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(12) **United States Patent**  
**Haught et al.**

(10) **Patent No.:** **US 10,267,797 B2**  
(45) **Date of Patent:** **Apr. 23, 2019**

(54) **DETECTION OF ORAL MICROBIAL VIRULENCE FACTORS**

(2013.01); *G01N 2333/705* (2013.01); *G01N 2333/70596* (2013.01); *G01N 2400/50* (2013.01)

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(58) **Field of Classification Search**

CPC ..... *G01N 33/566*  
See application file for complete search history.

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(56) **References Cited**

U.S. PATENT DOCUMENTS

5,175,089 A 12/1992 Fine et al.  
5,738,985 A 4/1998 Miles et al.  
2007/0160544 A1 7/2007 Sreenivasan  
2009/0047240 A1 2/2009 Johnson et al.  
2012/0019735 A1 1/2012 Zuidema et al.  
2017/0119643 A1 5/2017 Haught et al.

(73) Assignee: **The Procter & Gamble Company**, Cincinnati, OH (US)

FOREIGN PATENT DOCUMENTS

CA 2323630 A1 8/1990  
EP 1971865 B1 4/2013  
GB 2481267 B 4/2016  
WO WO1995013094 A1 5/1995  
WO WO2008042279 A2 4/2008  
WO WO2011091366 A2 7/2011  
WO WO2014098868 A1 6/2014

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(21) Appl. No.: **15/939,382**

(22) Filed: **Mar. 29, 2018**

(65) **Prior Publication Data**

US 2018/0231546 A1 Aug. 16, 2018

**Related U.S. Application Data**

(62) Division of application No. 15/148,663, filed on May 6, 2016, now Pat. No. 9,970,934.

(60) Provisional application No. 62/309,110, filed on Mar. 16, 2016, provisional application No. 62/157,659, filed on May 6, 2015.

(51) **Int. Cl.**

*C12Q 1/00* (2006.01)  
*G01N 33/566* (2006.01)  
*G01N 33/50* (2006.01)  
*C12Q 1/6897* (2018.01)  
*G01N 33/579* (2006.01)  
*A61K 8/02* (2006.01)  
*A61K 8/21* (2006.01)  
*A61K 8/36* (2006.01)  
*A61K 8/39* (2006.01)  
*A61K 8/42* (2006.01)  
*A61K 8/49* (2006.01)  
*A61Q 11/00* (2006.01)

(52) **U.S. Cl.**

CPC ..... *G01N 33/566* (2013.01); *A61K 8/0204* (2013.01); *A61K 8/21* (2013.01); *A61K 8/361* (2013.01); *A61K 8/39* (2013.01); *A61K 8/42* (2013.01); *A61K 8/4926* (2013.01); *A61Q 11/00* (2013.01); *C12Q 1/6897* (2013.01); *G01N 33/5041* (2013.01); *G01N 33/5044* (2013.01); *G01N 33/5091* (2013.01); *G01N 33/579* (2013.01); *A61K 2800/74* (2013.01); *C12Q 2600/136* (2013.01); *G01N 2333/195*

OTHER PUBLICATIONS

13837M International Search and Written Opinion for PCT/US2016/031349 dated Jul. 18, 2016.

13838M Search Report and Written Opinion for PCT/US2016/031350 dated Jul. 18, 2016.

Chuang, TH1 et al., "Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 5 and hTLR9", *Eur. Cytokine Netw.* Sep. 2000, 11(3):372-8).

Darveau, R.P. et al., "Porphyromonas gingivalis Lipopolysaccharide Contains Multiple Lipid A species That Functionally Interact with Both Toll-Like Receptors 2 and 4", *Infection and Immunity*, vol. 72, No. 9, Sep. 1, 2004, pp. 5041-5051.

Dongari-Bagtzoglou et al., "Production of inflammatory mediators and cytokines by human gingival fibroblasts following bacterial challenge", *J. Periodont. Res.* 1996; 31:90-98.

Henkel et al. "Toxins from Bacteria", *EXS* (2010) 100:1-30.

(Continued)

*Primary Examiner* — Albert M Navarro

(74) *Attorney, Agent, or Firm* — Parker D. McCrary

(57) **ABSTRACT**

Methods for detecting and quantifying toxins present in the oral cavity. The methods may include providing a biological sample, providing reporter cells expressing one or more Toll like receptors, exposing the cells to the biological sample, measuring the EC50 value of the lipopolysaccharide on activation of a Toll like receptor, quantification of the lipopolysaccharide in the biological sample.

**9 Claims, 31 Drawing Sheets**

**Specification includes a Sequence Listing.**

(56)

**References Cited**

## OTHER PUBLICATIONS

Hong, Sun Woong et al., "Lipoteichoic acid of *Streptococcus mutans* interacts with Toll-like receptor 2 through the lipid moiety for induction of inflammatory mediators in murine macrophages", *Molecular Immunology*, vol. 57, pp. 284-291, 2014.

Huang, Li-Yun et al., "Use of Toll-Like Receptor Assays to Detect and Identify Microbial Contaminants in Biological Products", *Journal of Clinical Microbiology*, Nov. 2009, p. 3427-3434.

Liebers, Verena et al. "Occupational Endotoxin-Exposure and Possible health Effects on Human", *American Journal of Industrial Medicine* 49(6):474-491 (2006).

Madianos, PN et al., "Generation of inflammatory stimuli: how bacteria set up inflammatory responses in the gingiva", *J. Clin. Periodontol.* 2005; 32(supplement 6): 57-71.

Mullarkey, M. et al. "Inhibition of endotoxin response by E5564, a novel toll like receptor 4 directed endotoxin antagonist", *Journal of Pharmacology and Experimental Therapeutics*, American Society for Pharmacology and Experimental Therapeutics, vol. 304, No. 3, Jan. 1, 2003, pp. 1093-1102.

Offenbacher, S et al., "Gingival transcriptome patterns during induction and resolution of experimental gingivitis in humans", *J. Periodontol.* Dec. 2009; 80(12): 1963-82.

Raetz et al. "Lipopolysaccharide endotoxin", *Annu. Rev. Biochem* (2002) 71:635-700.

Rock, FL et al., "A family of human receptors structurally related to *Drosophila* Toll", *Proc. National Academy Science*, Jan. 20, 1998, 95:588-93.

Shapira, L. et al., "Effect of Amine- and Stannous Fluoride on Human Neutrophil Functions in vitro", *J. Dent. Res.* 73 (7): 1381-1386, Jul. 1997.

Sulamain, Ali et al. "Corresponding Author: Bacterial Endotoxin Released by Different Types of Mouthwash", *World Applied Sciences Journal*, Jan. 1, 2012, pp. 305-309.

Takeuchi, et al., "A novel member of an expanding toll-like receptor family", *Gene*, Apr. 29, 1999, 231(1-2): pp. 59-65.

Wood, Stewart J. et al., "Anti-Endotoxin Agents. 1. Development of a Fluorescent Probe Displacement Method Optimized for High-Throughput Identification of Lipopolysaccharide-Binding Agents", May 1, 2004, retrieved from Internet on Jun. 23, 2016: URL:<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1540690/pdf/nihms2661.pdf>.

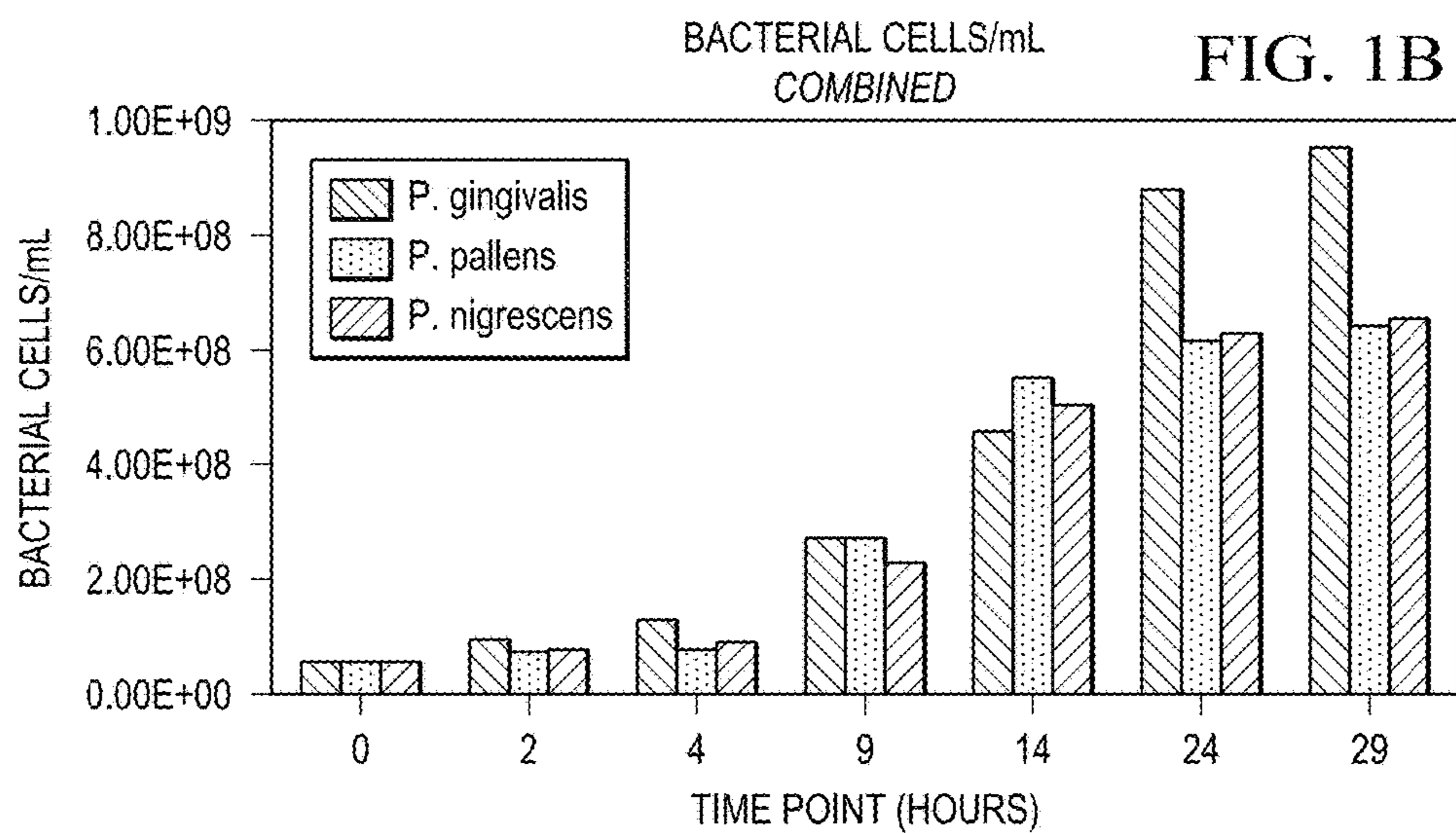
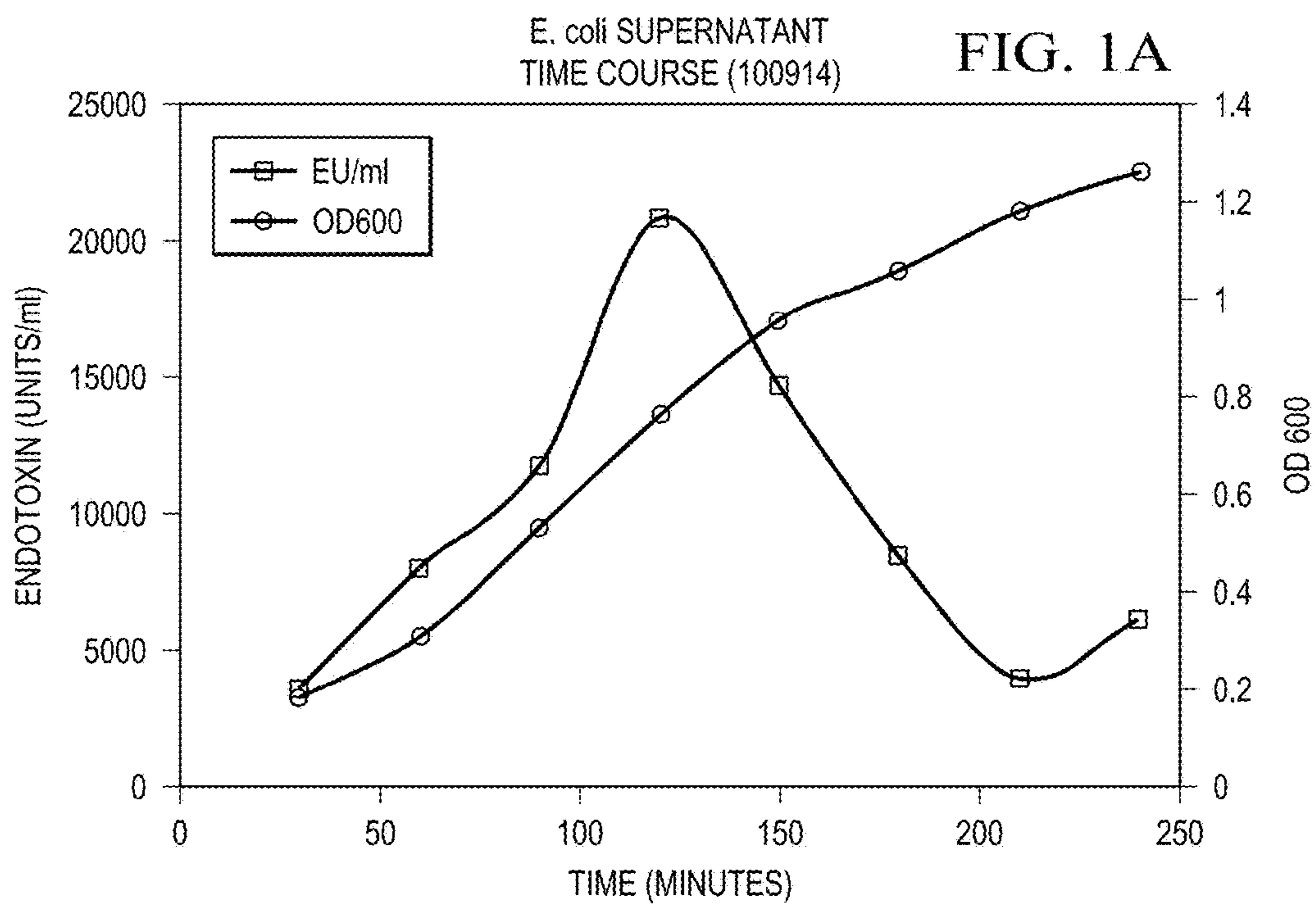
Yokoyama, Yuji et al., "Systemic Immune Response to Streptococcal and Staphylococcal Lipoteichoic Acids in Children with Recurrent Tonsillitis", *Acta Otolaryngol (Stockh)* 1996; Suppl 523: 108-111.

Zhang, Hongwei et al., "Lipoprotein Release by Bacteria: Potential Factor in Bacterial Pathogenesis", *Infection and Immunity*, Nov. 1998, vol. 66, No. 11, pp. 5196-5201.

All Office Actions from U.S. Appl. No. 15/148,663, filed May 6, 2016—See Private Pair.

All Office Actions from U.S. Appl. No. 15/149,044, filed May 6, 2016—See Private Pair.





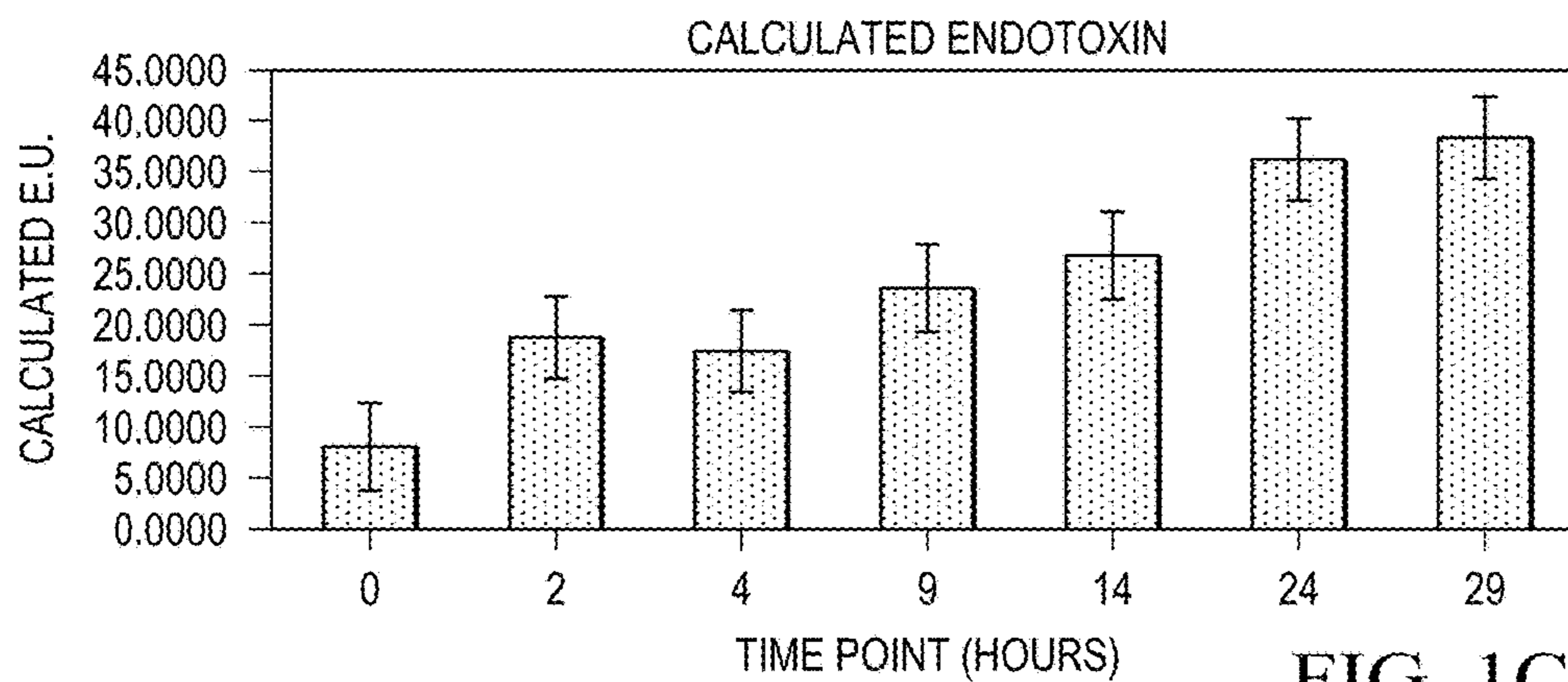


FIG. 1C

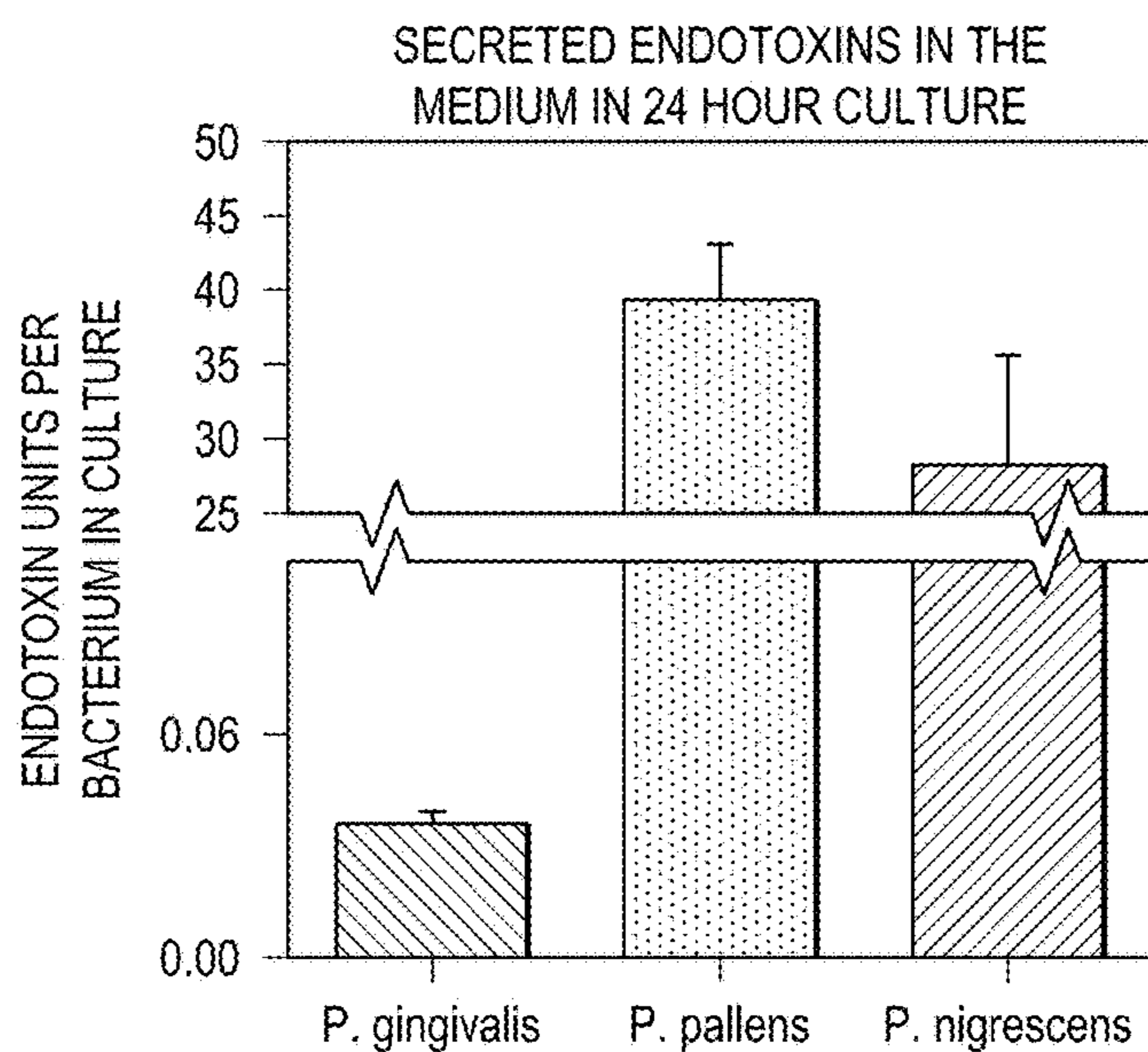


FIG. 1D

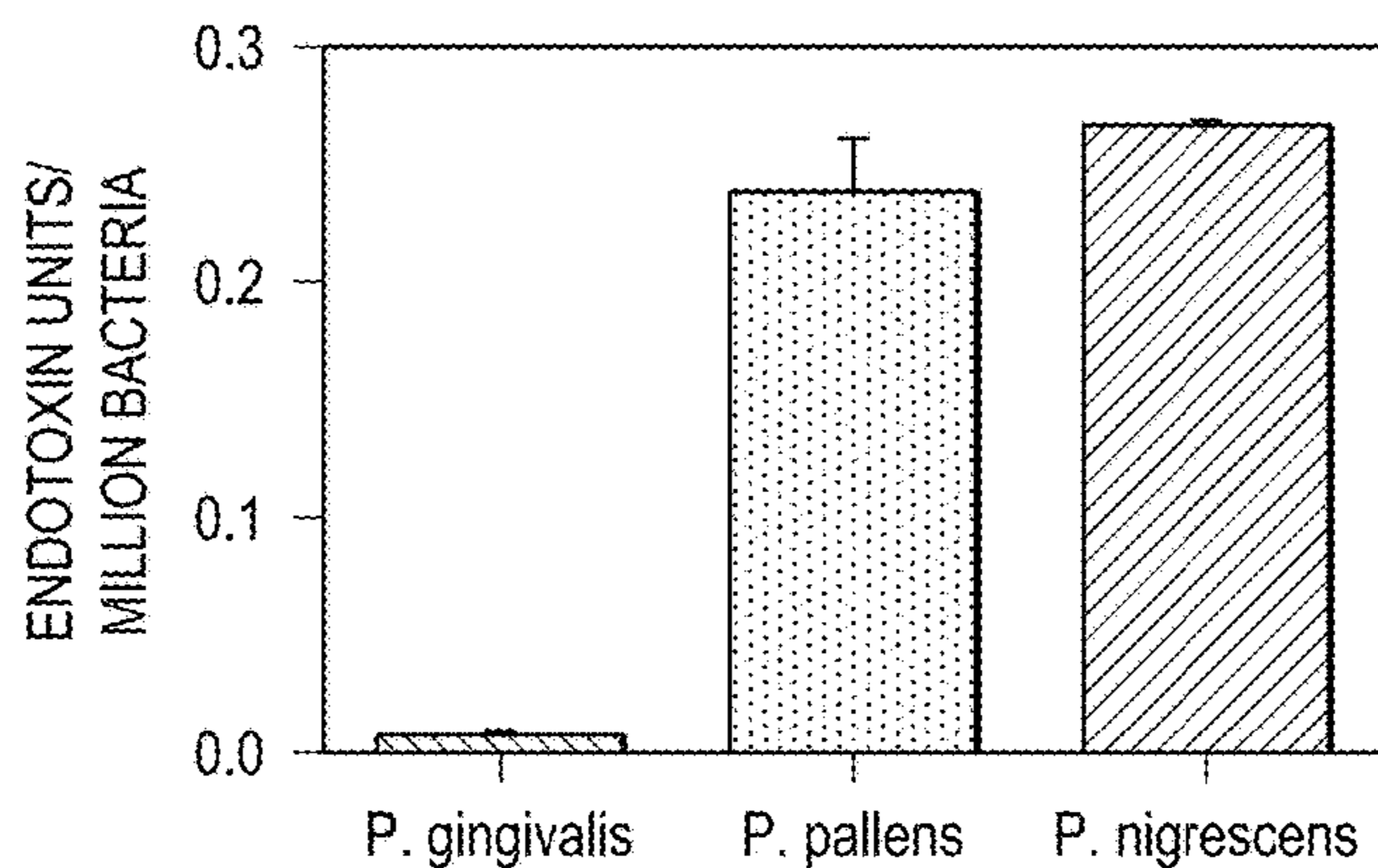


FIG. 1E

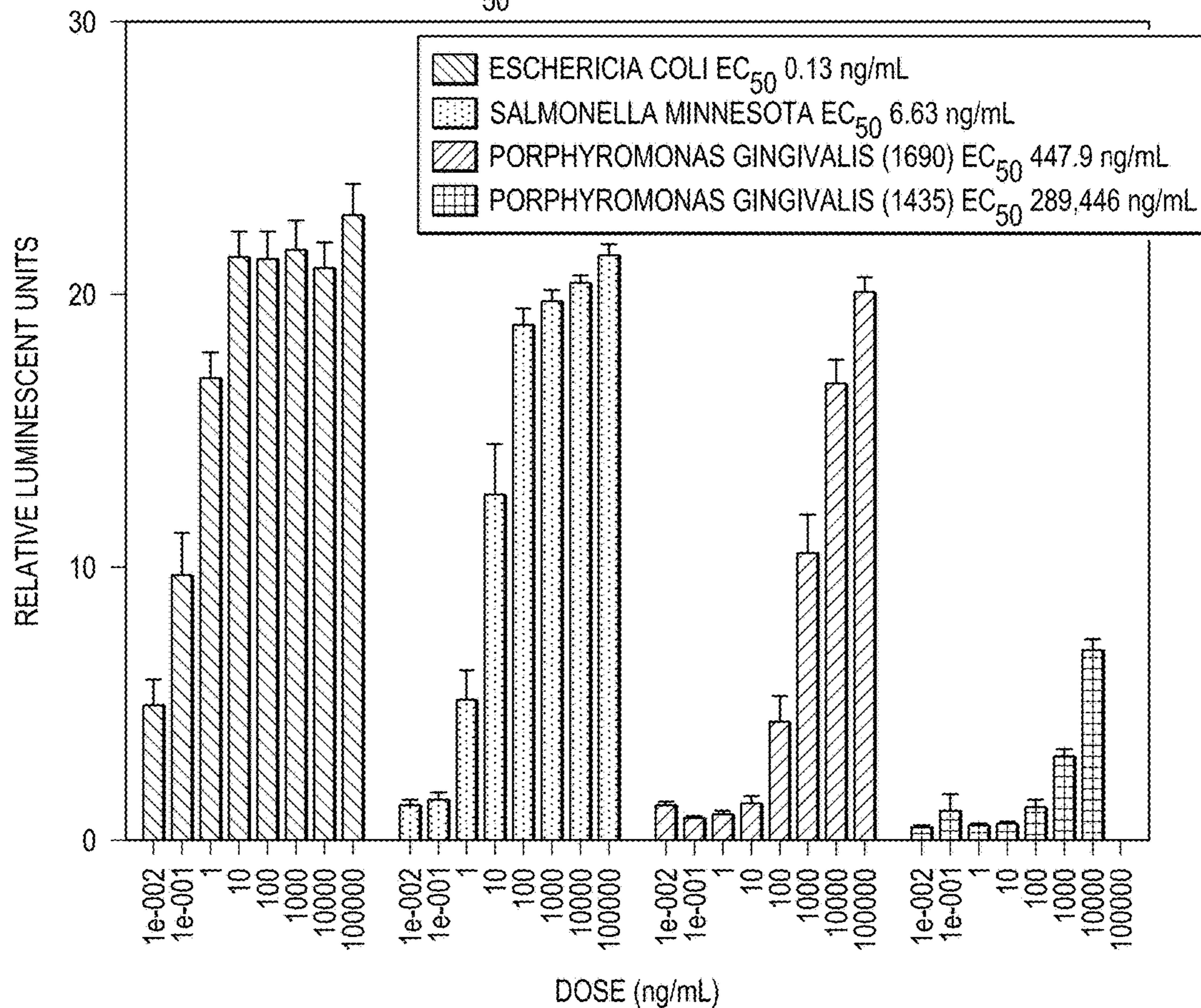


FIG. 1F

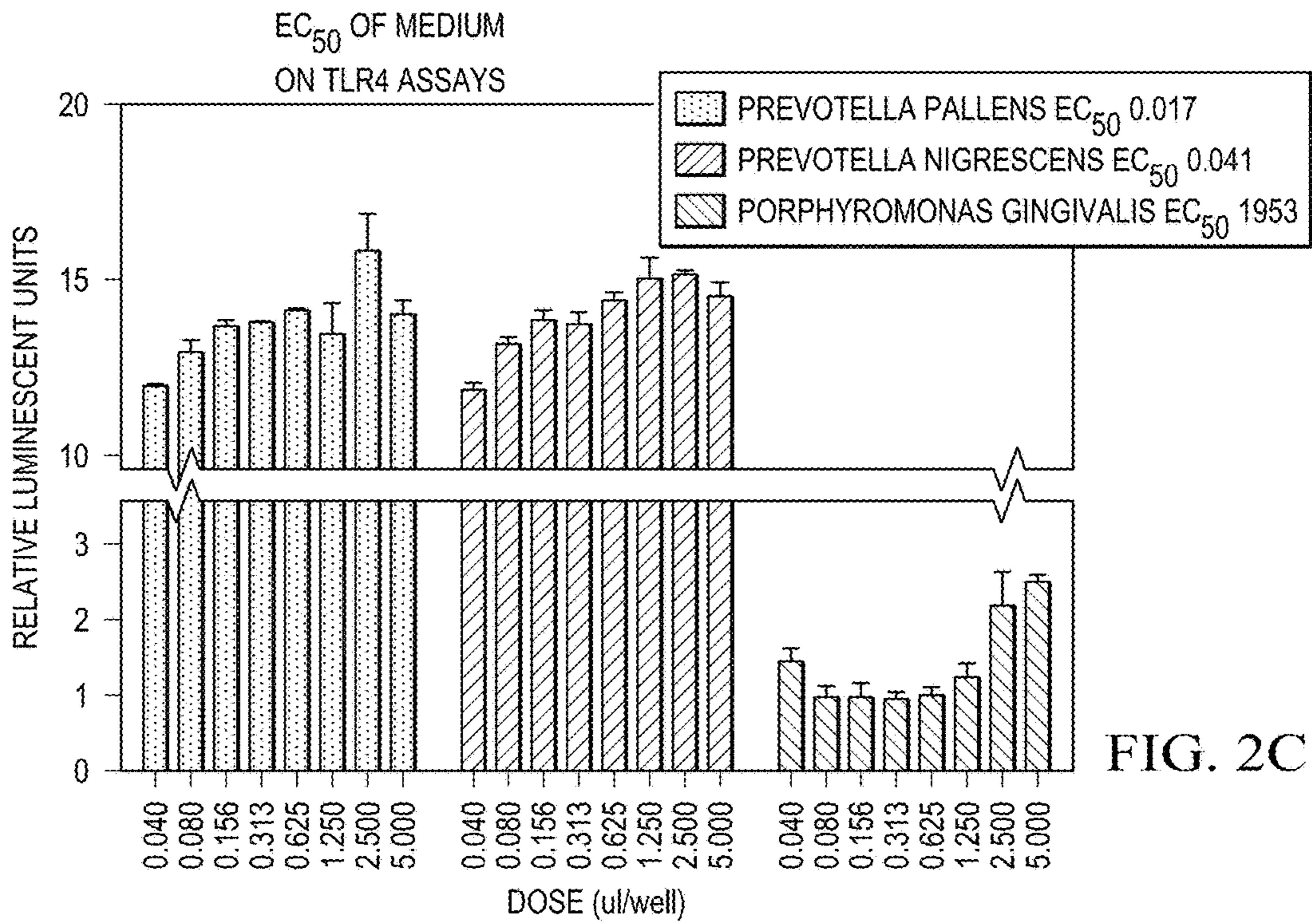
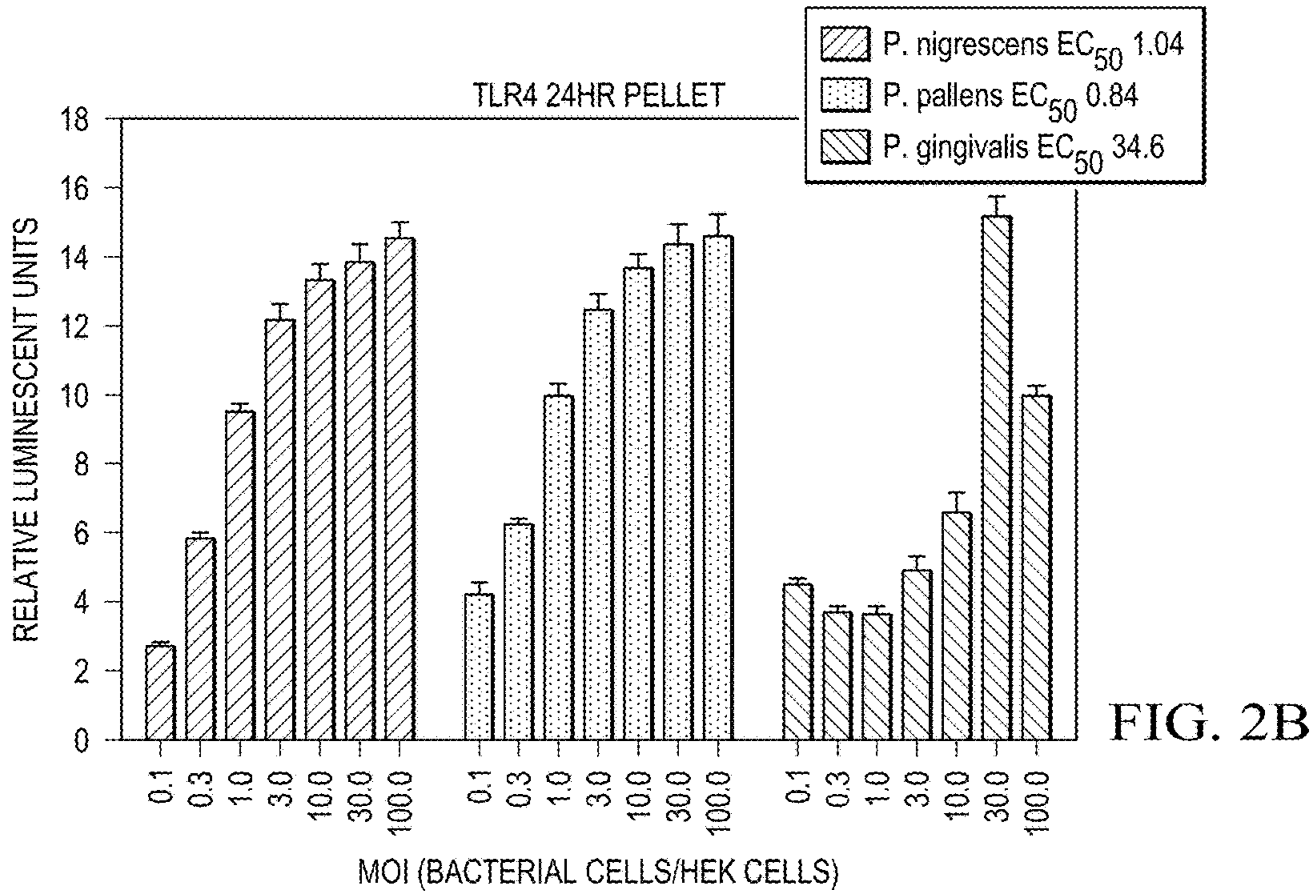


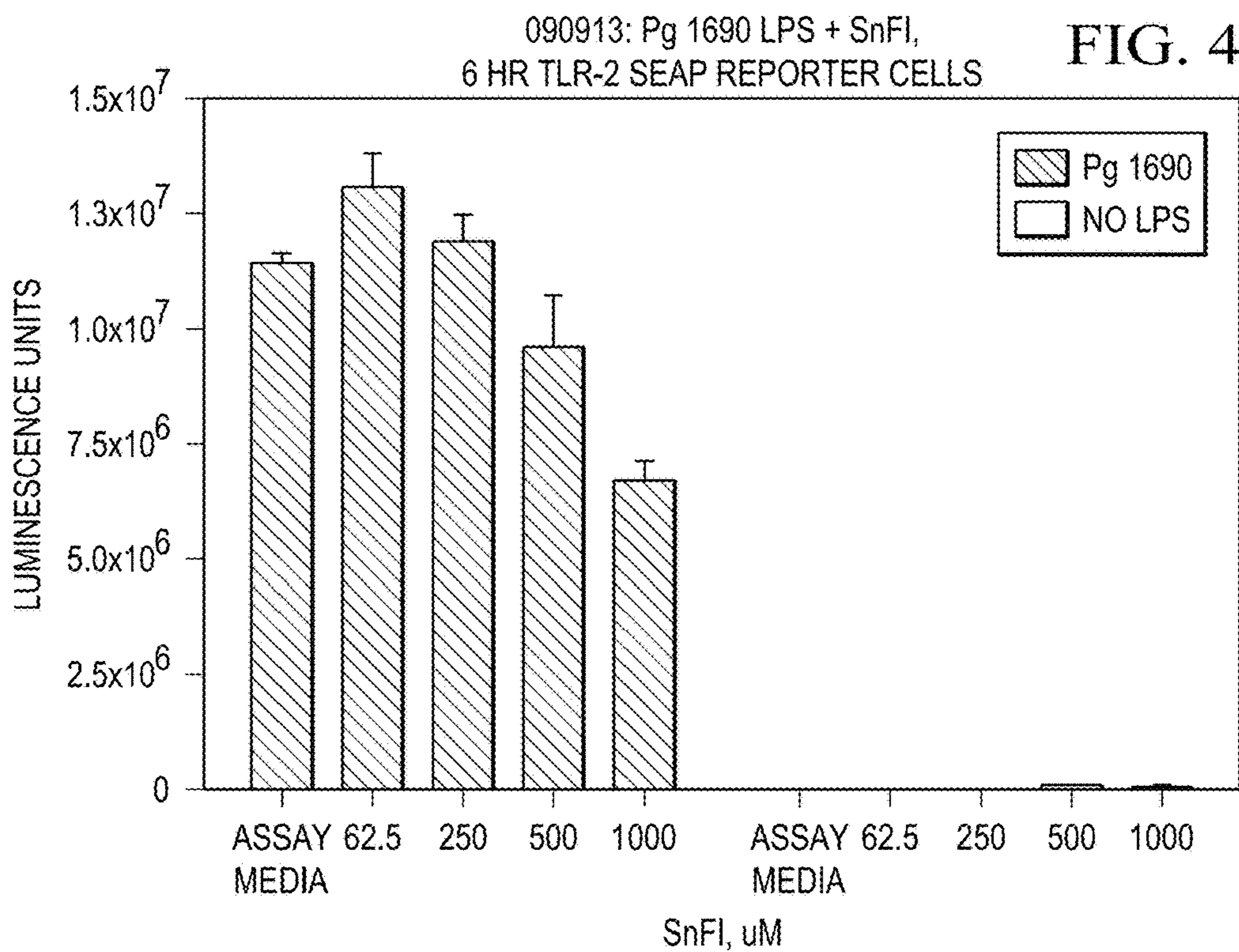
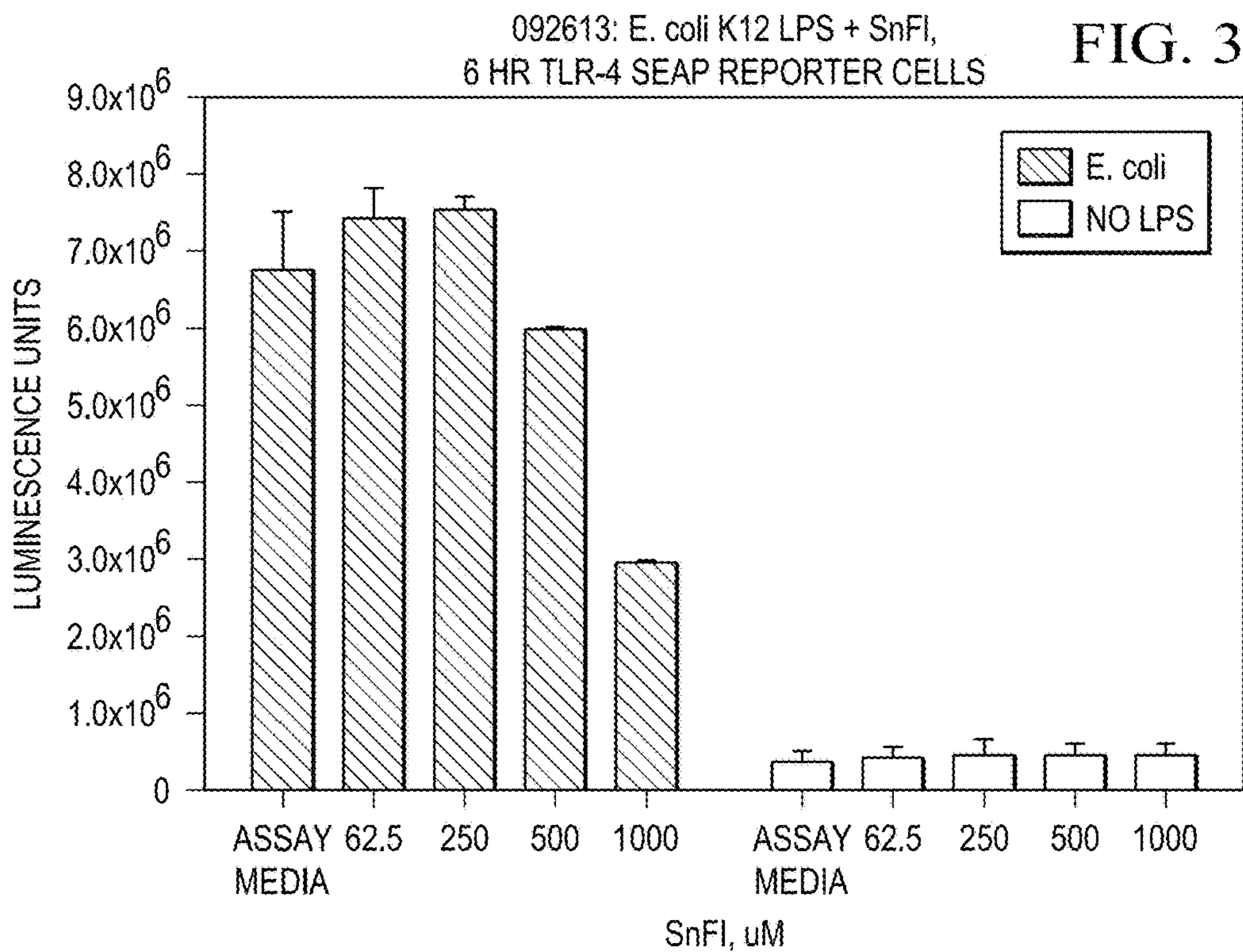
EC<sub>50</sub> OF LPS ON TLR4 ASSAYS

