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(54) Title: METHOD, KIT AND PRODUCT FOR DISEASE DIAGNOSIS

(57) Abstract

A method, product and kit for diagnosing disease utilizing nucleic acid hybridization. The nucleic acid probe is non-radioactive and comprises a reporter molecule bound to a nucleic acid sequence complementary to the nucleic acid sequence of the target disease organism. The probe is hybridized to the sequence of the target disease organism and the reporter molecule is detected after exposure to UV light. The method is used to detect the AIDS, HSV, CMV, HBV and HAV viruses as well as bacteria such as cholera, mycoplasma and chlamydia.

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"METHOD, KIT AND PRODUCT FOR DISEASE DIAGNOSIS"

Field of the Inventions

This invention relates to disease diagnosis and particularly to a non-radioactive method and apparatus in the form of diagnostic materials which are part of a diagnostic kit and a product which is used to diagnose the direct presence in a sample of a living disease organism, such as the AIDS virus, HSV, CMV, HBV , HAV and FLU viruses, and bacteria such as cholera, mycoplasmas, chlamydia and other invasive body organisms which cause disease, and which are usually infectious.

Background of the Inventions

Extensive progress has been made in recent years in molecular virology as a direct result of the expansion of our knowledge of molecular biology in general and as a consequence of the development of new elegant methods in recombinant DNA technology. Seperately these molecular biology techniques also, helped in analysing the structure, organization of individual genes or genomes.

Viral diagnostic methods are utilized by doctors and

clinical laboratories and hospitals and diagnostic aids have generally been developed by these organizations personnel. DNA related techniques are not being widely practiced outside research laboratories. Molecular techniques for viral diagnosis are urgently needed, providing they are sensitive, specific, rapid and easy to use. The development of such techniques and diagnostic kits will make it easier to be employed at the doctor's office or at clinical laboratories.

The clinician uses methods for viral diagnosis are laborious, time consuming or their sensitivity is in question (R1-R9). [R1 and R2 refer to the references identified in the Appendix hereto, as the designation (R...) identifies all references there identified. These references are specifically incorporated in there entirety herein.] The great number of viral diseases however, remains without any detection beyond clinical prognosis of the patients themselves. To the average practitioner the diagnosis of a patient's febrile illness is "probably a virus infection" if everything else failed.

The need for more adequate preventive medicine, and disease management, and the recent advancement in virus therapy research have made it urgent to have viral diagnostic tests, that are rapid, sensitive, safe and easy to use.

Those references cited in the Appendix are considered to be general background prior art references which have been specifically selected as illustrative of the closest techniques known to me, but these do not teach the present preferred inventions. No reference is known which suggests or which suggests combination with other references which might be combined with it to provide a base from which the method, apparatus and product described herein are known to me. Direct organism diagnosis by non-radioactive molecular biological

techniques as described herein were not known before my conception, yet background publication referred to herein may be used to understand the implications of my invention and to effect the results thereof for specific applications.

It is recognized that in research laboratories some progress had been made in the field of molecular diagnosis utilizing the exquisite specificity and sensitivity that can be obtained by nucleic acid hybridization. These procedures routinely employ DNA or obligodeoxyribonucleotide probes of high specific radioactivity coupled with autoradiographic or fluorographic detection methods (R3-R9). In spite of these analytical tools, there are some limitations and disadvantages associated with the use of radioactive probes, especially for routine application in clinical medicine. The short half-life of the used radioisotope, the personal safety, and the isotope disposal problems make it desirable to have a nonradioactive detection method providing the sensitivity is not compromised in detecting viruses in clinical specimens. Recently, HSV was reported as detected by others in clinical specimens by DNA hybridization using radioactive probes (R22). This prior attempt to detect a virus by DNA hybridization differs from the present inventions in a number of important respects, even if it were modified in light of the description of other aspects of my inventions. The use of radioactive probes is not suitable for use in doctor's offices or clinical laboratories and hospitals and offers risks which are not tolerable, as the risk of personal safety makes such detection unsuitable.

Summary of the Inventions

The preferred embodiment of my inventions provide a safe non-radioactive identifier product probe which will directly link (hybridize) to a virus or bacterial or other organism's DNA or rRNA. This linkage is used for

indicating the presence or absence of the suspected invasive organism. The probe or the identifier product is a DNA fragment or oligodeoxyribonucleotide complementary to the viral genome or to the rRNA sequence in other organisms, such as bacteria. This fragment would be supplied to a reporter or marker molecule and a marked probe created as the non-radioactive identifier product probe. In my preference this molecule would be fluorophore. Thus the signal of the reporter or marker identifer product probe would be visualized by ultraviolet light. This probe-fluorophore linked product is used to identify chlormetrically the presence thereof on a matrix where the genome or the rRNA of the organism obtained from a clinical sample is immobolized on a matrix. The methods elaborated in the detailed description are also be subject to automation for sample manipulation and in the recording of the nonradioactive chlormetric/fluorecent signal. Representative of what DNA linked products may identify are the AIDS virus, the HSV, CMV, HBV , HAV and FLU viruses and other bacterial organisms such as mycoplasmas, chlamydias, cholera and other invasive organisms.

The method employed in order to obtain the identifier product probe and the identified organism-manipulation products and the apparatus used therefor are described in detail in the description hereinafter. In addition, the method of diagnosis includes providing in the form of a diagnostic kit and method of its use as way that the suspect organisms can be linked to a specific probe, such as a virus or bacterial protein and thus identified and used for having the identification specifically and non-radioactively visualized by light emission and color identification.

A further understanding of the present inventions may be had by reference to the following descriptions with reference to the accompanying drawings in which:

Figure 1 is a block diagram of the basic method,

Figure 2(a) is a schematic representation of a probe's creation, while Figure 2(b) shows schematically how a sandwich matrix is created, and,

Figure 3 shows a sample target organism,

Figure 4 shows a marked target organism,

Figure 5A represents a slot matrix used in the immobilization method,

Figure 5B represents a sandwich fragment matrix used in the sandwich method,

Figure 6A represents schematically a method of obtaining the target marked hybrid by the sandwich method,

Figure 6B represents an alternative way of obtaining the marked target material by the sandwich method, and

Figure 7 is a schematic sectional view of a probe dispensing device.

Further Detailed Description

The main objective of the inventions described herein is to provide viral diagnostic kits to be used at the doctor's office or in a clinical laboratory or a hospital. Representative of the group of kits which may be made by those skilled in the art after a full understanding of the present inventions are those described herein including developing diagnositc kits for 1) AIDS, 2) HSV, 3) CMV, 4) HBV and HAV and 5)FLU viruses as well as bacteria and other organisms, including those described herein. The application of the inventions has use in the field of diagnosis and has potential not only on viral and bacterial diseases diagnosis in man but also in animals, plants and in testing industrial microbial contaminations. For bacteria and like disease organisms, it should be noted that the organisms, other than viruses,such as cholera, mycoplasma, chlamydia, etc., the target molecule would be ribosomal RNA (rRNA) since these cells contain ribosomes and thus the target rRNA

molecules would be amplified thousands of times. For viruses I prefer to employ genome detection (DNA/RNA).

