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(12) United States Patent
Ding et al.**(10) Patent No.: US 6,645,724 B1**
(45) Date of Patent: Nov. 11, 2003**(54) ASSAYS FOR ENDOTOXIN****(75) Inventors: Jeak Ling Ding**, Singapore (SG); **Bow Ho**, Singapore (SG)**(73) Assignee: National University of Singapore**, Singapore (SG)**(*) Notice:** Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.**(21) Appl. No.: 09/287,368****(22) Filed: Apr. 7, 1999****Related U.S. Application Data****(63)** Continuation-in-part of application No. 09/201,786, filed on Dec. 1, 1998, now abandoned, which is a continuation-in-part of application No. 09/081,767, filed on May 21, 1998, now abandoned.**(60)** Provisional application No. 60/058,816, filed on Sep. 19, 1997.**(51) Int. Cl.⁷** **A61K 39/02**; G01N 33/53; C12P 21/06; C12N 15/09; C12N 15/64**(52) U.S. Cl.** **435/7.1**; 424/236.1; 435/91.4; 435/219; 435/252.3; 435/320.1; 435/348; 435/69.1; 435/69.3; 436/546; 530/384; 530/390.1; 530/391.5; 530/391.7; 936/14; 936/16; 936/22; 936/31**(58) Field of Search** 424/236.1, 7.1, 424/69.3; 435/91.4, 219, 252.3, 320.1, 348; 436/546; 530/384, 390.1, 391.5, 391.7; 936/14, 16, 22, 31**(56) References Cited****U.S. PATENT DOCUMENTS**5,336,610 A * 8/1994 Esmon et al. 435/212
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Primary Examiner—Lynette R. F. Smith*Assistant Examiner*—Ja-Na Hines**(74) Attorney, Agent, or Firm**—Birch, Stewart, Kolasch & Birch, LLP**(57) ABSTRACT**

The horseshoe crab, *Carcinoscorpius rotundicauda* Factor C cDNA (CrFC21) has been cloned into a shuttle baculoviral vector and another vector suitable for expression in insect cells. The recombinant baculoviral DNA was then transfected into the insect cells for expression of recombinant Factor C. Recombinant Factor C was found to be immunoreactive and is capable of binding both free and bound/immobilized lipid A. It is enzymatically active when triggered by LPS. The rFC is probably of the two-chain form, being cleaved into the heavy and light chains after activation by Gram negative bacterial endotoxin. As low as 0.01 pg (0.001 ng/ml) of LPS was detectable by the rFC, thus, indicating its potentials as a novel generation of “limulus amoebocyte lysate.”

