



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

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| <b>(54) Title:</b> MONOCLONAL ANTIBODY CAPABLE OF BINDING TO PNA/NUCLEIC ACID COMPLEXES<br><br><b>(57) Abstract</b><br><p>Monoclonal antibodies capable of binding to complexes formed between PNA (<u>P</u>eptide <u>N</u>ucleic <u>A</u>cid) and nucleic acids, particularly to PNA/DNA or PNA/RNA complexes, are described. The preferred monoclonal antibodies are capable of binding to PNA/DNA or PNA/RNA complexes, but not to single-stranded PNAs, double-stranded nucleic acids or single-stranded nucleic acids. PNAs are newly developed, not naturally occurring compounds of which some have a polyamide backbone bearing a plurality of ligands such as naturally occurring nucleobases attached to the backbone through a suitable linker. Some PNAs have been shown to possess a surprisingly high affinity for complementary nucleic acid forming very stable and specific complexes. Such PNAs are thus suitable as hybridization probes for detection of nucleic acids. The antibodies now provided render these PNAs very usable as hybridization probes. The antibodies provided are useful in the capture, recognition, detection, identification or quantitation of nucleic acids in biological samples, via their ability to react with PNA-nucleic acid complexes.</p> |           |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |

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**Monoclonal antibody capable of binding to PNA/nucleic acid complexes**

The present invention relates to monoclonal antibodies capable of binding to complexes formed between PNA (Peptide Nucleic Acid) and nucleic acids.

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PNAs are newly developed, not naturally occurring compounds of which some have a polyamide backbone bearing a plurality of ligands such as naturally occurring nucleobases attached to the backbone through a suitable linker. Some PNAs have been shown to possess a surprisingly high affinity for complementary nucleic acids forming very stable and specific complexes. Such PNAs are thus suitable as hybridization probes for detection of nucleic acids. In accordance with the present invention, antibodies are provided which render such PNAs very usable as hybridization probes.

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15 These antibodies are useful in the capture, recognition, detection, identification or quantitation of nucleic acids in biological samples, via their ability to bind to PNA-nucleic acid complexes.

**BACKGROUND OF THE INVENTION**

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The capture, recognition, detection, identification or quantitation of one or more chemical or biological entities is useful in the fields of recombinant DNA, human and veterinary medicine, agriculture and food science, among others. In particular, these techniques can be used to detect and identify etiologic agents such as bacteria and virus, to screen bacteria for antibiotic resistance, to aid in the diagnosis of genetic disorders and to detect cancerous cells.

25

The state-of-the-art nucleic acid hybridization assay techniques generally involve hybridization with a labelled form of a complementary nucleic acid probe. Hybridization between a particular base sequence of a nucleic acid in a sample and a labelled probe is determined by detection of the labelled complexes. The preparation of labelled probes generally involves the enzymatic incorporation of radiolabelled or modified nucleotides or chemical modification of the probe to attach or form a detectable chemical group. Preparation of labelled probes is often time consuming and expen-

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sive and has to be carried out without destroying the ability of the probe to detectably hybridize with its complementary sequence.

Reagents for direct detecting a nucleic acid duplex formed as a result of hybridiza-  
5 tion between the sample and a nucleic acid probe and thereby avoid the chemical  
labelling of the used probes, would facilitate detection.

The generation of specific polyclonal antibodies that will bind double-stranded nucleic  
acids but not single-stranded nucleic acids is complicated by the fact that polyclonal  
10 antisera raised against double-stranded nucleic acids may contain antibodies that  
will cross-react with single-stranded nucleic acids. Polyclonal antisera may also  
contain naturally occurring antibodies to single-stranded nucleic acids or antibodies  
to single-stranded nucleic acids arising as a result of break down of the immunogen  
used for the immunization.

15 By use of monoclonal antibody technology, antibodies may be selected so as to  
possess a desired affinity and specificity.

From US 4,623,627 and US 4,833,084, monoclonal antibodies are known which bind  
20 to duplexes formed between a conventional nucleic acid probe and a target nucleic  
acid.

In WO 92/20702, the term PNA is used to describe compounds having a non-cyclic  
backbone and bearing a plurality of ligands such as naturally occurring nucleobases  
25 attached to the backbone through a suitable linker. PNAs in which the backbone is  
structurally homomorphous with the deoxyribose backbone such as PNAs com-  
prising polymerized N-(2-aminoethyl)glycine units, wherein the glycine is connected  
to naturally occurring nucleobases by a linker, are able to hybridize to nucleic acid  
having a base sequence that is complementary to the base sequence of the PNA so  
30 as to form stable PNA-nucleic acid complexes (Egholm et al., Nature, Vol 365, 566-  
568 (1993)).

Such PNAs have been shown to bind strongly to complementary nucleic acid  
sequences. The melting temperature,  $T_m$ , of the complexes formed between such  
35 PNAs and complementary nucleic acids is typically 1-2°C higher per base than the

$T_m$  value for a comparable duplex formed between a DNA or RNA probe and a nucleic acid target.  $T_m$  is defined as the temperature at which half of the strands of a nucleic acid duplex are dissociated or denatured.

- 5 In accordance with the present invention, novel monoclonal antibodies are provided which are able to recognize, bind and detect complexes formed between PNA and nucleic acid.

#### SUMMARY OF THE INVENTION

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One aspect of the present invention is monoclonal antibodies that are capable of binding to complexes formed between PNAs and nucleic acids.

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Apart from sharing the feature of base pairing, PNA/nucleic acid complexes and nucleic acid duplexes, such as DNA/DNA or DNA/RNA duplexes, possess substantially different properties in that the PNA of a preferred PNA/nucleic acid complex comprises polymerized N-(2-aminoethyl)glycine units rendering the PNA achiral and non charged as opposed to the corresponding strands of a nucleic acid duplex, wherein the backbone is a sequence of nucleotides containing one anion for each phosphate group. The ensuing steric and conformational differences between the two types of compounds makes it absolutely unpredictable whether antibodies binding specifically to PNA/nucleic acid complexes could be made available.

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- Other aspects of the invention are monoclonal antibodies that are capable of binding to complexes formed between PNA and DNA or between PNA and RNA.

25

In preferred embodiments, the monoclonal antibodies capable of binding to complexes formed between PNAs and nucleic acids do not bind to single-stranded PNAs, double-stranded nucleic acids or single-stranded nucleic acids.

