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(54) Title: LABELLING ARTICLES IT IS WISHED TO AUTHENTICATE

(57) Abstract

An item or substance it is wished to authenticate is labelled by means of a predetermined macromolecular first compound (signal compound) without divulging the identity of the first compound, to which first compound a complementary second compound is capable of binding such as to enable the presence of the first compound to be revealed and thus the genuineness of the item or substance verified.

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# DESCRIPTION

## LABELLING ARTICLES IT IS WISHED TO AUTHENTICATE

This invention relates to labelling articles to authenticate them.

Many areas of the modern world are faced with major problems associated with proof of authenticity. Counterfeit items whether mass produced as in the manufacturing world or forgeries as in the art world cause confusion and loss of revenues to genuine producers. In terms of world trade within the industrialised nations it is estimated that the actions of bogus counterfeit manufacturers result in multi-billion dollar losses. It is of particular concern to manufacturers of prestige, high value articles such as watches, electrical goods, high fashion clothes. The sale of counterfeit articles either in the producing country or abroad is a major trading difficulty for the industrial world. It is apparent that high volume, low cost products are also subject to counterfeiting and fraudulent sale.

In addition there are many reasons why individuals, corporations, public bodies and governments might wish to label authentic goods for reasons other than counterfeit control. For example, to monitor the flow of genuine articles along distribution and sales networks in order to be able to determine the ultimate fate of an article or item. This might provide information on the efficiency of a particular distribution network compared

with another, and in this case clearly has little to do with counterfeiting. The principle, however, remains the same: labelling an item in an unequivocal manner and by a system which enables the labelled item to be categorically identified.

There is a need for a system which can be applied to a variety of working modes to provide a totally secure protection system which itself cannot be counterfeited and cannot therefore be abused to protect counterfeit, forged items. Such a system under appropriate operating conditions could be used to monitor, control and regulate the trading and sale of genuine articles with a degree of protection for both the manufacturer and the consumer. There is a need for a totally secure system designed to achieve three major protective functions:

(a) to protect genuine manufacturers from the fraudulent sale of bogus, copied items by copying manufacturers who have no right to be manufacturing and selling these copies;

(b) to protect unaware customers from the witting or unwitting sale of fraudulent, copied, counterfeited articles; and

(c) to help governments and all appropriate governmental agencies monitor, regulate, control, police and prosecute where appropriate the trading of counterfeit items whether they are individually or mass counterfeited.

A new approach has now been developed to the

problem of authenticating articles. The present invention takes account of two major facts. Firstly, it takes advantage of the ability to detect the present or absence of molecules, such as DNA or protein per se by simple chemical analytical procedures. These types of procedures are plus/minus tests indicating whether or not DNA (or other macromolecule) is present. There is no discrimination between different DNA molecules from different sources, i.e. from different organisms. The resolution of the system may, however, be considerably improved by the second fact, namely by taking advantage of the ability of the whole sequence or substituent parts of compounds such as nucleic acids and proteins to be recognised unequivocally and thus reveal the genuineness of an article.

Accordingly, the present invention provides a method of labelling an item or substance it is wished to authenticate, which method comprises labelling the item or substance by means of a predetermined macromolecular first compound without divulging the identity of the first compound, to which first compound a complementary second compound is capable of binding such as to enable the presence of the first compound to be revealed and thus the genuineness of the item or substance verified.

The invention also provides a method of determining the authenticity of an item or substance, which method comprises;

i) determining whether the item or substance is labelled by means of a predetermined macromolecular first compound by means of a complementary second compound capable of binding to the first compound such as to enable the presence or absence of the first compound to be revealed and thus

ii) determining genuineness of the item or substance.

For convenience, we refer here to the first compound as a signal compound. The signal compound may comprise a sequence, for example of bases in the case of a nucleic acid and amino acid residues in the case of a protein, to which the complementary second compound binds. We refer to this sequence here as the signal sequence.

It is therefore now possible to label an item or substance or group of items or substances for which strict protection is desired in such a way that each can be recognized as genuine unequivocally and with predetermined levels of confidence. The invention may depend on the authorised labelling of genuine articles with a tag, for example, which carries one or more types of signal compound in such quantities, form and type that the genuineness of the tag (and hence the item or substance to which the tag is associated) can only be determined by the originator (producer, designer) of the tag.

Any substance or item (article) may be labelled. The technique of the invention may be applied to luxury goods such as watches, perfume and clothes; pharmaceuticals

and other chemicals such as fertilisers, herbicides and pesticides; films and recordings; bank notes; art works; documents such as passports; and machinery and parts such as parts for cars.

