KALEN Biomedical, LLC

Certified Nonpyrogenic Lipoproteins

Introduction

Kalen Biomedical, LLC (Kalen) has had several recent inquiries from our clients regarding information on the endotoxin levels in our products. Although all our products are sterilized by ultra-filtration and periodically checked for endotoxin, we conducted an assessment of the production of each product.

The results suggested that, with continual endotoxin testing and a few changes to our production methodology, Kalen would avoid potential sources of contamination and produce a product that we could certify as nonpyrogenic**.** This expanded quality control program includes the examination of incoming supplies and materials, intermediate steps in our production process, and our final products. A critical component of this program is testing for endotoxin at multiple time points in the production process. Of course this adds to the complexity, time, and cost to produce a certified nonpryogenic product. However certain of Kalen's clients find it beneficial, and cost effective, to use certified nonpyrogenic products in their research.

Kalen will continue to produce our normal line of high quality lipoproteins and lipoprotein derivatives for those clients who do not require certified nonpyrogenic material.

Sources of Endotoxin Contamination

Endotoxin is a lipopolysaccharide (LPS) commonly from gram negative bacterial cell walls. It is a heat stable molecule comprised of a lipid portion and a variable sugar chain. The LPS from *E. coli* is used as a reference, but potency varies from different preparations. The Food and Drug Administration (FDA) defined the Endotoxin Units (EU) as the activity compared to a standard, currently EC-6.

The most common and problematic source of contamination is distilled or deionized water. Once endotoxin has contaminated potential products, laboratory solutions or glass/plastic ware it is almost impossible to remove. It must be avoided from the beginning.

The components of laboratory media, all glassware and plastic ware must be nonpyrogenic. Media additives, such as sera and their derivatives and test agents (such as lipoproteins and their derivatives) are also common sources of endotoxin contamination. Sterilization of these items by autoclave, irradiation, or gas exposure usually does not destroy the endotoxin.

19640 Club House Road • Suite 415 • Montgomery Village, MD 20886
Email: info@Kalenbiomed.com • Contact: Kathy Foxx
Tel: 301.355.6155 • Mobile: 301.908.8947 • Fax: 301.355.6156

Nonpyrogenic Criteria and Client Endotoxin Requirements

The generally accepted threshold level for pyrogenicity is 0.5 EU/mL of a reference endotoxin (1). At this level (dosed at 0.5 EU per kg) approximately one-half of test rabbits will show a temperature increase of 0.55 ° C in 180 minutes.

To determine if this level was below where endotoxin begins to interfere with our client's assays and cultures, we spoke to several of our clients, briefly surveyed the literature, and used manufacture's listed values.

As expected, the range of acceptable endotoxin levels varied greatly by the type of biological assay as well as by the researcher, so we focused on the strictest criteria, the lowest endotoxin levels. Endotoxin levels were variously expressed as nanograms, picograms, or EU. The denominators for these values were expressed as per milliliter (mL), per milligram (mg), or per microgram (µg) of lipoprotein protein.

To consolidate these different systems to unified values we converted endotoxin weights (nanograms, etc) to the unit of potency, EU. The Endotoxin Unit, described as 10 EU per nanogram of the FDA reference standard EC-6, was used to convert weight to EU.

Acceptable Endotoxin Levels

Item	EU/mL
Plastic culture vessels (extracts)	0.5
Commercial cell culture media certified at <0.1 ng/ml	<1.0
Macrophage cultures	0.8
Endothelial Cell Cultures	<0.5

It was apparent that the 0.5 EU/mL threshold level for pyrogenicity could also be used to set the maximum endotoxin level acceptable for in vitro biological studies (<0.5 EU/mL).

The *Limulus* Assay

The chromogenic, kinetic assay for quantitating Gram-negative bacterial endotoxin uses a lysate of amebocytes from the hemolymph of the horseshoe crab *Limulus*. In the presence of endotoxin, a cascade of proteolytic factors in the lysate is activated, see Figure 1 (2). These factors cleave a synthetic peptide substrate to produce free p-nitroaniline (pNA). The production of pNA is measured by absorbance of the solution at 405 nm. Using the kinetic method, the time required to produce a threshold level of pNA is measured. The greater the quantity of endotoxin present, the shorter the time that is required to attain this threshold. A series of dilutions of a reference endotoxin is used as a "standard curve" to calculate the quantity of endotoxin in the unknown samples.

Endotoxin Test Kit

Kalen uses the *Limulus* Amebocyte Lysate (LAL) assay kit from Associates of Cape Cod, Inc. (East Falmouth, MA). According to the manufacturer, this kit, when used according to U.S. Food and Drug Administration guidelines, may be used for the end-product testing of "human injectable drugs (including biological products), animal injectable drugs, and medical devices" (2). The LAL test is recommended for the quantitation of endotoxin in raw materials used in production, including water, and for in-process monitoring of endotoxin levels.