30

In one of these embodiments, the monoclonal antibody is capable of binding to a complex formed between PNA and DNA, but not to PNA/RNA complexes, double-stranded DNA, DNA/RNA duplexes, single-stranded PNA or single-stranded nucleic acids.

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In another of these embodiments, the monoclonal antibody is capable of binding to a complex formed between PNA and RNA, but not to PNA/DNA complexes, double-stranded DNA, DNA/RNA duplexes, single-stranded PNAs or single-stranded nucleic acids.

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Monoclonal antibodies for which the specificity of the epitope(s) recognised to a higher degree is dictated by the conformation of the PNA/nucleic acid complexes than by the specific sequence of the PNA/nucleic acid complex are also part of the invention.

10

The monoclonal antibodies described herein are obtainable from a hybridoma using generally known techniques. A selected host animal is immunized with a complex formed by contacting a PNA with a nucleic acid. Lymphocytes that secrete antibodies are taken from the immunized animal and are fused with myeloma cells to produce hybridomas. Hybridoma cells producing antibodies which bind to PNA-nucleic acid complexes are selected. These selected hybridomas are subcloned to assure monoclonality of the secreted antibody. Suitable complexes for immunization, are complexes formed between PNA having a backbone of N-(2-aminoethyl)glycine units and DNA or RNA.

20

Various methods for detecting a particular nucleic acid sequence in a test sample are additional aspects of the invention, whereby the present antibodies are useful in the capture, recognition, detection, identification or quantitation of one or more chemical or biological entities.

25

The antibodies may be very useful in the human and veterinary field. It is contemplated that the present antibodies will be very suitable to detect the presence of or the amount of infectious agents in humans such as chlamydial or gonococcal organisms or infections with Epstein Barr virus or papillomavirus. The present antibodies are also useful in the general field of cytogenetics such as chromosome painting.

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The invention also provides a kit containing a monoclonal antibody according to the invention, which antibody is optionally labelled, a PNA sequence capable of forming a complex with the nucleic acid sequence to be detected and a visualisation system.

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## DESCRIPTION OF THE SPECIFIC EMBODIMENTS

As used herein, the term "monoclonal antibody" is intended to include whole, intact antibodies, antibody fragments, or in general any antibody-derived substance that comprises at least one antibody combining site having the characteristics described herein. Antibodies of any of the known classes and subclasses of immunoglobulins are encompassed, e.g., IgG, IgM, and so forth, as well as active fragments such as the Ig fragments conventionally known as Fab, F(ab'), and F(ab')<sub>2</sub>.

- 10 The term "nucleic acid" covers a nucleotide polymer composed of subunits, which are either deoxyribonucleosides or ribonucleosides joined together by phosphodiester bridges between the 5'-position of one nucleoside and the 3'-position of another nucleoside. They may be DNA or various types of RNA. The terms "bases" and "nucleobases" are used interchangeably for pyrimidine and purine bases of nucleic acids and PNAs.

The PNAs are synthesized according to the procedure described in "Improved Synthesis, Purification and Characterization of PNA Oligomers", Presented at the 3rd Solid-Phase Symposium, Oxford UK, Aug. 31-Sept. 4, 1994 or the PNAs were obtained from PerSeptive Biosystems (Framingham, MA, USA)

The PNA-nucleic acid complex used for immunization may suitably be a complex between PNA and DNA or between PNA and RNA. Since both nucleic acid and PNA are devoid of diversified peptide sources, both nucleic acid complexes and PNA/-nucleic acid complexes would be expected to be essentially non-immunogenic in normal host animals (i.e. animal which are not prone to generate auto-antibodies against nucleic acid) when injected *per se*. Whereas in these circumstances the conventional conjugation of the non-immunogenic antigen to a carrier foreign to the host animal generally has been found to be impractical and laborious and furthermore induce a risk of creating structural changes in the antigen, an immune response towards nucleic acid complexes has been elicited by immunizing normal host animals with non-covalent, ionic complexes formed between the poly-anionic nucleic acid complex and a poly-cationic protein derivative, particular a methylated albumin or globulin species (US 4,623,627, US 4,833,084).

It has now surprisingly been found that antibodies directed against PNA/DNA complexes can be raised by immunizing a normal host animal with a mixture comprising a PNA/DNA complex and a non-derivatized protein heterologous to the host animal, such as ovalbumin. This technology can also be applied to immunization with PNA/-  
5 RNA complexes.

A PNA-DNA complex can be prepared by contacting double-stranded or single-stranded DNA with a PNA molecule having a base sequence that is complementary to all or part of the DNA sequence, heating the mixture to form single-stranded molecules  
10 and allowing the mixture to cool slowly to room temperature. A PNA-RNA complex might be prepared by contacting RNA with a PNA molecule having a base sequence that is complementary to all or part of the RNA sequence, heating the mixture and allowing the mixture to cool slowly to room temperature. Complex formation may be characterized by determining  $T_m$  of the complex or by performing electrophoresis in  
15 polyacrylamide gels.

A suitable quantity of one of the PNA-nucleic acid complexes is mixed with an adjuvant and a carrier. Examples of suitable carriers are KLH (Keyhole Limpet Hemocyanin), ovalbumin and dextrans.  
20

The monoclonal antibodies can be harvested from the secretions of hybridoma cells produced by somatic cell hybridization techniques originated from the work of Köhler and Milstein, *Nature*, 256, 495 (1975). This technique is well-known and has undergone various refinements and improvements. Details are described in references such as: Waldmann, M. C. H. "Production of murine monoclonal antibodies" in *Monoclonal Antibodies*, Beverly, P. C. L., ed., Churchill Livingstone, London, 1986; Melchers, F. et al. in Preface of "Lymphocyte Hybridomas" in *Current Topics in Microbiology and Immunology* 81, Springer-Verlag (New York 1978), IX-XVII; and Yelton, D. E. et al, "Plasmacytomas and Hybridomas" in *Monoclonal Antibodies*,  
25 Kennett et al, eds., Plenum Press, New York, 1980, 3-17.  
30

Lymphocytes producing antibodies against PNA-nucleic acid complexes may be obtained from various sites, e.g. the lymph nodes, spleen or peripheral blood. The selected lymphocytes are preferably spleen cells from a host animal, e.g. a mouse or  
35 a rat, preferably a mouse, which has been immunized with a PNA-nucleic acid



complex such as complexes between PNA and DNA or between PNA and RNA. A mouse may be immunized intraperitoneally, subcutaneously or intravenously with a mixture of a PNA/DNA complex, wherein the PNA in preferred embodiments comprises polymerized N-(2-aminoethyl)glycine units, ovalbumin and a suitable adjuvant.

