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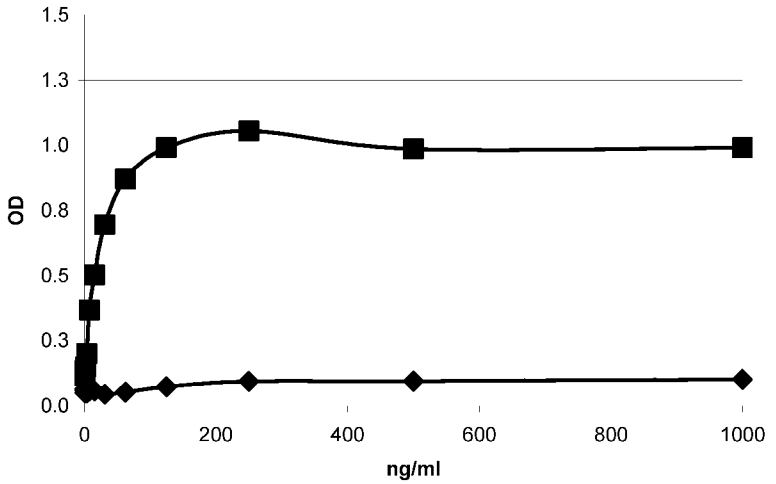

Fig. 2

(57) **Abstract**: Herein is reported a method for determining an antibody suitable for immunohistochemical staining of tissue samples comprising the following steps a) cultivating immunogen presenting cells in an adhesion enhancing compound coated cultivation vessel, b) incubating the cultivated cells with a cross-linking solution, c) incubating the formalin treated cells with a conditioning buffer, d) applying energy to the conditioned cells, eg in the form of microwaves, e) incubating the energy treated cells with a first antibody, f) determining the binding of the first antibody to the energy treated cells with a second antibody binding to the first antibody and thereby determining an antibody suitable for immunohistochemical staining of tissue samples.

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MICROWAVES FOR IMMUNOHISTOCHEMICAL ANALYSIS

Herein is reported a method for the generation of antibodies, such as immunohistochemistry suited antibodies, wherein the method can be used for the generation of therapeutic as well as diagnostic antibodies, thus, eliminating the need for different immunization methods for the generation of e.g. immunohistochemistry suited and therapeutic antibodies.

Background of the Invention

To preserve clinical samples for extended time periods as well as to prepare these samples for immunohistochemical and histopathological staining methods tissue-specific structures have to be maintained. For tissue processing generally cross-linking organic chemicals are employed. Cross-linking chemicals normally distort surface epitopes of cell surface presented molecules. The distorted epitopes differ substantially from the non-processed epitopes. Thus, antibodies binding to the not-processed epitope normally do not bind to the processed epitope.

Therapeutic antibodies bind to naturally occurring, not-processed epitopes and generally cannot be used for tissue based immunohistochemical methods.

For the production of immunohistochemically (IHC) suited and therapeutic antibodies different approaches are currently used. This results in double workflows to be performed. For the generation of IHC suited antibodies experimental animals are immunized by the application of peptides or short chain polypeptides. For the generation of therapeutic antibodies experimental animals are immunized with DNA, polypeptides or entire cells presenting the antigen. In contrast to the generation of therapeutic antibodies with not-processed, native immunogen the epitope variability is strongly reduced for the generation of IHC suited antibodies.

Antigen retrieval for immunohistochemistry is reported in D'Amico, F., et al., J. Immunol. Meth. 341 (2009) 1-18. In WO 2006/138694 a rabbit monoclonal antibody against ID1 protein is reported. A method for producing comprehensive anti-surface antibody wherein the antigen used is a microorganism fixed by a protein crosslinking and fixation reagent is reported in WO 2010/071237.

Summary of the Invention

It has been found that with the method of antigen fixation and retrieval as reported herein it is possible to screen antibodies that are suitable of immunohistochemical staining. By the combination of these two processes it can be accounted for the fact that the presented antigens is in a way comparable to the preparation of clinical samples.

It has been found that the above can be effected by the application of thermal energy to the sample in order to improve the antigen retrieval step.

Herein is reported as one aspect the use of thermal energy for the treatment of a chemically cross-linked cell prior to staining in an immunohistochemical analysis.

In one embodiment the thermal energy is in form of microwaves.

Herein is reported a method for the generation of antibodies, e.g. of IHC suited antibodies, with a method that can also be used for the generation of therapeutic antibodies, thus, eliminating the need for different methods for the generation of IHC suited and therapeutic antibodies.

By employing a method as reported herein it is possible to select e.g. IHC suited antibodies directly from the crude cultivation supernatant of hybridoma cells, or from the crude cultivation supernatant of B-cells obtained from an immunized experimental animal, or from antibodies obtained by cell-display, phage-display, or bacterial-display technologies. The immunization is in one embodiment by cell-, polypeptide- or DNA-immunization. Thus, it is reported herein a method for the generation of IHC suited antibodies by using native, not-processed immunogens.

Currently used test methods for identifying IHC suited antibodies for the staining of tissue samples is not straight forward and requires labor intensive antibody characterization. With the method as reported herein it is possible to characterize antibodies for their IHC suitability in a high throughput manner. This increases the possibility of identifying an IHC suited antibody.

An aspect as reported herein is a method for determining an antibody suitable for immunohistochemical staining of tissue samples comprising the following steps:

a) incubating a recombinant cell expressing an immunogen with a solution comprising a cross-linking agent,

b) applying energy to the cell, and

c) determining the binding of an antibody to the cell and thereby determining an antibody suitable for immunohistochemical staining.

In one embodiment of all aspects as reported herein the energy is thermal energy. In one embodiment the thermal energy is microwave.

In one embodiment the microwave has a wavelength of about 12 cm.

In one embodiment energy of 200 W to 1200 W is applied. In one embodiment the energy is about 800 W.

In one embodiment the energy is applied non-modulated and continuously during the application time.

In one embodiment the energy is applied for about 6 minutes. In one embodiment the energy is applied in intervals of three times two minutes.

In one embodiment of all aspects as reported herein the cross-linking agent is a chemical cross-linking agent. In one embodiment the chemical cross-linking agent is selected from the group comprising formaldehyde, paraformaldehyde, formalin, glutaraldehyde, dimethyl adipimidate, dimethyl suberimidate, osmium tetroxide, potassium dichromate, chromic acid, potassium permanganate, and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid-glutamic acid buffer-organic solvent mixture.

In one embodiment the cross-linking agent is formaldehyde in phosphate buffered saline. In one embodiment the solution is a 10 % (w/v) formalin solution.

In one embodiment the method comprises the following steps:

a) cultivating immunogen presenting recombinant cells in a cultivation vessel or on a glass coverslip, whose surface is coated with a surface adhesion enhancer or cell trapping agent,

b) incubating the cultivated recombinant cells with a solution comprising a cross-linking agent,

c) incubating the cross-linked cells with a conditioning buffer,

d) applying energy to the conditioned cross-linked cells,

e) incubating the energy treated, conditioned, cross linked cells with a first antibody,

f) determining the binding of the first antibody to the cells with a second antibody binding to the first antibody and thereby determining an antibody suitable for immunohistochemical staining of tissue samples.

The following embodiments are specific embodiments of all aspects as reported herein.

In one embodiment the cross-linking agent is a chemical cross-linking agent.

In one embodiment the energy is thermal energy.

In one embodiment the thermal energy is microwave.

In one embodiment the surface adhesion enhancer is selected from gelatin, collagen (such as collagen type I), poly-D-lysine, poly-L-lysine, fibronectin, laminin, or polyvinyl formal.

In one embodiment about $1*10^3$ to $15*10^3$ cells are cultivated. In one embodiment about $4*10^3$ cells are cultivated.

In one embodiment the cells are cultivated for 1 to 4 days. In one embodiment the cells are cultivated for 3 days.

In one embodiment the incubating with the solution is for 30 minutes to 90 minutes at room temperature. In one embodiment the incubating with the solution is for about 45 minutes.

In one embodiment the incubating with the first antibody is for 10 minutes to 120 minutes at room temperature. In one embodiment the incubating with the first antibody is for about 90 minutes.

In one embodiment the incubating with the first antibody is in the presence of about 0.5 % BSA (v/v).

In one embodiment the second antibody is an anti-rabbit IgG antibody conjugated to a detectable label, or an anti-mouse IgG antibody conjugated to a detectable

label, or an anti-rat IgG antibody conjugated to a detectable label, or an anti-goat IgG antibody conjugated to a detectable label, or an anti-sheep IgG antibody conjugated to a detectable label, or an anti-hamster IgG antibody conjugated to a detectable label, or an anti-human IgG antibody conjugated to a detectable label.

