

METHODS AND KITS FOR DETECTING ENDOTOXIN

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit under 35 U.S.C. §119(e) of U.S. Provisional
5 Application No. 63/641,556, filed May 2, 2024, and U.S. Provisional Application No.
63/501,972, filed May 12, 2003. The entire contents of the aforementioned applications are
incorporated by reference herein.

SEQUENCE LISTING STATEMENT

10 This application hereby incorporates by reference the material of the electronic
Sequence Listing filed concurrently herewith. The material in the electronic Sequence
Listing is submitted as an XML filed entitled "TP385919WO1.xml" created on May 6, 2024,
which has a file size of 12.0 KB (12,288 bytes), and is herein incorporated by reference in its
entirety.

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FIELD

This disclosure concerns an improved amebocyte lysate-based workflow for the
detection of endotoxin, and kits for detecting endotoxin activity.

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BACKGROUND

Horseshoe crab amebocyte lysates are widely used as a simple and sensitive assay for
the detection of endotoxin lipopolysaccharide (LPS), the membrane component of gram-
negative bacteria, in biological samples. When endotoxin encounters the amebocyte lysate, a
series of enzymatic reactions results in the activation of Factor C, Factor B, and proclotting
25 enzyme. The activated enzyme catalyzes a cleavage event in the assay substrate to produce a
detectable signal, such as a fluorescent or chromogenic signal, allowing for the detection and
quantitation of endotoxin in the sample. Although a variety of kits for detecting endotoxin
are commercially available, a need exists for an improved workflow with fewer steps that
retains or exceeds the sensitivity and specificity of existing methods.

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SUMMARY

Disclosed herein are improved methods and kits for the specific and sensitive
detection of endotoxin. In particular, described is an efficient endpoint assay that uses
amebocyte lysate (*e.g.*, *Limulus* or *Tachypleus* amebocyte lysate) to detect and quantify

endotoxin in samples, such as biological samples (*e.g.*, samples containing antibodies or other proteins, nucleic acid molecules, vaccines, cells and/or cell culture media), medical devices, or environmental samples (*e.g.*, water, soil, and/or plant material).

Provided herein are methods for detecting endotoxin in a sample. In some aspects, the method includes providing a plurality of endotoxin standards ranging in endotoxin concentration; providing a working solution that includes amebocyte lysate and a labelled peptide substrate susceptible to cleavage upon activation of proclotting enzyme in the amebocyte lysate in the presence of endotoxin; adding each of the plurality of endotoxin standards to empty wells of a reaction vessel that has been pre-heated to an appropriate assay temperature; adding the sample to one or more empty wells of the pre-heated reaction vessel; adding the working solution to the wells of the reaction vessel containing the plurality of endotoxin standards and to the wells of the reaction vessel containing the sample; heating the reaction vessel containing the plurality of endotoxin standards, sample and working solution to allow for cleavage of the labelled peptide substrate; adding a stop solution (such as acetic acid) to each well containing the plurality of standards, sample and working solution to stop the reaction between endotoxin present in the sample and proclotting enzyme present in the amebocyte lysate; and detecting the presence of the label released from the labelled peptide substrate upon activation of proclotting enzyme in the amebocyte lysate by endotoxin. In some examples, the working solution contains about 110 nM to about 260 nM of the labelled peptide substrate and the amebocyte lysate enzyme V_{max} is 0.07 to 0.26 absorbance units of p-nitroaniline (pNA) per minute with 1 endotoxin unit (EU)/mL of endotoxin. In some examples, the method further includes determining the amount of endotoxin in the sample. In specific examples, the V_{max} of the amebocyte lysate is about 0.07 to about 0.14, such as about 0.10.

In another aspect, the method of detecting endotoxin in a sample includes providing a working solution that includes amebocyte lysate and a labelled peptide substrate susceptible to cleavage upon activation of proclotting enzyme in the amebocyte lysate in the presence of endotoxin; adding the sample to empty wells of a reaction vessel that has been pre-heated to an appropriate assay temperature; adding the working solution to the wells of the reaction vessel containing the sample; heating the reaction vessel containing the sample and working solution to allow for cleavage of the labelled peptide substrate; adding a stop solution (such as acetic acid) to each well containing the sample and working solution to stop the reaction between endotoxin present in the sample and proclotting enzyme present in the amebocyte lysate; and detecting the presence of the label released from the labelled peptide substrate

upon activation of proclotting enzyme in the amebocyte lysate by endotoxin. In some examples, a plurality of endotoxin standards ranging in endotoxin concentration are evaluated in the endotoxin assay prior to, concurrently, or after the sample is tested for the presence of endotoxin.

5 Also provided herein are kits for detecting endotoxin. In some aspects, the kit includes an endotoxin standard(s) (such as a lyophilized endotoxin standard), amebocyte lysate (such as lyophilized amebocyte lysate (LAL)), and labelled peptide substrate. In some examples, the peptide substrate is labelled with a fluorophore. In some examples, the kit further includes endotoxin-free DMSO, endotoxin-free water, a reaction vessel, acetic acid,
10 pipette tips, and/or instructional material.

The foregoing and other features of this disclosure will become more apparent from the following detailed description of several aspects which proceeds with reference to the accompanying figures.

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BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A-1B: Two-step workflow for detection of endotoxin. Shown are the results of a classic amebocyte lysate endpoint endotoxin assay using endotoxin standards of 1.0 EU/mL, 0.5 EU/mL, 0.1 EU/mL, 0.01 EU/mL and 0.0 EU/mL, two incubation steps, and a
20 fluorescently-labelled peptide substrate. Amebocyte lysate was incubated with 50 µl of each endotoxin control for 30 minutes at 37°C. Endotoxin detection reagent was subsequently added and incubated at 37°C for 4 minutes. Results were read on a fluorescent plate reader and reported as relative fluorescence units (RFU) (FIG. 1A). The results shown in tabular form that the r^2 (RSQ) value was greater than 0.980 and the z value was above 0 (FIG. 1B).

25 **FIG. 2:** One-step workflow for detection of endotoxin without optimization. Amebocyte lysate, endotoxin standard and endotoxin detection reagent (a fluorescently-labelled peptide substrate) were incubated at 37°C in a single step. Readings were taken at multiple timepoints and RSQ and z scores were calculated. The results in tabular form show that none of time points resulted in a Z score above 0 and a $RSQ \geq 0.980$.

30 **FIGS. 3A-3B:** One-step workflow with optimization of amebocyte lysate concentration and a lower concentration of labelled peptide substrate. Endotoxin standards and a fluorescently-labelled peptide substrate at a concentration of 185 nM were incubated with one of two different concentrations of amebocyte lysate (75% and 100% of standard

amebocyte lysate concentration, corresponding to Vmax of 0.10 and 0.17 absorbance units of p-nitroaniline (pNA) per minute with 1 EU/mL of endotoxin for the original reagent, respectively). The results show that the best separation was achieved using a standard amebocyte lysate concentration (FIG. 3A, top curve), but the curve was better (RSQ values) for the diluted amebocyte lysate (FIG. 3A, bottom curve). The diluted amebocyte lysate had a log RSQ greater than 0.980 and a Z-score above 0 (FIG. 3B).

FIGS. 4A-4B: One-step workflow with optimized concentration of labelled peptide substrate and amebocyte lysate. Endotoxin standards were incubated with 185 nM labelled peptide substrate and diluted (75%) amebocyte lysate (Vmax of 0.10 absorbance units of pNA/min with 1 EU/mL of endotoxin) for 0 to 60 minutes. FIG. 4A shows results of a first study with readings taken at 5-minute intervals; FIG. 4B shows results from a second study with readings taken at 1-minute intervals. The results showed RSQ values above 0.980 and Z-scores above 0 for several of the time points.

FIG. 5: Flow-chart of an exemplary endotoxin assay workflow, such as that described in Examples 7 and 9.

FIGS. 6A-6C: Endotoxin detection assay results using the workflow shown in FIG. 5. Endotoxin standards were incubated with 185 nM labelled peptide substrate and diluted (75%) amebocyte lysate (Vmax of 0.10 absorbance units of pNA/min with 1 EU/mL of endotoxin) for 24 minutes and results were read using a fluorescent plate reader. FIG. 6A is a table showing the expected and calculated EU/ml of each standard. The RSQ value and Z score were 0.9991 and 0.21, respectively. FIG. 6B is a graph plotting Log RFU versus sample concentration (EU/mL). FIG. 6C is a graph showing expected versus calculated endotoxin concentration.

FIGS. 7A-7D: Endotoxin detection assay results using three different plate readers and the QUIBIT Flex Fluorometer. Endotoxin standards were incubated with 185 nM labelled peptide substrate and diluted (75%) amebocyte lysate for 15-25 minutes and results were read using an Agilent BIOTEK (FIG. 7A), SPECTRAMAX (FIG. 7B), or VARIOSKAN LUX (FIG. 7C) plate reader or the QUIBIT Flex Fluorometer (FIG. 7D). Similar RSQ values were obtained for each instrument (0.996-0.998) and all Z scores were above 0 (0.58 to 0.88).

FIG. 8: Flow-chart of an exemplary endotoxin assay workflow, such as that described in Examples 8 and 10.

FIGS. 9A-9C: Data processing for QUBIT endotoxin detection assay. The background-corrected data was plotted (FIG. 9A), and then a log transformed linear

regression was performed to determine the correlation coefficient (r) (FIG. 9B). After confirming $r \geq 0.980$, the data was fit to a polynomial model for increased accuracy. Concentrations were then calculated and plotted against the expected results (FIG. 9C).

FIGS. 10A-10B: Results of an endotoxin detection assay using a sample volume of 50 μ L. Endotoxin standard samples were incubated with 185 nM labelled peptide substrate and diluted (75%) amebocyte lysate for 20 minutes. (FIG. 10A) Tables showing the expected and calculated endotoxin concentrations (EU/mL). This assay had a RSQ value of 1.000 and a Z score of 0.61. (FIG. 10B) Graph showing the relative fluorescent units (RFU) for each endotoxin sample concentration.