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For fusion, myeloma cells of various animal origin can be used, for example, myeloma cells from mice, rats or humans. However, for reasons of genetic stability, it may be preferred to fuse lymphocytes and myeloma cells derived from the same animal species and most preferably from the same strain of such animal species.

10

Murine lymphocytes and myeloma cells are most commonly used. The myeloma cells are preferably from the BALB/c strain, most preferred is myeloma cell line P3-X63-Ag.8.

15

Fusion of the lymphocyte and myeloma cells to form hybridomas and selection of antibodies are accomplished by methods known per se. The selected hybridomas are cultured *in vitro* for an appropriate time period and aliquots of the culture fluid are drawn off to provide monoclonal antibody-rich fractions. Monoclonal antibodies as described herein may be recovered from these fractions by purification using methods known per se for purification of monoclonal antibodies.

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The present antibodies have a high degree of specificity for PNA-nucleic acid complexes. No significant degree of binding to double-stranded nucleic acids, double-stranded PNAs, single-stranded PNAs or single-stranded nucleic acids is observed. The specificity of the epitope(s) recognised by the present antibodies appears to be dictated by the conformation of the PNA-nucleic acid complex rather than by the specific sequence of the PNAs or the nucleic acids.

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The clones obtained produced antibodies capable of binding specifically to PNA/DNA complexes. Only insignificant reaction with single-stranded PNAs or DNA or double-stranded DNA or double-stranded PNA or PNA/RNA was observed with these clones.

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A high specificity and affinity of the present antibodies give significant advantages when used in the isolation, detection and quantitation of complexes formed between a PNA and a nucleic acid to be detected in a biological sample. Thus, antibodies

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having a high specificity for PNA/DNA complexes are particularly valuable in PNA based analysis for identifying infectious agents in humans such as chlamydial or gonococcal organisms or in animals. These antibodies are also very useful in the general field of cytogenetics such as specific detection of chromosomes.

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Antibodies according to the invention having a high specificity and affinity for PNA/-RNA complexes are particularly useful in PNA based analysis, for example for identifying mRNA or rRNA sequences.

10 Depending on the particular use, the antibody may be coupled with a detectable label such as enzymatically active groups like coenzymes, enzyme inhibitors and enzymes themselves, fluorescent labels, chromophores, luminescent labels, specifically bindable ligands such as biotin or haptens.

15 A method for detecting a particular nucleic acid sequence in a sample using the antibodies described herein may comprise the steps of

(a) forming a complex between the particular nucleic acid sequence to be detected in the sample and a PNA having a base sequence that is sufficiently complementary to the base sequence of the nucleic acid sequence to be detected so as to form complexes, said complexes having at least one epitope for an antibody as described herein,

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(b) contacting any complex that is formed between the PNA sequence and the nucleic acid sequence to be detected with an antibody as described herein, and

25

(c) determining the presence of antibody-PNA-nucleic acid complexes.

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The PNA sequence may suitably be immobilized onto a solid support prior to the contact with the sample containing the nucleic acid sequence to be detected, or the antibody may be immobilized onto a solid support prior to contact with the PNA-nucleic acid complex.

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If the nucleic acid sequences to be detected are present in an immobilized state in a biological specimen, a method may be used which comprises the steps of

- 5                   (a)     forming a complex between the particular nucleic acid sequence to be detected in the specimen and a PNA having a base sequence that is sufficiently complementary to the base sequence of the nucleic acid sequence to be detected so as to form complexes, said complexes having at least one epitope  
10                   for an antibody as described herein,
- (b)     contacting any complex that is formed between the PNA sequence and the nucleic acid sequence to be detected with an antibody as described herein, and  
15                   (c)     determining the presence of antibody-PNA-nucleic acid complexes.

In a method wherein the initial step is an immobilization of the nucleic acid sequence to be detected, the method suitably comprises the steps of

- (a)     immobilizing the nucleic acid sequence to be detected to a solid support,
- 25                   (b)     forming a complex between the particular nucleic acid sequence to be detected in the sample and a PNA having a base sequence that is sufficiently complementary to the base sequence of the nucleic acid sequence to be detected so as to form complexes, said complexes having at least one epitope  
30                   for an antibody as described herein,
- (c)     contacting any complex that is formed between the PNA sequence and the nucleic acid sequence to be detected with an antibody as described herein, and

- (d) determining the presence of antibody-PNA-nucleic acid complexes.

Examples of applications of the present antibodies are described below.

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A kit for carrying out the described methods or other methods using the present antibodies may in addition to the present antibody in labelled or unlabelled form contain a PNA sequence that is complementary to all or part of the nucleotide sequence to be detected and a visualisation system. The visualisation system may  
10 comprise an enzyme-conjugate (e.g. an enzyme conjugated antibody or an enzyme conjugated streptavidin) and a suitable substrate. The conjugate may have a reactivity to mouse immunoglobulin epitopes in cases where the unlabelled form of the present antibody is used or to hapten groups such as biotin, fluorescein or peptide in cases where the present antibody has been labelled with hapten groups. The sub-  
15 strate system of the kit may be selected to form a soluble coloured reaction product in cases where the PNA/nucleic acid complex is measured in an ELISA format or the substrate system may be selected to form an insoluble coloured reaction product in cases where the PNA/nucleic acid complex is measured in a biological sample or on a membrane.

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Application 1: Hybridization and detection in solution

A nucleic acid sequence of interest can be determined in solution by contacting the sample containing the nucleic acid with a PNA having a base sequence that is sufficiently complementary to the base sequence of the nucleic acid of interest so as  
25 to form complexes followed by contact with an antibody as described herein, recognising the PNA-nucleic acid complexes but not free PNA or nucleic acids. These reactions will result in a large complex which may be detected e.g. in a turbidimetric assay format.

30 Application 2: Solution hybridization and detection after immobilization

A nucleic acid sequence of interest can be determined by contacting it with a PNA having a base sequence that is sufficiently complementary to the base sequence of the nucleic acid of interest so as to form complexes. The formed complexes are, while still in solution, contacted with a labelled antibody as described herein. The  
35 formed PNA-nucleic acid-antibody complex is then captured using an antibody as

described herein which e.g. has been immobilized onto a solid support. Unbound materials are washed off and the amount of bound PNA-nucleic acid-antibody complex is determined via detection of the label on the antibody.