In one embodiment the first antibody is diluted in the range of from 1:100 to 1:100,000.

In one embodiment the anti-goat IgG antibody is diluted about 1:9000.

In one embodiment the anti-mouse IgG is diluted about 1:4000.

In one embodiment the detectable label is selected from dyes, luminescent labeling groups, luminescent metal complexes, and radioisotopes.

In one embodiment the conjugation is a chemical conjugation via N-terminal and/or ε-amino groups (lysine), ε-amino groups of different lysins, carboxy-, sulfhydryl-, hydroxyl-, and/or phenolic functional groups of the amino acid backbone of the antibody, and/or sugar alcohol groups of the carbohydrate structure of the antibody or via a specific binding pair.

In one embodiment the first antibody is conjugated to digoxigenin and linking to the detectable label is performed via a second antibody against digoxigenin.

In one embodiment the cultivation vessel is a multi-well plate.

A further aspect as reported herein is a kit comprising

- a glass cover slip, a 96 well multi-well plate, or a 384 multi-well plate with poly-D-lysine coated surface,
- a 10 % (w/v) formalin solution,
- an anti-rabbit IgG antibody conjugated to POD, and/or an anti-mouse IgG antibody conjugated to POD, and/or an anti-rat IgG antibody conjugated to POD.

One aspect as reported herein is a method for producing an antibody specifically binding to an antigen comprising the following steps:

a) immunizing an experimental animal with a recombinant cell expressing the antigen on its surface, whereby the cell has been incubated with a cross-linking agent and whereby energy has been applied to the cell prior to the immunization,

b) cultivating a cell comprising a nucleic acid, that encodes the antibody specifically binding to the antigen, whereby the nucleic acid has been obtained from an immune cell recovered from the immunized experimental animal, and

c) recovering the antibody from the cell or the cultivation medium and thereby producing an antibody specifically binding to an antigen.

Detailed Description of the Invention

Until now no high-throughput suited method for the identification of IHC suited antibodies has been reported. In the state of the art the use of Western blot is discussed, which is work intensive and not suited for high-throughput characterization.

Herein is reported a method in which the used immunogen is prior to the selection of the antibody pre-treated by incubation with formalin and a heat step. The immunogen can be a cell presenting the immunogen or a native polypeptide.

In more detail the method comprises in one embodiment the following: The wells of a multi-well plate are coated with poly-D-lysine or any other surface adhesion enhancer or cell trapping agent. In each well about $4*10^3$ cells are cultivated for about three days. Thereafter the cultivation medium is removed, the cells are washed and incubated with formalin for 45 minutes at room temperature. After the incubation the cells are washed with a citrate buffered solution (pH 6.0). Finally energy in form of microwaves is applied to the cells for about 6 minutes.

One embodiment as reported herein is a method for determining an antibody suitable for immunohistochemical staining of tissue samples comprising the following steps:

a) incubating a cell expressing an immunogen with a solution comprising a chemical cross-linking agent,

b) applying thermal energy to the cell, and

c) determining the binding of an antibody to the cell and thereby determining an antibody suitable for immunohistochemical staining.

In one embodiment the method comprises the following steps:

a) cultivating immunogen presenting cells in a cultivation vessel or on a glass coverslip, whose surface is coated with a surface adhesion enhancer or cell trapping agent,

b) incubating the cultivated cells with a solution comprising a chemical cross-linking agent,

c) incubating the cross-linked cells with a conditioning buffer,

d) applying thermal energy to the conditioned cross-linked cells,

e) incubating the energy treated, conditioned, cross linked cells with a first antibody,

f) determining the binding of the first antibody to the cells with a second antibody binding to the first antibody and thereby determining an antibody suitable for immunohistochemical staining of tissue samples.

In one embodiment the binding of the first antibody to the cells is detected at 37 °C. In another embodiment the cells are washed after the binding of the first antibody to remove non-specifically bound antibody. In another embodiment the first antibody is a cultivation supernatant of an antibody producing cell or obtained from a display library. In also an embodiment the antibody producing cell is a B-cell or a hybridoma cell.

Also an aspect as reported herein is a method for producing an antibody comprising the following steps:

a) selecting an antibody producing cell by
   - cultivating antigen or immunogen presenting cells in a cultivation vessel, which is coated with any cell trapping agent such as poly-D-lysine,
   - incubating the cultivated cells with a solution comprising a chemical cross-linking agent,
   - incubating the cells with a conditioning buffer,

- applying thermal energy to the conditioned cells,
- incubating the energy treated cells with a solution comprising a first antibody,
- determining the binding of the first antibody to the cells with a second antibody binding to the first antibody and thereby selecting an antibody producing cell,

b) optionally determining the nucleic acid encoding the antibody produced by the selected cell,

c) cultivating a mammalian cell comprising a nucleic acid encoding the antibody produced by the selected cell,

d) recovering the antibody from the cell or the cultivation medium and thereby producing the antibody.

Another aspect as reported herein is a method for producing an antibody specifically binding to an antigen comprising the following steps:

a) immunizing an experimental animal with a cell expressing the antigen on its surface, whereby the cell has been incubated with a chemical cross-linking agent and whereby thermal energy has been applied to the cross-linked cell,

b) cultivating a mammalian cell comprising a nucleic acid, whereby the nucleic acid encodes the antibody specifically binding to the antigen, and whereby the nucleic acid has been obtained from an immune cell recovered from the immunized experimental animal, and

c) recovering the antibody from the cell or the cultivation medium and thereby producing an antibody specifically binding to an antigen.

All the following embodiments are embodiments of all aspects as reported herein. In one embodiment the first antibody is obtained from the cultivation supernatant of a B-cell, or a hybridoma cell, or by any other antibody producing procedure. In another embodiment the mammalian cell is a CHO cell or a HEK cell. In one embodiment the mammalian cell is selected from the group of mammalian cells comprising CHO cells (e.g. CHO K1, CHO DG44), BHK cells, NS0 cells, SP2/0 cells, HEK 293 cells, HEK 293 EBNA cells, PER.C6® cells, and COS cells. In one embodiment the applying of thermal energy is an applying of microwaves. In a

further embodiment energy of 800 W is applied for 6 minutes. In also an embodiment about $4*10^3$ cells are cultivated for 3 days. In a further embodiment the solution is a 10 % (w/v) formalin solution. In an embodiment the incubating with the solution is for about 45 minutes at room temperature. In still another embodiment the incubating with the first antibody is for about 90 minutes at room temperature in the presence of about 0.5 % BSA (v/v). In also an embodiment the second antibody is an anti-rabbit IgG antibody conjugated to a detectable label, or an anti-mouse IgG antibody conjugated to a detectable label, or an anti-rat IgG antibody conjugated to a detectable label, or an anti-goat IgG antibody conjugated to a detectable label, or an anti-sheep IgG conjugated to a detectable label, or an anti-hamster IgG antibody conjugated to a detectable label, or an anti-human IgG antibody conjugated to a detectable label. In a further embodiment the anti-rabbit IgG antibody is diluted about 1:9000. In a further embodiment the anti-mouse IgG is diluted about 1:4000. In one embodiment the detectable label is selected from dyes, luminescent labeling groups, luminescent metal complexes, and radioisotopes. In still another embodiment the conjugation is a chemical conjugation via N-terminal and/or ε-amino groups (lysine), ε-amino groups of different lysins, carboxy-, sulfhydryl-, hydroxyl-, and/or phenolic functional groups of the amino acid backbone of the antibody, and/or sugar alcohol groups of the carbohydrate structure of the antibody or via a specific binding pair. In also an embodiment the first antibody is conjugated to digoxigenin and linking to the detectable label is performed via a second antibody against digoxigenin. In a further embodiment the cultivation vessel is a multi-well plate or a glass cover slip. In also an embodiment the multi-well plate is a 96-well plate or a 384-well plate.

The term “microwave” denotes an electromagnetic wave that has a wavelength of from one meter to one millimeter. Alternatively a microwave can be defined by its frequency which is in the range of from 300 MHz (0.3 GHz) to 300 GHz. In one embodiment the microwave has a wavelength of from 10 cm to 14 cm. In a further embodiment the microwave has a wavelength of about 12 cm.

The term “applying energy” denotes the application of energy to a sample either in a modulated or a in a non-modulated way. By the application of energy in a non-modulated way the energy is applied constantly, i.e. the same amount of energy is applied in consecutive identical time units independently of the span of the time units. By the application of energy in a modulated way the energy is applied wavelike, i.e. in consecutive identical time units different amounts of energy are applied.

