PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:
G01N 33/543	A1	WO 96/23228
		(43) International Publication Date: 1 August 1996 (01.08.96)

(21) International Application Number: PCT/GB96/00152

(22) International Filing Date: 25 January 1996 (25.01.96)

(30) Priority Data:

9501416.3	25 January 1995 (25.01.95)	GB
9501415.5	25 January 1995 (25.01.95)	GB
9501622.6	27 January 1995 (27.01.95)	GB

(71) Applicant *(for all designated States except US)*: INTERNATIONAL MUREX TECHNOLOGIES CORPORATION [CA/US]; 3075 Northwoods Circle, Norcross, GA 30071 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: DUNCAN, Richard, Julian, Stuart [GB/GB]; Murex Diagnostics Limited, Central Road, Temple Hill, Dartford, Kent DA1 5LR (GB).

(74) Agents: SILVESTON, Judith et al.; Abel & Imray, Northumberland House, 303-306 High Holborn, London WC1V 7LH (GB).

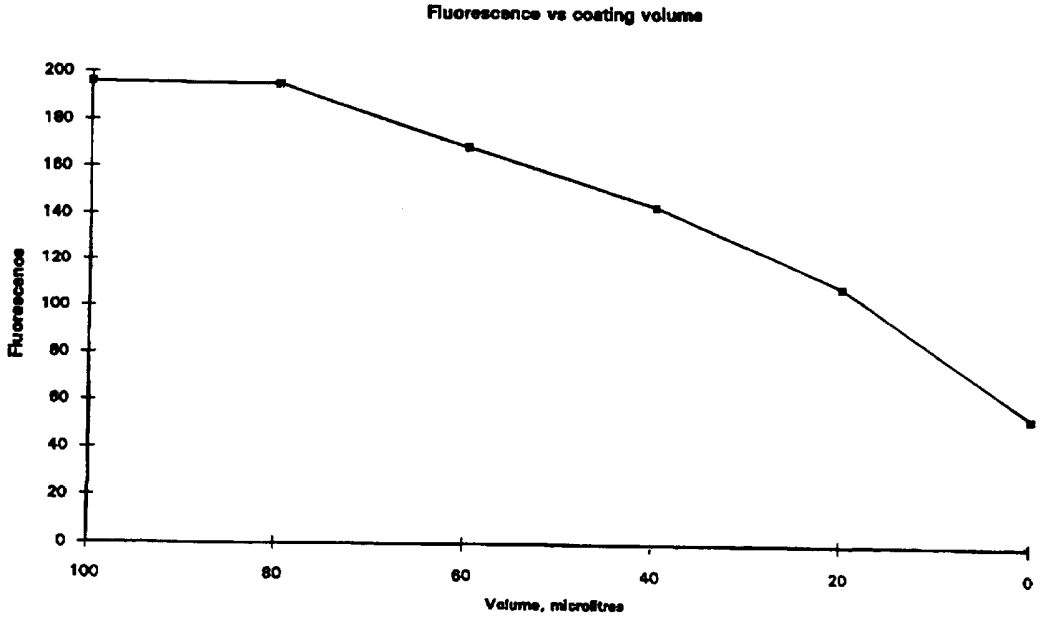
(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: IMPROVEMENTS TO IMMUNOASSAYS



(57) Abstract

A method for detecting the presence of a capture component immobilized on the capture zone of a solid phase for use in an immunoassay or another specific binding pair assay, wherein (a) the capture component is labelled with a label capable of producing a detectable signal directly or indirectly, and the signal is detected before or after the assay is carried out; or (b) after the assay has been carried out, there is performed a further test which comprises contacting the solid phase with an agent that is capable of interacting with the capture component or the captured analyte of the immunoassay or other specific binding pair assay and is also capable of producing a detectable signal directly or indirectly, and detecting the signal. The method enables the elimination of false negative results caused by incomplete or inadequate coating of the capture zone with capture component.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

IMPROVEMENTS TO IMMUNOASSAYS

The present invention relates to improvements in immunoassays and analogous assays for other specific binding pairs, especially assays to be carried out on samples of body fluids and other samples obtained from humans and non-human animals.

There are essentially two types of immunoassay format, one in which an antibody or antigen is captured selectively onto a capture zone of a solid phase from a sample under investigation (heterogeneous phase or "capture" assays), and another, a homogeneous phase assay format, in which the antibody or antigen under investigation is detected in solution. (Unless stated otherwise, the term "antigen" as used herein includes haptens.) Assays for specific binding pairs other than antibodies and antigens, for example, for receptors and their ligands, may be carried out analogously to immunoassays. The present invention is concerned with capture assays.

In a capture assay, the solid phase on which the capture occurs should be capable of being washed in some way and is, for example, of a plastics or polymeric material, for example, of nitrocellulose, polyvinyl chloride, polystyrene, polyamide, polyvinylidene fluoride or another synthetic polymer. The solid phase may be formed into microtitre wells, beads, dipsticks, aspiration tips, electrodes and optical devices. The solid phase may be particles, for example, so-called "latex" particles, stabilised blood or red blood cells, bacterial or fungal cells, spores, gold or other metallic sols, and proteinaceous colloids. The size of particles is generally from about 0.02 to 5 microns, and beads generally larger, for example, from about 2 mm to 10 mm. Alternatively, the solid phase may be a slide or "tile" having coated hollows. Slides, cards and tiles may be used as immunoassay vessels for agglutination assays.

Microtitre plates and beads of polymeric material are particularly convenient, especially for large-scale use, since automated systems are available commercially for handling such test formats.

Other solid phases that may be used include laminar solid phases, for example, membranes, sheets and strips, for example, of a porous, fibrous or bibulous material, for example, of nylon, polyvinyl chloride or another synthetic polymer, of a natural polymer, for example, cellulose, of a derivatized natural polymer, for example, cellulose acetate or nitrocellulose, or of glass fibres. Paper, for example, diazotized paper may be used. Films and coatings, for example, of fibrous or bibulous material, for example, as described above, may be used as the solid phase.

