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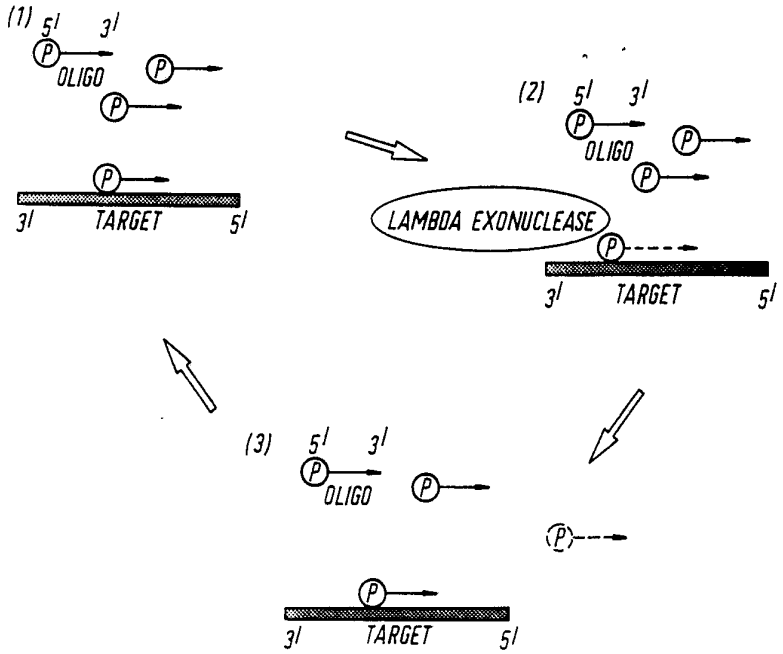

(57) Abstract

An assay method in which a target nucleic acid sequence is detected using a labelled probe comprising a complementary nucleic acid sequence under conditions where the target and probe hybridise and a nuclease requiring double stranded DNA for activity degrades the probe faster than the target. A cycling effect leads to large amount of degraded probe being formed, relative to the amount of target, providing an amplification of detectable degradation products. Also described are new oligonucleotides and kits.

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Assay for specific nucleic acid sequences

This invention relates to an assay method for the detection of specific nucleic acid sequences, to an optionally labelled oligonucleotide and to kits comprising components suitable for use in the method.

In recent years considerable research effort has been devoted to the development of assay methods for the detection of specific nucleic acid sequences. Such methods find wide application in the fields of medicine, veterinary science and food testing. In particular in the medical field they are used in the detection of microorganisms associated with diseases such as AIDS, syphilis and other sexually transmitted diseases and in the detection of genes or mutations associated with particular diseases such as cancer, cystic fibrosis and some cardiovascular diseases. They increasingly find application in identity testing. Their increasing application means that there exists a continuing need to develop new and improved assay methods.

Previously used assay methods depend upon the use of probes comprising nucleic acid sequences which are complementary to the specific nucleic acid sequences to be detected, i.e. together with the nucleic acid sequences to be detected the probe sequences will form sections of double stranded DNA. Thus the complementary nucleic acid sequences of the probes will hybridise with the corresponding nucleic acid sequences specific to the bacteria or other materials whose presence is to be detected and the occurrence of hybridisation is detected by a suitable means, frequently autoradiography or a fluorescence technique. For instance in order to detect strains of Salmonella in a mixed bacterial culture a probe comprising a nucleic acid sequence complementary to a sequence specific to Salmonella strains could be used and the hybridisation of the probe sequence with the target sequence in the Salmonella strains could be detected by a suitable means.

A significant disadvantage of many assay methods presently employed is that the detection systems which they use lead to a requirement for immobilisation of hybridised DNA. Methods which enabled the assays to be performed in solution would represent a considerable improvement. Additionally, several existing assay methods dependent upon the very sensitive polymerase chain reaction (PCR) technique are often complicated, requiring many cycles comprising several temperature changes over an extended period of time.

According to the present invention we provide an assay method for the detection of a specific nucleic acid sequence in which a medium is tested to detect material comprising a target nucleic acid sequence by contacting it with a probe comprising a nucleic acid sequence complementary to the target sequence, under conditions such that hybridisation of any target and probe complementary sequences occurs to produce double stranded nucleic acids, wherein the hybridisation occurs in the presence of a nucleolytic agent, which agent requires double stranded nucleic acids for activity, and which agent degrades the probe when hybridised with the target sequence to produce a degradation product, and detecting any degradation product.