The diagnostic kits have been provided to meet the following main criteria: 1) nonradioactive, 2) sensitivity, 3) specificity, 4) speed, 5) easy to use, and 6) cost effective.

Prospect Viral Diseases may be identified after a full understanding of the following description.

I. HTLV-III is the etiological agent for acquired immune deficiency syndrome (AIDS). The molecular biology of this newly discovered virus has been greatly elucidated in the last couple of years including the complete nucleotide sequence of the virus genome (R12-R18). During the virus propagation it affects and kills the lymphocytes. It is found in the blood and in other bodily lymphocytes and the patient is usually infected by other viruses (R19-R20). It is found in the blood and in other bodily fluids. A test is now available from Abbott Laboratories but this test is based on detecting antibodies produced by the patient to the HTLV-III virus. Thus, this test would indicate whether or not a person has been exposed to the virus. However, the test does not reveal whether an antibody-positive individual has AIDS or even if a person harbor the virus itself and is capable of transmitting the disease to others. Moreover, it is understood that this test is not 100 percent accurate, and that FDA Commissioner Frank Young has recently estimated that about 17 percent of the individuals who test positive for the virus will be "false positives". Some health specialists also are understood to have stated that not everyone who is infected with the virus will have antibodies, and accordingly the test would fail to identify these individuals. Presently, antibody tests for AIDS, are believed to give confusing results because of inadequate fractions, or infections which have not created antigens

for one reason or another.

In this respect, the preferred type of tests may be considered "second generation tests" since they detect the virus genetic machinery directly. Therefore, this type of testing may be employed as an essential confirmatory test or it may be employed as the first diagnostic test.

The need for virus direct testing, such as for AIDS, is great since it is estimated that there are about 2,300 blood banks in the USA alone that would have to make 80-100 million tests a year in order to screen for infectious diseases. However, the kits and procedures described herein are for use in the doctor's office, as well as in clinical laboratories and hospitals, and before and after reaching the blood bank.

II. Herpes Simplex Virus (HSV) results in Herpes genitalis which has become an increasingly prevalent sexually transmitted disease (CCD report, 1982). It is estimated that 1 in 20 adults in USA were infected annually. Rapid and accurate diagnosis of HSV infection is desirable to permit counseling, management of pregnancy, and the use of newly available chemotherapeutic agents.

HSV culture is the standard diagnosis test, but is expensive, time consuming, and unavailable in many diagnosis laboratories. Cytological or histopathological diagnosis is helpful in some cases, but the sensitivity of such examinations is generally 50 percent or less (R21). The serologic tests now readily available do not even distinguish prior nongenital HSV infection from genital herpes (R22). Recently, HSV was reported as detected by others in clinical specimens by DNA hybridization using radioactive probes (R22). This prior attempt to detect as virus by DNA hybridization differs from the present inventions in a number of important

respects. The use of radioactive probes is not suitable for use in doctor's offices or clinical laboratories and hospitals and offers risks which are not tolerable.

III. Viral Hepatitis is a major public health problem occurring endemically in all parts of the world. In man hepatitis A, hepatitis B and yellow fever virus are the most important causes of acute inflammation and necrosis of the liver. Inflammation of the liver is also associated with other viruses such as cytomegalovirus (CMV) and Epstein-Barr (EB). However, the term viral hepatitis commonly referred to hepatitis caused by virus type A and B (R23).

A. Hepatitis B Virus (HBV) is essentially transmitted by blood transfusion or by parental route (such as pregnancy, lactation) (R19). HBV DNA has been reported to integrate into the host cell genome and it is believed that the integration of viral DNA is interrelated with appearance and/or progression of human hepatocellular carcinoma (R22) which may arise as a rare sequel to the hepatitis. HBV DNA was clearly demonstrated to be present in nuclear and cytoplasmic fractions of infected human cells (R7). Recently, it has been reported that nonhepatic tissues (placenta and kidney) of people exposed to HBV infection contains the virus genome (R25) and a model for its integuation in hepatocellular carcinoma was proposed (R26).

A variety of laboratory techniques are available for detecting HBV, but again their sensitivity, and, specifity is in question.

B. Hepatitis A Virus (HAV) is known as the infectious or epidemic hepatitis. It spreads usually by person-to-person contact and major outbreaks result most frequently from facial contamination of food and water. A number of serological tests for HAV have been developed by others including immunoelectron microscopic immune hemagglutination, complement fixation, radioimmunoassay

and enzyme-linked immunosorbent techniques (R11,R23). The complete RNA genome of the human HAV has been published including its gene organization (R27).

IV. Cytomegalovirus (CMV) is common in the general population. It was reported that between 30 and 100 percent of adults show serological evidence of prior CMV infection. CMV is understood as a major cause of disease in newborn infants and immunosupressed patients (R8, R28, R29). Congenital CMV infection occurs in about 1 percent of all infants born in the United States and results in sensorineural hearing loss and/or mental retardation in children (R29). It was demonstrated recently that breast milk of patients contained CMV (R30). Premature infants with prolonged hospitalization in intensive care nurseries are believed to be at high risk of developing severe CMV (R31). The noncongenital type, among patients receiving immunosurpressive therapy for allograft operations up to 100 percent may be expected to show CMV in their urine (R8), and it is also, common among AIDS patients (R20).

The diagnosis of CMV infection presently depends on detection of a serological rise in titer of antibody to CMV antigen or isolation of virus in tissue culture. Although urine cultures of some congenitally infected infants may be identified as positive within several days, many cultures for CMV take six weeks or longer before being identified as positive. CMV DNA was detected in urine specimen using 32p-labelled DNA probes (R8, R28, R32). This probe detection method differs from the present inventions.

In a manner similar to the disgnosis of other viruses, FLU strains may be indentified by their genomes by use of the method according to my inventions. This will be useful in screening for specific strains.

The approach used in the direct diagnosis of a suspect sample and in maximizing the resultant signal

indicating detection result in a new non-radioactive direct diagnosis method and includes the described embodiments of the inventions which involve:

I. The creation of non-radioactive probe identifiers of DNA/RNA of viral genomes or rRNA for other organisms.

II. The provision of a stable matrix for fixing DNA, RNA, or rRNA of sample material.

III. The nonradioactive detection of sample's nuclear acids after efficient immobilization of viral genome (DNA/RNA) or other organisms rRNA on matrix by means of nuclear hybridization using identifier product probe which is complementary in sequence to the genome or rRNA of the organism that needs to be identified.

IV. The development of simple protocol to follow and supplying in addition to the safety aspect will make the kit usable by nonspecialist i.e. at doctor's office or in a clinical lab.

V. The creation of a visualization effect for identification of the organism-probe identified product.

The preferred embodiments result in simplifying the procedures for the manipulation of the samples.

I. Labeling of DNA can be accomplished by a variety of methods which will be described in accordance with my preferred embodiment a probe is created and labeled. A DNA probe is basically labeled fragment of DNA and is complementary to the target (e.g. virus) genome. There are two general ways which may be used to prepare DNA fragments:

A. By the chemical synthesis of DNA oligodeoxyribonucleotides. This subject has been recently reviewed (R33). Several types of automated machines for the chemical synthesis of oligonucleotides are already available in the market.

