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1 **RAPID ASSAY FOR DETECTION OF ENDOTOXINS**

2 **CROSS REFERENCE TO RELATED APPLICATION**

3 This application is a continuation-in-part of parent patent application entitled
4 **OPTICAL IMMUNOASSAY FOR MICROBIAL ANALYTES USING NON-SPECIFIC**
5 **DYES** of **Frances S. Ligler et al.** designated by **Serial No. 08/102,933** and **Navy Case No.**
6 **75,315** and filed in the U.S. Patent and Trademark Office on **August 6, 1993**, which
7 parent application is still pending before the U.S. Patent and Trademark Office, and
8 which patent application is incorporated herein by reference in its entirety and for all
9 purposes.

10 **BACKGROUND OF THE INVENTION**

11 **FIELD OF THE INVENTION**

12 The present invention relates to an apparatus and method for detecting
13 endotoxin.

14 **DESCRIPTION OF THE RELATED ART**

15 All gram-negative bacteria and many fungi have endotoxin as a major constituent
16 of their cell surface. Most gram-positive bacteria have a similar, endotoxin-like molecule
17 as a constituent of the cell surface. In general, bacterial endotoxins partially consist of a
18 **highly variable outer region** and a **conserved inner core**. The **variable outer region** is
19 composed of repeating oligosaccharide (sugar) units comprising the O-ANTIGENIC
20 region. The OUTER CORE and the O-ANTIGEN region, found within the cell walls
21 of various gram negative bacterial species, show species differentiation among endotoxins.

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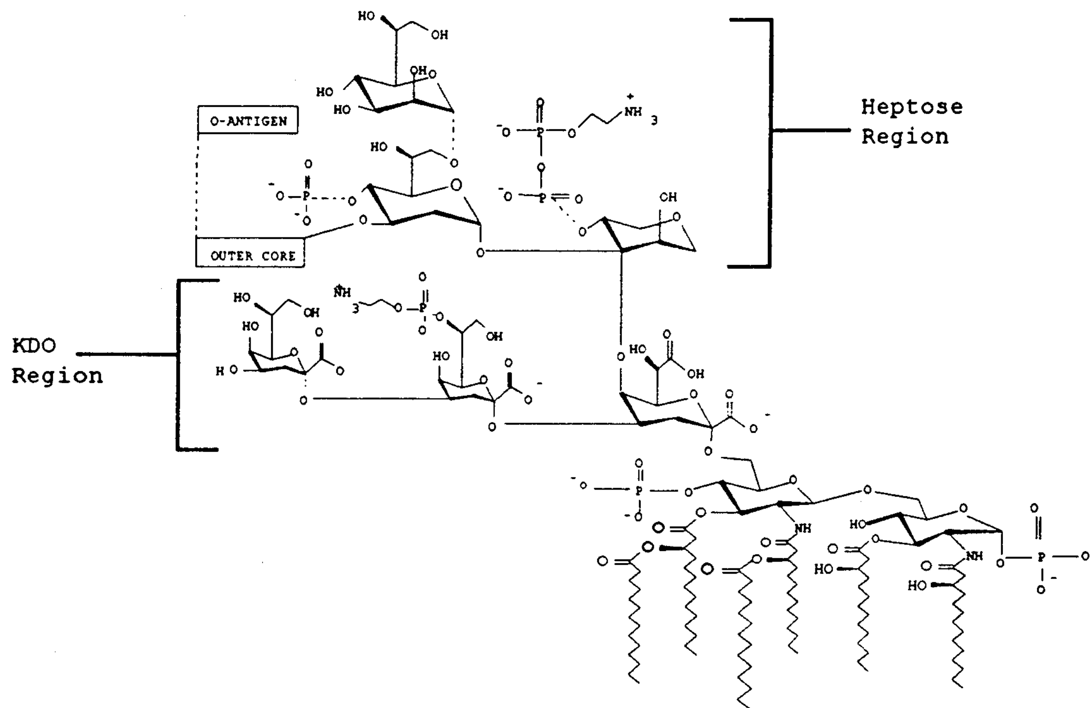
1 Bacterial endotoxins also consist of a **relatively constant, conserved inner core region.**
2 The **conserved inner core** consists of the KDO region and the heptose region. The lipid
3 A moiety (also a conserved portion of the endotoxin) is the toxic region of the endotoxin
4 found in the cell surface of gram-negative bacteria. An exemplary endotoxin is shown
5 below:
6



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1 Endotoxin is an extremely powerful stimulator of the immune system. The
2 devastating effects of bacterial infections and septicemia are in large part due to
3 endotoxin. Mortality rates, due to septicemia are high, 60% or more. The most effective
4 treatment of endotoxin related problems is early detection in real time with high
5 sensitivity. Additionally, endotoxin is a significant contaminant in food products and
6 pharmaceutical products. Accurate determination of endotoxin concentration is required
7 prior to product distribution. To satisfy the requirements of industrial production,
8 endotoxin assays must be accurate, rapid and cost effective.

9 Levin and Bang observed in the horseshoe crab (Limulus polyphemus) that blood
10 coagulation was a consequence of gram-negative bacterial infections. **See Levin, J., Bang,**
11 **F.B., 19 THROMBOS. DIATH. HAEMORRH., 186-197 (1968)**, incorporated by reference
12 herein in its entirety and for all purposes. When an extract of the horseshoe crab blood
13 was prepared and tested, a gelation reaction was observed in the presence of endotoxin.
14 Levin and Bang postulated that endotoxin mediated the gelation reaction of horseshoe
15 crab blood. They further postulated that the gelation reaction was initiated
16 enzymatically. The enzyme responsible for initiating the gelation reaction was identified
17 as limulus amebocyte lysate (LAL).

18 The reaction mechanism for the gelation reaction in horseshoe crab blood involves
19 the activation of a proclotting enzyme by **Ca^{2+}** and **endotoxin.** The **activated proclotting**
20 **enzyme** catalyzes the hydrolytic cleavage of coagulogen (a clottable protein of 215 amino
21 acid residues) into polypeptide subunits. **Clotting** occurs following the cleavage of the

1 215 amino acid coagulogen protein into a soluble peptide of 45 amino acid residues and
2 an insoluble peptide (coagulin) of approximately 170 amino acid residues. The insoluble
3 170 amino acid peptide, coagulin, undergoes polymerization to form a stable clot or gel.
4 The presence of a gel or clot indicates the presence of endotoxin. The formation of a
5 gel or clot is used in what is known as the limulus amebocyte lysate method (LAL). The
6 LAL method and its variations are the most commonly used endotoxin detection
7 methods.

8 The standard LAL tests are of two types, gelation and chromogenic. Both assays
9 are based on the enzyme cleavage reaction of coagulogen, but, in the chromogenic assay,
10 a color is produced during the cleavage step. Thus, the presence of the color instead of
11 a clot or gel indicates the presence of endotoxin.

12 The limitations of these LAL assays include limitations of specificity, limitations of
13 interfering substances and limitations of reproducibility. The LAL techniques require an
14 enzymatic reaction to detect the presence of endotoxin. Hence, substances that inhibit or
15 stimulate enzymatic cleavage of the 215 amino acid coagulogen protein will lead to false-
16 negative or false-positive results, respectively. In samples, such as serum or blood, there
17 are several factors known to interfere with the LAL method. For example, the LAL
18 enzyme cascade is inhibited by antibiotics, hormones, heavy metals, amino acids,
19 alkaloids, carbohydrates, plasma proteins, enzymes, electrolytes and B-1,3-D-glucan. **See**
20 **Satoshi, M., Masahiro, N., Taizo, W., Tadashi, S. and Tetsuya, T., 198 ANALYTICAL**
21 **BIOCHEMISTRY 292-297 (1983)**, incorporated by reference herein in its entirety and for all

1 purposes. For example, false positive gelation can be caused by thrombin,
2 thromboplastin, RNA, RNAase, trypsin, trypsin-like enzymes, lipotechoic acid and
3 peptidoglycan fragments. False negative results (blocking gelation) can be caused by
4 trypsin inhibitors, EDTA, other calcium binding reagents, high salt concentrations and
5 semi-synthetic penicillins. **See European Patent No. EP 0 265 127 A1**, incorporated
6 herein by reference in its entirety and for all purposes.

7 Another detection strategy is the sandwich enzyme linked immunosorbent assay
8 (ELISA). This assay (ELISA) involves immobilizing an antibody specific for a conserved
9 region of the endotoxin (**e.g.** the KDO region). Using ELISA, the endotoxin
10 immobilized (**i.e.** captured) by a first antibody is detected by using a second antibody
11 attached to another antigenic site of the endotoxin and a chromogenic enzyme. The
12 sensitivity of this technique is about **1 μg/ml.** This test is time consuming, requiring over
13 4 hours.

