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Advanced Recombinant Cascade Reagent PyroSmart NextGen® for Bacterial Endotoxins Test as Described in the Pharmacopeias

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The bacterial endotoxins test (BET) is a method of detection and quantification of bacterial endotoxin in injectable drugs and medical devices using amoebocyte lysate reagents sourced from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). Three manufacturers have launched three different types of recombinant reagents, and recently the United States, European and Japanese Pharmacopeias have been evaluating the equivalency of these reagents to natural amoebocyte lysate reagents. Several studies suggested that the recombinant reagents are very similar to amoebocyte lysate reagents, however there is potential for improvement. Previous reports indicate that recombinant reagents have two issues: extremely low endotoxin potency determination for *Helicobacter pylori* GU2 and high levels of interference from Heparin Calcium impacting endotoxin recovery. A new recombinant cascade reagent (rCR), PyroSmart NextGen®, recently introduced to the market has been developed to solve these issues. PyroSmart NextGen® has demonstrated higher reactivity to *H. pylori* GU2 and a lower level of interference from Heparin Calcium than other existing recombinant reagents. Additionally, the analytical capability and suitability of PyroSmart NextGen® has been demonstrated when applied to the BET as described in the US, European and Japanese pharmacopeias. PyroSmart NextGen® has also shown comparability to amoebocyte lysate reagents by demonstrating its ability to detect autochthonous endotoxin in water and in *Escherichia coli* culture. Overall, this study has verified that the rCR, PyroSmart NextGen® is a suitable alternative to amoebocyte lysate reagents.

Key words bacterial endotoxins test, recombinant cascade reagent, lysate reagent

INTRODUCTION

To assure patient safety, the testing of parenteral pharmaceuticals and medical devices for pyrogens is mandated.[1–3] The presence of pyrogens such as endotoxins derived from the cell walls of gram negative bacteria can induce fever which can lead to life threatening septic shock in severe cases.[4–8] The original FDA approved test was the rabbit pyrogen test, but this has largely been replaced by the Bacterial Endotoxins Test (BET), also known as the Limulus amebocyte lysate (LAL) assay. The reagent used for the LAL assay, known as limulus amoebocyte lysate reagent (lysate reagent), is comprised of the enzymatic cascade factors isolated from the blood of the horseshoe crab.[9–11] The cascade is initiated by endotoxin binding to a serine protease zymogen, factor C. Upon binding, factor C is autocatalytically converted to activated factor C, which subsequently converts factor B to activated factor B, followed by activation of the proclotting enzyme to the clotting enzyme.[12] At the end of the cascade, the clotting enzyme cleaves the endogenous coagulogen leading to coagulin and gel formation.[9]

The LAL assay provides a quantitative method for determining the amount of endotoxin present from remnant components of Gram-negative bacteria cell walls. There are four different LAL assays, the first is the gel-clot assay which was approved by the FDA to supplement the Rabbit Pyrogen Test in 1977. LAL assay methodologies advanced further with the turbidimetric and colorimetric techniques applied to the endpoint and kinetic chromogenic assays in the late 1980's and early 1990's. The kinetic chromogenic assay uses a chromogenic peptide that releases the yellow-colored *p*-nitro aniline when cleaved by the clotting enzyme.[11] These are the only methods currently approved by the FDA and filed in the three pharmacopeias for BET.

Major components of the lysate reagents are sourced from natural animal resources. There are three inherent issues that arise from using this natural resource. The first is the biological lot-to-lot variability of lysate reagents,[13–15] and secondly the potential interference from alternative pathways which are mediated by the presence of factor G in the hemocytes. This alternative pathway is activated by the binding of (1→3)-β-D-glucan from components in the fungal cell wall which may lead to a false positive result for endotoxin due to activation of the proclotting enzyme by the factor G cascade.[11-12] The final consideration is the environmental impact on harvesting the horseshoe crab and collecting its blood.[16-18] A solution to these issues would be the use of a recombinant reagent. In 2003, a recombinant factor C (rFC) fluoromet-

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ric assay from Cambrex Bio Science Walkersville (LONZA) (MD, USA) was introduced to the market, followed by another rFC reagent, EndoZyme® from Hyglos GmbH (bioMérieux) (Munich, Germany)[19]. These rFC reagents include only one zymogen protease (factor C) from the *Carcinoscorpius rotundicauda* or *Tachypleus tridentatus*, respectively. In 2015, PyroSmart® was introduced as the first recombinant cascade reagent (rCR) to have three enzymatic coagulation factors including rFC, recombinant factor B (rFB) and recombinant proclotting enzyme (rPCE) using the cloned genes derived from *T. tridentatus*.[20] The availability of these three types of recombinant reagent has resulted in the US, European and Japanese Pharmacopeias evaluating the comparability of recombinant reagents to lysate reagents. Many studies have found that the recombinant reagents have very similar characteristics to lysate reagents.[21–29] However, one research study in Japan found that unlike lysate reagents, both rFC reagents had almost no reactivity, whereas PyroSmart® had slight reactivity to endotoxin from *Helicobacter Pylori* GU2.[29] Another study found that both rFC reagents could not detect endotoxin added to Heparin Calcium even when diluted to the maximum valid dilution (MVD). The rCR reagent, PyroSmart® was able to detect endotoxin in Heparin Calcium after it was diluted below the MVD.[27]

PyroSmart NextGen® is the successor to PyroSmart® through the collaboration with Associates of Cape Cod, Inc. and Seikagaku Corporation using cloned genes derived from the *Limulus polyphemus*. It was developed to resolve the issues mentioned above and was introduced to the market as another rCR in April 2021. PyroSmart NextGen®, was evaluated for performance and analytical characteristics using linearity, accuracy, precision, range, quantitation limit and specificity as required by the ICH Q2 guideline and USP 1225. The endotoxin recoveries were compared in 27 injectable and bulk drugs determined by five lysate reagents, two rFC reagents, PyroSmart® and the new PyroSmart NextGen®. The endotoxin potencies for several purified and crude bacterial endotoxin strains were also compared using the same reagents mentioned above. The results from this study and the comparability of measuring endotoxin to lysate reagents will support the use of PyroSmart NextGen® as a suitable alternative reagent when performing the BET outlined in the pharmacopeias.