CRL Exhibit 1032
CRL v. Seikagaku

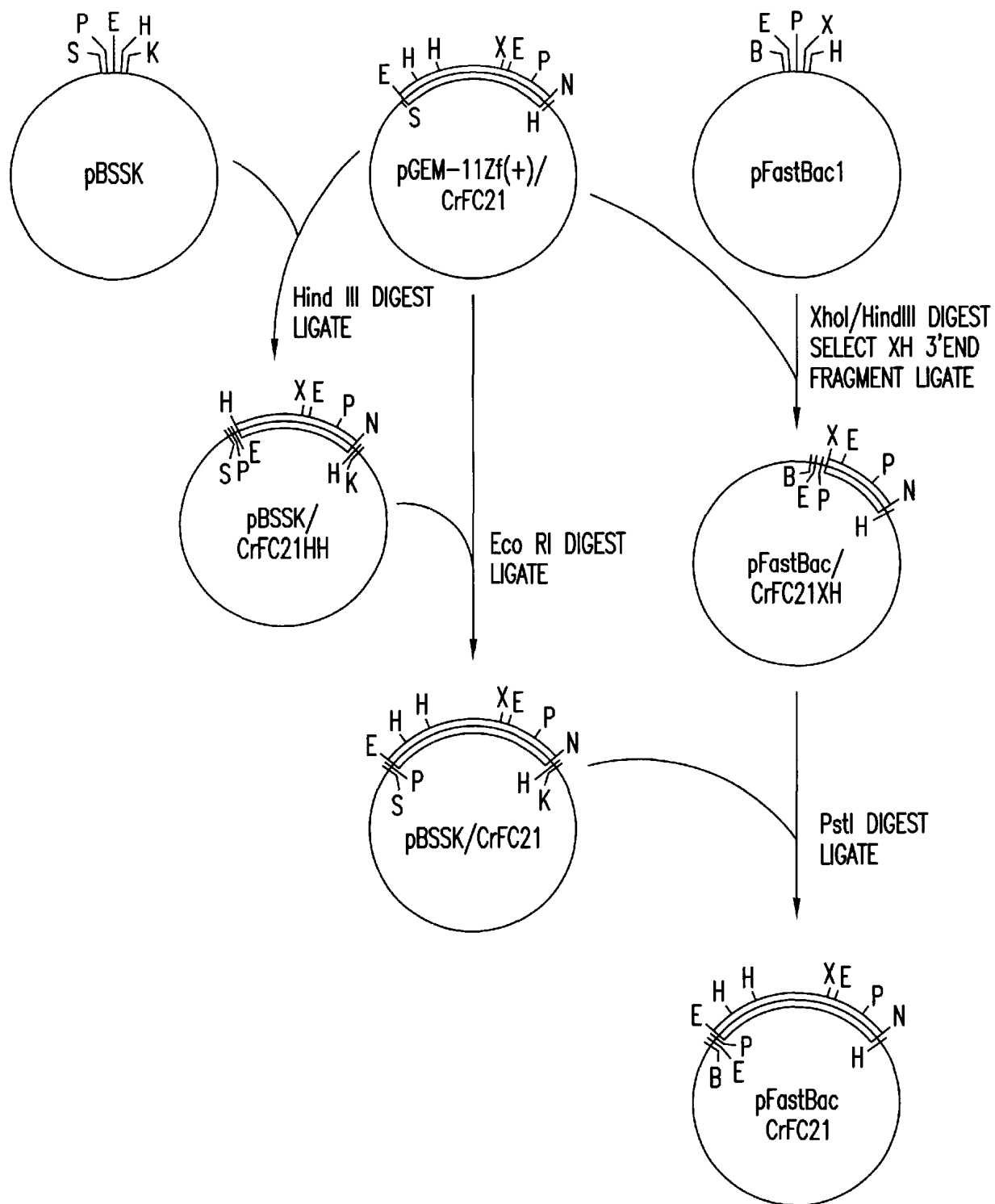


FIG.1A

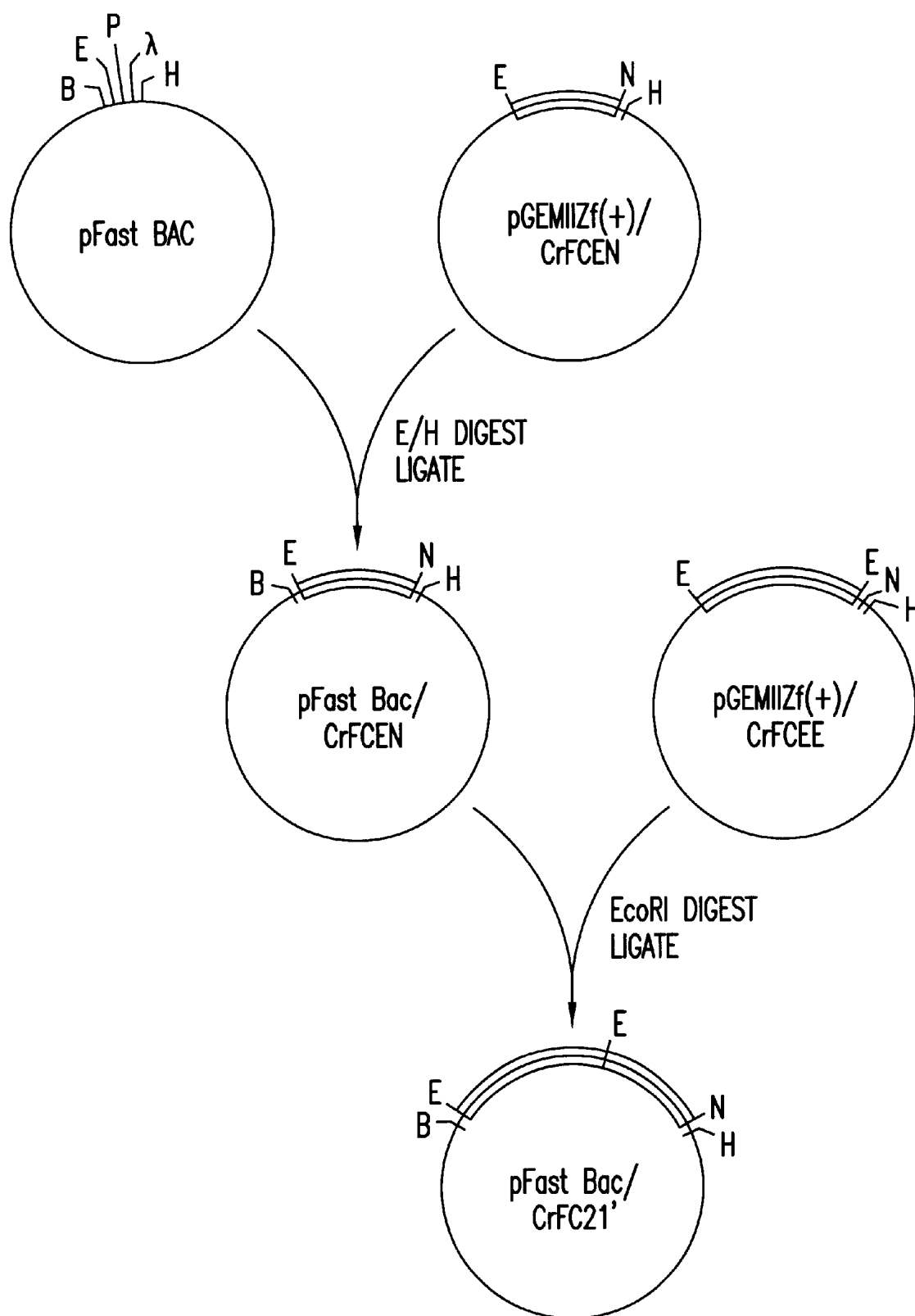


FIG.1B

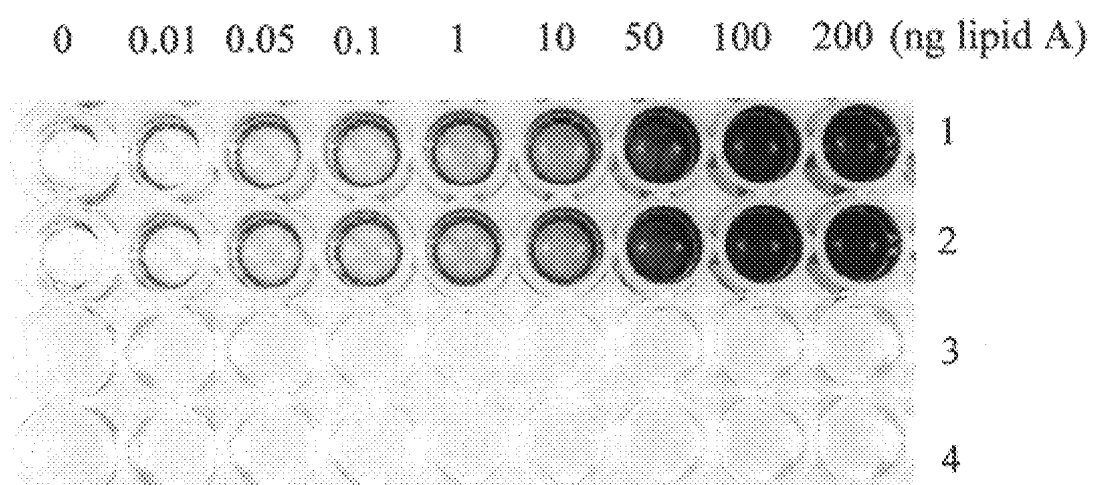


FIG.2

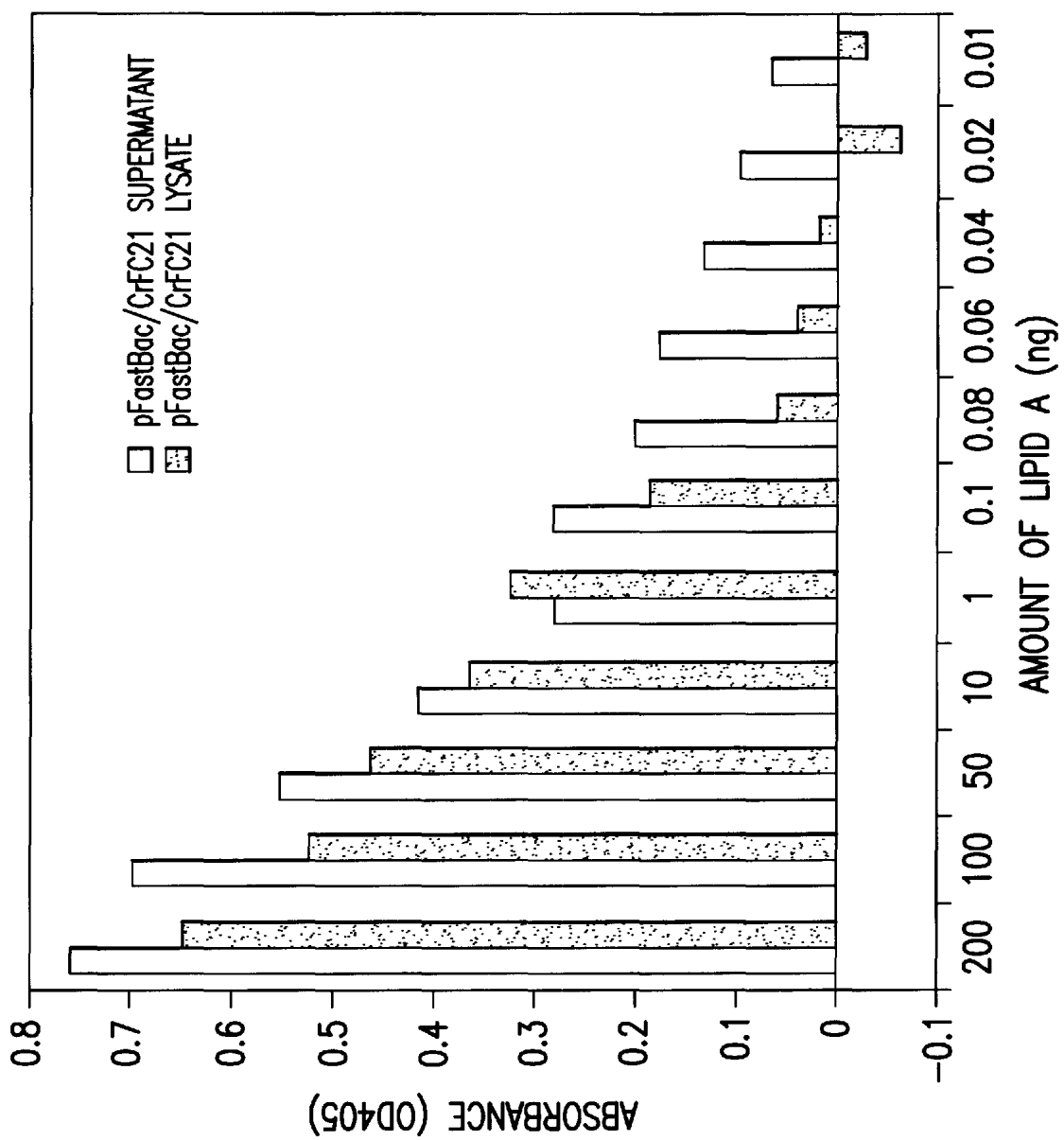


FIG.3A

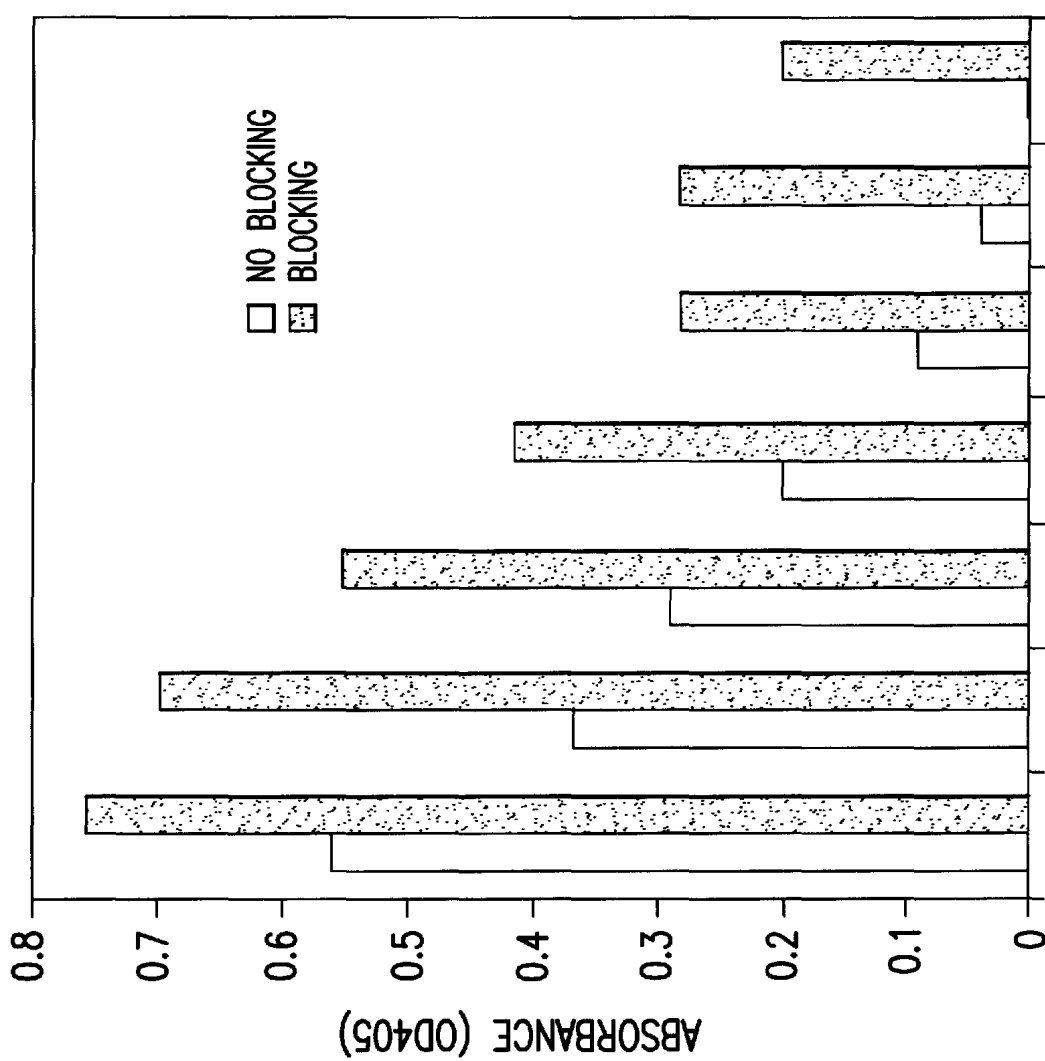


FIG.3B

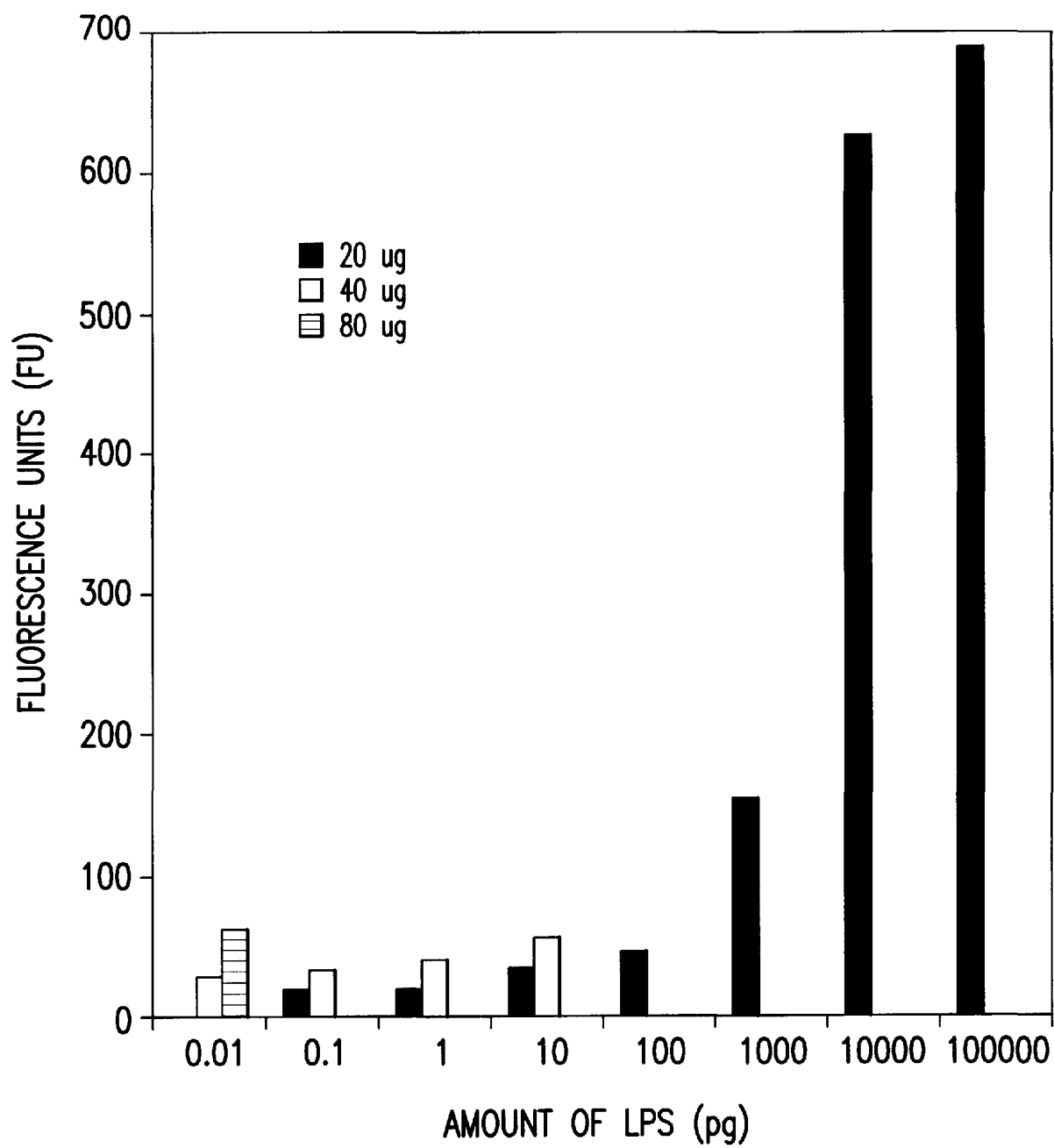


FIG.4

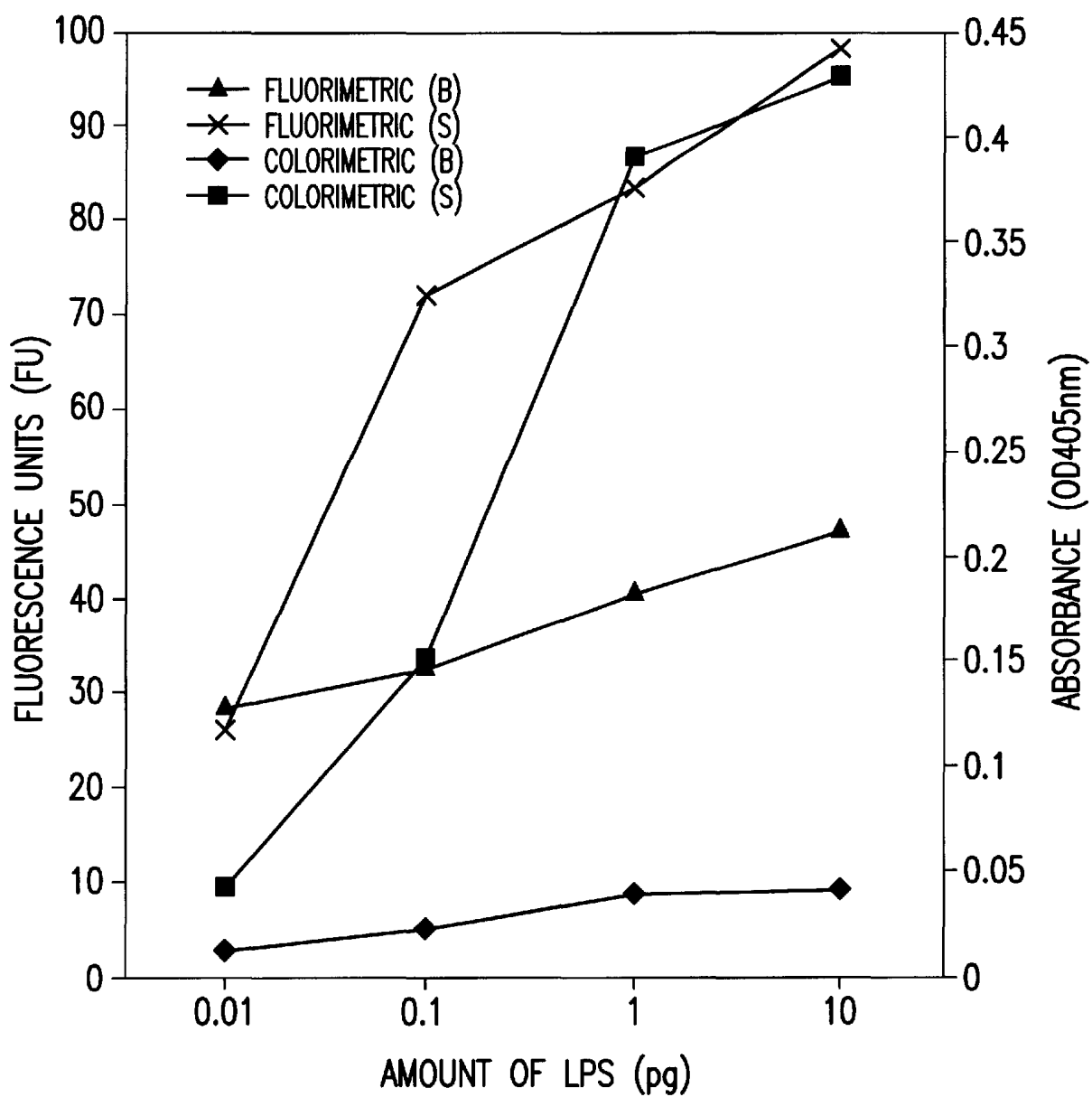


FIG.5