14 There are several limitations of the ELISA assay used in the detection of
15 endotoxin. Endotoxin has low affinity for ELISA plates. The lack of endotoxin affinity
16 diminishes sensitivity. When a first antibody is immobilized onto an ELISA plate and
17 endotoxin is bound by (**i.e.** captured by or immobilized by) the first antibody, there
18 appears to be significant interference with the binding of a secondary antibody to the
19 immobilized endotoxin, used in the detection of endotoxin. Finally, the sensitivity of
20 ELISA assays (in the **1μg/ml** range) is far below that of the LAL assays (**1 ng/ml** for
21 chromogenic LAL assay) and higher than clinically relevant concentrations of endotoxin

1 of about **1 ng/ml.**

2 Other variations of the LAL assays have been developed. These involve
3 combining the LAL assay with an enzyme linked immunosorbent assay (ELISA). A
4 capture antibody (**i.e.** a polyclonal first anti-endotoxin antibody) for the oligosaccharide
5 region of an endotoxin is immobilized on a microtiter plate. Endotoxin is introduced
6 over the ELISA microtiter plate. The bound endotoxin is detected by using the
7 chromogenic LAL system. Sensitivity is in the range of **2 pg/ml** in PBS and **10 pg/ml** in
8 diluted plasma. **See Mertsola, J., Cope, L.D., Munford R.S., McCracken, G.H. and**
9 **Hansen, E.J,** ***Detection of Experimental Haemophilus influenzae Type b Bacteremia and***
10 ***Endotoxemia by Means of an Immunolimulus Assay*****, 164 THE JOURNAL OF INFECTIOUS**
11 **DISEASES 353-358 (1991)**, incorporated by reference herein in its entirety and for all
12 purposes. **See Mertsola et al.,** ***Specific Detection of Haemophilus influenzae Type b***
13 ***Lipooligosaccharide by Immunoassay*****, 28 (12) JOURNAL OF CLINICAL MICROBIOLOGY pp.**
14 **2700-2706 (December 1990)**, incorporated by reference herein in its entirety and for all
15 purposes. The combination ELISA/LAL assay requires at least 12-24 hours to complete.

16 In the combination ELISA/LAL assay systems, the limitations are cumulative.
17 The endotoxin is first bound to an ELISA microtiter plate via a reaction with an antibody
18 or other capture molecule and the endotoxin is then detected using the chromogenic
19 LAL assay. The substances that interfere with the LAL assay also interfere with the
20 combination ELISA/LAL assay. In addition, the LAL reaction portion of the assay
21 requires a minimum of 40 minutes to 2 hours to perform and requires that serum be

1 removed prior to the addition of the enzyme because serum components may inhibit the
2 enzyme activity. the LAL assay does not reliably quantitate the amount of endotoxin
3 present. Detection of endotoxin in serum is five times (5x) less sensitive than detection
4 of endotoxin in buffer.

5 An antibody-based test reported to have higher sensitivity than ELISA is the latex
6 immunoassay technique. In this assay, latex beads are coated with a monoclonal
7 antibody (Ab1) specific for the O-9 determinant of endotoxin. The beads are then
8 incubated with a solution containing lipopolysaccharide (LPS; synonym for endotoxin). A
9 magnetic bead coated with another monoclonal antibody (Ab2) specific for a different
10 antigenic site of an endotoxin is added to the LPS solution surrounding the latex beads
11 coated with Ab1. In the presence of LPS, the magnetic beads (coated with Ab2)
12 complex with the latex beads (coated with Ab1) , via the LPS, and the latex beads are
13 sedimented by the use of a magnet. The quantitation of LPS is based on the turbidity of
14 the solution remaining after sedimentation of the magnetic beads (i.e. measuring the
15 latex beads still remaining in solution after sedimentation). The sensitivity varies based
16 on incubation time from 5 - 30 minutes. Sensitivities of **0.9-25 ng/ml** were reported. A
17 serious disadvantage of this assay is that it is inhibited by serum and by high
18 concentrations of endotoxin. **See Lim, P., 135 JOURNAL OF IMMUNOLOGICAL METHODS**
19 **257-261 (1990)**, incorporated by reference herein in its entirety and for all purposes.

20 **U.S. Patent No. 5,057,598** (Pollack et al.) discloses the use of monoclonal
21 antibodies for the immunological detection of endotoxin or endotoxin bearing organisms.

1 **See Pollack et al. (U.S. Pat. No. 5,057,598)**, incorporated by reference herein in its
2 entirety and for all purposes. Pollack et al. states, at column 18, lines 24-28, that
3 detection of endotoxin can be carried out in hours compared with detection of endotoxin
4 based on standard microbiological or cultural methods in days. Clearly, a detection
5 method that works in a time shorter than hours would be advantageous.

6 The oldest and best known test for endotoxin is the rabbit pyrogen test. This
7 assay has a low sensitivity, is expensive and is plagued with reproducibility problems since
8 different rabbits have different sensitivities to endotoxin challenge. Additionally, animal
9 tests are very time consuming and, therefore, of limited application in a clinical setting.

10 The immunoassays for endotoxin previously described are all sandwich
11 immunoassays which include the binding of two proteins to the endotoxin molecule. In
12 general, sandwich assays are the preferred approach for the detection of large molecule,
13 whereas competition assays are used for the detection of small molecules with only a
14 single protein binding site. Another sandwich assay for endotoxin, reported by **Connelly**,
15 uses lipopolysaccharide binding proteins of amebocyte lysates and labelled detection
16 reagents. **See U.S. Patent No. 4,906,567**, incorporated herein by reference in its entirety
17 and for all purposes. The general scheme used by **Connelly** involves holding
18 lipopolysaccharide binding proteins from one or more various organisms (See Col. 4,
19 lines 62-68, U.S. Patent No. 4,986,567 of Connelly) within the wells of microtiter plates
20 for about 2 hours followed by washing in PBS (phosphate buffered saline), followed by
21 holding BSA (bovine serum albumin) within the same microtiter plate wells for about 1

1 hour, followed by introduction of an endotoxin containing sample (or sample suspected of
2 containing endotoxin) and holding the sample for about 30 minutes to about 1 hour
3 within the same wells, followed by introduction of a horseradish peroxidase conjugated to
4 an LPS antibody via the heterobifunctional linking agent N-succinimidyl-4-(N-
5 maleimidomehtyl)cyclohexane-1-carboxylate (SMCC) and holding the LPS antibody-
6 peroxidase conjugate within the same wells for for about 30 - 60 minutes, followed by
7 washing in PBS and introducing a chromogenic substrate, tetramethylbenzidine (TMB)
8 into the same wells and holding for about another 10 - 15 minutes before taking an
9 optical density measurment at 630 nm. **See Connelly** at Examples 1, 2, 3, 4 and 5. **Id.**

10 In all of the **Connelly** examples, the pH is maintained at 9.0 or less and the time
11 to reading the optical density (OD) from the time when the sample containing LPS (or
12 suspected of containing LPS) is first intrtoduced into the microtiter plate wells is between
13 about 1 1/6 hours (1 hour, 10 min.--simultaneous or staggered addition of example 5 of
14 Connelly at Col 12, lines 15-35) to about 3 1/6 hours (3 hours, 10 min.--sequential
15 addition of example 4 of Connelly at Cols. 10 and 11).

16 **European Patent (EP 0 265 127) of Harvey and Wilson** describes a method and
17 apparatus for the detection of endotoxin using either polymyxin, an octapeptin, or other
18 similar cyclic peptides. An assay is carried out wherein the amount of a polymyxin-
19 endotoxin conjugate (hereinafter, polymyxin B-LPS conjugate) formed is quantitated.
20 The amount of the polymyxin B-LPS conjugate formed is quantitated by attaching a label
21 to either the polymyxin B or to the endotoxin. The labelled polymyxin B-LPS conjugate

1 is then measured. At page 7, lines 27-31 of **EP 0 265 127** it is stated that:

2 3 4 5 6
> In one form of the assay, the analyte which contains LPS and a standard, labelled, LPS preparation **compete** for a limited amount of immobilized polymyxin B, and the amount of label bound to the polymyxin B is then quantitated.

7 (Emphasis added.) From the above quoted language, it appears at first glance that the
8 analyte (containing LPS or suspected of containing LPS) and the standard, labelled, LPS
9 preparation are **simultaneously** placed in proximity to the immobilized polymyxin B
10 wherein the analyte LPS and the standard, labelled LPS **compete** for binding to the
11 polymyxin B. However, upon closer examination of Examples 1, 2, 3, and 4 of **EP 0 265**
12 **127**, it appears that the analyte LPS and the standard, labelled, LPS preparation are **not**
13 added simultaneously. Instead, the analyte LPS and the standard, labelled, LPS
14 preparation are added **consecutively** in proximity to the exemplary immobilized
15 polymyxin B. The requirement that the sample and labelled reagent be added
16 sequentially causes the assay to be inherently slower than an assay involving
17 simultaneous addition of analyte and labelled endotoxin. Examples 1-4 describe slow,
18 multistep reactions.

19 Example 1 of **EP 0 265 127** describes a process wherein the following steps are
20 executed:

21 (1) binding capacity of immobilized polymyxin B for LPS is determined by
22 using isotopically labelled LPS (^{14}C LPS);
23 (2) incubating test analyte solution (containing LPS or suspected of containing

1 LPS) with immobilized polymyxin B;

2 (3) adding a known quantity of isotopically labelled LPS (^{14}C LPS) to the
3 mixture of step (2);

4 (4) measuring the amount of isotopically labelled LPS in solution;

5 (5) subtracting the amount of isotopically labelled LPS in solution (**i.e.** unbound
6 isotopically labelled LPS) from the total amount of ^{14}C LPS introduced in
7 step (3) to determine the amound of ^{14}C LPS bound to the immobilized
8 polymyxin B; and

9 (6) subtracting the amount of ^{14}C LPS bound to the immobilized polymyxin B
10 determined in step (5) from the binding capacity of the immobilized
11 polymyxin B in step (1) to determine the amount of analyte LPS present.

12 The net result of step (6) indicates the amount of analyte LPS present and bound to the
13 immobilized polymyxin B.

14 Example 2 of **EP 0 265 127** describes a process wherein the following steps are
15 executed:

16 (1) incubating analyte LPS samples with a known quantity of polymyxin B
17 alkaline phospatase conjugate in sufficient excess to promote binding
18 between all the analyte LPS and the polymyxin B-alkaline phosphatase
19 conjugate;

20 (2) incubating the mixture of step (2) with an immobilized, standard LPS
21 preparation to bind the unbound excess of the polymyxin B alkaline

1 phosphatase conjugate from step (1);

2 (3) rinsing the preparation of step (2);

3 (4) measuring the amount of the excess polymyxin B-alkaline phosphatase

4 conjugate of step (1) now bound to the immobilized, standard LPS

5 preparation of step (2); and

6 (5) subtracting the amount of excess polymyxin B alkaline phosphatase

7 determined in step (4) from the total polymyxin B alkaline phosphatase

8 conjugate used in step (1) to determine the amount of analyte LPS present.

