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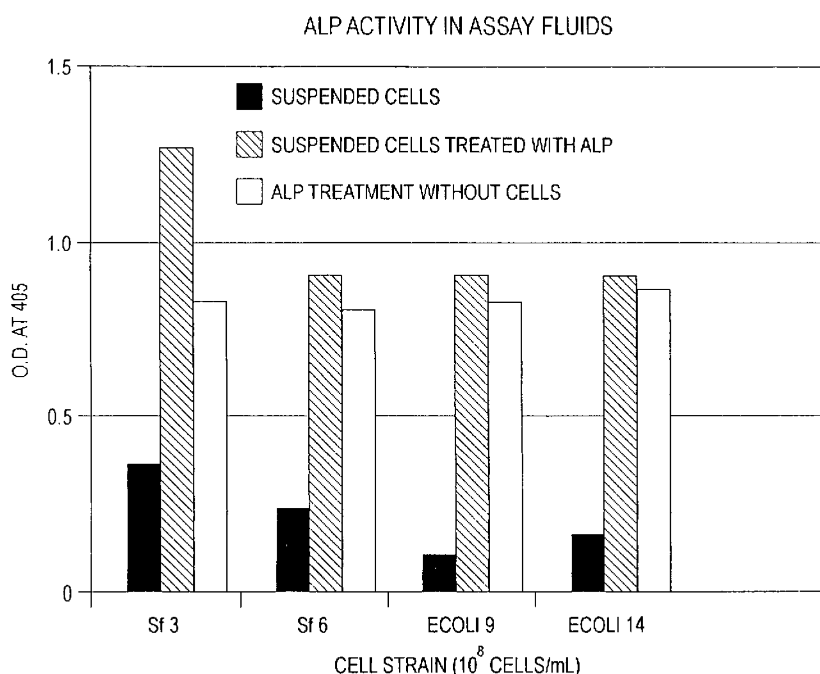
(54) **Title:** BACTERIAL TEST METHOD BY GLYCATED LABEL BINDING



(57) **Abstract:** A method for measuring the bacteria content of fluids such as urine and blood, in which a glycoprotein or glycopeptide is attached to the bacteria and a label attached to or inherent to the glycoprotein or glycopeptide provides a means for determining the amount of bacteria present. A preferred glycoprotein is alkaline phosphatase, which is an enzyme capable of attaching to all bacteria present in the fluid sample and inherently includes a label moiety in that color can be developed by addition of known reagents.

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BACTERIAL TEST METHOD BY GLYCATED LABEL BINDING

Background of the Invention

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This invention relates generally to methods for detecting bacteria in fluids, particularly in biological specimens. More specifically, the invention relates to rapid methods for detecting bacteria in urine and other fluids with improved accuracy compared to those currently available. Although analysis of urine is of particular
10 interest, other fluids, such as blood, serum, water, and the like may be analyzed using the methods of the invention.

A rapid test for bacteria is desirable, for example by using dry test strips of the sort now used for various purposes. At present, urine test strips are used to screen samples and rule out those which do not require laboratory assessment. However, the
15 current tests, such as measurement of nitrites and leukocytes, are not capable of rapidly providing accurate results. Often, many false results are obtained, causing unnecessary laboratory followup analyses. About 50% of a hospital laboratory's workload involves urine specimens and about 90% of these specimens are cultured and analyzed for total and gram negative bacteria. However, only about 10% of urine samples which are
20 cultured for detection of bacteria are actually found to test positive. Clearly, an accurate prescreening of urine could greatly reduce the number of samples sent to the laboratory for analysis.

The market penetration of the presently available test strips is not large, in part because the tests produce false positive results, as later determined by laboratory
25 followup analysis. Thus, a test strip which provides rapid and accurate determination of the presence of bacteria would reduce costs and make it possible to treat bacteria in a patient immediately, rather than waiting for laboratory results.

The present inventors were investigating methods by which bacteria could be detected accurately. One potential approach involved finding substances that could bind
30 to bacteria and then be detected and measured so that the amount of bacteria present could be determined. The problem can be stated as follows: How do substances bind to bacteria and which substances exhibit the properties needed for accurate measurements to be made? The binding should be specific to the bacteria. Non-specific binding can obscure the results since it can vary unpredictably and provide inaccurate results.

Antibodies are recognized as having the ability to attach to bacteria and it was believed that if ALP (alkaline phosphatase), which can be used to detect by color development materials to which it is bound, could be attached to another substance capable of attaching itself to bacteria, it would be possible to measure the amount of bacteria present. At first, experiments indicated that the ALP was bound to bacteria in a non-specific manner and therefore it was considered to present a problem to the development of a reliable method of measuring the amount of bacteria present in a sample. Further investigation was directed toward eliminating non-specific binding of ALP so that only the ALP attached to substances which could bind to bacteria would be measured. Surprisingly, it was found that the belief that the ALP was non-specifically bound to bacteria was not correct and that in fact, it did bind to bacteria, leading to the present invention. As will be seen below, ALP is a preferred substance for measuring the amount of bacteria, but other substances can be used, particularly glycopeptides and glycoproteins.

Related literature and patents

Methods for rapid testing for bacteria are known, but they differ from the method of the present invention. In one method, an immunoassay for detecting lipopolysaccharides from Gram negative bacteria such as E. Coli, Chlamydia or Salmonella uses a lipopolysaccharide binding protein or an antibody having specific binding affinity to the liposaccharide analyte as a first or second binding reagent (see WO 00/60354 and US 5,620,845). In US 5,866,344 other immunoassays are described for detecting polypeptides from cell walls. Proteins can be purified in a method using polysaccharide binding polypeptides and their conjugates (see US 5,962,289; US 5,340,731; and US 5,928,917). In US 5,856,201 detection of proteins using polysaccharide binding proteins and their conjugates is disclosed. The methods described in the above differ from those of the present invention, as will be seen in the discussion of the present invention below.

The methods which are based on liposaccharide antibodies or binding proteins do not provide a measure of the total bacteria present. They also do not use a glycopeptide or glycoprotein to bind to a bacteria cell. The methods based on polypeptides require antibodies to bind to the bacteria cell wall rather than using glycopeptides or glycoproteins. The methods based on polysaccharide binding polypeptides require the

fusion of short sequences of polypeptides onto analytes of interest and employ non-glycated polypeptides to bind to a polysaccharide.

Glycoproteins have been shown to bind to various biomolecules. For example, glycoproteins on a fungus cell surface have been shown to bind to host proteins. Also, 5 glycoproteins excreted from epithelial cells have been shown to bind to lipids and the binding of glycoproteins to carbohydrates is well known. All such interactions of glycoproteins are dependent on many factors, such as ionic strength and pH, and the affinity of the individual proteins for the biomolecules. However, the use of glycoproteins in assays for measurement of bacteria content has not been described 10 heretofore.

Glycoprotein receptors have been isolated on human monocyte cells. Two binding proteins extracted from the cell walls of human monocytes have been shown to have an affinity of $9x10^{+6}$ for binding fructosyllysine (lysyl peptides glycated with glucose) with 10,000 active binding sites per cell. These receptor protein sites are 15 thought to belong to the family of RNA-binding proteins and to be involved in the aging process by binding age related proteins such as glycated albumin. However, the prior art on glycoprotein receptors does not teach that receptors on the cell walls could be used for the detection of cells. There is no means provided for signal generation, whether by color particle or enzymatic reaction that can be used as a measure of the count or 20 detection of cells.

Bacteria are known to attach to host tissue, often by adhesion of bacterial cell membrane to extra-cellular matrix proteins of the host. This binding is known to occur through several modes of interaction, by glycoaminoglycans, collagens, proteins and integrins on their surface. Thus, the cell surface, including bacterial cell surfaces, can be 25 visualized as a mosaic of molecules capable of binding to proteins of the host tissues as well as receptor sites of the host.

The interaction between bacterial cells and glycoproteins is known generally, but the binding of specific glycopeptides to a bacterial cell has not been disclosed. Bacterial cell adhesion has been described to extra-cellular matrix proteins such as fibronectin and 30 lamin. This binding was shown to occur between the cell adhesions and glycated groups on the proteins. Similar results have been shown with connective tissue proteins and bacterial cells. Polypeptide and carbohydrate structures of glycoproteins can vary greatly and the chemical structures of glycopeptides and glycoproteins are often unknown, such as those which bind bacterial cells.

Methods for measuring binding of glycoproteins to bacterial cells have been described; however, the measurement of bacteria by glycopeptide or glycoprotein binding has not. More particularly, binding of glycopeptides or glycoproteins which are enzymes or are attached to detection labels has not been disclosed.

5 The binding of cell walls to alkaline phosphatase (ALP) is known, but at the present time, it is not possible to assign a precise function to any alkaline phosphatase other than the catalysis of the hydrolysis of phosphomonoester. It is known that tissue damage causes a release of these ALP iso-enzymes providing clinical significance.

Certain ALP iso-enzymes are known to be membrane-bound. Intestinal, liver, 10 bone, kidney and placental alkaline phophatase iso-enzymes are examples of enzymes that are known to be membrane bound to cell walls, including dipeptidylpeptidase, aminopeptidases such as alanine aminopeptidase, endopeptidase, gamma-glutamyl transferase, lactase, alpha-D-glucosidases, hydrolases such as glycosidase and 5' nucleotidase. Cell membrane binding for ALP is known to occur through a C-terminal 15 glycan-phosphatidyl-inositol anchor in which the long chain triglycerides of the anchor are incorporated into the lipoprotein membrane. The C-terminal glycan-phosphatidylinositol anchor is absent from the ALP produced by E Coli bacteria and the ALP from E Coli is considered to be a soluble enzyme. Thus, binding of ALP to E Coli in the present invention would have to occur by another mechanism.

20 ALP has been used in some diagnostic applications. For example, ALP has been used in an immunoassay diagnostic test as a label for the immunoassay; see US 5,225,328. However, it has not been used in a dry phase test without an antibody for detection of bacteria.

The present inventors have discovered that all bacteria cells have the ability to 25 bind certain glycoproteins through multiple binding sites. As a result of this discovery, they have found that such glycoproteins can be used in test strips having the ability to detect all bacteria present with accuracy, as will be seen in the detailed discussion of the invention which follows.

30 Summary of the Invention

In one aspect, the invention is a method for measuring the bacteria content of a fluid, typically a biological fluid, in which an effective amount of a glycoprotein or glycopeptide is reacted with bacteria in a sample of the fluid, the glycoprotein or glycopeptide being labeled with a detectable moiety. Any excess of the glycoprotein

which has not been reacted with bacteria is separated, after which the amount of the label moiety is measured and related to the amount of bacteria present in the sample. In a preferred embodiment, the glycoprotein or glycopeptide is alkaline phosphatase (ALP) and a reagent is added to develop color indicating the presence of ALP bound to bacteria. The association (binding) constant of the glycoprotein to bacteria should be at least 10^{+6} and the number of binding sites at least 100.

In preferred embodiments, the proteins have been glycated and generally include serum proteins, immunoglobulins, oxygen-binding proteins, fibrous proteins, intercellular enzymes, hormones, and secreted enzymes and inhibitors. Examples of serum proteins are albumin, prealbumin, transferrin, retinol binding proteins and beta-2 macroglobulin. Immunoglobulins may include IgG, IgA and IgM. Oxygen-binding proteins may include peroxidase, hemoglobin and myoglobin. Fibrous proteins may include collagens, fibrinogen and myosin. Examples of intra cellular enzymes include glutamate dihydrogenase, ALP, and lacate dehydrogenase. Representative hormones include insulin, growth hormone, and glucagon. Secreted enzymes and inhibitors may include protease inhibitors, alpha-1-microglobulin, trypsenogen, lysozyme, and alpha-1-acid glycoprotein.

Carbohydrate monomer units which may be attached to proteins maybe galactose (GAL), mannose (MAN), glucose (GLC), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), sialic acids (SA), fucose, and xylose.

Representative glycopeptides include Y-Ser-X, Y-Thr-X, Y-Asn-X-Ser, Y-Asn-X-Thr, and Gly-X-Hyl-Y where X may be any amino acid and Y may be Man, Gal, Glu, SA, GlcNAc, GalNAc, fucose or xylose.

