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(54) Title: A METHOD AND REAGENT COMBINATION FOR THE DIAGNOSIS OF MICROORGANISMS BY SANDWICH HYBRIDIZATION OF NUCLEIC ACIDS

(57) Abstract

Microbial diagnostic method based on a sandwich hybridization of nucleic acids on a solid carrier, as well as a reagent combination for utilization in the method. The method described in the invention can be used to identify, from a single sample containing nucleic acids of the microbes or microbial groups to be diagnosed, after first rendering the said nucleic acids single-stranded, all the microbes or microbial groups by adding to the sample two nucleic acid reagents for each microbe or microbial group to be diagnosed, of which one nucleic acid reagent is attached to a solid carrier in the single-stranded form and the other contains a completely different nucleic acid fragment, from the same microbe or microbial group, which is labelled with an established marker. The recognizing nucleic acid fragment hybridizes to a complementary single-stranded nucleic acid from the sample and the hybrid thus formed on the particulate carrier becomes labelled when the complementary labelled nucleic acid fragment anneals to the single-stranded nucleic acid originating from the sample. Because the labelled nucleic acid reagents do not alone hybridize with the solid carrier, only those carriers containing nucleic acid reagents corresponding to nucleic acids in the sample become labelled. The label is measured using established methods.

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A METHOD AND REAGENT COMBINATION FOR THE DIAGNOSIS OF MICROORGANISM
BY SANDWICH HYBRIDIZATION OF NUCLEIC ACIDS

The invention relates to a method for the diagnosis of

5   microbes based on sandwich hybridization of nucleic acids to
a solid carrier and to the combination of reagents used in
the method.

In traditional microbial diagnostics the presence of a

10   microbe in a given sample is demonstrated by isolation of
the microbe in question. After enrichment cultivation the
microbe is identified either on the basis of its biochemical
properties or using immunological methods. This type of
diagnosis requires that the microbe in the sample is viable.

15   Identification by isolation is moreover a laborious method,
which in the case of viruses may require 4 - 6 weeks.

The purpose of this invention is to provide a diagnostic
method in which the presence of a microbe in a sample is

20   demonstrated by the identification of its genetic material,
the nucleic acid, with the aid of the sensitive and specific
nucleic acid hybridization technique. In itself, nucleic
acid hybridization is an old and well known method for
investigating the identity of nucleic acids. Complementary

25   nucleic acid strands have the ability to form a tight double-
stranded structure according to the rules of base pairing,
and the resulting hybrid can be separated from the residual
single-stranded nucleic acid.

30   Some methods based on the identification of nucleic acid(s)
have already been applied to microbial diagnostics.
Enterotoxigenic Escherichia coli have been identified from
faecal samples by colony hybridization using the gene for
toxin production as a probe. Positive hybridization is

35   demonstrated by autoradiography (Moseley, S.L. et al., J.
Infect. Dis. (1980) 142, 892-898). Colony hybridization is

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based on the method originally developed by Grünstein and
Hogness (Proc. Natl. Acad. Sci. USA (1975) 72, 3961-3965).
Hybridization has also been used as a method to distinguish
between Herpes simplex virus type 1 and type 2 (Brautigam,
5   A.R. et al., J. Clin. Microbiol (1980) 12, 226-234),
however, not in rapid diagnostics but in typing of the virus
after enrichment cultivation. In this method the double-
stranded hybrid is separated from the fraction of nucleic
acids remaining single-stranded in the solution by affinity
10   chromatography.

It was recently published that DNA from cells infected with
Epstein-Barr virus (the sample) was after appropriate
pretreatment fixed directly onto the filters. The nucleic
15   acid in question is identified by hybridizing the filters
with the radioactive probe and positive hybridization is
detected by autoradiography (Brandsma, I. and Miller, K.
(1980) Proc, Natl. Acad. Sci. USA 77, 6851-6855).

20   A similar method as above is described in the DE-patent
publication No. 2,950,295 and in the Lancet Oct. 10, 1982,
pp. 765-767. In the DE-patent publication No. 2,950,295 a
method for the production of a radioactively labelled DNA
probe by aid of recombinant DNA techniques is described.
25   This DNA probe is made from the hepatitis B virus. In the
Lancet the use of this probe in a method to detect hepatitis
B virus is described. In this method the DNA from the sample
is transferred to a nitrocellulose filter and the fixed
hepatitis DNA from the sample is detected by using the probe
30   as a reagent.

Because two reagents are used, our method is much more
specific than the method described above and makes it
possible to construct different kits by aid of which
35   different microbes or microbial groups can be detected from
the same sample without disturbing the specifity of the test

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for each microbe or microbial group in question. In the
method accroding to the invention, two different nucleic
acid fragment reagents - one attached to a solid carrier,
the other labelled with a suitable label - are used and the
5 sample DNA is in the liquid state.

The invention described herein is based on the sandwich
hybridization technique (Dunn, A.R. and Hassell, J.A. (1977)
Cell 12, 23-36), which simplifies the handling of the sample
10 and the detection of the hybrid. For this reason the
technique is particularly suitable for diagnostic use.

In the method described in this invention all the desired
microbes or microbial groups can be identified from one and
15 the same sample that contains denatured single-stranded
nucleic acid strands of the microbes or microbial groups to
be identified, without sample division. The method requires
two nucleic acid reagents for each microbe or group of
microbes to be identified. The reagents are two separate
20 nucleic acid fragments derived from the genome of the
microbe to be identified, which have no sequence in common,
but preferably are situated close to each other in the
genome. The reagents can be prepared directly from the
microbial genomes or by using the established recombinant
25 DNA techniques. Of the two nucleic acid fragments, one is
fixed to a solid carrier, preferably a nitrocellulose
filter, after being denatured, and the other also in
single-stranded form is labelled with a suitable label. When
these nucleic acid reagents, two different reagents for each
30 microbe or microbial group to be identified, are placed in
contact with the single-stranded nucleic acids to be
identified in the sample, the nucleic acids anneal to the
complementary nucleic acid fragments on the solid carrier.
The hybrids thus formed on the carrier become labelled after
35 annealing to the labelled complementary nucleic acid frag-
ments. The labelled nucleic acid fragments do not alone

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hybridize to the nucleic acid fragments attached to the
carrier, but only to the corresponding single-stranded
nucleic acids originating from the sample. Thus only those
carriers to which the complementary nucleic acids from the
5   sample have hybridized can become labelled. These carriers
are easily washed and the label measured by established
methods.