In one embodiment the energy is applied in a defined volume. In one embodiment energy of 200 W to 1200 W is applied in a volume of 18 l. In a further embodiment energy of from 11 W/l to 67 W/l is applied. In a further embodiment about 44.5 W/l are applied.

In one embodiment the energy is applied for about six minutes in total. In another embodiment the energy is applied in three intervals of two minutes each.

The term “thermal energy” comprises all kind of energy that result in an increase of the total kinetic energy, i.e. all kinds of energy that effect disordered motion and increase of chemical bond vibrations. In other words the thermal energy results in a temperature increase, e.g. in a cell to which it is applied. An example of thermal energy is radiation (such as microwaves, and infrared waves, i.e. with wavelengths of from 10 µm to 50 cm).

The term “recombinant cell” or “a cell recombinantly expressing” or “a recombinantly expressing cell” denote a prokaryotic or eukaryotic cell that has been transfected with a nucleic acid encoding a polypeptide, e.g. an antigen or immunogen. The recombinant cell produces this polypeptide and presents it either on its cell surface or secretes it into the surrounding cultivation medium. In one embodiment the recombinant cell produces a membrane-bound antigen or immunogen. In one embodiment the recombinant cell is a bacterial cell or a mammalian cell.

The term "antibody" denotes a protein consisting of one or more polypeptide(s) substantially encoded by antibody genes. The recognized antibody genes include the different constant region genes as well as the myriad variable region genes. Antibodies may exist in a variety of formats, including, for example, Fv, Fab, and $F(ab)_2$ as well as single chains (scFv) or diabodies.

An antibody in general comprises two so called light chain polypeptides (light chain) and two so called heavy chain polypeptides (heavy chain). Each of the heavy and light chain polypeptides contains a variable domain (variable region) (generally the amino terminal portion of the polypeptide chain) comprising binding regions that are able to interact with an antigen. Each of the heavy and light chain polypeptides comprises a constant region (generally the carboxyl terminal portion). The constant region of the heavy chain mediates the binding of the antibody i) to cells bearing a Fc gamma receptor (FcγR), such as phagocytic cells, or ii) to cells bearing the neonatal Fc receptor (FcRn) also known as Brambell receptor. It also

mediates the binding to some factors including factors of the classical complement system such as component (C1q).

The variable domain of an antibody's light or heavy chain in turn comprises different segments, i.e. four framework regions (FR) and three hypervariable regions (CDR).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e. the individual antibodies comprised in the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to polyclonal antibody preparations, which include different antibodies directed against different antigenic sites (determinants or epitopes), each monoclonal antibody is directed against a single antigenic site on the antigen. In addition to their specificity, monoclonal antibodies are advantageous in that they may be synthesized uncontaminated by other antibodies. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies and is not to be construed as requiring production of the antibody by any particular method. Thus, in one embodiment the antibody is selected from a polyclonal antibody or a monoclonal antibody. In another embodiment the antibody is a monoclonal antibody.

The term "antibody solution" as used within the current application denotes an aqueous, buffered solution containing a complete antibody, an antibody fragment, or an antibody conjugate. This solution may be, e.g., a culture supernatant, or a column chromatography eluate, or a polished antibody solution.

The term "therapeutic antibody" relates to any antibody preparation which is intended for use in a human being. Preferably such therapeutic antibody will be a monoclonal antibody. Further preferred such monoclonal antibody will be obtained from a great ape or be a human monoclonal antibody or a humanized antibody. Preferably, it will be a human monoclonal antibody. Also preferred such therapeutic monoclonal antibody will be a humanized monoclonal antibody.

The term "buffered solution" denotes a solution in which changes of pH due to the addition or release of acidic or basic substances is leveled by a buffer substance. Any buffer substance resulting in such an effect can be used. In one embodiment pharmaceutically acceptable buffer substances are used, such as e.g. phosphoric

acid or salts thereof, acetic acid or salts thereof, citric acid or salts thereof, morpholine, 2-(N-morpholino) ethanesulfonic acid or salts thereof, histidine or salts thereof, glycine or salts thereof, or tris (hydroxymethyl) aminomethane (TRIS) or salts thereof. Especially preferred is phosphoric acid or salts thereof, or acetic acid or salts thereof, or citric acid or salts thereof, or histidine or salts thereof. Optionally the buffered solution may comprise an additional salt, such as e.g. sodium chloride, sodium sulphate, potassium chloride, potassium sulfate, sodium citrate, or potassium citrate.

The conjugation of an antibody to its conjugation partner can be performed by different methods, such as passive adsorption, chemical binding, or binding via a specific binding pair. The term "conjugation partner" denotes e.g. solid supports, polypeptides, detectable labels, members of specific binding pairs. In one embodiment the conjugation of the capture and/or tracer antibody to its conjugation partner is performed by chemically binding via N-terminal and/or ε-amino groups (lysine), ε-amino groups of different lysins, carboxy-, sulfhydryl-, hydroxyl-, and/or phenolic functional groups of the amino acid backbone of the antibody, and/or sugar alcohol groups of the carbohydrate structure of the antibody. In one embodiment the antibody is conjugated to its conjugation partner via a specific binding pair. In a further embodiment the antibody is conjugated to digoxigenin and linking to the detectable label is performed via an antibody against digoxigenin.

Chromogens (fluorescent or luminescent groups and dyes), enzymes, NMR-active groups or metal particles, haptens, e.g. digoxigenin, are examples of "detectable labels". The detectable label can also be a photoactivatable crosslinking group, e.g. an azido or an azirine group. Metal chelates which can be detected by electrochemiluminescense are also in one embodiment signal-emitting groups, with particular preference being given to ruthenium chelates, e.g. a ruthenium $(bispyridyl)_3^{2+}$ chelate. Suitable ruthenium labeling groups are described, for example, in EP 0 580 979, WO 90/05301, WO 90/11511, and WO 92/14138. For direct detection the labeling group can be selected from any known detectable marker groups, such as dyes, luminescent labeling groups such as chemiluminescent groups, e.g. acridinium esters or dioxetanes, or fluorescent dyes, e.g. fluorescein, coumarin, rhodamine, oxazine, resorufin, cyanine and derivatives thereof. Other examples of labeling groups are luminescent metal complexes, such as ruthenium or europium complexes, enzymes, e.g. as used for ELISA or for

CEDIA (Cloned Enzyme Donor Immunoassay, e.g. EP-A-0 061 888), and radioisotopes.

Indirect detection systems comprise, for example, that the detection reagent, e.g., the detection antibody is labeled with a first partner of a bioaffine binding pair. Examples of suitable binding pairs are hapten or antigen/antibody, biotin or biotin analogues such as aminobiotin, iminobiotin or desthiobiotin/avidin or Streptavidin, sugar/lectin, nucleic acid or nucleic acid analogue/complementary nucleic acid, and receptor/ligand, e.g., steroid hormone receptor/steroid hormone. Preferred first binding pair members comprise hapten, antigen and hormone. Especially preferred are haptens like digoxin and biotin and analogues thereof. The second partner of such binding pair, e.g. an antibody, Streptavidin, etc., usually is labeled to allow for direct detection, e.g., by the labels as mentioned above.

The term “antigen” denotes a protein determinant, also called an immunogen, capable of specific binding to an antibody. Antigens usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and non-conformational antigens are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

The term “cell” denotes a cell into which a nucleic acid, e.g. encoding a polypeptide to be expressed, can be or is introduced / transfected. The term „cell“ includes both prokaryotic cells, which are used for propagation of plasmids, and eukaryotic cells, which are used for the expression of a nucleic acid. In one embodiment the eukaryotic cells are mammalian cells. In a further embodiment the mammalian cell is selected from the group of mammalian cells comprising CHO cells (e.g. CHO K1, CHO DG44), BHK cells, NS0 cells, SP2/0 cells, HEK 293 cells, HEK 293 EBNA cells, PER.C6® cells, and COS cells. As used herein, the expression "cell" includes the subject cell and its progeny. Thus, the words "transformant" and "transformed cell" include the primary subject cell and cultures derived there from without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Variant progeny that have the same function or biological activity as screened for in the originally transformed cell are included.

The term „about“ denotes that the following value is the center of a range of +/- 10 % of that value.

