Method for detecting carcinomas in a solubilized cervical body sample

The present invention relates to a method for the early diagnosis of carcinomas and their preliminary stages, which comprises determining the overexpression of a cell cycle regulatory protein in a solubilized body sample. The present invention is particularly directed to a method for detecting cervical carcinomas, cervical intraepithelial neoplasias, or cervical carcinomas in-situ from a solubilized cervical body sample of a human subject, by solubilizing the cervical body sample in a lysis buffer, and determining the overexpression of cyclin dependent kinase inhibitor p16 in the solubilized cervical sample. The invention also concerns a test kit usable for this purpose as well as an in-vitro diagnostic device.
Description

[0001] The present invention relates to a method for the early diagnosis of carcinomas as well as their preliminary stages, particularly carcinomas of the upper respiratory tract or the anogenital tract, from a solubilized body sample.

BACKGROUND OF THE INVENTION

[0002] Preventive programs have been offered for the most differing carcinomas since the middle of the fifties. Regarding cervical carcinomas, they are based mainly on the morphological and cytological examination of cytosmears of the cervix uteri, what is called the Pap test, which is made on the basis of gynecological routine examinations at regular intervals in women from the 20th year on. By means of the morphology of the cells, the smears are divided into various intensity degrees of dysplastic cellular changes. According to Pap I-V, these intensity degrees are referred to as normal, mild dysplasia, fairly serious dysplasia, serious dysplasia and invasive carcinoma, respectively. If the Pap test leads to a striking result, a small biopsy will be taken and subjected to a histopathologic examination, by which the kind and intensity of the dysplasia are determined and classified as cervical intraepithelial neoplasia (CINI-III).

[0003] In spite of all preventive programs, cervical carcinomas that lead to 400,000 new cases per year are the most frequent carcinomas in women. This is inter alia due to the fact that up to 30 % of the results of the Pap test are false-negative.

[0004] In conventional screening for cervical carcinoma, swabs are used for detection of neoplastic lesions of the cervix uteri. In the screening procedure, different kinds of lesions have to be distinguished. Causes for lesions may for example be inflammations (due to infectious agents or physical or chemical damage) or preneoplastic and neoplastic changes. In morphological examinations the lesions of different characteristics are sophisticated to distinguish. Thus, for examination of swabs cytologists and pathologists have to be especially trained, and even experienced examiners have a high inter- and intra-observer variance in the assessment of a diagnosis based on cytological specimens. In general, the result of the examination is based upon the subjective interpretation of diagnostic criteria by the examining pathologist/cytologist. As a result, the rate of false positive and false negative results in the screening tests remains unsatisfying high.

[0005] However, the reproducibility of the examination results may be enhanced by the use of supporting molecular tools. Yet the problem with the preservation and preparation of the samples may not be overcome by just additionally using molecular markers. One further complication when performing cytological or histological examinations for screening purposes and especially when applying methods for the detection of molecular markers originates from strict precautions in preserving the samples from causing artefacts or improper results.

[0006] This is in part due to the instability of the cell-based morphological information and in part to the instability of the molecular markers to be detected during the tests. If the samples are not prepared, transported or stored in an appropriate manner, the cell-based information, or even the molecular information may be lost, or may be altered. So the diagnosis may be impossible, or may be prone to artefacts. For example, the interpretation of biopsies or cytological preparations is frequently made difficult or impossible by damaged (physically or bio-/chemically) cells. Furthermore regarding tissue samples or biopsies, the preservation of molecular constituents of the samples, which are subject to a rapid turnover, is sophisticated due to the time passing by until penetration of the total sample by appropriate preservatives.

[0007] As shown above, the morphologically supported diagnostic methods performed routinely in the art show two major disadvantages. Firstly, the methods are highly dependent on individual perception of the examiners. Secondly, the morphological information is quite sensitive to decay processes and thus to production of artefacts after preparation of the samples. Both aspects contribute to improper reproducibility of the results.

[0008] Therefore, it is the object of the present invention to provide a method by which cervical carcinomas can be diagnosed early and reliably. In addition, a differentiation should be possible by this method with respect to benign inflammatory or metaplastic changes from dysplastic preneoplasias. Moreover, the present invention provides methods for the detection of carcinomas on a biochemical basis from solubilized samples.

SUMMARY OF THE INVENTION

[0009] The present invention is directed to a method for detecting cervical carcinomas, cervical intraepithelial neoplasias, or cervical carcinomas in-situ from a solubilized body sample of a human subject. The method comprises the steps of: (a) obtaining a cervical body sample from a human subject, (b) solubilizing the cervical body sample in a lysis buffer, and (c) determining the overexpression of cyclin dependent kinase inhibitor p16 in the solubilized cervical sample by comparing the level of cyclin dependent kinase inhibitor p16 within said solubilized cervical sample with the level present in a solubilized healthy human cervical sample.

[0010] The present invention is also directed to an in-vitro diagnostic device comprising antibodies directed against...
cyclin dependent kinase inhibitor p16 fixed on solid carriers, for measuring p16 in a solubilized sample.

The present invention is further directed to a test kit for determining the level of cyclin dependent kinase inhibitor p16 comprising antibodies directed against cyclin dependent kinase inhibitor p16 and a lysis buffer for solubilization of a body sample.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the OD values returned in an ELISA test detecting the level of p16 in solubilized cervical samples; for experimental details see Example 1.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the applicant's insights that cell cycle regulatory proteins are overexpressed in many carcinomas, e.g. carcinomas of the upper respiratory tract or anogenital carcinomas, particularly cervical carcinoma, and preliminary stages of these carcinomas, respectively. Examples of the cell cycle regulatory proteins are cyclins. Cyclin-dependent kinases which regulate the cyclins are to be mentioned particularly. Cyclin-dependent kinase inhibitors which, in turn, regulate the cyclin-dependent kinases, are to be mentioned even more particularly. Examples of the cyclin-dependent kinase inhibitors are the proteins p14, p15, p16, p19, p21 and p27. The applicant has found that the intensity of cell cycle regulatory protein overexpression correlates with the degree of cell dysplasia.