The method according to this invention can in principle be
10 used for the identification of all either DNA- or RNA-
containing organisms, such as viruses, bacteria, fungi and
yeasts. The method has the specific advantage of permitting
identification of all the bacteria and viruses possibly in
question at the same time and from the same sample,
15 regardless of whether the microbes contain DNA or RNA. By
suitable combination of reagents it is possible to develop
"kits" such as each microbe to be identified has its own
solid carrier equipped with a tag and labelled nucleic acid
reagent. All the filters included in the reagent combination
20 can be added to the sample simultaneously, along with the
labelled nucleic acid reagents. When hybridization has taken
place, the solid carriers are washed and their labelling is
measured. The only carrier to become labelled is the one
containing sequences complementary to the microbial genome
25 in the investigated sample.

The combination of method and reagents can be used eg. in
medical microbiology, veterinary microbiology, food hygiene
investigations and microbial diagnostics of plant diseases.
30 Suitable sample materials are eg. all animal and plant
tissue homogenates, patient secretions such as blood, faeces
and nasal and urethral mucous. It can be estimated that the
method is sufficiently sensitive to detect the microbe
levels normally present in clinical samples. Preliminary
35 enrichment of the microbe present in the sample by
cultivation is of course possible before the identification

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test and in some cases would be essential. The method is
also suitable for the investigation of samples from which
the microbe can no longer be cultivated, but which contain
considerable amounts of microbial debris (eg. after the
5   commencement of antibiotic treatment), or when cultivation
of the microbe is particularly laborious and difficult (eg.
anaerobic bacteria, which are present in large numbers in
suppurative samples in the case of infections caused by
anaerobes.

10
The method could be adapted in the form of the following
reagent combinations, "kits", parts of which could of course
be used separately:

15   Respiratory infections:

     a)   Bacteria: β-haemolytic streptococci (A-group),
             Haemophilus influenzae, Pneumococci, Mycoplasma
             pneumoniae, mycobacteria

20
     b)   Viruses: Influenza A, Influenza B, Parainfluenza 1-3
             Respiratory syncytial virus, adenoviruses, corona
             viruses, rhinoviruses

25   Diarrhoeas:

     a)   Bacteria: salmonellae, shigellae, Yersinia entero-
             colitica, enterotoxigenic E. coli, Clostridium
             difficile, campylobacteria

30
     b)   Viruses: rotaviruses, parvoviruses, adenoviruses,
             enteroviruses

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Veneral diseases:

a)   Bacteria: <u>Neisseria gonorrhoeae</u>, <u>Treponema pallidum</u>,
        <u>Chlamydia trachomatis</u>

5

b)   Viruses: Herpex simplex-virus

c)   Yeasts: <u>Candida albicans</u>

10 d)   Protozoa: <u>Trichomonas vaginalis</u>

Sepsis:

a)   Bacteria: β-haemolytic streptococci (A-group),
15   pneumococci, enterobacteria as a single group

Food hygiene:

a)   Bacteria: Salmonella and <u>Clostridium perfringens</u>
20

Depending on the choice of reagents the specificity of the
test can be limited to a defined microbial genus (eg.
salmonellas) or to a whole microbial group eg. entero-
bacteriaceae by choosing identifying reagents from the area
25 of a common gene.

The nucleic acid reagents required in the sandwich
hybridization technique described in this invention are
produced by recombinant DNA technology. In the following the
30 reagent production and test procedure for example 1 are
described.

Reagents

35 Adenovirus type 2 (strain deposited at KTL, i.e. the
National Public Health Institute, Helsinki) was cultivated

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and purified and DNA was isolated (Petterson, U. and
Sambrook, J. (1973) J. Mol. Biol. $\underline{73}$, 125-130) (referred to
hereafter as Ad_2-DNA). The DNA was digested with BamHI-
restriction enzyme (BRL, i.e. Bethesda Research

5   Laboratories) that cuts the DNA into four reproducable
fragments. Of these four fragments two were inserted to the
BamHI-site of the vector plasmid pBR 322 (BRL) with the aid
of T4-ligase (BRL). (The fragments were not separated before
ligation, but the insert added to the plasmid was in each

10 case identified only after cloning). Subsequently the
bacterial host ($\underline{E.\ coli}$ HB101 (K12) gal⁻, pro⁻, leu⁻, hrs⁻,
hrm⁻, recA, str^r, F⁻) (obtained from KTL) was transformed
with the plasmid DNA composed of recombinant plasmids, i.e.
molecules that had accepted fragments of the adenovirus-DNA

15 (Cohen, S.N. et al. (1972) Proc. Natl. Acad. Sci. USA $\underline{69}$,
2110-2114). Among the transformed bacterial clones such as
most probably contained the recombinant plasmid were chosen.
Ampicillin and tetracycline resistance genes are transferred
to the bacterium by the pBR322-plasmid (Bolivar F. et al.