Labelling may be achieved in a variety of ways. A signal compound may be incorporated directly into an item or substance, for example during its manufacture. Alternatively, the signal compound may be attached such as by an adhesive. The adhesive may comprise the signal compound. The signal compound may also be included in a material such as a paint or ink which is applied to an item or substance. The contents of an item or substance may include the signal compound.

An article may be labelled by means of a tag to which a signal compound is attached. The signal compound may be protected by means of a clear plastics sheet. The tag may be directly attached to the article or incorporated directly as part of the structure of the article. Alternatively, the tag may be otherwise associated with the article such as loose in a box in which the article is packaged or included amongst documentation accompanying the article. For some articles, for example papers, the signal compound may be attached directly.

The signal compound is a compound to which a complementary second compound can bind so as to enable the presence of the signal compound to be detected. The signal compound is a macromolecule such as a nucleic acid or a

protein. An item or substance may be labelled by means of the signal compound in the form of such a macromolecule, i.e. the naked macromolecule. The macromolecule may be a synthetic one or one derived from a natural source. In the case of DNA, therefore, the genomic DNA or DNA from a partial restriction endonuclease digest thereof may be used.

Alternatively, however, the item or substance may be labelled by means of the signal compound in the form of spores or other resistant stages of an organism able to withstand the conditions of use as a label. In the case of DNA and (in the case of some viruses) RNA, the signal compound may be all or part of the genome of an organism. Especially microbes, therefore, such as viruses, bacteria and fungi could be employed as a label. They may be employed in small but measurable quantities. Examples of spores are spores of species of Bacillus such as B. subtilis, B. cereus or B. megaterium or of species of fungi such as Penicillium or Aspergillus. In these cases, the DNA or RNA would be contained in a living system which could be reisolated and the DNA or RNA identified. Sperm DNA, for example salmon sperm DNA (Sigma D-1501) may be used as the signal compound as well.

An array of different signal compounds, each occupying a discrete area, may be provided. Alternatively, an array of discrete areas of the same signal compound may be provided, in which case different complementary second

compounds may be used to bind with different signal sequences within the signal compound at each area. This applies particularly where the signal compound is a nucleic acid. Preferably the signal compound is a nucleic acid, DNA or RNA.

At a simple level, it may be sufficient for labelling purposes simply to detect the presence or absence of a macromolecule. Thus, the determination of the presence of DNA as a naked molecule or as part of a living system may be sufficient for certain purposes. The presence of DNA can be detected by using non-specific chemical agents which bind to the DNA, such as ethidium bromide, acridine orange or bis-benzimide (H33258, Hoechst dye 33258). In the case of ethidium bromide, this compound can not be detected under normal visual light wavelengths. Labelling may therefore be achieved by providing DNA and ethidium bromide together. Their presence can subsequently be detected by ultraviolet irradiation. This gives a simple method of labelling but does not permit high levels of uniqueness and therefore security to be obtained. It is a plus/minus test.

The uniqueness of DNA to each species and, indeed, each strain within a species together with the technical capacity to recognize quickly, unequivocally and accurately unique DNA molecules provides the basis to label articles of any sort and any origin, both animate and inanimate, in such a way that the labelled article may be

identified. This provides a more sophisticated form of labelling than a simple plus/minus test.

For each strain of organism, the DNA or RNA molecules are unique. Different strains of the same species differ by virtue of small variations in sequences of bases. For example, it is possible to recognise the DNA of different species and different strains of the same species by examining the DNA with labelled DNA probes. Specially constructed probes can be used which comprise a fragment of DNA which has been randomly derived from the genomic DNA of the organism in question. Synthetic DNA proves may be employed, as may bacteriophage probes such as an M-13 probe construction. Alternatively, a plasmid probe can be used. The probe DNA can hybridise with a matching DNA sequence which may be contained within a different DNA molecule.

In the present invention, therefore, an item or substance may be labelled with a signal DNA. The signal DNA comprises a sequence capable of hybridising with a specific probe DNA. This sequence is the signal sequence. Both the signal DNA and the probe DNA are kept secret. Where analysis of a labelled item or substance by means of the probe DNA reveals the signal sequence of the signal DNA, the item or substance is genuine. If not, the item or substance is not authentic. The probe DNA is of course labelled in such a way as to reveal whether or not hybridization has occurred, for example with a radioactive

or enzyme label, by biotinylation or by photobiotinylation.

The signal DNA may comprise one or more different signal sequences. Thus separately a given DNA signal molecule may be used several times on the same tag to give a unique signal depending on which individual specific DNA probe is used. Preferably, repeats of a signal sequence are present. This increases the sensitivity of the signal sequence to the probe DNA. Preferably, the signal DNA is the genomic DNA of a microorganism, although in a simple system a short DNA sequence such as one obtained synthetically may be employed. Viral or bacterial genomic DNA may be used, as may a partial restriction endonuclease digest of a genomic DNA. Plasmids may be employed.

