Binding of hydrophobic antigens to surfaces

Boas, Ulrik

Publication date:
2017

Document Version
Publisher's PDF, also known as Version of record

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 March 2017 (16.03.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/042303 A1

(51) International Patent Classification:
C07K 17/06 (2006.01) *G01N 33/543* (2006.01)
C07K 17/14 (2006.01)

(21) International Application Number:
PCT/EP2016/071240

(22) International Filing Date:
8 September 2016 (08.09.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
15184294.5 8 September 2015 (08.09.2015) EP

(71) Applicant: **DANMARKS TEKNISKE UNIVERSITET** [DK/DK]; Anker Engelunds Vej 101 A, 2800 Kgs. Lyngby (DK).

(72) Inventor: **BOAS, Ulrik**; Prinsesse Charlottesgade 5, 4.th., 2200 Copenhagen N (DK).

(74) Agent: **PLOUGMANN VINGTOFT A/S**; Rued Langgaards Vej 8, 2300 Copenhagen S (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) Title: BINDING OF HYDROPHOBIC ANTIGENS TO SURFACES

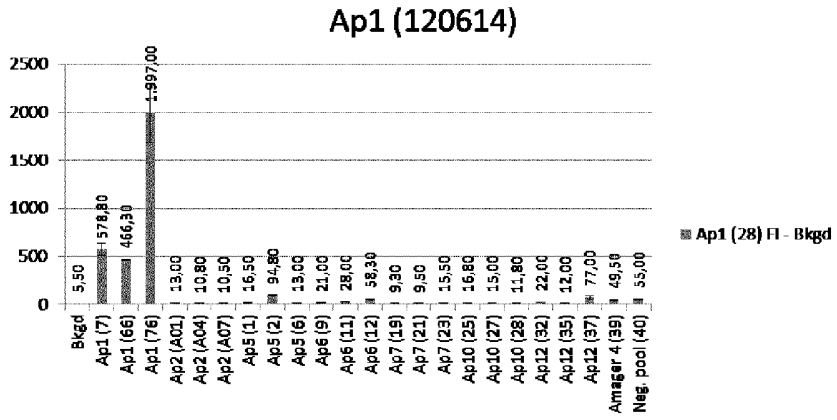


FIG. 2A

(57) Abstract: A first aspect of the present invention is a method of detecting antibodies comprising the steps of: i) providing a first group of beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups, ii) contacting said first group of beads with a first hydrophobic antigen to provide a first group of bead-antigen conjugates by adsorption of the first hydrophobic antigen on the first group of beads, iii) isolating said bead- antigen conjugates, iv) contacting said bead-antigen conjugates with a sample to bind antibodies therein to provide bead-antigen-antibody conjugates, and v) detecting said bead-antigen-antibody conjugates. Further aspects include an antibody detection kit, a bead-antigen conjugate and a composition comprising at least two different groups of bead-antigen-conjugates.

WO 2017/042303 A1

Binding of hydrophobic antigens to surfaces

Technical field of the invention

The present invention relates to bio-conjugate chemistry methods for providing surfaces that are useful within the field of diagnostics, particularly sero-diagnostics. In particular, the present invention relates to the preparation of a functionalized bead scaffold capable of adsorbing a plurality of hydrophobic antigens. The functionalized beads comprising hydrophobic antigens are useful in diagnostic arrays for the detection of *e.g.* bacterial antibodies.

Background of the invention

When a foreign organism infects the body, the immune system responds by producing antibodies against antigens exposed on the surface of the intruding organism. Fast and reliable detection of such antibodies is therefore a key parameter in the diagnostic of health threatening conditions. Screening of antibodies in serum samples is typically based on presentation of known bacterial or viral antigens.

Antigens may be represented by *e.g.* lipopeptides, glycolipids or capsular polysaccharides. One type of highly immunogenic antigens from the glycolipid group are lipopoly-saccharides (LPS). This class of antigens are prevalent in the outer membrane of bacteria, especially in gram-negative bacteria. They are amphiphilic molecules comprising a lipid part covalently attached to a polysaccharide part via a single polysaccharide unit, which is typically a 2-keto-3-deoxynononic acid (Kdn) residue, or a 2-keto-3-deoxy-D-mannooctonic acid (Kdo) residue. The polysaccharide part comprises an outer and inner region and an O-Chain (O-antigen). The O-chain is highly immunogenic and serotype specific, and LPS are therefore good antigens for immunochemical assays for serological detection of antibodies.

Thus, the detection of antibodies may be provided via the binding of antigens to various surfaces, thereby allowing immobilization of antibodies on said surfaces and subsequent detection. In many established immunochemical assays, the

antigens are covalently coupled to a support, a coupling that may result in loss of sensitivity or activity. Thus, there are disadvantages of covalent coupling of antigens to surfaces, including *e.g.* modifications to the antigen which may decrease affinity to target antibodies, and also tedious chemical modifications making rapid production of *e.g.* multiple surfaces presenting different antigens highly time consuming. This makes use of covalent immobilization of antigen to surfaces a highly time consuming process particularly for multiplex analysis.

Thus, WO 2013/060335 discloses a method of immobilizing de-lipidated LPS antigens on surfaces such as beads. A method involving covalent binding of the antigen to *e.g.* beads is disclosed and has the disadvantages as described above.

Methods of purification by non-covalently binding bacterial lipopolysaccharide from solution are described in connection with chromatographic removal of LPS in serum. Hirayama and Sakata, J. Chromatogr. B. (2002), 419-432 used the multivalent effect of hydrophobicity and ionic interactions of cationic polymers to remove LPS from solution and WO 2010/036133 disclosed a technique relying on lipid interactions for non-covalently binding of bacterial LPS. Similarly, Takahashi et al, J. Immunol. Methods. (1992), 67-71 utilized an ELISA format technique based on poly-L-lysine surfaces to capture LPS from solution.

Furthermore, Takahashi et al, J. Immunol. Methods. (1992), 67-71 used the bound LPS to screen for antibodies against the specific type of immobilized LPS antigen. The disclosure involves the use of poly-lysine coated micro-plates, while nothing is taught regarding beads coated with the substituted alkyls for effective antigen binding. Furthermore, nothing was disclosed about simultaneous screening for multiple antibodies using beads.

Hence, a versatile sero-diagnostic assay providing versatile antigen-coated beads capable of *e.g.* high-throughput screening for multiple sources of infection by presentation of a multitude of native antigens would be of great value. Furthermore, it would be beneficial if such a method could be based on an easy to use template that would allow regularly skilled personnel, such as laboratory technicians, to independently design and perform multiplex analysis of serum samples.

Summary of the invention

Thus, an object of the present invention relates to the establishment of a method for improved screening for antibodies via non-covalent adsorption of antigens.

In particular, it is an object of the present invention to provide easy to use template beads to which hydrophobic antigens of interest can be adsorbed and subsequently mixed, essentially forming a scaffold for serological screening that solves the above mentioned problems of the prior art with respect to providing a screening platform capable of rapid analysis and of identifying simultaneously a multitude of different antibodies for high-throughput processing and diagnostics of serum samples.

The inventor has surprisingly found that hydrophobic adsorption of hydrophobic antigens to alkyl chain coated beads yields a serological scaffold, which can consistently recognize antibodies out of serum samples, both with higher sensitivity and lower background signal than other known methods.

Another preferred aspect of the present invention is a method of detecting antibodies comprising the steps of:

i. providing a first group of beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups,
ii. contacting said first group of beads with a first hydrophobic antigen to provide a first group of bead-antigen conjugates,
iii. isolating said bead-antigen conjugates,
iv. contacting said bead-antigen conjugates with a sample to bind antibodies therein to provide bead-antigen-antibody conjugates, and
v. detecting said bead-antigen-antibody conjugates.

A further aspect of the present invention is an antibody detection kit comprising:

- at least one group of beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups,
- optionally at least one composition comprising a hydrophobic antigen, and

- optionally instructions for performing the method according to claim 1.

Another aspect of the present invention relates to a bead-antigen conjugate comprising:

- beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups, and
- a hydrophobic antigen.

Another aspect of the present invention relates to a composition comprising at least two individual groups of bead-antigen conjugates each individually comprising:

- beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups, and
- a hydrophobic antigen,

wherein the hydrophobic antigen is different in each group.

Apart from providing an easy to use high-throughput scaffold for detection of antibodies and particularly simultaneous detection of a multitude of antibodies within a serum sample, the present invention comes with several additional advantages. It is a highly versatile method in the sense that all types of hydrophobic antigens can be easily incorporated in multiplex screening, simply by adding the hydrophobic antigen of interest to the solution of functionalized beads. Modification of antigens, including particularly de-lipidation may be avoided, resulting in a much easier production of the bead-antigen conjugates of the present invention.

Brief description of the figures

Figure 1A-B shows a schematic representation of the chemical reaction of a carboxylic acid modified bead with A) N,N-diethylethylenediamine (DEDA) to form a 'DEDA bead' (Fig. 1A) and B) 5-amino-1-pentanol to form a 'Bead 2' (Fig. 1B). Both couplings are performed using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (NHS) activation in aqueous solution.

Figure 2A-F shows the detection of antibodies against different serotypes of *Actinobacillus Pleuropneumoniae* (Ap) in a multiplex Bead 2 assay. Ap antibodies were tested from a panel of serum sample from pigs that were experimentally infected with a single Ap serotype. It is apparent that Ap 1, 2, 5, 6, 10 and 12 (Fig. 2A, B, C, D, E and F, respectively) antibodies are detected with high signal-to-noise ratio and close to zero false-positive signal.

Figure 3A-C shows the utilization of the DEDA bead assay for detection of Ap 5 antibodies from a panel of serum sample from pigs that were experimentally infected with a single Ap serotype. Antibodies are detected with high signal-to-noise ratio and close to zero false-positive signal.

Figure 4 shows the utilization of the DEDA bead assay for detection of Ap 6 antibodies from a panel of serum sample from pigs that were experimentally infected with a single Ap serotype. This figure exemplifies that cross-reactivity between antibodies can occur (here Ap 6 and Ap 8).

Figure 5A-D shows a comparison between *Lawsonia* antigen bound by adsorption to Bead 2 (Fig. 5A-B) and EDC coupled *Lawsonia* antigen beads (Fig. 5C-D). Both constructs effectively detects *Lawsonia* antibodies. However, higher P/N values are achieved by adsorbing *Lawsonia* antigen to 'Bead 2'.

Figure 6 shows the utilization of the DEDA bead assay for detection of *Salmonella Typhimurium* in a panel of samples from pigs that were experimentally infected with a single Ap serotype. Antibodies against *Salmonella Typhimurium* are detected with high signal-to-noise ratio and close to zero false-positive signal.

Figure 7 shows the utilization of the DEDA bead assay for detection of *Salmonella Choleraesuis* in a panel of samples from pigs that were experimentally infected with a single Ap serotype. Antibodies against *Salmonella Choleraesuis* are detected with high signal-to-noise ratio and close to zero false-positive signal.

Figure 8A-C shows a comparison between Ap 5 antibody detection by coupling of Ap5 antigen to DEDA beads (Fig. 8A) and coupling of Ap 5 antigen to PLL beads (Fig. 8B). Detection of Ap 5 antibodies by traditional ELISA technique (Fig. 8C) is

also shown as reference. Ap 5 DEDA beads are easy to handle and results in the highest antibody sensitivity.

The present invention will now be described in more detail in the following.

Detailed description of the invention

Definitions

Prior to discussing the present invention in further details, the following terms and conventions will first be defined:

Antibody

In the present context, an antibody is a protein that specifically binds a corresponding antigen. Antibodies may particularly stem from the immune system of *e.g.* mammals, and may be directed towards antigens related to foreign bodies including particularly bacteria. Thus, antibodies may be antibodies directed towards bacterial antigens, which are typically presented on the surface of said bacteria.

Bead

In the present context, a bead is a nano- or microscopic surface build from one or more materials and can have a variety of sizes and take many shapes, *e.g.* spherical shells or solid spheres.

Polymer bead

In the present context, a polymer is a chemical compound composed of repetitive sequences of identical units and can be either naturally occurring or synthetic, *e.g.* polystyrene, sepharose or latex. A polymer bead may be functionalized with a moiety suitable for coupling of additional molecules to the polymer bead, *e.g.* esters, amides and ketones. Furthermore, polymer beads could be utilized in for example well-plates or other laboratory equipment used for biological assays.

Nanoparticle

In the present context, a nanoparticle is a nanoscopic surface build from one or more materials, such as gold, co-block polymers, dendrimeric polymers or metal colloids. They can have a variety of sizes and take many shapes, *e.g.* spherical

shells, solid spheres, rods and fibers. The surface modifications of nanoparticles may determine their properties. In particular, the surface modifications can regulate stability, solubility, and interactions with other molecules in solution. Nanoparticles often have desirable properties due to the large surface area to volume ratio of the nanoparticle and are therefore widely used as scaffolds in a span of assays.

Alkyl group

In the present context, an alkyl group may be an alkane missing one hydrogen, *i.e.* with the general chemical formula C_nH_{2n+1}. The alkyl may be linear or branched and can be substituted with functional groups such as amine, ammonium, ether or hydroxyl groups. Since the alkyl groups of the present invention may be optionally substituted they may in the present context also include *e.g.* alkylene groups, *i.e.* where both ends of the alkyl group is substituted (*e.g.* $-CH_2-CH_2-CH_2-$ or propylene). Particularly C_1-C_{10} alkyl groups may include alkyl or alkylene groups having from 1-10 carbon atoms. Examples include methyl/methylene, ethyl/ethylene, propyl/propylene, butyl/butylene, isobutyl/isobutylene, pentyl/pentylene, etc.

Hydrophobic antigen

In the present context, a hydrophobic antigen is a molecule recognized by a specific antibody, wherein the antigen comprises a moiety comprising a hydrophobic part, such as a lipid moiety. A lipid is in the present context a small amphiphilic molecule that exists for instance in cellular lipid membranes or in connection with proteins as functional modifications. A non-exhaustive list of hydrophobic antigens includes glycolipids, lipopeptides, and capsular polysaccharides.

Bead-antigen conjugate

In the present context, a bead-antigen conjugate is a bead derivative that is non-covalently bound to an antigen. The conjugate may be formed using *e.g.* hydrophobic interactions, hydrophilic interactions, ionic interactions, van der walls forces, hydrogen bonding, and particularly also combinations hereof.

Hydrophobic interactions

In the present context, hydrophobic interactions refers to the interaction between two hydrophobic moieties of two separate molecules. A hydrophobic moiety is a nonpolar part of a molecule, such as a long chain of carbons. When exposed to an aqueous medium, hydrophobic moieties will adsorb to each other to minimize the energy of the system. In the present context, a hydrophobic interaction may be between one hydrophobic moiety located on a bead and another hydrophobic moiety located on the antigen to be immobilized on the bead. Specifically, the hydrophobic moieties of the bead and antigen may be carbon chains. The carbon chains may be substituted with functional groups to facilitate other interactions in addition to hydrophobic interactions. The strength of the hydrophobic interaction depends amongst others on the number of carbon atoms of the hydrophobic moiety. Thus, the longer the carbon chains are, the stronger the hydrophobic interaction will be between two hydrophobic moieties. In the present invention, the beads may be C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups.