9 An assay time of 1 hour plus the time necessary for scintillation counting was required.

10 Sensitivity was 10 μg/ml. The statement was made that increasing the specific activity of

11 the ^{14}C-LPS would increase sensitivity of the assay. However, increasing specific activity

12 also increases bacground so that the gain from such an improvement in the labelled

13 reagent is rarely greater than a factor of 10. In addition, radiolabels may be hazardous

14 to an inexperienced user and involve undesirable problems of disposal as hazardous

15 waste.

16 Example 3 of **EP 0 265 127** describes a process wherein the following steps are

17 executed:

18 (1) incubating a limited excess amount of immobilized polymyxin B with

19 analyte LPS to bind all analyte LPS;

20 (2) incubating an excess of standard, LPS-alkaline phosphatase conjugate with

21 the mixture of step (1) and removing excess standard LPs-alkaline

1 phosphatase by rinsing;

2 (3) measuring the amount of standard LPS-alkaline phosphatase conjugate
3 bound in step (2) to the immobilized polymyxin B; and

4 (4) subtracting the amount of the immobilized LPS-alkaline phosphatase
5 conjugate bound in step (2) from the total amount of immobilized
6 polymyxin B to determine the amount of analyte LPS bound in step (1).

7 It is difficult to imagine how Example 3 is characterized as a "displacement ELISA" (see
8 p. 10, line 21) since it is stated at p. 8, line 25 that LPS can block the binding of ^{14}C LPS
9 to polymyxin B/S4B. *See* EP **0 265 127**.

10 Example 4 of **EP 0 265 127** describes a process wherein the following steps are
11 executed:

12 (1) immobilizing analyte LPS and rinsing;

13 (2) binding polymyxin B-alkaline phosphatase conjugate to immobilized analyte
14 LPS of step (1); and

15 (3) measuring the amount of labelled polymyxin B attached to the immobilized
16 analyte LPS of step (1) to determine the amount of analyte LPS present in
17 an analyte sample.

18 Examples 2-4 provide neither data nor experimental details. ELISA assays are
19 generally less sensitive than radioimmunoassays using the same reagents and same
20 general approach. Thus, the approaches described in examples 2-4 would not be
21 expected to produce sensitivity greater than about 10 μg/ml. The use of enzymes as

1 labels also involve problems of interferents from the sample matrix, increasing
2 background response over time during the assay, and increasing instability of the enzyme
3 label during storage.

4 In all of the assay formats described, European Patent (**EP 0 265 127**) has several
5 drawbacks:

6 (1) washing steps are required in Examples 2, 3, and 4;
7 (2) lengthy incubation steps are required in Examples 1, 2, 3, and 4, so that all
8 assays require more than 1 hour to perform;
9 (3) a radioactive label is used in example 1;
10 (4) enzyme labelled LPS or enzyme labelled polymyxin B is used in Examples
11 2, 3, and 4; and
12 (5) analyte samples used are **extracts from**, for example, body fluids (see p. 7,
13 line 33 of **EP 0 265 127**).
14 (6) no evidence of sensitivity or potential sensitivity greater than μg/ml is
15 provided.

16 Thus, there remains a need for an endotoxin assay with high sensitivity to
17 concentrations **as low as about 1 ng/ml** which can be used in non-homogeneous samples
18 such as serum or saliva, which has no requirement for enzymes or radiolabels, which
19 requires very little manipulation by the operator, which is rapid, which can be used with
20 intact cells, cell fragments or solubilized cells, which can be used on a wide variety of
21 clinical and environmental samples, which requires minimal or no sample preparation,

1 which can be used in a wide variety of environments, structural forms and conditions,
2 which can be used rapidly (between about 15 seconds to about 10 minutes) to test for the
3 presence of endotoxin and which can be adapted to determine the specific type of
4 endotoxin detected.

5 **SUMMARY OF THE INVENTION**

6 It is, therefore, an object of this invention to provide a rapid test for endotoxin.

7 It is another object of this invention to provide a rapid test for endotoxin which
8 can be performed in the presence of serum or other biological samples including, but not
9 limited to, urine, saliva, and mucus.

10 It is yet another object of this invention to provide a test for endotoxin which has
11 no requirement for enzymes.

12 It is a further object of this invention to provide an assay requiring very little
13 manipulation by the operator.

14 It is yet a further object of this invention to provide a rapid test for endotoxin
15 which is sensitive to quantities as low as 1 ng/ml.

16 It is even a further object of this invention to provide a rapid test for endotoxin in
17 intact cells, cell fragments or purified endotoxin.

18 It is an additional object of this invention to provide a rapid test for endotoxin in
19 aqueous samples, for example, from the environment or manufacturing processes.

20 It is an additional object of this invention to provide a rapid test for endotoxin in
21 a wide variety of environments, structural forms and conditions.

1 It is an additional object of this invention to provide a rapid test for endotoxin
2 and, if desired, to diagnose the specific type of endotoxin detected.

3 These and other objects of the presently claimed invention are accomplished by a
4 process which is fast, sensitive, requires no enzyme linked detection systems and can be
5 performed successfully with non-homogeneous samples containing endotoxin. The
6 claimed assay relies on the binding of an endotoxin to a suitable capture molecule and
7 the competitive detection of the capture molecule-endotoxin complex without the use of
8 enzymatic reactions to visualize or enhance detection. For example, a fluorescent dye
9 may be used to label the capture-molecule endotoxin complex and form an exemplary
10 capture molecule-endotoxin-fluorescent label complex. Detection of the exemplary
11 fluorescent label may be carried out by detecting a fluorescence signal obtained from the
12 evanescent wave region of a fiber optic waveguide probe wherein the capture molecule-
13 endotoxin-label complex is immobilized on the surface of the sensing portion of the fiber
14 optic waveguide probe.

15 **DETAILED DESCRIPTION OF THE DRAWINGS**

16 **Fig. 1** is a concentration-response curve obtained from the direct binding of
17 increasing concentrations of fluorescently labelled *E. coli* 0128:B12-endotoxin to a
18 capture molecule, polymyxin B, the capture molecule being covalently bound to the
19 surface of a combination tapered fiber optic waveguide probe.

20 **Fig. 2** is a concentration-response curve obtained from the binding of increasing
21 concentrations of fluorescently labelled *E. coli* 0128:B12-endotoxin to a capture molecule,

1 goat IgG, the capture molecule being covalently bound to the surface of a combination
2 tapered fiber optic waveguide probe.

3 **Fig. 3** is a concentration-response curve obtained from the binding of 17ng/ml of
4 fluorescently labelled *E. coli* EH100 Ra mutant endotoxin to a capture molecule, limulin
5 lectin, the capture molecule being immobilized on to the surface of a combination
6 tapered fiber optic waveguide probe. The response is measured in microvolts (μVolts).

7 **Fig. 4** is a concentration-response curve obtained from the binding of increasing
8 concentrations of fluorescently labelled *E. coli* 0128:b12 endotoxin to a capture
9 molecule, polymyxin B, the capture molecule being immobilized on to the surface of a
10 combination tapered fiber optic waveguide probe. The response is measured in
11 microvolts (μVolts) which response varies proportionately with the concentration of
12 endotoxin present in the sample tested.

13 **Fig. 5** is a concentration-response curve obtained from the competitive binding of
14 increasing concentrations of unlabelled *E. coli* 0128:b12 endotoxin in the presence of
15 fluorescently labelled *E. coli* 0128:b12 endotoxin. The capture molecule was polymyxin B

16 **Fig. 6** is a concentration response curve obtained from the binding of increasing
17 concentrations of fluorescently labelled *E. coli* 0128:b12 endotoxin to a capture molecule,
18 polymyxin B, the capture molecule being immobilized on to the surface of a fiber optic
19 waveguide. The response is measured in microvolts (μVolts) which response varies
20 proportionately with the concentration of endotoxin present in the sample tested and
21 each point is an average of triplicate determinations.

1 **Fig. 7** is a concentration-response curve obtained from the competitive binding of
2 increasing concentrations of unlabelled *E. coli* 0128:b12 endotoxin in the presence of
3 fluorescently labelled *E. coli* 0128:b12 endotoxin. The capture molecule was polymyxin
4 B.

5 **DETAILED DESCRIPTON OF THE PREFERRED EMBODIMENTS**

6 The following detailed description of the invention is provided to aid those skilled
7 in the art in practicing the present invention. However, the following detailed description
8 of the invention should not be construed to unduly limit the present invention.
9 Variations and modifications in the embodiments discussed may be made by those of
10 ordinary skill in the art without departing from the scope of the present inventive
11 discovery.

12 Broadly, the competetive assay of the presently claimed invention comprises:
13 (1) one or more endotoxin capture molecules immobilized upon a substrate;
14 (2) a standard sample of one or more labelled endotoxin molecules;
15 (3) a means for detecting the presence of one or more endotoxin capture molecule-
16 labelled endotoxin molecule complexes (ECM-LEM complexes), said one or more
17 ECM-LEM complexes forming in the presence of one or more unlabelled analyte
18 endotoxin molecules (AEM); and
19 (4) a means for measuring the quantity of ECM-LEM complexes formed.

20 The capture molecules include but are not limited to antibodies, lectins, cell
21 receptors, antibiotics, endotoxin binding proteins, or specifically engineered peptides

1 referenced in ***Random Peptide Libraries: A Source of Specific Protein Binding Molecules*** by
2 **J.J. Devlin et al.**, published in SCIENCE, **Vol. 24, pp. 404-405 (1990)**, incorporated hererin
3 by reference in its entirety and for all purposes. Antibodies and antibiotics (such as
4 polymyxin B) may be preferred simply because of their specificity, availability and
5 stability following immobilization.

