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(54) Title: METHODS OF CONCENTRATING MICROORGANISMS USING AFFINITY SEPARATION

(57) Abstract

A method for concentrating a particular microorganism or microorganisms of interest in a sample is provided and effected by contacting the sample with a matrix to which is bound an affinity receptor specific for the particular microorganism or microorganisms, the affinity receptor and the matrix being selected so as to allow capture of the microorganism or microorganisms to the matrix via the affinity receptor when present in the sample at a concentration of ultra low levels, thereby obviating the need for a prolonged pre–enrichment step, and in some cases obviating altogether the need for a pre–enrichment step, of the microorganism or microorganisms in the sample.

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METHODS OF CONCENTRATING MICROORGANISMS USING AFFINITY SEPARATION

FIELD AND BACKGROUND OF THE INVENTION

The invention relates to methods for the capture and concentration of microorganism or microorganisms using affinity separation for the specific concentration and detection of a particular microorganism or microorganisms present in samples at ultra low levels from samples of large or small volumes. Advantageously, the methods are useful to concentrate and/or detect microorganisms present in samples at ultra low levels. In a preferred embodiment cellulose binding domain- or protein-receptor conjugates are employed to affinity bind a particular microorganism or microorganisms to a cellulosic or chitin matrix.

Citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention.

There is a tremendous effort being made to develop rapid microbial testing to meet the needs of the food, medical, veterinary and environmental industries. The food industry, for example, needs rapid microbial testing to approve or reject raw, semi-processed or processed materials and determine whether or not to release a held batch of product.

Furthermore, with the increasing implementation of hazard analysis and critical control point programs by the food industry, the demand for rapid microbial testing has been steadily on the rise (Rules and Regulations, Department of Agriculture Food Safety and Inspection Service, "Pathogen Reduction; Hazard Analysis and Critical Control Point (HACCP) Systems" (1996) 61 Federal Register 38806).

Rapid microbiological methods such as nucleic acid probe hybridization and immunological assays have advanced dramatically, shortening the time required for the detection of pathogens in meats and other foods. However, these methods most often require concentrations of target microorganism of 10^4 to 10^6 cells per ml or more (Blackburn et al., 1994, Lett. Appl. Microbiol. 19:32-36; Swaminathan et al., 1994, Ann. Rev. Microbiol. 48:401-426; and Tian et al., 1996, J. Food Prot. 59:1158-1163).

Foods which are contaminated by bacterial pathogens usually have low numbers of bacteria so that an enrichment step is required prior to the application of a rapid detection assay or even a selective culture method.

PCR-based assays have the potential to overcome the need for long

selective enrichment steps due to their ability to detect and identify pathogens in the presence of large numbers of background flora (K. Venkateswaran et al., 1997, Appl. Environ. Microbiol. 63:4127-4131). Nevertheless, when target pathogen concentrations are low, i.e., less than 10^3 colony forming units per gram (CFU/g), current PCR procedures require 6 to 18 hours of enrichment prior to PCR amplification in order to detect such target pathogens. This enrichment step not only brings the target pathogen, which may be present at levels of less than 1 cell per ml or gram of food (Swaminathan et al., 1994, Ann. Rev. Microbiol. 48:401-426), to PCR detectable levels, but also allows for samples to be diluted or filtered to reduce or partially remove PCR inhibitory food components while bringing the target pathogen to a concentration which is detectable (K. Venkateswaran et al., 1997, Appl. Environ. Microbiol. 63:4127-4131).

The possibility of selectively concentrating target bacteria from a food sample, other than by culture methods, while removing background flora would have a tremendous impact on rapid food testing saving time and increasing sensitivities of existing detection techniques. In culture methods, selective reagents are necessary in order to inhibit the growth of competing microorganisms but they also partially inhibit target bacteria, ultimately increasing the time it requires to achieve detectable levels of the target pathogen. Furthermore, selection media inhibit the "resuscitation" (reviving the viability) of damaged bacteria present, e.g., in meat samples, due to freeze thaw cycles, inhibitory food components, or desiccation during processing. Similarly, consecutive enrichment steps are needed in order to dilute compounds present in many foods which are inhibitory to the target bacteria and in some cases the target bacteria do not revive or revive very slowly. Zero tolerance standards require the detection of these damaged pathogens even when they are present at minute levels. Selective concentration of target bacteria would enable rapid enrichment to be carried out without the addition of selective reagents which are necessary in order to inhibit the growth of background flora, but which also partially inhibit target bacteria and also eliminate the need for successive enrichment steps needed to dilute inhibitory compounds both of which ultimately increase the time it takes, to achieve detectable levels of target pathogen. Inhibition or removal of competing microorganisms and inhibitory compounds found in food samples is highly desirable and some times necessary before efficient resuscitation of the target pathogens can be successfully effected.

In summary, efficient methods for selective concentration of a particular pathogen(s) from foods would not only shorten the overall time required for detection of these pathogens, but would allow for more sensitive detection due to more efficient resuscitation of damaged bacteria. Furthermore, the ability to collect larger more representative samples while selectively concentrating a specific pathogen from these samples would greatly increase the probability of detecting a pathogen in foods when they are present at extremely low concentrations.

Solid Phase Immunoassays:

The need to develop rapid on-line microbial testing protocols emphasizes the need to develop methods, other than those requiring time consuming incubation steps, to enrich target bacteria by noncultural concentration of the microorganisms from a food sample being tested. Solid-phase immunoassays have found increasing application in diagnostic microbiology due to their high specificity. The specificity of an immunoassay is determined by the antibody or antigen which has been immobilized onto a solid support and by the specificity of the support itself. The specificity of an immunoassay, carried out on a solid support, is enhanced by the ability to remove, after completion of the immunocapture step, unwanted material from the antigen antibody immune complex. This wash step will be facilitated if the solid support itself possesses a 'non-sticky' surface, i.e., microflora and other substances found in a sample are not bound to solid support in a non-specific manner. Many substances have been used as solid supports for immobilization of antibody or antigen including nitrocellulose, nylon, and agarose beads.

The success of an immunoassay in detecting the presence of a particular microorganism(s) in a sample depends upon the presence of the microorganism above a critical concentration. This concentration is dictated by the sensitivity of the immunoassay which will be used to determine if the microorganism is present in the sample. In most cases, immunoassays require culturing of the sample prior to performing the assay, which culturing functions to dramatically increase the total number of the microorganisms present in the sample. Most often a selective enrichment step is used to culture the target organism where compounds, for example, antibiotics, which inhibit background flora, or a particular nutritional requirement (or the absence of), are included/excluded in a medium, for optimizing the growth of the target organism. Other methods, such as manipulation of physical conditions of the medium i.e., elevated

temperature of the growth media, may also be used as means for selection. In all of these cases, the goal is to give a relative advantage to the target organism as compared to background flora.

Often the inhibitory compounds or conditions have an inhibitory effect, though to a lesser degree, on the target organism itself, especially when these are injured and need to be resuscitated prior to rapid cell multiplication.

U.S. Patent No. 5,415,997 describes a method for the detection of a particular microorganism in a mixed population which overcomes the need for selective media by using an immunoimmobilization technique followed by non-selective growth and immunoassay, or release of the microorganism by cleavage of the antibody-microorganism bond and subsequent growth on non-selective media. The success of this immunoassay in detecting the presence of a particular microorganisms is dependent on the pre-enrichment step which allows the target microorganism to reach the critical concentration which will ensure that some of these organisms will be captured by the immobilized antibodies.

Although effective in removing the need for selective enrichment media, this technique requires for success a critical concentration as dictated by the sensitivity of standard ELISA immunoassay of approximately 10^3 CFU/ml of a target microorganism, e.g., *Salmonella typhimurium.*

Classic methods of concentrating microorganisms:

The United States Department of Agriculture Food Safety and Inspection Service ("USDA-FSIS") has developed a series of both presumptive and confirmatory tests for detection of *Escherichia coli* (*E. coli*), strain O157:H7. The presumptive test involves culturing 25 grams of homogenized food samples in enrichment medium and plating on specialized agar plates of Sorbitol MacConkey ("SMAC"). In using this technique the speed of the assay is sacrificed in order to achieve high sensitivity and definitive identification, rendering such testing schemes as impractical for large-scale routine food industry testing programs. The USDA assay is, as pointed out, slow but is regarded as definitive due to its extreme sensitivity level of less than or equal to 3 CFU/g of food (Sharar et al., 1995, United States Dept. of Agriculture, Food Safety and Inspection Service: Protocol for isolation and identification of *Escherichia coli* O157:H7. Revision 4 of Laboratory Communication # 38. U.S. Dept. of Agriculture, Washington, D.C.). Such an extremely high sensitivity is necessary to detect the extraordinary low infectious dose of *E. coli* O157:H7

thought to initiate disease (Szabo et al., 1990, Appl. Environ. Microbiol. 56:3546-3549).

Immuno Magnetic Separation:

Sensitivity of enrichment techniques can be enhanced by the use of Immuno Magnetic Separation ("IMS") of *E. coli* O157:H7 prior to subculture (Chapman et al., 1993, Vet. Rec. 133:171-172 and Wright et al., 1994, Epidemiol. Infect. 113:31-39). Inclusion of an IMS step in the pre-enrichment step followed by isolating the microorganism by subculturing on Sorbitol MacConkey Agar ("SMAC"), increased the sensitivity 100 fold from 2000 CFU/10g beef to a detection limit of 20 CFU/10g beef after the sample had been enriched for several hours (Wright, et al., 1994, Epidemiol. Infect. 113:31-39).

In bovine fecal samples which were artificially inoculated with 10^3, 10^2 , 10 , 1 and 0.1 *E. coli* O157:H7 organisms per ml, IMS did not increase sensitivity of detection compared to direct culture and enrichment/subculture in two out of twelve *E. coli* O157 strains which were tested after six hours pre-enrichment, IMS, and then finally plating on SMAC supplemented with 0.05 mg/L Cefixime and 2.5 mg/L Potassium tellurite ("CT-SMAC") plates with additional 18 hour incubation at 37 °C (Chapman et al., 1997, Appl. Environ. Microbiol. 63:2549-2553).

In fact, in one of these two samples, even at the highest level of inoculation i.e., 10^3 CFU/ml, detection was not possible at all with the IMS technique. In the remaining 10 *E. coli* O157 strains, IMS improved detection such that fecal suspension samples which were inoculated with between 10^3 CFU/ml and 1 CFU/ml were shown to be positive for *E. coli* O157:H7. However, it is critical to emphasize that the positive results which were achieved by IMS were made possible only after 6 hours of pre-enrichment in selective enrichment culture media. During this 6 hour pre-enrichment step it can be assumed that the concentration of *E. coli* O157:H7 increased by two to three orders of magnitude to a final concentration of *E. coli* O157:H7 of greater than 10^3 CFU/ml even at the lowest detectable inoculum level, 1 CFU/ml.

Standard protocols for IMS have demonstrated that between 11 % and 41 % of *E. coli* O157 cells can be isolated from 1 ml of a 6 hour pre-enrichment medium in which the concentration of presumptive positive in these cultures was between 4.4×10^4 and 4.8×10^5 CFU/ml (Tomoyasu, 1998, Appl. Environ. Microbiol. 64:376-382).

The percent capture by IMS of *Salmonella* bacteria cells present at low concentrations in growth media is approximately 10 % (Hanai, et al., 1997, Appl. Environ. Microbiol. 63:775-778). Furthermore, capture and detection by IMS of *Salmonella* bacteria is possible only when the concentration of bacteria present in sample is at least 10^3 CFU/ml.

Similar results are seen with *E. coli* O157 in which another application of IMS has been used in Immunomagnetic-Electrochemiluminescent (IM-ECL) detection of *E. coli* O157:H7 in foods and environmental water samples to perform rapid detection of this bacteria with a sensitivity of approximately 1000 CFU/ml in the various food and environmental matrices which were tested (Yu and Bruno, 1996 Appl. Environ. Microbiol. 62:587-592). The IM-ECL assay, however, is inhibited by milk. This inhibition effect was observed with whole, nonfat and low-fat (2 %) milk samples in which IM capture is apparently impeded by the entrapment of magnetic beads and bacteria in lumps of lipid or casein.

Other Concentration Techniques:

Hydroxyapatite ("HA") has been used to concentrate bacteria from foods and bovine feces because of its ability to adhere to whole bacterial cells (Berry et al., 1997, Appl. Environ. Microbiol. 63:4069-4074). However, this technique works poorly when the pathogen is present at concentrations less than 10^3-10^4 CFU/ml and when inhibitory substances such as those which are found in feces are present. Furthermore, HA has a limited concentration effect with certain pathogens such as, for example, *E. coli* O157:H7 even when this pathogen is present at high concentrations.

In another method, antibody-coated cellulose sponges have been used to enhance the isolation of *Salmonella* from 10-fold pre-enriched samples obtained from contaminated pig, poultry, and cattle farms, poultry hatcheries, animal feed mills, and processed animal proteins (Davies and Wray, 1997, Letts. in Appl. Microbiol. 25:246-248). The use of polyvalent "O" antiserum enhanced the specificity of the *Salmonella* captured. However, this method has the drawback of requiring an 18 hour pre-enrichment step followed by growth of the captured bacteria on an Rappaport medium (MSRV) plate for an additional 18-24 hours. This cellulose sponge method does not provide a rapid method of concentration and detection required by the food processing industry and requires a pre-enrichment step.

The inclusion of a longer enrichment step or a secondary enrichment step in conventional methods of concentrating bacteria will increase the

concentration per ml of a particular type of bacteria and, therefore, the overall sensitivity of a test being used to determine the presence of a bacteria in a sample. However, the longer incubation periods will significantly add to the time and resources needed to perform the test. Highly specific capture coupled with concentration of relatively large sample volumes is the ideal solution for highly sensitive, specific as well as rapid testing of food, in particular milk samples, for example, and biological, clinical and environmental samples.

Cellulose:

Chemically modified cellulose has been very useful for immobilization of proteins such as endo-beta-glucosidase, an enzyme that is used for wine and fruit juice treatments (Shoseyov et al., 1990, J. Agric. Food Chem. 38:1387-1390). However, immobilization requires the chemical modification of the cellulose which has detrimental effects on the matrix, making flow qualities unsuitable for industrial work and resulting in a fluffy compressible material that is not suitable for applications involving packed columns.

Objects of the Invention:

A rapid, highly sensitive method of concentrating and detecting microorganisms in a sample is needed in the art. Such a method of concentrating microorganisms would be useful for detecting very low numbers of microorganisms in large volumes of dilute samples, encountered especially in the food, environmental, veterinary and medical industries.

Such method would be particularly useful for detecting microorganisms in samples containing ultra low levels of microorganisms in samples, for example, in samples with as few as 0.00025, 0.001, 0.008 or <1 CFU/ml and up to about 10^3 CFU/ml.

One of the objects of the present invention is to provide advantageously useful methods of concentrating or concentrating and detecting particular microorganisms in samples.

Another object of the present invention is to provide a cellulose substrate that is not chemically modified and that can be used as a highly suitable matrix for immobilization of microorganisms by means of attaching a CBP- or CBD-receptor conjugate to the cellulose substrate. The cellulose possesses the advantage that the resulting solid matrix is natural, inert and non-toxic (having required no "chemical modifications"). It is further advantageous because the resulting solid matrix retains its physical properties, and has a low non-specific affinity for most proteins. Hence it is

a "non-sticky" matrix and microorganisms that are not "target" microorganisms are not non-specifically bound. Further, it is relatively low priced. At present, cellulose prices are 100-500 fold lower than those of, for example, glutathione-Sepharose and IPTG-Sepharose, making cellulose an attractive, inexpensive matrix that can be used safely in food and pharmaceutical industries.

SUMMARY OF THE INVENTION

The present invention provides methods for concentrating a particular microorganism or microorganisms of interest in a sample. In addition, the present invention provides a prefiltration method and device, which are advantageously used in combination with the concentration methods of the present invention, as further detailed hereinunder.

According to one aspect a method according to the present invention for concentrating a particular microorganism or microorganisms of interest in a sample is effected by contacting the sample with a matrix to which is bound an affinity receptor specific for the particular microorganism or microorganisms, the affinity receptor and the matrix being selected so as to allow capture of the microorganism or microorganisms to the matrix via the affinity receptor when present in the sample at a concentration of ultra low levels, thereby obviating the need for a prolonged pre-enrichment step of the microorganism or microorganisms in the sample.

As used herein, the term "ultra low level" means a sample having a low concentration of microorganisms of interest, i.e., with at least 0.00025, 0.001, 0.008 or <1 CFU/ml and up to about 10^3 CFU/ml of the microorganisms.

The methods can also include a washing step to remove unbound material of the sample from the matrix.

The methods can also include a step for enriching the concentrated microorganism(s) *in situ* by addition of a culture medium to the matrix or by enriching the concentrated microorganism(s) *in vitro* by transferring the microorganism(s) from the matrix to a culture medium.

The method can also include a step of performing an assay to detect any microorganisms that bind to the matrix.

According to further features in preferred embodiments of the invention described below, the matrix is selected from the group consisting of natural and synthetic matrices.

According to still further features in the described preferred embodiments the matrix is a polysaccharide matrix.

According to still further features in the described preferred embodiments the polysaccharide matrix is selected from the group consisting of a cellulosic matrix, an agarose matrix, a chitin matrix, a starch matrix and a matrix of cellulose, agarose, chitin and starch derivatives.

According to still further features in the described preferred embodiments the matrix is a coated matrix.

According to still further features in the described preferred embodiments the matrix is a synthetic matrix selected from the group consisting of a polypropylene matrix, polyester matrix, a polyamide matrix, a polyethylene matrix, an acrylamide matrix, a methacrylate matrix, a sepharose matrix, a polystyrene matrix and matrices which are derivatives of the above matrices.

According to still further features in the described preferred embodiments the matrix is in a form selected from the group consisting of beads, threads, a cloth, a woven material, a non-woven material, a membrane, a powder, a foam and a sponge.

According to still further features in the described preferred embodiments the matrix is selected from the group consisting of a porous matrix and a non-porous matrix.

According to still further features in the described preferred embodiments the affinity receptor is directly bound to said matrix via covalent or non-covalent interactions.

According to still further features in the described preferred embodiments the affinity receptor forms a part of a conjugate or fusion including a counterpart moiety having affinity to the matrix.

According to still further features in the described preferred embodiments the counterpart moiety is a matrix binding peptide.

According to still further features in the described preferred embodiments the matrix binding peptide is a polysaccharide binding protein or domain.

According to still further features in the described preferred embodiments the polysaccharide binding protein or domain is a cellulose binding protein or domain or a maltose binding protein.

According to preferred embodiments the methods of the present invention are effected by incubating a solid sample with a liquid to form an extract, and contacting the extract with a cellulosic or chitin matrix to which

is bound a cellulose binding protein (CBP) - or domain (CBD) - receptor conjugate specific for the microorganism. In a preferred embodiment, the sample is a sample having ultra low level(s) – of microorganism(s).

According to additional preferred embodiments the methods of the present invention are effected by contacting a sample with a cellulosic or chitin matrix to which is bound a cellulose binding protein (CBP)- or cellulose binding domain (CBD) receptor conjugate specific for the microorganism(s) and enriching a concentrated microorganism(s) by addition of a culture medium with or without antibiotics to the matrix. This embodiment may further comprise performing an assay to detect any microorganisms that bind to the CBP- or CBD-receptor conjugate bound to the matrix. In a preferred embodiment, the sample is a sample having ultra low level(s) of microorganism(s).

Capture using affinity receptor concentration methods is a user friendly technique for microbial detection with a sensitivity level of at least 0.00025 CFU/ml food, biological, and environmental samples. In other embodiments, the sensitivity level is at least 0.001 CFU/ml at least 0.008 CFU/ml or <1 CFU/ml.

The invention has utility in concentrating microorganisms in samples, particularly dilute samples, in order to detect the microorganisms by any means known in the art.

Such methods of concentration provide improved means of concentrating microorganisms in food, environmental, or biological, such as medical or veterinary, samples.

The present invention has a number of advantages over previously described concentration methods.

For example, the use of cellulosic fabric as a matrix, as well as other matrices, allow for larger volumes of liquids (up to 10 liters) to be passed with relatively high flow rates as compared to DYNAL® DYNALBEADS ® procedure, which is limited to very small volume samples, i.e., about 1 ml. The low non-specific binding of the matrix achieves very low background levels.

In certain preferred embodiments, the present invention is able to capture microorganisms present at very low concentrations by use of high surface area cellulosic or chitin matrix, such as, but not limited to gauze.

The present invention is also able to bind, without any modifications of the matrix, antibodies specifically resulting in a highly specific capture/concentration device.

Furthermore, the present invention provides for the ability to selectively capture microorganisms and then enrich *in situ* the captured microorganisms on the matrix without hindering the ability of the microorganisms to replicate and, in fact, enhancing the growth of the microorganism.

The physical properties of the matrix employed according to the present invention enable its performance under conditions that Immuno Magnetic Separation (IMS) perform poorly, i.e., in the presence of food samples containing milk and food samples containing bacteria at concentrations lower than 10^3 CFU/ml.

According to preferred embodiments of the present invention the concentration methods are further effected by a prefiltration step which is designed for the removal of debris in a form selected from the group consisting of particulates, fatty materials, starchy materials and proteinaceous materials from the sample. Such removal is of greater importance when (i) larger volumes of samples need to be processed; (ii) low levels of the particular microorganism or microorganisms are present in the sample; (iii) inhibitory compounds are present in the sample; and (iv) the level of particulate matter present in the sample inhibits the ability to process the sample. Measures are taken to ensure substantially no removal of the particular microorganism or microorganisms upon prefiltration. Any suitable filtering device with one or more filtering layers of selected properties can be employed to implement the prefiltration step according to the present invention, depending on the sample processed.

A presently preferred filtering device in accordance to the teachings of the present invention, which is designed to perform the above prefiltration task, comprises (a) a housing defining a space having an inlet and an outlet forming a filtration path therein; (b) a first filter layer including a cotton filter being within the housing in the filtration path; (c) a second filter layer including a polyester filter being within the housing in the filtration path; and (d) a third filter layer including a polypropylene filter being within the housing in the filtration path.

According to another embodiment the filtering device comprises a housing defining a space having an inlet and an outlet forming a filtration path therein; (b) a first filter layer including a polyurethane filter being

within the housing in the filtration path; (c) a second filter layer including a non-woven filter being within the housing in the filtration path; and (d) a third filter layer including a polypropylene filter being within the housing in the filtration path. Preferably it further comprises (e) a fourth filter layer including a hollow body of woven mesh being within the housing in the filtration path.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention herein described, by way of example only, with reference to the accompanying drawings, wherein:

Figure 1 is an illustrative diagram of one mode of operation of a method of the present invention using an immunoconcentrator ("IC"), such as cellulose binding domain immunoconcentrator ("CBD-IC") which contains am appropriate matrix, such as cellulosic or chitin matrix to which is bound a microorganism affinity receptor, such as a CBP- or CBD microorganism affinity receptor conjugate.

Figure 2 is an illustrative diagram of an automated mode of operation of a method of the present invention using an IC.

Figure 3 is a photograph of a denaturing SDS-PAGE gel showing the binding of the N-succinimidyl 3-(2-pyridydithio)propionate) ("SPDP") CBD-goat IgG conjugate to AVICELL™. Lane 1, protein markers (10 - 225 Kd); lane 2, CBD-conjugate loaded (conjugate solution before binding to cellulose); lane 3, unbound conjugate (supernatant solution from wash step solutions); and lane 4, bound conjugate (proteins which were bound to the cellulose). H and L signify the Heavy and Light peptide chains of the IgG.

Figure 4 is a graph showing the bifunctional activity of the CBD-anti-*E. coli* O157:H7 conjugate. CBD-anti-*E. coli* O157:H7 was bound to cellulose beads in phosphate buffered saline with 0.05 % TWEEN™ 20 ("PBS-TWEEN™ 20"). *E. coli* O157:H7 antigen was added (0.5 ml at 10^3, 10^4, or 10^5 CFU/ml), incubated for 15 minutes and washed with PBS-TWEEN™ 20. Anti-*E. coli* O157:H7-HRP (horse radish peroxidase) was added, incubated for 15 minutes at room temperature, and washed with PBS-TWEEN 20. 0.2 ml K-Blue™ substrate was added and incubated for 15 minutes at room temperature. The substrate was transferred into a fresh 10 tube and 0.2 ml 1M H_2SO_4 was added. The absorbance of the sample were read using a spectrophotometer at 450 nm.

Figures 5a and 5b are photographs of denaturing SDS - polyacrylamide gels, 15 % and 7.5 %, respectively, showing the difference in specific binding of native goat IgG (lanes 1-3) compared to the CBD-IgG conjugate (lanes 4-6). Figure 5a (15 % SDS-PAGE) and Figure 5b (7.5 % SDS-PAGE); Lane 1, IgG alone; Lane 2, supernatant of unbound proteins; Lane 3, IgG that bound to cellulose; Lane 4, molecular weight markers; Lane 5, CBD-goat IgG; Lane 6, supernatant having unbound conjugate; Lane 7, conjugate that bound to cellulose; Lane 8, molecular weight markers.

Figure 6 is a graph depicting the capture of bacteria. PBS solution containing 2480 CFU *E. coli* O157:H7 was drawn into and expelled from a CBD-IC device using a standard pipettor. Capture of bacteria by a single cycle is compared to 5 cycles. Control was the device without CBD-anti-*E. coli* O157:H7 in which bacteria were cycled 5 times through the device. Devices were washed once with 5 ml PBS after the capture step.

Figure 7 is a graph depicting the selective capture of *Salmonella typhimurium* from a 1 ml sample having 129 CFU. The control was a cellulose containing device with no CBD-anti-*Salmonella* conjugate (control, left bar) and the test device contained cellulose bound CBD-anti-*Salmonella* IgG conjugate (CBD-anti-*Salmonella,* right bar).

Figure 8 is a graph showing a comparison of the immunoconcentration of *E. coli* O157:H7. PBS solution containing 590, 76 or 6 CFU of *E. coli* O157:H7 was cycled twice through a device containing a CBD-anti-*E. coli* O157:H7 conjugate bound to cellulose using a standard pipettor. The Control was the device without the bound CBD-anti-*E. coli* O157:H7. The devices were washed once with 5 ml of PBS after the capture step.

Figure 9 is a graph showing the effect of different wash solutions on the capture of *E. coli* O157:H7. Capture of 1770 CFU *E. coli* O157:H7 is shown when 5 ml saline, PBS or PBS-TWEEN™ 20 was used for the wash step. The results show that all wash solutions produced a high percent of capture when the CBD-IgG conjugates were used, but much less without the conjugates. The specificity of the matrix is demonstrated by the controls in which a device without CBD-anti-*E. coli* O157:H7 was used.

Figure 10 is a graph showing the detection of *E. coli* O157:H7 from 1 ml of PBS by ELISA after capture by a device with a CBD-anti-*E. coli* O157:H7 IgG conjugate and *in situ* enrichment. PBS solution containing 22 CFU *E. coli* O157:H7 was cycled once through a device using standard

pipettor. Devices were washed once with 5 ml PBS-TWEEN™ 20 and 5 ml PBS after the capture step. Captured bacteria were enriched *in situ* for 4 or 5 hours after the wash step and then analyzed by ELISA. The optical density was converted into CFU/ml using a standard curve.

Figure 11 is a graph showing detection of *E. coli* O157:H7 from ground beef samples by ELISA after CBD-IC immunoconcentration and *in situ* enrichment. 225 ml PBS buffer was added to 25 grams of ground beef samples which were previously spiked with 44 CFU *E. coli* O157:H7. Samples were stomached in stomacher classic 400 unit (IUL USA Inc. Erlanger Kentucky) at 1500 rpm for 2 minutes and filtered before being passed through a CBD-IC device. Unspiked samples were used for the control. Captured bacteria were enriched *in situ* after the wash step for 4 or 5 hours and then analyzed by ELISA. The optical density was converted into CFU/ml based on a standard curve.

Figure 12 is a graph depicting the detection of *E. coli* O157:H7 in 250 ml of meat extract inoculated with 20 or 200 CFU *E. coli* O157:H7 and captured by the CBD-anti-*E. coli* O157:H7-IC gauze device (using gauze as the cellulosic matrix) followed by *in situ* enrichment and detection by ELISA.

Figure 13 is a graph showing a comparison of the detection of *E. coli* O157:H7 in 1 ml of meat extract inoculated with 40 CFU *E. coli* O157:H7 and loaded on the CBD-anti-*E. coli* IC gauze device by consecutive cycles or incubated with DYNAL® DYNABEADS®-anti-*E. coli* O157 followed by 6 hours *in situ* enrichment and detection by ELISA. Control trials were performed using samples without O157:H7 inoculum.

Figure 14 is a graph showing a comparison between CBD-IC and DYNAL® DYNABEADS® methods for the capture of *E. coli* O157:H7 from samples of fresh milk containing 1%, 2% or 3% fat.

Figure 15 is a graph showing the capture of *E. coli* O157:H7 in a sample of ultra-high temperature ("UHT") long-life homogenized milk containing 1 % fat. 250 ml samples were spiked with 5 CFU or 60 CFU of *E. coli* O157:H7, passed through CBD-IC followed by *in situ* enrichment for 5 hours, and analyzed by ELISA. For the control, unspiked samples were used.

Figure 16 is a graph showing a comparison of the capture of *E. coli* O157:H7 in a sample of fresh homogenized milk containing 3 % fat. 250 ml samples were spiked with 30 CFU *E. coli* O157:H7, passed through a

CBD-IC, followed by *in situ* enrichment for 5 hours and ELISA analysis. For the control, unspiked samples were used.

Figure 17 is a graph showing the results of the capture of *E. coli* O157:H7 from ground beef extract samples. Extracts were obtained from 25 grams of ground beef that were spiked with 2 CFU or 7 CFU *E. coli* O157:H7 and then pre-enriched in modified EC medium ("mEC") (Difco) without Novobiocin. The samples were then passed through CBD-IC followed by *in situ* enrichment for 5 hours and analyzed by ELISA. For the control, unspiked samples were used.

Figure 18 is a graph showing the results of the capture of *E. coli* O157:H7 from PBS by a maltose binding protein (MBP)-antibody-immunoconcetrator (IC) containing an α-amylose coated matrix. MBP-Ab-IC cartridges were used in triplicates to capture viable *E. coli* O157:H7 cells from a 1 ml PBS solution containing 100 CFU's. After capture and wash steps the wash solutions were combined and bacterial concentration was determined by a filter method using GN-6 METRICEL™ on mENDO agar LES medium and overnight incubation at 37 °C. Cartridges containing MBP without conjugated antibody (Ab) served as control.

Figure 19 is a graph showing the results of the capture of *E. coli* O157:H7 from PBS by a maltose binding protein (MBP)-antibody-immunoconcetrator (IC) containing an α-amylose coated matrix. MBP-Ab-IC cartridges were used in duplicates to capture viable *E. coli* O157:H7 cells from 250 ml PBS solution containing 100 CFU's. After capture and wash steps the wash solutions were combined and bacterial concentration was determined by a filter method using GN-6 METRICEL™ on mENDO agar LES medium and overnight incubation at 37 °C. Cartridges without MBP-Ab antibody served as control.

Figure 20 is a graph showing the results of the capture of *E. coli* O157:H7 from 25 ml PBS by a CBD-antibody cellulose beads complex. CBD-IC beads cartridge was used to capture viable *E. coli* O157:H7 from 25 ml PBS solution containing 52 CFU (for ORBICELL™) or 110 CFU (for VISKASE®). After capture and wash steps the wash solutions were combined and bacterial concentration was determined by filter method using GN-6 METRICEL™ on mENDO agar LES medium and overnight incubation at 37 °C. Beads, after wash step, were collected with 1 ml PBS and plated onto mENDO agar LES. In previous experiments control ORBICELL™ beads (without CBD-Ab) were shown to have no capture effect on viable *E. coli* O157:H7.

Figure 21 is a graph showing the results of the capture of *S. typhimurium* from 250 ml meat extract by a CBD-antibody demonstrating the sensitivity of CBD-IC without a pre-enrichment step. Samples of 250 ml stomached meat extract were spiked with 200, 100, 50, 15 or zero CFU *S. typhimurium* and then passed through a CBD-IC anti-*Salmonella* device. Captured *Salmonella* were then enriched at 37 °C for six hours with 120 µl of M-Broth without Novobiocin. Samples were then analyzed by ELISA using monoclonal anti-*S. typhimurium*. All experiments were performed in duplicates. Numbers in parenthesis beneath the bar indicate number of positive samples.

Figure 22 is a graph showing the results of the capture of *S. typhimurium* from 250 ml meat extract by a CBD-antibody following a brief pre-enrichment in a non-selective medium, demonstrating the sensitivity of CBD-IC for *S. typhimurium*. Samples of 250 ml stomacher meat extract were spiked with 28, 14, 7 or zero CFU *S. typhimurium*, pre-enriched for 1 hour at 37 °C using Buffered Peptone Water and then passed through CBD-IC anti-*Salmonella* device. Captured *Salmonella* cells were enriched at 37 ° C for 7 hours with 120 µl of M-Broth without Novobiocin. Samples were then analyzed by ELISA using monoclonal anti *S. typhimurium*. Fraction in parentheses represents number of positives out of number of trials.