B. By cleavage of the desired DNA fragments. I prefer to use fragments cloned in a plasmid, by the appropriate restriction enzyme(s) followed by the

purification of such fragment from the rest of the DNA. Many of these techniques were developed by me and/or used for molecular biology purposes including DNA sequence analysis (R34-R41).

I prefer the chemical method for its simplicity and cost for the production of the desired, although, alternative DNA fragments are useful and could be used in conjunction with the synthesized oligonucleotides. In other arts there has been published literature which identifies techniques which have been used in these other non-diagnostic arts for labeling DNA fragments. The most commonly used methods in labeling short DNA fragments or oligodeoxyribonucleotides are by γ - (^{32}P or ^{35}S) using T4 polynucleotide kinase (R42). It has been suggested that longer DNA fragments could also be nicktranslated by incorporating α - (^{32}P or ^{35}S) deoxyribonucleotides in the DNA (R43). More recently, random priming of DNA was used to label DNA (oligolabeling) using synthetic oligodeoxyribonucleotides (R44). These methods have been used mainly as a research tool and may be considered as useful for comparative assessments of efficiency with the preferred embodiments of my invention.

It is recognized that recently, there has been some published work for the nonradioactive labeling of DNA. As an example, Biotin-labelled DNA has been utilized for visualization of bacterial DNA. This method is based on the fact that biotin (vitamin H) can strongly bind (non covalent) with egg white glycoprotein (MW68,000) or the non gycosylated protein strepavidin (MW60,000) produced by streptomyces avidini which will lead to the formation of a high affinity complex (Kd is about 10^{-15}) (R45). This complex can easily be visualized/by enzymes that are commercially available (e.g. peroxidase, and alkaline phosphatase). Enzymatic methods for labeling DNA with biotin have been reported (R46) using biotinylated TTP

derivatives in a nicktranslation reaction. Also, the labeling of oligodeoxynucleotides with biotin by primer extension reaction using E.coli DNA polymerase I (klenow fragment) has been reported (R47). It has been suggested that RNA could be labelled with biotin by T4 ligase induced modification of 3 terminus utilizing a biotinylated ADP analog (R48). Recently, chemical methods for the synthesis of biotinylated dexoyribonucleotides have been reported (R49-R52).

Both the enzymatic and the chemical methods for the synthesis of biotinylated DNA have been used to detect single genes or virus genomes in clinical specimens (R49,R50,R53). These methods, however, still need to be changed in order to increase the signal when small clinical samples (containing less virus) are used simultaneously the time for hybridization and visualization could and should be shortened as provided by the utilization of the inventions provided herein.

B. The Direct DNA Enzyme Cross Linking Method to bind of DNA. This method (R54) is based on: a) the fusion of an enzyme (e.g. peroxidase, alkaline phosphatase) to a synthetic polyamine carrying many primary amino groups (poythyleneamine) using benzoquinone as crosslinking reagent, b) the modified enzyme can now be able to bind electrostatically to polyaneon (DNA) due to its small, positively charged tail, and c) the ionic binding between the macromolecular components is essential for a successful crosslinking reaction leading to covalent bonds. The enzyme could be covalently bound to DNA using gluteraldehide. The enzyme(s) is visualized then by standard enzyme reactions. Although this method may be employed as part of the method of my invention it is considered as a secondary embodiment of the specific aspect for which it is used and not the most preferred embodiment for while promising (using method HBV genome was detected (R54)), it still requires elaborate reagent

preparations.

C. Fluorescent Conjugated DNA is employed in a preferred embodiment of my invention. This method is very promising as optimized and modified for non-radioactive diagnostic purposes. This embodiment utilizes fluorescent tagged DNA probes for diagnostic application. This method was employed by others in an experiment to automate DNA sequence analysis (R55). It is based on the synthesis of oligonucleotides which contain an aliphatic amino group at their 5' end. In accordance with the best mode of my invention, this amino group is utilized to synthesize several fluorescent derivatives in accordance with my invention. I have modified this method to make it applicable for non-radioactive diagnosis of viral DNA and for creation of specific probes. This approach is used to produce multiple viral diagnosis kits by attaching different fluorescent groups producing different colors for different probes thereby detecting different pathogens in to the same sample.

II. The immobilization of samples of DNA is accomplished by fixing the DNA of a sample on a stable matrix. There are different methods which have been used for the immobilization of nucleic acids on solid support or membranes (R56).

The most commonly used methods to immobilize nucleic acids could be summarized as follows:

A. Methods based on the blotting or the transfer of DNA bands from electrophoretic gels (R39). This method generally is not preferred as suitable for our purposes since it is time consuming; nucleic acids of the sample have to be purified, and only a few samples can be processed at the same time. However, the efficiency and sensitivity of this method was recently improved (R58).

B. The dot blot method simply immobilizes the samples' nucleic acids by blotting directly on the membranes or by filtering the sample through the membrane

while the samples' DNA are retained by the membrane filter (R59, R60). This method has some advantages in as far as it can employ a large number of samples simultaneously. It does not require elaborate purification of samples' nucleic acids and it does not require prior electrophoresis. While this method has been used to retain DNA samples in research it is not known to have been used as an element for a method and apparatus for diagnosis of disease bodies in a sample. It may be employed as an alternate embodiment for the broad aspects of my inventions.

C. Sandwich hybridization method. This method requires two nonoverlapping DNA fragments complementary to the virus genome, in addition to the viral genome itself for the signal to develop (R61, R62). One of the two fragments (oligodeoxyribonucleotide) is immobilized on solid support and the other fragment is mixed with the sample. Only if the clinical sample contains the corresponding virus genome would a signal develop.

Viral genome has been detected in clinical samples utilizing this method using radioactive labelled probes (R8).

This sandwich method was modified recently by using resin (sephicryl S-500) to immobilize the DNA instead of membrane support (R63).

In the best mode of my inventions the sandwich hybridization method has to be modified to use nonradioactive probes and to be more suitable for crude clinical samples. It is also changed to increase the efficiency of binding DNA molecules by providing solid support, preferably in the form of membranes. There exist suitable commercial membranes for binding DNA and RNA. Nylon membranes could be modified to increase their binding abilities of macromolecules (R64). Such membranes may be used to give colormatic reading as a step for automation of the diagnosis process. Resins

such as sephicroyl S-500 may be used alternatively.

III. Sample Manipulation:

Clinical samples usually have a lot of contaminating molecules (protein, salts, etc..). However, the ideal method for detecting any pathogen's genome should not require elaborate manipulations and should not compromise the detection signal.

Viral genomes has been detected by DNA hybridization in clinical samples such as urine (R8,R28,R32), stool (R10,R65,R66), cotton swab specimens (R22), mouthwashes (R67), blood serum (R11,R68), blood leucocytes (R69), and semen (R70). It has become evident to me from the recent published work that virus genomes can be detected in clinical samples. However, further improvements of the sample manipulation are required. The followings are examples of alternative sample manipulation methods to be used.

