

A Recombinant Factor C Procedure for the Detection of Gram-negative Bacterial Endotoxin

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ABSTRACT This *Stimuli* article addresses a modified photometric procedure for the detection of gram-negative bacterial endotoxin (lipopolysaccharide). This procedure is based on the activation of recombinant Factor C (rFC), the endotoxin-sensitive protease that initiates the traditional *Limulus* Amebocyte Lysate (LAL) cascade. The rFC procedure described in this *Stimuli* article is a single-step, quantitative endpoint procedure that measures the enzyme-mediated cleavage of a fluorogenic substrate. This study shows that the rFC procedure is equivalent to LAL in its ability to measure endotoxin and has a quantitation range comparable to that of quantitative photometric LAL procedures. However, unlike LAL, which uses an extract from the blood cells of the horseshoe crab *Limulus polyphemus* that contains Factor C, the rFC procedure uses an rFC cloned from the horseshoe crab, *Carcinoscorpius rotundicauda*, thereby reducing the number of false-positive results involving glucans.

RATIONALE

The majority of parenteral drugs and implantable medical devices are tested for gram-negative bacterial endotoxin using reagents prepared from circulating amebocytes found in the blood of the American horseshoe crab, *Limulus polyphemus*. Variations of this procedure are described in *US Pharmacopeia (USP) General Chapter Bacterial Endotoxins Test* (85) (1). This chapter describes the gel-clot *Limulus* Amebocyte Lysate (LAL) procedure and the various kinetic and endpoint photometric LAL procedures. A comparison of a kinetic turbidimetric LAL, kinetic chromogenic LAL, and the proposed recombinant quantitative photometric procedure that is not subject to false-positive reactions involving glucans, is the subject of this *Stimuli* article. This alternative procedure has been successfully validated according to the requirements described in *USP General Information Chapter Validation of Compendial Procedures* (1225) (2).

Mechanism of Reaction

The protease cascade and rationale of traditional LAL procedures have been extensively investigated (3–7). Factor C (FC), the first component in the LAL cascade, is a protease zymogen that is activated by endotoxin binding (5, 6, 8). Following a series of cascading events, a dose-dependent response is seen in the presence of endotoxin (Figure 1a, LAL–Endotoxin). In the kinetic turbidimetric variation of the LAL procedure, in the presence of endotoxin the protein coagulogen is converted to coagulin, resulting in a dose-dependent increase in turbidity. In the kinetic chromogenic version of the LAL procedure, a synthetic chromogenic substrate is cleaved to produce a yellow color. An alternative LAL-based pathway triggered by glucans can also invoke a similar response, thus causing a false-positive result (Figure 1, LAL–Glucan).