Ionic interaction

In the present context ionic interactions are interactions between charged moieties in molecules, such as interactions between anions and cations, i.e. a negative charge attracting a positive charge. Particularly, ionic interactions may include salt formation between anions and cations.

Adsorption

In the present context, adsorption means the adhesion of e.g. a molecule or biomolecule to e.g. a solid surface. In the present context, adsorption does not include chemisorption, i.e. covalent bond formation between e.g. the molecule and the surface. In the present context, the adsorption is mainly due to hydrophobic and electrostatic interactions, but may also include other known molecular interactions such as van der wall forces, ionic interactions etc.

Bead-antigen-antibody conjugate

In the present context, a bead-antigen-antibody conjugate is a bead-antigen conjugate as defined above further comprising a bound antibody, via said antigen.

Thus, it is a bead derivative that is non-covalently bound to an antigen binding a specific antibody.

Isolation

In the present context, isolation or isolating constitutes a technique for separating a specific entity from a solution or from other entities in a solution containing multiple entities. The entities may for example be the beads or bead-antigen conjugates of the present invention. Examples of such techniques could be but are not limited to magnetism, centrifugation, size exclusion, cell sorting and dialysis.

Sample

In the present context, a sample is any aqueous biological solution, which could possibly contain antibodies, *e.g.* a mammalian serum sample. Alternatively, a sample could also originate from a non-mammalian solution, *e.g.* a solution of wastewater.

In order to provide a more efficient method for detection of antibodies in for example serum samples, the present inventors have surprisingly found that the non-covalent adsorption of certain antigens to beads enables a facile method for the detection of antibodies, which eliminates or reduces some of the problems present in the prior art, involving time-consuming methods where antigens are chemically adapted and covalently bound, or wherein detection of multiple antibodies in one sample requires the preparation of many unique beads.

Therefore, a first aspect of the present invention is a method of detecting antibodies comprising the steps of:

i. providing a first group of beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups,
ii. performing non-covalent adsorption of a first hydrophobic antigen on said first group of beads provided in step i) to provide a first group of bead-antigen conjugates,
iii. isolating said bead-antigen conjugates,
iv. contacting said bead-antigen conjugates with a sample to bind antibodies therein to provide bead-antigen-antibody conjugates, and

v. detecting said bead-antigen-antibody conjugates.

Another preferred aspect of the present invention is a method of detecting antibodies comprising the steps of:

i. providing a first group of beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups,
ii. contacting said first group of beads with a first hydrophobic antigen to provide a first group of bead-antigen conjugates
iii. isolating said bead-antigen conjugates,
iv. contacting said bead-antigen conjugates with a sample to bind antibodies therein to provide bead-antigen-antibody conjugates, and
v. detecting said bead-antigen-antibody conjugates.

To provide a durable, flexible and reliable method, it is necessary to choose a surface scaffold that allows for easy customization of the surface and simultaneously provide high stability of the composition in solution. Consequently, in one preferred embodiment of the invention, the beads are selected from the group consisting of polymer beads and nanoparticles, preferably polymer beads.

One particular advantage of the present invention is that it allows for the facile preparation of several different bead-antigen conjugates very easily using similar bead scaffolds, which are rapidly conjugated by non-covalent adsorption with different antigens.

In one embodiment of the present method step i) is defined as providing a first group of beads comprising a surface modified with C_1-C_{10} alkyl groups selected from the group consisting of amine, ammonium, ether and hydroxyl groups.

In another embodiment the first group of beads are directly contacted with said first hydrophobic antigen, i.e. without any moieties, linkers or other molecules between the bead and the antigen.

Thus in one embodiment of the present invention the method as described herein is applied, wherein steps i) and ii) are repeated with a second and/or further

group of beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups and a second and/or further hydrophobic antigen to provide second and/or further group of bead-antigen conjugates. The groups of beads used may vary for example in fluorescent marker used, thereby providing for unique bead-antigen combinations, providing for enhanced detection upon conjugation with antibodies and possibly secondary antibodies comprising further markers.

To allow the facile detection of multiple antibodies the method may comprise a step of mixing said first bead-antigen conjugate with said second and/or further bead-antigen conjugates. Contacting this mixture with a sample will then allow for simultaneous detection of multiple antibodies therein, *i.e.* multiplex analysis. A multiplex assay is a type of assay that simultaneously measures multiple analytes (dozens or more) in a single run/cycle of the assay. It is distinguished from procedures that measure one analyte at a time.

The present inventor have found that specific types of substituents on beads provides for effective and strong non-covalent binding of hydrophobic antigens on the surface of said beads. Particularly, the inventor has found that the combination of alkyl/alkylene groups and more polar groups such as amines, hydroxyls and ammoniums provides for a suitable binding strength, while avoiding that the beads form highly undesirable aggregates. In addition, it was surprisingly found that these particular substituents provided sufficiently strong binding to avoid detectable antigen exchange between beads, which would be detrimental to multiplex analysis. These criteria are fulfilled by the C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups of the present invention.

Without being bound by theory, the strong binding and negligible antigen exchange between beads of the present bead-antigen conjugates are the result of multiple interactions between the beads and the antigen. Thus, the alkyl chains of the beads hydrophobically interact with the hydrophobic moiety of the antigen as described above. In one embodiment of the present invention, the hydrophobic moiety of the antigen is the alkyl chains of the lipid part of LPS. In addition to the hydrophobic interactions, the amine, ammonium, ether and/or hydroxyl groups of

the beads interact ionically with charged moieties on the antigen. In one embodiment of the present invention, the charged moieties of the antigen is the negative charges of the phosphor groups of the lipid part of LPS.

Thus, in an embodiment, the hydrophobic antigen is adsorbed on the bead through hydrophobic and ionic interactions.

A preferred embodiment of the present invention relates to the method as described herein, wherein said first hydrophobic antigen is adsorbed on said first group of beads to provide said first group of bead-antigen conjugates.

Another preferred embodiment of the present invention relates to the method as described herein, wherein said adsorption does not include formation of a covalent bond between said first hydrophobic antigen and said first group of beads.

Yet another embodiment of the present invention relates to the method as described herein, wherein said first group of beads are non-covalently attached to said first hydrophobic antigen to provide said first group of bead-antigen conjugates.

Still another embodiment of the present invention relates to the method as described herein, wherein said first hydrophobic antigen is adsorbed or non-covalently attached to said first group of beads through hydrophobic interactions.

An even further embodiment of the present invention relates to the method as described herein, wherein said first hydrophobic antigen is adsorbed or non-covalently attached to said first group of beads through hydrophobic and ionic interactions.

In another embodiment, LPS is adsorbed on the bead through hydrophobic and ionic interactions.

In a preferred embodiment, LPS is adsorbed on the bead through hydrophobic interactions between the alkyl chains of the bead and the alkyl chains of the lipid

part of LPS and ionic interactions between amine or ammonium groups of the beads and negative charges of the phosphor groups of the lipid part of LPS.

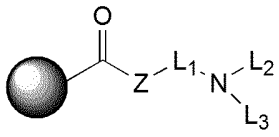
In one embodiment, the C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups are linear. In another embodiment, they may be branched. The groups may comprise a mixture of linear and branched C_1-C_{10} alkyl groups. As mentioned, the alkyls may be alkylenes or a mixture of alkyls and alkylenes.

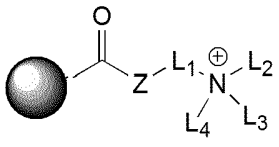
In one embodiment, the C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups form a dendrimer. Dendrimers are well-known repetitively branched molecules, and may comprise *e.g.* alkyls with amines as branching points.

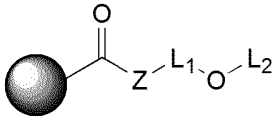
In a particularly preferred embodiment, the amine, ammonium, ether and/or hydroxyl groups of the present invention are selected from the group consisting of primary amines, secondary amines, tertiary amines, quaternary ammoniums, primary hydroxyl groups, ethers, or a mixture thereof. In dendrimers tertiary amines are primarily used as branching points.

In a typical embodiment of the present invention a commercially available bead is provided, which may optionally be tagged with a marker, such as a fluorescent marker. The bead may be provided with for example activated ester groups to provide for easy addition of substituents, but other methods of substitution are also available. Thus, in a preferred embodiment, the C_1-C_{10} alkyl groups of the present invention are coupled to the beads via a moiety selected from the group consisting of esters, thioesters, amides, thioamides, ketones, and aldehydes, with esters and amides being particularly preferred.

In one advantageous embodiment of the present invention, the beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups are beads according to formula (I), (II), or (III):


(I)


(II)


(III)

Wherein

- Z is a bond or selected from the group consisting of O and NR_1,
- L_1 is a C_1-C_{10} alkylene group,
- L_2, L_3 and L_4 independently are selected from hydrogen or an optionally substituted C_1-C_{10} alkyl group, and
- R_1 is selected from the group consisting of hydrogen and methyl.

The optional substituent on the C_1-C_{10} alkyl group may preferably be selected from the group consisting of hydroxy, alkoxy, amine, alkylamine, ammonium and alkylammonium.

In one embodiment, Z is NH. In another embodiment, L_1 is a C_1-C_9 alkylene, such as a C_1-C_8 alkylene, a C_1-C_7 alkylene, a C_1-C_6 alkylene, a C_2-C_5 alkylene, such as a C_3-C_5 alkylene group, the most preferred being a C_2-C_5 alkylene group. L_1 may preferably be a straight chain alkylene. Preferably, the optionally substituted C_1-C_{10} alkyl group of L_2, L_3 and L_4 are C_1-C_9 alkyl, such as a C_1-C_8 alkyl, a C_1-C_7 alkyl, a C_1-C_6 alkyl, a C_2-C_5 alkyl, such as a C_3-C_5 alkyl groups. The compounds of formula (I), (II) or (III) may constitute dendrimers in a preferred embodiment.

In yet another embodiment, the C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups are not polylysines.

In one embodiment of the invention, the hydrophobic antigens of the present invention may be molecules comprising a hydrophobic or amphiphilic moiety, such as a lipidated part. Thus, in an alternative embodiment the antigen is a amphiphilic antigen. Such moieties may be assigned to the antigens by *e.g.* post translational modifications.

The hydrophobic and/or amphiphilic antigens of the present invention may particular be antigens related to bacteria. Thus, in one embodiment, the hydrophobic and/or amphiphilic antigen is selected from the group consisting of a lipopeptide, glycolipid or capsular polysaccharide.

Glycolipids are particularly preferred as they are a common form of bacterial antigens and thus useful for the detection of infective diseases in *e.g.* samples from animals, such as mammals. A particularly preferred glycolipid is a lipopolysaccharide. In a preferred embodiment, the glycolipid is a lipopolysaccharide isolated from bacteria, preferably gram-negative bacteria. Thus, in another preferred embodiment, the glycolipid is a lipopolysaccharide isolated from gram-negative bacteria. Preferably, said lipopolysaccharide is isolated from bacteria genera selected from the group consisting of *Actinobacillus sp, Salmonella sp, Lawsonia sp, Mycoplasma sp, Haemophilus sp, Escherichia coli ssp, Klebsiella sp, Vibrio sp, Bordetella sp, Pseudomonas sp, Chlamydia sp, Neisseria sp, Shigella sp, Proteus sp, Brucella sp, Streptobacillus sp, Yersenia sp, Legionella sp, Serratia sp, Pasteurella sp* and *Mannheimia sp.*

In a particular embodiment, said lipopolysaccharide is derived from *Actinobacillus pleuropneumoniae* (Ap) serotypes selected from the group Ap 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15.

One advantage of the present invention is that the antigens isolated from bacteria are directly applicable for non-covalent binding to the beads without further modification, such as *e.g.* de-lipidation which is often used in the prior art. Thus, preferably said hydrophobic antigen is in a native form.

The beads of the present invention function as non-soluble carriers for the antigens and antigen-antibody binding pairs, which may thus be separated from

solution. Further, they may include tags or markers to aid in detection. In a preferred embodiment, the beads of the present invention are polymer beads made from a polymer selected from the group consisting of polystyrene, polypropylene, crosslinked polyethyleneglycol, sepharose and latex. Mixtures of polymer types may also be employed. In another embodiment of the invention, the beads are nanoparticles made from a material selected from the group consisting of gold, co-block polymers, dendrimeric polymers and metal colloids. In order to facilitate isolation of beads and conjugates hereof from solution the beads may include a means for isolating them from solution. For example, the beads may be magnetic. They may alternatively be insoluble. Their lack of solubility may provide for isolation by filtration. Other isolation methods may include centrifugation, where the density difference between bead and solution is employed. Preferably, the beads may comprise a barcode/marker or tag, such as a fluorescent, luminescent, size, magnetic, spin-label, nucleotide/DNA or peptide marker.

The sample of the present invention may be any sample comprising antibodies. Typically a biological sample is tested, *e.g.* to detect antibodies towards infective diseases and/or bacteria. Samples may be serum samples, preferably mammalian serum samples. In a particular embodiment, the sample may be a porcine serum sample. In another embodiment, the sample may be a serum sample from a species with an origin selected from the group consisting of porcine, bovine, avian or human.

As mentioned under the description of the beads of the invention, several methods for isolation of the bead conjugates from *e.g.* the sample solution may be employed. Thus, in one embodiment, said bead-antigen conjugates and/or bead-antigen-antibody conjugates are isolated from solution according to a method selected from magnetism, centrifugation, size exclusion, cell sorting and dialysis.

The beads may be contacted with the hydrophobic antigen in aqueous solution. Furthermore, such a contact between beads and hydrophobic antigen may be completed in less than 5 h, such as less than 4 h, 3 h, such as less than 2 h. As

such, attachment between beads and hydrophobic antigen may be completed in less than 5 h, such as less than 4 h, 3 h, such as less than 2 h.

The adsorption of antigen onto the surface of the modified beads of the present invention may be performed in a number of ways. Preferably, the non-covalent adsorption of hydrophobic antigen to the beads is performed in aqueous solution. Preferably, the non-covalent adsorption of hydrophobic antigen to the beads is completed in less than 10 h, such as less than 5 h, such as less than 4 h, 3 h, such as less than 2 h.

The detection step of the present invention may be performed in several ways and may depend on the nature of the beads used and optional further markers used to identify individual bead-antigen-antibody conjugates. In a preferred embodiment, the beads may include fluorescent markers or tags, while the bead-antigen-antibody conjugates may be further tagged by *e.g.* a secondary antibody comprising *e.g.* a different fluorescent tag. This provides for a unique dual-colored tag, which allows for selective detection of beads comprising antibodies. Thus, in a preferred embodiment, the isolated bead-antigen-antibody conjugates are detected and identified in an apparatus suitable for quantification of fluorescent signals. Said apparatus suitable for quantification of fluorescent signals may be selected from the group consisting of spectrometers, microscopes and flow cytometers. In addition, as described above, the isolated bead-antigen-antibody conjugates are detected and identified using a ratio of two fluorescent signals. Therefore, the isolated bead-antigen-antibody conjugate may preferably be contacted with a second fluorescent label. In a particularly preferred embodiment, the isolated bead-antigen-antibody conjugate is contacted with a biotinylated secondary antibody and subsequently contacted with streptavidin-phycoerythrin (SA-PE).