- 5 Alternatively, the PNAs having a base sequence that is sufficiently complementary to the base sequence of the nucleic acid of interest so as to form complexes may carry a label, e.g. biotin, a fluorescent label, or other moieties which are suitable for catching of PNA-nucleic acid complexes. Unbound materials is washed off and the amount of bound PNA-nucleic acid-antibody complex is determined either via  
10 detection of the label on the antibody or by using a secondary antibody detection system.

#### Application 3: Capture assay

- A traditional capture assay comprises the steps: recognition, capture and detection  
15 and may be composed in various ways. One example of such assay is described below.

- An antibody capable of binding to a PNA-nucleic acid complex is immobilized onto a solid support, e.g. onto an ELISA plate. PNA and sample are mixed and allowed to  
20 react in solution in the wells of the ELISA-plate. If complexes between the PNA and the sample nucleic acids are formed, these complexes will be captured by the immobilized antibody. Unbound materials are washed off and the amount of bound PNA-nucleic acid-antibody complex is determined. The capture step may also be based on other recognisable moieties than a PNA-nucleic acid complex. Such moieties  
25 could e.g. be biotinylated PNAs or PNAs labelled with other haptens, peptides, or polypeptides.

- In some assay formats, two or more of the steps indicated above may be performed simultaneously.  
30

#### Application 4: Detection of PNA/nucleic acid complexes immobilized onto a solid support

- Complexes formed between PNAs and nucleic acids in which either the PNA or the nucleic acid initially was immobilized onto a solid support can be detected by the  
35 antibody described herein. This detection can be performed either directly using such

an antibody conjugated to an enzyme, a fluorescent marker or another signal generating system, or indirectly using one of the secondary detection systems commonly used for detecting antibodies bound to a target. The solid support considered should be understood in a very broad sense like e.g. nylon or nitrocellulose membranes (Southern or Northern blots), a tissue section, cell smears, cytopins or chromosome spreads (*in situ* hybridization), or a plastic surface (an ELISA format).

This system has the advantage that the normally very extensive washing procedures included in these technologies can be significantly reduced since non-specifically bound PNAs, being single-stranded, will not give rise to a signal as the antibody only recognises PNA forming complexes with nucleic acids.

#### Application 5: Biosensor systems

Detection and quantification of nucleic acids in a biological sample may be performed using a biosensor system such as the BIAcore biosensor system from Pharmacia. The interaction of biomolecules with an immobilized ligand on a sensor chip is measured at the surface using evanescent light. The system includes a sensor chip to which the ligand can be immobilized in a hydrophilic dextran matrix, a miniaturised fluids cartridge for the transport of analytes and reagents to the sensor surface, a SPR (surface plasmon resonance) detector, an autosampler and system control and evaluation software. Specific ligands are covalently immobilized to the sensor chip through amine, thiol or aldehyde chemistry or biospecifically by e.g. biotin - avidin interaction.

The antibody as described herein may be coupled to a sensor chip of the biosensor-system used, e.g. to a dextran layer of a sensor chip in a BIAcore system. A sample is mixed with PNA and incubated so that a complex is formed between the nucleic acid in the sample and PNA having a base sequence that is sufficiently complementary to the base sequence of the nucleic acid of interest so as to form complexes.

The sample is passed through the flow system of the biosensor system and the antibody coupled to the sensor chip will bind specifically to the PNA-nucleic acid complexes if such complexes have been formed. Based on the SPR detection employed by the biosensor system, this binding will generate a signal depending on the amount of materials bound to the surface.

#### Application 6: Detection of bound PNA in cells

Under suitable conditions, PNAs may be able to penetrate the cell-wall of living or fixed cells, e.g. cell-lines, hemopoietic cells, and animal/human tissues (important in therapeutic applications). It may be important to be able to detect PNAs that have  
5 hybridized to different targets in the individual cells. In such cases, labelling of the PNAs with haptens or other reporter molecules may not be advantageous as this may inhibit or interfere with the penetration of the PNAs into the cells. The detection of PNAs hybridizing to a target by either immunohistochemistry (in frozen or fixed tissue biopsies) or by flow-cytometry (e.g. on cells treated with detergent, acetone or  
10 alcohol) are important. It is also advantageous to be able to detect binding and/or tissue distribution of PNA's added to a cell culture or administered to a living animal. Such detection is made possible with an antibody provided as described herein.

In the following examples, specific embodiments of the present invention are given.  
15 These examples are not intended to limit the invention in any way.

### EXAMPLES

#### EXAMPLE 1

20

##### PNA/nucleic acid complexes and single chains

PNAs comprising polymerized N-(2-aminoethyl)glycine units to which nucleobases are attached through a methylenecarbonyl linker, were synthesized and purified as described in "Improved Synthesis, Purification and characterization of PNA Oligomers", presented at the 3rd Solid-Phase Symposium, Oxford UK, Aug. 31-Sept. 4,  
25 1994, and by M. Egholm et al., J. Am. Chem. Soc. 114, 1895-1897 (1992) and M. Egholm et al., J. Chem. Soc. chem. Commun. 800-801 (1993), or such PNAs were obtained from PerSeptive Biosystems. The base sequence of the PNA used is preferably virtually non-self-complementary in order to avoid self-hybridization in the  
30 PNA molecule. The number of purines and pyrimidines is approximately equal to allow formation of a double helix configuration rather than a triple helix configuration.

DNA sequences were synthesized on an abi 381A DNA synthesizer from Applied Biosystems Inc using a standard 381A cycle/procedure. The monomers used were  
35 standard  $\beta$ -cyanoethyl phosphoramidites for Applied Biosystems Synthesizer. RNA

sequences were purchased from "DNA Technology Aps, Science Park Aarhus, Gustav Wieds Vej 10, DK-8000 Aarhus.