It is not essential for the nucleolytic agent to be added to the medium before the target has been contacted with the probe, but for convenience it is preferred.

The nucleolytic agent is preferably an exonuclease but other agents, chemical or enzymatic agents, which degrade the hybridised probe faster than the target can be envisaged. As will be appreciated, the faster the hybridised probe is degraded relative to the target the more degraded probe will be produced, and for this reason it is preferred that the probe is degraded at least twice as fast, more preferably at least ten times, especially more than one hundred times faster than the target. It is most preferred that the target is not substantially degraded by the nucleolytic agent in the region at which the probe hybridises.

Hereinafter the invention will be described in terms of an assay method using an exonuclease as nucleolytic agent, that is to say using an exonucleolytic agent.

The exonucleolytic agent used in the assay method of the invention may be any exonuclease which requires double stranded nucleic acids for activity and degrades the hybridised probe faster than the target to which it is hybridised.

A preferred exonuclease is lambda exonuclease, a readily available exonuclease, but other exonucleases, including 5' to 3' and 3' to 5' exonucleases, are also suitable. Lambda exonuclease is a 5' to 3' exonuclease requiring a 5' phosphate group and double stranded nucleic acids for activity. We expect that oligonucleotides (with 5' phosphate groups) are only be degraded by lambda exonuclease to their constituent mononucleotides if they are annealed to their complementary sequences to form double stranded nucleic acids. Other suitable exonuclease enzymes may require other terminal groups for example 3' phosphate groups or hydroxyl groups for activity.

A preferred assay method of the invention is for detecting the presence or absence of a target nucleic acid sequence in a medium which comprises the steps of:

(a) adding an oligonucleotide probe, which is preferably labelled, to

the medium, which probe comprises a nucleic acid sequence complementary to the target, and the probe hybridises with the target when present;

(b) degrading the hybridised probe by means of an exonuclease which requires double stranded nucleic acids for activity and degrades the hybridised probe faster than said target; and

(c) detecting the presence or absence of degraded probe.

The method used for detecting the presence or absence of degraded probe will depend upon the nature of the probe, and suitable methods will be apparent to persons skilled in the art. For example where the probe is labelled by a fluorophore or chemiluminescent label then degraded probe may be detected by observing its

fluorescence or luminescence; similarly, where the probe is labelled using a radioactive label degraded probe can be detected using known techniques such as autoradiography.

When an excess of a suitable probe oligonucleotide is mixed with a target nucleic acid sequence, such as DNA, in the presence of an exonuclease in the medium of the assay method of the invention a cycling effect occurs which enables a small amount of a target nucleic acid sequence to produce a relatively large quantity of degraded probe oligonucleotide sequences. This produces an amplification effect which is an important advantage of the method of the invention.

This amplification effect is illustrated diagrammatically for lambda exonuclease by the three steps shown in Figure 3 of the accompanying drawings in which:-

In step (1) a target comprising the target nucleic acid sequence to be detected is shown in the presence of probe oligonucleotides having 5' phosphate groups and comprising complementary probe nucleic acid sequences, one of which probe oligonucleotides has hybridised with the target sequence.

In step (2) a lambda exonuclease attacks the probe component of the double stranded DNA formed by the hybridised target and probe, degrading the hybridised probe but leaving the target intact.

In step (3) the degraded probe is released from the target and a further undegraded probe oligonucleotide hybridises with the target sequence, and the cycle is repeated. The various fragments of the degraded probe are detected by a means available during operation of the method.

In carrying out the assay method of the invention, for example as part of a medical test, a urogenital swab or a blood or urine sample or other biological sample can be taken, subjected to suitable further treatment, for instance by heating, and then made up into a suitable medium for treatment by the assay method. The assay method is then carried out to produce an effect which can be detected

by suitable means. The exact conditions, e.g. temperature, at which the assay method is carried out will depend upon the exonuclease enzyme which is used, for instance when lambda exonuclease is used a suitable temperature is 37°C and a suitable pH is 9.

Any suitable means may be used to detect the various fragments of the degraded labelled probe produced in the assay method of the invention.