As explained, the present invention has the particular advantage of providing an assay highly suitable to multiplex analysis and detection of antibodies. Thus, in a preferred embodiment of the method of the present invention, a multitude of groups of beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine and/or hydroxyl groups and hydrophobic antigens are combined to form a plurality of bead-antigen conjugates. Each individual group of bead-antigen

conjugates may comprise many beads, such as *e.g.* 100-10.000 beads, preferably 100-5.000 beads, 100-2000 beads, such as preferably 500-1500 beads. Preferably, each individual group of bead-antigen conjugates are present in equal numbers. Up to 100 different groups of bead-antigen conjugates may be employed, such as preferably 1-80 groups, 1-60 groups, 1-40 groups, 1-30 groups, 2-20, groups such as preferably 2-15 groups of bead-antigen conjugates.

The method of the present invention may preferably be used for sero-diagnostics. Particularly human or porcine sero-diagnostics. In one embodiment, the present invention may be used for porcine, bovine, avian or human sero-diagnostic. In another embodiment, the method of the present invention may be used for sero-diagnostics of a serum sample from a species with an origin selected from the group consisting of porcine, bovine, avian or human.

The method of the present invention may advantageously be performed via the use of an antibody detection kit.

Thus, a second aspect of the present invention is an antibody detection kit comprising:

- at least one group of beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups,
- optionally at least one composition comprising a hydrophobic antigen, and
- optionally instructions for performing the method according to claim 1.

In a preferred embodiment, the at least one composition comprising a hydrophobic antigen is included in the kit. In another embodiment, the bead-antigen conjugates may already be formed in the kit, and thus said kit comprises at least one composition comprising a group of bead-antigen conjugates. In another embodiment, at least two groups of beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups are included. In a further embodiment, at least two compositions comprising a hydrophobic antigen are included. Finally, at least two compositions comprising a group of bead-antigen conjugates may be included.

It is noted that the embodiments defined for the method of the present invention also applies to the kit when applicable.

The present invention also involves a bead-antigen conjugate. Thus, a third aspect of the present invention is a bead-antigen conjugate comprising:

- beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups, and
- a hydrophobic antigen,

wherein said hydrophobic antigen is non-covalently adsorbed to said bead.

A preferred version of the third aspect of the present invention relates to a bead-antigen conjugate comprising:

- beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups, and
- a hydrophobic antigen.

An embodiment of the present invention relates to the bead-antigen conjugate as described herein, wherein said hydrophobic antigen is adsorbed on said beads.

Another embodiment of the present invention relates to the bead-antigen conjugate as described herein, wherein said adsorption does not include formation of a covalent bond between said hydrophobic antigen and said beads.

Yet another embodiment of the present invention relates to the bead-antigen conjugate as described herein, wherein said beads are non-covalently attached to said hydrophobic antigen.

In a preferred embodiment, the bead-antigen conjugate is for use in the detection of antibodies.

A fourth aspect of the present invention is a composition comprising at least two individual groups of bead-antigen conjugates each individually comprising:

- beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups, and
- a hydrophobic antigen,

wherein said hydrophobic antigen is non-covalently adsorbed to said bead, and wherein the hydrophobic antigen is different in each group.

A preferred version of the fourth aspect of the present invention relates to a composition comprising at least two individual groups of bead-antigen conjugates each individually comprising:

- beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups, and
- a hydrophobic antigen,

wherein the hydrophobic antigen is different in each group.

An embodiment of the present invention relates to the composition as described herein, wherein said hydrophobic antigen is adsorbed on said beads.

Another embodiment of the present invention relates to the composition as described herein, wherein said adsorption does not include formation of a covalent bond between said hydrophobic antigen and said beads.

Still another embodiment of the present invention relates to the composition as described herein, wherein said beads are non-covalently attached to said hydrophobic antigen.

It is noted that the embodiments defined for the method of the present invention also applies to the bead-antigen conjugate and the composition when applicable.

All patent and non-patent references cited in the present application, are hereby incorporated by reference in their entirety.

The invention will now be described in further details in the following non-limiting examples.

Examples

Abbreviations:

DEDA: N,N-diethylethylenediamine

EDC: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide

NHS: N-hydroxysulfosuccinimide

SA-PE: Streptavidin-Phycoerythrin

MWCO: Molecular weight cut-off

Example 1a: Beads

Commercially available beads of different chemical compositions were used as a starting point for synthesis of functionalized beads capable of adsorbing hydrophobic antigens from an aqueous solution. In particular, beads of 0.3-10 µm made of polystyrene, polypropylene and sepharose were employed as starting material. The beads were purchased functionalized with carboxyl groups, amino groups or hydroxyl groups, to enable coupling of alkyl groups to the bead surface. Furthermore, the beads were either plain or magnetic and/or fluorescent.

Example 1b: Nanoparticles

In some embodiments of the invention, the beads were replaced by nanoparticles selected from the group consisting of gold, silver, cadmium sulfide, cadmium selenide, latex, dendrimers. These nanoparticles contained mercapto acids and/or mercapto amines that enabled further modifications of the nanoparticle surface.

Example 2a: DEDA microbeads - Protocol for the modification of corresponding carboxy-beads with C_1-C_{10} alkyl groups comprising amine groups

1. A commercial suspension of magnetic beads (Bio-Rad, Bio-Plex Pro/Mag-Plex Magnetic COOH beads, 064) were vortexed/stirred and 2 mL was transferred to an Eppendorf tube (Protein low bind) using a pipette with a cut pipette tip.
2. 1mL MilliQ water was added to the Eppendorf tube.
3. N,N-diethylethylenediamine (DEDA) (460 µL, 3.2 mmol, MW 118.2, d 0.83) was added and the Eppendorf tube was shaken/turned until all crystals had dissolved. The shaking was performed to avoid precipitation/colloid formation in solution.

4. NHS (300 mg, 3.2 mmol) were added and the tube was shaken/turned until all crystals had dissolved.
5. The bead suspension was cooled on an ice bath.
6. EDC (600 mg, 3.2 mmol, MW 191) was added in the cold solution and the tube was shaken/turned until all crystals had dissolved.
7. The bead suspension was kept on ice bath for 10 min.
8. The mixture was shaken overnight at room temperature.
9. The beads were washed 3 times with 4 mL MilliQ water by
    a. vortexing the bead suspension to homogenize and
    b. retain modified beads in the tube by the use of a Bio-Rad magnet for a minimum of 3 minutes and
    c. removal of the supernatant
10. After removal of the final supernatant, the beads were suspended in 4mL MilliQ water.

See Fig. 1a for a schematic overview of the formation of DEDA beads.

Example 2b: "Bead 2" beads – Protocol for the modification of corresponding carboxy-beads with C_1-C_{10} alkyl groups comprising hydroxy groups

1. A commercial suspension of magnetic beads (Bio-Rad, Bio-Plex Pro/Mag-Plex Magnetic COOH beads, 026 and 045) were vortexed/stirred and 100 uL were transferred to two 15 mL Falcon tubes (one Falcon tube for 026 and one for 045 beads, respectively) using a pipette with a cut pipette tip.
2. 200 µL MilliQ water was added to each Falcon tube.
3. 5-amino-1-pentanol (15 mg, 0.16 mmol, MW 103) was added and the Falcon tube was shaken/turned until all crystals had dissolved. The shaking was performed to avoid precipitation/colloid formation in solution.
4. EDC (30 mg, 0.16 mmol, MW 191) and NHS (15 mg, 0.16 mmol, MW 115) were added and the Falcon tube was shaken/turned until all crystals had dissolved.
5. The mixture was shaken overnight at room temperature.
6. The beads were transferred to an Eppendorf tube (Protein low bind) using a pipette with a cut pipette tip.
7. The mixture was washed 3 times with MilliQ water by the use of Bio-Rad magnet equipment.

8. After removal of the final supernatant the beads were suspended in 200 µL MilliQ water.

See Fig. 1b for a schematic overview of the formation of bead-2 beads.

Example 3: Preparation of lipopolysaccharide (LPS) antigens

LPS was prepared by fermentation of the parent bacteria and purification according to the procedure published in E.S. Jauho *et al.* (J. Immunol. Meth., 2000).

Example 4: Adsorption of lipopolysaccharide antigens to modified polymer beads (*e.g.* DEDA beads or "beads 2")

The following equipment were used for coupling of LPS. Sonication bath (Bandelin, Sonorex Digitec), Bio-Rad magnet for 1.5 mL and 5 mL Eppendorf tubes, 1.5 mL and 5 mL LoBind Eppendorf tubes, hemacytometer (Bürker türk), microscope (Leica DMRB, 10x/0.22 PH1 lense)

Samples with a variety of antigens for subsequent binding to the modified beads were prepared according to table 1.

Table 1: Antigen type and dilution for bead modification

Ap serotype	Bead region	Antigen*	LPS/CP batch	Dilution
Ap 1	28	LPS	15. april 02	1 ul + 1 ml MQ
Ap 5	64	CP	155 (1)	100 ul + 900 ul MQ
Ap 6	45	LPS	146/150	1 ul + 1 ml MQ
Ap 7	37	LPS	134 (1)	1 ul + 1 ml MQ
Ap 10	43	LPS	122 (6&7)	1 ul + 1 ml MQ
Ap 12	62	LPS	142 (1)	1 ul + 1 ml MQ

* LPS: Lipopolysaccharide in solution, CP: Capsular polysaccharide.

MES Buffer (0,1 M 2-(N-morpholino ethanesulfonic acid (MES), pH 5,0) was prepared by dissolving 4.88g MES in MilliQ (250mL) and adding approx. 5 drops 5N NaOH, followed by sterile filtration.

Coupling of LPS to the modified beads were accomplished by the following protocol:

1. The modified beads were vortexed and sonicated (20 sec).
2. Beads were counted in a hemacytometer.
3. $8x10^5$ beads were transferred to a 1.5mL LoBind Eppendorf tube.
4. Beads were placed in BioRad Magnet equipment for 60 sec and the supernatant was carefully removed with a pipette.
5. Beads were suspended in MES buffer (50 µL), and vortexed and sonicated (20sec).
6. LPS solution (150 µL) were added to the beads.
7. The sample was incubated at room temperature for 30 min in the dark.
8. LPS solution (200 µL) were added to the sample, followed by vortexing and incubation at room temperature for 30 min in the dark.
9. Step 8 was repeated two times.
10. The sample was placed in Bio-Rad magnet equipment for 60 sec and the supernatant was carefully removed with a pipette.
11. MilliQ water (1000 µL) was added to the sample.
12. Step 10 and 11 were repeated 5 times.
13. After removal of the supernatant on the Bio-Rad magnet equipment, the beads were suspended in MilliQ water (150 µL).
14. Bead concentration was calculated by use of a hemacytometer and the following equation: $\text{Number of}\frac{\text{beads}}{\text{mL}} = (\frac{\text{Bead count in 4 wells}}{4} \times \text{dilution factor} \times 1000)$
15. The LPS modified beads were stored in the dark at 4 °C.

Example 5: Contacting samples with bead-antigen conjugates

Aliqots of magnetic beads coupled with the antigen in question were vortexed and each bead suspension was diluted to give a concentration of 5 x 10^4 beads/mL using an equal amount of dilution buffer (PBS, 0.05% Tween, 1% BSA, 0.5M NaCl). The beads were mixed,vortexed and sonicated, and 25 µL of this bead mixture is added to each well of a 96 well plate. Hereafter 25 µL swine serum

samples diluted in dilution buffer were added to the bead mixture in each well. The plates were incubated 40 min at room temperature on a plate shaker.

After incubation, the plate was washed with washing buffer (PBS, 0.05% Tween) using an Elx405 Biotek washing robot. 25uL Biotin conjugated canine anti-swine IgG (Sigma Aldrich) diluted in BSA-Tween buffer (PBS, 0.05% Tween, 1% BSA) was added to each well and the bead mixtures were incubated for 20 min at room temperature on plate shaker. Unbound biotin conjugated IgG was removed by washing using the washing robot. 25uL Streptavidin-PE (Life Tech) diluted in BSA-Tween buffer was added and the plate was incubated for 20 min. The plate was washed with the washing robot and then 100uL washing buffer was added and the plate was shaken for ca. 1 min.

Example 6: Detection of bead-antigen-antibody conjugates

The plate was analyzed by a plate reader (Bio-Plex 200 Reader from Bio-Rad) which was adjusted to analyze a minimum of 50 beads of a bead set in 50 µL bead solution. Bound swine serum sample antibodies was measured in median fluorescent intensity (MFI) where MFI for background (MFI measured for beads incubated without a serum sample) was subtracted. For each sample, the S/P% was calculated using the following formula:

$$\frac{\text{MFI(serum sample)} - \text{MFI (negative control serum}}{\text{MFI (positive control serum)} - \text{MFI (negative control serum)}} \times 100$$

Additionally, the data is in some cases represented by a P/N value, which is the MFI of the sample divided by the MFI for the negative control: P/N = MFI sample/MFI negative control.

Example 7: "Bead 2" results - *Actinobacillus Pleuropneumoniae* (Ap), different serotypes in Ap multiplex Bead 2 assay

Bead-antigen conjugates consisting of Bead 2 and LPS antigen originating from different serotypes of Ap (1, 2, 5, 6, 10 and 12) were prepared according to example 2b and example 4. The six versions of bead-antigen conjugates were mixed and contacted with a panel of serum samples from pigs that were experimentally infected with only a single Ap serotype. Subsequent to binding of antibodies specific to the bead-antigen conjugates, biotinylated anti-pig IgG were added followed by addition of SA-PE.

Amount of bound antibody was evaluated by the fluorescent read-out corresponding to the unique two color-code assigned to each antigen (combination of bead and SA-PE fluorescence). Fig. 2A-F depicts the results of each of the serum samples corresponding to Ap 1, 2, 5, 6, 10 and 12. The mixture of bead-antigen conjugates were capable of detecting the single Ap serotype in each pig serum sample with minimal occurrence of false positives.

Example 8: Ap 5-DEDA beads results

Bead-antigen conjugates consisting of DEDA beads with adsorbed LPS Ap 5 antigen (Fig. 3A-C) were prepared according to example 2a and example 4. The bead-antigen conjugates were contacted with a panel of serum sample from pigs that were experimentally infected with a wide selection of antigens.

Detection and quantification of bound antibodies were performed as described in example 7. The bead-antigen conjugates were capable of detecting antibodies corresponding to the single Ap 5 serotype within the panel of pig serum sample and distinguish the sample from other bacteria genus.