FIG. 2A











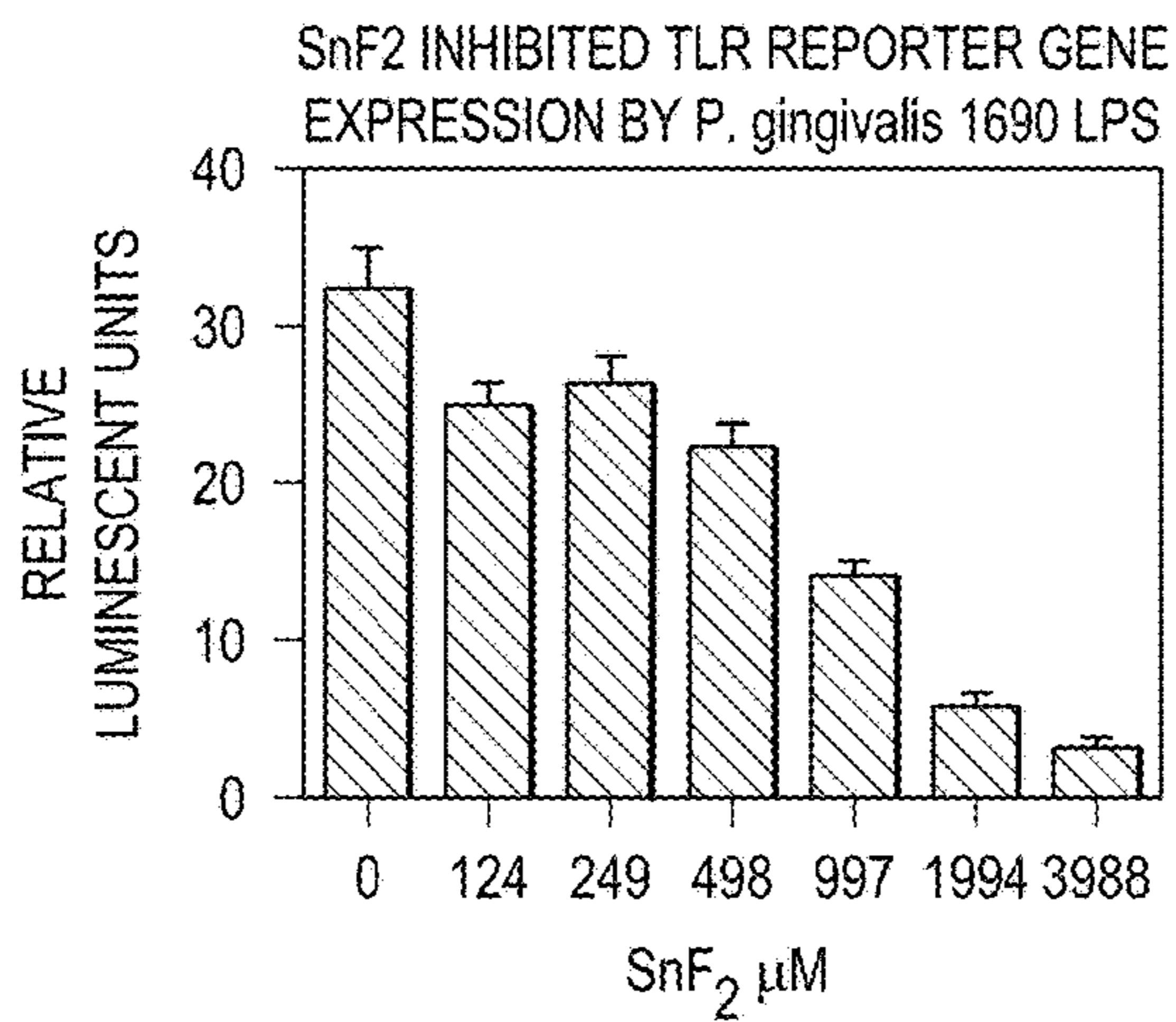


FIG. 5

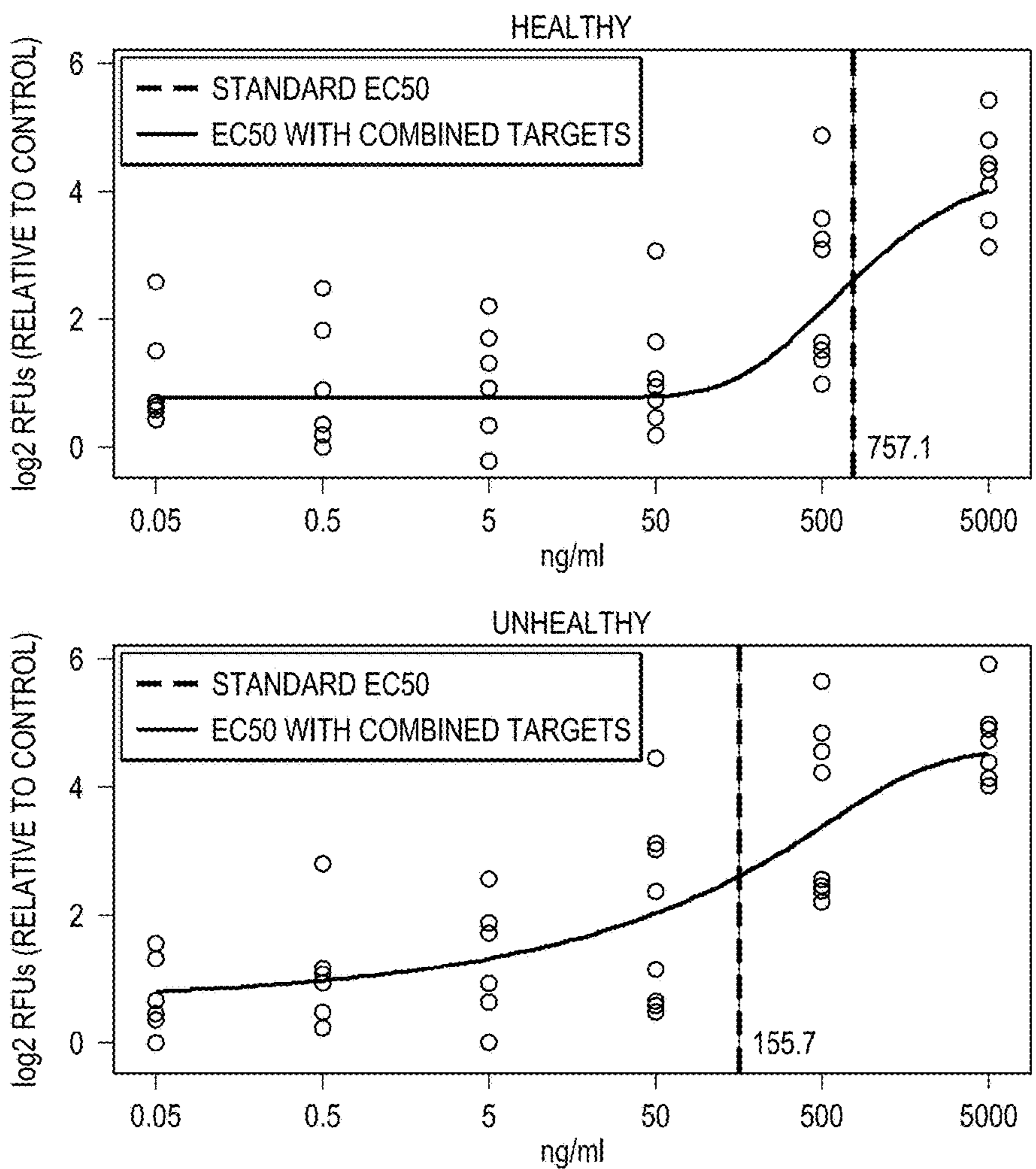


FIG. 6



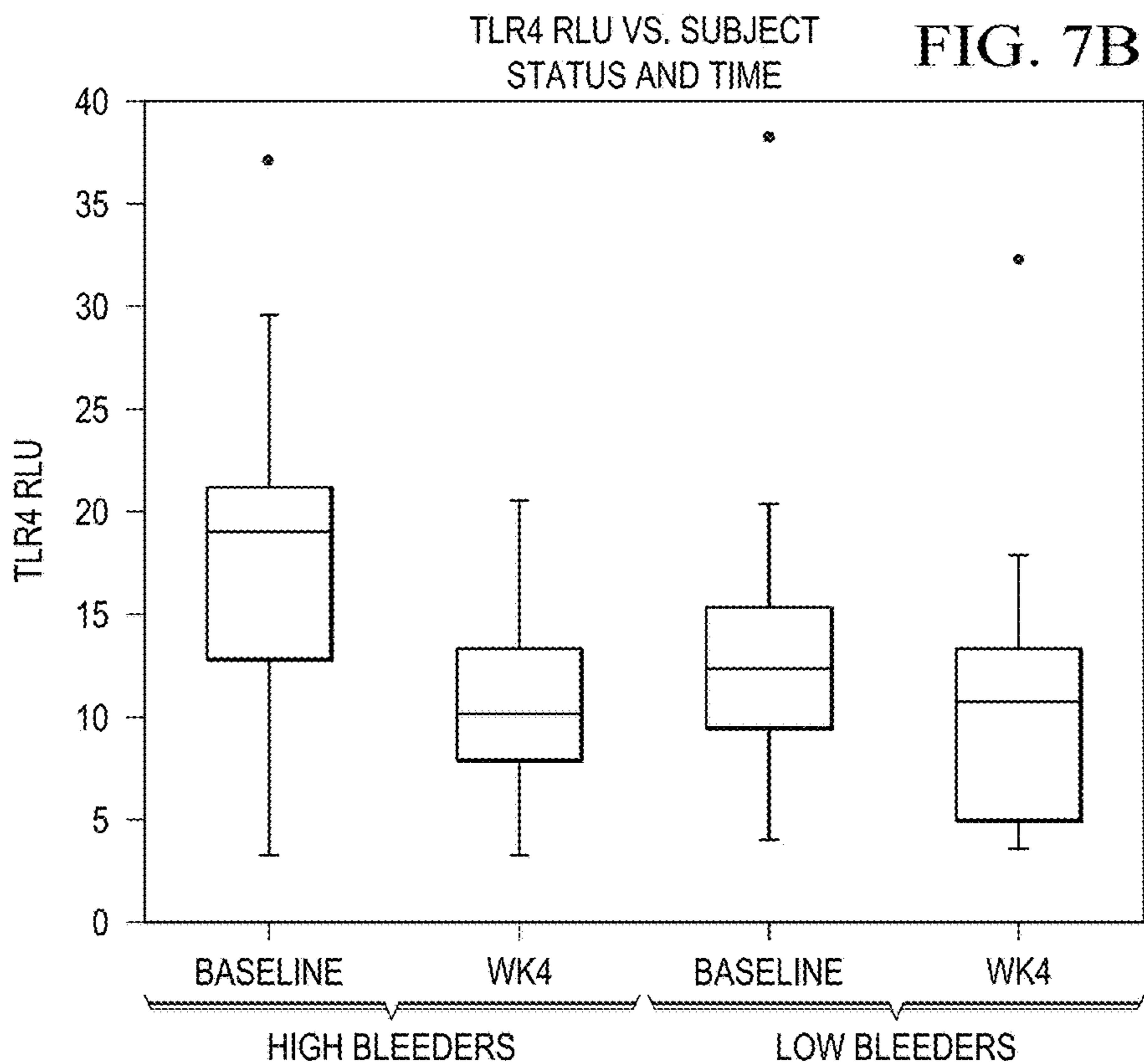
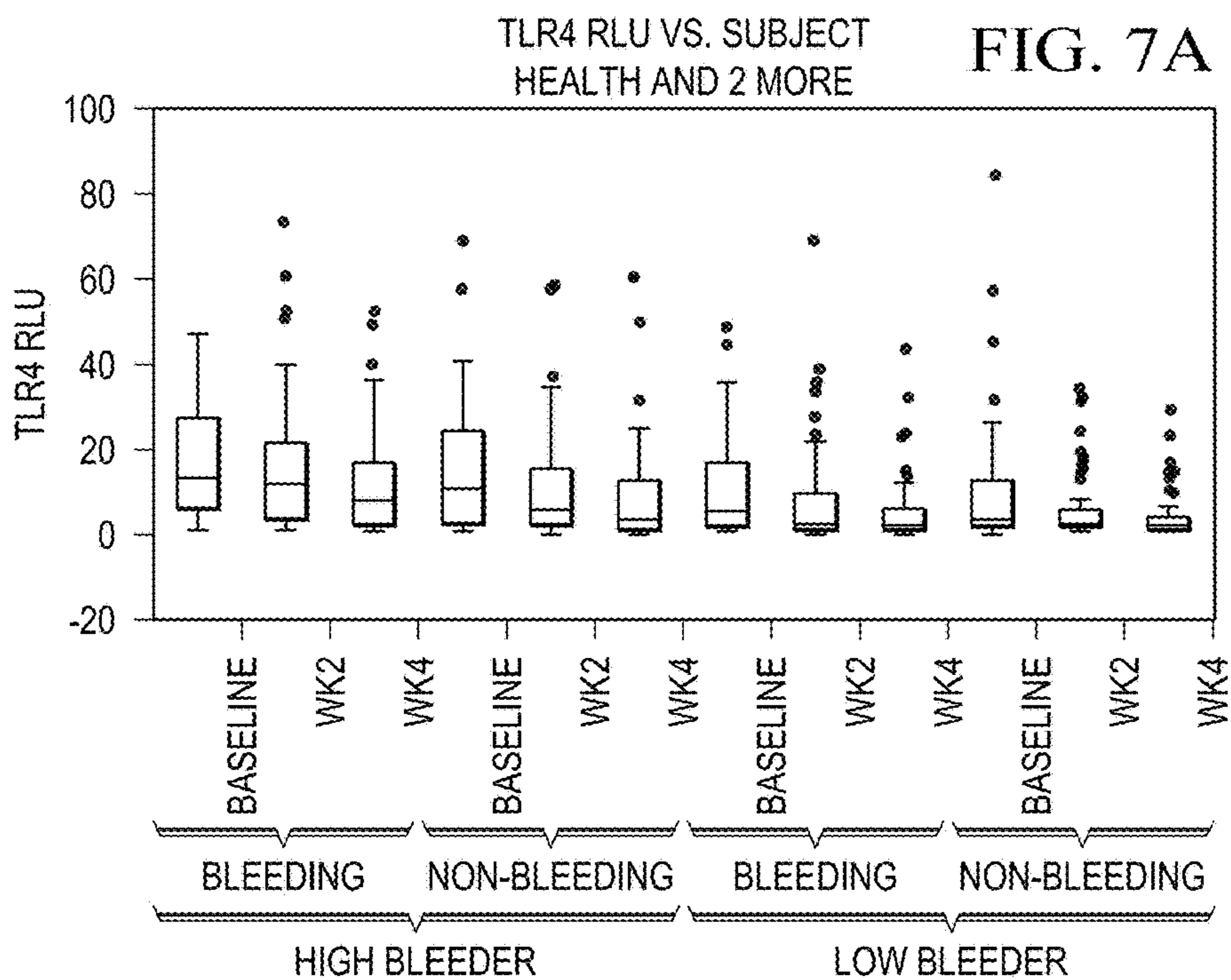


FIG. 8A

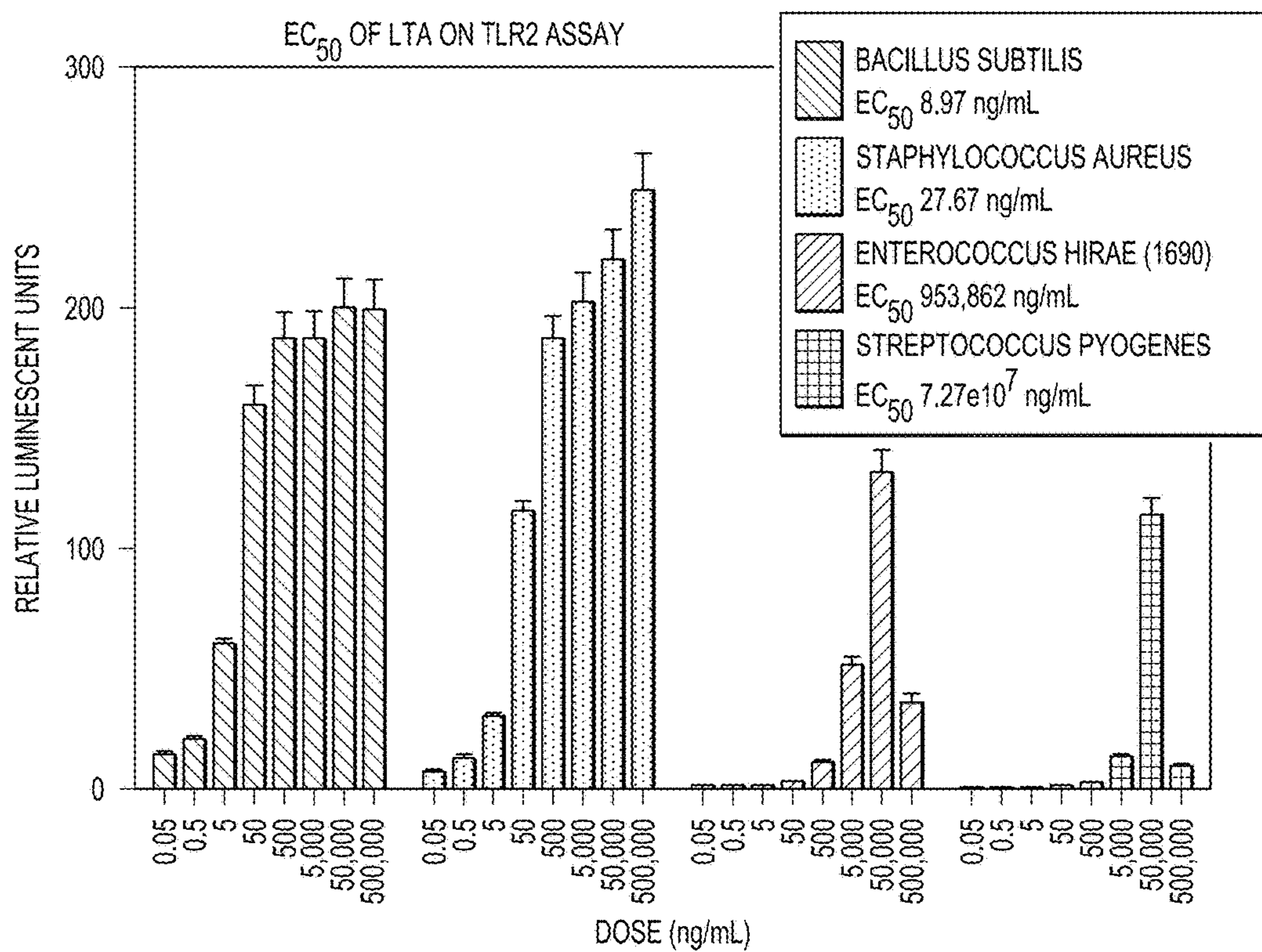
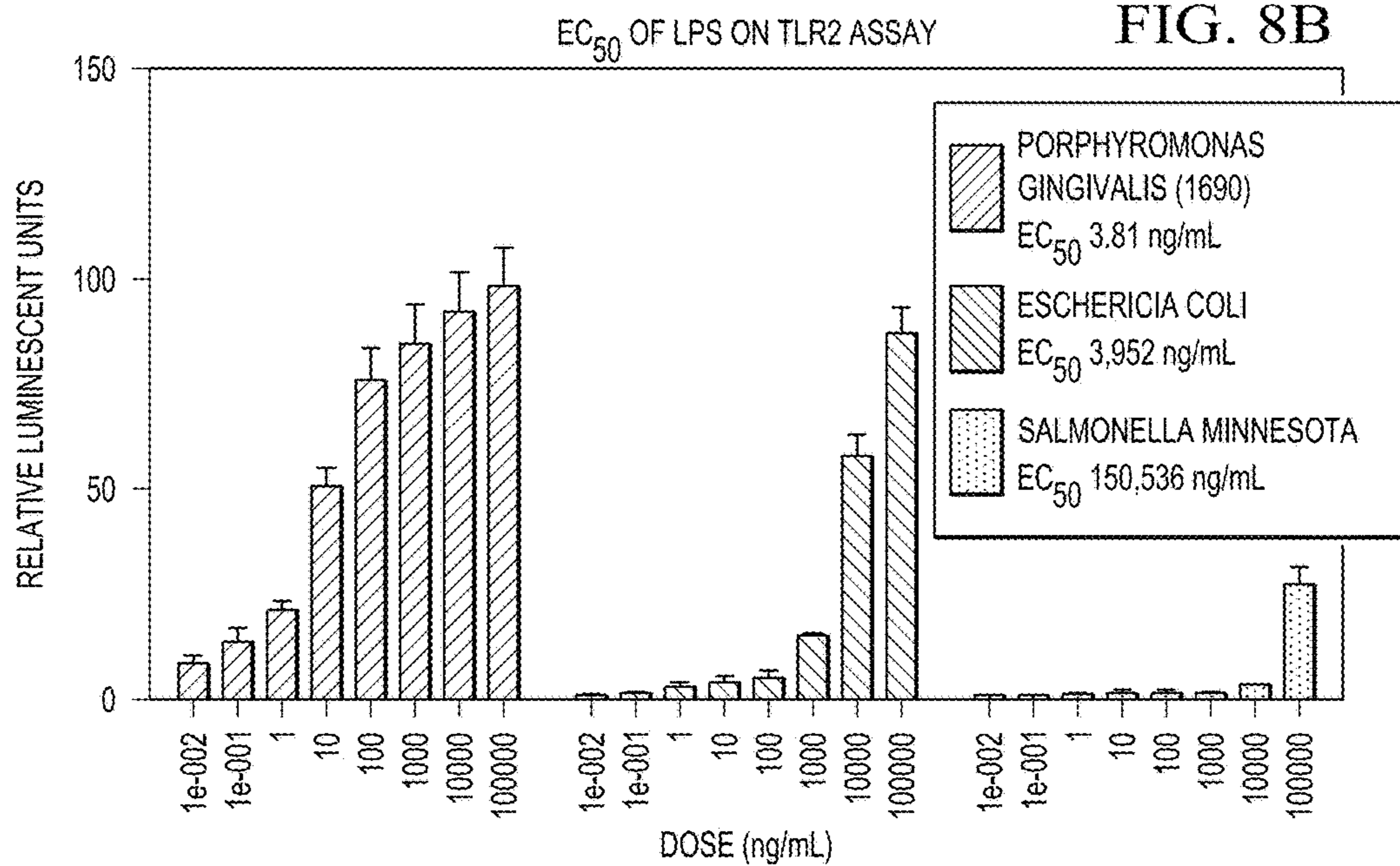


FIG. 8B





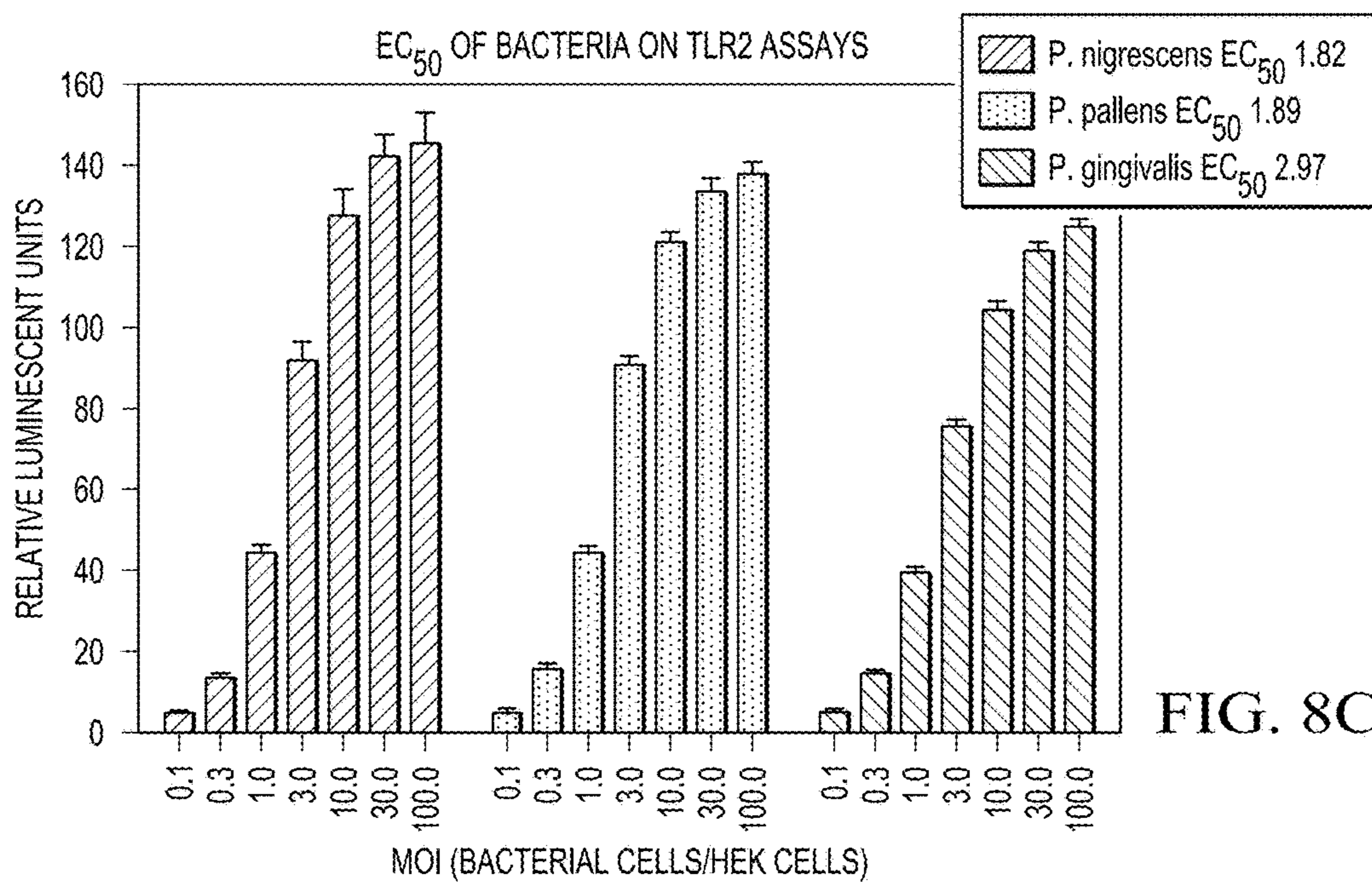


FIG. 8C

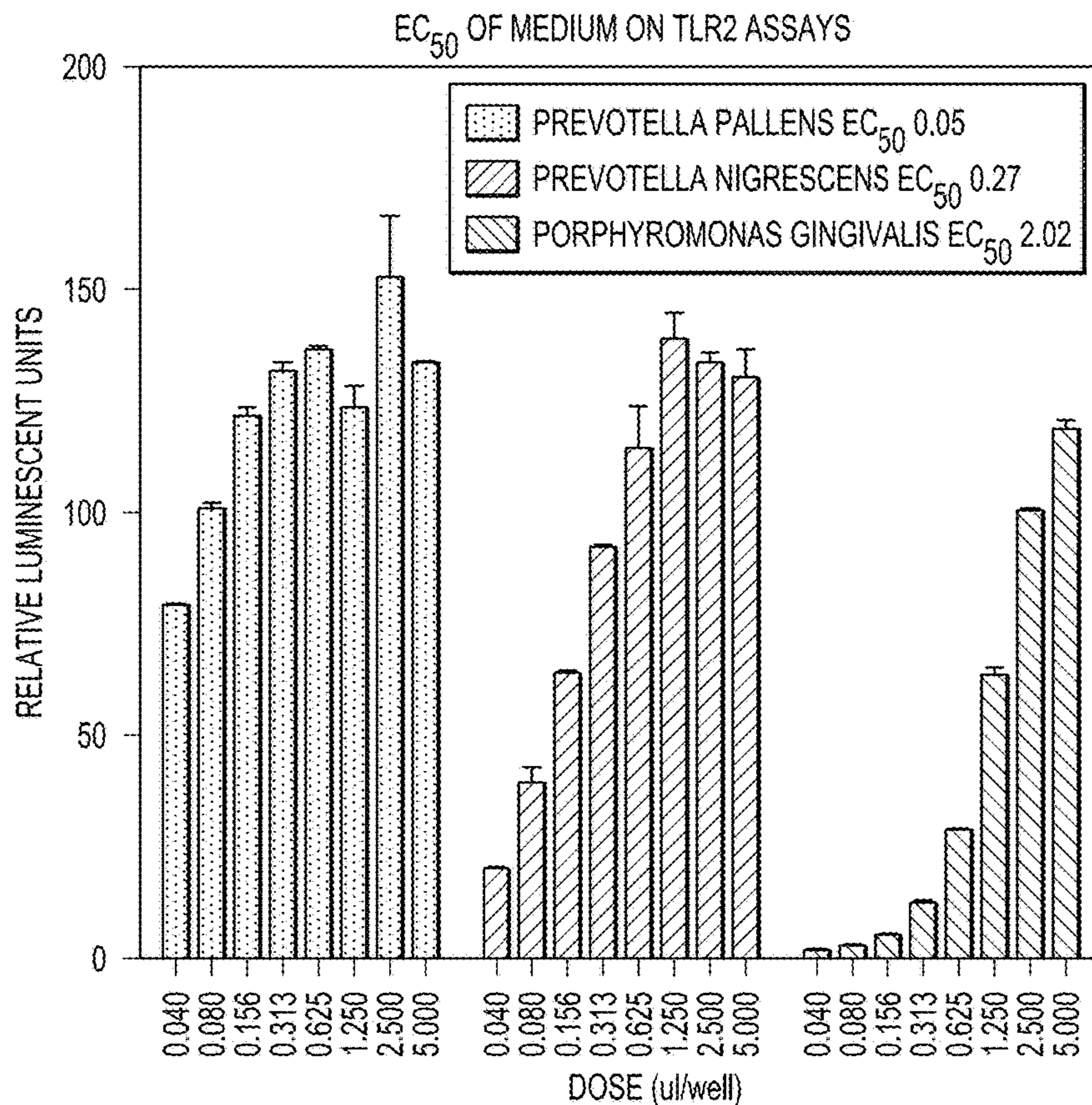


FIG. 8D

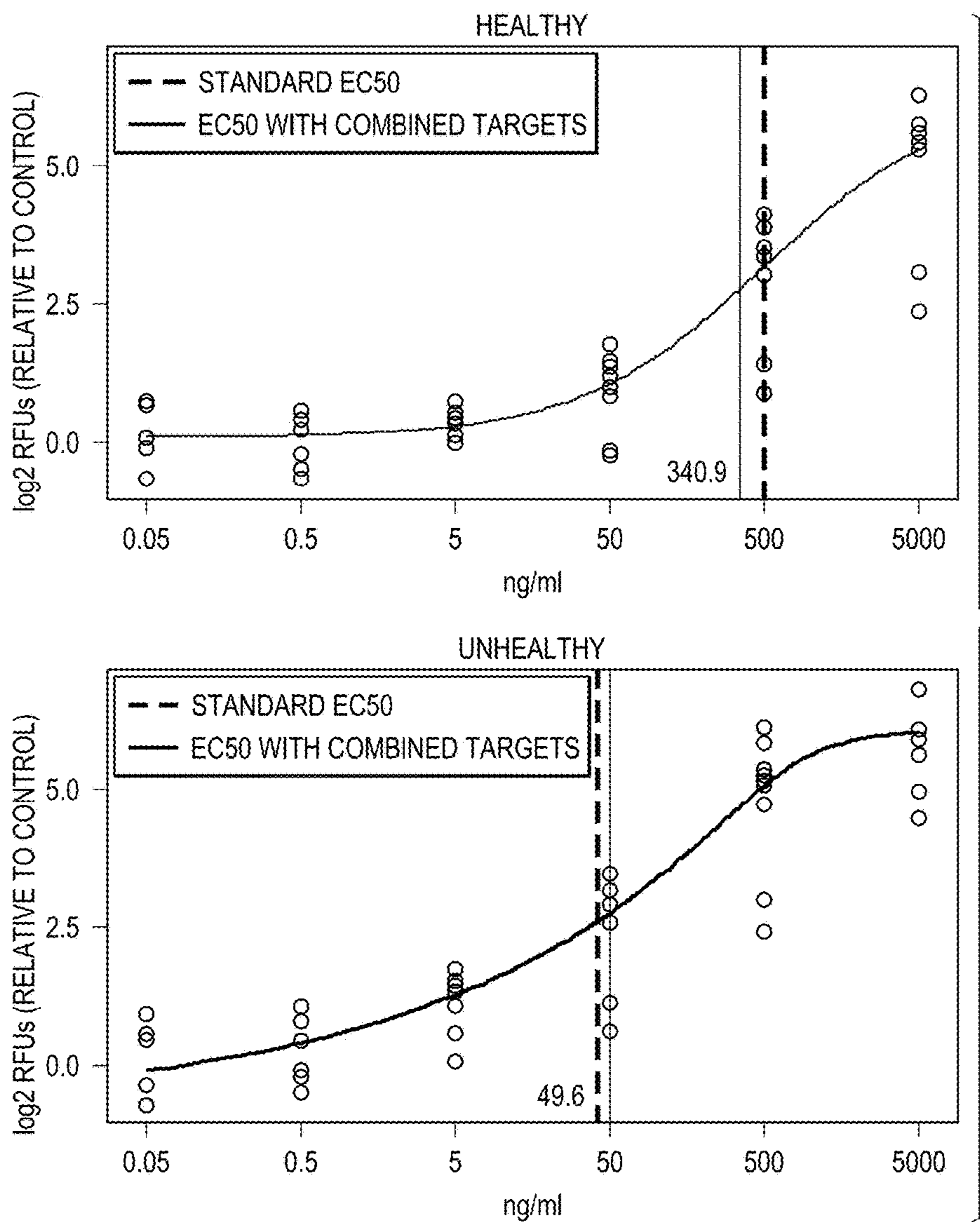


FIG. 9



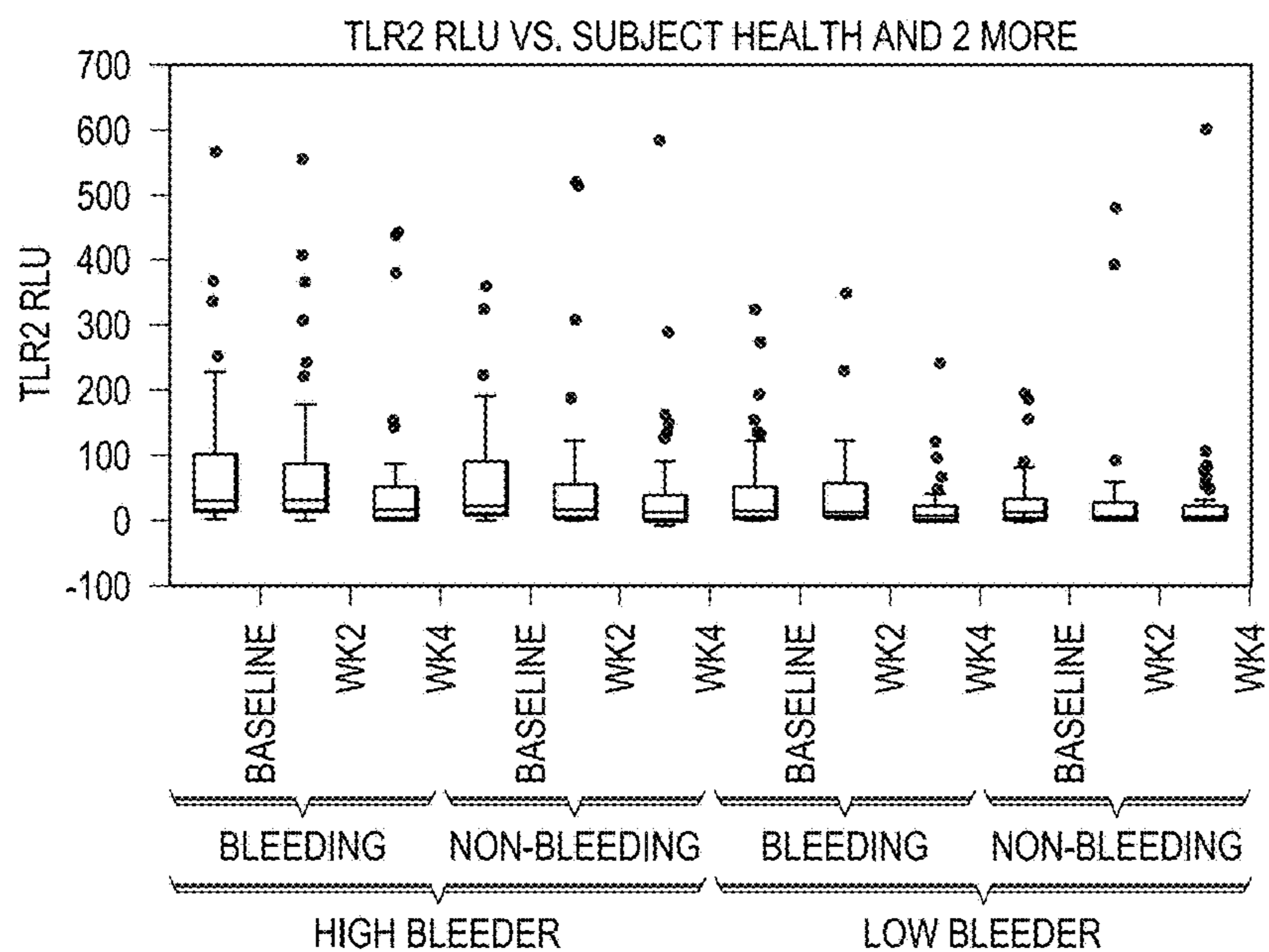


FIG. 10A

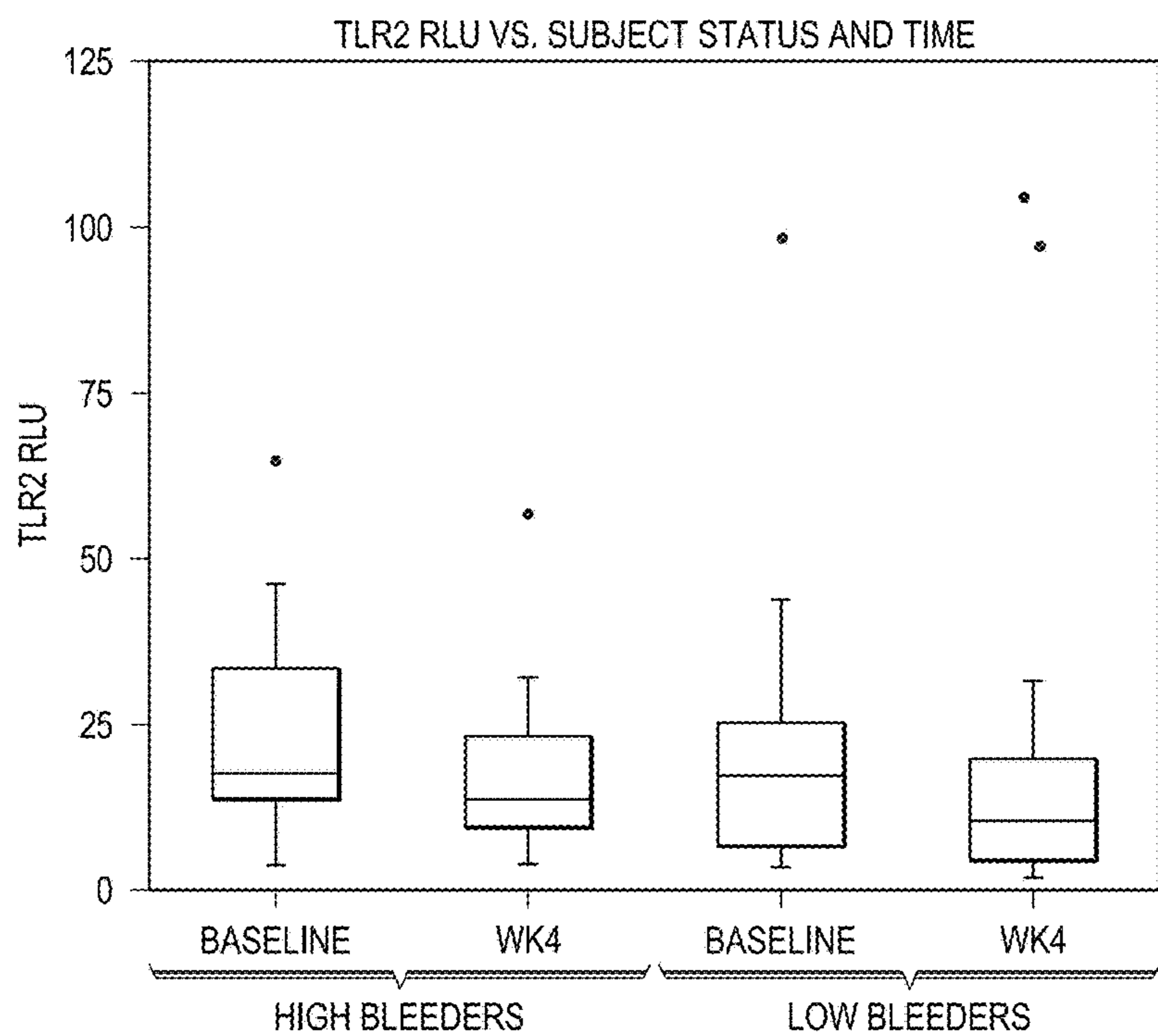


FIG. 10B

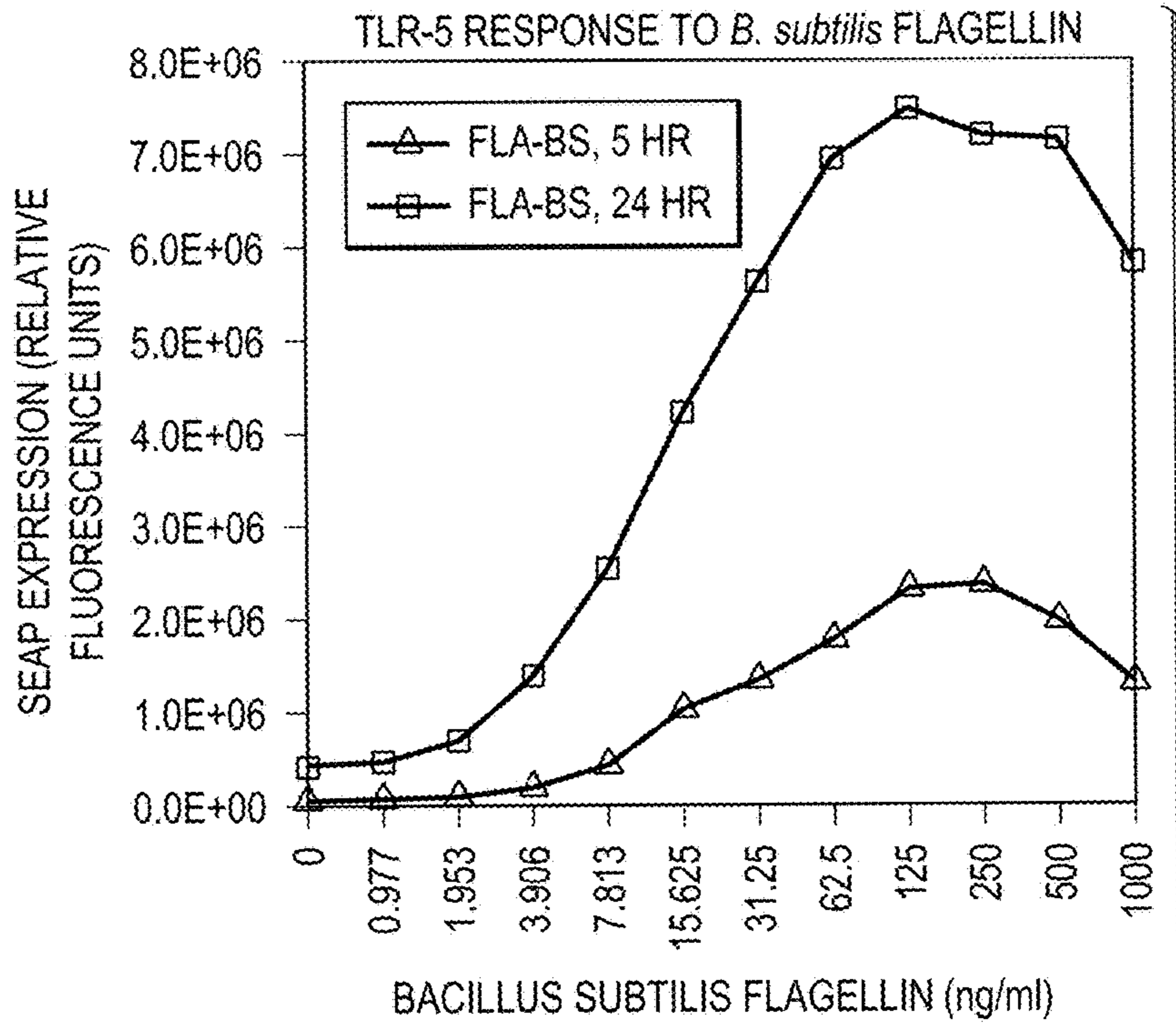
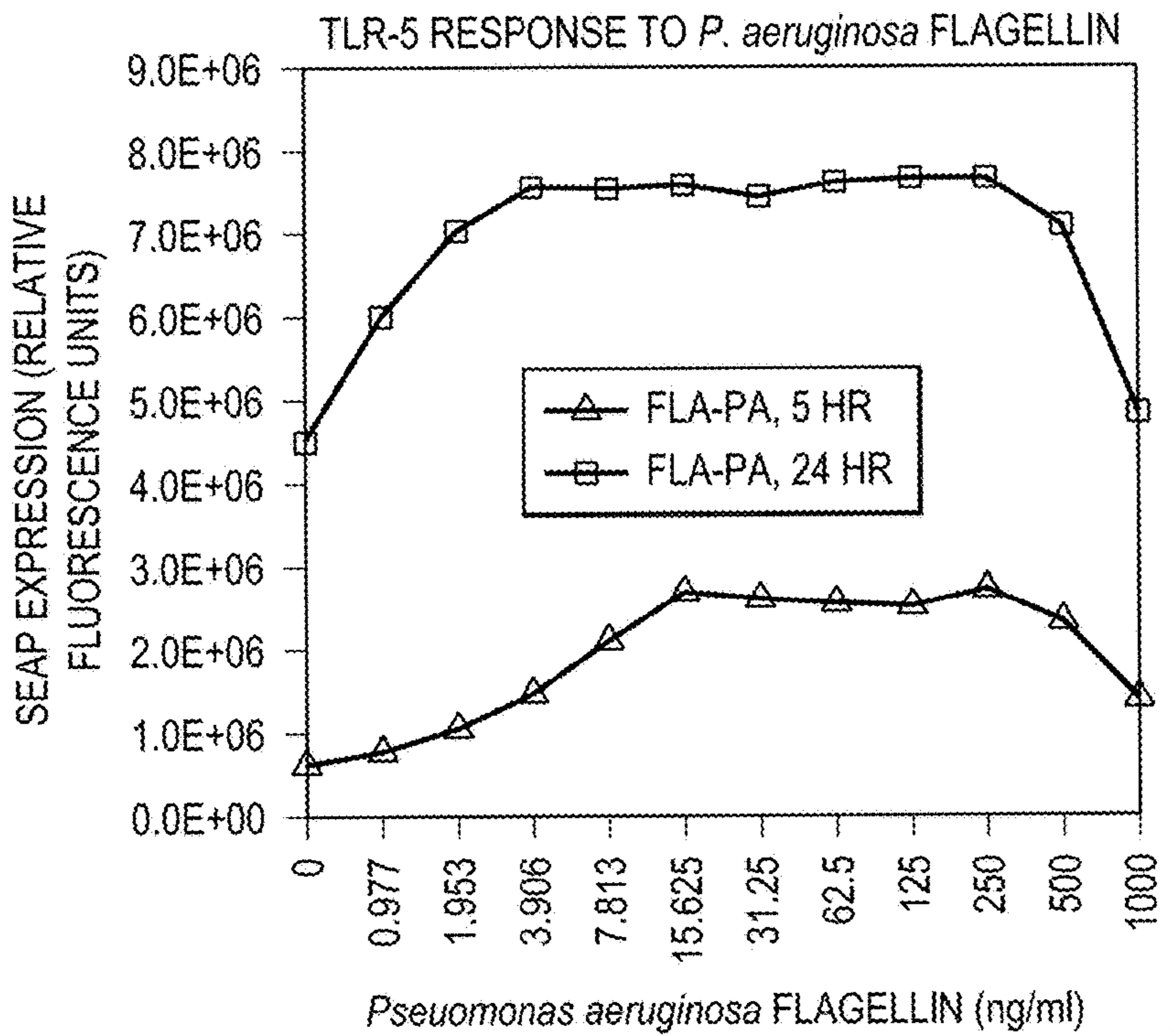