Figure 23a is a graph showing the results of capture of *E. coli* O157:H7 onto a polyester matrix. One ml PBS solution containing 91 CFU *E. coli* O157:H7 was cycled 5 times through an immunoconcentrator device using a standard pipettor. Following capture, devices were washed twice with 5 ml PBS. Captured bacteria were calculated by subtracting the number of expelled bacteria from the inoculum.

Figure 23b is a graph showing the results of capture of *E. coli* O157:H7 onto a polyester matrix and subsequent enrichment. One ml PBS solution containing 91 CFU *E. coli* O157:H7 was cycled 5 times through an immunoconcentrator device using a standard pipettor. Following capture, devices were washed twice with 5 ml PBS. Captured bacteria were then enriched *in situ* for 5.5 hours and were analyzed by ELISA.

Figure 24 is a graph showing the results of capture of *E. coli* O157:H7 onto a polyester matrix and subsequent enrichment. One ml PBS solution containing 113 CFU *E. coli* O157:H7 was cycled 5 times through an immunoconcentrator device using a standard pipettor. Following capture, devices were washed twice with 5 ml PBS. Captured bacteria were then enriched *in situ* for 5 hours and were analyzed by ELISA.

Figure 25 is a graph showing the results of capture of *E. coli* O157:H7 onto a polyester matrix and subsequent enrichment. 250 ml Buffered Peptone Water solution containing 91 CFU *E. coli* O157:H7 was passed through an immunoconcentrator device. Following capture, devices were washed twice with 5 ml PBS. Captured bacteria were calculated by subtracting the number of bacteria that were not captured from the input. Captured bacteria amplified *in-situ* after wash step were analyzed by ELISA.

Figure 26 is a graph showing the results of capture of *E. coli* O157:H7 onto a polyester matrix and subsequent enrichment. 25 grams ground beef stomached in 225 ml Buffered Peptone Water solution was spiked with 136 or 0 CFU *E. coli* O157:H7, filtered through a SPUNTECH ™ filter and passed through an immunoconcentrator containing IgG-polyester matrix. Following capture, devices were washed twice with 5 ml PBS-TWEEN™ 20 and then captured bacteria were enriched using 150 µl mEC media for 5 hours at 37 °C and were analyzed by ELISA.

Figures 27a-c are cross sectional views of three preferred embodiments of prefiltration devices according to the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention encompasses methods for concentrating particular microorganism or microorganisms of interest from a sample. The methods of the present invention are effected by contacting the sample with a matrix to which is bound an affinity receptor specific for the microorganism(s). Advantageously, according to the present methods, the sample can be a sample having ultra low level(s) of microorganism(s) with as few as 0.00025, 0.001, 0.008 or <1 CFU/ml or as many as 10^3 CFU/ml of the microorganism(s) of interest. One advantage of the methods according to the present invention is that these methods obviate the need for a prolonged (e.g., > 8 or 10 hours, overnight) pre-enrichment step, and in some cases obviates the need for any pre-enrichment, of the microorganism(s) prior to their concentration by the method. The invention further relates to a prefiltration method and device, which are advantageously used in combination with the concentration methods of the present invention, as further detailed hereinunder.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of the components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

Matrix selection:

Any matrix can be employed according to the present invention for concentrating a particular microorganism or microorganisms of interest. A matrix according to the present invention is selected such that in combination with an appropriate affinity receptor which is capable of specifically binding the microorganism, which affinity receptor is boundable to the matrix, an affinity concentrator device is obtainable, which device efficiently captures the particular microorganism(s) even if present in a very low level in the analyzed sample, thereby obviating the need for prolonged pre-enrichment steps prior to concentration. It will be appreciated that affinity receptors, antibodies in particular, can be readily immobilized onto a variety of natural and synthetic matrices. Further considerations for matrix selection include (i) the ability of the matrix to efficiently pass large volumes of particulated liquid without clogging; and (ii) low or no inherent affinity for the microorganism(s) in the sample being tested.

It will be appreciated that having these criteria in mind, one ordinarily skilled in the art would be able to select and test the performance of a variety of matrices and to experimentally or rationally conclude whether a specific matrix is operable and characterized as described herein.

For example, the ordinary artisan will select a matrix (i) to which an antibody can be directly or indirectly immobilized; (ii) which is not clogged upon passage of a large volumes (e.g., 2 ml - 10 liters) of a particulated liquid, such as a solution including particulated meat; and (iii) which has low or substantially no inherent affinity for the microorganism(s) in the sample being tested.

Thus, a matrix operable with the microorganism(s) concentration methods of the present invention include both natural and synthetic matrices. Examples of natural matrices operable with the microorganism(s) concentration methods of the present invention include polysaccharide

matrices, such as, but not limited to, a cellulosic matrix, an agarose matrix, a chitin matrix, a starch matrix and derivatives thereof. The matrix according to the present invention can also be a coated matrix. A coated matrix includes a structural core and a coat to which the affinity receptor is bound (immobilized). Examples of synthetic matrices operable with the microorganism(s) concentration methods of the present invention include synthetic polymers, such as, but not limited to, a polypropylene matrix, polyester matrix, a polyamide matrix, a polyethylene matrix, an acrylamide matrix, a methacrylate matrix, a sepharose matrix, a polystyrene matrix and matrices which are derivatives of the above matrices. Methods of binding affinity receptors, such as antibodies or other peptides, to any of the above matrices are well known in the art and can include, as further detailed hereinunder binding via a coupled counterpart moiety having specific affinity to the matrix or component thereof, binding via covalent interactions and/or binding via what is known in the art as non-specific interactions. Examples are provided hereinunder, preferred protocols are provided in a plurality of laboratory manuals, including, G.T. Hermanson et al. (1992) "Immobilized Affinity Ligand Techniques", Academic Press, San Diego. Further detail relating to synthetic matrices such as polyester are found in U.S. Patent No. 5,196,757, which is incorporated herein by reference.

The matrix employed with the microorganism(s) concentration methods of the present invention can be of a plurality of forms, including, but not limited to beads, threads, a cloth, a woven material, a non-woven material, a membrane, a powder, a foam or a sponge. Such forms are well known in the art for both synthetic and natural matrices.

It is appreciated that for most applications non-porous matrices are preferred since such matrices are known to have low or no affinity to bacteria and are also less prone to clogging. However, porous matrices having pours which are small enough so as to prevent the entry of bacteria thereto, e.g., pours in the nanometer range, can be readily employed to effect the microorganism(s) concentration methods of the present invention.

Preferred matrices according to the present invention include those matrices to which a moiety having affinity thereto is known. Such a moiety can be conjugated or fused to the affinity receptor to thereby assist in binding (immobilizing) the affinity receptor to the matrix. Such a moiety, which is referred to herein also as a counter moiety is preferably a matrix

binding peptide, such as, but not limited to, and as further detailed hereinunder, a polysaccharide binding protein or domain.

According to preferred embodiments of the present invention the matrix used in the microorganism concentration methods are preferably made of cellulosic or chitin material. The cellulosic material can be in any of a variety of forms, for example, but not limited to, crystalline cellulose, such as AVICELL™ (FMC Corp., Philadelphia, PA), porous and non-porous cellulose beads and bacterial microcrystalline cellulose; a mix of crystalline and amorphous cellulose such as cellulose sponges, cotton fibers or "balls", and cotton or cellulose gauze; non-woven and woven viscose fibers, or amorphous cellulose such as phosphoric acid swollen cellulose and other solid matrices coated with cellulose or cellulose derivatives such as cellulose acetate or ethyl hydroxyl ethyl cellulose ("EHEC"), etc. The chitin material can be, for example, but not limited to, granular chitin from crab shells (Sigma Chemical Co., St. Louis, MO) or chitin beads. Most preferably, the cellulosic or chitin matrix is a material that will not clog upon filtering large volumes of sample.

Matrix binding peptides:

As used herein in the specification and in the claims section below, a "matrix binding peptide" includes peptides e.g., proteins and domains (portions) thereof, which are capable of affinity binding to the matrix of choice. The phrase thus includes, for example, peptides screened for their matrix binding activity out of a library, such as a peptide library or a DNA library (e.g., a cDNA expression library or a display library). Yet, the phrase also includes peptides designed and engineered to be capable of binding to a matrix and/or units thereof.

Such peptides include amino acid sequences that are derived from a matrix binding region, of e.g., a polysaccharide binding protein or a polysaccharide binding domain e.g., of a polysaccharidase. The polysaccharide binding peptide can include any amino acid sequence or a glycoprotein derivative thereof which binds to an oligosaccharide polymer, for example, the polysaccharide binding domain or protein can be derived from a polysaccharidase, a binding domain of a polysaccharide binding protein or a protein screened for and isolated from a peptide library or a protein designed and engineered to be capable of binding to a polysaccharide or saccharide units thereof. The polysaccharide binding domain or protein can be naturally occurring or synthetic. Suitable polysaccharidases from which a polysaccharide binding domain or protein

may be obtained include β-1,4-glucanases. In a preferred embodiment, a polysaccharide binding domain or protein from a cellulase or scaffoldin is used. Typically, the amino acid sequence is essentially lacking in the hydrolytic activity of the polysaccharidase, but retains the substrate binding activity. The amino acid sequence preferably has less than about 10 % of the hydrolytic activity of the native polysaccharidase; more preferably less than about 5 %, and most preferably less than about 1 % of the hydrolytic activity of the native polysaccharidase, ideally no activity altogether.

The polysaccharide binding domain or protein can be obtained from a variety of sources, including enzymes and other proteins which bind to polysaccharides which find use in the subject invention.

In Table 5 below are listed those binding domains which bind to one or more soluble/insoluble polysaccharides including all binding domains with affinity for soluble glucans (α, β, and/or mixed linkages). The N1 cellulose-binding domain from endoglucanase CenC of *C. fimi* is the only protein known to bind soluble cellosaccharides and one of a small set of proteins which are known to bind any soluble polysaccharides. Also, listed in Tables 1 to 4 are examples of proteins containing putative β -1,3-glucan-binding domains (Table 1); proteins containing Streptococcal glucan-binding repeats (Cpl superfamily) (Table 2); enzymes with chitin-binding domains (Table 3), and starch-binding domains (Table 4). Scaffoldin proteins which include a cellulose binding domain protein such as that produced by *Clostridium cellulovorans* (Shoseyov *et al.*, PCT/US94/04132) can also be used for preparing a polysaccharide binding domain or protein. Several fungi, including *Trichoderma* species and others, also produce polysaccharidases from which polysaccharide binding domains or proteins can be isolated. Additional examples can be found in, for example, Microbial Hydrolysis of Polysaccharides, R. A. J. Warren, Annu. Rev. Microbiol. 1996, 50:183-212; and "Advances in Microbial Physiology" R. K. Poole, Ed., 1995, Academic Press Limited, both are incorporated by reference as if fully set forth herein. In addition, U.S. Pat. No. 5,643,758 teaches a maltose binding protein capable of binding to the polysaccharide α-amylose.

Table 1

Overview of proteins containing putative β-1,3 glucan-binding domains

Source (strain)	Protein	accession No.	Ref[1]
Type I			
B. circulans (WL-12)	GLCA1	P23903/M34503/JQ0420	1
B. circulans (IAM 1165)	BglH	JN0772/D17519/S67033	2
Type II			
Actinomadura sp. (FC7)	XynII	U08894	3
Arthrobacter sp. (YCWD3)	GLCI	D23668	9
O. xanthineolytica	GLC	P22222/M60826/A39094	4
R. faecitabidus (YLM-50)	RP I	Q05308/A45053/D10753	5a,b
R. communis	Ricin	A12892	6
S. lividans (1326)	XlnA	P26514/M64551/JS07986	7
T. tridentatus	FactorGa	D16622	8

B. : *Bacillus*, *O.* : *Oerskovia*, *R. faecitabidus* : *Rarobacter faecitabidus*, *R. communis*: *Ricinus communis*, *S.* : *Streptomyces*, *T.* : *Tachypleus* (Horseshoe Crab)

[1]**References:**

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3) Harpin *et al.* (1994) *EMBL Data Library*
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5b) Shimoi *et al.* (1992) *J. Biochem* **110**, 608-613
6) Horn *et al.* (1989) Patent A12892
7) Shareck *et al.* (1991) *Gene* **107**, 75-82
8) Seki *et al.* (1994) *J. Biol. Chem.* **269**, 1370-1374
9) Watanabe *et al.* (1993) *EMBL Data Library*

Table 2

Overview of proteins containing Streptococcal glucan-binding repeats (Cpl superfamily)

Source	Protein	Accession No.	Ref.[2]
S. downei (sobrinus) (0MZ176)	GTF-I	D13858	1
S. downei (sobrinus) (MFe28)	GTF-I	P11001/M17391	2
S. downei (sobrinus) (MFe28)	GTF-S	P29336/M30943/A41483	3
S. downei (sobrinus) (6715)	GTF-I	P27470/D90216/A38175	4
S. downei (sobrinus)	DEI	L34406	5
S. mutants (Ingbritt)	GBP	M30945/A37184	6
S. mutants (GS-5)	GTF-B	A33128	7
S. mutants (GS-5)	GTF-B	P08987/M17361/B33135	8
S. mutants	GTF-B$^{3'}$-ORF	P05427/C33135	8
S. mutants (GS-5)	GTF-C	P13470/M17361/M22054	9
S. mutants (GS-5)	GTF-C	not available	10
S. mutants (GS-5)	GTF-D	M29296/A45866	11
S. salivarius	GTF-J	A44811/S22726/S28809 Z11873/M64111	12
S. salivarius	GTF-K	S22737/S22727/Z11872	13
S. salivarius (ATCC25975)	GTF-L	L35495	14
S. salivarius (ATCC25975)	GTF-M	L35928	14
S. pneumoniae R6	LytA	P06653/A25634/M13812	15
S. pneumoniae	PspA	A41971/M74122	16
Phage HB-3	HBL	P32762/M34652	17
Phage Cp-1	CPL-1	P15057/J03586/A31086	18
Phage Cp-9	CPL-9	P19386/M34780/JQ0438	19
Phage EJ-1	EJL	A42936	20
C. difficile (VPI 10463)	ToxA	P16154/A37052/M30307 X51797/S08638	21
C. difficile (BARTS W1)	ToxA	A60991/X17194	22
C. difficile (VPI 10463)	ToxB	P18177/X53138/X60984 S10317	23,24
C. difficile (1470)	ToxB	S44271/Z23277	25,26
C. novyi	a-toxin	S44272/Z23280	27
C. novyi	a-toxin	Z48636	28
C. acetobutylicum (NCIB8052)	CspA	S49255/Z37723	29
C. acetobutylicum (NCIB8052)	CspB	Z50008	30
C. acetobutylicum (NCIB8052)	CspC	Z50033	30
C. acetobutylicum (NCIB8052)	CspD	Z50009	30

[2]References:

1) Sato *et al.* (1993) *DNA sequence* **4**, 19-27
2) Ferreti *et al.* (1987) *J. Bacteriol.* **169**, 4271-4278
3) Gilmore *et al.* (1990) *J. Infect. Immun.* **58**, 2452-2458

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24) von Eichel-Streiber *et al.* (1992) *Mol. Gen. Genet.* **233**, 260-268
25) Sartinger *et al.* (1993) *EMBL Data Library*
26) von Eichel-Streiber *et al.* (1995) *Mol. Microbiol.* **In Press**
27) Hofmann *et al.* (1993) *EMBL Data Library*
28) Hofmann *et al.* (1995) *Mol. Gen. Genet.* **In Press**
29) Sanchez *et al.* (1994) *EMBL Data Library*
30) Sanchez *et al.* (1995) *EMBL Data Library*

New polysaccharide binding domains or proteins with interesting binding characteristics and specificities can be identified and screened for in a variety of ways including spectroscopic (titration) methods such as: NMR spectroscopy (Zhu *et al.* Biochemistry (1995) 34:13196-13202, Gehring *et al.* Biochemistry (1991) 30:5524-5531), UV difference spectroscopy (Belshaw *et al.* Eur. J. Biochem. (1993) 211:717-724), fluorescence (titration) spectroscopy (Miller *et al. J. Biol. Chem.* (1983) 258:13665-13672), UV or fluorescence stopped flow analysis (De Boeck *et al.* Eur. J. Biochem. (1985) 149:141-415), affinity methods such as affinity electrophoresis (Mimura *et al.* J. chromatography (1992) 597:345-350) or affinity chromatography on immobilized mono or oligosaccharides, precipitation or agglutination analysis including turbidimetric or nephelometric analysis (Knibbs *et al.* J. Biol. Chem. (1993) 14940-14947), competitive inhibition assays (with or without quantitative IC50 determination) and various physical or physico-chemical methods including differential scanning or isothermal titration calorimetry (Sigurskjold *et al. J. Biol. Chem.* (1992) 267:8371-8376; Sigurskjold *et al.* Eur. J. Biol. (1994) 225:133-141) or comparative protein stability assays (melts) in the absence or presence of oligo saccharides using thermal CD or fluorescence spectroscopy.

The K_a for binding of the polysaccharide binding domains or proteins to oligosaccharide is at least in the range of weak antibody-antigen extractions, i.e., $\geq 10^3$, preferably 10^4, most preferably 10^6 M^{-1}. If the binding of the polysaccharide binding domain or protein to the oligosaccharide is exothermic or endothermic, then binding will increase or decrease, respectively, at lower temperatures, providing a means for temperature modulation of the immobilization step.

Table 3
Overview of enzymes with chitin-binding domains

Source (strain)	Enzyme	Accession No.	Ref.[3]
Bacterial enzymes			
Type I			
Aeromonas sp. (No10S-24)	Chi	D31818	1
Bacillus circulans (WL-12)	ChiA1	P20533/M57601/A38368	2
Bacillus circulans (WL-12)	ChiD	P27050/D10594	3
Janthinobacterium lividum	Chi69	U07025	4
Streptomyces griseus	ProteaseC	A53669	5
Type II			
Aeromonas cavia (K1)	Chi	U09139	6
Alteromonas sp (0-7)	Chi85	A40633/P32823/D13762	7
Autographa californica (C6)	NPH-128[a]	P41684/L22858	8
Serratia marcescens	ChiA	A25090/X03657/L01455/P07254	9
Type III			
Rhizopus oligosporus (IFO8631)	Chi1	P29026/A47022/D10157/S27418	10
Rhizopus oligosporus (IFO8631)	Chi2	P29027/B47022/D10158/S27419	10
Saccharomyces cerevisiae	Chi	S50371/U17243	11
Saccharomyces cerevisiae (DBY939)	Chi1	P29028/M74069	12
Saccharomyces cerevisiae (DBY918)	Chi2	P29029/M7407/B41035	12
Plant enzymes			
Hevein superfamily			
Allium sativum	Chi	M94105	13
Amaranthus caudatus	AMP-1[b]	P27275/A40240	14, 15
Amaranthus caudatus	AMP-2[b]	S37381/A40240	14, 15
Arabidopsis thaliana (cv. colombia)	ChiB	P19171/M38240/B45511	16
Arabidopsis thaliana	PHP[c]	U01880	17

Brassica napus	Chi	U21848	18
Brassica napus	Chi2	Q09023/M95835	19
Hevea brasiliensis	Hev1[d]	P02877/M36986/A03770/A38288	20, 21
Hordeum vulgare	Chi33	L34211	22
Lycopersicon esculentum	Chi9	Q05538/Z15140/S37344	23
Nicotiana tabacum	CBP20[e]	S72424	24
Nicotiana tabacum	Chi	A21091	25
Nicotiana tabacum (cv. Havana)	Chi	A29074/M15173/S20981/S19855	26
Nicotiana tabacum (FB7-1)	Chi	JQ0993/S0828	27
Nicotiana tabacum (cv. Samsun)	Chi	A16119	28
Nicotiana tabacum (cv. Havana)	Chi	P08252/X16939/S08627	27
Nicotiana tabacum (cv. BY4) 26,27,29	Chi	P24091/X51599/X64519//S13322	
Nicotiana tabacum (cv. Havana)	Chi	P29059/X64518/S20982	26
Oryza sativum (IR36)	ChiA	L37289	30
Oryza sativum	ChiB	JC2253/S42829/Z29962	31
Oryza sativum	Chi	S39979/S40414/X56787	32
Oryza sativum (cv. Japonicum)	Chi	X56063	33
Oryza sativum (cv. Japonicum)	Chi1	P24626/X54367/S14948	34
Oryza sativum	Chi2	P25765/S15997	35
Oryza sativum (cv. Japonicum)	Chi3	D16223	
Oryza sativum	ChiA	JC2252/S42828	30
Oryza sativum	Chi1	D16221	32
Oryza sativum (IR58)	Chi	U02286	36
Oryza sativum	Chi	X87109	37
Pisum sativum (cv. Birte)	Chi	P36907/X63899	38
Pisum sativum (cv. Alcan)	Chi2	L37876	39
Populus trichocarpa	Chi	S18750/S18751/X59995/P29032	40
Populus trichocarpa (H11-11)	Chi	U01660	41
Phaseolus vulgaris (cv. Saxa)	Chi	A24215/S43926/Jq0965/P36361	42
Phaseolus vulgaris (cv. Saxa)	Chi [f]	P06215/M13968/M19052/A25898	43,44,45
Sambucus nigra	PR-3	Z46948	46
Secale cereale	Chi	JC2071	47
Solanum tuberosum	ChiB1	U02605	48
Solanum tuberosum	ChiB2	U02606	48
Solanum tuberosum	ChiB3	U02607/S43317	48
Solanum tuberosum	ChiB4	U02608	48
Solanum tuberosum (cv. Maris Piper)	WIN-1[g]	P09761/X13497/S04926	49
Solanum tuberosum (cv. Maris Piper)	WIN-2[g]	P09762/X13497/S04927	49
Triticum aestivum	Chi [h]	S38670/X76041	50
Triticum aestivum	WGA-1$_h$	P10968/M25536/S09623/S07289	51,52
Triticum aestivum	WGA-2$_h$	P02876/M25537/S09624	51,53
Triticum aestivum	WGA-3	P10969/J02961/S10045/A28401	54
Ulmus americana (NPS3-487)	Chi [i]	L22032	55
Urtica dioica	AGL	M87302	56
Vigna unguiculata (cv. Red caloona)	Chi1	X88800	57

[a]NHP : nuclear polyhedrosis virus endochitinase like sequence; Chi : chitinase, [b]anti-microbial peptide, [c]pre-hevein like protein, [d]hevein, [e]chitin-binding protein, [f]pathogenesis related protein, [g]wound-induced protein, [h]wheat germ agglutinin, [i]agglutinin (lectin).

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Table 4

Overview of enzymes containing starch-binding domains

Source (strain)	Enzyme	Accession No.	Ref.[4]
A. awarori (var. *kawachi*)	AMYG	P23176/D00427/JT0479	1, 2
A. niger (T21)	AMYG	S73370	3
A. niger -A. awamori	AMYG1/G2	P04064/A90986/A29166/X00712/ X00548	4,5,6
		K02465	7,8,9
A. oryzae	AMYG (GLAA)	P36914/JQ1346/D01035/S75274/ D01108	10, 11
A. Shirousamii	AMYG (GLA)	P22832/JQ0607/D10460	12
Bacillus sp. (B1018)	AMY[a]	P17692/M33302/D90112/S09196	13
Bacillus sp. (TS-23)	a-AMY	U22045	14
Bacillus sp. (1-1)	CGT	P31746/S26399	15
Bacillus sp. (6.63)	CGT	P31747/X66106/S21532	16
Bacillus sp. (17-1)	CGT	P30921/M28053/A37208	17
Bacillus sp. (38-2)	CGT	P09121/M19880/D00129/S24193	18, 19
Bacillus sp. (1011)	CGT	P05618/A26678/M17366	20
Bacillus sp. (DSM5850)	CGT	A18991	21
Bacillus sp. (KC 201)	CGT	D13068	15, 22
B. cereus (SPOII)	b-AMY	A48961/P36924/S54911	23
B. circulans (8)	CGT	P30920/X68326/S23674	24
B. circulans (251)	CGT	X78145	25
B. Licheniformis	CGTA	P14014/X15752/S15920	26
B. macerans (IFO 3490)	CGTM (CDG1)	P04830/X5904/S31281	27
B. macerans (IAM 1243)	CGT	M12777	28
B. macerans	CGT (CDG2)	P31835/S26589	29
B. ohbensis	CGT	P27036/D90243	30
B. stearothermophilus	AMYM[b]	P19531/M36539/S28784	31
B. stearothermophilus (NO2)	CGT	P31797/X59042/S26588/X59043/ X59404/S31284	32
C. rolfsii (AHU 9627)	AMYG2	D49448	33
D. discoideum	ORF	S15693/X51947	34
H. grisea (var. *thermoidea*)	GLA1	M89475	35
H. resinae (ATCC20495)	GAMP	Q03045/X68143/X67708/S31422/ S33908	36-38
K. pneumoniae (M5A1)	CGT	P08704/M15264/A29023	39
N. crassa (74-OR23-1A)	GLA-1	P14804/X67291/S13711/S13710/ S36364	40, 41
P. saccharophila (IAM1504)	MTA[c]	P22963/X16732/S05667	42
Pseudomonas sp. (KO-8940)	AMF-1[d]	D10769/JS0631/D01143	43
P. stutzeri (MO-19)	AMYP[c]	P13507/M24516/A32803	44
S. griseus (IMRU 3570)	AMY	P30270/X57568/S14063	45
S. limosus (S.albidoflavus)	AML	P09794/ M18244/B28391	46
S. violaceus (S. venezuela) (ATCC15068)	AML	P22998/M25263/JS0101	47
Th. curvata (CCM 3352)	TAM[e]	P29750/X59159/JH0638	48
Th. thermosulfurogenes [f] (DSM3896/EM1)	AMYA	P26827/X54654/X54982/ S17298/S37706	49
Th. thermosulfurogenes (ATCC 33743)	AMYB	P19584/M22471/A31389	50

[a]Raw-starch digesting amylase, [b]Maltogenic α-amylase, [c]Maltotetraose-forming amylase (1,4-α-maltotetrahydrolase), [d]Maltopentaose-forming amylase, [e]thermostable α-amylase, [f]formerly *Clostridium thermosulfurogenes*. AMYG, GAM and GLA: glucoamylase, AMY or AML: alpha-amylase, CGT: β-cyclodextrin glycosyltransferase or cyclomaltodextrin glucanotransferase, ORF: open reading frame. *A.*: *Aspergillus*, *B.*: *Bacillus*, *C.*: *Corticium*, *D.*: *Dictiostelium*, *H. grisea*: *Humicola grisea*, *H. resinea: Hormoconis resinae* (*Amorphotheca resinae*), *K.*: *Klebsiella*, *N.*: *Neurospora*, *S.*: *Streptomyces*, *Th. curvata: Thermomonospora curvata*, *Th.*: *Thermoanaerobacter*.

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Table 5
Sources of polysaccharide binding domains

Binding Domain	Proteins Where Binding Domain is Found
Cellulose Binding Domains[1]	β-glucanases (avicelases, CMCases, cellodextrinases) exoglucanses or cellobiohydrolases cellulose binding proteins xylanases mixed xylanases/glucanases esterases chitinases β-1,3-glucanases β-1,3-(β-1,4)-glucanases (β-)mannanases β-glucosidases/galactosidases cellulose synthases (unconfirmed)
Starch/Maltodextrin Binding Domains	α-amylases[2,3] β-amylases[4,5] pullulanases glucoamylases[6,7] cyclodextrin glucotransferases[8-10] (cyclomaltodextrin glucanotransferases) maltodextrin binding proteins[11]
Dextran Binding Domains	*(Streptococcal)* glycosyl transferases[12] dextran sucrases (unconfirmed) *Clostridial* toxins[13,14] glucoamylases[6] dextran binding proteins
β-Glucan Binding Domains	β-1,3-glucanases[15,16] β-1,3-(β-1,4)-glucanases (unconfirmed) β-1,3-glucan binding protein
Chitin Binding Domains	chitinases chitobiases chitin binding proteins (see *also* cellulose binding domains) Heivein

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Once the most appropriate polysaccharide binding peptide for a particular application has been identified, the polysaccharide binding protein or domain can be prepared by transforming into a host cell a DNA construct comprising DNA encoding the appropriate polysaccharide binding moiety. The phrase "polysaccharide binding peptide" intends an amino acid sequence which comprises at least a functional portion of the polysaccharide binding region of a polysaccharidase or a polysaccharide binding protein. The phrase further relates to glycoprotein derivatives of such amino acid sequences. The phrase further relates to a polypeptide screened for its polysaccharide binding activity out of a library, such as a peptide library or a DNA library (e.g., a cDNA library or a display library). By "functional portion" is intended an amino acid sequence which binds to an oligosaccharide polymer of interest.

The techniques used in isolating polysaccharidase genes, such as a cellulase gene, and genes for polysaccharide binding proteins are known in the art, including synthesis, isolation from genomic DNA, preparation from cDNA, or combinations thereof. (See, U.S. Pat. Nos. 5,137,819; 5,202,247; and 5,340,731) The sequences for several polypeptide binding domains, which bind to soluble oligosaccharides are known (See, Figure 1 of PCT/CA97/00033, WO 97/26358). The DNAs coding for a variety of polysaccharidases and polysaccharide binding proteins are also known. Various techniques for manipulation of genes are well known, and include restriction, digestion, resection, ligation, *in vitro* mutagenesis, primer repair, employing linkers and adapters, and the like (see Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989).

The amino acid sequence of a polysaccharidase also can be used to design a probe to screen a cDNA or a genomic library prepared from mRNA or DNA from cells of interest as donor cells for a

polysaccharidase gene or a polysaccharide binding protein gene. By using the polysaccharidase cDNA or binding protein cDNA or a fragment thereof as a hybridization probe, structurally related genes found in other microorganisms can be easily cloned. Particularly contemplated is the isolation of genes from organisms that express polysaccharidase activity using oligonucleotide probes based on the nucleotide sequences of genes obtainable from an organism wherein the catalytic and binding domains of the polysaccharidase are discrete, although other polysaccharide binding proteins also can be used (see, for example, Shoseyov, *et al.*, Proc. Nat'l. Acad. Sci. (USA) (1992) 89:3483-3487).

Probes developed using consensus sequences for the binding domain of a polysaccharidase or polysaccharide-binding protein are of particular interest. The β-1,4-glycanases from *C. fimi* characterized to date are endoglucanases A, B, C and D (CenA, CenB, CenC and CenD, respectively), exocellobiohydrolases A and B (CbhA and CbhB, respectively), and xylanases A and D (Cex and XylD, respectively) (see Wong *et al.* (1986) Gene, 44:315; Meinke *et al.* (1991) J. Bacteriol., 173:308; Coutinho *et al.*, (1991) Mol. Microbiol. 5:1221; Meinke *et al.*, (1993) Bacteriol., 175:1910; Meinke *et al.*, (1994) Mol. Microbiol., 12:413; Shen *et al.*, Biochem. J., in press; O'Neill *et al.*, (1986) Gene, 44:325; and Millward-Sadler *et al.*, (1994) Mol. Microbiol., 11:375). All are modular proteins of varying degrees of complexity, but with two features in common: a catalytic domain (CD) and a cellulose-binding domain (CBD) which can function independently (see Millward-Sadler *et al.*, (1994) Mol. Microbiol., 11:375; Gilkes *et al.*, (1988) J. Biol. Chem., 263:10401; Meinke *et al.*, (1991) J. Bacteriol., 173:7126; and Coutinho *et al.*, (1992) Mol. Microbiol., 6:1242). In four of the enzymes, CenB, CenD, CbhA and CbhB, fibronectin type III (Fn3) repeats separate the N-terminal CD from the C-terminal CBD. The CDs of the enzymes come from six of the families of glycoside hydrolases (see Henrissat (1991) Biochem. J., 280:309; and Henrissat *et al.*, (1993) Biochem. J., 293:781); all of the enzymes have an N- or C-terminal CBD or CBDs (see Tomme *et al.*, Adv. Microb. Physiol., in press); CenC has tandem CBDs from family IV at its N-terminus; CenB and XylD each have a second, internal CBD from families III and II, respectively. Cex and XylD are clearly xylanases; however, Cex, but not XylD, has low activity on cellulose. Nonetheless, like several other bacterial xylanases (see Gilbert *et al.*, (1993) J. Gen. Microbiol., 139:187), they have CBDs. *C. fimi*

probably produces other β-1,4-glycanases. Similar systems are produced by related bacteria (see Wilson (1992) Crit. Rev. Biotechnol., 12:45; and Hazlewood *et al.*, (1992) J. Appl. Bacteriol., 72:244). Unrelated bacteria also produce glycanases; *Clostridium thermocellum,* for example, produces twenty or more β-1,4-glycanases (see Beguin *et al.*, (1992) FEMS Microbiol. Lett., 100:523). The CBD derived from *C. fimi* endoglucanase C N1, is the only protein known to bind soluble cellosaccharides and one of a small set of proteins that are known to bind any soluble polysaccharides.