Example 9: Ap 6-DEDA beads results

Bead-antigen conjugates consisting of DEDA beads with adsorbed LPS Ap 6 antigen (Fig. 4) were prepared according to example 2a and example 4. The bead-antigen conjugates were contacted with a panel of serum sample from pigs that were experimentally infected with a wide selection of antigens.

Detection and quantification of bound antibodies were performed as described in example 7. The bead-antigen conjugates detected not only the single Ap 6 serotype antibodies but also the Ap 8 serotype antibodies within the panel of pig serum samples, however, Ap8/Ap6 cross-reactivity is a generally occurring phenomenon.

Example 10: Bead 2 with bound *Lawsonia* antigen tested and compared to EDC coupled *Lawsonia* antigen beads.

Bead-antigen conjugates consisting of 'Bead 2' beads with adsorbed *Lawsonia* antigen (Fig. 5A-B) were prepared according to example 2b and example 4. Bead-antigen conjugates formed by either EDC coupling of *Lawsonia* antigen to

unmodified beads (according to the procedure published by Iihara et al. Jpn. J. Infect. Dis. (2007), 230-234) or coupling to 'Bead 2' beads (Fig. 5C-D) according to example 4. The two bead-antigen conjugate constructs were contacted with a panel of serum sample from pigs that were experimentally infected with a selection of antigens. Detection and quantification of bound antibodies were performed as described in example 7. Both constructs effectively detected *Lawsonia* antibodies. However, higher P/N values were achieved by adsorption of *Lawsonia* antigen to 'Bead 2'.

Example 11: *Salmonella Typhimurium* on DEDA beads

Bead-antigen conjugates consisting of DEDA beads with adsorbed *Salmonella Typhimurium* antigen (Fig. 6) were prepared according to example 2a and example 4. The bead-antigen conjugates were contacted with a panel of serum sample from pigs that were experimentally infected with a wide selection of antigens. Detection and quantification of bound antibodies were performed as described in example 7. The bead-antigen conjugates were capable of detecting antibodies corresponding to the *Salmonella Typhimurium* antigen within the panel of pig serum sample and distinguish the sample from other bacteria genus. Antibodies against *Salmonella Typhimurium* were detected with high signal-to-noise ratio and close to zero false-positive signal.

Example 12: *Salmonella Cholerasuis* antigen on DEDA beads

This example is identical to example 11, only now the antigen on the DEDA beads were *Salmonella Cholerasuis* antigen (Fig. 7). The bead-antigen conjugates were capable of detecting antibodies corresponding to the *Salmonella Cholerasuis* antigen within the panel of pig serum sample and distinguish the sample from other bacteria genus. Antibodies against *Salmonella Cholerasuis* were detected with high signal-to-noise ratio and close to zero false-positive signal.

Example 13: DEDA latex particles (blue and red colored) - Protocol for the modification of corresponding carboxy-Latex particles

1. 500 µL 10% Latex particles (420nm) were put in an Eppendorf tube.
2. 500 µL MQ water was added
3. N, N-Diethylene diamine (230uL) was added
4. NHS (150mg) was added
5. The mixture was cooled on an ice bath

6. EDC (300mg) was added and the reaction mix was kept on ice bath for 10 minutes
7. Shaking 16 h at r.t.
8. Transfer to Vivaspin (MWCO 3000), an addition of MQ to a volume of 15 mL
9. Centrifuging for 12 minutes (5000rpm) removed almost all supernatant
10. The Vivaspin procedure above was performed three times.
11. After removal of the supernatant, the residual latex beads were suspended in MQ water (5mL), thus yielding a 1 % suspension of DEDA-Latex beads.

Example 14: Adsorption of glycolipid antigens from mycoplasma to DEDA-Latex polymer particles

1. Mycoplasma glycolipid antigen (25 µL, 1mg/mL solution) was added to 1% DEDA-Latex particles in MQ (500 µL)
2. Sonication for 5 minutes
3. Incubation 16 h at room temperature
4. Centrifuging (2minutes, 10.000 rpm)
5. Removal of the supernatant
6. Centrifuging/supernatant removal was done three times
7. Resuspension in MQ water (500 µL), yielding a 1% suspension of Mycoplasma-Latex particles
8. The Latex particles were sonicated 20 min prior to use

Example 15: Comparison of beads of the invention to poly-L-lysine (PLL) beads

Coupling of Ap5 CP antigen to poly-L-lysine beads:

Tube A (Alkaline buffer (4mL 0.01% with 0.001% phenolphthalein)): NaOH (0.1g) was dissolved in MilliQ water (10mL). 1 mL of this solution was diluted in Milli Q water (99mL). 40mL of this solution was mixed with 40 µL phenolphthalein (1% in ethanol). After thorough vortexing this solution of transferred to four tubes (A1-A4), 1 mL solution in each.

Tube B: Cyanuric chloride (1mg) in four tubes (B1-B4)

Tube C: Poly-L-lysine (10mg, the compound should be a dry crystalline otherwise do not apply) was dissolved in MQ water (10mL). This solution was transferred to four tubes (C1-C4), where C1: Ap5 (10mg/mL), C2: Ap5 (20mg/mL), C3:Ap5 (50mg/mL), C4: Ap5 (100mg/mL).

Alkalizing the polysaccharide (e.g. lipopolysaccharide): Transfer 200 µL LPS solution to tube A and mix for 10 sec (pink solution)

Activation: The solution in tube A was transferred to tube B and vortexed for 10 sec. until the solution was colorless.
Coupling of antigen to poly-L-lysine: The content of tube B was transferred to tube C and vortexed for 20 sec, and put in the cold (4 °C) overnight.
The LPS-poly-L-lysine conjugates were desalted on a PD10 column.

Coupling of LPS-poly-L-lysine conjugate to carboxylated beads:
Carboxy-beads (64 µL, 8 x 10^5 beads) were transferred to LoBind Eppendorf tube, and the tube was put in the BioRad magnet equipment for 60 sec and the supernatant was carefully removed. The beads were resuspended in 64uL activation buffer (0.1M NaH_2PO_4, pH 6.2) and vortexed followed by sonication for 20 sec. 10 µL NHS solution (0.01g in 200 µL activation buffer) was added followed by addition of 10 µL EDC solution (50mg/mL in activation buffer). The mixture was vortexed at low speed. Incubation for 20 min in the dark. The tube was put in the BioRad magnet for 60 sec and the supernatant was removed. The activated beads were washed with 200 µL PBS. The LPS-Polylysine conjugate in PBS (500 µL) and vortex the bead suspension. Incubation (rotation) for 2 h in the dark at room temperature. The tube was placed in the BioRad magnet equipment for 60 sec and the supernatant carefully removed. The beads were washed with 500 µL and the supernatant removed using the magnet as previously described. The beads were suspended in blocking buffer (PBS-Tween-BSA) and vortexed mildly for 15 sec. The beads were incubated in the dark for 30 min. The supernatant was removed on the magnet and the beads were suspended in 50 µL storage buffer (PBS-TBN) and vortexed for 20 sec. The bead concentration was determined on a hemocytometer. The antigen-coupled beads were stored at 2-8 °C in the dark.

In addition to the Ap 5 DEDA beads (Fig. 8A) gives a higher read out (higher sensitivity), compared to the Ap 5 PLL beads (Fig. 8B), the latter furthermore agglutinate heavily, which make bead counting much more slow, therefore, much larger amounts/numbers of beads are needed to give a reliable and sensitive read out. With Ap 5 PLL beads the coupling efficiency also varies much more compared

to the Ap 5 coupling to DEDA beads. The Ap 5 DEDA beads also show superior Ap 5 antibody sensitivity compared to traditional ELISA technique (Fig. 8C).

References

- Hirayama and Sakata, J. Chromatogr. B. (2002), 419-432
- WO 2010/036133
- Takahashi et al, J. Immunol. Methods. (1992), 67-71
- WO 2013/060335
- E.S. Jauho, U. Boas, C. Wiuff, K. Wredstrøm, B. Pedersen, L.O. Andresen, P.M.H.Heegaard, M.H. Jakobsen, New technology for regiospecific coupling of polysaccharide antigens in ELISA for serological detection, J. Immunol. Meth. 242 (2000) 133–143.
- Iihara et al. Jpn. J. Infect. Dis. (2007), 230-234

Claims

1. A method of detecting antibodies comprising the steps of:
    i. providing a first group of beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups,
    ii. contacting said first group of beads with a first hydrophobic antigen to provide a first group of bead-antigen conjugates,
    iii. isolating said bead-antigen conjugates,
    iv. contacting said bead-antigen conjugates with a sample to bind antibodies therein to provide bead-antigen-antibody conjugates, and
    v. detecting said bead-antigen-antibody conjugates.

2. The method according to claim 1, wherein said first hydrophobic antigen is adsorbed on said first group of beads to provide said first group of bead-antigen conjugates.

3. The method according to claim 2, wherein said adsorption does not include formation of a covalent bond between said first hydrophobic antigen and said first group of beads.

4. The method according to claim 1, wherein said first group of beads are non-covalently attached to said first hydrophobic antigen to provide said first group of bead-antigen conjugates.

5. The method according to claims 2-4, wherein said first hydrophobic antigen is adsorbed or non-covalently attached to said first group of beads through hydrophobic interactions.

6. The method according to claims 2-4, wherein said first hydrophobic antigen is adsorbed or non-covalently attached to said first group of beads through hydrophobic and ionic interactions.

7. The method according to any of the preceding claims, wherein said beads are selected from the group consisting of polymer beads and nanoparticles.

8. The method according to any of the preceding claims, wherein steps i) and ii) are repeated with a second and/or further group of beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups and a second and/or further hydrophobic antigen to provide second and/or further group of bead-antigen conjugates.

9. The method according to any of the preceding claims, comprising a step of mixing said first bead-antigen conjugate with said second and/or further bead-antigen conjugates.

10. The method according to any of the preceding claims, wherein the C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups are linear.

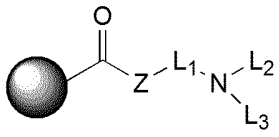
11. The method according to any of the preceding claims, wherein the C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups are branched.

12. The method according to any of the preceding claims, wherein the C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups form a dendrimer.

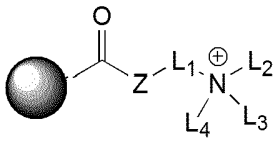
13. The method according to any of the preceding claims, wherein the amine, ammonium, ether and/or hydroxyl groups are selected from the group consisting of primary amines, secondary amines, tertiary amines, quaternary ammoniums, primary hydroxyl groups, ethers, or a mixture thereof.

14. The method according to any of the preceding claims, wherein the C_1-C_{10} alkyl groups are coupled to the beads via a moiety selected from the group consisting of esters, thioesters, amides, thioamides, ketones, and aldehydes.

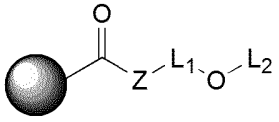
15. The method according to any of the preceding claims, wherein the beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups are beads according to formula (I), (II), or (III):



(I)



(II)



(III)

wherein

- Z is a bond or selected from the group consisting of O and NR_1,
- L_1 is a C_1-C_{10} alkylene group,
- L_2, L_3 and L_4 independently are selected from hydrogen or an optionally substituted C_1-C_{10} alkyl group, and
- R_1 is selected from the group consisting of hydrogen and methyl.

16. The method according to claim 15, wherein the optional substituent on the C_1-C_{10} alkyl group is selected from the group consisting of hydroxy, alkoxy, amine, alkylamine, ammonium and alkylammonium.

17. The method according to any of claims 15-16, wherein Z is NH.

18. The method according to any one of claims 15-17, wherein L_1 is a C_2-C_5 alkylene group.

19. The method according to any one of claims 15-18, wherein L_1 is a straight chain alkylene.

20. The method according to any of the preceding claims, wherein the C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups are not polylysines.

21. The method according to any of the preceding claims, wherein the hydrophobic antigen is selected from the group consisting of a lipopeptide, glycolipid or capsular polysaccharide.

22. The method according to any of the preceding claims, wherein the hydrophobic antigen is a glycolipid.

23. The method according to claims 21-22, wherein the glycolipid is a lipopolysaccharide.

24. The method according to claims 21-23, wherein the glycolipid is a lipopolysaccharide isolated from bacteria.

25. The method according to any one of claims 23-24, wherein said lipopolysaccharide is isolated from bacteria genera selected from the group consisting of *Actinobacillus sp, Salmonella sp, Lawsonia sp, Mycoplasma sp, Haemophilus sp, Escherichia coli ssp, Klebsiella sp, Vibrio sp, Bordetella sp, Pseudomonas sp, Chlamydia sp, Neisseria sp, Shigella sp, Proteus sp, Brucella sp, Streptobacillus sp, Yersenia sp, Legionella sp, Serratia sp, Pasteurella sp* and *Mannheimia sp.*

26. The method according to any one of claims 23-25, wherein said lipopolysaccharide is derived from *Actinobacillus pleuropneumoniae* (Ap) serotypes selected from the group Ap 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15.

27. The method according to any of the preceding claims, wherein said hydrophobic antigen is in a native form.

28. The method according to any of the preceding claims, wherein the beads are polymer beads made from a polymer selected from the group consisting of polystyrene, polypropylene, crosslinked polyethyleneglycol, sepharose and latex.

29. The method according to any of the preceding claims, wherein the beads are nanoparticles made from a material selected from the group consisting of gold, co-block polymers, dendrimeric polymers and metal colloids.

30. The method according to any of the preceding claims, wherein the beads are magnetic.

31. The method according to any of the preceding claims, wherein the beads comprise a fluorescent marker.

32. The method according to any of the preceding claims, wherein said sample is a mammalian serum sample.

33. The method according to any of the preceding claims, wherein said sample is a porcine serum sample.

34. The method according to any of the preceding claims, wherein said bead-antigen conjugates and/or bead-antigen-antibody conjugates are isolated from solution according to a method selected from magnetism, centrifugation, size exclusion, cell sorting and dialysis.

35. The method according to any of the preceding claims, wherein contacting said first group of beads with a first hydrophobic antigen is performed in aqueous solution.

36. The method according to any of the preceding claims, wherein contacting said first group of beads with a first hydrophobic antigen is completed in less than 5 h, such as less than 4 h, 3 h, such as less than 2 h.

37. The method according to any of the preceding claims, wherein the isolated bead-antigen-antibody conjugates are detected and identified in an apparatus suitable for quantification of fluorescent signals.

38. The method according to claim 37, wherein said apparatus suitable for quantification of fluorescent signals is selected from the group consisting of spectrometers, microscopes and flow cytometers.

39. The method according to any of the preceding claims, wherein the isolated bead-antigen-antibody conjugates are detected and identified using a ratio of two fluorescent signals.

40. The method according to any of the preceding claims, wherein the isolated bead-antigen-antibody conjugate is contacted with a second fluorescent label.

41. The method according to any of the preceding claims, wherein the isolated bead-antigen-antibody conjugate is contacted with a biotinylated secondary antibody and subsequently contacted with streptavidin-phycoerythrin (SA-PE).