FIG. 11





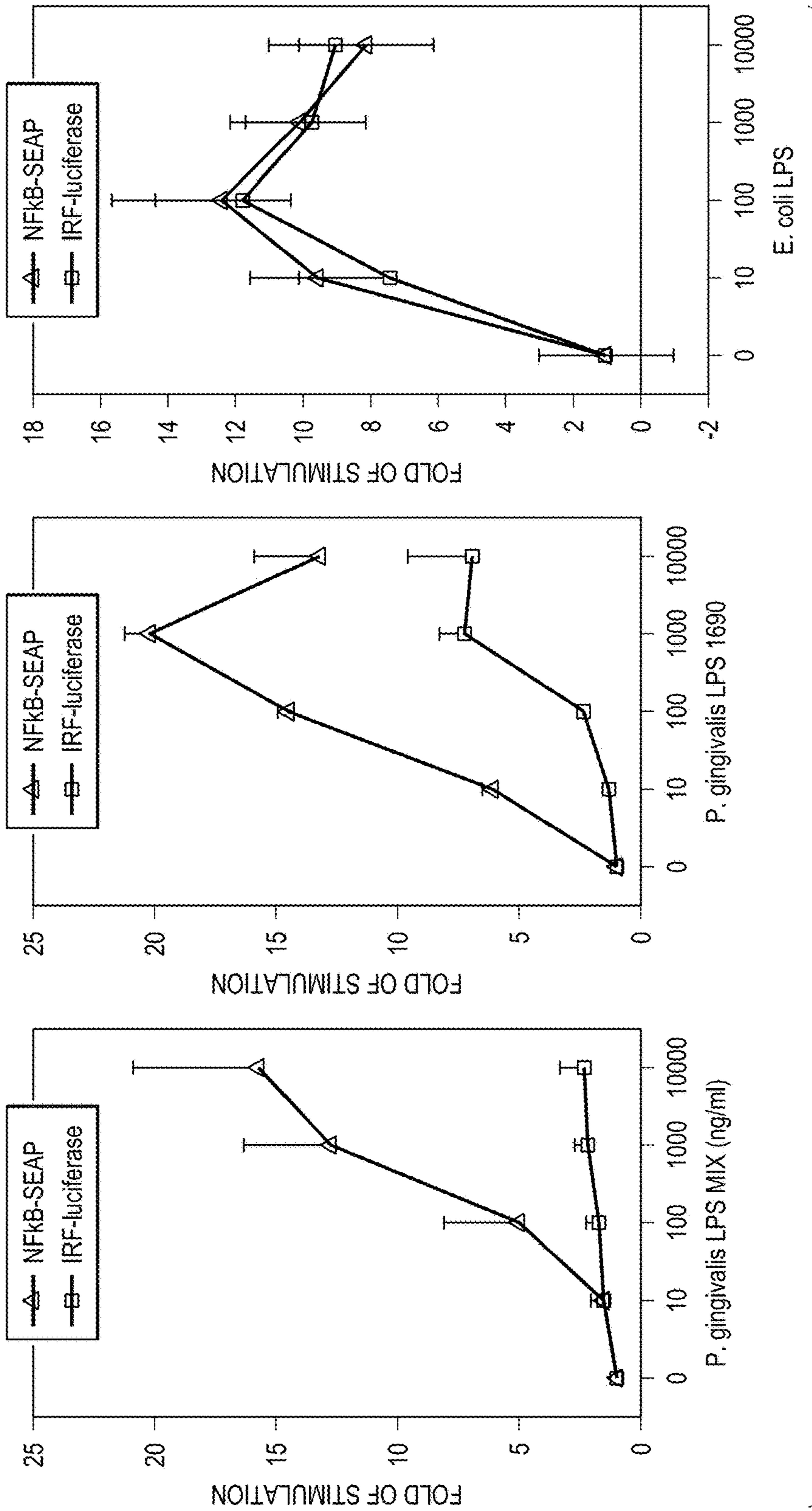


FIG. 12A

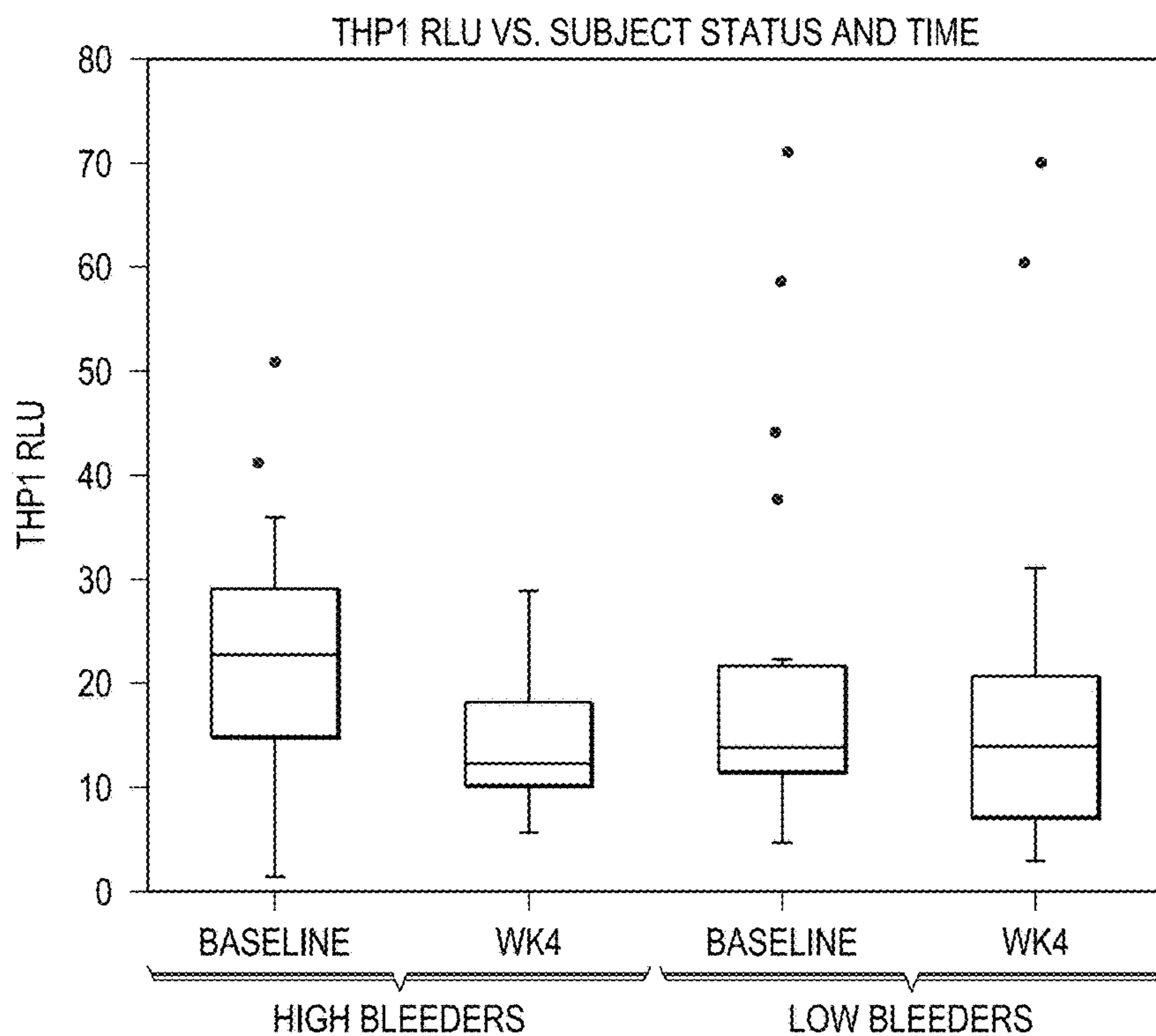


FIG. 12B

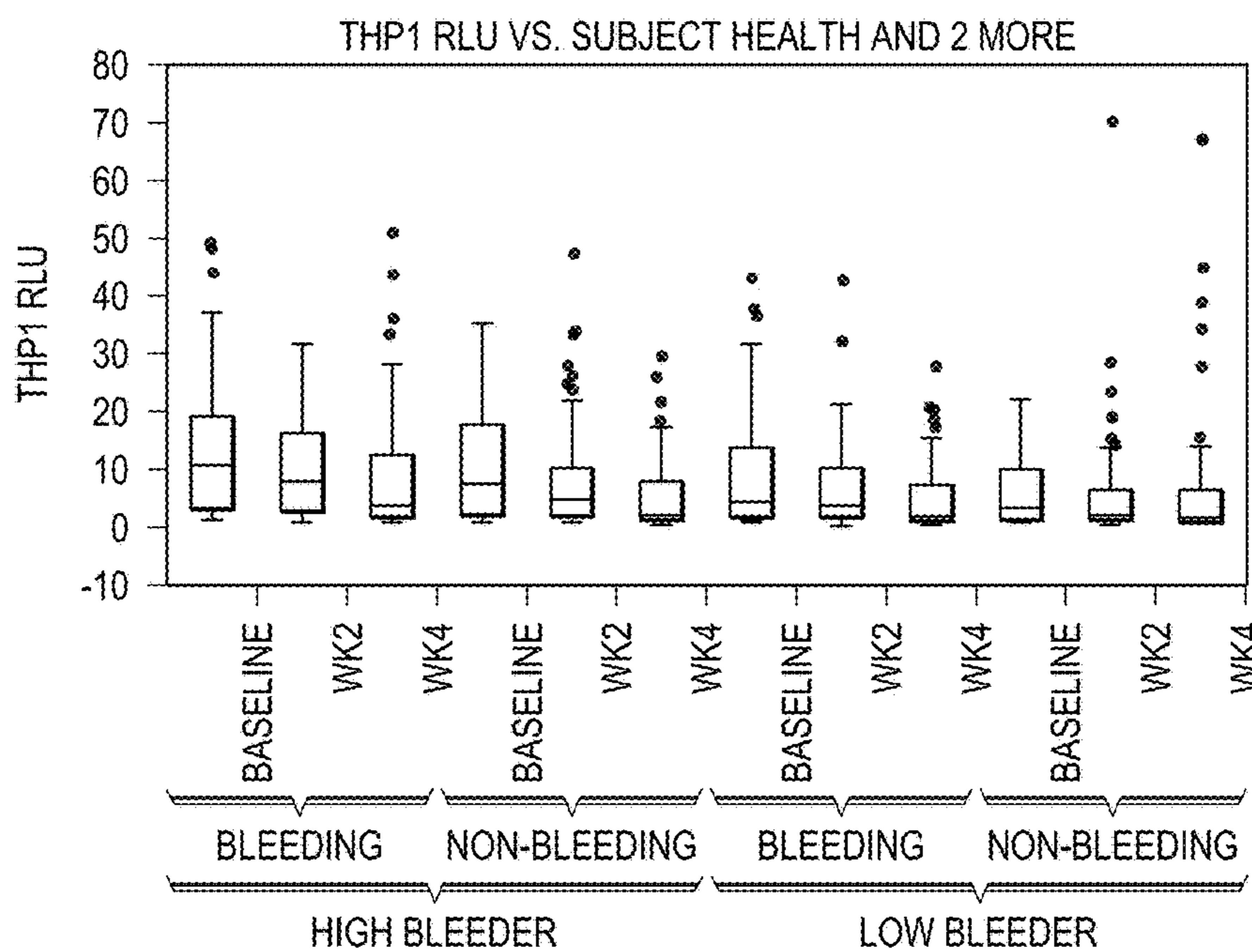


FIG. 12C



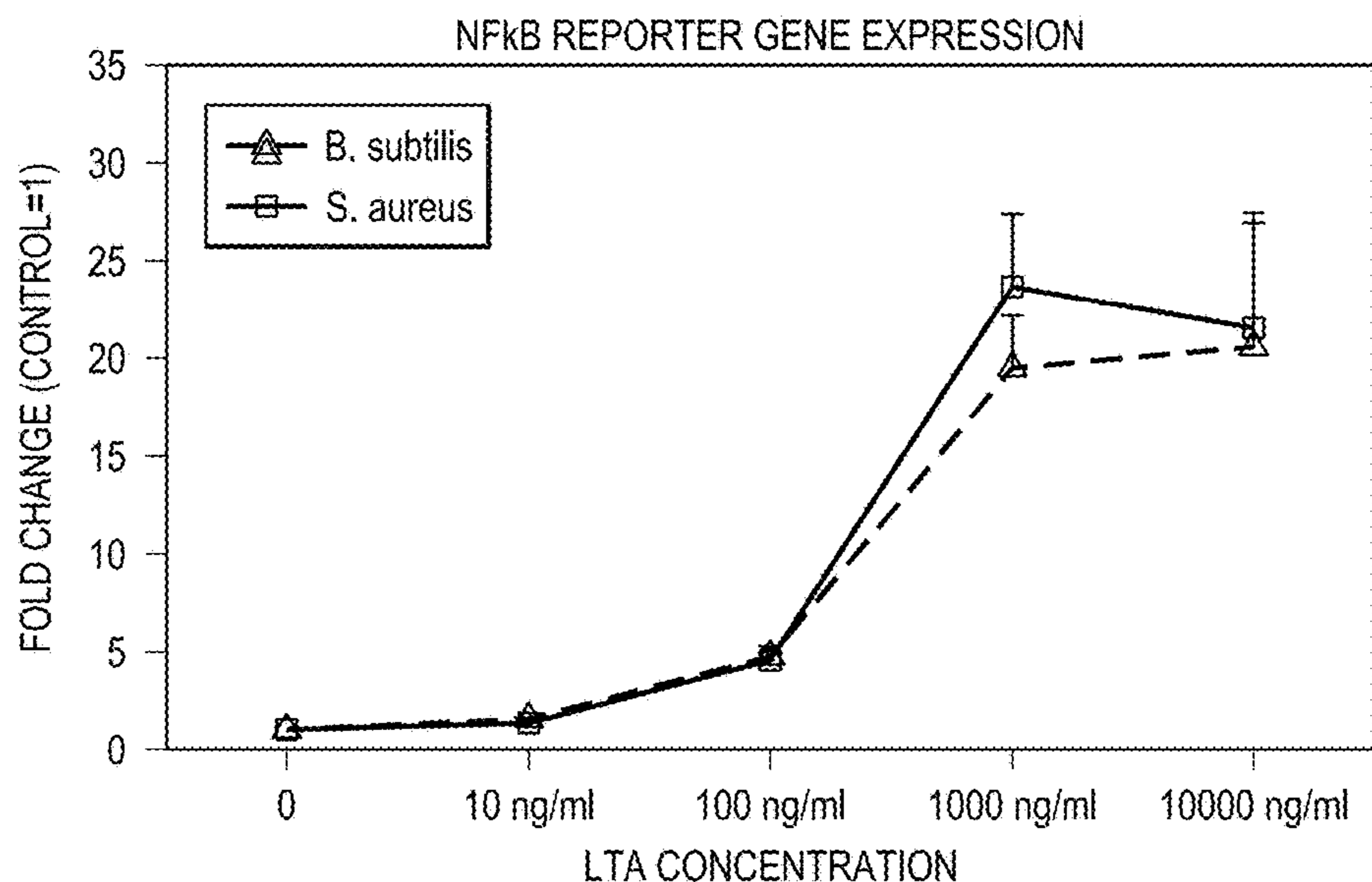


FIG. 13

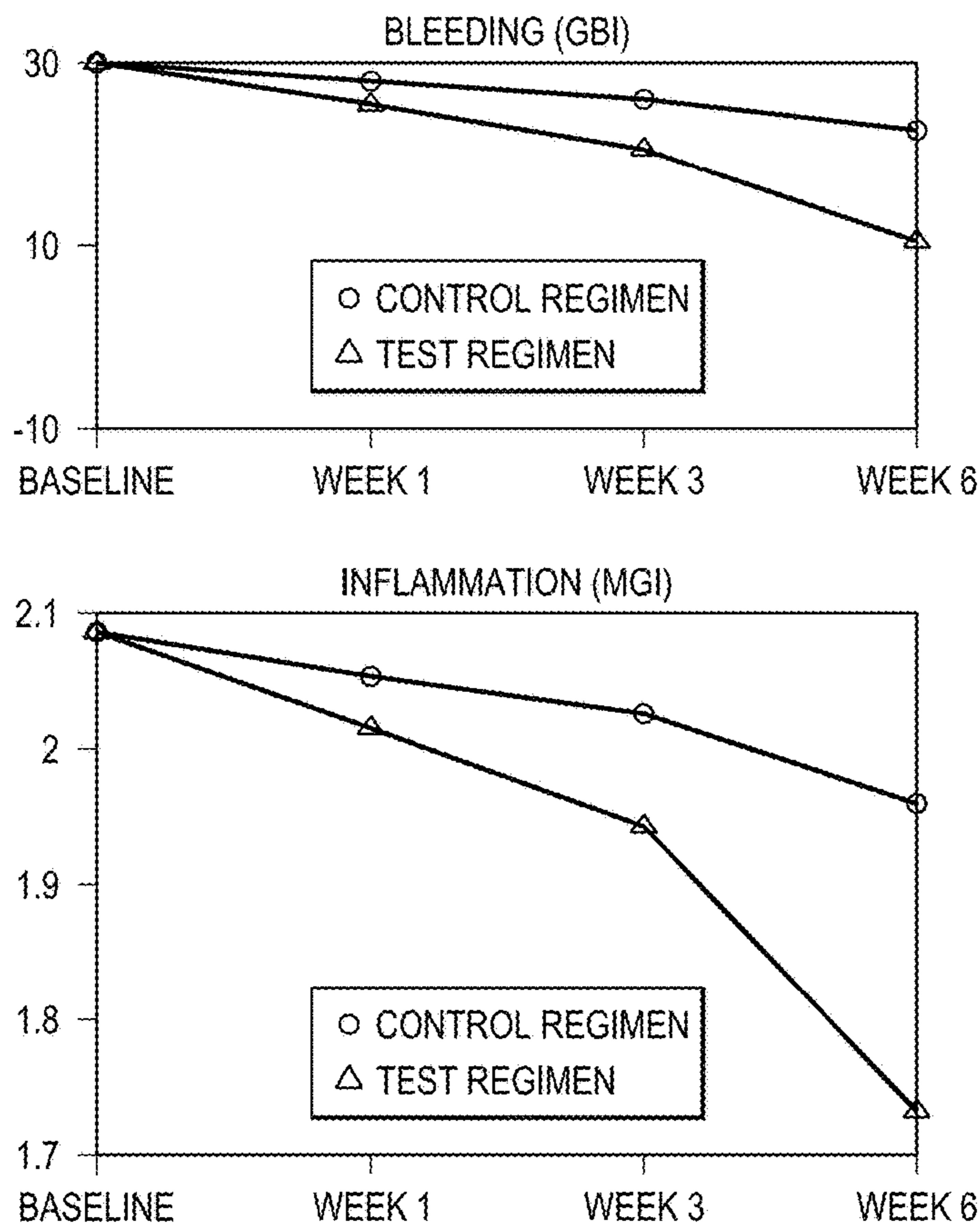


FIG. 14

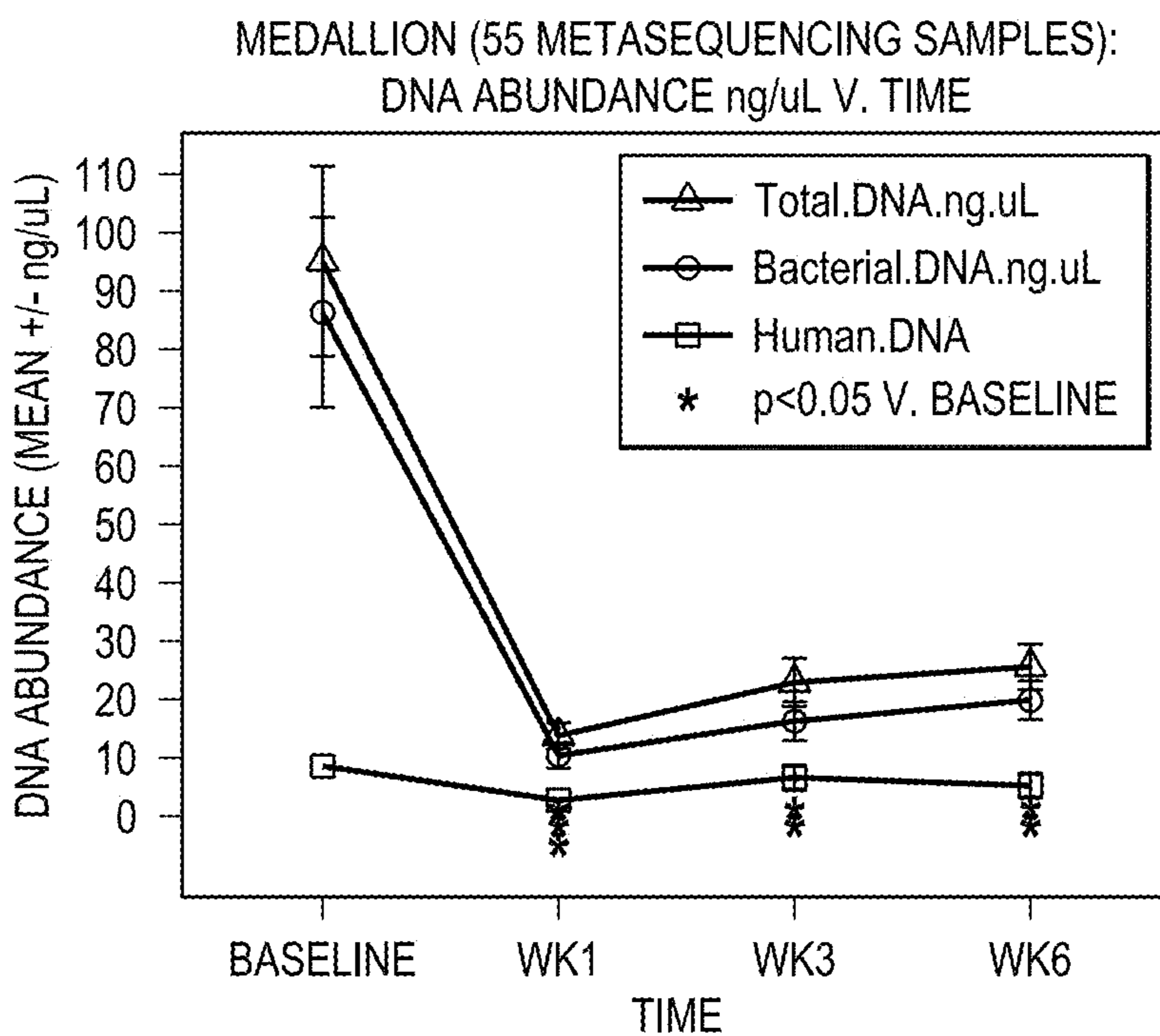


FIG. 15

FIG. 16A-1  
FIG. 16A-2  
FIG. 16A-3

FIG. 16A



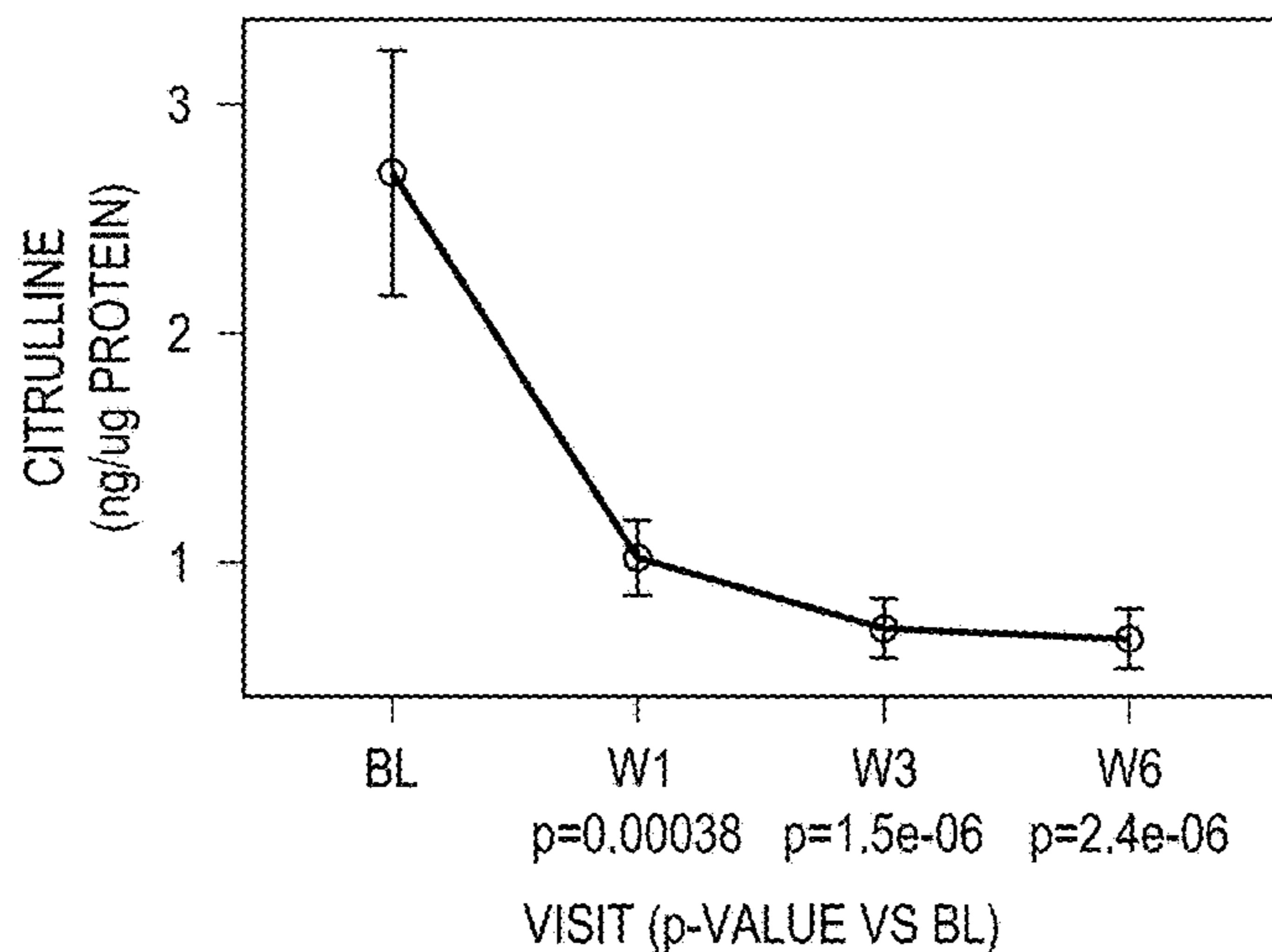


FIG. 16

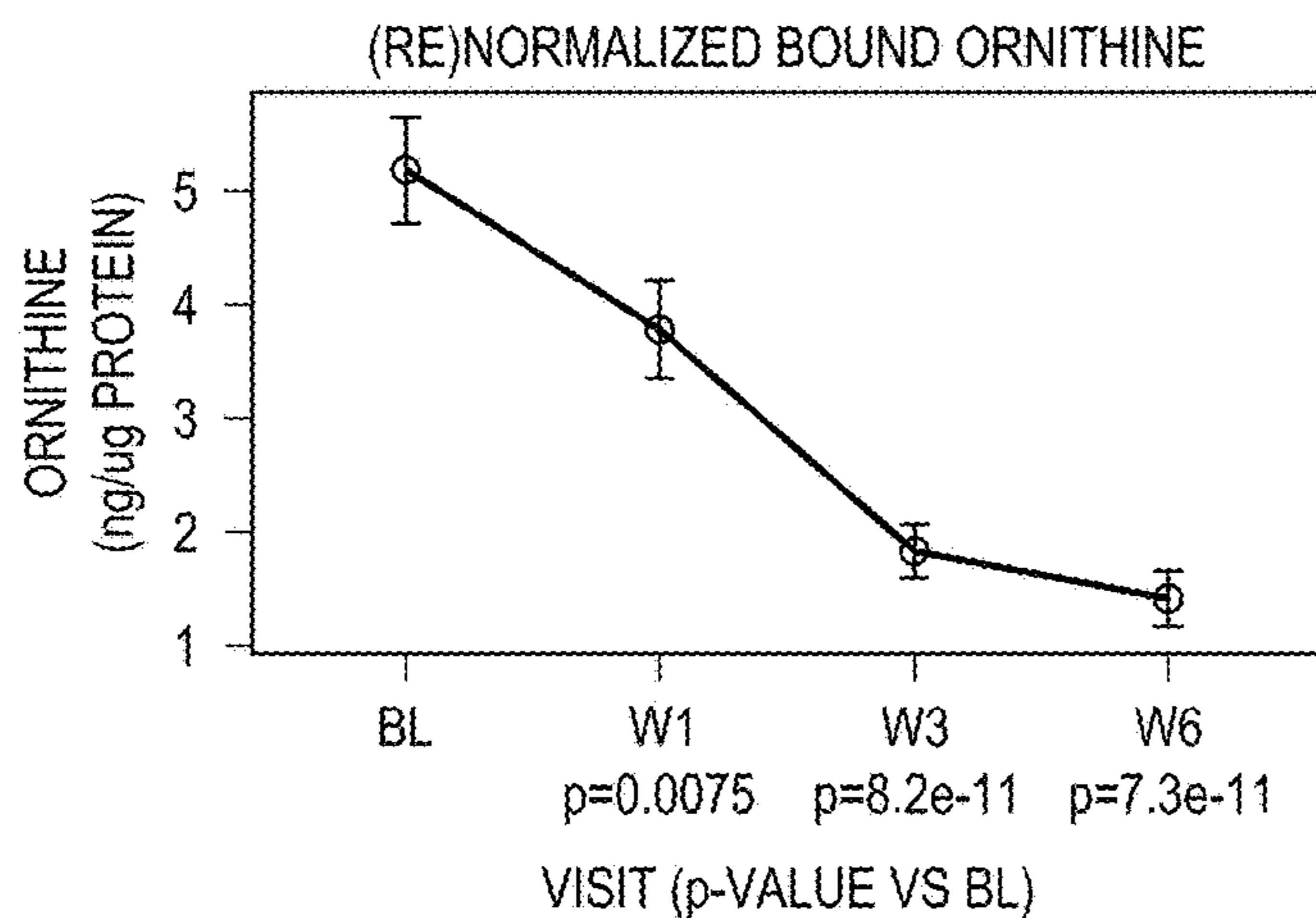


FIG. 17

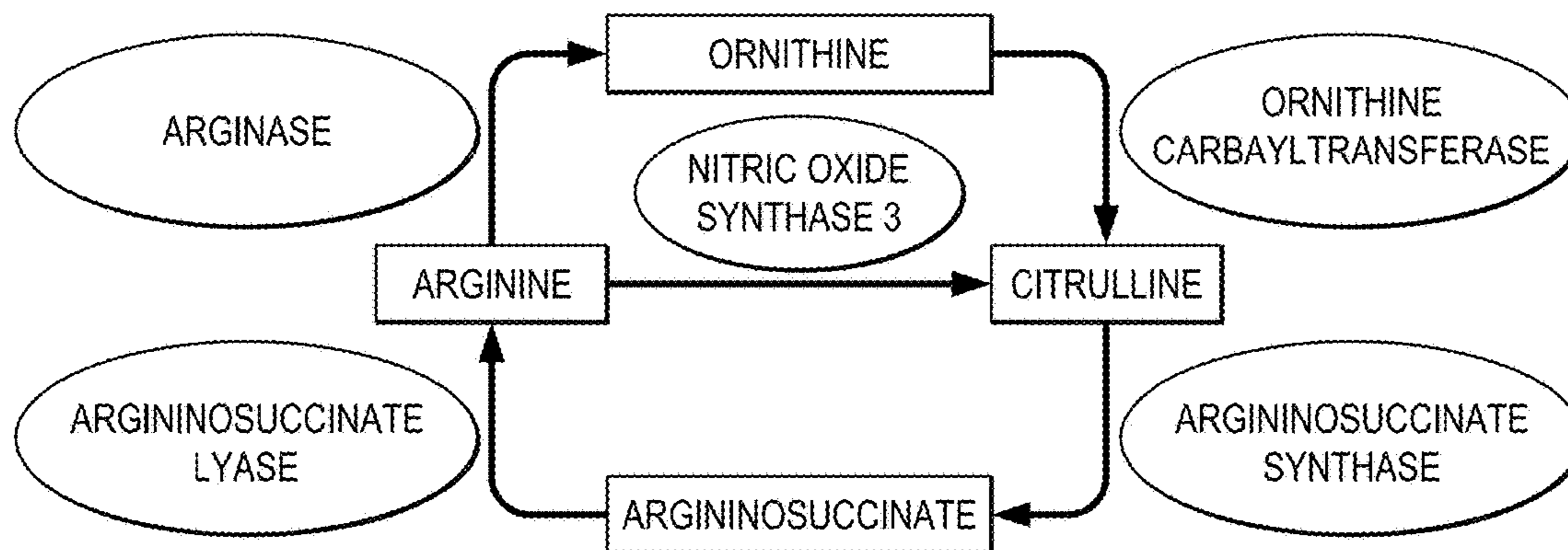


FIG. 18

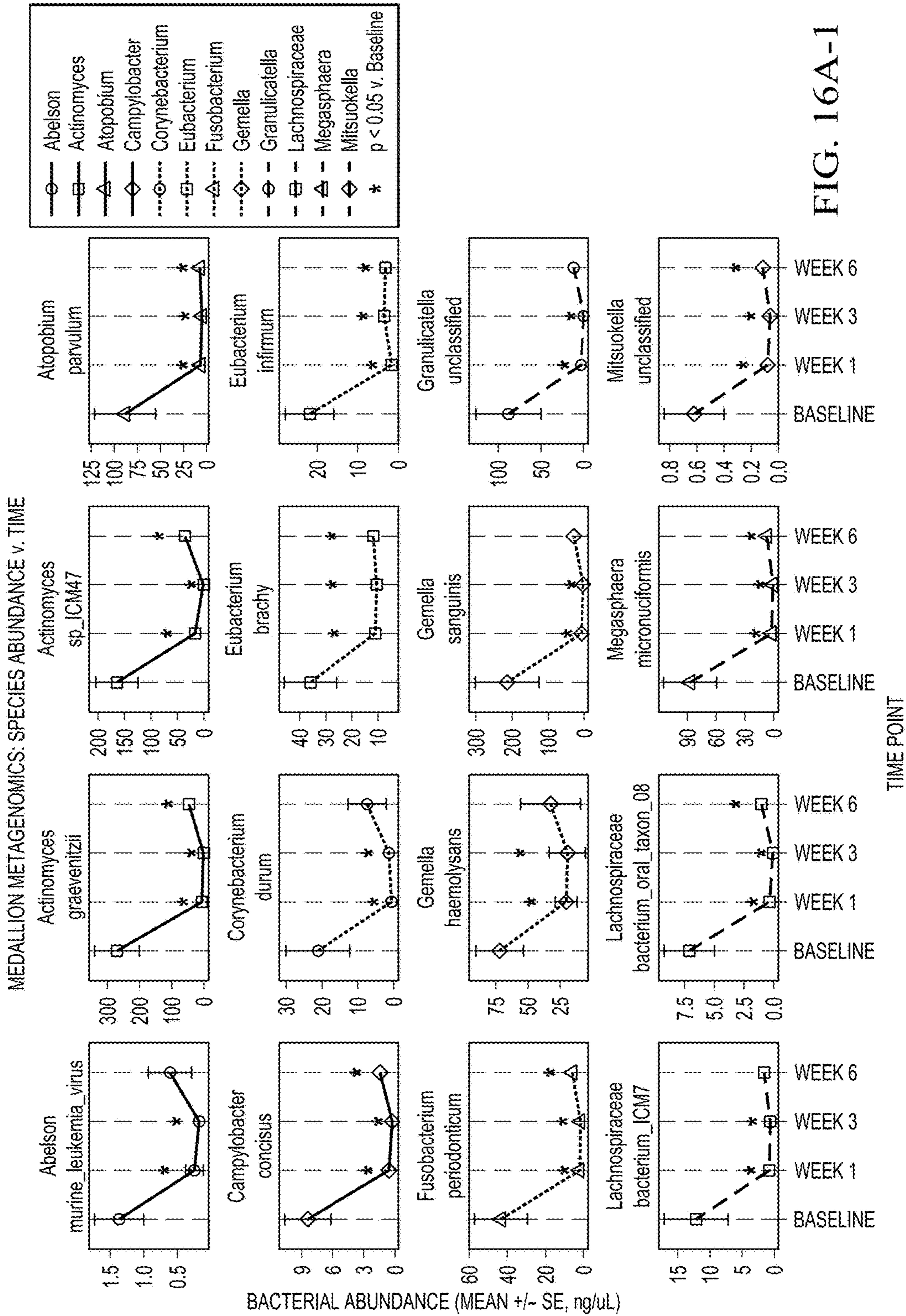


FIG. 16A-1



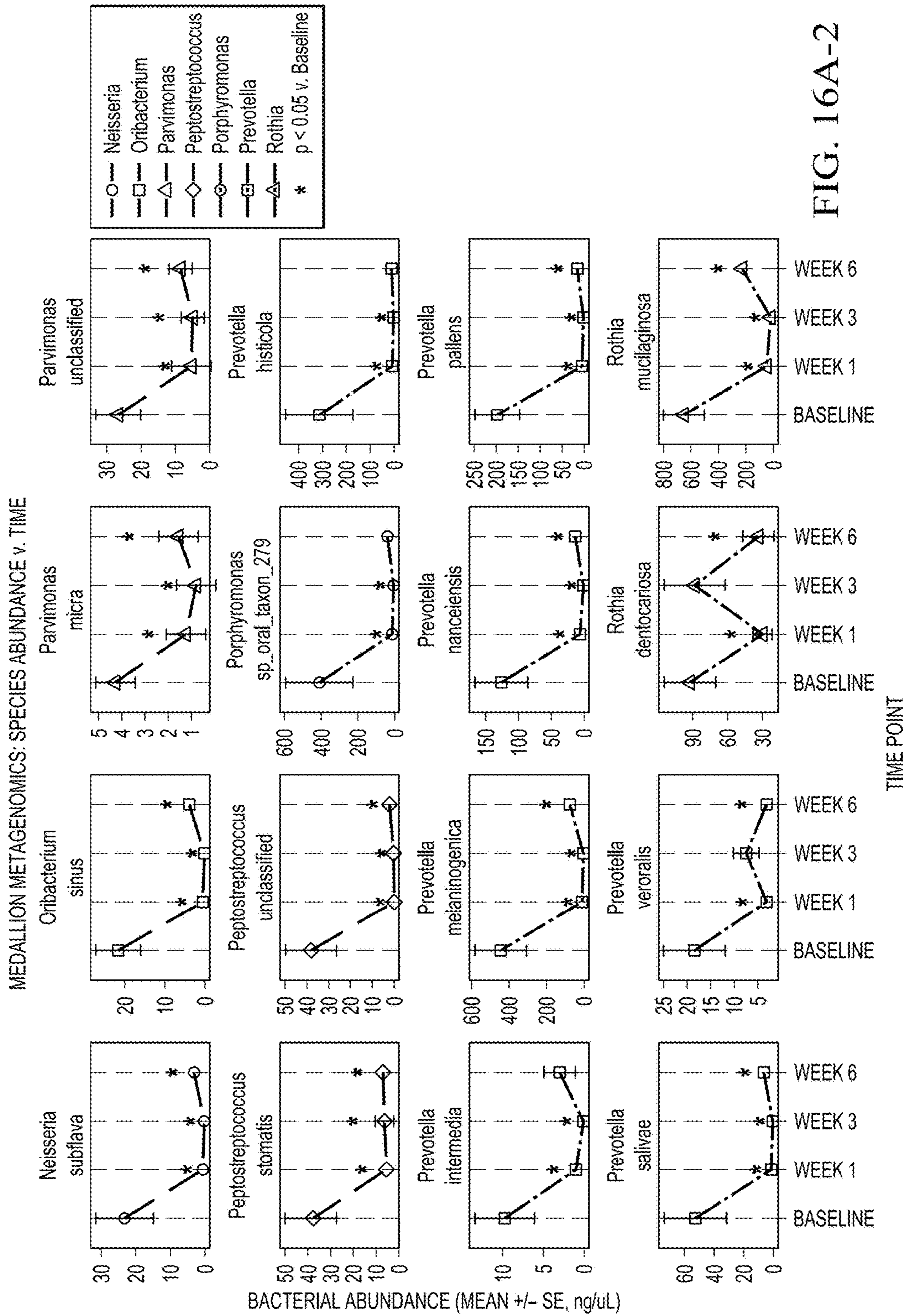