FIGS. 11A-11B: Results of an endotoxin detection assay using a sample volume of 5 μ L. Endotoxin standard samples were incubated with 185 nM labelled peptide substrate and diluted (75%) amebocyte lysate for 21 minutes. (FIG. 11A) Tables showing the expected and calculated endotoxin concentrations (EU/mL). This assay had a RSQ value of 0.9996 and a Z score of 0.54. (FIG. 11B) Graph showing the relative fluorescent units (RFU) for each endotoxin sample concentration.

FIGS. 12A-12B: Results of endotoxin detection assays using different users, dye lots and endotoxin control standard. (FIG. 12A) Tables showing the results of an assay performed by user #1, using dye lot A, 25 EU endotoxin control, and lot 1 LAL. The RSQ value for this assay was 0.99998 and the Z score was 0.56. (FIG. 12B) Tables showing the results of an assay performed by user #2, using dye lot B, 35 EU endotoxin control, and lot 1 LAL. The RSQ value for this assay was 0.99994 and the Z score was 0.57.

FIGS. 13A-13B: Results of endotoxin detection assays using different users, dye lots and endotoxin control standard. (FIG. 13A) Tables showing the results of an assay performed by user #1, using dye lot A, 25 EU endotoxin control, and lot 2 LAL. The RSQ value for this assay was 0.99996 and the Z score was 0.19. (FIG. 13B) Tables showing the results of an assay performed by user #2, using dye lot B, 35 EU endotoxin control, and lot 2 LAL. The RSQ value for this assay was 0.0.9997 and the Z score was 0.58.

FIGS. 14A-14B: Results of endotoxin detection assays using different dye lots and LAL lots. (FIG. 14A) Tables showing the results of an assay performed by user #1, using dye lot A, 25 EU endotoxin control, and lot 1 LAL (incubation time 20 minutes). The RSQ value for this assay was 0.99998 and the Z score was 0.56. (FIG. 14B) Tables showing the results of an assay performed by user #1, using dye lot B, 25 EU endotoxin control, and lot 2

LAL (incubation time 21 minutes). The RSQ value for this assay was 0.99996 and the Z score was 0.19.

FIGS. 15A-15B: Results of endotoxin detection assays using five different QUBIT FLEX instruments. (FIG. 15A) Table showing the expected and calculated endotoxin concentrations for each QUBIT FLEX instrument (QUBIT-1, QUBIT-2, QUBIT-3, QUBIT-4, QUBIT-5) using LAL lot 1 (incubation time 20 minutes). (FIG. 15B) Table showing the expected and calculated endotoxin concentrations for each QUBIT FLEX instrument (QUBIT-1, QUBIT-2, QUBIT-3, QUBIT-4, QUBIT-5) using LAL lot 2 (incubation time 21 minutes).

FIG. 16: Flow-chart of a method embodiment under the present disclosure.

FIG. 17: Flow-chart of a method embodiment under the present disclosure.

DETAILED DESCRIPTION

I. Introduction

Disclosed herein is an efficient endpoint assay that uses amebocyte lysates to quantitate endotoxin in biological or environmental samples. Amebocyte lysates are widely used as a simple and sensitive assay for the detection of endotoxin lipopolysaccharide (LPS), the membrane component of Gram-negative bacteria. When endotoxin encounters the amebocyte lysate, a series of enzymatic reactions results in the activation of Factor C, Factor B, and proclotting enzyme. The activated clotting enzyme catalyzes a cleavage event in the assay substrate to produce a strong detectable (such as fluorescent) signal. After stopping the reaction, the resulting signal can be measured on a microplate reader (such as a fluorescent microplate reader) or a fluorometer. The resulting signal is proportional to the endotoxin concentration in the sample and allows quantification from 0.01-10.0 EU/mL depending on the sample volume used.

The disclosed endotoxin assay workflow is advantageous because only a single incubation step is required – the sample, amebocyte lysate, and indicator reagent (*e.g.*, a fluorescently-labelled peptide substrate) are combined in a single incubation step. A single step is desirable to minimize user error and to improve the speed of the assay. These features are in contrast to classic amebocyte lysate-based assays, which require incubation of the sample with amebocyte lysate for about 15-45 minutes, after which the indicator reagent is added. The one-step incubation workflow was achieved by reducing the concentration of amebocyte lysate and reducing the concentration of the labelled peptide substrate, relative to currently available amebocyte lysate-based endpoint endotoxin assays. Furthermore, the

assay disclosed herein permits the use of different sample input volumes, ranging from 1 μ L to 50 μ L, including 5 μ L, 25 μ L, and 50 μ L, which enables detection of endotoxin at concentrations of 0.10-10.0 EU/mL, 0.02-2.0 EU/mL, and 0.01-1.0 EU/mL, respectively.

Without wishing to be bound by theory, previous amebocyte lysate-based assays may not have been successful using a single incubation step because the assays involve an interdependent reaction cascade (one reaction needs to occur before the next can proceed) and the previously used concentrations of peptide substrate and amebocyte lysate may not have allowed sufficient build-up of required precursors/reactants.

10 II. Abbreviations

DMSO	dimethyl sulfoxide
EU	endotoxin unit
LAL	lyophilized amebocyte lysate
LPS	lipopolysaccharide
15 pNA	p-nitroaniline
RFU	relative fluorescence units

III. Summary of Terms

Unless otherwise noted, technical terms are used according to conventional usage. Definitions of many common terms in molecular biology may be found in Krebs *et al.* (eds.), *Lewin's genes XII*, published by Jones & Bartlett Learning, 2017. As used herein, the singular forms "a," "an," and "the," refer to both the singular as well as plural, unless the context clearly indicates otherwise. For example, the term "a sample" includes singular or plural samples and can be considered equivalent to the phrase "at least one sample." As used herein, the term "comprises" means "includes." It is further to be understood that any and all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for descriptive purposes, unless otherwise indicated. Although many methods and materials similar or equivalent to those described herein can be used, particular suitable methods and materials are described herein. In case of conflict, the present specification, including explanations of terms, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. To facilitate review of the various aspects, the following explanations of terms are provided:

Amebocyte lysate: An extract of blood cells (amebocytes) from a horseshoe crab (e.g., *Limulus polyphemus*, *Tachypleus tridentatus*, *Tachypleus gigas*, or *Carcinoscorpius rotundicauda*). In some aspects herein, the amebocyte lysate is lyophilized amebocyte lysate (LAL).

5 **Chromophore:** A molecule that absorbs particular wavelengths of visible light, thereby conferring color upon the chromophore. In some aspects herein, a peptide is labeled with a chromophore, such as at the N-terminus or C-terminus. Non-limiting examples of chromophores that can be used with the methods and kits disclosed herein include, but are not limited to, p-nitroaniline (pNA), 2,4-dinitrophenyl (Dnp), β -naphthylamide (β NA), β -naphthyl ester, p-nitrophenyl ester (Onp), and thiobenzyl ester (SBzl).

10 **Proclotting enzyme:** An enzyme found in the blood of horseshoe crabs (e.g., *Limulus polyphemus* or *Tachypleus tridentatus*) that is activated in the presence of endotoxin (to produce clotting enzyme). The horseshoe crab has a primitive but highly sensitive response to endotoxin. In the presence of endotoxin, horseshoe crab amebocytes (blood cells) degranulate, leading to the activation of an enzymatic cascade that involves three serine protease zymogens (factor C, factor B and proclotting enzyme z) and coagulogen (a clottable protein). Endotoxin activates zymogen factor C to produce factor C, which activates zymogen factor B to produce factor B, which in turn converts proclotting enzyme to clotting enzyme (Maloney *et al.*, *PLoS Biol* 16(1):e2006607, 2018).

20 **Detect:** To determine if a particular agent or analyte (e.g., endotoxin) is present or absent, and in some examples further includes quantification of the agent/analyte (e.g., endotoxin) if detected.

25 **Endotoxin:** Lipopolysaccharide (LPS) found in the outer membrane of Gram-negative bacteria. The lipid A component of LPS is responsible for the endotoxin activity of Gram-negative bacteria. In some aspects herein, the endotoxin is from a Gram-negative bacterial species of the genus *Escherichia*, *Shigella*, *Salmonella*, *Campylobacter*, *Neisseria*, *Haemophilus*, *Corynebacteria*, *Citrobacter*, *Chlamydia*, *Brucella*, *Pseudomonas*, *Helicobacter*, or *Vibrio*.

30 **Fluorophore:** A chemical compound, which when excited by exposure to a particular wavelength of light, emits light (fluoresces), for example at a different wavelength than that to which it was exposed. Also encompassed by the term “fluorophore” are luminescent molecules, which are chemical compounds that do not require exposure to a particular wavelength of light to fluoresce; luminescent compounds naturally fluoresce.

Therefore, the use of luminescent signals eliminates the need for an external source of electromagnetic radiation, such as a laser. An example of a luminescent molecule includes, but is not limited to, aequorin (Tsien, *Ann Rev Biochem* 67:509, 1998).