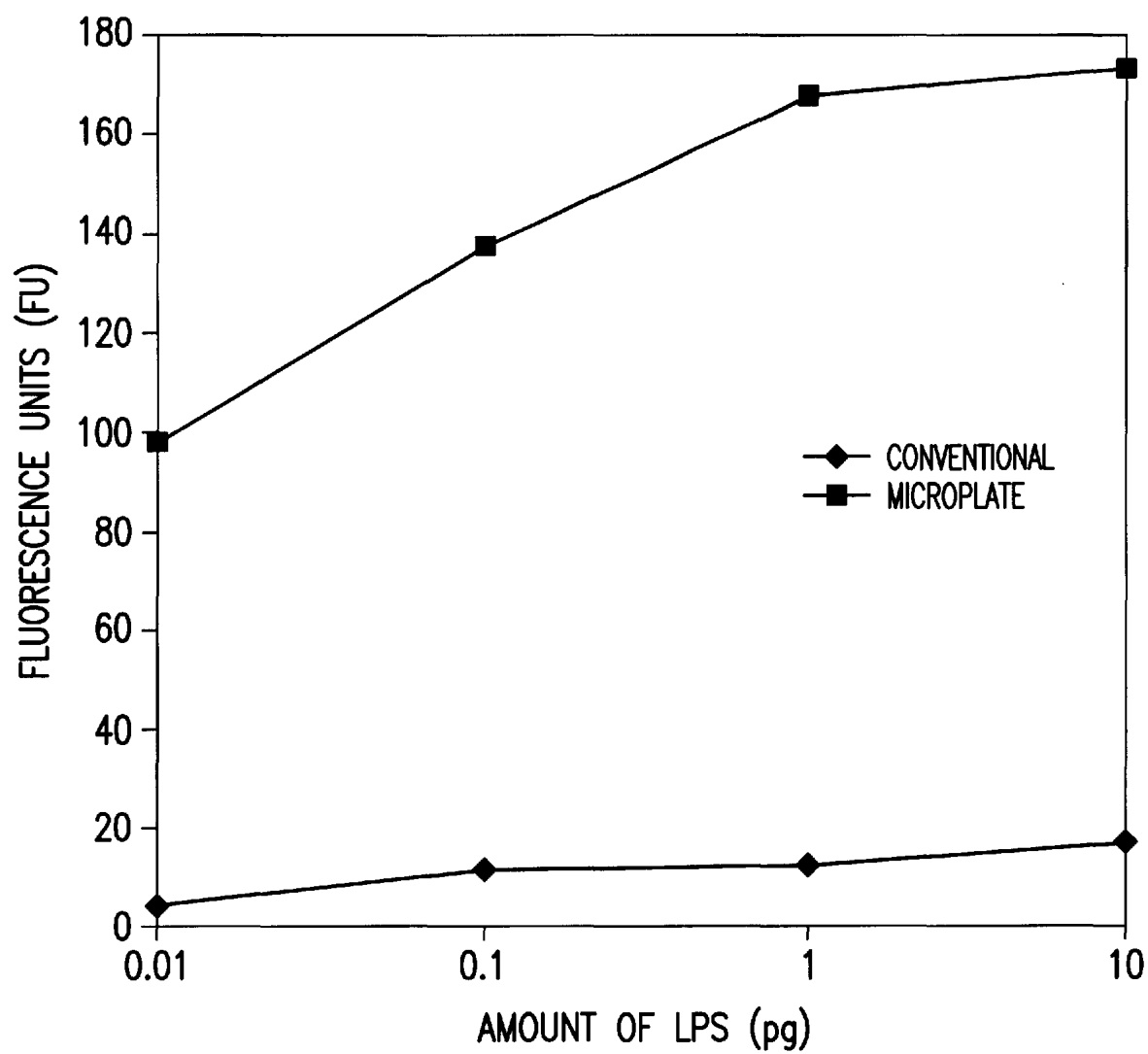


FIG.6

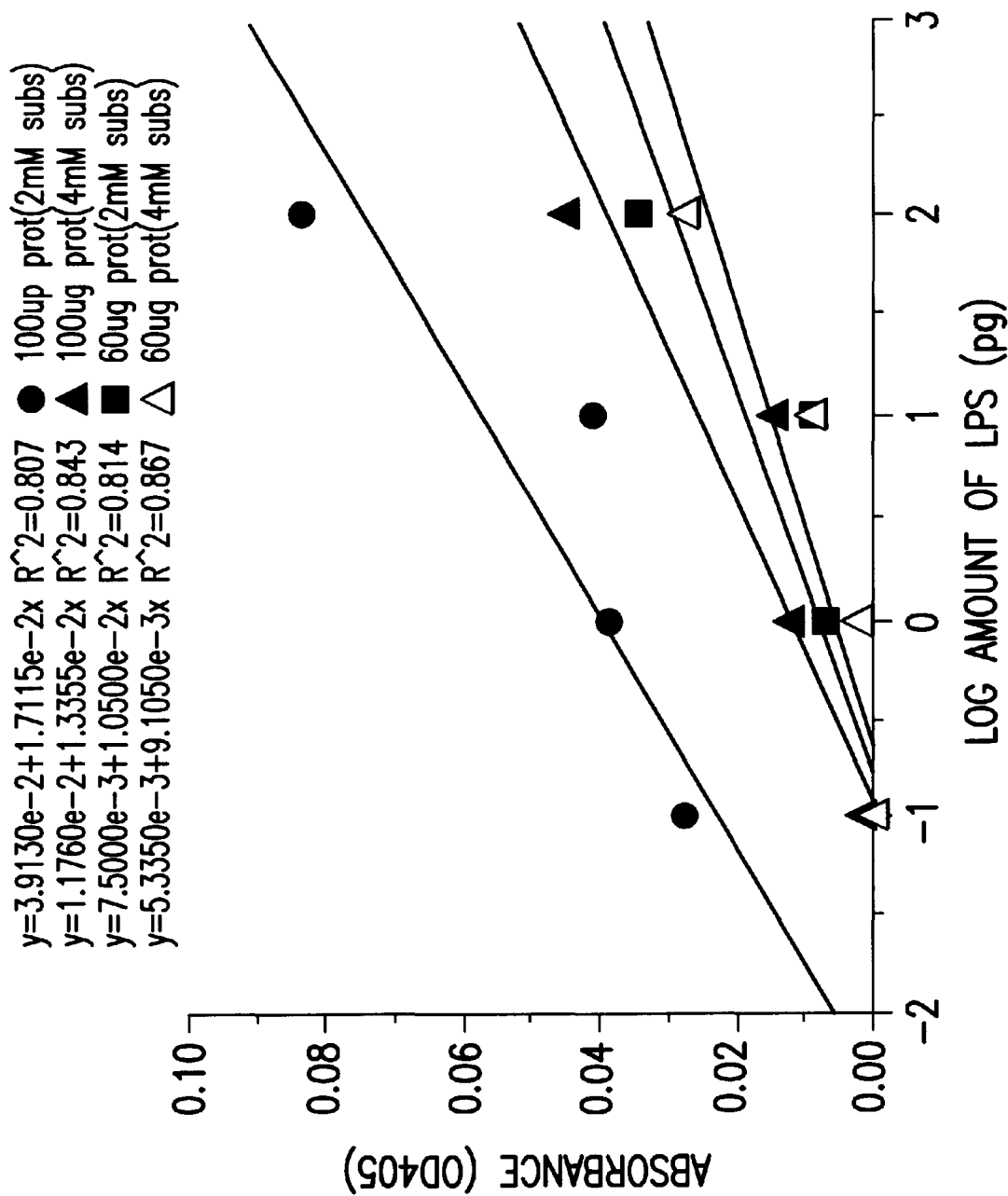


FIG.7

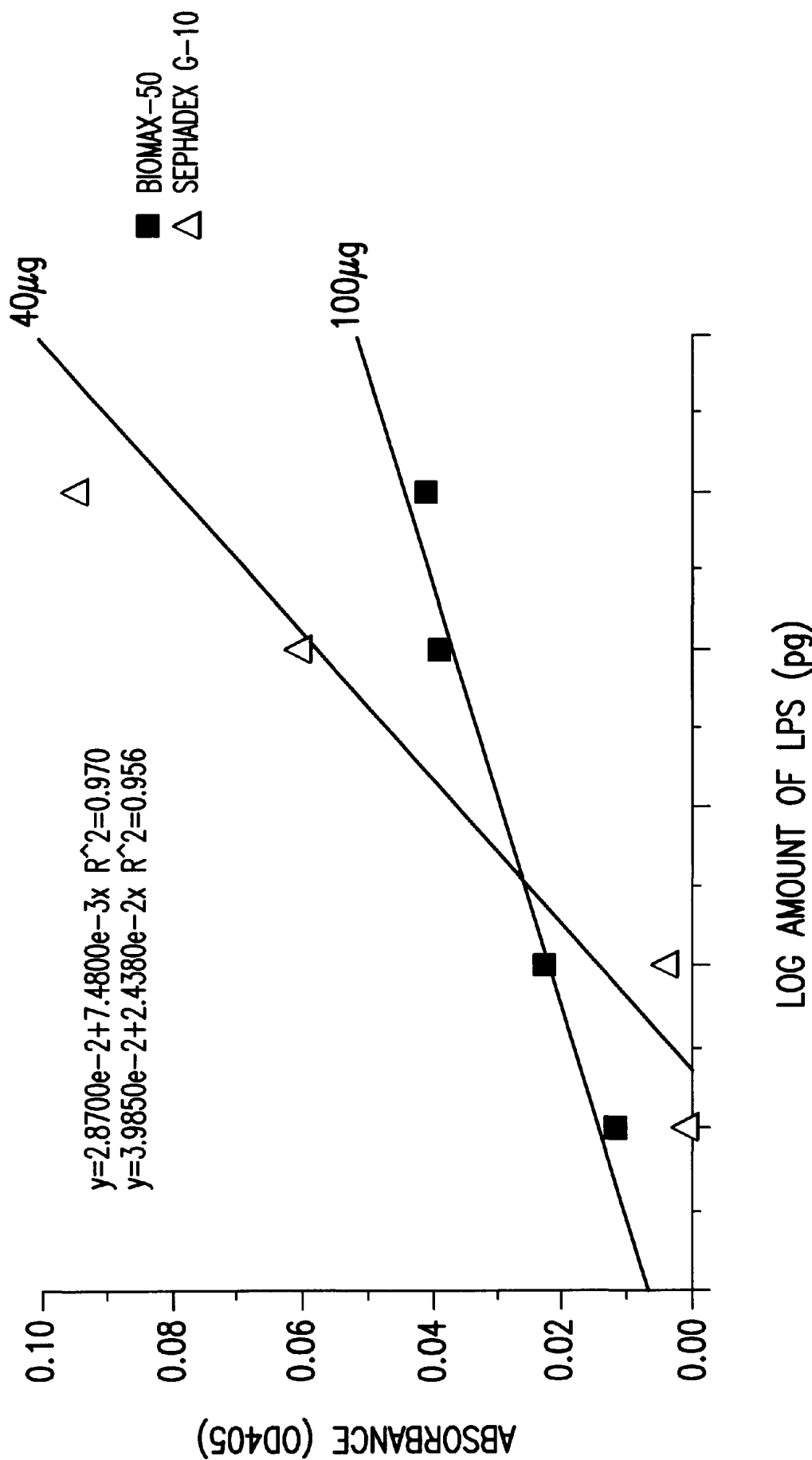


FIG.8

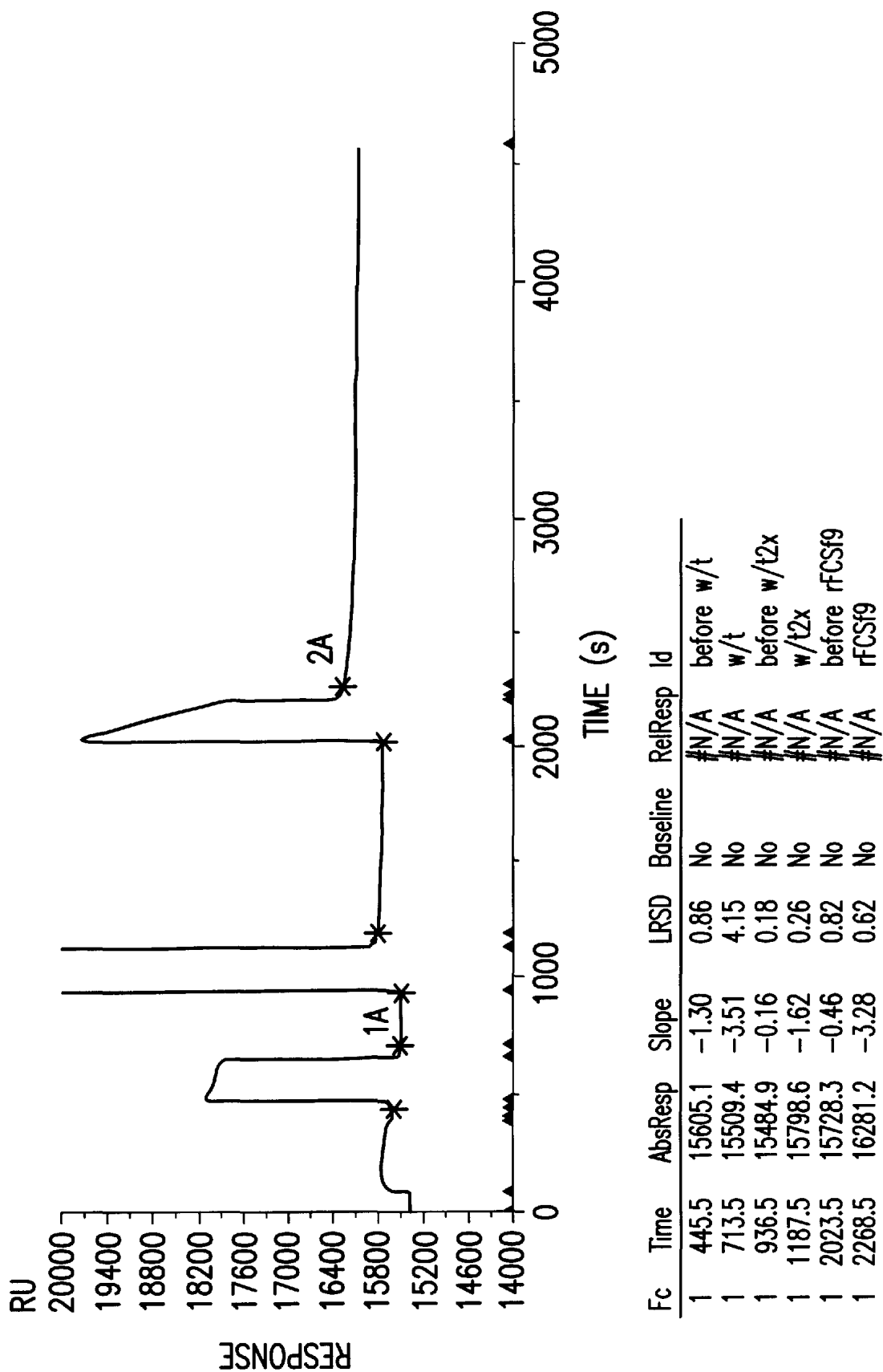


FIG.9

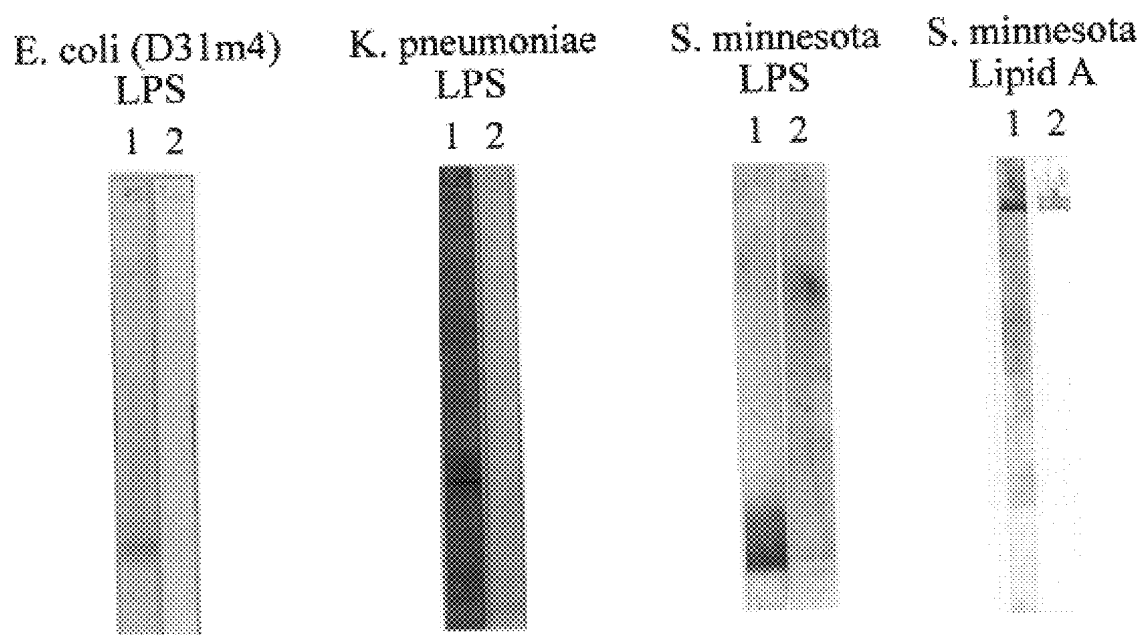


FIG.10

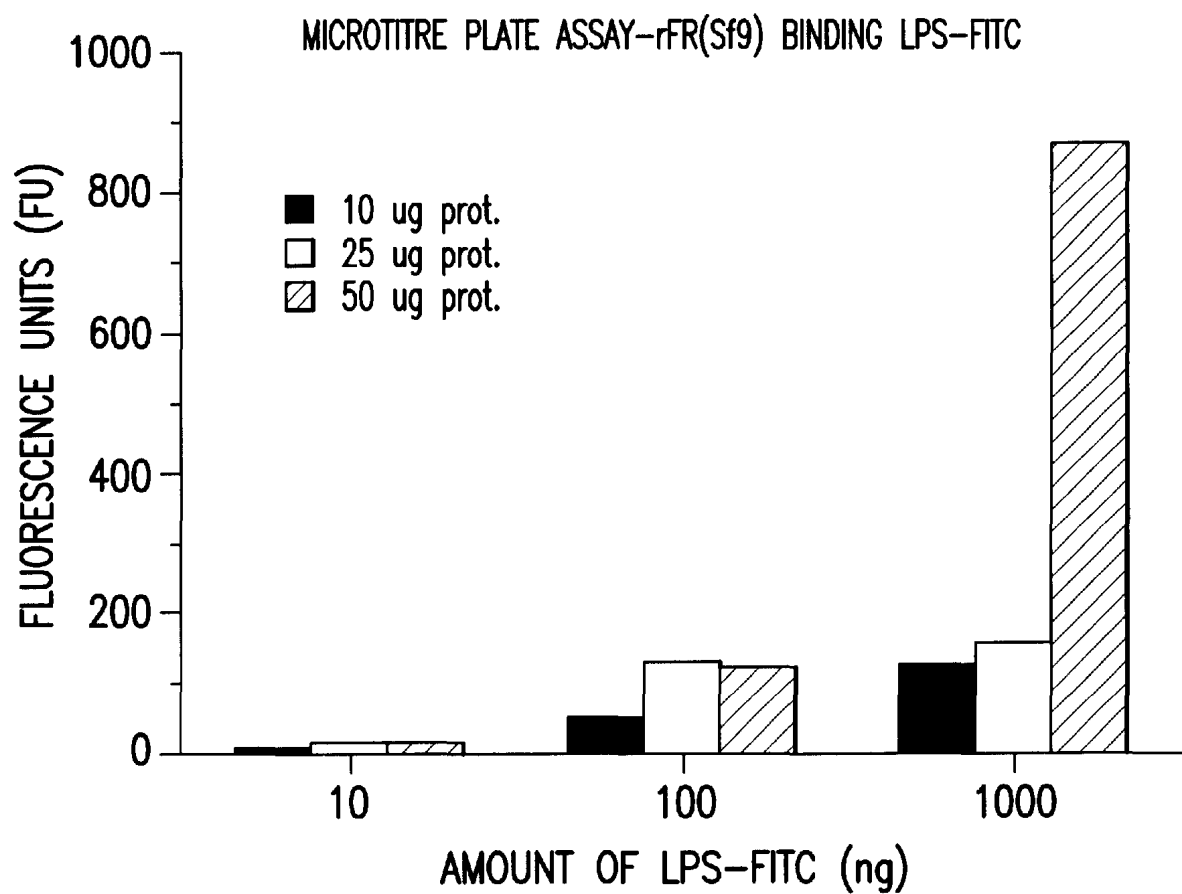


FIG.11

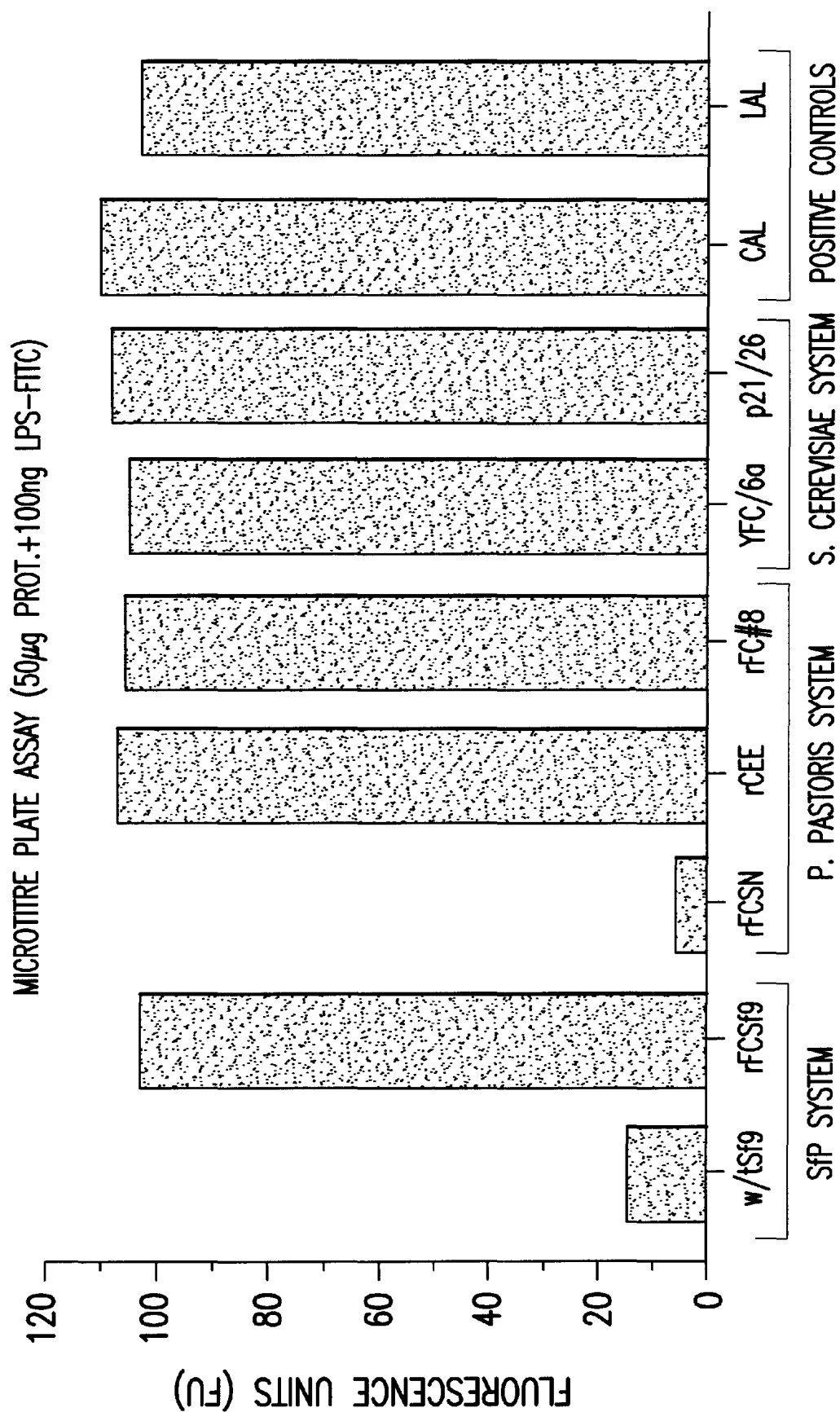


FIG.12

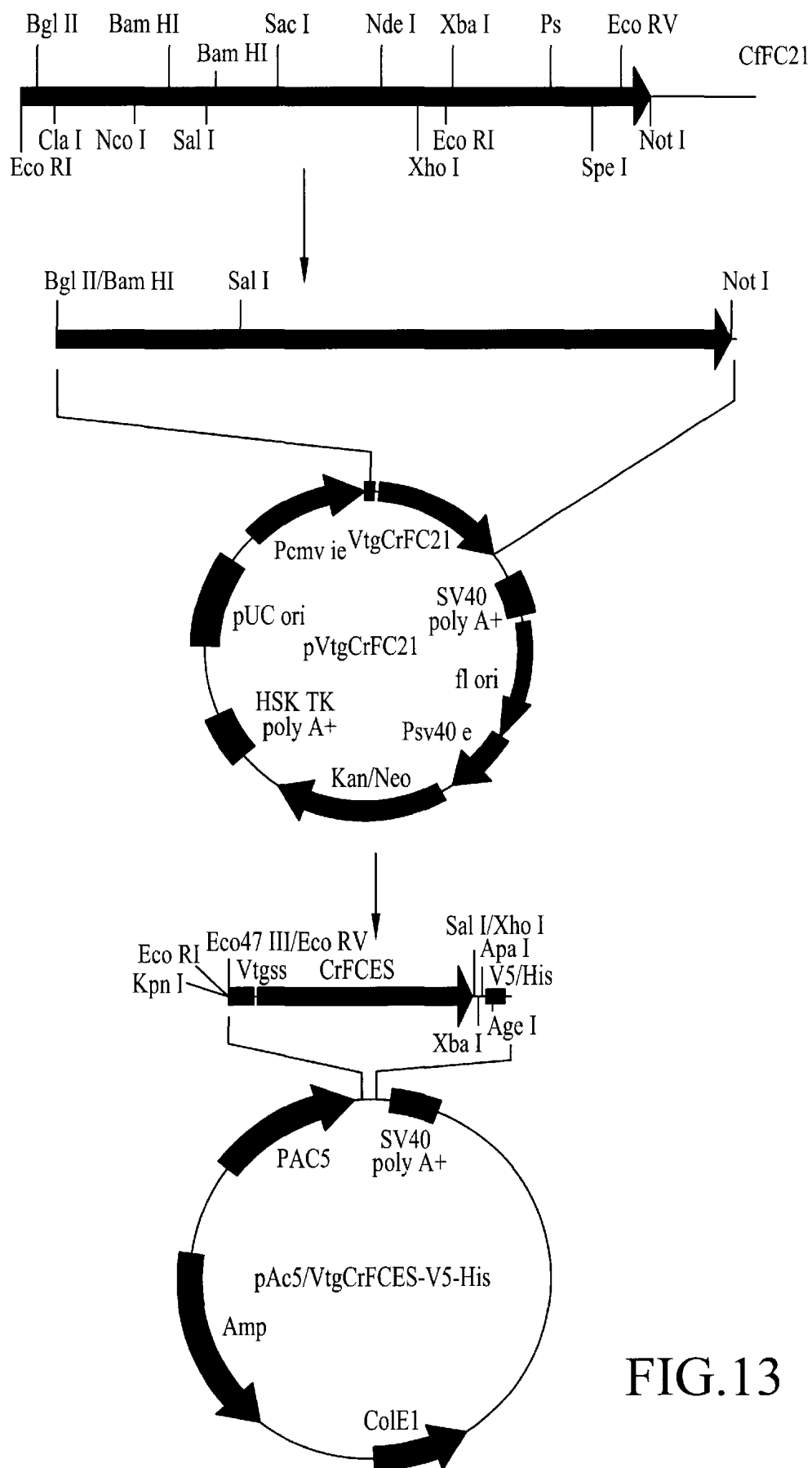


FIG.13

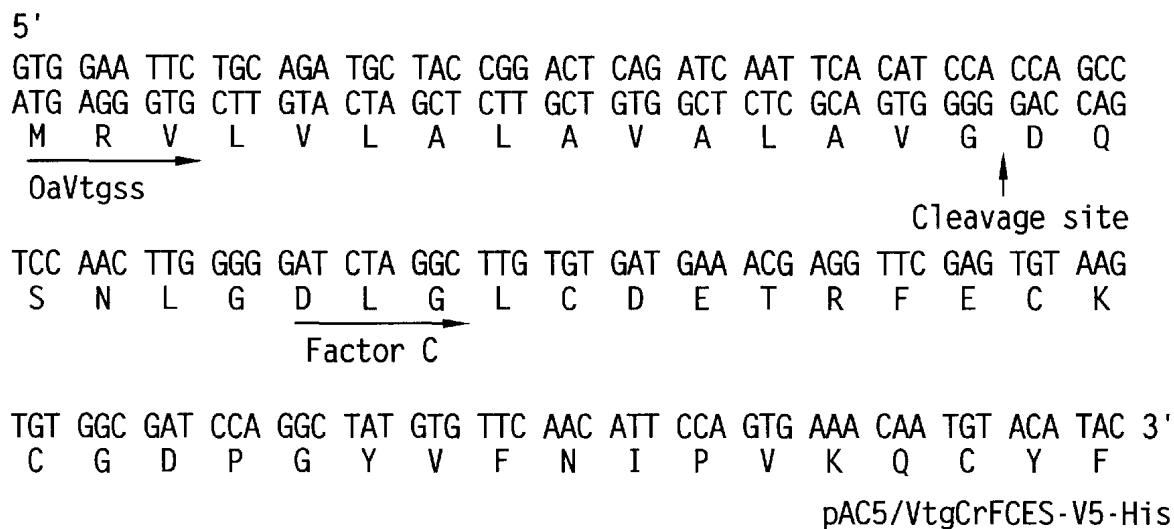