FIG. 16A-2

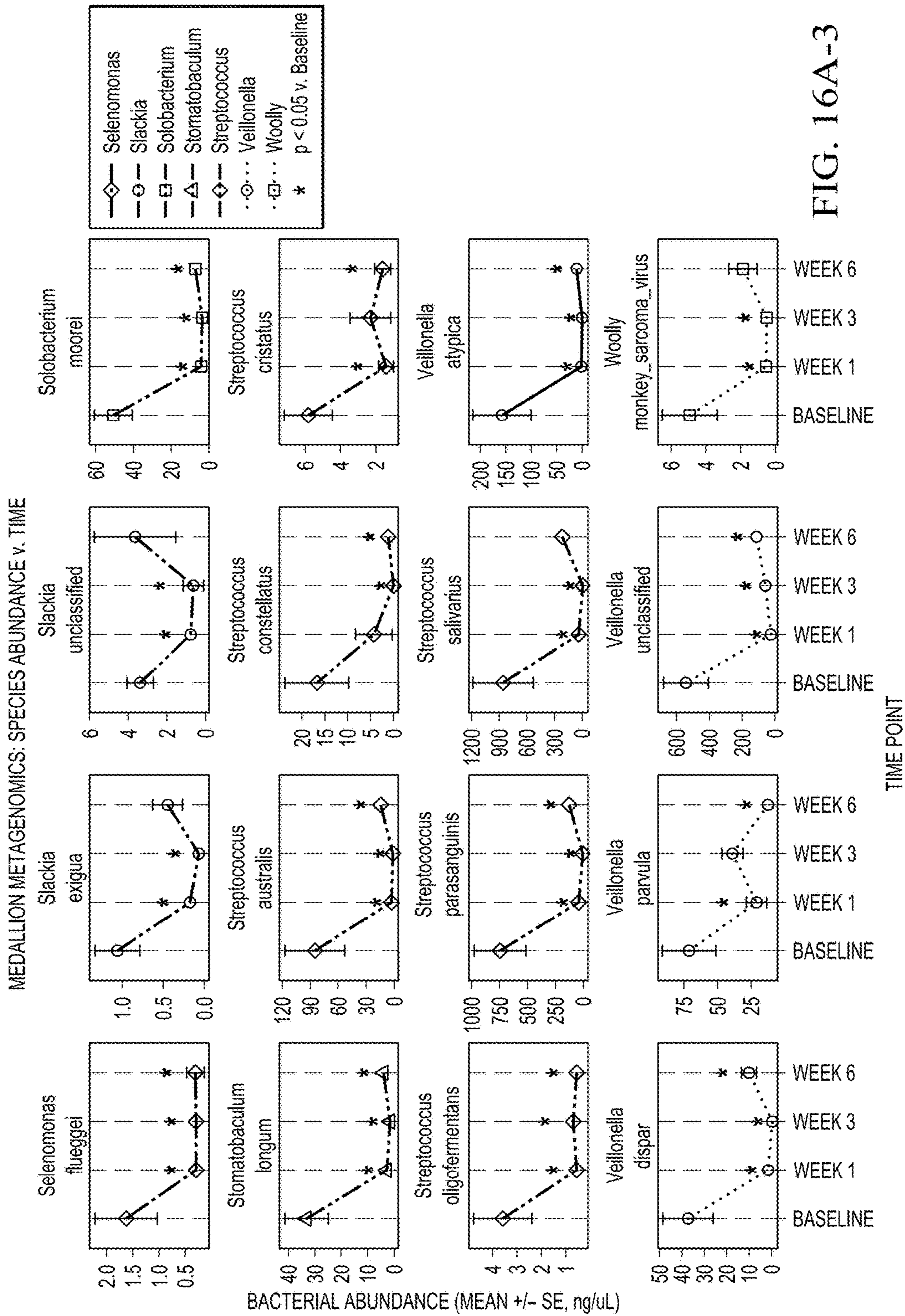
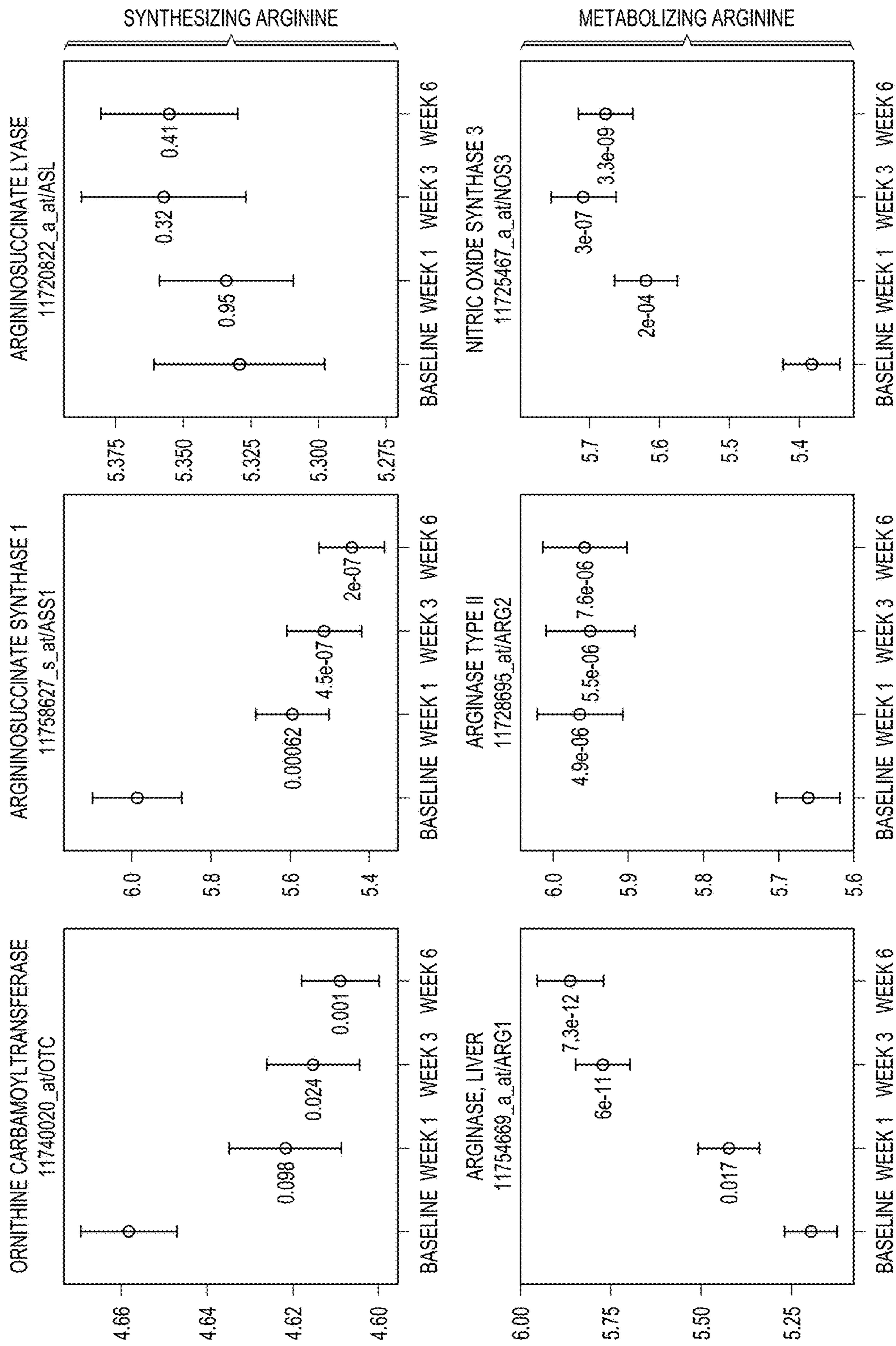


FIG. 16A-3



FIG. 19



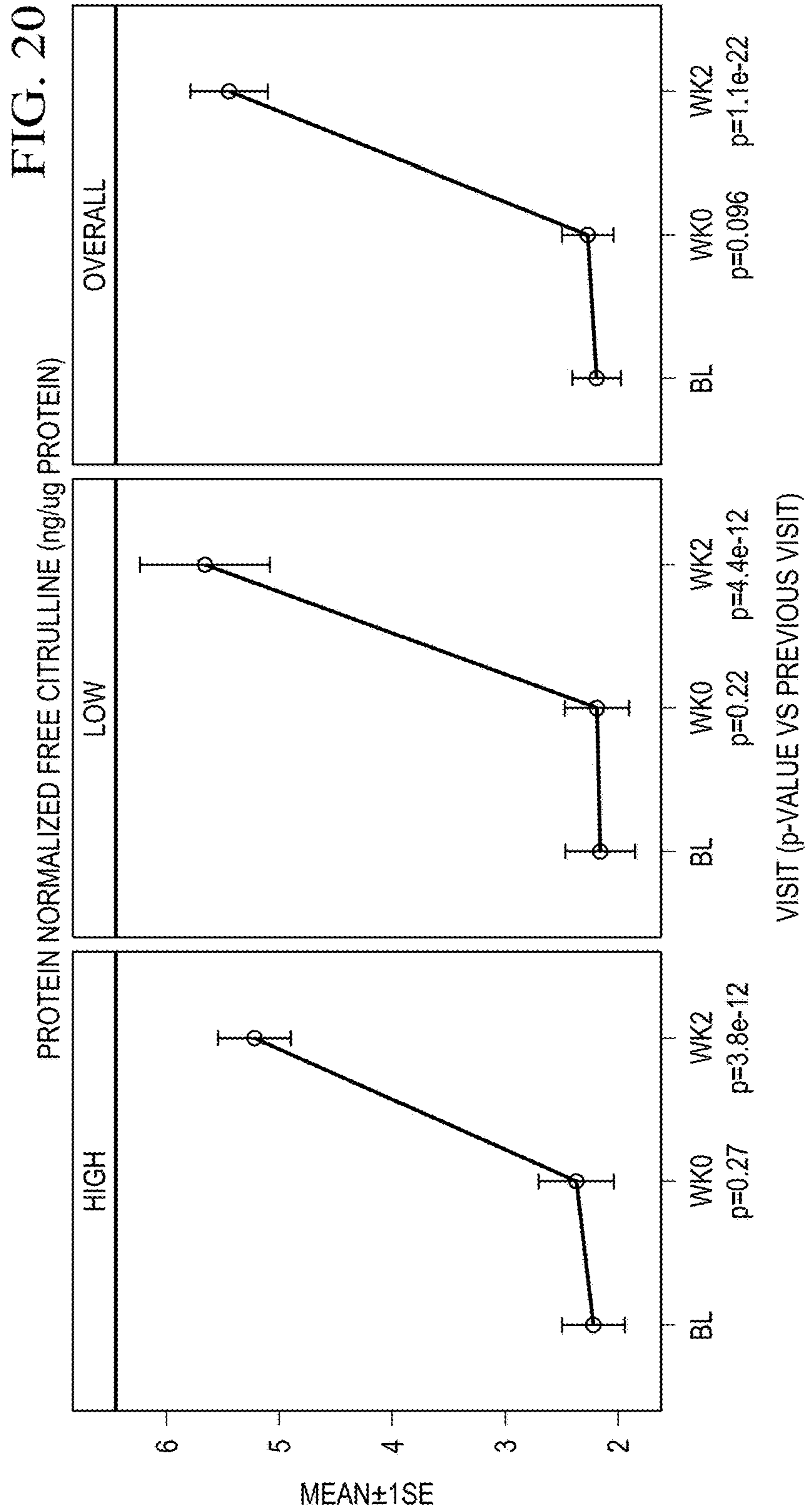
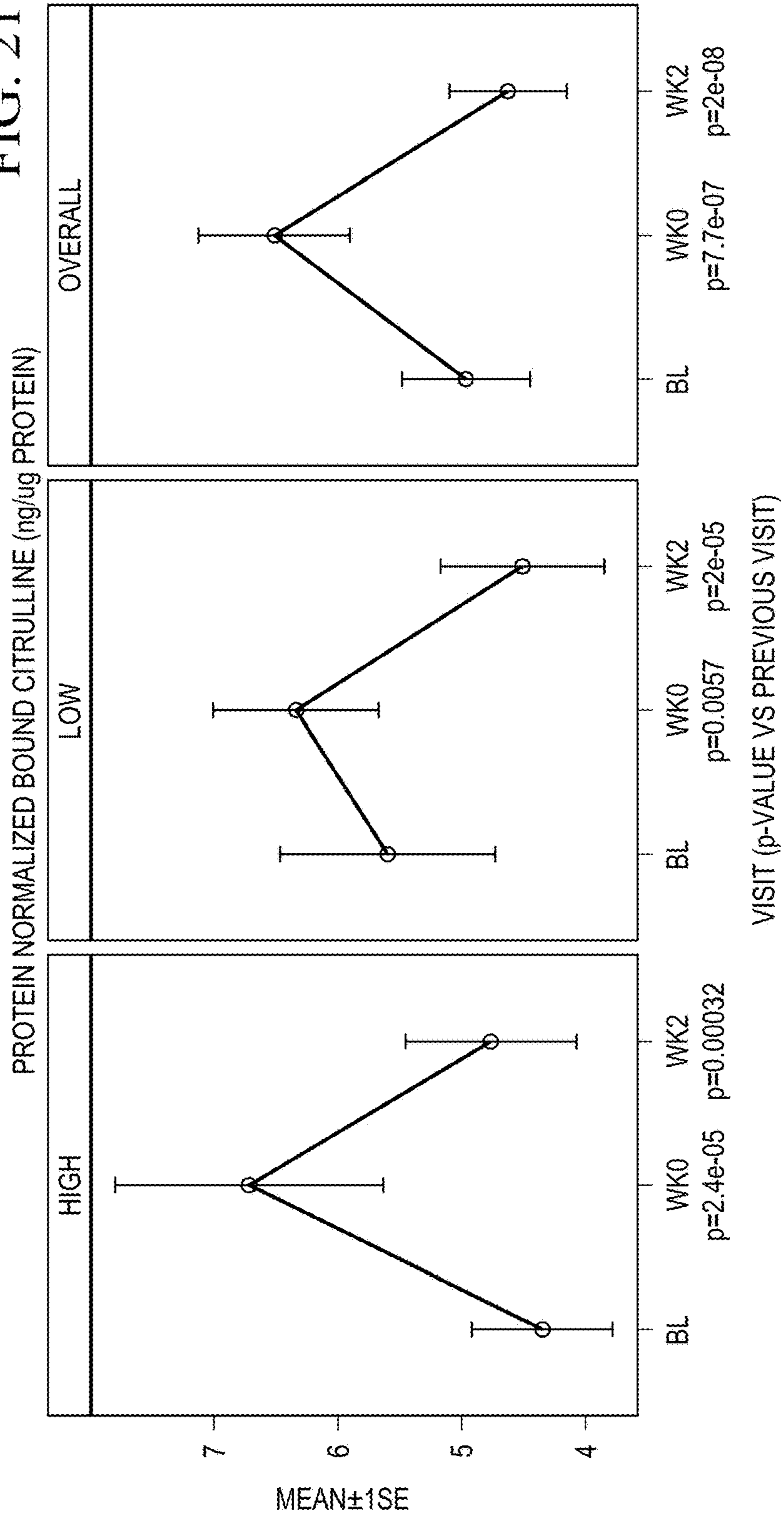




FIG. 21



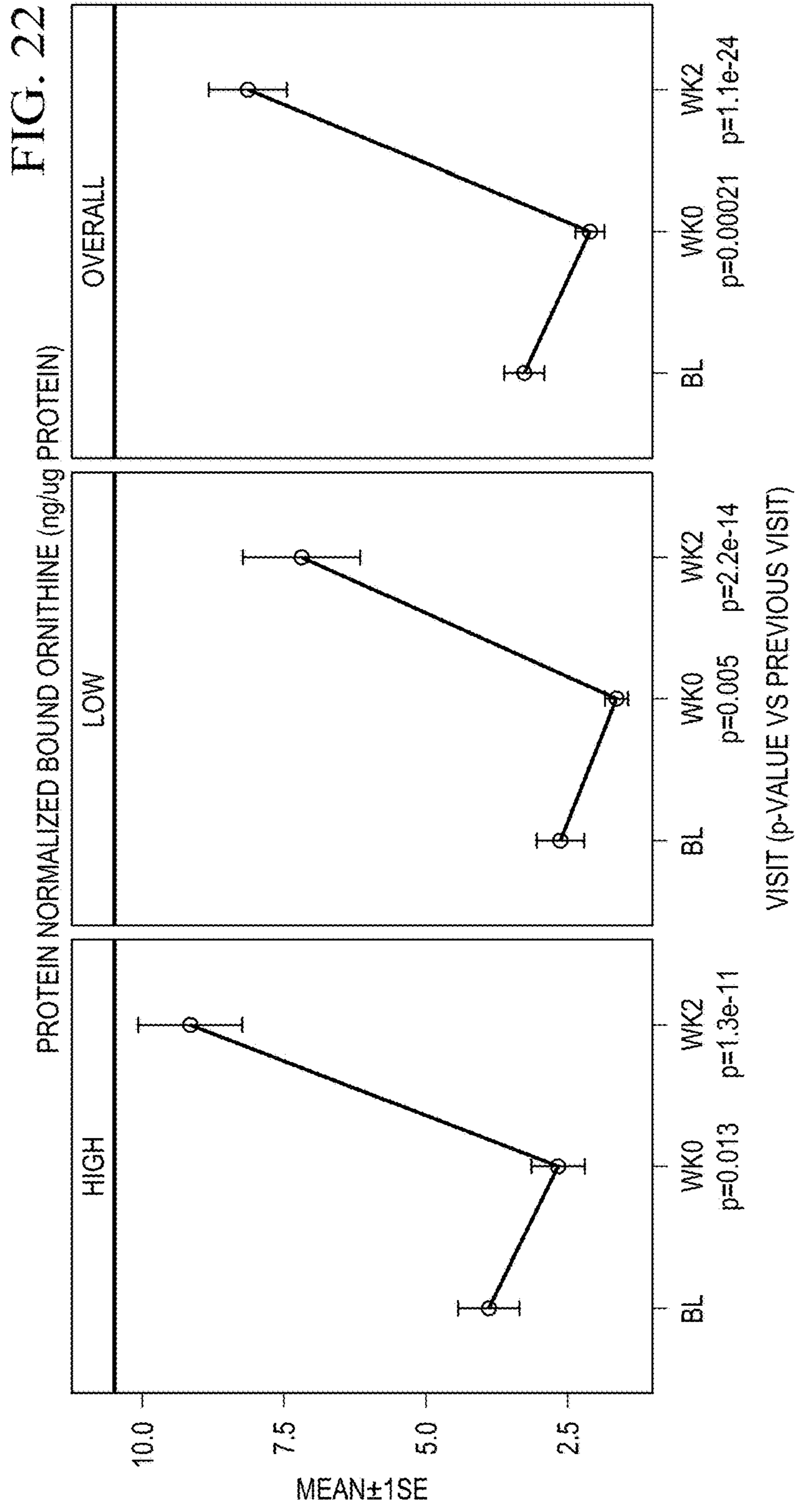


FIG. 23

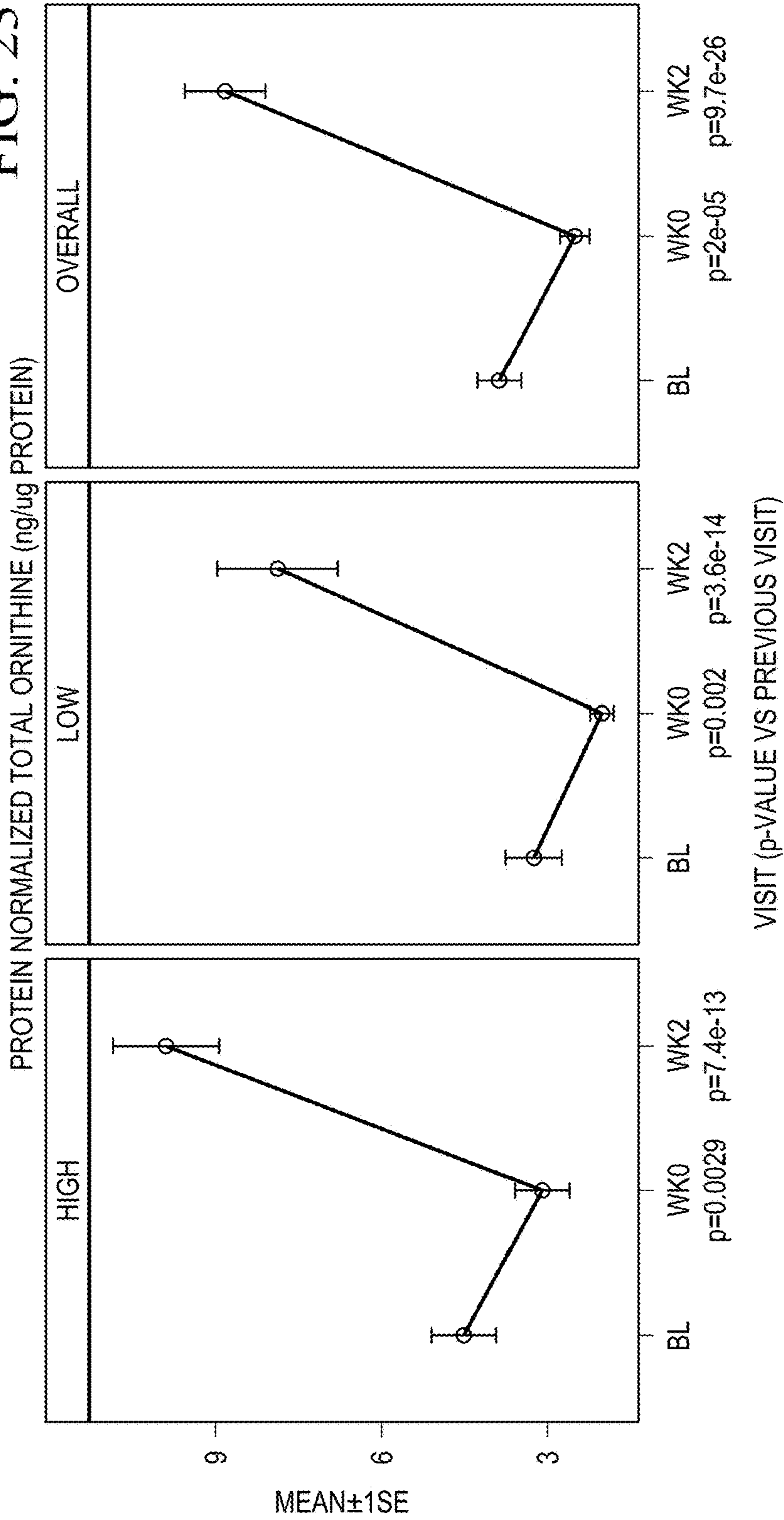




FIG. 24

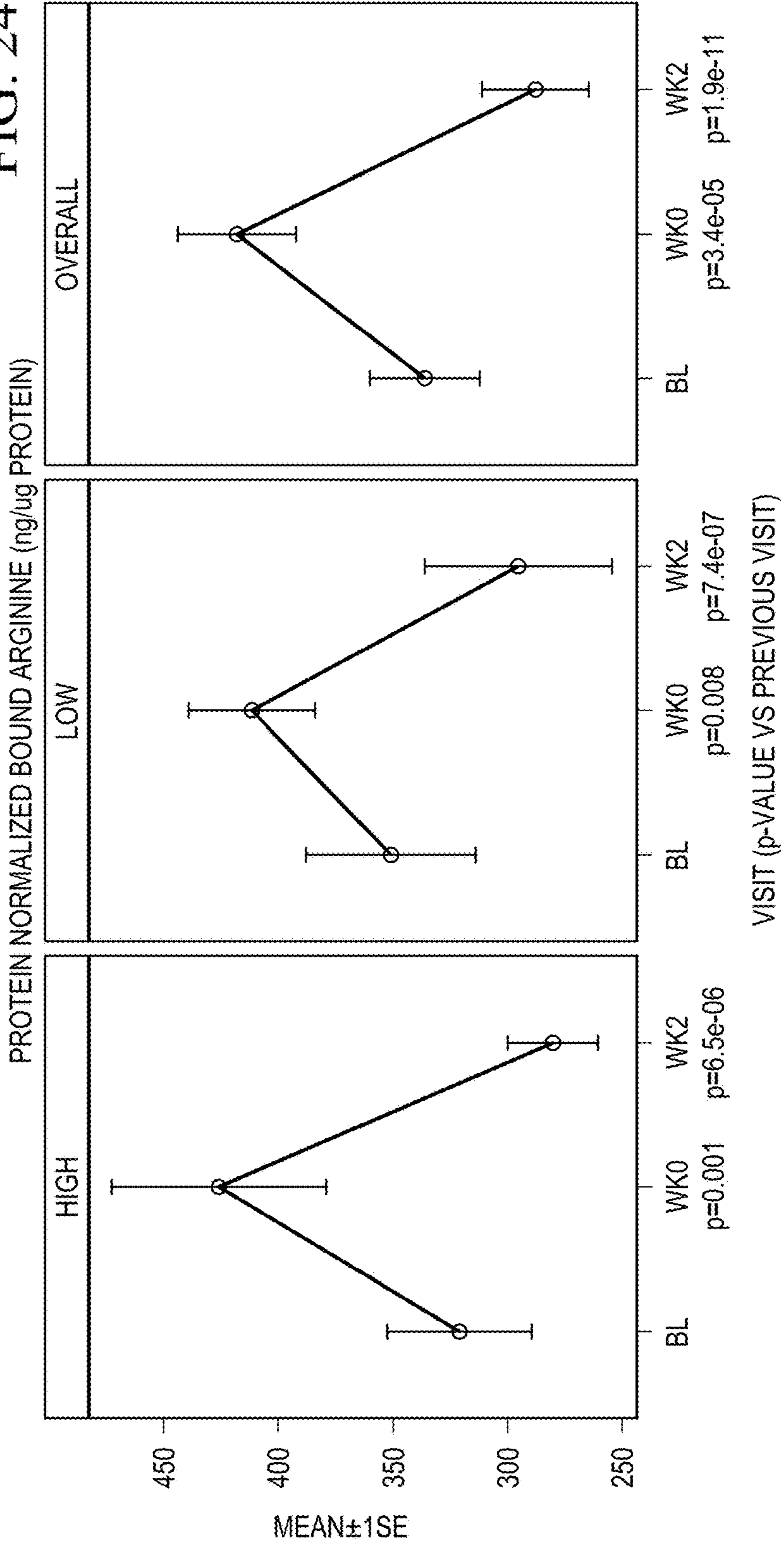
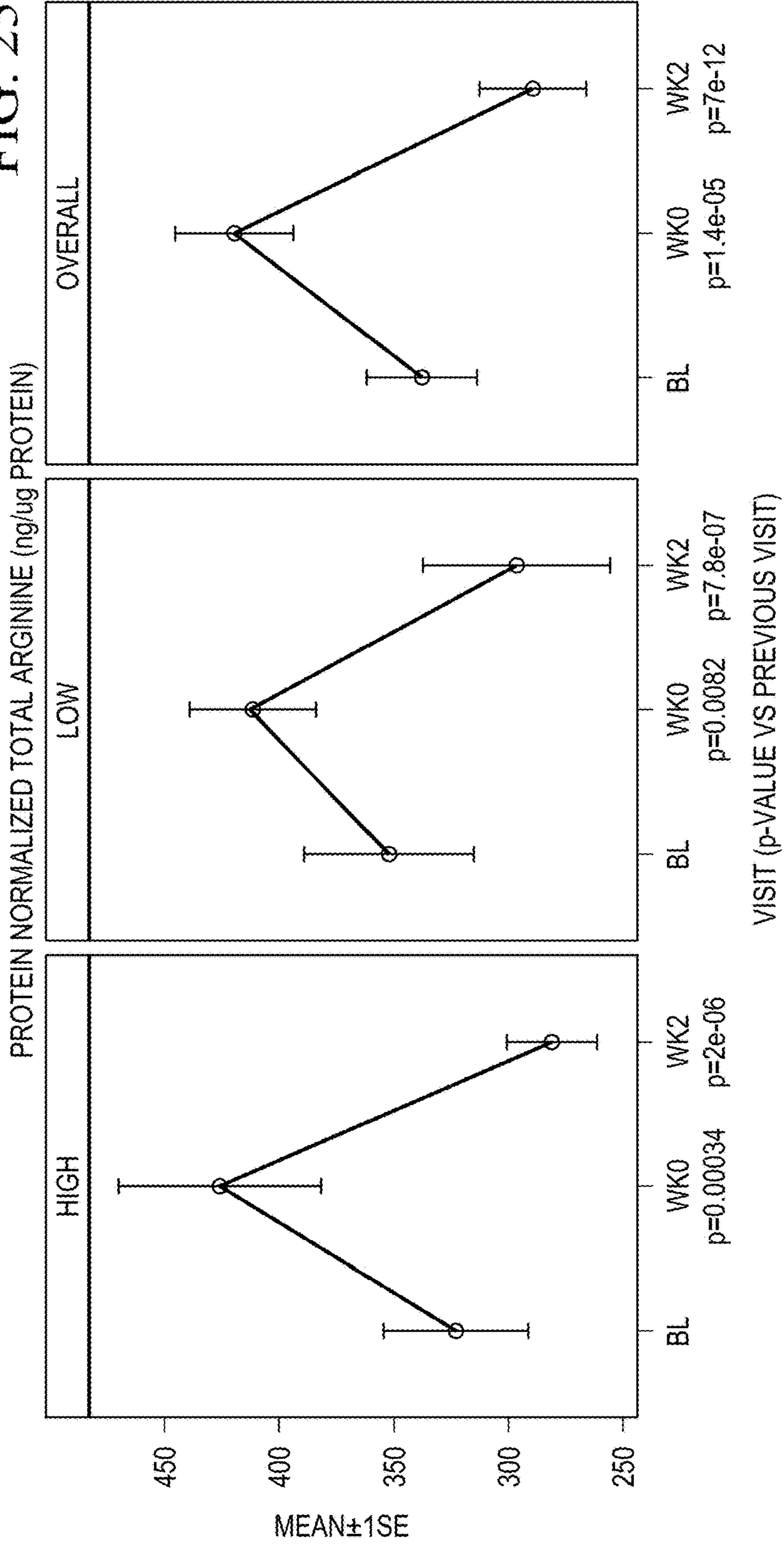


FIG. 25



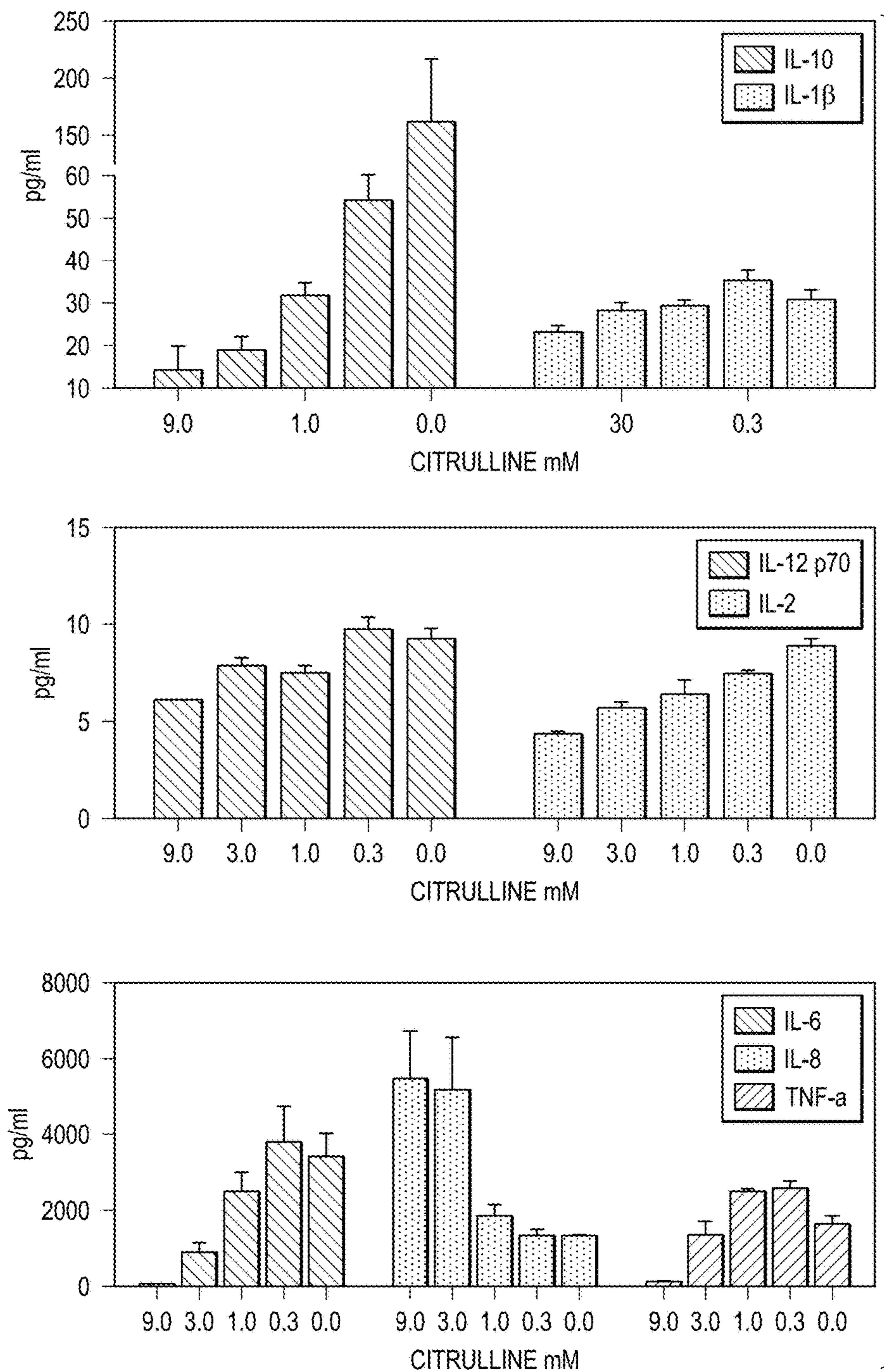


FIG. 26



INHIBITION OF BODIPY TR  
CADAVERINE BY BINDING TO LTA

FIG. 27

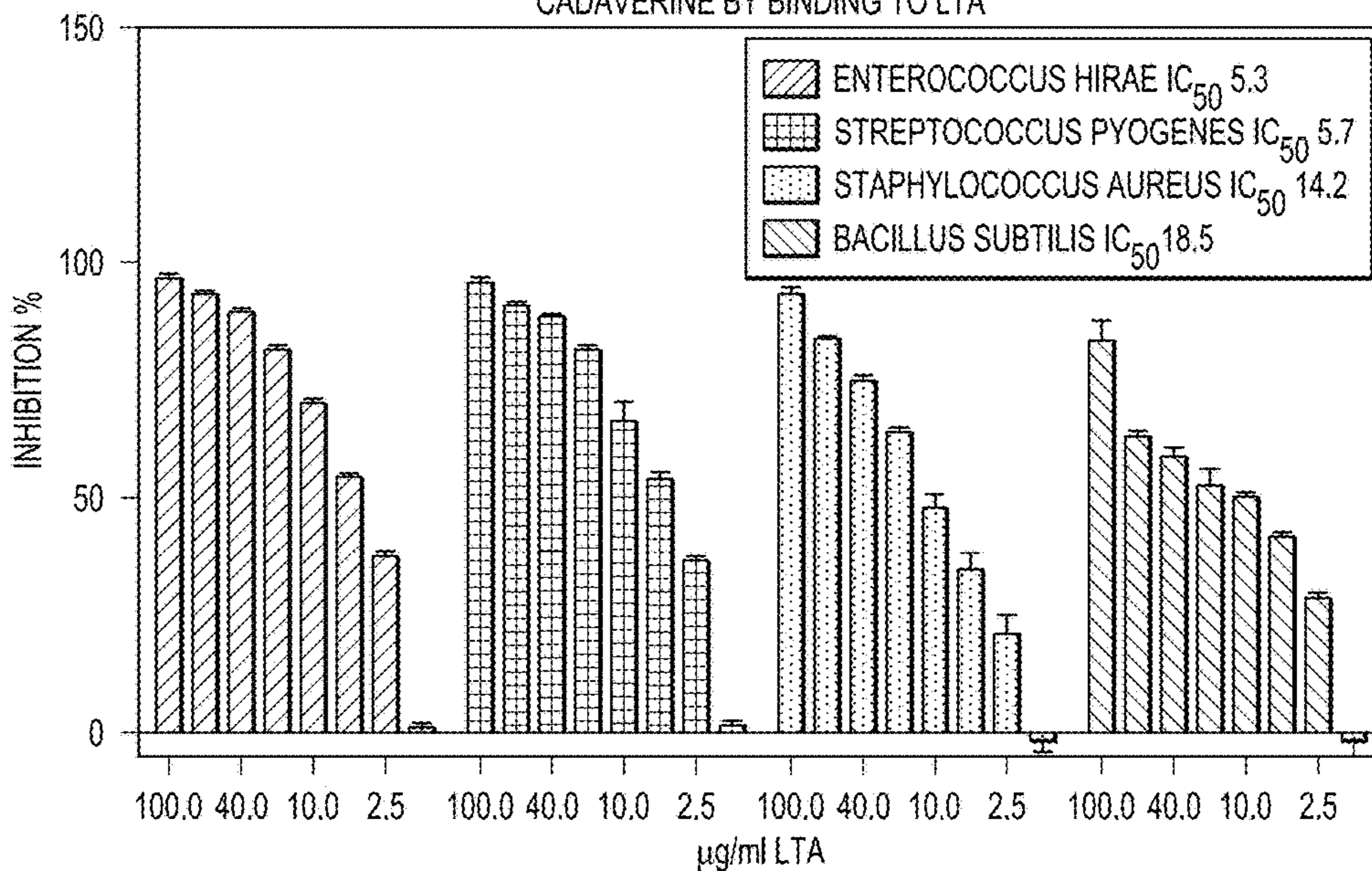
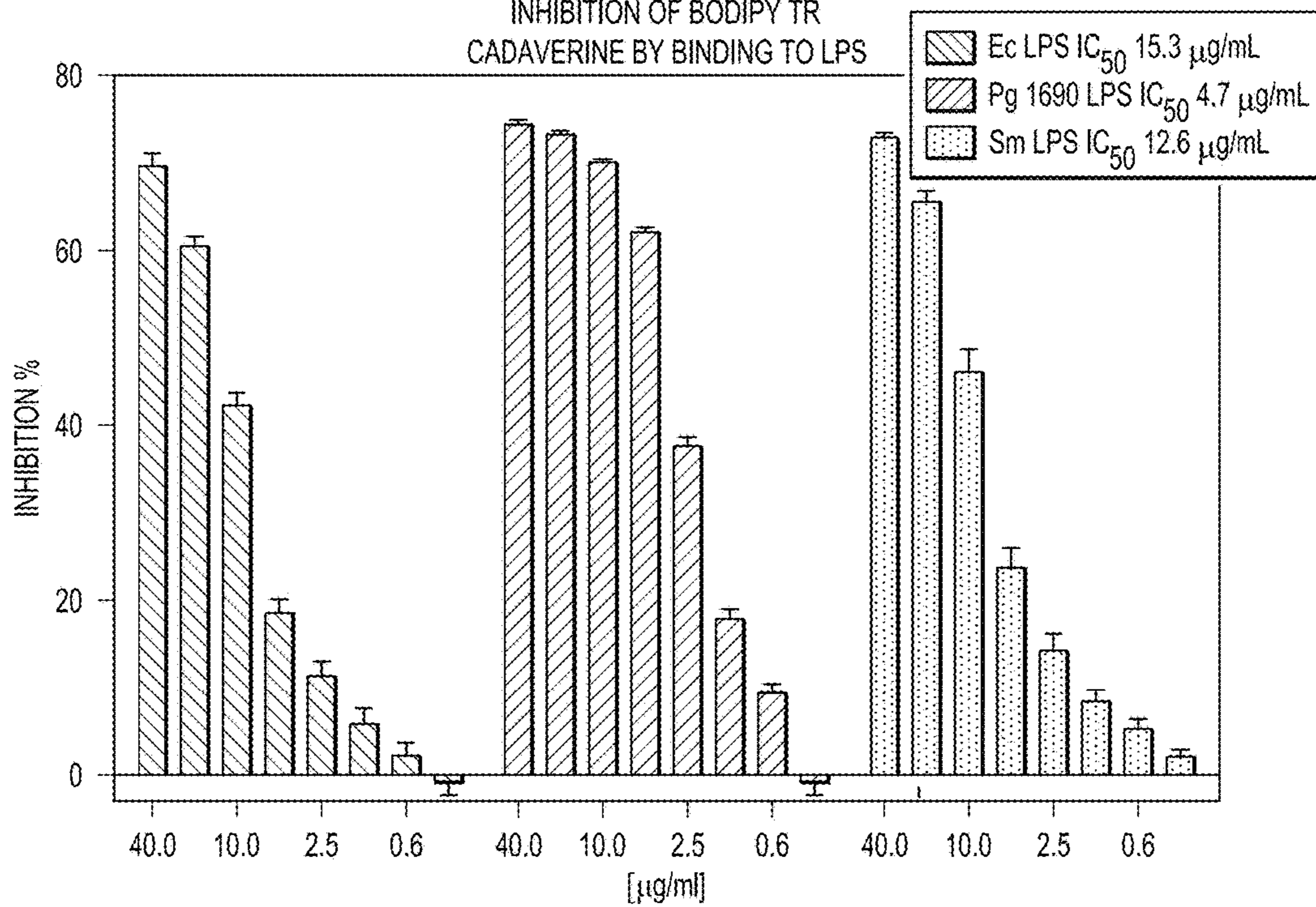