It is to be understood that the above examples of solid phases are given by way of illustration only, and the invention is not limited to the use of such solid phases. The invention may be practised on any solid phase suitable for use in immunoassays.

A solid phase, for example, a membrane, sheet, strip, film or coating, may be incorporated in a device for the determination of multiple or, more generally, single samples.

An assay device may incorporate the solid phase within a rigid support or a housing, which may also comprise some or all of the reagents required for carrying out an assay.

A laminar solid phase may be used for a so-called "membrane" assay, for example, a dot-blot or Western blot assay.

In a capture assay, the capture component is immobilized in a predetermined "capture zone". The capture zone may be substantially the entire surface of the solid phase, for example, the inner surface of a microwell, or the outer surface of a bead or other particle. For use in membrane assays and in some assay devices, however, the capture zone is generally a defined region that is less than the total surface of the solid phase for example, one or more dots, stripes or other defined areas of predetermined size and shape.

When the substance under investigation is an antigen, the capture component is generally a polyclonal or monoclonal antibody that forms a complex with that antigen. When the

substance under investigation is an antibody, the capture component may be an antigen that binds that antibody species selectively.

As an alternative to the selective capture of the antibody species of interest by an antigen, the capture component may be any substance that is capable of capturing antibodies non-selectively (an "immunoglobulin capture" assay). A representative proportion of all antibodies is captured, and then the antibody species of interest is detected by means of a labelled component that interacts specifically with that antibody species. The labelled component is generally a labelled antigen that binds specifically with the antibody species of interest. Suitable capture components for an immunoglobulin capture assay include, for example, protein A, protein G, anti-Fc antibodies, anti-IgG, anti-IgM and anti-IgA antibodies.

The immunoassay techniques described herein in connection with antibodies and antigens can be applied analogously to the detection and/or determination of members of specific binding pairs other than antigens and antibodies, especially receptors and their ligands, and references herein to immunoassays also include other specific binding pair assays, unless specified otherwise. Examples of ligands are hormones, for example, insulin and glucagon, amino acid transport proteins, viral coat proteins, molecules involved in neurotransmission, for example, adrenergic and cholinergic transmitters, serotonin and dopamine, adrenalin, histamine and cytokines. In some cases, where an antibody to a ligand or a receptor can be produced, it may it may preferable to determine a ligand or a receptor by a conventional immunoassay. If it is difficult or impossible to produce an antibody, or if it is desirable for any other reason, a ligand or receptor may be determined in a capture assay analogous to a capture immunoassay. In such an assay, either the ligand or the receptor may be immobilised on the capture zone of a solid phase. Agonists and antagonists of ligands may also be determined in such a system.

The term "capture component" accordingly denotes any substance that is immobilized on a capture zone of a solid phase for use in a heterogeneous phase (capture) assay for an antigen or hapten, antibody, or member of another specific binding pair, for example, a ligand or receptor, or an agonist or antagonist of a ligand.

Numerous books and review articles describe the theory and practice of immunoassays. Advice is given, for example, on the characteristics and choice of solid substrates for capture assays, on methods and reagents for coating solid substrates with capture components, on the nature and choice of labels, and on methods for labelling components. An example of a standard textbook is "ELISA and Other Solid Phase Immunoassays, Theoretical and Practical Aspects", Editors D.M. Kemeny & S.J. Challacombe, published by John Wiley, 1988. Such advice may also be applied to assays for other specific binding pairs.

Testing of samples of body fluids and of solid samples, for example, of cells or tissue, obtained from a body for is carried out routinely. Testing is carried out in two main contexts. One is often called "clinical" testing and involves testing samples of body fluids and solid samples in a clinical context, for example, for the purpose of diagnosing disease and disorders, monitoring the course of disease and/or monitoring treatment in an individual. Another major type of testing is the screening of donated blood in order to maintain a supply of blood and blood products free from contamination by pathogenic organisms.

Most immunoassays and other specific binding pair assays performed in clinical laboratories and blood screening laboratories are expected to give negative results. Normally a negative result is shown by a lack of a specific signal being generated in the test system. Unfortunately this lack of specific signal could be either because the sample is actually negative or because the test system has in some way failed. Modern immunoassays allow the user to check that the correct type of sample has been added, and that all the other

reagents have been added or used in the correct volumes and sequence. For example, our "Sample Addition Monitor" system (WO93/25905) enables the user to be certain that a sample has been added to an immunoassay vessel, for example, microwell, to which it should be added, and eliminates false negatives resulting from absence of sample. Controls also enable timings, temperatures and functionality of nearly all components of an immunoassay system to be validated. Thus operator error is minimised and controllable.

There still remains the problem that during manufacture it is possible that the capture zone of the solid phase on which an assay is to be conducted failed to be coated appropriately with the capture component of the system. In the production of coated microtitre plates, manufacturers check that each well in a microtitre plate is filled with reagent at each stage of manufacture, either colorimetrically, gravimetrically or by some physical measurement of liquid level in the well. Similar checks are carried out in the coating of beads and other particles. While each of the measures outlined above shows that a well has been filled with reagent (and other quality assurance measures show that the correct material has been placed in the well) none will show whether or not the material which is intended to coat the well has actually bound to the surface of each individual well. Binding failure could be, for example, because an individual well became dirty during plate moulding, contaminated in store or during pre-manufacture stages, exposed to contamination during the binding phase, or even replaced with an unauthorised strip of wells during the coating or quality assurance procedures. Similar considerations apply to beads and other particles and also to other solid phases, for example, laminar solid phases for use in blot assays or in assay devices.