A “nucleic acid” as used herein, refers to a polymeric molecule consisting of individual nucleotides (also called bases) a, c, g, and t (or u in RNA), for example to DNA, RNA, or modifications thereof. This polynucleotide molecule can be a naturally occurring polynucleotide molecule or a synthetic polynucleotide molecule or a combination of one or more naturally occurring polynucleotide molecules with one or more synthetic polynucleotide molecules. Also encompassed by this definition are naturally occurring polynucleotide molecules in which one or more nucleotides are changed (e.g. by mutagenesis), deleted, or added. A nucleic acid can either be isolated, or integrated in another nucleic acid, e.g. in an expression cassette, a plasmid, or the chromosome of a host cell. A nucleic acid is likewise characterized by its nucleic acid sequence consisting of individual nucleotides. The nucleic acid can be build up of DNA-fragments which are either isolated or synthesized by chemical means. The nucleic acid can be integrated into another nucleic acid, e.g. in an expression plasmid or the genome/chromosome of a eukaryotic host cell. Plasmid includes shuttle and expression plasmids. Typically, the plasmid will also comprise a prokaryotic propagation unit comprising an origin of replication (e.g. the ColE1 origin of replication) and a selectable marker (e.g. ampicillin or tetracycline resistance gene), for replication and selection, respectively, of the plasmid in prokaryotes.

To a person skilled in the art procedures and methods are well known to convert an amino acid sequence, e.g. of a polypeptide, into a corresponding nucleic acid sequence encoding this amino acid sequence. Therefore, a nucleic acid is characterized by its nucleic acid sequence consisting of individual nucleotides and likewise by the amino acid sequence of a polypeptide encoded thereby.

A "polypeptide" is a polymer consisting of amino acids joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 20 amino acid residues may be referred to as "peptides", whereas molecules consisting of two or more polypeptides or comprising one polypeptide of more than 100 amino acid residues may be referred to as “proteins”. A polypeptide may also comprise non-amino acid components, such as carbohydrate groups, metal ions, or carboxylic acid esters. The non-amino acid components may be added by the cell, in which the polypeptide is expressed, and may vary with the type of cell. Polypeptides are defined herein in terms of their amino acid backbone structure or

the nucleic acid encoding the same. Additions such as carbohydrate groups are generally not specified, but may be present nonetheless.

In one embodiment the cultivation vessel is a multi-well plate. In a further embodiment the cultivation vessel is a 384-well multi well plate.

In one embodiment about 5000 cells are seeded per cultivation vessel. In one embodiment the cells are seeded on an area of about 3.3 square millimeters. In a further embodiment the cells are grown for about three days. In another embodiment the cells are 100 % confluent growing cells.

In one embodiment the cultivation medium is removed from the incubated cells prior to the application of the energy and the cells are overlaid with a buffered solution. In one embodiment the cells are overlaid with about 25 µl of a buffered solution.

The following examples and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1 Non-binding of IHC suited antibody to wt-MCF-7 cells which do not express CDCP1 on the cell surface. Diamonds: #4115 cell signaling rabbit antibody; squares: anti-CDCP1 antibody.

Figure 2 Non-binding of IHC suited antibody to MCF-7 cells expressing CDCP1 but binding of not-IHC suited antibodies to MCF-7 cells expressing CDCP1. Diamonds: #4115 cell signaling rabbit antibody; squares: anti-CDCP1 antibody.

Figure 3 Non-binding of IHC suited antibody to wt-MCF-7 cells not expressing CDCP1 and non-binding of not-IHC suited antibodies to wt-MCF-7 cells not expressing CDCP1. Diamonds: #4115 cell signaling rabbit antibody; squares: anti-CDCP1 antibody.

Figure 4 Binding of IHC suited antibody to MCF-7 cells expressing CDCP1 and being process with a method as reported herein and non-binding of not-IHC suited antibodies to MCF-7 cells expressing CDCP1 and being treated with a method as reported

herein. Diamonds: #4115 cell signaling rabbit antibody; squares: anti-CDCP1 antibody.

Figure 5 Screened for IHC suitability with the method as reported herein: Exemplary results for rabbit derived anti-CDCP1 antibodies. Antibodies 3 and 5 are suited for IHC application whereas antibody 2 cannot be recommended. Diamonds: #4115 cell signaling rabbit antibody; squares: rabbit anti-CDCP1 antibody #5; circles: rabbit anti-CDCP1 antibody #3; filled triangles: different buffer, rabbit anti-CDCP1 antibody; "X": different buffer, mouse anti-CDCP1 antibody; open triangles: anti-CDCP1 antibody.

Figure 6 Screened for IHC suitability with the method as reported herein. Exemplary results for mouse derived anti-CDCP1 antibodies. Antibody 4 might be suitable for an IHC application whereas antibodies 1 and 6 cannot be recommended. Diamonds: #4115 cell signaling rabbit antibody; squares: mouse anti-CDCP1 antibody #4; open squares: different buffer, rabbit anti-CDCP1 antibody; filled circles: different buffer, mouse anti-CDCP1 antibody; filled triangle: mouse anti-CDCP1 antibody #1; open circles: anti-CDCP1 antibody.

Figure 7 Screened for IHC suitability with the method as reported herein. Exemplary results for mouse derived anti-HER3 antibodies. Antibody #119 is suited for IHC application. Triangles: rabbit anti-HER3 antibody #119; squares: mouse anti-HER3 antibody #376. Example 1.

Example 1

General method

In each well of a 384 well multi-well plate, which had been coated with poly-D-lysine, about $4*10^3$ to $5*10^3$ cells were seeded (seeding area about 3.3 square millimeters) and cultivated for about three days until a confluency of about 100 % was obtained. The supernatant was removed, e.g. by aspiration. Thereafter 25 µl of a 10 % (w/v) formalin solution were added to each well. After incubation for 45 minutes at room temperature the supernatant was removed. To each well 25 µl of citrate buffer, pH 6.0, were applied. The multi-well plate was thereafter treated for 6 minutes (3x2 minutes) with microwaves of 800 W and a wavelength of 12 cm. The treatment was with non-modulated energy application in a volume of 18 l. In

the volume of 18 l to at most 15 multi well plates in piles of at most 5 plates each the energy of 800 W was applied for 6 minutes. After the microwave incubation the wells were washed three times with 90 µl PBS-T buffer. For labeling 12.5 µl of a PBS buffer with 1 % (v/v) BSA and 12.5 µl of a solution of the primary antibody were added to each well and incubated for 1.5 hours at room temperature. After the incubation the wells were washed three times with 90 µl PBS-T buffer. For detection 25 µl of a secondary anti-rabbit/mouse/rat antibody POD conjugate were added and incubated for one hour (goat-anti-rabbit IgG dilute 1:9000 / goat anti-mouse IgG dilute 1:4000). Afterwards the wells were washed three times with 90 µl PBS-T buffer. For color formation 25 µl of a TMB solution were added and the extinction was determined at 370/492 nm or at 450/620 nm after the addition of 1 M hydrochloric acid.

For the determination of the binding of antibodies to wild-type cells and antigen presenting cells the cells were grown on Chamber-Slides (BD Biosciences) until confluency was achieved. After the cells have grown on the entire surface of the slide the cells were de-watered by treatment with different ethanol solutions. Afterwards the de-watered cells were treated with formalin for 10 minutes.

After the fixation with formalin an antigen-retrieval was performed as reported in Yamashita, S., Prog. Histochem. Cytochem. 41 (2007) 141-200. Thereafter the antibody was applied to the surface of the slide and incubated for 1 hour at high humidity. The binding of the antibody to the cell surface was determined by a detection antibody conjugated to the fluorescent label Alexa-Fluor 568.

Example 2

Comparative Example – binding to not-processed cells

Wild-type MCF-7 cells, which do not express CDCP1 on the cell surface, show no binding to the IHC suited antibody (cell signaling antibody) as well as the not IHC suited antibody (Figure 1).

MCF-7 cells expressing CDCP1 on the cell surface show no binding to the IHC suited antibody (cell signaling antibody) but show binding of the not IHC suited antibody (Figure 2).

Example 3

Binding to processed cells

Wild-type MCF-7 cells, which do not express CDCP1 on the cell surface, show no binding to the IHC suited antibody (cell signaling antibody) as well as the not IHC suited antibody (Figure 3) as also in Example 1.

MCF-7 cells expressing CDCP1 on the cell surface which have been processed with the method as reported herein show binding to the IHC suited antibody (cell signaling antibody) but show no binding of the not IHC suited antibody (Figure 4).

Example 4

Immunization of rabbits and mice with CDCP1 derived immunogens

An immunization of rabbits and mice with a CDCP1 derived immunogen was performed. The B-cells isolated from the experimental animals were fused to yield hybridoma cells. The hybridoma cell supernatants were screened for IHC suitability with the method as reported herein.

Exemplary results for rabbit derived antibodies are shown in Figure 5. Antibodies 3 and 5 are suited for IHC application whereas antibody 2 cannot be recommended.

Exemplary results for mouse derived antibodies are shown in Figure 6. Antibody 4 might be suitable for an IHC application whereas antibodies 1 and 6 cannot be recommended.