A. The use of 2-butanol to concentrate nucleic acids of the samples. It was observed that butanol concentrate diluted DNA samples (R71). I have also used this method for similar purposes, and I prefer this as a best mode for DNA. It has been recently used to concentrate CMV DNA from urine sample (R8).

B. Polyethylen glycol either to concentrate the virus particles or virus genomes is an alternative method. This has been used extensively in molecular biology for similar purposes (R34).

C. The use of alkaline solutions and chemical detergents to lyse the virus particles as well as denaturate its double strands for molecular hybridization purposes is an alternative method.

D. The use of Guanidium Thiocyanate as a strong deproteinizing agent and nuclease inhibitor is preferred for RNA. It has been used extensively in the isolation of mRNA (R72). Guanidium Thiocyanate does not seem to affect nylon membranes.

IV. Diagnostic procedures are important considerations in the effective direct non-radioactive diagnosis of disease. The procedure and number of steps that have to be used by the doctor/clinician has to be reduced to a minimum. Basically, these steps in accordance with the best mode of practicing my inventions involve:

A. The deposit of a sample containing the virus genome on an appropriate specifically preactivated matrix;

B. Denaturating the sample genome complementary strands and adding the specific identifier probe(s);

C. Colormetric fluorometric visualization of any identifier probe-identified organism product hybridization if the probe is complementary to the target genome, resulting a positive signal which may be visualized. (This positive signal would mean that the sample has the virus or other body for which the probe was made.)

In accordance with the preferred mode of my inventions the method of diagnosis employs a diagnosis kit which employs specifically developed probes for the virus or other disease body and employs a modified sandwich method or alternatively a dot blot method in such a way that the DNA fragment(s) or rRNA complimentary to the suspect genome is chemically linked to a solid support matrix.

The sandwich method is more suitable for crude clinical samples and is the most preferred mode at the present time. The sandwich method is modified such that DNA fragment(s) (oligodeoxyribonucleotides) complementary to the virus genome is chemically linked or crosslinked to a solid support. For other purposes genomes have been so linked. (R73) In my preferred embodiment the DNA may be crosslinked to membranes by ultraviolet light. This increases efficiency and

sensitivity of detection providing the support membrane or resin is treated afterwards to minimize any non-specific background noise. Alternatively the dot slot hybridization method may be used to detect viral genomes in crude extract. The direct dot blot method may be used in applying sample nucleic acids to filter membranes, and this method may be used to allow multiple samples to be tested simultaneously.

The sandwich method is modified for this purpose. Multiple viral genomes in the same sample may be detected by the sandwich method by employing different immobilized DNA fragments on the same membrane in different slots and by the use of different probe fragments in the hybridization mix. It is frequent for imunosuppressed patients and other patients (such as AIDS victims) to have multiple infections, and accordingly, the use of different hybridization fragments in the same mixture allows multiple screening.

the

The efficiency and sensitivity of detection is increased by treating the support (membrane or resin) afterwards to minimize any non-specific background noise. Such solid supports are stable for months and are supplied as part of the diagnostic kit. A probe fragment (rRNA or DNA) complementary to the desired genome prepared and is non-radioactively labeled and packaged seperately as a part of the diagnostic kit.

Upon use, diagnosis is obtained by mixing the probe fragment with the sample genome and the solid support (with attached complementary fragment). If there is a chemical bond established in a hybridization reaction, the signal will develop, but, because a chemical bond is required, this will develop only if the sample has a viral or other body's genome complementary to both fragments.

In the sandwich hybridization method, upon mixing

the probe fragment with the sample genome and the solid support (which an attached complementary fragment to the virus genome but not overlapping with the probe fragment) in a hybridization reaction, the signal will develop only if the sample has a viral genome complementary to both DNA fragments.

In the dot/slot method hybridization is carried out by using the probe and the immobolized genome direction in a hybridization reaction. The signal will develop if the viral genome complementary to the probe fragment is present.

The non-radioactive probe in the preferred embodiment of my invention is created by preparing a desired complementary probe fragment or oligonucleotide with an automatic DNA synthesizer and covalently attaching fluorophores to the chemically synthesized oligonucleotide(s) that is complementary to the target genome. Automatic DNA synthesizers which can created a suitible probe fragment(s) such as a probe chain AGCTACCGAA (or like chain characteristic of a chain or the target species or strain) can be used in the creation of the probe chain. These devices are commercially available from suppliers such as Beckman Instruments, Inc., P.O.Box 10200, Palo Alto, California 94304, U.S.A. or Genetic Design, 111 School Street, Watertown, MA 02172 U.S.A.. Suitable fluorophores are commercially available from Aldrich Chemical Co. 940 West Saint Paul Ave. Milwaukee, Wisconsin 53233 U.S.A and Sigma Chemical Company, P.O. Box 14508, St. Louis, Mo. 63170, U.S.A.

The base composition of DNA (Deoxyribonucleic acid) and rRNA (Ribonucleic acid) is characteristic of the organism (species or strain). Under UV light various of the fluorophore probes will fluorescence. Thus utilizing the techniques described here, either a fluorescence or ultraviolet microscope, in case of <u>in situe</u> tissue diagnosis, and the a kit described herein is all that is

needed to detect the fluorescence as a signal as a fluorescence color under ultraviolet light. The genome of organisms each have the specific base composition of a sequence of deoxyribonucleotides or ribonucleotides linked with phosphodiester bonds usually in double strands. The strands are stabilized together by hydrogen bonds. These hydrogen bonds could be broken, thus the strands are separated by heating, alkaline, or other chemicals. I create a probe chain chemically as by the chemical synthesis methods of digonucletides (R33) and using chemical methods of other researchers to add an identifier moiety to the probe (DNA fragment/oligonucleotide) (R50, R51, R52, R55). Enzymatic detection methods will be colorometric and visual, while if the identifier molecule is a fluorophobe a U.V. light is used to visualize the fluorecent signal which matches or complementary at a fragment section the specific chain of the DNA or RNA of the species or strain that is to be identified. Such a probe chain might be AGCTTTGC. etc.(A=Alenine,G=Guanine,C=Cytsine,T=Thymine).

In order to provide this reaction, the matrix is exposed to the sample and the to be indentified sequence denatured and left on the matrix. The matrix which is preferred membrane filters that retain DNA/RNA and resist damage such as nylon membranes or modified nylon membranes to increase their efficiency in binding nuclear acids. These filters are commercially available from Schleicher & Schuell, Keen, New Hampshire 03431 U.S.A., or Bio-Rad, Chemical Division, 2200 Wright Avenue, Richmond, California 94804 or from other companies such as NEN or Amersham for example. The resin sephicryl S-500 and like resins are preferred for automation. Sephicryl resins may be obtained from Pharmacia, Inc., 800 Centennial Avenue, Piscataway, New Jersey 08854 U.S.A. This matrix is packaged as a part of the kit when the sandwich method is utilized DNA fragment

(oligonibonucleotides), is crosslinked to the membrane (R73), the membrance is then treated to minimize back ground noise. The probe is placed in a aqueous solution, and in a capsule which can be penetrated by a plunger dispenser. An example of such a dispenser is shown in Figure 3 in which the dispenser is in the form of a hypodermic syringe which has a capsule penetrating point 31 on the needle's syringe end. The capsule under pressure from the syringe plunger forces the capsule against the penetrating point to rupture the capsule which is dispensed through the needle onto the matrix in a hybridization medium. This hybridization step is done in sealed plastic bags. In the sandwich method it is also intended to supply the matrix prepared and presented as above in the sealed bag that contains the hybridization medium and the probe(s). The sample then is injected in the bag which is then resealed to accomplish hybridization. After washing with provided solutions to remove the probe residue, the matrix is then exposed to UV light, and if fluorescence occurs the target is identified by that fluorescence of the hybrid identified product resulting from the target-probe bond.