Up to now it has seemed satisfactory merely to check that the solid phase, for example, each well or bead, had been treated appropriately during manufacture. The present invention provides methods and means to ensure that the

coating component is actually present on the capture zone of the solid phase, and also that it is functional.

The present invention provides a method for detecting the presence of a capture component immobilized on a capture zone of a solid phase for use in an immunoassay or other specific binding pair assay for an analyte of interest, wherein

(a) the capture component is labelled with a label capable of producing a detectable signal directly or indirectly, and the signal is detected before or after the assay is carried out; or

(b) after the assay has been carried out, there is performed a further test which comprises contacting the solid phase with an agent that is capable of interacting with the capture component or the captured analyte of the immunoassay or other specific binding pair assay and is also capable of producing a detectable signal directly or indirectly, and detecting the signal.

The signal produced may be detected qualitatively, quantitatively or semi-quantitatively (i.e. the term "detect" as used herein includes "determine"). Absence of the signal indicates that the capture component is not present on the capture zone of a solid phase, or is not present in an adequate amount. A signal lower than expected indicates incomplete or inadequate immobilization of capture component, that is to say, the amount of immobilized capture component is less than expected. The method according to the invention therefore provides a valuable method for eliminating from immunoassays or other specific binding pair assays false negative results due to manufacturing defects.

When the capture component itself is labelled with a label that is capable of producing a detectable signal, either indirectly or especially directly, it is a simple matter for the manufacturer to detect the signal on completion of manufacture to ensure that the labelled capture molecules are located as expected on the solid phase, for example, in a microwell, on a bead or on a capture zone of a

laminar solid phase.

Alternatively or in addition, the test to check for the presence of capture component on the capture zone of a solid phase may be carried out by the user of the solid phase, either before or after performing the immunoassay (or other specific binding pair assay) itself. If no signal is detected, or the signal is lower than expected for a particular solid phase, that solid phase should not be used for the intended assay. If the test is carried out after the assay itself has been performed on a sample, the result of that assay should be ignored and the assay should be repeated.

The capture component may be labelled before it is applied to the capture zone of a solid phase, or labelling may be carried out after the capture zone of a solid phase has been coated with unlabelled component. It is generally preferable to label the capture component before it is immobilized, since labelling in situ may result in non-specific binding of the label to the solid phase.

The present invention accordingly provides a solid phase for use in an immunoassay or another specific binding pair assay, which solid phase comprises a capture component immobilized on a capture zone, characterised in that the capture component is labelled with a label capable of producing a detectable signal directly or indirectly.

The present invention further provides a method for producing a solid phase for use in an immunoassay or another specific binding pair assay, which comprises

(a) immobilizing on a capture zone of the solid phase a capture component that is labelled with a label capable of producing a detectable signal directly or indirectly, or

(b) immobilizing a capture component on a capture zone of the solid phase and labelling the immobilized capture component with a label capable of producing a detectable signal directly or indirectly.

Small molecules that produce (or emit) a predetermined detectable signal without changing the biological properties of the labelled molecule are well known and are widely used in immunoassays and other specific binding pair assays. The detectable signal may be optical, radioactive or physico-chemical and may be produced when the label is stimulated or excited, as in the case with fluorescent labels. (The term "produce" used herein includes "emits".) Alternatively, the signal may result from agglutination, a diffraction effect or a birefringence effect.

Examples of directly detectable labels include, for example, radioactive markers; fluorescent markers, for example, fluorescamine, rhodamine or fluorescein; and coloured markers, for example, dyes and coloured particles, for example, gold sol. Further examples of directly detectable labels, for example, magnetically resonant and electroactive species, and methods for labelling molecules that can be used as capture components according to the present invention are described in the literature of the art, including standard text books, for example, Kemeny & Challacombe loc. cit, scientific publications and patent specifications.

Coloured and fluorescent labels are generally preferable to and more commercially acceptable than radiolabels. The present invention relates to both immunoassays (RIAs) and the more commercially important enzyme immunoassays (EIAs). For EIAs, radiolabels are generally unsuitable for labelling the capture component. In the case of an RIA a coloured or fluorescent label is preferred for labelling the capture component. If a radiolabel is used for the capture component in an RIA, it should emit a different type of radiation from that of the radiolabel for the assay itself.

Coloured labels and fluorescent labels may be read either by eye or by machine. Fluorescence may be determined by eye using an ultra-violet lamp and scanning the solid phase while looking for fluorescent and non-fluorescing units or regions. Determining the label using a machine has the

advantage that a permanent record of the reading may be obtained.

Fluorescent labels have an advantage over coloured labels of less potential interference with later steps in the immunoassay, which may involve coloured reagents and/or colour changes. They also have the further advantage that fluorescence is a much more sensitive technique than colorimetry. Fluorescent labels are therefore generally the preferred choice.

The signal produced by the label may be detected (or determined) qualitatively, quantitatively or semi-quantitatively. Fluorescent labels are particularly suitable for semi-quantitative and quantitative determinations, as shown in Figure 1 of the accompany drawing. Figure 1 is a graph of fluorescence readings of microwells against coating volumes for fluorescein-labelled antibodies used to coat the microwells. The fluorescence readings were made on dry microwells after the coating process had been completed.

When the coating component has been labelled with a label that can be detected directly, especially a coloured or fluorescent label, the manufacturer of a coated solid phase can easily incorporate a detection step after completion of the conventional coating process, for example, after the product has been dried and before it is packaged, in order to check that the coating component is present as required. The user of the solid phase may check for the detectable marker and hence the capture component on the solid phase before use i.e. while it is still in the dry state, in addition to or as an alternative to a manufacturer's quality control detection step.