20 (1977) Gene $\underline{2}$, 95-113). Bacteria containing the recombinant
plasmid are, however, sensitive to tetracycline, because the
BamHI-restriction site is within the tetracycline gene and
the foreign DNA inserting into this region destroys the
gene. The insert of the plasmid was characterized after

25 plasmid enrichment by determining the size of the
restriction fragments after BamHI digestion using agarose
gel electrophoresis. The adjacent BamHI D- and C-fragments
of the Ad_2-DNA (cf. gene map) were chosen as reagents
(Söderlund, H. et al. (1976) Cell $\underline{7}$, 585-593). The desired

30 recombinant plasmids, Ad_2C-pBR322, KTL No. E231 and
Ad_2D-pBR322, KTL No. EH230, were cultivated and purified as
has been described in the literature (Clewell, D.B. and
Helinski, D.R. (1969) Proc. Natl. Acad. Sci. USA $\underline{62}$,
1159-1166).

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The recombinant plasmid Ad$_2$D-pBR322 was used as the filter
reagent. It is not necessary for the present application to
remove the plasmid sequences, because the sample does not
contain pBR322-sequences. However, for radioactive labelling
5   the nucleic acid was separated from pBR322-DNA after BamHI-
digestion with the aid of agarose gel electrophoresis. The
C-fragment was isolated from LGT-agarose (Marine Colloids,
Inc.) by phenol extraction or electro-elution (Wieslander,
L. (1979) Anal. Biochem. 98, 305-309) and concentrated by
10   ethanol precipitation.

It is particularly expedient to subclone the nucleic acid
fragment chosen for labelling in a separate vector, in order
to avoid the hybridization background resulting from the
15   direct hybridization with the filter of the residual plasmid
sequences, contaminating the labelled nucleic acid reagent.
The single-stranded DNA-phage M13 mp7 (BRL), to which DNA
fragments obtained by BamHI-digestion can easily be
transferred, could be used as an optimal vector (Messing, J.
20   et al. (1981) Nucleic Acids Res. 9, 309-323).

Attachment of DNA to the filter

The recombinant plasmid Ad$_2$D-pBR322 was denatured to a
25   single-stranded form and nicked randomly at several sites by
treatment with 0.2 N NaOH (5 min. 100°C), whereafter the DNA
was chilled and, immediately prior to transference to the
filter, neutralized and pipetted to the transfer solution,
4 x SSC medium on ice (SSC = 0.15 M NaCL, 0.015 M Sodium
30   citrate). The filters (Schleicher and Schüll BA85 nitro-
cellulose) were thoroughly wetted in 4 x SSC solution (about
2 h) before the application of DNA. The DNA is attached to
the filter in a dilute solution (0.5-1.0 μg/ml) by sucking
the solution through the filter in a weak vacuum. The filter
35   is capable of absorbing DNA up to about 180 μg/cm^2 (Kafatos,
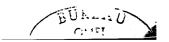F.C. et al. (1979) Nucleic Acids Res. 7, 1541-1552). We have

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used DNA-concentrations of between 0.5 μg DNA/2.5 cm
diameter of filter and 1.0 μg DNA/0.7 cm diameter of filter.
After DNA-filtration the filters are washed in 4 x SSC,
dried at room temperature and finally baked in vacuum oven
5   at 80°C for 2 h, after which the DNA on the filters remains
stable and the filters can be stored for long periods at
room temperature (Southern, E.M. (1975) J. Mol. Biol. 98,
503-517).

10   Labelling of the radioactive nucleic acid fragment

The radioactive label used was the ^{125}I-isotope. This
isotope can be detected using γ-counters, which are
available in most large laboratory units. The half-life of
15   the isotope is 60 days, for which reason the utilization
period of ^{125}I-labelled reagents is about 4 months.

"Nick-translation" labelling

20   The principle of this method is to displace one of the
nucleotides in the nucleic acid with a radioactive one, upon
which the whole DNA molecule becomes labelled. This is
carried out according to the method published by Rigby,
P.W.J. et al. (J. Mol. Biol. (1977) 113, 237-251). In the
25   reaction the DNA becomes radioactively labelled when the
solution contains ^{125}I-labelled deoxynucleoside triphosphate
as substrate, in this case ^{125}I-dCTP (Radiochemical Centre,
Amersham: >1500 Ci/mmol). Under optimal conditions a
specific activity of 10^9 cpm/μg DNA can be obtained. The
30   labelled DNA is purified from nucleotides remaining in the
reaction mixture by simple gel filtration, eg. using BioGel
P30 (BioRad).

10

Other labelling methods

The single-stranded nucleic acid reagent produced in M13
mp7-phage is labelled by chemical iodination, in which the
5   reactive ^{125}I is added covalently to the nucleic acid
(Commenford, S.L. (1971) Biochemistry 10, 1993-2000, Orosz,
J.M. and Wetmur, J.G. (1974) Biochemistry 13, 5467-5473).
Alternatively, the nucleic acid can be made radioactive by
endlabelling with radioactive nucleotides by the terminal
10   transferase (Roychoudhury, R. and Wu, R. (1980) Meth.
Enzymol. 65, 43-62).

The reagent preparation described above relates to microbes
of which the genetic material is in the form of DNA. In the
15   case of RNA viruses the cloning of genome fragments takes
place in such a way that at first a DNA copy (cDNA) of the
virus RNA has been made with the aid of reverse tran-
scriptase, followed by DNA-polymerase to copy the second DNA
strand, thereafter the DNA is cloned as described above
20   (Salser, W. (1979) in Genetic Engineering, Ed. A.M.
Chakrabarty, CRC Press, pp. 53-81).

The most suitable cloning method is chosen depending on the
microbe used. The hosts as well as the vectors can vary.
25   Possibilities include the λ-phage as vector, other plasmids,
cosmids, cloning eg. in Bacillus subtilis bacteria, etc.
(Recombinant DNA, Benchmarck Papers in Microbiology, Vol.
15, Eds. K.J. Denniston and L.W. Enqvist, Dowden, Hutchinson
and Ross, Inc. (1981); Ish-Horowicz, D. and Burke, J.F.
30   (1981) Nucleic Acids Res. 9, 2989-2998).