FIG. 28

INHIBITION OF BODIPY TR  
CADAVERINE BY BINDING TO LPS



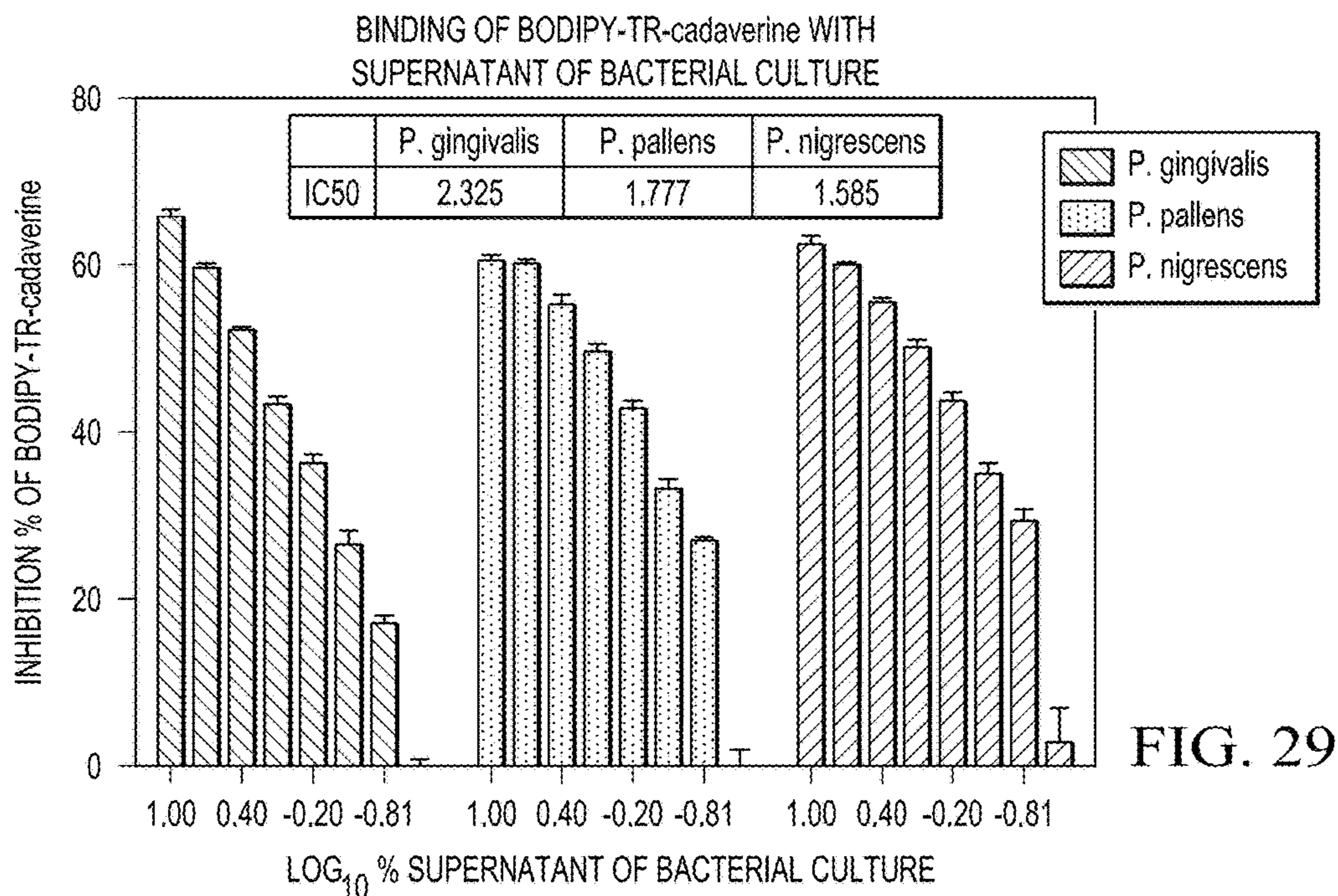


FIG. 29

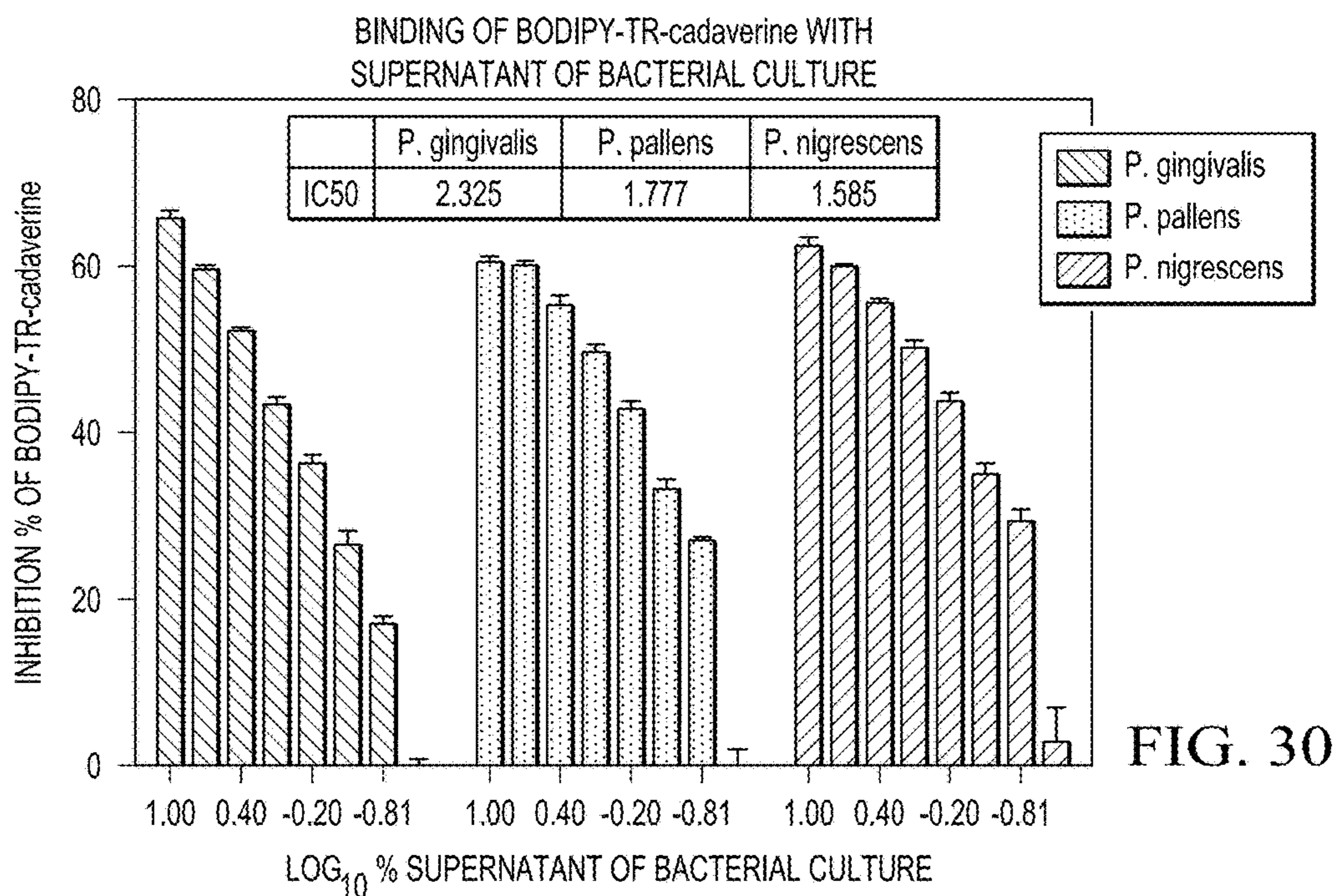


FIG. 30



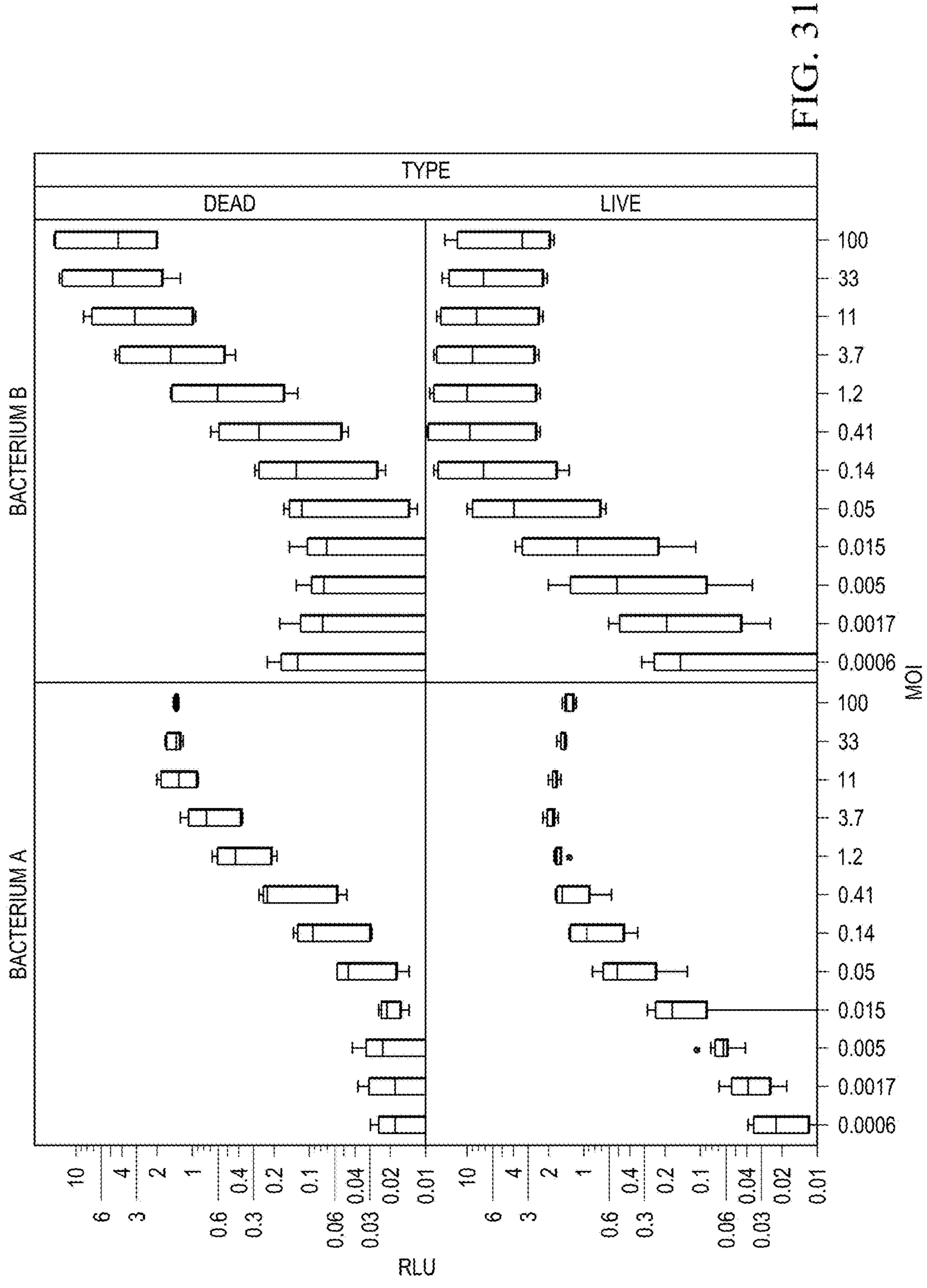


FIG. 31



## DETECTION OF ORAL MICROBIAL VIRULENCE FACTORS

### FIELD OF THE INVENTION

The invention relates to a method for detecting and quantifying bacterial virulence factors in oral samples, and for identifying agents for detoxifying oral virulence factors and a method for determining the effectiveness of detoxifying agents.

### BACKGROUND OF THE INVENTION

The toxins of bacterial cell walls (Henkel et al, *EXS*. (2010) 100: 1-29) have been associated with health related issues, such as septic shock, fever and malaise (V. Liebers et al., *Am J Ind Med*. (2006) 49(6): 474-91). Examples of Gram-negative cell wall toxins associated with health concerns are endotoxins, such as lipopolysaccharide (LPS), peptidoglycans, and fimbriae; and Gram-positive cell wall toxins associated with health concerns are lipoteichoic acid (LTA) and peptidoglycans. There are many other bacterial toxins, such as enterotoxins and exotoxins, as reported in Henkel et al, *EXS*. 2010; 100: 1-29. For the oral environment, the LPS and LTA seem to be the dominant drivers of a bacterial induced immune response, or at least the best characterized. The immune response mounted by the body in response to these toxins depends on the origin of the toxin and the exposure history of the individual to said toxin. The LPS is a component of Gram-negative bacteria that is different from strain to strain, as has been illustrated with the differences in virulence of *E. coli* (Raetz and Whitfield *Annu. Rev. Biochem* (2002) 71:635-700). LPS is composed of a lipid A fraction, core region, and may have an O-antigen. The Lipid A fraction's fatty acid composition has been shown to determine its virulence in response to its interaction with the Toll-like 4 (TLR4) receptor. The LTA has been linked to various inflammatory responses (Y. Yokoyama, et al., *Acta Otolaryngol Suppl*. (1996) 523:108-111) and associated with Toll-like receptor 2 (TLR2) activation. It is widely believed that only the lysed bacteria liberate LPS that can initiate an inflammatory response (CA2323630). However, Zhang et al. showed that growing bacteria secrete LPS at a level in proportion to their growth phase (H. Zhang et al. (1998) *Infection & Immunity*, 66(11), 5196-5201). Therefore, even a small fraction of the plaque left on the teeth after brushing could seed the inflammatory cascade due to the release of LPS from the Gram negative bacteria present in the plaque.

Methods of detecting specific microbial species have been demonstrated in the art. In US Pub. No. 2012/019735A1, methods were proposed to distinguish disease-causing bacteria via spectrophotometric methods. Though they were able to show the presence of specific microbes, their invention would not allow the user to determine the virulence level of a specific site. Further, their method requires the microbes to be cultured in the lab in order to obtain a sufficient quantity of LTA or LPS for detection. Thus, their invention lacks the ability to detect the non-culturable species present, nor would it allow for measures of toxicity of biological samples.

In U.S. Pat. No. 5,175,089, the use of the *Limulus* amoebocyte lysate (LAL) endotoxin (LPS) assay was applied to the determination of the amount of endotoxin in the periodontal pocket. Though they were able to show overall amounts of endotoxin present, they lacked the ability to differentiate diseased versus healthy endotoxin and they

were unable to quantify the level of virulence of the endotoxin. Further, their invention limited them to the Gram-negative endotoxin, as the LTA is not detectable via the LAL kit.

In US Pub. No. 2009/0047240, the chaperonin 10 (Cpn10) was used to modulate the clustering of Cpn10 in a cell line (murine RAW264) with labeled antibodies. Though they showed TLR-4, 7, and 9 reporter genes in an HEK cell line, their system would not allow for a more sensitive or low level detection needed for microbial populations with weaker activating LPS, since those genes were under the control of the NFkB binding sites only (a minimal promoter). Their system lacks the sensitivity needed to differentiate biological systems with multiple microbial species and no dominant organism present. Further, their system needs strong NFkB activators to overcome the weak promoter used in their system, thus unable to pick up weaker TLR LPS agonists, such as LPS from *Porphyromonas gingivalis*. Additionally, their system lacked the ability to detect TLR3 agonists, which would be deleterious to the characterization of an inflammatory disease, such as gingivitis.

US Pub. No. 2007/0160544 describes a method for determining orally deleterious bacteria. Their method calls for contacting a gingival cell with bacteria or a bacterial component and measuring an inflammatory marker. According to US Pub. No. 2007/0160544, the presence of a marker indicates inflammation and the labeling of a bacterium as deleterious. Conversely, they say that the absence of a marker indicates the bacterium is not a problem. Though they cited Toll-like receptors, which were known in the art as part of the pathway to generate cytokines, their method would have only allowed for determining the presence of a cytokine.

Since oral cells contain one or more of the receptors to which a bacterial virulence factor would activate, screening on the individual receptors requires the use of engineered cells, such as reporter cells containing the receptor gene of interest. What further complicates the use of native oral cells, such as gingival cells, is that the expression and activation of a receptor, such as a Toll-like receptor, is specific to the function of the cell. Gingival cells are less likely to respond to bacterial virulence factors, due to their constant contact with microbes in the dental plaque. Thus the need exists to have engineered cells where a direct response can be measured via a reporter system.

In addition to quantifying the virulence of microbial components and byproducts, there also exists a need for an in vitro screen of the inflammatory potential of organic and inorganic molecules, which would allow for pharmacokinetic parameters to be determined.

### SUMMARY OF THE INVENTION

A method of determining bacterial virulence in an oral cavity is provided that includes providing a reporter cell expressing at least one toll-like receptor; providing a sample of oral matter; combining the sample of oral matter and the reporter cell; and measuring the toll-like receptor activation. The method may also include the additional steps of providing another sample of oral matter from an individual who used an oral care composition prior to providing another sample of oral matter; combining the another sample of oral matter and the reporter cell; measuring the toll-like receptor response; and comparing the toll-like receptor response from the sample of oral matter and another sample of oral matter.



A method for determining the virulence of lipopolysaccharide comprising providing a lipopolysaccharide; providing a reporter cell expressing at least one Toll-like receptor; combining the reporter cell with the lipopolysaccharide; measuring the toll-like receptor activation; and quantifying the lipopolysaccharide.

A method for determining the virulence of lipoteichoic acid comprising providing a lipoteichoic acid; providing a reporter cell expressing at least one Toll-like receptor; combining the reporter cell with the lipoteichoic acid; measuring the toll-like receptor activation; and quantifying the lipoteichoic acid.

The present invention includes methods of improving and/or resolving the state of gingival inflammation utilizing molecules, peptides, or proteins/enzymes that bind to, alter, or chemically modify bacterial virulence factors and/or host response mechanisms. The methods provide a means of quantifying the level of lipopolysaccharide of oral tissues; and utilizes TLR-4 and TLR-2 reporter cells line combined with the detection LPS via a fluorescence assay, such as BODIPY TR cadaverine, or endotoxin detection assay, assay to assign potency and quantification of LPS. The invention also includes assays and protocols which enable communication and demonstrations to consumers and dental professionals utilizing the TLR reporter cells.

The present invention includes methods for determining the potency of lipopolysaccharide comprising: a) providing a lipopolysaccharide sample; b) providing reporter cells expressing one or more Toll-like receptors; c) exposing the cells to the lipopolysaccharide sample; d) measuring the EC50 value of the lipopolysaccharide on activation of a Toll-like receptor; e) quantification of the lipopolysaccharide.

The present invention also includes isolating lipopolysaccharide from a growth culture of Gram negative bacteria. A lipopolysaccharide may be isolated from a biological sample. The biological sample includes, but is not limited to, an oral plaque, saliva, gingival brush samples. Toll-like receptor reporter gene assays, such as TLR4-SEAP and/or TLR2-SEAP, may be used to detect and quantify bacterial toxins, including but not limited to endotoxins, in a biological sample.

A BODIPY TR cadaverine assay may be used to detect and quantify lipopolysaccharide in a biological sample. In addition, LAL (the Limulus ameobocyte lysate assay) assay or endotoxin detection assay may be used to detect and quantify the lipopolysaccharide in a biological sample.

The present invention may be directed toward a method for determining the potency of an oral biofilm comprising: a) providing a biofilm sample; b) providing reporter cells expressing one or more Toll like receptors; c) exposing the cells to the biofilm sample; d) measuring the EC50 value of the biofilm activation of a Toll like receptor; e) quantification of the lipopolysaccharide. The biofilm may be an oral plaque, including but not limited to subgingival plaque, marginal or gumline plaque, supragingival plaque.

The present invention may also be directed toward a method for determining the potency of virulence in an oral sample comprising: a) providing an oral sample; b) providing reporter cells expressing one or more Toll-like receptors; c) exposing the cells to the oral sample; d) measuring the EC50 value of the biofilm activation of a Toll like receptor; e) quantification of the lipopolysaccharide. The oral sample may include saliva, oral lavage or gingival crevicular fluid.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A is a graph showing secretion of LPS during bacterial growth in culture.

FIG. 1B is a graph showing time course of bacterial growth in culture medium.

FIG. 1C is a graph showing secretion of LPS during bacterial growth in culture.

FIG. 1D is a graph showing secretion of LPS during bacterial growth in culture at 24 hours. *P. pallens* and *P. nigrescens* released more LPS into the medium than *P. gingivalis*.

FIG. 1E is a graph showing the amount of LPS per bacterium during bacterial growth in culture at 24 hours. *P. pallens* and *P. nigrescens* contain more LPS than *P. gingivalis*.

FIG. 1F is a picture showing the taking of gingival plaque samples using Periopaper.

FIG. 2A is a graph showing cell culture and virulence determination of lipopolysaccharide from different bacteria.

FIG. 2B is a graph showing activation of TLR4 reporter gene in culture.

FIG. 2C is a graph showing activation of TLR4 reporter gene in culture.

FIG. 3 is a graph showing stannous fluoride inhibition of TLR4 reporter gene expression.

FIG. 4 is a graph showing stannous fluoride prevention of *P. gingivalis* LPS from activating TLR2 reporter gene expression.

FIG. 5 is a graph showing percentage reduction of *P. gingivalis* LPS on activation of TLR-4 by stannous fluoride.

FIG. 6 is a graph showing EC50 determination of subgingival plaques that activate TLR4-SEAP signal transduction. Dental plaques from unhealthy sites had a smaller EC50 than those from healthy sites, suggesting the dental plaques from unhealthy sites contain more virulence factors.

FIG. 7A is a graph showing TLR4 Evaluation of Human Subgingival Plaque (Healthy vs. Gingivitis).

FIG. 7B is a graph showing TLR4 Evaluation of Human oral lavage (Healthy vs. Gingivitis), and

FIG. 8A is a graph showing cell culture and virulence determination of LTA from different bacteria.

FIG. 8B is a graph showing cell culture and virulence determination of LPS from different bacteria.

FIG. 8C is a graph showing cell culture and virulence determination of LPS from different bacteria.

FIG. 8D is a graph showing cell culture and virulence determination of LPS from different bacteria.

FIG. 9 is an EC50 graph showing determination of subgingival plaques that activate TLR2-SEAP signal transduction.

FIG. 10A is a graph showing TLR2 Evaluation of Human Subgingival Plaque (Healthy vs. Gingivitis).

FIG. 10B is a graph showing TLR2 Evaluation of Human oral lavage (Healthy vs. Gingivitis).

FIG. 11 is a graph showing cell culture and virulence determination of flagellins from different bacteria.

FIG. 12A is a graph showing LPS Stimulated TLR Activity in Human Monocytes THP-1.

FIG. 12B is a graph showing Evaluation of Human oral lavage (Healthy vs. Gingivitis) using the IRF-Luc reporter gene in THP-1 cells.

FIG. 12C is a graph showing Evaluation of Human Subgingival Plaque (Healthy vs. Gingivitis) in THP-1 cells using the NFkB reporter gene.

FIG. 13 is a graph showing detection of LTA of different bacteria in THP1 dual reporter cells.

FIG. 14 is a graph showing clinical measurements.

FIG. 15 is graphs showing decreases in bacterial abundance in the supragingival plaques during six weeks of regimen treatment.



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FIG. 16 is a graph showing citrulline concentrations in Buccal-brush samples over a six-week regimen treatment.

FIG. 16A is a figure showing the relationship between FIG. 16A-1, FIG. 16A-2, and FIG. 16A-3.

FIG. 16A-1 is graphs showing decreases in bacterial abundance in the supragingival plaques during six weeks of regimen treatment.

FIG. 16A-2 is graphs showing decreases in bacterial abundance in the supragingival plaques during six weeks of regimen treatment.

FIG. 16A-3 is graphs showing decreases in bacterial abundance in the supragingival plaques during six weeks of regimen treatment.

FIG. 17 is a graph showing protein bound ornithine in Buccal brush samples during treatment.

FIG. 18 is a diagram showing enzymes in the ornithine, citrulline and arginine cycle.

FIG. 19 is a graph showing expression of ornithine, citrulline and arginine genes during a 6-week treatment.

FIG. 20 is a graph showing citrulline concentrations increased in Buccal-brush samples in experimental gingivitis.

FIG. 21 is graphs showing protein bound citrulline in Buccal brush samples in experimentally induced gingivitis.

FIG. 22 is graphs showing concentrations of protein bound ornithine in Buccal-brush samples in experimentally induced gingivitis.

FIG. 23 is graphs showing concentrations of total ornithine in Buccal-brush samples in experimentally induced gingivitis.

FIG. 24 is graphs showing concentrations of protein bound arginine in Buccal-brush samples in experimentally induced gingivitis.

FIG. 25 is a graph showing concentrations of total arginine in Buccal-brush samples in experimentally induced gingivitis.

FIG. 26 is a graph showing citrulline inhibited cytokine production stimulated by *P. gingivalis* LPS 1690 in THP-1 cells.

FIG. 27 is graphs showing bacterial LTA reduces fluorescent intensity of BC in a dose dependent manner.

FIG. 28 is graphs showing LPS reduces the fluorescent intensity of BC in a dose-dependent manner.

FIG. 29 is graphs showing the supernatant of bacterial culture reduces the fluorescent intensity of BC in a dose-dependent manner.

FIG. 30 is graphs showing bacteria reduce the fluorescent intensity of BC in a dose-dependent manner.

FIG. 31 is graphs showing EC50 values.

#### DETAILED DESCRIPTION OF THE INVENTION

As disclosed herein, it was surprisingly discovered that one or more methods could be used to detect and quantify the virulence in subgingival and supragingival plaques, thus distinguishing healthy gingivae from an inflamed site suffering from the symptoms of gingivitis. The state of health of the gingivae can be directly related to the level of bacterial toxins present, for example, endotoxins, and thus a reduction of these toxins, as discussed herein, in the oral cavity, as determined by screening on non-gingival engineered cells, would improve overall oral health. Additionally, the way an individual responds to the virulence factors can be quantified utilizing the individual's metabolic pathways, such as by quantifying products of the urea cycle.

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Gingivitis is defined per the FDA monograph (12 CFR Part 356, Vol. 68, No. 103 (2003)) as "An inflammatory lesion of the gingiva that is most frequently caused by dental plaque. Gingivitis is characterized by tissue swelling and redness, loss of stippling (a normal state in which the surface of healthy gingiva is comprised of small lobes), glossy surface, and increased tissue temperature. The gingiva also may bleed upon gentle provocation, such as tooth brushing or may bleed spontaneously. Gingivitis is usually not painful." Within the monograph, plaque is defined as being composed of multiple bacterial species. Those species exert a constant inflammatory pressure on the host tissues.

When the inflammation progresses to the state of gingivitis, there exists a need to quantify how severe the gingivitis is and how effective treatments from oral hygiene products are in reducing the inflammatory response. The reduction in inflammatory response due to activation of membrane bound receptors across the gingival cells is termed detoxification; and measuring the level of detoxification, which is lacking in the art, is needed to educate consumers on the efficacy of their oral hygiene.

Pathogenesis of gingivitis involves both bacteria and host responses. The present invention discloses methodologies measuring the virulence factors in the dental plaques in vitro, and also measuring effects of virulence factors on gingival tissues in vivo. These methodologies allow understanding of what virulence factor types are present in the dental plaques, and how the host responds. Importantly, the measuring of virulence factors provides a detailed assessment on the severity of gingivitis in terms of virulence factors of the microbes in dental plaques and the health status of the host. In addition, these methods help evaluate the effectiveness of a technology in preventing and treating gingivitis.

The present invention includes a methodology comprising one or more of the following steps: (1) using the endotoxin detection assay kit or BODIPY-TR cadaverine method to quantify total LPS and LTA present in a biological sample and to detect technologies that inhibit the endotoxin detection assay; (2) using the Toll like receptor assays to determine potency of purified virulence and oral dental plaques, and to measure the efficacy of technologies that neutralize toxicity of virulence factors; (3) using meta-sequencing to identify and quantitate bacteria in supragingival plaques, (4) measuring ornithine and citrulline in gingival swab samples to determine the healthy status of gingival tissue, (5) measuring protein and mRNA levels to determine the levels of host responses, and determining citrulline activities in inhibiting LPS-induced production of proinflammatory cytokines.

The above-mentioned methods and chemistry may be applied in a strip form to the outer surface of the tooth and gumline. The strip may contain color or fluorescence reagents to interact with the virulence factors present and thus allow for semi-quantitative determination of the virulence present.

This would allow for rapid assessment of the level of severity of gingivitis and/or periodontal disease or to ascertain the effectiveness of oral products. This execution of the described methods allows for consumers to ascertain the state of their dental health at home or allows for professionals, such as dentists, to rapidly measure the state of a patient's oral health.

The methods described above can be used to determine the cellular impact of organic and inorganic molecules, as long as there is an interaction between the molecule and the targeted receptor. Further, the methods could be used to



ascertain if a molecule would cause an irritation or inflammatory response. The receptor in question would be used in a reporter system, as described herein, and the molecular impact of the molecule in question determined. The EC50 value of the molecule in question would be determined using the receptor associated with the biological response, thus reducing or eliminating the need to do animal testing.

All percentages and ratios used hereinafter are by weight of total composition, unless otherwise indicated. All percentages, ratios, and levels of ingredients referred to herein are based on the actual amount of the ingredient, and do not include solvents, fillers, or other materials with which the ingredient may be combined as a commercially available product, unless otherwise indicated.

All measurements referred to herein are made at 25° C. (i.e. room temperature) unless otherwise specified

As used herein, the word “include,” and its variants, are intended to be non-limiting, such that recitation of items in a list is not to the exclusion of other like items that may also be useful in the materials, compositions, devices, and methods of this invention.

As used herein, the word “or” when used as a connector of two or more elements is meant to include the elements individually and in combination; for example X or Y, means X or Y or both.

By “personal care composition” is meant a product, which in the ordinary course of usage is applied to or contacted with a body surface to provide a beneficial effect. Body surface includes skin, for example dermal or mucosal; body surface also includes structures associated with the body surface for example hair, teeth, or nails. Examples of personal care compositions include a product applied to a human body for improving appearance, cleansing, and odor control or general aesthetics. Non-limiting examples of personal care compositions include hair coloring compositions, oral care compositions, after shave gels and creams, pre-shave preparations, shaving gels, creams, or foams, moisturizers and lotions, cough and cold compositions, leave-on skin lotions and creams, shampoos, conditioners, shower gels, bar soaps, toilet bars, antiperspirants, deodorants, depilatories, lipsticks, foundations, mascara, sunless tanners and sunscreen lotions.

By “oral care composition”, as used herein, is meant a product, which in the ordinary course of usage, is not intentionally swallowed for purposes of systemic administration of particular therapeutic agents, but is rather retained in the oral cavity for a time sufficient to contact dental surfaces or oral tissues. Examples of oral care compositions include dentifrice, mouth rinse, mousse, foam, mouth spray, lozenge, chewable tablet, chewing gum, tooth whitening strips, floss and floss coatings, breath freshening dissolvable strips, or denture care or adhesive product. The oral care composition may also be incorporated onto strips or films for direct application or attachment to oral surfaces.

The term “dentifrice”, as used herein, includes tooth or subgingival-paste, gel, or liquid formulations unless otherwise specified. The dentifrice composition may be a single phase composition or may be a combination of two or more separate dentifrice compositions. The dentifrice composition may be in any desired form, such as deep striped, surface striped, multilayered, having a gel surrounding a paste, or any combination thereof. Each dentifrice composition in a dentifrice comprising two or more separate dentifrice compositions may be contained in a physically separated compartment of a dispenser and dispensed side-by-side.

The term “teeth”, as used herein, refers to natural teeth as well as artificial teeth or dental prosthesis.

Virulence factors are molecules produced by pathogenic microbes that contribute to the pathogenicity of the organism and enable them to invade and proliferate in the host, and evade host immune surveillance. Virulence factors include, but are not limited to the following: Gram positive and Gram negative cell wall components, such as lipopolysaccharide and lipoteichoic acids, bacterial DNA, flagellin, peptidoglycan, adhesins, invasins, and antiphagocytic factors, hemolysins, bacterial metabolites, fimbriae, outer membrane vesicles, bacterial proteins or bacterial enzymes. A reduction in virulence or “detoxification”, based on reduced activation of Toll-like receptors, can be used to measure the effectiveness of various treatments.

The term “detoxification” or “detoxify” or “detox” as used herein, refers to the neutralization, reduction, or removal of microbial virulence factors as measured by a reduction in the activation of a receptor known to be responsive with a virulence factor from an engineered cell, such as a reporter cell line. In certain embodiments a determination of virulence is assigned based on the activation of one or more of the Toll-like receptors.

The present invention includes obtaining a sample of oral matter. The oral matter can include gum-line plaque, subgingival plaque, supragingival plaque, interstitial plaque, gingival crevicular fluid (GCF), gingival biopsy, saliva, or tongue swab. The oral matter may be obtained by any method known in the art, for example, subgingival plaque sample may be collected physically by scraping or by using paper points. The plaque may be collected off of the tooth beneath gums from the sulcus, the developed periodontal pocket, or at the gumline. For example, each paper point can be placed in the pocket between the tooth and the gingiva for 10 seconds. After 10 seconds, a paper point can be removed and placed into a pre-labeled 1.5 ml tube with 700 µl phosphate-buffered saline. The sampling procedure can be repeated with three more paper points. After all four paper points are collected, the 1.5 ml tube will be closed, vortexed for 30 seconds and placed on dry ice until the samples are stored in a -80° C. freezer. Other methods of collection could include a mechanical device to help release the plaque from the tooth surface, such as a sonic scaler. The oral matter may be obtained both before and after treatment of an oral site from which the oral matter is obtained. An oral site from which the oral matter is obtained includes host tissues and bacterial matters. Further treatment of an oral site may be more than once and may include multiple different treatments, for example a regimen, such as brushing teeth followed by mouthrinse. In addition to obtaining oral matter before and after completed treatments, oral matter may be obtained between separate treatments, for example between the brushing of teeth followed by the use of mouth rinse.

The sample of oral matter is combined with a Toll-like receptor in a reporter cell. Examples of Toll-like Receptors that can be used in the present invention include TLR 2, TLR 4, TLR 5, and TLR 9. Human TLR cDNA (TLR 1, 2, 3, 4, and 5) was first cloned in 1998 and their sequences were published (Rock F L, Hardiman G, Timans J C, Kastelein R A, Bazan J F. A family of human receptors structurally related to *Drosophila* Toll. Proc Natl Acad Sci USA. 1998 Jan. 20; 95:588-93). Rock et al. discovered that cytoplasmic domains of *Drosophila* Toll and human interleukin 1 receptors shared high sequence homology, and hypothesized that both molecules trigger related signaling pathways tied to the nuclear translocation of Rel-type transcription factors. They cloned a class of putative human receptors with a protein architecture that is similar to *Drosophila* Toll in both intra- and extracellular segments. They hypothesized that the five



human Toll-like receptors—named TLRs 1-5—are probably the direct homologs of the fly molecule and, as such, could constitute an important and unrecognized component of innate immunity in humans. Soon after the DNA sequences for TLR1 to 5 were published, other TLR DNA sequences were also revealed. For example, TLR6 cDNA sequence was reported in 1999 (Takeuchi O1, Kawai T, Sanjo H, Copeland N G, Gilbert D J, Jenkins N A, Takeda K, Akira S. A novel member of an expanding toll-like receptor family. *Gene*. 1999 Apr. 29; 231(1-2):59-65). cDNA sequences of human TLR7, TLR8 and TLR9 were reported in 2000 (Chuang TH1, Ulevitch R J. Cloning and characterization of a sub-family of human toll-like receptors: hTLR7, hTLR8 and hTLR9. *Eur Cytokine Netw*. 2000 September; 11(3):372-8).

SEQ ID NO	Sequence
1	Human Toll-Like Receptor 1 Amino Acid sequence
2	Human Toll-Like Receptor 2 Amino Acid sequence
3	Human Toll-Like Receptor 3 Amino Acid sequence
4	Human Toll-Like Receptor 4 Amino Acid sequence
5	Human Toll-Like Receptor 5 Amino Acid sequence
6	Human Toll-Like Receptor 6 Amino Acid sequence
7	Human Toll-Like Receptor 7 Amino Acid sequence
8	Human Toll-Like Receptor 8 Amino Acid sequence
9	Human Toll-Like Receptor 9 Amino Acid Sequence

A sequence listing that sets forth the amino acid sequences for SEQ ID NO: 1 to 9 herein is being filed concurrently with the present application as an ASCII text file titled “13837M\_AA\_Sequence\_Listing\_ST25.” The ASCII text file was created on 29 Mar. 2016 and is 68 Kbytes in size. In accordance with MPEP § 605.08 and 37 CFR § 1.52(e), the subject matter in the ASCII text file is incorporated herein by reference.

TLR proteins can form heterodimers or homodimers. There are 10 TLR genes identified in humans. Their gene products form homodimers or heterodimers in cell membranes. For example, TLR1 can form heterodimers with TLR2. Similarly, TLR6 also can assemble a heterodimer with TLR2. On other hand, TLR4 forms a homodimer. Toll-like receptors (TLRs) are a class of proteins that play a key role in the innate immune system as well as the digestive system. They are membrane-spanning, non-catalytic receptors usually expressed in cells such as macrophages, dendrite cells, and gingival epithelial cells that recognize structurally conserved molecules derived from microbes. Once these microbes have breached physical barriers such as the skin or intestinal tract mucosa or oral epithelial cells they are recognized by TLRs, which activate immune cell responses. Toll-like receptors were targeted because they are the primary sensors of virulence factors produced by the microbes in the dental plaques. TLR 1, 2, 4, 5 and 6 are expressed in the cell plasma membranes, providing easy access for the virulence factors to be sensed by the host receptors. TLR 3, 7, 8, and 9 are located on the membranes of endosomes; and as virulence factors are phagocytosed into the cells, they also gain access to TLR 3, 7, 8 and 9

A reporter cell refers to a eukaryotic cell, such as, but not limited to, HEK 293T, human monocyte (THP1), Chinese hamster ovary (CHO) cell, murine cells, or monkey kidney epithelial (Vero) cells engineered to express a predetermined number of TLR receptors, for example a single TLR receptor; which is in contrast to gingival cells that express multiple functional TLR receptors. Thus, one type of engineered reporter cells respond to only one type of virulence

factors in the dental plaques. In contrast, gingival cells express several types of functional TLRs, and can't be used to identify a single type of virulence factor in dental plaques. The output from gingival cells is the sum of various virulence factors in the dental plaques. HEK 293T cells can be used as reporter cells, as they are easy to maintain and have similar gene expression profiles to oral epithelial cells, making them a closer match to the gene expression of gingival cells, such that the results will mirror in vivo results. In contrast to naturally occurring gingival cells the reporter cells of the present invention are easy to maintain in the lab, and stable in phenotypes. Further, the reporter cells make detection of virulence factors simpler, are more reproducible, increase accuracy, provide higher throughput are more specific and more quantifiable.

Individual TLR receptor genes are stably transfected to HEK 293 cells as described by Invivogen (/PDF/HEK\_Blue\_hltr4\_TDS.pdf). HEK-Blue™ hTLR4 Cells are designed for studying the stimulation of human TLR4 (hTLR4) by monitoring the activation of NF-kB. HEK-Blue™ hTLR4 Cells were obtained by co-transfection of the hTLR4 gene, the MD-2/CD14 co-receptor genes and a secreted embryonic alkaline phosphatase (SEAP) reporter gene into HEK293 cells. The SEAP reporter gene is placed under the control of an IL-12 p40 minimal promoter fused to five NF-kB and AP-1-binding sites (Supplement 1. HEK-Blue™ hTLR4 Cells SEAP Reporter 293 cells expressing the human TLR4 gene, Catalog # hkb-hltr4, Version #15C04-MM (/PDF/HEK\_Blue\_hltr4\_TDS.pdf). As compared to measuring an immune response the reporter genes of the present invention allow rapid, specific and reproducible measurements of virulence factors.

The level of receptor activation can be determined by any method known in the art for the type of reporter gene used. For example if an NFkB-SEAP reporter gene is used, one could measure the production of SEAP in the culture medium. The reporter cells can be treated with virulence factors, or dental plaque matters collected before or after treatments. Expression of the reporter gene will be stimulated and SEAP secreted into the medium when stimulated by virulence factors. The level of reporter gene product SEAP can be readily measured with commercial kits, and will be proportional to the amount of particular types of virulence factors. Similarly, if an NFkB-luciferase, NFkB-beta-lactamase, or other reporter genes are used, available kits can be used to measure the reporter gene products.

The potency can then be determined based on parameters such as, EC50 and fold of stimulation. EC50 provides a measurement on the amount of virulence factors needed to mount an inflammatory response, and the fold of stimulation is indicative of the severity of inflammatory responses the virulence factors cause. The EC50 is used to determine potency; wherein “potency” as defined by the Merck Manual, refers to the concentration (EC50) of a chemistry required to produce 50% of the chemistry's maximal effect as depicted by a graded dose-response curve. EC50 equals Kd (Dissociation constant, which is a measure of 50% of the substance in question bound to the receptor) when there is a linear relationship between occupancy and response. Often, signal amplification occurs between receptor occupancy and response, which results in the EC50 for response being much less (ie, positioned to the left on the abscissa of the log dose-response curve) than Kd for receptor occupancy. Potency depends on both the affinity of a compound for its receptor, and the efficiency with which a compound-receptor interaction is coupled to response. The dose of a compound required to produce an effect is inversely related to potency.



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In general, low potency is important only if it results in a need to administer a compound in large doses that are impractical. Quantal dose-response curves provide information on the potency of a compound that is different from the information derived from graded dose-response curves. In a 5 quantal dose-response relationship, the EC50 is the dose at which 50% of individuals exhibit the specified quantal effect.

In the present invention, the activities of endotoxins or lipopolysaccharides can be measured using an endotoxin 10 detection assay, or LAL assay. The Limulus Amebocyte Lysate (LAL) test has been used to detect LPS. LAL is derived from the blood cells, or amebocytes, of the horseshoe crab, *Limulus polyphemus*. At present, some main endotoxin detection agents are derived from recombinant 15 proteins. Thereinafter, endotoxin detection assay and LAL assay are used interchangeably.