Examples of suitable binding domains are shown in Figure 1 of PCT/CA97/00033 (WO 97/26358), which presents an alignment of binding domains from various enzymes that bind to polysaccharides and identifies amino acid residues that are conserved among most or all of the enzymes. This information can be used to derive a suitable oligonucleotide probe using methods known to those of skill in the art. The probes can be considerably shorter than the entire sequence but should at least be 10, preferably at least 14, nucleotides in length. Longer oligonucleotides are useful, up to the full legnth of the gene, preferably no more than 500, more preferably no more than 250, nucleotides in length. RNA or DNA probes can be used. In use, the probes are typically labeled in a detectable manner, for example, with ^{32}P, ^{3}H, biotin, avidin or other detectable reagents, and are incubated with single-stranded DNA or RNA from the organism in which a gene is being sought. Hybridization is detected by means of the label after the unhybridized probe has been separated from the hybridized probe. The hybridized probe is typically immobilized on a solid matrix such as nitrocellulose paper. Hybridization techniques suitable for use with oligonucleotides are well known to those skilled in the art. Although probes are normally used with a detectable label that allows easy identification, unlabeled oligonucleotides are also useful, both as precursors of labeled probes and for use in methods that provide for direct detection of double-stranded DNA (or DNA/RNA). Accordingly, the term "oligonucleotide probe" refers to both labeled and unlabeled forms.

Generally, the binding domains identified by probing nucleic acids from an organism of interest will show at least about 40 % identity (including as appropriate allowances for conservative substitutions, gaps for better alignment and the like) to the binding region or regions from which the probe was derived and will bind to a soluble β-1,4 glucan with

a K_a of $\geq 10^3$ M^{-1}. More preferably, the binding domains will be at least about 60 % identical, and most preferably at least about 70 % identical to the binding region used to derive the probe. The percentage of identity will be greater among those amino acids that are conserved among polysaccharidase binding domains. Analyses of amino acid sequence comparisons can be performed using programs in PC/Gene (IntelliGenetics, Inc.). PCLUSTAL can be used for multiple sequence alignment and generation of phylogenetic trees.

In order to isolate the polysaccharide binding protein or a polysaccharide binding domain from an enzyme or a cluster of enzymes that binds to a polysaccharide, several genetic approaches can be used. One method uses restriction enzymes to remove a portion of the gene that codes for portions of the protein other than the binding portion thereof. The remaining gene fragments are fused with expression control sequences to obtain a mutated gene that encodes a truncated protein. Another method involves the use of exonucleases such as *Bal*31 to systematically delete nucleotides either externally from the 5' and the 3' ends of the DNA or internally from a restricted gap within the gene. These gene deletion methods result in a mutated gene encoding a shortened protein molecule which can then be evaluated for substrate or polysaccharide binding ability. Appropriate substrates for evaluating binding activity include those for the enzymes are listed in Tables 1-5 above, as well as the carbohydrates listed in Table 6 below.

Table 6

Uncharged Polysaccharides* Compounds	Charged Polysaccharides	Low Molecular Weight
Dextran	Na carboxymethyl dextran	Dextrins derived from cellulose (Cellotriose, cellotetraose, etc.)
Hydroxypropyl dextran	Na carboxymethyl xylotriose, etc.	Xylose, xylobiose, cellulose
Carboxymethyl dextran	Na dextran sulfate	Maltodextrins and derivatives
Maltodextrin	DEAE dextran	
Arabinogalactan	Polygalacturonic acid (pectin)	
Hydroxypropyl starch		
Amylopectin		
Methyl cellulose		
Hydroxyethyl cellulose		
Ethylhydroxyethyl cellulose		
Carboxymethyl cellulose		
Hydroxypropyl cellulose		
Ficoll		
Carboxymethyl starch		
Hydroxyethyl starch		
Pullulan		
Chitin		

* Polymers can be crude or purified.

According to preferred embodiments of the present invention a cellulose binding protein or cellulose binding domain is employed as a counterpart moiety having affinity to the matrix of choice and thus serves as a matrix binding peptide, as this term is defined hereinabove. Any cellulose binding protein or cellulose binding domain may be used in the present invention. The term "cellulose binding protein" ("CBP") refers to any protein, polypeptide or peptide including a glycoprotein, which specifically binds to cellulose or hemicellulose or chitin. The cellulose binding protein may or may not have cellulose or cellulolytic activity. The term "cellulose binding domain" ("CBD") refers to any protein, polypeptide or peptide, including a glycoprotein, which is a region or portion of a larger protein, said region or portion which binds specifically to cellulose or hemicellulose or chitin. The cellulose binding domain (CBD) may be a part or portion of a cellulase, xylanase or other polysaccharidase, e.g., a chitinase, etc., a sugar binding protein such as maltose binding protein, or scaffoldin such as CbpA of *Clostridium celluvorans, etc.* Many cellulases and hemicellulases (e.g. xylanases and mannases) have the ability to associate

with their substrates. These enzymes typically have a catalytic domain containing the active site for substrate hydrolysis and a carbohydrate-binding domain or cellulose-binding domain (herein generally designated "CBD") for binding the insoluble cellulosic or hemicellulosic matrices. The CBD may also be from a non-catalytic polysaccharide binding protein. To date, more than one hundred cellulose-binding domains (CBDs) have been classified into at least thirteen families designated I-XIII (Tomme et al., 1995, "CelluloseBinding Domains: Classification and Properties", in ACS Symposium Series 618 Enzymatic Degradation and Insoluble Carbohydrates, pp. 142-161, Saddler and Penner eds., American Chemical Society, Washington, D.C. (Tomme I) ; Tomme et al., 1995, Adv. Microb. Physiol, 37:1 (Tomme II); and Smant et al., 1998, Proc. Natl. Acad. Sci U.S.A. 95:4906,-4911) (incorporated herein by reference). Any of the CBDs described in Tomme I or II or any variants thereof, any other presently known CBDs or any new CBDs which may be identified can be used in the present invention. As an illustrative, but in no way limiting example, the CBP or CBD can be from a bacterial, fungal, slime mold, or nematode protein or polypeptide. For a more particular illustrative example, the CBD is obtainable from *Clostridium cellulovorans, Clostridium cellulovorans, or Cellulomonas fimi* (e.g., CenA, CenB, CenD, Cex). In addition, the CBD may be selected from a phage display peptide or peptidomimetic library, random or otherwise, using cellulose or any other polysaccharide or other matrix as a screening agent. (See Smith, 1985, Science 228:1315-1317 and Lam, 1991, Nature 354:82-84). Furthermore, the CBD may be derived by mutation of a portion of a protein, polypeptide or peptide, including a glycoprotein, which binds to a polysaccharide other than cellulose (or hemicellulose), such as a chitinase, which specifically binds chitin, or a sugar binding protein such as maltose binding protein, rendering said portion capable of binding to cellulose. In any event, the CBD binds cellulose or hemicellulose. Shoseyov and Doi (1990, Proc. Natl. Acad. Sci. USA 87:21.92-2195) isolated a unique cellulose-binding protein (CbpA) from the cellulose "complex" of the cellulolytic bacterium *Clostridium cellulovorans.* This major subunit of the cellulose complex was found to bind to cellulose, but had no hydrolytic activity, and was essential for the degradation of crystalline cellulose. The CbpA gene has been cloned and sequenced (Shoseyov et al., 1992, Proc. Natl. Acad. Sci. USA 89:3483-3487). Using PCR primers flanking the cellulose-binding domain (herein, this specific CBD is designated "cbd") of CbpA, the latter

was successfully cloned into an overexpression vector that enabled overproduction of the approximately 17 kDa CBD in *Escherichia* coli. The recombinant CBD exhibits very strong affinity to cellulose and chitin (U.S. Pat. No. 5,496,934; Goldstein et al., 1993, J. Bacteriol. 175:5762; PCT International Publication WO 94/24158, all are incorporated by reference as if fully set forth herein).

In recent years, several CBDs have been isolated from different sources. Most of these have been isolated from proteins that have separate catalytic, i.e., cellulose and cellulose binding domains, and only two have been isolated from proteins that have no apparent hydrolytic activity but possess cellulose-binding activity (Goldstein et al., 1993, J. Bacteriol. 175:5762-5768; Morag et al., 1995, Appl. Environ. Microbiol. 61-1980-1986). Greenwood et al. (1989, FEBS Lett. 224:127-131) fused the cellulose binding region of *Cellulomonas fimi* endoglucanase to the enzyme alkaline phosphatase. The recombinant fusion protein retained both its phosphatase activity and the ability to bind to cellulose. For more descriptions of cellulose binding fusion proteins, see U.S. Patent No. 5,137,819 issued to Kilburn et al., and U.S. Patent No. 5,719,044 issued to Shoseyov et al. both incorporated by reference herein.

Receptors and microorganisms:

As used herein, an "affinity receptor" (anti-ligand) includes any compound or composition which recognizes a particular spatial and/or polar organization of a ligand compound or composition, e.g., an epitopic or determinant site or a complementary binding site.

Illustrative receptors include, but are not limited to, immunoglobulins or antibodies or antigen binding portions thereof, such as Fv, F(abl)2, Fab fragments, single chain antibodies, chimeric or humanized antibodies, complementary determining regions of antibodies; protein A; protein G; protein L; enzymes and non-catalytic fragments thereof; protein receptors; avidin or streptavidin; lectin binding proteins; oligosaccharides; nucleic acids and polynucleotides; oligolipids; etc.

As will be understood easily by those skilled in the art, nucleic acids, oligonucleotides and polynucleotides which are complementary to one another can serve as receptor or anti-ligand and ligand in the methods of the present invention.

The receptors used in the present methods are capable of specifically or preferentially binding to a microorganism or microorganisms of interest.

The microorganisms that can be concentrated or concentrated and detected using the methods of the present invention include, but are not limited to, bacteria, viruses, fungi, protozoans, and nematodes.

For example, the bacteria can be *Escherichia* coli, *Salmonella, Campylobacter, Legionella, Clostridium, Pseudomonas, Listeria, Staphylococcus, Bacillus, Shigella, Mycobacteria, Bordetella, Streptococcus, Helicobacter,* etc.

The viruses include, for example, but are not limited to, viruses of the families: *Poxviridae, iridoviridae, Herpes viridae, Adenoviridae,* Papovarviridae, and Retroviridae, such as the Acquired Immune Deficiency Syndrome (AIDS) virus, etc.

Fungi which can be concentrated and detected by the present invention include, for example, *Aspergillus, Blastomyces, Candida* (such as yeast), *Coccidioides, Cryptococcus* and *Histoplasma,* etc.

The protozoan groups that can be detected by the present invention include, for example, *Rhizopoda* (e.g., amoeba such as *Entamoeba histolytica,* and *Dientamoeba fragilis*), *Mastigophora* (flagellates) (e.g., *Giardia larablia)*, *Ciliatea* (ciliates) (e.g. *Balantidium coli*) and *Sporozoa* (e.g., *Isospora, Cryptosporidium).*

For further examples of microorganisms, see, *Microbiology,* 4th ed., Davis et al., 1990, J.B. Lippincott Co., Philadelphia, PA; and *Fundamental Virology,* 3rd ed., 1996, Lippencott-Raven Publishers, Philadelphia, PA. See also, *Zinsser Microbiology* 19th ed., Joklik et al., 1988, Appleton & Lange, Norwalk, Conn., or any other available textbook on microbiology available to those skilled in the art.

Antibodies:

The present invention can utilize serum immunoglobulins, polyclonal antibodies or fragments thereof or monoclonal antibodies or fragments thereof. Preferably the antibodies are raised against a surface antigen of a microorganism. More preferably, the surface antigen is a flagellar protein or lipopolysaccharide. Preferably the antibodies are highly specific for the target microorganism and substantially do not react with other microorganisms found in sample being testing. Monoclonal antibodies or purified fragments of the monoclonal antibodies having at least a portion of an antigen binding region, including such as Fv, F(abl)2, Fab fragments (Harlow and Lane, 1988 Antibody, Cold Spring Harbor), single chain antibodies (U.S. Patent 4,946,778), chimeric or humanized antibodies (Morrison et al., 1984, Proc. Natl. Acad. Sci. USA 81:6851; Neuberger et

al., 1984, Nature 312:604-8) and complementarily determining regions (CDR) may be prepared by conventional procedures. Purification of these serum immunoglobulins antibodies or fragments can be accomplished by a variety of methods known to those of skill including, precipitation by ammonium sulfate or sodium sulfate followed by dialysis against saline, ion exchange chromatography, affinity or immunoaffinity chromatography as well as gel filtration, zone electrophoresis, etc. (see Goding in, Monoclonal Antibodies: Principles and Practice, 2nd ed., pp. 104-126, 1986, Orlando, Fla., Academic Press).

Binding (immobilizing) a receptor to a matrix:

Many methods have been used to couple receptors (binding proteins/peptides) to natural and synthetic matrices. These include chemical conjugation involving activation of matrix and/or ligand, streptavidin-biotin interactions, passive adsorption, and reversible and irreversible binding via CBD's and other saccharide binding proteins. Matrices can be natural supports, such as agarose, cellulose and controlled pore glass and silica. Matrices can also be synthetic supports such as acrylamide derivatives, methacrylate derivatives, polystyrene and its derivatives. Some examples of chemical and bio-interaction based coupling of peptides, antibodies and immunoglobulin binding proteins to various matrices follow.

Kohn J. and Wilchek M. (1982, Biochem. Biophys. Res. Commun. 107(3): 878-874) describes mechanisms of activation by CNBr which can be used for coupling to natural supports such as polysaccharide matrices as well as synthetic polymers that contain hydroxyl groups. Periodate oxidized cross linked agarose has been used to immobilize proteins such as Human IgG (Domen *et al.*, 1990, J. Chromatogr. 510: 293-302). Antibodies and their fragments can also be coupled by selectively reducing disulfides in the hinge region with 2-mercaptoethylamine to form free sulfhydryls which in turn can be immobilized to sulfhydryl-reactive supports designed to couple these groups such as a iodoacetyl activated agarose or other bromoacetyl, maleimide, divinylsulfone-activiated matrix of choice which may or may not be modified by spacer molecules (Hermanson G.T., Krishna Mallia A.K., Smith P.K. ("Immobilized Affinity Ligand Techniques", 1992, Academic Press, San Diego)).

N-hydroxy succinimide esters (NHS esters) can also be used to activate supports containing hydroxyl groups. The reagent, *N,N'*-disuccinimidyl carbonate (DSC) has been used for this purpose (Miron T. and Wilchek M., 1987, Methods Enzymol. 135: 84-90; Miron T. and

Wilchek M., 1993, Bioconjug. Chem. 4(6): 568-569; Wilchek M *et al.*, 1994, Bioconjug. Chem. 5(5): 491-492)

Although non covalent passive adsorption can be used to couple antibodies to porous and nonporous surfaces (Tijssen P. 1985, *In* "Practice and Theory of Enzyme Immunoassays," pp. 297. Elsevier, NY.) such as polystyrene and polystyrene/divinylbenzene, specific modification and coupling chemistry can provide covalently bound molecules. Electrophilic aromatic substitution reactions such as brief treatment with chlorosulphonic acid yield an amine-reactive surface for ligand immobilization. Hermanson G.T., Krishna Mallia A.K., Smith P.K. ("Immobilized Affinity Ligand Techniques, 1992, Academic Press, San Diego) summarized methods used to generate functional groups on polystyrene by nitration followed by reduction to an aromatic amine. The aromatic amine can then be converted into an isocyanate, isothiocyanate, or a diazonium salt for reaction with ligands.

Polyacrylamide beads have also been used to immobilize ligands. Hermanson G.T., Krishna Mallia A.K., Smith P.K. ("Immobilized Affinity Ligand Techniques, 1992, Academic Press, San Diego) summarized methods in which polyacrylamide can be activated by gluteraldehyde providing a multivalent aldehyde-containing intermediate useful for coupling amines via reductive amination. Other acrylamide derivatives include Trisacryl, Sephacryl, Ultrgel AcA and Azylactone which can also be used to immobilize ligands.

Methacrylate derivatives such as TSK-Gel Toyopearl resin (Tosoh Corp. Japan) contain numerous hydroxyl groups within their matrix structure which are easily activated by CNBr, *N,N'*-carbonyl diimidazole (CDI) (Hearn M.T.W., 1987, Methods Enzymol. 135: 102-117), tresyl, tosyl, epoxy and DVS. Alternatively, modification with glycidol followed by periodate oxidation can be used to couple amines of proteins. Other methacrylate derivatives that can be used to couple ligands include HEMA and Eupergit as reviewed by Hermanson G.T., Krishna Mallia A.K., Smith P.K. ("Immobilized Affinity Ligand Techniques, 1992, Academic Press, San Diego).

Many methods have been used to couple receptors (binding proteins/peptides) to cellulosic matrices. These include, for example, chemical conjugation, streptavidin-biotin interactions, passive adsorption, and reversible and irreversible binding via CBD's. Some examples of chemical coupling and, streptavidin-biotin interactions follow:

Ferrua B, Maiolini R, Masseyeff R, J. Immunol. Methods, 1979, 25(1):49-53 teach coupling of gamma-globulin to microcrytstalline cellulose by periodate oxidation.

Hadge, D. Allerg Immunol (Leipz) 1990;36(4):299-307 described a method in which 25 mg of bovine IgG (BGG) was coupled to packed bead cellulose activated by 5-norbornene-2.3-dicarboximido carbonochloridate to obtain BGG-immunosorbents with 4.6 to 4.9 mg BGG/ml matrix. The use of these immunosorbents for affinity chromatography resulted in the isolation of one milligram of pure rabbit anti-BGG antibodies by means of about 4.6 mg of BGG coupled to the cellulose.

Turkova J, Petkov L, Sajdok J, Kas J, Benes MJ, J. Chromatogr. 1990, Feb 2 (500):585-93 described a biospecific sorbent for the isolation of ovalbumin antibodies involving the preparation of coupled ovalbumin via its periodate-oxidized carbohydrate moiety to bead cellulose modified with adipic acid dihydrazide. The anti-ovalbumin IgG fraction isolated on this sorbent from immune rabbit serum contained only antibodies against protein determinants of ovalbumin. Thus, when these IgG were immobilized through their carbohydrate moieties to cellulose beads it became possible to prepare a biospecific sorbent for concanavalin A by oriented adsorption of ovalbumin. Ovalbumin was specifically adsorbed via its protein moiety and its carbohydrate part remained free for interaction with concanavalin A.

al-Abdulla IH, Mellor GW, Childerstone MS, Sidki AM, Smith DS, J. Immunol. Methods, 1989, Sep 1, 122(2):253-8 compared the periodate and 1,1'-carbonyldiimidazole activation methods with the cyanogen bromide procedure for coupling antibodies to magnetizable cellulose/iron oxide solid-phase particles. Fluoroimmunoassays for quinine, primaquine, normetanephrine and cannabinoids were employed to assess the binding properties of such coupled solid phases. The cyanogen bromide and 1,1'-carbonyldiimidazole methods gave similar products in most cases, while the specific binding capacity of periodate coupled particles was between two and five times lower. Nevertheless, comparable standard curves could be obtained with solid phase coupled by each method. The periodate and 1,1'-carbonyldiimidazole methods are acceptable alternatives, notably for laboratories lacking the facility to handle the toxic cyanogen bromide.

Metelitsa DI, Pliugacheva EI, Ermolenko IN, Liubliner IP, Kaputskii FN, Pratsenko VE, Prikl. Biokhim. Mikrobiol. 1992, Jul-Aug, 28(4):531-8 performed covalent binding of bovine liver catalase to phosphate-cellulose

matrices (gauze, granules, and paper with various surface density) and to acetate-cellulose porous membranes of different productivity. The capacity of the catalase binding to the molded cellulose carriers maximum concentration of the bound catalase after periodate oxidation of the carriers at room temperature was determined.

Englebretsen DR, Harding DR, Pept. Res. 1994, Nov-Dec, 7(6):322-6 used aminopropyl derivatized Perloza beaded cellulose acylated with alpha-bromoacetic anhydride to give alpha-bromo-acetamidopropyl Perloza. (N-Acetyl)-Cys-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, the 7 C-terminal amino acids of the decapeptide luteinizing hormone-releasing hormone with a cysteine added to the N-terminus, was synthesized using Fmoc chemistry. The purified peptide (1.35-1.9 eq) was coupled to alpha-bromoacetamidopropyl Perloza in 0.1 M $NaHCO_3$ solution, pH 8.3, for 1-2 hours. The peptide was anchored to the support via a thioether linkage. Analysis of the peptide-Perloza conjugate indicated near-quantitative displacement of support-bound bromine by the peptide. The peptidic affinity matrix was able to bind ovine antibodies to luteinizing hormone-releasing hormone (LHRH). Thioether immobilization offers directed, chemically stable, high-yield anchoring of synthetic peptides onto a chromatographic support. The high reaction efficiency means there is little waste of valuable synthetic peptide.

Beddows CG, Mirauer RA, Guthrie JT, Biotechnol. Bioeng. 1980, Feb, 22(2):311-21 teach immobilized beta-Galactosidase and other enzymes on p-amino-carbanilated derivatives of cellulose and methylol cellulose using the diazo method and through gluteraldehyde. The p-amino-carbanilated cellulose (CTAC) was used to immobilize beta galactosidase, glucose oxidase, trypsin, pepsin, and papain.

Le KD, Gilkes NR, Kilburn DG, Miller RC Jr, Saddler JN, Warren RA, Enzyme Microb. Technol. 1994, Jun, 16(6):496-500 produced a fusion protein, Sta-CBDCex, which comprises streptavidin with a cellulose-binding domain (CBDCex) fused to its C Terminus. This fused protein was produced in the cytoplasm of Escherichia coli, where it formed inclusion bodies. Renatured Sta-CBDCex, recovered from the inclusion bodies, adsorbed to AVICEL™, a microcrystalline cellulose. The cellulose-bound Sta-CBDCex in turn bound biotinylated alkaline phosphatase or biotinylated beta-glucosidase. The immobilized beta-glucosidase remained fully active during 2 weeks of continuous column operation at 50 °C.

Krysteva MA, Yotova LK, J. Chem. Technol. Biotechnol. 1992, 54(1):13-8 teach artificial multienzyme complexes which were prepared by covalent binding to polysaccharide structures which have been activated with urea and formaldehyde. In this manner double enzyme complexes of glucose oxidase and catalase, a glucose oxidase and invertase, have been prepared by immobilization onto cellulose fabric. The resulting multienzyme systems were highly active and stable, making them suitable for use in measuring the concentrations of glucose in solutions.

Krysteva MA, Blagov SR, Sokolov TT, J. Appl. Biochem. 1984, Oct-Dec, 6(5-6):367-73 teach a variation on the previous method in which cellulose and microcrystalline cellulose are treated consecutively with sodium periodate and urea. The interaction of urea derivatives with formaldehyde results in highly reactive groups, capable of further condensation with the amino acid residues of the proteins. The condensation of chymotrypsin, pepsin, and ovomucoid with such activated matrices has been studied in the pH interval 2 to 10. Differences have been found in the binding properties of basic and acid proteins. Satisfactory values have been obtained concerning the relative enzymatic and inhibitory activity of the immobilized products with respect to high- and low-molecular substrates. Chymotrypsin, immobilized on microcrystalline cellulose matrix, is found to manifest better catalytic properties compared with chymotrypsin immobilized on cellulose matrix. A probable sequence of the stages of chemical activation of the matrices and covalent binding of the proteins to them has been proposed. The main advantages of the proposed method consist of the high reactivity of the binding group in a wide pH range, its suitable length, and its easy synthesis.

Vlasov LG, Tolstykh PI, Ignatiuk TE, Razzakov ON, Antibiot Khimioter 1988 Nov, 33(11):848-50 teach the preparation of immobilized forms of lysozyme by its covalent binding on dialdehyde cellulose and polycaproamide fibers as woven and knitted fabrics respectively. The preparations were estimated by the content of protein and bacteriolytic activity. The lysozyme activity per 1 gram of the carrier and the protein content on dialdehyde cellulose were several times higher than those on polycaproamide while the specific activity of lysozyme on the polycaproamide carrier was somewhat higher than that on dialdehyde cellulose. The effect of the immobilized lysozyme in treatment of purulent wounds was studied on albino rats. It was shown that the periods of the wound healing with the use of the immobilized lysozyme were shorter than

those with the use of native lysozyme. Cytological and morphological investigation of the wound wall confirmed the higher efficacy of the lysozyme immobilized forms in treatment of purulent wounds as compared to the use of the native enzyme.

Conjugating or fusing a counter moiety having affinity to the matrix of choice to the affinity receptor:

Many methods are known in the art to conjugate or fuse (couple) molecules of different types, including peptides. These methods can be used according to the present invention to couple a counter moiety having affinity to the matrix of choice to the affinity receptor selected to recognize a particular microorganism to be concentrated from a sample, to thereby assist in binding (immobilizing) the affinity receptor to the matrix.

CBP or CBD or for that matter any other peptide can be conjugated or fused to a receptor using any conjugation method known to one skilled in the art. The peptide can be conjugated to a receptor, for example, an antibody of interest, using a 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (also called N-succinimidyl 3-(2pyridyldithio) propionate) ("SDPD") (Sigma, Cat. No. P-3415), a gluteraldehyde conjugation procedure or a carbodiimide conjugation procedure. The peptide can also be fused to a receptor using recombinant DNA techniques known to one skilled in the art to make fusion proteins, e.g., CBD or CBP fusion proteins.

SPDP conjugation:

Any SPDP conjugation method known to those skilled in the art can be used. For example, in one illustrative embodiment, a modification of the method of Cumber et al. (1985, Methods of Enzymology 112: 207-224) as described below, is used.

A peptide, such as CBD or CBP, (1.7 mg/ml) is mixed with a 10-fold excess of SPDP (50 mM in ethanol) and the receptor is mixed with a 25-fold excess of SPDP in 20 mM sodium phosphate, 0.10 M NaCl pH 7.2 and each of the reactions incubated, e.g., for 3 hours at room temperature. The reactions are then dialyzed against PBS.

The peptide is reduced, e.g., with 50 mM DTT for 1 hour at room temperature. The reduced peptide is desalted by equilibration on G-25 column (up to 5 % sample/column volume) with 50 mM KH_2PO_4 pH 6.5. The reduced peptide is combined with the SPDP-receptor in a molar ratio of 1:10 receptor:peptide and incubated at 4 °C overnight to form a peptide-receptor conjugate, e.g., CBD-receptor conjugate.

Gluteraldehyde conjugation:

Conjugation of a peptide (e.g., CBP or CBD) with a receptor can be accomplished by methods known to those skilled in the art using gluteraldehyde. For example, in one illustrative embodiment, the method of conjugation by G.T. Hermanson (1996, "Antibody Modification and Conjugation, in Bioconjugate Techniques, Academic Press, San Diego) described below, is used.

The receptor and the peptide, e.g., CBD, (1.1 mg/ml) are mixed at a 10-fold excess with 0.05 % gluteraldehyde in 0.1 M phosphate, 0.15 M NaCl pH 6.8, and allowed to react for 2 hours at room temperature. 0.01 M lysine can be added to block excess sites. After-the reaction, the excess gluteraldehyde is removed using a G-25 column equilibrated with PBS (10 % v/v sample/column volumes)

Carbodiimide conjugation:

Conjugation of a peptide with a receptor can be accomplished by methods known to those skilled in the art using a dehydrating agent such as a carbodiimide. Most preferably the carbodiimide is used in the presence of 4-dimethyl aminopyridine. As is well known to those skilled in the art, carbodiimide conjugation can be used to form a covalent bond between a carboxyl group of peptide and an hydroxyl group of a receptor (resulting in the formation of an ester bond), or an amino group of a receptor (resulting in the formation of an amide bond) or a sulfhydryl group of a receptor (resulting in the formation of a thioester bond).

Likewise, carbodiimide coupling can be used to form analgous covalent bonds between a carbogroup of a receptor and an hydroxyl, amino or sulfhydryl group of the peptide. See, generally, J. March, Advanced Organic Chemistry: Reaction's, Mechanism, and Structure, pp. 349-50 & 372-74 (3d ed.), 1985. By means of illustration, and not limitation, the peptide is conjugated to a receptor via a covalent bond using a carbodiimide, such as dicyclohexylcarbodiimide. See generally, the methods of conjugation by B. Neises et al. (1978, Angew Chem., Int. Ed. Engl. 17:522; A. Hassner et al. (1978, Tetrahedron Lett. 4475); E.P. Boden et al. (1986, J. Org. Chem. 50:2394) and L.J. Mathias (1979, Synthesis 561).

Peptide-receptor fusions:

In other specific embodiments, the peptide, such as, CBP or CBD, can be expressed as a fusion, or chimeric protein product, comprising the peptide joined via a peptide bond to a receptor. Such a chimeric product

can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences of the peptide and the receptor to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art such as a recombinant protein or displayed on a phage. For example, see generally Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual 2nd ed. (Cold Spring Harbor Laboratory Press, New York). Alternatively, such a chimeric product or fusion can be made by protein synthesis techniques, e.g., by use of a peptide synthesizer.

Methods of concentrating and/or methods of detection:

The present invention provides methods of concentrating a particular microorganism(s) of interest in a sample, comprising contacting the sample with a matrix, such as, but not limited to, a cellulosic or chitin matrix, to which is bound an affinity receptor or receptor conjugate, such as, but not limited to, a cellulose binding protein (CBP) - receptor or cellulose binding domain (CBD) - receptor conjugate, specific for the microorganism(s). In a particular embodiment, the sample is a dilute sample containing at least 0.0025 CFU/ml and up to 10^3 CFU/ml of microorganism(s). In other embodiments, the dilute sample contains at least 0.001 CFU/ml, at least 0.008 CFU/ml or <1 CFU/ml.

In another embodiment, the present methods for concentrating microorganisms can further comprise washing the matrix to remove unbound materials. In more particular embodiments, the wash solution, for example, can be saline (0.85 % NaCl), phosphate buffered saline (PBS), or PBS with 0. 05 % TWEEN° 20. Illustrative examples using a washing step are presented in the Examples section that follows.

In another embodiment of the present invention, the methods for concentration can further comprise a step for enriching the concentrated microorganism(s) . The enrichment can be performed *in situ* by addition of culture medium to the matrix and incubation at an appropriate temperature so that captured microorganisms will multiply and optionally be recaptured by the affinity receptor which is bound to the matrix. Illustrative examples of *in situ* enrichment are presented in the Examples section that follows.

In one embodiment, the present methods for concentrating microorganisms can further comprise washing the matrix to remove unbound materials after the capture and enrichment steps, by, foe example, phosphate buffered saline (PBS) optionally supplemented with 0.05 % TWEEN ™ 20.

Alternatively, the enrichment of the concentrated microorganism(s) can be performed outside of the immunoconcentrator or *in vitro* by transferring the microorganisms to culture medium in another container or test tube. In particular embodiments, the culture medium is with or without antibiotics. In a preferred embodiment, the culture medium is without antibiotics. In yet another embodiment, the microorganisms are released from the matrix using any method known in the art, including but not limited to, use of a competing receptor, etc., and then the microorganisms are cultured *in vitro* in another container or test tube.

In another embodiment, the microorganism(s) are captured by a matrix to which is bound an affinity receptor or receptor conjugate, such as, but not limited to, a CBP-receptor- or CBD-receptor-conjugate, optionally enriched in a culture medium, and then detected using an appropriate detection method of choice, as further detailed hereinunder. As exemplified in the Examples section, certain detection methods demonstrate such high sensitivity, e.g. 1-10 CFU's in a volume of less than 1 ml, such that the post capture enrichment step is not essential when these methods are employed for detection.

In yet another embodiment, using samples having an extremely low level of microorganisms (less than 20 CFU/25g sample), the sample is pre-enriched for a brief period of 1-5 hours and then the microorganisms are captured with a concentrator, such as a CBP or CBD concentrator ("CBP-C" or "CBD-C") comprising cellulosic or chitin matrix to which is bound a CBP - or CBD - receptor conjugate or fusion, enriched in a culture medium, and then detected using an appropriate detection method of choice.

In a more particular embodiment, a CBP or CBD immunoconcentrator ("CBP-IC" or "CBD-IC") is used which comprises a cellulosic or chitin matrix to which is bound a CBP- or CBD-antibody conjugate.

Further, the present invention can be performed manually or using methods and devices for automation (See, e.g., Figures 1 and 2).

The present invention can further comprise methods for detecting any microorganisms such as, but not limited to, methods of detection selected from immunoassays, polymerase chain reaction, plating the microorganisms on selective media, other nucleic acid signal amplification methods, methods using a biosensor or an electrochemical detector, etc.

In one embodiment, the method of the present invention is performed by contacting a sample with a receptor bound matrix in a single pass in

which the sample flows through the matrix once in a single direction. Alternatively, the method is performed by contacting a sample using multiple passes. In the multiple pass embodiment, a sample flows through the matrix is "recirculated" and passes through the matrix, i.e., multiple passes in a single direction. In another embodiment, the method of the present invention is performed by contacting a sample with a receptor bound matrix in a single cycle in which the sample is drawn up into the matrix and expelled, thereby, passing over the matrix twice in two directions. Alternatively, the method can be performed using multiple cycles. As illustrated below, the amount of bound microorganisms can be increased by contacting the sample with the receptor bound matrix in multiple cycles.

