A validation study of Qubit and Quant-iT Endotoxin Detection Assays as an alternative method for bacterial endotoxin testing

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Introduction

Bacterial endotoxin testing (BET) is the process of detecting endotoxins, a frequent contaminant of recombinant proteins and nucleic acids purified from gram-negative bacteria such as E. coli. Chromogenic and turbidimetric assays that use Tachypleus or Limulus Amebocyte Lystate (TAL/LAL) are commonly used methods for BET. While this technique is recognized in the United States Pharmacopeia (USP) Chapter <85>, there are 'alternative methods' described in USP Chapter <1085> that can provide key benefits. USP Chapter <1225> defines the validation criteria required for alternative test methods to meet the standards set by pharmacopeial organizations. While each country has their own regulatory guidelines, the United States Pharmacopeia guidelines for BET are aligned with the European and Japanese Pharmacopeias.

Here we describe the key benefits of the Qubit™ and Quant-iT™ Endotoxin Detection Assays, and provide a summary of the validation process and results from a validation experiment.

Differentiated Assay Benefits

Qubit and Quant-iT Assays deliver comparable results to chromogenic LAL assays yet offer several benefits over these methods:

- High sensitivity and broad dynamic range – detect as little as 0.01 EU/mL to 10.0 EU/mL
- Flexible – suitable for wide range of samples, including protein, antibody, or nucleic acid samples
- Compatibility – can be used with a fluorescence microplate reader or benchtop fluorometer
- Easy to use – when paired with the Qubit Flex Fluorometer, calculations are performed automatically reducing the potential for error

Validation Process

While Qubit and Quant-iT Endotoxin Detection Assays rely on amebocyte lysates, the pharmacopeial guidelines currently consider fluorogenic detection as an 'alternative method'. As a result, additional validation steps are required to show that the results from these assays are comparable to those achieved by established TAL/LAL compendial methods.

Endotoxin detection assays are considered a category II analytical method. For this category, USP <1225> and International Council for Harmonization (ICH) Q2B require users to demonstrate specificity, precision, accuracy, linearity, detection limit, quantification limit, range, and robustness.

Materials and Methods

All tests were carried out using a reference standard endotoxin (*E. coli* 0113:H10:K LPS) with pyrogen-free materials.

Qubit and Quant-iT Endotoxin Detection Assays (Cat. Nos. Q32891 and Q32892) were tested for performance across the quantification ranges alongside various other commercially available endotoxin assays. First, the Qubit and Quant-iT assays were tested for robustness using three lots of material and evaluated for precision, accuracy, and linearity. Next, samples were analyzed with established compendial methods using chromogenic LAL-based assays. Lastly, samples were evaluated against other alternative test methods. In all cases, protocols from each manufacturer were followed and the same samples were used for each assays testing.

Metric	Evaluation	Acceptance Criteria
Accuracy	Calculated as the percent recovery of a known amount of 'spiked-in' analyze in the sample and involves comparing the new procedure results with those achieved using a previously validated method.	Results should be within 50-200% of the known sample concentration and within 25% of the expected value.
Precision	Degree of agreement between replicates measurements	Coefficient of variance (CV) < 15%
Linearity	The standard curve of multiple lots must adhere to a linear model	Linear correlation coefficient, *r*, ≥ 0.980 for the standard curve that contains a blank, low, medium, and high concentration.
Specificity	Ability to detect endotoxin in the presence of components that may be present in routine samples	Known impurities, when present within reported tolerances, must not interfere with results (50-200% of known concentration)
Range*	The upper and lower limits of the assay to quantify endotoxin while meeting the needs for accuracy, precision, and linearity.	Verified by confirming that the low and high detection values can meet ≤ 25% relative error (accuracy), ≤15% CV (precision), and *r* ≥ 0.980 (linearity).
Detection Limit*	The lowest sample value that can be detected	Z score ≥ 0 where the background and lowest sample concentration are determined, and the average and standard deviation of those measurements are determined to be separable with 95% confidence.
*Specific to user application needing to at least address the endotoxin release limit.		