Example 5

Immunization of rabbits with HER3 derived immunogens

An immunization of rabbits with a HER3 derived immunogen was performed. The B-cells isolated from the experimental animals were fused to yield hybridoma cells. The hybridoma cell supernatants were screened for IHC suitability with the method as reported herein.

Exemplary results are shown in Figure 7. Antibody #119 is suited for IHC application.

Patent Claims

1. Use of thermal energy in form of microwaves for the treatment of a chemically cross-linked cell prior to staining in an immunohistochemical analysis.

2. A method for determining an antibody suitable for immunohistochemical staining of tissue samples comprising the following steps:

   a) incubating a cell recombinantly expressing an antigen with a solution comprising a chemical cross-linking agent,
   b) applying thermal energy to the cell, and
   c) determining the binding of an antibody to the cell and thereby determining an antibody suitable for immunohistochemical staining.

3. The method according to claim 2, characterized in comprising the following steps:

   a) cultivating antigen or immunogen presenting recombinant cells in a cultivation vessel or on a glass coverslip, whose surface is coated with a surface adhesion enhancer,
   b) incubating the cultivated recombinant cells with a solution comprising a chemical cross-linking agent,
   c) incubating the cross-linked cells with a conditioning buffer,
   d) applying thermal energy to the conditioned cross-linked cells,
   e) incubating the energy treated, conditioned, cross linked cells with a first antibody,
   f) determining the binding of the first antibody to the cells with a second antibody binding to the first antibody and thereby determining an antibody suitable for immunohistochemical staining of tissue samples.

4. A method for producing an antibody specifically binding to an antigen comprising the following steps:

   a) immunizing an experimental animal with a cell recombinantly expressing the antigen on its surface, whereby the cell has been incubated with a chemical cross-linking agent and whereby thermal energy has been applied to the chemically cross-linked cell,

b) cultivating a cell comprising a nucleic acid, whereby the nucleic acid encodes the antibody specifically binding to the antigen, and whereby the nucleic acid has been obtained from an immune cell recovered from the immunized experimental animal, and

c) recovering the antibody from the cell or the cultivation medium and thereby producing an antibody specifically binding to an antigen.

5. The method according to claim 4, characterized in comprising the further step:

a-1) selecting an antibody producing cell by

- cultivating antigen or immunogen presenting recombinant cells in a cultivation vessel, which is coated with a surface adhesion enhancer,
- incubating the cultivated recombinant cells with a solution comprising a chemical cross-linking agent,
- incubating the cells with a conditioning buffer,
- applying thermal energy to the conditioned cells,
- incubating the energy treated cells with a solution comprising a first antibody,
- determining the binding of the first antibody to the cells with a second antibody binding to the first antibody and thereby selecting an antibody producing cell.

6. The method according to any one of claims 4 or 5, characterized in comprising the further step:

a-2) determining the nucleic acid encoding the antibody produced by the cell.

7. The method according to any one of claims 2 to 6, characterized in that the applying thermal energy is the applying of thermal energy in form a microwaves.

8. The method according to any one of claims 3 or 5 to 7, characterized in that the conditioning buffer is a citrate buffer with a pH value of about 6.

9. The use or the method according to any one of the preceding claims, characterized in that the chemical cross-linking agent is selected from the group comprising formaldehyde, paraformaldehyde, formalin,

glutaraldehyde, dimethyl adipimidate, dimethyl suberimidate, osmium tetroxide, potassium dichromate, chromic acid, potassium permanganate, and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid-glutamic acid buffer-organic solvent mixture.

10. The use or the method according to any one of the preceding claims, characterized in that energy of about 800 W is applied for about 6 minutes.

11. The method according to any one of claims 3 or 5 to 10, characterized in that the surface adhesion enhancer is selected from gelatin, collagen (such as collagen type I), poly-D-lysine, poly-L-lysine, fibronectin, laminin, or polyvinyl formal.

12. The method according to any one of claims 2 or 5 to 11, characterized in that about $4*10^3$ antigen or immunogen presenting cells are cultivated for about 3 days.

13. The use or the method according to any one of the preceding claims, characterized in that the incubating with the chemical cross-linking agent is for about 45 minutes.

14. The method according to any one of claims 3 or 5 to 13, characterized in that the incubating with the first antibody is in the presence of about 0.5 % BSA (v/v) for 90 minutes.

Fig. 1

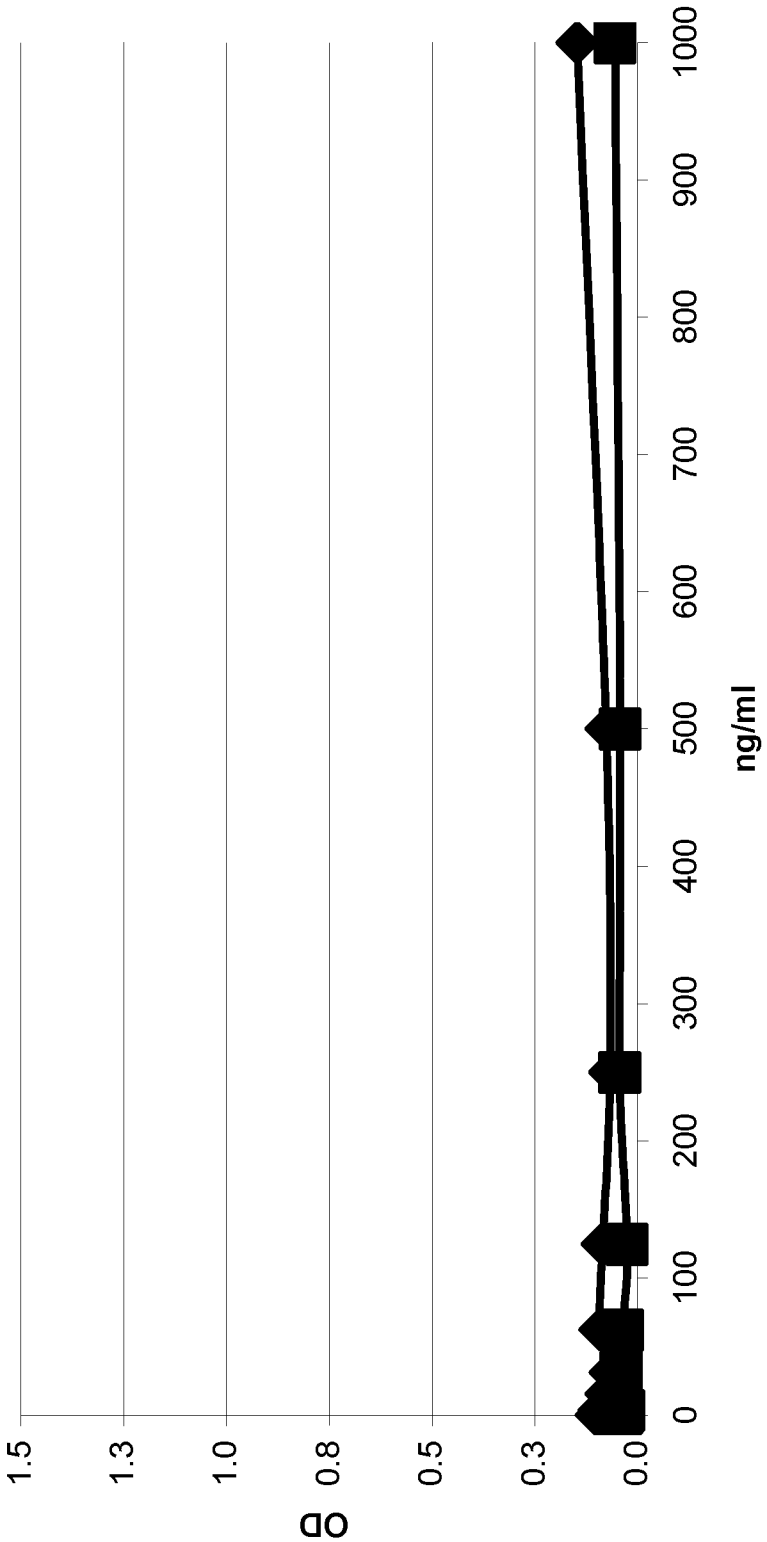
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Fig. 2