Label moieties which may be added to glycoproteins include radioactive, fluorescent, electroactive, chem-luminescent, enzyme antibody, and particulate labels. Blocking compounds may be included, such as members of the group consisting of polymers, non-glycated proteins, non-glycated polypeptides and polysaccharides. Cations may be added, especially zinc, copper and iron to increase the binding of the glycoprotein or glycopeptide to bacteria.

In another aspect, the invention is a dry test method for measuring the bacteria content of a fluid wherein a glycoprotein or glycopeptide containing a label moiety is bound to the bacteria and the label moiety measured to determine the bacteria content of the fluid sample.

Brief Description of the Drawings

FIG. 1 illustrates the results of Example 1

FIG. 2 illustrates additional results of Example 1.

FIG. 3 illustrates the results of Example 4.

5 FIG. 4 illustrates the results of Example 7.

FIG. 5 illustrates the results of Example 7.

FIG. 6 illustrates the results of Example 8.

FIG. 7 illustrates the results of Example 8.

FIG. 8 illustrates the effect of pH on ALP activity.

10 FIG. 9 illustrates the effect of pH on ALP activity.

FIG. 10 illustrates the effect of different cations on ALP binding.

FIG. 11 illustrates the effect of different cations on ALP binding.

While the invention is susceptible to various modifications and alternative forms, specific embodiments have been shown by way of examples described in detail herein.
15 It should be understood, however, that the invention is not intended to be limited to the embodiments disclosed, rather, the invention is defined by the appended claims.

Description of the Preferred Embodiments

Glycoproteins and Glycopeptides

20 Both glycoproteins and glycopeptides are composed of amino acids with peptide linkages and carbohydrates. Generally glycoproteins have higher molecular weights than glycopeptides. Glycoproteins and glycopeptides can be attached to bacteria through charge attraction and shape to molecules on the cell wall. As will be seen in the examples below, the amount of the glycoprotein or glycopeptide bound to bacteria cells will vary
25 depending on several factors, including the molecular structure, presence of metals, ionic strength, and pH of the environment.

Glycoproteins, in which one or more carbohydrate units have been attached covalently to the protein, are a widely varied group of biomolecules. Most secretory proteins, and their fragments, are glycoproteins, as are components of membranes such as
30 cell receptors, where the carbohydrates are involved in cell to cell adhesion.

Examples of proteins that can be glycated include serum proteins (e.g., albumin, pre-albumin, transferrin, retinol binding protein, beta-2-macroglobuin), immunoglobulins (e.g., IgG, IgA, IgM), oxygen-binding (e.g., peroxidase, hemoglobin, myoglobin), fibrous protein (e.g., collagens, fibrinogen, myosin), intra cellular enzymes (e.g., glutamate

dehdrogenase, ALP, lacate dehdrogenase), hormones (e.g., insulin, growth hormone, glucagon) and secreted enzymes and inhibitors (e.g., protease inhibitors, alpha-1-microglobulin, trypsinogen, lysozyme, alpha-1-acid glycoprotein).

The carbohydrate monomer units that are commonly attached to proteins include galactose (Gal), mannose (Man), glucose (Glu), N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (Gal NAc), sialic acids (SA), fucose and xylose. The carbohydrate chains occur with a wide variety of lengths and structures, but some typical structures encountered are Man-GlcNAc-, GalNAc(Gal)(SA)-, Man(Man(Man)$_2$) (Man(Man))-GlcNAc -GlcNAc-, Man((Man-GlcNAc-Gal-SA)$_2$-GlcNAc -GlcNAc- and those listed in Table 2 below.

The carbohydrate chains are generally attached to proteins and peptides via the hydroxyl groups of serine (Ser) or threonine (Thr) amino acid residues, the amide N atom of asparagine (Asn) side chains or through hydroxy-lysine (Hyl) residues. The particular Ser and Thr residues O-glycosylated do not appear to occur in unique amino acid sequences, therefore Ser or Thr can be connected to any aminoacid, such as Ser-X, Thr-X, where X can be any amino acid. The glycosylation of Hyl residues occurs in a characteristic sequence -Gly-Y-Hyl-Z-Arg-, where Y and Z are any amino acids. The Asn residues N-glycosylated occur in the sequence of -Asn-X-Ser- or -Asn-X-Thr-, where X may be any of the normal amino acids, other than Pro.

One particularly effective glycoprotein is alkaline phosphatase (ALP). It has the advantage of being capable of binding to bacteria and inherently providing a label moiety which can be developed by addition of known reagents, a technique used in immunoassay diagnostic tests. The amount of the glycoprotein will depend upon the amount of the bacteria present in the sample; for example, when bacteria is present, a certain amount of glycoprotein will be dependent on the number of binding sites and strength of the binding constant. With a given glycoprotein and bacteria cell type the binding sites are fixed and the amount of glycoprotein bound is directly proportional to the amount of bacteria present.

Label Moieties

Alkaline phosphatase is particularly useful, as mentioned above, since it inherently provides a label. Other glycoproteins or glycopeptides may not have the inherent ability to serve as a label as well as binding to the bacteria. Thus, in those instances, label moieties may be added so that the amount of the glycoprotein or glycopeptide can be

measured to indicate the amount of the bacteria present. Examples of such label moieties which may be useful include colorimetric, radioactive, fluorescent, electroactive, chem-luminescent, enzyme antibody, and particulate labels.

5 Additional Components

The method of the invention may be applied in dry test strips familiar to those skilled in the art, or in wet test methods such as those described in the examples below. Depending on the specific technique, buffering compounds, substrates for the glycoprotein or glycopeptide, enzyme amplification compounds, and other additives such
10 as blocking compounds may be present.

It has been discovered that adding specific transition state metals increase protein binding to bacteria cell walls. While not required, the use of specific transition state metals increases the sensitivity of an assay based on glycated protein binding to bacteria.

In a particularly preferred embodiment of the invention, such metals are used to
15 increase the response of the labeling moiety. Various metals have been evaluated. Of these, zinc, copper, and iron have been found to have a beneficial effect, particularly zinc, as will be seen in the examples below.

Substrates for ALP include the phosphate esters of the following organic groups, primary and aliphatic alcohol, sugars, sugar alcohols, phenols, naphthols and nucleosides.
20 Examples of substrates forming visual color include naphthol-AS-BI-phosphate, naphthol-AS-MX-phosphate, p-nitrophenol phosphate phenylphosphate (PPNP), indoxylphosphate, e.g., bromo-chloro-indolyl-phosphate (BCIP), phenolphthalein phosphate, thymolphthalein monophosphate and diphosphate, beta-naphthylphosphate, dicyclohexylammonium salt of PPNP for stability, thymolphthalein monophosphate,
25 phenolphthalein diphosphate, carboxyphenyl phosphate, beta-glycerophosphate and beta-glycerolphosphate. Examples of fluorescent substrates for ALP include methylfluoresceine alpha-naphthyl phosphate. Alkaline phosphatase can be measured by a wide range of chemiluminescent and bioluminescent substrates. Examples of chemiluminescent substrates for ALP include adamantyl 1,2-dioetane aryl phosphate, 5-
30 bromo-4-chloro-3-indolyl phosphate, phenacyl phosphate, NADP, ascorbic acid 2-O-phosphate, cortisol-21-O phosphate, N,N' -dimethyl-9,9' bisacridinium dinitrate, indolyl derivatives, e.g., 5-bromo-4chloro-3-indolyl phosphate disodium salt (BCIP-2Na), D-luciferine-O-phosphate and adamanyl 1,2-dioxetane aryl phosphate (AMPPD).

Various buffers, both non-transphosphorylating and those of varying degrees of transphosphorylating property have been used for ALP determinations (i.e., Carbonate, 2-amino-2-methyl-1-propanol and diethanolamine). Buffers commonly utilized for ALP include ethylaminoethanol (pKa 9.9), diethanolamine (pKa 8.7), tris- (hyroxymethyl) aminomethane (pKa 7.8), 2-amino-2-methyl-1-propanol MAP (pKa 9.3), 2-amino-2-methyl-1,3-propanediol (pKa 8.6), sodium carbonate, sodium bicarbonate (pKa 9.9), glycyl-glycine (pKa 8.2), glycine (pKa 9.6), and barbital (pKa 7.44) with activity measured at pH ranges of 7 to 10.

Additional additives such as enzyme co-factors may be used to enhance the reaction conditions for enzymes. Mannitol and other alcohols can be used to increase ALP substrate rates. In the case of ALP, at least one equivalent of Zn, Ca and Mg metal for each ALP molecule will be present to provide catalytic activity and possibly also for maintenance of the native enzyme structure. Enzyme inhibitors are also often used to modulate enzyme assay ranges and mask interference. In the case of ALP, known inhibitors include cysteine, EDTA and thioglycolic acid , L-phenylalanine, L-homoarginine, L-tryptophane, L-leucine, levamisol and imidazole. It is also known that salts such as sodium chloride can be used to control enzymes. It is also known that surfactants such as sodium dodecyl sulfate and bile acids modulate enzyme assay ranges and sensitivity.

Enzyme amplification systems can also be used to increase detection limits for enzyme assays. Several enzyme amplification methods for the detection of alkaline phosphatase are known. These include the formation of formazan (INT-violet colorimetrically or resazurin fluorimetrically) through enzyme systems (e.g., diaphorase and alcohol deyhydrogenase) that employ NAD co-factor and rely on ALP to dephosphorylate NADP enzyme to produce NAD. For example, nicotinamide adenine dinucleotide phosphate (NADP) conversion to NAD^+ by ALP has been used for amplification. The NAD^+ compound was then reduced to NADH by alcohol dehydrogenase in the presence of ethanol included in the reaction medium. In turn, NADH in the presence of diphorase was converted back into NAD with simultaneous reduction of tetrazolium salt also present in the medium. This resulted in an accumulation of colored soluble formazen dye, proportional to the concentration of NAD^+ generated by AP. The newly formed NAD^+ is recycled many times, resulting in a 100-fold increase in sensitivity.

Blocking compounds selected from the group consisting of polymers, non-glycated proteins, non-glycated polypeptides, and polysaccharides may be included in order to reduce interference or improve color. Interference is improved by preventing non-specific binding by interfering substances to bacteria by instead binding interfering substances to the blocking compound. Color is improved by acting as a spreading layer which allows color to be uniform in dry reagents.

Test Methods

The use of glycoproteins for the detection of bacteria can be applied to a variety of test methods. The methods require combining a glycoprotein with sample to be assayed, separating the glycoprotein bound to bacteria from free unbound glycoprotein and measuring bound or free glycoprotein. Such steps can be accomplished through a variety of fluid handling analyzers such as centrifugal, microfluidic devices, chromatography strips, filtration and microplate readers, to name a few.

The effectiveness of glycoproteins for the detection of bacteria is measured in the same way for all test methods. Effectiveness is measured by obtaining a bacteria detection signal that is three standard deviations from the signal obtained in the absence of bacteria.

In order for the glycoproteins to be effective at detecting 1000 bacteria cell/mL, the association constant must be at least 10^{+6} and the number of binding sites at least 100. These measures of the bind strength for glycoprotein to bacteria and of the number of binding sites for glycoprotein to bacteria allow a sufficient bacteria detection signal. The 1000 bacteria cell/mL detection limit is the minimal clinically desired threshold. A sufficient background reading for the glycoprotein binding to other specimen components, e.g., other proteins, must be an association constant of less than 10^{+4}. Using ALP as a representative example, a binding constant of $5x10^{+6}$ and the number of binding sites was estimated to be 590.