One skilled in the art would know and be able to empirically determine the number of cycles or passes to perform in order to capture the microorganism(s) to concentrate them sufficiently to detect the microorganism(s).

The present methods can be performed using a variety of sample volumes as further illustrated below. For example, the methods of concentration can utilize sample volumes ranging from 1, 20, 250, 650 ml and larger volumes, such as many liters. In a preferred embodiment, the sample volume is 2 ml or less. In another preferred embodiment, the sample volume is 2 ml to 4 liters. In yet another preferred embodiment, the sample volume is 2 ml to 10 liters. For example, liquid samples of 250 ml to 4 L are used from samples such as milk, carcass wash, juices, composite liquid extracts (e.g., stomached samples) and samples of ready to eat foods such as baby foods.

In an embodiment of the invention, volumes smaller than 2 ml can be cycled through a receptor bound matrix at least 5 times to achieve nearly 100 % specific capture of the microorganism(s) present in the sample.

The steps of the methods of the present invention can be performed in any of a variety of orders. In one embodiment, the microorganisms are captured by a receptor containing concentrator followed by detection of the microorganisms using an appropriate detection method of choice.

In another embodiment of the present invention, the method of concentrating a particular microorganism(s) in a sample comprises contacting a sample with a matrix, such as cellulose or chitin matrix, to which is bound a receptor or a receptor conjugate or fusion, such as CBP-receptor or CBD-receptor conjugate or fusion, and enriching the

concentrated microorganism(s) with a culture medium with or without antibiotics, and may further comprise performing a method of detecting the microorganism.

In any of the above embodiments, the sample can be a liquid extract of a solid sample.

In yet another embodiment, the method of concentrating a particular microorganism(s) of interest comprises contacting a liquid with a solid sample to create an extract which is passed through a concentrator according to any of the embodiments described herein. The solid sample can be mixed with a liquid in any way known to those skilled in the art in order to extract microorganisms from the solid sample into the liquid.

For example, mixing methods can include, but are not limited to, "stomaching", sonicating and high speed vibrating. The term "stomaching" is a well known term in the field of food microbiology and it means to blend a solid sample with a liquid to obtain a liquid sample which contains a microorganism population representative of the population of microorganisms present in the original sample and which has been extracted, nearly in its entirety into the liquid. For example, the "stomaching" is performed using a device such as the Stomacher Classic 400 Unit (IUL USA Inc., Erlanger, Kentucky). The extract can then be recirculated through the solid sample and through the matrix as many times as desired to concentrate the microorganism(s) .

For example, in a more particular embodiment, a solid sample, such as ground beef, is placed in a stomacher bag to which is added a liquid. The liquid and solid sample are mixed or "stomached" in order to form an extract of said sample, said extract containing any microorganism(s) in the solid sample. The extract is passed through a matrix to which is bound an affinity receptor specific for microorganism(s) of interest, e.g., *E. coli*, etc. The extract flows through the matrix back into the stomacher bag containing the solid sample to further extract more microorganism(s). The extract is recycled as many times as desired through the solid sample and the matrix.

By way of example, and not limitation, a solid sample of 20, 25, 50, 65 or 375 grams is extracted with a liquid, e.g. at w/w or vol/vol ratio of about 1:10. The solid and liquid are "stomached" to produce a homogeneous slur. In one embodiment, directly after stomaching, the liquid sample is contacted with a matrix to which is bound an affinity receptor.

In another embodiment, the liquid sample obtained after stomaching is pre-enriched for 1-5 hours before contacting the sample with the matrix to which is bound an affinity receptor.

Further, in yet another embodiment, the enrichment occurs simultaneously as the liquid sample is contacted with the matrix to which is bound an affinity receptor.

Lastly, in an alternative embodiment, the enrichment occurs in the matrix after the microorganisms are captured.

In another embodiment, multiple stomacher bags containing homogeneous samples are connected to the same matrix so that larger volumes of liquid sample are used in order to capture the microorganism(s).

Prefiltration of samples:

The ability to produce samples which have had their solid (particulates) and/or fatty content greatly reduced while retaining nearly 100 % of the bacterial content in the filtrate is an advantageous step in concentrating microorganisms as described herein, chiefly because it prevents clogging and assists in capture. As a direct consequence it is possible to process large volumes of solid and fat containing samples, such as food samples, containing a dilute antigen, such as pathogens.

As a result of the prefiltration step these samples can be readily filtered through receptor-coated matrices as described herein, which allow the concentration of antigens, including viable bacterial cells, on the matrix and their subsequent detection. This is especially important when the growth of viable bacteria or the resuscitation of damaged bacterial cells are inhibited by the presence of inhibitory compounds in a sample containing low levels of the organism of interest.

The ability to process an entire sample, of large volume, on receptor-coated matrix allows for the capture, concentration and isolation of a large percent of the target cells from the total sample and thus removing such cells from inhibitory compounds and other microflora, enabling their rapid growth and or resuscitation in a non-inhibitory, defined, growth media of choice.

The ability to concentrate cells depends on the stickiness (i.e., the extent that the matrix binds to organisms and compounds other than that which the affinity receptor which has been bound to the matrix is specific) and the nominal pore size of the receptor-coated matrix, since for effective concentration the sample solution must be passed through the capture

surface while enabling a relatively high percentage of targets to be captured and the subsequent removal of unwanted compounds.

Thus, a prefiltration step is preferably employed prior to the implementation of the concentration methods of the present invention.

A filter device incorporating a plurality of filter materials has been devised to effect prefiltration according to the present invention. Such a filtering device is directed at removal of fats, starchy materials, proteinaceous materials and particulates while retaining the flora of a filtered sample, e.g., a "stomached" food sample.

Historically, removal of fats, starchy materials, proteinaceous materials and particulates involves complex filtration systems in addition to other physical separation means, such as centrifugation, for extracting desired elements of the sample. For optimal results of the concentration methods of the present invention, especially when low level of microorganisms are traced for, or when only small sample volumes are available, it is essential that only a minimal liquid and/or number of target organisms will be lost while prefiltering the sample for the removal of unwanted material.

As further detailed hereinunder, the filtering device according to the present invention includes several layers of different types of filtration media, each separate layer, in fact, performs a particular function or functions of the separation process.

Several embodiment of the filtering device according to the present invention, which is referred to hereinbelow as device **50**, are illustrated in Figures 27a-c.

Device **50** according to the present invention includes a housing **52**. Housing **52** can be made of two or more integrating parts, so as to enable reuse thereof after replacing some or all of the filters therein. Housing **52** is formed with an inlet **54** and outlet **56** for allowing solutions in and out of device **50** by, for example gravitational force. Housing **52** serves to accommodate a plurality of filter layers each including a dedicated filter material as further detailed below.

According to one embodiment, as shown in Figure 27a, three basic filter layers are employed. The order and type of these filter layers may vary with different food types in order to effect the best possible prefiltration for immunoconcentration. One filter layer includes a cotton based non-woven fabric **58**. This material is very hydrophilic and acts as a primary absorbent of the sample and also as a depth filter to trap out the

larger solids and prevent such solids of progressing beyond to other filter layers.

Another filter layer includes a polyester non-woven material **60** which is only slightly hydrophobic and acts both as a second stage solid trap and also as a trap of fatty substances contained in the filtered sample.

Still another layer is a slightly hydrophilic polypropylene felt material **62** which provides the final stage of the solid/particulate removal. Being of polypropylene fibres which are inherently hydrophobic, the fibres substantially do not absorb aqueous liquids. Since the liquid content of the sample provides the desired filtrate and is only adsorbed onto the surface of the fibres, the liquid is readily and effectively washed through this final, fine polypropylene filter material with minimal losses.

According to another embodiment, as shown in Figures 27b-c, three or four filter layers are employed.

One filter layer includes an open cell, reticulated polyurethane foam block (e.g., cylinder) **64**, which is designed to retain much of the substantially lipid or fatty content of the sample, starch compounds, as well as to act as a primary separator of the solids content.

Another filter layer includes a non-woven filter material **66** which would typically be of polyester or cellulose fibres.

The final filtration stage according to this embodiment of the present invention is performed by polypropylene felt material **62**, as in the embodiment shown in Figure 27a. A spacer **68** can be employed to create an air space **70** between any of the filters employed.

A fourth filter layer is shown in Figure 27c to include a hollow body **72** made of a woven mesh of nylon or polyester fibers. This filter provides a coarse filtration step to remove relatively large solids without providing any significant reduction in flow of liquid through filtering device **50**.

Applications:

The present invention finds uses in various applications, including but not limited to those listed herein below.

Food industry:

The methods of the present invention can be used to provide rapid food contamination tests for any food-borne microorganism, including, but not limited to, *Escherichia coli, Salmonella, Listeria, Campylobacter, Shigella, Staphylococcus, etc.* In particular, the microorganism is *E. coli* O157:H7.

The rapid tests can be used to test any foods, including, but not limited to, meats, fruits, fruit juices, vegetables, vegetable products, dairy products such as milk, grain products and spices. The rapid tests can also be used to test Sponge Rinse fluid which is generated from Cattle, Swine and Turkey Carcass testing as well as the Carcass Rinse fluid generated from the Whole Chicken bird rinse method described by the HACCP procedures (Appendices E-G, 61 Federal Register 38917-38945).

Medical industry:

The methods of the present invention can be used to provide rapid medical including clinical or veterinary tests for microorganisms, such as bacteria, viruses, fungi such as yeast, protozoans, or nematodes. For example, the present invention can be used to provide a rapid immunoconcentration method for detecting contaminants such as bacteria or viruses in medical products such as plasma, clotting factors, drugs etc. The present invention can also be used in the clinical setting for immunoconcentrating and detecting microorganisms from samples from patients such as stool, as well as body fluids such as blood, urine, saliva and other bodily fluids or solubilized patient samples for use in hospitals or in a doctor's office. For example, the present invention can be used for the rapid detection of urinary tract infections.

Environmental industry:

The methods of the present invention can be used to provide rapid concentration and detection tests for contamination in environmental samples such as water, waste water or industrial effluent, air (with the aid of an impinger for example), and soil samples. By way of example, and not limitation, the present invention can be used to concentrate and detect water supply contaminants such as *Legionella*, *Clostridium, Pseudomonas,* and coliforms. The present invention can also be used to detect microorganism contamination in waste water such as *Escherichia coli, Salmonella, Campylobacter, etc.*

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

EXAMPLE 1

Binding of a CBD-conjugate to a cellulose matrix

The following example describes the binding of a CBD-IgG conjugate to the cellulose matrix AVICELL™ 200 or to cotton gauze.

Materials and methods:

Preparation of bacteria:

Stock solutions of viable *E. coli* O157:H7 bacteria (ATCC No. 43895) were prepared by growing cultures overnight at 37 °C on Tryptic Soy Agar (Difco) slants. Each slant was loaded with 3.0 ml saline solution (0.85 % NaCl) and the media was suspended by mixing using a vortex. The resulting suspension was transferred to a fresh test tube and serially diluted with saline to prepare solutions with the desired bacterial concentrations of 5×10^1, 5×10^2, 5×10^3 and 5×10^4 CFU/ml (colony forming units). The exact concentration of these solutions was confirmed by plating 50 and 100 µl of the solution estimated to be 5×10^2 onto Tryptic Soy Agar (Difco) or where indicated onto mENDO agar LES (Difco Laboratories, Detroit, Michigan), incubating overnight at 37 °C, and counting the CFU's.

Stock solutions of *Salmonella typhimurium* (ATCC No. 14028) were prepared by growing cultures overnight at 37 °C in Tryptic Soy Broth ("TSB") (Difco) medium with rotation. One part of the overnight culture was diluted with 24 parts fresh TSB and incubated approximately one hour until the optical density at 600 nm (OD_{600}) was 0.3. This solution was then serially diluted with saline solution (0.85 % NaCl) to the desired concentrations.

Antibodies:

The antibodies (IgG) used in the following Examples are as follows: Goat anti-*E. coli* O157:H7 (Cat. No. 01-95-90) and goat anti-*Salmonella* CSA-I (Cat. No. 01-91-99) were obtained from KPL (Gaithersburg, Maryland). Rabbit anti-*Salmonella* sp. antibodies were obtained from Virostat (Maine). Monoclonal (Clone M-32242) anti *Salmonella typhimurium* antibodies were obtained from, Fitzgerald, Concord, MA).

Preparation of a CBD immunoconcentrator (CBD-IC):

(a) SPDP conjugation procedure:

The cellulose binding domain from *Clostridium cellulovarans* (CBD) was prepared by dissolving the CBD in water at a concentration of 2-3 mg/ml.

The SPDP solution is prepared as follows: 5 mg SPDP 3-(2-pyridyldithio) propionic acid N-hydroxysuccinimide ester (also called N-Succinimidyl 3-(2-pyridydithio)propionate) ("SPDP") (Cat. No. P-3415, Sigma Chemical Co., St. Louis, Missouri) was dissolved in 300 µl ethanol (50 mM).

The SPDP conjugation reactions were performed as follows: 1.7 mg/ml CBD (0.1 µmol) was mixed with a 10 molar excess of SPDP solution (30 µl of 50 mM SPDP in ethanol) and the IgG (2 mg/ml) was mixed with a 25-fold molar excess of SPDP in PBS.

The reactions were incubated for 3 hours at room temperature. Phosphate buffered saline ("PBS") (20 mM phosphate, 150 mM NaCl, pH 7.2) was added to a final volume of 1 ml. The mixture was then dialyzed against PBS.

The CBD was reduced with 50 mM dithiothrietol (DTT) for 1 hour at room temperature. The reduced CBD was desalted using a G-25 column (up to 5 % sample/column volume ratio) which was equilibrated with 50 mM KH_2PO_4, pH 6.5. To produce the CBD-IgG conjugate, the reduced CBD was then mixed with the SDPD-IgG conjugate in a 1:10 molar ratio IgG:CBD and incubated overnight at 4 °C.

Conjugation of CBD with IgG was evaluated by (i) binding the conjugate to AVICELL™ 200 in the presence of TWEEN™ 20 (0.05%) (to reduce nonspecific binding); and (ii) an Enzyme Linked Immunosorbant Assay (ELISA) as set forth below.

The CBD-IgG conjugate was stored with 0.05 % sodium azide at 4 ° C.

ELISA:

The Enzyme Linked Immunosorbant Assay was performed as follows: E.I.A./R.I.A. flat bottom, high binding plates (Costar, Cat. No. 3590) were coated with anti-*E. coli* O157:H7 1 µg/ml in PBS, by adding 100 µl in each well and incubating for 2 hours at 37 °C. The plates were washed three times with PBS-TWEEN™ 20. The plates were blocked using 1 % BSA/PBS by adding 200 µl into each well and incubating for 1 hour at 37 °C. The plates were then washed three times with 0.05% PBS-TWEEN

™ 20. *E. coli* O157:H7 were added from 0 to 10^6 CFU/ml in PBS-TWEEN ™ 20 in 100 µl per well and incubated for 1 hour at 37 °C. The plates were washed three times with PBS-TWEEN™ 20. 100 µl of anti-*E. coli* O157:H7-HRP were added at a dilution of 1:16,000 in PBS-TWEEN™ 20 (0.05 %) and incubated for 1 hour at 37 °C. The plates were washed three times with PBS-TWEEN™ 20. 100 µl of K-Blue™ substrate was added and incubated for 30 minutes at room temperature. The reaction was stopped by adding 100 µl of 1M H_2SO_4. The results were determined by reading the absorbance at 450 nm.

Preparation of cellulosic matrix:

A piece of folded gauze (Nissan, medical gauze, 17 threads per sq. cm. lot 0012) was boiled in 1 % $NaHCO_3$ 1:15-20 weight per volume for 30 minutes. The gauze was washed four times in distilled water and three times in double distilled water. Each washing was performed by stirring the gauze in water for 15 minutes followed by wringing it and patting on paper before hanging it to dry. Gauze prepared in this fashion was cut into 50 mg quadrants and packed into the bottom end of a pipette tip (QSP, 111-N, white) or concentrator device.

Binding of conjugate onto gauze:

150 µl of conjugate (diluted 1:6 with PBS TWEEN™ 20 to a final concentration of 0.17 mg/ml) was added to the gauze inside the immunoconcentrator device or pipette tip, incubated for 15 minutes at 37 ° C, cooled to room temperature, washed once with 5 ml of PBS-TWEEN™ 20 and then washed with 5 ml of PBS. The immunoconcentrator ("IC") was then ready for use. For a control IC, 150 µl of PBS TWEEN™ 20 was added to gauze without a CBD-IgG conjugate.

Binding of conjugate onto AVICELL™ 200:

2 µg CBD-IgG conjugate was applied to 1 mg AVICELL™ 200 (FMC Corp. N. V., Belgium) in PBS-TWEEN™ 20 buffer, and incubated with rotation at room temperature for 1 hour. The remaining buffer was collected and the AVICELL™ was washed three times with PBS. In order to determine the amount of proteins (CBD-IgG conjugate) which were bound to the AVICELL™ the beads were placed in Sample Application Buffer ("SAB") (50 % glycerol, 700 mM β-mercaptoethanol, 20 % SDS, 0.02 % (w/v) Bromophenol Blue, and 375 mM Tris buffer, pH 8.8) and the proteins were released by boiling the AVICELL™-CBD-IgG mixture. The released proteins were then analyzed by SDS-PAGE.

Samples of the remaining CBD-IgG conjugate mixed with the AVICELL™ 200, the unbound CBD-IgG conjugate, and the bound CBD-IgG conjugate were analyzed using SDS-PAGE (15 % acrylamide).

Results:

The results of the CBD-IgG conjugate binding to AVICELL™ 200 are shown in Figure 3 which shows that the CBD-IgG conjugate binds to the AVICELL™ cellulose matrix and can be released by boiling the AVICELL™-CBD-IgG complex in Sample Application Buffer containing β -mercaptoethanol. Lane 2 shows a sample of the conjugate loaded onto the CBD-IgG AVICELL™ 200 and lane 4 shows the CBD-IgG eluted from the AVICELL™ 200 matrix. Lane 3 contains the unbound conjugate (supernatant solution from wash step solutions).

EXAMPLE 2

Gluteraldehyde conjugation of CBD-IgG and binding assay to beaded cellulose 50-80 μm

The following example describes the conjugation of a CBD to an antibody using gluteraldehyde and the specific binding of the CBD-IgG conjugate to the cellulose matrix of beaded cellulose 50-80 μm.

Materials and methods:

The CBD from *Clostridium cellulovorans* was conjugated with affinity purified Goat anti-*E. coli* O157:H7 IgG (KPL, Cat. No. 01-95-90) using the following procedure.

Goat anti-*E. coli* IgG (0.9 mg/ml) was mixed with a 10 fold molar excess of CBD (1.1 mg/ml) with 0.05 % gluteraldehyde (Gluteraldehyde 25 % solution, Sigma Chemical Co., St. Louis, MO; Cat. No. G-5882) in 0.1 M phosphate buffer (0.1 M phosphate, 0.15 M NaCl, pH 6.8) for 2 hours at room temperature. L-lysine (Sigma, St. Louis, MO; Cat. No. L-6027) was added to a final concentration of 0.01 M as a blocking agent to block excess reactive sites. The CBD-IgG conjugate was purified and the excess gluteraldehyde was removed using a G-25 column equilibrated with PBS (10 % v/v; sample/column volume).

The binding assay of the CBD-IgG conjugate was performed by mixing 1 μg of the CBD-IgG conjugate with 10 mg beaded cellulose 50-80 μm (Sigma Chemical. Co., St. Louis, MO) in PBS-TWEEN™ 20 and incubating for 1 hour at room temperature. The beaded cellulose was then washed three times with PBS.

The amount of bacteria captured was measured by ELISA as follows: To determine the capturing ability of the CBD conjugate adsorbed to the beaded cellulose, *E. coli* O157:H7 heat killed standard (0.5 ml at 0, 10^3, 10^4 or 10^5 CFU/ml) was added to the matrix and incubated for 15 minutes at room temperature. The column was washed three times with PBS-TWEEN™ 20. Then 15.6 ng of affinity purified goat anti-*E. coli*-O157:H7-HRP (horseradish peroxidase labeled), 0.1 mg/ml (KPL, Cat. No. 04-95-4) was added and incubated for 15 minutes at room temperature. The column was washed three times with PBS-TWEEN™ 20. 0.2 ml of K-blue™ substrate (Neogen Corp., Lexington, KY) was added and the mixture was incubated for 15 minutes at room temperature in the dark. The substrate was transferred into a fresh tube and 0.2 ml 1M H_2SO_4 was added. The absorbance was determined with a spectrophotometer at 450 nm. The positive control was *E. coli* O157:H7, 7 x 10^9 CFU/ml (KPL, Cat. No. 50-95-90).

Results:

The results of this example are illustrated in Figure 4 which shows the bifunctional activity of the CBD-anti-*E. coli* O157:H7 conjugate. The CBD-IgG conjugate bound to the beaded cellulose was still capable of binding the *E. coli* in the sample.

EXAMPLE 3

Evaluation of the specific binding of CBD-conjugate to cellulose

The following example illustrates the specific binding of a CBD-goat IgG conjugate to cellulose. The non-specific binding (i.e., removable with a saline detergent solution, e.g., PBS-TWEEN™ 20) of native goat IgG to cellulose is shown for comparison.

Materials and methods:

10 mg of cotton gauze were mixed with approximately 15 µg CBD-goat IgG conjugate or native goat IgG in final volume of 0.5 ml PBS-TWEEN™ 20 and incubated for 1 hour at room temperature with inversion. The cellulose gauze was washed three times with 1 ml PBS-TWEEN™ 20 and the cellulose gauze was pelleted by centrifugation before each wash step. The supernatant was discarded and the cellulose gauze was resuspended with 50 µl Sample Application Buffer with: β-mercaptoethanol. After boiling for 10 minutes the supernatant was analyzed using 15 % SDS-PAGE (Figure 5a) and 7.5% SDS-PAGE (Figure 5b).

Results:

The results are illustrated in Figures 5a and 5b which show that in contrast to native IgG (which did not efficiently bind at the concentrations employed), the CBD- IgG conjugate specifically bound to the cellulose matrix (See Figures 5a and 5b, compare lanes 3 and 7). The 15 % gel shows the different IgG fractions i.e., heavy (HC) and light (LC) chains of the CBD-IgG conjugate that were loaded onto the cellulose matrix and that specifically bound. The 7.5 % gel shows the high molecular weight conjugate more clearly than the 15 % gel.

EXAMPLE 4

High capture rate of bacteria by cycling sample

The following example illustrates the nearly quantitative capture of *E. coli* O157:H7 from a 1 ml sample using the CBD immunoconcentration method. The CBD-IC without IgG was used as control.

Materials and methods:

One milliliter of model PBS solution containing 2.48 x 103 CFU/ml of *E. coli* O157:H7 was drawn into the SPDP conjugate CBD-IC by standard action of lab pipettor using either 1 cycle or 5 cycles in an up and down manner. Each cycle took approximately 30 seconds to complete. After completion of the capture cycles, the CBD-IC was washed twice with 4.5 ml PBS. The amount of bacteria captured by the device was calculated by subtracting the amount of bacteria in the wash solutions from the original inoculum number. One ml of expelled model solution was added to 9 ml of wash solution and the bacterial concentration was determined by the membrane filter using GN-6 METRICEL™ (Gelman Sciences Inc., MI) on mENDO agar LES medium and incubated overnight at 37 °C.

GN-6 METRICEL™ 0.45 micron membranes are mixed cellulose ester membranes and are used in the isolation and enumeration of bacteria in wastewater, drinking water by the Membrane Filtration Technique and other analytical and general laboratory filtration needs. GN-6 METRICEL ™ was used to filter the wash steps during the capture protocol in order to enumerate the number of bacteria which are not captured during the process. The filter is then transferred to a petri dish with mENDO agar LES together with bacteria which have been trapped by the 0.45 micron filter coliforms including *E. coli* which when grown on this medium produce black colonies that are easily identified on the white background of the METRICEL™ membrane.

Results:

The results are illustrated in Figure 6 which shows that over 60 % of the bacteria were captured using a single cycle through the CBD-IgG immunoconcentrator, and over 90 % of the bacteria were captured using five cycles through the CBD-IgG immunoconcentrator.

EXAMPLE 5

Selective capture of Salmonella typhxmurxum

The following example illustrates the selective immunocapture and concentration of *Salmonella typhimurium* from a 1 ml solution.

Materials and methods:

Rabbit anti-*Salmonella* sp. antibodies were obtained from Virostat (Maine) and conjugated to CBD according to the gluteraldehyde procedure as described above. One ml PBS solution containing 1.29 x 10^2 CFU/ml was drawn into a CBD-IC by 5 cycles using standard action of lab pipettor in an up and down manner. It took approximately 30 seconds to complete each cycle. After completion of capture cycles the CBD-IC was washed with 2 x 5 ml PBS-TWEEN™ and 2 x 5 ml PBS. The amount of bacteria captured by the device was calculated by subtracting the amount of bacteria in wash solutions from original inoculum number. One ml of expelled model solution was added to 20 ml wash and bacterial concentration was determined by filter method using GN-6 METRICEL™ on Xylose Lysine Decarboxylase Agar (XLD: LAB M Ltd., Lancashire, UK) and overnight incubation at 37 °C.

Results:

The results are illustrated in Figure 7 which shows that the concentrator device with CBD-anti-*Salmonella* conjugate was able to capture approximately 50 % of the bacteria in a 1 ml sample as compared to less than 12 % capture for a cellulose concentrator device with no CBD-anti-*Salmonella* conjugate.

EXAMPLE 6

Immunoconcentration of bacteria at low concentrations

The following example demonstrates the immunoconcentration of low concentrations of bacteria from one milliliter samples.

Materials and methods:

One ml of PBS solution containing 590, 73, or 6 CFU/ml was drawn into the CBD-IC by standard action of lab pipettor using 2 cycles in an up and down manner. After completion of the capture cycles the CBD-IC was washed twice with 4.5 ml PBS. The amount of bacteria captured by the device was calculated by subtracting the amount of bacteria in wash solutions from original inoculum number. One ml of expelled model solution was added to 9 ml of wash solution and bacterial concentration was determined by filter method using GN-6 METRICEL™ or mENDO agar LES medium and incubated overnight at 37 °C. Gauze containing bacteria which have been captured by the CBD-IgG can be plated onto growth media and incubated overnight. Each CFU which was captured will give rise to a distinct "colony" on the gauze itself. These "colonies" grow very rapidly overnight and can spread out quickly covering entire surface of the gauze making it impossible to enumerate CFU's. However, when the number of bacteria which have been captured is less than 25-30 CFU's it is possible to count these "colonies". The sample containing 6 CFU/ml, the matrix was plated directly on mENDO agar LES medium in order to count and confirm the number of bacteria that were captured.

Results:

The results are illustrated in Figure 8 which shows that the CBD-IgG immunoconcentration method captures 80 % or more of the bacteria. The CBD-IC method can detect as few as 6 CFU/ml from a sample solution.

The CBD-IC method has been used to capture as few as 1 CFU/ml and greater than 10^6 CFU/ml. Furthermore, the system is indifferent to volumes such that 1 CFU can be placed in large volumes (e.g., 25 or 100 ml to many liters) with the same capture rate as in 1 CFU per 1 ml.

EXAMPLE 7

Specificity of immunoconcentration using the CBD immunoconcentration method

The following example demonstrates the specificity of immunoconcentration of *E. coli* O157:H7 from a 1 ml sample.

Materials and methods:

One ml of model PBS solution containing 1.77 x 10^3 CFU/ml *E. coli* O157:H7 was drawn into a CBD-IC having no CBD-IgG conjugate or CBD-anti-*E.* coli O157:H7 conjugate in five cycles by standard action of lab pipettor in an up and down manner. Each cycle took approximately 30

seconds to complete. After completion of the capture cycles the CBD-IC was washed twice with 4.5 ml of either saline solution (0.85 % NaCl), PBS or PBS-TWEEN™ 20. The amount of bacteria captured by the device was calculated by subtracting the amount of bacteria in wash solutions from number of bacteria in the original inoculum. Bacterial concentration in the wash solution (to which was added 1 ml of expelled model solution) was determined by filter method using GN-6 METRICEL™ on mENDO agar LES medium and overnight incubation at 37 °C as described above.

Results:

The results are illustrated in Figure 9 which shows that saline solution, PBS or PBS-TWEEN™ 20 can be used as a wash solution without affecting the ability of CBD-anti-*E. coli* O157:H7 to bind to the *E. coli* O157:H7 in a sample. Moreover, the PBS-TWEEN™ 20 wash solution functioned better than the saline or PBS alone to remove nonspecifically bound *E. coli.*

CBDs bind specifically to cellulose in PBS-TWEEN™ 20 while most other proteins bind negligibly under these conditions. These conditions effectively eliminate the nonspecific binding of other proteins to the cellulose matrix. Once the CBD-IgG conjugate is bound to the cellulose matrix, the CBD moiety is removed only by the most severe denaturing conditions by 6 M guanidine HCl, 5 M urea or boiling at 100 °C. CBD conjugate that remains bound in the presence of the detergent is most likely to be bound via its CBD moiety and not by the antibody. The antibody is then available to specifically bind to the microorganism(s) in the sample.

EXAMPLE 8

Capture and in situ enrichment in CBD-IC

The following example illustrates the capture and enrichment of bacteria from a 1 ml pure culture model solution containing 22 CFU of *E. coli* O157:H7.

Materials and methods:

Preparation of model solutions:

The pure culture model saline solution containing 22 CFU/ml of *E. coli* O157:H7 was prepared by serial dilution of an overnight culture after estimating the concentration of bacteria by measuring absorbance at 650 nm using a spectrophotometer. The final bacterial concentration was determined by filter method using GN-6 METRICEL™ on mENDO agar LES medium and incubation overnight at 37 °C as described above.

Immunocapture:

Bacteria were immunocaptured from the solution by drawing the solution into an immunoconcentrator device (two part plastic device which was machined from white acetal plastic) using a 1 ml standard pipettor. Inoculated solutions were drawn into the device and then expelled by standard pipettor action and repeated so that each sample passed through the device twice. The device was then placed in a rack and disengaged from the pipettor. The CBD-IC was washed once with 5 ml PBS-TWEEN™ 20 and then with 5 ml PBS by passing the wash solution through the device from top to bottom. In certain instances the wash fluid was collected for further analysis by the filter method described above to determine percent of bacteria that were not immunocaptured by the CBD-IC. The percentage of captured bacteria was determined by analyzing certain CBD-ICs by plating the gauze matrix thereof directly onto mENDO agar LES medium and incubating overnight at 37 °C.

CBD-IC enrichment procedure:

After completion of the wash step, 250 µl of Modified EC (mEC) (Difco) medium (without Novobiocin) which was previously dispensed into sterile plastic tubes, was drawn into the device by standard pipettor action to draw the medium into chamber of the CBD-IC. The tube containing the CBD-IC device was then placed into a 37 °C incubator. After incubation the tube was heated in a heat block to 100 °C for ten minutes to kill the bacteria. The growth media containing the heat killed bacteria were then expelled from the IC by reverse pipettor action and used for an ELISA evaluation. All solutions were tested in duplicate or triplicate for each enrichment time point tested.

Results:

The results of this example are illustrated in Figure 10 which shows that *E. coli* O157:H7 at the low concentration of 22 CFU/ml can be detected with only 4 hours of post capture enrichment.

EXAMPLE 9

Detection of E. coli O157:H7 in ground beef by ELISA after CBD-IC and in situ enrichment

Materials and methods:

Eight individual 25 gram samples of minced ground beef (Lot 980302) were inoculated with 1 ml of PBS containing 44 CFU *E. coli* O157:H7 and then stomached in 225 ml PBS for 2 minutes. The meat

extract samples were filtered through a 100 % cotton fabric filter (manufactured by SPUNTECH Industries, Ltd., Israel) by placing the fabric in a 12 cm diameter funnel and then passing the stomached sample through this filter. The samples were transferred to a reservoir and passed through a CBD-IC. The total time to pass the entire sample through the device was 30 minutes at room temperature.

After immunoconcentration the CBD-IC device was detached from sample apparatus and washed twice with 5 ml PBS-TWEEN™ 20 (0.05 %) and twice with 5 ml PBS.

300 µl of mEC medium were placed into a sterile plastic tube for each device. The device was attached to a standard 1 ml pipettor and the medium was drawn into the device which was then detached from the pipettor and placed into the tube for incubation. The sample was incubated at 37 °C for 4 or 5 hours.

After incubation, the tubes were placed in a boiling water bath for 10 minutes to kill the bacteria.

The device was attached to a pipettor and the medium was expelled into a tube. 200 µl PBS were added to each device and the remaining bacteria were expelled using a pipettor.

A total of 400 µl of bacterial solution was recovered from the device and 50 µl samples were used for an ELISA analysis.

Results:

The results are presented in Figure 11. The two sets of bars represent *in situ* enrichment times of 4 and 5 hours. This example shows that the CBD-IC is capable of performing immunocapture as well as effective immunoconcentration of *E. coli* O157:H7 present in meat samples. Most significant is the ability of the CBD-IC to concentrate pathogen organisms when their concentration is less than 0.2 CFU/ml.

