FUJIFILM

Predictive Oncology

Study report

EndoPrep™ as a sample treatment system to be used with FUJIFILM PYROSTAR™ ES-F/Plate

January 2024

Timothy Francis
FUJIFILM

Chrysanty Weaver, Ph.D.
Predictive Oncology

David Johnson, Ph.D.
Predictive Oncology

Authors contributed equally

Introduction

Sub-nanogram levels of endotoxin can trigger immune responses and alter the phenotype and function of many cells including monocytes, neutrophils, dendritic cells, hepatocytes, vascular and respiratory epithelium, and arterial smooth muscle cells. Thus, accurate quantitation of endotoxin is crucial for production of samples for biological use. The Food and Drug Administration (FDA) enforces established guidelines set by the U.S. Pharmacopeia (USP) or Association for the Advancement of Medical Instrumentation (AAMII) for allowable endotoxin content in injectable drugs. This guidance comes as published monograph limits or as calculated by pyrogenic dose guidance. [FDA, 2012]. The Limulus Amebocyte Lysate (LAL) test is recognized by the U.S. Pharmacopeia (USP) for determining endotoxin levels in many drug and device products.

The effects of protein interference in endotoxin detection assays have been advised on by the USP and detailed through research. Since the cascade reaction of the LAL chemistry involves a cascade of protease enzymes, proteins that cause potent interference to the LAL cascade are protease or protease inhibitor proteins. Catalyzing proteins often resist denaturation and have a potent effect in small concentrations. Dilution and heat treatment, the traditionally recommended procedures for overcoming protein interference, are often ineffective.

The cascade reaction of LAL initiates the first serine protease precursor (Factor C) from its inactivated form; this in turn activates the second serine protease precursor (Factor B). The activated Factor B stimulates the clotting enzyme, converting it from a proclotting enzyme. The clotting enzyme cleaves peptide bonds within coagulogen to yield coagulin, an insoluble protein the precipitates from solution. The rate at which turbidity causes a threshold absorbance value to be met results in an activation time. The relationship between activation time and endotoxin concentration allows for calculation of relative endotoxin concentration. Protease and protease inhibiting proteins can respectively shorten or lengthen the activation time, leading to enhancement or inhibition of the assay.

EndoPrep™ is a sample treatment system that neutralizes the inhibitory effects of proteins/peptides on endotoxin and results in more accurate detection and quantification of endotoxin. To accomplish this, the sample to be tested is subjected to an hour-long treatment with the EndoPrep™ digestion enzyme. Predictive Oncology (POAI) and FUJIFILM Wako have validated that a 100-fold dilution after the treatment is sufficient to subsequently remove the digestion enzyme's effects on the PYROSTAR™ ES-F LAL protein cascade. The regulatory standards allow and welcome treatment such as this that will not interfere with the LAL protein cascade. This white paper demonstrates the successful use of EndoPrep™ to remove protein interference in the LAL cascade with 3 of 4 interfering proteins and the need to evaluate the EndoPrep™ with each specific protein product to determine appropriate use of the product.

Materials

- PYROSTAR™ ES-F/Plate (FUJIFILM Wako Cat. # WPEPK4-20015)
- EndoPrep™ (Predictive Oncology Cat. # EDP-4001.01)
- Bioclean plate, 96 well (FUJIFILM Wako Cat. # 293-35221-HS)
- Pyrogen-free dilution tubes
- LAL Reagent Water (LRW)
- Plate reader
- Proteins: bovine serum albumin (EMP Millipore Cat. # 126579), bovine IgG (MP Biomedicals Cat. # 08641401), human hemoglobin (Sigma Cat. # H7379)

Benefits

- Increased detection accuracy with most interfering biologics
- Minimizes inhibition and enhancement effects of peptides and proteins on endotoxin detection.
- Easy to use and validate using the Example PYROSTAR™ – EndoPrep™ protocol.
- Compatible with LAL and recombinant factor C assays for most biologics

Methods

Spike Test

Four proteins were evaluated for protein interference in the LAL reaction and for the ability of EndoPrep™ to reduce interference. The full detailed method to evaluate EndoPrep™ and PYROSTAR™ ES-F/Plate with USP <1085> - Method Suitability Testing are included in the Example Template PYROSTAR™ EndoPrep™ Protocol. Each example protein product was spiked with Control Standard Endotoxin to simulate endotoxin contamination as recommended by USP <1085>. Recovered endotoxin detection was measured both directly and with the EndoPrep™ sample treatment. The percentage of the original spike detected by the PYROSTAR™ LAL reaction was compared between EndoPrep™ treatment and untreated control samples.

Reconstitution of Reagents

- Control Standard Endotoxin (CSE) was reconstituted with the volume of LAL reagent water (LRW) indicated on the Certificate of Analysis included with the PYROSTAR™ ES-F/Plate kit to yield a solution of 1000 EU/ml. The vial was vortexed for 15 minutes.
- LAL Reagent was reconstituted with LRW according to PYROSTAR™ ES-F/Plate kit instruction.
- POAI protease solution was reconstituted with POAI digestion buffer according to EndoPrep™ kit instruction.