This discription may be further understood with reference to the accompanying drawings in which Figure 1 represents the overall method steps. Thus in Figure 1, block 11 represents the steps involved in creation of a fluorescent-bonded probe, and step 12 represents the step of packaging the fluorescent-bonded probe. Step 13 involves obtaining the sample, for example a source of blood 14 or urine 15. Step 16 represents the step of obtaining the sample target organism. Step 17 represents the step of applying the target organism to the matrix container and fixing the target organism to the matrix. Step 18 is the step of mixing the marked-bonded probe such as the preferred fluorophore or halogen marked probe (or other form of probe having color staining or

visualization creation) with sample on the matrix and if a bonding reaction occurs creating a mutation hybrid probe-target bond. Step 19 is the step of the method under which visualization-by the eye (10) occurs, as in the instance illustrated by a simple microscope (20) and ultraviolet light source (21).

Figure 2 represents a schematic representation of the creation of a probe such as a DNA fragment, oligonucleotide, or oligodeoxyribonucleotide represented as probe base AGATACCGAA (22), and the creation of a fragment thereof ATACCG (23), after elimination of the underscored AG and AA. This fragment is mixed with color identification or light emitting reporter or marker molecule source material, such as the preferred flurophore (24), for example Flurescein isothiocyanate, NBD-F and a marked probe fragment (F)ATACCG (25) created. This is preferable packaged in a container (26) such as a small vial with a rubber cap. Alternatively it is packaged in the same plastic bag with the matrix, with an attached matrix fragment, with or without the hybridization mix in the same bag (not shown in Figure 2).

In a similar manner a binding fragment chain ATCC (28) as shown in Figure 2(b) when coupled to a matrix (29) for the sandwich method creates a bonding base matrix (30) for the target organism.

Figure 3 shows a sample target organism being obtained, for example from a blood sample or urine sample (31) by a nucleic acid concentration process (32) to create nucleic acid sample material (33). The target organism is for example GCTACCGATGTCGGTAT (33') and in the instance illustrated is found in the sample material (33). The step 17 representing the application of the target organism to the matrix and fixing the target organism to the matrix differs depending upon the method employed. In Figure 3 the sandwich method is illustrated

at the left of the dotted vertical line while the immobolization method is illustrated at the right of the dotted vertical line. In accordance with the sandwich method, the matrix (29) has a bonding fragment (30') attached to the matrix to form the bonding matrix (30), and in the dot method example of the immobilization method there is direct bloting attachment of the sample GCTACCGATGTCGGTAT (33') to the matrix (29).

In order to mark the sample (33') hybridization material and the reporter or marker probe are also provided as illustrated schematically by the vertical dotted line and marked probe (25) introduction step (34). When the probe (25) is introduced into container having the sample matrix bound organism (33') which in this instance is the target chain, a hybridization bonding of the fragment (25) and the organism (33') occurs, creating the target organism which is marked for visualization effect by the flurophobe labeled probe-target bond (36).

Figure 4A and 4B show in detail the attachment of the marked probe to the sample target organism and to the matrix, as represented at the bottom of Figure 3. This is obtained by supplying the target bearing matrix with the flurophore marked probe GCCATTA(F). A hybridization medium is utilized to provide the environment in which hybridization of the marked probe and the target genome occurs. If there is a match the hybrid reaction occurs and the bonding (36) labels the target (33') on the matrix. The sample target organism is washed and if there is a hybrid reaction which has occrued the marked target will remain on the matrix.

Figure 5A and Figure 5B illustrate matrix packages. In the immobilization method, the matrix (29) is packaged in sheets to fit in a slot sample applicator (40). As an example of this slot sample application Fig. 5A shows the matrix membrane (nylon or resin) (29) between an upper slot bearing surface plate (41) and a backing substrate

(42), both made of either plastic or glass. There are a plurality of slots (43) in the surface plate in order to permit the sample to attach to the matrix.

In Figure 5B the sandwich method is illustrated. In this instance a matrix fragment CGTA exists in a coating which is attached to the matrix (29). The matrix is then preferably pretreated to minimize any background noise and dried and packaged for individual sample manipulation. Alternatively it can be packaged in plastic bags shown by dotted line (45) containing the hybridization media mix or in plastic bags wich are seperate from the plastic bag which carries the hybridization mix.

Figure 6A and Figure 6B represent alternative methods of use of the sandwich method. In Figure 6A the matrix (29) with bonding fragment coating, if used, are packaged in a plastic bag (45) along with, alternatively, the reporter probes (25). The plastic bag can also contain the hybridization medium, as well as the probes. The sample (33') is injected into the plastic bag, as by a syringe (47), and the bag is resealed. The probes in the plastic bag can be introduced at a time after the hybridization medium is added to the bag.

Alternatively, a matrix with a dried fragment can be packaged in a container (48). Hybridization media (49) is provided in a container, as is the probe material (25) all as part of one of the kit packages (50). Into this package or along with it, the probes can be mixed, together with the hybridization medium. Normally in this alternative, the hybridization medium is prepared, then the prepared sample, and the probes and the hybridization medium, is added to a bag containing the matrix.

Figure 6A and 6B both permit use of a sealed frangible probe containing container shown in a schematic sectional view of the frangible container (51) for the color producing probe (25) which is inserted in and as

shown contained in the dispensing device (52) in Figure 7. The probe is sealed in container (51) helps to prevent contamination. The dispensing needle (53) has at its base (54) within the syringe dispenser (30) detent points (55) which when forced against (as by the action of the plunger (56)) the container (51) breaks it and allow the syringe to dispense the probe. Alternatively, the probe can be contained in another kind of container such as a micropipette with a dispensing stopper plunger, or in a rubber stopped container.

It will be appreciated that the matrix may be formed on a microscope slide 22 with cover 23 which can removed to introduce the tagged probe. A matrix 24 is positioned within the slide onto which the target material is affixed by introduction and a denaturization process.

It will be appreciated by those skilled in the art that various modifications and rearrangements can be made to the apparatus and methods described. The probes can be tailored to specific screening criteria. After an understanding of this discription by those skilled in the art such persons will adapt and modify and improve upon the various specific illustrations described herein without departing from the scope of the claimed inventions.