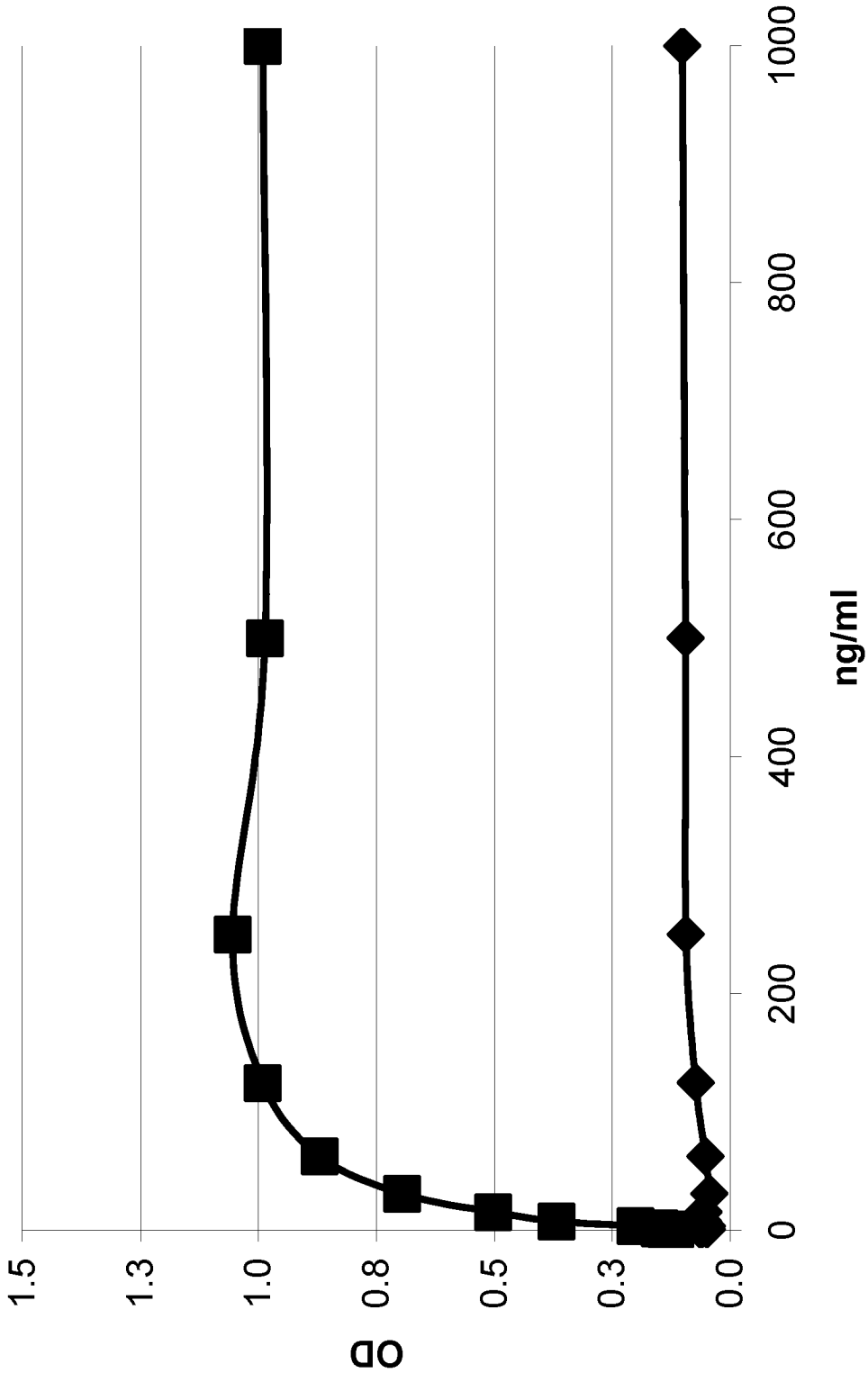

Fig. 3

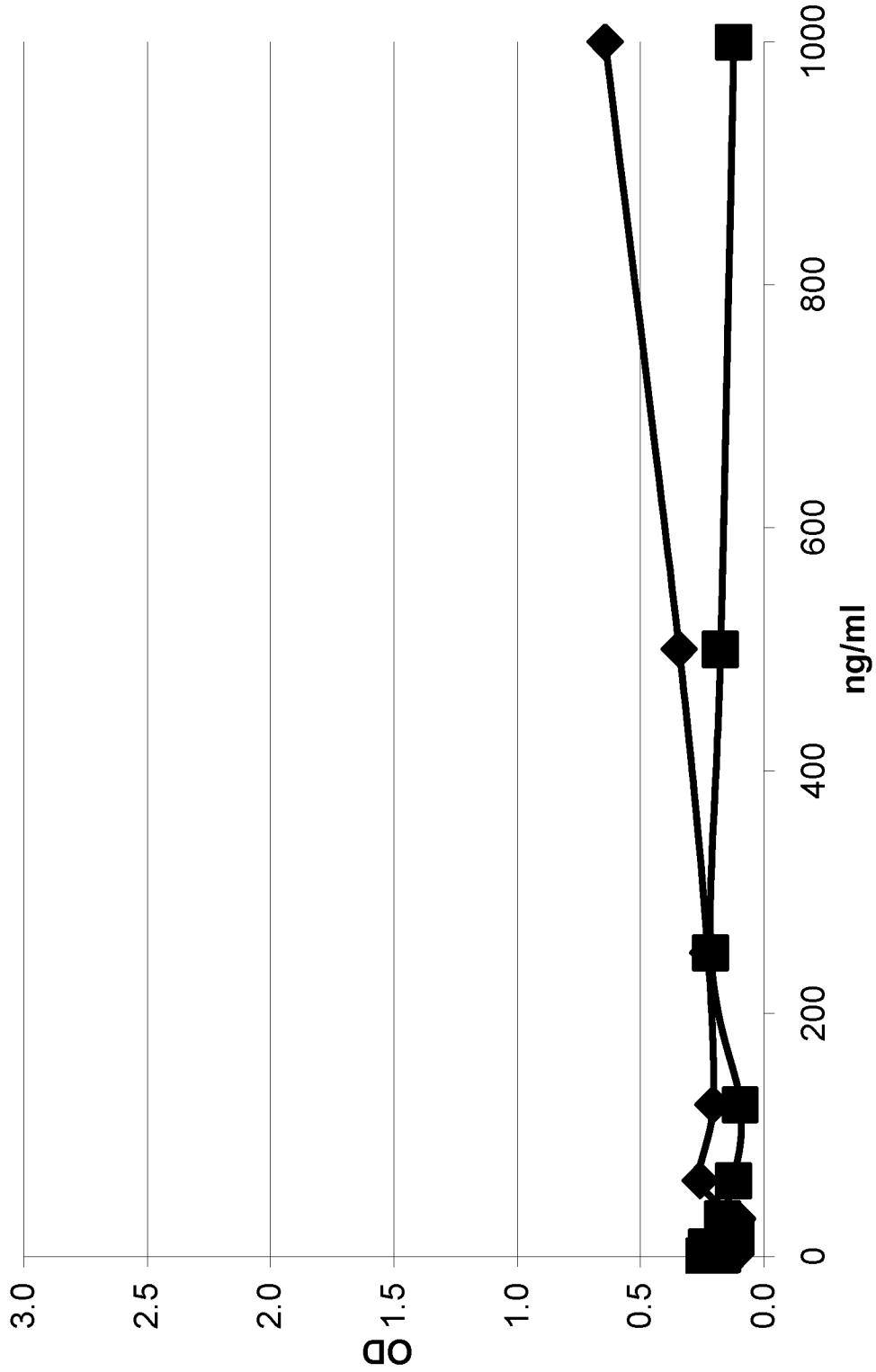

Fig. 4

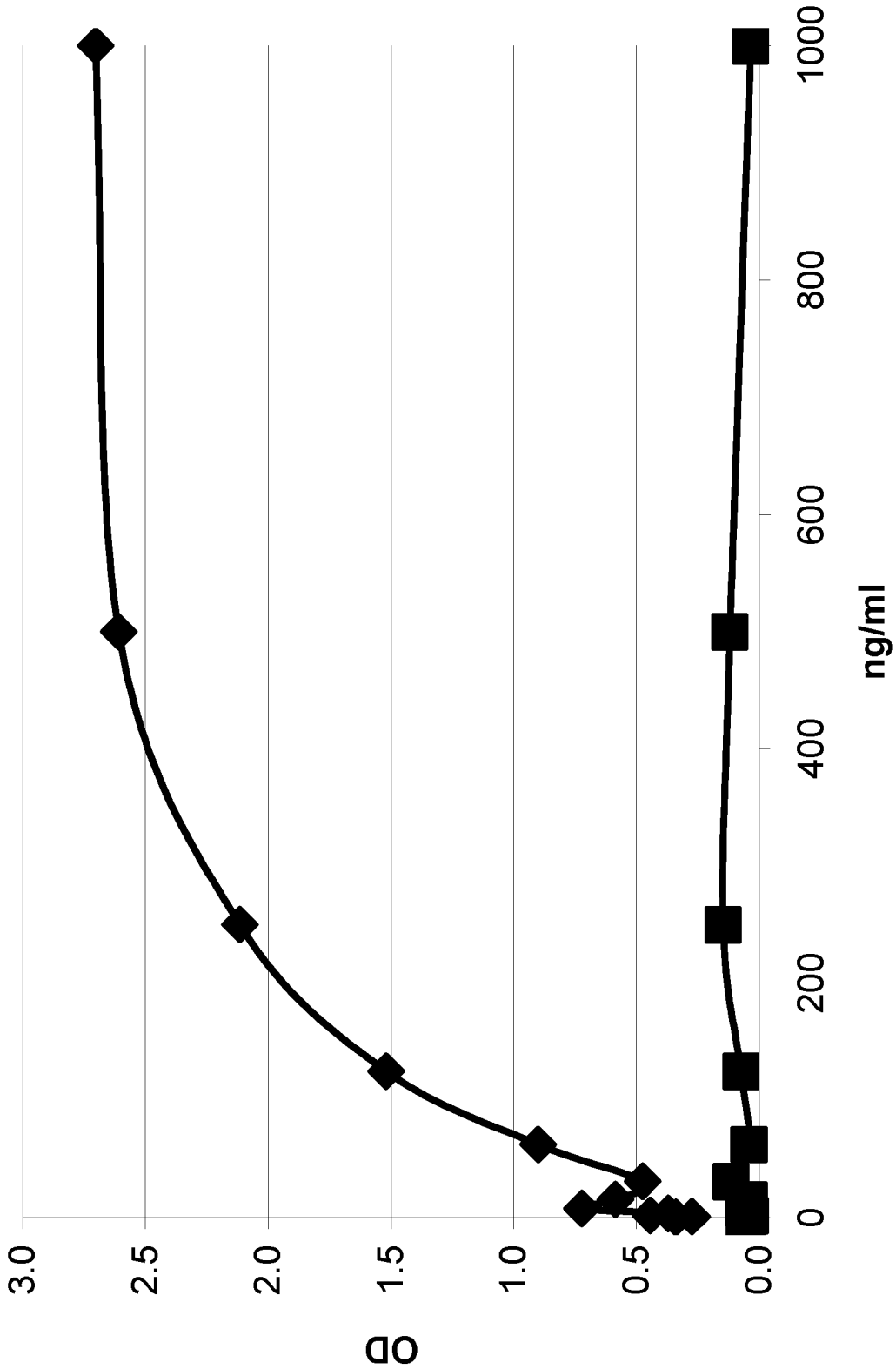