6 The capture molecule can be adsorbed or covalently bound to the substrate.
7 Procedures for immobilizing capture molecules onto a substrate (**e.g.** solid surface) are
8 given in **U.S. Patent No. 5,077,210** of **Ligler et al.**, incorporated herein by reference in its
9 entirety and for all purposes.

10 Exemplary capture molecules, such as antibiotics, lectins, antibodies, and
11 endotoxin binding proteins, are covalently immobilized on a substrate carrying exemplary
12 surface reactive groups such as hydroxyl groups. Exemplary substrates (e.g. solid
13 supports) have or can be modified to have surface reactive groups such as hydroxyl
14 groups which can be reacted with a capture molecule for direct crosslinking or with a
15 silane or alkyl thiol film for indirect crosslinking. The substrate onto which the capture
16 molecule is immobilized depends only on the type of system used to quantify the amount
17 of unlabelled analyte endotoxin molecules present. Exemplary substrates may include
18 slides, beads (magnetic, synthetic or natural polymers), optical fibers, metal films, and
19 cuvettes (quartz, glass, silica). The exemplary surface of the substrate may be smooth,
20 flat, curved, round, rough, or with or without edges. Suitable substrates are preferably
21 inorganic substrates including but not limited to silicon, glass, silica, quartz, metal oxides,

1 organic polymers, and the like which can be for example optical fibers, wires, wafers,
2 films, discs or planar surface, microscope slides, or beads. Generally, the solid surfaces
3 (substrates) have or can be modified to have functional groups such as surface hydroxyl
4 groups that react with exemplary silanizing reagents or exemplary metal oxide groups
5 reactive with exemplary alkyl thiol reagents.

6 Endotoxin has several chemically reactive groups including primary amines and
7 carboxyl groups. Such amine and carboxyl groups allow for label attachment at various
8 sites on the endotoxin. In a preferred embodinent, the type of label used in the presently
9 claimed invention must generate a signal at the surface of an optical waveguide.
10 Examples include fluorophores, colorimetric dyes, metal chelates or carbonyls,
11 electrochemiluminescent labels, or luminescent labels. A label is selected so that a signal
12 characteristic of a labelled endotoxin molecule when the labelled endotoxin molecule is
13 bound to an endotoxin capture molecule, forming an endotoxin capture molecule--
14 labelled endotoxin molecule (ECM-LEM) complex, can be detected in both the presence
15 and absence of a competing endotoxin capture molecule--unlabelled analyte endotoxin
16 molecule (ECM-AEM) complex.

17 The immobilization of the ECM can be via covalent or noncovalent chemistries.
18 The objective is to affix the ECM to a substrate surface in such a way that the capture
19 molecule retains its biological activity while remaining fixed to the substrate surface for
20 the purpose of quantitation. For example, immobilization of an exemplary endotoxin
21 capture molecule (ECM) may be carried out upon the surface of the exemplary substrate

1 having a surface coated with an exemplary heterobifunctional crosslinking agent. For
2 example, polymyxin B may be immobilized onto the exemplary substrate (having a
3 surface coated with an exemplary heterobifunctional crosslinking agent) at a
4 concentration of about 1-10 mg/ml of polymyxin B in an exemplary 0.1M NaBorate
5 solution at an exemplary pH of 9.0. The prepared exemplary substrate is suspended in
6 the polymyxin B solution for about 30 min. The pH of the exemplary solution is then
7 neutralized using HCl. The exemplary substrate is then washed several times with PBS
8 to remove any unreacted exemplary polymyxin B and to further neutralize the exemplary
9 substrate surface.

10 Once the exemplary substrate has an exemplary capture molecule immobilized
11 upon its surface, the process of the presently claimed competitive assay is carried out.
12 The process of the presently claimed invention comprises:

13 (1) immobilizing one or more ECM upon a substrate;
14 (2) introducing at time = t_0, a standard sample of one or more labelled
15 endotoxin molecules and an analyte sample containing or suspected of
16 containing one or more analyte endotoxin molecules over the one or more
17 immobilized ECMs to form one or more ECM-LEM complexes and, if any
18 analyte endotoxin molecules are present in the analyte sample, to form one
19 or more endotoxin capture molecule--analyte endotoxin molecule (ECM-
20 AEM) complexes;
21 (3) measuring at time = t_1 the amount of one or more ECM-LEM complexes

1 formed wherein $t_4 \leq (t_1 - t_0) \leq t_3$ minutes wherein t_4 is a time = t_4 , t_3 is a
2 time = t_3, wherein t_4 is about 0.05 to about 0.20 minutes and t_3 is a time
3 selected from the group consisting of about 10 minutes, 5 minutes, 4
4 minutes, 3 minutes, 2 minutes, 1 minute, 0.5 minute and 0.25 minute,
5 respectively; and
6 (4) calculating the amount, if any, of the ECM-AEM complexes formed.
7 The formation of the ECM-AEM complexes at the surface of a waveguide is
8 measured using the waveguide to collect the signal generated by the label. The signal
9 can be a change in refractive index, a phase shift in the light, fluorescence, luminescence,
10 absorbance, respectively. Depending upon the type of signal generated, various means of
11 quantitating the signal known in the art may be used. The device may or may not need a
12 light source to excite the label. If needed, the excitation light can travel down the
13 waveguide or excite the light from outside the waveguide.
14 Having described the invention, the following examples are given to illustrate
15 specific applications of the invention, including the best mode now known to perform the
16 invention. These specific examples are not intended to limit the scope of the invention
17 described in this application.
18 **Examples**
19 **Fluorimeter and Waveguide Configuration used for Examples**
20 The fluorimeter and waveguides used in the examples described here were
21 laboratory prototypes. A similar fluorimeter operating at 635 nm is commercially

1 available (Research International, Woodinville, WA). Exemplary suitable dyes for use in
2 conjunction with fluorimeter from Research International are the sulfoindocyanine dyes
3 (cyanine based dyes **e.g.** Cy5 dyes) described by **Mujumdar et. al.** in the paper entitled
4 *Cyanine Dye Labeling Reagents: Sulfoindocyanine Succinimidyl Esters*, **BIOCONJUGATE**
5 **CHEMISTRY, Vol. 4, No. 2, pp105-111 (March/April 1993)**--incorporated herein by
6 reference in its entirety and for all purposes, the dyes being available from Biological
7 Detection Systems, Inc. located in Pittsburgh, Pennsylvania. For details of the device and
8 waveguide construction, *see* L.C. Shriver-Lake, G.P. Anderson, J.P. Golden and F.S.
9 Ligler, ***The effect of Tapering the Optical Fiber on Evanescent Wave Measurements* 25**
10 **ANALYTICAL LETTERS 7, pp. 1183-1199** (1992), incorporated by reference herein in its
11 entirety and for all purposes. ***See*** J.P. Golden, L.C. Shriver-Lake, G.P. Anderson, R.B.
12 Thompson and F.S. Ligler, ***Fluorometer and Tapered Fiber Optic Probe for Sensing in the***
13 ***Evanescent Wave*** 31 OPTICAL ENGINEERING NO. 7, **pp. 1458-1462 (July 1992),**
14 incorporated by reference herein in its entirety and for all purposes. ***See*** G.P. Anderson,
15 J.P. Golden and F.S. Ligler, ***A Fiber Optic Biosensor: Combination Tapered Fibers Designed***
16 ***for Improved Signal Acquisition*, 8 BIOSENSORS & BIOELECTRONICS, pp. 249-256 (1993),**
17 incorporated by reference herein in its entirety and for all purposes.
18 The detection optics in the fluorimeter used in these experiments were encased in
19 a light-proof metal enclosure to reduce the effects of ambient light and electromagnetic
20 influence on the detector circuitry. Key components include optics for launching and
21 collecting the light mounted on kinematic mounts. A laser light source was selected for

1 its moderate power, stability, narrow excitation bandwidth and efficient light coupling into
2 the fiber. The exemplary rhodamine-based fluorescent labels (e.g. TRITC) used with the
3 sensor in the examples described here were excited at 514 nm and emit in the 570 (± 50
4 nm) nm range where there is little intrinsic fluorescence in most clinical and
5 environmental samples. A 514 nm laser beam from an air-cooled 50-mW argon ion laser
6 (Omnichrome 532, Chino, California) was launched into the most proximal end of the
7 cladded fiber. The laser was adjusted to a 12-mW output to minimize bleaching of the
8 fluorophores bound to the distal end of the optical fiber . The line filter (Melles Griot)
9 removed plasma lines from the laser source. The laser beam passed through a chopper,
10 a dichroic mirror and a spherical lens (f/1, one inch focal length bioconvex lens;
11 Newport Corporation) onto the proximal end of the optical fiber. Approximately, 8
12 degrees of the fiber's numerical aperture of 23 degrees were filled. The collected
13 fluorescence signal from the distal end of the optical fiber traveled the reverse path to
14 the dichroic mirror where it was reflected through a longpass filter (KV550) onto a
15 silicon photodiode. The chopper and photodiode (EG&G Judson) were connected to a
16 lock-in amplifier (LIA, Stanford Research Systems, Sunnyvale, California) and computer
17 for phase sensitive detection via chopper controller. The photodiode was selected rather
18 than a photomultiplier tube because of low cost, reliability and compatibility with the
19 lock-in amplifier. The data, measured as μV, were collected using the laptop computer.
20 The fiber optic waveguide used in the examples was made from a length of step-
21 index plastic clad silica optical fiber (200 μm diameter core, Quartz et Silice, Quartz

