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Evaluation of Recombinant Cascade Reagent PyroSmart NextGen® and Limulus Amebocyte Lysate Equivalency in a Plate and Tube Reader for Bacterial Endotoxins Testing

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PyroSmart NextGen® is a recombinant cascade reagent (rCR) for the detection and quantification of bacterial endotoxins developed using cloned genes derived from the *Limulus polyphemus* horseshoe crab genome. Requirements for use of this alternative reagent include analysis of analytical performance, method suitability, and test result equivalency to Limulus amoebocyte lysate (LAL) reagents used in the compendial Bacterial Endotoxins Test (BET). The plate reader evaluation has been expanded to address two long-standing user preferences, with the inclusion of the tube reader method increasing the sensitivity of endotoxin detection from 0.005 EU/mL to 0.001 EU/mL. The utilization of PyroSmart NextGen® with the two different instrument types also allows for a more comprehensive equivalency analysis. Furthermore, the comparison results demonstrate that PyroSmart NextGen® detects equivalent levels of autochthonous endotoxin in water samples when compared to LAL reagents. Overall, this study provides the first large-scale example of equivalency analysis utilizing a robust rCR and has verified that PyroSmart NextGen® meets the expectations for alternative reagents.

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

INTRODUCTION

A key component of medical device and injectable drug testing is the detection and quantification of high-pyrogenicity endotoxins from the cell walls of Gram-negative bacteria. The standard procedure for detecting endotoxin is the Bacterial Endotoxins Test (BET), which is harmonized across three pharmacopeias: the Japanese Pharmacopeia (JP), the United States Pharmacopeia (USP), and the European Pharmacopeia (Ph. Eur.). It utilizes Limulus amoebocyte lysate (LAL) reagents manufactured in accordance with regulations of competent authorities such as the United States Food and Drug Administration (FDA).[1–3] LAL reagents are comprised of serine protease cascade factors isolated from horseshoe crab blood.[4–6] However, BET end-user demand has seen a shift in recent years towards more sustainable testing solutions. There are now two types of commercially available recombinant reagents: recombinant Factor C reagent (rFC) containing only recombinant Factor C, and recombinant cascade reagent (rCR) containing recombinant factor C, recombinant factor B, and recombinant proclotting enzyme.

According to the JP, USP, and Ph. Eur., methods using either rFC or rCR are considered alternative to LAL when testing per monographs, but there is no oversight by a competent authority on the manufacturing of recombinant reagents. Additionally, there have been many discussions surrounding different approaches to the implementation of alternative analytical procedures. For example, USP *General Notices and Requirements* 6.30 states that alternative reagents must be validated for analytical performance according to USP <1225> and results must be equivalent or better when compared to LAL reagents.[7] The FDA *Guidance for Industry* indicates that alternative reagents should meet the method suitability requirements of USP <85>, and equivalency can be demonstrated by comparing LAL and alternative test results of samples containing endotoxin.[8] This study aims to evaluate PyroSmart NextGen® against these requirements for alternative assays.

PyroSmart NextGen®, the successor of PyroSmart®, was developed by Associates of Cape Cod, Inc., and Seikagaku Corporation to improve the reactivity to endotoxin from *Helicobacter Pylori* GU2 and to overcome the assay interference from Heparin Calcium. This reagent has previously met the requirements for method validation with a plate reader instrument.[9] For a comprehensive analysis, the performance and analytical characteristics of PyroSmart NextGen® in a tube reader were evaluated here according to the ICH Q2 guideline and USP <1225>.[10,11] This study is a critical extension of PyroSmart NextGen® testing because it validates the utilization of a tube reader, with a five-fold sensitivity increase from a detection limit of 0.005 to 0.001 EU/mL. Addition of the tube reader method expands the analytical capability of PyroSmart NextGen® and demonstrates its robustness.

This study included equivalency testing of over 100 filtered water samples known to have detectable levels of autochthonous endotoxin with PyroSmart NextGen® and two LAL reagents using the plate and tube reader methods. However, equivalency does not yet have strictly defined guidelines for analysis or acceptance criteria. Therefore, the data collected in this study was evaluated using three different comparison methods and in-house criteria. This approach provides a comprehensive

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analysis of equivalency and an example for future studies.

MATERIALS AND METHODS

Endotoxin USP reference standard endotoxin (USP-RSE) was purchased from the United States Pharmaceutical Convention (MD, USA).