FIG.14A

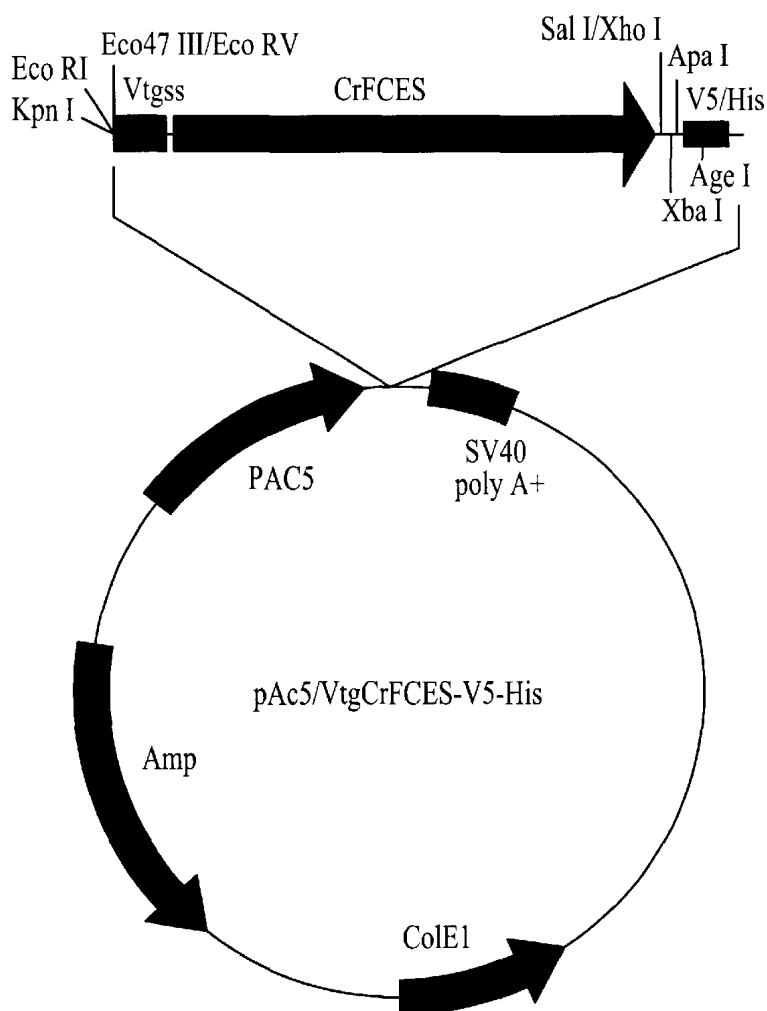


FIG.14B

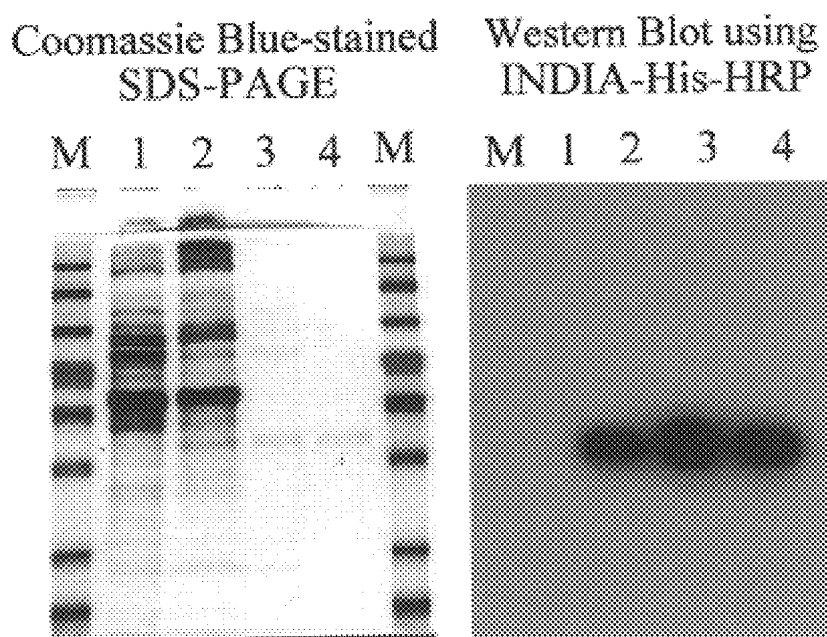


FIG.15A

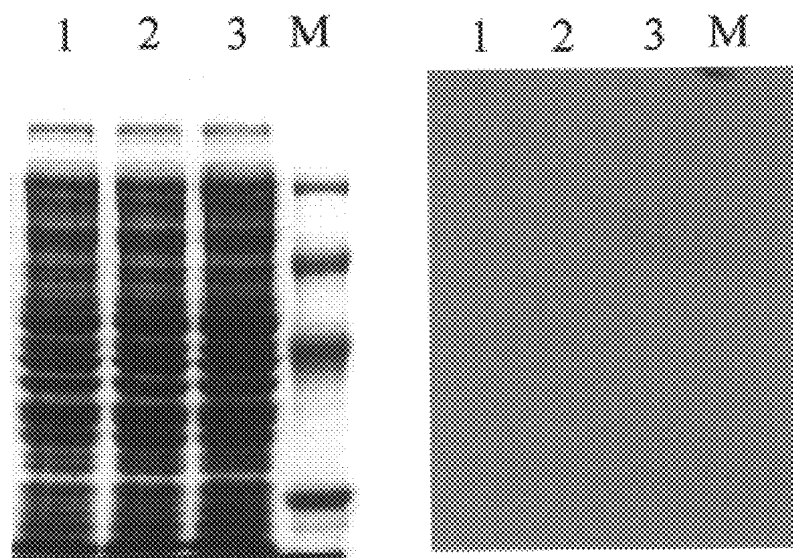


FIG.15B

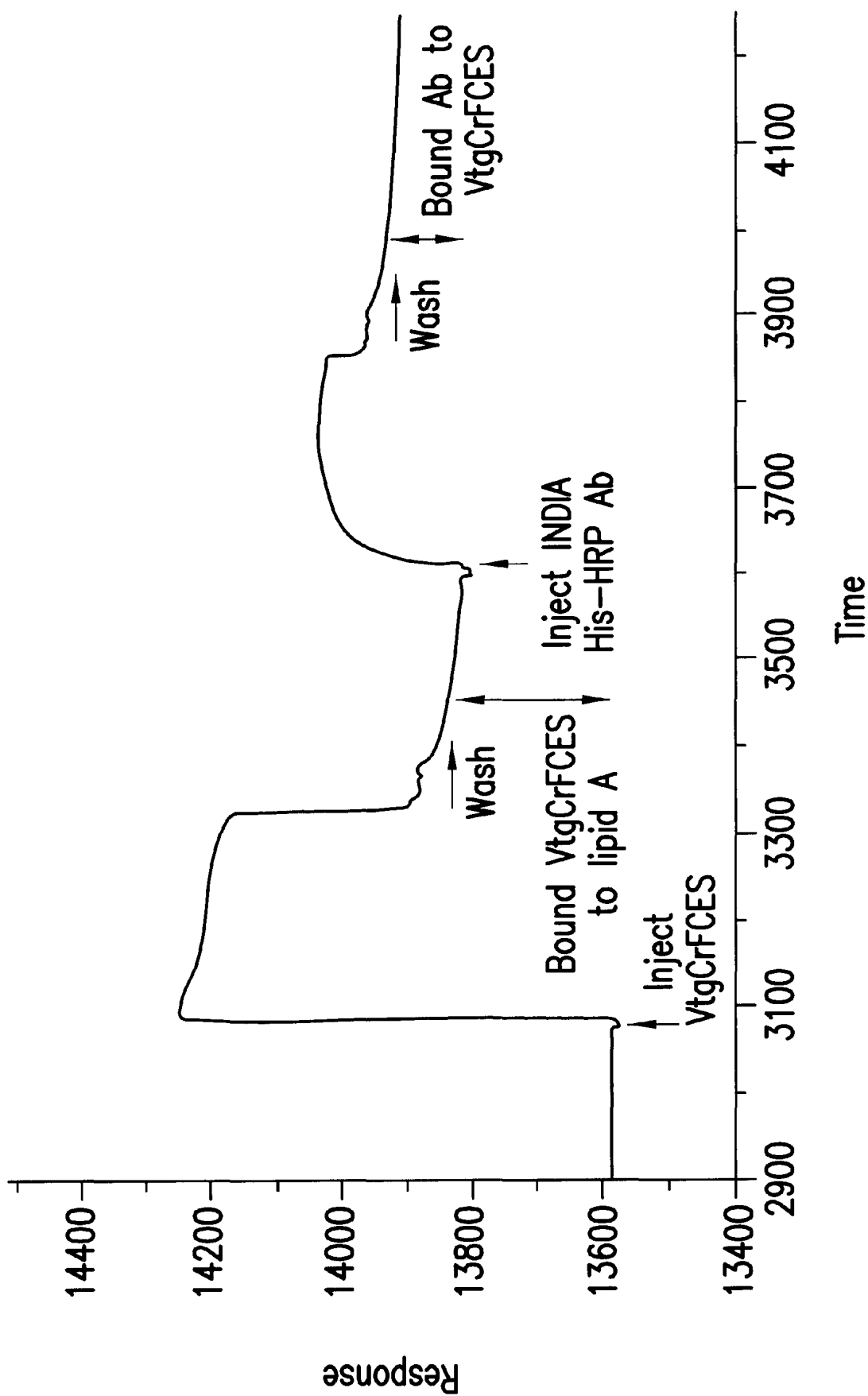


FIG.16

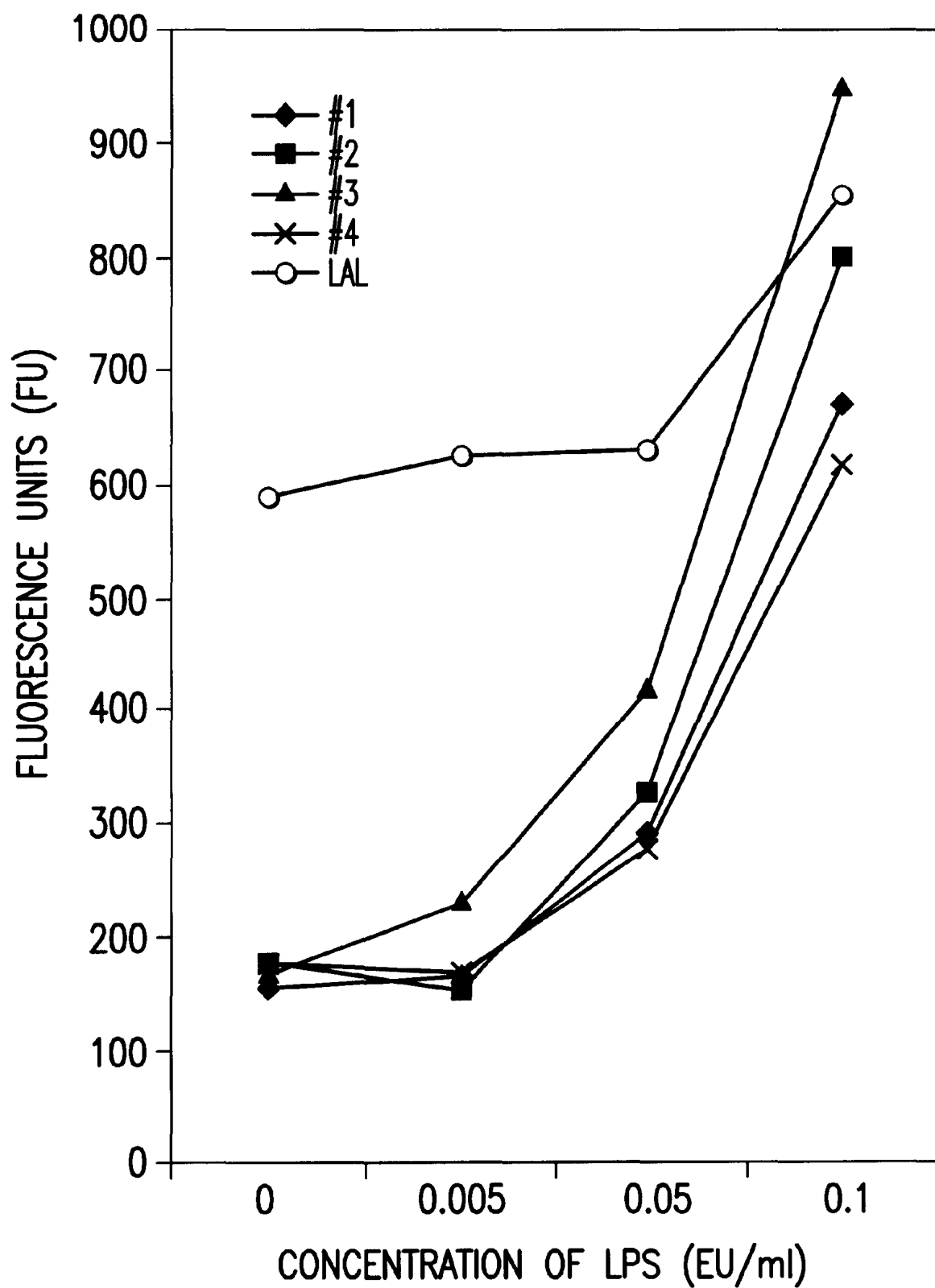


FIG.17

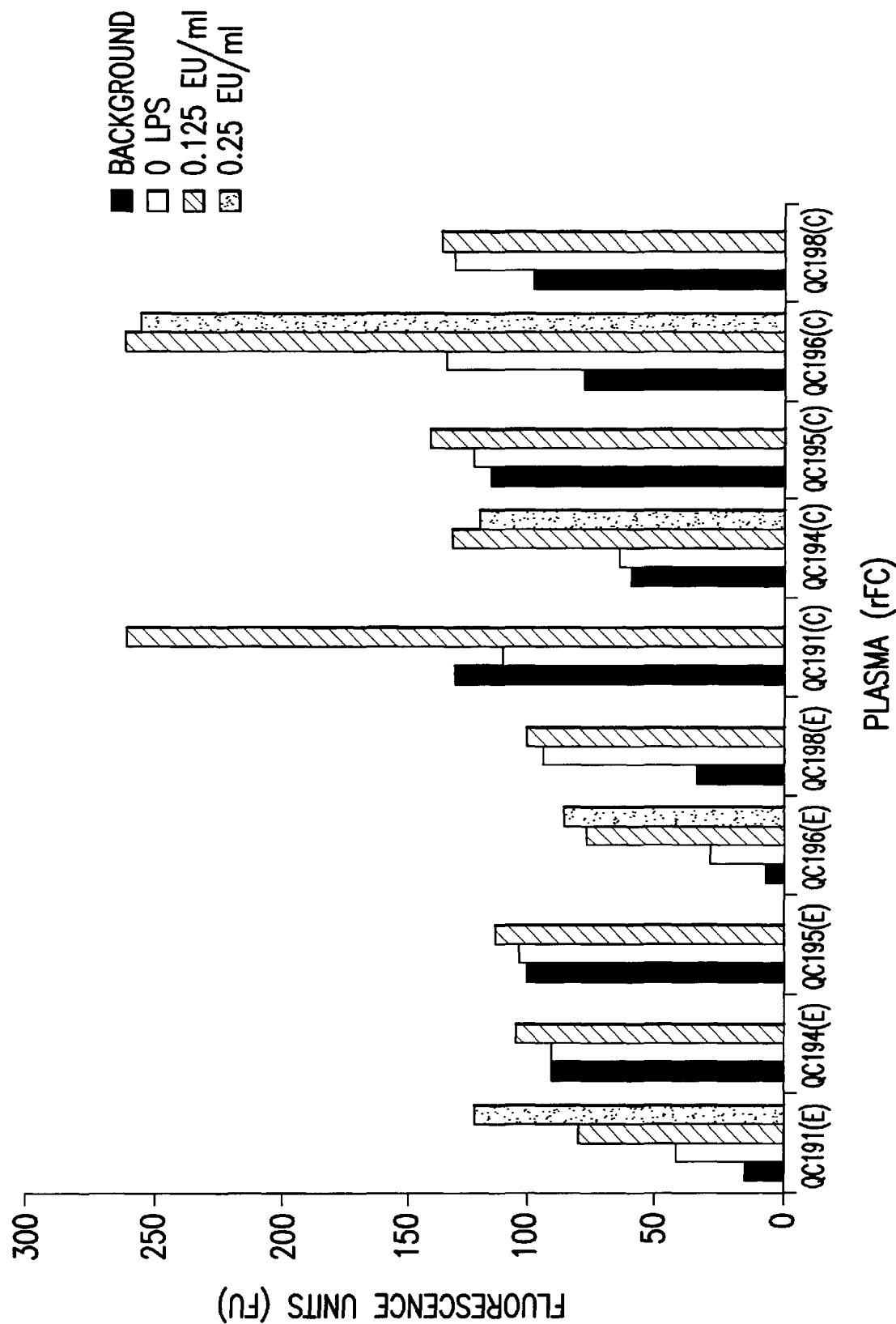


FIG.18A

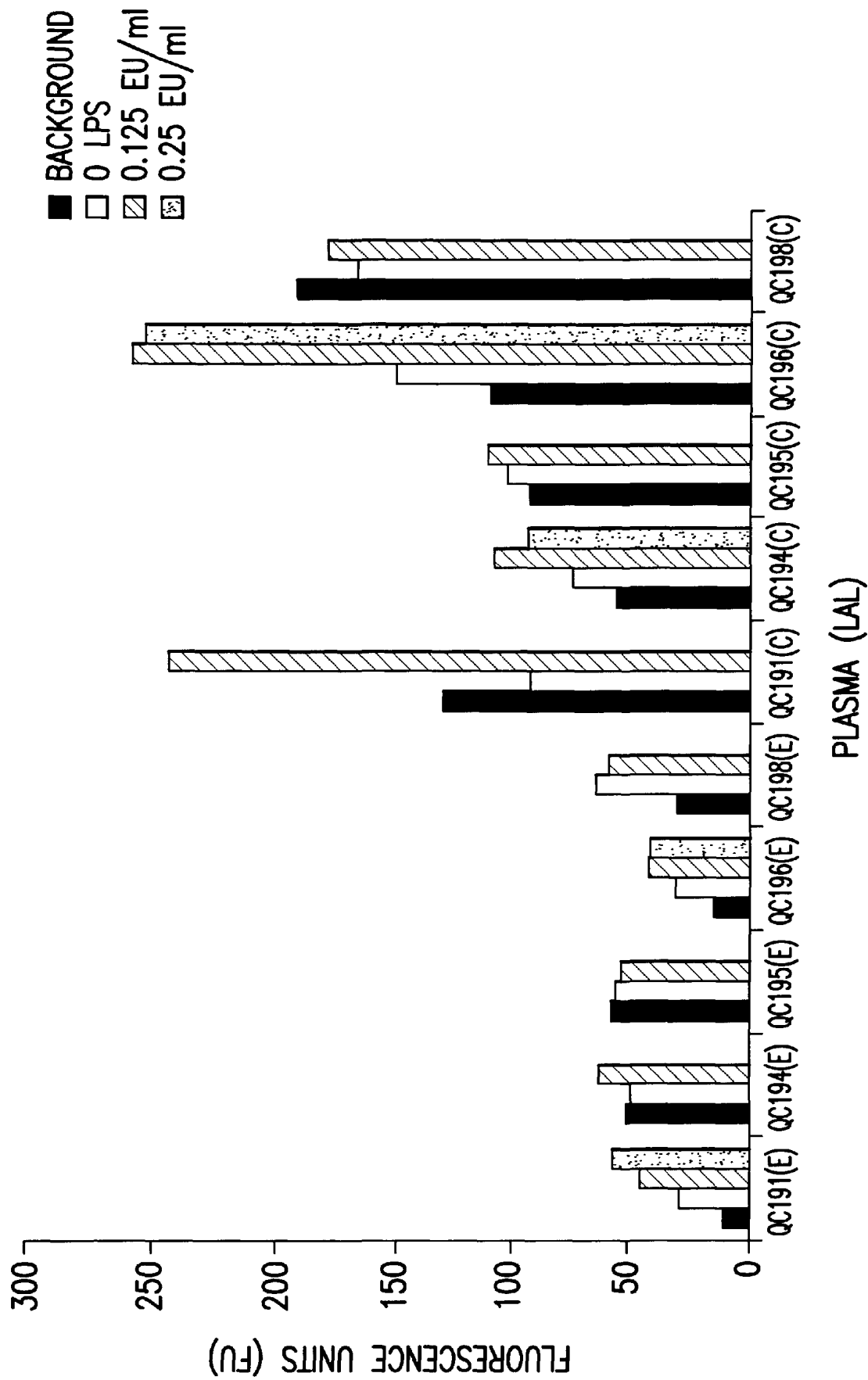


FIG.18B

FIG. 19A
PLASMA (rFC)