According to the invention, the applicant's insights are used for a method for the early diagnosis of carcinomas and their preliminary stages, which comprises determining the overexpression of cell cycle proteins in a body sample.

According to the invention, cytological or histological examination procedures may be substituted by the use of molecular markers. Such markers may e.g. be used in immuno-histochemical staining reactions, or in the course of in-situ hybridization reactions. Combinations of morphological examinations and immuno-histochemical staining reactions based on marker molecules, characteristic for carcinoma of the cervix uteri, may lead to enhanced results. The morphologic examination remains laborious and time consuming and thus expensive, even when supported by the molecular methods, that make the results more reliable. Additionally, the diagnosis on a morphologically cell based level is, even when supported by molecular parameters, subject to individual perception of the morphology by individual examiners. Thus the diagnosis is dependent on the person, that performs the examinations.

The inventors moreover could show that in specific cases molecular markers may be used as diagnostic tools without further support by cell based morphological examinations. Methods for diagnosis of carcinomas on a molecular level only, without the support of cell based information, are restricted to cases, where markers or levels of markers are non-ambiguously specific for the condition to be characterized. This is especially true, if the markers are non-human substances. For example detection of viral infections may be carried out in solutions of samples, because the markers characteristic for the presence of viruses in tissues do not occur in unaffected human tissues.

However, the inventors found that human cyclin dependent kinase inhibitor p16 may serve as a marker for carcinomas in biochemical marker based detection procedures although it is a cell cycle regulatory protein being expressed at low levels in any normally proliferating human cell in certain stages of the cell cycle.

"p16" or "cyclin dependent kinase inhibitor p16" as used herein refers to cyclin dependent kinase inhibitor p161NK4a (also denominated as CDKN2 or MTS1) the gene of which is located in chromosomal region 9p21. p16 was first described in Serrano, M., et al., Nature, 1993 Dec 16; 366(6456):704-7. The terms "p16" or "cyclin dependent kinase inhibitor p16" in all their grammatical forms as used in the context of the present invention refers to nucleic acid as well as polypeptide molecules. "p16" or "cyclin dependent kinase inhibitor p16" thus comprises e.g. RNA (mRNA, hnRNA, etc.), DNA (cDNA, genomic DNA, etc.), proteins, polypeptides, proteoglycans, glycoproteins and the respective fragments of these molecules.

The level of p16 refers to a semiquantitative as well as a quantitative value regarding the amount of the p16 present in a sample. A quantitative value may e.g. be represented in terms of a concentration. A semiquantitative value may be expressed in terms of a scale of levels e.g. undetectable levels, low levels, intermediate levels, high levels or any other suitable mode. The level of p16 may also be represented in terms of a dependent parameter such as the intensity of a signal generated in an assay format in response to the presence of p16. In certain embodiments the level of p16 may also refer to a qualitative determination of the presence of p16.

Due to the expression of cyclin dependent kinase inhibitor p16 in certain benign cell types present in cervical specimens, the diagnosis of dysplasias based on the level of cyclin dependent kinase inhibitor p16 without additional information on the cellular morphology seem to be difficult or impossible. It was known in the art that up to 30% of metaplastic cells, which may be present in cervical swabs, are immunoreactive for cyclin dependent kinase inhibitor p16 at a moderate to high level. Moreover, endometrial cells that may under certain circumstances be present in cervical swabs are positive for p16. In cytological or histological testing procedures, this fact does not influence the diagnosis, because the cell types may easily be distinguished from dysplastic cells with respect to their cellular morphology. (one
Appendix E

According to the present invention, the body samples may be solubilized in any suitable solvent. Such solvents may for example be aqueous solutions of chaotropic agents such as e.g. urea, GuaSCN, Formamid, of detergents such as anionic detergents (e.g. SDS, N-lauryl sarcosine, sodium deoxycholate, alkyl-aryl sulphonates, long chain (fatty) alcohol sulphates, olefine sulphates and phosphonates, alpha olefine sulphates and phosphonates, sulphated monoglycerides, sulphated ethers, sulphosuccinates, alkane sulphonates, phosphate esters, alkyl isethionates, sucrose esters), cationic detergents (e.g. cetyl trimethylammonium chloride), non-ionic detergents (e.g. Tween 20, Nonidet P-40, Triton X-100, NP-40, Igepal CA-630, N-Octyl-Glucosid) or amphoteric detergents (e.g CHAPS, 3-Dodecyl-dimethylammonio-propane-1-sulfonate, Lauryldimethylamine oxide) and/or of alkali hydroxides such as e.g. NaOH or KOH. Generally any suitable liquid may be used as a solvent in the lysis buffer of the present invention. The liquid may
be organic or inorganic and may be a pure liquid, a mixture of liquids or a solution of substances in the liquid and may contain additional substances to enhance the properties of the solvent. In certain embodiments, where lysis of cells may be achieved without the use of detergents, hyper- or hypotonic solutions or buffers or simply water or an organic liquid may be used as solvent. Any liquid, that is suited to solubilize the cellular components of body samples in total or in parts may be regarded as a lysis buffer as used herein. Thus lysis buffers as used herein need not contain buffer substances or have buffer capacity.

[0029] In one embodiment, the solvent is designed, so that cells, cell debris, nucleic acids, polypeptides, lipids and other biomolecules potentially present in the raw sample are dissolved. In further embodiments of the present invention, the solvent may be designed to assure differential lysis of specific components of the body sample, leaving other components undissolved.

[0030] The solution for dissolving the body sample according to the present invention may furthermore comprise one or more agents that prevent the degradation of components within the raw samples. Such components may for example comprise enzyme inhibitors such as proteinase inhibitors, RNAse inhibitors, DNAse inhibitors, etc. In one embodiment of the present invention, the sample is lysed directly in the form obtained from test-individuals. Proteinase inhibitors may e.g. comprise inhibitors of serine proteinases, inhibitors of cysteine proteinases, inhibitors of aspartic proteinases, inhibitors of metalloproteinases, inhibitors of acidic proteinases, inhibitors of alkaline proteinases or inhibitors of neutral proteinases.