Water Samples One hundred and two water samples were collected from ten towns in Barnstable County, MA, USA and two towns in Pennsylvania, USA. Each water sample had been filtered through home-grade filtration systems available at each location. The samples were stored at 2–8°C for up to ten days based on a stability study (data not shown).

LAL Reagents Pyrochrome® and Pyrotell®-T with Glucashield® buffer were obtained from Associates of Cape Cod, Inc. (MA, USA).

Recombinant Reagent PyroSmart NextGen® was obtained from Associates of Cape Cod, Inc. (MA, USA).

Endotoxin Assays Endotoxin was quantified using recombinant and LAL reagents. All reagents were tested according to their Instructions for Use. The onset time assay mode was used, which measures the time required to reach a threshold optical density. The standard curve was constructed by plotting the log-converted onset time (Y-axis) against the log-converted standard concentration (X-axis) and was used to determine the endotoxin concentration in samples.

Analytical Characteristics of PyroSmart NextGen® in a Tube Reader PyroSmart NextGen® in a tube reader was evaluated for accuracy, precision, specificity, quantitation limit, linearity, and range according to the ICH Q2 guideline and USP <1225>.[10,11] The ICH guideline M10 Bioanalytical Method Validation and Study Sample Analysis and a previous study were referenced when assessing all acceptance criteria.[9,12] This study included 16 assays tested by two analysts using two tube reader instruments over two days with two reagent lots. Preliminary tube reader testing of PyroSmart NextGen® demonstrated a sensitivity of 0.001 EU/mL. Therefore, a ten-fold series of USP-RSE standard curve dilutions from 1 to 0.001 EU/mL with eight replicates each were measured and analyzed.

Equivalency of PyroSmart NextGen® and LAL Reagents Equivalency was evaluated through testing of various filtered water samples with two LAL reagents and PyroSmart NextGen®. Pyrochrome® (chromogenic) was compared to PyroSmart NextGen® in a plate reader, and Pyrotell®-T (turbidimetric) was compared to PyroSmart NextGen® in a tube reader. The water samples were tested by all four methods within the same eight-hour period. Ten-fold standard curves specific to each assay method were generated using USP-RSE (0.01 to 10 EU/mL for the plate reader, and 0.001 to 1 EU/mL for the tube reader). The samples were diluted to a previously determined non-interfering dilution of 50-fold and tested with positive product controls (PPCs) at an endotoxin concentration in the middle of the standard curve. Data analysis was performed utilizing "relative recovery", which is defined as the sample endotoxin concentration determined by PyroSmart NextGen® as a percentage of the endotoxin detected in the same sample determined by an LAL reagent. It is calculated according to the following equation: ([Sample endotoxin concentration determined by recombinant (EU/mL)] ÷ [Sample endotoxin concentration determined by LAL (EU/mL)] x 100).[13] For LAL reagent comparison, the chromogenic LAL reagent results were divided by the turbidimetric LAL reagent results when calculating relative recovery. Linear regression analysis was performed for each method, which involves plotting the sample endotoxin concentration results determined by PyroSmart NextGen® on the Y axis and the sample endotoxin concentration results determined by the LAL reagent on the X axis. Bland-Altman plots were used to illustrate the percent difference against the average endotoxin concentration in samples, which provides specific detail regarding the differences between the reagents.[14]

RESULTS

Analytical Characteristics of PyroSmart NextGen® All analytical test results of the PyroSmart NextGen® tube reader method validation satisfied the acceptance criteria (Table 1). The Quantitation Limit was determined to be 0.001 EU/mL, which increases the reagent sensitivity five-fold compared to the plate reader assay. With the results from a previous study, the analytical performance and suitability of PyroSmart NextGen® as an alternative to LAL reagents have been confirmed.[9]

Equivalency of PyroSmart NextGen® and LAL Reagents A large sample size was tested to provide statistical significance of the resulting data.[13] Out of the 102 samples tested, a total of 68 samples tested using the plate reader method and 74 samples tested using the tube reader method had valid PPC recoveries within 50–200% and were therefore included in further data analysis. Because the valid plate reader samples were sometimes different from the valid tube reader samples, separate analyses were performed for each method. As depicted in Fig. 1, 91% of plate reader samples and 80% of tube reader samples have relative recovery results within 50–200%. Prior to further evaluation, a normality test was performed, and it was determined that the sample endotoxin concentration data should be logarithmically transformed for linear regression and Bland-Altman plot analysis. The linear regression comparison of samples tested in a plate reader resulted in a slope of 0.9873 with a 95% confidence interval (CI) of 0.8779 to 1.097, whereas tube reader testing had a slope of 1.073 with a CI of 0.9769 to 1.170 (Fig. 2). For the Bland-Altman plots illustrated in Fig. 3, the plate reader testing resulted in a bias of −21.43 and 97% of samples were within the 95% upper and lower limits of agreement (LOA), while the tube reader testing had 97% of the data within these limits and a bias of −64.58.