Standard Curve, Sample Setup

Endotoxin samples for the standard curve were produced by serial dilution of Control Standard Endotoxin (CSE) included in the PYROSTAR™ ES/F Plate kit (Table 1). The initial protein concentrations, before spike, were set to 5 mg/ml except for the lower solubility Sushi3 peptide, set to 1 mg/ml. Endotoxin spikes were added to give a final concentration of either 0.1 or 1 EU/ml.

Initial Endotoxin (EU/ml)	Volume Endotoxin (ml)	Volume LRW (ml)	Final Endotoxin (EU/ml)
1000 (CSE)	0.2	1.8	100
100	0.2	1.8	10
10	0.2	1.8	1
1	0.2	1.8	0.1
0.1	0.2	1.8	0.01

Table 1. Serial dilution of Control Standard Endotoxin

Procedure for protein/peptide neutralization with EndoPrep™

The EndoPrep™ (EP) kit consists of EP Digestion Buffer and EP Protease Solution. Aliquots of 270 µl of each protein sample were mixed with 30 µl of the EP Protease Solution and vortexed for 30 seconds. A sample containing 30 µl of EP Digestion Buffer instead of the EP Protease Solution was included in each set of experiments to determine baseline endotoxin measurement without digestion. After mixing, the tubes were covered with Parafilm and incubated in a 37°C water bath for the indicated times. After treatment, the samples were diluted 1:100 with endotoxin-free water and endotoxin was measured using the PYROSTAR™ ES/F Plate kit. The dilution resulting from addition of the EndoPrep™ Protease Solution and subsequent dilution with endotoxin-free water was included in the calculation of the results given.

Results

The use of EndoPrep™ with PYROSTAR™ demonstrates the ability to reduce protein interference with a variety of common proteins. Figure 1 shows that with Sushi3 (known to bind endotoxin), hemoglobin, and IgG, EndoPrep™ improves detection of endotoxin during the spike recovery test. With the exception, BSA, a spike test demonstrates variability that confirms EndoPrep™ should not be used. Proper spike testing, described above, determines product suitability for the application.

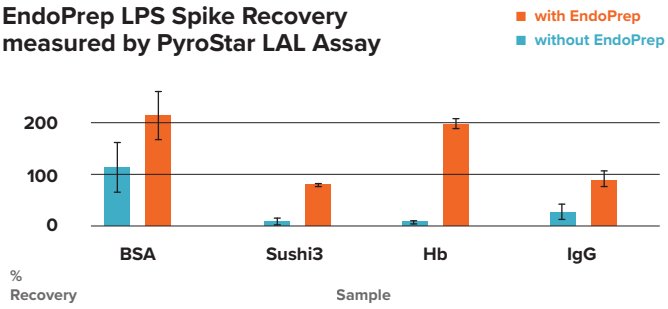

Table 2. LPS Spike Recovery measured by PYROSTAR™ LAL Assay with and without EndoPrep™ treatment.

Conclusions

Interference of a product with an LAL assay is a well-known confounding factor which is addressed by the Test for Interfering Factors in the U.S. Pharmacopoeia (USP). The EndoPrep™ sample preparation kit is designed to prevent interference (inhibition or enhancement) due to proteins with the PYROSTAR™ ES-F/Plate Assay. The procedure defined in this white paper is a rapid challenge test to determine the ability of EndoPrep™ to prevent interference with the LAL assay. The results presented here demonstrate that the EndoPrep™ kit can reduce interference (inhibition and enhancement) of a variety of protein products. This makes the EndoPrep™ kit a valuable tool when used with the FUJIFILM PYROSTAR™ ES-F/Plate assay to achieve compliance with USP standards.

The LAL assay is a complex protein interaction pathway that is known to be enhanced or inhibited by the presence of protein samples, including pharmaceutical products. Use of the EndoPrep™ kit can reduce these interactions. However, use of the kit must be validated with a particular protein sample. The spike test evaluation should be performed in replicates compliant with the USP's guidance on method suitability testing for LAL reagent determine variability of protein interaction. Confirmed validation of the EndoPrep™ kit provides a potential solution for interference of a particular protein product with the LAL assay.

References

- RM- 411 PYROSTAR™ ES-F Multi Package Insert (with CSE)
- RM-412 PYROSTAR™ ES-F Single Package Insert (with CSE)
- USP <85> Bacterial Endotoxins Test
- USP <1085> Guidelines on Endotoxins Testing
- USP <161> Medical Devices-Bacterial Endotoxin and Pyrogen Tests
- FDA Guidance for Industry: Pyrogen and endotoxins testing: questions and answers, 2012.
- ANSI/AAMI ST72:2019 Bacterial Endotoxins - Test methods, routine monitoring, and alternatives to batch testing
- Predictive Oncology EndoPrep™ Instruction Booklet
- Example Template PYROSTAR™ - EndoPrep™ Protocol

Predictive Oncology

FUJIFILM

91 43rd Street
Suite 110
Pittsburgh, PA 15201

(412) 432-1500

www.predictive-oncology.com