The signal sequence of the signal DNA may be a sequence predetermined by the availability of a probe capable of hybridising with it. It may be a sequence intrinsic to the DNA molecule. Alternatively, a signal sequence may be introduced specifically into or mixed with a DNA molecule. The size of the signal sequence should be such as to enable a detectable response to a probe DNA to be elicited. For handling reasons, a signal sequence of from 1 to 10 kbp is suitable.

Various levels of confidence may be achieved that an item or substance labelled with a signal DNA is genuine. The degree of confidence may be increased by using two or more signal DNAs. An item or substance may be labelled by means of discrete areas each of a different signal DNA. A

signal DNA may be applied mixed with another DNA, termed here a maze DNA. The presence of the maze DNA complicates the task of a person attempting to determine the correct signal sequence of a signal DNA.

The invention may be applied as follows. A range of different microorganisms are separately grown. For each microorganism the genomic DNA is extracted and a set of different probes produced, using standard molecular biology techniques, by cloning randomly short sequences of the genomic DNA produced by digestion with a particular restriction endonuclease. This produces probes which will recognize by hybridization the DNA of that microbial species from which the DNA can be obtained. Thus the probe DNAs define the signal sequences which in principle must be possessed only by the signal DNAs.

It is unlikely that the general probes will hybridize with the DNA of different species but this can be easily checked by attempting to hybridize the probes against different sources of DNA. Any probes which do cross-hybridize can be discarded. Using a similar procedure strain specific probes can be obtained by examining hybridization against DNAs from different strains of the same species. Those which do not cross-hybridize will be specific for a certain strain of a given species. The sets of probes are labelled and stored for future use.

Tags may be prepared by placing signal DNAs at defined positions on an appropriate support material such

as paper, purified cellulose or cellulose acetate sheets, or nylon-based membranes such as Hybond C or Hybond N (Amersham International plc). A signal DNA may be applied either separated from the rest of a microorganism or in the form of a treated cell suspension or paste. The DNA can be applied either as dots or bands, each dot or band being DNA from a different microorganism or containing a different signal DNA. The DNA is attached to the support material in a form capable of hybridising with a probe DNA. Thus, the DNA will generally be denatured to render it single-stranded. Typically, the amount of signal DNA applied in each discrete area is from 10 pg to 10 ug. Signal RNA may be used instead of DNA. Spores of bacteria and fungi may be used directly, as may virus particles.

The tags prepared in this way are attached to items or substances which it is wished to authenticate. To determine the authenticity of any item or substance labelled with such a tag, the tag is processed using the stored set of labelled probe DNAs. The probe DNAs are brought into contact with the tag and bind to the signal sequence of each signal DNA. Determination of hybridization according to a pre-set pattern confirms the genuineness of the labelled item or substance.

A very high degree of confidence can be achieved in this way that the item or substance is genuine. For example, suppose a tag comprises six rows of three dots each of DNA, that each DNA is different and that 1000

different sources of DNA giving at least 1000 unique signal sequences are available to make the tags. The probability that a forger could independently reproduce any given row of three dots drawn at random from a pool of 1000 separate and distinct DNAs is 1 in $10^9$, in the case that the forger knows which 1000 DNAs have been used to create the pattern. Since there is no direct way in which any forger can even begin to discover this, unless he has a complete set of diagnostic DNA probes for DNA from a much larger sample of living organisms, there is no direct way of calculating the probability of a forger being able to reproduce the pattern unless the "worst case" example is taken in which the forger knows which 1000 DNAs have been chosen to form the basis for the technique. In this case for a complete tag of 18 dots the probability of a forger being to reproduce the correct array is 1 in $10^{54}$. Clearly any confidence limit can be chosen depending on the number of signal DNAs available to the maker of the tag and the number of signal DNAs applied to the tag.