The probe preferably comprises 10 to 100 bases, more preferably 15 to 50, especilly 20 to 30 bases. As will be understood the term "bases" refers to the bases found in nucleic acids, such as U, A, T, G and C.

When the enzyme degrades in the 5' to 3' direction a label should be at or near the 3' terminus whilst when the enzyme degrades in the 3' to 5' direction a label should be at or near the 5' terminus.

As will be appreciated, the probe is labelled in such a way that it is detectable, for example using any of the labels used in biochemistry, such as by a chemiluminscer or fluorophore, a radioactive label, or other detectable chemical substance such as an active substrate, co-factor or enzyme donor peptide for a second enzyme system. The radioactive label preferably contains a radioactive isotope of a carbon, nitrogen, sulphur, iodine or phosphorus atom, particularly ^{32}P, which may form part of a nucleotide. An example of a chemiluminescser is luciferin, and fluorescein is an example of a fluorophore.

Suitable means for detecting the degradation of probe oligonucleotides in the assay method include separation of the products on polyacrylamide gels and preferably either:

(a) directly detecting degradation by the incorporation of a fluorophore on a suitable terminal nucleotide (3' in the case of lambda exonuclease) and measuring the change in fluorescence polarisation as the probe containing the fluorophore is degraded from large to small [for example, as described in our European Patent Application EP 0382433]; or

(b) designing the probe oligonucleotide such that degradation results in release of an active substrate, co-factor or enzyme donor peptide for a second enzyme system: such as the release of NAD followed by a standard oxidation-reduction reaction, or the release of a peptide derived from beta-galactosidase which combines with an apoenzyme to give the holoenzyme capable of generating a signal from appropriate substrate [for example as described in Clin. Chem., 32/9, 1637-41, (1986)].

The assay method of the invention can be used to detect specific sequences on either double or single stranded nucleic acid.

In the case of a double stranded target the specific sequence must be made available for hybridisation to the probe oligonucleotide, this can be achieved by conventional methods such as denaturation. The use of additional oligonucleotides either side of the oligonucleotide probe, known in the art as "blocking oligonucleotides", can alleviate the potential problem of target which is normally double stranded re-annealing. The use of multiple probe oligonucleotides can also serve to enhance the sensitivity of the assay method as well as (if arranged consecutively) preventing re-annealing of a double stranded target.

We believe that lambda exonuclease may have some activity against single-stranded DNA, although this is far less than the activity against double-stranded DNA (Sriprakash et al, J. Biol Chem. 250, 5438-45, 1975). Our experiments would suggest that this activity arises because of short regions of self-complementarity, particularly at the 5' end, in some oligonucleotide sequences. Thus this "single-stranded" activity can be minimised by careful oligonucleotide design, avoiding even short regions of self-complementarity at the 5' end.

The assay method of the invention has the advantage that it does not require the use of a large number of temperature changes over an extended period of time.

According to a further aspect of the invention there is provided a kit for detecting the presence or absence of a target nucleic acid sequence which comprises:

(1) a probe comprising a nucleic acid sequence complementary to a region of the target nucleic acid sequence; and

(2) a nuclease which requires double stranded nucleic acids for activity and which degrades the probe, when hybridised with the target, faster than said target.

It is preferred that the kit also contains an appropriate pH buffer, or a protocol for carrying out the assay method of the invention or both. The precise nature of the pH buffer depends upon the properties of the nuclease, and will be selected such that it is capable of buffering a solution to a pH at which the nuclease is active. For example, a kit containing a nuclease requiring a pH of 9 for optional activity would preferably contain a pH buffer which maintain a pH of 8 - 10, especially pH 8.5 to 9.5, more especially around pH 9.

The preferred probe and nuclease are as hereinbefore described in relation to the assay method of the invention.

Brief description of the drawings

Figure 1A is an autoradiograph showing the effects of varying lambda exonuclease concentrations (measured in Units) on varying quantities of oligonucleotide 1377.

Figure 1B is an autoradiograph showing the effect of varying lambda exonuclease concentrations on varying quantities of oligonucleotide 1125 (as defined hereinafter).

Figure 2 is an autoradiograph showing the effect of lambda exonuclease on single stranded oligonucleotide probe in the presence of varying amounts of its target complementary strand.

Figure 3 illustrates the amplification effect provided by the preferred assay method of the present invention. This Figure is hereinbefore discussed in more detail.