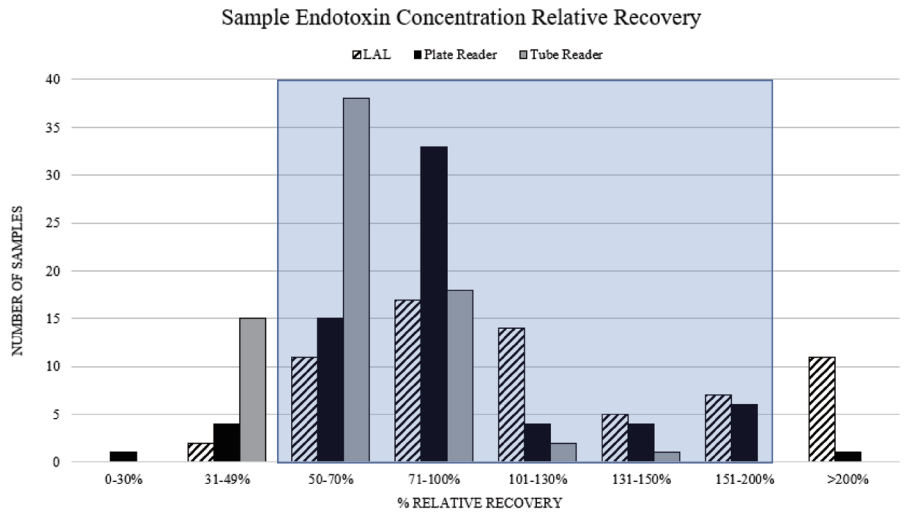
DISCUSSION

Requirements for the use of alternative reagents for BET include demonstration of analytical performance per USP <1225> and method suitability per USP <85>. Once these have been evaluated, multiple comparison methods should be used to determine whether the alternative reagent is equivalent to LAL reagents using product samples containing detectable levels of autochthonous endotoxin.[7,8] A common method of evaluating equivalency is relative recovery, which is a calculation of the percent difference between sample endotoxin concentrations determined by two methods. There is no defined criterion for acceptable relative recovery, thus the USP maximum reagent variability of 50–200% was utilized as a reference.[13] If all samples (100%) have relative recovery results within 50–200%, this indicates complete equivalency between two methods. However, comparison of the LAL reagents in this study resulted in only 54 out of 67 relative recovery results

Table 1. Assessment of PyroSmart NextGen® Analytical Characteristics in a Tube Reader According to the ICH Q2 Guideline

Analytical Characteristics	Results		Acceptance Criteria
	Onset Assay Mode		
1. Linearity (absolute value, correlation coefficient)	**0.001–1 EU/mL** Minimum 0.997 Maximum 0.999		$\|r\| \geq 0.980$
2. Accuracy (recovery)	**EU/mL** 0.001 0.01 0.1 1.0	**Min–Max (%)** 84–101 99–119 111–129 83–94	50–200%
3. Precision			
3-1 Repeatability (CV)	**EU/mL** 0.001 0.01 0.1 1.0	**Min–Max (%)** 7–32 5–14 3–30 3–7	CV ≤ 35% 0.001 EU/mL CV ≤ 30% 0.01–1 EU/mL
3-2 Intermediate Precision (95% CI for CV)	**EU/mL** 0.001 0.01 0.1 1.0	**Min–Max (%)** 15–18 8–9 11–13 5–7	CV ≤ 35% 0.001 EU/mL CV ≤ 30% 0.01–1 EU/mL
4. Range	0.001–1 EU/mL		Precision, accuracy, and linearity at suitable level
5. Quantitation Limit	**At 0.001 EU/mL** Accuracy: 84–101% Repeatability: 7–32%		The lowest concentration of Et that can be quantitatively determined with suitable precision and accuracy

Note: Reproducibility (multiple locations) and specificity were analyzed, and the results met the acceptance criteria in a previous study not included here.