In some aspects herein, a peptide is labeled with a fluorophore, such as at the N-terminus or C-terminus. Exemplary fluorophores include, but are not limited to, xanthene, fluorescein, rhodamine, rhodol, roseamine, carbopyranone, indole, indacene, borapolyazaindacene, furan, benzofuran, cyanine, benzocyanine, benzopyrilium, pyrene, coumarin, styryl, squaraine, resorufin, anthraquinone, acridine and benzophenoxazine. Examples of fluorophores suitable for use with the disclosed endotoxin assays, include, but are not limited to, 6-carboxyfluorescein (FAM), tetrachlorofluorescein (TET), tetramethylrhodamine (TMR), hexachlorofluorescein (HEX), JOE, 6-carboxy-X-rhodamine (ROX), CAL FluorTM, PulsarTM, QuasarTM, Texas RedTM, Texas Red-X, CyTM3, CyTM5, BODIPYTM (boron-dipyrromethene), Alexa FluorTM dyes (such as Alexa FluorTM 488), Oregon GreenTM, and Endotoxin Green. Other examples of fluorophores that can be used in the methods disclosed herein are provided in U.S. Patent No. 5,866,366. These include, but are not limited to: 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid, acridine and derivatives such as acridine and acridine isothiocyanate, 5-(2'-aminoethyl)amino-naphthalene-1-sulfonic acid (EDANS), 4-amino-N-[3-vinylsulfonyl]phenyl]-naphthalimide-3,5 disulfonate (Lucifer Yellow VS), N-(4-anilino-1-naphthyl)-maleimide, anthranilamide, Brilliant Yellow, coumarin and derivatives such as coumarin, 7-amino-4-methylcoumarin (AMC, Coumarin 120), 7-amino-4-trifluoromethylcoumarin (Coumarin 151); cyanosine; 4',6-diaminidino-2-phenylindole (DAPI); 5', 5"-dibromopyrogallol-sulfonephthalein (Bromopyrogallol Red); 7-diethylamino-3-(4'-isothiocyanatophenyl)-4-methylcoumarin; diethylenetriamine pentaacetate; 4,4'-diisothiocyanatodihydro-stilbene-2,2'-disulfonic acid; 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; 5-[dimethyl-amino]naphthalene-1-sulfonyl chloride (DNS, dansyl chloride); 4-(4'-dimethyl-aminophenylazo)benzoic acid (DABCYL); 4-dimethylaminophenylazophenyl-4'-isothiocyanate (DABITC); eosin and derivatives such as eosin and eosin isothiocyanate; erythrosin and derivatives such as erythrosin B and erythrosin isothiocyanate; ethidium; fluorescein and derivatives such as 5-carboxyfluorescein (FAM), 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF), 2'7'-dimethoxy-4'5'-dichloro-6-carboxyfluorescein (JOE), fluorescein, fluorescein isothiocyanate (FITC), and QFITC (XRITC); fluorescamine; IR144; IR1446; Malachite Green isothiocyanate; 4-methylumbelliferone; ortho cresolphthalein; nitrotyrosine; pararosaniline; Phenol Red; B-phycoerythrin; o-phthalaldehyde; pyrene and derivatives such as pyrene, pyrene butyrate

and succinimidyl 1-pyrene butyrate; Reactive Red 4 (Cibacron® Brilliant Red 3B-A); rhodamine and derivatives such as 6-carboxy-X-rhodamine (ROX), 6-carboxyrhodamine (R6G), lissamine rhodamine B sulfonyl chloride, rhodamine (Rhod), rhodamine B, rhodamine 123, rhodamine X isothiocyanate, sulforhodamine B, sulforhodamine 101 and sulfonyl chloride derivative of sulforhodamine 101 (Texas Red); N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); tetramethyl rhodamine; tetramethyl rhodamine isothiocyanate (TRITC); riboflavin; rosolic acid and terbium chelate derivatives. See also U.S. Patent Nos. 5,627,027; 5,486,616; 5,569,587; 5,569,766; and 5,800,996. In additional examples, the fluorophore includes fluorescently-labeled nanodots, fluorescently-labeled carbon dots, or quantum dots, or fluorescently-labeled oligonucleotide nanostructures (such as those disclosed in U.S. Patent No. 11,674,901).

Label: A compound or composition that is conjugated (*e.g.*, covalently linked) directly or indirectly to another molecule (such as a peptide) to facilitate detection of that molecule or to facilitate detection of enzymatic activity (such as enzymatic activity that results in cleavage of the label from a substrate, such as a peptide substrate). Specific non-limiting examples of labels include fluorescent and fluorogenic moieties (*e.g.*, fluorophores), chromogenic moieties, haptens (such as biotin, digoxigenin, and fluorescein), affinity tags, and radioactive isotopes (such as ^{32}P , ^{33}P , ^{35}S , and ^{125}I). In some aspects herein, the label includes a fluorophore or a chromophore.

Lipopolysaccharide (LPS): Large molecules comprised of a lipid and a polysaccharide, which are part of the outer membrane of Gram-negative bacteria. LPS is composed of O-antigen (also referred to as O-polysaccharide chain), core oligosaccharide (which includes an outer core and an inner core), and lipid A. Endotoxin activity is found in the lipid A component of LPS.

Peptide substrate: In the context of the present disclosure, a “peptide substrate” is a short peptide of at least 3 amino acids in length (such as at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 amino acids in length) conjugated to a label, such as a fluorescent or chromogenic label. The peptide substrate is cleavable in the presence of activated clotting enzyme, thereby releasing the label. In some aspects herein, the peptide substrate has the formula: $\text{R}_1\text{-A}_1\text{-A}_2\text{-A}_3\text{-A}_4\text{-B-R}_2$ (SEQ ID NO: 1), where R_1 is hydrogen, a blocking aromatic hydrocarbon or acyl; A_1 is an L- or D-amino acid selected from Ile, Val or Leu; A_2 is Glu or Asp; A_3 is Ala or Cyc; A_4 is Arg; B is an -NH- or -O-linkage; and R_2 is a label (such as a fluorescent or chromogenic label). In other examples, the peptide substrate has the formula Ac-Ile-Glu-Ala-Arg-label (SEQ ID NO: 2). Peptide

substrates for endotoxin assays are known (see, *e.g.*, EP0074761). Specific, non-limiting examples of peptide substrates are provided in the table below.

N-Benzoyl-Ile-Glu-Ala-Arg-pNA	SEQ ID NO: 3
N-Benzoyl-Ile-Glu-Ala-Arg-4-Methoxy- β -naphthylamide	SEQ ID NO: 4
N-Benzoyl-Ile-Glu-Cys-Arg-pNA	SEQ ID NO: 5
N-Benzoyl-Ile-Glu-Cys-Arg-4-Methoxy- β -naphthylamide	SEQ ID NO: 6
Acyl-Ile-Glu-Ala-Arg-pNA	SEQ ID NO: 7
Acyl-Ile-Glu-Ala-Arg-4-Methoxy- β -naphthylamide	SEQ ID NO: 8

Plurality: Any number that is more than one. In some embodiments herein, a
 5 “plurality of endotoxin standards” means at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, or at least 8 endotoxin standards.

Polypeptide, peptide, and protein: Polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has
 10 been modified; for example, modified with disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation, such as conjugation with a labeling component (such as a fluorophore or chromogen). As used herein, the term "amino acid" includes natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. The term “residue”
 15 or “amino acid residue” includes reference to an amino acid that is incorporated into a protein, polypeptide, or peptide.

Reaction vessel: Any container suitable for holding the samples to be analyzed for the presence of endotoxin according to the methods disclosed herein. Reaction vessels include, but are not limited to, multi-well plates, microfuge tubes (such as polypropylene
 20 tubes), test tubes, cartridges, and the like. In some examples, the reaction vessel is made of glass or plastic. In specific examples, the reaction vessel is a multi-well plate, such as a PIERCE Endotoxin-Free Plate Strip (Thermo Fisher Cat. No. A43888 or NC9217517). In other specific examples, the reaction vessel includes polypropylene tubes, such as QUBIT FLEX pyrogen-free tube strips (Thermo Fisher Cat No. Q32893).

Sample: Any sample that contains or could contain endotoxin. Samples can be from, for example, an animal, a cell, a plant, a laboratory reagent (such as cell culture media), a medical device, or the environment. In some aspects, the sample is a biological sample, such

as a biological sample that includes nucleic acids (*e.g.*, DNA, cDNA, or RNA), proteins (*e.g.*, antibodies), cells and/or cell culture media. Other examples of biological samples include, but are not limited to, fluid samples (such as bodily fluids), cell samples, aspirate samples and/or tissue samples. Specific biological samples include, but are not limited to, sputum, saliva, mucus, nasal wash, serum, urine, blood, plasma, feces, cerebral spinal fluid (CSF), bronchoalveolar lavage (BAL) fluid, nasopharyngeal samples, oropharyngeal samples, biopsy samples, needle aspirates, and tissue sections. Samples may be concentrated or diluted before analysis. Environmental samples can include, for example, water, plant material, vegetation and/or soil.

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IV. Improved Endotoxin Assay Workflow

The present disclosure describes an efficient and sensitive endpoint assay that uses amebocyte lysates to detect and quantitate endotoxin. The disclosed endotoxin assay workflow provides an improvement over currently available endotoxin detection methods because only a single incubation step is required – the sample, amebocyte lysate and indicator reagent (*e.g.*, a fluorescently-labelled peptide substrate) are incubated together in a single step. In contrast, classic amebocyte lysate-based assays require incubation of the sample with amebocyte lysate prior to incubation with the indicator reagent. The one-step incubation workflow was achieved by reducing the amount of amebocyte lysate and reducing the concentration of the labelled peptide substrate, relative to currently available amebocyte lysate-based endpoint endotoxin assays.

Provided herein are methods for detecting endotoxin in a sample. In some aspects, the method includes providing a plurality of endotoxin standards ranging in concentration; providing a working solution comprising amebocyte lysate and a labelled peptide substrate susceptible to cleavage upon activation of proclotting enzyme in the amebocyte lysate in the presence of endotoxin; adding each of the plurality of endotoxin standards to empty wells of a reaction vessel that has been pre-heated to an appropriate assay temperature (such as about 36°C to about 38°C, such as 37°C); adding the sample to one or more empty wells of the pre-heated reaction vessel; adding the working solution to the wells of the reaction vessel containing the plurality of standards and to the wells of the reaction vessel containing the sample; heating the reaction vessel containing the plurality of standards, sample and working solution to allow for cleavage of the labelled peptide substrate; adding a stop solution to each well containing the working solution to stop the reaction between endotoxin present in the sample and proclotting enzyme present in the amebocyte lysate; and detecting the presence of

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the label released from the labelled peptide substrate upon activation of proclotting enzyme in the amebocyte lysate by endotoxin.