As an alternative to a label that produces a signal directly, the capture component may carry a label that is capable of producing a signal indirectly. A particularly preferred example of an indirect label is an enzyme that is capable of giving rise to a measurable or otherwise detectable change of any sort, particularly one in which the enzyme catalyses a detectable colour or fluorescence change

in the presence of a suitable substrate. Such enzyme systems are widely used in enzyme-linked immunoassays (EIAs) and are well known per se, see for example, Kemeny & Challacombe loc. cit. (which also describes other direct and indirect labels). Examples of typical enzyme systems are those using alkaline phosphatase, ß-galactosidase, urease or peroxidase, for example, horse-radish peroxidase.

When the immunoassay (or other specific binding pair assay) itself is an enzyme-linked assay, the same enzyme system may be used in the capture component detection step, but it may be preferable to use an enzyme that catalyses a different reaction.

In a further example of an indirect label system, the capture component is labelled with biotin, which is capable of producing a detectable signal on reaction with avidin or streptavidin that is itself labelled with a direct or indirect label.

In an immunoassay for an antigen, for example hepatitis B surface antigen (HBsAg), the manufacturer would normally coat the capture zone of a solid phase, for example, microwells or beads, with antibodies which react with the antigen. Labelling the antibody with a label that produces (emits) a detectable signal, especially a direct label, for example, a fluorescent label, enables measurement of the label as a last stage during manufacture. Alternative or in addition, the signal may be read by the user prior to using the wells or beads for the intended assay or after carrying out the intended assay.

Similarly with the so-called "immunoglobulin capture" format, where an anti-human antibody is used as the capture component for antibodies of interest, the anti-human antibody may be labelled. With the "antigen sandwich" format for detection of antibodies, the plate-coating antigen may be labelled.

Instead of detecting a direct or indirect label bound to the capture component itself, the solid phase may be contacted (after the immunoassay or other specific binding

pair assay) with an agent that is capable of interacting with the capture component or the captured analyte and is also capable of producing a detectable signal, directly or indirectly. In this embodiment of the invention, the user of the assay will carry out the confirmatory test.

The agent that is capable of interacting with a capture component may be a binding partner for the capture component. When the capture component is an antigen, the binding partner is generally an antibody (polyclonal or monoclonal) that binds selectively to that antigen. When the capture component is an antibody, the binding partner may be an antigen or may be a second antibody that will bind to the capture antibody, for example, the second antibody may be an anti-species antibody e.g. if the capture antibody is a sheep antibody, the second antibody may be an anti-sheep antibody. Similar considerations apply to other types of binding partners, for example, ligands and their receptors. The binding partner is labelled with a label capable of producing a signal directly or indirectly.

According to a further embodiment of the present invention, after the immunoassay or other specific binding pair assay for an analyte of interest has been carried out, the solid phase is contacted with an agent that is capable of interacting with the captured analyte and is also labelled with a label capable of producing a detectable signal directly or indirectly. This embodiment is particularly useful for immunoglobulin capture assays.

In the case of immunoassays or other specific binding pair assays carried out on samples from humans, the agent capable of interacting with the captured analyte is preferably a directly or indirectly labelled binding partner for the capture analyte and is especially a directly or indirectly labelled anti-human antibody.

The agent that interacts with the capture component or the captured analyte is labelled with a label capable of producing a detectable signal directly or indirectly. The signal may be an optical, radio-active or physico-chemical

signal. The agent may be labelled with a label that is detectable as such, for example, a dye, coloured particle, radiolabel, electroactive species, magnetically resonant species or fluorophore. Alternatively the agent may be provided with a label that produces a signal indirectly, for example, by labelling the agent with biotin or with an enzyme that is itself capable of giving rise to a measurable change of any sort. Alternatively, the signal may result from agglutination, a diffraction effect or a birefringence effect involving the agent. Examples of direct labels and of indirect labels are given above. For the reasons given above, there are advantages in using a coloured or fluorescent species as the entity that provides the detectable signal.

For all the embodiments of the present invention except those in which the capture component is labelled with a label that is detectable directly, it is necessary to use reagents in addition to those required for the immunoassay or other specific binding partner assay in order to produce a detectable signal.

Accordingly, the present invention provides a kit that comprises component(s) for the detection of a capture component immobilized on the capture zone of a solid phase by a method according to the present invention and optionally also comprises component(s) required for the immunoassay or other specific binding pair assay for the analyte of interest.

For example, in the case where the capture component is labelled with an indirect label, for example an enzyme, a substrate for the enzyme may be provided; when the capture component is labelled with biotin, labelled avidin or streptavidin may be provided. If the avidin or streptavidin is labelled with an indirect label, for example an enzyme, the appropriate substrate may also be provided. When the capture component or captured analyte is to be detected after the immunoassay or other specific binding pair assay, for example, by interaction with an agent capable of producing a detectable signal, that agent may be provided, together with

any further reagents required. For example, the agent may be a directly labelled specific binding partner, in which case no further reagents are required. If the specific binding partner is labelled with an enzyme or with biotin, then further reagents may be provided as described above.

A kit may also comprises further reagents, selected for example, from diluents, buffers, wash solutions, stop solutions, standard and control solutions.

The present invention is particularly concerned with enzyme immunoassays (EIAs). As is well known, an EIA involves the use of a component that carries an enzyme capable of catalysing a detectable reaction, especially a reaction with a substrate that results in a colour or fluorescence change. In a capture (heterogeneous phase) assay the enzyme-labelled component forms a complex with another component that is bound (directly or indirectly) to the capture zone of a solid phase. Addition of the substrate results in a detectable, for example, colour change. It is conventional to add a so-called "stop solution" (often a strong acid) to stop the colour reaction. The signal, for example, colour or fluorescence, is then observed by eye or read by machine. After completion of the assay, the various liquid and solid components are generally thrown away. In a capture (heterogeneous phase) RIA, the solid phase is generally disposed of after measurement of any bound radiolabel.

