Sensitive and flexible bacterial endotoxin testing with the EndoZyme® II Recombinant Factor C Assay using the Synergy™ HTX Microplate Reader

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EndoZyme® II is a bacterial endotoxin test for pharmaceuticals and medical devices as well as their in-process intermediates and raw materials. The assay relies on recombinant horseshoe crab Factor C (rFC), an exact synthetic copy of the endotoxin-sensitive enzyme naturally harboured by the blood of horseshoe crabs. Compared with methods sourced from these animals, rFC methods offer better specificity, flexibility, lot-to-lot consistency and, importantly: a sustainable and secure source. This article covers how the EndoZyme II assay is established, optimised and validated using the Synergy™ HTX Multi-Mode Microplate Reader with Gen5™ software.

Introduction

Endotoxin consists of lipopolysaccharides (LPS) that are ubiquitous among Gram-negative bacteria. Hence, endotoxin can be found in any place inhabited by these microbes, for instance contaminated water systems. Since endotoxin is a pyrogen, i.e. a fever-inducing agent, it may cause severe immune reactions upon contact with the systemic circulation of a patient. Therefore, injectable pharmaceuticals and implantable medical devices must be tested for endotoxin to guarantee patient safety. To this end, bacterial endotoxin tests (BET), such as the recombinant Factor C (rFC) assay EndoZyme II, are used. rFC is an exact synthetic copy of the endotoxin-detecting enzyme harboured by the blood cells, specifically amebocytes, of horseshoe crabs. In EndoZyme II, rFC is activated by endotoxin and in turn processes a fluorogenic substrate. The more endotoxin rFC detects, the stronger the increase in fluorescence. This signal development is measured by a fluorescence microplate reader such as the Synergy™ HTX from BioTek Instruments, Inc.
Classical bacterial endotoxin tests (BETs) like the Limulus Amebocyte Lysate (LAL) test still rely on sourcing reagents from the blood of horseshoe crabs that are currently considered as vulnerable and their population is in decline [1]. LAL quality may vary across individual animals and seasons. Furthermore, they show false-positive reactions in the presence of beta-glucans. In contrast to the classical BETs, EndoZyme II is entirely sustainable, more consistent across lots (Figure 2 B) and specific to endotoxin, but nevertheless equivalent or better in terms of sensitivity and the determination of endotoxin concentration.

**Assay handling and data analysis**

For performing EndoZyme II, initially, all kit components are brought to room temperature. The supplied control standard endotoxin (CSE) is reconstituted in a volume of water as labelled on the respective bottle and the Certificate of Analysis. Serial dilutions of the standard and, if necessary, the samples are prepared. 100 µL of each standard dilution, the negative control and the samples are transferred onto a microplate, at least in duplicate. Defined amounts of the CSE are spiked into aliquots of the samples as positive product controls. The plate is heated to 37°C in the Synergy HTX. Subsequently, 100 µL assay reagent, consisting of previously mixed eight parts Assay Buffer, one part Substrate and one part rFC-Enzyme, are added to the filled wells of the microplate. The Synergy™ HTX accommodates the microplate, shakes it for 15 seconds, incubates it at 37±0.5°C and measures fluorescence intensity with excitation at 380/20 nm and emission at 440/40 nm in relative fluorescence units (RFU) at several time points. For all filled wells, the difference in RFU between the time point zero and 60 minutes is calculated (dRFU, Figure 1A and B).

Furthermore, the blank dRFU are subtracted from all other dRFU (net dRFU) to adjust the instrument-specific baseline (Figure 1C). For calculating the endotoxin activity of standards, samples and positive controls in endotoxin units per millilitre (EU/mL), a linear equation is fit to the logarithm of each net dRFU plotted against the logarithm of the EU/mL of each standard point in a standard series (Figure 2A). This is the standard curve which must demonstrate a correlation coefficient (r) of ≥ 0.980 as defined by the internationally harmonized Pharmacopoeia chapters on the bacterial endotoxin test [2–4]. Per the same regulation, the positive product control (PPC) must show an endotoxin spike recovery of 50 – 200 % of the expected concentration. Generally, EndoZyme II’s linearity is very high up to 5 EU/mL (r ≥ 0.998), in turn guaranteeing excellent accuracy.

![Figure 1](image1.png)  
**Figure 1** | EndoZyme II reaction kinetics. A The fluorescence intensity of three replicates of 0.005 EU/mL with EndoZyme II reagents was measured over time and quantified in relative fluorescence units (RFU). B The difference in RFU from time point zero (dRFU) was calculated for each of the same replicates of 0.005 EU/mL and each measurement time point, respectively. C The average dRFU of blank (water) and 0.005 EU/mL was calculated for each time point.

![Figure 2](image2.png)  
**Figure 2** | EndoZyme II standard curves. A The standard curve of EndoZyme II lot 18434 demonstrates the linear correlation of endotoxin concentration and fluorescence intensity increase in net dRFU. Error bars denoting the standard deviation of each standard. B Standard curves from different EndoZyme II lots are compared.
**Instrument and software set-up**

Setting up the Synergy™ HTX is simple and fast. It only requires connecting the device to a Windows PC via USB, installing the co-delivered analysis software Gen5™ and choosing the instrument from a list in the software. Gen5™ Secure is a 21 CFR part 11-compliant version for regulated environments of the pharmaceutical and medical device industry.