In another aspect, the method of detecting endotoxin in a sample includes providing a working solution that includes amebocyte lysate and a labelled peptide substrate susceptible to cleavage upon activation of proclotting enzyme in the amebocyte lysate in the presence of
5 endotoxin; adding the sample to empty wells of a reaction vessel that has been pre-heated to an appropriate assay temperature; adding the working solution to the wells of the reaction vessel containing the sample; heating the reaction vessel containing the sample and working solution to allow for cleavage of the labelled peptide substrate; adding a stop solution (such
10 as acetic acid) to each well containing the sample and working solution to stop the reaction between endotoxin present in the sample and proclotting enzyme present in the amebocyte lysate; and detecting the presence of the label released from the labelled peptide substrate upon activation of proclotting enzyme in the amebocyte lysate by endotoxin. In some examples, a plurality of endotoxin standards ranging in endotoxin concentration are evaluated
15 in the endotoxin assay prior to, concurrently, or after the sample is tested for the presence of endotoxin.

FIG. 5 shows a flow-chart of an exemplary method (500) of detecting endotoxin according to the present disclosure. Step 510 is preheating pyrogen-free multi-well plate to about 37°C. Step 520 is reconstituting endotoxin control standard and preparing endotoxin
20 standard dilution series for use in creating an endotoxin concentration standard curve. Step 530 is adding samples and standards to wells of the multi-well plate and continuing to preheat at about 37°C. Step 540 is preparing working solution comprising amebocyte lysate and labelled peptide substrate reagent. Step 550 is adding working solution to the wells of the multi-well plate containing the standards or the sample and incubating at 37°C. Step 560 is
25 adding stop solution after the lot-specific incubation time. Step 570 is detecting the signal of the label released from the labelled peptide substrate as indicated by the read-out on a microplate reader.

FIG. 8 shows a flow-chart of an exemplary method (800) of detecting endotoxin according to the present disclosure. Step 810 is preheating pyrogen-free assay tubes to about
30 37°C. Step 820 is reconstituting endotoxin control standard and preparing endotoxin standard dilution series for use in creating an endotoxin concentration standard curve. Step 830 is adding samples and standards to assay tubes and continuing to preheat at about 37°C. Step 840 is preparing working solution comprising amebocyte lysate and labelled peptide substrate reagent. Step 850 is adding working solution to the assay tubes containing the

standards or the sample and incubating at 37°C. Step 860 is adding stop solution after the lot-specific incubation time. Step 870 is detecting the signal of the label released from the labelled peptide substrate as indicated by the read-out on a fluorometer such as a QUBIT Flex Fluorometer.

5 FIG. 16 shows a flow-chart of an exemplary method (1600) for detecting endotoxin under the present disclosure. Step 1610 is providing a plurality of endotoxin standards ranging in exotoxin concentration for creating a standard curve. Step 1620 is providing a working solution comprising amebocyte lysate and a labelled peptide substrate susceptible to cleavage upon activation of proclotting enzyme in the amebocyte lysate in the presence of
10 endotoxin. Step 1630 is adding each of the plurality of endotoxin standards to empty wells of a pre-heated reaction vessel. Step 1640 is adding the sample to one or more empty wells of the pre-heated reaction vessel. Step 1650 is adding the working solution to the wells of the reaction vessel containing the plurality of standards and to the wells of the reaction vessel containing the sample. Step 1660 is heating the reaction vessel containing the plurality of
15 standards, sample and working solution to allow for cleavage of the labelled peptide substrate. Step 1670 is adding a stop solution to each well containing the working solution. Step 1680 is detecting the presence of the label released from the labelled peptide substrate upon activation of proclotting enzyme in the amebocyte lysate by endotoxin.

 FIG. 17 shows a flow-chart of an exemplary method (1700) for detecting endotoxin
20 under the present disclosure. Step 1710 is providing a working solution comprising amebocyte lysate and a labelled peptide substrate susceptible to cleavage upon activation of proclotting enzyme in the amebocyte lysate in the presence of endotoxin. Step 1720 is adding the sample to one or more empty wells of the pre-heated reaction vessel. Step 1730 is adding the working solution to the wells of the reaction vessel containing the sample. Step
25 1740 is heating the reaction vessel containing the sample and working solution to allow for cleavage of the labelled peptide substrate. Step 1750 is adding a stop solution to each well containing the working solution. Step 1760 is detecting the presence of the label released from the labelled peptide substrate upon activation of proclotting enzyme in the amebocyte lysate by endotoxin.

30 In some aspects of the disclosed methods, the working solution contains about 110 nM to about 260 nM of the labelled peptide substrate. In some examples, the working solution contains about 125 nM to about 250 nM, about 150 nM to about 220 nM, about 175 nM to about 200 nM, or about 180 to about 190 nM of the labelled peptide substrate. In particular examples, the working solution contains about 180 nM, about 181 nM, about 182

nM, about 183 nM, about 184 nM, about 185 nM, about 186 nM, about 187 nM, about 188 nM, about 189 nM or about 190 nM. In one non-limiting example, the working solution contains 185 nM of the labelled peptide substrate.

In some aspects of the disclosed methods, the amebocyte lysate in the working
5 solution has an enzyme Vmax of 0.07 to about 0.26 absorbance units of p-nitroaniline (pNA) per minute with 1 endotoxin unit (EU)/mL of endotoxin. In some examples, the amebocyte lysate enzyme Vmax is about 0.07 to about 0.14, about 0.10 to about 0.24, about 0.12 to about 0.22, about 0.14 to about 0.20, about 0.16 to about 0.18, about 0.10 to about 0.14, or about 0.10 to about 0.17 absorbance units of pNA per minute with 1 EU/mL of endotoxin. In
10 specific non-limiting examples, the amebocyte lysate enzyme Vmax is 0.07, 0.08, 0.09, 0.10, 0.11, 0.12, 0.13, 0.14, 0.15, 0.16, 0.17, 0.18, 0.19, 0.20, 0.21, 0.22, 0.23, 0.24, 0.25 or 0.26 absorbance units of pNA per minute with 1 EU/mL of endotoxin.

The sample to be tested for the presence of endotoxin can be any type of sample, such as a sample suspected of containing endotoxin. In some aspects, the sample is a biological
15 sample, such as a sample containing antibodies, proteins, nucleic acid molecules, vaccines, cells, and/or cell culture media. In other aspects, the sample is an environmental sample, such as a sample containing water, plant material or other vegetation, or soil.

In some aspects, the sample is added to the one or more wells of the reaction vessel in a total volume of 50 μ L. In some examples, at least two, at least three or at least four
20 replicates of each sample are added to individual wells of the reaction vessel.

The volume of the sample can vary but is typically in a total volume of 50 μ L when added to the reaction vessel. In some examples, the 50 μ L sample volume is 5 μ L of sample diluted with 45 μ L of endotoxin-free water; 25 μ L of sample diluted with 25 μ L endotoxin-free water; or 50 μ L of undiluted sample.

In some aspects, the plurality of endotoxin standards includes at least two, at least three, at least four, at least five, or at least six endotoxin standards of varying concentrations. In some examples, the plurality of endotoxin standards ranges in concentration from about 10.0 endotoxin units (EU)/mL to about 0.0 EU/ml, about 5.0 EU/m to about 0.0 EU/ml, about 2.5 EU/ml to about 0.0 EU/ml, or about 1.0 EU/mL to about 0.0 EU/ml. In one non-limiting
25 example, the plurality of endotoxin standards includes four standards with endotoxin concentrations of 1.0 EU/mL, 0.1 EU/mL, 0.01 EU/mL and 0.00 EU/mL, respectively.
30

In some aspects, about 50 μ L (such as about 40, about 45, about 50, about 55, or about 60 μ L) of each of the plurality of endotoxin standards is added to the wells of the

reaction vessel. In some examples, at least two, at least three, at least four or at least five replicates of each endotoxin standard are added to empty wells of the reaction vessel. In particular examples, three replicates of each endotoxin standard are added to empty wells of the reaction vessel.

5 In some aspects, the reaction vessel containing the plurality of endotoxin standards and sample is heated at a temperature of about 36°C, about 37°C or about 38°C for about 5 to about 15 minutes, such as about 8 to about 12 minutes, such as about 10 minutes prior to adding the working solution. In some examples, the reaction vessel containing the plurality of endotoxin standards and sample is heated at a temperature of 37°C for 10 minutes.

10 In some aspects, the labelled peptide substrate is dissolved in endotoxin-free DMSO prior to combining with the amebocyte lysate. However, the labelled peptide substrate can be dissolved in other solvents, such as water, buffer or an alcohol (such as ethanol or methanol).

 In some aspects, about 80 µL to about 120 µL of the working solution is added to each well containing the plurality of endotoxin standards and each well containing the
15 sample. In some examples, about 80 µL, about 90 µL, about 100 µL, about 110 µL or about 120 µL of the working solution is added to each well. In specific examples, 100 µL of the working solution is added to each well.

 In some aspects, the plurality of endotoxin standards, sample and working solution are heated at about 36°C, about 37°C or about 38°C for a pre-determined amount of time
20 sufficient to allow for cleavage of the labelled peptide substrate. The pre-determined amount of time is dependent upon the enzymatic activity in the amebocyte lysate, which varies by lot. The enzymatic activity of the amebocyte lysate can be determined by Vmax using pNA and endotoxin (1 EU/mL). The assay is incubated at 37°C and observed at 405 nm absorbance reading once every minute. The observed absorbance is plotted against time (minutes) and
25 the maximum slope at each time is determined (absorbance units per minute). In some examples, the pre-determined amount of time sufficient to allow for cleavage of the labelled peptide substrate is about 15 to about 45 minutes, about 15 to about 40 minutes, about 20 to about 45 minutes, about 20 to about 35 minutes, about 20 to about 40 minutes, about 25 to about 35 minutes, about 20 to about 30 minutes, or about 15 to about 25 minutes. In specific
30 non-limiting examples, the pre-determined amount of time sufficient to allow for cleavage of the labelled peptide substrate is about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 22, about 23, about 24, about 25, about 26, about 27, about 28, about 29,

about 30, about 31, about 32, about 33, about 34, about 35, about 36, about 37, about 38, about 39 or about 40 minutes.