[0031] Further preparations of the sample, such as separation of insoluble components, preserving and transporting of the cells. Thus the cellular components of the raw samples are included in a single sample solution.

[0032] The preparation of a sample for use in a method as disclosed herein may also comprise several steps of further preparations of the sample, such as separation of insoluble components, isolation of polypeptides or nucleic acids, preparation of solid phase fixed peptides or nucleic acids or preparation of beads, membranes or slides to which the molecules to be determined are coupled covalently or non-covalently.

[0033] The expression "determining the overexpression of cell cycle regulatory proteins" comprises any methods which are suited for detecting the expression of cell cycle regulatory proteins or their encoding mRNAs and an amplification of the corresponding genes, respectively. In order to determine an overexpression, the body sample to be examined is compared with a corresponding body sample which originates from a healthy person. Such a sample can be present in a standardized form.

[0034] The comparison with normal healthy body samples may be achieved by different methods. In one embodiment of the present invention, the comparison may be performed directly by including a control reaction with non-diseased tissue or cell sample. This non-diseased tissue or cell samples may be provided from a healthy person or from non-diseased regions of the human subject under examination or from cell culture cells known to show the properties of non-diseased cells with respect to cyclin dependent kinase inhibitor p16 expression. In another embodiment, the comparison may be performed indirectly by comparing the level of cyclin dependent kinase inhibitor p16 within the sample under investigation to a level of cyclin dependent kinase inhibitor p16 known to be present in normal healthy samples. The knowledge about the level for normal healthy tissue or cell samples may be derived from a representative number of testing or from scientific publication providing information the expression level of cyclin dependent kinase inhibitor p16 in normal healthy cells. Comparison may be performed by employing a value for the concentration of the p16 protein or nucleic acids; otherwise a characteristic value depending on the protein or nucleic acid concentration such as the optical density under defined reaction conditions may be employed. Otherwise the known value may be represented by a surrogate control such as a peptide or a recombinant protein. Thus the level of p16 present in normal healthy samples may be represented by a control sample of a recombinant protein or a peptide in the testing procedure.

[0035] The level of p16 in a healthy human cervical sample can be determined from a standardized sample solution. A standardized sample solution may comprise a solution of a solubilized pool of normal cell or normal tissue samples. The sample pool may, e.g., be a pool of cytological specimens with pre-assessed normal diagnosis from a screening population, or a pool of normal cells obtained from histological specimens. Furthermore, a pool of normal cells may be obtained from tissue culture of normal cervical epithelial cells. The sample solution may, e.g., be standardized with respect to the content of cells per ml sample solution. Any other parameter for standardization may be applied. The sample solution may e.g. be provided in a standardized form to ensure stability and reproducibility of the test results. In certain embodiments such solution may be provided as a component of the kit for comparison or calibration purposes.

[0037] In certain embodiments, the step of comparing the level of cyclin dependent kinase inhibitor p16 present in
a patient sample to a level known to be present in a normal healthy body sample is embodied as employing a cut-off value or threshold value for the concentration of p16. The cut-off in this context is a value (e.g. for the concentration of p16 protein given in e.g. mg/ml or for the optical density measured under defined conditions in an ELISA test) which is suited to separate normal healthy samples from diseased samples. e.g. all samples giving values above the cut-off value are considered to be dysplastic, whereas the samples giving value below the cut-off value are considered to be healthy.

[0038] In certain embodiments, the threshold or cut-off may be set in a way to separate high grade dysplasias from all less severe stages of dysplasias. In other embodiments, the cut-off may be defined to differentiate healthy samples from dysplasias including precursory stages. It is thus possible to tailor the testing format in order to fit different tasks such as early detection of lesions and even precursors of the lesions or detection of lesions that deserve immediate therapy.

[0039] The (over) expression of cell cycle regulatory proteins can be detected on a nucleic acid level and protein level, respectively. Regarding the detection on a protein level, it is possible to use e.g. antibodies which are directed against cell cycle regulatory proteins. These antibodies can be used in the most varying methods such as Western blot, ELISA or immunoprecipitation. It may be favorable for the antibodies to be fixed on solid carriers such as ELISA plates, reaction vessels, beads, spheres, membranes, colloidal metals (e.g. gold), porous members, surfaces of capillaries (e.g. in flow through test), test strips or latex particles.

[0040] In certain embodiments of the present invention, the detection of the marker molecules is performed from a solution of dissolved body samples. Therefore detection may be carried out in solution or using reagents fixed to a solid phase.

[0041] A solid phase as used in the context of the present invention may comprise various embodiments of solid substances such as planar surfaces, particles (including micro-, nano-particles or even smaller particles). In certain embodiments, particles may be provided as spheres, beads, colloids, or the like.

[0042] The fixation of reagents to the solid phase in a test kit or an in-vitro diagnostic device may be carried out via direct fixation or via indirect fixation. Direct fixation may be carried out by covalent binding, non-covalent binding, association, or adsorption to surfaces. Indirect fixation may be carried out through binding of the antibody to agents which themselves are directly fixed to solid phases. Binding agents, for example, include avidin, streptavidin, biotin, digoxigenin, antibodies or the like.

[0043] The detection of one or more molecular markers may be performed in a single reaction mixture or in two or more separate reaction mixtures. The detection reactions for several marker molecules may for example be performed simultaneously in multi-well reaction vessels. The detection reaction for marker molecules may comprise one or more further reactions with detecting agents either recognizing the initial marker molecules or preferably recognizing the prior molecules (e.g. primary antibodies) used to recognize the initial markers. The detection reaction further may comprise a reporter reaction indicating the level of the markers characteristic for cell proliferative disorders or the normalization markers.

[0044] The detection reaction for detecting the level of cyclin dependent kinase inhibitor p16 in solubilized samples may be carried out in solution or with reagents fixed to solid phases. In certain embodiments, the detection reaction may be carried out in solution; such procedures may comprise any methods suited for the detection of molecular interactions (binding of an antibody or similar binding agent to an antigen) in solution. The methods for determination of molecular interaction (change in conductivity, mass changes, light-, UV-, IR-, magnetic spectrometric changes, plasmon resonance, etc.) are known to those of skill in the art. In certain embodiments the detection may comprise a method where a complex of detection reagent bound to antigen is adsorbed to a solid phase for detection purpose. Thus, non-covalent bonding of the analytes to solid phases in the course of the detection reaction or even before starting the detection reaction may be used in a method according to the present invention.