- A. A method of determining bacterial virulence in an oral cavity comprising:
  - a. providing a reporter cell expressing at least one toll-like 20 receptor;
  - b. providing a sample of oral matter;
  - c. combining the sample of oral matter and the reporter cell;
  - d. measuring the toll-like receptor activation. 25
- B. The method of paragraph A, wherein the toll-like receptor is at least one of TLR1 to TLR9.
- C. The method according to paragraph A or B, wherein the reporter cell expresses at least of one TLR2 or TLR4.
- D. The method according to any one of paragraphs A to C, 30 wherein the toll-like receptor activates a reporter gene, preferably wherein the reported gene is a secreted embryonic alkaline phosphatase (SEAP) reporter gene.
- E. The method according to any one of paragraphs A to D, wherein the sample of oral matter is at least one of 35 gum-line plaque, subgingival plaque, supragingival plaque, interstitial plaque, gingival crevicular fluid (GCF), gingival biopsy, saliva, or tongue swab, preferably wherein the sample of oral matter comprises virulence factor that is at least one of Gram positive cell wall 40 components, Gram negative cell wall components, bacterial DNA, flagellin, peptidoglycan, bacterial metabolites, fimbriae; outer membrane vesicles bacterial proteins, or bacterial enzymes.
- F. The method of paragraph E, wherein the sample of oral 45 matter comprises virulence factor that is at least one of lipopolysaccharide or lipoteichoic acid.
- G. The method according to any of paragraphs A to F comprising the additional steps of:
  - e. providing another sample of oral matter from an 50 individual who used an oral care composition prior to providing another sample of oral matter;
  - f. combining the another sample of oral matter and the reporter cell;
  - g. measuring the toll-like receptor response;
  - h. comparing the toll-like receptor response from the sample of oral matter and another sample of oral matter.
- H. The method of paragraph G, where the sample of oral matter and the another sample of oral matter are from the 60 same individual.
- I. A method for determining the virulence of lipopolysaccharide comprising:
  - a. providing a lipopolysaccharide;
  - b. providing a reporter cell expressing at least one Toll- 65 like receptor;
  - c. combining the reporter cell with the lipopolysaccharide;

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- d. measuring the toll-like receptor activation;
- e. quantifying the lipopolysaccharide.
- J. The method of paragraph I, wherein the Toll-like receptor activation is determined by measuring at least one of 5 EC50 or fold of stimulation.
- K. The method according to paragraph I or J, wherein the Toll-like receptor is at least one of TLR1 to TLR9.
- L. The method according to any of paragraphs I to K, wherein the reporter cell expresses at least one of TLR2 10 or TLR4.
- M. The method according to any of paragraphs I to L, wherein the Toll-like receptor activates a reporter gene.
- N. The method according to paragraph M, wherein the reported gene is a secreted embryonic alkaline phosphatase (SEAP) reporter gene. 15
- O. The method according to any of paragraphs I to N, wherein the lipopolysaccharide is quantified using at least one of limulus amebocyte lysate assay or BODIPY TR cadaverine assay.

## EXAMPLES

## Example 1—Determination of Secreted Lipopolysaccharide by Bacteria

Growth of bacteria: A 1 ml aliquot of a 24 hour culture of *E. coli* ATCC 8739 was used to inoculate 100 ml of Luria-Bertani (LB) broth in a 250 ml baffled flask. This culture was then incubated at 37° C. with agitation (220 rpm) and sampled at 30 minute intervals. Samples were assessed for turbidity (OD<sub>600</sub>) in a SpectraMax platereader M3 (Molecular Devices, Sunnydale, Calif.), which is one method of monitoring the growth and physiological state of microorganisms. The sample turbidity was then recorded and the samples were centrifuged at 5000 RPM for 10 min at room temperature. The supernatant, thereinafter referred to as “supernatant of bacterial culture”, was subsequently analyzed for LPS content using the procedure as described below.

Twenty ml aliquots of MTGE broth (Anaerobe Systems, Morgan Hill, Calif.) were inoculated with *P. gingivalis* ATCC 33277, *P. pallens* ATCC 700821, or *P. nigrescens* ATCC 25261. These cultures were incubated overnight in a Whitley A45 Anaerobic Workstation (Don Whitley Scientific, Frederick, Md.) at 37° C. with an 85:10:5 N<sub>2</sub>:CO<sub>2</sub>:H<sub>2</sub> gas ratio. One ml aliquots of these starter cultures were then used to inoculate 99 ml of membrane-Tryptone Glucose Extract (m-TGE) broth in a 250 ml baffled flask. These cultures were then incubated under agitation (200 rpm) as 40 previously described and sampled at regular intervals. Samples were assessed for turbidity (OD<sub>600</sub>) in a Tecan Infinite m200 Pro (Tecan Trading AG, Switzerland) and then centrifuged at 16,100×g for 10 min at room temperature. Supernatants were decanted and passed through a 0.22 μm 50 filter prior to analysis for LPS content.

In the experiment, only OD600 was measured. For the sake of consistency in following experiments, we converted OD600 readings into bacterial numbers, even though the relationship between OD600 readings and bacterial numbers 60 is varied for each bacterium. The number of bacteria was estimated based on spectrophotometer readings at OD<sub>600</sub> (OD<sub>600</sub> of 1.0=8×10<sup>8</sup> cells/ml).

The Limulus Amebocyte Lysate Assay (LAL) is an assay to determine the total amount of lipopolysaccharide (LPS) in the sample tested (Pierce LAL Chromogenic Endotoxin Quantitation Kit, ThermoFischer Scientific, Waltham, Mass.). The assay was performed following manufacturer's 65



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instruction. Ninety-six-well microplates were first equilibrated in a heating block for 10 min at 37° C. Fifty µl each of standard or sample was dispensed into the microplate wells and incubated with plate covered for 5 min at 37° C. Then 50 µl LAL was added to each well. Plates were shaken gently and incubated for 10 min at 37° C. 100 µl of chromogenic substrate was added and incubated for 6 min at 37° C. Finally, 50 µl Stop Reagent was added and the absorbance was measured at 405-410 nm on Spectramax M3 platereader (Molecular Device, Sunnyvale, Calif.).

FIGS. 1A, 1C, and 1D show the ability of microbes to shed LPS as part of their normal growth cycle. This data shows the need to deliver chemistry to the subgingival plaque to effectively mitigate the LPS, since tooth brushing generally does not remove the subgingival plaque.

The LPS, as measured by the LAL kit reported in endotoxin unit per ml (EU/ml), was shed by the bacteria (*E. coli* K12) as depicted in FIG. 1A. The growth media began to be depleted of complex sugars around 120 minutes, as reflected in the bacterial growth curve in FIG. 1B, where the LPS shedding started to decline. This data gave a reason to believe that a mature biofilm/plaque could supply a constant level of LPS to the host cells, if food sources were present. The LPS would then have the ability to induce an inflammatory response from the host cells.

Importantly, LPS are secreted into the supernatant of bacterial culture (FIG. 1D). LPS also exists in bacterial walls (FIG. 1E). Again, this data further enforce the need to deliver chemistry to the subgingival plaque to effectively mitigate the LPS, since tooth brushing generally does not remove the subgingival plaque.

#### Example 2—LAL Assay to Quantify Virulence Factors in Dental Plaques

Seven panelists, with at least three bleeding sites, took part in the testing. A licensed dental hygienist collected subgingival plaque samples. Samples were taken at the tooth/gum interface (buccal surfaces only) using care to avoid contact with the oral soft tissues. Six subgingival plaque sites were sampled from each panelist (3 healthy and 3 unhealthy sites). Unhealthy teeth had bleeding sites with pockets greater than 3 mm and healthy sites had no bleeding with pocket depth less than 2 mm. Prior to sampling, panelists were instructed to abstain for 12 hours from oral hygiene and refrain from eating, chewing gum, drinking (except small sips of water). Next, panelists had their marginal plaque collected with a curette at the sampling sites. Then, from the same site, subgingival plaque samples were collected with 3 consecutive paper points as shown in FIG. 1F. The sampling sites were isolated with cotton rolls and gently air-dried. Paper points (PROFLOW incorporated, Amityville, N.Y.) were gently placed for 10 seconds into the pocket until a minimum of resistance was felt. After 10 seconds, paper points were removed and placed into pre-labeled 1.5 ml tubes. The same sampling procedure was repeated with 2 more paper points (paper points go into separate tubes). The first, second and third sample paper points from a healthy site of all panelists were pooled separately into three tubes, labeled as paper point 1, 2 and 3, respectively. Similarly the unhealthy site samples were also pooled.

TABLE 1 showed that unhealthy dental plaques contained more endotoxins than the healthy dental plaques. One ml PBS was added to each pooled sample in the 1.5 ml tube. Bacteria were lysed in a MolBio Fast Prep bead beater (MP Biomedicals, Santa Ana, Calif.). Samples were centrifuged

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for 10 min at 10,000 RPM at 4° C., supernatants were collected and analyzed with LAL assay kits following manufacturer's instruction as described in EXAMPLE 1.

TABLE 1

Protein concentrations and endotoxin levels in the pooled dental plaque samples.	
Dental plaque	Endotoxin (endotoxin unit)
Healthy paperpoint 1 sub plaque	1284
Healthy paperpoint 2 sub plaque	476
Healthy paperpoint 3 sub plaque	361
Healthy Marginal Plaque	23180
Unhealthy paperpoint 1 sub plaque	3371
Unhealthy paperpoint 2 sub plaque	1732
Unhealthy paperpoint 3 sub plaque	16111
Unhealthy Marginal Plaque	80277

It was expected that the marginal plaques in unhealthy sites had more endotoxins than those in the healthy sites (TABLE1) within the same subjects. Three samples were taken from subgingival pockets with three paper points sequentially, named paper point 1, 2 and 3. Again, the subgingival plaques taken by the paper point 1 had more endotoxins in the unhealthy sites than in the healthy sites (TABLE 1). The same is true for the samples taken by paper point 2 and 3. Importantly, dental plaques in the unhealthy subgingival pockets possessed more endotoxins than plaques from healthy pockets. This may explain why unhealthy gingiva are prone to bleeding upon probing.

#### Example 3—Technology Development Using the LAL Assay Kit

The LAL assay, as described in EXAMPLE 1, was modified for development of technology which inhibits LPS from activating a proenzyme in the LAL assay. The Thermo Scientific Pierce LAL Chromogenic Endotoxin Quantitation Kit is a quantitative endpoint assay for the detection of LPS, which catalyzes the activation of a proenzyme in the modified Limulus Amebocyte Lysate (LAL). The activated proenzyme then splits p-Nitroaniline (pNA) from the colorless substrate, Ac-Ile-Glu-Ala-Arg-pNA. The product pNA is photometrically measured at 405-410 nm. If SnF2 binds to LPS, the latter can't react with the proenzyme in the LAL kit. Consequently, the proenzyme is not activated, and the colorless substrate Ac-Ile-Glu-Ala-Arg-pNA will not split and no color product is produced. *P. gingivalis* LPS 1690 (1 ng/ml), or *E. coli* LPS (1 ng/ml), and stannous fluoride and other materials (50 and 500 µM), as listed in TABLE 2, were dissolved in endotoxin-free water. Then 50 µl LAL was added to each well. Plates were shaken gently and incubated for 10 min at 37° C. 100 µl of chromogenic substrate was added and incubated for 6 min at 37° C. Finally, 50 µl Stop Reagent was added and the absorbance was measured at 405-410 nm on Spectramax M3 plate reader (Molecular Device, Sunnyvale, Calif.).

As shown in TABLE 2, SnF2 and some other compounds inhibited LPS activities in LAL assays



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TABLE 2

Inhibition of LPS activities on LAL Assays				
Samples	Inhibition of LAL activity %			
	P. gingivalis LPS 1690 1 ng/ml		E. coli LPS 1 ng/ml	
	500 uM	50 uM	500 uM	50 uM
Tin (II) fluoride	60	49	92	87
stannous chloride	48	21	89	65
Cetylpyridinium chloride monohydrate	103	77	103	46
Chlorhexidine	102	38	97	57
zinc citrate, dihydrate	104	57	104	82
zinc lactate	58	0	66	0
potassium oxalate	8	0	1	6
Triclosan (irgasan)	0	0	10	0
1-Hydroxypyridine-2-thione zinc salt	0	0	2	6
sodium fluoride	0	0	4	5
Carboxymethyl cellulose sodium	0	0	2	0

#### Example 4—Binding of BODIPY-TR-Cadaverine to LPS and Displacement of LPS Binding by Stannous Fluoride

In addition to LAL quantification of LPS, the BODIPY method can be utilized to assess the level of LPS. Detoxifying technologies are able to target and neutralize bacterial virulence factors, such as LPS and LTA. To develop such LPS and LTA sequestration technologies, a high throughput screening was employed to identify molecules that disrupt the activation of a Toll-like receptor by LPS and LTA, and other virulence factors. The high throughput screen utilizes the fluorescent dye BODIPY-TR-cadaverine 5-(((4-(4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxy)acetyl)amino)pentylamine hydrochloride (BC fluorescent dye), purchased from Life Technologies (Carlsbad, Calif.), as described previously by Wood, Miller and David (2004) (Comb Chem High Throughput Screen 2004 May; 7(3):239-49. Anti-endotoxin agents. 1. Development of a fluorescent probe displacement method optimized for the rapid identification of lipopolysaccharide-binding agents.). The experiment was carried out at 22° C. *E. coli* LPS (15 µg/ml) was mixed in a well of a 96-well solid black flat bottom plate (Corning Incorporated, Corning, N.Y.) with SNF2 and cetylpyridinium chloride in 30 µl of 50 mM Tris buffer at pH 7.4 for 10 mM, and then 20 µl of 60 µM BC fluorescent dye was added right before fluorescence measurement at 22° C., or room temperature. Fluorescence measurements were performed in a SpectraMax M3 automated 96 well plate reader (Molecular Device, Sunnyvale, Calif.). The excitation wavelength was 580 nM and the fluorescence emission was measured at 620 nM.

As shown in TABLE 3, leading anti-gingivitis technologies, such as stannous fluoride, displaced LPS from BODIPY TR cadaverine.

TABLE 3

Displacement of LPS from binding to BODIPY TR cadaverine		
Concentrations mM	Displacement %	
	Stannous fluoride	Cetylpyridinium chloride
0.16	2.45	9.98
0.31	2.79	31.11

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TABLE 3-continued

Displacement of LPS from binding to BODIPY TR cadaverine		
Concentrations mM	Displacement %	
	Stannous fluoride	Cetylpyridinium chloride
0.63	12.69	68.05
1.25	49.44	91.12

#### Example 5—Cell Culture and Virulence Determination of Lipopolysaccharide from Different Bacteria

Reporter gene cell lines, human HEK 293T cells, were purchased from Invivogen of San Diego, Calif. The HEK 293T cells were stably transfected with at least two exogenous genes, a TLR4 structural gene, and a SEAP reporter gene, which is under the control of NFκB transcriptional factors. The cell line is named here as TLR4-SEAP. The reporter gene encodes a secreted enzyme, called embryonic alkaline phosphatase or SEAP. The SEAP reporter is placed under the control of the interferon-β minimal promoter fused to five NFκB and AP-1-binding sites. Furthermore, the TLR4-SEAP cell line also contains a CD14 co-receptor gene, which is needed to transfer LPS to TLR4 receptors. The recombinant TLR binds its ligand, or distinct pathogen-associated molecule, initiates a chain of responses, leading to recruitment of NFκB and AP1 transcription factors to the reporter gene promoter, which induce expression of SEAP.

Cell culture and treatment: 500,000 gene reporter cells were grown and maintained in 15 ml growth medium, comprised of DMEM medium supplemented with 10% fetal calf serum in T75 flasks for three days at 37° C., 5% CO<sub>2</sub>, and 95% humidity. For treatment, wells of a 96-well plate were seeded with 10,000 cells/well in 100 µL of growth medium. The cells were incubated for 72 hours at 37° C., 5% CO<sub>2</sub>, and 95% humidity until day 4. On day 4, medium was changed to assay medium (90 µl), which is the DMEM medium without fetal calf serum. LPS, bacteria and the culture medium of bacterial growth, as described in EXAMPLE 1, were first resolved or mixed with the assay medium. 10 µl of the bacteria, LPS and culture medium of bacterial growth were added to the TLR4-SEAP cells. Samples were taken 24 hours later, following addition of LPS, bacteria, and culture medium. Expression of the reporter gene (SEAP) was quantified with a commercially available kit (SEAP Reporter Gene Assay of Cayman Chemical Co., Ann Arbor, Mich.).

EC50 was calculated using GraphPad Prism software (GraphPad Software, La Jolla, Calif.). Samples with lower EC50 are more potent in activating the TLR4 reporter gene than those with higher EC50. As shown in FIG. 2A, LPS from *E. coli* has lower EC50 than *P. gingivalis*, thus, was far more potent than *P. gingivalis* (Pg). *Salmonella Minnesota* LPS is not as potent as that of *E. coli*, but is far more potent than those of *P. gingivalis* LPS 1690 and 1435. Each species of bacteria produces multiple forms of LPS. Each form of LPS from the same species of bacteria has different potency in stimulating TLR4-downstream signaling pathways. For example, Pg 1690 LPS is more potent than Pg1435/50. LPS is a component in bacterial cell walls. Likely, *E. coli* cell wall is more virulent in inducing production of proinflammatory cytokines in host cells than *P. gingivalis* when they are in direct contact with host blood cells. *P. gingivalis* had



far higher EC50 than *P. pallens* and *P. nigrescens* as shown in FIG. 2B in stimulating TLR4 reporter gene expression, suggesting that *P. pallens* and *P. nigrescens* are more likely to promote production of proinflammatory cytokines in host cells than *P. gingivalis*.

Bacteria release LPS into the supernatant of bacterial culture. As shown in FIG. 2C, the supernatant of *P. pallens* has an EC50 that is similar to that of *P. nigrescens*, but far lower than that of *P. gingivalis*, in stimulating expression of TLR4 reporter gene. Again, those results imply that the products of *P. pallens* and *P. nigrescens* are more likely to promote production of proinflammatory cytokines in host cells than those of *P. gingivalis*.

#### Example 6—Development of Technology for Inhibition of LPS Effects on Stimulating Expression of NFkB-Regulated Reporter Genes

Stannous fluoride is a leading anti-gingivitis technology in P&G toothpaste products. Tests were conducted to understand whether stannous fluoride could reduce LPS's ability to trigger proinflammatory responses in host cells. TLR4-SEAP reporter cells were prepared using the same conditions as described in EXAMPLE 5 in the presence or absence of LPS. Production of SEAP was quantified also as described in EXAMPLE 5.

FIG. 3 shows the effect of stannous at various concentrations from 62.5 uM to 1,000 uM on 100 ng/ml *E. coli* LPS, as reported by activation of TLR-4. At stannous concentrations of 500 uM or higher, the level of *E. coli* induction of TLR-4 was decreased.

FIG. 4 shows the effects of stannous at various concentrations from 62.5 uM to 1,000 uM on *P. gingivalis* LPS, as reported by activation of TLR-2. At stannous concentrations of 1000 uM, the level of *P. gingivalis* induction of TLR-2 was decreased.

The data in FIG. 5 shows reduction of LPS activity by the stannous ion, from a stannous fluoride salt. The data showed that stannous fluoride, at 1.6 mM and 3.2 mM, reduce about 50% of *P. gingivalis* LPS (500 ng/ml) activation on the TLR4 reporter system (One asterisk means  $P < 0.05$ , two asterisks mean  $P < 0.01$ ).

#### Example 7—Cell Culture and Virulence Determination of EC50 in Activation of TLR4-SEAP Signal Transduction by Clinical Samples

The method described in EXAMPLE 5 is effective at determining the potency of LPS from different bacteria. The same method was used to determine the EC50 of clinical samples, as described in EXAMPLE 2. As shown in FIG. 6, dental plaques from unhealthy sites had a smaller EC50 than those from healthy sites, suggesting the dental plaques from unhealthy sites contain more virulence factors.

The same method described in EXAMPLE 5 was used to examine the clinical samples in another study. A clinical study was conducted to evaluate sample collection methods and measurement procedures. It was a controlled, examiner-blind study. Forty panelists met the inclusion criteria, wherein in order to be included in the study, each panelist must:

- Provide written informed consent to participate in the study;
- Be 18 years of age or older;
- Agree not to participate in any other oral/dental product studies during the course of this study;

Agree to delay any elective dentistry (including dental prophylaxis) until the study has been completed;

Agree to refrain from any form of non-specified oral hygiene during the treatment periods, including but not limited to the use of products such as floss or whitening products;

Agree to return for all scheduled visits and follow study procedures;

Must have at least 16 natural teeth;

Be in good general health, as determined by the Investigator/designee based on a review of the health history/update for participation in the study.

For Unhealthy Group (high bleeder group):

Have at least 20 bleeding sites (sites with a score of 1 or 2 on the GBI index); Have minimum 3 sampling sites with bleeding and pocket depth  $> 3$  mm but not deeper than 4 mm;

Have minimum 3 sampling sites without bleeding and with pocket depth  $< 2$  mm

For Healthy Group (low bleeder group):

Have maximum 3 bleeding sites (sites with a score of 1 or 2 on the GBI index);

No pockets deeper than 2 mm. Twenty (20) panelists were qualified as healthy—with up to 3 bleeding sites and with all pockets less than or equal to 2 mm deep and twenty (20) panelists were qualified as unhealthy—with greater than 20 bleeding sites with at least 3 pockets greater than or equal to 3 mm but not deeper than 4 mm with bleeding, and at least 3 pockets less than or equal to 2 mm deep with no bleeding for sampling. All panelists had up to 6 sites identified as "sampling sites." The "sampling sites" had supragingival and subgingival plaque collected at Baseline, Week 2 and Week 4. Subgingival plaque samples were taken from a gingival sulcus from the pre-identified sites. Prior to sample collection, the site had supragingival plaque removed with a curette. The site was dried and subgingival plaque samples were collected with another dental curette (e.g., Gracey  $^{13/14}$ ,  $^{15/16}$ ,  $^{11/12}$ ,  $7/8$ ,  $^{1/2}$ .) Each Gracey curette is designed to adapt to a specific area or tooth surface. For example, Gracey  $^{13/14}$  is designed to adapt to the distal surfaces of posterior teeth. Samples from each site were placed in a pre-labeled 2.0 ml sterile tube containing 300  $\mu$ l of DPBS buffer with about 50 of sterile 1 mm glass beads. Samples were stored at 4° C. The subgingival samples were stored at -80° C. until analyzed. The samples were thawed at room temperature and dispersed in a TissueLyser II (Qiagen, Valencia, Calif., USA) at 30 shakes per second for 3 min. Protein concentrations of the dispersed subgingival samples were measured using a Pierce microBCA Protein kit (ThermoFisher Scientific, Grand Island, N.Y., USA) following the manufacturer's instruction.

Oral lavage samples were collected at wake up (one per panelist) by rinsing with 4 ml of water for 30 seconds and then expectorating the contents of the mouth into a centrifuge tube. These samples were frozen at home until they were brought into the site in a cold pack. Each panelist collected up to 15 samples throughout the study. Saliva samples were frozen at -70° C. from submission.

All panelists were given investigational products: Crest® Pro-Health Clinical Gum Protection Toothpaste (0.454% stannous fluoride) and Oral-B® Indicator Soft Manual Toothbrush. Panelists continued their regular oral hygiene routine, and did not use any new products starting from the baseline to the end of four week treatment study. During the



four week treatment period, panelists brushed their teeth twice daily, morning and evening, in their customary manner using the assigned dentifrice and soft manual toothbrush.

The subgingival plaques from the above clinical study were applied to the TLR4 reporter cells in a procedure as described in EXAMPLE 5. FIG. 7A shows the results of a four-week study of 40 panelists going from baseline out over four weeks of treatment with Crest ProHealth Clinical toothpaste. The subgingival plaque samples in bleeding sites on the high bleeders group stimulated high expression of TLR4 reporter gene. More virulence in a sample elicits higher RLU (relative luminescent units) readings in the TLR4 reporter gene assay. As shown in FIG. 7A, the baseline samples of the high bleeders group had higher RLU than those of the low bleeders on both the bleeding and non-bleeding sites. After treatment with Crest® Pro-Health Clinical Gum Protection Toothpaste (0.454% stannous fluoride) and Oral-B® Indicator Soft Manual Toothbrush for four weeks, the virulence was reduced at week 4 in both high and lower bleeders groups at both bleeding and non-bleeding sites.

The oral lavage samples were applied to the TLR4 reporter cells in a procedure as described in EXAMPLE 5. As shown in FIG. 7B, oral lavage (Healthy vs. Gingivitis) samples were evaluated in the TLR4-SEAP reporter assay. The baseline samples of the high bleeders group had higher RLU than those of the low bleeders. After treatment with Crest® Pro-Health Clinical Gum Protection Toothpaste (0.454% stannous fluoride) and Oral-B® Indicator Soft Manual Toothbrush for four weeks, the virulence was reduced at week 4 in the high bleeder group.

#### Example 8—Cell Culture and Virulence Determination of Lipoteichoic Acids from Different Bacteria

The reporter gene cell lines, human HEK 293T cells, were purchased from Invivogen of San Diego, Calif. The HEK 293T cells were stably transfected with at least two exogenous genes, a TLR2 structural gene, and SEAP reporter gene which is under the control of NFκB transcriptional factors. The cell line is named here as TLR2-SEAP. The reporter gene encodes a secreted enzyme, called embryonic alkaline phosphatase or SEAP. The SEAP reporter is placed under the control of the interferon-β minimal promoter fused to five NFκB and AP-1-binding sites. Furthermore, a CD14 co-receptor gene was transfected into the reporter gene cells expressing TLR2, as CD14 has been identified as a co-receptor for TLR2 ligands to enhance the TLR response. The CD14 co-receptor is needed to transfer LTA to TLR2 receptors. The recombinant TLR2 binds its ligand, or distinct pathogen-associated molecule, initiates a chain of responses, leading to recruitment of NFκB and AP1 transcription factors to the reporter gene promoter, which induce expression of SEAP.

Cell culture and treatment: 500,000 gene reporter cells were grown and maintained in 15 ml growth medium, comprising DMEM medium supplemented with 10% fetal calf serum in T75 flasks for three days at 37° C., 5% CO<sub>2</sub>, and 95% humidity. For treatment with LTA, wells of a 96-well plate were seeded with 10,000 cells/well in 100 μL of growth medium. The cells were incubated for 72 hours at 37° C., 5% CO<sub>2</sub>, and 95% humidity until day 4. On day 4, medium (100 μL) was changed to DMEM medium without fetal calf serum. LTA, LPS and bacterial cells, as described in EXAMPLE 7, were added. Samples were taken 24 hours later, following addition of samples. Expression of the

reporter gene (SEAP) was quantified with a commercially available kit (SEAP Reporter Gene Assay of Cayman Chemical Co., Ann Arbor, Mich.).

As shown in FIGS. 8A, 8B, 8C and 8D, LTA, LPS, bacteria and the supernatant of bacterial culture could bind to TLR2 and activate TLR2 downstream signaling pathways in a dose-dependent manner. As shown in FIG. 8A, *B. subtilis* (BS) LTA is more potent than that of *Enterococcus hirae*. As shown in FIG. 8B, *P. gingivalis* LPS also activated expression of the TLR2 reporter gene. For example, Pg1690, as shown in FIG. 8B, activated TLR2-SEAP signal transduction, and stimulated SEAP production. But as shown in FIG. 8B, *E. coli* LPS did not activate the TLR2-SEAP reporter cells. It should also be noted that *P. pallens*, *P. nigrescens* and *P. gingivalis* have similar EC50 in stimulating expression of TLR2 reporter gene (FIG. 8C). However, the released TLR2 ligands from the three different bacteria have very different EC50 on activation of TLR2 reporter gene (FIG. 8D).

#### Example 9—Cell Culture and Virulence Determination of EC50 in Activation of TLR2-SEAP Signal Transduction by Clinical Samples

The method described in EXAMPLE 8 is effective in determining the EC50 of LTA and other TLR2 ligands from different bacteria. The same method was used to determine the EC50 of clinical samples, as described in EXAMPLE 2. As shown in FIG. 9, dental plaques from unhealthy (bleeding) sites had smaller EC50 than those from healthy (non-bleeding) sites, suggesting the dental plaques from unhealthy sites contain more virulence factors.

Clinical samples as described for FIG. 7A of EXAMPLE 7 were examined using the TLR2-SEAP reporter gene assay. The results are shown in FIG. 10A. The subgingival samples in unhealthy (bleeding) sites from the unhealthy group (high bleeders) had more virulence factors than other sites. The baseline samples of the high bleeders group had higher RLU than those of the low bleeders on both the bleeding and non-bleeding sites. After treatment with Crest® Pro-Health Clinical Gum Protection Toothpaste (0.454% stannous fluoride) and Oral-B® Indicator Soft Manual Toothbrush for four weeks, the virulence was reduced at week 4 in both high and low bleeders groups at both bleeding sites.

The clinical samples as described for FIG. 7B of EXAMPLE 7 were examined using the TLR2-SEAP reporter gene assay. As shown in FIG. 10B, oral lavage (Healthy vs. Gingivitis) was evaluated. After treatment with Crest® Pro-Health Clinical Gum Protection Toothpaste (0.454% stannous fluoride) and Oral-B® Indicator Soft Manual Toothbrush for four weeks, the virulence was reduced at week 4 in the high bleeder group.

#### Example 10—Cell Culture and Virulence Determination of Flagellins from Different Bacteria

The reporter gene cell lines, human HEK 293T cells, were purchased from Invivogen of San Diego Calif. The HEK 293T cells were stably transfected with two exogenous genes, a TLR5 structural gene, and SEAP reporter gene which is under the control of NFκB transcriptional factors. The cell line was named as TLR5-NFκB-SEAP. The reporter gene encodes a secreted enzyme, called embryonic alkaline phosphatase or SEAP. The SEAP reporter is placed under the control of the interferon-β minimal promoter fused to five NFκB and AP-1-binding sites. The recombinant TLR5 binds



to its ligand, or distinct pathogen-associated molecule, and initiates a chain of responses leading to recruitment of NFkB and AP1 transcription factors to the reporter gene promoter, which induce expression of SEAP.

Cell culture and treatment: 500,000 gene reporter cells were grown and maintained in DMEM medium supplemented with 10% fetal calf serum in T75 flasks for three days at 37° C., 5% CO<sub>2</sub>, and 95% humidity. For treatment with flagellin, wells of a 96-well plate were seeded with 10,000 cells/well in 100 µL of growth medium. The cells were incubated for 72 hours at 37° C., 5% CO<sub>2</sub>, 95% humidity until day 4 after cells were seeded onto wells of a 96-well plate. On day 4, medium (100 µL) was changed to DMEM medium without fetal calf serum. *S. subtilis* and *S. aureus* flagellin were added at a range of concentration from 0.97 ng to 1 µg/ml. Samples were taken at 6 and 24 hours later after adding flagellin. Expression of reporter gene (SEAP) was quantified with a commercially available kit (SEAP Reporter Gene Assay of Cayman Chemical Co., Ann Arbor, Mich.).

FIG. 11 shows both flagellins (*B. subtilis* and *P. aeruginosa*) could activate TLR5 downstream signaling pathways. Flagellin from *B. subtilis* stimulated TLR5-SEAP signaling transduction in a dose-dependent manner. The EC<sub>50</sub> of flagellin on TLR5 was 0.02 ng/ml at 5 hours and 0.014 ng/ml at 23 hours. The EC<sub>50</sub> of flagellin from *Pseudomonas aeruginosa* on TLR5 was 0.0049 at 5 hours and 0.0019 at 23 hours. Flagellin from *P. aeruginosa* was more potent on stimulating TLR5 signaling pathways.

#### Example 11—Cell Culture and Virulence Determination of LPS from Different Bacteria in THP1 Dual Reporter Cells

Bacterial cell wall and membrane components are recognized by TLR2. TLR2 recognizes the microbial motifs PGN (peptidoglycan)/lipoproteins/dectin and LPS. TLR1 and TLR6 form heterodimers with TLR2 and bind to triacylated lipoproteins and diacylated lipoproteins, respectively. THP1 NFkB-SEAP and IRF-Lucia™ Reporter Monocytes were purchased from Invivogen, San Diego, Calif. THP1-Dual cells were derived from the human THP-1 monocyte cell line by stable integration of two inducible reporter constructs. THP1-Dual cells feature the Lucia gene under the control of an ISG54 (interferon-stimulated gene) minimal promoter in conjunction with five interferon-stimulated response elements. THP1-Dual cells also express a SEAP reporter gene driven by an IFN- $\beta$  minimal promoter fused to five copies of the NF- $\kappa$ B consensus transcriptional response element and three copies of the c-Rel binding site. As a result, THP1-Dual cells allow the simultaneous study of the NFkB pathway, by monitoring the activity of SEAP, and the interferon regulatory factor (IRF) pathway, by assessing the activity of Lucia (IRF-Luc). Both reporter proteins are readily measurable in the cell culture supernatant. This THP-1 cell line possesses functional TLR1, TLR2, TLR4, TLR5, TLR6 and TLR8, purchased from Invivogen. TLR4 senses LPS from Gram-negative bacteria while TLR5 recognizes bacterial flagellin from both Gram-positive and Gram-negative bacteria, TLR8 detects long single-stranded RNA.

Culture and treatment: The THP1-dual cells were cultured in 15 ml growth medium (RPMI 1640 with 10% heat-inactivated fetal bovine serum) in a T75 flask at 37° C. and 5% CO<sub>2</sub>. Cells were passed every 3 to 4 days by inoculating 300,000-500,000 cells/ml into a fresh T75 flask with 15 ml of fresh growth medium. To determine the effect of bacterial

components on reporter gene expression, wells in 96-well plates were seeded at 100,000 cells in 90 µl of growth medium. 10 µl of bacterial wall and membrane components, or heat-killed whole bacteria, were added to each well. After incubation for 18 hours at 37° C. and 5% CO<sub>2</sub>, secreted luciferase and SEAP were quantified with commercially available assay kits (QUANTI-Luc of Invivogen, San Diego, Calif. for luciferase; SEAP Reporter Gene Assay of Cayman Chemical Co., Ann Arbor, Mich. for SEAP).

DHP1-dual reporter cells were treated with three different preparations of LPS as shown in FIG. 12A. All three LPS (ng/ml) activated production of NFkB-SEAP reporter genes in a dose-dependent manner. In addition, Pg 1690 LPS and *E. coli* LPS also stimulated expression of the IRF-luciferase reporter gene. TLR4 ligands, upon binding to TLR4 receptors, activate at least two signaling pathways. One is a common pathway NFkB-SEAP, which can be activated by all TLR ligands upon binding to their specific receptors. For example, TLR2 ligand, LTA, can bind to TLR2 receptors and activate the NFkB-SEAP signaling pathway. Similarly, TLR4 ligand, LPS, upon binding to TLR4 receptors, also is able to activate the NFkB-SEAP signaling transduction. As shown in FIG. 12A, *E. coli* LPS is a more potent ligand than *P. gingivalis* 1690 LPS on activation of both NFkB-SEAP and IRF-luciferase signaling transduction. THP-1 cells produce several functional TLR receptors. And all TLR receptors can activate the NFkB pathway, thus promoting expression of the NFkB-SEAP reporter gene. The reading of NFkB-SEAP is the collective actions of all TLR receptors, such as TLR2, TLR1, TLR6 and TLR4. All LPS from different bacteria stimulated NFkB-SEAP reporter gene. IRF-luciferase reporter gene, on the other hand, is driven by a limited number of TLR receptors, primarily TLR3, TLR4, TLR7, TLR8 and TLR9. Both *P. gingivalis* LPS 1690 and *E. coli* LPS stimulated expression of IRF-luciferase in a dose-dependent fashion.

The THP-1 reporter cells were used to examine the clinical samples as described for FIG. 7B of EXAMPLE 7. As shown in FIG. 12B, oral lavage (Healthy vs. Gingivitis) was evaluated using the IRF-Luc reporter gene in THP-1 cells. After treatment with Crest® Pro-Health Clinical Gum Protection Toothpaste (0.454% stannous fluoride) and Oral-B® Indicator Soft Manual Toothbrush for four weeks, the virulence was reduced at week 4 in both high and lower bleeders groups.

The THP-1 reporter cells were used to examine the clinical samples as described for FIG. 7A of EXAMPLE 7. As shown in FIG. 12C, the subgingival Plaque (Healthy vs. Gingivitis) was examined using the NFkB reporter gene in THP-1 cells. The baseline samples of the high bleeders group had higher RLU than those of the low bleeders. After treatment with Crest® Pro-Health Clinical Gum Protection Toothpaste (0.454% stannous fluoride) and Oral-B® Indicator Soft Manual Toothbrush for four weeks, the virulence was reduced at week 4 in the bleeding sites in both high and lower bleeders groups.

#### Example 12—Cell Culture and Virulence Determination of LTA from Different Bacteria and Biofilms in THP1 Dual Reporter Cells

THP1 dual reporter cells also express TLR2, TLR1 and TLR6 receptors. Bacterial cell wall and some membrane components are recognized by TLR2, TLR1 and TLR6. TLR2 recognizes the microbial motifs PGN (peptidoglycan)/lipoproteins/dectin and LPS. To determine whether LTA from different bacteria have different effects on stimu-



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lating NFkB-SEAP reporter gene expression in the THP1 dual reporter cells, the cells were prepared and treated in the same procedures as described in EXAMPLE 11. As shown in FIG. 13, LTA from both *B. subtilis* and *S. aureus* had similar potency in promoting reporter gene expression in the THP1 dual reporter cells.

Example 13—Distinguishing Bacterial Components from Different Bacteria in Human Primary Gingival Epithelial Cells

Primary human gingival epithelial cells were purchased from Zen-bio (Research Triangle Park, N.C.), and maintained in 15 ml of growth medium (CellnTec medium supplemented with CellnTec Growth Supplement, purchased from CellnTec Advanced Cell Systems AG, Bern, Switzerland) in T75 flasks at 37° C. under a 5% CO<sub>2</sub> atmosphere. As the experiment was done at 24 h, 48 h and 72 h time point and with assay media, CellnTec medium alone or with supplements, six 96 well plates were seeded with 7,500 cells/well in 100 µl of CELLnTEC growth medium at 37° C. under a 5% CO<sub>2</sub> atmosphere. The growth medium was changed to assay medium right before adding LPS or bacterial DNA. For example, if the assay medium was CellnTec medium without supplements, 100 µl of CellnTec medium was added without supplements in each well. If the assay medium was the growth medium, 100 µl of growth medium was added to each well. The *P. gingivalis* LPS and bacterial DNA were added to the cells. At 24 h, 48 h and 72 h, medium was collected for analysis. Cytokines were measured using Elisa kits from Meso Scale Discovery (Rockville, Md.), as per the manufacturer's instructions.

As shown in TABLE 4, human primary gingival epithelial cells were treated with *P. gingivalis* DNA at 0, 0.3, 1 and 2 µg/ml. The cultures were harvested at 24 and 48 hours after treatment and six proinflammatory cytokines (interferon-γ, IL-1β, IL-2, IL-10, IL-12p70 and TNF-α) were analyzed using ELISA kits from Meso Scale Discovery. As shown in TABLE 4, expression of interferon-γ, IL-1β, IL-2, IL-10, IL-12p70 and TNF-α was low, or almost undetectable (each value is the mean of three replicate in one experiment). They were not viable biomarkers in distinguishing bacterial DNA. The results in TABLE 4 illustrate the lack of inflammatory response from human gingival epithelial cells, thus demonstrating the need to use an engineered cell.

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TABLE 4

Screening of Human Gingival Epithelial Cells for Inflammatory Markers treated with <i>P. gingivalis</i> DNA							
Production of cytokines (pg/ml) in primary human gingival epithelial cells treated with <i>P. gingivalis</i> genomic DNA							
	<i>P. gingivalis</i> genomic DNA	<i>P. gingivalis</i>			<i>P. gingivalis</i> genomic DNA	<i>P. gingivalis</i>	
		24 hours	48 hours			24 hours	48 hours
Interferon gamma	0 ug	0	0.25	IL-1 beta	0 ug	0	0.5
	0.3 ug	0	0.33		0.3 ug	0	0.33
	1 ug	0	0		1 ug	0	0
	2 ug	0.33	0.67		2 ug	0	0
IL-10	PG DNA	0.5	1	IL-12p70	PG DNA	0	0.25
	0 ug				0 ug		
	PG DNA	0.67	0.33		PG DNA	0.67	0
	0.3 ug				0.3 ug		
	PG DNA	0	0		PG DNA	0	0.67
	1 ug				1 ug		
IL-2	PG DNA	0.33	0.33	TNF-alpha	PG DNA	0	0.33
	2 ug				2 ug		
	PG DNA	0.5	1		PG DNA	1	1.5
	0 ug				0 ug		
	PG DNA	0	0.67		PG DNA	1.67	1.33
	0.3 ug				0.3 ug		
IL-2	PG DNA	0.33	1	IL-2	PG DNA	0.67	1
	1 ug				1 ug		
	PG DNA	0.33	0.67		PG DNA	1.33	1.33
	2 ug				2 ug		

Human primary gingival epithelial cells were treated with *P. gingivalis* LPS at 0, 0.3, 1 and 2 µg/ml in the procedures described above. As shown in TABLE 5, again, expression of interferon-γ, IL-1β, IL-2, IL-10, IL-12p70 and TNF-α was low, or almost undetectable. They were not viable biomarkers in distinguishing bacterial LPS in primary human gingival epithelial cells. The results in TABLE 5 further illustrate how human gingival cells were not sufficiently sensitive to bacterial virulence factors for an assay, and thus the need to utilize a cell line capable of demonstrating a dose dependent response to virulence factors.

TABLE 5

Screening of Human Gingival Epithelial Cells for Inflammatory Markers treated with <i>P. gingivalis</i> LPS								
Production of cytokines (pg/ml) in primary human gingival epithelial cells treated with <i>P. gingivalis</i> LPS								
		24 h			24 h		48 h	
		24 h	48 h		24 h	48 h		
Interferon gamma	PG IPS 0 ug	0.00	0.25	IL-1 beta	PG IPS 0 ug	0.00	0.50	
	PG LPS 0.3 ug	0.00	0.00		PG LPS 0.3 ug	0.00	0.67	
	PG LPS 1 ug	0.33	0.33		PG LPS 1 ug	0.00	0.00	
	PG LPS 2 ug	0.00	0.00		PG LPS 2 ug	0.00	1.00	
IL-10	PG IPS 0 ug	0.50	1.00	IL-12p70	PG IPS 0 ug	0.00	0.25	
	PG LPS 0.3 ug	0.67	0.67		PG LPS 0.3 ug	0.00	0.00	
	PG LPS 1 ug	0.00	0.67		PG LPS 1 ug	0.00	0.33	
	PG LPS 2 ug	0.33	0.67		PG LPS 2 ug	0.67	0.00	
IL-2	PG IPS 0 ug	0.50	1.00	TNF-alpha	PG IPS 0 ug	1.00	1.50	
	PG LPS 0.3 ug	0.33	0.67		PG LPS 0.3 ug	1.33	0.67	
	PG LPS 1 ug	0.33	1.00		PG LPS 1 ug	2.00	1.33	
	PG LPS 2 ug	1.33	1.00		PG LPS 2 ug	0.67	1.33	



Example 14—Gingivitis-Enriched Bacteria Reduced  
in Abundance in Six Weeks of Gingivitis Treatment

A randomized, two-group clinical study was conducted with 69 panelists (35 in the negative control group and 34 in the test regimen group). Panelists were 39 years old on average, ranging from 20 to 69, and 46% of the panelists were female. Treatment groups were well balanced, since there were no statistically significant ( $p \geq 0.05$ ) differences for demographic characteristics (age, ethnicity, gender) or starting measurements for Gingival Bleeding Index (GBI); mean=29.957 with at least 20 bleeding sites, and Modified Gingival Index (MGI); mean=2.086. All sixty-nine panelists attended each visit and completed the treatment process. The following treatment groups were compared over a 6-week period:

Test regimen: Crest® Pro-Health Clinical Plaque Control (0.454% stannous fluoride) dentifrice, Oral-B® Professional Care 1000 with Precision Clean brush head and Crest® Pro-Health Refreshing Clean Mint (0.07% CPC) mouth rinse. Control regimen: Crest® Cavity Protection (0.243% sodium fluoride) dentifrice and Oral-B® Indicator Soft Manual toothbrush.

Dental plaques were collected from the same panelists in the test regimen in the clinical study as described in EXAMPLE 2. A supragingival sample was taken from each panelist with a sterile curette at the tooth/gum interface, using care to avoid contact with the oral soft tissue. Plaques were sampled from all available natural teeth (upper arch only) until no plaque was visible. Following sampling, the plaque samples were placed into a pre-labeled (panelist ID, sample initials, visit, and date) Eppendorf tube with 1 ml of PBS/Glycerol buffer and about 50 of sterile 1 mm glass beads, stored on ice until all samples were collected. The samples were then transferred to a  $-70^{\circ}$  C. freezer for storage until further processing. Genomic DNA was isolated from supragingival plaque samples using QIAamp® genomic DNA kits (Qiagen, Germany) following manufacturer's instruction. Metasequencing was carried out at BGI Americas Corporation (Cambridge, Mass.). All data were analyzed at Global Biotech of Procter & Gamble Company in Mason, Ohio.

