Creation of an In-house Naturally Ocurring Endotoxin Preparation for Use in Endotoxin Spiking Studies and LAL Sample Hold Time Analysis

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Abstract

Detection of endotoxin that may be present in biopharmaceutical products is critical to patient safety. Although the endotoxin molecule itself is highly stable, various factors such as matrix composition, storage temperature and container makeup can affect its stability in manufacturing samples that are collected and subsequently assayed for endotoxin content. Pfizer in Andover, MA developed a procedure for creating an in-house naturally occurring endotoxin (NOE) preparation to assess the stability of endotoxins across various matrices, temperatures and containers. The use of NOE provides benefits over using commercially available Control Standard Endotoxin (CSE), such as increased laboratory flexibility and control, and has been used by Pfizer to assess endotoxin stability over time in various matrices and temperatures.

Introduction

Endotoxin is a lipopolysaccharide structure located in the cell wall of Gram-negative bacteria. Since endotoxins belong to a group of fever-causing substances called pyrogens, parenteral drug products that may contain endotoxin can elicit a pyrogenic reaction in patients. The FDA has established a pyrogenic threshold of 5-endotoxin units/kilogram (EU/kg) of body weight. Endotoxin exposure beyond this level may induce fever, shock, and death. It is critical that endotoxin levels are monitored and controlled in biomanufacturing processes and products for reasons of safety and compliance. The *Limulus* Amebocyte Lysate (LAL) assay is often used to measure the level of endotoxins in biological products. Testing occurs at a variety of points throughout the purification process, as well as in bulk drug substance and finished drug product. Effective endotoxin testing is critical to the safety of a product, and as such it is crucial that any endotoxins that may be present throughout the manufacture and release of a product be accurately detected.

Although it is generally accepted that the endotoxin molecule itself is highly stable, various matrices, temperatures and/or storage containers may affect the ability of the *Limulus* Amebocyte Lysate (LAL) assay to detect the presence of endotoxin. At Pfizer Specialty Care in Andover, MA, a variety of studies were initiated to study these effects. In order to measure the effect of time and/or temperature on the recovery of endotoxins, it was necessary to add endotoxins into the starting biopharmaceutical samples. Since there were multiple matrices and temperatures being assessed, a large volume of endotoxin was required to complete these studies.

Control Standard Endotoxin (CSE) is a type of endotoxin that is commercially available. CSE is used to prepare standard curves and positive product controls (PPCs) for the LAL assay. CSE is prepared by licensed LAL vendors as a reagent from a purified *E. coli* strain and contains fillers in the formula. As such, CSE is not representative of endotoxin that may be present during an actual contamination event. Also, CSE is generally not available in high EU/mL formulations. Naturally occurring endotoxin (NOE) is produced by Gram-negative bacteria and is filtered but not further processed, making it representative of a contamination event. Additionally, since it can be grown to very high EU/mL concentrations, it is suitable for use in spiking studies.

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Selection of Organisms

A variety of Gram-negative organisms were screened for production of NOE in order to determine potential candidates for use in generating an NOE preparation. All NOE solutions were made by preparing a lawn culture on Tryptic Soy Agar (TSA) and incubating at 32°C for 24-48 hours. Colonies were picked from the plate, inoculated into Tryptic Soy Broth (TSB) and incubated at 32°C in a shaking incubator overnight. The resulting culture was centrifuged, filtered through a 0.45 µm filter and tested for endotoxin using the kinetic chromogenic LAL assay. Table 1-1 indicates the resulting EU/mL for each organism assessed.

Previous NOE spiking studies at the Pfizer Andover, MA site using *Escherichia coli* and *Serratia marcescens* preparations were useful since they produced high EU/mL stock solutions that remained stable over long periods of time. Organisms that produce high concentrations of endotoxin are particularly beneficial for use in spiking studies as these preparations allow for flexibility of testing both low and high spiking concentrations.

Because the preparations produce endotoxin concentrations that are significantly higher than levels seen during routine use, the concentration of laboratory prepared NOE stocks must be confirmed prior to each use in order to prepare an accurate spiking solution.

LAL Validation vs. LAL Spiking Studies

When LAL assays are validated for routine use during product manufacture and release, endotoxin in the form of CSE is spiked into already diluted sample as the PPC in order to evaluate matrix inhibition/enhancement. Validation activities focus on the interfering factors test, which assesses whether the sample matrix may be interfering with endotoxin detection by determining the PPC spike recovery (i.e., the amount of endotoxin spiked into the PPC must be recovered at 50-200%). A sample dilution is selected where the spike recovery falls within the acceptable PPC spike recovery range and also aligns with the parameters set in USP Chapter <85>.