These considerations apply too to a further level at which the invention may be applied. This depends upon examination of the minute differences in organism identity due to DNA restriction enzyme polymorphisms. How to do this is disclosed in our WO-A-86/02101. This provides a method of determining whether a first and a second organism, the identity of one of which is known, are identical, which method comprises:

(i) digesting genomic DNA of the first organism with one or more restriction endonuclease;

(ii) separating by electrophoresis the DNA fragments thus obtained;

(iii) determining the positions of the fragments thus-separated which hybridise with one or more first labelled probes ("hybridisation patterns", "band patterns" or "maps"), the or each probe comprising a fragment of DNA which has been derived randomly from the genomic DNA of an organism of the same species as the first or second organism; and

(iv) comparing the positions of the fragments thus-determined with the positions of DNA fragments which bind to the or each said first probe, which have been produced from genomic DNA of the second organism by digestion of genomic DNA of the second organism with the or each said restriction endonuclease and which have been subjected to electrophoresis in an identical manner to the DNA fragments obtained from genomic DNA of the first organism;

steps (i) to (iv) being effected using an amount of probe DNA and one or more restriction endounucleases such that sufficient bands are revealed by the hybridisation in step (iii) to achieve a probability (X) of $10^{-12}$ or less that, when the comparison in step (iv) reveals that the two organisms appear identical, the two organisms will have failed to have been distinguished as

genuinely different and unrelated as determined by:-

$$X = F^q \qquad (1)$$

wherein q is the number of positions revealed by the probing in step (iii) and F is a fraction, representative of the proportion of DNA fragments which are identical between restriction endonuclease digests of genomic DNA of pairs of independently-obtained organisms of the same species as the first and second organisms, by

(a) digesting separately using the same restriction endonuclease genomic DNA of a number of independently-obtained organisms of the same species as the said first and second organisms sufficient to obtain a F value representative of the species and, optionally, dividing each digest into portions;

(b) subjecting to electrophoresis side-by-side on a gel the digest, or a portion of the digest, for each of the independently-obtained organisms;

(c) probing the gel using a second labelled probe comprising a fragment of DNA derived randomly from the genomic DNA of an organism of the said species;

(d) comparing the hybridisation patterns on the gel thus revealed for pairwise combinations of the independently-obtained organisms; and

(e) optionally repeating steps (b) to (d) for one or more further portion of the digest for each of the independently-obtained organisms but using a said second labelled probe comprising a different said fragment of DNA

each time.

In other words, q is the total number of common positions of DNA digest fragments revealed by the pairwise comparison of the first and second organisms in step (iv), when the two organisms have identical maps.

Preferably, steps (i) to (iv) are effected by

(i') digesting separately genomic DNA of the first organism and genomic DNA of the second organism with the same restriction endonuclease and, optionally, dividing each digest into portions;

(ii') subjecting to electrophoresis side-by-side on a gel the digest, or a portion of the digest, for each organism;

(iii') probing the gel using a said first labelled probe and comparing the hybridisation patterns for the two organisms thus-revealed; and

(iv') optionally repeating steps (ii') and (iii') for one or more further portion of the digest for each organism but using a said first labelled probe comprising a different said fragment of DNA each time.

The procedure of steps (i') to (iv') can be effected two or more times using a different restriction endonuclease each time.

The method of WO-A-86/02101 depends upon the degree to which the failure to detect a difference between hybridisation patterns for two organisms can be taken as evidence of identity. Applying that method to the present

invention, a signal DNA (first organism) is applied to a tag typically in an amount of from 25 to 250 ug. The tag is attached to an item or substance which it is wished to authenticate. To determine the authenticity of the article, the signal DNA is removed from the tag, for example by electroeluting, and compared with genuine signal DNA (second organism) which has been stored. The signal sequences of the signal DNA are the sequences which hybridise with the probes employed in determining hybridisation patterns according to WO-A-86/02101. The signal sequences may be predetermined. The probes may have been constructed previously and stored. Alternatively, the probes may be prepared at the time the supposed signal DNA from a label and genuine signal DNA are compared.

By utilising the method of WO-A-86/02101, one signal DNA can be analysed to a predetermined level of confidence that it is unequivocally from a particular strain of a given species. Thus for each DNA added, a confidence limit can be given that it is from a particular strain. For a confidence of 1 chance in $3 \times 10^{62}$, for example, that identity will be claimed falsely, the chance that a fradulent tag manufacturer will make a tag with two correct signal DNAs is $9 \times 10^{124}$.

The signal compound may alternatively be a protein. Here, the invention relies on the variation of amino acid sequences of different proteins and the ability to recognize such sequences. Thus, an antigen-antibody

system may be employed. Where the signal compound acts as an antigen, the signal sequence is an antigenic determinant thereof which is recognized by an antibody. Where the signal compound acts as an antibody, the signal sequence is the sequence of the antibody via which it binds to a corresponding antigen. An idiotype-anti-idiotype system may be used in which both antigen and antibody molecules are immunoglobulins. One immunoglobulin has a binding specificity for the antigen-binding site of the other.