EXAMPLE 10

Bacterial capture by cellulose--CBD-anti-E. coli O157:H7 conjugate vs. cellulose anti-E. coli O157:H7

The following example presents a comparison of the binding of *E. coli* O157:H7 by cellulose bound CBD-anti-*E. coli* O157:H7 conjugate or cellulose bound anti-*E. coli* O157:H7.

Materials and methods:

50 mg of cotton gauze or cellulose beads (ORBICELL™) (Accurate Polymers, Ltd., Illinois) were incubated with CBD-IgG, IgG, or PBS alone

each in the presence of TWEEN™ 20 (0.05 %) and washed twice with 10 ml PBS-TWEEN™ 20. One ml of PBS-TWEEN™ 20 containing 800 CFU *E. coli* O157:H7 was added to the cellulose matrix, incubated for 5 minutes, and then the textile/beads were washed with 10 ml PBS-TWEEN™ 20 and 10 ml PBS. The washed cellulose as well as all wash solutions were plated on mENDO agar LES plates. Colonies of *E. coli* O157:H7 were counted after an overnight incubation of plates at 37 °C.

Results:

Bacterial capture was calculated as the percentage of the *E. coli* colonies obtained from wash solutions compared to the initial amount of bacteria passed over the cellulose matrix (% of O157:H7 bacteria captured from 800 CFU in 1 ml PBS).

Table 7

	CBD-IgG	IgG	PBS-TWEEN ™ 20
Textile	42 %	16 %	0 %
Cellulose beads	50 %	0-12 %	0 %

As shown in Table 7, the cellulose bound CBD-IgG conjugate captured a greater percentage of bacteria as is compared to the cellulose matrix with only native IgG, or PBS-TWEEN™ 20 alone.

EXAMPLE 11

Comparison between DYNAL® and CBD immunoconcentrator (CBD-IC) procedures for E. coli O157:H7 capture and detection from a meat extract

The following example presents a comparison of the capture and isolation of *E. coli* O157:H7 by DYNAL® beads and the CBD-immunoconcentrator.

Materials and methods:

(a) CBD-IC procedure:

25 grams of ground beef were homogenized in 225 ml of PBS using a stomacher. The extract was filtered twice through a cotton fiber (SPUNTECH™-80 g/m). The extracts were inoculated with 0, 20 or 200 CFU of *E. coli* O157:H7. The experiment was performed in triplicate for each inoculum level. The 225 ml inoculated extracts were passed through the CBD-anti-*E. coli* O157:H7 gauze IC device during a 30 minutes period, and washed with 10 ml of PBS-TWEEN™ 20 and then 10 ml of PBS.

The washed gauze containing tips were transferred into a 10 ml tube and 120 µl of enrichment mEC medium were drawn into the device followed by a 6 hour incubation at 37 °C. The tubes containing the enriched textile tips were boiled for 10 minutes in a water bath, and then the mEC medium containing bacteria was expelled into an eppendorf tube. The device was then washed once with 120 µl of PBS. The PBS wash was added to the mEC medium to produce a sample adapted for ELISA testing. The presence of *E. coli* O157 in 50 µl of the sample (enriched medium and PBS wash) was detected by ELISA using HRPanti-*E. coli* O157:H7 (KPL). Optical density of the substrate (K-Blue™, Neogen Corp., Lexington, KY) was measured in an ELISA reader. Results are summarized in Figure 12.

(b) DYNAL® procedure:

The DYNAL® procedure was performed according to manufacturer's protocol as follows: 25 grams of ground beef were extracted using 225 ml of pre-enrichment medium of buffered peptone water ("BPW") (Difco, Cat. No. 1810-17-9) using a stomacher. The extract was inoculated with 0, 20, 200, 1000 or 10,000 CFU similar to the CBD-IC protocol above and incubated for 6 hours at 37 °C in a standard stomacher bag.

One ml of enriched meat extract was incubated for 30 minutes with DYNABEADS™ magnetic anti-*E. coli* O157:H7 beads with continuous mixing according to manufacturer's recommended protocol (Dynal-Product No. 710.03; printed 05/97; Rev. No. 01). Tubes containing extract and magnetic beads were placed in a magnetic stand to concentrate beads before each wash step. Beads were washed twice with 1 ml PBS-TWEEN™ 20 and the beads were plated on Sorbitol MacConkey Agar (SMAC) and SMAC supplemented with Cefixime and potassium tellurite, 0.05 mg/L and 2.5 mg/L (CT-SMAC), respectively. The plates were incubated 18-24 hours at 37 °C.

Since *E. coli* O157:H7 does not ferment sorbitol within 24 hours and, therefore, appears as colorless colonies on SMAC and CT-SMAC and are resistant to tellurite and cefixime, the overnight grown colonies were examined for white sorbitol negative (presumptive O157 positive) vs. red color sorbitol positive (negative-non-O157 colonies). Both white and red colonies were confirmed by Oxoid Latex O157 agglutination test as O157 positive or negative, respectively. The Oxoid *E. coli* O157 latex test will demonstrate by slide agglutination *E. coli* strains possessing the O157 serogroup antigen. One drop of test O157 latex is mixed with a loop of

bacteria from a sorbitol negative colony and rocked gently for one minute to observe whether or not agglutination occurs. Agglutination indicates that the colony was *E. coli* O157.

Results:

No positive colonies were detected, using DYNAL® protocol, in the inoculated samples when inoculum level was 20 and 200 CFU/225 ml extract, as well as in the negative controls which were not inoculated. This is probably due to the fact that more than 300 negative red colonies were captured by DYNAL® beads and, upon plating, appeared in both inoculated and non-inoculated sample plates. This high density of Sorbitol positive red colonies make the identification of presumptive positive nearly impossible when inoculum level was 20 and 200 CFU/per 225 ml (25 g ground beef). However, when inoculum levels of 1000 and 10,000 CFU/225 ml extract (25 g ground beef) were tested, then positive O157 colonies were identified on CT-SMAC plates which were then confirmed by latex agglutination. Plating of the O157 bacteria used for inoculum on the CT-SMAC plates resulted in white positive colonies as expected, thus confirming appropriate plating procedure. In contrast the CBD-IC protocol detected *E. coli* O157:H7 at all tested levels of inoculum, e.g., 20 and 200 CFU's/225 ml sample.

Conclusions:

The CBD-IC procedure was able to detect 200 CFU and as low as 20 CFU inoculated *E. coli* O157:H7 bacteria from 250 ml meat extract samples as shown in Figure 12 by incorporating a post-capture enrichment procedure..

In contrast, the detection and isolation of 200 CFU *E. coli* O157:H7 using the DYNAL® beads procedure was not possible due to the presence of a high background of microflora in the 225 ml samples after 6 hours of pre-enrichment.

EXAMPLE 12

Comparison between DYNAL® and CBD-IC for the capture and detection of E. coli O157:H7 using the post immunocapture enrichment procedure

The following example describes a comparison between DYNAL® beads and the CBD-IC for the detection of *E.* coli O157:H7, ensuing the post immunocapture enrichment procedure.

Materials and methods:

One ml of meat extract prepared as in the previous Example was inoculated with 40 CFU of *E. coli* O157:H7 and loaded on the CBD-anti-*E. coli* IC gauze device using 5 consecutive cycles, or the sample was incubated with DYNAL®-anti-*E. coli* O157 beads. The capture step for both the CBD-IC procedure and the DYNAL® procedure were carried out in 1 minute.

The IC was washed in 5 ml of PBS-TWEEN™ 20 and 5 ml of PBS. The DYNAL® beads were washed as described in the previous Example using the magnet apparatus. An enrichment step was performed with the IC as described above. The DYNAL® beads were incubated in 120 µl of mEC medium in eppendorff tubes for 5 or 6 hours.

The IC samples were treated as in the previous Example. The enriched sample DYNAL® beads was boiled for 20 minutes, followed by addition of 120 µl of PBS. 50 µl of the DYNAL® sample were tested for *E. coli* O157 as described for the IC.

The capture capabilities (% of O157:H7 bacteria captured from total present in 1 ml meat extract sample) of the CBD-IC and the DYNAL® beads were also determined by removing the gauze from the CBD-IC device after the wash step, and plating the gauze or DYNAL® beads onto CT-SMAC plates. Positive white colonies present after an overnight incubation at 37 °C were counted. Presumptive positives were confirmed by the Oxoid agglutination test. Determination of captured bacteria was performed in triplicate and in parallel to the above *in situ* enrichment.

Results:

The results of this example show that the DYNAL® DYNABEADS ® were less effective at detecting *E. coli* O157:H7 from 1 ml of an inoculated meat extract then the CBD-IC, as shown using the post-capture *in situ* enrichment and ELISA procedure (see Figure 13); and also by plating of the textile matrix and the DYNAL® DYNABEADS® beads after the wash step.

The capture capabilities (% of O157:H7 bacteria captured from 40 CFU present in 1 ml meat extract sample) of the CBD-IC was 38-58 % of the total bacteria which was much higher than the capture percent of 17-27 % for the DYNAL® DYNABEADS® beads.

EXAMPLE 13

Comparison of the capture of E. coli O157 in milk by the DYNAL® method and the CBD-IC method

The following example describes a comparison of capturing *E. coli* O157:H7 from milk samples between the DYNAL® DYNABEADS® and CBD-IC methods.

Materials and methods:

(a) CBD-IC procedure:

200 CFU of *E. coli* O157:H7 were inoculated into 1 ml samples of fresh homogenized milk having 1 %, 2 %, and 3 % fat. The milk samples were drawn into CBD-IC in 5 cycles by standard action of lab pipettor in an up and down manner. Each cycle took approximately 30 seconds to complete.

After completion of the capture cycles the CBD-IC was washed with 4.5 ml of PBS-TWEEN™ 20 and then 4.5 ml of PBS. The amount of bacteria captured by the device was calculated by subtracting the amount of bacteria in the wash solutions from the number of bacteria in the original inoculum.

The bacterial concentration in the wash solutions (to which 1 ml of expelled model solution was added) was determined by filter method using GN-6 METRICEL™ on mENDO agar LES medium incubated at 37 °C.

(b) DYNAL® DYNABEADS® beads procedure:

200 CFU *E. coli* O157:H7 were inoculated into 1 ml samples of fresh homogenized milk having 1 %, 2 %, and 3 % fat. *E. coli* O157:H7 bacteria were captured from the milk samples by Immunomagnetic Separation as described in the DYNAL® DYNABEADS® beads anti-*E. coli* O157 protocol. In brief, each 1 ml of spiked milk sample was incubated for 30 minutes with DYNAL® magnetic anti-*E. coli* O157:H7 DYNABEADS®, and then washed twice with PBS-TWEEN™ 20 while using the magnet to concentrate the beads onto the side of the tube before each wash step. The beads were then plated on SMAC selective agar plates in order to enumerate the number of captured bacteria.

Results:

The results of this example are set forth in Figure 14. The CBD-IC method is indifferent to the percent fat present in different milk samples as exhibited by the high percent capture of *E. coli* O157:H7 in the samples with 1 %, 2 % and 3 % fat. The DYNAL® DYNADEADS® method displayed a certain degree of difficulty in carrying out the manufacturers

protocol due to poor concentration of beads by the action of the magnet in samples with 3 % fat and to a lesser degree in samples having 2 % fat. Beads in these samples tended to collect at the bottom of the eppendorf tube despite the magnetic effect on the beads thereby making it difficult to recover the beads during the wash steps. Nevertheless, DYNAL® beads did capture *E. coli* O157:H7 in the milk samples at all fat concentrations although at a lower efficiency than the CBD-IC method.

EXAMPLE 14

Detection of E. coli O157:H7 in milk by ELISA after CBD-IC and in situ enrichment

The following example describes the ELISA detection of *E. coli* O157:H7 captured from 1 % fat UHT long life homogenized and 3% fat fresh homogenized milk samples by CBD-IC followed by *in situ* enrichment.

Materials and methods:

5 CFU or 60 CFU of *E. coli* O157:H7 were inoculated into 250 ml samples of 1 % Fat UHT long life homogenized milk and 30 CFU *E. coli* O157:H7 were inoculated into 250 ml samples of 3 % fat fresh homogenized milk. The milk samples were passed through a CBD-IC by gravity. The total time to pass the entire sample through the device was 25 minutes at room temperature. After immunoconcentration the device was detached from the sample reservoir apparatus and washed twice with 5 ml PBS-TWEEN™ 20 (0.05 %) and twice with 5 ml PBS. 120 µl of mEC medium were placed into a sterile plastic tube for each device. The device was attached to standard 1 ml pipettor and the medium was drawn into device from the tube. The device was then detached from the pipettor and placed into the tube for incubation. The incubation was carried out for five hours at 37 °C.

After incubation, the tubes were placed in a boiling water bath for ten minutes to kill the bacteria therein. The device was reattached to the pipettor and the medium was expelled back into a tube. 120 µl of PBS were then drawn into the device in order to expel and pool the remaining bacteria.

A total of 200 µl of the bacterial solution was recovered from the device and 50 µl used for ELISA analysis as described above.

Results:

The results of the example are set forth in Figures 15 and 16. The control sample was not spiked with bacteria. Figure 15 shows that the

CBD-IC was able to concentrate the bacteria from 1 % fat milk samples following *in situ* enrichment when the inoculum was 60 CFU per 250 ml (the numbers of bacteria in the enriched sample are in Figure 15 above the sample bar). However, when 5 CFU were inoculated into 250 ml 1 % fat milk *E.* coli O157:H7 was detected in only one of three samples. The negatives may have been due to poor distribution of the bacteria in the inoculum, resulting in samples which were not spiked, or may be due to lack of capture of the few CFU's present. Figure 16 shows that the CBD-IC was able to concentrate bacteria from a sample of 3 % fat milk samples. Following *in situ* enrichment the bacteria were detectable by ELISA (the number of bacteria in the enriched sample is above sample bar in Figure 16). The CBD-IC method can be used to capture and concentrate *E. coli* O157:H7 from large volume samples such as 250 ml of milk having 1 % and 3 % fat.

EXAMPLE 15

Capture of E. coli O157:H7 from ground meat extract: pre-enrichment, CBD-IC and in situ enrichment

The following example describes the capture of *E. coli* O157:H7 from ground beef samples that were pre-enriched, concentrated and enriched *in situ*.

Materials and methods:

Twenty grams of beef samples were diluted in a standard stomacher bag with 225 ml mEC and were stomached for 2 minutes. The extract was then filtered twice through a cotton filter (SPUNTECH) and spiked with either 2 CFU of 7 CFU *E. coli* O157:H7. For the control, meat samples without *E. coli* O157:H7 were used. All treatments were carried out in triplicate. The extract samples were pre-enriched by incubating them for 3 hours at 37 °C and then passing them through a CBD-IC at a rate of approximately 7 ml per minute. After immunoconcentration the CBD-IC device was detached from the sample reservoir apparatus and was washed twice with 5 ml of PBS-TWEEN™ 20 (0.05 %) and twice with 5 ml of PBS.

120 µl of mEC medium was placed into a sterile plastic tube for each device. The device was attached to a standard 1 ml pipettor and the medium was drawn into the device from the tube. The device was then detached from the pipettor and placed into the tube for incubation. The tube was incubated at 37 °C for 5 hours. After incubation, the tubes were placed in a boiling water bath for ten minutes to kill the bacteria therein. The

device was reattached to a pipettor and the medium was expelled back into the tube.

120 µl of PBS were drawn into each device in order to expel and pool the remaining bacteria. 50 µl of this solution containing bacteria was analyzed by ELISA as described above.

Results:

The results of the example are set forth in Figure 17. Extremely low concentrations of bacteria were isolated and detected from meat samples by pre-enrichment followed by immunoconcentration using the CBD-IC device, and *in situ* enrichment. This process was carried out entirely without the use of selective reagents such as antibiotics.

EXAMPLE 16

Capture of E. coli O157:H7 by a maltose binding protein (MBP)-antibody conjugate - small volume

The following example demonstrates that microorganisms can be captured onto an amylose coated matrix by a MBP-antibody conjugate.

Materials and Methods:

Preparation of an immunoconcentrator employing maltose binding protein-antibody conjugate (MBP-Ab-IC):

(i) SPDP Conjugation procedure:

1.8 mg MBP (6.0 mg/ml, Maltose Binding Protein-New England Biolabs, Inc. Product No. 800-44L) were mixed with 20-fold molar excess of SPDP (68 mM, MW 312.4 g/mol, N-Succinimidyl 3-(2-pyridyldithio) propionate, Cat. No. P-3415, Sigma) in ethanol. Similarly, 2.2 mg IgG (1.5 mg/ml, KPL, affinity purified antibody Goat anti-*E. coli* O157:H7, Cat. No. 95-90-10, Lot No. TH077) were mixed with 25-fold molar excess of SPDP (68 mM) in ethanol for two hours at room temperature. Thereafter both reactions were dialyzed against PBS, overnight, at 4 °C, and the precipitates were spun down.

The IgG was then reduced by addition of 1 M DTT to a final concentration of 50 mM and incubated for one hour at room temperature. The reduced IgG was then desalted on Sephadex G-25 column (up to 5 % sample/column volume ratio) by equilibration with 50 mM KH_2PO_4, pH 6.5. The reduced SPDP-IgG was mixed with the SPDP-MBP in molar ratio of 1:2.9 IgG:MBP at 4 °C overnight. The precipitate was spin down and stored at 4 °C until use.

(ii) ***Preparation of amylose coated gauze matrix:***

1.3 grams of amylose (Corn Practical grade: Approximately 70 %, Cat. No. A-7043, Sigma) per 100 ml of distilled water were dissolved and boiled. 50 mg pre-cut gauze quadrants were added to the boiling amylose solution. 1-2 minutes later amylose coated gauze quadrants were removed from the boiling amylose solution and were air dried at room temperature. Excess amylose was then washed from the gauze by submerging the coated gauze in 1 liter of Tris buffer pH 7.4 at 60 °C, washing 5 minutes with a stirrer and repeating the wash step two more times, each time using a fresh buffer. Finally, each individual quadrant was washed with 1 ml Tris buffer pH 7.4 and air dried at room temperature.

Preparation of a MBP-Ab-IC capture cartridge:

NaCl was added to the MBP-Ab conjugate to a final concentration of 0.2 M. For control, MBP was dialyzed against 50 mM KH_2PO_4, pH 6.5, with 0.2 M NaCl and then diluted with the same buffer to a final concentration of 0.1 mg/ml. 150 µl of conjugate (MBP-Ab, final concentration 0.1 mg protein/ml as determined by the Bradford method) or MBP alone was added onto a gauze prepacked into a cartridge, incubated for 15 minutes at 37°C, cooled to room temperature and washed twice with 5 ml of PBS. The MBP-Ab-IC and MBP-IC were then ready for use.

Capture and wash step:

One ml of PBS containing 100 CFU's *E. coli* O157:H7 was drawn into the MBP-Ab-IC or the MBP-IC by standard action of lab pipettor, 5 cycles, in an up and down manner. Following the capture step, the cartridge was washed with 10 ml PBS solution. The amount of bacteria captured by the device was calculated by subtracting the amount of bacteria in wash solutions from original inoculum number. One ml of expelled model solution was added to 10 ml wash solution and bacterial concentration was determined by a filter method using GN-6 METRICEL™ (Gelman Sciences Inc., MI) on mENDO agar LES medium and incubated overnight at 37 °C.

Experimental Results:

The results of the example are set forth in Figure 18. These results show that MBP-Ab bound to amylose coated fabric can be used to capture *E. coli* O157:H7 from model solutions.

EXAMPLE 17

Capture of E. coli O157:H7 by a maltose binding protein (MBP)-antibody conjugate - large volume

The following example demonstrates that microorganisms present at low concentrations (140 CFU/250 ml) can be captured and concentrated on an amylose coated matrix by a MBP-antibody conjugate.

Materials and Methods:

MBP-Ab-IC was prepared as described in the Example above. For control, amylose coated matrix was used without MBP-Ab complex.

Capture and wash step: 250 ml of PBS containing 140 CFU's *E. coli* O157:H7 were passed through MBP-Ab-IC or control IC by gravity at a flow rate of 8 ml per minute. Following the capture step, the cartridge was washed with 10 ml PBS solution and this solution was combined with the 250 ml flow through solution. The amount of bacteria captured by the device was calculated by subtracting the amount of bacteria in flow through and wash solutions, combined, from the original inoculum number. The number of CFU in these solutions was determined by filter method using GN-6 METRICEL™ on mENDO agar LES medium and overnight incubation at 37°C

Experimental Results:

The results of the example are set forth in Figure 19. These results show that MBP-Ab bound to amylose coated fabric can be used to capture and concentrate *E. coli* O157:H7 from model solutions when target bacteria are present at low concentrations in large volume samples.

EXAMPLE 18

Capture of E. coli O157:H7 from 25 ml PBS by a CBD-antibody cellulose beads complex

The following example demonstrates that microorganisms can be captured on cellulose bead matrix by a CBD-antibody conjugate.

Materials and Methods:

Preparation of a CBD-IC cellulose beads capture cartridge:

25 µl CBD-IgG conjugate prepared as described in Example 1 above were diluted to 200 µl with PBS and added to 100 mg of ORBICELL™ beads (45-75 µm, non-porous, Accurate Polymers, Ltd.) or VISKASE® beads (VISKASE® - Applied Technologies Group - spherical cellulose beads-wide diameter range approx. 30-200 µm, non-porous) and incubated for 1 hour with inversion at room temperature. The beads were then loaded into a 1 ml column with a frit at the bottom and then washed with 5 ml PBS.

Capture and wash step: 25 ml of PBS containing 52 CFU or 110 CFU *E. coli* O157:H7 for ORBICELL™ and VISKASE®, respectively, were loaded into a 50 ml reservoir and then passed through the CBD-Ab cellulose bead column. Following the capture step, the cartridge was

washed with 5 ml PBS-TWEEN™ 20 (0.05 %) and then with 7 ml PBS solution. The beads were then removed from the column with 1 ml PBS, which was then plated together with the beads directly onto mENDO agar LES for overnight incubation and plate count. The amount of bacteria captured by the device was calculated by counting the number of colonies that grew on the plates containing the beads as well as by subtracting the amount of bacteria in wash solutions from original inoculum number. The 12 ml wash solutions were collected and bacterial concentration was determined by filter method using GN-6 METRICEL™ on mENDO agar LES medium and overnight incubation at 37 °C.

Experimental Results:

The results of the example are set forth in Figure 20, demonstrating that CBD-Ab-ORBICELL™ and CBD-Ab-VISKASE® complexes capture and concentrate *E. coli* O157:H7 from PBS model solutions. There is a discrepancy between the number of bacteria captured by VISKASE® beads as determined by subtracting non-captured from input as opposed to CFU that grew on plated beads. The reason for this is unknown but it may be due to more than 1 bacteria being captured to a single bead. The exact size of these beads is unknown but the range is much greater than the ORBICELL ™ beads and includes beads of up to 200 μm.

These results show that CBD-Ab bound to cellulose beads can be used to capture and concentrate bacteria from dilute solutions of target microorganisms.

EXAMPLE 19

Detection of Salmonella typhimurium in meat extracts at different spike levels

The following example illustrates the selective immunocapture and concentration of *Salmonella typhimurium* from meat extracts.

Materials and methods:

Preparation of a CBD-IC capture cartridge: 50 mg cotton gauze was packed into a CBD-IC cartridge and then 150 μl of a solution containing CBD-anti *Salmonella* sp. gluteraldehyde conjugate (Lot. No. GM-980719) diluted 1:6 in PBS was loaded into the cartridge, incubated for 15 minutes at 37°C, cooled to room temperature and washed twice with 5 ml of PBS-TWEEN ™ 20 and then washed twice with 5 ml of PBS.

Capture and detection:

25 grams of ground beef samples (Lot. No. SC 73098) were diluted with 225 ml PBS (to yield total extract volume of about 250 ml) and stomached for 2 minutes. The stomached samples were filtered through a SPUNTECH filter and then 200 ml extract aliquots were spiked with 200, 100, 50, 15 or zero CFU (duplicates at each level) of *Salmonella typhimurium*. The spiked samples were passed through a CBD-IC anti *Salmonella* sp. device. The CBD-IC was then washed with 10 ml PBS-TWEEN™ 20 and then with 10 ml PBS. 120 µl of M-Broth without Novobiocin was added to the device and *Salmonella* was enriched by incubation at 37 °C for six hours. After amplification the sample was boiled to kill the captured bacteria and thereafter expelled into a collection tube. Additional 120 µl PBS were added, expelled and pooled with the previous portion in the collection tube. ELISA was performed on the pooled sample, 50 µl per well, using Monoclonal (Clone M-32242) anti *Salmonella Typhimurium*. As controls served meat extracts without spike.

Experimental results:

The results of the example are set forth in Figure 21 demonstrating that for spike levels equal or greater than 50 CFU per 250 ml beef extract, all trials were positive. However, at 15 CFU per 250 ml beef extract only one of the three trials resulted in a positive ELISA assay. These results demonstrate that the CBD-IC can be used to capture, concentrate and detect *Salmonella* sp. when these organisms are found at extremely low concentrations in highly particulated food samples.

EXAMPLE 20

Sensitivity of CBD-IC for S. typhimurium with one hour pre-enrichment step

The following example illustrates the selective immunocapture and concentration of *Salmonella typhimurium* from meat extracts following brief pre-enrichment.

Materials and methods:

Capture and wash step:

225 ml buffered peptone water were added to 25 grams of ground beef (Lot. No. Rub 81398) which was then stomached for one minute using a SEWARD 400 stomacher. The stomached beef sample was then filtered through a cotton filter (SPUNTECH cotton) and the extract was inoculated with 28, 14, 7 or zero CFU's *S. typhimurium* and, pre-enriched for 1 hour at

37 °C and then passed through CBD-IC anti-*Salmonella* device (prepared as described above). Following the capture step, the cartridge was washed once with 10 ml PBS-TWEEN™ 20 (0.05 %) and then with 10 ml of PBS from top to bottom. 120 µl of M-Broth without Novobiocin was added to the device and the *Salmonella* were enriched at 37 °C for seven hours. After enrichment the sample was boiled and expelled into a collection tube. Additional 120 µl PBS were added, expelled and pooled with the previous portion in the collection tube. ELISA was performed on the pooled sample, 50 µl per well, using Monoclonal (Clone M-32242) anti *Salmonella Typhimurium*. Controls were meat without spike.

Results:

The results of the example are set forth in Figure 22, demonstrating that *Salmonella Typhimurium* can be captured, concentrated and detected for spike levels as low as 7 CFU per 225 ml beef extract. These results demonstrate that the CBD-IC can be used to capture, concentrate and detect *Salmonella* sp. When these organisms are found at extremely low concentrations in highly particulate food samples and that sensitivity can be enhanced by incorporating a short pre-enrichment step using a non-specific growth medium.

EXAMPLE 21

Capture and detection of E. coli O157:H7 in 1 ml PBS samples using polyester bound IgG

The following example illustrates the selective immunocapture and concentration of E. coli O157:H7 from 1 ml PBS using IgG bound to polyester matrix.

Materials and methods:

Preparation of a polyester IgG-IC capture cartridge:

15 mg polyester hydrophobic synthetic polymer foam (Dec 632/45 ppi reticulated polyester 23-27 Kg/M^3 density, Recticel Declon, UK) were packed into an IC cartridge (6 mm in diameter x 10 mm) and then 150 µl of a solution containing or 40 µg anti *E. coli* O157:H7 goat antibodies in PBS (IgG-IC) were loaded into the cartridge and incubated at room temperature either overnight or for one hour and washed three times with 1 ml of PBS.

Capture and Detection:

One ml of PBS containing 91 CFU's *E. coli* O157:H7 was drawn into the IgG-IC by standard action of lab pipettor, 5 cycles, in an up and down manner. Following the capture step, the cartridge was washed with 10 ml

PBS solution. The amount of bacteria captured by the device was calculated by subtracting the amount of bacteria in wash solutions from original inoculum number. One ml of expelled model solution was added to 10 ml wash and bacterial concentration was determined by filter method using GN-6 METRICEL on mENDO agar LES medium and overnight incubation at 37 °C.

Enrichment:

For each device, 150 µl of mEC medium were placed into a sterile plastic tube. The device was attached to a standard 1 ml pipettor and the medium was drawn into the device from the tube. The device was then detached from the pipettor and placed into the tube for incubation. Incubation was carried out at 37 °C for a period of 5.5 hours. After incubation, each device was placed in a boiling bath for ten minutes to kill the bacteria. The device was then reattached to the pipettor and the medium was expelled back into the tube. 100 µl of PBS were drawn into the device in order to expel and pool the remaining bacteria. A total of 200 µl of bacterial solution was recovered from the device and 50 µl were then used for ELISA analysis.

Experimental results:

The results of the example are set forth in Figures 23a-b, demonstrating that the polyester hydrophobic synthetic polymer foam can be used to immunocapture, enrich and detect *E. coli* O157:H7 when these organisms are found at low concentrations in model solutions.

EXAMPLE 22

Capture and detection of E. coli O157:H7 in 1 ml PBS samples using polyester bound polyester bound anti O157:H7 IgG

The following example illustrates the selective immunocapture and concentration of E. coli O157:H7 from 1 ml PBS using IgG bound to polyester matrix.

Materials and methods:

Preparation of a polyester IC cartridge:

50 mg CELLONA® (100 % polyester padding, Lohmann GmbH & Co. KG, Neuwied, Germany) were packed into an IC cartridge (15 mm x 30 mm) and then 150 µl of a solution containing 40 µg anti *E. coli* O157:H7 goat antibodies in PBS were loaded into the cartridge (IgG-IC) and incubated at room temperature for 1 hour. Then, each device was washed three times with 1 ml of PBS.

Capture and detection: 1 ml of PBS containing 113 CFU's *E. coli* O157:H7 was drawn into the IgG-IC by a standard action of a lab pipettor, 5 cycles, in an up and down manner. Following the capture step, the cartridge was washed with 10 ml PBS solution. The amount of bacteria captured by the device was calculated by subtracting the amount of bacteria in the wash solutions from the original inoculum number. One ml of expelled model solution was added to 10 ml wash solution and the bacterial concentration was determined by a filter method using GN-6 METRICEL™ on mENDO agar LES medium and overnight incubation at 37 °C.

Enrichment:

For each device, 150 µl of mEC medium were placed into a sterile plastic tube. The device was attached to a standard 1 ml pipettor and the medium was drawn into the device from the tube. The device was then detached from the pipettor and placed into the tube for incubation. Incubation was carried out at 37 °C for a period of 5 hours. After incubation, each device was placed in a boiling bath for ten minutes to kill the bacteria. The device was then reattached to the pipettor and the medium was expelled back into the tube. 100 µl of PBS were drawn into the device in order to expel and pool the remaining bacteria. A total of 200 µl of bacterial solution was recovered from the device and 50 µl were then used for ELISA analysis.

Results:

The results of the example are set forth in Figure 24, demonstrating that a high percentage, 50 %, of the 113 CFU present in the 1 ml of the model PBS solution were captured. These results demonstrate that the synthetic polyester polymer CELLONA® can be used to immunocapture *E. coli* O157:H7 cells when these organisms are found at low concentrations in a model solution.

EXAMPLE 23

Capture and detection of E. coli O157:H7 in 250 ml modified Buffered Peptone Water (mBPW) samples using polyester bound anti O157:H7 IgG

The following example illustrates the selective immunocapture and concentration of *E. coli* O157:H7 from 250 ml mBPW using IgG bound to a polyester matrix.

Materials and methods:

Preparation of a polyester IgG-IC capture cartridge:

50 mg CELLONA® (100 % polyester padding, Lohmann GmbH & Co. KG, Neuwied, Germany) were packed into an IC cartridge (15 mm x 30 mm) and then 150 µl of a solution containing 20 µg anti *E. coli* O157:H7 goat antibodies (IgG, KPL, affinity purified antibody Goat anti-*E. coli* O157:H7 Cat. No. 95-90-10, Lot No. TH077) in PBS were loaded into the cartridge (IgG-IC) and incubated at room temperature for 1 hour. Then, each device was washed three times with 1 ml of PBS. For control 50 mg CELLONA® polyester incubated with PBS alone was used.

Capture and wash step:

225 ml mBPW (BACTO® Modified Buffered Peptone Water: Difco Laboratories, Detroit MI) were inoculated with 91 CFU's of *E. coli* O157:H7 and then passed through the cartridges by a gravity pass over 30 minutes. Following the capture step, the cartridge was washed twice with 5 ml of PBS.

Enrichment:

For each device, 150 µl of mEC medium were placed into a sterile plastic tube. The device was attached to a standard 1 ml pipettor and the medium was drawn into the device from the tube. The device was then detached from the pipettor and placed into the tube for incubation. Incubation was carried out at 37 °C for a period of 5 hours. After incubation, each device was placed in a boiling bath for ten minutes to kill the bacteria. The device was then reattached to the pipettor and the medium was expelled back into the tube. 100 µl of PBS were drawn into the device in order to expel and pool the remaining bacteria. A total of 200 µl of bacterial solution was recovered from the device and 50 µl were then used for ELISA analysis.