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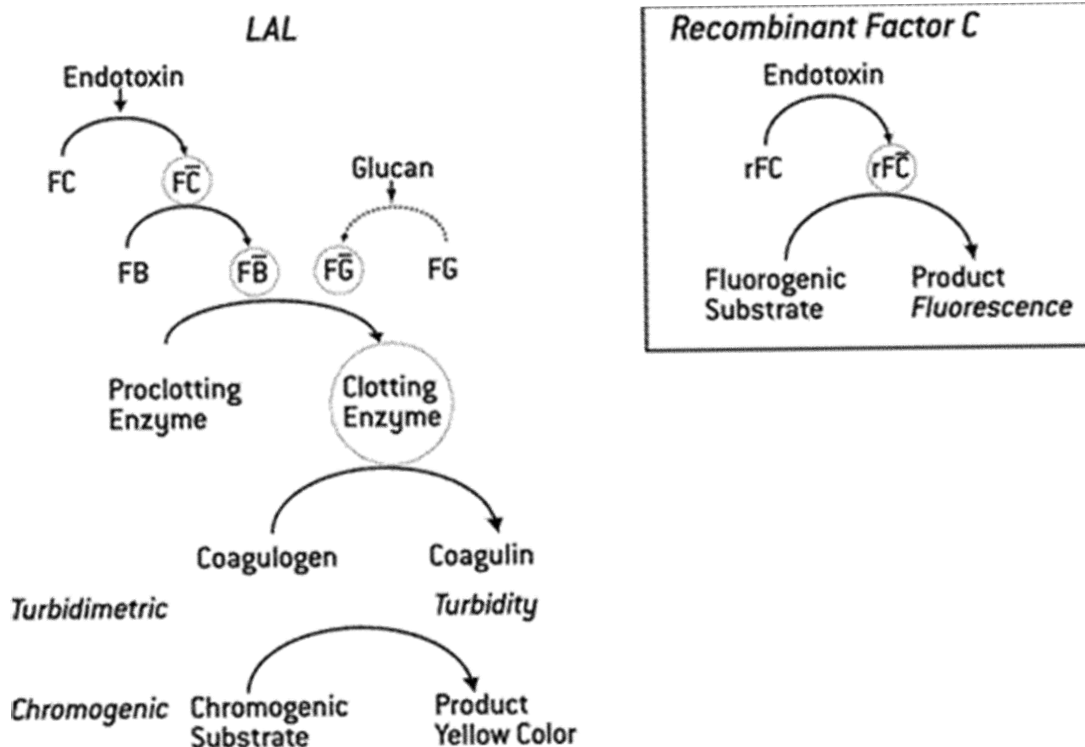


Figure 1a. LAL procedure. Figure 1b. (insert) rFC procedure.

The rFC procedure (Figure 1b) utilizes a single protein as its active ingredient, rFC, cloned from the horseshoe crab *Carcinoscorpius rotundicauda* (8–9). In the rFC procedure endotoxin binding activates rFC. This activated molecule then acts to cleave a fluorogenic substrate, resulting in the generation of a fluorogenic compound. The rFC endotoxin detection reaction is not subject to false-positive reactions involving glucans (Figure 1b, Recombinant Factor C). The rFC protein derived from *C. rotundicauda* shares a 90.5% identical amino acid sequence with the native Factor C protein derived from *L. polyphemus* (unpublished data).

ANALYTICAL PROCEDURE

Precaution: All materials used in this procedure must be endotoxin free.

Recombinant FC is used in a single-step, fluorogenic endpoint procedure to measure endotoxin. One hundred microliters of water blank, standard, and sample are added to a microplate. The microplate is then pre-incubated in a fluorescent microplate reader at $37^{\circ} \pm 1^{\circ}\text{C}$ for 10 min, followed by addition of 100 μL rFC/substrate working reagent to each well. A time zero reading is taken (excitation/emission wavelengths 380/440 nm). The microplate is further incubated at $37^{\circ} \pm 1^{\circ}\text{C}$ for 60 min in the fluorescent microplate reader. At elapsed incubation time of 60 min, the microplate is read a second time (excitation/emission wavelengths 380/440 nm). Corrected fluorescence (fluorescence reading at 60 min minus fluorescence reading at time zero) of endotoxin

standards and samples are then normalized by the corrected fluorescence of the water blank. The log of the normalized fluorescence of the endotoxin standards is plotted vs the log of endotoxin concentration. Generation of the standard curve is performed using linear regression (least squares). The log increase in fluorescence (net change in relative fluorescence units) vs the log endotoxin concentration is linear over the 0.01 to 10 EU/mL range (EU = endotoxin unit). Endotoxin in samples is predicted using the endotoxin standard curve.