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APPENDIX

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What is claimed is:

Claim 1. A method of creating a non-radioactive diagnostic probe for disease organisms, comprising

selecting a desired chain DNA fragment or oligodeoxyribonucleotide which is complementary to the genome or to the rRNA sequence of a target disease organism and providing said desired chain as part of a primary probe chain, and modifying the primary probe chain by non-radioactive chemical alteration to form a probe which will produce a visualization or colorometric effect.

Claim 2. A method according to claim 1 wherein the probe chain is complementary to a viral genome.

Claim 3. A method according to claim 1 where the probe chain is complementary to a genome of a virus or a group of viruses including AIDS, HSV, CMV, HBV, HAV, FLU viruses, and strains thereof.

Claim 4. A method according to claim 1 wherein a plurality of probe chains of complementary to different species or strains are created, and marked with specific identifier molecules.

Claim 5. A method according to claim 1 wherein the probe chain is complementary to an rRNA sequence of a target organism or strain.

Claim 6. A method according to claim 1 wherein the non-radioactive chemical alteration includes the addition to the probe of at least one molecule which will produce a visualization effect by light emission and/or color identification.

Claim 7. A method according to claim 6 wherein said molecule is a fluroprobe or halogen containing molecule.

Claim 8. A method according to claim 1 wherein the primary probe chain is chemically created by chemical synthesis of digonucletides.

Claim 9. A method according to claim 1 wherein the primary probe chain is created by breaking strands of

deoxyribonucleotides or ribonucleotides.

Claim 10. A method according to claim 9 wherein the probe chain base probe chains are broken by heating, and/or exposure to alkaline, or other chemicals to create the primary probe chain.

Claim 11. A method of non-radioactive direct diagnosis of the presence of an organism, comprising,

providing DNA and/or RNA complexes of a species or strain of the organism and segregating one or more of the complexes as a target, subjecting the said target to the presence of a tagged non-radioactive probe having a reporter moiety and having a specific fragment of a target disease organism nucleic acid chain and permitting a hybrid bonding reaction to occur if the target has a complementary fragment which will bond with the tagged non-radioactive probe.

Claim 12. A method according to claim 11 wherein said tagged non-radioactive probe is a diagnostic probe for disease organisms, comprising

a desired chain fragment which matches a DNA fragment or oligodeoxyribonucleotide or rRNA sequence of a target disease organism chain and which said desired chain fragment is part of a primary probe chain which has been modified by non-radioactive chemical alteration to form a probe which will produce a visualization effect.

Claim 13. A method according to claim 11 wherein said tagged non-radioactive probe is a probe with a fragment complementary to a DNA fragment or oligodeoxyribonucleotide complementary to a viral genome or to the rRNA sequence of a pathogen and the reporter moiety of the probe is adapted to produce a visualization or colorometric effect.

Claim 14. A method according to claim 11 wherein said target disease organism nucleic acid chain is bound to a matrix adapted to hold the target disease organism nucleic acid chain and hold it in position for a

hybridization reaction with said tagged non-radioactive probe.

Claim 15. A method according to claim 14 wherein said matrix has bound to it a binding fragment complementary to the target disease organism nucleic acid chain which is adapted to bond to the target disease organism nucleic acid chain and hold it in position for a hybridization reaction with said tagged non-radioactive probe.

Claim 16. A method according to claim 11 wherein said tagged non-radioactive probe and target disease organism nucleic acid chain are immersed in a hybridization media and in said media a hybridization reaction between the probe and nucleic acid chain will occur if there is a chain complementary match, thereby bonding the reporter moiety of the probe to the target chain.

Claim 17. A method according to claim 11 wherein after there has been a time elapse sufficient to permit a hybridization reaction to occur between the probe and target chain to bind the reporter moiety to the target chain and create a reporter probe-target chain hybrid, any unbound probes and other contaminent material are removed from the reporter probe-target chain hybrid so as to permit verification of the presence of the chain hybrid.

Claim 18. A method according to claim 17 wherein the chain hybrid is exposed to light or an enzymatic reaction to cause a visualizaton effect or colorometric signal to occur which when recognized as matching a known visulaization effect or colormetric signal of the reporter moiety identifies the hybrid chain as being present and having a target chain, thereby identifying the target pathogen as being present in the test sample being diagnosed.

Claim 19. A method according to claim 11 where there

are provided a plurality of different probe fragments complementary to chains of different organisms.

Claim 20. A matrix for direct diagnosis of a disease pathogen comprising,

a substrate meterial and a fragement of a nucleic acid chain of a target pathogen.

Claim 21. A matrix according to Claim 20 wherein there are different fragments of nucleic acid chains of different target pathogens provided.

Claim 22. A matrix according to claim 20 wherein said disease pathogen is selected from a group including the AIDS, HSV, CMV, HBV , HAV and FLU viruses and bacteria such as cholera, mycoplasmas, chlamydia and other invasive body organisms which cause disease and which are usually infectious.

Claim 23. A method according to claim 11 wherein the primary probe chain is complementary to a pathogen selected from a group including the AIDS virus, HSV, CMV, HBV and HAV viruses, FLU viruses and bacteria such as cholera, mycoplasmas, chlamydia and other invasive body organisms which cause disease, and which are usually infectious.

Claim 24. A non-radioactive probe for the diagnosis of the direct presence of an organism by linkage with the DNA or RNA chain of the organism, comprising,

a tagged non-radioactive probe having a probe chain fragment which matches a fragment of a target organism chain and which said probe chain fragment has been modified by non-radioactive chemical alteration to form a probe with an added reporter moiety which will produce a visualization effect.

Claim 25. A non-radioactive probe according to claim 24 wherein said reporter moiety has a molecule obtained from fluorophore or hologen compound which will produce a visualization effect or colormetric signal.

Claim 26. A kit for the direct diagnosis of the

presence of an organism in a sample comprising,

a non-radioactive probe for the diagnosis of the direct presence of an organism by linkage with the DNA or RNA chain of the organism, comprising,

a tagged non-radioactive probe having a probe chain fragment which matches a fragment of a target organism chain and which said probe chain fragment has been modified by non-radioactive chemical alteration to form a probe with an added molecule which will produce a visualization effect or colorometric signal, and

a container for said probe.

Claim 27. A kit according to claim 26, including, a solid support matrix.

Claim 28. A kit according to claim 27 wherein said solid support matrix is a membrane.

Claim 29. A kit according to claim 27 wherein said matrix is a resin.

Claim 30. A kit according to claim 27 wherein said matrix is a nylon membrane.

Claim 31. A kit according to claim 27 wherein said matrix is adapted to bond to a target organism chain.

Claim 32. A kit according to claim 28 wherein said matrix has bonded thereto a short chain fragment which will hybridize with a chain of a target organism.

Claim 33. A kit according to claim 32 wherein said short chain fragment includes a fragment of a nucleic acid.

Claim 34. A kit according to claim 26, including a hybridization medium.

Claim 35. A kit according to claim 26 wherein said container is frangible.

Claim 36. A kit according to claim 26 wherein said container is held by a dispensing element.