42. The method according to any of the preceding claims, wherein a multitude of groups of beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine and/or hydroxyl groups and hydrophobic antigens are combined to form a plurality of bead-antigen conjugates.

43. The method according to any of the preceding claims, wherein said method is used for sero-diagnostics.

44. An antibody detection kit comprising:

- at least one group of beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups,
- optionally at least one composition comprising a hydrophobic antigen, and
- optionally instructions for performing the method according to claim 1.

45. The antibody detection kit according to claim 44, comprising at least one composition comprising a hydrophobic antigen.

46. The antibody detection kit according to any of claims 44-45, comprising at least one composition comprising a group of bead-antigen conjugates.

47. A bead-antigen conjugate comprising:

- beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups, and
- a hydrophobic antigen.

48. The bead-antigen conjugate according to claim 47, wherein said hydrophobic antigen is adsorbed on said beads.

49. The bead-antigen conjugate according to claim 48, wherein said adsorption does not include formation of a covalent bond between said hydrophobic antigen and said beads.

50. The bead-antigen conjugate according to claim 47, wherein said beads are non-covalently attached to said hydrophobic antigen.

51. A composition comprising at least two individual groups of bead-antigen conjugates each individually comprising:

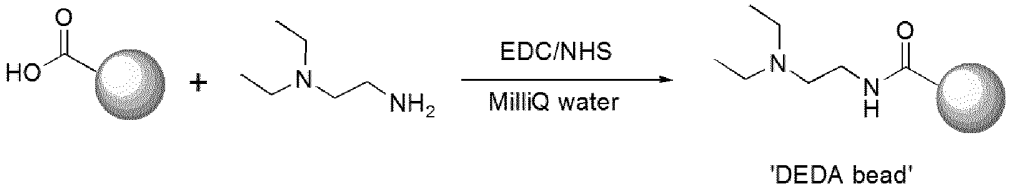
- beads comprising a surface modified with C_1-C_{10} alkyl groups comprising amine, ammonium, ether and/or hydroxyl groups, and
- a hydrophobic antigen,

wherein the hydrophobic antigen is different in each group.

52. The composition according to claim 51, wherein said hydrophobic antigen is adsorbed on said beads.

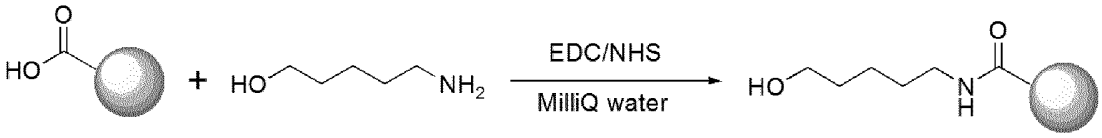
53. The composition according to claim 52, wherein said adsorption does not include formation of a covalent bond between said hydrophobic antigen and said beads.

54. The composition according to claim 51, wherein said beads are non-covalently attached to said hydrophobic antigen.



'DEDA bead'

FIG. 1A



'Bead 2'

FIG. 1B

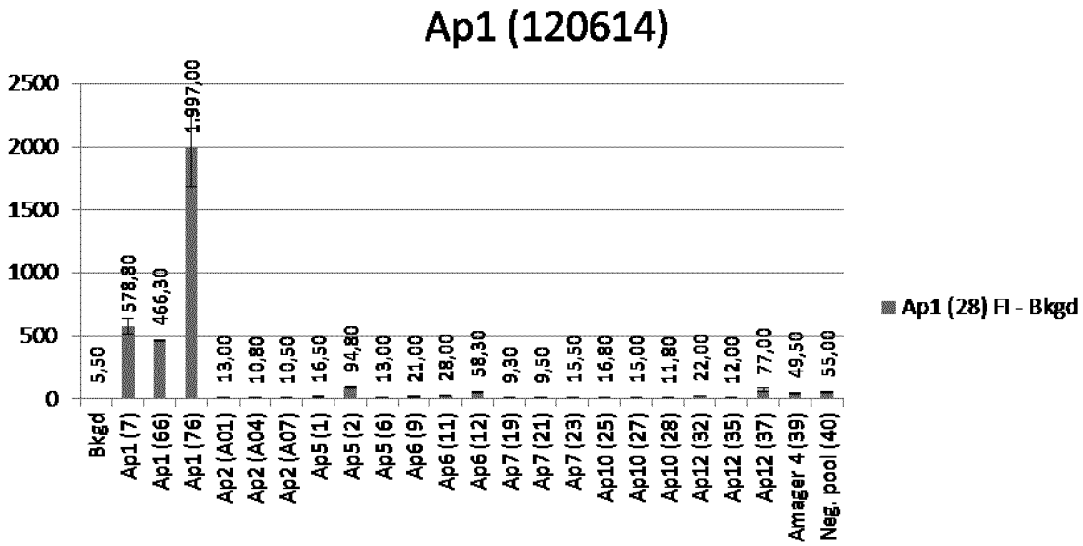

Ap1 (120614)
2500
2000
1500
1000
500
0
5,50
578,80
466,30
1.997,00
13,00
10,80
10,50
16,50
94,80
13,00
21,00
28,00
58,30
9,30
9,50
15,50
16,80
15,00
11,80
22,00
12,00
77,00
49,50
55,00
Bkgd
Ap1 (7)
Ap1 (66)
Ap1 (76)
Ap2 (A01)
Ap2 (A04)
Ap2 (A07)
Ap5 (1)
Ap5 (2)
Ap5 (6)
Ap6 (9)
Ap6 (11)
Ap6 (12)
Ap7 (19)
Ap7 (21)
Ap7 (23)
Ap10 (25)
Ap10 (27)
Ap10 (28)
Ap12 (32)
Ap12 (35)
Ap12 (37)
Amager 4 (39)
Neg. pool (40)
Ap1 (28) FI - Bkgd

FIG. 2A

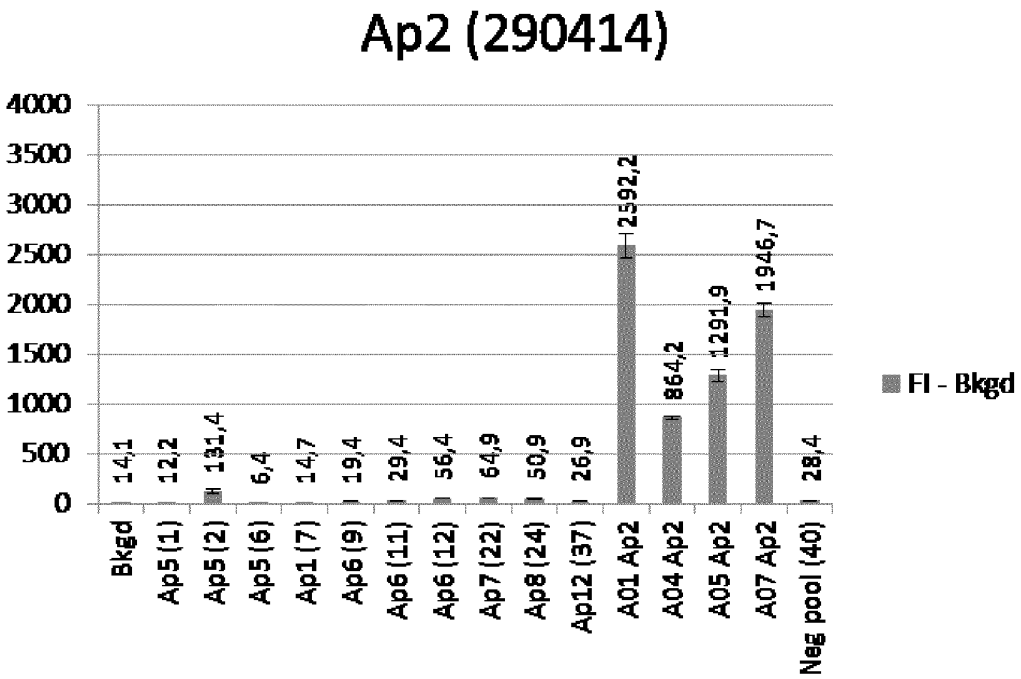

Ap2 (290414)
4000
3500
3000
2500
2000
1500
1000
500
0
14,1
12,2
131,4
6,4
14,7
19,4
29,4
56,4
64,9
50,9
26,9
2592,2
864,2
1291,9
1946,7
28,4
Bkgd
Ap5 (1)
Ap5 (2)
Ap5 (6)
Ap1 (7)
Ap6 (9)
Ap6 (11)
Ap6 (12)
Ap7 (22)
Ap8 (24)
Ap12 (37)
A01 Ap2
A04 Ap2
A05 Ap2
A07 Ap2
Neg pool (40)
FI - Bkgd

FIG. 2B

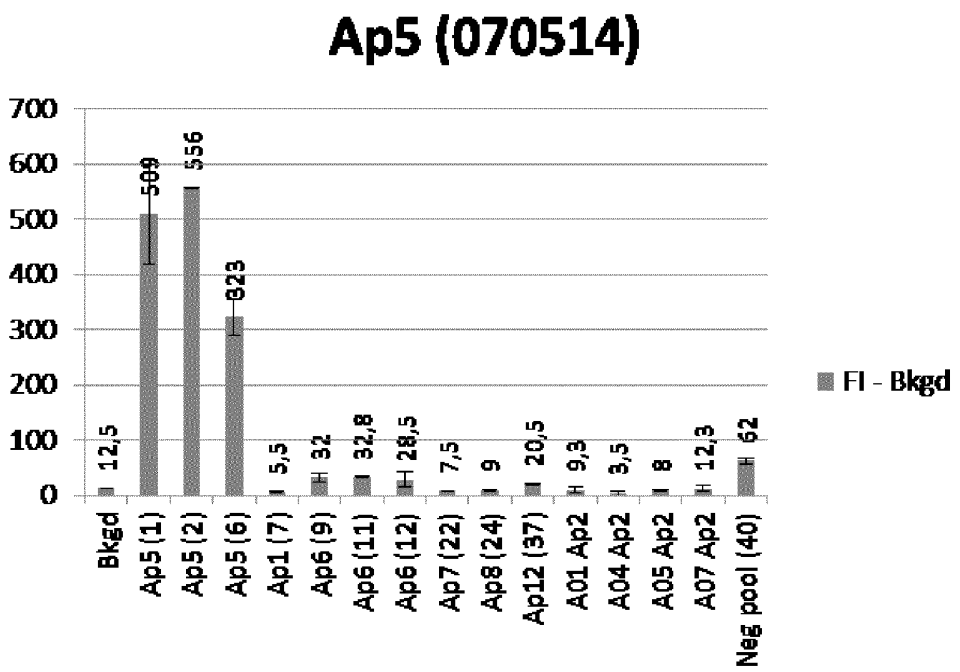

Ap5 (070514)
700
600
500
400
300
200
100
0
12,5
509
556
323
5,5
32
32,8
28,5
7,5
9
20,5
9,3
3,5
8
12,3
62
Bkgd
Ap5 (1)
Ap5 (2)
Ap5 (6)
Ap1 (7)
Ap6 (9)
Ap6 (11)
Ap6 (12)
Ap7 (22)
Ap8 (24)
Ap12 (37)
A01 Ap2
A04 Ap2
A05 Ap2
A07 Ap2
Neg pool (40)
FI - Bkgd

FIG. 2C

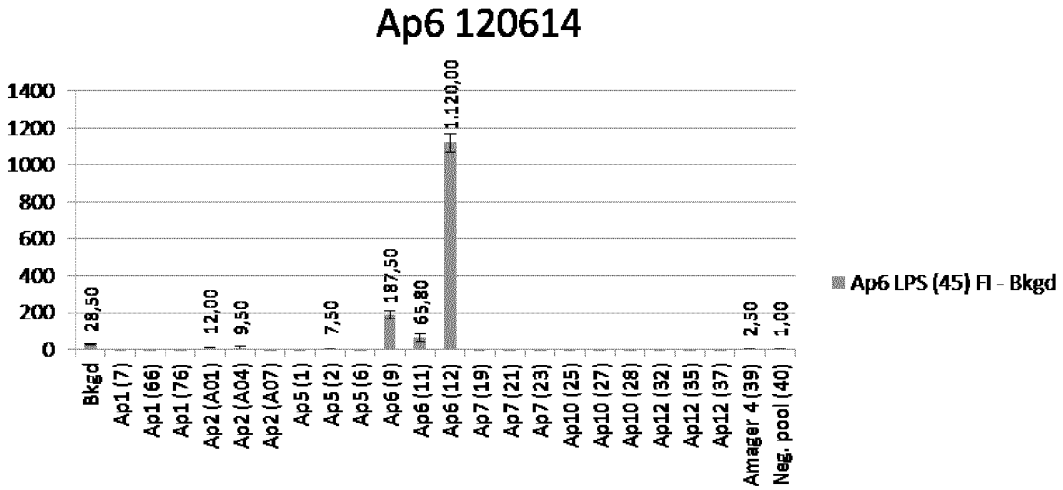

Ap6 120614
1400
1200
1000
800
600
400
200
0
28,50
12,00
9,50
7,50
187,50
65,80
1.120,00
2,50
1,00
Bkgd
Ap1 (7)
Ap1 (66)
Ap1 (76)
Ap2 (A01)
Ap2 (A04)
Ap2 (A07)
Ap5 (1)
Ap5 (2)
Ap5 (6)
Ap6 (9)
Ap6 (11)
Ap6 (12)
Ap7 (19)
Ap7 (21)
Ap7 (23)
Ap10 (25)
Ap10 (27)
Ap10 (28)
Ap12 (32)
Ap12 (35)
Ap12 (37)
Amager 4 (39)
Neg. pool (40)
Ap6 LPS (45) FI - Bkgd

FIG. 2D

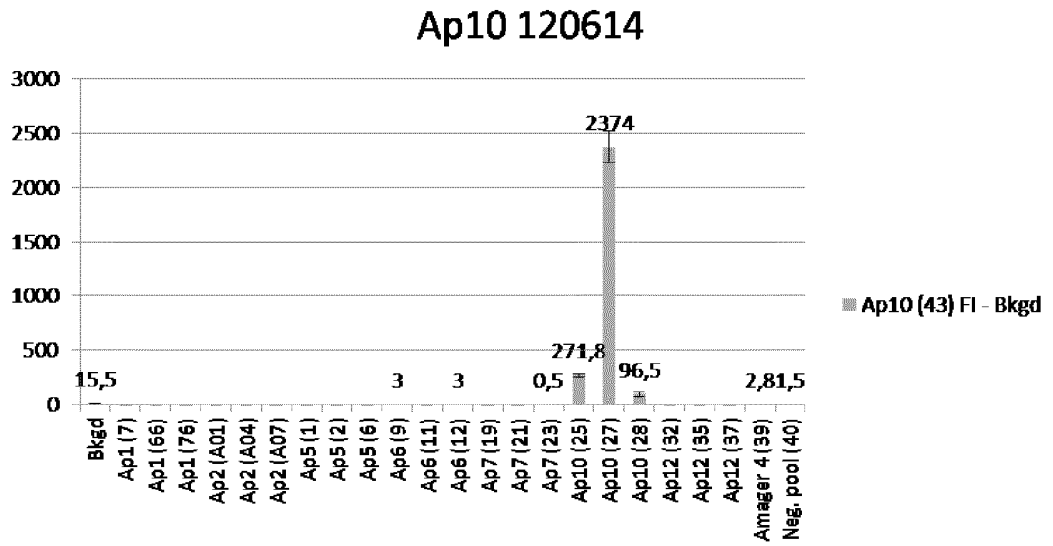

Ap10 120614
3000
2500
2000
1500
1000
500
0
15,5
3
3
0,5
271,8
2374
96,5
2,81,5
Bkgd
Ap1 (7)
Ap1 (66)
Ap1 (76)
Ap2 (A01)
Ap2 (A04)
Ap2 (A07)
Ap5 (1)
Ap5 (2)
Ap5 (6)
Ap6 (9)
Ap6 (11)
Ap6 (12)
Ap7 (19)
Ap7 (21)
Ap7 (23)
Ap10 (25)
Ap10 (27)
Ap10 (28)
Ap12 (32)
Ap12 (35)
Ap12 (37)
Amager 4 (39)
Neg. pool (40)
Ap10 (43) FI - Bkgd