DATA ELEMENTS

Specificity

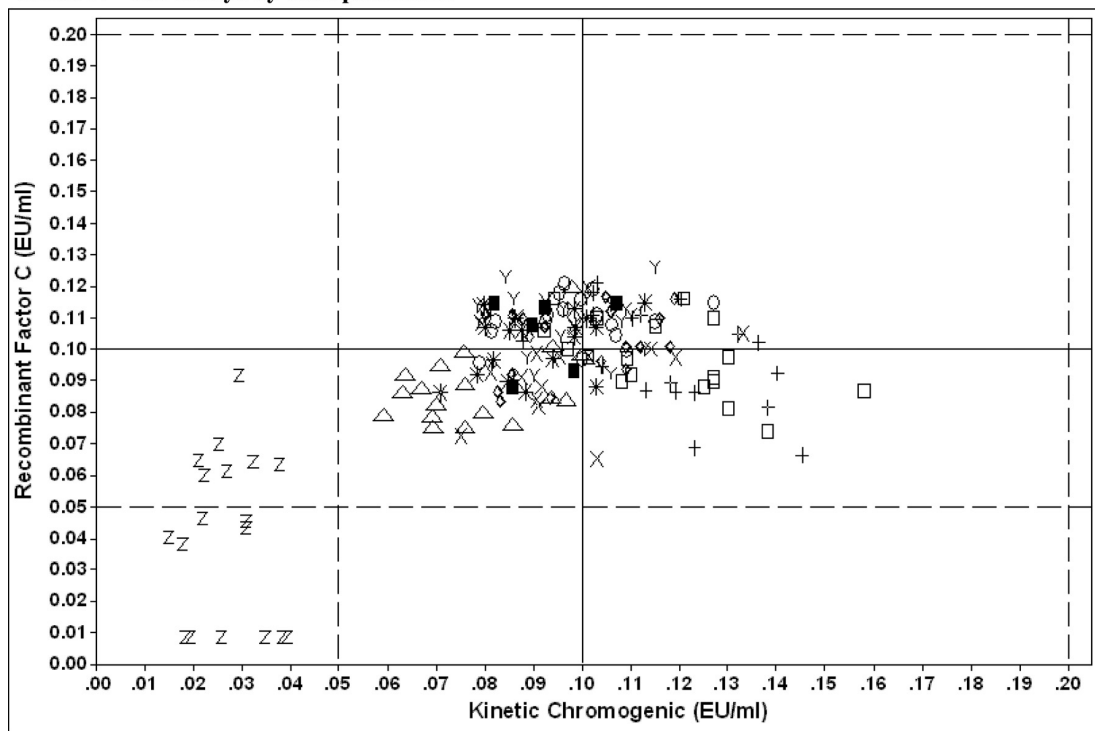
As defined in (1225), specificity is the “ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.” In the case of analytical procedures for impurities, specificity may be established “by spiking the drug substance or product with appropriate levels of impurities.”

A multicenter study (6 centers, 3 analysts/center) was initiated to test 10 pharmaceutical or related products using both the rFC and the kinetic chromogenic LAL procedures. Products were tested at maximum valid dilution (MVD), MVD/2, and MVD/10 (see (85) for further details), except for Water for Injection, which was tested undiluted. Parallel samples were tested with endotoxin spiked to a final concentration of 0.1 EU/mL. One hun-

dred sixty-eight measurements were made with the unspiked samples across the 6 study sites for each procedure. For both the rFC and kinetic chromogenic procedures, no detectable endotoxin was observed in any of the unspiked samples (data not shown). This was expected because all test samples were pharmaceuticals released to the marketplace (except for hemodialy-

sate). For measurements of samples spiked to a nominal value of 0.1 EU/mL, spike recoveries in the range of 0.05 to 0.20 EU/mL (50%–200%) are considered acceptable, or “passing” according to requirements in (85). Except for the Human Albumin (HA) samples, all of the measurements made with each procedure were within the acceptable range of 0.05 to 0.20 EU/mL (Figure 2).