1 Products, Tuckerton, Delaware) with a connector on the proximal end to facilitate
2 replacement and alignment. The distal end was modified to perform biochemical assays
3 in the evanescent wave. At the distal end of the fiber, 12.5 cm of cladding was stripped
4 away from the core by removal of the buffer and cladding with a razor blade. Residual
5 cladding was removed by immersing this end of the optical fiber in concentrated
6 hydrofluoric acid (HF) for 1 minute. This distal end portion of the fiber was the sensing
7 region on which the capture molecules were immobilized. The combination taper probe
8 (**i.e.** the combination of a first short tapered section and a second longer, shallower
9 tapered section) was prepared by slowly immersing the declad distal end of the fiber into
10 concentrated HF, using a computer controlled stepper motor. Two to three centimeters
11 of the distal, unclad end of the fiber was first lowered into the concentrated HF acid.
12 The distal, unclad end of the fiber was further lowered at a constant rate of about 0.53
13 cm/minute for the next 9 cm to create the gradually tapered section . The distal, unclad
14 fiber was even further lowered into the concentrated HF acid for another 1 cm at a
15 constant rate of about 0.045 cm/minute to create the more steeply tapered section. This
16 relatively steep tapered section was tapered from the original 100 μm radius down to 63
17 μm. The taper dimensions were measured with a calibrated optical microscope. ***See***
18 G.P. Anderson, J.P. Golden and F.S. Ligler, ***A Fiber Optic Biosensor: Combination Tapered***
19 ***Fibers Designed for Improved Signal Acquisition*****, 8 BIOSENSORS & BIOELECTRONICS, pp.**
20 **250-251 and Fig. 1 at pp. 250 (1993)**, incorporated by reference herein in its entirety and
21 for all purposes.

1 For immobilization of the capture molecules, the distal unclad tapered fiber
2 having surface hydroxyl groups was immersed in a 2% solution of
3 mercaptopropyltrimethoxysilane (MTS) dissolved in toluene for 30 minutes under N_2.
4 Thereafter, the fiber was rinsed with toluene also under N_2. The silanized fiber was then
5 placed in a 2 mM solution of a heterobifunctional crosslinking agent, γ-
6 maleimidobutyryloxy succinimide ester, for 1 h. The fiber was then rinsed in phosphate
7 buffered saline (PBS) at pH 7.4. Lastly, the distal end of the fiber is suspended in a
8 solution of 0.05 mg/ml of lectin, 0.05mg/ml solution of IgG antibody for endotoxin, 0.02
9 mg/ml of endotoxin neutralizing protein (ENP), 0.01μg/ml of polymyxin B, 0.1μg/ml of
10 polymyxin B, 1.0mg/ml of polymyxin B, or 10.0mg/ml of polymyxin B, respectively, for 2
11 h. The fibers with immobilized capture molecules are stored in endotoxin-free PBS at
12 4°C. See Bhatia et al., Analytical Biochemistry, 178, 408-413 (1989), incorporated herein
13 by reference in its entirety and for all purposes.

14 After the immobilization of the capture molecules, the entire uncladded portion of
15 the exemplary combination tapered fiber optic waveguide was sealed into a flow
16 chamber constructed from an exemplary shortened 200 μl capillary tube and tee
17 connectors, having a total length of 12 -13 cm. The distal end of the fiber was glued
18 outside the chamber, allowing only light from the evanescent wave to enter the fiber
19 surrounded by the solution within the tee connection capillary. Both ends of the capillary
20 tube were sealed with hot-melt glue, with the clad region of the fiber extending into the
21 proximal portion of the apparatus. The sample to be tested was introduced over the

1 distal end of the optical fiber via sample inlet and exited at sample outlet. Use of this
2 capillary tube apparatus allows one to test a given analyte sample solution over the
3 optical waveguide and to wash the waveguide before introducing another analyte sample
4 solution.

5 **Example 1**

6 Labelled endotoxin was prepared and the binding of the labelled endotoxin to the
7 capture molecule immobilized on the waveguide was measured. In this example,
8 polymyxin B was immobilized on the fiber using a solution of 10mg/ml polymyxin B as
9 described above.

10 A standard sample of labelled endotoxin molecules was made according to the
11 following procedure. A known amount of endotoxin (for example, 1 mg/ml) was
12 dissolved in 5.0 ml of a 0.1M NaBorate solution at pH 10.5 and vortexed vigorously for at
13 least 15 minutes at room temperature. A fluorescent label, tetramethyl isothiocyanate
14 (TRITC, Sigma), was added to the vortexed solution to a w/w concentration of 1:100 of
15 endotoxin:TRITC. The TRITC/endotoxin sample was incubated at 37 °C in the dark for
16 4 h with intermittent vortexing . The TRITC labelled endotoxin was dialyzed against
17 several changes of 0.15 M NaCl. Any unbound TRITC was removed by passing the
18 endotoxin solution over a Sephadex G-25 column (Sigma). The standard sample of
19 labelled endotoxin was collected in fractions and the ratio of endotoxin to TRITC
20 calculated. Molar ratios of fluorophore to endotoxin of approximately **0.8:1** to about
21 **1.5:1** **(i.e.** molar conc. of fluorophore : molar conc. of endotoxin) were considered

1 acceptable for use in this example. There were two different endotoxins used, the E.Coli
2 EH 100 Ra mutant endotoxin (lacking the O-antigenic region) and from E.Coli 0128:B12
3 endotoxin, respectively.

4 The TRITC labelled standard sample of endotoxin was further used according to
5 the following procedure. Labelled endotoxin was reconstituted in PBS which contained
6 either 2 mg/ml of bovine serum albumin (BSA) or 0.1 % Triton X-114. The labelled
7 endotoxin standard sample was introduced through the capillary tube containing the fiber
8 optic probe coated with the immobilized capture molecule. Binding was observed from
9 about 0-2 minutes at 30 second intervals. The excitation laser beam (514nm) was blocked
10 between evanescent wave signal measurements to avoid photobleaching.

11 **Fig. 1** depicts response curves obtained from the direct binding of increasing
12 concentrations of fluorescently labelled *E. coli* 0128:B12 endotoxin to a capture molecule,
13 polymyxin B, the capture molecule being covalently bound to the surface of a fiber optic
14 waveguide. Note that the fluorescent label used is tetramethyl rhodamine-5-
15 isothiocyanate (TRITC). The labelled endotoxin standard was introduced through the
16 capillary tube containing the fiber optic waveguide coated with the immobilized capture
17 molecule. Binding signals were measured at 30 second intervals. The excitation laser
18 beam (514 nm) was blocked between signal measurements to avoid photobleaching. The
19 signal measured from the standard solutions of labelled endotoxin was determined using
20 a different fiber waveguide for each recording. The response is measured in microvolts
21 (μVolts) which response varies proportionately with the concentration of endotoxin

1 present in the sample tested. The curves plot the change in fluorescence at the
2 waveguide surface as a function of the concentration of fluorescently labelled *E. coli*
3 0128:B12-endotoxin in solution.
4
5 **Example 2.**
6 In order (a) to demonstrate the binding of labelled endotoxin by a different
7 capture molecule and (b) to demonstrate the quantitation of signal produced by
8 increasing concentrations of the labelled endotoxin, waveguides coated with anti-
9 endotoxin antibody were exposed to increasing concentrations of labelled endotoxin.
10 **Fig. 2** is a concentration-response curve obtained from the binding of increasing
11 concentrations of fluorescently labelled *E. coli* 0128:B12-endotoxin to a capture molecule,
12 goat IgG antibody specific for *E. coli* 0128:B12 endotoxin, the capture molecule being
13 covalently bound to the surface of a fiber optic waveguide. Note that the fluorescent
14 label used is tetramethyl rhodamine-5-isothiocyanate (TRITC). The response is
15 measured in microvolts (μVolts) which response varies proportionately with the
16 concentration of endotoxin present in the sample tested. The curve is a plot of change in
17 fluorescence as a function of the concentration of fluorescently labelled *E. coli* 0128:B12
18 endotoxin in PBS + 2 mg/ml BSA. The complex of antibody-endotoxin-TRITC was
19 detected as it formed at the waveguide surface.
20 **Example 3.**
21 In order to demonstrate the binding of labelled endotoxin to yet another capture

1 molecule on the surface of the waveguide, the waveguide was coated with limulin lection.
2 **Fig. 3** is a time-response curve obtained from the binding of 17 ng/ml of fluorescently
3 labelled *E. coli* Eh100 Ra mutant endotoxin to a capture molecule, limulin lectin, the
4 capture molecule being immobilized on to the surface of a fiber optic waveguide. The
5 response is measured in microvolts (μVolts). The complex of limulin lectin-endotoxin-
6 TRITC was detected as it formed at the surface of the waveguide and within 1 minute of
7 after the addition of the TRITC labelled endotoxin.
8 **Example 4.**
9 In order to demonstrate a quantitative dose-response relationship between the
10 concentration of labelled endotoxin put over the waveguide and the signal produced,
11 waveguides were coated with polymxyin B as the capture molecule. **Fig. 4** is a
12 concentration-response curve obtained from the binding of increasing concentrations of
13 fluorescently labelled *E. coli* 0128:B12 endotoxin to a capture molecule, polymyxin B, the
14 capture molecule being immobilized on to the surface of a combination tapered fiber
15 optic waveguide probe. The response is measured in microvolts (μVolts) which response
16 varies proportionately with the concentration of endotoxin present in the sample tested.
17 Under the conditions of the assay used to obtain **Fig. 4**, the capture molecule, polymyxin
18 B, was immobilized onto the surface of a fiber optic waveguide probe and the assay was
19 conducted in PBS + 2 mg/ml BSA. The complex of polymyxin B-endotoxin-TRITC was
20 detected as it formed at the surface of the waveguide. The response shows percent
21 signal (compared to the signal produced on each fiber for 500 ng/ml of TRITC-

1 endotoxin) as a function of increasing concentrations of fluorescently labelled endotoxin
2 from 12.5 ng/ml to 500 ng/ml.