[0045] A probe for the detection of the marker molecules may be any molecule, that specifically binds to said marker molecules. The probe may for example be an antigen binding agent such as antibodies (monoclonal or polyclonal), antibody fragments or artificial molecules comprising antigen binding epitopes, DNA or RNA binding molecules such as proteins or nucleic acids. Nucleic acids binding to other nucleic acids may for example be oligonucleotides for detection purposes or primers. A molecule is said to recognize another molecule if it specifically interacts with that molecule. Specific interaction may for example be specific binding to or of the other molecule. The term “antibody” in all its grammatical forms shall in the context of the present invention refer generally to antigen binding molecules including but not limited to monoclonal and polyclonal antibodies, fragments of antibodies, antigen binding epitopes, mini-antibodies, peptidomimetics with antigen-binding properties, anticalines and diabodies.

[0046] The reporter reaction may be any event producing a signal in response to the presence of the marker or to the binding of a specific probe to the marker. For example, a reaction producing a colored compound, a fluorescent compound, a light emitting compound, a radiation emitting compound, or the concentration of one or more of these compounds to a detectable concentration in a predefined area of a testing device may serve as reporter reaction. (one sentence deleted as it was not applicable in the present invention)
Applicable formats for the detection reaction according to the present invention may be blotting techniques, such as Western-Blot, Southern-blot, Northern-blot. The blotting techniques are known to those of ordinary skill in the art and may be performed for example as electro-blots, semidry-blots, vacuum-blots or dot-blots. Furthermore immunological methods for detection of molecules may be applied, such as for example immunoprecipitation or immunological assays, such as EIA, ELISA, RIA, FIA (fluorescent immunoassay) lateral flow assays (using porous members or capillaries), immunochromatographic strips, flow through assays, latex agglutination assays etc. In nucleic acid based approaches hybridisation or amplification techniques may be applied.

Immunooassays for use in the invention may comprise competitive as well as non-competitive immunoassays, such as sandwich assays.

In certain embodiments of the invention, immunochemical or nucleic acid based testing may be performed using a testing device for clinical laboratories. Such testing device may comprise any device suitable for immunochemical or nucleic acid based testing including any format such as point of care testing devices as well as bench top or laboratory devices. The devices may be e.g. provided as open or closed platform systems. The system may be based on any suitable methodology such as microtiter plates, multwell plates, flow through or lateral flow systems, microchip or array based systems or bead or membrane based systems. The detection methods employed may comprise any methods known to those of skill in the art useful for immunochemical or nucleic acids based detection reactions. Such detection systems may be e.g. luminescence systems (electroluminescence, bioluminescence, photoluminescence, radioluminescence, chemiluminescence, electrochemoluminescence), fluorescence based systems, conductivity based detection systems, radiation (light, UV, X-ray, gamma etc.), plasmon resonance (e.g. Surface Plasmon Resonance SPR) or any other known method.

The term porous member as used herein shall generally apply to any three dimensional arrangements of porous substances. Such porous member may e.g. comprise compounds as membranes, beads or other.

By means of the present invention it is possible to diagnose carcinomas early, i.e. in their preliminary stages.

A further subject matter of the present invention relates to a kit for carrying out a method according to the invention. Such a kit comprises e.g.:

(a) a reagent for detecting the expression of a cell cycle regulatory protein, e.g. an antibody directed against such a protein or a nucleic acid coding for such a protein and parts thereof, respectively,

(b) a lysis buffer for solubilization of the body sample,

(c) conventional auxiliary agents, such as buffers, carriers, markers, etc., and optionally

(d) an agent for control reactions, e.g. a cell cycle regulatory protein, a nucleic acid coding for such a protein and parts thereof, respectively, or a preparation of cells.

Furthermore, one or several of the individual components may be present. For example, the detection reagent and as other reagents fixed to a solid phase may be present.

Generally, the lysis buffer may be any suitable solvent known to those of skill in the art. The lysis buffer for use in the kit may, for example, be organic or aqueous solutions of chaotropic agents such as e.g. urea, GuaSCN, Formamid, of detergents such as anionic detergents (e.g. SDS, N-lauryl sarcosine, sodium deoxycholate, alkyl-aryl sulphonates, long chain (fatty) alcohol sulphates, olefine sulphates and sulphonates, alpha olefine sulphates and sulphonates, sulphated monoglycerides, sulphated ethers, sulphosuccinates, alkane sulphonates, phosphate esters, alkyl isethionates, sucrose esters), cationic detergents (e.g. cetyl trimethylammonium chloride), non-ionic detergents (e.g. Tween 20, Nonidet P-40, Triton X-100, NP-40, Igepal CA-630, N-Octyl-Glucosid) or amphoteric detergents (e.g CHAPS, 3-Dodecyl-dimethylammonio-propane-1-sulfonate, Lauridymethylamine oxide) and/or of alkali hydroxides such as e.g. NaOH or KOH. In certain embodiments, where lysis of cells may be achieved without the use of detergents, hyper- or hypotonic solutions or buffers or simply water or an organic liquid may be used as solvent. Any liquid, that is suited to solubilize the cellular components of body samples in total or in parts may be regarded as a lysis buffer as used herein. Thus lysis buffers as used herein need not contain buffer substances or have buffer capacity.

To obtain optimal results of the assay, the pH of a lysis buffer that can be directly applied to the assay system is around neutral. The pH of the lysis buffer is within the range of 4 to 10. In certain embodiments, the pH is in a range from 5 to 9. In a preferred embodiment, the pH is in a range from 6 to 8. In a more preferred embodiment, the pH is in the range from 6.5 to 7.5.