FIG. 2E

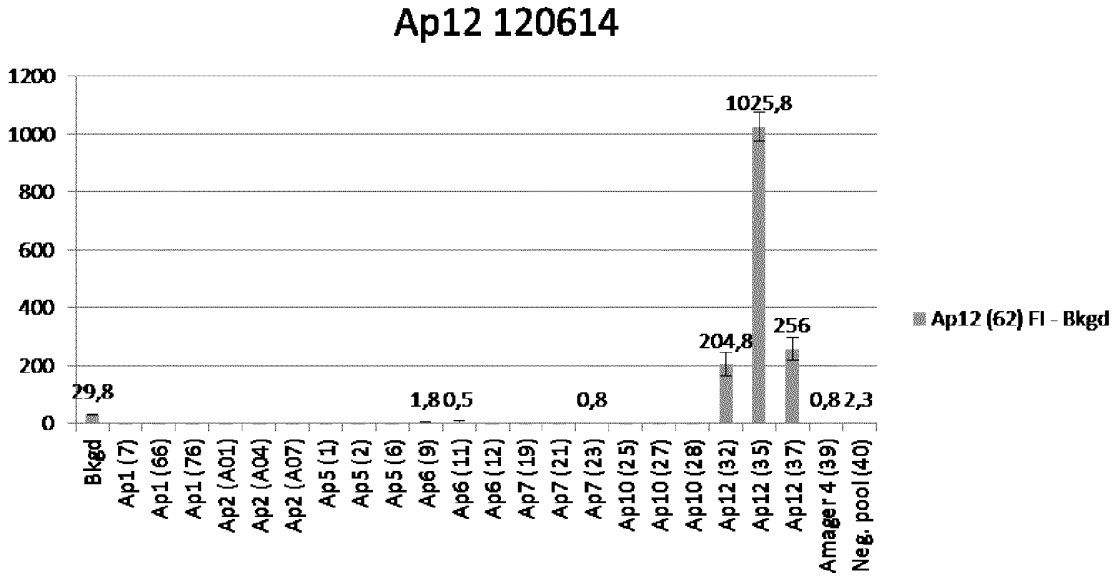

Ap12 120614
1200
1000
800
600
400
200
0
29,8
1,8
0,5
0,8
204,8
1025,8
256
0,8
2,3
Bkgd
Ap1 (7)
Ap1 (66)
Ap1 (76)
Ap2 (A01)
Ap2 (A04)
Ap2 (A07)
Ap5 (1)
Ap5 (2)
Ap5 (6)
Ap6 (9)
Ap6 (11)
Ap6 (12)
Ap7 (19)
Ap7 (21)
Ap7 (23)
Ap10 (25)
Ap10 (27)
Ap10 (28)
Ap12 (32)
Ap12 (35)
Ap12 (37)
Amager 4 (39)
Neg. pool (40)
Ap12 (62) FI - Bkgd

FIG. 2F

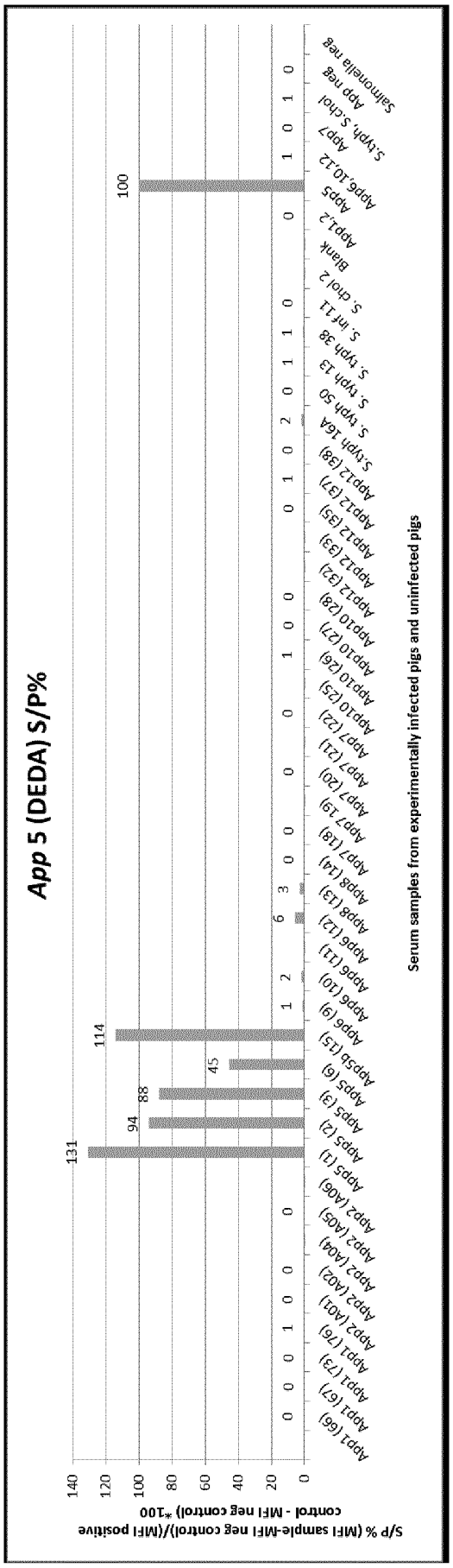

App 5 (DEDA) S/P%
S/P % (MFI sample-MFI neg control)/(MFI positive control - MFI neg control) *100
Serum samples from experimentally infected pigs and uninfected pigs
0
20
40
60
80
100
120
140
App1 (66)
App1 (67)
App1 (73)
App1 (76)
App2 (A01)
App2 (A02)
App2 (A04)
App2 (A05)
App2 (A06)
App5 (1)
App5 (2)
App5 (3)
App5 (6)
App5b (15)
App6 (9)
App6 (10)
App6 (11)
App6 (12)
App6 (13)
App8 (14)
App7 (18)
App7 19)
App7 (20)
App7 (21)
App7 (22)
App10 (25)
App10 (26)
App10 (27)
App10 (28)
App12 (32)
App12 (33)
App12 (35)
App12 (37)
App12 (38)
S.typh 16a
S. typh 50
S. typh 13
S. typh 38
S. inf 11
S. chol 2
blank
App1,2
App5
App6.10.12
App7
S.typh. S.chol
App neg
Salmonella neg
131
94
88
45
114
100

FIG. 3A

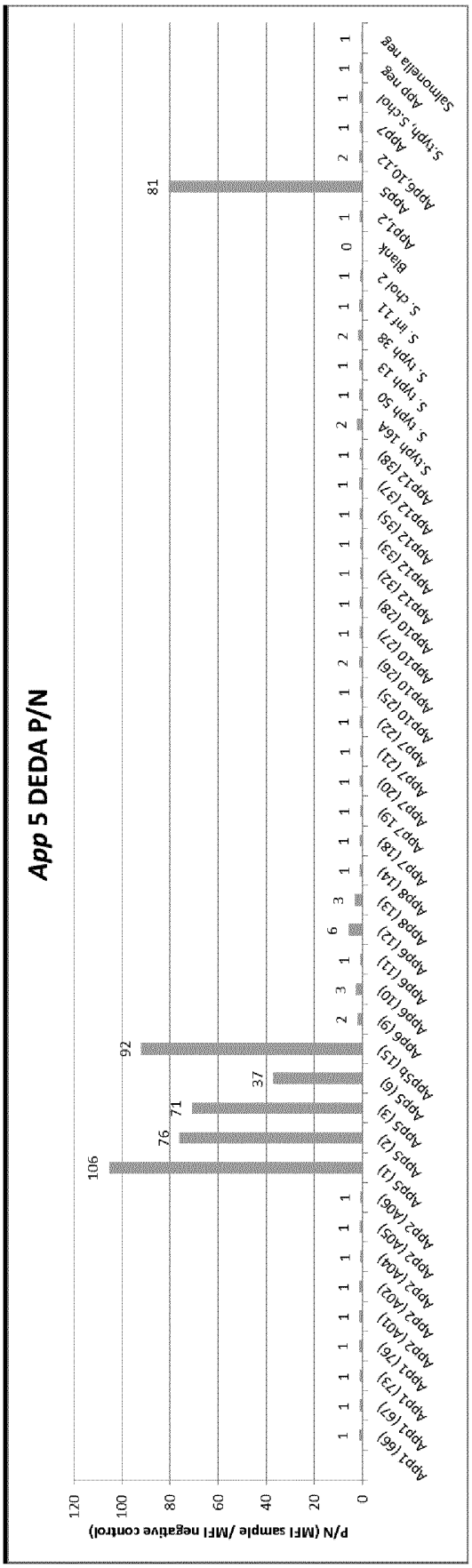

App 5 DEDA P/N
P/N (MFI sample /MFI negative control)
0
20
40
60
80
100
120
106
76
71
37
92
81
App1 (66)
App1 (67)
App1 (73)
App1 (76)
App2 (A01)
App2 (A02)
App2 (A04)
App2 (A05)
App2 (A06)
App5 (1)
App5 (2)
App5 (3)
App5 (6)
App5b (15)
App6 (9)
App6 (10)
App6 (11)
App6 (12)
App8 (13)
App8 (14)
App7 (18)
App7 19)
App7 (20)
App7 (21)
App7 (22)
App10 (25)
App10 (26)
App10 (27)
App10 (28)
App12 (32)
App12 (33)
App12 (35)
App12 (37)
App12 (38)
S.typh.16A
S.typh.50
S. typh 13
S. typh 38
S. inf.11
S. chol 2
blank
App1.2
App5
App6.10.12
App7
S.typh, S.chol
App neg
Salmonella neg

FIG. 3B

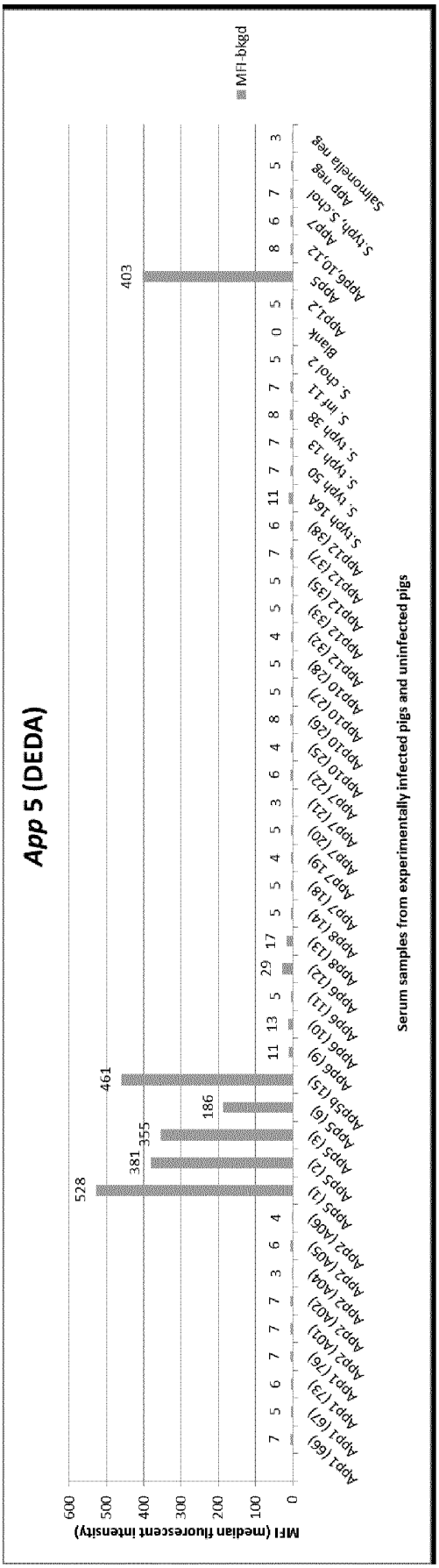

App 5 (DEDA)
MFI-bkgd
MFI (median fluorescent intensity)
Serum samples from experimentally infected pigs and uninfected pigs
App1 (66)
App1 (67)
App1 (73)
App1 (76)
App2 [A01]
App2 [A02]
App2 [A04]
App2 [A05]
App2 [A06]
App5 (1)
App5 (2)
App5 (3)
App5 (6)
App5b (15)
App6 (9)
App6 (10)
App6 (11)
App6 (12)
App8 (13)
App8 (14)
App7 (18)
App7 19
App7 (20)
App7 (21)
App7 (22)
App10 (25)
App10 (26)
App10 (27)
App10 (28)
App12 (32)
App12 (33)
App12 (35)
App12 (37)
App12 (38)
S. typh 16A
S. typh 50
S. typh 13
S. typh 38
S. Inf 11
S. chol 2
Blank
App1.2
App5
App6,10,12
App7
S.typh. S.chol
App neg
Salmonella neg
600
500
400
300
200
100
0
528
381
355
461
186
403
11
13
5
29
17
5
7
6
7
7
7
3
6
4
5
4
5
5
3
6
4
8
5
5
4
5
5
6
7
5
7
11
7
6
8
7
5
0
5
8
6
7
5
3