3 **Example 5.**

4 Once the signal measured from the standard solution of labelled endotoxin was
5 determined, the change in signal caused by a competitive binding of unlabelled endotoxin
6 and the standard endotoxin solution was measured. Note that the subsequent signal was
7 produced by a comingled solution of the standard sample of labelled endotoxin and the
8 analyte sample of the unlabelled endotoxin flowing concomittantly over the fiber optic
9 probe held within the capillary tube, the probe being prepared using polymxyxin B at 10
10 ng/ml.

11 **Fig. 5** is a concentration-response curve obtained from the competitive binding of
12 increasing concentrations of unlabelled *E. coli* 0128:B12 endotoxin in the presence of
13 fluorescently labelled *E. coli* 0128:B12 endotoxin. The fluorescently labelled and
14 unlabelled endotoxin were dissolved in PBS + 2 mg/ml BSA. The fluorescent label used
15 was TRITC. Increasing concentrations of unlabelled endotoxin (0-250 ng/ml) were added
16 to a standard concentration of TRITC-endotoxin of 200 ng/ml. The response is
17 measured in microvolts (μVolts) and each point is an average of triplicate determinations
18 and each point is standardized to % inhibition of signal from a 100% response (100%
19 response is wherein no unlabelled endotoxin is present in solution and 200 ng/ml of
20 TRITC-endotoxin is present in solution of PBS + 2 mg/ml BSA). Under the conditions
21 of the competitive assay used to obtain **Fig. 5**, the capture molecule, polymyxin B, was

1 immobilized onto the surface of a fiber optic waveguide probe and the assay was
2 conducted in PBS + 2 mg/ml BSA. The complex of polymyxin B-endotoxin-TRITC was
3 detected at the surface of the fiber optic waveguide. The response is standardized to
4 show % inhibition of signal as a function of increasing concentrations of unlabelled
5 endotoxin from 0 to 250 ng/ml. Note that 10% of the expected fluorescent signal (**i.e.**
6 100% response, *supra*) is inhibited at a concentration of 12.5 ng/ml of unlabelled
7 endotoxin in solution.

8 **Example 6**

9 The affect of serum on endotoxin binding and on the competitive assay for
10 endotoxin was determined according to the following procedure.

11 A concentration response curve obtained from the binding of increasing
12 concentrations of fluorescently labelled *E. coli* 0128:B12 endotoxin to a capture molecule,
13 polymyxin B, the capture molecule being immobilized on to the surface of a combination
14 tapered fiber optic waveguide probe is depicted in **Fig. 6**. The response is measured in
15 microvolts (μVolts) which response varies proportionately with the concentration of
16 endotoxin present in the sample tested and each point is the mean $\pm$ standard error of
17 triplicate determinations. Under the conditions of the assay used to obtain **Fig. 6**, the
18 capture molecule, polymyxin B, was immobilized onto the surface of a fiber optic
19 waveguide probe and the assay was conducted in PBS + 2 mg/ml BSA containing 0%
20 serum, 1% serum, 5% serum, or 20% serum. The complex of polymyxin B-endotoxin-
21 TRITC was detected at the surface of the fiber optic waveguide. The figure shows the

1 percent signal as a function of increasing concentrations of fluorescently labelled
2 endotoxin from 12.5 ng/ml to 500 ng/ml. Note that 25 ng/ml of endotoxin-TRITC can be
3 detected in 20% serum and 12.5 ng/ml was detected in 1% serum.

4 **Example 7**

5 **Fig. 7** is a concentration-response curve obtained from the competitive binding of
6 increasing concentrations of unlabelled *E. coli* 0128:B12 endotoxin in the presence of
7 fluorescently labelled *E. coli* 0128:B12 endotoxin. The fluorescently labelled and
8 unlabelled endotoxin were dissolved in PBS + 2 mg/ml BSA containing 0% serum, 1%
9 serum, 5% serum or 20% serum, respectively. The fluorescent label used was TRITC.
10 Increasing concentrations of unlabelled endotoxin (0-250ng/ml) were added to a standard
11 concentration of TRITC-endotoxin at a concentration of 200 ng/ml. The response is
12 measured in microvolts (μVolts) and each point is an average of triplicate determinations
13 and each point is standardized to percent inhibition of signal from a 100% response
14 (100% response is wherein no unlabelled endotoxin is present in solution and 200 ng/ml
15 of TRITC-endotoxin is present in solution of PBS + 2 mg/ml BSA). Under the
16 conditions of the competitive assay used to obtain **Fig. 7**, the capture molecule, polymyxin
17 B, was immobilized onto the surface of a fiber optic waveguide and the assay was
18 conducted in PBS + 2 mg/ml BSA. The complex of polymyxin B-endotoxin-TRITC was
19 detected as it formed at the waveguide surface. The response is standardized to show
20 percent inhibition of signal as a function of increasing concentrations of unlabelled
21 endotoxin from 0 to 250 ng/ml. The curve for 0% serum (not shown) was identical to

1 that for 1% serum. Note the expected fluorescent signal (**i.e.** as compared to 100%

2 response, *supra*) is inhibited at a concentration of 25 ng/ml of unlabelled endotoxin in

3 solution.

4

INVENTORS' NAME: Ligler et al.
Serial No.:08/

PATENT APPLICATION
Navy Case No.:75,797

1 ## ABSTRACT

2 The presently claimed invention is an apparatus and method for the detection of

3 endotoxin via a competitive assay.