The invention is illustrated by the following Example in which a capital letter U means Units:-

Example

Materials and methods.

(1) 3' Labelling of Oligonucleotides

10 pmoles of oligonucleotide
1 microlitre of ^{32}P ddATP
1OU terminal transferase
in 30 microlitres 140 mM sodium cacodylate pH 7.5,
1 mM cobalt chloride
100 micromoles dithiothreitol
Incubated at 37°C for thirty minutes
Add 1OU of terminal transferase
100 pmoles ddATP
Incubated at 37°C for thirty minutes
Reaction stopped by the addition of 2 microlitres of 0.5M EDTA
Diluted with water to 0.025 pmole/microlitre

(2) 3' Blocking of Oligonucleotides

25 nmoles of oligonucleotide
25U terminal transferase
500 mmoles ddATP
in 1.0 ml 140 microlitres sodium cacodylate pH 7.5
1 mM cobalt chloride
100 micromoles dithiothreitol
Incubated at 37°C for sixty minutes
Reaction stopped by the addition of 5 microlitres 0.54 EDTA
Diluted with water to 250 pmole/microlitre,
5 pmole/microlitre and 0.25 pmol/microlitre

(3) Exonuclease Reaction Conditions

All reactions were carried out in 50 microlitres exonuclease buffer (67 mM glycine/sodium hydroxide pH 9.0, 2 mM magnesium chloride), and incubated for fifteen minutes at 37°C. Lambda exonuclease was added to these reactions at varying dilutions, also made in exonuclease buffer. Reactions were stopped by the addition of 25 microlitres of 95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol.

(4) Visualization of results

25 microlitres of the reaction products were heated to 75°C before electrophoresis on a 10% polyacrylamide, 0.25% bis-acrylamide gel, containing 4% urea. The gel was electrophoresed at 1500V for one hour before application of the reaction product. After application of the reaction product the gel was electrophoresed at 1500V for approximately two hours until good separation of the bromophenol blue marker and the xylene cyanol marker had been achieved. The gel was then fixed in 10% methanol, 10% acetic acid for twenty minutes before being dried onto a piece of filter paper. The gel was then autoradiographed overnight.

(5) Oligonucleotides used in this Example

Oligonucleotide 1125

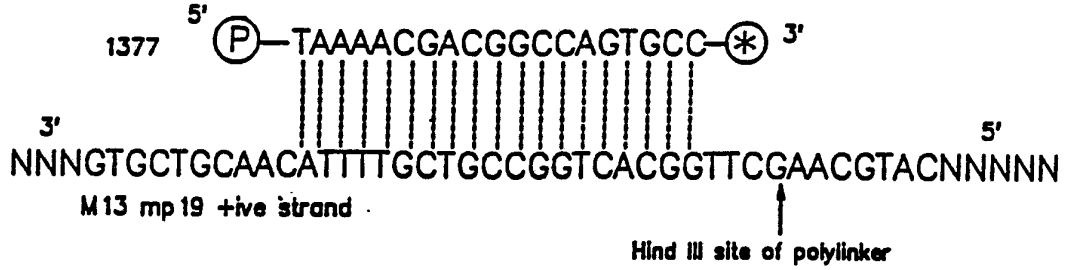
5' pCGAATCGTAACCGTTCGTACGAGAATCGCT 3'

Oligonucleotide 1377

5' pTAAAACGACGGCCAGTGCC 3'

Formula 1 illustrates the binding of labelled oligonucleotide 1377 on the positive strand of M13 mp19.

Formula 1



wherein the 5' encircled capital P is a phosphate group and the 3' encircled star is a label, for example ^{32}P-dd ATP or a fluorophore.

Both oligonucleotide 1125 and 1377 had 5' chemically attached phosphate groups and had been purified by application to a Mono Q column. Oligonucleotide 1125 is a Vectorette universal primer used in Chemical Genetics' UK Patent Application No.8818020.3.

Oligonucleotide 1377 is complementary to the positive strand of M13 mp19 DNA and has been specifically designed for use in

the exonuclease cycling assay (see Formula 1).

(6) Effect of Exonuclease on single stranded oligonucleotide

0.05 pmoles of labelled oligonucleotide was added to either 0.45 pmoles, 4.95 pmoles or 49.95 pmoles of 3' blocked oligonucleotide. Lambda exonuclease was added at concentrations of 10U, 1U, 0.1U, 0.01U or 0.001U. Digestion reactions and visualization of the products were carried out as described above.