Examples of Lysis Buffers may for example be selected from the compositions given in Table 1.
In certain situations, the cyclin dependent kinase inhibitor p16 can be degraded in the solubilized samples and may thus not be detected. This is particularly true, if the samples are directly transferred to a lysing medium and stored therein for a certain period of time. To prevent degradation, lysis buffer may furthermore comprise one or more agents that prevent the degradation of components within the raw samples. Such components may for example comprise enzyme inhibitors such as proteinase inhibitors, RNAse inhibitors, DNAse inhibitors, etc. The inhibitors may e.g. comprise proteinase inhibitors selected from the compositions given in Table 2.

### Table 2:

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>class of inhibited protease</th>
<th>concentration</th>
<th>Solubility in water</th>
<th>stability in water</th>
<th>p16 stabilization in mtm lysis buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>Serine</td>
<td>0.6-2 µg/ml</td>
<td>Very good</td>
<td>good</td>
<td>no</td>
</tr>
</tbody>
</table>
For stabilization purpose, the lysis buffer may also comprise bulk protein (e.g. albumin such as bovine serum albumin or calf serum albumin or other bulk proteins) to compete in degradation with the sample proteins. The bulk proteins may e.g. be present in combination with proteinase inhibitors or may be added instead of proteinase inhibitors. In one embodiment, the solvent may be selected to be compatible with the assay (e.g. ELISA) performance, so that solubilized samples may directly be applied to the assay.

In some embodiments of the present invention, the lysis buffer may be tailored in order to enable for the setting of a specific cut-off value.

One aspect of the invention relates to an in-vitro diagnostic device. An in-vitro diagnostic device according to the present invention is characterized by solid phase fixed detection reagents specific for a cyclin dependent kinase inhibitor. In one embodiment, the detection reagents are specific for cyclin dependent kinase inhibitor p16.

In the art, there are some in-vitro diagnostic devices employing reagents for the detection of cyclin dependent kinase inhibitor p16 in histological or cytological specimens. These in-vitro diagnostic devices are cell-based detection devices that detect the p16 antigen in cells or tissues, not in solubilized samples. p16 being an intracellular antigen, it may only be accessible to detection reagents in solution after permeabilization of cells. Thus, the in-vitro diagnostic application of reagents for detection of cyclin dependent kinase inhibitor p16 known in the art excludes the fixation of the detection reagents to a solid phase. The art have not taught the design of test kits or in-vitro diagnostics containing p16-fixed solid phase detection reagents. An approach for assessing diagnosis on the basis of solubilized samples seemed not viable from the art and has not been suggested before.

It is thus an aspect of the present invention to provide an in-vitro diagnostic device comprising probes directed against cyclin dependent kinase inhibitor p16\textsuperscript{NK4a} fixed to a solid phase allowing assessment of diagnosis of carcinomas and their precursor lesions in a solubilized sample. In certain embodiments of the present invention, the probes may e.g. comprise antibodies or fragments thereof directed against p16\textsuperscript{NK4a} protein. It is an advantage of the in-vitro diagnostic devices of the present invention to allow for easy and economic assessment of diagnosis of carcinomas and their precursor lesions. The test may be suited for screening purposes as well as for diagnostic purposes and may be applied in primary diagnosis as well as in monitoring of disease course. The in-vitro diagnostic devices may in certain embodiments be applicable for use in clinical laboratories, for point of care testing or even for self testing.

The in-vitro diagnostic devices comprising solid phase fixed reagents for the detection of cyclin dependent kinase inhibitor p16 may be useful for the detection of various different carcinoma-entities and their respective precursor lesions. The in-vitro diagnostic devices may be applied for analysis of any kind of lysed body samples.

The antibodies can be fixed to the solid phase via direct fixation or via indirect fixation. Direct fixation can be done by covalent or non-covalent binding or association to surfaces. Indirect fixation can be done through binding of the antibody to agents which themselves are directly fixed to solid phases. Such agents may comprise antibodies or...
other binding agents like avidin, streptavidin, biotin, digoxigenin or the like.

The in-vitro diagnostic devices envisaged in the invention are selected from the group consisting of

- an ELISA device comprising antibodies directed against cyclin dependent kinase inhibitor p16 fixed to ELISA plates, ELISA stripes or ELISA wells;
- a lateral flow test device, comprising antibodies directed against cyclin dependent kinase inhibitor p16 fixed to test strips, colloidal gold particles or latex particles;
- a flow through assay device, comprising antibodies directed against cyclin dependent kinase inhibitor p16 fixed to a porous member, or to the surface of capillaries;
- a latex agglutination assay device, comprising antibodies directed against cyclin dependent kinase inhibitor p16 fixed to latex particles; and
- an immunoassay device, comprising antibodies directed against cyclin dependent kinase inhibitor p16 fixed to beads, membranes, or microspheres.

The ELISA devices may be of any kind known to those of skill in the art. These devices comprise devices for sandwich ELISA formats, for competitive ELISA formats and any other ELISA formats.

Lateral flow assay devices for use as an in-vitro diagnostic device according to the present invention are any lateral flow assay devices employing at least one reagent binding to cyclin dependent kinase inhibitor p16 fixed to a solid phase. Such devices may employ various mechanisms for visualization of the test result. In certain embodiments, the tests may employ secondary detection reagents directed against cyclin dependent kinase inhibitor p16 or another component participating in the test couples to detectable moieties. The detectable moieties may comprise colloidal gold, (colored) latex particles and others.

Flow through assay devices for use in the present invention may comprise devices based on capillaries or on porous members (such as membranes, beads or other three dimensional arrangements of porous substances). Depending on the embodiment the size of pores or capillaries need to adjusted to ensure optimal flow conditions.

By means of the present invention, it is possible to diagnose carcinomas early. In particular, preliminary stages of carcinomas can be detected early. It must also be emphasized that it is possible to make a differentiation with respect to benign inflammatory or metaplastic changes of dysplastic preneoplasias. Another characteristic is that the results obtained by a method according to the invention are not subject to a subjective evaluation, so that e.g. false-negative results and false-positive results of a Pap test or histological preparations can be avoided. In addition, the present invention distinguishes itself by rapid and simple handling, so that it can be used for extensive screening measures, particularly also in third-world countries. Thus, the present invention represents an important contribution to today's diagnostics of cancerous diseases.

