

Tab. 1. Percentage of PPC recovery (0.5 EU/mL) in different samples in ENDOZYME® II GO compared to two LAL assays. Samples and spiked samples were tested in duplicate replicates (n = 2). Red font color highlights invalid PPC recovery.

PRECISION

Precision was assessed based on the coefficient of variation (CV) calculated on the back-calculated concentrations of the standard curves. The rFC test showed a higher intra-assay standard precision in comparison to the LAL tests due to accurately pre-loaded CSE (Tab. 2).

Nominal conc. [EU/mL]	ENDOZYME II GO CV(EU/mL) [%]	LAL1 CV(EU/mL) [%]	LAL2 CV(EU/mL) [%]
5	1.6	3.0	6.8
0.5	3.0	4.1	5.6
0.05	2.1	3.7	3.2
0.005	6.2	7.1	19.3

Tab. 2. Coefficient of variation (CV) of back-calculated CSE standard curve concentrations (EU/mL) determined in different endotoxin assays. Each standard (0.005–5 EU/mL) was measured in fourfold determination (n = 4).

EXCLUSIVITY

The endotoxin detection of ENDOZYME II GO occurs with endotoxin-specific rFC. As expected, zymosan, a 1,3-beta-glucan, only produced a false-positive signal with the LAL tests, but not with the rFC assay (Tab. 3).

	ENDOZYME II GO		LAL1		LAL2	
Conc. zymosan [mg/mL]	Mean EU/mL	PPC recovery [%]	Mean EU/mL	PPC recovery [%]	Mean EU/mL	PPC recovery [%]
0.01	< 0.005	98	6.6	**2250**	2.8	**484**
0.001	< 0.005	96	0.27	**479**	0.43	**286**

Tab. 3. False-positive signals and recovery of spiked endotoxin (PPC = 0.5 EU/mL). Samples were tested in duplicates (n = 2).

LIMIT OF QUANTIFICATION

The limit of quantification was evaluated using three different reagent lots of ENDOZYME II GO and correlated to time to result. After 15 minutes, 0.05 EU/mL were detected and within 55 minutes 0.005 EU/mL (Fig. 4.).

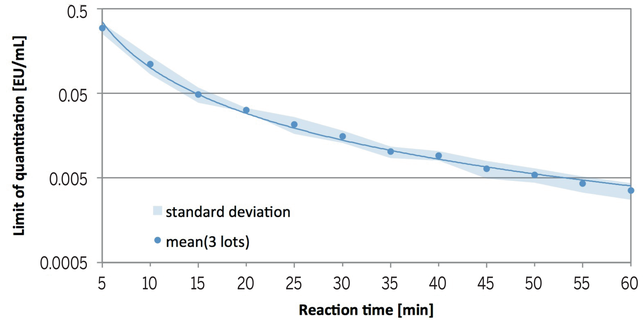

Fig. 4. Correlation between the reaction time and the limit of quantification, here the sum of the mean of the blank and 10 times its standard deviation, converted into EU/mL based on the standards above this sum. Three reagent lots were used three times, each, with 12 blanks and 2 standards of each endotoxin concentration (0.005 –50 EU/mL). The depicted standard deviation was calculated for all lots (n = 3), based on the mean limit of quantification of each lot.

CONCLUSIONS

The new method based on rFC - ENDOZYME II GO - was shown to perform equivalent or better than the tested LAL tests, with:

- **Higher rate of valid results in various samples**
- **Higher intra-assay precision**
- **No false-positive results from β-Glucans**

From a workflow point of view, our new method requires **half of the handling time** in comparison to conventional microplate-based endotoxin tests.

The GOPLATE **helps reduce the risk of human error** and invalid results by eliminating the manual preparation of standard dilutions and PPCs.

Hands-on time reduction combined with shorter time to result, e.g. for water testing, provides a **high-throughput solution.** Furthermore, the simplified workflow facilitates automation.