- The PNA and DNA sequences may be labelled or unlabelled and may optionally contain one or more linker units, preferably one or two linker units wherein the two linker units are attached end to end. Linkers are in all cases written as "-link-" independently of it being labelled PNA or DNA sequences or the number of linker units added.
- 5
- 10 PNA can be labelled with biotin in the following way: a linker comprising one or two units of 2-(aminoethoxy)ethoxy acetic acid (AEEA) is attached to the PNA on the resin (see above), and biotin is attached in the following way. Two solutions were used. The first solution contained 0.1 M biotin in 5% s-collidin in DMF with 0.2 M of N-ethyldicyclohexylamine and the second solution contained 0.18 M HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) in DMF. The two solutions were mixed in a ratio of 2 to 1 and the mixture was left for approximately one minute before it was combined with the resin to which the PNA with one or two units of AEEA were attached.
- 15
- 20 For biotin labelling of DNA the following two procedures can be used. For labelling in the 5' end of the DNA oligomers a linker (Spacer phosphoramidite, Clontech Laboratories) was connected to the 5'-OH of the oligomer and then reacted with a biotin labelling reagent (Biotin CE phosphoramidite, 22-0001-35, Cruachem Limited). For labelling in the 3' end of the oligomers the DNA synthesis was started from a Biotin-CPG support (3'-Biotin-ON CPG cat # RP-5225-2 K.J. Ross Petersen, Agern Alle 3, DK-2970 Hørsholm). The first reagent was a linker (Spacer phosphoramidite, Clontech Laboratories, Inc.) and the monomer reagents were added for synthesising the oligomer.
- 25
- 30 All PNA sequences are written from the amino-terminal end which is denoted "H-" (corresponding to the 5'-end in DNA) to the C-terminal end which is denoted "CONH<sub>2</sub>" (corresponding to the 3'-end in DNA). All DNA sequences are written from the 5'-end to the 3'-end. The following test complexes/compounds were used:



H12. An unlabelled PNA/DNA complex (the immunogen) comprising a 45-mer DNA sequence (U1) and 3 units of a 15-mer PNA sequence (U2).

The base sequence of the 45-mer DNA (U1) was as follows:

5'-GCA AAT GCT CTA GGC GCA AAT GCT CTA GGC GCA AAT GCT  
CTA GGC-3'.

The base sequence of the 15-mer PNA (U2) was as follows:

H-GCC TAG AGC ATT TGC-CONH<sub>2</sub>

L1. A single stranded 45-mer DNA sequence (ssDNA) with a biotin attached to the 5'-end of the DNA sequence.

The base sequence of the 45-mer DNA (L1) was as follows:

5'-Bio-link-GCA AAT GCT CTA GGC GCA AAT GCT CTA GGC GCA AAT  
GCT CTA GGC- 3'.

L2. A 45-mer DNA sequence (L2) with a biotin attached to the 3'-end of the DNA sequence.

The base sequence of the 45-mer DNA (L2) was as follows:

5'-GCA AAT GCT CTA GGC GCA AAT GCT CTA GGC GCA AAT GCT  
CTA GGC-link-Bio-3'.

H2. A PNA/DNA complex comprising a 45-mer DNA sequence (L2) and 3 units of a 15-mer PNA sequence (U2) wherein biotin is attached to the 3'-end of the 45-mer DNA sequence. Apart from the biotin moiety coupled to the DNA sequence, this complex corresponds to the complex used for immunization.

The base sequence of the 45-mer DNA (L2) was as follows:

5'-GCA AAT GCT CTA GGC GCA AAT GCT CTA GGC GCA AAT GCT  
CTA GGC-link-Bio-3'.

The base sequence of the 15-mer PNA (U2) was as follows:

H-GCC TAG AGC ATT TGC-CONH<sub>2</sub>

L4. A single stranded 15-mer PNA sequence (ssPNA) with a base sequence corresponding to the 15-mer PNA in the complex used for immunization, and labelled with biotin in the 5' end. The base sequence of this 15-mer PNA (L4) was as follows:

Bio-link-GCC TAG AGC ATT TGC-CONH<sub>2</sub>

- L3. A 15-mer DNA sequence (L3) wherein a biotin is attached to the 5'-end of the DNA sequence. The base sequence of this 15-mer DNA (L3) was as follows:
- 5 5'-Bio-link-GCA AAT GCT CTA GGC-3'
- H3. A PNA/DNA complex comprising a 15-mer DNA sequence (L3) and a 15-mer PNA sequence (U2) wherein biotin is attached to the 5'-end of the 15-mer DNA sequence.
- 10 The base sequence of this 15-mer DNA (L3) was as follows:  
5'-Bio-link-GCA AAT GCT CTA GGC-3'  
The base sequence of the 15-mer PNA (U2) was as follows:  
H-GCC TAG AGC ATT TGC-CONH<sub>2</sub>
- 15 H4. A DNA/DNA complex comprising a 15-mer DNA sequence (L3) and a complementary 15-mer DNA sequence (U4). The biotin is attached to the 5'-end of the L3 15-mer DNA sequence.
- The base sequence of this 15-mer DNA (L3) was as follows:  
5'-Bio-link-GCA AAT GCT CTA GGC-3'
- 20 The base sequence of the 15-mer DNA (U4) was as follows:  
5'-GCC TAG AGC ATT TGC-3'
- H20. A large DNA/DNA complex resembling the immunogen and consisting of a 45-mer DNA sequence (L2), labelled with biotin at the 3'-end, and 3 units of
- 25 a 15-mer DNA sequence (U4). The sequences of the two chains in this complex is given above (in relation to the description of H2 and H4).
- L5. A 20-mer PNA sequence wherein the PNA is labelled with biotin at the amino-terminal end. The base sequence of the 20-mer PNA (L5) was as follows:
- 30 Bio-link-CGG CCG CCG ATA TTG GCA AC-CONH<sub>2</sub>
- H7. A PNA/DNA complex comprising a 20-mer PNA sequence and a 20-mer DNA having a base sequence that is different from the sequence of the

complex used for immunization and wherein the PNA is labelled with biotin at the amino-terminal end.

The base sequence of the 20-mer PNA (L5) was as follows:

Bio-link-CGG CCG CCG ATA TTG GCA AC-CONH<sub>2</sub>

5 The base sequence of this 20-mer DNA (U6) was as follows:

5'-GTT GCC AAT ATC GGC GGC CG-3'

L6. A 17-mer DNA sequence with a biotin attached at the 5'-end.

The base sequence of the 17-mer DNA (L6) was as follows:

10 5'-Bio-link-ATT GTT TCG GCA ATT GT-3'

H8. A PNA/DNA complex comprising a 17-mer PNA sequence and a 17-mer DNA sequence wherein the base sequence is different from the complexes previous described, but related to the complex H9 described below. The DNA strand of this complex is labelled with biotin at the 5'-end.