The invention is illustrated further by the following examples, which are not to be construed as limiting the invention in scope or spirit to the specific procedures described in them.

EXAMPLES

Example 1: Detection of Cervical Intraepithelial Neoplasia in an ELISA test format.

33 cervical swabs provided in a lysis buffer were subjected to ELISA based detection of overexpression of cyclin dependent kinase inhibitor p16 in solutions prepared from the cells contained in the swabs. The ELISA testing was performed as follows:

(A) Cell Lysis

Cervical swab brushes were given into 15 ml vessels, containing 2 ml of mtm lysis buffer (2% Triton X-100, 0.4% SDS, 0.6mM PMSF in PBS). Cervical cells present in the brush were lysed for at least 20h. The lysates of the cervical swab samples were then transferred in 2 ml tubes and were centrifuged at 4°C (15 min at 28,000 x g (16.600rpm HighspeedCentrifuge JEC Multi RF)); -Supernatant was transferred to a fresh tube. The supernatant may be stored at -20 °C.
(B) Performing the ELISA

Coating of ELISA-plates

[0074] Stock-solution of p16 specific antibody clone mtm E6H4 was diluted in PBS to give ready-to-use coating solution.

[0075] 50µl of the coating solution was added to each well of the ELISA plates.

[0076] For coating, the plates were incubated overnight at 4°C.

[0077] Coating solution was removed from the ELISA plates and the plates were rinsed using an automated ELISA washer as follows:

- 7 x 250µl washing buffer (0.1% Tween20 (v/v) in PBS)

after removing remnants of the washing buffer, 300µl blocking buffer (2% BSA in PBS) was added to each well. Plates were incubated for 1h on a rocking device at ambient temperature.

Incubation with samples

[0078] After removing the blocking buffer, 100µl of the lysed cell sample was added to each well. Lysates of HeLa-cells were used as positive control;

[0079] For purpose of calibration of the test, different concentrations of recombinant p16 protein (0 pg/ml, 50 pg/ml, 100 pg/ml, 200 pg/ml, 400 pg/ml, 800 pg/ml) were included in the test.

[0080] Samples were incubated for 1 h at room temperature.

[0081] Thereafter washing was performed on an automated ELISA washer as follows:

- 7 x 250µl washing buffer. The remaining buffer was removed.

Incubation with detection antibody

[0082] Working solution of biotinylated secondary antibody clone mtm D7D7 specific for p16 protein was prepared by dilution of stock solution.

[0083] 100µl of working solution was added to each well. After incubation for 1h at RT, antibody solution was removed and ELISA plates were washed by an automated ELISA washer

- 7 x with 250µl washing buffer.

Detection

[0084] Streptavidin-HRP-polymers (1mg/ml) were pre-diluted 1:10. (4µl +36µl incubation buffer); Final incubation solution was prepared by dilution 1:300 in incubation buffer (0,1% BSA in PBS) to a final concentration of 0,33 µg/ml.

[0085] 100µl of this solution were added to each well and incubated for 1 h at RT.

[0086] Thereafter, the buffer was removed and the plates were washed manually with 200 µl washing buffer per well 5 times.

Substrate incubation

[0087] TMB-substrate was equilibrated to 25°C for 1h in the dark.

[0088] 100µl of substrate solution was added to each well.

[0089] The ELISA plates were incubated at 25°C for exactly 15 min in the dark. Then the reaction was stopped by addition of 80 µl 2.5M H2SO4.

[0090] Within 5 min. after stopping the reaction, OD 450 nm was determined. After evaluation of the results, each sample returned a value for the OD.

[0091] Results of this experiment are given in Figure 3. The ELISA results were compared to the diagnostic results of a Papanicolaou test (PAP test, cervical cytology) from the same patients. The cervical cytology were evaluated according to the Munich Classification II (1990). Pap II encompasses benign cells, cervicitis and metaplasia, Pap IV encompasses severe dysplasia and carcinoma in situ. It turned out that samples returning an OD greater than 0.9 in the ELISA correspond to samples, that are classified as dysplastic by the conventional cytopathological PAP test.

[0092] Applying OD 0.9 as threshold for the evaluation of the samples, the ELISA results may be reported as follows:
The ELISA test is positive in all samples (100%) from women having severe dysplasia and is negative in all 30 samples (100%) of women having no dysplasia.

Using the threshold evaluated in these experiments, cytological specimens of 300 patients were tested in the presented ELISA testing format. In this experiments the specimens identified as being dysplastic by cytological examination may also be identified as dysplastic in the ELISA testing format.

The results show, that the quantification of p16 protein in solubilized patient samples allows to detect dysplasias from the samples. The diagnosis in the present example is based on the comparison of the level of p16 determined in a specific patient sample to the level known to be present in normal non-dysplastic samples. The comparison is carried out in the testing format by applying a threshold value for the OD determined in the ELISA above which the sample is to be classified as positive.

Example 4: Detection of Cervical Intraepithelial Neoplasia in an Lateral Flow test format.

Nine cervical swabs provided in PreservCyt (Cytyc Corporation Boxborough, Mass.) solution have been subjected to conventional PAP testing and simultaneously to lateral flow based detection of overexpression of cyclin dependent kinase inhibitor p16 in solutions prepared from the cell suspensions obtained from the swabs. The lateral flow testing was performed as follows:

(A) Cell Lysis

10 ml of the cell suspensions from the individual cervical swab samples provided as PreservCyt™ fixed materials were transferred to a 15 ml reaction vessel. The samples were centrifuged 15 min at ambient temperature at 1500 x g (3000rpm, Heraeus Varifuge, rotor 8074); supernatant was discarded, and remaining methanol allowed to evaporate (15 min at ambient temperature); the pellet was dissolved in 500 µl Lysisbuffer and transferred to a 1.5 ml reaction vessel. The solution was centrifuged at 4°C (15 min at 28000 x g (16600rpm Microcentrifuge Biofuge fresco)); Supematant was transferred to a fresh tube. Supernatant may be stored at -20°C.