MATERIALS AND METHODS

Purified and Crude Bacterial Endotoxins United States pharmacopeia reference standard endotoxin (USP-RSE) was purchased from the United States Pharmaceutical Convention (MD, USA). Samples of endotoxin derived from *Escherichia coli* O55:B5, *Salmonella minnesota* R595 Re and *Salmonella typhimurium* were obtained from the List Biological Laboratories, Inc. (CA, USA) and endotoxin derived from *Serratia marcescens* and *Pseudomonas aeruginosa* 10 were purchased from Sigma-Aldrich (MO, USA). Endotoxin derived from *Helicobacter pylori* GU2 was obtained from LPS Laboratory (Akita, Japan).

Water Samples The deionized water was collected from six sampling points in a building in the Central Research Laboratory of Seikagaku Corporation, and two laboratories in two Universities in Japan. The bottled water was purchased in Japan. Pachyman, (1→3)-β-D-glucan (BG), was prepared from *Poria cocos*.[30] Water for injection, WFI, purchased from Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan) and laboratory reagent water from Associates of Cape Cod, Inc. (MA, USA) was used for this study.

Lysate Reagents Kinetic-QCL™ and reconstitution liquid β-G-Blocker was purchased from Lonza (MD, USA). Limulus ES-II was purchased from FUJIFILM Wako Pure Chemical Corporation (Tokyo, Japan) and Endochrome-K™ with Endotoxin specific reconstitution buffer was purchased from Charles River Laboratories (MA, USA). Endospecy® ES-50M was supplied by the Seikagaku Corporation (Tokyo, Japan) and Pyrochrome® with Glucashield® reconstitution buffer was obtained from Associates of Cape Cod, Inc. (MA, USA).

Recombinant Reagents PyroSmart NextGen® was obtained from Associates of Cape Cod, Inc. (MA, USA) and PyroSmart® was supplied by Seikagaku Corporation (Tokyo, Japan). PyroGene™ was purchased from Lonza and EndoZyme® II was purchased from bioMérieux (Munich, Germany).

Endotoxin Assays Endotoxin was detected and quantified using recombinant and lysate reagents. All reagents were tested and used according to their Instructions for Use (IFU) using turbidimetric, fluorometric or chromogenic principals. Three assay modes were used for analysis: endpoint, rate and onset. The rate assay measures the mean rate (Vmean: mAbs/min) of color development over time in the assay. The standard curve is constructed by plotting Vmean (Y-axis) against the standard concentration (X-axis) and is used to calculate the endotoxin concentration in samples. The onset time assay measures the time required to reach a threshold OD. The standard curve is constructed by plotting the log converted onset time (Y-axis) against the log converted standard concentration (X-axis) and is used to determine endotoxin concentrations in samples. Both rate and onset time assays were applied to PyroSmart® and PyroSmart NextGen®. The onset time assay was applied to Kinetic-QCL™, Limulus ES-II, Endochrome-K™ and Pyrochrome® and the rate assay to Endospecy® ES-50M. The endpoint assay was applied in PyroGene™ and EndoZyme® II.

Potency of Endotoxin from Different Bacterial Strains USP-RSE was employed as a standard and a two-fold dilution series was prepared with the following concentrations: 0.1, 0.05, 0.025, 0.0125 and 0.00625 endotoxin unit (EU)/mL additionally, a ten-fold dilution series was prepared with the following concentrations:50, 5, 0.5, 0.05 and 0.005 EU/mL. These standard dilutions were used for the rate and onset time assays for PyroSmart NextGen®. The standard dilutions for the other reagents were prepared according to their IFU's. Endotoxins derived from *E. coli* O55:B5, *P. aeruginosa* 10 and *S. marcescens* were dissolved in water. In cases where the endotoxin was insoluble in water, such as with *S. minnesota* R595 Re and *S. typhimurium*, 0.1%(v/v) triethylamine (TEA) was used first to dissolve the endotoxin followed by 0.2 mol/L Tris-HCl buffer, pH 8.0 to neutralize the solution and subsequent dilutions were done with water. Solubilized endotoxin was diluted by two-fold or ten-fold and tested using the reagents mentioned above. The determined endotoxin value (EU/mL) was divided by the concentration of each dilution (ng/mL) to calculate the potency (EU/ng) and the mean potency was calculated from the average potency of each endotoxin dilution within the range of the standard curve.

Analytical Characteristics of PyroSmart NextGen® PyroSmart NextGen® was evaluated according to the ICH Q2 guideline and USP 1225 and analytical performance including accuracy, precision, specificity, quantitation limit, linearity,

and range, was assessed.[31] The acceptance criteria described in a previous study[27] as well as the Guideline on Bioanalytical Method Validation M10 (Ligand Binding Assay) [32] were referenced when assessing the criteria for linearity, accuracy, repeatability, intermediate precision, reproducibility, range, quantitation limit and specificity. The analytical characteristics of the rate and onset time assays were evaluated after changing five different factors: analyst, plate reader, day, facility and PyroSmart NextGen® lot. This study included 4 analysts, 3 plate readers used over three days with 2 lots of reagents at two facilities (Seikagaku Corp. and Associates of Cape Cod, Inc.) for a total of 34 rate and 28 onset assay measurements. A series of USP-RSE standard curve dilutions with eight replicates (rate assay: 0.1, 0.05, 0.025, 0.0125, 0.00625 EU/mL, onset assay: 50, 5, 0.5, 0.05, 0.005 EU/mL) were prepared and measured with PyroSmart NextGen®.

Linearity –The correlation coefficients determined from standard curve regression analysis for both assay modes were evaluated from a total of 48 assays, which were tested over three days by four analysts at two different facilities with two different lots.

Accuracy – The recovery of endotoxin for each standard curve from linear regression analysis was used for calculating accuracy. The minimum and maximum accuracy of each endotoxin concentration for both test modes was evaluated. An average of eight replicates were included for this measurement for a total of 48 assays, tested over three days by four analysts at two different facilities with two different lots.

Repeatability – The average endotoxin concentration measured at each point on the standard curve was used to calculate the coefficient of variation (%CV). Eight replicates were measured for each standard on the regression line. Forty-eight rate and onset assays were performed over three days by four analysts at two facilities with two different lots.

Intermediate Precision – The standard deviation and %CV were determined from the concentration replicates of the 48 rate and onset assays performed over three days by three analysts at one company. The upper and lower variance was determined using CHI square analysis.

Reproducibility – Standard deviation and %CV was determined from the concentration replicates of 24 rate and onset assays performed over three days by two analysts at two companies with two lots. The recovered concentration %CV and multi curve regression analysis was used for the analysis.

Range – The lowest and highest endotoxin concentrations from standard curves that have acceptable levels of accuracy, repeatability, intermediate precision and linearity are used to determine the range. Forty-eight assays were performed over three days by four analysts at two different facilities with two different lots.