Fig. 5

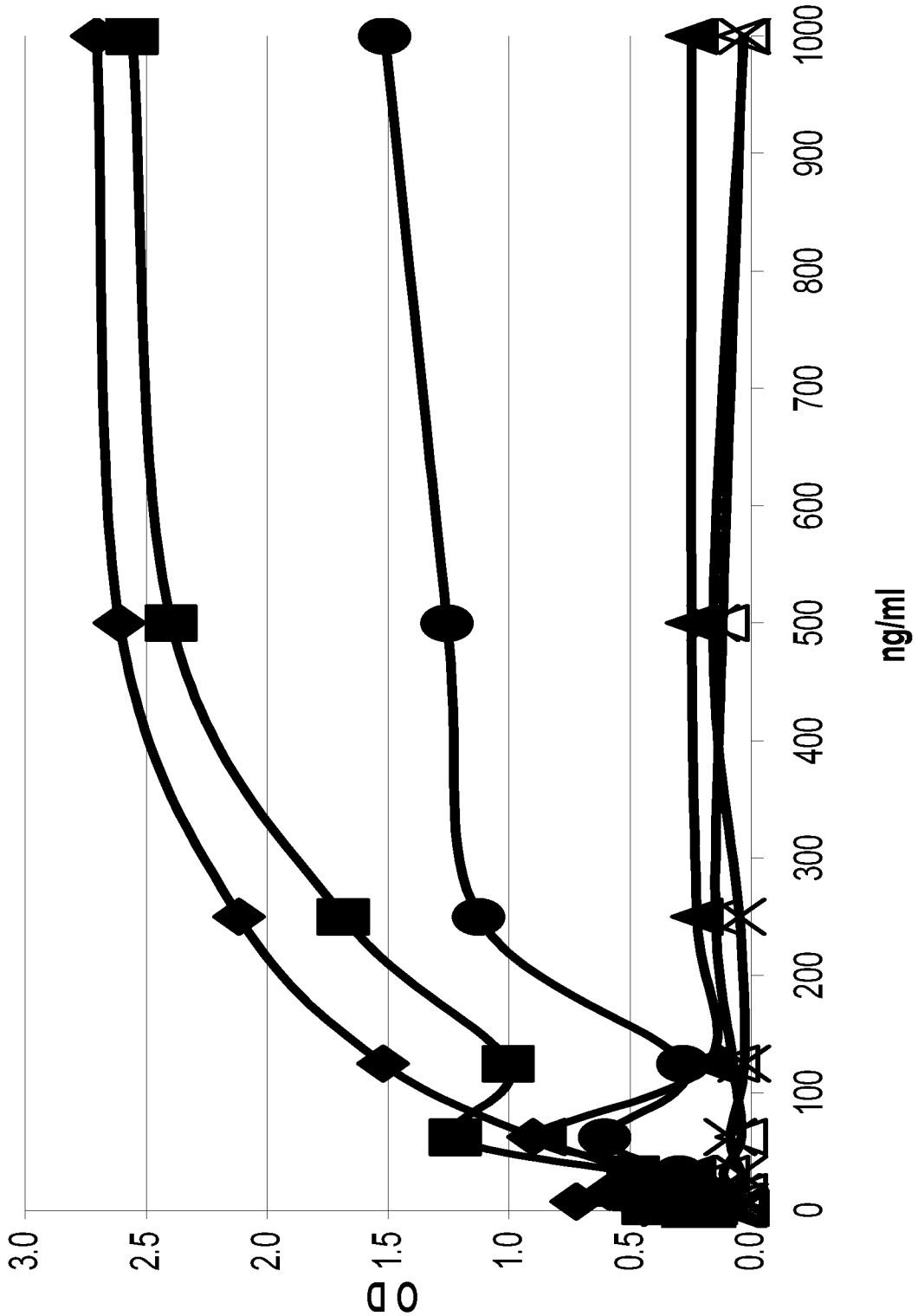
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Fig. 6