According to the present invention, however, either the confirmatory assay is carried out before the assay by the manufacturer and/or by the user of the assay or, alternatively, after the assay itself. In the latter case the solid phase on which an assay (either EIA or RIA) for the analyte of interest has been carried out is not discarded but further reactions or tests are carried out to determine whether capture component is present, if it is present in an adequate amount, and/or if it is functional. It is generally (although not always) necessary to wash the solid phase (after discarding any liquid, for example, the coloured

contents of a microwell, or a tube in which a bead has been assayed). The solid phase is then contacted with the agent that is capable of interacting with the capture component or the captured analyte and also of producing a detectable signal and is also contacted, simultaneously or sequentially, with any further reagents that are required to produce the detectable signal.

As described above, the agent capable of producing the signal may be a binding partner for the capture component or captured analyte and may be labelled with a label that itself produces (emits) a detectable signal, for example, a coloured particle or a fluorescent label. In that case, generally after the solid phase has been washed, the agent may be simply incubated with the solid phase, any agent remaining unbound after the incubation is washed off, and the solid phase is then examined for the presence of the signal. For example, a coloured or fluorescent label may be determined by eye or, at the appropriate wavelength, by machine.

Instead of carrying a label that itself produces (emits) a detectable signal, a binding partner for the capture component may carry an indirect label, that is to say, a label that requires a further reagent for the production of a detectable signal. Such an indirect label is generally an enzyme capable of catalysing a reaction involving production of a detectable signal, generally a colour change or a change in fluorescence.

In such a case, after the assay for the analyte of interest has been carried out, the solid phase is generally washed, and is then incubated with the enzyme-labelled binding partner for the capture component and with the appropriate substrate for the enzyme in the same manner as a conventional EIA is carried out.

The capture component may be determined indirectly via the captured analyte, using a directly or indirectly labelled binding partner for the captured analyte, such an assay may be carried out analogous to the method described above for the determination of the capture component itself.

If the agent that is capable of interacting with the capture component or the captured analyte is labelled with biotin, labelled avidin or streptavidin is used to provide a detectable signal.

In a further embodiment, the capture component itself is labelled with a label capable of producing a signal indirectly. That label may be an enzyme capable of catalysing a reaction that gives rise to a detectable signal. In that case, either before or after the assay for the analyte of interest has been carried out, the solid phase is incubated with an appropriate substrate in the conventional manner for an EIA. The solid phase is then washed and the assay itself is carried out. If the test for capture component is carried out after the immunoassay or other specific binding pair assay itself, the solid phase is washed after the assay and the solid phase incubated with the substrate for the enzyme.

An indirect label for the capture component may be biotin, in which case the agent capable of producing a detectable signal may be avidin or streptavidin, labelled with a direct or indirect label, as described above for direct and indirect labels for binding partners for a capture component. In that case, before the assay for the analyte of interest is carried out, the solid phase is incubated with the labelled avidin or streptavidin. The direct or indirect label may then be determined as described above for binding partners for a capture component. The solid phase is generally washed before carrying out the main assay. Alternatively, the biotin assay is carried out after the main assay, generally after the solid phase has been washed.

Coloured labels and fluorescent labels, used as direct labels for any labelled component in any of the methods described above may be read either by eye or by machine, as may enzyme-catalysed colour or fluorescence changes. Fluorescence may be determined by eye using an ultra-violet lamp and scanning the solid phase while looking for fluorescent and non-fluorescing units, for example,

individual microwells, or regions, for example, capture zones or laminar solid phases. Determining a coloured label or the product of a colour reaction using a machine has the advantage that a permanent record of the reading may be obtained.

Capture component may be present on the capture zone of a solid phase but not functional. For example, the capture component may have become contaminated such that it is present in the correct amount on the solid phase but it is unable to form a complex with the analyte of interest.

If the capture component is not functional, a negative result will be obtained if an attempt is made to determine the capture component via the captured analyte, because none will be present. Analogously, if the method of the present invention is carried out using a binding partner for the coating component, a negative result will be obtained because none will be bound.

The present invention accordingly provides a method of determining the presence of functional capture component immobilized on a capture zone of a solid phase used for an immunoassay or other specific binding pair assay for an analyte of interest wherein, after the immunoassay or other specific binding pair assay for the analyte has been carried out, there is performed a further test wherein the solid phase is contacted with a binding partner for the capture component or for the captured analyte, the binding partner being capable, directly or indirectly, of producing a detectable signal.

Accordingly, the absence of signal in this method of the present invention indicates that the capture component is not present on the capture zone of a solid phase, is not present in an adequate amount, or is not functional. A signal lower than expected indicates incomplete or inadequate immobilization of capture component or inadequate functionality. A negative result in the assay for the analyte should therefore be ignored and the assay repeated.

In capture assays, including assays to which the present

invention relates, the capture component is immobilized on a predetermined capture zone of the solid phase. For example, the capture component may be coated onto the entire surface of the solid phase, for example, over the inner surface of a microwell or over the outer surface of a bead or other particle. Alternatively the capture component may be immobilized on defined regions of the solid phase only, for example, in dots, stripes or other defined areas of predetermined size and shape, for example, in the case of laminar solid phases to be used for blot assays.

Methods for coating antigen and antibody capture components on solid phases are well known and are described in the literature of the art, for example, in Kemeny & Challacombe, loc. cit. The reaction conditions used will vary depending on the nature and composition of the solid phase.

In general an antibody or antigen (or member of another specific binding pair) is dissolved at an experimentally determined optimum concentration, usually between about 0.5 and 5 micrograms/ml, in an optimum pH buffering medium, for example, a sodium carbonate/sodium bicarbonate buffer, for example, at about pH 9 to 10, for example, pH 9.6; sometimes a pH around 5 is better, and allowed to adsorb passively to the solid phase. Sometimes chemical bonding is used to bind the reagent more firmly. The capture component is generally allowed to bind to the solid phase for at least 30 minutes, and usually overnight, then the solid phase is washed, generally with a pH buffering solution. After the binding (or bonding) step, a solution which will block any unfilled binding sites is added. The blocking agents is generally a protein, for example, albumin or casein, but other materials, including non-proteinaceous materials can be used. After a further incubation, generally more than about 30 minutes, not usually overnight, the solid phase is finally washed and allowed to dry. It is then ready for use.