Clinical measurements: Bleeding sites (GBI) were decreased in the test regimen significantly on week 1, 3 and 6 in comparison to the control regimen (FIG. 14). Similarly, Inflammation (MGI) grades also decreased in the test regimen (FIG. 14).

Genomic DNA of the supragingival plaques in the test regimen was sequenced. As shown in FIG. 15, abundance of

certain bacteria in the supragingival plaques changed in the six week treatments. Certain bacteria, such as *Porphyromonas* sp oral taxon 279 and *Prevotella pallens*, were decreased in weeks 1 and 3 (FIG. 15). The amount of each bacterial species was plotted over the four time periods of the treatment. The amount of certain bacteria, such as *Peptostreptococcus stomatis* and *Prevotella intermedia*, was reduced during the six week of treatment as shown in FIG. 15. This is also shown in FIG. 16A-1, FIG. 16A-2, and FIG. 16A-3.

Example 15—Production of Cytokines,  
Chemokines and Other Bioactive Proteins  
Decreased as Gingivitis Symptoms were Alleviated  
During 6 Week of Treatment

In the same clinical study as described in EXAMPLE 14, gingival-brush samples were collected from the same panelists as in EXAMPLE 14. Before sampling, panelists rinsed their mouths for 30 seconds with water. A dental hygienist then sampled the area just above the gumline using a buccal swab brush (Epicentre Biotechnologies cat.# MB100SP). The swab was immediately placed into 1 ml extraction buffer [PBS, 0.25M NaCl, 1x Halt™ Protease Inhibitor Single-Use Cocktail (Lifetechnologies, Grand Island, N.Y.)] in a 1.5 ml Eppendorf tube vortexed for 30 seconds, and immediately frozen on dry ice and stored in a  $-80^{\circ}$  C freezer until analysis. The samples were taken out of freezer, thawed and extracted by placing the samples on a tube shaker for 30 minutes at  $4^{\circ}$  C. The tubes were centrifuged at 15000 RPM for 10 min in Eppendorf Centrifuge 5417R (Eppendorf, Ontario, Canada) to pellet any debris. The extract (800  $\mu$ l) was analyzed for protein concentrations using the Bio-Rad protein assay (BioRad, Hercules, Calif.).

Forty proteins were measured in the gingival samples using V-PLEX Human Biomarker 40-Plex Kit (Meso Scale Diagnostics Rockville, Md.). The assay was performed following the manufacturer's instruction.

Among the proteins measured in the gingival samples, most proteins in the Proinflammatory Panel 1 (human), Cytokine Panel 1 (human), Chemokine Panel 1 (human), Angiogenesis Panel 1 (human), and Vascular Injury Panel 2 (human) had significant changes in their abundance during the 6-week treatment (TABLE 6). Those include FN- $\gamma$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF- $\alpha$ , GM-CSF, IL-5, IL-16, IL-7, IL-12/IL-23p40, IL-1 $\alpha$ , VEGF-A, IL-17A, IL-15, TNF- $\beta$ , IL-8 (HA), MCP-1, MCP-4, Eotaxin, IP-10, MDC, Eotaxin-3, TARC, MIP-1 $\alpha$ , MIP-1 $\beta$ , VEGF-C, VEGF-D, Tie-2, Flt-1/VEGFR1, PIGF, FGF (basic), SAA, CRP, VCAM-1, and ICAM-1.

TABLE 6

	Changes in abundance of proteins in the gingival-brush samples							
	Mean				$\alpha = 0.05$			
	Baseline	Week 1	Week 3	Week 6	Baseline	Week 1	Week 3	Week 6
ICAM-1	16.035	12.209	10.090	9.767	A	B	B, C	C
IL-1 $\alpha$	3.554	2.331	2.181	1.891	A	A, B	B, C	C
IL-1 $\beta$	53.666	35.575	24.295	24.440	A	B	C	C
TNF- $\beta$	0.0013	0.0010	0.0008	0.0007	A	B	C	C
IL-12p70	0.172	0.148	0.118	0.127	A	A, B	C	B, C
IL-13	0.805	0.762	0.624	0.648	A	A, B	C	B, C
IL-4	0.127	0.115	0.090	0.096	A	A, B	C	B, C
IL-5	0.004	0.003	0.002	0.003	A	B	C	B, C
CRP	15.637	12.743	12.385	5.809	A	A	A	B
Eotaxin	0.077	0.064	0.059	0.059	A	A, B	B	B
GM-CSF	0.010	0.008	0.008	0.008	A	B	B	B



TABLE 6-continued

Changes in abundance of proteins in the gingival-brush samples								
	0.530	0.446	0.378	0.386	A	A, B	B	B
IFN $\gamma$	0.530	0.446	0.378	0.386	A	A, B	B	B
IL-10	0.875	0.490	0.423	0.244	A	A, B	B	B
IL-15	0.005	0.003	0.003	0.003	A	B	B	B
IL-16	0.466	0.345	0.342	0.295	A	B	B	B
IL-6	0.196	0.192	0.168	0.150	A	A	A, B	B
IL-7	0.004	0.003	0.003	0.003	A	B	B	B
IL-8	856.276	652.066	567.361	572.602	A	B	B	B
MCP-1	0.053	0.047	0.039	0.039	A	A, B	B	B
MDC	0.399	0.407	0.345	0.339	A	A	B	B

	Mean				$\alpha = 0.05$			
	Baseline	Week 1	Week 3	Week 6	Baseline	Week 1	Week 3	Week 6
SAA	7.039	6.905	6.092	5.162	A	A	A, B	B
Tie-2	0.273	0.239	0.267	0.221	A	A, B	A	B
VCAM-1	4.971	3.706	3.156	2.892	A	B	B	B
VEGF	0.625	0.511	0.478	0.480	A	B	B	B
VEGF 2	0.772	0.661	0.620	0.582	A	B	B	B
VEGF-D	0.057	0.052	0.051	0.045	A	A, B	A, B	B
VEGF-C	0.145	0.149	0.125	0.137	A, B	A	B	A, B
TARC	0.020	0.029	0.019	0.019	A	B	A	A
bFGF	0.020	0.015	0.012	0.013	A	A	A	A
Eotaxin-3	0.095	0.108	0.091	0.094	A	A	A	A
Flt-1	0.390	0.518	0.433	0.415	A	B	A, B	A
IL-12p40	0.039	0.031	0.028	0.031	A	A	A	A
IL-2	0.166	0.199	0.210	0.162	A	A	A	A
IL-8 (HA)	47.508	44.362	41.260	39.119	A	A	A	A
IP-10	0.540	1.688	0.740	0.606	A	A	A	A
MCP-4	0.023	0.023	0.020	0.022	A	A	A	A
MIP-1 $\alpha$	0.091	0.091	0.084	0.080	A	A	A	A
MIP-1 $\beta$	0.091	0.100	0.110	0.094	A	A	A	A
TNF $\alpha$	2.009	2.067	2.021	1.670	A	A	A	A

#### Example 16—One Hundred Seventy Metabolites were Identified in Gingival Samples

The same gingival-brush samples as described in EXAMPLE 15 were used for metabolomic analyses. Fourteen panelists were selected randomly from each treatment group to determine if any metabolite concentrations were changed in gingival samples during the first 3 weeks of treatment. Both baseline and week 3 samples were sent to Metabolon, Inc. (Durham, N.C.) for metabolomic measure-

ment. 170 metabolites were identified and quantified. As shown in TABLE 7, some metabolite concentrations were changed during the first 3 weeks of treatment. Citrulline concentrations in the gingival samples were reduced after three weeks of treatment in the treatment regimen group. Similarly, ornithine was also reduced in the treatment regimen group after three weeks of treatment. Reduction of citrulline and ornithine was likely associated with alleviation of gingivitis.

TABLE 7

Comparison of metabolites in gingival brush samples between baseline and week 3 during gingivitis treatment						
Biochemical Name	Baseline mean	3 week mean	3 week/baseline	p-value	q-value	Mass
13-HODE + 9-HODE	1.0877	0.7088	0.65	0.0601	0.1338	295.2
1-arachidonoylglycerophosphoethanolamine	1.2294	0.8274	0.67	0.038	0.1035	500.3
1-oleoylglycerophosphoethanolamine	0.7378	1.0747	1.46	0.0767	0.1548	478.3
2-methylbutyrylcarnitine (C5)	1.7769	0.6997	0.39	0.0034	0.0546	246.1
adenosine 5'-monophosphate (AMP)	1.4092	0.8451	0.6	0.0295	0.0956	348.1
alanine	0.8721	1.102	1.26	0.0318	0.0973	115.9
arginylleucine	1.4447	0.6819	0.47	0.0084	0.0777	288.3
arginylphenylalanine	0.9616	0.3335	0.35	0.0119	0.0777	322.2
asparagylleucine	0.9295	0.6122	0.66	0.0698	0.1465	246.2
citrulline	1.0147	0.71	0.7	0.0104	0.0777	176.1
deoxycarnitine	3.2381	0.6088	0.19	0.0003	0.0168	146.1
EDTA	1.5985	0.8384	0.52	0.0138	0.0777	291.1
erythritol	1.625	0.8085	0.5	0.0582	0.1325	217
fructose	1.9933	1.1106	0.56	0.0847	0.1605	217
glutamine	1.2459	0.8366	0.67	0.0374	0.1035	147.2
glutathione, oxidized (GSSG)	1.0161	1.4669	1.44	0.087	0.1605	613.1



TABLE 7-continued

Comparison of metabolites in gingival brush samples between baseline and week 3 during gingivitis treatment						
Biochemical Name	Baseline mean	3 week mean	3 week/ baseline	p-value	q-value	Mass
glycerol	1.3783	0.8308	0.6	0.0391	0.1035	205
lauryl sulfate	1.685	0.8623	0.51	0.0397	0.1035	265.2
leucine	1.2158	0.9359	0.77	0.0613	0.1338	132.2
leucylleucine	0.9505	0.4393	0.46	0.0251	0.0877	245.1
lysylleucine	1.2009	0.5275	0.44	0.0036	0.0546	260.2
lysylphenylalanine	1.1682	0.4563	0.39	0.0095	0.0777	294.3
maltose	0.8727	1.4481	1.66	0.022	0.0877	204.1
maltotriose	1.0456	1.8347	1.75	0.0858	0.1605	204
mannitol	1.3004	0.7982	0.61	0.042	0.107	319.1
ornithine	1.2916	0.7069	0.55	0.0367	0.1035	141.9
palatinitol	1.4395	0.8272	0.57	0.0782	0.1549	204
phosphate	1.4008	0.8376	0.6	0.0208	0.0877	298.9
proline	1.405	0.99	0.7	0.0033	0.0546	116.1
propionylcarnitine	1.2565	0.7688	0.61	0.0201	0.0877	218.2
pyroglutamine	1.3424	0.7873	0.59	0.0136	0.0777	129.2
serylisoleucine	1.1753	0.7169	0.61	0.0814	0.1583	219.2
spermidine	1.1613	0.8678	0.75	0.0687	0.1465	146.2
succinate	1.2929	0.8113	0.63	0.0754	0.1548	247
threonylleucine	1.1513	0.4931	0.43	0.0044	0.0594	231.2
threonylphenylalanine	1.7693	0.918	0.52	0.0233	0.0877	267.2
trehalose	2.3563	0.9084	0.39	0.0054	0.0647	361.2
tryptophan	1.1518	0.9089	0.79	0.0487	0.1185	205.1
tyrosine	1.383	1.0299	0.74	0.0161	0.0787	182.1
valine	1.1598	0.9271	0.8	0.0304	0.0956	118.1
valylvaline	0.9347	0.8231	0.88	0.0508	0.1207	215.2
X-13671	0.5035	0.918	1.82	0.0545	0.1267	315.3
X-14588	1.3647	0.8378	0.61	0.024	0.0877	151
X-16103	1.3643	0.8461	0.62	0.0297	0.0956	99.3
X-17266	1.3158	0.576	0.44	0.0003	0.0168	530.4
X-17375	1.4785	0.8387	0.57	0.0189	0.0877	357.1
X-18472	0.6138	1.1441	1.86	0.0011	0.0405	827.1
X-18779	1.3756	0.8035	0.58	0.0162	0.0787	209.1
X-19607	1.5237	0.7167	0.47	0.002	0.0537	366.1
X-19609	1.3284	0.7721	0.58	0.016	0.0787	204
X-19612	1.3896	0.7843	0.56	0.01	0.0777	427.2
X-19613	1.3412	0.7535	0.56	0.0099	0.0777	429.3
X-19614	1.3378	0.7343	0.55	0.0454	0.113	570.1
X-19807	1.3478	0.8411	0.62	0.0244	0.0877	93
X-19808	1.3348	0.8368	0.63	0.0254	0.0877	95
X-19850	1.3576	0.7519	0.55	0.011	0.0777	334.2
X-19857	1.3357	0.8032	0.6	0.038	0.1035	230
X-20000	1.2784	0.7536	0.59	0.0133	0.0777	81.2

#### Example 17—Citrulline was Reduced in Regimen Treatment Over 6 Weeks

Quantitation of citrulline and ornithine from the extracts of the Gingival-brush samples was conducted using gradient hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC/MS/MS). Gingival-brush samples were obtained from the same human panelists in the clinical study as described in EXAMPLE 14, and were placed into extraction buffer as described in EXAMPLE 15. The supernatants were subject to both HILIC/MS/MS and BCA analysis. For free citrulline and ornithine analysis, the extracts of the Gingival-brush samples were analyzed either directly (50  $\mu$ l undiluted sample solution) in 50/50 acetonitrile/ultra-pure water with 0.754% formic acid or diluted fivefold. For total citrulline and ornithine analysis, the extracts of the Gingival-brush samples were first hydrolyzed using 6 N HCl (50  $\mu$ L of extract with 450  $\mu$ L of 6N HCl), no shaking, and placed on a hot plate at 110° C. for 16 hours. The hydrolyzed samples were then dried down under vacuum at room temperature (Savant speedvac of Lifetechnology, Grand Island, N.Y.) and then reconstituted in 1 ml of

dilution solution (50/50 acetonitrile/ultra-pure water with 0.754% formic acid) for analysis. The standards and the samples were analyzed using gradient hydrophilic interaction liquid chromatography with tandem mass spectrometry (HILIC/MS/MS). Analytes and the corresponding ISTDs (stable isotope labeled internal standard) were monitored by electrospray ionization (ESI) in positive mode using the selected-reaction-monitoring schemes shown in TABLE 8. A standard curve was constructed by plotting the signal, defined here as the peak area ratio (peak area analyte/peak area ISTD), for each standard versus the mass of each analyte for the corresponding standard. The mass of each analyte in the calibration standards and Gingival-brush extract samples were then back-calculated using the generated regression equation. The concentration of protein bound citrulline or ornithine was calculated as the result of subtracting the concentration of free citrulline or ornithine from the concentration of total citrulline or ornithine, respectively. The result was reported as the concentration of citrulline or ornithine or the result was standardized by dividing by the amount of citrulline or ornithine by the amount of the total proteins that were found in the extract.



TABLE 8

Multiple Reaction Monitoring (MRM) transitions for analytes and their corresponding stable isotope labeled internal standards			
Analytes	MRM	Internal Standards	MRM
Citrulline	176 → 159	d <sub>7</sub> -Citrulline	181 → 164
Ornithine	133 → 70	d <sub>6</sub> -Ornithine	139 → 76

All samples from all panelists of the Test regimen [Crest® Pro-Health Clinical Plaque Control (0.454% stannous fluoride) dentifrice, Oral-B® Professional Care 1000 with Precision Clean brush head and Crest® Pro-Health Refreshing Clean Mint (0.07% CPC) mouth rinse] were analyzed. As shown in FIG. 16, citrulline levels reduced rapidly in the first week of treatment, and then continued to decline gradually in weeks 3 and 6 of treatment. These results are consistent with clinical observations, where gingival bleeding sites (GBI) and the gingival inflammation (MGI) were reduced over the 6-week treatment period.

#### Example 18—Levels of Proteins Containing Ornithine Decreased Over 6 Week Treatment

The same samples as described in EXAMPLE 17 were analyzed using procedures as described in EXAMPLE 17. Gingivitis was treated for 6 weeks. Baseline (BL) represents diseased status. Symptoms of gingivitis were alleviated from week 1 to week 6 treatments. Protein bound ornithine (the difference between total and the free ornithine) was higher in gingivitis as shown in FIG. 17. Protein bound ornithine was reduced gradually as gingivitis was decreased in severity.

#### Example 19—Expression of Enzymes in the Ornithine-Citrulline-Arginine Cycle was Changed in Gingival Samples During the 6 Week Treatment

Gingival samples were collected as described in EXAMPLES 15, from the same panelists as in EXAMPLE 15, and were used to examine the expression of genes during 6 weeks of treatments with Test regimen [Crest® Pro-Health Clinical Plaque Control (0.454% stannous fluoride) dentifrice, Oral-B® Professional Care 1000 with Precision Clean brush head and Crest® Pro-Health Refreshing Clean Mint (0.07% CPC) mouth rinse] and Control regimen [Crest® Cavity Protection (0.243% sodium fluoride) dentifrice and Oral-B® Indicator Soft Manual toothbrush].

After harvesting the samples, the brush was completely immersed in the RNAlater solution (1 ml in a 1.5 ml Eppendorf tube) for keeping RNA from degrading during transport and storage (Qiagen, Valencia, Calif.). The micro-centrifuge tubes were vortexed/mixed for 30 seconds, immediately frozen on dry ice, stored and transferred on dry ice to the lab for biomarker analysis. RNA isolation and microarray analysis were performed as described previously in a publication (Offenbacher S, Barros S P, Paquette D W, Winston J L, Biesbrock A R, Thomason R G, Gibb R D, Fulmer A W, Tiesman J P, Juhlin K D, Wang S L, Reichling T D, Chen K S, Ho B. *J Periodontol.* 2009 December; 80(12):1963-82. doi: 10.1902/jop.2009.080645. Gingival transcriptome patterns during induction and resolution of experimental gingivitis in humans).

The ornithine-citrulline-arginine cycle consists of four enzymes (FIG. 18). The main feature of the cycle is that three amino acids (arginine, ornithine, and citrulline) can be

converted to each other. The first enzyme is ornithine transcarbamoylase, which transfers a carbamoyl group from carbamoyl phosphate to ornithine to generate citrulline. This reaction occurs in the matrix of the mitochondria. Expression of ornithine transcarbamoylase was reduced in the treatment (FIG. 19). The second enzyme is argininosuccinate synthetase. This enzyme uses ATP to activate citrulline by forming a citrullyl-AMP intermediate, which is attacked by the amino group of an aspartate residue to generate argininosuccinate. This and subsequent two reactions occur in the cytosol. Again, expression of argininosuccinate synthetase decreased during the treatment. The third enzyme is argininosuccinate lyase, which catalyzes cleavage of argininosuccinate into fumarate and arginine. The last enzyme is argininase. Argininases cleave arginine to produce urea and ornithine. In a contrast to the decreased expression of ornithine transcarbamoylase and argininosuccinate synthetase genes, argininase I and II increased (FIG. 19).

Arginine is also a substrate for nitric oxide synthase, which oxidizes arginine to produce citrulline and nitric oxide. Expression of nitric oxide synthase gene increased too (FIG. 19).

#### Example 20—Citrulline Increased in Gingival Samples in Experiment Gingivitis

Experimental gingivitis: Another clinical study was carried out to determine whether citrulline is increased in experimentally induced gingivitis in healthy human panelists. This was a case-control study enrolling 60 panelists. The study population included two groups as follows: Group 1 or high bleeders group, thirty (30) panelists with at least 20 bleeding sites, where bleeding is a GBI site score of 1 or 2 at baseline. Group 2 or low bleeders group, thirty (30) panelists with 2 or less bleeding sites, where bleeding is a GBI site score of 1 or 2.

The study consisted of two Phases: Health/Rigorous Hygiene Phase with dental prophylaxis, polishing and rigorous oral hygiene; and Induced Gingivitis Phase without oral hygiene. At the Screening visit, panelists underwent an oral soft tissue assessment and had a gingivitis evaluation (Modified Gingival Index (MGI) and Gingival Bleeding Index (GBI). At Visit 2 qualifying panelists received an oral soft tissue exam followed by a gingivitis evaluation and gingival plaques and gum swabs were collected for the qPCR, protein and RNA host biomarker analysis. Following that, all panelists received dental prophylaxis and entered the Health/Rigorous Hygiene Phase, lasting two weeks. After two weeks of rigorous hygiene, panelists entered the Induced Gingivitis Phase, lasting for three weeks. Oral soft tissue exams and gingivitis were re-evaluated and all samples (gum swabs) were collected at Baseline, WK0 and WK2.

Gingival sample collection—A gum swab was collected from each side of the upper arch using the procedures as described in EXAMPLE 15. Gum swabs were collected close to the gum line from the buccal sites only (preferably from four adjacent teeth—preferably from premolar and molar areas). Panelists rinsed for 30 seconds with 15 ml of Listerine rinse to clean the surface of sampling area. After the Listerine rinse, panelists rinsed for 30 seconds with 20 ml of water. Following that, selected sites were isolated with a cotton roll and gently dried with an air syringe and two gum swabs were taken with collection brushes/swabs from the gingiva region close to the gumline of the selected teeth. The samples were placed in a pre-labeled (panelist ID, sample ID, visit, and date) 1.5 ml micro-centrifuge tube



containing 800  $\mu$ l DPBS (Dulbecco's phosphate-buffered saline) (Lifetechnologies, Grand Island, N.Y.) with protease inhibitors, including AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride) 2 mM, aprotinin 0.3  $\mu$ M, Bestatin 130  $\mu$ M, EDTA (Ethylenediaminetetraacetic acid) 1 mM, E-64 1  $\mu$ M, and leupeptin 1  $\mu$ M. The vials were vortexed/mixed for 30 seconds, immediately frozen on dry ice, stored and transferred on dry ice to the lab for biomarker analysis. Samples from three visits were analyzed using the procedures described in EXAMPLE 17, and shown in FIG. 20. Those three visits were baseline, Week 0, (right after the Health/Rigorous Hygiene Phase and before the induced gingivitis phase) and week 2 (at the end of Induced Gingivitis Phase). Free citrulline levels were low in both the high and low bleeders groups at the baseline and week 0, but rose quickly in the induced gingivitis in both groups at week 2.

#### Example 21—Levels of Proteins Containing Citrulline Decreased in Experimentally Induced Gingivitis

The same procedures were used as described in EXAMPLE 17. The samples were the same as described in EXAMPLE 20. Protein bound citrulline was lower at the baseline than that at week 0 in both high and low bleeders groups as shown in FIG. 21 in gingival tissue. It was low in experimental gingivitis in both groups at week 2.

#### Example 22—Levels of Proteins Containing Ornithine Increased in Gingival Samples in Experiment Gingivitis

The same clinical samples from experimental gingivitis (EXAMPLE 20) were analyzed using the procedures described in EXAMPLE 17. The bound ornithine was the lowest at week 0 (FIG. 22) in both groups. Its levels at the baseline were higher than those at week 0. The bound ornithine reached peaks when gingivitis was induced in both groups at week 2. Also it is worth noting the total ornithine (Free and protein bound ornithine) was increased in the induced gingivitis (FIG. 23) in both groups.

#### Example 23—Levels of Proteins Containing Arginine Decreased in Gingival Samples in Experimentally Induced Gingivitis

The same procedures were used as described in EXAMPLE 17. The samples were the same as described in EXAMPLE 20. The protein bound arginine was the lowest in induced gingivitis (FIG. 24) in both groups. Its levels were higher in WK0 than at Baseline in both groups. The total arginine in the gingival brush samples displayed the same patterns as the protein bound one (FIG. 25).

#### Example 24—Citrulline Inhibited LPS Stimulated Production of Proinflammatory Cytokine in Human THP-1 Cells

Citrulline was purchased from Sigma-Aldrich (St. Louis, Mo.). THP1-Dual™ cells were purchased from Invivogen (San Diego, Calif.). Cells were cultured following the manufacturer's instruction, as described in EXAMPLE 11. For treatment, 0.3 mM to 9 mM of citrulline were first added to the culture medium. Then, 300 ng/ml of *P. gingivalis* LPS 1690 were added 60 minutes later. After 24 hours of treat-

ment, media was collected and analyzed for cytokine production using 9-plex kit (Meso Scale Diagnostics Rockville, Md.).

*P. gingivalis* LPS 1690 stimulated cytokine production, as shown in FIG. 26. Citrulline inhibited *P. gingivalis* LPS 1690 effects on proinflammatory cytokine production in a dose-dependent manner. Those cytokines include IL-6, TNF- $\alpha$ , IL-12p70, IL-10, IL-2, IFN-r and IL-1 $\beta$ .

#### Example 25—Binding of BODIPY-TR-Cadaverine to Bacteria and their Products

Bacteria and their products can activate TLR2 and TLR4 reporter genes as described in EXAMPLES 5 and 8. Here, experiments were carried out to determine whether different bacteria and their products have different IC<sub>50</sub> in reducing fluorescent intensity of BODIPY-TR-cadaverine, hereinafter referred to as BC, in a procedure as described in EXAMPLE 4. Briefly, high throughput screen utilizes the fluorescent dye BODIPY-TR-cadaverine 5-(((4-(4,4-difluoro--(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)phenoxy)acetyl)amino)pentylamine hydrochloride (BC fluorescent dye), purchased from Life Technologies (Carlsbad, Calif.), as described previously by Wood, Miller and David (2004) (Comb Chem High Throughput Screen 2004 May; 7(3):239-49. Anti-endotoxin agents. 1. Development of a fluorescent probe displacement method optimized for the rapid identification of lipopolysaccharide-binding agents.). The experiment was carried out at room temperature. *E. coli* LPS (15  $\mu$ g/ml) was mixed in a well of a 96-well solid black flat bottom plate (Corning Incorporated, Corning, N.Y.) with SnF<sub>2</sub> and cetylpyridinium chloride in 30  $\mu$ l of 50 mM Tris buffer at pH 7.4 for 10 min, and then 20  $\mu$ l of 60  $\mu$ M BC fluorescent dye was added right before fluorescence measurement at 21° C., or room temperature. Fluorescent measurements were performed in a SpectraMax M3 automated 96 well plate reader (Molecular Device, Sunnyvale, Calif.). The excitation wavelength was 580 nM and the fluorescence emission was measured at 620 nM. The half maximal inhibitory concentration (IC<sub>50</sub>) is a measure of the effectiveness of bacterial products inhibiting BC emitting fluorescence. It indicates how much of a bacterial material is needed to inhibit BC fluorescence by half using GraphPad Prism software (GraphPad Software, La Jolla, Calif.). As shown in FIGS. 27 and 28, each bacterial product decreases the fluorescent intensity of BC in a dose-dependent manner. Similarly, the whole bacterial cells and the supernatant of bacterial growth also reduced the fluorescence of BC in a dose-dependent fashion (FIGS. 29 and 30).

#### Example 26—Determination of Bacterial Virulence

Growth of bacteria: Two bacteria, Bacterium A and Bacterium B, were cultured in Tryptic Soy Broth medium (Sigma-Aldrich, St. Louis, Mo.) at 37° C. with shaking at 200 rpm. The bacteria were harvested at 24 hours, and suspended in 0.5 ml of phosphate-buffered saline, labeled "live". Half ml of "Live" bacteria was transferred to a 1.5 ml microtube, and heated to 80° C. for 30 min. The heat-treated bacteria were labeled "Heat-Inactivated", or "Dead".

Measurement of TLR responses in THP-1 gene reporter cells (NFkB-SEAP): The Live and Heat-Inactivated bacteria were applied to THP-1 cells as described in EXAMPLE 11. As shown in FIG. 31, EC<sub>50</sub> of Bacterium A and B on activation of NFkB-SEAP reporter gene in THP-1 cells was determined. Both Live and Heat-inactivated (Dead) bacteria stimulated expression of the NFkB-SEAP reporter gene.



Bacterium B had a lower EC50 than Bacterium A in activating expression of the NFkB-SEAP reporter gene.

Cytokine production and measurement: Human peripheral blood mononuclear cells (hPBMC) were obtained from All Cells company (All Cells, Alameda, Calif.) as Leukapheresed blood. Leukapheresed blood was mixed with an equal part of DMEM+glutaGRO supplemented with 9.1% fetal bovine serum and 1% penicillin/streptomycin (Thermo Fisher, Waltham, Mass.). hPBMC were isolated from the 1:1 mixture of blood and culture medium by collecting the buffy coat of a centrifuged Histopaque®-1077 (Sigma-Aldrich, St. Louis, Mo.) buffer density gradient. The cells (200,000 cells) were cultured in 200 µl of DMEM+glutaGRO supplemented with 9.1% fetal bovine serum and 1% penicillin/streptomycin, and treated with Live and Heat-Inactive bacteria (6,250,000 colony-forming units). The medium was harvested at 24 hours after adding the bacteria, and analyzed for proinflammatory cytokines in a kit following manufacturer's instruction (Meso Scale Diagnostics, Rockville, Md.).

As shown in TABLE 9, both live bacterium A and B stimulated production of cytokines in hPBMC. Bacterium B was far more potent than Bacterium A in promoting production of IFN-γ, IL-10, IL-12p70, IL-1β, IL-6, IL-8 and TNF-α in hPBMC.

Statistics	Cytokines	Live Bacterium A	Live Bacterium
Mean	IFN-γ	867.16	12734.36
Mean	IL-10	68.35	412.51
Mean	IL-12p70	23.75	253.47
Mean	IL-1β	2300.86	6969.31
Mean	IL-2	44.24	65.17
Mean	IL-6	3431.05	9963.84
Mean	IL-8	65742.91	70357.25
Mean	TNF-α	3710.66	13825.49
Std Dev	IFN-γ	488.11	8200.13
Std Dev	IL-10	33.56	314.23

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Statistics	Cytokines	Live Bacterium A	Live Bacterium
Std Dev	IL-12p70	14.71	283.33
Std Dev	IL-1β	1569.72	7691.93
Std Dev	IL-2	32.97	36.29
Std Dev	IL-6	2212.62	6552.12
Std Dev	IL-8	18689.24	13669.28
Std Dev	TNF-α	2503.25	8302.26

The dimensions and values disclosed herein are not to be understood as being strictly limited to the exact numerical values recited. Instead, unless otherwise specified, each such dimension is intended to mean both the recited value and a functionally equivalent range surrounding that value. For example, a dimension disclosed as "40 mm" is intended to mean "about 40 mm."

Every document cited herein, including any cross referenced or related patent or application and any patent application or patent to which this application claims priority or benefit thereof, is hereby incorporated herein by reference in its entirety unless expressly excluded or otherwise limited. The citation of any document is not an admission that it is prior art with respect to any invention disclosed or claimed herein or that it alone, or in any combination with any other reference or references, teaches, suggests or discloses any such invention. Further, to the extent that any meaning or definition of a term in this document conflicts with any meaning or definition of the same term in a document incorporated by reference, the meaning or definition assigned to that term in this document shall govern.

While particular embodiments of the present invention have been illustrated and described, it would be obvious to those skilled in the art that various other changes and modifications can be made without departing from the spirit and scope of the invention. It is therefore intended to cover in the appended claims all such changes and modifications that are within the scope of this invention.

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Met Arg Gln Thr Leu Pro Cys Ile Tyr Phe Trp Gly Gly Leu Leu Pro  
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 Phe Gly Met Leu Cys Ala Ser Ser Thr Thr Lys Cys Thr Val Ser His  
 20 25 30  
 Glu Val Ala Asp Cys Ser His Leu Lys Leu Thr Gln Val Pro Asp Asp  
 35 40 45  
 Leu Pro Thr Asn Ile Thr Val Leu Asn Leu Thr His Asn Gln Leu Arg  
 50 55 60  
 Arg Leu Pro Ala Ala Asn Phe Thr Arg Tyr Ser Gln Leu Thr Ser Leu  
 65 70 75 80  
 Asp Val Gly Phe Asn Thr Ile Ser Lys Leu Glu Pro Glu Leu Cys Gln  
 85 90 95  
 Lys Leu Pro Met Leu Lys Val Leu Asn Leu Gln His Asn Glu Leu Ser  
 100 105 110  
 Gln Leu Ser Asp Lys Thr Phe Ala Phe Cys Thr Asn Leu Thr Glu Leu  
 115 120 125



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His Leu Met Ser Asn Ser Ile Gln Lys Ile Lys Asn Asn Pro Phe Val  
 130 135 140

Lys Gln Lys Asn Leu Ile Thr Leu Asp Leu Ser His Asn Gly Leu Ser  
 145 150 155 160

Ser Thr Lys Leu Gly Thr Gln Val Gln Leu Glu Asn Leu Gln Glu Leu  
 165 170 175

Leu Leu Ser Asn Asn Lys Ile Gln Ala Leu Lys Ser Glu Glu Leu Asp  
 180 185 190

Ile Phe Ala Asn Ser Ser Leu Lys Lys Leu Glu Leu Ser Ser Asn Gln  
 195 200 205

Ile Lys Glu Phe Ser Pro Gly Cys Phe His Ala Ile Gly Arg Leu Phe  
 210 215 220

Gly Leu Phe Leu Asn Asn Val Gln Leu Gly Pro Ser Leu Thr Glu Lys  
 225 230 235 240

Leu Cys Leu Glu Leu Ala Asn Thr Ser Ile Arg Asn Leu Ser Leu Ser  
 245 250 255

Asn Ser Gln Leu Ser Thr Thr Ser Asn Thr Thr Phe Leu Gly Leu Lys  
 260 265 270

Trp Thr Asn Leu Thr Met Leu Asp Leu Ser Tyr Asn Asn Leu Asn Val  
 275 280 285

Val Gly Asn Asp Ser Phe Ala Trp Leu Pro Gln Leu Glu Tyr Phe Phe  
 290 295 300

Leu Glu Tyr Asn Asn Ile Gln His Leu Phe Ser His Ser Leu His Gly  
 305 310 315 320

Leu Phe Asn Val Arg Tyr Leu Asn Leu Lys Arg Ser Phe Thr Lys Gln  
 325 330 335

Ser Ile Ser Leu Ala Ser Leu Pro Lys Ile Asp Asp Phe Ser Phe Gln  
 340 345 350

Trp Leu Lys Cys Leu Glu His Leu Asn Met Glu Asp Asn Asp Ile Pro  
 355 360 365

Gly Ile Lys Ser Asn Met Phe Thr Gly Leu Ile Asn Leu Lys Tyr Leu  
 370 375 380

Ser Leu Ser Asn Ser Phe Thr Ser Leu Arg Thr Leu Thr Asn Glu Thr  
 385 390 395 400

Phe Val Ser Leu Ala His Ser Pro Leu His Ile Leu Asn Leu Thr Lys  
 405 410 415

Asn Lys Ile Ser Lys Ile Glu Ser Asp Ala Phe Ser Trp Leu Gly His  
 420 425 430

Leu Glu Val Leu Asp Leu Gly Leu Asn Glu Ile Gly Gln Glu Leu Thr  
 435 440 445

Gly Gln Glu Trp Arg Gly Leu Glu Asn Ile Phe Glu Ile Tyr Leu Ser  
 450 455 460

Tyr Asn Lys Tyr Leu Gln Leu Thr Arg Asn Ser Phe Ala Leu Val Pro  
 465 470 475 480

Ser Leu Gln Arg Leu Met Leu Arg Arg Val Ala Leu Lys Asn Val Asp  
 485 490 495

Ser Ser Pro Ser Pro Phe Gln Pro Leu Arg Asn Leu Thr Ile Leu Asp  
 500 505 510

Leu Ser Asn Asn Asn Ile Ala Asn Ile Asn Asp Asp Met Leu Glu Gly  
 515 520 525

Leu Glu Lys Leu Glu Ile Leu Asp Leu Gln His Asn Asn Leu Ala Arg  
 530 535 540

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Leu Trp Lys His Ala Asn Pro Gly Gly Pro Ile Tyr Phe Leu Lys Gly  
 545 550 555 560  
 Leu Ser His Leu His Ile Leu Asn Leu Glu Ser Asn Gly Phe Asp Glu  
 565 570 575  
 Ile Pro Val Glu Val Phe Lys Asp Leu Phe Glu Leu Lys Ile Ile Asp  
 580 585 590  
 Leu Gly Leu Asn Asn Leu Asn Thr Leu Pro Ala Ser Val Phe Asn Asn  
 595 600 605  
 Gln Val Ser Leu Lys Ser Leu Asn Leu Gln Lys Asn Leu Ile Thr Ser  
 610 615 620  
 Val Glu Lys Lys Val Phe Gly Pro Ala Phe Arg Asn Leu Thr Glu Leu  
 625 630 635 640  
 Asp Met Arg Phe Asn Pro Phe Asp Cys Thr Cys Glu Ser Ile Ala Trp  
 645 650 655  
 Phe Val Asn Trp Ile Asn Glu Thr His Thr Asn Ile Pro Glu Leu Ser  
 660 665 670  
 Ser His Tyr Leu Cys Asn Thr Pro Pro His Tyr His Gly Phe Pro Val  
 675 680 685  
 Arg Leu Phe Asp Thr Ser Ser Cys Lys Asp Ser Ala Pro Phe Glu Leu  
 690 695 700  
 Phe Phe Met Ile Asn Thr Ser Ile Leu Leu Ile Phe Ile Phe Ile Val  
 705 710 715 720  
 Leu Leu Ile His Phe Glu Gly Trp Arg Ile Ser Phe Tyr Trp Asn Val  
 725 730 735  
 Ser Val His Arg Val Leu Gly Phe Lys Glu Ile Asp Arg Gln Thr Glu  
 740 745 750  
 Gln Phe Glu Tyr Ala Ala Tyr Ile Ile His Ala Tyr Lys Asp Lys Asp  
 755 760 765  
 Trp Val Trp Glu His Phe Ser Ser Met Glu Lys Glu Asp Gln Ser Leu  
 770 775 780  
 Lys Phe Cys Leu Glu Glu Arg Asp Phe Glu Ala Gly Val Phe Glu Leu  
 785 790 795 800  
 Glu Ala Ile Val Asn Ser Ile Lys Arg Ser Arg Lys Ile Ile Phe Val  
 805 810 815  
 Ile Thr His His Leu Leu Lys Asp Pro Leu Cys Lys Arg Phe Lys Val  
 820 825 830  
 His His Ala Val Gln Gln Ala Ile Glu Gln Asn Leu Asp Ser Ile Ile  
 835 840 845  
 Leu Val Phe Leu Glu Glu Ile Pro Asp Tyr Lys Leu Asn His Ala Leu  
 850 855 860  
 Cys Leu Arg Arg Gly Met Phe Lys Ser His Cys Ile Leu Asn Trp Pro  
 865 870 875 880  
 Val Gln Lys Glu Arg Ile Gly Ala Phe Arg His Lys Leu Gln Val Ala  
 885 890 895  
 Leu Gly Ser Lys Asn Ser Val His  
 900

<210> SEQ ID NO 4  
 <211> LENGTH: 798  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

Met Glu Leu Asn Phe Tyr Lys Ile Pro Asp Asn Leu Pro Phe Ser Thr  
 1 5 10 15



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Lys Asn Leu Asp Leu Ser Phe Asn Pro Leu Arg His Leu Gly Ser Tyr  
 20 25 30  
 Ser Phe Phe Ser Phe Pro Glu Leu Gln Val Leu Asp Leu Ser Arg Cys  
 35 40 45  
 Glu Ile Gln Thr Ile Glu Asp Gly Ala Tyr Gln Ser Leu Ser His Leu  
 50 55 60  
 Ser Thr Leu Ile Leu Thr Gly Asn Pro Ile Gln Ser Leu Ala Leu Gly  
 65 70 75 80  
 Ala Phe Ser Gly Leu Ser Ser Leu Gln Lys Leu Val Ala Val Glu Thr  
 85 90 95  
 Asn Leu Ala Ser Leu Glu Asn Phe Pro Ile Gly His Leu Lys Thr Leu  
 100 105 110  
 Lys Glu Leu Asn Val Ala His Asn Leu Ile Gln Ser Phe Lys Leu Pro  
 115 120 125  
 Glu Tyr Phe Ser Asn Leu Thr Asn Leu Glu His Leu Asp Leu Ser Ser  
 130 135 140  
 Asn Lys Ile Gln Ser Ile Tyr Cys Thr Asp Leu Arg Val Leu His Gln  
 145 150 155 160  
 Met Pro Leu Leu Asn Leu Ser Leu Asp Leu Ser Leu Asn Pro Met Asn  
 165 170 175  
 Phe Ile Gln Pro Gly Ala Phe Lys Glu Ile Arg Leu His Lys Leu Thr  
 180 185 190  
 Leu Arg Asn Asn Phe Asp Ser Leu Asn Val Met Lys Thr Cys Ile Gln  
 195 200 205  
 Gly Leu Ala Gly Leu Glu Val His Arg Leu Val Leu Gly Glu Phe Arg  
 210 215 220  
 Asn Glu Gly Asn Leu Glu Lys Phe Asp Lys Ser Ala Leu Glu Gly Leu  
 225 230 235 240  
 Cys Asn Leu Thr Ile Glu Glu Phe Arg Leu Ala Tyr Leu Asp Tyr Tyr  
 245 250 255  
 Leu Asp Asp Ile Ile Asp Leu Phe Asn Cys Leu Thr Asn Val Ser Ser  
 260 265 270  
 Phe Ser Leu Val Ser Val Thr Ile Glu Arg Val Lys Asp Phe Ser Tyr  
 275 280 285  
 Asn Phe Gly Trp Gln His Leu Glu Leu Val Asn Cys Lys Phe Gly Gln  
 290 295 300  
 Phe Pro Thr Leu Lys Leu Lys Ser Leu Lys Arg Leu Thr Phe Thr Ser  
 305 310 315 320  
 Asn Lys Gly Gly Asn Ala Phe Ser Glu Val Asp Leu Pro Ser Leu Glu  
 325 330 335  
 Phe Leu Asp Leu Ser Arg Asn Gly Leu Ser Phe Lys Gly Cys Cys Ser  
 340 345 350  
 Gln Ser Asp Phe Gly Thr Thr Ser Leu Lys Tyr Leu Asp Leu Ser Phe  
 355 360 365  
 Asn Gly Val Ile Thr Met Ser Ser Asn Phe Leu Gly Leu Glu Gln Leu  
 370 375 380  
 Glu His Leu Asp Phe Gln His Ser Asn Leu Lys Gln Met Ser Glu Phe  
 385 390 395 400  
 Ser Val Phe Leu Ser Leu Arg Asn Leu Ile Tyr Leu Asp Ile Ser His  
 405 410 415  
 Thr His Thr Arg Val Ala Phe Asn Gly Ile Phe Asn Gly Leu Ser Ser  
 420 425 430

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Leu Glu Val Leu Lys Met Ala Gly Asn Ser Phe Gln Glu Asn Phe Leu  
                   435                                  440                                  445

Pro Asp Ile Phe Thr Glu Leu Arg Asn Leu Thr Phe Leu Asp Leu Ser  
                   450                                  455                                  460

Gln Cys Gln Leu Glu Gln Leu Ser Pro Thr Ala Phe Asn Ser Leu Ser  
 465                                  470                                  475                                  480

Ser Leu Gln Val Leu Asn Met Ser His Asn Asn Phe Phe Ser Leu Asp  
                                   485                                  490                                  495

Thr Phe Pro Tyr Lys Cys Leu Asn Ser Leu Gln Val Leu Asp Tyr Ser  
                                   500                                  505                                  510

Leu Asn His Ile Met Thr Ser Lys Lys Gln Glu Leu Gln His Phe Pro  
                   515                                  520                                  525

Ser Ser Leu Ala Phe Leu Asn Leu Thr Gln Asn Asp Phe Ala Cys Thr  
                   530                                  535                                  540

Cys Glu His Gln Ser Phe Leu Gln Trp Ile Lys Asp Gln Arg Gln Leu  
 545                                  550                                  555                                  560

Leu Val Glu Val Glu Arg Met Glu Cys Ala Thr Pro Ser Asp Lys Gln  
                                   565                                  570                                  575