Example 1

Bacteria assay by binding of Intestinal Alkaline Phosphatase

Bacterial cells (10^6 to 10^8 cells/mL) were washed twice with water after centrifugation to separate the cells into a packed pellet from supernatant liquid. The washed cells in pellet form were suspended in 40 μL water and 10 μL of aqueous bovine

intestinal alkaline phosphatase (ALP) was added (2 μg or 10,000 Units). The mixture was left at room temperature for 30 minutes and then centrifuged, after which the bacterial pellets were washed with water 4-5 times (50 μL). All the washing supernatants were combined. A blank without cells was diluted in the same way. The final pellets were suspended in 50 μl water and both supernatants and cell suspensions were assayed for detection of ALP binding using 2.5 μl of 0.005 M para-nitrophenol phosphate (PNPP) in Tris or EPPS buffer at pH 7.5. The hydrolysis of the substrate results in yellow (PNPP) or blue-green (BCIP) color that is directly proportional to the amount of ALP bound to the bacteria. Alkaline phosphatase (ALP) activity was tested using common substrates such as BCIP (bromo-chloro-indolyl-phosphate) forming a blue/green color in Tris buffer, pH 7.5. After 10 minutes at room temperature the samples were read in a plate reader (Biotek Powerwave Absorbance Reader) at a wavelength of 405 nm. The parallel set of bacteria was run without addition of ALP as controls.

Intestinal ALP binding to bacteria cells was observed. In Figure 1, the striped bars show that suspended cells after ALP treatment and washings had more intestinal ALP activity than untreated cells (the solid bars). The solid bars do show that suspended cells not treated with intestinal ALP did have some ALP activity, believed to be from native ALP in the bacteria. As a control, the ALP activity of the treatment solutions show the maximum activity expected without contribution from native ALP.

Figure 1 demonstrates intestinal ALP binding to all bacterial strains tested. Both gram positive bacteria such as *Staphylococcus aureus (Sf)* strains # 3 and #6 and gram negative bacteria such as *Escherichia Coli (E. Coli)* strains # 9 and 14 were found to bind the ALP. Again the striped bars being significantly larger than the solid bars demonstrate this. Figure 2 shows that the amount of ALP bound or activity generated is directly proportional to the amount of bacteria cells present. The ALP activity of the suspended cell increased with increasing amounts of cells.

The mechanism of the binding of ALP to the bacterial cells is not fully understood, but it is believed that glycated peptides in ALP or other glycoproteins are binding to the protein receptors anchored in the cell wall or are binding the peptidoglycon membrane. Both gram positive and gram negative bacteria are known to have protein receptors in their outer membranes. For gram negative bacteria, the outer lipopolysaccharide membrane has receptor proteins. For gram positive bacteria the outer peptidoglycon membrane has receptor proteins.

Example 2

Bacteria assay by binding of non-glycated protein to bacteria

5 As a control, an enzymatic protein lacking glycation, beta-galactosidase, was tested for binding to bacteria cell walls. The bacteria from both *Staph.* and *E.coli* were tested for beta-galactosidase binding. The beta-Galactosidases (20 mU) were added to saline suspensions of 10^8 cells/ mL of both bacteria and were assayed as well as the pellets (cells re-suspended in water) and supernatants after spinning the bacteria using 10 dimethylacridinium B-D-galactose (DMAG) as the substrate. The assay to determine the amount of enzyme was to add 10 μL of aqueous DMAG (0.5 mM) and 5 μL of aqueous tris buffer (1M) adjusted to pH 7.5 or test bacteria (10^7 cells) and H_2O to 100 μl. Bright yellow color of DMAG changes to light green to dark blue in 5-30 minutes (with beta-galactosidase in 5 min) which is read at 634 nm on a plate reader.

15

Beta-D- galactosidase is a non-glycoprotein and non-membrane protein. In these experiments, beta-D- galactosidase did not bind bacteria and no measurement of bacteria was possible.

20

Example 3

Bacteria assay by binding of glycated proteins to bacteria

Bacterial cells (1 to 4.5 x 10^7 cells/mL) were washed twice with water after 25 centrifugation to separate cells into a packed pellet from the supernatant liquid. The washed cells in pellet form were suspended in 20 ul of N-2-hydroxyethyl piperazine-N'-[3-propane sulfonic acid] EPPS buffer (50 mM at pH 8.0) and 30 μL of water. Glycated protein(s) (2-40 μg) were added. In some cases a glycated protein (2-40 μg) and bovine intestinal alkaline phosphatase (ALP) (2 μg or 10,000 Units) were added and the binding 30 of the glycated protein measured by the reduction of binding of ALP.

The mixture of glycated protein and bacterial cells was left at 25° C for 15 minutes. The mixture was then centrifuged at 30,000 rpm for 30 minutes after which the bacterial cells formed a pellet at the bottom of the tube and were washed with water 4-5 times (50 μL). Centrifugation allows separation of glycoprotein bound to the bacteria 35 cells from unbound glycated protein(s).

After washing, the bacterial pellets were suspended in 50 μL of borate buffer (25 mM at pH 9.0). A 5 μL aliquot of the suspension was assayed for detection of ALP

binding by adding 5 μL of para-nitrophenol phosphate (PNPP, 100 mM), 50 μL sodium borate buffer (25 mM at pH 9.0) and 140 μL of water. The hydrolysis of the PNPP substrate resulted in a yellow color. The color is read at 405 nm using a ELISA plate reader between 15-30 min. The absorbance is directly proportional to the amount of
5 ALP bound to the bacteria cell adhesions for glycated groups.

Various glycated and non-glycated proteins were tested for binding to bacteria (See Table 1). Albumin, prealbumin, alpha-1-antitrypsin, alpha-1-microglobulin, retinol binding protein, alpha-1-acid glycoprotein, alpha-2-glycoprotein, transferrin, Tamm-Horsfall glycoprotein and immunoglobulins were all known glycated proteins as
10 received from suppliers. Hemoglobin, lysozyme, and myoglobin are all known non-glycated proteins as received from suppliers. All proteins were found to be binding the bacteria cell by measurements of bound protein using comassie brilliant blue.

Only a protein binding to the cell adhesions for glycated groups causes the inhibition of the binding of ALP by bacteria. A protein binding to the cell adhesions for
15 glycated groups provides a positive number in Table 1. For example, albumin prevented 50% of ALP from binding to E. Coli bacteria. As seen in Table 1 all glycated proteins inhibited the binding of ALP by bacteria. Non-glycated proteins such as hemoglobin, myoglobin and lysozyme did not inhibit the binding of ALP. As a control, three non glycated polypeptides (polyarginine, polylysine, polyhistidine) were tested and not found
20 to inhibit ALP activity.

Table 1

Demonstration of binding of glycated proteins to bacteria

Added protein	*E.coli*	*S. faec.*
Albumin	50%	61%
Prealbumin	50%	57%
Tamm-Horsfall Glycoprotein	40%	49%
alpha-1-Antitrypsin	84%	74%
Myoglobin (non-glycated)	NI	NI
Hemoglogin (non-glycated)	NI	NI
Transferrin	75%	75%
Retinol Binding Protein	81%	83%
alpha-1-Acid glycoprotein	86%	90%
beta-2-Glycoprotein	74%	61%
Lysozyme (non-glycated)	NI	NI
IgG, IgA, IgM and Fragments	63%	71%
Polylysine, poly arginine poly histidine	NI	NI

5

*NI= no inhibition

10 As can be seen, glycated proteins can bind to bacteria and be used to determine the amount of bacteria present in a sample. A determination of the amount of bound and/or free glycated proteins label can be done in several ways.

ALP is an example of a glycated protein having enzymatic functionality and generating a signal, as demonstrated in Example 1. Other examples of enzymatic 15 glycated proteins include acid phosphatase, fucosidase, phospholipase, glucocerebrosidase, hydrolase, arylsufatase A, amylases, cellobiohydrolase, and peroxidase.

Alternatively, glycated proteins may be labeled to provide a signal indicating the amount which has been attached to bacteria, for example the comassie brilliant blue used 20 in Example 3. Other labels could be a chromogen, an enzyme antibody with label, or a particle such as gold sol or colored latex. Common labels include radioactive, fluorescent, electroactive or chemi-luminescent compounds, enzymes, and particulates.

Blocking additives can be used to block competing reactions and reduce interference or act as spreading agents. Examples are the non-binding glycoproteins of 25 Example 3. Others are polymers such as poly (vinyl pyrrolidone) or polyvinyl alcohol and proteins such as casein, gelatin, albumin, hydrophobic cellulose, and polysaccharides.

Example 4

Bacteria assay by binding of ALP iso-forms to bacteria

5 Bacterial cells (1 to 4.5 x 10^7 cells/mL) were washed twice with water after centrifugation to separate cells into a packed pellet from the supernatant liquid. The washed cells in pellet form were suspended in 20 μL of EPPS buffer (50 mM at pH 8.0) and 30 μL of water. Hemoglobin (20 μg) was added as a blocking additive. Alkaline phosphatase (ALP) (100 mUnits) from intestine, placenta, and bacteria sources were 10 added.

The mixture of glycated protein and bacterial cells was left at 25° C for 15 minutes. The mixture was then centrifuged at 30,000 rpm for 30 minutes after which the bacterial cells formed a pellet at the bottom of the tube and were washed with water 4-5 times (50 μL). Centrifugation allows separation of glycoprotein bound to the bacteria 15 cells from unbound glycated protein(s).

After washing, the bacterial pellets were suspended in 50 μL of sodium tetraborate buffer (25 mM at pH 9.5). A 5 μL aliquot of the suspension was assayed for detection of ALP binding by adding 5 μL of para-nitrophenol phosphate (PNPP, 100 mM), 50 μL sodium borate buffer (25 mM at pH 9.0) and 140 μL of water. The 20 hydrolysis of the PNPP substrate resulted in a yellow color. The color is read at 405 nm using an ELISA plate reader between 15-30 min and the absorbance is directly proportional to the amount of ALP bound to the bacteria cell adhesions for glycated groups. The results are illustrated in Figure 3.

A comparison of ALP isozymes from placenta, bacterial and intestine sources 25 allows an understanding of what glycosylation is needed for binding. The ISO forms of intestinal, liver, bone, and placental ALP have differences in carbohydrate structures and amount of sialic acid. Intestinal ALP lacks terminal sialic acids on its carbohydrate chains while placenta and bacterial have sialic acid residues. Bacterial ALP lacks a membrane binding glycophospholipid portion present in the mammalian ALP. Placenta 30 ALP contains fucose, mannose and galactose while intestinal ALP has a high hexose and hexoamine content.

According to Figure 3, the glycophospholipids are not requirements for glycoprotein binding to bacteria as the bacterial ALP binds bacteria but lacks the glycophospholipid. All ALP bound to bacteria to some extent although placenta ALP

exhibited the lowest enzyme activity as well as lowest binding to bacteria. This result supported our belief that certain degrees of glycosylation are better binders for bacteria.

Polylysine-conjugated intestinal ALP was also found to bind bacteria. The conjugation of ALP with a non-glycated peptide was not found to inhibit binding to
5 bacteria and could provide linker arms for labels.

Example 5

Bacteria assay in presence of carbohydrates, polysaccharides, glycopeptides and lectins
10

Bacterial cells (1 to 4.5 x 10^7 cells/mL) were washed twice with water after centrifugation to separate cells into a packed pellet from the supernatant liquid. The washed cells in pellet form were suspended in 20 μl of EPPS buffer (50 mM at pH 8.0) and 30 μL of water. Hemoglobin (20 μg) was added as a blocking additive. Alkaline
15 phosphatase (ALP) (100 mUnits) from bovine intestine and 15 μg of simple carbohydrates or proteoglycan or lectins, were added.

The mixture of glycated protein and bacterial cells was left at 25° C for 15 minutes. The mixture was then centrifuged at 30,000 rpm for 30 minutes after which the bacterial cells formed a pellet at the bottom of the tube and were washed with water 4-5
20 times (50 μL). Centrifugation allows separation of glycoprotein bound to the bacteria cells from unbound glycated protein(s).

After washing, the bacterial pellets were suspended in 50 μL of sodium tetraborate buffer (25 mM at pH 9.5). A 5 uL aliquot of the suspension was assayed for detection of ALP binding by adding 5 μL of para-nitrophenol phosphate (PNPP, 100 mM), 50 μL
25 sodium borate buffer (25 mM at pH 9.0) and 140 μL of water. The hydrolysis of the PNPP substrate resulted in a yellow color. The color is read at 405 nm using a ELISA plate reader between 15-30 min and the absorbance is directly proportional to the amount of ALP bound to the bacteria cell adhesions for glycated groups.