Results:

The results of the example are set forth in Figure 25, demonstrating that 15 % of the 91 CFU which were present in the 250 ml sample were captured by IC. These results demonstrate that the synthetic polyester polymer can be used to immobilize capture agents in order to concentrate, enrich and detect *E. coli* O157:H7 when these organisms are found at extremely low concentrations in large volumes of the model solution.

EXAMPLE 24

Concentration and detection of E. coli O157:H7 in ground beef extracts using polyester bound anti O157:H7 IgG

The following example illustrates the selective immunocapture and concentration of *E. coli* O157:H7 from 225 ml ground beef extracts using IgG bound to a polyester matrix.

Materials and methods:

Preparation of a polyester IC capture cartridge:

50 mg CELLONA® (100 % polyester padding, Lohmann GmbH & Co. KG, Neuwied, Germany) were packed into an IC cartridge (15 mm x 30 mm) and then 150 µl of a solution containing 20 µg anti *E. coli* O157:H7 goat antibodies (IgG, KPL, affinity purified antibody Goat anti-*E. coli* O157:H7 Cat. No. 95-90-10, Lot No. TH077) in PBS were loaded into the cartridge (IgG-IC) and incubated at room temperature for 1 hour. Then, each device was washed three times with 1 ml of PBS. For control 50 mg CELLONA® polyester incubated with PBS alone was used.

Capture and wash step:

225 ml mBPW (BACTO® Modified Buffered Peptone Water: Difco Laboratories, Detroit MI) were added to 25 grams of ground beef which was then stomached for one minute using a SEWARD 400 stomacher. The stomached beef sample was then filtered through a cotton filter (SPUNTECH™ cotton) and the extract was inoculated with 0 CFU or 136 CFU's of *E. coli* O157:H7 and then passed through the cartridges by a gravity pass over 30 minutes. Following the capture step, the cartridge was washed twice with 5 ml of PBS TWEEN™ 20 (0.05 %).

Enrichment:

For each device, 150 µl of mEC medium were placed into a sterile plastic tube. The device was attached to a standard 1 ml pipettor and the medium was drawn into the device from the tube. The device was then detached from the pipettor and placed into the tube for incubation. Incubation was carried out at 37 °C for a period of 5 hours. After incubation, each device was placed in a boiling bath for ten minutes to kill the bacteria. The device was then reattached to the pipettor and the medium was expelled back into the tube. 100 µl of PBS were drawn into the device in order to expel and pool the remaining bacteria. A total of 200 µl of bacterial solution was recovered from the device and 50 µl were then used for ELISA analysis.

Results:

The results of the example are set forth in Figure 26, demonstrating that a portion of the 136 CFU present in the meat extract were captured and amplified during the 5 hour incubation period, whereas the matrix is specific and that the sample itself is negative. These results demonstrate that the synthetic polyester polymer can be used to immobilize capture agents in order to concentrate, enrich and detect *E. coli* O157:H7 when these organisms are found at extremely low concentrations in large volumes of meat extract solutions containing particulate matter.

EXAMPLE 25

Capture and concentration of E. coli O157:H7 at low concentrations in 225 ml modified Buffered Peptone Water (mBPW) samples using CBD-anti-O157:H7 IgG

The following example illustrates the selective immunocapture and concentration of *E. coli* O157:H7 present at low concentrations in 225 ml mBPW using CBD-anti-O157:H7 IgG bound to a gauze matrix.

Capture and wash step:

225 ml mBPW (BACTO® Modified Buffered Peptone Water: Difco Laboratories, Detroit MI) were inoculated with between 1-9 CFU's of *E. coli* O157:H7 or between 10 and 19 CFU's and then passed through cartridges by a gravity pass over 30 minutes. Following the capture step, the each cartridge was washed twice with 5 ml PBS-TWEEN™ 20 and then with 5 ml PBS by passing the wash solution through the device from top to bottom. The wash fluid was collected and added to the 225 ml flow through and further analyzed by the filter method described above to determine percent of bacteria that were not immunocaptured by the CBD-IC. The percentage of captured bacteria was determined by plating the gauze matrix directly onto mENDO agar LES medium and incubating overnight at 37 °C followed by counting CFU's which formed.

Results:

The results of the example are set forth in Table 8, demonstrating that in 78 % of the samples which contained between 1-9 CFU's per 225 ml and in 100 % of the samples which contained between 10-19 CFU's per 225 ml at least 1 CFU was captured by the CBD-IC device.

These results demonstrate that the CBD-IC device can be used to capture *E. coli* O157:H7 found at low concentrations in large volumes of

model media solution in order to concentrate the pathogen for further analysis.

Table 8

Capture of O157:H7 from 225 ml mBPW		
CFU's per 250 ml	Total number of samples	Number of positives samples*
1-9	9	7 (78 %)
10-19	9	9 (100 %)

* Positive defined as at least 1 CFU captured on matrix

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications cited herein are incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1. A method of concentrating a particular microorganism or microorganisms in a sample, the method comprising the step of contacting said sample with a cellulosic or chitin matrix to which is bound a cellulose binding protein (CBP) -receptor or cellulose binding domain (CBD) -receptor conjugate specific for said microorganism or microorganisms.

2. The method of claim 1, wherein said sample contains from at least 0.008 CFU/ml to about 10^3 CFU/ml of the microorganism or microorganisms.

3. The method of claim 1, wherein said sample contains from at least 0.001 CFU/ml to about 10^3 CFU/ml of the microorganism or microorganisms.

4. The method of claim 1, wherein said sample contains from at least 0.00025 CFU/ml to about 10^3 CFU/ml of the microorganism or microorganisms.

5. The method of claim 1, further comprising the step of washing said matrix to remove unbound material from said matrix.

6. The method of claim 1, further comprising the step of enriching the concentrated microorganism or microorganisms *in situ* by addition to said matrix of a culture medium with or without antibiotics.

7. The method of claim 6, wherein the culture medium is without antibiotics.

8. The method of claim 1, further comprising the step of performing a method of detecting any microorganisms that bind to said CBP-receptor or CBD-receptor conjugate bound to said matrix.

9. The method of claim 8, wherein the method of detecting any microorganisms is an immunoassay, or a nucleic acid signal amplification method.

10. The method of claim 9, wherein the nucleic acid signal amplification method is a polymerase chain reaction.

11. The method of claim 1, wherein said matrix is a cellulose fabric or thread, cellulose beads or chitin beads, or a cellulose sponge or thread.

13. The method of claim 11, wherein said cellulose fabric is gauze.

14. The method of claim 1, wherein said sample is contacted with said matrix in a single pass.

15. The method of claim 1, wherein said sample is contacted with said matrix in a single cycle.

16. The method of claim 1, wherein said sample is contacted with said matrix in more than one cycle.

17. The method of claim 1, wherein said sample is in a volume of 2 ml or less.

18. The method of claim 1, wherein said sample is in a volume of 2 ml to 4 liters.

19. The method of claim 1, wherein said sample is in a volume of 2 ml to 10 liters.

20. The method of claim 1, wherein said cellulose binding domain is obtainable from a bacterial, fungal, slime mold, or nematode protein or polypeptide.

21. The method of claim 1, wherein said cellulose binding domain is obtainable from a phage display library, a combinatorial library or a nucleic acid library.

22. The method of claim 1, wherein said cellulose binding domain is obtainable from a *Clostridium cellulovorans, Clostridium thermocellum or Cellulomonas fimi* protein or polypeptide.

23. The method of claim 20, wherein said cellulose binding domain is obtainable from a *Clostridium cellulovorans* protein or polypeptide.

24. The method of claim 1, wherein the microorganism is a bacterium, virus, fungus, protozoan, or nematode.

25. The method of claim 24, wherein the bacterium is an *Escherichia, Salmonella, Listeria, Campylobacter, Legionella, Clostridium, Pseudomonas, or Shigella.*

26. The method of claim 1, wherein the microorganisms are coliforms.

27. The method of claim 1, wherein the sample is a liquid extract of a solid sample.

28. A method of concentrating a particular microorganism or microorganisms in a sample, comprising the step of contacting said sample with a cellulose or chitin matrix to which is bound a cellulose binding protein (CBP)-receptor or cellulose binding domain (CBD)-receptor conjugate specific for said microorganism or microorganisms to concentrate said microorganism or microorganisms and enriching the concentrated microorganism or microorganisms by addition of a culture medium with or without antibiotics to said matrix.

29. The method of claim 28, further comprising the step of performing a method of detecting any microorganism or microorganisms that binds to said CBP-receptor or CBD-receptor conjugate bound to said matrix.

30. The method of claim 8, wherein the method of detecting a microorganism is performed using a biosensor.

31. The method of claim 25, wherein the bacterium is *Escherichia coli.*

32. The method of claim 31, wherein the *E. coli, is E.* coli 0157:H7.

33. The method of claim 25, wherein the bacterium is a *Salmonella sp.*

34. A method of concentrating a particular microorganism or microorganisms in a sample, comprising the step of contacting said sample with a matrix to which is bound an affinity receptor specific for said particular microorganism or microorganisms, said affinity receptor and said matrix being selected so as to allow capture of said microorganism or microorganisms to said matrix via said affinity receptor when present in said sample at a concentration of at least 0.00025 CFU/ml to about 10^3 CFU/ml, thereby obviating the need for a prolonged pre-enrichment step of said microorganism or microorganisms in said sample.

35. The method of claim 34, wherein said concentration is of at least 0.001 CFU/ml to about 1 CFU/ml.

36. The method of claim 34, wherein said matrix is selected from the group consisting of natural and synthetic matrices.

37. The method of claim 34, wherein said matrix is a polysaccharide matrix.

38. The method of claim 37, wherein said polysaccharide matrix is selected from the group consisting of a cellulosic matrix, an agarose matrix, a chitin matrix, a starch matrix and a matrix of cellulose, agarose, chitin and starch derivatives.

39. The method of claim 34, wherein said matrix is a coated matrix.

40. The method of claim 34, wherein said matrix is a synthetic matrix selected from the group consisting of a polypropylene matrix,

polyester matrix, a polyamide matrix, a polyethylene matrix, an acrylamide matrix, a methacrylate matrix, a sepharose matrix, a polystyrene matrix and matrices which are derivatives thereof.

41. The method of claim 34, wherein said matrix is in a form selected from the group consisting of beads, threads, a cloth, a woven material, a non-woven material, a membrane, a powder, a foam and a sponge.

42. The method of claim 34, wherein said matrix is selected from the group consisting of a porous matrix and a non-porous matrix.

43. The method of claim 34, wherein said affinity receptor is directly bound to said matrix via covalent or non-covalent interactions.

44. The method of claim 34, wherein said affinity receptor forms a part of a conjugate or fusion including a counterpart moiety having affinity to said matrix.

45. The method of claim 44, wherein said counterpart moiety is a matrix binding peptide.

46. The method of claim 45, wherein said matrix binding peptide is a polysaccharide binding protein or domain.

47. The method of claim 46, wherein said polysaccharide binding protein or domain is a cellulose binding protein or domain.

48. The method of claim 47, wherein said cellulose binding protein or domain is from a bacterial, fungal, slime mold, or nematode.

49. The method of claim 45, wherein said matrix binding peptide is from a phage display library, a combinatorial library or a nucleic acid library.

50. The method of claim 46, wherein said cellulose binding protein or domain is from a species selected from the group consisting of

Clostridium cellulovorans, Clostridium thermocellum and Cellulomonas fimi.

51. The method of claim 34, wherein said concentration of said microorganism or microorganisms in said sample is from at least 0.001 CFU/ml to about 10^3 CFU/ml.

52. The method of claim 34, wherein said concentration of said microorganism or microorganisms in said sample is from at least 0.008 CFU/ml to about 10^3 CFU/ml.

53. The method of claim 34, further comprising the step of washing said matrix to remove unbound material from said matrix.

54. The method of claim 34, further comprising the step of enriching the concentrated microorganism or microorganisms *in situ* by addition to the matrix of a culture medium selected from the group consisting of an antibiotics-containing culture medium and an antibiotics-free culture medium.

55. The method of claim 54, wherein said culture medium is an antibiotics-free culture medium.

56. The method of claim 34, further comprising the step of performing a method of detecting any microorganisms that bind to said affinity receptor bound to said matrix.

57. The method of claim 56, wherein said method of detecting any microorganisms is selected from the group consisting of an immunoassay and a nucleic acid signal amplification method.

58. The method of claim 57, wherein said nucleic acid signal amplification method is a polymerase chain reaction.

59. The method of claim 56, wherein said method of detecting any microorganisms is effected by a biosensor.

60. The method of claim 34, wherein said sample is contacted with said matrix in a single pass.

61. The method of claim 34, wherein said sample is contacted with said matrix in a single cycle.

62. The method of claim 34, wherein said sample is contacted with said matrix in more than one cycle.

63. The method of claim 34, wherein said sample is in a volume of 2 ml or less.

64. The method of claim 34, wherein said sample is in a volume of 2 ml to 4 liters.

65. The method of claim 34, wherein said sample is in a volume of 2 ml to 10 liters.

66. The method of claim 34, wherein said microorganism is selected from the group consisting of a bacterium, a virus, a fungus, a protozoan and a nematode.

67. The method of claim 66, wherein said bacterium is of a genus selected from the group consisting of *Escherichia, Salmonella, Listeria, Campylobacter, Legionella, Clostridium, Pseudomonas* and *Shigella.*

68. The method of claim 34, wherein said microorganisms are coliforms.

69. The method of claim 34, wherein said sample is a liquid extract of a solid sample.

70. The method of claim 1, further comprising a prefiltration step for removal of debris in a form selected from the group consisting of particulates, fatty materials, starchy materials and proteinaceous materials from said sample.

71. The method of claim 34, further comprising a prefiltration step for removal of debris in a form selected from the group consisting of particulates, fatty materials, starchy materials and proteinaceous materials from said sample.

72. A filtering device comprising:

(a) a housing defining a space having an inlet and an outlet forming a filtration path therein;

(b) a first filter layer including a cotton filter being within said housing in said filtration path;

(c) a second filter layer including a polyester filter being within said housing in said filtration path; and

(d) a third filter layer including a polypropylene filter being within said housing in said filtration path.

73. A filtering device comprising:

(a) a housing defining a space having an inlet and an outlet forming a filtration path therein;

(b) a first filter layer including a polyurethane filter being within said housing in said filtration path;

(c) a second filter layer including a non-woven filter being within said housing in said filtration path; and

(d) a third filter layer including a polypropylene filter being within said housing in said filtration path.

74. The filtering device of claim 73, further comprising:

(e) a fourth filter layer including a hollow body of woven mesh being within said housing in said filtration path.

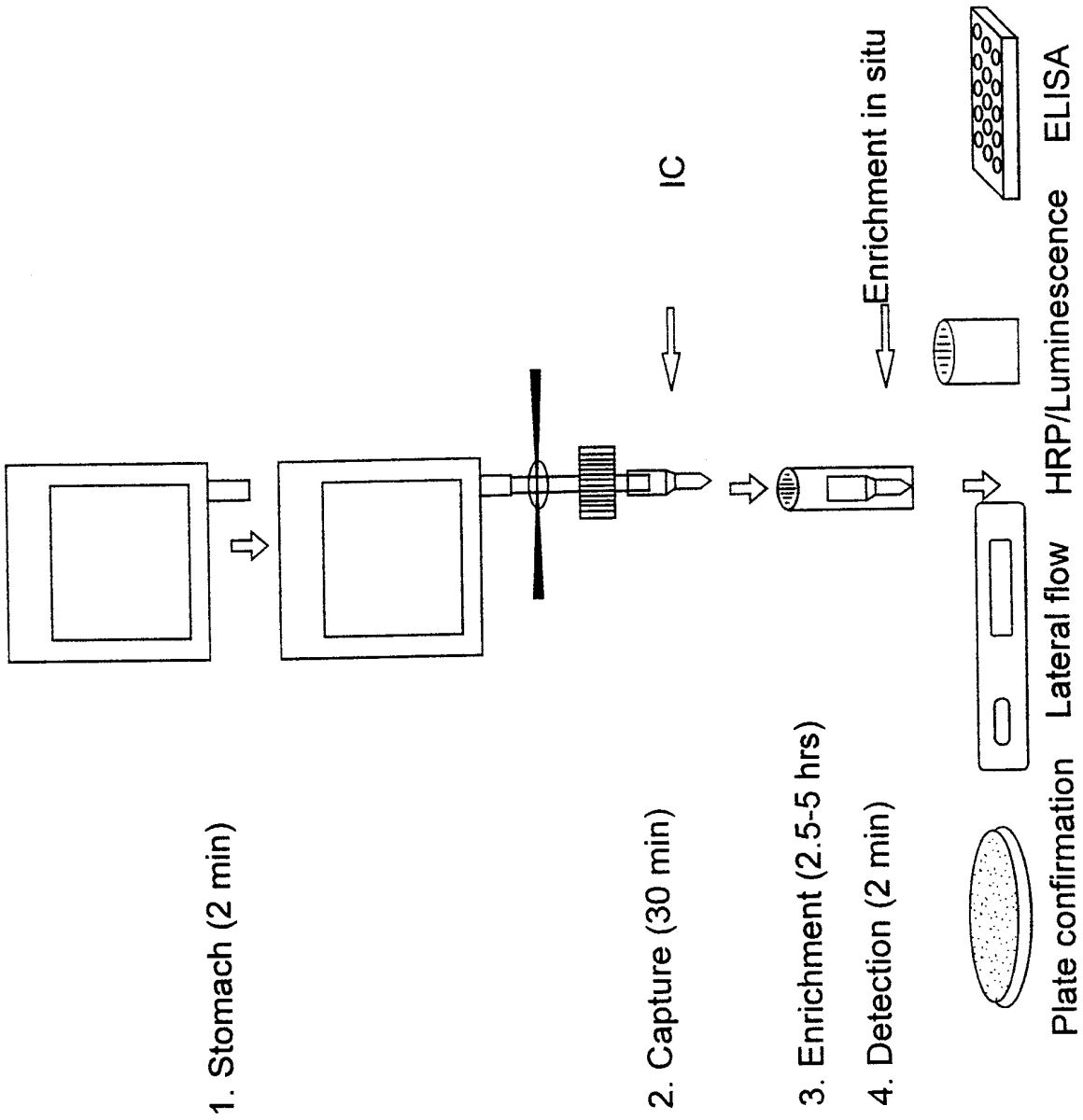

1. Stomach (2 min)
2. Capture (30 min)
IC
3. Enrichment (2.5-5 hrs)
Enrichment in situ
4. Detection (2 min)
Plate confirmation
Lateral flow
HRP/Luminescence
ELISA

Fig. 1

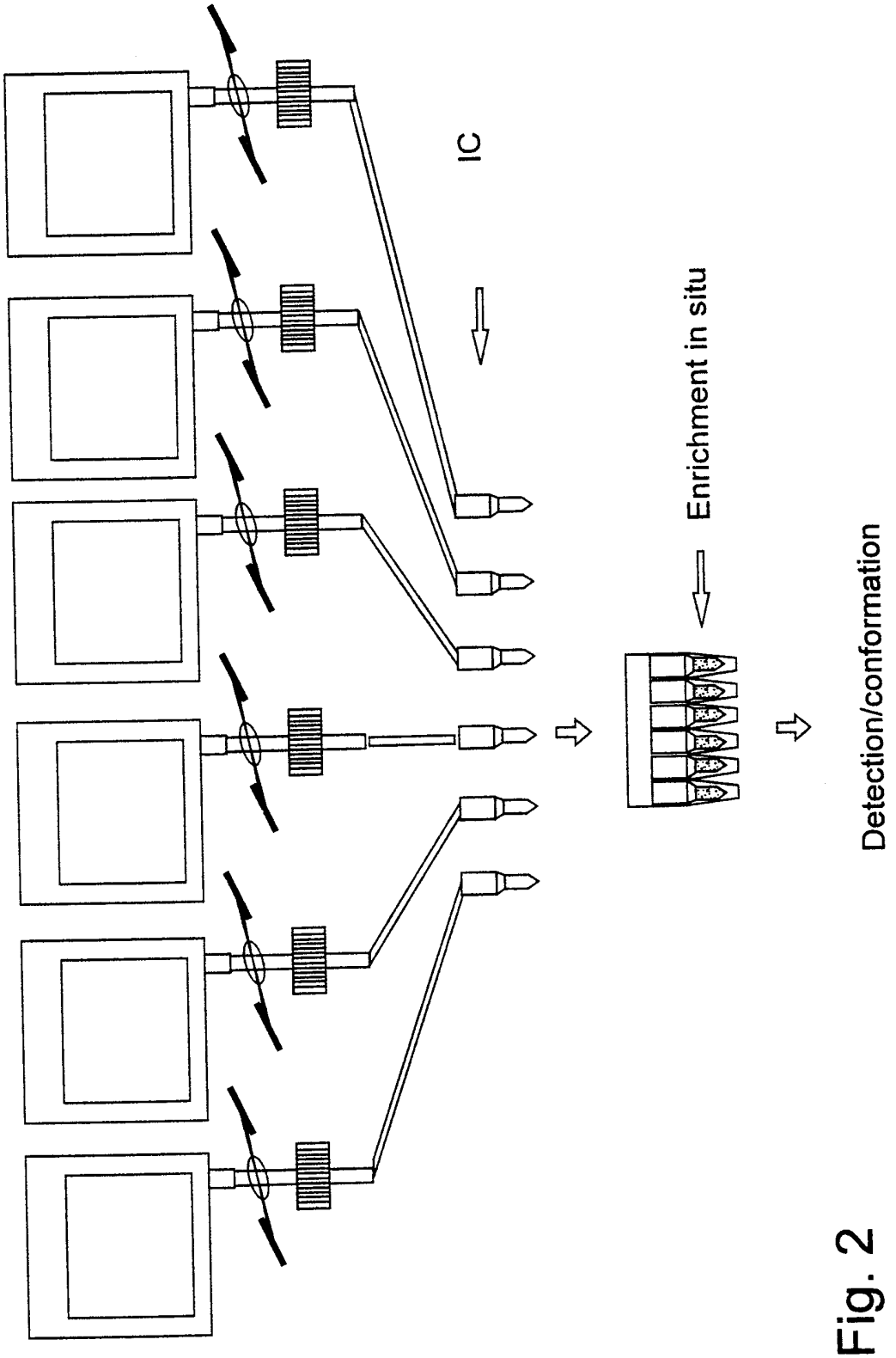


Fig. 2

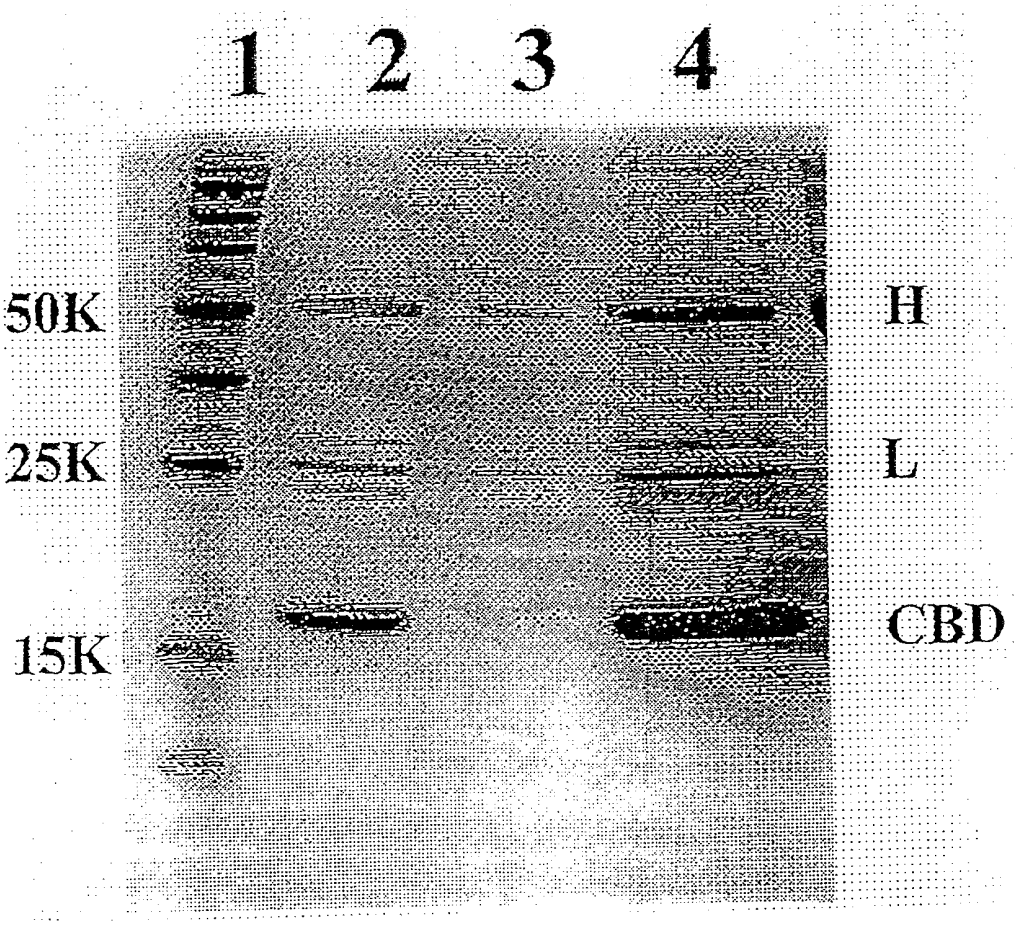


Fig. 3

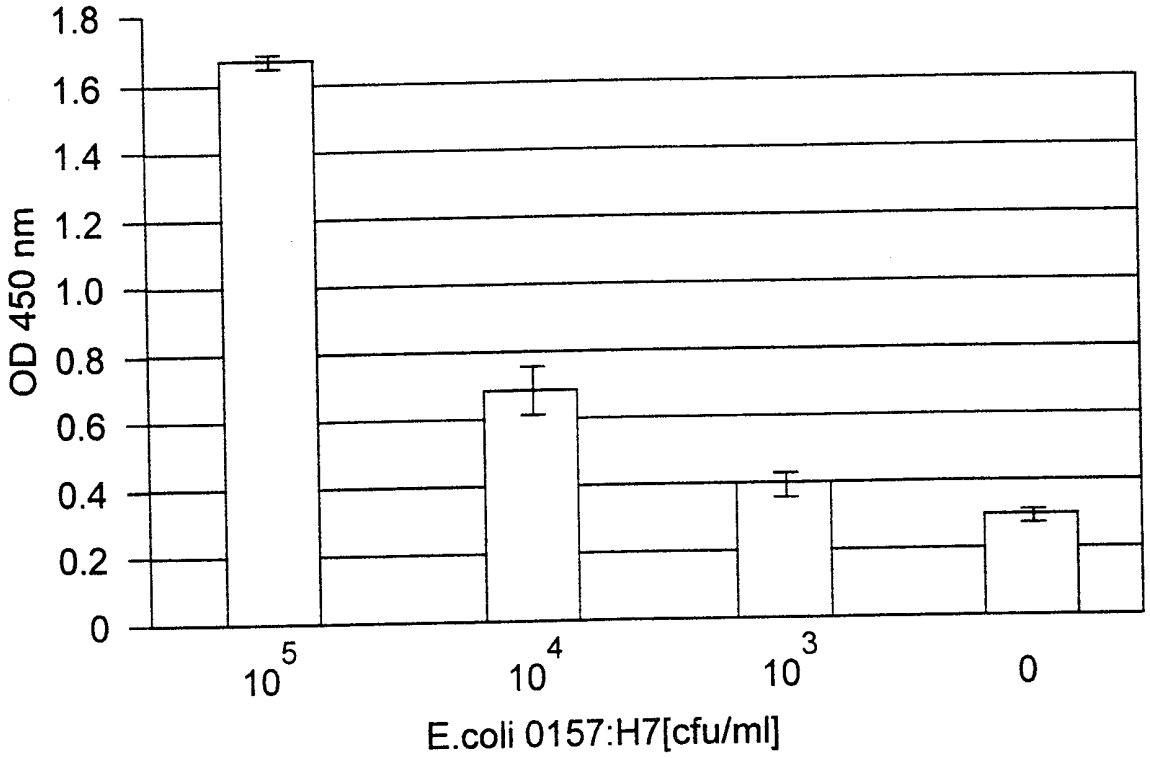
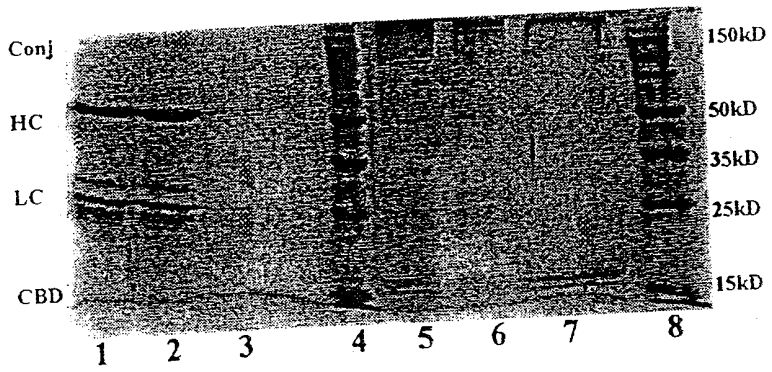

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
OD 450 nm
10^5
10^4
10^3
0
E.coli 0157:H7[cfu/ml]

Fig. 4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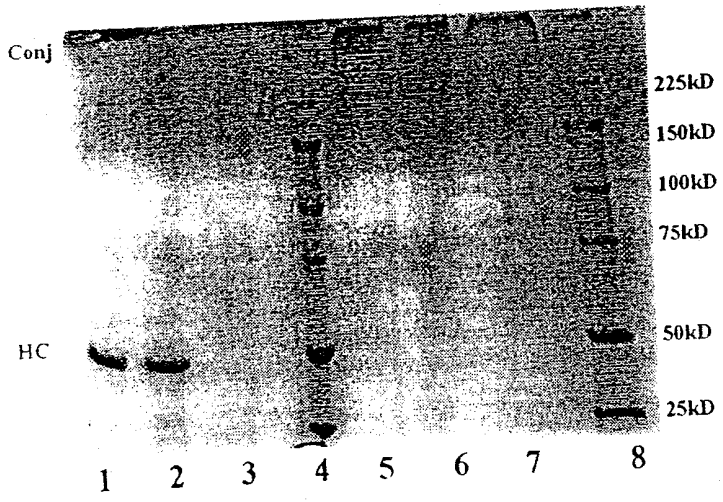
LC
CBD
150kD
50kD
35kD
25kD
15kD
1 2 3 4 5 6 7 8

Fig. 5A


Conj
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225kD
150kD
100kD
75kD
50kD
25kD
1 2 3 4 5 6 7 8

Fig. 5B

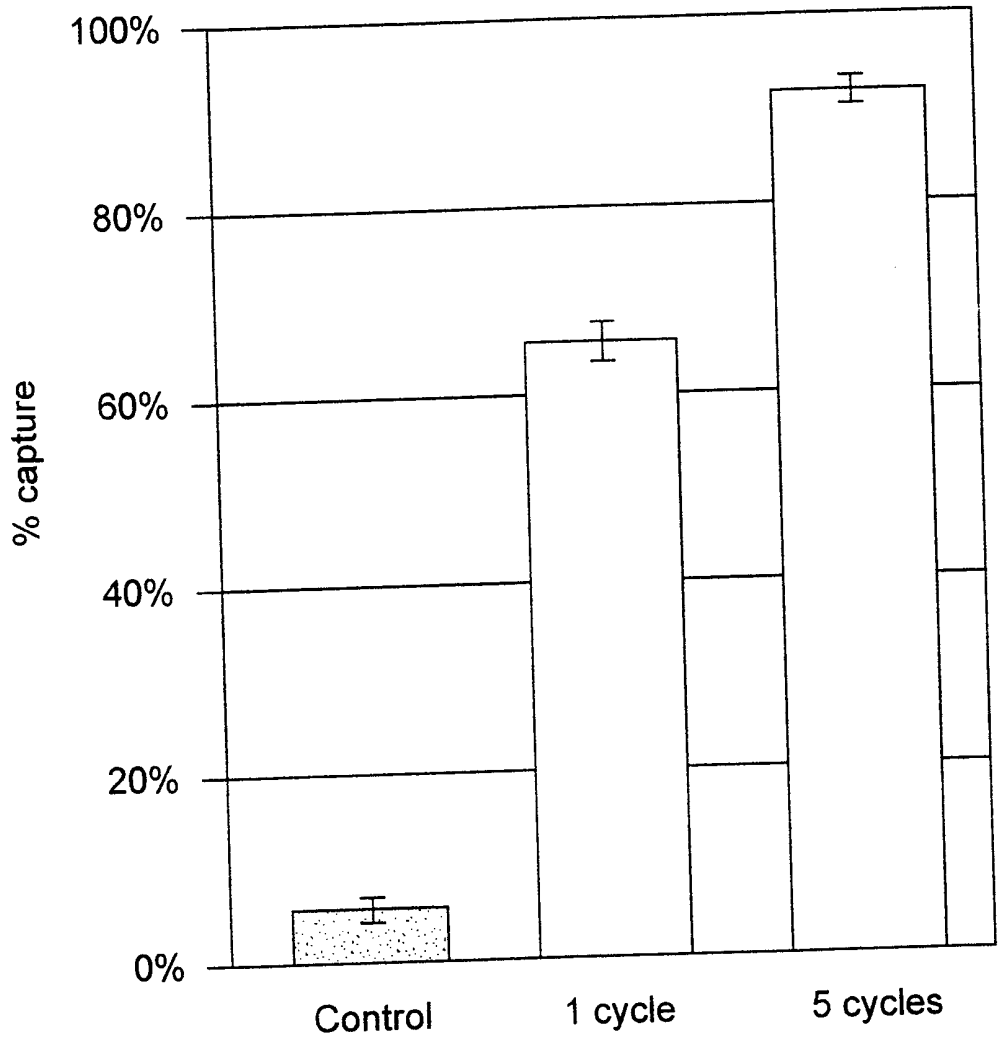

Fig. 6

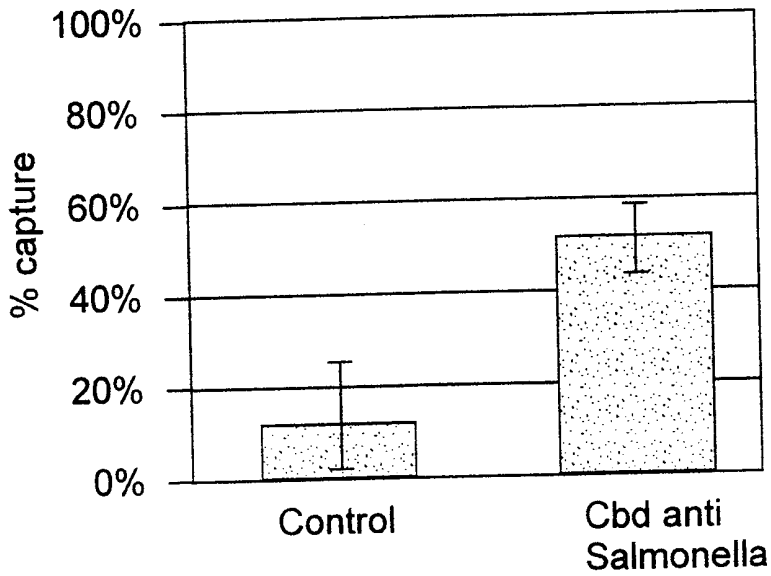
100%
80%
60%
40%
20%
0%
% capture
Control
Cbd anti Salmonella

