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Development of endotoxin specific LAL reagent and modifications of EIA Quant^{TM} microplate reader for BET testing

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Abstract
The main aim of this study is to utilize the chromogenic substrate BOC-Val-Leu-Gly-Agr-pNA, the commercially available LAL reagent was altered to develop a kinetic chromogenic LAL reagent. With the help of Zwittergent 3-14, an LAL reconstitution buffer sensitive to endotoxins was produced. A commercially available EIA QuantTM ELISA microplate reader was customized to carry out kinetic measurements to determine the time taken to attain the optical density of the samples. Plotting a standard line as the log of the time required to reach the optical density (seconds) against the log of the endotoxin concentration (EU/mL), it was found that the least two dilution out of 5 serial dilutions were not detectable in the modified microplate reader.

Keywords: endotoxin, Limulus amebocyte lysate, chromogenic substrate and Absorbance microplate reader

Introduction
At an anticipated CAGR of 11.5 %, the size of the worldwide pyrogen testing market is expected to increase from USD 767.1 million in 2020 to USD 1843.4 million by 2028. Testing for bacterial endotoxins will be a crucial aspect of quality control to guarantee patient security. The bacterial endotoxin can be lethal if it enters the patient circulation in high enough amounts to induce dangerous symptoms including fever and septic shock. Because of this, it is critical to check the endotoxin concentration of medications and medical equipment before they are used or implanted. In essence, the Limulus Amebocyte Lysate (LAL) assay test verifies the safety of sterile pharmaceutical and medical items for human use. The LAL assay test is the most used method for endotoxin testing. Various methods, including as gel clot, kinetic chromogenic, and kinetic turbidimetric tests, can be used to achieve this. The LAL test is frequently regarded as specific for LPS and is highly sensitive to endotoxin. However, it has been demonstrated that some specific glucose polymers are LAL reactive when present in sufficient amounts (ng to mg per mL). Among these glucans, β- (1,3)-D-glucans, although non pyrogenic in nature, can readily react with the G factor present in the amebocyte lysate. Roslansky *et.al.* reported that Zwittergent 3-14 can inhibit the reaction of amebocyte lysate with glucans and increase the sensitivity of amebocyte lysate towards endotoxin.

The chromogenic process relies on the appearance of color following the dissolution of a synthetic peptide chromogen complex. The quantitative link between endotoxin concentration and chromophore release at the conclusion of an incubation time, which is the foundation of the end point chromogenic test technique. The kinetic chromogenic assay is a technique for determining the amount of time required to get the reaction mixture's absorbance to a specific level or the pace at which color develops. Endotoxin-containing samples react with Limulus Amebocyte Lysate (LAL) by activating its coagulating enzyme, which then interacts with an inert synthetic substrate. This is made up of a short peptide connected to the p-nitroaniline (pNA), a chromophore molecule at its c-terminus. The coagulating enzyme triggers the release of the yellow pNA molecule once the cascade enzymatic process is triggered. The amount of endotoxins present in samples is directly correlated with the color development.

To measure the levels of endotoxin in samples, a microplate reader is used to measure the time it takes to reach absorbance. For using kinetic chromogenic LAL reagnets, a microplate reader which can do kinetic measurements is required. Thus, Meril's commercially available Merilyzer EIA QuantTM Microplate reader has been modified for the testing of the modified kinetic chromogenic LAL reagents. We hereby report the development of an endotoxin- specific kinetic chromogenic LAL reagent by modifying a commercially available LAL reagent. In addition, we modified the Meril's Merilyzer EIA QuantTM Microplate reader to identify the time required to reach the absorbance of the sample.

Materials and Methods

All glassware was depyrogenated using hot air oven for a minimum of 3 hr at 180°C before using in the test proceedings. LAL reagent water (LRW) was prepared using double distilled water and ultrafiltration and was confirmed to have less than 0.001 endotoxin unit (EU)/ml by the LAL test.