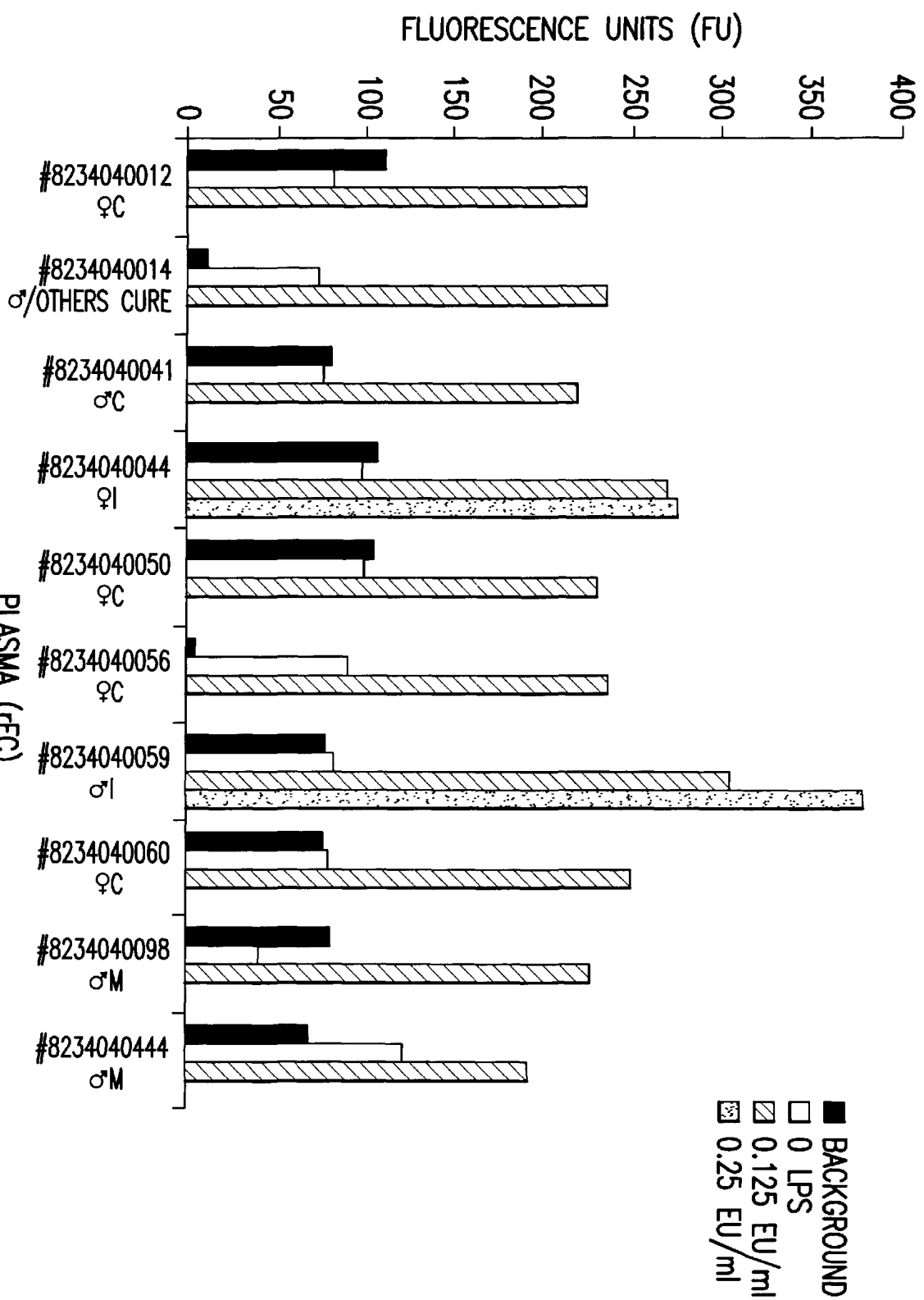


FIG. 19B
PLASMA (LAL)

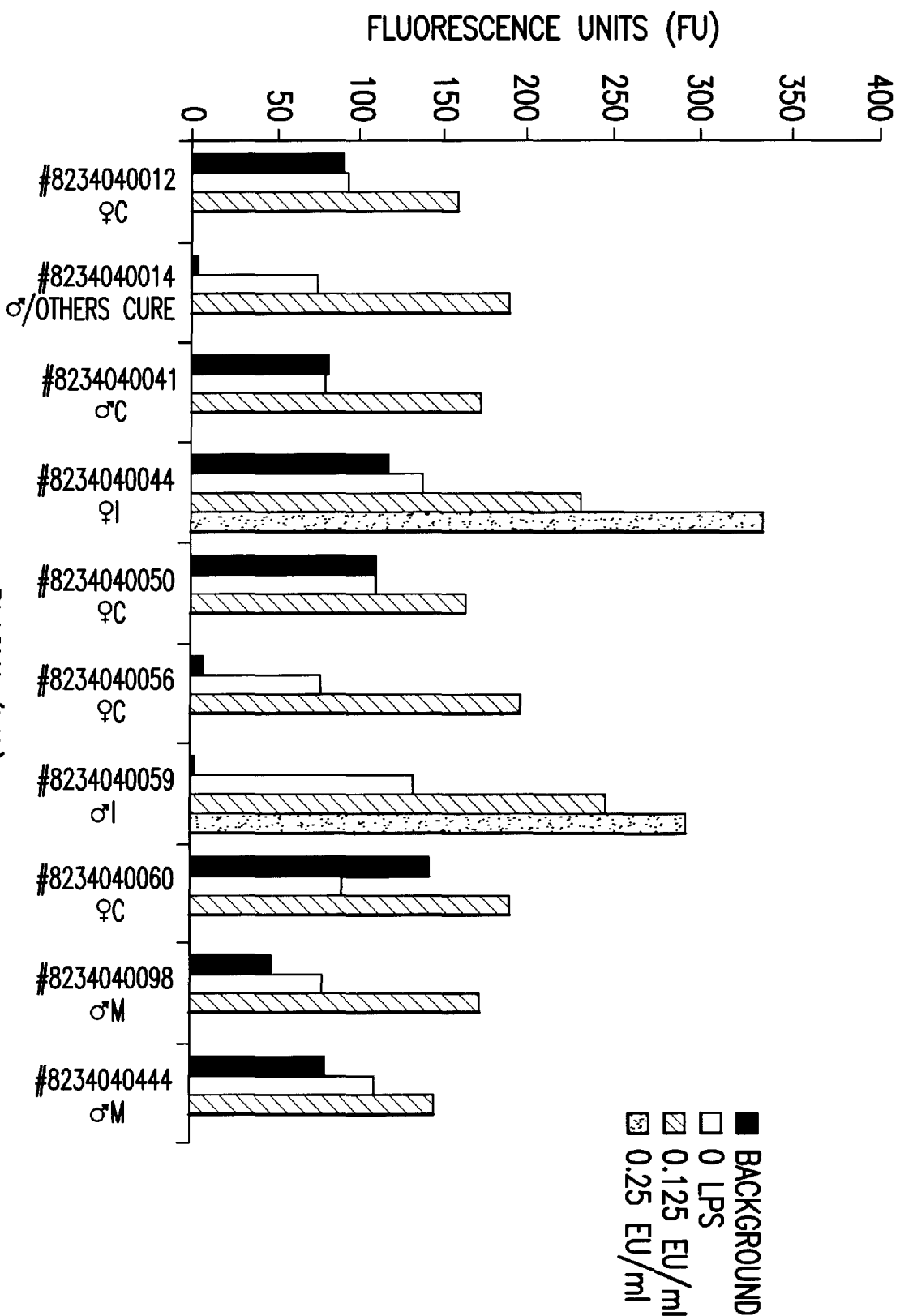


FIG. 20A

PLASMA (rFC)

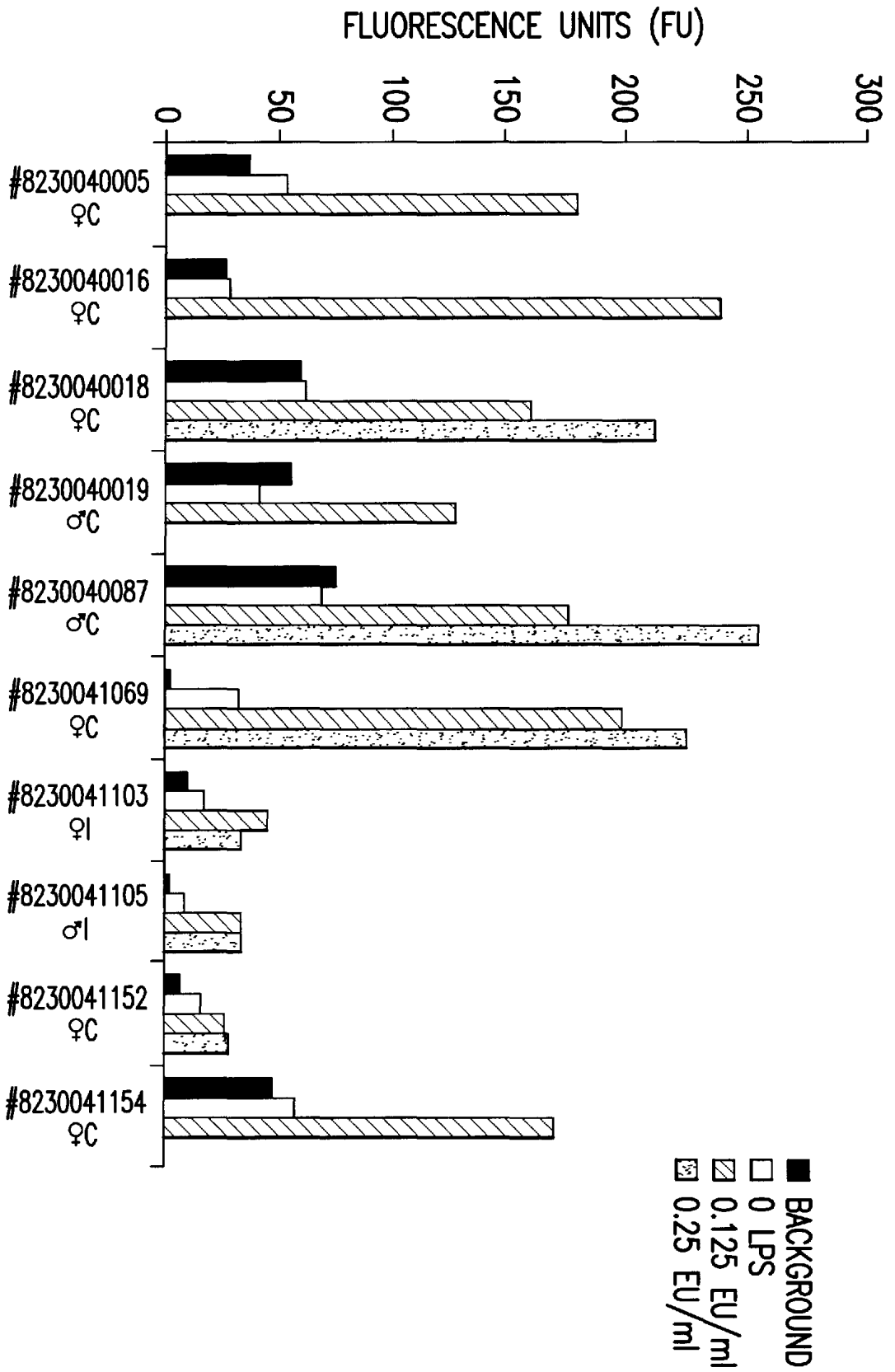
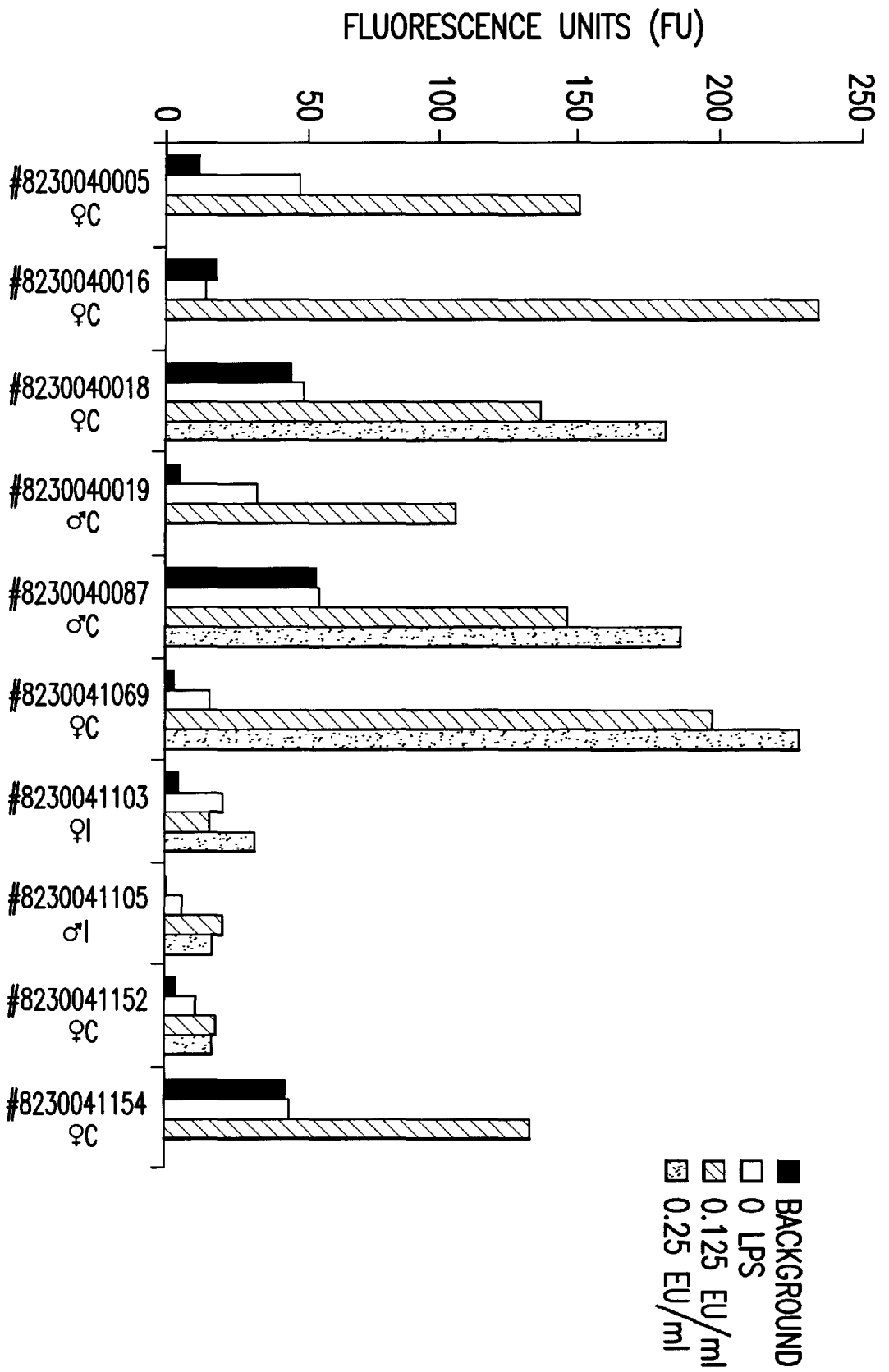


FIG. 20B
PLASMA (LAL)



ASSAYS FOR ENDOTOXIN

RELATED APPLICATIONS

The present application is a Continuation-In-Part of Ser. No. 09/201,786, filed Dec. 1, 1998 now abandoned, which in turn is a Continuation-In-Part of Ser. No. 09/081,767, filed May 21, 1998 now abandoned which in turn relies upon Provisional Application No. 60/058,816, filed Sep. 19, 1997. Each of these applications is hereby incorporated in their entirety by reference.

FIELD OF THE INVENTION

The present invention relates to recombinant Factor C (rFC) of a horseshoe crab, produced in an insect cell system. The invention also relates to vectors for producing the protein by recombinant DNA methods and to methods for using the recombinant Factor C to detect endotoxins in a sample or for removal of endotoxins from a sample by affinity methods.

THE RELATED ART

The amoebocytes of horseshoe crabs contain an efficient coagulation cascade system which is activated by endotoxin, also known as lipopolysaccharide (LPS) from Gram negative bacteria. The enzymatic components of the coagulation cascade and the molecular events responsible for the subsequent gelation of the amoebocyte lysate have been characterized in *Tachypleus tridentatus*¹ and *Carcinoscorpius rotundicauda*^{2,3,4}. Factor C has been shown to be the intracellular endotoxin-sensitive serine protease that initiates the coagulation cascade system⁵.

By spiking, the *Limulus* amoebocyte lysate (LAL) test detects femtogram levels of LPS⁶. Owing to its extreme sensitivity, the amoebocyte lysate, in particular, the LAL has been developed into a commercial assay for widespread use in the detection of pyrogenic LPS in drugs and other pharmaceutical products^{7,8}. This assay is based on the LPS-induced coagulation reaction of the lysate, culminating in formation of a gel clot. However, (a) the possible lack of specificity due to 1–3 β -D glucan and (b) the batch-to-batch variation in the sensitivity of commercial lysate to LPS, due to seasonal and geographical differences in the starting material⁹ has prompted our laboratory to employ recombinant DNA technology to genetically-engineer Factor C as an alternative source of novel “limulus lysate” for endotoxin detection.

cDNAs encoding Factor C have been cloned^{1,10,15}. There are six potential glycosylation sites in the amino acid sequence of the Factor C from *Carcinoscorpius rotundicauda* (CrFC)^{10,15}. Cloned cDNA encoding CrFC has been expressed in *E. coli*¹¹ and also in yeast expression systems^{12,24}. The rFC obtained from yeast was found to be immunoreactive and capable of binding LPS, although only limited amounts of rFC produced in yeast were soluble^{13,14}. Also, it was found that LPS could not activate the enzymatic activity of yeast rFC, thus, a direct enzyme-based LPS detection is not possible using rFC produced in yeast¹⁴.

Since the early 1970s, the diagnostic potential of LAL for endotoxemia has been recognized to be extremely important for timely and effective treatment. Thus, many studies have been conducted to improve the sensitivity of endotoxin assays by changing the formulation of the LAL and assay methodology²⁵. Since blood (plasma) components interfere with the test, various methods to remove inhibition and/or

gelation clot method had been criticized as being subjective, semiquantitative and prone to variations in interpretations by different workers. Thus, advent of an improved fluorimetric assay for using LAL in LPS detection of plasma would be desirable. Even more desirable would be the compatibility of this assay for both LAL and a recombinant Factor C (rFC) in their sensitivity to LPS. Furthermore, this demonstrates the possible diagnostic utility of rFC for endotoxemia.

SUMMARY OF THE INVENTION

The present inventors believed that expression in insect cells rather than in a prokaryotic or simple eukaryotic expression system is suitable for producing rFC with full biological activity. Furthermore, horseshoe crabs and insects belong to the same phylum, Arthropoda, and so insect cells might more closely resemble the cells of the horseshoe crab than yeast cells in their physiology and biochemistry. Thus, rFC produced in insect cells might more closely resemble the protein as purified from the horseshoe crab and retain the bioactivity of having a serine protease activity activated by LPS.