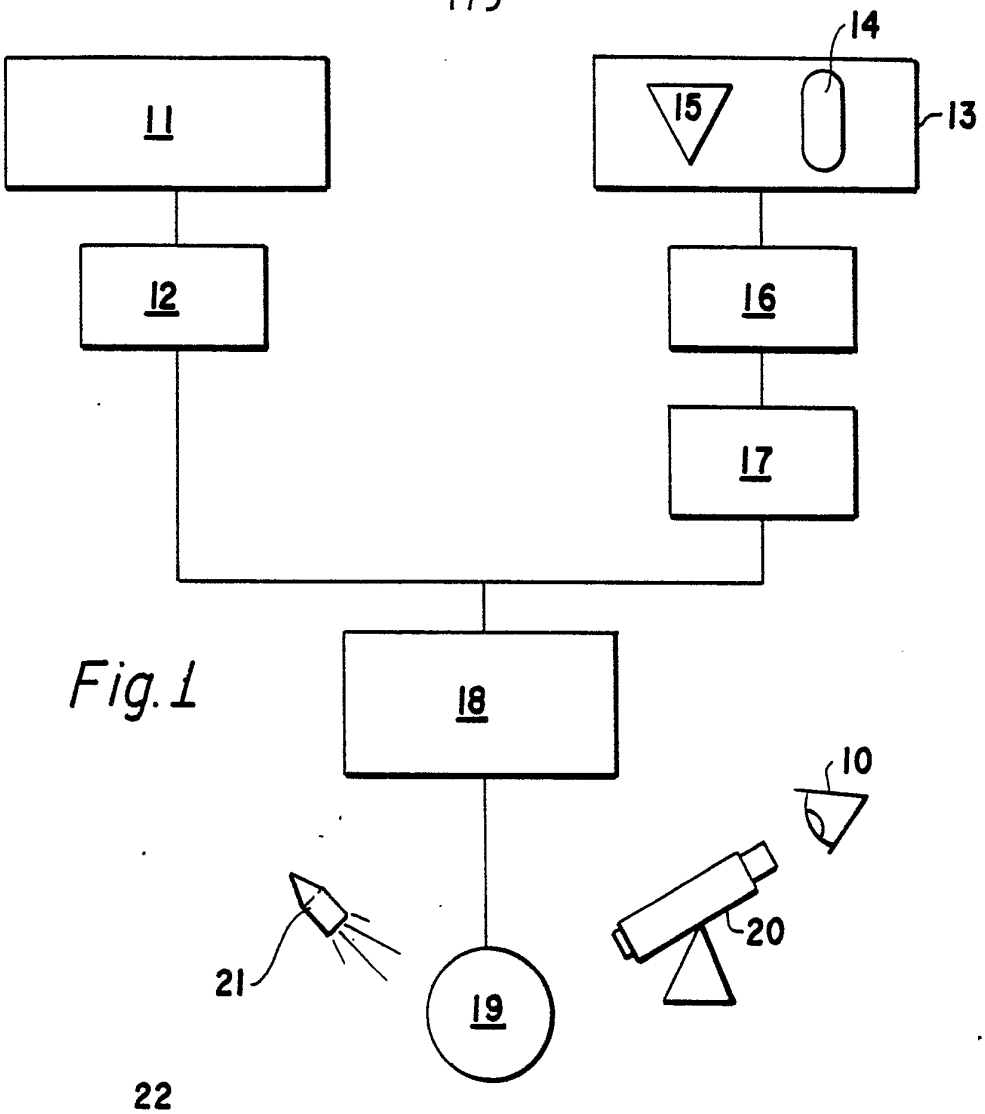

Fig. 1

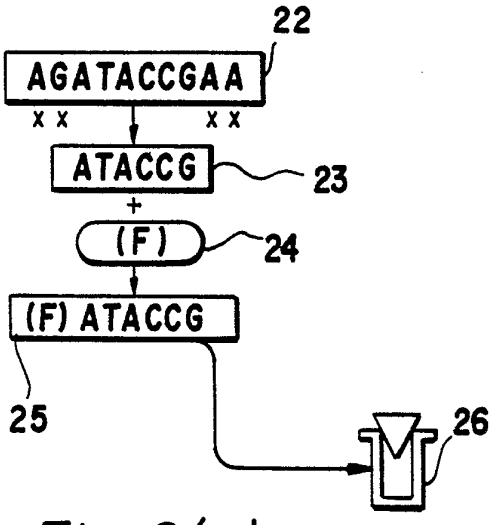

Fig. 2(a)

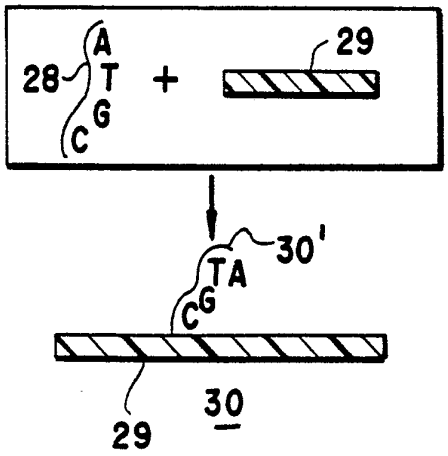

Fig. 2(b)