The binding of ALP to bacteria was shown by an absorbance of 1.8 to 2.0 in Table
30 2 in the absence of carbohydrates, proteoglycans, and lectins. The monosaccharides (simple carbohydrates) including Glucose, Mannose, Galactose and Sialic acid did not produce any effect on bacteria binding of ALP (all sources). Therefore simple carbohydrates are not involved in the binding and are not suitable as bacterial binders for attachment to detection labels. This also supports the need for glycopeptides or
35 glycoproteins as binders rather than simple glyco-units.

Polysaccharides weakly inhibited the bacteria binding of ALP to a degree depending on the repeating carbohydrate unit. These results show that polysaccharides are involved in the binding of bacteria with ALP. Polysaccharides with N-acetylgalactosamine were more inhibitory and likely contained residual peptide units. By
5 contrast lipopolysaccharide (LPS) was without any effect for the sources tested (B4 and B8 from 2 different serotypes of E.coli). Lipopolysaccharide contains Lipid A and O-antigen on the outer structure and does not expose its polysaccharide core.

Lipoteichoic acid is an example of polysaccharides with repeating carbohydrate and amino acid (Hyl) units. The structure of the polysaccharide varies with the source of
10 LTA. Structures with and without N-acetylgalactosamine are known. In our results LTA (S. sanguis) strongly inhibits the bacteria bound ALP activity, whereas, depending on the source, varying or lack of inhibition was observed. Teichoic acid with repeating carbohydrate and amino acid (Hyl) units itself was found equally inhibitory. This supports our belief that the binding of glycopeptides to bacteria involves carbohydrate and
15 amino acid components.

Lectins are proteins found in plant seeds which bind polysaccharides and monsaccharides attached to peptides. As seen in Table 2 lectins inhibited the bacteria binding of ALP depending on the polysaccharide unit that the lectin bound. These results also support the involvement of glycopeptides in the binding of bacteria and the ALP.
20 The lectin binds the glyco group of ALP and prevents it from reacting with bacteria. Since several of the lectins are active but only bind one type of glyco group, several types of glyco peptide groups can cause binding of ALP to bacteria.

Table 2

Additional carbohydrates, proteoglycan, and lectins	*E.coli*	*S. faec.*
None	1.8	2.0
Simple carbohydrate		
Glucose (β-D-Glucose)	1.8	2.4
Galactose (Gal or β-D-Galactose)	2.0	2.0
Fucose	2.0	2.3
Mannose (Man)	1.7	2.4
Sialic Acid (N-Acetyleneuaminic Acid)	2.0	1.7
Muramic Acid	1.8	2.1
GlcNAc (N-Acetyl-β-D-Glucosamine)	2.0	2.0
GalNAc (N-Acetyl-β-D-Galactosamine)	1.8	1.9
Glucuronic acid	1.9	2.0
Iduronic acid	1.9	2.0
Polysacharide		
Chondroitin sulfate A (repeating GalNAc & glucuronic)	1.1	1.3
Chondroitin sulfate B (repeating GalNAc & iduronic acid)	0.9	0.5
Hyaluronic Acid (repeating GlcNAc & glucuronic acid)	1.8	2.0
Lipopolysaccharide	1.8	2.0
Glycopeptide		
Lipoteichoic acid (from S. sanguis)	0.2	0.2
Lectins that bind glycopeptides		
Euonymus Europeus (Gal-Gal)	1.6	1.8
Bauhinia Purpurea (Gal-GalNAc)	0.3	0.4
Maackia Amurensis (Sialic Acid)	0.1	0.1
Concanavalin A (Man,Glc)	0.0	0.1
Caragana Arborescens (GalNAc)	0.8	1.0

5 A glycated protein or glycated peptide can be attached to a label or as part of the label in several ways. The data in Example 5 shows that the glycated portion can be a polysaccharides or a monosaccharide attached to at least one peptide. Examples of polysaccharides or monsacharides include those in Table 2.

Example 6

Alternative separation method of glycated proteins bound to bacteria

5 Bacteria bound to alkaline phosphatase (ALP) can be separated using a membrane (low protein binding Nylon 66 Loprodyne) on backed microtiter plates (Nunc Nalge International).

The loprodyne-membrane-backed plates were treated with 1 or 2% detergent (Tween 20 or TritonX305) in water or buffers (TBS: Tris, 25mM, pH 7.6 containing 150 10 mM NaCl or KCO3: 0.1M, pH 9.6) overnight at room temperature. Blocking solutions were vacuum filtered. Bacteria suspensions (10^7 cells, 100 µl) in saline were combined with 50 µl EPPS buffer (0.05M, pH 8.1) and 50 µl H_2O containing 20 mU ALP. The combined solution was incubated for 15 min at 37° C on a shaker and then added to the loprodyne-membrane-backed plate.

15 The solution was vacuum filtered leaving bacteria adhered on the membrane and then washed twice with 2% Tween20 in water. To the washed membrane, 200 µl of H_2O with 50 µl Glycine (0.05M, pH 10.4) and containing 1mM PNPP were added and the color formed due to the bacteria bound ALP read at 405nm.

20 **Table 3**

Condition	**Bacteria Binding to ALP (O.D. at 405nm)**		
ALP concentration	1.0 mU	2.0 mU	5.0 mU
No Bacteria	0.04	0.05	0.16
Plus Bacteria	0.13	0.30	0.83

25

The separation of E. Coli with bound ALP from unbound ALP is shown by a size exclusion membrane in Example 6 and by centrifugation in Examples 1-5. The size of E. 30 Coli is 1 x 1 x 2 µm and any membrane, filter or device trapping particles of this size would be acceptable. These include microfluidic devices, filters, column chromatography and chromatography strips. The mass of E. Coli is 1.6×10^{-12} gm/cell and any membrane, filter or device trapping a mass of this size would be also acceptable.

Example 7

Effect of Divalent Cations in Protein Binding to Bacteria

5

Bacterial cells (1 to 4.5 x 10^7 cells/mL) were washed twice with water after centrifugation to separate cells into a packed pellet from the supernatant liquid. The washed cells in pellet form were suspended in 20 μL of EPPS buffer (50 mM at pH 8.0) and 30 μL of water. Bovine intestinal alkaline phosphatase (ALP) (2 μg or 10,000 Units)
10 was added and 0.2 mM of several cations.

The mixture of glycated protein and bacterial cells was left at 25° C for 15 minutes. The mixture was then centrifuged at 30,000 rpm for 30 minutes after which the bacterial cells formed a pellet at the bottom of the tube and were washed with water 4-5 times (50 μL). Centrifugation allows separation of glycoprotein bound to the bacteria
15 cells from unbound glycated protein(s).

After washing, the bacterial pellets were suspended in 50 μL of borate buffer (25 mM at pH 9.0). A 5 μL aliquot of the suspension was assayed for detection of ALP binding by adding 5 μL of para-nitrophenol phosphate (PNPP, 100 mM), 50 μL sodium borate buffer (25 mM at pH 9.0) and 140 μL of water. The hydrolysis of the PNPP
20 substrate resulted in a yellow color. The color was read at 405 nm using an ELISA plate reader between 15-30 min; the absorbance is directly proportional to the amount of ALP bound to the bacteria cell.

Table 4

25

Condition	O.D. at 405 nm	
	No ALP	With ALP
No cation	**0.18**	**0.30**
+ $CaCl_2$ (1 mM)	0.23	0.36
+ $MgCl_2$ (1 mM)	0.20	0.44
+ $ZnCl_2$ (0.2 mM)	**0.23**	**0.53**

30

As seen in Table 4, Zn^{2+} (0.2 mM) resulted in significantly higher binding of ALP to the bacteria. Concentration dependent binding study has been performed in the presence of increasing concentration of zinc with S. faecalis strain as shown in Figure 4.
35 Result indicated that optimum binding occurs at 1 mM zinc concentration. The studies have been continued with different strains of bacteria in the presence of 1 mM Zn^{2+} and the ALP binding could be monitored even at $5x10^6$ bacteria concentration (Fig.5).

Example 8

Effect of Various Cation on ALP Binding to Bacteria

Bacterial cells (1 to 4.5 x 10^7 cells/mL) were washed twice with water after centrifugation to separate cells into a packed pellet from the supernatant liquid. The washed cells in pellet form were suspended in 20 μL of EPPS buffer (50 mM at pH 8.0) and 30 μL of water. Bovine intestinal alkaline phosphatase (ALP) (2 μg or 10,000 Units) was added and 0.2 mM of each cation.

The mixture of glycated protein and bacterial cells was left at 25° C for 15 minutes. The mixture was then centrifuged at 30,000 rpm for 30 minutes after which the bacterial cells formed a pellet at the bottom of the tube and was washed with water 4-5 times (50 μL). Centrifugation allows separation of glycoprotein bound to the bacteria cells from unbound glycated protein(s).

After washing, the bacterial pellets were suspended in 50 μL of borate buffer (25 mM at pH 9.0). A 5 μL aliquot of the suspension was assayed for detection of ALP binding by adding 5 μL of para-nitrophenol phosphate (PNPP, 100 mM), 50 μL sodium borate buffer (25 mM at pH 9.0) and 140 μL of water. The hydrolysis of the PNPP substrate resulted in a yellow color. The color was read at 405 nm using an ELISA plate reader between 15-30 min; the absorbance is directly proportional to the amount of ALP bound to the bacteria cell.

Zinc dependency of all the protein binding to bacterial cell wall had been observed as mentioned before. The effect of various cations (2 mM) on the binding of bovine intestinal mucosa ALP (Biozyme) to different bacteria is shown in Figure 6. In addition to zinc, Cu^{2+}, Fe^{2+} and Fe^{3+} also seem to stimulate ALP- binding in both Gram-positive and Gram-positive strains of bacteria (Sf – Staph. faec.; Ec: E. coli). Figure 6 also indicates total inhibition of ALP activity in the presence of EDTA (10 mM). Zinc had been used for continuing ALP binding studies. Alkaline phosphatase binding to bacteria seems to be very dependent on the presence of cations as seen in Figure 6. The data in Figure 7 shows the binding of various glycated proteins in absence and presence of zinc which clearly demonstrates cation dependency of all the proteins tested for binding to both Gram-positive and Gram-negative bacterial cell wall. The amount of ALP used for this study was so small it can only be detected by measuring enzymatic activity.

Example 9

Optimum Conditions for Concentration of Zn in ALP Binding

The human placental ALP activity is comparable to ALP from other sources
5 when assayed in glycine buffer as seen in Figures 8-9. Among three cations effective for binding of ALP to the bacteria, zinc was the best metal when the ALP-bound bacteria (both Staph and E.coli) were assayed in glycine buffer, pH 10.0 (Figs. 10-11). The binding was conducted at pH 8.0 in EPPS buffer.

In Figures 8-11, the following abbreviations are used:

10 B1BZ = bovine intestinal from a first vendor

B1Si = bovine intestinal from a second vendor

HPL = human placenta

Bact = bacterial

What is Claimed Is:

2

1. A method for measuring the bacteria content of fluids comprising:

4 a. binding an effective amount of a glycoprotein or glycopeptide with bacteria contained in a sample of fluid, said glycoprotein or glycopeptide having

6 a label to indicate its presence;

b. separating excess unbound glycoprotein or glycopeptide from said

8 fluid sample after reacting said glycoprotein or glycopeptide with bacteria in said sample in step (a);

10 c. measuring the amount of said label remaining after separating said excess unbound glycoprotein or glycopeptide of (b); and

12 d. determining the bacteria content of said sample as related to the amount of said label measured in step (c).

14

2. A method of Claim 1 wherein said glycoprotein or glycopeptide has a

16 binding constant to bacteria of at least 10^6 and at least 100 binding sites.

18 3. A method of Claim 1 wherein said glycoprotein is at least one member of the group consisting of serum proteins, immunoglobulins, oxygen-binding

20 proteins, fibrous proteins, intra cellular enzymes, hormones, and secreted enzymes and inhibitors.

22

4. A method of Claim 3 wherein said serum proteins are selected from the

24 group consisting of albumin, prealbumin, transferrin, retinol binding protein, and beta-2-macroglobulin.

26

5. A method of Claim 3 wherein said immunoglobulins are selected from the

28 group consisting of IgG, IgA, and IgM.

30 6. A method of Claim 3 wherein said fibrous proteins are selected from the group consisting of collagens, fibrinogens and myosin.