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Performance of the test

Sample treatment

5   The microbial nucleic acid to be investigated must be
     released from the microbe itself and also from the infected
     cells, after which it must be denatured to the single-
     stranded form. Virus genomes can be liberated by treating
     the sample material with 1 % sodium dodecylsulphate (SDS)
10   and destroying the proteins protecting the genome by
     proteinase K-treatment (1 mg/ml, 37°C, 60 min). Bacterial
     samples must in addition be broken down using lysozyme- and
     EDTA-treatment.

15   If the sample contains large quantities of viscous high-
     molecular weight cellular-DNA, this must be sheared at a few
     sites in order to reduce its viscosity, eg. by sonication or
     by passing the sample a few times through a fine needle.

20   Hybridization

     Hybridization takes place eg. in 50 % formamide (deionized,
     stored at -20°C), in 4 x SSC Denhardt solution (Denhardt,
     D.T. (1966) Biochem. Biophys. Res. Commun. 23, 641-646)
25   containing 1 % SDS and 0.5 mg/ml DNA (salmon sperm or calf
     thymus) at 37°C and usually overnight for 16-20 hours. The
     filters chosen for the test are incubated in a suitable
     vessel, to which the hybridization mixture is added and the
     hybridization is started. The hybridization mixture contains
30   (a) the pretreated sample to which is added the radioactive
     nucleic acid reagent(s) that have been denatured together by
     boiling for 5 minutes followed by quick cooling at 0°C; (b)
     concentrated formamide-, SSC- and Denhardt-solutions, which
     are pipetted to the denatured and cooled nucleic acid
35   mixture (a). After mixing, the hybridization mixture is
     pipetted to the filters in the hybridization vessel. After

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hybridization the filters are carefully washed and counted
individually in the Υ-counter.

The invention is clarified in the following with the aid of
5   some practical examples.

Example 1

Detection of adenovirus by the sandwich hybridization method
10 (Table 1)

The details of the test are clarified in the text to Table
1. The sandwich hybridization method can detect virus-DNA
from a solution, but the viral genome can equally well be
15 detected from infected cells.

The hybridization background is measured in a tube
containing only the filter and the labelled nucleic acid
reagent, without the sample. The background is caused by the
20 pBR322 sequences occuring in the labelled nucleic acid
reagent. These sequences hybridize directly with the filter
without the sample mediating it. The filters containing calf
thymus and no DNA are used in the test as controls,
indicating on the one hand the specificity of hybridization
25 and on the other the level of the nonspecific background
arising eg. from insufficient washing.

In the following tables the background due to the reagents
has been subtracted from the cpm-values hybridized to the
30 filters.

13

Table 1

Adenovirus test

Sample	Filters (cpm)		
	Adeno 1)	Calf thymus 2)	Blank 3)
Adenovirus type 2-DNA (BRL) (500 ng)	9000	49	-
HeLa-cells (6 x 10⁵) infected with adenovirus	8200	-	-

Filters:
1) Ad₂D-pBR322-plasmid, 2 µg
2) Calf thymus DNA 1. µg (Boehringer Mannheim)
3) Blank (no DNA)

Labelled nucleic acid reagent:
Ad₂-BamHI C-fragment, purified, specific activity
90 x 10⁶ cpm/µg (200 000 cpm ¹²⁵I/reaction)

Hybridization:
50 % formamide, 4 x SSC
Denhardt solution, containing 0.5 mg/ml salmon sperm DNA and
1 % SDS, 37°C, 16 h

Washing:
0.1 % SSC, room temperature, 40 min

Samples:
Adenovirus type 2 DNA (BRL)
Infection with type 2 adenovirus took place in HeLa-cells.
The cells were then disrupted by treatment with 1 % SDS,
followed by digestion with 1 mg/ml proteinase-K-enzyme
(Sigma) for 30 min 37°C. Before denaturation the sample was

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passed through a fine needle. The values appearing in the
table have been corrected by subtraction of the reagent
background, obtained by carrying out a similar hybridization
but without sample.

5

Example 2

Detection of an RNA-virus with the aid of sandwich
hybridization   (Table 2)

10

The model RNA-virus used was the Semliki Forest virus (proto-
type strain, obtained from the London School of Hygiene and
Tropical Medicine), of which the genome is single-stranded
RNA. Using the virus genome as a template cDNA was produced,
15 which was cloned into the PstI site of pBR322 plasmid as
described by Garoff et al. (Proc. Natl. Acad. Sci. (1980)
USA 77, 6376-6380). The recombinant plasmid thus obtained is
pKTH312 KTL No. EH 232. The insert of this plasmid
originating from the virus genome is about 1400 nucleotides
20 long and is from the structural protein area, approximately
from nucleotide 200 to nucleotide 1600 when numbering is
started from the beginning of the structural genes (Garoff,
H. et al. 1980). For the production of reagent the whole
recombinant plasmid pKTH312 was linearized with EcoRI
25 restriction enzyme (BRL) (the sequence originating from the
Semliki Forest virus does not contain recognition sites for
the EcoRI-enzyme), and the linearized plasmid was cut into
two fragments using XhoI-enzyme (BRL). The restriction site
of the latter was located with the Semliki Forest virus
30 sequence. The larger EcoRI-XhoI-fragment A (about 3900 base
pairs) was attached to the filter and the smaller fragment B
(about 1850 base pairs) was labelled with ^{125}I using the
nick translation technique.

15

Both free Semliki Forest virus and virus-infected cells were
used as samples in this test. In both cases the virus-
specific nucleic acids of the sample were composed entirely
of RNA.