LAL validation studies are typically designed to consider the effect of the sample matrix after the sample has been diluted to a level whereby interference is no longer seen in the assay, as determined by the recovery of the PPC spike into the diluted sample. In this case, the endotoxin in the PPC only interacts with the sample after dilution, not at the onset.

To determine the effect the sample matrix has on endotoxin, the endotoxin must interact with the undiluted sample matrix prior to performing the routine LAL assay. Using NOE to initially spike undiluted sample better represents a contamination event, since the source of such a contamination would most likely be from bacterial sources and not from a processed endotoxin source such as CSE.

Table 1-1: Organisms Assessed for NOE production

Organism	EU/mL
Klebsiella pneumoniae	36,789
Salmonella typhimurium	15,062
Moraxella osloensis	12,934
Proteus mirablis	26,779
Stenotrophomonas maltophilia	35,564
Sphingomonas paucimobilis	<50
Escherichia coli	28,075
Serratia marcescens	56,830
Acinetobacter genomospecies	4,640

The main difference between the role of the PPC spike and NOE spike is that the PPC spike provides information as to whether the sample matrix is interfering with the detection measures of the assay itself, while the NOE spike provides information on the direct interaction of the sample matrix with endotoxin. Figure 1 illustrates the difference between routine testing and NOE spiking studies using the kinetic LAL assay.

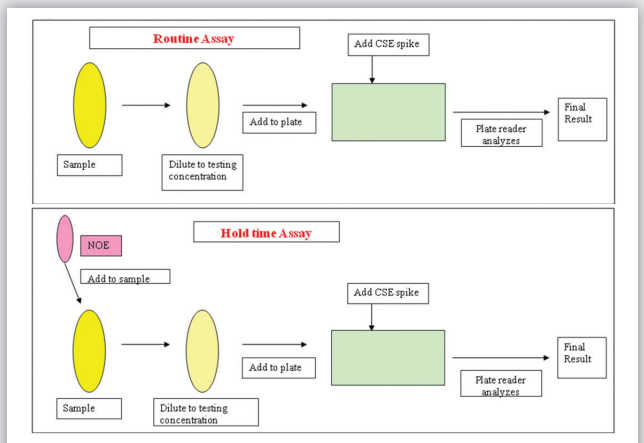

Figure 1. Routine LAL Assay vs. NOE Spiking Study

Design of Hold Time Studies

In many cases, LAL samples are delivered to the testing laboratory and held for some period of time prior to initiating testing. Studies should be performed to evaluate the impact of this hold time on the recovery of endotoxins in a particular sample type. NOE is useful for these studies since high EU/mL stocks can be easily prepared and stored by the laboratory, making it relatively simple to create spiked samples at various endotoxin concentrations

Hold time studies are performed by spiking NOE into undiluted samples prior to performing the LAL assay. The spiked samples are assayed immediately to determine the starting concentration, and aliquots are stored and assayed at set time points. By spiking NOE into undiluted sample, the effect of the product and the matrix on any endotoxin that may be present may be determined, both at the initial time point and over the storage time and temperature for the sample. The kinetic LAL assay is used in these studies since the results are quantitative, allowing for a determination of the endotoxin amount at each time point.

Table 1-2 shows the results of a hold time study using NOE spiked into two different buffers that were held at 2-8°C for 3 weeks, and Table 1-3 shows the results of a hold time study using NOE spiked into the same two buffers, this time held at -80°C for 3 months These results indicate that endotoxin may be recovered from the buffer matrices over the entire time period of the studies at both temperatures.

Initial Time Point Testing and Use of NOE for Troubleshooting

An important consideration for hold time studies is confirmation of the amount of endotoxin initially spiked. When the initial time point is assayed

Table 1-2: NOE Spiking Study with Buffers at 2-8°C

Sample	Results (EU/mL)					
	T0 (initial timepoint)	T1 (1 day)	T2 (2 days)	T3 (1 week)	T4 (2 weeks)	T5 (3 weeks)
Buffer A	55	46	46	44	39	41
Buffer B	61	54	53	42	45	48