Validation of Qubit and Quant-iT Assays

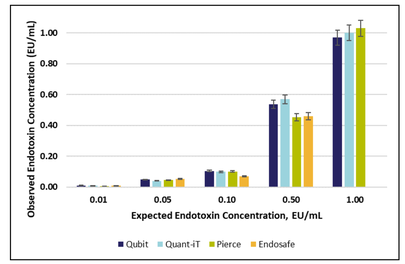
Three lots of the Qubit and Quant-iT Endotoxin Detection Assays were evaluated for key performance metrics as defined by testing criteria for category II analytical methods. For each lot, five concentrations were tested across the dynamic range of the assay as well as a background. For each sample, four replicates were tested. Spike tests were performed with pDNA (Cat. No. SD0041) at 1 µg/mL using an expected concentration of 0.5 EU/mL. Negative controls were assessed using 50 uL of endotoxin-free water as a sample. The data is summarized below.

Metric	Criteria	Results		
		Lot 1	Lot 2	Lot 3
Accuracy	Error ≤ 25% vs expected value	0.01 ±6% 0.05 ±7% 0.10 ±4% 0.50 ±9% 1.00 ±5%	0.01 ±7% 0.05 ±6% 0.10 ±2% 0.50 ±9% 1.00 ±3%	0.01 ±10% 0.05 ±3% 0.10 ±8% 0.50 ±3% 1.00 ±3%
Precision	CV ≤ 10% among replicates	0.01 ±8% 0.05 ±2% 0.10 ±4% 0.50 ±8% 1.00 ±3%	0.01 ±9% 0.05 ±6% 0.10 ±2% 0.50 ±9% 1.00 ±3%	0.01 ±10% 0.05 ±3% 0.10 ±8% 0.50 ±6% 1.00 ±3%
Linearity	*r* ≥ 0.980	0.99998	0.99996	0.9997
Negative control	Blank ≤ 0.075 EU/mL	0.008 EU/mL	0.0004 EU/mL	0.0024 EU/mL
Spike Control	50 ≤ x ≤ 200%	102%	103%	98%

Comparison to Existing Methods

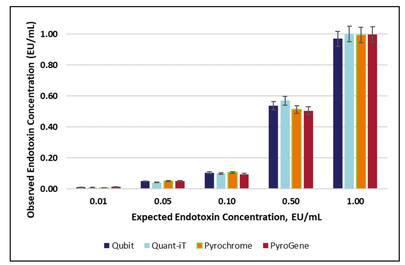
Two assays that currently fall into the compendial method qualification for endotoxin testing were assayed against the new Qubit and Quant-iT assays using the same samples in order to compare results. The Pierce™ Chromogenic Endotoxin Quant Kit (A39553), an endpoint chromogenic LAL-based assay was tested at 5 concentrations over the detection range of the assay. For all concentrations tested with the Qubit and Quant-iT assays, results were within 10% of the expected endotoxin concentration. Average sample concentrations measured with Qubit and Quant-iT assays were also within 50-200% of measurements made with other assays - with most comparisons ranging between 80-120%.

Additionally, the Charles River EndoSafe® nexgen-PTS™ system, with 0.50 – 0.05 EU/mL detection was tested. This assay features a kinetic read system that is chromogenic and based on LAL. The results from this assay were consistent among replicates and within range of the testing criteria. As with the previous testing, the results were consistent between the different assay types tested.



Comparison to Other Alternative Methods

Additional testing was performed to compare the Qubit and Quant-iT assays with other alternative testing methods. The Lonza PyroGene® Assay was tested, which uses fluorescence as a readout along with recombinant factor C as an alternative to amebocyte lysate. Additionally, the PyroSmart NextGen® assay from Associates of Cape Cod which features a recombinant cascade reagent was tested. For all concentrations tested with the Qubit and Quant-iT assays, results were within 10% of the expected endotoxin concentration. Average sample concentrations measured with Qubit and Quant-iT assays were also within 50-200% of measurements made with other assays - with most comparisons ranging between 80-120%.



Conclusion

There are a variety of current methods to quantify bacterial endotoxins. Alternative methods to compendial bacterial endotoxin testing assays can be used if they provide advantages in terms of accuracy, sensitivity, precision, selectivity, or adaptability to automation or computerized data reduction. United States Pharmacopeia (USP) <1225> and International Council for Harmonization (ICH) Q2B provide well-structure guidance on validating testing for alternative methods. Qubit and Quant-iT Endotoxin Detection Assays demonstrate performance comparable to existing compendial assays when validated using USP <1225> and ICH Q2B recommendations.

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