An antibody-antigen system may be used as follows. Antibodies are prepared by immunising groups of animals each with a variety of different, non-cross-reacting antigens, such as an array of different haptens. Serum from such animals is collected, and the antibodies raised against individual antigens are purified. The antibodies are then immobilised on a suitable substrate such as cellulose nitrate or a nylon-based substrate for example Hybond C or Hybond N. A patterned array of points of antibody application is built up as desired. The non-specific binding properties of the binding membrane are then blocked. The membrane is dried, sealed under plastic, and associated with the item or substance it is wished to authenticate. For decoding, the specific antigen or hapten is applied to the tag. Binding is detected using an appropriate detection system.

The security provided by the present invention depends upon the particular coding system of signal

compound(s) being kept secret. A predetermined signal compound is used for the labelling but its identity is not divulged. Items and substances are labelled so that it is not apparent from visual inspection which signal compound(s) are being used. However, a manufacturer, wishing to label securely his product so that any counterfeit product may readily be identified, may do so by publicly disclosing that it is labelled according to the present invention or not disclosing this fact.

In the former instance, the manufacturer can declare by advertising that all genuine products are protected by signal compound(s), for example in the form of a tag. The tag may form a detachable, prominent label on which will appear any desired printed information, in particular the batch code number for the manufacturer's particular, unique tag. Following purchase of a product, a customer may mail the tag for processing. Fraudently printed tags can therefore be readily identified.

In the latter instance, a manufacturer may secretly apply a tag to his product, without declaring its presence. Sampling of product from retail shops by manufacturer's representatives posing as normal customers will allow the manufacturer of the tags to discover whether or not counterfeit products are appearing.

Whichever way is adopted, each tag may be unique and possess different signal compounds. Alternatively, a batch of tags with the same signal compounds may be used.

In the case of using signal DNAs, a tag may be designed to operate at both levels described previously.

The following Examples illustrate the invention.

Example 1: Plus/Minus test for DNA of Lactobacillus plantarum BTLS1 (NCIB 12156)

Lactobacillus plantarum BTLS1 is described in WO-A-86/02101. It was deposited on 25th September 1985 at the National Collection of Industrial (now, and Marine) Bacteria, Aberdeen, GB under accession number NCIB 12156. Genomic DNA was obtained from BTLS1 as described in WO-A-86/02101. The genomic DNA was denatured by heating at 100$^{o}$ for 5 minutes.

An aliquot of denatured DNA in solution was applied to a tag of Hybond C or N from the opposite side of the tag in order to draw down the DNA onto the material of the tag. The tag was then dried for 30 minutes at 37$^{o}$C. The denatured DNA was then covalently coupled to the tag by baking at 80$^{o}$C for up to 4 hours or by exposure of the tag to ultraviolet light (304 nm) for 30 minutes. Some of the tags were protected by covering with flexible, clear PVC (ICI plc) or Saran Wrap (Dow Corning). Also, cotton and nylon fabrics (of shirts) were labelled with the denatured DNA. 100 ul of the solution of denatured DNA were added to each fabric which was then dried in air. 5 ng to 5 ug quantities of DNA were applied to the tags and fabrics.

Subsequently, the plastics films were removed

from the tags to which they had been applied. The tags were stained with a solution of less than 5 ug $ml^{-1}$ ethidium bromide. Under ultraviolet radiation (304 nm), a red-orange fluorescence was observed. This meant that the ethidium bromide had bound to the DNA. In a similar fashion, the areas of the shirt cotton and nylon fabrics to which the DNA had been applied was stained with the ethidium bromide solution. Again, fluorescence was observed to reveal the presence of the DNA.

Example 2: Preparation of a tag using intrinsic, single or low copy number signal sequences

1. Preparation of the signal and probe DNA

1.1. Select the microbial sources of DNA.

1.2. Prepare and purify genomic DNA by standard methods giving clean, high molecular weight DNA.

1.3. Select specific sequences for probes by restriction enzyme digestion of the DNA (e.g. with Sau3A), followed by size fractionation of the DNA by electrophoresis in 1% agarose gels, from which fragments of convenient size can be electroeluted [see McDonnel M.W. et al (1977) J. Molec. Biol. 110 119] and cloned into a suitable plasmid vector. These clones can then be tested for homology with other sources of DNA using such tests as the rapid 'dot blot' procedure of Kafatos C. et al. (1979) Nucleic Acids Research 7. 1541, according to which it will be possible to select sequences hybridising uniquely to the desired source DNA. Alternatively, such probes can be

identified with greater economy of effort using enrichment techniques, such as the deletion enrichment technique of Lamar E.E. and Palmer E. (1984) Cell 37. 171-177.

1.4. Following selection of suitable probes the signal DNA is prepared in quantity using standard plasmid preparation techniques, extracted and purified to give high molecular weight DNA.