If the capture component is to be immobilized on a capture zone that is less than the total surface of the solid

phase, for example, if the capture zone is dots, stripes or predetermined shapes on a laminar solid phase, the capture component may be painted or printed onto the capture zone. A further method of applying capture components in the case where a laminar solid phase comprises a number of capture zones each with a different capture component, for example, as in Western blots, is to apply a mixture of capture components to the solid phase and to separate the capture components by electrophoresis.

If the capture component carries a label, either a direct label, for example, a coloured or fluorescent label, or an indirect label, for example, an enzyme or biotin, it may be labelled before it is applied to the solid phase, or labelling may be carried out after coating with unlabelled component. It is preferable to label the capture component before it is immobilized, since labelling in situ may result in non-specific binding of the label to the solid phase.

The signal produced by the label may be determined qualitatively, quantitatively or semi-quantitatively. Fluorescent labels are particularly suitable for semi-quantitative and quantitative determinations, as shown in Figure 1 of the accompany drawing. Figure 1 is a graph of fluorescence readings against coating volumes for fluorescein-labelled antibodies used to coat microwells.

As indicated above, the absence of signal in a method of the present invention indicates that the capture zone of a solid phase has not been coated with the capture component or that an inadequate amount of capture component is present or that the capture component is not functional. A signal lower than that expected indicates incomplete or inadequate coating of the capture zone or inadequate functionality of the capture component. A negative result in the previous assay for the analyte of interest should therefore be ignored and the assay replaced. As indicated above, the present invention provides a valuable method of eliminating false negative results due to manufacturing defects.

The following Examples illustrate the invention:

EXAMPLE 1

Fluorescence labelling

The method of Mann K.G. and Fish, W.W. Methods in Enzymology 26 (1972) p28 was used:

Sheep anti-human IgG was mixed with fluorescein isothiocyanate at an experimentally determined optimum concentration ratio (which was about 80:1, at a protein concentration of about 5 mg/ml), allowed to react overnight, and then the excess fluorescent reagent was removed by gel filtration.

Plate coating

Antibody labelled with fluorescein isothiocyanate as described above was brought to concentration of 5 microgram/ml in a carbonate/bicarbonate buffer pH 9.6. Various volumes of the labelled antibody solution (0, 20, 40, 60, 80 and 100 ml) were added to wells of a commercially available microtitre plate (Nunc, Maxisorb; Nunc products are available from Life Technologies, PO Box 35, Washington Road, Abbotts Inch Industrial Estate, Paisley, Renfrewshire, TA3 4EF, Scotland). Wells A1-A7, B1-B7, C1-C7, D1-D7, E1-E7, F1-F7, G1-G7 and H1-H7 were used.

Wells A8-A12, B8-B12, C8-C12, D8-D12, E8-E12, F8-F12, G8-G12 and H8-H12 of the plate were incubated with 100 μl of the antibody-containing solution, with the exception of wells B12, C9, C11, E8, E10, F12 and G10, which were intentionally left empty.

The plate was incubated overnight at room temperature, and then free sites on the wells were blocked. The plates were then drained and dried at room temperature.

Fluorescence reading

Fluorescence of the dry microwells was measured using a Dynatech plate reading filter fluorimeter. The wavelengths for fluorescein are activation at 490nm with fluorescence at 535nm.

Results:

The results obtained are presented in Table 1 below and in Figure 1 of the accompanying drawings. Table 1 sets out the fluorescence readings obtained for the plate (wells A1-H12) and indicates the volume of antibody-containing solution used in the wells. Figure 1 is a graph showing fluorescence of the dry wells vs. coating volume.

Referring to Table 1, it can be seen from the fluorescence values obtained for the wells that had been incubated with the various different volumes of labelled antibody solution (wells A1-A7, B1-B7, C1-C7, D1-D7, E1-E7, F1-F7, G1-G7 and H1-H7) that the fluorescence is directly related to the coating volume and hence the amount of capture component present in the wells. These results presented are presented graphically in Figure 1.

The results presented in Table 1 relating to the wells A8-A12, B8-B12, C8-C12, D8-D12, E8-E12, F8-F12, G8-G12 and H8-H12 show clearly that those wells that had been incubated with 100 μl of antibody-containing solution show a high level of fluorescence, while those that had been left empty intentionally (wells B12, C9, C11, E8, E10, F12 and G10) show substantially no fluorescence. The absence of signal therefore corresponds directly with absence of capture component. The results shown in Figure 1 demonstrate that the level of fluorescence observed is related to the amount of capture component present.

Accordingly, the method of the present invention may be used for qualitative, semi-quantitative or quantitative determination of a capture component on a capture zone of a solid phase for use in an immunoassay or another specific binding pair assay.

TABLE 1

Antibody volume	0	100	80	60	40	20	0	100 (with some intentionally missed wells)				
	1	2	3	4	5	6	7	8	9	10	11	12
A	64	149	147	124	133	106	59	156	153	160	171	159
B	53	172	171	133	117	94	57	156	153	171	143	54
C	62	257	262	173	132	98	54	209	43	197	44	231
D	57	220	184	175	173	102	54	249	172	163	197	172
E	60	238	231	198	175	125	52	54	181	47	254	175
F	51	200	227	193	171	128	52	237	182	176	213	49
G	66	202	185	210	123	120	53	249	292	50	225	195
H	58	133	151	144	130	100	51	149	205	201	230	272
Mean	59	196	195	169	144	109	54					
Std. Dev.	5.2	42.9	41.1	31.8	24.4	13.2	2.7					

EXAMPLE 2

Coating of microwells

Some microwells of a 96-well microtitre plate (Nunc, Maxisorb), were coated with a mixture of polyclonal rabbit antihuman IgG (DAKO) and polyclonal mouse antihuman IgM (DAKO). (Nunc products are available from Life Technologies, PO Box 35, Washington Road, Abbotts Inch Industrial Estate, Paisley, Renfrewshire, TA3 4EF, Scotland, and DAKO products from DAKO, 16 Manor Courtyard, Hughenden Avenue, High Wycombe, Bucks HP3 5RE, England.) The remaining microwells were coated with a mixture of HIV-1 and HIV-2 antigens (HIV-1 recombinant protein comprising core and envelope antigens from the CBL-1 isolate of HIV-1 (Sattentau Q.J. et al. (1986) Science 234 1120), HIV-2 peptide comprising gp36 envelope antigen).