(7) Effect of Exonuclease on single stranded oligonucleotide in the presence of the complementary strand

M13 mp19 single-stranded DNA (positive strand) was diluted to 2.5 pmole, 0.8 pmole, 280 fmole, 30 fmole, 10 fmole, 3.3 fmole, 1.1 fmole and 380 amole. 0.05 pmole of labelled 1377 and 4.95 pmole of 3' blocked 1377 were added to these along with 0.1U of lambda exonuclease. Digestion reactions and visualization of the products were carried out as described earlier.

The results obtained in the Example are described below.

Oligonucleotide Design:

Oligonucleotide 1125 is a PCR primer used in Chemical Genetics and was initially selected because it was available with a terminal 5' phosphate group. In the role for which it was designed it is preferred that 3' complementarity is not present, if the formation of primer dimers is to be avoided. However, in the assay described here 5' complementarity is to be avoided if non-specific degradation is not to be a problem. Examination of the structure of oligonucleotide 1125 reveals that the most stable configuration would contain 5' complementarity as shown in Formula (2):

Formula (2)

```
3'   TCGCTAAGAGCATGCTTGCCAATGCTAAGC      5'
       ||| ||     ||  ||     || |||
 5'    CGAATCGTAACCGTTCGTACGAGAATCGCT      3'
```

Furthermore, the Tm of this structure is predicted to be 48°C. Thus at the reaction temperature employed here (37°C) a large proportion of this oligonucleotide could be expected to be in a suitable form for degradation by lambda exonuclease.

Oligonucleotide 1377 was specifically designed from a 40 base sequence complementary to the positive strand of M13 mp19 by examining the self-complementarity of all available 20mer oligonucleotides in this region. Examination of the sequence of oligonucleotide 1377 reveals the most stable self-complementary structures have 5' overhangs, as shown in Formulae (3) and (4), and thus are relatively immune from degradation by lambda exonuclease:

Formula (3)

```
3'      CCGTGACCGGCAGCAAAAT      5'
        |  ||||  |
5'      TAAAACGACGGCCAGTGCC      3'
```

Formula (4)

```
3'           CCGTGACCGGCAGCAAAAT    5'
             |||   |||
5'      TAAAACGACGGCCAGTGCC         3'
```

Furthermore, the Tm of these structures is predicted to be 24°C, that is 13°C lower than the reaction temperature, and thus a large proportion of this oligonucleotide would be in single stranded form.

Effect of exonuclease on single-stranded oligonucleotide

Both oligonucleotides 1125 and 1377 were prepared with a 5' terminal phosphate group. This was achieved by conventional automated DNA synthesis on an ABI3 80B DNA synthesiser, using the reagent "phosphate-ON" (Clontech). Before use the oliginucleotides were radiolabelled by the addition of radioactive ddAMP to their 3' termini.

Figure 1A shows the effects of varying lambda exonuclease concentrations (measured in Units) on varying quantities of oligonucleotide 1377. There were 10 Units used in lanes 5 and 10; 1 Unit used in lanes 1, 6 and 11; 0.1 Unit used in lanes 2, 7 and 12; 0.01 Unit used in lanes 3, 8 and 13; 0.001 Unit used in lanes 4, 9 and 14; and no exonuclease in lane 15. Lanes 1 to 4 used 0.5

pmoles of oligonucleotide 1377, lanes 5 to 9 used 5pmoles of oligonucleotide 1377, and lanes 10-14 used 50 pmoles of oligonucleotide 1377. Lane X is not relevant.

From Figure 1A it can be seen that 5 pmoles of oligonucleotide 1377 is degraded by 1 Unit of lambda exonuclease, but not by 0.1 Unit (lanes 6 & 7).

Figure 1B shows the effects of varying lambda exonuclease concentrations on varying quantities of oligonucleotide 1125. There were 10 Units used in lane 1; 1 Unit in lane 2; 0.01 Unit in lane 3; 0.01 Unit in lane 4; 0.001 Unit in lane 5; and no exonuclease in lane 6. All lanes used 5pmoles of oligonucleotide 1125.

From Figure 1B it can be seen that 5 pmoles of oligonucleotide 1125 is at least partially degraded by as little as 0.001 Unit of exonuclease (lane 5). Thus careful design of the probe oligonucleotide, avoiding 5' self-complementarity, can minimise non-specific degradation.