In some aspects, the stop solution includes acetic acid. In some examples, the stop solution is 25% acetic acid in water. In other examples, the stop solution is another acid, such as HCl or citric acid, such as 25% HCl or 25% citric acid in water.

In some aspects, the label of the labelled peptide substrate is a fluorescent label. In some examples, the fluorescent label is a fluorophore. In some examples, the fluorophore includes, but is not limited to, xanthene, fluorescein, rhodamine, rhodol, roseamine, carbopyranone, indole, indacene, borapolyazaindacene, furan, benzofuran, cyanine, benzocyanine, benzopyrilium, pyrene, coumarin, styryl, squarine, resorufin, anthraquinone, acridine and benzophenoxazine. In some examples, fluorescent label is detected using a fluorometer or fluorescent plate reader.

In other aspects, the label of the labelled peptide substrate is a chromogenic label. In some examples, the chromogen includes pNA, 5-amino-2-nitrobenzoic acid (ANBA), 7-amino-4-methoxycoumarin (ANC), quinonylamide (QUA), dimethyl 5-aminoisophthalate (DPA), or a derivative thereof. In some examples, the chromogenic label is detecting using a spectrophotometer or a microplate reader.

The reaction vessel used with the claimed methods can vary, but generally contains multiple wells for including replicates of each endotoxin standard and replicates of the sample(s) to be tested. The reaction vessels are endotoxin-free to avoid contamination. In some aspects, the reaction vessel is a multi-well plate, such as a 96-well plate. In other aspects, the reaction vessel is a plurality of polypropylene tubes (such as a tube strip) or a plurality of glass tubes. In other aspects, the reaction vessel is a cartridge.

In some aspects of the disclosed methods, the method further includes determining the amount of endotoxin in the sample. In some examples, determining the amount of endotoxin in the sample includes comparing the amount of label released by the sample to the amount of label released by the plurality of endotoxin standards.

In some aspects, the method has a sensitivity of >0.01 EU/mL of endotoxin.

In some aspects, the method further includes calculating regression of the plurality of endotoxin standards using log transformed concentration and relative fluorescent unit (RFU) data and performing linear regression fit to calculate the correlation coefficient. In some aspects, the calculating steps are performed by one or more algorithms installed on the fluorometer. In some examples, the correlation coefficient is greater than or equal to 0.980.

In some aspects, the method further includes calculating endotoxin concentration in the sample. In some examples, calculating endotoxin concentration in the sample includes quadratic curve fitting (see Examples 7, 9 and 10).

5 V. Kits for Detecting Endotoxin

Also provided herein are kits for carrying out the disclosed methods for detecting endotoxin.

In some aspects, the kit includes an endotoxin standard (such as a lyophilized endotoxin standard). In some examples, the endotoxin standard includes 10 to 50 endotoxin
10 units (EU). The endotoxin standard can be used to prepare a plurality of endotoxin standards of varying concentration.

In some aspects, the kit includes (or further includes) amebocyte lysate, such as lyophilized amebocyte lysate (LAL). In some examples, the LAL (or reconstituted LAL) has an enzyme Vmax of about 0.07 to about 0.14, such as about 0.07, about 0.08, about 0.09,
15 about 0.1, about 0.11, about 0.12, about 0.13 or about 0.14. In particular examples, the enzyme Vmax is 0.1.

In some aspects, the kit includes (or further includes) a labelled peptide substrate, such as a peptide substrate labelled with a fluorophore or a chromogen. In some examples, the peptide substrate has the formula $R_1-A_1-A_2-A_3-A_4-B-R_2$ (SEQ ID NO: 1), where R_1 is
20 hydrogen, a blocking aromatic hydrocarbon or acyl; A_1 is an L- or D-amino acid selected from Ile, Val or Leu; A_2 is Glu or Asp; A_3 is Ala or Cys; A_4 is Arg; B is an -NH- or -O-linkage; and R_2 is a label (such as a fluorescent or chromogenic label). In other examples, the peptide substrate has the formula Ac-Ile-Glu-Ala-Arg-label (SEQ ID NO: 2), wherein the label is a fluorophore or a chromogen. In specific examples, the peptide substrate comprises
25 SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7 or SEQ ID NO: 8.

In some examples, the fluorophore includes a xanthene, fluorescein, rhodamine, rhodol, roseamine, carbopyranone, indole, indacene, borapolyazaindacene, furan, benzofuran, cyanine, benzocyanine, benzopyrilium, pyrene, coumarin, styryl, squarine, resorufin,
30 anthraquinone, acridine or benzophenoxazine. In some examples the fluorophore is a fluorescein or a rhodamine.

In some examples, the chromogen includes pNA, 5-amino-2-nitrobenzoic acid (ANBA), 7-amino-4-methoxycoumarin (ANC), quinonylamide (QUA), dimethyl 5-aminoisophthalate (DPA), or a derivative thereof.

The kit can include one or more additional items to carry out the claimed methods of endotoxin detection. In some examples, the kit further includes endotoxin-free DMSO, endotoxin-free water, reaction vessel(s), acetic acid (such as 25% acetic acid in water), pipette tips, and/or instructional material. In particular examples, the reaction vessel is a multi-well plate (such as a 96-well plate), a cartridge, or a set of polypropylene tubes (such as a strip of polypropylene tubes).

EXAMPLES

The following examples are provided to illustrate particular features of certain aspects of the disclosure, but the scope of the claims should not be limited to those features exemplified.

Example 1: Two-step workflow

This example describes an amebocyte lysate-based endotoxin assay performed using a standard two-step incubation.

Classic amebocyte lysate-based endpoint assays use two incubation steps: (1) amebocyte lysate is incubated with sample for a lot-specific period of time; and (2) endotoxin detection reagent is added and incubated with the lysate/sample mixture.

The target detection range is 0.01 to 1.0 EU/mL, thus a background that is indistinguishable from the 0.01 EU/mL signal is not viable. This can be measured and reported as a “z score” where results greater than 0 are considered viable and results below 0 are considered to have signals that are not distinguishable from background. This calculation is based on raw RFU signals. A correlation coefficient (measured as r^2 or RSQ) below 0.980 is also not viable. In order for an endotoxin assay workflow to be considered viable, both the background separation and the correlation coefficient tests (RSQ) need to pass.

In this study, 50 μ l of each endotoxin control having various endotoxin concentrations, measured in EU/mL, was incubated with amebocyte lysate for 30 minutes at 37°C. Endotoxin detection reagent was subsequently added and incubated at 37°C for 4 minutes. Results were read on a fluorescent plate reader. As shown in FIGS. 1A-1B, the r^2 value was greater than 0.980 and the z value was above 0, indicating the assay was viable.

Example 2: One-step workflow without optimization

This example describes an amebocyte lysate-based endotoxin assay workflow with a single incubation step.

This assay was performed as described in Example 1, except that endotoxin control samples (0 to 1 EU/mL), amebocyte lysate and the endotoxin detection reagent were incubated together (at 37°C) in a single step. Readings were taken at a number of different timepoints (from 0 to 45 minutes) and RFQ and z scores were calculated. As shown in FIG. 2, no time points met passing criteria (*i.e.*, Z score above 0 and RSQ ≥0.980).

Example 3: Optimization of labelled substrate peptide concentration

This example describes an endotoxin assay workflow with a single incubation step, but with a variable amount of endotoxin detection reagent, which is a fluorescently labelled peptide substrate.

This study was performed to determine whether using a lower concentration of peptide substrate would decrease the background signal, thereby improving lower-end endotoxin concentration discrimination. Assay conditions were constant, except for the amount of input substrate. In this assay, 100% corresponds to 370 nM of peptide substrate; 50% corresponds to 185 nM of peptide substrate; and 25% corresponds to 92.5 nM of peptide substrate. The results are shown in Table 1.

Table 1. Optimization of peptide substrate concentration

	25%	50%	100%
RSQ	0.871	0.922	0.907
Log RSQ	0.978	0.984	0.980
Z-Score	-0.11	0.57	0.35

These results demonstrated that using less of the labelled peptide substrate, particularly 50% of the standard concentration, improved all tested assay metrics over benchmark conditions.

Example 4: Optimization of amebocyte lysate concentration

This example describes an endotoxin assay workflow with a single incubation step, but with a variable amount of amebocyte lysate concentration.

This study was performed to determine the effect of different concentrations of amebocyte lysate. It was hypothesized that using a lower concentration of lysate would decrease the reaction rate, improving the relationship between the high and low sample concentrations. Three different lysate concentrations were used in this study – 75%, 100% and 125% of standard lysate concentration, corresponding to V_{max} of 0.10, 0.17 and 0.19. All other assay conditions were constant. The results are shown in Table 2.

Table 2. Optimization of amebocyte lysate concentration

	75%	100%	125%
RSQ	0.980	0.946	0.943
Log RSQ	0.954	0.994	0.993
Z-Score	0.31	0.24	0.63

These results demonstrate that using higher concentrations of the amebocyte lysate increased background compared to benchmark conditions. In contrast, using 75% of the standard concentration (corresponding to V_{max} 0.10), resulted in an RSQ of 0.980 and a Z-score of 0.31, which indicate the assay is viable.

Example 5: Optimizing amebocyte lysate concentration with lower concentration of peptide substrate

This example describes an endotoxin assay workflow with a single incubation step, a lower peptide substrate concentration (as described in Example 3) and either standard or diluted amebocyte lysate.