15 The base sequence of the 17-mer DNA (L6) was as follows:

5'-Bio-link-ATT GTT TCG GCA ATT GT-3'

The base sequence of the 17-mer PNA (U7) was as follows:

H-link-ACA ATT GCC GAA ACA AT-CONH<sub>2</sub>

20

L7. A 17-mer PNA sequence labelled with biotin at the amino-terminal end.

The base sequence of the 17-mer PNA (L7) was as follows:

Bio-link-ACA ATT GCC GAA ACA AT-CONH<sub>2</sub>

25 H9. A PNA/DNA complex comprising a 17-mer PNA sequence and a DNA sequence wherein the base sequence is different from the complexes previous described, but related to the complex H8 described above. The PNA strand of this complex is labelled with biotin at the amino-terminal end.

The base sequence of the 17-mer DNA (U8) was as follows:

30 5'-ATT GTT TCG GCA ATT GT-3'

The base sequence of the 17-mer PNA (L7) was as follows:

Bio-link-ACA ATT GCC GAA ACA AT-CONH<sub>2</sub>

L8. A 19-mer PNA sequence labelled with biotin at the amino-terminal end.

35 The base sequence of the 19-mer PNA (L8) was as follows:

Bio-link-TTC AAC TCT GTG AGT TGA A-CONH<sub>2</sub>

- 5 H10. A PNA/RNA complex comprising a 19-mer PNA sequence (L8) and a 19-mer RNA sequence (U9) with a base sequence complementary to the PNA sequence. The PNA strand of this complex is labelled with biotin at the amino-terminal end.

The base sequence of the 19-mer PNA (L8) was as follows:

Bio-link-TTC AAC TCT GTG AGT TGA A-CONH<sub>2</sub>

The base sequence of the 19-mer RNA (U9) was as follows:

- 10 5'-UUC AAC UCA CAG AGU UGA A-3'

- 15 H22. A PNA/DNA complex comprising a 19-mer PNA sequence (L8) and a 19-mer DNA sequence (U26) with a base sequence complementary to the PNA base sequence. The PNA strand of this complex is labelled with biotin at the amino-terminal end.

The base sequence of the 19-mer PNA (L8) was as follows:

Bio-link-TTC AAC TCT GTG AGT TGA A-CONH<sub>2</sub>

The base sequence of the 19-mer DNA (U26) was as follows:

- 20 5'-TTC AAC TCA CAG AGT TGA A-3'

- 25 H29. A PNA/DNA complex comprising a 15-mer PNA sequence (U27) and a 30-mer DNA sequence (L11). The 30-mer DNA sequence is labelled with biotin in the 3'-end. The PNA sequence is complementary to the central part of the DNA sequence resulting in single-stranded DNA overhangs both 5'- and 3'- to the PNA/DNA complex.

The base sequence of the 30-mer DNA (L11) was as follows:

5'-GCT GAC GTT CCG CAC ATG TCA ACC ATA TGT-link-Bio-3'

The base sequence of the 15-mer PNA (U27) was as follows:

- 30 H-link-GTT GAC ATG TGC GGA-CONH<sub>2</sub>.

- H30. A PNA/DNA complex comprising a 45-mer DNA sequence (L12) and 3 units of a 15-mer PNA sequence (U13) wherein biotin is attached to the 5'-end of the 45-mer DNA sequence.

The base sequence of the 45-mer DNA (L12) was as follows:

Bio-link-TCC GCA CAT GTC AAC TCC GCA CAT GTC AAC TCC GCA  
CAT GTC AAC-3'.

The base sequence of the 15-mer PNA (U13) was as follows:

H-GTT GAC ATG TGC GGA-CONH<sub>2</sub>.

5

H31. A PNA/DNA complex comprising a 45-mer DNA sequence (L13) and 3 units  
of a 15-mer PNA sequence (U13) wherein biotin is attached to the 3'-end of  
the 45-mer DNA sequence.

The base sequence of L13 is identical to the base sequence of L12 above.

10

Thus the sequence of L13 is as follows:

5'-TCC GCA CAT GTC AAC TCC GCA CAT GTC AAC TCC GCA CAT  
GTC AAC-link-Bio-3'.

The sequence of the 15-mer PNA (U13) was as follows:

H-GTT GAC ATG TGC GGA-CONH<sub>2</sub>.

15

H32. A PNA/PNA complex (dsPNA) comprising two 17-mer PNA sequences, L7  
and U28, wherein biotin is attached to the 5'-end of L7.

The base sequence of L7 is as follows:

Bio-link-ACA ATT GCC GAA ACA AT-CONH<sub>2</sub>.

20

The base sequence of U28 is as follows:

H-ATT GTT TCG GCA ATT GT- CONH<sub>2</sub>.

dsDNA. Apart from the different single-stranded PNA or DNA sequences and the  
complexes indicated above, also double-stranded DNA (dsDNA) comprising  
25 fragments of calf thymus DNA (Sigma D-1501; converted to fragments  
comprising from 200 to 1000 bp) have been used to test the specificity of  
these monoclonal antibodies.

The PNA/nucleic acid complexes are prepared by, in a suitable buffer (e.g. 50 mM  
30 Tris-HCl, pH 7.6, 50 mM NaCl), mixing the nucleic acid with PNA having a base  
sequence that is complementary to all or a part of the nucleic acid sequence, heating  
the mixture to form single-stranded molecules and allowing the mixture to cool slowly  
to room temperature.

35

Complex formation was characterized as described below

T<sub>m</sub> determinations: T<sub>m</sub> measurements of PNA/DNA and PNA/RNA complexes were performed in a Lambda 2S UV/VIS spectrometer (Perkin Elmer) equipped with a "cell holder" with a heating facility (Peltier heating element).

- 5 For the preparation of a final amount of 2 nmol complex in 3 mL 50 mM Tris-HCl, pH 7.6, 50 mM NaCl buffer, a suitable amount of each strand and the buffer are mixed in a 3.6 mL NUNC CryoTube. The mixture is heated to 95°C for 10 minutes and the allowed to cool slowly to room temperature (3 to 4 hours). Approximately 2.8 mL is transferred to a 3 mL quartz cuvette with a lid and a stirring magnet. The
- 10 temperature of the cuvette is increased at a speed of 0.2°C/minute, starting at 20°C and ending at 95°C. Absorbance at 260 nm is measured continuously. The T<sub>m</sub> value is determined as the top point of the first derivative of the melting curve.