The present invention relates to genetic engineering of a bioactive rFC, which unequivocally exhibits full biological functionality. It is capable of specifically recognizing and binding LPS and lipid A in both free and immobilized forms. Interference from 1–3 β -D-glucan, which switches on the alternate pathway in the coagulation cascade in conventional LAL, is not anticipated in assays of the present invention that use only Factor C as the LPS-binding, serine protease enzyme. Both the LPS-activated enzymatic assays of rFC and the enzyme linked immunosorbent assay (ELISA) lipid A binding assay could be formulated into a rapid high throughput mass screening test for LPS. Thus, a novel generation of “limulus amoebocyte lysate” has been invented, being capable of rapid and sensitive diagnosis and removal of subpicogram levels of endotoxin. The invention provides a standardized and convenient source of enzyme-based diagnostic reagent for detection of the ubiquitously contaminating endotoxin in pharmaceutical products. This inexhaustible supply of genetically-engineered Factor C can be easily standardized to circumvent batch-to-batch variations in sensitivity to LPS, a problem faced by the conventional LAL industry. Furthermore, the ability of the rFC of the invention to protect mice from endotoxemia, as well as its bacteriostatic activity, adds to its value in in vivo applications. Furthermore, the availability of rFC obviates the need for routine harvesting of the horseshoe crab for procurement of their amoebocyte lysate, and therefore, conserves this endangered “living fossil”.

The present inventors have succeeded in expressing biologically active rFC using recombinant baculoviruses and other vectors appropriate for expression of heterologous DNA in insect host cells. The rFC obtained is enzymatically active. Thus, expression of rFC in insect cells is a convenient and economical source of rFC protein for use in rapid, sensitive, specific and quantitative determination of LPS in pharmaceutical products and other biological fluids.

Thus, the present invention comprises purified rFC that is enzymatically active. The phrase “enzymatically active” means that the Factor C protein has the biological activity of binding LPS or lipid A, being activated as to its serine protease activity upon LPS or lipid A binding. Enzymatically active rFC will induce coagulation of an amoebocyte lysate and will also cleave synthetic substrates such as, but not limited to, Boc-Val-Pro-Arg-MCA, Mu-Val-Pro-Arg-

The present invention is also embodied in a method for producing substantially purified, enzymatically active rFC. The method comprises expressing DNA encoding a Factor C protein having the enzymatic activity described above in a culture of insect cells, then isolating the enzymatically active Factor C protein. The isolation preferably includes an ultrafiltration step. The purification preferably also includes a step of gel-filtration chromatography on a matrix having an exclusion limit of 100 kilodaltons. The gel filtration is preferably applied after the ultrafiltration. The exclusion limit of the gel filtration matrix can vary substantially; an effective matrix will provide at least about a 4-fold increase in the serine protease activity of an ultrafiltered crude preparation as measured by the fluorometric assay described herein.

The present invention also encompasses host-vector systems for expressing enzymatically active rFC. The host cells in these embodiments of the invention are insect cells, preferably lepidopteran cells. The vectors in these embodiments support replication of inserted DNA in insect cells and expression of heterologous DNA in insect cells. The vectors are preferably baculovirus or plasmid vectors. The heterologous DNA is sufficient to encode a Factor C enzyme of a horseshoe crab, preferably of the genus *Carcinoscorpius*, *Tachyplesus* or *Limulus*. The recombinant Factor C will preferably at least have the LPS binding activity of Factor C and more preferably will have both LPS binding activity and serine protease activity. Preferred heterologous DNA is a polynucleotide having the sequence shown in SEQ ID NO:1 or SEQ ID NO:3.

The present invention is also embodied in assays for endotoxin comprising contacting a sample to be assayed for the presence of endotoxin or LPS or Lipid A with enzymatically active rFC according to the invention and measuring the serine protease activity of the rFC. The amount of serine protease activity of the rFC will reflect its activation due to binding of LPS or Lipid A or of another endotoxin known in the art to bind to Factor C of a horseshoe crab. The serine protease activity is conveniently measured by any method known in the art but is preferably measured by a chromogenic or fluorogenic method. In such a method formation of a product from a substrate by cleavage of the substrate by the serine protease activity of the rFC, resulting in a change in color or in fluorescence emission, is measured. Preferred substrates for such a chromogenic or fluorogenic assay are N-t-BOC-Val-Pro-Arg-MCA, Mu-Val-Pro-Arg-AFC and Boc-Val-Pro-Arg-pNA.

Additional embodiments of the invention include immunologic methods for assaying the presence of Lipid A or LPS or endotoxin in a sample. These methods of the invention rely upon binding of antibody that specifically binds to Factor C and subsequent detection or quantitation of the amount of the Factor C-antibody complex. In a preferred embodiment, the sample to be assayed is contacted with immobilized antibody that specifically binds to Lipid A or LPS or endotoxin as the ligand to form immobilized ligand. The immobilized ligand is then contacted with rFC according to the present invention to form immobilized rFC. Then the immobilized rFC is contacted with a second antibody that specifically binds the rFC. Finally, the presence or preferably the amount of the rFC-second antibody complex is determined. This determination can be performed by any method typical in the art such as a third antibody that binds the second antibody, perhaps through its Fc portion, or the like. In an alternate embodiment of this aspect of the invention, the second antibody is omitted and the enzymatic

In another embodiment of the invention, the specific binding of LPS or lipid A to rFC is employed in a BIA-CORE™ assay (Pharmacia Biotech). By immobilizing the rFC on the substrate plate of the BIA-CORE™ apparatus, the presence of LPS or lipid A in a sample can be detected. Optimization of the amount of the rFC to be immobilized for a given load of LPS in a sample is considered within the skill of the ordinary practitioner. The BIA-CORE™ apparatus is operated in accord with the manufacturer's instructions.

Also, the present invention is embodied in methods for removal of endotoxin from a sample, wherein immobilized rFC is contacted with the sample, under conditions such that endotoxin in the sample binds the immobilized rFC, then the bound endotoxin is separated from the sample.

BRIEF DESCRIPTION OF DRAWINGS

FIGS. 1A–B: Cloning of CrFC21 cDNA into the baculovirus expression vector, pFastBac I™ (SEQ ID NO:7). Two constructions of the same plasmid were done. (1A) A 2.3 kb Hind III fragment from pGEM11Zf(+)/CrFC21¹⁵ was cloned into HindIII linearized pBluescript™ SK(+) (pBSSK) to give pBSSK/CrFC21/HH. This was then digested with Eco RI and ligated with the 2.3 kb Eco RI fragment from pGEM11Zf(+)/CrFC21 to regenerate recombinant pBSSK/CrFC21. Separately, a 1.3 kb Xho I/Hind III fragment, derived from pGEM11Zf(+)/CrFC21, was cloned into XhoI/HindIII digested pFastBac I™ to give pFastBac/CrFC21XH. The final full-length construct, pFastBac/CrFC21 was generated when the 2.9 kb Pst I CrFC fragment from pBSSK/CrFC21 was ligated to Pst I linearized pFastBac/CrFC21/XH.

(1B) The pFastBac I™ vector and an intermediate CrFC subclone of pGEM11Zf(+)/CrFCEN were digested with Eco RI and Hind III. The liberated CrFCEN insert was ligated directionally into the linearized pFastBac I™ vector to yield pFastBac/CrFCEN. pFastBac/CrFCEN was then digested with Eco RI, as was another vector comprising DNA encoding Factor C, pGEM11Zf(+)/CrFCEE. The insert released from pGEM11Zf(+)/CrFCEE was ligated into the linearized pFastBac/CrFCEN. The clone having the proper orientation was selected by restriction analysis and designated pFastBac/CrFC21'.

FIGS. 2A–2D: Immunoblot analysis of Sf9 rFC. (2A) Reducing SDS PAGE of 5 µg total protein of cell lysate and culture supernatant harvested at 24 and 48 h post-induction (p.i.). (2B) Comparison of reducing and non-reducing SDS PAGE analyses of rFC from 72 h p.i. The results indicate that rFC is probably a double-chain form of Factor C as further proven by the LPS-treated (lane 2) and -untreated (lane 1) rFC under reducing (2C) and non-reducing (2D) conditions. Western Blots (FIGS. 2C and 2D) were developed using a horseradish peroxidase system.

FIG. 2: ELISA lipid A-binding assay of rFC. A gradation of increasing intensity of color development of the enzymatically-hydrolyzed product is seen from 0.01 to 200 ng lipid A. Rows 1 & 2 contain 10 µg total protein per well of culture supernatant from pFastBac/CrFC21 infected Sf9 cells after 72 h p.i. Rows 3 & 4 are controls containing 10 µg total protein per well of culture supernatant from wild-type ACMNPV-infected Sf9 cells after 72 h p.i.

FIGS. 3A–3B: ELISA lipid A-binding assay of rFC. (3A), The histogram illustrates a quantification of the lipid A based on the absorbance at OD_{405nm} of 72 h, culture supernatant (10 µg) and cell lysate (20 µg) after their reaction with 0.01 to 200 ng of lipid A. The results were normalized with wild

consistently showed higher efficacy of binding lipid A even though a lower total amount of protein (10 μ g) was used. (3B) Blocking of excess sites with 0.2% BSA effectively removed the non-specific background binding and results, in higher net absorbance readings.

FIG. 4: Biological Activity of BIOMAXTM purified rFC. The rFC was enriched by BIOMAXTM-50 ultrafiltration and this protein sample was reacted with a range of absolute amounts of LPS (0.01–10 000 pg). Both 40 and 80 μ g amounts of rFC showed enzymatic activity with 0.01 pg LPS.

FIG. 5: Fluorimetric and calorimetric assays of LPS using purified rFC. In both the fluorimetric and calorimetric assays, further purification of BIOMAXTM-purified rFC by SEPHADEXTM G-100 dramatically improved the sensitivity of rFC to LPS. The conventional tube method of fluorimetric assay was compared with the microcolorimetric assay for both the BIOMAXTM sample (B) and SEPHADEXTM G-100 purified sample (S). Amounts of rFC used were 40 μ g and 100 μ g for the fluorimetric and calorimetric assays, respectively.

FIG. 6: Comparison of tube and plate fluorimetric assays. A comparison of the fluorescence readings was made between the conventional tube method and the microtiter plate method using 40 μ g of the SEPHADEXTM G-100 purified rFC.

FIG. 7: Colorimetric assay for LPS-induced Factor C enzyme activity of rFC. A comparison is made of the amounts of culture supernatant proteins (60 and 100 μ g) containing rFC, and the concentrations (2 and 4 mM) of the calorimetric substrate, Boc-Val-Pro-Arg-pNA. It is observed that as low as 0.01 pg endotoxin was optimally detected with 100 μ g culture supernatant at 2 mM pNA substrate.

FIG. 8: Colorimetric assay of LPS using purified rFC. Similar to the fluorimetric assay, the calorimetric test also showed that the SEPHADEXTM G-100 purified rFC exhibited improved sensitivity to LPS, where 40 μ g of purified rFC (instead of 100 μ g of BIOMAXTM-purified rFC) was sufficient to detect subpicogram levels of LPS.

FIG. 9: Binding of rFC to lipid A assayed by BIACORETM bioassay. Binding between rFC produced from pFastBac/CrFC21 and immobilized lipid A (*E. coli* D31m4) was assayed using the BIACORETM X biosensor (Pharmacia Biotech). BIOMAXTM and SEPHADEXTM G-100 purified supernatants of cultures of Sf9 cells infected with wild-type baculovirus did not show any background binding to the immobilized lipid A (plateau 1A). On the other hand, the rFC from the culture supernatant of Sf9 cells infected with pFastBac/CrFC21 specifically bound the immobilized lipid A with a net activity of 553 Response Units (plateau 2A). The protein samples, each at 1 mg/ml, were injected at 10 μ l/min for 3 minutes over the ligand monolayer that was previously immobilized on a HPA chip¹⁴.

FIG. 10: rFC from Baculovirus binds LPS and lipid A from several Gram negative bacterial species. LPS from three different species of bacteria, *E. coli*, *K. pneumoniae*, and *S. minnesota*, and lipid A from *S. minnesota*, were separated by electrophoresis and electroblotted onto an ImmobilonTM PVDF membrane. Each LPS/lipid A strip was incubated with rFC from pFastBac/CrFC21 (lane 1) or control culture supernatant from wild-type AcMNPV-infected Sf9 cells (lane 2). The results show that rFC binds LPS/lipid A from different species of Gram negative bacteria.

FIG. 11: Microtiter plate-immobilized rFC for detection

protein containing rFC derived from baculoviral system (rFC Sf9) immobilized on a 96-well microtiter plate was capable of specifically recognizing and binding subpicogram levels of FITC-conjugated LPS. The efficacy of binding/detection of a range of LPS by various amounts of rFC protein immobilized onto the microtiter plate is shown.

FIG. 12: Microtiter plate-immobilized rFC from various yeasts binds to LPS. Immobilized rFC derived from yeast (*P. pastoris*: rFC#8 {pHILD2/CrFC21} and rFCEE {pHILD2/CrFC21EE}; *S. cerevisiae*: YFC/6a {YepSec1/CrFC26 Δ 6a} and P21/26 {pEMBLyex4/CrFC21/26}). Native Factor C in *Carcinoscorpius amoeboocyte* lysate, LAL (50 μ g protein) were used as positive controls. There is consistency in the efficiency of recognition of LPS-FITC and its binding to the immobilized rFC. The negative controls were w/tSf9 (wild-type Sf9 cells infected with AcMNPV DNA alone) and rFCSN (rFC derived from a control yeast recombinant clone devoid of the LPS binding domain).

FIG. 13: Construction of pAc5/VtgCrFCES-V5-His. The full-length CrFC 21 cDNA was released by a Bgl II and Not I double digest. The released fragment was subcloned into the Bam HI and Not I sites of VtgEGFP. Consequently, the Bgl II/Bam HI site is destroyed. Then the LPS-binding domain of CrFC21 was released by digestion with Eco47 III and Sal I (cutting an internal site). The fragment was subcloned into the Eco RV and Xho I sites of pAc5/V5-HisA to give pAc5/VtgCrFCES-V5-His. Both Eco47 III/Eco RV and Sal I/Xho I sites are destroyed. FIG. 14: Map of pAc5/VtgCrFCES-V5-His. FIG. 14A shows the amino acid and nucleotide sequences at the junction of the vitellogenin signal sequence (Vtgss) and the CrFCES cDNA. FIG. 14B shows the complete map of the vector. The Vtgss portion of the construct is described in detail in co-pending application Ser. No. 60/106,426. The nucleotide junctions were determined by sequencing using the Ac5 forward primer and pcDNA3.1/BGH reverse primer. The CrFCES is cloned in-frame with respect to Vtgss (at the 5' end) and V5-His (at the 3' end). The secreted VtgCrFCES protein was also purified via affinity chromatography (TalonTM, Clontech) under denaturing conditions (6M urea, 250 mM NaCl and 20 mM sodium phosphate buffer, pH7.0). The protein was transferred to a PVDF membrane in transfer buffer (10% methanol, 10 mM CAPS, pH 11.0) and its N-terminal amino acid sequence determined. Using the Vtgss secretory signal, only a single cleavage point was identified (indicated by the arrow). Thus, Vtgss allows the homogenous production of secreted heterologous recombinant protein.

FIGS. 15A–15B: Distribution of secreted VtgCrFCES protein.

15A—culture medium

Lane M: BenchmarkTM prestained marker
Lane 1: control medium (30 μ g)
Lane 2: VtgCrFCES medium (not purified 30 μ g)
Lane 3: VtgCrFCES (affinity purified; 1 μ g)
Lane 4: VtgCrFCES (ISOpriome purified; 1 μ g)

Left panel, Coomassie Blue-stained gels; right panel, Western blotting using INDIA-His-HRP.

15B—cell lysate;

Lane M: Bio-rad prestain marker
Lane 1: control cell lysate (30 μ g)
Lane 2: VtgCrFCES cell lysate (transient; 30 μ g)
Lane 3: VtgCrFCES cell lysate (stable; 30 μ g)

Left panel, Coomassie Blue-stained gels; right panel, Western blotting using INDIA-His-HRP.

The VtgCrFCES (hereinafter “VtgCrFCES”) protein was

SuperSignal HisProbe™ Western Blotting Kit (Pierce). The secreted VtgCrFCES was purified to homogeneity by isoelectric focusing (ISOPrime™, Hoeffer) resulting in a single protein band having a molecular weight identical to that of the protein isolated by affinity column chromatography. The ISOPrime™-purified protein was not denatured even when purified in pyrogen-free water. No VtgCrFCES was detected in the cell lysate. The purification process was made easy and more effective by the presence of VtgCrFCES in the culture medium.

FIG. 16: Lipid A binding properties of ISOPrime™-purified VtgCrFCES. Immobilization of lipid A to the HPA sensor chip has been described earlier (14).

FIG. 17: FIG. 17 shows a plot of the fluorescence units (FU) of the product of the Factor C enzymatic reaction after 10 µg of LAL or rFC was pre-activated by increasing amounts of LPS. The microfluorimetric detection of a range of concentrations of LPS by LAL in comparison to rFC from different production batches (Nos. 1, 2, 3 and 4) is shown.

FIGS. 18A–18B: FIGS. 18A and 18B: Use of rFC (18A) and LAL (18B) in microfluorimetric detection of LPS in spiked human cord blood. Cord blood was apyrogenically obtained in either EDTA (E) or citrate (C) buffer as anticoagulants. Replicates of each plasma sample were spiked with 0.125 or 0.25 EU/ml of LPS prior to their use in the microfluorimetric assay. The background shows unspiked plasma assayed in the absence of substrate. This is to test for the background level of fluorescence that may be given off by the endogenous components of the plasma. The 0 LPS was used as a negative control to compare with the LPS-inducibility of Factor C in the rFC when tested with the other two LPS-spiked plasma.

FIGS. 19A–19B: Use of rFC (19A) and LAL (19B) for detection of LPS in 2-day old citrated human plasma. A visibly good correlation can be seen in the sensitivity of detection by rFC and LAL.

FIGS. 20A and 20B: Use of rFC (20A) and LAL (20B) for detection of LPS in 7-day old citrated human plasma. A visibly good correlation can be seen again in the sensitivity of detection by rFC and LAL. Aging of the plasma samples at 4° C. did not appear to affect the microfluorimetric assay for LPS.

DETAILED DESCRIPTION OF THE INVENTION

The present application relates to rFC of the horseshoe crab.

A preferred horseshoe crab that can serve as a source of DNA or mRNA for producing the rFC of the invention is *Carcinoscorpius rotundicauda* (CrFC). The present invention relates especially to expression of rFC by means of baculovirus host-vector systems. The present application also relates to a fluorometric assay for endotoxin that makes use of the rFC expressed by recombinant DNA methods.

cDNAs encoding Factor C proteins from *Carcinoscorpius rotundicauda* have been previously described^{10,15}. rFC from *Carcinoscorpius rotundicauda* (rCrFC) has been produced in vitro by coupled transcription/translation systems^{10,15}. However, the present invention resides partly in the development of in vivo systems, especially using insect cells as the host cell, for efficient production of rFC by expression of cloned DNA.

Also, the protection of rFC from activation and subsequent self-proteolysis by binding of endotoxin which may be present in solutions used in isolation of the protein is

(Me₂SO, DMSO) is added to solutions which are used during the purification process. Even greater protection of the rFactor C is achieved by also adding an agent effective for chelating divalent metal ions to the purification solutions.

cDNAs appropriate for expression in the presently-described system can be cDNAs encoding Factor C of any horseshoe crab. Two representative nucleotide sequences are presented as SEQ ID NO:1 and SEQ ID NO:3 (encoding the amino acid sequences of SEQ ID NOS:2 and 4). A composite DNA sequence, assembled from incomplete cDNA fragments, encoding the Factor C of *Tachyplesus tridentatus* is disclosed by Muta et al.¹

For use in the LPS binding assays and LPS removal according to the invention, the Factor C can be produced by any method typical in the art, but is preferably made in a eukaryotic host cell. Production of rFC in yeast host-vector systems is described in reference 16. As it has been the Inventors' recent experience that Factor C produced in yeast lacked serine protease activity, rFC for use in enzymatic activity-based assays is preferably produced by a baculovirus host-vector system.