Relative Recovery Range	Percentage of Plate Reader Samples	Percentage of Tube Reader Samples	Percentage of LAL Samples
<50%	7% (5 of 68)	20% (15 of 74)	3% (2 of 67)
50-200%	91% (62 of 68)	80% (59 of 74)	81% (54 of 67)
>200%	1% (1 of 68)	0% (0 of 74)	16% (11 of 67)

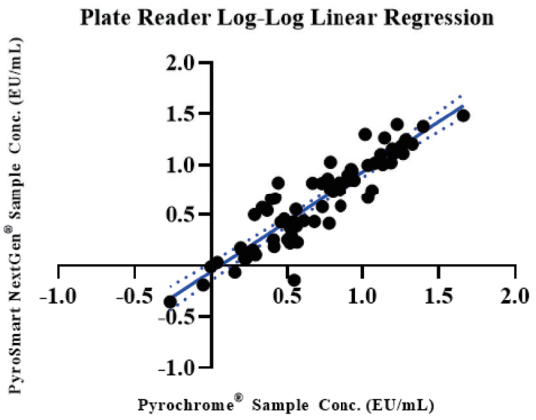
Fig. 1. Summary of Relative Recovery Analysis for Sample Endotoxin Concentration Results Using a Plate Reader, Tube Reader, and a Comparison of Sample Results Tested by the Two LAL Reagents

The graph represents the number of samples that have relative recovery results within each defined range. The blue box highlights all of the data with results within 50–200%.

(81%) within 50–200% (Fig. 1). This data is limited to two specific LAL reagents, hence the percentage of samples with relative recovery results within 50–200% may decrease when considering all types of FDA-licensed LAL reagents (29 total). Furthermore, the evaluation of three separate studies analyzing the endotoxin detected in water samples resulted in an average of 63% of relative recovery results within 50–200%. However, a fourth study illustrated that a significant number of samples had underestimated levels of endotoxin when tested by all recombinant methods.[13] Therefore, as an example to help detect this underestimation when evaluating recombinant reagents, the in-house criterion applied here is at least 70% of relative recovery results should be within 50–200%. PyroSmart NextGen® met this criterion with both methods.

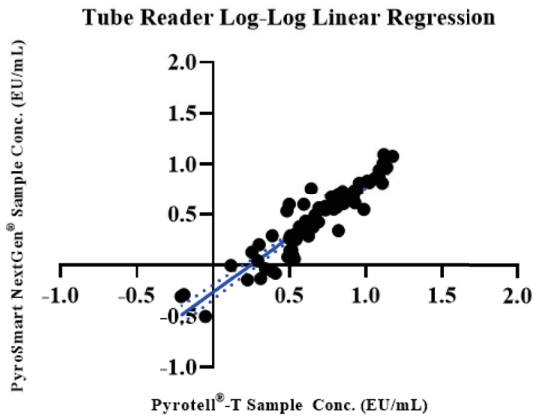
Linear regression analysis is another method of assessing agreement between two sets of data.[15,16] A slope of 1 indicates complete result equivalency, and LAL assay variability allows for results between 50–200% (slope varies from 0.5 to 2.0). However, previous linear regression data comparing endotoxin potencies determined by three LAL reagents resulted in

(a)



Parameter	Result
Slope	0.9873
95% CI of Slope	0.8779 to 1.097
R squared	0.8311

(b)

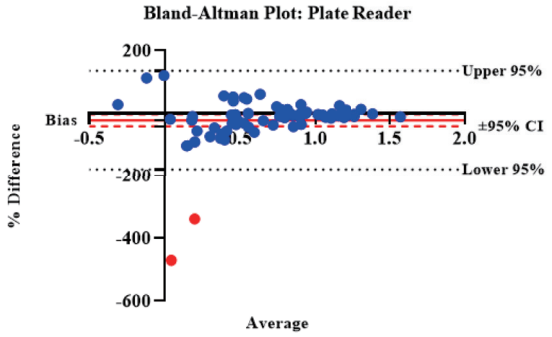


Parameter	Result
Slope	1.073
95% CI of Slope	0.9769 to 1.170
R squared	0.8725

Fig. 2. (a) Linear Regression Analysis of the Endotoxin Concentration in Samples Tested by Pyrochrome® Compared to Those Tested by PyroSmart NextGen®, Both Performed Using a Plate Reader Instrument. (b) Linear Regression Analysis of the Endotoxin Concentration in Samples Tested by Pyrotell®-T Compared to Those Tested by PyroSmart NextGen®, Both Performed Using a Tube Reader Instrument

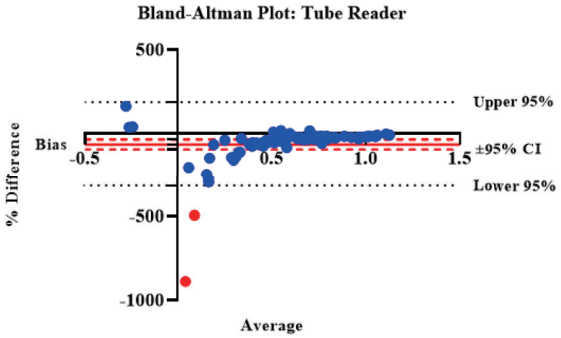
The solid blue lines represent the slope, and the dotted blue lines are the 95% confidence interval (CI) of the slope.