Quantitation Limit (QL) – The quantitation limit is defined as the lowest concentration of endotoxin that can be quantitatively determined with suitable precision and accuracy. The onset quantitation limit was determined from twenty-four onset assays performed by four analysts over three days at two companies with two lots. Each onset assay was also analyzed for the ability of the 0.005 EU/mL standard concentration to meet the acceptance criteria for repeatability and accuracy.

Specificity – Two series of USP-RSE dilutions for both assay modes were prepared with and without addition of 200 pg/mL BG. The presence of 200 pg/mL BG can produce a false positive reaction which is similar to the reactivity induced by approximately 5 EU/mL endotoxin in lysate reagents. Measurements were made in quadruplicate and the mean endotoxin concentration was used for regression curve analysis and comparison. Two lots of PyroSmart NextGen® were tested on two days by one analyst at one facility.

Recovery of USP-RSE Added in Various Injectable and Bulk Drugs Recovery tests were performed in accordance with the procedure for testing interfering factors described in the BET in the Pharmacopeias. Various injectable and bulk drugs were diluted with water, and then standard endotoxin was added at a concentration in the middle point of the standard curve according to the inhibition/enhancement test for BET. Endotoxin recovery was calculated by subtracting the diluted parenteral drug sample from the sample with added endotoxin. The injectable and bulk drugs used in this study were determined to be free from interfering factors when the endotoxin recovery is within 50% and 200%. The minimum dilution factor that shows neither inhibition nor enhancement is defined as the Non-Interfering-Dilution (NID). Optimization of the onset time assay for PyroSmart NextGen® was required to appropriately measure endotoxin in Heparin Calcium only (see Table 2).

Detection of Autochthonous Endotoxin in Water and *E. coli* O113:H10:K Negative Culture Supernatant The comparability of recombinant reagents to lysate reagents was evaluated by examining water and a culture supernatant of *E. coli* O113:H10:K negative samples containing detectable levels of autochthonous endotoxin.

RESULTS

Potency of Endotoxin from Different Bacterial Strains Potency determined by PyroSmart NextGen® was compared to other recombinant and lysate reagents using six different strains of gram-negative bacteria as shown in Fig. 1. The F-test and t-test (P = 0.05) determined there is no significant difference between the six data sets generated with the recombinant reagents and five data sets generated with the lysate reagents with the only exception being *Helicobacter pylori* GU2 endotoxin. The potencies of *H. pylori* GU2 determined by the lysate reagents became bipolar. Endospecy® ES-50M, Pyrochrome® and the chromogenic reagent 1 (KCA 1) showed higher potencies while the turbidimetric reagent (KTA) and the chromogenic reagent 2 (KCA 2) showed lower potencies. The potency determined by PyroSmart NextGen® for *H. pylori* GU2 is significantly higher than the potency determined by both rFC reagents and more consistent with the potencies determined by the lysate reagents.

Analytical characteristics of PyroSmart NextGen®

Linearity – All standard curves meet the calculated correlation coefficient acceptance criteria ($|r| \geq 0.980$) as described in USP <85> (Table 1).

Accuracy – The minimum accuracy for the rate assay is 84% and 71% for the onset assay. The maximum accuracy for the rate assay is 125% and 140% for the onset assay. The accuracy in both assay modes satisfied the acceptance criteria (50–200%) as described in USP <85>.

Repeatability – The minimum and maximum repeatability was 3 to 21% for the rate assay and 4 to 30% for the onset assay. All results satisfied the acceptance criteria as described in Table 1.

Intermediate Precision – The minimum and maximum

Table 1. Assessment of PyroSmart NextGen® Analytical Characteristics According to ICH Q2 Guideline

Analytical Characteristics	Results				Acceptance Criteria
	Rate Assay Mode		Onset Assay Mode		
1. Linearity (absolute value, correlation coefficient)	**0.00625–0.1 EU/mL** Minimum 0.998 Maximum 1.000		**0.005–50 EU/mL** Minimum 0.996 Maximum 0.999		$\|r\| \geq 0.980$
2. Accuracy (recovery)	**EU/mL**	**Min–Max (%)**	**EU/mL**	**Min–Max (%)**	50–200%
	0.00625	84–125	0.005	71–88	
	0.0125	98–109	0.05	87–138	
	0.025	94–105	0.5	75–140	
	0.05	93–101	5.0	106–118	
	0.10	100–102	50	75–95	
3. Precision					
3-1 Repeatability (CV)	**EU/mL**	**Min–Max (%)**	**EU/mL**	**Min–Max (%)**	CV ≤ 25% 0.00625 EU/mL
	0.00625	7–21	0.005	8–30	CV ≤ 20% 0.0125–0.10 EU/mL
	0.0125	5–16	0.05	6–25	
	0.025	6–12	0.5	7–23	CV ≤ 35% 0.005 EU/mL
	0.05	3–14	5.0	4–12	CV ≤ 30% 0.05–50 EU/mL
	0.10	3–18	50	5–16	
3-2 Intermediate Precision (95% CI for CV)	**EU/mL**	**Min–Max (%)**	**EU/mL**	**Min–Max (%)**	CV ≤ 25% 0.00625 EU/mL
	0.00625	15–19	0.005	20–24	CV ≤ 20% 0.0125–0.10 EU/mL
	0.0125	9–11	0.05	12–15	
	0.025	8–10	0.5	16–19	CV ≤ 35% 0.005 EU/mL
	0.05	7–9	5.0	7–9	CV ≤ 30% 0.05–50 EU/mL
	0.10	7–9	50	10–12	
3-3 Reproducibility (95% CI for CV)	**EU/mL**	**Min–Max (%)**	**EU/mL**	**Min–Max (%)**	CV ≤ 25% 0.00625 EU/mL
	0.00625	15–17	0.005	19–22	CV ≤ 20% 0.0125–0.10 EU/mL
	0.0125	9–11	0.05	12–14	
	0.025	8–10	0.5	15–17	CV ≤ 35% 0.005 EU/mL
	0.05	7–8	5.0	7–9	CV ≤ 30% 0.05–50 EU/mL
	0.10	7–8	50	10–12	
4. Range	0.00625–0.1 EU/mL		0.005–50 EU/mL		Precision, accuracy and linearity at suitable level
5. Quantitation Limit	Not Assessed		**At 0.005 EU/mL** Accuracy: 71–88% Repeatability: 6–30%		The lowest concentration of Et that can be quantitatively determined with suitable precision and accuracy
6. Specificity (Reactivity with Beta Glucan)	**Results from regression analysis (95% CI)** Intercept: −0.901–0.150 Slope: 0.909–1.020		**Results from regression analysis (95% CI)** Intercept: 5.923–113.0 Slope: 0.924–1.006		Not reactive to Beta Glucan