- Acrylamide gel electrophoresis: The complex formation was also tested by running
- 15 the complexes in a 20% polyacrylamide gel in TBE buffer (89 mM Tris-borate, 2 mM EDTA). The complexes were transferred to Nytran 13N filter paper (Schleicher & Schuell). Complexes were visualised in accordance with the label of the complex. Complexes containing either a biotin or a fluorescein label were visualised using alkaline phosphatase (AP) conjugated streptavidin or anti fluorescein antibody,
- 20 respectively. Unlabelled complexes were visualised either directly in the polyacrylamide gel by staining with ethidium bromide or by use of a polyclonal PNA/nucleic acid antibody as described in WO 95/17430 followed by a secondary antibody, e.g. swine anti-rabbit/AP. Bound AP conjugates were visualised using the chromagen mixture NBT/BCIP.

25

Dot blot: A dilution row of a complex (from 20 ng to 2 ng per dot) was spotted onto a Nytran 13N filter paper (Schleicher & Schuell). Visualisation were performed as described above.

## 30 EXAMPLE 2

### **Production of monoclonal antibodies**

#### Preparation of immunogen and immunization

The antigen used for immunization, the PNA/DNA complex H12, was prepared by mixing the following in a total volume of 2 mL:

35

0.939 mg the 45-mer polydeoxyribonucleotide (DNA)

1.145 mg the 15-mer PNA sequence

50 mM Tris-HCl, pH 7.5

50 mM NaCl

5

This mixture was heated to 92°C in a heating block and allowed to cool slowly to room temperature. The final concentration of the PNA/DNA complex was 1.04 mg/mL. Complex formation was tested as described in Example 1.

- 10 To the PNA/DNA complex prepared at a concentration of 1.04 mg/mL ovalbumin was added to a concentration of 250 mg/mL. This mixture was further mixed with Freund's incomplete adjuvant at a ratio of 1:1 v/v and was used for immunizing female BALB/c mice intraperitoneally or subcutaneously five times with an interval of approximately three weeks.

15

#### Production of hybridomas

- Three days before the fusion was planned the mouse was immunized intraperitoneally without adding adjuvant to the mixture of the PNA/DNA complex and ovalbumin. The fusion was carried out using standard fusion procedures with spleen cells from the immunized mouse and the myeloma cell line P3-X63-Ag.8 as fusion partner.
- 20

#### Screening of hybridomas:

- Initial screening took place 14 days after the fusion and 50 clones were selected as giving a positive signal in an ELISA system as described below and using a H2 complex in the second layer. H2 is a biotinylated version of the PNA/DNA complex used for immunization. Positive clones were cultivated and supernatants were re-tested in the ELISA systems described below. Clonality was ensured by limited dilution of the cells followed by screening of the supernatants in ELISA. Hybridomas were grown under standard conditions in RPMI 1640 medium supplemented with 10% foetal calf serum.
- 25
- 30

#### Test of specificity:

- Identification of specific antibody activity was based on results obtained with different PNA/nucleic acid complexes. Microtiter plates (MaxiSorp, Nunc) coated with streptavidin were incubated with biotinylated PNA/DNA complexes at a concentration
- 35

of 100 ng/ml in THT, for one hour at room temperature. After washing with THT (Denley WeWash 4) supernatant from the hybridomas or dilutions of these in 10% foetal calf serum were added to separate wells, and incubated as a standard for 2 hours at room temperature. The supernatants were diluted 2 fold as follows 1:2, 1:4, 5 1:8, 1:16, 1:32, 1:64 and 1:128. Detection of binding antibodies were performed with HRP conjugated rabbit anti mouse IgG (DAKO) and OPD (o-phenylenediamine) (DAKO) according to the manufactures instructions. Detection reactions were stopped after 10-15 minutes with 1 M H<sub>2</sub>SO<sub>4</sub>, and finally the plates were read at OD<sub>492</sub> (Molecular Devices). All washings between different steps were done with 10 THT. The complexes/compounds used for testing the specificity are described in Example 1.

#### Test results

The supernatants were tested in the test systems described above. The supernatant 15 from two of the clones of fusion F86 (1B11 and 1G12) showed preferential binding to PNA/DNA complexes as shown in Table 1. The optical density at 492 nm obtained after diluting the supernatant 1:2 is shown in tests of supernatant using the test complexes/compounds shown and which are described in Example 1. The background values (measured by omitting the antibody) was below 0.150 and has 20 not been subtracted from the OD values given in Table 1. (nd denotes not determined).



TABLE 1

| Test complexes/<br>compounds | Number of bases in<br>the complex | supernatant from<br>clone F86 1B11 | Supernatant from<br>clone F86 1G12 |
|------------------------------|-----------------------------------|------------------------------------|------------------------------------|
| H2 (PNA/DNA)                 | 3×15 / 45                         | 3.609                              | 1.521                              |
| H3 (PNA/DNA)                 | 15 / 15                           | 0.144                              | nd                                 |
| H7 (PNA/DNA)                 | 20 / 20                           | 3.052                              | 1.805                              |
| H8 (PNA/DNA)                 | 17 / 17                           | 0.150                              | 1.136                              |
| H9 (PNA/DNA)                 | 17 / 17                           | 0.985                              | 0.517                              |
| H22 (PNA/DNA)                | 19 / 19                           | 0.210                              | nd                                 |
| H29 (PNA/DNA)                | 15 / 30                           | 0.861                              | nd                                 |
| H30 (PNA/DNA)                | 3×15 / 45                         | 0.215                              | nd                                 |
| H31 (PNA/DNA)                | 3×15 / 45                         | 0.172                              | nd                                 |
| H4 (DNA/DNA)                 | 15 / 15                           | 0.310                              | nd                                 |
| H20 (DNA/DNA)                | 3×15 / 45                         | 0.220                              | nd                                 |
| H32 (PNA/PNA)                | 17 / 17                           | 0.124                              | nd                                 |
| dsDNA                        |                                   | 0.106                              | 0.097                              |
| L4 (ssPNA)                   | 15                                | 0.318                              | 0.111                              |
| L5 (ssPNA)                   | 20                                | 0.239                              | 0.110                              |
| L7 (ssPNA)                   | 17                                | 0.338                              | 0.146                              |
| L8 (ssPNA)                   | 19                                | 0.208                              | nd                                 |
| L1 (ssDNA)                   | 45                                | 0.217                              | nd                                 |
| L2 (ssDNA)                   | 45                                | 0.142                              | nd                                 |
| L6 (ssDNA)                   | 17                                | 0.270                              | 0.075                              |
| L3 (ssDNA)                   | 15                                | 0.02                               | 0.061                              |
| H10 (PNA/RNA)                | 19 / 19                           | 0.168                              | nd                                 |

As shown in Table 1, the supernatants from the two clones F86 1B11 and F86 1G12 reacted with most of the PNA/DNA complexes tested, although with varying intensities. The base sequence of the PNA/DNA complexes tested varied as shown in Example 1 and the results obtained showed that the antibody response is not or  
5 only to a minor degree dependent on the base sequence. Only insignificant reaction was seen with single-stranded PNA or DNA or double-stranded DNA or PNA or PNA/RNA.