(a)



Parameter	Result
Bias	-21.43
95% CI of Bias	-40.80 to -2.062
% of Data Points within 95% LOA	97% (66 of 68)

(b)



Parameter	Result
Bias	-64.58
95% CI of Bias	-94.46 to -34.69
% of Data Points within 95% LOA	97% (72 of 74)

Fig. 3. (a) Bland-Altman Plot Analysis of the Endotoxin Concentration in Samples Tested in a Plate Reader with Pyrochrome® Compared to Those Tested with PyroSmart NextGen®. (b) Bland-Altman Plot Analysis of the Endotoxin Concentration in Samples Tested in a Tube Reader with Pyrotell®-T Compared to Those Tested with PyroSmart NextGen®

A bias (solid red line) of zero indicates that the two methods have identical results. The red dotted lines are the 95% confidence interval (CI) of that bias. The red data points indicate that they are outside of the 95% upper and lower limits of agreement (LOA, black dotted lines).

slopes ranging from 0.611 to 1.463 with an average of 1.03.[17] Therefore, the criterion applied here is the linear regression slope should be within 0.7 and 1.3 to ensure equivalency and encompass typical assay variability for each method. As shown in Fig. 2, the criteria for the slope were met for both the plate reader and tube reader comparisons.

The ICH guideline M10 indicates that Bland-Altman plots are also appropriate for determining whether two methods are equivalent.[12] They provide more specific details when compared to the agreement determined by linear regression analysis because the percent difference is plotted against the average of the sample endotoxin concentration results. The bias between these mean differences signifies whether one assay has higher results than another, thus a bias line of zero equates to 100% equivalency. The plots also estimate upper and lower LOA, which include 95% of the differences between the two methods. Compared to a linear regression slope, the Bland Altman LOA are stronger indicators of method equivalency because they specifically evaluate whether the differences between methods are significant rather than simply determining the level of agreement.[18] The criterion used to demonstrate equivalency in this instance is at least 95% of all data

points should fall within this interval. Comparison between PyroSmart NextGen® and LAL reagents for both the plate and tube reader methods met this criterion.

As exemplified here, alternative recombinant methods must have evidence to support equivalency to LAL reagents. When choosing which recombinant reagent to validate and implement, product quality and development aspects should also be examined using a risk-based approach. All documentation should be available for review to determine if risk management was included as part of the recombinant reagent product development, and a Design Control or Quality by Design approach should be used to ensure a product quality equivalent to that of LAL reagents. The recombinant reagent should be manufactured under an appropriate quality management system that meets International Organization for Standardization and/or current Good Manufacturing Practice standards. This quality management system should be applied to the establishment and maintenance of the master cell bank with standardized procedures to ensure supply sustainability. A consistent supply of the same product is essential, as any changes to the recombinant product by the manufacturer can greatly impact the test results. For an end-user, implementation requirements as well as quality product development and manufacturing of the recombinant reagent are essential details that should be considered.

The incorporation of the tube reader method enhances the capabilities of PyroSmart NextGen® by increasing the assay sensitivity five-fold when compared to the plate reader assay, and by providing more options for the end-user. Additionally, it allows for direct equivalency analysis with various LAL reagents. Comparison of PyroSmart NextGen® and two LAL reagents has met all criteria stated here, hence the PyroSmart NextGen® reagent is considered equivalent for both the plate reader and tube reader methods. These results and the method validation data provide sufficient evidence that the expectations for alternative assays per USP *General Notices and Requirements* 6.30 and the FDA *Guidance for Industry* have been met.[7,8] The development and manufacturing processes of the reagent also meet all quality standards described. Therefore, PyroSmart NextGen® is an ideal candidate when choosing a recombinant reagent for implementation.

Conflict of interest Kelley, Stevens, Marchessault, Akiyoshi are employees of Associates of Cape Cod, Inc. Jahngen is a consultant to Associates of Cape Cod, Inc.

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