The mixture of antibodies or of antigens was brought to a concentration of 5 microgram/ml in a carbonate/-bicarbonate buffer pH9.6. The solution was incubated in the microwells overnight at room temperature, and then free sites on the wells were blocked. The microtitre plates were then drained and dried at room temperature.

Immunoassay for HIV-1+2

100µl of diluted samples (10 µl sample and 90µl sample diluent) were added to wells of the microtitre plate and incubated under humid conditions at 37°C for 60 minutes. Some of the samples were HIV-1 positive samples, some were HIV-2 positive, and some were HIV-1+2 negative. The wells were then washed thoroughly five times with a wash solution, each wash step involving removal of the contents of each well by aspiration, filling the well with wash solution (glycine borate buffer containing Tween), and soaking for 30 seconds. After the final wash step the contents of the well were removed and the wells were inverted and tapped dry on a paper towel or tissue. 50µl of a working strength solution of the HIV-1 and HIV-2 conjugates (HIV-1 recombinant protein comprising core and envelope antigens from the CBL-1 isolate

of HIV-1 (Sattentau Q.J. et al. (1986) Science 234 1120) conjugated to HRP (horse-radish peroxidase and HIV-2 peptide comprising gp36 envelope antigen, conjugated to HRP) in HEPES buffer containing bovine serum albumin and detergents were added to the wells either singly or in combination as described below and the plate was incubated at 37°C for 30 minutes under humid conditions. After a further wash step as described above, 100µl of substrate solution containing TMB (3,3',5,5'-tetramethylbenzidine) and hydrogen peroxide was added to each well, the plate was incubated at 37°C under humid conditions for 30 minutes and the reaction was then stopped using 50µl of 2M sulphuric acid. The absorbance in the wells was recorded at 450nm with 690nm as the reference wavelength.

Determination of capture component

Method:

After the absorbance of the wells had been read in the immunoassay for HIV, the presence or absence of capture component (a mixture of rabbit antihuman IgG and mouse antihuman IgM) on individual microwells was determined as follows:

The wells were washed with the wash solution described above, then the wells were each filled with 50 µl of a solution of peroxidase-labelled antibodies directed against the capture antibodies, that is to say, a mixture of peroxidase-labelled anti-rabbit and anti-mouse antibodies. The mixture was incubated in the wells for five minutes, then the wells were washed with the same wash solution as before, peroxidase substrate (TMB) was added and, after incubation for 10 minutes, the reaction was stopped using 2M sulphuric acid. The absorbance was read spectrophotometrically at 450nm with 690nm as the reference wavelength. (The time between stopping the colour reaction of the immunoassay itself and washing to begin the determination of the capture component is not critical but should be minimised and is preferably less than 30 minutes.)

Results:

The results are presented in the following Table 2:

TABLE 2

Probe	Wells: HIV 1+2		Wells: GMAC		
Conjugate diluent	0.015	0.014	0.012	0.014	0.015
anti-mouse	0.288	0.322	2.570	2.421	2.396
anti-mouse	0.241	0.242	2.542	2.387	2.079
anti-mouse	0.235	0.243	2.413	2.311	2.211
anti-rabbit	0.073	0.093	2.223	2.425	2.305
anti-rabbit	0.067	0.079	2.708	2.764	2.498

It can be seen that results obtained for the capture component test in those wells that were coated with the antibody capture component (anti-human IgG and anti-human IgM mixture) are greater by a factor of about 10 than the results for those wells that were coated with HIV antigens. Surprisingly, this result was observed regardless of whether the wells had contained HIV-positive or HIV-negative samples.

These results demonstrate that the method of the present invention may be used to determine the presence or absence of capture component on a capture zone of a solid phase used for an immunoassay. The efficacy and reliability of this method has been confirmed by large-scale trials carried out by blood banks during screening of donated blood.

CLAIMS

1. A method for detecting the presence of a capture component immobilized on the capture zone of a solid phase for use in an immunoassay or another specific binding pair assay, wherein
(a) the capture component is labelled with a label capable of producing a detectable signal directly or indirectly, and the signal is detected before or after the assay is carried out; or
(b) after the assay has been carried out, there is performed a further test which comprises contacting the solid phase with an agent that is capable of interacting with the capture component or the captured analyte of the immunoassay or other specific binding pair assay and is also capable of producing a detectable signal directly or indirectly, and detecting the signal.

2. A method as claimed in claim 1, wherein a direct label is a coloured label or a fluorescent label.

3. A method as claimed in claim 1, wherein an indirect label is an enzyme capable of catalysing a detectable change or is biotin.

4. A method as claimed in any one of claims 1 to 3, wherein the agent capable of interacting with the capture component or with the captured analyte is a binding partner therefor.

5. A solid phase for use in an immunoassay or another specific binding pair assay, which solid phase comprises a capture zone on which capture component is immobilized, characterised in that the capture component is labelled with a label capable of producing a detectable signal directly or indirectly.

6. A solid phase as claimed in claim 5, wherein the capture

zone is a defined region less that the total surface of the solid phase.