1.5 Any of the signal DNA's can be transformed if desired into single stranded RNA derivatives, which hybridise more rapidly with DNA than does single stranded DNA, and which cannot be cut with restriction enzymes, by using the SP series of vectors described by Butler E.T. & Chamberlain M.H. (1982) J. Biol. Chem. 257 5772.

2. Preparation of the tag

2.1. For each of the dots on the tag matrix, apply 5 to 10 ug of each selected source of signal DNA (or any sufficient amount) to its chosen site on the tag membrane. Denture the DNA and covalently couple it to the membrane for example Hybond C or Hybond N.

2.2. Dry and seal the membrane.

3. Decoding of a tag

3.1 Reference is made to a data bank recording the essential features of the tags prepared for an article in question. A printed number on a tag can be used to identify which DNAs were employed and how they were distributed on the signal dot matrix. A mixture of the appropriate probes or one probe at a time is used to verify

the authenticity of the tag by specific hybridisation of these probes to the sites on the recovered tag to which authentic DNA would have been applied.

Example 3: Preparation of a tag using maze DNA and muli-copy signal DNA

1. Preparation of maze DNA

1.1. Select the microbial sources of DNA

1.2. Grow the organisms providing the DNA

1.3. Extract the DNA and purify it by standard methods to give high molecular weight DNA.

1.4. Sonicate the DNA to yield a population of molecules whose median molecular weight is equal to that of the chosen signal DNA.

1.5. Dry and seal the membrane.

2. Preparation of the signal DNA

2.1. Select the microbial sources of DNA. These may or may not be the same as the sources of maze DNA.

2.2. Prepare and purify genomic DNA by standard methods giving clean, high molecular weight DNA.

2.3. Select specific sequences for probes by restriction enzyme digestion of the DNA (eg with Sau3A), followed by size fractionation of the DNA by electrophoresis in 1% agarose gels, from which fragments of convenient size can be electroeluted [see McDonnel M.W. et al (1977) J. Molec. Biol. 110 119] and cloned into a suitable plasmid vector. These clones can then be tested for homology with other sources of DNA using such tests as

the rapid 'dot blot' procedure of Kafatos C. et al. (1979) Nucleic Acids Research 7. 1541, according to which it will be possible to select sequences hybridising uniquely to the desired source DNA. Alternatively, such probes can be identified with greater economy of effort using enrichment techniques, such as the deletion enrichment technique of Lamar E.E. and Palmer E. (1984) Cell 37. 171-177.

2.4. Following selection of suitable probes the signal DNA is prepared in quantity using standard plasmid preparation techniques. Where the signal DNA is required for addition either to homologous maze DNA, or to heterologous maze DNA it must be further purified by excision of the specific signal sequence using an appropriate restriction enzyme, followed by separation of the signal DNA from the residual probe DNA by standard methods. Such purified signal DNA is then ready for addition to sonicated maze DNA of equivalent median molecular weight.

2.5. Any of the signal DNA's can be transformed if desired into single stranded RNA derivatives, which hybridise more rapidly with DNA than does single stranded DNA, and which cannot be cut with restriction enzymes, by using the SP series of vectors described by Butler E.T. & Chamberlain M.H. (1982) J. Biol. Chem. 257 5772.

3. Preparation of a tag

3.1. Mix in the signal DNA with the maze DNA to give 40 to 100 copies of individual units of signal DNA for

each unit of maze DNA. For each of the dots on the tag matrix, apply 50 to 100 ng of one of the mixtures of DNA (or any sufficient amount) to its chosen site on the tag membrane. Denature the DNA and covalently couple it to the membrane for example Hybond C or Hybond N.

3.2. Dry and seal the membrane.

4. Decoding of a tag

4.1. Reference is made to a data bank recording the essential features of the tags prepared for an article in question. A printed number on a tag can be used to identify which DNAs were employed and how they were distributed on the dot matrix. A mixture of the appropriate probes or one probe at a time is then used to verify the authenticity of the tag by specific hybridisation of these probes to the sites on the recovered tag to which authentic DNA would have been applied.

Example 4: Preparation of a tag using intrinsic, multiple copies of the same signal sequences

Four probes were prepared from the genomic DNA of Lactobacillus plantarum BTLS1. These probes were pBTL8, pBTL23, pBTL29 and pBTL30. The preparation of these probes is described in WO-A-86/02101. These probes were labelled as described there. The probes were tested for homology with other sources of DNA by the rapid 'dot blot' procedure. None hybridised significantly with DNA from any of the genera or species tested especially under the conditions of high stringence which were used as described

in WO-A-86/02101. There is no reason to suspect that they would hybridise with non-Lactobacillus plantarum DNA. Thus, these four probes are unique to a given species and all four could be used to detect on L. plantarum DNA. Further analysis in fact showed that probe pBTL29 only hybridised significantly with DNA of L. plantarum BTLS1.