Note: N = 168 assays by each procedure



Symbol legend:

- | | |
|--------------------------------------|------------------------------------|
| ■ Water for Injection | △ Gentamicin Sulfate Injection USP |
| + Lactated Ringer's Injection USP | ○ Insulin Human Injection USP |
| × 0.9% Sodium Chloride Injection USP | ★ Vancomycin HCl USP |
| ◇ 50% Dextrose Injection USP | Y Erythropoietin |
| Z Albumin (Human) USP 25% Solution | □ Hemodialysate |

Figure 2. rFC vs kinetic chromogenic LAL: comparison of results for 10 products spiked to 0.1 EU/mL.

With the kinetic chromogenic procedure, all 18 measurements on the HA sample were outside the acceptable range and below 0.05 EU/mL. With the rFC procedure, 7 of the HA measurements were within the acceptable range, and 11 were outside and below 0.05 EU/mL. HA is known to be inhibitory in the LAL-endotoxin reaction due to proteinacious components in the preparation. Other means of circumventing this inhibitory property

of HA were not included as part of this study, offering a more direct examination of any inhibitory/enhancement effects across the two analytical procedures.

The two procedures were further compared by determining for each procedure the percent of assay results that were within $\pm 25\%$ of the nominal 0.1 EU/mL spike. Table 1 shows that a significantly ($P < 0.01$) greater proportion of the rFC results (85.7%) were within $\pm 25\%$ of the target concentration than were the proportion of chromogenic results (75.0%).

Table 1. rFC and Chromogenic Assay Results within ± 25% of Spike Value

Note: N = 168 assay results by each procedure		
	Number and % of Results within ± 25% of 0.1 EU/mL Spike	Number and % of Results beyond ± 25% of 0.1 EU/mL Spike
rFC	144 85.7%	24 14.3%
Chromogenic	126 75.0%	42 25.0%

The proportion of results within ± 25% of 0.1 EU/mL is greater for the rFC procedure than for chromogenic results; *P* < 0.01, 1-tailed Fisher’s Exact Test.

LAL-reactive Material

LAL-reactive Material (LRM) consists of cellulosic material derived from sources such as Cuprophan hemodialysis filters (CF Capillary Flow Dialyzer, Model CF23, Baxter, Deerfield, IL). LRM contains glucans of various linkages, and the β-1,3 linkage is a significant contributor to the LRM false-positive responses in LAL-based procedures (10). With an LRM preparation prepared according to the procedure described by Pearson et al. (11), the rFC assay has been previously shown to have no significant response to LRM when compared to an LAL-based kinetic chromogenic procedure (12).

Response to Different Endotoxin Sources

A comparison was made regarding the response of rFC, kinetic chromogenic LAL, and turbidimetric LAL photometric procedures to different sources of purified endotoxin. Sources tested were: EC-6 (the internationally recognized Reference Standard Endotoxin which is distributed by USP and is derived from *Escherichia coli* stain O113:H10:K(-), *E. coli* O55:B5, *Pseudomonas aeruginosa* F-D Type 1, and *Salmonella minnesota* R595 (Re). Three assays were performed for each endotoxin–procedure combination. The results are summarized in Figure 3 and Table 2.