This assay used 50 μ l of endotoxin sample and 185 nM of peptide substrate with either a standard amebocyte lysate concentration (100%) or a diluted amebocyte lysate concentration (75%). The results are shown in FIGS. 3A-3B. While the best separation was found with the standard amebocyte lysate concentration (FIG. 3A, top curve), the curve overall was better (RSQ values) for the diluted amebocyte lysate (FIG. 3A, bottom curve). The diluted amebocyte lysate had a log RSQ greater than 0.980 and a Z-score above 0 (FIG. 3B).

Example 6: Improved one-step workflow using optimized concentrations of peptide substrate and amebocyte lysate

This example describes an endotoxin assay workflow with a single incubation step, an optimized peptide substrate concentration (as described in Example 3) and an optimized amebocyte lysate concentration (as described in Examples 4 and 5).

In this assay, endotoxin samples (50 μ L) ranging from 0.0 to 1 EU/mL were pre-heated to 37°C in a 96-well microplate. A solution of fluorescent peptide substrate (peptide concentration of 185 nM) and amebocyte lysate (Vmax of 0.10) was added. The plate was maintained at 37°C for the duration of the assay. The fluorescent signal was monitored at 480nm/520nm excitation/emission over time. FIG. 4A shows results of a first study with sampling at five-minute intervals, while FIG. 4B shows results of a second study with sampling at one-minute intervals (FIG. 4B). Specific assay metrics, including the RSQ (square of the correlation coefficient) separation of background from signal (z score) were calculated. An RSQ > 0.980 and a z score > 0.0 was considered passing. The RSQ was calculated for the raw data (x = endotoxin concentration vs RFU) as well as the log RSQ where the data was log transformed (log sample concentration and log RFU). As shown in FIG. 4A, several of the time points showed passing criteria (RSQ above 0.980 and Z-score above 0, shown in bold font in FIG. 4A). In FIGs. 4A and 4B, failed metrics are annotated with an asterisk while the bolded box designates the window where all incubation times passed the desired specifications.

Example 7: Endotoxin detection assay using a fluorescent substrate and a microplate reader

This example describes an exemplary assay that can be performed to detect and quantify endotoxin in a sample. The assay can be performed using 50 μ L, 25 μ L or 5 μ L of input sample, which allows for the detection of endotoxin at a concentration of 0.01-1.0 EU/mL, 0.02-2.0 EU/mL, or 0.10-10.0 EU/mL, respectively. An overview of the assay workflow is shown as Method 500 in FIG. 5.

Materials

- Endotoxin reagent containing a fluorescently-labelled peptide substrate
- Endotoxin-free DMSO
- Lyophilized endotoxin standard containing 10-50 EU of endotoxin from *E. coli* (0111:B4)
- Lyophilized amebocyte lysate

- Endotoxin-free water
- Pyrogen-free pipette tips and pipettors
- Pyrogen-free 96-well microtiter plate (reaction vessel)
- Endotoxin-free glass test tubes
- 5 • Stable temperature heat block with 96-well plate adaptor
- Plate mixer
- Vortex mixer
- Acetic acid, 25%

10 **Preparation of Sample and Standards**

The endotoxin-free water, DMSO and endotoxin standard are brought to room temperature and the heat block is pre-warmed to $37\pm 1^{\circ}\text{C}$.

- The endotoxin standard is reconstituted with room temperature, endotoxin-free water to make a 10 EU/mL solution. The solution is mixed vigorously for 10 minutes at ~1500 rpm. A serial dilution of the endotoxin solution is performed to yield four different standards: 1.0 EU/mL, 0.1 EU/mL, 0.01 EU/mL and 0.0 EU/mL.

- 15 A volume of 50 μL of each endotoxin standard solution is added to a pyrogen-free 96-well plate using the desired number of replicates (such as $n\geq 3$). The samples are mixed vigorously prior to pipetting.

- 20 A total volume of 50 μL of each sample is added to empty wells of the 96-well plate. If using 5 μL or 25 μL of sample, pyrogen-free water is added to yield a final volume of 50 μL . Replicates of each sample can be included. If needed, the pH of the samples to be tested is adjusted to pH 6-8 using endotoxin-free 0.1 M NaOH or 0.1 M HCl.

- Components of undiluted serum can interfere with the assay. A serum sample must be free of red blood cells. The sample is optionally heat-shocked at 70°C for 15 minutes.

- 25 The 96-well plate containing the standards and sample(s) is pre-heated at $37\pm 1^{\circ}\text{C}$ for 10-20 minutes using a heat block.

Preparation of Working Solution

- 30 The endotoxin reagent is dissolved in 80 μL of endotoxin-free DMSO and mixed vigorously. The lyophilized amebocyte lysate is reconstituted with endotoxin-free water and mixed gently to dissolve the powder. The working solution is made by combining 30 μL of

the DMSO-endotoxin reagent to the rehydrated amebocyte lysate. The two components are mixed by inversion.

Assay Steps

- 5 The multi-well plate is kept at $37\pm 1^{\circ}\text{C}$ for the duration of the assay. Working solution (100 μL) is added to each well containing a standard or sample and mixed by tapping the plate several times or using a plate mixer. The plate is incubated for the lot-specific time (about 15-25 minutes). The reaction is stopped by adding 50 μL of stop solution (25% acetic acid in water) to each well. For consistent results, the stop solution is
- 10 added to the wells in the same order as the amebocyte lysate was added. The contents of the multi-well plate are mixed by gently tapping the plate or using a plate mixer.

Data Acquisition and Analysis

- 15 The multi-well plate is read using a fluorescent plate reader, with excitation at 490 nm and emission at 525 nm, using ± 10 nm bandwidth and top read optics. A software analysis program is used to obtain a regression for the standards. The analysis can be performed using log transformed concentration and RFU data, followed by a linear regression fit to verify that the correlation coefficient, r , is greater than or equal to 0.980. Once verified, sample concentrations are obtained using a log-transformation on the concentration and background
- 20 corrected RFUs using a quadratic curve fitting. A linear curve fitting is optionally done prior to quadratic curve fitting to confirm that the correlation coefficient, r , is satisfactory.

Exemplary Results

Standard	Concentration	Avg RFU	Corrected Avg RFU	log Concentration	log RFU
STD1	0.00 EU/mL	8.4	0.0	NA	NA
STD2	0.01 EU/mL	15.1	6.7	-2	0.827
STD3	0.10 EU/mL	96.5	88.1	-1	1.945
STD4	1.00 EU/mL	614.5	606.1	0	2.783

- 25 The curve-fitting model is used to determine the unknown sample concentrations. The following example is provided using the log transformed data from the above table and quadratic curve fitting.

$$\text{Sample EU/mL} = 10^{[-b - \sqrt{b^2 - 4*a*(c-\text{STD1})}]/2a}$$

$$a = -0.140$$

b = 0.697

c = 2.783

Standard	Concentration	Calculated Concentration
STD1	0.00 EU/mL	NA
STD2	0.01 EU/mL	0.010 EU/mL
STD3	0.10 EU/mL	0.100 EU/mL
STD4	1.00 EU/mL	1.000 EU/mL

5

Example 8: Endotoxin detection assay using a fluorescent substrate and a QUBIT Flex fluorometer

This example describes an exemplary assay that can be performed to detect and quantify endotoxin in a sample using the QUBIT Flex Fluorometer. The assay can be performed using 50 µL, 25 µL or 5 µL of input sample, which allows for the detection of endotoxin at a concentration of 0.01-1.0 EU/mL, 0.02-2.0 EU/mL, or 0.10-10.0 EU/mL, respectively. An overview of the assay workflow is shown as Method 800 in FIG. 8.

Materials

- Endotoxin reagent containing a fluorescently-labelled peptide substrate
- Endotoxin-free DMSO
- Lyophilized endotoxin standard containing 10-50 EU of endotoxin from *E. coli* (0111:B4)
- Lyophilized amebocyte lysate
- Endotoxin-free water
- Acetic acid, 25%
- Disposable pyrogen-free glass tubes
- PCR tube strip mixer
- Pyrogen-free pipette tips and pipettors
- Pyrogen-free tube strips (reaction vessel)
- Stable temperature heat block with microfuge tube inserts
- Vortex mixer

Preparation of Sample and Standards

The endotoxin-free water, DMSO and endotoxin standard are brought to room temperature and the heat block is pre-warmed to $37\pm1^{\circ}\text{C}$.

The endotoxin standard is reconstituted with room temperature, endotoxin-free water to make a 10 EU/mL solution. The solution is mixed vigorously for 10 minutes (*e.g.*, at
5 <1500 rpm). A serial dilution of the endotoxin solution is performed to yield four different standards: 1.0 EU/mL, 0.1 EU/mL, 0.01 EU/mL and 0.0 EU/mL.

The standard solutions are mixed vigorously prior to pipetting. A volume of 50 μL of each endotoxin standard solution is added to empty tubes of the tube stripe (in duplicate or quadruplicate) in the following locations:

Tubes 1 & 2	Tubes 3 & 4	Tubes 5 & 6	Tubes 7 & 8
0 EU/mL (STD1)	0.01 EU/mL (STD2)	0.1 EU/mL (STD3)	1.0 EU/mL (STD4)

10

A total volume of 50 μL of each sample is added to appropriate tubes of the tube strip. If using 5 μL or 25 μL of sample, pyrogen-free water is added to yield a final volume of 50 μL . Replicates of each sample can be included. If needed, the pH of the samples to be tested is adjusted to pH 6-8 using endotoxin-free 0.1 M NaOH or 0.1 M HCl.

15 Components of undiluted serum can interfere with the assay. A serum sample must be free of red blood cells. The sample is optionally heat-shocked at 70°C for 15 minutes.

The tube strip(s) containing the standards and sample(s) is pre-heated at $37\pm1^{\circ}\text{C}$ for 10-20 minutes using a heat block.

20 Preparation of Working Solution

The endotoxin reagent is dissolved in 80 μL of endotoxin-free DMSO and mixed vigorously. The lyophilized amebocyte lysate is reconstituted with 4.4 mL endotoxin-free water and mixed gently to dissolve the powder. The working solution is prepared by combining 30 μL of the DMSO-endotoxin reagent to the rehydrated amebocyte lysate. The
25 two components are mixed by inversion.