Table 1-3: NOE Spiking Study with Buffers at -80°C

Sample	Results (EU/mL)					
	T0 (initial timepoint)	T1 (1 day)	T2 (1 month)	T3 (2 months)	T4 (3 months)	T5 (3 weeks)
Buffer A	55	42	50	49	46	41
Buffer B	61	54	51	52	52	48

directly after NOE spiking, consideration must be taken as to whether the sample matrix has an immediate effect on endotoxin recovery. To demonstrate that there is no sample matrix interference, an identical spike into LAL reagent water (LRW) is performed. Table 1-4 illustrates the use of this LRW spiking confirmation. Three different NOE spiking levels were used in this study, indicated by the target NOE spike amount. The actual amount recovered in EU/mL is indicated in the columns under test sample and LRW, are well within the LAL assay variability, and indicate that there is no interference from the sample matrix.

Table 1-4: LRW Spiking Confirmation

Target NOE spike	Test sample (EU/mL)	LRW (EU/mL)
50 EU/mL	32	30
100 EU/mL	67	47
200 EU/mL	124	111

The importance of performing this spiking confirmation in LRW for the initial time point is clearly illustrated in the following study (Table 1-5). NOE was spiked into a test sample and assayed immediately to determine the starting NOE concentration. NOE was spiked in three different concentrations, indicated by the target NOE spike. The endotoxin recovery was below the limit of detection of the assay at the dilution tested for all three spike levels. However, because an LRW confirmation was not performed, it is difficult to determine whether there was a dilution or assay error, or actual matrix or product interference.

Table 1-5: Test Sample NOE Spiking without LRW Confirmation

Target NOE spike	Test Sample (EU/mL)
50 EU/mL	< 50
100 EU/mL	< 50
200 EU/mL	< 50

The study was repeated at two different spiking levels, with an LRW confirmation sample performed side-by-side with the test sample (Table 1-6). The addition of the LRW confirmation sample clearly illustrates that there is sample matrix interference, leading to lack of endotoxin detection in the test sample.

In this case, multiple studies were performed to determine the cause of the sample matrix interference. The use of laboratory-prepared NOE

Table 1-6: Test Sample NOE Spiking with LRW Confirmation

Target NOE spike	Test Sample (EU/mL)	LRW (EU/mL)
100 EU/mL	< 50	73
200 EU/mL	< 50	250

stocks led to increased efficiency in the laboratory's ability to perform multiple studies in short periods of time, and allowed for the use of spiking concentrations from 5 EU/mL up to 3500 EU/mL. Table 1-7 shows data from a study with a different test sample where the NOE spike was very low (5-40 EU/mL). In this case, endotoxin recovery was consistent between the test sample and the LRW confirmation sample indicating no matrix interference.

Table 1-7: Test Sample NOE Spiking at Low EU/mL Concentrations

Target NOE spike	Test sample (EU/mL)	LRW (EU/mL)
5 EU/mL	4	4
20 EU/mL	15	20
40 EU/mL	27	29

Preparations from several different organisms were used as well, adding robustness and variability to the data collected. This highlights an additional benefit of using in-house preparations of NOE, since stocks from multiple organisms can be quickly prepared and used when desired.

Table 1-8 shows some of the data generated using the same test sample from Tables 1-5 and 1-6, with spikes from a high EU/mL NOE producer (*S. marcescens*) and a low EU/mL NOE producer (*A. genomospecies*). Both were spiked at a target of 200 EU/mL and showed similar recovery, further indicating that the test sample was interfering with the recovery of endotoxin, rather than any assay errors or issues with the a particular source of endotoxin.

Table 1-8: Spiking with Multiple NOE Producers

NOE used	Target NOE spike	Test Sample (EU/mL)	LRW (EU/mL)
S. marcescens	200 EU/mL	62	121
A. genomospecies	200 EU/mL	< 50	175

Conclusion

The data presented in this report is used to illustrate the importance of testing various matrices, temperatures and storage times for interference in endotoxin recovery for samples that will be held in the laboratory for any length of time prior to testing. It is especially important to understand the interaction between endotoxin and the sample matrix at the initial time point, since any matrix inhibiton that may be present would be identified at this time. The use of laboratory-prepared NOE stocks greatly increased the flexibility, efficiency and range of testing options available when performing this testing or troubleshooting matrices that demonstrated inhibition when NOE was spiked into the test sample matrix. When encountering matrix inhibition at the initial time point, alternate testing methods would need to be developed, and NOE is extremely useful in this case as well, since confirmation of successful endotoxin recovery is critical to any alternate method that would be implemented.