(B) Performing the Lateral Flow Assay

Applying capture antibody to membrane

Stock solution of p16 specific antibody clone mtm E6H4 was diluted in TBS (containing 1% bovine serum albumin) to give ready-to-use spotting solution with a final concentration of 1 mg antibody/ml. The ready-to-use solution was spotted onto nitrocellulose membrane at 30 µl/30 cm. Whatman wicks were attached to one end of the nitrocellulose and dipsticks are dried for 1 hour at 37°C. Then they were allowed to equilibrate at room temperature and cut into 4mm width dipsticks.

Preparation of Conjugate solution

Stock-solution of p16 specific antibody clone mtm D7D7, conjugated to colloidal gold (40 nm particle size) was diluted in TBS (containing 1% bovine serum albumin) to give ready-to-use detection antibody solution with a final concentration of 1.0 OD at 520nm.

Incubation with samples

Then 20 µl of the lysed cell samples were added to 20 µl ready-to-use detection antibody solution in a microtiter well and mixed. Dipstick, coated with capture antibody clone E6H4 was added to the well, sample was soaked and run to completion. The signal was read while the dipstick is still wet.
Results

[0101] In our testing format, 2 samples (samples 1 and 2) classified as PAP IVa by PAP staining and therefore representing dysplastic cells, gave clearly visible purple bands in the area of spotted capture antibody. In contrast, no band was detected for the other 7 samples (samples 3-9), classified as PAP II-III by PAP staining and therefore representing normal cells.

[0102] ELISA was performed by the same protocols given in Example 1. The results are shown in Table 4.

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[0103] The invention, and the manner and process of making and using it, are now described in such, full, clear, concise and exact terms as to enable any person skilled in the art to which it pertains, to make and use the same. It is to be understood that the foregoing describes preferred embodiments of the present invention and that modifications may be made therein without departing from the scope of the present invention as set forth in the claims. To particularly point and distinctly claim the subject matter regarded as invention, the following claims conclude this specification.

Claims

1. A method for detecting cervical carcinomas, cervical intraepithelial neoplasias or cervical carcinomas in-situ from a solubilized sample of a human subject, the method comprises the steps of:
   (a) obtaining a cervical body sample from a human subject,
   (b) solubilizing the cervical body sample in a lysis buffer, and
   (c) determining the overexpression of cyclin dependent kinase inhibitor p16 in the solubilized cervical sample by comparing the level of cyclin dependent kinase inhibitor p16 within said solubilized cervical sample with the level present in a solubilized healthy human cervical sample.

2. The method according to Claim 1, wherein the level of cyclin dependent kinase inhibitor p16 in the healthy human cervical body sample is provided as a predetermined value to set up a threshold for the detection procedure.

3. The method according to Claim 1, wherein the level of cyclin dependent kinase inhibitor p16 in a healthy human cervical sample is determined from a standardized sample solution, or from a representative number of healthy human cervical samples.

4. The method according to any one of the Claim 3, and wherein the determination of the level of cyclin dependent kinase inhibitor p16 in a healthy human cervical sample is carried out:
   a. in the course of the detection procedure,
   b. upon calibration of the detection system,
   c. once for each lot of detection reagents, or
d. as a standard value for the detection method.
5. The method according to any one of the Claims 1 to 3, wherein the cervical body sample is swab, smear, aspirate, biopsy, preserved cytological specimen, histological specimen, fixed cell preparation or fixed tissue preparation.

6. The method according to any one of the Claims 1 to 5, wherein the cervical body sample is solubilized

   a. immediately after obtaining the sample,
   b. after storage and/or transport in a storage buffer, or
   c. after transport in a transportation buffer.

7. An in-vitro diagnostic device comprising antibodies directed against cyclin dependent kinase inhibitor p16 fixed on solid carriers, for detecting p16 in a solubilized sample.

8. The in-vitro diagnostic device according to Claim 7, which is selected from the group consisting of:

   a. an ELISA device comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to ELISA plates, ELISA stripes or ELISA wells;
   b. a lateral flow test device, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to test strips, colloidal gold particles or latex particles;
   c. a flow through assay device, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to a porous member, or to the surface of capillaries;
   d. a latex agglutination assay device, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to latex particles;
   e. an immunoassay device, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to beads or membranes; and
   f. an immunoassay device, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to microspheres.

9. A test kit for determining the level of cyclin dependent kinase inhibitor p16 comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 and a lysis buffer for solubilization of a body sample.

10. The test kit according to Claim 9, wherein the lysis buffer comprises at least one composition selected from the group consisting of chaotropic agents, anionic detergents, cationic detergents, non-ionic detergents, amphoteric detergents, and alkaline compositions.

11. The test kit according to Claims 9 or Claim 10, wherein the lysis buffer comprises at least one composition selected from the group consisting of a proteinase inhibitor, a DNAse inhibitor, and an RNAse inhibitor.

12. The test kit according to Claim 11, wherein the proteinase inhibitor is selected from the group consisting of inhibitors to serine proteinases, inhibitors to cysteine proteinases, inhibitors to aspartic proteinases, inhibitors to metallo proteinases, inhibitors to acidic proteinases, inhibitors to neutral proteinases, and inhibitors to alkaline proteinases.

13. The test kit according to any one of the Claims 9 to 12, wherein the lysis buffer comprises at least one non-ionic detergent and at least one proteinase inhibitor.

14. The test kit according to Claim 13, wherein the lysis buffer contains Triton X-100 and at least one inhibitor of serine proteinases.

15. The test kit according to any one of the Claims 9 to 14, further comprising at least one marker molecule for carrying out a positive control reaction, reagents and buffers commonly used for carrying out the detection reaction.

16. The test kit according to any one of the Claims 9 to 15, further comprising a recombinant p16 protein, fragments thereof or peptides derived from p16 as marker molecules for carrying out a positive control reaction.

17. A test kit comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed on solid carriers, for detecting p16 in a solubilized sample.