7. A method for producing a solid phase for use in an immunoassay or another specific binding pair assay, which comprises

(a) immobilizing on a capture zone of the solid phase a capture component that is labelled with a label capable of producing a detectable signal directly or indirectly, or

(b) immobilizing a capture component on a capture zone of the solid phase and labelling the immobilized capture component with a label capable of producing a detectable signal directly or indirectly.

8. A method as claimed in claim 7, wherein the capture zone is less than the total surface area of the solid phase.

9. A kit that comprises component(s) for the detection of a capture component immobilized on the capture zone of a solid phase by a method as claimed in any one of claims 1 to 4 and optionally also comprises component(s) required for the immunoassay or other specific binding pair assay for the analyte of interest.

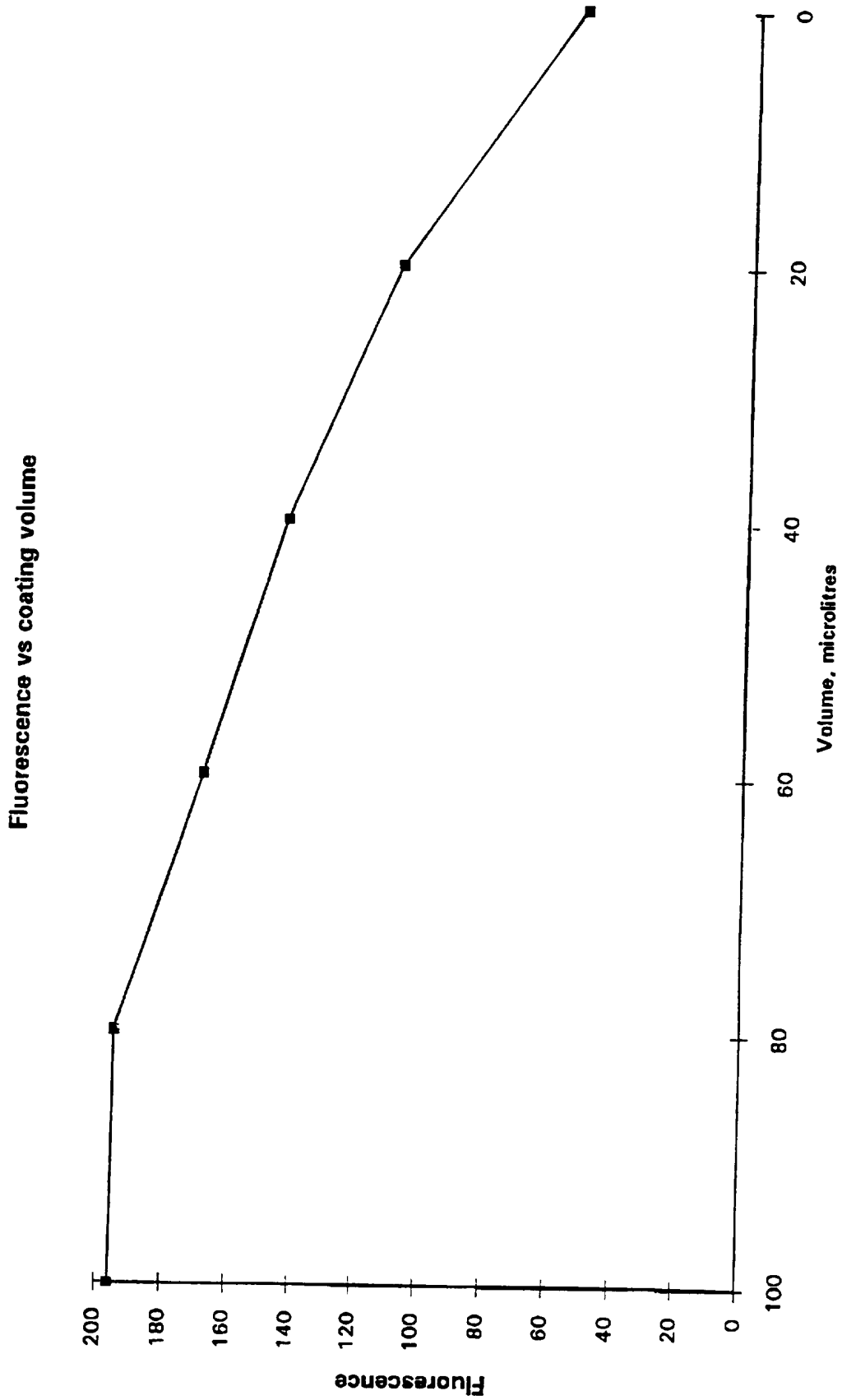

Fluorescence vs coating volume
Fluorescence
200
180
160
140
120
100
80
60
40
20
0
Volume, microlitres
0
20
40
60
80
100

FIGURE 1

INTERNATIONAL SEARCH REPORT

International Application No
PC./GB 96/00152

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/543

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 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	WO,A,93 25905 (INT MUREX TECH CORP ;DUNCAN RICHARD JULIAN STUART (FR)) 23 December 1993 see the whole document ---	1-4,9
Y	EP,A,0 101 912 (GENETIC DIAGNOSTIC CORP) 7 March 1984 see the whole document -----	1-4,9

☐ 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	Date of mailing of the international search report
23 May 1996	11. 06. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Hoekstra, S

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 96/00152

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9325905	23-12-93	AU-B-	4342693	04-01-94
		CA-A-	2137238	23-12-93
		DE-T-	643832	13-07-95
		EP-A-	0643832	22-03-95
		ES-T-	2068790	01-05-95
		JP-T-	7507634	24-08-95
EP-A-0101912	07-03-84	US-A-	4477576	16-10-84
		US-A-	4606855	19-08-86
		AU-B-	1728883	02-02-84
		JP-A-	59072059	23-04-84
		SE-A-	8304145	27-01-84