"Stringent conditions" for hybridization are those that provide for hybridization of sequences having less than 15% mismatch, preferably less than 10% mismatch, most preferably 0% to 5% mismatch. Exemplary of such conditions, using probes of 50 bases or longer, are an aqueous solution of 0.9 M NaCl at 65° C.; an aqueous solution of 0.98 M NaCl, 20% formamide at 42–45° C. The conditions will vary according to the length of the probe, its G+C content and other variables as known to the skilled practitioner¹¹. Exemplary wash conditions following hybridization are an aqueous solution of 0.9 M NaCl at 45–65° C., preferably 55–65° C. Lower salt, or addition of an organic solvent such as formamide, in the wash buffer will increase the stringency of the condition as known in the art.

A preferred hybridization condition is at 42° C. in 50% formamide, 5×SSC, 1×Denhardt's solution, 20 mM phosphate buffer, pH 6.5, 50 µg/ml calf thymus DNA, 0.1% SDS. Salt and temperature conditions equivalent to the hybridization conditions employed can be calculated from the following equation¹⁸:

$$T_m = 81.5^\circ \text{C.} - 16.6(\log_{10}[\text{Na}^+]) + 0.41(\% \text{G+C}) - 0.63(\% \text{formamide}) - (600/l),$$

where l = the length of the hybrid in base pairs.

A preferred washing condition is in 1×SSC, 0.1% SDS washing solution at room temperature, followed by washing at high stringency with 0.1×SSC, 0.1% SDS at 42° C. and 2× with 0.1× SSC/0.1% SDS for 15 min. each at 42° C.

EXAMPLE 1

Recombinant Constructs of CrFC cDNA in a Baculovirus Expression Vector Plasmids and Sf9 Cell Culture

Sf9 insect cells were maintained as a monolayer culture in serum-free SF 900 II SFM medium supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin (Life Technologies, Inc.) in a humidified incubator (Forma, USA) at 27° C. The plasmid pFastBac I™ and the competent DH10Bac *E. coli* were from Life Technologies, Inc., USA. Construction of pFastBac/CrFC21, Transposition Into *E. coli* and Transfection Into Sf9 Insect Cells.

The strategy for cloning CrFC21 into the pFastBac I™

in FIG. 1. The recombinant plasmids were verified by restriction enzyme digestion. The 5' cloning sites were further confirmed by dideoxynucleotide sequencing using the forward primer designed from the -44 position of the polyhedrin promoter region, before they were used for transfection in insect cells. PCR and Southern analyses of the pFastBac/CrFC21 DNA confirmed the authenticity of recombinant baculoviruses.

The CrFC21 cDNA¹⁰ from pGEM11Zf+/CrFC21¹¹ was recloned in two steps into pBluescript II SK+ (pBSSK), to yield pBSSK/CrFC 21. Further manipulations using pBSSK/CrFC21 and the baculoviral expression vector, pFastBac I™ were carried out using standard methods to clone full-length CrFC21, thus, yielding the recombinant construct, pFastBac/CrFC21 (FIG. 1). pFastBac/CrFC21 was transformed into competent *E. coli*, DH10Bac, and cultured in LB agar containing 50 µg/ml kanamycin, 7 µg/ml gentamycin, 10 µg/ml tetracycline, 30 µl of 2% X-gal and 40 µg/ml of IPTG. Screening¹⁹ for positive clones involved the use of the 2.3 kb ³²P-CrFC21/EE fragment as probe¹⁰. The recombinant bacmid DNA was isolated and transfected into Sf9 cells.

EXAMPLE 2

Expression of rFC in Insect Host Cells

Rapid Microtiter-plate Plaque Assay

Early log phase recombinant Sf9 cells were seeded at 6.5×10^4 cells per well. The culture was incubated in a sealed bag at 27° C. for 1 h. Meanwhile, the virus stock was serially diluted 10-fold with SFM containing 10% FBS to give final dilutions of 10^{-2} to 10^{-4} . The BacPak™ Baculovirus Rapid Titer Kit (Invitrogen) was used for plaque assay. It is an immunoassay which uses a primary monoclonal antibody raised to an AcMNPV envelope glycoprotein (gp64). A secondary goat anti-mouse HRP-conjugated antibody enables visualization of the infected cells as blue-stained viral plaques or foci seen under the light microscope. The virus titer (pfu/ml) was calculated based on the following formula:

(Average no. of foci per well $\times$ dilution factor $\times 40$) $\times 2$ where 40 represents the inoculum volume normalization factor.

Scale-up of Infection of Sf9 Cells for Production of rFC

The culture supernatant from the 6-well plates was harvested and the viral stock was amplified by re-infection of Sf9 cells grown in 25 cm² flasks, using a multiplicity of infection (MOI) of 0.1–1.0. In such cultures, the viral stock reached a titer of 2×10^7 pfu/ml. Aliquots of this viral stock were re-inoculated at a MOI of 5–10 into Sf9 cells grown in 15 ml SFM medium in 75 cm² flasks. The volume of the viral inoculum was determined using the formula:

$$\frac{(\text{total no. of cells}) \times (\text{MOI in pfu/cell})}{(\text{viral titer in pfu/ml})}$$

Subsequently, Sf9 cells were passaged twice and conditioned to grow in suspension in 100 ml SFM medium, in spinner flasks (Bellco, USA). At the mid log phase of growth, the viral stock from the 75 cm² flask cells was inoculated at a MOI of 5–10. In the same manner, the cell culture volume was scaled up further in increasingly larger spinner flasks of 250, 500 and 1000 ml, infected with proportionally increasing volumes of viral stock at the same

Preparation of Protein Samples From Recombinant Baculovirus-infected Sf9 Cells.

(a) Cell lysate: Sf9 cells infected with the recombinant baculovirus at a MOI of 5–10 were harvested at 24, 48 and 72 h p.i. The cells were washed 3 times with pyrogen-free PBS and centrifuged at 3000 $\times$ g for 10 min at 4° C. during each cycle of washing. The cell pellet was resuspended in 2–3 volumes of PBS and subjected to 5 cycles of freeze-thawing at -80° C. and 37° C., respectively. The cell debris was removed by centrifuging at 14000 $\times$ g for 10 min at 4° C. The supernatant containing the soluble protein fraction was stored at -20° C. This supernatant represents the cell lysate.

(b) Culture supernatant: At the respective times of harvest, the cell medium was collected and centrifuged at 3000 $\times$ g for 10 min at 4° C. to remove any cells or cell debris. The medium was then concentrated 10-fold by centrifugation through a BIOMAX™-50 kDa cutoff ultrafree membrane (Millipore) at 2000 $\times$ g for 20 min or more. The total proteins present in the cell lysate and culture supernatant were quantified by Bradford assay²⁰. Partial purification of rFC was carried out at 4° C. by gel filtration chromatography through SEPHADEX™ G-100 (e.g. 1.5 $\times$ 90 cm), using 0.05 M Tris-HCl (pH 7.5) containing 0.154 M NaCl. Fractions of 1 ml were collected and the void volume peak was concentrated. The protein concentration and Factor C enzyme activity were assayed for the resulting rFC. This preparation is henceforth referred to as "gel filtration-purified rFC".

Western Immunoblot Detection of rFC

Five µg of each cell lysate, or culture supernatant, harvested from 24, 48 and 72 h p.i. was analyzed on 10% SDS-PAGE gels, under denaturing conditions²¹. The electrophoretically-resolved bands were then transferred onto Immobilon™ PVDF membrane (Millipore, USA). The membrane was washed in PBS for 30 min, and blocked in 1% skimmed milk-PBS for 1 h followed by overnight incubation with rabbit anti-Factor C antibody diluted 1:500 in 0.2% Tween-20-PBS containing 1% BSA. Horseradish peroxidase-conjugated secondary goat anti-rabbit antibody, diluted 1:10000, was subsequently incubated with the membrane. For visualization of protein bands, the membrane was treated with SUPERSIGNAL™ chemiluminescent substrate (Pierce, USA) for 5–10 min, followed by 3 min exposure of the membrane to an X-ray film.

The Western analysis revealed 3 bands of immunoreactive rFC proteins of 132, 88 and 44 kDa, expressed by pFastBac/CrFC21 recombinant baculoviruses at 24, 48 and 72 h post infection, pi. At 24 h p.i., rFC was observed in the culture supernatant, but not in the cell lysate. The 48 h and 72 h p.i. culture supernatant showed increasing amounts of rFC. The rFC in the cell lysate started to appear as a faint 132 kDa band only at 48 h p.i., and reached a substantial level at 72 h p.i. The immunoblot thus showed that the bulk of the rFC produced was released from the infected Sf9 cells into the medium. This is probably due to lysis of the infected cells, which released the recombinant protein. On ultracentrifugation at 100,000 $\times$ g for 1 h at 4° C., rFC was found to be in the soluble fraction.

The results show that rFC protein was expressed correctly under the direction of the viral late promoter from the polyhedrin gene using the native translation start site from the CrFC cDNA. As there are six potential glycosylation sites in the CrFC cDNA sequence¹⁰; the protein band of 132 kDa represents the intact glycosylated form of Factor C. The 88 and 44 kDa proteins are likely the activated products of rFC whose molecular sizes correspond closely to the heavy and light chains, respectively, of double-chain Factor C³. Autoactivation could have occurred in the presence of picogram levels of ubiquitous endotoxin during the prepa-

of rFC from 72 h p.i., electrophoresed under reducing and non-reducing conditions of SDS-PAGE, the 88 and 44 kDa bands became more prominent under reducing conditions. Under non-reducing conditions, LPS-activated rFC still retained its 132 kDa band, thus indicating the double-chain form of rFC³. The presence of a double chain form, of rFC was further proven when the BIOMAXTM-purified rFC was pre-incubated with LPS before Western blotting. Under reducing condition, the LPS-treated rFC showed activated products of 88 and 44 kDa which were absent in the untreated rFC sample. However, under non-reducing conditions, the 132 kDa band was intact for both the LPS-treated and -untreated rFC.

EXAMPLE 3

RFC Binds Lipid A, the Biologically-potent Component of LPS

ELISA to Determine Lipid A Binding by rFC

In order to visualize and test the ability of rFC to specifically bind the biologically potent component of LPS, diphenylphosphoryl lipid A (*E. coli* K12, D31M4, List Biologicals, Inc., USA) ranging from 0.01 to 100 ng in 100 μ l volumes was immobilized onto 96-well Nunc IMMUNO-PLATESTM (PolySorp). The immobilization was carried out overnight at room temperature. Unbound lipid A was removed and the plates were washed 6 times with wash buffer containing 0.01% Tween 20 and 0.01% thimerosal in PBS. The excess sites were blocked for 1 h at room temperature with the same buffer containing 0.2% BSA, after which the wells were again washed 6 times. Aliquots of 100 μ l of BIOMAXTM 50-treated rFC from culture supernatant containing 20 μ g total protein was then added to the wells and incubated overnight at room temperature. Unbound rFC was removed, and the wells were washed 6 times in wash buffer. This was followed by addition of aliquots of 100 μ l of 1:500 diluted rabbit anti-Factor C antibody and incubation was continued for 2 h at 37° C. Subsequently, the wells were washed 6 times in the wash buffer before addition of 100 μ l aliquots of 1:2000 diluted goat anti-rabbit antibody conjugated with horseradish peroxidase. After washing the wells 6 times, 1 mg/ml of substrate ABTS, 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)] diammonium salt (Boehringer Mannheim) was added in 200 μ l aliquots and incubated at room temperature for 15 min. The formation of a green product was quantified by reading its absorbance at 405 nm. rFC binding the immobilized lipid A results in positive color formation via the ELISA test.

The ELISA test for lipid A-binding indicates that, rFC is capable of specifically recognizing and binding to immobilized lipid A and hence, it could be used in the detection of endotoxin. With increasing amounts of lipid A in the wells, there is an increasing intensity of color development in the ABTS product (FIG. 2). Compared to the cell lysate, the 72 h p.i. culture supernatant consistently yielded more efficacious rFC for detection of lipid A (FIG. 3A). Furthermore, it was observed that blocking of excess sites in the wells with 0.2% BSA removed non-specific background binding and drastically improved the specificity of lipid A binding (FIG. 3B). This assay indicates that rFC can be used for mass screening of pharmaceutical products for LPS contamination, with the capability of quantifying LPS. This efficacy is comparable to the commercially available natural lysate derived from the *Limulus* or *Tachypleus* amoebocyte lysate.

EXAMPLE 4

Immobilized rFC Can be Used to Detect/remove LPS in a Sample

Two hundred μ l samples containing either control wild-

or partially-purified rFC samples (obtained by BIOMAXTM-ultrafiltration), diluted in PBS to 10, 25 or 50 μ g total protein per 200 μ l were coated/immobilized onto each of the wells of a 96-well microtiter plate (NUNC, USA). The plates were left overnight at 4° C. Unbound protein was removed from the wells, and 200 μ l of 0.2% BSA (depyrogenized by ultrafiltration) dissolved in PBS was added to the wells for 1 h at 37° C., to block unoccupied sites. The wells were washed 3 times with PBS. This was followed by addition of 200 μ l FITC-conjugated LPS (*E. coli* 055:5B, List Biological Labs, USA) to the wells. The plate was incubated at 37° C. for 1 h, after which each well was washed 6x with PBS. The fluorescence was read at EX_{495nm} and Em_{525nm} using LS-50B Spectrofluorimeter (Perkin Elmer).

Wells coated with 10, 25 or 50 μ g of partially purified proteins containing rFC showed increasing efficiency of binding LPS-FITC. Blocking of the wells with 0.2% BSA reduced the background fluorescence reading, indicating improvement in the specificity of binding of LPS to the immobilized rFC. Immobilization of negative control proteins (w/tSf9: wild-type Sf9 cell culture supernatant of Sf9 cells infected with AcNMPV DNA alone, and rFC SN: yeast rFC derived from the truncated recombinant rFC devoid of LPS-binding domain described in ref. 16, to the wells did not capture or bind LPS, thus indicating the specificity of recognition of LPS by the immobilized rFC.

EXAMPLE 5

The Baculoviral rFC is Enzymatically Activated by LPS

Fluorimetric and Colorimetric Assays for LPS-activated rFC Enzyme Activity

As a proenzyme, Factor C becomes catalytically activated by trace levels of LPS. Thus, conversion of its enzymatic substrate to product indicates the presence of LPS. rFC samples present in the crude cell lysate and culture supernatant were used for analysis of LPS-activated Factor C enzyme activity by using two different substrates. The first, in a conventional tube assay format, is based on a modification of the fluorimetric assay of Iwanaga et al.²² Using rFC obtained from a 72 h p.i. culture supernatant, 10 μ g total protein in a volume of 0.1 ml was mixed with 1.9 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1 M NaCl and 0.05 M CaCl₂. The mixtures were preincubated with 0.01 to 100 μ g of LPS (*E. coli* 055:B5, Sigma) at 37° C. for 1 h before addition of 15 μ l of 2 mM fluorimetric substrate, Boc-Val-Pro-Arg-MCA (Sigma). Incubation was continued for 30 min and the reaction was terminated with 0.1 ml glacial acetic acid. The product AMC was read in Fluorescence Units (FU) at EX_{380nm} (slit 10 nm) and Em_{460nm} (slit 5 nm) using a Perkin Elmer Luminescence Spectrophotometer (LS-50B). For multiple samples, this assay was routinely scaled down to 96-well microtiter plate assay. Briefly, the microassay involved 1 h pre-incubation of LPS with rFC in a volume of 100 μ l, followed by addition of 1.5 μ l of 2 mM fluorimetric substrate and 100 μ l of 100 mM Tris-HCl, pH 8.0, containing 0.2 M NaCl and 0.05 M CaCl₂ and further incubation for 30 min at 37° C. before termination of the reaction with 10 μ l of glacial acetic acid. The fluorescence was read in a 96-well microtiter plate reader module.

The second enzymatic assay for LPS involved a modification of the calorimetric test²³ where preincubation of culture supernatant proteins with LPS ranging from 0.01 to 10 pg was carried out at 37° C. for 1 h. The reaction volume was scaled down to 200 μ l in 0.1 M Tris-HCl (pH 8.0)

50 μ l of 2 mM of a calorimetric substrate, Boc-Val-Pro-Arg-p-nitroanilide (Seikagaku, Japan). Incubation at 37° C. was resumed for 1 h before termination of the reaction with 28 μ l of glacial acetic acid. This substrate is hydrolyzed by rFC to produce pNA that was measured calorimetrically at OD_{405nm}.

From 24 to 48 to 72 h p.i., there was progressively increasing trend in the enzymatic activity of rFC in supernatants of cultures of insect cells transformed with the construct of Example 1, as indicated by the increase in fluorimetric units of the AMC product hydrolyzed from Boc-Val-Pro-Arg-MCA substrate. A comparison of the amount of total proteins present in the cell lysate (Lysate: 50 μ g) and culture supernatant (Sup: 5 μ g) illustrates that the culture supernatant from 72 h p.i. contained rFC that is >5–10 fold more effective in LPS detection. Twenty μ g of BIOMAX™-purified rFC was able to detect 0.01 ng LPS. Using 40 to 80 μ g of this protein, the detection limit could be easily extended to LPS levels below 0.01 pg or 0.001 ng/ml (FIG. 4). Purification of rFC by chromatography through SEPHADEX™ G-100 yielded enzymatic activity of even higher sensitivity to LPS (FIG. 5). It is envisaged that more elaborate purification of rFC following the methods covered in reference 24 would vastly improve the efficacy of the rFC for endotoxin detection. Furthermore, when the fluorimetric assay was modified to ~200 μ l, using a 96-well microtiter plate, the sensitivity to LPS was improved by 10-fold (FIG. 6). This was directly attributable to the removal of background fluorescence by gel filtration.

Furthermore, the LPS-activated rFC enzyme assay was also conveniently quantifiable by a calorimetric assay with the Boc-Val-Pro-Arg-pNA substrate. The sensitivity to LPS was 0.1 pg (0.01 ng/ml) with 100 μ g of BIOMAX™-50-treated culture supernatant when 2 mM of the pNA substrate was employed (FIG. 7). Similar to the fluorimetric assay, the calorimetric test also showed that the SEPHADEX™

G-100-purified rFC exhibited improved sensitivity to LPS, where 40 μ g of purified rFC (instead of 100 μ g of BIOMAX™ rFC) was sufficient to detect subpicogram levels of LPS (FIG. 8). Use of gel filtration-purified rFC resulted in a 4-fold increase in sensitivity to LPS. A direct comparison of the 2 microassays revealed that with gel filtration-purified rFC, the calorimetric assay achieved sensitivity to LPS comparable to the fluorimetric assay. Thus, using the scaled down, yet improved sensitivity assay for LPS detection, high throughput screening of samples can be conveniently achieved by either the calorimetric or fluorimetric assay using the 96-well microtiter plate assays. This enables rapid and mass screening of samples with limited volumes.

EXAMPLE 6

Fusion of Vtgss to LPS-binding Domain of CrFC Gene (CrFCES) for Expression and Secretion of Recombinant ES Protein From Drosophila Cells

The *O. aureus* vitellogenin gene secretory signal (Vtgss) was fused upstream to the EcoRI-Sall cDNA fragment encoding the LPS-binding domain of *Carcinoscorpius rotundicauda* Factor C CrFCES (10). The construct was inserted into the pAc5/V5-His vector backbone (Invitrogen, see FIG. 13).