18. The test kit according to Claim 17, which is selected from the group consisting of:
a. an ELISA kit comprising antibodies, fragments thereof or antigen binding agents directed against cyclin
dependent kinase inhibitor p16 fixed to ELISA plates, ELISA stripes or ELISA wells;
b. a lateral flow test kit, comprising antibodies, fragments thereof or antigen binding agents directed against
cyclin dependent kinase inhibitor p16 fixed to test strips, colloidal gold particles or latex particles;
c. a flow through assay kit, comprising antibodies, fragments thereof or antigen binding agents directed against
cyclin dependent kinase inhibitor p16 fixed to a porous member, or to the surface of capillaries;
d. a latex agglutination assay kit, comprising antibodies, fragments thereof or antigen binding agents directed
against cyclin dependent kinase inhibitor p16 fixed to latex particles;
e. an immunoassay kit, comprising antibodies, fragments thereof or antigen binding agents directed against
cyclin dependent kinase inhibitor p16 fixed to beads or membranes; and
f. an immunoassay kit, comprising antibodies, fragments thereof or antigen binding agents directed against
cyclin dependent kinase inhibitor p16 fixed to microspheres.

Amended claims in accordance with Rule 86(2) EPC

1. An in-vitro method for detecting cervical carcinomas, cervical intraepithelial neoplasias or cervical carcinomas in-
situ from a solubilized sample of a human subject, the method comprises the steps of:
   (a) solubilizing a cervical body sample obtained from a human subject in a lysis buffer, and
   (b) determining the overexpression of cyclin dependent kinase inhibitor p16 in the solubilized cervical sample
   by comparing the level of cyclin dependent kinase inhibitor p16 within said solubilized cervical sample with
   the level present in a solubilized healthy human cervical sample.

2. The method according to Claim 1, wherein the level of cyclin dependent kinase inhibitor p16 in the healthy human
   cervical body sample is provided as a predetermined value to set up a threshold for the detection procedure.

3. The method according to Claim 1, wherein the level of cyclin dependent kinase inhibitor p16 in a healthy human
   cervical sample is determined from a standardized sample solution, or from a representative number of healthy
   human cervical samples.

4. The method according to any one of the Claim 3, and wherein the determination of the level of cyclin dependent
   kinase inhibitor p16 in a healthy human cervical sample is carried out:
      a. in the course of the detection procedure,
      b. upon calibration of the detection system,
      c. once for each lot of detection reagents, or
      d. as a standard value for the detection method.

5. The method according to any one of the Claims 1 to 3, wherein the cervical body sample is swab, smear, aspirate,
   biopsy, preserved cytological specimen, histological specimen, fixed cell preparation or fixed tissue preparation.

6. The method according to any one of the Claims 1 to 5, wherein the cervical body sample is solubilized
   a. immediately after obtaining the sample,
   b. after storage and/or transport in a storage buffer, or
   c. after transport in a transportation buffer.

7. A test kit for determining the level of cyclin dependent kinase inhibitor p16 comprising antibodies, fragments thereof
   or antigen binding agents directed against cyclin dependent kinase inhibitor p16 and a lysis buffer for solubilization
   of a body sample.

8. The test kit according to Claim 7, wherein the lysis buffer comprises at least one composition selected from the
   group consisting of chaotropic agents, anionic detergents, cationic detergents, non-ionic detergents, amphoteric
   detergents, and alkaline compositions.

9. The test kit according to Claim 7 or Claim 8, wherein the lysis buffer comprises at least one composition selected
   from the group consisting of a proteinase inhibitor, a DNAse inhibitor, and an RNAse inhibitor.
10. The test kit according to Claim 9, wherein the proteinase inhibitor is selected from the group consisting of inhibitors to serine proteinases, inhibitors to cysteine proteinases, inhibitors to aspartic proteinases, inhibitors to metallo proteinases, inhibitors to acidic proteinases, inhibitors to neutral proteinases, and inhibitors to alkaline proteinases.

11. The test kit according to any one of the Claims 7 to 10, wherein the lysis buffer comprises at least one non-ionic detergent and at least one proteinase inhibitor.

12. The test kit according to Claim 11, wherein the lysis buffer contains Triton X-100 and at least one inhibitor of serine proteinases.

13. The test kit according to any one of the Claims 7 to 12, further comprising at least one marker molecule for carrying out a positive control reaction, reagents and buffers commonly used for carrying out the detection reaction.

14. The test kit according to any one of the Claims 7 to 13, further comprising a recombinant p16 protein, fragments thereof or peptides derived from p16 as marker molecules for carrying out a positive control reaction.

15. A test kit comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed on solid carriers, for detecting p16 in a solubilized sample.

16. The test kit according to Claim 15, which is selected from the group consisting of:
   a. an ELISA kit comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to ELISA plates, ELISA stripes or ELISA wells;
   b. a lateral flow test kit, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to test strips, colloidal gold particles or latex particles;
   c. a flow through assay kit, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to a porous member, or to the surface of capillaries;
   d. a latex agglutination assay kit, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to latex particles;
   e. an immunoassay kit, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to beads or membranes; and
   f. an immunoassay kit, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to microspheres.

17. The test kit according to Claim 15 or Claim 16 being an in-vitro diagnostic device.

18. The in-vitro diagnostic device according to Claim 17, which is selected from the group consisting of:
   a. an ELISA device comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to ELISA plates, ELISA stripes or ELISA wells;
   b. a lateral flow test device, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to test strips, colloidal gold particles or latex particles;
   c. a flow through assay device, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to a porous member, or to the surface of capillaries;
   d. a latex agglutination assay device, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to latex particles;
   e. an immunoassay device, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to beads or membranes; and
   f. an immunoassay device, comprising antibodies, fragments thereof or antigen binding agents directed against cyclin dependent kinase inhibitor p16 fixed to microspheres.
Figure 1:
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**INCOMPLETE SEARCH**

The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.

Claims searched completely:

Claims searched incompletely:

Claims not searched:

Reason for the limitation of the search:

Claims 1-6 are directed to a diagnostic method that involves treatment of the human/animal body (Article 52 (4) EPC). All claims have been searched.

Place of search: MUNICH

Date of completion of the search: 11 November 2003

Examiner: Diez Schlereth, D
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For more details about this annex: see Official Journal of the European Patent Office, No. 12/82.
This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EOP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

11-11-2003

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