32

7. A method of Claim 3 wherein said oxygen-binding proteins are selected

34 from the group consisting of peroxidase, hemoglobin and myoglobin.

2 8. A method of Claim 3 wherein said intra cellular enzymes are selected from the group consisting of glutamate hydrogenase, ALP, and lactate
4 dehydrogenase.

6 9. A method of Claim 3 wherein said hormones are selected from the group consisting of insulin, growth hormone, and glucagon.
8

10. A method of Claim 3 wherein said secreted enzymes and inhibitors are
10 selected from the group consisting of protease inhibitors, alpha-1-macroglobulin, typsinogen, lysozyme, and alpha-1-acid glycoprotein.
12

11. A method of Claim 1 wherein said glycoprotein or glycopeptide is an
14 enzyme.

16 12. A method of Claim 11 wherein said glycoprotein or glycopeptide is an enzyme selected from the group consisting of alkaline phosphatase, acid
18 phosphatase, fucosidase, mannosidase, hexaminidase, alpha-galactosidase, phospholipase, hyaluronidase, glucocerebrosidase, hydrolase, arylsulfatase A,
20 amylases, cellobiohydrolase, and peroxidase.

22 13. A method of Claim 12 wherein said enzyme is alkaline phosphatase (ALP).
24

14. A method of Claim 13 wherein said ALP is intestinal ALP.
26

15. A method of Claim 1 wherein said glycoprotein or glycopeptide is a
28 glycoprotein.

30 16. A method of Claim 1 wherein said glycoprotein or glycopeptide is a glycopeptide.
32

17. A method of Claim 16 wherein said glycopeptide contains at least one
34 peptide and one carbohydrate.

2 18. A method of Claim 17 wherein said glycopeptide is at least one member of the group consisting of Y-Ser-X, Y-Thr-X, Y-Asn-X-Ser, Y-Asn-X-Thr, and
4 Gly-X-Hyl-Y

Where: X is an amino acid and Y is Man, Gal, Glu, SA, GlcNAc,
6 GalNAc, fucose or xylose.

8 19. A method of Claim 13 wherein ALP is measured by adding as a reagent PNPP.

10

20. A method of Claim 19 wherein the color developed by said reagent is read
12 at a wavelength of 405nm.

14 21. A method of Claim 1 wherein said glycoprotein or glycopeptide has a label selected from the group consisting of radioactive, fluorescent, electroactive,
16 chemi-luminescent, enzyme antibody, and particulate labels.

18 22. A method of Claim 21 wherein said label is a particle selected from the group consisting of latex beads and gold sols.

20

23. A method of Claim 21 wherein said label is comassie brilliant blue.

22

24. A method of Claim 1 further comprising adding to said sample blocking
24 compounds selected from the group consisting of polymers, non-glycated proteins, non-glycated polypeptides, and polysaccharides.

26

25. A method of Claim 1 further comprising at least one cation capable of
28 increasing the binding of said glycoprotein or glycopeptide to bacteria.

30 26. A method of Claim 25 wherein said cation is at least one member of the group consisting of zinc, copper and iron.

32

27. A method of Claim 26 wherein said cation is zinc.

34

28. A device for measuring the bacterial content of fluids comprising:

2 a. a glycoprotein or glycopeptide labeled to provide a means for detecting said glycoprotein or glycopeptide;

4 b. a structural support for said labeled glycoprotein or glycopeptide, whereby said labeled glycoprotein or glycopeptide can be brought into contact

6 with a sample of said fluid.

8 29. A device of Claim 28 wherein said glycoprotein or glycopeptide has a binding constant to bacteria of at least 10^6 and at least 100 binding sites.

10

30. A device of Claim 28 wherein said glycoprotein is at least one member of

12 the group consisting of serum proteins, immunoglobulins, oxygen-binding proteins, fibrous proteins, intra cellular enzymes, hormones, and secreted

14 enzymes and inhibitors.

16 31. A device of Claim 28 wherein said glycopeptide is at least one member of the group consisting of Y-Ser-X, Y-Thr-X, Y-Asn-X-Ser, Y-Asn-X-Thr, and

18 Gly-X-Hyl-Y.

Where: X is an amino acid and Y is Man, Gal, Glu, SA, GlaNAc,

20 GalNAc, fucose or xylose.

22 32. A device of Claim 28 wherein said glycoprotein or glycopeptide has a label selected from the group consisting of radioactive, fluorescent, electroactive,

24 chemi-luminescent, enzyme, and particulate labels.

26 33. A device of Claim 28 wherein said labeled glycoprotein is ALP.

28 34. A device of Claim 28 further comprising at least one cation capable of increasing the binding of said glycoprotein or glycopeptide to bacteria.