Data Analysis Details
1. 3 days, 4 analysts, 2 Kits, 24 Rate assays, 24 Onset assays, Associates of Cape Cod, Inc. and Seikagaku, Corporation
2. 3 days, 4 analysts, 2 Kits, 24 Rate assays, 24 Onset assays, Associates of Cape Cod, Inc. and Seikagaku, Corporation
3-1. 3 days, 3 analysts, 2 Kits, 18 Rate assays, 18 Onset assays, Associates of Cape Cod, Inc.
3-2. 3 days, 3 analysts, 2 Kits, 18 Rate assays, 18 Onset assays, Associates of Cape Cod, Inc.
3-3. 3 days, 4 analysts, 2 Kits, 24 Rate assays, 24 Onset assays, Associates of Cape Cod, Inc. and Seikagaku, Corporation
4. 3 days, 4 analysts, 2 Kits, 24 Rate assays, 24 Onset assays, Associates of Cape Cod, Inc. and Seikagaku, Corporation
5. 3 days, 4 analysts, 2 Kits, 24 assays, Associates of Cape Cod, Inc. and Seikagaku, Corporation
6. 2 days, 1 analyst, 2 Kits, 4 Rate Assays, 4 Onset assays, Associates of Cape Cod, Inc.

repeatability was 7 to 19% for the rate assay and 7 to 24% for the onset assay. All results satisfied the acceptance criteria as described in Table 1.

Reproducibility – The minimum and maximum reproducibility was 7 to 17% for the rate assay and 7 to 22% for the onset assay. All results satisfied the acceptance criteria as described in Table 1.

Range – The repeatability, intermediate precision, accuracy, and linearity are at suitable levels within the range of 0.00625 to 0.1 EU/mL for the rate assay and 0.005 to 50 EU/mL for the onset assay.

Quantitation Limit (QL) – As shown in Table 1, the accuracy (71–88%) and repeatability (6–30%) at 0.005 EU/mL for the onset assay satisfied all acceptance criteria. These results indicate that the quantitation limit for the onset time assay is at the lowest standard (0.005 EU/mL).

Specificity – Specificity is defined as demonstrating no reagent reactivity in the presence of (1→3)- β-D-glucan. Regression curve analysis for both assay modes was performed on two lots comparing performance with and without BG (Fig. 2). The 95% CI for the slope and intercept of the regression lines include "1" and "0" indicating endotoxin concentrations with and without BG are not statistically different.

All analytical test results in method validation of the PyroSmart NextGen® satisfied the acceptance criteria and confirmed its analytical performance and suitability as an alternative reagent to the BET in the pharmacopeias.

Recovery of USP-RSE Added in Various Injectable and Bulk Drugs Interference from various injectable drugs in reactivity of PyroSmart NextGen® and other recombinant and lysate reagents was examined. Additionally, Aciclovir, Insulin, PBS and WFI, were tested. Endotoxin recovery was measured using four recombinant reagents and five lysate reagents. The minimum non-interfering dilution factors (NID) are shown in Table 2. The data illustrates that PyroSmart® and PyroSmart NextGen® have similar NIDs for twenty-six of the twenty-seven drugs tested, including Aciclovir, Insulin, PBS and WFI with the only exception being Heparin Calcium. In this case, PyroSmart NextGen® had an NID of 128 in the rate assay and 512 in the onset assay whereas PyroSmart® had an NID of

Table 2. Non-Interfering-Dilution (NID) of Injectable Drugs Tested with Recombinant Reagents and Lysate Reagents