Effect of exonuclease on single-stranded oligonucleotide in the presence of the complementary strand

Figure 2 shows the effect of lambda exonuclease on single-stranded oligonucleotide probe in the presence of varying amounts of its target complementary strand. In lanes 1 to 10 there was used 0.1 Units of lambda exonuclease, and in all lanes there were 5 pmoles of single-stranded probe oligonucleotide. The concentrations of target sequence in lanes 1 to 11 were as follows: lane 1, 2.5 pmoles; lane 2, 0.8 pmoles; lane 3, 280 fmoles; lane 4, blank; lane 5, 30 fmoles; lane 6, 10 fmoles; lane 7, 3 fmoles; lane 8, 1.1 fmoles; lane 9, 380 amoles; lane 10, none; lane 11, none and no exonuclease.

We determined the maximum enzyme concentration under which minimal non-specific degradation of the probe oligonucleotide (1377) occurred: 5 pmoles probe oligonucleotide; 0.1 Units of exonuclease (Figure 1A, lane 7). These conditions were used to examine the

effect of a range of concentrations of target sequence on the degradation of the probe oligonucleotide in the presence of lambda exonuclease (Figure 2). Specific degradation was detectable in the presence of 1.1 fmoles of target (Lane 8); apparently similar levels of degradation were detectable for amounts of target ranging from 2.5 pmoles to 30-10 fmoles (Lanes 1, 2, 3, 5 & 6). These amounts of target are 166-500 fold less than the amount of probe oligonucleotide, indicating that cycling, as outlined in Figure 3, is occurring.

In our experiments only 1 % of the probe oligonucleotide was radiolabelled; increasing the proportion of labelled probe and optimisation of the conditions should allow the sensitivity of this novel assay to be significantly increased.

CLAIMS

1. An assay method for the detection of a specific nucleic acid sequence in which a medium is tested to detect material comprising a target nucleic acid sequence by contacting it with a probe comprising a nucleic acid sequence complementary to the target sequence, under conditions such that hybridisation of any target and probe complementary sequences occurs to produce double stranded nucleic acids, wherein the hybridisation occurs in the presence of a nucleolytic agent, which agent requires double stranded nucleic acids for activity, and which agent degrades the probe when hybridised with the target sequence to produce a degradation product, and detecting any degradation product.

2. An assay method according to Claim 1 wherein the nucleolytic agent is added to the medium after the target has been contacted with the probe.

3. An assay method according to any one of the preceding claims wherein the nucleolytic agent is an exonuclease.

4. An assay method for detecting the presence or absence of a target nucleic acid sequence in a medium which comprises the steps of:

(a) adding an oligonucleotide probe to the medium, which probe comprises a nucleic acid sequence complementary to the target, and

the probe hybridises with the target when present;

(b) degrading the hybridised probe by means of an exonuclease which requires double stranded nucleic acids for activity and degrades the hybridised probe faster than said target; and

(c) detecting the presence or absence of degraded probe.

5. An assay method according to Claim 3 or Claim 4 wherein the exonuclease is lambda exonuclease.

6. An assay method according to any one of the preceding claims wherein the probe is labelled by a chemiluminescer, a fluorophore, a radioactive label, or other detectable chemical substance.

7. An assay method according to Claim 6 wherein the probe is labelled by a radioactive label.

8. A kit for detecting the presence or absence of a target nucleic acid sequence which comprises:

(1) a probe comprising a nucleic acid sequence complementary to a region of the target nucleic acid sequence; and

(2) a nuclease which requires double stranded nucleic acids for activity and which degrades the probe, when hybridised with the target, faster than said target.