30

35. A device of Claim 34 wherein said cation is zinc.

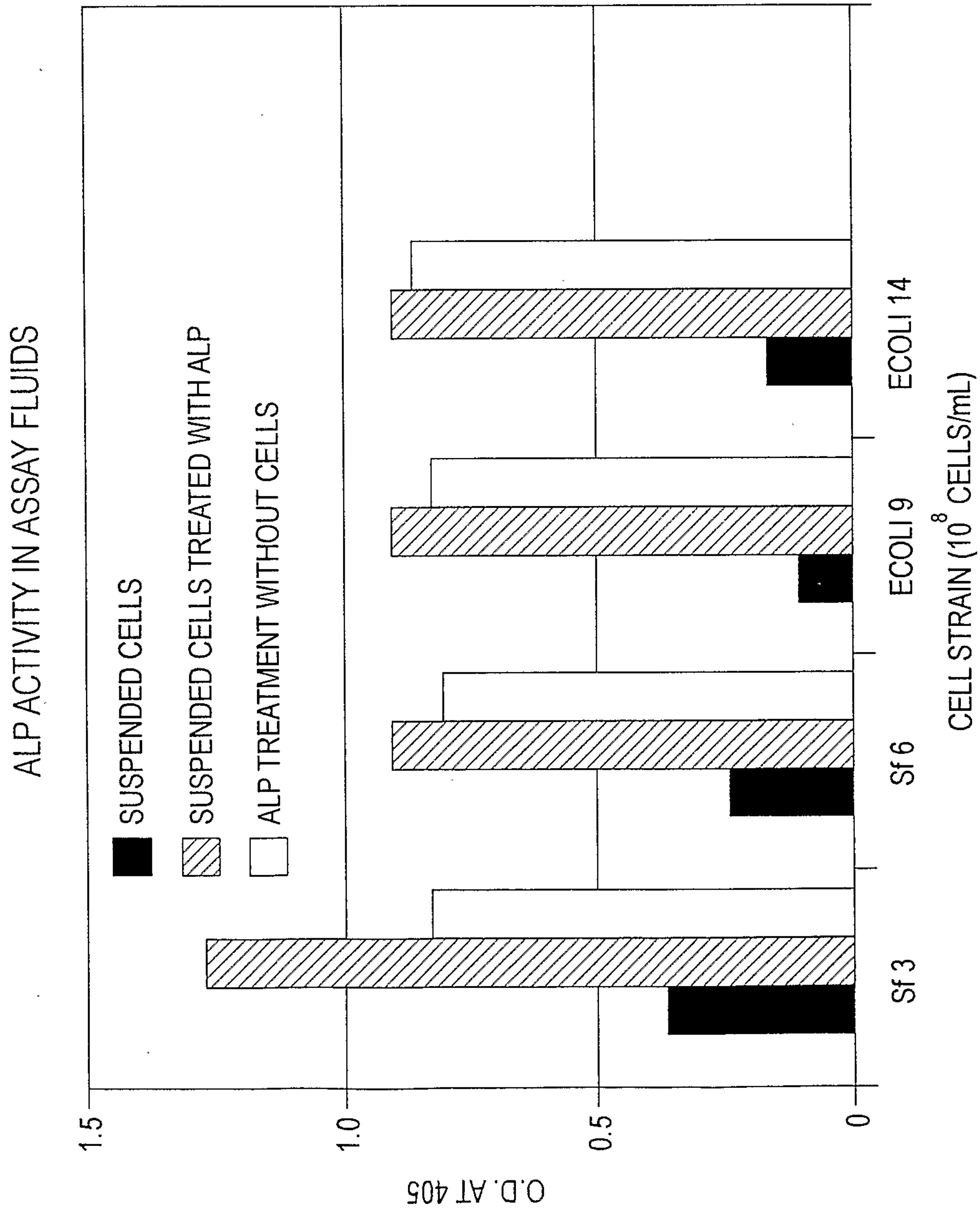


FIG. 1

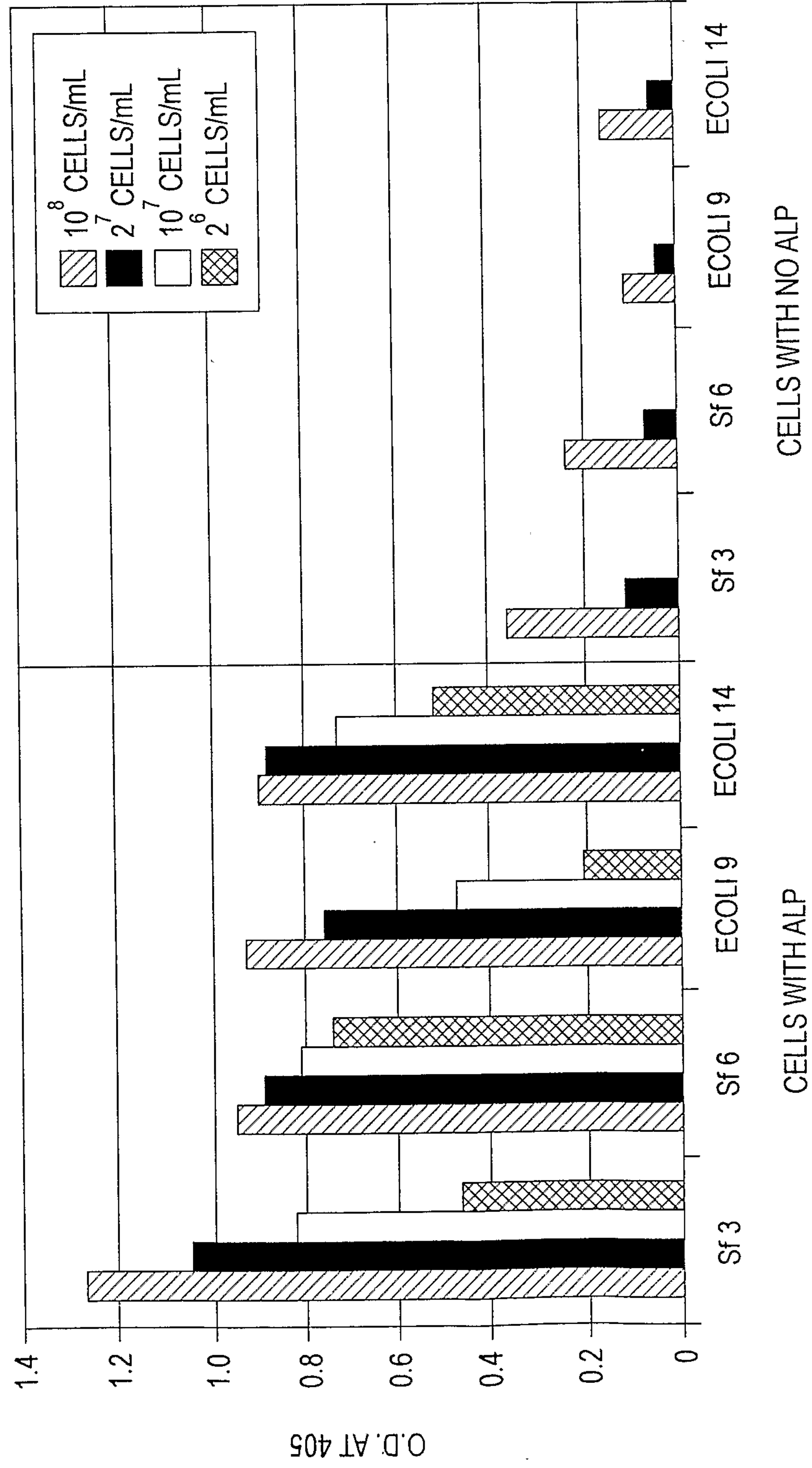


FIG. 2

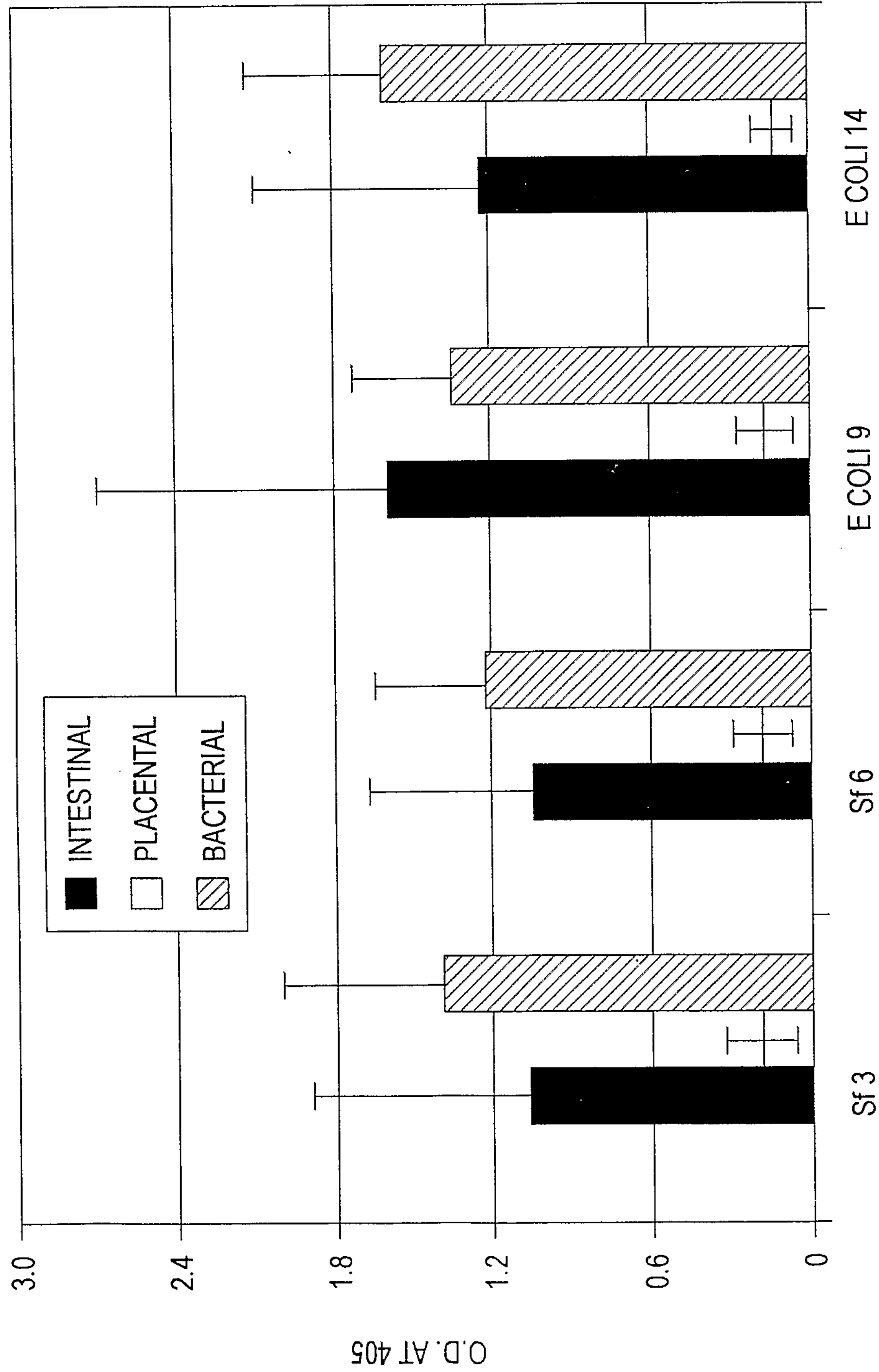


FIG. 3

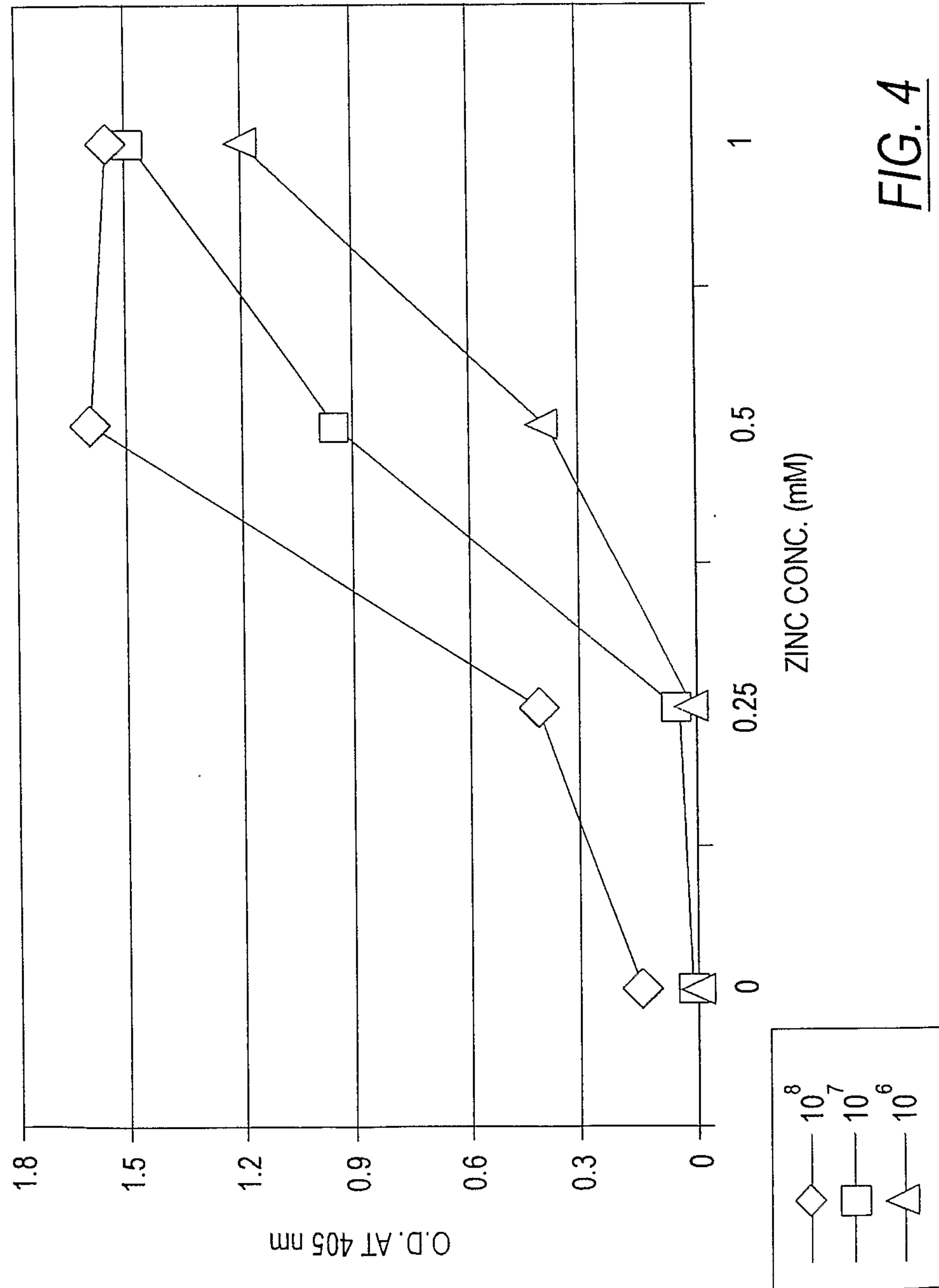


FIG. 4

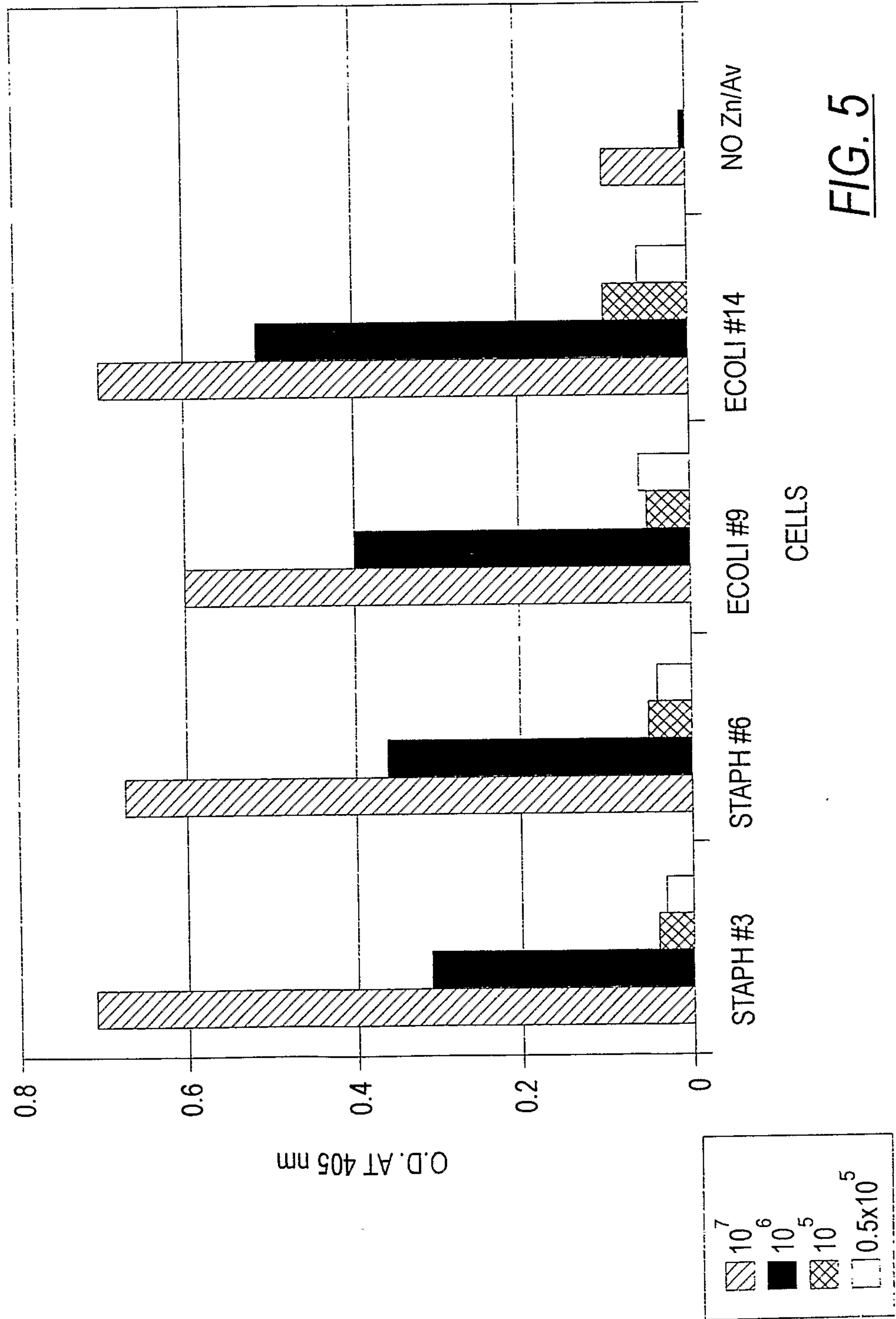


FIG. 5

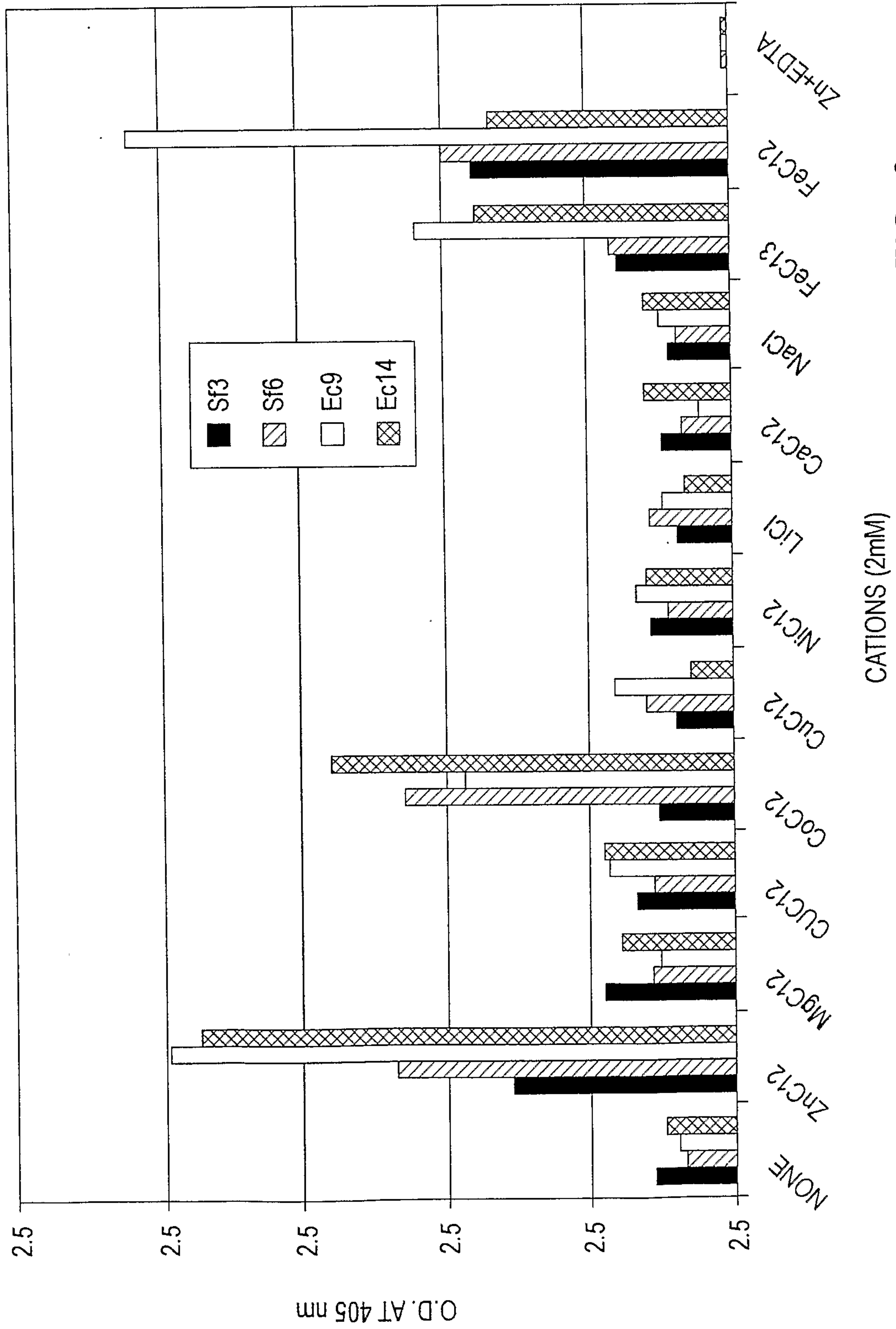


FIG. 6

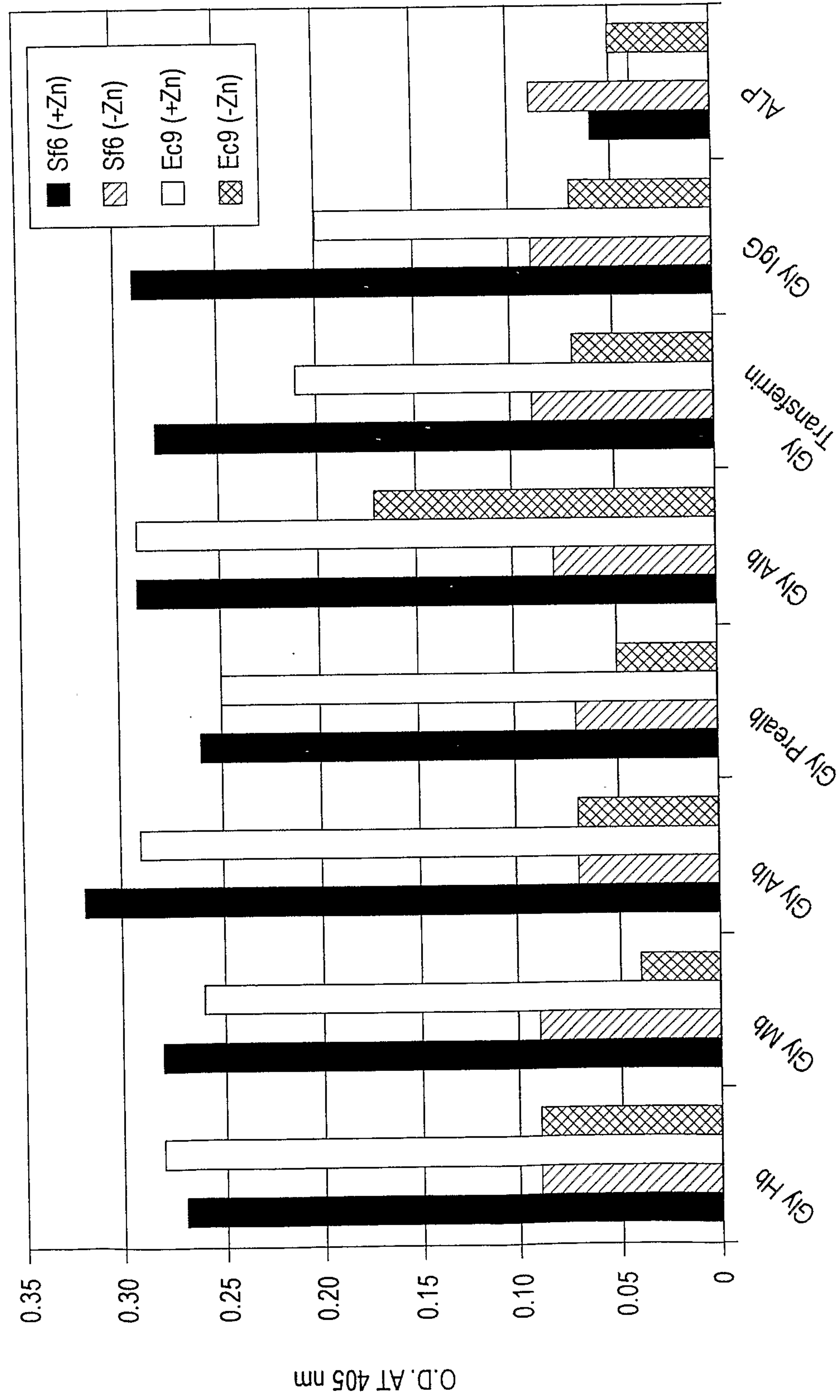


FIG. 7

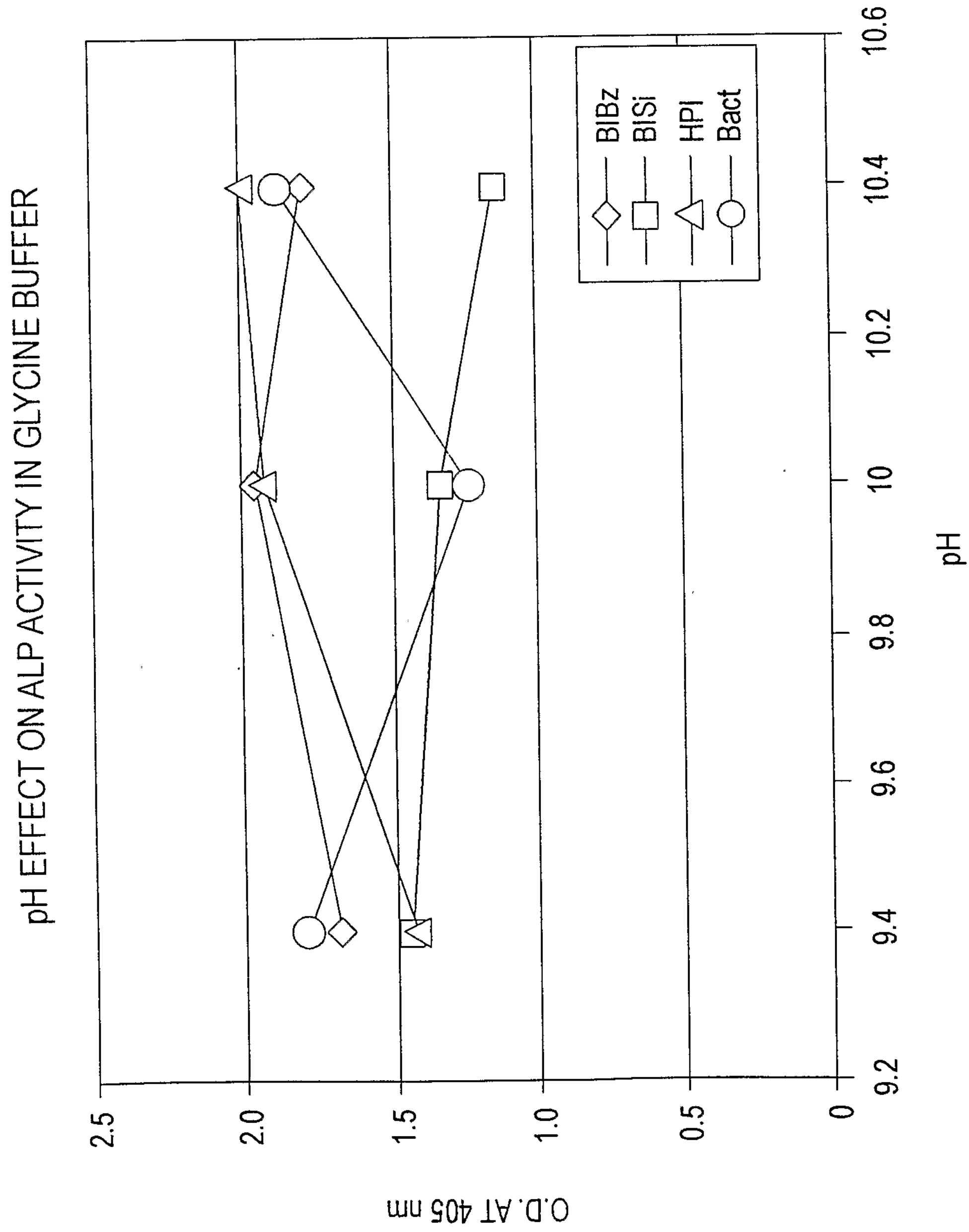


FIG. 8

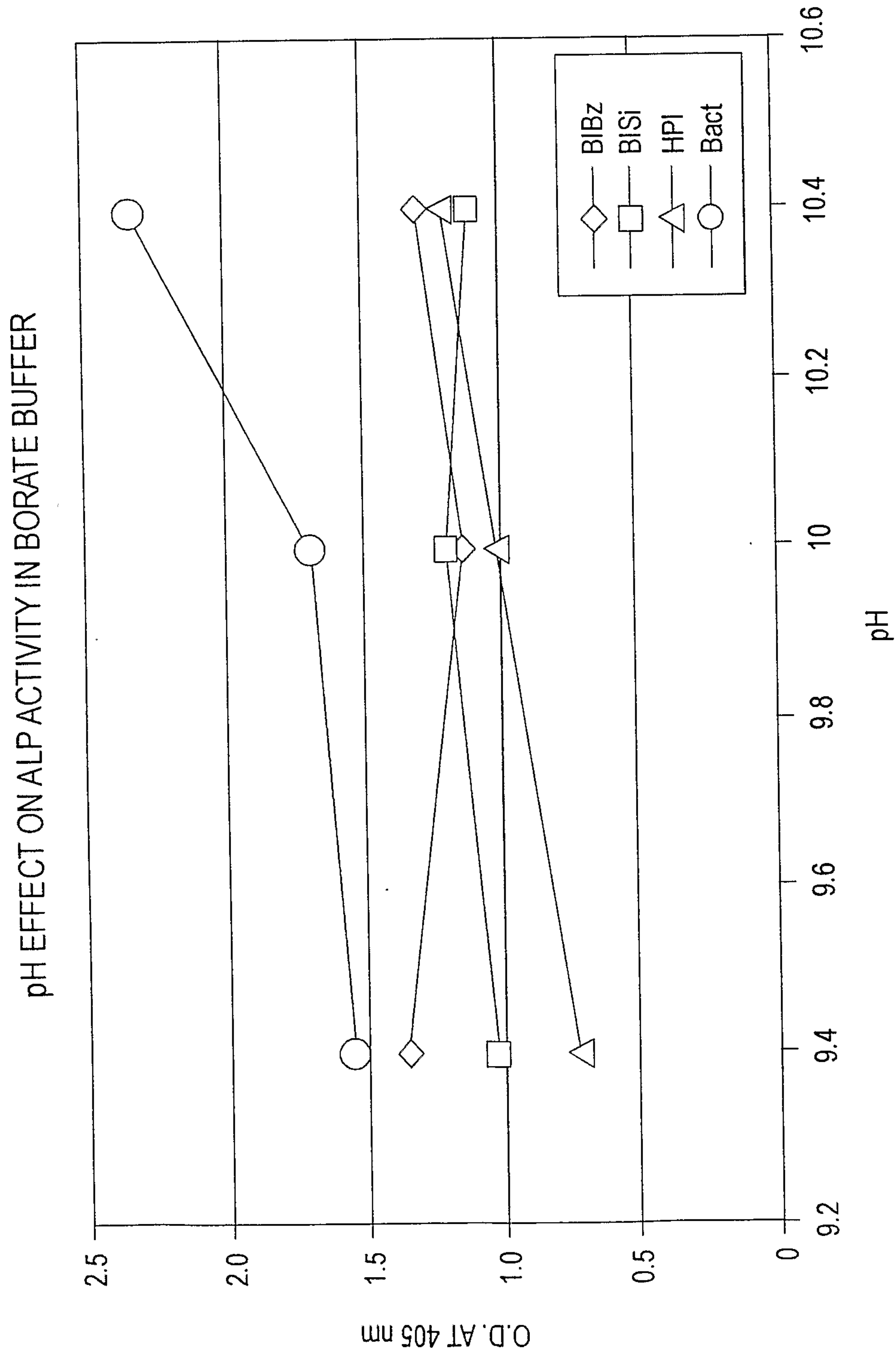


FIG. 9

FIG. 10

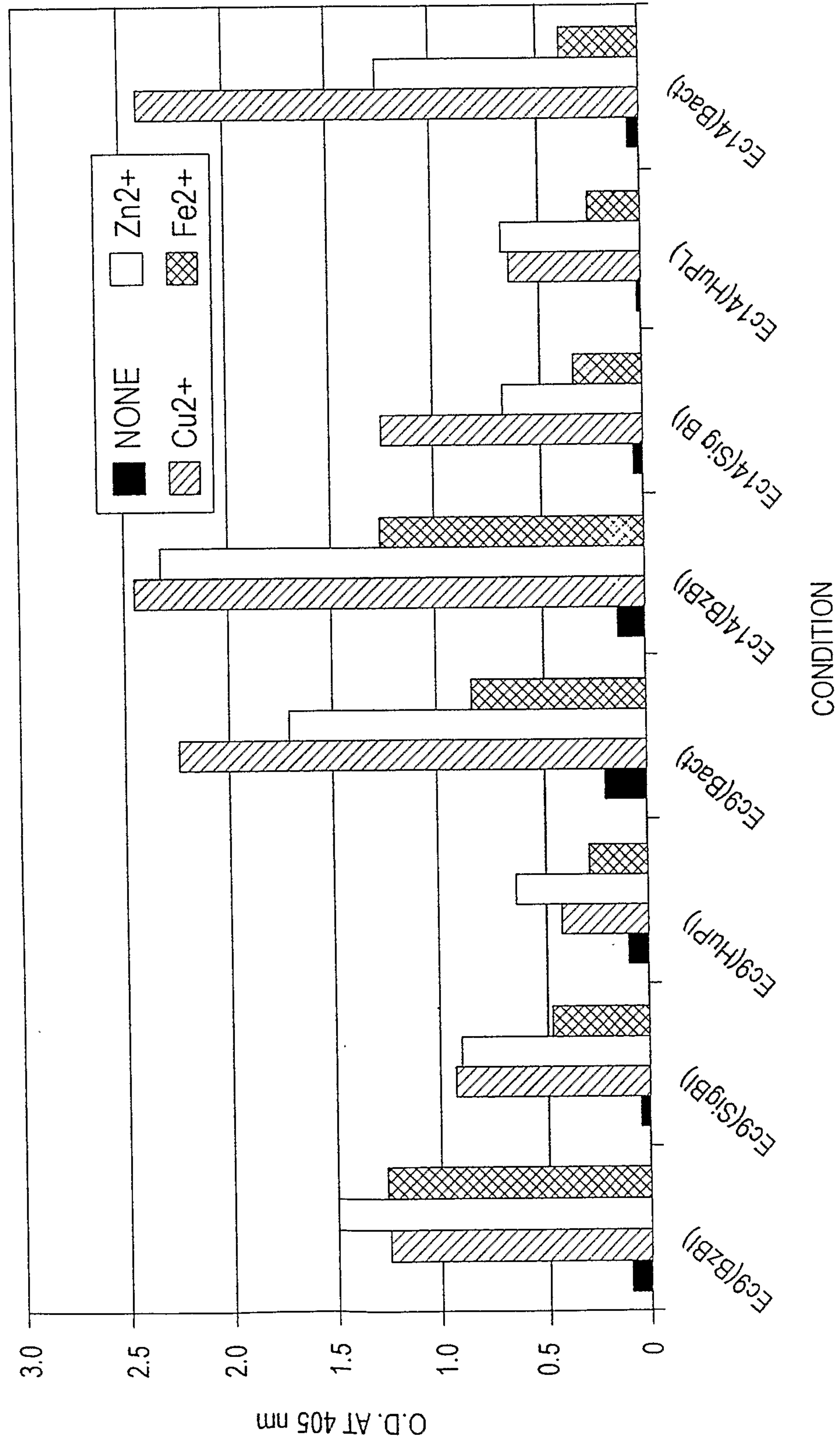
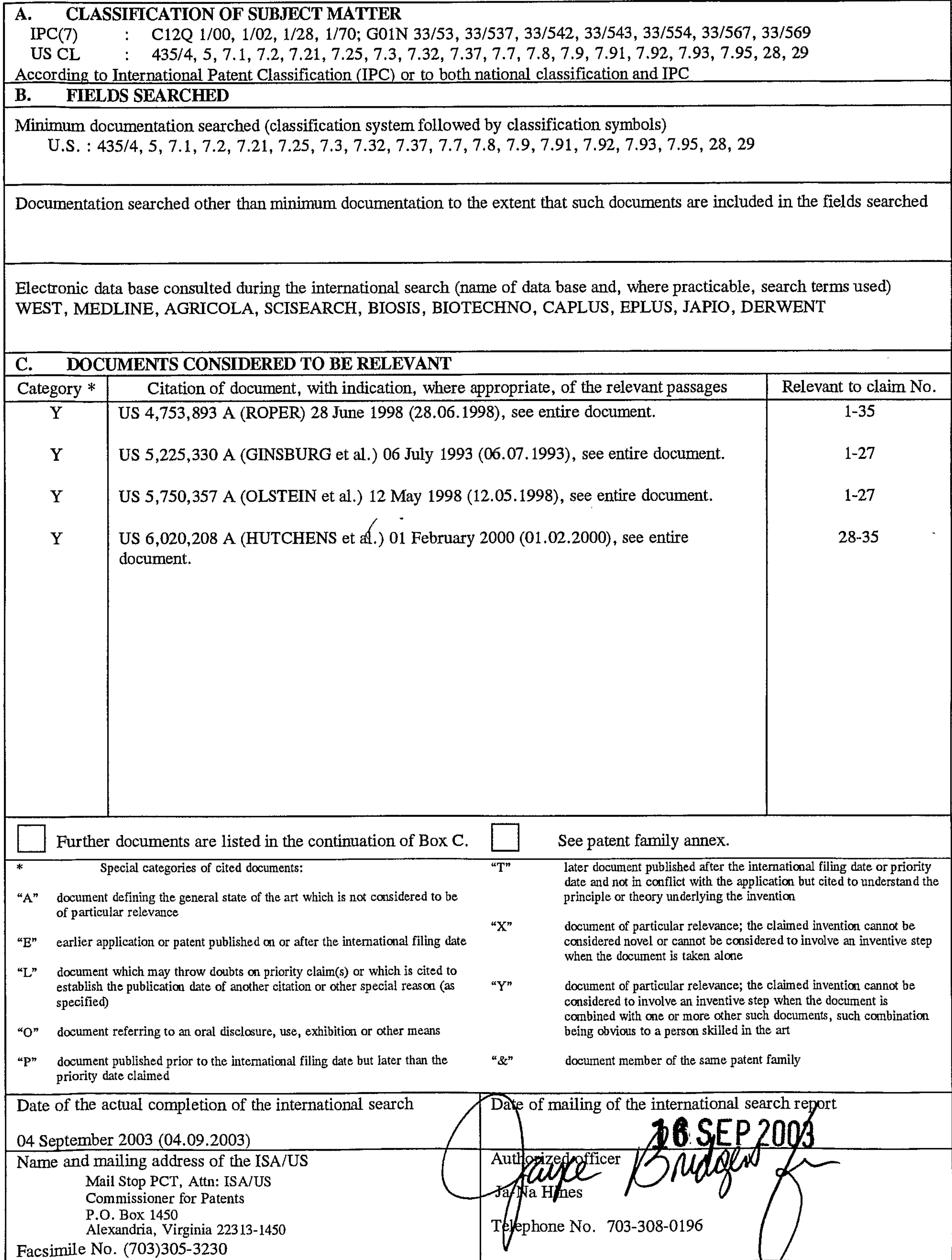


FIG. 11



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/17688