Parenteral drugs	Stock solution conc.	Release limit	NID (upper line) / *MVD (lower line)*										
			Recombinant reagents						Limulus lysate reagents				
			PyroSmart NextGen®		PyroSmart®		rFC 1[1]	rFC 2[1]	ES-50M[1]	KTA[1]	Pyrochrome®	KCA 1[1]	KCA 2[1]
			Rate	Onset	Rate	Onset	Fluorescein endpoint		Rate	Onset			
Aminophylline Injection	25 mg/mL	0.6 EU/mg	16 *2400*	8 *3000*	8 *2400*	16 *3000*	8 *3000*	2[2] *3000*	8 *2400*	8 *1920*	16 *3000*	4 *3000*	8 *3000*
Idarubicin Hydrochloride for Injection	1 mg/mL	8.9 EU/mg	512 *1424*	512 *1780*	256 *1424*	512 *1780*	128 *1780*	32 *1780*	128 *1424*	8 *1139*	512 *1780*	32 *1780*	16 *1780*
Calcium Chloride Injection	55.5 mg/mL	0.30 EU/mg	64 *2,664*	128 *3,330*	32 *2,664*	64 *3,330*	256 *3,330*	512 *3,330*	128 *2,664*	16 *2,131*	32 *3,330*	128 *3,330*	128 *3,330*
10% Sodium Chloride Injection	10%	3.6 EU/mL	4 *576*	8 *720*	4 *576*	8 *720*	8 *720*	1 *720*	8 *576*	2 *461*	2 *720*	16 *720*	4 *720*
Xylitol Injection	5%	0.50 EU/mL	1 *80*	1 *100*	1 *80*	1 *100*	1 *100*	1 *100*	1 *80*	1 *64*	1 *100*	1 *100*	1 *100*
Sodium Citrate Injection for Transfusion	100 mg/mL	5.6 EU/mL	1 *896*	1 *1,120*	2 *896*	1 *1,120*	256 *1,120*	512[3] *1,120*	4 *896*	4 *717*	16 *1,120*	64 *1,120*	16 *1,120*
Digoxin Injection	0.25 mg/mL	200 EU/mg	32 *8,000*	32 *10,000*	16 *8,000*	16 *10,000*	16 *10,000*	16 *10,000*	32 *8,000*	32 *6,400*	16 *10,000*	8 *10,000*	16 *10,000*
Dimorpholamine Injection	15 mg/mL	5.0 EU/mg	16 *12,000*	32 *15,000*	16 *12,000*	16 *15,000*	8 *15,000*	2 *15,000*	16 *12,000*	8 *9,600*	8 *15,000*	16 *15,000*	16 *15,000*
Isotonic Sodium Chloride solution	-	0.50 EU/mL	1 *80*	1 *100*	1 *80*	1 *100*	1 *80*	1 *100*	1 *100*	1 *64*	1 *100*	1 *100*	1 *100*
Cefazolin Sodium for Injection	333 mg/mL	0.05 EU/mg	32 *2,677*	32 *3,333*	32 *2,677*	32 *3,333*	128 *3,333*	128 *3,333*	64 *2,677*	32 *2,133*	32 *3,333*	64 *3,333*	64 *3,333*
Tazobactam	450 mg/mL	0.04 EU/mg	128 *2,880*	128 *3,600*	128 *2,880*	128 *3,600*	128 *3,600*	512 *3,600*	256 *2,880*	128 *2,304*	128 *3,600*	64 *3,600*	128 *3,600*
Sodium Bicarbonate Injection	0.833 mEq/mL	5.0 EU/mEq	8 *666*	8 *833*	8 *666*	8 *833*	128[3] *833*	256[3] *833*	8 *666*	8 *533*	8 *833*	8 *833*	8 *833*
Dehydrocholic Acid Injection	100 mg/mL	0.30 EU/mg	32 *4,800*	64 *6,000*	64 *4,800*	64 *6,000*	256 *6,000*	512[3] *6,000*	64 *4,800*	64 *3,840*	64 *6,000*	32 *6,000*	64 *6,000*
Doxorubicin Hydrochloride for Injection	10 mg/mL	2.50 EU/mg	1,024[3] *4,000*	512[3] *5,000*	512[3] *4,000*	1,024[3] *5,000*	512[3] *5,000*	256[3] *5,000*	32 *4,000*	16 *3,200*	16 *5,000*	32 *5,000*	32 *5,000*
Nicardipine Hydrochloride Injection	1 mg/mL	8.33 EU/mg	16 *1,333*	16 *1,666*	16 *1,333*	16 *1,666*	64 *1,666*	32 *1,666*	16 *1,333*	16 *1,066*	16 *1,666*	16 *1,666*	8 *1,666*
Nicotinic Acid Injection	50 mg/mL	3.0 EU/mg	16 *24,000*	32 *30,000*	16 *24,000*	32 *30,000*	16 *30,000*	8 *30,000*	16 *24,000*	16 *19,200*	32 *30,000*	8 *30,000*	16 *30,000*
Papaverine Hydrochloride Injection	40 mg/mL	6.0 EU/mg	512 *38,400*	512 *48,000*	256 *38,400*	256 *48,000*	128 *48,000*	128 *48,000*	64 *38,400*	64 *30,720*	256 *48,000*	128 *48,000*	128 *48,000*
Vancomycin Hydrochloride Injection	100 mg/mL	0.25 EU/mg	64 *4,000*	64 *5,000*	64 *4,000*	128 *5,000*	128 *5,000*	32 *5,000*	32 *4,000*	128 *3,200*	128 *5,000*	128 *5,000*	128 *5,000*
Piperacillin Sodium for Injection	200 mg/mL	0.04 EU/mg	64 *1,280*	64 *1,600*	64 *1,280*	128 *1,600*	64 *1,600*	64 *1,600*	128 *1,280*	64 *1,024*	64 *1,600*	32 *1,600*	64 *1,600*
Famotidine Injection	10 mg/mL	15 EU/mg	8 *24,000*	8 *30,000*	8 *24,000*	16 *30,000*	4 *30,000*	8 *30,000*	4 *24,000*	8 *19,200*	16 *30,000*	16 *30,000*	8 *30,000*
Glucose Injection	50%	0.50 EU/mL	4 *80*	8 *100*	4 *80*	8 *100*	8 *100*	4 *100*	8 *80*	4 *64*	8 *100*	4 *100*	8 *100*
Heparin Calcium	25000 unit/mL	0.003 EU/unit	128 *12,000*	512 *15,000*	4,000 *12,000*	4000[4] *15,000*	> 60,000[3] *15,000*	> 60,000[3] *15,000*	128 *12,000*	8 *9,600*	16 *15,000*	4,000 *15,000*	64 *15,000*
D-Mannitol Injection	20%	0.50 EU/mL	1 *80*	2 *100*	1 *80*	1 *100*	2 *100*	1 *100*	4 *80*	2 *64*	4 *100*	1 *100*	4 *100*
Aciclovir	25 mg/mL	N/A	2	4	4	4	128	256	8	8	4	256	4
Insulin	100 unit/mL	N/A	1	1	1	1	16[3]	64[3]	2	1	2	1	1
PBS (Dulbecco)	-	-	1	1	1	1	1	2	1	1	1	1	1
WFI	-	-	1	1	1	1	1	1	1	1	1	1	1

1) rFC = Recombinant Factor C, ES-50M = Endospecy® ES-50M, KTA = Kinetic Turbidimetric Reagent, KCA = Kinetic Chromogenic Reagent
2) Recoveries were lower than 50% (out of the criteria) when diluted by from 32 to 2048
3) Shadow: Deviate from the range of NID with limulus lysate reagents more than four-fold
4) Sixty minute assay was performed

4,000 for each assay.

The NIDs in the rate and onset assays for the twenty-seven samples tested with both rCRs were lower than or fell within the minimum and maximum range of the NIDs determined by the five lysate reagents. This data suggests that both rCRs and naturally derived lysate reagents have similar resistivity to inhibiting and enhancing factors in samples. When comparing recombinant reagents, the rFC reagents showed higher inhibi-