- **Chromogenic LAL Reagent preparation:** Chromogenic substrate (BOC-Val-Leu-Gly- Agr–pNA) was dissolved in LRW to a final concentration of 2.2 µmol/ml. The final solution was filtered through 0.22 µm filter. The lyophilised LAL reagent (Dynamiker Biotechnology, China) was dissolved in 2.6 ml of chromogenic substrate solution and vortexed for 10 minutes. The resultant solution was lyophilised and stored at 2 to 8°C until used.

- **Endotoxin Specific LAL Reconstitution Buffer:** 0.05 M Tris/MgCl2/NaCl buffer was prepared by adding 2.00 g magnesium chloride hexahydrate, 4.50 g sodium chloride and 6.06 g Tris buffer (powder form) in a 1000 ml LRW maintain a pH range of 7.0-7.5. The buffer was sterilized using autoclave and stored at 0-4°C till further use. In a vial, 2.6 ml 0.05 M Tris/MgCl2/NaCl buffer was taken and 0.16 mg of Zwittergent 3- 14 was added under Laminar Air Flow (LAF) and mixed well using vortex. The resultant solution was depyrogenated using ultrafiltration and stored at 0-4°C till further use.

- **Controlled Standard Endotoxin (CSE):** CSE *E.coli* 055:B5 (1.0 EU/mL) was purchased from Dynamiker Biotechnology, China.

- **Microplates:** Flat-bottom, polystyrene microplates were used (Dynamiker Biotechnology, China).

Microplate Absorbance Reader

The commercially available Merilyzer EIA QuantTM ELISA Microplate Reader (Meril Diagnostics Pvt. Ltd.) was modified for use with chromogenic LAL reagents. A heating block was added above the microplate area in the reader to provide incubation from both the top and bottom as the commercial unit comes with bottom heating block only. As a result, this addition will provide uniform incubation and help improve the accuracy of your results.

Fig 1: Merilyzer EIA Quant^{TM} Microplate reader

Sample Testing with endotoxin specific LAL reagent

In reusable depyrogenated test tubes, endotoxin was serially diluted in the range of 1.00 to 0.01 EU/ml. In microplate wells, 0.1 ml samples of each dilution were dispensed. Chromogenic LAL was reconstituted with LRW and vortexed for 60 seconds to uniformly disperse the suspension. The microplate was filled with 0.1 ml of Chromogenic LAL. The plates were placed in a modified Merilyzer EIA QuantTM Microplate reader, and the reaction was allowed to run for 60 minutes. The time at which the color reached an optical density was calculated by the computer. To calculate the endotoxin concentrations of the unknowns, a standard line was plotted as the log of the time taken (seconds) versus the log of the endotoxin concentration (EU/mL).

Result and Discussion

A modified kinetic chromogenic LAL reagent was developed by adding a chromogenic substrate (BOC-Val-Leu-Gly-Agr–pNA) to a commercially available LAL reagent and the sensitivity of the lysate to react with endotoxin was increased by adding Zwittergent 3-14 in the reconstitution buffer made using LRW. The samples were run in the modified Merilyzer EIA QuantTM Microplate reader and the reaction was allowed to run for 60 minutes. Even though the LAL test has been made more sensitive, faster, and accurate, there are still some improvements that need to be made.

The standard line (Fig. 2) was obtained by plotting the log of endotoxin concentration versus log of time taken. This standard line was run in duplicate and had a r value of 0.9988. The linear regression curve was found with temperatures ranging from 36.1-36.9 oC. During the procedure, the last two endotoxin dilutions were not detected. Table 1 shows the reaction time for different endotoxin concentrations.