5

Table 2

Detection of Semliki Forest virus with the aid of the
sandwich hybridization method

Sample	Filters (cpm)		
	Semliki Forest virus 1)	Calf thymus 2)	Blank 3)
Semliki Forest virus 30 µg	3340	–	33
Cells infected with Semliki Forest virus (5 x 10^5)	2698	8	10
Non-infected cells	10	5	8

Filters:
25 1) EcoRI-XhoI-fragment A (1.2 µg) of the pKTH312 plasmid
    2) Calf thymus DNA 1 µg
    3) Blank (no DNA)

Labelled nucleic acid reagents:
30 EcoRI-XhoI-fragment B of the plasmid pKTH312, specific
    activity 90 x 10^6 cpm/µg DNA (200 000 cpm ^{125}I/reaction).

Hybridization:
As described in Table 1

35

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Washing:
As described in Table 1

Samples:
5   Semliki Forest virus (30 μg) was disrupted with SDS before
the test. The infected cells were handled as described in
Table 1. The infection with Semliki Forest virus was carried
out in BHK-21 cells.

10 The values given in the table have been corrected for
reagent background, obtained from a similar hybridization
without sample.

Example 3

15

A virus sample in which the viral messenger RNA is detected
with the aid of the sandwich hybridization method   (Table 3)

The sandwich hybridization reagents were produced from SV40-
20 virus DNA (BRL) by cutting the DNA into two parts using
PstI-enzyme (Boehringer Mannheim) as described by Lebowitz
and Weissman (Curr. Topics in Microbiol. Immunol. 87,
43-172) and the fragments were isolated and purified by
agarose gel electrophoresis. Fragment A (4000 base pairs)
25 was radioactively labelled with ^{125}I by nick translation and
fragment B (1220 base pairs) was attached to the filter.

The DNA fragments were chosen so that each contained areas
coding for both early and late messengers. Thus fragment B
30 contains about 700 bases from the structural protein gene
VP1 and over 600 bases from the gene for early messengers.
Because the DNA of SV40 virus is in itself a covalently
closed ring, it cannot be detected by the test before
linearization. Therefore, when infected cells are used as
35 the sample it is possible to test how well the method is
adaptable to the detection of RNA copies of the viral

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genome. As can be seen from the results in Table 3, the test
is excellently suited to the investigation of infected
cells. The table also demonstrates that the same reagents
can be used to investigate both the viral DNA and mRNA made
5   from it.

Table 3

Detection of SV40-virus by the sandwich hybridization
10 technique

Sample	Filters (cpm)		
	SV40 1)	Calf thymus 2)	Blank 3)
Test 1 SV40 viral DNA (50 ng) (linearized)	20061	159	104
No sample	–	–	–
Test 2 CV1-cells infected with SV40-virus 40 h after infection (10^6 cells)	30814	294	580
Non-infected cells	–	–	–

Filters:
    1) The shorter fragment PstI B (0.2 µg) of the circular
30 SV40-virus DNA digested with PstI-restriction enzyme
    2) Calf thymus DNA 1 µg
    3) Blank (no DNA)

Labelled nucleic acid reagent:
35 The longer PstI A-fragment of the SV40-virus DNA, specific
    activity 28 x 10^6 cpm/µg DNA (200 000 cpm ^{125}I/reaction)

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Hybridization:
As described in Table 1
The hybridization time is 40 h

5   Washing:
As described in Table 1

Samples:
SV40-virus DNA (BRL) was linearized with EcoRI restriction
10 enzyme (BRL). CV1-cells (Biomedical Centre, Uppsala
University) were infected with SV40-virus (obtained from
Janice Y. Chou and Robert G. Martini, NIH, Bethesda) and the
cells were harvested 40 h after infection. Treatment of the
sample was as described in Table 1.
15

The values presented in the table have been corrected for
reagent background, obtained from a similar hybridization
carried out without sample.

20 Example 4

Detection of Bacillus amyloliquefaciens by sandwich
hybridization   (Table 4)

25 The reagents were fragments of the α-amylase gene of B.
amyloliquefaciens E 18 (Technical Research Centre of
Finland, VTT), which were isolated for the purpose of this
test from the recombinant plasmid pKTH10 (Palva, I. et al.
(1981) Gene, 15, 43-51) by treatment with restriction enzyme
30 and subsequent agarose gel electrophoresis. The fragments
used for this test were the ClaI-EcoRI fragment area of the
α-amylase gene (460 base pairs) (ClaI Boehringer Mannheim)
and the EcoRI-BamHI fragment (1500 base pairs). The
EcoRI-BamHI fragment was attached to the filter and the
35 ClaI-EcoRI fragment was radioactively labelled with ^{125}I by
nick translation.

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As can be seen from Table 4, the <u>B. amyloliquefaciens</u> in a sample was identifiable by sandwich hybridization on the basis of the single α-amylase gene. <u>E. coli</u> gave a negative result in this test (indistinguishable from the background).

Table 4

<u>Bacterial diagnostics by sandwich hybridization</u>

Sample	Filters (cmp)		
	α-amylase 1)	Calf thymus 2)	Blank 3)
pKTH10-plasmid-DNA (linearized) 1 μg	5773	47	-
No sample	-	-	-
<u>E. coli</u> HB101 (10^9)	-	-	-
<u>Bacillus amyloliquefaciens</u> (3×10^9)	3377	-	-
<u>Bacillus amyloliquefaciens</u> (10^9)	2871	-	-

<u>Filters:</u>
1) The EcoRI-BamHI fragment of the α-amylase gene from plasmid pKTH10, 0.35 μg
2) Calf thymus DNA, 1 μg
3) Blank (no DNA)

<u>Labelled nucleic acid reagent:</u>
The ClaI-EcoRI fragment of the α-amylase gene from plasmid pKTH10, specific activity 35×10^6 cpm/μg (200 000 cpm ^{125}I/reaction)

20

Hybridization:
As described in Table 1

Washing:
5 As described in Table 1

Samples:
Bacterial samples were treated with lysozyme (67 μg/ml) for
30 min at 37°C; 5 mM EDTA was added to E. coli samples, too.
10 After the treatment SDS was added to all the samples (final
concentration 2 %), which were then passed twice through a
fine needle to reduce their viscosity before being denatured
by boiling as described in the text relating to handling of
samples.