Genomic DNA of L.plantarum BTLS1 was covalently coupled to a tag of Hybond C or a tag of Hybond N as described in Example 1. Tags sealed with a plastics film as described in Example 1 were prepared as described there. Subsequently, the plastics film sealing the tag was removed to enable analysis of each tag.

In order to use the probes to detect the presence of signal L. plantarum DNA, the probes were labelled with either [$^{35}$S]-dCTP (deoxycytidine 5'-$\alpha$[$^{35}$S]-thiotriphosphate) or Renz-labelled (Nucleic Acid Research 12, 3435-34444, 1984) probes, i.e. probes linked to the enzyme horseradish peroxidase.

The tags were washed in prehybridisation buffer (6x SSC; 5x Denhardt's solution - 0.1% (w/v)-ficoll-400, 0.1% (w/v) bovine serum albumin, 0.1% (w/v) polyvinylpyrrolidone in 1x SSC-; and 0.5% (w/v) SDS) containing 20 ug ml$^{-1}$ denatured herring sperm DNA (Sigma Chemical Co.) for 3 hr in heat-sealed polyethylene bags (Pifco Ltd). Hybridisations were carried out at 65$^{o}$C in fresh prehybridisation buffer and labelled DNA probes were added, after denaturation, to a final concentration of

10-40 ng $ml^{-1}$. Sealed polyethylene bags containing the hybridisation reactions were incubated at 65°C for 16 h. After hybridisation the filters were washed twice in 2x SSC at 65°C for 15 min, once in 2x SSC, 0.1% (w/v) SDS at 65°C for 30 min and finally in the stringency wash which was either 0.1xSSC or 1xSSC at 65°C for 10 min. Filters were then blotted dry using Whatman 3MM chromatography paper.

The presence of homologous signal DNA was detected either by autoradiography as described in WO-A-86/02101 or by a colourimetric enzymic reaction (Renz et al). For the former method, the position of homologous signal DNA on the tag appeared as a dark spot on the x-ray film. Where there was no binding of non-homologous DNA no spots were detected. Similarly, the presence of hybridisation of signal L. plantarum DNA and probe DNA with the Renz technique resulted in the deposition of a black product of the enzyme reaction.

Example 5: Preparation of a tag for a higher level of security

1. Preparation of tag

1.1 High molecular weight double stranded DNA is prepared from a number of microorganisms whose DNA has been uniquely identified according to the procedure described in WO-A-86/02101.

1.2 Up to 250 ug of chosen DNA is applied to a defined site measuring approximately 250$mm^2$ on the tag membrane, from which intact DNA can subsequently be

eluted.

2. Decoding of tag

2.1. Reference is made to a data bank recording the essential features of the tags prepared for an article in question. A printed number can be used to identify which signal DNA was employed. Analysis proceeds by effecting electroelution of the site on the recovered tag at which authentic DNA would have been applied followed by analysis according to the method of WO-A-86/02101.

CLAIMS

1. A method of labelling an item or substance it is wished to authenticate, characterised in that the item or substance is labelled by means of a predetermined macromolecular first compound (signal compound) without divulging the identity of the first compound, to which first compound a complementary second compound is capable of binding such as to enable the presence of the first compound to be revealed and thus the genuineness of the item or substance verified.

2. A method according to claim 1, wherein labelling is achieved by incorporating the signal compound directly into the item or substance, by attaching the signal compound to the item or substance, by applying to the item or substance a material in which the signal compound is included or by including the signal compound in the contents of the item or substance.

3. A method according to claim 1, wherein an article is labelled by means of a tag to which the signal compound is attached.

4. A method according to claim 3, wherein the tag is directly attached to the article, is incorporated directly in the article as part of the structure of the article or is otherwise associated with the article.

5. A method according to claim 1, wherein the item or substance is labelled by means of the signal compound in the form of a macromolecule.

6. A method according to claim 1, wherein the item or substance is labelled by means of the signal compound in the form of spores of a bacterium or fungus or in the form of a virus.

7. A method according to claim 1, wherein the signal compound is a nucleic acid.

8. A method according to claim 7, wherein the nucleic acid is DNA.

9. A method according to claim 8, wherein the DNA comprises one or more signal sequences capable of hybridising with a specific DNA probe.

10. A method according to claim 8, wherein the said DNA is mixed with another DNA.

11. A method according to claim 8, wherein the DNA is the genomic DNA of a microorganism or DNA from a partial restriction endonuclease digest thereof.