Assay Steps

The tube strip is kept at $37\pm1^{\circ}\text{C}$ for the duration of the assay. Working solution (100 μL) is added to each tube containing a standard or sample and thoroughly mixed (such as by
30 using a vortex with a tube-strip adaptor and vortexing at speeds fast enough to create a visible vortex). The plate is incubated for the lot-specific time (about 15-25 minutes). The reaction

is stopped by adding 50 μ L of stop solution (25% acetic acid in water) to each tube. For consistent results, the stop solution is added to the wells in the same order as the amebocyte lysate was added. The contents of the tubes are mixed by vortex. The results are read on the QUBIT Flex Fluorometer.

5

Example 9: Validation of the endotoxin detection assay using a microplate reader

This example describes results obtained using the materials and endotoxin detection assay protocol described in Example 7 and shown in FIG. 5.

10 Preparation of Standards

Endotoxin-free water, DMSO and endotoxin standard were brought to room temperature and a heat block was pre-warmed to $37\pm 1^\circ\text{C}$. Endotoxin standard was reconstituted with room temperature, endotoxin-free water to make a 10 EU/mL solution. The solution was mixed vigorously for 10 minutes at ~ 1500 rpm. A serial dilution of the endotoxin solution was performed to yield four different standards: 1.0 EU/mL, 0.1 EU/mL, 0.01 EU/mL and 0.0 EU/mL. A volume of 50 μ L of each endotoxin standard solution was added to a pyrogen-free 96-well plate using three or more replicates. The samples were mixed vigorously prior to pipetting. The 96-well plate containing the standards was pre-heated at $37\pm 1^\circ\text{C}$ for 10-20 minutes using a heat block.

20

Preparation of Working Solution

The endotoxin reagent was dissolved in 80 μ L of endotoxin-free DMSO and mixed vigorously. The lyophilized amebocyte lysate was reconstituted with endotoxin-free water and mixed gently to dissolve the powder. The working solution was made by combining 30 μ L of the DMSO-endotoxin reagent to the rehydrated amebocyte lysate. The two components were mixed by inversion.

25

Assay Steps

The multi-well plate was kept at $37\pm 1^\circ\text{C}$ for the duration of the assay. Working solution (100 μ L) was added to each well containing a standard and mixed by tapping the plate several times or using a plate mixer. The plate was incubated for the lot-specific time (15-25 minutes). The reaction was stopped by adding 50 μ L of stop solution (25% acetic acid in water) to each well. For consistent results, the stop solution was added to the wells in

30

the same order as the amebocyte lysate was added. The contents of the multi-well plate were mixed by gently tapping the plate or using a plate mixer.

Data Acquisition, Analysis and Results

5 The multi-well plate was read using a fluorescent plate reader (Tecan Infinite M1000 PRO), with excitation at 490 nm and emission at 525 nm, using ± 10 nm bandwidth and top read optics. A software analysis program was used to obtain a regression for the standards. The analysis was performed using log transformed concentration and RFU data, followed by a linear regression fit to verify that the correlation coefficient, r , was greater than or equal to 10 0.980. Once verified, concentrations were obtained using a log-transformation on the concentration and background corrected RFUs using a quadratic curve fitting. A linear curve fitting was done prior to quadratic curve fitting to confirm that the correlation coefficient, r , was satisfactory. The curve-fitting model was used to determine the concentrations of endotoxin. The results showed that the expected concentration and calculated concentration 15 of endotoxin were very similar (FIGS. 6A-6C), demonstrating the accuracy of this assay.

In an additional study, endotoxin standards were tested using the same protocol described above, and the results were obtained using three different plate readers (Agilent BIOTEK, SPECTRAMAX and VARIOSKAN LUX), as well as the QUBIT Flex (an 8-well fluorometer). The results showed that the expected and calculated concentrations of 20 endotoxin were highly similar regardless of the instrument used for reading the results (FIGS. 7A-7D). The accuracy, precision, linearity and background separation of each plate reader and the QUBIT Flex are summarized in Table 3.

25 **Table 3. Summary of results obtained using three different plate readers and Qubit Flex**

Metric	QUBIT Flex	VARIOSKAN LUX	Agilent BIOTEK	SPECTRAMAX
Accuracy (error vs. expected value)	0.01 $\pm$ 6%	0.01 $\pm$ 1%	0.01 $\pm$ 2%	0.01 $\pm$ 3%
	0.05 $\pm$ 7%	0.05 $\pm$ 7%	0.05 $\pm$ 7%	0.05 $\pm$ 15%
	0.10 $\pm$ 4%	0.10 $\pm$ 6%	0.10 $\pm$ 2%	0.10 $\pm$ 13%
	0.50 $\pm$ 9%	0.50 $\pm$ 4%	0.50 $\pm$ 13%	0.50 $\pm$ 7%
	1.00 $\pm$ 5%	1.00 $\pm$ 4%	1.00 $\pm$ 8%	1.00 $\pm$ 6%
Precision	0.01 $\pm$ 8%	0.01 $\pm$ 7%	0.01 $\pm$ 2%	0.01 $\pm$ 8%
	0.05 $\pm$ 2%	0.05 $\pm$ 5%	0.05 $\pm$ 2%	0.05 $\pm$ 3%

Metric	QUBIT Flex	VARIOSKAN LUX	Agilent BIOTEK	SPECTRAMAX
(variation between replicates)	0.10 $\pm$ 4%	0.10 $\pm$ 7%	0.10 $\pm$ 2%	0.10 $\pm$ 1%
	0.50 $\pm$ 8%	0.50 $\pm$ 3%	0.50 $\pm$ 8%	0.50 $\pm$ 2%
	1.00 $\pm$ 3%	1.00 $\pm$ 3%	1.00 $\pm$ 1%	1.00 $\pm$ 1%
Linearity (<i>r</i>)	0.996	0.998	0.997	0.996
Background separation (Z score)	0.88	0.58	0.64	0.63

Example 10: Validation of endotoxin detection assay using a QUBIT fluorometer

This example describes results obtained using the materials and endotoxin detection assay protocol described in Example 8 and shown in FIG. 8.

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Preparation of Standards

Endotoxin-free water, DMSO and endotoxin standard were brought to room temperature and a heat block was pre-warmed to 37 $\pm$ 1°C. The endotoxin standard was reconstituted with room temperature, endotoxin-free water to make a 10 EU/mL solution.

- 10 The solution was mixed vigorously for 10 minutes (at <1500 rpm). A serial dilution of the endotoxin solution was performed to yield four different standards: 1.0 EU/mL, 0.1 EU/mL, 0.01 EU/mL and 0.0 EU/mL. The standard solutions were mixed vigorously prior to pipetting. A volume of 50 μ L of each endotoxin standard solution was added to empty tubes of the tube stripe (in duplicate or quadruplicate). The tube strip containing the standards was
- 15 pre-heated at 37 $\pm$ 1°C for 10-20 minutes using a heat block.

Preparation of Working Solution

- The endotoxin reagent was dissolved in 80 μ L of endotoxin-free DMSO and mixed vigorously. The lyophilized amebocyte lysate was reconstituted with 4.4 mL endotoxin-free
- 20 water and mixed gently to dissolve the powder. The working solution was prepared by combining 30 μ L of the DMSO-endotoxin reagent to the rehydrated amebocyte lysate. The two components were mixed by inversion.

Assay Steps

- 25 The tube strip was kept at 37 $\pm$ 1°C for the duration of the assay. Working solution (100 μ L) was added to each tube containing a standard and thoroughly mixed using a vortex

with a tube-strip adaptor and vortexing at speeds fast enough to create a visible vortex. The plate was incubated for the lot-specific time (15-25 minutes). The reaction was stopped by adding 50 μ L of stop solution (25% acetic acid in water) to each tube. For consistent results, the stop solution was added to the wells in the same order as the amebocyte lysate was added.

- 5 The contents of the tubes were mixed by vortex. The results were read on a QUBIT Flex Fluorometer.

Results

- Data processing for the QUBIT endotoxin detection assay was performed by one or
10 more algorithms integrated in the fluorometer. Briefly, the on-instrument data processing included first plotting the background-corrected data (FIG. 9A), and then a log transformed linear regression was performed to determine the correlation coefficient (r) (FIG. 9B). After confirming $r \geq 0.980$, the data was fit to a polynomial model for increased accuracy. Concentrations were then calculated and plotted against the expected results (FIG. 9C). The
15 results demonstrate that the calculated concentrations are nearly identical to the expected concentrations, indicating the accuracy of the assay.

- Further studies were conducted to test different sample sizes, user variability, endotoxin sources, and dye lots. Using the protocol described above and in Example 8, 50 μ L sample sizes were tested, using 4 sample replicates per concentration, lot 1 LAL, and an
20 incubation time of 20 minutes. The results are shown in FIGS. 10A-10B. The same assay was conducted using a sample size of 5 μ L, four replicates per concentration, lot 2 LAL, and an incubation time of 21 minutes. The results are shown in FIGS. 11A-11B. These results demonstrate that accurate results were obtained regardless of the sample size.

- Additional studies evaluated the endotoxin detection assay using two different users
25 (user #1 and user #2), two different endotoxin control standards (concentration of 25 EU and 35 EU), two different dye lots (dye lot A and dye lot B), and two different lots of LAL (lot 1 and lot 2). Four sample replicates were used per concentration of endotoxin standard. The results using lot 1 LAL are shown in FIGS. 12A-12B and the results using lot 2 LAL are shown in FIGS. 13A-13B. In addition, FIGS. 14A-14B show the results of the endotoxin
30 detection assay performed by user #1, using the 25 EU endotoxin control, and either lot 1 LAL combined with dye lot A (FIG. 14A) or lot 2 LAL combined with dye lot B (FIG. 14B). These data demonstrate that the one-step incubation endotoxin detection assay provides

consistent results despite changes in LAL lots, dye lots, users and the endotoxin control standard concentration.