Table 1: Variable Endotoxin concentration and its reaction time

EU Concentration	Mean Reaction Time	CV%	Calculated Value
1 EU/mL	1365.67	1.27	0.98028
0.5EU/mL	1701.16	2.5	0.52032
0.25 EU/mL	2208.75	2.66	0.24507
0.05 EU/mL	>3600	0	0.19289
0.01 EU/mL	>3600	0	0.19289

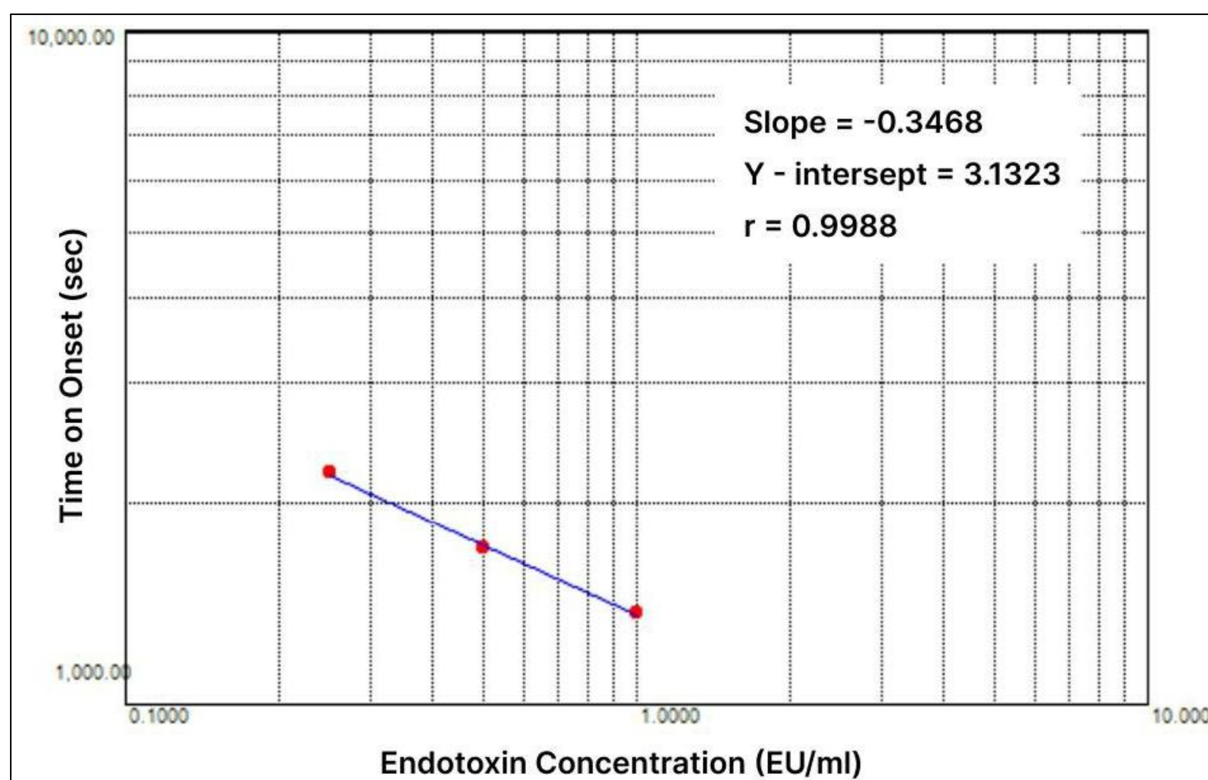

Fig 2: log of the time taken (seconds) versus the log of the endotoxin concentration (EU/mL).

The standard line equations in two experiments conducted on different days were remarkably consistent. The slopes ranged from -0.3468 to -0.3520, the y intercepts from 3.1695 to 3.1323, and the r values were all 0.99 or higher (data not shown).

Conclusion

After making changes to a commercially available LAL reagent by adding chromogenic substrate and increasing the sensitivity of the amebocyte lysate reaction with endotoxin using Zwittergent 3-14, the modified reagent was only able to produce results for 3 out of 5 control dilutions. More work is needed to be done in order to perfect the modified LAL reagent and the Merilyzer EIA QuantTM ELISA Microplate Reader. The main concern right now is that the reagent isn't working as accurately as it should, so more investigation needs to be done to make it more effective. Furthermore, the microplate reader requires improvement; currently, it is not picking up on all of the data from the reagent tests. To optimize both the reagent and the microplate reader, further studies has to be performed.

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