15

The values appearing in the table have been corrected for
reagent background, obtained from a similar hybridization
without sample.

20 Example 5

An example of a reagent combination kit based on the
sandwich hybridization method (Table 5)

25 The samples investigated in this test were cells infected by
three viruses (adenovirus, SV40 virus and Herpes simplex
virus) and a sample containing Bacillus amyloliquefaciens
bacteria. The following reagents were all simultaneously
added to each sample, 5 filters, each containing one type of
30 DNA from SV40 virus, adenovirus, Bacillus amyloliquefaciens
α-amylase gene and calf thymus, as well as a filter
containing no DNA at all; in addition 200 000 cpm of each of
the following labelled nucleic acid reagents: SV40 virus-,
adenovirus- and α-amylase gene DNA-reagent.

35

21

Our example shows that it is possible, without division or
dilution of the sample, to investigate simultaneously a
suitable series of microbes by adding the reagent
combination to the sample. The sample may contain both viral
and bacterial nucleic acid. The filters can be recognized by
a sign (= mark, tags), which identifies the sequence it
contains and tags, which microbe was attached/hybridized to
it. The signs can be numbers or letters, eg. 1 or SV40 2 or
Ad etc. or other signs as *for SV40 or Δ for AD or O for
Bacillus.

Table 5

A kit based on the filter hybridization technique

Sample	Filters (cpm)				
	SV40 1)	Adeno 2)	α-amylase 3)	Calf thymus 4)	Blank 5)
Cells infected with SV40 virus (10^6)	18390	2	13	22	31
Cells infected with adenovirus type 2 (6×10^5)	–	8750	5	13	–
Cells infected with Herpes simplex virus (10^6)	–	–	–	5	13
Bacillus amyloliquefaciens (10^9)	15	8	6500	16	5
Non-infected cells	–	–	–	–	–

22

Filters:
1) As in Table 3
2) As in Table 1
3) As in Table 4
5   4) Calf thymus DNA, 1 μg
5) Blank (no DNA)

Labelled nucleic acid reagents:
SV40 virus as in Table 3
10 Adenovirus as in Table 1
α-amylase gene as in Table 4

Hybridization:
As in Table 1

.15

Washing:
As in Table 1

Samples:
20 Cell samples infected with SV40 virus and adenovirus have
been described in Tables 3 and 1, respectively.

10^6 Vero cells were infected with Herpes simplex virus type
1. The cells were harvested 20 h post infection as
25 cytopathic effect could be observed. The sample was treated
as described for adenovirus infected cells (Table 1).

Bacillus amyloliquefaciens sample:
As in Table 4

30

The values in the table are corrected for reagent
background, obtained by carrying out a similar hybridization
without sample.

23

Example 6

Detection of Escherichia coli by sandwich hybridization
(Table 6)

5

The reagents were prepared from the ompA-gene (outer
membrane protein A -gene) of Escherichia coli.

The hybrid plasmids pKTH40 and pKTH45, used as starting

10   material, were prepared from the pTU100 plasmid described by
Henning et al. (1979) Proc. Natl. Acad. Sci. USA 76,
4360-4364.

The plasmid pKTH45 (deposited at KTL, i.e. National Public

15   Health Institute, Helsinki No. ...), used as a filter
reagent, was composed of 740 base pairs from 5' -terminal
end of the ompA-gene inserted into the pBR322-plasmid.

The plasmid pKTH40 contains 300 base pairs from the 3'

20   -terminal end of the ompA-gene and the immediately following
1700 base pairs from the genome of E. coli. The pKTH40
plasmid was cleaved with the BamHI restriction enzyme to
receive the DNA fragment of E. coli, which contains the 1700
base pairs mentioned above. This fragment was transferred to

25   the single-stranded bacteriophage M13mp7 according to the
methods described by Messing et al. (1981), Nucl. Acids Res.
9, 309-321, Heidecker et al. (1980), Gene 10, 69-73, Gardner
et al. (1981), Nucl. Acids Res. 9, 2871-2888. The recombi-
nation-phage mKTH1207 (deposited at KTL No. ...) was

30   labelled with ^{125}I-isotope as described on page 10 under the
heading "Other labelling methods" and was used as a probe in
the sandwich hybridization method.

DNA from disrupted E. coli cells, as well as isolated,

35   purified DNA from E. coli, can be detected by sandwich
hybridization as shown in Table 6.

24

Table 6

Detection of Escherichia coli by sandwich hybridization

Sample	Filters (cpm)		
	ompA 1)	Calf thymus 2)	Blank 3)
E. coli K12 HB101 DNA a) 2 x 10⁷	282	-	-
E. coli K12 HB101 DNA a) 2 x 10⁸	2206	-	-
E. coli K12 HB101 Cells b) 2 x 10⁷	1113	-	-
E. coli K12 HB101 Cells b) 2 x 10⁸	2327	12	5
a) number of DNA-molecules b) number of cells			

Filters:
1) pKTH45 plasmid 1.088 μg (2 x 10^{11} molecules)
2) Calf thymus DNA 1.088 μg
3) Blank (no DNA)

Labelled nucleic acid reagents:
mKTH1207, specific activity 8 x 10^7 cpm/μg DNA
(200 000 cpm/reaction)

Hybridization:
4 x SCC, 1 x Denhardt solution without BSA (bovine serum
albumin), 0.25 % SDS, 200 μg/ml Herring sperm DNA, 17.5 h,
+65°C

25

<u>Washing:</u>
As described in Table 1

<u>Samples:</u>

5   <u>E. coli</u> K12 HB101 -DNA was isolated according to the Marmur-
method described by Marmur (1961) J. Mol. Biol. <u>3</u>, 208-218.
DNA was denatured at 7mM NaOH, +100°C, 5 min.