Thus, the present monoclonal antibodies have a high degree of specificity for  
10 PNA/nucleic acid complexes. The specificity of the epitope(s) recognized by the present antibodies appears to a high degree to be dictated by the conformation of the PNA/nucleic acid complex rather than by the specific base sequence of the PNA/nucleic acid complex.

15 The immunoglobulin class of the supernatants were tested in standard ELISA system for determination of the Ig class. The antibodies from both clones were found to be of the IgM type.

From another fusion of spleen cells from a mouse immunized as described in  
20 Example 2 and the fusion partner P3-X63-Ag.8, two clones F88 1C10 and F88 6E5 were obtained. The reactivity of these clones were in agreement with the results obtained with clone F86 1B11. These clones, F88 1C10 and F88 6E5, were also found to be of the IgM type.

25 Clone F86 1B11 has been deposited with ECACC on May 31, 1995 (deposit number ECACC 95053111.)

Although PNA comprising a N-(2-aminoethyl)glycine backbone has been used in the present work, this should not be taken as a limitation. It is expected that PNA with  
30 other types of backbone can be used in a similar way as long as the PNA is capable of forming stable complexes with nucleic acids.

The disclosure in Danish patent application No 718/95 from which this application claims priority are incorporated by reference.

## CLAIMS

1. Monoclonal antibody characterized in that it is capable of binding to complexes formed between PNA (Peptide Nucleic Acid) and nucleic acids.  
5
2. Monoclonal antibody according to claim 1, characterized in that it does not bind to single-stranded PNAs, double-stranded nucleic acids, or single-stranded nucleic acids.
- 10 3. Monoclonal antibody according to claim 2, characterized in that it binds to a complex formed between PNA and DNA, but not to PNA/ RNA complexes, double-stranded DNA, DNA/RNA-duplexes, single-stranded PNAs or single-stranded DNA.
- 15 4. Monoclonal antibody according to claim 3, characterized in that it is obtainable by immunizing a host animal with a PNA/DNA-complex, wherein the PNA has a backbone of N-(2-aminoethyl)glycine, fusing antibody producing cells from said animal with neoplastic cells to produce hybridomas, selecting hybridomas that produce an antibody that binds to complexes formed  
20 between PNA and DNA, culturing such hybridomas and optionally purifying the antibodies.
5. Monoclonal antibody according to claim 2, characterized in that it is obtainable by immunizing a host animal with a PNA/RNA-complex, wherein  
25 the PNA has a backbone of N-(2-aminoethyl)glycine, fusing antibody producing cells from said animal with neoplastic cells to produce hybridomas, selecting hybridomas that produce an antibody that binds to complexes formed between PNA and RNA, culturing such hybridomas and optionally purifying the antibodies.
- 30 6. Monoclonal antibody according to any of the claims 1 to 5 in detectably labelled form.
7. A hybridoma cell line capable of producing a monoclonal antibody according  
35 to any of the claims 1 to 5.

8. A hybridoma cell line capable of producing a monoclonal antibody according to claim 4, wherein the hybridoma cell line is ECACC 95053111.
- 5 9. Monoclonal antibody obtainable from a hybridoma cell line according to claim 7 or 8.
- 10 10. Use of a monoclonal antibody according to any of the claims 1 to 6 or 9 for detection of a PNA/nucleic acid complex formed between a particular nucleic acid sequence to be detected in a sample and PNA capable of forming a complex with said particular nucleic acid sequence.
- 15 11. A kit for detecting a particular nucleic acid sequence in a sample, said kit containing antibody according to any of the claims 1 to 6 or 9, a PNA sequence that is capable of forming a complex with the nucleic acid sequence to be detected and a visualisation system.

# INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

|                                                                                                                                                                                   |                                    |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------|
| <b>A. The indications made below relate to the microorganism referred to in the description</b><br>on page <u>24</u> , line <u>25</u>                                             |                                    |
| <b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>                            |                                    |
| Name of depositary institution<br>ECACC - European Collection of Animal Cell Cultures                                                                                             |                                    |
| Address of depositary institution (including postal code and country)<br><br>CAMR<br>Porton Down<br>Salisbury<br>England                                                          |                                    |
| Date of deposit<br>31 May 1995 (31.05.95)                                                                                                                                         | Accession Number<br>ECACC 95053111 |
| <b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span> |                                    |
|                                                                                                                                                                                   |                                    |
| <b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)                                                                 |                                    |
|                                                                                                                                                                                   |                                    |
| <b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)                                                                                                      |                                    |
| The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")              |                                    |
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# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/DK 95/00485

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C07K16/44 C12N5/20 G01N33/577

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)  
IPC 6 C07K C12N 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 practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                | Relevant to claim No. |
|------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Y          | EP,A,0 135 159 (CETUS CORPORATION) 27<br>March 1985<br>cited in the application<br>see page 12, line 24 - page 14, line 30<br>see claims<br>---                                                                                                                                   | 1-11                  |
| Y          | NATURE,<br>vol. 365, no. 6446, 7 October 1993 LONDON,<br>GB,<br>pages 566-568,<br>M. EGHOLM ET AL. 'PNA hybridizes to<br>complementary oligonucleotides obeying the<br>Watson-Crick hydrogen-bonding rules.'<br>cited in the application<br>see the whole document<br>---<br>-/-- | 1-11                  |

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in 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 document 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

29 March 1996

Date of mailing of the international search report

22.04.96

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Nooij, F

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/DK 95/00485

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                              | Relevant to claim No. |
|------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
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