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 one aspect, provided herein is a method for detecting endotoxin in a sample, comprising: 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 110 nM to 260 nM of the labelled peptide substrate and the amebocyte lysate enzyme Vmax is 0.07 to 0.26 absorbance units of p-nitroaniline (pNA) per minute with 1 endotoxin unit (EU)/mL of endotoxin; adding the sample to empty wells of a reaction vessel that has been pre-heated to a temperature of 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 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. In another aspect, provided herein is a kit for detecting endotoxin, comprising: lyophilized endotoxin standard; lyophilized amebocyte lysate (LAL) with a Vmax of 0.07 to 0.14; and labelled peptide substrate at a concentration of 295 nM to 445 nM; optionally, wherein the LAL has a Vmax of about 0.10; optionally, wherein the labelled peptide substrate is at a concentration of about 370 nM. In some examples, 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 Vmax 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 Vmax of the amebocyte lysate is about 0.07 to about 0.14, such as about 0.10.

In another example, 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.

Also provided herein are kits for detecting endotoxin. In some examples, 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, 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 examples which proceeds with reference to the accompanying figures.

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 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).

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.

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

CLAIMS

1. A method for detecting endotoxin in a sample, comprising:

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 110 nM to 260 nM of the labelled peptide substrate and the amebocyte lysate enzyme Vmax is 0.07 to 0.26 absorbance units of p-nitroaniline (pNA) per minute with 1 endotoxin unit (EU)/mL of endotoxin;

adding the sample to empty wells of a reaction vessel that has been pre-heated to a temperature of 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 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.

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 that has been pre-heated to a temperature of 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 solution at 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.

3. The method of claim 1 or claim 2, wherein the working solution contains 150 nM to 220 nM of the labelled peptide substrate;

optionally, wherein the working solution contains about 185 nM of the labelled peptide substrate;

optionally, wherein the working solution contains amebocyte lysate with an enzyme Vmax of 0.07 to 0.14, or about 0.10.

4. The method of any one of claims 1-3, wherein the sample comprises antibodies, proteins, nucleic acid molecules, cells, cell culture media, water, plant material or soil.

5. The method of any one of claims 1-4, wherein the sample is added to the one or more wells of the reaction vessel in a total volume of 50 µL;

optionally, wherein the 50 µL sample volume is:

5 µL of sample diluted with 45 µL of endotoxin-free water;

25 µL of sample diluted with 25 µL endotoxin-free water; or

50 µL of undiluted sample.

6. The method of any one of claims 2-5, wherein the plurality of endotoxin standards ranges in concentration from 10.0 endotoxin units (EU)/mL to 0.0 EU/ml, 5.0 EU/m to 0.0 EU/ml, 2.5 EU/ml to 0.0 EU/ml, or 1.0 EU/mL to 0.0 EU/ml;

optionally, 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.

7. The method of any one of claims 2-6, wherein 50 µL of each of the plurality of endotoxin standards is added to the wells of the reaction vessel;

optionally, wherein each of the plurality of endotoxin standard is added in three replicates to the reaction vessel.

8. The method of any one of claims 2-7, wherein the reaction vessel containing the plurality of endotoxin standards and sample is heated at a temperature of 36-38°C for about 10 minutes prior to adding the working solution.

9. The method of any one of claims 2-8, wherein approximately 100 µL of the working solution is added to each well containing the plurality of endotoxin standards and each well containing the sample.

10. The method of any one of claims 2-9, wherein the plurality of endotoxin standards, sample and working solution are heated at 36-38°C for a pre-determined amount of time sufficient to allow for cleavage of the labelled peptide substrate;

optionally, wherein the pre-determined amount of time sufficient to allow for cleavage of the labelled peptide substrate is 15 to 40 minutes;

optionally, wherein the pre-determined amount of time sufficient to allow for cleavage of the labelled peptide substrate is 15 to 25 minutes.

11. The method of any one of claims 1-10, wherein the stop solution comprises acetic acid;

optionally, wherein the acetic acid is 25% acetic acid in water.

12. The method of any one of claims 1-11, wherein the label is a fluorescent label or a chromogenic label.

13. The method of claim 12, wherein the label is detected using a fluorometer, a fluorescent plate reader, a spectrophotometer, or a microplate reader.

14. The method of any one of claims 1-13, wherein the reaction vessel is a multi-well plate, a cartridge, or a plurality of polypropylene tubes.

15. The method of any one of claims 1-14, further comprising determining the amount of endotoxin in the sample;

optionally, 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;

optionally 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.

16. The method of any one of claims 1-15, wherein the method has a sensitivity of >0.01 EU/mL of endotoxin.

17. The method of claim 15, wherein the correlation coefficient is greater than or equal to 0.980.

18. The method of any one of claims 1-17, further comprising calculating endotoxin concentration in the sample;

optionally wherein calculating endotoxin concentration in the sample comprises quadratic curve fitting.

19. A kit for detecting endotoxin, comprising:

lyophilized endotoxin standard;

lyophilized amebocyte lysate (LAL) with a Vmax of 0.07 to 0.14; and

labelled peptide substrate at a concentration of 295 nM to 445 nM;

optionally, wherein the LAL has a Vmax of about 0.10;

optionally, wherein the labelled peptide substrate is at a concentration of about 370 nM.

20. The kit of claim 19, further comprising one or more of endotoxin-free DMSO, endotoxin-free water, a reaction vessel, acetic acid, pipette tips, and instructional material;

optionally, wherein the reaction vessel comprises a multi-well plate, polypropylene tubes, or a cartridge.