FIG. 3C

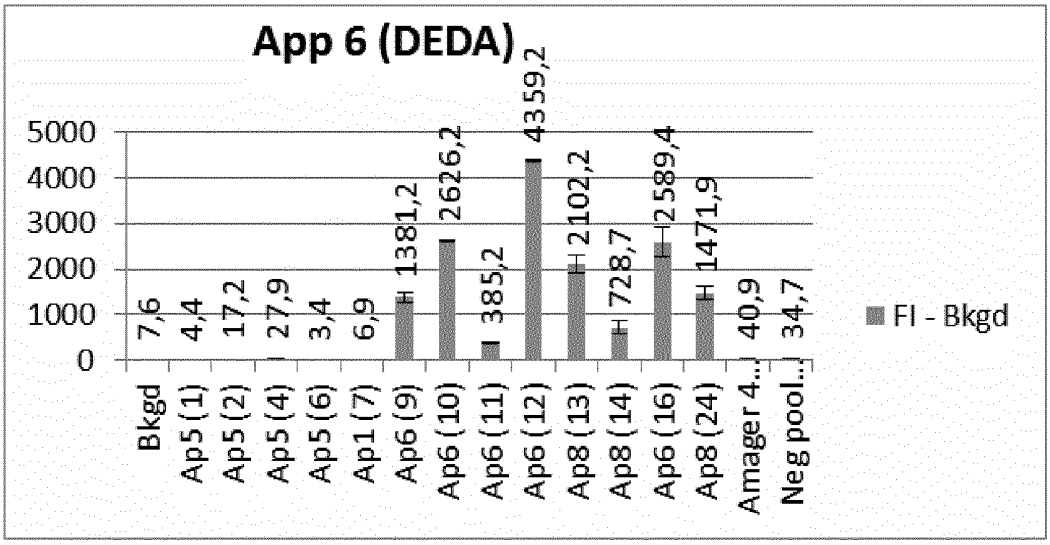

App 6 (DEDA)
5000
4000
3000
2000
1000
0
7,6
4,4
17,2
27,9
3,4
6,9
1381,2
2626,2
385,2
4359,2
2102,2
728,7
2589,4
1471,9
40,9
34,7
Bkgd
Ap5 (1)
Ap5 (2)
Ap5 (4)
Ap5 (6)
Ap1 (7)
Ap6 (9)
Ap6 (10)
Ap6 (11)
Ap6 (12)
Ap8 (13)
Ap8 (14)
Ap6 (16)
Ap8 (24)
Amager 4...
Neg pool...
FI - Bkgd

FIG. 4

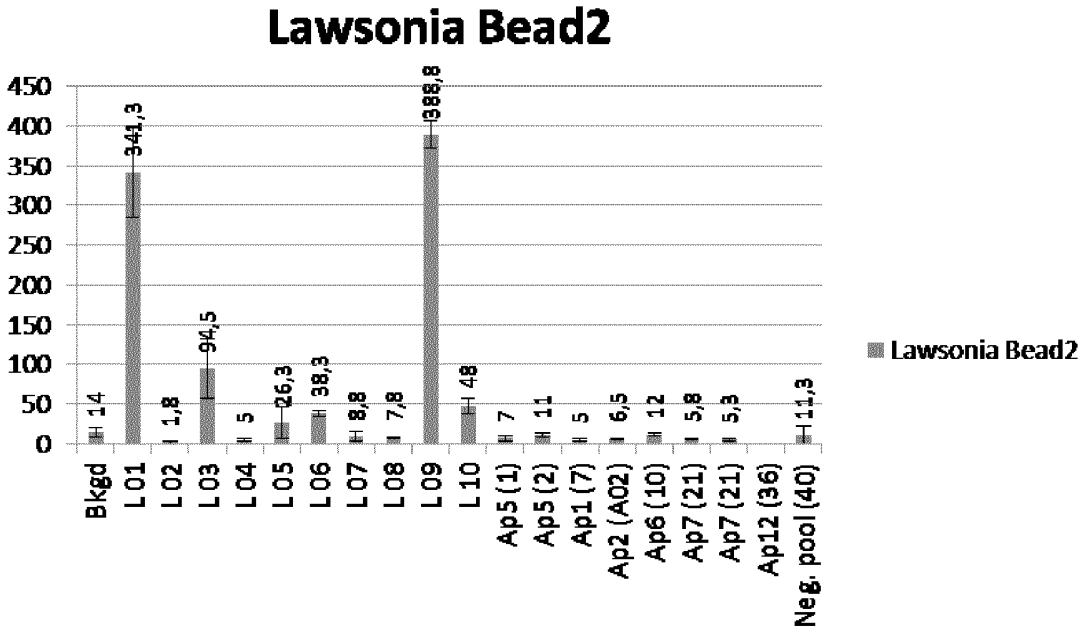

Lawsonia Bead2
450
400
350
300
250
200
150
100
50
0
14
341,3
1,8
94,5
5
26,3
38,3
8,8
7,8
388,8
48
7
11
5
6,5
12
5,8
5,3
11,3
Bkgd
L01
L02
L03
L04
L05
L06
L07
L08
L09
L10
Ap5 (1)
Ap5 (2)
Ap1 (7)
Ap2 (A02)
Ap6 (10)
Ap7 (21)
Ap7 (21)
Ap12 (36)
Neg. pool (40)
Lawsonia Bead2

FIG. 5A

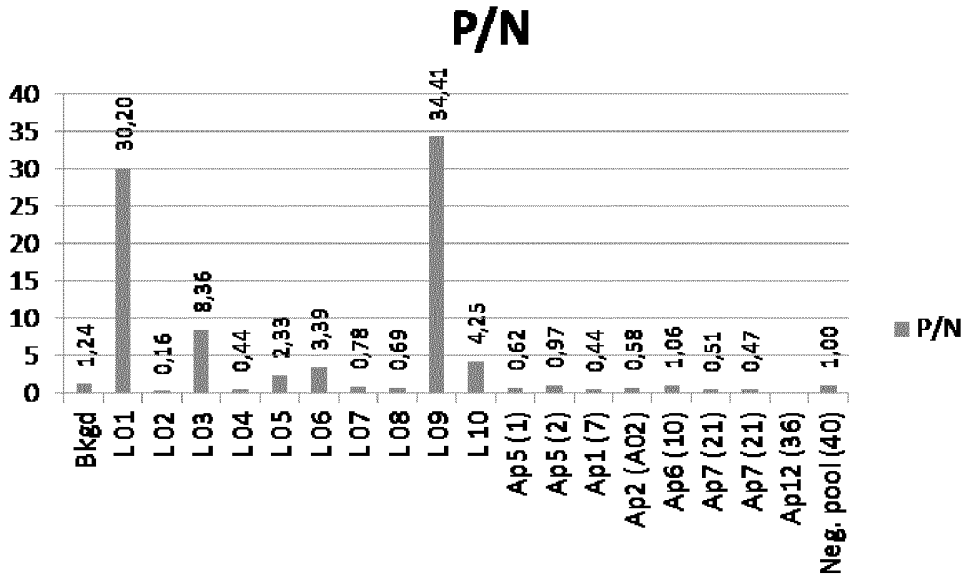

P/N
40
35
30
25
20
15
10
5
0
1,24
30,20
0,16
8,36
0,44
2,33
3,39
0,78
0,69
34,41
4,25
0,62
0,97
0,44
0,58
1,06
0,51
0,47
1,00
Bkgd
L01
L02
L03
L04
L05
L06
L07
L08
L09
L10
Ap5 (1)
Ap5 (2)
Ap1 (7)
Ap2 (A02)
Ap6 (10)
Ap7 (21)
Ap7 (21)
Ap12 (36)
Neg. pool (40)
P/N

FIG. 5B

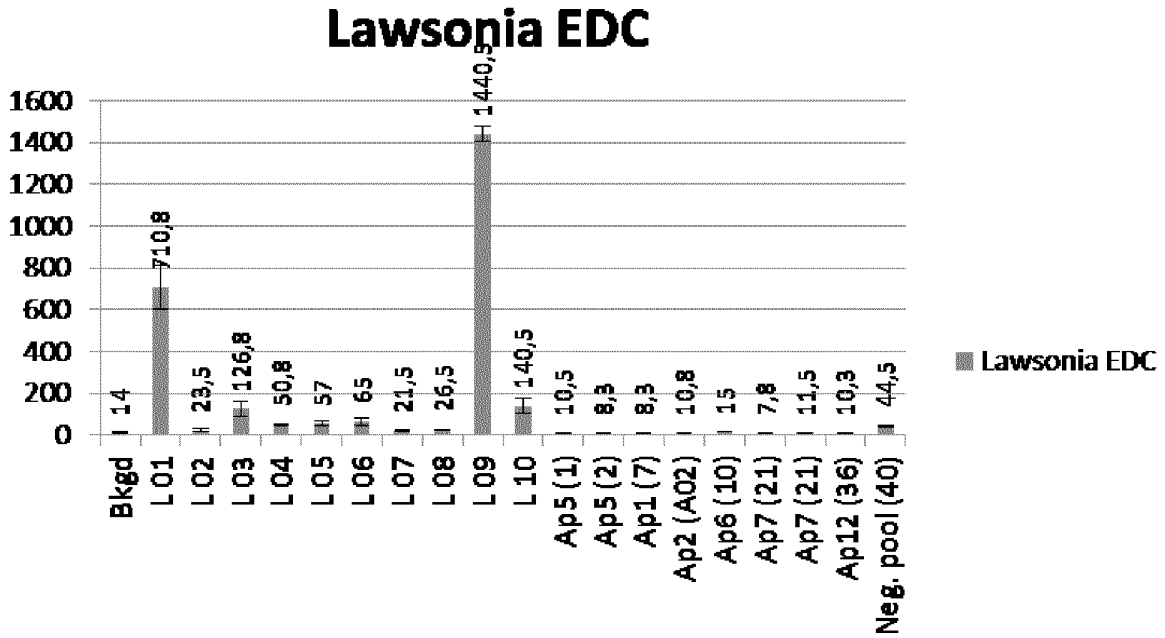

Lawsonia EDC
1600
1400
1200
1000
800
600
400
200
0
14
710,8
23,5
126,8
50,8
57
65
21,5
26,5
1440,5
140,5
10,5
8,3
8,3
10,8
15
7,8
11,5
10,3
44,5
Bkgd
L01
L02
L03
L04
L05
L06
L07
L08
L09
L10
Ap5 (1)
Ap5 (2)
Ap1 (7)
Ap2 (A02)
Ap6 (10)
Ap7 (21)
Ap7 (21)
Ap12 (36)
Neg. pool (40)
Lawsonia EDC

FIG. 5C

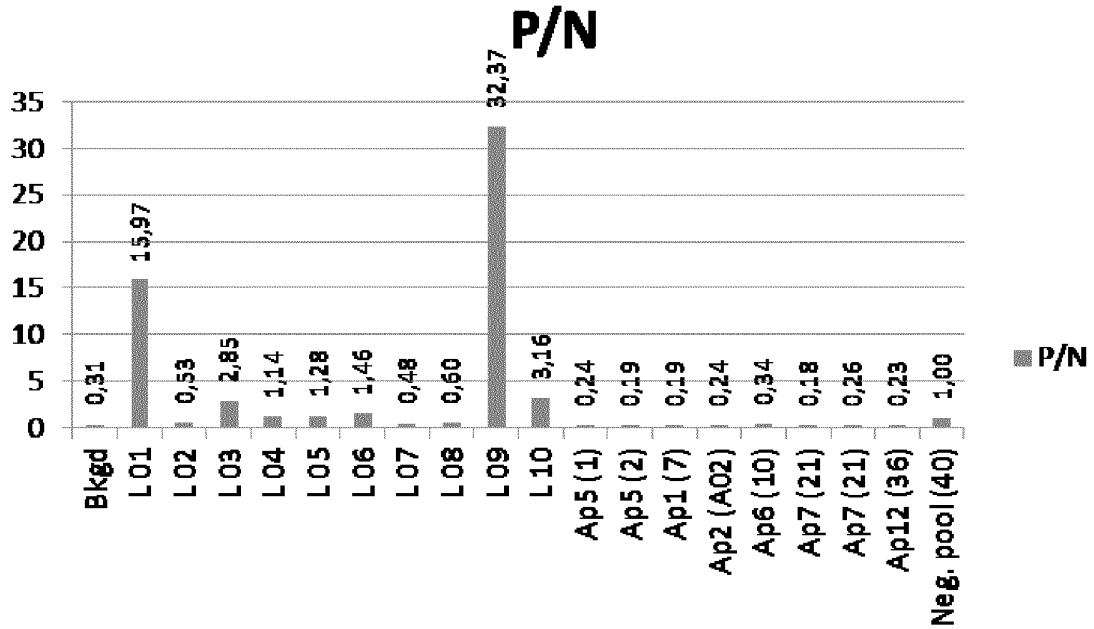

P/N
35
30
25
20
15
10
5
0
0,31
15,97
0,53
2,85
1,14
1,28
1,46
0,48
0,60
32,37
3,16
0,24
0,19
0,19
0,24
0,34
0,18
0,26
0,23
1,00
Bkgd
L01
L02
L03
L04
L05
L06
L07
L08
L09
L10
Ap5 (1)
Ap5 (2)
Ap1 (7)
Ap2 (A02)
Ap6 (10)
Ap7 (21)
Ap7 (21)
Ap12 (36)
Neg. pool (40)
P/N