Fig. 7

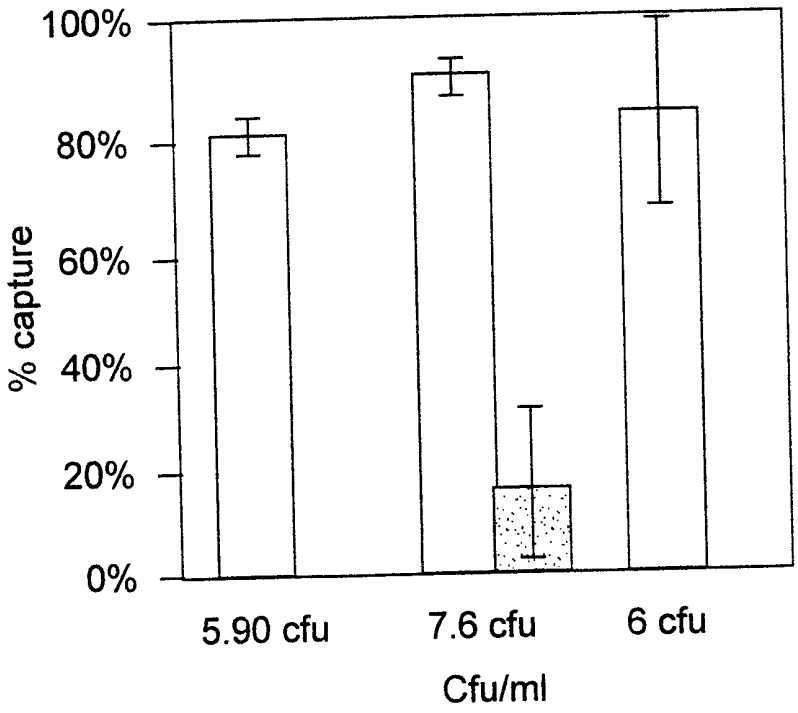
100%
80%
60%
40%
20%
0%
% capture
5.90 cfu
7.6 cfu
6 cfu
Cfu/ml
CBD anti E coli: 0.157 H7
Control

Fig. 8

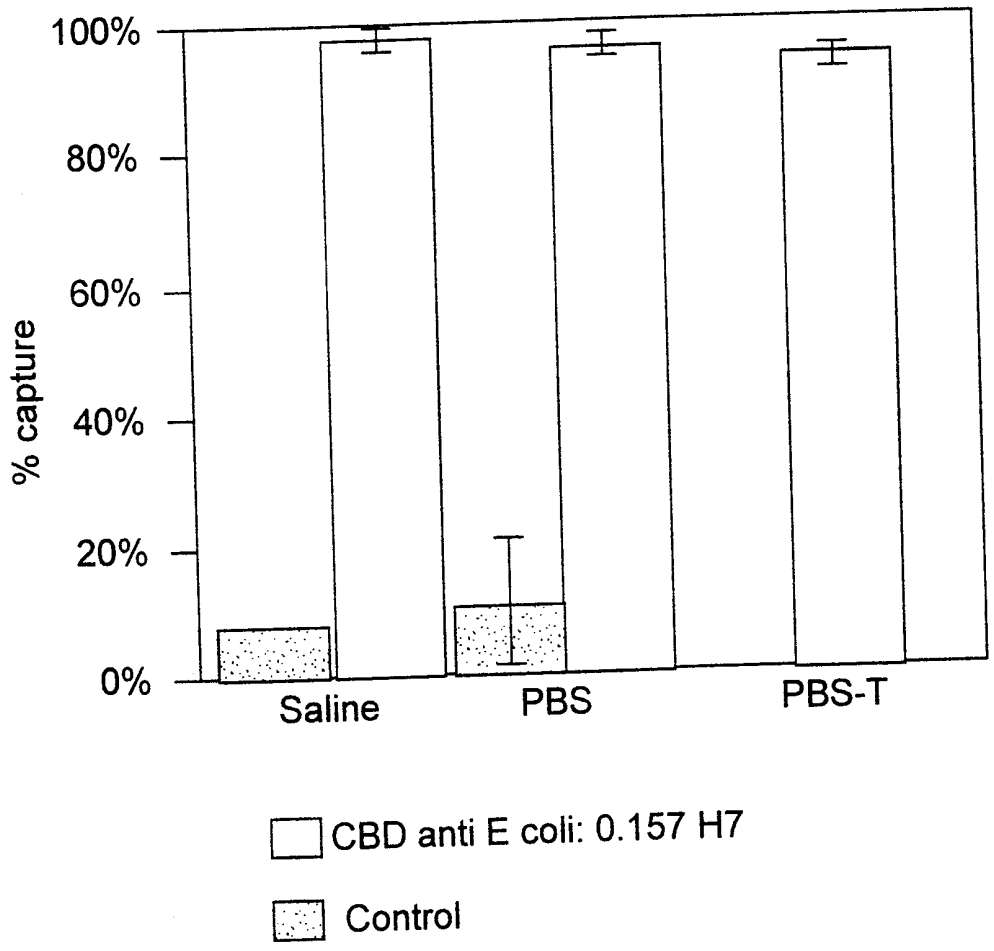
100%
80%
60%
40%
20%
0%
% capture
Saline
PBS
PBS-T
CBD anti E coli: 0.157 H7
Control

Fig. 9

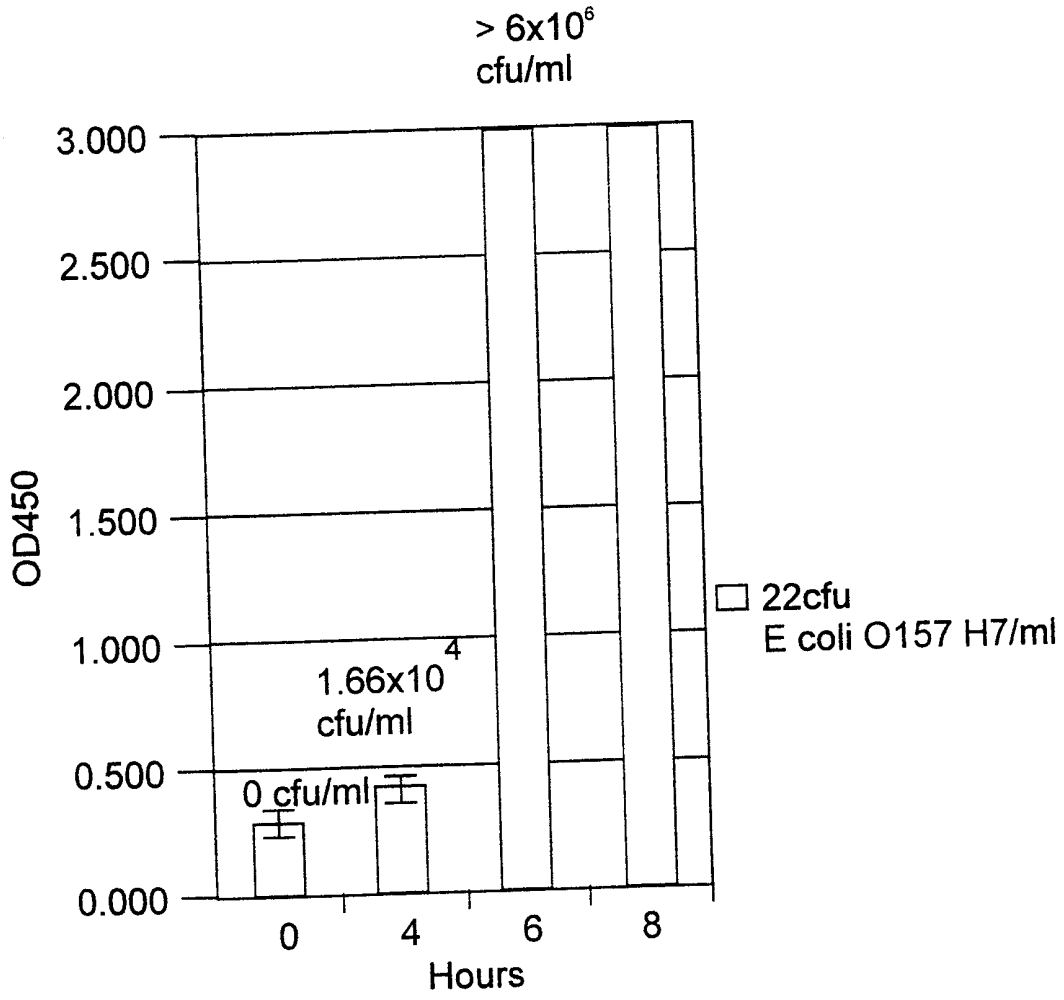
> 6x10^6 cfu/ml
3.000
2.500
2.000
1.500
1.000
0.500
0.000
OD450
1.66x10^4 cfu/ml
0 cfu/ml
0
4
6
8
Hours
22cfu E coli O157 H7/ml

Fig. 10

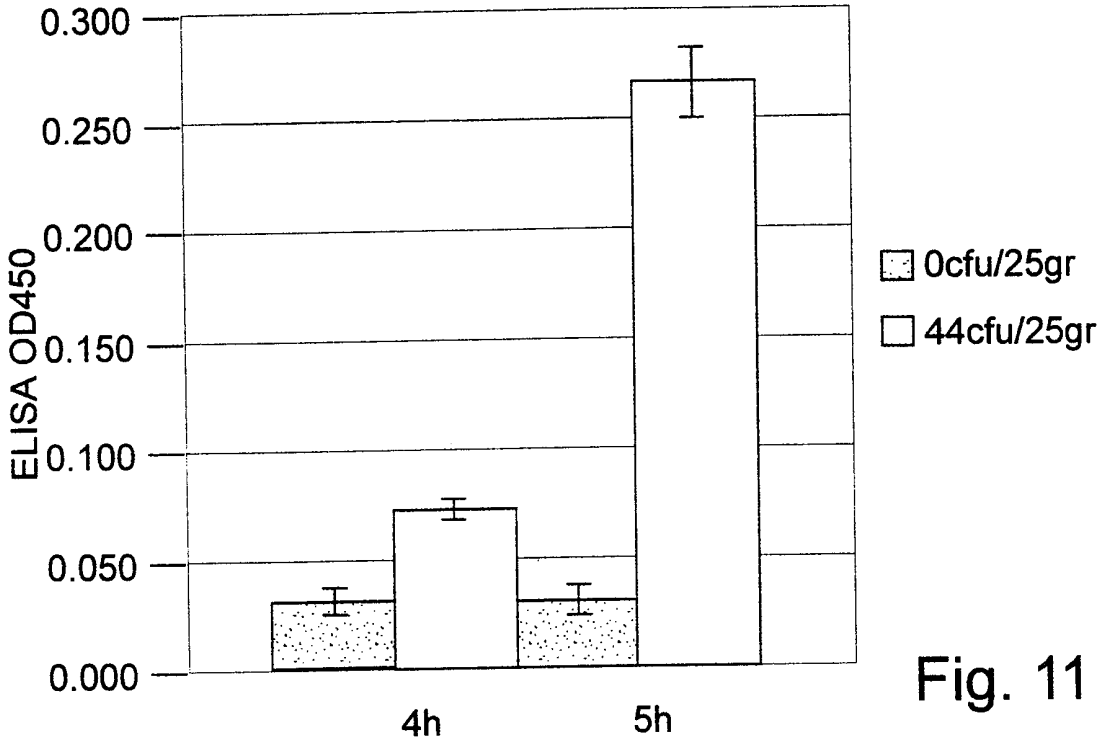
0.300
0.250
0.200
0.150
0.100
0.050
0.000
ELISA OD450
0cfu/25gr
44cfu/25gr
4h
5h

Fig. 11

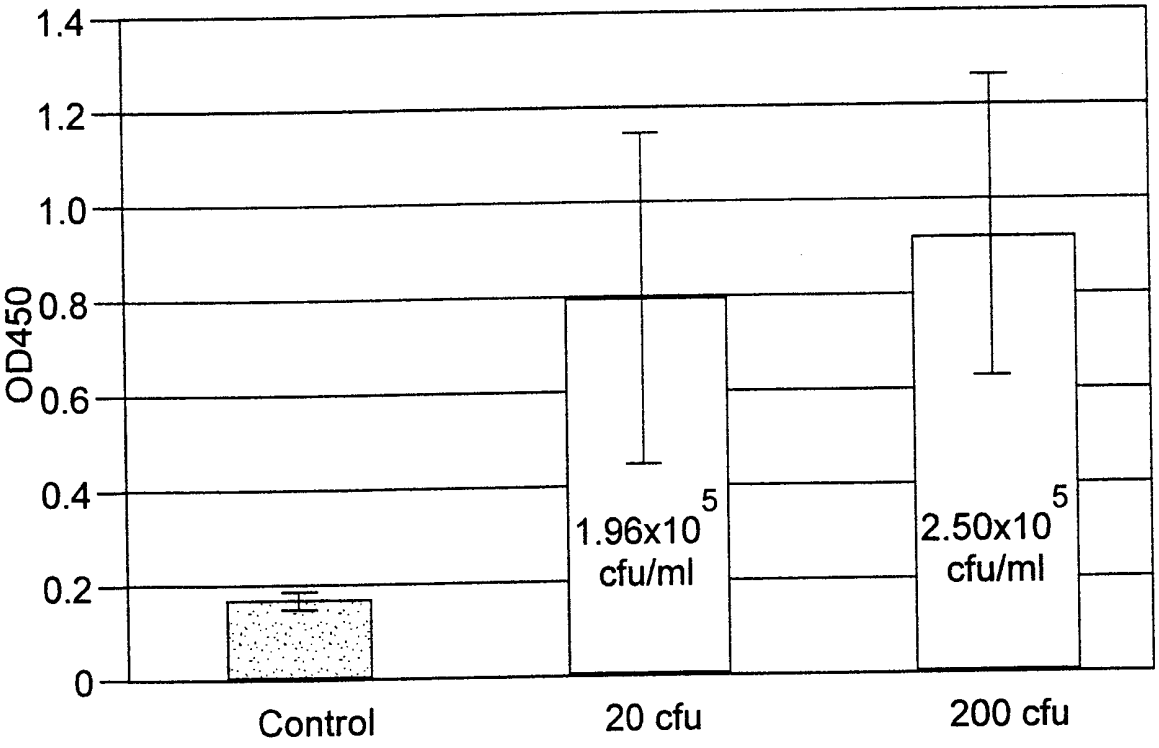
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
OD450
1.96x10 5 cfu/ml
2.50x10 5 cfu/ml
Control
20 cfu
200 cfu

Fig. 12

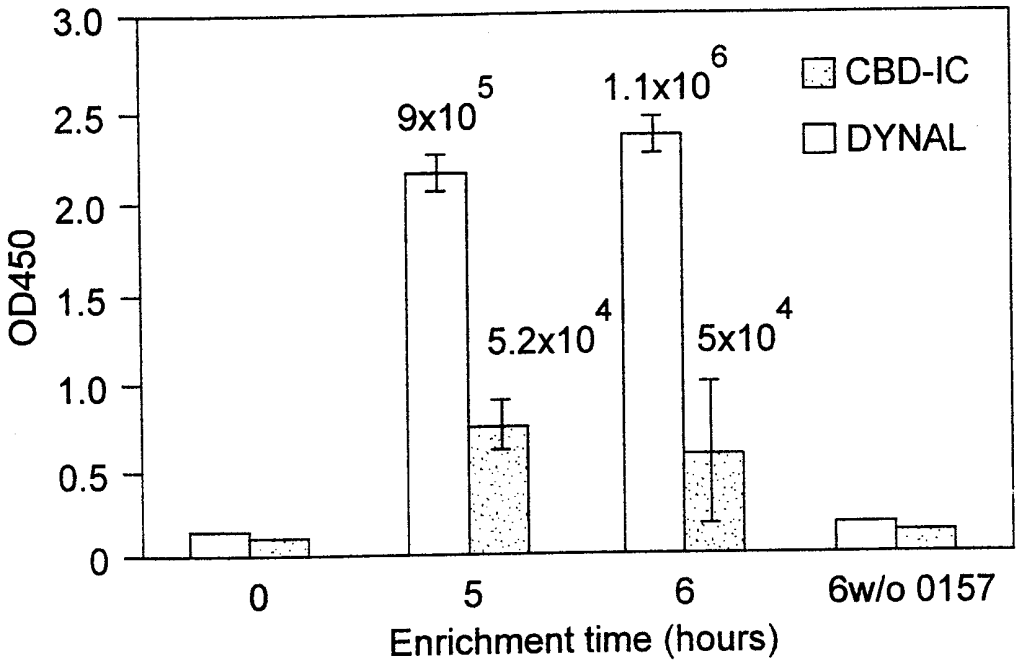
3.0
2.5
2.0
1.5
1.0
0.5
0
OD450
9x10^5
5.2x10^4
1.1x10^6
5x10^4
CBD-IC
DYNAL
0
5
6
6w/o 0157
Enrichment time (hours)

Fig. 13

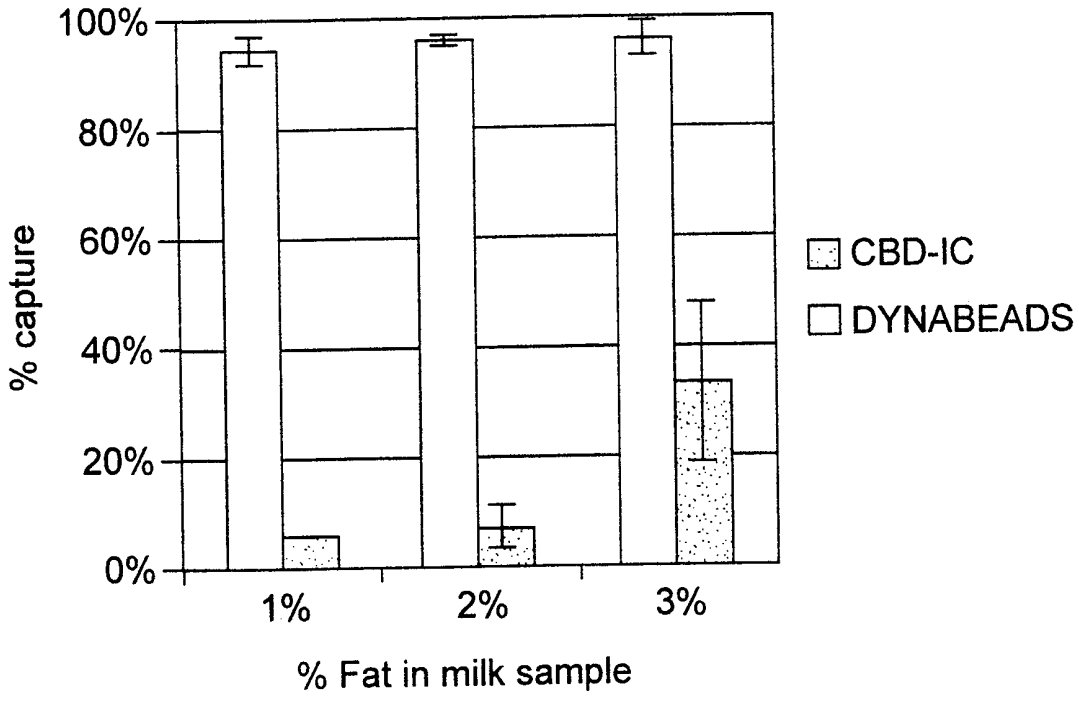
100%
80%
60%
40%
20%
0%
% capture
CBD-IC
DYNABEADS
1%
2%
3%
% Fat in milk sample

Fig. 14

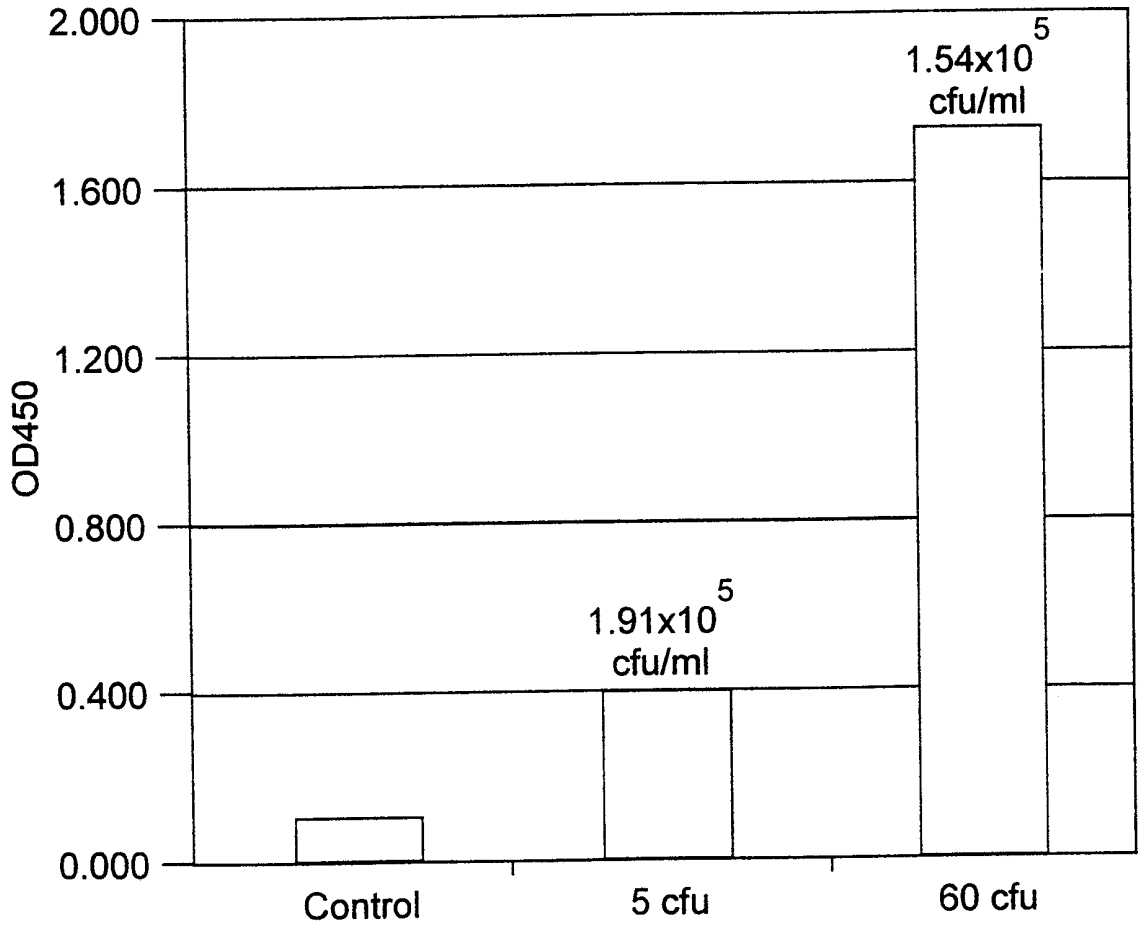
2.000
1.600
1.200
0.800
0.400
0.000
OD450
1.54x10 5
cfu/ml
1.91x10 5
cfu/ml
Control
5 cfu
60 cfu

Fig. 15

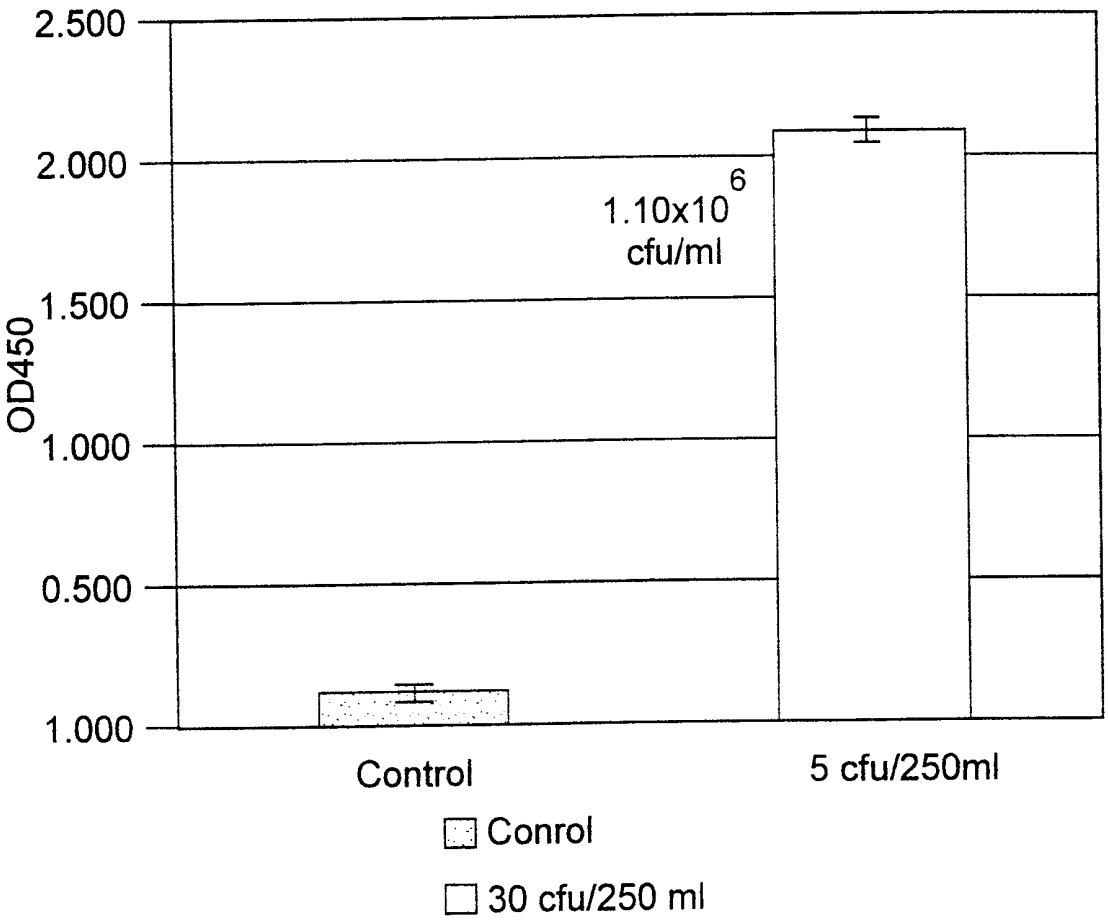
2.500
2.000
1.500
1.000
0.500
1.000
OD450
1.10x10 6
cfu/ml
Control
5 cfu/250ml
Conrol
30 cfu/250 ml

Fig. 16

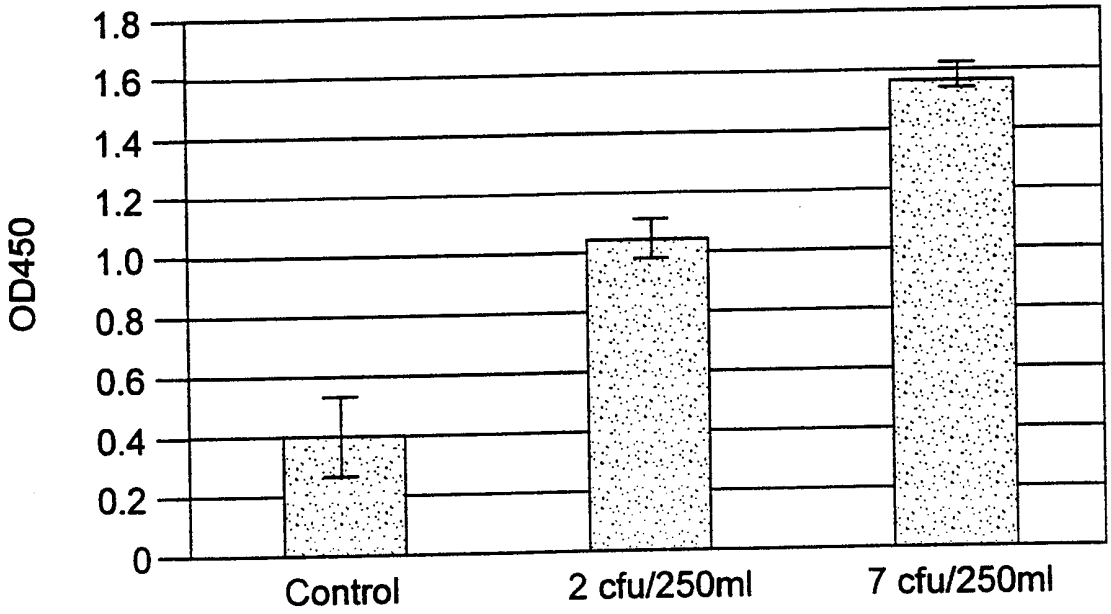
OD450
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Control
2 cfu/250ml
7 cfu/250ml

Fig. 17

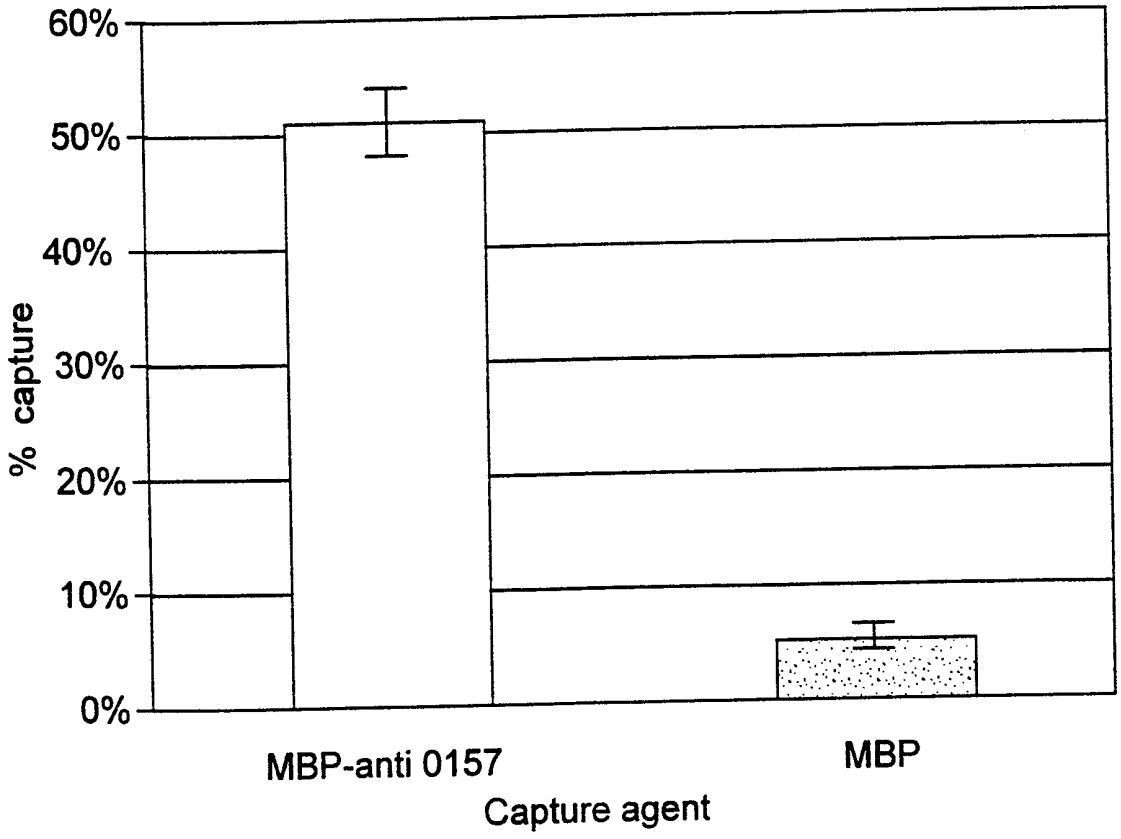
% capture
60%
50%
40%
30%
20%
10%
0%
MBP-anti 0157
MBP
Capture agent