The cells were treated with lysozyme (500 µg/ml), EDTA
10   (70 mM +37°C, 30 min), SDS (0.25 %, +65°C) and the free DNA
was denatured by boiling at 14 mM NaOH, +100°C, 5 min).

The values presented in the table have been corrected for
reagent background obtained from a similar hybridization
15   without sample.

PATENT CLAIMS

1. A microbial diagnostic method, based on sandwich
hybridization of nucleic acids on a solid carrier, to
5   identify microbes or microbial groups, in which method two
different nucleic acid reagents with no sequences in common,
but preferably situated close to each other in the genome of
the given microbe or microbial group, are used for the
identification of that microbe or microbial group in a given
10 sample, one of the nucleic acid reagents being a single-
stranded nucleic acid fragment attached to a solid carrier
and the other being a single-stranded nucleic acid fragment
labelled with a suitable marker, the method being
c h a r a c t e r i z e d   in that the nucleic acid
15 reagents are brought into contact with the nucleic acids of
.   all the microbes or microbial groups in the undivided
sample, where the sample nucleic acids are first, by known
methods, rendered single-stranded, upon which the sample
nucleic acids hybridize to the complementary nucleic acid
20 fragments, themselves attached to the solid carrier and the
carrier-attached hybrids become labelled due to the
subsequent annealing of labelled nucleic acid fragments to
the sample nucleic acid fragments, after which the carrier-
attached label is measured by established methods.
25

2. A reagent combination to be used in the method according
to patent claim 1,   c h a r a c t e r i z e d   in that it
includes two nucleic acid reagents for each microbe or
microbial group to be identified, and the necessary two
30 different nucleic acid fragments, which have been made from
different parts being preferably near each other of the
microbe or microbial group for the reagents, have been
produced either directly from the microbial genome or using
established recombinant DNA techniques, and these group- or
35 species-specific nucleic acid fragments have been rendered

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single-stranded, and one has been attached to a solid
carrier and the other labelled with a suitable marker.

3. A reagent combination according to claim 2,
5 c h a r a c t e r i z e d   in that for the identification
of adenovirus it includes the adenovirus recombinant plasmid
$Ad_2DpBR322$ as a solid carrier attached nucleic acid reagent
and the adenovirus Ad_2-BamHI C-fragment as a labelled
nucleic acid reagent.
10

4. A reagent combination according to claim 2,
c h a r a c t e r i z e d   in that for the identification
of Semlike Forest virus it includes the EcoRI-XhoI fragment
A of the pKTH312 plasmid as a solid carrier attached nucleic
15 acid reagent and the EcoRI-XhoI fragment B of the pKTH312
plasmid as a labelled nucleic acid reagent.

5. A reagent combination according to claim 2,
c h a r a c t e r i z e d   in that for the identification
20 of SV40 virus it includes the PstI B fragment of SV40 virus
as a solid carrier attached nucleic acid reagent and the
PstI A fragment of SV40 virus as a labelled nucleic acid
reagent.

25 6. A reagent combination according to claim 2,
c h a r a c t e r i z e d. in that for the identification
of Bacillus amyloliquefaciens it includes the EcoRI-BamHI
fragment of the α-amylase gene of the Bacillus
amyloliquefaciens plasmid pKTH10 as a solid carrier attached
30 nucleic acid reagent and the ClaI-EcoRI fragment of the
α-amylase gene of the Bacillus amyloliquefaciens plasmid
pKTH10 as a labelled nucleic acid reagent.

7. A reagent combination according to claim 2,
35 c h a r a c t e r i z e d   in that E. coli is identified
using the plasmid pKTH45 as a solid carrier attached nucleic

28

acid reagent and the recombination-phage mKTH1207 as a
labelled nucleic acid reagent.

8. A reagent combination according to claims 2, 3, 5 and 6,
5   c h a r a c t e r i z e d   in that for the identification
and distinction of different microbes, viruses as well as
bacteria, such as adenovirus, SV40 virus and <u>Bacillus
amyloliquefaciens</u>, from the same sample containing nucleic
acids originating from different microbes, the combination
10   includes the $Ad_2DpBR322$ recombinant plasmid of adenovirus,
the PstI B fragment of SV40 virus and the EcoRI-BamHI
fragment of the α-amylase gene of the <u>Bacillus
amyloliquefaciens</u> plasmid pKTH10 as solid carrier-attached
nucleic acid reagents, and the Ad_2-BamHI C fragment of
15   adenovirus, the PstI A fragment of SV40 virus and the
ClaI-EcoRI fragment of the α-amylase gene of the <u>Bacillus
amyloliquefaciens</u> plasmid pKTH10 as labelled nucleic acid
reagents.

AMENDED CLAIMS
(received by the International Bureau on 07 March 1983 (07.03.83)) ·

(amended)   1. A microbial diagnostic method, based on sandwich
hybridization of nucleic acids on a solid carrier, to
identify microbes or microbial groups, in which method two
different nucleic acid reagents with no sequences in common,
but preferably situated close to each other in the genome of
the given microbe or microbial group, are used for the .
identification of that microbe or microbial group in a given
sample, one of the nucleic acid reagents being a single-
stranded nucleic acid fragment attached to a solid carrier
and the other being a single-stranded nucleic acid fragment
labelled with a suitable marker, the method being
c h a r a c t e r i z e d   in that for the simultaneous
identification of different microbes or microbial groups a
plurality of nucleic acid reagents are brought into contact
with the nucleic acids of all the microbes or microbial
groups in the undivided sample, where the sample nucleic
acids are first, by known methods, rendered single-stranded,
upon which the sample nucleic acids hybridize to the
complementary nucleic acid fragments, themselves attached to
the solid carrier and the carrier-attached hybrids become
labelled due to the subsequent annealing of labelled nucleic
acid fragments to the sample nucleic acid fragments, after
which the carrier-attached label is measured by established .
methods.