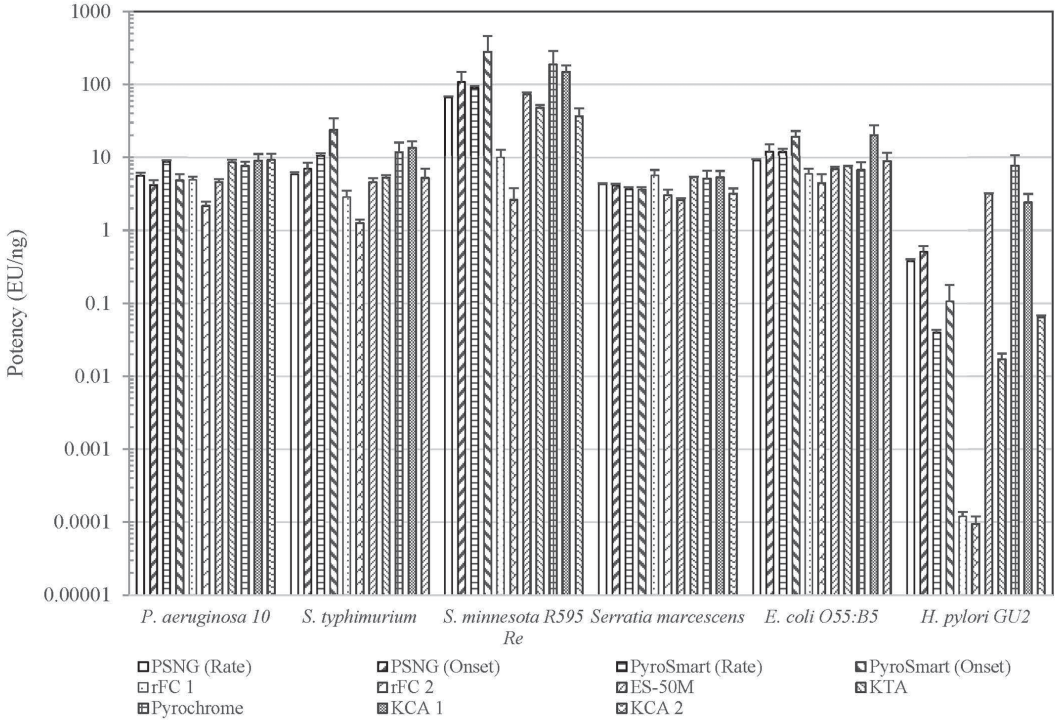

Fig. 1. Comparison of the Potency of Different Endotoxins Measured with Four Recombinant Reagents and Five Limulus Lysate Reagents

Potency is expressed as EU/ng using USP reference standard endotoxin (RSE) as a reference. Standard deviation is expressed with error bars. PSNG (Rate): PyroSmart NextGen® in rate assay, PSNG (Onset): PyroSmart NextGen® in onset assay, PyroSmart® (Rate): PyroSmart® in rate assay, PyroSmart® (Onset): PyroSmart® in onset assay, rFC1: recombinant Factor C (rFC) reagent 1, rFC2: recombinant Factor C (rFC) reagent 2, ES-50M: Endospecy® ES-50M, KTA: Kinetic Turbidimetric reagent, KCA: Kinetic Chromogenic reagent

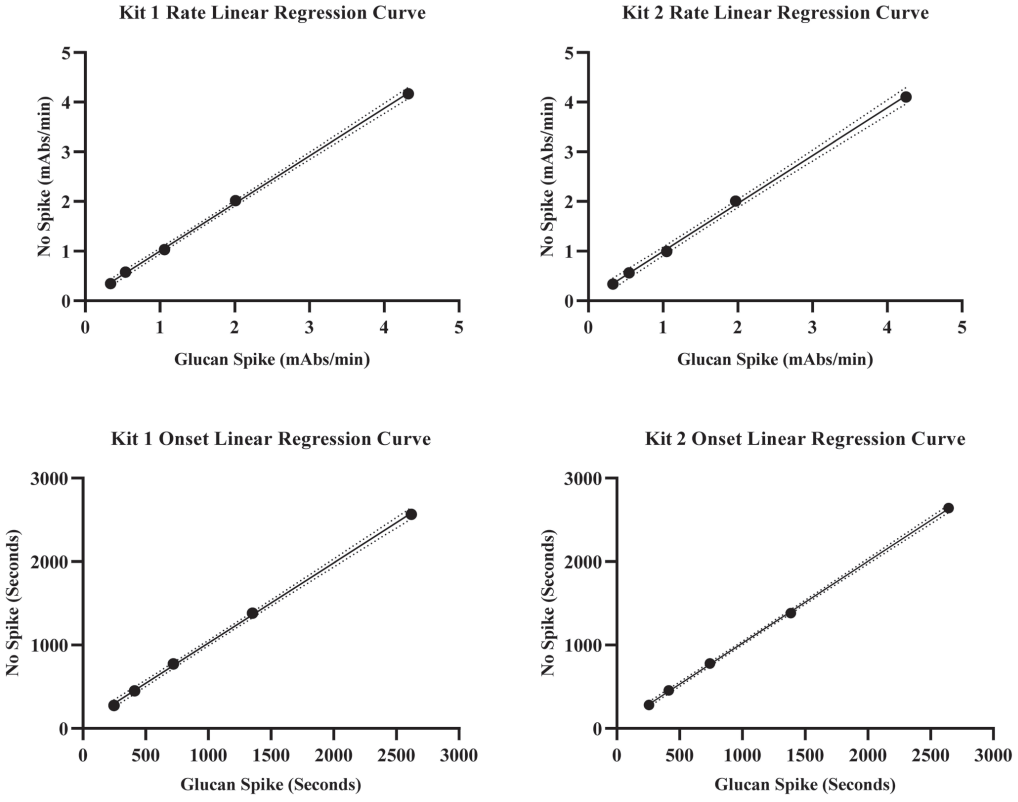

Fig. 2. Regression Analysis of the Reactivity of PyroSmart NextGen® to Endotoxin in the Presence and Absences of (1→3)- β-D Glucan

Top: Rate Assay, Bottom: Onset Assay

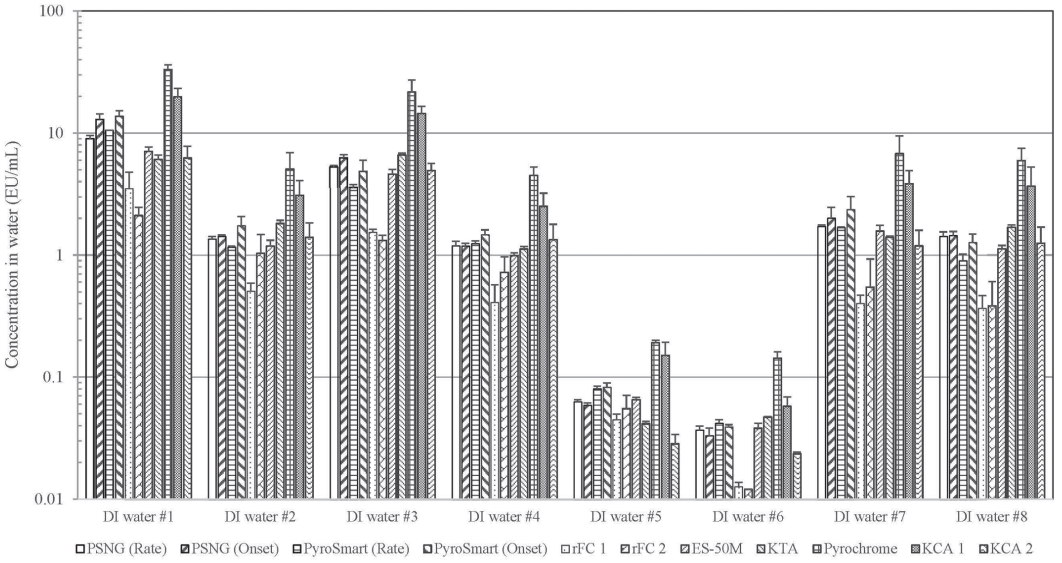

Fig. 3. Comparison of the Autochthonous Endotoxin Level in Deionized Water Collected from Eight Places Measured with Four Recombinant Reagents and Five Limulus Lysate Reagents