Gly Met Pro Val Leu Ser Leu Asn Ile Thr Cys Gln Met Asn Lys Thr  
                                   580                                  585                                  590

Ile Ile Gly Val Ser Val Leu Ser Val Leu Val Val Ser Val Val Ala  
                   595                                  600                                  605

Val Leu Val Tyr Lys Phe Tyr Phe His Leu Met Leu Leu Ala Gly Cys  
                   610                                  615                                  620

Ile Lys Tyr Gly Arg Gly Glu Asn Ile Tyr Asp Ala Phe Val Ile Tyr  
 625                                  630                                  635                                  640

Ser Ser Gln Asp Glu Asp Trp Val Arg Asn Glu Leu Val Lys Asn Leu  
                                   645                                  650                                  655

Glu Glu Gly Val Pro Pro Phe Gln Leu Cys Leu His Tyr Arg Asp Phe  
                                   660                                  665                                  670

Ile Pro Gly Val Ala Ile Ala Ala Asn Ile Ile His Glu Gly Phe His  
                   675                                  680                                  685

Lys Ser Arg Lys Val Ile Val Val Val Ser Gln His Phe Ile Gln Ser  
                   690                                  695                                  700

Arg Trp Cys Ile Phe Glu Tyr Glu Ile Ala Gln Thr Trp Gln Phe Leu  
 705                                  710                                  715                                  720

Ser Ser Arg Ala Gly Ile Ile Phe Ile Val Leu Gln Lys Val Glu Lys  
                                   725                                  730                                  735

Thr Leu Leu Arg Gln Gln Val Glu Leu Tyr Arg Leu Leu Ser Arg Asn  
                                   740                                  745                                  750

Thr Tyr Leu Glu Trp Glu Asp Ser Val Leu Gly Arg His Ile Phe Trp  
                   755                                  760                                  765

Arg Arg Leu Arg Lys Ala Leu Leu Asp Gly Lys Ser Trp Asn Pro Glu  
                   770                                  775                                  780

Gly Thr Val Gly Thr Gly Cys Asn Trp Gln Glu Ala Thr Ser  
 785                                  790                                  795

<210> SEQ ID NO 5  
 <211> LENGTH: 858  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

Met Gly Asp His Leu Asp Leu Leu Leu Gly Val Val Leu Met Ala Gly  
 1                  5                                  10                                  15



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Pro Val Phe Gly Ile Pro Ser Cys Ser Phe Asp Gly Arg Ile Ala Phe  
 20 25 30  
 Tyr Arg Phe Cys Asn Leu Thr Gln Val Pro Gln Val Leu Asn Thr Thr  
 35 40 45  
 Glu Arg Leu Leu Leu Ser Phe Asn Tyr Ile Arg Thr Val Thr Ala Ser  
 50 55 60  
 Ser Phe Pro Phe Leu Glu Gln Leu Gln Leu Leu Glu Leu Gly Ser Gln  
 65 70 75 80  
 Tyr Thr Pro Leu Thr Ile Asp Lys Glu Ala Phe Arg Asn Leu Pro Asn  
 85 90 95  
 Leu Arg Ile Leu Asp Leu Gly Ser Ser Lys Ile Tyr Phe Leu His Pro  
 100 105 110  
 Asp Ala Phe Gln Gly Leu Phe His Leu Phe Glu Leu Arg Leu Tyr Phe  
 115 120 125  
 Cys Gly Leu Ser Asp Ala Val Leu Lys Asp Gly Tyr Phe Arg Asn Leu  
 130 135 140  
 Lys Ala Leu Thr Arg Leu Asp Leu Ser Lys Asn Gln Ile Arg Ser Leu  
 145 150 155 160  
 Tyr Leu His Pro Ser Phe Gly Lys Leu Asn Ser Leu Lys Ser Ile Asp  
 165 170 175  
 Phe Ser Ser Asn Gln Ile Phe Leu Val Cys Glu His Glu Leu Glu Pro  
 180 185 190  
 Leu Gln Gly Lys Thr Leu Ser Phe Phe Ser Leu Ala Ala Asn Ser Leu  
 195 200 205  
 Tyr Ser Arg Val Ser Val Asp Trp Gly Lys Cys Met Asn Pro Phe Arg  
 210 215 220  
 Asn Met Val Leu Glu Ile Leu Asp Val Ser Gly Asn Gly Trp Thr Val  
 225 230 235 240  
 Asp Ile Thr Gly Asn Phe Ser Asn Ala Ile Ser Lys Ser Gln Ala Phe  
 245 250 255  
 Ser Leu Ile Leu Ala His His Ile Met Gly Ala Gly Phe Gly Phe His  
 260 265 270  
 Asn Ile Lys Asp Pro Asp Gln Asn Thr Phe Ala Gly Leu Ala Arg Ser  
 275 280 285  
 Ser Val Arg His Leu Asp Leu Ser His Gly Phe Val Phe Ser Leu Asn  
 290 295 300  
 Ser Arg Val Phe Glu Thr Leu Lys Asp Leu Lys Val Leu Asn Leu Ala  
 305 310 315 320  
 Tyr Asn Lys Ile Asn Lys Ile Ala Asp Glu Ala Phe Tyr Gly Leu Asp  
 325 330 335  
 Asn Leu Gln Val Leu Asn Leu Ser Tyr Asn Leu Leu Gly Glu Leu Tyr  
 340 345 350  
 Ser Ser Asn Phe Tyr Gly Leu Pro Lys Val Ala Tyr Ile Asp Leu Gln  
 355 360 365  
 Lys Asn His Ile Ala Ile Ile Gln Asp Gln Thr Phe Lys Phe Leu Glu  
 370 375 380  
 Lys Leu Gln Thr Leu Asp Leu Arg Asp Asn Ala Leu Thr Thr Ile His  
 385 390 395 400  
 Phe Ile Pro Ser Ile Pro Asp Ile Phe Leu Ser Gly Asn Lys Leu Val  
 405 410 415  
 Thr Leu Pro Lys Ile Asn Leu Thr Ala Asn Leu Ile His Leu Ser Glu  
 420 425 430

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Asn Arg Leu Glu Asn Leu Asp Ile Leu Tyr Phe Leu Leu Arg Val Pro  
           435                                  440                                  445

His Leu Gln Ile Leu Ile Leu Asn Gln Asn Arg Phe Ser Ser Cys Ser  
       450                                  455                                  460

Gly Asp Gln Thr Pro Ser Glu Asn Pro Ser Leu Glu Gln Leu Phe Leu  
 465                                  470                                  475                                  480

Gly Glu Asn Met Leu Gln Leu Ala Trp Glu Thr Glu Leu Cys Trp Asp  
                                   485                                  490                                  495

Val Phe Glu Gly Leu Ser His Leu Gln Val Leu Tyr Leu Asn His Asn  
                                   500                                  505                                  510

Tyr Leu Asn Ser Leu Pro Pro Gly Val Phe Ser His Leu Thr Ala Leu  
                                   515                                  520                                  525

Arg Gly Leu Ser Leu Asn Ser Asn Arg Leu Thr Val Leu Ser His Asn  
       530                                  535                                  540

Asp Leu Pro Ala Asn Leu Glu Ile Leu Asp Ile Ser Arg Asn Gln Leu  
 545                                  550                                  555                                  560

Leu Ala Pro Asn Pro Asp Val Phe Val Ser Leu Ser Val Leu Asp Ile  
                                   565                                  570                                  575

Thr His Asn Lys Phe Ile Cys Glu Cys Glu Leu Ser Thr Phe Ile Asn  
                                   580                                  585                                  590

Trp Leu Asn His Thr Asn Val Thr Ile Ala Gly Pro Pro Ala Asp Ile  
                                   595                                  600                                  605

Tyr Cys Val Tyr Pro Asp Ser Phe Ser Gly Val Ser Leu Phe Ser Leu  
       610                                  615                                  620

Ser Thr Glu Gly Cys Asp Glu Glu Glu Val Leu Lys Ser Leu Lys Phe  
 625                                  630                                  635                                  640

Ser Leu Phe Ile Val Cys Thr Val Thr Leu Thr Leu Phe Leu Met Thr  
                                   645                                  650                                  655

Ile Leu Thr Val Thr Lys Phe Arg Gly Phe Cys Phe Ile Cys Tyr Lys  
                                   660                                  665                                  670

Thr Ala Gln Arg Leu Val Phe Lys Asp His Pro Gln Gly Thr Glu Pro  
       675                                  680                                  685

Asp Met Tyr Lys Tyr Asp Ala Tyr Leu Cys Phe Ser Ser Lys Asp Phe  
       690                                  695                                  700

Thr Trp Val Gln Asn Ala Leu Leu Lys His Leu Asp Thr Gln Tyr Ser  
 705                                  710                                  715                                  720

Asp Gln Asn Arg Phe Asn Leu Cys Phe Glu Glu Arg Asp Phe Val Pro  
       725                                  730                                  735

Gly Glu Asn Arg Ile Ala Asn Ile Gln Asp Ala Ile Trp Asn Ser Arg  
       740                                  745                                  750

Lys Ile Val Cys Leu Val Ser Arg His Phe Leu Arg Asp Gly Trp Cys  
       755                                  760                                  765

Leu Glu Ala Phe Ser Tyr Ala Gln Gly Arg Cys Leu Ser Asp Leu Asn  
       770                                  775                                  780

Ser Ala Leu Ile Met Val Val Val Gly Ser Leu Ser Gln Tyr Gln Leu  
 785                                  790                                  795                                  800

Met Lys His Gln Ser Ile Arg Gly Phe Val Gln Lys Gln Gln Tyr Leu  
                                   805                                  810                                  815

Arg Trp Pro Glu Asp Leu Gln Asp Val Gly Trp Phe Leu His Lys Leu  
                                   820                                  825                                  830

Ser Gln Gln Ile Leu Lys Lys Glu Lys Glu Lys Lys Lys Asp Asn Asn  
                                   835                                  840                                  845

Ile Pro Leu Gln Thr Val Ala Thr Ile Ser



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850 855

<210> SEQ ID NO 6  
 <211> LENGTH: 796  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 6

Met Thr Lys Asp Lys Glu Pro Ile Val Lys Ser Phe His Phe Val Cys  
 1 5 10 15

Leu Met Ile Ile Ile Val Gly Thr Arg Ile Gln Phe Ser Asp Gly Asn  
 20 25 30

Glu Phe Ala Val Asp Lys Ser Lys Arg Gly Leu Ile His Val Pro Lys  
 35 40 45

Asp Leu Pro Leu Lys Thr Lys Val Leu Asp Met Ser Gln Asn Tyr Ile  
 50 55 60

Ala Glu Leu Gln Val Ser Asp Met Ser Phe Leu Ser Glu Leu Thr Val  
 65 70 75 80

Leu Arg Leu Ser His Asn Arg Ile Gln Leu Leu Asp Leu Ser Val Phe  
 85 90 95

Lys Phe Asn Gln Asp Leu Glu Tyr Leu Asp Leu Ser His Asn Gln Leu  
 100 105 110

Gln Lys Ile Ser Cys His Pro Ile Val Ser Phe Arg His Leu Asp Leu  
 115 120 125

Ser Phe Asn Asp Phe Lys Ala Leu Pro Ile Cys Lys Glu Phe Gly Asn  
 130 135 140

Leu Ser Gln Leu Asn Phe Leu Gly Leu Ser Ala Met Lys Leu Gln Lys  
 145 150 155 160

Leu Asp Leu Leu Pro Ile Ala His Leu His Leu Ser Tyr Ile Leu Leu  
 165 170 175

Asp Leu Arg Asn Tyr Tyr Ile Lys Glu Asn Glu Thr Glu Ser Leu Gln  
 180 185 190

Ile Leu Asn Ala Lys Thr Leu His Leu Val Phe His Pro Thr Ser Leu  
 195 200 205

Phe Ala Ile Gln Val Asn Ile Ser Val Asn Thr Leu Gly Cys Leu Gln  
 210 215 220

Leu Thr Asn Ile Lys Leu Asn Asp Asp Asn Cys Gln Val Phe Ile Lys  
 225 230 235 240

Phe Leu Ser Glu Leu Thr Arg Gly Pro Thr Leu Leu Asn Phe Thr Leu  
 245 250 255

Asn His Ile Glu Thr Thr Trp Lys Cys Leu Val Arg Val Phe Gln Phe  
 260 265 270

Leu Trp Pro Lys Pro Val Glu Tyr Leu Asn Ile Tyr Asn Leu Thr Ile  
 275 280 285

Ile Glu Ser Ile Arg Glu Glu Asp Phe Thr Tyr Ser Lys Thr Thr Leu  
 290 295 300

Lys Ala Leu Thr Ile Glu His Ile Thr Asn Gln Val Phe Leu Phe Ser  
 305 310 315 320

Gln Thr Ala Leu Tyr Thr Val Phe Ser Glu Met Asn Ile Met Met Leu  
 325 330 335

Thr Ile Ser Asp Thr Pro Phe Ile His Met Leu Cys Pro His Ala Pro  
 340 345 350

Ser Thr Phe Lys Phe Leu Asn Phe Thr Gln Asn Val Phe Thr Asp Ser  
 355 360 365

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Ile Phe Glu Lys Cys Ser Thr Leu Val Lys Leu Glu Thr Leu Ile Leu  
 370 375 380

Gln Lys Asn Gly Leu Lys Asp Leu Phe Lys Val Gly Leu Met Thr Lys  
 385 390 395 400

Asp Met Pro Ser Leu Glu Ile Leu Asp Val Ser Trp Asn Ser Leu Glu  
 405 410 415

Ser Gly Arg His Lys Glu Asn Cys Thr Trp Val Glu Ser Ile Val Val  
 420 425 430

Leu Asn Leu Ser Ser Asn Met Leu Thr Asp Ser Val Phe Arg Cys Leu  
 435 440 445

Pro Pro Arg Ile Lys Val Leu Asp Leu His Ser Asn Lys Ile Lys Ser  
 450 455 460

Val Pro Lys Gln Val Val Lys Leu Glu Ala Leu Gln Glu Leu Asn Val  
 465 470 475 480

Ala Phe Asn Ser Leu Thr Asp Leu Pro Gly Cys Gly Ser Phe Ser Ser  
 485 490 495

Leu Ser Val Leu Ile Ile Asp His Asn Ser Val Ser His Pro Ser Ala  
 500 505 510

Asp Phe Phe Gln Ser Cys Gln Lys Met Arg Ser Ile Lys Ala Gly Asp  
 515 520 525

Asn Pro Phe Gln Cys Thr Cys Glu Leu Arg Glu Phe Val Lys Asn Ile  
 530 535 540

Asp Gln Val Ser Ser Glu Val Leu Glu Gly Trp Pro Asp Ser Tyr Lys  
 545 550 555 560

Cys Asp Tyr Pro Glu Ser Tyr Arg Gly Ser Pro Leu Lys Asp Phe His  
 565 570 575

Met Ser Glu Leu Ser Cys Asn Ile Thr Leu Leu Ile Val Thr Ile Gly  
 580 585 590

Ala Thr Met Leu Val Leu Ala Val Thr Val Thr Ser Leu Cys Ile Tyr  
 595 600 605

Leu Asp Leu Pro Trp Tyr Leu Arg Met Val Cys Gln Trp Thr Gln Thr  
 610 615 620

Arg Arg Arg Ala Arg Asn Ile Pro Leu Glu Glu Leu Gln Arg Asn Leu  
 625 630 635 640

Gln Phe His Ala Phe Ile Ser Tyr Ser Glu His Asp Ser Ala Trp Val  
 645 650 655

Lys Ser Glu Leu Val Pro Tyr Leu Glu Lys Glu Asp Ile Gln Ile Cys  
 660 665 670

Leu His Glu Arg Asn Phe Val Pro Gly Lys Ser Ile Val Glu Asn Ile  
 675 680 685

Ile Asn Cys Ile Glu Lys Ser Tyr Lys Ser Ile Phe Val Leu Ser Pro  
 690 695 700

Asn Phe Val Gln Ser Glu Trp Cys His Tyr Glu Leu Tyr Phe Ala His  
 705 710 715 720

His Asn Leu Phe His Glu Gly Ser Asn Asn Leu Ile Leu Ile Leu Leu  
 725 730 735

Glu Pro Ile Pro Gln Asn Ser Ile Pro Asn Lys Tyr His Lys Leu Lys  
 740 745 750

Ala Leu Met Thr Gln Arg Thr Tyr Leu Gln Trp Pro Lys Glu Lys Ser  
 755 760 765

Lys Arg Gly Leu Phe Trp Ala Asn Ile Arg Ala Ala Phe Asn Met Lys  
 770 775 780

Leu Thr Leu Val Thr Glu Asn Asn Asp Val Lys Ser



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785                      790                      795  
 <210> SEQ ID NO 7  
 <211> LENGTH: 1049  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens  
 <400> SEQUENCE: 7  
 Met Val Phe Pro Met Trp Thr Leu Lys Arg Gln Ile Leu Ile Leu Phe  
 1                      5                      10                      15  
 Asn Ile Ile Leu Ile Ser Lys Leu Leu Gly Ala Arg Trp Phe Pro Lys  
                     20                      25                      30  
 Thr Leu Pro Cys Asp Val Thr Leu Asp Val Pro Lys Asn His Val Ile  
                     35                      40                      45  
 Val Asp Cys Thr Asp Lys His Leu Thr Glu Ile Pro Gly Gly Ile Pro  
                     50                      55                      60  
 Thr Asn Thr Thr Asn Leu Thr Leu Thr Ile Asn His Ile Pro Asp Ile  
 65                      70                      75                      80  
 Ser Pro Ala Ser Phe His Arg Leu Asp His Leu Val Glu Ile Asp Phe  
                     85                      90                      95  
 Arg Cys Asn Cys Val Pro Ile Pro Leu Gly Ser Lys Asn Asn Met Cys  
                     100                      105                      110  
 Ile Lys Arg Leu Gln Ile Lys Pro Arg Ser Phe Ser Gly Leu Thr Tyr  
                     115                      120                      125  
 Leu Lys Ser Leu Tyr Leu Asp Gly Asn Gln Leu Leu Glu Ile Pro Gln  
                     130                      135                      140  
 Gly Leu Pro Pro Ser Leu Gln Leu Leu Ser Leu Glu Ala Asn Asn Ile  
 145                      150                      155                      160  
 Phe Ser Ile Arg Lys Glu Asn Leu Thr Glu Leu Ala Asn Ile Glu Ile  
                     165                      170                      175  
 Leu Tyr Leu Gly Gln Asn Cys Tyr Tyr Arg Asn Pro Cys Tyr Val Ser  
                     180                      185                      190  
 Tyr Ser Ile Glu Lys Asp Ala Phe Leu Asn Leu Thr Lys Leu Lys Val  
                     195                      200                      205  
 Leu Ser Leu Lys Asp Asn Asn Val Thr Ala Val Pro Thr Val Leu Pro  
                     210                      215                      220  
 Ser Thr Leu Thr Glu Leu Tyr Leu Tyr Asn Asn Met Ile Ala Lys Ile  
 225                      230                      235                      240  
 Gln Glu Asp Asp Phe Asn Asn Leu Asn Gln Leu Gln Ile Leu Asp Leu  
                     245                      250                      255  
 Ser Gly Asn Cys Pro Arg Cys Tyr Asn Ala Pro Phe Pro Cys Ala Pro  
                     260                      265                      270  
 Cys Lys Asn Asn Ser Pro Leu Gln Ile Pro Val Asn Ala Phe Asp Ala  
                     275                      280                      285  
 Leu Thr Glu Leu Lys Val Leu Arg Leu His Ser Asn Ser Leu Gln His  
                     290                      295                      300  
 Val Pro Pro Arg Trp Phe Lys Asn Ile Asn Lys Leu Gln Glu Leu Asp  
 305                      310                      315                      320  
 Leu Ser Gln Asn Phe Leu Ala Lys Glu Ile Gly Asp Ala Lys Phe Leu  
                     325                      330                      335  
 His Phe Leu Pro Ser Leu Ile Gln Leu Asp Leu Ser Phe Asn Phe Glu  
                     340                      345                      350  
 Leu Gln Val Tyr Arg Ala Ser Met Asn Leu Ser Gln Ala Phe Ser Ser  
                     355                      360                      365

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Leu Lys Ser Leu Lys Ile Leu Arg Ile Arg Gly Tyr Val Phe Lys Glu  
 370 375 380

Leu Lys Ser Phe Asn Leu Ser Pro Leu His Asn Leu Gln Asn Leu Glu  
 385 390 395 400

Val Leu Asp Leu Gly Thr Asn Phe Ile Lys Ile Ala Asn Leu Ser Met  
 405 410 415

Phe Lys Gln Phe Lys Arg Leu Lys Val Ile Asp Leu Ser Val Asn Lys  
 420 425 430

Ile Ser Pro Ser Gly Asp Ser Ser Glu Val Gly Phe Cys Ser Asn Ala  
 435 440 445

Arg Thr Ser Val Glu Ser Tyr Glu Pro Gln Val Leu Glu Gln Leu His  
 450 455 460

Tyr Phe Arg Tyr Asp Lys Tyr Ala Arg Ser Cys Arg Phe Lys Asn Lys  
 465 470 475 480

Glu Ala Ser Phe Met Ser Val Asn Glu Ser Cys Tyr Lys Tyr Gly Gln  
 485 490 495

Thr Leu Asp Leu Ser Lys Asn Ser Ile Phe Phe Val Lys Ser Ser Asp  
 500 505 510

Phe Gln His Leu Ser Phe Leu Lys Cys Leu Asn Leu Ser Gly Asn Leu  
 515 520 525

Ile Ser Gln Thr Leu Asn Gly Ser Glu Phe Gln Pro Leu Ala Glu Leu  
 530 535 540

Arg Tyr Leu Asp Phe Ser Asn Asn Arg Leu Asp Leu Leu His Ser Thr  
 545 550 555 560

Ala Phe Glu Glu Leu His Lys Leu Glu Val Leu Asp Ile Ser Ser Asn  
 565 570 575

Ser His Tyr Phe Gln Ser Glu Gly Ile Thr His Met Leu Asn Phe Thr  
 580 585 590

Lys Asn Leu Lys Val Leu Gln Lys Leu Met Met Asn Asp Asn Asp Ile  
 595 600 605

Ser Ser Ser Thr Ser Arg Thr Met Glu Ser Glu Ser Leu Arg Thr Leu  
 610 615 620

Glu Phe Arg Gly Asn His Leu Asp Val Leu Trp Arg Glu Gly Asp Asn  
 625 630 635 640

Arg Tyr Leu Gln Leu Phe Lys Asn Leu Leu Lys Leu Glu Glu Leu Asp  
 645 650 655

Ile Ser Lys Asn Ser Leu Ser Phe Leu Pro Ser Gly Val Phe Asp Gly  
 660 665 670

Met Pro Pro Asn Leu Lys Asn Leu Ser Leu Ala Lys Asn Gly Leu Lys  
 675 680 685

Ser Phe Ser Trp Lys Lys Leu Gln Cys Leu Lys Asn Leu Glu Thr Leu  
 690 695 700

Asp Leu Ser His Asn Gln Leu Thr Thr Val Pro Glu Arg Leu Ser Asn  
 705 710 715 720

Cys Ser Arg Ser Leu Lys Asn Leu Ile Leu Lys Asn Asn Gln Ile Arg  
 725 730 735

Ser Leu Thr Lys Tyr Phe Leu Gln Asp Ala Phe Gln Leu Arg Tyr Leu  
 740 745 750

Asp Leu Ser Ser Asn Lys Ile Gln Met Ile Gln Lys Thr Ser Phe Pro  
 755 760 765

Glu Asn Val Leu Asn Asn Leu Lys Met Leu Leu Leu His His Asn Arg  
 770 775 780

Phe Leu Cys Thr Cys Asp Ala Val Trp Phe Val Trp Trp Val Asn His



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785	790	795	800
Thr Glu Val	Thr Ile Pro Tyr Leu Ala	Thr Asp Val Thr Cys Val Gly	
	805	810	815
Pro Gly Ala His Lys Gly Gln Ser Val Ile Ser Leu Asp Leu Tyr Thr	820	825	830
Cys Glu Leu Asp Leu Thr Asn Leu Ile Leu Phe Ser Leu Ser Ile Ser	835	840	845
Val Ser Leu Phe Leu Met Val Met Met Thr Ala Ser His Leu Tyr Phe	850	855	860
Trp Asp Val Trp Tyr Ile Tyr His Phe Cys Lys Ala Lys Ile Lys Gly	865	870	875
		880	885
Tyr Gln Arg Leu Ile Ser Pro Asp Cys Cys Tyr Asp Ala Phe Ile Val	885	890	895
Tyr Asp Thr Lys Asp Pro Ala Val Thr Glu Trp Val Leu Ala Glu Leu	900	905	910
Val Ala Lys Leu Glu Asp Pro Arg Glu Lys His Phe Asn Leu Cys Leu	915	920	925
Glu Glu Arg Asp Trp Leu Pro Gly Gln Pro Val Leu Glu Asn Leu Ser	930	935	940
Gln Ser Ile Gln Leu Ser Lys Lys Thr Val Phe Val Met Thr Asp Lys	945	950	955
			960
Tyr Ala Lys Thr Glu Asn Phe Lys Ile Ala Phe Tyr Leu Ser His Gln	965	970	975
Arg Leu Met Asp Glu Lys Val Asp Val Ile Ile Leu Ile Phe Leu Glu	980	985	990
Lys Pro Phe Gln Lys Ser Lys Phe Leu Gln Leu Arg Lys Arg Leu Cys	995	1000	1005
Gly Ser Ser Val Leu Glu Trp Pro Thr Asn Pro Gln Ala His Pro	1010	1015	1020
Tyr Phe Trp Gln Cys Leu Lys Asn Ala Leu Ala Thr Asp Asn His	1025	1030	1035
Val Ala Tyr Ser Gln Val Phe Lys Glu Thr Val	1040	1045	

<210> SEQ ID NO 8  
 <211> LENGTH: 1041  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8

Met Glu Asn Met Phe Leu Gln Ser Ser Met Leu Thr Cys Ile Phe Leu	1	5	10	15
Leu Ile Ser Gly Ser Cys Glu Leu Cys Ala Glu Glu Asn Phe Ser Arg	20	25	30	
Ser Tyr Pro Cys Asp Glu Lys Lys Gln Asn Asp Ser Val Ile Ala Glu	35	40	45	
Cys Ser Asn Arg Arg Leu Gln Glu Val Pro Gln Thr Val Gly Lys Tyr	50	55	60	
Val Thr Glu Leu Asp Leu Ser Asp Asn Phe Ile Thr His Ile Thr Asn	65	70	75	80
Glu Ser Phe Gln Gly Leu Gln Asn Leu Thr Lys Ile Asn Leu Asn His	85	90	95	
Asn Pro Asn Val Gln His Gln Asn Gly Asn Pro Gly Ile Gln Ser Asn	100	105	110	

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Gly Leu Asn Ile Thr Asp Gly Ala Phe Leu Asn Leu Lys Asn Leu Arg  
 115 120 125

Glu Leu Leu Leu Glu Asp Asn Gln Leu Pro Gln Ile Pro Ser Gly Leu  
 130 135 140

Pro Glu Ser Leu Thr Glu Leu Ser Leu Ile Gln Asn Asn Ile Tyr Asn  
 145 150 155 160

Ile Thr Lys Glu Gly Ile Ser Arg Leu Ile Asn Leu Lys Asn Leu Tyr  
 165 170 175

Leu Ala Trp Asn Cys Tyr Phe Asn Lys Val Cys Glu Lys Thr Asn Ile  
 180 185 190

Glu Asp Gly Val Phe Glu Thr Leu Thr Asn Leu Glu Leu Leu Ser Leu  
 195 200 205

Ser Phe Asn Ser Leu Ser His Val Pro Pro Lys Leu Pro Ser Ser Leu  
 210 215 220

Arg Lys Leu Phe Leu Ser Asn Thr Gln Ile Lys Tyr Ile Ser Glu Glu  
 225 230 235 240

Asp Phe Lys Gly Leu Ile Asn Leu Thr Leu Leu Asp Leu Ser Gly Asn  
 245 250 255

Cys Pro Arg Cys Phe Asn Ala Pro Phe Pro Cys Val Pro Cys Asp Gly  
 260 265 270

Gly Ala Ser Ile Asn Ile Asp Arg Phe Ala Phe Gln Asn Leu Thr Gln  
 275 280 285

Leu Arg Tyr Leu Asn Leu Ser Ser Thr Ser Leu Arg Lys Ile Asn Ala  
 290 295 300

Ala Trp Phe Lys Asn Met Pro His Leu Lys Val Leu Asp Leu Glu Phe  
 305 310 315 320

Asn Tyr Leu Val Gly Glu Ile Ala Ser Gly Ala Phe Leu Thr Met Leu  
 325 330 335

Pro Arg Leu Glu Ile Leu Asp Leu Ser Phe Asn Tyr Ile Lys Gly Ser  
 340 345 350

Tyr Pro Gln His Ile Asn Ile Ser Arg Asn Phe Ser Lys Leu Leu Ser  
 355 360 365

Leu Arg Ala Leu His Leu Arg Gly Tyr Val Phe Gln Glu Leu Arg Glu  
 370 375 380

Asp Asp Phe Gln Pro Leu Met Gln Leu Pro Asn Leu Ser Thr Ile Asn  
 385 390 395 400

Leu Gly Ile Asn Phe Ile Lys Gln Ile Asp Phe Lys Leu Phe Gln Asn  
 405 410 415

Phe Ser Asn Leu Glu Ile Ile Tyr Leu Ser Glu Asn Arg Ile Ser Pro  
 420 425 430

Leu Val Lys Asp Thr Arg Gln Ser Tyr Ala Asn Ser Ser Ser Phe Gln  
 435 440 445

Arg His Ile Arg Lys Arg Arg Ser Thr Asp Phe Glu Phe Asp Pro His  
 450 455 460

Ser Asn Phe Tyr His Phe Thr Arg Pro Leu Ile Lys Pro Gln Cys Ala  
 465 470 475 480

Ala Tyr Gly Lys Ala Leu Asp Leu Ser Leu Asn Ser Ile Phe Phe Ile  
 485 490 495

Gly Pro Asn Gln Phe Glu Asn Leu Pro Asp Ile Ala Cys Leu Asn Leu  
 500 505 510

Ser Ala Asn Ser Asn Ala Gln Val Leu Ser Gly Thr Glu Phe Ser Ala  
 515 520 525

Ile Pro His Val Lys Tyr Leu Asp Leu Thr Asn Asn Arg Leu Asp Phe



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530				535				540							
Asp	Asn	Ala	Ser	Ala	Leu	Thr	Glu	Leu	Ser	Asp	Leu	Glu	Val	Leu	Asp
545					550					555					560
Leu	Ser	Tyr	Asn	Ser	His	Tyr	Phe	Arg	Ile	Ala	Gly	Val	Thr	His	His
				565					570					575	
Leu	Glu	Phe	Ile	Gln	Asn	Phe	Thr	Asn	Leu	Lys	Val	Leu	Asn	Leu	Ser
				580				585				590			
His	Asn	Asn	Ile	Tyr	Thr	Leu	Thr	Asp	Lys	Tyr	Asn	Leu	Glu	Ser	Lys
		595					600					605			
Ser	Leu	Val	Glu	Leu	Val	Phe	Ser	Gly	Asn	Arg	Leu	Asp	Ile	Leu	Trp
	610					615					620				
Asn	Asp	Asp	Asp	Asn	Arg	Tyr	Ile	Ser	Ile	Phe	Lys	Gly	Leu	Lys	Asn
625					630					635					640
Leu	Thr	Arg	Leu	Asp	Leu	Ser	Leu	Asn	Arg	Leu	Lys	His	Ile	Pro	Asn
				645					650					655	
Glu	Ala	Phe	Leu	Asn	Leu	Pro	Ala	Ser	Leu	Thr	Glu	Leu	His	Ile	Asn
			660						665				670		
Asp	Asn	Met	Leu	Lys	Phe	Phe	Asn	Trp	Thr	Leu	Leu	Gln	Gln	Phe	Pro
		675					680					685			
Arg	Leu	Glu	Leu	Leu	Asp	Leu	Arg	Gly	Asn	Lys	Leu	Leu	Phe	Leu	Thr
	690					695					700				
Asp	Ser	Leu	Ser	Asp	Phe	Thr	Ser	Ser	Leu	Arg	Thr	Leu	Leu	Leu	Ser
705					710					715					720
His	Asn	Arg	Ile	Ser	His	Leu	Pro	Ser	Gly	Phe	Leu	Ser	Glu	Val	Ser
				725					730					735	
Ser	Leu	Lys	His	Leu	Asp	Leu	Ser	Ser	Asn	Leu	Leu	Lys	Thr	Ile	Asn
			740						745				750		
Lys	Ser	Ala	Leu	Glu	Thr	Lys	Thr	Thr	Thr	Lys	Leu	Ser	Met	Leu	Glu
		755					760					765			
Leu	His	Gly	Asn	Pro	Phe	Glu	Cys	Thr	Cys	Asp	Ile	Gly	Asp	Phe	Arg
	770					775					780				
Arg	Trp	Met	Asp	Glu	His	Leu	Asn	Val	Lys	Ile	Pro	Arg	Leu	Val	Asp
785					790					795					800
Val	Ile	Cys	Ala	Ser	Pro	Gly	Asp	Gln	Arg	Gly	Lys	Ser	Ile	Val	Ser
			805						810					815	
Leu	Glu	Leu	Thr	Thr	Cys	Val	Ser	Asp	Val	Thr	Ala	Val	Ile	Leu	Phe
			820					825					830		
Phe	Phe	Thr	Phe	Phe	Ile	Thr	Thr	Met	Val	Met	Leu	Ala	Ala	Leu	Ala
		835					840					845			
His	His	Leu	Phe	Tyr	Trp	Asp	Val	Trp	Phe	Ile	Tyr	Asn	Val	Cys	Leu
	850					855					860				
Ala	Lys	Val	Lys	Gly	Tyr	Arg	Ser	Leu	Ser	Thr	Ser	Gln	Thr	Phe	Tyr
865					870					875					880
Asp	Ala	Tyr	Ile	Ser	Tyr	Asp	Thr	Lys	Asp	Ala	Ser	Val	Thr	Asp	Trp
				885					890					895	
Val	Ile	Asn	Glu	Leu	Arg	Tyr	His	Leu	Glu	Glu	Ser	Arg	Asp	Lys	Asn
			900					905					910		
Val	Leu	Leu	Cys	Leu	Glu	Glu	Arg	Asp	Trp	Asp	Pro	Gly	Leu	Ala	Ile
		915					920					925			
Ile	Asp	Asn	Leu	Met	Gln	Ser	Ile	Asn	Gln	Ser	Lys	Lys	Thr	Val	Phe
	930					935					940				
Val	Leu	Thr	Lys	Lys	Tyr	Ala	Lys	Ser	Trp	Asn	Phe	Lys	Thr	Ala	Phe
945					950					955					960





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275			280			285									
Leu	Val	Leu	Lys	Asp	Ser	Ser	Leu	Ser	Trp	Leu	Asn	Ala	Ser	Trp	Phe
	290						295				300				
Arg	Gly	Leu	Gly	Asn	Leu	Arg	Val	Leu	Asp	Leu	Ser	Glu	Asn	Phe	Leu
	305				310					315					320
Tyr	Lys	Cys	Ile	Thr	Lys	Thr	Lys	Ala	Phe	Gln	Gly	Leu	Thr	Gln	Leu
				325					330					335	
Arg	Lys	Leu	Asn	Leu	Ser	Phe	Asn	Tyr	Gln	Lys	Arg	Val	Ser	Phe	Ala
			340					345					350		
His	Leu	Ser	Leu	Ala	Pro	Ser	Phe	Gly	Ser	Leu	Val	Ala	Leu	Lys	Glu
		355						360					365		
Leu	Asp	Met	His	Gly	Ile	Phe	Phe	Arg	Ser	Leu	Asp	Glu	Thr	Thr	Leu
	370						375				380				
Arg	Pro	Leu	Ala	Arg	Leu	Pro	Met	Leu	Gln	Thr	Leu	Arg	Leu	Gln	Met
	385				390					395					400
Asn	Phe	Ile	Asn	Gln	Ala	Gln	Leu	Gly	Ile	Phe	Arg	Ala	Phe	Pro	Gly
				405					410					415	
Leu	Arg	Tyr	Val	Asp	Leu	Ser	Asp	Asn	Arg	Ile	Ser	Gly	Ala	Ser	Glu
			420					425					430		
Leu	Thr	Ala	Thr	Met	Gly	Glu	Ala	Asp	Gly	Gly	Glu	Lys	Val	Trp	Leu
		435						440					445		
Gln	Pro	Gly	Asp	Leu	Ala	Pro	Ala	Pro	Val	Asp	Thr	Pro	Ser	Ser	Glu
	450						455				460				
Asp	Phe	Arg	Pro	Asn	Cys	Ser	Thr	Leu	Asn	Phe	Thr	Leu	Asp	Leu	Ser
	465				470					475				480	
Arg	Asn	Asn	Leu	Val	Thr	Val	Gln	Pro	Glu	Met	Phe	Ala	Gln	Leu	Ser
			485						490					495	
His	Leu	Gln	Cys	Leu	Arg	Leu	Ser	His	Asn	Cys	Ile	Ser	Gln	Ala	Val
			500					505					510		
Asn	Gly	Ser	Gln	Phe	Leu	Pro	Leu	Thr	Gly	Leu	Gln	Val	Leu	Asp	Leu
		515					520						525		
Ser	His	Asn	Lys	Leu	Asp	Leu	Tyr	His	Glu	His	Ser	Phe	Thr	Glu	Leu
	530						535				540				
Pro	Arg	Leu	Glu	Ala	Leu	Asp	Leu	Ser	Tyr	Asn	Ser	Gln	Pro	Phe	Gly
	545				550					555					560
Met	Gln	Gly	Val	Gly	His	Asn	Phe	Ser	Phe	Val	Ala	His	Leu	Arg	Thr
				565					570					575	
Leu	Arg	His	Leu	Ser	Leu	Ala	His	Asn	Asn	Ile	His	Ser	Gln	Val	Ser
			580					585					590		
Gln	Gln	Leu	Cys	Ser	Thr	Ser	Leu	Arg	Ala	Leu	Asp	Phe	Ser	Gly	Asn
		595					600					605			
Ala	Leu	Gly	His	Met	Trp	Ala	Glu	Gly	Asp	Leu	Tyr	Leu	His	Phe	Phe
	610						615				620				
Gln	Gly	Leu	Ser	Gly	Leu	Ile	Trp	Leu	Asp	Leu	Ser	Gln	Asn	Arg	Leu
	625				630					635					640
His	Thr	Leu	Leu	Pro	Gln	Thr	Leu	Arg	Asn	Leu	Pro	Lys	Ser	Leu	Gln
				645					650					655	
Val	Leu	Arg	Leu	Arg	Asp	Asn	Tyr	Leu	Ala	Phe	Phe	Lys	Trp	Trp	Ser
			660					665					670		
Leu	His	Phe	Leu	Pro	Lys	Leu	Glu	Val	Leu	Asp	Leu	Ala	Gly	Asn	Gln
		675					680						685		
Leu	Lys	Ala	Leu	Thr	Asn	Gly	Ser	Leu	Pro	Ala	Gly	Thr	Arg	Leu	Arg
	690						695				700				

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Arg Leu Asp Val Ser Cys Asn Ser Ile Ser Phe Val Ala Pro Gly Phe  
 705 710 715 720  
 Phe Ser Lys Ala Lys Glu Leu Arg Glu Leu Asn Leu Ser Ala Asn Ala  
 725 730 735  
 Leu Lys Thr Val Asp His Ser Trp Phe Gly Pro Leu Ala Ser Ala Leu  
 740 745 750  
 Gln Ile Leu Asp Val Ser Ala Asn Pro Leu His Cys Ala Cys Gly Ala  
 755 760 765  
 Ala Phe Met Asp Phe Leu Leu Glu Val Gln Ala Ala Val Pro Gly Leu  
 770 775 780  
 Pro Ser Arg Val Lys Cys Gly Ser Pro Gly Gln Leu Gln Gly Leu Ser  
 785 790 795 800  
 Ile Phe Ala Gln Asp Leu Arg Leu Cys Leu Asp Glu Ala Leu Ser Trp  
 805 810 815  
 Asp Cys Phe Ala Leu Ser Leu Leu Ala Val Ala Leu Gly Leu Gly Val  
 820 825 830  
 Pro Met Leu His His Leu Cys Gly Trp Asp Leu Trp Tyr Cys Phe His  
 835 840 845  
 Leu Cys Leu Ala Trp Leu Pro Trp Arg Gly Arg Gln Ser Gly Arg Asp  
 850 855 860  
 Glu Asp Ala Leu Pro Tyr Asp Ala Phe Val Val Phe Asp Lys Thr Gln  
 865 870 875 880  
 Ser Ala Val Ala Asp Trp Val Tyr Asn Glu Leu Arg Gly Gln Leu Glu  
 885 890 895  
 Glu Cys Arg Gly Arg Trp Ala Leu Arg Leu Cys Leu Glu Glu Arg Asp  
 900 905 910  
 Trp Leu Pro Gly Lys Thr Leu Phe Glu Asn Leu Trp Ala Ser Val Tyr  
 915 920 925  
 Gly Ser Arg Lys Thr Leu Phe Val Leu Ala His Thr Asp Arg Val Ser  
 930 935 940  
 Gly Leu Leu Arg Ala Ser Phe Leu Leu Ala Gln Gln Arg Leu Leu Glu  
 945 950 955 960  
 Asp Arg Lys Asp Val Val Val Leu Val Ile Leu Ser Pro Asp Gly Arg  
 965 970 975  
 Arg Ser Arg Tyr Val Arg Leu Arg Gln Arg Leu Cys Arg Gln Ser Val  
 980 985 990  
 Leu Leu Trp Pro His Gln Pro Ser Gly Gln Arg Ser Phe Trp Ala Gln  
 995 1000 1005  
 Leu Gly Met Ala Leu Thr Arg Asp Asn His His Phe Tyr Asn Arg  
 1010 1015 1020  
 Asn Phe Cys Gln Gly Pro Thr Ala Glu  
 1025 1030

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What is claimed is:

1. A method of determining bacterial virulence in an oral cavity comprising:

- a. providing a reporter cell expressing at least one toll-like receptor;
- b. providing a sample of oral matter;
- c. combining the sample of oral matter and the reporter cell;
- d. measuring the toll-like receptor activation;
- e. providing another sample of oral matter from an individual who used an oral care composition prior to providing another sample of oral matter;

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- f. combining the another sample of oral matter and the reporter cell;
  - g. measuring the toll-like receptor response;
  - h. comparing the toll-like receptor response from the sample of oral matter and another sample of oral matter.
2. The method of claim 1, wherein the toll-like receptor is at least one of TLR1 to TLR9.
3. The method of claim 1, wherein the reporter cell expresses at least of one TLR2 or TLR4.
4. The method of claim 1, wherein the toll-like receptor activates a reporter gene.



5. The method of claim 4, wherein the reporter gene is a secreted embryonic alkaline phosphatase (SEAP) reporter gene.

6. The method of claim 1, wherein the sample of oral matter is at least one of gum-line plaque, subgingival plaque, 5 supragingival plaque, interstitial plaque, gingival crevicular fluid (GCF), gingival biopsy, saliva, or tongue swab.

7. The method of claim 1, wherein the sample of oral matter comprises virulence factor that is at least one of Gram positive cell wall components, Gram negative cell wall 10 components, bacterial DNA, flagellin, peptidoglycan, bacterial metabolites, fimbriae; outer membrane vesicles bacterial proteins, or bacterial enzymes.

8. The method of claim 7, wherein the sample of oral matter comprises virulence factor that is at least one of 15 lipopolysaccharide or lipoteichoic acid.

9. The method of claim 1, where the sample of oral matter and the another sample of oral matter are from the same individual.

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