FIG. 5D

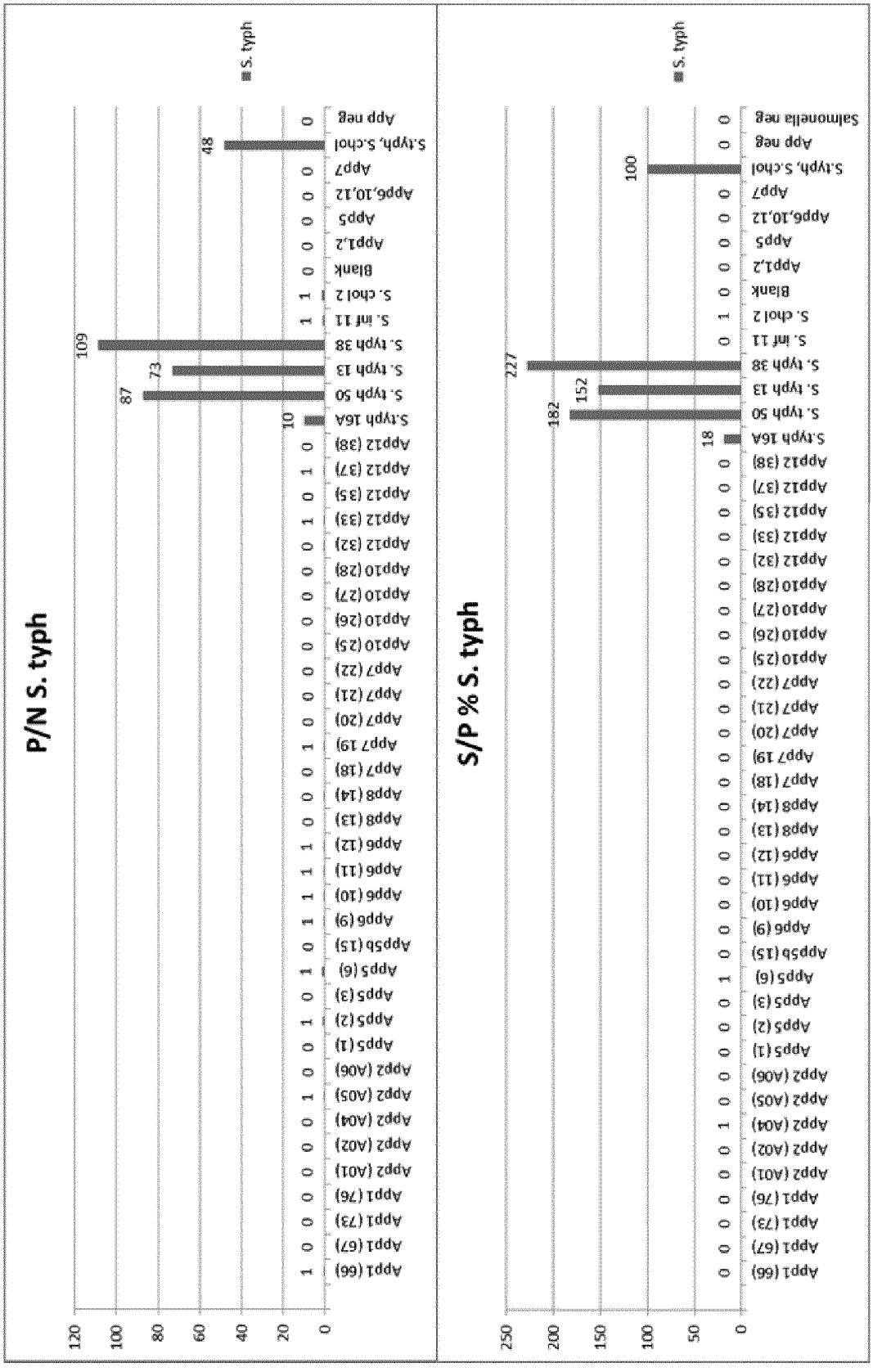

P/N S. typh
S. typh
App1 (66)
App1 (67)
App1 (73)
App1 (76)
App2 (A01)
App2 (A02)
App2 (A04)
App2 (A05)
App2 (A06)
App5 (1)
App5 (2)
App5 (3)
App5 (6)
App5b (15)
App6 (9)
App6 (10)
App6 (11)
App6 (12)
App8 (13)
App8 (14)
App7 (18)
App7 19)
App7 (20)
App7 (21)
App7 (22)
App10 (25)
App10 (26)
App10 (27)
App10 (28)
App12 (32)
App12 (33)
App12 (35)
App12 (37)
App12 (38)
S.typh 16A
S. typh 50
S. typh 13
S. typh 38
S. inf 11
S. chol 2
Blank
App1,2
App5
App6,10,12
App7
S.typh, S.chol
App neg
S/P % S. typh
Salmonella neg

FIG. 6

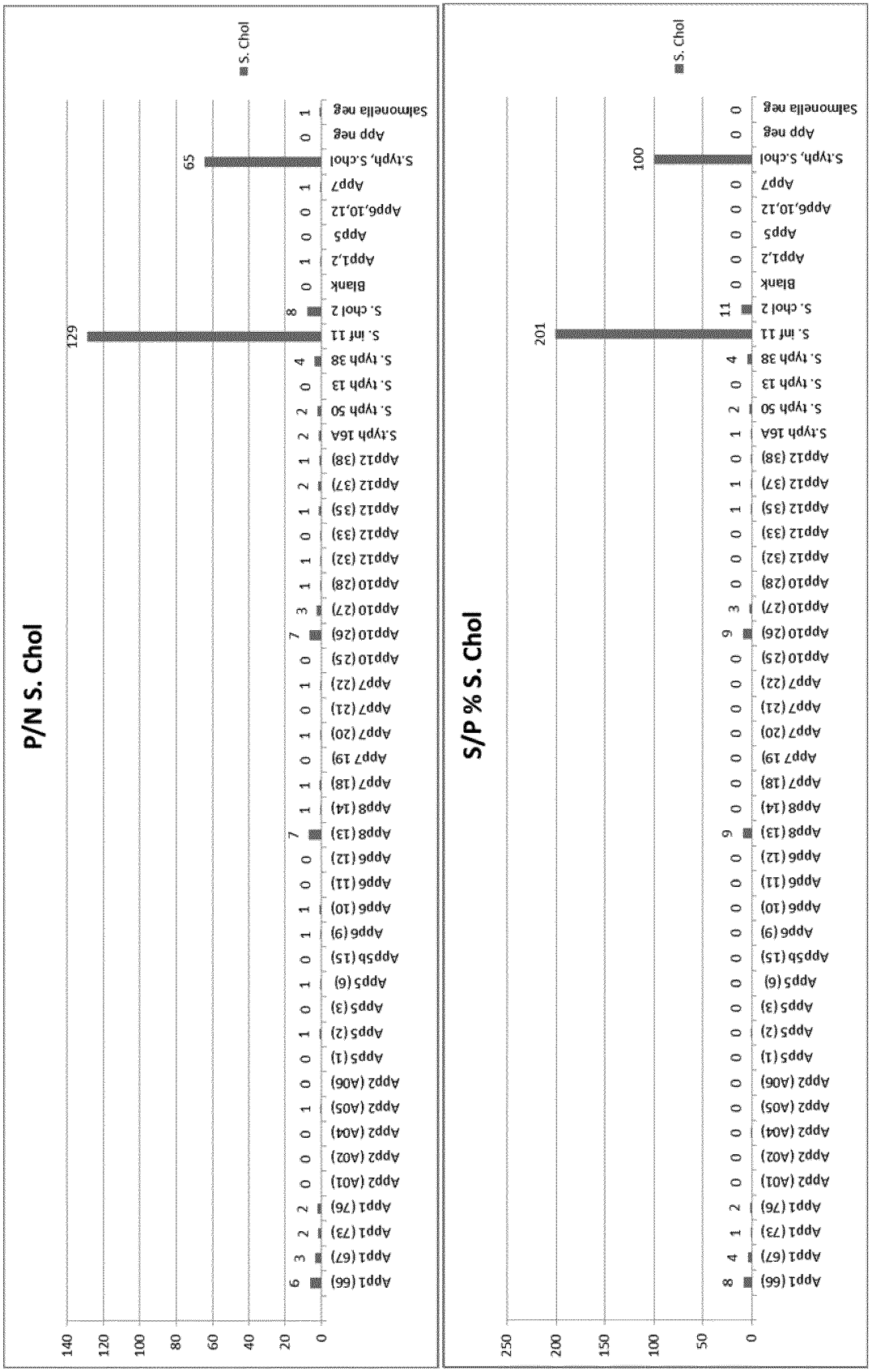

P/N S. Chol
S. Chol
S/P % S. Chol
S. Chol
Salmonella neg
App neg
S.typh, S.chol
App7
App6,10,12
App5
Blank
App1,2
S. chol 2
S. inf 11
S. typh 38
S. typh 13
S. typh 50
S.typh 16A
App12 (38)
App12 (37)
App12 (35)
App12 (33)
App12 (32)
App10 (28)
App10 (27)
App10 (26)
App10 (25)
App7 (22)
App7 (21)
App7 (20)
App7 19)
App7 (18)
App8 (14)
App8 (13)
App6 (12)
App6 (11)
App6 (10)
App6 (9)
App5b (15)
App5 (6)
App5 (3)
App5 (2)
App5 (1)
App2 (A06)
App2 (A05)
App2 (A04)
App2 (A02)
App2 (A01)
App1 (76)
App1 (73)
App1 (67)
App1 (66)

FIG. 7

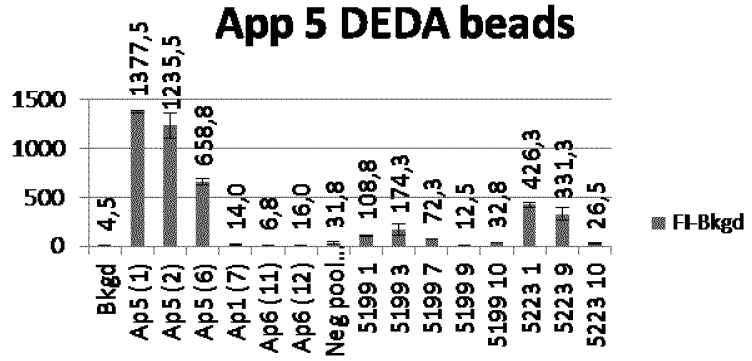


Fig. 8A

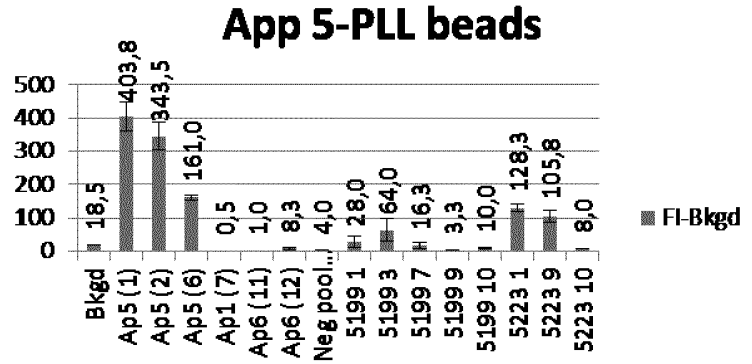


Fig. 8B

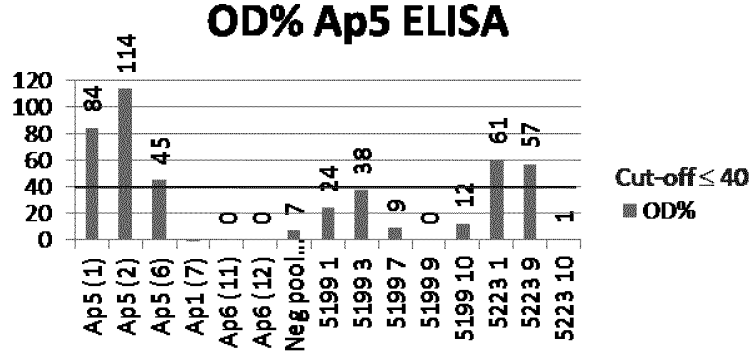


Fig. 8C

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/071240

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K17/06 C07K17/14 G01N33/543
ADD.

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)
C07K G01N

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

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

EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 2013/060335 A1 (UNIV DANMARKS TEKNISKE [DK]) 2 May 2013 (2013-05-02) cited in the application pages 1,11,12; claims 1-25; figure 3; examples 1-3 -----	1-14, 20-43 15-19
X Y	BOAS ULRIK ET AL: "Method to conjugate polysaccharide antigens to surfaces for the detection of antibodies", ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC, NEW YORK, vol. 465, 27 July 2014 (2014-07-27), pages 73-80, XP029085093, ISSN: 0003-2697, DOI: 10.1016/J.AB.2014.07.005 the whole document -----	1-14, 20-28, 30-43 15-19,29
	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"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)

"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

"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

"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

"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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 October 2016	13/12/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Schmidt-Yodlee, H

4

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JAUHO E S ET AL: "New technology for regiospecific covalent coupling of polysaccharide antigens in ELISA for serological detection", JOURNAL OF IMMUNOLOGICAL METHODS, ELSEVIER SCIENCE PUBLISHERS B.V.,AMSTERDAM, NL, vol. 242, no. 1-2, 28 August 2000 (2000-08-28), pages 133-143, XP004210717, ISSN: 0022-1759, DOI: 10.1016/S0022-1759(00)00248-9 cited in the application abstract; figure 1	15-19
Y	WO 2013/028996 A1 (BAYLOR RES INST [US]; ZURAWSKI GERARD [US]; ZURAWSKI SANDRA [US]) 28 February 2013 (2013-02-28) claims 1,9; figures 1A, 1B	29
Y	US 4 140 662 A (RECKEL RUDOLPH P ET AL) 20 February 1979 (1979-02-20) column 2 - column 3; claims 1-18	15-19
A	CN 101 493 469 A (CHINESE ACAD INSP & QUARANTINE [CN]) 29 July 2009 (2009-07-29) claims 1-15	1-43
A	I LEROUGE ET AL: "O-antigen structural variation: mechanisms and possible roles in animal/plant-microbe interactions", FEMS MICROBIOLOGY REVIEWS, vol. 25, no. 5, 1 December 2001 (2001-12-01), pages 0-47, XP055307918, AMSTERDAM; NL ISSN: 0168-6445, DOI: 10.1016/S0168-6445(01)00070-5 abstract section 4.1.2 "Chemical basis of LPS changes"	1-43
A	WO 2010/036133 A1 (INST IMMUNOLOGII I TERAPII DOS [PL]; LIPINSKI TOMASZ [PL]; RYBKA JACEK) 1 April 2010 (2010-04-01) cited in the application the whole document	1-43

-/--

4

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LAURA A. MCALLISTER ET AL: "Synthesis and Application of a Novel Ligand for Affinity Chromatography Based Removal of Endotoxin from Antibodies", BIOCONJUGATE CHEMISTRY., vol. 18, no. 2, 1 March 2007 (2007-03-01), pages 559-566, XP055307944, US ISSN: 1043-1802, DOI: 10.1021/bc0602984 the whole document -----	1-43
A	GOMES A G ET AL: "Clearance of host cell impurities from plasmid-containing lysates by boronate adsorption", JOURNAL OF CHROMATOGRAPHY, ELSEVIER SCIENCE PUBLISHERS B.V, NL, vol. 1217, no. 15, 9 April 2010 (2010-04-09), pages 2262-2266, XP026971433, ISSN: 0021-9673 [retrieved on 2010-02-16] the whole document -----	1-43

4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2016/071240

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search 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 No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

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

see additional 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 fees, this Authority did not invite payment of additional fees.

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.:

1-43

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-43

   method of detecting antibodies according to claim 1
   ---

2. claims: 44-46

   antibody detection kit
   ---

3. claims: 47-50

   bead-antigen conjugate
   ---

4. claims: 51-54

   composition comprising at least two individual groups of bead-antigen conjugates
   ---

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/071240

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2013060335 A1	02-05-2013	NONE	
WO 2013028996 A1	28-02-2013	AR 088428 A1	11-06-2014
		TW 201314208 A	01-04-2013
		US 2013053271 A1	28-02-2013
		WO 2013028996 A1	28-02-2013
US 4140662 A	20-02-1979	AR 217685 A1	15-04-1980
		AT 361636 B	25-03-1981
		AU 3431278 A	27-09-1979
		BE 865195 A1	22-09-1978
		BR 7801779 A	19-12-1978
		CA 1089764 A	18-11-1980
		DE 2812845 A1	28-09-1978
		DK 128078 A	26-09-1978
		ES 467190 A1	01-08-1979
		FR 2384789 A1	20-10-1978
		GB 1600434 A	14-10-1981
		IT 1111074 B	13-01-1986
		JP S53118517 A	17-10-1978
		NL 7803209 A	27-09-1978
		NO 781039 A	26-09-1978
		NZ 186763 A	26-08-1980
		SE 7803241 A	26-09-1978
		US 4140662 A	20-02-1979
		ZA 7801719 B	28-11-1979
CN 101493469 A	29-07-2009	NONE	
WO 2010036133 A1	01-04-2010	EP 2328927 A1	08-06-2011
		ES 2560779 T3	22-02-2016
		PL 217758 B1	29-08-2014
		US 2011230648 A1	22-09-2011
		WO 2010036133 A1	01-04-2010