Fig. 18

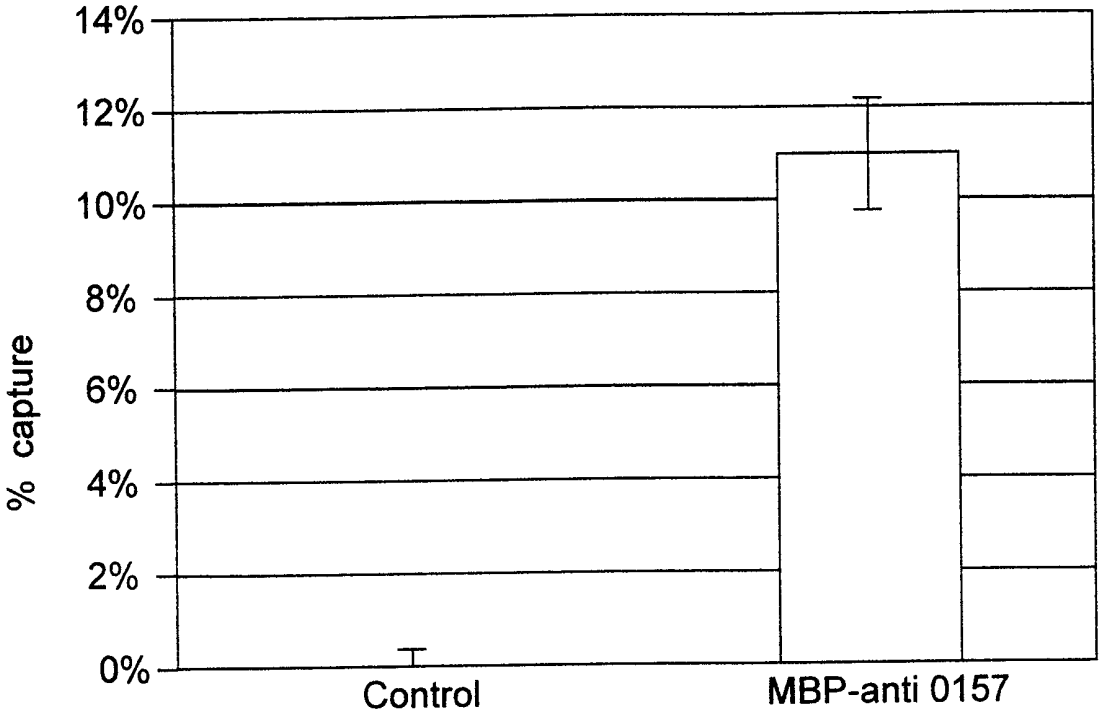
14%
12%
10%
8%
6%
4%
2%
0%
% capture
Control
MBP-anti 0157

Fig. 19

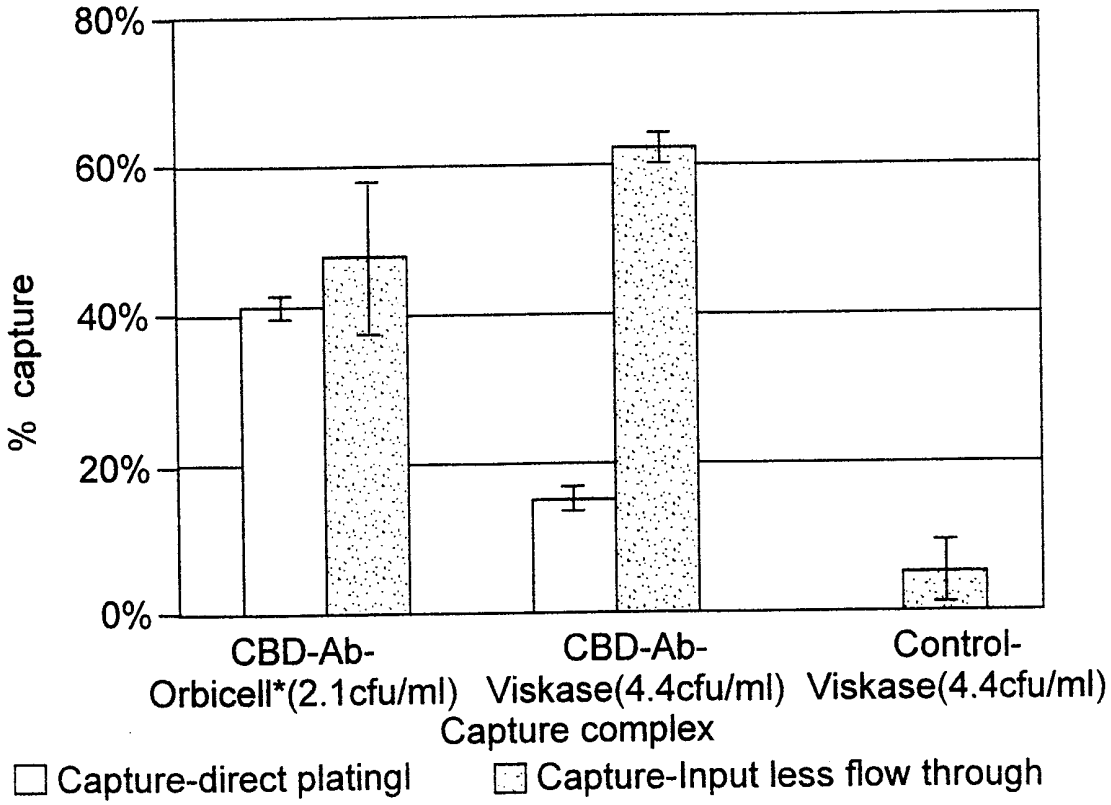
80%
60%
40%
20%
0%
% capture
CBD-Ab-Orbicell*(2.1cfu/ml)
CBD-Ab-Viskase(4.4cfu/ml)
Control-Viskase(4.4cfu/ml)
Capture complex
Capture-direct platingl
Capture-Input less flow through

Fig. 20

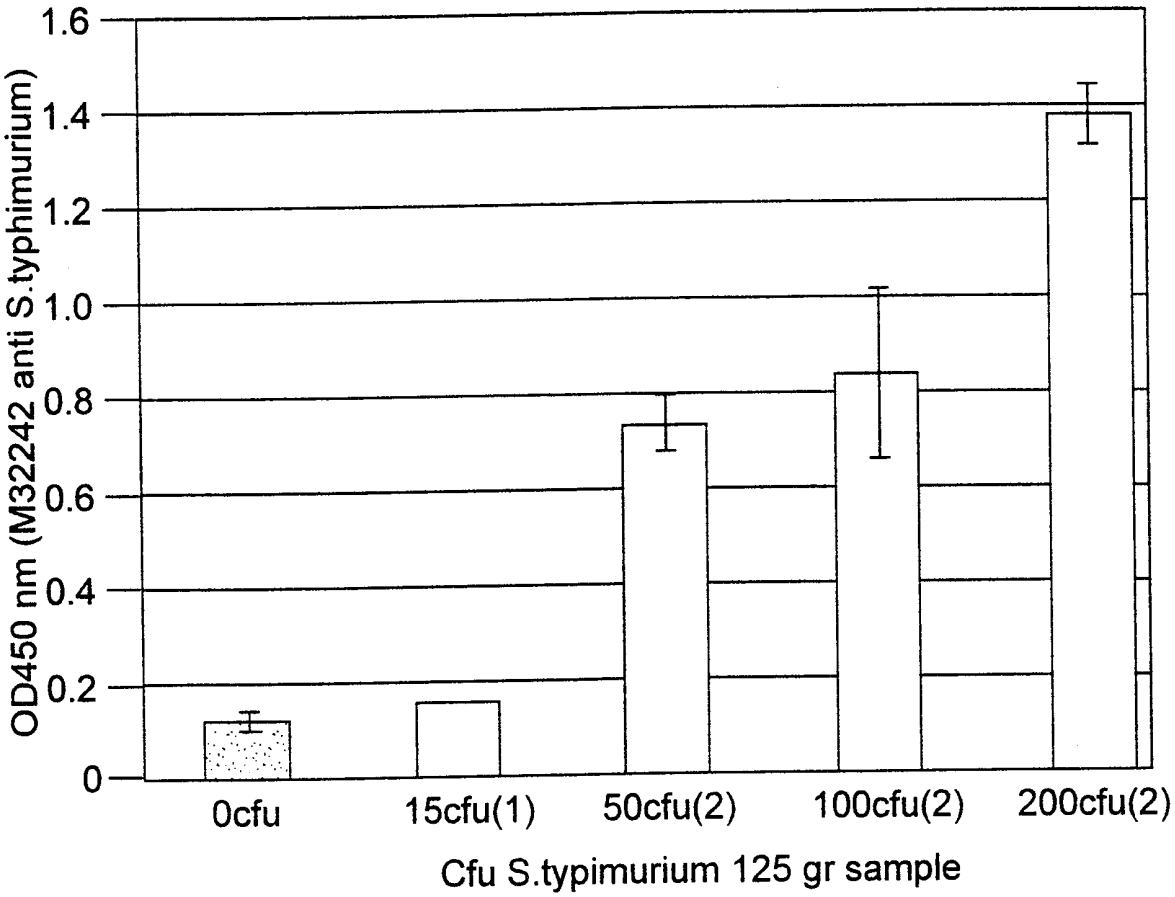
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
OD450 nm (M32242 anti S.typhimurium)
0cfu
15cfu(1)
50cfu(2)
100cfu(2)
200cfu(2)
Cfu S.typimurium 125 gr sample

Fig. 21

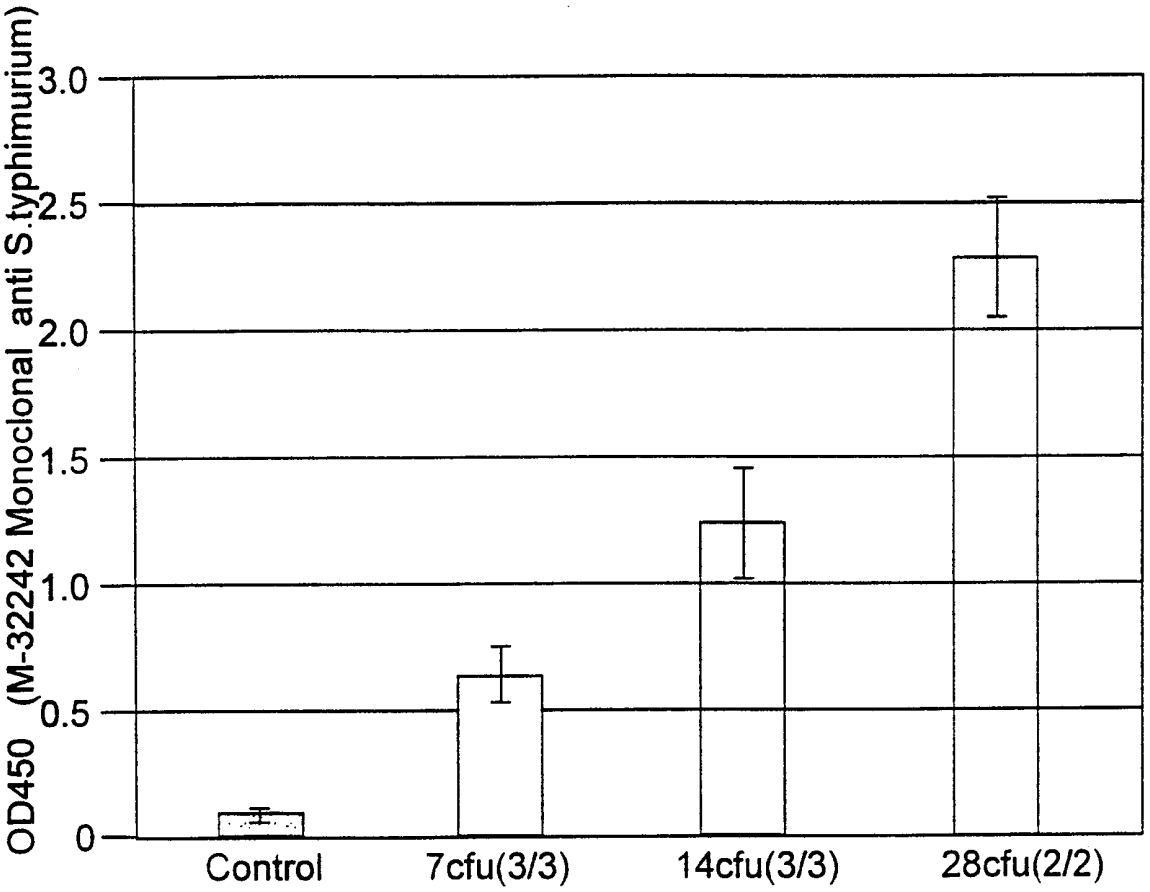
OD450 (M-32242 Monoclonal anti S.typhimurium)
3.0
2.5
2.0
1.5
1.0
0.5
0
Control
7cfu(3/3)
14cfu(3/3)
28cfu(2/2)

Fig. 22

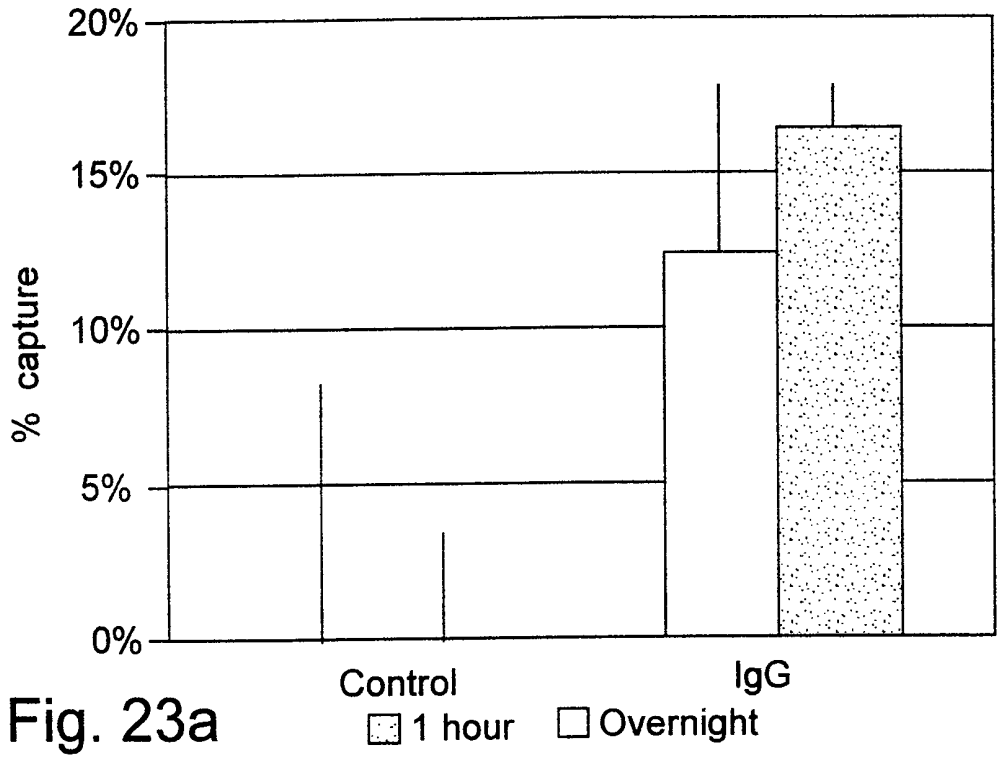

Fig. 23a

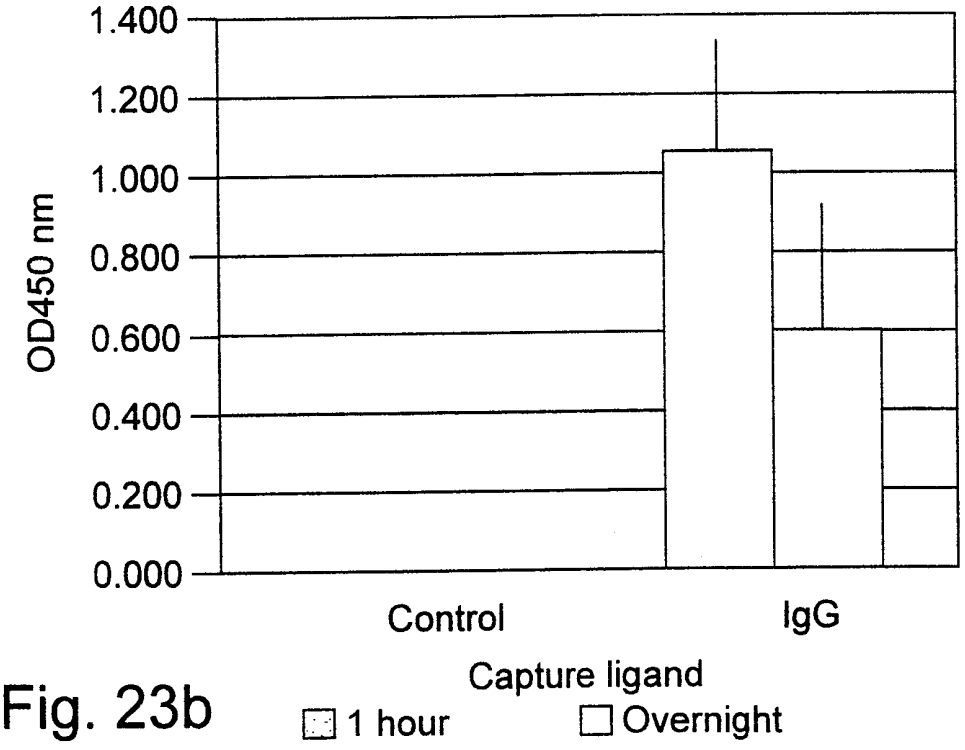

Fig. 23b

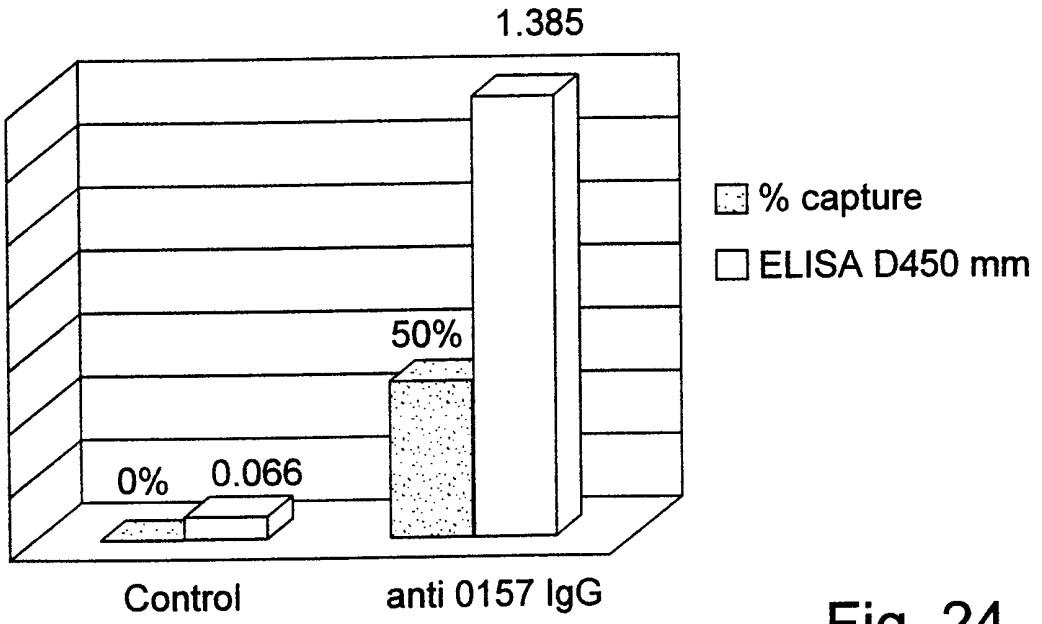
1.385
% capture
ELISA D450 mm
50%
0%
0.066
Control
anti 0157 IgG

Fig. 24

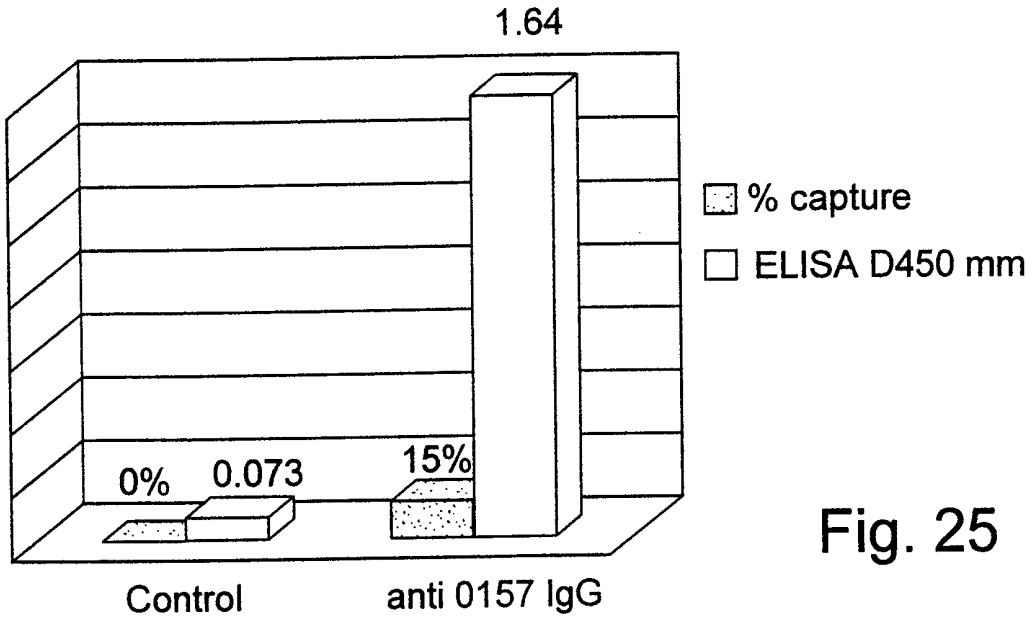
1.64
% capture
ELISA D450 mm
15%
0%
0.073
Control
anti 0157 IgG

Fig. 25

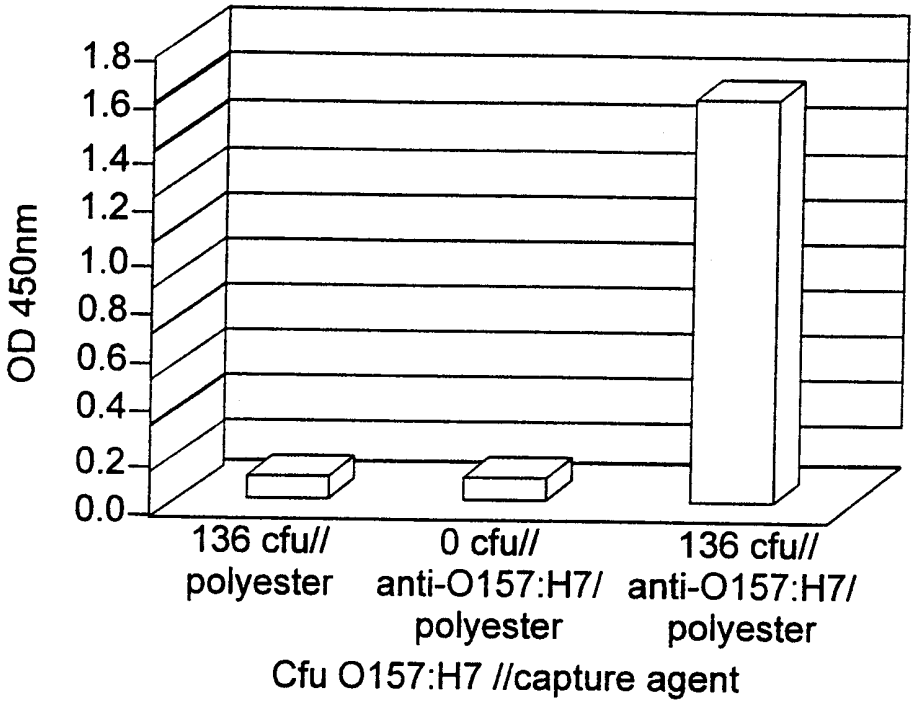
OD 450nm
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
136 cfu// polyester
0 cfu// anti-O157:H7/ polyester
136 cfu// anti-O157:H7/ polyester
Cfu O157:H7 //capture agent

Fig. 26

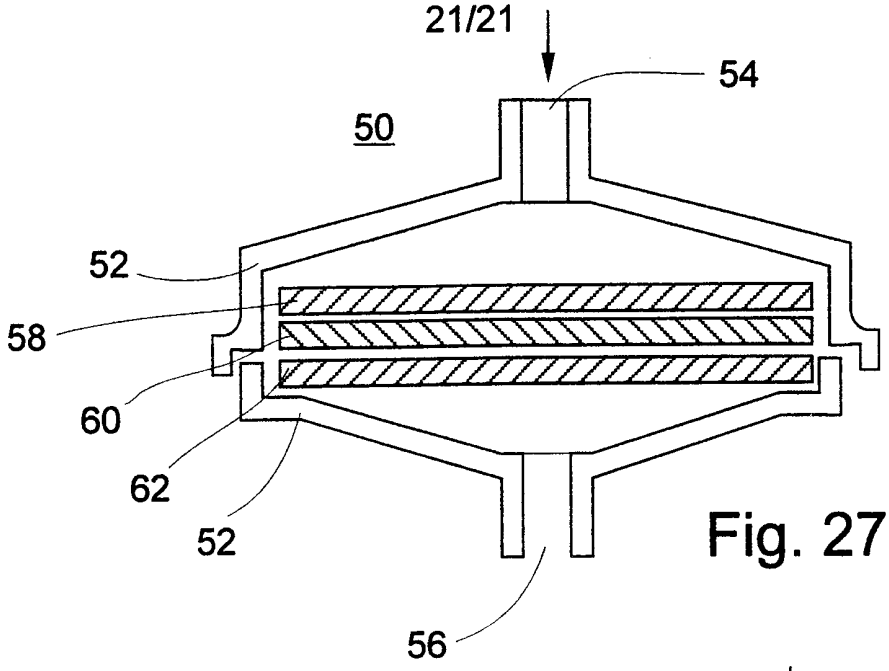

Fig. 27a

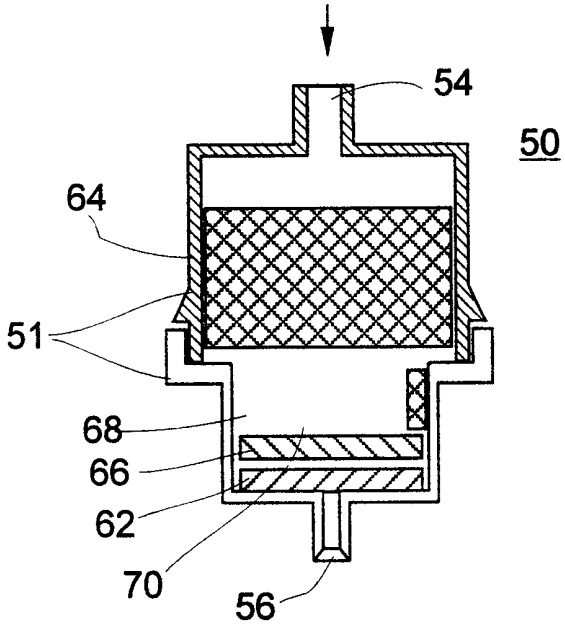

Fig. 27b

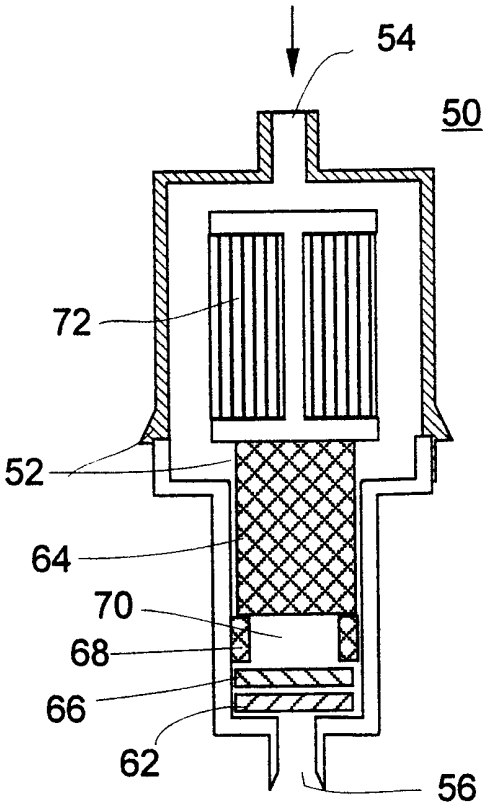

Fig. 27c

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17589

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
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. : Please See Extra Sheet.

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

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, CAS ONLINE, DIALOG, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,415,997 A (ATRACHE et al.) 16 May 1995, col. 3, line 28-col. 8, line 60.	1-11, 13-71
Y	US 5,719,044 A (SHOSEYOV et al.) 17 February 1998, col. 20, lines 22-34.	1-11, 13-71
Y	US 5,496,934 A (SHOSEYOV et al.) 05 March 1996, col. 4, line 43-col. 9, line 19.	1-11, 13-71
Y,P	US 5,856,201 A (SHOSEYOV et al.) 05 January 1999, col. 3, line 65-col. 9, line 57.	1-11, 13-71
A	US 4,250,256 A (WIELINGER et al.) 10 February 1981, col. 2, line 40-col. 3, line 15.	1-11, 13-71

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

06 NOVEMBER 1999

Date of mailing of the international search report

19 NOV 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer

PENSEE T. DO

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17589

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,232,838 A (NELSON et al.)03 August 1993, col. 2, line 66-col. 5, line 25.	1-11, 13-71
A	US 5,314,855 A (THORPE et al.) 24 May 1994, col. 3, lines 14-55.	1-11, 13-71
A	US 5,681,712 A (NELSON) 28 October 1997, col. 1, line 40-col. 2, line 51.	1-11, 13-71
A	US 5,587,286 A (PAHUSKI et al.) 24 December 1996, col. 3, line 51-col. 6, line 57.	1-11, 13-71
A,P	US 5,846,783 A (WU et al.) 08 December 1998, col. 4, line 50-col. 6, line 14.	1-11, 13-71
A	US 5,820,767 A (KANE et al.) 13 October 1998, col. 3, line 60-col. 12, line 25.	1-11, 13-71
A,P	US 5,837,452 A (CLARK et al.) 17 November 1998, col. 3, line 24-col. 7, line 49.	1-11, 13-71
A	US 5,807,694 A (ZAWISTOWSKI) 15 September 1998, col. 2, line 35-col. 3, line 67.	1-11, 13-71
A	US 4,563,418 A (WARD, JR.) 07 January 1986, col. 2, lines 16-59.	1-11, 13-71

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17589

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-11, 13-71

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/17589

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

G01N 21/77, 33/53, 33/44, 33/567, 33/554, 33/569, 33/537, 33/543, 33/531, 33/566, 33/545, 33/549; C12N 1/12, 1/20, 1/14, 1/16, 1/18, 1/10; C12M 1/00; C12Q 1/68, 1/24, 1/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 7.1, 7.2, 7.21, 7.32, 7.35, 7.37, 7.6, 7.9, 7.91, 7.92, 7.93, 7.94, 7.95, 30, 34, 252.1, 252.8, 252.7, 254.1, 258.1, 287.8, 287.9, 962, 969; 436/501, 518, 530, 535, 543, 532, 531, 85, 170, 823, 825

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

435/6, 7.1, 7.2, 7.21, 7.32, 7.35, 7.37, 7.6, 7.9, 7.91, 7.92, 7.93, 7.94, 7.95, 30, 34, 252.1, 252.8, 252.7, 254.1, 258.1, 287.8, 287.9, 962, 969; 436/501, 518, 530, 535, 543, 532, 531, 85, 170, 823, 825

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-11, 13-71, drawn to a method of concentrating a particular microorganism(s) in a sample.
Group II, claim(s) 72-74, drawn to a filtering device.

The inventions listed as Groups I-II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the device does not require the use of the claimed method.

There is no claim numbered as claim 12.