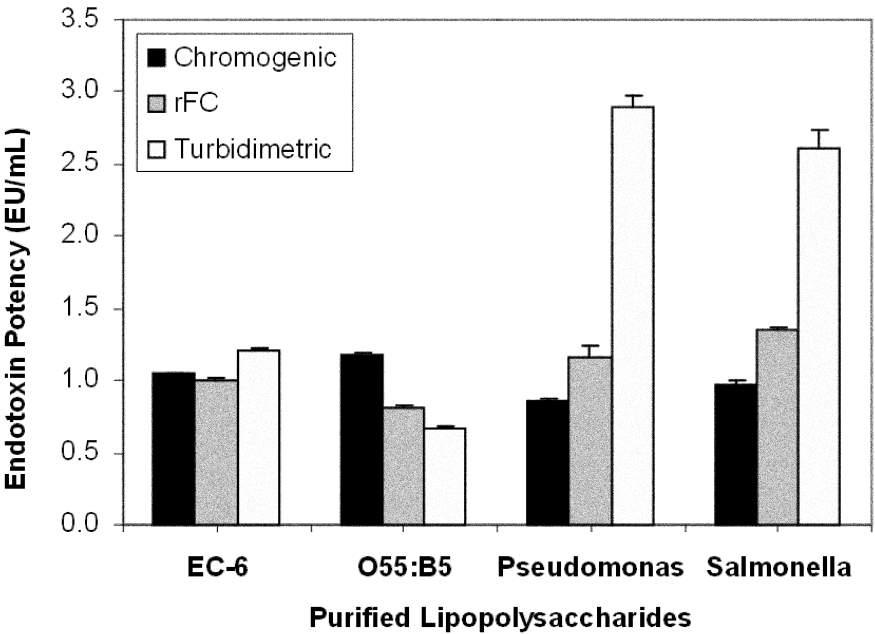


Figure 3. Endotoxin response to different source materials.

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Table 2. Results for Different Endotoxin Source Materials

Endotoxin Source	Procedure	Mean ^a (EU/mL)	Standard Error (EU/mL)	Mean Comparisons ^b
EC-6	Chromogenic	1.04	0.012	Turbidimetric Mean > Chromogenic and rFC Mean ($P < 0.05$)
	rFC	1.00	0.017	
	Turbidimetric	1.20	0.015	
O55:B5	Chromogenic	1.18	0.023	Chromogenic Mean > rFC Mean > Turbidimetric Mean ($P < 0.05$)
	rFC	0.81	0.021	
	Turbidimetric	0.67	0.007	
Pseudomonas	Chromogenic	0.86	0.014	Turbidimetric Mean > rFC Mean > Chromogenic Mean ($P < 0.05$)
	rFC	1.16	0.071	
	Turbidimetric	2.90	0.081	
Salmonella	Chromogenic	0.98	0.029	Turbidimetric Mean > rFC Mean > Chromogenic Mean ($P < 0.05$)
	rFC	1.36	0.015	
	Turbidimetric	2.61	0.131	

^a N = 3 assays for each endotoxin–procedure combination.

^b Multiple comparisons among each pair using Student's *t*-statistic.

The two LAL-based procedures gave statistically significant different mean responses for all four endotoxin sources. The turbidimetric response was greater than the chromogenic response for all sources except O55:B5, where the chromogenic response was greater.

For the O55:B5, *Pseudomonas*, and *Salmonella* sources the rFC mean response was significantly different from the two LAL-based procedures but fell between the two. With EC-6 there was not a significant difference between the rFC and chromogenic procedures, but both of these were significantly less than the turbidimetric results although the magnitude of the difference was small.

Recombinant FC recognizes endotoxin from different source materials as do other photometric procedures, demonstrating equivalency to the LAL-based procedures.

Precision

As defined in (1225), the precision of an analytical procedure “is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogeneous sample. The precision of an analytical method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements.”

Comparative precision testing was performed with the rFC and kinetic chromogenic procedures at three test centers, by three analysts at each test center. Each analyst measured endotoxin samples from the three regions of the 0.01 to 10 EU standard curve (low = 0.0316 EU/mL, medium = 0.316 EU/mL, and high = 3.16 EU/mL) over the course of three different days.

Table 3 summarizes the precision study results. Note that total variation [measured standard deviation or percent coefficient of variation (%CV)] from this study is the sum of the variation among replicates within assay, variation among days, variation among analysts, and variation among locations. For the rFC procedure, observed total CV was 20% for the low concentration, 18% for the medium concentration, and 11% for the high concentration. Each of these is less than the corresponding CVs obtained with the kinetic chromogenic procedure. A significant difference between procedures was not detected for the low and medium concentrations, but at the high concentration rFC total variation was significantly less than chromogenic total variation ($P < 0.02$).