Vitellogenin is the yolk protein of the oviparous and ovoviviparous animals. It is exclusively synthesized in the liver, secreted and finally deposited in the eggs. The secretion of vitellogenin is directed by its secretory signal. The

amino acids) is challenging because it is rich in cysteine residues and requires the correct formation of 9 disulfide bonds for full biological activity. The recombinant protein (VtgssCrFCES) is synthesized using the Drosophila Expression System™. Stable recombinant Drosophila cell lines were obtained and the distribution of the protein was identified by Western-blotting using very sensitive chemiluminescent detection. The recombinant protein was also purified to homogeneity for N-terminal amino acid sequencing.

Precise cleavage between Vtgss and the adjoining mature fusion CrFCES protein was demonstrated by N-terminal amino acid sequencing of VtgCrFCES. The data show that Vtgss is cleaved at a single point (FIG. 14A). The absence of multiple cleavages suggests that Vtgss directs homogeneous production of secreted heterologous protein from Drosophila cells.

Results of the Western Blot show that practically all of VtgCrFCES is secreted into the culture medium (FIGS. 15A, 15B). No intracellular VtgCrFCES was detected using the very sensitive Chemiluminescent Western Blot. The fusion of Vtgss to the LPS-binding domain resulted in its secretion whereas the native signal of Factor C targets it intracellularly. The presence of VtgCrFCES in the culture medium allows simple purification from either batch or continuous culture.

The LPS-binding properties of the secreted protein are retained as shown by plasmon resonance studies, using the BIACORE™ in vivo biosensor apparatus (FIG. 16). The protein secreted from the Drosophila cells binds LPS despite addition of 6 Vtgss-derived amino acids to the N-terminus of the CrFCES protein. Thus, Drosophila cells can be used to express and secrete correctly folded CrFCES. Functional Vtgss truncated after the aspartic acid residue that is the carboxy-terminus of the cleavage site can be used to reduce the number of extra amino terminal residues.

EXAMPLE 7

Evaluation of Microfluorimetric Detection of LPS in Aqueous Solution and Human Plasma by rFC and LAL

The microfluorimetric assay for LPS-activated Factor C of Example 5 produces a 10-fold improvement in the sensitivity of detection of LPS. In the present example, the same assay was employed for LPS detection in human plasma by conventional Limulus amoebocyte lysate (LAL), and the efficacy was compared with that of rFC. Briefly, LPS was pre-incubated with LAL or rFC in a volume of 100 μ l, followed by addition of 1.5 μ l of 2 mM fluorimetric substrate (Boc-Val-Pro-Arg-MCA, Sigma) and 100 μ l of 10.0 mM Tris-HCl, pH 8.0, containing 0.2 M NaCl and 0.05 M CaCl₂, then further incubated for 30 min at 37° C. before termination of the reaction with 10 μ l of glacial acetic acid. The fluorescence of the released product, AMC, was read in a 96-well microtiter plate reader module using a Perkin Elmer Luminescence Spectrophotometer (LS-50B) at EX_{380nm} and Em_{460nm}.

The results, presented in FIGS. 17–20 and Table 1 show that the new improved protocol for microfluorimetric assay of LPS using rFC or LAL can be used as a sensitive method of detection of LPS in, for example, aqueous solutions and human plasma.

FIG. 17 shows a plot of the fluorescence units (FU) of the product of the Factor C enzymatic reaction after 10 μ g of LAL or rFC was pre-activated by increasing amounts of

trations of LPS by LAL in comparison to rFC from different production batches (Nos. 1, 2, 3 and 4) is shown.

FIGS. 18A and 18B show a comparison between (i) effective of EDTA-containing plasma (E) over citrated plasma (C) in LPS detection by (ii) rFC and by conventional LAL. In these tests, plasma derived from cord blood was spiked with LPS, and the resulting LPS-spiked plasma samples were, used in the microfluorimetric assay employing Factor C from either rFC or LAL. The results show that first, citrate is a recommended anticoagulant to use for deriving plasma for the purpose of LPS-detection by either rFC or LAL. Second, there is consistency in the level of sensitivity of LPS-detection by rFC and LAL.

FIGS. 19A and 19B show a comparison between detection of LPS by 10 μ g of rFC (15A) and 10 μ g of LAL (15B) using 2-day old plasma samples derived by citrate treatment. There is close visual correlation observed between samples measured using rFC and LAL.

FIGS. 20A and 20B show another comparison between detection of LPS by 10 μ g of rFC (16A) and 10 μ g of LAL (16B) using 7-day old plasma samples derived by citrate treatment. Again, close correlation can be seen between LPS detection in samples using rFC and LAL.

Table 1 shows examples of the use of the microfluorimetric assay for LPS detection by LAL over a one year period. Aging of LAL preparation shows a progressive increase in the blank (0 EU/ml LPS) values of FU, suggesting slight autoactivation of the LAL over time. There is a corresponding increase in most cases in the FU at 0.125 EU/ml LPS. With the exception of the November 13 case, there was a 33% loss of sensitivity of LPS-induced increase in FU over time.

TABLE 1

CASE EXAMPLES WHERE NEW MICROFLUORIMETRIC ASSAY WAS APPLIED TO LAL SAMPLES

Date	LAL (amount in μ g)	LPS (0 EU/ml)	LPS (0.125 EU/ml)	Fold
Jan. 14, 1998	10	16.3	208.0	12.8
Feb. 25, 1998	10	16.1	299.8	18.6
Aug. 3, 1998	10	54.9	562.7	10.2
Nov. 13, 1998	10	73.1	261.8	3.6
Nov. 18, 1998	10	84.2	748.9	8.9
Dec. 22, 1998	10	69.7	591.4	8.5

The invention being thus described, modification of the invention with respect to various materials and methods will be apparent to one of ordinary skill in the art. Such modifications are to be considered as falling within the scope of the invention, which is defined by the claims hereinbelow.

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SEQUENCE LISTING

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Asn	Ser	Gly	Gly	Ala	Val	His	Val	Val	Asn	Asn	Gly	Pro	Tyr	Ser	Asp	
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Ile	Gly	Leu	His	Arg	Leu	Asp	Ala	Glu	Lys	Pro	Phe	Ile	Trp	Glu	Leu	
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Gly Arg Ser Asp Ser Pro Arg Ser Pro Phe Ile Trp Asn Gly Asn Ser		
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Thr Glu Ile Gly Gln Trp Pro Trp Gln Ala Gly Ile Ser Arg Trp Leu		
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Ala Asp His Asn Met Trp Phe Leu Gln Cys Gly Gly Ser Leu Leu Asn		
	850	855 860
Glu Lys Trp Ile Val Thr Ala Ala His Cys Val Thr Tyr Ser Ala Thr		
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Ala Glu Ile Ile Asp Pro Asn Gln Phe Lys Met Tyr Leu Gly Lys Tyr		
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Tyr Arg Asp Asp Ser Arg Asp Asp Asp Tyr Val Gln Val Arg Glu Ala		
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Leu Glu Ile His Val Asn Pro Asn Tyr Asp Pro Gly Asn Leu Asn Phe		
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Asp Ile Ala Leu Ile Gln Leu Lys Thr Pro Val Thr Leu Thr Thr Arg		
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Val Gln Pro Ile Cys Leu Pro Thr Asp Ile Thr Thr Arg Glu His Leu		
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Lys Glu Gly Thr Leu Ala Val Val Thr Gly Trp Gly Leu Asn Glu Asn		
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Asn Thr Tyr Ser Glu Thr Ile Gln Gln Ala Val Leu Pro Val Val Ala		
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Ala Ser Thr Cys Glu Glu Gly Tyr Lys Glu Ala Asp Leu Pro Leu Thr		
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Val Thr Glu Asn Met Phe Cys Ala Gly Tyr Lys Lys Gly Arg Tyr Asp		
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Ala Cys Ser Gly Asp Ser Gly Gly Pro Leu Val Phe Ala Asp Asp Ser		
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Arg Thr Glu Arg Arg Trp Val Leu Glu Gly Ile Val Ser Trp Gly Ser		
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 <223> OTHER INFORMATION: any n or Xaa = Unknown

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Val Asp Leu Gly Leu Cys Asp Glu Thr Arg Phe Glu Cys Lys Cys Gly	
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Asp Pro Gly Tyr Val Phe Asn Ile Pro Val Lys Gln Cys Thr Tyr Phe	
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Tyr Arg Trp Arg Pro Tyr Cys Lys Pro Cys Asp Asp Leu Glu Ala Lys	
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Asp Ile Cys Pro Lys Tyr Lys Arg Cys Gln Glu Cys Lys Ala Gly Leu	
80 85 90	
gat agt tgt gtt act tgt cca cct aac aaa tat ggt act tgg tgt agc	338
Asp Ser Cys Val Thr Cys Pro Pro Asn Lys Tyr Gly Thr Trp Cys Ser	
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Gly Glu Cys Gln Cys Lys Asn Gly Gly Ile Cys Asp Gln Arg Thr Gly	
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Ala Cys Ala Cys Arg Asp Arg Tyr Glu Gly Val His Cys Glu Ile Leu	
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Lys Gly Cys Pro Leu Leu Pro Ser Asp Ser Gln Val Gln Glu Val Arg	
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Asn Pro Pro Asp Asn Pro Gln Thr Ile Asp Tyr Ser Cys Ser Pro Gly	
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Phe Lys Leu Lys Gly Met Ala Arg Ile Ser Cys Leu Pro Asn Gly Gln	
175 180 185	
tgg agt aac ttt cca ccc aaa tgt att cga gaa tgt gcc atg gtt tca	626
Trp Ser Asn Phe Pro Pro Lys Cys Ile Arg Glu Cys Ala Met Val Ser	
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Ser Pro Glu His Gly Lys Val Asn Ala Leu Ser Gly Asp Met Ile Glu	
205 210 215	
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Gly Ala Thr Leu Arg Phe Ser Cys Asp Ser Pro Tyr Tyr Leu Ile Gly	
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Gln Glu Thr Leu Thr Cys Gln Gly Asn Gly Gln Trp Asn Gly Gln Ile	
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Pro Gln Cys Lys Asn Leu Val Phe Cys Pro Asp Leu Asp Pro Val Asn	
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cat gct gaa cac aag gtt aaa att ggt gtg gaa caa aaa tat ggt cag	866
His Ala Glu His Lys Val Lys Ile Gly Val Glu Gln Lys Tyr Gly Gln	
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Phe Pro Gln Gly Thr Glu Val Thr Tyr Thr Cys Ser Gly Asn Tyr Phe	
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Leu Met Gly Phe Asp Thr Leu Lys Cys Asn Pro Asp Gly Ser Trp Ser	
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Ile His Cys Pro Ala Gly Cys Ser Leu Thr Ala Gly Thr Val Trp Gly	
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aca gcc ata tac cat gaa ctt tcc tca gtg tgt cgt gca gcc atc cat	1154
Thr Ala Ile Tyr His Glu Leu Ser Ser Val Cys Arg Ala Ala Ile His	
365 370 375	
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Ala Gly Lys Leu Pro Asn Ser Gly Gly Ala Val His Val Val Asn Asn	
380 385 390 395	
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Gly Pro Tyr Ser Asp Phe Leu Gly Ser Asp Leu Asn Gly Ile Lys Ser	
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gaa gag ttg aag tct ctt gcc cgg agt ttc cga ttc gat tat gtc cgt	1298
Glu Glu Leu Lys Ser Leu Ala Arg Ser Phe Arg Phe Asp Tyr Val Arg	
415 420 425	
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Ser Ser Thr Ala Gly Lys Ser Gly Cys Pro Asp Gly Trp Phe Glu Val	
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gac gag aac tgt gtg tac gtt aca tca aaa cag aga gcc tgg gaa aga	1394
Asp Glu Asn Cys Val Tyr Val Thr Ser Lys Gln Arg Ala Trp Glu Arg	
445 450 455	
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Phe Ile Trp Glu Leu Met Asp Arg Ser Asn Val Val Leu Asn Asp Asn	
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Tyr Met Asp Ile Gln Asp Gln Leu Gln Ser Val Trp Lys Thr Lys Ser	
540 545 550 555	
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Cys Phe Gln Pro Ser Ser Phe Ala Cys Met Met Asp Leu Ser Asp Arg	
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Asn Lys Ala Lys Cys Asp Asp Pro Gly Ser Leu Glu Asn Gly His Ala	
575 580 585	
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Thr Leu His Gly Gln Ser Ile Asp Gly Phe Tyr Ala Gly Ser Ser Ile	
590 595 600	
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Arg Tyr Ser Cys Glu Val Leu His Tyr Leu Ser Gly Thr Glu Thr Val	
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aca aga gaa cac ttg aag gag gga aca tta gca gtg gtg aca ggt tgg Thr Arg Glu His Leu Lys Glu Gly Thr Leu Ala Val Val Thr Gly Trp 895 900 905			2738
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gtc	agc	tg	ggc	agt	ccc	agt	gga	tgt	ggc	aag	gcg	aac	cag	tac	ggg	3026	
Val	Ser	Trp	Gly	Ser	Pro	Ser	Gly	Cys	Gly	Lys	Ala	Asn	Gln	Tyr	Gly		
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ggc	ttc	act	aaa	gtt	aac	gtt	ttc	ctg	tca	tg	att	agg	cag	ttc	att	3074	
Gly	Phe	Thr	Lys	Val	Asn	Val	Phe	Leu	Ser	Trp	Ile	Arg	Gln	Phe	Ile		
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 <220> FEATURE:
 <223> OTHER INFORMATION: any n or Xaa = Unknown

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Tyr	Lys	Arg	Cys	Gln	Glu	Cys	Lys	Ala	Gly	Leu	Asp	Ser	Cys	Val	Thr		
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Pro	Lys	Cys	Ile	Arg	Glu	Cys	Ala	Met	Val	Ser	Ser	Pro	Glu	His	Gly		
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Leu	Val	Phe	Cys 260	Pro	Asp	Leu	Asp	Pro 265	Val	Asn	His	Ala	Glu	His	Lys
Val	Lys	Ile	Gly 275	Val	Glu	Gln	Lys 280	Tyr	Gly	Gln	Phe	Pro 285	Gln	Gly	Thr
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Thr 305	Leu	Lys	Cys	Asn 310	Pro	Asp	Gly	Ser	Trp	Ser 315	Gly	Ser	Gln	Pro	Ser 320
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Asp	Phe	Leu	Asp 340	Asp	Val	Gly	Glu	Pro 345	Val	Arg	Ile	His	Cys 350	Pro	Ala
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Glu 370	Leu	Ser	Ser	Val	Cys 375	Arg	Ala	Ala	Ile	His	Ala 380	Gly	Lys	Leu	Pro
Asn 385	Ser	Gly	Gly	Ala 390	Val	His	Val	Val	Asn	Asn 395	Gly	Pro	Tyr	Ser	Asp 400
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Lys	Ser	Gly 435	Cys	Pro	Asp	Gly 440	Trp	Phe 440	Glu	Val	Asp 445	Glu	Asn	Cys	Val
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Thr 465	Asn	Met	Ala	Ala 470	Arg	Leu	Ala	Val	Leu	Asp 475	Lys	Asp	Val	Ile	Pro 480
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Ser	Phe	Ala	Cys	Met 565	Met	Asp	Leu	Ser	Asp 570	Arg	Asn	Lys	Ala	Lys 575	Cys
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What is claimed is:

1. An assay for endotoxin, said assay comprising:

- a) contacting a sample to be assayed, wherein said sample comprises a peptide cleavable to produce a chromogenic or fluorogenic moiety, with a recombinant Factor C of a horseshoe crab, wherein said recombinant Factor C is obtained from an insect host cell culture and has protease activity; and
- b) measuring the amount of said chromogenic or fluoro-

age of said moiety from said peptide is indicative of the presence of said endotoxin in said sample.

2. The assay of claim 1, wherein said peptide is N-t-Boc-Val-Pro-Arg-MCA, Mu-Val-Pro-Arg-AFC, or Boc-Val-Pro-Arg-pNA and wherein said assay is conducted in a volume of about 200 μ l.

3. The assay of claim 2, wherein said insect host cell is a lepidopteran cell.

4. The assay of claim 2, wherein said recombinant Factor

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5. The assay of claim 2, wherein said recombinant Factor C comprises a polypeptide having 75% or greater sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, and the amino acid sequence of Factor C of *Tachypleus tridentatus*.

6. The assay of claim 1, wherein said insect host cell is a lepidopteran cell.

7. The assay of claim 1, wherein said recombinant Factor C is obtained from a dipteran host cell culture.

8. The assay of claim 1, wherein said recombinant Factor C comprises a polypeptide having 75% or greater sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, and the amino acid sequence of Factor C of *Tachypleus tridentatus*.

9. The assay of claim 1, wherein said peptide is N-t-Boc-Val-Pro-Arg-MCA or Mu-Val-Pro-Arg-AFC.

10. The assay of claim 1, wherein said recombinant Factor C is obtained from a Sf9 host cell culture.

11. An assay for endotoxin, said assay comprising:

- a) contacting a sample to be assayed, said sample having a volume of about 200 μ l and comprising a peptide cleavable to produce a chromogenic or fluorogenic moiety, with a recombinant Factor C of a horseshoe crab, wherein said recombinant Factor C is produced using a vector comprising a cDNA encoding Factor C, is obtained from an insect host cell culture, and has protease activity; and
- b) measuring the amount of said chromogenic or fluorogenic moiety cleaved from said peptide, wherein cleavage of said moiety from said peptide is indicative of the presence of said endotoxin in said sample.

12. The assay of claim 11, wherein said cDNA encoding Factor C comprises a nucleic acid encoding a polypeptide having 75% or greater sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, and the amino acid sequence of Factor C of *Tachypleus tridentatus*.

13. The assay of claim 12, wherein said nucleic acid is a cDNA encoding Factor C of *Limulus*.

14. The assay of claim 13, wherein said recombinant Factor C is obtained using a baculovirus-derived host-vector system.

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15. The assay of claim 12, wherein said nucleic acid encodes the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4, or the amino acid sequence of *Tachypleus tridentatus*.

16. The assay of claim 15, wherein said recombinant Factor C is obtained using a baculovirus-derived host-vector system.

17. The assay of claim 12, wherein said recombinant Factor C is obtained using a baculovirus-derived host-vector system.

18. The assay of claim 11, wherein said recombinant Factor C is obtained using a baculovirus-derived host-vector system.

19. The assay of claim 11, wherein said insect cell is a dipteran cell.

20. The assay of claim 1, wherein said recombinant Factor C is obtained from a lepidopteran host cell culture.

21. An assay for endotoxin, said assay comprising:

- a) mixing a sample to be assayed with a recombinant Factor C of a horseshoe crab, wherein said recombinant Factor C is obtained from an insect host cell culture and has protease activity, and incubating the mixed sample;
- b) adding to the incubated, mixed sample a peptid that is cleavable to produce a chromogenic or fluorogenic moiety and continuing incubation to allow cleavage of the peptide by Factor C activated by endotoxin in said sample; and
- c) measuring the amount of said chromogenic or fluorogenic moiety cleaved from said peptide, wherein cleavage of said moiety from said peptide is indicative of the presence of said endotoxin in said sample.

22. The assay of claim 21, performed in a volume of about 200 μ l.

23. The assay of claim 21, wherein the incubation at step a) is performed for at least 1 hour and wherein the incubation at step b) is performed for at least one-half hour.

24. The assay of claim 21, wherein said recombinant Factor C is produced using a baculovirus based host vector system.

25. The assay of claim 21, wherein said recombinant Factor C is obtained from a lepidopteran host cell culture.

* * * * *