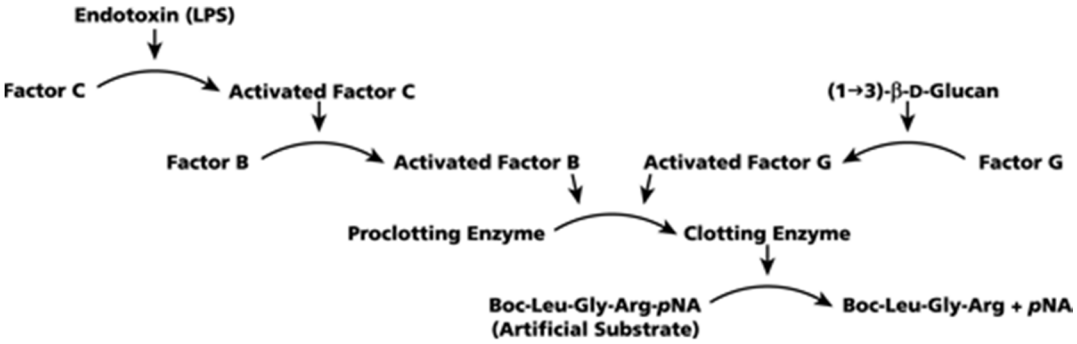
We qualified this method for use in our laboratory. In our hands the lower limit of quantification (the lowest positive result on our standard curve) is either 0.005 or 0.0025 EU/mL, depending upon the individual assay.

Matrix Effects and Complications

A large number of substances and conditions are known to interfere (inhibit or enhance) with the assay. A common strategy for reducing the interference is to dilute the material in water that is known to be free of endotoxin. The trick is to balance the dilution of the interfering substance and still accurately quantitate the diluted endotoxin. Preliminary testing determines the range where the endotoxin (if present) will be detected and the interfering substances will no longer interfere.

An alternate pathway for activation of the *Limulus* proteolytic cascade is known to exist in the presence of (1-3)-beta-D-glucan (Fig.1). Materials that have been exposed to cellulose (viz. dialysis tubing or filter membranes) often have this glucan present. Our analyses are conducted using the manufacturer's suggested blocking buffer to mitigate the interference from this pathway.

Figure 1 (from reference 2)



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Many proteins from blood products will also interfere with the cascade of proteolytic factors in this assay. Our samples are diluted with endotoxin free water and heat inactivated to reduce the interference from these proteins.

Internal Control

To determine if any of our samples express these matrix effects that interfere with the assay (either inhibition or enhancement) Kalen analyzes each sample with and without a spike of a known concentration of endotoxin. The spike recovery is determined and used to ensure that any interference is negligible. The manufacturer's suggested valid range of spike recovery is 50% to 200%.

Results

The endotoxin assay Kalen uses expresses the analytical results in EU/mL. Most Kalen lipoprotein products are sold by milligrams of protein, and many at 1 mg/mL. For consistency, we will express the assayed level of endotoxin in our products as EU/mg.

The spike recoveries and endotoxin results are provided for recent preparations of two of Kalen Biomedical's new CNP products below: Certified NonPyrogenic Low Density Lipoprotein (CNP LDL) and Certified NonPyrogenic Medium Oxidized LDL (CNP MOx)

Endotoxin Test Results for Certified NonPyrogenic Lipoprotein Products

Product	Preparation	Protein Concentration (mg/mL)	Percent Spike Recovery	EU/mg
CNP LDL	A	5	89	< 0.5
	B	5	86	< 0.5
	C	3	103	< 0.5
	D	5	78	< 0.5
	E	4	57	< 0.5
	F	5	62	< 0.5
	G	1	99	< 0.5
	H	1	105	< 0.5
CNP MOx	I	1	97	< 0.5
	J	1	102	< 0.5
	K	1	106	< 0.5
	L	1	81	< 0.5
	M (REJECTED)	1	116	0.5
	N	1	75	< 0.5
	O	1	104	< 0.5

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Summary

Kalen Biomedical now offers Low Density Lipoprotein and Oxidized Low Density Lipoprotein products that have endotoxin levels less than 0.5 EU/mg as determined by the Associates of Cape Cod, Inc. (East Falmouth, MA) Limulus Amebocyte Lysate (LAL) assay kit.

References

1. Hoffman, et. al., 2005, Journal of Immunological Methods 298: 163-173.
2. Fungitell® Assay, Associates of Cape Cod, Inc. http://www.acciusa.com/clinical/fungitell/index.html.
3. *Limulus* Amebocyte Lysate Package Insert, Associates of Cape Cod, Inc. Rev005 June 2007.

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