12. A method according to claim 8, wherein the DNA is in the form of a plasmid.

13. A method according to claim 8, wherein the DNA is a synthetic DNA.

14. A method according to claim 1, wherein an array of different signal compounds, each occupying a discrete area, is provided.

15. A method according to claim 1, wherein an array of discrete areas of the same signal compound is provided.

16. A method according to claim 1, wherein an

item or substance selected from luxury goods, pharmaceuticals and other chemicals, films and recordings, bank notes, art works, documents and machinery and parts is labelled.

17. A method according to claim 1, further comprising subsequently determining whether the item or substance is labelled by means of the signal compound by means of the complementary second compound so as to enable the presence or absence of the first compound to be revealed and thus determining the genuineness of the item or substance.

18. A method of determining the authenticity of an item or substance, characterised in that

(i) whether the item or substance is labelled by means of a predetermined macromolecular first compound ("signal compound") is determined by means of a complementary second compound capable of binding to the first compound such as to enable the presence or absence of the first compound to be revealed and thus

(ii) the genuineness of the item or substance is determined.

19. A method according to claim 18, wherein the presence or absence of the signal compound is detected.

20. A method according to claim 18, wherein a labelled nucleic acid probe capable of binding to the signal compound is contacted with an area occupied by the signal compound when the item or substance is genuine and

determining whether the probe does hybridise with the first compound at this area.

21. A method according to claim 20, wherein the probe is a DNA probe.

22. Use of a nucleic acid as a label for an item or substance.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 87/00242

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: G 09 F 3/00; // C 12 Q 1/68

## II. FIELDS SEARCHED

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| IPC[4] | G 09 F |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

| Category [10] | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| Y | US, A, 3861886 (MELOY) 21 January 1975 see column 3, lines 36-55; column 7, lines 5-30; column 10, lines 5-35; column 11, lines 39-50 | 1,2,4-6, 17-19 |
| A | -- | 16 |
| Y | EP, A, 0133671 (MILES LABS. INC.) 6 March 1985 see pages 6-25 | 1,2,4-6, 17-19 |
| A | -- | 7-15,20-22 |
| A | US, A, 4390452 (MINNESOTA MINING & MANUFACTURING CO.) 28 June 1983 see the whole document | 1-4,16 |
| A | -- EP, A, 0111340 (INTEGRATED GENETICS, INC.) 20 June 1984 see the whole document | 1,2,4-15, 17-22 |
| A | -- US, A, 3733178 (ERIKSON) 15 May 1973 see column 1, lines 50-68; column 2, ./. | 1-4,16-19 |

* Special categories of cited documents: [10]

"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

"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

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 18th June 1987 | 28 JUL 1987 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | M. VAN MOL |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|---|---|---|
| | lines 57-65; columns 4-6 | |
| | -- | |
| A | US, A, 3772200 (MINNESOTA MINING & MANUFACTURING CO.) 13 November 1973 see abstract; column 6, lines 5-20 | 16 |
| | -- | |
| A | US, A, 4387112 (BLACH) 7 June 1983 see abstract | 1,2,4,16 |
| | -- | |
| A | US, A, 4363965 (SOBERMAN et al.) 14 December 1982 see abstract | 1,2,4,16 |
| | -- | |
| A | Nucleic Acids Research, volume 13, no. 3, February 1985, IRL Press Limited, (Oxford, GB), A.C. Forster et al.: "Non-radioactive hybridization probes prepared by the chemical labelling of DNA and RNA with a novel reagent, photobiotin", see pages 745-761 | |
| | -- | |
| P,A | WO, A, 86/02101 (BIOTECHNICAL LTD) 10 April 1986 cited in the application | |

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ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 87/00242 (SA 16773)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 08/07/87

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| US-A- 3861886 | 21/01/75 | None | | |
| EP-A- 0133671 | 06/03/85 | AU-A- | 3138784 | 07/02/85 |
| | | JP-A- | 60100056 | 03/06/85 |
| US-A- 4390452 | 28/06/83 | None | | |
| EP-A- 0111340 | 20/06/84 | JP-A- | 59122499 | 14/07/84 |
| | | US-A- | 4588682 | 13/05/86 |
| | | CA-A- | 1211058 | 09/09/86 |
| US-A- 3733178 | 15/05/73 | None | | |
| US-A- 3772200 | 13/11/73 | US-A- | 3897284 | 29/07/75 |
| US-A- 4387112 | 07/06/83 | None | | |
| US-A- 4363965 | 14/12/82 | None | | |
| WO-A- 8602101 | 10/04/86 | JP-T- | 62500423 | 26/02/87 |
| | | EP-A- | 0227678 | 08/07/87 |