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April 14, 1995

FIGURE 1

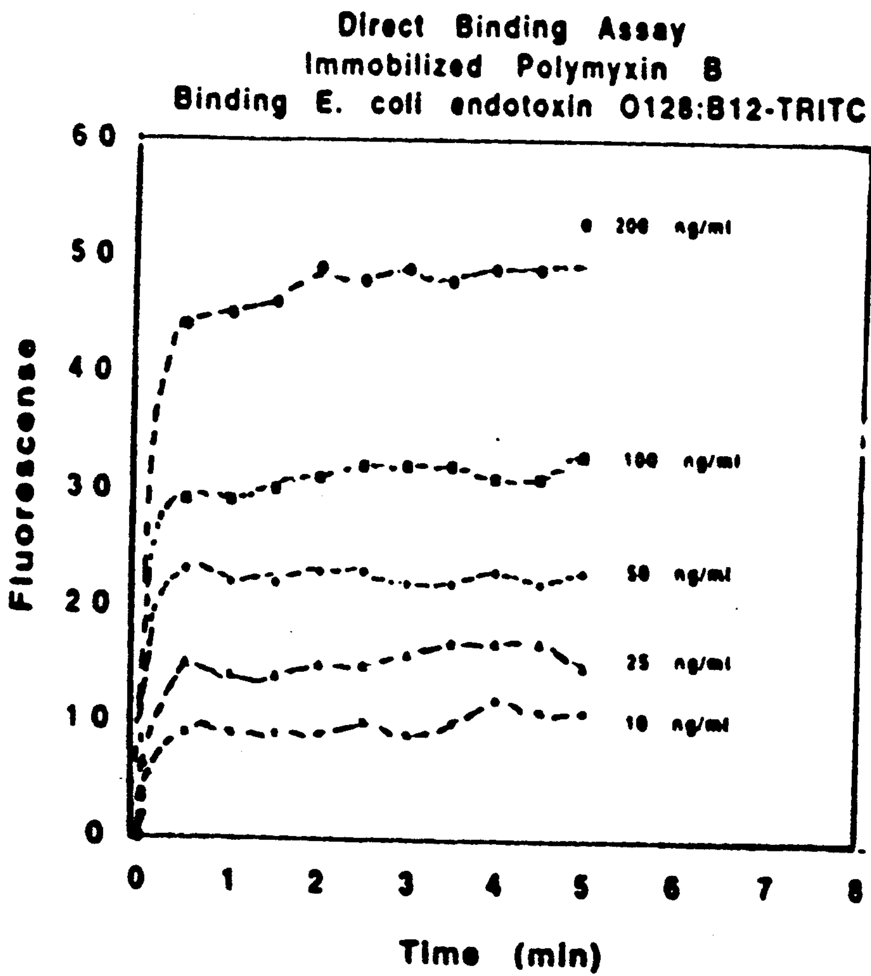


FIGURE 2

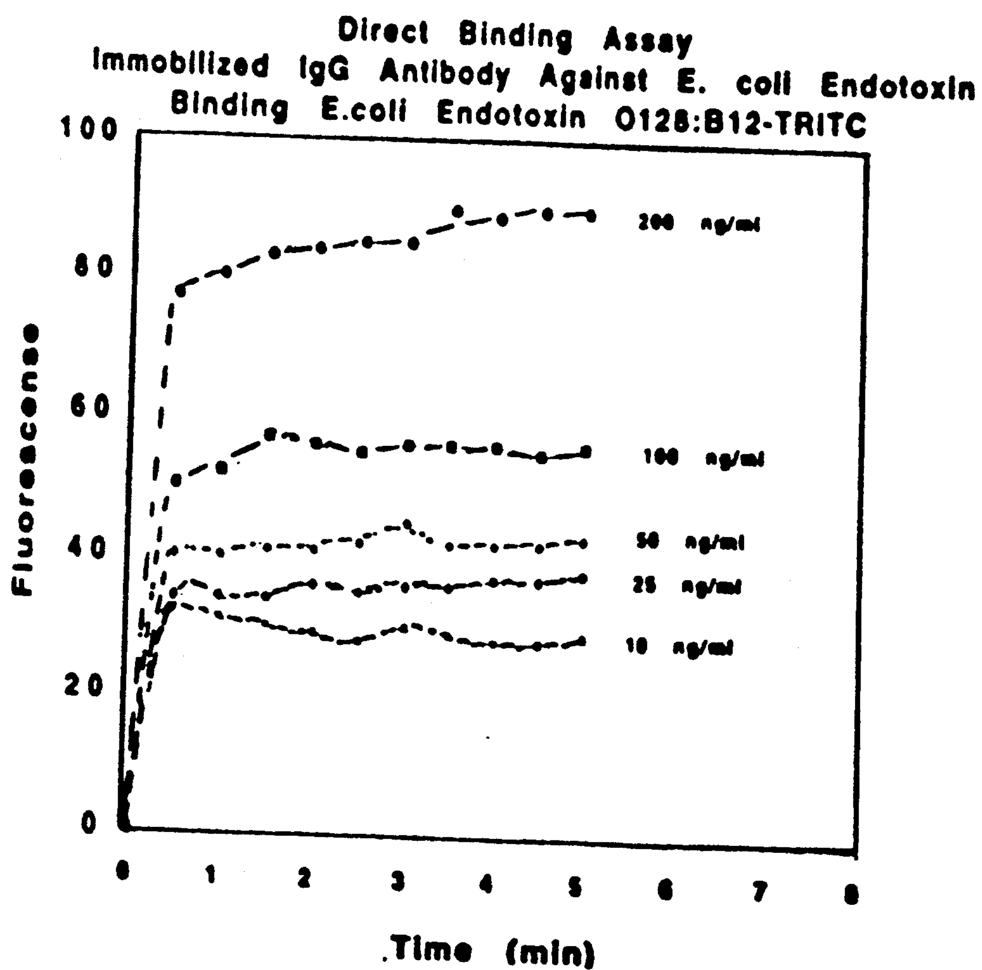


FIGURE 3

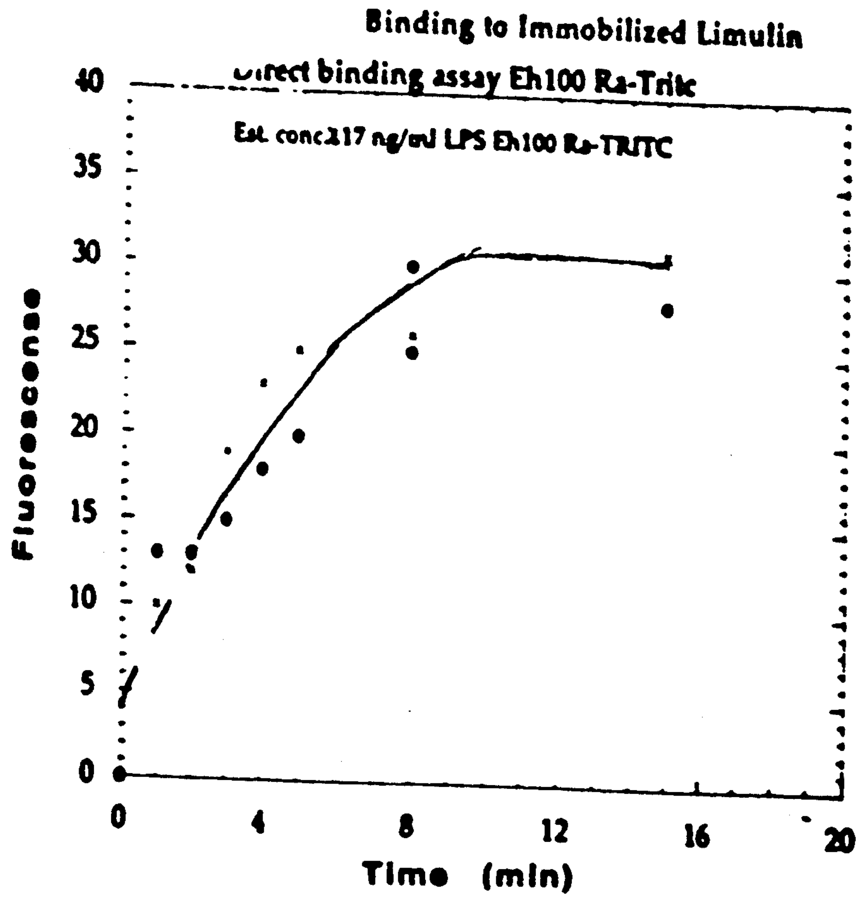


FIGURE 4