Endotoxin level is expressed as EU/mL using USP reference standard endotoxin (RSE) as a reference. Standard deviation is expressed with error bars. PSNG (Rate): PyroSmart NextGen® in rate assay, PSNG (Onset): PyroSmart NextGen® in onset assay, PyroSmart® (Rate): PyroSmart® in rate assay, PyroSmart® (Onset): PyroSmart® in onset assay, rFC1: recombinant Factor C (rFC) reagent 1, rFC2: recombinant Factor C (rFC) reagent 2, ES-50M: Endospecy® ES-50M, KTA: Kinetic Turbidimetric reagent, KCA1: Kinetic Chromogenic reagent 1, KCA2: Kinetic Chromogenic reagent 2

tion when detecting endotoxin in Heparin Calcium, Aciclovir and Insulin than the two rCRs.

Detection of Autochthonous Endotoxin in Water and *E. coli* O113:H10:K Negative Culture Supernatant The autochthonous endotoxin concentration in deionized- and commercially available bottled water samples, and a culture supernatant of *E. coli* O113:H10:K negative was measured by recombinant and lysate reagents (Fig. 3 and 4, respectively). The F-test and t-test ($P = 0.05$) found no significant difference in the detectability of endotoxin in each sample by the recombinant or lysate reagents.

The endotoxin levels detected by both assay modes in samples tested with PyroSmart NextGen® fell within the minimum and maximum range of the five lysate reagents. PyroSmart® detected all autochthonous endotoxin in the onset assay at levels falling within the minimum and maximum range of the five lysate reagents. The detectability of autochthonous endotoxin using the PyroSmart® rate assay was lower in four water samples (DI water #2, 3 and 8, and bottled water) than the five lysate reagents. The detected levels were slightly lower than the lowest level of the lysate reagents by 1.8% (#2; 1.161 EU/mL vs Endospecy® ES-50M 1.182 EU/mL) to 22.4% (#3; 3.578 EU/mL vs Endospecy® ES-50M 4.610 EU/mL). Both rFC reagents detected endotoxin within the minimum and maximum range of those detected by lysate reagents in two out of ten samples, *E. coli* O113:H10:K negative culture supernatant and deionized water #5. The detected endotoxin levels determined with rFC1 and rFC2 reagents were lower than the lowest level detected by the lysate reagents. For example, the minimum difference in rFC1 was 42.5% (#1; 3.490 EU/mL vs KTA 6.067 EU/mL) and the maximum was 67.6% (#8; 0.364 EU/mL vs Endospecy® ES-50M 1.122 EU/mL). The minimum difference in rFC2 was12.5% (#2; 1.034 EU/mL vs Endospecy® ES-50M 1.182 EU/mL) and the maximum was 71.4% (#3: 1.317 EU/mL vs Endospecy® ES-50M 4.610 EU/mL).

DISCUSSION

Endotoxin derived from different bacterial strains had highly variable potencies (0.017 EU/ng of *H. pylori* GU2 versus 47.9 EU/ng of *S. minnesota* R595 Re with KTA) (Fig. 1), which have also been reported in previous literature.[22,33–38] This variance can be attributed to different lipid A moiety structures and polysaccharide length. Endotoxin potency is also highly variable among lysate reagents from 2.0-fold (*P. aeruginosa* 10; Endospecy® ES-50M 4.55 EU/ng vs KCA2 9.27 EU/ng) to 452-fold (*H. pylori* GU2; KTA 0.017 EU/ng vs Pyrochrome® 7.69 EU/ng). This same variability is also observed among the different recombinant reagents. All potencies of the six endotoxins tested with the PyroSmart NextGen® in rate assay fell within the minimum and maximum potency range of the five lysate reagents. The same is true for the PyroSmart NextGen® in onset assay apart from *P. aeruginosa* 10 endotoxin which fell outside the minimum and maximum potency range of the five lysate reagents. The potency of *P. aeruginosa* 10 endotoxin (4.18 EU/ng) was slightly lower than the lowest potency (4.55 EU/ng) determined by the lysate reagent, Endospecy® ES-50M, by only 8.1%. This data suggests that PyroSmart NextGen® can determine endotoxin potencies that are comparable to lysate reagents. The reactivity of PyroSmart NextGen® (Rate: 0.38 EU/ng, Onset time: 0.48 EU/ng) to *H. pylori* endotoxin is of great significance because it shows pronounced improvement compared to PyroSmart® (Rate: 0.04 EU/ng, Onset time: 0.11 EU/ng) by 9.5-fold with the rate assay and 4.4-fold with the onset assay, and furthermore was much higher than those of rFC1 (0.00012 EU/ng) and rFC2 (0.00009 EU/ng) by approximately 3,600 and 4,800-fold, respectively. The improved potency of *H. pylori* endotoxin may be related to the 3-factor cascade reaction as opposed to the single factor reaction in the rFCs as well as the different DNA sequences used to clone each of the recombinant factors used in this

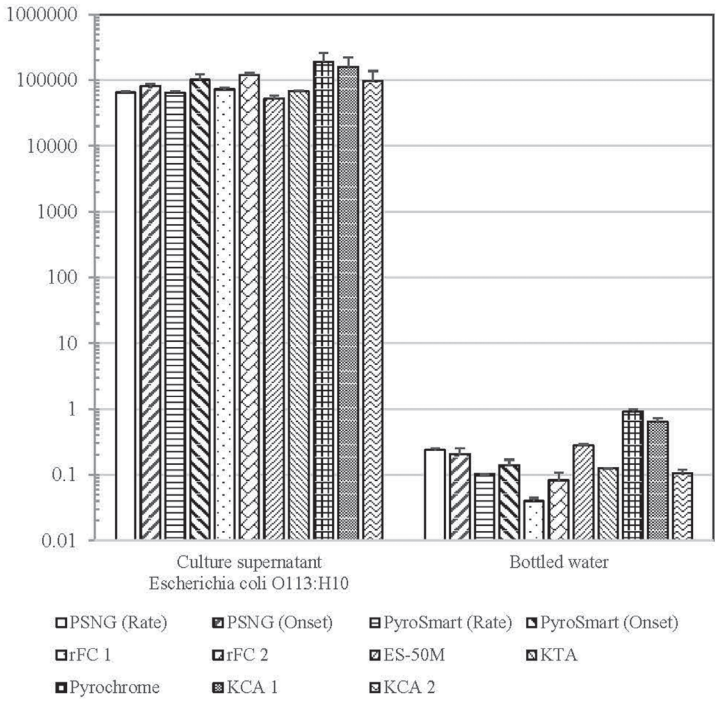

Fig. 4. Comparison of the Autochthonous Endotoxin Level in a Commercially Available Bottled Water and in Supernatant of *E. coli* O113:H10:K (-) Culture with Four Recombinant Reagents and Five Limulus Lysate Reagents