Table 3. Precision Study Analysis Summary

rFC		Low Sample 0.0316 EU/mL			Medium Sample 0.316 EU/mL			High Sample 3.16 EU/mL		
Location	N	Mean (EU/mL)	Standard (EU/mL)	%CV ^a	Mean (EU/mL)	Standard (EU/mL)	%CV	Mean (EU/mL)	Standard (EU/mL)	%CV
1	18	0.0291	0.0045	14	0.403	0.049	16	3.52	0.32	10
2	18	0.0276	0.0050	16	0.365	0.052	16	3.45	0.34	11
3	18	0.0365	0.0056	18	0.410	0.058	18	3.65	0.40	13
Over Locations	54	0.0311	0.0051 ^b	16	0.392	0.053	17	3.54	0.35	11
			0.0063 ^c	20		0.056	18		0.36 ^d	11
Chromogenic		Low Sample 0.0316 EU/mL			Medium Sample 0.316 EU/mL			High Sample 3.16 EU/mL		
Location	N	Mean (EU/mL)	Standard (EU/mL)	%CV ^a	Mean (EU/mL)	Standard (EU/mL)	%CV	Mean (EU/mL)	Standard (EU/mL)	%CV
1	18	0.0350	0.0033	10	0.446	0.060	19	3.22	0.54	17
2	18	0.0327	0.0069	22	0.403	0.066	21	3.02	0.42	13
3	18	0.0420	0.0067	21	0.416	0.064	20	3.41	0.49	16
Over Locations	54	0.0366	0.0059 ^b	19	0.421	0.063	20	3.22	0.49	16
			0.0070 ^c	22		0.065	21		0.50 ^d	16

^a All %CVs were calculated using the known concentrations of the samples rather than the measured mean responses.^b Pooled within location standard deviation.^c Total standard deviation over locations.^d For the High sample, rFC total variation is significantly less than chromogenic total variation ($P < 0.02$). There is not a significant difference in total variation for the Low and Medium samples.

Accuracy of Individual Results

As defined in (1225), the accuracy of an analytical procedure "is the closeness of test results obtained by that procedure to the true value. The accuracy of an analytical procedure should be established across its range."

Accuracy of individual assay results was assessed using the data from the precision study, which used three known concentrations of EC-6 (the current Reference Standard Endotoxin) as an endotoxin source. The three samples tested had concentrations of 0.0316 EU/mL, 0.316 EU/mL, and 3.16 EU/mL and were tested at three locations, by a total of nine different analysts, and over three different days by each analyst.

As a measure of "closeness" of individual assay results to the known concentration, each assay result was classified as either within $\pm 25\%$ of the known sample concentration (i.e., "close") or beyond $\pm 25\%$. Table 4 shows that at each of the three sample concentrations, the rFC procedure had a greater proportion of assay results within $\pm 25\%$ of the known concentration than did the chromogenic procedure. The difference, however, was not statistically significant.

Table 4. Accuracy Relative to Known Concentration^a

		rFC	Chromogenic
0.0316 EU/mL Sample	Mean standard deviation	0.0311 EU/mL 0.0063 EU/mL	0.0366 EU/mL 0.0070 EU/mL
	% of results within ± 25% of known concentration^b	81.5%	74.1%
0.316 EU/mL Sample	Mean standard deviation	0.392 EU/mL 0.056 EU/mL	0.421 EU/mL 0.065 EU/mL
	% of results within ± 25% of known concentration^b	44.4%	29.6%
3.16 EU/mL Sample	Mean standard deviation	3.54 EU/mL 0.36 EU/mL	3.22 EU/mL 0.50 EU/mL
	% of results within ± 25% of known concentration^b	92.6%	87.0%

^a Results were obtained from the precision study (see *Table 3*) and are based on N = 54 assays of each sample, by each procedure.

^b The differences between procedures are not significant based on Fisher's Exact Test.

Linearity

As defined in <1225>, the linearity of an analytical procedure "is its ability to elicit results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range."