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12Q 1/00, 1/02, 1/28, 1/70; G01N 33/53, 33/537, 33/542, 33/543, 33/554, 33/567, 33/569
US CL : 435/4, 5, 7.1, 7.2, 7.21, 7.25, 7.3, 7.32, 7.37, 7.7, 7.8, 7.9, 7.91, 7.92, 7.93, 7.95, 28, 29
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/4, 5, 7.1, 7.2, 7.21, 7.25, 7.3, 7.32, 7.37, 7.7, 7.8, 7.9, 7.91, 7.92, 7.93, 7.95, 28, 29

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST, MEDLINE, AGRICOLA, SCISEARCH, BIOSIS, BIOTECHNO, CAPLUS, EPLUS, JAPIO, DERWENT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,753,893 A (ROPER) 28 June 1998 (28.06.1998), see entire document.	1-35
Y	US 5,225,330 A (GINSBURG et al.) 06 July 1993 (06.07.1993), see entire document.	1-27
Y	US 5,750,357 A (OLSTEIN et al.) 12 May 1998 (12.05.1998), see entire document.	1-27
Y	US 6,020,208 A (HUTCHENS et al.) 01 February 2000 (01.02.2000), see entire document.	28-35

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
04 September 2003 (04.09.2003)	16 SEP 2003

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Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	JaNa Hines Telephone No. 703-308-0196

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专利名称(译)	通过糖化标记结合的细菌测试方法		
公开(公告)号	EP1549759A1	公开(公告)日	2005-07-06
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[标]申请(专利权)人(译)	拜尔健康护理有限责任公司		
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摘要(译)

一种测量诸如尿液和血液的液体的细菌含量的方法，其中糖蛋白或糖肽附着于细菌，并且附着于糖蛋白或糖肽的固有标记提供了用于确定存在的细菌量的手段。优选的糖蛋白是碱性磷酸酶，其是能够附着于流体样品中存在的所有细菌的酶，并且固有地包括标记部分，其中该颜色可以通过添加已知试剂来显色。