(amended)   2. A plurality of reagent combinations to be used in the
method according to patent claim 1,   c h a r a c t e r -
i z e d   in that it includes two nucleic acid reagents for
each microbe or microbial group to be identified, and the
necessary two different nucleic acid fragments, which have
been made from different parts being preferably near each
other of the microbe or microbial group for the reagents,
have been produced either directly from the microbial genome
or using established recombinant DNA techniques, and these

group- or species-specific nucleic acid fragments have been
rendered single-stranded, and one has been attached to a
solid carrier and the other labelled with a suitable marker.

(amended) 3. A plurality of reagent combinations according to claim
2, c h a r a c t e r i z e d   in that for the
identification of adenovirus it includes the adenovirus
recombinant plasmid Ad₂DpBR322 as a solid carrier attached
nucleic acid reagent and the adenovirus Ad₂-BamHI C-fragment
as a labelled nucleic acid reagent.

(amended) 4. A plurality of reagent combinations according to claim
2, c h a r a c t e r i z e d   in that for the
identification of Semlike Forest virus it includes the
EcoRI-XhoI fragment A of the pKTH312 plasmid as a solid
carrier attached nucleic acid reagent and the EcoRI-XhoI
fragment B of the pKTH312 plasmid as a labelled nucleic acid
reagent.

(amended) 5. A plurality of reagent combinations according to claim
2, c h a r a c t e r i z e d   in that for the
identification of SV40 virus it includes the PstI B fragment
of SV40 virus as a solid carrier attached nucleic acid
reagent and the PstI A fragment of SV40 virus as a labelled
nucleic acid reagent.

(amended) 6. A plurality of reagent combinations according to claim
2, c h a r a c t e r i z e d   in that for the
identification of Bacillus amyloliquefaciens it includes the
EcoRI-BamHI fragment of the α-amylase gene of the Bacillus
amyloliquefaciens plasmid pKTH10 as a solid carrier attached
nucleic acid reagent and the ClaI-EcoRI fragment of the
α-amylase gene of the Bacillus amyloliquefaciens plasmid
pKTH10 as a labelled nucleic acid reagent.

(amended) 7. A plurality of reagent combinations according to claim 2,

c h a r a c t e r i z e d   in that <u>E. coli</u> is identified
using the plasmid pKTH45 as a solid carrier attached nucleic
acid reagent and the recombination-phage mKTH1207 as a
labelled nucleic acid reagent.

(amended) 8.   A plurality of reagent combinations according to claims
2, 3, 5 and 6,   c h a r a c t e r i z e d   in that for the
identification and distinction of different microbes,
viruses as well as bacteria, such as adenovirus, SV40 virus
and <u>Bacillus amyloliquefaciens</u>, from the same sample
containing nucleic acids originating from different
microbes, the combination includes the Ad$_2$DpBR322
recombinant plasmid of adenovirus, the PstI B fragment of
SV40 virus and the EcoRI-BamHI fragment of the α-amylase
gene of the <u>Bacillus amyloliquefaciens</u> plasmid pKTH10 as
solid carrier-attached nucleic acid reagents, and the
Ad$_2$-BamHI C fragment of adenovirus, the PstI A fragment of
SV40 virus and the ClaI-EcoRI fragment of the α-amylase gene
of the <u>Bacillus amyloliquefaciens</u> plasmid pKTH10 as labelled
nucleic acid reagents.

INTERNATIONAL SEARCH REPORT

International Application No   PCT/FI82/00038

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ²

According to International Patent Classification (IPC) or to both National Classification and IPC 3

C 12 Q 1/68,   G 01 N 33/58

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System	Classification Symbols
IPC 3	C 12 Q 1/68,00,16,70, G 01 N 33/58,60,48,50,52
US Cl	435:6, 5, 172

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁶

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	Chemical Abstracts Vol 93 (1981), abstract No 65229w, Methods Enzymol. 1980, 65 (Nucleic Acids Pt I) 468-78.	1
A	Chemical Abstracts Vol 79 (1973), abstract No 50606d, Proc. Nat. Acad. Sci. USA 1973, 70(6), 1697-1700.	1
A	GB, A, 2 019 408 (INSTITUT PASTEUR) 31 October 1979	1
A	GB, A, 2 034 323 (INSTITUT PASTEUR) 4 June 1980	1
P	US, A, 4 302 204 (WAHL G M and STARK G R) 24 November 1981	1
E	DE, A1, 3 211 311 (BETHESDA RESEARCH LABORATORIES INC) 7 October 1982	1
A	Derwent´s Abstract No 85535B/47, SU 649 751	1, 7

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  "&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ³
1982-12-29	1983 -01- 1 3

International Searching Authority ¹	Signature of Authorized Officer ¹⁰
Swedish Patent Office	C-O Gustafsson

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
E	EP, A1, 0 062 286 (ALBERT EINSTEIN COLLEGE OF MEDICINE OF YESHIVA UNIVERSITY) 13 October 1982	1
E	EP, A2, 0 063 879 (YALE UNIVERSITY) 3 November 1982	1
A	J. Inf. Dis. Vol 142, No 6, pp 892-98, published December 1980 (MOSELEY S L et al) "Detection of Enterotoxigenic Escherichia coli by DNA Colony Hybridization".	1, 7
A	J. Clin. Microbiol. Vol 12, No 2, published August 1980 (BRAUTIGAM A R et al), "Rapid Typing of Herpes Simplex Virus Isolates by Deoxyribonucleic Acid: Deoxyribonucleic Acid Hybridization".	1