Endotoxin level is expressed as EU/mL using USP reference standard endotoxin (RSE) as a reference. Standard deviation is expressed with error bars. PSNG (Rate): PyroSmart NextGen® in rate assay, PSNG (Onset): PyroSmart NextGen® in onset assay, PyroSmart® (Rate): PyroSmart® in rate assay, PyroSmart® (Onset): PyroSmart® in onset assay, rFC1: recombinant Factor C (rFC) reagent 1, rFC2: recombinant Factor C (rFC) reagent 2, ES-50M: Endospecy® ES-50M, KTA: Kinetic Turbidimetric reagent, KCA1: Kinetic Chromogenic reagent 1, KCA2: Kinetic Chromogenic reagent 2

study.[39] Compared to lysate reagents, the potency of endotoxin from *H. pylori* determined by PyroSmart NextGen® fell within the range of 1/10 the minimum and 10-fold the maximum to those determined with KCA1 (2.42 EU/ng), Endospecy® ES-50M (3.18 EU/ng) and Pyrochrome® (7.69 EU/ng). This data suggests that out of all the recombinant reagents used in this study, only PyroSmart NextGen® has the closest reactivity to the three lysate reagents when determining the potency of *H. pylori* endotoxin.

All minimum non-interfering dilution factors (NIDs) for the twenty-seven injectable and bulk drugs measured with PyroSmart NextGen® as well as PyroSmart® were less than the MVDs of the respective drugs (Table 2). Furthermore, both PyroSmart NextGen® and PyroSmart® had similar reactivity to endotoxin in twenty-six of the twenty-seven samples tested when compared to the five lysate reagents. In comparison to the other recombinant reagents, rFC1 was susceptible to interference in four samples, and rFC2 was susceptible to interferences in six samples. This data suggests that both rCR, PyroSmart NextGen® and PyroSmart® are more resistant to the inhibition and/or enhancement of endotoxin recovery in the samples than both rFC reagents. This testing also shows that PyroSmart NextGen® is capable of mitigating the strong inhibitory interference of Heparin Calcium on endotoxin recovery and becomes capable of detecting endotoxin after diluting it by just 128-fold for the rate assay or 512-fold for the onset assay. Both rate and onset PyroSmart NextGen® assays had NIDs that fell within the minimum and maximum NID (8–4,000-fold) range for the lysate reagents. In comparison, both rFC1 and rFC2 could not detect endotoxin in Heparin Calcium diluted to the MVD. PyroSmart® was capable of measuring endotoxin in Heparin Calcium once diluted 4,000-fold which is the upper limit of the minimum and maximum NID range determined by the lysate reagents, and this NID was approximatly10-fold greater than the NID determined by PyroSmart NextGen®.

This data is consistent with findings in a previous study[27], and the three enzymatic factors present in the rCRs as opposed to the single enzymatic factor present in the rFCs may reduce interference from Heparin Calcium. Heparin Calcium has been reported to interact with factor C and factor B,[40-41] therefore, Heparin Calcium may induce attenuation of the interaction between endotoxin and factor C. Moreover, factor B has been known to bind to endotoxin along with factor C and assist the cascade reaction from factor C to the proclotting enzyme.[42] Therefore, the presence of factor B in PyroSmart NextGen® and PyroSmart® may mitigate the interference from Heparin Calcium. The differences in NID of PyroSmart NextGen® and PyroSmart® may be attributable to the different host cells used to produce some recombinant factors since resistance to interference varies depending on host cells.[20]

The comparability of recombinant reagents to lysate reagents was investigated based on the different detection levels of autochthonous endotoxin in eight deionized and one commercially available bottled water sample along with one sample of *E. coli* O113: H10: K negative culture supernatant (Fig. 3 and 4). PyroSmart NextGen® in both rate and onset assays detected autochthonous endotoxin in all samples, and all of them fell within the minimum and maximum range of the levels detected by lysate reagents. Both rFC1 and rFC2, detected autochthonous endotoxin that fell within the range of the lysate reagents in one out of eight deionized water samples

and the culture supernatant. The PyroSmart® in onset assay detected autochthonous endotoxin in all samples falling within the range of the lysate reagents. However, the PyroSmart® rate assay detected autochthonous endotoxin in 5 out of 8 deionized water samples and the culture supernatant with values falling within the range of the lysate reagents. The improved consistency observed in the ability of the rCRs and lysate reagents to detect autochthonous endotoxin compared to the rFCs may be attributable to the existence of the coagulation cascade reaction present in the rCRs and lysate reagents. It is unclear why PyroSmart NextGen® is superior to PyroSmart® in terms of its ability to detect autochthonous endotoxin, but this may be due to the different ratios of recombinant factors and other components in each reagent.

In conclusion, the recombinant cascade reagent PyroSmart NextGen® has demonstrated its superiority over the other recombinant reagents and is a suitable alternative reagent for performing the BET in the Pharmacopeias. This study has demonstrated compliance with the ICH Q2 guideline, USP <1225> and <85>, equivalency to lysate reagents and improved reactivity to *H. pylori* endotoxin compared to rFC reagents and its predecessor PyroSmart®. Additionally, PyroSmart NextGen® has no reactivity to (1→3)-β-D-glucan which is a well-known trigger for alternative reaction pathways present in lysate reagents making it endotoxin specific. Moreover, incorporation of PyroSmart NextGen® into the BET will significantly contribute to sustainability and conservation efforts currently underway to preserve the global horseshoe crab population.

Conflict of interest Stevens, Kelley, D'Ordine, Akiyoshi and Jahngen are employees of Associates of Cape Cod, Inc., Ogura, Mizumura and Oda are employees of Seikagaku Corporation.

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