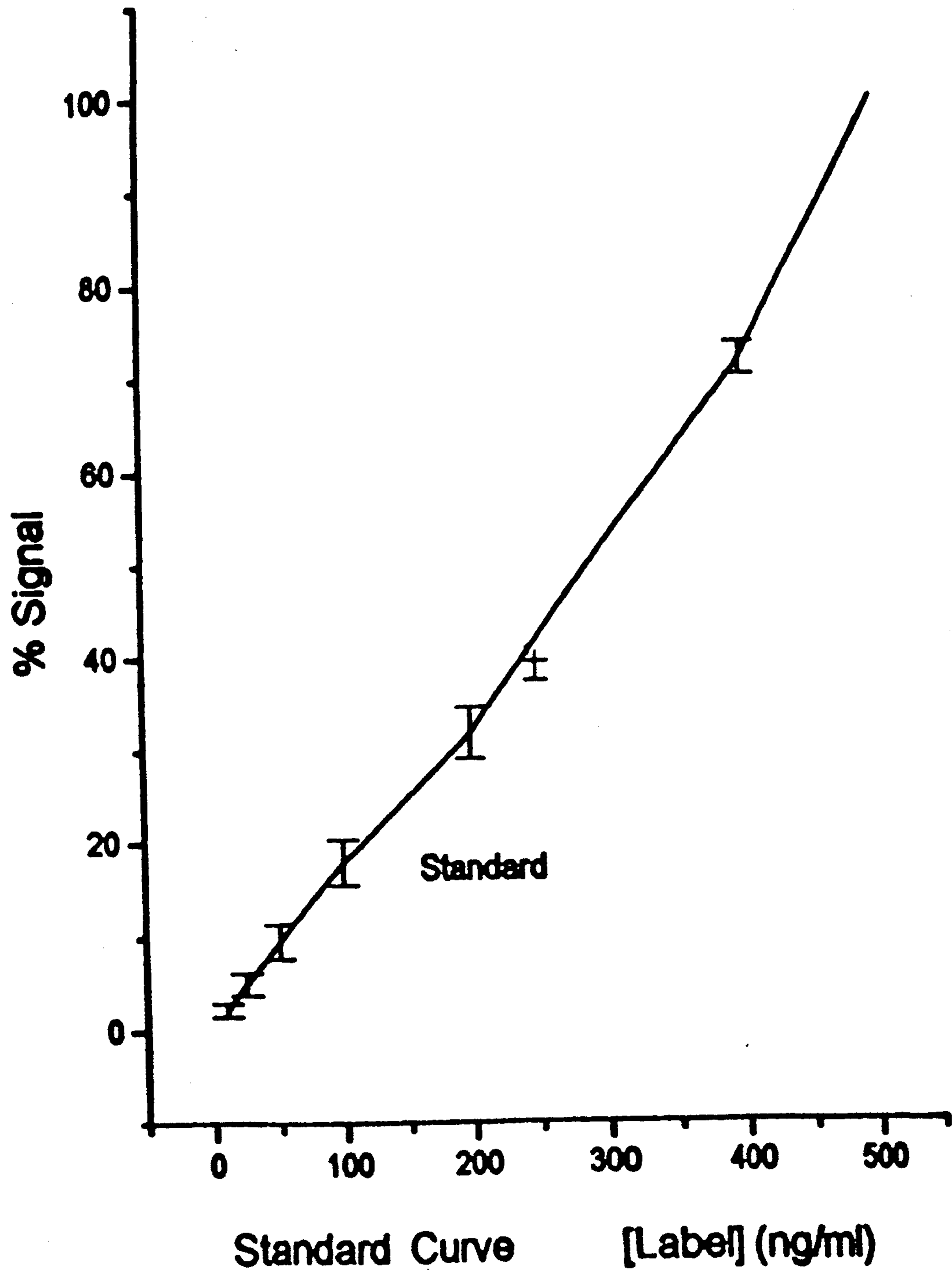
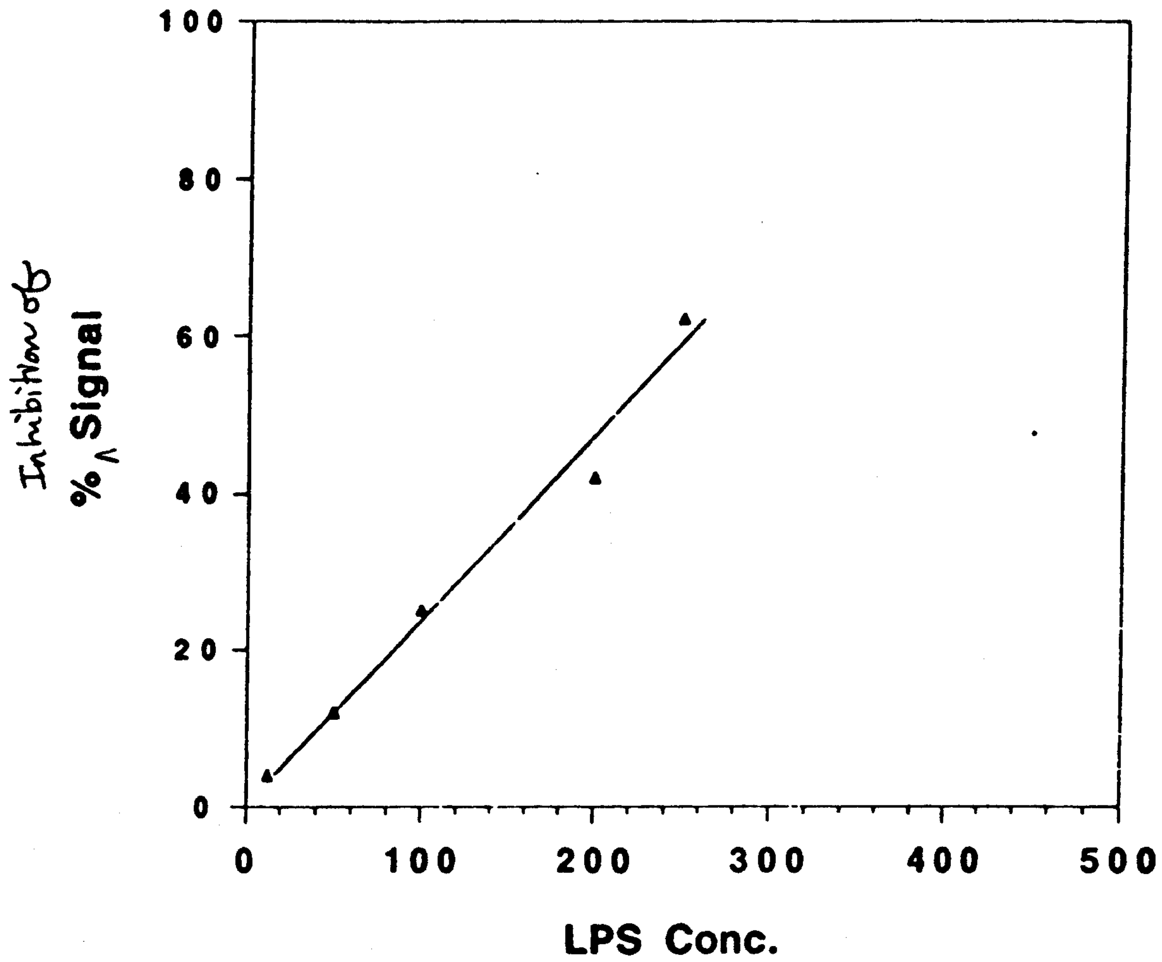
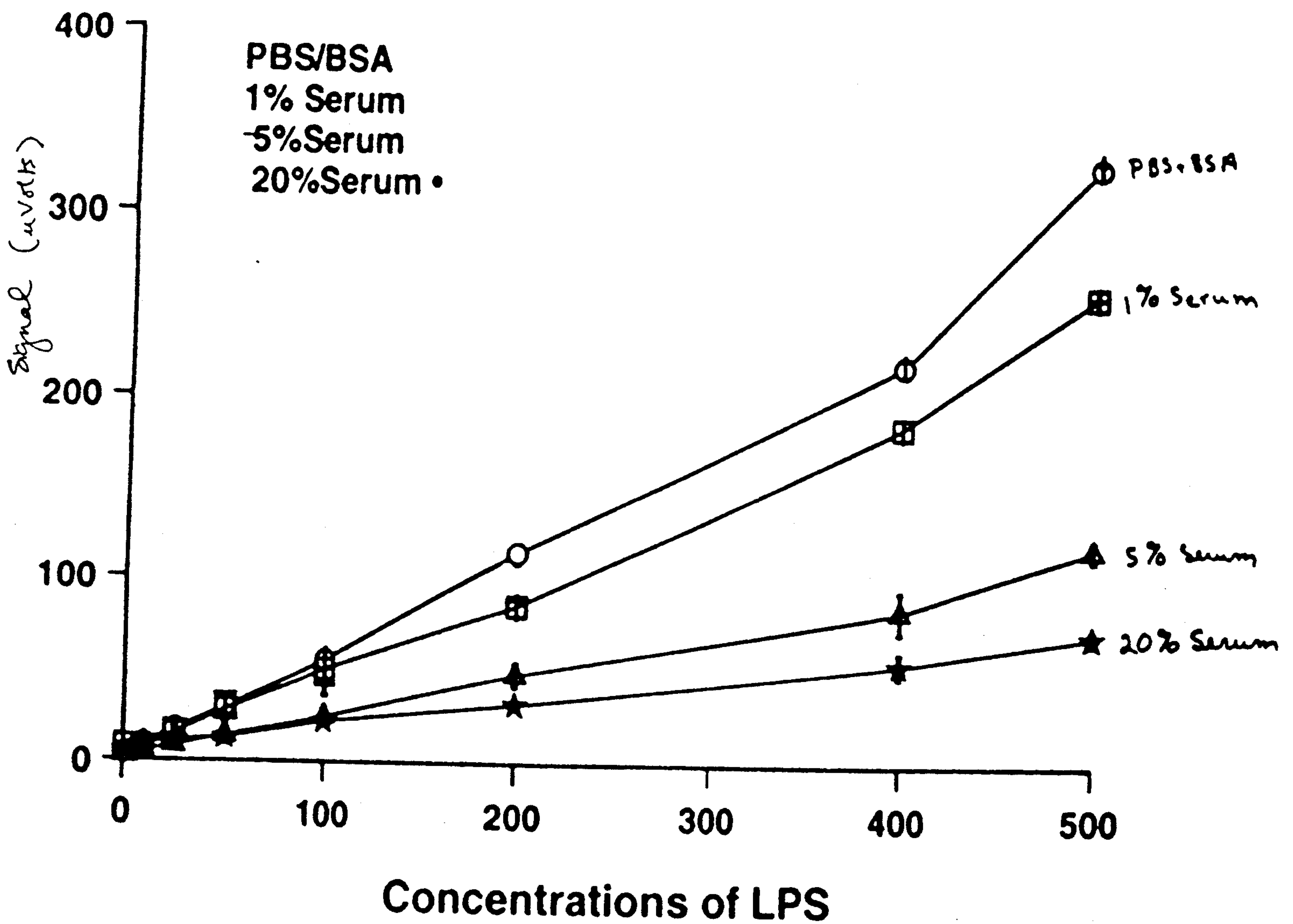


FIGURE 5



Final [TRITC-LPS] = 200 ng/ml

FIGURE 6



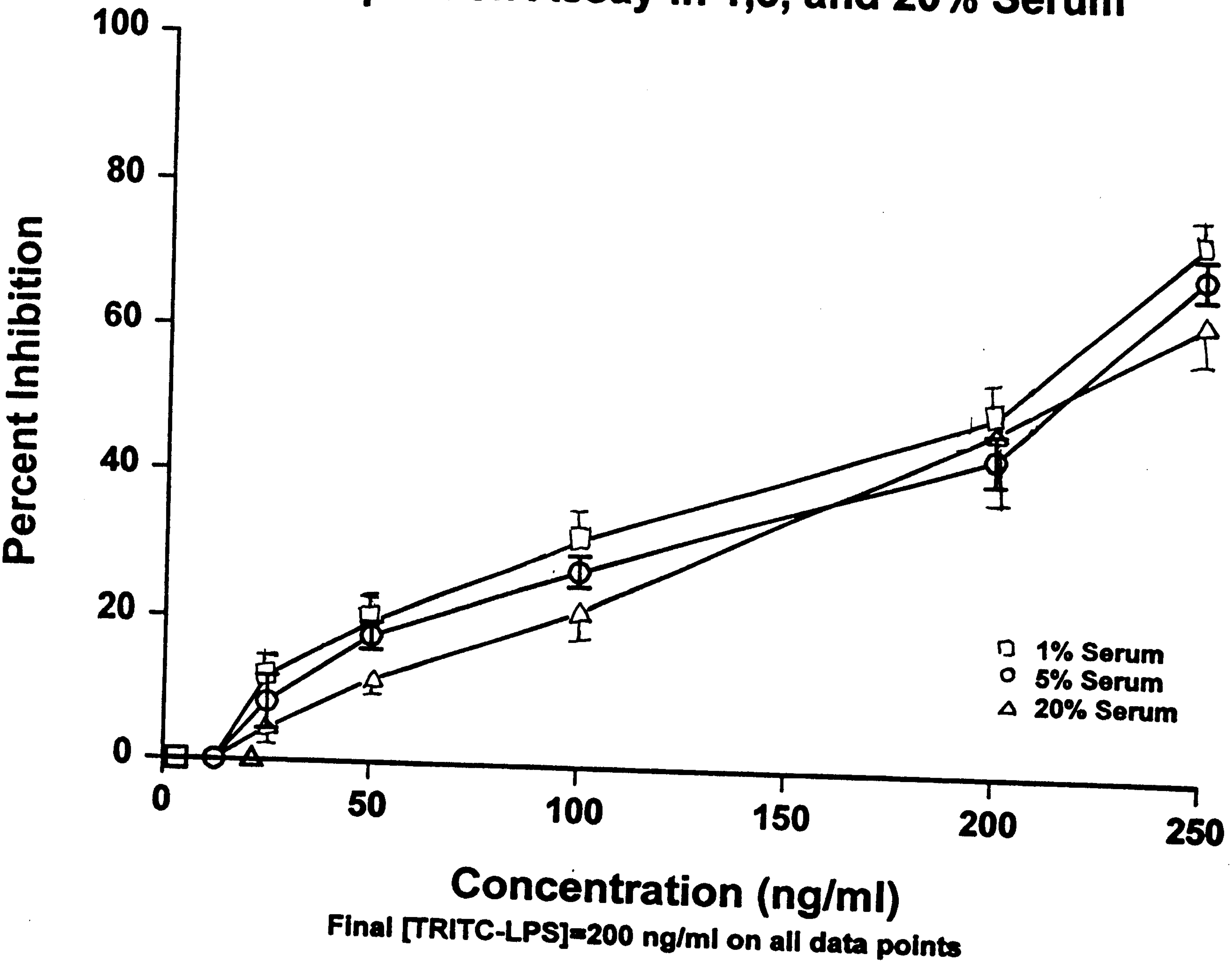


FIGURE 7