9. A kit according to Claim 8 in which the nuclease is an exonuclease.

FIG. 1A

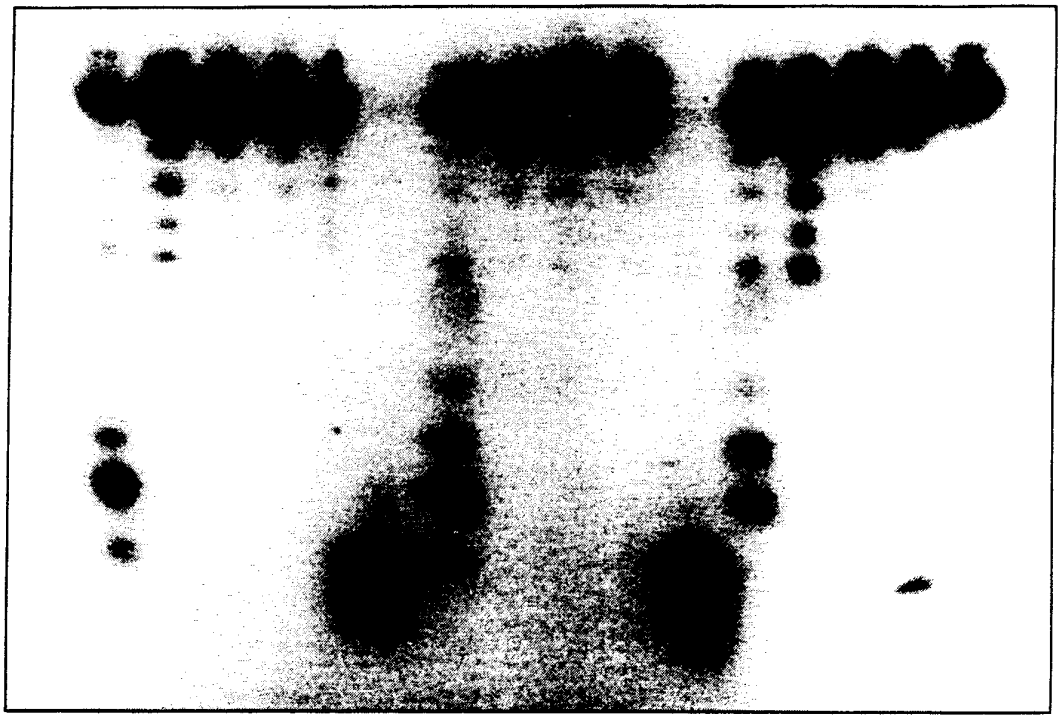


FIG. 1B

FIG.2

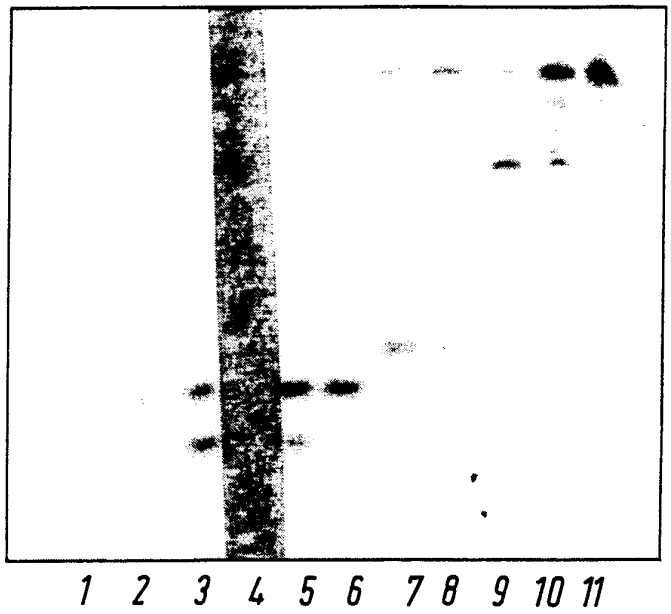
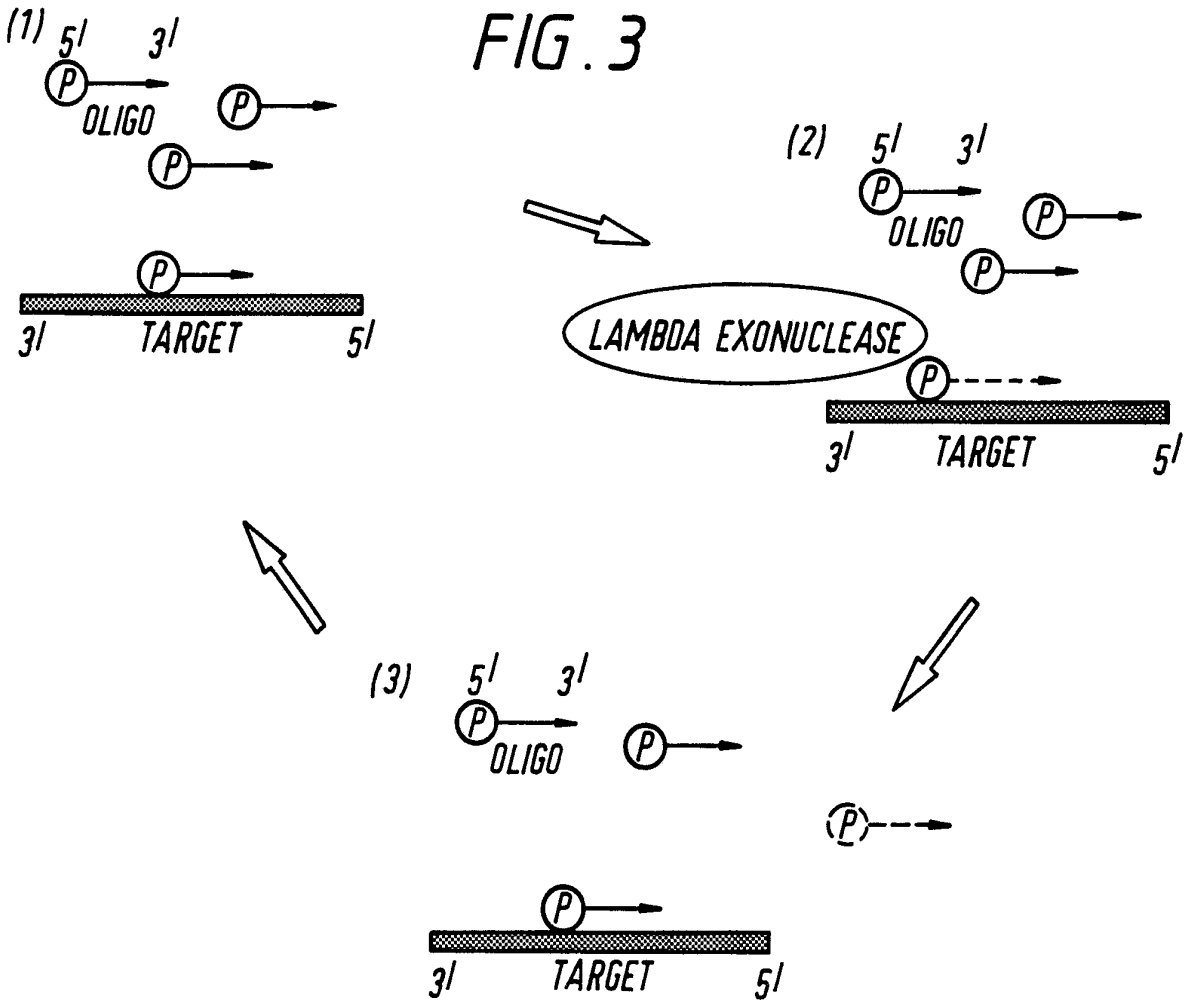


FIG.3



INTERNATIONAL SEARCH REPORT

International Applica. No PCT/GB 9100696

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

C 12 Q 1/68 C 12 Q 1/44 ·

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 12 Q

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 °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	US-A-4 725 537 (FRITSCH et al.) 16th February 1988, see abstract; claim 1 ---	1,2,4,6 -8
X	US-A-4 775 619 (URDEA) 4th October 1988, see abstract; claim 1 ---	1,2,4,6 -8
A	EP-A-0 200 057 (ALLIED CORP) 5th November 1986, see abstract; claims 1-8 ---	1,2
X	WO-A-8 909 284 (UNIVERSITY OF IOWA RESEARCH FOUNDATION) 5th October 1989, see abstract; figure 2 ---	1,2,4,6 -8
X	WO-A-8 910 415 (MEIOGENICS INC.) 2nd November 1989, see abstract; figure 1 ---	1,2,4,6 -8
X	EP-A-0 142 299 (FUJIREBIO K.K.) 22nd May 1985, see abstract; claim 1 --- -/-	1,2,4,6 -8

° Special categories of cited documents :[10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23-07-1991	19. 09. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Danielle van der Haas

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.
X	EP-A-0 123 513 (AMERSHAM INT. Plc.) 31st October 1984, see abstract	1,2,4,6 -8

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9100696 SA 47186

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 06/09/91
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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