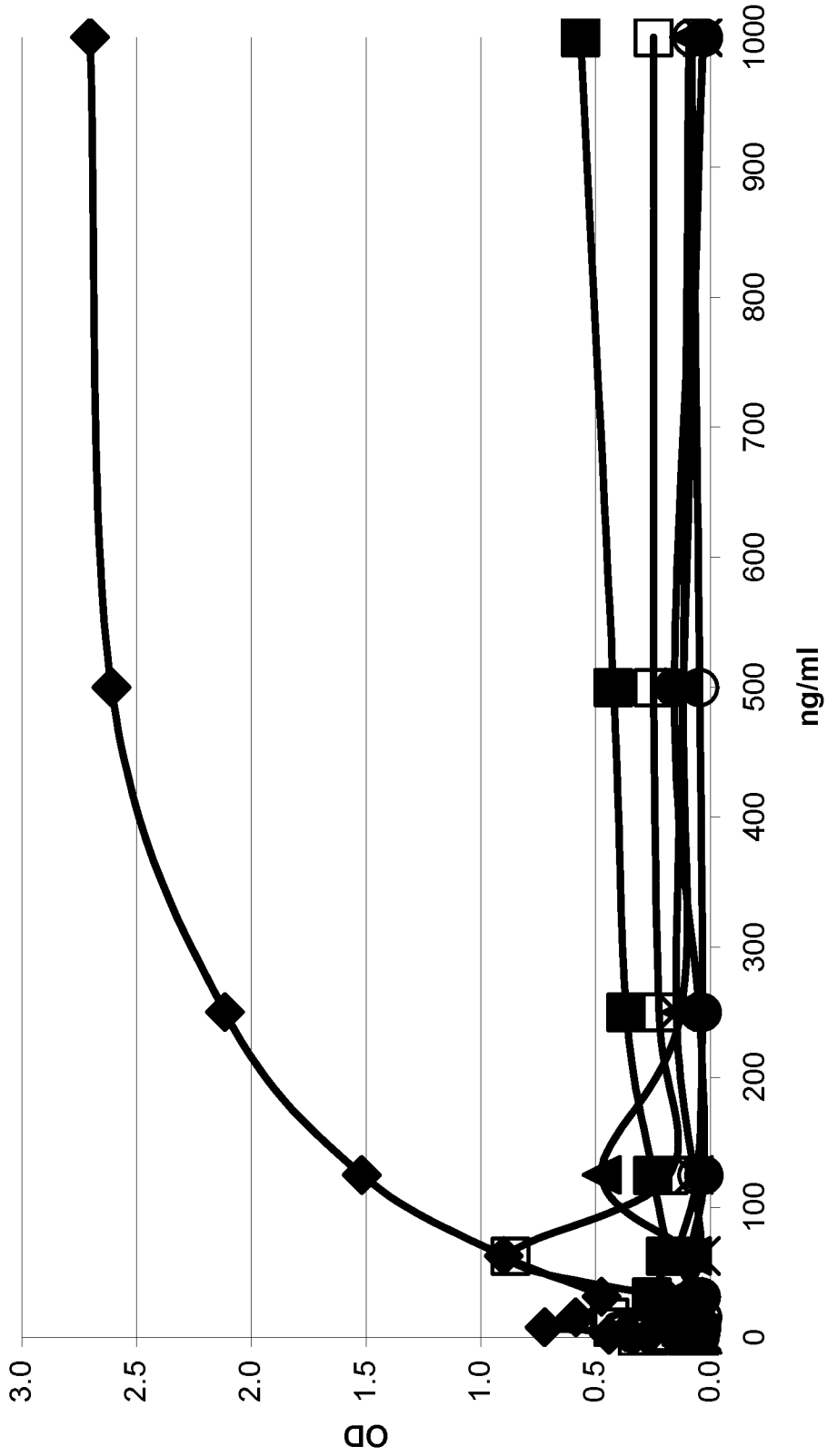
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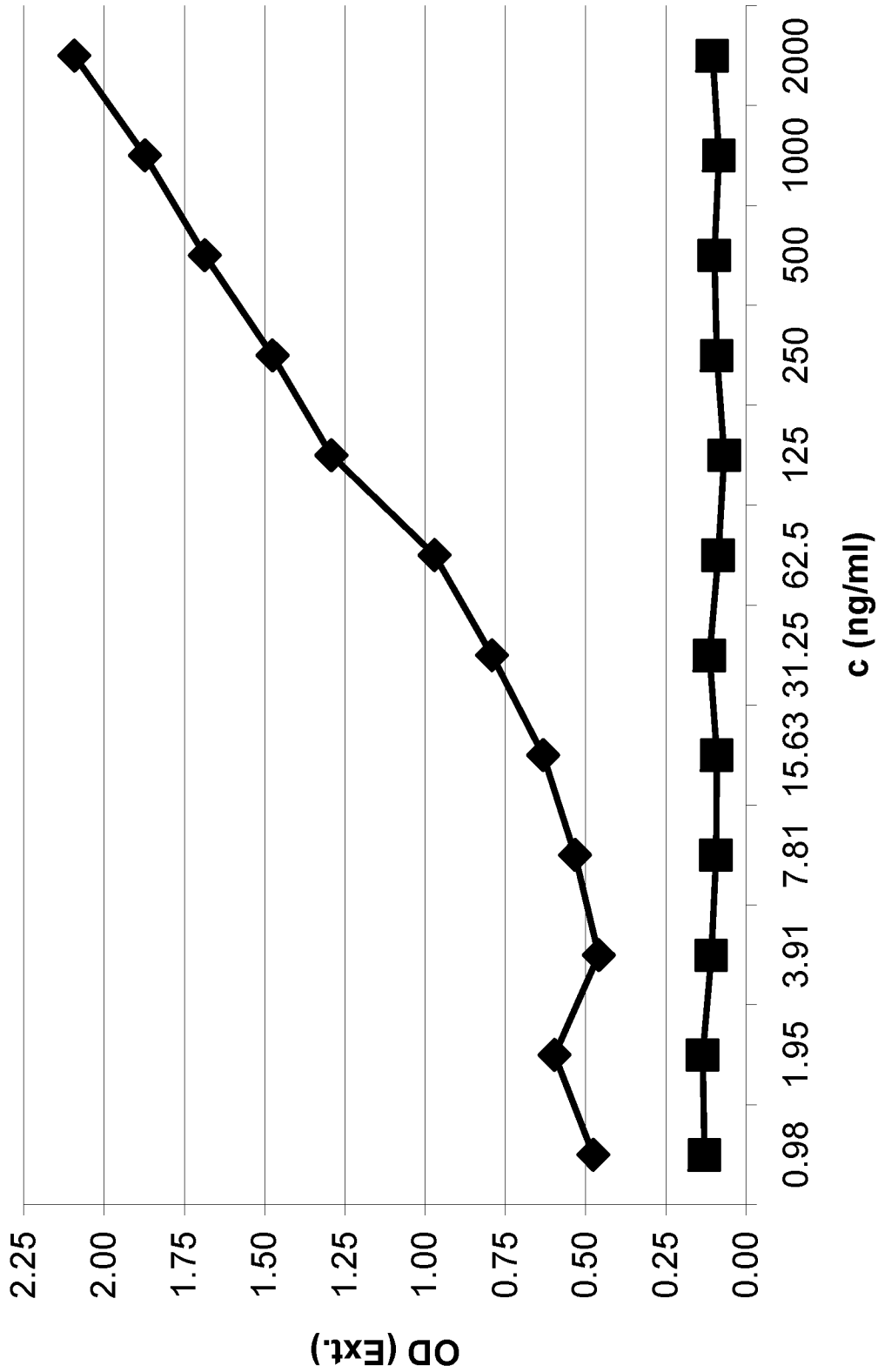
Fig. 7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62.5
125
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500
1000
2000
c (ng/ml)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2012/057052

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(completely); 9, 10, 13(partially)

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.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/057052

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/18 C07K16/28 C07K16/32 G01N1/44
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)
C12Q 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, EMBASE, 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	WO 2006/037993 A2 (AUVATION LTD [GB]; MURRAY GRAEME IAN [GB]; TELFER COLIN MATHESON [GB];) 13 April 2006 (2006-04-13) abstract page 29, line 23 - line 25 page 35, line 1 - line 15 page 41, line 5 - line 15 ----- -/--	1,9,10

[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
30 May 2012	12/09/2012

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	Mulder, Lonneke

2

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.
X	ANTHONY S.-Y. LEONG ET AL: "Accelerated immunohistochemical staining by microwaves", THE JOURNAL OF PATHOLOGY, vol. 161, no. 4, 1 August 1990 (1990-08-01), pages 327-334, XP055026746, ISSN: 0022-3417, DOI: 10.1002/path.1711610409 abstract; figures 4,5 section 'materials and methods'	1,9,10, 13
X	TERESA ELENA MUÑOZ ET AL: "Microwave-assisted immunostaining: a new approach yields fast and consistent results", JOURNAL OF NEUROSCIENCE METHODS, vol. 137, no. 2, 1 August 2004 (2004-08-01), pages 133-139, XP055026731, ISSN: 0165-0270, DOI: 10.1016/j.jneumeth.2004.02.020 abstract; tables 1,2 section materials and methods	1,9,10, 13
X	LYSKA L. EMERSON ET AL: "A Comparison of Immunohistochemical Stain Quality in Conventional and Rapid Microwave Processed Tissues", AMERICAN JOURNAL OF CLINICAL PATHOLOGY, vol. 125, no. 2, 29 December 2005 (2005-12-29), pages 176-183, XP055026745, ISSN: 0002-9173, DOI: 10.1309/GN6QCBMLLEATLK2M abstract; table 3 section materials and methods	1,9,13
X	BOON M E ET AL: "Microwaves for immunohistochemistry", MICRON, PERGAMON, OXFORD, GB, vol. 25, no. 2, 1 January 1994 (1994-01-01), pages 151-170, XP024447385, ISSN: 0968-4328, DOI: 10.1016/0968-4328(94)90040-X [retrieved on 1994-01-01] abstract page 158-159 section III C	1,9,13

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2012/057052

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006037993 A2	13-04-2006	NONE	

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(completely); 9, 10, 13(partially)

   Use of antigens displayed on cross-linked cells comprising the application of thermal energy, wherein the thermal energy is in the form of microwaves.

   ---

2. claims: 2, 3(completely); 7-14(partially)

   Method relating to antigens displayed on cross-linked cells, the method comprising the application of thermal energy, wherein the method comprises a step of testing if an antibody is suitable for IHC.

   ---

3. claims: 4-6(completely); 7-14(partially)

   Method relating to antigens displayed on cross-linked cells, the method comprising the application of thermal energy, wherein the method comprises a step of producing an antibody recognising the antigen.