System variability was also tested by performing the endotoxin detection assay described in Example 8 using five different QUBIT FLEX instruments (referred to as
5 QUBIT-1, QUBIT-2, QUBIT-3, QUBIT-4, AND QUBIT-5) and two different LAL lots (lot 1, t = 20 minutes; lot 2, t = 21 minutes). The results for Lot 1 and Lot 2 are shown in FIG. 15A and FIG. 15B, respectively. The results were consistent among the five different instruments for both LAL lots.

Taken together, these results demonstrate the validity of the one-step endotoxin
10 detection assay and demonstrate that the assay is sensitive and specific regardless of LAL lot, dye lot, user, sample volume, or instrument used to read the assay results.

It will be apparent that the precise details of the methods or compositions described may be varied or modified without departing from the spirit of the described aspects of the
15 disclosure. We claim all such modifications and variations that fall within the scope and spirit of the claims below.

INCORPORATION BY REFERENCE

All publications, patents, and patent applications mentioned in this specification are
20 herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

CLAIMS

1. A method for detecting endotoxin in a sample, comprising:
providing a working solution comprising amebocyte lysate and a labelled peptide
5 substrate susceptible to cleavage upon activation of proclotting enzyme in the amebocyte
lysate in the presence of endotoxin, wherein the working solution contains about 110 nM to
about 260 nM of the labelled peptide substrate and the amebocyte lysate enzyme Vmax is
about 0.07 to about 0.26 absorbance units of p-nitroaniline (pNA) per minute with 1
endotoxin unit (EU)/mL of endotoxin;
10 adding the sample to empty wells of a reaction vessel that has been pre-heated to a
temperature of about 36-38°C;
adding the working solution to the wells of the reaction vessel containing the sample;
heating the reaction vessel containing the sample and working solution at about 36-
38°C to allow for cleavage of the labelled peptide substrate;
15 adding a stop solution to each well containing the working solution to stop the
reaction between endotoxin present in the sample and proclotting enzyme present in the
amebocyte lysate; and
detecting the presence of the label released from the labelled peptide substrate upon
activation of proclotting enzyme in the amebocyte lysate by endotoxin, thereby detecting
20 endotoxin in the sample.
2. The method of claim 1, further comprising:
providing a plurality of endotoxin standards ranging in concentration;
adding each of the plurality of endotoxin standards to empty wells of a reaction vessel
25 that has been pre-heated to a temperature of about 36-38°C;
adding the sample to one or more empty wells of the pre-heated reaction vessel;
adding the working solution to the wells of the reaction vessel containing the plurality
of standards and to the wells of the reaction vessel containing the sample;
heating the reaction vessel containing the plurality of standards, sample and working
30 solution at about 36-38°C to allow for cleavage of the labelled peptide substrate;
adding a stop solution to each well containing the working solution to stop the
reaction between endotoxin present in the sample and proclotting enzyme present in the
amebocyte lysate; and

detecting the presence of the label released from the labelled peptide substrate upon activation of proclotting enzyme in the amebocyte lysate by endotoxin, thereby detecting endotoxin in the sample.

- 5 3. A method for detecting endotoxin in a sample, comprising:
 providing a plurality of endotoxin standards ranging in concentration;
 providing a working solution comprising amebocyte lysate and a labelled peptide
 substrate susceptible to cleavage upon activation of proclotting enzyme in the amebocyte
 lysate in the presence of endotoxin, wherein the working solution contains about 110 nM to
10 about 260 nM of the labelled peptide substrate and the amebocyte lysate enzyme Vmax is
 about 0.07 to about 0.26 absorbance units of p-nitroaniline (pNA) per minute with 1
 endotoxin unit (EU)/mL of endotoxin;
 adding each of the plurality of endotoxin standards to empty wells of a reaction vessel
 that has been pre-heated to a temperature of about 36-38°C;
15 adding the sample to one or more empty wells of the pre-heated reaction vessel;
 adding the working solution to the wells of the reaction vessel containing the plurality
 of standards and to the wells of the reaction vessel containing the sample;
 heating the reaction vessel containing the plurality of standards, sample and working
 solution at about 36-38°C to allow for cleavage of the labelled peptide substrate;
20 adding a stop solution to each well containing the working solution to stop the
 reaction between endotoxin present in the sample and proclotting enzyme present in the
 amebocyte lysate; and
 detecting the presence of the label released from the labelled peptide substrate upon
 activation of proclotting enzyme in the amebocyte lysate by endotoxin, thereby detecting
25 endotoxin in the sample.

4. The method of any one of claims 1-3, wherein the working solution contains
 about 150 nM to about 220 nM of the labelled peptide substrate.

- 30 5. The method of any one of claims 1-4, wherein the working solution contains
 about 185 nM of the labelled peptide substrate.

6. The method of any one of claims 1-5, wherein the working solution contains
 amebocyte lysate with an enzyme Vmax of about 0.07 to about 0.14, or about 0.10.

7. The method of any one of claims 1-6, wherein the sample is suspected of containing endotoxin.

5 8. The method of any one of claims 1-7, wherein the sample comprises antibodies, proteins, nucleic acid molecules, cells, cell culture media, water, plant material or soil.

9. The method of any one of claims 1-8, wherein the sample is added to the one
10 or more wells of the reaction vessel in a total volume of 50 μ L.

10. The method of claim 9, wherein the 50 μ L sample volume is:
5 μ L of sample diluted with 45 μ L of endotoxin-free water;
25 μ L of sample diluted with 25 μ L endotoxin-free water; or
15 50 μ L of undiluted sample.

11. The method of any one of claims 2-10, wherein the plurality of endotoxin standards ranges in concentration from about 10.0 endotoxin units (EU)/mL to about 0.0 EU/ml, about 5.0 EU/m to about 0.0 EU/ml, about 2.5 EU/ml to about 0.0 EU/ml, or about
20 1.0 EU/mL to about 0.0 EU/ml.

12. The method of any one of claims 2-11, wherein the plurality of endotoxin standards comprises four standards with endotoxin concentrations of 1.0 EU/mL, 0.1 EU/mL, 0.01 EU/mL and 0.00 EU/mL, respectively.
25

13. The method of any one of claims 2-12, wherein 50 μ L of each of the plurality of endotoxin standards is added to the wells of the reaction vessel.

14. The method of any one of claims 2-13, wherein each of the plurality of
30 endotoxin standard is added in three replicates to the reaction vessel.

15. The method of any one of claims 2-14, wherein the reaction vessel containing the plurality of endotoxin standards and sample is heated at a temperature of about 36-38°C for about 10 minutes prior to adding the working solution.

5 16. The method of any one of claims 1-15, wherein the labelled peptide substrate is dissolved in endotoxin-free DMSO prior to combining with the amebocyte lysate.

17. The method of any one of claims 2-16, wherein approximately 100 µL of the working solution is added to each well containing the plurality of endotoxin standards and
10 each well containing the sample.

18. The method of any one of claims 2-17, wherein the plurality of endotoxin standards, sample and working solution are heated at about 36-38°C for a pre-determined amount of time sufficient to allow for cleavage of the labelled peptide substrate.

15 19. The method of claim 18, wherein the pre-determined amount of time sufficient to allow for cleavage of the labelled peptide substrate is about 15 to about 40 minutes.

20. The method of claim 18, wherein the pre-determined amount of time sufficient
20 to allow for cleavage of the labelled peptide substrate is about 15 to about 25 minutes.

21. The method of any one of claims 1-20, wherein the stop solution comprises acetic acid.

25 22. The method of claim 21, wherein the acetic acid is 25% acetic acid in water.

23. The method of any one of claims 1-22, wherein the label is a fluorescent label.

24. The method of claim 23, wherein the fluorescent label is detected using a
30 fluorometer or fluorescent plate reader.

25. The method of any one of claims 1-22, wherein the label is a chromogenic label.

26. The method of claim 25, wherein the chromogenic label is detecting using a spectrophotometer or a microplate reader.

5 27. The method of any one of claims 1-26, wherein the reaction vessel is a multi-well plate or a cartridge.

28. The method of any one of claims 1-26, wherein the reaction vessel is a plurality of polypropylene tubes.

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29. The method of any one of claims 1-28, further comprising determining the amount of endotoxin in the sample.

30. The method of claim 29, wherein determining the amount of endotoxin in the sample comprises comparing the amount of label released by the sample to the amount of label released by the plurality of endotoxin standards.

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31. The method of any one of claims 1-30, wherein the method has a sensitivity of >0.01 EU/mL of endotoxin.

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32. The method of any one of claims 1-31, further comprising calculating regression of the plurality of endotoxin standards using log transformed concentration and relative fluorescent unit (RFU) data and performing linear regression fit to calculate the correlation coefficient.

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33. The method of claim 32, wherein the correlation coefficient is greater than or equal to 0.980.

34. The method of any one of claims 1-33, further comprising calculating endotoxin concentration in the sample.

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35. The method of claim 34, wherein calculating endotoxin concentration in the sample comprises quadratic curve fitting.

36. A kit for detecting endotoxin, comprising:
lyophilized endotoxin standard;
lyophilized amebocyte lysate (LAL) with a V_{max} of about 0.07 to about 0.14; and
labelled peptide substrate at a concentration of about 295 nM to about 445 nM.
- 5
37. The kit of claim 36, wherein the LAL has a V_{max} of about 0.10.
38. The kit of claim 36 or claim 37, wherein the labelled peptide substrate is at a
concentration of about 370 nM.
- 10
39. The kit of any one of claims 36-38, further comprising one or more of
endotoxin-free DMSO, endotoxin-free water, a reaction vessel, acetic acid, pipette tips, and
instructional material.
- 15
40. The kit of claim 39, wherein the reaction vessel comprises a multi-well plate,
polypropylene tubes, or a cartridge.