A series of 18 rFC standard curves were generated as part of the 6-site multicenter study. Each curve included four standards (0.01, 0.1, 1.0, and 10 EU/mL) in repli-

cates of three. The log net change in RFU (difference of the 60-min time point minus the zero time point corrected for the blank) was plotted against log endotoxin concentration to generate a standard curve. *Table 5* summarizes the linear regression statistics from the 18 curves.

Table 5. rFC Linear Regression Summary Statistics^a

Standard Curve Characteristic	Mean	CV%	Range	
			Minimum	Maximum
Correlation	0.996	0.1%	0.995	0.998
Slope	0.865	1.9%	0.831	0.901
Y-intercept	3.398	1.1%	3.307	3.450

^a Based on 18 standard curves, by six analysts at six sites.

The correlation coefficient was greater than 0.995 for all 18 standard curves, meeting FDA requirement (≥ 10.9801) for LAL-based procedures (13). Standard curve slopes and y-intercepts were also highly consistent among the 18 standard curves, with CVs of 1.9% and 1.1% respectively.

Range

As defined in (1225), the range of a procedure “is validated by verifying that the analytical procedure provides acceptable precision, accuracy, and linearity when applied to samples containing analyte at the extremes of the range as well as within the range.” Data obtained from the precision study (Table 3) show similar variation between the rFC and kinetic chromogenic procedures. The total variation of the rFC procedure was either the same as or lower than the kinetic chromogenic procedure for the three regions (low, middle, and high) of the standard curve. For the three regions the accuracy of the rFC procedure was equivalent to that of the kinetic chromogenic procedure (Table 4).

Quantitation Limit

According to (1225), the quantitation limit is the “lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. The quantitation limit is expressed as the concentration of analyte in the sample.”

We evaluated three distinct lots of rFC enzyme to determine the quantitation limit of the rFC procedure. Reference Standard Endotoxin EC-6 was used to prepare the endotoxin standard curve (0.01 to 10 EU/mL). Three separate assays were performed, each with one of the three rFC enzyme lots. Five separate samples of both LAL Reagent Water (control blank) and 0.01 EU/mL EC-6 endotoxin were tested as unknowns in replicates of three with each of the three enzyme lots.

Table 6 contains a summary of the results. The assay results on the 0.01 EU/mL sample show acceptable precision and accuracy, demonstrating that the quantitation limit is at least 0.01 EU/mL. Furthermore, the 99% tolerance intervals for assays on the water blank and 0.01 EU/mL-samples do not overlap, indicating that assays on a sample with 0.01 EU/mL are clearly distinct from assays on a blank sample.

Table 6. rFC Quantitation Limit Summary Statistics

	Number of Assay Results	Mean (EU/mL)	Standard Deviation (EU/mL)	%CV	99% Tolerance Interval (95% Confidence)
0.01 EU/mL sample	15	0.009	0.0007	7.8%	0.006–0.012 EU/mL
Blank water sample	15	0.002	0.0008	n/a ^a	0–0.005 EU/mL

^a n/a = not applicable

CONCLUSION

The rFC procedure for the detection of gram-negative bacterial endotoxin has been shown to be equivalent to LAL in its ability to quantifiably measure endotoxin and has a quantitative range comparable to that of quantitative photometric LAL procedures. However, unlike the LAL endotoxin procedure that uses Factor C derived from horseshoe crabs, the rFC procedure uses a recombinant form of Factor C. It provides another means of detecting endotoxin by other than traditional LAL procedures. The rFC does not contain the glucan-sensitive Factor G, and therefore the rFC-based procedure is not subject to false positives that can be caused by β -1,3-glucans (12). It utilizes material of nonanimal origin as its active ingredient. The requirements for validation of Compendial Methods as specified in (1225) for specificity, precision, accuracy, linearity, range, and quantitation limit have been met for the rFC procedure and are equivalent to current USP procedures for endotoxin measurement.

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