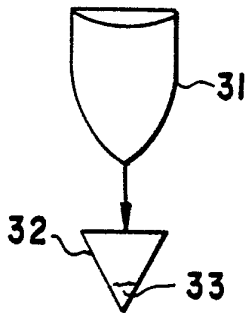

Fig. 3

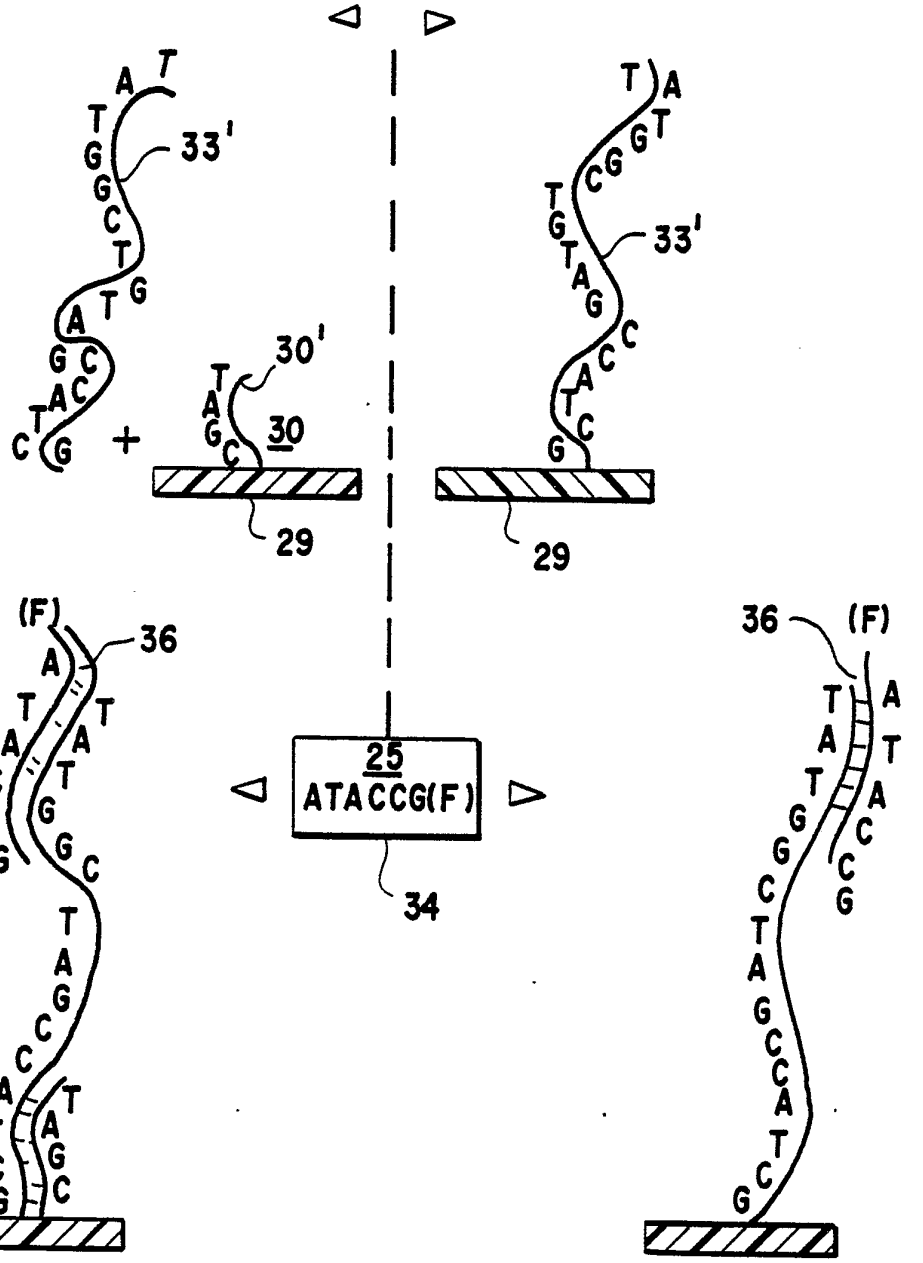

Fig. 4A

Fig. 4B

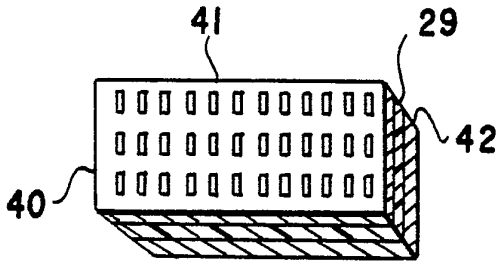

Fig. 5A

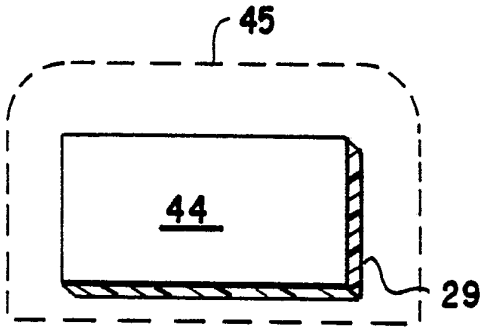

Fig. 5B

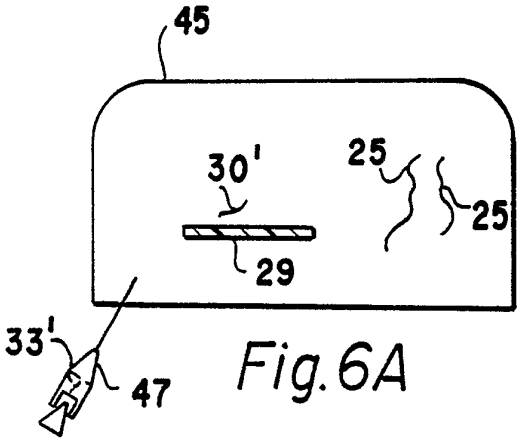

Fig. 6A

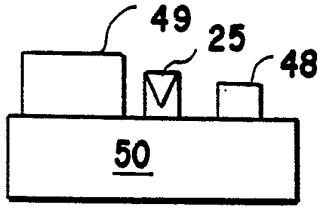

Fig. 6B

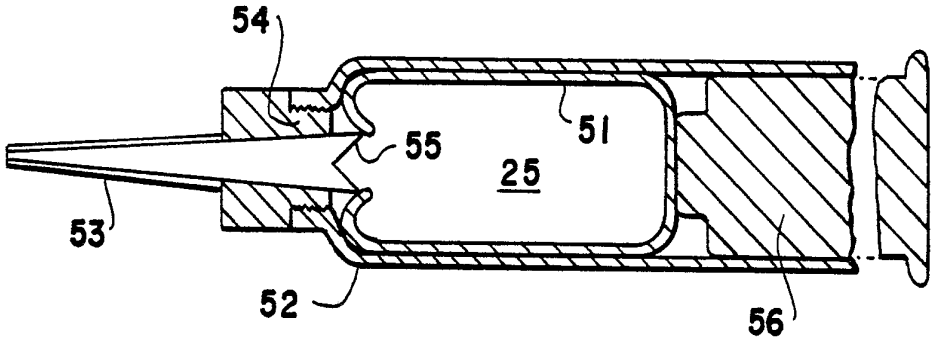

Fig. 7

INTERNATIONAL SEARCH REPORT

International Application No PCT/US86/02594

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

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

INT. CL[4]: C12Q 1/68
U.S. CL : 435/6

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	435/6,5,29,188,810 935/78; 436/530,800,805,811 536/27

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Computer Search: Lexpat

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]
P,X Y	US,A, 4,581,333 (KOURILSKY ET AL) 08 April, 1986 see column 2, lines 47-66; column 3, lines 29-37 and 43-46, column 4, lines 56-61.	1,2,6-12, 14-18,24, 25 3-5,13,19-23
Y	US,A, 4,358,535 (FALKOW ET AL) 09 November 1982, see column 2, lines 36-42 and 62-69, column 3, lines 4-10 and 39-45	1-36

* Special categories of cited documents: [15]

"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 [2]	Date of Mailing of this International Search Report [2]
30 January 1987	10 FEB 1987
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	Jeremy M. Jay

Form PCT/ISA/210 (second sheet) (October 1981)

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]
X Y	Chemical Abstracts, Volume 102, No. 5, issued 04 February 1985 (Columbus, Ohio, USA) J.G. Stavrianopoulos et al, "Methods and structures employing non-radioactive chemically-labeled polynucleotide probes" see page 282, column 2, the abstract No. 42494u, Eur. Pat. Appl. EP 117,440 (Eng).	1,2,6,8-12,14-18,24,25 3-5, 7,13, 19-23,26-36
X Y	Biochemical Society Trans actions Vol. 12, issued April 1984 (Essex, England) J. L. Woodhead et al, "Non-radioactive gene-specific probes" see pages 279 and 280.	1,2,6,8-12, 14-18,24,25 3-5,7,13,19-23,26-36