For qualifying the instrument, we recommend directly running EndoZyme II assays. The readout of fluorescence intensity is relative and depends on the sensitivity of the light detector, the so-called gain. Ideally, the gain is adjusted to yield a dRFU of 5000 for an endotoxin concentration of 0.5 EU/mL. A serial dilution of EndoZyme II’s control standard endotoxin, applied in triplicate, should show good linearity, i.e. r of minimum 0.980 (see above). Meanwhile, a single dilution of the standard, e.g. 0.5 EU/mL, should demonstrate appropriate uniformity across the entire plate, i.e. a dRFU coefficient of variation (CV) of maximum 25% for a total 96 replicates.

bioMérieux provides Gen5™ templates, so-called protocols, for the above-mentioned qualification trials as well as for routine endotoxin testing. Users only need to enter trial-specific information such as the reagent lot number, the list of sample identities and their respective dilution factors. Each protocol specifies all measurement parameters for the Synergy™ HTX, extracts the RFU and processes them. Finally, it returns all relevant endotoxin testing data in customizable reports that can be printed, signed and filed. The Synergy™ HTX can also contain a module that measures light absorbance at flexible wavelengths, a so-called monochromator. Thus, users who intend to gradually switch to EndoZyme II may also use it for any classical BET or other absorbance-based assays.

**Assay optimisation**

For BETs, the quantitation limit is commonly termed sensitivity. In classical BETs, the sensitivity is limited by the background activity of the reagents, i.e. reaction in the absence of endotoxin. EndoZyme II, however, does not display such background activity. Hence, the sensitivity mostly depends on the measurement background of the instrument and the assay reaction time. Indeed, the standard protocol of EndoZyme II exhibits a state-of-the-art sensitivity of 0.005 EU/mL after 60 minutes reaction time. Non-interfering samples such as water, do not have to be tested at overly sensitive levels. For instance, water for injection has an endotoxin limit of 0.25 EU/mL. After a reaction time of only 15 min, EndoZyme II clearly resolves 0.05 EU/mL (Figure 3A), sufficient for such a sample. On the other hand, after 120 minutes, the assay can reliably resolve as little as 0.001 EU/mL. Such a high sensitivity is particularly important for medical devices. The higher the sensitivity, the higher the volume of water used for rinsing medical devices before testing the water for endotoxin. Thus, bacterial endotoxin testing can cover larger devices or more
devices within a single water pool. Furthermore, pharmaceuticals exhibiting strong test interference can be tested in higher and in turn less interfering dilutions.

In fact, the sensitivity of EndoZyme II does not only depend on the reagents, but also on the instrument, here the Synergy HTX. Increasing the gain (Figure 3B) and the number measurements per data point (Figure 3C) can also enhance the sensitivity. In fact, different sets of light filters can be used for fluorescence excitation and emission reading, but 380/20 and 440/40 nm represent the respective optima (Figure 3D).

When EndoZyme II is validated for testing further products, a test for interfering factors with several product dilutions can be used to establish, if necessary, the dilution which allows for 50 - 200 % spike recovery. Using this dilution or non-interfering undiluted product, accuracy, precision, quantitation limit, linearity and range can be demonstrated by a single method suitability test.

bioMérieux provides validation protocols to capture user testing results for both initial validation and method suitability, thus reducing the documentation effort for EndoZyme II users.

Figure 4 | Influence of different hold times of samples and reagents on EndoZyme II accuracy. EndoZyme II assay reagent was incubated for different hold times and added to 0.15 EU/mL, which was also incubated for different hold times in the assay plate. Each combination of assay reagent and endotoxin sample hold time was tested in triplicate. A standard curve from 0.005 to 5 EU/mL with 10 min hold time of both assay reagent and endotoxin was used for endotoxin concentration quantitation.

Conclusion

EndoZyme II is a sensitive, flexible and sustainable bacterial endotoxin test with an unprecedented lot-to-lot consistency that is ideally used with the Synergy HTX. The assay can be optimised for the user’s specific needs. Finally, the validation is straightforward and concluded within a few days.

Method validation

For establishing bacterial endotoxin testing using EndoZyme II, a recognized alternative to the LAL test, the assay needs to be validated as a quantitative analytical procedure [5,6,7] following exactly the same methodology as classical BETs as per global Pharmacopoeias [2-4]. This encompasses the demonstration of suitable accuracy, precision, specificity, quantitation limit, linearity, range and robustness. Accuracy, precision, linearity and range can be evaluated by a single set up that is repeated four times. Analysts, days and reagent lots are cross-varied for this purpose. The quantitation limit only needs to be demonstrated once for two reagent lots each. Robustness has sufficiently been tested by bioMérieux. Therefore, the user does not need to provide respective data. Specificity has already comprehensively been shown to be equivalent between classical and recombinant BETs such as EndoZyme II by the Japanese Pharmaceuticals and Medical Devices Agency (PMDA). Their multi-centre ring study [8] featured three assays per category and a total 29 endotoxin samples from diverse bacterial strains and of different extent of purification. Thus, the user only needs to show that endotoxin can be quantified in their test samples with acceptable accuracy and precision.
References


